The Identification and Determination of Vitamin A and β -Carotene in an Animal-fat Mixture

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The investigation of the biological effects of vitamin A has been in progress in our laboratory for several years. During the course of our experiments it was found that the inclusion of a mixture of four parts of beef fat and one part of mutton fat, supplied at the 10% level, provided a vitamin A-like material detectable by bioassay. Initially we were unable to obtain specific chemical or physical tests for vitamin A and carotene, but an improvement in technique enabled us subsequently to do so. The chemical analysis of the fat was undertaken shortly after the appearance of the conflicting reports of Lowe & Morton (1953) and of Herb, Riemen-schneider, Kaunitz & Slanetz (1953). While our work was in progress, a paper by Ames & Harris (1954) confirmed the presence of vitamin A in lard, as originally suggested by Herb *et al.* (1953).

It is the object of this study, first, to add further confirmation of the presence of vitamin A in beef and mutton fat and, secondly, to describe an improved method for the concentration of trace amounts of vitamin A and of β -carotene from animal fat.

It would seem that vitamin A may be of wide-spread occurrence in the depot fats of mammals. The methods which we have used for the concentration of trace amounts of vitamin A and β -carotene are probably generally applicable to such fats.

EXPERIMENTAL AND RESULTS

Beef fat-mutton fat mixture. This was a 4:1 (w/w) mixture commercially rendered by a local butcher. In addition, two samples of beef fat from the same fresh carcass were kindly supplied by Mr I. H. Chayen of British Glues and Chemicals Ltd., London. These were (i) a sample rendered by steam in the usual way, and (ii) a sample rendered in cold water by the impulse-rendering technique (Chayen & Ashworth, 1953).

Biological assay of vitamin A. The assays were carried out by an improved method which will be published shortly. The beef fat-mutton fat mixture assayed at 5.3 i.u./g. and distillate B at 130–140 i.u./g. Both beef fat samples (i) and (ii) above contained 5 i.u. of vitamin A/g., so that it would appear that no vitamin A is lost during normal steam rendering.

Chemical assay of vitamin A in beef fat-mutton fat mixture. The fat (50 g.) was saponified by the method of Boldingh & Drost (1951). The ethanolic solution was diluted with cold water until its ethanol content was about 35% (v/v), and was then extracted with peroxide-free ether (1 × 500 ml.; 2 × 250 ml.). The combined ethereal extracts were washed with boiled-out distilled water (5 × 30 ml.) and evaporated. All these operations were carried out in nitrogen and in the presence of a little quinol. Water was removed from the residue under water-pump vacuum at room temp. The residue (approx. 0.16 g.) was taken up in dry ether (5.0 ml.) and dry hexane (15.0 ml.) added. The solution was allowed to stand for 30 min. so that the cholesterol could crystallize out. A portion (2 ml.) of this solution was then chromatographed on alumina (Peter Spence Ltd., Widnes, grade H, heated for 3 hr. at 105°) by the method of Thompson, Ganguly & Kon (1949), and the vitamin A content of eluate (c) after evaporation in nitrogen was determined by the Carr-Price method. Several experiments gave a value of 5.2 i.u. of vitamin/g. fat. The solids from eluate (c) showed E_{max} at 325 m μ . in 96% (v/v) aqueous ethanol. Because of interfering substances the vitamin A content of the fat calculated from the optical density at this wavelength was about double that obtained by the Carr-Price method.

The above procedure was carried out on saponified fat from which the unsaponifiable portion had been removed and cholesterol plus known amounts of vitamin A added. Theoretical recovery of vitamin A was obtained.

Molecular distillation of rendered fat. The procedure was similar to that of Herb *et al.* (1953). The rendered beef fat-mutton fat mixture (4:1, 36 kg.) was distilled in kilogram quantities in a 5 in. rotating-disk molecular still (Distillation Products Inc., Rochester, N.Y.) at 220°. The distillate (6330 g., 18%) contained most of the original vitamin A-like activity and was redistilled to give the following fractions. Distillate A: distilling up to 130° (211 g. $\equiv$ 0.6% of original fat). Distillate B: distilling at 130–200° (747 g. $\equiv$ 2.1% of original fat). Residue C: not distilled. Distillate B was by far the richest in vitamin A-like activity, as measured by biological tests on rats; it appeared to contain the bulk of the yellow colour of the original fat.

Saponification of distillate B. Distillate B was saponified by the method of Boldingh & Drost (1951). The ethanolic solution was then diluted with water until its ethanol content was 35% (v/v) and extracted continuously with pentane in an efficient liquid-liquid extractor for 8 hr. Evaporation of the solvent yielded 3.6% of unsaponifiable material (0.075% of original fat) as an orange-yellow solid containing much crystalline cholesterol. A solution of this unsaponifiable material in CHCl_3 gave with the SbCl_5

reagent an intense fleeting blue colour showing an absorption band at 620 $m\mu$. In ethanolic solution, the unsaponifiable material showed no peak at 324 $m\mu$; there was only general unresolved background absorption.

Reversed-phase partition chromatographic experiments.

(a) Vitamin A-cholesterol mixtures: a jacketed column, 1.5 cm. in diameter and kept at 35°, was used. Non-wetting kieselguhr (35 g.) and *n*-heptane (31.5 ml.) (cf. Howard & Martin, 1950; Silk & Hahn, 1954) were thoroughly mixed by shaking and added to 60% (v/v) aqueous methanol; the resultant slurry was vigorously boiled under water-pump vacuum to expel air and then packed into the column already half-filled with degassed 60% (v/v) aqueous methanol. Packing was accomplished by gravity, a plunger being used to break up any lumps. A mixture (50 mg.) of vitamin A and cholesterol (1:25) in 75% (v/v) aqueous methanol equilibrated with heptane was poured on to the column which was then developed with the same solvent mixture. The vitamin A came quickly through the column, whereas the cholesterol remained behind. The developing solvent was then changed to 90% (v/v) aqueous methanol equilibrated with heptane. Water was added to the eluate, which was then extracted with $CHCl_3$. The $CHCl_3$ extract gave the sensitive Liebermann-Burchard test for cholesterol.

(b) Unsaponifiable material from distillate B: the unsaponifiable material from 30 g. of distillate B was shaken with pentane (50 ml.) and mixed with one-seventh of the non-wetting kieselguhr-*n*-heptane mixture [prepared as in (a)] kept back for the purpose. The mixture was evaporated at room temp. under water-pump vacuum until the residue was coarse and granular. To the residue was added 60% (v/v) aqueous methanol (50 ml.) and the whole was then boiled *in vacuo* to remove air. The resulting slurry was poured on top of the column prepared as in (a) from the remaining six-sevenths of the kieselguhr-*n*-heptane. On settling, the kieselguhr column was about 70 cm. long. A siphon delivering 3.2 ml. samples (sufficient to fill the 1 cm. cells of a Beckman DU spectrophotometer) was attached to the bottom of the column. The column was developed with 60% (v/v) aqueous methanol equilibrated with heptane until the initial cloudiness of the eluate cleared; 75% (v/v) aqueous methanol equilibrated with heptane was then used. The optical density readings of the eluates (at 324 $m\mu$.) were measured and it was found that the initial background values were below 0.05. These values rose sharply when the vitamin A began to come off the column; those eluates absorbing strongly at 324 $m\mu$ were collected separately, a middle fraction being bulked. The amount of vitamin A present was calculated from the volumes and optical densities of the appropriate eluates. The ultraviolet absorption spectrum of the middle bulked fraction was identical in shape with that of pure vitamin A from 324 $m\mu$. onwards; there was, however, increased absorption over the range 220–324 $m\mu$., but this increase was quite small above 300 $m\mu$. In the above manner a figure of 3.6 mg. of vitamin A/100 g. of distillate B was found, i.e. 120 i.u./g. When all of the vitamin A had been removed from the column, the latter was washed with pentane and the yellow eluate was collected for later examination for the presence of carotenes (see below).

Conversion of vitamin A into anhydrovitamin A. The middle bulked fraction of eluate above was diluted with an equal quantity of water and extracted continuously for 2 hr. with pentane in an efficient liquid-liquid extractor. After

removal of solvent from the extract, the residue was taken up in dry ethanol and its ultraviolet absorption curve in the range 340–400 $m\mu$. determined. A portion of the solution was treated with dry ethanolic HCl until it was 0.033 N with respect to HCl. After 15 min. at 30°, this solution was neutralized with ethanolic KOH and its absorption curve in the 340–400 $m\mu$. range measured. From this was subtracted the curve obtained before treatment with HCl. This experiment was repeated with crystalline vitamin A. The resulting curves are shown in Fig. 1. The absorption maxima found under these conditions were about 3 $m\mu$. lower than those recorded by Shantz, Crawley & Embree (1943) and by Ames & Harris (1954). The absorption quotients found by us for anhydrovitamin A from synthetic crystalline vitamin A were: 351 $m\mu$./368 $m\mu$. = 0.55 and 389 $m\mu$./368 $m\mu$. = 0.78. Shantz *et al.* (1943) recorded values of 0.68 and 0.87 respectively. The quotients for the vitamin A concentrate from the fat (obtained as described above) were: 351 $m\mu$./368 $m\mu$. = 0.52 and 389 $m\mu$./368 $m\mu$. = 0.76.

Alumina chromatography of the vitamin A-enriched fraction from the reversed-phase partition chromatogram. With a view to obtaining material suitable for infrared identification, several attempts were made to purify further the vitamin A-enriched fraction by chromatography on alumina. Trial experiments with pure vitamin A indicated that good recoveries were obtained with alumina containing 4% of water and with ether-pentane (1:9, v/v) as eluent.

In another experiment reversed-phase partition chromatography of an unsaponifiable fraction from distillate B afforded 53.9 mg. of material containing 2.3 mg. of vitamin A (by absorption measurement at 324 $m\mu$.), i.e. the preparation contained 4.25% of vitamin A. After solution in pentane, this was poured on to a column of alumina containing 4% water (5 g., 7.5 cm. $\times$ 1.0 cm.) prepared in pentane. Elution with ether-pentane (1:9, v/v) mixture afforded 8.7 mg. of material containing 1.3 mg. of vitamin A, i.e. 15% of vitamin A. Although some concentration had

Fig. 1. Absorption curves in ethanol of anhydrovitamin A from fat (●—●) and from crystalline vitamin A (○---○).

been effected, there was still too much extraneous matter present in the final preparation for identification of the vitamin A by infrared spectroscopy.

Examination of the carotenes of distillate B. The combined yellow pentane eluates from several reversed-phase chromatograms (see above) representing 200 g. of distillate B were bulked, concentrated and filtered from solid cholesterol. The filtrate was evaporated to dryness *in vacuo* and then dissolved in hexane and chromatographed on alumina (activity I-II, Brockman's scale) contained in a tube 2 cm. in diameter. Four distinct zones appeared, namely, zone 1 (9.5 cm.) pink; zone 2 (0.5 cm.) brown; zones 3 and 4 (total of 4 cm.) bright yellow. The eluate was slightly yellow. The extracts from zones 1 and 2 were combined; they had absorption maxima at 424, 449 and 474 m μ . (in hexane) and still contained much cholesterol. The combined extract was chromatographed twice on Ca(OH)₂ and then finally on alumina (activity I-II). In the final alumina chromatogram a column 2.7 cm. in diameter was used; development with benzene-hexane mixture (1:1, v/v) gave three zones, namely, zone A (5 cm.) brown-pink; zone B (10 cm.) pale pink; zone C (6 cm.) white. The extract from zone B was dissolved in hexane and its ultraviolet absorption spectrum determined; it gave a curve identical with that of β -carotene which had been treated in precisely the same way (see Fig. 2). From the intensity of absorption it was found that 0.39 mg. of β -carotene was obtained from 200 g. of distillate B; this is equivalent to a vitamin A activity in distillate B of 3 i.u./g. Other carotenes were present in small

amounts, but these were not investigated. Further confirmation of the identity of the β -carotene was obtained by carrying out a mixed chromatogram on alumina with an authentic specimen. Only one unresolved zone formed.

DISCUSSION

The present studies indicate that vitamin A is present in our beef fat-mutton fat mixture and also in beef fat. This fact has been demonstrated by both chemical and biological assay. In addition, the active factor has been considerably concentrated but our product was still too impure for identification by infrared spectroscopy. The amount of vitamin A we have found is considerably greater than the value (0.2-0.5 i.u./g.) recorded by Henry, Goodwin, Kon & Morton (1941) for commercial beef dripping. According to Herb *et al.* (1953) lard contains 0.5-2 i.u. of vitamin A/g., which is of the same order as that we have found in the beef fat-mutton fat mixture. Recently Pierse (1954) has found vitamin A in human fat to the extent of about 7 i.u./g., together with appreciable amounts of β -carotene.

SUMMARY

1. The vitamin A activity (as indicated by bio-assay) of a mixture of rendered beef and mutton fats (4:1) has been shown to be due entirely to vitamin A, together with a minor amount of β -carotene.
2. An enormous concentration of the vitamin A in the unsaponifiable portions was effected by means of reversed-phase partition chromatography.

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Fig. 2. Absorption curves in hexane of β -carotene from fat (●—●) and from crystalline β -carotene after chromatography on alumina (---●).