review

The biosynthesis and metabolism of carotenoids and retinol (vitamin A)

JAMES ALLEN OLSON

Department of Biochemistry, University of Florida College of Medicine, Gainesville, Florida

SUMMARY Acetate, by condensation and decarboxylation reactions, is converted to isopentenyl pyrophosphate, which condenses to form C_{20} terpenol pyrophosphates. These latter compounds condense to yield the initial C_{40} carotenoid precursor which is presumably phytoene. By a series of dehydrogenation, cyclization, isomerization, and hydration reactions, various acyclic and alicyclic carotenoids are formed. Subsequently, hydroxylation, epoxidation, and oxidation–reduction reactions may occur. Several carotenoids and β-apocarotenals may be converted into retinol in mammalian tissues. Thereafter, retinol may be esterified, oxidized to retinal and retinoic acid, isomerized, and further metabolized. This review has stressed the route by which these transformations occur and the characteristics of the enzymes involved rather than nutritional, functional, or chemical aspects of carotenoids.

I. INTRODUCTION

THE PURPOSE of this review is to summarize knowledge of the biosynthetic pathways and the metabolic transformations of carotenoids and retinol derivatives, and to discuss briefly their function in the living organism. Clearly, many aspects of carotenoids and retinol derivatives will not be considered, such as the distribution of carotenoids in nature (1), chemical procedures for the synthesis of an ever-increasing number of analogues of carotenoids and retinol (2–4), the nutritional literature on retinol requirements, excess, and deficiency (5, 6), and in large part, the proposed specific functions of these pigments in photoreception (7, 8). Some aspects of the biosynthesis of carotenoids (9, 10) and of the transformation and function of retinol (11) have been recently treated. The biosynthesis of carotenoids and their metabolism have been reviewed by T. W. Goodwin in his recent book on *The Biosynthesis of Vitamins and Related Compounds* (11*a*). A useful compilation of contemporary work on retinol metabolism and function was presented at a Symposium on Vitamin A and Its Metabolism, in honor of Professor P. Karrer, and is published in *Vitamins and Hormones*, Vol. 18, 1960.

The nomenclature recommended by the International Union of Pure and Applied Chemistry (12) is employed in this review. The term *carotenoid* is used for all polyisoprenoid structures with extensive conjugated double bond systems. *Polyene* is employed to denote colorless, more saturated precursors of the carotenoids. Retinol, retinal, and retinoic acid are used for vitamin A alcohol, vitamin A aldehyde, and vitamin A acid, respectively. β-Apocarotenals and β-apocarotenoic acids are derivatives of β-carotene which lack one β-ionone ring and possess a terminal aldehyde or carboxyl group in the specified position. The numbering system employed for β-carotene and retinol is given in Fig. 1, and the formulas of several β-apocarotenals are depicted in Fig. 3.

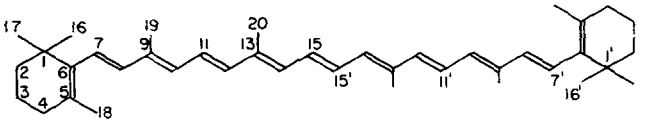
II. THE BIOSYNTHESIS OF CAROTENOIDS

A. The Formation of a C_{20} Precursor

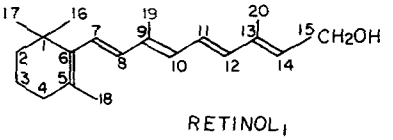
Initially, information concerning the early stages of carotene biosynthesis came largely from experiments with radioactive acetic acid. Grob and Bütler demonstrated that C^{14}-labeled acetate was incorporated into β-carotene in *Mucor hiemalis*, and that the labeling pattern in β-carotene was characteristic of compounds formed by the condensation of isoprene units (13, 14). Although Grob and Goodwin observed no randomization of the label of acetate-2-C^{14} in β-carotene synthesized by *Mucor* and *Phycomyces blakesleeanus* (14, 15), some randomization has been noted by others (16, 17). The search for the

active isoprenoid compound in sterol and carotene biosynthesis led ultimately to mevalonic acid, which proved to be extremely active in sterol biosynthesis (18). Subsequently, the incorporation of radioactive mevalonic acid into the carotenoids of *Mucor* (19), *P. blakesleeanus* (20–22), *N. crassa* (23), *E. gracilis* (24), ripening tomatoes (25, 26), *C. pyrenoidosa* (26), *B. trispora* (26), and carrot root preparations (27) was demonstrated. The labeling pattern in the isolated carotenoid, when determined, was similar to that found in squalene.

Early attempts to demonstrate the biosynthesis of carotenoids in cell-free preparations were discouraging (28). Subsequently the conversion of radioactive acetate to lycopene in tomato homogenates (29) and in cell-free carrot preparations (27) was observed. Although acetate



β-CAROTENE



RETINOL

FIG. 1. Formulas for β-carotene and retinol.

is often a poor substrate, cell-free preparations of a *Staphylococcus aureus* mutant, *P. blakesleeanus*, and tomatoes incorporate mevalonic acid, β-hydroxy-β-methylglutaric acid, 5-phosphomevalonic acid, and isopentenyl pyrophosphate into various polyenes and carotenoids (30–34). The cofactor requirements for the biosynthesis of polyenes from two- or five-carbon precursors are in accord with the generally accepted pathway for polyisoprenoid synthesis (Fig. 2). In addition to ATP, a divalent metal ion, and reduced pyridine nucleotide, coenzyme A was required when acetate was the substrate, but not when mevalonic acid, mevalonic 5-phosphate, or isopentenyl pyrophosphate were employed. Manganese was more effective in the bacterial system, but magnesium or manganese was active with *P. blakesleeanus* (33). Ethylenediaminetetraacetic acid inhibited incorporation, and nicotinamide, presumably by inhibiting the destruction of pyridine nucleotides, augmented the incorporation of mevalonate into phytoene (30). FAD, which is not implicated in the biosynthesis of squalene, was highly stimulatory in the formation of phytoene by *S. aureus* (31). NADP and NADH were required for the incorporation of mevalonate into β-carotene, whereas NAD inhibited (33). Divalent copper also stimulated this reaction. The biosynthesis of phytoene proceeded well in the absence of oxygen, in accord with the presumed pathway given in Fig. 2 (31).

That the pyrophosphates of geranyl geraniol or possibly geranyl linalool are the immediate precursors of the first C_{40} polyene is supported by several lines of evidence (35–39). When cell-free preparations of *P. blakesleeanus*, carrots, or tomatoes were incubated with farnesyl pyrophosphate, the addition of mevalonic acid or isopentenyl pyrophosphate appreciably stimulated the synthesis of polyenes (35–37). In addition, geranyl linalool and geranyl geraniol pyrophosphates have been isolated from these incubation mixtures (38, 39).

B. The formation of a C_{40} Compound

By analogy with the condensation of farnesyl pyrophosphate to yield squalene (40), geranyl geraniol pyrophosphate might well condense to form the C_{40} analogue, lycopersene (41). In support of this reaction, Grob et al. (39) reported that geranyl geraniol pyrophosphate was converted to lycopersene by extracts of *N. crassa* in the presence of NADPH. Squalene and lycopersene were separated, and most of the C^{14} label was found in the lycopersene zone. Later, the natural occurrence of lycopersene in *N. crassa* was reported (42).

On the other hand, Davies et al. (43) were unable to repeat Grob's work. No trace of lycopersene was found in extracts of *N. crassa* grown on diphenylamine, nor was mevalonic acid-2-C^{14} incorporated into lycopersene, although other polyenes contained radioactivity. Lycopersene could not be detected either in diphenylamine-inhibited cultures of *Rhodospirillum rubrum*, nor in a 2 kg sample of leaf lipid that was examined carefully for polyenes. Whereas the concentration of phytoene in the sample was 10 μg per 100 g of fresh tissue, the lycopersene concentration was less than 3×10^{-4} μg per 100 g (44, 45). In addition, mevalonic acid was not incorporated into lycopersene when maize seedlings were incubated with radioactive mevalonic acid (44, 45). Porter reported similar results. When preparations of tomato and carrot plastids and enzymes were incubated with labeled terpenol pyrophosphates, or with isopentenyl pyrophosphate, the polyene fraction was labeled. Upon purification by alumina chromatography, reduction of the lycopersene fraction to lycopersane, and isolation of the latter by gas–liquid chromatography (GLC), no radioactivity was found in lycopersane (36, 37). Differences in cofactor requirements for squalene and phytoene formation have also been noticed. The coenzyme NADPH is required for squalene synthesis (40), and presumably would be required for lycopersene synthesis (39). With whole and solubilized plastid preparations of tomato, however, the formation of phytoene from terpenol pyrophosphates was somewhat stimulated by NADP, but inhibited by NADPH (37). These effects

are interesting in that phytoene should be formed from terpenol pyrophosphates without a net change in oxidation state. Thus, although the bulk of evidence favors phytoene as the initial condensation product, lycopersene might yet be an intermediate, possibly bound to an enzyme in minute amounts.

The position of phytoene in the biosynthesis of carotenoids has been the subject of controversy. If cultures of mold or bacteria are incubated with diphenylamine, phytoene and other polyenes accumulate in large quantities and carotenoid synthesis is greatly depressed. Upon removal of diphenylamine, carotenoids are formed at the expense of many of the polyenes, but the phytoene concentration does not change (46, 47). One explanation of these findings is that phytoene is a side-product of carotene biosynthesis. Alternatively, phytoene may be a normal intermediate in carotene synthesis, but after being removed from active synthetic sites, it may not be metabolized (47). Recently, the enzymatic conversion of phytoene to phytofluene by isolated tomato plastids has been clearly demonstrated by Beeler and Porter (48). Radioactive phytoene was prepared biosynthetically and characterized by reduction to lycopersane. The phytofluene formed was chromatographed in the presence of carrier phytofluene, reduced to lycopersane, rechromatographed, and finally studied by gas–liquid chromatography. Neither oxygen nor NAD was required for this conversion. Since intact plastids were employed, adequate quantities of some hydrogen acceptor must have been present. The conversion of phytoene into δ-carotene by extracts of *S. aureus* has also been reported (49). Air was required for this conversion, but the characterization of the substrate and product was by no means as careful as in the previous case. The conversion of radioactive phytofluene to xanthophylls and epoxides of carotenoids by isolated chloroplasts of maize has also been reported (50). In this instance, the phytofluene was carefully purified, but the products were only separated by column chromatography and were not further characterized before counting.

Oxygen itself is not required for many oxidative reactions in carotene synthesis. Anaerobic organisms synthesize appreciably amounts of carotenoids (51), and an aerobic organism, *Mycobacterium*, formed ζ-carotene and neurosporene from phytoene and phytofluene in the absence of oxygen, provided that a suitable electron acceptor was present (52). The cyclization of radioactive lycopene to β-carotene in isolated chloroplasts has been demonstrated, and the reverse reaction has been shown with tomato parenchymatous tissue (53). In this case, labeled substrates were carefully purified and the products were characterized by chromatography and repeated crystallization. Supporting data were reported by Godner and Rotfarb (54).

The probable sequence of intermediates leading from the early C_{40} condensation product to β-carotene is also given in Fig. 2. Uncertain intermediates, such as lycopersene, are given in parentheses. This sequence is also supported by a number of kinetic studies on the disappearance of polyenes and the appearance of more unsaturated carotenoids (47, 55, 56), and by genetic data (9). In addition, the relative specific activities of carotenes synthesized from labeled terpenol pyrophosphates by tomato plastids are in accord with this scheme, with the exception that the specific activity of β-carotene was higher than that of lycopene (57). Thus, an alternative route from neurosporene to β-carotene via β-zea-carotene might exist which bypasses lycopene (9, 58). Whether compartmentalization of lycopene is present in this case is uncertain, and further evidence is required to support the lycopene bypass pathway. The concept of desaturation as a major pathway for β-carotene biosynthesis was originally suggested by Zechmeister (59), was developed further by Porter and Lincoln (60), was abetted by recent structural work on polyenes (61), and was evolved into its present form by Porter and others.

Additional studies have been conducted on the pathway for the biosynthesis of acyclic carotenoids in the photosynthetic bacteria, *Rhodospirillum rubrum* and *Rhodospirillum gelatinosa* (10, 46, 62), under anaerobic conditions. Spirilloxanthin is the major end product. Many of the presumed intermediates in this biosynthetic pathway have been isolated and characterized by Jensen (63–68). Largely based on structural considerations and on the kinetics of appearance and disappearance of various intermediates, the biosynthetic scheme for the purple bacteria has been defined (Fig. 2). The points of departure of these pathways from the major route in plants and fungi are the neurosporene and lycopene steps. Apparently the order of dehydration at the 3′ and 7′ positions and of cyclization or hydration in the terminal portion of the chain varies with the species involved. As pointed out by Jensen (10, 62), four types of reactions are involved in the formation of anaerobic acyclic carotenoids, two of which involve dehydrogenation, one hydration, and one methylation. Further evidence of an enzymatic type would be welcome in confirming these suggested pathways.

III. METABOLISM OF CAROTENOIDS

A. Hydroxylation

The importance of light and oxygen for the formation of highly unsaturated carotenoids and of xanthophylls from phytoene and phytofluene has been demonstrated in many microorganisms (69, 70). Although oxygen probably acts only as the ultimate electron acceptor in

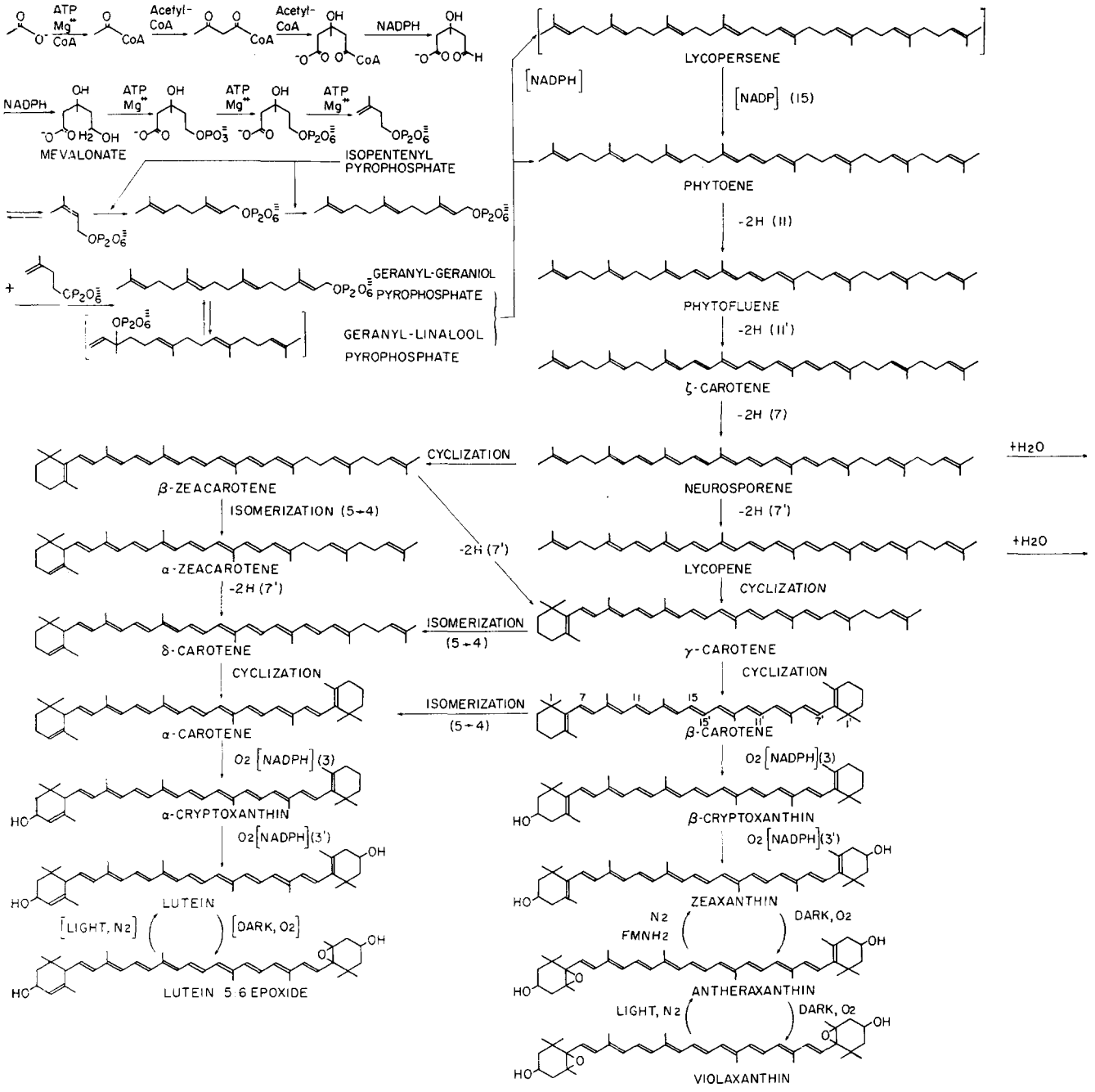


FIG. 2. A composite of postulated pathways for carotenoid biosynthesis. Possible intermediates which have not been identified or clearly implicated are given in parentheses. (*Fig. 2 continued on opposite page*)

polyene synthesis (52), its direct involvement in the hydroxylation of carotenoids has been shown in bacteria, leaves, and several algae (10, 56, 71–73). More recently, the incorporation of molecular oxygen labeled with O^{18} into the hydroxyl groups of lutein, violaxanthin, and neoxanthin in *Chlorella vulgaris* and into spheroidenone in *R. rubrum* was demonstrated (74, 75). Oxygen from water did not appear in the hydroxyl groups of these compounds. On the other hand, the introduction of hydroxyl groups into carotenoids of the photosynthetic purple bacterium, *Rhodospirillum rubrum*, grown anaerobically, proceeds by the hydration of terminal double bonds (10, 62). The possible dehydroxylation of xanthophylls into carotene has also been suggested (76).

B. Epoxidation

The major carotenoid pigments of leaves are hydroxy and epoxy derivatives of α- and β-carotene. Recently Sapozhnikov (73) reported that the epoxide, violaxanthin, decreases and that lutein increases in leaves under anaerobic conditions in the light, whereas the reverse reaction occurs under oxygen in the dark. Subsequent

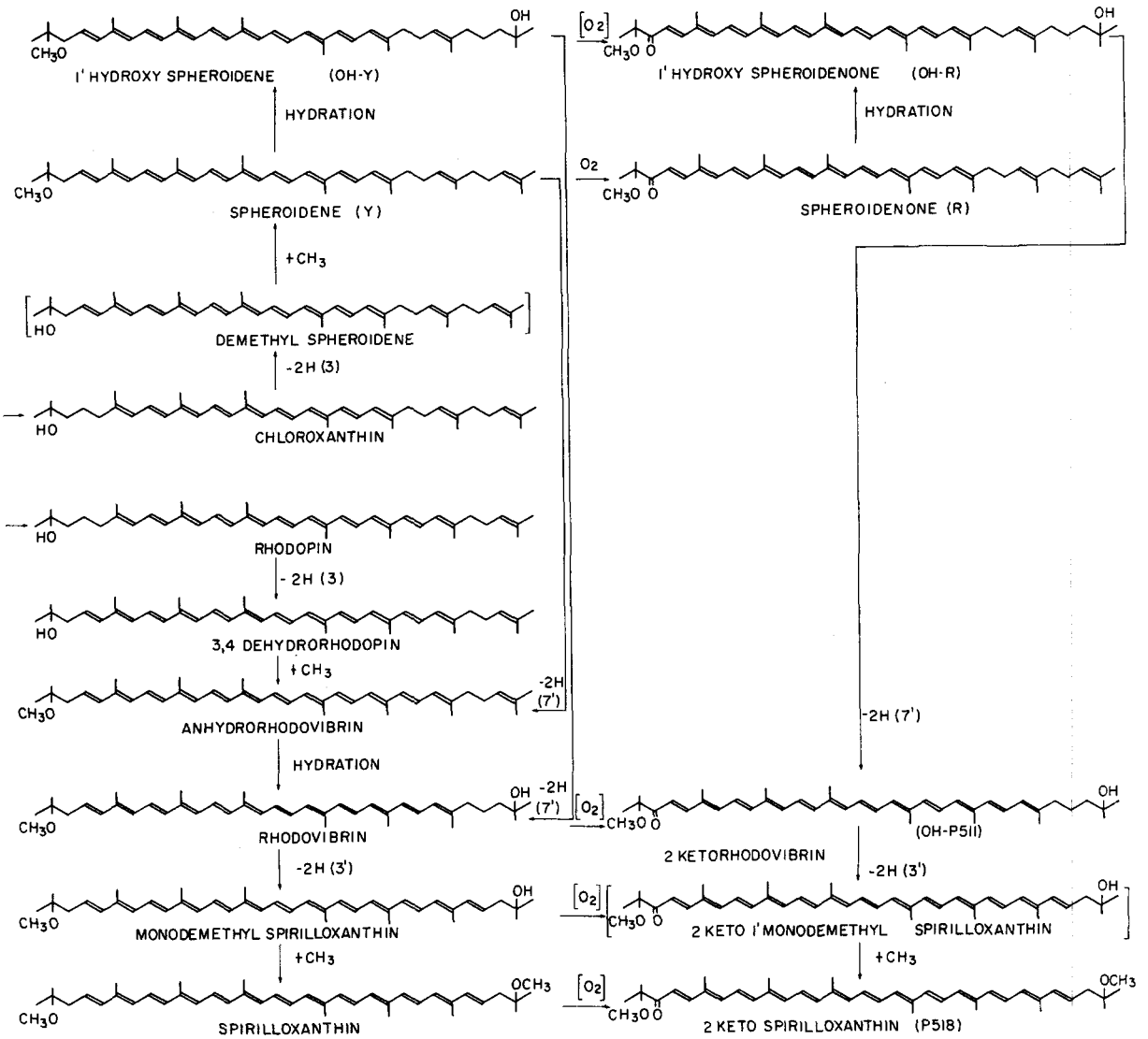


Fig. 2. (*Concluded*)

studies from the Russian group have attempted to clarify the nature of these reactions (77–79). Studying the same reaction in spinach and lima bean leaves, Yamamoto et al. (80) showed that violaxanthin was initially converted to the monoepoxycarotenoid, antheraxanthin, and then to zeaxanthin in the anaerobic light reaction. The light reaction was more easily demonstrated than the dark reaction. They suggested that the α-carotene and β-carotene series were separate at the level of epoxidation and de-epoxidation. These reactions are included in Fig. 2.

The origin of the oxygen atom in the carotene epoxides is somewhat uncertain. On the basis of a small contribution from H_2O^{18} (04.1 atom of O^{18}) in the trihydroxy monoepoxide neoxanthin (81), Yamamoto et al. (74) suggested that the epoxide oxygen probably came from water, in contrast to the hydroxyl of xanthophyll which was derived from molecular oxygen. In other similar cases, however (82, 83), epoxide formation at olefinic bonds requires molecular oxygen and is directly analogous to the hydroxylase reaction at saturated carbon atoms. Further studies on the origin of oxygen in carotenoid epoxides would, therefore, be welcome.

Similar effects of light and darkness on reversible epoxidation have been observed in *Euglena gracilis* (84). Subsequently, Krinsky has also shown that lyophilized

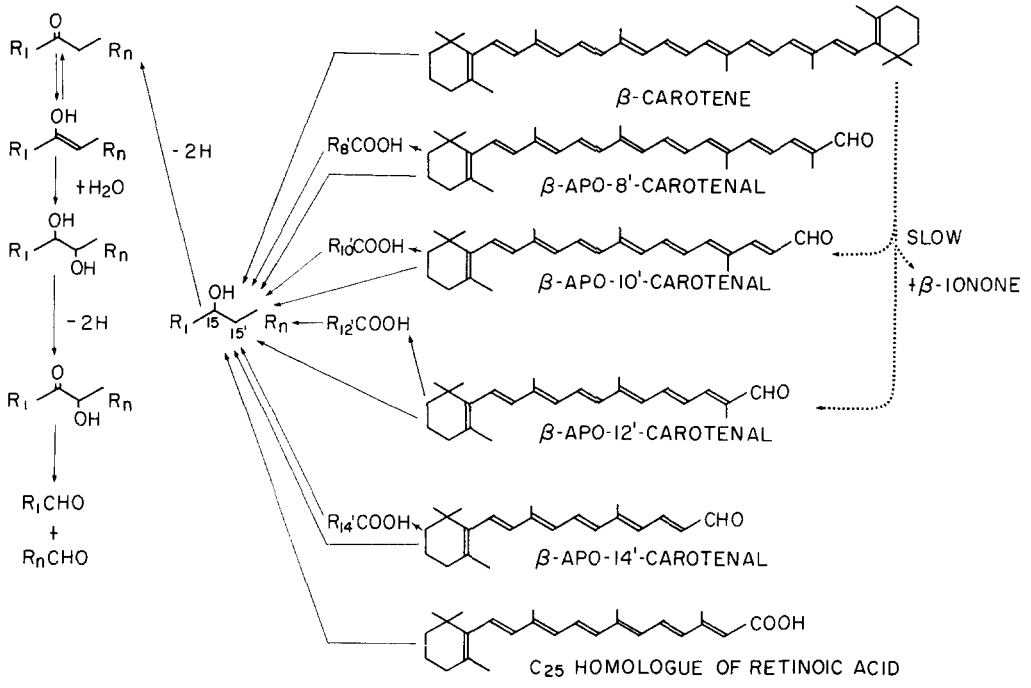


FIG. 3. A possible pathway for the conversion of β-carotene and β-apocarotenals to retinal.

cells of *Euglena* will convert antheraxanthin to zeaxanthin only in the presence of NADPH, FMN, and a heat-stable cellular component. $FMNH_2$ replaced NADPH and FMN.[1] In addition, 5- and 5′-hydroxylated xanthophylls may be intermediates between zeaxanthin and antheraxanthin and between the latter and violaxanthin.[1] De-epoxidation is inhibited by oxygen (85).

C. *Isomerization*

Many carotenoids with *cis* double bonds exist in nature (2, 4). The biosynthetic relationship between these *cis* isomers and the predominant all-*trans* carotenoids and polyenes is uncertain. Porter has suggested that pro-neurosporene arises from ζ-carotene and can be converted either to neurosporene or to prolycopene (9). A possible role of chlorophyll in carotene isomerization has been suggested (86). Many *cis* polyenes isomerize rapidly in the presence of light alone, and iodine catalysis of this isomerization has been studied in detail (4). An evaluation of the physiological significance of these forms must await further work. As yet no carotenoid isomerase has been isolated and studied.

D. *Oxidation–Reduction*

In addition to the carotenoids and polyenes shown in Fig. 2, many isomers and further oxidation products exist. The formation of ketonic groups from hydroxyls presumably involves a classical dehydrogenase with a requirement for pyridine nucleotide. Astaxanthin, the 4,4′-diketo analogue of zeaxanthin, probably arises from the oxidation of β-cryptoxanthin or zeaxanthin (87). With the exception of the epoxidation of xanthophylls and the transformation of alcohols to ketones few, if any, of these oxidative reactions are reversible. The reduction of conjugated double bond systems to yield isolated double bonds would not be favored chemically. Hydroxylation and hydration reactions take place largely in peripheral double bonds or at unsaturated sites rather than in the conjugated double bond system. By the same token, carotenoid acids seem to have their carboxyl groups on terminal portions of the molecule. The biosynthesis of acidic carotenoids has not been studied in detail.

[1] Krinsky, N. I., personal communication.

E. *Degradation*

In autumn leaves the striking color changes from green to red or yellow are caused by a disappearance of chlorophyll (1). In addition, β-carotene disappears and xanthophylls of the leaf become more esterified (87 *a*). The major pigments of the leaf, lutein, violaxanthin, and neoxanthin, are converted to mono- and diesters (88). Lutein 3-linoleate, but not 3′-linoleate, has been identified in fall leaves (88 *a*). The conjugated chromophore of the carotenoids is seemingly unaffected by leaf fading.

IV. BIOSYNTHESIS OF RETINOL

Since Moore first demonstrated that β-carotene is metabolized to retinol in the animal body (89), considerable interest has been shown in the possible mechanism of this conversion. Simple comparison of the two molecular species indicates that β-carotene might be cleaved by central fission to give two molecules of retinol, and this idea was supported by the relative biological activity

of a series of carotene isomers in growth tests. In many nutritional studies, however, β-carotene was only half as active as retinol on a weight basis, which suggested that only one molecule of retinol was formed per molecule of β-carotene. In consideration of the fact that a series of β-apocarotenals was highly active biologically, Glover (90) postulated that one molecule of retinol arises from one molecule of β-carotene by cleavage of the conjugated chain adjacent to one β-ionone ring followed by sequential oxidative removal of fragments containing two to five carbon atoms. Much of the work on this problem up to 1960 has been reviewed by Glover (91) and Moore (92).

Recent studies on the conversion have favored the central cleavage hypothesis. When β-carotene was injected into duodenal jejunal loops of rat intestine, retinol ester accumulated in the mucosal cells. Very few labeled compounds of low molecular weight or acidic nature were present in comparison with the amount of retinol ester formed (93). In addition, when rat intestine was incubated in vitro in a closed system, β-carotene was largely converted to retinol ester, and very few other products, including CO_2, contained radioactivity.[2] Although the stoichiometry in these experiments is not exact, the results strongly favor central cleavage rather than stepwise oxidation as the major pathway for retinol formation. Since the formation of small amounts of β-apocarotenals would not have been detected in these experiments, some stepwise oxidation may have occurred. β-Ionone has been reported to appear during β-carotene metabolism by intestinal tissue (94).

A postulated scheme for the conversion of β-carotene into retinol is given in Fig. 3. Intermediates which occur in β-carotene cleavage are present in small amounts in gut and have not been identified. Anaerobically, the intestine is unable to form retinol ester from β-carotene,[2] and a coupled oxidation-reduction may be necessary to disrupt the conjugation of the central chain. This postulated oxidative attack on the central double bond of the β-carotene molecule is in line with purely chemical studies in which peroxide (95) and osmium tetroxide (96) have been used to cleave β-carotene to retinal.

The involvement of the β-apocarotenals remains to be explained. Carotenals have been found in intestinal tissue and in a number of higher plants (97, 98). The biological activity and metabolism of a series of β-apocarotenals and carotenoic acids have been carefully examined by Glover (91). Of this group of compounds, 3-apo-12′-carotenal and its acid are most active biologically, and indeed, are more active for growth than β-carotene (99). On the other hand, β-carotene was superior to all members of the apocarotenal group in the storage of liver retinol (91). In addition, when radioactive β-carotene was fed, the specific activity of the carotenal derivatives was lower than that of liver retinol ester. Glover concluded that the stepwise oxidative pathway is probably of minor importance in gut.

Little is known of the pathway from β-apocarotenal to retinal. In all probability, the enzyme which initially attacks β-carotene at the 15 position also attacks the carotenal derivatives in a similar manner. Subsequent oxidative reactions would ultimately yield one mole of retinal and an aldehydic or acidic fragment from β-apocarotenal (Fig. 3). The isomers, α- and γ-carotene, might be attacked similarly. To my knowledge the conversion of one β-apocarotenal to another has not been demonstrated, and hence the proposed pathway is feasible. The immediate products of this proposed cleavage have not been identified, although a five-carbon compound may result from the metabolism of β-apocarotenal (99). With present in vitro methods, this suggestion can readily be verified experimentally.

In view of the use of β-apocarotenals as coloring agents for food (99 *a*), the subsequent metabolism of these compounds has evoked interest. The major product of their metabolism, other than retinol, is the corresponding carotenoic acid (91, 100). Both in the rat and in the hen's egg, an appreciable amount of carotenoic acid is produced when β-apo-8′-carotenal is the sole nutrient source of carotenoid. In the egg, the major portion of the carotenoid acid is esterified. Other acidic products also form (100). Small amounts of carotenoic acids, which presumably are products of carotenoid metabolism, have also been demonstrated in mushrooms, carrots, and spinach (100).

The site in the mammalian body at which β-carotene is converted to retinol has interested many workers. Moore has reviewed the pertinent literature in a thoughtful, thorough way (92). Thompson and his co-workers firmly established that the intestine is a major site for this cleavage reaction (101, 102), and subsequent studies have confirmed and extended these findings by the use of both in vivo and in vitro methods (103, 104). However, upon removal of the intestine and a number of other organs of the rat, the increase in retinol concentrations in plasma and liver after an intravenous injection of β-carotene was not affected appreciably (105, 106). The lung and liver have been particularly mentioned as possible sites of conversion. However, in a dog heart–lung preparation, β-carotene was not converted at an appreciable rate to retinol (107). Recently, the formation of retinol ester from β-carotene has been demonstrated in the isolated perfused rat liver (108). In the rat, the conversion rate for intestine was approximately twice as great as for liver on the basis of the whole organs, and four to seven times as great per gram wet weight of

[2] Olson, J. A., unpublished observations.

tissue (108). Both organs converted carotene into retinol at a much faster rate than that required to satisfy the nutritional requirements. After Pollard and Bieri demonstrated the oxidation of β-carotene to unidentified products by hemolyzed blood (109), McGillivray suggested that β-carotene might be cleaved into β-apocarotenals in blood (110). No evidence in support of this suggestion was obtained with the perfused liver system (108). Thus, whether organs other than the intestine and liver are capable of forming retinol from carotene is as yet uncertain.

Since appreciable amounts of intermediates do not accumulate in the conversion of β-carotene to retinol in intact isolated tissues, analysis of this sytem with cell-free preparations of the intestine and other organs would be most helpful. Thus far results have not been too encouraging. Liver homogenates have been reported to convert β-carotene into retinol in very low yield (111). After incubation of β-carotene with intestinal homogenates, retinol ester, β-ionone, and a retinal protein complex were reported to appear (94). The addition of NAD and FAD together stimulated the reaction, although neither cofactor was effective separately (112). Reddy and Thomas have also claimed that β-carotene is cleaved to retinol in sucrose homogenates of cow duodenum (113). In these instances, the Carr-Price reaction, thin-layer chromatography, and spectra were employed to measure the content of retinol. On the other hand, others have had difficulty in demonstrating this reaction in a number of tissue preparations (114–116). Although the conversion of β-carotene into retinol ester by intestinal sections in vitro has been readily demonstrated (104), cell-free preparations have always been inactive in the author's laboratory.[2] Although some radioactivity invariably appeared in the retinal and retinol ester fractions after incubation, purified derivatives of these compounds were devoid of label. Further studies in this area are clearly requisite.

The general metabolism of labeled radioactive β-carotene and its distribution in tissues of the rat and chick have been investigated in several laboratories. After the administration of 200 μg of uniformly labeled β-carotene to rats by stomach tube, radioactivity appeared in most organs of the body, and particularly in the liver (117). Interestingly, the adrenal gland contained appreciable amounts of the given dose, and the concentration of radioactivity per unit weight of adrenal tissue was higher than in any other organ. The nonsaponifiable fraction of most organs contained most of the radioactivity, although some appeared in the acidic fraction as well. In the pituitary gland the acidic fraction was larger than the nonsaponifiable fraction. Most of the nonsaponifiable fraction was identified as retinol with smaller amounts of β-carotene present. Acidic fractions were not characterized. The average amounts of radioactivity in the expired CO_2 for a group of animals which had been sacrificed at various times up to 24 hours after dosing was 12% of the administered dose. Less extensive experiments conducted by others (118, 119) yielded analogous results, except that the conversion of administered radioactivity to CO_2 was only 2–6%. In more recent studies, the metabolism of β-carotene labeled with tritium, as well as with C^{14}, has been studied (120).

The question arises whether carotene is cleaved only to retinol, or is metabolized directly to other breakdown products by a different route. Several lines of evidence suggest that the major pathway of carotene metabolism, if not the only one, is its transformation into retinol. The rate of release of $C^{14}O_2$ in rats was similar when either labeled retinal or β-carotene was administered (91). In addition, few products other than retinol and its ester were formed when labeled β-carotene was incubated with intestinal sections in vitro or in the perfused liver system (93, 108). Indeed, small amounts of polar products were also formed when retinol-C^{14} was incubated with intestinal sections.[2]

Throughout the years, the presence of an unsubstituted β-ionone ring and a suitable attached conjugated system have been firmly established as essential requirements for biological activity. Hence, the report that the diacetate of astaxanthin (3,3′-dihydroxy, 4,4′-diketo-β-carotene) was converted to retinol by rat's eyes incubated in vitro was greeted with some interest (121, 122). In addition, *retro*-retinol$_2$, which seems to be 3-ethoxy-*retro*-retinol$_1$, also has weak biological activity (123, 123 *a*). A careful extension of this work is well warranted.

V. THE METABOLISM OF RETINOL

A. Esterification and Hydrolysis

Retinol in foodstuffs and in tissues appears largely in the form of its ester, and to some extent as the free alcohol. Esterases of many kinds exist in tissue secretions and in cells, and considerable attention has been paid to the enzymes which catalyze the hydrolysis and resynthesis of retinol ester. In a number of tissues, retinol esterase is associated with the microsomal fraction. The optimum for hydrolysis is pH 8.6, and acetate esters are generally hydrolyzed more rapidly than esters of long-chain fatty acids. Interestingly, taurocholic acid stimulates the hydrolysis of retinol palmitate, but not of retinol acetate, whereas the nonionic detergent, Tween 20, inhibits the hydrolysis of retinol palmitate.

The synthesis of retinol esters has also been demonstrated in various tissue homogenates. Again, the microsomal fraction contains most of the esterase activity, but the pH optimum is between 5 and 7. ATP, coenzyme A, and bile salts do not stimulate ester formation, al-

though sulfhydryl compounds activate the enzyme in some cases. The synthetic activity is inhibited by bile salts and by other detergents. A summary of the properties of various retinol esterases is given in Table 1 (124–135).

The relative activites given in Table 1 are somewhat difficult to compare because they are drawn from many different sources in which different reaction conditions were employed. However, liver clearly has the highest hydrolytic activity toward retinol acetate, but little or no activity toward long-chain fatty esters. With the exception of the liver, which had hydrolytic activity but no demonstrable synthetic activity, and the pigment layers of the eye, which esterified retinol but apparently did not cleave it, all other tissues were able to carry out both reactions. Since perfused retinol and retinal quickly appear as retinol ester in a perfused liver system, liver certainly has the capability of carrying out this synthetic reaction (136). Apparently the enzyme is quite labile in tissue preparations. In view of the well documented fact that liver homogenates are unable to hydrolyze retinol palmitate (124–129, 131), High has suggested that liver retinol ester might be mobilized as such and transported to the kidney or to other organs for hydrolysis (126). Quite possibly, the hydrolytic activity of liver is as labile as the synthetic activity, and hence is present but has not been observed.

Hydrolytic reactions involving water-soluble compounds proceed largely to completion, and for the synthesis of carboxylic ester bonds, activation of the carboxyl group is usually necessary. In this case, the synthesis of retinol ester from retinol and free fatty acid does not require the usual cofactors, ATP and coenzyme A. If this reaction occurred under anhydrous conditions, however, the formation of carboxylic esters would be favored. Since retinol ester is lipophilic and, hence, must be adsorbed on a protein or dispersed in a micelle, water might well be excluded from the vicinity of the substrate molecule. Within lipid micelles, the ionization constant for a carboxyl group may be as high as pH 7 (137), and hence, condensation might tend to occur more rapidly at a lower pH. The enzyme behaves in an analogous way: the pH optimum for condensation is acidic, whereas that for hydrolysis is alkaline. The manner in which taurocholate stimulates the hydrolysis of retinol palmitate, but inhibits the esterification process,

TABLE 1 PROPERTIES OF RETINOL ESTERASES

Tissue	Species	Cell Site*	Relative Activity with: Acetate	Relative Activity with: Palmitate	Optimal pH	*Km*	Cofactors or Activators†	Inactivators or Inhibitors‡	References
Hydrolytic activity									
Liver	Rat	Mc	High	0	8.6 (6.6)	$2–6 \times 10^{-4}$ M	...	Acetone-drying, heavy metals, quinine, etc.	124–129, 131
	Chick	Mc	High	0	...	3×10^{-4}	...	...	129, 131
		N	High	0	...	1.9×10^{-4}	...	...	129, 131
Pancreas	Rat	...	High	Medium	...	...	TC(P)	Acetone-drying	127, 128
Intestinal mucosa	Rat	Mc	Medium	Low	8.6	...	TC(P)	NaF, DFP, Tween 20 (P)	124, 127, 128, 130
Kidney	Rat	...	Medium	Low	...	...	TC(P)	...	126–128, 130
Spleen	Rat	...	Medium	Low	...	...	TC(P)	...	126–128, 130
Plasma	Rat, man Rabbit	...	Low	0	...	...	TC(P)	...	126, 127, 130, 134
Erythrocytes	Rat	...	0	0	...	...	...	...	126, 127, 130, 134
Eye, retina	Cow	...	Medium	Low	...	...	...	...	132
Eye, pigment layer	Cow	...	0	0	...	...	...	...	132
Muscle, intestine	Rat	...	...	Low	...	...	...	...	130
Synthetic Activity									
Liver	Rat	...	0	0	...	...	...	...	125, 126, 130
Pancreas	Rat	...	...	High	5–7	...	*Not* A, CoA, BA	...	130, 133
	Chick	...	Medium	High	5.4–5.8	$4–6 \times 10^{-4}$	*Not* A, CoA, BA	*Not* PCMB	133
	Rabbit	...	...	High	5–7	...	*Not* A, CoA, BA	...	133
Intestine	Rat	Mc	...	High	6.6	...	*Not* A, CoA	TC, Tween 20	130
Kidney	Rat	...	...	Low	...	...	...	...	125, 126, 130
Spleen	Rat	...	...	Low	...	...	...	...	130
Plasma	Rat	...	...	Low	...	...	...	...	126, 130
Eye, retina	Cow	R	...	Medium	...	...	*Not* A, CoA, Cys, palmitate	Tween 80, digitonin	132
Eye, pigment layer	Cow	R	...	Medium	...	...	SH compounds, *not* A, palmitate	Tween 80, PCMB	132
Muscle	Rat	...	...	Medium	...	...	...	...	135

* Cell site: Mc, microsomes; N, nuclei; R, particulate residue.
† Cofactors or activators: TC, taurocholate; (P), effect observed with palmitate ester; A, ATP; BA, bile salt; Cys, cysteine.
‡ Inactivators: DFP, diisopropyl fluorophosphate; PCMB, *p*-chloromercuribenzoate.

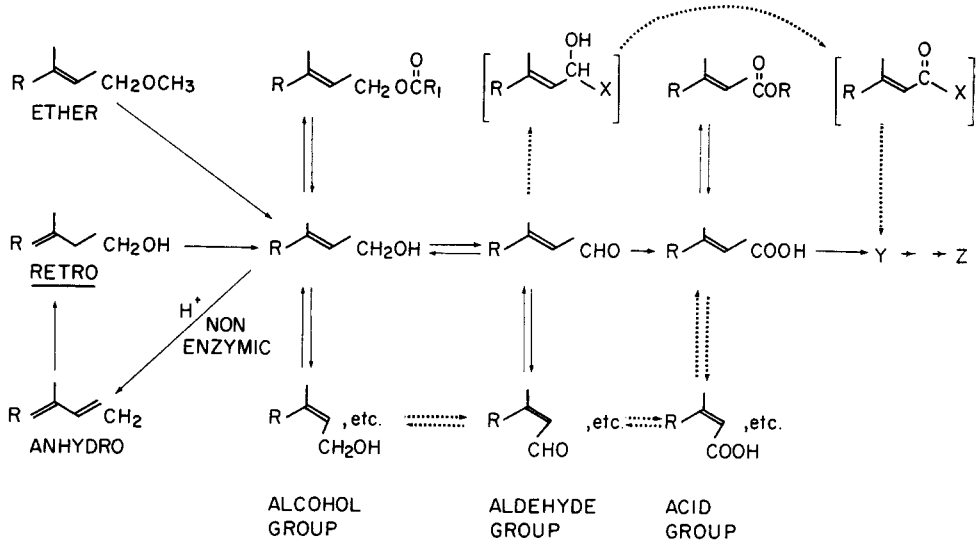


FIG. 4. Pathways of retinol metabolism.

is unknown. Since bile acids form very small micelles (138) in contrast with the Tween series of nonionic detergents (139), differences in the effect of these detergents on enzymatic reactions might be expected.

Similar effects of bile acids have been observed with the analogous enzyme, cholesterol esterase. Pancreatic cholesterol esterase requires taurine-conjugated bile acids, but is independent of ATP and coenzyme A (140), whereas the liver enzyme requires ATP and coenzyme A for esterification (141). Whereas the former enzyme has been highly purified in soluble form (140), the latter is a component of the microsomes, similar to vitamin A esterase. The reaction in rat liver microsomes is not straightforward, since ATP and CoA are much more effective than acyl CoA esters in the esterification of cholesterol (142). The importance of substrate dispersion in enzyme activity has been indicated in many other studies (143–145).

In view of the uncertain characterization of many esterases, the relationship among cholesterol esterase, retinol esterase, and pancreatic and liver esterases has been carefully examined by Ganguly and his colleagues (127–131 *a*). As a result of differences in substrate specificity, stability, and separation of the enzymes by agar gel electrophoresis, adsorption on calcium phosphate gel, and ammonium sulfate fractionation, they concluded that the three enzymes are distinct. On the basis of the different action of detergents and inhibitors on the hydrolase and synthetase activities of retinol esterase, Ganguly also feels that these two activities may reside in different enzymes. Before any definitive statement can be made concerning this point, however, the enzymes should be purified more highly, and the role of substrate dispersion in enzyme activity should be better evaluated.

The suggestion of Gray et al. (146), made many years ago, that retinol ester in food is extensively hydrolyzed to the free alcohol before absorption into the intestinal cells, has been well confirmed (147, 148). Regardless of the form of vitamin A fed, the ester in mucosal cells is largely palmitate, even when long-chain unsaturated fatty acids are present (135). In a careful study of the fatty acid composition of retinol esters in the retina, Futterman and Andrews (149) found mainly palmitic acid, smaller amounts of stearic acid, and traces of the C_{16} and C_{18} monoenic fatty acids. Although a relatively large percentage of total lipid of the retina is composed of C_{20} and C_{22} polyenic fatty acids, little or none of these was found in retinol ester. As in the intestinal mucosa (135), the distribution of fatty acids in retinol ester is more selective than might be expected from the known specificity of isolated retinol esterase.

The high biological activity of retinyl methyl ether prompted Thompson and Pitt to study its conversion into retinol in vivo (150). After feeding daily doses of retinyl methyl ether to vitamin A-deprived rats, 22% of the ether fed was found as retinol of the liver. The exact enzymatic requirements for this conversion are unknown. A general scheme of retinol metabolism is given in Fig. 4.

B. Conversion of Retinol to Retinal

Soon after Morton identified Wald's retinene as retinal (151), the biological reduction of retinal to retinol was studied in homogenates of frog and cattle retinas (152, 153). Reduced NAD was shown to be required (152). Since both crude rabbit liver extracts and crystalline horse liver alcohol dehydrogenase catalyze retinol oxidation, the general involvement of alcohol dehydrogenase in this interconversion was suggested (154, 155). The

known conversion of retinal to retinol by the intestinal mucosa in vivo also suggested alcohol dehydrogenase action (156) and explained the high biological activity of the aldehyde (157).

Few enzymes have been studied as intensively as crystalline horse liver alcohol dehydrogenase (158). The liver enzyme does not possess a high substrate specificity and acts on a large number of straight and branched chain aliphatic and aromatic alcohols. Although NAD is the preferred cofactor, other pyridine nucleotides react at low rates. Unlike the liver enzyme, the enzyme isolated from yeast will not oxidize retinol or other branched chain alcohols. With the liver enzyme, the equilibrium constant for retinol oxidation is 3.3×10^{-9} M (155). Hence, at neutral pH, the rate of reduction of retinal is considerably greater than that of retinol oxidation.

Recently, rat liver retinal reductase has been studied in some detail (139). The enzyme was purified threefold from the nonparticulate supernatant fraction of perfused rat liver. The pH optimum of the enzyme toward retinal is 5.9, and the Michaelis constants for retinal and reduced NAD were 2.2 to 2.9×10^{-4} M, and 1.8×10^{-5} M, respectively. The enzyme was quite unstable below pH 7.0, and was rapidly inactivated at 53°. Some interesting effects of salt on the activity of the enzyme were observed. Retinal reduction was stimulated by sodium chloride or ammonium sulfate, whereas acetaldehyde reduction was inhibited by salts. In addition, Tween 80 and sodium lauryl sulfate inhibited retinal reducing activity, but did not affect acetaldehyde reduction or ethanol oxidation. Interestingly, identical effects of salt and detergents were observed with crystalline horse liver alcohol dehydrogenase, which is known to be a single-enzyme protein. Thus, the interaction of a given enzyme with a water-soluble and micellar substrate seems to be affected by different factors. By the use of fractionation and inactivation tests, Zachman concluded that alcohol dehydrogenase of rat liver and retinal reductase were, indeed, the same enzyme (139).

The reversible reduction of retinal to retinol occurs in skin, intestine, and liver (156), and probably takes place in other organs as well. Alcohol dehydrogenase of fish liver has a specificity similar to that of horse liver, but the enzymes from bacteria and wheat germ are more similar to the yeast enzyme in specificity (158). Retinal reductase of other mammalian tissues has not been studied in detail.

Recently, Futterman has implicated reduced triphosphopyridine nucleotide in the visual cycle of the retina (159). Reduced NADP was found to be more effective than reduced NAD as an electron donor for the reduction of retinal by washed residues of visual cell outer segments. Since the hexose monophosphate shunt is apparently the major pathway for glucose oxidation in these preparations, a coupled reaction between the NADP reducing steps of the hexose monophosphate shunt and NADPH oxidation by retinal reductase was readily observed. When the coupled reaction was employed, NAD itself was relatively inactive. Futterman concludes that NADPH is used physiologically in retinal reduction, and that the glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase reactions may supply at least 60% of the required NADPH. The retinal reductase of outer retinal segments is insoluble, unlike the liver enzyme, and apparently is less specific with respect to pyridine nucleotides.

C. Isomerization

All isomers of retinol that have been tested have biological activity. Analogous isomers of retinal and retinoic acid are also biologically active as measured by growth assays and vaginal smear tests. In the case of retinol and retinal but not of retinoic acid, the storage of retinol ester in the rat liver has proved to be a convenient bioassay procedure. These data are summarized in Table 2

TABLE 2 BIOLOGICAL POTENCY OF GEOMETRIC ISOMERS OF RETINOL DERIVATIVES WITH RESPECT TO ALL-TRANS RETINOL$_1$ ACETATE

Isomer	Retinol Acetate	Retinal	Retinoic Acid	References
Retinol$_1$				
All-*trans*	100% (G, LS)	91% (G, LS)	10 (G), 8 (V), 50 (G), 128% (g)	160–162, 164–167
13-mono-*cis*	75 (G, LS), 68 (V)	93 (LS)	4 (V) 24 (g)	160–163, 165, 166
9-mono-*cis*	21 (G, LS)	19 (G, LS)	1.2 (V) 10 (g)	160–162, 165, 166
9-*cis*,13-*cis*	24 (G, LS)	17 (LS)	7 (g)	160–162, 166
11-mono-*cis*	24 (G, LS)	47 (G, LS)	...	160–162
11-*cis*,13-*cis*	15	31	...	160
Retinol$_2$				
All-*trans*	51 (V), 112 (MV)	57 (V), 116 (MV)	...	168
13-mono-*cis*	36 (V)	...	...	168
9-mono-*cis*	14 (V)	...	...	168

The method used to assay the isomers is included in parentheses: G, rat growth; LS, rat liver storage; V, rat vaginal smear; MV, modified rat vaginal smear; g, check growth.

(160–168). The variable results obtained with retinoic acid are probably due to the nature of the vehicle employed in dosing. The lower values, 8% and 10%, were observed when retinoic acid was given in oil, whereas the higher values were obtained when retinoic acid was suspended in a detergent. Indeed, when micellar solutions of retinoic acid were injected intraperitoneally, values of 141% were found (167). The appreciable activity observed with most of the isomers suggest either that they are isomerized to all-*trans* retinol, or that the isomer itself has biological activity.

When the all-*trans*, 9-*cis* or 9-*cis*,13-*cis* retinol acetate is fed to rats, an equilibrium mixture of isomers appears in the liver (169). When 11-*cis* retinol is fed, 10% of the administered dose is found in the liver, largely in the all-*trans* form (170, 171). Some isomerization of orally administered isomers occurs in the stomach, but transformation of the *cis* isomers to the all-*trans* form seems to take place largely in the liver (172, 173). Preliminary studies have been conducted on retinol isomerases prepared from various tissues (174). Isomerase activity was found in all cell fractions of liver, intestine, and kidney, but a combined mitochondria–microsome fraction was most active. Liver particulate fractions converted 11-*cis* and 13-*cis* retinol in an aqueous Tween 80 suspension into the all-*trans* form. The 11-*cis* isomer was converted more rapidly than the 13-*cis* compound. When all-*trans* retinol was incubated with the same preparation, about 7% of *cis* isomers formed. All incubations were conducted in the dark. Intestinal preparations and kidney particulate fractions were able to convert the 11-*cis* isomer to the all-*trans* compound at $^1/_2$ and $^1/_5$ the rate of liver tissue, respectively, but were inactive in isomerizing the 13-*cis* compound. Since the biological potency of the 13-*cis* isomer is considerably higher than that of the 11-*cis* isomer (Table 2), the isomerase activities of various tissues toward given isomers are not in line with their biological effects. Since the absorption of the *cis* isomers seems to be relatively rapid, the reason for their lower biological activity has not yet been elucidated. In general, the *cis* isomers seem to act by isomerization to the all-*trans* form rather than by functioning directly in some growth-promoting biological system. The specificity of various isomerases has not been well defined, and a great deal of straightforward enzymological study must yet be conducted.

TABLE 3 PROPERTIES OF ENZYMES OXIDIZING RETINAL

	Aldehyde Oxidase	Aldehyde Dehydrogenase
Source	Hog liver, rat liver	Rabbit liver
Purification	6-fold	2-fold
Michaelis constants		
Retinal	1.65×10^{-3} M	1.4×10^{-6} M
NAD	...	10^{-5} M
pH optimum	7.2	10
Relative rate	$0.42\left(\frac{\text{Retinal}}{CH_3CHO}\right)$	$0.29\left(\frac{\text{Retinal}}{C_2H_5CHO}\right)$
Activation energy	...	14.6 kcal/mole
Activators	...	Diethylstilbestrol, cortisone, estrone, dehydroisoandrosterone
Inhibitors	*p*-Chloromercuribenzoate, atabrine, metal chelators	Progesterone, androsterone, testosterone, deoxycorticosterone
Other sources	Rabbit liver, rat intestine, rat kidneys, pigeon liver	Rabbit kidney and small intestine, calf liver
References	187, 187 *a*	188–190

The best studied of the vitamin A isomerases is the 11-*cis* retinal isomerase of the retina (175). Hubbard extracted a soluble enzyme from cow retina and from frog pigment layer preparations which catalyzed the reversible isomerization of all-*trans* retinal to 11-*cis* retinal. The enzyme was inactive with the 9-*cis* isomer, and probably with the 13-*cis* isomer as well. When the reaction was run in the dark, the equilibrium mixture contained 5% of the 11-*cis* isomer and 95% of the all-*trans* isomer. In the light, approximately 32% of the 11-*cis* isomer was present at pseudoequilibrium. The enzyme was maximally active at an alkaline pH, the Michaelis constant was about 2×10^{-5} M, and the Q_{10} of the reaction was 2 in the dark and 1 in the light. The enzyme was specific for the aldehyde isomers and did not act on either 11-*cis* retinol or all-*trans* retinol. Since the isomerase reaction in the dark is relatively slow, and the equilibrium is unfavorable, some other undefined system, either separate or coupled with the isomerase physiologically, is presumably acting in the intact eye (176).

The photoisomerization of rhodopsin may also be considered as an enzymatic process. In this case, rhodopsin and isorhodopsin might be considered as enzyme–substrate complexes between rhodopsin and the appropriate isomer of retinal (175, 176). The initial event in rhodopsin dissociation is the isomerization of bound 11-*cis* retinal to bound all-*trans* retinal (176), and the initial product is a highly excited, unstable molecule, prelumirhodopsin (177–179). At low temperatures, reversible isomerization of retinal from 11-*cis* through all-*trans* to 9-*cis* may occur in combination with the opsin molecule (178, 179). No further attempt will be made here to consider the elegant work of Wald, Hubbard, and their colleagues on the events of the visual cycle, which has been recently reviewed (176, 178, 180).

D. Dehydration and Hydration

Retinol is readily dehydrated in the presence of acid to yield anhydroretinol, which contains a *retro* system of conjugated double bonds (181). This dehydration reac-

tion has not been demonstrated in biological systems and is essentially irreversible. Nevertheless, anhydroretinol does have 0.4% of the biological activity of all-*trans* retinol (182). When anhydroretinol is fed to rats, a new compound, *retro*-retinol, appears in the liver in small amounts. *retro*-Retinol has 7% of the biological activity of all-*trans* retinol in growth tests (182). When *retro*-retinol is fed to vitamin A deficient rats, 7.4% of the administered dose can be isolated from the liver as all-*trans* retinol and about 2% is found as unchanged *retro*-retinol (183). Apparently, the biological activity of anhydroretinol is due to its hydration to *retro*-retinol and isomerization to all-*trans* retinol in the animal. Anhydro-retinol$_2$ and its presumed hydrate, *retro*-3-ethoxyretinol, also have slight biological activity, and may be transformed to the all-*trans* form by an analogous sequence of reactions (123, 123 *a*).

E. The Conversion of Retinal to Retinoic Acid

Almost twenty years ago, retinoic acid was shown to have 10% of the growth-stimulating activity of all-*trans* retinol when given in oil, but almost equal activity when given as the sodium salt (164, 184). The biological activity of various isomers of retinoic acid is given in Table 2. Unlike most other derivatives of retinol, retinoic acid is not converted to all-*trans* retinol in the animal body. Even after massive doses of retinoic acid, no retinol is found in the tissues (166, 185). Furthermore, Dowling and Wald showed that rats fed retinoic acid grew normally, but became very night blind and sustained pathological changes in the retina (186).

That liver aldehyde oxidase and aldehyde dehydrogenase are capable of converting retinal to retinoic acid was shown independently by Dmitrovskii (187), Elder and Topper (188, 189), and Futterman (190). In addition, milk xanthine oxidase also is capable of converting retinal to retinoic acid (190). A summary of the properties of retinal-oxidizing enzymes is given in Table 3. Elder has suggested that aldehyde dehydrogenase is more important physiologically than aldehyde oxidase because its Michaelis constant is more in keeping with the probable concentration of retinal in liver.

The control of aldehyde dehydrogenase by steroid hormones is also of interest. The estrogenic compound, diethyl stilbestrol, shifts the *Km* for retinal from 1.4×10^{-6} M to 7.1×10^{-6} M at a concentration of 10^{-5} M, and also increases the maximum velocity. Thus, diethyl stilbestrol is inhibitory at concentrations of retinal below 3×10^{-6} M, but is stimulatory above this concentration (189). Some natural sterols were less effective in activating aldehyde dehydrogenase, and still other sterols were inhibitory. The importance of these sterol-sensitive reactions to a control of retinol metabolism is still very uncertain.

Indeed, retinoic acid has not yet been unambiguously identified as a product of retinol metabolism. The finding that retinal is converted to retinoic acid in rat jejunum (187) was not observed in chick intestine (197). From rather limited spectrophotometric evidence, retinoic acid has been reported as a metabolite of β-carotene in rat intestinal homogenates (199). On the other hand, Wright was unable to trap radioactive retinoic acid when C^{14}-labeled retinol acetate was injected into rats or incubated with normal pig adrenal homogenates (191). Similarly, Wolf has been unable to find radioactive retinoic acid after feeding labeled retinol and carrier retinoic acid to rats.[3] Possibly retinal may form a hemimercaptal or Schiff base which is rapidly oxidized through the corresponding retinoyl derivative to some subsequent metabolite without yielding appreciable amounts of free retinoic acid. On the other hand, retinoic acid may form at a relatively slow rate, but may subsequently be oxidized or conjugated at a very rapid rate. This suggestion is favored by the fact that retinoic acid does not accumulate in appreciable quantities in tissues even when large doses of the acid are given (192–195). By the use of improved methods of analysis, Jurkowitz has succeeded in identifying retinoic acid in human plasma after the administration of large doses of the free acid (196). With these improved methods, retinoic acid was found in the plasma, liver, and intestinal wall of chicks when 3 mg doses were given, but not when daily doses of 50 to 100 μg were administered (197). Even with these high doses, retinoic acid disappeared rapidly, and by 18 hours only traces were found (197). By the use of gas chromatographic methods for the analysis of methyl retinoate (198), Dunagin has been able to isolate and characterize small amounts of free and esterified retinoic acid from the liver and bile of rats 3 to 5 hours after the intravenous administration of medium doses of retinoic acid.[4] Methyl retinoate, upon incubation with rat liver homogenate, is readily hydrolyzed to the free acid.[5]

Thus, retinoic acid, either free or in some bound or activated form, seems to be a metabolically active intermediate in retinol metabolism. The fact that retinoic acid, unlike retinol, is not stored, is not converted to retinol, but yet is biologically active in growth has raised many questions concerning its metabolism and mode of action.

F. The Metabolism of Retinoic Acid

When radioactive retinol is injected intraperitoneally into rats, about 5% appears in the carbon dioxide within 24 hours (200). In addition, appreciable amounts of radioactivity are found in the urine, feces, carcass, liver,

[3] Wolf, G., personal communication.
[4] Dunagin, P. E., Jr., personal communication.
[5] Crum, R., and J. A. Olson, unpublished observations.

intestine, and other organs. A large portion of these metabolites was found in the saponifiable and water-soluble fractions. Wolf characterized two of the major excretory products of urine by analysis of their functional groups. Although retinoic acid was not suspected as an intermediate of retinol metabolism at that time, these metabolites are probably derived from retinoic acid. Subsequently, attempts have been made to define the metabolic pathway for retinoic acid and to associate physiological function with its intermediary metabolites. This investigation is still in its infancy. A summary of the properties of various metabolites of retinol and of retinoic acid is given in Table 4 (200–205). Most of the isolated metabolites are more polar than retinoic acid, but are extractable by ether from acidic solution. In most instances, only small amounts of the various metabolites have been isolated, and the fractions obtained must certainly be grossly contaminated with other substances. Hence, physical and chemical characterization of them at the present time is quite uncertain. A notable exception is Wolf's WS fraction, which was crystallized as the dinitrophenylhydrazone (200).

Zachman's recent observation that 20 to 40% of the injected dose of radioactive retinol or retinoic acid appears in the bile of bile ductcannulated rats within 4 to 8 hours may give further impetus to the characterization of these metabolites (205). Zachman further showed that bile metabolites from both retinol and retinoic acid are readily reabsorbed from the rat intestine, and that about $^1/_3$ of the intraduodenal dose was re-excreted in the bile within 24 hours. Thus, the oft-observed presence of metabolites of retinol and retinoic acid in the intestinal lumen (197, 200)[3] may well result from the enterohepatic circulation of retinol metabolites rather than from digestive processes in the gut.

Apparently a number of metabolites form, if the preliminary information given in Table 4 is a suitable guide, and the elucidation of their structures and the definition of the metabolic pathway are a challenge to interested investigators.

Several metabolites of retinoic acid metabolism are biologically active, either in the sulfurylase assay or in the classical growth test (Table 4). The difficult task of defining the biological function of retinoic acid in terms of chemical structure and molecular action seems to be progressing at a slow but certain pace.

VI. FUNCTIONS OF CAROTENOIDS AND RETINOL DERIVATIVES

Although this review is largely devoted to the biosynthesis and metabolism of carotenoids and retinol, the possible functions of carotenoids and retinol should be briefly mentioned. Carotenoids are universally present in photosynthetic structures, but normal photosynthetic reac-

TABLE 4 PROPERTIES OF METABOLITES OF RETINOL AND RETINOIC ACID

Substrate	Species	Fluid or Tissue	Designation	Properties				References
				Solubility	Chemical	Physical	Biological	
Retinol-2-C^{14}	Rat	Urine	WS	Water, not ether	Ketone, $C_{11}H_{14}O_4$?	Carboxyl ester	...	200, 201
			WES	Water and ether	Unsaturation, hydroxyl aldehyde	Conjugated aldehyde	...	200, 201
		Serum α_2-globulin	...	*Not* petroleum ether after ethanol	...	...	...	202
Retinol-6,7-C^{14}	Rat	Mainly liver supernatant fraction	...	Ether after KOH treatment	...	...	...	203
Retinoic acid-6,7-C^{14}	Rat	Stomach wall, liver	334	...	...	λ_{max} = 334 mμ	Active in sulfurylase assay	204
Retinoic acid-6,7-C^{14}	Chick	Mainly intestinal contents and excreta; also intestine, liver, and plasma	C	Acid ether	No $SbCl_3$ reaction	...	Prevents eye symptoms	197
			D	Aqueous after acid ether extraction	...	...	Inactive	197
Retinoic acid-6,7-C^{14}	Rat	Liver supernatant fraction	Acidic lipid factor	Butanol, then acid ether	...	...	Active in sulfurylase assay	Footnote 3
		Intestine	5	Acid ether after KOH, then chromatography	...	λ_{max} = 252 mμ Infrared-OH	Active in rat growth, not in vision	Footnote 3
		Intestine	8	Same as above	...	...	Inactive	Footnote 3
Retinol-6,7-C^{14}	Rat	Bile	I	Eluted from anionic exchange resin by methanol	...	λ_{max} = <300 mμ	Inactive in rat growth	205
			III	By 10–100% acetic acid	...	...		
Retinoic acid-6,7-C^{14}	Rat	Bile, liver, intestine	I	Same as above	...	λ_{max} = 350 mμ	...	205, footnote 4
			III		...	λ_{max} = 360 mμ	...	

tions occur in their absence. For example, photosynthetic electron transfer reactions occur normally in carotenoidless mutants of *Rhodospirillum* (206), and the photoinduced electron paramagnetic resonance signal in photosynthetic bacteria requires the presence of chlorophyll, but not of carotenoids (207). On the other hand, carotenoids may contribute to photosynthetic reactions by transferring absorbed energy to chlorophyll (208). Some involvement in the electron transfer process is suggested by the changes in the absorption spectra of carotenoids which are induced by light and oxygenation (206). In some organisms, carotenoids may play an important role as accessory pigments in the enhancement reaction (8). Carotenoids may also serve as photoreceptors in phototropic systems, although the evidence favoring this suggestion is hardly conclusive (209). Quite apart from the possible involvement of carotenoids in photosynthesis, Stanier has suggested that they may protect the cell from photodestruction and photokilling (210).

In keeping with the association of carotenoids with photosynthetic processes in microorganisms and plants, 11-*cis* retinal, bound to opsin, functions in the well known visual cycle (176, 178). Retinoic acid is not active in the visual cycle nor is it converted to 11-*cis* retinal. However, Wolf and his collaborators have clearly implicated retinoic acid and its derivatives in the synthesis of mucopolysaccharides (211), and more specifically, in the activation of sulfate to phosphoadenosine phosphosulfate (212). Ganguly has shown that the sulfurylation of phenols is also inhibited in tissues of retinol-deficient rats, but is restored by the addition of retinol or retinoic acid (213). Corticosterone synthesis is also depressed in retinol deficiency, and the rate-limiting reaction seems to be the 11β-hydroxylation of deoxycorticosterone (214). Either retinol or retinoic acid restored the enzyme to normal levels in adrenal homogenates. No clear relation has yet been demonstrated between the role of retinoic acid in mucopolysaccharide synthesis and its role in corticosterone formation.

With respect to the hypervitaminotic state, Lucy, Dingle, and their collaborators have published extensively in support of their hypothesis that retinol penetrates lipoprotein membranes and causes increased permeability and decreased stability of the membranous structure. A recent paper may serve as a key to this literature (215).

VII. FINAL COMMENTS

The pathways of carotene biosynthesis have been defined in some detail during the past few years. Understanding of the pathway for sterol biosynthesis, and elucidation of the structures of many carotenoids and polyenes, have been most helpful in the definition of a reasonable sequence for the formation of carotenoids in plants, fungi, and several photosynthetic bacteria. Information on the enzymes involved in these pathways is scant, however, and much work is yet needed on the preparation of suitable cell-free systems and characterization of individual enzymatic steps. Although reactions of only a few types account for the vast majority of transformations which occur among the polyene and carotenoid groups, little knowledge exists at present concerning the number of enzymes involved, their specificity, and their cofactor requirements.

Of considerable interest is the large number of oxygenase reactions which have been associated with carotene metabolism. Furthermore, the reversible epoxidation of xanthophylls is of considerable interest, particularly in view of the possible function of epoxides as oxygen carriers in plants. The pathway for the degradation of normal carotenoid end products such as β-carotene and spirilloxanthin has received little attention. Microorganisms do not oxidize these compounds readily, and their presence in many geological deposits suggests that they are relatively resistant to biological transformation as well as highly stable under proper conditions.

The major pathway for the transformation of β-carotene into retinol has been fairly well established as a central cleavage type of reaction, but the details of the reaction sequence are still unclear. As in the case of carotene biosynthesis, suitable cell-free systems have not been readily prepared, and appreciable amounts of intermediates between β-carotene and retinol do not accumulate. It seems likely that the conversion of partially degraded β-carotene molecules such as the β-apocarotenals into retinol proceeds in a manner analogous to β-carotene cleavage rather than by stepwise oxidation of small two- or three-carbon entities.

Several enzymes which are known to act on retinol derivatives, namely alcohol dehydrogenase, aldehyde dehydrogenase, aldehyde oxidase, and xanthine oxidase, have a common property of broad specificity. An apparent exception is retinol esterase, which is not identical with cholesterol esterase and other tissue esterases. The action of these enzymes on lipophilic substrates which are dispersed in micellar solution is strongly affected by salts and the nature of the detergent. Elucidation of the manner in which bile acids, other detergents, and salts affect these reactions would be most welcome.

Most geometrical isomers and biologically active analogues of retinol are converted to all-*trans* retinol in the animal body. The activity of these compounds in stimulating growth and in other biological assays can therefore be attributed to retinol or to one of its metabolites. An exception is retinoic acid, which is not converted to retinal or retinol, but yet has equivalent biological

activity in growth. Unlike retinal and retinol, retinoic acid does not accumulate in the liver, but is converted to metabolites which appear in the liver, bile, intestine, and other organs. The structure of these metabolites and their possible role in mucopolysaccharide synthesis, steroid synthesis, and membrane structure are being intensively studied in several laboratories.

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