VITAMIN A ISOMERS IN ANIMAL LIVERS

by

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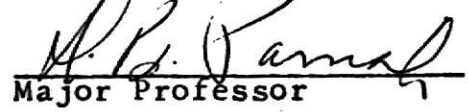

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I. INTRODUCTION

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

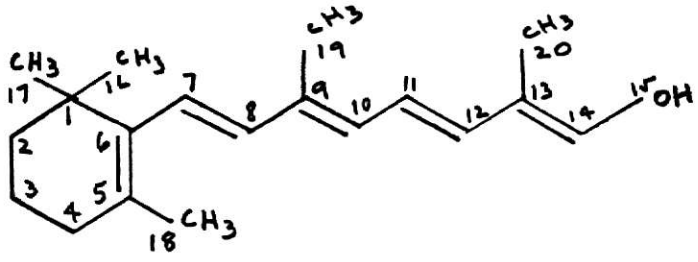
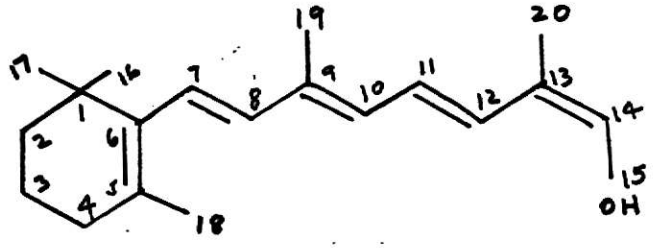


Fig. 1.



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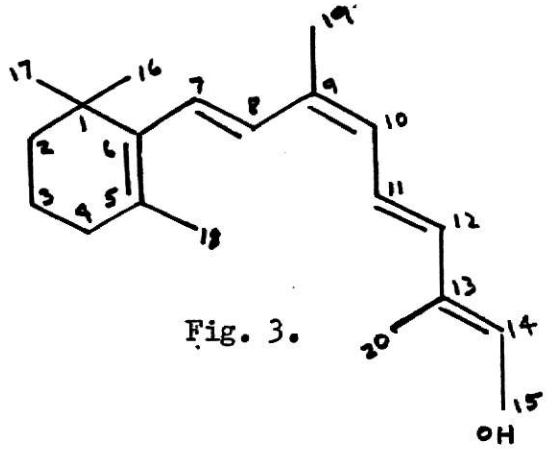

Fig. 3.

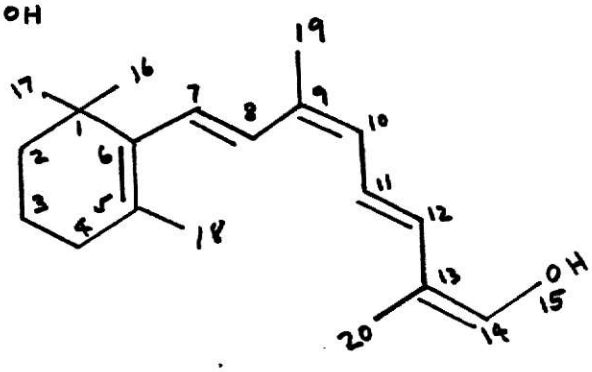

Fig. 4.

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

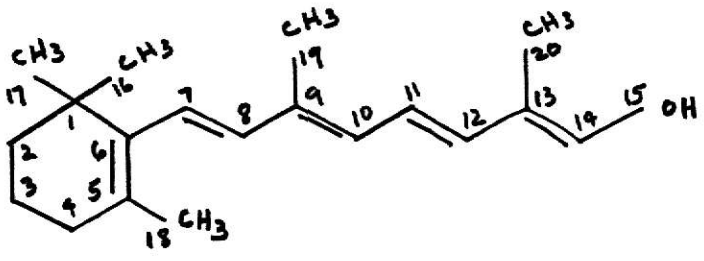

Fig. 1.

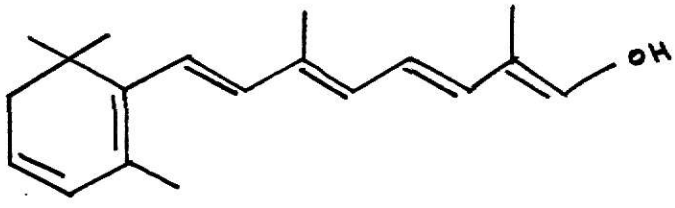

Fig. 2.

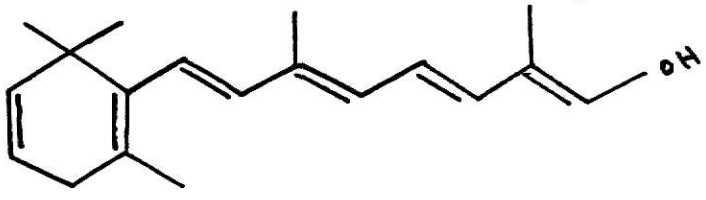

Fig. 3.

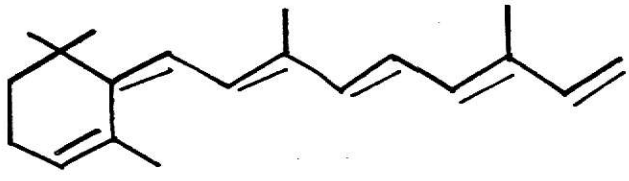

Fig. 4.

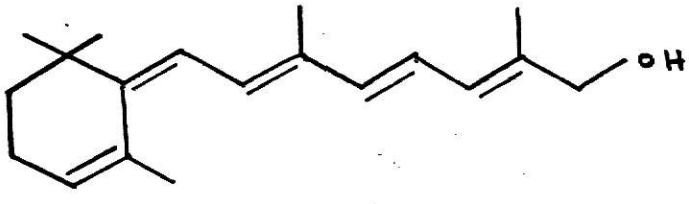

Fig. 5.

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

absorption curve of the unsaponifiable fraction of the sample. Spectrophotometric methods do not give accurate results on mixtures containing vitamin A isomers, artifacts or certain impurities, and there may be little relationship between biological and spectrophotometric properties. Modifications have been introduced to improve the results by spectrophotometric assays, such as determination before and after destructive irradiation (90); the vitamin A absorption is the difference of the readings before and after irradiation. It is assumed, but not necessarily true, that vitamin A is the only substance affected upon exposure to light. Critical factors include length of irradiation time and wavelength of the light. Other authors (91) suggested a spectrophotometric method for vitamin A based on conversion of retinol to anhydrovitamin A, stating as the advantage that no other compound will interfere with the absorptions characteristic of the anhydro form at 351, 371 and 390 nm. Chromatography, a rather lengthy process, is apparently not necessary if this procedure is followed, but small traces of water can cause appreciable losses of vitamin A.

Morton and Stubbs, 1946 and 1948 (92-94), stated that "irrelevant absorption" was found which was due to vitamin A_2, kitol, anhydrovitamin A_1 and A_2, oxidation products of vitamin A, carotenoids and unidentified colored compounds, as well as intermediates and artifacts which occur as a result of vitamin A decomposition during storage or extraction, especially in highly concentrated oils. Deterioration also occurs during transit.

Hydrolysis of the sample, and purification through chromatography aid in the assay, but even then a correction for irrelevant absorption is necessary.

A correction formula was developed by Morton and Stubbs based on the assumption that irrelevant absorption at 310 and 334 nm (in isopropyl alsohol) will be horizontal at a level of 6/7 of the intensity of E max. at 325 nm. The difference between the uncorrected and the corrected curve is assumed to represent the spectra of substances other than vitamin A.

Observations on precision and reproducibility of the Morton-Stubbs three-point correction have been reported (95-98). The correction is based on the assumption that absorption curves of natural forms of vitamin A are indistinguishable from that of pure all-trans retinol and that UV absorption curves of materials other than vitamin A in natural oils have three linear points at certain wavelengths. There is evidence that these assumptions are not always correct. It has been shown that comparatively small departures from these conditions may be associated with appreciable loss of accuracy.

Cama, Collins and Morton (99) reported that, although for a wide range of products the $E^{1\%}_{1\,cm}$ value at 325-328 nm on the oil or unsaponificable fraction is a good guide to potency, it is necessary to take full account of the presence of anhydrovitamin A_1 and A_2, kitol, epoxides and other substances for an accurate assay. Except with low potency oils it usually is safer to work on the whole oil because the correction applied to unsaponificable

materials tends to give low results. Also of great importance is the determination of the neo/all-*trans* ratio, which is measured as the maleic value (MV).

Bioassays for vitamin A isomers. The potency of vitamin A in preparations can be determined by bioassay (100). Among the types of bioassay, the most highly regarded when dealing with vitamin A isomers, is the liver-storage assay, in which the amount of vitamin A stored in livers of the animals previously deprived of the vitamin is determined (36, 101, 102). Results obtained in these studies show essentially a linear response over a dose range of 500 to 10,000 units. When the US Reference Standard was fed, 67% of the ingested dose was found stored in the liver.

Physico-chemical methods for determining vitamin A isomers. The opsin assay (103-106) has been used for estimation of specific vitamin A isomers. In this method the 9-*cis* and the 9,13-di-*cis* isomers in hydrolyzed extracts are determined. The retinols are purified on alumina, oxidized to aldehydes with MnO_2, and reacted with opsin (protein of retina). The specific reaction of the 9-*cis* retinols with opsin to give isorhodopsin with an adsorption maximum at 487 nm makes the determination possible. Application of this procedure to liver oils from fish, cattle, sheep, pigs and rats showed 9-*cis* isomer contents ranging from 1 to 30% of the total vitamin A (105).

Knobloch and Krenova 1969 (107) published on the quantitative determination of vitamin A isomers by an optical absorbance gradient method. The determination is based on separation by chromatography on a silica gel column and elution with 24:1 to 9:1 pentane-ether mixtures. Absorbance of each eluate is measured at 325 nm and plotted against volume of fraction collected; planimetry of the areas under the curves gives the concentration of each isomer. Five isomers of vitamin A were claimed to have been separated by this technique.

The most commonly used method for the determination of vitamin A isomers is that of Ames and Lehman (28). The reaction of vitamin A with maleic anhydride characterizes those isomers in which both the 13-14 and the 11-12 bonds are trans. Vitamin A is reacted with maleic anhydride for 16 hours under controlled conditions, and the maleic value is calculated from the proportion of unreacted vitamin. All-trans vitamin A and the 9-cis-isomer have a MV of zero; the adducts formed do not give blue color with $SbCl_3$.

III. METHOD

A. Instruments and Apparatus

Evelyn Photoelectric Colorimeter. Electro-Nite Co., Rubicon Instruments Division, Comply and Decatur Roads, Philadelphia, Penn., 19154. The instrument has a direct-reading, deflection-type galvanometer, filters to transmit light at 620 nm, and matched absorption tubes.

Rapid delivery pipet. For $SbCl_3$ reagent (Parrish and Caldwell, 108).

Chromatography tubes. 15 x 170 mm, sealed to a tube 5 x 100 mm.

Ultraviolet light. Long wavelength, such as UVL-22, 3660 Angstrom units, Ultraviolet Products, San Gabriel, California.

Refluxing apparatus. All-glass condenser and 300-ml boiling flasks with five joints; six units in series, with steam as the source of heat.

Separatory funnels. Five hundred and 250 ml. Lubricate lightly. Lubricant should not be soluble in alcohol or hexane, nor contain alcohol or hexane soluble substances that will absorb ultraviolet light.

Vacuum evaporator. A water aspirator and 50 to 55° water bath. A suitable assembly consists of a Corning flask of appropriate size, sealed to a connecting tube and male joint to fit one arm of 2-mm bore, 3-way stopcock (85, Fig. 2).

Spectrophotometer. Beckman, Model DU, with voltage regulator and provided with silica cells.

Other glassware. Glass-stoppered volumetric flasks (200, 25 and 10 ml), volumetric transfer pipets and glass funnels.

B. Reagents

Potassium hydroxide. Reagent grade, 50% aqueous solution prepared daily prior to determination.

Alcohol. 95% methanol, aldehyde free (Schiff's test).

Hexane. Commercial grade Skellysolve B, b.p. 64 to 68°.

Sodium sulfate. Anhydrous, powdered, reagent grade.

Chloroform. Reagent grade. Purify if necessary by distillation, discarding first and last 10%.

Antimony trichloride (Carr-Price, reagent). Crystals, ACS reagent grade, Allied Chemicals, Code 1369. Use 1/4 lb bottle; weigh 100 gm, transfer to dark bottle, using glass funnel; add about 400 ml reagent-grade chloroform, warm to dissolve, and dilute to 500 ml with chloroform. Add 3% acetic anhydride (analytical reagent), filter and store in a dark bottle with ground-glass stopper.

Potassium-sodium tartrate (Rochelle salt). N. F. powder. Ten percent solution for cleaning glassware that has been in contact with the Carr-Price reagent. Fifty percent HCl also is suitable.

USP vitamin A reference standard. 34.4 mg trans-vitamin A acetate (equivalent to 30.0 mg trans-vitamin A alcohol) per gm. (Equivalent to 100,000 USP units per gm.) U. S. Pharmacopeial Convention, 4630 Montgomery Ave., Bethesda, Md., 20014.

Adsorbent. Neutral alumina, grade 1, M. Woelm, Germany. Deactivated to 5% moisture. Before use, allow moisture to diffuse and heat of hydration to dissipate for three hours after adding water. Store in small, tightly closed containers to avoid further change in moisture content.

Acetone-in-hexane solution. Acetone (reagent) freshly diluted with hexane to concentrations of 2, 4 and 15%.

Benzene. Reagent grade.

Maleic anhydride ACS, certified, 10% solution in benzene. Discard after one week.

C. Procedure

Fourteen chicken livers were obtained from the Department of Biochemistry, Animal Nutrition Laboratory; four beef livers, one sheep liver, and one pig liver were obtained from the Department of Animal Science and Industry, Kansas State University; and two chicken livers, one beef liver, three calf livers, and four pig livers were from supermarkets in Manhattan. Livers were kept frozen until sampled for analysis. None of the samples were stored for more than three days before the first analysis.

Some liver samples were given treatments prior to hydrolysis to study effect on isomerization and relative biopotencies. Some livers were exposed to visible light for 4 hours, and to higher temperatures (50° for 24 hours); others were analyzed before and after refrigeration for 6 weeks, and before and after storage for 3 weeks in darkness at room temperature; certain liver samples were analyzed before and after 1.5, 3, 4 and 5 months' storage in a frozen condition. Isomerization and relative biopotencies were determined in the liver extracts after exposure to iodine[3] for 2 or 4 hours, and light plus iodine for 2 hours.

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[3]One drop of approximately 5% iodine in benzene added to 10 ml of benzene extract containing vitamin A.

In earlier work, storage of vitamin A in animal livers as an equilibrium mixture of isomers (9) could not be verified (109). Ames[4] suggested that perhaps chicks selectively lose all-*trans* retinol from the liver storage when changed to a vitamin A low or depletion diet, resulting in a relative increase in the proportion of vitamin A *cis*-isomers. To study this possibility, an experiment was set up in which chicks fed a richly-supplemented vitamin A diet for 6 weeks were depleted by feeding a vitamin A-free diet for 8 weeks. The vitamin A content of livers of some chicks was analyzed for isomer distribution at the beginning of the depletion period and content in livers of others after 3 and 8 weeks.

The general procedure used for vitamin A was modified from the AOAC method for mixed feeds (77) as outlined in the following:

Sampling and hydrolysis. Frozen livers were sliced thinly and mixed. Random samples of about 20 to 30 gm were weighed accurately in duplicate and placed in 300 ml boiling flasks, except in the case of chicken livers where the whole liver was used as a single sample. At all times samples were protected from direct sunlight and the work was carried out under subdued laboratory light. Quantities and order of adding the reagents were slightly modified based on results obtained in preliminary work. Distilled water (10 ml) was added to the sample, followed by 10 ml of freshly prepared 50% KOH and 70 ml of 95% ethanol.

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[4] S. R. Ames personal communication.

The reagents and sample were mixed thoroughly and refluxed for 30 minutes, swirling the flasks 3 or 4 times during refluxing to insure complete hydrolysis.

Extraction. The neck of the refluxing apparatus was rinsed with 5 ml of water, which together with the solution of hydrolyzed sample was transferred to a separatory funnel. The flasks were rinsed with 10 ml of water and the rinsing also was added to the solution in the separatory funnel. One hundred ml of hexane was added and the separatory funnel was shaken for 60 seconds for extraction, followed by two similar 50-ml extractions. After shaking, enough time was allowed for the layers to separate, and the bottom layer was withdrawn. If emulsification occurred, a few ml of absolute alcohol or a few crystals of anhydrous sodium were added to the separatory funnel and it was swirled again. If the extract was highly pigmented, additional hexane extractions were used until all extractable pigments were removed, which was used as an indication of completeness of extraction of hexane-soluble substances, including vitamin A.

The three or more extractions were collected together in a separatory funnel, and three successive washings (100 ml and two 50 ml each)[5] with distilled water followed. The first washing was only by gentle swirling and inverting the separatory funnel. The second and third washings were by shaking gently.

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[5] pH of the last washing was checked for complete removal of base.

Any emulsion formed was retained in the hexane solution. After washing, the hexane solution was allowed to stand 10 to 15 minutes and any water separating was drained carefully. Anhydrous sodium sulfate (2-3 gm) was added to the hexane solution and the separatory funnel shaken. After standing for 3 to 5 minutes the solution was filtered through a small cotton plug held in a funnel placed in the top of a volumetric flask of appropriate size. The solution was diluted to volume with hexane.

Chromatography. An aliquot of the sample was chromatographed through 5% deactivated neutral alumina. Vitamin A content per ml of extract was determined in the whole extract prior to chromatography, so that an estimate could be made of the quantity of extract to chromatograph for a satisfactory determination of maleic anhydride value and vitamin A by the $SbCl_3$ and by the spectrophotometric methods. If the volume of extract required was larger than 25 ml, the extract was evaporated under vacuum to reduce the volume to 25 ml. Chromatography was done in subdued light. The column was eluted with 0, 2, 4 and 15% acetone-hexane solutions to obtain various fractions of the sample. Elution rate was about 40 to 60 drops per minute. Separation of the fractions was followed by observing the color of different bands and by fluorescence using the long-wavelength ultraviolet light.

Different fractions were collected and measured separately. Retinol, the fraction eluted with 15% acetone-hexane, fluoresced

green. A quantitative determination of vitamin A was performed on this fraction.

Carr-Price reaction (38). A calibration curve was prepared as follows: a solution of USP Reference Standard vitamin A was prepared by accurately weighing about 0.1 gm of the oil. After adding 0.25 ml of cotton seed oil, 12 ml of distilled water, 5 ml of 50% KOH, and 25 ml 95% ethanol it was hydrolyzed 30 minutes. Vitamin A was extracted using the same technique as on samples. Dilutions of standard vitamin A were made to prepare a series of at least five chloroform solutions containing from one to 10 µg of vitamin A per ml, which gave transmittances in the 20 to 85% range.

Chloroform (1 ml) was pipetted to a colorimeter tube which was placed in the colorimeter holder. Nine ml of $SbCl_3$ reagent was added from a rapid delivery pipet. With this solution in the light path, the galvanometer was set at a 100% transmittance, using the 620 nm filter. The operation was repeated in duplicate for each chloroform solution of the standard and percent transmittance was recorded from the galvanometer at the pause point 3 to 5 seconds after adding the reagent. All readings were converted to OD or absorbance by the formula ($2 - \log G_{620}$), where G_{620} means galvanometer reading (transmittance) at 620 nm. This value is commonly called "L value" when using the Evelyn colorimeter. Absorbance was plotted on the X axis and µg of vitamin A per tube on the Y axis. In the absorbance range from approximately 0.1 to 0.6, the data plot as a straight line.

The value of the tangent to the calibration curve was calculated. It is used as the factor to convert sample absorbance to vitamin A. The factor obtained was 13.5 (or 45 when results are expressed in USP units since 1 μg of vitamin A is assumed to be equivalent to 3.33 USP units).

Sufficient sample extract was used to contain from 7 to 15 units of vitamin A. The proper portion was evaporated to dryness at 50° under vacuum provided by the aspirator. The residue was dissolved immediately in 1 ml of $CHCl_3$.

Samples and standard solutions were treated in the same manner. If the extract contained enough pigments to give a yellow color in visible light, correction was made for carotene pigment (77) prior to the $SbCl_3$ reaction.

Units of vitamin A in the sample were calculated as follows:

$$\text{USP units of vitamin A} = \text{Absorbance} \times \frac{X}{1} \times \frac{45}{\text{wt of sample in gm}}$$

X = Volume of total extract.

Determination of maleic value. The maleic anhydride method for determining vitamin A isomers (27) is based on the reaction of vitamin A with maleic anhydride (44, 18, 28) carried out as follows:

a) Determine total vitamin A as described under III C.

b) Evaporate to dryness under vacuum at 50 to 55° an aliquot of the sample containing at least 2500 USP units of vitamin A. Redissolve in 10 ml of benzene and divide in two 5-ml portions. Place each portion in 10-ml flasks.

c) Dilute 1 to 10-ml volume with benzene (solution A), and the other to 10 ml with 10% maleic anhydride solution (solution B). Stopper, mix, and incubate each for 16 hours at 25°.

d) Dilute 2 ml of solution A to 25 ml with chloroform (sample A). Dilute 2 ml of solution B to 10 ml with chloroform (sample B). Do the same with tubes of both the A and the B series. Determine vitamin A in 1 ml of each solution as in III C.

e) Convert all transmittancies to absorbance (L value) and calculate vitamin A in series A and B, as in III C.

f) Calculate recovery (R) as follows:

$$\frac{\text{Units of vitamin A in solution B}}{\text{Units of vitamin A in solution A}} \times 100 = R\ (\%)$$

R = Recovery in %.

g) Determine maleic values (MV) as follows:

$$\frac{R-1.1}{88.4} \times 100 = MV$$

The factors 1.1 and 88.4 have been developed to calculate maleic values in hydrolyzed vitamin A (vitamin A alcohol) knowing the percentage recovery (R) (28).

h) Estimate relative biopotency (RBP) by the following regression equation or from prepared table (27, 28),

$$RBP = 99.5 - 0.2\ (MV) - 0.051\ (MV)^2 + 0.000768\ (MV)^3$$

Spectrophotometric determination of vitamin A with Morton-Stubbs correction (37). Ultraviolet absorption curves also were determined on the unsaponifiable and chromatographed fractions of the samples. The data were used to calculate vitamin A content for comparison with results by the colorimetric method and

relative biopotency calculations as indications of purity of extracts.

For spectrophotometric determinations, a portion of the sample extract containing from 7 to 15 USP units of vitamin A was evaporated under vacuum and redissolved in isopropyl alcohol. Absorption curves were run on the retinol fraction and other fractions collected during chromatography. Corrected absorptions at 325 nm were obtained by applying the following formula to data at the wavelengths indicated:

$$A_{corrected} = 7(A_{325}) - 4.375\ (A_{334}) - 2.625\ (A_{310})$$

A = Absorbance at indicated wavelengths.

Derivation of the factors in the formula was reported by Morton and Stubbs (93).

The USP units of vitamin A are given by:

$$\frac{A_{corrected}}{L \times C} \times 5700 \times \frac{1}{0.3} = \text{USP units of vitamin A per gm}$$

$A_{corrected}$ = Corrected absorbance at 325 nm.

L = Length of light path in cm (width of cell).

C = Concentration of sample in gm per 1000 ml of isopropyl alcohol solution.

5700 = Factor for conversion from spectroscopic to gravimetric units.

0.3 = Factor to convert grams to USP units.

IV. RESULTS

The USP Reference Standard (all-*trans* vitamin A) and 13-*cis* retinol (Distillation Products, Inc.) were analyzed for maleic values and relative biopotencies (RBP) following the same

procedure as for liver samples. The RBP obtained was 99.5% for the all-*trans* retinol and 72% for the 13-*cis* retinol, in general agreement with published values for these isomers (6, 22).

In general, RBP of vitamin A in fresh livers were high, ranging from 91% to 100% (Table 1). Analyses by the Carr-Price method were in duplicate. Therefore, values for methods 1 and 2 are available in duplicate, except for chicken livers (Appendix, Table 2). Values in Table 1, however, are from single determinations so that all comparisons by three different methods are from the same liver extract. Samples 16, 17 and 22 had relative biopotencies of 91%. These were the lowest biopotencies found on samples at the time received. Samples 16 and 17 were from 15-month-old Hereford-Angus cross steers raised in a commercial feedlot, and sample 22 was a calf liver from a supermarket. Sample 14, from a 15-month-old Angus steer, raised in a feedlot, was almost depleted of vitamin A. Since only six USP units of vitamin A per gm were found in that liver, the maleic value was not determined because more vitamin A is required for the determination.

In general, chicken livers gave higher values for vitamin A and relative biopotencies than other liver samples. Most chicken livers were from birds in the Department of Biochemistry, Animal Nutrition laboratories, and were fed controlled diets. Pig, sheep and calf livers had higher vitamin A content and higher RBP than beef livers, except for one sample of beef liver from the supermarket. Animals raised in the feedlots did not have appreciable quantities of vitamin A in the livers.

Table 1

Comparison of vitamin A data by three different methods.

Source of liver sample (species)	Sample No.	Months of storage frozen	Method 1 Carr-Price[1] USP units/gm	MV[2]	RBP[3] %	Method 2 Biopotency[4] USP units/gm	Method 3 UV[5] USP units/gm
	1		923	3	99	914	958
	2		864	4	98	847	851
	3		882	2	99	874	836
	4		849	2	99	841	798
	5		930	2	99	921	827
Chicks	23		554	5	98	543	575
	24		983	7	96	944	--
	25		896	6	97	869	788
	26		872	9	95	828	799
	9		250	4	98	245	371
	10		192	2	99	190	217
	11		84	1	100	90	80
	14		6	--	--	--	8
	14	5	2	--	--	--	--
	16		36	11	91	33	33
Beef	16	5	17	8	95	16	16
	17		57	11	91	52	53
	17	4	37	9	95	35	32
	18		61	7	95	58	58
	18	4	61	10	94	57	27
	15		266	6	97	258	264
Sheep	15	5	78[6]	11	92	70	65
	13		446	3	98	437	446
	12		208	5	98	204	345
	20[7]		484	6	96	465	482
Pig	20	2nd day	360	5	98	342	--
	27		250	8	95	238	218
	28		215	5	98	210	196
	19		151	4	98	148	148
	19	4	93	8	95	88	86
	21		260	10	94	244	202
Calf	21	1.5	185	4	98	181	182
	22		224	11	91	214	198
	22	3	140	11	91	129	122

[1]In this table and hereafter, 1 ug of vitamin A by the Carr-Price method is assumed equivalent to 3.3 USP vitamin A units.

[2]Maleic values.

[3]Relative biopotency.

[4]As calculated from RBP.

[5]USP XVII, with Morton-Stubbs correction.

[6]Highly autolyzed after 5 mo storage, where found to be incompletely frozen.

[7]This liver not frozen; refrigerated only before analysis.

Carr-Price vitamin A values were generally higher than UV vitamin A values, but the difference was small or moderate in most cases. Biopotencies, as calculated from RBP data, were, of course, lower than values given directly by the $SbCl_3$ reaction. The spectrophotometric values were higher than those by the Carr-Price method in only a few cases (six out of thirty).

Traces of anhydrovitamin A, as determined from shapes of spectrophotometric absorption curves, were found in all chicken livers and in pig liver sample 20, which had not been frozen.

In samples given special treatments (samples 19, 21 and 22) some isomerization apparently took place. Sample 19 (Table 2) had an initial RBP of 98%, which dropped to 96% when the determination was made after 6 weeks of refrigeration, 95% when the sample was stored in a frozen condition for 4 months, and 82% when the sample was stored in darkness at room temperature for 3 weeks. Sufficient anhydrovitamin A also was developed that it could be detected spectrophotometrically but not enough to record. In sample 21 (Table 3) calculated relative biopotencies were slightly higher after livers were exposed to light or 50°. When the sample was kept frozen for 6 weeks, relative biopotency dropped from 98 to 94% on exposure to light and to 90% on exposure to 50° for 24 hours. Both before and after storage in a frozen condition, small quantities of anhydroretinol developed (2 to 5%). During chromatography this substance was eluted with hexane only. It fluoresces orange under UV light and has characteristic absorption maxima at 351, 371 and 392 nm (Fig. 1).

Table 2

Effect of different treatments on liver vitamin A relative biopotencies, calf liver 19.

Storage variable	Carr-Price USP units/ gm	RBP %	Biopotency USP units/ gm[a]	UV USP units/ gm[b]
Initial determination	151[b]	98	148	148
After 3 weeks room temperature[c]	97	82	80	58
	105	82	85	
Average	101	82	83	
After 6 weeks refrigeration[d]	95	96	91	89
	95	96	91	
Average	95	96	91	
After 4 months frozen storage[d]	93	95	88	86
	109	95	103	
Average	97	95	95	

[a]Calculated from RBP values.

[b]USP with Morton-Stubbs correction. Samples not run in duplicate.

[c]Protected from light.

[d]Traces of anhydroretinol developed on refrigeration, as shown by UV spectra.

Table 3

Effect of different treatments on liver vitamin A relative biopotencies, calf liver 21.

Treatments	Carr-Price USP units/ gm	RBP %	Biopotency USP units/ gm[a]	UV USP units/ gm[b]
Initial determination	260	93	242	202
	242	94	228	
Average	251	94	236	
Exposed to light 4 hours	214	98	210	213
	186	96	179	
Average	200	97	194	
Exposed to 50°/ 24 hours[c]	188	97	182	168
	115	95	109	
Average	152	96	146	
After 6 weeks storage frozen				
Initial determination	185	98	181	182
	115	98	174	
Average	152	98	177	
Exposed to light 4 hours[d]	196	92	180	178
	221	95	210	
Average	208	94	196	
Exposed to 50°/ 24 hours[e]	177	88	156	167
	158	92	145	
Average	167	90	150	

[a]Calculated biopotency from RBP data.

[b]USP with Morton-Stubbs correction. UV samples not run in duplicate.

[c]Traces of anhydroretinol developed.

[d]About 2% of total vitamin A as anhydroretinol; not present when sample was fresh.

[e]About 5% of total vitamin A as anhydroretinol; only traces present when liver was fresh.

Fig. 1

Absorbance curves of chromatographically separated fractions from extract of liver sample 22 stored in a frozen condition for 6 weeks.

——— Retinol in extract exposed to light for 4 hours at room temperature (eluted with 15% acetone in hexane).

—·—·— Retinol in extract exposed to 50° for 24 hours in darkness (eluted with 15% acetone in hexane).

..... Anhydroretinol in extract exposed to light for 4 hours at room temperature (eluted with hexane).

- - - - - - - - Anhydroretinol in extract exposed to 50° for 24 hours in darkness (eluted with hexane).

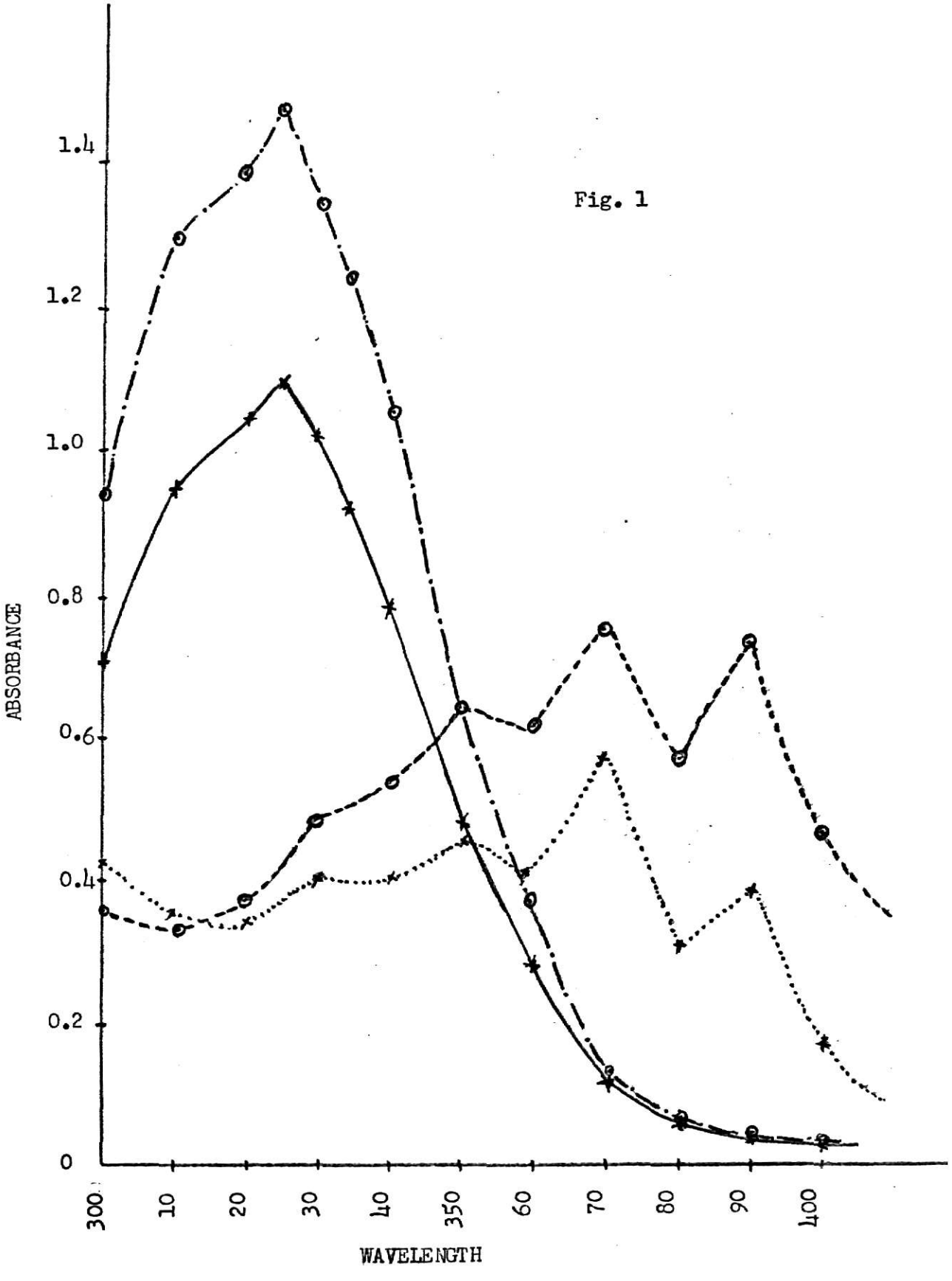
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
ABSORBANCE
300
10
20
30
40
350
60
70
80
90
400
WAVELENGTH

Fig. 1

More of this vitamin A derivative was found in livers kept frozen 6 to 20 weeks prior to treatments than in livers analyzed prior to storage.

In liver 22 (Table 4), vitamin A RBP was 92% in fresh liver and 93% after 5-months storage in a frozen condition. Isomerization occurred when samples of liver 22 were held at 50° for 24 hours in darkness, RBP dropping to 87% and then to 83% when portions of the same sample were exposed to light for 4 hours. No anhydroretinol was detected in this sample. Even if a small quantity of anhydroretinol were present, it would have been difficult to detect because the sample was highly pigmented and carotenoid absorption was found somewhat in the 350-390 nm range.

Special treatments also were given to vitamin A-containing extracts of livers to study the effect on isomerization of vitamin A in such solutions. Two drops of approximately 5% iodine in benzene were added to the vitamin A extracts in benzene and they were allowed to stand for 2 hours. Then maleic values were determined. Relative biopotency dropped from 98% to 78%. When treatment continued for 4 hours, a drop from 97% to 70% was found. In extracts treated with iodine plus exposure to light for 2 hours a complete isomerization and/or destruction apparently occurred since biopotency dropped from 97% to 0% (Table 5).

Chickens fed a basal diet (110) supplemented with higher-than-normal levels of vitamin A for 6 weeks were changed to a vitamin A-depletion diet to check on a suggestion[6] that the

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[6] S. R. Ames, personal communication.

Table 4

Effect of different treatments on liver vitamin A relative biopotencies, calf liver 22.

Treatments	Carr-Price USP units/ gm	RBP %	Biopotency USP units/ gm[a]	UV USP units/ gm[b]
Initial determination	224	91	204	198
	243	92	224	
Average	234	92	215	
Exposed to light 4 hours	162	86	139	145
	180	80	144	
Average	171	83	142	
Exposed to 50°/ 24 hours	158	89	140	116
	162	85	138	
Average	160	87	139	
After 5 months frozen storage	140	92	129	122
	178	94	167	
Average	159	93	148	

[a]Calculated biopotency from RBP data.

[b]USP with Morton-Stubbs correction. UV samples not run in duplicate.

Table 5

Special treatments of liver extracts.

Liver sample No.	Treatment	RBP before treatment %	RBP after treatment %
19	Iodine/2 hours	98	78
20	Iodine/2 hours	97	70
20	Iodine/4 hours	97	57
20	Iodine plus light/2 hours	97	0
22	Iodine/2 hours	92	86

reason an equilibrium mixture of vitamin A isomers was found in the liver in some earlier work (9) possibly resulted from selective metabolism of the trans form of vitamin A. In the present experiment original relative biopotencies, as shown by assay of livers from control chickens, were 95% to 97%. Maleic values and relative biopotencies were determined after 3 and after 8 weeks of the depletion period. Relative biopotencies were similar to those at the start of the depletion period, indicating that most of the vitamin A present in liver was essentially the all-trans retinol form (Table 6), and that little cis-isomer was present in the liver.

Table 6

Effect of a depletion diet on relative biopotencies of livers of chickens previously fed a higher-than-normal vitamin A-supplemented diet for six weeks.

Depletion time (weeks)	Liver sample No.	Carr-Price USP units/ gm	RBP %	Biopotency USP units/ gm	UV USP units/ gm[a]
0	25	896	97	869	788
0	26	872	95	828	799
3	29	620	97	601	569
3	30	948	95	900	809
8	31	607	96	583	613
8	32	661	97	642	581
8	33	857	96	823	833

[a]USP with Morton-Stubbs correction.

UV spectroscopic values usually were lower than but in the same general range as Carr-Price values. Ultraviolet spectra

indicated trace quantities of anhydroretinol developed during the depletion period. Chromatographic fractions eluted with 4% acetone in hexane showed absorption maxima in the 250-290 nm range and at 340 nm, which might be interpreted as resulting from vitamin D (Fig. 2). Absorption curves of fractions obtained by chromatography of extracts after the 8-week depletion were similar to those in Fig. 2.

V. DISCUSSION

Total vitamin A in beef livers analyzed was found to be lower than in all other samples analyzed, which is not surprising because the cattle were in feedlots for various periods of time and little information is available concerning vitamin A supplementation to those animals.

In general, livers of chickens from the Department of Chemistry, Animal Nutrition Laboratories, had the highest total vitamin A. Those chickens received a normal growing diet (111). The exceptions were chicken livers 9 and 10 which were obtained from local supermarkets. The total vitamin A in sheep, pig and calf livers was intermediate between the values obtained for beef and chicken livers.

The high relative biopotencies of vitamin A found in the normal (untreated) livers in this study indicate that normally the all-*trans* vitamin A form is the one stored in the liver, with at most only small amounts of the *cis*-isomers. That is in agreement with Braekman (13) who fed all-*trans* vitamin A and

Fig. 2

Absorbance curves of fractions separated by chromatography of extract of liver sample 30 from a chicken fed a vitamin A-depletion diet for 3 weeks.

——— Retinol in extract from liver of a chicken fed a depletion diet for 3 weeks (eluted with 15% acetone-hexane).

—.—. Anhydroretinol (traces only) in extract from liver of a chicken fed a depletion diet for 3 weeks (eluted with hexane).

- - - - - - - - Impurity (possibly vitamin D) in extract from liver of a chicken fed a depletion diet for 3 weeks (eluted with 2% acetone-hexane).

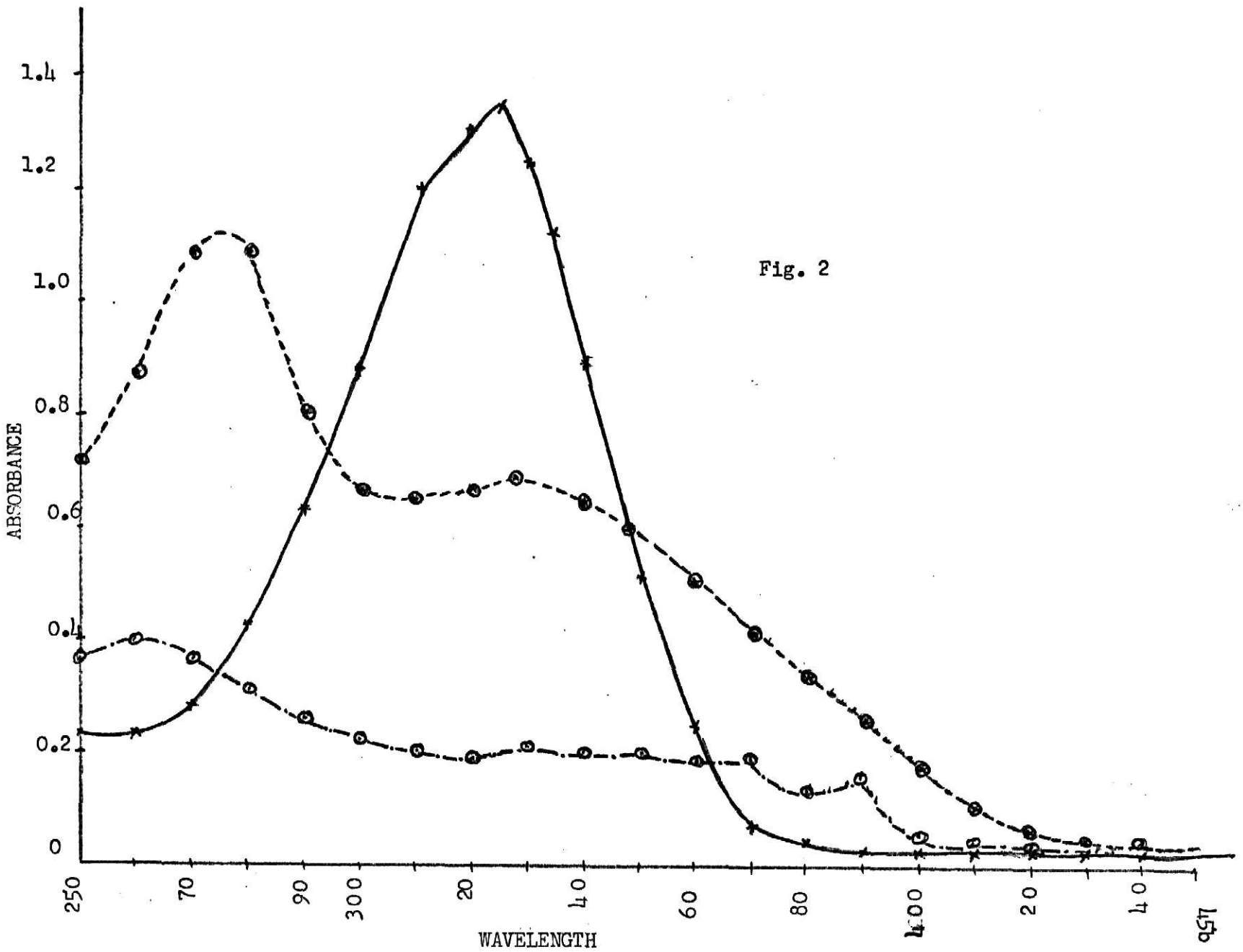
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
ABSORBANCE
250
70
90
300
20
40
60
80
400
20
40
450
WAVELENGTH

Fig. 2

THE PRECEDING PAGE WAS NUMBERED PAGE 45B, AND SHOULD HAVE BEEN NUMBERED PAGE 45A.

THIS IS AS RECEIVED FROM CUSTOMER.

neovitamin A to chicks and found that the group receiving all-trans vitamin A had low values for neovitamin A, indicating that no isomerization took place. The group fed neovitamin A, however, showed some isomerization to the all-trans retinol. Both findings indicated that all-trans is the natural form of vitamin A present in the livers of chickens. Braekman et al. (32) also reported that fresh livers of rats and chicks were low in slow-reacting isomers (cis-isomers), provided the animals were not fed appreciable amounts of such isomers.

The feeding of a vitamin A-depletion diet to chickens previously fed a vitamin A-supplemented diet for six weeks apparently had no effect on relative biopotencies of the liver vitamin A. Thus, there was no selective loss of all-trans retinol from their livers, as suggested by Ames.[7] Instead, high relative biopotencies of 97% and 95% were found in two livers at the end of the third week, and 96% and 97% in livers at the end of the 8-week depletion period. These values were in the same range as the initial values. It is possible that the changes suggested by Ames might have been found if a longer depletion time were used.

When special treatments which have been found to induce isomerization of vitamin A were applied to livers, some changes in RBP were observed. The initial vitamin A biopotency of liver 19 was 98% but it is changed to 96% after 6 weeks refrigeration, to 95% when the liver was stored in a frozen condition for

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[7] S. R. Ames personal communication.

4 months, and to 82% when the liver was left in the dark at room temperature for 3 weeks. This was in good agreement with the report (32) that isomerization occurred when livers from rats were stored at room temperature for 2 to 7 days, whereas storage at 4^{o} or lower produced only small amounts of isomers.

In livers 21 and 22, which were exposed to light for 4 hours at room temperature, there was a slight isomerization of vitamin A, as indicated by decreases in relative biopotencies from 98% and 91% to 94% and 83%, respectively, and from 98% and 92% to 90% and 87%, respectively, when livers were exposed to 50^{o} for 24 hours. This change in RBP of vitamin A in liver 22 upon exposure to light was detected only after the liver had been stored previously for 6 weeks in a frozen condition; no appreciable changes were found when the experiment was carried out on fresh liver tissue. These findings appear to substantiate those of Baba (16) who found that isomerization of liver oils was not very intense at 150^{o} for 30 minutes but was 50% after holding at 240^{o} for 30 minutes.

Isomerization also occurred when liver extracts were treated with iodine or iodine plus light. The relative biopotencies of three samples dropped from 98% to 78%, from 97% to 70% and from 92% to 86% in a 2-hour treatment, and the RBP of one sample dropped from 97% to 57% in a 4-hour treatment. Iodine plus light for 2 hours reduced RBP from 97% to 0%, indicating total loss of vitamin A activity or destruction of vitamin A. These results are similar to those of Zechmeister (14) who produced <u>cis</u>-isomers

in carotenoids with light or light plus iodine, as indicated by appearance of maxima at 260 and 320 nm in the UV spectra. Kardys (17) also used iodine to treat a mixture of synthetic vitamin A of low biological activity, converting it to an isomeric mixture with increased biological activity.

The effect of iodine and iodine plus light caused greater isomerization of liver vitamin A extracts, than did exposure of livers to light at room temperature, or to 50° for 24 hours in darkness.

When livers were stored under refrigeration, or at room temperature, some isomerization occurred, but the percentage loss of total vitamin A was greater than changes in degree of isomerization.

VI. SUMMARY

A study was made of biopotency (true vitamin A activity) of vitamin A stored in animal livers. Of the four most common isomers of vitamin A, the all-trans retinol has the highest activity and is assigned a relative biopotency (RBP) of 100%, followed by 13-*cis* retinol, 75%, and the 9-*cis* and the 9,13-di-*cis* retinols, about 25%.

The Carr-Price colorimetric method was used for the determination of total vitamin A, and that method along with the maleic anhydride method was used for determination of vitamin A isomers and relative biopotencies of the vitamin A of the livers.

The USP XVII spectroscopic method also was used throughout the study for comparison with other assays.

Under normal conditions, the predominant form of vitamin A in livers assayed was the all-*trans* retinol, with only small or insignificant quantities of *cis*-isomers. The data indicated that if livers are stored at low temperatures and protected from light for short to moderate periods of time, the biopotency of the vitamin A should be high, 91% to 100%.

Some isomerization of vitamin A was found when livers were stored either at room temperature or exposed to light, or stored at an elevated temperature (50°), or when extracts were exposed to the action of iodine or light.

No change in RBP of vitamin A in chicken livers was observed during the 8-week period in which a vitamin A-depletion diet was fed.

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APPENDIX

Appendix Table 1

Additional data on source of livers.

Sample No.	Source of liver samples (species)	Source of animal	Diet (if known)	Age (mo) (if known)	Weight of animal (lb) (if known)
1	Chick	Biochemistry[a]	Purina	4	---
2	"	"	"	"	---
3	"	"	"	"	---
4	"	"	"	"	---
5	"	"	"	"	---
9	"	Supermarket	---	---	---
10	"	"	---	---	---
11	Beef	"	---	---	---
12	Pig	"	---	---	---
13	"	Animal Sci.[b]	---	---	200
14	Beef	" "	feedlot	15	---
15[c]	Sheep	" "	---	---	150
16	Beef	" "	---	15	935
17	"	" "	feedlot	18	1000
18	"	" "	"	18	1000
19[d]	Calf	Supermarket	---	---	---
20[e]	Pig	"	---	---	---
21[d]	Calf	"	---	---	---
22[d]	"	"	---	---	---
23	Chick	Biochemistry	basal diet	3.5	---
24	"	"	"	3.5	---
25	"	"	suppl. feed	1.5	---

Appendix Table 1 (continued).

Sample No.	Source of liver samples (species)	Source of animal	Diet (if known)	Age (mo) (if known)	Weight of animal (lb) (if known)
26	Chick	Biochemistry	suppl. feed	1.5	---
27	Pig	Supermarket	---	---	---
28	"	"	---	---	---
29	Chick	Biochemistry	depletion feed	2.25	---
30	"	"	"	2.25	---
31	"	"	"	4.5	---
32	"	"	"	4.5	---
33	"	"	"	4.5	---

[a]Animal Nutrition Laboratory.

[b]Meat Laboratory, Dept. of Animal Science and Industry.

[c]Frozen for 3 months prior to assay.

[d]Exposed to special treatment also.

[e]Never frozen, just refrigerated.

Appendix Table 2

Data on duplicate determinations of vitamin A by two different methods of assay.

Specie source of liver sample	Sample No.	Method 1 Carr-Price USP units/gm	RBP %	Method 2 Biopotency USP units/gm
Beef	11	84 97	100	84 97
Pig	12	206 209	98	202 204
Pig	13	427 446	98	423 437
Beef	14	6 5	--	8 6
Sheep	15	160 175	97	158 172
Beef	16	36 38	91	33 35
Beef	17	57 55	91	52 50
Beef	18	61 52	95	57 51
Calf	19	152 150	98	149 148
Pig	20	484 398	96	464 386
Calf	21	260 242	94	242 227
Calf	22	224 243	91	204 224

VITAMIN A ISOMERS IN ANIMAL LIVERS

by

DALIA MERCEDES AGUILAR

B. S., University of Havana, Cuba, 1951

AN ABSTRACT OF A MASTER'S THESIS

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Manhattan, Kansas

1970

The conjugated double bond system in the vitamin A structure gives rise to isomerism of the cis-trans type. Of the 32 theoretical isomers of vitamin A that are possible, only four are commonly found in food, food products and supplements. The all-trans form is assigned a relative biopotency of 100% since it has the highest true physiological value. The other isomers have lower relative biopotencies, ranging from 75% for the 13-cis retinol to only about 25% for the other cis-derivatives. Biopotency of a vitamin A sample is dependent on the relative proportions of the various isomers present.

The purpose of this study was to determine relative biopotencies of vitamin A in livers of several species. Extracts from 16 chicken livers, 8 beef livers (including 3 calf livers), one sheep liver and 5 pig livers were used in the experiments. These extracts were analyzed for total vitamin A and for vitamin A isomer as determined from maleic values, a method developed by Ames and Lehman in 1960. Total vitamin A was determined by the USP XVII spectrophotometric method with the Morton-Stubbs correction for "irrelevant absorption," and by the Carr-Price colorimetric method.

Little isomerization of vitamin A was found in fresh or properly refrigerated liver samples; relative biopotencies ranged from 91% to 100%, indicating that the most common form of vitamin A in livers of these animals is the most active all-trans form.

Isomerization of vitamin A and decreases in relative biopotencies were found when special treatments were given to the livers. Livers were assayed after receiving the following

treatments: refrigeration for 6 weeks, storage at room temperature in the dark for 3 weeks, frozen for periods of 1.5 to 5 months, exposed to light for 4 hours at room temperature, and held at 50° for 24 hours in darkness. The greatest decrease in relative biopotency (approximately 10%) was found in livers stored for 3 weeks at room temperature.

Effect of iodine for 2 and 4 hours and iodine plus light for 2 hours on liver extracts also was studied. Treating the extracts of vitamin A with iodine for 2 hours caused relative biopotencies to decrease 7% to 20%, but iodine plus light for 2 hours caused relative biopotency to drop to 0%.

Chickens that were fed a higher than normal vitamin A-supplemented diet for 6 weeks were changed to a vitamin A-depletion diet. Maleic values were determined on liver of those chickens after 0, 3 or 8 weeks depletion. The relative biopotencies of vitamin A in those livers is the all-trans retinol form, and that depletion of liver vitamin A did not cause an increase in cis-isomers or relative biopotencies.