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GUIDELINES FOR THE ERADICATION OF VITAMIN A DEFICIENCY AND XEROPHTHALMIA

VI. Recent Advances in the Metabolism and Function of Vitamin A and Their Relationship to Applied Nutrition

A REPORT OF THE INTERNATIONAL VITAMIN A CONSULTATIVE GROUP (IVACG)

The purpose of the International Vitamin A Consultative Group (IVACG) is to guide international activities aimed at reducing vitamin A deficiency in the world. The group offers consultation and guidance to various operating and donor agencies who are seeking to reduce vitamin A deficiency and its accompanying blindness. As part of this service, IVACG has prepared guidelines and recommendations for:

—Assessing the regional distribution and magnitude of vitamin A deficiency;

—Developing intervention strategies and methodologies to combat vitamin A deficiency;

—Evaluating effectiveness of implemented programs on a continuing basis so that the evaluation of the effectiveness of intervention techniques is a continuing and dynamic procedure;

—Research needed to support the assessment, intervention and evaluation of programs.

Other task force reports of the International Vitamin A Consultative Group (IVACG) which have been published in *Guidelines for the Eradication of Vitamin A Deficiency and Xerophthalmia* are:

I. Assessment of Vitamin A Status (1977)

II. Selection of Intervention Strategies (1977)

III. Evaluation of Selected Vitamin A Intervention Strategies (1977)

IV. Research Needs in Present and Future Vitamin A Programs (1977)

V. Recommendations of IVACG Concerning Research and Development Needs (1977)

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RECENT ADVANCES IN THE METABOLISM AND FUNCTION OF VITAMIN A AND THEIR RELATIONSHIP TO APPLIED NUTRITION

A REPORT OF THE
INTERNATIONAL VITAMIN A CONSULTATIVE GROUP
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INTRODUCTION

Vitamin A deficiency, often in association with protein-energy malnutrition, parasitic infestation and diarrheal disease, is a major nutritional problem among preschool children in many areas of the world. Quite apart from its molecular role in the light-sensing molecules in the eye, vitamin A is required for normal growth and reproduction, is involved in resistance to infection, and modulates the differentiation of epithelial cells. Acute vitamin A deficiency, although affecting many tissues of the body, is clinically and socially most significant in its disruption of the cornea of the external eye. By a series of little-understood changes, the surface of the cornea becomes dry and rough, and then ultimately breaks down partially or completely, leading to permanent scarring or irreversible loss of sight. In the world today, 20,000 to 100,000 young children go blind each year from a lack of vitamin A in their diets. A more precise figure is unavailable, since mortality is extraordinarily high among those who become blind.

Underlying the clinical signs leading to these tragic sequelae are a number of molecular changes, some well defined and some not, which result when vitamin A is absent from the diet. By knowing more about the chemical roles which vitamin A plays in living processes, we might understand why the lack of this vitamin causes the disruption of epithelial tissues, might define better ways of assessing vitamin A status, might better treat eye lesions caused by avitaminosis A, and might explain why certain kinds of nutritional deficiencies are associated together.

This sixth task force report of the International Vitamin A Consultative Group, therefore, has the rather ambitious objective of relating our current understanding of the absorption, metabolism and function of vitamin A to practical problems of vitamin A deficiency and strategies for minimizing it. The task proved to be troublesome for two major reasons, namely 1) that relationships clarified by the use of sophisticated instrumentation in well-controlled animal or clinical experiments cannot readily be applied to human malnutrition in a local ambient, and 2) that severe human malnutrition almost always has multiple causes, which confound the effects due to the absence of any single nutritional factor. As an example of the first problem, a skilled technician can quantitatively measure with relative ease the rate and extent of dark adaptation in trained adults by use of the electro-retinogram in a properly equipped physiological laboratory. In contrast, the utilization of such sophisticated equipment on frightened crying children in a remote village, even if the equipment could be safely transported there and made to operate, poses insurmountable problems. So on the one hand, scientists explore the frontiers of knowledge in well equipped laboratories with little concern for the application of their new-found concepts to practical situations, and on the other, nutritionists and public health workers apply familiar conventional procedures in their work, despite their limitations, with little attention given to developing new ways of approaching the complex task of nutritional assessment. Our report tries to bridge this gap.

We have started with nomenclature, and then continued with a summary of the present state of knowledge relative to vitamin A absorption, metabolism and function. Throughout the report data on humans are given whenever possible, but much of our basic knowledge about vitamin A is necessarily derived from animal experimentation. Although species differences undoubtedly exist, *qualitative* findings relative to vitamin A tend to be consistent from one species to another. We delve rather deeply into the biochemistry of vitamin A in these early sections, but try throughout to give a brief current summary of our understanding of specific topics as well as to cite a few useful recent references as an introduction to the literature. Since our thinking about nutrition and about strategies for combating deficiency are greatly influenced by our knowledge of metabolism, any serious new approach must be based on sound scientific knowledge.

But let us take a hypothetical case. We know that about 50% of a dose of orally ingested vitamin A is normally stored in the liver and that the body reserve is depleted at a certain rate on a diet low in vitamin A. If indeed the efficiency of storage could be increased to 90% and the rate of depletion cut to 1/3, a single dose of vitamin A might protect a child for a six-fold longer period. Thus, in the large dose distribution programs, doses of vitamin A might be given to an individual child only one

in two to three years instead of every four to six months, thereby improving effectiveness and cutting program costs significantly. Unfortunately we cannot now design such a system, but the main point is that a logical approach to this problem clearly depends on basic knowledge about metabolic control mechanisms.

The function of vitamin A in vision, i.e., in rod and cone cell structure, is well known. Although the retina of the eye can be disrupted in severe vitamin A deficiency, the cornea is the tissue most seriously affected by vitamin A malnutrition in humans. The changes which occur are complex and have not as yet been satisfactorily defined biochemically. A reduction in glycoprotein synthesis and secretion is probably involved, however, but other cellular defects are also present. In truth, vitamin A deficiency causes a vast array of metabolic abnormalities, and one of the thorniest scientific problems of the past decade has been the sorting out of those pathways which are primarily affected.

The applied section deals with many of the issues which most interest and concern nutritionists and public health workers. Infants enter the world with a very marginal reserve of vitamin A, due mainly to efficient placental control on the amount of vitamin A which can be transported from mother to fetus. During the next two crucial years, good nutrition is particularly important. With respect to the amount of vitamin A needed, recommended dietary allowances are at best a guide, and the requirement for a given child depends on growth rate, a balanced intake of nutrients and the presence of disease. Of particular note is the fact that close nutritional relationships exist among vitamin A, protein, iron, vitamin E and zinc, all of which tend to be limiting nutrients in many areas of the world. Infection exacerbates malnutrition, and it is not surprising that both specific and general interactions exist between vitamin A and immunological defense mechanisms.

We conclude our report with a discussion of methodology, not with the intent of defining specific procedures, but rather of dealing with some relatively new methods and with some questions of interpretation. And finally, we engage in a "brain-storming" session on possible new approaches to vitamin A status.

We hope that this report will not only be informative, but also will stimulate further study in the difficult interface between basic knowledge and the realities of the malnourished child.

SUMMARY

The purpose of this report is to summarize current knowledge about the absorption, metabolism and function of vitamin A, and insofar as possible, to apply this knowledge for the improvement of vitamin A nutriture.

Let us start with definitions. Vitamin A is a generic term which includes all compounds with the biological activity of retinol. One μg all-*trans* retinol = 3.33 International Units (IU) of all-*trans* vitamin A_1 alcohol. In converting all sources of vitamin A and carotenoids in the diet to a single unit, the term *retinol equivalent* is used. Generally one μg retinol is assumed to be equivalent to six μg β-carotene or to 12 μg mixed dietary carotenoids. Retinol binding protein (RBP), which is the primary carrier for retinol in the plasma, can exist as *holo*-RBP, i.e., a 1:1 molar complex with retinol, and as *apo*-RBP, which is free of retinol. A more suitable way of expressing plasma concentrations of retinol and RBP is μMoles/liter rather than the commonly used terms of μg/dl and μg/ml, respectively.

Vitamin A and its provitamins must be provided from foods. After ingestion, dietary carotenoids and vitamin A are liberated into fatty globules in the stomach, dispersed by bile salts, hydrolyzed and incorporated into mixed micelles in the intestinal lumen and absorbed into mucosal cells. Dietary protein and fat as well as bile enhance their digestion and absorption. Carotenoids are cleaved mainly to retinaldehyde but also to other fragments within intestinal cells, and the retinaldehyde is primarily reduced to retinol. Although some retinol is transported via the portal circulation and in low density lipoproteins as the free alcohol form, most is esterified in intestinal cells with palmitic acid or with other long chain fatty acids and is then transported as an ester in low density lipoproteins of the lymph.

After leaving intestinal cells, retinyl esters in low density lipoproteins of the plasma are probably hydrolyzed by esterases on liver cell membranes. Intracellularly, unesterified retinol is bound by a specific cellular retinol binding

protein (cRBP) in the cytosol. Retinol is then esterified and transferred into a soluble lipoglycoprotein aggregate which serves as a storage form. Retinyl esterhydrolase, which is tightly bound to the lipoglycoprotein aggregate, hydrolyzes retinyl ester to retinol, which then forms a complex with a specific retinol binding protein (*apo*-RBP). Plasma RBP and cRBP are quite different proteins.

Apo-RBP binds retinol in a 1:1 molar complex in the cytoplasm, and the resulting *holo*-RBP is taken up by the Golgi fraction and secreted into the blood. Although *holo*-RBP forms a 1:1 molar complex with prealbumin in humans, the two proteins are apparently secreted separately by the liver. After transfer of retinol to cells of target tissues, *apo*-RBP is degraded in the kidney. When associated with prealbumin, the half life of *holo*-RBP in human plasma is 11-16 hours. Hormones, and particularly estrogens, markedly affect *holo*-RBP levels in plasma. Inasmuch as target cells specifically bind RBP and not retinol, the ability of vitamin A analogues to interact with RBP markedly affects their biological activities.

Holo-RBP in plasma binds very tightly to specific cell surface receptors, which recognize the protein moiety of RBP. During the transport process across the cell plasma membrane, only the retinol is internalized. A number of intracellular retinol and retinoic acid binding proteins have been described, but their distinct functions remain to be shown.

Within cells vitamin A is always associated with cellular proteins or lipid-rich structures. Some common metabolic reactions of retinol are esterification, oxidation to retinaldehyde, isomerization, conjugation with glucuronic acid and phosphorylation. Retinaldehyde is oxidized to retinoic acid, which may be further oxidized and chain shortened, mainly by 2 or 3 carbon steps. The ring system is also oxidized but not cleaved. Although much effort has been given to the identification of biologically active metabolic derivatives of vitamin A, none has yet been found with activity greater than that of the vitamin itself.

A portion of the ingested vitamin A is directly excreted in the feces without absorption. Of absorbed vitamin A, about 60% is stored in the liver and about 40% is rapidly metabolized and excreted in the feces and urine. Vitamin A stored in the liver is mainly used for the formation of *holo*-RBP, which transports retinol to other tissues, although some vitamin A conjugates (glucuronides) are excreted in the bile and some oxidized chain-shortened products are excreted in the urine. A significant portion of the retinol released as conjugates and as an RBP complex is recycled back to the liver. These recycling processes may be of critical importance when the vitamin A status is poor.

Cells of the retinal pigment epithelium are the avenue of entry of plasma retinol to photoreceptor cells of the retina. Retinyl ester is stored in the pigment epithelial cells whereas photoreceptor cells contain 11-*cis* retinaldehyde, the visual pigment chromophore and retinol. A complex transport cycle exists between photoreceptor and pigment epithelial cells. Within photoreceptor cells retinol and retinaldehyde are interconverted as are all-*trans* and 11-*cis* isomers. Outer tips of the disc membrane assemblies of rod and cone cells are shed periodically in a diurnal cycle to make way for newly synthesized discs at the base of the outer segments. The shed tips are phagocytized by pigment epithelial cells.

Vitamin A deficiency causes dryness of the eye, partially due to interference with normal mucus production by conjunctival goblet cells as well as to undefined cellular changes in the corneal epithelium. Ulceration and perforation of the cornea may be caused by release of collagenase and/or by decrease in production of a collagenase inhibitor in serum, α_1-macroglobulin.

Vitamin A is also necessary for maintenance of the normal mucociliary function of epithelial tissues. Biochemical investigations have shown that RNA synthesis is affected by the vitamin in a variety of tissues. Moreover, the biosynthesis of some glycoproteins is decreased in vitamin A deficiency and enhanced upon administration of excessive doses of the vitamin, *in vivo* and *in vitro*, in intact cell systems. Epithelial tissues phosphorylate retinol to yield retinyl phosphate, a compound which functions as an acceptor and donor of specific sugar residues to glycoconjugates. Retinoic acid is also effective in glycoprotein biosynthesis, but its molecular mode of action is unclear at present.

Retinoids are effective preventive agents of chemical carcinogenesis in the skin, the respiratory tract, the breast and the bladder. They also affect cultured transformed fibroblasts by

altering their saturation density and adhesion in the direction of normal growth. This effect is reversible upon removal of the retinoid. Thus both epithelial and mesenchymal cell systems need vitamin A for their normal functions.

Concern about vitamin A nutriture starts well before birth. In experimental animals, birth defects or interruption of pregnancy can result from either vitamin A deficiency or hypervitaminosis A. During development of the fetus, the placenta regulates the passage of vitamin A to the fetus, probably by its recognition of maternal *holo*-RBP. In spite of a relatively constant intrauterine ambient, the range of plasma retinol values and liver reserves of vitamin A in human fetuses is quite large. Whereas plasma retinol values tend to remain relatively constant during gestation (12-40 weeks), liver vitamin A concentrations tend to peak around 28 weeks and then to decrease until term. Regardless of the vitamin A status of the mother, the newborn infant has only marginal reserves of vitamin A.

From an experimental standpoint, the recommended dietary allowances for vitamin A from various countries are in the right range for adults, but are less firmly based for children and other groups. Defects in the plasma transport, cellular uptake and intracellular utilization of vitamin A, as well as a lack of the dietary vitamin, may affect the appearance of signs of deficiency. Plasma levels of retinol which are necessary for the normal function of various tissues are not known. Experimental animals with a low vitamin A intake have a poor appetite, are listless and grow slowly, even when free of infection. Inasmuch as similar symptoms are seen in nutritionally disadvantaged children in their local ambients, subclinical vitamin A deficiency may often contribute to the syndrome of the generally malnourished child.

As a result of vitamin A deficiency as well as other reasons, vitamin A concentrations may be depressed in the plasma and *apo*-RBP levels may be elevated in the liver. When a small dose of vitamin A is given orally, plasma *holo*-RBP concentrations rise. When liver reserves and plasma level are initially low, the rise is significant, rapid and sustained. Other conditions give different responses. Thus a new parameter, the relative dose response (RDR), might be useful in distinguishing subclinical vitamin A status from other factors which depress plasma retinol levels.

From the turnover and utilization rates of endogenous vitamin A, the daily intake of vitamin A required to maintain total body stores at a specified level can be calculated. The importance of recycling mechanisms in conserving vitamin A in the body thus becomes clear. In several cases which are considered, the calculated intakes agree reasonably well with the recommended daily allowances for that age group. Thus the use of *in vivo* kinetic analysis seems appropriate both in extending our knowledge about nutritional interactions affecting vitamin A metabolism as well as in better defining vitamin A requirements.

Vitamin A requirements are increased by diseases which reduce the intestinal absorption of carotenoids and vitamin A, increase the rate of tissue metabolism or reduce the efficiency of retinol transport and utilization. Thus diseases which affect the intestine, pancreas, liver and kidneys most markedly alter vitamin A needs. Plasma retinol levels are usually depressed in disease, but in some cases, such as in nephrosis, may be increased.

Vitamin A has been called the "anti-infective" vitamin because its deficiency gives rise to a wide variety of infections in experimental animals. Many aspects of the immune response may be depressed in vitamin A deficiency, namely non-specific systems like mucus production and phagocytosis, humoral immunity and cell-mediated immunity. In contrast to the increased susceptibility to infection noted in the deficiency state, a large dose of vitamin A serves as an effective adjuvant, enhances phagocytosis, shortens the survival time of allogenic skin grafts and changes the surface characteristics of cells. On the other hand, a large dose of retinoic acid inhibits interferon synthesis and a large dose of retinaldehyde blocks complement-induced hemolysis.

Children with vitamin A deficiency commonly suffer as well from protein calorie malnutrition and an inadequate intake of other nutrients. The increased susceptibility to infection noted in vitamin A deficient children, therefore, is probably due to multiple causes of a nutritional and socioeconomic nature.

Just as vitamin A deficiency affects infections, so also do infections influence the transport and utilization of vitamin A. Plasma values

of retinol, RBP and albumin are significantly lowered by infections, and the rate of vitamin A metabolism is greater. Of various infections acute hepatitis and measles have the most marked affect, followed by upper respiratory infections, chicken pox and bronchitis. In contrast, diarrhea tends to increase plasma retinol levels, mainly because of dehydration and resultant hemo-concentration. Chronic parasitic infestation may also lower plasma retinol values, probably by interfering with the intestinal absorption of carotenoids and vitamin A.

Acute protein deficiency interferes with carotenoid and vitamin A absorption in the intestine and with the cleavage of carotenoids into vitamin A in the intestinal mucosa. In addition, the synthesis of *apo*-RBP, PA and the storage lipoglycoprotein for retinyl ester in the liver is depressed. Since both plasma PA and RBP have short half lives, their plasma concentrations serve as sensitive indicators of inadequate protein nutriture. Inasmuch as a poor vitamin A status commonly accompanies PEM, vitamin A supplements should be included from the outset in dietary therapy for the condition. Vitamin A deficiency also affects protein metabolism, both by reducing the synthesis and release of RBP from the liver as well as by impairing nitrogen utilization. The plasma *apo*-RBP found in vitamin A deficiency is biologically inactive, e.g., is no longer able to bind retinol.

Dietary fat markedly stimulates the absorption of carotenoids and vitamin A, in all likelihood by enhancing bile secretion into the intestine. Thus a low fat diet in pregnant or lactating women (<20g/day) or in children (~5g/day) will markedly reduce carotenoid absorption.

In the intestinal lumen ionic iron may catalyze the oxidative destruction of vitamin A and carotenoids. When chelated in heme or with other proteins in tissues, iron is much less active in this regard. On the other hand, vitamin A may facilitate the release of iron from the liver for use in hemoglobin synthesis. Whether or not a functional relationship exists, anemia and vitamin A deficiency seem to be correlated epidemiologically. By virtue of its anti-oxidant properties, vitamin E may protect vitamin A and carotenoids from oxidation in the intestinal lumen. At the tissue level, vitamin A absorption and storage are depressed in vitamin E deficiency but are not enhanced much above normal by large doses of vitamin E. Although the symptoms of zinc deficiency and vitamin A deficiency are similar in many respects, no clear molecular interaction has been shown. Nonetheless, vitamin A seems to be spared in zinc deficient animals, probably as a result of the lower growth rate of these animals.

Inasmuch as protein malnutrition and vitamin A deficiency are commonly associated clinically, a separate evaluation of protein status by the measurement of plasma prealbumin is useful. In the handling of blood samples for vitamin A assay, samples should be kept cool and plasma or serum separated off as soon as possible. Samples should either be analyzed promptly, which is preferable, or else be stored under nitrogen in the frozen state. Retinol in organic solvent extracts of plasma can be readily analyzed by the Carr-Price reaction or by fluorescence or other properly standardized procedures. In case retinyl ester is present in significant amounts, chromatographic separation of the ester from free retinol may be necessary.

Of approximately 400 naturally occurring carotenoids, only about 20 serve as provitamins. The principal provitamins are hydrocarbons (α-, β- and γ-carotenes) and cryptoxanthin. The abundant polar carotenoids of plants are generally inactive. Carotenoids found in the plasma are an indicator of the recent intake of carotenoid-containing foods and not a useful measure of vitamin A status. Usually 15-60% of total plasma carotenoids consist of provitamins, but the percentage might vary from very low to high values. Carotenoids of plasma normally contribute only around one percent of a sufficient total body reserve of vitamin A. As a function of food intake, very high values of plasma carotenoids are found among certain individuals or in some societies, and very low values are sometimes associated with low fat intakes or malabsorption syndromes. When plasma carotene concentrations are high, special precautions must be observed in determining plasma vitamin A values.

Although total plasma RBP can most sensitively be measured by radio-immunoassay, radial immuno-diffusion assay is more suitable for field studies. Extreme care must be taken,

however, in using antibody concentrations suitable for the amount of RBP being measured. Since a significant quantity of inactive *apo*-RBP circulates in the plasma of vitamin A-deficient children, the data obtained must also be interpreted with caution.

Holo-RBP, the physiologically active form of retinol, may be sensitively measured by the fluorescence emitted from the holo-RBP zone of a polyacrylamide gel after electrophoresis. This procedure, however, requires sophisticated instrumentation and frequent standardization.

The possibility of using other available biological samples as indicators of vitamin A status is discussed.

NOMENCLATURE

Abstract: Vitamin A is a generic term which includes all compounds with the biological activity of retinol. One μg all-*trans* retinol = 3.33 International Units (IU) of all-*trans* vitamin A_1 alcohol. In converting all sources of vitamin A and carotenoids in the diet to a single unit, the term *retinol equivalent* is used. Generally one μg retinol is assumed to be equivalent to six μg β-carotene or to 12 μg mixed dietary carotenoids. Retinol binding protein (RBP), which is the primary carrier for retinol in the plasma, can exist as *holo*-RBP, i.e., a 1:1 molar complex with retinol, and as *apo*-RBP, which is free of retinol. A more suitable way of expressing plasma concentrations of retinol and RBP is μMoles/liter rather than the commonly used terms of μg/dl and μg/ml, respectively.

* * * *

The nomenclature of vitamin A and related compounds has undergone many modifications since E. B. McCollum and Maude Davis called this new growth factor *fat-soluble A* in 1913. With a focus on growth response, the International Unit (I.U.) was established, and later, with the purification of vitamin A alcohol and the determination of its structure, one μg all-*trans* vitamin A_1 alcohol was equated with 3.33 I.U. In a major international revision of vitamin A nomenclature in the 1960s, the term *retinol* was introduced for vitamin A_1 alcohol, and *retinaldehyde* (or retinal) for vitamin A_1 aldehyde.

In dealing with multiple forms of vitamin A active substances in the diet, the term *retinol equivalents* was later devised. Although the relative biological effectiveness of β-carotene and customary mixtures of carotenoids in food products depends on a variety of conditions, a much used convention is that one μg all-*trans* retinol is equivalent to six μg all-*trans* β-carotene or to 12 μg of mixed carotenoids from dietary sources. In order to avoid ambiguity and to clarify the nature of vitamin A intake, the total daily intake in μg retinol equivalents, the μg retinol ingested as pre-formed vitamin A and the μg retinol equivalents derived from carotenoids should all be expressed, as well as the method of calculating the retinol equivalents from dietary carotenoids.

The identification of a specific plasma carrier for retinol, retinol binding protein, which in man has a molecular weight of 21,000 daltons, has elicited much interest in the past decade. Retinol binding protein normally carries one mole of retinol per mole protein, and furthermore, largely forms a 1:1 complex with prealbumin (MW 52000) in human plasma. These interactions, as well as the specific binding of retinol binding protein to cell surfaces, emphasize the *molecular* aspects of vitamin A transport and function. The term *holo*-RBP denotes the 1:1 molar complex of retinol and RBP, whereas *apo*-RBP is the protein alone without bound retinol.

Plasma vitamin A concentrations are conventionally expressed as μg retinol per 100 ml plasma or serum, e.g., μg retinol/deciliter, retinol binding protein concentrations as μg/ml, and liver vitamin A concentrations as μg retinol equivalents per gram wet weight, inasmuch as retinyl esters are the major storage form. In order to emphasize the important molar relationships between retinol and its carrier protein, plasma retinol and retinol binding concentrations might better be expressed as μMoles per liter, and tissue concentrations as μM/kg, or its equivalent, nano-Moles per gram (nM/g).

Thus, equivalent values would be: 1 μM retinol/l = 286 μg/l or 28.6 μg/100 ml, and 1 μM RBP/l = 21 mg RBP/l or 21 μg/ml. Plasma concentrations of retinol and RBP in equivalent units are given in Table I.

This suggested change to molar units is in keeping with the general trend of expressing nutritional units as standard chemical concen-

TABLE I

Expression of Plasma Vitamin A and Retinol Binding Protein Concentrations in Various Units and the Corresponding Micromolarity

RETINOL			RBP
μM/l	μg/dl	I.U./dl	μg/ml
0.3	8.6	28.6	6.3
0.6	17.2	57.2	12.6
1.0	28.6	95.3	21.0
1.5	42.9	143.0	31.5
2.0	57.2	190.7	42.0
2.5	71.5	238.3	52.5

trations. Despite the greater utility of these molar expressions, however, the present report has used the more conventional terms, largely because of their common use in the nutritional literature and the greater familiarity of nutritionists with them.

METABOLISM

Absorption from the intestine

Abstract: Dietary carotenoids and vitamin A are liberated into fatty globules in the stomach, dispersed by bile salts, hydrolyzed and incorporated into mixed micelles in the intestinal lumen and absorbed into mucosal cells. Dietary protein and fat as well as bile enhance their digestion and absorption. Carotenoids are cleaved mainly to retinaldehyde but also to other fragments within intestinal cells, and the retinaldehyde is primarily reduced to retinol. Although some retinol is transported via the portal circulation and in low density lipoproteins as the free alcohol form, most is esterified in intestinal cells with palmitic acid or with other long chain fatty acids and is then transported as an ester in low density lipoproteins of the lymph.

* * * *

Digestion and dispersion in the stomach and intestine: After the ingestion of food, the provitamin carotenoids of vegetable tissues and preformed vitamin A of animal tissues are released from associated proteins by the proteolytic action of pepsin in the stomach and of chymotrypsin and trypsin in the small intestine. During this stage the liberated carotenoids and vitamin A are dissolved in fatty globules which then pass into the lumen of the duodenum. These globules then encounter the bile salts, pancreatic lipase and phospholipase A_2, which release long chain fatty acids, monoglycerides and diglycerides from the dietary triglycerides and fatty acids and lysolecithin from the dietary phospholipids and biliary lecithin. Vitamin A esters also are hydrolyzed at this stage by esterase derived from the intestine or pancreas. These lipid products of digestion then interact with bile salts and cholesterol to form soapy aggregates with a diameter of about 20 Å, termed mixed micelles, which solubilize the vitamin A and β-carotene. These mixed micelles diffuse into the glycoprotein layer surrounding the microvilli or brushborder of the mucosal cells, where they come into contact with the cell membranes. The components of the micelles, except the bile salts, which are poorly absorbed in the upper intestine, then individually penetrate the lipid phase of the mucosal cell membranes to reach the cytoplasm.

Factors affecting the dispersion of lipids: The efficiency of dispersion of vitamin A and β-carotene is affected by the presence or absence of other components in the diet as well as by

the general nutritional status of the subject. Soluble protein and the peptides derived therefrom are surface active agents which assist in the dispersion of lipids within the intestinal lumen while at the same time providing hydrophobic centers to which the non-polar β-carotene may bind during transfer to the plasma membrane. The presence of increasing amounts of protein up to 20-40% in the diet also aids the intracellular formation of retinaldehyde from β-carotene.

Fat in the diet provides the vehicle for transporting the vitamin A and carotenoids from the stomach into the intestinal lumen and is the source of some of the digestion products which take part in micelle formation. Some dietary lipids such as seed oils may also contain α-tocopherol, which has a protective antioxidant effect on vitamin A.

An ample supply of bile salts is necessary not only for solubilizing the apolar β-carotene, but more specifically for stimulating pancreatic lipase and assisting β-carotene to penetrate the plasma membrane. Thus, the uptake of β-carotene and retinol is higher from bile salt micelles than from dispersions of synthetic detergents. The rates of absorption of β-carotene and retinol increase linearly with the concentration of bile salts up to about 10 mM and then level off. Even though both carotene and retinol reach maximal rates of uptake at the same bile salt concentration, the absorption rate for carotenoids under all conditions is much slower than that of retinol. During fat absorption the normal concentration of bile salts in the intestinal lumen is around 10 mM, well above its critical micelle concentration of 4 mM.

Intracellular phase: Within the mucosal cell β-carotene is acted on by a carotenoid dioxygenase, which oxidatively cleaves the molecule mainly at the central 15-15′ double bond to form two molecules of retinaldehyde, but also to some degree at excentric double bonds to yield small amounts of β-apo-carotenals. The latter compounds may be oxidized further to yield retinaldehyde or, after conversion to the corresponding β-apocarotenoic acid by aldehyde oxidase, may be progressively transformed to retinoic acid. Most of the retinaldehyde is reduced promptly to retinol by retinaldehyde reductase, which is different from alcohol dehydrogenase. The equilibrium favors greatly the alcohol form of the vitamin. A small portion of the aldehyde is oxidized in the cytosol to sodium retinoate, which is water-soluble and can pass into the portal blood for transport to the liver.

During its passage through the intestinal epithelium, up to 75% of the retinol, irrespective of source, becomes esterified with long chain fatty acids. The palmitate ester is mainly formed, with smaller quantities of the stearate, oleate and other fatty acyl esters. A cellular retinol binding protein may participate in the transport of retinol across the cell, or at least to the esteratic site on the endoplasmic reticulum. The retinyl ester synthetase and the fatty acid biosynthesis complex may well be localized close to each other on the same membrane.

Significant amounts of unesterified retinol can be absorbed directly via the portal vein, probably in micelles with other fatty acids or bound to albumin. A further portion enters the lymphatic system along with retinyl esters dispersed in the lipid phase of chylomicra. When radioactively labelled retinol is given to rats, unesterified retinol appears in the lymph within half an hour of oral dosing and before the ester is detected. The specific activity of free retinol initially found in the liver was also higher than that of the ester fraction. Nevertheless, an oral dose of retinol is largely transported in ester form in the low density lipoprotein fraction (chylomicra) of the lymph, from which it enters the blood stream.

That portion of the β-carotene which escapes oxidation in the intestinal cells is also distributed among the various lipoproteins of the lymph, with the low density group carrying the highest proportion.

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Uptake and storage in the liver

Abstract: Retinyl esters in low density lipoproteins of the plasma are probably hydrolyzed by esterases on liver cell membranes. Intracellularly, unesterified retinol is bound by a specific cellular retinol binding protein (cRBP) in the cytosol. Retinol is then esterified and transferred into a soluble lipoglycoprotein aggregate which serves as a storage form. Retinyl ester hydrolase, which is tightly bound to the lipoglycoprotein aggregate, hydrolyzes retinyl ester to retinol, which then forms a complex with a specific retinol binding protein (*apo*-RBP). Plasma RBP and cRBP are quite different proteins. The retinol RBP complex (*holo*-RBP) enters vesicles derived from the Golgi body and is then secreted into the plasma.

* * * *

Retinyl esters in the low density lipoproteins from the lymph enter the liver sinusoids and come into contact with the parenchymal cell membranes. Lipoprotein lipase degrades the triglycerides in the low density lipoproteins and esterases of the liver cell membranes hydrolyze the retinyl esters. The extent to which retinyl esters may cross liver cellular membranes by pinocytosis is not yet known.

Retinol liberated by esterase action crosses the parenchymal liver cell membrane directly. The remaining vitamin A in the lipoprotein fraction may be taken up by pinocytosis. *Plasma* retinol-binding protein does not appear to be involved in this process, inasmuch as retinol attached to RBP exchanges with excess retinol *in vitro* only very slowly, with a t ½ of about three hours. On the other hand, *cellular* retinol-binding protein may well be the acceptor within the parenchymal cell which transports the retinol to the endoplasmic reticulum, where it is again esterified, largely to the palmitate ester, and transferred into a soluble complex macromolecule made up of various lipids, several polypeptide chains and carbohydrates linked covalently to the protein.

The retinyl compounds of the complex, which account for two percent of the total weight of the macromolecule, consist of retinyl-esters (96%) and unesterified retinol (4%). This storage complex also contains a tightly bound retinyl ester hydrolase, which is able to hydrolyze retinyl ester localized in the same aggregate. Esterase activity is enhanced by an acceptor for unesterified retinol such as serum albumin or *apo*-RBP. Thus, upon hydrolysis, retinol may be directly transferred from its binding site in the storage complex to its specific plasma binding protein. Although isolated as a soluble aggregate, the storage complex is probably enclosed within vesicles in the intact cell.

Vitamin A is normally stored in the liver parenchymal cell and not the Kupffer cells of the reticulo-endothelial system as was thought at one time. When large doses of vitamin A are given, however, perisinusoidal cells of the liver, called Ito cells, take up much of the dose. These Ito cells may also differentiate into fibroblasts. Whether these cells play an important physiological role in vitamin A storage under normal conditions is still unclear. β-Carotene can be stored in liver fatty globules and in adipose fat around extrahepatic tissues. The β-carotene stored in liver can be slowly converted to retinol.

The distribution of retinol within the subcellular fractions of liver cells has been reexamined in relation to retinol-binding protein. As noted above, retinyl esters are located mainly in the storage complex, with only small proportions distributed in the cytosol and microsomes. Plasma RBP synthesized in the liver is mainly bound to the membranous components, including the Golgi fraction. Plasma RBP freshly synthesized in the endoplasmic reticulum apparently collects retinol from the storage complex before being packaged into secretory vesicles for transport to the plasma membrane and eventual export. The total amount of unesterified retinol present in the cell is about ten times the amount bound to plasma retinol binding protein. Although most of the unes-

terified retinol is in the storage complex, some may be present in the lipid phase of membranes and the remainder in the cytosol fraction bound to cellular retinol binding protein. Purified mitochondria contain neither retinol nor its ester. These processes are summarized in Figure I.

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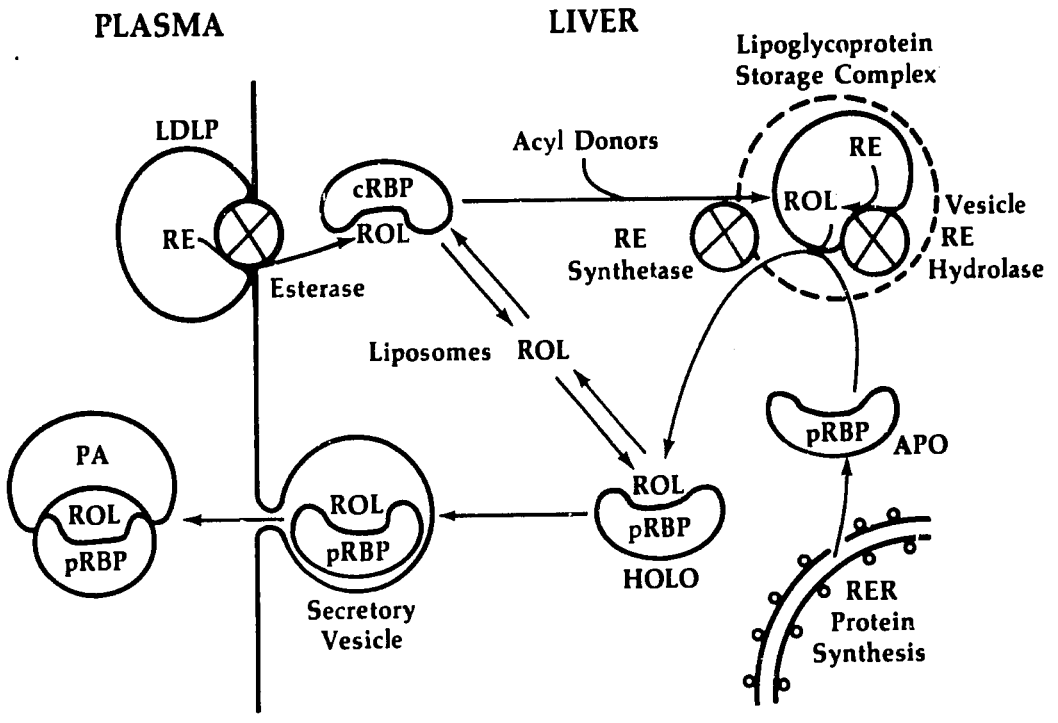
Transport in plasma

Abstract: A specific, small (MW 21,000) binding protein for retinol (*apo*-RBP) is synthesized in the liver. This *apo*-RBP binds retinol in a 1:1 molar complex in the cytoplasm, and the resulting *holo*-RBP is taken up by the Golgi fraction and secreted into the blood. Although *holo*-RBP forms a 1:1 molar complex with prealbumin in humans, the two proteins are apparently secreted separately by the liver. After transfer of retinol to cells of target tissues, *apo*-RBP is degraded in the kidney. When associated with prealbumin, the half life of *holo*-RBP in human plasma is about 16 hours. Hormones, and particularly estrogens, markedly affect *holo*-RBP levels in plasma. Inasmuch as target cells specifically bind RBP and not retinol, the ability of vitamin A analogues to

FIGURE I



Uptake, storage and release of vitamin A from liver cells. Abbreviations are: ROL, retinol; RE, retinyl ester; cRBP, cellular retinol-binding protein, pRBP, plasma retinol-binding protein.

interact with RBP markedly affects their biological activities.

* * * *

Whereas absorbed dietary retinol is carried to the liver mainly as its ester in complex with lipoproteins, delivery of the alcohol to the target tissues is more highly controlled by its transport on a specific retinol-binding protein (RBP). The latter, which is a single polypeptide of approximately 21,000 daltons, contains a hydrophobic cleft into which the relatively planar retinol molecule fits. RBP forms a 1:1 complex in plasma with thyroxine-binding prealbumin (PA) composed of four subunits of 13,000 daltons. The association constant (Ka) for the PA-*holo*-RBP complex is about 10^6M.

The formation of this large complex may be of physiological significance in man in preventing the smaller retinol-binding protein and hence the vitamin from rapid loss by ultrafiltration through the glomerular membranes of the kidney. It is known, however, that RBP and the vitamin can pass through the glomerulus and into the kidney tubules where both are readily reabsorbed by the endothelial cells in the proximal region. Furthermore, animals like the bovine, in which an RBP-PA complex is not found, have no difficulty in maintaining plasma levels of RBP and vitamin A. Most of the metabolism of the protein occurs in the kidney. The reabsorbed vitamin returns to the liver via the lymphatic system.

Mechanism of secretion from the liver: RBP is associated with particulate material in the Golgi fraction as well as elsewhere in the cytoplasm. Prior to secretion retinol-binding protein is packaged in secretory vesicles which ultimately fuse with the plasma membrane and secrete their contents into the plasma. The vesicles are thought to be channelled to the plasma membrane by the microtubules, since *holo*-RBP secretion is inhibited by colchicine, a disruptor of tubulin formation.

Although RBP associates with PA in human plasma and both proteins are synthesized in the liver, the two proteins are in all likelihood secreted independently and then bind together in the plasma.

Factors affecting the concentration of RBP in plasma. In studies conducted in Europe, the mean concentration of plasma RBP for adult males is higher (47μg/ml) than that for females (42 μg/ml). The mean concentration for European children is much less, remaining in the 20 to 30 μg/ml range until about ten years of age, when it commences to increase with the onset of puberty to the adult level. This phenomenon also occurs in the polyestrus rat. In rats the RBP level tends to overshoot the normal adult range with the development of their gonads before settling back to adult values. In seasonal breeding animals the plasma level of RBP increases cyclically about threefold from the concentration occurring in the anestrus phase to that obtaining in the estrus phase of their development. The increased supply of RBP in plasma is associated with the rapid differentiation and growth of gonadal tissues. These changes occur in both sexes and are independent of shorter term changes which take place during the estrus cycle in the female. It has also been observed that contraceptives cause the plasma RBP level to be significantly higher in females. It would appear, therefore, that the secretion of RBP from the liver is under hormonal control, but the detailed mechanism is not yet understood.

The total molar concentration of RBP in plasma measured by immuno-assay invariably exceeds by five to 15% the molar concentration of retinol as determined by color test or fluorescence. This surplus *apo*-RBP, which has already given up its retinol to target tissues, is apparently unable to bind retinol because of a change in conformation due to the loss of amide groups or possibly of a C-terminal amino acid. On the other hand, since retinol dispersed on Celite can exchange with retinol in *holo*-RBP and since retinol is readily extracted from *holo*-RBP by using a non-polar solvent, loss of the C-terminal amino acid of RBP is probably not essential for the transfer of bound retinol to a target cell membrane.

This *apo*-RBP does not bind to prealbumin and appears to be metabolized more rapidly than the native protein by the kidney. Since the fraction of *apo*-RBP increases in retinol-deficient subjects, the estimation of total RBP by immunodiffusion assay will give a value considerably higher than that of the functional *holo*-RBP. Nonetheless, the immunodiffusion assay is a useful indicator of vitamin A deficiency, particularly when the latter is serious.

These assays, which require only a simple, easily portable apparatus, can also be run in situations where a spectrophotometer is not available. Whenever possible, however, retinol should also be estimated.

Turnover: Human *holo*-RBP, when in association with pre-albumin, has a half life of 11-16 hours, whereas prealbumin has a half life of two to four days. In the rat, these half-lives are increased about 50% in protein deficiency.

Transport in relation to function: Transport of retinol by RBP has an important controlling effect on its function. The analogue of vitamin A, α-retinol, is readily absorbed by the animal and stored in the liver. Although biologically inactive when administered *in vivo*, it is biologically active when properly presented to retinol deficient tissues *in vitro*. Its biological inactivity when given *in vivo* is due to the fact that it is unable to bind to RBP, and hence cannot be transported to the target tissues.

Retinoic acid, which also possesses some biological activity, is transported in plasma bound to albumin. There may, however, also be present in plasma a carrier protein for retinoic acid which is similar to the one found inside liver and tumor cells.

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Uptake by cells and intracellular transport

Abstract: *Holo*-RBP of plasma binds very tightly to specific cell surface receptors, which recognize the protein moiety of RBP. During the transport process across the cell plasma membrane, only the retinol is internalized. A number of intracellular retinol and retinoic acid binding proteins have been described, but their distinct functions remain to be shown.

* * * *

A specific cell surface receptor for plasma retinol binding protein on target cells: In *holo*-RBP the retinol ligand is tightly bound inside plasma RBP and cannot be "recognized" as such. Thus the retinol-RBP complex and not retinol alone is recognized by the target tissue.

Target cells, e.g., the pigment epithelial cells in the vertebrate eye, possess a specific cell surface receptor for plasma RBP. This cell surface receptor binds *holo*-RBP with great affinity; the association constant (Ka) is estimated at $\geqq 2 \times 10^{11}$ M and the second order rate constant at about 10^{8} M^{-1} S^{-1} (at 22°). Binding to the receptor is specific for plasma RBP. Prealbumin, a protein that is tightly bound to serum RBP in many animals, is not necessary for the binding of plasma RBP to the cell surface receptor. In keeping with this observation, the binding of RBP to the receptor ($Ka > 10^{11}$ M) is greater than to prealbumin ($Ka \simeq 10^{6}$ M).

Retinal pigment epithelial cells have about $5 \cdot 10^{4}$ cell surface receptors for plasma RBP per cell. The pigment epithelial cells, which are a monolayer of cells interposed between the systemic blood supply and the neural retina, have this receptor only on one side of the cell: that side which faces the systemic blood supply. The localization of the cell surface receptor on target cells of other tissues is not known at this time. Probably the density (number per unit area) of the surface receptor on cells of other

tissues is lower than on pigment epithelial cells, thus making both its detection and its surface localization more difficult.

The retinol ligand is not required for the binding of RBP to the cell surface receptor; *apo*-RBP is also bound, but less firmly than *holo*-RBP. The cell surface receptor also will bind a synthetic complex of retinoic acid and *apo*-RBP. Thus, the cell surface receptor recognizes only the protein part of the retinol-plasma RBP complex.

Physiological transport of ligand into target cell: Plasma RBP, although essential for the binding of a retinol-containing complex to the cell surface receptor of the target cell, is not transported across the cell membrane. By double labeling experiments together with autoradiography, and by studies on the displacement of plasma RBP from the cell surface receptor, only retinol is found to be transported across the cell membrane while *apo*-RBP stays behind. The cell surface does not discriminate against an analog ligand as long as it is associated with plasma RBP. Thus cells may take up retinoic acid from an artificially reconstituted retinoic acid-plasma RBP complex. On the other hand, neither retinol nor retinoic acid is transported into cells as a complex with serum albumin.

During the transport of retinol from *holo*-RBP into isolated pigment epithelial cells, some of the *apo*-RBP dissociates from the cell surface and some is partially degraded, presumably by proteolytic enzymes. Whether this process is observed only in isolated cell preparations, where proteolytic enzymes may be released from broken cells, or is the normal fate of "discarded" *apo*-RBP remains to be investigated.

Intracellular transport: Retinol which is taken up by cells in the above described process can be detected inside the cells in three forms: 1) as the "free" ligand, 2) as a complex with small (MW about 15000) proteins and 3) as a complex with a high molecular weight lipoglycoprotein. Several other low molecular weight proteins that bind retinol and retinoic acid when the ligands are added to the cell sap have also been described. The role of these intracellular binding proteins in the function of vitamin A is still unclear.

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Intracellular metabolism

Abstract: Within cells vitamin A is always associated with cellular proteins or lipid-rich structures. Some common metabolic reactions of retinol are esterification, oxidation to retinaldehyde, isomerization, conjugation with glucuronic acid, and phosphorylation. Retinaldehyde is oxidized to retinoic acid, which may be further oxidized and chain shortened, mainly by two or three carbon steps. The ring system is also oxidized but not cleaved. Although much effort has been given to the identification of biologically active metabolic derivatives of vitamin A, none has yet been found with activity greater than that of the vitamin itself.

* * *

Within cells vitamin A is not free in solution, like glucose or phosphate ion, but is associated either with specific cellular proteins, enzymes or lipid-rich aggregates and membranes. The physiological substrate for the enzymatic transformation of vitamin A, then, is the vitamin in a complex and not a free molecule in solution. As already mentioned, retinol is readily esterified and the retinyl esters hydrolyzed by most, but certainly not by all, cells. Retinol

may also be reversibly oxidized to retinaldehyde and then irreversibly to retinoic acid in most cells. In some tissues, such as the retina, very specific enzymes exist for the former reaction. Retinol may also be phosphorylated to a compound which plays a role in glycoprotein synthesis, as will be elaborated later.

Isomerases exist in the gut, liver and eye for the interconversion of the *trans* and various *cis* forms. Both retinol and retinoic acid are conjugated to β-glucuronides in the endoplasmic reticulum of the intestine and liver, but not in the retina. Oxidation of the trimethyl cyclohexene ring to the four-keto derivative occurs in the liver, and the oxidative cleavage of the side-chain, probably by the release mainly of two and three carbon units, takes place in the kidney and possibly in the liver.

Much attention has been given to a search for a physiologically active metabolite of vitamin A, i.e., some metabolic derivative which retains the full activity of vitamin A in growth and differentiation. Although many modified compounds have been detected, none has yet been isolated whose chemical structure and mode of action have been rigorously established.

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Excretion

Abstract: A portion of the ingested vitamin A is directly excreted in the feces without absorption. Of absorbed vitamin A, about 60% is stored in the liver and about 40% is rapidly metabolized and excreted in the feces and urine. Vitamin A stored in the liver is mainly used for the formation of *holo*-RBP, which transports retinol to other tissues, although some vitamin A conjugates (glucuronides) are excreted in the bile and some oxidized chain-shortened products are excreted in the urine. A significant portion of the retinol released as conjugates and as an RBP complex is recycled back to the liver. These recycling processes may be of critical importance when the vitamin A status is poor.

* * * *

A summary of major pathways of vitamin A metabolism is given in Figure II. Ingested vitamin A is treated as follows: 1) a small portion ($\leqq$20%) is not absorbed from the intestine, and hence is excreted rapidly in the feces; 2) an appreciable amount (20-50%) is absorbed but then, after being conjugated or oxidized, is excreted within several days either in the feces or the urine; and 3) a significant portion (30-60%) is stored, largely in ester form, in the liver. Thus, of a given dose of vitamin A, 40-70% is excreted within about a week, and the remainder is stored in the liver with smaller amounts in other tissues.

Stored vitamin A is metabolized more slowly and by two major routes: 1) the release of retinol from the liver as a retinol binding protein complex, which is ultimately dissociated and/or degraded both in the peripheral epithelial tissues and in the kidney; and 2) the conversion of stored retinyl ester both to conjugated forms of vitamin A which are released into the bile and to oxidized forms which are excreted in the urine.

The kinetics of vitamin excretion are determined by three major factors:

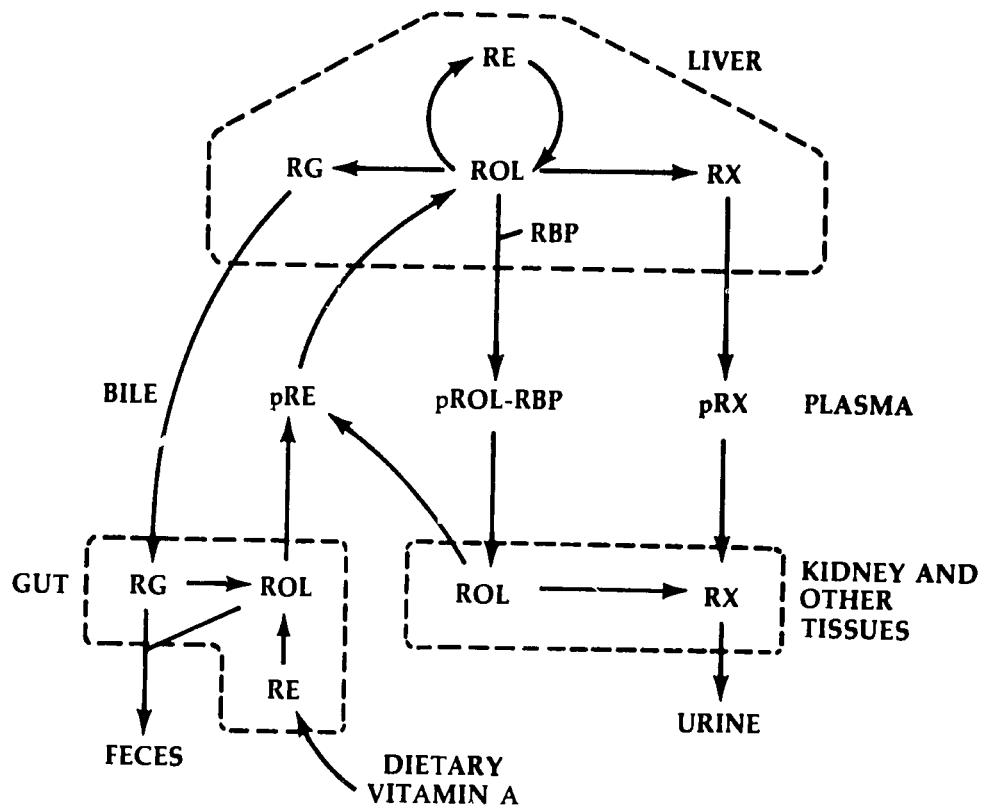
1) a relatively constant release of retinol from the liver as an RBP complex to replace that utilized by peripheral tissues. From the rate at which RBP is removed from the plasma ($t\ ½ = 11$-16 hrs.), a fractional metabolic rate, i.e., the fraction lost or replaced per day, of 2.7 can be calculated. Thus, replacing the complex of retinol and RBP which is metabolized each day will take about three mg of retinol in the adult. A portion of this amount may be derived, as indicated below, from recycled retinol.

2) a first-order release of retinol from the liver as a function of total liver stores, both as conjugated forms, such as β-glucuronides, in the bile and as oxidized metabolites in the urine. The excretion rate will, therefore, be faster when liver concentrations are high and will be slower when liver stores are low. In adult males, the mean half-life for the turnover of

vitamin A from the total body pool is about 140 days, whereas in children it is probably shorter. Thus, an adult with an ample total body reserve of 200 mg would release about one mg of retinol per day in various metabolic products. Inasmuch as this excretion mechanism is sensitive to the total body pool of vitamin A, it serves as a buffering device to conserve vitamin A when liver stores are low and to eliminate it when liver stores or intake is high.

3) recycling of released vitamin A. Some of the conjugated forms of vitamin A released into the bile are reabsorbed from the gut and transported back to the liver, thereby allowing for the reutilization of metabolized retinol. Similarly, retinol initially released as an RBP complex is also transported in part back to the liver from the kidney and probably from other tissues. The form in which retinol is recycled is probably as retinyl ester in association with low density lipoproteins. Recycling mechanisms, which normally account for a significant portion of the total amount of vitamin A mobi-

FIGURE II



Dynamics of vitamin A recycling and excretion. Abbreviations are: ROL, retinol; RE, retinyl ester; RG, retinyl glucuronide; RX, biologically inactive oxidation products; RBP, plasma retinol-binding protein. The prefix p denotes plasma components. Thus p ROL-RBP denotes *holo*-RBP.

lized from the liver each day, may become critically important during periods of vitamin A deprivation.

Excreted metabolites of vitamin A may be either biologically active or inactive. Active excreted metabolites consist mainly of conjugated forms of retinol or retinoic acid, such as the β-glucuronides. Retinoic acid is a normal oxidation product of retinol which retains biological activity for growth but is not active in vision or in the differentiation of some tissues. Inactive metabolities are largely chain-shortened oxidation products in which one or more carbon atoms have been chopped off the long unsaturated chain. In addition, the trimethyl cyclohexane ring of vitamin A may be oxidized to a 4-keto derivative. These inactive products cannot be converted back to vitamin A, nor are they stored in the liver or other tissues. Once formed, most are excreted rapidly in the urine.

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FUNCTION

The retina

Abstract: Cells of the retinal pigment epithelium are the avenue of entry of plasma retinol to photoreceptor cells of the retina. Retinyl ester is stored in the pigment epithelial cells whereas photoreceptor cells contain 11-*cis* retinaldehyde, the visual pigment chromophore, and retinol. A complex transport cycle exists between photoreceptor and pigment epithelial cells. Within photoreceptor cells retinol and retinaldehyde are interconverted as are all-*trans* and 11-*cis* isomers. Outer tips of the disc membrane assemblies of rod and cone cells are shed periodically in a diurnal cycle to make way for newly synthesized discs at the base of the outer segments. The shed tips are phagocytized by pigment epithelial cells.

* * * *

The only established function of retinol in the retina is to serve as the precursor of 11-*cis* retinaldehyde, the chromophore of all known visual pigments. Visual pigments are structural proteins that form an integral part of the outer segment discs or membranes of photoreceptor cells. The photoreceptor outer segment is the only site in the eye where visual pigments are found. Retinol, retinaldehyde and retinyl esters take part in a complex mobile interaction and transport between cells of the retinal pigment epithelium and the photoreceptor cells. The interplay between these ligands and vision is only dimly understood. The paragraphs that follow focus on the facts that are mostly agreed upon by researchers in the field.

The retinal pigment epithelium, which is interposed between the systemic blood supply and the neural retina (including photoreceptor cells) seems to be the avenue of entry of retinyl compounds into the retina. Since all-*trans* retinol is the only ligand which is bound *in vivo* to serum RBP, it seems almost certain that this is the form which is taken up by the pigment epithelium. Once inside the pigment epithelium, almost all of the retinol is stored as the ester. Since the ligand of visual pigments is 11-*cis* retinaldehyde, it is clear that retinyl ester, in its delivery from the pigment epithelium to the photoreceptors, must be hydrolyzed to retinol, oxidized to the aldehyde, and then isomerized to the 11-*cis* conformation. The sites and sequence of these steps are not known, although a great deal of work has been done in this area.

What is clear is the following. The photoreceptor cells contain about four percent of the total vitamin A in the eye. Essentially all of the vitamin A in these cells is in the form either

of 11-*cis*-retinaldehyde or of retinol. Thus the photoreceptors neither store retinyl esters nor seem capable of synthesizing them. Some 60% of the retinol in dark-adapted photoreceptors is due to the 11-*cis* form. Photoreceptors can, however, oxidize 11-*cis* retinol to 11-*cis* retinaldehyde. The questions that are not yet answered are the manner and the form in which retinol is transferred from the pigment epithelium to the photoreceptors and from the photoreceptors back to the pigment epithelium following illumination. The reactions that isomerize all-*trans* retinol to 11-*cis* retinol or all *trans*-retinaldehyde to 11-*cis* retinaldehyde also have not been well characterized. There have been suggestions that the conversion of all-*trans* to 11-*cis* isomers is also a photoreaction similar to the photoisomerization of 11-*cis* to all-*trans* retinal during illumination of visual pigments. It is not clear at this time, however, whether or not photoisomerization of an all-*trans* to an 11-*cis* form actually takes place *in vivo* as a natural mechanism.

It has been known for about ten years that retinal rod photoreceptors renew themselves by periodically shedding their outer tips while growing new disc membranes at the base of the rod photoreceptor outer segment. The shed tips are taken up through a phagocytic process by the pigment epithelial cells. The pigment epithelium in turn degrades the various constituents of the phagocytized outer segments. It was shown recently that rod outer segments, which mediate night vision and contain the visual pigment rhodopsin, shed their tips at the beginning of a light period. On the other hand, cone outer segments, which mediate color vision and are operational under bright illumination, shed their tips at the beginning of the dark period. It seems clear from these results that periods of dark illumination provide a signal for a daily physiological rhythm in photoreceptor renewal. Light or darkness are not necessary as such for renewal of photoreceptors, however, since animals that are kept in light or darkness for longer than 12 hour cycles will still go on shedding their photoreceptor tips at a 12 hour interval. Thus light presumably acts only as a synchronization mechanism for this diurnal phenomenon of photoreceptor renewal. The mechanism of this link between photoreceptor cell shedding and visual pigment has not yet been elucidated.

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The cornea

Abstract: Vitamin A deficiency causes dryness of the eye, partially due to interference with normal mucus production by conjunctival goblet cells as well as to undefined cellular changes in the corneal epithelium. Ulceration and perforation of the cornea may be caused by release of collagenase and/or by decrease in production of a collagenase inhibitor in serum, α_1-macroglobulin.

* * * *

Although vitamin A is essential for maintaining the integrity of the cornea, no satisfactory biochemical mechanism has been deduced that would explain its function. Vitamin A deficiency gives rise to several biochemical changes in the cornea, but whether these changes are only indicative of the pleiotropic reaction of corneal cells or have a direct causal relationship to the observed pathology is yet unclear.

Vitamin A deficiency, which is often accompanied clinically by protein and calorie malnutrition, gives rise to several changes in the cornea. The eye becomes dry (xerophthalmic) due to interference with tear production, and the corneal epithelial cells become keratinized and lose their normal ordered architecture. Ultimately corneal ulceration and perforation of the cornea may occur.

Some of the changes observed early in vitamin A deficiency are possibly caused by disruption of the normal three layers of the tear film. A mucus layer, which lies next to the corneal epithelium and which seems to be produced by conjunctival goblet cells, is reduced in quantity. This reduction in the amount of mucus in tears is apparently caused by pathology in the conjunctival goblet cells. The corneal ulceration seen later may be caused by reduced

protection of the epithelium due to dryness of the eye. Concomitantly the activity of the proteolytic enzyme collagenase increases in the cornea. The active collagenase found in keratomalacia probably comes from both epithelial and stromal cells. It is not clear at this time whether the observed collagenase activity is the result of release from injured cells, or is actually the cause of the corneal ulcer. Plasma normally contains a collagenase inhibitor, alpha-macroglobulin, which is depressed in severe vitamin A deficiency. In vitamin A deficiency in animals the incorporation of some sugars into glycoproteins is also reduced. The relationship of these observations to the clinically observed changes, however, remains to be clarified.

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Systemic actions of vitamin A

Abstract: Vitamin A is necessary for maintenance of the normal mucociliary function of epithelial tissues. Biochemical investigations have shown that RNA synthesis is affected by the vitamin in a variety of tissues. Moreover, the biosynthesis of some glycoproteins is decreased in vitamin A deficiency and enhanced upon administration of excessive doses of the vitamin, *in vivo* and *in vitro*, in intact cell systems. Epithelial tissues phosphorylate retinol to yield retinyl phosphate, a compound which functions as an acceptor and donor of specific sugar residues to glycoconjugates. Retinoic acid is also effective in glycoprotein biosynthesis, but its molecular mode of action is unclear at present. Retinoids are effective preventive agents of chemical carcinogenesis in the skin, the respiratory tract, the breast and the bladder. They also affect cultured transformed fibroblasts by altering their saturation density and adhesion in the direction of normal growth. This effect is reversible upon removal of the retinoid. Thus both epithelial and mesenchymal cell systems need vitamin A for their normal functions. Whether one or several biochemical mechanisms are involved is unclear at present.

* * * *

Morphological aspects: Alterations in the maintenance of normal differentiative processes and functions are evident manifestations of deficiency of the fat-soluble nutrient, vitamin A. Such alterations are most easily detected in epithelial tissues, which in deficiency gradually lose their capacity to biosynthesize mucus and in some cases, such as in the respiratory mucosa, the conjunctival-corneal epithelium and the lining of the taste buds, are replaced by a layer of keratinocytes. Other epithelial tissues such as the intestinal mucosa, the liver, the germinal epithelium of the testis and the cells of the retina are also altered profoundly, but they are not replaced by a layer of keratinocytes. Thus, keratinization does not appear to be a necessary and specific result of vitamin A deficiency, but it occurs in some epithelia as a consequence of injury and lack of function.

Effect of vitamin A on RNA biosynthesis: Inasmuch as vitamin A deficiency causes replacement of rows of mucus-secreting epithelial cells in some tissues by a layer of keratinizing cells, the possibility that vitamin A directs epithelial cell differentiation by controlling the synthesis of RNA molecules involved in the formation of cell-specific proteins has been seriously probed.

That vitamin A stimulates the synthesis of RNA in the small intestine of vitamin A deficient rats both *in vivo* and *in vitro* was shown over a decade ago. Although different portions of the intestine are stimulated to different degrees, the nuclear-RNA fraction of deficient cells shows a consistent increase upon treatment with vitamin A. Another RNA fraction which is depressed in mucosal cells in vitamin A deficiency is transfer-RNA.

The liver reacts similarly. Within a few minutes after potassium retinoate is injected into vitamin A deficient rats, the formation of a nuclear RNA fraction increases over two-fold.

Studies with other epithelial tissues have confirmed and extended the above observations. In epithelial cells of the hamster trachea, for example, the rate of synthesis of high molecular weight RNA is depressed in vitamin A deficiency, and in mouse epidermal cells cultured in a vitamin A-free medium both RNA and DNA formation are stimulated by the addition of vitamin A and its analogues. Indeed the latter cell system has been ingeniously employed as a biological assay technique for vitamin A derivatives, inasmuch as the stimulation of RNA formation is dose-dependent.

Fast growing tissues, such as regenerating liver and estrogen-primed chick oviduct, are also sensitive to vitamin A. In vitamin A deprivation the amount of RNA and DNA in the tissue decreases and growth falters.

Molecular role of vitamin A in glycoprotein synthesis: As mentioned earlier, retinol is phosphorylated in a variety of tissues and in cultured cells. Retinyl phosphate is synthesized from retinol by hamster and rat liver *in vivo*, and is also found both in cultured hamster intestinal cells (35 ng/g) and in cultured mouse epidermal cells. Its ultraviolet absorption maximum is identical to that of retinol (absorption maximum at 325 nm), and it yields anhydroretinol as the product of mild alkaline hydrolysis. Retinyl phosphate has also been synthesized chemically along with other phosphorylated retinoids, such as the mixed anhydrides of retinoic acid and phosphoric acid and of 13-*cis* retinoic acid and phosphoric acid.

Phosphorylated retinol is present in membrane structures along with its glycosylated derivative mannosyl-retinyl-phosphate. This latter compound is formed *in vivo* by the action of a mannosyl transferase present in rat and hamster liver, in cultural epidermal cells and in human platelets. This mannosyl transferase is localized in membranes of the endoplasmic reticulum. Consequently, mannosyl-retinyl-phosphate is also synthesized by microsomal systems, but to a lesser extent than by intact cells.

A second mannosyl transferase, which uses another isoprenologue, dolichyl-phosphate, as a substrate, is also localized in the endoplasmic reticulum. Since this enzyme is much more active in microsomal systems than in the intact cell, the two lipid intermediates mannosyl-retinyl-phosphate and mannosyl-dolichyl-phosphate may well reside in different domains of the membrane and may have different functions.

In rat liver mannosyl-retinyl-phosphate may act as a cellular intermediate for the glycosylation of specific glycoproteins. Because of this involvement of retinol in mannosyl transfer reactions, it was not surprising to find that a severe deficiency of vitamin A is accompanied by a 79% decrease in the amount of macromolecularly-bound mannose in hamster liver. As mentioned earlier, the concentration of at least one glycoprotein, the α_1-macroglobulin of plasma, was also found to be decreased in vitamin A-deficient rats.

Thus retinol is phosphorylated to retinyl phosphate, which forms an integral part of the endoplasmic reticulum and carries out specific glycosylation reactions. Retinoic acid is also active in restoring glycoprotein biosynthesis to normal levels when administered to vitamin A-depleted hamsters. Little is known, however, of the mechanism by which retinoic acid replaces retinol in this function.

Effects of vitamin A and retinoids on transformed cells: Both natural and synthetic derivatives of vitamin A (retinoids) function as preventive agents in the development of epithelial cancer. These compounds are also active in reversing squamous metaplastic changes induced by vitamin A deficiency in cultured hamster tracheas.

In addition to their activity as preventive agents, retinoic acid and retinol also inhibit the growth of established transformed cell lines. For example, cultured transformed mouse fibroblasts (L-929 cells), upon treatment with retinoic acid (5 μg/ml), grow less well and reach a saturation density one fourth that of their untreated controls. This effect appears to be due to a restored contact-dependent inhibition of growth rather than to toxicity or increased nutritional requirements of treated cells. The same phenomenon has been observed in a variety of other transformed cell lines.

In addition to the restoration of contact inhibition of growth to transformed cells, the adhesion of cells to the tissue culture surface and to each other is profoundly increased by retinoids at concentrations as low as 0.1 μg/ml. In the presence of retinol only 5-10% of trans-

formed mouse fibroblasts (3T12-3 cells) are released from the tissue culture dish by low concentrations of EDTA, whereas in its absence 95% are released.

These two phenomena, the restoration of contact inhibition of growth and increased adhesion, may reflect a modification of the transformed phenotype in the direction of normal growth, and could therefore explain some of the inhibitory effects of vitamin A on the growth of some transplanted tumors.

Since not all cell lines respond to retinoid treatment, however, it would be of interest to investigate whether responsiveness to retinoids is mediated by specific binding proteins at the surface of tumor cells. Since a variety of normal, embryonic and transformed tissues of mesenchymal and epithelial origin contain cellular binding proteins for vitamin A, the effectiveness of vitamin A in nhibiting tumor cells might be predicted from assays for these specific binding proteins.

Neoplastic transformation, or at least its clinical manifestations, usually appears late in life. Thus, along with other factors, the availability of retinoids in older persons may well influence the expression of the transformed phenotype *in vivo*. Consequently, low levels of vitamin A in the liver, impaired plasma transport, or defective uptake by cells may well allow promoters of carcinogenesis to be more active.

It thus appears that vitamin A is functional in a variety of cellular systems of epithelial and mesenchymal origin. It is not clear at present whether such diverse functions may be explained by a common biochemical reaction. Similarly, although the biochemical reactions and linkages in which retinoids are involved in a variety of systems may be the same, their phenotypic expression may differ as a result of the nature of the specific systems on which they act.

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APPLIED ASPECTS

Prenatal considerations

Abstract: Birth defects or interruption of pregnancy can result from either vitamin A deficiency or hypervitaminosis A. During development of the human fetus, the placenta regulates the passage of vitamin A from the mother to the fetus, probably by its recognition of maternal *holo*-RBP. In spite of a relatively constant intrauterine ambient, the range of plasma retinol values and liver reserves of vitamin A in human fetuses is quite large. Whereas plasma retinol values in the fetus tend to remain relatively constant during gestation (12-40 weeks), liver vitamin A concentrations tend to peak around 28 weeks and then to decrease until term. Regardless of the vitamin A status of the mother, the newborn infant has only marginal reserves of vitamin A.

* * * *

Vitamin A deficiency during pregnancy has marked effects on the development of the fetus. Although known effects of vitamin A deficiency on fetal development are necessarily derived from animal experimentation, similar interactions in all likelihood also occur in humans. In experimental animals two major effects of vitamin A deficiency are noted: resorption or abortion of the fetus and the appearance of birth defects. Early in development placental abnormalities appear and the fetal circulatory system fails to develop. At term abnormalities of the eye are often observed, from complete absence (anophthalmos) of the eyeball through microphthalmos to various kinds of severe histological defects. Harelip and abnormalities of the genito-urinary tract are common, and many other tissues of the fetus may be affected. Truly deficient pregnant females usually resorb the fetus, whereas those who ingest a small amount of vitamin A often carry the malformed fetus to term. Thus, the vitamin A status of the mother is important in the normal development of the fetus.

The dietary intake of carotenoids and vitamin A is low among poor pregnant women in many parts of the world. Furthermore, mean plasma retinol values of these women (30 μg/dl) tend to fall significantly during the third trimester (20 μg/dl). As a consequence both the plasma values and the liver stores of neonates born to malnourished women are considerably lower than those of well-nourished mothers. Thus, otherwise normal infants are handicapped at birth by very limited reserves of vitamin A. After birth, plasma retinol values of lactating mothers recover to normal levels. Interestingly, neither the duration of lactation nor the birth order of the nursed baby seems to affect maternal plasma retinol values. Traditional dietary restrictions exercised during pregnancy and lactation, however, tend to lower intakes of carotenoids and vitamin A, which must ultimately affect the nursing child.

On the other hand, too much vitamin A can also cause a lot of trouble. Again, much of the knowledge we possess is derived from animal experimentation. Hypervitaminosis A inhibits implantation of the fertilized ovum and causes uterine hemorrhage and fetal resorption early in pregnancy. The neural crest often fails to close. In newborn animals from hypervitaminotic dams, birth defects are also commonly observed, such as lack of skull closure over the brain (anencephaly), harelip, cleft palate and cataracts. Microcephaly, a deranged central nervous system and hypoplastic adrenals were noted in a malformed fetus born to a woman who ingested 150,000 IU of vitamin A daily from the nineteenth through the fortieth day of gestation. It must be stressed that chronic hypervitaminosis A is only produced by very large doses of vitamin A, about 1000 times the daily requirement, which are continued over weeks or months and are associated with chronic high plasma vitamin A levels ($\geqq$ 200 μg/dl). *Holo*-RBP does not change appreciably in hypervitaminosis A; most of the circulating vitamin A, therefore, is lipoprotein-bound retinyl ester. Since such high doses are rarely if ever present in a normal diet, only the chronic use of vitamin A supplements of high potency might give rise to reproductive abnormalities.

During development of the fetus, the placenta serves an important role in modulating the transfer of vitamin A from the mother. In all likelihood the actual transferring agent is

a complex between retinol and a specific plasma carrier, retinol binding protein (RBP), which is formed in the mother's liver and carried to the placenta in the maternal blood in association with prealbumin. Although we are not sure of the exact way in which vitamin A is transferred across the placenta, an identical retinol-RBP complex is found in the fetal circulation. Early in gestational development, the intact complex of retinol and retinol binding protein may pass directly into the fetal circulation from the maternal blood. In birds as well, *holo*-RBP passes freely into the egg yolk. At a later stage, the formation of retinol binding protein begins in the fetal liver, and thereafter most circulating fetal RBP is probably derived from that source. Since the concentration of the RBP complex in either maternal or fetal plasma does not change much, even when other forms of vitamin A, such as retinyl ester, are present in large amounts in the maternal plasma, the specific carrier and placental transfer functions of RBP may also protect the fetus from an overdose of vitamin A.

Rather surprisingly, the concentration of vitamin A in the plasma and liver of developing human fetuses varies within relatively wide limits (2-110 μg/dl plasma and 1-52 μg/g for liver in one study), in spite of the relatively constant intrauterine environment. In regard to gestational age, plasma retinol concentrations remain relatively constant from 12 weeks to term, perhaps with a slight downward slope, whereas liver concentrations tend to increase until the 28th week and then to fall until term. This decrease in the liver concentration of vitamin A in the third trimester is probably due to a rapid fetal growth rate. Nonetheless, the total amount of vitamin A in the human fetus becomes maximal at term.

The plasma concentration of vitamin A in human fetuses, as in adults, bears no predictable relationship to the total body reserve except at very low levels (< 5 μg retinol/dl). Furthermore, the plasma level in the newborn is usually about 50-70% that in the maternal plasma, largely because the steady-state level of retinol binding protein is lower in the fetus.

Because of the placental regulation of vitamin A transport and the rapid growth of the fetus during the last trimester, newborn children have a relatively small vitamin A reserve. The median liver concentration of retinol in neonates from a dozen or so studies in many countries is around 15 μg/g, and the total body pool is roughly two mg of retinol. This amount of vitamin A, using a reasonable linear utilization rate of 80 μg/day, would last for only 25 days. Thus, newborn infants are clearly in a *marginal* vitamin A status, and definitely need a substantial intake of vitamin A both to meet demands for growth as well as to augment their body reserves. Colostrum is a very good source of vitamin A (100-300 μg retinol/dl), and breast milk is a good source (50 μg retinol/dl). In fact, the recommended daily allowance for infants of 420 μg retinol/day is based on the presumed ingestion of 840 ml of breast milk/day by healthy infants. Newborn infants, of course, do not consume that volume of breast milk. In addition, both the mean concentration of vitamin A in breast milk and the mean daily volume are appreciably lower (~20 μg/dl) in many countries of the world than the values cited above.

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Status of Mothers and Infants in Poor Communities of India. *Trop. Pediat.* 61: 262, 1962.

Recommended dietary allowances, tissue requirements and marginal vitamin A status

Abstract: From an experimental standpoint, the recommended dietary allowances for vitamin A from various countries are in the right range for adults but are less firmly based for children and other groups. Defects in the plasma transport, cellular uptake and intracellular utilization of vitamin A, as well as a lack of the dietary vitamin, may affect the appearance of signs of deficiency. Plasma levels of retinol, which are necessary for the normal function of various tissues, are not known. Experimental animals with a low vitamin A intake have a poor appetite, are listless and grow slowly, even when free of infection. Inasmuch as similar symptoms are seen in nutritionally disadvantaged children in their local ambients, subclinical vitamin A deficiency may often contribute to the syndrome of the generally malnourished child.

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In the United States the recommended daily allowances of retinol equivalents for various groups are as follows: infants, 420 μg; children 0.5 to four years old, 400 μg; adult females, 800 μg; adult males and pregnant women, 1000 μg; and lactating women, 1200 μg. Other countries have made similar recommendations. The allowance for infants is based on the amount of vitamin A ingested by healthy infants from the breast milk of their well nourished mothers, whereas that for adults is based on the daily intake of vitamin A which maintains plasma vitamin A concentrations in depleted subjects above 20 μg retinol per deciliter plasma. Values for other groups have been extrapolated from these two norms. In a recent repletion study conducted with vitamin A-depleted adult volunteers, 300 μg of retinol were required to correct most symptoms of vitamin A deficiency, 600 μg to correct all symptoms and to raise plasma retinol concentrations above 20 μg/dl and 1200 μg to raise plasma retinol concentrations to 30 μg/dl. Currently, recommended allowances for adults seem therefore to be in the right range, whereas those for infants and children are based on less firm data.

Clinical signs and symptoms of vitamin A deficiency occur when target tissues are undersupplied with the vitamin. Too little vitamin A in the blood is the most likely cause for an inadequate supply, but depressed concentrations or alterations in membrane receptor sites or intracellular binding proteins may be contributing factors in some cases. Night blindness is usually the first specific symptom of vitamin A deficiency in young children. The plasma level of vitamin A below which night blindness appears has not been defined, however, mainly because quantitative methods for measuring dark adaptation or for measuring rhodopsin levels are not applicable in small children. Nonetheless, nightblindness is likely to occur when plasma vitamin A levels are below 10 μg/dl in children and may occur when they are below 30 μg/dl in adults. Considerable individual variation has been observed in adults; impaired adaptation may occur, for example, even when the plasma retinol level is above 30 μg/dl. On the other hand, color vision is much less affected by vitamin A deficiency.

At the present time, it is not possible to associate precise blood levels with impaired functions in tissues other than the retina. Indeed, the blood level of vitamin A required to meet functional needs is likely to vary from tissue to tissue in accord both with the physiological activity of the tissue and its specific requirement for vitamin A. Histological changes generally do not occur in animals when plasma levels are above 20 μg/dl, but are found to a varying degree in different tissues when plasma levels are below 10 μg/dl. When liver stores approach depletion levels (< 10 μg/g) in rats on a diet containing very little vitamin A, plasma vitamin A levels fall to the 10-20 μg/dl range. These rats, although not showing obvious eye signs of deficiency, are prone to severe infections, have a reduced appetite, inefficiently metabolize protein and grow at a reduced rate. When rats are raised under germ-free conditions on diets lacking in vitamin A, they eat less and grow at a rate slower than normal. Although showing some changes in the external eye, they nonetheless survive well beyond the time that conventionally reared rats on the same vitamin A-deficient diet have died. Therefore, rats with subclinical vitamin A nu-

TABLE II

Suggested Guidelines for Assessment of Relative Levels of Vitamin A Nutrition in Young Children

Retinol Status	Dietary Intake (retinol equivalents μg/day)	Liver (μg/g)	Plasma (μg/dl)	Clinical Signs
Adequate	>400	>20	>20	None
M A R G I N A L				Possibly contributes to decreased growth, appetite and resistance to infection
Critical	<200	<10	<10	Xerophthalmia
Clinical Deficiency				Nightblindness Conjunctival-xerosis Corneal xerosis Corneal ulceration Keratomalacia

trition living under germ-free conditions are still "at risk" in terms of their overall growth response.

Children living in disadvantaged communities often show symptoms analogous to those seen in experimental animals which are subclinically deficient in vitamin A. Obviously non-specific symptoms such as poor appetite, reduced growth rate, listlessness and an increased incidence of infection cannot be attributed solely to inadequate vitamin A nutrition. Supportive dietary and biochemical data, such as those shown in Table II, are needed to implicate marginal vitamin A status in the syndrome. Nonetheless, inadequate vitamin A nutrition, quite apart from the specific signs of acute deficiency, may well contribute to the above-cited symptoms of the disadvantaged child, and perhaps to the other health risks as well.

The "at risk" concept, as applied to the health and nutrition of young children, has been defined by a committee of the International Union of Nutritional Sciences as a "major, identifiable biological or environmental circumstance or event which increases the risk of severe illness." The parallels noted between animal studies and human populations suggest that liver stores below the range of 10 μg/g wet weight and plasma levels in the range of

10-20 μg/dl define an "at risk" child population for which preventive programs deserve consideration. Unfortunately, simple non-invasive procedures are not currently available for determining liver reserves of vitamin A in children, and plasma levels of vitamin A in the cited range may result from reduced protein intake, infection and other factors in addition to marginal vitamin A status. Nonetheless, the effects of marginal intake of vitamin A on the optimal growth and development of young children must be appreciated.

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Stimulation of plasma retinol levels by small doses of vitamin A

Abstract: As a result of vitamin A deficiency as well as other reasons, vitamin A concentrations may be depressed in the plasma and *apo*-RBP levels may be elevated in the liver. When a small dose of vitamin A is given orally, plasma *holo*-RBP concentrations rise. When liver reserves and plasma level are initially low, the rise is significant, rapid and sustained. Other conditions give different responses. Thus a new parameter, the relative dose response (RDR), might be useful in distinguishing subclinical vitamin A status from other factors which depress plasma retinol levels.

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As the liver reserves of vitamin A are depleted in children on a diet low in vitamin A, the plasma concentration of *holo*-RBP falls from the normal value of 20-30 μg/ml to very low levels. Nonetheless, *apo*-RBP continues to circulate in the plasma at a concentration of about 12 μg/ml. Similar changes have been noted in rats.

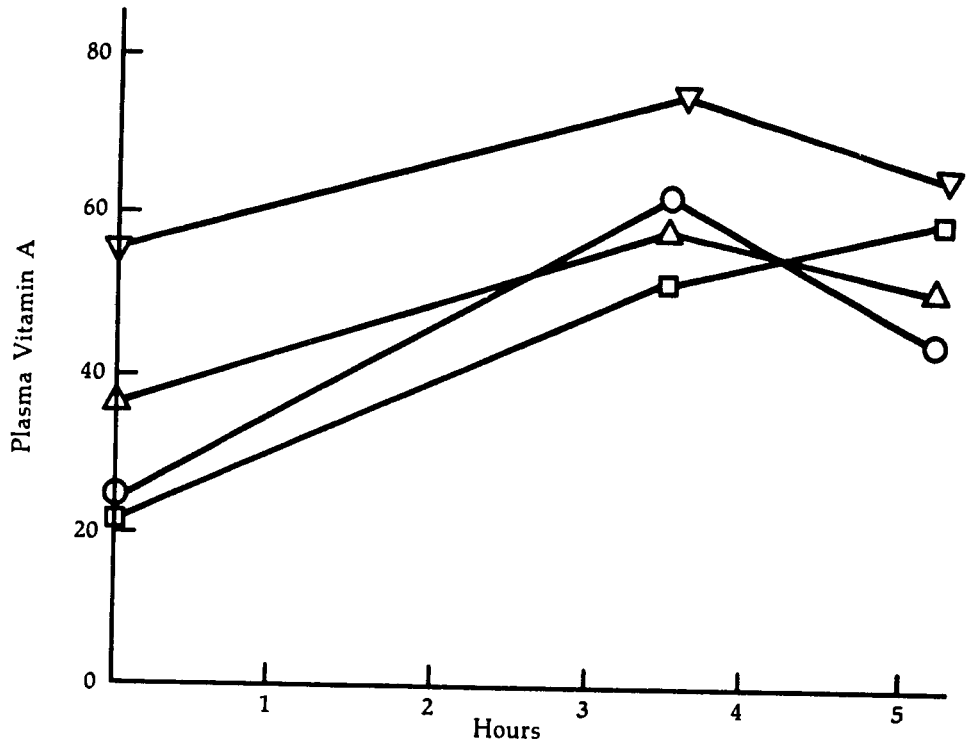
When either vitamin A-depleted children or rats are given a single small oral dose of vitamin A under fasting conditions, a surge in the plasma level of *holo*-RBP occurs which peaks at four to six hours after dosing. The plasma level of vitamin A reached in five hours and the level sustained over the subsequent 24 hours is related to the amount of vitamin A given. When small doses are given to children, for example, *holo*-RBP levels fall for five to 24 hours and then rise slowly to normal levels in a week. Daily feeding of a small dose to previously depleted rats in the absence of protein deficiency will sustain normal plasma levels indefinitely but may not lead to a buildup of liver reserves. When protein energy malnutrition (PEM) complicates the deficiency in children and rats, an immediate post-dosing surge in *holo*-RBP is followed by a return to low pre-dosing plasma levels. Marasmic children show a greater surge than do those with kwashiorkor. As the protein deficit is corrected and RBP synthesis is reestablished, however, the plasma level slowly rises into the normal range. The magnitude of the plasma response and the time period over which maximum plasma levels are sustained are dependent on the dose of vitamin A given, the relative level of vitamin A in the liver reserve and the initial fasting blood level.

As noted earlier, no practical field method to assess subclinical vitamin A deficiency now exists. Plasma levels of vitamin A are commonly in the 10-20 μg/dl range, well below normal (≧ 30 μg/dl) but still above the level characteristic of depletion (< 10 μg/dl).

Plasma levels of vitamin A may be depressed by inadequate vitamin A intake or for a variety of other reasons. Since vitamin A is in a steady state in the plasma, separate feedback controls must exist for RBP synthesis and for the formation and release of *holo*-RBP. Thus the response of the liver to small oral doses of vitamin A may provide a practical method for the assessment of subclinical vitamin A nutrition in a local ambient.

The availability of endogenous or dietary retinol may well be the rate-limiting factor in maintaining normal plasma *holo*-RBP levels in subclinical vitamin A deficiency, but should not be rate-limiting when plasma levels of *holo*-RBP are lowered by other factors. Thus the surge in *holo*-RBP which follows the administration of a small oral dose of vitamin A may specifically reflect liver stores of vitamin A. When the reserves are below that critical level which triggers *holo*-RBP secretion, *apo*-RBP would be expected to accumulate in the liver. Some *apo*-RBP might accumulate for other reasons as well. Although the release of accumulated hepatic RBP following a small dose of vitamin A would be expected in both situations, the rise would only be sustained and normal steady state plasma levels would be reached in

FIGURE III



The response of plasma retinol levels to a small oral dose of vitamin A at zero time in rats differing in vitamin A status. ▽, satisfactory plasma and liver levels, RDR <50%; △, satisfactory plasma level with any liver reserve, RDR <50%; ○, low plasma level but satisfactory liver reserve, RDR <50%; □, low plasma and liver reserves, RDR >50%. Satisfactory plasma values were taken as ≧30 μg retinol/dl; satisfactory liver reserves were taken as ≧10 μg/g.

individuals who initially were subclinically deficient in vitamin A. In individuals with low plasma levels who are not depleted in vitamin A, a return to this lower steady-state level would be expected soon after dosing.

The relative response to the dose (RDR) is calculated as a percentage from the absolute rise in plasma retinol between zero and five hours post-dosing divided by the level reached at five hours. Thus,

$$\text{RDR}\ (\%) = \frac{A_5 - A_0}{A_5} \times 100$$

where A_5 = plasma retinol level, in μg/dl, at five hours after dosing and A_0 = plasma retinol level, in μg/dl, in the fasting subject before dosing. A five-hour interval is chosen since most vitamin A-sufficient individuals have reached peak post-dosing levels before this time and their plasma levels are declining. Typical patterns of the plasma response to a small oral dose of vitamin A in rats differing in relative vitamin A states are given in Figure III.

The relative dose response (RDR) has been tested in weanling rats by depriving them of a dietary source of vitamin A and challenging them with a small oral dose during progressive stages of depletion. An RDR above 50% occurred only when plasma levels were less than 30 μg/dl and liver reserves were less than ten μg/g. Plasma levels below 30 μg/dl because of chronic inadequate protein nutrition, growth limitation or other factors not related to depleted liver vitamin A stores were invariably associated with an RDR of less than 50%. Animals with initial plasma levels above 30 μg/dl did not show an RDR above 50%, even when the liver reserve was less than ten μg/g. Hence, the RDR assessment clearly distinguished rats with lowered plasma levels *and* reduced liver stores from those with lowered plasma levels but *without* reduced stores. The RDR assessment, however, did not identify rats with reduced liver stores which had plasma levels greater than 30 μg/dl.

This procedure has not as yet been tested in children who live under the usual complex ecologic conditions associated with hypovitaminosis A. These children frequently have plasma levels in the range of 10-30 μg/dl. Confounding factors known to lower plasma vitamin A levels include multiple concurrent nutrient deficits and physiological, physical and social stresses of various kinds. Children *acutely* deficient in protein fail to respond to an oral dose of vitamin A because they lack the amino acids needed to synthesize RBP. When protein is provided to protein-deficient children, plasma levels of vitamin A rise, but only after a period of time in excess of the five hours used in the RDR assessment. Whether children *chronically* undernourished with respect to protein and calories will respond to the RDR assessment approach has not yet been explored.

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The dynamics of vitamin A utilization and its relation to vitamin A requirements

Abstract: From the turnover and utilization

rates of endogenous vitamin A, the daily intake of vitamin A required to maintain total body stores at a specified level can be calculated. The importance of recycling mechanisms in conserving vitamin A in the body thus becomes clear. In several cases which are considered, the calculated intakes agree reasonably well with the recommended daily allowances for that age group. Thus the use of *in vivo* kinetic analysis seems appropriate both in extending our knowledge about nutritional interactions affecting vitamin A metabolism as well as in better defining vitamin A requirements.

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By using knowledge about the utilization rates of vitamin A for various processes, we can approach the requirement for vitamin A in a different way; namely, to assess the amount of vitamin A which must be ingested in order to maintain the total body reserve at a given level. Factors which influence these utilization rates, such as age, sex, pregnancy, lactation, infection and the like, might also be evaluated in a quantitative way and included in the calculation. Let us take the example of a 60 kg adult with a liver vitamin A concentration of 100 μg/g, which is roughly the median value for healthy adults from many countries. Since the liver weighs about three percent of the body weight and contains roughly 90% of the total vitamin A in the body, the total body reserve of retinol would be 200 mg. Since the mean half-life for vitamin A in adult liver is about 140 days, stored vitamin A should be replaced at a rate of 0.5% of the total body reserve per day, or 1000 μg. If 75%, 50% and 25% of the ingested vitamin A is stored, dietary intakes of 1330, 2000 and 4000 μg retinol, respectively, would be needed to maintain a total body reserve of 200 mg. Since this reserve is five-to ten-fold greater than the minimal acceptable reserve, these calculated dietary intakes are considerably higher than the estimated RDA of about 1000 μg retinol/day for adults.

If the same adult has a liver vitamin A concentration of only 10 μg/g, the total body reserve would be around 20 mg. At such relatively low levels, the dominant utilization process would be the constant formation of a retinol-RBP complex rather than the first-order replacement rate. Since the plasma volume would be about 2.2 liters, the total amount of retinol circulating on RBP would be about 1.1 mg. At a fractional metabolic rate for RBP of 2.7 per day, three mg of retinol would be used daily for *holo*-RBP formation. Thus the total body pool of vitamin A would be utilized in a seven-day period. Clearly, significant recycling of vitamin A back to the liver must occur. Under these conditions as well, the fractional metabolic rate for plasma RBP may well be lower and in all likelihood the irreversible oxidation of retinol in peripheral tissues is slower.

Finally, let us consider a three-year-old child weighing 15 kg with a liver retinol concentration of 20 μg/g. The total body reserve of retinol would be ten mg. The replacement rate might be calculated with respect to both liver turnover and RBP synthesis. by using an estimated half life of 50 days for liver retinol, the fractional metabolic rate would be 1.4% of the total body pool per day, or 140 μg. If the efficiency of storage is 50%, the child should ingest 280 μg retinol per day. At a plasma RBP concentration of 20 μg/ml, the total plasma RBP pool would amount to 11.1 mg, which would bind 150 μg retinol. If replaced 2.7 times daily, retinol used daily for *holo*-RBP synthesis would be 410 μg. A significant portion of that retinol would be recycled back to the liver. However, if *no* recycling takes place and the storage efficiency is 50%, 820 μg retinol should be ingested daily to maintain the steady state. These calculated dietary intake values of 280 and 810 μg retinol are again in accord with the RDA of 400 μg retinol for 0.5 to 4-year-old children.

The above calculations are not intended as a definitive expression of the required intakes of vitamin A for different age groups or reserve levels, but rather as examples of a different approach to the definition of vitamin A needs. The examples also point out the need to obtain more information about the turnover of vitamin A in the liver and of RBP in the plasma as a function of age and stress and of the extent of recycling under various conditions. The fact that calculated values based on the concept agree reasonably well with those obtained empirically bodes will for the use of these procedures in the future.

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Vitamin A requirements and disease

Abstract: Vitamin A requirements are increased by diseases which reduce the intestinal absorption of carotenoids and vitamin A, increase the rate of tissue metabolism or reduce the efficiency of retinol transport and utilization. Thus diseases which affect the intestine, pancreas, liver and kidneys most markedly alter vitamin A needs. Plasma retinol levels are usually depressed in diseased states; but in some cases, such as nephrosis, they may be increased.

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Diseases influence the requirements for vitamin A by altering absorption efficiency, the rate of tissue metabolism, or the efficiency of utilization. In general, plasma retinol levels are depressed in syndromes involving defective absorption or transport of lipids, in infectious processes with or without pyrexia, in some chronic debilitating diseases, in disorders that increase the metabolic rate, in diseases that affect hepatic function and in conditions that interfere with normal renal function. Knowledge of the circumstances necessary for the efficient absorption of carotenoids and vitamin A from food and knowledge of the metabolism of vitamin A transport proteins provides the basis for understanding how diseases influence requirements.

The efficiency absorption of the highly nonpolar carotenoids, and to a lesser extent the more polar preformed vitamin, is dependent upon the effective digestion and absorption of dietary lipids. Chronic lipid malabsorption syndromes that result from ineffective lipolysis (e.g., pancreatitis or pancreatic insufficiency), defective micelle formation (e.g., biliary cirrhosis or bile acid insufficiency) or from atrophy of or a reduction in mucosal surface area (e.g., sprue, chronic diarrhea or acute protein-calorie malnutrition) limit the availability of preformed vitamin A and precursor carotenes from food sources. If a vitamin A supplement is not provided to persons with these conditions a deficiency may in time develop.

Diseases that increase the rate of tissue metabolism increase requirements for vitamin A. This is evidenced by a lowered plasma level in hyperthyroidism and febrile diseases. Presumably the decline in plasma level is due to an increased rate of catabolism of vitamin A in tissues.

The efficiency of utilization of vitamin A, and therefore its requirement, is affected by both liver and kidney diseases. The liver is both the site of synthesis of retinol-binding protein and the major storage organ for vitamin A. Whether retinol is derived from dietary or endogenous sources, the available evidence indicates that retinol binding to RBP occurs within the liver. Parenchymal cell disease such as hepatitis, therefore, may limit the synthesis of *apo*-RBP, the formation of prealbumin, the synthesis of the storage lipoglycoprotein and the release of retinol from stored retinyl esters. Since the molar ratio of *apo*-RBP to *holo*-RBP and of *holo*-RBP to prealbumin are normal in liver disease, the overall hepatic synthesis of protein is reduced whereas control mechanisms seem to remain intact.

Another disease in which vitamin A metabolism has been studied and found to influence vitamin A requirements is cystic fibrosis. This condition is characterized by an inefficient absorption of lipids by the intestine secondary to defective hydrolysis, lipolysis and micelle formation. Hepatic function is also impaired, although the nature of the primary hepatic defect has not been defined. Whatever the underlying cause, the synthesis or release of *holo*-RBP is subnormal in cystic fibrosis.

The kidneys are a major pathway of RBP catabolism. Free uncomplexed RBP or *holo*-

RBP are small enough molecules to be filtered by the glomeruli, whereas PA and the PA-*holo*-RBP complex are not. The tubule cells efficiently reabsorb and catabolize filtered RBP or *holo*-RBP, and very little retinol or RBP normally appears in the urine. In tubule dysfunction that results in proteinuria of whatever etiology, however, large amounts of RBP are lost in the urine. In contrast to tubular dysfunction, renal diseases that depress glomerular filtration cause abnormally high circulating levels of vitamin A and an elevated molar ratio of RBP to vitamin A.

Ophthalmic disorders such as retinitis pigmentosa do not appear to be related directly to vitamin A intake. In retinitis pigmentosa, for example, plasma concentrations of retinol and *holo*-RBP are normal. Vitamin A metabolism in chronic disease states such as cancer, diabetes and coronary heart disease has not been extensively studied. Disorders of the circulatory system in which the flow of blood is reduced through the liver and kidney, however, will impair the clearance of RBP and result in elevated plasma levels. Thus in future studies, not only vitamin A intake and storage, but also the integrity of the plasma transport system, the uptake of retinol by target cells and the dynamics of vitamin A utilization should be explored.

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Vitamin A and infection

Abstract: Vitamin A has been called the "anti-infective" vitamin because its deficiency gives rise to a wide variety of infections in experimental animals. Many aspects of the immune response may be depressed in vitamin A deficiency, namely non-specific systems like mucus production and phagocytosis, humoral immunity and cell-mediated immunity. In contrast to the increased susceptibility to infection noted in the deficiency state, a large dose of vitamin A serves as an effective adjuvant, enhances phagocytosis, shortens the survival time of allogenic skin grafts and changes the surface characteristics of cells. On the other hand, a large dose of retinoic acid inhibits interferon synthesis and a large dose of retinaldehyde blocks complement-included hemolysis.

Children with vitamin A deficiency commonly suffer as well from protein calorie malnutrition and an inadequate intake of other nutrients. The increased susceptibility to infection noted in vitamin A-deficient children, therefore, is probably due to multiple causes of a nutritional and socioeconomic nature.

Just as vitamin A deficiency affects infections, so also do infections influence the transport and utilization of vitamin A. Plasma values of retinol, RBP and albumin are significantly lowered by infections, and the rate of vitamin A metabolism is greater. Of various infections, acute hepatitis and measles have the most marked effect, followed by upper respiratory infections, chicken pox and bronchitis. In contrast, diarrhea tends to increase plasma retinol levels, mainly because of dehydration and resultant hemo-concentration. Chronic parasitic infestation may also lower plasma retinol values, probably by interfering with the intestinal

absorption of carotenoids and vitamin A.

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Fifty years ago Green and Mellanby called vitamin A the "anti-infective vitamin," largely because vitamin A-deficient rats were particularly afflicted by abscesses of the salivary glands and by infections of the alimentary tract, kidney and bladder. An increased susceptibility to lung infections in vitamin A-deficient animals has been noted by others. More recently vitamin A-deficient rats maintained in a germ-free condition or treated with antibiotics have been shown to survive for much longer periods than do deficient rats in a conventional ambient.

In children with vitamin A deficiency, the incidence and severity of infection tends to be greater than in normal children. Since protein calorie malnutrition as well as the inadequate intake of other nutrients are often associated with vitamin A deficiency in children, the relative importance of the lack of various dietary factors as causative agents in the process of infection is difficult to assess. Nonetheless, several interrelationships between vitamin A and infection might be considered, namely, 1) the effects of the deficiency on specific immunological processes, 2) the effects of large doses of vitamin A on various aspects of the immune response, and 3) the effects of infection on the transport and utilization of vitamin A. Stress must be placed on the marked difference between changes which occur in a *deficient* state and are corrected by *small* amounts of vitamin A, and changes which are induced in *normal* animals by *large* pharmacological doses of vitamin A.

Immunological defense mechanisms can be considered as a group of several interacting systems: 1) non-specific mechanisms, such as mucus production and non-specific phagocytosis; 2) the specific humoral system, in which plasma cells derived from B-lymphocytes produce circulating antibodies against a given antigen; 3) cell mediated immunity, which involves immunologically active T-lymphocytes; and 4) the complement system of plasma, which induces the lysis of cells with bound humoral antibody. Interactions among these systems include both synergistic and antagonistic relations among B-lymphocytes, T-lymphocytes and macrophages.

In vitamin A-deficient rats the number of mucus-secreting cells markedly decreases, particularly in the upper intestine. The general process of keratinization in the trachea, salivary gland ducts, skin and elsewhere is also accompanied by a reduction both in mucus cell number and in mucus secretion. The fact that reduced amounts of the immunoglubulin IgA are found in intestinal secretions of vitamin A-deficient children may well be caused by a reduced number of intestinal goblet cells. Reduced mucus secretion, therefore, may well facilitate the entry of bacteria and other infective agents into the child.

The most dramatic effects of vitamin A deficiency on the humoral system have been observed in deficient chickens. Lymphocyte and plasma cell populations in the Bursa of Fabricius and nasal and paranasal lymphoid glands are depleted. Subsequent infection with Newcastle disease virus further depletes plasma cell and bursal lymphocyte populations, and the overall resistance to infection is markedly reduced. In vitamin A-deficient children the γ-globulin fraction of plasma is lowered, but in this case protein calorie malnutrition might well be a contributing factor.

Cell mediated immunity, which is markedly impaired in severe protein calorie malnutrition, is also affected by vitamin A deficiency. In vitamin A-deficient rats the mitogenic response of splenic lymphocytes, which are roughly an equal mixture of B and T cells, to concanavalin A, phytohemaglutinin (PHA) and *E. coli* lipopolysaccharide is depressed to about 30% of normal levels. In children with clinical signs of vitamin A deficiency the number of circulating T-lymphocytes is lowered and the bacteriocidal activity of the plasma leukocyte fraction is reduced.

Total serum hemolytic complement is depressed in children suffering from kwashiorkor, due both to reduction in several factors of the complement system as well as to increased serum anti-complement activity. In protein-deficient rats a similar change occurs except that anti-complement activity is absent. Interestingly, vitamin A deficiency alone gives *higher* than normal complement values in rats, which offsets the depressing effects of protein deficiency on total serum hemolytic complement activity.

Large pharmacological doses of vitamin A

and its analogues have marked effects on the immune system. Firstly, vitamin A serves as an effective adjuvant in rendering bovine γ-globulin immunogenic in mice and in stimulating seven-fold the number of hemaglutinin forming cells in the spleen of mice or chicks challenged with sheep red blood cells. Phagocytosis of *E. coli* is also enhanced. In the cell-mediated system the survival time of allogenic skin grafts is shortened by large doses of vitamin A, possibly due to its stimulation of cytotoxic T-lymphocytes. Vitamin A also has therapeutic and prophylactic effects in mice inoculated with viruses, and inhibits the growth of cultured murine leukemic cells *in vitro*. Retinol, which is known to associate physically with membranes and lipid bi-layers, may well act in cell mediated immunity by changing the surface properties of allogeneic or tumor cells sufficiently to allow their recognition by macrophages or T-lympocytes. Indeed, retinol has been shown to have a neuraminidase-like effect on the surfaces of ascites tumor cells and human lymphocytes. On the other hand, large doses of vitamin A can also have suppressive effects; retinoic acid prevents interferon synthesis in tissue culture cells challenged with Newcastle disease virus, for example, and retinaldehyde strongly inhibits the lysis of sensitized sheep erythrocytes with guinea pig complement.

Infections tend to lower the concentrations of vitamin A, RBP and albumin in the plasma of both children and adults, whereas globulin levels are raised. In a group of 24 children from two to seven years of age, the mean plasma values of retinol, RBP and albumin during infection were 82%, 90% and 90% respectively, of those found during the recuperation phase. In children, measles has a particularly striking effect, lowering mean plasma retinol, RBP and albumin values to 50%, 76% and 89% respectively, of those in uninfected control children. With respect to other childhood diseases, plasma retinol levels were only 72% of normal during upper respiratory infections, 87% of control values during chicken pox and 94% of normal during bronchitis. During severe diarrhea in children, plasma retinol and RBP values were often *higher* than normal, presumably due to marked dehydration and hemo-concentration. Acute hepatitis, which has been primarily studied in adults, also lowers plasma vitamin A values dramatically, i.e., to about 30% of normal. Indeed, plasma retinol concentrations were inversely related to serum activities of glutamate-pyruvate transaminase, an enzyme commonly used as an indicator of liver damage.

In all types of infection plasma retinol values are more affected than RBP levels. During infection plasma carotene concentrations tended to change in the same manner as plasma retinol values. Changes in blood carotenoids, however, reflect modification of the dietary pattern or malabsorption caused by the disease rather than the dynamics of retinol released from the liver and its tissue utilization.

When RBP levels fall to roughly 50% of normal levels in adults with chronic diseases of the intestine or liver, dark adaptation is somewhat impaired. Severe infections may therefore interfere with the proper utilization of vitamin A and give rise to a localized tissue deprivation of the vitamin, even when total body stores are adequate. If the deprivation occurs among cells involved in the immune response, a vicious cycle may be established in which a marginal vitamin A status may allow the development of an infection, which may further impair the vitamin A status of lymphocytes and macrophages, which in turn may increase the severity of the infection.

Parasitic infestation may also influence plasma retinol values. In human adults infested with intestinal flukes *(Fasciolopsis)*, for example, the mean plasma retinol value was only 30% that of normals, whereas hookworm *(Ancyclostoma)* or liver fluke *(Opisthorchis)* infestations tended to lower mean plasma retinol levels to about 80% of normal values. The absorption of vitamin A in children suffering from ascariasis or giardiasis is also impaired. In most instances intestinal parasites primarily act by reducing vitamin A and carotene absorption. Over an extended period, liver stores of vitamin A are depleted and plasma retinol values fall.

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Nutritional relationships with protein and fat

Abstract: Acute protein deficiency interferes with carotenoid and vitamin A absorption in the intestine and with the cleavage of carotenoids into vitamin A in the intestinal mucosa. In addition, the synthesis of *apo*-RBP, PA and the storage lipoglycoprotein for retinyl ester in the liver is depressed. Thus, plasma prealbumin or RBP levels are better indicators of inadequate protein nutriture than is the plasma albumin concentration. Inasmuch as a poor vitamin A status commonly accompanies PEM, vitamin A supplements should be included from the outset in dietary therapy for the condition. Vitamin A deficiency also affects protein metabolism, both by reducing the synthesis and release of RBP from the liver and by impairing nitrogen utilization. The plasma *apo*-RBP found in vitamin A deficiency is biologically inactive, e.g., is no longer able to bind retinol. Dietary fat markedly stimulates the absorption of carotenoids and vitamin A. Thus, a low fat intake in pregnant or lactating women (< 20g/day) or in children (~ 5g/day) markedly reduces carotenoid utilization.

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Acute protein deficiency interferes with vitamin A metabolism both indirectly by generally depressing the synthesis and release of proteolytic and lipolytic enzymes of the gut and pancreas and directly by limiting the synthesis of plasma transport proteins by the liver. Thus, proteins, lipids, carotenoids and retinyl ester in the diet are hydrolyzed more slowly, and micelle formation in the intestinal lumen is impaired. Since the secretion of glycoproteins from the goblet cells is lowered, both the diffusion layer which traps the micelles around the mucosal cell membrane as well as the viscosity of the lumen fluid are reduced. Thus, micelles containing retinol and its provitamins are not so readily brought into contact with the intestinal mucosae in the optimal region for absorption. Finally, the mucosae and the underlying lamina propria layer of the villus are less well developed, so that the overall mechanisms of absorption become severely depressed. In spite of these marked changes in digestion and absorption, a substantial portion of a large oral dose of vitamin A is still absorbed in children with PEM. Carotenoid utilization is particularly affected by PEM. Not only is carotenoid absorption reduced, but its cleavage to retinaldehyde by carotenoid dioxygenase in the intestinal mucosa is depressed. Since most children with PEM largely depend on carotenoids as a dietary source of vitamin A, this relationship has considerable practical significance.

Inasmuch as both RBP and PA contain a high proportion of aromatic and other essential amino acids, subjects on a protein diet of poor quality are unable to synthesize the specific carrier protein well. In experiments with rats on a rice diet, the plasma concentration of RBP is proportionally depressed more severely than is albumin, which obviously has a deleterious effect on retinol transport to tissues. A number of studies have also been made of RBP and prealbumin levels in preschool children suffering from protein energy malnutrition. The approximate range of values found in cases of kwashiorkor, marasmus-kwashiorkor and marasmus are given in Table III. Clearly, the

prealbumin concentration in plasma is a better parameter for assessing protein status than is the albumin concentration.

Upon the initiation of therapy, both *holo*-RBP and PA levels in the plasma of PEM children increase in a correlated way to normal levels over a two to four week period. Although an increase in plasma *holo*-RBP may occur in the absence of vitamin A supplementation, acute signs of vitamin A deficiency have been precipitated by treating both acute and mild forms of PEM with vitamin A-free protein and calorie-rich foods. Therefore vitamin A should be an essential ingredient in all foods used for PEM therapy, whether or not signs of vitamin A deficiency are noted.

Just as protein deficiency markedly affects vitamin A nutriture, so also does vitamin A deficiency influence protein metabolism. As the reserves of vitamin A in the liver dwindle, the amount of circulating *holo*-RBP in the plasma falls to very low levels. Even though plasma retinol concentrations are very low, the concentration of *apo*-RBP in vitamin A-deficient or retinoate-treated animals remains at about 12 μg/ml, i.e., about ¼ of the total RBP concentration in normal animals. Concomitantly, the *apo*-RBP concentration in the liver increases about four-fold. When retinol is readministered orally, *holo*-RBP is rapidly released into the bloodstream, reaches a peak value well above the normal steady state in four to six hours, and then ultimately falls to the normal range. As mentioned previously, the rapid release of *holo*-RBP in response to a small oral dose of vitamin A might be useful in assessing subclinical states of vitamin A deficiency.

Interestingly, some *apo*-RBP is secreted by the liver and circulates in the plasma even in deficient children and experimental animals. This *apo*-RBP is not able to bind retinol, however, and hence must be modified metabolically in some way.

Another effect of vitamin A deficiency on protein metabolism is an impairment in nitrogen balance. Vitamin A-deficient experimental animals grow less well than normal animals on a given force-fed diet and excrete more nitrogen as urea in the urine. Thus vitamin A deficiency and protein deficiency serve to exacerbate the imbalances caused by the other. It is not surprising, therefore, to find the most acute effects of both deficiencies when they are concomitant.

Fat in the diet has a marked stimulatory effect on the absorption of carotenoids and vitamin A. Fat is known is enhance the secretion of cholecystokinin from the duodenum, which in turn stimulates gall bladder contrac-

TABLE III

Concentrations of retinol, retinol-binding protein and prealbumin in plasma of preschool children with kwashiorkor (K), marasmic kwashiorkor (MK) or marasmus (M) before and two weeks after therapy.

Group	Retinol	Retinol binding protein		Prealbumin	Albumin
	μg/dl	Holo (μg/ml)	Total	μg/ml	g/dl
Initial condition					
K	0 — 10	0 — 8	10 — 15	35 — 75	1.5 — 2.0
M-K	0 — 10	0 — 10	15 — 25	40 — 80	1.8 — 2.5
M	5 — 20	5 — 20	25 — 35	85 — 120	2.5 — 3.3
After therapy					
K			30 — 40	80 — 100	2.0 — 2.5
M-K	20	20 — 30	30 — 40	80 — 120	2.3 — 3.0
M			35 — 45	100 — 200	2.8 — 3.6
Normal	34 — 48	25 — 35	35 — 45	150 — 250	3.6 — 4.3

tion. Since conjugated bile salts are absolutely required for carotenoid absorption and important both for retinyl ester cleavage and for retinol absorption, the role of fat in vitamin A absorption is fairly well defined. In areas of the world where vitamin A deficiency is common, fat intake also tends to be very low. A low fat intake (<20g/day) in pregnant and lactating women will tend to lower carotenoid absorption and reduce total body stores of vitamin A. The dietary restrictions practiced by pregnant and lactating women in some societies, i.e., the exclusion of eggs and meats from the diet and the avoidance of certain fruits and vegetables, will further lower vitamin A reserves and reduce vitamin A and carotene levels in breast milk. Preschool children in some developing societies ingest five grams of fat or less per day, which unquestionably interferes with carotenoid absorption. The addition of some fat (18g) to the low-fat diets greatly enhances carotenoid absorption from green leafy vegetables, as evidenced by a rapid increase in serum vitamin A values. Although certain types of fat have been reported to stimulate carotenoid absorption more than others, these differences are small relative to the changes mentioned above.

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Nutritional interactions with iron, vitamin E and zinc

Abstract: In the intestinal lumen ionic iron may catalyze the oxidative destruction of vitamin A and carotenoids. When chelated in heme or with other proteins in tissues, iron is much less active in this regard. On the other hand, vitamin A may facilitate the release of iron from the liver for use in hemoglobin synthesis. Whether or not a functional relationship exists, anemia and vitamin A deficiency seem to be correlated epidemiologically. By virtue of its anti-oxidant properties, vitamin E may protect vitamin A and carotenoids from oxidation in the intestinal lumen. At the tissue level, vitamin A absorption and storage are depressed in vitamin E deficiency but are not enhanced much above normal by large doses of vitamin E. Although the symptoms of zinc deficiency and vitamin A deficiency are similar in many respects, no clear molecular interaction has been shown. Nonetheless, vitamin A seems to be spared in zinc-deficient animals, probably as a result of a lower growth rate.

* * * *

Because preformed and precursor vitamin A-active compounds are easily oxidizable, other nutrients that act as pro- or antioxidants affect vitamin A requirements and its metabolism. Among those nutrients which have received the most study are iron and vitamin E. The effects are thought to occur both in the luminal environment during digestion and absorption and at the cellular level in tissues.

Iron acts as a pro-oxidant when present in its ionic form. Binding to heme, transferrin, myoglobin, ferritin or hemosiderin reduces its oxidant activity. Hence, uncomplexed ferric iron in the intestinal milieu may facilitate oxidation of both preformed and precursor vitamin A-active compounds. Uncomplexed ferrous iron is unlikely to be present in the more alkaline medium of the small intestine, since ferrous iron is largely chelated to other substances while in the acidic medium of the stomach.

At the cellular level, vitamin A may facilitate the availability of iron for the synthesis of

hemoglobin. Epidemiologic data from human nutrition surveys also suggest a correlation between the prevalence of anemia and of vitamin A deficiency.

Vitamin E (tocopherols) is an effective lipid-soluble antioxidant in the intestinal lumen, and presumably serves a similar function in tissues as well. The intraluminal antioxidant activity is well documented in animal studies, whereas intracellular interactions depend on the vitamin E status of the subject. For example, vitamin E-deficient animals absorb and store vitamin A more poorly and deplete existent liver reserves of vitamin A more rapidly than do normal animals. On the other hand, the addition of vitamin E to an oral preparation of vitamin A did not significantly improve the retention of vitamin A in malnourished children. Nonetheless, an interaction at one or both levels is the basis for including vitamin E in high potency, prophylactic oral preparations of vitamin A used in intervention programs.

A linkage between zinc and vitamin A metabolism has also been suggested. Signs of both deficiencies are similar, i.e., loss of appetite, decreased growth and epithelial keratinization. In zinc-deficient animals plasma vitamin A levels are low but return to normal upon the administration of zinc. Subsequent studies in weanling and pregnant rats have indicated that zinc is not directly involved in the release of *holo*-RBP. Rather, zinc deficiency seems to act indirectly on vitamin A metabolism by depressing growth and hence reducing the tissue requirements for vitamin A. Zinc is not a component of RBP, but because of its general role in protein synthesis, may limit the synthesis of both RBP and PA.

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METHODOLOGY

Abstract: Inasmuch as protein malnutrition and vitamin A deficiency are commonly associated clinically, a separate evaluation of protein status by the measurement of plasma prealbumin is useful. In the handling of blood samples for vitamin A assay, samples should be kept cool and plasma or serum separated off as soon as possible. Samples should either be analyzed promptly, which is preferable, or else be stored under nitrogen in the frozen state. Retinol in organic solvent extracts of plasma can be readily analyzed by Lewis acid reagents, by fluorescence or by other properly standardized procedures. In case retinyl ester is present in significant amounts, chromatographic separation of the ester from free retinal may be necessary.

Of around 400 naturally occurring carotenoids, only about 20 serve as provitamins. The principal provitamins are hydrocarbons ($\alpha-$, $\beta-$ and $\gamma-$ carotenes) and cryptoxanthin. The abundant polar carotenoids of plants are generally inactive. Carotenoids found in the plasma are an indicator of the recent intake of carotenoid-containing foods and are not a useful measure of vitamin A status. Usually 15-60% of total plasma carotenoids consist of provitamins, but the percentage might vary from very low to high values. Carotenoids of plasma normally contribute only around one percent of a sufficient total body reserve of vitamin A. As a function of food intake very high values of plasma carotenoids are found among certain individuals or in some societies, and very low values are sometimes associated with low fat intakes or malabsorption syndromes. When plasma carotene concentrations are high, special precautions must be observed in determining plasma vitamin A values.

Although total plasma RBP can most sensi-

tively be measured by radio-immunoassay, radial immuno-diffusion assay is more suitable for field studies. Extreme care must be taken, however, in using antibody concentrations appropriate to the amount of RBP being measured. Since a significant quantity of inactive *apo*-RBP circulates in the plasma of vitamin A-deficient children, the data obtained must also be interpreted with caution.

Holo-RBP, the physiologically active form of retinol, may be sensitively measured by the fluorescence emitted from the *holo*-RBP zone of a polyacrylamide gel after electrophoresis. This procedure, however, requires sophisticated instrumentation and frequent standardization.

The possibility of using other available biological samples as indicators of vitamin A status is discussed.

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In assessing the nutritional status of a community, it is necessary to obtain biochemical evidence from blood analyses to correlate with clinical observations and the standard anthropometric measurements of growth, weight, etc. In the past, plasma albumin has been monitored as an indicator of the state of protein nutrition and plasma retinol has been monitored as a measure of vitamin A status. It is now apparent that the concentration of prealbumin, β-transferrin and retinol-binding protein, plasma proteins with shorter half lives than albumin, responds more quickly to changes in the dietary protein intake. These proteins are therefore considered better indicators both in assessing protein status and following the recovery of malnourished patients after therapy. Prealbumin is probably the best of this group because it does not appear to be directly affected by the intake of other nutrients (e.g., retinol and iron) as are the binding proteins. In regard to vitamin A status, total plasma retinol-binding protein (RBP) and *holo*-RBP are now being assayed in addition to, or as an alternative to, the measurement of total retinol in plasma by either colorimetric or fluorometric methods.

Sampling of blood

Most of the newer procedures for assaying the protein moieties are microanalytical in character and require only a few microliters of sample. These procedures can, therefore, be applied to the small volumes of blood usually available (100-200 μl) from field studies. Where large blood samples are obtained in more comprehensive clinical studies, the sample volume is of course not a problem. Whatever the sample size, all samples taken should be collected in sterile containers and stored in an insulated cool box at 5°C pending transfer to a laboratory refrigerator. If an anticoagulant is used, the blood cells should be separated off as soon as possible. The plasma should then be frozen in sealable plastic containers and stored at −20°C if the analysis is not going to be carried out immediately.

Where storage of the samples over a long period is unavoidable, the sample tubes and their container should be outgassed with N_2 and sealed to minimize any oxidative degradation of retinol. The addition of 0.1% sodium azide as a protein preservative is also helpful.

Retinol analysis

If the sample is taken from a postprandial subject or from one recently given a massive dose of retinol, precautions must be taken to eliminate the possible interference of retinyl esters in assaying retinol following extraction of total lipids from the serum or plasma. Retinol can be readily separated from retinyl esters chromatographically on a small column of either water-weakened alumina or silicic acid, the absorption capacity of which has been standardized previously for the eluting solvents to be used. The column must be screened from the light during the separation process. After collecting the retinol fraction, a tiny drop of arachis oil, which contains the natural antioxidant α-tocopherol, may be added before removing the solvent under nitrogen. Alternatively, a small amount of the antioxidant BHT (0.01%) may be added to the solvent used in the chromatography. The addition of one or two drops of acetic anhydride to the extract before making the retinol fraction up to a known volume helps to remove any traces of residual water which could interfere with the color test.

The amount of retinol present is usually estimated in chloroform with trifluoracetic acid or trichloroacetic acid. In view of the speed of this reaction, it is advisable to carry out the assay so that the optical density of the mixture

can be read within 10-15 seconds of adding the reagent. A recording spectrophotometer is helpful in that the recorder can be set up and started at the time of mixing to enable peak color development and its decay to be followed rapidly. This enables accurate reproducible comparison of standards and unknowns to be made. Replicate tests should be carried out.

Carotenoid analysis

Carotenoids are a class of highly conjugated polyisoprenoid pigments, most of which contain 40 carbon atoms and are symmetrical around the central (15,15') double bond. Around 400 natural carotenoids of known structure have been characterized from a wide variety of plants, animals and micro-organisms. Humans cannot synthesize carotenoids, and hence whatever carotenoids are found in human tissue are derived either directly from the diet or are metabolites of dietary compounds. For nutritional purposes, carotenoids can be divided into those which can be cleaved to vitamin A (provitamins), and consequently are biologically active, and those which cannot. Of the hundreds of known carotenoids, only about 20 serve as provitamins.

The best known provitamin is the hydrocarbon all-*trans* β-carotene, which can be oxidatively cleaved at its central double bond to yield two molecules of retinaldehyde. Other important provitamins are the hydrocarbons α-carotene, γ-carotene and 3-hydroxy β-carotene, or cryptoxanthin. Most of the common carotenoids of plants, such as the dihydroxy compounds lutein and zeaxanthin and the acyclic compound lycopene, are not biologically active.

Carotenoids in foods are absorbed reasonably well (30-60%) in healthy human beings, and are transported in a low density lipoprotein of the plasma to tissues, where they are stored. Although some carotenoids are found in the liver, many are deposited in adipose tissue, where they are largely dissolved in the fat globules. Within a week or so after the ingestion of a large dose of carotenoids, the plasma carotenoid value returns to its baseline level. Much of the absorbed β-carotene and other provitamins is converted to vitamin A, whereas the biologically inactive carotenoids are metabolized and excreted by ill-defined pathways. Unlike vitamin A, no specific mechanisms exist for the mobilization and transport of carotenoids from their deposition sites, i.e., adipose tissue, to the plasma. Thus plasma carotenoids are useful as an indicator of the recent dietary intake of carotene-containing foods, but give no information about vitamin A status. In healthy subjects, plasma carotenoid and vitamin A values are not correlated.

It is not surprising, therefore, to find much more variability in plasma carotenoid levels than in plasma retinol concentrations, which are under homeostatic control. Average plasma carotenoid values in well nourished subjects from various countries vary from 80 μg/dl to over 250 μg/dl. The normal range is often taken as 40-100 μg/dl, but this is merely a statistical measure which is not helpful in evaluating vitamin A status.

The composition of the carotenoids found in the plasma is a function of the carotenoid composition of recently ingested food, the relative absorption efficiency of various compounds, their relative rate of metabolism in the intestinal mucosa and their relative rates of clearance from the plasma. Clearly, the percentage of biologically active carotenoids in the plasma cannot be readily predicted, and values from 15% to 60% h ve been reported. Although chromatographi systems exist for separating most carotenoids, the isolation of fractions containing hydrocarbons, monohydroxylated derivatives and more polar carotenoids is usually satisfactory for nutritional purposes. The hydrocarbon fraction with a peak absorbancy around 450 nM consists mainly of biologically active carotenoids, the monohydroxy fraction of some, and the more polar fraction of very few provitamins.

Carotenoids of the plasma contribute very little to the total body stores of vitamin A, as shown in the following calculation. A three-year-old 15 Kg child with a total body reserve of 10 mg retinol will have a liver vitamin A store of 20 μg/g. If the total carotenoid concentration is 100 μg/dl, of which 40% is β-carotene or equivalent provitamins and the plasma volume is 500 ml, the total amount of β-carotene equivalents in the plasma will be 200 μg. If one assumes that the biological activity of these plasma carotenoids is 50% that of retinol, the total retinol equivalent would be 100 μg, or 1% of an acceptable total body reserve. When the liver reserve of vitamin A is depleted (< 5 μg/g), plasma carotenoids may make up a

larger portion (five to ten percent) of the total body reserve. These children, however, are still very much at risk and in urgent need of treatment with vitamin A.

Three special cases might now be considered. Individuals who routinely ingest large amounts of carotenoids from natural sources such as red palm oil, tomato juice, carrot juice, or spinach puree, often have very high values of plasma carotenoids, e.g., 600-3000 μg/dl. This condition may be accompanied by a generalized xanthosis, which is most pronounced in the palms of the hands and soles of the feet. Outside of the jaundiced appearance, the condition is innocuous clinically and disappears with a change of diet. Quite understandably, vitamin A deficiency has never been found among hypercarotenemic persons.

Some persons, including children, have fairly high plasma carotenoid levels (150-400 μg/dl) and apparently low vitamin A values (0-15 μg/dl). These values might logically coexist if the plasma carotenoids contain few or no provitamins, lipidemia also exists, the blood sample is taken soon after an infrequent carotenoid-rich meal, liver function is severely impaired, or vitamin A values are depressed because of severe protein-calorie malnutrition or infection. But generally a technical problem exists, inasmuch as the carotenoids yield a blue color which is over-corrected in the Carr-Price or related assays, or quench the vitamin A fluorescence in the fluorescence assay. The UV irradiation assay (Bessey-Lowry) is less affected by carotenoids per se, but may be influenced by accompanying polyenes such as phytoene and phytofluene. The latter also affects the fluorescence assay. In such cases separation of carotenoids from retinol by chromatography on silica gel or alumina before assay is essential.

The third case concerns persons with very low plasma carotenoid values, which might be due either to the absence of carotene-containing foods in the diet or to poor intestinal absorption. The latter might be due to chronic conditions, such as diets very low in fat, severe intestinal parasitization, or fat malabsorption (steatorrhea), or to acute infections of the alimentary tract. If plasma vitamin A values are acceptable and no clinical signs are present, there is clearly no cause for alarm. But if plasma vitamin A values are also low, the low carotene values reinforce one's concern for the vitamin A status of the child.

The measurement of carotenoids is easy and should accompany the vitamin A analysis of plasma, both as a separate indicator of the recent intake of carotene-containing foods and as a precaution against the calculation of incorrect vitamin A values. The absorbancy of lipid extracts of plasma is measured at 450 nm in hexane or ethanol or at 466 nm in chloroform, and the concentration of carotenoids as β-carotene is calculated by use of an $E^{1\%}_{1cm}$ of 2500. Although conventionally calculated using the absorption coefficient and peak wavelength of β-carotene, one must always bear in mind that *total mixed carotenoids* are being estimated, and quite possibly very little if any β-carotene is present.

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Analysis of total retinol-binding protein

The most sensitive method of determining the total amount of retinol-binding protein in plasma is the radio immunoassay procedure. This assay can only be reasonably organized, however, in a large clinical or research laboratory with the appropriate facilities for preparing the radioactive iodinated standards and for assaying the radioactivity. The less-sensitive but perfectly adequate radial immuno-diffusion

assay is the most convenient procedure for use in smaller laboratories without elaborate facilities.

The radial immuno-diffusion assay, however, must be carried out with extreme care to give reliable results. The commercially available agar plates containing specific antibodies to RBP are ideally suitable for determining concentrations of the protein in the normal adult range of values from 30 to 70 μg/ml plasma. They are less well suited for the much lower concentrations in the 5-15 μg/ml range found in the severely malnourished child, mainly because the precipitin rings formed do not extend much beyond the edge of the sample well. For this lower range, plates of agar can be prepared containing a lower concentration of added antiserum which enables precipitin rings to be formed with a larger diameter.

Visualization of these rings can be improved by immersing the gel in one percent tannic acid for five to 15 minutes, or by soaking the gel in phosphate buffered saline overnight and then drying it on filter paper before staining the rings with Coomassie or Kenacid blue dye. Alternatively, with modest electrophoresis facilities, rectangular agar plates containing specific antiserum can be prepared to enable retinol-binding protein to be assayed by the Laurell electroimmunophoresis technique.

In the preparation of one's own plate, however, extreme care must be exercised to ensure that the molten agar is poured on to a prewarmed properly levelled surface of glass or plastic so that a uniform thickness of gel will result on cooling. Again the standards used to cover the range of unknowns should be made up to have roughly the same total overall protein content. In the case of PEM children, in whom the total plasma protein concentration is only about half that of normal adults, a range of sub-standards prepared by diluting normal adult sera two-fold and containing different known amounts of RBP are satisfactory. Pure RBP solutions to which three percent pure bovine serum albumin has been added may also be used. The size of the wells may be made smaller, i.e., on the order of two millimeter diameter, so that they contain only a 2-3 μl sample. A plot of the square of the precipitin ring diameter versus concentration of RBP gives a straight line with an intercept on the Y-ordinate. Values for standards should be chosen to bridge the range of unknown values expected.

The assay of total RBP suffers from the lack of distinction between *holo*-RBP and *apo*-RBP. Even in acute vitamin A deficiency, *apo*-RBP levels may be 30-40% of normal values. Since this *apo*-RBP is a modified protein which is unable to bind retinol, it gives a false positive reaction in this test relative to vitamin A status.

Similar procedures can be used for the assay of prealbumin. The plasma concentration of this protein is much higher than RBP, however, so blood samples should be diluted from two- to eight-fold before assay depending on the expected range of concentrations.

Analysis of holo-RBP

Retinol bound in its physiologically active form in plasma may be assayed directly by scanning the fluorescence of the *holo*-RBP zone in the pherogram of plasma upon irradiating zones of the polyacrylamide disc gel by a narrow beam of ultraviolet light. This is the only procedure which solely measures the physiological form of vitamin A in the plasma.

A modified chromoscan has been used for this purpose. Depending on the concentration of *holo*-RBP in the plasma, accurate values can be obtained with only five to 20 μl samples of plasma. It is advisable, however, to run samples of a known control sub-standard routinely alongside the unknowns to ensure that the original calibration factors are applicable. This precaution guards against instrument errors arising through unavoidable changes in the UV source or detector phototubes of the instrument.

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Thoughts on other approaches to an evaluation of vitamin A status

Ideally the nutritional status of a child might be accurately and rapidly assessed without drawing blood samples, plucking hair, collecting urine, conducting complicated analyses, or recourse to any other time-consuming, invasive or costly process. Although all approaches to a valid assessment of nutritional status—whether clinical, dietary, physiological or biochemical—purport to achieve that goal, all known procedures fall short for a variety of reasons. In the case of vitamin A deficiency, the major confounding factors include the rarity of signs specifically related to the deficiency, the non-specificity of a variety of more frequent indicators, the difficulty and cost of using sophisticated instrumentation and techniques with the infant and very young child in the field, and the multiple etiology of most nutritional deficiencies. Perhaps the safest course is the use of multiple indicators which, if found in a certain constellation, might indicate that vitamin A inadequacy is contributing to a generalized malnutrition syndrome.

Biochemical methods currently used to evaluate vitamin A status largely involve the plasma. Other biological samples may of course be collected, such as hair, nails, saliva, sweat, sebum, tears, urine and feces. Cells of certain kinds—such as white blood cells, red blood cells, skin, the buccal mucosa, adipose tissue cells and corneal cells—also might be obtained without undue difficulty. In order to broaden the horizon of scientists attempting to devise better or supportive procedures for assessing vitamin A nutriture, we have compiled a list of biological systems which, for reasons too detailed to be included here, might well be affected by vitamin A status. These systems are:

1. The agglutination of red blood cells by lectins.
2. The glycopeptide pattern of urine.
3. The amino acid pattern in blood or urine.
4. α_1-Macroglobulin and other glycoproteins in plasma.
5. The fatty acid composition and the metabolic activities of adipose tissue.
6. The tensile strength of hair.
7. The cells and glycoproteins found in saliva.
8. The excretion of vitamin A metabolities in the urine and feces.
9. The relative amount of keratin or pre-keratin, as detected by specific stains in skin or corneal cells.
10. Surface receptors for RBP on epithelial cells.
11. The lipid composition of cellular membranes.
12. The immune response in lymphocytes.
13. RNA synthesis in cells upon exposure to vitamin A.

If any of these systems proves to be useful as an assay method for vitamin A status, or indeed for a malnutrition syndrome to which vitamin A lack specifically contributes, our efforts in making these suggestions will be well rewarded.

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