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APPENDIX A

THE ELECTRON TRANSPORT CHAIN AND OXIDATIVE PHOSPHORYLATION

Most of the usable energy obtained from the breakdown of carbohydrates or fats is derived by *oxidative phosphorylation*, which takes place within mitochondria. For example, the breakdown of glucose by glycolysis and the citric acid cycle yields a total of four molecules of ATP, ten molecules of NADH, and two molecules of $FADH_2$. Electrons from NADH and $FADH_2$ are then transferred to molecular oxygen, coupled to the formation of an additional 32 to 34 ATP molecules by oxidative phosphorylation. Electron transport and oxidative phosphorylation are critical activities of protein complexes in the inner mitochondrial membrane. Which ultimately serve as the major source of cellular energy as shown in Figure 61 (Karp, 1997; Cooper, 2000).

The Electron Transport Chain (Cooper, 2000)

During oxidative phosphorylation, electrons derived from NADH and $FADH_2$ combine with O_2, and the energy released from these oxidation/ reduction reactions is used to drive the synthesis of ATP from ADP. The transfer of electrons from NADH to O_2 is a very energy-yielding reaction, with $\Delta G^{\circ\prime} = -52.5$ kcal/mol for each pair of electrons transferred. To be harvested in usable form, this energy must be produced gradually, by the passage of electrons through a series of carriers, which constitute the *electron transport chain*. These carriers are organized into four complexes in the inner mitochondrial membrane. A fifth protein complex then serves to couple the energy-yielding reactions of electron transport to ATP synthesis.

Electrons from NADH enter the electron transport chain in complex I, which consists of nearby 40 polypeptide chains. These electrons are initially transferred from NADH to flavin mononucleotide and then, through an iron-sulfur carrier, to co-enzyme Q—and energy-yielding process with $\Delta G^{\circ\prime} = -16.6$ kcal/mol. Coenzyme Q (also called ubiquinone) is a small, lipid-soluble molecule that carries electrons from complex I through the membrane to complex III, which consists of about ten polypeptides. In complex III, electrons are transferred from cytochrome *b* to cytochrome *c*—an energy-yielding reaction with $\Delta G^{\circ\prime} = -10.1$ kcal/mol. Cytochrome *c*, a peripheral membrane

protein bound to the outer face of the inner membrane, then carries electrons to complex IV (cytochrome oxidase), which they are finally transferred to O_2 ($\Delta G^{\circ\prime}$ = -25.8 kcal/mol).

A distinct protein complex (complex II), which consists of four polypeptides, receives electrons from the citric acid cycle intermediate, succinate. These electrons are transferred to $FADH_2$, rather than to NADH, and then to coenzyme Q. From coenzyme Q, electrons are transferred to complex III and then to complex IV as already described. In contrast to the transfer of electrons from NADH to coenzyme Q at complex I, the transfer of electrons from $FADH_2$ to coenzyme Q is not associated with a significant decrease in free energy and, therefore, is not coupled to ATP synthesis. Consequently, the passage of electrons derived from $FADH_2$ through the electron transport chain yields free energy only at complexes III and IV.

The free energy derived from the passage of electrons through complexes I, III, and IV is harvested by being coupled to the synthesis of ATP. Importantly, the mechanism by which the energy derived from these electron transport reactions is coupled to ATP synthesis is fundamentally different from the synthesis of ATP during glycolysis or the citric acid cycle. In the latter cases, a high-energy phosphate is transferred directly to ADP from the other substrate of an energy-yielding reaction. For example, in the final reaction of glycolysis, the high-energy phosphate of phosphoenolpyruvate is transferred to ADP, yielding pyruvate plus ATP. Such direct transfer of high-energy phosphate groups does not occur during electron transport. Instead, the energy derived from electron transport is coupled to the generation of a proton gradient across the inner mitochondrial membrane. The potential energy stored in this gradient is then harvested by fifth protein complex, which couples the energetically favorable flow of protons back across the membrane to the synthesis of ATP.

Chemiosmotic Coupling (Cooper, 2000)

The mechanism of coupling electron transport to ATP generation, *chemiosmotic coupling*, is a striking example of the relationship between structure and function in cell biology. It is now recognized as a general mechanism of ATP generation, operating not only in mitochondria but also in chloroplasts and in bacteria, where ATP is generated via a proton gradients across the plasma membrane.

Electron transport through complexes I, III, and IV is coupled to the transport of proton out of the inner of the mitochondrion. Thus, the energy-yielding reactions of electron transport are coupled to the transfer of protons from the matrix to the intermembrane space, which establishes a proton gradient across the inner membrane. Complexes I and IV appear to act as proton pumps that transfer protons across the membrane as a result of conformational changes induced by electron transport. In complex III, protons are carried across the membrane by coenzyme Q, which accepts protons from the matrix at complexes I or II and releases them into the intermembrane space at complex III. Complexes I and III each transfer four protons across the membrane per pair of electrons. In complex IV, two protons per pair of electrons are pumped across the membrane and another two protons per pair of electrons are combined with O_2 to form H_2O within the matrix. Thus, the equivalent of four protons per pair of electrons are transported out of the mitochondrial matrix at each of these three complexes. This transfer of protons from the matrix to the intermembrane space plays the critical role of converting the energy derived from the oxidation/ reduction reactions of electron transport to the potential energy stored in a proton gradient.

Because protons are electrically charged particles, the potential energy stored in the proton gradient is electric as well as chemical in nature. The electric component corresponds to the voltage difference across the inner mitochondrial membrane, with the matrix of the mitochondrion negative and the intermembrane space positive. The corresponding free energy is given by the equation:

$$\Delta G = -F\Delta V$$

Where F is the Faraday constant and ΔV is the membrane potential. The additional free energy corresponding to the difference in proton concentration across the membrane is given by the equation:

$$\Delta G = RT \ln \frac{[H^+]_i}{[H^+]_o}$$

where $[H^+]_i$ and $[H^+]_0$ refer, respectively, to the proton concentrations inside and outside the mitochondria.

In metabolically active cells, protons are typically pumped out of the matrix such that the proton gradient across the inner membrane corresponds to about one pH unit, or a tenfold lower concentration of protons within mitochondria. The pH of the mitochondrial matrix is therefore about 8, compared to the neutral pH (approximately 7) of the cytosol and intermembrane space. This gradient also generates an electric potential of approximately 0.14 V across the membrane, with the matrix negative. Both the pH gradient and the electric potential drive protons back into the matrix from the cytosol, so they combine to form an electrochemical gradient across the inner mitochondrial membrane, corresponding to a ΔG of approximately -5 kcal/mol per proton.

Because the phospholipid bilayer is impermeable to ions, protons are able to cross the membrane only through a protein channel. This restriction allows the energy in the electrochemical gradient to be harnessed and converted to ATP as a result of the action of the complex V (or ATP synthase) in oxidative phosphorylation. ATP synthase is organized into two structurally distinct components, F_0 and F_1, which are linked by a slender stalk. The F_0 portion spans the inner membrane and provides a channel through which protons are able to flow back from the intermembrane space to the matrix. The energetically favorable return of protons to the matrix is coupled to ATP synthesis by the F_1 subunit, which catalyzes the synthesis of ATP from ADP and phosphate ions (P_i). The flow of protons through F_0 drives the rotation of F_1, which acts as a rotary motor to drive ATP synthesis.

It appears that the flow of four protons back across the membrane through F_0 is required to drive the synthesis of one molecule of ATP by F_1, consistent with the proton transfers at complexes I, III, and IV each contributing sufficient free energy to

the proton gradient to drive the synthesis of one ATP molecule. The oxidation of one molecule of NADH thus leads to the synthesis of three molecules of ATP, whereas the oxidation of $FADH_2$, which enters the electron transport chain at complex II, yields only two ATP molecules.

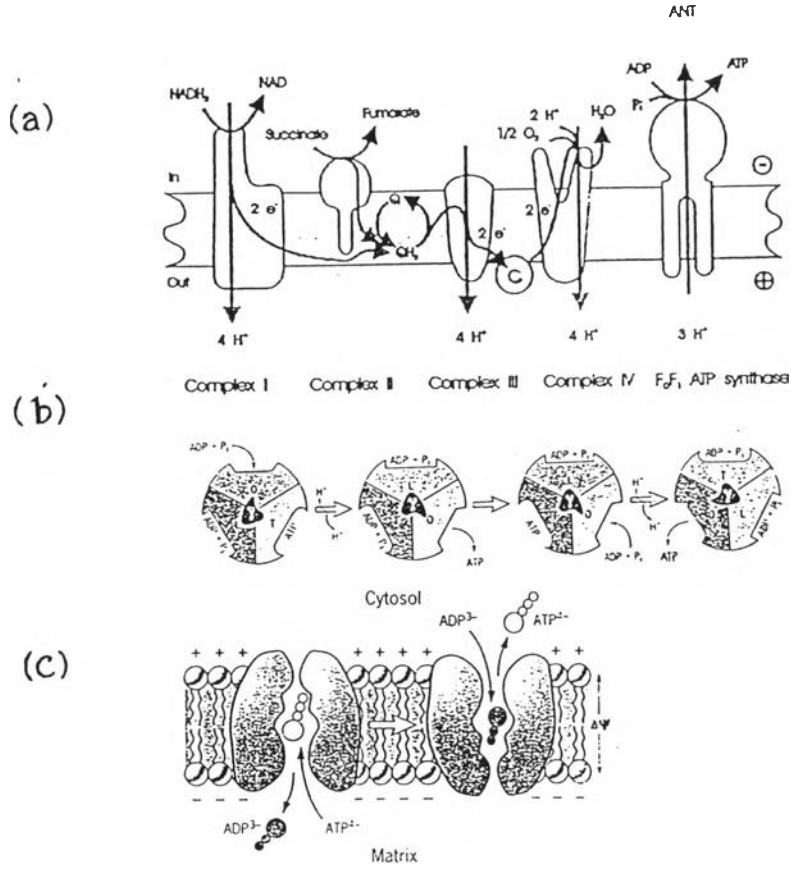

Figure 61 Energy production by mitochondria. (a) The electron transport chain components involved in oxidative phosphorylation are located within the mitochondrial inner membrane: C, cytochrome *c*; Q, ubiquinone; Pi, inorganic phosphate. (b) Schematic drawing showing changes at all three catalytic sites of subunits in F_1 portion of ATP synthase. The movement of protons through the F_0 portion of the enzyme causes the rotation of γ subunit and conformational changes of the catalytic subunits through different states: O, open conformation, in which the affinity for substrates is greatly decreased; L, loose conformation, in which substrates are loosely bound; and T, tight conformation, in which the affinity for substrates increased, causing them to be tightly bound to the catalytic site. (c) The adenine nucleotide translocator (ANT) (Karp, 1997).

Transport of Metabolites Across the Inner Membrane (Cooper, 2000)

In addition to driving the synthesis of ATP, the potential energy stored in the electrochemical gradient drives the transport of small molecules into and out of mitochondria. For example, the ATP synthesized within mitochondria has to be exported to the cytosol, while ADP and P_i need to be imported from the cytosol for ATP synthesis to continue. The electrochemical gradient generated by proton pumping provides energy required for the transport of these molecules and other metabolites that need to be concentrated within mitochondria.

The transport of ATP and ADP across the inner membrane is mediated by an integral membrane protein, the adenine nucleotide translocator (ANT), which transports one molecule of ADP into the mitochondrion in exchange for one molecule of ATP transferred from the mitochondrion to the cytosol. Because ATP carries more negative charge than ADP (-4 compared to -3), this exchange is driven by the voltage component of the electrochemical gradient. Since the proton gradient establishes a positive charge on the cytosolic side of the membrane, the export of ATP in exchange for ADP is energetically favorable.

The synthesis of ATP within the mitochondrion requires phosphate ions (P_i) as well as ADP, so P_i must also imported from the cytosol. This is mediated by another membrane transport protein, which imports phosphate ($H_2PO_4^-$) and exports hydroxyl ions (OH^-). This exchange is electrically neutral because both phosphate and hydroxyl ions have a charge of -1. However, the exchange is driven by the proton concentration gradient; the higher pH within mitochondria corresponds to a higher concentration of hydroxyl ions, favoring their translocation to the cytosolic side of the membrane.

Energy from the electrochemical gradient is similarly used to drive the transport of other metabolites into mitochondria. For example, the import of pyruvate from the cytosol (where it is produced by glycolysis) is mediated by a transporter that exchanges pyruvate for hydroxyl ions. Other intermediates of the citric acid cycle are able to shuttle between mitochondria and the cytosol by similar exchange mechanisms.

APPENDIX B

Oxidative Stress in Erythrocytes

Why is the erythrocyte particularly susceptible to oxidant stress? The oxygen-binding protein hemoglobin constitutes more than 90% of erythrocyte protein. Indeed, hemoglobin is packed so tightly into the cell that, in the case of sickle-cell anemia, it can aggregate and distort the shape of the entire cell. The erythrocyte probably encounters higher oxygen tensions than any other cell in the body, other than the cells of the lung. Most drugs and toxicants enter the body via the blood, and thus the erythrocyte cannot avoid exposure to xenobiotics. Since the mature erythrocyte is non-nucleated, protein synthesis cannot be carried out, and thus protein damage inevitably results in loss of cell function. Indeed, the red blood cell lives in the circulation for a few months, at most, before being removed in the spleen and degraded (Josephy, 1997).

What are the targets for oxidative damage to the red blood cell? First, hemoglobin itself is susceptible. The oxygen-carrying capacity of hemoglobin is due to the ability of the ferrous iron center in the heme prosthetic group to coordinate molecular oxygen without undergoing complete electron transfer:

$$Fe^{2+}O_2 \leftrightarrow Fe^{2+}O_2 \text{(fast)} \rightarrow Fe^{3+} + O_2^{\cdot-} \text{(slow)}$$

Native hemoglobin is ferrous, Fe^{2+}; ferric heme, the usual oxidation state of most other heme proteins, is ineffective as an oxygen carrier. Hemoglobin repeatedly binds and releases oxygen without undergoing chemical oxidation. Nevertheless, hemoglobin does become oxidized to the ferric form at a measurable rate (Winterbourn, 1990). Ferric hemoglobin (i.e., at least one of the four heme groups oxidized) is known as methemoglobin. In a healthy individual, about 1% of total hemoglobin is methemoglobin (Josephy, 1997).

Since the cirulation time of a red blood cell from the lungs to the tissues, and back to the lungs, occurs in a few seconds, but the lifetime of the cell is about 140 days, biochemical mechanisms must exist to deal with methemoglobin formation.

Besides hemoglobin, other enzymes and proteins in the erythrocyte are potential targets for oxidative damage. The membrane of the erythocyte is composed primarily

of phospholipid protein; it combines great mechanical strength, required to remain intact throughout its traverse of the circulatory system, with the fluidity required to maintain the rheological properties the blood. Damage to the membrane lipids or proteins may lead, for example, to inactivation of the Na^+/K^+ ATPase (Na^+/K^+ pump), which plays a critical role in regulating cytosolic ionic composition and cell volume. Disruption of the membrane of the erythrocyte, a possible consequence of severe oxidative stress, causes release of the hemoglobin into the blood plasma; such hemolysis is potentially fatal, since plasma hemoglobin interferes with the function of the kidneys (Josephy, 1997).

In addition, a short detour from the glycolytic pathway are the synthesis and degradation of 2,3-diphosphoglycerate (2,3-DPG), which is a molecule that controls the electron transport in erythrocytes and plays an essential role in basic metabolism. In erythrocytes, 2,3-DPG concentrations are very sensitive to the energy demand of the cell (Mulquiney and Kuchel, 1999; Stryer, 1995). (See Figure 62)

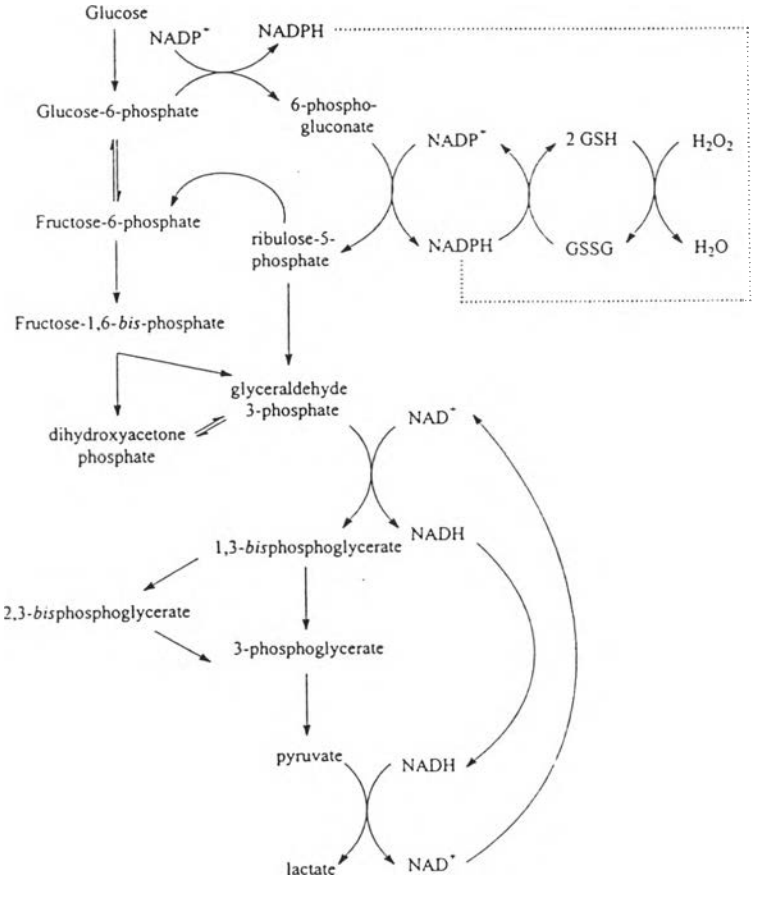

Figure 62 Glucose metabolism in the red blood cell. (Josephy, 1997)

Chemical Induction of Methemoglobin

A remarkably wide variety of chemical agents can induce methemohlobin formation. Some agents [e.g., ferricyanide, $Fe(CN)_6^{3-}$] are only active when added directly to hemoglobin *in vitro*. The negatively charged complex ion is unable to cross the erythrocyte cell membrane. Other agents–for example, nitrobenzene are only active *in vivo*; metabolic bioactivation converts these agents into the species responsible for methemoglobin formation. Nitrite (NO_2^-). *N*–hydroxyarylamines, and hydrogen peroxide are active both *in vivo* and *in vitro*. Several of these agents can be clinically significant. For example, exposure to nitrate fertilizers can be high in agricultural areas, notably in children drinking well-water; the nitrate can be reduced to nitrite by intestinal microflora. Nitrite is used per se as a preservative for meat. An outbreak of acquired methemoglobinemia in Dublin, Ireland, was traced to the application of excessive nitrite to pork by the local butcher (Walley and Flanagan, 1987). Acquired methemoglobinemia can also be induced by various drugs, such as phenylhydrazine and primaquine (George et al., 1966; Watkins et al., 1985; Christopher, White, and Eaton, 1990; Morais Mda and Augusto, 1993).

In view of the diversity of the chemical structures of methemoglobinemia-inducing agents, one should not expect that a single mechanism accounts for their activities. The list includes both oxidizing (ferricyanide, hydrogen peroxide) and reducing (nitrite, *N*-hydroxyarylamines) compounds.

Mechanisms of Methemoglobin Formation

The simplest interpretation of the mechanism of hemoglobin oxidation, given earlier, is one-electron transfer from dioxygen to iron: $Fe^{2} + O_2 \rightarrow Fe^{3+} + O_2^{\cdot-}$. However, this is clearly not the whole story. Hemoglobin is a tetramer of heme-containing subunits, so the complete stoichiometry of oxidation should account for all four. Also, the fate of the superoxide product of the first step must be considered. In the erythrocyte, superoxide dismutase and catalase (or glutathione peroxidase) will convert $O_2^{\cdot-}$ to oxygen and water. In a model system of pure hemoglobin, the methemoglobin formation has been shown in the following scheme (Watkins et al., 1985):

$$Fe^{2+}O_2 \rightarrow Fe^{3+} + O_2^- \quad (1)$$

$$Fe^{2+}O_2 + O_2^- + 2H^+ \rightarrow Fe^{3+} + O_2 + H_2O_2 \quad (2)$$

$$Fe^{2+}O_2 + H_2O_2 \rightarrow Fe^{3+} + OH^- + HO^{\cdot} + O_2 \quad (3)$$

$$Fe^{2+}O_2 + HO^{\cdot} \rightarrow Fe^{3+} + OH^- + O_2 \quad (4)$$

$$\textit{Net:} \quad 4(Fe^{2+}O_2) + 2H^+ \rightarrow 4Fe^{3+} + 2OH^- + 3O_2$$

The first step is the one-electron transfer reaction. In step 2, the superoxide anion and the heme iron reduce the heme-bound dioxygen to hydrogen peroxide, yielding ferric heme and dioxygen. This process, whereby a reducing agent (in this case, superoxide) generates methemoglobin and hydrogen peroxide, by unleashing the oxidizing power of the hemoglobin-bound oxygen molecule, is very significant: It demonstrates that a reducing species can cause oxidative stress. This mechanism underlies the apparently paradoxical methemoglobin induction by reducing species, such as nitrite and many drugs (Castro, Wade, and Belser, 1978). Step 3 is a form of Fenton chemistry: production of hydroxyl radical from hydrogen peroxide and ferrous iron. In step 4, this hydroxyl radical oxidizes one more ferrous heme unit.

These results, obtained with purified hemoglobin solutions, illustrate the potential for hemoglobin autoxidation to generate all the reactive species (O_2^-, H_2O_2, $HO^{\cdot}$) associated with radiation damage and oxidative stress. In the intact red cell, these species will either participate in hemoglobin oxidation (as shown above), be detoxified by antioxidant enzymes and metabolic antioxidants, or lead to oxidative damage of other red cell consituents, including the cell membrane.

Metabolic Reduction of Methemoglobin

Since autoxidation of hemoglobin is unavoidable, even in the absence of xenobiotic stress, and hemoglobin cannot be replaced by the red cell, methemoglobin must be reduced enzymatically back to the ferrous state. In addition, the superoxide flux generated by autoxidation must be dealt with, or oxidative stress will destroy vital cellular functions, such as membrane integrity (Thornalley and Stern, 1985). Both of these metabolic challenges require reducing power.

The red blood cell (like the brain) relies exclusively on blood glucose for energy. It has no mitochondria, and therefore it cannot utilize the Krebs cycle. Certainly, this greatly limits the energy-generating capacity of the cell, since oxidative phosphorylation cannot occur. Glucose metabolism takes place by glycolysis (Embden-Meyerhof pathway) to pyruvate or by the hexose monophosphate shunt pathway (see Figure 62).

Glycolysis, from glucose to lactate, is a redox-balanced process; glucose is split into two three-carbon fragments. The NADH generated by glyceraldehyde-3-phosphate dehydrogenase is consumed by lactate dehydrogenase (reduction of pyruvate to lactate). This is the standard route of anaerobic glycolysis. On the other hand, if another mechanism exists for regeneration of NAD^+ from NADH then glycolysis can terminate at pyruvate. The reduction of methemoglobin provides such an avenue. NADH is the main source of reducing equivalents for regeneration of ferrous hemoglobin from methemoglobin.

Cytochrome b_5 and NADH-Cytochrome b_5 Reductase

A study of Gibson (1948) revealed that red cells treated with amyl nitrite reduced the resulting methemoglobin when subsequently incubated with glucose. Cells from an individual with congenital methemoglobinemia failed to do so. Iodoacetic acid, a well-known glycolytic inhibitor, stopped the glucose-dependent reduction observed in the normal cells. Lactate could also drive the reduction, and the lactate-dependent reaction was iodoacetate-insensitive. This evidence was consistent with an NADH-dependent process, since iodoacetate inhibits glyceraldehyde-phosphate dehydrogenase, the NADH-generating step in glycolysis. Lactate dehydrogenase, acting in the reverse direction to glycolysis, also generates NADH, but is insensitive to iodoacetate.

The NADH-dependent reduction of methemolobin was first demonstrated in a cell free system by Scott and Griffith (1959). NADH-methemoglobin reductase activity could, in principle, be assayed directly by monitoring NADH-dependent reduction of methemoglobin. A simple colorimetric assay was devised. The artificial electron acceptor dichloroindophenol (DCIP) is purple in its oxidized form and is colorless is the reduced "leuco" form. DCIP rapidly redox-equilibrates with the hemoglobin/methemoglobin couple, so reduction of DCIP (600 nm) can be used to

monitor methemoglobin reduction. Nitrite-treated hemolyzate from normal individuals catalyzed the NADH-dependent reduction of DCIP. Nitrite-treated hemolyzate from persons with congenital methemoglobinemia did not do so. The enzyme catalyzing the observed NADH-dependent reduction of DCIP was named diaphorase.

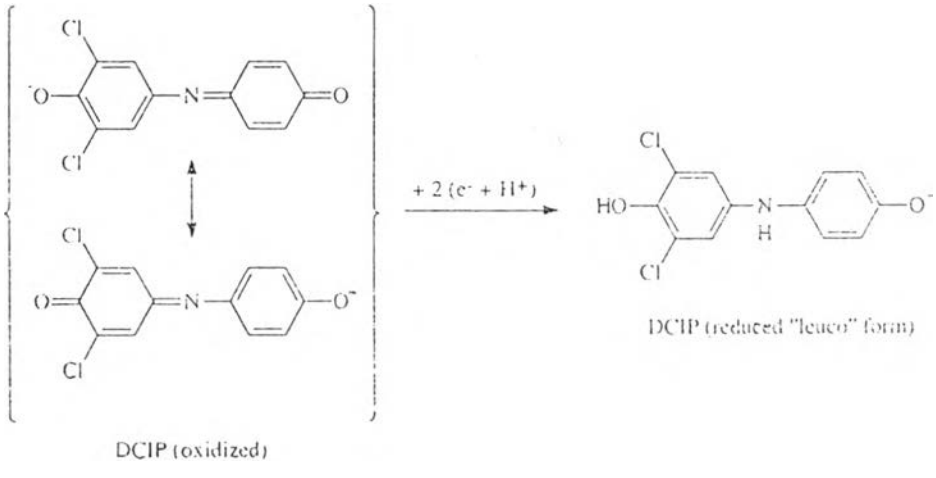

Figure 63 NADH-dependent reduction of DCIP (Josephy, 1997).

The key breakthrough in elucidation of the enzymology of methomoglobin reduction was the discovery that cytochrome b_5 is an intermediary electron carrier (Hultquist and Passon, 1971). The enzyme which accepts electrons from NADH was then named as NADH-cytochrome b_5 reductase, rather than "diaphorase," the old term for an enzyme capable of reducing dyes (Cadenas, 1995). Reducing equivalents from NADH are carried through an electron-transport chain, via the flavoprotein (NADH-cytochrome b_5 reductase) and the cytochrome (b_5), to methemoglobin as the terminal electron acceptor:

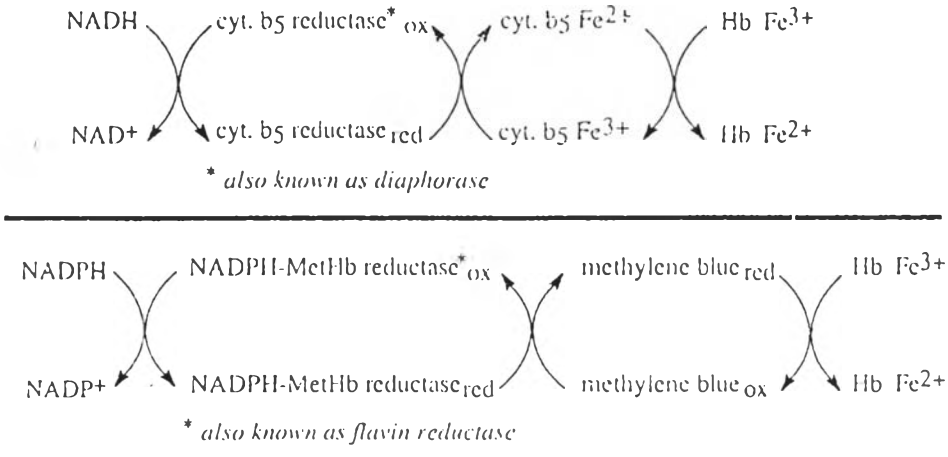

Figure 64 Pathways of methemoglobin reduction: NADH-dependent and NADPH-dependent (Josephy, 1997).

NADPH-Dependent Reduction of Methemoglobin

About 10% of the flux of glucose catabolism in the red blood cell occurs via the pentose phosphate pathway (Palsson, Narang, and Joshi, 1989). The oxidative steps of the pathway (catalyzed by glucose-6-phosphate dehydrogenate and 6-phosphogluconate dehydrogenate) generate NADPH. In the red cell, this pathway operates primarily to generate NADPH, which can maintain glutathione in the reduced form. The carbohydrate product of the oxidative reactions, ribulose-5-phosphate, is then converted to fructose-6-phosphate and glyceraldehyde-3-phosphate, which consequently reenter the glycolytic pathway to pyruvate (Stryer, 1995) (see Figure 62).

Glucose-6-phosphate dehydrogenase (G6PD) deficiency is a very common inborn error of metabolism leading to lowered synthesis of NADPH (Senozan and Thielman, 1991; Beutler et al., 1991). In this condition, the impariment of NADPH generation would be expected to cause depletion of reduced glutathione and reduced ability to scavenge superoxide and hydrogen peroxide. In addition, erythrocyte catalase contains bound NADPH, which protects the enzyme against peroxide-induced inactivation. Reduced catalase activity in G6PD-deficient cells may also be the critical factor in oxidant sensitivity (Scott, Wagner, and Chiu, 1993).

APPENDIX C

FACScan flow cytometer system (Beckton Dickinson, 1994)

FACScan flow cytometer is desigend for applications ranging from routine clinical to advanced research. It analyzes cells as they pass one at a time through a focused laser beam. FACScan can measure up to five parameters-forward light scatter (FSC), side light scatter (SSC), and three fluorescence parameters (FL1, FL2, FL3). And FACScan can measure the pulse height, area, and width of each fluorescence parameter.

FACScan analyzes cells as they travel in a moving fluid stream past a fixed laser beam. As a cell passes in front of the laser, several measurements are made based on the physical characteristics of the cell. These characteristics, which pertain to how the cell scatters the laser light and emits fluorescence, provide us with information about the cell's size, internal complexity, and relatively fluorescence intensity. This information is collected and transmitted to the computer.

The FACScan flow cytometer is designed for a wide range of applications. FACScan is for in vitro diagnostic use for enumeration of leukocyte (non-blast) subsets or reticulocytes. In addition, FACScan can be used for the following research applications: DNA cell-cycle analysis, platelet studies, three-color immunofluorescence applications, chromosome classification, or intracellular ionized calcium measurements.

Overview of Various FACScan Applications

1. Immunophenotyping

Immunophenotyping, one of the most frequency performed and widely accepted techniques in flow cytometry, is used for identification and differentiation of subpopulations of leukocytes based on their surface antigens. Although lymphocyte subpopulations are most commonly studied, any cell type can be analyzed virtually.

Cells are incubated with a monoclonal antibody (e.g., CD3, which reacts with T cells) that is tagged with a fluorescent marker (fluorochrome). If the antibody is specific to an antigen site on the cell, the antibody and its corresponding fluorochrome bind to the cell. The staining process can be a simple, one-step (direct immunofluorescence) or two-step (indirect immunofluorescence) procedure. After the completion of the staining procedure, the cell sample is analyzed on the FACScan.

By viewing the FSC vs SSC parameter, cell populations can be distinguish based on cell size and internal complexity. A specific subpopulation may be selected by drawing a gate around it before analyzing the fluorescence parameters.

1.1. Direct Immunofluorescence

Direct immunofluorescence staining involves incubating the white blood cells with a monoclonal antibody conjugated to a fluorochrome. This antibody, which is specific for a particular antigen site on a cell, binds to the surface antigens on the cell. A wash step removes any unbound antibody. Typically, a sample is stained with a combination of two fluorochrome-labeled antibodies (e.g., CD3 FITC and CD8 PE). This staining allows the measurement of two immunofluorescence properties simultaneously and is referred to as two-color staining.

1.2. Indirect Immunofluorescence

Indirect immunofluorescence staining is similar to direct staining except the staining takes place in two steps rather than one. Cells are incubated with a primary monoclonal antibody. After any unbound antibody is washed away, the cells are incubated with a second-step reagent that is specific to the primary antibody and conjugated to a fluorochrome.

1.3. Three-Color Immunofluorescence

Three-color fluorescence is an efficient way of analyzing three immunofluorescence properties from one sample tube. Cells are incubated with three different conjugated monoclonal antibodies. The sample shown was stained with CD3 FITC, CD4 PE, and CD8 PerCP.

2. DNA Cell-Cycle Analysis

Quantitative and qualitative DNA measurements of cell nuclei can be helpful in studying the biological behavior of tumors. The two principle calculations– cell-cycle analysis and DNA index– can help to determine how tumor cells differ from normal, healthy cells. Cell-cycle analysis gives an estimation of the percentage of cells within each phase of the cell cycle. For example, a very high S phase in a tumor cell population suggests the tumor is rapidly proliferating. The DNA index is the ratio of DNA content of the tumor cells divided by the DNA content of normal control cells. A ratio not equal to one suggests that the tumor has an abnormal DNA content.

Propidium iodide, a specific nucleic acid dye, is used to stain the cell or cell nucleus to measure DNA content.

3. Reticulocyte Enumeration

Reticulocytes are young red blood cells that contain residual organelles (i.e., ribosomes and mitochondria). The presence of these organelles differentiates reticulocytes from mature erythrocytes. The organelles contain residual ribonucleic acid (RNA) and deoxyribonucleic acid (DNA), which is absent from mature erythrocytes. Enumerating reticulocytes is perhaps the best way to evaluate abnormal red blood cell production in a variety of disorders.

4. Platelets

Platelet activation, adhesion, and aggregation responses are functions directly related to the platelet membrane or their internal granules. Characterizing and measuring the membrane glycoproteins responsible for these functions are valuable when investigating suspected platelet disorders.

5. Intracellular Calcium

Calcium ions, serving as intracellular messengers, flow across the membranes of cells to mediate cellular responses to external stimuli. Typically, a kinetic experiment is performed where cells are located with an appropriate calcium probe, stimulated with a mitogenic lectin, then analyzed on the FACScan. The results show the changes in intracellular calcium over time.

The Cell Cycle Analysis

The determination of DNA content is one of the most common flow cytometry applications used by researchers to monitor the growth kinetics of cells and to quantitate DNA content abnormalities and kinetics in tumor populations. Proliferating cells progress through several phases before they undergo cell division. These phases are referred to as G_1, S, G_2, and M. Cells not proliferating (resting) are referred to as G_0 cells.

The DNA content of cells are considered in the various phases. In both the G_0 and G_1 phases, cells have the DNA content as 2C (having two copies of chromosomes). Cell replication begins in S phase when DNA is synthesized and the DNA content in the nucleus doublets. These cells are now referred to as 4C (having four copies of chromosomes). The DNA content of the cell remains at 4C until the cell undergoes mitosis or cell division in M phase. Here, two daughter cells are formed.

Nuclei analyzed for DNA content are typically stained with propidium iodide (PI), which binds stoichiometrically to DNA.

APPENDIX D

Recipes for SDS gel electrophoresis and washing protocol

1. 12% Resolving Gel (Lower Gel) Preparation for Tris-glycine SDS-Polyacrylamide Gel Electrophoresis

Solution Components	10 ml per Gel Mold
Distilled H_2O	3.3 ml
30% Acrylamide mix (Protogel®)	4.0 ml
1.5 M Tris (pH 8.8)	2.5 ml
10% SDS	0.1 ml
10% Ammonium persulfate	0.1 ml
TEMED	0.004 ml

2. 5% Stacking Gel Preparation for Tris-glycine SDS-Polyacrylamide Gel Electrophoresis

Solution Components	6 ml per Gel Mold
Distilled H_2O	4.1 ml
30% Acrylamide mix (Protogel®)	1.0 ml
1.0 M Tris (pH 6.8)	0.75 ml
10% SDS	0.06 ml
10% Ammonium persulfate	0.06 ml
TEMED	0.006 ml

3. Preparation for Sample Buffer

Solution Components	Volume of 50 ml
Tris	0.38 g
Glycerol	7.5 ml
10% SDS	10.0 ml
β-mercaptoethanol	2.5 ml
Bromophenol blue	0.025 g
Distilled H_2O to 50 ml, pH to 8.3 with HCl	

4. Preparation for Transfer Buffer

Solution Components	Volume of 1000 ml
Tris HCl (25 mM) pH 6.8	3.03 g
Glycine (192 mM)	14.4 g
Methanol (20%)	200.0 ml
Distilled H_2O to 1000 ml, pH to 7.6 with HCl	

5. Preparation for Blocking Buffer

Solution Components	Volume of 1000 ml
Milk	50 g
Tween 20 (0.1%)	1.0 ml
PBS to 1000.0 ml	

6. Preparation for Staining Solution

Solution Components	Volume of 100 ml
Commassie brilliant blue R250	0.25 g
Methanol	45.0 ml
Glacial acetic acid	10.0 ml
Distilled H_2O	45.0 ml

7. Washing Protocol Step 1

2 rinses in PBS-milk-tween	
1 rinse in PBS-milk-tween	for 10 minutes
2 rinses in PBS-milk-tween	for 5 minutes
1 rinse in PBS-tween	for 5 minutes

8. Washing Protocol Step 2

2 rinses in PBS-milk-tween	
1 rinse in PBS-milk-tween	for 10 minutes
2 rinses in PBS-milk-tween	for 5 minutes
1 rinse in PBS-tween	for 5 minutes
1 rinse in PBS	for 5 minutes

BIOGRAPHY

Miss Tipicha Posayanonda was born on June 29, 1973 in Bangkok, Thailand. She received her Bachelor's Degree of Science in Pharmacy in 1995 from the Faculty of Pharmaceutical Sciences, Chulalongkorn University, Thailand. She got a scholarship from the Thai Government to continue her Master's degree and received her Master of Public Health in Toxicology from the School of Public Health, University of Michigan, Ann Arbor, U.S.A. in 1998.

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