CHAPTER - 1

INTRODUCTION
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During the nineteenth century, scientists found that the main constituents present in different foods were carbohydrates, fats, proteins, minerals and water. At the beginning of the 20th century, feeding experiments were conducted on mice by some physiologists, with synthetic diets, based on blends of pure carbohydrates, proteins, fat and mineral salts. The results showed that animals would not grow on such synthetic diets. It was evident that natural foods contained certain unknown substances which are essential for the growth of animals. These were called vitamins. It was recognized quite early that vitamin-A had an unique anti-infective property (1), but its linkage to immunological mechanisms remained unknown for many years.

Discovery of vitamins and increasing knowledge about their role in nutrition aroused much medical and public interest in 1920s and 1930s. Before the discovery of antibiotics, the outcome of severe infections often depended on the excellence of supportive care, which included a fully nutritious diet.

One of the earliest associations with dietary deficiencies and pathology is probably the link between the curative effects of liver applied to the eyes of night-blind Egyptians during the time of Pharaohs. Later in 1892, it was reported that children who suffered from measles or whooping cough were likely to develop xerophthalmia. In 1925, Wolbach and Howe (2) showed that rats fed diets devoid of retinol had thymic atrophy. In 1930 (3), it was shown that dietary carotenoids could protect against bacterial infection. In
1932, Ellison (4) reported that measles, which infects the respiratory epithelium resulted in epithelial changes, similar to those seen in vitamin-A deficient animals.

**Nomenclature and some chemical structures**

With regard to retinoids, the IUPAC-IUB joint commission on biochemical nomenclature, has defined retinoids as "a class of compounds consisting of four isoprenoid units, joined together in a head to tail manner. All retinoids may be formally derived from monocyclic parent compound containing five carbon double bonds and a functional group at the terminus of the cyclic position. To avoid confusion with previously used names in this field, no parent hydrocarbon is named (5).

In recent years, the term retinoid has been taken to include both the naturally occurring compounds with vitamin-A activity and synthetic analogs with or without biological activity of retinol (vitamin-A). Carotenoids are not included in this group of compounds. The commission further recommended that the term vitamin-A should be used as a generic descriptor for retinoids that exhibit qualitatively the biological activities of retinol. This term should be used in derived terms, such as vitamin-A activity, vitamin-A deficiency and vitamin-A antagonist.

**Chemical structures**

All trans-retinol (vitamin-A) alcohol is the parent vitamin-A compound, occurring mostly in the form of palmitic acid ester.
All trans-retinol

Retinyl Palmitate

Retinal

Retinoic acid
The numbering system is adopted by IUPAC system.

The aldehyde form, retinal, is found in the retina of the eye and the acid form, retinoic acid, is a metabolite and a highly active form of vitamin-A.

**Beta-carotene**

Vitamin-A is a unique vitamin in that it exists in the plant world only in the form of precursor compounds or provitamins, but principally as β-carotene. This is a member of a large class of naturally occurring carotenoids. Olson (8) listed about 50 compounds in the carotenoid family with vitamin-A activity.

Moore (9) demonstrated that rats fed massive amounts of carotenoids extracted from carrots deposited large amounts of vitamin-A in their liver. Karrer et al (10) established the chemical relationship between β-carotene and vitamin-A.

Carotenoids act as antioxidants by quenching free radicals (11). They also modify the host immune system by increasing the response of T-lymphocytes or thymocytes to PHA (12, 13). Since, β-carotene increases the population of natural killer cells and T-helper cells, these cells, may be useful in acquired immuno deficiency syndrome (AIDS), where immune cells are low in number and defective in nature (14). Trial experiments with carotenoids have shown that they inhibit the replication of AIDS virus (15, 16). β-carotene has been shown to reduce the risk of cancer (17, 18), increase resistance to bacterial infection (19), protect against photosensitivity associated with genetically inherited skin diseases, such as erythropoietic
protoporphyria (20, 21). Numerous reports have demonstrated the ability of carotenoids to modulate cell proliferation, differentiation and transformation to neoplasia (22-24). \( \beta \)-carotene has been shown to help in the secretion of a novel cytokine with cytotoxic capacity, when human peripheral blood mononuclear cells are cultured with \( \beta \)-carotene (25). Carotenoids are present in human skin and their concentrations are decreased following the exposure to UV light (26-28). Epidemiological survey has shown that individuals with high carotenoid intakes have low risk of developing cataracts (29).

**Absorption of carotenoids**

Little is known about the efficiency of intestinal absorption of provitamin-A carotenoids, like \( \alpha \), \( \beta \), \( \gamma \) isomers and cryptoxanthin and their conversion to retinol. Carotene is absorbed by passive diffusion and in humans between 5 and 50\% is absorbed (30). Absorption requires the presence of fat and bile salts to form micelle. It is a process of passive diffusion (31), presence of fatty acids promotes the split of \( \beta \)-carotene to retinal. It is generally assumed that in humans consuming a normal diet, one-sixth (on a weight basis) of dietary \( \beta \)-carotene and one-twelfth of other pro-vitamin carotenoids are absorbed and converted to retinol in enterocytes (32). The conversion of \( \beta \)-carotene to retinol is an important biological process. Although retinol has been synthesized chemically by several procedures in recent years (33), human requirement of vitamin-A depends largely on dietary sources of provitamins. After intestinal absorption, carotenoids, including \( \beta \)-carotene are taken up by chylomicrons and enter the blood stream via the lymph, ultimately appearing in plasma in the low density lipoprotein
and finds its way into the adipose tissue, where it is stored (34). Absorption of carotenoids is an age dependent process, being more complete in adults than in children (35). Vitamin-E protects carotenes against oxidative destruction (36). The enzymatic mechanism responsible for intestinal conversion of β-carotene to retinol has been a subject of controversy (37, 38). Work by Dmitrovski (37) indicates that carotene may be cleaved, both centrally by 15, 15 dioxygenase as originally reported (39, 40), as well as peripherally by retinaldehyde reductase. It has been reported that retinal bound to the intestinal cellular retinol binding protein, may be reduced by a membrane bound microsomal enzyme (41).

Absorption, storage and metabolism of retinol

"Vitamin-A" is the term used for all compounds that exhibit biological activity of retinol, whereas "retinoids" is a term that includes the natural form of vitamin-A as well as many synthetic analogs of retinol, whether or not they have the biological activity. The main dietary source of vitamin-A are pro-vitamin-A carotenoids from vegetables, preformed retinyl esters and retinol from animal tissues.

Studies on retinol absorption began as early as 1960 (42). All that could be said at that time was that dietary retinyl esters undergo extensive hydrolysis in the lumen of small intestine of rats and other species, and that vitamin-A is absorbed through the lymphatic system, as its esters. Since then, considerable progress has been made regarding several aspects of absorption of vitamin-A. In the past, absorption of vitamin-A meant absorption of retinol and its esters, but now it includes absorption of retinal and retinoic acid also. Moreover, later work has shown that
vitamin-A is not absorbed through the lymphatic system alone. Significant amount is being absorbed through the portal route as well. Retinyl esters from the diet are hydrolyzed in the intestines before absorption into enterocytes. Pancreatic lipase has been assumed to be responsible for the hydrolysis, because patients with pancreatic insufficiency have reduced absorption of vitamin-A, ingested as retinyl palmitate (43). Ganguly (44) has suggested that retinyl ester hydrolase and cholesteryl ester hydrolase, are the two enzymes responsible for the hydrolysis of dietary retinyl esters and cholesteryl esters. These enzymes are found to be distinct from each other and from pancreatic lipase. Hydrolases are situated on the outer surface of the mucosal cell membrane, and are oriented in such a way that they can come into direct contact with its substrate. Recent work suggests that in rats, the long chain retinyl esters are hydrolyzed by an enzyme from the brush border of enterocytes (45). Retinol in physiological concentrations is apparently absorbed by facilitated diffusion, whereas at pharmacological concentration, it is absorbed by passive diffusion. Absorption of retinol is probably less than 75% and is dependent upon the quantity and quality of fat and vitamin-E in the diet (46). The actual form of vitamin-A entering the intestinal cells is retinol itself (47). The presence of free polyunsaturated fatty acids and retinoic acid, inhibits the absorption of retinol, possibly by competing with retinol for a carrier protein (48). Ong (49) found that retinol complexed to CRBP (II) was esterified by lecithin: retinol acetyltransferase and that CRBP (II) may therefore play a major role in the normal carrier-mediated absorption of retinol.
Uptake of chylomicron retinyl esters in peripheral tissues

Most retinyl esters present in the chylomicrons remain with the particles when they are converted to chylomicron remnants in the peripheral blood circulation. The rate of entry into the lymph vessels is increased by the presence of bile salts in high concentrations or at high pH. The retinyl esters (mainly palmitate) enter the lacteals of the intestine along with chylomicrons and are then carried to the liver via the lymph and thoracic duct (50). The liver clears the chylomicron remnants in which the retinyl esters are transported, bound to lipoproteins. Thus, for a short while after a meal, retinyl esters bound to lipoprotein are found in the blood before they are stored in the liver. Extra-hepatic uptake of remnants may be important in the delivery of fatty acids, sterols, retinol and carotenoids to adipose tissue, skeletal muscle, kidney and bone marrow (51, 52). Although the uptake into kidney is at first slow, the concentration of recoverable vitamin-A amounts to 12% in 72 hrs. The free retinol in kidney probably originates from the small amount of retinol bound to RBP (holo RBP) in plasma, that is not attached to pre-albumin. This moiety is filtered through glomeruli of the kidney and reabsorbed by the tubules, and after metabolic degradation of the RBP, appears as free retinol.

It has been reported that retinol content is increased in the bone marrow of man, after a large oral dosage of retinyl palmitate (53). Hussain et al (54) showed that retinol disappeared rapidly from the bone marrow of the marmoset.
In vitamin-A sufficient rats, most of the chylomicron remnant retinyl esters can be taken up by the hepatocytes, and appear to be rapidly (2-4 hrs) transferred as retinol to perisinusoidal stellate cells in the liver (Fig-1)

**Uptake of chylomicron-remnant retinyl esters by liver**

In mammals, 50-80% of the body’s total retinol is normally present in the liver (55, 56). Stellate cells contain about 90% of the liver total retinol, which is adequate to last for several months. Only when retinol concentration is very low in the liver (less than 1-5 nmole/g tissue) does the proportion of liver vitamin-A in hepatocytes become appreciable (57).

Once chylomicron-remnant retinyl esters have been taken up by liver parenchymal cells, a rapid hydrolysis takes place most probably catalyzed by a plasma membrane retinyl ester hydrolase (58).

Retinol binds to RBP, presumably in the endoplasmic reticulum, and RBP retinol is then transferred to the golgi compartment for secretion (59). This secretion is influenced by vitamin-A status: in vitamin-A deficient animals, secretion is reduced, such that plasma concentrations of RBP and retinol are decreased. However, vitamin-A repletion leads to an immediate increase in secretion of RBP-retinol from the liver (59). Once it was assumed that retinol leaves the blood plasma and is taken up by tissues and irreversibly utilized. However, Vahlquist (60) indicated that retinol may be recycled to the blood. This work was further verified by other workers in rats (61-63).
Fig-1 Vitamin-A absorption, transport, storage and metabolism

Storage of retinol

Liver accounts for 70-90% of body's retinol stores. Studies have shown that vitamin-A resides in parenchymal cell (hepatocytes) within 1 hr. After feeding labelled vitamin, only about 4% of the total stored retinyl ester was found in Kupffer cells (64). After this work, Wake (65) and Yamamoto et al (66) reported that although not in Kupffer cells, vitamin-A was stored in the fat storing or stellate cells of the liver. These cells contain lipid droplets and the number of droplets as well as their vitamin-A content increased with vitamin-A feeding (67). Chemical analysis of these lipid droplets from rat liver stellate cells revealed that 12 to 65% of the total lipid mass is retinyl esters, depending on the vitamin-A status of the animal. Triglycerols comprise between 35 to 50% of the lipid mass, which does not seem to be related to dietary fat or vitamin-A (55). Robinson and Kuwabara (68) found a dose related increase in the number and size of these droplets in mouse liver, while Popper (69) reported that these stellate cells were the last cells to lose vitamin-A fluorescence on depletion. Further studies (70) showed that when labelled retinyl ester was administered to a normal rat, the level of retinyl esters in parenchymal cells of liver elevated, while that in plasma dropped. After 2 hr, the level of labelled esters in non-parenchymal liver cells rose, while that of parenchymal cells fell. It implies that retinyl esters are initially transported from the plasma to parenchymal cells and then to non-parenchymal cells for storage. An interesting finding was that in vitamin-A deficient rats, the retinyl esters remained in the parenchymal cells and were not transported to the non-parenchymal cells (71).
The reduced accumulation of retinol in stellate cells of vitamin-A depleted animals may also be explained by reduced lecithin retinol acyltransferase (LRAT) activity (72). Retinoic acid was more potent than retinol for the rise in LRAT activity, which increased 10 times more in non-parenchymal cells than in parenchymal cells (73).

After liver, the second most important organ for vitamin-A storage is kidney. It is known that a portion of holo RBP, filters through the glomeruli and is reabsorbed by the tubules, where retinol is liberated, while RBP is degraded (60). Kidney not being a prime storage organ for retinol plays a negligible role in the initial uptake of retinyl esters arriving in the circulation with chylomicrons (51).

Another storage site for retinol is the pigment epithelium of the retina. Here, the retinol enters from the circulation as holo RBP and becomes esterified before being stored in the lipid droplets. In the rat 96% of vitamin-A of the retina is stored in phosphatidyl ethanolamine in ester form in equilibrium with retinol and retinal of the rods and cones. With increased intake of vitamin-A, there is a concomitant increase in the pigment epithelium lipid droplets containing retinyl esters (68).

Vitamin-E is essential for vitamin-A storage, both in the pigment epithelium and in liver. In a study, it was found that in rats maintained on a vitamin-A free and vitamin-A marginal diet showed liver vitamin-A levels of 10% of those maintained on a vitamin-E adequate diet and vitamin-A marginal diet (74).
In liver, stored vitamin-A esters are bound to a high molecular weight lipo-protein complex, first described by Heller (75). This complex is found in the soluble fraction of liver cytosol and contains 3% vitamin-A, of which 96% exists in the ester and 4% exists in the free retinol form. The lipo-protein complex not only stores retinyl esters but also has the capacity to hydrolyze them and transfer the liberated retinol to its serum carrier protein, RBP (76).

**Mobilization of retinol from storage sites**

**Retinol Binding Protein (RBP)**

The transport of lipo-soluble vitamin-A alcohol (retinol) from its storage sites to the target tissues is accomplished by a specific transport protein, the retinol binding protein (RBP) (77, 78). RBP is associated with thyroxine binding transthyretin in plasma. RBP is a single polypeptide chain of approximately 21 KDa, and has a binding site for retinol. The identification and partial characterization of retinal pigment epithelial membrane receptor for plasma RBP has been reported (79). Further studies have shown that retinol can also be released from its carrier to liposomes or to plasma membranes without interaction between RBP and cell surface receptors (80-82).

Retinol can also be dissociated *in vitro*, from RBP complex by treatment with organic solvents (83).

The removal of retinol from RBP leaves a larger empty space in the hydrophobic interior of the protein molecule, which trigger conformational changes in RBP (84). Recent data suggest that apo-RBP can bind to stellate cells, make a complex with stellate cell retinol and then be released into the circulation as RBP-retinol complex (85).
In a study, it was shown that approximately 50% of plasma retinol enters to kidneys, about 20% to liver and the remaining 30% to other tissues (86). Studies have further shown that many extrahepatic tissues including the kidneys, contain mRNA for RBP (87). Rats contain a considerable amount of body vitamin-A store and that adipose cells may produce and secrete retinol bound to RBP (88). Consequently, adipose tissue may contribute substantially to the body’s production and pools of RBP, and play an important role in recycling of retinol.

**Metabolism of retinol**

Studies have shown that retinoids are metabolized by different enzymes, when complexed with binding proteins (73, 89-92). Four steps are involved in the intracellular metabolism of retinol.

a) **Esterification of retinol**

Retinol is a fat soluble compound with hydrophilic hydroxyl group, which partitions into the membranes. To ensure its smooth transportation and function in the cells and to limit its concentration in the membranes, retinol is either bound to binding proteins or is esterified with long chain fatty acids. Enterocytes and mammary gland cells esterify retinol for transport to lymph and milk, whereas liver stellate cells and retinal pigment epithelial (RPE) cells, esterify retinol to store vitamin-A in lipid droplets. Lecithin: retinol acyl-transferase is the main intestinal enzyme esterifying retinol under normal conditions. However, acyl coA: retinol acyltransferase may be important when large doses of retinol enter the intestinal cells. The activity of
the enzyme lecithin retinol acyl transferase is low in retinol deficiency (93). The esterification of all trans retinol by LRAT in RPE cells is linked to the direct conversion of all-trans retinyl esters to 11-cis retinol by an isomerase (94).

b) **Activation of retinol to metabolites**

There are strong indications that the retinoids may alter gene expression at the transcription level and are derived from recently discovered group of transcription enhancing retinoic acid receptors (RARs) (95-99). Retinoid isomerization may be important not only in vision but also in transcription regulation, by binding to regulatory sequence termed as the RA response element (100-102).

It has been suggested that retinoic acid cannot substitute all effects of retinol in growth regulation. Retinol is metabolized by many cells to 14-hydroxy-4, 14-retro retinol (103). Retinoic acid synthesis occurs involving two distinct steps, (i) dehydrogenation of retinol to retinal by alcohol dehydrogenase and (ii) a further oxidation of retinal to retinoic acid.

c) **Retinoylation of cell proteins**

It has been reported that nuclear proteins in several cell lines are retinoylated *in vitro* via a thioester bond in a dose dependent manner (104). The number of retinoylated proteins is small, but includes important proteins like the regulatory sub-units of cAMP dependent protein kinase (105), cytokeratins (106), and ribonucleotide reductase (107).
Todate three proteins, growth hormones, laminin and retinoic acid have been identified whose synthesis is stimulated by RAR binding to RA and then to the hormone response element of genes (108).

d) Catabolism of retinol

Many metabolites of retinol are formed and some of them have been identified (109). Glucuronides, formed from retinol, are excreted in bile and urine (110). The major intermediate product of retinol catabolism is retinoic acid. Once formed, retinoic acid cannot be converted to retinal or retinol. Other retinoids include all trans retinoic acid, 12-cis-4-oxo retinoic acid and all trans retinoyl p-glucuronide (109-112). It is not known whether these retinoids simply reflect retinoid catabolism or whether they have a physiological role in vitamin-A action.

In the liver, retinol is conjugated with glucuronic acid and oxidized to the aldehyde and acid forms. The oxidized forms and the glucuronides are excreted in the bile and approximately 30% of retinol glucuronide is re-cycled back to the liver in an enterohepatic circulation of vitamin-A. Glucuronides remaining in the bowel are hydrolysed by enteric bacteria and excreted in the faeces (113).

Functions of retinol

Knowledge about retinol has advanced dramatically during the last few years. A major breakthrough came with the discovery of nuclear retinoid receptors that regulate gene expression by binding to short DNA sequence in the vicinity of target genes. Most of the extra-visual functions
of retinol seem to be mediated via these newly discovered receptors.

Compounds like retinoic acid and retinol are more like hormones than vitamins (114). Retinoic acid influences the pattern of early development and alters the process of cell differentiation. The diverse effects of retinoic acid reflects its regulation of specific proteins including various hormones and growth factor (115).

**Function in differentiation through action on cell nucleus**

One of the earliest observations concerning the function of vitamin-A was that it controls epithelial cell differentiation. In epithelial cell culture, the vitamin directs differentiation to diminish expression of stratified squamous epithelia (116, 117). Omri and Chytil (118) described the interaction of retinol with the genome with respect to mRNA. Another form of differentiation also involving vitamin-A is found in the development of the embryo.

**Action on cell surface**

Vitamin-A acts on cell membrane. Membrane is ultimately derived from endoplasmic reticulum and golgi apparatus by fusion with existing plasma membrane. One can assume that retinol has a major function in influencing cell membrane in general. This function cannot be attributed to retinoic acid. Yaar et al (119), showed that retinoic acid, when added to keratinocytes in culture, inhibited the S-S cross-linking in keratin and the differentiation of the cell membrane.
Function in glycosylation

Cell surface function of vitamin-A is likely to be mediated by the principal cell surface membrane constituents, the glycoproteins. Retinol plays an important role in glycoprotein synthesis. Glycoproteins are important as components of surfaces of epithelial cells and epithelial mucin (120). Vitamin-A maintains the receptor glycoproteins of lysosomal membranes (121).

Lotan et al (122) showed a stimulation by retinoic acid in the synthesis of fibronectin on the surface of a human carcinoma cell line. Sasak et al (123) found an increase in cell substratum adhesiveness with retinoic acid. This was accompanied by a change in glycoprotein. Karl and Gris (124) showed a stimulation in the secretion of androgen binding protein, a glycoprotein, from sertoli cells of rat testis in culture.

Most glycoproteins affected by retinol are found to be N-glycosidically linked. A necessary intermediate step in the formation of glycoproteins is the synthesis of oligosaccharide lipids. The mannose chains of oligosaccharides, linked to asparagine of the protein core, through two glucosamine units, are built up on dolichyl pyrophosphate. Dolichol is anchored to the lipid of the endoplasmic reticulum where mannose addition occurs which is mediated by retinol (125).

Function in the retina

The physiological function of vitamin-A is well known. George Wald (126) was awarded the Nobel Prize for medicine in
1967 for his discovery of the role of vitamin-A in the visual cycle. About 1% of our daily intake of vitamin-A is used by the retina. Vitamin-A alcohol is oxidized to vitamin-A aldehyde in the epithelium of the rods by alcohol dehydrogenase. In the visual function, vitamin-A is combined with a hexose and hexosamine containing protein opsin to form the visual pigment, called rhodopsin. Functionally 11-cis retinaldehyde is the active form of vitamin moiety of rhodopsin that combines with opsin.

In the visual cycle the incident light initiates a series of photo-chemical changes in rhodopsin. The re-synthesis of rhodopsin is isomer specific requiring the 11-cis isomer of retinene. In the dark the pigment is regenerated. In this process retinine aldehyde is isomerized back to the cis-form. Some of the vitamin is degraded in the overall process.

**Function in cell mediated immunity**

Retinol plays a significant role in maintaining the integrity of the mucous membrane, which serves as a barrier to infections. Retinoic acid was found to enhance natural killer cell activity (127). The increased activity of natural killer cells resulting from retinoic acid supplementation may be due to the alteration in the cell surface structure and an increase in cell surface receptor expression (128). Retinol also improves the function of macrophages. Improvement of phagocytic function of alveolar macrophages in particular was noted in rats, while retinoic acid improved the activity of peritoneal macrophages in mice (129).
Function in growth

Retinol has an important role in growth although the cellular mechanisms are only beginning to be understood (130). The retinoic acid receptor shares a common gene sequence with the thyroid hormone nuclear receptor (131). As a result retinoic acid may act in conjunction with thyroid hormone to regulate growth hormone (132). A metabolic link between growth hormone induced growth and retinol has been suggested (133). Retinol stimulates the growth of epithelial cells of skin, trachea and cornea in culture.

Function in reproductive system

Retinol is essential for normal reproduction in males and females. Retinol but not retinoic acid maintains general epithelium of spermatocytogenesis (134), whereas retinoic acid helps in the biosynthesis of testosterone and estrogen and in the process of fertilization and implantation.

Function as a free radical scavenger

Free radicals are highly reactive molecules that contain one or more unpaired electrons (135). These molecules can cause tissue damage by reacting with polyunsaturated fatty acids in cell membranes, nucleotides in DNA and sulphhydryl bonds in proteins (136). There is increasing evidence implicating free radicals in the pathogenesis of a number of infections (137).

The body's major defence mechanism against free radical damage is the anti-oxidants or free radical scavengers. One of the powerful scavenging agents is vitamin-A acting as an antioxidant (138).
Vitamin-A aids many biological functions in vivo, retinol supporting all of them. Retinaldehyde uniquely supports the visual cycle. Retinoic acid is the most potent of naturally occurring retinoids in promoting differentiation in several model systems.

**Retinol deficiency**

Vitamin-A or retinol is an essential nutrient for man and all mammalian species. Deficiency of this vitamin results in retardation of growth, reproduction, resistance to infections, and a host of mild to severe biochemical and clinical signs, depending upon the degree of insufficiency. Retinol deficiency is often associated with protein energy malnutrition, parasitic infestation and diarrheal disease. The synergistic effect of retinol deficiency and infection may be responsible for excessive childhood morbidity and mortality.

**Xerophthalmia, morbidity and mortality**

For many years the problem of retinol deficiency in developing countries emphasized the increased risk of xerophthalmia, which includes all ocular manifestations of vitamin-A deficiency (139). Green and Mellanby (140), showed that when animals were given retinol-free diet all the animals died with some infective or pyrogenic lesions.

Retinol deficiency is a major cause of childhood morbidity and mortality in the developing world (141). Retinol deficient children have a reduced antibody response to infections, which may be a possible cause of increased morbidity and mortality (142).
Host resistance to infections

A number of reports have appeared in the literature emphasizing the association between retinol deficiency and increased susceptibility to infectious diseases (143-145). Wolbach and Howe (2) showed that in retinol deficiency mucus producing epithelial cells were replaced by keratin producing cells. Recently it has been shown that the number of goblet cells in the epithelia of duodenum are reduced in retinol deficiency (146). Impaired antibody production has been shown in retinol deficient animals (147, 148). The impaired antibody response in retinol deficiency could be the result of a defect in either B-cells, T-cells or both (149). In vitro studies of cell mediated immunity have shown that mitogenic responses are reduced in retinol deficiency (150). Retinol deficient mice showed delayed hypersensitive responses to the skin contact antigen (151). Decrease in the number of B-cells has been found in the spleen of retinol deficient rats, which also suffered from protein energy malnutrition (152). Retinol deficiency results in lower weight of the bursa of Fabricus indicating changes in the production of B and T cells (149). The T-cells of vitamin-A deficient mice produced excess interferon-gamma which inhibits B-cell growth and proliferation of T-cells that secrete interleukin-4 and interleukin-5 (153). Reduced interleukin-5 secretion decreases the development of eosinophils in retinol deficient mice (154, 155).

Weight growth

The link between retinol deficiency and growth was established as early as 1913 (156). Cartilaginous growth occurs within the epiphypseal growth plates of long bones and
compact bone remodelling is achieved by osteoclasts on bone surfaces. These processes determine the length and shape of the bones.

It has been shown that retinol deficient rats have inactive osteogenesis, dense calcification and irregular trabecular tunnelling in the epiphyses of individual long bones (157). Later studies also reported depressed endochondral activity and shorter bones (158). Vitamin-A metabolites have been shown in vitro, to act directly on bone resorption by osteoclasts (159).

Other lesions that appear at the weight plateau stage include an elevation in cerebrospinal fluid pressure, reduced visceral growth and loss of body fat (160).

**Mucosal surface**

Vitamin-A is important in maintaining the integrity of epithelial surfaces. In vitamin-A deficiency there is stratification of the cells, followed by squamous metaplasia and eventual keratinization (161). These changes are accompanied by widespread desquamation of cells and decreased mucous production which allow the infection to set in. Chandra et al (162) noted increased bacterial adhesion to respiratory epithelium among children with retinol deficiency. In a study by Ongsakul (163), it was observed that the kinetics of blood clearance of injected *E.Coli* and *in vitro* phagocytic activity of polymorphonuclear leucocytes were markedly reduced.
Other effects

It has been shown that retinol deficient rats have impaired DNA synthesis and reduced turnover of epithelial cells (164). Retinol deficiency also delays the biotransformation of amino pyrine to benzoic acid (165).

Retinol deficiency in patients with liver disease is very common (166). Fat malabsorption is a common feature in intestinal and pancreatic diseases and may be an important factor in causing retinol deficiency (167).

Retinol deficiency leads to decreased synthesis of glycoproteins (168) and lowers the activity of certain xenobiotic metabolizing enzymes (169).

Retinol deficient female animals can conceive but they resorb the foetus. In case of birds, Thompson et al (170) showed that eggs from retinol deficient hens, although fertile, failed to develop beyond first two days of fertilization.

Retinol deficiency is associated with a wide range of consequences from blinding xerophthalmia to apparent compromises in growth, decreased resistance to infection, ultimately leading to death.

Epidemiological survey

The importance of vitamin-A deficiency as the cause of blindness following xerophthalmia in many areas of the third world is well recognised. It is estimated that more than 10 million children develop xerophthalmia each year and half of
them become blind. It has been proposed that vitamin-A supplementation should be practised as a regular feature of the Expanded Programmes for Immunization of the World Health Organization (WHO), United Nations (International), UNICEF etc.

Studies have shown that widespread prevalence of retinol deficiency is due to an unsatisfactory vitamin-A nutritional status of the mother during pregnancy and lactation, habit of delayed supplementation and the poor vitamin-A content of supplementary foods on which the infants and children are weaned (171, 172).

Hypovitaminosis is a nutritional problem common to tropical and sub-tropical regions of the world. WHO has stressed the urgency of the problem in North-East Brazil and other developing countries and recommended combative measures at both the agricultural and public health levels.

Studies of Oomen et al (173) indicate a wide geographical distribution of vitamin-A deficiency. It is particularly associated more with inhabitants suffering from protein calorie malnutrition.

Vitamin-A deficiency is one of the most important public health problems in India. The incidence is particularly high among pre-school children belonging to poor income groups. Results of several diet surveys have shown that the dietary intake of vitamin-A is grossly inadequate in poor socio-economic groups and is a major etiological factor (174-176). Community studies have shown that retinol deficiency leads to urinary bladder stones, colon cancer, anaemia (177), measles (178) and decreased immunity (179).
Almost 15,000 to 40,000 morbidly obese people undergo jejuno-ileal bypass surgery in the United States alone. As a result of this, retinol deficiency is more prevalent among these people (180). Of the two studies conducted in India, one study (181) reported no significant reduction in mortality after high dose supplementation with vitamin-A, whereas another study (182), reported a 54% reduction in mortality in pre-school children receiving small weekly doses of vitamin-A.

**Effect of retinol deficiency on structure and function of membranes**

Apart from the visual function, retinol deficiency manifests its effect on the structure and function of cell membranes. Retinol deficiency leads to the degeneration of the myelin sheath, which is a constituent of nerve axon cell membranes, disrupts the stability of liver lysosomal membrane in rats (183). Roels (184) found that erythrocyte membranes of the rats were swollen and distorted in retinol deficiency. The lipid and the protein bi-layer structure of the cell membranes is affected due to retinol deficiency (185). Retinol deficiency alters the activity of drug metabolising enzymes located in the microsomal membrane (186). Retinol *in vitro*, increases the permeability and the fluidity of the natural and reconstituted membranes (187). Liver lysosomal membrane released more ribonuclease from retinol deficient rat (188). Retinol is essential for the normal functioning of rat kidney and plasma membranes (188). Mitochondrial membranes were found to be swollen in retinol deficient state (189). Membranes are the sites of biochemical actions for vitamins, especially retinol. Lipoprotein membranes of the cells are hence greatly affected by retinol deficiency as
it leads to structural changes within the membrane. The presence of retinol in cell membranes was confirmed by Mack (188).

Membrane bound enzymes

Every living cell has at least one membrane in order to differentiate it from its surrounding environment. Every eukaryotic cell has a membrane bound compartment which allows life processes to be segregated. Cell membranes are selectively permeable to the surrounding media. The need for cells to maintain a constant internal milieu is important for life. Cell membranes play an important role in separating cell’s content from its surrounding. Membranes create and maintain electro chemical gradient. All cellular membranes have a bilayer of amphiphilic lipid molecules where proteins are integrated. Lipids and proteins are usually capable of moving laterally within the plane of the membrane indicating that membranes are not static but dynamic entities. Such a motion is important for the transduction of signals across them. Enzymes form an integral part of cell membranes. Some of the enzymes associated with the cell membranes are Na⁺-K⁺ ATPase, alkaline phosphatase and 5’ nucleotidase.

Sodium Potassium Activated Adenosine Triphosphatase (Na⁺ K⁺ATPase)

A "Pump" is involved for the active transport of ions across the cell membrane. The "Pump" is driven by ATP hydrolysis e.g. H⁺ATPase, Na⁺K⁺ATPase etc (190). Na⁺ K⁺ATPase (E.C. 3.6.1.3) is a plasma membrane enzyme that catalyses the active transport of Na⁺ and K⁺ across the cell membrane. The pump works against the uphill cation gradient
and utilizes energy in the form of ATP. It is found virtually in all the cells of the animal kingdom.

The Na⁺K⁺ATPase or Na⁺K⁺ pump, extrudes sodium in exchange for potassium. In all the cells except erythrocytes, the cell membrane is more permeable to potassium than to sodium. Na⁺K⁺ATPase is inhibited by a class of drugs, the cardiac glycoside e.g. ouabain, that are elaborated as poisons. The Na⁺K⁺ATPase has been extensively studied because it is involved in cell physiology and is important for the treatment of heart failure and arrhythmias (191). It is important also because of its close link between the active transport of Na⁺ and K⁺, along with the transport of some sugars and amino acids. The pump is also a major energy utilizing process in the cell (192) and plays an important role in mitochondrial respiration. The "pump" is responsive to hormones e.g. insulin, aldosterone, ADH and thyroxine (193).

Na⁺K⁺ATPase has two protein sub-units, α and β. A small hydrophobic protein has also been proposed to be a part of the active complex (191). The α subunit contains the binding site for ATP and cardiac glycosides. It is generally referred to as the "catalytic sub-unit". The β subunit is a glycoprotein, which is required for the enzymatic activity. The β subunit has never been separated from the α subunit with irreversible loss of function. The Na⁺K⁺ATPase is integrated in the membrane with ATP binding site facing the cytoplasm, while ouabain binding site facing the extracellular space.

Coupled electro neutral movement of Na⁺, K⁺ and Cl⁻ occurs nearly in all tissue types including epithelia,
excitable tissue, red cells (194) and Ehrlich ascites (195, 196). Na⁺ K⁺ATPase is present in high concentrations in kidney (197) and brain (198). In the kidney the "pump" is responsible for resorption of cations (199), while it is responsible for nerve conduction in the brain (200).

Kaplay (201) reported that the ouabain sensitive Na⁺-K⁺ATPase is increased in erythrocyte membrane of children suffering from kwashiorkor and in the kidneys of protein energy malnourished rats. However, no such increase in Na⁺-K⁺ATPase activity was observed in patients with marasmus.

A potent mercury chelating component is present in Na⁺-K⁺ATPase enzyme which may be the target for mercurial diuretics and mercury-like inhibitors extracted from body fluids or tissues (202). It is reported that Na⁺ K⁺ATPase activity is decreased in rat kidney, brain and erythrocytes in alloxon diabetic rats (203). Na⁺ K⁺ATPase is also inhibited by antipsychotic drugs. Inhibition seems to be due to the changes in fatty acid composition of lipid and microviscosity of the membrane (204). Growth retardation of nutritionally dwarfed patients is associated with decreased erythrocyte Na⁺ K⁺ATPase activity, without other biochemical evidence of malnutrition (205). Inhibition of Na⁺ K⁺ATPase impairs the extrusion of sodium in exchange for potassium across the baso-lateral cell membrane leading to the accumulation of sodium and decrease of intracellular potassium activity to depolarization and accumulation of anions e.g. chloride (206), resulting in the swelling of the cell membrane (207). In the liver cell-volume is maintained evenly when the Na⁺K⁺ATPase is inhibited by ouabain, presumably due to extrusion of electrolytes and water by exocytosis (208).
The ubiquity of distribution and variety of functions attributed to ATPase suggests its basic importance in membrane function.

**ALKALINE PHOSPHATASE**

Alkaline phosphatase (E.C. 3.1.3.1) is a membrane bound enzyme and its activity is indicative of the membrane function (209). Alkaline phosphatase is an integral enzyme known to be intimately associated with the hydrophobic core of intestinal microvillus membrane. Its activity is assessed (followed) to study the compositional changes in membrane lipids, which affect the membrane functions (210).

Under certain nutritional stress conditions the activity of alkaline phosphatase is altered (211). Alkaline phosphatase is synthesized in the endoplasmic reticulum.

Phosphatases act as catalysis for the hydrolysis of alkaline phosphatases at high pH. It has been found that there is a low degree of homology between type 1 and type 2A protein phosphatases with alkaline phosphatase. (212, 213). On the other hand, significant homology exists between human placental and *E.coli* alkaline phosphatases (214). Soluble alkaline phosphatase in serum is utilized as a diagnostic tool for several disease. The enzyme has been characterized and its properties well studied (215). Alkaline phosphatase from bone, liver, kidney and intestine have been partially purified. Activity of urea-resistant neutrophil alkaline phosphatase is an effective maternal blood marker for Down’s Syndrome (216). Retinyl acetate increases the alkaline phosphatase activity in mouse skin when applied topically (217). Alkaline phosphatase activity may vary with age, sex
and hormonal status (218). Schiele et al (219) showed that liver and bone isoenzymes were higher in post menopausal women. Increased alkaline phosphatase activity has been reported in patients with diabetes mellitus (220).

Owing to its wide distribution, alkaline phosphatase is one of the important membrane marker enzymes.

**5’Nucleotidase**

5’Nucleotidase (E.C.3.1.3.5) plays an important role in physiological, biochemical and regulatory processes, hydrolyses purine and pyrimidine nucleoside monophosphates to their respective nucleoside and inorganic phosphate (221). 5’ Nucleotidase is a membrane bound enzyme, which flows from the endoplasmic reticulum to the plasma through Golgi apparatus (222). There is a constant exchange of this enzyme between the cell surface and the cytoplasmic membrane (223).

A deficiency of this enzyme leads to immune dysfunction (224). A decrease in 5’ nucleotidase activity was observed in elderly subjects (225). Administration of zinc has been shown to improve the activity of this enzyme (226). Sphingomyelin-rich bilayer membrane is reported to be rich in 5’nucleotidase activity (227). Phosphatase plays an important role in anchoring the enzyme to the membrane (228).

In an earlier study, it has been shown that in rats the activity of 5’ nucleotidase increases during myelination (229). Another study suggested that 5’nucleotidase is localized in the distal processes of oligodendroglia in the rat brain (230). The activity of 5’ nucleotidase has been associated with the maturity of the cells (231) and hormonal regulations (232).
All these observations suggest that 5' nucleotidase has immense physiological significance.

**Food Additives**

By definition, food additive is any substance added to food intentionally or non-intentionally, in small quantities to improve its appearance, flavour, texture or storage properties (233). These include dyes, pigments, colouring matter, spices or herbs. Spices were among the first food additives used not only to enhance the taste of the food, but also to disguise the taste of the spoiled foods (234). But now, spices are used to enhance the colour, appearance and taste of Indian curries, but even then some Indian curries are concocted with medicinal advantages in mind (235). Of all the spices, turmeric is one of the widely consumed spice and is well known for its medicinal properties. Curcumin, the active principle of turmeric exhibits a wide range of physiological and pharmacological properties (236). Curcumin is also used as a natural food colour.

**Curcumin structure**

![Curcumin structure]

**Chemical name:**
1,7 bis 4 hydroxy 3 methoxy 1,6 heptadiene 3,5 dione

**Chemical formula:**
\[\text{CH}_3\text{OC}_6\text{H}_3\text{CH: CO}\]_2\text{CH}_2
Composition of turmeric

Turmeric has a number of useful constituents. It has a high content of potassium. It also contains calcium, phosphorus, iron, vitamin A and nicotinic acid. An important constituent is curcumin, the pigment which imparts yellow colour to turmeric. Turmeric also contains some essential oils. The following Table shows the proximate composition of turmeric:

<table>
<thead>
<tr>
<th>Composition of turmeric (g per 100g) (237)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Moisture</td>
</tr>
<tr>
<td>Protein</td>
</tr>
<tr>
<td>Fat</td>
</tr>
<tr>
<td>Minerals</td>
</tr>
<tr>
<td>Fibre</td>
</tr>
<tr>
<td>Carbohydrates</td>
</tr>
<tr>
<td>Vitamin A equivalent</td>
</tr>
</tbody>
</table>

Turmeric is used for cleaning the wounds and pimples, washing inflamed eyes and to cure sprains. Apart from this, turmeric is also used as a home remedy for respiratory disease e.g. influenza, bronchitis, bronchial asthma, cold and cough. It is also prescribed for abdominal gas, fever, liver disease, urinary disease and to cure intestinal worms (238). Curcumin, the active principle of turmeric possesses antimicrobial and antioxidant properties (239). It is known for its hypocholesteremic and hypoglycemic activity in humans and rats (240). Till now, no such evidence exists regarding the use of spices for curing/combating the ill effects of retinol deficiency.
Foods containing pre-formed vitamin-A are expensive and hence are beyond the reach of the poor. Spices like turmeric are used all over the world, particularly in India by all socio-economic groups and because turmeric/curcumin and vitamin-A (retinol) share some of the common properties like being anti-inflammatory, antioxidant having conjugated structures and being lipid soluble, the present investigation was undertaken to study the efficacy of curcumin or turmeric in combating the ill effects of experimentally induced retinol deficiency.
SCOPE OF THE PRESENT INVESTIGATION

Retinol deficiency is a serious health problem primarily affecting the children in developing countries. Retinol supports vision, growth, cellular differentiation, bone development, reproduction and immunity. Retinol plays an important role in maintaining the activity of membrane bound enzymes.

Turmeric is one of the most commonly used spices in India. Curcumin, the active principle of turmeric, shares its anti-oxidant and anti-inflammatory properties with retinol. Moreover both retinol and curcumin have conjugated structure, and are lipid soluble. Apart from other beneficial effects of turmeric on health, the juice of turmeric is believed to cure purulent opthalmia.

The present study was undertaken based on the hypothesis that curcumin or turmeric may combat the ill-effects of retinol deficiency.

To study the effect of retinol deficiency on membrane bound enzymes, weanling male albino rats were made retinol deficient by feeding a synthetic diet devoid of retinol. Three microsomal enzymes viz., Na⁺ K⁺ATPase, alkaline phosphatase and 5' nucleotidase from liver, kidney, spleen and brain were studied. Results showed that retinol deficiency altered the activities of these enzymes. Chemical and physical probes like lectins, detergents and freezing and thawing reacted with the enzymes differently.
Retinol deficient membranes were more prone to lipid peroxidation and had reduced essential fatty acid levels.

The retinol deficient rats were fed 0.1% curcumin or 0.1% turmeric in the diet, to study whether curcumin or turmeric would revert some of the altered biochemical parameters, observed in retinol deficiency. The results revealed that curcumin or turmeric was able to revert the altered parameters closer to the normal values. Turmeric fed group showed a better response than the curcumin fed group.