THE DETERMINATION OF VITAMIN A AND CAROTENE IN SMALL QUANTITIES OF BLOOD SERUM

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For purposes of nutritional surveys and experimental studies, it was found necessary to have a method for measuring vitamin A and carotene on 0.1 ml. or less of serum in order that finger blood might be utilized or that undue amounts of blood need not be withdrawn from small experimental animals. Existing methods require at least 1 ml. of serum (1–4) and therefore necessitate venipuncture, which is time-consuming and for which consent is often difficult to obtain from subjects, particularly children, of a nutritional survey. It was also felt necessary to establish a procedure whereby large numbers of analyses could be performed without the expenditure of undue analytical time.

The usual Carr-Price (5) (antimony trichloride) reaction for vitamin A presents great difficulties when attempts are made to adapt it to small scale work. The volatility of the solvents used, petroleum ether and chloroform, makes the necessary manipulations very difficult and even slight evaporation of the chloroform results in condensation of moisture, with resultant turbidity from the antimony trichloride reagent. Furthermore, the evanescent nature of the blue color obtained renders colorimetry very difficult on a small scale. Therefore, attention was directed to the measurement of vitamin A by its absorption in the ultraviolet (328 mμ) in spite of the fact that the color intensity is only about one-third as great as with the antimony trichloride reagent. The direct ultraviolet absorption of vitamin A has been greatly limited in analytical usefulness except for measurements of high potency oils, owing to its non-specificity, since other compounds likely to be present contribute to the absorption at 328 mμ (6, 7). However, Little (8) has partially circumvented this difficulty by measuring the absorption before and after irradiation with ultraviolet light of wave-lengths 310 to 400 mμ, which destroyed chiefly vitamin A in the oils and tissues tested. Little's paper (8) gives references to those who previously made use of this principle on a limited scale and in a variety of ways for the analysis of foodstuffs. Chevallier *et al.* (9) have used an irradiation method in measurement of vitamin A in larger volumes of serum. However, the possibilities of this technique have never been fully explored, particularly in reference to blood analysis.

By utilizing a destructive irradiation technique it has been possible to develop a satisfactory method for measuring vitamin A and carotene in 60 c.mm. (0.06 ml.) of serum. Even smaller volumes of serum (35 c.mm.) can be used, if greater attention is paid to technical details. With this procedure, one analyst can measure the vitamin A and carotene in at least 50 sera in a working day.

The proposed method depends on (1) saponification and extraction of the vitamin A and carotene from serum on a micro scale with solvents of low volatility; (2) measurement of the light absorption of the small volumes at 328 and 460 mμ; (3) destruction of the vitamin A absorption at 328 mμ without affecting the absorption of other compounds at this wave-length; and (4) remeasurement of the absorption at 328 mμ.

Reagents and Apparatus—

1. 1 N KOH in 90 per cent ethyl alcohol (1 volume of 11 N KOH plus 10 volumes of absolute alcohol). The reagent should be prepared the day it is used. If color develops rapidly or if the reagent gives a blank, the alcohol should be refluxed with KOH and redistilled before use.

2. Kerosene-xylene mixture (1:1). Xylene, C.P., and odorless (water-white) reagent kerosene (obtainable from Eimer and Amend, New York).

3. Test-tubes 10 cm. × 3 mm.; 20 cm. lengths of tubing, 3.0 to 3.5 mm. internal diameter, are cleaned by boiling in half concentrated nitric acid, rinsed, dried, and divided in the middle with a hot, narrow, blast lamp flame to yield two tubes ready for use. Pyrex tubes have been used but presumably soft glass would be satisfactory and easier to seal in the flame.[1]

4. Soft glass tubes similar to those described above but only 4 cm. long and 2.5 to 3.0 mm. internal diameter. These are made and cleaned in the same fashion.[1]

5. Lang-Levy constriction pipettes, 60 c.mm. (10, 11). It is desirable to have the upper constriction quite small to permit the pipetting of the organic solvents with low surface tension. If the tip is slender and the bend in the end very short, it will facilitate the measurement of samples into the long narrow tubes.

6. General Electric B-H4 mercury discharge lamp with purple envelope and with its special transformer.

7. Arrangement for irradiating samples in the soft glass tubes (Fig. 1). When the racks are in position around the lamp, the brightest part of the light source should be opposite the lower half of the tubes so that this portion of the tubes receives full illumination. The shadow of the electrode support must not fall on any tube. A moderate air current from a fan must be used to keep the tubes cool.

[1] It has been found easier to make new tubes than to clean old ones, since after one end is sealed, cleaning is somewhat laborious owing to the narrow bore.

8. A Beckman spectrophotometer fitted with a micro attachment and 2 mm. quartz cuvettes (12). (The micro attachment and cells are obtainable from the Pyrocell Manufacturing Company, 207 East 84th Street, New York 28.)

9. Racks about 5 × 5 × 2 inches to hold 100 long tubes. These may be made from wire screen (two pieces of ½ inch mesh and one piece of ⅛ inch mesh) or from sheet metal.

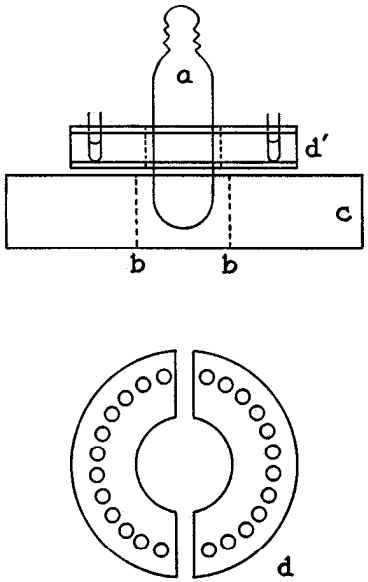

FIG. 1. Arrangement for ultraviolet irradiation. The mercury lamp (*a*) is held vertically in a clamp, base up, with the other and extending 3 or 4 cm. into a hole 8 cm. in diameter, *b-b*, in a large block of wood, *c*, which serves as a base. Semicircular racks (*d*, *d'*) are provided for holding the glass tubes in a circle equidistant from the lamp (6 cm. from the center of the lamp). These racks may be made from pieces of ½ inch plywood held about 2 cm. apart, with the upper piece drilled to hold the tubes. Twenty or thirty holes may be drilled in each rack along a semicircular line.

10. The head of an eightpenny nail is cut off, and the nail is slightly flattened for a distance of 10 or 15 mm. and inserted in a small high speed hand drill (*e.g.*, Handee grinder, Chicago Wheel Manufacturing Company, Chicago) with the end projecting about 20 mm.

Procedure

Into the long slender tubes are put 60 c.mm. of serum and 60 c.mm. of alcoholic KOH. If the solutions do not run to the bottom, they are sent

down by a whipping motion. Mixing is accomplished by touching the side of the tube near the bottom to the whirling nail in the motor drill which has been mounted in a clamp with the nail up. The tube is immersed (along with others in a rack) in a water bath at 60° for 20 minutes, cooled, and 60 c.mm. of the kerosene-xylene mixture are added. Extraction is accomplished by holding the tubes at about a 45° angle against the whirling nail in such a manner that the contents are violently agitated for 10 or 15 seconds. They are then centrifuged 10 minutes at 3000 R.P.M. The tubes should be at room temperature or a little below before they are centrifuged. Each tube is cut with a file just above the kerosene-xylene layer and this layer is pipetted into the special narrow Beckman cuvette (12); all the solution possible is used, but with great care to avoid any of the aqueous layer which would cause turbidity. The pipetting is best accomplished with a fine tipped constriction pipette of about 50 or 60 c.mm. volume. However, this pipette need not be calibrated. The constriction acts merely as a brake to prevent the sample from being accidentally drawn up too far in the pipette.

Readings are made at 460 and 328 mμ. The sample is then removed to a short soft glass tube (item (4) above) and irradiated along with the other samples with the B-H4 lamp. The lamp must have been turned on at least 10 minutes prior to the beginning of the irradiation. The necessary irradiation time (30 to 60 minutes) should be determined by trial with known vitamin A solutions. Irradiation is applied for 6 or 8 times as long as is found to be necessary to destroy 50 per cent of the vitamin A in pure solutions. After irradiation, a second reading at 328 mμ is taken. In order to eliminate the danger of a turbidity of unestablished origin which sometimes develops and which may be so slight as to be unnoticeable and yet serious enough to cause real error, the pipette used to transfer the sample back into the cuvette after the irradiation is rinsed before each sample with anhydrous propionic acid for one-third to one-half of its length below the constriction. This procedure neither adds significantly to the analytical time nor changes the volume enough to cause error. If necessary or desired, the propionic acid may be added prior to the first reading, since its incorporation does not affect the course of irradiation.[3]

Calculation—$E_{460} \times 480$ = micrograms per cent of carotene. (E_{328} − E irradiated 328) × 637 = micrograms per cent of vitamin A. E = optical density with a cell having a 1 cm. light path = 2 minus log per cent trans-

[3] A substitute for propionic acid, which may possibly be more satisfactory, is a 1:1 mixture of xylene and 2-methyl-2,4-pentanediol. The addition of a great excess of propionic acid may in some cases itself induce turbidity, whereas pentanediol appears to be miscible with serum extracts in all proportions. The xylene is added to reduce viscosity.

mission with such a cell. Optical density is given directly on the Beckman spectrophotometer. If the volumes of serum and kerosene-xylene are not equal, these must be multiplied by (volume of kerosene) — (xylene)/(volume of serum).

The factor of 637 for vitamin A is based on an $E^{1\%}_{1\,cm.}$ for vitamin A palmitate in alcohol of 1720 at 328 mμ, calculated as free alcohol (13). Since vitamin A ester has only 96 per cent as much absorption in kerosene-xylene and still has 3 per cent of its initial absorption after irradiation, and since furthermore the absorption is reduced 2 per cent owing to the necessity of using a wide spectral band (8 mμ), the net $E^{1\%}_{1\,cm.} = 1720 \times 0.96 \times 0.97 \times 0.98 = 1570$. 1,000,000/1570 = 637. The extinction coefficient of the vitamin A ester was used rather than that of the free alcohol, since most of the vitamin A in serum is esterified and saponification is quite incomplete. The factor of 480 for carotene was obtained by measuring the absorption of β-carotene (Smaco) in kerosene-xylene ($E^{1\%}_{1\,cm.} = 2080$).

If desired, the volume of serum and reagents may be increased or decreased proportionately. The ratio of serum to alcohol must be kept constant, but the amount of kerosene-xylene may be varied independently.

DISCUSSION

Saponification and extraction are conveniently carried out in the long slender tubes which prevent undue evaporation during saponification and which give sufficient fluid depth to facilitate subsequent removal of the organic solvent layer. Saponification presents no problem and it scarcely prolongs the analytical time, since it is as easy to add alcoholic KOH as it is to add alcohol alone, which must be added in any event, and as many as 100 samples may be saponified at once in a single water bath. The saponification is not complete, glycerides are only partially hydrolyzed, and the same is probably true for vitamin A esters. However, the alkaline treatment accomplishes its purpose; *viz.*, facilitation of vitamin A extraction and the removal of interfering materials.

If a motor stirrer such as is described above is not available, mixing and extracting may be accomplished by adding a 1 cm. length of 0.041 inch diameter stainless steel wire (from the Newark Wire Cloth Company, Newark, New Jersey) and shaking. Mild agitation suffices to mix the alcohol with the serum, and after adding the kerosene-xylene, the tubes are sealed at the upper end in a flame and shaken vigorously. Up to 50 tubes may be shaken together by hand (200 or 300 times). For a large series of analyses this technique is as rapid as the one given above; however, it is necessary to take great care to prevent any serum from wetting the top of the tube, which would result in charring when the tubes are sealed off and thereby jeopardize the analysis.

A major problem was to find a solvent which would completely extract vitamin A and carotene from serum and which would permit manipulation of small volumes without undue evaporation. Petroleum ether was completely unsuited owing to its volatility; toluene was an improvement; xylene was still better; and kerosene showed practically no evaporation but failed to extract vitamin A or carotene quantitatively. A 1:1 mixture of kerosene and xylene was found to have such a low volatility that evaporation could be ignored, and recovery experiments from serum demonstrated the quantitative extraction of both pigments.

Measurement of light absorption in small volumes with the Beckman spectrophotometer has been described (12). Since the samples are transferred, after the first readings, from the absorption cuvettes to small tubes for irradiation and transferred back to the cuvettes for the second readings, there is danger that so much of the sample might be lost during the manipulations as to leave an insufficient volume for the second readings. Such loss can be prevented by using slender tipped transfer pipettes and making sure that no more than a trace of liquid is left in either cuvette, irradiation tube, or transfer pipette.

Destruction of Vitamin A—When vitamin A or vitamin A ester in kerosene-xylene is irradiated with ultraviolet light, absorption at 328 mμ rapidly decreases, leaving a residual absorption of approximately 3 per cent. However, when an unsaponified serum extract is irradiated in a quartz or Pyrex tube with an unfiltered mercury vapor lamp, the absorption at 328 mμ falls and then rises higher than its initial value. Evidently while vitamin A is being destroyed, other substances are being converted into more highly absorptive materials. This phenomenon was delayed but not completely prevented by restricting the irradiation to the wave-lengths between 310 and 400 mμ, as recommended by Little (8). Little used a Corning No. 986 filter and an aqueous potassium acid phthalate solution in front of the light source to accomplish this purpose. It has been found more convenient to utilize a light source encased in a purple envelope (General Electric B-H4), which essentially cuts out wave-lengths longer than 400 mμ, and to place the samples in ordinary soda lime ("soft") glass tubes which cut off wave-lengths shorter than 310 mμ. If, in addition to filtering the light, the serum is saponified before extraction, the absorption at 328 mμ falls to a plateau which remains unchanged with further irradiation. Saponification has also been found necessary in order to effect complete extraction of vitamin A. With saponification, the light filters are perhaps unnecessary, but it has been felt desirable to retain them as a precautionary measure. However, if the soft glass used is of such a composition as to make prolonged irradiation necessary to destroy vitamin A, Pyrex tubes may be substituted.

Fig. 2 furnishes evidence that under the prescribed conditions it is only vitamin A which is destroyed by the irradiation and that new absorbing materials are not formed. A serum extract was irradiated for 0, 9, and 60 minutes, and the absorption curves were measured between 305 and 400 mμ. The readings at 9 and 60 minutes were then subtracted from the

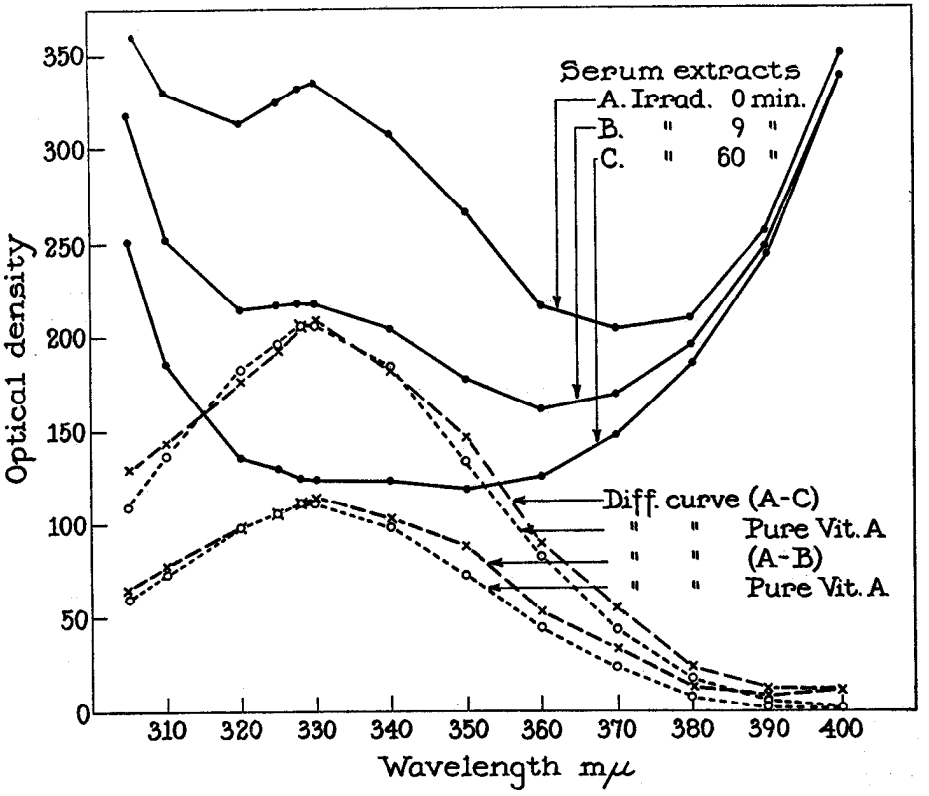

FIG. 2. Effect of irradiation on absorption of serum extracts. Absorption curves of serum extracts before and after irradiation with ultraviolet light (310 to 420 mμ), and difference curves for serum extracts and pure vitamin A solution, obtained by subtracting the values for the absorption curves before and after irradiation.

readings at zero time to obtain difference curves representing the change in absorption induced by irradiation. These difference curves have been compared with difference curves calculated from the change in the absorption of pure vitamin A solutions induced by prolonged irradiation. It will be seen that the curves for pure vitamin A and serum extract difference nearly coincide. This is strong evidence that the absorption changes are attributable to vitamin A destruction only.

Comparison with Antimony Trichloride (Carr-Price) Method—When comparisons were made between the micromethod described above and the antimony trichloride method as usually applied to serum (1–4), it was found that a number of the sera gave much higher values by the micromethod. At least part of the discrepancy was traced to certain inadequacies of the Carr-Price method as usually applied to serum. The sources of error in the antimony trichloride method proved to be (*a*) incomplete extraction of vitamin A from unsaponified serum (added vitamin A can, however, be completely extracted without saponification) and (*b*) the presence of unknown materials in some extracts which inhibited the color formation

TABLE I

Effect of Saponification on Carotene and Vitamin A Values (Antimony Trichloride Method)

The results are given in micrograms per cent.

Serum treatment	Serum 1	Serum 2	Serum 3	Serum 4	Serum 5	Serum 6
Vitamin A						
A. Saponified; calculated from internal standard	45	55	62	65	69	111
B. Saponified; calculated from pure standard	42	52	57	67	65	92
C. Not saponified; calculated from pure standard	37	45	50	57	51	78
D. (C) as % of (A)	82	82	87	88	88	70
Carotene						
E. Saponified	124	232	159	171	104	176
F. Not saponified	122	189	155	166	104	169

with antimony trichloride. Comparison of the figures in Lines A and B of Table I clearly shows the importance of the use of internal standards in serum vitamin A analysis; otherwise an error is introduced owing to the inhibitory effects of materials in serum extracts on the development of the color. In Line A the vitamin A content of the serum was calculated on the basis of the increment of color produced by adding pure vitamin A to an aliquot of the serum extract immediately before color development. In Line B the vitamin A values were calculated from the color intensity produced in pure solution. A comparison of the figures in Lines B and C illustrates the effects of previous saponification on the extractability of the vitamin A from the serum.[3] It will be noted from Line D that the combined errors due to the above causes amount to from 12 to 30 per cent.

[3] The lower values in Line C are in part due to incomplete extraction and in part due to a greater inhibition of color development without saponification.

The presence of inhibitory materials in natural products which influence the rate and extent of color development resulting from the antimony trichloride reaction has long been known (14–16). Oser, Melnick, and Pader (17) have recently emphasized the value of the use of an internal standard to decrease the error resulting from this effect in analysis of oils and foodstuffs. Likewise, the importance of saponification as a means of eliminating these inhibitory materials and in aiding extraction has been previously pointed out in connection with food analysis. Analogous information in relation to serum analysis has not been satisfactory.

Yudkin (18) reported that saponification is unnecessary for the determination of vitamin A in serum, whereas others have found it to be necessary (19, 20). It is possible that the saponification employed by Yudkin resulted in the destruction of tocopherol. If this were the case, part of the vitamin A would be destroyed during the evaporation of the petroleum ether extracts and any benefits of saponification would be obscured. Pett and LePage (20) observed that vitamin A values increased with mild saponification and decreased with more drastic alkaline treatment, and in this laboratory it has been found that after prolonged saponification higher results are obtained by the Carr-Price method if tocopherol is incorporated in the petroleum ether used for extraction. With milder saponification, or none at all, the presence of tocopherol is without effect on the results. In the majority of methods in current usage for the determination of vitamin A in serum, saponification is omitted.

In order to obviate these difficulties, a modified antimony trichloride procedure was used for making comparisons with the proposed micromethod. 7 ml. of serum were saponified with 1 N KOH in 90 per cent ethyl alcohol for 20 minutes at 60°. Extracts were then made by shaking with 7 ml. of petroleum ether (b.p. 30–60°) containing 1 mg. per cent of α-tocopherol (to prevent danger of loss of vitamin A during evaporation of the extracts). Triplicate determinations were made with 1 ml. aliquots of the petroleum ether extract. The carotene was measured by absorption at 460 mμ in the Coleman model 6 spectrophotometer. The petroleum ether was then evaporated, the residue taken up in 0.1 ml. of chloroform plus 0.01 ml. of acetic anhydride, and 1 ml. of 25 per cent antimony trichloride in chloroform was added. Measurements were made at 15 seconds at 620 mμ with the same instrument. Corrections were made for the contribution of carotene to the color, assuming that all of the 460 mμ absorption was due to β-carotene. In addition, internal standards were included in triplicate by substituting 0.1 ml. of standard vitamin A solution in $CHCl_3$ for the pure $CHCl_3$. These values were utilized in computing the results. The microdeterminations were made as previously described with 60 c.mm. of serum. Eleven sera were analyzed by both the macro- (antimony trichloride) and microprocedures for vitamin A and carotene. The

averages of all the determinations were in good agreement (Table II) by the two methods. The standard deviation between individual values measured both ways was 5 γ per cent for vitamin A and 7 γ per cent for carotene (one carotene value omitted in calculating the standard deviation). This is additional proof of the reliability of the proposed micromethod for the measurement of vitamin A in serum.

Use of Different Serum Volumes and Reproducibility—Although it is recommended that 60 to 100 c.mm. of serum be used for analysis, it is possible to obtain valid data with as little as 35 c.mm. if somewhat more

TABLE II

Comparison between Micro- and Macromethods for Vitamin A and Carotene

The results are given in micrograms per cent.

Serum No.	Vitamin A		Carotene	
	Micro*	Macro† (Carr-Price)	Micro*	Macro‡
1	106	111	183	176
2	74	68	185	171
3	66	69	110	104
4	56	52	91	87
5	55	62	159	159
6	52	51	179	171
7	51	51	131	122
8	50	55	198	232
9	46	43	89	83
10	43	40	100	97
11	39	45	123	124
Average.....	58	59	141	139

* Proposed method with 0.06 ml. of serum.

† Modified antimony trichloride procedure with 1 ml. of serum.

‡ Petroleum ether extract of 1 ml. of serum.

attention is given to analytical details. A number of sera were analyzed in replicate by the proposed procedure with 35, 60, and 100 c.mm. samples. The samples were treated with volumes of alcoholic KOH equal to the serum volumes and were then extracted with 40, 60, and 100 c.mm., respectively, of kerosene-xylene. Nine to twenty-eight samples were measured at each volume level. The standard deviations were 1, 1, and 2 γ per cent, respectively, for vitamin A, and 1, 1, and 1 for carotene.

Table III shows that essentially the same absolute values are obtained when different amounts of serum are used for analysis. Three sera were analyzed in triplicate at each volume level.

Effect of Storage—In making analyses for nutritional surveys it is most

convenient if samples can be collected in the field, transported to a central laboratory, and analyzed at a later date. In this case one must be assured of the keeping quality of samples and of the storage conditions compatible with the stability of the substances to be measured. To obtain the necessary information, two serum samples were stored at various temperatures in a number of sealed tubes and analyzed after 1 and after 4.5 months for vitamin A and carotene by the proposed micromethod. There was no detectable change after 1 month at either 4° or −20° in either vitamin A or carotene. At room temperature, however, the vitamin A had fallen to about 45 per cent of its initial value and the carotene to 10 per cent or less. After 4.5 months at −20° there was no significant change in vitamin A and the carotene had only fallen by about 6 per cent. At 4° the carotene

TABLE III

Vitamin A and Carotene Values with Different Volumes of Serum

	Serum 1			Serum 2			Serum 3		
Volume of serum, *c.mm.*	35	60	100	35	60	100	35	60	100
Vitamin A, γ %	74	75	74	49	47	50	50	52	50
Carotene, γ %	131	132	132	95	98	96	139	138	138

All measurements made in triplicate.

was almost gone in both samples, one vitamin A value was unchanged, and the other had fallen 40 per cent. It seems permissible to conclude that in serum carotene is more unstable than is vitamin A, and that sera may be stored for several weeks at 4° or several months at −20° without prejudice to the results.

SUMMARY

1. A method is described for measuring the vitamin A and carotene in 60 c.mm. of serum, an amount easily obtainable from the finger. Since, in addition, one analyst can perform at least 50 determinations in a working day, it appears to fulfil the requirements for a nutritional survey method or for studies on small animals.

2. The method has been compared with a modified Carr-Price (antimony trichloride) macroprocedure which gave essentially the same carotene and vitamin A values as the micromethod.

3. Low values for vitamin A were obtained with the Carr-Price method as usually performed on serum. Several factors which appear to be responsible for these low results are discussed.

4. Data are given on the keeping qualities of vitamin A and carotene in stored sera.

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