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A Method for the Determination of Carotene and Vitamin A in Human Blood Serum

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In the course of a study of serum vitamin A and carotene amongst the population of Ruanda-Urundi, we found it necessary to examine the antimony trichloride technic.

There is a very great deal of literature on the subject of the Carr-Price test for the determination of serum vitamin A and carotene, and extensive literature surveys and discussions of this method can be found in several handbooks and papers.[1-6] Very few authors give a complete description of the method used, whereas in this technic, details may be very important and may greatly influence the results. For example, the purity or the lack of purity of the solvents employed (of the chloroform in particular) may cause many difficulties and errors. Widely varying figures have been reported in the literature as "normal" values for vitamin A concentrations, and we believe this may be, at least partly, due to differences in the technics used for the determinations by the various workers. We have therefore decided to give a detailed account of the method we used.

To determine the reproducibility of the vitamin A and carotene determinations, a series of sera were analyzed twice. It was found that the difference in the results between duplicates did not exceed 5 per cent for both carotene and vitamin A. The recovery of known amounts of vitamin A to serum is better than 95 per cent.

APPARATUS

For the routine determinations, a Model 14 Coleman Universal Spectrophotometer was used, with Coleman type 14-302 round cuvettes (19 x 105 mm), pairs of which had been matched in our laboratory to within less than 0.1 per cent transmission. A 14-214 (PC4) filter was used in this spectrophotometer for all the work described here. All readings on the Coleman 14 were taken from the transmission figures on the galvanometer scale.

For checking the purity of the standards and the solvents a Unicam SP 500 Photo-electric Quartz Spectrophotometer was used, with square quartz cuvettes having a 10 mm light path (accurate to within ±0.02 mm), pairs of which had been matched in our laboratory to within less than 0.1 per cent transmission. Red low-actinic glassware was used throughout the procedure (spectral transmission 0–12 per cent in the range from 3,000 to 6,000 angstrom). The temperature in the laboratory where the work was done was kept at 20° C.

SOLVENTS AND REAGENTS

Commercially available "laboratory reagent" type solvents are usually not good enough for spectrophotometry and additional precautions have to be taken before using these solvents. The solvents used for this work were purified according to the following procedures, some of which are in use in the Centraal Instituut voor Voedings Onderzoek, Utrecht, Holland, and were kindly communicated to us by Dr. C. Engel.

(1) *Ethanol*

Analytical reagent grade absolute ethyl alcohol was refluxed for 30 minutes in the presence of 5–10 g of aluminum powder and 5–10 g of KOH (analytical reagent) per liter of alcohol. After refluxing for 30 min, the mix-

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ture was distilled through a 68-cm long column and the light and heavy fractions were discarded; each of these fractions represented 10 per cent of the original total volume of the alcohol.

The ethanol gave a transmission reading of 100 per cent at the wavelengths used in our work when checked against bi-distilled water.

(2) *Petroleum Ether*

Analytical reagent grade petroleum ether, boiling range 40–60° C, was refluxed for 2 hours in the presence of 60 ml concentrated H_2SO_4 per liter of petroleum ether. After cooling, the sulfuric acid layer was removed (separating funnel). This operation was done three times altogether with fresh sulfuric acid.

The petroleum ether was washed in a separating funnel with distilled water until the wash waters no longer showed an acid reaction to Congo-red test paper.

The petroleum ether was dried overnight over anhydrous magnesium sulfate; the magnesium sulfate was filtered off and the solvent was distilled through a 68-cm long column in the presence of a few pellets of sodium hydroxide (analytical reagent grade).

Light and heavy fractions each representing 5 per cent of the total starting volume were discarded.

The petroleum ether gave 100 per cent transmission reading when checked against bi-distilled water at the required wavelengths.

(3) *Chloroform*

Analytical grade chloroform was washed three times with a 10 per cent aqueous solution of sodium thiosulfate. It was then dried overnight over anhydrous calcium chloride, and filtered. The filtered chloroform was shaken with finely powdered anhydrous sodium thiosulfate of "analytical reagent" grade, and filtered again. The chloroform was fractionated, using a 68-cm long column. "Light" and "heavy" fractions each representing about 20 per cent of the total starting volume were discarded. Particular care was taken to use perfectly dry apparatus and to avoid strong light during all operations. Half a per cent (by volume) of absolute ethyl alcohol (purified for spectrophotometric work) was added to the chloroform after the fractionation. We found it better not to use recovered chloroform.

(4) *Antimony Trichloride Reagent*

British Drug Houses antimony trichloride solution in chloroform was used. This solution comes sealed in brown glass 500 ml ampules and contains 86.1 per cent chloroform and 13.6 per cent $SbCl_3$ (corresponding to 8.85 per cent w/w Sb_2O_3). The reagent was measured with the British Drug Houses automatic antimony trichloride pipette specially made for this purpose. We checked the accuracy of the calibration of these pipettes.

STANDARDS USED FOR ESTABLISHING THE CALIBRATION CURVES

(1) *Vitamin A*

For vitamin A, we used the standard solution prepared for the first international collaborative experiment organized in 1955 by the Vitamin Commission of the Food Division of the Applied Chemistry Section of the International Union of Pure and Applied Chemistry. We are very grateful to this organization for donating the standard. It is pure all-trans synthetic vitamin A acetate made by l'Alimentation Equilibrée (France), Distillation Products (U.S.A.), and Hoffmann-La Roche (Switzerland) mixed and dissolved in oil. The potency of that solution as determined by the international collaborative experiment is 98,800 I.U./g of oil.

We repeated this international collaborative test, following exactly the procedure which had been used for that experiment and which was given to us by the chairman of the Vitamin Commission, using our Unicam SP500 Photoelectric Quartz Spectrophotometer.

The results we obtained were in excellent agreement with those found in the collaborative test and quite near the maximum of the Gauss distribution curve of the results from the 36 laboratories who had taken part in that test. This confirmed the reliability of our apparatus,

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technic, reagents and vitamin A standard.

(2) *Beta-Carotene*

Pure all-trans β-carotene kindly given to us by Hoffmann-La Roche (Switzerland) was used. This product, after drying in a high vacuum drying pistol, gave an E-value (1 g/liter, 1 cm) of 251.8 at 451 mμ in *n*-hexane.

It had absorption maxima in the same solvent at 451 and 479 mμ and an absorption minimum at 468 mμ.[2]

We used British Drug Houses "Hexane fraction from petroleum special for spectroscopy" which gave 100 per cent transmission when checked against bi-distilled water at the required wavelengths.

TECHNIC USED FOR THE DETERMINATION OF VITAMIN A AND CAROTENE IN BLOOD SERUM

Three ml of serum were pipetted into a 15 ml centrifuge tube. Three ml of freshly prepared KOH N/1 in 90 per cent ethanol were added very slowly with shaking.

The mixture was saponified under reflux for 20 minutes at 50–60° C in a water bath. It was cooled and 6 ml of petroleum ether 40–60° C were added. The tube was then shaken very vigorously by hand for 10 minutes. It was centrifuged for 1 minute at 500 r.p.m., 5 ml of the petroleum ether layer were pipetted off into a Coleman cuvette, containing 2 ml of petroleum ether. These 2 ml of petroleum ether were added to bring the level in the cuvettes up to the required height for the light beam.

The transmission of this solution was read on the Coleman 14 spectrophotometer at 452 mμ. The solvent was then evaporated from the Coleman cuvette by warming it in a water bath at 50° to 60° C while gently blowing nitrogen into the tube. The last traces of solvent were removed by blowing nitrogen at 20° C. The residue was immediately taken up in 1 ml of chloroform.

The cuvette was then placed in position in the Coleman-14 spectrophotometer. The zero of the instrument was adjusted by inserting the paired cuvette containing 1 ml of chloroform in the light beam and adding 6 ml of the antimony trichloride reagent and one drop of acetic anhydride to it while in position in the instrument. (This zero-setting was repeated before every determination with fresh chloroform and antimony trichloride.) Six ml of the antimony trichloride reagent and one drop of acetic anhydride were added very rapidly to the test cuvette after it had been placed in the light beam. The transmission was read at 620 mμ as soon as the galvanometer spot stopped for a few seconds on the scale.

CALIBRATION CURVE FOR BETA-CAROTENE

Ten dilutions of the pure all-trans beta carotene were made in petroleum ether (boiling range 40–60° C). Direct transmission readings were taken at 452 mμ on the Model 14 Coleman Universal Spectrophotometer. They are plotted against the concentration as ⊙ on graph No. 1. To check whether any β-carotene was lost in saponifying, 13 experiments were done in the following way: 1 ml of each of the petroleum ether solutions of varying concentrations was pipetted into a centrifuge tube, 3 ml of bi-distilled water and 3 ml of KOH N/1 in 90 per cent ethanol were added and the procedure used for serum was applied, adding 5 ml of petroleum ether instead of 6 ml.

The tranmission readings at 452 mμ were plotted on graph 1 as X.

We concluded from this, that saponification did not influence the carotene-content.

The best-fitting curve was calculated from the experimental points using the least squares method.

It was found to be $\log T = 2.0000 - 0.0030938\,C$ and is shown on graph No. 1.

CALIBRATION CURVE FOR VITAMIN A

The vitamin A calibration curve is the lower curve shown on graph No. 2.

A series of dilutions of the vitamin A standard in absolute ethanol were made. One ml of each of those solutions of known vitamin A concentration was pipetted into a 15 ml centrifuge tube. Two ml of bi-distilled water were added. This gave an aqueous layer of approximately 3 ml.

This was done in order to work under the same circumstances as for the vitamin A determination in the serum, where 3 ml of serum were

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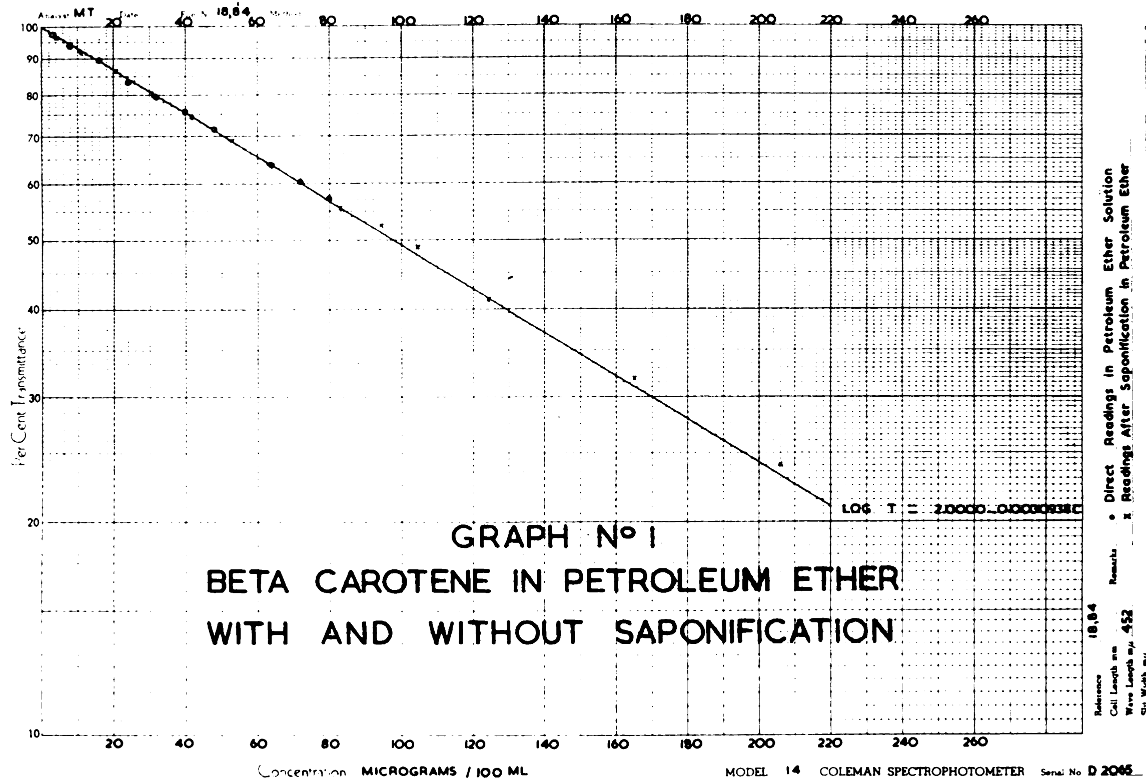

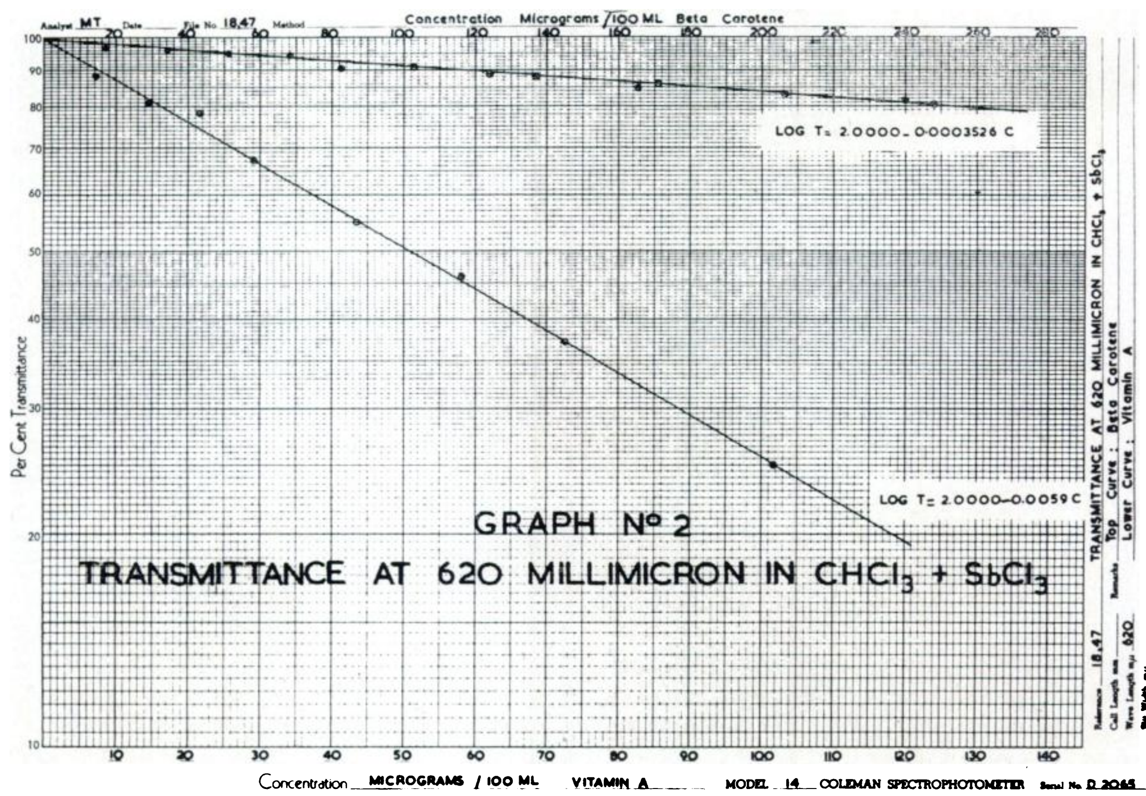

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used. The same technic as used for serum was then applied.

The transmission at 620 mμ was read and plotted on the graph. The curve was drawn using the equation $\log T = 2.0000 - 0.0059$ C. This equation was obtained from the experimental data by means of the least squares method.

METHOD OF CALCULATING CAROTENE AND VITAMIN A CONCENTRATIONS IN THE SERUM

(1) *Carotene*

A table was drawn up enabling us to read directly the carotene concentration in μg per 100 ml of serum from the transmission reading obtained on the spectrophotometer. For this, the concentrations of carotene in the petroleum ether solution obtained from the formula $\log T = 2.0000 - 0.0030938$ C had to be multiplied by 2.8; indeed, as is clear from the description of the method used for the determination of vitamin A and carotene in the serum, the extract from $^5/_6$ of 3 ml of serum was dissolved in 7 ml of petroleum ether for reading the transmission on the spectrophotometer.

(2) *Vitamin A*

(A) Correction of the vitamin A transmission reading at 620 mμ for carotene interference at that wavelength. Antimony trichloride in chloroform solution reacts with carotene and the absorption of the reaction product at 620 mμ interferes with the vitamin A determination which is done at the same wavelength.

To determine the extent of this interference, a series of solutions of the β-carotene standard in petroleum ether were made, aliquot portions of it were evaporated from the Colemen cuvettes as described above, taken up in chloroform and antimony trichloride reagent, and the transmissions were read at 620 mμ. The experimental points thus found are shown as ⊙ on graph No. 2 (top curve).

The best-fitting curve was calculated from the experimental points using the least squares method. It was found to be $\log T = 2.0000 - 0.0003526$ C and is the top curve on graph No. 2. A second series of solutions of the β-carotene standard in petroleum ether was made, aliquot portions of it were evaporated from centrifuge tubes as described above, and exactly the same procedure was than applied to the residues remaining in the centrifuge tube (after adding 3 ml of bi-distilled water to each tube). as is described for the serum (i.e., saponification, extraction, transmission reading at 452 mμ, evaporation, transmission reading at 620 mμ, in antimony trichloride reagent). It was found that here again, saponification, following the procedure used for the serum analysis, did not influence the transmission readings at 620 mμ of β-carotene in antimony trichloride reagent.

To correct the transmission reading taken on the serum extract at 620 mμ, for the carotene interference, the following method was applied:

The carotene concentration in the serum was calculated from the transmission reading at 452 mμ by means of graph No. 1.

The density at 620 mμ corresponding to this carotene concentration was obtained from the top curve on graph No. 2. This density was subtracted from the density of the serum extract as read on the spectrophotometer at 620 mμ.

(B) Calculation of the vitamin A concentration from the corrected transmission at 620 mμ.

A table was drawn up enabling us to read directly the vitamin A concentration in μg per 100 ml of serum from the density value at 620 mμ, corrected for carotene interference as indicated above. For this, the concentrations of vitamin A in the antimony trichloride reagent, obtained from the equation $\log T = 2.0000 - 0.0059$ C corresponding to the lower curve shown in graph No. 2, had to be multiplied by 2.8 because of the difference between the concentrations in the serum and the concentration of the solution in the cell in the spectrophotometer.

SUMMARY

A method for determining total carotene and vitamin A in human serum has been described in detail. The usual procedure of protein precipitation with ethanol, saponification and petroleum ether extraction was followed. Total carotene was measured by a transmission reading at 452 mμ. Vitamin A was

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determined according to the Carr-Price method with transmission reading at 620 mμ and correction for carotene interference.

Solvents, standards, reagents, and apparatus used have been described because they may greatly influence the results. The calibration graphs and details of the method for calculating the results have been given.

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