VITAMIN A ISOMERS IN ANIMAL LIVERS

by

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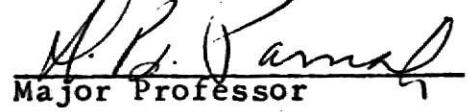

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TABLE OF CONTENTS

I. INTRODUCTION

For some 60 years vitamin A has been recognized as one of the required factors in animal diets. Vitamin A is an important growth factor. In its absence deficiency symptoms appear especially in epithelial tissues. Xerophthalmia, which may lead to blindness, formerly was a common condition among poor people, and night blindness often was found among adults. Today, with a better understanding of nutrition and the use of supplements in diets, the situation has improved.

The total vitamin A content of a food or feed by the customary methods of assay may not yield useful analytical data, since it is the physiological activity of the vitamin--the availability to the animal organism--that is of real concern. When the International or USP unit is used to express vitamin A content, it means the "activity" of 0.344 mg of crystalline all-*trans* retinyl acetate (0.3 mg of retinol). Biopotency of vitamin A is related to activity and not to weight. Biopotency of vitamin A expresses true nutritional value; it is based on an activity value of 100% for all-*trans* vitamin A, the predominant form in both natural and synthetic products.

Due to the conjugated double bond system in the vitamin A molecule, isomerism of the *cis-trans* type is possible and any modification in the characteristic conjugated double bond system of the all-*trans* retinol results in a loss of biological activity of the vitamin. Theoretically, 32 isomers of vitamin A are possible. The all-*trans* and three *cis-trans* forms (13-mono-*cis*,

9-mono-cis and the 9,13-di-cis, with lower biopotencies) are those most commonly found in commercial preparations and are of greatest concern (Plate I).

The cis-isomers, together with other vitamin A forms, such as vitamin A_2, anhydrovitamin A_1 and A_2, oxidation products of vitamin A, carotenoids, kitol, unidentified molecules, and small amounts of intermediates contribute to "irrelevant absorptions," giving incorrect and high values for retinol when the customary assay methods are used.

The purpose of this investigation was to determine the relative proportions of the commonly found vitamin A isomers in some animal livers and the resulting biopotencies of the liver vitamin A. In addition, studies were made on effect of light, temperature, and length of storage time on those values. Hexane extracts of livers were treated with iodine and exposed to light to observe isomeric changes that might occur under such conditions.

II. REVIEW OF LITERATURE

A. Cis-Isomers

Isomerization. In 1939, Smith (1) reported that it seemed likely that the vitamin A in an oil or concentrate existed as a mixture of geometrical isomerides, and that on irradiation, energy is absorbed causing a change in the proportion of isomerides.

EXPLANATION OF PLATE I

The all-*trans* and most common *cis*-isomers of retinol.

Fig. 1. All-*trans* retinol.

Fig. 2. 13-mono-*cis* retinol.

Fig. 3. 9-mono-*cis* retinol.

Fig. 4. 9,13-di-*cis* retinol.

PLATE I

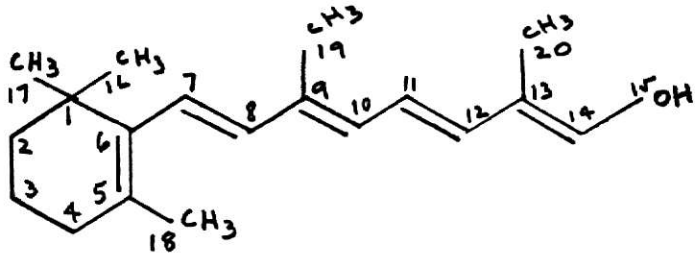
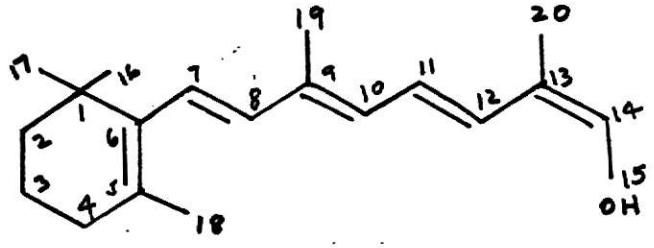


Fig. 1.



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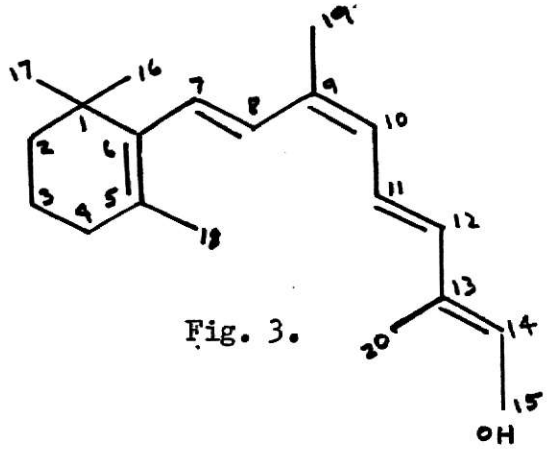

Fig. 3.

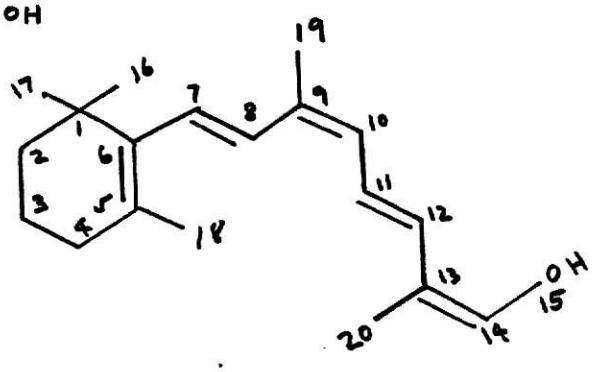

Fig. 4.

There are uncertainties (2) in reports of investigations on vitamin A. Due to differences in photometers used, samples, and techniques followed, two investigators seldom report the same analytical results for the same sample even in the same solvent by the same general methods. Results differ when different physico-chemical or biological assays are used. Among the factors responsible for the different results is presence of the cis-isomers of retinol, each with slightly different UV spectra or Carr-Price reaction product, but with wide differences in biopotencies of some of them.

The biopotencies of vitamin A isomers have been studied (3-8). All investigators have reported lower biopotencies for the cis-isomers than for the all-trans. Relative biopotencies of the cis-isomers, as percentages of the biopotency for all-trans-retinol, as given by Ames (6), are:

	Relative biopotencies	
	Retinyl Acetate %	Retinal %
all-trans[1]	100	91
13-mono-cis	75	93
11-mono-cis	24	47
9-mono-cis	21	19
9,13-di-cis	24	17

[1] Here and hereafter, the older nomenclature for vitamin A isomers has been changed to the nomenclature according to Comission on Nomenclature of Biological Chemistry, Intern. Union of Pure and Applied Chemistry. J. Am. Chem. Soc. 82:5575, 1960.

Isomerization in vivo. When vitamin A-depleted albino rats were supplemented orally with 3,000 to 7,000 units of all-trans, 9-mono-cis, or 9,13-di-cis vitamin A acetate, the relative biopotencies of vitamin A stored were 64%, 28%, and 24%, respectively (9). The rats apparently isomerized some all-trans vitamin A and stored a mixture of all trans and less active cis-isomers other than neovitamin A (13-cis). The 9-cis and 9,13-di-cis isomers were isomerized partly to the all-trans vitamin A form. Evidently vitamin A was stored in the liver as an equilibrium mixture of isomers independent of the isomer fed.

Plack (10) and Murray, Stainer and Campbell (11) reported on isomerization of 11-cis and 13-cis vitamin A to the all-trans form in rats fed those cis-isomers. The results of the experiments suggest that, although the cis-isomers were absorbed, they were converted, presumably in the liver, to 80 to 90% of all-trans vitamin A in the case of the 11-cis isomer and in significantly high concentrations in the case of the 13-cis form. Baba (12) also observed in vivo that the type of vitamin A stored in the liver was hardly influenced by distribution of retinol isomers administered, as well as by the ratio of isomer distribution of vitamin A in the feed, although maleic values of liver vitamin A in vitro decreased when a "slow reacting" isomer was added.

Isomerization of vitamin A in chickens was studied by Braekman (13), who fed isomers of all-trans vitamin A ester, all-trans vitamin A alcohol, and neovitamin A alcohol (13-mono-cis). Those receiving the all-trans forms usually showed such low

values for neovitamin A as to indicate that no isomerization took place; neovitamin A, however, apparently isomerized to all-trans vitamin A. Isomerization appears to take place in the intestinal wall as well as in the liver. The results again indicate that all-trans vitamin A is the natural form present in the liver.

Factors affecting isomerization. Zechmeister (14) treated all-trans carotenoids with light or light plus iodine, finding that the extinction coefficients at 260 and 320 nm, as well as in the visible range, diminished, but that a new maximum developed at a longer wavelength (between 320 and 380 nm) where all-trans carotenoids show little absorption. The new maximum has been called the "cis-peak." Theoretically, each possible stereoisomer is present in an equilibrium mixture, even if only in minute amounts. Zechmeister (14) found that visible light caused more isomerization than ultraviolet light, and iodine catalyzed conversion of all-trans vitamin A into an equilibrium mixture containing cis-isomers. Spontaneous isomerization also can occur at room temperature and on heating or refluxing in absence of catalysts. Acids likewise catalyze isomerization. To avoid this, samples were kept cool and protected from light. Deuel (15) reported that vitamin A was isomerized by light and iodine. Development of the cis-peak was slow in the presence of iodine but rapid when samples were exposed to iodine plus light. Baba (12) found different values for the biopotencies of vitamin A in fish liver oils, which were the consequences of isomerization. He (16) studied factors that affected the rate of isomerization

of vitamin A samples and found: a) isomerization by heat treatment was not remarkable. Heating at 150° was not effective. Heating at 240° resulted in a 50% isomerization after a 30-minute treatment; b) little isomerization on two months storage at room temperature; and c) presence of liver tissue increased isomerization fivefold.

Mixtures of synthetic vitamin A isomers of low biopotencies isomerized to all-trans and neovitamin A (higher biopotencies forms) by treatment with iodine in the presence of pyridine (17).

A method describing the synthesis of geometric isomers of vitamin A using the reaction with maleic anhydride and interconversion by catalytic isomerization has been reported (18).

Characteristic of the cis-isomers. Spectrophotometric data on cis-isomers have been determined by a number of investigators. Much of this data has been collected by Kofler and Rubin (19). The cis-isomers are hypochromic with respect to the all-trans compounds, and, in general, they have maxima absorption peaks at shorter wavelengths; an exception is the 13-cis derivative, which is hypochromic but has the absorption maximum at a longer wavelength (328 nm) than the all-trans isomer. Cis-isomers generally have more or less distinct peaks in the UV region (250-290 nm), which are absent in the all-trans compounds (20). Ames (21) stated that when a vitamin A product has a high blue color to USP ratio and a low biopotency, it is due to the presence of cis-isomers.

Neovitamin A, the 13-cis isomer, constitutes 35% of the total vitamin A present in a number of common fish liver oils (22). Thus, physical and chemical properties of the 13-cis isomer, especially its biological potency, are of commercial as well as theoretical interest. Presence of neovitamin A also has been reported by others (23, 24). The absorption curve is similar to that of trans vitamin A, but the maximum is at 328 nm instead of at 325 nm, as for the all-trans retinol. Robenson and Baxter (22) found an average value of $E^{1\%}_{1\ cm}$ (328 nm) of 1645 for five preparations of neovitamin A. They also reported that neovitamin A is less readily attacked by atmospheric oxygen than is the all-trans form. Treatment of neovitamin A with a solution of HCl in ethanol yielded an anhydro compound which appeared to be identical with anhydrovitamin A. They also found that neovitamin A has only 75% the biopotency of the all-trans form on a molar basis, and that the all-trans and neovitamin A aldehydes have biopotencies of approximately 91% that of the all-trans acetate.

Formation of maleic anhydride adducts. In 1939 Pauling (25) reported that the 9-10 and the 13-14 double bonds in the vitamin A structure were "stereochemically effective" and could exist readily in either the trans or cis configuration, and that other isomers are unlikely because they would be sterically hindered.

Meunier and Jounmeteau (26) studied cis-trans isomerism. They found that the proportion of vitamin A present in the trans form, as estimated by the reduction of intensity of the $SbCl_3$ reaction after treatment with maleic anhydride, was 18 to 42% in

the shark oils and concentrates and 45 to 55% in oils from red tunny fish. The rate of addition of maleic anhydride to form an adduct which gives no blue color with $SbCl_3$ (27, 28) is the basis for the most used method for detecting vitamin A isomers.

Maleic anhydride forms a condensation product of the Diels-Alder type with those vitamin A isomers in which both the 13-14 and the 11-12 double bonds are trans (22). Details of the procedure have been described (2). The isomers of vitamin A can be distinguished as the fast reacting group: all-trans, 9-mono-cis, and possibly 7-mon-cis, and the slow reacting group: 11-mono-cis, 13-mono-cis, 9,13-di-cis and 11,13-di-cis (18). Since apparently little or no 11-cis and 11,13-di-cis isomers are found in vitamin A supplements, the maleic value measures the amount of unreacted 13-mono-cis and 9,13-di-cis. Formulas to calculate the maleic values (MV) and relative biopotencies (RBP) have been published (29). The biopotency of the mixture is calculated as follows:

$$\text{Biopotency} = \frac{\text{RBP x Chemical potency by } SbCl_3 \text{ detn.}}{100}$$

The method has been applied to mixtures of vitamin A isomers from both synthetic and natural products (29).

The alcohol form of vitamin A reacts faster with maleic anhydride than the ester forms (30). After allowing the reaction to continue for three hours at 25° in a water bath, at which time some 99% of the "fast reacting" components has combined with maleic anhydride, the "slow reacting" isomers can be isolated from the reaction mixture of vitamin A isomers by adding ethanol-KOH, water, and extracting with light petroleum ether.

A method for fast and slow reacting components in a mixture has been developed by Black (31). For equal Carr-Price response, the biological potencies of the vitamin A from different species of Crustacea were 32, 35, 44, 25 and 29% that of all-trans vitamin A.

Fresh livers from rats contain little slow reacting vitamin A isomers, if the animal was not fed appreciable amounts of such isomers (32). Results on chicks indicate similar conditions. The feeding experiments indicated that the slow reacting isomers of vitamin A in the livers of rats may be derived primarily from the diets available to the experimental animal and that the fast reacting isomers are the naturally occurring forms in the livers of rats and chicks. Lambertsen, Myklestad and Braekman (33) reported the presence of only small amounts of neovitamin A in fresh fish livers.

Vitamin A stereoisomers labeled with ^{14}C were prepared by Isler et al. (34). Because of "Pauling hindrance" the cis-isomers are unstable and labeling does not seem to be possible for all the cis-isomers.

Aqueous multivitamin drop formulations, using different samples of all-trans palmitate were prepared (35). These samples were assayed after 3, 6, 9 and 12 months storage at 37^{o}, and after 9, 12 and 15 months storage at 25^{o} by the maleic anhydride technique for isomers, and by 3 methods for total vitamin A. Results indicated that during storage of the multivitamin preparations, all-trans vitamin A isomerized to a mixture containing

not only the all-trans and 13-cis forms, but also significant quantities of 9-mono-cis and 9,13-di-cis forms, which have much lower biological activities. Results differed by the three assay methods. The largest deterioration in vitamin A content was found when the rat liver-storage bioassay (36) was used; intermediate changes were shown by the USP spectrophotometer assay (37) and less deterioration was detected by the $SbCl_3$ blue-color assay (38). The different results indicate isomerization of vitamin A. All four isomers form the same blue color with $SbCl_3$, but the three cis-isomers have lowered USP values.

Studies of the biological potency of isomerized vitamin A palmitate in aqueous multivitamin dispersions indicated that relative biopotencies varied inversely with maleic values (29). Results of that assay seemed to indicate that the all-trans vitamin A palmitate isomerizes more rapidly to the 13-cis isomer, which has 75% of the biopotency of the all-trans vitamin A, than to the 9-cis isomers, which has only about 23% the biopotency of all-trans vitamin A. The isomerization of all-trans vitamin A palmitate in aqueous multivitamin dispersions appeared to reach an equilibrium, since even after prolonged storage the relative biopotencies did not drop below 60 to 70%.

Murray and Campbell (39) reported on the biological potencies of acetate and aldehyde forms of all-trans, 9-mono-cis, and 13-mono-cis vitamin A and on the acetate form of 9,13-di-cis vitamin A, as estimated by the rat vaginal smear method. Results were similar to those found by Ames (36) by growth and liver-storage methods.

Since the biological potencies of the 9-cis acetate and aldehyde and the 9,13-di-cis acetate isomers were relatively low, it followed that both the USP XVII and the $SbCl_3$ methods would give too high potencies for samples containing appreciable amounts of 9-cis and 9,13-di-cis isomers. On the average, values by the USP XVII assay were 2% high for both types of samples and those by the $SbCl_3$ method, were 17 and 12% high for oily and aqueous preparations, respectively, as compared to bioassays.

The assays for vitamin A in margarine have been reviewed (40). This review reported the effects to be expected from each of five methods of assay when commercial sources of vitamin A that contain cis-isomers are analyzed. If all-trans is the only form of the vitamin A present, all methods[2] should give the same results.

B. Effect of Non-Retinol Interfering Substances on Vitamin A Biopotency Determination

Among the vitamin A derivatives that interfere with the retinol assay is the 3-4 dehydroretinol (vitamin A_2), the predominant form of vitamin A in fresh water fishes and the subject of study by many workers (41-45). Vitamin A_2 is similar in structure to vitamin A_1, except for one more double bond; the absorption maxima is at 351 nm in the UV, and at 693-695 nm with

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[2]These methods are: $SbCl_3$ blue-color (Assoc. Vitamin Chemists); USP method (Spectrometry with Morton-Stubbs correction); growth rate of rats (Federal Std. of Identity); colorimetry with chromatography (AOAC); and spectrometry with various methods for correction blank absorption (NAMM).

$SbCl_3$. Vitamin A_2 gives a brownish UV fluorescence against a dark violet background (46). It is often mistaken for vitamin A_1 that has maxima at 325 nm in the UV and at 620 nm with $SbCl_3$. Pure vitamin A_2 alcohol has a biological potency of 40% of that of vitamin A_1 alcohol (47). Gillam et al. (43) reported an absorption maximum between 340 and 350 nm for vitamin A_2 compared with the 325-328 nm for marine fish liver oils. They also stated that some liver oils of fresh water fish may be toxic. Other authors (48) reported that massive doses of vitamin A_2 were less well tolerated by rats than equal doses of vitamin A_1. Partition chromatography may be used to separate vitamins A_1 and A_2 in fish lipid unsaponifiable matter (36).

Vitamin A aldehyde and ester derivatives of vitamin A_2 have been encountered (49-52), which certainly interfere in the analysis for all-trans retinol. When vitamin A is oxidized to aldehyde, the biological activity is preserved, but when oxidized to the epoxide form the activity is lost (45). The possibility of biological activity surviving oxidation in foodstuffs may, therefore, depend on the direction taken by the oxidation.

A new congener of vitamin A_2 has been reported (53) which is the 2-dehydroretinol. It was found during chromatography of liver oils of "Bagarious bagarious" fish. The absorption bands were at 433, 408, 388, 368, 350, 310, 296 and 284 nm. The compound was obtained also by treating 3-dehydroretinol with dehydrating agents.

A non-vitamin A active substance found in liver extracts, with UV absorption similar to that of vitamin A, was reported as early as 1934 by Castle et al. (54). It was prepared by treating retinol with N/30 HCl in absolute alcohol (55). The reaction was thought to be one of cyclization, but the properties described for the compound indicate dehydration instead. The compound is now called anhydrovitamin A. The absorption spectra has maxima at 351, 371 and 392 nm, with $E^{1\%}_{1\,cm}$ values of 2500, 3650 and 3180, respectively. The absorption band at 392 nm is always well defined but the maximum at 351 nm is a pronounced peak in some samples and only a sharp inflection in other samples. The $SbCl_3$ reaction product of anhydroretinol had an $E^{1\%}_{1\,cm}$ (620 nm) of 5500. This value is somewhat higher than that of vitamin A_1 (4800). The biological activity of anhydroretinol is only 0.4% that of all-trans retinol (56).

Utilization of anhydroretinol by the vitamin A deficient rat was very poor (57). It was not absorbed or stored to any measurable extent. Probably the small growth-promoting activity of anhydrovitamin A was due to one or more derivatives formed in vivo, probably by a hydroxylation mechanism (58). The error in assaying samples containing anhydrovitamin A by the $SbCl_3$ method is greater than by the UV absorption at 325 nm. Anhydrovitamin A is readily separated from vitamin A through column chromatography.

Shantz (56) and Embree and Shantz (59) reported the formation of another vitamin A derivative, retrovitamin A, when vitamin A remained in contact with an acid-alcohol reagent for

extended periods of time. Whereas anhydrovitamin A is formed after exposures from 10 to 15 minutes, the other derivative required from 10 to 16 hours. Retrovitamin A also was formed by treating anhydrovitamin A with HCl or $SbCl_3$, and by treating retinyl acetate with concentrated hydrobromic acid (60). The absorption spectra of retrovitamin A, compared to that of anhydrovitamin A, is shifted about 20 nm towards shorter wavelengths, with maxima reported at 332, 348 and 366 nm, respectively. A biopotency of 12% was found for retrovitamin A acetate. This derivative appeared in commercial samples of liquid multivitamin preparations together with anhydrovitamin A but was not separate by chromatography like anhydroretinol (61). It was concluded that anhydrovitamin A and retrovitamin A may occur in pharmaceutical products in amounts that interfere with the assay of vitamin A by the USP XVII and British Pharmacopeia methods.

Olson (20) reported that the retro-system once formed, does not shift back to the normal conjugated system of vitamin A, although hydration of the terminal double bond may take place. (See Plate II for formulas of compounds discussed.)

There are indications of in vivo conversion of retroretinol to retinol (62), but the conversion is not efficient, as suggested by the low biological potency of retrovitamin A. Retrovitamin A was prepared from retinyl acetate by treatment with concentrated hydrobromic acid (63); separation of the retro form was accomplished by chromatography on 5% deactivated alumina.

EXPLANATION OF PLATE II

Vitamin A and some vitamin A derivatives.

Fig. 1. All-*trans* retinol (vitamin A_1).

Fig. 2. 3,4 dehydroretinol (vitamin A_2).

Fig. 3. 2-dehydroretinol.

Fig. 4. Anhydroretinol.

Fig. 5. Retroretinol.

PLATE II

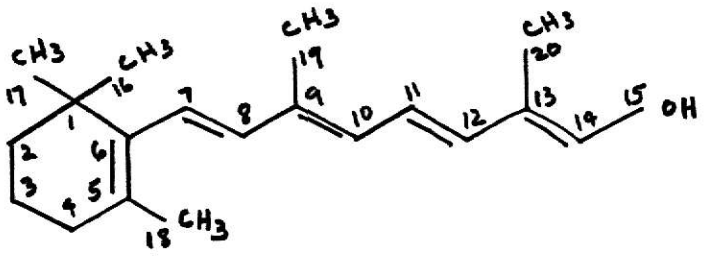
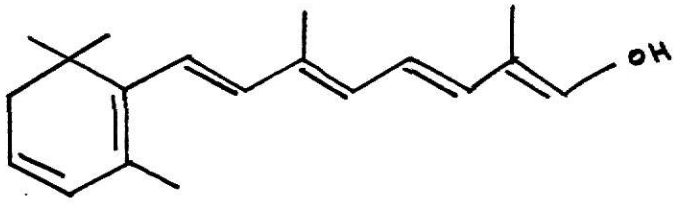


Fig. 1.



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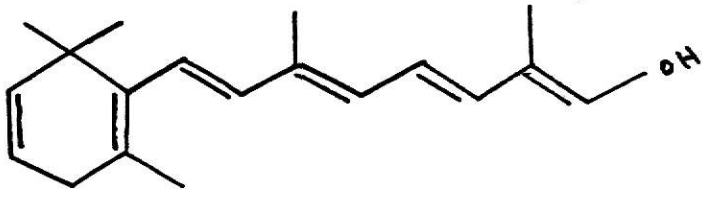

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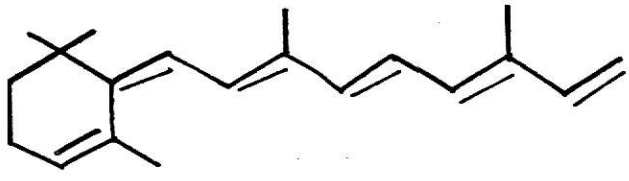

Fig. 4.

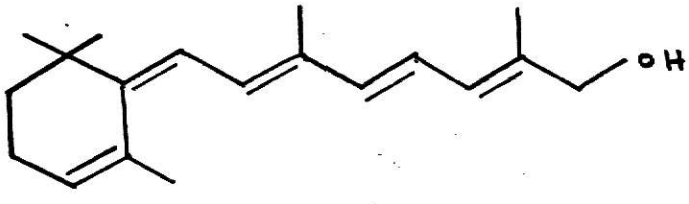

Fig. 5.

C. Methods for Determination of Total Vitamin A and Vitamin A Isomers

Alumina for chromatography. Studies on assays of vitamin A with and without chromatography (64-68) have been carried out to obtain vitamin A extracts sufficiently free of interfering substances, including vitamin A derivatives and isomers, that satisfactory assays are obtained (68, 69). Alumina for chromatography has been evaluated with respect to particle size (70) and adsorptive activity for optimum control of the chromatographic column. Proper deactivation of alumina used is also important, since anhydroretinol is formed by insufficiently deactivated alumina (71). Catalytic dehydration of alcohols over alumina (72, 73) to form ethers and olefins, and conversion of alcohols to ethers also has been reported (74). These reactions might occur in vitamin A.

Colorimetric methods for total vitamin A. In 1925, Rosenheim and Drummond (75) studied color reactions for vitamin A determination. Carr and Price (38) extended those studies and developed a method which, with some modifications, is still in use today. The sensitiveness of that method, as well as the effect of light on stability of the blue color complex formed with the vitamin, has been reported (76).

A commonly used procedure for colorimetric determination of vitamin A is that in Official Methods of Analysis (77), which is based on the blue color formed with vitamin A and $SbCl_3$ having an absorption maximum at 620 nm. The colorimetric assay usually is

preceded by hydrolysis with alcoholic KOH, extraction, and chromatography on alumina. Other color producing reagents, such as trifluoroacetic acid and ferric chloride, also have been used and some advantages and disadvantages discussed (78). Methods have been published for the assay of vitamin A using dichlorohydrin (79-82) as the reagent, and still others (83, 84) using Superfiltrol and polyenes (85) on acid earths.

Spectrophotometric methods for total vitamin A. The methods are based on the absorption maximum of vitamin A in isopropanol at 325 nm. A polar solvent (alcohol) results in higher spectrophotometric values than does a nonpolar solvent (hydrocarbon), and a shift of the peak toward shorter wavelengths (86, 87, 88), especially for samples of unsaponifiable fractions of oils or vitamin A alcohol. The vitamin A concentration must be controlled to keep the absorbance (OD) between 0.2 and 0.8 for highest precision in the determination.

Strohecker and Henning (89) stated that "when pure vitamin A is present in a mixture, the spectrophotometric method is the ideal one; but if the sample of vitamin A contains other substances absorbing in the same region as all-trans retinol, there will be high interference in the assay." They pointed out that most of the interfering substances present in vitamin A oils can be removed by hydrolysis and extraction. Decomposition products of retinol developed when preparations containing vitamin A are kept for some time, particularly under certain conditions of temperature and pH. Decomposition products markedly affect the