UNIT 3

# Fat-Soluble Vitamins

## TABLE OF CONTENTS

There are four vitamins that are soluble only in fat solvents and not in water. As such, these vitamins are found in lipid extracts of tissues and foods. They are named alphabetically in the order of their discovery.

## I. VITAMIN A

Vitamin A was the first vitamin identified as an essential micronutrient needed by humans. Although it has been recognized as a chemical entity for about 60 years, foods rich in this vitamin have long been prescribed as treatment for night blindness. Ancient Egyptian physicians recommended the consumption of ox or chicken liver for people unable to see at night. In India it was recognized that inadequate diets were related to night blindness and, in France, that diets consisting of sugar, starch, olive oil, and wheat gluten fed to animals resulted in ulcerated corneas. Mori, in Japan, reported on the curative power of cod-liver oil in the treatment of conjunctivitis and, later, Hopkins in the U.S., reported on the importance of whole milk in such treatments. During the 1920s, Osborne and Mendel at Yale and McCollum's group in Wisconsin identified a substance in cod-liver oil, egg yolk, and butterfat that cured night blindness and which was essential for normal growth. They called this substance "fat soluble A."

**Table 1 Nomenclature of Major Compounds in the Vitamin A Group**

| Recommended Name | Synonyms |
|---|---|
| Retinol | Vitamin A alcohol |
| Retinal | Vitamin A aldehyde, retinene, retinaldehyde |
| Retinoic acid | Vitamin A acid |
| 3-Dehydroretinol | Vitamin $A_2$ (alcohol) |
| 3-Dehydroretinal | Vitamin $A_2$ aldehyde; $retinene_2$ |
| 3-Dehydroretinoic acid | Vitamin $A_2$ acid |
| Anhydroretinol | Anhydrovitamin A |
| Retro Retinol | Rehydrovitamin A |
| 5,6-Epoxyretinol | 5,6-Epoxyvitamin A alcohol |
| Retinyl palmitate | Vitamin A palmitate |
| Retinyl acetate | Vitamin A acetate |
| Retinyl β-glucuronide | Vitamin A acid β-glucuronide |
| 11-*cis*-Retinaldehyde | 11-*cis* or Neo b vitamin A aldehyde |
| 4-Ketoretinol | 4-Keto vitamin A alcohol |
| Retinyl phosphate | Vitamin A phosphate |
| β-Carotene | Provitamin A |
| α-Carotene | Provitamin A |
| γ-Carotene | Provitamin A |

## A. Structure and Nomenclature

Vitamin A is not a single compound. It exists in several forms and is found in a variety of foods such as liver and highly colored vegetables. The IUPAC-IUB Commission on Biochemical Nomenclature has proposed the following rules for naming the compounds having vitamin A activity. The parent substance, all-*trans* vitamin A alcohol, is designated "all-*trans* retinol." Derivatives of this compound are named accordingly. In Table 1 are listed the major vitamin A compounds.

In foods of animal origin the vitamin usually occurs as the alcohol (retinol). However, it can also occur as an aldehyde (retinal) or as an acid (retinoic acid). In foods of plant origin, the precursor to the vitamin is associated with the plant pigments and is a member of the carotene family of compounds. These latter compounds can be converted to vitamin A in the animal body and are known as provitamins. Of the carotenes, β-carotene is the most potent.

Figure 1 gives the structure of vitamin A as all-*trans* retinol. Some of the biologically important compounds having vitamin A activity are shown in Figure 2.

Note that all of these compounds have a β-ionone ring to which an isoprenoid chain is attached. This structure is essential if a compound is to have vitamin activity. If any substitutions to the chain or ring occur, then the activity of the compound as vitamin A is reduced. For example, substitution of methyl groups for the hydrogen on carbon 15 of the side chain results in a derivative that has

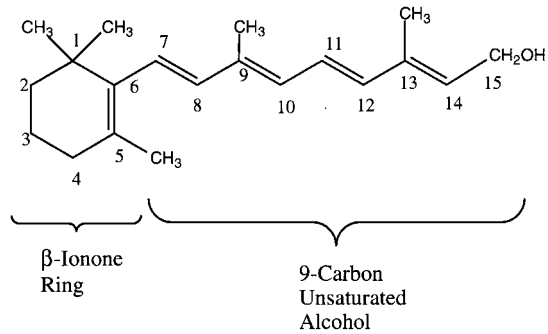



**Figure 1** Structure of all-*trans* retinol.

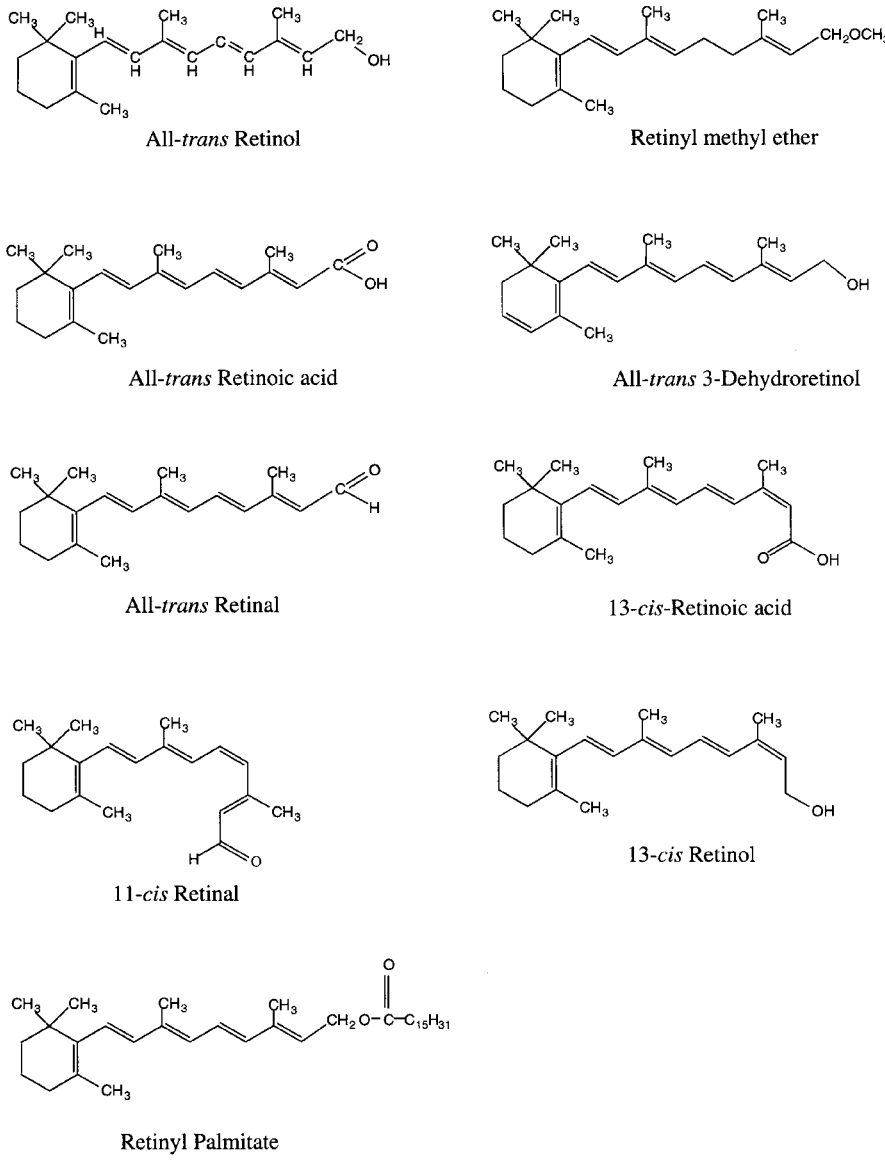



**Figure 2** Structures of Vitamin A compounds.

no vitamin activity. However, the preparation of a methyl ester or other esters at carbon 15 results in a very stable compound with full vitamin activity. In addition to improving the chemical stability of the compound, these ester forms confer an improved solubility in food oils. These vitamin ester forms are frequently used in food products for vitamin enrichment.

If the side chain is lengthened or shortened, vitamin activity is lost. Activity is also reduced if the unsaturated bonds are converted to saturated bonds or if the side chain is isomerized. Oxidation of the β-ionone ring and/or removal of its methyl groups likewise reduces vitamin activity. Some of these substituted or isomerized forms are potent therapeutic agents. For example, 13-*cis* retinoic acid has been used in the treatment of certain kinds of cancer. Other analogs, notably the fluoro and chloro derivatives, have been synthesized with the hope of providing chemotherapeutic agents for the treatment of certain skin diseases and cancer.

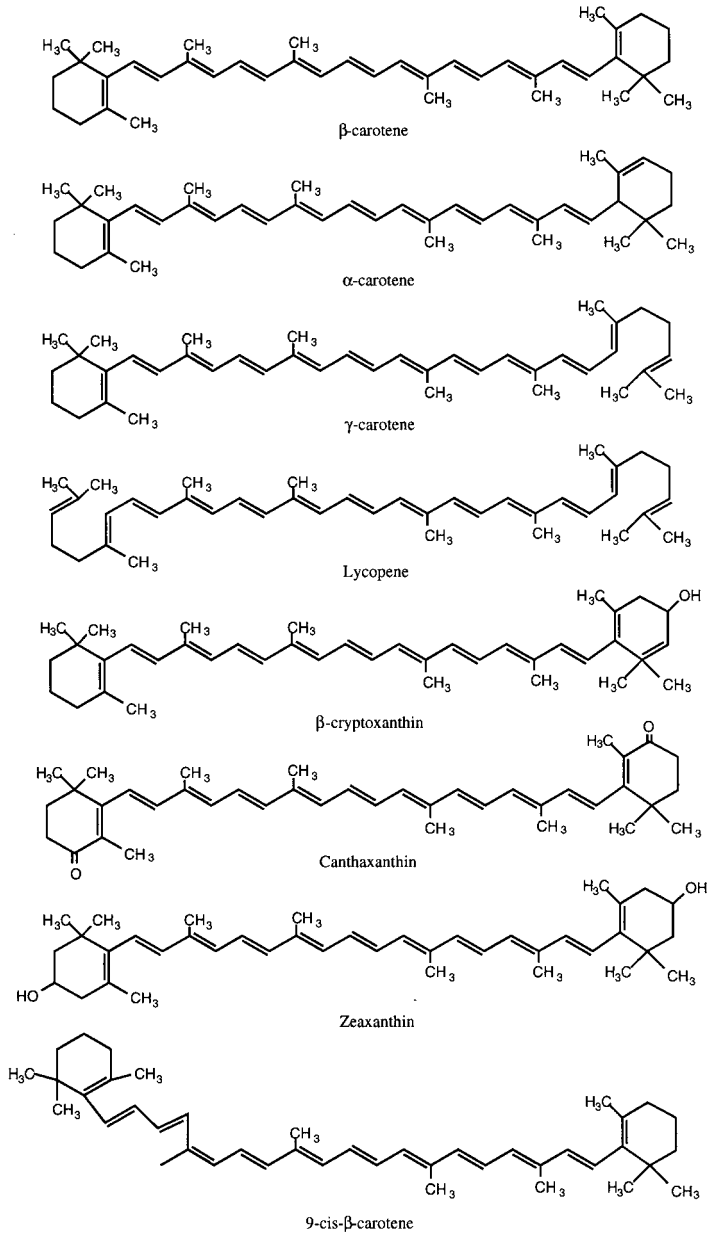



**Figure 3** Structures of carotenes having vitamin A activity.

The provitamin A group consists of members of the carotene family. Shown in Figure 3 are the structures of some of these compounds. Also shown are structures of related compounds that, although highly colored, have little potential as a precursor of retinol. More than 600 members of the carotenoid family of pigments exist. However, only 50 or so can be converted (or degraded) into components that have vitamin activity. All these compounds have many conjugated double bonds and thus each can form a variety of geometric isomers. β-Carotene, for example, can assume a cis or a trans configuration at each of its double bonds and in theory could have 272 isomeric forms. The asymmetric carotene, α-carotene, can, in theory, appear in 512 forms. The vitamin A

**Table 2 Carotenoids With Vitamin A Activity**

| Compound | Relative Potency |
|---|---|
| β-Carotene[a] | 100 |
| α-Carotene | 53 |
| γ-Carotene | 43 |
| Cryptoxanthin | 57 |
| Lycopene | 0 |
| Zeaxanthin | 0 |
| Xanthophyll | 0 |

[a] Reference compound for subsequent compounds. Note that β-carotene compared to all-*trans* retinol is only half as active.

activity of the provitamin members of the carotene family is variable. Theoretically, β-carotene should provide two molecules of retinol. However, in living systems this does not always happen. The β-carotene content of food varies with the growing conditions and the post-harvest storage of the food. In addition, the digestibility of the food affects the availability of the vitamin. Even when fully available, β-carotene and other provitamin A compounds may not be absorbed efficiently and, further, the enzymes responsible for cleaving the β-carotene into two equal parts may not be active. In general, the β-carotene molecule will provide about 50% of its quantity as vitamin A. During its cleavage by the enzyme β-carotenoid 15,15′-dioxygenase, there is some oxidative conversion of the cleavage product to retinal and some oxidation to retinoic acid. This retinoic acid is rapidly excreted in the urine. Other carotenes are less potent than β-carotene, due not only to a decrease in their absorption, but also to their chemical structures, which do not meet the requirements described above for vitamin activity. These compounds are listed in Table 2. Note that some of these compounds, i.e., xanthophylls and lycopenes, have no vitamin activity even though they are highly colored and are related chemically to β-carotene.

## B. Chemical Properties

Through the careful work of Karrer and associates, the structures of both β-carotene and all-*trans* retinol were determined. It was only after this work was completed that it was realized that β-carotene was a precursor for retinol.

With the structures now known, the next step was the crystallization of the compounds. This was accomplished for vitamin A from fish liver by Holmes and Corbet. Ten years later, in 1947, Arens and van Drop and also Isler et al. were able to synthesize pure all-*trans* retinol. The chemical synthesis of β-carotene was achieved shortly thereafter. With the crystallization, structure identification, and synthesis came the understanding of the physical and chemical properties of these compounds and the development of techniques to measure their presence in food. All-*trans* retinol is a nearly colorless oil and is soluble in such fat solvents as ether, ethanol, chloroform, and methanol. While fairly stable to the moderate heat needed to cook foods, it is unstable to very high heat, to light, and to oxidation by oxidizing agents. Alpha-tocopherol or its acetate (vitamin E), through its role as an antioxidant, prevents some of the destruction of retinol.

The above properties of vitamin A allow for the removal of the vitamin from foods by fat-solvent extraction and its subsequent determination using agents such as antimony trichloride (the Carr-Price reaction), which produce a blue color. The intensity of the color is directly proportional to the amount of retinol in the material being analyzed. More recently, the development of high resolution (high pressure) chromatography (HPLC) has made possible the separation and quantification of each of the vitamers A. This technique is very sensitive and can detect microgram amounts of the vitamers. β-Carotene is also soluble in fat solvents such as acetone or ethanol. It is bright yellow in color and it, too, is stable to moderate heat but unstable to light or oxidation.

HPLC is used to separate and quantitate the members of the large carotenoid family. Several isomers and derivatives (Figure 2) of retinol have been identified. The isomerization of all-*trans* retinol to a mixture of its isomers is enhanced by high temperatures, ultraviolet light, and iodine or iron. While the isomerization of all-*trans* retinol to 11-*cis* retinol is an important *in vivo* reaction for the maintenance of the visual cycle, the production of isomers outside of the body through the application of high heat, light, and oxidizing agents results in a loss in biological activity of the original compounds. For example, retinyl acetate, if irradiated in hexane, forms an asymmetrical dimer (kitol) which has less than 1% of its original activity. This dimer has been isolated from whale-liver oil and some investigators have suggested that, in this species, the kitol could serve as a storage form of the vitamin. However, whether the kitol found in the whale liver was produced enzymatically or was consumed by the whale as a contaminant of its food supply has not been determined. In any event, for most species, vitamin A is stored in the liver not as a kitol but as an ester with a fatty acid (usually palmitate) in a lipid-protein complex.

Retinal, the aldehyde, combines with various amines to form Schiff bases. This reaction is important to the formation of rhodopsin within the visual cycle (see Figure 4). If mineral oil is present with retinal and the amine, the retinal may first form a hemiacetal which then reacts with a variety of amines to form the Schiff base. All-*trans* retinal also reacts with both the sulfhydryl and amino groups of cysteine or the amino group of tryptophan to form a five-membered thiazolidine ring. The formation of the thiazolidine ring is enhanced if formaldehyde is present. The resulting compound has a red color which is bleached upon exposure to light. This reaction, similar to the bleaching of rhodopsin (the visual pigment in the eye) with light exposure, does not utilize the same amino acid that is used in rhodopsin. In the latter, retinal binds to the epsilon amino group of lysine rather than the amino group of tryptophan or cysteine. Bleaching occurs when the energy transmitted by the light causes a shift in the electrons within the *N*-retinyl lysine. The same principle is involved in the loss of color with light exposure of *N*-retinyl cysteine and *N*-retinyl tryptophan. Interestingly, the many retinyl-amino acid complexes possible in the laboratory apparently do not form in the body. This is probably due to the limited amounts of both the free vitamin and the free amino acids. Both are usually bound or complexed to some other compound and are not readily available for this reaction.

In nature, vitamin A and carotene are bound to proteins, particularly the lipoproteins. Indeed, the vitamin is stored as an ester in those tissues (notably the liver) which synthesize lipoprotein. Usually, retinol is covalently bound to palmitic acid as well as being associated with a protein. The vitamin also complexes with other proteins as part of its mechanism of action at the subcellular level.

Retinol and its derivatives are soluble in nonpolar solvents, but not in water. However, retinol or retinyl esters can be made miscible in water with the use of detergents such as Tween 80™ (polyoxyethylene sorbitan monooleate). The resulting aqueous micellar suspension allows for the preparation of aqueous multivitamin supplements that are then useful for infants, small children, or persons unable to swallow a capsule. Studies of the absorption of vitamin A in this form, compared to the absorption of vitamins dissolved in an oil, have shown that this form of the vitamin is readily absorbed. This is particularly important for those persons having a fat absorption problem as in pancreatitis, celiac disease, idiopathic steatorrhea, biliary disease, or short gut syndrome.

Aqueous preparations are also useful to the food processor wishing to fortify or enrich a particular food product. Some food formulations would not be compatible with an oil-based vitamin A preparation, whereas an aqueous preparation combines very well with the ingredients in the food.

## C. Biopotency

The varying potencies of the different vitamin A compounds have, in the past, contributed confusion to the literature. This has been resolved by the establishment of a standard definition for vitamin A. One international unit (IU) of vitamin A is defined as the activity of 0.3 μg of all-*trans*

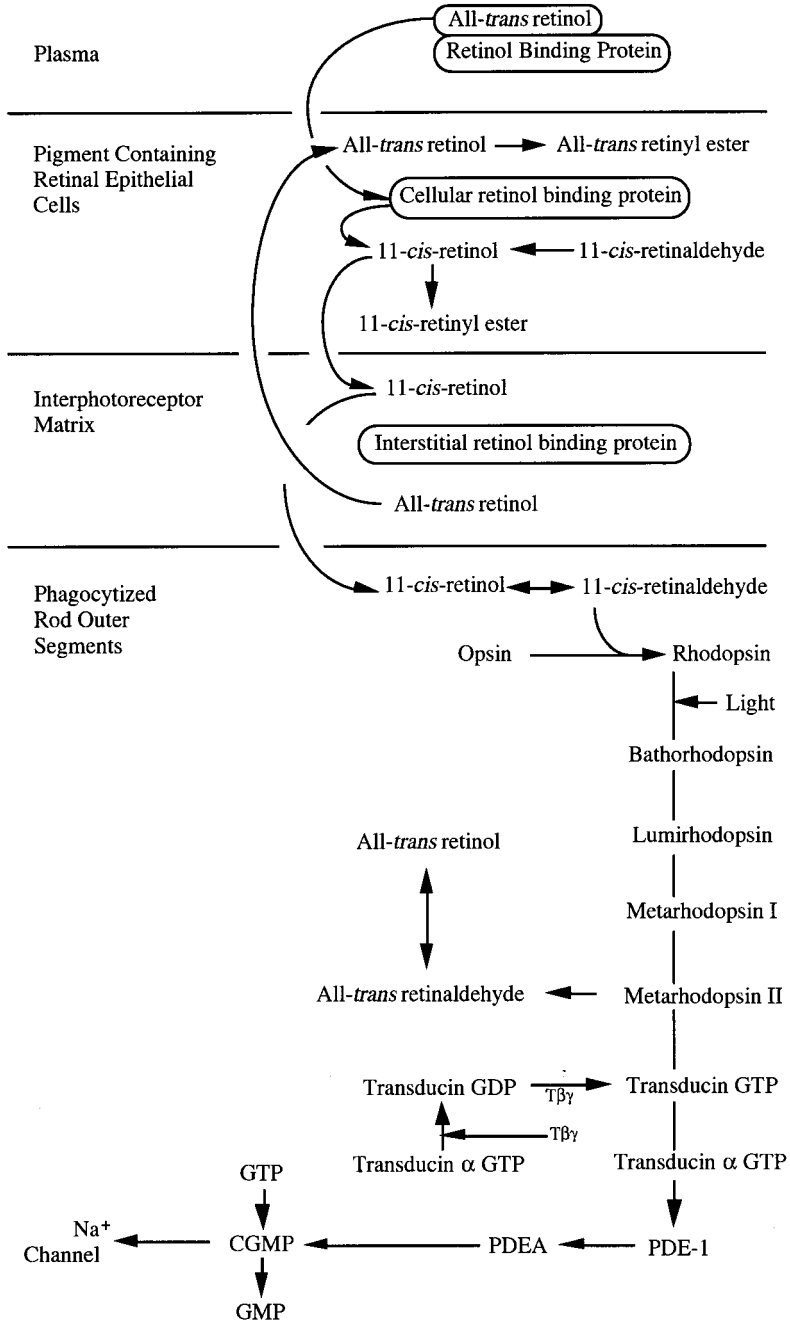



**Figure 4** The visual cycle.

retinol. Because β-carotene must be converted to retinol to be active, and because this conversion is not 100% efficient, 1 μg of all-*trans* β-carotene is equivalent to 0.167 μg of all-*trans* retinol. For other members of the carotene family, appropriate (as per Table 2) correction factors must be applied to determine the vitamin A activity in terms of retinol equivalents (RE). In general, however, 1 mg of retinol is roughly equivalent to 6 mg β-carotene and 12 mg of mixed dietary carotenes. The use

**Table 3 Retinol Equivalents (RE) for Humans**

| Compound | μg/IU | IU/μg | RE/μg |
|---|---|---|---|
| All-*trans* retinol | 0.300 | 3.33 | 1.00 |
| All-*trans* retinyl acetate | 0.344 | 2.91 | — |
| All-*trans* retinyl palmitate | 0.549 | 1.82 | — |
| All-*trans* β-carotene | 1.800 | 0.56 | 0.167 |
| Mixed carotenoids | 3.600 | 0.28 | 0.083 |

From Olson, J.A., *Handbook of Vitamins*, 2nd ed., Machlin, L.J., Ed., Marcel Dekker, New York, 1990. With permission.

of the term retinol equivalents allows for the calculation of the vitamin A activity found in a variety of foods, each containing one or more vitamin A compounds. For example, if 100 g of a given food contained 100 mg of α-carotene, which has 53% of the potency of β-carotene, the retinol equivalence would be $100 \times 0.53 \times 0.167$ or 8.851. Thus, this food would provide 8.851 retinol equivalents or 29.5 IU of vitamin A (8.851/0.3) per 100 g. Most tables of food composition give the vitamin A content in terms of IU; however, the reader should be aware of how these units are derived and realize that corrections must be made to allow for availability and efficiency of conversion of the provitamin to the active vitamin form, all-*trans* retinol. Table 3 lists the retinol equivalents (REs) for major vitamin A sources.

## D. Sources

Vitamin A and its carotene precursors are present in a variety of foods. Red meat, liver, whole milk, cheese, butter, and fortified margarine are but a few of the foods containing retinol. Carotene-rich foods include the highly colored fruits and vegetables such as squash, carrots, rich green vegetables (peas, beans, etc.), yellow fruits (peaches, apricots), and vegetable oils.

## E. Metabolism of Vitamin A

### *1. Absorption*

The availability of the vitamin depends largely on the form ingested and on the presence and activity of the system responsible for its uptake. Figure 5 schematically shows the route of retinol absorption. It is generally accepted that all-*trans* retinol is the preferred form for absorption. Retinal and retinoic acid are less well absorbed although both will disappear from the intestinal contents at a rate commensurate with an active transport system. Absorption requires the presence of food lipid and bile salts. Carotenoids are absorbed less efficiently, via diffusion. There is no active carrier for carotene within the luminal cell. Carotene is oxidatively cleaved to retinal, which is reduced to retinol and to small amounts of longer-chain β-apocarotenoids. Both central and exocentric cleavages are known to occur. The cleavage enzymes are very unstable and are rapidly inactivated. Two enzymes are involved in central cleavage; the first, β-carotene-15,15′-dioxygenase, catalyzes the cleavage of the central double bond to yield two molecules of retinal. The second, retinal reductase, catalyzes the reduction of retinal to retinol. This reaction is shown in Figure 6. The conversion of β-carotene to Vitamin A is not 100% efficient; thus, 2 mol of retinol are not obtained from 1 mol of β-carotene.

In order for the first enzyme to work, the β-carotene must be solubilized. This means that bile salts and some lipid must be present. Oxygen is also required since this is a dioxygenase type of reaction. Carotene dioxygenase is present in the soluble cell fraction and has been isolated from rat, hog, and rabbit intestinal tissue and characterized. The enzyme of the second reaction, retinal reductase, is also a soluble mucosal cell enzyme. It requires the presence of either NADPH or NADH as a donor of reducing equivalents. It is not particularly specific for the resulting retinal as

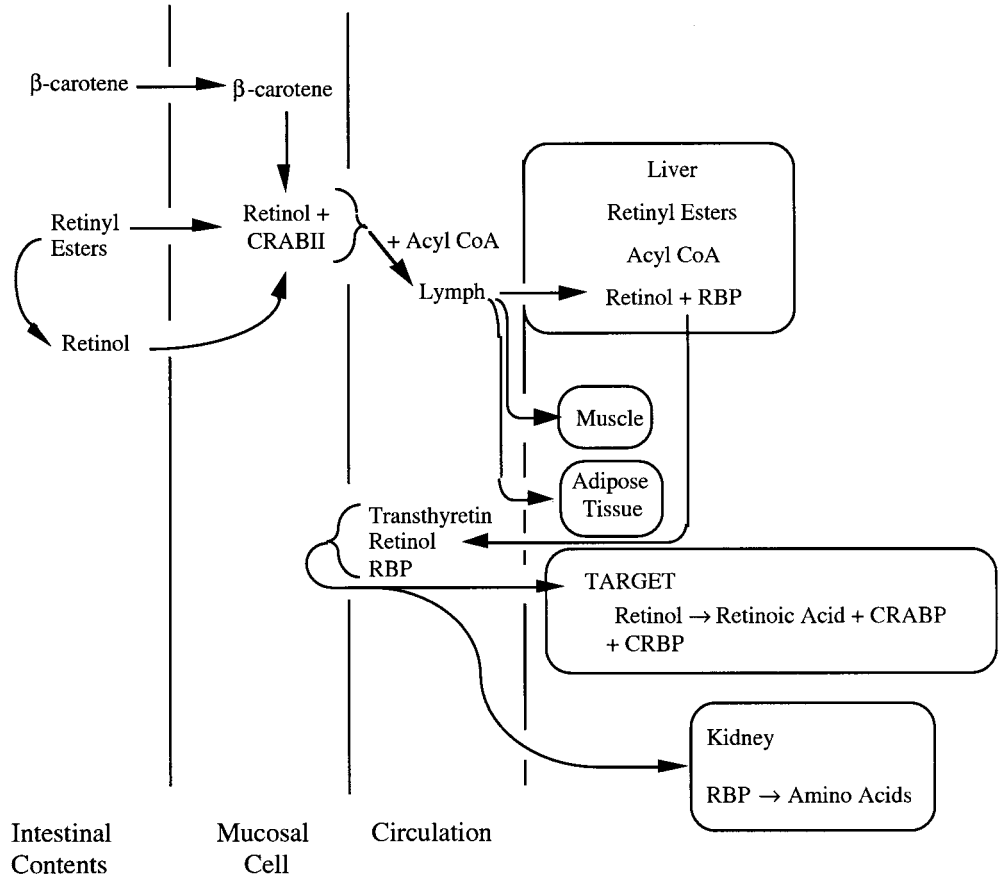



**Figure 5** Absorption and subsequent transport of vitamin A.

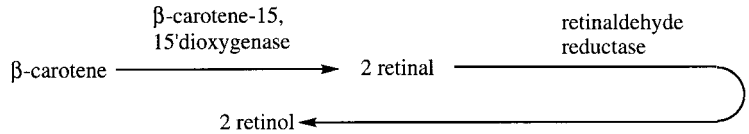



**Figure 6** Central cleavage of β-carotene.

it will catalyze the reduction of several short- and medium-chain aliphatic aldehydes in addition to retinal.

There are two pathways for retinol esterification. In the first, an acyl CoA-independent reaction is used. This involves a complex between retinol and Type II cellular retinol binding protein (CRBP II). As retinol intake increases there is a corresponding increase in the activity of the enzyme (acyl CoA retinol acyl transferase), which catalyzes the formation of this complex. Cellular retinol binding protein (CRBP II) is found only in the cytosol of the intestinal mucosal cell and, interestingly, its synthesis is influenced by the intake of particular fatty acids. Suruga et al. have shown that unsaturated fatty acids, particularly linoleic and α-linolenic acid, enhance CRBP II mRNA transcription. These fatty acids also enhance S14 and retinoic acid receptor (RAR) transcription in the adipocyte. Thus, it would appear that vitamin A uptake by the mucosal cell is dependent on dietary fat, not only because of its influence on absorption but also because of its role in enhancing the synthesis of the mucosal cell CRBP II.

The second pathway is an acyl CoA-dependent pathway whereby retinol is bound to any protein, not just the specific CRBP II mentioned above. In both pathways, the retinol is protein-bound prior to ester formation. As mentioned in Section I.A, the esterification of retinol results in a more lipophilic material readily soluble in the lipids of the cell. This, in turn, facilitates its movement into the cell and its metabolic function as well as its storage.

Retinoic acid, like retinal and retinol, is rapidly absorbed by the mucosal cells. However, it is also rapidly excreted (rather than stored, as are the other vitamin forms) in the urine; thus, this form is not as useful as the alcohol or aldehyde forms as a dietary ingredient.

In food, all-*trans* retinol is usually esterified to long-chain fatty acids. Palmitic acid is the most common of these fatty acids. The ester is hydrolyzed in the intestine by a specific esterase which, in some species, is activated by the bile salt, sodium taurocholate. After the ester has been hydrolyzed, the resulting retinol is actively transported (carrier mediated) into the mucosal cell where it is then reesterified to a long-chain fatty acid (palmitic acid) and incorporated into lymph chylomicrons. The absorption of vitamin A thus follows the same route as the long-chain fatty acids of the dietary triacylglycerides, cholesterol, and cholesterol esters. The chylomicrons are absorbed by the lacteals of the lymphatic system and enter the circulation where the thoracic duct joins the circulatory system at the vena cava. Once in the vascular compartment, the triacylglyceride of the retinol-containing chylomicron is removed, leaving the protein carrier, retinol, and cholesterol. Whereas the triacylglycerides are removed from the chylomicrons primarily by the extrahepatic tissues, retinol is removed primarily by the liver. In the liver, hydrolysis and reesterification occur once again as the vitamin enters the hepatocyte and is stored within the cell, associated with droplets of lipid. Interestingly, retinoic acid is absorbed not via the lymphatic system but by the portal system, and does not accumulate in the liver. Retinoic acid, because it is not stored, represents only a very small percentage of the body's vitamin A content.

As mentioned previously, β-carotene is converted to retinol in the mucosal cells of the intestine. Dietary retinal may also be converted to retinol and all forms of the vitamin are transported to the liver as part of the chylomicrons.

### 2. Transport

It has become apparent that a specific protein is needed for subsequent transport of retinol from the liver to the peripheral target tissues. This protein, called retinol binding protein (RBP), was first isolated by Kanai et al. in 1968. RBP is synthesized in the liver (Figure 5). It is a single polypeptide chain (21,000 Da) and possesses a single binding site for retinol. The mobilization of vitamin A from the liver requires this protein. It binds, on a one-to-one basis, to one molecule of retinol. The usual level of binding protein in plasma is about 40 to 50 μg/ml. However, the level of this protein is responsive to nutritional status. In protein-malnourished children it is depressed while in vitamin A-deficient individuals it is elevated. Patients with renal disease have elevated levels of RBP and may be at risk of developing vitamin A toxicity if vitamin A intake is above normal.

In the patient with renal disease, the increase in the binding protein is probably due to the decreased capacity of the kidney to remove the protein. The kidney is the main catabolic site for RBP. Where protein intakes are low, binding protein levels are also low, thus explaining the simultaneous observations of symptoms of protein and retinol deficiency in malnourished children. This association is due to the inability of the liver in the protein-malnourished child to synthesize the retinol binding protein, thus the child is unable to utilize the hepatic vitamin stores. Once protein is restored to the diet, symptoms of both deficiency syndromes will disappear. If dietary retinol (or its precursors) is lacking, serum RBP levels will fall while hepatic levels rise. Within minutes after retinol is given to a deficient individual, these changes are reversed: serum levels rise while hepatic levels fall. These observations provide clear evidence of the importance of this binding protein in the utilization of retinol. Compounds which influence the levels of this binding protein influence

**Table 4 Retinol Binding Proteins**

| Acronym | Protein | Molecular Weight (Da) | Location | Function |
|---|---|---|---|---|
| RBP | Retinol binding protein | 21,000 | Plasma | Transports all-*trans* retinol from intestinal absorption site to target tissues |
| CRBP | Cellular retinol binding protein | 14,600 | Cells of target tissue | Transports all-*trans* retinol from plasma membrane to organelles within the cell |
| CRBP II | Cellular retinol binding protein Type II | 16,000 | Absorptive cells of small intestine | Transports all-*trans* retinol from absorptive sites on plasma membrane of mucosal cells |
| CRABP | Cellular retinoic acid binding protein | 14,600 | Cells of target tissue | Transports all-*trans* retinoic acid to the nucleus |
| CRALBP | Cellular retinal binding protein | 33,000 | Specific cells in the eye | Transports 11-*cis* retinal and 11-*cis* retinol as part of the visual cycle |
| IRBP | Interphotoreceptor or interstitial retinol binding protein | 144,000 | Retina | Transports all-*trans* retinol and 11-*cis* retinal in the retina extracellular space |
| RAR | Nuclear retinoic acid receptor, 3 main forms ($\alpha$, $\beta$, $\gamma$) | | All cells<br>$\alpha$ — Liver<br>$\beta$ — Brain<br>$\gamma$ — Liver, kidney, lung | Binds retinoic acid and regions of DNA having the GGTCA sequence |
| RXR | Nuclear retinoic acid receptors, multiple forms | | | |

the mobilization and excretion of retinol. Estrogens increase the level of this protein whereas cadmium poisoning, because it increases excretion, reduces the level of this protein. In plasma, vitamin A circulates bound to the retinol binding protein which, in turn, forms a protein-protein complex with transthyretin, a tetramer that also binds thyroxine in a 1:1 complex. Because this complex contains the vitamin and thyroxine there is an association of thyroid status and vitamin A status.

Once the RBP complex arrives at the target tissue, it must then bind to its receptor site on the cell membrane. Receptors for retinol and retinoic acid have been identified on the cell membranes of a variety of cells. The retinol is then released from the serum binding protein and transferred into the cell, where it is then bound to intracellular binding proteins. Of interest are the observations that these binding proteins, while similar to the binding protein in the serum, are highly specific for the different vitamin A forms. The CRBP is the cellular retinol binding protein (14,600 Da) while CRABP is the cellular retinoic acid binding protein (14,600 Da), CRALBP is the retinal binding protein (33,000 Da), and IRBP is the interphotoreceptor or interstitial retinol binding protein (144,000 Da). The latter is found only in the extracellular space of the retina. As described earlier, CRBP II is the retinol binding protein found in the mucosal cells of the small intestine. The presence of these binding proteins in different tissues is highly variable. Shown in Table 4 are the features and functions of these binding proteins.

While the bulk transport of the various vitamers A occurs via the chylomicrons which are released into the lymph, there seems to be no specific protein within the chylomicron that has a special affinity for this vitamin. However, once the chylomicron remnants (the remains of the chylomicrons which have lost some of their lipids to the muscle and the adipose tissue) are taken up by the liver, the special binding proteins have their effects. The chylomicron remnant retains most of its original vitamin A content as vitamin A esters. These esters are stored in the hepatocyte or are released into the circulation bound to RBP following hydrolysis.

### 3. Degradation and Excretion

The early Nobel Prize-winning work of Wald et al., which showed that retinol must be converted to retinal before the vitamin can function in vision, led the way for other workers to investigate the further metabolism of the various active forms. Since vitamin A not only maintains the visual cycle but is also necessary for growth, epithelial cell differentiation, skeletal tissue development, spermatogenesis, and the development and maintenance of the placenta, and because each of these functions requires a specific form of the vitamin, it appears that each of these tissues has specific structural requirements. Retinal involvement in vision has been demonstrated, and it appears that retinol but not retinal or retinoic acid is required for the support of reproduction, and that all of the three forms will support growth and cell differentiation. That the three forms are interchangeable in the latter function suggests that retinoic acid is the active form for this function. Both retinol and retinal can be converted to retinoic acid but the reverse reactions are not possible, and only in specific tissues such as the retina are retinol and retinal interconvertible. From studies using radioactively labeled retinal, retinol, and retinoic acid, it is apparent that retinoic acid is the common metabolic intermediate of the vitamin A group. Once retinol is mobilized from hepatic stores, transported to its target tissue via the retinol binding protein, and transferred to its intracellular active site via the intracellular retinol binding proteins, it is then utilized either as retinol or retinal or converted to retinoic acid. Studies of the excretion patterns of labeled retinol and retinoic acid revealed that retinol was used more slowly than retinoic acid. Label from retinoic acid was almost completely recovered within 48 hr of administration, whereas more than 7 days were required to recover even half of the label from the retinol.

The use of retinoic acid, isotopically labeled in several different locations, allowed Roberts and DeLuca to determine the metabolic pathway of retinoic acid. The label from retinoic acid was recovered as $^{14}CO_2$ from the expired air as well as from $^{14}C$-labeled decarboxylated metabolites and the β-ionone ring lacking part of its isoprenoid side chain. The structures of all the metabolites which appear in the urine and feces are not known.

## F. Functions of Vitamin A

### 1. Protein Synthesis

Some of the earliest reports of retinol deficiency included observations of the changes in epithelial cells of animals fed vitamin A-deficient diets. Normal columnar epithelial cells were replaced by squamous keratinizing epithelium. These changes were reversed when vitamin A was restored to the diet. Epithelial cells, particularly those lining the gastrointestinal tract, have very short half-lives (in the order of 3 to 7 days) and as such are replaced frequently. Thus, in vitamin A deficiency, changes in these cells as well as in other cells having a rapid turnover time, indicate that the vitamin functions at the level of protein synthesis and cellular differentiation. Studies of protein synthesis by mucosal cells from deficient and control animals indicated that the vitamin is involved directly in protein synthesis both at the transcriptional and translational level. Such a role for retinol is supported by observations of an alteration in messenger RNA synthesis in vitamin A-deficient animals.

Table 5 lists a number of cellular proteins that have retinoic acid as an influence on their synthesis or activation. Also listed are gene products that require either a cis- or trans-acting retinoic acid-containing transcription factor. Retinol is converted to retinoic acid which regulates cell function by binding to intracellular retinoic acid receptors. Two distinct families of nuclear receptors have been identified. Each of these families, called RAR and RXR, have multiple forms and both are structurally similar to the receptor that binds the steroid and thyroid hormones. These receptors function as ligand-activated transcription factors (see Table 4) that regulate mRNA transcription.

**Table 5 Gene Products Influenced by Retinoic Acid**

**Proteins That Have Their Synthesis Increased or Decreased Due to RA-Receptor Effect on the Transcription of Their mRNA**

| | |
|---|---|
| Growth hormone | Neuronal cell |
| Transforming growth factor β2 | Calcium binding protein, Calbindin |
| Transglutaminase | Ornithine decarboxylase |
| Phosphoenolpyruvate carboxykinase | Osteocalcin |
| Gsα | Insulin |
| Alcohol dehydrogenase | |
| t-Plasminogen activator | |
| Glycerophosphate dehydrogenase | |

**Retinoic Acid Receptor Binding Proteins That Function in mRNA Transcription**

| |
|---|
| 1,25-$(OH)_2D_3$ receptors |
| Retinoic acid receptors β |
| cfos[a] |
| Progesterone receptors[a] |
| Zif 268 transcription factor |
| AP-2 transcription factor |
| MSH Receptors |
| Interleukin 6-receptors[a] |
| Interleukin 2-receptors |
| EGF receptors (corneal endothelium) |
| EGF receptors (corneal epithelium)[a] |
| Peroxisomal proliferator-activated receptors |

[a] The activity of these proteins are suppressed when the RA-receptor is bound to them.

They bind to regions of DNA that have a GGTCA sequence. In some instances, these factors stimulate transcription and in other instances they suppress the process. There is also an interaction of vitamin A with other vitamins. For example, the synthesis of the calcium binding protein, calbindin, is usually regulated by vitamin D. This protein, found in the intestinal mucosal cells and the kidney, is also found in the brain. In the brain its synthesis is regulated by retinoic acid rather than vitamin D. In vitamin A-deficient brain cells, additions of retinoic acid increased the mRNA for calbindin and calbindin synthesis. Additions of vitamin D were without effect. The retinoic acid receptor contains zinc finger protein sequence motifs which mediate its binding to DNA. The carboxyl terminal of the receptor functions in this ligand binding. Retinoic acid binding to nuclear receptors sets in motion a sequence of events that culminates in a change in transcription of the cis-linked gene. That is, proteins are synthesized, and these proteins bind to regions of the promoter adjacent to the start site of the DNA that is to be transcribed. Such binding either activates or suppresses transcription and, as a result, there are corresponding increases or decreases in the mRNA coding for specific proteins. This, in turn, leads to changes in cell function. Table 6 lists a number of enzymes that have been reported to be affected by the deficient state. In each of these instances it could be assumed that the reason for the change in activity could be explained by the effect of vitamin A on the synthesis of these enzymes. Where there is an increase (or decrease) it is likely that transcription, and hence synthesis, is influenced (or kept within normal bounds) when vitamin A intake is adequate.

Recently, several investigators have reported on the need for retinoic acid by the insulin-secreting β cells of the pancreas. The mechanism of action of retinoic acid in this cell type is far from clear. Insulin release is a process that depends on the glucokinase sensing system which, in turn, depends on an optimal supply of ATP. If there is an ATP shortfall the insulin release mechanism will falter and diabetes mellitus may develop. An ATP shortfall can be the result of one or more mutations

**Table 6 Enzymes That Are Affected by Vitamin A Deficiency**

| Enzyme | Reaction | Effect |
|---|---|---|
| ATPase | ATP ↔ ADP + Pi | Increase |
| Arginase | L-Arginine → ornithine + urea | Increase |
| Xanthine oxidase | Hypoxanthine →→ uric acid | Increase |
| Alanine amino transferase | L-Alanine α-ketoglutarate→ pyruvate + L-glutamate | No change |
| Aspartate amino transferase | L-Aspartate + α-ketoglutarate → oxaloacetate + L-glutamate | No change |
| Vitamin A palmitate hydrolase | Vitamin A palmitate → vitamin A + palmitic acid | No change |
| Vitamin A ester synthetase | Vitamin A + fatty acid →ester | No change |
| $\Delta^{5,3}$ β-Hydroxysteroid dehydrogenase | Removal of $H^+$ group from progesterone, glucocorticoid, estrogen, testosterone | |
| 11β-Steroid hydroxylase | Synthesis of steroid hormones | Decrease |
| ATP sulfurylase | ATP + $SO_4$ → adenyl sulfate + $PPi^{-3}$ | Decrease |
| Sulfotransferase | Transfers sulfuryl groups to -O and -N of suitable groups. Synthesis of mucopolysaccharides | |
| L-γ-Gulonolactone oxidase | L-Gulonolactone → L-ascorbic acid | Decrease |
| *p*-Hydroxyphenol pyruvate oxidase | *p*-Hydroxyphenyl pyruvate → homogentisic acid | Decrease |

in the genes encoding the components of OXPHOS and diabetes mellitus can be observed in persons or animals having a mutation in the mitochondrial genome. Many years ago, vitamin A-deficient rats were found to have reduced OXPHOS activity, but it is only recently that vitamin A (as retinoic acid) has been suggested to play a role in OXPHOS gene expression, thereby linking this vitamin to OXPHOS, insulin release, and diabetes mellitus.

There is another aspect of vitamin A nutriture that is of importance when the function of this vitamin is considered. This concerns the structure and function of the nuclear retinoic acid binding protein, the RA receptor. Should this receptor not be synthesized, as can occur in the absence of retinoic acid, the whole cascade of events dependent on the binding of the RA-receptor complex will not occur. Further, should the receptor itself be aberrant in amino acid sequence, both its capacity to bind RA and its affinity for specific regions of the DNA will be affected. In this instance it is easy to understand how cellular differentiation would be affected. Indeed, several investigators have suggested that this could explain the occurrence of congenital defects in a number of species where early embryonic development of the spinal column and the heart might be due to abnormal RA-receptor binding, with the subsequent result of defective differentiation, organ formation, and organ function.

This role for the vitamin, although lacking biochemical mechanistic detail, was among the first functions recognized by early investigators. An excellent paper on retinoid signaling and the generation of cellular diversity in the embryonic mouse spinal cord has recently been published, as has a paper on retinoid signaling in the developing mammal (see articles by Chien et al., Soprano et al., Dutz and Sandell, and the review by DeLuca).

### *2. Reproduction and Growth*

The role of vitamin A in the growth process is related to its function in RNA synthesis as described above. Animals fed vitamin A-deficient diets do not eat well, and their poor growth may stem from their inadequate intakes of not only vitamin A but also the other essential nutrients. As mentioned previously, vitamin A is responsible for the maintenance of the integrity of the epithelial tissues. Since the taste buds are specialized epithelial cells, feeding a deficient diet probably results in a change in the structure and function of these taste buds, resulting in a loss in appetite. As well, other epithelial cells are also affected, particularly those cells which secrete lubricating and digestive fluids in the mouth, stomach, and intestinal tract. The lack of lubrication due to atrophy of these important cells would certainly affect food intake and, hence, result in poor growth. In addition, reduction in food intake itself imposes a stress on the growing animal, and stress, with its attendant

hormonal responses such as an increase in thyroid hormone release and an increase in adrenal activity, would have profound influences on protein turnover and energy utilization and, of course, growth.

As mentioned, the role of the vitamin in reproduction relates to its role in RNA and protein synthesis. Ornithine decarboxylase, an enzyme that closely correlates to cell division and tissue growth, has recently been identified as a protooncogene. Ornithine decarboxylase (ODC) is the first rate-limiting enzyme in the biosynthesis of polyamines which are essential for cell growth. Retinoic acid suppresses the transcription of ODC mRNA and by doing so serves as a "brake" on uncontrolled cell growth. This function of retinoic acid on ODC transcription is counterbalanced by retinoic acid-estrogen receptor binding. The latter enhances ODC mRNA transcription. Not only would the growth of a fertilized ova be affected in this manner, but also, through its effects on protein synthesis, vitamin A could affect the synthesis of enzymes needed to produce the steroid hormones which regulate and orchestrate the reproduction process. Several of the enzymes listed in Table 6 are involved in the synthesis of these hormones. Of the enzymes listed, three (ATPase, arginase, and xanthine oxidase) relate primarily to energy or protein wastage, as would be expected to increase in a deficient animal. The transferase and the enzymes of retinol metabolism are unchanged, while enzymes for the synthesis of mucopolysaccharides are decreased.

Observations of increases in the phospholipid content of a variety of cellular and subcellular membranes in vitamin A-deficient rats suggest that enzymes of lipid metabolism are also affected. Cholesterol absorption is increased in the deficient rat and this increase may in turn affect phospholipid synthesis and membrane phospholipid content since mammalian membranes consist largely of cholesterol and phospholipids. Changes in membrane composition conceivably could explain the increased susceptibility of deficient animals to infection, but equally likely is the reduction in the protective effect of a normal intact epithelium which acts as a physical barrier to the pathogenic organisms, and the reduction in the synthesis of antibodies and antibody-forming cells in the spleen of vitamin A-deficient animals.

The secondary characteristics of vitamin A deficiency are probably related to the role of the vitamin in protein synthesis, as described above. The primary characteristics of decreased adaptation to darkness, poor growth, reduced reproductive capacity, xerophthalmia, keratomalacia, and anemia are all related.

## 3. Vision

Of the various functions vitamin A serves, its role in the maintenance of adaptation to darkness was the first to be fully described on a molecular basis. When animals are deprived of vitamin A, the amount of rhodopsin declines. This is followed by decreases in the amount of the protein, opsin. Rhodopsin is present in the rod cells of the retinas of most animals. The synthesis of rhodopsin and its subsequent bleaching was elucidated by Wald and others. The process is shown in Figure 4. All-*trans* retinol is transported to the retina cell, transferred into the cell, and converted to all-*trans* retinal. All-*trans* retinal is isomerized to the active vitamin, 11-*cis* retinal, which combines with opsin to form rhodopsin. Rhodopsin is an asymmetric protein with a molecular weight of about 38,000 Da. It has both a hydrophilic and a hydrophobic region with a folded length of about 70 Å. It spans the membrane of the retina via seven helical segments that cross back and forth and comprises about 60% of the membrane protein. The light-sensitive portion of the molecule resides in its hydrophobic region. When rhodopsin is exposed to light it changes its shape. The primary photochemical event is the very rapid isomerization of 11-*cis* retinal to a highly strained form, bathorhodopsin. Note that the alcohol (retinol) and the aldehyde (retinal) are interchangeable with respect to the maintenance of the visual function.

Retinoic acid is ineffective primarily because there are no enzymes in the eye to convert the retinoic acid to the active 11-*cis* retinal needed for the formation of rhodopsin.

The formation of iodopsin in the cones involves 11-*cis* retinol and the photochemical isomerization of the 11-*cis* isomer triggers the visual process. In the rhodopsin breakdown process, an

electrical potential arises and generates an electrical impulse which is transmitted via the optic nerve to the brain. That 11-*cis* retinal is also involved in color vision (the responsibility of the cones) has been suggested; however, the mechanism of this involvement has not been fully explored. Three major cone pigments have been identified that have absorption maxima of 450, 525, and 550 nm, respectively. Whether these are single pigments or a mixture and whether one or more contain 11-*cis* retinal has not been determined.

### G. Hypervitaminosis A

Because the vitamin is stored in the liver, it is possible to develop a toxic condition when very high (10 times normal intake) levels of the vitamin are consumed. As early as 1934, reports appeared in the literature of vitamin A intoxication in humans, rats, and chicks. In chicks, the most obvious clinical signs are a reduced growth rate, an encrustation of the eyelids, and a reddening of the corners of the mouth. In rats, bone fractures are observed. These bone fractures may be related to the unusual brittleness of the bone in hypervitaminosis. In humans, hypervitaminosis A is characterized by increased intracranial pressure resulting in headaches, blurring of vision, and in young children, a bulging fontanel. Hair loss and skin lesions, anorexia, weight loss, nausea, vomiting, vague abdominal pain, and irritability are common symptoms. In experimental animals, excess vitamin A intake during gestation results in congenital malformations in the young. Because of the limitation in the conversion of β-carotene to retinol, vitamin A intoxication is less likely with large intakes of carotene; however, reports of yellowing of the skin of persons consuming large amounts of carrot juice have appeared. This yellowing is likely due to the deposition of carotene and associated pigments in the subcutaneous fat.

### H. Recommended Dietary Allowances

Adult requirements for retinol have been estimated using a variety of techniques. Individuals vary in their needs for the vitamin. Reports in the literature indicate that requirements may be increased by fever, infection, cold, hyperthyroidism, chemical toxicants, and excessive exposure to sunlight. In addition, the genetic heritage of the individual introduces an additional measure of variability in the needs of population groups.

To determine an individual's vitamin A requirement, that individual must first be deprived of all dietary sources of the vitamin until the first signs of deficiency appear. Because the liver stores the vitamin, this depletion period may be as long as two years for the human. Once depleted, graded amounts of the vitamin are restored to the diet until signs of the deficiency disappear. When a steady state is achieved, that level of intake is the requirement. Obviously, such a procedure is not practical for large numbers of people. Because of the time and expense involved in determining requirements, recommended intakes are used. These are shown in Table 7. These are estimates of the requirements of large population groups based on detailed studies (as per above) of a small group of subjects. The recommendation is usually twice the determined requirement so as to allow for variability in need. As more data are collected on human subjects, the safety factor may be reduced and the recommended intake changed. Table 7 gives the U.S. recommended dietary allowances for humans in retinol equivalents.

## II. VITAMIN D

### A. Overview

Just as night blindness has been recognized for centuries as a disease treatable by diet (vitamin A), the classical disease of vitamin D deficiency, rickets, has been evident since ancient

**Table 7 Recommended Dietary Allowances (RDA) for Vitamin A**

| Group | Age | RDA (Retinol Equivalents) |
|---|---|---|
| Infants | Birth–6 months | 375 |
| | 7–12 months | 375 |
| Children | 1–3 | 400 |
| | 4–6 | 500 |
| | 7–10 | 700 |
| Males | 11–14 | 1000 |
| | 15–18 | 1000 |
| | 19–24 | 1000 |
| | 25–50 | 1000 |
| | 51+ | 1000 |
| Females | 11–14 | 800 |
| | 15–18 | 800 |
| | 19–24 | 800 |
| | 25–50 | 800 |
| | 51+ | 800 |
| Pregnancy | | 800 |
| Lactation | 1st 6 months | 1300 |
| | 2nd 6 months | 1200 |

times. Historians do not agree as to when the first symptoms of vitamin D deficiency were evident. Some suggest that the stooped appearance of the Neanderthal Man (ca. 50,000 B.C.) was due to an inadequate vitamin D intake rather than being characteristic of a low evolutionary status. Evidence of rickets in skeletons from humans of the Neolithic Age, the first settlers of Greenland, and the ancient Egyptians, Greeks, and Romans has been reported.

The first detailed descriptions of the disease are found in the writings of Dr. Daniel Whistler of Leiden, Netherlands and Professor Francis Glisson in the mid-1600s. Beyond these descriptions and the acceptance of rickets as a disease entity, little progress was made until the late 1800s when it was suggested that the lack of sunlight and "perhaps" poor diet were related to the appearance of bone malformation. It was frequently reported that infants born in the spring and dying the following winter did not have any symptoms of rickets, whereas infants born in the fall and dying the next spring had rickets. Funk, in 1914, suggested that rickets was a nutrient deficiency disorder. This was verified by the brilliant work of Sir Edward Mellanby. Mellanby constructed a grain diet which produced rickets in puppies. When he gave cod-liver oil, the disease did not develop. At that time, Mellanby did not know that there were two fat-soluble vitamins (A and D) in cod-liver oil, and he thought that he was studying the antirachitic properties of vitamin A. Not until the two vitamins were separated and identified was it realized that Mellanby's antirachitic factor was vitamin D. The recognition of vitamin D as a separate entity from vitamin A came from the work of McCollum and associates in 1922. In a landmark paper, McCollum reported the results of his work on the characterization of vitamin A. He described the vulnerability of the vitamin to oxidation and the fact that the antirachitic factor remained even after the cod-liver oil was aerated and heated and the antixerophthalmic factor (vitamin A) was destroyed.

Although the importance of sunlight had long been recognized in the prevention and treatment of rickets, the relationship of ultraviolet light to the dietary intake of vitamin D was not appreciated until Steenbeck, and also Goldblatt et al., demonstrated that ultraviolet light gave antirachitic properties to sterol-containing foods if these foods were incorporated into diets previously shown to produce rickets. From this point on, the research concerning vitamin D, as it was so-named by McCollum and associates, became largely chemical in nature. It has only been within the last decade or so that work on vitamin D has elucidated its mechanism of action.

**Table 8 D Vitamers Are Produced from Provitamin Forms When These Precursor Forms Are Exposed to Ultraviolet Light. These Are Not Active Until They Are Hydroxylated at Carbons 1 and 25**

| Precursor | D Vitamer |
|---|---|
| Ergosterol | $D_2$ (Ergocalciferol) |
| 7-Dehydrocholesterol | $D_3$ (Cholecalciferol) |
| 22,23-Dehydroergosterol | $D_4$ |
| 7-Dehydrositosterol | $D_5$ |
| 7-Dehydrostigmasterol | $D_6$ |
| 7-Dehydrocompesterol | $D_7$ |

## B. Structure and Nomenclature

Like vitamin A, vitamin D is not a single compound. The D vitamins listed in Table 8 are a family of 9,10-secosteroids which differ only in the structures of their side chains. Figure 7 shows some of these different structures. There is no $D_1$ because when the vitamins were originally isolated and identified, the compound identified as $D_1$ turned out to be a mixture of the other D vitamins rather than a separate entity.

Since the other D vitamins were already described and named, the $D_1$ designation was deleted from the list. All the D vitamin forms are related structurally to four-ring called compounds cyclopentanoperhydrophenanthrenes, from which they were derived by a photochemical reaction. The official nomenclature proposed for vitamin D by IUPAC-IUB Commission on Biochemical Nomenclature relates the vitamin to its steroid nucleus. Each carbon is numbered using the same system as is used for other sterols such as cholesterol. This is illustrated in Figure 8. The numbering system of the four-ring cholesterol structure is retained even though the compound loses its B ring during its conversion to the vitamin.

The chief structural prerequisite of compounds serving as D provitamins is the sterol structure which has an opened B ring that contains a $D_{5,6}$ conjugated double bond. No vitamin activity is possessed by the compound until the B ring is opened.

This occurs as a result of exposure to ultraviolet light. In addition, vitamin activity is dependent on the presence of a hydroxyl group at carbon 3 and upon the presence of conjugated double bonds at the 10-19, 5-6, and 7-8 positions. If the location of these double bonds is shifted, vitamin activity is substantially reduced. A side chain of a length at least equivalent to that of cholesterol is also a prerequisite for vitamin activity. If the side chain is replaced by a hydroxyl group, for example, the vitamin activity is lost. The potency of the various D vitamins is determined by the side chain. $D_5$, for example, with its branched 10-carbon side chain, is much less active with respect to the calcification of bone cartilage than is $D_3$ with its 9-membered side chain.

Of the compounds shown in Figure 7, the most common form is that of $D_2$, ergocalciferol, so-called because its parent compound is ergosterol. Ergosterol can readily be prepared from plant materials and, thus, serves as a commercially important source of the vitamin. Vitamin $D_3$, cholecalciferol, is the most important member of the D family because it is the only form which can be generated *in vivo*. Cholesterol, from which cholecalciferol takes its name, serves as the precursor. The 7-dehydrocholesterol at the skin's surface is acted upon by ultraviolet light and is converted to vitamin $D_3$. Here, then, is the connection between diet, sunshine, and rickets sought many years ago when rickets was prevalent in young children. In the absence of sunshine this conversion does not take place. Recall the dress patterns of the people of the eighteenth and nineteenth century. Children (as well as adults) wore many layers of clothing that shielded the skin from ultraviolet light. This practice severely restricted vitamin D synthesis.

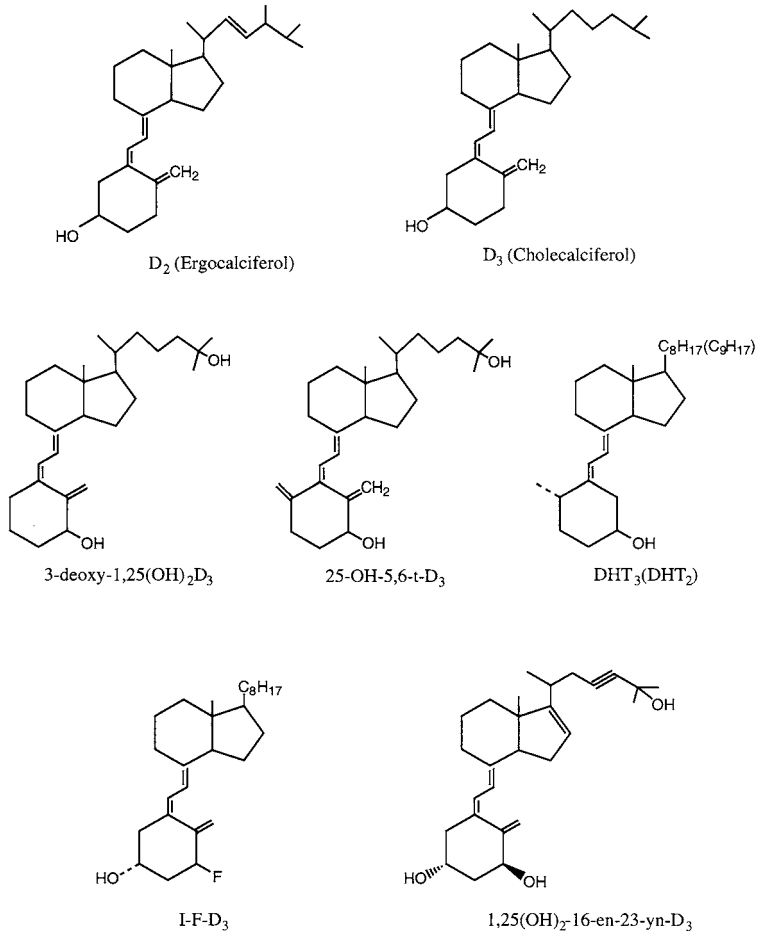



**Figure 7** Compounds with vitamin D activity. Not all of these compounds have identical activity.

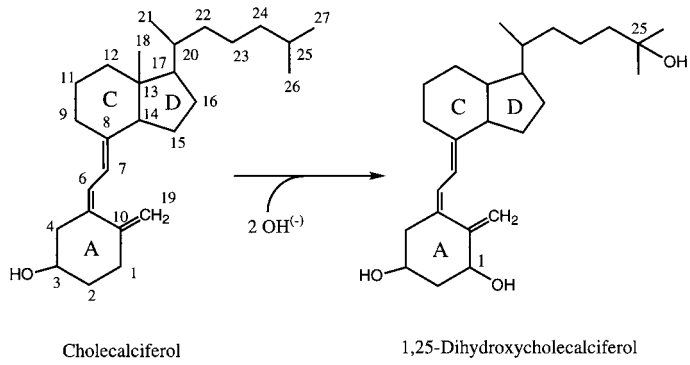



**Figure 8** Chemical structure of vitamin D showing carbon numbers of the basic structure. The rings are labeled A, C, and D after the ring letters of cholesterol. When activated, a hydroxyl group is added at carbons 1 and 25 to form 1,25-dihydroxycalciferol.

**Table 9 Physical Characteristics of Vitamins $D_2$ and $D_3$**

| Vitamin | Number Double Bonds | Melting Point | UV Absorption Maximum | Molar Extinction Coefficient | Optical Rotation |
|---|---|---|---|---|---|
| $D_2$ | 4 | 121°C | 265 nm | 19,400 | $\alpha \frac{20}{D}$ + 106° |
| $D_3$ | 3 | 83–85°C | 264–265 | 18,300 | $\alpha \frac{20}{D}$ + 84.8° |

Most mammals can convert both $D_2$ and $D_3$ to the active principles (1,25-dihydroxyergocalciferol and 1,25-dihydroxycholecalciferol) which are responsible for D's biological function. Birds seem unable to make this conversion using $D_2$ or the resulting hydroxylation product. It is also possible that the conversion product is either rapidly degraded and/or excreted. Thus, birds must be supplied with $D_3$ rather than $D_2$ as the vitamin of choice. It has been estimated that for birds, $D_2$ has only one-tenth the biological activity of $D_3$ on a molar basis.

## C. Physical and Chemical Properties

The history of vitamin D would not be complete without mentioning the careful work of a Frenchman, Charles Tanret, who isolated and characterized a sterol from fungus-infected rye which he called ergosterol. The melting point, optical rotation, and elemental composition of ergosterol reported by Tanret in 1889 were identical to those reported by Windaus more than 30 years later. Windaus and associates were able to elucidate the structures of ergosterol and ergocalciferol. However, their complete structures were not verifiable until the techniques of X-ray analysis and infrared spectroscopy were developed.

The precursors and the vitamins are sterols which are members of the nonsaponifiable lipid class. At room temperature, they are white to yellowish solids with relatively low melting points. The various structural and physical characteristics of $D_2$ and $D_3$ are listed in Table 9. Under normal conditions, $D_3$ is more stable than $D_2$; however, both compounds undergo oxidation when exposed to air for periods of 24 to 72 hr. When protected from air and moisture and stored under refrigeration, oxidation of the vitamin can be minimized. In acid solutions, the D vitamins are unstable. However, in alkaline solutions they are stable. All the D vitamins are moderately soluble in fats, oils, and ethanol, and very soluble in fat solvents such as chloroform, methanol, and ether. All of the vitamers are unstable to light. In the dry form the vitamers are more stable than when in solution. Stability in solution can be enhanced by the presence of such antioxidants as α-tocopherol and vitamin A.

Although the D vitamins are not soluble in water, they, like the A vitamins, can be made miscible with water through the use of detergents or surfactants. However, because of the vitamins' vulnerability to oxidation, such solutions are very unstable. This is due to the wide dispersion of the vitamin molecules in water which has oxygen dissolved in it. Some protection against this oxidation can be provided if α-tocopherol (vitamin E) is added to the solution. Other chemical alterations can result in decreased vitamin potency as well. Saturation of any of the double bonds or the substitution of a chloride, bromide, or mercaptan residue for the hydroxyl group attached to carbon 3 results in a loss of vitamin activity.

## D. Biopotency

The comparative potency of the D vitamers depends on several factors: (1) the species consuming the vitamers; and (2) the particular function assessed. With respect to species specificity, in mammalian species both the $D_2$ and $D_3$ are equivalent and both would be given a value of 100 if rickets prevention was used as the functional criterion. However, should these two vitamers be compared in chicks as preventers of rickets, $D_2$ would be given a value of perhaps 10 while $D_3$ would be 100. In this instance it is clear that species differ in their use of these two vitamers. A

related sterol, dihydrotachysterol, a product of irradiated ergosterol, would have only 5 to 10% of the activity of ergocalciferol. In contrast, the activated forms of $D_3$ (25-hydroxy and 1,25-dihydroxycholecalciferol) are far more potent (2 to 5 times and 5 to 10 times, respectively) than their parent vitamer, $D_3$. The synthetic analog of $D_3$, 1α-hydroxycholecalciferol, likewise has 5 to 10 times the potency of cholecalciferol. There are other vitamin D analogs that have selective biological activity and may have use as therapeutic agents. The analog 3-deoxy-1,25-dihydroxycholecalciferol is far more active as an agent to promote intestinal calcium uptake than as an agent to promote bone calcium mobilization. This is also true for the analog, 25-hydroxy-5,6-cholecalciferol.

The reverse effects, increased bone calcium mobilization rather than increased intestinal calcium absorption, have been shown for analogs having a longer carbon chain at carbon 20 and/or having a fluorine attached at carbon 3 (see Figure 7). Cell differentiation, another vitamin D function, is markedly enhanced by the addition of a hydroxyl group at carbon 3, an unsaturation between carbons 16 and 17, and a triple bond between carbons 22 and 23. This analog has greater activity with respect to cell differentiation than for intestinal calcium uptake and bone calcium mobilization.

## E. Methods of Assay

Because mammals require so little vitamin D and because so few foods contain the vitamin, methods for its determination have to be sensitive, reliable, and accurate. A wide variety of assays have been developed that are capable of quantifying fairly well the amount of vitamin D in a test substance. These assays can be divided roughly into two groups: chemical and biological. Biological assays, with few exceptions, are usually more sensitive than chemical assays because so little of the vitamin is required by animals. The smallest amount of vitamin detectable by the biological methods is 120 ng or 0.3 nmol, whereas with the chemical methods the smallest amount detectable is approximately 9 times that or 2.6 nmol. The exception to this comparison is the technique which utilizes high pressure liquid chromatography followed by ultraviolet absorption analysis. This technique can measure as little as 5 ng or 1/24th that of the bioassay techniques. Gas chromatography is also very sensitive, especially if the chromatograph is equipped with an electron capture detector. Using this technique, as little as 50 pg of the vitamin can be detected. This degree of sensitivity is needed for the detection of tissue vitamin levels since, aside from vitamin $B_{12}$, vitamin D is the most potent of the vitamins. Only small amounts are needed and so only small amounts will be found in those tissues requiring the vitamin.

Table 10 summarizes the main methods that have been used for the detection of biological levels of vitamin D. Under the chemical assay techniques, note that a variety of color reactions can be used in vitamin D quantification. These color reactions are possible because the vitamin contains several rings which can react with a variety of compounds in solution and produce a color. The intensity of the color is directly related to the quantity of the vitamin in solution.

While these colorimetric methods are relatively easy to perform, they have several drawbacks. First, and most important, the color reaction is possible because of the ring structure; many sterols have this same ring structure but few have vitamin activity. Thus, colorimetric methods are not specific enough to permit true vitamin quantification in a mixture. The second drawback is that there must be sufficient vitamin in the test substance to react with the color reagent to produce a measurable color change. This requires instrumentation that is able to measure these changes. In general, this degree of sensitivity is missing in most instruments designed to measure colorimetric changes.

The ring structure of vitamin D, although common to many different sterols, can be utilized very well in assay techniques where the sterols are first separated and then assayed. The vitamin D sterol ring structure has a characteristic ultraviolet absorption spectra. At 264 to 265 nm, the intensity of the light absorbed is directly proportional to the quantity of vitamin D present. Sterols can be separated from the lipid component of a sample by saponification. The nonsaponifiable lipids (the sterols) can be further separated by digitonin precipitation. Vitamin D and its related

**Table 10 Summary of Methods Used in Determining Vitamin D Content of Tissues and Foods**

| Method | Sensitivity[a] (nmoles) | Usual Working Range (nmoles) | Comments |
|---|---|---|---|
| | **Chemical Methods** | | |
| Colorimetric[b] | | | |
| Aniline-HCl | No values given | No values given | Devised in 1925. |
| Antimony chloride | 3.2 | 3.2–6.5 | Used primarily to assess pharmaceutical preparations. |
| Trifluoroacetic acid | 2.6 | 1–80 | |
| Ultraviolet absorption | 2.6 | 2.6–52 | A solution with 5.47 nmol of vitamin D will have an absorbance of 0.10 at 264 nm. |
| Ultraviolet fluorescence | 2.6 | 2.6–26 | Based on the property of acetic anhydride-sulfuric acid induced fluorescence of the vitamin. |
| Gas chromatography | 0.1 pmol | 0.01–10 | Based on use of an electron capture detector. |
| High pressure liquid chromatography | 0.01 | 0.05–100 | |
| Gas chromatography-mass spectrometry | 0.01 | 0.01–50 | Equipment not widely available due to expense. Can separate and quantify individual vitamers in a mixture. |
| | **Biological Methods** | | |
| Rat line test | 0.03 | 0.03–0.07 | Time consuming — requires 7 days of feeding after rats become rachitic. |
| Chick test | 0.13 | 0.006–15 | Requires 21 days of feeding. |
| Intestinal $Ca^{2+}$ absorption | *In vivo* 0.33 | 0.125–25 µg | Requires 1 day. |
| | *In vitro* 0.66 | 250–1000 | Requires 1 day. |
| Bone $Ca^{2+}$ mobilization | 0.32 | 0.125–25 µg | Requires 1 day. |
| Body growth | 0.06 | 50–1250 | Requires 21–28 days. |
| Immunoassay of calcium-binding protein | 0.0025 | 1 ng | Requires 1 day. |

[a] Defined as the least amount of vitamin detectable by the method.
[b] Other color reagents have also been used.

sterols will not precipitate, whereas cholesterol and the other four-ring sterols will. If the remaining supernatant containing the vitamin D components is then fractionated using chromatographic techniques, the resulting fractions can be assayed according to the amount of ultraviolet light absorbed.

The ultraviolet light absorption characteristic can also be used to determine the extent of conversion of provitamin D to $D_2$ or $D_3$. Ergosterol or cholesterol can be irradiated until the resultant compound exhibits the typical absorption spectra characteristic of the vitamin. Of course, just as ultraviolet light is needed for this conversion, one must remember that light in excess will destroy the vitamin and its usefulness will be lost.

There are a number of chemical assay techniques useful for the determination of vitamin D activity in biological samples and a number of biological assay techniques which can be used. The advantages of the bioassay are those of sensitivity and specificity. The disadvantages are those of time, expense, and accuracy. The basis of the bioassay is the idea that the physiological effect is quantifiable and, theoretically, the magnitude of the vitamin effect is in direct proportion to the amount of vitamin D in the test substance. For many years, the standard bioassay for vitamin D was the rat line test first devised by McCollum in 1922. This test consists of depleting rats of their vitamin D stores and then treating them with graded amounts of the test substance for one week.

The rats are then killed, the bones of the forepaws excised and cleaned of adhering tissue, sliced longitudinally, and placed in a solution of silver nitrate. The silver nitrate is absorbed by the areas of the bone where calcium has recently been deposited. These regions will turn black upon exposure to light. The resultant black line is then measured and compared to lines obtained from rats fed known amounts of vitamin D. Thus, the line test is based on the activity of the vitamin in promoting calcium uptake by the bone. While this is probably a good method to estimate vitamin activity in a given substance, it has the pitfall of not distinguishing between the various D forms. The user of this method also must assume that bone calcium deposition is the vitamin's most important function; this is not always true.

Use of the line test to assess vitamin content of human foods is probably acceptable since rats and humans can use all forms of D similarly. However, use of the rat line test to assess the D content of feeds for chickens would present problems due to the fact that the chickens do not use $D_2$ and $D_3$ equally well. As pointed out earlier, chickens need $D_3$ in their diets rather than $D_2$. Other bioassays listed in Table 10 also are based on a physiological/metabolic parameter which assumes that the quantity of the vitamin in a test substance is proportional to the activity of the system being assessed. Tests such as the intestinal calcium uptake test, the bone calcium mobilization test, the body growth test, and the calcium binding protein assay all have been devised and published. They all have the advantage of assessing the biological potency of the vitamin D contained in the test substance and all are sensitive to quantities of D likely to be present in biological (as opposed to pharmacological) preparations.

## F. International Units (IU)

It was from the use of the bioassay techniques that the definition of the international unit (IU) was developed. One IU was defined as the smallest amount of vitamin required to elicit a physiological response, i.e., the calcification of bone. As it was used in this context, with the rat as the reference animal, 1 mg of vitamin D was equivalent to 40,000 IU of the vitamin. In 1931, the World Health Organization of the League of Nations adopted as their international standard of reference for vitamin D the activity of 1 mg of a reference solution of irradiated ergosterol. At the time this definition was developed, researchers were not aware of the different D vitamins nor were they aware of the species specificity for the vitamin form. Later, as knowledge about the vitamin increased, the definition was changed such that the present definition developed by Nelson in 1949 uses cholecalciferol, $D_3$, as the reference standard — 1.0 g of a cottonseed oil solution of $D_3$ contains 10 mg of the vitamin or 400 USP units. Thus, the potency of the different D vitamins are related to the most important biologic compounds in the D family.

## G. Metabolism of Vitamin D

### *1. Absorption*

Prior to the understanding and elucidation of the conversion of cholesterol to cholecalciferol and then to its activation as 1,25-dihydroxycholecalciferol, considerable attention was given to the mechanisms for the intestinal absorption of vitamin D. It was found that dietary vitamin D was absorbed with the food fats and was dependent on the presence of the bile salts. Any disease which resulted in an impairment of fat absorption likewise resulted in an impairment of vitamin D absorption. Absorption of the vitamin is a passive process which is influenced by the composition of the gut contents. Vitamin D is absorbed with the long-chain fatty acids and is present in the chylomicrons of the lymphatic system. Absorption takes place primarily in the jejunum and ileum. This has a protective effect on vitamin D stores since the bile, released into the duodenum, is the chief excretory pathway of the vitamin; reabsorption in times of vitamin need can protect the body

from undue loss. However, in times of vitamin excess, this reabsorptive mechanism may be a detriment rather than a benefit. The vitamin is absorbed in either the hydroxylated or the nonhydroxylated form.

While many of the other essential nutrients are absorbed via an active transport system, there is little reason to believe that absorption of vitamin D is by any mechanism other than passive diffusion. The body, if exposed to sunlight, can convert the 7-dehydrocholesterol at the skin's surface to cholecalciferol, and this compound is then metabolized: first in the liver to 25-hydroxycholecalciferol and further in the kidney producing the active principle, 1,25-dihydroxycholecalciferol. Because the body, under the right conditions, can synthesize *in toto* its vitamin D needs, and because it needs so little of the vitamin, there appears to be little reason for the body to develop an active transport system for its absorption. However, in the person with renal disease, the synthesis of 1,25-dihydroxycholecalciferol is impaired and, in this individual, intestinal uptake of the active form is quite important. Oral supplements can be used to ensure adequate vitamin D status.

Because the body can completely synthesize dehydrocholesterol and convert it to $D_2$ or $D_3$, and then hydroxylate it to form the active form, an argument against its essentiality as a nutrient can be developed. In point of fact, because the active form is synthesized in the kidney and from there distributed by the blood to all parts of the body, this active form meets the definition of a hormone, and the kidney, the site of synthesis, meets the definition of an endocrine organ. Thus, whether vitamin D is a nutrient or a hormone is dependent on the degree of exposure to ultraviolet light. Lacking exposure, vitamin D must be provided in the diet and thus is an essential nutrient.

## 2. Transport

Once absorbed, vitamin D is transported in nonesterified form bound to a specific vitamin D binding protein. This protein (DBP) is nearly identical to the α-2-globulins and albumins with respect to its electrophoretic mobility. All of the forms of vitamin D (25-hydroxy-$D_3$, 24,25-dihydroxy-$D_3$, and 1,25-dihydroxy-$D_3$) are carried by this protein which is a globulin with a molecular weight of 58,000 Da. Its binding affinity varies with the vitamin form. DNA sequence analysis of DBP shows homology with a fetoprotein and serum albumin. DBP also has a high affinity for actin, but the physiological significance for this cross reactivity is unknown. The transcription of the DBP is promoted by a vitamin D-receptor protein transcription factor. Thus, there is a complete loop of a transport protein needed for vitamin D, and which in turn is dependent on vitamin D for its synthesis.

## 3. Metabolism

Once absorbed or synthesized at the body surface, vitamin D is transported via DBP to the liver. Here it is hydroxylated via the enzyme vitamin D hydroxylase at carbon 25 to form 25-hydroxycholecalciferol. Figure 9 illustrates the pathway from cholesterol to 1,25-dihydroxycholecalciferol. With the first hydroxylation a number of products are formed, but the most important of these is 25-hydroxycholecalciferol. The biological function of each of these metabolites is not known completely. As mentioned, the hydroxylation occurs in the liver and is catalyzed by a cytochrome P-450-dependent mixed-function monooxygenase. This enzyme has been found in both mitochondrial and microsomal compartments. It is a two-component system which involves flavoprotein and a cytochrome P-450 and is regulated by the concentration of ionized calcium in serum. The hydroxylation reaction can be inhibited if $D_3$ analogs having modified side chains are infused into an animal fed a rachitic diet and a $D_3$ supplement.

25-Hydroxy-$D_3$ is then bound to DBP and transported from the liver to the kidney where a second hydroxyl group is added at carbon 1. This hydroxylation occurs in the kidney proximal tubule mitochondria and is catalyzed by the enzyme 25-OH-$D_3$-1α-hydroxylase. This enzyme has

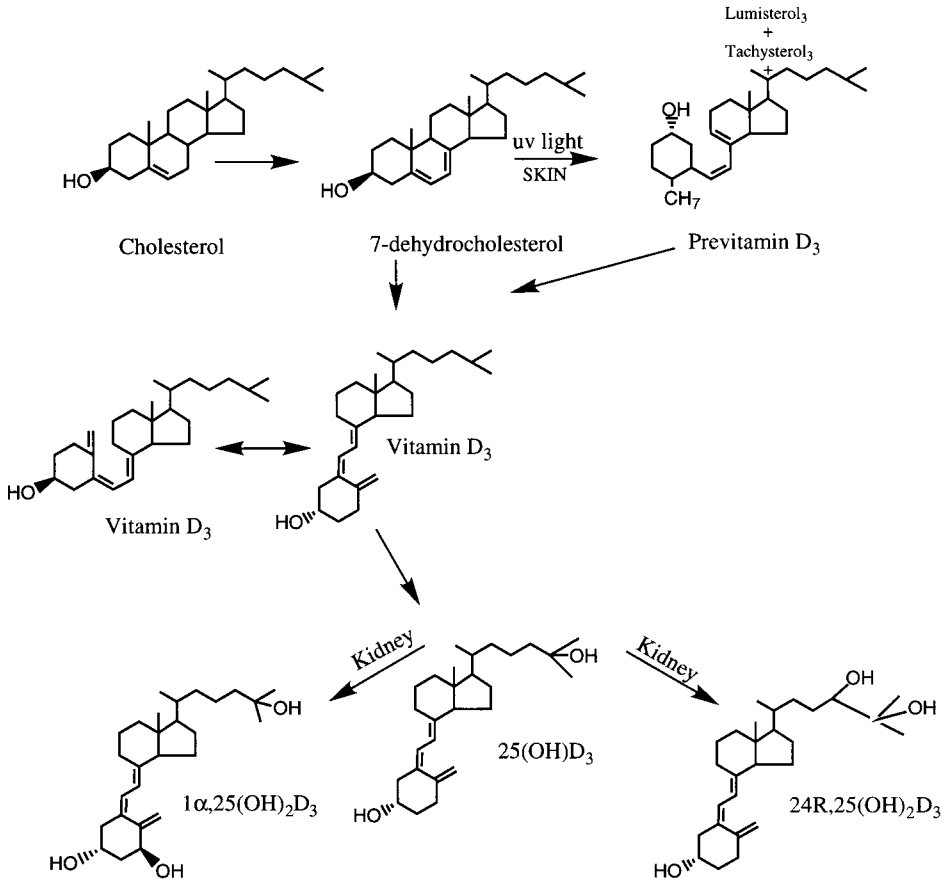



**Figure 9** Synthesis of active 1,25-dihydroxycholecalciferol using cholesterol as the initial substrate. Several isomers can result when previtamin D or 7-dehydrocholesterol is converted to $D_3$, cholecalciferol.

been characterized as a three-component enzyme involving cytochrome P-450, an iron-sulfur protein (ferredoxin), and ferredoxin reductase. The reductant is $NADPH_2$. Considerable evidence has shown that 1,25-dihydroxycholecalciferol is the active principle that stimulates bone mineralization, intestinal calcium uptake, and calcium mobilization. Because this product is so active in the regulation of calcium homeostasis, its synthesis must be closely regulated. Indeed, product feedback regulation exists with respect to the activity of this enzyme and control is also exercised at the level of its mRNA transcription. In both instances, the level of 1,25-dihydroxycholecalciferol (the product) negatively affects 1α-hydroxylase activity and suppresses mRNA transcription of the hydroxylase gene product. In addition, control of the hydroxylase enzyme is exerted by the parathyroid hormone, PTH. When plasma calcium levels fall, PTH is released and this hormone stimulates 1α-hydroxylase activity while decreasing the activity of 25-hydroxylase. In turn, PTH release is down-regulated by rising levels of 1,25-dihydroxycholecalciferol and its analog, 24,25-dihydroxycholecalciferol. Insulin, growth hormone, estrogen, and prolactin are additional hormones that stimulate the activity of the 1α-hydroxylase. The mechanisms that explain these stimulatory effects are less well known and are probably related to their effects on bone mineralization as well as on other calcium-using processes.

Just as several metabolites are formed in the 25-hydroxylase reaction, a number of products also result with the second hydroxylation. Some of these transformations are shown in Figure 10. Also shown are the degradative products found primarily in the feces. These products appear in

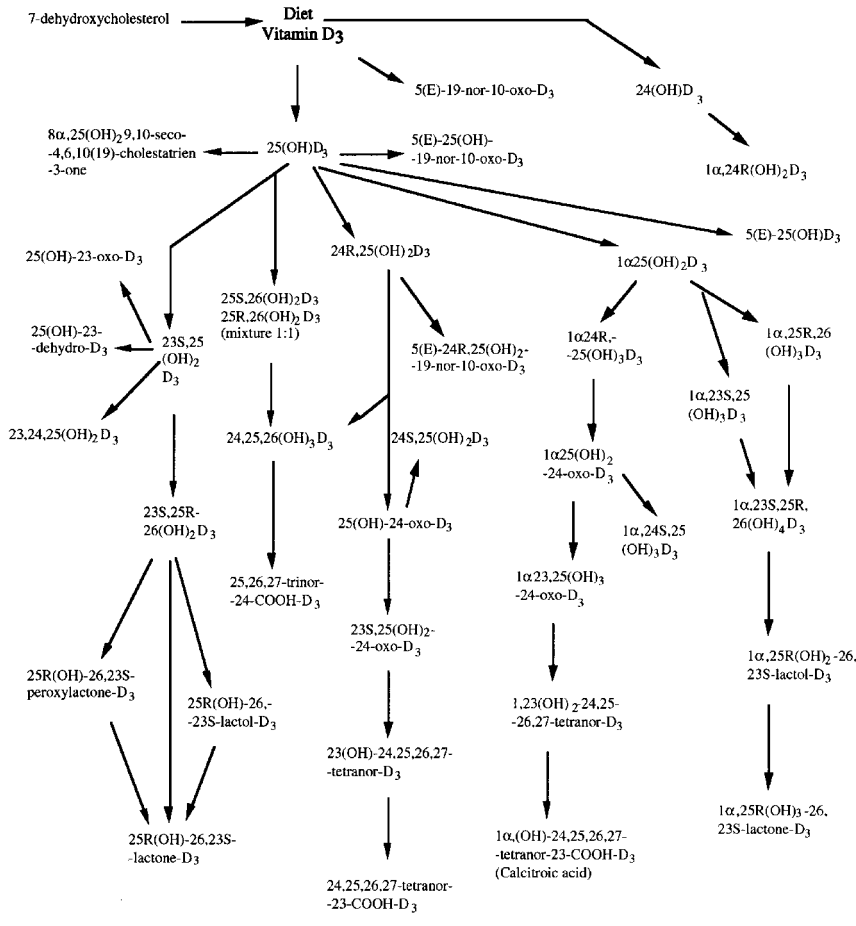



**Figure 10** Pathways for vitamin $D_3$ synthesis and degradation.

the feces because of biliary transport from the liver to the small intestine and subsequent degradation by enteric flora.

$D_3$ is subject to other metabolic reactions as well (Figure 10). Whether these metabolites have specific functions with respect to mineral metabolism is not fully understood. Instead of 1,25-dihydroxy-$D_3$, 24,25-dihydroxy-$D_3$ may be formed and may serve to enhance bone mineralization and embryonic development, and to suppress parathyroid hormone release. 24,25-Dihydroxy-$D_3$ arises by hydroxylation of 25-hydroxy-$D_3$. When 25-OH-$D_3$-1α-hydroxylase activity is suppressed, 24,25-hydroxylation is stimulated. This hydroxylase is substrate inducible through the mechanism of increased enzyme protein synthesis and has been found in kidney, intestine, and cartilage. 24,25-Dihydroxy-$D_3$ may represent a "spillover" metabolite of $D_3$. That is, a metabolite is formed when excess 25-hydroxy-$D_3$ is present in the body. Other $D_3$ metabolites such as 25-hydroxy-$D_3$-26,23-lactone also can be regarded as spillover metabolites, since measurable quantities are observed under conditions of excess intake. While 24,25-dihydroxy-$D_3$ does function in the bone mineralization process, it is not as active in this respect as is 1,25-dihydroxy-$D_3$.

The question of whether a hydroxy group at carbon 25 is a requirement for vitamin activity has been posed since several $D_3$ metabolites lack this structural element. Studies utilizing fluoro-substituted $D_3$ showed conclusively that while maximal activity is shown by the 1,25-$D_3$ compound, vitamin activity can also be shown by compounds lacking this structure. In part, the structural requisite for vitamin activity may relate to the role the 25-hydroxy substituents play in determining

the molecular shape of the compound. This shape must conform to the receptor shape of the cellular membranes in order for the $D_3$ to be utilized. Specific intracellular receptors for 1,25-dihydroxy-$D_3$ have been found in parathyroid, pancreatic, pituitary, and placental tissues. All these tissues have been shown to require $D_3$ for the regulation of their function. For example, in $D_3$ deficiency, pancreatic release of insulin is impaired. Insulin release is a calcium-dependent process which, by inference, means that there must be a calcium-binding protein whose synthesis requires the vitamin.

As can be seen in Figure 10, several pathways exist for the degradation of the active 1,25-dihydroxycholecalciferol. These include oxidative removal of the side chain, additional hydroxylation at carbon 24, the formation of a lactone (1,25 $OH_2$ $D_2$-26,23-lactone), and additional hydroxylation at carbon 26. While 25-hydroxycholecalciferol can accumulate in the heart, lungs, kidneys, and liver, 1,25 dihydroxycholecalciferol does not accumulate. The active form is not stored appreciably but is found in almost every cell and tissue type.

## 4. Function

Until the recognition of the central role of the calcium ion in cellular metabolic regulation, it was thought that vitamin D's only function was to facilitate the deposition of calcium and phosphorus in bone. This concept developed when it was recognized that the bowed legs of rickets was due to inadequate mineralization in the absence of adequate vitamin D intake or exposure to sunlight.

Studies of calcium absorption *in vivo* by the intestine revealed that D-deficient rats absorb less calcium than D-sufficient rats and that rats fed very high levels (10,000 IU/day) absorbed more calcium than did normally fed rats. These observations of the effects of the vitamin on calcium uptake led to work designed to determine the mechanism of this effect. It was soon discovered that vitamin D (1,25-dihydroxy-$D_3$) served to stimulate the synthesis of a specific gut cell protein that was responsible for calcium uptake. This protein, called the intestinal calcium binding protein (calbindin), was isolated from the intestine and later from brain, bone, kidney, uterus, parotid gland, parathyroid glands, and skin. Several different calcium binding proteins have been found but not all of these binding proteins are vitamin D dependent. That is, once formed, their activity with respect to calcium binding is unaffected by vitamin deficiency. Most, however, are dependent on vitamin D for their synthesis. Thus, these calcium-binding proteins are molecular expressions of the hormonal action of the vitamin.

As animals age, the levels of the calcium-binding protein fall. Yet, when calcium intake levels fall, the synthesis and activity of the binding proteins rise. This mechanism explains how individuals can adapt to low calcium diets. Interestingly, calcium deprivation stimulates the conversion of cholecalciferol to 25-hydroxycholecalciferol in the liver and to 1,25-dihydroxycholecalciferol in the kidney. Aging, however, seems to affect this regulatory mechanism. As humans age, they are less able to absorb calcium and may develop osteomalacia, a condition analogous to rickets in children and characterized by demineralization of the bone. In patients with osteomalacia, intestinal absorption of calcium is decreased, but when 1,25-dihydroxy-$D_3$ is administered, calcium absorption is increased. It would appear, therefore, that one of the consequences of aging is an impaired conversion of 25-hydroxy-$D_3$ to 1,25-dihydroxy-$D_3$, and since less of the latter is available, less calcium binding protein is synthesized. Measures of calcium-binding protein in aging rats, using an immunoassay technique, have shown that this is indeed the case.

Vitamin D also increases intestinal absorption of calcium by mechanisms apart from the synthesis of calcium-binding protein. It does this as part of its general tropic effect as a steroid on a variety of cellular reactions. Vitamin D causes a change in membrane permeability to calcium at the brush border, perhaps through a change in the lipid (fatty acid) component of the membrane. It stimulates the $Ca^{2+}Mg^{2+}$ ATPase on the membrane of the cell wall, increases Krebs cycle activity, increases the conversion of ATP to cAMP, and increases the activity of the alkaline phosphatase

enzyme. All these effects in the intestinal cell are independent of the vitamin's effect on calcium-binding protein synthesis.

In addition to its role in calcium absorption, vitamin D serves to induce the uptake of phosphate and magnesium by the brush border of the intestine. The effect on phosphate uptake is independent of its effect on calcium absorption and is due to an effect of the vitamin on the synthesis of a sodium-dependent membrane carrier for phosphate. The effect of vitamin D on magnesium absorption is incidental to its effect on calcium absorption, since the calcium-binding protein has a weak affinity for magnesium. Thus, if synthesis of the calcium-binding protein results in an increase in calcium uptake, it also results in a significant increase in magnesium uptake.

### *a. Regulation of Serum Calcium Levels*

Serum calcium levels are closely regulated in the body so as to maintain optimal muscle contractility and cellular function. Several hormones are involved in this regulation: 1,25-dihydroxy-$D_3$ produced by the kidney, parathyroid hormone released by the parathyroid gland, and thyrocalcitonin released by the thyroid C cells. Each has a specific function with respect to serum calcium levels and all three are interdependent. Vitamin $D_3$ increases blood calcium by increasing intestinal calcium uptake and decreases blood calcium by increasing calcium deposition in the bone. In the relative absence of vitamin D, parathyroid hormone increases serum calcium levels by increasing the activity of the kidney 1$\alpha$-25-hydroxylase with the result of increasing blood levels of 1,25-dihydroxy-$D_3$ and through enhancing bone mineral mobilization and phosphate diuresis. Parathyroid hormone in the presence of vitamin D has the reverse action on bone. When both hormones (parathormone and 1,25-$D_3$) are present, bone mineralization is stimulated. Even though the parathyroid hormone stimulates the production of 1,25-dihydroxy-$D_3$, $D_3$ does not stimulate parathyroid hormone release. Thyrocalcitonin serves to lower blood calcium levels through stimulating bone calcium uptake, and its effect is independent of parathyroid hormone yet is dependent on the availability of calcium from the intestine. If serum calcium levels are elevated through a calcium infusion, thyrocalcitonin will be released and stimulate bone calcium uptake even in animals lacking both parathyroid hormone and $D_3$.

### *b. Mode of Action at the Genomic Level*

The process of vitamin D (1,25-dihydroxycholecalciferol)-receptor binding to specific DNA sequences follows the classic model for steroid hormone action. Like vitamin A, vitamin D binds to a receptor protein (vitamin D receptor protein, VDR) in the nucleus. This receptor protein is a member of the steroid hormone receptor superfamily. The receptor protein then acquires an affinity for specific DNA sequences located upstream from the promoter sequence of the target gene. These specific DNA sequences are called response elements. The receptor protein consists of a structure in which two zinc atoms are coordinated in two finger-like domains. The N-terminal finger confers specificity to the binding while the second finger stabilizes the complex. When bound, transcription of the cognate protein mRNA is activated. Several response elements have been identified, with each being specific for a specific gene product. As shown in Figure 11, the response elements have in common imperfect direct repeats of six base pair half elements separated by a three-base-pair spacer. Affinity of the nuclear receptor protein for vitamin D is modified by phosphorylation. Two sites of phosphorylation have been found and both are on serine residues. One site is located in the DNA binding region at serine 51 between two zinc-finger DNA binding motifs, and if phosphorylated by protein kinase C (or a related enzyme) DNA binding is reduced. The second site is located in the hormone-binding N-terminal region at serine 208. It has the opposite effect. When phosphorylated, probably by casein kinase II or a related enzyme, transcription is activated.

The vitamin-protein receptor complex that binds to the DNA consists of three distinct elements: 1,25-dihydroxycholecalciferol (the hormone ligand), the vitamin receptor, and one of the retinoid

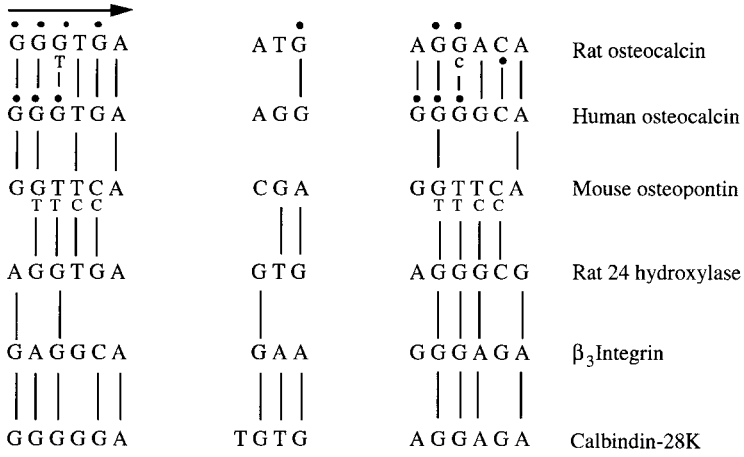



**Figure 11** Vitamin D response elements for osteocalcin, osteopontin, $D_3$-24-hydroxylase, $\beta_3$ integrin, and calbindin 28K. Circles above bases indicate guanine residues that have been shown by methylation interference experiments to be protected upon protein binding to the vitamin D responsive element. The small letters below the large ones indicate points at which base substitutions have been found and which abolish responsiveness to vitamin D. (Adapted form Whitfield, G.K. et al., *J. Nutr.,* 125, 1690, 1995. With permission.)

X receptors (RXRα). Here is an instance where, once again, a vitamin D-vitamin A interaction occurs. The 9-*cis* retinoic acid can attenuate the induction of the transcriptional activation that occurs when the vitamin D-receptor complex binds to the vitamin D responsive element. Perhaps 9-*cis* retinoic acid has this effect because when it binds to the retinoid X receptor, it blocks or partially occludes the binding site of the vitamin D-protein for DNA. Figure 12 illustrates the mode of vitamin D action at the genomic level. To date, the nuclear vitamin D receptor has been found in 34 different cell types and it is quite likely that it is a universal nuclear component. Probably this is because every cell has a need to move calcium into or out of its various compartments as part of its metabolic control systems. Thus, calcium-binding proteins whose synthesis is vitamin D dependent are needed. Among the proteins thus far identified are osteocalcin, vitamin D binding protein, osteopontin, 24-hydroxylase-β3-interferon, calbindin, prepro PTH, calcitonin, Type I collagen, fibronectin, bone matrix GLA protein, interleukin 2 and interleukin γ, transcription factors (GM-CSF, c-myc, c-fos, c-fms), vitamin D receptors, calbindin $D_{28x}$ and $_{9x}$, and prolactin. The transcription of the genes for each of these proteins are affected or regulated by vitamin D.

## H. Vitamin D Deficiency

As discussed earlier, bone deformities are the hallmark of the vitamin D-deficient child while porous brittle bones are indications of the deficiency in the adult. In view of the fact that the liver can store large quantities of the previtamin, it is difficult to visualize how the disorder can develop. Rickets was very common in the U.S. prior to the enrichment of milk and other food products. In part, rickets developed because heavy clothing shielded the skin from the ultraviolet rays of the sun. Lacking this exposure, the only other source was food, and because so few foods contain significant quantities of the vitamin, deficiency states developed.

Today, osteomalacia, the adult form of rickets, can be due to inadequate intake or exposure to sunlight and also can be due to disease or damage to either the liver or kidney. As pointed out in Section II.G of this unit, both these organs are essential for the conversion of cholecalciferol to 1,25-dihydroxy-$D_3$. If either organ is nonfunctional in this respect, the deficient state will develop. On rare occasions, the deficient state will develop not because of any lack of dietary D or sunlight or because of kidney or liver damage, but through a genetic error in which the 1,25-hydroxylase

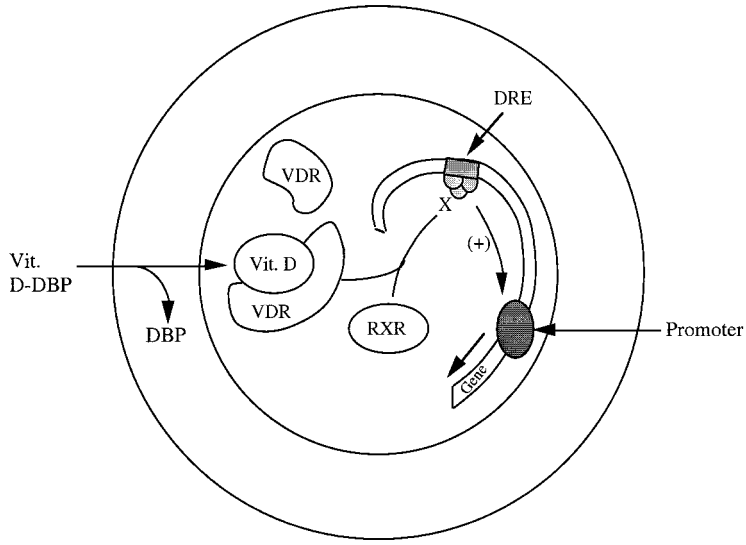



**Figure 12** Schematic representation showing vitamin D bound to vitamin D binding protein (DBP) entering the nucleus, binding to receptor (VDR), complexing with retinoid receptor (RXR), and then binding to the vitamin D responsive elements (DRE) as a trans-acting factor enhancing the transcription of a variety of calcium-binding proteins.

enzyme is missing and the 1,25-dihydroxy-$D_3$ cannot be synthesized. In individuals so afflicted, 1,25-dihydroxy-$D_3$ must be supplied to prevent the deficiency state from developing.

1,25-Dihydroxy-$D_3$ must also be provided to the anephritic patient, since these patients cannot synthesize this hormone. Until the realization that the kidney served as the endocrine organ for 1,25-dihydroxy-$D_3$ synthesis, renal disease was almost always accompanied by a disturbed calcium balance, osteomalacia, and osteoporosis.

## I. Hypervitaminosis D

Since previtamin D is fat soluble, like vitamin A, it can be stored. The storage capacity of the liver for the D precursor is much less than its capacity for A and toxic conditions can develop if large amounts of D (in excess of need and storage capacity) are consumed over extended periods of time. Because D's main function is to facilitate calcium uptake from the intestine and tissue calcium deposition, excess D in the toxic range (1,000 to 10,000 IU/day) will result in excess calcification, not only of bone but of soft tissues as well. Renal stones, calcification of the heart, major vessels, muscles, and other tissues have been shown in experimental animals as well as in humans. Seelig has described a series of patients who were unusually sensitive to vitamin D either *in utero* or in infancy. These patients had multiple abnormalities in soft tissues and bones and were mentally retarded. Whether excess D intakes provoke mild, moderate, or severe abnormalities is related not only to the individual's genetic background but also to his/her calcium, magnesium, and phosphorus intake. If any of these are consumed in excess of the others, vitamin D intoxication becomes more apparent.

## J. Recommended Dietary Allowances

Because the active $D_3$ hormone can be synthesized in the body, an absolute requirement is difficult to determine. Few foods naturally contain sufficient preformed vitamin D. In the absence of synthesis *in vivo*, preformed vitamin D must be added to the diet. This is done almost to excess

**Table 11 Recommended Dietary Allowances (RDA) for Vitamin D**

| Group | Age (years) | RDA (μg/day) |
|---|---|---|
| Infants | 0–6 months | 7.5 |
| | 7–12 months | 10 |
| Children | 1–3 | 10 |
| | 4–6 | 10 |
| | 7–10 | 10 |
| Males | 11–14 | 10 |
| | 15–18 | 10 |
| | 19–24 | 10 |
| | 25–50 | 5 |
| | 51+ | 5 |
| Females | 11–14 | 10 |
| | 15–18 | 10 |
| | 19–24 | 10 |
| | 25–50 | 5 |
| | 51+ | 5 |
| Pregnancy | — | 10 |
| Lactation | — | 10 |

in the U.S. Milk is fortified with 10 μg per quart and other food products such as margarine are also fortified with the vitamin. Due to this fortification, infantile rickets is almost unknown today. The level of supplementation for milk was selected based on the concept that the growing child should drink one quart of milk a day in order to optimize growth. Adults usually do not require vitamin supplementation unless they are either pregnant or lactating. Pregnant or lactating females are recommended to consume 10 μg vitamin D per day. Table 11 gives the recommended dietary allowances for vitamin D.

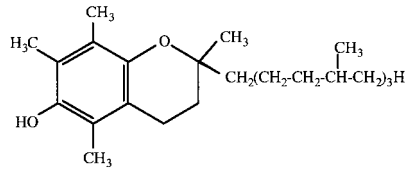
## III. VITAMIN E

### A. Overview

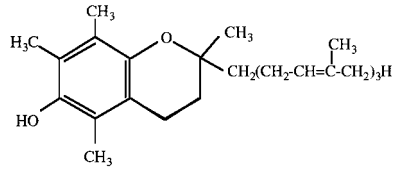
While much of the excitement of the "vitamin discovery era" was devoted to the work on vitamins A and D, Evans and Bishop in 1922 discovered an unidentified factor in vegetable oil which, if lacking from the diet, resulted in reproductive failure. The term tocopherol was proposed by Emerson for this factor because of its role in reproduction. Rats fed diets lacking this vitamin failed to reproduce. The name tocopherol comes from the Greek work *tokos*, meaning childbirth, *pherein*, meaning to bring forth, and *ol*, the chemical suffix meaning alcohol. Vitamin E, the name suggested by Evans and Bishop, is commonly used for that group of tocol and tocotrienol derivatives having vitamin E activity. The most active of these is α-tocopherol (Figure 13). Much of the earlier work was devoted to tocopherol's role in reproduction such that its function as an antioxidant was ignored. However, in the last 30 years considerable attention has been given to this role in metabolism. It is recognized that vitamin E is an essential nutrient for many animal species. The level of vitamin E in plasma lipoproteins and in the phospholipids of the membrane around and within the cell are dependent on vitamin E intake as well as on the intake of other antioxidant nutrients and on the level of dietary polyunsaturated fatty acids.

### B. Structure and Nomenclature

The most active naturally occurring form of vitamin E is D-α-tocopherol. Other tocopherols have been isolated having varying degrees of vitamin activity. Figure 13 shows the molecular

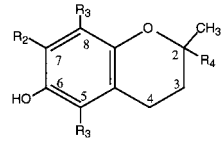



α-Tocopherol (5, 7, 8 Trimethyltocol)




α -Tocotrienol (5, 7, 8 Trimethyltrienol)

**Figure 13** Basic structure of vitamin E.




$R_1R_2R_3 = CH_3$ or H

$R_4 = CH_2(CH_2CH_2\underset{\displaystyle CH_3}{\underset{|}{C}}HCH2)_3H$ (Tocols)

or

$CH_2(CH_2CH = \underset{\displaystyle CH_3}{\underset{|}{C}}— CH_2)_3H$ (Tocotrienols)

α-Tocol or Tocotrienol have $R_1,R_2,R_3 = CH_3$

β-Tocol or Tocotrienol have $R_1,R_3 = CH_3$; R = H

γ-Tocol or Tocotrienol have $R_2,R_3 = CH_3$; $R_1$ = H

δ-Tocol or Tocotrienol have $R_3 = CH_3$; $R_1R_2$ = H

ε-Tocol or Tocotrienol have $R_1 = CH_3$; $R_2,R_3$ = H

ζ–Tocol or Tocotrienol have $R_1,R_2 = CH_3$; $R_3$ = H

η–Tocol or Tocotrienol have $R_2 = CH_3$; $R_1,R_3$ = H

**Figure 14** Structures of naturally occurring compounds having vitamin E activity.

structures of α-tocopherol and α-tocotrienol. Figure 14 shows the other tocopherols and tocotrienols and their relationship to α-tocopherol. To have vitamin activity, the compound must have the double ring structure (chromane nucleus) as shown in Figures 13 and 14 and must also have a side chain attached at carbon 2 and methyl groups attached at carbons 5, 7, or 8. α-Tocopherols have methyl groups attached at all three positions and represent the most active vitamin compounds. β-Tocopherols have methyl groups attached to carbons 5 and 8; γ-tocopherols have methyl groups attached at carbons 7 and 8, and δ-tocopherols have only one methyl group attached at carbon 8. If the side chain attached to carbon 2 is saturated, then the compound is a member of the tocol family of compounds; if unsaturated, it belongs to the tocotrienol family. All forms have a hydroxyl group at carbon 6 and a methyl group at carbon 2. Other forms, ε, ζ, and η, have their methyl groups at carbon 5, or 5 and 7, or 7, respectively. Naturally occurring vitamin E is in the D form

**Table 12 Commercially Available Products Having Vitamin E Activity**

| Form | Units of activity/mg |
|---|---|
| DL-α-Tocopheryl acetate (all-rac) | 1.00 |
| DL-α-Tocopherol (all-rac) | 1.10 |
| D-α-Tocopheryl acetate (RRR) | 1.36 |
| D-α-Tocopheryl acid succinate (all-rac) | 1.49 |
| DL-α-Tocopheryl acid succinate (all-rac) | 0.89 |
| D-α-Tocopheryl acid succinate (RRR) | 1.21 |

*Note:* RRR — only naturally occurring stereoisomers bear this designation.

whereas synthetic vitamin E preparations are mixtures of the D and L forms. Both tocols and trienols occur as a variety of isomers. There are commercially available products usually marketed as acetate or succinate esters. The ester form does not usually occur in nature. Table 12 lists some of these commercially available forms.

## C. International Units and Methods of Analysis

The international unit (IU) of vitamin E activity uses the activity of 1 mg of DL-α-tocopherol acetate (all-rac) in the rat fetal absorption assay as its reference standard. Even though D-α-tocopherol is 36% more active than the DL form, the latter was selected as the reference substance because it is more readily available as a standard of comparison. This choice may be a poor one because of the lack of validation of the fetal rat absorption assay as a true test of vitamin E potency. The fetal resorption test uses female vitamin E-depleted virgin rats. These rats are mated to normal males and given the test substance. After 21 days, the number of live fetuses and the number of dead and resorbed ones are counted. The potency of the test material is compared to a known amount of DL-α-tocopherol. Other tests have also been devised and are based on other functions of vitamin E. These tests may yield more reliable comparisons and may result in a redefinition of the IU. Tests of biopotency include the red cell hemolysis test and tests designed to evaluate the potency of the test substance in preventing or curing muscular dystrophy. Using DL-α-tocopherol as the standard with a value of 100, β-tocopherol has a value of 25 to 40 on the fetal resorption test, 15 to 27 on the hemolysis test, and 12 on the preventative muscular dystrophy test using the chicken as the test animal. γ-Tocopherol is even less potent, with values of 1 to 11, 3 to 20, and 5 for the same tests, respectively. The other tocopherols and tocotrienols are even less potent by comparison. Burton and Traber have reviewed the biopotency of vitamin E isomers as antioxidants.

Biochemical methods which utilize changes in enzyme activity rather than functional tests such as the rat fetal resorption test allow for the comparison of the different E vitamin forms. For example, plasma pyruvate kinase, hepatic glutathione peroxidase, and muscle cyclooxygenase activity are reduced in vitamin E-deficient rats. However, a true dose-response curve showing intake vs. changes in enzyme activity patterns, which in turn precede tissue changes, does not clearly provide a basis for biopotency. These enzyme activity studies are not as sensitive with respect to vitamin intake and potency as one would like.

Chemical analyses using thin-layer chromatography, gas-liquid chromatography, and high performance liquid chromatography (HPLC) are now available. These methods are very sensitive and can separate and quantify the various isomers in food, plasma, blood cells, and tissues. The HPLC method is the one of choice because sample preparation is minimal. Amounts of the isomers in the nanogram range can be detected and quantified.

## D. Chemical and Physical Properties

The tocopherols are slightly viscous oils that are stable to heat and alkali. They are slowly oxidized by atmospheric oxygen and rapidly oxidized by iron or silver salts. The addition of acetate

**Table 13 Characteristics of the Major Tocopherols**

| Compound | Color | Boiling Point (°C) | Molecular Weight (Da) | Absorption Maxima (nm) | Extraction (Ethanol) |
|---|---|---|---|---|---|
| DL-α-Tocopherol | Colorless to pale yellow | 200–220 | 430.69 | 292–294 | 71–76 |
| D-α-Tocopherol | Colorless to pale yellow | — | 430.69 | 292–294 | 72–76 |
| DL-α-Tocopherol acetate | Colorless to pale yellow | 224 | 472.73 | 285.5 | 40–44 |
| D-α-Tocopheryl acetate | Colorless to pale yellow | — | 472.73 | 285.5 | 40–44 |

**Table 14 Vitamin E Content of a Variety of Foods**

| Food | α-Tocopherol (μg/g) | Food | α-Tocopherol (μg/g) |
|---|---|---|---|
| Corn oil | 159 | Pork | 4–6 |
| Olive oil | 100 | Chicken | 2–4 |
| Peanut oil | 189 | Almonds | 270 |
| Wheat germ oil | 1194 | Peanuts | 72 |
| Palm oil | 211 | Oatmeal | 17 |
| Soft margarine | 139 | Rice | 1–7 |
| Milk | 0.2–1.1 | Wheat germ | 117 |
| Butter | 10–33 | Apple | 3 |
| Lard | 2–38 | Peach | 13 |
| Eggs | 8–12 | Asparagus | 16 |
| Fish | 4–33 | Spinach | 25 |
| Beef | 5–8 | Carrots | 4 |

or succinate to the molecule adds stability towards oxidation. The tocopherols are insoluble in water but soluble in the usual fat solvents. Ultraviolet light destroys the vitamin activity. Table 13 gives the properties of four of the most potent tocopherols.

## E. Sources

The tocopherols have been isolated from a number of foods. Almost all are from the plant kingdom, with wheat germ oil being the richest source. European wheat germ oil contains mostly β-tocopherols while American wheat germ oil contains mostly α-tocopherols. Corn oil contains α-tocopherols and soybean oil δ-tocopherols. Olive and peanut oil are poor sources of the vitamin. Some animal products such as egg yolk, liver, and milk contain tocopherols but, in general, foods of animal origin are relatively poor sources of the vitamin. Table 14 provides values of tocopherols in a variety of foods. Vegetable oils vary from 100 μg/g (olive oil) to nearly 1200 μg/g (wheat germ oil). Some of the animal products shown in this table have a range of values given because of seasonal variations due to differences in intakes of the animal from which these foods come.

## F. Metabolism

### *1. Absorption and Transport*

Because of its lipophilicity, vitamin E, like the other fat-soluble vitamins, is absorbed via the formation of chylomicrons and their uptake by the lymphatic system. The tocopherols are transported as part of the lipoprotein complex. Absorption is relatively poor and it is unlikely to involve a protein carrier-mediated process. In humans, studies of labeled tocopherol absorption have shown that less than half of the labeled material appears in the lymph and up to 50% of the ingested vitamin may appear in the feces. Efficiency of absorption is enhanced by the presence of food fat in the intestine. The use of water-miscible preparations enhances absorption efficiency, particularly

in those individuals whose fat absorption is impaired, i.e., persons with cystic fibrosis or biliary disease. The commercially prepared tocopherol acetate or palmitate loses the acetate or palmitate through the action of a bile-dependent, mucosal-cell esterase prior to absorption. The pancreatic lipases, bile acids, and mucosal-cell esterases are all-important components of the digestion and absorption of vitamin E from food sources. The same processes required for the digestion and absorption of food fat apply here for the tocopherols. Absorption through penetration of the apical plasma membrane of the enterocytes of the brush border is maximal in the jejunum.

There are some species differences in the process in that mammals absorb the vitamin as part of a lipoprotein complex (chylomicrons) into the lymph whereas birds have the vitamin transported directly into the portal blood. In addition, there are gender differences in absorption efficiency: females are more efficient than males. Unlike the food fats (cholesterol and the acylglycerides), hydrolysis is not followed by reesterification in the absorption process. To date, a specific tocopherol transport protein in the blood has not been identified or described. It appears that the tocopherols are bound to all of the lipid-carrying proteins in the blood and lymph. An excellent antioxidant, vitamin E serves this function very well as it is being transported (from enterocyte to target tissue) with those lipids that could be peroxidized and thus require protection. Some cardiovascular researchers have suggested that one important function of vitamin E is to prevent the peroxidation of lipids in the blood which would, in turn, suppress possible endothelial damage to the vascular tree and thus suppress some of the early events in plaque formation. Whether this hypothesis about the role of vitamin E in preventing such degenerative disease is true remains to be proven.

### *2. Intracellular Transport and Storage*

Although no specific transport protein has been found for the tocopherols in blood and lymph, there appears to be such a protein within the cells. A 30-kDA α-tocopherol-binding protein has been found in the hepatic cytosol and another 14.2-kDa one in heart and liver that specifically binds to α-tocopherol and transfers it from liposomes to mitochondria. No doubt we will also find that either this or another low molecular weight protein transfers the vitamin to the nucleus. Having the vitamin in these two organelles protects them from free radical damage. One of the targets of free radicals is the genetic material, DNA, while another is the membrane phospholipid. In either instance, damage to these vital components could be devastating. The smaller of the two binding proteins is similar in size to the intracellular fatty acid-binding protein, FABP. This protein also binds some of the eicosanoids but not α-tocopherol. The other tocopherols (β, γ, etc.) are not bound to the tocopherol-binding proteins to the same extent as α-tocopherol, nor are these isomers retained as well.

Tocopherols are found in all of the cells in the body, with adrenal cells, pituitary cells, platelets, and testicular cells having the highest concentrations. Adipose tissue, muscle, and liver serve as reservoirs and these tissues will become depleted should intake levels be inadequate to meet the need. The rate of depletion with dietary inadequacy varies considerably. Since its main function is as one of several antioxidants, other nutrients which also serve in this capacity can affect vitamin depletion. The intake of β-carotene and ascorbic acid and the polyunsaturated fatty acids can markedly affect the rate of use of α-tocopherol as an antioxidant. Increased intakes of β-carotene and ascorbic acid protect the α-tocopherol from depletion, whereas increased intakes of polyunsaturated fatty acids drive up the need for antioxidants. A further consideration is the intake of selenium. This mineral is an integral part of the glutathione peroxidase system that suppresses free radical production. In selenium-deficient animals, the need for α-tocopherol is increased, and vice versa. The α-tocopherol-deficient animal has a greater need for selenium. In addition, α-tocopherol protects against iron toxicity in another instance of a mineral-vitamin interaction. In this situation, high levels of iron drive up the potential for free radical formation and this can be overcome with increases in vitamin E intake.

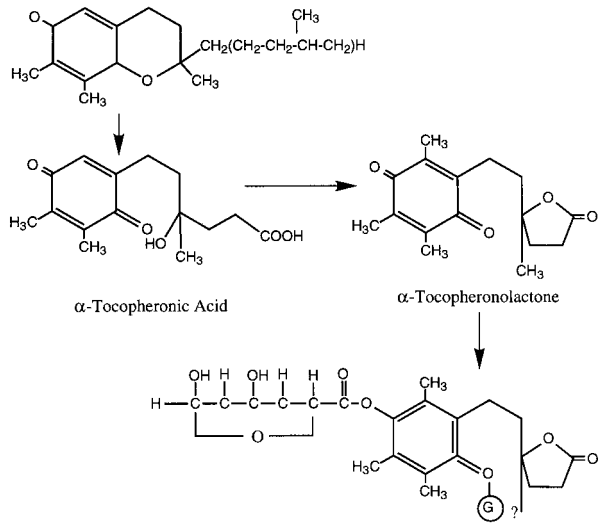



**Figure 15** Excretory pathway for the tocopherols. These compounds are found in the feces.

### 3. Catabolism and Excretion

Upon entry into the cell very little degradation occurs. Usually less than 1% of the ingested vitamin (or its metabolite) appears in the urine. Compounds called Simon's metabolites appear in the urine. These are glucouronates of the parent compound. The degradation and excretion via the intestine is shown in Figure 15.

### 4. Function

As mentioned, the main function of vitamin E is as an antioxidant. This function is shared by β-carotene, ascorbic acid, the selenium-dependent glutathione peroxidase, and the copper-manganese- and magnesium-dependent superoxide dismutases.

Peroxides of fatty acids, amino acids, and proteins are highly reactive materials that can damage cells and tissues. The phospholipids of the membranes within and around the cells are the most vulnerable to this peroxidation because they contain fewer saturated fatty acids than the stored triacylglycerols within the cell. Phospholipids usually contain an unsaturated fatty acid (arachidonate) at carbon 2, a saturated fatty acid at carbon 1, and a phosphate intermediate at carbon 3. In erythrocytes, the selenoenzyme, glutathione peroxidase, protects hemoglobin and the cell membrane from peroxide damage. The enzyme works to maintain glutathione levels, thus regulating the redox state of the cells. In so doing, this enzyme protects hemoglobin and the cell membrane by detoxifying lipid hydroperoxides to less toxic fatty acids, preventing the initial free radical attack on either the hemoglobin protein or the membrane lipids. Vitamin E potentiates glutathione peroxidase action by serving as a free radical scavenger, thus preventing lipid hydroperoxide formation.

Glutathione peroxidase has been found in cells other than red blood cells. It is present in adipose tissue, liver, muscle, and glandular tissue and its activity is complementary to that of catalase, another enzyme which uses peroxide as a substrate. Together, these enzymes and vitamin E protect the integrity of the membranes by preventing the degradation, through oxidation, of the membrane lipids. This function of vitamin E is seen more clearly in animals fed high levels of polyunsaturated fatty acids. As the intake of these acids is increased a larger portion is incorporated into the membrane lipids, which in turn become more vulnerable to oxidation. Unless protected against

oxidation, the functionality of the membranes will be impaired and, if uncorrected, the cell will die. Disturbances in the transport of materials across membranes has been shown in liver with respect to cation flux. Liver slices from E-deficient animals lost the ability to regulate sodium/potassium exchange and calcium flux. Investigators have shown a decline in mitochondrial respiration in vitamin E-deficient rats. Such a decline probably represents a decline in the flux of ADP or calcium into the mitochondria to stimulate oxygen uptake by the respiratory chain. Such a decline would permit more oxygen to remain in the cytosol to further stimulate lipid oxidation. In addition to peroxidative damage to the membrane, there is also damage to the DNA, with the possible result of aberrant gene products. Thus, a whole cascade of responses to vitamin E insufficiency can be envisioned. Interestingly, in diseases manifested by an increased hemolysis of the red cells and a decreased ability of the hemoglobin to carry oxygen, red cell vitamin E levels are low. This has been shown in patients with sickle-cell anemia and in patients with cystic fibrosis. In patients with sickle-cell anemia, the low vitamin E level in the erythrocytes is accompanied by an increased level of glutathione peroxidase activity. It has been suggested that the increase in enzyme activity was compensatory to the decrease in vitamin E content.

Vitamin E and zinc have been found to have interacting effects in the protection of skin lipids. In zinc-deficient chicks, supplementation with vitamin E decreased the severity of the zinc deficiency state, suggesting that zinc also may have antioxidant properties or that there may be an interacting effect of zinc with the vitamin.

In addition to the above main function of vitamin E, there are other roles for this substance. One involves eicosanoid synthesis. Thromboxane ($TXA_2$), a platelet aggregating factor, is synthesized from arachidonic acid (20:4) via a free-radical-mediated reaction. This synthesis is greater in a deficient animal than in an adequately nourished one. Vitamin E enhances prostacyclin formation and inhibits the lipooxygenase and phospholipase reactions. This effect is secondary to the vitamin's role as an antioxidant. As mentioned, phospholipase $A_2$ is stimulated by lipid peroxides. Other secondary functions also are related to its antioxidant function. Oxidant damage to DNA in bone marrow could explain red blood cell deformation that typifies vitamin E deficiency as well as explain the fragility (due to membrane damage) of these cells. In turn, this would explain why enhanced red cell fragility is a characteristic of the deficient state.

Steroid hormone synthesis as well as spermatogenesis, both processes that are impaired in the deficient animal, could be explained by the damaging effects of free radicals on membranes and/or DNA, which are corrected by the provision of this antioxidant vitamin.

## G. Hypervitaminosis E

Even though vitamin E is a fat-soluble vitamin like A and D, there is little evidence that high intakes will result in toxicity in humans. Due to inefficient vitamin uptake by the enterocyte, excess intake is excreted in feces. However, E toxicity has been produced in chickens. It is characterized by growth failure, poor bone calcification, depressed hematocrit, and increased prothrombin times. These symptoms suggest that the excess E interfered with the absorption and/or use of the other fat-soluble vitamins since these symptoms are those of the A, D, and K deficiency states. This suggests that proponents of megadoses of vitamin E as treatment for heart disease, muscular dystrophy, and infertility (amongst other ailments) may unwittingly advocate the development of additional problems associated with an imbalance in fat soluble vitamin intake due to these large E intakes.

## H. Deficiency

One of the first deficiency symptoms recorded for the tocopherols was infertility, followed by the discovery that white muscle disease, a peculiar muscle dystrophy, could be reversed if vitamin E

**Table 15 Vitamin E Deficiency Disorders**

| Disorder | Species Affected | Tissue Affected |
|---|---|---|
| | **Reproductive Failure** | |
| Female | Rodents, birds | Embryonic vascular tissue |
| Male | Rodents, dog, birds, monkey, rabbit | Male gonads |
| Hepatic necrosis[b] | Rat, pig | Liver |
| Fibrosis[b] | Chicken, mouse | Pancreas |
| Hemolysis[a,c] | Rat, chick, premature infant | Erythrocytes |
| Anemia | Monkey | Bone marrow |
| Encephalomalacia[a,c] | Chick | Cerebellum |
| Exudative diathesis[b] | Birds | Vascular system |
| Kidney degeneration[a,b] | Rodents, monkey, mink | Kidney tabular epithelium |
| Steatitis[a,c] | Mink, pig, chick | Adipose tissue |
| | **Nutritional Myopathies** | |
| Type A muscular dystrophy | Rodents, monkey, duck, mink | Skeletal muscle |
| Type B white muscle disease[b] | Lamb, calf, kid | Skeletal and heart muscle |
| Type C myopathy[b] | Turkey | Gizzard, heart |
| Type D myopathy | Chicken | Skeletal muscle |

[a] Increased intake of polyunsaturated acids potentiate deficiency.
[b] Can be reversed by addition of selenium to the diet.
[c] Antioxidants can be substituted for vitamin E to cure condition.

was provided. Later it was recognized that selenium also played a role in the muscle symptom. Listed in Table 15 are the many symptoms attributed to inadequate vitamin E intake. All of these symptoms are related primarily to the level of peroxides in the tissue or to peroxide damage to either the membranes and/or DNA.

### I. Recommended Dietary Allowance

Because of the interacting effects of vitamin E with selenium and other antioxidants, the requirement for the vitamin has been difficult to ascertain. It has been estimated that the average adult consumes approximately 15 mg/day but the range of intake is very large. As mentioned earlier, vitamin E requirements are larger when polyunsaturated fat intake is increased. Fortunately, foods containing large supplies of these polyunsaturated fatty acids also contain large quantities of vitamin E. The vitamin E to polyunsaturated fatty acid intake ratio should be 0.6. Table 16 provides the RDA for humans.

## IV. VITAMIN K

### A. Overview

Even though vitamin K was one of the last fat-soluble vitamins discovered, its existence was suspected as early as 1929. In that year, Henrik Dam was studying cholesterol biosynthesis and observed that chickens fed a semisynthetic sterol-free diet had numerous subcutaneous hemorrhages. Hemorrhages were observed in other tissues as well, and when blood was withdrawn from these birds, it had a prolonged clotting time. At first it was thought that these were symptoms of scurvy in birds, but the addition of vitamin C did not cure the disorder. It was then thought that the hemorrhages characterized the bird's response to a dietary toxin. This hypothesis was also disproved. Finally, it was shown that the inclusion of plant sterols prevented the disease and thus the disease was shown to be a nutrient deficiency.

**Table 16 Recommended Dietary Allowances (RDA) for Vitamin E**

| Group | Age | RDA (mg α-tocopherol equivalents) |
|---|---|---|
| Infants | 0–6 months | 3 |
| | 7–12 months | 4 |
| Children | 1–3 | 6 |
| | 4–6 | 7 |
| | 7–10 | 7 |
| Males | 11–14 | 10 |
| | 15–18 | 10 |
| | 19–24 | 10 |
| | 25–50 | 10 |
| | 51+ | 10 |
| Females | 11–14 | 8 |
| | 15–18 | 8 |
| | 19–24 | 8 |
| | 25–50 | 8 |
| | 51+ | 8 |
| Pregnancy | | 10 |
| Lactation | 1st 6 months | 12 |
| | 2nd 6 months | 11 |

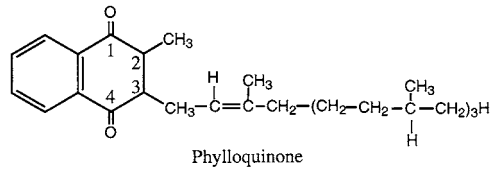
Because the condition was characterized by a delayed blood clotting time and because it could be cured or prevented by the inclusion of the nonsaponifiable sterol fraction of a lipid extract of alfalfa, it was named the antihemorrhagic factor. Dam proposed that it be called vitamin K. The letter K was chosen from the German word, koagulation.

## B. Structure and Nomenclature

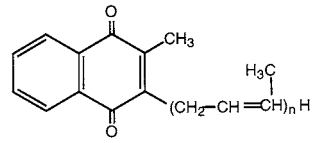
Subsequent to its recognition as an essential micronutrient, vitamin K was isolated from alfalfa and from fish meal. The compounds isolated from these two sources were not identical, so one was named $K_1$ and the other $K_2$. Almquist was the first to show that the vitamin could be synthesized by bacteria. He discovered that putrefied fish meal contained more of the vitamin than nonputrefied fish meal. It was also learned that bacteria in the intestine of both the rat and the chicken synthesized the vitamin, thus ensuring a good supply of the vitamin if coprophagy (eating feces) was permitted.

These early studies thus provided the reason to suspect that there was more than one form of the vitamin. A large number of compounds, all related to a 2-methyl-1,4-naphthaquinone possess vitamin K activity (Figure 16). Compounds isolated from plants have a phytyl moiety at position 3 and are members of the $K_1$ family of compounds. Phylloquinone [2-methyl-3-phytyl-1,4-naphthaquinone (II)] is the most important member of this family. The K vitamins are identified by their family and by the length of the side chain attached at position 3. The shorthand designation uses the letter K with a subscript to indicate family, and a superscript to indicate the side-chain length. Thus, $K_2^{20}$ indicates a member of the family of compounds isolated from animal sources having a 20-carbon side chain. The character of the side chain determines whether a compound is a member of the $K_1$ or $K_2$ family. $K_1$ compounds have a saturated side chain whereas $K_2$ compounds have an unsaturated side chain. Chain lengths of the $K_1$ and $K_2$ vitamins can vary from 5 to 35 carbons.

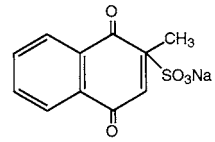
A third group of compounds is the $K_3$ family. These compounds lack the side chain at carbon 3. Menadione is the parent compound name and it is a solid crystalline material menadione sodium bisulfite (a salt), as shown in Figure 16. Other salts are also available. These salts are water soluble and thus have great use in diet formulations or mixed animal feeds. Clinically useful is menadiol sodium diphosphate. The use of this compound must be very carefully monitored as overdoses can




Phylloquinone




Menaquinone




Menadione Sodium Bisulfite

**Figure 16** Structures of vitamin $K_1$ (phylloquinone), $K_2$ (menaquinone), and $K_3$ (menadione, a synthetic vitamin precursor that is converted to $K_2$ by the intestinal flora).

result in hyperbilirubinemia and jaundice. These $K_3$ compounds can be synthesized in the laboratory. When consumed as a dietary ingredient, the quinone structure is converted by the intestinal flora to a member of the $K_2$ family.

There are several structural requirements for vitamin activity: there must be a methyl group at carbon 2 and a side chain at carbon 3, and the benzene ring must be unsubstituted. The chain length can vary; however, optimal activity is observed in compounds having a 20-carbon side chain. $K_1$ and $K_2$ compounds with similar side chains have similar vitamin activities.

The vitamin can exist in either the cis or trans configuration. All-*trans*-phylloquinone is the naturally occurring form whereas synthetic phylloquinone is a mixture of the cis and trans forms.

## C. Biopotency

The various compounds with vitamin activity are not equivalent with respect to potency as a vitamin. The most potent compound of the phylloquinone series is the one with a 20-member side chain. Compounds having fewer or greater numbers of carbons are less active. Table 17 provides this comparison. The most potent compound in the menaquinone series is the one with a 25-member unsaturated side chain.

## D. Chemical and Physical Properties

Phylloquinone ($K_1^{20}$) is a yellow viscous oil. The physical state of menaquinone ($K_2^{20}$) depends on its side-chain length. If the side chain is 5 or 10 carbons long it is an oil, if longer it is a solid. Menadione ($K_3$) is a solid. All three families of compounds are soluble in fat solvents. Menadione can be made water soluble by converting it to a sodium salt. All the vitamin K compounds are stable to air and moisture but unstable to ultraviolet light. They are also stable in acid solutions but are destroyed by alkali and reducing agents. These compounds possess a distinctive absorption spectra because of the presence of naphthaquinone ring system.

**Table 17 Comparative Potency of Various Members of the Phylloquinone and Menaquinone Families of Vitamin K**

| Side Chain Length (# carbons) | Family | |
|---|---|---|
| | Phylloquinone | Menaquinone |
| 10 | 10 | 15 |
| 15 | 30 | 40 |
| 20 | 100 | 100 |
| 25 | 80 | 120 |
| 30 | 50 | 100 |
| 35 | — | 70 |

*Note:* Phylloquinone, $K_1^{20}$, is given a value of 100 and the remaining compounds are compared to this compound with reference to its biological function in promoting clot formation.

## E. Chemical Assays

Many different assays have been proposed for vitamin K but they all face the same problem: vitamin K is normally present in very low concentrations and numerous interfering substances such as quinones, chlorophyll, and carotenoid pigments are usually present.

Several colorimetric methods have been developed. One of the first, known as the Dam-Karrer reaction, is the measurement of the reddish-brown color that forms when sodium ethylate reacts with vitamin K. In another method, menadione, as the sodium sulfite, is reduced and then titrated to a green endpoint with ceric sulfate. In yet another, menadione is converted to its 2,4-dinitrophenylhydrazone when heated with 2,4-dinitrophenylhydrazine in ethanol. An excess of ammonia causes the solution to become blue-green with an absorption maxima at 635 nm.

As mentioned earlier, the K vitamins have a characteristic ultraviolet absorption spectrum. If the material is sufficiently pure, it can be identified and quantitated. Vitamin K has also been quantitated by a thin-layer and gas chromatographic technique. The menadione content of foodstuffs as been determined with high performance liquid chromatography (HPLC). This technique has been used to determine the blood and tissue levels of the vitamin in humans. As little as 0.5 mmol/l has been detected in the blood of newborns and adults. Regardless of the assay method used, the analytical procedure should include protection of the samples from light. All of the vitamers are sensitive to ultraviolet light and will decompose if exposed. Care also should be exercised with respect to pH. The K vitamers are sensitive to alkali but are relatively stable to oxidants and heat. They can be safely extracted using vacuum distillation.

## F. Bioassays

One of the earliest biotechniques for measuring vitamin K content of foods uses the chick. This method is sensitive to 0.1 μg phylloquinone per gram of diet. In this assay, newly hatched chicks are fed a K-free diet for 10 days and thus made deficient. They are then fed a supplement containing the assay food. The prothrombin level of the blood is then compared with a standard curve resulting from the feeding of known amounts of phylloquinone.

Instead of measuring prothrombin concentration, plasma prothrombin times can be measured. Prothrombin time is an indirect and inverse measurement of the amount of prothrombin in the blood: an increase in prothrombin time signifies a decrease in prothrombin concentration. The technique commonly used is a modification of the one-stage method developed by Quick. Blood removed from a patient or animal is immediately oxalated; oxalate binds the calcium and prevents prothrombin from changing into thrombin. Later, an excess of thromboplastic substance (obtained from rabbit or rat brain) and calcium are added to the plasma and the clotting time of the plasma

is noted; this time is the prothrombin time. The normal prothrombin time is approximately 12 s; however, the actual time depends to a large extent on the exact procedure employed. To be valid, the "pro time" of a vitamin K-deficient animal must be compared to that of a normal individual.

## G. Biosynthesis

Although not too much is known about the biosynthesis of phylloquinone in plants, apparently the synthesis occurs at the same time as that of chlorophyll. The vitamin concentration is richest in that part of the plant that is photosynthetically active: carrot tops are a good source, but not the root; peas sprouted in light contain more than peas sprouted in the dark; and the inner leaves of cabbage have about one-fourth less vitamin than the outer leaves.

Menaquinone is synthesized by intestinal bacteria in the distal small intestine and in the colon. Martius and Esser found that chicks fed a vitamin K-deficient diet and then given menadione ($K_3$) for long periods of time will synthesize menaquinone ($K_2^{20}$). Apparently, this synthesis is more complete at small physiological doses than at high doses, such as are used when vitamin K is given as an antidote to dicumarol. Because of intestinal biosynthesis, the experimental animals used in K deficiency studies should be reared in cages that prevent coprophagy.

All of the natural forms of the K vitamins can be stored in the liver. Menadione is not stored as such but is stored as its conversion product, menaquinone. Menadione metabolism by the liver occurs at the expense of the redox state. When menadione is metabolized by $Ca^{2+}$-loaded mitochondria, there is a rapid oxidation and loss of pyridine nucleotides and a decrease in ATP level. The effects of menadione on $Ca^{2+}$ homeostasis are probably initiated by NAD(P)H: (quinone-acceptor) oxido-reductase. Since it is an oxidant, large amounts of menadione have been shown to alter the surface structure and reduce the thiol content of the liver cell. Because of these changes, menadione is cytotoxic in large quantities. This may explain the induction of jaundice in the newborn of mothers given large doses of menadione just prior to delivery. This once popular obstetric practice has been discontinued.

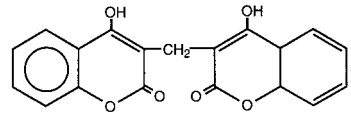
Using labeled vitamin, it was found that rats stored the vitamin in the liver — 50% of the vitamin was found bound to the endoplasmic reticulum. When cis and trans forms were compared, the biologically active trans isomer was found in the rough membrane fraction (endoplasmic reticulum) whereas the inactive cis isomer was found in the mitochondria.

## H. Antagonists, Antivitamins

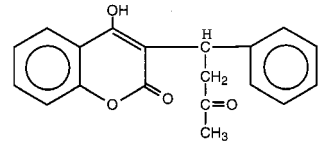
Blood coagulation can be inhibited by a variety of agents that are clinically useful. Oxalates, heparin, and sodium citrate are but a few of these. These compounds work by binding one or more of the essential ingredients for clot formation. One group of anticoagulants are those which act by antagonizing vitamin K in its role in prothrombin carboxylation. These compounds, shown in Figure 17, are members of a quinone family of compounds. The first of these is 3,3′-methyl-bis-(4-hydroxycoumarin), called dicumarol. It was isolated from spoiled sweet clover and was shown to cause a hemorrhagic disease in cattle. Dicumarol has been found very useful in the clinical setting as an anticoagulant in people at risk for coronary events. It is marketed as Coumarin. Another use is as a rodenticide, marketed as Warfarin®. The structure of Warfarin is also shown in Figure 17. A third group of antivitamin K compounds are the 2-substituted 1,3-indandiones. These are useful as rodenticides but because they can cause liver damage they are not used in the clinical setting.

## I. Sources

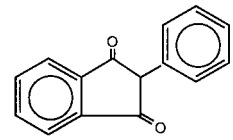
Phylloquinone serves as the major source of dietary vitamin K in humans. Alfalfa has long been recognized as being most plentifully supplied with vitamin K. In general, studies of the vitamin K content of common foods, as determined by the chick bioassay method, reveal that green




Dicumarol
(Coumarin)




Warfarin




2 phenyl-1,3-indandione

**Figure 17** Structures of several compounds that are potent vitamin K antagonists.

leafy vegetables contain large quantities of vitamin K, meats and dairy products intermediate quantities, and fruits and cereals small quantities.

## J. Absorption

Under normal physiologic conditions, most nutrients are absorbed before they reach the colon. In large measure, this is true of the K vitamins. However, vitamin K can be absorbed very well by the colon. This is an advantage to the individual since it ensures the uptake of K that is synthesized in the lower intestinal tract by the gut flora. The mechanism by which the K vitamins are absorbed, and the rate at which this occurs, is species dependent. Species such as the chicken, which have a rapid gut passage time, absorb the vitamin more rapidly than do species such as the rat which have a long gut passage time. The absorption of $K_1$ and $K_2$ analogs is generally thought to occur via an active, energy-dependent transport process, whereas $K_3$ (menadione) analogs are absorbed by passive diffusion.

The absorption of $K_1$ and $K_2$ requires a protein carrier and, again, species differ in the saturability of the carrier. In rats, the carrier is saturated at far lower vitamin concentrations than occurs in chickens. These differences in carrier saturability led early investigators to suggest that vitamin K absorption was a passive process. Subsequent studies using *in vitro* techniques or using labeled vitamin given *in vivo* showed that absorption was indeed an active process.

In contrast to the absorption of phylloquinone and the menaquinones, menadione appears to be absorbed primarily in the large intestine where the gut bacteria have converted it to a form with a side chain. Without the side chain the absorption of $K_3$ is a passive process. Biosynthesis by the gut flora is an adequate source of biologically active vitamin K under normal conditions, thus making it difficult to obtain a K deficiency in humans and experimental animals. However, under conditions of stress, such as hypoprothrombinemia induced by coumarin-type anticoagulants, intestinal synthesis does not produce enough of the vitamin to overcome the effects of the drug. Proof of the importance of colonic absorption is seen in the improvement in prothrombin times in chicks

and infants when given the vitamin rectally. Excessive intakes of menadione can be harmful due to the quinone structure, which is an oxidant. Quinones can uncouple oxidative phosphorylation.

Absorption of the K vitamins is dependent on the presence of lipid which stimulates the release of bile and pancreatic lipases. As lipids are absorbed into the lymphatic system, so too are the K vitamers. If there is any impairment in the lipid absorption process, less vitamin K will be absorbed. For example, patients with biliary obstruction have been shown to absorb substantially less vitamin K than normal subjects.

Phylloquinone absorption shows a diurnal rhythm. In rats, the highest rate of absorption is at midnight; the lowest is at 6 a.m. This coincides with the rats' eating pattern. The rat is a nocturnal feeder consuming most of its food between 8 p.m. and midnight. Estrogen enhances the absorption of phylloquinone for both intact and castrated males. Castrated rats are more susceptible to uncontrolled hemorrhage due to coumarin than are intact female rats. Female rats also synthesize more prothrombin than do male rats.

## K. Metabolism and Function

Historically, vitamin K has been regarded as a vitamin with a single function: the coagulation of blood. While the concept of this function is true, we now know that it serves as an essential cosubstrate in the post-translational oxidative carboxylation of glutamic acid residues in a small group of proteins, most of which are involved in blood coagulation. These proteins are the blood clotting factors II, VII, IX, and X, a calcium-binding bone protein (bone Gla protein), osteocalcin, and plasma proteins C and S.

Blood coagulation is not a single one-step phenomenon. Rather, it involves several phases which must interdigitate if a clot is to be formed. Four phases have been identified: (1) the formation of thromboplastin, (2) the activation of thromboplastin, (3) the formation of thrombin, and (4) the formation of fibrin. Following injury, a blood clot is formed when the blood protein fibrinogen is transformed into an insoluble network of fibers (fibrin) by the reaction cascade illustrated in Figure 18. The change of fibrinogen to fibrin (phase 4) is catalyzed by thrombin which itself must arise from prothrombin. Prior to activation by the protease factor Xa, both the prothrombin and the protease are adsorbed onto the phospholipids of the damaged cells by way of calcium bridges. Without carboxylation, these bridges will not form and the adherence or adsorption of the prothrombin to the phospholipids of the injured cell walls does not take place. The phospholipids are not only important to the binding of prothrombin to the injured cell wall, but are also important determinants of carboxylase activity. Phosphatidyl choline has been found to be an essential component of the carboxylase enzyme system. When depleted of phospholipid the enzyme loses activity; when repleted, its activity is restored. The synthesis of thrombin from prothrombin (phase 3) is catalyzed by prothrombinase, active preaccelerin, and the phospholipid, cephalin. These factors, in turn, are activated by a combination of blood and tissue convertins which represent or reflect the synthesis and activation of the thromboplastin complex (phases 1 and 2). The synthesis and activation of the thromboplastin complex requires several factors which have been named and identified. These factors, studied by different groups, were given different names, and these different names lent considerable confusion to the understanding of the coagulation process. To clarify the literature, it was decided by the International Committee for Standardization of the Nomenclature of Blood Clotting Factors to recommend the use of a numerical system for designating the various factors. This numbering system is given in Table 18 along with some of the other names in use and the function of each in the coagulation process. Note that four of the factors are proteins whose synthesis is dependent on vitamin K. These proteins, prothrombin (factor II), proconvertin (factor VII), the Christmas factor (factor IX), and the Stuart-Prower factor (factor X) are all calcium dependent. That is, calcium ions must be present for their activation and participation in the coagulation cascade (Figure 18). Note, too, that the four proteins which are vitamin K dependent are synthesized in the liver, hence the reason why the liver concentrates and stores this vitamin.

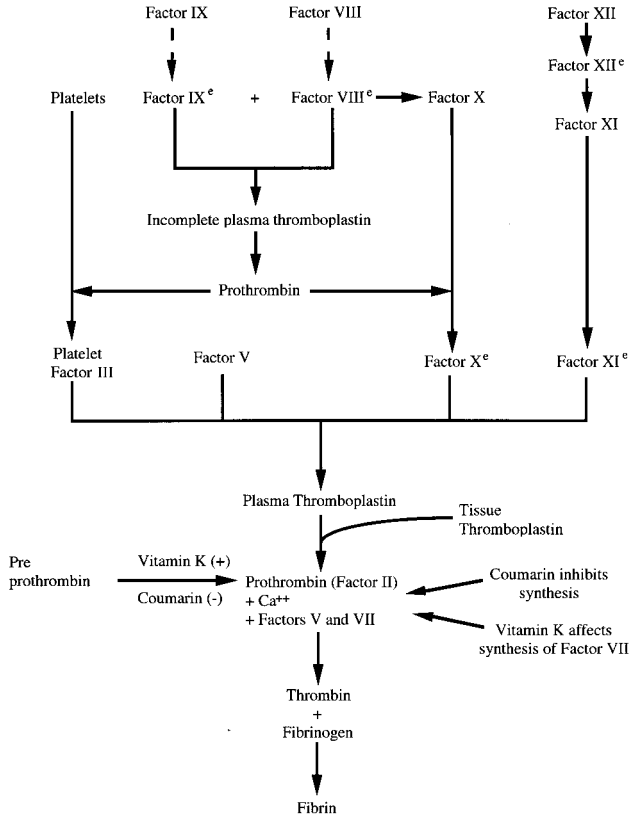



**Figure 18** Formation of a clot.

The function of vitamin K is as a cosubstrate in the post-translational oxidative carboxylation of proteins at selected glutamic acid residues. This is a cyclic process (Figure 19). As the vitamin serves as a cosubstrate, it is metabolized by the hepatic microsomes. There are two pathways for vitamin K reduction: (1) the epoxi-quinone cycle, and (2) DT diaphorase and microsomal dehydrogenase. The latter pathway is important for counteracting coumarin toxicity. The metabolism of vitamin K is dependent on adequate intakes of niacin and riboflavin, which are components of the redox systems important in the transfer of reducing equivalents. Vitamin K is reduced to the hydroquinone (vitamin $KH_2$) with NADH as the coenzyme (NADH is a niacin-containing coenzyme). The K quinone reacts with $CO_2$ and $O_2$ in the microsomal carboxylation of glutamyl residues and is then oxidized to form an epoxide. The epoxide is converted back to the quinone form by an epoxide reductase. The reductase is a two-component cytosolic enzyme which catalyzes the reduction of the vitamin K epoxide using a thiol reductant (dithiothreitol *in vitro*) as either a primary or secondary source of reducing equivalents. Warfarin® (coumarin), an antivitamin that binds to the epoxidase and reductase, interferes with both the reduction of vitamin K to the hydroquinone and the conversion of the epoxide back to the original compound.

Vitamin K epoxide can substitute for vitamin K in glutamate carboxylation and prothrombin synthesis. This similarity is probably due to the unhindered conversion of the epoxide to the hydroquinone. The epoxidation reaction is coupled to the carboxylation of the glutamic acid residues protruding from peptides having clusters of this amino acid. Aspartyl residues can also be carboxylated. The epoxidation reaction can be coupled to the oxidation of other peptides as well; however, only those having the clusters of glutamic acid will be oxidatively carboxylated. Unless the proteins

**Table 18 Blood Clotting Factors**

| Factor | Name | Function | Remarks |
|---|---|---|---|
| I | Fibrinogen | Provides the structural network upon which clot is formed. | |
| II | Prothrombin | Precursor of thrombin, a proteolytic enzyme which causes fibrinogen to lose one or more peptides and polymerize. | Vitamin K dependent, synthesized in liver. |
| III | Thromboplastin | Serves to stimulate prothrombin conversion to thrombin. | Not a single compound. Includes factors IX, XI, XII, X, V. |
| IV | Calcium | Combines with prothrombin in presence of agents. | Oxalate acts as an anticoagulant by binding $Ca^{++}$. |
| V | Labile factor proaccelerin accelerator (Ac) globule | Has thromboplastic activity. Released by collagen and not ADP; proceeds normally when prostaglandin synthesis is inhibited. | Disappears when plasma is heated or stored with oxalate. In the activated form, may function as the Ka receptor on the platelet membrane. |
| VII | Proconvertin serum prothrombin conversion accelerator (SPCA), cothromboplastin, autoprothrombin I. | Has thromboplastic activity. | Vitamin K dependent. |
| VIII | Antihemophilic factor, antihemophilic globulin(AHG) | Activates factor X; associated with platelet membranes. May have a role in the adherence of platelets to subendothelium. | Deficiency of this factor is the cause of the classic form of hemophilia A. The vonWillebrand factor is a carrier of factor VIII. |
| IX | Plasma thromboplastic component (PTC), Christmas factor | Stimulates conversion of prothrombin to thrombin. | Vitamin K-dependent deficiency results in hemophilia or Christmas disease, named after first patient to have disease. |
| X | Stuart-Prower factor | Stimulates conversion of prothrombin to thrombin. | Vitamin K-dependent glycoprotein. Gene for human factor X is essentially identical with that for factor IX and Protein C[162a]. |
| XI | Plasma thromboplastin antecedent (PTA) | Has thromboplastic activity. | Patients with PTA are mild hemophiliacs. |
| XII | Hageman factor | Has thromboplastic activity. | Deficiency does not result in hemophilia. |
| XIII | Laki-Lorand factor (LLF) Fibrin stabilizing factor | Stabilizes fibrin network; is a proenzyme in the plasma activated by thrombin and which catalyzes the formation of covalent lysyl bonds between the chains of fibrin. | |
| | Platelet thromboplastic factor | Catalyzes formation of platelet factor. | Appears when platelets disintegrate. |

From Walsh, P.N., *Fed. Proc.,* 40:2086, 1981. With permission.

are carboxylated they are unable to bind calcium. For example, the precursor of prothrombin, acarboxyprothrombin has, within the first 33 amino acids at the amino terminal end, 10 tightly clustered glutamic acid residues and binds less than 1 mol of calcium per mole of protein. When carboxylated, the glutamic acid residues are converted to carboxyglutamic acid residues, and now each mole of the prothrombin protein can bind 10 to 12 mol of calcium. Thus, the carboxylated glutamic acid-rich region serves an important function in clot formation.

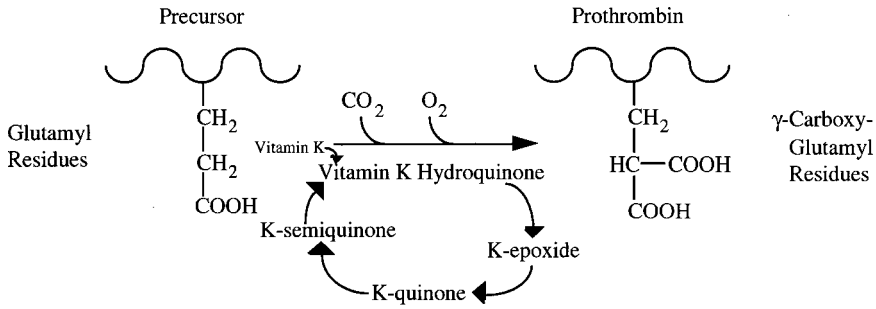



**Figure 19** The vitamin K-dependent carboxylation reaction coupled with the epoxi-quinone cycle.

Proteins other than those involved in the coagulation process have also been shown to be vitamin K dependent. Carboxylated proteins have been found in bone matrix. Up to 20% of the noncollagenous proteins (or 1 to 2% of the total bone protein) is a carboxylated protein called osteocalcin. Osteocalcin is synthesized in bone tissue. Bone microsomes are responsible for the post-translational vitamin K-dependent oxidative carboxylation reaction. The synthesis of osteocalcin is highest during rapid growth periods and coincides with detectable bone mineralization. The osteocalcin appears prior to mineralization and, like the blood coagulation proteins, shows a remarkable avidity for calcium. It will also bind (in order of preference after calcium) magnesium, strontium, barium, and lanthanide. In addition to its ability to bind divalent ions, osteocalcin also binds strongly to hydroxyapatite, the major calcium phosphate salt of the bone. Thus, osteocalcin serves a major role in bone mineralization and this role can not be performed unless its glutamic acid residues have been carboxylated.

Studies of embryonic chicks treated with Warfarin® showed a decrease in both osteocalcin and mineralization. Warfarin-treated animals, in contrast, do not show this same effect; osteocalcin levels are low but bone mineralization is normal. Because the overwhelming effects of vitamin K deficiency induced via Warfarin treatment results in lethal bleeding, the secondary effect on bone mineralization is not observed unless embryonic tissues are used. Evidence of Warfarin injury has been reported in humans. Mothers consuming coumarin during their pregnancy have given birth to infants having defective bone development such as stippled epiphyses, “saddle” nose, punctate calcifications, and frontal bossing.

Another vitamin K-dependent protein involved in the regulation of bone calcium homeostasis is bone Gla protein (BGP). It is a 49-residue protein secreted by osteosarcoma cells having an osteoblastic phenotype and appears in calcifying tissues 1 to 2 weeks after mineral deposition, and at the approximate time that the maturation of bone mineral to hydroxyapatite is thought to occur. The synthesis and secretion of BGP from osteosarcoma cells is regulated by vitamin D. The renal cortex also contains a microsomal vitamin K-dependent oxidative carboxylation system. Its function is to produce a γ-carboxy-glutamic acid-rich protein which serves to bind calcium. It is located in the renal tubule and may function importantly in the conservation of calcium. Unfortunately, these proteins may be entirely too active in calcium uptake and kidney stones may result. Renal stones have been found to contain a unique carboxylated peptide in addition to calcium oxalate. Whether this peptide was responsible for the stone accumulation or was attracted to it because of its high calcium content is not known.

In summary, then, vitamin K functions as a cosubstrate in the production of unique calcium-binding proteins in the blood, bone, and kidney. Further research may reveal the presence of other K-dependent proteins which serve to translocate the calcium ion from one cellular compartment to another. However, evidence of their existence is not now in hand.

In addition to its function as described above, vitamin K serves a role in the inhibition of the carcinogenic properties of benzopyrene. Menadione inhibits aryl hydrocarbon hydroxylase, thus reducing the levels of carcinogenic and mutagenic metabolites in the cell with a resultant reduction in tumor formation. Vitamin $K_1$ and $K_2$ have the opposite effect. The different effects probably relate to the previously described effects of these forms on redox state, calcium ion status, and ATP production — all of which are involved in tumorogenesis.

## L. Deficiency

Due to the fact that intestinal synthesis of vitamin K usually provides sufficient amounts of the vitamin to the body, primary vitamin K deficiency is rare. However, secondary deficiency states can develop as a result of biliary disease which results in an impaired absorption of the vitamin. Deficiency can also occur as a result of long-term broad-spectrum antibiotic therapy, which may kill the vitamin K-synthesizing intestinal flora, or as a result of anticoagulant therapy using coumarin (Warfarin®) which, as shown in the previous section, interferes with the metabolism and function of the vitamin. The primary characteristic of the deficiency state is a delayed or prolonged clotting time. Deficient individuals may have numerous bruises indicative of subcutaneous hemorrhaging in response to injury. A very sensitive test for deficiency status has been developed. This test uses the measurement of under-γ-carboxylated prothrombin as determined by immunochemical detection and by a decreased urinary excretion of γ-carboxyglutamic acid. This test could be quite useful in assessing not only vitamin K status but also the efficacy of anticoagulant therapy. The objective of such therapy is to slightly impair clotting to prevent vascular occlusions but yet avoid hemorrhages. However, there may be consequences to such therapy.

In women, osteoporosis as well as atherosclerosis are major health concerns. The former has been thought to be related to the loss of estrogen action on bone homeostasis, while the latter involves calcium deposits in the fatty streaks of the vascular system. Although these two conditions may be independent, there is presently some thought that they are not. Indeed, they may be linked via the action or loss of action of vitamin K in the maintenance of calcium homeostasis. Some Dutch workers have shown that markers of bone formation increase whereas bone resorption markers decrease with the administration of pharmacological doses of vitamin K. In addition, these scientists have reported the presence of abdominal aorta atherosclerotic calcifications associated with marginal vitamin K status, as well as reduced bone mass. These observations suggest that there is a relationship between the two processes.

The bone Gla protein is similar to matrix Gla protein (MGP) which inhibits bone mineral resorption as well as inhibits vascular mineralization. Likely, MGP synthesis is vitamin K dependent, and likely its deficiency is the coordinator of the two disorders, osteoporosis and atherosclerosis.

Newborn infants, because they have not yet established their K-synthesizing intestinal flora, have delayed coagulation times. For many years, it was common practice to give the mother an injection of menadione just prior to delivery in order to rectify the newborn's problem. However, it then became apparent that this prophylactic therapy was having no effect on the infant with respect to prothrombin time and, in addition, some infants became jaundiced with this treatment. As a result, vitamin K administration to women just prior to delivery is no longer a routine practice in obstetrics.

## M. Recommended Dietary Allowance

Although the gut flora can usually synthesize sufficient vitamin to meet the nutritional need, the Food and Nutrition Board of the National Academy of Science, National Research Council has made recommendations for a daily intake. These are shown in Table 19.

**Table 19 Recommended Dietary Allowances (RDA) for Vitamin K**

| Group | Age | RDA (μg) |
|---|---|---|
| Infants | 0–6 months | 5 |
| | 7–12 months | 10 |
| Children | 1–3 | 15 |
| | 4–6 | 20 |
| | 7–10 | 30 |
| Males | 11–14 | 45 |
| | 15–18 | 65 |
| | 19–24 | 70 |
| | 25–50 | 80 |
| | 51+ | 80 |
| Females | 11–14 | 45 |
| | 15–18 | 55 |
| | 19–24 | 60 |
| | 25–50 | 65 |
| | 51+ | 65 |
| Pregnancy | — | 65 |
| Lactation | — | 65 |

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## Vitamin K


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