# CHAPTER II

# BACKGROUND INFORMATION

## 2.1 Egg yolk cholesterol

### 2.1.1 Yolk composition

Approximately 33% of yolk is solid. There are three major constituents: triglyceride-rich lipoproteins, lipovitellin and phosvitin. Minor components comprise immunoglobulins, serum albumin and binding proteins for varieties of vitamins (e.g. thiamine, riboflavin and biotin) (Griffin et al., 1985). Over 95% of yolk cholesterol is associated with the yolk triglyceride-rich lipoproteins. The remainder is bound to lipovitellin, a protein/lipid complex that contains about 20% lipid of which only 4% is cholesterol (Cook, 1986; Noble, 1987). Most cholesterol in yolk is in non-esterified form, although about 20% is present as cholesterol esters in hens fed normal commercial diets (Noble, 1987). Most studies on yolk cholesterol involved measurements of total cholesterol content. Although in recent years, there has been considerable discussion about the adequacy of methods for measuring yolk cholesterol, there now seems to be general agreement that eggs from commercial flocks typically contain about 200 mg cholesterol/egg (Beyer and Jensen, 1989; Van Elsyk et al., 1991).

### 2.1.2 Formation of yolk

The mechanisms involve in yolk formation have been described in detail by Griffin et al. (1985). The major yolk precursors-vitellogenin and the triglyceride-rich lipoproteins are synthesized in the liver of the laying hen and transported in the plasma to the ovary. At any one time, only about 5-7 follicles in the ovary are in the final phase of rapid growth. The thecal layers of these follicles are very well vascularized with unusually permeable capillaries that allow the plasma to leak out into the surrounding tissues, but potential yolk precursors have to pass through several layers in the follicle wall before they

are taken up into the yolk (Figure 2.1). They first have to cross the basal lamina, a connective tissue layer that surrounds the oocyte and acts as a coarse filter preventing passage of large plasma components. The inability of the large chylomicron particles secreted by the avian intestine to enter the basal lamina effectively excludes fat of immediate dietary origin from yolk (Griffin and Perry, 1985). Yolk precursors then pass between the granulosa cells surrounding the oocyte and bind to the oocyte plasma membrane (Griffin, 1992).
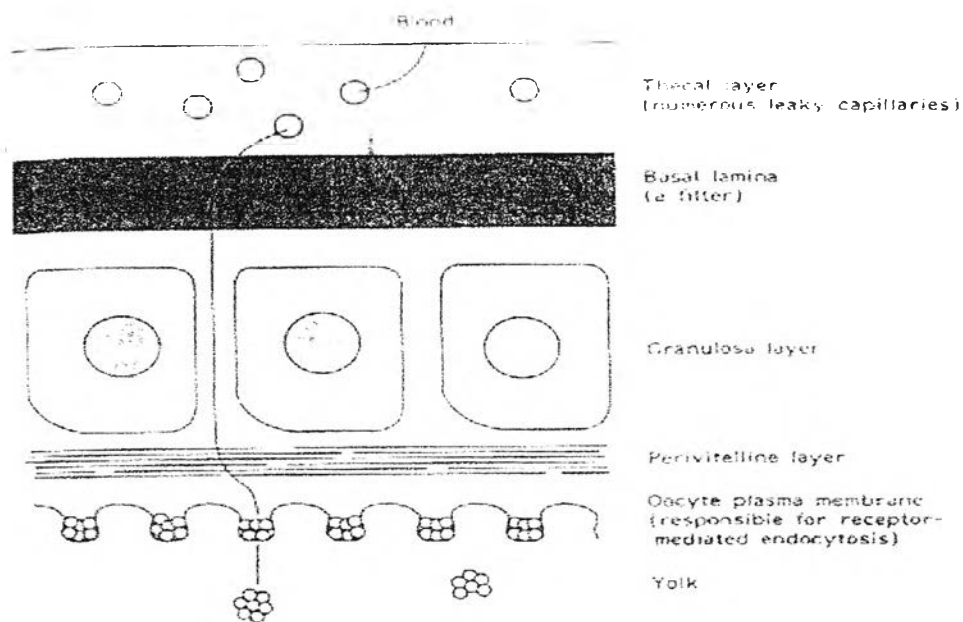


Figure 2.1 Cross-section through the wall of the ovarian follicle (Griffin, 1985).

In Figure 2.1, potential yolk components have to pass out of the thecal layer, through the basal lamina, between the granulose cells before binding to the oocyte plasma membrane and uptake into yolk by receptor-mediated endocytosis (Griffin, 1985). Binding to a sufficient number of receptors induces the formation of coated pits. These then form endocytic vesicles and their incorporation into the oocyte allows the precursors to be deposited to form the yolk. The triglyceride-rich lipoproteins in transit to the yolk are large enough to be visualized with the electron microscope and the sequence of events involved in endocytosis at the oocyte plasma membrane has been clearly demonstrated in a series of electron micrographs by Perry and Gilbert (1979) (Figure 2.2). The developing oocytes grow

from about 3 to 25 mm in diameter in 5-6 days and this requires a high rate of endocytosis that can only be achieved through a rapid recirculation of plasma membrane receptors (Nimpf et al., 1989).
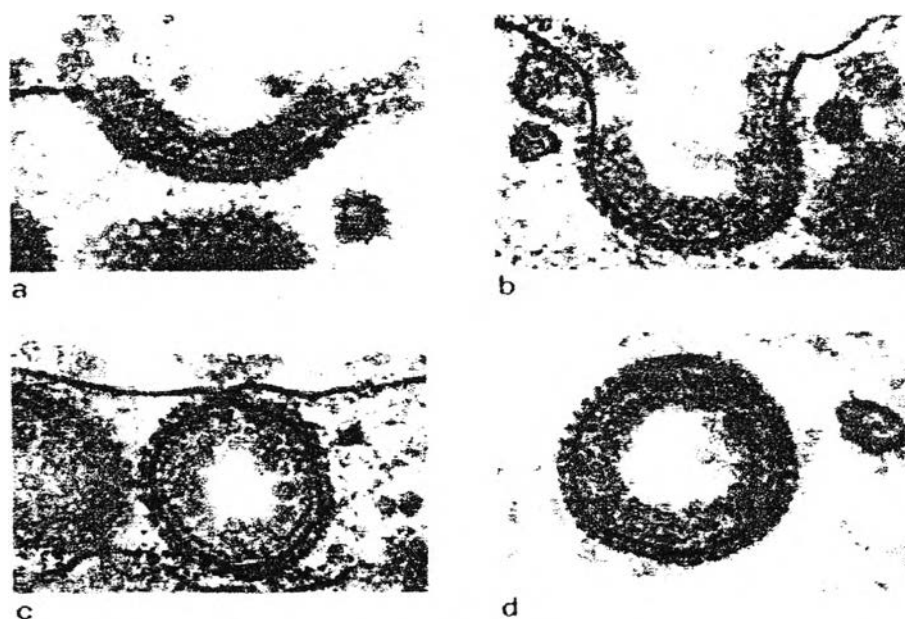


Figure 2.2 Uptake of intact triglyceride-rich lipoproteins across the oocyte plasma membrane (Perry and Gilbert, 1979).

In Figure 2.2, lipoproteins first bind to receptors on the plasma membrane (a). This induces the formation of a coated pit (b) and membrane fusion (c); creating an endocytic vesicle that is now within the oocyte (d). Electron micrographs courtesy of M. Perry. Comparison of concentrations in plasma and yolk suggest that there is an active uptake into the oocyte of triglyceride-rich lipoproteins, vitellogenin and at least some of the vitamin-binding proteins and immunoglobulin classes (Griffin et el., 1985). An obvious interpretation of this early evidence was that yolk composition is in large part determined by the relative proportions of specific receptors on the oocyte plasma membrane (Stifani et al., 1990). The binding of vitellogenin to the receptor appears to be via the lipovitellin part of the molecule rather than the phosvitin moieties (Stifani et al., 1987). The single receptor involved is distinct from the LDL receptor present on other cell types in birds and mammals (Hayashi et al., 1989).

### 2.1.3 Synthesis of egg lipid

The average egg of 60 g contains approximately 6 g of lipid, which is almost wholly confined to the yolk. Maintenance of egg output necessitates, therefore, the transport and turnover of enormous quantities of lipid (Gilbert, 1971), which far exceed that able to be absorbed from the diet. The metabolic effort required to sustain the supply of lipid for yolk formation is thus achieved by a unique and highly organized synthesis and transport system. The increasing in the levels of phospholipids and free cholesterol are not observed, but kinetic studies have suggested that their turnover within the liver is significantly increased (Taurog et al., 1944). The accumulation of lipid in the liver largely occurs through a stimulation of fatty acid and lipid synthesis, in contrast to mammals, is predominantly associated with the liver rather than the adipose tissue (Leveille et al., 1975). Total plasma lipid concentration increases from 200-500 mg/100 ml in immature hen to a level may exceed 200 mg/100 ml. The changes in the plasma lipid concentration which accompany egg laying are associated almost wholly with a dramatic rise in the concentration of the triglyceride-rich lipoproteins, very low density lipoprotein fraction as a result of increased synthesis within the liver (Kudzma et al., 1975).

### 2.1.4 Egg lipid composition

Almost all the lipid of the yolk exists in the lipoprotein form, which may the readily be separated in to distinct classes by a range of physical and chemical methods (Cook, 1968; Gornall and Kuksis, 1973). The overall lipid: protein ratio of the yolk is about 2:1. Extractable lipid accounts for 33% of the total weight of the yolk and 60-65% of its dry matter content. The proportions of the major individual lipid fraction are listed in Table 2.1. This and subsequent data on egg lipid composition are from a range of a published analysis, including those of the authors (Rhodes and Lea, 1957; Privett et al., 1962).

Table 2.1 Proportion of major lipids in yolk (% weight of total yolk).

| Lipid | Weight of total yolk (%) | Phospholipids | Weight of total yolk (%) |
|---|---|---|---|
| Cholesteryl esters | 1.3 | Phophatidylethanolamine | 23.9 |
| Triglycerides | 63.1 | Phosphatidylserine | 2.7 |
| Free fatty acids | 0.9 | Phosphatidylcholine | 69.1 |
| Free cholesterol | 4.9 | Sphingomyelin | 1.0 |
| Phospholipids | 29.7 | Ohters | 3.2 |

As would be expected from their plasma origin, the major yolk lipid fractions is triglyceride, which is accompanied by a substantial quantity of phospholipid; the only other major component is free cholesterol. Other extractable ' lipid-like ' substance, e.g. pigments, may be present in the yolk but in very low proportions. Phosphatidylcholine and phosphatidylethanolamine are the major phospholipid components.

The fatty acid compositions of the major lipid fractions are given in Table 2.2. The fatty acids listed account for the majority of the total fatty acids with the small percentage of C14, C15, C17 and C20 fatty acids. As can be seen, oleic acid is the major fatty acid in the lipid fractions; with palmitic and stearic acids accounting for up to half the total, substantial levels of linoleic acid are also present. The phospholipid fraction contains a high level of other polyunsaturated fatty acids (Noble and Cocchi, 1990).

Table 2.2 Fatty acid compositions (major fatty acids, % by weight of total yolk) of the cholesteryl esters, triglycerides and total phospholipid fractions of the yolk.

| Fatty acid | Carbon | Cholesteryl ester | Triglycerides | Phospholipids |
|---|---|---|---|---|
| Palmitic | 16 | 29.1 | 24.5 | 28.4 |
| Palmitoleic | 16:1 | 1.0 | 6.6 | 1.9 |
| Stearic | 18 | 9.5 | 6.4 | 14.9 |
| Oleic | 18:1 | 40.1 | 46.2 | 29.5 |
| Linoleic | 18:2 | 18.0 | 14.7 | 13.8 |
| Linolenic | 18:3 | 0.3 | 1.1 | 0.3 |
| Arechidonic | 20:1 | 0.9 | 0.3 | 6.2 |
| Docasahexaenoic (DHA) | 22:6 | 0.5 | <0.2 | 4.1 |

### 2.1.5 Plasma lipoprotein structure

Triglyceride-rich lipoproteins isolated from the plasma of mammals and birds have a common structure, with a core of triglyceride and cholesterol esters and a surface layer of phospholipid, cholesterol and specific apoproteins (Figure 2.3). The lipoproteins synthesized by the livers of laying hens are unusually small and regular in size, with a mean diameter of about 30 ± 5 nm. They are much smaller than the triglyceride-rich lipoproteins synthesized by the liver of immature hens or male chickens (or those produced by mammals) and this seems to be a specific adaptation to allow them to pass readily through the basal lamina layer in the ovarian follicle wall (Griffin and Perry, 1985).

The incorporation of intact lipoproteins into the oocyte by receptor-mediated endocytosis means that cholesterol content of yolk is determined by the cholesterol content of the individual yolk lipoprotein particles and not by the cholesterol concentrations in the plasma. In another way, the cholesterol content of yolk is dependent on lipoprotein composition not concentration (Griffin, 1992). Unfortunately, free cholesterol is not a casual component of triglyceride-rich lipoproteins. In contrast, the esterified cholesterol in yolk

lipoproteins is associated with the lipid core. The role of the small amount of cholesterol that is associated with vitellogenin is unknown, but it may be an inevitable consequence of secretion of a lipophilic molecule.
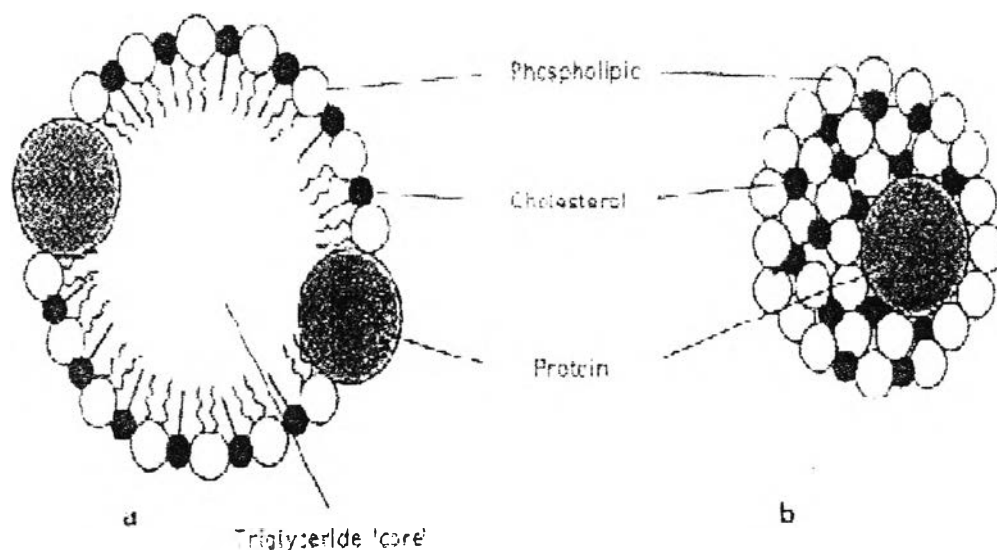


Figure 2.3 Structure of triglyceride-rich lipoproteins, with non-esterified cholesterol, phospholipid and apoproteins combining to stabilize the lipoprotein surface and triglyceride and cholesterol esters forming the 'core'. A, Section; b, surface view.

### 2.1.6 Relationship between plasma and yolk cholesterol concentrations

There is a relationship between egg yolk and plasma cholesterol levels (Hargis, 1988), however, scientist found little or no correlation between the two. Bacon et al. (1973) measured the uptake of radioactively labeled very low-density lipoprotein (VLDL) into turkey yolk and found that it had no correlation with plasma VLDL concentration. In vitro studies have demonstrated that the affinity of the receptors on the oocyte plasma membrane is high, with reports of half maximal binding of vitellogenin at about 100 µg/ml (Stifani et al., 1987) and of VLDL at about 50 µg apoprotein/ml (Perry et al., 1985). Concentrations of yolk precursors in the plasma of laying hens are normally at least ten-fold greater than these values and, as a consequence, the oocyte receptors are always readily saturated. The formation of coated pits probably requires occupation of a critical number of receptors and

even if plasma concentrations of precursors were very low, this is likely to influence the rate of endocytosis but not the content of each endocytic vesicle (Griffin, 1992).

### 2.1.7 Lipoprotein assembly and the origin of yolk cholesterol

Apo-B and cholesterol esters are synthesized in the rough endoplasmic reticulum of hepatocytes and the phospholipid and triglyceride components of VLDL originate in the smooth endoplasmic reticulum, assembly must involve a number of steps (Griffin, 1992). The esterification of cholesterol in the smooth endoplasmic recticulum provides sufficient substrate to bind to the lipophilic sites on the apo-B molecule as it is synthesized. The plasma origin of lipoprotein non-esterified cholesterol is uncertain. Intracellular membrane has very low levels of cholesterol (Reinhart, 1990) and most of the cholesterol in cells is present in the plasma membrane. Kahn et al. (1989) have reported that lipoprotein cholesterol originates from a rapidly turning over pool distinct from that in the plasma membrane. At least in the rat, most of lipoprotein cholesterol is incorporated into lipoproteins after their initial assembly.

The cholesterol content of VLDLs in rat liver perfusates tends to be lower than those of VLDLs isolated from the plasma (Hamilton et al., 1991). Part of the cholesterol in circulating VLDL may therefore derive from intracellular membrane, the hepatocyte plasma membrane during the process of secretion and transfer from red blood cell plasma membranes after their entry into the circulation (Van Meer, 1989; Reinhart, 1990). No information is available about cholesterol exchange in laying hens. However, even if triglyceride-rich lipoproteins were synthesized by laying hen liver with low levels of cholesterol, their half-life in the circulation was approximately 2 h (Bacon et al., 1978). This may provide sufficient time to allow reduces substantial transfer of cholesterol from circulating erythrocytes. Studies on model systems have shown that affinity for cholesterol is better with smaller particles and for those enriched in dipalmitoylphosphatidylcholine and sphingomyelin (Phillips et al., 1987). Laying hen triglyceride-rich lipoproteins have relatively low concentrations of sphingomyelin or disaturated phospholipid (Noble, 1987) and there

would seem to be little scope for altering their affinity for cholesterol by manipulating surface phospholipid composition (Griffin, 1992).

### 2.1.8 Yolk cholesterol esters

Cholesterol esters are core components of triglyceride-rich lipoproteins; it appears to be much than non-esterified cholesterol (Griffin, 1992). Decreases in cholesterol content of VLDL after feeding oil to rats (Parks et al., 1989) or lovastatin to human patients (Arad et al., 1990), for examples, were due to substantial reductions in cholesterol ester content; lipoprotein non-esterified cholesterol content was unaffected by either treatment. Some of the small reductions reported in cholesterol content of egg yolk may have been achieved through reductions in cholesterol ester content. Elkin and Rogler (1989), for example, reported an 11% reduction in total cholesterol content of egg yolk (mg cholesterol/g yolk) after feeding lovastatin to hens for 35 days. Lovastatin is an effective drug that lowers the blood cholesterol by inhibiting the production of cholesterol in the liver. About 20% of yolk cholesterol in eggs is in the form of cholesterol esters (Noble, 1987), all of the decrease reported could have been due to a reduction in yolk cholesterol ester content.

### 2.1.9 Substitution of egg yolk cholesterol

The feeding of laying hens with plant sterols, for example, can cause a gradual replacement of egg yolk cholesterol with sitosterol. Similarly, the inhibition of cholesterol synthesis with probucal leads to the gradual replacement of cholesterol in the egg by its immediate metabolic precursor, desmosterol (Griffin, 1992). Sitosterol is sufficiently similar to cholesterol to be able to substitute for cholesterol in lipoproteins without altering their size or general composition (Hidaka et al., 1990). Cholesterol is an important component of the plasma membrane of cells, where it influences membrane permeability and the activity of membrane-bound enzymes. Cholesterol is a precursor of isoprenoids and precursor in the synthesis of a number of steroid hormones with key roles in controlling reproduction. Other sterols cannot substitute for cholesterol in these roles, and it is perhaps not surprising that the gradual replacement of cholesterol in laying hen tissues by desmosterol eventually leads

to the cessation of egg production (Burgess et al., 1962). The physiological consequences of replacement of cholesterol by plant sterols have been studied most extensively in the clinical disorder of sitosterolaemia.

## 2.2 Fat composition of crude palm oil

Oil Palm is a plant in "Palmae or Receae" and "Cocoideae". It is devided into 3 types; Elaeis guineensis (African oil palm), Elaeis oleifera (South Amerigan Oil Palm) and Elaeis odora (American Oil Palm). Palm oil comprise high amount of saturated fatty acids. Concentrations of various free fatty acids in crude palm oil are shown in Table 2.3.

Table 2.3 Concentrations of free fatty acid in crude palm oil.

| Free fatty acid | Free fatty acid ratio (%) |
|---|---|
| **Saturated fatty acid** | |
| Lauric acid (C12) | Trace |
| Myristic acid (C14) | 2 |
| Palmitic acid (C16) | 43 |
| Stearic acid (C18) | 7 |
| Arachidonic acid (C20) | Trace |
| Total | 52 |
| **Unsaturated fatty acid** | |
| Oleic acid (C18:1) | 39 |
| Linoleic acid (C18:2) | 9 |
| Linoleneic acid (C18:3) | Trace |
| Total | 48 |

## 2.3 Fat soluble vitamin E in palm oil

Vitamin E is one the most important phytonutrients in edible oils. It consists of eight naturally occurring isomers, a family of four tocopherols (alpha, beta, gamma and delta) and four tocotrienols (alpha, beta, gamma and delta) homologues as shown in Figure 2.4 (IUPAC-IUB Joint Commission on Biochemical Nomenclature, 1982).

Alpha-tocopherol is the most abundant form of vitamin in nature (Sheppard et al., 1993). It has the highest biological activity based on fetal resorption assay (Bunyan et al., 1961; Weiser et al., 1986; Weiser et al., 1996), and it can reverse vitamin E deficiency symptoms in humans (Brin et al., 1986; Kohlschutter et al., 1988; Schuelke et al., 1999). Natural vitamin E is a combination of tocopherols and tocotrienols (Ab Garpor, 1993). The major form of vitamin E in crude palm oil is tocotrienol. It was recently shown that tocotrienol lower the levels of blood cholesterol and LDL cholesterol in human and experimental animals (Qureshi et al., 1991b).
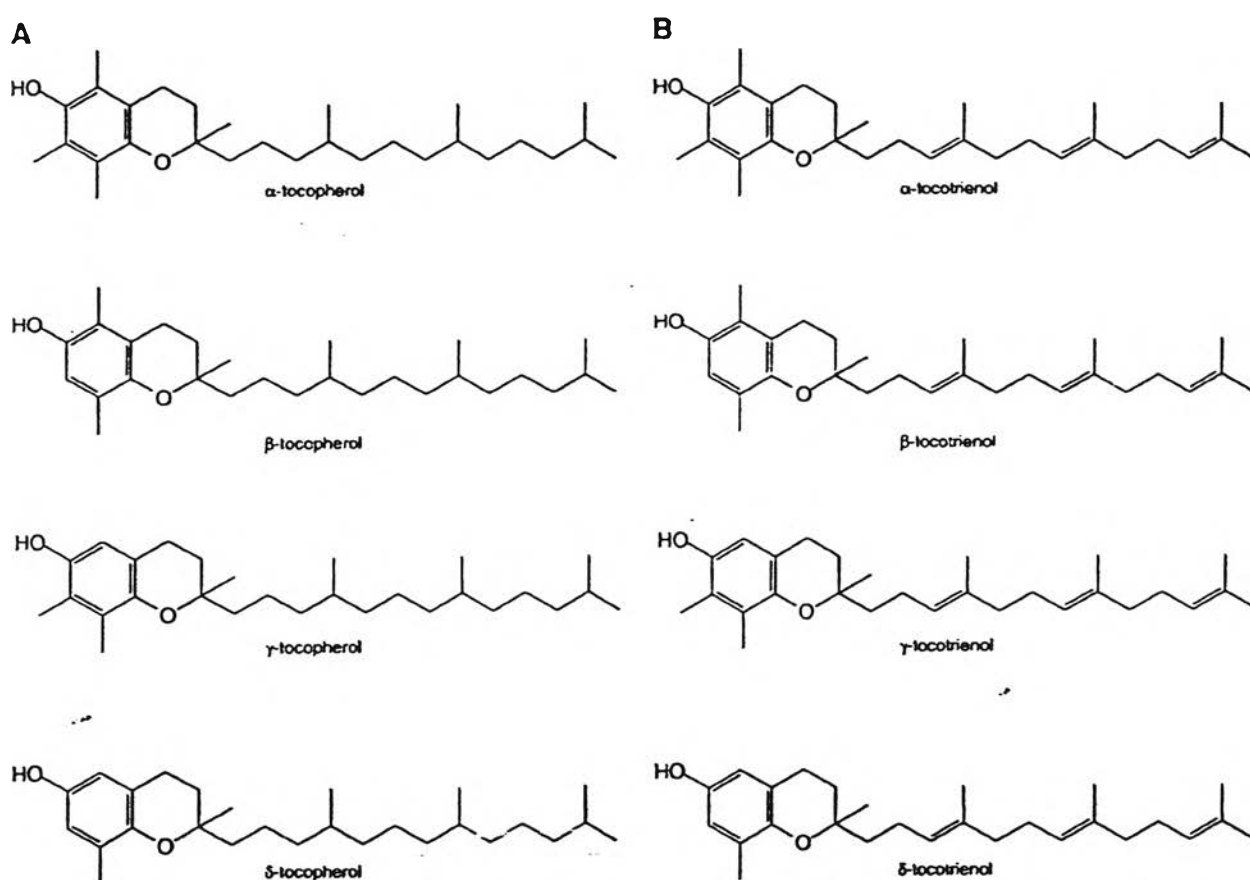


Figure 2.4 Naturally occurring forms of vitamin E (IUPAC-IUB Joint Commission on Biochemical Nomenclature, 1982).

Vitamin E is present in crude palm oil (600-1000 ppm) and in refined palm oil (470-670 ppm), together with β-carotene and vitamin A. The major homologues of palm oil vitamin E are α-tocopherol, α-tocotrienol, γ-tocotrienol and δ-tocotrienol. Tocopherols are found in polyunsaturated vegetable oils and in the germ of cereal seeds, whereas

tocotrienols are found in the aleurone and subaleurone layers of cereal seeds and in palm oil. γ-Tocotrienol is a predominant composition in crude palm oil as shown in Table 2.4. The percentage of palm fatty acid distillate (PFAD) tocotrienols and tocopherols is approximately 0.48%. Tocopherol predominates in certain oils; such as corn oil, soybean oil, and olive oil whereas the tocotrienol series predominates in palm oil, rice bran oil, and barley oil. Table 2.5 shows the composition of tocopherols and tocotrienols in commonly consumed oils (Qureshi and Qureshi, 1993).

Table 2.4 Composition of vitamin E in crude palm oil.

| Compound | % |
|---|---|
| α-tocopherol | 22 |
| α-tocotrienol | 20 |
| γ-tocotrienol | 46 |
| δ-tocotrienol | 12 |

Source: Ab Garpor (1993)

In the literature survey of tocotrienols and tocopherols in oils and fats, it is found that tocotrienols are present in only a few of these oils, as shown in Table 2.6 (Gapor et al., 1983). The data from Table 2.4 indicate that crude palm oil is the richest source of tocotrienols and it is also the most practical source of tocotrienols currently. In 1992, Evans and Bishop discovered vitamin E as a micronutrient essential for reproduction in rats. It was rediscovered as factor II and placed in the context of cellular antioxidant systems, together with sulfur amino acids (factor I) and selenium (factor III). Vitamin E subsequently proved to be effective in preventing lipid peroxidation and other radical driven oxidative events (Esterbauer et al., 1991).

Table 2.5 Tocopherols and tocotrienols in different oils and fats.

| Oil and fats | Tocopherols (ppm) | | | | | Tocotrienols (ppm) | | | | | Total |
| | $\alpha$-T | $\beta$-T | $\gamma$-T | $\delta$-T | %-T | $\alpha$-T$_3$ | $\beta$-T$_3$ | $\gamma$-T$_3$ | $\delta$-T$_3$ | %-T$_3$ | T+ T$_3$ |
| | | | | | | | | | | | (ppm) |
| Corn oil | 112 | 50 | 602 | 18 | 100 | - | - | - | - | 0 | 782 |
| Soybean oil | 101 | - | 593 | 264 | 100 | - | - | - | - | 0 | 958 |
| Rice oil | 124 | 40 | 50 | - | 22 | 184 | 21 | 570 | - | 78 | 989 |
| Palm oil | 279 | - | 61 | - | 31 | 274 | - | 398 | 69 | 69 | 1081 |
| MD-RBD[a] | 144 | 30 | 75 | 60 | 32 | 402 | 14 | 180 | 60 | 68 | 965 |
| (barley oil) | | | | | | | | | | | |
| Olive oil | 51 | - | - | - | 100 | - | - | - | - | 0 | 51 |
| Coconut oil | 5 | - | - | 6 | 31 | 5 | 1 | 19 | - | 69 | 36 |
| Lard | 12 | - | 7 | - | 73 | 7 | - | - | - | 27 | 26 |

[a] Molecular distilled-refined, bleached, and deodorized (Sheppard et al., 1993)

Table 2.6 Tocopherol (T) and tocotrienols (T$_3$) in palm fatty acid distillate (PFAD).

| Feedstuff of refining | Total T and T3 in PFAD (ppm) | | |
| | Average | Range | No. of sample [a] |
| Crude Palm oil | 3973 | 744-8191 | 26 |
| Palm olein | 3391 | 1081-7122 | 19 |
| Palm stearin | 1379 | 162-2408 | 4 |

[a] Samples were collected from 14 refineries (Sheppard et al., 1993)

## 2.3.1 Absorption and transport of vitamin E

Absorption of vitamin E is related to intestinal fat digestion and is facilitated by bile and pancreatic lipase (Ullrey, 1981). Whether presented as free alcohol or as ester, most vitamin E is absorbed as the alcohol. The balance studies indicated that much less vitamin E is absorbed, or retained in the body than vitamin A. Vitamin E recovered in feces from a test dose was found to range from 65 to 80% in human, rabbit, and hen, although in chicks, it was reported approximately about 25%. It is not known how much fecal vitamin E

represents unabsorbed tocopherol and how much may come via secretion in the bile. The latter usually has tocopherol content similar to that of blood plasma.

The natural tocopherol is subjected to destruction in the digestive tract to some extent, whereas the acetate ester form is not. Much of the acetate is readily split off in the intestinal wall and the alcohol is reformed and absorbed, thereby permitting the vitamin to function as a biological antioxidant. Any acetate form absorbed or injected into the body evidently is converted there to the alcohol form. Lipid, including tocopherol, must be emulsified and solubilized before their absorption across the brush-border membrane of the enterocyte. Emulsification beings in the stomach by predominantly mechanical forces that break up large emulsion particles into smaller particle. Within the small intestine, chyme mixes with pancreatic and biliary secretions, which are necessary for the efficient absorption of tocopherol. Pancreatic lipase is necessary for the hydrolysis of triglyceride in the small intestine to monoglycerides and fatty acids and is able to transport tocopherol across the unstirred water layer to the brush-border membrane of the enterocyte.

Once tocopherol has been solubilized within bile salt micelles and transported across the unstirred water layer, the micelle comes into contact with the absorptive brush-border membrane of the enterocyte (Traber et al., 1993) by passive diffusion. Thus, the process is non-saturable, non carrier-mediated, and unaffected by metabolic inhibitors and does not require energy (Gallo-Torres, 1980; Hollander et al., 1975). The gastrointestine and related organs in domestic fowl are different from mammals. The pancreas is a pale yellow organ located within the duodenal loop, although part of it may be found outside the loop. It is relatively small in carnivores and granivores but large in piscivores and insectivores.

### 2.3.2 Plasma transport and distribution of vitamin E

Vitamin E is transported into the blood by the plasma lipoproteins (Behrens et al., 1982; Haga et al., 1982) and erythrocytes (Gallo-Torres, 1980). There is no evidence for the existence of a specific vitamin E plasma carrier protein (Bjorneboe et al., 1990; Burton and Traber, 1990). This is in contrast to the transport of vitamin A by retinal binding

protein, which apparently mediates the delivery of retinal to target tissue by a receptor pathway (Blomhoff et al., 1990). There are two important consequences resulting from the transport of vitamin E in lipoproteins. First, circulating polyunsaturated fatty acids (PUFAs) and other lipids are protected from free-radical attack by vitamin E (Esterbauer et al., 1987). Secondly, plasma vitamin E concentrations do not entirely depend on dietary intake but vary with those of the lipoproteins. This is manifested by high correlations between plasma concentrations of tocopherol and total lipids or cholesterol (Widhalm et al., 1985). Thus, when lipoprotein concentrations of tocopherol and total lipids or cholesterolemia, vitamin E concentrations are also increased (Lambert and Mourot, 1984). Because of the relationship between plasma tocopherol and lipid concentrations, vitamin E status is generally expressed in relation to circulating lipids.

Normal plasma vitamin E concentrations in humans range from 11 to 37 µmol/L (Farrell, 1988; Farrell, 1980; Horwitt et al., 1984). When plasma lipids are taken into account, the lower limits of normal are 1.6 µmol $\alpha$-tocopherol/mmol lipid (0.8 mg/g) or 2.5 µmol $\alpha$-tocopherol/mmol cholesterol (Horwitt et al., et al., 1972). Both $\alpha$- and $\gamma$-tocopherols are present in the blood of humans and experimental animals. Plasma $\alpha$-tocopherol is usually about 5 to 10 fold higher than $\gamma$-tocopherol, despite the fact that most diets are rich in $\gamma$-tocopherol (Handelman et al., 1985).

### 2.3.3 Distribution within plasma lipoproteins

There are four main lipoprotein classes: (1) chylomicrons, (2) very low-density lipoproteins (VLDL), (3) low-density lipoproteins (LDL), and (4) high-density lipoproteins (HDL). The distribution of $\alpha$-tocopherol in lipoproteins does not pararell with individual lipid classes, such as triglycerides, cholesterol, or phospholipids, indicating that the vitamin does not share the same metabolic fate as any one these lipids. Furthermore, the lipoprotein distribution of $\alpha$ -tocopherol does not reflect the relative importance of individual lipoprotein classes for the transport of vitamin E. The transfer of tocopherol between lipoproteins is not assisted by the neutral lipid transfer protein, which promotes the exchange of cholesteryl ester for triglyceride between HDL and the triglyceride-rich lipoproteins, that is,

chylomicrons and VLDL (Granot et al., 1988). Following its absorption, vitamin E is secreted from the intestine incorporated in chylomicrons. These are then catabolized by the action of lipoprotein lipase and bound to the surface of the endothelial limiting of the capillary walls (Nelsson-Ehle et al., 1980). Since the delivery of dietary α-tocopherol by chylomicrons fluctuates with the load of absorbed vitamin E, hepatic VLDL are important for maintaining α-tocopherol plasma concentrations (Cohn et al., 1988; Traber et al., 1990a; Traber et al., 1990b). Vitamin E also partitions between lipoproteins during the catabolism of chylomicrons and VLDL (Bjorneboe et al., 1987; Traber et al., 1988). Some tocopherol is probably transferred with the excess surface material from chylomicrons and VLDL to HDL during the hydrolysis of triglycerides. Transfer of tocopherol from HDL to other lipoproteins could then occur, enriching the lipoprotein fractions with the vitamin (Traber et al., 1993).

### 2.3.4 Hepatic tocopherol binding protein

A tocopherol binding protein (31 KD) has been described in rat liver (Behrens and Madere, 1982; Catignani and Bieri, 1977) but not in intestine (Catignani and Bieri, 1977). The tocopherol binding protein discriminates between the form of vitamin E in the liver in vivo and that this protein is involved in the incorporation of RRR-α-tocopherol into nascent VLDL (Traber et al., 1993).

## 2.4 Absorption and distribution in tissue of tocopherols and tocotrienols

### 2.4.1 Absorption and distribution in tissue of tocopherols

Tocopherols are carried by the plasma lipoproteins, mechanism that provide tissues with lipids from the lipoproteins pathways for the movement of lipids from lipoproteins to tissues, including lipases, uptake by lipoprotein receptor-mediated endocytosis, and receptor-independent uptake, as well as spontaneous transfer and exchange reactions (Traber et al., 1993). Exchange of tocopherols between lipoproteins and membranes has long been recognized as a process by which vitamin E can be transferred to cells. Spontaneous transfer and exchange of α-tocopherol between lipoprotein and either red

blood cells (Bjornson et al., 1975; Kayden and Bjornson, 1972) or liposomal membranes (Massey, 1984). Because the erythrocyte lacks both lipoprotein receptors and lipoprotein lipase activity, these cells are likely to depend on transfer and exchange mechanisms for the adjustment of their tocopherol concentration in vivo (Traber et al., 1993).

### 2.4.2 Absorption and distribution in tissues of tocotrienols

The antioxidant efficacy of tocotrienols in membranes is higher than that of tocopherol, although their uptake and distribution after oral ingestion are less than that of $\alpha$-tocopherol. However, tocotrienol could still be detected in the postprandial plasma of humans, and tocotrienols were found in all classes of lipoproteins (Hayes et al., 1993). The liver contains a transfer protein that preferentially enriches VLDL with $\alpha$-tocopherol (Arita et al., 1995). Therefore, the liver discriminates between tocopherols and tocotrienols secretes tocopherol preferentially by $\alpha$-tocopherol transfer protein $(\alpha\text{-TTP})^3$ (Ouahchi et al., 1995). However, it is important to note that tocotrienols being to a family of plant phenolic compounds, which have a brief and transient nature with respect to their metabolism, i.e., compared with $\alpha$-tocopherol, they are inferior with regard to tissue retention and half-life. Tocotrienols penetrate rapidly through skin, and its topical application is an efficient means with which to enrich skin with vitamin E (Traber et al., 1998).

## 2.5 Physiological effect of crude palm oil

Palm vitamin E helps to protect unsaturated fatty acids in the body against free radical formation, which can cause damage to blood, cells and tissues. Several animals and human studies have shown the association between vitamin E, an antioxidant vitamin, with a reduced risk of cardiovascular diseases (Riemersa et al., 1991). Palm vitamin E (TRF; tocotrienol rich freaction), alpha-, gamma-, and delta-tocotrienol have been shown to suppress cholesterol biosynthesis and reduce total serum cholesterol and LDL cholesterol levels in hypocholesterolemic chickens (Quresshi et al., 1986; Qureshi et al., 1988). It has also been reported that vitamin E has anticancncer properties in animal model and the result suggest that tocotrienols show the potential to have an edge over tocopherols in terms

of anti-cancer (Nesaretnam et al., 1992). Palm vitamin E meets all the properties that are associated with other vitamin E preparations. In addition a number of advantageous effects of the tocotrienols present in palm oil have become evident. These included greater antioxidant properties, which are coupled with possible cholesterol lowering and anticancer effects.

## 2.6 Tocotrienol hypocholesterolemic effect and antioxidant effect

### 2.6.1 Tocotrienols: novel hypercholesterolemic agents with antioxidant properties

The mevalonate pathway has $\beta$-hydroxy-$\beta$-methylglutaryl coenzyme A (HMG-CoA) reductase is catalyzes the rate-determining step in the biosynthesis of cholesterol (Siperstein and Fagon, 1966). Under certain conditions, HMG-CoA synthase controls the rate of cholesterogenesis (Ramachandran et al., 1978). Most of the early studies on HMG-CoA reductase were performed with the yeast enzyme. HMG-CoA reductase catalyzes the two-step reduction of (4R, 3S)-HMG-COA (thioester) to (2R, 3R)-mevalonate (alcohol) in the presence of 2-mol NADPH, as shown in Figure 2.5.

The HMG-CoA and NADPH bind to the reductase, and a hydride ion transfer from the A side of NADPH produces a hemithioacetal of mevalonate and CoA, which is the enzyme-bound intermediate. After replacement of NADP$^+$ with NADPH, the mevalonate hemithioacetal cleaves into mevalonate and CoA, which remain bound to the enzyme. During the second reductive step, there is a hydride transfer from NADPH that reduces mevalodate to mevalonate, and the products mevalonate, CoA, and finally NADP$^+$ leave the enzyme (Qureshi et al., 1976). The second reductive step, particularly the release of NADP$^+$ from the enzyme, is the rate-determining step. This study predicted that group $X$ and $Y$ on the enzyme act as acid-base catalysts to assist in the direct transfer of a hydride ion between the nucleotide and substrate (Qureshi et al., 1976).
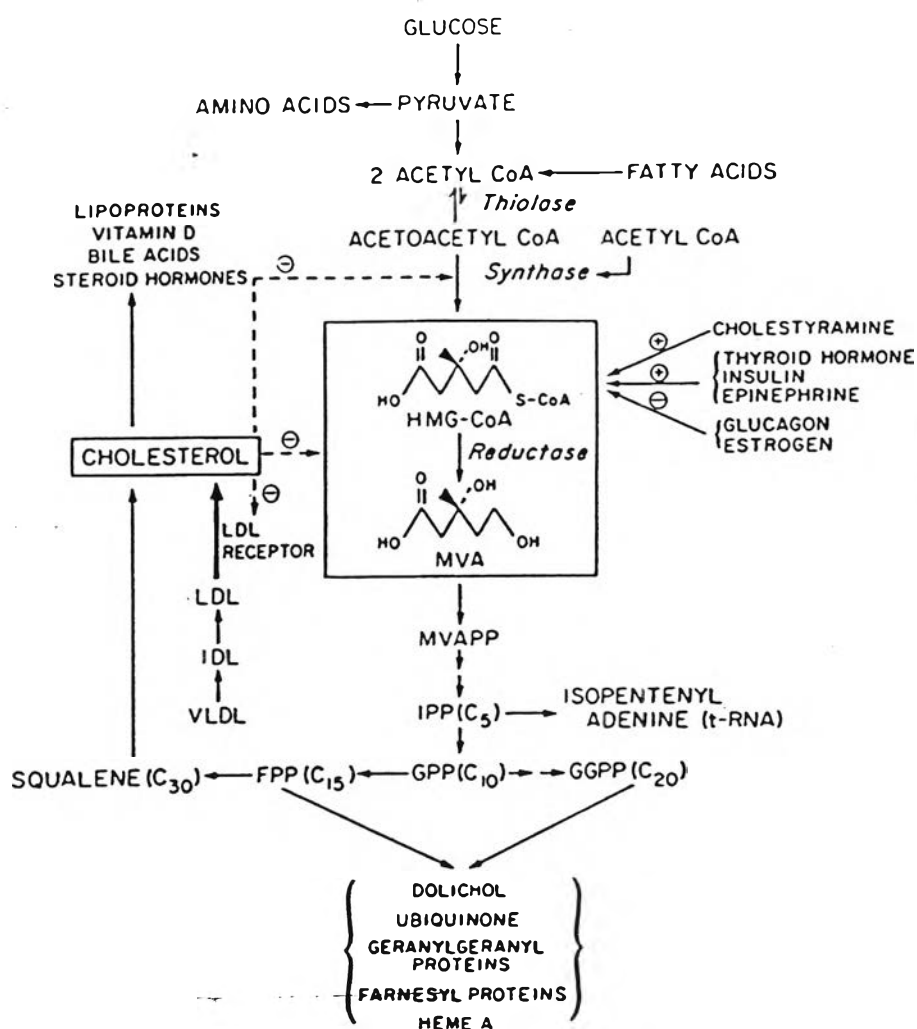
Figure 2.5 The regulation of the HMG-CoA reductase (Qureshi and Porter, 1981).

In Figure 2.5, the initial step involve in the pathway to the biosynthesis of cholesterol, starting from acetyl CoA to isopentenyl pyrophosphate, was reviewed by Qureshi and Porter (1981). The feedback regulation of the reductase was reviewed by Goldstein and Brown (1990). HMG-CoA, $\beta$-hydroxy-$\beta$-methylglutaryl coenzyme A; MVA, mevalonic acid; MVAPP, mevalonate pyrophosphate; IPP, isopentenyl pyrophosphate; LDL; GPP, geranyl pyrophosphate; FPP, farnesyl pyrophosphate; LDL, low-density lipoprotein; IDL, intermediated-density lipoproteins; and VLDL, very low-density lipoproteins.

HMG-CoA reductase is regulated by various nutritional and hormonal factors (Dugan, 1981; Geden et al., 1986). It is also regulated by LDL receptors, as shown in Figure 2.5. Cholesterol is obtained from two sources, by biosynthesis staring with acetyl CoA and by receptor-mediated uptake of plasma LDL. Both processes are controlled by the sterol-mediated feedback repression of the genes for HMG-CoA synthesis, HMG-CoA reductase, and the LDL receptor (Goldstein and Brown, 1990). The posttranscriptional regulation of HMG-CoA reductase is accomplished by one of the nonsterol isoprenoids (Goldstein and Brown, 1990). The elegant study of Brown and Goldstein (1983) showed that the uptake of LDL-bound cholesterol in macrophages occurs through receptor-mediated endocytosis.

This uptake results in the suppression of endogenous cholesterol biosynthesis, enhances rate of intracellular cholesterol esterification, and reduces the number of high-affinity LDL receptors expressed on the surface of liver cells (Brown and Goldstein, 1983). Subjects with homozygous familial hypercholesterolemia have a total or near total deficiency of LDL receptors and also have a tendency for premature coronary artery disease (Goldstein and Brown, 1983). The accelerated degradation of HMG CoA reductase requires a nonsterol isoprenoid as well as sterol. Sterol accelerates reductase degradation in part by diverting this mevalonate into a nonsterol regulatory product (Goldstein and Brown, 1990). Most of the drugs available for hypercholesterolemia are aimed to reduce cholesterol and LDL cholesterol levels. Most of these drugs have some side effects, however, and there mechanism of action at the cellular level is not known (Illingworth, 1987).

### 2.6.1.1 Mechanism of action for the hypocholesterolemic effect of the tocotrienols

The tocotrienols seem to have a profound effect on the biosynthesis of cholesterol. The tocotrienols are very effective in lowering blood cholesterol and LDL cholesterol levels by suppressing HMG-CoA reductase. It has been established that the suppression of HMG-CoA reductase requires two regulators: cholesterol delivered by receptor-mediated uptake of LDL and a nonsterol product derived from mevalonate. The former is expressed

predominantly through changes in the rate of transcription of the HMG-CoA reductase mRNA or by degradation of the protein (Goldstein and Brown, 1990).

The exact mechanism by which the tocotrienols act at the cellular level has not yet established. The LDL receptor protein in HepG2 cell membranes is not co-suppressed by γ-tocotrienol, in contrast to the effects of 25-hydroxycholesterol. γ-Tocotrienol does not decrease the level of HMG-CoA reductase mRNA in HepG2 cells. These results suggest that the tocotrienols inhibit sterolgenesis by suppressing HMG-CoA reductase through a novel posttranscriptional mechanism. Squalene transfer and epoidation are also modulated by γ-tocotrienol. Squalene transfer and formation of squalene epoxide increase and the production of squalene dioxide decreases using microsomes from HepG2 cells treated with 10 μM γ-tocotrienol versus controls (Parker and Clark, 1991). It is conceivable that the tocotrienols are transported in the blood in association with low and high-density lipoproteins, as has been reported for the tocopherols (Bjornson et al., 1976; Haga et al., 1982; Parker and Clark, 1991). The tocotrienols may prevent the oxidation of lipoproteins. Tocotrienols may also bind to other proteins; as some as those described for α-tocopherol (Behrens and Madere, 1982; Catignani and Bieri, 1977). The hydroxyl group on the chromatin ring may be important in the binding.

### 2.6.1.2 Vitamin E therapy: prevention of atherogenesis

Dietary constituent that are import to control atherogenesis include fatty acid (PUFA, mFA, and SFA), cholesterol, antioxidant vitamin, trace elements, and fiber.

Sites where plasma cholesterol level can be controlled include:

1. The LDL receptor, which is a critical link between plasma and intracellular sterol pools.

2. Interruption of the enterohepatic circulation of bile acids.

3. The intestinal mucosa, which is limited in the number of sites available for sterol absorption.

4. The enzymes responsible for cholesterol homeostasis: HMG-CoA reductase, acyl CoA-cholesterol acyltransferase, cholesterol 7α-hydroxylase (activates hepatocyte LDL receptor and extracts LDL cholesterol from the circulation).

5. Protection of LDL from oxidative attack, oxLDL goes to scavenger receptors in the macrophages.

There are several theoretical supports on the benefits of vitamin E (Palmvitee) supplementation in hypercholesterolemia and subsequent cardiovascular diseases. In addition to those involved in LDL cholesterol metabolism, the inhibitory effect of vitamin E on platelet adhesion (Steiner, 1983) eicosanoid metabolism, aryl sulfatase B inhibition, and platelet-vascular interactions (Betteridge, 1987) may help to retard the formation of arterial thrombosis. The supplementation doses has been from 1 to 4 capsules/day, corresponding to 18-72 mg $\alpha$-tocopherol and 42-168 mg $\alpha$-, $\gamma$-, and $\delta$-tocotrienols.

Palmvitee supplementation was shown to (Figure 2.6.)

1. Reduce serum total cholesterol and LDL cholesterol.

2. Increase serum $\alpha$-tocopherol concentration.

3. Decrease apo B concentration.

4. Decrease thromboxane concentration.

5. Decrease platelet factor IV concentration.

Palmvitee supplementation was shown not to:

1. Increase liver-derived serum enzymes.

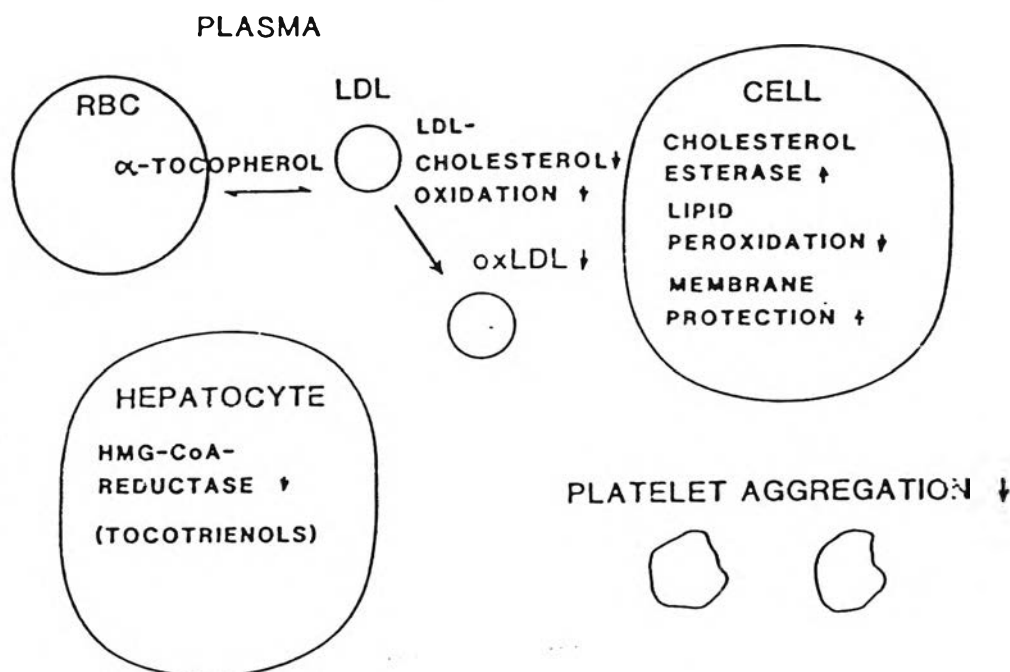2. Result in subjective or objective side effects.

Figure 2.6 Basic compartments (hepatocyte, plasma, LDL, red blood cell (RBC), cell, and platelets) and events in which vitamin E ($\alpha$-tocopherol and tocotrienols) controls the risk of atherogenesis (Steriner, 1983).

### 2.6.2 Antioxidant function of tocopherols and tocotrienols

Even though the mechanism of physiological activity of vitamin E is not clearly understood, it is likely that at least some of the biological activities that have been demonstrated are due to its antioxidant function. $\gamma$-Tocopherol is more effective than $\alpha$-tocopherol in preventing lipid peroxidation induction in vitro (Burton et al., 1983). Other reports have suggested that $\alpha$-tocopherol is more effective than $\gamma$-tocopherol. It is now believed (Chipault, 1962) that relative effectiveness depends on the experimental conditions. $\alpha$-Tocopherol homologues with shorter hydrocarbon tails manifested remarkably higher efficiency in habiting lipid peroxidation in different natural membranes and in liposome compared to $\alpha$-tocopherol (kagan et al., 1990). Although no difference in radical-scavenging activity between $\alpha$-tocopherol and $\alpha$-tocotrienol was found in hexane, the activity of $\alpha$-tocotrienol in scavenging peroxyl radicals is 1.5-fold higher in liposome

compared with α-tocopherol (Serbinova et al., 1991). Vitamin E does not work in isolation from other antioxidants; rather it is part of an interlinking set of redox antioxidant cycles, which has been termed the " antioxidant net work " (Figure 2.7). It is hypothesized that vitamin E acts catalytically, i.e., it is efficiently reduced free radical (chromanoxyl) form, which arise after quenching lipid radicals, to return back to its reduced native state (Serbinova et al., 1991).
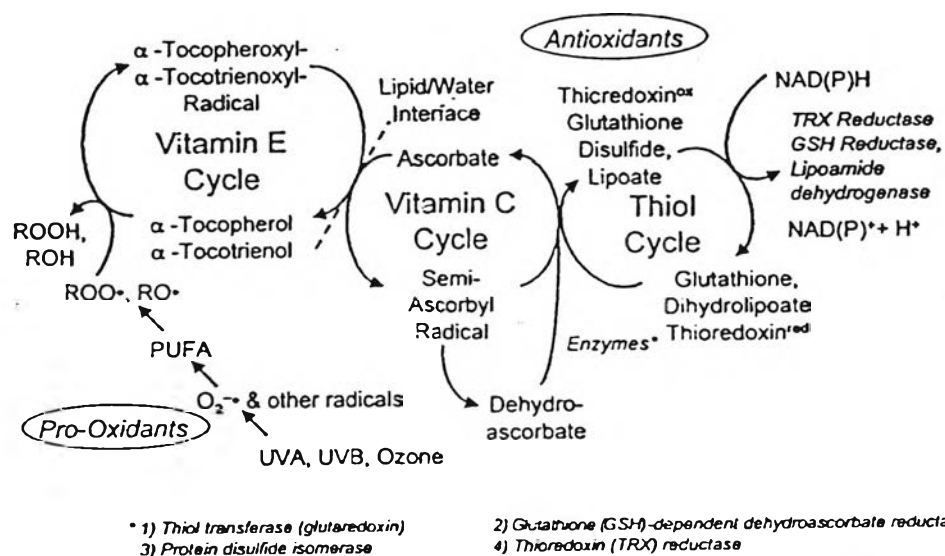


* 1) Thiol transferase (glutaredoxin)
3) Protein disulfide isomerase

2) Glutathione (GSH)-dependent dehydroascorbate reductase
4) Thioredoxin (TRX) reductase

**Figure 2.7** The antioxidant network showing the interaction among vitamin E, vitamin C and thiol redox cycles (Serbinova et al., 1991).