

CHAPTER 2

REVIEW LITERATURE

2.1 Gamma oryzanol (γ -oryzanol)

2.1.1 Chemistry

Gamma oryzanol is a mixture of sterol esters of ferulic acid and was first isolated in **rice bran oil (RBO)** by Kaneko and Tsuchiya in the early 1950s (Kaneko and Tsuchiya, 1954). The 10 components of γ -oryzanol were identified as Δ -7-stigmasteryl ferulate, stigmasteryl ferulate, cycloartenyl ferulate, 24-methylene cycloartenyl ferulate, Δ -7-campestenyl ferulate, campesteryl ferulate, Δ -7-sitostenyl ferulate, sitosteryl ferulate, compestanyl ferulate, and sitostanyl ferulate, see Figure 1. Three of these, cycloartenyl ferulate, 24-methylenecycloartenyl ferulate, and campesteryl ferulate, were major components of γ -oryzanol (Xu and Godber, 1999).

The composition of γ -oryzanol depends on the rice variety, some RBO contains cycloartenol instead of 24-methylene cycloartanol. Crude RBO contain 1.56 % γ -oryzanol (Norton, 1995). Some Japanese publications have claimed that oryzanol has various pharmacological effects and therefore is used for medical uses (Sayre and Saunders, 1990 and Wheeler and Garleb, 1991). However, these claims are not supported by scientific studies with one exception, namely that of plasma cholesterol reduction in animals and men (Wheeler and Garleb, 1991). As for fatty acid esters of phytosterols, this cholesterol reduction might be caused by the sterol moiety of γ -oryzanol, which is split off from the ferulic acid part in the small intestine by cholesterol esterase (Swell *et al.*, 1954 and Fujiwara *et al.*, 1983). Ferulic acid is known to be taken up and excreted along with its metabolites in the urine (Fujiwara *et al.*, 1983). Few studies suggested also that ferulic acid might have hypolipidemic effects (Sharma, 1980; Srinivasan and Satyanarayana, 1988; Seetharamaiah and Chandrasekhara, 1993). Ferulic acid has antioxidant activity, and a decrease in lipid peroxides has been reported in studies with γ -oryzanol (Graf, 1992 and Wada *et al.*, 1981). The highest antioxidant activity was found for 24-methylenecycloartenyl ferulate, and

all three γ -oryzanol components had higher activity than that of any of the four vitamin E components. Because the quantity of γ -oryzanol is up to 10 times higher than that of vitamin E in rice bran, γ -oryzanol may be a more important antioxidant of rice bran in the inhibiting of cholesterol oxidation than vitamin E, which has been considered to be the major antioxidant in rice bran. Furthermore, ferulic acid may have anticarcinogenic properties through the inhibition of N-nitroso compounds formation and has been found to suppress benzo[a]pyrene-induced neoplasia in the forestomach of mice (Kuenzig *et al.*, 1984 and Wattenberg *et al.*, 1980)

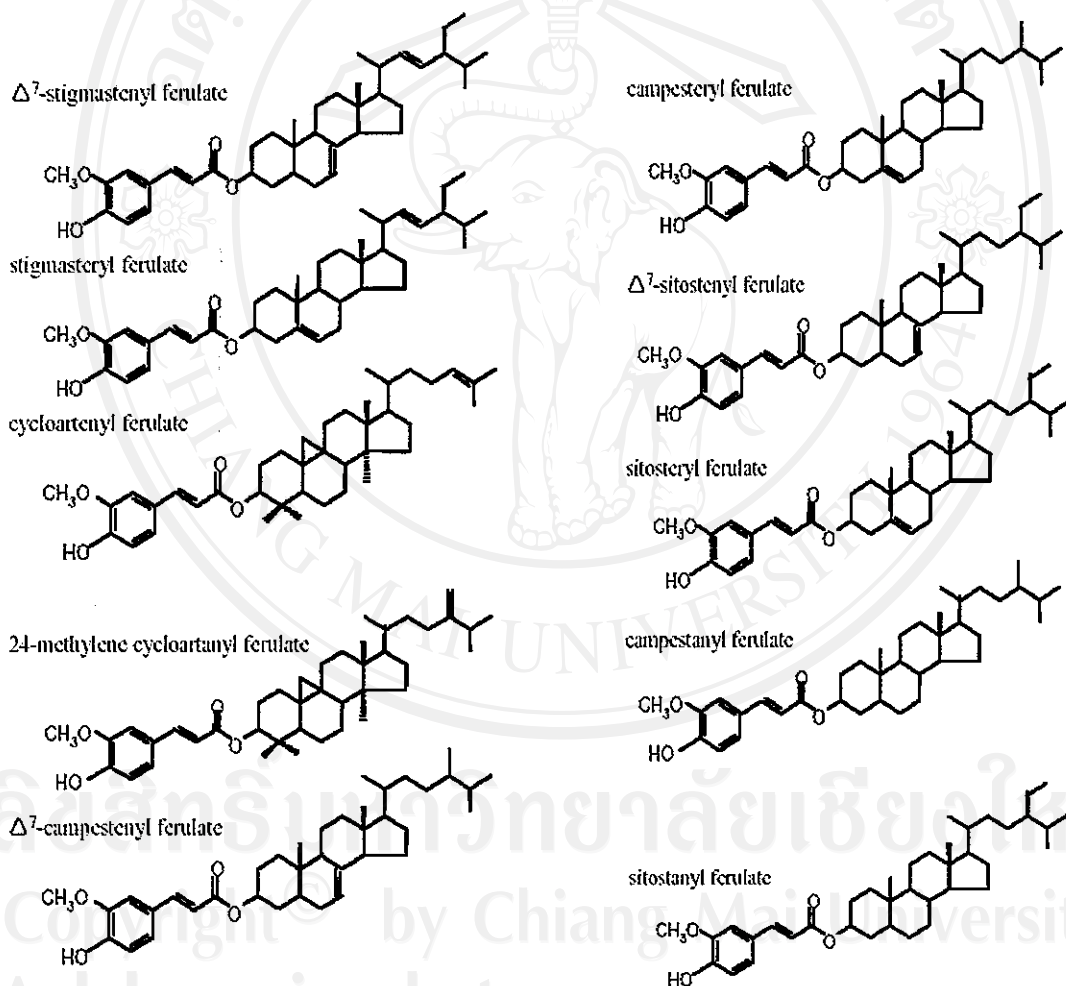


Figure 1. Molecular structure of γ -oryzanol components (Xu and Godber, 1999).

2.1.2 Source of γ -oryzanol

Rice contains 0.6-3.5 % oil. The oil concentrated of the rice grain is primarily in the germ but is also presented in the bran as well. About 80% of the lipids of brown rice are in the bran and polishing. About one third of lipid is in the embryo. Rice bran and germ contain the oil referred to as RBO. Bran contains 15-20 % oil. Triglycerides, the main component of crude RBO, make up approximately 80 % of the oil. The primary fatty acid major of typical RBO is oleic acid, see Table 1.

Table 1. Composition of rice bran oil

Fatty acid	Chain length: No. of double bonds	Per cent
Myristic	14:0	0.1-1.0
Palmitoleic	16:1	0.2-0.6
Stearic	18:0	1.0-3.0
Oleic	18:1	40.0-50.0
Linoleic	18:2	20.0-42.0
Linolenic	18:3	0.0-1.0
Arachidic	20:0	0.0-1.0

(Adapted from Marshall and Wadsworth, 1994)

The minor constituents of oil consist of phospholipids, glycolipids, sterol, waxes, and tocopherols, see Table 2. The broad class of unsaponifiable matter of RBO consists of approximately 42 % sterols, 24 % higher alcohols, 20 % ferulic acid esters, 10% hydrocarbons, and 2 % unknown. The unsaponifiables are generally represented by sterol fraction of the oil. These include free sterols, sterol esters, sterylglucosides, and acylsteryl glycosides. β -Sitosterol is the most abundant sterol present. γ -oryzanol, present at 0.96-2.9 % of bran oil, is a ferulic acid ester of triterpenoid alcohols and presents in sterol fraction removed primarily during caustic refining of the oil (Marshall and Wadsworth, 1994). Crude RBO contain 1.56 % γ -oryzanol

(Norton, 1995). Karladee *et al.*, (2003) and Pongpichan *et al.*, (2004) reported that the purple glutinous rice bran contain a higher amount of γ -oryzanol than white rice bran.

Table 2. Composition of crude rice bran oil

Composition	Per cent
Triglycerides	80
Phospholipids	2
Glycolipids	1
Sterols	5
Waxes	2-5

(Adapted from Marshall and Wadsworth, 1994)

RBO is characterized by its relatively high content of unsaponifiable material. The content of the unsaponifiable material in refining edible RBO is regulated by the Japan Agricultural Standard, to be lower than 5 %. This value is considerably higher than that of other vegetable oils, lower than 1–1.5 %. The most characterized component of RBO is γ -oryzanol, the ferulate esters of triterpene alcohols, see Table 3 (Itoh *et al.*, 1973a and b). The content of γ -oryzanol differs with source of RBO, ranging from 115 to 780 ppm, depending on the degree and possibly the method of processing (Rogers *et al.*, 1933). Cycloartenol and 24-methylene cycloartanol are the major component terpene alcohols.

Table 3. Sterol and triterpene contents in different edible oils

mg/100 gm oil					
Oil	Campesterol	Stigmasterol	Cycloartanol	Cycloartenol	24-Methylene cycloartanol
Rice bran	560	271	106	428	494
Safflower	45	31	1	34	7
Corn	410	110	4	8	11
Sunflower	31	31	-	29	16
Cottonseed	17	4	-	10	17
Sesame	117	62	4	62	107
Soybean	72	72	-	156	8
Peanut	36	21	1	11	16

(Adapted from Itoh *et al.*, 1973a and b)

2.1.3 Determination of γ -oryzanol

Use of **high performance liquid chromatography (HPLC)** method has been established that γ -oryzanol is a mixture of several components (Diack and Saska, 1994; Norton, 1995; Rogers *et al.*, 1993; Evershed *et al.*, 1988), but depending on the chromatographic approach taken, different numbers of individual components have been identified. Gamma oryzanol was separated into two fractions by Diack and Saska (1994) using normal-phase HPLC and a column packed with spherical silica, but each fraction contained at least two or more constituents and it was not possible to identify and quantify the individual components using this approach. Norton (1995) used a reverse-phase approach and was able to identify five individual components of γ -oryzanol. Six components of γ -oryzanol were identified using reverse-phase HPLC by Evershed *et al.*, (1988) and Rogers *et al.*, (1993). Therefore, Xu and Godber, (1999) successfully separated and identified ten components of γ -oryzanol from rice bran oil using preparative normal-phase HPLC to concentrate γ -oryzanol and to reduce interfering substances and reverse-phase HPLC to isolate and collect each component. These ten components were identified as Δ -7-stigmastenyl ferulate,

stigmasteryl ferulate, cycloartenyl ferulate, 24-methylenecycloartanyl ferulate, Δ -7-campestenyl ferulate, campesteryl ferulate, Δ -7-sitostenyl ferulate, sitosteryl ferulate, campestanyl ferulate and sitostanyl ferulate see Figure 1 for chemical structures. The three major components among these are cycloartenyl ferulate, 24-methylenecycloartanyl ferulate and campesteryl ferulate. In this study γ -oryzanol was concentrated using reversed phase HPLC.

2.2 Free radical

Free radical is any species capable of independent existence that contains one or more unpaired electrons. An unpaired electron is one occupies an atomic or molecular orbital by itself. A superscript dot after the formula is usually causes free radical species. Radical can be formed when a covalent bond is broken if one electron from each of the pair shared remains with each atom, a process known as homolytic fission. If A and B are two atoms covalently bonded (representing the electron pair), (Halliwell and Gutteridge, 1999). Homolytic and heterolytic fission can be written as:



Figure 2. Proces of homolytic and heterolytic fission (Halliwell and Gutteridge, 1999).

2.2.1 Reactive oxygen species (ROS)

ROS are normal oxidant by-products of aerobic metabolism and under normal metabolic conditions about 2-5 % of O_2 consumed by mitochondria is converted to ROS (Boveris and Chance, 1973). ROS include three major radical species: superoxide anion ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), highly reactive hydroxyl radical ($\cdot OH$), and peroxy radicals (ROO^{\cdot}) (Cross *et al.*, 1987). Superoxide anion is formed as a result of the leakage of electrons from mitochondrial electron transport and the reduction of molecular oxygen (O_2); this occurs primarily at Complex I (NADH dehydrogenase) (Turrens and Boveris, 1980), and Complex III (Ubiquinone-cytochrome b-c1 complex) (Turrens *et al.*, 1985). Hydrogen peroxide is generated spontaneously by the

dismutation of superoxide anion, under the effects of SOD (McCord and Fridovich, 1969). The intracellular levels of H_2O_2 are normally less than 100 nM, and this species can easily diffuse through cellular membranes, but H_2O_2 is much less reactive than superoxide anion (Chance *et al.*, 1979). Hydroxyl radicals are the most reactive form of ROS produced in mammalian cells, their production is catalyzed by transition metals (e.g., Fe [II] ions generated via reduction of Fe [III] by $O_2^{\cdot -}$), in the presence of hydrogen peroxide. Hydroxyl radicals are highly reactive at the site of their production (Halliwell and Gutteridge, 1984). In addition to mitochondria, other potential sources of superoxide anion, H_2O_2 , and $\cdot OH$ are the endoplasmic reticulum (e.g., NADH cytochrome c reductase; cytochrome P-450) and the nuclear membrane (Freeman and Crapo 1982; Goepfert *et al.*, 1995).

2.2.2 Interactions of ROS with lipids

Lipid peroxidation is a form of oxidative damage that occurs in cell membranes when unsaturated fatty acids react with excess levels of ROS (e.g., $\cdot OH$ radicals or a transition metal/oxidant complex) to form both fatty acid radicals and lipid hydroperoxide ($LOOH^{\cdot}$) (Burton and Traber, 1990). This process is thought to proceed by radical-mediated abstraction of a hydrogen atom from a methylene carbon on PUFA or PUFA side chain. The resulting carbon-centered radical may then undergo molecular rearrangement followed by interaction with molecular oxygen to form peroxy radical (McCall and Frei, 1999). Lipid hydroperoxides cause reversible alterations to membrane structure and function (Girotti, 1998), and are a source of highly reactive aldehydes (e.g., **4-hydroxynonenal**, HNE; **malondialdehyde**, MDA). The cell can be protected from lipid peroxidation by intracellular antioxidant processes. Cellular SOD, GSH and CAT remove cellular superoxides and peroxides prior to their reaction with metal catalysts. ROS initiated peroxidative chain reactions that escaped the actions of the above enzymatic defenses are terminated by chain breaking antioxidants, including water soluble vitamin C, urate, glutathione, and lipid-soluble α -tocopherol, β -carotene, and Ubiquinone (Roth, 1997). Vitamins such as α -tocopherol are localized in the phospholipid bilayers of a membranes, while flavonoids are likely to be concentrated at the interface between the phospholipid bilayers and the water phase because of their hydrophilicity. Flavonoids can scavenge free radicals

involving (LOO^\bullet) and ($\text{}^\bullet\text{OH}$), depending on number and site of the hydroxyl group and double bonds in their structures (Terao and Piskula, 1999).

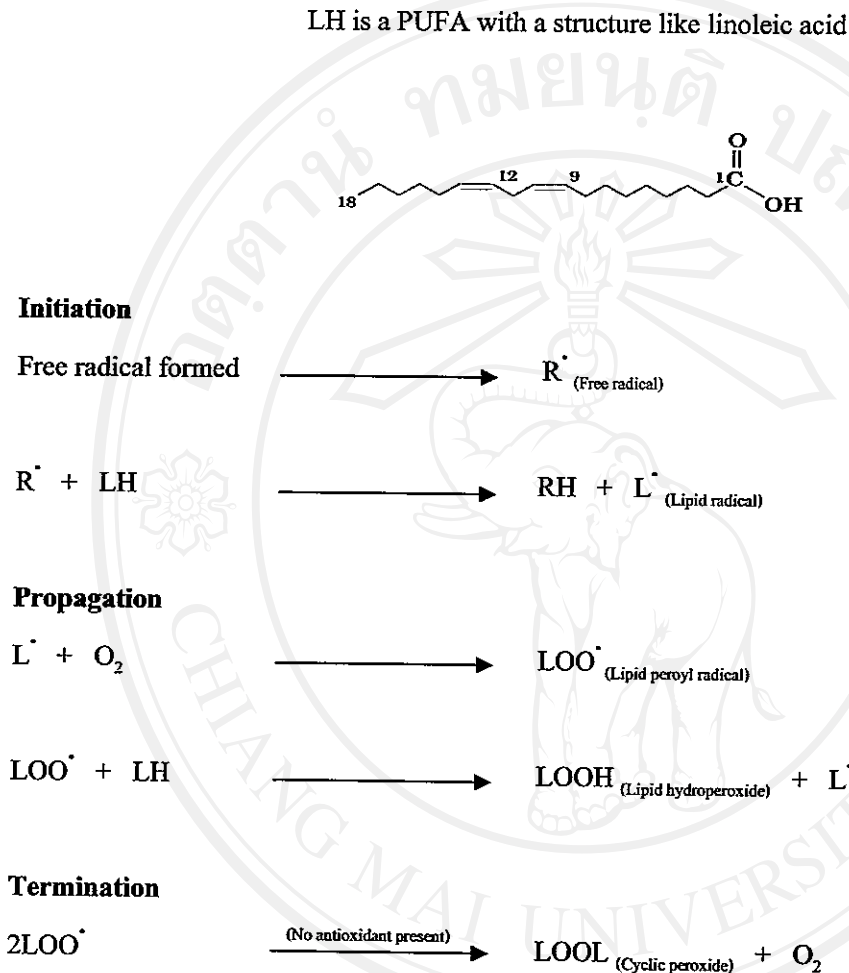


Figure 3. Lipid peroxidation chain reaction (Macrae *et al.*, 1993).

2.3 Lipid bilayer of cell membrane

Membranes are boundaries of living cells. They are composed of large amounts of lipid and protein and small amount of carbohydrate. Lipid plays an important role forming a bilayer around cells. To create membrane bilayer, phospholipids associate their hydrophobic parts in opposite directions, acting as the inner and outer surfaces (Plummer, 1989). Thus protein and carbohydrate can integrate with these lipid bilayer structures. Types and functions of cells

determine proportions of the membrane's components. Examples of the composition of some membranes are shown in Figure 4.

Membranes act as cell barriers which selectively in out transport cell vital agents such as oxygen and nutrients. Moreover, there are various molecules embedded on cell membrane (Saalmüller and Bryant, 1994). These involve a number of vital and survival processes inside the body. For example, each immune cell possess a variety of **cell surface antigens** (CDs), immunoglobulins (Igs) and **major histocompatibility complex** (MHC) e.g. these molecules act as receptors, signal transducers and antigen recognitors. If cell membranes are changed, these molecules and their functions are affected.

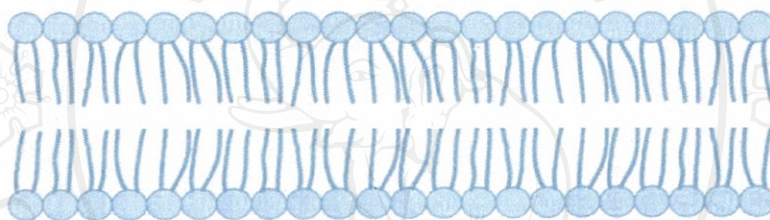


Figure 4. Structure of cell membrane (Voet and Voet, 1995).

2.4 Oxidative stress and the immune system

Oxidative stress, resulting from cumulative damage caused by ROS, is present throughout life and thought to be a major contributor to the aging process. The immune system is particularly vulnerable to oxidative damage because many immune cells produce those reactive compounds as part of the body's defense mechanisms. Higher organisms have evolved a variety of antioxidant defense systems to prevent the generation of ROS or intercept any that are generated. Enzymes such as SOD, GSH and CAT can safely decompose peroxides, particularly hydrogen peroxide produced during the "respiratory burst" involved in killing invading microorganisms, and SOD can intercept or "scavenge" free radicals. However, the food we eat provides us with a large amount of our body's total supply of antioxidants in the form of various micronutrients and "non-nutrients" (Hughes, 2001). Oxidative stress in terms of a two-pan balance, with ROS in one pan and antioxidants in the other, then tipping the balance in favor of the ROS is thought to be a major contributor to several degenerative disorders. Indeed, it is

probably crucial to keep the balance between ROS and antioxidants as level as possible, from as early an age as possible, to prolong the onset of, if not prevent, many age-related disorders.

The immune system appears to be particularly sensitive to oxidative stress. Immune cells rely heavily on cell-to-cell communication, particularly via membrane-bound receptors; to work effectively see in Figure 5. Cell membranes are rich in PUFA acids that, if peroxidized, can lead to a loss of membrane integrity, altered membrane fluidity, (Baker and Meydani, 1994), and result in alterations in intracellular signaling and cell function. It has been shown that exposure to ROS can lead to a reduction in cell-membrane receptor expression (Gruner *et al.*, 1986), see in Figure 5. In addition, the production of ROS by phagocytic immune cells can damage the cells themselves if they are not sufficiently protected by antioxidants.

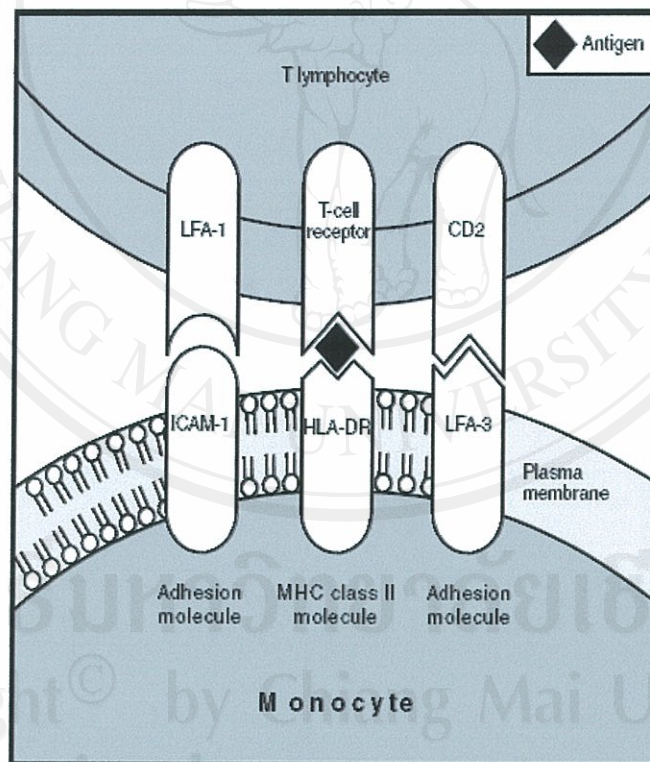


Figure 5. Cell-surface molecules involved in initiating cell-mediated immune responses. HLA-DR, human-leukocyte-associated antigen; ICAM-1, intercellular adhesion molecule-1; LFA, leukocyte-function-associated antigen; MHC, major histocompatibility complex (Hughes, 2001).

2.5 An overview of the immune system

The immune system is composed of a large and complex set of widely distributed elements. Distinctive characteristics of the immune system include specificity, memory, mobility, replicability, and cooperativity. Specificity and memory are characteristics of lymphocytes. Various specific and nonspecific elements of the immune system demonstrate mobility; these include T and B-lymphocytes, immunoglobulins, complement, and hematopoietic cells. In addition, specific and nonspecific cellular components of the immune system can replicate. Cooperativity is a required feature if optimal function of the immune system is to occur. Cooperative interaction involves specific cellular elements, cell products, and nonlymphoid elements (Turgeon, 1996).

The function of the immune system is to recognize self from non-self and to defend the body against nonself. Such a system is necessary for survival in all living organisms. The distinction of self from nonself is made by an elaborate, specific recognition system. Specific elements of the immune system are the lymphocytes. The immune system also has non-specific effector mechanisms that usually amplify the specific functions. Nonspecific components of the immune system include mononuclear phagocytes, polymorphonuclear leukocytes, and soluble factors (e.g., complement), (Turgeon, 1996).

2.5.1 Natural immunity

Healthy individuals protect themselves against microbes by means of many different mechanisms. These include physical barriers, phagocytic cells and eosinophils in blood and tissues, a class of lymphocytes called **natural killer** (NK) cells, and various blood-borne molecules. Natural immunity is characterized as a nonspecific mechanism. If a microorganism penetrates the skin or mucosal membranes, a second line of cellular and humoral defense mechanisms becomes operational. The elements of the natural resistance include phagocytic cells, complement, and acute inflammatory reaction. Despite their relatively lack of specificity, these components are essential because they are largely responsible for natural immunity to many environmental microorganisms. Phagocytic cells, which engulf invading foreign materials, constitute the major cellular component. Complement proteins are the major humoral (fluid) component of natural immunity. Other substances of the humoral component are lysozymes and

interferon, which are sometimes described as natural antibiotics. Interferon is a family of proteins produced rapidly by many cells in response to viral infection; it blocks the replication of virus in other cells (Turgeon, 1996). Phagocytes primarily **polymorphonuclear neutrophils (PMN)** and macrophages are primarily responsible for ingesting particles/pathogens and breaking them down in their phagolysosomes. When a particle attaches to the surface of the PMN, it activates the NADP oxidase system, resulting in the sequential conversion of a molecule of oxygen to superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and finally, hypochloride ions (OCl^-). A second important bactericidal pathway involves the singlet oxygen (1O_2). Both the 1OH and 1O_2 radicals are unstable and will react with bacterial lipids to form bactericidal hydroperoxides. However, it should be mentioned that these free radicals, when released into the extracellular environment, may be harmful to immune cells and the surrounding tissues (Chew, 1996).

2.5.2 The mucosal immune system

Mucosal epithelia cover an area at least 200 times that of the skin. This extensive and vulnerable surface barrier is protected by numerous innate mechanisms in intimate cooperation with adaptive (acquired) mucosal immunity. The main humoral mediators of this local first-line immune system are **secretory immunoglobulin A (SIgA)** and **secretory immunoglobulin M (SIgM)**; the former class of antibodies constitutes the largest non-inflammatory defense system of the body (Russell *et al.*, 1989 and Brandtzaeg *et al.*, 1999). The secretory antibody system performs immune exclusion by inhibiting colonization of pathogens and penetration of harmful soluble antigens. Interestingly, innocuous proteins and components of commensal bacteria do not only stimulate the secretory antibody system, but also activate suppressive mechanisms collectively called "oral tolerance" when induced via the gut (Brandtzaeg, 1996 ; 1998). The mucosal surfaces of the gastrointestinal and respiratory tracts, like the skin, are colonized by lymphocytes and accessory cells in order to respond optimally to ingested and inhaled antigens. In the mucosa of the gastrointestinal tract, lymphocytes are found in large numbers in three main regions; within the epithelial layer, scattered throughout the lamina propria, and in organized collections in the lamina propria such as Peyer's patches. Cells at each site have distinct phenotypic and functional characteristics. The intestinal lamina propria contains a mixed population of immune cells. These include T lymphocytes, most of which are $CD4^+$ and have the

phenotype of activated cells. It is likely that T cells initially recognize and respond to antigens in regional mesenteric lymph nodes and migrate back to the intestine to populate the lamina propria. The lamina propria also contains large numbers of activated B lymphocytes and plasma cells, as well as macrophages, dendritic cells, eosinophils, and mast cells (Abbas *et al.*, 2000). The **mucosal associated lymphoid tissue (MALT)** has been well defined in the gastro-intestinal, respiratory and nasal tracts, with the inductive sites of aggregated lymphoid tissues (such as Peyer's patches in the gut) and effector sites of the respective mucosal tissues (Gowans and Knight, 1964; Mestecky *et al.*, 1994; McDermott and Bienenstock, 1979; McGhee *et al.*, 1999; and Weisz-Carrington *et al.*, 1987). Genital immunity within the MALT was documented by both oral and nasal immunization inducing antibodies in the genital tract in mice (McDermott *et al.*, 1979). A significant finding was that genital or rectal immunization elicits not only local mucosal but also systemic T and B cell immune responses (Lehner *et al.*, 1992; 1993; Belyakov *et al.*, 1998 and Eriksson *et al.*, 1999).

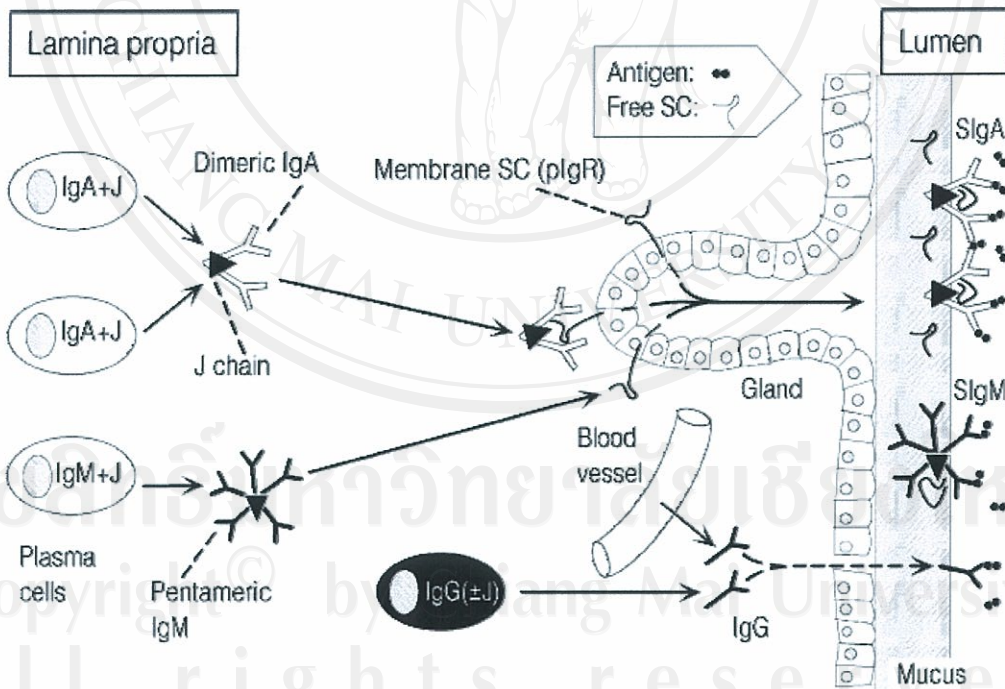
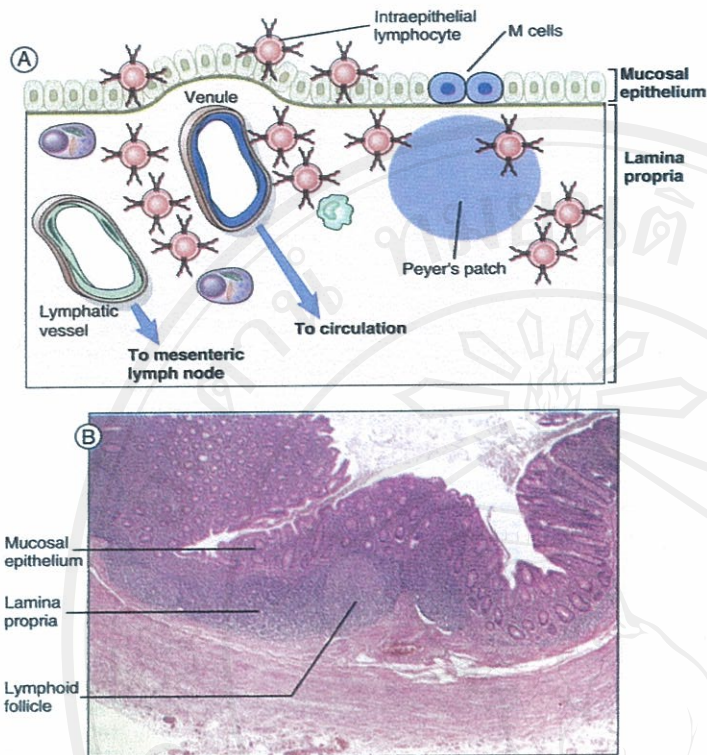


Figure 6. Model for external transport of J chain-containing dimeric IgA and pentameric IgM by pIgR, expressed basolaterally as membrane SC on glandular epithelial cells; secretory immunoglobulin A (SIgA), secretory immunoglobulin M (SIgM), membrane secretory component (SC), polymeric Ig receptor (pIgR), J(junction) (Adapted from Brandtzaeg, 2003).



A. Schematic diagram of the mucosal immune system in gastrointestinal tract

B. Photomicrograph of the mucosal immune system in gastrointestinal tract

Figure 7. The mucosal immune system (Adapted from Abbas *et al.*, 2000).

2.5.3 Adaptive immunity

If a microorganism overwhelms the body's natural resistance, a third line of defensive resistance exists. Acquired or adaptive immunity is a more recently evolved mechanism. It allows the body to recognize, remember, and respond to a specific stimulus, an antigen. Adaptive immunity can result in the elimination of microorganisms and in the recovery from disease, and it frequently leaves the host with specific immunologic memory. This condition of memory or recall, acquired resistance, allows the host to respond more effectively if reinfection with the same microorganism occurs.

Adaptive immunity, like natural immunity, is composed of cellular and humoral components. The major cellular component of this mechanism is the lymphocyte; the major humoral component is the antibody. Lymphocytes selectively respond to nonself materials, antigens, which leads to immune memory and a permanently altered pattern of response or adaptation to the environment. The majority of the actions of the two categories of the adaptive

response, humoral-mediated and cell-mediated immunity are exerted by the interaction of antibody with complement and the phagocytic cells of natural immunity, and of T cells with macrophages (Turgeon, 1996).

2.5.3.1 Humoral-mediated immunity

If specific antibodies have been formed to antigenic stimulation, they are available to protect the body against foreign substances. The recognition of foreign substances and subsequent production of antibodies to these substances are the specific means by which infection is acquired if the antibodies are formed by the host or received from another source. These two types of acquired immunity are called active and passive immunity, respectively.

Active immunity can be acquired by natural exposure in response to an infection or natural series of infections, or it may be acquired by an intentional injection of antigen, vaccination, is an effective method of stimulating antibody production and memory (acquired resistance) without suffering from the disease. Suspensions of antigenic materials used for immunization are varied and may be of animal or plant origin. These products may be composed of living suspensions of weak or attenuated cells or viruses, killed cells or viruses, or extracted bacterial products such as the altered and no-longer-poisonous toxoids used to immunize against diphtheria and tetanus. The selected agents should stimulate the production of antibodies without clinical signs and symptoms of disease in an immunocompetent (a host that is able to recognize a foreign antigen and build specific antigen-directed antibodies) and cause permanent antigenic memory (Turgeon, 1996).

The antibodies produced in a humoral response to antigenic stimulus are heterogeneous in specificity and may include all immunoglobulin classes. This heterogeneous response is owing to the fact that most antigens have multiple antigenic determinants that trigger off the activation of different B cells. Therefore, the serum of any mammal contains a heterogeneous mixture of immunoglobulin molecules. The specificities of these immunoglobulin molecules will reflect the organism's past antigenic exposure and history.

The first antibody produced in response to a primary exposure of an immunogen is IgM. When the immunogen is persistent or the host is re-exposed to the immunogen other classes of antibody may be produced as well as IgM. The body compartment in which the immunogen is

presented can determine the predominant antibody isotype produced (e.g., IgA in the gastrointestinal tract). In general, primary exposure to an immunogen stimulates the production of IgM initially, followed by the appearance of IgG, (Crowther, 1995) see in Figure 8.

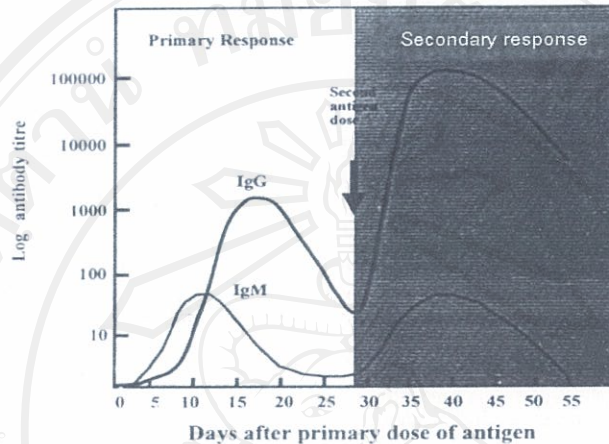


Figure 8. Anamnestic responses following second administration of antigen. Primary response following initial antigen dose has a lag phase. On secondary stimulation, there is an almost immediate rise in titer and higher levels of antibodies are achieved that are mainly IgG (Crowther, 1995).

2.5.3.2 Cell-mediated immunity

Cell-mediated immunity consists of immune activities that differ from antibody-immunity. Lymphocytes are the unique bearers of immunologic specificity, which depends on their antigen receptors. The full development and expression of immune responses, however, require that nonlymphoid cells and molecules primarily act as amplifiers and modifiers. Cell-mediated immunity is moderated by the link between T lymphocytes and phagocytic cells (e.g., monocytes-macrophages), (Turgeon, 1996). B type of lymphocyte can probably respond to a native antigenic determinant of the appropriate “fit”; a T type of lymphocyte responds to antigens presented by other cells in the context of MHC proteins (Turgeon, 1996). T type of lymphocyte does not directly recognize the antigens of microorganisms or other living cells such as allografts (a graft of tissue from a genetically different member of the same species, e.g., a human kidney), but rather when the antigen is present on the surface of an antigen-presenting cell, the

macrophage. Antigen-presenting cells (APCs) were at first thought to be limited to cells of mononuclear phagocyte system. Recently, other kinds of cells (e.g., endothelial and glial), have been demonstrated to have the ability of “present” antigens (Turgeon, 1996).

Lymphocytes are immunologically active through various types of direct cell-to-cell contact and by the production of soluble factors. Nonspecific soluble factors are made by or act of various elements of the immune system. These molecules are collectively called cytokines. Some mediators that act between leukocytes are called interleukins (Turgeon, 1996).

2.6 Antioxidants and the immune system

Antioxidants are important to animal health. They function as antioxidants to remove harmful free radicals produced through normal cellular activity and from environmental stressors, thereby maintaining the structural integrity of immune cells. In addition, they may regulate cellular events. Therefore antioxidants are important to animal health by enhancing immunity (Chew, 1996).

2.6.1 Antioxidant enzymes

The primary antioxidant defense is enzymatic, involving SOD, GSH and CAT. The enzyme SOD decomposes superoxide radicals by converting them to hydrogen peroxide plus oxygen. GSH and CAT are enzymes that decompose peroxides, particularly hydrogen peroxide. SOD are two forms: a mitochondria enzyme, which contains manganese, and a cytosolic enzyme, which contains copper and zinc. GSH contains Se while CAT contains iron (Hughes, 2002).

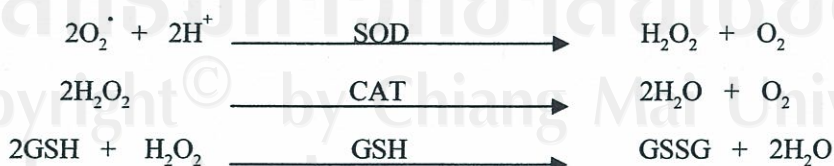


Figure 9. Antioxidant enzymes reaction mechanisms (Hughes, 2002).

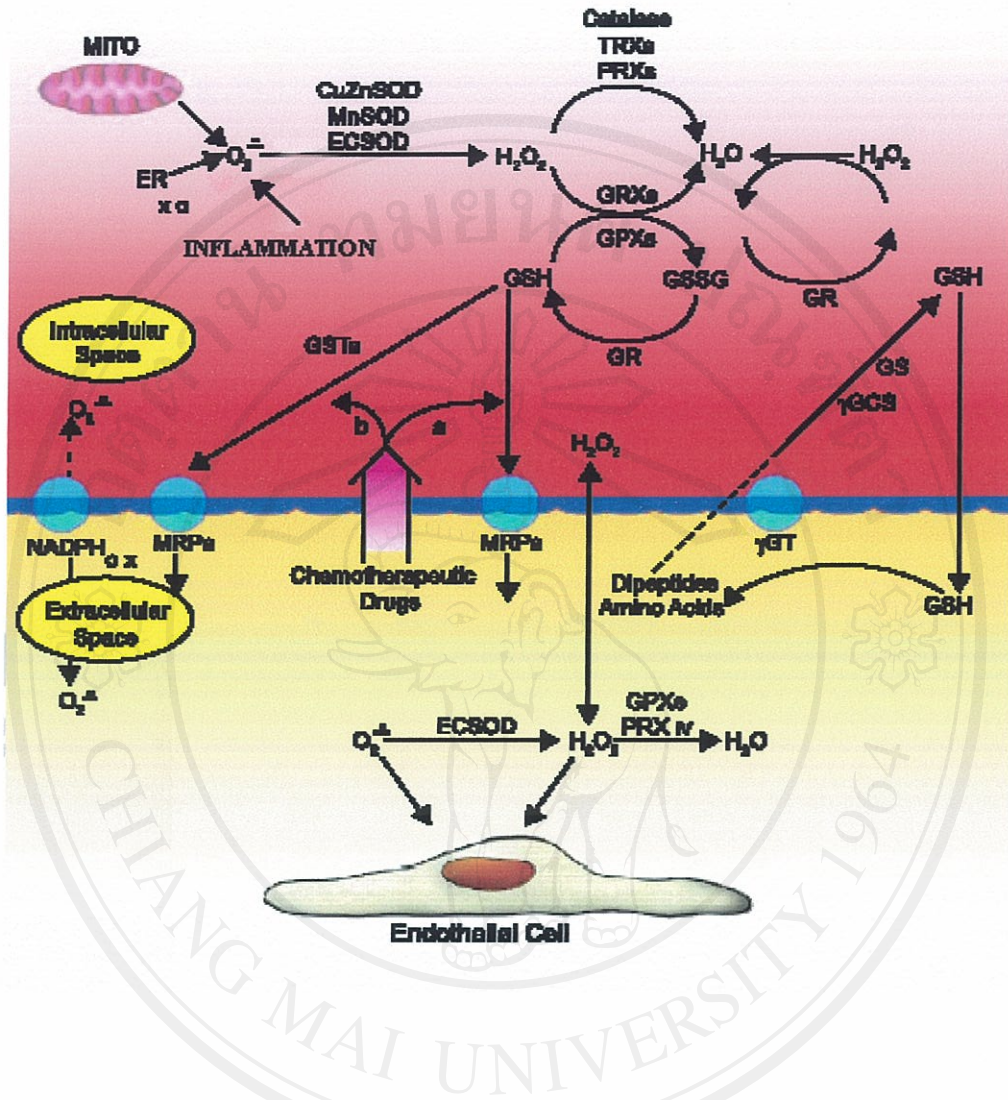


Figure 10. Multiple antioxidant pathways participate in the scavenging of superoxide and hydrogen peroxide in the intracellular and extracellular spaces. ER, endoplasmic reticulum; TRXs, thioredoxins (and thioredoxin reductases); PRXs, peroxiredoxins (thioredoxin peroxidase); GRXs, glutaredoxins; GPXs, classical (intracellular) glutathione peroxidase; γ GT, γ -glutamyl transpeptidase; catalase; GR, glutathione reductase; γ GCS, γ -glutamyl cysteine synthetase (glutamate cysteine ligase); GSH, reduced glutathione; GSSG, oxidized glutathione; and MRP, multidrug resistant protein. MRPs have also been called GSH-conjugate carriers and GS-X pumps. ECSOD, extracellular glutathione peroxidase (GPXe), and peroxiredoxin IV (PRX IV) (Kinnula and Crapo, 2003).

2.6.2 Natural antioxidants

Highly reactive free radicals are generated from normal cellular metabolism and from the ingestion / inhalation of environmental pollutants and drugs. These free radicals, when allowed to accumulate, are capable of destroying the integrity of cellular membranes, enzymes and nuclear DNA. Antioxidants serve to stabilize these highly reactive free radicals, thereby maintain the structural and functional integrity of cells. Therefore, antioxidants are very important to the health and production capacity of animals. Among the antioxidants normally consumed by animals, the antioxidant vitamins are of particular importance. These naturally occurring antioxidant vitamins include those vitamins A, E and C and carotenoids (Chew, 1996), and γ -oryzanol has been suggested to possess the capability of antioxidant functionality (Duve and White, 1991).

2.6.2.1 Vitamin A

The incidence of infection is greatly increased in animals with vitamin A deficiency (Jackman and Gibbs, 1968; Nelson *et al.*, 1968; Scrimshaw *et al.*, 1968). Vitamin A deficient rodents and chickens showed decreased cellularity of the spleen and thymus (Bang *et al.*, 1973; Davis and Sell, 1983; Butera and Krakowka, 1986). Both *in vitro* and *in vivo* studies, reported that vitamin A increased mitogen-induced lymphocyte proliferation (Chew, 1987). Mitogen-induced splenic lymphocyte proliferation was reduced in rats deficient in vitamin A (Nauss *et al.*, 1979 and Mark *et al.*, 1983). Retinol and retinoic acid stimulated the proliferation of bovine blood lymphocytes (Daniel *et al.*, 1986) *in vitro*. However, high concentrations of retinol *in vitro* suppressed human T cell supplemented with retinoic acid and retinyl palmitate (Dennert and Lotan, 1978; Tomita *et al.*, 1985). Retinol stimulated NK cell activity in mice (Fraker *et al.*, 1986) while retinyl acetate failed to produce similar enhancement in mouse thymocytes and spleenocytes (Moriguchi *et al.*, 1985).

Blood antibody titers were stimulated in vitamin A supplemented mice immunized with antigens (Jurin and Tannock, 1972; Cohen and Cohen, 1973). Mice supplemented with retinoic acid had secondary IgG and IgE responses to ovalbumin (Barnett, 1983). Rats fed a vitamin A deficient diet had decreased local intestinal fluid but not serum IgA secretion (Sirsinha *et al.*,

1980). The number of splenic plaque-forming cells in response to sheep RBC was increased (Cohen and Cohen, 1973) or unaltered in mice fed vitamin A (Dennert and Lotan, 1978).

Phagocytosis of opsonized and non-opsonized particles by peritoneal macrophages was enhanced in mice supplemented with high levels of retinyl palmitate (Moriguchi *et al.*, 1985). Retinol and retinoic acid had increased phagocytic and killing activity (Tjoelker *et al.*, 1986).

2.6.2.2 Vitamin E

Vitamin E is the major antioxidant in blood. Vitamin E reacts with peroxy radicals produced from PUFA in the membrane phospholipids or lipoproteins to yield a stable lipid hydroperoxide.

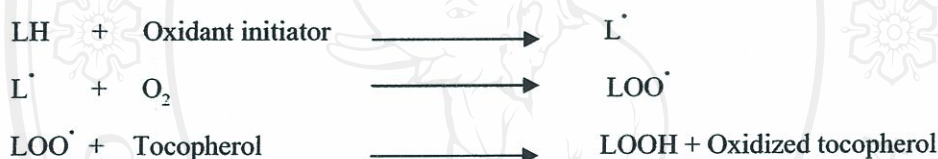


Figure 11. Vitamin E reactions in the scavenging of Lipid peroxy radicals (Chew, 1996).

Through this biochemical reaction, vitamin E effectively reduces harmful lipid free radicals and thereby protects tissues from free radical attack. This antioxidant activity of vitamin E in preventing lipid peroxidation is one of the possible mechanisms by which vitamin E enhances immune response.

Vitamin E supplementation increased the resistance of chicks against *Escherichia coli* (Heinzerling *et al.*, 1974) and of lambs against Chlamydia pathogen (Stephens *et al.*, 1979). The role of vitamin E in modulating cellular and non-cellular host defense has been reported. Weanling pigs fed a diet deficient in vitamin E and **selenium** (Se) had lower mitogen-induced lymphocyte proliferative response when cells were incubated with autologous serum (Lessard *et al.*, 1991). Similarly, peripheral lymphocytes obtained from sows fed a diet deficient in vitamin E or in vitamin E and Se had reduced mitogen-induced proliferation during the immediate prepartum and postpartum periods (Wuryastuti *et al.*, 1993). The authors (Bendich *et al.*, 1986) fed diets deficient in vitamin E but otherwise adequate in all essential nutrients showed decreased

T and B cell response. The authors (Bendich *et al.*, 1986) observed that the immune system was affected well before there were signs of overt vitamin E deficiency. Similarly, rats fed a diet deficient in vitamin E and Se had lower mitogen-induced lymphocyte proliferative response (Eskew *et al.*, 1985). A positive correlation between splenic vitamin E concentration and mitogen-induced splenic lymphocyte proliferation was reported in mice (Mbawuike *et al.*, 1982). Mice deficient in vitamin E and Se had lower NK cell-mediated cytotoxicity and T-lymphocyte-mediated cytotoxicity (Meeker *et al.*, 1985).

Vitamin E also modulates humoral immunity. Pigs supplemented with vitamin E and Se had increased immunoglobulin production (Peplowski *et al.*, 1980). Young and old C57BL/6 male mice were given a diet containing a high dose of vitamin E and its effect on the immune system was examined before and after the exposure to restraint stress, the results in the present study suggested that the vitamin E treatment was effective in the prevention of immunological decline of young mice before and after the exposure to the stress (Wakikawa *et al.*, 1999). Vitamin E deficient laboratory animals showed depressed number of plaque-forming cells (Corwin and Xhloss, 1980; Gebremichael *et al.*, 1984). Injection of vitamin E and Se into sheep simultaneously with *Brucella ovis* (Afzal *et al.*, 1984) or *Clostridium perfringens* (Tengerdy *et al.*, 1983) showed enhancement of antibody response. Lambs supplemented with vitamin E and challenged with parainfluenza virus had higher serum antibody titers after a secondary challenge (Reffett *et al.*, 1988). Similarly, steers supplemented with vitamin E and Se had higher antibody response to *Pasteurella haemolytica* vaccination (Droke and Loerch, 1989). Increased antibody response from vitamin E supplementation also occurred by way of transfer of immunoglobulins from the dam to the offspring as shown in sows (Hayek *et al.*, 1989) and in hens through the yolk sac (Jackson *et al.*, 1978 and Nockels, 1979).

The importance of vitamin E in regulating phagocyte function also has been reported. The phagocytic and bactericidal activities of colostral PMN, isolated from sows fed a vitamin E deficient or a vitamin E and Se deficient diet during gestation were suppressed (Wuryastuti *et al.*, 1993). Similarly, PMN from vitamin E deficient rats had lower phagocytic activity, decreased chemotaxis and protection against autooxidative damage (Harris *et al.*, 1980). Vitamin E administration increased the phagocytic activity of the reticuloendothelial system in mice (Heinzerling *et al.*, 1974). PMN from dairy cows injected with vitamin E and Se had enhanced

phagocytic and bactericidal activity as compared with Se deficient cows (Gyang *et al.*, 1984). Vitamin E and Se also may guard against peroxidation of arachidonic acid (Likoff *et al.*, 1981; Lawrence *et al.*, 1985) and thereby alter arachidonic acid metabolism in animals (Bryant and Bailey, 1980; Aziz and Klesius, 1986). This mechanism could account for the observed enhanced immune response by maintaining the functional integrity of immune cells and tissues.

2.6.2.3 Vitamin C

Vitamin C may serve to reduce the tocopheroxyl radical, thereby restoring the radical scavenging activity of vitamin E (Niki, 1987). Bendich *et al.*, (1984) reported that higher concentrations of vitamin E in lungs from guinea pigs supplemented with vitamin C as compared with those not given vitamin C.

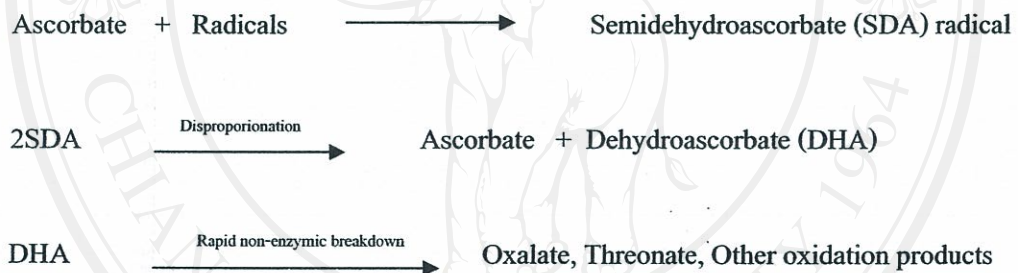


Figure 12. Vitamin C reactions in the scavenging of radicals (Halliwell and Gutteridge, 1999).

Vitamin C supplemented at the rate of 330 mg kg⁻¹ reduced mortality and pericarditis in chicks infected with *Escherichia coli* (Gross *et al.*, 1988). The amount of vitamin C needed for this protective effect increased with higher environmental stress level. Similarly, calves supplemented with vitamin C had a lower incidence of scouring (Cummins and Brunner, 1989). Vitamin C supplementation decreased the rate of mortality in channel catfish infected with *Edwardsiella ictaluri*, a bacterium that causes enteric septicemia (Li and Lovell, 1985). In the latter study, vitamin C deficient catfish had lower antibody response to *Edwardsiella ictaluri* antigen, decreased complement activity and depressed PMN phagocytic activity. However, the bactericidal activity of the peripheral PMN was not affected. Vitamin C deficiency reduced (Derubertis *et al.*, 1974; Fraser *et al.*, 1978) or had no significant effect (Bendich *et al.*, 1984) on lymphocyte mitogenesis

in guinea pigs. Dietary vitamin C supplementation increased concentrations of blood immunoglobulins in dairy calves in one study (Blair and Cummins, 1984) but not in another (Cummins and Brunner, 1989). In guinea pigs, vitamin C was shown to be important in maintaining normal primary and secondary antibody responses (Kumar and Axelrod, 1969). Chicks fed megadoses (1 % of diet) of vitamin C did not show enhanced delayed type hypersensitivity, graft vs. host rejection, or antibody response to sheep red blood cells (McCorrkle *et al.*, 1980). However, adult birds fed the vitamin C had higher antibody titers against *Brucella abortus* whereas chicks had suppressed concentrations of the antibody.

Vitamin C is found in high concentrations in blood leukocytes of that (Moser, 1987). The high concentration of vitamin C in PMN is essential for its function (Anderson and Lukey, 1987). Also, the protective effect of vitamin C may partly be mediated through its ability to reduce circulating glucocorticoids (Basu and Schorahm, 1982). Dietary vitamin C reduces circulating glucocorticoids in chickens (Nockels *et al.*, 1973). Also the suppressive effect of the corticoids on PMN function in cattle is alleviated with vitamin C supplementation (Roth and Kaeberle, 1985).

2.6.2.4 Carotenoids

Carotenoids include a group of over 600 pigmented compounds found in nature. A few of these possess provitamin A activity. Seifter *et al.*, (1981) reported a marked stimulatory action of β -carotene on the growth of the thymus gland and a large increase in the number of small thymic lymphocytes. In contrast, retinoic acid supplementation resulted in a reduced number of thymic lymphocytes and delayed skin graft rejection response. Tomita *et al.*, (1987) reported that mice fed β -carotene had augmented tumor immunity against syngeneic fibrosarcoma cells. They further demonstrated that the action of β -carotene was specific against the antigens.

Carotenoids function as antioxidants by deactivating reactive chemical species such as free radicals, singlet oxygen and photochemical sensitizers (Burton, 1989). For example, β -carotene functions as a chain-breaking antioxidant in a lipid environment. The peroxy radicals (ROO \cdot) formed from lipids (especially polyunsaturated phospholipids) are very damaging to cells.



Figure 13. Carotenoids reactions in the scavenging of radical (Chew, 1996).

The extensive system of double bonds makes carotene and other carotenoids susceptible to be attacked by peroxy radicals, resulting in the formation of inactive products. Through their antioxidant actions, carotenoids could directly or indirectly regulate immune function. Both *in vitro* and *in vivo* studies have reported the enhancement of host defense functions (Chew, 1993a) and reproduction (Chew, 1993b) in animals and humans supplemented with carotenoids. Holstein cows supplemented with β -carotene had increased mitogen-induced lymphocyte proliferation during the peripartum period (Heirman *et al.*, 1990). Vitamin A did not have a similar response profile. Likewise, blood lymphocytes isolated from Holstein cows during the peripartum period (Daniel *et al.*, 1990b) and from non-lactating pregnant heifers (Daniel *et al.*, 1986) and incubated *in vitro* with β -carotene showed higher mitogen-induced lymphocyte proliferation than unsupplemented cultures. Retinol had no effect on lymphocyte proliferation, Tjoelker *et al.*, (1988b) reported that β -carotene inhibited lymphocyte proliferation *in vitro* in dairy cows immediately prior to and after drying off. The discrepancy likely reflects differences in the concentration of β -carotene used in the incubation mixture, the concentrations of blood β -carotene and vitamin A of the lactating cows. The effects of blood β -carotene and vitamin A concentrations and lactational status of the cow on the *in vitro* effects of β -carotene on lymphocyte proliferation have been demonstrated (Tjoelker *et al.*, 1990). The stimulatory activity of carotenoids on lymphocyte blastogenesis has similarly been demonstrated in pigs (Hoskinson *et al.*, 1989, Hoskinson *et al.*, 1992) and in rats (Bendich and Shapiro, 1986). Increased numbers of helper and inducer T lymphocytes have been reported in human adults given oral β -carotene supplementation (Alexander *et al.*, 1985; Watson *et al.*, 1991).

In vitro, β -carotene enhanced the bactericidal activity of bovine blood and mammary PMN isolated from cows during the peripartum period (Daniel *et al.*, 1990a). Similarly, blood

PMN isolated from Holstein cows fed β -carotene had higher bacterial killing ability during the peripartum period (Michal *et al.*, 1994). The incensement of bacterial killing could be accounted for partly by increased myeloperoxidase activity in the PMN. Tjoelker *et al.*, (1988a) reported that β -carotene stimulated PNM phagocytic and bacterial killing ability in dairy cows before and after drying off. Incontrast, retinol acid generally decreased phagocytosis and had no effect on kelling activity. Blood PNM from cows supplemented with β -carotene during the period around dry off maintained their phagocytic ability as opposed to the decreased phagocytic ability observed with PMN from cows fed only preformed vitamin A (Tjoelker *et al.*, 1990). Schwartz *et al.*, (1990) reported increased cytochrome oxidase and peroxidase activities in macrophages incubated with canthaxanthin, β -carotene, and α -carotenecompared with incubation with 13-cis retinoic acid. The stimulatory activity of canthaxanthin was greater than observed with β -carotene and α -carotene. Phagocytosis also was stimulated by these carotenoids, even though to a lower degree. All of these changes indicate increased respiratory bursts by the macrophages when they are exposed to carotenoids. Carotenoids may regulate immune cell function by protecting them against reactive oxygen species, by altering membrane fluidity, by increase gap-junctional intercellular communication, and by inhibiting arachidonic acid oxidation.

2.6.2.5 γ -oryzanol

Gamma oryzanol is not a single compound but instead comprises a variety of ferulic acide esters called α , β and γ -oryzanol. The triterpene alcohol components of a typical γ -oryzanol consist primarily of cycloartenol and 24-methylenecycloartanol but they also include other minor sterols, such as campestanol, stigmastanol, β -sitosterol, cycloartanol and cholesterol. Gamma oryzanol, is of substantial commercial significance in Japan as a food and medical antioxidant, especially when used in synergy with α -tocopherol, γ -oryzanol markedly inhibits the oxidation of rice bran oil, malondialdehyde generation during iron-mediated microsomal lipid peroxidation. Due to its excellent emulsifying properties and high UV absorption, γ -oryzanol also is the active ingredient in various cosmetic soaps. Furthermore, γ -oryzanol has been suggested to possess the capability of antioxidant functionality (Duve and White, 1991). All components exhibited significant antioxidant activity in the inhibition of cholesterol oxidation. The antioxidant activity of γ -oryzanol has been evaluated in a few models using chemicals to

induce oxidation and measuring the generation of oxidized products. One of them is a cholesterol oxidation model accelerated by **2,2'-azobis (2-methylpropionamide) dihydrochloride (AAPH)**, which was established to compare the inhibitory capability of γ -oryzanol and vitamin E on production of oxidized cholesterol (Xu and Godber, 2001). The antioxidant activity was determined in terms of the degree to which the generation of seven oxidized cholesterol products (7- α - and - β -hydroperoxycholesterol, 5,6- α - and - β -epoxycholesterol, 7- α - and - β -hydroxycholesterol and 7-ketcholesterol) were prevented in the presence of three major components of γ -oryzanol (cycloartenyl ferulate, 24-methylene cycloartenyl ferulate and campesteryl ferulate) or vitamin E components (α -tocopherol, γ -tocopherol, α -tocotrienol, γ -tocotrienol). The results suggested that 24-methylene cycloartenyl ferulate had the highest antioxidant activity and all three major components of γ -oryzanol had higher antioxidant activity than vitamin E components (Xu and Godber, 2001). Another assessment of antioxidant activity of γ -oryzanol using AAPH as a peroxy radical generator utilized an oxygen radical absorbance capacity assay (Huang, 2002). This assay used cyclic (α -1,4)-linked oligosaccharides of α -D-gluco-pyranose, which have both hydrophilic and hydrophobic regions, to prepare lipophilic antioxidants such as vitamin E isomers and γ -oryzanol, and fluorescein as a target of free radical attack. Antioxidant activity was determined as the degree to which the fluorescence decay of fluorescein caused by AAPH was inhibited over time. Similarly, their results showed that γ -oryzanol possessed the greatest antioxidant activity compared to other antioxidants tested including α -tocopherol, γ -tocopherol, δ -tocopherol and tocotrienols, while α -tocopherol acetate had zero effect. It seemed that γ -oryzanol had the greatest antioxidant activity towards peroxy radicals generated by AAPH and it was proposed that the antioxidant mechanism of γ -oryzanol might be due to its intramolecular hydrogen-bonded methoxyphenols, where hydrogen donation would occur more readily. It has also been postulated that the phenol group is the key functional group for antioxidant activity and that steric hindrance around the phenol group decreases antioxidant activity. For example, in their model of the antioxidant activity of tocopherols, Huang (2002) found that relative order of antioxidant activity was $\alpha < \gamma < \delta$ and the reason was explained relative to the number of methyl groups *ortho* to the phenol group where α has two, γ has one and δ has zero. The theory has also been approached in a review of the antioxidant chemistry of ferulic acid (Graf, 1992). It was explained that the antioxidant activity of ferulic acid is due to its phenolic hydroxyl group that has

hydrogen donating property to scavenge a reactive radical and form a phenoxy radical. This radical is then stabilized resonantly because the unpaired electron can be delocalized across the entire molecule. The second phenolic hydroxyl group can enhance the radical-scavenging property by providing additional resonance stabilization and form quinone. The methoxyl group of ferulic acid partially destabilizes the phenoxy radical and impairs its antioxidant activity (Graf, 1992).

In addition, rice bran oil have demonstrated an ability to improve the plasma lipid pattern of rodents, rabbits, non-human primates and human, reducing total plasma cholesterol and triglyceride concentration and increasing the high density lipoprotein cholesterol level. Other potential properties of rice bran oil and γ -oryzanol, studied both *in vitro* and in animal models, include modulation of pituitary secretion, inhibition of gastric acid secretion, antioxidant action and inhibition of platelet aggregation (Cicero and Gaddi, 2001). Ferulic acid may have anticarcinogenic properties through the inhibition of the formation of N-nitroso compounds, and has been found to suppress benzo[a]pyrene-induced neoplasia in the forestomach of mice (Kuenzig *et al.*, 1984 and Wattenberg *et al.*, 1980). Furthermore, γ -oryzanol reduced serum cholesterol in mice (Nakamura, 1996). This effect has been studied further in rats and hamsters (Deckere and Korver, 1996). Amounts of γ -oryzanol lower than or equal 0.2 % of diet decreased serum cholesterol in animals made hypercholesterolemic by dietary cholesterol. Gamma oryzanol decreased liver cholesterol (Seetharamaiah and Chandrasekhara, 1993 and Seetharamaiah and Chandrasekhara, 1989). Cycloartenol is one of the main sterols of γ -oryzanol and has been shown to decrease serum cholesterol (Norton, 1995). However, no effect was found, possibly because of the presence of 1 % sitosterol or the use of safflower oil in the diet. Cycloartenol did decrease liver cholesterol. In addition, another main sterol of γ -oryzanol, 24-methylene cycloartanol, was found to be less effective than cycloartenol (Ikeda and Nakashima-Yoshida Sugano, 1985).

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2.7 Enzyme-Linked Immunosorbent Assay (ELISA)

2.7.1 Direct ELISA

Antigen (Ag) attached to the solid phase is reacted directly with an enzyme-labeled antiserum. This has the disadvantage that all sera raised against different antigens have to be labeled. Thus, this is a poor assay if was used to detect antigen from “crude” samples (containing a high concentration of contaminating substances), since low levels of antigen attach to wells owing to competition for plastic sites by such contaminants. This is a typical assay for use in the estimation of the titer of enzyme-labeled antispecies conjugates. The scheme is shown diagrammatically in Figure 14 (Crowther, 1995).

2.7.2 Indirect ELISA

This is extensively used for the detection and/or titration of specific antibodies from serum samples. The specificity of the assay is directed by the antigen of the solid-phase, which may be highly purified and characterized or relatively crude and noncharacterized. After addition and incubation of the antigen, the wells are washed to get rid of unbound antigen. Serum containing antibodies against this antigen can then be added and diluted in a buffer that prevents the nonspecific adsorption of protein for any free sites on the solid phase not occupied by the antigen (Blocking buffer).

Sera, may be added as a single dilution (Common in epidemiological testing of large number of sera) or as a dilution range. After incubation, the wells are washed to get rid of unbound antibodies. Bound antibody is then detected after incubation, with a single dilution of antispecies antibody conjugated to an enzyme. This is diluted in blocking buffer. The amount of specific antibody binding to the antigen is quantified after addition of color development reagents (enzyme substrate or substrate/ dye combination). The scheme is shown diagrammatically in Figure 15 (Crowther, 1995).

2.7.3 Sandwich ELISA

Antibody is attached to the solid-phase, usually as an IgG fraction of the whole serum. A constant dilution of antibody is attached to the solid phase, and after incubation, unabsorbed antibody is washed away. Antigen at a single dilution or as a dilution range is then added, in

a buffer that prevents nonspecific binding of the serum proteins to any available plastic sites. After incubation, unbound antigen is washed away. Bound antigen is then detected by the addition of enzyme-labeled antibody specific for the “trapped” or “captured” antigen after addition of color development reagents. Figure 16 show the scheme diagrammatically (Crowther, 1995).

2.7.4 Competition ELISA

Competition ELISA involves the adsorption of antibodies to the solid phase. After washing away unadsorbed antibodies, the antigen labeled with enzyme and samples were added. This is pretitrated so that the antigen is saturated, and no free antigenic sites are available for further antibody combination. After washing and development of the assay, the replacement is observed as a decrease in the color expected (that found in control wells containing no competing serum). The scheme is illustrated in Figure 17 (Crowther, 1995).

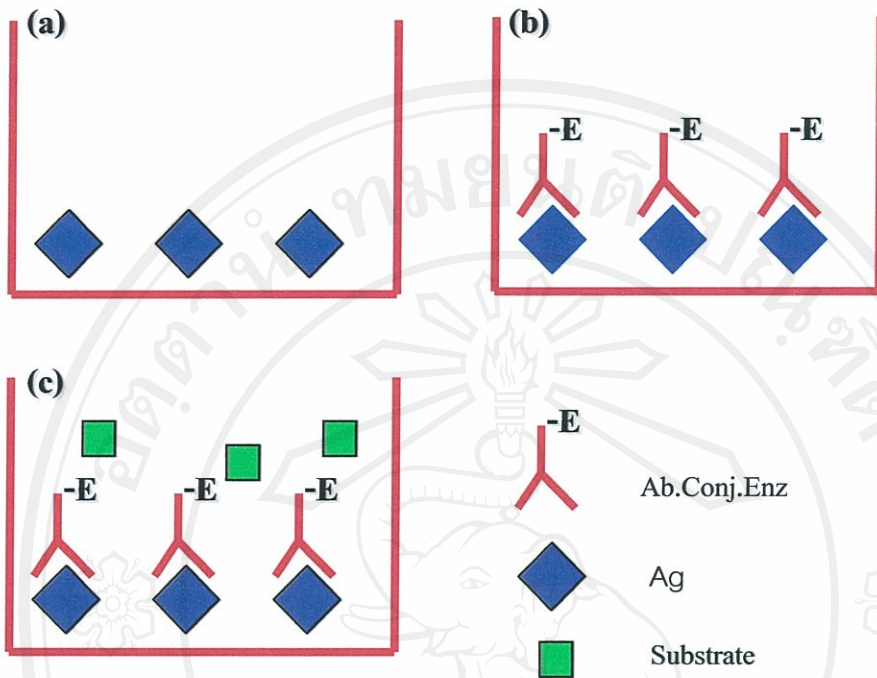


Figure 14. Direct ELISA. Antigen is attached to the solid phase (a). After washing, enzyme-labeled antibodies are added (b). After an incubation period and washing, the substrate is added (c) and the color allowed developed (Crowther, 1995).

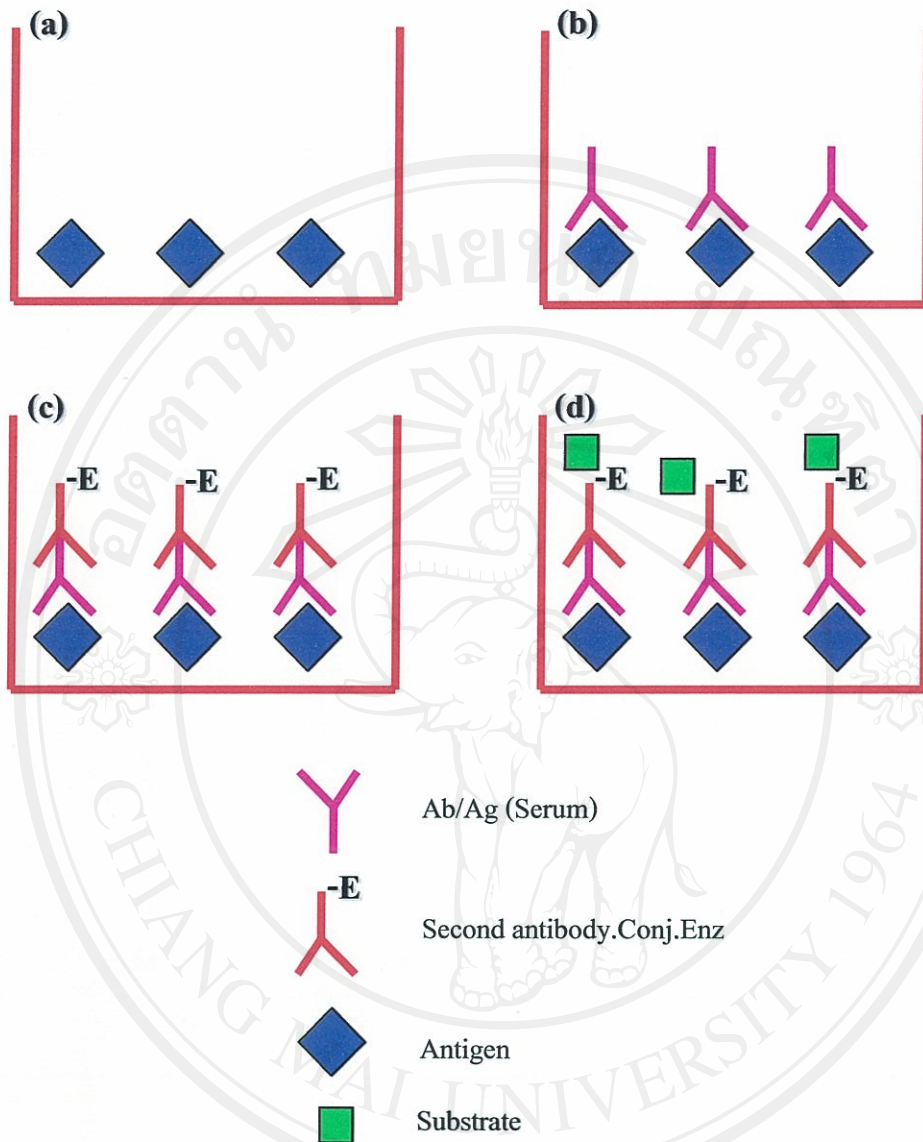


Figure 15. Indirect ELISA. Antigen attached to the solid phase (a), any bound antibodies (b), addition of an antispecies antiserum labeled with enzyme (c). After the substrate is added (d) and the color allowed to developed (Crowther, 1995).

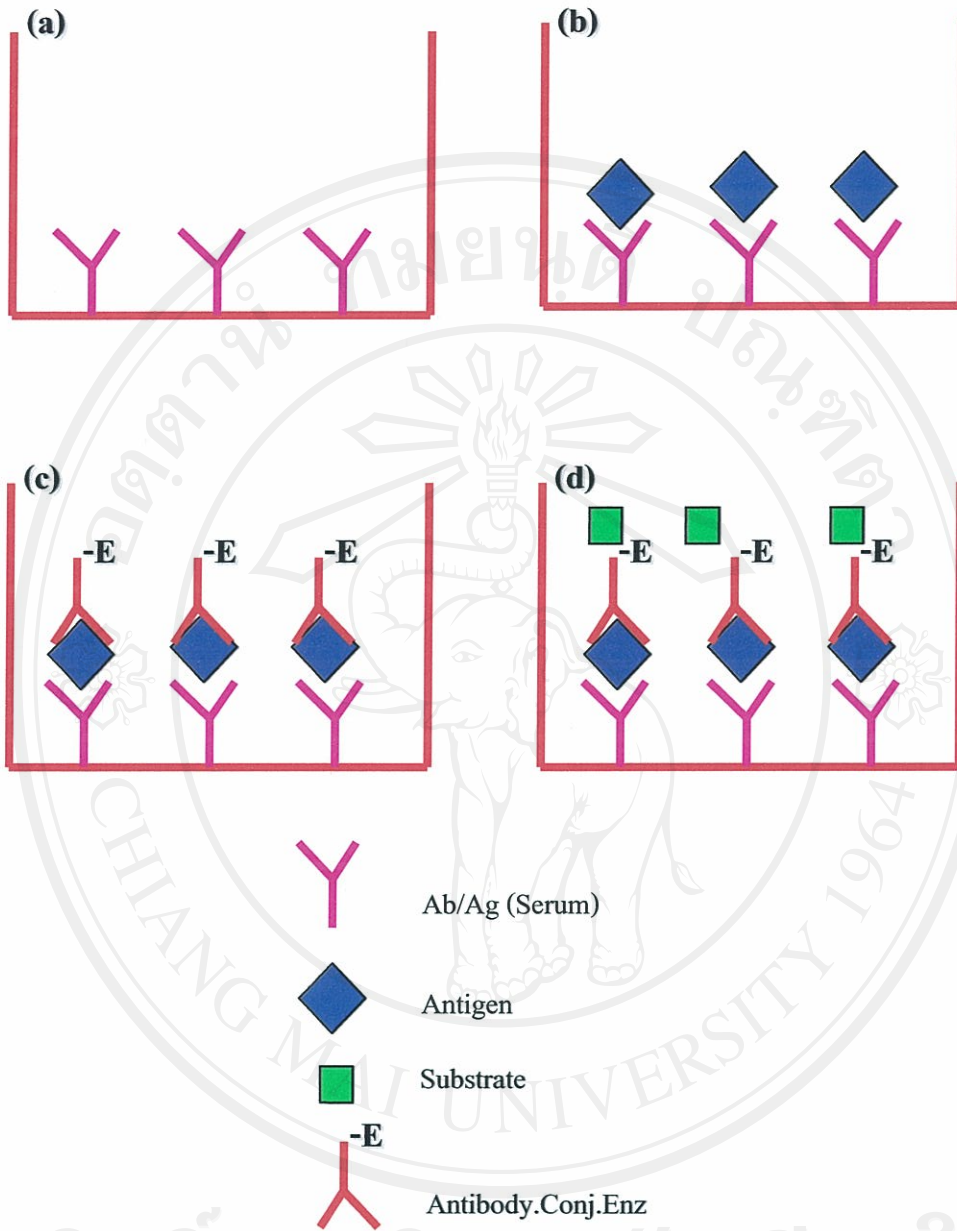


Figure 16. Sandwich ELISA. This system exploits the antibodies attached to the solid phase (a), to capture antigen (b). This then detected using an enzyme-labeled serum specific for the antigen. The detecting antibody is labeled with enzyme (c). After the substrate is added (d) and the color allowed to developed (Crowther, 1995).

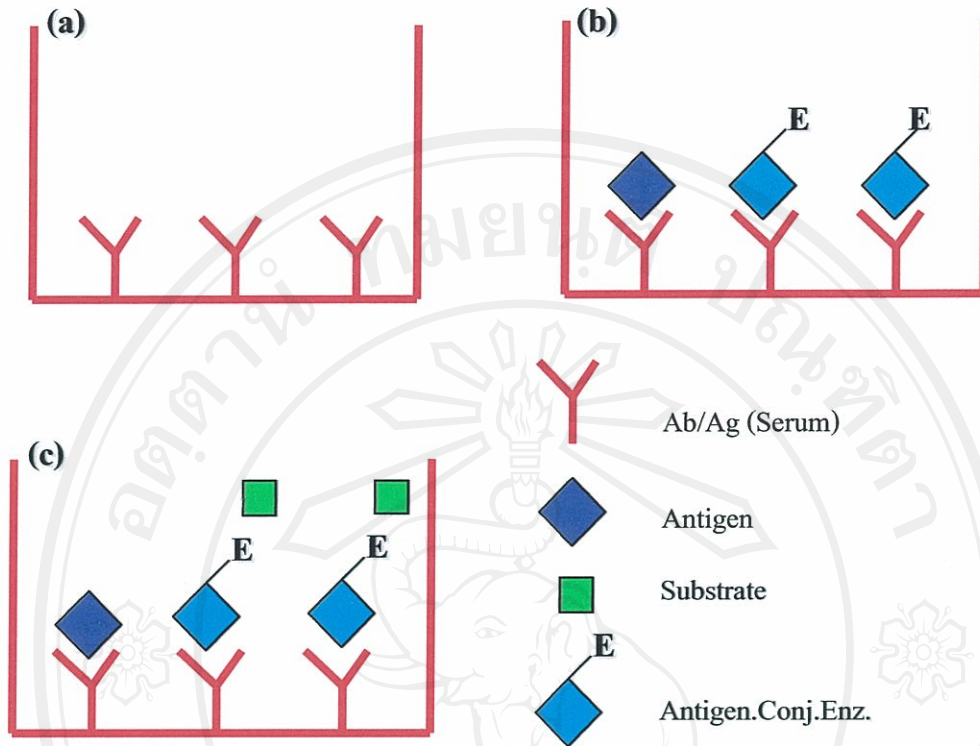


Figure 17. Competitive ELISA. Antibody was attached to the solid phase (a). Then washing away unabsorbed antibodies, the antigen labeled with enzyme was added (b). After the substrate is added and the color allowed to developed (c) (Crowther, 1995).