Chapter 9

Application to Physiology and Pharmacology

Abstract

Functions of living cells are regulated by charged or polar signaling small molecules called ligands. Some ligands can change the conformation of the ion channels in the cell membrane when they bind specifically to the receptors and open the channels for the specific ion. The selective permeability of membrane causes osmosis and the membrane potential. Axon of nerve can generate action potentials when the membrane potential is raised above the threshold, and conducts its action potential along the axon to the terminal. In the nucleus of the living cell there are chromosomes made of DNA. Chemical structure of DNA is a double-stranded polymer of four kinds of nucleotides, and the genetic code on DNA is carried out by m-RNA which provides the basic instructions for production of proteins in the cytoplasm. Because enzymes are proteins, their catalytic activity might be changed if the code marked on DNA is changed by mutation that could cause diseases.

9.1 The Cell Membrane

The phospholipid bilayer forms the basic structure of the membrane. Each phospholipid molecule has a polar head that is charged and two nonpolar hydrocarbon tails from fatty acids. A polar head is *hydrophilic.* It is so called since it attracts polar water molecules. Nonpolar tails are *hydrophobic*, which avoid water and align in the center of the two layer membrane. The polar heads are exposed to water both inside and outside of the cell. The nonpolar tails lie between the polar heads making sandwich-like structure. This self-orienting property of phospholipids leads the biological membrane into closed spherical structures and reseals it by itself when torn out. Libosome is made up of the phospholipid bilayer membrane. It is considered the simplest form of a biological cell. Majority of membrane phospholipids have one unsaturated none

polar hydrocarbon chain which kinks their tails whereby increasing the space between them and membrane fluidity as shown in Fig. 9.2.

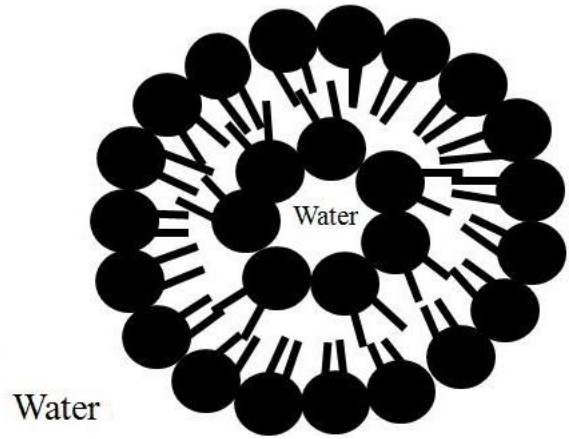

Figure 9.1 *Libosome, is the simplest form of the cell made of the phospholipid bilayer membrane.*

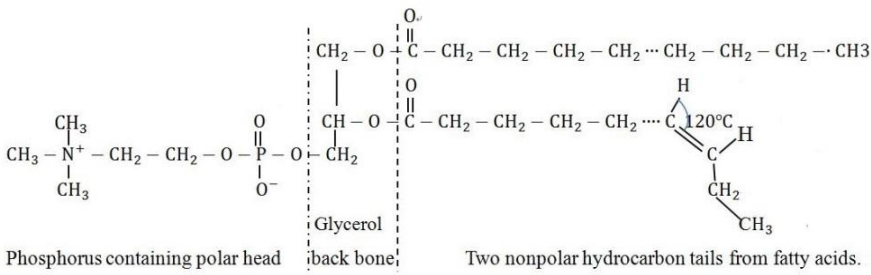

Figure 9.2 *Phosphatidylcholine is one of important phospholipid molecules.*

Cholesterol fills the space created by the phospholipid tails. The hydroxyl group of cholesterol interacts with the polar head group of membrane phospholipids, while the tetracyclic carbon skeleton and the hydrocarbon chain are embedded in the membrane alongside the nonpolar tails of the phospholipids. The bulky structure of the tetracyclic ring of cholesterol contributes to add solidity to the cell membrane. It changes membrane quality of being fluid, and enables animal cells to protect membrane integrity while

changing shape and moving about. In this structural role, cholesterol reduces the permeability of the cell membrane to neutral polar solutes and ions. Only gases such as oxygen, carbon dioxide and nitrogen, and lipid soluble small nonpolar molecules can pass through the phospholipid bilayer membrane. Ions and polar molecules cannot diffuse through it. Ions and polar molecules require channels or transporters which are made of columnar proteins in order to pass through the membrane. In general channels have two states, open and closed as seen in the voltage gated channels. Only in the open state channels act as pores for the selected ions. On the other hand, a transporter forms an intermediate complex with the solute, and a subsequent conformational change in the transporter causes translocation of the solutes to the other side of the membrane as described for Na^+-K^+ ATPase in section 9.6.

Example 9-1 This is phosphate containing polar head of phosphatidylcholine. A) Indicate charged atoms which attract ions and polar molecules. B) Indicate hydrogen acceptors that make hydrogen bond with water.

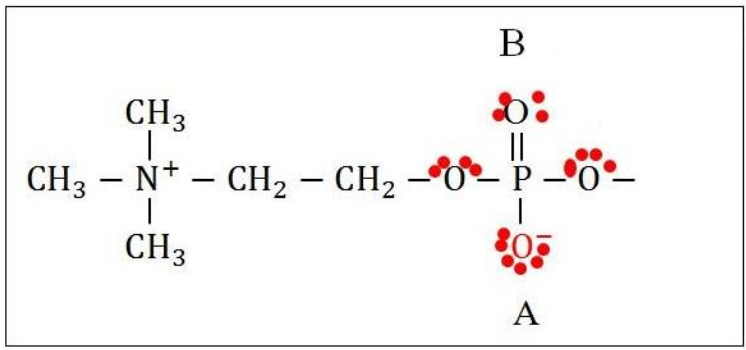

Figure* 9.*3 *Phosphate containing polar head of phosphatidylcholine.*

The electron configuration of P (15) is [N]$3s^2 3p^3$. n = 3, and l = 0, 1, 2. It has empty d-orbitals which means more than eight electrons can be held around the central atom.

Expanded valence shells are more common when the central atom, P is bonded to small highly electronegative atoms such as O.

A) Positively Charged atom: N^+ attracts anions and water molecules to aggregate around it.

Negatively charged atom: O^- attracts cations, and water molecules to aggregate around it.

B) All oxygen atoms with lone pairs will accept hydrogen atom of water to make hydrogen bonds.

9.2 Shape of protein in Aqueous Solution

Proteins and peptides are amino acid polymers. An amino group from one amino acid forms an amide bond with the carboxyl of a second amino acid. Some amino acids have hydrophobic side chains and others have hydrophilic side chains. In the aqueous environment the hydrophobic core regions of the protein move inside while hydrophilic polar side chains face outside to form hydrogen bond with polar molecules like water, and charged side chains facing outside attract some ions and some water molecules. Hence, an unfolded protein becomes globular in water.

Once the amino acid sequence of the protein was determined, computer programs can estimate the shape of the protein.

Amino acids which have nonpolar chains are glycine (Gly), alanine (Ala), valine (Val), leucine (Leu), isoleucine (Ile), methionine (Me), phenylalanine (Phe), tryptophan (Trp), and proline (Pro).

Amino acids which have polar side chain are serine (Ser), threonine (Thr), cysteine (Cys), tyrosine (Tyr), asparagine (Asn), glutamine (Gln).

Amino acids which have negatively charged side chain are aspartic acid (Asp), glutamic acid (Glu).

Amino acids which have positively charged side chain are lysine (Lys), arginine (Arg), histidine (His).

Question 9-1 Can you predict the shape of the protein in the membrane is globular or columnar if we know its sequence of amino acid.

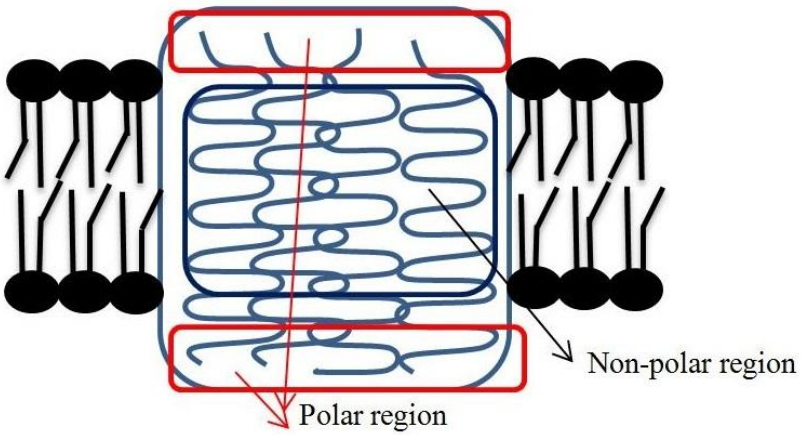

Figure 9.4 *Shape of protein in the membrane.*

Answer: columnar

9.3 Transmembrane Proteins

Transmembrane proteins are firmly inserted into the phospholipid bilayer. Spanning the entire width of the membrane, they protrude on both side of the cell. They have both hydrophilic and hydrophobic regions. The hydrophilic sites interact with water inside and outside the cell, while the hydrophobic sites interact with lipid tails and cholesterols inside the membrane.

Columnar proteins make up about half of the cell membrane by mass and are responsible for the specialized membrane functions. 1) Some *transmembrane*

proteins are *aquaporins* through which water can move in and out according to the differences of concentration of water soluble solutes from both sides of the membrane. 2) Some transmembrane proteins are pumps by which ions moved across cell membranes against electrochemical potential using energy supplied by ATP.

3) Some transmembrane proteins are simple channels for a specific small solute to diffuse from high to low concentration. 4) Some transmembrane proteins have a receptor on an ion channel. The receptors are called *ionotropic* (*ligand-gated ion channel*) *receptors*. Acetylcholine (ACh) at a *neuromuscular junction* (junction of a motor nerve axon terminal and a skeletal muscle fiber) and in a *autonomic ganglion* (a mass of nerve cell bodies at junction of the first and second neuron of autonomic nervous system) and others such as glutamate, serotonin (5HT), and gamma-aminobutyric acid (GABA) in the *central nervous system* are examples of *neurotransmitters*. They are signaling chemicals of nervous system released from synaptic vesicles formed in the axon terminals of nerves, bind specifically to the receptors on the ion channels in the postsynaptic membrane of the target cell such as a skeletal muscle fiber or a nerve cell and the binding changes the conformation of the pore columnar protein and opens the channel for ions such as Na^+, Cl^-, Ca^{2+}, and K^+ to be able to pass through it. 5) Some transmembrane proteins have a receptor coupled with a G protein. The receptors are called *metabolic* (*G protein-coupled*) *receptors*. When G protein-coupled receptors in the membrane of smooth muscle fiber, glands, and cardiac muscle fiber, bind selectively to *hormones* such as epinephrine released from adrenal medulla, ACTH, and glucagon, or the *neurotransmitters* such as acetylcholine secreted from post ganglionic axon of parasympathetic nerves, norepinephrine released from autonomic nerves, and dopamine from dopaminergic nerves in central nervous system, or *other chemical messengers* such as odorants, they relay signals down to the cell interior and operate via

second messengers such as cyclic AMP. 6) Some receptors act as enzyme. When hormones such as insulin bind to the receptor, it activates receptor's protein kinase protrusion inside the cell which phosphorylates insulin response substrates, triggering a cascade of chemical responses inside the cell.

Question 9-2 There are some liposomes which contain 1% Na^+Cl^- aqueous solution in the cell and only have water channels in the membrane. If we immerse them in the 0.5% Na^+Cl^- aqueous solution in a large container, what will happen? Calculate ΔG of dilution for Na^+ at 25℃.

Answer: Concentration of Na^+ in the liposome is 1%. Concentration of Na^+ in the container is 0.5%.

From Eq. 7.3, $\Delta G_{dil} = \Delta H_{dil} - T\Delta S_{dil} = -T\Delta S_{dil} = -RTln\frac{[\text{initial concentration}]}{[\text{final concentration}]}$

$$= -RT\ ln\frac{1}{0.5} = -8.315\ \text{J}\cdot\ \text{K}^{-1}\cdot\text{mol}^{-1}\cdot 298\ \text{K}\ \cdot 0.693 = -1.72\ \text{kJ}\cdot\text{mol}^{-1}.$$

Water will flow into the cell and the cell swells until the concentration of Na^+ inside equals 0.5%. Some cells may expand and burst. Water moves across a semipermeable membrane from the region of lower solute concentration to the region of higher solute concentration until the solute concentration becomes equal on both sides.

9.4 Discovery of Aquaporins

In 1991 when Peter Agre, a hematologist and his assistants were studying to produce the antibody in rabbits to *the denatured partially purified Rh polypeptide*, they accidentally discovered the peptide, which was later identified as aquaporin. But instead the antibody produced did not react with the core Rh polypeptide with 32 kDa, it reacted only with a 28 kDa polypeptide contaminated in their Rh preparations. A Dalton (Da), atomic mass unit is defined as 1/12 of the atomic weight of a ^{12}C. They found this 28 kDa

polypeptide was abundant in the membrane of red cells and kidney, and was a tetrameric membrane-spanning protein suggesting that it is a channel protein. But what might be its function? His clinical mentor, J. C. Parker suggested that red cells and renal tubules were very permeable to water and recommended him to consider its role in membrane as water channel. The first experiment was carried out using the frog oocytes which were known to have very low water permeability. Control oocytes were subjected to be injected water. Test oocytes were injected 2 ng of mRNA encoding the 28 kDa protein which was prepared from the cloned cDNA from an erythroid library. After three days of the protein synthesis, the test oocytes acquired the 28 kDa protein in its membrane. Dr. Agre and his collaborator put both control and test oocytes in *distilled water*. The results were that the control oocytes failed to swell, while the test oocytes expanded. To confirm the function of this protein, libosomes were reconstituted into synthetic libosome vesicles with the purified protein called aquaporin and examined by the electron microscope. Membrane surface of the untreated libosomes were smooth. However, the membranes reconstituted with the protein contained intramembranous particles. The control untreated liposomes shrank in the hypertonic buffer in one half seconds. However, the liposomes synthesized with the aquaporins shrank rapidly in about 20 milliseconds in the same solution. They also attempted to check acid permeation, but it was not detected.

Question 9-3 1) Why they chose frog oocytes for this experiment? Once they made the antibody to the 28 kDa protein, the examined tissues was treated with the labeled antibody to look for the protein. 2) Other than red blood cell, which tissue showed large amount of the labeled antibody?

Answer: 1) The frog oocytes were known to have very low water permeability. 2) Kidney

9.5 Osmotic Pressure

We can make up liposomes with the aquaporins artificially so that water can only pass through the membrane. When the cell containing NaCl is put in water, the pressure inside the cell becomes higher than outside due to osmosis. We will calculate the osmotic pressure below.

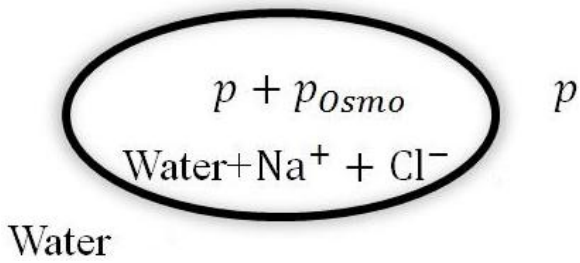

Figure 9.5 *Osmotic pressure in the cell.*

At equilibrium: $\Delta G = 0$

$$G_{sol}(p + p_{Osmo}, T) - G_{water}(p, T) = 0 \tag{9.1}$$

For dilution of *one mole of water* from Eq.7.3,

$$\Delta G_{dil} = G_{sol}(p + p_{Osmo}, T) - G_{water}(p + p_{Osmo}, T)$$

$$= -RT\ ln\ \frac{initial\ concentration}{final\ concentration} = RT\ ln\ X_{water}$$

$$X_{water} = \frac{\text{The number of moles of water}}{\text{The number of moles of water and NaCl}}$$

$$G_{sol}(p + p_{Osmo}, T) = G_{water}(p + p_{Osmo}, T) + RT\ ln\ X_{water} \tag{9.2}$$

Substituting"$G_{water}(p + p_{Osmo}, T) + RT\ ln\ X_{water}$" for $G_{sol}(p + p_{Osmo}, T)$ in Eq.9.1,

$$G_{water}(p + p_{Osmo}, T) + RT\ lnX_{water} - G_{water}(p, T) = 0$$

$$\Delta G = G_{water}(p + p_{Osmo}, T) - G_{water}(p, T) = -RT\ lnX_{water} \tag{9.3}$$

At constant T, $dG = -SdT + Vdp = Vdp$ for reversible process from Example 1.6.

$$\Delta G = \int_p^{p+p_{osmo}} \bar{V}_{water}\, dp = \bar{V}_{water} p_{osmo} \tag{9.4}$$

where $\bar{V}_{water}$ is volume of water per one mole.

From Eq.9.3 and Eq.9.4, for one mole of water,

$$\bar{V}_{water} p_{osmo} = -RT ln\, X_{water}. \tag{9.5}$$

Using Talor's approximation,

$$ln\, X_{water} = ln(1 - X_{\mathrm{Na^+Cl^-}}) \approx -X_{\mathrm{Na^+Cl^-}} = \frac{-n_{\mathrm{Na^+Cl^-}}}{n_{water} + n_{\mathrm{Na^+Cl^-}}} \approx \frac{-n_{\mathrm{Na^+Cl^-}}}{n_{water}}.$$

where n_{water} is the number of moles of water and $n_{\mathrm{Na^+Cl^-}}$ is the number of moles of NaCl in the cell. Then Eq.9.5 becomes,

$$\bar{V}_{water} p_{osmo} = \frac{V_{water}}{n_{water}} p_{osmo} = -RT\, \frac{-n_{\mathrm{Na^+Cl^-}}}{n_{water}}.$$

$$V_{water} \approx V_{solution}$$

$$p_{osmo} V_{solution} = RT n_{\mathrm{Na^+Cl^-}} \tag{9.6}$$

where $V_{solution}$ is the volume of solution. It looks like the ideal gas law.

9.6 Primary Active Transport

Primary active transport is the process in which ions are moved across cell membranes against electrochemical gradients using energy supplied by ATP. Action of Na^+-K^+ pump is an important example of primary active transport. Actually it is the enzyme called Na^+-K^+ ATPase that pumps Na^+ out and K^+ into the cell respectively against the gradient with the use of ATP which gives off the terminal phosphate to the enzyme only when in the presence of Na^+ during the transport process. This phosphorylation was found

experimentally on a negatively charged Asp (Aspartic acid) residue of the enzyme as a highly reactive aspartyl phosphate intermediate. The key that the enzyme acts as the pump is the conformational change associated with the changes of Na^+ and K^+ affinity to their binding sites when the highly reactive phosphate intermediate releases the activated energy and relaxes to the low energy state. Concentration of Na^+ is higher outside the cell whereas concentration of K^+ is higher inside the cell. Na^+-K^+ pump drives three Na^+ out against the gradient while at the same time pumping two K^+ in from outside. This process is continuous and requires energy from ATP. The ATP-driven Na^+-K^+ pump works against electrochemical gradients. The concentration difference between the cell membrane for each ion and the electric potential between the cell membrane are considered as the outcome of the action of Na^+-K^+ pump, and the selective permeability of the membrane for each ion as explained later in 9.7. The permeability of the membrane in its resting state to Na^+ is very low. At first, 3 Na^+ inside the cell bind to the pump protein. Binding of Na^+ promotes phosphorylation of the enzyme by ATP. The formation of aspartyl phosphate intermediate of the enzyme causes the conformational change of the enzyme and the decreased Na^+ affinity to the binding site, expelling 3 Na^+ ions to the outside against the gradient. Extracellular 2 K^+ ions bind to the pump protein which now has an increased K^+ affinity. The K^+ binding triggers hydrolysis of the phosphate residue and a release of the phosphate from the pump. Then the pump protein returns to its original shape with increasing Na^+ but decreasing K^+ affinity to the binding sites. Two K^+ ions are able to leave the pump and enter the cell. The Na^+ binding sites are now ready to bind intracellular 3 Na^+ again.

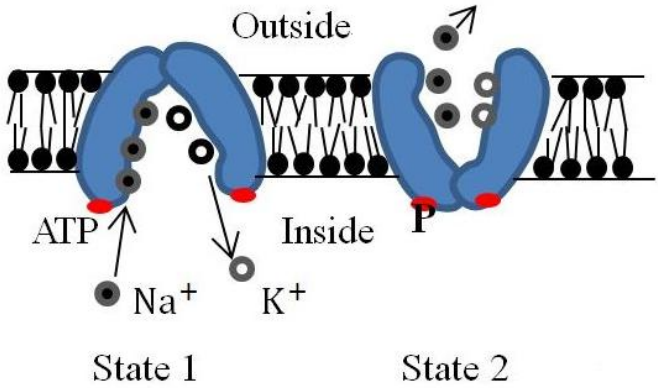

Figure 9.6 *Binding of* Na^+ *promotes phosphorylation of* Na^+-K^+ *ATPase by ATP and expels 3 sodium ions outside at the same time due to the conformational change associated with decreasing* Na^+ *affinity to the binding site.*

State 1 has an inward-facing high-affinity Na^+ binding site. The enzyme is not phosphorylated at State 1.

State 2 has an outward-facing high-affinity K^+ binding site. The enzyme is phosphorylated at State 2.

Binding of K^+ causes the enzyme to release the phosphate and return to its original shape, and expels 2 potassium ions inside at the same time.

9.7 Resting Membrane Potential

The action of the Na^+-K^+ pump and selective permeability of the membrane for ions create the concentration difference for each ion, and electrochemical potential both sides of the cell. The Na^+-K^+ is continuously pumping Na^+ out from inside of the cell, and Na^+ is hard to return since the permeability of the membrane in its resting state to Na^+ is very low. Thus it creates the concentration difference of Na^+, and then the potential between both sides of the cell. K^+ pumped in from outside is able to move through the membrane more easily because the membrane is somewhat permeable to K^+ in the

resting state, but the flowing out is restricted due to the opposing electrical potential. When the chemical and electrical gradients are equal in magnitude, the ion is said to be in electrochemical equilibrium, and the membrane potential that is established at equilibrium is called the *equilibrium potential* for that ion under the existing concentration gradient.

In human, extracellular fluid contains Na^+ 140 mM and K^+ 4.4 mM. Intracellular fluid contains Na^+ 15 mM and K^+ 140 mM.

Example 9-2 Initially $(emf)^\circ_{cell}$ was 0 due to no concentration gradients for Na^+ and K^+ ions through the membrane of the cell. $(emf)^\circ_{cell}$ was zero initially. Now we created the ion concentration gradient of Na^+ and K^+, respectively between the outside and inside of the cell. When equilibrium was obtained, we got the following concentrations of each ion due to the selective permeability and the electrical potential arose. The extracellular fluid contained $[Na^+]_{eq}$ = 140 mM and $[K^+]_{eq}$ = 4.4 mM. The intracellular fluid contained $[Na^+]_{eq}$ = 15 mM and $[K^+]_{eq}$ = 140 mM.

Calculate the equilibrium potentials at 37℃ under the following conditions. a) The membrane is 100% permeable to K^+ and 0% permeable to Na^+. b) The membrane is 0% permeable to K^+ and 100% permeable to Na^+.

$(emf)^\circ_{cell} = \frac{RT}{nF} ln\, K$ from Nernst equation 7.19.

a) At equilibrium, net outflow of K^+ is balanced by the opposing membrane potential.

$$(emf)^\circ_{cell} = \frac{RT}{nF} ln\, K = \frac{(8.315\ \mathrm{J \cdot K^{-1} \cdot mol^{-1}})(310\mathrm{K})}{96490\ \mathrm{C \cdot\ mol^{-1}}} ln \frac{[\mathrm{K^+}]_{out\ eq}}{[\mathrm{K^+}]_{in\ eq}}$$

$$= 0.0267\ ln \frac{4.4}{140} = 0.0267 \times (-3.46) = -92\ \mathrm{mV}$$

b) At equilibrium, net inflow of Na^+ is balanced by the opposing membrane potential.

$$(emf)^{\circ}_{cell} = \frac{RT}{nF} ln \frac{[Na^+]_{out\ eq}}{[Na^+]_{in\ eq}} = 0.0267 \times ln \frac{140}{15}$$

$$= 0.0267 \times 2.23 = 59.6 \text{ mV}$$

Question 9-4 If the extracellular concentration of Ca^{2+} is 10^5 times higher than the intracellular concentration what is $(emf)^{\circ}_{cell}$ caused by the Ca^{2+} gradient? T = 37℃.

Answer: $(emf)^{\circ}_{cell} = \frac{RT}{nF} ln\, K = \frac{(8.315\ \text{J}\cdot\text{K}^{-1}\cdot\text{mol}^{-1})(310\text{K})}{2\times 96490\ \text{C}\cdot\text{mol}^{-1}} ln \frac{[Ca^{2+}]_{out\ eq}}{[Ca^{2+}]_{in\ eq}}$

$$= 0.0134 \times 5 \times \ln 10 = 153\text{mV}.$$

9.8 Goldman Equation

To calculate the true membrane potential, we have to modify Nernst equation to add some terms for other permeant ions. Taking account of all ions which are not equally permeant, the membrane potential, $(emf)^{\circ}_{cell}$ is calculated based on the concentrations and permeabilities of all ions that move across the membrane. Goldman equation is a modified Nernst equation.

$$(emf)^{\circ}_{cell} = \frac{RT}{nF} ln\, K = \frac{RT}{nF} ln \frac{P_K[K^+]_{out\ eq} + P_{Na}[Na^+]_{out\ eq} + P_{Cl}[Cl^-]_{in\ eq}}{P_K[K^+]_{in\ eq} + P_{Na}[Na^+]_{in\ eq} + P_{Cl}[Cl^-]_{out\ eq}} \quad (9.7)$$

where P_K, P_{Na}, and P_{Cl} are potassium, sodium, and chloride permeability respectively.

Because Cl^- has a negative charge, $[Cl^-]_{in}$ is in the numerator and $[Cl^-]_{out}$ is in the denominator.

If the membrane is permeable only to K^+, then P_{Na} and P_{Cl} are both zero. Goldmann equation becomes Nernst equation for K^+.

It is useful to reformulate Goldman equation in terms of permeability of each ion relative to that of K^+, because K^+ is the most permeant ion.

$$(emf)^{\circ}_{cell} = \frac{RT}{nF} ln\, K = \frac{RT}{nF} ln \frac{[K^+]_{out\ eq} + P_{Na}/P_K\,[Na^+]_{out\ eq} + P_{Cl}/P_K\,[Cl^-]_{in\ eq}}{[K^+]_{in\ eq} + P_{Na}/P_K\,[Na^+]_{in\ eq} + P_{Cl}/P_K\,[Cl^-]_{out\ eq}} \quad (9.8)$$

where P_{Na}/P_K is permeability of Na^+ relative to that of K^+ and P_{Cl}/P_K is permeability of Cl^- relative to that of K^+.

Question 9-5 $P_K : P_{Na} : P_{Cl} = 1.00 : 0.04 : 0.45$. In human, extracellular fluid contains Na^+ 140 mM, K^+ 4.4 mM and Cl^- 105 mM, and intracellular fluid contains Na^+ 15 mM, K^+ 140 mM and Cl^- 7.0 mM at resting state. Calculate the resting membrane potential using Goldman equation. T = 37℃

Answer: $(emf)^{\circ}_{cell} = \frac{RT}{nF} ln \frac{[K^+]_{out\ eq} + P_{Na}/P_K\,[Na^+]_{out\ eq} + P_{Cl}/P_K\,[Cl^-]_{in\ eq}}{[K^+]_{in\ eq} + P_{Na}/P_K\,[Na^+]_{in\ eq} + P_{Cl}/P_K\,[Cl^-]_{out\ eq}}$

$$= 0.0267 \times ln \frac{4.4 + 0.04 \times 140 + 0.45 \times 7.0}{140 + 0.04 \times 15 + 0.45 \times 105}$$

$$= 0.0267 \times ln \frac{13.15}{187.9} = 0.0267 \times (-2.66) = -71.0\text{mV}$$

This is very close to the measured value, -70 mV.

9.9 Action Potential

The membrane potential at resting state is about – 70 mV in human. If there are *voltage gated* Na^+ and K^+ *channels* in the cell membrane, and the number of open state of each voltage gated channel increases as the potential is raised above a certain value, an increase in the permeability for each ion will generate new potential called an action potential. The *action potential* arises when the potential is raised above the threshold, -55 mV, which is a reversal of membrane potential with a total change of about 100 mV from – 70 mV to + 30 mV. If we accept the following assumption, a sharp curve of action potential is easily explainable. When the potential becomes over the

threshold, suddenly many voltage gated Na^+ channels open and its number of open state increases sharply until all of them open in one mms and then falls abruptly, whereas the number of open voltage gated K^+ channels increases gradually until all of them open in 1.5 mms with the time lag of 0.5 mms and falls gradually as shown in Fig. 9.7. At first a sharp increase in Na^+ inflow raises the potential steeply. And then when the Na^+ inflow stops, the time lagged increase in K^+ outflow causes the potential to move backward to the initial state.

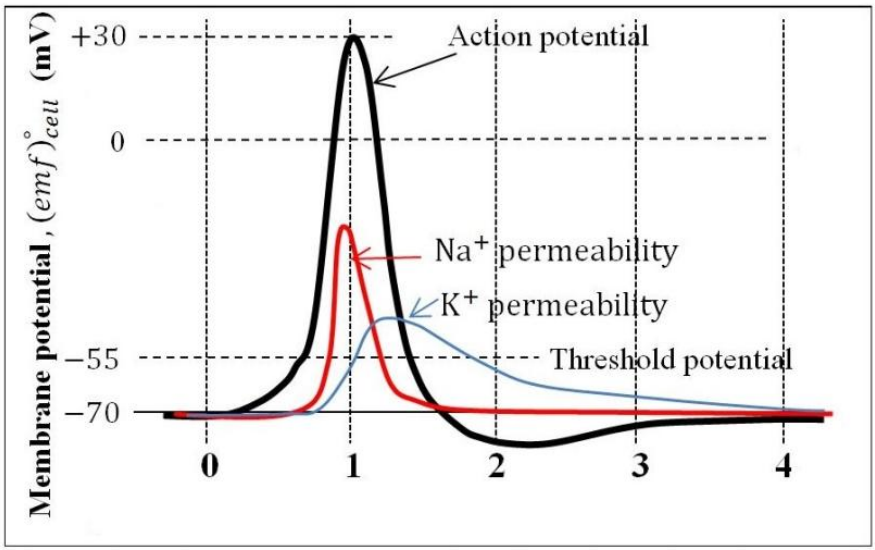

Figure 9.7 *A steep action potential arise with a sharp increase in number of opening of voltage gated* $\boldsymbol{Na^+}$ *channels in the cell membrane. The changes of* $\boldsymbol{Na^+}$ *and* $\boldsymbol{K^+}$ *permeability depend on their number of opening voltage gated channels.*

Depolarization is defined as that the membrane potential becomes less negative toward zero. The change in the membrane potential from -70 to -55 mV is depolarization. By convention, depolarization also includes the state in which the membrane potential moves above zero.

Repolarization is defined as movement of the membrane potential to the initial resting state.

Hyperpolarization is defined as that the membrane potential becomes more negative than the resting potential.

Question 9-6 Explain why repolarization occur?

Answer: When the potential is raised above the threshold, the number of open Na^+ channels rises sharply initially and then falls steeply, while that of open K^+ channels is increasing gradually and decreasing slowly as shown in Fig. 9.6. K^+ keeps flowing out while inflow of Na^+ drops sharply, which cause inside the cell less positive.

Example 9-3 Let's calculate the relative permeability of Na^+ to K^+ at the maximum membrane potential of + 30 mV.

We assume $P_K : P_{N_a} : P_{Cl} = 1.00 : x : 0.45$.

$$(emf)^{\circ}_{cell} = 0.030$$

$$= \frac{RT}{nF} ln \frac{[K^+]_{out\ eq} + P_{N_a}/P_K\,[Na^+]_{out\ eq} + P_{Cl}/P_K\,[Cl^-]_{in\ eq}}{[K^+]_{in\ eq} + P_{Na}/P_K\,[Na^+]_{in\ eq} + P_{Cl}/P_K\,[Cl^-]_{out\ eq}}.$$

$$0.030 = 0.0267 \times ln \frac{4.4 + 140x + 0.45 \times 7.0}{140 + 15x + 0.45 \times 105} = 0.0267 \times ln \frac{7.55 + 140x}{187.25 + 15x}$$

$$1.12 = ln \frac{7.55 + 140x}{187.25 + 15x}$$

$$e^{1.12} = 3.065 = \frac{7.55 + 140x}{187.25 + 15x}$$

$$574 + 46x = 7.55 + 140x \qquad x = 6.03$$

Relative permeability of Na^+ to K^+ at the maximum potential is 6.03. It was 0.04 at resting state. At the maximum potential, the relative permeability of Na^+ to K^+ becomes 151 times larger. The action potential is the outcome of

changes of the relative permeability in the cell membrane. The increasing number of open channels makes the membrane more permeable to those ions.

9.10 Graded Potential

Graded potentials are the localized potentials which can be either less negative or more negative compared to the resting potential. Once formed, they are decaying over distance. Gradient potentials vary in size which is proportional to the intensity of stimulus. A ligand-gated Na^+ ion channels open when acetylcholine (ACh) binds to the receptor on the ion channel. More ACh binding opens more channels for Na^+ to flow in due to conformational changes of the pore protein, which leads to greater depolarization with a resultant generation of action potential. A ligand-gated Cl^- ion channels open when GABA binds to the receptor on the channel. The binding of more GABA opens more channels for Cl^- to flow in, which causes hyperpolarization with a resultant inhibition of activity in the central nervous system. Increasing permeability of K^+ by opening a ligand-gated K^+ ion channels by GABA will also cause hyperpolarization.

Summary of Generation of Action Potential

The selective permeability of membrane causes osmosis and the membrane potential. In order to generate action potential, two conditions are required. The one is that there should be both voltage gated Na^+ and K^+ channels in the tissue cell membrane. The second is that the depolarization above threshold potential, – 55 mV must be obtained by stimulating more ligand-gated Na^+ ion channels simultaneously or stimulate them repeatedly in a short interval to get the additive potential.

9.11 Tissues with Voltage Gated Channels

9.11.1 Nerve Cell

Anatomically, a nerve cell (*neuron*) consists of dendrites, a cell body, an axon, and axon terminals. *Dendrites and a cell body* have ligand-gated ion channels which have a receptor for binding to a signaling chemical called a *neurotransmitter* (a ligand of nervous system). A neurotransmitter is a little molecule that binds specifically to the receptor. Once it binds, it allows the channel to open. Dendrites and a cell body are considered as a *receptive region* since dendrites receive excitatory, and a cell body receives inhibitory stimulus from outside respectively. *Axon* can generate action potentials since it has voltage gated Na^+ and K^+ channels. The site close to its cell body is *axon hillock* which is a trigger zone. Axon also conducts its action potential in the way that action potentials are renewed at each point along the axon. Axon terminals releases neurotransmitters, and are said to be *secretary region*.

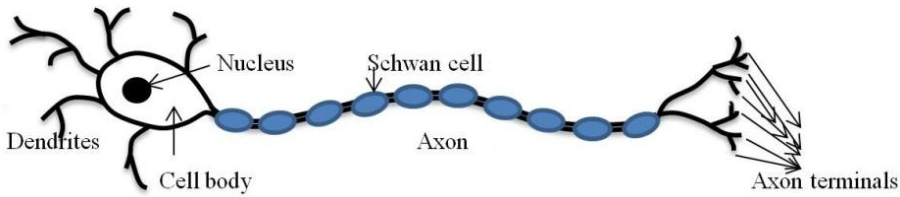

Figure 9.8 *A motor nerve cell innervate skeletal muscle. Its axon has Schwan cells which increase action potential propagation velocity by decreasing electric capacity of membrane.*

The capacitance is proportional to the dielectric constant, *K* that is a property of a non-conducting material referred to as dielectrics. *K* of air is nearly one. Since the cell membrane is made of a non-conducting material, the capacitance of an axon is expressed in terms of geometry of the membrane as follows.

Capacitance $= \epsilon \frac{A}{d}$ where $\epsilon = K\epsilon_0$ is the permittivity of the dielectric material and ϵ_0 is called the permittivity of free space. A is membrane surface

area, and d is width of membrane including a Schwan cell. The capacitance of an axon of a motor neuron is low due to the insulation by Schwan cells. If the capacitance decreases, the responsiveness increases.

Example 9-4 1) Calculate the capacitance of an axon of 1 cm long and the radius of 10 μm. Dielectric constant(K) of the membrane is set to be 3. 2) Estimate change of the concentration of sodium ions in the cell as a result of one action potential.

The membrane of an axon is modeled as a cylindrically shaped parallel-plate capacitor. The separation of the plates is the thickness of the membrane which is about 10^{-8}m.

1) The area of the cylinder of radius (r) and length (l) is

$$\text{A}= 2\pi rl \approx 6.28 \times (10^{-5}\text{m}) \times (0.01\ \text{m}) \approx 6.2 \times 10^{-7}\text{m}^2.$$

$$\text{Capacitance} = K\epsilon_0 \frac{A}{d} \approx 3 \times \left(8.85 \times \frac{10^{-12}\text{C}^2}{\text{N}} \cdot \text{m}^2\right) \frac{6.2 \times 10^{-7}\text{m}^2}{10^{-8}\text{m}}$$

$$\approx 1.6 \times 10^{-9}\text{F}$$

2) Since the voltage changes from -70 to +30 mV, the total change is about 100 mV. The amount of charge that moves in is

$$Q = CV \approx (1.6 \times 10^{-9}\text{F})(0.1\ \text{V}) = 1.6 \times 10^{-10}\ \text{C}.$$

Each sodium ion carries 1.6×10^{-19} C.

The number of Na^+ that flow across the membrane is

$$Q/e \approx 1.6 \times 10^{-10}\ \text{C}/1.6 \times 10^{-19}\ \text{C} = 10^9.$$

The volume of the cylindrical axon is

$$\text{Volume} = \pi r^2 l = 3.14 \times (10^{-5})^2 \times 0.01 = 3.14 \times 10^{-12}\ \text{m}^3$$

The number of Sodium ions contained in one m^3 of the intracellular fluid in its resting state is

$$[Na^+]_{eq} = 15 \text{ mM} = 15\times 10^{-3}\frac{\text{mol}}{\text{L}} = 15\frac{\text{mol}}{\text{m}^3}$$
$$= 15 \times 6.02 \times 10^{23} \text{ ions/m}^3 \approx 90 \times 10^{23} \text{ ions/m}^3.$$

The cylindrical axon contains $90 \times 10^{23} \times 3.14 \times 10^{-12} = 2.83 \times 10^{13}$ sodium ions.

About 10^9 sodium ions flow in across the membrane per one action potential passing, which will not change the concentration inside of the axon significantly.

9.11.2 Muscle Fibers and Receptors

Skeletal muscle fiber has striated appearance. Muscle fibers contain many myofibrils. The nerve cells that activate skeletal muscle fibers are called somatic *motor neurons* or motor neurons of voluntary nervous system. The axon of each motor neuron divides as it enters the muscle, and each axon ending gives off several short branches called axon terminals that collectively form a *neuromuscular junction*. Each skeletal muscle fiber has only one neuromuscular junction. A motor neuron has its secretary regions at axon terminals. Stimulation of a motor nerve results in the release of acetylcholine (ACh) from the muscle in Ringer's solution as shown Fig. 9.8. Adding of ACh in the same solution also produces muscular contraction similar to that elicited by stimulation of motor nerve. The experiments suggested us that at the neuromuscular junctions ACh might be secreted from axon terminals. The muscle fiber of sarcolemma (membrane) at the junctions will have millions of ACh receptors. The binding of released ACh to receptors on the muscle fiber membrane results in a depolarization of the membrane which will induce the

muscular contraction. Poor absorption and low lipophilicity are due to a positive charge on quaternary ammonium.

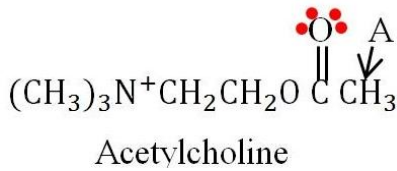

Figure 9.9 *Poor absorption and low lipophilicity are due to a positive charge on quaternary ammonium. ACh is attracted by the catalytic anionic site of acetylcholinesterase.*

However experiments showed the effects of the stimulation were quickly disappeared, which suggested that the secreted ACh should be breakdown quickly.

The receptors are considered as ionotropic (ligand-gated ion channel) receptors. When ACh is attracted due to its positive charge and binds site A to the ionotropic receptor, it will induce the conformational change of the channel protein by electrostatic force between the channel protein and a positively charged ACh molecule. With the resultant increase in permeability of sodium ions, the inflow of sodium ions consequently causes a depolarization of the resting membrane potential. The receptor is called a nicotinic acetylcholine receptor since these receptors are activated by nicotine. The membrane except the area at a neuromuscular junction has voltage gated Na^+ and K^+ channels. Once action potential is generated at the membrane area close to the junction, the transmission of action potential will occur along the cell membrane of the muscle fiber. And later it was found that action potential down the T shaped tubules causes the voltage-sensitive tubule proteins to change shape. This shape change opens the Ca^{2+} release channels of the sarcoplasmic reticulum (specialized endoplasmic reticulum of muscle cells) allowing massive amounts

of Ca^{2+} to flow into the cytosol (fluid portion of the cytoplasm) and contraction of myofibrils arises.

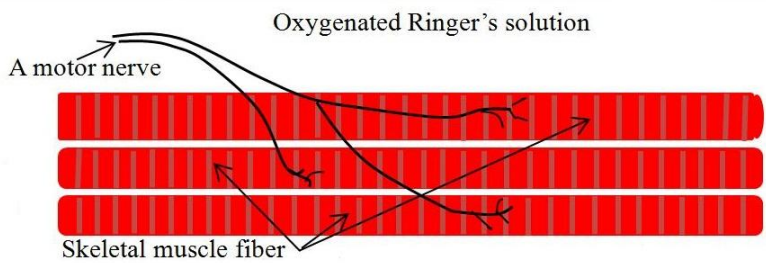

Figure 9.10 *Skeletal muscle innervated by a motor nerve in a glass container.*

We make a preparation of a motor nerve terminated on a skeletal muscle in oxygenated Ringer's solution (an aqueous solution of calcium chloride, sodium chloride, and potassium chloride that isotonic to the tissues). In order to contract the muscle, action potential has to be generated in the each muscle fiber. There are number of ways to generate action potential in muscle fibers.

1) Give small injections of depolarizing current in the axon of a motor neuron. Since there are voltage gated channels in the axon membrane, the stimuli will cause an action potential. When the action potential reach the terminal, ACh will be released.

2) Drop ACh in the Ringer's solution

3) Give small injections of depolarizing current in some muscle fibers.

Cardiac Muscle has striated appearance. Muscle fibers contain many myofibrils, and contract by the sliding filament mechanism like skeletal muscle. In contrast to skeletal muscle, cardiac muscle fibers are shorter and interconnected. Cardiac muscle has innervation of post ganglionic parasympathetic and sympathetic nerves. The membranes of adjacent cardiac muscle fibers interlock each other. Thus, a heart muscle fiber does not need to have an individual innervation. Sinoatrial (SA) node, atrioventricular (AV) node,

and the His-Purkinje system generate action potential intrinsically and cause rhythmic contractions of heart muscle. Nerve impulses cannot initiate contraction of cardiac muscle. Instead, they can modulate the intrinsic contraction generated by SA node.

If you electrically stimulate the vagus nerve which is parasympathetic nerve, the heart slows down beating. The experiment that Dr. Loewe did in 1921 was to connect two frog hearts to the same glass container of oxygenated Ringer's solution. The solution perfusing the first heart was applied to the second heart. When he stimulated the nerve to frog heart A, it reduced beating, but a little while later heart B also reduced beating. The experiment suggested that there was a diffusible substance moving from heart A to heart B. The diffusible substance reduced heart A and heart B beating was called the vagus stuff. We now know that the vagus stuff is ACh. Adding of ACh in the same solution also reduces heart beating. It acts on a particular type of acetylcholine receptor called a muscarinic acetylcholine receptor which is entirely different from the nicotinic acetylcholine receptors. These muscarinic acetylcholine receptors are activated by the mushroom toxin, muscarine.

If ACh acted on ligand-gated ion channel receptor, depolarization will occur which would generate action potential leading to Ca^{+} outflow from sarcoplasmic reticulum with a resultant contraction of the cardiac muscle. However Ach induces a negative inotropic effect on hearts that is a decrease in the force of muscular contractions. When T. Watanabe was a student in 1971, he did many experiments to explain the ACh effects on rat heart measuring the ratio of inactivated form of *phosphorylase* compared to the active form. Experimental data suggested a decrease in second messenger, cyclic AMP. Unfortunately, he could not give enough evidence to explain negative inotropic effects and his paper was not accepted. Today we know these muscarinic

acetylcholine receptors are metabolic (G protein-coupled) receptors. The basic functions of muscarinic cholinergic receptors are mediated by interactions with G proteins. Stimulation of M_2 cholinergic receptors leads to interaction with the certain G proteins (G_i and G_0) with a resulting inhibition of *adenylyl cyclase* which leads to a decrease in *cyclic AMP* followed by its activation of K^+ channels, and inhibition of voltage-gated Ca^{2+} channels. These effects result in hyperpolarization and inhibition of excitable membranes in heart. We will consider its detail in 9.12.

Smooth muscle is spindle-shaped cells with one centrally located nucleus and no externally visible striations. It is found mainly in the walls of hollow organs. Smooth muscle has innervations of post ganglionic parasympathetic and sympathetic nerves. Like cardiac muscle, Ach secreted from post ganglionic parasympathetic nerve terminal binds to the muscarinic cholinergic receptors which are metabolic G protein-coupled receptors. Its functions are mediated by interactions with G proteins. M_3 cholinergic receptors are abundant in smooth muscle and glands. G_{11} and G_{13} proteins are responsible for stimulation of phospholipase C activity. Hydrolysis of one of membrane phospholipids, phosphatidylinositol produces inositol triphosphate. Inositol triphosphate causes release of intracellular Ca^{2+} from the endoplasmic reticulum of smooth muscle fiber with a resultant contraction of smooth muscle.

9.12 Intracellular Messenger, Cyclic AMP

Adenylyl cyclase is activated by Gs protein. And the enzyme converts ATP to cyclic AMP. Adenosine Triphosphate becomes cyclic Adenosine Monophosphate due to transferring of a high-energy phosphate bond to a hydroxyl group on the sugar molecule. Cyclic AMP is a key intracellular messenger.

Negatively charged cyclic AMP binds to a kinase and activates it by changing its conformation of the kinase. A typical kinase transfers a high energy phosphate bond from ATP to a serine side chain on a protein. Serine contains a hydroxyl group on the side chain and so the hydrogen of a hydroxyl group is replaced by a phosphate group. This phosphate group has a negative charge and it can change conformation of a protein by the coulombic force. A kinase phosphorylates a protein and a phosphatase removes the phosphate group from the phosphorylated protein and brings the protein back to the original hydroxyl form.

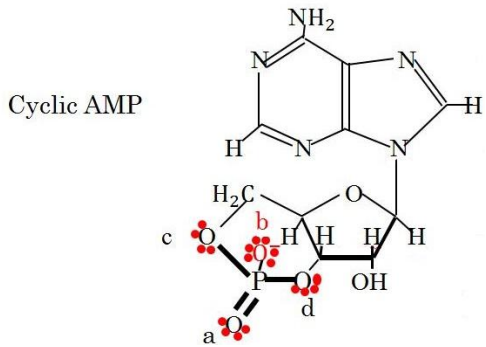

Figure 9.11 *Cyclic AMP is negatively charged and has lone pairs on oxygen atoms.*

Sigma bond around P: O_a- P: $\sigma(O_a\ 2sp^2, P\ 3sp^3)$,

P- O_b: $\sigma(P\ 3sp^3, O_b\ 2sp^2)$, P- O_c: $\sigma(P\ 3sp^3, O_c\ 2sp^3)$,

P- O_d: $\sigma(P\ 3sp^3, O_d\ 2sp^3)$.

Geometry around P: tetrahedral 109.5°. Geometry of around O_c, O_d: bent 104.5°<109.5°.

The phosphorylated protein in this case is an ion channel, i.e. calcium activated potassium channel. It repolarizes and stops firing normally if it is not phophorylated. If the calcium activated K^+ channel is phosphorylated by the kinase, it is partially inhibited as a result of its conformational change. If cyclic AMP increases, the K^+ channels lose its function partially and K^+

permeability decreases. A decrease in K^+ permeability causes cardiac muscle more excitable.

Stimulation of M_2 cholinergic receptors by Ach leads to interaction with G_i proteins with a resulting inhibition of adenylyl cyclase which leads to a decrease in cyclic AMP followed by activation of the K^+ channels. An increase in K^+ permeability results in repolarization of the excitable membranes. These effects are most clear in myocardium, where a decrease in cyclic AMP and its activation of the K^+ channels explain negative inotropic effects of Ach on heart.

Question 9-7 Norepinephrine(NE) and Epinephrine(EPI) are neurotransmitters of post ganglionic sympathomimetic nerve. EPI(80%) and NE(20%) are circulating hormones secreted from adrenal medulla. Stimulation of β adrenergic receptors by EPI or NE leads to interaction with Gs proteins with a resulting *activation of adenylyl cyclase*. Decrease in K^+ permeability cause cardiac muscle more excitable. What do you expect the effects on heart function if you inject a small quantity of EPI or NE in the circulating blood to heart?

Answer: Because EPI has a potent stimulant of β adrenergic receptors. NE has poor stimulant of β adrenergic receptors. EPI increases heart rate, stroke volume, cardiac output, arrhythmias, and coronary blood flow. NE increases stroke volume, arrhythmias, and coronary blood flow, but not heart rate and stroke volume.

9.13 Inhibition of Acetylcholinesterase

Acetylcholine is synthesized from acetyl-CoA and choline by choline acetyltransferase. Acetylcholine(ACh) is secreted from axon terminals of a cholinergic nerve. In the peripheral nervous system after ACh binds to the ionotropic nicotinic receptors of skeletal muscle to induce contraction and adrenal

medulla to secrete EPI, or metabolic muscarinic receptors of cardiac muscle to reduce inotropic action and smooth muscle to induce the muscle contraction, its effects should be quickly ended by the enzymatic breakdown within the time limits before the next nervous impulse is coming. Its enzyme that clears ACh from site of action is called acetylcholinesterase(AChE) which is located in the synaptic clefts. The destruction of ACh to acetic acid and choline by the enzyme prevents *continued skeletal muscle fiber contraction* in the absence of additional nervous stimulation, and *prolonged negative inotropic effects on heart muscle*, and *continued contraction of smooth muscle* of trachea, sphincter and ciliary muscles of eye, stomach and intestinal muscles, and urinary bladder.

Inhibition of AChE prevents the removal of ACh from the synaptic clefts which will cause continuous skeletal and smooth muscle contraction and prolonged negative inotropic effects on hearts.

When Ach is attracted by the catalytic anionic site of AChE, it binds and forms a tetrahedral intermediate followed by formation of the acetyl enzyme with liberation of choline, and rapid hydrolysis of the acetyl enzyme with return to the original state.

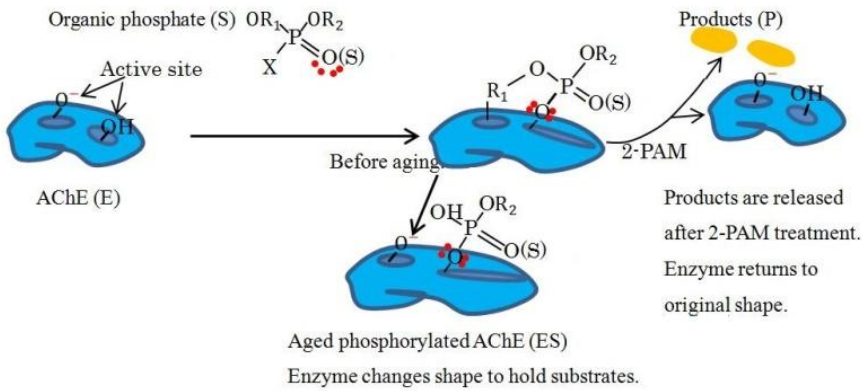

Figure 9.12 *Inhibition of AChE by organophosphates and treatment by 2-PAM. AChE has two active sites. Enzyme-substrate complex(ES) is the phosphorylated enzyme intermediate. Sarin is extremely toxic because it has F as X.*

We know some inhibitors of AChE such as edrophonium classified as quaternary alcohols, neostigmine as carbamate esters, parathion and sarin as organophosphates. They react with AChE, and bind more tightly to the enzyme than ACh. They form the enzyme intermediate, and sit active site of the enzyme, and prevent Ach from binding and breaking down. Therefore they can serve for as competitive inhibitors.

AchE reversible inhibors: Edrophonium and neostigmine.

Neostigmine reacts with AChE and forms the dimethyl carbamylated enzyme intermediate. Slow hydrolysis of the intermediate prevents ACh from binding. Its half-life is 1-6 hrs.

AchE irreversible inhibors: Organophosphates such as isoflurophate, parathion, malathion, and sarin.

Parathion had used as an insectcide, but now replaced by more safer malathion. Sarin is extremely toxic nerve gas because it has F as X and O instead of S. Hydrolysis of the phosphorylated enzyme intermediate is very slow. It goes aging. The aged phosphorylated intermediate is resistant to hydrolysis and reactivation. Pralidoxime (2-PAM) is used for the treatment of organophosphate poisoning by regenerating free enzyme if it is given before aging.

Question 9-8 An adult of crop duster calls because he has lost control over his chronic twitch and he has now problems with blurry vision and control of his bowels and bladder. What treatment is suggested?

Answer: His problems with blurry vision and control of his bowels and bladder seem to be the prolonged effects of ACh. Ask the patient if he is using the insecticide such as organophosphates. The measurement of the activity of AChE in serum will be carried out for the diagnosis. The treatment of organophosphate poisoning is an injection of atropine as a blockade of muscarinic receptors and 2-PAM to regenerate free enzyme of AChE.

9.14 Role of Adenosine Triphosphate (ATP) in Cell Metabolism

Glucose is the most important energy source for the body cells. Energy released during glucose catabolism is coupled to the synthesis of adenosine triphosphate. ATP provides a form of energy that is immediately usable by all body cells. ATP has three phosphate groups, and each phosphate group has a negative charge and repels each other. So ATP is very unstable reactive molecule with many nonbonding electrons, but has two high-energy phosphate bonds which are necessary to overcome the repulsion as shown in Fig. 9.12. ATP releases energy when it loses a phosphate group. Giving off its terminal phosphate group with high bond energy to other molecule, ATP becomes more stable molecule called adenosine diphosphate (ADP). On the other hand a molecule obtains that high bond energy when the phosphate released from ATP by hydrolysis is attached to the molecule. This process is called a phosphorylation as seen in Na^+-K^+ pump in section 9.6. In this way *ATP transfers energy when the cellular work needs energy*.

Examples of the cellular work driven by energy from ATP are as follows.

ATP phosphorylates some transmembrane proteins, and the activated transporter can transport solutes across cell membrane against electrochemical potential.

ATP phosphorylates and activates contractile proteins called myosin in smooth muscle cells so that the cell can shorten.

ATP phosphorylates key reactants, and proceeds chemical reactions by providing energy.

Highly reactive ATP is known as one of units of RNA, and deoxy-ATP shown in Fig. 9.14 is known as one of units of DNA.

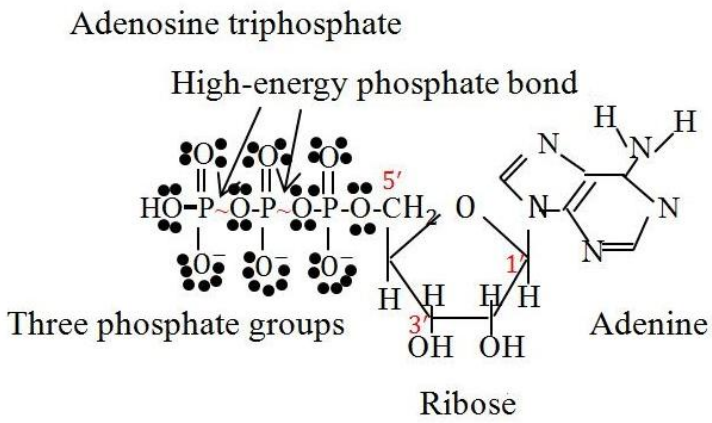

Figure 9.13 *ATP has three negatively charged phosphate groups and very unstable due to repulsive forces among the groups. It becomes stable when giving off the terminal phosphate group to other molecule.*

9.15 Nucleic Acids (DNA and RNA)

The nucleic acids are composed of carbon, oxygen, hydrogen, nitrogen, and phosphorus. The nucleic acids include two classes of molecules, *deoxyribonucleic acid* (DNA) and *ribonucleic acid* (RNA). They are the largest molecules in the body. The structural units of nucleic acids are called nucleotides. Each nucleotide consists of three components which are a nitrogen containing base, a pentose sugar, and three negatively charged phosphate groups. The bases are adenine (A), guanine (G), cytosine (C), thymine (T), and uracil (U). Adenine and guanine are two ring bases called purines, whereas cytosine, thymine, and uracil are single ring bases called pyrimidines. DNA and RNA are both composed of nucleotides, but they differ in many respects. *DNA* is found in the nucleus of the cell. It constitutes the genes. It replicates before a cell divides, which means the genetic information in the descendant cells is identical. The genetic code on DNA provides the basic instructions for building proteins in the body. A technique called DNA fingerprinting analyzes tiny samples of DNA taken from blood, or other body tissues, and shows the results as a genetic barcode that distinguishes each of us from others. DNA is a long,

double-stranded polymer of nucleotides. The bases in DNA are A, G, C, and T, and its pentose sugar is 2′-deoxyribose. How these nucleotides are combined? They are polymerized by the DNA polymerase. The bond is called a phosphodiester bond between the 3′-hydroxyl group of a deoxyribose and the 5′-phosphate. When the bond forms, the two phosphates leave. A new nucleotide is always added to the terminal of growing strand at the free 3′- hydroxyl group of the deoxyribose.

Alternating sugar and phosphate components of each chain form the backbones of the ladder shaped DNA. Its two nucleotide chains are held together horizontally by hydrogen bonds between bases. The purine A pairs with the pyrimidine T. The purine G pairs with the pyrimidine C. The whole molecule is coiled into a spiral staircase-like structure called a *double helix*. The two chains of nucleotides run in opposite directions.

Rosalind Franklin's work on the *X-ray diffraction images of DNA* led to the model of the *DNA double helix* by Francis Crick and James Watson in 1953. According to Francis Crick, her data and research were keys in determining the structure. James Watson affirmed this opinion later.

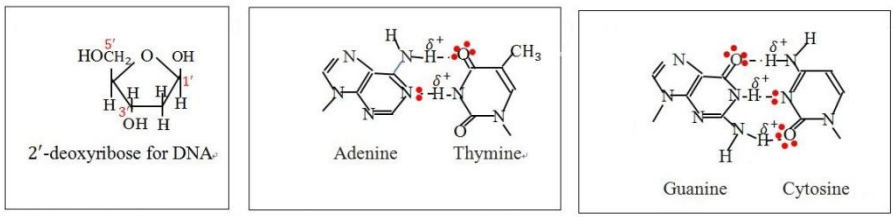

Figure 9.14 *Components of a nucleotide, a pentose sugar and nitrogen containing bases.*

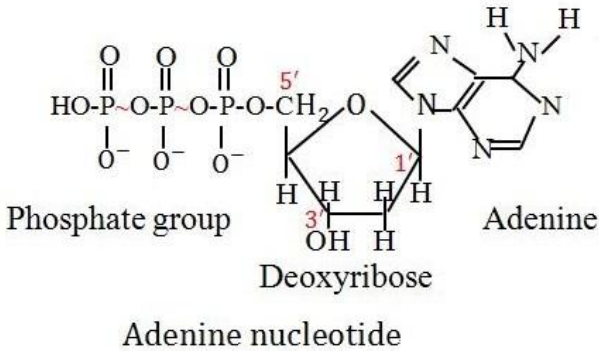

Figure 9.15 *This adenine nucleotide is deoxyadenosine triphosphate (dATP) which has a three negatively charged phosphate groups and a free OH group at* **3′** *of deoxyribose, and a very unstable molecule.*

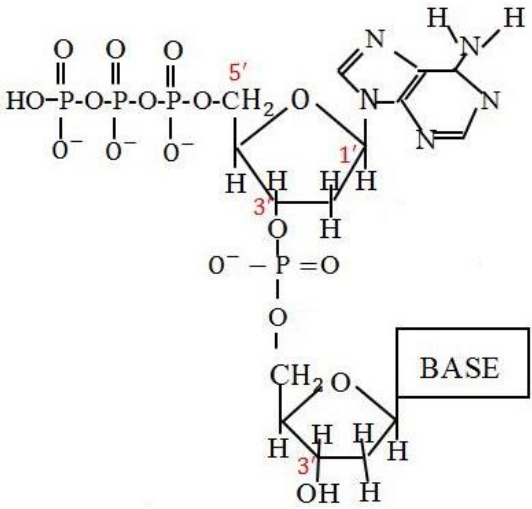

Figure 9.16 *Nucleotides are held together by a strong phosphodiester bond using its own high energy each nucleotide brings. When the bond forms, two phosphate groups leave. New nucleotide is always added to the growing strand at the free* **3′***- hydroxyl group of the deoxyribose by the polymerase.*

RNA is located chiefly in the cytoplasm outside the nucleus. A gene is defined as a segment of a DNA molecule that carries instructions for one protein. *Messenger RNA* (mRNA) carries out the code orders for the particular protein synthesis from the gene in the nucleus into the cytoplasm. Each code is three

bases called a triplet which specifies a particular amino acid. The complementary base sequence on mRNA is read and translated into proteins by *ribosomal RNA* in ribosomes. RNA molecules are single strand of nucleotides which permits the formation of diverse structures that is straight or folded. RNA bases include A, G, C, and U in place of T, and its suger is ribose. Uracil (U) has no methyl group that thymine (T) has. *MicroRNA* molecules present in the nucleus control genetic expression by shutting down genes or altering the expression.

Question 9-9 Describe structural differences between DNA and RNA.

9.16 Semiconservative Replication of DNA

In order to pass the identical copies of the cell genes on to each of offspring, its DNA should be replicated exactly before a cell divedes. To start the replication, the DNA helices should be unwinded from the histons of the nucleasomes. Each polynucleotide strand serves as a template for polymerizing free nucleotides dissolved in the nucleoplasm to synthesize a new complementary strand by DNA polymerase. Since each new DNA molecule consists of one old and one new polynucleotide strand, the DNA replication is called semiconservative replication. In 1957 Matthew Meselson and Franklin W. Stahl confirmed this replication in the experiment on the distribution of parental atoms among progeny DNA molecules using nitrogen isotopic labels. They first cultured bacterial *E. coli* cells to grow and divide in the presence of ^{15}N in a medium. After many rounds of cell division, they transferred the cells to a medium containing ^{14}N. After allowing the cells to grow and complete two rounds of replication and division, they separated the newly formed DNA from the parental DNA by means of density-gradient centrifugation. The lysed cells containing ^{14}N and ^{15}N were centrifuged at 44770 rpm for 24 hrs in CsCl solution, and the DNA molecules present were driven by the centrifugal force into the region where the cesium chloride solution density is equal to their own

buoyant density. The banding of DNA is detected by ultraviolet absorption photographs. After one round replication and division, they found that all DNA molecules had incorporated both ^{14}N and ^{15}N. After two rounds of replication is completed, they found half DNA molecules contained only ^{14}N and half DNA molecules contained both ^{14}N and ^{15}N.

Once the template DNA gets a primer to start, DNA polymerase adds a nucleotide to 3'OH of the end of a primer and synthesizes the new strand in the 5' to 3' direction. The template DNA is only read in the 3' to 5' direction. In 1967, Okazaki and their colleagues suggested the discontinuous replication in the bottom strand shown in Figure 9.16.

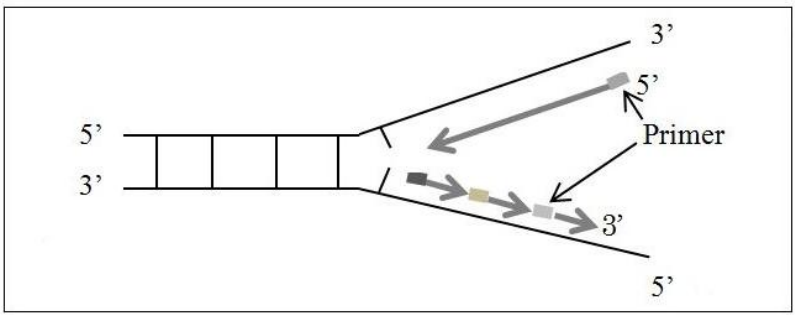

Figure 9.17 *Okazaki fragments of the bottom strand are tied up by the enzyme called a ligase.*

9.17 Protein Synthesis in the Living Cells

Proteins are made up of amino acids. A gene is defined as a segment of a DNA molecule that carries instructions for one peptide chain. There are four kinds of bases, A, G, T, and C on DNA. Each sequence of three bases specifies a particular amino acid. AAA is for phenylalanine, AGA for serine, and TAC for Methionine. These rules are called the genetic code. Protein synthesis involves three steps. One step is transcription in which the gene on DNA is encoded in *mRNA*. *Three-base sequence* on mRNA is called a *codon*. The

second step is RNA processing that removes introns from mRNA. *Introns* are noncoding segments that range 60 to 100,000 nucleotides. The third step is translation in which the information carried by mRNA is decoded by the ribosome to assemble proteins.

9.17.1 Transcription

The promoter is a special DNA sequence that specifies where RNA synthesis starts and which DNA strand serves as the template strand. The *prokaryotic promoter* has the sequence which is recognized by RNA polymerase. RNA polymerase binds to the promoter, and starts transcription. In eukaryotes gen-activating chemical called the *transcription factors* help position RNA polymerase at the promoter. Without the transcription factors RNA polymerase cannot bind to the promoter. Transcription involves the transfer of information of a DNA's base sequence to the complementary base sequence of a RNA molecule. RNA polymerase reads a DNA template at start codon and synthesizes a complementary strand of RNA in 5' to 3' direction. Genetic information is now encoded in the RNA by means of a specific sequence of three consecutive nucleotides (codon) that specifies a particular amino acid in a protein or starts or stops protein synthesis. *The start codon on RNA is AUG and stop codon on RNA is either UAA or UAG or UGA.* Each codon is complementary to the corresponding triplet in the DNA from which it was transcribed. When the polymerase reaches a special base sequence called a terminate signal, transcription ends. Eukaryotic genes contain noncoding base sequences called *introns*. The coding regions are called *exons*. After transcription the newly formed pre-mRNA is detached, its introns are removed, exons are joined in a process called *splicing*, and bases are added at both ends. Then it leaves the nucleus via a nuclear pore. Near the 3' end of *pre-mRNA* and after the stop codon there is the sequence AAUAAA called terminator corresponding terminate signal. This sequence signals for an enzyme to cut the

pre-mRNA, and after this cutting, 3' *poly-A tail* (a 100 to 300 sequence of adenine nucleotides) is added to 3' end of the pre-mRNA. This tail is considered to be important because it helps mRNA leave the nucleus via a nuclear pore, and protects the mRNA from degradation. On the other hand, in prokaryotes genes contain no introns, and so no splicing is needed. Since there is no nuclear envelope, the translation of mRNA begins in the ribosomes near the chromosome without processing of the RNA.

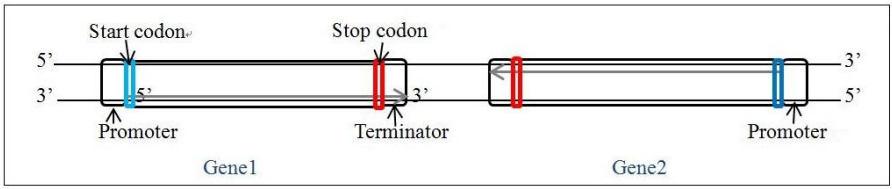

Figure 9.18 *The promoter specifies where mRNA synthesis starts and which DNA strand serves as the template strand. To synthesize RNA in the 5' to 3' direction going from left to right, RNA polymerase reads the bottom strand of DNA in the 3' to 5' direction. To synthesize RNA in the 5' to 3' direction going from right to left, RNA polymerase reads the top strand in the 3' to 5' direction.*

9.17.2 Translation

Ribosomes are made up of proteins and ribosomal RNA. The ribosome reads the messenger RNA in the 5' to 3' direction and translates into the particular protein. When the ribosome reads the three-base sequence on mRNA, it calls for the amino acid carried by the corresponding transfer RNA. Each transfer RNA brings each specified amino acid dissolved in the cytosol to a large ribosomal subunit. At the large subunit of the ribosome, ribosomal RNA acts as a *ribozyme*, catalyzing peptide bond formation of these amino acids. The ribosome starts translation at the first AUG and terminates at UAA, UAG, or UGA. UCC codes for serine, and UUU codes for phenylalanine.

Table 9.1 *Genetic information is encoded in mRNA by means of a specific sequence of three consecutive nucleotides (codon) that specifies a particular amino acid in a protein or starts or stops protein synthesis. For example, if the codon on mRNA is AUG, AUG codes for methionine and UAA codes for stop. Alanine (Ala), Arginine (Arg), Asparagine (Asn), Aspartic acid (Asp), Cysteine (Cys), Glutamic acid (Glu), Glutamine (Gln), Glycine (Gly), Histidine (His), Isoleucine (Ile), Leucine (Leu), Lysine (Lys), Methionine (Met), Phenylalanine (Phe), Proline (Pro), Serine (Ser), Threonine (Thr), Tryptophan (Trp), Tyrosine (Tyr), Valine (Val).*

First Base	Second Base								Third Base
	U		**C**		**A**		**G**		
U	UUU	Phe	UCU	Ser	UAU	Tyr	UGU	Cys	**U**
	UUC	Phe	UCC	Ser	UAC	Tyr	UGC	Cys	**C**
	UUA	Leu	UCA	Ser	UAA	Stop	UGA	Stop	**A**
	UUG	Leu	UCG	Ser	UAG	Stop	UGG	Trp	**G**
C	CUU	Leu	CCU	Pro	CAU	His	CGU	Arg	**U**
	CUC	Leu	CCC	Pro	CAC	His	CGC	Arg	**C**
	CUA	Leu	CCA	Pro	CAA	Gln	CGA	Arg	**A**
	CUG	Leu	CCG	Pro	CAG	Gln	CGG	Arg	**G**
A	AUU	Ile	ACU	Thr	AAU	Asn	AGU	Ser	**U**
	AUC	Ile	ACC	Thr	AAC	Asn	AGC	Ser	**C**
	AUA	Ile	ACA	Thr	AAA	Lys	AGA	Arg	**A**
	AUG	Met	ACG	Thr	AAG	Lys	AGG	Arg	**G**
G	GUU	Val	GCU	Ala	GAU	Asp	GGU	Gly	**U**
	GUC	Val	GCC	Ala	GAC	Asp	GGC	Gly	**C**
	GUA	Val	GCA	Ala	GAA	Glu	GGA	Gly	**A**
	GUG	Val	GCG	Ala	GAG	Glu	GGG	Gly	**G**

Question 9-10 The complete sequence of a small mRNA encoding a small protein is shown below. Predict the peptide encoded by this mRNA. mRNA = 5' AUAGAUGAACCCCCAAGCCUAGGAG 3'

Answer: Met-Asn-Pro-Gln-Ala. The ribosome starts translation at the first AUG. UAG is a stop codon.

9.18 Transfection of Foreign DNA into Host Cells and Restriction Enzymes

Restriction enzymes of bacteria catalyze the cleavage of DNA such as those injected by a phage (a virus that infects bacteria). Bacteria acquired those enzymes in order to defend themselves against such invasions. Each restriction enzyme cuts DNA at a specific recognition sequence. For example the enzyme EcoRI and DraI cut DNA only where the following paired sequence in Fig. 9.18 is encountered. Today some 276 enzymes are known.

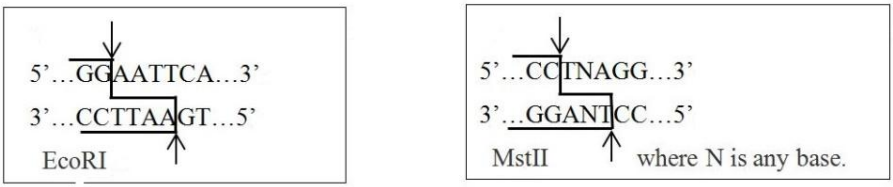

Figure 9.19 *Restriction enzymes cut DNA bonds between 3'OH of one nucleotide and 5'phosphate of the next one at the specific restriction site.*

Adding methyl groups to certain bases at the recognition sites on the bacterial DNA blocks the restriction enzyme and protects the bacterial DNA from being cut by themselves. Those methylases in the host might also modify the few phage DNA that escape the restriction. The few phages that have escaped one strain of bacteria can then grow well on a new host. DNA molecules of different species cut by the same restriction enzyme can be pasted together by the enzyme called a ligase.

9.19 Plasmids as Vectors

A vector is a DNA molecule used as a vehicle to artificially carry foreign genetic materials into another cell, where it can be replicated and expressed. A vector is a tiny size in comparison to the host chromosomes. A vector must have the origin of replication to replicate independently in the host cell, a recognition

sequence for a restriction enzyme allowing the vector to be cut and combined with the foreign DNA, and a marker gene such as a penicillin or ampicillin resistance gene that tells us the presence in the host cell when cultured in the medium containing each antibiotics. Bacteria have acquired additional many small circular chromosomes, i.e. plasmids to defend themselves against yeast invasions. A plasmid has been used as a vector.

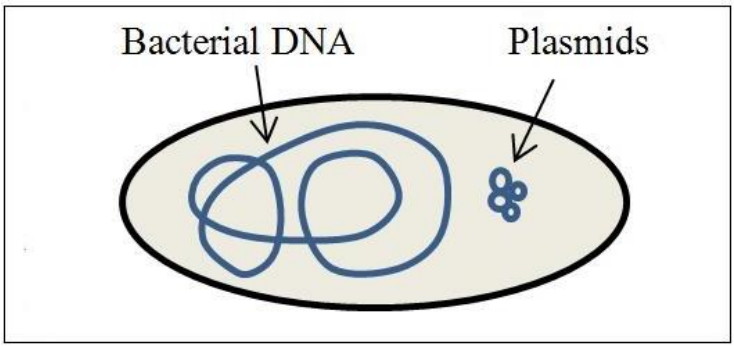

Figure 9.20 *A plasmid is a small DNA molecule physically separated from a genomic DNA and can replicate independently. They are commonly found in bacteria as small circular double-stranded DNA molecules.*

9.20 Polymerase Chain Reaction

In 1983 Kerry Mullis demonstrated the polymerase chain reaction (PCR) technic which made it possible to amplify genes million of times. It has been used in diagnosis of diseases, gene cloning and gene identification. The PCR is based on repetition of alternate temperature change of low 70℃ and high 95℃ in the reaction process. We first synthesize two short DNA primers that correspond the terminal ends of the DNA of our interest. PCR requires forward and reverse primers. Besides of the primers we need the double-stranded DNA to be amplified, DNA polymerase, and all deoxyribonucleoside triphosphates (dNTPs); i.e. deoxyadeosine triphosphate (dATP), deoxycytidine triphosphate (dCTP), deoxyguanosine triphosphate (dGTP), and deoxythymidine triphosphate (dTTP).

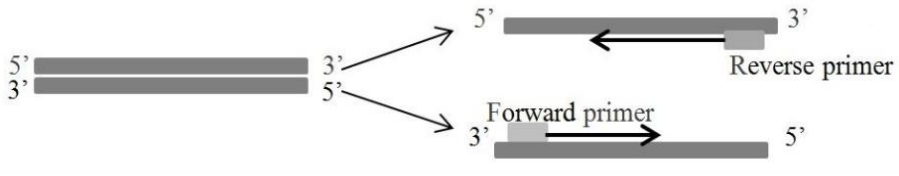

Figure 9.21 *The polymerase reaction with forward and reverse primers at 70 ℃.*

A DNA molecule of our interest should be heated to 95℃ to dissociate hydrogen bonds of a double-stranded DNA to obtain the denatured single-stranded DNAs. At this high temperature ordinary DNA polymerase becomes denatured and has no function. Thomas Brock and his colleagues found a bacterium called Thermus aquaticus living in the hot springs at Yellowstone. They discovered that T. aquaticus had all heat resistant enzymes including DNA polymerase that functioned at 95℃, and the enzyme has been used in PCR.

To initiate the reaction, two kinds of short synthesized single-stranded DNA are added in large quantities to a water solution containing of the double-stranded DNA to be amplified. These two are complementary to both ends of the DNA. Besides those, we need the DNA replication enzyme called DNA polymerase that has been found in T. aquaticus and all kinds of dNTPs in abundance. The solution is then heated to 95 ℃. The double-stranded DNA in a sample dissociates into the single-stranded, but no polymerase chain reaction occurs at this high temperature. The temperature is then lowered to 70 ℃. The primers go hydrogen bonding with the target single-stranded DNA to form partial double-stranded DNA. The polymerase replicates this partial double-stranded DNA by elongating the DNA primers until a complete double-stranded DNA is formed. Then the temperature is raised to 95 ℃ to create single-stranded DNA from the target DNA which is now doubled. The temperature is lowered again. The polymerization proceeds with an abundance of primers and dNTPs. The target DNA is doubled every time the temperature is

raised and lowered. The DNA portions bracketed by the two primers are amplified by repeating this process over and over again.

9.21 DNA Sequencing Reaction

DNA sequencing is the process of determining the precise order of nucleotides within a DNA molecule. Genomic DNA is fragmented into random pieces cut by restriction enzymes from bacteria or mechanical shearing method, transfected into bacteria by vectors, and cloned as a gene library. DNA from individual bacterial clones is sequenced and the sequence is assembled by using overlapping DNA regions. In a DNA sequencing reaction, we need many template DNA molecules, large amounts of DNA primers to provide the free 3' OH for the start of DNA synthesis, many DNA polymerase to catalyze the synthesise of the DNA, and all kinds of dNTPs in abundance. Besides of these reagent, we need small amounts of defective dNTPs to stop the chain reaction when it goes in the last end of synthesized DNA chain. Defective dNTPs are such dNTPs that have no free 3' OH to react. We call them ddNTPs (dideoxyribonucleoside triphosphates) which are ddATP, ddCTP, ddGTP, and ddTTP. Absence of OH at 3' position means no additional nucleotides can be added to it. Various molecules with different lengths of the DNA will be produced after the polymerizing reaction, and those newly formed DNA length will be determined by where the dideoxynucleotides are attached at the end of the polymerizing chain.

Each ddNTP is tagged a different fluorescent group as a mark that emits a different color in order to visualize the synthesized DNA fragments under a laser beam of ultraviolet light after the separation by capillary electrophoresis. Shorter DNA fragment moves to the cathode faster than longer ones when the voltage is applied since molecules are negatively charged due to a phosphate group.

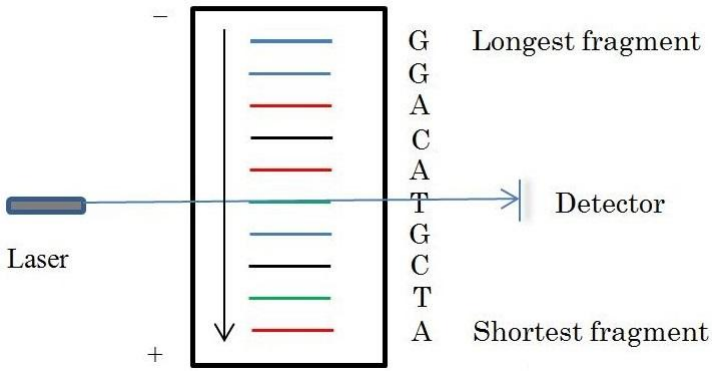

Figure 9.22 *Separation of the different length of synthesized DNA fragments by electrophoresis.*

9.22 Reverse Transcription Polymerase Chain Reaction

Reverse transcription polymerase chain reaction (RT-PCR) is used for the detection of mRNA in tissue levels or finding the specific protein-coding gene. Reverse transcribing the mRNA of our interest into its DNA complement (cDNA) is carried out by the enzyme called *reverse transcriptase*. Since messenger RNAs do not last long in the cytoplasm and present in small amounts, the newly synthesized cDNA is in small amounts, and has to be amplified using traditional PCR for the detection.

The first step in cDNA production is to extract mRNA from a tissue and hydrogen-bond to the primer called oligo dT (a string of thymine nucleotide). The reverse transcriptase synthesizes DNA using mRNA as a template. The produced cDNA is complementary to the mRNA and is different from the genomic DNA. Quantitative RT-PCR is considered to be the most powerful, sensitive, and quantitative assay for the detection of mRNA in tissue levels. It is frequently used in the finding a specific gene for the defective protein from diseases.

Question 9-11 How do we find a specific gene?

Answer: A DNA copy of mRNA from a tissue can be made by reverse transcriptase. The complimentary DNA is different from the genomic DNA in that cDNA does not contain a promoter, introns and terminator. A high degree of similarity to the cDNA (a copy of the messenger RNA which encodes the base sequence for an unknown structure of a protein) is strong evidence that the region of a target genome is the protein-coding gene. And from the base sequence we can determine the structure of the protein.

Question 9-12 How do we make insulin in bacteria?

Answer: To produce human insulin in bacterial cells, the plasmid that is chosen should have a bacterial promoter, and the source DNA which should be a wild type of cDNA which is a DNA copy of mRNA for insulin.

Bacteria cannot express the genomic DNA for insulin because bacteria won't splice the pre-mRNA. We use cDNA instead of using a genomic DNA since cDNA won't have any introns. However cDNA has no sites for RNA polymerase to bind to start transcription. You have to attach a bacterial promoter to a plasmid. Besides, we need to add ribosome-binding sequence, and the terminator to the plasmid, and then the bacteria that incorporate the plasmid start transcribing cDNA. They will produce mRNA which will be translated in the ribosome.

If we had a DNA copy of mRNA that is extracted from beta cells in the pancreas and could attach it to a plasmid with a bacterial promoter, ribosome-binding sequence, and terminator, bacteria that incorporated the plamid would make mRNA which could be translated to a protein. The bacteria is cultured on a piece of filter paper, and the colony which produce insulin can be recognized by the labeled antibody of insulin. We can produce therapeutic quantities of insulin by cloning.

9.23 Mutations

Mutations occur as a result of the natural instability of the bases in DNA. It occurs spontaneously or is caused by certain mutagens. At certain base pairs where cytosine residues have been methylated to 5-methylcytosine is one of the most susceptible sites of mutation. When an unmethylated cytosine residue loses its amino group, uracil residue is formed as shown in Figure 9.22. A DNA repair system removes this abnormal base and replaces it with cytosine. If 5-methylcytosine loses its amino group, the result of its loss is thymine that is a natural base of DNA and so repair system won't work as shown in Figure 9.23. When DNA replicates, a half of the daughter DNA becomes mutant. Such mutation might alter the conformation of an active site of an enzyme or receptor.

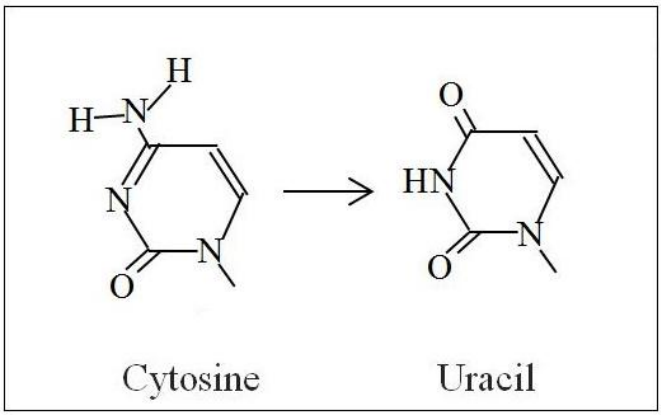

Figure 9.23 *When cytosine residue in one of DNA strand loses its amino group, uracil residue is formed. A DNA repair system replaces the abnormal base with cytosine residue.*

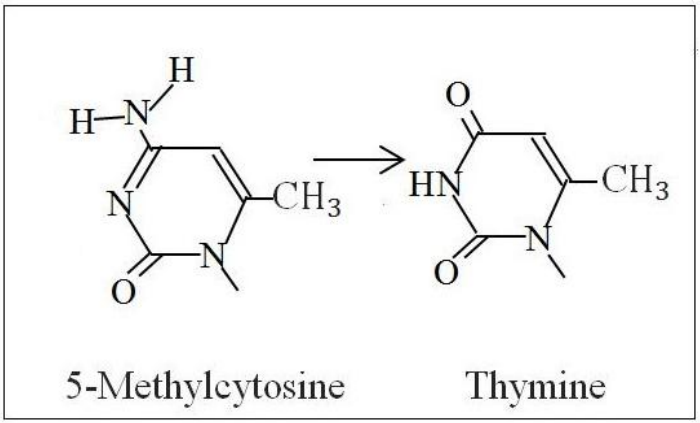

Figure 9.24 *When 5-methylcytosine residue loses its amino group, thymine residue is formed, and it is not repaired. Half of its daughter DNA is mutant.*

Most cells in the body are regulated to divide only when they are exposed to extracellular stimulus such as growth hormones. When DNA are damaged by spontaniously or mutagens, cancer cells emerge by mutations, and devide continuously and form tomors without controls. Epitherial and bone marrow cells divide more ofton, and therfore such cells are especially susceptible to cancer since there is less time for DNA repair system to fix the abnormal DNA bases before the next repliction occurs. If you bite your tongue often, that part of tongue is exposed to growth hormone continuously and has more chance to get tumor. Because enzymes are proteins, their catalytic activity might be changed if the code marked on DNA is changed by mutation that could cause diseases.

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