CHAPTER II

LITERATURE REVIEW

A. The color of skin and pigment system
The color of the skin (1,2)

Four skin colors are produced by normal skin pigment such as red, yellow, brown, and blue. Exogenous pigment produced carotenoids (yellow), endogenous pigment produced melanin (brown) in the dermis. Red is occurred by oxygenated hemoglobin in capillaries and also blue is occurred by reduced hemoglobin in the vanules of the dermis [4]. The skin colors are different in each part of each person. For instance, in black people, the abdomen is the darkest and the lumbar region is the lightest. In white people, the upper thigh is the darkest and the lumbar region is the lightest. Females are generally lighter than males [5]. Major color determinant is melanin, racial and ethnic difference in skin color are related to the number, size, shape, distribution, and degradation of melanin-containing organelles called melanosomes. These are products of specialized exocrine glands derived from neural crest, called melanocytes, which contain nerve cell-like dendrites. In the skin, melanocytes ramify their dendrites to the keratinocytes so that they transfer melanin-containing melanosome to the epidermis. Melanin is a virtually insoluble polymer of high molecular weight and can attach to a protein.

Melanin pigmentation of human skin is divided in two components. [4]

1. **Constitutive skin color** is the amount of melanin pigmentation generated in the absence of sun exposure and other influences.

2. **Facultative skin color or tan** is composed of the short-lived intermediate tanning (IT) and delayed tanning (DT). Both of them come from direct exposure of the skin to the ultraviolet light (UVL); facultative color is reversible. The increase in pigmentation can also result from endocrine causes, such as those that occur during pregnancy, and from an interaction of light hormonal effects, as seen in melasma (cholasma) and in Addison's disease.

From categorization by chemical composition, melanin is divided into

1. **Eumelanin,** a brown polymer derived from the conversion of amino acid, tyrosine by tyrosinase to alkali-insoluble brown product [6].

2. **Phaeomelanin,** a yellow–red alkali-soluble pigment derived from the intermediate of the conversion of tyrosine, dopaquinone, combined with cysteine to form 5-S-cysteinyl dopa and then phaeomelanin [6].

Each epidermal melanocyte secretes melanosome into a finite number of neighboring keratinocytes; single melanocyte supplies melanosomes to a group of about 36 keratinocytes. Fitzpatrick and co-worker call this partnership of melanocytes and neighboring group of keratinocyte an " epidermal melanin unit" (Figure 1).

Skin color has a complex process ranging from the molecules with are relevant to melanin synthesis. Skin color does not depend upon the number of the melanocytes, but upon the organization within the epidermal melanin unit. Hair and skin color are the result of key events in these processes, which include [4,7]:

1. The migration of the melanoblast from the neural crest.
2. The differentiation of melanoblast to form epidermal melanocytes.
3. The formation of the melanosomes.
4. Synthesis of melanin and its deposition on melanosomes.
5. The transfer of melanosomes to keratonocytes, either as aggregate or discrete particles or a complex.
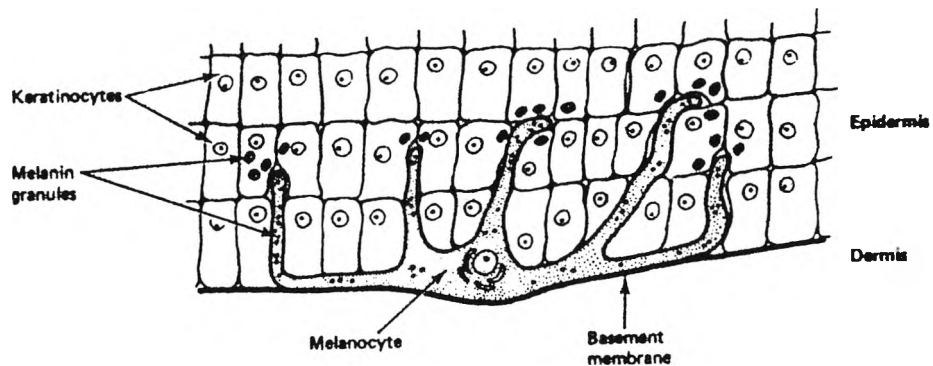6. The degradation of melanosomes within the keratinocytes.

Figure 1. Epidermal melanin unit. Epidermal melanocytes are situated in the basal layer. Melanin is formed within the melanocytes and then transferred into the neighboring keratinocytes. In this way, the melanin provides protection against UV radiation. In the epidermis each melanocyte is normally associated with 36 keratinocytes and together from the epidermal melanin unit [7].

Pigment system

Melanocytes

Melanocytes (Figure 2) are defined as the cells capable of synthesizing tyrosinase enzyme, which necessarily converse from tyrosine to dopa, including melanosomes that synthesize and deposite of melanin. Melanocytes have a round to oval nucleus, numerous mitochondria and vesicles, microfilament (5-7nm), intermediate filaments (10nm), and microtubules (25nm) in the cytoplasm. There are abundant smooth endoplasmic reticulum, rough endoplasmic reticulum, and a Golgi apparatus, all of which are necessary to synthesis proteins.

Masson calls the processes in which the melanocytes discharge melosome into keratinocyte a 'cytocrine activity'. In humans, the melanocyte not only deposites on dermal-epidermal junction, hair, follicles, and dermis, but is also found in mucous membranes, eye, inner ear, cochlea, and vestibular system. Messon called the melanocytes, which retain the melanin they have synthesized, but cannot discharge their melanosome to the surrounding "continent melanocytes". Continent melanocytes (dermal melanocytes) generally appear in extracutaneous sites. In contrast, the melanocytes that can transfer pigment granules to keratinocytes are called "secretory melanocyte". The latter are usually found in epidermis and hair follicles [9]. Like all secretory cells, they have a well-developed endoplasmic recticulum and Golgi complex also [9]. Thus, the epidermal melanocytes are the representative melanocytes in mammals. In vertibrates, there are a large number of melanocytes do not transfer melanosomes to any other cells after synthesis, but contain them through out the life of the cell. These are called melanophores.

The population of melanocytes is not constant throughout life. In the beginning, there are around 800-1000 pigment cells/sq mm and constantly remain until mid adulthood (30-39 years). Thereafter, there is a progressive decrease in population density as a function of age. Moreover, during age, they tend to change with numerous factors, for instance, sunlight increases the melanin population; in contrast, cold, severe thermal burns and age-related factors decrease in melanocyte

density. In fact, melanocytes seem to decrease beginning in fifth decade of life in all parts of the body. Thus, we can see that hair begin turn gray in the fourth and fifth decades of life. Furthermore, with age, the older melanocytes are found larger and more dendritic than younger ones [8].
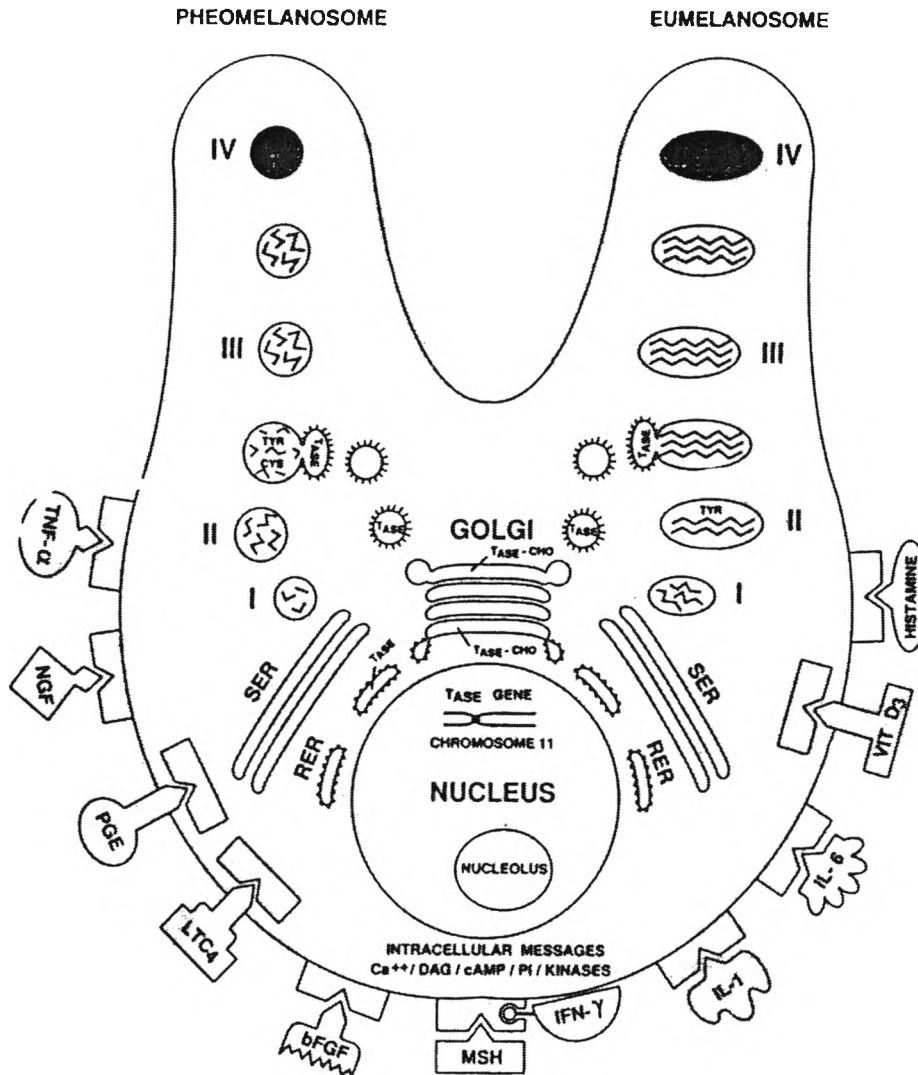
Figure 2. Schematic drawing of melanocyte.

Melanocytes development

Melanocytes in skin and other organs are derived from the neural crest. The dorsal side of the embryo thickens to form a neural plate in the second week of gestation (Figure 3) [8]. The plate develops a groove that becomes a neural tube and later becomes a spinal cord. At weeks 3-4, neural crest cells are located between the neural tube and excessively in epidermis. By week 5 of gestation, neural crest cells migrate through the mesoderm to locate in other tissues. Some of these become melanoblasts (melanocytes precursors). By electron microscopy, melanocytes containing melanosomes are detected in dorsal dermis by week 7 of gestation. After week 7, the population density of the epidermal melanocytes is found to excessively increase.
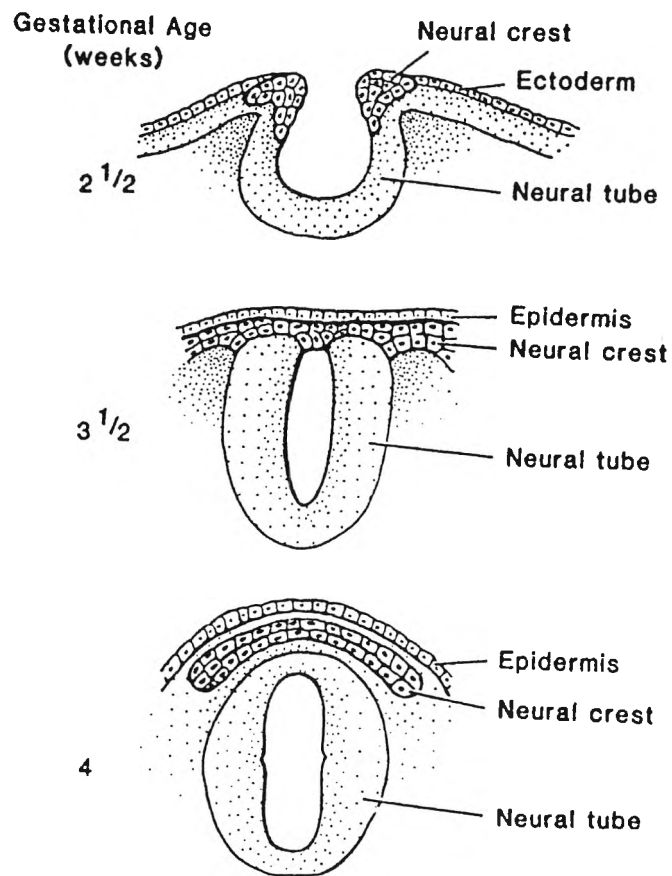
Figure 3. **Schematic drawing showing the formation of the neural crest cells between week 2 and 4 of gestation. The neural crest begins (week 2 ½) as a group of cells along the neural groove. By week 4 the neural crest cells are located between the epidermis and the neural tube [8].**

Melanosomes

Melanosome formation and Melanogenesis

The characteristic ultrastructural feature of melanocytes is the cytoplasmic melanosome. Premelanosomes are formed by a dilation of smooth endoplasmic recticulum. There are two types of melanosomes.

1. **Eumelanosomes**, which contain brown/black pigment, are oval in shape. In their substructures, they have a banded lamella distribution of protein in which eumelanin is deposited.

2. **Phaeomelanosomes**, which contain red pigment, are round in shape. In their substructures, they have a haphazard array distribution of protein which phaeomelanin is deposited.

Melanogenesis occurs in melanocytes, which are found in the epidermal basal layer. When melanocytes are stimulated by numerous inflammatory/ immune cytokines such as interleukin-1 (IL1), IL3, IL6, interferon-γ, prostaglandins, leukotrienes, including prostaglandin E, arachidonic acid, and α-MSH; Enzyme tyrosinase (Tase) is translated from tyrosinase gene (T_{ASE}) on chromosome 11 to the rough endoplasmic reticulum (RER), then translocated to Golgi apparatus. (see Figure2) The enzyme tyrosinase is glycosylated (T_{ASE}.CHO) as it passes from the cis to trans surface of the Golgi apparatus. The mature tyrosinase enzyme and some melanin precursors such as 5,6-dihydroxy indole, dihydroxy-indole-2-carboxylic acid pinch off the Golgi in clathrin-coated vesicles (illustrated as T_{ASE} in spiked vesicles) and translocate through the cytoplasm to fuse with melanosomes that are formed from the smooth endoplasmic reticulum (SER). Stage I melanosomes (premelanosomes) have some structural features of melanosomes. Stage II melanosomes are fully formed with well-defined protein substructures but contain no melanin. Tyrosine (TYR) enters the melanosomes at this stage. After fusion of the tyrosinase enzyme (T_{ASE}) with the melanosome, eumelanin formation begins (stage III) and is completed shortly after stage IV. At this point, the melanosome is replete with eumelanin. The same cell can form phaeomelanin via another pathway. Then, melanosomes are transferred by pinching off the tip of the melanocytes, which is phagocytized by keratinocytes [table 1] [6,7,8,9]

It is now postulated that there are at least three or four isoenzyme forms of tyrosinase representing different stages in the formation of active enzyme. The isoenzyme T_3 is thought to be a newly synthesized molecule. When they combine with sialic acid and neutral sugars, they converted T_1 and finally T_4 which is complex to the melanosomal memebrane [7].

Table 1. Four Stages of Melanosome Formation

Stage	Melanosome	Features
I	Premelanosome	Round in shape. No organized substructure. A few filaments may be present.
II	Melanosome	Eumelanosomes are oval and have a regular substructure. Phaeomelanosomes are round with an irregular substructure. No melanin is evident in either.
III	Melanosome	Partially melanized eumelanosome or Phaeomelanosome.
IV	Melanosome	Fully melanized

In melanosome, copper containing enzyme tyrosinase catalyzes two distinct reactions: the hydroxylation of tyrosine to 3,4-dihydroxy phenylalanine (DOPA), and subsequent oxidation to dopaquinone [10]. Three isoenzyme(T_1, T_2, T_3) forms of active tyrosinase have been isolated from mouse melanoma. While T_1 and T_2 with similar molecular weights of 66,600 and 56,700 are soluble, T_3 is insoluble. Recently, it has been found that not only tyrisinase enzyme is involved in melanogenesis, but the tyrosinase-related proteins (TRP-1) and (TRP-2) as well [5].

Eumelanin, the dark-brown pigment, is oxidized through a series of steps. After dopaquinone is cyclized to leucodopachrome in a rapid, non-enzymatic reaction. A rapid oxidation of leucodopachrome, catalyzed by dopaquinone, gives a red pigment dopachrome. Then dopachrome is converted to 5,6-dihydroxyindole (DHI) by decarboxylation or in smaller amounts, to 5,6,-dihydroxyindole-2-carboxylic acid (DHICA) by tautomerization. The first reaction has recently been postulated by Barter et al., about new enzyme, dopachrome oxidoreductase which is thought to convert dopachrome to 5,6 dihydroxyindole [7]. The later reaction is catalyzed by a dopachrome tautomerase, originally called dopachrome conversion factor. Next, it is further oxidized to give eumelanin polymer (see Figure 4A, 4B). The final step of conversion of DHI, DHICA or mixed oligomers to eumelanin is little understood. However, the enzyme involved in the reaction from dopachrome to melanin is still debatable [10,11].

According to Prota (1980), Phaeomelanin, the yellow or red pigment, comes from the encounter of dopaquinone with either cysteine or glutathione (GSH) resulting in the formation of common intermediate, 5-S-cysteinyldopas (cys-dopas) which is further quickly oxidized into the benzothiazines and transformed to phaeomelanin (Figure 5)[7]. The cysteinyldopas could also result from the combination of glutathione with dopaquinone by α-glutamyltransferase to corlorless glutathionedopa [7].
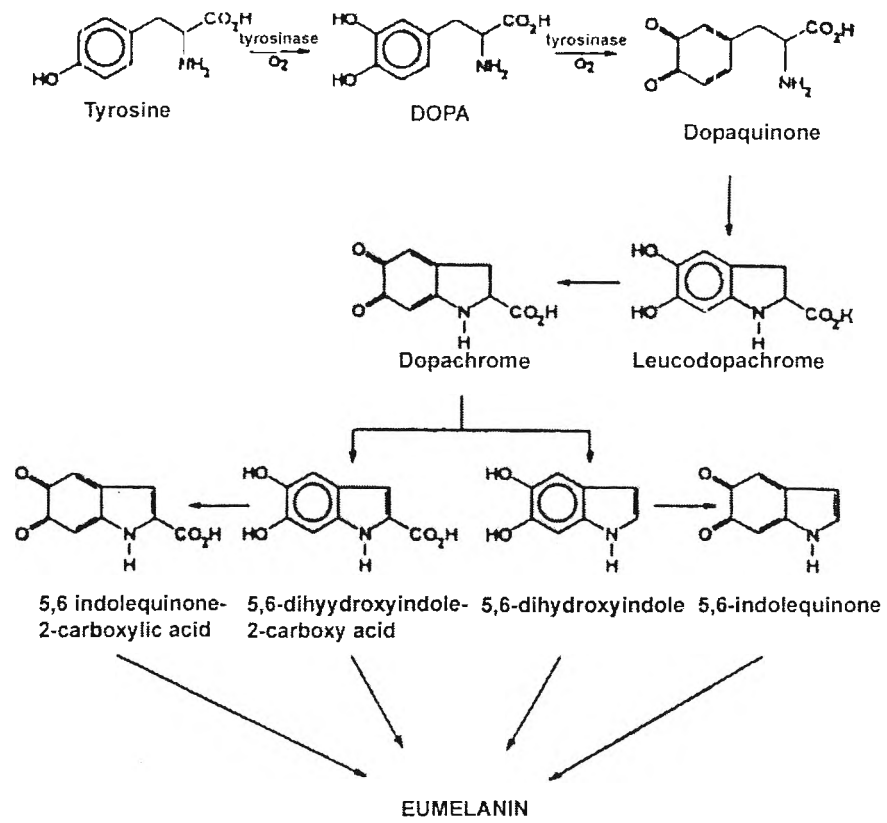
Figure 4A. Schematic outline of the eumelanin synthesis

CO_2

DHI

DHI OLIGOMERS

DOPACHROME

MELANIN(S)

TRP-2

DHICA

DHICA OLIGOMERS

(P) = PEROXIDASE; (N) = METAL IONS; (T) = TYROSINASE
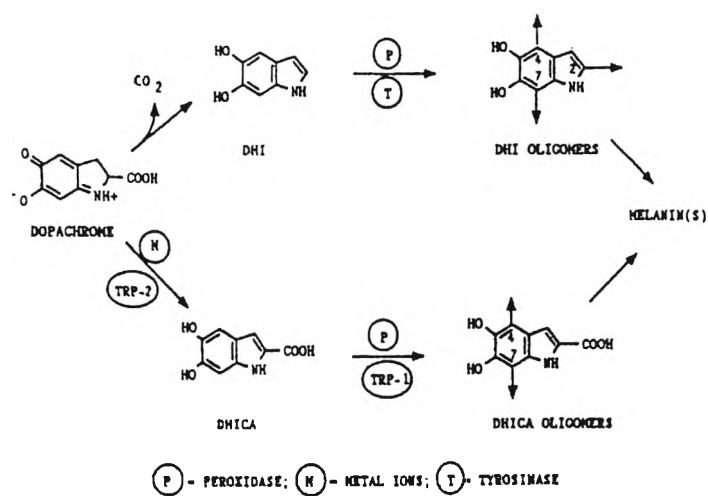
Figure 4B. Schematic outline of the later stages of the melanogenesis
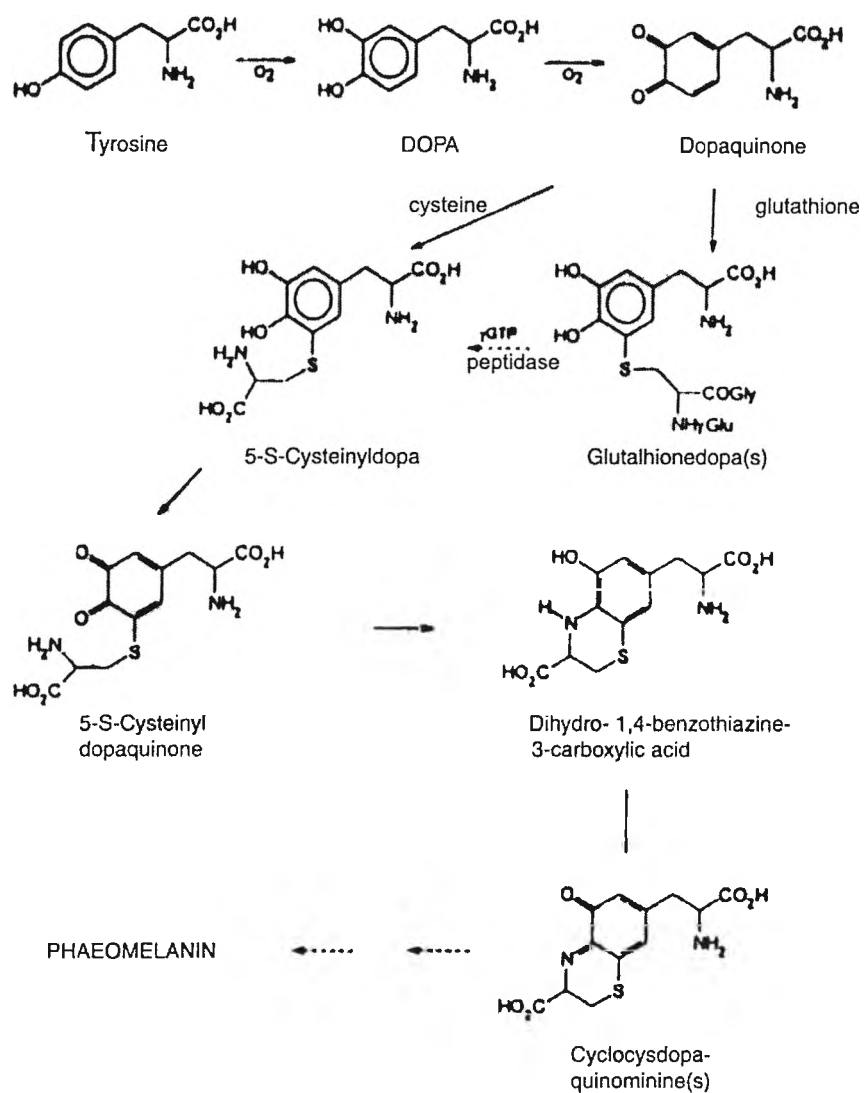
Figure 5. Schematic outline of the synthesis of the phaeomelanin

Melanosome movement

The mechanism of the melanosome movement within the melanocyte is still unclear. However, from the studies in fish and amphibian (fog skin), three possible mechanisms have been proposed:

1. A change in the intracellular current flow resulting from local differences in membrane potential between the central and peripheral parts of the cell (the electrophoresis theory) [7,9]

2. The ionic release or exchange of membrane-bound ions (the ion-exchange theory) [7,9]

3. Sol and gel transformation which cause changes in hydrostatic pressure of cytoplasm (the sol-gel transformation theory)

Recently, a new hypothesis called "the microtubule theory" has been proposed [9]. In this theory, the movement of melanosome may occur within channels surrounded by microtubules. Moreover, Jimbow and coworkers found the melanocytes characteristically contain 100-A° filament which are involved not only in the elongation of the dendrite, but also in the movement of melanosome.

Transfer of melanosomes to keratinocytes

Melanosomes, which are transferred from melanosome to keratinocytes, are found either discrete particles (nonaggregates) or aggregates of two or more particles within membrane-limited vesicle [9]. These structures are called "melanosome complex". In the Caucasoid (white) skin, melanosome complex in kelatinocytes appears smaller and predominantly aggregates whereas Negroid (black) skin and in the dark hair of all races, melanosome are large and remain discrete. Difference in melanosome size that is observed in various races and skin type is shown in table 2 [10].

The processes in which melanosomes transferred from the tip of dendrite into melanosome and dispersed singly or as a complex have 2 explanations. Firstly, melanocytic dendrite contacts the cell membrane of the keratinocyte which then pinch

off its tips containing melanosomes and encloses both within a phagocytic vacuole. This postulate presents that, at least initially, the phagocytic vacuole posses a double membrame-one of keratinicyte origin, the other of melanocytic origin. Another postulates present that melanosomes are secreted extracellularly from the tips of the melanocytic dendrite and then engulfed by the keratinocytes [9].

Table 2.   Relationship among skin color, size and distribution pattern of melanosomes and skin type classification*

Skin color	Size of melanosomes	Tyrosinase activity in melanocytes	Distribution of melanosomes in epidermal keratinocytes	Approximate number of melanosomes per basal keratinocyte*	Skin type**
Heavily pigmented skin of African, American Negroes and Australian Aborigines	0.7-0.8 μm 0.3-0.4 μm	Marked	Single, Nonaggregated	400 ± 35	VI
Moderately pigmented skin of Mongoloids (American Indians)	0.5-0.7 μm 0.2-0.4 μm	Moderate	Mixed Nonaggregated as well as aggregated	250 ± 50	V VI
Moderately pigmented skin of Caucasoid (East Indians, Italians, Egyptians)	0.5-0.7 μm 0.2-0.4 μm	Moderate	Predominantly aggregated	200 ± 5	II III
Lightly pigmented skin of Caucasoid (fair skinned Americans, British, French, Germans)	0.4-0.6 μm 0.2-0.4 μm	Weak	Predominantly aggregated	100 ± 50	I II III

*   Based on random calculations of 50 keratinocytes in basal layer.

** Estimate of sensitivity to UVB erythema based on the history of sunburn and capacity of tan.

Degradation of melanosomes [7,9]

Melanosomes are subjected to degradation by lysosome enzymes. The specific enzymes involved in this process are still unclear. Because the melanosome of Negroids are larger than Caucasoids, degradation seems to take a longer time.

Melanin Functions [8]

Melanin is known as a filter and protects cell by absorption of UV light penetrating to the epidermis. Because the smaller proportion of the UV can reach the basilar layer, the amount of DNA damage is decreased by 50% by the presence of melanin in the epidermis [8].

The generation of radical oxygen (oxygen with unpair electron) or singlet oxygen (electrons located in high energy orbital) by ultraviolet light including the abundance of normal radical found in the skin such as hydrogen peroxide, hydroxyl radicals, and superoxides bring about mutation, DNA damage, cancers and cell death. Not only UV light, but also all forms of the inflammation produce a variety of high energy oxygens such as arachidonic acid, prostaglandins, and leukotrienes. However, the body cells have several mechanisms or scavengers such as ascorbic acid, glutathione, and carotene to eliminate high-energy oxygen. Melanocytes are thought to be a scavenger and radical remover from the surrounding cell such as keratinocytes and Langerhans cell by oxidation of tyrosine to form melanin. By this way, radical and toxic by-products are removed [8].

Regulation of melanogenesis [5,8,12]

Ultraviolet Light [8,5]

Ultraviolet light, especially UV B (290-320 nm) is the most effective tanning spectrum. Two types of pigmentation of the skin in humans occur in response to sun exposure [5]. One is immediate pigment darkening (IPD), referred as a Meirowsky phenomenon. This hyperpigment is induced by long wave UV light. It is temporary, rapidly induced, and soon recovered. Another is the increased pigmentation that follows the erythema response. This is the delayed tanning reaction (DT) and can be seen 48-72 hr. after exposure to UV Light [8].

A classification of sun-reactive skin type based on sunburn and tanning history (Table 3) is now generally used. In people with type III or IV who tan well, there is a significant increase in the quantity of melanin within the epidermis after UVB because melanosomes increase in exposure to number and larger size, but this mechanism is not known. However, the studies of Friedmann and Gilchrest in culture suggest that the cell have internal photoreceptor responsible for generating oxygen radicals when activated by UVB. After exposure to UVB, epidermis tends to release eicosanoid such as prostaglandin. Prostaglandin E (PGE) and Prostaglandin D (PGD) enhance melanin formaiton whereas Prostaglandin A (PGA) suppresses tyrosinase activity.

UVA (320-400nm) can also induce tanning by inducing melanin synthesis and the transfer of melanosomes to adjacent keratinocytes. Unlike UVB exposure, the only number of melanosomes increase with constant size [8].

Table 3. Classification of Sun-reactive Skin Types:

Skin type	Sun sensitivity	Pigmentary response
I	Very sensitive, always burn easily	Little or no tan
II	Very sensitive, always burn	Minimal tan
III	Sensitive, burn moderately	Tan gradually(light brown)
IV	Moderately sensitive, burn minimally	Tan easily (brown)
V	Minimally sensitive, rarely burn	Tan darkly (dark brown)
VI	Insensitive, never burn	Deeply pigmented (black)

Inflammation

People who have the type I and type II skin do not tan well; different from type III and IV who rapidly respond to most inflammatory stimuli such as mild thermal burns, cold, mechanical abrasions, wound healing, or dermatitis of any form because of lacking the essential processes to produce pigment.

Melanotropin

A precursor protein, proopiomelanocortin, in the pituitary produce variety of peptides cleaved into smaller peptides such as α-MSH, adrenocorticotropic hormone (ACTH), β lipotropin, β-MSH, and endorphin. β-MSH probably is not a biologically relevant molecule in humans. α-MSH is said to be important to hyperpigmentation observed in patients with adrenal insufficiency. In tissue culture, melanoma cells and human melanocytes respond to α-MSH by increasing tyrosinase activity and melanin synthesis. Moreover, intracellular cyclic AMP concentrations and cyclic AMP-dependent accompanied with calcium ions or formation of prostaglandin found to rapidly increase after adding of α-MSH [8]. Mechanistically, the effect of MSH is commonly explained as due to binding of MSH to a specific receptor on the melanocyte membrane. This results in the activation of cyclic AMP-independent protein kinase and subsequent phosphorelation of one or more proteins to initiate the so-called cascade of events leading to an increase in tyrosinase and subsequent stimulation of melanogenesis (Figure 6)[11].
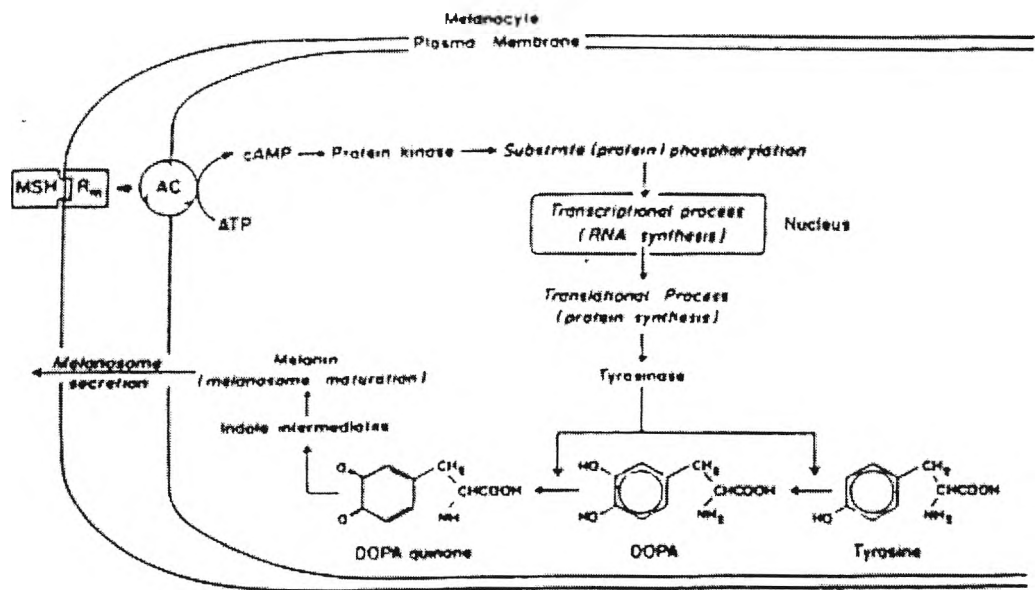
Figure 6. Model for MSH activation of melanocyte tyrosinase and
melanin synthesis (Hadley, 1988)

Prostaglandins and Leukotrienes

Epidermis tends to release eicosanoids such as prostaglandin E (PGE) and Prostaglandin D (PGD) which enhance melanogenesis whereas Prostaglandin A (PGA) suppresses melanogenesis [8]. In melanoma cells, Leukotriene C_4 (LTC_4) act as a mitogen by stimulating melanocytes.

1,25-Dihydroxy Vitamin D_3

When the skin is exposed to Ultra violet light, 7-dehydrochloresterol in the keratinocytes is converted into cholecalciferol. This compound further activates enzyme phospholipase A_2 and causes the release of arachidonic acid before conversion into vitamin D_3. At present, a vitamin D_3 receptor is found on normal human melanocytes. The dihydroxylated vitamin D and 25-hydroxy vitamin D_3 appear to suppress tyrosinase activity of human melanocytes [10].

Interleukins, Lymphokines, and Interferon

There is a report by Swope and co-workers that IL-1 can inhibit melanogenesis effect by decreasing tyrosinase activity both in murine melanoma cells and in normal human melanocytes growing in culture. IL-1 also blocks the melanizing effect of α-MSH, PEG, cyclic AMP, and other agents. This later observation suggests melanocytes have a receptor for IL-1. Not only IL-1 can decrease in tyrosinase and proliferation, but also IL-6 and tumor necrosis factor-α can. Nevertheless, This mechanism by which these lymphokines alter the pigmentary system is under investigation. Besides, Interferon-γ is reported from Kameyama and co-workers that probably alter the surface receptors for MSH resulting in exhibits synergistic melanogenesis when combination with α-MSH.

Basic Fibroblast Growth Factor (bFGF)

It is synthesized by keratinocytes and released in epidermis. In culture, bFGF is necessary for the maintenance of normal melanocytes. The receptor of bFGF is also found at the surface of melanocytes.

Nerve Growth Factor

The receptor of Nerve Growth Factor is also found at the surface of melanocytes. It may be important in the migration of melanocytes.

Histamine

From the laboratories, mast cell product shows melanized stimulation by increasing of tyrosinase activity when histamine is added to melanoma culture.

B. Whitening Agents

Because tyrosinase plays a key role in melanin biosynthesis, many people have tried to find substances that can either block the synthesis of tyrosinase or inhibit its activity so that the formation of melanogenesis is prevented. Moreover, they tried to combine several compounds with different mechanisms to achieve the most efficient and least toxic skin lightening effect. Some of the whitening ingredients can be described, each having a different of action and interfering in different ways in the processes of tanning as described above.

1. Suppression of the formation of tyrosinase

Only a few active ingredients are recently known to have tyrosinase ability suppression. The best known active is natural L (+) lactic acid and its lactate salt.

Lactic acid and Lactates [13,14,15]

Lactic acid, one of AHA (α-hydroxy acid), was discovered in 1780 by the Swedish chemist C.W. Scheele and is officially known as 2-hydrohypropionic acid [13]. Lactic occurs in two forms of optical active molecule, the L(+) form (natural lactic) which is found in human body and produced by fermentation procedures and metabolic reaction, and the synthetic form ((50:50) L(+) : D(-)). In addition, L(+) lactic acid is also present in blood, muscles, organs, hair and skin. The D(-) forms can also be produced selectively via fermentation by certain strains of bacteria [14]. The benefits of natural L(+) and lactates to skin have been known for ages. Cleopattra used to take baths containing goat milk and long ago women washed their faces with

wine. Although unaware of the principles, they took advantage of the beneficial properties of the lactic acid and lactate.

Over the years, scientists have acquired a better understanding of the structure of the skin, the compounds present and their mechanism of action. One important discovery was the group of skin compound known as the Natural Moisturizing Factor (NMF), which maintain the water in the skin. Sodium lactates have been hypothesized to be part of skin's own natural moisturizing system. Several clinical and *in vitro* studies have shown that topically applied sodium lactate will be absorbed by the skin and lead to moisturized, softened skin. Furthermore, in the cosmetic industry, lactic acid and its salts are added into formulation for a good buffer, effective humectants, natural preservative, safe, and act as a part of NMF. It is further known that L(+) lactic acid is used in treatment of some types of acne since it is effective against the propiobacterium acne. In the Takahashi et al showed that sodium lactate retains the water longer in the stratum corneum than the skin and that it gave higher skin-softening [15].

Lactic acid used as exfoliative and skin whitening agent

More recently, the skin lightening properties of lactic acid and lactates have become obvious. Several studies and publications have reported the whitening properties of natural L(+) lactic acid and lactates. At low concentration (5%), it stimulates epidermal turnover of cells (exfoliation) and has potential to mediate long term skin improvement; this occurred at low pH. At pH of skin level (5-5.5), the exfoliative action of lactic acid is only modest because most of the lactic acid is available in its salt form (sodium lactate or other salt of lactic acid). Next to that, Stiller et al found that lactate reduces age spots on photodamaged skin whereas glycolic acid did not show a significant effect. These confirm that the performance of lactates go beyond the exfoliative action. If formulated at low pH, It is presumed that the exfoliate action of lactic acid is by speeding of the epithelial cells from the lower epidermis to the surface [16]. Since much of the melanin resides in the epithelial cells of the epidermis; it is possible that pigmentation will be reduced.

When used at higher levels, it has a positive effect on the treatment of photo damaged skin. Some published reports suggested that lactic acid has a whitening

effect not only via stimulating epidermal turnover, but also inhibiting the formation of tyrosinase [16], which may contribute to the overall activity of composition. It has been supported by W.P Smith that high concentration of AHAs (12% lactic acid) can induce changes in both the epidermis and dermis while lower concentration only delivers superficial effect [16]. This author also demonstrates that the ability to deliver the skin changes is likely to come from alteration of the skin barrier. This author further shows the synergistic activity in combination of 8.8% L(+) lactic acid and 1% vitamin C in the treatment color of age spot[16]. Moreover, it has been supported by the study of van Rijsbergen. He also shows that a combination of L(+) lactic acid with vitamin C reveals a higher synergistic effect against age-spots than single vitamin C or lactic acid. They further suggest that lactates in combination with tyrosinase inhibiting agents like vitamin C, vitamin B₃, arbutin, kojic acid or licorice may enhance the skin lightening effect of a formulation [15].

Other properties of lactic acid and lactates

In a whitening formulation, lactic and lactates can add three other important properties to the formulation.

1. Lactic acid and lactates are one of the most effective moisturizers. The moisturizing effect of lactic acid and its sodium salt is directly related to the water holding capacity of the lactate molecule. Lactate shows a high water-holding capacity available in the market. The experiment of W. P Smith shows a significantly better hydrating skin when applied with both L(+) lactic acid and D(-) lactic acid than glycolic acid [17].

2. Lactates are able to increase the natural ceramide level in the skin. L(+) lactates stimulate ceramide biosynthesis leading to an increase in stratum corneum ceramide levels which result in superior lipid barrier and more effective resistance against xerosis.

3. Lactic acid is a mild and effective AHA. In combination with whitening properties it could potentially boost the whitening process.

4. Lactic acid can use as a combination.

Nowadays, a large-scale clinical trial is being conducted in the Philippines among dark-skin Asians comparing the whitening efficacy of lactate with the whitening performance of other frequently used ingredients. The *in vitro* study of

Zuidoff and Rijsbergen by using melanoderm as a human epidermis model to compare the tyrosinase inhibitive/suppressive action of 3% lactic acid with other whitening agents which comprise 1% kojic acid, 1% MAP (Magnesuim ascorbyl phosphate), and 1% ascorbic acid. The study indicates that Ascorbic acid showed only a very modest performance whereas lactic acid and kojic acid showed a dramatic decrease in tyrosinase activity over other whitening agents. Thereafter, they study in vivo whitening efficacy on 7 formulations between 8 %lactates (sodium lactate 60%), 1% arbutin, 0.3% licorice, 1% kojic cid, arbutin + sodium lactate, licorice + sodium lactate, and kojic acid + sodium lactate. From the study, it can be seen that the formulations containing sodium lactate (even single sodium lactate formulation) continue to achieve a reduction in pigmentation after 6 to 12 weeks. Then, After 12 weeks, the combination of sodium lactate + licorice, sodium lactate + arbutin as well as a single lactate formulation are the best performing whitening formulations [13,14].

2. Inhibition of the activity of tyrosinase

Skin-whitening ingredients that can inhibit the activity of tyrosinase are in general highly soluble in water. Their mechanisms are based on an alteration of active center of enzyme tyrosinase, thus inducing its activity. In most cases the ingredients are extracted from natural sources such as bearbery (arbutin leaves), licorice root (glycerhizinic acid), citrus fruits (ascorbic acid) or fermented carbohydrates (kojic acid, gamma-pyron compound). The disadvantage of these ingredients is in general their high price and instability in formulations in some cases [13].

Arbutin (hydroquinone-beta-D-glucopyranoside)[17,18]

Arbutin, the active component of the crude drug *uvae ursi* folium described in the Japanese Pharmacopia, is a hydroquinone glycoside[17]. The chemical structure is shown below:
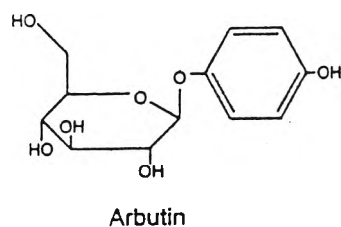
Figure 7. The chemical structure of arbutin

In the 18th century, Arbutin was first used in the medical area as an anti-inflammatory and anti-bacterial ingredient. More recently, it has been known very well as a skin-lightening agent used for depigmentation. Arbutin inhibits the formation of melanin pigment by inhibiting melasomal tyrosinase activity, rather than the suppression of the synthesis and expression of this enzyme. Arbutin has been used in combination with other ingredients to improve the activity for cosmetic used or for preventing skin pigmentation [19].

In a study by Maeda and Fukuda, neonatal human melanocytes are used for *in vitro* assay of depigmenting agents, such as hydroquinone, linoleic acid, arbutin, kojic acid, and ascorbic acid, by comparison of their effects on melanin synthesis. The report indicated that arbutin can reduce the amount of melanin up to 75%. This effect was about 1/100 of hydroquinone, but stronger than kojic and ascorbic acid while linoleic acid seem to have no tyrosinase inhibitory activity at non-cytotoxic ranges [18].

There are also the studies of Azizah which compare the melanin reduction of 3% arbutin + 0.005% licorice extract containing cream, 1% kojic acid + 1% liposome loaded vitamin C containing cream, and 3% MAP + 0.05% licorice extract containing cream. The result obtained show that the cream contained 3% arbutin + 0.005% licorice extract was better in decreasing the number of melanin (3.41%), while the cream containing 3% MAP + 0.05% licorice extract was better in increasing lightness. However, the result also showed the erythema effect from the application of 3% arbutin + 0.005% licorice extract containing cream more significant compared to

other cream whereas 1% kojic acid + 1% liposome loaded vitamin C showed the minimum effect of erythema [19].

Kojic acid (5-hydroxy-4-pyran-one-2-methyl) [18,21]

Kojic acid (Figure 8), a fungal metabolic product, has been used as a skin whitening agent in skin care products marketed in Japan since 1988. The chemical structure is shown below:
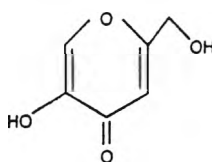
Kojic acid

Figure 8. The chemical structure of kojic acid

It was first isolated from *Aspergillus* in 1907. Kojic acid is used in concentrations ranging from 1-4%. At this concentration level, Kojic acid suppresses free tyrosinase, mainly by chelating the copper in tyrosinase. Indeed, it has been shown significantly to enhance neutrophile phagocytosis and lymphocyte proliferation stimulated by phytophemagglutinin. Melanocytes treated with kojic acid become nondendritic with decreased melanin content. Additionally, it scavenges oxygen species that are released excessively from cells or generated in tissue or blood [22].

In Japan, it is used in non-prescription skin-care products up to a concentration of 1%. Usually, it is used at the highest concentration to allow maximum percutaneous absorption and thus therapeutic activity. Because it is widely used in some countries, particulary in Japan, the oral safety has been studied.

In the study of Shibuya et al. in investigating mutagenicity in kojic acid by the Ames test, it was concluded that although kojic acid is a weak mutagen in bacteria, it is nonmutagenic in the eukaryotic system either *in vivo* or *in vitro*.

However, true adverse effects after human oral ingestion have not been demonstrated. Nakagawa et al noted that no sign of relapse dermatitis or any other adverse on sensitized patients upon ingestion of food containing kojic acid. However, they also indicated that topical application many induce allergic contact dermatitis with sensitized patients because the patients using one or more kojic acid containing products have a high frequency of contact dermatitis. Thus, they postulated that kojic acid was considered to have a high sensitizing potential [21].

Recently, in vitro whitening efficacy was evaluated by Majmudar et al. In these studies, kojic acid (1%), Lactic acid (3%), and 1% Magnesium ascorbyl phosphate (MAP) in nonionic aqueous base were applied for three days on melanoderm. These studies suggest that all whitening agents and base were not toxic. Moreover, kojic acid and lactic acid presented better inhibition more than MAP. The studies also indicated that kojic acid in an anhydrous form could induce more skin whitening than an aqueous form [23].

Lim conducted a non placebo controlled study to test 2% kojic acid in a gel containing 2% glycolic acid and 2% hydroquinone in 40 Chinese women who had epidermal melasma for 12 weeks. Half of a face was treated with above formulation while another treated with identical containing no drug formulation. Result showed similar improvement in melasma on both sides. More than half (60%) of the melasma cleared in side receiving kojic acid, whereas less than half (48%) cleared in side denied kojic acid; in particular, two patients had complete clearance only in the kojic acid-treated side. However, the improvement did not show a statistical difference between the formulations.

Evelyn suggested that the inhibitory effects on tyrosinase activity of kojic acid ester (kojic dipalmitate) had a better effect than kojic acid and hydroquinone when measuring the coloration of various melanin intermediates and thus tyrosinase activity. Additionally, the human patch test on kojic dipalmitate showed it to be completely non-irritating [24].
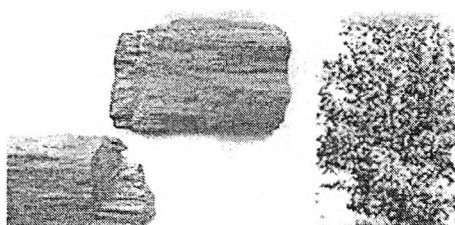
Artocarpus Lakoocha extracts [25,26]

Figure 9. The picture of *Artocarpus Lakoocha* heartwood

Scientific name	*Artocarpus lakoocha* Roxb.
Family	Moraceae
English name	-
Local name	Ma-Haad, Kayae, Tapang, Ma-Haadbaiyai, Had
Clinical efficacy	3-5g of Puag-Haad are used as an anthelminthic such as Taenia saginata and worms in pig and dogs.
Safty	When received 200 or 100 mg two interval dose every 10 hr, it was found no toxicity to liver and spleen. Moreover, it found that no toxic to any organ, when feeded 1.25g/Kg to mice.
Puag-Haad	Dried aqueous extract of *Artocarpus lakoocha* heartwood
Description	yellowish powder

(Artocarpus lakoocha Roxb., family Moraceae)(Figure 9) is a plant commonly found in many parts of South and Southeast Asia. Actually, it was used to treat worms for many years, but recently, the discovery of new application brings it to a new whitening agent. Sritularak et al. [3] found that the extract from Puag-Haad stem had the ability to inhibit tyrosinase activity *in vivo*. They screened plant extracts in vitro for their ability to inhibit tyrosinase activity using a commercially available tyrosinase derived from mushroom. One of the plant extracts tested, Puag-Haad (*Artocarpus lakoocha*), expressed strong tyrosinase inhibition which was greater than many well-known tyrosinase inhibitors like kojic acid and arbutin.

The active compounds in the stem extract of Puag-Haad are 2,4,3',5'-tetrahydroxystilbene (compound 1) and 4,3',5'-trihydroxystilbene (compound 2) expressing IC_{50} of 1.5 µM and 946.6 µM, respectively (Figure 10). The activity of compound 1 is much greater than kojic acid (IC_{50} = 26 µM) and arbutin (IC_{50} = 1.2 mM) [3].
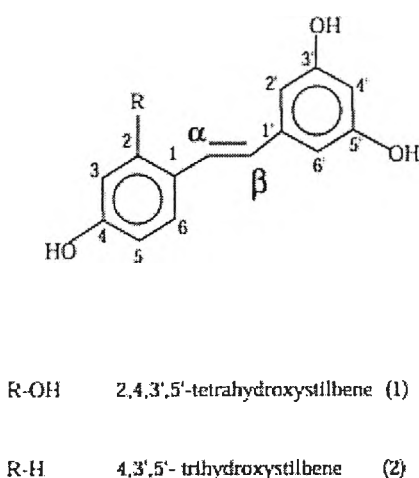

R-OH   2,4,3',5'-tetrahydroxystilbene (1)

R-H   4,3',5'- trihydroxystilbene (2)

Figure 10. The structures of compound 1 and 2 in Puag-Haad

(A. lakoocha) extract

From the preliminary *in vivo* data by Pengrungruangwong [27] using guinea pigs as a model, it was found that 0.5% and 1% of dried stem Ma-Haad extract (locally called Puag-Haad) dissolved in propylene glycol could significantly reduce melanin concentration on the back of guinea pig skin (p< 0.05). Furthermore, the effect of 0.5% and 1% extract was found to be similar, indicating the possible saturation of enzyme tyrosinase by the active ingredients in the extract. Moreover, 0.5% extract also produced greater *in vivo* skin whitening effect in guinea pigs than 3% kojic acid (p<0.05). Thus, it would be interesting to know if further lowering the concentration of the same extract to 0.25% would still exhibit significant skin whitening activity.

Mulberry extract

Mulberry extract can be obtained from the roots of the mulberry tree. Their major components are isoprenyl flavoniods and coumarin. These have been used medicinally for skin whitening as well as for antimicrobial action, as a diuretic, and to eliminate fever and coughing [28]. Recently, the paper mulberry has been also studied for the whitening effect. The active compound, 5-[3-(2,4-dihydrohyphenyl) propyl]-3,4-bis(3-methy-2-butenyl)-1,2-benzenediol, was isolated from the root bark of paper mulberry and tested for effective tyrosinase inhibition. The obtained data showed the tyrosinase inhibition effect overcome ascorbic acid, kojic acid, and hydroquinone. The $IC_{50,}$ the concentration causing 50% inhibition of activity of tyrosinase, was reported to be 0.396% compared to 5.5% for HQ and 10% for kojic acid. Indeed, it was considered a nonirritant substance when tested for sensitizing potential at either 24 hours or 48 hours by performing a patch test using 1% paper mulberry extract [3,22,29].

Ascorbic acid and its derivatives [21]

Ascorbic acid may inhibit melanin production by reducing o-quinone, so that melanin cannot be performed by the action of tyrosinase until all vitamin C is oxidized. Because vitamin C is quickly oxidized and decomposes in aqueous solution, it is not generally useful as a depigmenting agent. The structure of vitamin C is shown below:
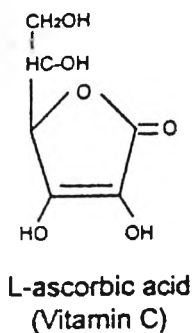
CH₂OH

HC-OH

HO   OH

**L-ascorbic acid
(Vitamin C)**

Figure 11. The chemical structure of vitamin C

Recently, stable derivatives of vitamin C have been synthesized to minimize this problem.   Magnesium-L-ascorbyl-2-phosphate (VC-PMG) is a vitamin C derivative that is stable in water, especially in neutral or alkali solution containing boric or it salt. VC-PMG is hydrolyzed by phosphatases of liver or skin to vitamin C and thus exhibits vitamin C reducing activity.

Kameyama et al. investigated the effect of VC-PMG on melanogenesis *in vitro* and *in vivo*. Results from their non-placebo-controlled study suggested the topical application of VC-PMG was significantly effective in lightening the skin in 19 of 34 patients with chloasma or senile freckles and in 3 of 25 subjects with normally pigmented healthy skin.

3. Direct reduction of melanin

The most known active ingredient based on this mechanism is hydroquinone.

Hydroquinone [21,22]

Hydroquinone is also a ubiquitous chemical readily available in cosmetic and nonprescription forms for skin lightening. The chemical structure of hydroquinone is shown below:
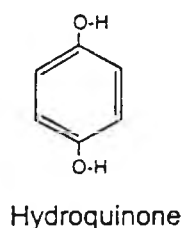
Figure 12. The chemical structure of hydroquinone

It is considered one of the most effective inhibitors of melanogenesis *in vitro* and *in vivo*. Hydroquinone causes reversible inhibition of cellular metabolism by affecting both DNA and RNA synthesis and degradation of melanosomes and destruction of melanocyte. Electron microscopic studies of black guinea pig skin treated with hydroquinone show the atomic consequences of this action:

(1) The melanosome structure is disturbed, resulting in decreased production or increase degradation of these organelles, or both.

(2) Hydroqinone exposure can ultimately lead to the degradation of melanocyte.

(3) Keratinocyte are spared, showing no apparent injury.

The most common drug used in the US as a skin lightener is hydroquinone (1.5 to 4% commercially, or up to 10% in custom-made product). Low concentrations are found in many cosmetics while the higher concentrations are prescription drugs.

Arndt and Fitzpatrick, in a non-placebo-controlled study, compare the efficacy of 2% and 5% hydroquinone cream for treatment of pigmentary disorder in 56 patients. Hydroquinone was a moderately effective depigmenting agent in 80% of cases. There was no efficacy difference between 2 concentrations, however the lower concentration was less irritating than the higher one.

In a non-placebo-controlled study, Fitzpatrick et al. evaluated the efficacy of a 2% cream of stabilized hydroquinone in 93 patients. Of those patients, 64% showed decreasing hypermelanosis.

The 2% Hydroquinone is readily available over the counter in various cosmetic preparations. However, for better efficacy, it is often combined with other ingredients for treatment of hyperpigmentation. The original Kligman formula involves compounding 5% HQ with 0.1% retinoic acid and 0.1% dexamethasone in hydrophilic ointment for the treatment melasma, ephelides, and postinflammatory hyperpigmentation. In contrast, they experienced poor results with each of the aforementioned as monotherapies. Indeed, Sanchez and Vazques postulated that the efficacy of hydroquinone might be improved when it is used in combination with other chemicals such as tretinoin, salicylic acid or corticosteriods.

As indicated above, not only the advantages of hydroquinones have been recognized, but they have been considered cytotoxic substances, with relatively high specificity to melanocytes as well. Adverse reactions associated with hydroquinone use include both acute and chronic complications. Among acute reaction are irritant dermatitis, nail discoloration, and postinflammatory hyperpigmentation. For these reasons, cosmetic formulations containing hydroquinone have been banned in several countries including Thailand. Hydroquinone used by people living in regions receiving a high degree of sunlight has been associated with development of ochronosis. This is a chronic skin condition characterized by appearance of sooty pigmentation commonly affecting the cheeks, forehead, and regions surrounding the eyes. European nations have stopped sales of hydroquinone depigmenting creams because of its mutagenic activity [30].

4. Other mechanisms

4.1 Inhibition of melanosomes transfer from melanocytes to keratinocyte

Vitamin B_3 comes in 2 principal forms, namely niacinamide (pyridine 3-carboxamide or nicotinamide) and niacin (pyridine 3-carboxylic acid or nicotinic acid). The amide form (nicotinamide) is already found to be an integral part of the coenzymes for metabolism, i.e., nicotinamide adenine dinucleotide (NAD) and nicotinamide adenine dinucleotide phosphate (NADP) [31,32]. As pharmacological action of niacinamide, high-dose niacinamide may be helpful in preventing type 1 (childhood-onset) diabetes and reducing symptoms of osteoarthritis. The action of niacinamide as a whitening agent is still totally unclear. Niacinamide (nicotinamide)

is reported to be active skin lightener, but scientific evidence of its effect is lacking [30]. However, there are reported by Barratt (unilever) et al. that topical treatment with niacinamide + sunscreen product (1% niacinamide, 1.25%parsol MCX, 0.4%parsol 1789) for 8-12 weeks on forearm whitened basal skin color vs. no treatment in Indian, Indonesian and Mexican subjects. It was of value to prove that the combination could lead to whiten the basal skin color [33].

Recently, the skin lightening effect of topical niacinamide has been studied. Hakozaki et al.[34] compared the products with and without niacinamide. The result of image anslysis demonstrated significant reduction of total hyperpigmented spot area and increase of L* value change from baseline for niacinamide-treated side vs. vehicle-treated side after 4 weeks of treatment. Interestingly, they also showed superior skin whitening efficacy of niacinamide plus sunscreen treated side compared with sunscreen alone (no significant in week 8th).

To investigate the mechanism action of niacinamide, These authors used a cell co –culture model and stained the melanosomes with succinimidyl ester of carboxy fluorescein diacetate (CFDA). Their experiment showed that niacinamide inhibited the transfer of melanosomes from melanocytes to keratinocytes. Moreover, Virador et al.[34] showed that niacinamide did not exhibit much inhibition of melanogenesis activity using purified tyrosinase in their assay. These results suggest that niacinamide may not be a potent tyrosinase inhibitor but may exhibit skin lightening effect via another novel yet not fully understood mechanism like the inhibition of melanosome transfer.

4.2 Suppression of production of Prostaglandins(PGs)

Tranexamic acid

Tranexamic acid (TA), or tran-4- aminomethyl cyclohexane carboxylic acid has a molecular formula of $C_8H_{15}NO_2$ with molecular weight of 157.214. Its appearance is white crystalline powder, odorless and bitter taste. It is freely soluble in water and glycial acid, very slightly soluble in ethanol, practically insoluble in ether

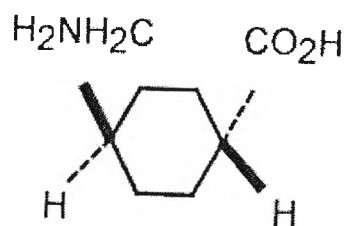and dissolves in sodium hydroxide TS [35]. Figure 13. shows the chemical structure of tranexamic acid.

Figure 13. The chemical structure of tranexamic acid.

In clinical application, tranexamic acid, known as an antifribrinolytic agent, competitively inhibits the activation of plasminogen in the fibrinolytic cycle ie. the conversion of plasminogen to plasmin [36]. The drug has affinity for the five lysine-binding sites of plasminogen. It thus promotes clot stability, and is useful as adjunctive therapy in haemophillia and some other bleeding disorders [37].

Recently, tranexamic acid has been studied as a whitening agent. In the studies of Maeda and Naganuma with Weiser-Maple guinea pigs induced by ultraviolet (UV) exposure(840mJ cm-2), skin pigmentation was clearly observed after seven day exposure and continued to increase to 29 days. Post-exposure applications of 2% and 3% solutions of tranexamic acid to the exposure regions prevented or inhibited the pigmentation process compared with the vehicle control. As plasmin is known to contribute to release of arachidonic acid (AA) and production of prostaglandins (PGs), the effects have been examined of tranexamic acid on AA-induced pigmentation in guinea pig skin. Topical application of tranexamic acid causes a dose-dependent decrease in AA-induced pigmentation. Thus this report indicated that tranexamic acid reduces melanocyte tyrosinase activity by suppressing the UV induced the production of prostaglandin, UV-induced melanogens, and thus melanogenesis [38,39].

C. Evaluating Skin-Lightening Agents [40]

In vitro evaluation

To develop effective ingredients, including skin lightener, reliable evaluation methods are essential. Certain tests should be used to determine lightener or effectiveness, such as the enzyme, cell-culture and tissue culture methods.

1. Enzyme method

Tyrosinase, commonly taken from mushrooms, is added to its substrate tyrosine or DOPA. Dopachrome is one of the intermediate substances in the melanin biosynthesis. By the dopachrome method, the red color of dopachrome can be detected by visible light. The potential tyrosinase inhibitor would show minimal dopachrome absorption [41]. Radioactive substrate can also be used.

2. Cell-culture method

Skin lightening agents are added to a culture medium to evaluate their inhibitory effect on melanogenesis in either mouse B-16 melanoma cells or human melanocytes. This method allows detection of not only inhibitory effect on enzyme activity of tyrosinase, but also on any process not related to tyrosinase, such as the inhibition of synthesis of the sugar side chain or transfer enzymes between organelles.

3. Tissue-culture method

This method is sometimes used to study the interaction of melanocytes with their neighboring cells and tissues. Dissected mouse follicles or guinea pig skin are commonly used.

In vivo evaluations [40]

Once results from *in vitro* methods prove promising, *in vivo* methods should be used to confirm the efficacy.

1. Effect on UV-induced pigmentation

Following UV irradiation, pigmentation changes on exposed area of either the shaved backs of guinea pig skin or the human forearm are assessed visually,

optically (by chromometer or mexameter) and histologically. Topical application of the test samples may start before or after the UV-induced pigmentation has subsided, depending on the test's objective.

2. Clinical tests

Placebo-controlled clinical tests determine a skin lightening efficacy to cure or improve facial pigmentary disorders. Researchers can also evaluate possible side effects and any other effects, such as the result of adding sunscreen.