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**Reprints of Selected Methods for  
the Analysis of Vitamin A and  
Carotenoids in Nutrition Surveys**

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**International Vitamin A  
Consultative Group (IVACG)**

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# Reprints of Selected Methods for the Analysis of Vitamin A and Carotenoids in Nutrition Surveys

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**I.**  
**Analysis of Plasma Vitamin A**

## Determination of Serum Retinol (Vitamin A) by High-Speed Liquid Chromatography

M. G. M. De Ruyter and A. P. De Leeuw

We report a fast and simple high-speed liquid-chromatographic assay for serum retinol. Only 100  $\mu$ l of serum is required. The lower detection limit is 50  $\mu$ g/liter; linearity was demonstrated up to 1.50 mg/liter. On analyzing a serum pool eight times, a CV of 2.5% was obtained. Values by this method are compared with results obtained by a fluorometric method [*Clin. Chem.* 16, 766 (1970)].

In the past 10 years most determinations for retinol in serum or plasma were based on molecular fluorescence properties of the retinol structure. However, presence of fluorescent components other than retinol raised problems of nonspecificity with serum samples. To overcome this problem Garry et al. (1) and Pollack et al. (2) introduced a chromatographic step before fluorometry. Other authors (3, 4) used correction formulas to compensate nonspecific fluorescent interferences. Recently Futterman et al. (5) proposed a direct fluorometric procedure (no extraction step required), taking advantage of the enhancement of the fluorescence of the retinol binding protein-retinol complex. As in the latter procedure the serum sample is diluted 100-fold and nonspecific fluorescence is successfully minimized. However, variation in the enhancement factor (mean value is 16.5 but varies from 13.6 to 20.9) with different serum samples may present a drawback.

Here, we describe a method that is specific, owing to the chromatographic step, and sensitive and fast, owing to the on-line ultraviolet detection at 328 nm and the omission of concentration steps. It can be easily performed if an isocratic liquid chromatograph is available.

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### Materials and Methods

#### Apparatus

A Varian 4100 liquid chromatograph equipped with a stop-flow injection system and a Variscan multiple wavelength detector are used. The column is filled with a 10- $\mu$ m irregular microparticulate silica (15 cm  $\times$  0.2 cm i.d.). All this is from Varian Benelux, Brussels.

#### Reagents

*Petroleum ether* (bp 40–60 °C), *methanol*, *isopropanol*, and *dichloromethane* were all analytical-grade reagents from Merck (Darmstadt, Germany) and were used without further purification.

*Retinol* (vitamin A alcohol) was of crystalline purity, from Fluka AG (Switzerland).

The *internal standard* [all-*trans*-9-(4-methoxy-2,3,6-trimethylphenyl)-3,7-dimethyl-2,4,6,8-tetraenol] was synthesized by reduction of ethyl-all-*trans*-9-(4-methoxy-2,3,6-trimethylphenyl)-3,7-dimethyl-2,4,6,8-tetraenoate (a gift from Hoffmann-La Roche, Basel, Switzerland) with  $\text{LiAlH}_4$ . The final solution of the internal standard in methanol used had an absorbance of 0.895 at 327 nm ( $\lambda_{\text{max}}$ ) in a 1.00-cm quartz cell.

The extraction solvent and the mobile phase have the same composition, i.e., petroleum ether/dichloromethane/isopropanol (80/19.3/0.7 by vol).

#### Procedure

Transfer 100  $\mu$ l of serum (or plasma), 15  $\mu$ l of internal standard solution and 100  $\mu$ l of methanol to a conical centrifuge tube (7.6  $\times$  0.9 cm). Mix the contents of the tube with a vortex-type mixer. Add 200  $\mu$ l of extraction solvent and cap the tube (polyethylene cap). Extract by interrupted mixing on the vortex-type mixer for 60 s. After centrifugation (3000 rpm, 2 min) inject 100  $\mu$ l of

**Table 1. Chromatographic Conditions**

Column:	15 X 0.2 cm i.d. MicroPak Si-10
Mobile phase:	petroleum ether/dichloromethane/ isopropanol (80/19.3/0.7 by vol)
Flow rate:	0.5 ml/min
Pressure:	10 kg/cm <sup>2</sup>
Detector	328 nm
wavelength:	
Detection	0.04 AUFS on recorder
sensitivity:	
Temperature:	ambient

the supernate on top of the column by use of a Hamilton 710 syringe, 100- $\mu$ l capacity.

### Results and Discussion

Chromatographic conditions are given in Table 1, and a typical chromatogram obtained for a serum extract to which a known amount of internal standard has been added is shown in Figure 1.

The procedure is standardized by adding different volumes of a methanolic retinol standard solution to water and analyzing these aqueous samples by the above procedure. A linear relationship between peak height ratios (peak height retinol/peak height internal standard) and retinol concentration was found over the range of 0–1.50 mg/liter (Figure 2). By using the same calibration curve ( $y = 0.0182x - 0.0067$ ) the concentration of retinol in a serum sample is easily determined after calculation of its peak height ratio.

Within-run precision of the method was obtained from eight replicate determinations of a serum pool. The mean value was 589  $\mu$ g/liter (SD 15  $\mu$ g/liter; CV 2.5%).

The analytical recovery of retinol from serum was determined by adding known quantities of retinol to serum and analyzing. Results (Table 2) ranged between 91 and 100%, quite acceptable for a trace analysis.

Background absorption of a retinol-free serum sample was estimated by irradiating an aliquot of a serum sample with long-wave ultraviolet light ( $>320$  nm) for 3 h; this treatment should destroy all retinol present. The irradiated serum was taken through the entire analysis procedure, and no residual absorption could be detected in the chromatogram at the elution time of retinol.

Identification of the serum retinol peak is made on the basis of retention time. To illustrate the specificity of the method, we performed an on-line recording of the absorption maximum of the eluted compound. Therefore the flow at the apex of the peak was interrupted and a continuous spectrum from 400 to 300 nm recorded. A maximum was found at 327 nm, which agrees closely with values reported in the literature (6). In addition to this, we determined the capacity ratios of several compounds with structures closely related to retinol. Data are given in Table 3. The only compound that might interfere with the analysis is retinoic acid, which

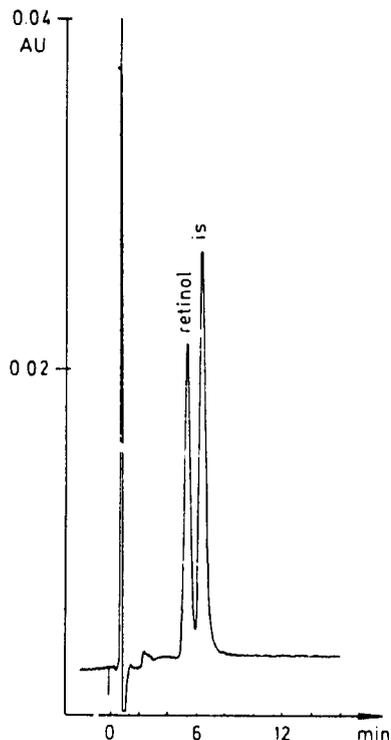


Fig. 1. Chromatogram of a serum extract to which internal standard has been added

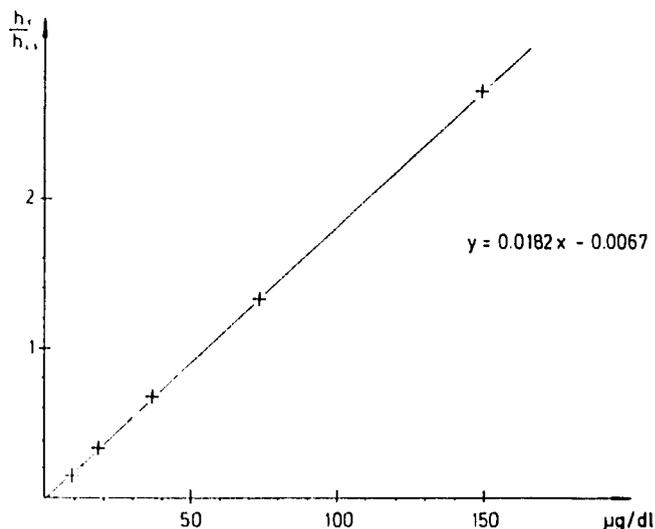


Fig. 2. Calibration curve for serum retinol (peak height ratios vs. retinol concentrations)

**Table 2. Analytical Recovery of Retinol from Serum**

Initial concn	Added	Measured	Recovered*	Recovery, %
		$\mu$ g/liter		
603	627	1201	598	95
603	502	1075	472	94
603	376	978	375	100
603	627	1171	568	91
603	502	1103	500	100
603	376	975	372	99

**Table 3. Capacity Ratios ( $K'$ ) of Compounds Related to Retinol**

$\beta$ -Carotene	0
Retinyl palmitate	0
Retinyl acetate	0
All-trans retinal	0.75
All-trans retinol	5.0
Internal standard	6.2
All-trans retinoic acid	5.2

has a  $K' = 5.2$ . However serum concentrations of retinoic acid, if present, are so low it would not be detected. Phytofluene, the nonspecific fluorescent compound that interferes with the fluorometric assay in sera, has absorption maxima at 331, 349, and 366 nm (3). As an apolar molecule it will elute together with  $\beta$ -carotene, which does not interfere with our assay.

We compared our high-speed liquid-chromatographic method with the fluorometric method of Garry et al. (1), which includes silicic acid chromatography as a clean-up step, analyzing 22 sera for retinol by both methods. The fluorometric method yielded a mean value of 820  $\mu\text{g/liter}$ , whereas 600  $\mu\text{g/liter}$  was found with our method. The correlation coefficient between the two methods was 0.779. The higher values obtained by the fluorometric method and the rather poor correlation between the two methods may be due to the technical difficulties we encountered in performing the fluorometric assay and (or) the presumptive higher specificity of our method.

In summary, high-speed liquid chromatography for serum retinol offers several advantages over the assays

currently in use. The column chromatography provides high specificity, and on-line ultraviolet detection at 328 nm makes the assay quite sensitive: only 100  $\mu\text{l}$  of serum is needed to obtain a detection limit of 50  $\mu\text{g}$  of retinol per liter. Sample preparation and chromatographic run demand an overall analysis time of less than 12 min, fast compared to other methods. Because few manipulations are needed the possibility of sample deterioration is minimized. Furthermore, the addition of an internal standard before the extraction improves the precision and compensates for possible losses caused by evaporation or spilling. The internal standard has been chosen on basis of its retention characteristics and its structural analogy to retinol. It elutes very close to the retinol peak and has the same  $\lambda_{\text{max}}$  as retinol, which makes it well suited for our purposes. The assay could also be performed without addition of internal standard. However a fixed-volume injection loop would be required to get reproducible injections, and the advantage of an assay where an internal standard is used would be lost.

### References

1. Garry, P. J., Pollack, J. D., and Owen, G. M., Plasma vitamin A assay by fluorometry and use of a silicic acid column technique. *Clin. Chem.* 16, 766 (1970).
2. Pollack, J. D., Owen, G. M., Garry, P. J., and Clark, D., Plasma Retinol assay by elution from silicic acid with *o*-cymene (*p*-isopropyl toluene). *Clin. Chem.* 19, 977 (1973).
3. Thompson, J. N., Erdody, P., Brien, R., and Murray, T. K., Fluorometric determination of vitamin A in human blood and liver. *Biochem. Med.* 5, 67 (1971).
4. Bubb, F. A., and Murphy, G. M., Determination of serum phytofluene and retinol. *Clin. Chim. Acta* 48, 329 (1973).
5. Futterman, S., Swanson, D., and Kalina, R. E., A new, rapid fluorimetric determination of retinol serum. *Invest. Ophthalmol.* 14, 125 (1975).
6. Roels, O. A., and Mahadevan, S., In *The Vitamins*, VI, P. Gyorgy and W. N. Pearson, Eds. Academic Press, N.Y. and London, 1967, p 143.

# Simultaneous determination of $\alpha$ -tocopherol and retinol in plasma or red cells by high pressure liquid chromatography<sup>1, 2</sup>

John G. Bieri, Ph.D., Teresa J. Tolliver, B.S., and George L. Catignani,<sup>3</sup> Ph.D.

**ABSTRACT**—This paper describes a rapid, microprocedure for the simultaneous determination of  $\alpha$ -tocopherol (vitamin E) and retinol (vitamin A) in plasma, and of  $\alpha$ -tocopherol alone in red cells since cells do not contain retinol. A total lipid extract from 0.1 ml plasma or 0.125 ml red cells and containing internal standards of  $\alpha$ -tocopheryl acetate and retinyl acetate is injected onto a high pressure liquid chromatograph with a reverse phase column developed with methanol-water. An ultraviolet detector with 280-nm filter is used. The chromatogram is complete in 8 min and the  $\alpha$ -tocopherol and retinol are quantitated by the peak height ratio method. Comparison of results with both plasma and red cells gave excellent agreement with conventional methods for these vitamins. The procedure should be particularly useful for clinical studies and nutrition surveys. *Am. J. Clin. Nutr.* 32: 2143-2149, 1979.

The recent application of high pressure liquid chromatography (HPLC) to analyses of biological material has provided the nutritional biochemist with a highly useful technique for a wide variety of applications. Advantages of HPLC over spectrophotometry, fluorometry, or gas chromatography include its rapidity, relative freedom from interfering impurities, nondestructive conditions, and simplified methodology applicable to very small samples. The feasibility of HPLC for analyzing blood plasma separately for vitamin A (retinol) and vitamin E ( $\alpha$ -tocopherol) have been described (1-5). In this report, we describe a rapid, microprocedure for the simultaneous determination of retinol and  $\alpha$ -tocopherol in plasma or serum and compare the results with other established methods used in nutritional studies. Application of the HPLC procedure to the analysis of  $\alpha$ -tocopherol in red cells is also described (red cells do not contain retinol).

## Materials

The HPLC instrumentation was from Waters Associates, Inc. (Milford, Mass.) and consisted of a model 6000 A solvent delivery system, a model U6K universal liquid chromatograph injector, and a model 440 absorbance detector. The latter was fitted with a 280 nm interference filter and was connected to a Tracor Westronics (Fort Worth, Texas) model FD-10A dual pen, 10 mv, 10 inch recorder run at 1 cm/min. The column was

3.9 mm  $\times$  30 cm stainless steel packed with micro Bondapak C-18. A guard column (precolumn) 3  $\times$  22 mm packed with Bondapak C-18 Corasil was attached to the primary column. The solvent was reagent grade methanol-water, 95:5, filtered through a 0.5- $\mu$  filter. Samples were injected with a 100- $\mu$ l gas syringe (Precision Sampling, Inc., Baton Rouge, La.). For spectrophotometric determinations of retinol and  $\alpha$ -tocopherol, the Giltford Star II (Oberlin, Ohio) and Beckman DU (Irvine, Calif.) spectrophotometers, respectively, were used. Hexane (nanograde), heptane (analytical reagent), and anhydrous ethyl ether (analytical reagent) were from Mallinckrodt, St. Louis, Mo. Reagent grade absolute methanol was from J. T. Baker, Phillipsburg, N.J., and pyrogallol was obtained from Eastman Organic Chemicals, Rochester, N.Y.

## Standards

Standard compounds were all-trans retinyl acetate and *D*- $\alpha$ -tocopherol (Eastman Organic Chemicals, Rochester, N.Y.) and *d*- $\alpha$ -tocopheryl acetate (General Biochemicals, Cleveland, Ohio). The retinyl acetate was purified by injecting 100 to 150  $\mu$ g into the HPLC and collecting the middle portion of the peak. The solvent was evaporated under nitrogen and the retinyl acetate dissolved in ethanol. The  $\alpha$ -tocopherol and  $\alpha$ -tocopheryl acetate did not require purification. Retinol was pre-

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pared from retinyl acetate by saponifying with alcoholic KOH in the presence of 5% pyrogallol, extracting into hexane, and purifying by chromatography on a 0.5 × 3 cm column of alumina weakened with 10% water. The column was developed with 2 ml 1% acetone in hexane followed by 5 ml 25% diethyl ether in hexane to elute the retinol. Standard stock solutions of these compounds were prepared in redistilled absolute ethanol and the concentrations checked spectrophotometrically. Dilutions of these stock standards, the working standards, were made regularly as indicated below. Extinction coefficients used were (1% solution) retinol, 1780 at 328 nm; retinyl acetate, 1710 at 328 nm;  $\alpha$ -tocopherol, 75.8 at 292 nm; and  $\alpha$ -tocopheryl acetate, 43.6 at 285 nm. The dilute  $\alpha$ -tocopheryl acetate working internal standard was stable for several months when stored at 4°C, but the retinyl acetate working internal standard showed skewing of the HPLC peak after 1 week. This solution was prepared weekly from the stock retinyl acetate solution which was stable for a month when kept at 4°C in the dark. The retinol and  $\alpha$ -tocopherol standards were kept for only a few days when determining the peak height ratios (below).

#### Standard curves

For quantitation, internal standards of retinyl acetate and  $\alpha$ -tocopheryl acetate were used. To prepare a standard curve for the peak height ratios (6), a constant amount of the acetate was combined with variable amounts of the free alcohol form of the vitamin to give solutions with a 3-fold weight ratio. These solutions of retinol and retinyl acetate, or  $\alpha$ -tocopherol and  $\alpha$ -tocopheryl acetate, were injected into the HPLC instrument and the peak height ratios measured. Ratios were the same whether the compounds were injected in ethanol or in methanol.

#### Analysis of plasma for $\alpha$ -tocopherol and retinol

Blood samples were from normal donors at the National Institutes of Health blood bank. For the analysis of blood plasma (or serum), 50  $\mu$ l of the internal standard of retinyl acetate solution in ethanol (0.8 to 1.2  $\mu$ g/ml) and 50  $\mu$ l of the internal standard  $\alpha$ -tocopheryl acetate solution in ethanol (40–50  $\mu$ g/ml) are pipeted into a 6 × 20 mm disposable glass test tube, using a Lang-Levy pipet. Plasma, 100  $\mu$ l, is added and the contents mixed vigorously on a vortex mixer for 10 sec. For extraction of the lipid, 100  $\mu$ l spectro grade hexane or heptane<sup>1</sup> are added and the contents mixed vigorously intermittently for 45 sec on a vortex mixer, making sure that the contents at the bottom of the tube are thoroughly extracted. The tubes are centrifuged to separate the phases and as much of the solvent as possible is carefully drawn off with a 75  $\mu$ l Lang-Levy pipet and transferred to a 3- or 5-ml conical centrifuge tube. The solvent is evaporated under a stream of nitrogen with the tube in a 60°C water bath. For injection into the chromatograph, the lipid in the centrifuge tube is dissolved in 25  $\mu$ l diethyl ether followed by 75  $\mu$ l methanol, with gentle mixing by finger tapping. About 90  $\mu$ l of the solution is injected, using a 10- $\mu$ l flush of methanol in the syringe. A flow rate of 2.5 ml/min was used with the detector set at 0.01 attenuation.

#### Analysis of red cells for $\alpha$ -tocopherol

Red cells are washed three times with 0.9% saline and a 50% suspension is prepared in saline containing 0.5% pyrogallol. The hematocrit is determined immediately for use in calculations. Of the suspension 0.5 ml is pipeted into a 12 × 100 mm screw cap or glass stoppered test tube followed by 1.5 ml cold methanol (cooled in a dry ice-acetone bath) added dropwise while slowly vibrating the tube on a vortex mixer. This precipitates the proteins in a fine suspension without clumping. To the tube are added 20  $\mu$ l  $\alpha$ -tocopheryl acetate standard (40 to 50  $\mu$ g/ml) and 2 ml heptane.<sup>2</sup> The tubes are shaken vigorously or mixed on a vortex mixer for 45 sec, then centrifuged to separate the phases. About 1 ml heptane is transferred to a 5-ml centrifuge tube, being careful not to take up any protein. The solvent is evaporated and the lipid injected as described above for plasma. The detector is operated at maximum sensitivity, i.e., 0.005 attenuation with a flow rate of 3.5 ml/min.

## Results

The reverse phase column with methanol:water as solvent gave good separation of the compounds generally considered to be of primary interest in the nutritional assessment of vitamins A and E (Fig. 1). Retinol emerged very shortly after the solvent peak and was well separated from its internal standard, retinyl acetate. Retinyl palmitate, a minor component of total plasma vitamin A, emerged after about 20 min but with this long retention time the small amount normally present in plasma was not detectable.  $\alpha$ -Tocopherol and  $\alpha$ -tocopheryl acetate were distinctly separated but  $\beta$ -tocopherol was not separated from  $\gamma$ -tocopherol. This combined peak is considered to be predominantly  $\gamma$ -tocopherol in human plasma (7). A peak presumed to be  $\alpha$ -tocopheryl quinone was found only in red cell extracts when pyrogallol was not included as an antioxidant.

There were no interfering peaks in plasma at the locations of the two internal standards, retinyl acetate and  $\alpha$ -tocopheryl acetate (Fig. 2). A chromatogram of the same normal plasma with added standards is shown in Figure 3. As can be seen, under the given conditions the chromatogram was completed

<sup>1</sup> Loss of solvent by evaporation is less with heptane, however, with the use of internal standards this problem is alleviated.

<sup>2</sup> Since 1 ml of the solvent must be evaporated, any contaminants will be amplified on the HPLC recording. We found hexane to have considerably more interfering material than heptane.

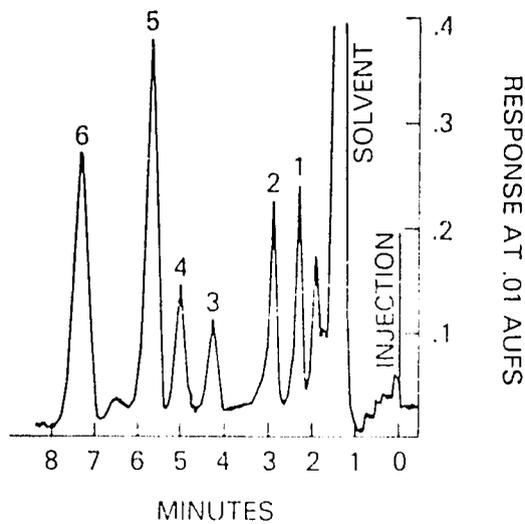


FIG. 1. HPLC chromatogram of compounds of interest in the analysis of plasma and red cells for  $\alpha$ -tocopherol and retinol. Peak 1, retinol; 2, retinyl acetate; 3,  $\alpha$ -tocopherylquinone; 4,  $\beta$  +  $\gamma$ -tocopherol; 5,  $\alpha$ -tocopherol; 6,  $\alpha$ -tocopheryl acetate. Conditions: methanol:water, 95:5; flow rate, 2.5 ml/min; detector with 280-nm filter and attenuation at 0.01 absorption units full scale (AUFS).

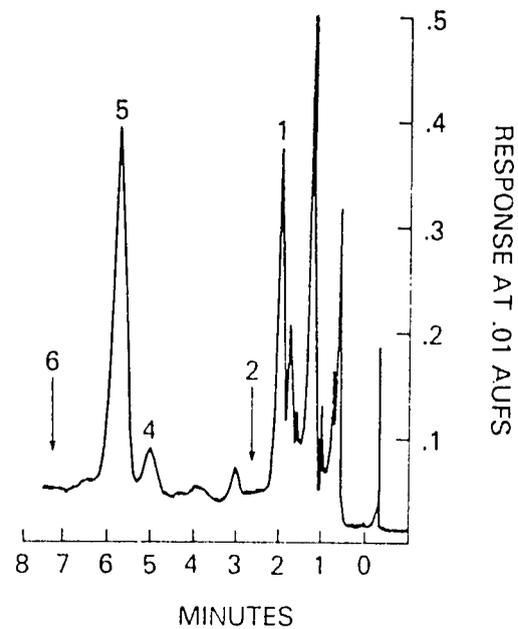


FIG. 2. Chromatogram of normal human plasma without internal standards. Conditions and peak identification as for Figure 1. Unmarked peaks are unidentified.

in about 8 min. Faster flow rates often did not give sufficient separation of retinol from the solvent peak.

Evidence suggesting that no other compound in human plasma has the same retention time as  $\alpha$ -tocopherol was obtained with blood from a patient with  $\alpha$ - $\beta$ -lipoproteinemia who had been taking a poorly utilized preparation of  $\alpha$ -tocopherol (Fig. 4). Only a barely detectable peak, calculated to be 60  $\mu$ g/dl, was found. In contrast, the retinol value, 21  $\mu$ g/dl, was in the low normal range. Similar evidence for the absence of an interfering peak at the retinol position was not possible with human blood, since patients who malabsorb are routinely treated with vitamin A and generally attain normal blood values. Analysis of plasma from a chronically retinol deficient rat maintained on retinoic acid, however, gave no peak at the retention time of retinol.

The 0.1-ml sample of plasma with the HPLC conditions given in Figure 1 gave peak heights for retinol and  $\alpha$ -tocopherol that ranged from 0.2 to 0.6 of the full recorder scale for normal plasmas. If necessary, a 50- $\mu$ l plasma sample size could be used with the

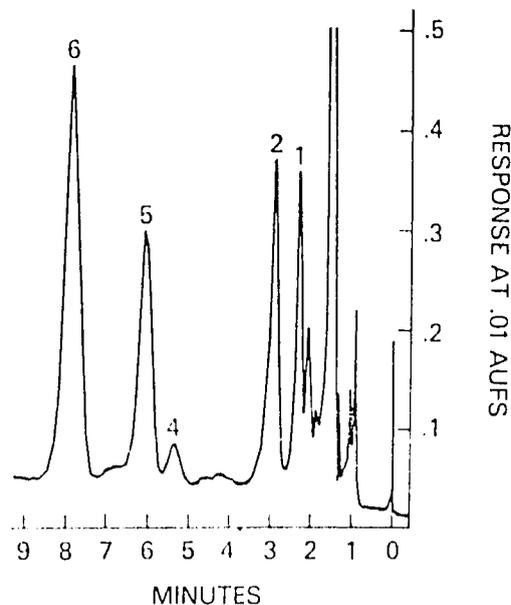


FIG. 3. Chromatogram of same normal human plasma as in Figure 2 but with internal standards of retinyl acetate and  $\alpha$ -tocopheryl acetate added. Conditions and peak identification as for Figure 1.

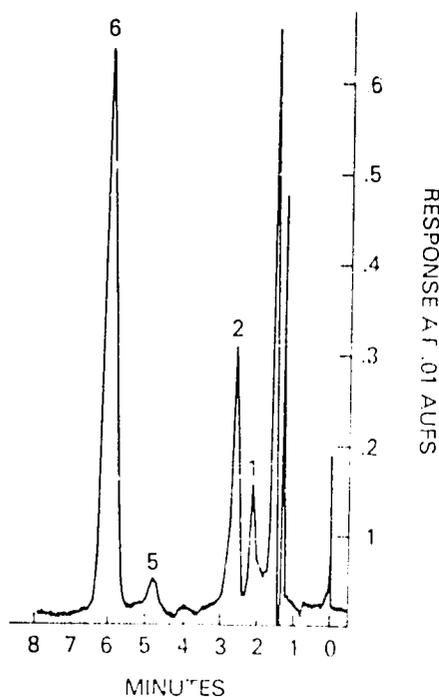


FIG 4. Chromatogram of plasma from a patient with  $\alpha$ - $\beta$ -lipoproteinemia, with added internal standards, showing barely detectable  $\alpha$ -tocopherol. Conditions as in Figure 1.

detector at its highest attenuation, but working with these smaller volumes is considerably more difficult.

Standard curves for peak height ratios for the pairs,  $\alpha$ -tocopherol:tocopheryl acetate and retinol:retinyl acetate are shown in Figure 5. The much greater extinction coefficient for retinol than for  $\alpha$ -tocopherol made the analysis about 20 to 25 times more sensitive for retinol. Thus, a 6 cm high peak was given by 0.024  $\mu$ g retinol and by 0.6  $\mu$ g  $\alpha$ -tocopherol under the conditions given in Figures 1 to 3.

A comparison of analyses of 14 human plasmas for retinol by HPLC and by the trifluoroacetic acid (TFA) method of Neeld and Pearson (8), and for  $\alpha$ -tocopherol by HPLC and by the Emmerie-Engel color reaction after thin layer separation (9) is given in Table I. For both retinol and  $\alpha$ -tocopherol the HPLC method gave results that were not significantly different by linear regression analysis from the macrocolorimetric procedures. The correlation coefficient ( $r$ ) was slightly higher for tocopherol than for retinol. It may have been anticipated that the HPLC

method would give slightly lower values for retinol than the TFA method, since the latter determines retinyl esters in addition to retinol. Probably the correction for carotenoid pigments was too large, thus giving low values for total vitamin A. The correction factor for carotenoids is made with  $\beta$ -carotene, whereas this is a minor component of human plasma carotenoids and the different carotenoids give varying color intensity with TFA (10).

The precision of the method for the same plasma determined on 10 consecutive days is given in Table 2. A slightly lower coefficient of variation was obtained for  $\alpha$ -tocopherol than for retinol but both were relatively low. Precision for the same day analysis of 10 aliquots of a second plasma gave for retinol a mean of 45.7  $\mu$ g/dl, SD = 1.9, coefficient of variation = 4.1%, and for  $\alpha$ -tocopherol a mean of 580  $\mu$ g/dl, SD = 32, coefficient of variation = 5.6% (data not shown). Recoveries of  $\alpha$ -tocopherol and retinol added in amounts about the same as those present in

TABLE I  
Comparison of the determination of plasma retinol and  $\alpha$ -tocopherol by HPLC and colorimetry<sup>a</sup>

Sample	Retinol		$\alpha$ -Tocopherol	
	HPLC	TFA <sup>b</sup>	HPLC	ELC <sup>c</sup>
	<i>ug/dl</i>			
1	70	55	882	942
2	63	54	710	703
3	85	67	1682	1835
4	50	27	684	690
5	66	46	926	996
6	48	55	810	839
7	78	71	1212	1320
8	70	84	1217	1328
9	37	32	612	513
10	34	29	570	569
11	46	31	836	896
12	41	33	506	513
13	40	35	677	681
14	25	29	422	461
$\bar{X}$	53.8 <sup>d</sup>	46.3 <sup>d</sup>	839.0 <sup>e</sup>	977.6 <sup>e</sup>
SE	4.8	4.9	90.2	104.2
$r$	0.833		0.996	

<sup>a</sup>Samples 1 to 8 were drawn with EDTA tubes. Samples 9 to 14 had an unspecified volume of acid-citrate dextrose added that diluted the plasma and resulted in generally lower values. All samples were done in duplicate. <sup>b</sup>Trifluoroacetic acid method (8). <sup>c</sup>Emmerie-Engel color reaction after one dimensional thin-layer chromatography (9). <sup>d</sup>Means are not significantly different ( $P > 0.3$ ). <sup>e</sup>Means are not significantly different ( $P > 0.8$ ).

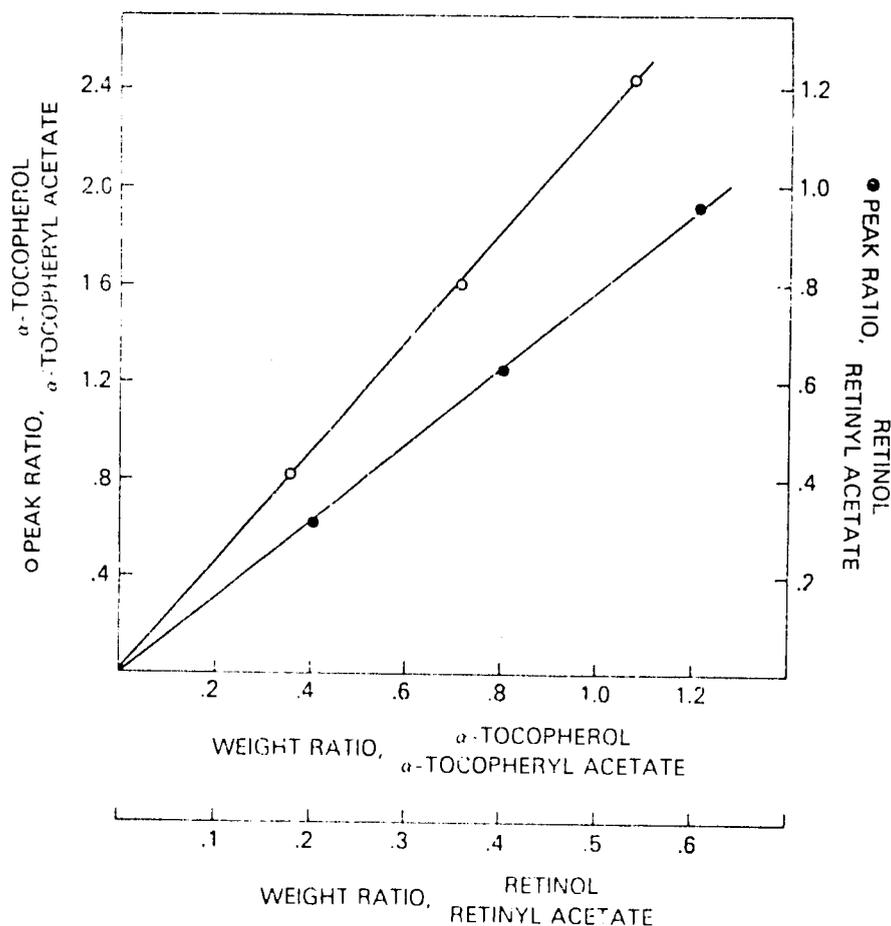


FIG. 5. Standard curves for peak height ratio versus weight ratio for retinol:retinyl acetate and  $\alpha$ -tocopherol: $\alpha$ -tocopheryl acetate. Conditions as in Figure 1

TABLE 2  
Precision of the HPLC procedure for determining plasma retinol and  $\alpha$ -tocopherol<sup>a</sup>

Day	Retinol	$\alpha$ -Tocopherol
	$\mu\text{g/dl}$	
1	34	447
2	34	438
3	31	449
4	32	447
5	30	440
6	31	440
7	32	467
8	30	454
9	31	445
10	31	430
$\bar{X}$	31.6	445.7
SD	1.4	10.0
Coefficient of variation (%)	4.4	2.0

<sup>a</sup> Same plasma run in duplicate on 10 consecutive days.

plasma were, respectively,  $100.7\% \pm 3.0$  and  $103.4\% \pm 5.2$  (means  $\pm$  SE for five trials).

The analysis of red blood cells for  $\alpha$ -tocopherol required a slightly larger volume than for plasma because red cells contain about one-fifth as much of the vitamin as does plasma (11, 12). A typical chromatogram of a red cell extract (Fig. 6) with added  $\alpha$ -tocopheryl acetate shows that only  $\alpha$ -tocopherol gave a significant peak. Analyses of nine human blood samples (Table 3) gave values in a range similar to that found previously by gas chromatographic (11), or spectrophotometric methods (12). Recovery of added  $\alpha$ -tocopherol was  $103.5\% \pm 5.2$  (mean  $\pm$  SE) when an amount equal to that present in the sample was added. A limiting factor on sample size was the amount of lipid that could be dissolved in ether:methanol. The described procedure permitted a clear or

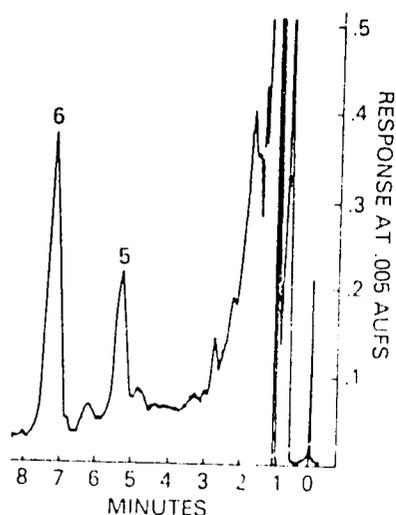


FIG. 6. Chromatogram of normal human red cells with internal standard of  $\alpha$ -tocopheryl acetate. Peak identification as in Figure 1. Conditions: methanol-water, 92:8, flow rate, 3.5 ml/min, detector with 280-nm filter and attenuation at 0.005 AUFS. Sample injected was equivalent to 0.125 ml of cells.

TABLE 3  
 $\alpha$ -Tocopherol concentration of normal red cells and plasma<sup>a</sup>

Sample	Red cells ug/ml	Plasma ug/ml	Red blood cells-plasma
1	168	993	0.169
2	154	953	0.157
3	146	735	0.199
4	164	686	0.239
5	139	717	0.194
6	290	1515	0.191
7	219	1080	0.203
8	199	811	0.245
9	235	1024	0.230
$\bar{X}$	190	949	0.203
SE	17	92	0.009

<sup>a</sup> Bloods from normal donors, drawn with EDTA and run in duplicate.

slightly cloudy solution for injection. Larger samples caused lipid, presumably cholesterol, to precipitate out of solution. Extraction of the red cell hemolysate without pyrogallol present invariably gave rise to a significant amount of an  $\alpha$ -tocopherol oxidation product with a retention time the same as that for  $\alpha$ -tocopherylquinone. Inclusion of sodium ascorbate or butylated hydroxy toluene in the red cell suspension did not prevent the oxidation.

## Discussion

The rapid simultaneous determination of retinol and  $\alpha$ -tocopherol in plasma should be of considerable usefulness in many clinical situations as well as in the nutritional assessment of normal subjects. We have used the method to determine the response to oral vitamin A and E supplements of patients with fat malabsorption as found in cystic fibrosis and  $\alpha$ - $\beta$ -lipoproteinemia. In the latter disease, the method has been used in current studies to monitor the response to several different aqueous vitamin preparations. In nutritional assessment, very few surveys have determined vitamin E because of difficulties in its analysis, even though it is well-documented that poor vitamin E status is prevalent in many poorly-nourished populations (13, 14). The long-known sparing effect of vitamin E on tissue vitamin A in animals (15) has recently been shown to operate in children (16). Application of the described simultaneous analysis of vitamins A and E should permit more detailed studies of their relationship in children.

The HPLC procedure has several advantages over other currently used methods for these vitamins. A simultaneous fluorometric procedure (17) determines total retinol and total tocopherols whereas HPLC separates retinol from its esters and also distinguishes between  $\alpha$ -tocopherol and the  $\beta$ ,  $\gamma$ -tocopherol pair. Fluorometric analyses are often plagued by spurious results from contamination (18). With HPLC, contamination is infrequent and if present is usually recognizable from the recording. Other procedures for analyzing  $\alpha$ -tocopherol in plasma are usually time-consuming and involve inherent losses. In the HPLC method for plasma, no oxidative conditions or separation steps are included that would lead to losses.

The use of internal standards, another advantage of our procedure, eliminates errors from pipeting or the evaporation of solvents. Care must be taken, however, to see that the composition of the dilute working standards does not change from either decomposition or evaporation. As noted in "Materials," change in the retinyl acetate standard was usually noted after 1 week by a shoulder on the HPLC peak. The  $\alpha$ -tocopheryl acetate

standard appeared to be stable for at least several weeks, but evaporation after 2 weeks resulted in increased concentration. For these reasons, we recommend that the working retinyl acetate standard be prepared from the stock solution weekly, and the working  $\alpha$ -tocopheryl acetate standard be prepared bi-weekly.

The usual oxidative loss of  $\alpha$ -tocopherol during the preparation of red cells for analysis has been noted previously and various measures used to prevent it (11, 12). Even though we did not saponify the cells, significant formation of an oxidation product of  $\alpha$ -tocopherol occurred during hemolysis and extraction. Only the inclusion of 0.5% pyrogallol in the saline solution used to dilute the cells was found adequate to prevent oxidative loss. The much smaller sample size and shortened analysis time make the procedure a marked improvement over previous methods for red cell  $\alpha$ -tocopherol (11, 12). An HPLC method for red cell tocopherols that appeared while this study was in progress (19), is similar to ours but includes saponification. 

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## References

1. DE RUYER, M. G. M., AND A. P. DE LEENHIER. Determination of serum retinol (vitamin A) by high-speed liquid chromatography. *Clin. Chem.* 22: 1593, 1976.
2. ABE, K., K. ISHIBASHI, M. OHMAI, K. KAWABE AND G. KATSUI. Determination of vitamin A in serum and liver by high-speed liquid chromatography. *Vitamins (Japan)* 51: 275, 1977.
3. ABE, K., AND G. KATSUI. Determination of tocopherols in serum by high-speed liquid chromatography. *Vitamins (Japan)* 49: 259, 1975.
4. NILSSON, B., B. JOHANSSON, I. JONSSON AND L. HOLMBERG. Determination of plasma  $\alpha$ -tocopherol by high-performance liquid chromatography. *J. Chromatog.* 145: 169, 1978.
5. DE LEENHIER, A. P., V. O. DE BEVERE, A. A. CRUYT AND A. F. CLAYS. Determination of serum  $\alpha$ -tocopherol (vitamin E) by high-performance liquid chromatography. *Clin. Chem.* 24: 585, 1978.
6. VANDER HEUVEL, W. J. A., AND A. G. ZACCHIEL. Gas-liquid chromatography in drug analysis. In: *Advances in Chromatography*, edited by J. C. Giddings, E. Grushka, J. Cazes, and P. R. Brown. New York: Marcel Dekker, Publisher, 1976, vol. 14, pp. 226-228.
7. BIERI, J. G., AND R. P. EVARIS. Tocopherols and polyunsaturated fatty acids in human tissues. *Am. J. Clin. Nutr.* 28: 717, 1975.
8. NOLLO, J. B., JR., AND W. N. PEARSON. Micro- and micromethods for the determination of serum vitamin A using trifluoroacetic acid. *J. Nutr.* 79: 454, 1963.
9. BIERI, J. G., AND E. L. PRIVAL. Serum vitamin E determined by thin-layer chromatography. *Proc. Soc. Exptl. Biol. Med.* 120: 554, 1965.
10. MELAKIS, D. S., W. W. C. READ, Z. L. AWDEH, AND M. F. HALIAS. Microdetermination of vitamin A and carotenoids in blood and tissue. In: *Methods of Biochemical Analyses*, edited by D. Glick. New York: Interscience Publishers, 1967, pp. 1-23.
11. BIERI, J. G., R. K. H. POLKKA AND E. L. PRIVAL. Determination of  $\alpha$ -tocopherol in erythrocytes by gas-liquid chromatography. *J. Lipid Res.* 11: 118, 1970.
12. KAYDEN, H. J., C. K. CHOW AND L. K. BJORNSSON. Spectrophotometric method for determination of tocopherol in red cells. *J. Lipid Res.* 14: 533, 1973.
13. RAHMANS, M. M., S. HOSSAIN, S. A. TALUKDAR, K. AHMAD AND J. G. BIERI. Serum vitamin E levels in the rural population of East Pakistan. *Proc. Soc. Exp. Biol. Med.* 117: 133, 1964.
14. OLSON, R. E. Effect of variations in protein and caloric intake on rate of recovery and selected physiological responses in Thai children with protein-calorie malnutrition. In: *Protein-Calorie Malnutrition*, edited by R. E. Olson. New York: Academic Press, 1975, pp. 277-295.
15. MOORE, T. *Vitamin A*. Amsterdam: Elsevier Publishing Co., 1957, p. 231.
16. JAGADEESAS, V., AND V. REDDY. Interrelationship between vitamins E and A: a clinical study. *Clin. Chim. Acta* 90: 71, 1978.
17. THOMPSON, I. N., P. ERDODY AND W. B. MAXWELL. Simultaneous fluorometric determinations of vitamins A and E in human serum and plasma. *Biochem. Med.* 8: 403, 1973.
18. SINGHAR, A. J., AND W. STAFFERY. Blood-collecting tube as a contamination source in vitamin E fluorometry. *Clin. Chem.* 24: 2073, 1978.
19. ISHIBASHI, K., K. ABE, M. OHMAI, K. KAWABE AND G. KATSUI. Determination of tocopherols in red blood cells by high-speed liquid chromatography. *Vitamins (Japan)* 51: 415, 1977.

## 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  $m\mu$ ) 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  $m\mu$  (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  $m\mu$ , 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  $m\mu$ ; (3) destruction of the vitamin A absorption at 328  $m\mu$  without affecting the absorption of other compounds at this wave-length; and (4) remeasurement of the absorption at 328  $m\mu$ .

*Reagents and Apparatus*

1. 1  $\times$  KOH in 90 per cent ethyl alcohol (1 volume of 11  $\times$  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.  $\times$  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.<sup>1</sup>

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.<sup>1</sup>

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-114 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.

<sup>1</sup> 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  $5 \times 5 \times 2$  inches to hold 100 long tubes. These may be made from wire screen (two pieces of  $\frac{1}{2}$  inch mesh and one piece of  $\frac{1}{8}$  inch mesh) or from sheet metal.

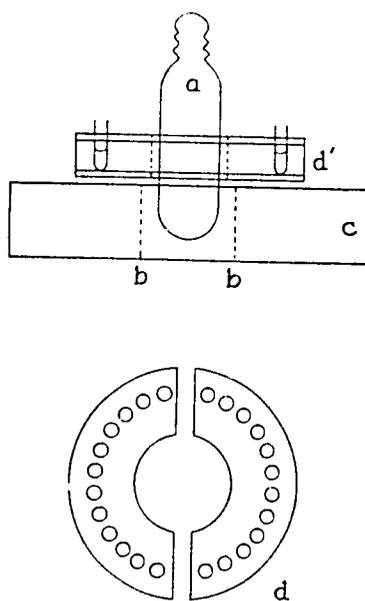


FIG. 1. Arrangement for ultraviolet irradiation. The mercury lamp (a) is held vertically in a clamp, base up, with the other end extending 3 or 4 cm. into a hole 8 cm. in diameter, *b-b*, in a large block of wood, *c*, which serves as a base. Semicircular racks (*d, d'*) are provided for holding the 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  $\frac{1}{4}$  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 (*c.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  $m\mu$ . 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  $m\mu$  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.<sup>2</sup>

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

<sup>2</sup> 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  $E_{1\text{cm.}}^{1\%}$  for vitamin A palmitate in alcohol of 1720 at 328  $m\mu$ , 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  $m\mu$ ), the net  $E_{1\text{cm.}}^{1\%} = 1720 \times 0.96 \times 0.97 \times 0.98 = 1570$ .  $1,000,000/1570 = 637$ . 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  $\beta$ -carotene (Smaco) in kerosene-xylene ( $E_{1\text{cm.}}^{1\%} = 2080$ ).

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  $m\mu$  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  $m\mu$  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  $m\mu$ , 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-111), which essentially cuts out wave-lengths longer than 400  $m\mu$ , and to place the samples in ordinary soda lime ("soft") glass tubes which cut off wave-lengths shorter than 310  $m\mu$ . If, in addition to filtering the light, the serum is saponified before extraction, the absorption at 328  $m\mu$  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  $m\mu$ . The readings at 9 and 60 minutes were then subtracted from the

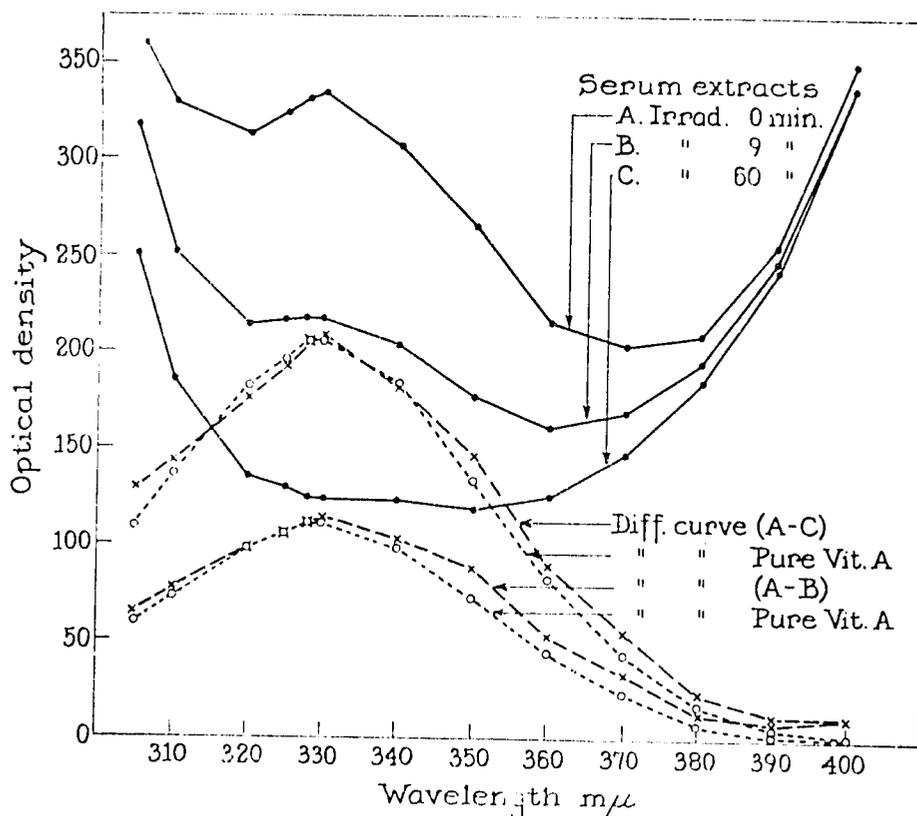


FIG. 2. Effect of irradiation on absorption of serum extracts. Absorption curves of serum extracts before and after irradiation with ultraviolet light (310 to 420  $m\mu$ ), and difference curves for serum extracts and pure vitamin A solution, obtained by subtracting the values for the absorption curves 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.<sup>3</sup> It will be noted from Line D that the combined errors due to the above causes amount to from 12 to 30 per cent.

<sup>3</sup> 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 micro-method. 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  $\alpha$ -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  $m\mu$  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  $m\mu$  with the same instrument. Corrections were made for the contribution of carotene to the color, assuming that all of the 460  $m\mu$  absorption was due to  $\beta$ -carotene. In addition, internal standards were included in triplicate by substituting 0.1 ml. of standard vitamin A solution in  $\text{CHCl}_3$  for the pure  $\text{CHCl}_3$ . 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  $\gamma$  per cent for vitamin A and 7  $\gamma$  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  $\gamma$  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  $4^{\circ}$  or  $-20^{\circ}$  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  $-20^{\circ}$  there was no significant change in vitamin A and the carotene had only fallen by about 6 per cent. At  $4^{\circ}$  the carotene

TABLE III  
*Vitamin A and Carotene Values with Different Volumes of Serum*

	Serum 1			Serum 2			Serum 3		
	35	60	100	35	60	100	35	60	100
Volume of serum, <i>c.mm.</i> . . . . .	35	60	100	35	60	100	35	60	100
Vitamin A, $\gamma$ % . . . . .	74	75	74	49	47	50	50	52	50
Carotene, $\gamma$ % . . . . .	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  $4^{\circ}$  or several months at  $-20^{\circ}$  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.

## BIBLIOGRAPHY

1. McCoord, A. B., and Luce-Clausen, E. M., *J. Nutr.*, **7**, 557 (1934).
2. Kimble, M. S., *J. Lab. and Clin. Med.*, **24**, 1055 (1939).
3. May, C. D., Blackfan, K. D., McCreary, J. F., and Allen, F. H., Jr., *Am. J. Dis. Child.*, **59**, 1167 (1940).
4. Josephs, H. W., *Bull. Johns Hopkins Hosp.*, **65**, 112 (1939).
5. Carr, F. H., and Price, E. A., *Biochem. J.*, **20**, 497 (1926).
6. Morton, R. A., *The application of absorption spectra to the study of vitamins, hormones and coenzymes*, London, 2nd edition (1942).
7. Oser, B. L., Melnick, D., and Pader, M., *Ind. and Eng. Chem., Anal. Ed.*, **15**, 717 (1943).
8. Little, R. W., *Ind. and Eng. Chem., Anal. Ed.*, **16**, 258 (1944).
9. Chevallier, A., Choron, Y., and Matheron, R., *Compt. rend. Soc. biol.*, **127**, 541 (1938).
10. Levy, M., *Compt. rend. trav. Lab. Carlsberg, Série chim.*, **21**, 101 (1936).
11. Bessey, O. A., Lowry, O. H., and Brock, M. J., *J. Biol. Chem.*, **164**, 321 (1946).
12. Lowry, O. H., and Bessey, O. A., *J. Biol. Chem.*, **163**, 633 (1946).
13. Baxter, J. G., and Robeson, C. D., *J. Am. Chem. Soc.*, **64**, 2407 (1942).
14. Norris, E. R., and Church, A. E., *J. Biol. Chem.*, **85**, 477 (1929-30).
15. Heilbron, I. M., Gillam, A. E., and Morton, R. A., *Biochem. J.*, **25**, 1352 (1931).
16. Corbet, R. E., Geisinger, H. H., and Holmes, H. N., *J. Biol. Chem.*, **100**, 657 (1933).
17. Oser, B. L., Melnick, D. D., and Pader, M., *Ind. and Eng. Chem., Anal. Ed.*, **15**, 724 (1943).
18. Yudkin, S., *Biochem. J.*, **35**, 551 (1941).
19. Lindqvist, T., *Acta med. Scand.*, suppl. 97 (1938).
20. Pett, L. B., and LePage, G. A., *J. Biol. Chem.*, **132**, 585 (1940).

# Macro- and Micromethods for the Determination of Serum Vitamin A using Trifluoroacetic Acid<sup>1</sup>

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The main problems encountered in the use of the Carr-Price (antimony trichloride) method for the determination of vitamin A have been well known since its original description (Carr and Price, '26). The antimony trichloride-chloroform reagent develops turbidity in the presence of trace amounts of moisture and the characteristic blue color formed by this reagent with vitamin A is subject to rapid fading. Despite these drawbacks, the Carr-Price reaction remains the method of choice for serum vitamin A determinations when sufficient blood can be obtained. On the micro-scale, however, the turbidity problem becomes unmanageable and for this reason Bessey et al. ('46) devised a micro-spectrophotometric method that has been widely employed. Dugan and Frigerio<sup>2</sup> have recently investigated the reactions of various Lewis acids with vitamin A and its derivatives. They found that trifluoroacetic acid (TFA) retained the sensitivity and specificity of the  $SbCl_3$  reaction but did not exhibit the turbidity and film-forming properties of the latter reagent in the presence of moisture. This property suggested to us that TFA might not only be used to replace  $SbCl_3$  in the conventional macro-determination of serum vitamin A but might also serve as the basis for a new simple micro technique. This paper reports the details of new macro- and micro- vitamin A procedures developed with the use of TFA as the chromogenic agent that offer certain advantages over methods now used.

## MATERIALS AND METHODS

### Reagents

*Ethanol, 95%*. Reagent grade was used without further purification.

*Petroleum ether*. Bottled, reagent grade benzene, boiling over a range of 38 to

56.9°C, was used without further purification for the micro- procedure. A special reagent grade petroleum ether,<sup>3</sup> boiling over a range of 30 to 40°C, was used without further purification for the macro- procedure.

*Carr-Price ( $SbCl_3$ ) reagent*. A 20% solution of  $SbCl_3$  in reagent grade chloroform was filtered repeatedly through anhydrous  $Na_2SO_4$  until clear and was then stored in a brown bottle over anhydrous  $Na_2SO_4$ .

*Trifluoroacetic acid (TFA) reagent*. One volume of trifluoroacetic acid<sup>4</sup> was mixed with 2 volumes of reagent grade chloroform just prior to use. This solution is stable for 4 hours.

*Acetic anhydride*. Reagent grade was used.

*Saponification mixture*. Ninety per cent ethanol was made 1.0 N in KOH by the addition of an appropriate weight of KOH pellets.

*$\beta$ -Carotene standard solution*.  $\beta$ -Carotene stored under nitrogen was used as a standard.<sup>5</sup> A 50-mg sample of this was dissolved in a few milliliters of chloroform and brought to a final volume of 100 ml with petroleum ether. One milliliter of this solution was then diluted to 100 ml with petroleum ether to form an intermediate standard. This solution is stable for only a few hours and should be made up just prior to use.

*Vitamin A standard solution*. The USP reference standard solution of vitamin A

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<sup>2</sup>Dugan, R. E., and N. A. Frigerio 1961 A.C.S. Abstracts, 160: 65c.

<sup>3</sup>Ligroine, Baker Chemical Company, Phillipsburg, New Jersey.

<sup>4</sup>Eastman Organic Chemicals no. 6287.

<sup>5</sup>Nutritional Biochemicals Corporation, Cleveland.

acetate in cottonseed oil<sup>6</sup> was used. This standard contains 34.4 mg of all *trans* vitamin A acetate/gm of solution. A 100-mg sample of this standard (containing 3.44 mg of vitamin A acetate) was dissolved in chloroform and diluted to 50 ml in a volumetric flask. This solution is stable for at least two days when refrigerated and kept protected from light.

#### Apparatus

A Coleman Jr. Spectrophotometer equipped with 10 × 75 mm cuvettes was used for the macro-analyses. The micro-analyses were carried out in a Beckman model DU spectrophotometer equipped with the micro-attachments described by Lowry and Bessey ('46). Microcuvettes measuring 2.5 × 10 × 25 mm were used.<sup>7</sup> For the micromethod extractions a special mixer<sup>8</sup> was used. The high-speed drill mixing device described by Bessey et al. ('46) also proved to be satisfactory. Lang-Levy constriction pipettes of 0.01-, 0.05-, 0.075-, and 0.100-ml capacities were used in the micromethod. Two-tenths-milliliter serological pipettes were drawn out to slender tips for use in pipetting petroleum ether in the micro-procedure.

#### Material for analysis

Human plasma for analysis was obtained from the Vanderbilt clinical laboratory or blood bank. It was usually necessary to pool plasma samples from the clinical laboratory to obtain enough for replicate analysis. Heparinized rat blood was obtained by heart puncture. Porcine and bovine blood was taken from individual animals at a local abattoir. In this instance, serum was used. All samples were kept frozen until analyzed.

#### Macro-procedure

This procedure is basically a modification of the methods of Kimble ('39) and Kaser and Stekol ('43) except that TFA is substituted for SbCl<sub>5</sub>.

Transfer in duplicate 2 ml of serum into 16 × 125 mm glass stoppered test tubes. Add, with mixing, 2 ml of 95% ethanol followed by 3.0 ml of petroleum ether (boiling range 30 to 40°C). Stopper and shake vigorously for two minutes to insure complete extraction of carotene and vitamin A. Centrifuge the tubes slowly

for three minutes. Carefully pipette off 2.0 ml of the petroleum ether (upper) layer and place it in a Coleman 75 × 100 mm cuvette. Stopper immediately with a cork and read the carotene at 450 mμ against a petroleum ether blank in the Coleman Jr. spectrophotometer. Remove the cuvette and evaporate the petroleum ether to dryness in a 35 to 40°C water bath. An impinging stream of nitrogen may be used to increase the rate of evaporation but this is not necessary. Take up the residue immediately in 0.1 ml of chloroform and add 0.1 ml of acetic anhydride. Set the Coleman spectrophotometer at 620 mμ to zero optical density with a blank consisting of 0.1 ml of chloroform and 1.0 ml of TFA reagent. Place the sample cuvette in the spectrophotometer, add 1.0 ml of TFA reagent and record the reading at the pause point or alternately, exactly 30 seconds after addition of the reagent. The former may be defined as the maximal "hesitation" reading that occurs after the galvanometer has recovered from its initial swing. The timed reading is to be preferred.

#### Standard curves and calculations

The β-carotene intermediate standard is diluted with petroleum ether to give solutions containing 0.5, 1.0, 1.5, and 2.0 μg of β-carotene/ml, respectively. The optical densities of these solutions are read at 450 mμ against a petroleum ether blank and a standard curve plotted. It should be mentioned that β-carotene is a notoriously labile compound even when extreme care is taken in its storage. Under our laboratory conditions, F = 6.8 in the following equation:

$$F = \frac{\mu\text{g carotene/ml}}{\text{optical density}}$$

Although it might be expected that this factor would vary slightly from laboratory to laboratory, any gross deviation should be cause to suspect the purity of the standard.

Since β-carotene also reacts with the TFA reagent to give a blue color, it is necessary to run a TFA — carotene stand-

<sup>6</sup> U.S. Pharmacopoeial Convention, 46 Park Avenue, New York 10, New York.

<sup>7</sup> Pyrocell Manufacturing Company, New York 28, New York.

<sup>8</sup> Cyclo-Mixer, Clay-Adams, Inc., New York 10, New York.

ard curve to permit calculation of a correction factor. Make up carotene standards in chloroform for this determination to contain 4.0, 8.0, and 10.0  $\mu\text{g}/\text{ml}$ . Place 0.1-ml aliquots in Coleman cuvettes, and carry out the TFA reaction as previously described. Under our laboratory conditions:

$$\text{OD}_{450} \times 0.300 = \text{OD}_{620}$$

Vitamin A standards are prepared from the stock standard solution to give solutions containing 6.8, 13.7, 20.6 and 34.4  $\mu\text{g}/\text{ml}$ , respectively. For preparation of the standard curve, pipette 0.1-ml aliquots of these standards into Coleman cuvettes for reaction with the TFA reagent. Under our laboratory conditions  $F = 4.49$  in the following equation:

$$F = \frac{\mu\text{g vitamin A/tube}}{\text{optical density}}$$

From the foregoing calculations and the volumes of reagents used, the amounts of  $\beta$ -carotene and vitamin A in the sample are calculated as

(1) carotene

$$\text{OD}_{450} \times 1020 = \mu\text{g carotene/100 ml serum}$$

(2) vitamin A

$$\text{OD}_{620} - (\text{OD}_{450} \times 0.300) \times 337 = \mu\text{g vitamin A/100 ml serum.}$$

The factors shown in the above equations should be derived by each operator, particularly in view of the fact that a new and relatively untested chromogen has been introduced. We have, however, found no differences in TFA — vitamin A color yields with 14 separate bottles of this reagent purchased at different times.

#### Micro- procedure

The micromethod is essentially the same as the macromethod except that it has been adapted to a much smaller scale. Pipette either 0.1 or 0.05 ml of serum into a 6  $\times$  50 mm test tube. Add an equal volume of 95% ethanol (Lang-Levy pipette) and 0.15 ml of petroleum ether (38 to 56.9°C). Stopper the tube immediately with a cork and extract the vitamin A and carotene by agitation with the special mixer<sup>9</sup> for 45 seconds. Centrifuge the tube for 10 minutes at 3,000  $\times g$  and transfer 0.10 ml of the petroleum ether layer to a micro-cuvette by means of a

Lang-Levy fine-tipped constriction pipette. Read the carotene optical density immediately at 450 m $\mu$  against a petroleum ether blank. Transfer as much of the sample as possible to a clean 6  $\times$  50 mm test tube and rinse the cuvette once with 0.05 ml of petroleum ether. Add the rinsing to the sample in the test tube. Evaporate to dryness in a 40 to 50°C water bath (5 minutes). Take up the residue in 0.01 ml of chloroform and add 0.10 ml of the TFA reagent with vigorous shaking. Transfer rapidly as much of the solution as possible to the micro-cuvette by means of a 0.10-ml Lang-Levy pipette. Take the vitamin A reading at 620 m $\mu$  against a TFA reagent blank exactly 30 seconds after the addition of the TFA reagent. It is essential that this step be carried out with great care, particularly with respect to the timing because the color fades.

Carotene and vitamin A standard curves are prepared using smaller aliquots of the same standards used in the macro- procedure.

#### RESULTS

Trifluoroacetic acid — chloroform mixtures were tested for vitamin A color yields in the proportions of 1:1, 1:2, 1:5, 1:10 and 1:20. The 1:2 and 1:5 mixtures gave about the same color yields but the other mixtures gave considerably less. The 1:2 mixture was used throughout this study.

The blue species produced by the interaction of TFA, and vitamin A in chloroform was found to exhibit maximal absorption of 616 m $\mu$  in accordance with the observations of Dugan.<sup>10</sup> After standing for about two hours the original blue color decayed to a pink with a maximal absorption at about 540 m $\mu$  but of greatly reduced sensitivity. No attempts were made to use this color analytically. Various fat-soluble substances (cholesterol, calciferol, vitamin E, vitamin K) gave no color with TFA when tested at concentrations greater than those normally found in sera. An impure sample of xanthophyl gave virtually no color at concentrations deemed to lie within the physiological range.

<sup>9</sup> See footnote 8.

<sup>10</sup> Dugan, R. E., 1962, personal communication.

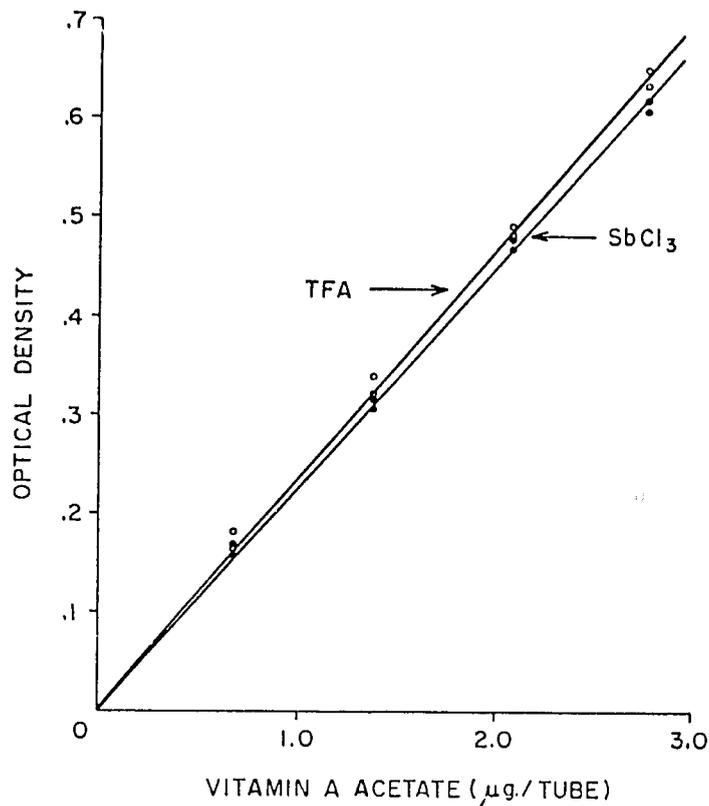


Fig. 1 Comparison of vitamin A standard curves obtained with antimony trichloride and trifluoroacetic acid. Readings were made in  $75 \times 100$ -mm cuvettes with a Coleman Jr. spectrophotometer at  $620 \text{ m}\mu$ .

Typical vitamin A acetate dose-response curves measured at  $620 \text{ m}\mu$  in the Coleman Jr. spectrophotometer with TFA or  $\text{SbCl}_3$  are shown in figure 1. The color yield with TFA was also slightly greater than that with  $\text{SbCl}_3$  with vitamin A alcohol and vitamin A acid. The yield of colored complex formed with vitamin A acid was less than half that with vitamin A acetate. This vitamin A acid—TFA complex formed slowly and was a deep purple rather than a typical Carr-Price blue.  $\beta$ -Carotene gave less than one-tenth the color yield of vitamin A at  $620 \text{ m}\mu$  with both reagents. The carotene color yield with TFA was slightly less than that with  $\text{SbCl}_3$ .

The stability of the colored species formed with TFA varied depending upon the type of cuvette used. Representative time-decay curves with vitamin A acetate and serum extracts are shown in figure 2.

In a micro-cuvette the color formed with a vitamin A acetate standard faded about 12% by 1 to 1.5 minutes after the initial reading, when as the same reaction in a Coleman cuvette faded by approximately 20%. Under the same conditions the colored species formed with a serum extract faded 10% in a micro-cuvette as opposed to 25% in a Coleman cuvette.

The TFA method was compared with the classic  $\text{SbCl}_3$  technique using human, rat, bovine and porcine sera (table 1). The two methods agreed quite well when human, rat, or porcine sera were analyzed. Generally TFA values were slightly higher. When bovine sera containing large amounts of carotene were analyzed, however, the vitamin A values obtained with TFA were markedly higher than those obtained with  $\text{SbCl}_3$ . When the carotene values were low (bovine samples 8 and 9), good agreement was obtained. This

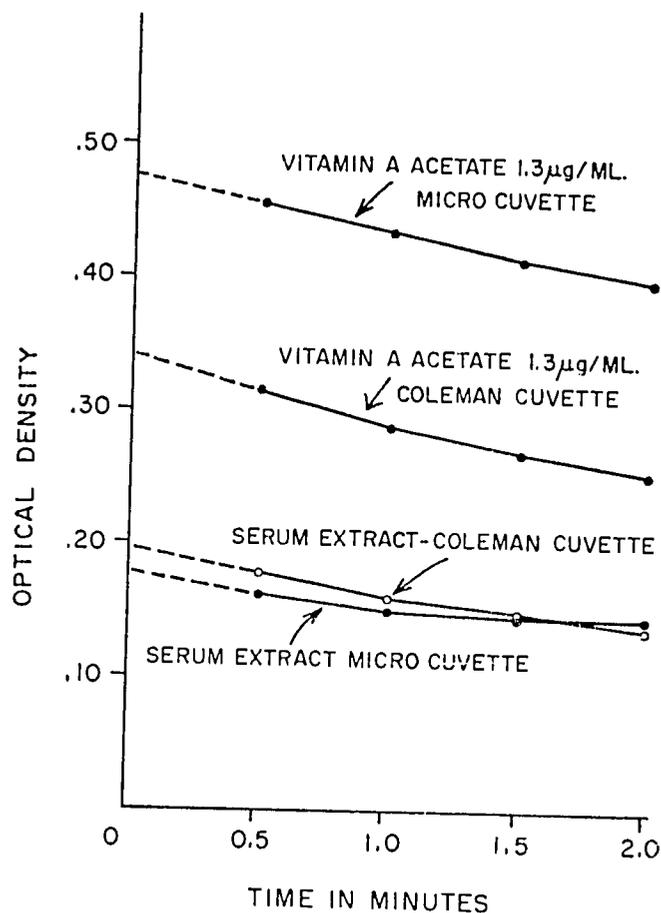


Fig. 2 Trifluoroacetic acid — vitamin A fading curves. Initial readings were taken exactly 30 seconds after addition of the TFA and then at the time intervals and in the instrument specified.

implies that the two chromogens react differently with the carotenoids or the other pigments normally present in bovine sera, or with both. Since serum carotene levels of 300  $\mu\text{g}/100\text{ ml}$  are but rarely encountered in the human, this does not militate against the routine use of TFA for the analysis of human sera.

Reproducibility studies were carried out with  $\text{SbCl}_3$  and TFA by analyzing the same serum sample repeatedly both on the same day and on different days (table 2). In both situations TFA performed slightly better than did  $\text{SbCl}_3$ . This may be a function of the  $\text{SbCl}_3$  moisture problem since amounts of turbidity, sufficient to affect the optical density readings, often escape the analyst's eye and might therefore result in greater variability.

Table 3 presents a comparison of vitamin A values determined by the usual procedure or after a preliminary saponification of the serum. The vitamin A values obtained after saponification were slightly higher than those obtained without saponification, but carotene levels after saponification were distinctly lower in several samples. Our results suggest, therefore, that there is some advantage to be gained by saponification, which is in accordance with the report of Bessey and Lowry ('46).

Recovery experiments were performed on a series of human sera using both the  $\text{SbCl}_3$  and TFA methods. These were determined in triplicate by comparing the increase in Carr-Price color resulting from the addition of vitamin A acetate to sam-

TABLE 1  
A comparison of  $SbCl_3$  and trifluoroacetic acid (TFA) plasma vitamin A values in various species.

Sample	Vitamin A		TFA
	Carotene	$SbCl_3$	
	$\mu g./100 ml$	$\mu g./100 ml$	
Bovine <sup>1</sup>			
1	848.6	40.9	55.2
2	823.4	38.9	56.1
3	476.3	21.6	35.2
4	744.5	36.9	49.9
5	722.4	27.3	41.1
6	772.9	31.9	36.8
7	1116.8	118.5	129.1
8	93.3	19.9	21.5
9	47.9	20.6	20.9
Mean	627.3	39.6	49.5
SD <sup>2</sup>	356.1	30.6	32.5
Rat			
1	5.6	30.3	31.5
2	0.0	48.6	46.4
3	1.8	25.9	24.9
4	12.5	38.6	43.0
Mean	4.9	35.8	36.4
SD	5.5	9.9	9.9
Human			
1	115.0	38.4	39.2
2	65.5	25.8	32.2
3	106.3	26.6	28.0
4	136.4	42.0	47.5
5	71.6	39.7	39.3
6	63.5	37.2	39.4
7	116.5	49.7	52.3
8	180.6	35.1	36.3
9	143.6	36.5	40.3
10	74.8	39.2	47.8
Mean	107.8	37.0	40.2
SD	39.5	7.0	7.4
Porcine <sup>1</sup>			
1	4.4	26.6	30.2
2	2.5	29.3	31.2
3	6.9	39.6	38.6
4	1.8	19.9	21.8
5	0.0	25.9	26.8
6	0.0	24.6	27.4
7	0.0	29.6	34.6
8	3.7	21.9	23.0
9	1.2	13.3	16.2
Mean	2.3	25.6	27.7
SD	2.3	7.3	6.8

<sup>1</sup> Serum.

<sup>2</sup> Standard deviation.

ples of known vitamin A content. The data in table 4 show that both methods gave good recoveries of the added vitamin A acetate.

In table 5 representative results obtained with the TFA macro- and micro-

methods are compared. The agreement of values was relatively good except in sample 24 which contained an unusually large amount of carotene.

Essentially the same values were obtained when 0.05 and 0.10 ml of serum were analyzed by the micromethod (table 6). In these analyses each serum was analyzed in triplicate at the two volume levels. The amount of alcohol added was equal to that of the serum but the same amount of petroleum ether (0.15 ml) was used for extraction.

#### DISCUSSION

The color reaction of TFA with vitamin A appears to be suitable for the measurement of vitamin A in serum since the values obtained in human, rat, and porcine sera are similar to those found with the Carr-Price reaction. The direct measurement of vitamin A with TFA in bovine sera without the prior removal of carotene requires further study before its validity can be assessed.

The use of TFA eliminates entirely the moisture problem encountered with  $SbCl_3$ . The TFA is also less toxic than  $SbCl_3$  and unlike the latter does not form a tenacious film on the cuvette.

The main drawbacks of the TFA method are the evanescent nature of the blue color and the extreme volatility of the solvents used. The former has been satisfactorily controlled by reading exactly 30 seconds after the addition of TFA to the serum extract. The low boiling range petroleum ether used in the macromethod was entirely too volatile for the micromethod but the higher boiling petroleum ether product (40 to 60°C) proved to be acceptable if the various manipulations were performed rapidly. Ambient temperature in our laboratory averaged 21 to 23°C during these studies. Hexane was also found to be a suitable extractant and possessed the advantage of being considerably less volatile than petroleum ether. It would undoubtedly be a more desirable solvent for use at ambient temperatures higher than ours. Evaporation of a hexane extract in a 40°C water bath took an unduly long period of time, but an impinging stream of nitrogen from a hypodermic

TABLE 2  
 Reproducibility of  $SbCl_3$  and trifluoroacetic acid (TFA) values for human serum vitamin A<sup>1</sup>

Experiment A			Experiment B		
Sample	$SbCl_3$	TFA	Sample	$SbCl_3$	TFA
	$\mu g/100\ ml$			$\mu g/100\ ml$	
1	47.6	51.9	11	48.6	45.2
2	48.6	51.4	12	48.9	53.9
3	50.9	48.3	13	49.6	51.1
4	48.9	49.6	14	45.2	51.7
5	51.2	49.2	15	46.6	49.6
6	50.2	50.2	16	50.2	52.1
7	50.6	48.9	17	41.6	50.5
8	48.2	49.2	18	43.9	52.1
9	44.9	48.6	19	40.9	53.6
10	51.2	48.6	20	42.6	51.7
Mean	49.2	49.8	Mean	45.8	51.1
SD <sup>2</sup>	2.0	1.9	SD	3.4	2.4
C <sup>3</sup>	4.0%	3.8%	C	7.4%	4.6%

<sup>1</sup> In experiment A, 10 aliquots of a pooled serum sample were analyzed simultaneously. In experiment B, an aliquot of the same pooled sample was analyzed daily for 10 consecutive days.

<sup>2</sup> Standard deviation.

<sup>3</sup> Coefficient of variation.

TABLE 3  
 Effect of saponification upon vitamin A values of human serum<sup>1</sup>

Sample	Saponified			Unsaponified		
	Carotene	Vitamin A		Carotene	Vitamin A	
		$SbCl_3$	TFA		$SbCl_3$	TFA
	$\mu g/100\ ml$	$\mu g/100\ ml$		$\mu g/100\ ml$	$\mu g/100\ ml$	
11	76.7	53.9	51.5	89.9	56.1	50.2
12	73.5	39.6	37.4	63.5	37.2	39.4
13	154.7		37.3	180.6		36.5
14	125.3		49.3	148.6		40.3
15	75.6		50.2	74.8		47.8

<sup>1</sup> Saponification was carried out at 60° for 20 minutes with 1 N alcoholic KOH.

TABLE 4  
 Vitamin A recovery from serum samples

Sample	Vitamin A content of serum	Vitamin A acetate added	Expected vitamin A	Actual vitamin A	Recovery
	$\mu g/100\ ml$	$\mu g/100\ ml$	$\mu g/100\ ml$	$\mu g/100\ ml$	%
			$SbCl_3$		
16	49.5	51.6	101.1	93.7	92.6
17	25.8	51.6	77.4	72.7	93.9
18	49.4	51.6	101.0	98.7	97.7
19	26.6	41.2	67.8	72.0	106.1
20	42.0	41.2	83.2	84.3	101.3
			Trifluoroacetic acid (TFA)		
16	50.4	51.6	102.0	99.6	97.6
17	32.2	51.6	83.8	82.1	97.9
18	55.5	51.6	107.1	99.9	93.2
19	28.0	48.6	76.6	73.1	95.4
20	47.5	48.6	96.1	92.7	96.4

TABLE 5  
Comparison of macro- and micromethods for vitamin A analysis using trifluoroacetic acid (TFA)

Sample	Macromethod		Micromethod	
	Carotene	Vitamin A	Carotene	Vitamin A
	$\mu\text{g}/100\text{ ml}$	$\mu\text{g}/100\text{ ml}$	$\mu\text{g}/100\text{ ml}$	$\mu\text{g}/100\text{ ml}$
21	109.3	44.0	112.3	42.4
22	138.7	24.4	140.8	28.3
23	116.2	40.7	113.0	41.1
24	283.0	28.2	264.4	36.9
25	202.4	35.7	191.7	41.3

TABLE 6  
Comparison of vitamin A levels obtained with different amounts of serum

Sample	0.050 ml serum		0.100 ml serum	
	Carotene	Vitamin A	Carotene	Vitamin A
	$\mu\text{g}/100\text{ ml}$	$\mu\text{g}/100\text{ ml}$	$\mu\text{g}/100\text{ ml}$	$\mu\text{g}/100\text{ ml}$
26	79.9	39.1	107.8	41.1
27	122.3	50.2	129.0	46.1
28	107.2	43.7	118.1	42.1
29	145.4	38.2	171.1	32.1

needle took it to dryness in about one minute. Heptane, benzene, and xylene were suitable extractants, but their higher boiling points made them not useful. Although much has been made of the possibility of oxidative loss of vitamin A during the evaporation step, we have been unable to demonstrate such a loss at 40°C even by the use of an impinging stream of air.

In our laboratory, saponification resulted in a slightly higher vitamin A value although not of the magnitude reported by Bessey et al. ('46) and Sobel and Snow ('47). It would therefore appear to be appropriate to carry out a saponification prior to extraction. Oser et al. ('43) recommend the use of an internal vitamin A standard to reduce the error introduced by the effect of inhibitory substances on color development. Bessey et al. ('46) found that vitamin A values were from 5 to 10% higher when calculated by this procedure than those taken from a conventional standard curve. Accordingly the use of an internal standard would increase somewhat the accuracy of the method, but in practice this approach would depend largely upon the availability of serum because duplicate samples are required. In nutrition surveys the usual quantity of blood available from finger-tip puncture

normally would not be sufficient for this purpose.

Attempts to make the method more rapid by the direct addition of undiluted TFA to serum extracts were not successful. Although the TFA--vitamin A color formed readily with vitamin A standards in both hexane and benzene, some serum extracts gave no color with TFA and, for unknown reasons, TFA was insoluble in hexane extracts of sera. Whereas  $\text{CHCl}_3$ ,  $\text{CCl}_4$ , and  $\text{C}_2\text{H}_2\text{Cl}_2$  gave full color yields with vitamin A and TFA, they failed to extract quantitatively vitamin A and carotene from the serum.

The TFA method offers its greatest advantage over existing methods at the micro-level. The only micromethod in common use is that of Bessey et al. ('46), which depends upon the measurement of the ultraviolet absorption of serum extracts at 328 m $\mu$  before and after irradiation with ultraviolet light. The main source of difficulty with this technique is that ultraviolet light not only destroys vitamin A but changes other substances that absorb at 328 m $\mu$  as well. This may introduce a serious error, especially when low vitamin A levels are being measured (Caster and Mickelsen, '55). The observations of Bieri and Schultze ('51) and

Sobel and Snow ('47) that hemolysis results in consistently high values for vitamin A and carotene when measured by this method were confirmed by Utley et al. ('58). These investigators reported that a rigid purification of the kerosene-xylene extraction mixture was necessary to insure reliable results and concluded that the Carr-Price method was the method of choice when sufficient serum was available. In addition to these shortcomings, the Bessey method is tedious and time consuming and requires an ultraviolet spectrophotometer and a special irradiation device. The TFA micromethod described here is about three times more sensitive than the spectrophotometric procedure, does not require elaborate purification or drying of solvents, is not affected by hemolysis and does not require the use of an ultraviolet spectrophotometer. Although an ultraviolet spectrophotometer was used in this study, a few trials with the Beckman Spinco Model 151 micro-spectrophotometer<sup>11</sup> suggest that this instrument could also be used. The TFA method is considerably more rapid than the Bessey procedure. One operator can easily analyze 20 samples in duplicate in three hours. It is, moreover, even more rapid than the macro-procedure because the solvent evaporation time is reduced from  $\pm 30$  minutes (macro-) to  $\pm 5$  minutes (micro-).

#### SUMMARY

A new method for the determination of plasma or serum vitamin A levels which uses trifluoroacetic acid (TFA) as the chromogen is described. This reagent produces a typical Carr-Price color but does not exhibit the turbidity or film-forming properties of  $SbCl_5$  in the presence of moisture. The new procedure gives values that agree well with those obtained by the Carr-Price method when rat, porcine, or human sera are analyzed. Significantly

higher values are obtained with TFA, however, when bovine sera were analyzed. A micro-modification of the method permitting the analysis of 50  $\mu$ l of serum is described and its advantages over existing methods are discussed.

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#### LITERATURE CITED

- Bessey, O. A., O. H. Lowry, M. J. Brock and J. A. Lopez 1946 The determination of vitamin A and carotene in small quantities of blood serum. *J. Biol. Chem.*, 166: 177.
- Bieri, J. G., and M. O. Schultze 1951 Micro-spectrophotometric determination of vitamin A and carotene in blood. *Arch. Biochem. Biophys.*, 34: 273.
- Carr, T. H., and E. A. Price 1926 Color reactions attributed to vitamin A. *Biochem. J.*, 20: 497.
- Caster, W. O., and O. Mickelson 1955 Serum vitamin A level: A critique of methods and significance. *Am. J. Clin. Nutrition*, 3: 409.
- Kaser, M., and J. A. Stekol 1943 A critical study of the Carr-Price reaction for the determination of  $\beta$ -carotene and vitamin A in biological materials. *J. Lab. Clin. Med.*, 28: 904.
- Kimble, M. S. 1939 The photocolormeter determination of vitamin A and carotene in human plasma. *Ibid.*, 24: 1055.
- Lowry, O. H., and O. A. Bessey 1946 The adaptation of the Beckman Spectrophotometer to measurements on minute quantities of biological materials. *J. Biol. Chem.*, 163: 633.
- Oser, B., D. Melnick and M. Pader 1943 Estimation of vitamin A in food products. *Ind. Eng. Chem. (Anal. ed.)*, 15: 724.
- Sobel, A. E., and S. D. Snow 1947 The estimation of serum vitamin A with activated glycerol dichlorohydrin. *J. Biol. Chem.*, 171: 617.
- Utley, M. H., E. R. Brodovsky and W. N. Pearson 1958 Hemolysis and reagent purity as factors causing erratic results in the estimation of vitamin A and carotene in serum by the Bessey-Lowry method. *J. Nutrition*, 66: 205.

<sup>11</sup> Beckman Instruments, Inc., Palo Alto, California.

VITAMIN A DETERMINATIONS IN HUMAN BLOOD: AN ANALYSIS OF METHODOLOGY<sup>1</sup>

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## SUMMARY

Colorimetric and fluorometric measurement approaches are evaluated for the determination of Vitamin A in blood plasma with and without fractionation by alumina column chromatography. Results for forty-one human plasma samples indicate that the two measurement approaches applied without the alumina separation step can be adequate for broad population surveys when properly controlled, but that the separation step is necessary to avoid interferences when reliable results are required for individual samples, especially for Vitamin A levels below  $0.2 \mu\text{g ml}^{-1}$ . Both measurement approaches give highly reliable results when preceded by alumina column chromatography.

## INTRODUCTION

The quantitative determination of vitamin A in either human plasma or serum is important for the evaluation of vitamin A status of individuals and populations. Although it is usually assumed that all of the major methods available [1-8] are reasonably reliable when used with care, there are few studies that systematically compare the different methodologies to determine which gives the most acceptable results, particularly in the near-deficiency range for humans ( $< 0.20 \mu\text{g ml}^{-1}$  calculated in retinol equivalent).

This paper presents the results of a critical evaluation of two commonly used approaches, namely, a colorimetric method described by Carr and Price [4] as modified by Neeld and Pearson [1] and a fluorometric method described by Thompson et al. [2,3]. The colorimetric method is based on the development of a blue color when Vitamin A is treated with trifluoroacetic acid (TFA) and the fluorescence method is based on fluorescence emission of Vitamin A with excitation and emission wavelengths at 330 and 480 nm respectively. Both methods were evaluated with and without alumina column chromatography [3] of plasma extracts as an independent basis for evaluating and comparing the accuracies of the colorimetric and fluorescence measurement methods.

## EXPERIMENTAL

### Reagents

Except as noted, all reagents were "analytical grade" and were used as purchased. Trifluoroacetic acid was obtained from two suppliers in five separate lots with no observable variations in blue color development or stability. Hexane (or its petroleum ether substitute) usually required special treatment [9] to reduce background fluorescence contamination before being used in the fluorometric procedure. Ethyl alcohol (either 95 or 100%) for plasma extractions and chloroform for the colorimetric procedure were distilled and stored [9]. Ethyl ether was treated with aqueous ferrous sulfate and distilled to remove peroxides

and antioxidant stabilizers, respectively [9]. The purified ether was stored in the dark over steel wool which had previously been washed with solvent until free of fluorescent contaminants.

Activated neutral alumina ( $Al_2O_3$ ) in fine granular form and designated "suitable for chromatography" was used for column fractionations after being checked for contamination by the colorimetric and fluorometric procedures and partially deactivated with water as described in the section on column chromatography.

#### Equipment

Time-dependent absorbance readings were determined with a recording Beckman Acta III spectrophotometer. Carotene values were determined from absorbance readings at 450 nm with a Beckman Spinco colorimeter using matched test-tube style cuvettes covered with aluminum foil to prevent evaporation of the volatile hexane and chloroform. The colorimeter was periodically checked by comparison with the spectrophotometer.

Fluorometric measurements were performed with a Hitachi Perkin Elmer MPF 2A spectrofluorometer with 10 mm x 10 mm square cuvettes used for column eluates and microcuvettes with a minimum usable volume of 0.35 ml used for unfractionated extracts. Care was taken throughout to prevent contact of samples, extracts, reagents, or column eluates with rubber, lubricants, and certain types of plastic to avoid fluorescent contamination. Concentrated samples of vitamin A standards required special

handling to avoid contamination of apparatus and glassware with the resulting possibility of spurious errors in later determinations.

### Samples

Human blood samples were drawn by syringe with heparin used as anticoagulant. Rat blood was obtained from stock white rats by cardiac puncture or decapitation with sodium citrate used as anticoagulant. All blood was centrifuged 5-10 min and the plasma transferred to glass screw-top vials with Teflon seals for freezing and storage in the dark. For the analysis of methodological variables, plasma was pooled and then subdivided into smaller identical fractions for multiple determinations on each sample. No changes in results were observed as a result of sample storage.

### Standards

Vitamin A standard was freshly prepared each day by partial hydrolysis of a retinyl acetate (ROAc) solution in oil obtained from USP Vitamin A Reference Standard capsules (USP, Inc., Rockville, MD). To about 0.15 ml of oil containing about 5 mg of ROAc in a conical glass stoppered centrifuge tube, 1 ml of water and 5 pellets (about 0.5 g) of potassium hydroxide were added. Contents of the tube were vortexed and placed in a boiling water bath, with occasional swirling, for 20 min. The hydrolyzed mixture was cooled, 1 ml of water and 5 ml of hexane added and the tube again vortexed for 2 min and centrifuged 5-10 min. The hexane (upper) layer, containing a mixture of ROH, ROAc, and a yellow residue from the oil, was used both as a source of ROH or ROAc

and as the concentrated mixture needed to determine the correct amount of water to be used for deactivation of alumina.

To produce a vitamin A standard, a solution of purified ROH (or ROAc) was obtained by applying about 0.5 ml of the hexane extract directly to a column containing 1 g of deactivated alumina. Chromatography was performed by eluting the column sequentially with 10 ml each of hexane containing 0, 2, 10, and 50% (v/v) of ethyl ether. Purified ROAc appears in the 2% ethyl ether fraction and ROH in the 50% ethyl ether fraction. The desired fraction was evaporated nearly to dryness at 40°C under a stream of N<sub>2</sub> in dim light, then rediluted with hexane to give a solution having an absorbance reading between 0.20 and 0.30 at 325 nm (A<sub>325</sub>) with a 1 cm light path, (ROH equivalent to about 1.0-1.5 μg ml<sup>-1</sup>). The absorbance reading and an absorptivity of 5.464 ml μg<sup>-1</sup> cm<sup>-1</sup> were used to compute the vitamin A concentration. For fluorometric standardization, three 1.0 ml portions of this standard solution were diluted to 25 ml with pure hexane and with 2 and 50% ethyl ether in hexane.

Pure crystalline all-trans-β-carotene was carefully weighed from a previously unopened ampule, dissolved in hexane, and diluted to about 5 μg ml<sup>-1</sup>. Concentrations were calculated either from dilution factors or from absorbance measurements at 450 nm (A<sub>450</sub>) measured with a Beckman spectrophotometer and an absorptivity in hexane of 3.971 ml μg<sup>-1</sup> cm<sup>-1</sup>.

### Colorimetric procedures

The procedure used in this study was a modified version of that of Neeld and Pearson [1]. Duplicate plasma samples (usually 0.5 ml) were extracted in glass-stoppered centrifuge tubes by addition of ethyl alcohol (either 95 or 100%) and hexane in the proportions 1:1:2 (v/v/v), respectively, followed by vigorous vortexing for 2 min and centrifugation for 5-10 min.

Carotene in the hexane extracts was estimated from the absorbance at 450 nm ( $A_{450}$ ). Hexane extracts and standards were then evaporated just to dryness at 40°C under a gentle stream of  $N_2$  in dim light. A 0.1 ml volume of a mixture of chloroform and acetic anhydride (1:1, v/v) was added immediately to dissolve and dehydrate the residue and the tubes were recapped immediately and taken to a darkened spectrophotometer room for subsequent processing. A 0.5 ml volume of a 1:1 (v/v) mixture of TFA and chloroform (prepared within 1 h of use) was added to 0.1 ml sample with a Pasteur pipet. The reaction mixture was immediately redrawn into the Pasteur pipet and transferred rapidly to a prepositioned microcuvette, in which  $A_{620}$  values were recorded either continuously or at 15 s ( $t_{15}$ ) and 30 s ( $t_{30}$ ) after the start of the reaction. Blank values were obtained for samples, standards, and reagents.

### Fluorometric procedure

Fluorescence values from plasma extracts when measured at wavelengths suitable for vitamin A determinations must be corrected for the non-specific contribution of other blood components [2,3,6,7,11]. In the procedure of Thompson et al. [2], a correction is made assuming that the carotenoid phytofluene is the only significant fluorometrically active contaminant and phytofluene extracted from tomatoes is used as a standard for determining the correction factor. In this study the pure hexane fractions obtained from alumina column chromatography of human plasma extracts were measured directly as an approximate phytofluene standard. No differences were observed in the fluorometric characteristics of standards prepared from multiple human or rat plasma samples in preliminary studies at the wavelengths used for the fluorometric procedure. The human plasma samples, however, contained much larger quantities of the fluorescent contaminant.

In all fluorescence measurements the emission wavelength used was 480 nm, the excitation wavelength 330 nm or 365 nm, and the emission and excitation bandwidths 8 nm each. A freshly prepared standard solution of ROH in hexane was used to adjust full-scale to be equivalent to a blank-corrected ROH concentration of 100 ng ml<sup>-1</sup> (in hexane) at 330 nm. The fluorescence reading for the standard ROH (designated  $F_{330}$ ) was recorded, the excitation wavelength readjusted to 365 nm without changing any other controls and the  $F_{365}$  value recorded. The corresponding  $F_{365}$  value for a hexane blank was obtained. After verifying the origi-

nal  $F_{330}$  scale expansion with the ROH standard, the  $F_{330}$  and  $F_{365}$  values for the phytofluene solutions obtained by column fractionation of human plasma extracts, were similarly obtained. Blank values were determined by using the pure hexane fraction from alumina columns eluted without sample. Because the ROH standard is unstable, a secondary standard of quinine sulfate (formula weight 782.96) of  $10 \text{ ng ml}^{-1}$  in  $0.1 \text{ N H}_2\text{SO}_4$  was standardized against standard ROH solutions and routinely used to calibrate the fluorometer at both 330 nm and 365 nm [2]. The observed ratio of  $F_{365}/F_{330}$  values for the quinine sulfate under these conditions was 1.23.

Appropriate equations relate the  $F_{365}$  and  $F_{330}$  values of samples, corrected for blanks, with those obtained for the ROH and phytofluene standards [2]. As determined in our laboratory, the  $F_{365}/F_{330}$  ratios for ROH and phytofluene, (designated A and P, respectively) were  $A = 0.33$  and  $P = 1.97$ , with the corresponding constants  $P/(P-A)$  and  $1/(P-A)$  equal to 1.20 and 0.61, respectively. Considering dilution factors in the extraction procedure, vitamin A concentration can be calculated for a plasma sample as:

Vitamin A ( $\mu\text{g ml}^{-1}$ ) =  $(0.0120 \times F_{330} - 0.0061 \times F_{365})$  where the  $F_{330}$  and  $F_{365}$  values are from the unfractionated plasma extract after correction for sample blanks.

For vitamin A quantitation by the fluorescence procedure, unfractionated plasma extracts and blanks were obtained in duplicate as in the previous procedure except that the proportions of plasma (either 0.05 or 0.5 ml), ethyl alcohol, and hexane were 1:1:10 (v/v/v), respectively. The larger proportion of hexane was necessary due to the lower detection limit of the fluorescence procedure as compared with the colorimetric procedure. Additional dilution of the extracts of some samples with very high vitamin A or phytofluene levels was required. Extractions were complete under these conditions as shown in preliminary studies by second extractions.

#### Alumina column chromatography procedure

The procedure was essentially that described by Thompson et al. [3]. Alumina columns with a bed diameter of about 5 mm and a height of about 4.3 cm were prepared by adding 1 g of partially deactivated alumina to columns partially filled with hexane to prevent formation of air bubbles.

The amount of water necessary for appropriate deactivation of alumina from different batches varied from 0.04-0.06 ml  $\text{g}^{-1}$ . To characterize each new batch, a precise quantity (usually 20 g) was weighed into each of six tightly stoppered tubes and increments (0.04-0.065 ml  $\text{g}^{-1}$ ) of water were added to give a graded series. The tubes were sha-

ken, equilibrated for at least 24 h, again shaken, and finally made into columns as described above. About 0.5 ml of the ROH-ROAc mixture in hexane from hydrolysis of the ROAc standard capsule was applied to each column and the columns were fractionated with hexane containing 0, 2, 10, and 50% (v/v) ethyl ether as described earlier. Each fraction from a column was viewed under ultraviolet light. The partially deactivated column that produced the milky-green fluorescence of vitamin A exclusively in the 2 and 50% ethyl ether fractions contained the proper amount of water.

Excess hexane was drained from columns before sample extracts were applied but at no time was the under surface permitted to go dry. Duplicate hexane extracts of samples were obtained as described for the colorimetric procedure except that 0.5 ml of ethyl alcohol and 1.5 ml of hexane were used. Extractions were complete under these conditions. One milliliter of the extract was evaporated just to dryness ( $N_2$ ,  $40^\circ C$ ), the residue immediately redissolved in about 1 ml of hexane and applied to the column with a small following rinse. Four hexane fractions of 10 ml each, containing 0, 2, 10 and 50% ethyl ether respectively, were collected in 10-ml volumetric flasks. Evaporation losses were replaced to volume with the appropriate solvent. The 0 and 10% ethyl ether fractions were regularly examined for vitamin A as a control of column operation.

Interfering contaminants either to the colorimetric or fluorescence measurements were found exclusively in the pure hexane fraction of plasma extracts. However, these hexane fractions did not account in either procedure for all of the contamination observed in unfractionated extracts, some apparently being retained permanently on the alumina.

Vitamin A in fractions obtained from column chromatography was most conveniently quantitated by fluorometry, a method which allows column fractions to be measured directly without further preparation. For these measurements the fluorometer conditions were as described earlier. A freshly prepared solution of vitamin A of known concentration (between 40-60 ng ml<sup>-1</sup>) in 2 and 50% ethyl ether was used to standardize the fluorometer for the corresponding column fractions. Blank values were obtained for each column fraction. The fluorescence yield of ROH was 1.09 and 1.28 times greater in a 2 and 50% ethyl ether, respectively, compared to ROH in pure hexane. No correction for fluorescent contaminants was required because spectral characteristics of 2 and 50% column fractions were indistinguishable from pure ROH in the respective solvent at wavelengths used in the fluorescence procedure.

The colorimetric procedure described earlier is an alternative to the fluorometric quantitation of fractionated plasma extracts. No correction for  $\beta$ -carotene was required because  $A_{450}$  values for concentrated solutions in hexane were zero, and time-dependent losses of  $A_{620}$  values were identical to pure vitamin A of the same concentration. However, though vitamin A values were identical by either the fluorometric or

colorimetric procedures, evaporation of the two 10-ml fractions required for the latter was time-consuming and only the fluorometric procedure was used for the data reported in this paper.

## RESULTS AND DISCUSSION

Preliminary results of an early experiment designed to select the most suitable TFA concentration for use in the colorimetric procedure are shown in Table 1 for a pooled sample of human plasma and for a standard. It is clear from the data that results for the pooled plasma and standard have different dependencies on TFA concentration, and experiments were designed to determine the origin of these differences.

It was observed in all of these studies that  $A_{620}$  values decreased linearly with time from the earliest measurement observed until at least 30 s after the reaction was initiated. Thus, initial  $A_{620}$  values were readily estimated for each reaction by extrapolation of  $A$  vs  $t$  data to  $t = 0$ .

Figure 1 shows that the greatest initial values for  $A_{620}$  using a retinyl acetate standard were developed in reaction mixtures containing the lowest concentration of TFA. However,  $A_{620}$  values developed by vitamin A become increasingly more stable at higher TFA concentrations. With  $\beta$ -carotene,  $A_{620}$  values increase both with increasing TFA concentration and with increasing time of color development. Sets of studies identical to those represented in Fig. 1 were performed with three additional (lower) concentrations of vitamin A and  $\beta$ -carotene. From these

studies it was seen, for any fixed interval of color development and any TFA concentration selected, that  $A_{620}$  values were directly proportional to the concentration of vitamin A or  $\beta$ -carotene. Thus, standard curves of  $A_{620}$  values for both standards, would always be linear for any combination of TFA concentration and measurement interval selected, leading to the impression (shown below to be false) that almost any set of conditions selected should function satisfactorily when applied to plasma extracts.

A similar set of studies was made using unfractionated plasma extracts. Results were completely reproducible for any one sample but highly divergent among different samples. All plasma samples, however, exhibited certain characteristics in common. Changes in  $A_{620}$  values were linear with time at least to  $(t_{30})$  for all but the lowest TFA concentration used, thereby allowing ready extrapolation of the  $A_{620}$  values to  $(t_0)$ . The  $A_{620}$  values were directly proportional to the quantity of vitamin A in the sample regardless of the time of blue color development or TFA concentration used, as long as both the time interval and reagent concentration were constant. Nevertheless, wide variations in stability and even direction of change of  $A_{620}$  values with time were found for different plasma extracts. The most common observation was that  $A_{620}$  values faded faster than that of standard vitamin A although  $A_{620}$  values of some samples increased with time in a manner similar to those of the  $\beta$ -carotene standard. After subtracting the changing  $\beta$ -carotene correction point by point over the life of the reaction, the residual  $A_{620}$

values, usually fell far more rapidly, and never less rapidly, than values of pure vitamin A standards at the same TFA concentrations. The outcome in almost all cases was a large and unanticipated bias toward low quantitative estimations, with the bias being reduced proportionately as the time of development of  $A_{620}$  values was shortened toward ( $t_0$ ).

When vitamin A values for unfractionated human plasma extracts determined using ( $t_0$ ) values in the colorimetric procedure were compared with values obtained for the same extracts after alumina column fractionation, a marked improvement was noted over values determined using ( $t_{15}$ ) or ( $t_{30}$ ) for all TFA concentrations. The values determined colorimetrically now clustered about the column values. However there was some residual dependence on TFA concentration, and there was always a large residual scatter, apparently sample-dependent, of up to  $\pm 0.10 \mu\text{g ml}^{-1}$  compared to the column value.

We find that rat plasma contains essentially no carotenoids that absorb at  $A_{450}$ . Rat plasma extract, therefore, offered a means of evaluating the degree to which inadequacies of the  $\beta$ -carotene correction might be contributing to the discrepancies noted. Three pooled samples of unfractionated rat plasma extracts were analyzed by the colorimetric and fluorometric procedures and compared with vitamin A values obtained after fractionation on alumina columns. Consistent results were obtained for all three pools, the principal difference being the total vitamin A present. For the three, colorimetric values for vitamin A under conditions of 42% TFA and ( $t_0$ ) were 0.220, 0.275 and 0.345  $\mu\text{g ml}^{-1}$ , and the corresponding column values were 0.225, 0.290 and 0.345  $\mu\text{g ml}^{-1}$ .

Results of the detailed colorimetric procedure and the alumina column fractionation for one such pool of rat plasma are presented in Fig. 2. These data reveal the same relative instability of  $A_{620}$  values in unfractionated rat plasma extracts as was seen in human plasma (Table 1). However in all three rat plasma samples, values for vitamin A in the colorimetric procedure with TFA concentrations above 40% were similar to values obtained after column fractionation. This finding has since been confirmed in many routine studies of rat plasma in our laboratory using ( $t_0$ ) colorimetric values and 40% TFA. The fluorometric procedure consistently gave slightly higher values for vitamin A than were obtained after column fractionation, a consistency which may be the result of a dietary or genetic factor affecting the phytofluene correction in this highly controlled population.

Figure 3 presents the results for 41 human plasma samples in which vitamin A levels determined by the colorimetric procedure are compared with values obtained after the column chromatographic procedure. Similar results were obtained when the fluorometric and column procedures were compared. Both the colorimetric and fluorometric procedures have roughly comparable characteristics for the quantitation of vitamin A in unfractionated extracts of human plasma samples, as long as the necessary precautions are taken in the colorimetric procedure. The regression equation relating vitamin A values (in  $\mu\text{g ml}^{-1}$ ) obtained by the colorimetric procedure to corresponding values obtained after chro-

matographic separation for these samples gave a slope =  $0.860 \pm 0.064$  and y-intercept =  $0.059 \pm 0.026$  while the equivalent values for the fluorometric procedure, for the same samples, were slope =  $1.05 \pm 0.042$  and y-intercept =  $0.015 \pm 0.018$ . The 95% confidence limits of both slopes and intercepts overlap. Standard error of estimates for these data sets were 0.047 and 0.043 respectively.

A slightly higher slope was seen in the fluorometric values from human samples compared to the values found after fractionation. This was consistent with the results observed for the rat plasma samples. A similar bias, of approximately the same magnitude, is seen in the data of Thompson et al. (ref. 2), providing a level of assurance that our use of the pure hexane fraction of human plasma extracts was equivalent to the use of tomato phytofluene in the original procedure of those authors. Certainly the procedure for obtaining the pure hexane fraction is more directly applicable and appears more appropriate. The slight observed upward bias in the fluorescence procedure would be relatively inconsequential in population surveys.

Of greater consequence for the use of either of these procedures is the scatter of both the colorimetric and fluorescent values, presumably introduced primarily by inadequacies of the  $\beta$ -carotene and phytofluene corrections, and which seems to be relatively independent of the vitamin A content of the plasma sample. The four samples having colorimetric values for vitamin A of between  $0.170$ - $0.185 \mu\text{g ml}^{-1}$  had column values of  $0.130$ ,  $0.135$ ,  $0.175$  and  $0.250 \mu\text{g ml}^{-1}$  (Fig. 3), and

fluorescence vitamin A values between 0.145-0.200  $\mu\text{g ml}^{-1}$ . A similar conclusion may be drawn with regard to the fluorometric procedure by reference to Fig. 4 of Thompson et al. [2]. This scatter may well be unacceptable, where the analyses are to be used as an indicator of vitamin A deficiency in individuals. A reasonable solution in larger studies to the apparently inherent inability of either method to give sufficiently accurate vitamin A determinations on individual unfractionated plasma extracts at low levels of vitamin A (below about 0.25  $\mu\text{g ml}^{-1}$ ) is to subject the expected small number of plasma samples exhibiting these low levels to the more extensive column fractionation procedure. Awdeh [12] also reported a bias toward low values for vitamin A determined colorimetrically on unfractionated plasma extracts which was corrected by preliminary column fractionation to remove carotenoids.

## CONCLUSIONS

The results presented indicate that the colorimetric procedure using TFA to determine vitamin A should contain a TFA reagent concentration of 40% to develop the full  $A_{620}$  values in the presence of natural plasma contaminants. This concentration is higher than that described in the original procedure [1]. In addition,  $A_{620}$  readings other than those obtained at the first moment of the reaction ( $t_0$ ) produce lower than expected values in unfractionated plasma extracts after correcting for  $\beta$ -carotene, because the color decay rate typically is

much more rapid than that of pure vitamin A standards.

To solve this problem  $A_{620}$  readings for all samples and standards are taken exactly 15 s and 30 s after the TFA reagent is added, ( $A_{t=15}$  and  $A_{t=30}$ , respectively) instead of the usual single reading (either at a pause point [10] or after 30 s). These values are extrapolated to the start of the reaction ( $A_{t=0}$ ) in some appropriate manner, such as by the equation  $A_{t=0} = A_{t=15} + [A_{t=15} - A_{t=30}]$ . All standard curves are constructed using  $A_{t=0}$  values also, and calculations of vitamin A content of samples are based solely on  $A_{t=0}$  values. Otherwise no changes of procedure or calculation are required from those previously suggested [1].

The problem of the inadequacy of the carotene corrections for unfractionated samples remains, even after taking the preceding precautions. In practice it is likely to be critical only for vitamin A determinations which fall below 0.20-0.25  $\mu\text{g ml}^{-1}$ .

The fluorometric procedure exhibited, in this and other studies and in that of the original authors [2], a slight systematic bias toward higher vitamin A values in unfractionated plasma extracts than was seen in the same extracts fractionated on alumina columns or determined colorimetrically. A fluorimetrically active fraction obtained from human plasma extracts after alumina column chromatography (the pure hexane fraction) used in these studies as the phytofluene standard simplified the original procedure and gave results indistinguishable from those obtained by Thompson et al. using a phytofluene standard

isolated from tomatoes. Deviations on unfractionated plasma extracts from those obtained after alumina column fractionation were similar to those observed for the colorimetric procedure.

In general, the alumina column fractionation procedure was lengthy, expensive and time-consuming for general use in surveys with large numbers of samples. Its use appears necessary, however, for those studies in which apparently low plasma vitamin A levels obtained from colorimetry or fluorometric determinations are to be correlated with the clinical vitamin A status of individuals, or where plasma retinol levels must be distinguished from plasma levels of retinyl esters.

#### Acknowledgements

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## REFERENCES

1. J.B. Neeld, and W.N. Pearson, *J. Nutr.*, 79 (1963) 454.
2. J.N. Thompson, P. Erdody, R. Brien and T.K. Murray, *Biochem. Med.*, 5 (1971) 67.
3. J.N. Thompson, P. Erdody and W.B. Maxwell, *Biochem. Med.*, 8 (1973) 403.
4. T.H. Carr and E.A. Price, *Biochem. J.*, 20 (1926) 497.
5. O.A. Bessey, O.H. Lowry, M.J. Brock and J.A. Lopez, *J. Biol. Chem.*, 166 (1946) 177.
6. P.J. Garry, J.S. Pollack and G.M. Owen, *Clin. Chem.*, 16 (1970) 766.
7. J. Selvaraj and T.P. Susheela, *Clin. Chem. Acta*, 27 (1970) 165.
8. J.G. Bieri, T.J. Tolliver and G.L. Catignani, *Am. J. Clin. Nutr.*, 32 (1979) 2143.
9. O.A. Roels and S. Mahadevan, *The Vitamins*, Academic Press, New York, 1967, p. 140.
10. D.W. Bradley and C.L. Hornbeck, *Biochem. Med.*, 7 (1973) 78.
11. B. Sivakumar, *Clin. Chim. Acta*, 79 (1977) 189.
12. Z.L. Awdeh, *Anal. Biochem.*, 10 (1965) 156.

TABLE 1

Effect of TFA concentration on  $A_{620}$  at 30 s for a pooled plasma sample.

TFA: CHCl <sub>3</sub> (v/v)	TFA in reaction mixture (%)	Absorbance (620 nm)			Vitamin A standard	Apparent vitamin A content ( $\mu\text{g ml}^{-1}$ )
		Sample extract	$\beta$ -carotene standard	Difference		
1:5	14	0.065	0.033	0.032	0.161	0.17
1:3	21	0.089	0.040	0.049	0.190	0.22
1:2	28	0.110	0.045	0.065	0.219	0.25
1:1	42	0.132	0.053	0.079	0.223	0.30
3:1	63	0.138	0.060	0.078	0.191	0.35
1:0	83	0.142	0.065	0.077	0.121	0.54

### FIGURE LEGENDS

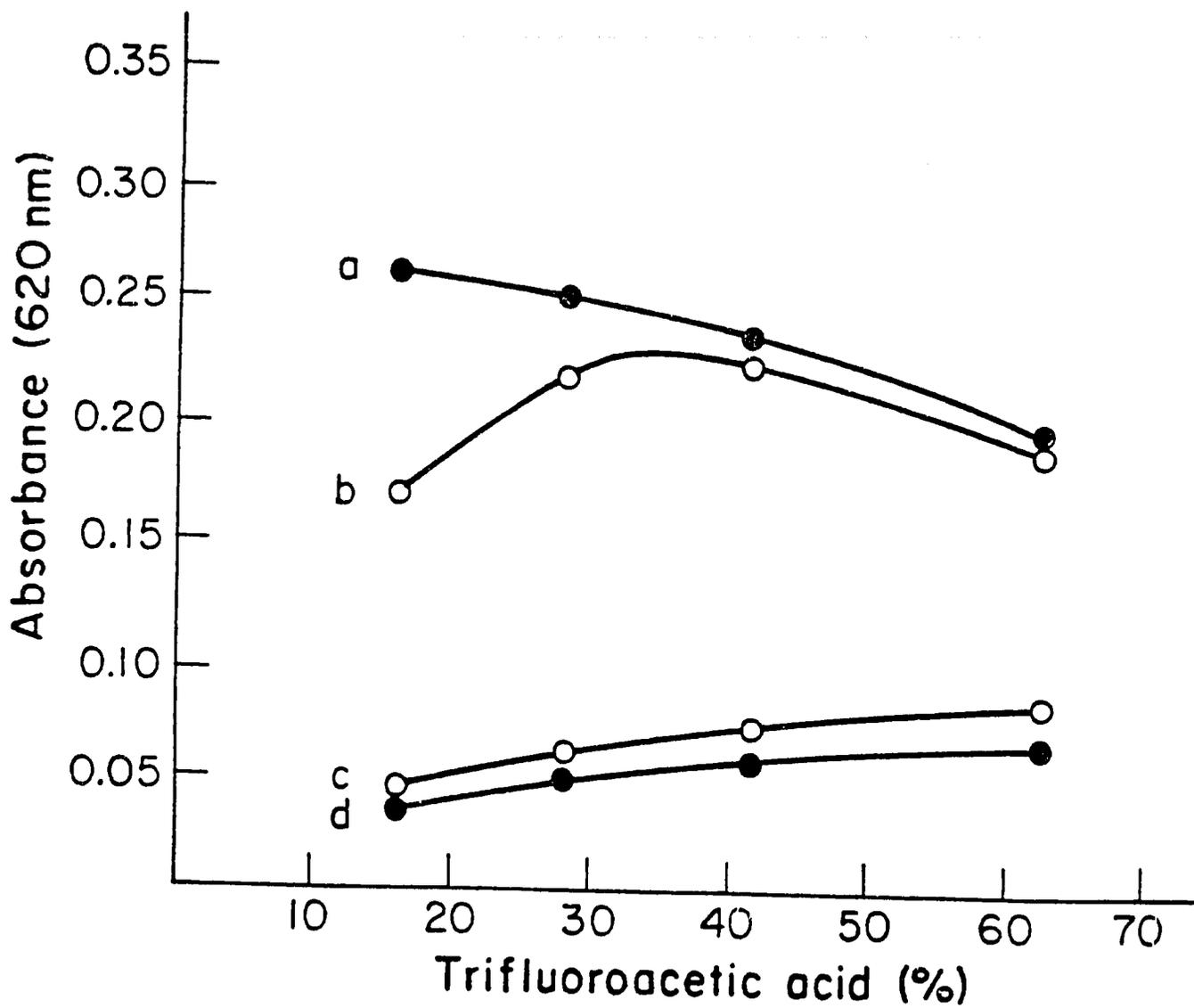
Fig. 1. Effects of TFA concentration on  $A_{620}$  response. Curves a and b: Retinyl acetate ( $0.5 \mu\text{g ml}^{-1}$ ) at  $t=0$  (a) and  $t=30$  s (b). Curves c and d:  $\beta$ -carotene ( $1 \mu\text{g ml}^{-1}$ ) at  $t=0$  (c) and  $t=30$  s (d).

Fig. 2. Effects of TFA concentration on the apparent vitamin A content of a single pooled sample of rat plasma. Curve a: values from  $A_{620}$  response at  $t_0$ . Curve b: values from  $A_{620}$  response at  $t_{30}$ . (-----) = vitamin A determined after fractionation on alumina columns.

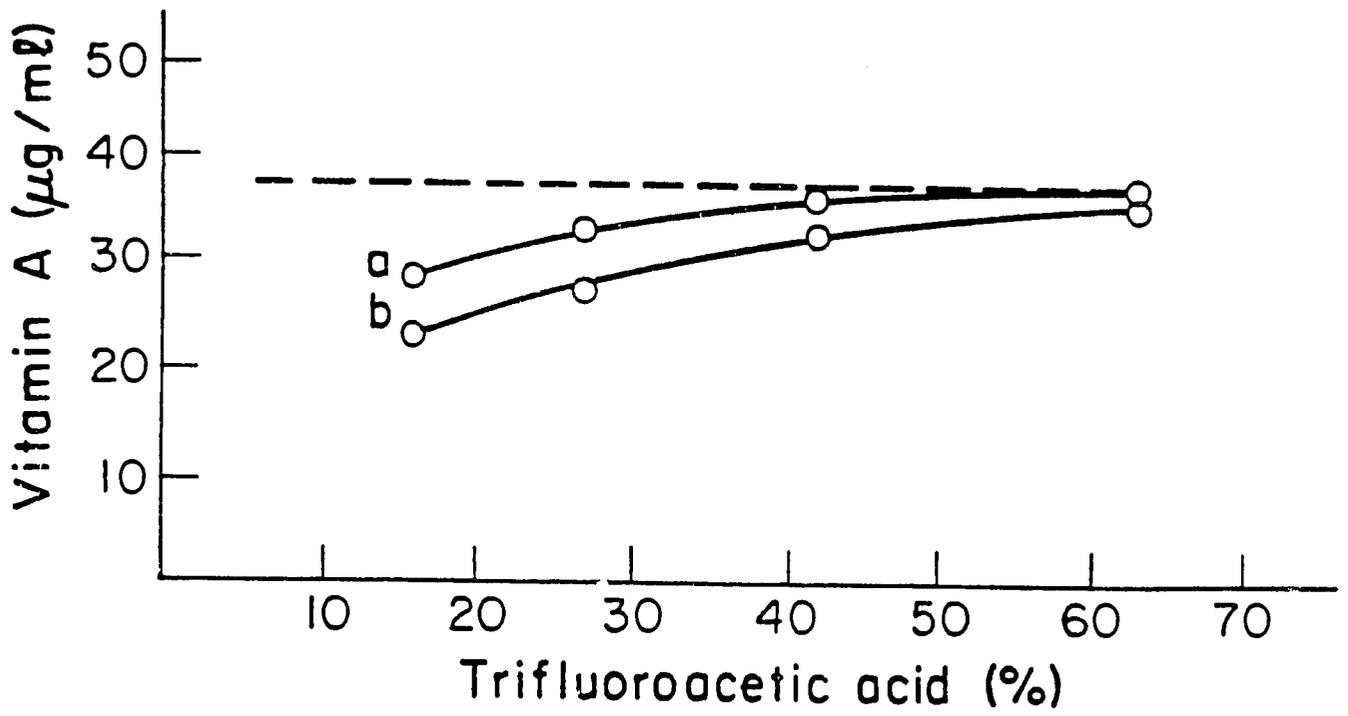
Fig. 3. Comparison of vitamin A values obtained for 41 samples of human plasma by the colorimetric procedure (b) with values for the same samples after alumina column chromatography (a) of their extracts. TFA concentration was 42%,  $A_{t=0}$  values used in the colorimetric procedure.

(O) = a single plasma sample analyzed in duplicate by both procedures.

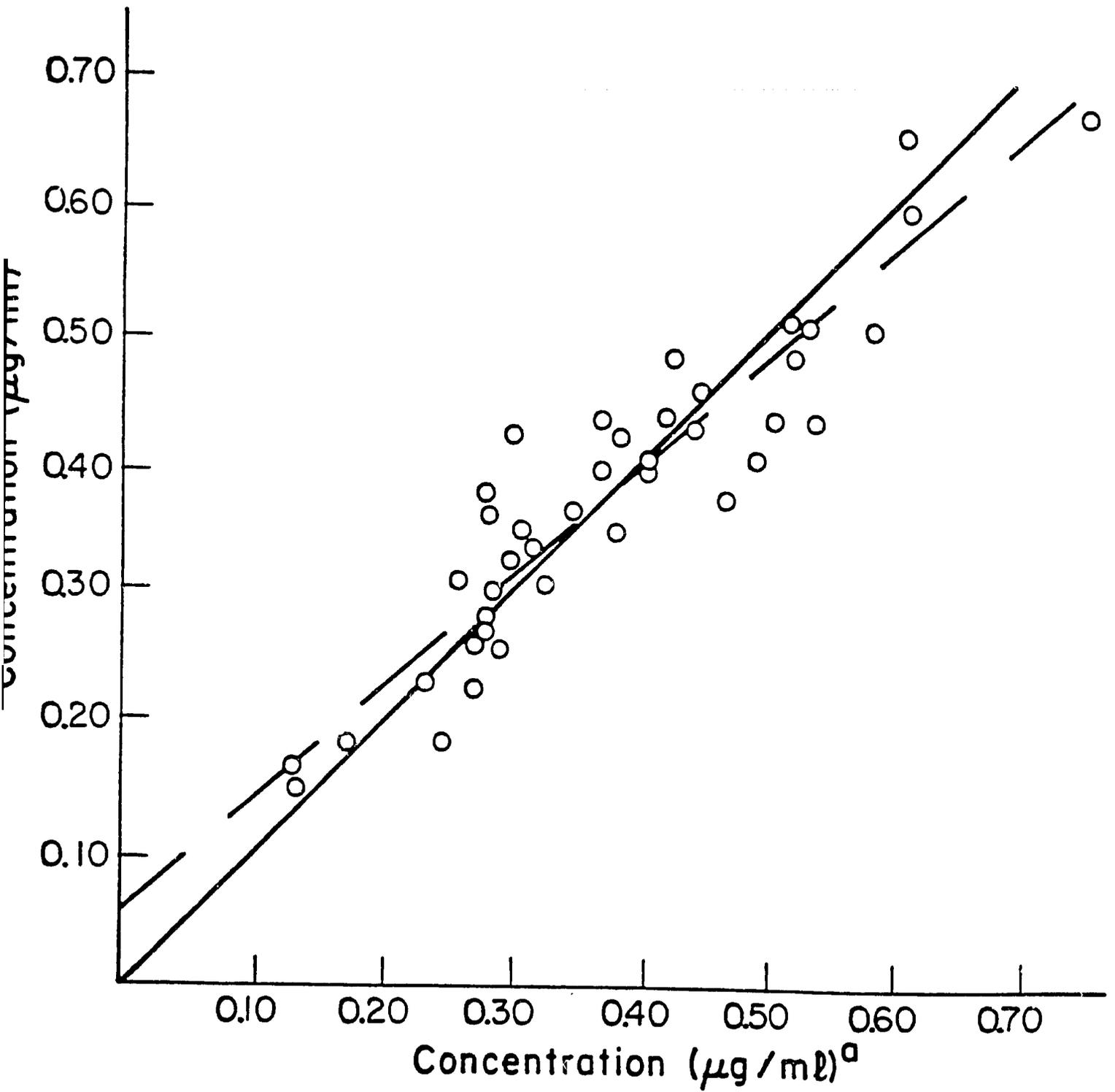
(——) = ideal line; (- - -) = least-squares line.



Loerch & Underwood Fig. 1.



Loerch & Underwood Fig. 2.



Loerch & Underwood Fig. 3.

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## Fluorometric Determination of Vitamin A in Human Blood and Liver

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Vitamin A (retinol) and its esters fluoresce in ultraviolet light, and analytical methods based on this property have been claimed to be simple, specific, and sensitive (1-6). Although the fluorometric assays seem to be ideally suited to large-scale clinical projects, such as surveys of vitamin A levels in human blood, they have not been widely used for these purposes up to the present time. Probably prospective users have discovered, as we did, that the published fluorometric procedures yield vitamin A values which are often overtly incorrect and sometimes more than double the true levels.

The fluorescence of human blood and liver lipids was therefore investigated and a major source of interference in the analytical methods was identified. Modified procedures for vitamin A analysis in blood and liver, which were accurate and yet still retained the speed and simplicity of previous fluorometric methods, were then devised and tested.

It is common knowledge, although poorly documented, that many derivatives and breakdown products of vitamin A fluoresce. The fluorescence spectra of several substances structurally related to vitamin A have been investigated to establish whether fluorometric methods for vitamin A are truly more specific than other procedures.

### MATERIALS AND METHODS

*Reagents.* Hexane (toxicographic grade, Anachemia Chemicals) and petroleum ether (pesticide quality, Matheson, Coleman & Bell) were distilled before use. Ethanol (95%) was distilled from KOH pellets, and diethyl ether (anhydrous, Mallinckrodt) was distilled over reduced iron. Aluminum oxide used for column chromatography (neutral AC7, BioRad) was washed with distilled ethanol followed by distilled ether and dried overnight at 120°. Silicic acid (silicAR CC-7, Mallinckrodt) was used as obtained.

*Centrifuge Tubes and Containers for Solvents.* Organic solvents were found to extract fluorescent substances from plastic caps, tubes, and bottles. Although the excitation maximum of this material was 315 nm and the emission maximum was near 360 nm, there was enough fluorescence to cause serious errors in the estimation of vitamin A when plastic vessels were employed during an analysis. Apparatus made entirely of glass was therefore used during all the analytical manipulations.

*Spectrophotofluorometers.* Most of the fluorescence measurements were made with an Aminco-Bowman spectrophotofluorometer equipped with a pen recorder and an IP21 photomultiplier. Slit widths (numbered 1-7) were 3, 2, 3, 3, 2, 3, and 2 mm, respectively. The photomultiplier microphotometer sensitivity was kept at the maximum setting and scale adjustments were made with the meter multiplier. It was found convenient to express fluorescence readings in arbitrary units, which were defined as the meter readings multiplied by 1000 times the setting of the meter multiplier.

In some experiments the spectra were measured with a Hitachi Perkin Elmer MPF-2A equipped with a R106 photomultiplier.

*Fluorescence Spectra.* Spectra of phytofluene and several vitamin A derivatives were recorded on the Hitachi Perkin Elmer instrument. Solutions were prepared in hexane with maximum absorbance values of 0.5 and then were diluted tenfold for the fluorescence measurements. The excitation spectra were recorded with bandwidths of 2 nm (excitation) and 20 nm (emission), and the emission spectra were recorded with bandwidths of 20 nm (excitation) and 4 nm (emission). The spectra were not corrected for instrument artifacts.

*Vitamin A Derivatives.* It is difficult to preserve retinol and its esters in pure form, and solutions rapidly deteriorate during storage. Convenient sources for preparing samples for fluorescence measurements were concentrates of retinyl acetate (1 million units/gm, Nutritional Biochemicals Corporation) which deposited large crystals of the ester when stored at 5°. When needed, portions of these crystals were removed with forceps and rinsed with cold petroleum ether. Retinol was obtained by saponification of a crystal of the acetate. It was purified by chromatography on alumina, essentially as described below for the analysis of blood.

Retinoic acid was used as obtained (crystalline, Distillation Products Industries), and the crystalline methyl ester was prepared from it according to the method of Robeson (7).

Retrovitamin A was obtained by saponification of retrovitamin A acetate, which was prepared from retinyl acetate (8). Anhydrovitamin

A was prepared from retinol (9). Retrovitamin A and anhydrovitamin A were purified by chromatography on columns of alumina but were not crystallized.

Retinyl methyl ether was crystalline (a gift from Hoffmann-La Roche and Co., Basel).

*Products of Irradiation of Retinyl Esters.* When alcoholic solutions of retinyl esters were exposed to ultraviolet light, there was a marked increase in fluorescence due to the formation of a highly fluorescent derivative (10-13 and unpublished observation). A purified sample of this substance was prepared by irradiating a solution of 150  $\mu$ g retinyl acetate in 50 ml methanol with ultraviolet light from a laboratory hand lamp (UVL 22, Ultraviolet Products Inc., Calif.) at a distance of 20 cm until the fluorescence at 340 nm excitation and 475 nm emission reached a maximum (13). The irradiation lasted 10 minutes and the intensity of the fluorescence from the solution increased almost fivefold. Peaks in the excitation spectrum changed to 345 and 365 nm. The solution was evaporated under reduced pressure and the residue was taken up in petroleum ether and chromatographed on a 10-gm column of alumina weakened with 5% water. The main fluorescent band was eluted with 10 ml petroleum ether.

*Isolation of Phytofluene.* Tomato juice and tomato ketchup were extracted by a procedure based on that of Koe and Zechmeister (14). For example, in one preparation 5 kg tomato ketchup was adjusted to pH 8 and stirred with an equal volume of methanol. After standing overnight the mixture was filtered through several layers of cheesecloth and squeezed dry. The aqueous methanol extract was discarded. The solid was stirred with 3 liters of 1:1 carbon tetrachloride and methanol and again filtered through cheesecloth. This extraction was repeated with another 2 liters of the solvent mixture, and the filtrates were combined and washed several times with water. The resulting clear deep-red solution was dried over sodium sulfate and evaporated under reduced pressure. The oily residue was dissolved in petroleum ether and chromatographed on a column of 100 gm alumina weakened with 2% water. The column was examined periodically with an ultraviolet lamp, and the main fluorescent band was eluted with 2% diethyl ether in petrol. An aliquot of this material was further purified by chromatography on a column of silicic acid. Using petroleum ether as eluant, phytofluene was separated from two yellow pigments which were eluted before and after the main colorless, but fluorescent, band.

*Simple Fluorometric Analysis of Blood.* Samples of blood plasma (0.2 ml) were analyzed according to four published methods (2, 3, 5, 6)

essentially as recommended in the original papers. However, in the procedure described by Kahan (2; p. 653) the plasma was diluted with 5 ml water to permit complete extraction of the retinol.<sup>1</sup>

*Fluorometric Analysis of Retinol and Retinyl Esters in Blood by Column Chromatography.* Plasma or serum (usually 1 ml) was shaken with an equal volume of ethanol and 5 volumes petroleum ether. The petrol extract was evaporated and redissolved in 10 ml petroleum ether and chromatographed on a column, 5 mm in diameter, of 1 gm alumina weakened with 5% water. The column was eluted with 10-ml portions of petrol, 2% diethyl ether in petrol, 10% diethyl ether in petrol, and finally 50% diethyl ether in petrol. Phytofluene was eluted in the first fraction, retinyl esters in the second, and retinol in the last. Fluorescence measurements were made directly on the 10-ml eluates. Standards of retinol for this estimation were therefore prepared in 50% diethyl ether in petrol.

*Fluorometric Analysis of Retinol and Phytofluene Mixtures by Means of a Correction Formula.* Phytofluene has fluorescence spectra which closely overlap those of retinol (see below), but as the shape of the excitation spectrum differs substantially from that of retinol, it is possible to calculate the proportions of the two substances in a mixture from fluorescence readings at two excitation wavelengths. The fluorescence of a mixture was measured at 475 nm using two excitation wavelengths (330 and 360 nm on our Aminco-Bowman<sup>2</sup>), and the correction formula was derived as follows. The fluorescence ( $x$ ) produced at 330 nm was assumed to equal  $a + p + B$ , where  $a$  was contributed by retinol,  $p$  by phytofluene, and  $B$  was a relatively small solvent blank. The latter was found to be constant between 320 and 375 nm. The fluorescence ( $y$ ) at 360 nm was similarly assumed to be  $Aa + Pp + B$ , where  $A$  and  $P$  are constants obtained from measurements of the ratio of the fluorescence at 360 and 330 nm in solutions of pure retinol and phytofluene, respec-

<sup>1</sup>In Kahan's published method, retinol and retinyl esters are extracted from 80-98% ethanol into cyclohexane. We have consistently confirmed that retinol is incompletely extracted by this procedure, as it is relatively soluble in 80% ethanol. In contrast to Kahan's report, we found that the aqueous alcohol phase should contain at least 40% water for efficient extraction of retinol into hexane or cyclohexane.

<sup>2</sup>Different wavelengths may be preferable with other instruments as the position of the peaks depends both on the machine and the slit widths. The latter should not necessarily be adjusted to provide maximum resolution as this could make the readings inconveniently sensitive to the wavelength setting. A suitable resolution gives an activation spectrum for phytofluene similar to that in Fig. 3. For example, the Hitachi Perkin Elmer MPF-2A was used with both the excitation and emission slits set for 8-nm bandwidths, the excitation wavelengths were 330 nm and 365 nm and the emission was measured at 480 nm.

tively. Solution of the simultaneous equations gave: fluorescence at 330 nm due to vitamin A

$$a = \frac{P}{P-1} (x - B) - \frac{1}{P-1} (y - B) \quad (1)$$

$$= K_1 x - K_2 y + K_3 \quad (2)$$

where  $K_1$ ,  $K_2$ , and  $K_3$  were constants.

The readings had to be corrected for day-to-day variations in the sensitivity of the spectrophotofluorometer. Neither phytofluene nor retinol was convenient as a standard because of instability. Instead, fluorescence readings (475 nm) at 330 nm ( $Q_1$ ) and 360 nm ( $Q_2$ ) excitation were made on a solution of quinine sulfate in 0.1 N  $H_2SO_4$  at the same time as the purified standards of retinol and phytofluene were examined in deriving the constants for the formula. At subsequent analyses, the readings on the quinine solution ( $q_1$  and  $q_2$ ) were repeated.<sup>3</sup> The vitamin A fluorescence was then calculated from

$$a = \frac{P}{P-1} \left( \frac{Q_1}{q_1} x - B \right) - \frac{1}{P-1} \left( \frac{Q_2}{q_2} y - B \right) \quad (3)$$

Application of the correction formula to the analysis of blood was as follows. Plasma or serum (0.2 ml) was homogenized for 30 seconds with 1 ml water, 1 ml ethanol, and 5 ml hexane in a 25-ml centrifuge tube using a Vortex mixer. After centrifugation an aliquot of the hexane layer was removed and fluorescence at 475 nm was measured at 330 and 360 nm excitation with the meter multiplier set at 0.001. In practice, the formula was adjusted to calculate the amount of vitamin A in micrograms directly. Thus, a solution of freshly chromatographed retinol in petrol was diluted until a reading between 0.3 and 0.6 was obtained at 325 nm in a spectrophotometer. The concentration ( $c$   $\mu\text{g}/\text{ml}$ ) was calculated from the  $E_{325}^{1\text{cm}}$  of 1530. This solution was diluted tenfold with hexane, and fluorescence readings at 475 nm were taken at 330 nm ( $d$ ) and 360 nm ( $e$ ) excitation with the meter multiplier set at 0.01. The value of A was calculated from  $e/d$  and a factor for converting fluorescence to micrograms of vitamin A per 100 ml serum was calculated from 25 ( $e/d$ ). A value for  $P$  was obtained similarly using a freshly chromato-

<sup>3</sup> Alternatively the instrument may be set to read 0 with the blank and to read 100 with the quinine standard at 360 nm. The calculation then simplifies to

$$a = \frac{P}{P-1} \left( \frac{Q_1}{q_1} x \right) - \frac{1}{P-1} y$$

A disadvantage of this method, however, is that serious changes in the performance of the instrument are not always obvious.

graphed solution of phytofluene. A value for  $B$  was obtained by measuring the fluorescence of a blank analysis using water in place of serum. A working formula was then obtained as: vitamin A ( $\mu\text{g}/100$  ml serum or plasma)

$$= \left[ \frac{P}{P-A} \left( \frac{Q_1}{q_1} x - B \right) - \frac{1}{P-A} \left( \frac{Q_2}{q_2} y - B \right) \right] 25 \frac{c}{d} \quad (4)$$

$$= K_1 \frac{x}{q_1} - K_2 \frac{y}{q_2} + K_3 \quad (5)$$

where  $K_1$ ,  $K_2$ , and  $K_3$  were constants.

*Separation of Retinol and Phytofluene in Blood by Partition Between Solvents.* Retinol is approximately equally soluble in 80% ethanol and hexane whereas phytofluene is almost insoluble in aqueous alcohol. The difference in solubility was used as the basis for a fluorometric determination of retinol in human blood. Plasma or serum (0.2 ml) was homogenized with 1 ml ethanol and 1 ml hexane and centrifuged. The hexane layer, which according to standards contained all of the phytofluene and approximately 40% of the retinol, was removed and discarded. The remaining aqueous ethanol phase was homogenized with 1 ml water and 5 ml hexane and centrifuged. Fluorescence due to the remaining retinol was measured on an aliquot of the separated hexane layer. A correction for the loss of retinol during the first extraction was obtained by passing a sample of freshly chromatographed retinol through the procedure.

*Determination of Retinol in Blood with SbCl<sub>5</sub>.* Plasma or serum (usually 3 ml) was shaken with an equal volume of ethanol and 3 volumes of petroleum ether. An aliquot of the petrol was evaporated, the residue was taken up in 1 ml chloroform, and 9 ml 25% SbCl<sub>5</sub> in chloroform was added. The blue color was measured immediately in an Evelyn colorimeter, using a 620-mμ filter, and compared with that of standards. When a correction for carotenoids was to be applied, the petrol extract was first evaporated to 5 ml and the absorbance at 450 mμ was measured in a spectrophotometer. The correction was calculated as suggested by Moore (15). Some samples were analyzed after saponification as described by Roels and Mahadevan (16).

*Fluorometric Analysis of Human and Rat Liver.* Samples of human liver (0.5 gm) were saponified by heating in a boiling water bath for 15 minutes in 15-ml centrifuge tubes with 0.5 ml 60% aqueous KOH and 1 ml 1% ethanolic pyrogallol. Water (1 ml) was then added and the unsaponifiable lipid was extracted by homogenizing the digest twice with 5 ml hexane. The two extracts were pooled and made up to 25 ml. This solution was diluted for direct fluorometric measurements.

In order to test the procedure the ultraviolet absorption spectrum of

the final extract was recorded. Also, an aliquot (10 ml) was evaporated and chromatographed on a 1-gm column of alumina as in the analysis of blood, and fluorescence and ultraviolet absorption measurements were made on the eluates.

The method was also tested by applying it to liver tissue obtained from 12 rats which had been reared on a vitamin A-free diet. These animals had low plasma levels of retinol (the mean value  $\pm$  SD was  $12.9 \pm 7.0$   $\mu\text{g}/100$  ml) but they showed no overt signs of deficiency disease. Samples of each liver (0.5 gm) were analyzed fluorometrically after saponification as described for human liver.

#### RESULTS

*Interfering Fluorescent Lipid in Human Blood.* Fluorometric assays on human blood serum and plasma, which were carried out according to the published procedures (2, 3, 5, 6), gave vitamin A values which were consistently higher than those obtained with the  $\text{SbCl}_3$  method, suggesting that human blood contained fluorescent lipids other than vitamin A. The fluorescence emission spectra of cyclohexane or hexane extracts of blood resembled that of retinol, but often the excitation spectra clearly indicated that a second fluorescent compound was present (Fig. 1). Pooled specimens of human blood plasma were homogenized with a mixture of ethanol and petrol, and the separated petrol extracts were chromatographed on columns of water-weakened alumina. Fluorescence emission was measured at 475 nm and four fractions from the chromatography gave significant fluorescence when irradiated with light between

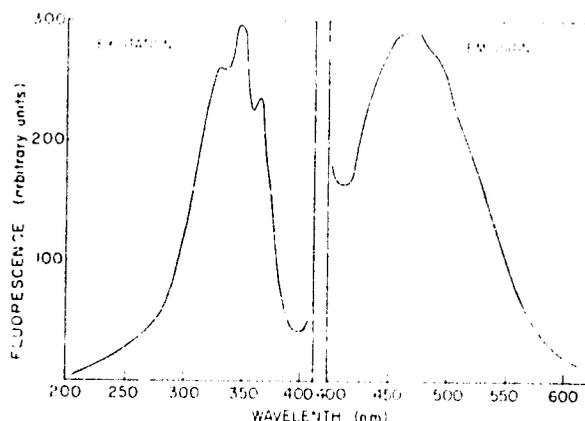


FIG. 1. Excitation and emission spectra of a cyclohexane extract of 0.2 ml human blood serum, which was prepared according to the method of Kahan (2). Spectra were measured in the Aminco-Bowman and are uncorrected.

300 and 400 nm. On columns weakened with 5% water, fluorescence was obtained from retinyl esters in the 2% ether in petrol fractions and from retinol in the 20 and 100% ether fractions. Fluorescence was also obtained from the petrol fraction. It had an emission spectrum similar to that of retinol, but the excitation spectrum had twin maxima at 350 and 370 nm with a shoulder at 335 nm (Fig. 2). The excitation spectrum and chromatographic properties of the petrol fraction were similar to those of phytofluene (see below), a carotenoid widely distributed in vegetable foods (14, 17-19), and comparison of the fluorescence spectra with those of phytofluene extracted from tomatoes revealed close agreement (Fig. 3).

*Fluorescence of Phytofluene.* The samples of phytofluene isolated from tomatoes were obtained as colorless solutions having ultraviolet absorption spectra similar to those described by previous investigators (14, 17-19) with maxima at 331, 349, and 366 nm. The fluorescence of phytofluene does not appear to have been studied hitherto. In the Aminco-

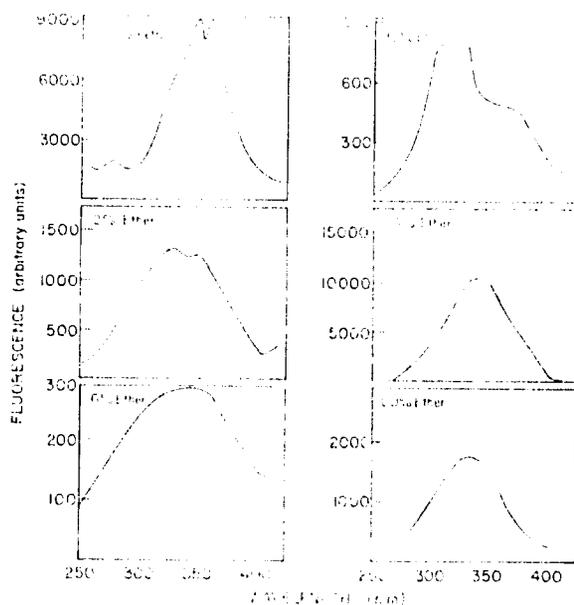


FIG. 2. Uncorrected excitation spectra of the 475-nm fluorescence emission (measured in hexane in the Aminco-Bowman) from eluates from an alumina column chromatography of an extract of 25 ml human plasma. The different scales have been chosen to show the shape of the spectra, and the fluorescence intensities were calculated from the total fluorescence units (see text) in each fraction. Thus most of the fluorescence was in the 100% petrol (phytofluene), 2% retinyl ester, 20% and 100% ether (retinol). Small amounts of other substances, perhaps artifacts, were detectable in the remaining fractions. The solvents were calculated to contribute approximately 20 fluorescence units to each fraction.

Bowman, with slits set up as described, the excitation spectrum was, as expected, similar to the ultraviolet absorption spectrum with loss of fine structure (Fig. 3). The emission spectrum had a broad maximum at 470-500 nm with poorly defined peaks at 470 and 487 nm.

In tomatoes, phytofluene is apparently mainly in a *cis* form which very readily undergoes isomerization to all *trans* (14, 17). It has been reported that this change increases the intensity of the fluorescence (17) and alters the shape of the absorption spectrum (14). Our preparations from tomatoes were considered to initially contain the *cis* form because irradiation produced an increase in fluorescence, but this increase was less than 20% and thus probably resulted in part from the increase in absorption (14).

The fluorescence properties of phytofluene and changes due to isomerization were studied primarily because of their relevance to the correction formula for vitamin A determinations. Increases in fluorescence intensity, however, have no effect on the constants used in the formula unless they are accompanied by changes in the relative fluorescence intensities at the two excitation wavelengths selected for the measurements. The ratio of the fluorescence intensities at 360 and 330 nm exci-

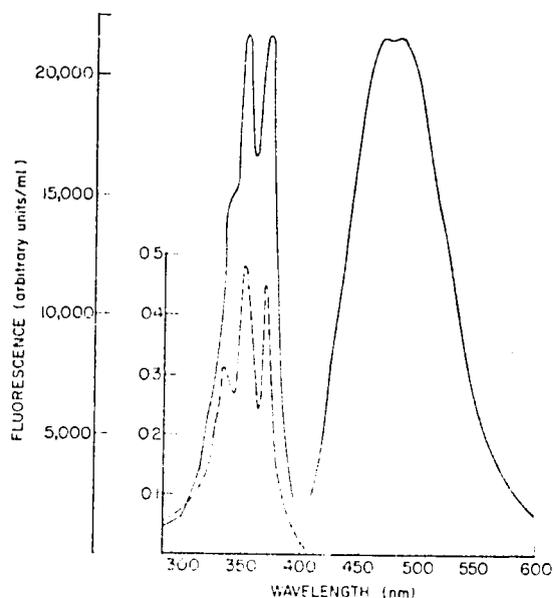


FIG. 3. Uncorrected excitation (left) and emission (right) spectra of phytofluene from tomatoes (solid line) compared with the absorption spectrum (broken line). The fluorescence spectra were measured in the Aminco-Bowman using a solution diluted to contain 430 fluorescence units/ml. Solvent:hexane.

tation (i.e.,  $P$ ) was therefore determined before and after isomerization with light. During isomerization the ratio with our instrument changed from 1.38 to 1.42; As it has not been possible to identify the configuration of phytofluene in blood, a rounded value (1.40) was used for  $P$  in the correction formula.

*Fluorescence of Vitamin A and Related Substances.* The fluorescence data for the substances examined are summarized in Table 1.

The excitation spectra were virtually identical to the ultraviolet absorption spectra except that low wavelength "end absorption" was absent. The only other noteworthy feature was the presence of three conspicuous peaks at 250, 260, 270 nm in the excitation spectrum of anhydrovitamin A, which accompanied the better known ones at longer wavelengths. These may have been *cis* peaks analogous to those known to occur in carotenoids.

The emission maxima sometimes indicated fine structure (Table 1), suggesting mirror image relationships with the excitation spectra, although the emission peaks were ill defined.

The relative fluorescence intensities depend upon the instrument settings and are thus to a certain degree arbitrary. In Table 1, the intensities are related to the ultraviolet absorption at the excitation wavelength and are then compared. This procedure avoids the use of  $E_{1cm}^{1\%}$  values, which sometimes are uncertain or unknown. The most intense fluorescence was obtained from phytofluene, whereas that from methyl retinoate was barely detectable. In general, the fluorescence was strong in the substances with five double bonds.

*Effect of Irradiation on Retinyl Acetate.* The fluorescent substance

TABLE 1  
FLUORESCENCE OF VITAMIN A AND RELATED SUBSTANCES

Substance	Chromophore <sup>a</sup>	Solvent	Fluorescence intensity <sup>b</sup>	Emission maximum (nm)
Retinyl acetate	5	Hexane	1.0	470
Retinyl methyl ether	5	Hexane	0.94	475
Retrovitamin A	5	Hexane	0.47	525, 505 <sup>c</sup>
Irradiation product	5	Hexane	3.68	500, 480 <sup>c</sup>
Phytofluene	5	Hexane	6.44	465, 490, <sup>c</sup> 520 <sup>c</sup>
Anhydrovitamin A	6	Hexane	0.08	530
Retinoic acid	6	Ethanol	0.08	470
Methyl retinoate	6	Ethanol	Trace	

<sup>a</sup> Number conjugated double bonds.

<sup>b</sup> Fluorescence reading  $\div$  absorbance; expressed relative to retinyl acetate.

<sup>c</sup> Shoulder.

produced by irradiation of retinyl acetate with ultraviolet light had an identical fluorescence excitation spectrum to that of the blood lipid in the Aminco-Bowman, and the ultraviolet absorption spectrum was similar to that of phytofluene with peaks at 329, 345, and 364 nm. However, the fluorescence emission of the irradiation product had a peak at 500 nm, whereas that from both phytofluene and the tissue lipid had a peak at 475 nm. On thin layer chromatography, the fluorescent tissue lipid and phytofluene had identical  $R_f$  values, whereas the irradiation product was more polar. Thus, with silicic acid plates and 1% ether in petrol as developing solvent, phytofluene and the lipid had a  $R_f$  of 0.8 and the irradiation product had a  $R_f$  of 0.2 which was equal to that of retinyl methyl ether.

*Analysis of Human Blood.* The fluorescence measurements on the extracts of blood were easily reproduced. Thus 10 determinations by the correction formula method on a sample of pooled blood averaged 65.0  $\mu\text{g}/100$  ml with a standard deviation of 1.8  $\mu\text{g}$  and a coefficient of variation of 2.8%. Accuracy was more difficult to assess as the true levels of vitamin A in the blood samples were not known. Blood samples were analyzed for vitamin A by the correction formula procedure and also by at least one of the alternative methods. These comparisons are summarized together in Table 2 and are shown individually in Figs. 4-8. The most accurate alternative method was considered to be that utilizing alumina column chromatography. Comparison of the results obtained by the correction formula and the column method revealed excellent agreement (Fig. 4). In some analyses the amount of water used in the extraction steps was reduced and as a result, low levels of retinol were ob-

TABLE 2  
COMPARISON OF RESULTS OF CORRECTION FORMULA METHOD WITH THOSE OF  
OTHER PROCEDURES

Figure	Procedure compared	No. of samples	Correlation coefficient	Ratio answers <sup>a</sup>
4	Column	28	0.96	1.06 $\pm$ 0.24
6	SbCl <sub>5</sub>	27	0.89	1.11 $\pm$ 0.24
7	SbCl <sub>5</sub> , carotene correction	27	0.84	0.91 $\pm$ 0.26
6	SbCl <sub>5</sub> , saponified	20	0.40	1.16 $\pm$ 0.20
7	SbCl <sub>5</sub> , carotene correction, saponified	20	0.61	0.77 $\pm$ 0.14
5	Partition	16	0.95	1.18 $\pm$ 0.09
8	Partition, corrected with formula	16	0.97	1.02 $\pm$ 0.06

<sup>a</sup> Mean value  $\pm$  S.E. of answer from compared procedure  $\div$  answer from correction formula.

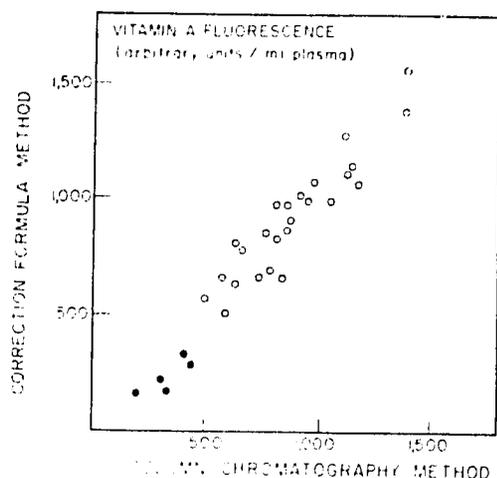


Fig. 4. Comparison of the results of vitamin A estimations using the correction formula with those obtained after chromatography on alumina. Results were expressed in arbitrary fluorescence units (see text; 1  $\mu\text{g}$  retinol is equivalent to approximately 1640 units). Open circles were results of the routine procedure. Filled circles were analyses on hexane extracts of blood obtained using the minimum amount of water needed for separation of the phases (see text).

tained with normal amounts of phytofluene. The purpose of this procedure was to extend the range of the results and to simulate retinol levels which conceivably might be encountered in samples from vitamin A-deficient donors.

In these tests the results were calculated in arbitrary fluorescence units at 330 nm excitation. In the column method the fluorescence of the retinyl ester fraction was added to that of the free retinol fraction as the

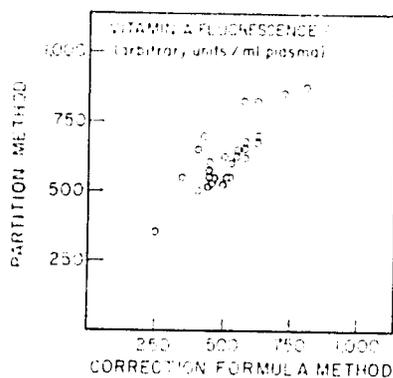


Fig. 5. Comparison of the results of the partition method with those of the correction formula method.

correction formula does not distinguish between them. The contribution of the ester fraction, however, was small and, for nutritional survey purposes, negligible.

The separation of phytofluene and retinol by partition between solvents was devised in an attempt to provide a rapid fluorometric determination of blood retinol that could be done with less expensive filter fluorimeters. The method usually gave satisfactory results but sometimes the answers were much higher than those obtained by the correction formula (Fig. 5). Examination of the fluorescence spectra of the final extracts revealed evidence of phytofluene in the offending samples which had not been removed during the first hexane extraction.

The  $SbCl_5$  assays differed from those by the correction formula method (Fig. 6) more than had those by the column chromatography method. Saponification or correction for carotenoids (Fig. 7) did not improve the correlations and, on the contrary, examination of individual results revealed that the carotenoid correction often increased the discrepancies.

*Analysis of Liver.* The ultraviolet absorption spectra of unsaponifiable lipids from normal human liver had peaks near 330 nm but often there was too much end absorption to permit an accurate estimation of the retinol content. Thus, it was necessary to chromatograph the unsaponifiable lipids in order to estimate retinol by absorption spectroscopy.

In contrast the fluorescence excitation spectra of the diluted unsaponifiable lipids did not have a low wavelength component equivalent to "end absorption" and chromatography was unnecessary; the retinol content

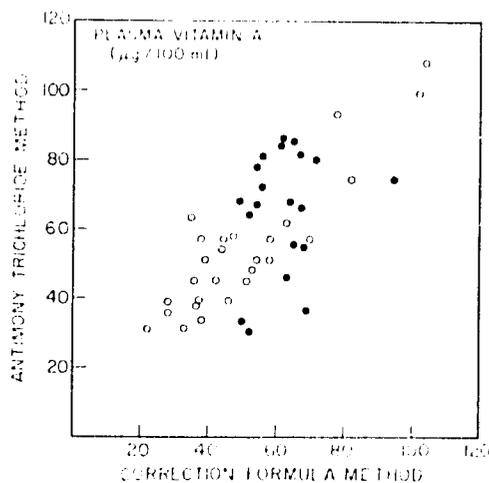


FIG. 6. Comparison of the results of the correction formula method with those of the antimony trichloride procedure either with (●) or without (○) saponification.

could be calculated directly by use of the correction formula. Column chromatography of the unsaponifiable lipids confirmed that fluorescence was due to retinol and relatively small amounts of phytofluene.

No fluorescent artifacts were obtained from the alkaline pyrogallol in blank saponifications.

The excitation spectra of the saponified lipids from livers of rats depleted of vitamin A had maxima near 330 nm, but the peaks were broad and according to the correction formula approximately one-half of the fluorescence was due to retinol. The retinol concentrations were calculated to be  $0.25 \pm 0.15 \mu\text{g}/\text{gm}$  (mean  $\pm$  SD); these values are not claimed to be accurate but they indicate the sensitivity of the method using a 0.5-gm sample.

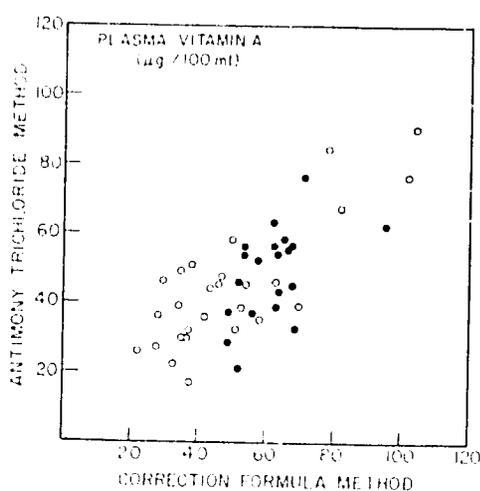
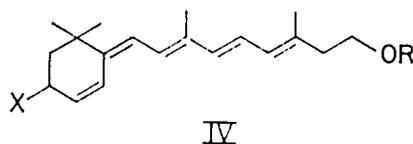
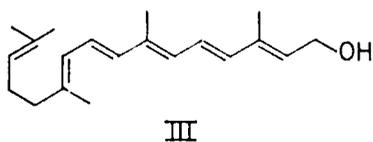
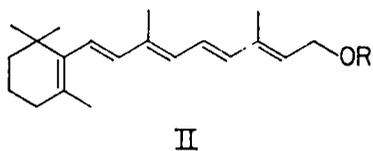
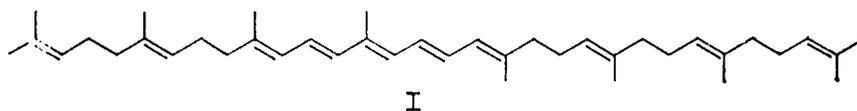


Fig. 7. The results of the correction formula method shown in Fig. 6 compared with those of the antimony trichloride procedure corrected for the presence of carotenoids either with (●) or without (○) saponification.

#### DISCUSSION

Fluorometric methods for determining vitamin A are more sensitive than other procedures, but they can give erroneous answers when applied to human blood and liver because there is a lipid in these tissues which fluoresces at the same wavelength as vitamin A. The interfering substance was identified as the carotenoid phytofluene (I) by its fluorescence and chromatographic behavior.

The excitation spectrum of the fluorescent lipid was identical to that of phytofluene and it corresponded to an ultraviolet absorption spectrum with three peaks near 331, 349, and 366 nm. Thus, the chromophore



possessed five conjugated double bonds and was not degraded by the spacial conflicts (20) which are alleged to result in a single maximum in the spectra of retinol and its derivatives (II; R = H, CH<sub>3</sub>, COCH<sub>3</sub>, etc.). A similar chromophore exists in other characterized substances besides phytofluene (21), but the blood lipid could be distinguished from all those examples which we have considered by its chromatographic properties and sometimes by its fluorescence emission.

For example,  $\gamma$ -vitamin A (III) chromatographs similarly to retinol. Furthermore, it is very unstable and it has not been obtained other than by chemical synthesis. There is no published information revealing whether or not it is fluorescent (22).

Retrovitamin A aldehyde, alcohol, and esters (IV; X = H; R = H, COCH<sub>3</sub>, etc.) are readily produced as artifacts from retinol and its esters during storage and analytical manipulations. They are strongly fluorescent and, as expected, have a fluorescence excitation spectrum similar to that of phytofluene. These derivatives do not, however, chromatograph on alumina columns with phytofluene but are eluted with 2, 10, and 50% ether in petrol.

Rehydrovitamin A<sub>2</sub> is also fluorescent (23) and it is thought to be an

ethoxyalcohol (IV; X = OC<sub>2</sub>H<sub>5</sub>; R = H); however, on chromatography it also is more polar than the blood lipid and phytofluene.

Isoanhydrovitamin A is the only substance known to us which has properties superficially similar to those of the blood lipid. It was first produced from retinol by prolonged treatment with anhydrous ethanolic hydrochloric acid (9). Two structures have been suggested (24), both of which are ethers with an alkoxy group on either the terminal carbon (IV; X = H; R = C<sub>2</sub>H<sub>5</sub>) or the one adjacent. Previous publications do not mention whether the compound is fluorescent, and unfortunately our attempts to prepare it using alcoholic hydrogen chloride, in common with those of other workers (12), have not been successful. This substance was at one time thought to be formed also during irradiation of retinyl esters with ultraviolet light, although doubts arose after subsequent investigations (12). As a retrovitamin A ether would chromatograph closely to phytofluene on alumina columns, we reinvestigated the products of irradiation of retinyl esters with the purpose of comparing them with the unidentified lipid. Our experiments confirmed the original suggestion (12) that a retrovitamin A ether is produced during irradiation. This substance was eluted from alumina columns in the petrol fraction with phytofluene, but it was easily demonstrated to be more polar than the blood lipid and phytofluene by thin-layer chromatography, and it had the same *R<sub>F</sub>* as retinyl methyl ether. Although its excitation spectrum was similar to that of phytofluene, the emission was at a longer wavelength (Table I). From both mobility on thin-layer chromatography and the emission spectrum, the lipid in human blood and liver was concluded to be a hydrocarbon and not a retrovitamin A ether.

Although many hydrocarbon structures were theoretically possible for the fluorescent tissue lipid, nutritional and biochemical considerations strongly supported the view that this substance was phytofluene. The latter occurs in foods with colored carotenoids, many of which are found in human and animal tissues. Fluorometric analysis of a large number of samples of human blood serum indicated that the interference in the determination of vitamin A could be explained by phytofluene levels rarely exceeding 20  $\mu\text{g}/100\text{ ml}$ ; this would be compatible with the normal mean total carotenoids of 1.40  $\mu\text{g}/100\text{ ml}$  (15).

The possibility of interference in the analysis for vitamin A from fluorescent lipids other than phytofluene has also been examined. Retrovitamin A derivatives are obvious sources as they accompany retinol and retinyl esters in lipid extracts of tissues, although they are probably entirely artifacts. Indeed, retro derivatives occur in commercial synthetic preparations of retinol and retinyl esters. As they fluoresce almost as much, and sometimes more than, the corresponding retinol derivatives

(Table 1), it is important that standards of the latter for fluorescence determinations should be purified immediately before use. This precaution does not seem to have been taken hitherto, and therefore previously published data concerning the fluorescence of retinol must be viewed with suspicion. The conversion of retinyl esters to the highly fluorescent retro ether, when irradiated in alcohol, can result in serious errors: the reaction can occur in the absence of deliberate irradiation and is apt to occur in chloroform-methanol mixtures. This popular solvent is therefore unsuitable for the extraction of tissue lipids for fluorescence determinations.

Other than retinol, retinyl esters, phytofluene, and retro derivatives we have not yet encountered significant amounts of lipids in extracts from rat and human tissues which fluoresce near 450-500 nm when excited between 300 and 400 nm. This convenient circumstance is almost to be expected. Although the relationships between molecular structure and fluorescence are not completely understood, a minimum requirement for fluorescence is the absorption of light. Lipids absorbing light maximally at wavelengths near 330 nm can be expected to have four to six conjugated double bonds, and retinol derivatives and carotenoids are the only examples known to occur in normal animal tissues. The fluorescence of these polyenes does not seem to have been studied systematically hitherto, but from published data and our own preliminary observations (Table 1) a few generalizations are possible. Thus at room temperature strong fluorescence is obtained from conjugated pentaenes, which include provitamin A derivatives and phytofluene. Similar fluorescence is obtained from retinol derivatives, which have a degraded pentaene chromophore. The addition of carbonyl groups to the pentaene chromophore, as in retinal and retinoic acid, almost abolishes fluorescence, at least at room temperature (25). Conjugated hexaenes are only weakly fluorescent: 3-dehydroretinol (vitamin A<sub>2</sub>) has a brown fluorescence and anhydrovitamin A fluoresces at 530 nm (Table 1). Conjugated chains with seven or more double bonds (i.e., colored carotenoids) have negligible fluorescence at room temperature (26) and strong fluorescence has not been reported in conjugated tetraenes ( $\gamma$ -vitamin A; 5,6-epoxy-vitamin A) or trienes (phytoene).

Thus among isoprenoid polyenes, fluorescence near 475 nm seems to be confined to the pentaenes, and when it occurs in conjugated structures of longer or shorter length, the emission is weak and it has a maximum at a different wavelength. One would expect that this characteristic property of conjugated pentaenes could be exploited to obtain a highly specific and sensitive assay for vitamin A. This has been substantiated by our experience of the application of spectrophotofluorometry to the

analyses of a variety of tissues. We have found the fluorometric assay to be superior to the antimony trichloride and other procedures in its convenience, sensitivity, specificity, and accuracy. It is not completely immune to interference, however, and the common preliminary purification steps, such as chromatography and saponification, cannot always be bypassed. In the analysis of normal liver, simple extracts with ethanol-ether mixtures could be used for the direct estimation of vitamin A. However, the crude extracts contained interfering fluorescent material (probably pyridoxine derivatives) which could be removed by washing with water. This and other interference was also easily eliminated by saponification, and chromatography of the unsaponifiable lipids demonstrated that retinol and a trace of phytofluene accounted for most of the remaining fluorescence. Saponification is, therefore, recommended as a preliminary step in the analysis of human liver.

The elimination of the interference due to phytofluene is necessary in the analysis of blood and it is sometimes desirable in the analysis of liver. It can be achieved by several column chromatography systems. We have found polyethylene glycol-celite columns (27) to be the most convenient as they can be reused and, when connected to a flowcell, automated. Alumina columns were chosen when phytofluene, retinol, and retinyl esters were to be measured separately.

The most rapid method for eliminating interference due to phytofluene made use of a correction formula. In general, caution is necessary with correction formulas as some exaggerate errors when they are applied to spectra containing unexpected components. This happens, for example, with the Morton-Stubbs correction (16). However, the construction of our formula is such that even when unidentified interference arises, the formula rarely aggravates the error. Thus the retinol fluorescence is calculated from Eq. 2, and when interference is present having fluorescence  $i_1$  at the first wavelength and  $i_2$  at the second, the error in the corrected determination is  $K_1 i_1 - K_2 i_2$ . The error when the formula is not used is  $i_1$ . The formula therefore reduces the error whenever

$$\left(\frac{K_1 + 1}{K_2}\right) i_1 > i_2 > \left(\frac{K_1 - 1}{K_2}\right) i_1.$$

which in practice is the most probable relationship.

The generalizations we have made concerning the fluorescence of isoprenoid polyenes have an important bearing on the fluorometric analysis for vitamin A because they imply that serious interference, if present, is most likely to arise from pentaenes. Fundamentally, our correction formula is based on the existence of an atypical pentaene chromophore

in retinol derivatives, and one of its virtues is that it corrects, at least approximately, for the presence of most pent-enes as they have excitation spectra which differ only slightly from that of phytofluene.

It was considered unsatisfactory to assess the accuracy of the correction formula method by comparing it with established procedures, such as the SbCl<sub>5</sub> and the Bessie-Lowry technique, because these methods are themselves inaccurate and unreliable. The SbCl<sub>5</sub> reaction requires careful timing, and, compared with fluorometric methods, it is insensitive. Furthermore, some, but not all, carotenoids and lipids interfere with the reaction and it is difficult to correct for this error (Ref. 15, pp. 59-62). Comparison of the results of the fluorometric method with those of the SbCl<sub>5</sub> test revealed differences which probably were mainly due to errors in the latter. Although a correction for carotenoids carried out in the recommended manner on the SbCl<sub>5</sub> procedure sometimes brought the answers closer together, with many samples it increased the discrepancy. Saponification was found to have a similarly inconsistent effect.

The Bessie-Lowry method, in which the ultraviolet absorption of an extract is measured before and after irradiation with ultraviolet light, was not compared directly. However, phytofluene, which absorbs light in the same region of the spectrum as retinol, was found to be rapidly destroyed during irradiation and thus it would be expected to interfere in a Bessie-Lowry assay. Phytofluene fluoresces more intensely than retinol (Table 1); thus interference due to phytofluene in the Bessie-Lowry technique can be calculated to be only a fraction of that experienced in the fluorometric method. It is evident, however, that retinol is not the only lipid in blood which is destroyed by irradiation, and in addition to phytofluene, there may be other nonfluorescent carotenoids which are removed. This possibility was suggested earlier by the results of tests applied to the Bessie-Lowry procedure by Caster and Mickelsen (28).

The most satisfactory alternative method for vitamin A determinations in blood was a new one involving chromatography of extracts on columns of alumina followed by fluorometric measurements on the eluates. The method enabled retinol, retinyl esters, and phytofluene to be measured separately, and yet it involved only one stage, immediately before chromatography, at which it was necessary to evaporate the lipid extracts to dryness. During chromatography some retinol was lost and fluorescent artifacts were taken up from the alumina and solvents; however, measurements on standards and blanks indicated that errors arising from these changes were negligibly small. The method was probably accurate therefore, as there were no other foreseeable sources of error.

The answers from the correction formula correlated well with those obtained by column chromatography, and this agreement is considered to be convincing evidence of the validity of the former. Thus, in comparisons with other procedures, it can be assumed that the discrepancies are due to errors in the compared method, rather than in the correction formula.

The correction formula was devised for use with a spectrophotofluorometer, but a similar procedure could probably be devised for a filter instrument using appropriate interference filters with narrow pass-bands. As an alternative, the method involving partition between solvents was tested as a potentially rapid procedure for blood analysis using a simple filter instrument. The results correlated reasonably well with those of the correction formula and the accuracy was judged to be at least as good as that of the antimony trichloride procedure. However, it did not always eliminate all of the interference, and with a few samples the results were too high; this may have been due to the presence of retroderivatives, hydroxylated forms of phytylfluene or phytylfluene in the final extract. Some difficulty was experienced in removing the small first hexane extract quantitatively and this could explain the incomplete removal of phytylfluene. The fluorescence data from each of the final extracts (which should have been due entirely to retinol) was therefore corrected for the presence of traces of phytylfluene using the formula. Significantly those samples which had given high values were those which were altered most by this correction procedure. These adjusted readings were then corrected for the recovery of retinol during the extractions and excellent agreement was obtained with the correction formula method applied to the original plasma samples (Fig. 8). This calculation, although of no practical value as a method of analysis, provided additional evidence for the validity of the correction formula, and confirmed that

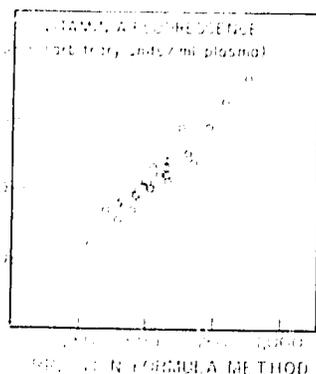


Fig. 8. The data shown in Fig. 5 after the results of the partition method had been corrected for the incomplete removal of phytylfluene (see text).

the high values obtained by the partition method were due to incomplete removal of either phytofluene or a substance with a similar spectrum.

Previous workers failed to detect the interference due to phytofluene in the fluorometric analysis of vitamin A in human blood because they neglected to make relevant tests of their procedures. Hansen and Warwick (3, 4) claimed to have demonstrated specificity by confirming that cholesterol, carotene, tocopherol, and calciferol did not interfere in their assay. The choice of these substances seems arbitrary and it is difficult to rationalize; although all of these are nutritionally and biochemically important, only carotene absorbs significant amounts of light above 310 nm and only tocopherol fluoresces. Hansen and Warwick found human blood samples had a mean value of 133  $\mu\text{g}$  vitamin A/100 ml which is far above the accepted normal value of 40  $\mu\text{g}$ /100 ml (12). Presumably their results were largely a measure of phytofluene.

Drujan, Castillan, and Guerrero (5) obtained normal values for human blood samples, which suggests that either their samples had lower than usual levels of vitamin A or that interference due to phytofluene was absent. They did not make direct comparisons with other accepted methods and thus it is impossible to decide between these interpretations.

Kahan (2) compared his fluorescence procedure with the antimony trichloride and ultraviolet absorption methods and obtained reasonable agreement. However, most of the blood used in these tests was obtained from donors previously dosed with large amounts of vitamin A, and as a result the vitamin A levels were considerably higher than those found in normal individuals. In the normal range ( $< 2$   $\mu\text{moles/liter}$ ; Fig. 3 Ref. 2) there appears to be no correlation between the methods. Kahan cited the absence of interference from hemoglobin, bilirubin, cholesterol, and other fat-soluble vitamins as evidence for specificity. He also claimed that "carotenoids" had no appreciable fluorescence, although clearly without justification. Destruction of vitamin A in blood extracts by ultraviolet irradiation left little residual fluorescence; however, many substances are removed by irradiation, including phytofluene.

Selvaraj and Susheela (6) combined fluorescence measurements with destructive irradiation and saponification in the analysis of blood. Their procedure would certainly be subject to interference from phytofluene. However, excellent correlation with the Bessie-Lowry method was obtained. It is possible perhaps as in the studies in Venezuela (5), that the phytofluene level in the diet of the donors was low.

#### SUMMARY

The fluorescence spectra of vitamin A derivatives and related substances have been examined and strong fluorescence was found to be

associated with the chromophore containing five conjugated double bonds. Simple fluorometric assays for vitamin A in human blood were found to be inaccurate because of interference from a fluorescent lipid, identified as the carotenoid phytofluene. A new rapid fluorometric method, which corrected for this interference by means of a formula, has been devised. The answers obtained by the new method correlated satisfactorily with those obtained by use of the antimony trichloride procedure and correlated even better with determinations made after isolation of the vitamin A by chromatography on columns of alumina. A fluorometric assay, involving preliminary saponification, has been developed for the analysis of human liver.

## REFERENCES

1. DE, N. K., *Indian J. Med. Res.* **43**, 3 (1955).
2. KAHAN, J., *Scand. J. Clin. Lab. Invest.* **18**, 679 (1966).
3. HANSEN, J. G., AND WARWICK, W. J., *Tech. Bull. Regist. Med. Techn.* **38**, 239 (1968).
4. HANSEN, J. G., AND WARWICK, W. J., *Tech. Bull. Regist. Med. Techn.* **39**, 70 (1969).
5. DRUJAN, B. D., CASTILLO, R., AND GUERRERO, E., *Anal. Biochem.* **23**, 44 (1968).
6. SELVARAJ, R. J., AND SUSHEELA, T. P., *Clin. Chim. Acta* **27**, 1 (1970).
7. ROBESON, C. D., U.S. Patent 2583594 (1952).
8. BEUTAL, R. H., HINKLEY, D. F., AND POLLAK, P. L., *J. Amer. Chem. Soc.* **77**, 5166 (1955).
9. SHANTZ, E. M., CAWLEY, J. D., AND EMBREE, N. D., *J. Amer. Chem. Soc.* **65**, 901 (1943).
10. SOBOTKA, H., KANN, S., AND LOEWENSTEIN, E., *J. Amer. Chem. Soc.* **65**, 1959 (1943).
11. SOBOTKA, H., KANN, S., AND WINTERNITZ, W., *J. Biol. Chem.* **152**, 635 (1944).
12. SOBOTKA, H., KANN, S., WINTERNITZ, W., AND BRAND, E. J., *J. Amer. Chem. Soc.* **66**, 1162 (1944).
13. KAHAN, J., *Acta Chem. Scand.* **21**, 2515 (1967).
14. KOE, B. K., AND ZECHMEISTER, L., *Arch. Biochem. Biophys.* **46**, 100 (1952).
15. MOORE, T., in "Vitamin A," Elsevier, Amsterdam, 1957.
16. ROELS, O. A., AND MAHADEVAN, S., in "The Vitamins," 2nd ed. (P. Gyorgy and W. N. Pearson, Eds.), Vol. VI, p. 139. Academic Press, New York, 1967.
17. PETRACEK, F. J., AND ZECHMEISTER, L., *J. Amer. Chem. Soc.* **74**, 184 (1952).
18. WALLACE, V., AND PORTER, J. W., *Arch. Biochem. Biophys.* **36**, 468 (1952).
19. DAVIS, J. B., JACKMAN, L. M., SIDMONS, P. T., AND WEEDON, B. C. L., *J. Chem. Soc.* 1961, 261.
20. ZECHMEISTER, L., in "Cis-trans Isomeric Carotenoids Vitamins A and Arylpolycenes," p. 126. Academic Press, New York, 1962.
21. SCHWIETER, U., AND ISLER, O., in "The Vitamins," 2nd ed. (W. H. Sebrell and R. S. Harris, Eds.), Vol. I, p. 5. Academic Press, New York, 1967.
22. MANGHAND, P. S., RUEGG, R., SCHWIETER, U., SIDMONS, P. T., AND WEEDON, B. C. L., *J. Chem. Soc.* 1965, 2019.

23. BAMJI, M. S., CAMA, H. R., AND SUNDARESAN, P. R., *J. Biol. Chem.* **237**, 2747 (1962).
24. OROSHNIK, W., *Science* **119**, 660 (1954).
25. THOMSON, A. J., *J. Chem. Phys.* **51**, 4106 (1969).
26. CHERRY, R. J., CHAPMAN, D., AND LANGELAAR, J., *Trans. Faraday Soc.* **64**, 2304 (1964).
27. MURRAY, T. K., *Proc. Soc. Exp. Biol. Med.* **111**, 609 (1962).
28. CASTER, W. O., AND MICKELSEN, O., *Amer. J. Clin. Nutr.* **3**, 409 (1955).

**II.**  
**Analysis of Liver Vitamin A**

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## A SIMPLE DUAL ASSAY FOR VITAMIN A AND CAROTENOIDS IN HUMAN LIVER

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### ABSTRACT

Samples of liver (1 g) are gently mashed with anhydrous sodium sulfate (2.5 g), covered with a given volume of chloroform (5.0 ml) and stored for 8-24 hr in tightly closed vials at 4°. Aliquots of the chloroform solution (0.02-0.3 ml) are analyzed both by a spectrophotometric procedure, using a correction formula for nonspecific absorption, and by the Carr-Price reaction using trichloroacetic acid. The time spent per assay is short, saponification, extraction and solvent transfer are eliminated, health hazards are minimized, and sensitivity is high (1 µg/g liver). Disparity between the two values obtained indicates the presence of extraneous factors in the extract. In a group of 37 human liver samples taken at autopsy, the median percentage difference between the two values was 7.7% over a range of retinol concentrations from 11 to 395 µg/g. In only 2 cases did values differ by more than 20%.

### INTRODUCTION

Assays of vitamin A in tissue extracts are often affected by the presence of reactive or inhibitory contaminants (1,2); e.g., phytofluene in fluorescence assays (3) and β-carotene in the Carr-Price assay (4). These contaminants either can be eliminated by chromatographic purification of vitamin A, or their effects can be minimized by the use of suitable correction formulas. In nutritional surveys, however, the former procedure is time consuming and costly, and the latter is often not applicable. Thus, in the routine use of any single method for vitamin A analysis, some values will be significantly in error.

Both to identify aberrant values and to minimize their effects in surveys of vitamin A concentrations in liver, a simple procedure has been developed in which two different properties of the vitamin A molecule, spectrophotometric absorbancy at 330 nm and the Carr-Price reaction, are measured in every sample. Inasmuch as both assays can be quickly carried out on the same extract without need for saponification, filtration, centrifugation or solvent transfer, the described procedure offers as well the advantages of rapidity, simplicity and high yield.

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## METHOD

Sample Storage. Samples of liver (5-10 g) are taken from the central portion of the right lobe at autopsy, placed in small snap-top containers, frozen at -20° and stored until analysis. Samples are usually analyzed within one week of collection.

Sample extraction. Approximately 1 g of frozen liver is quickly weighed on a tared piece of aluminum foil to the nearest 10 mg, placed in a 9 ml screw-top vial, gently mashed against the side of the vial together with 2.5 g of anhydrous sodium sulfate by use of a spatula and covered with 5.0 ml of chloroform. After gentle mixing, the vial is hermetically sealed and placed at 0° overnight; i.e., 8-24 hr. During this period, the chloroform phase forms a clear 1-2 cm layer above the caked residue.

Analysis of vitamin A by absorbance spectrophotometry. An aliquot (0.30 ml) of the chloroform extract is diluted to a total of 3.0 ml with ethanol, mixed and read in a 1-cm cuvette (4-ml capacity) in a Zeiss PMQ-111 spectrophotometer at 280, 330, 380 and 450 nm. Contaminant absorption in the ultraviolet region, traces of turbidity in the sample and  $\beta$ -carotene end-absorption are corrected by the formula:

$$\text{Corr. } A_{330} = 0.5 (2.27 \times A_{330} + 0.17 \times A_{450} - A_{280} - A_{380}) \quad (\text{Eq. 1})$$

Then,  $\mu\text{g retinol/g liver} =$

$$\frac{\text{corrected } A_{330} \times \text{dilution factor (e.g., 50 in the cited case)}}{0.1835 \times \text{sample weight (g)}} \quad (\text{Eq. 2})$$

And,  $\mu\text{g carotenoids/g liver} =$

$$\frac{A_{450} \times \text{dilution factor (e.g., 50 in the cited case)}}{0.25 \times \text{sample weight (g)}} \quad (\text{Eq. 3})$$

Analysis of vitamin A by the Carr-Price reaction. An aliquot (0.20 ml) of the same chloroform extract is placed in a 1-cm cuvette, 1.8 ml of freshly prepared 30% trichloroacetic acid in anhydrous chloroform is quickly and forcefully pipetted into the cuvette, and the absorbancy at 620 nm is measured at its maximum, usually 10 sec thereafter. If the absorbancy is very low (0.06) and does not decrease over 20-30 sec, the vitamin A content is taken as zero. The amount of vitamin A in the test is determined from a standard curve run at the same time with reference retinol acetate. When appreciable amounts of carotenoids are present, vitamin A is corrected in the following way:

$$\text{Corr. retinol } (\mu\text{g/g}) = \text{observed retinol } (\mu\text{g/g}) - \frac{\text{carotenoids } (\mu\text{g/g})}{20} \quad (\text{Eq. 4})$$

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### Comments on the procedure

1. The method requires approximately 10 min for the preparation of each extract and an average of 20 min for conducting both analyses. Duplicates invariably agree within 1%.
2. When fresh samples of human liver are used, gloves should be worn. Frozen liver samples should be weighed quickly, and all materials in contact with the sample should be decontaminated in ethanol before disposal.
3. Mashing should continue only to a point at which the sodium sulfate is reasonably well mixed with the liver paste. The mixture should not be ground to a fine powder because: a) some of the fine powder may escape as an aerosol and thereby pose a health hazard, and b) the chloroform layer becomes cloudy with fine particles, which adversely affect the assay and are difficult to remove. Ideally, the liver and sodium sulfate should cake together in the bottom of the vial during extraction with a crystal clear chloroform layer above it.
4. Manual pipetters are used for all transfers, never suction by mouth.
5. Fresh solutions of trichloroacetic acid are warmed to room temperature before use inasmuch as Carr-Price readings are about 50% higher with the cold reagent.

Materials. Reagent grade (A.C.S.) chemicals (namely, anhydrous sodium sulfate, chloroform stabilized with 0.75% absolute ethanol, absolute ethanol and white and dry crystalline trichloroacetic acid) were always used. Crystalline all-trans retinyl acetate was obtained from Distillation Products, Inc. (Eastman), and Arovit, a Hoffmann-LaRoche formulation of retinyl palmitate in an aqueous micellar solution, was used as a secondary standard. Red palm oil (Tropicana, Oldesa) was a commercial Brazilian product. Solvents were not distilled or further purified. Trichloroacetic acid was stored at 0°, and vitamin A products were kept under nitrogen at -20°.

Reagents. Vitamin A standard All-trans retinyl acetate (5.0 mg) was dissolved in 100 ml absolute ethanol. After a further 1:10 dilution with ethanol, the concentration of vitamin A in µg retinol per ml was calculated by use of an  $E_{1\text{cm}}^{1\%}$  of 1.55 at 330 nm. A first dilution of retinyl acetate (50 µg/ml), when stored in a refrigerator at +4°C, could be used as a standard for about a week. Nonetheless, the concentration of retinol was calculated each day from the absorbancy at 330 nm. The stock solutions, upon appropriate dilution with chloroform (not ethanol), were used for defining at least 3 points on the Carr-Price standard curve.

30% Trichloroacetic acid (TCA) in chloroform. Trichloroacetic acid (6g) was dissolved in 12 ml chloroform, warmed to room temperature, and diluted to 20 ml. As noted by others (5), the concentration of TCA

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is not critical; identical color yields were found with 22-35% TCA. Because TCA solutions deteriorate rapidly in the light (4,6) and lose 10-20% of their chromogenic capability per day even when stored at 0° in the dark, fresh TCA solutions were made up daily and were shielded from light before use.

### RESULTS

Extraction method. When comparable 1-g samples of frozen bovine liver were finely ground with 5 g anhydrous Na<sub>2</sub>SO<sub>4</sub> and left under 10 ml of a given solvent overnight at 0°, the yields (mean ± S.D.) of vitamin A in µg retinol/g were comparable; namely, diethyl ether (414 ± 25 µg/g), petroleum ether (429 ± 17 µg/g) and chloroform (420 ± 15 µg/g). By just mashing the sample gently in sodium sulfate, the color yield was 95% that of grinding; indeed, the color yield was about 80% of maximum when the liver sample was just covered with Na<sub>2</sub>SO<sub>4</sub> and a solvent for 18 hr.

In mashed samples, the extraction of vitamin A was 95% complete in 8 hr, and of carotenoids was nearly 100% complete in 3 hr. Thereafter, retinol concentrations remained constant for at least 24 hr. These extraction rates are roughly comparable to those reported for direct ethyl ether extraction (7, 8). In keeping with these past studies, a weight ratio of Na<sub>2</sub>SO<sub>4</sub> to liver samples of 2 to 5 gave the best results. Since 1-g samples of liver were normally used, a small glass scoop was fashioned, which contained 2.5 g Na<sub>2</sub>SO<sub>4</sub> when full.

To avoid loss of solvent during the 8-24 hr extraction period, the vials must be hermetically sealed. Glass screw-top vials (1.4 cm I.D. x 6 cm or 2 cm I.D. x 5 cm) equipped with conventional plastic-coated cardboard gaskets in Bakelite covers proved satisfactory, provided that chloroform was never in direct contact with the gasket. Ground-glass stoppers or pop-top containers were leaky, even when taped. Essentially no change in the relative volumes of the phases occurred during equilibration.

Standard curves. The absorbancy in ethanol at 330 nm was linear with the concentration of vitamin A at least to 1.0 under these conditions of assay. A slight downward curvature occurred in the Carr-Price assay, however, at retinol concentrations above 1 µg/ml. Consequently, standards of 0.5, 1 and 2 µg retinol/ml were run with each set of samples. The Carr-Price color yield varied slightly (+3%) from day to day, with an average  $E_{1\text{cm}}^{1\%}$  of 4400 at 620 nm, about 12% below maximal reported values (2).

Correction formula for spectrophotometric assays. Inasmuch as liver extracts contain a variety of compounds that absorb in the ultraviolet, the absorbancy at 330 nm must be suitably corrected (9). In the spectrum of pure vitamin A in ethanol, the absorbancy ratio of 280 nm to 330 nm is 0.21 (designated as a) and of 380 nm to 330 nm is 0.06 (designated as b). With  $\beta$ -carotene or red palm oil, the absorbancy ratio of 380 nm to 450 nm is 0.17 (denoted as c). Retinol does not absorb at 450 nm, nor does  $\beta$ -carotene absorb appreciably at 330 nm (<5% of 450 nm absorption).

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Sample turbidity (T) is most easily detected at 380 nm, where few lipid soluble constituents of liver absorb. Since turbidity values were small, the same correction was made at all four wavelengths. Carotenoids were expressed as  $\mu\text{g}$   $\beta$ -carotene by using an  $E_{1\%}^{1\text{cm}}$  at 450 nm of 2500 in ethanol. Inasmuch as the absorption of ultraviolet-absorbing compounds other than vitamin A is assumed to increase linearly from zero at 380 nm to 280 nm, the nonspecific absorption (NSA) at 330 nm would be 0.5 that at 280 nm.

At each of the wavelengths selected, the observed absorbancies consist of the following components:

$$A_{280} = \underline{a} \times \text{corr. } A_{330} + \text{NSA} + T \quad (\text{Eq. 5})$$

$$A_{330} = \text{corr. } A_{330} + 0.5 \text{ NSA} + T \quad (\text{Eq. 6})$$

$$A_{380} = \underline{b} \times \text{corr. } A_{330} + \underline{c} \times \text{corr. } A_{450} + T \quad (\text{Eq. 7})$$

$$A_{450} = \text{corr. } A_{450} + T \quad (\text{Eq. 8})$$

Use of the observed absorbancies at 330 nm and 450 nm in equations 5 and 7 in place of the corrected values had only a marginal effect on the calculated values. Although different assumptions might be made (e.g., changing the slope of the nonspecific absorption line between 380 and 280 nm), the above formulas have worked best over a wide range of liver retinol concentrations.

Equations 5-8 with the above-cited simplifications can then be solved for corrected  $A_{330}$ ; namely,

$$\text{corr. } A_{330} = \frac{(2 + \underline{a} + \underline{b}) A_{330} + \underline{c} \times A_{450} - A_{280} - A_{380}}{2} \quad (\text{Eq. 9})$$

Substitution of the determined values of  $\underline{a}$ ,  $\underline{b}$  and  $\underline{c}$  yields Eq. 1.

Correction for carotenoids in the Carr-Price assay. Carotenoids react rather slowly with Lewis acids to give a somewhat stable blue complex with maximal absorption at 590 nm (1). Under the described conditions of assay, 4 mg of mixed carotenoids in a sample of red palm oil gave an optical density at 620 nm, which was equivalent to 0.2  $\mu\text{g}$  retinol. Thus, vitamin A concentrations determined in this assay were corrected by equation 4. This correction of 5% is smaller than those (10% and 12%) used by others (1, 4) because vitamin A readings were taken in the assay at a maximum and before the carotenoid color was fully developed.

Recovery experiments. When retinyl acetate (177  $\mu\text{g}$  as retinol) in a tiny volume of chloroform was added to a liver sample containing 5.0  $\mu\text{g}$  retinol just before mashing, the recovery of added retinol was  $99.6\% \pm 0.5\%$  ( $n=3$ ).

Comparison with saponification procedures. Essentially equivalent liver samples containing approximately 120  $\mu\text{g}$  retinol were either

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extracted directly by the described method or were homogenized in water, saponified in 9% KOH and 90% ethanol at 60° for 30 min under nitrogen, extracted thrice with petroleum ether, evaporated under nitrogen and dissolved in chloroform. The ratio of retinol obtained by the direct extraction method to that obtained by saponification was  $114\% \pm 4.4\%$ , in accordance with the experience of others (7, 8).

Comparison of retinol values obtained by the spectrophotometric and Carr-Price assays. In a group of 37 autopsy specimens of human liver analyzed by the dual assay procedure, the range of values was 11-395  $\mu\text{g/g}$ . The median percentage difference between the two values, relative to the largest value, was 7.7%. The distribution of percentage differences was: <5%, 14; 5-10%, 11; 10-15%, 6; and >15%, 6. Two of the samples gave values that differed greatly, 47 and 28  $\mu\text{g/g}$  in one case and 6 and 19  $\mu\text{g/g}$  in the other. In both cases, anomalous peaks were found that grossly affected the spectrophotometric value. The mean ratios were not demonstrably influenced by the absolute concentration of retinol in the liver. Excluding the two anomalous cases, the mean percentage differences were: 10-20  $\mu\text{g/g}$ : 7.6% (n=7); 20-50  $\mu\text{g/g}$ : 6.4% (n=12); 50-100  $\mu\text{g/g}$ : 7.5% (n=10); and >100  $\mu\text{g/g}$ : 6.0% (n=6), with an overall mean of 7.5%. Among the 37 cases, the spectrophotometric value was higher than the Carr-Price value in 20 cases, lower in 15 and identical in 2.

Liver concentrations of retinol below 10  $\mu\text{g/g}$  must be considered separately. In 12 samples analyzed by this procedure, 5 had no detectable vitamin A by either assay procedure. In the other 7, the range of differences between the two assays was 0.8-4.1  $\mu\text{g/g}$ , with a mean difference of 2.5  $\mu\text{g/g}$ . In all but one of these latter cases, however, the Carr-Price value was higher than the spectrophotometric value.

### DISCUSSION

A novel aspect of the present procedure is that two assays of different kinds are run on each extract. Inasmuch as any single assay of crude extracts is plagued by nonspecific reactions or by inhibitors, the dual assay gives a good indication of the variability produced by extraneous factors. When the agreement between values is close, one feels confident that the value obtained has validity. On the other hand, when the values are disparate, other techniques might be used to obtain more precise information. The only alternative to dual analysis is careful, high-resolution chromatographic procedures, which are both costly and time consuming.

The present procedure was developed as a tool for nutritional surveys, for which reasonable estimates of retinol concentrations in liver satisfy the needs of the study. The defined spectrophotometric procedure unquestionably gives only an estimate inasmuch as the nature of ultraviolet-absorbing compounds found in a wide variety of liver samples will unquestionably differ. Nonetheless, the spectrophotometric estimate generally agreed very closely with the Carr-Price value over a wide range of liver retinol concentrations. This agreement was particularly gratifying at very low retinol values where maximal error in the spectrophotometric reading would be expected.

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The principle of the method (i.e., simple extraction and dual analysis) can be readily modified to meet specific needs. Since petroleum ether, for example, is fully as effective an extractant as ethyl ether or chloroform under the defined conditions of extraction, a dual assay involving absorbance spectrophotometry and fluorescence rather than the Carr-Price assay might also be developed.

### ACKNOWLEDGMENTS

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### REFERENCES

1. Moore, T., Vitamin A, Elsevier Publ. Co., Amsterdam, 1957, p. 40-66.
2. Olson, J. A., in *Newer Methods of Nutritional Biochemistry*, Vol. 2 (A. A. Albanese, editor) Academic Press, Inc., New York, 1965, p. 348-357.
3. Thompson, J. H., Erdody, P., Brien, R. and Murray, T. K. Fluorometric determination of vitamin A in human blood and liver. *Biochem. Med.* 5: 67-89 (1971).
4. Neeld, J. B., and Pearson, W. N. Macro- and Micromethods for the Determination of Serum Vitamin A using Trifluoroacetic Acid. *J. Nutr.* 79: 454-462 (1963).
5. Bayfield, R. F. Colorimetric Determination of Vitamin A with Trichloroacetic Acid. *Anal. Biochem.* 39: 282-287 (1971).
6. Subramanyam, G. B., and Parrish, D. B. Colorimetric reagents for determining vitamin A in feeds and foods. *J. Assoc. Off. Anal. Chem.* 59: 1125-1130 (1976).
7. Ames, S. R., Riskey, H. A. and Harris, P. L. Simplified Procedure for Extraction and Determination of Vitamin A in Liver. *Anal. Chem.* 26: 1378-1381 (1954).
8. Hinds, F. G., Peter, A. P. and Richards, G. E. Methods of Extracting Vitamin A from Liver. *J. Anim. Sci.* 27: 1678-1681 (1968).
9. Morton, R. A., and Stubbs, A. L. Studies in Vitamin A. 4. Spectrophotometric determination of vitamin A in liver oils: correction for irrelevant absorption. *Biochem. J.* 42: 195-203 (1948).

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**III.**  
**Separation of Retinol, Retinyl Esters and Carotenoids**

## SHORT COMMUNICATIONS

### Separation of Vitamin A from Carotenoids in Micro Samples of Serum

Severe vitamin A deficiency (xerophthalmia) is an important cause of blindness in young children in many parts of the Near and Far East. Repeated determinations of serum vitamin A levels on these sick children necessitate micro samples. The method of Bessey *et al.* (1), in which the optical density of vitamin A is measured at 328  $m\mu$ , is subject to many errors (2). A more recent method (3), suitable for micro samples, depends on the blue color formation with trifluoroacetic acid resulting from both carotenoids and vitamin A, with a correction applied for the amount of carotenoids present. As this correction is derived from a pure  $\beta$ -carotene standard, the accuracy of the value obtained for vitamin A depends on the carotenoids being  $\beta$ -carotene, since other carotenoids give varying intensities of color with the chromogen. It is known that blood carotenoids are a reflection of dietary carotenoids (4). Foodstuffs vary widely in their carotenoid composition. We have encountered cases showing only minimal clinical evidence of vitamin A deficiency with extremely low serum levels of the vitamin in the presence of high levels of carotenoids. With large quantities of material it is possible to separate vitamin A from the carotenoids by column chromatography, but on a micro scale separation by paper or thin-layer chromatography has resulted in low recoveries. A simple and rapid method of separation is described here and its usefulness illustrated in the study of human vitamin A deficiency being undertaken in this laboratory, the results of which will be presented elsewhere.

*Method.* Capillary glass tubes, 100  $\times$  2 mm, of the type used for melting point determinations, were partly closed at one end by a sintered glass plug formed by filling each capillary tube to a depth of 2 mm with glass powder and heating it in a cool flame for a few seconds. Excess glass powder was tapped out. About 100 such capillaries were tightly packed into a glass tube, one end of which was closed with a cork. The space above the capillaries was filled with the absorbent and the tube vibrated in a vertical position by holding it against a rapidly rotating drill. Complete filling with uniform packing was achieved in about 10 min. The

adsorbent found most useful for separating the individual carotenoids was alumina (200 mesh). Silicic acid (325 mesh) gave better separation of vitamin A from the carotenoids.

Serum (0.1 ml) and 0.1 ml of 5% alcoholic potassium hydroxide were pipetted into a 50 x 5 mm tube, and thoroughly mixed. The tube was then placed in a water bath at 60 C for 20 min. After cooling, 0.15 ml of petroleum ether (boiling range 40-50 C) was added. The contents were thoroughly mixed by holding the tube against a rapidly rotating deid for 1 min, after which the tube was centrifuged; 0.1 ml of the petroleum layer was transferred to another tube and the volume reduced under a stream of nitrogen at room temperature to about 0.01 ml. When the sintered glass end of the capillary column was placed into the solution the sample was absorbed on the capillary column. The walls were washed with 0.01 ml of petroleum ether and the washing similarly absorbed into the column. The column was developed by standing it upright in a small tube containing petroleum ether. The solvent front reached the top of the adsorbent in about 10 min.

When using silicic acid (325 mesh), vitamin A was concentrated in a narrow band near the bottom of the column ( $R_f$  0.05) while  $\beta$ -carotene traveled to the top of the column ( $R_f$  0.8). Other unidentified yellow pigments were frequently found between these two bands. The section of the capillary column containing the vitamin A was cut off and extracted with chloroform, and vitamin A determined by reading the intensity of the blue color produced with trifluoroacetic acid reagent (3). Recoveries from capillary columns of known quantities of vitamin A under such conditions in the presence of varying amounts of  $\beta$ -carotene were better than 90% (see Table I).

This procedure was carried out on 12 samples of serum, 6 containing high levels of carotenoids and low levels of vitamin A, and the other 6

TABLE I  
RECOVERY OF VITAMIN A FROM CAPILLARY COLUMNS IN THE PRESENCE  
OF VARYING AMOUNTS OF  $\beta$ -CAROTENE

	Vitamin A content of sample, $\mu\text{g}$	$\beta$ -Carotene content of sample, $\mu\text{g}$	Vitamin A recovered, $\mu\text{g}$	% Recovery
1	0.032	0	0.030	94
2	0.055	0	0.054	98
3	0.110	0	0.111	101
4	0.023	0.015	0.023	100
5	0.057	0.077	0.056	98
6	0.065	0.075	0.063	97
7	0.128	0.150	0.124	97

containing low levels of both carotenoids and vitamin A as measured by the method of Neeld and Pearson (3). The values for vitamin A found by these two methods are compared in Table 2.

TABLE 2  
COMPARISON OF VITAMIN A ESTIMATIONS BEFORE AND AFTER SEPARATION  
ON CAPILLARY COLUMNS

Sample No.	Method of Neeld and Pearson		Method described
	Carotenoids, $\mu\text{g } \%$	Vitamin A, $\mu\text{g } \%$	Vitamin A, $\mu\text{g } \%$
1	70	0	19
2	94	0	6
3	70	1	13
4	107	3	4
5	81	3	14
6	143	2	8
7	14	0	1
8	17	11	8
9	14	19	16
10	24	0	5
11	11	3	5
12	26	7	7

These results show that the carotenoids present in serum can cause falsely low values to be calculated for vitamin A. This effect is most apparent and the error is more serious when the vitamin A content is low. The method is also being applied to the separation of micro quantities of other lipids present in serum.

#### ACKNOWLEDGMENT

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#### REFERENCES

1. BESSEY, O. A., LOWRY, O. H., BROCK, M. J., AND LOPEZ, J. A., *J. Biol. Chem.* **166**, 177 (1946).
2. UTELY, M. H., BRODOVSKY, E. R., AND PEARSON, W. N., *J. Nutr.* **66**, 205 (1958).
3. NEELD, J. B., AND PEARSON, W. N., *J. Nutr.* **79**, 454 (1963).
4. GOODWIN, T. W., "The Comparative Biochemistry of the Carotenoids," p. 229. Chapman and Hall, London, 1952.

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# Simultaneous Determination of Retinol and Retinyl Esters in Serum or Plasma by Reversed-Phase High-Performance Liquid Chromatography

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We propose a single-run liquid-chromatographic determination, with ultraviolet detection at 330 nm, for serum retinol and retinyl esters. The vitamin A derivatives are extracted according to the Bligh-Dyer procedure. With 200  $\mu$ l of serum, the lower detection limit is 50  $\mu$ g/liter for retinol and about 100  $\mu$ g/liter for retinyl esters. Within-run precision (CV) was 2.3% for retinol, 4.3% for retinyl palmitate. Day-to-day precision (CV,  $n = 20$ ) for retinol was 4.9% during a month. The method can be used for the assessment of vitamin A absorption tests and for the determination of serum retinol (normal, subnormal, and above-normal concentrations). Serum retinyl esters can only be measured in conditions where concentrations exceed 100  $\mu$ g/liter.

**Additional Keyphrase:** *vitamin A absorption tests*

The importance of the simultaneous determination of the free and esterified forms of vitamin A alcohol in serum has been shown by Goodman et al. (1, 2). They demonstrated that hypervitaminosis A in human beings, as in laboratory animals, was characterized by increased serum concentrations of retinyl esters, even in conditions where retinol concentrations were normal.

The determination of retinol and retinyl esters is usually performed by column-chromatographic separation followed by fluorometry (1, 2) or by batch separation on alumina followed by colorimetry with trifluoroacetic acid (3).

We describe here a high-performance liquid-chromatographic method for simultaneously determining retinol and retinyl esters by reversed-phase chromatography, with on-line ultraviolet detection at 330 nm. A similar method was recently reported by Abe et al. (4), using fluorometry for on-line detection. We reported a method for the specific determination of serum retinol by adsorption high-performance liquid chromatography (5).

## Materials and Methods

### Chromatographic Equipment

We used a Varian 4100 liquid chromatograph equipped with a Varichrom multiple-wavelength detector (Varian Associates, Palo Alto, Calif.) and a Valco CV-6-UHPA-N60 injection valve

with a 50- $\mu$ l loop (Valco Instr. Co., Houston, Tex.) All analyses were performed on a 15  $\times$  0.32-cm column packed with 10- $\mu$ m RSII, C<sub>18</sub> HL (octadecylsilica with 18% bonded organic material). The column tubing (Lichroma SS) and packing material were obtained from RSL, St. Martens-Latem, Belgium. The column was packed in our laboratory by a slurry technique (6) under the following conditions: slurry liquid, tetrachloromethane; slurry concentration, 40 g/liter; packing pressure, 20.7 MPa (3000 psi); pump, Varian 8500; pressurizing liquid, methanol. The column had an initial efficiency of 5750 plates at a flow-rate of 0.1 ml/min (reduced plate height  $h = 2.6$  at reduced velocity  $v = 3.6$ ) measured for retinol ( $k' = 0.97$ ) with methanol as eluent. It was operated at ambient temperature.

Other equipment included a Rotary Evapo-Mix (Buchler Instrument Div., Div. Searle Diagnostics Inc., Fort Lee, N. J. 07024), an ultrasonic bath, a bench-top centrifuge, a double-beam ultraviolet spectrophotometer (Pye Unicam SP 1800, Pye Unicam, Cambridge, UK), and 14  $\times$  100 mm Teflon-lined screw-capped centrifuge tubes.

### Reagents

Methanol and chloroform were analytical-grade reagents from Merck, Darmstadt, Germany, and were used without further purification.

All-*trans*-retinol and all-*trans*-retinyl palmitate were of crystalline purity from Sigma Chemical Co., St. Louis, Mo. 63178. Retinyl propionate, the internal standard, was obtained from A.E.C., 03600 Commeny, France. Retinyl stearate, retinyl oleate, retinyl linoleate, retinyl myristate, and retinyl laurate were synthesized by reaction of retinol with the corresponding acyl chlorides (7). The reaction products were purified on a Lobar RP<sub>8</sub> column (Merck), with methanol as eluent. The concentration of the Vitamin A solutions used in this work were determined from their generally accepted molar absorptivity values (8, 9).

### Procedure

**Sample extraction.** Transfer 200  $\mu$ l of serum (or plasma) to a centrifuge tube; add 0.6 ml of water, 2.0 ml of methanolic solution of internal standard, 137  $\mu$ g/liter, and 1.0 ml of CHCl<sub>3</sub>. After thorough mixing for 1 min and standing for 5 min, add 1.0 ml of water and 1.0 ml of CHCl<sub>3</sub>. Mix gently and centrifuge (1500  $\times g$ , 5 min). Transfer the CHCl<sub>3</sub> (lower) phase to an evaporation tube and evaporate under reduced pressure. Dissolve the residue in 100  $\mu$ l of CH<sub>3</sub>OH/CHCl<sub>3</sub> (4/1 by vol). After sonication for 10 min, inject 50  $\mu$ l of the solution on top of the column.

**Chromatography.** Methanol is used as the mobile phase at

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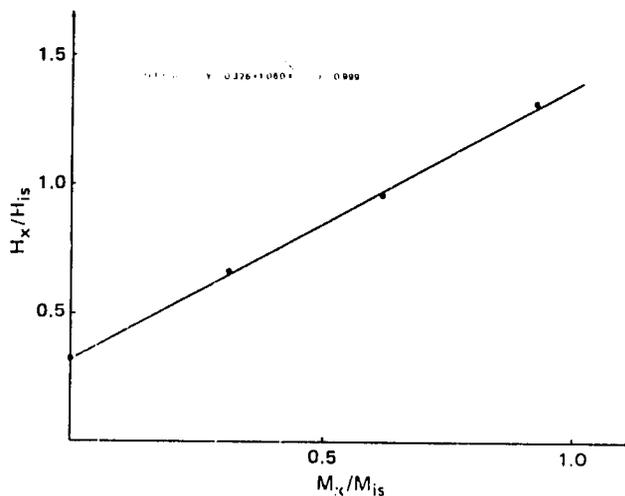


Fig. 1. Calibration curve for retinol (peak-height ratios vs. mass ratios)

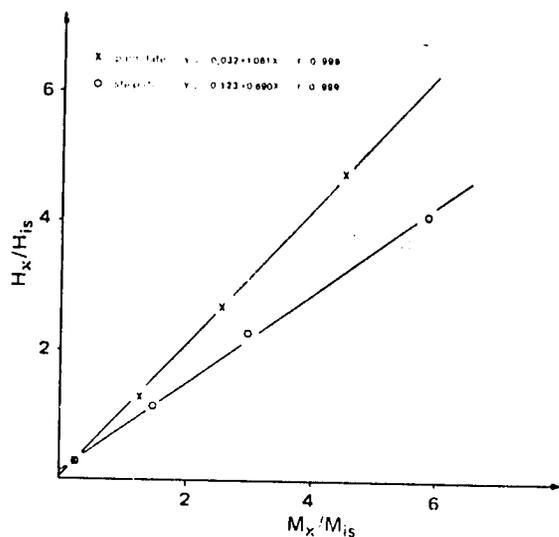


Fig. 2. Calibration curves for retinyl palmitate (upper) and for retinyl stearate (lower) (peak height ratios vs. mass ratios)

a flow rate of 1 ml/min, and the effluent is monitored at 330 nm. Variable detection sensitivities are used in order to register the peaks in-scale. The most frequent settings were 0.1 A full-scale for the first 4 min (detection of retinol and retinyl propionate) and 0.01 A full-scale to continue the run (detection of the higher retinyl esters).

**Quantitation.** The procedure is standardized by adding retinol, retinyl palmitate, and retinyl stearate to samples of a serum pool and taking these through the entire procedure. Peak heights of retinol and retinyl propionate (internal standard) are measured and recalculated to a 0.1 A full-scale setting, whereas the peak heights of the higher retinyl esters are measured and converted to a 0.01 A full-scale setting. From values obtained, the peak height ratio is calculated [analyte/retinyl propionate (internal standard)]. Working curves (Figures 1 and 2) for each analyte relate peak height ratio to mass ratio. The working curve for retinol (Figure 1) is used after subtraction of the intercept with the y-axis. This intercept represents the endogenous retinol concentration of the serum pool used for standardization. No significant intercept is found for retinyl palmitate or retinyl stearate (Figure 2) because the normal concentrations in serum are too low to be detected. Serum concentrations in the unknown samples are easily determined after measurement of the peak height ratios by use of these working curves.

Table 1. Analytical Precision

	CV %	n	$\bar{x}$ , $\mu\text{g/liter}$
<i>Within-day</i>			
Retinol	2.0	11	720
Retinyl palmitate	4.3	11	6460
Retinyl stearate	6.0	11	4150
<i>Day-to-day</i>			
Retinol	4.9	20	650

Table 2. Extraction Recovery

Substance	$\bar{x}$ , %	SD, %	CV, %	n	Range, $\mu\text{g/liter}$
Retinol	97.0	6.1	6.3	8	160–1300
Retinyl palmitate	96.6	7.0	7.3	7	160–3200

Table 3. Serum Vitamin A Concentrations of Three Normal Adults 4 h after Ingestion of 200 000 int. units of Vitamin A Palmitate (with a Meal)

Sex	Retinol	Retinyl palmitate $\mu\text{g/liter}$	Retinyl stearate
♂	760	1820	800
♂	710	840	500
♀	540	1000	700

## Results

Peak height ratio and mass ratio were linearly related over the range of 0–0.93 for retinol (corresponding to concentrations of 0–984  $\mu\text{g/liter}$ ) and 0–5.8 for retinyl esters (corresponding to concentrations of 0–5.50 mg/liter) (Figures 1 and 2).

**Reproducibility.** We determined within-day precision of the method by performing 11 replicate analyses on a serum sample from a subject to whom 300 000 int. units of vitamin A was administered. Day-to-day precision for retinol was estimated by analyzing serum samples from a serum pool (stored in darkness at  $-18^\circ\text{C}$ ) during a one-month period ( $n = 20$ ). The results are shown in Table 1.

**Extraction recovery.** This was estimated by analyzing serum samples supplemented with retinol (in amounts giving concentrations ranging from 160 to 1300  $\mu\text{g/liter}$ ) and with retinyl palmitate (160 to 3200  $\mu\text{g/liter}$ ). The internal standard was added to the  $\text{CHCl}_3$  layer after extraction. Results are given in Table 2.

**Assessment of a vitamin A absorption test.** To three subjects, 200 000 int. units of vitamin A palmitate was orally administered together with a light meal. Blood was sampled 4 h later and the plasma analyzed according to our procedure (Table 3). A typical chromatogram obtained from such an experiment is shown in Figure 3.

**Identity of the serum peaks.** Peaks were identified on the basis of their retention characteristics. Retention times of the serum peaks matched those from a standard mixture containing the different vitamin A derivatives. A chromatogram of such a standard mixture is shown in Figure 4. Capacity ratios ( $k'$ ) of retinol and retinyl esters are given in Table 4. Absorption maxima of the serum peaks were also determined by injecting a fixed amount, with a fixed-volume (10  $\mu\text{l}$ ) loop, of a serum extract at different wavelength settings and recording the peak heights. The absorption maxima ( $325 \pm 5$

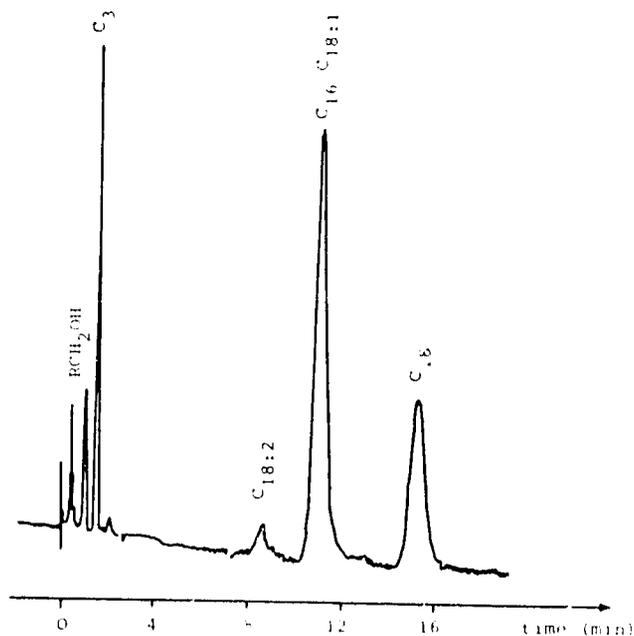


Fig. 3. Chromatogram of a serum extract from a subject 4 h after receiving 300 000 int. units of Vitamin A palmitate  
 $RCH_2OH$  = retinol;  $C_3$  = retinyl propionate (int. std.);  $C_{18:2}$  = retinyl linoleate;  $C_{18:1}$ ,  $C_{16}$  = retinyl oleate, retinyl palmitate;  $C_{18}$  = retinyl stearate

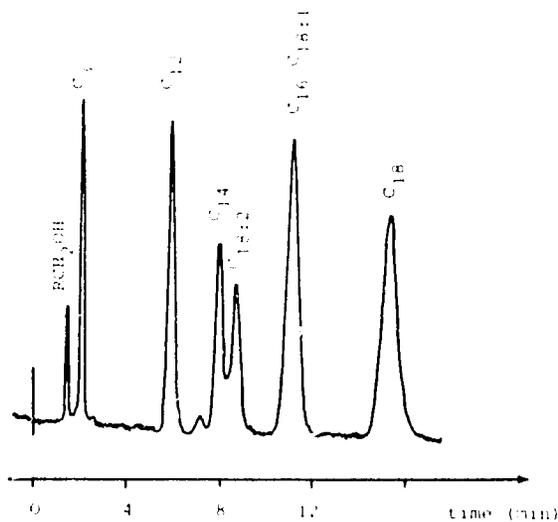


Fig. 4. Chromatogram of a standard mixture containing retinol ( $RCH_2OH$ ), retinyl propionate ( $C_3$ ), retinyl laurate ( $C_{12}$ ), retinyl myristate ( $C_{14}$ ), retinyl linoleate ( $C_{18:2}$ ), retinyl palmitate ( $C_{16}$ ), retinyl oleate ( $C_{18:1}$ ), and retinyl stearate ( $C_{18}$ )

Table 4. Capacity Ratios ( $k'$ ) of Retinol and Retinyl Esters on a 10- $\mu$ m RSIL  $C_{18}$  HL Column<sup>a</sup>

	$k'$
Retinol	0.97
Retinyl propionate	1.89
Retinyl laurate	7.17
Retinyl myristate	10.13
Retinyl linoleate	11.11
Retinyl palmitate	14.28
Retinyl oleate	14.49
Retinyl stearate	20.27

<sup>a</sup>  $k'$  is the ratio of the weight of the solute (sample) in the stationary phase to that in the mobile phase of the column. It is a retention parameter.

nm) were identical for all peaks and agreed with values reported in the literature for retinol and retinyl esters (9).

## Discussion

According to previous work on retinyl ester composition after vitamin A intake (7), four different esters should be found in lymph. With our method the chromatogram (Figure 3) of a serum extract, containing retinyl esters formed *in vivo*, showed only four peaks with absorption at 330 nm. The first peak ( $RCH_2OH$ ) is also present in a serum extract of a fasting person, and is retinol. Apart from retinyl propionate ( $C_3$ ) used as internal standard<sup>3</sup> three retinyl ester peaks are found. The first ester peak ( $C_{18:2}$ ) is very small and is probably retinyl linoleate. The second ( $C_{16}$ ,  $C_{18:1}$ ) is a composite peak containing retinyl palmitate and retinyl oleate. These two esters cannot be separated by reversed-phase chromatography on octadecyl silica with methanol as eluent. To resolve them, a more selective system should be used. Reversed-phase chromatography with  $AgNO_3/CH_3OH$  as mobile phase is being investigated in our laboratory and seems quite promising. The third ester peak ( $C_{18}$ ) is retinyl stearate.

The use of heavily loaded silica (18% organic material bonded) is imperative for the simultaneous determination of retinol and retinyl esters. It allowed us to use pure methanol as mobile phase. If lower loadings of organic phase are used, water must be added to the eluent to cause retention of retinol. However, the increased water content of the mobile phase can cause precipitation of the esters. Furthermore, the residue of the serum extract will not dissolve in a  $CH_3OH/H_2O$  mixture. Even with pure methanol, the residue is not completely dissolved and the analytes are not quantitatively brought into solution. Therefore, we deliberately add 20%  $CHCl_3$  to the injection solvent and sonicate for 10 min. This results in complete dissolution of the residue but decreases the column efficiency for early eluting peaks, a decrease that fortunately does not alter the resolution too much.

Abe et al. (1) developed two methods for determining retinol and retinyl palmitate, one based on reversed-phase chromatography and the other on adsorption chromatography. To overcome the problems on reversed-phase systems mentioned above, they used a mixture of isopropanol/ethanol/water as mobile phase and fluorometry for detection. Due to the specificity of the fluorescence detector, retinol has probably not to be retained on the column to afford specificity. Unfortunately, their paper only reports retention times and not  $k'$ -values, and thus this assumption cannot be verified. The use of the fluorescence detector also allowed them to use an adsorption system for determination of low concentrations of retinyl palmitate. Apart from the fact that the palmitate is probably unretained, they did not include retention times for phytofluene, a highly fluorescent compound that interferes with fluorometric assays, as reported by Thompson et al. (10).

We had to use the Bligh-Dyer (11) extraction to get a high extraction recovery for retinyl esters. We failed to achieve reproducible and quantitative extraction with a hexane procedure as reported by other workers (2-4).

For the assessment of vitamin A absorption tests, this method has the advantage that it does not require a blood sample before ingestion of the vitamin. Serum retinol concentrations are unaffected whether or not vitamin A has been ingested. The ingested vitamin A is present in the serum only in the esterified form. In serum from normal fasting persons, the concentration of retinyl esters is too low to be measured, so the test can be interpreted by the concentration of retinyl esters present in serum after ingestion. The advantage of a single venepuncture and only one analysis over analysis of two samples taken at 4-h interval is obvious.

In summary, reversed-phase high-performance liquid chromatography for determination of retinol and retinyl esters offers several advantages over other assays now in use. The column chromatography with on-line ultraviolet detection at 330 nm provides specificity and permits the determination of both free and esterified vitamin A in serum. The method is rather sensitive, because only 200  $\mu$ l of serum is necessary to obtain detection limits of 50  $\mu$ g/liter for retinol and  $\pm$ 100  $\mu$ g/liter for retinyl esters. The addition of an internal standard (retinyl propionate) before the extraction improves the precision and compensates for possible losses during manipulation. Use of a fluorescence detector would improve the procedure, because a fivefold increase in sensitivity together with a better specificity can be expected. However, fluorescence detection is more subject to problems than is the usual absorption detection technique.

## References

1. Mahlia, A. K., Smith, J. E., and Goodman, De W. S., Metabolism of retinol-binding protein and vitamin A during hypervitaminosis A in the rat. *J. Lipid Res.* **16**, 180 (1975).
2. Smith, F. R., and Goodman, De W. S., Vitamin A transport in human Vitamin A toxicity. *N. Engl. J. Med.* **294**, 805 (1976).
3. Vahlquist, A., A simplified technique for determination of the vitamin A composition in biological fluids. *Int. J. Vitam. Nutr. Res.* **44**, 375 (1974).
4. Abe, K., Ishibashi, K., Ohmae, M., et al., Determination of Vitamin A in serum and liver by high-speed liquid chromatography. *Vitamins* **51**, 272 (1977). (In Japanese).
5. De Ruyter, M. G. M., and De Leenheer, A. P., Determination of serum retinol (vitamin A) by high-speed liquid chromatography. *Clin. Chem.* **22**, 1593 (1976).
6. Webber, T. J. N., and McKerrel, E. H., Optimisation of liquid chromatography: performance on columns packed with microparticulate silicas. *J. Chromatogr.* **122**, 243 (1976).
7. Huang, H. S., and Goodman, De W. S., Vitamin A and carotenoids. I. Intestinal absorption and metabolism of  $^{14}$ C-labeled vitamin A alcohol and  $\beta$ -carotene in the rat. *J. Biol. Chem.* **240**, 2839 (1965).
8. A. E. C. (03600 Commeny, France) data sheet on Axerofluid\*, propionate de vitamine A, propionate de r tynyle.
9. Roels, O. A., and Mahadevan, S., in *The Vitamins*, II, P. Gyorgy and W. N. Pearson, Eds., Academic Press, New York and London, 1967, p. 143.
10. Thompson, J. N., Erdody, P., Brien, R., and Murray, T. K., Fluorometric determination of vitamin A in human blood and liver. *Biochem. Med.* **5**, 67 (1971).
11. Bligh, E. G., and Dyer, W. J., A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.* **37**, 911 (1959).

## UPTAKE OF RETINOL, RETINOL-BINDING PROTEIN AND THYROXINE-BINDING PREALBUMIN BY EGG YOLK OF JAPANESE QUAIL

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**Abstract** 1. The water soluble fraction of quail egg yolk was found to contain retinol-binding protein (RBP) and thyroxine-binding prealbumin (TBPA) immunochemically similar to their counterparts in domestic fowl blood. Yolk RBP concentration was below half that of plasma, whereas TBPA concentration was about the same in both fluids.

2. Less than 4% of yolk retinol was attached to RBP (holoRBP) and the holoprotein accounted for about half the total RBP.

3. At oviposition, the yolk content of retinol, but not that of holoRBP, was correlated with holoRBP concentration in blood plasma.

### INTRODUCTION

Most of the water-soluble (livetin) proteins of avian egg yolk resemble in immunochemical and electrophoretic properties their counterparts in blood plasma. Of the major plasma proteins, albumin,  $\gamma$ -globulin and transferrin have been found by several groups (see McIndoe & Culbert, 1979, for a bibliography) in the livetin of domestic fowl egg yolk. Of the minor protein constituents of avian plasma found more recently in egg yolk are the two carrier proteins involved in vitamin A transport in the blood, retinol-binding protein (RBP) and thyroxine-binding prealbumin (TBPA). This was first confirmed for domestic fowl (Heller, 1976) and later for duck (Sreekrishna & Cama, 1978). The uptake of vitamin A from the maternal blood circulation by the developing oocyte is a process essential for the maintenance of normal reproduction in the domestic fowl (Thompson *et al.*, 1969) because the embryos of eggs laid by vitamin A deficient-hens fail to develop beyond day 2 unless vitamin A derivatives, of which the most potent is retinol, are injected into the eggs before incubation.

In Japanese quail (*Coturnix coturnix Japonica*), the supply of retinol transported in the blood bound to the RBP-TBPA complex appears to be under hormonal control because there is a specific rise in RBP concentration (Heaf & Glover, 1979; Glover *et al.*, 1980) as the birds enter a period of maximal retinol utilisation during the reproductive seasons of spring and summer. Towards the end of this period, the plasma concentration of holoRBP (RBP with retinol attached) declines whereas the concentration of total RBP (the sum of holoRBP and apoRBP, i.e. RBP without retinol attached) remains high for a further 2-3 weeks before it too falls to a minimum in the autumn. This contrasting change in the two forms of RBP in the blood at the end of the reproductive

season in the quail has been attributed to a combination of maturing oocytes removing retinol from the blood leaving the apo form of the carrier protein in the circulation and a steep fall in holoRBP secretion from the liver (Heaf & Glover, 1979). The effect of these two processes is to increase the ratio of apoRBP to holoRBP in the blood. However, if the uptake of retinol by the quail oocyte is similar to that of the domestic fowl which is reported to produce egg-yolk with all the vitamin A attached to the RBP-TBPA complex (Heller, 1976), the accumulation of apoRBP in the plasma of laying quail at the end of the reproductive season must arise by an alternative process.

The work reported here was undertaken in order to provide a better understanding of the vitamin A uptake process in the maturing avian oocyte. Experiments were carried out to measure retinol, holoRBP, total immunoreactive RBP and prealbumin in the egg yolk of Japanese quail and to determine the influence of the maternal plasma holoRBP concentration on the uptakes of both retinol and holoRBP by the yolk.

### MATERIALS AND METHODS

Eggs were obtained within 24 hr of their being laid by 6-12 month old Japanese quail housed one bird to a cage with free access to Turkey Super Starter Crumbs (J. Bibby & Co. Ltd, Preston). Where comparisons of yolk and maternal plasma constituents were made, individual eggs were marked to identify the donor. Selected analyses were performed on single eggs collected from each of 43 quail.

Blood (100  $\mu$ l) was collected into heparinised glass capillary tubes by making a small hole with a syringe needle in a superficial brachial wing vein where it passes over the humeral radial ulnar joint. Plasma, separated by centrifugation after sealing one end of the capillary tube in a flame, was assayed immediately for holoRBP.

#### Isolation of yolk fractions

Individual yolks were separated from the white, placed in a preweighed stoppered vial, and the weight of the yolk was determined. A value of unity was used for the density of yolk which was found by experiment to be 1.032. An

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amount of phosphate buffered saline (PBS, 0.15 M NaCl, 0.02 M phosphate, pH 7.5) equal in volume to the yolk was added to the vial, the yolk membrane was ruptured with a spatula and the stoppered vial was shaken vigorously for 1 min to disperse the yolk contents in the PBS. Approximately 1 ml of this crude yolk homogenate was centrifuged at 16,000 *g* for 5 min at room temperature in a micro-centrifuge (Quickfit Instrumentation, Stone, Staffs.). The supernatant which was slightly cloudy with some colloidal material was used immediately for the determination of holoRBP, immunoreactive RBP and prealbumin. The water contents of native yolk, crude yolk homogenate and the supernatant from centrifuged yolk homogenate were determined by drying weighed amounts of the homogenate and the supernatant to constant weight in an oven at 105 °C.

#### Determination of retinol

To 1 ml of crude yolk extract, sampled with a microsyringe (Unimetrics Corporation, Anaheim, California), was added 1 ml ethanol in a 5 ml glass stoppered test tube. After shaking vigorously for 30 sec 2 ml light petroleum (b.p. 40–60 °C) peroxide free diethyl ether (70% 30%) mixture containing 1 mg butylated hydroxy toluene (BHT, British Drug Houses Ltd, Poole) was added. The mixture was then shaken for 2 min and the emulsion which formed was separated into two distinct layers in the tube by centrifugation at approx. 50 *g*. The upper organic layer was transferred to a conical glass tube and the extraction was repeated as before by adding a second 2 ml portion of solvent mixture. The combined solvent extracts were evaporated to dryness at room temperature in the dark under a stream of nitrogen. 0.5 ml cyclohexane was then added to dissolve the lipid extract which was then drawn into a clean dry 1 ml microsyringe, taking care to avoid drawing up air. After recording the volume of the extract it was stored in a stoppered vial surrounded by aluminium foil to exclude light, at 4 °C. A standard solution of retinol was prepared by dissolving 1 mg retinol (Sigma Chemical Co., London) in 10 ml cyclohexane containing arachis oil (10 g/l) and BHT (0.5 g/l). The exact concentration of retinol was determined from the u.v. absorbance of a 1:20 dilution of the standard in spectroscopically pure ethanol, using  $\epsilon_{325}$  52480. Standards and extracts were spotted with a microsyringe (10  $\mu$ l, Hamilton, Bonaduz, Switzerland) 2 cm from one edge of a 20 × 20 cm thin-layer plate coated with silica gel G (Merck, Darmstadt) to a depth of 0.25 mm. Six standard spots in the range 0.05–0.25  $\mu$ g retinol were distributed across the plate, alternating with six 5  $\mu$ l applications of extracts (duplicate determinations of three yolk extracts). The plate was developed in a solvent mixture (Kahan, 1967) of light petroleum (b.p. 40–60 °C) peroxide free diethyl ether acetic acid (90:10:1) containing BHT 5 g/l, for a distance of 14 cm from the origin. After drying the plate the single row of fluorescent spots was located by brief exposure of the plate to u.v. light and marking either end of the row. The determination of the retinol content of the spots was performed immediately on a Scanning Thin Layer Reflectance Densitometer (Carl Zeiss, Germany) which had been prepared ready for use. Spots were scanned in the direction of plate development with a monochromator wavelength of 330 nm, a slit width of 0.2 mm and a slit length of 10 mm which was slightly larger than the diameter of the spots. The areas of the recorder peaks for the spots were determined by triangulation and a calibration curve was drawn for the standard spots. The retinol contents of the extract spots were read from the calibration curve and using the values obtained for the volumes of the lipid extract and the crude yolk homogenate, the retinol content of the yolk was calculated.

#### Determination of TBPA and RBP

HoloRBP was determined by scanning fluorimetry of the green retinol u.v. fluorescence of holoRBP separated from

other proteins on polyacrylamide gels (Glover *et al.*, 1974). 50  $\mu$ l of supernatant of yolk homogenate in which was dissolved 5 mg sucrose or 5  $\mu$ l plasma mixed with the same amount of a 200 g/l solution of sucrose in PBS was applied directly to the gel. The assay was standardised by similar treatment of 20  $\mu$ l portions of a human serum pool stored in small aliquots under nitrogen at -20 °C, for which the holoRBP concentration was accurately determined. The retinol fluorescence yield of pure human and quail RBP have been found to be identical.

Plasma immunoreactive RBP and TBPA were determined by radial-immunodiffusion (Manem *et al.*, 1965) after dilution of one part of plasma with nine parts of PBS containing 30 g/l bovine serum albumin (BSA/PBS) for RBP assay and with 19 parts of BSA/PBS for TBPA assays. The colloid in the yolk homogenate supernatant rendered this preparation unsuitable for the radial-immunodiffusion method of protein assay because of the difficulty experienced in washing material other than the immunoprecipitate from the agarose prior to staining. Yolk immunoreactive RBP and TBPA were therefore determined by immunoelectrophoresis (Laurell, 1966). The antisera used were prepared as described elsewhere (Heaf & Glover, 1979; El-Sayed *et al.*, 1980). One part of yolk homogenate supernatant was diluted with one part of BSA/PBS for the assay of RBP and with four parts of BSA/PBS for the assay of TBPA. A single set of standards for use in both immunoassays was prepared by diluting quail serum, in which the RBP and TBPA concentrations had been determined using the pure proteins isolated from chicken serum (Heaf & Glover, 1979; El-Sayed *et al.*, 1980) for reference, with BSA/PBS to obtain a range of concentrations from 5–50  $\mu$ g/ml.

#### Calculations

Plasma retinol was not assayed directly but the amount in each sample was calculated assuming that all the retinol in quail plasma is bound in a 1:1 complex with holoRBP and that the molecular weight of quail RBP is 20,500. For the purpose of making comparisons between the concentrations of the specific proteins in both yolk and plasma it was convenient to obtain their yolk concentrations in relation to yolk water because of the high proportion of insoluble material in yolk. Using values found for individual yolk weights, and for the water content of the crude homogenates and homogenate supernatants the amounts of the specific proteins present were expressed as: (1) per whole yolk, (2) per unit weight of whole yolk and (3) per unit volume of water in whole yolk. During the yolk retinol determination the water contents were not measured. The figure obtained for retinol in terms of yolk water is calculated from a value for the water content of quail egg yolk of 0.487 ml/g ( $\pm$  0.011 SD) which is the mean of all determinations.

## RESULTS AND DISCUSSION

The egg yolk of Japanese quail was found to contain material which cross reacts with specific antisera raised against RBP and TBPA isolated from the blood of domestic fowl. These antisera also cross react with RBP and TBPA in quail plasma, thus providing the basis for their specific assay in this species (Heaf & Glover, 1979; El-Sayed *et al.*, 1980). In addition, the polyacrylamide gel electrophoresis of livetin fraction of yolk yielded a single u.v. fluorescent band, identical in colour and electrophoretic mobility to the holoRBP band of quail and domestic fowl plasma. These findings show that Japanese quail egg yolk, like that of domestic fowl (Heller, 1976) and duck (Sreekrishna & Cama, 1978), contains both TBPA and RBP

Table 1. Retinol, retinol-binding protein (RBP) and thyroxine-binding prealbumin (TBPA) in the blood plasma and egg yolk of Japanese quail

	Blood		Egg yolk	
	$\mu\text{mol/l. plasma}$	$\text{nmol/yolk}$	$\text{nmol/g yolk}$	$\mu\text{mol/l. yolk water}$
Retinol	$7.07 \pm 1.95$ (18)	$120 \pm 48$ (27)	$33.2 \pm 2.1$ (23)	$69.5 \pm 25.1$ (23)
HoloRBP	$7.07 \pm 1.95$ (18)	$4.93 \pm 1.95$ (25)	$1.25 \pm 0.52$ (25)	$2.57 \pm 1.06$ (25)
Total RBP	$10.5 \pm 2.66$ (9)	$9.28 \pm 4.93$ (10)	$2.35 \pm 1.09$ (10)	$4.75 \pm 2.34$ (10)
TBPA	$5.26 \pm 0.91$ (10)	$11.9 \pm 2.06$ (15)	$3.06 \pm 0.33$ (10)	$6.31 \pm 0.67$ (10)

\* Plasma retinol values were calculated from corresponding holoRBP values (see text). Values are mean  $\pm$  SD with the number of quail used shown in brackets.

and that a form of RBP is present with retinol attached.

The thin-layer chromatography system used on yolk lipid extracts to separate retinol from its esters with fatty acids (Kahan, 1967) showed single spots which had the characteristic green u.v. fluorescence of retinol and which migrated the same distance from the origin as the pure retinol standards. These observations provided the basis for the specific assay of retinol and it was not possible to detect retinyl esters on the tlc plates. No attempts were made to increase the sensitivity of the system to demonstrate the presence of retinyl esters in quail eggs because the free alcohol comprises about 90% of the total retinol in avian eggs before they are incubated (Neff *et al.*, 1949) and the esters therefore represent only a minor proportion, as is the case in blood plasma (Hoch & Hoch, 1946). The retinol content of quail egg yolk (Table 1) was found to be very similar to that of the domestic fowl (Moore, 1957). Some of this retinol remains attached to RBP but it can be calculated from the holoRBP content of quail yolk (Table 1), assuming the molecular weight of quail RBP to be similar to the value of 20,500 found for domestic fowl (Abe *et al.*, 1975), that this accounts for only about 4% of the total retinol present in yolk. Furthermore, by using Triton X-100 in the extraction of holoRBP, which has been shown to be effective in releasing membrane bound RBP in liver extracts (Glover *et al.*, 1974), a value of  $31.7 (\pm 3.9 \text{ SD}) \mu\text{g/ml}$  was found for the holoRBP concentration of yolk homogenate supernatant compared with a value of  $32.1 (\pm 10.3) \mu\text{g/ml}$  without Triton. This shows that additional RBP bound retinol is unlikely to be present in association with the globular membranes found in avian egg yolk (Vadehra *et al.*, 1977). In addition, the total immunoreactive RBP (Table 1), which was almost double the value for holoRBP in quail egg yolk, indicates that if a) RBP entered the yolk originally in the form of holoRBP this would only account for about 8% of the retinol present. It therefore appears that more than 90% of the retinol in quail egg yolk occurs without its plasma carrier protein.

The retinol content of egg yolk is strongly dependent on the availability of vitamin A in the diet of the hen (Parrish *et al.*, 1950), and this is most likely to be mediated by the concentration of holoRBP in the blood circulation. This hypothesis was tested by measuring the holoRBP content of the maternal plasma on the same day as the eggs were laid. Figure 1a shows the highly significant correlation between maternal plasma holoRBP concen-

tration and retinol content of the egg. However, there is surprisingly no correlation between the concentrations of holoRBP in both plasma and yolk water (Fig. 1b). This indicates that there is no coupling between the uptake of retinol and that of its carrier protein. A comparison of the plasma concentrations of retinol and RBP with those of yolk water (Table 1) further supports this view. The plasma concentrations are close to the corresponding concentrations in terms of plasma water because plasma contains only about 5% of dissolved solids, and the values for plasma can therefore be compared directly with those for yolk water to determine whether a change in concentration has taken place during uptake into the

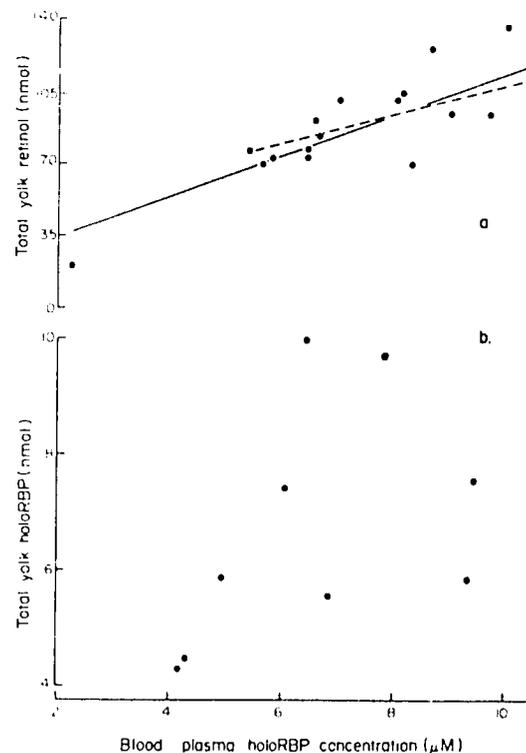


Fig. 1. Relationships between the total egg yolk content of retinol (a) or holoRBP (b) and the concentration of holoRBP in the blood plasma sampled from the corresponding hens with 24 hr of the eggs having been laid. Panel (a) shows linear regression of yolk retinol on plasma holoRBP for all birds (unbroken line,  $r = 0.8$ ,  $P < 0.001$ ) and for all except the bird with abnormally low values for plasma and yolk (broken line,  $r = 0.65$ ,  $P < 0.01$ ).

yolk. Whereas retinol was about 10 times more concentrated in yolk than in plasma, the concentrations of holoRBP and total RBP were less than half those of plasma. These data indicate that either retinol alone is accumulated, possibly by a process similar to that found in other tissues such as the retina (Maraini *et al.*, 1977) and serosal surface of the small intestine (Rask & Peterson, 1976) or following uptake of the intact retinol RBP complex, retinol is selectively retained whereas the carrier protein returns to the plasma. Whichever is the case, the developing follicle must have an acceptor system with an affinity for retinol similar to RBP itself. The form of retinol storage within newly formed yolk, whether in association with the lipoproteins or with another specific binding protein, has not been investigated.

In contrast to RBP, the mean concentration of TBPA, which complexes with RBP in plasma was found to be approximately the same concentration in yolk water as in plasma (Table 1). Differences in the relative uptakes of other plasma proteins such as albumin and  $\gamma$  globulin have been reported previously for the domestic fowl (Patterson *et al.*, 1962). The difference in relative uptake indicating a partial exclusion of RBP from yolk may result from its involvement in the process of retinol uptake possibly by its binding to an RBP receptor. Whereas in plasma the molar ratio of TBPA to holoRBP was about 0.7, in yolk it was above unity at a value of 2.5. These ratios indicate that in yolk but not in plasma, all the holoRBP can form a 1:1 complex with TBPA. In the plasma of the laying quail it is possible that the lower affinity binding sites on TBPA may participate in complexing RBP.

Although RBP and TBPA are present in domestic fowl yolk (Heller, 1976) the uptake of these carrier proteins in relation to retinol have not been measured quantitatively in this species. It is therefore not possible at present to say whether quail and domestic fowl differ significantly in respect of the retinol transport system from blood to yolk. However, such a difference is unlikely in view of their close relationship in evolution.

The normal frequency of egg laying of quail kept under the conditions described in Materials and Methods is one egg per day. This therefore means that these quail showed a daily loss of retinol from the body of about 34  $\mu$ g. This amount of retinol is about 2-3 times that present at any one time in the blood circulation, and when added to the retinol utilisation by other tissues of the body indicates that the half life of holoRBP secreted by the liver is somewhat less in the laying quail than the value of about 7 hr reported for the cynomolgus monkey and rat (Vahlquist, 1972; Muhikal & Glover, 1974). We have suggested elsewhere (Heaf & Glover, 1979) that it is the uptake of retinol from its complex with RBP by yolk that is the primary cause of the relatively high apoRBP concentration present in the plasma of the laying quail. The clearance and metabolism of apoRBP by the kidney presumably has a secondary role in that the process is adequate to keep the proportion of apoRBP in plasma produced out of the breeding season at 10-15% of the total RBP, but is unable to deal more rapidly with the additional amount formed during laying.

## SUMMARY AND CONCLUSIONS

The Japanese quail egg yolk, like that of domestic fowl and duck, was found to contain the two proteins, RBP and TBPA, which are involved in the transport of vitamin A. Although they are present in all three avian species studied so far and are likely to be present in the yolk of all avian species, their role may be of lesser importance in yolk than in plasma for several reasons: (1) most, if not all, livetin proteins have been shown to originate from plasma and in quail yolk there was no evidence for a specific concentration of RBP or TBPA; (2) all the retinol in plasma is attached to RBP whereas in quail yolk less than 4% was present as the complex; (3) the total yolk retinol, but not RBP, was found to be correlated with the plasma RBP concentration, thus indicating that the specificity of the uptake process is for retinol and not for its carrier protein.

A possible function for the small amount of RBP and TBPA present in yolk may be to prime the retinol transport system in the early stages of embryogenesis until a supply of the proteins from the embryonic liver becomes available.

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## REFERENCES

- ABE T., MUTO Y. & HOSOYA N. (1975) Vitamin A transport in chicken plasma: isolation and characterisation of retinol-binding protein (RBP), prealbumin (PA) and RBP-PA complex. *J. Lipid Res.* **16**, 200-210.
- EL-SAYED M., HEAF D. J., & GLOVER J. (1980) Effect of photoperiod on thyroxine binding prealbumin in Japanese quail. *Gen. Comp. Endocr.* In press.
- GLOVER J., JAY C. & WHITE G. H. (1974) Distribution of retinol binding protein in the tissues. *Vitam. Horm.* **32**, 215-235.
- GLOVER J., HEAF D. J. & LARGE S. (1980) Seasonal changes in plasma retinol-binding holoprotein in Japanese quail. *Br. J. Nutr.* **43**, 357-366.
- GLOVER J., MONLEY L., MUHICAL H. & WESTON S. (1974) Micro-method for fluorimetric assay of retinol-binding protein in blood plasma. *Clinica Chim. Acta* **50**, 371-380.
- HEAF D. J. & GLOVER J. (1979) Circannual changes in plasma concentrations of retinol binding protein and luteinizing hormone in male and female Japanese quail. *J. Endocr.* **83**, 475-482.
- HELLER J. (1976) Purification and evidence for the identity of chicken plasma and egg yolk retinol binding protein prealbumin complex. *Dev. Biol.* **51**, 1-8.
- HOCH H. & HOCH H. (1946) The state of vitamin A in human serum. *Br. J. exp. Pathol.* **27**, 316-328.
- KAHAN J. (1967) Thin layer chromatography of vitamin A metabolites in human serum and liver tissue. *J. Chromat.* **30**, 506-513.
- LAURELL C.-B. (1966) Quantitative estimation of proteins by electrophoresis in agarose gel containing antibodies. *Analyt. Biochem.* **15**, 45-52.
- MANCINI G., CARBONARA A. O. & HEREMANS J. F. (1965) Immunochemical quantitation of antigens by single radial immunodiffusion. *Immunochemistry* **2**, 235-254.
- MARAINI G., OTTONELLO S., GOZZOLI F. & MERLI A. (1977) Identification of membrane protein binding the retinol in retinal pigment epithelium. *Nature* **265**, 68-69.

- MCINDOE W. M. & CULBERT J. (1979) The plasma albumins and other livetin proteins in egg yolk of the domestic fowl. *Int. J. Biochem.* **10**, 659-663.
- MOORE T. (1957) In *Vitamin A*, Ch. 21, p. 257. Elsevier, London.
- MUHLAL H. & GLOVER J. (1974) Effects of dietary deficiencies of protein and retinol on the plasma level of retinol-binding protein the rat. *Br. J. Nutr.* **32**, 549-558.
- NEFF A. W., PARRISH D. B., HUGHES J. S. & PAYNE L. F. (1949) The state of vitamin A in eggs. *Archs Biochem.* **21**, 315-320.
- PARRISH D. B., WILLIAMS R. N., HUGHES J. S. & PAYNE L. F. (1950) Transfer of vitamin A from the yolk to the chick embryo during incubation. *Archs Biochem.* **29**, 1-6.
- PATTERSON R., YOUNGER J. S., WEDGE W. O. & DIXON F. J. (1962) The metabolism of serum proteins in the hen and chick and secretion of serum proteins by the ovary of the hen. *J. gen. Physiol.* **45**, 501-513.
- RASK L. & PETERSON P. A. (1976) *In vitro* uptake of vitamin A from the retinol-binding plasma protein to mucosal epithelial cells from the monkeys small intestine. *J. biol. Chem.* **251**, 6360-6366.
- SREEKRISHNA K. & CAMA H. R. (1978) Vitamin A transport for embryonic development. Characterisation of retinol-binding protein and prealbumin from avian egg-yolk. *Ind. J. Biochem. Biophys.* **15**, 255-259.
- THOMPSON J. N., HOWELL J. McC., PITT G. A. J. & McLAUGHLIN C. (1969) The biological activity of retinoic acid in the domestic fowl and the effects of vitamin A deficiency on the chick embryo. *Br. J. Nutr.* **23**, 471-490.
- VADHRA D. V., BAIN J. M. & BURLEY R. W. (1977) Lipid-protein globules of avian egg yolk. Isolation and properties of globules stable in concentrated sodium chloride solution. *Biochem. J.* **166**, 619-624.
- VANLQVIST A. (1972) Metabolism of the vitamin A transporting protein complex. Turnover of retinol-binding protein, prealbumin and vitamin A in a primate (*Macaca Irus*). *Scand. J. clin. Lab. Invest.* **30**, 349-360.

**IV.**  
**Analysis of Plasma Retinol-Binding Protein**

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## MICRO-METHOD FOR FLUORIMETRIC ASSAY OF RETINOL-BINDING PROTEIN IN BLOOD PLASMA

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### Summary

1. Holo retinol-binding protein was assayed by irradiating the pherogram of the plasma with ultraviolet light and measuring its yellow green fluorescence in the  $\alpha_2$ -globulin zone.

2. The level of holo retinol-binding protein in normal adults was found to be  $47.5 \pm 1.3$  S.E.)  $\mu\text{g/ml}$ .

3. The mean level of total retinol-binding protein in one group of normal patients was  $48.3 \mu\text{g/ml}$  compared to  $42.3 \mu\text{g/ml}$  for the holo protein indicating that some apoprotein is usually present in human plasma.

### Introduction

A micro-method for the determination of retinol-binding protein (RBP) in human plasma by radioimmunoassay has been described previously by Smith et al. [1]. This uses specific rabbit antiserum to RBP and  $^{125}\text{I}$ -labelled RBP. The method is more sensitive than the classical extraction and colorimetric procedures but the reagents take some time to prepare and do not keep indefinitely. The antiserum also reacts with apo- as well as holoRBP, hence in cases of retinol deficiency where some apoprotein may be present, the amount of retinol bound to the protein would still have to be determined separately. Although the early procedures [2] for determining retinol in the lipid extract from serum by antimony trichloride [3] or trifluoroacetic acid [4], have been considerably improved in sensitivity through the use of micro-cells in modern rapid recording spectrophotometers, these are inherently subject to appreciable error on account of the instability of the chromogens formed and the lack of specificity of the reagents and are, therefore, not reliable on a micro-scale (requiring less than  $50 \mu\text{l}$  plasma). These difficulties are overcome to a considerable extent by using the more sensitive fluorimetric procedure for retinol devised by Thompson et al. [5], but this assay is affected by some interference

from the fluorescence of variable amounts of other substances such as phytofluene and from colour quenching by other carotenoids which may be present in the lipid extracts from the serum so that corrections have to be applied.

The new procedure described below is specific for the physiologically-active form of retinol attached to RBP. In devising it, advantage is taken of three main features of holoRBP: (i) the fluorescence of retinol attached to its specific carrier protein is more than 10 times greater than that of free retinol in hydrocarbon solvent [6,7], (ii) the low molecular weight of RBP allows it to be separated electrophoretically in gels free from other large molecular weight lipoproteins which would interfere with the fluorescence emitted by it and, (iii) retinol bound to its carrier protein is much more stable than free material [7].

Consequently, by first separating RBP from other serum proteins by disc-gel electrophoresis on transparent polyacrylamide, it is possible to assay fluorimetrically the retinol attached to its carrier protein with considerable accuracy. Only very small amounts of serum or plasma are required comparable with those used in colorimetric methods.

## Experimental

### *Disc-gel electrophoresis*

This is carried out at 4°C in standard disc-gel tubes 6 mm internal diameter which are filled to within 2 cm of the top with 5% w/v polyacrylamide resolving gel containing 0.75 M Tris-HCl buffer, pH 8.9, and 5% (w/v) sucrose. A further 1 cm of 3% (w/v) concentrating-gel containing 0.25 M Tris-HCl at the same pH is placed on top. A known volume of serum or plasma samples (20–50  $\mu$ l) previously prepared to contain 10% (w/v) sucrose is then subjected to electrophoresis on these gels in the Shandon apparatus, with constant current at 4 mA tube for 90 min in the dark. The gels are subsequently removed and placed in turn in the 10 cm  $\times$  1 cm quartz cell of the Chromoscan instrument for scanning with UV light (300–400 nm). The gel is covered with distilled water. It is important to transfer the gels only immediately before assay in order that the RBP may not be lost by diffusion.

### *Estimation of fluorescence*

The optical arrangement of the standard instrument in the fluorescence mode of operation is not sufficiently good to differentiate the low level of fluorescence due to RBP above the background fluctuations of the photomultiplier and recording system "electronic noise". The collection of the fluorescence from resolved components in the gel was improved by inserting an additional lens and green filter (465 nm) adapter between the cell holder and the photomultiplier compartment as indicated in Fig. 1. This modification ensured a more efficient collection of the fluorescence by the PM tube so that the level of current generated was brought into the capacity range of the electronic amplification system. Insertion of the lens adapter in the sample beam also means that the horizontally moving cell carriage has to be set at a fixed height. It was arranged that the gel immersed in distilled water within the quartz cell (8 mm wide) was irradiated optimally as it traverses the centre of the fixed narrow beam of UV light collimated by the slit (1 cm long  $\times$  1 mm wide). The

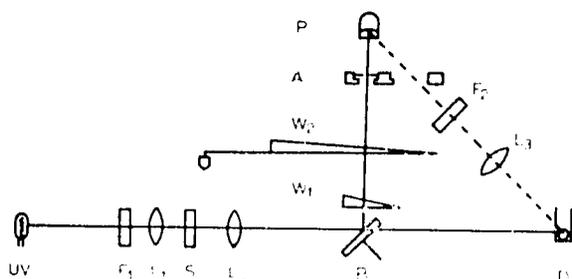


Fig. 1. Diagram of optical system in Chromosean modified for measurement of fluorescence. The filter  $F_2$  and lens 3 are combined in one adapter for fitting to the standard instrument. P, Photomultiplier tube; UV, Ultraviolet lamp; A, 3 mm diam. aperture;  $F_1$ , Wood's glass filter;  $W_1$ , Baseline control wedge;  $F_2$ , Filter passing 465 nm;  $W_2$ , Balancing wedge;  $L_1$ ,  $L_2$ ,  $L_3$ , lenses; B, Beam splitter; S, 0.5 mm slit; D, Disc-gel in quartz cell.

weak background fluorescence of the polyacrylamide gel and quartz cell is so balanced against the reference beam using the manually adjustable wedge and collimating discs in the latter so that the recorder trace for a blank gel runs about 5 cm from the right-hand edge of the paper (the polarity switch within the instrument is left in "Normal" position). The most suitable cam for use in the automatic system driven by the motor balancing the reference and sample beams was found to be 5-0771D.

Some quartz cells give background traces with one or two undulations in them. For ease of measurement of recorded peaks, it is better to select those cells with a linear background over the region opposite that corresponding to the RBP zone under assay (see Fig. 2A). The peak area for RBP fluorescence is measured by triangulation and double peak areas obtained by multiplying heights times widths of bases (converted to double area/100  $\mu$ l fluid applied to gel) are plotted against the quantity of RBP in the gel (expressed as the concentration of RBP in the serum or test fluid in  $\mu$ g/ml or as the amount of retinol  $\mu$ g/100 ml). The relative molecular weights of human RBP: retinol are 77:1 approximately. In order to eliminate any errors due to possible uneven running of the protein in the gel it was found convenient to scan the gel a second time after rotating it through 180° and then taking a mean value of the peak areas obtained.

#### *Colorimetric detection of retinol in gel fractions*

Excised fluorescent zones from gel pherograms were extracted with a small volume of ethanol by kneading the small segment of gel between the fingers covered with a polythene glove. The alcohol extract was diluted to 50% with water and the lipid taken into light petroleum. The latter was evaporated off and the residual lipid dissolved in 2 ml chloroform and aliquots taken for retinol colour test using trifluoroacetic acid reagent [4].

#### *Isolation of RBP*

RBP for preparing the rabbit antiserum and for use as a reference standard in the immunoassay was isolated by procedures outlined previously by Kirby et al. [8]. The final RBP preparation in 0.1 M Tris buffer, pH 8.0, runs as a single

zone, corresponding to a protein with 22000 molecular weight, (a) in the ultracentrifuge, (b) in disc-gel electrophoresis when complexed with sodium dodecyl sulphate and (c) on a standardised Sephadex G100 column. The degree of saturation of the protein with retinol was invariably greater than 85% and qualitatively the preparation contained both fast and slow moving holoprotein components [9]. The RBP was stored in buffer at pH 8.0 and  $-20^{\circ}$ .

#### *Preparation of anti-serum to human RBP*

RBP dissolved in 1 ml buffer at pH 8 (concentration 0.5–1.0 mg/ml) was added to an equal volume of Freund's adjuvant with stirring and the premix emulsified before injection into rabbits at five sites (1 ml/site) at monthly intervals as described by Kirby [10]. A reasonable specific antibody titre was observed about 2 weeks after the first dose and small amounts of blood could be drawn as required from the animals at suitable intervals after booster doses. The blood was allowed to clot and the serum separated off for isolation of the  $\gamma$ -globulin fraction. This antiserum was kept at  $4^{\circ}$  and used for the various tests. The antiserum to prealbumin was obtained from Boehringerwerke (Hoechst Pharmaceuticals). This, too, was raised in the rabbit.

#### *Immunological methods*

Immunodiffusion was carried out according to the procedure of Ouchterlony [11] in 1% agarose gel containing 0.05 M Tris-HCl buffer, pH 7.4. Quantitative estimation of RBP in serum, chromatographic fractions etc. was determined using the procedure of Mancini et al. [12]. Assays were carried out in duplicate. The basic standard RBP solutions contained approximately 40  $\mu\text{g}/\text{ml}$ . Whole serum was used without dilution.

#### *Immuno-electrophoresis*

In order to obtain greater sensitivity and more intense precipitation lines, the following procedure was adopted for qualitatively detecting RBP and PA in electrophoretograms. An electrophoretogram of 40  $\mu\text{l}$  serum was prepared in a standard disc-gel of polyacrylamide. The gel (6 mm diameter) was then laid across an agarose plate between two parallel slots cut about 12–15 mm apart. One slot was filled with a solution of rabbit antiserum to RBP and the other with that to PA. Diffusion was allowed to take place overnight as before. The zones of precipitation were clearly visible in the agarose gel 24 h later.

## Results

A typical fluorescence scan of adult human serum is presented in Fig. 2B alongside a similar trace for a blank gel (Fig. 2A) and a photograph (Fig. 2C) to the same scale of the gel stained for proteins. Four peaks are present, one of which at the origin arises from fluorescence of components (including possibly retinyl esters and phytofluene) in high molecular weight  $\beta$ -lipoproteins and chylomicra which do not penetrate easily into the small pores of the gel. This fluorescence usually has a bluish tint. The second peak from the origin is due to yellow-green fluorescence typical of RBP which runs in that position. This zone has an electrophoretic mobility corresponding to  $\alpha_2$ -globulin. The third peak

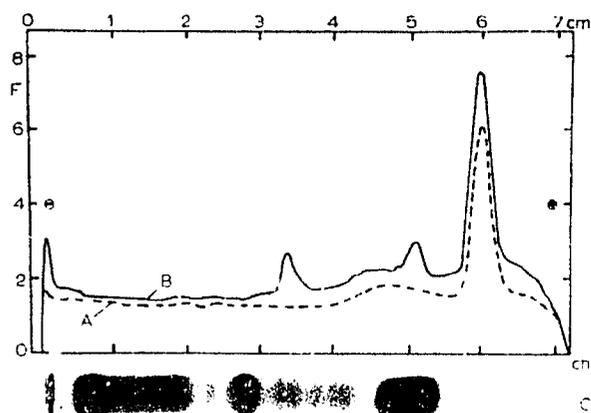


Fig. 2. Fluorescence scans of disc-gel pherograms: (A) Control blank and (B) human serum shown alongside the latter gel stained for protein (C).

arises from materials associated with albumin zone. This also has a pale green tint but does not contain retinol. The fourth peak fluoresces bright blue and has the highest mobility running close to the buffer front. It has not been investigated yet but the fluorescence derives from an impurity in the gel and not the serum. It serves as a useful marker for prealbumin whose mobility corresponds closely to it. Retinol is also absent from this zone. The identification of the second fluorescence peak with RBP was made by examining qualitatively the material extracted from the above zones in a set of 16 gels. The  $\alpha_2$ -globulin zones were cut out of the gels, kneaded and homogenised in a small volume of physiological saline, allowed to stand for an hour and the mixture centrifuged to precipitate small gel-fragments. The fluorescence of the supernatant extract was examined in an MPF2A Hitachi-Perkin Elmer fluorospectrophotometer using the sensitive micro-cell assembly. The  $\lambda_{\text{max}}$  of excitation and emission spectra were found to be 332 nm and 456 nm as shown in Fig. 3. and identical with those obtained for pure RBP. In order to have more material

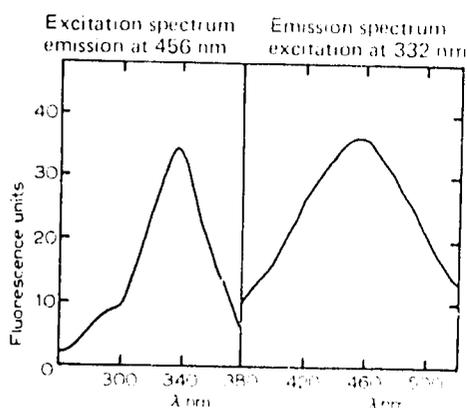


Fig. 3. Excitation and emission spectra of retinol-binding protein extracted from  $\alpha_2$ -globulin zone of pherogram of human plasma proteins. Excitation  $\lambda_{\text{max}} = 330$  nm with shoulder at 293 nm. Emission  $\lambda_{\text{max}} = 456$  nm.

for this type of analysis, 2 ml of human serum were first chromatographed on a small column (5 cm  $\times$  8 mm) of the dipolar ion-exchange support arginine—Sephacrose 6B (Porath and Fornstedt [13]). The RBP containing fraction was eluted from the column and concentrated to approximately 0.25 to 0.2 volume of the original serum. Electrophoresis of this concentrate enabled a larger amount of the material in  $\alpha_2$ -globulin zone containing the yellow green fluorescence to be separated and extracted for qualitative examination in the fluorospectrophotometer. The excitation and emission spectra obtained are those shown in Fig. 3. Again a separate set of 8 gel-electrophoretograms of serum were run and the excised fluorescent zones examined for retinol as previously described. Only this lipid extract from the  $\alpha_2$ -zone gave a positive green colour with trifluoroacetic acid reagent indicative of retinol. No colour was given by similar portions of the gel from either the albumin or prealbumin regions. Again, the  $\alpha_2$ -fluorescent material is absent from pherograms of plasma from retinol-deficient children suffering from kwashiorkor.

Further confirmation that the protein portion of RBP was present in this  $\alpha_2$ -zone was obtained through the use of its specific antiserum. Two disc-gel electrophoretograms of the same serum were prepared. One was stained for proteins and the other was placed on top of an agar plate into which the resolved protein components could diffuse against the specific antisera to RBP and PA, respectively, placed in adjacent slots. The immuno-precipitins formed in the agar are shown (alongside the stained proteins) in Fig. 4. It is clear that there is only one RBP zone and one PA zone in the electrophoretogram and that the former has the same relative mobility to the latter as the yellow green

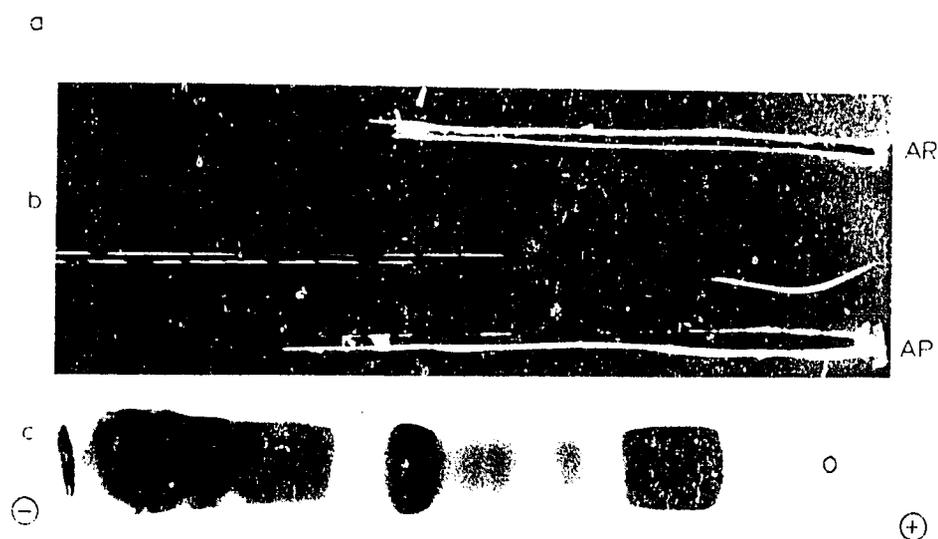


Fig. 4. Fluorescence scan (a) of disc-gel pherogram of human plasma set alongside precipitin zones formed in agarose plate (b) when the separated proteins as shown in the similar stained gel (c) were allowed to diffuse into the agarose against the specific antisera to RBP (upper slot-AR) and PA (lower slot-AP).

TABLE I  
IDENTIFICATION OF RBP WITH GREEN-FLUORESCENT BAND IN  $\alpha_2$ -GLOBULIN REGION OF DISC-GEL ELECTROPHORETOGRAM ON 5% POLYACRYLAMIDE

Test Sample	Distance from origin (cm)		Relative mobilities
	Green-fluorescent zone	Pre-albumin	
Serum 1	2.55	4.7	0.54
2	2.68	4.9	0.55
RBP/PA concentrate 1	2.85*	5.1	0.56
2	2.85	5.1	0.56
	Specific Immunoprecipitation by:		
	Anti-RBP	Anti-PA	
Serum 3	3.3	5.8	0.57

\* Fluorescence  $\lambda$  max 456 nm. Excitation  $\lambda$  max 332 nm.

fluorescent zone to PA. The values obtained with 3 different samples of serum are given alongside two results for concentrates of RBP with PA in Table I. In the quantitative work, all assays were carried out in duplicate and the amount of the RBP present in individual samples determined from the mean area of the RBP fluorescence peaks obtained in the two scans as described above. The mean difference between the latter values for a large number of samples was found to be 5% ( $\pm$  0.8 S.E.). The linearity of the assay procedure was checked for increasing volumes (10 to 60  $\mu$ l) of human plasma resolved in the electrophoretogram. The values obtained for peak areas were plotted against volume of plasma used as shown in Fig. 5. The fluorescence recorded in the Chromoscan is clearly linearly related to volume of plasma up to 50  $\mu$ l; beyond that the yield of fluorescence is proportionately less due either to self absorption effects on account of the thickness of the gel or to less efficient penetration of

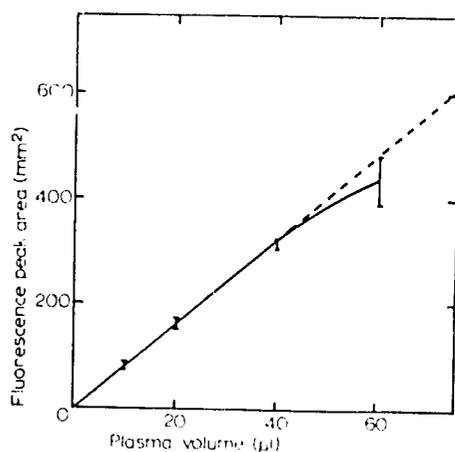


Fig. 5. Graph of relationship between the quantity of RBP in plasma as measured by area of fluorescence peak in the scan of the pherogram against the volume of sample subjected to electrophoresis on polyacrylamide gel.

TABLE II  
REPRODUCIBILITY OF FLUORESCENCE ASSAY OF FREE RETINOL IN SERUM

Sample No.	Vol. ( $\mu$ l)	No. of gels	Fluorescence Peak Area (A)		Retinol ( $\mu$ g/100 ml)
			A ( $\text{mm}^2 \times 2$ ) (Mean $\pm$ S.E.)	A / 100 $\mu$ l	
1	20	8	278 $\pm$ 4.5	1390	51
2	40	8	370 $\pm$ 10.6	925	34
3*	40	6	454 $\pm$ 14.8	1135	44

\* This sample was diluted 10-fold before analysis. The area obtained is approximately 10% lower than the value ( $1270 \text{ mm}^2$ ) found for the undiluted sample.

the UV light into the increasing amount of protein within the narrow band about 2 to 3 mm wide.

The reproducibility of the technique was tested by determining the amount of RBP in the same plasma eight successive times using 20- $\mu$ l portions for one sample and 40- $\mu$ l portions for another. The results are presented in Table II and show that the technique is reproducible to within 2--3% S.E.

A series of results obtained using 20- $\mu$ l portions of human plasma were plotted against the concentrations of free retinol present in the plasma samples as determined on lipid extracts from much larger amounts (20 ml) chromatographed on weakened alumina according to the procedure of Huang and Goodman [14]. The retinol-containing fraction from the column was assayed using trifluoroacetate. The standard curve shown in Fig. 6 was obtained. The slope of this holds for the particular fixed geometry of the light source and cell carriage and sensitivity setting (9) of the instrument, all of which have to be maintained. Any modification of these or of thickness of gel used would give a curve with slightly different slope. Some of the lower values on the standard graph were obtained by assaying diluted plasma. The average value of retinol for 20

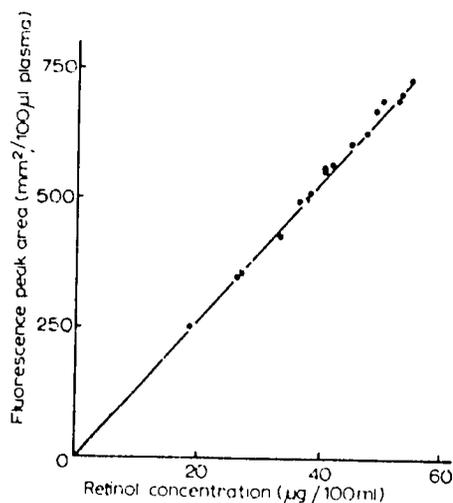


Fig. 6. Standard curve showing the direct relationship between the area of fluorescence peak and the amount of RBP in plasma.

TABLE III  
THE CONCENTRATIONS OF TOTAL RBP AND HOLO-RBP IN NORMAL ADULT HUMAN PLASMA

Sample No.	Total RBP (a) by Immunoassay ( $\mu\text{g/ml}$ )	Holo-RBP (b) by Fluorimetry ( $\mu\text{g/ml}$ )	Apo-RBP (a-b) ( $\mu\text{g/ml}$ )
1	49	49	0
2	50	46	4
3	47	37	10
4	54	52	2
5	47	31	16
6	42	43	0
7	51	36	15
8	46	44	2
Mean	48.3	42.3	6.0

samples which included plasma from both sexes but mostly male, was found to be  $47.7 \mu\text{g}/100\text{ml}$ . The values for RBP obtained in the above fluorescence assay are compared with the values obtained for the protein moiety by the immunoassay method of Mancini et al. [12] in Table III. It can be seen that there is usually a small amount of the apoprotein present in normal adult plasma.

### Discussion

For colorimetric procedures 3-ml samples of plasma are usually taken in order to obtain sufficient lipid extract for chromatography and to provide reasonably acceptable optical density readings with standard 1-cm cuvettes. Some semi-micro procedures have been developed in recent years to enable the total retinol in 0.5 ml of serum to be assayed colorimetrically and a fluorescent procedure [5] has recently been described which requires only 0.2 ml. All these methods, however, are subject to considerable error in view of the lability of free retinol on exposure to air or light during the extraction and assay process. Thus, for deficiency cases where the levels of vitamin A in plasma are often lower than one-fifth the normal adult level, the above micro-procedures are appreciably limited in their useful application especially if chromatography of the lipid extract is also done. Yet in many nutritional investigations, the colorimetric procedures have still had to be used.

In this new procedure the retinol remains attached to its carrier protein throughout the analysis where it is known to be more stable and less subject to uncontrolled oxidation. Again during the separation stage of the analysis, it is kept in the dark at  $4^\circ\text{C}$  reducing further the chance of spurious losses and in the final assay is only momentarily exposed to light at room temperature.

The sensitivity of the procedure is such that a  $40 \mu\text{l}$  sample of plasma containing as little as 1 to 2 ng retinol, i.e. equivalent to 2.5 to  $4 \mu\text{g}$  retinol/100 ml plasma (e.g. from an extremely deficient subject) can be assayed with less than 10% error as can be seen from the series of results obtained with 10-fold diluted serum (Table II). This is of the same order of sensitivity as that of the radioimmunoassay described by Smith and colleagues [1] and for the

direct immunoassay of RBP by the simpler diffusion procedure of Mancini *et al.* [12].

Inspection of many fluorescence traces of the RBP zone in the disc-gel electrophoretogram and the UV-irradiated gel itself shows that the RBP is a single normally distributed band and not a doublet such as one finds in similarly prepared traces of purified RBP which contains a mixture of fast and slower moving components. It would seem that mainly one type of holoprotein is present in the original serum and that the faster moving components detected in the purified RBP are artifacts of the isolation procedure as has been suggested by Raz *et al.* [9]. Occasionally, however, small shoulders can be seen on some of the fluorescence peaks and in these cases perhaps a small percentage of the RBP may have been present in a form with higher electrophoretic mobility, but equally well these could possibly be caused by some unevenness in the application of the plasma sample to the gel or in the electrophoresis run.

The results in Table III show that there is usually some apoRBP present. However, when 10 such samples were shaken with a 5-fold excess of retinol dispersed on Celite, there was no increase in their amounts of holoRBP indicating that the apo-component was unable to bind retinol. On the other hand, in other control experiments to be reported fully later plasma was extracted with heptane to remove retinol almost completely from the RBP. When the plasma was shaken with retinol the holoprotein was readily regenerated. Clearly most of the apoprotein circulating in normal plasma must be denatured or partially metabolised rendering it incapable of carrying the vitamin.

The above rapid assay procedure which is specific for holoretinol-binding protein should prove useful clinically. This protein has a rapid turnover rate [15] and disorders of the liver and kidney affect the normal steady state level markedly [16].

## References

- 1 F.R. Smith, A. Raz and DeW. S. Goodman, *J. Clin. Invest.*, 49 (1970) 1754.
- 2 M.S. Kimble, *J. Lab. Clin. Med.*, 24 (1939) 1055.
- 3 F.H. Carr and F.A. Price, *Biochem. J.*, 20 (1926) 497.
- 4 J.R. Neeld and W.N. Pearson, *J. Nutr.*, 79 (1963) 454.
- 5 J.N. Thompson, P. Erdody, R. Brien and T.K. Murray, *Biochem. Med.*, 5 (1971) 67.
- 6 DeW. S. Goodman and R.B. Leshe, *Biochim. Biophys. Acta*, 260 (1972) 670.
- 7 S. Futterman and J. Heller, *J. Biol. Chem.*, 247 (1972) 5168.
- 8 W. Kirby, G.H. White and J. Glover, *Biochem. J.*, 123 (1971) 31 P.
- 9 A. Raz, T. Shtrator and DeW. S. Goodman, *J. Biol. Chem.*, 245 (1970) 1903.
- 10 W. Kirby, Ph.D. Thesis, University of Liverpool, 1971.
- 11 O. Ouchterlony, *Progr. Allergy*, 6 (1962) 30.
- 12 G. Mancini, A.O. Carbonara and J.F. Heremans, *Immunochemistry*, 2 (1965) 235.
- 13 J. Porath, and N. Fornsstedt, *J. Chromatog.*, 51 (1970) 479.
- 14 H.S. Huang and DeW. S. Goodman, *J. Biol. Chem.*, 240 (1965) 2839.
- 15 A. Vahlquist, *Scand. J. Clin. Lab. Invest.*, 30 (1972) 349.
- 16 F.R. Smith and DeW. S. Goodman, *J. Clin. Invest.*, 50 (1971) 2426.

# Retinol-binding protein in serum of xerophthalmic, malnourished children before and after treatment at a nutrition center<sup>1</sup>

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**ABSTRACT**—Retinol-binding protein and prealbumin levels have been measured in the serum of children who are both malnourished and show clinical signs of xerophthalmia. The more severe the xerophthalmia the lower the concentration of retinol-binding protein and prealbumin in serum. When a dose of 100,000 IU of water-miscible retinol palmitate was injected, the retinol-binding protein did not reach a normal level within 24 hr as might have been expected if deficiency of vitamin A were the sole factor. A high protein diet containing a carotene together with further vitamin A brought the retinol-binding protein and prealbumin levels to normal within about a week. Thirty-four children were admitted to the Nutrition Rehabilitation Centre, of these, nine (average age 3 years and 2 months) who stayed for a month or more, showed a median weight gain of 1.7 kg in the first month. But this rate of growth slowed down on return home. Of the children 12 with corneal xerophthalmia in one or both eyes were observed for a sufficient length of time for an assessment to be made of the effect of treatment on vision. None became blind but one had seriously impaired vision and one other lost one eye. The remaining children have normal vision or vision that although impaired will still enable them to lead an active life. We suggest that the children needed protein from which to synthesize retinol-binding protein before normal retinol transport could be re-established. (*Am J Clin Nutr*, 30: 1968-1973, 1977)

Retinol is released from the liver combined 1:1 with retinol-binding protein (RBP) and this compound circulates in the blood combined with prealbumin (PA), (1-3). The level of retinol in the blood is significantly correlated with the levels of RBP and PA (1) and all are diminished in the blood of severely malnourished children (4-6). Rats which are deficient solely in vitamin A have low RBP in blood but four times the normal level of RBP in the liver. This is reversed when such rats are given a single oral dose of vitamin A. The blood RBP rises to normal within a few hours and stays normal for at least 15 days, while the liver RBP falls (7).

Xerophthalmic children are rarely deficient solely in vitamin A. They are almost always severely malnourished as well. In some, the dominating factor seems to be retinol deficiency and, in others, protein is also necessary in order to synthesize RBP (1, 5, 6, 8). We have studied the RBP and PA concentrations in the serum of children

admitted to the Nutrition Rehabilitation Centre (NRC), Madurai, S. India (9) who were severely malnourished and showed conjunctival or corneal signs of xerophthalmia. We estimated serum RBP and PA concentrations on their first arrival at the ophthalmic out-patients department of the Government Erskine Hospital and, whenever possible, 24 hr later, after an injection of 100,000 IU of water-miscible retinol palmitate, and again at varying times there-

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after. The questions set initially were: what are the levels of RBP and PA in such children and does injection of retinol palmitate raise the level of RBP to normal within 24 hr as it might be expected to do if there were no limiting factor(s) other than retinol deficiency. Furthermore, does the cheap, locally available and culturally acceptable vegetarian diet supplied at the NRC allow synthesis of RBP to proceed.

**Methods**

Thirty-four children (average age 2 years and 3 months) were initially examined on arrival and, after the blood sample ( $I_0$ ) had been taken from the arm vein, they were injected with 100,000 IU of water-miscible retinol palmitate and their general medical treatment was organised. They were then admitted with their parent to the NRC and fed. The children were seen daily as out-patients, and further doses of vitamin A were given as considered necessary by the medical staff. Blood samples were taken again, if possible after 24 hr and at intervals until the child returned home. Some came back 1 to 2 months later and a further blood sample was taken to see if home food maintained RBP and PA levels in serum.

Classification of xerophthalmia was that recommended by W.H.O. (10).

Food at the NRC consisted of 6 meals/snacks a day, supplying 140 kcal, 4g vegetable protein per kilogram and 5 mg  $\beta$  carotene per day. At the start less than this was eaten. The diet is based on food the parents are used to, can buy in the local markets, and can probably afford for their children. The protein sources were ragi, bajra, wheat, rice, whole ground nut meal, green gram, and red gram. Meals were arranged so that both cereals and pulses were fed at each meal, in an attempt to avoid limiting amino acids. The  $\beta$  carotene came from carrots, leaves of the drumstick tree (*Moringa oleifera*) tree for the pickling, amaranth, and other dark green leafy vegetables in season. Oil and jaggery, the local crude sugar that is rich in iron, supplied taste and calories.

RBP and PA were estimated by the method of single radial immunodiffusion (11) using plates and standard human serum from Hoechst, W. Germany. The diameters of the precipitin rings were read after 48 hr on the bench. Samples of 5  $\mu$ l of undiluted,  $1/2$  and  $1/3$  dilutions of the standard serum were tested on each plate together with 5  $\mu$ l samples of undiluted test serum for RBP. Undiluted and  $1/2$  dilutions of test serum were used in the estimation of PA.

The radial immunodiffusion method measures both apo- and holo-RBP. Some samples were taken to Britain in ice for measurement of total RBP as well as of holo-RBP (12) at the Biochemical Dept, University of Liverpool. Using a normal sample volume of 20  $\mu$ l plasma, holo-RBP is clearly measurable down to 0.5  $\mu$ g holo-RBP per ml of plasma (12). With vitamin A-deficient plasma, 40  $\mu$ l are usually taken, so reducing the detection level to less than 0.25  $\mu$ g/ml, equivalent

to retinol concentration of 0.33  $\mu$ g/(1 IU retinol) 100 ml.

Determination of statistical significance was made using the nonparametric Wilcoxon test (13). The results are expressed as Mean  $\pm$  SEM.

**Results**

Young children have less RBP in their blood than adults (14). Three well-nourished Indian children, who however showed Bitot spots, had RBP levels in their serum of 35, 26, 35  $\mu$ g/ml and PA of 145, 180, 140  $\mu$ g/ml. These are similar to those of well-nourished English children.

The children we examined at Madurai were all malnourished. Thirteen were less than half their expected weight/age on the Gomez scale (15), 14 were between 50 to 60% and seven were between 61 to 75%. Nearly all were cases of marasmus or of marasmic kwashiorkor. Their average age was 2 years and 3 months. Twenty-one showed X2, corneal xerosis or X3, corneal xerosis plus ulceration. Nine showed X1B, conjunctival xerosis and Bitot spot (10), and four had no eye lesions.

The median value of RBP in the serum of 28 children on admission ( $I_0$ ) was 15  $\mu$ g/ml. The  $I_0$  value of RBP in 9 children with conjunctival xerophthalmia (X1) was  $22.1 \pm 2.35$   $\mu$ g/ml and in 19 children with X2, X3 corneal xerophthalmia was  $13.5 \pm 0.81$   $\mu$ g/ml serum,  $P < 0.001$ . Figure 1 records the individual data.

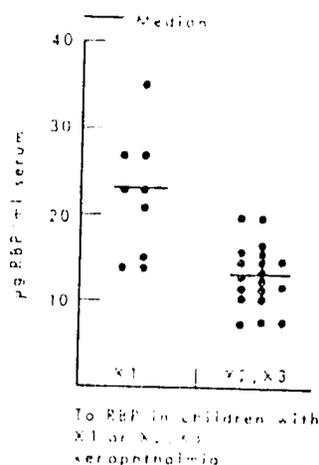


FIG. 1. RBP at admission ( $I_0$ ) in children with X1 or X2, X3 xerophthalmia.

The rise in RBP that accompanied treatment at the NRC is shown in Figure 2. Each subsection compares the RBP of the same children, at the start and after the specified time; the children in each subsection overlap but are not identical because it was not always possible to obtain a blood sample from each child during each particular period.

There is obviously no quick return to a normal level of RBP after injection of 100,000 IU of vitamin A but rather a steady progress to reach normality between 4 to 13 days of treatment with some overshoot to within a high normal range for well-nourished children. The difference between RBP on admission ( $T_0$ ) and at  $T_{200}$  was statistically significant,  $P = 0.005$  and between  $T_0$  and  $T_{140-7 \text{ days}}$ ,  $P = 0.001$ . The level of RBP fell somewhat after 1 to 2 months at home but not to a deficient level.

A few serum samples were taken back to Britain in ice for estimation of holo-RBP (12) as well as of total RBP (Table 1). Laboratory tests on the stability of RBP were carried out with human plasma stored in sterile vials, such as were used for the transport of the samples. No detectable losses of retinol bound to RBP occurred in samples stored at  $-20^\circ\text{C}$  for up to 10 weeks and at  $-4^\circ\text{C}$  for 1 to 2 weeks. Some slight losses (5 to 10%) of bound retinol occurred after 4 to 6 weeks at  $-4^\circ\text{C}$ . Samples stored in ice for the short transit time between India and England should not therefore have lost any holo-RBP.

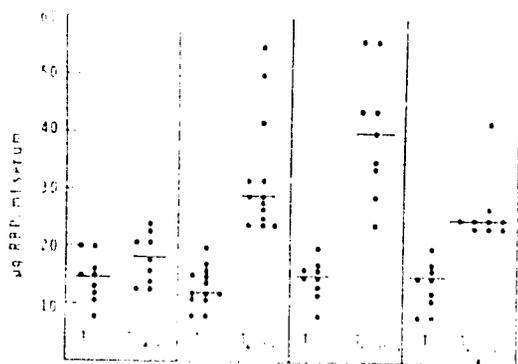


FIG. 2. Increase of serum RBP with time, treatment and after follow-up. Values are given for children on admission ( $T_0$ ) and for the same children after the specified time interval. — Median.

Total RBP, whether estimated at Madurai or after transport to England was in fair agreement at all time intervals ( $T_0$ ,  $T_{21-7}$  and  $T_{5-10 \text{ days}}$ ). Holo-RBP increased in line with the total. The one normal child investigated had normal holo-RBP both at  $T_0$  and  $T_{200}$ . These correspondences give reasonable confidence that transport had not materially altered the values of either holo- or total RBP. We have frequently encountered low total RBP and zero holo-RBP values in samples of plasma from severely vitamin A and protein-deficient children in other countries. Total serum retinol levels were not measured on the samples from India.

We estimated prealbumin as an indicator of malnutrition and specifically of protein deficiency (6). The concentration of PA was low at the time of admission (Fig. 3). There was a significant difference between the PA concentration in the serum of children admitted with X1 ( $101.6 \pm 7.0 \mu\text{g/ml}$ ) and those with X2, X3 ( $76.2 \pm 4.4 \mu\text{g/ml}$ ),  $P = 0.01$ . The levels of PA and RBP were correlated with each other throughout the range seen before and during treatment.

All children were weighed on admission, and at weekly intervals. Only 15 stayed more than two weeks at the NRC. Nine (average age 3 years and 2 months) who stayed at least one month, showed a median gain of 1.2 kg in the first month. This rate of gain slowed down on return home. Two children, treated as out-patients because their parents refused admission, showed little or no gain in weight during 2 to 3 months (Fig. 4).

Assessment of the final effect of treatment on vision was difficult as many of the children stayed only a short time. Of the children 12 who were admitted with corneal xerophthalmia (X2, X3) in one or both eyes had reasonably complete and long-term notes. Table 2 shows the effect of treatment on their eyes. No child became blind but one has seriously impaired vision and one other is blind in one eye.

## Discussion

It has been suggested (1, 2, 5) that a functional deficiency of vitamin A may be

TABLE 1  
RBP in serum of severely malnourished children with X2, X3A, xerophthalmia, before and after dosage with vitamin A and on an improved diet

Age	Nutrition diagnosis	RBP ( $\mu\text{g/ml}$ )								
		At outset			24 Hr after dose plus diet			5-19 Days after dosing plus diet		
		Madurai Liverpool			Madurai Liverpool			Madurai Liverpool		
		Total	Total	Holo	Total	Total	Holo	Total	Total	Holo
2.5 <sup>a</sup>	IV <sup>b</sup> /MK <sup>c</sup> , X3A <sup>d</sup>	20	17	14	25	28	30	56	53	48
5	IV/M, X3A	9	7	0 <sup>e</sup>	15	18	19	29	24	23
2	III <sup>f</sup> /M, X2 <sup>g</sup>	23			23	27	25	38	29	18
2	III/M, X3A	20	9	0 <sup>e</sup>	24	20	19	41	53	58
2.5	III/M, X3A				31	23	23	49	41	31
2.5	IV/M X2	15	11	0 <sup>e</sup>	21	22	21	29	37	33
2	IV/MK, X3A	11	12	0 <sup>e</sup>	15	18	20	55		56
6	Normal	35	36	29	38	38	38			

<sup>a</sup> This child received Vitamin A before reaching the Nutrition Centre. <sup>b</sup> Nutrition grade IV is < 50% and grade III is < 60% normal weight for age. <sup>c</sup> Marasmus, kwashiorkor. <sup>d</sup> X2 is corneal xerosis, X3A is corneal xerosis with ulceration. <sup>e</sup> Less than 0.5  $\mu\text{g/ml}$ .

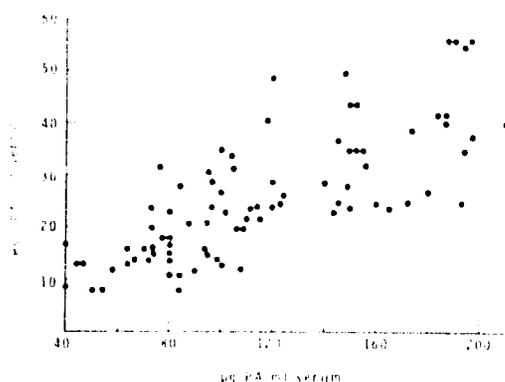


FIG. 3. Relation between levels of RBP and of PA before and during treatment and after follow-up. All values listed.

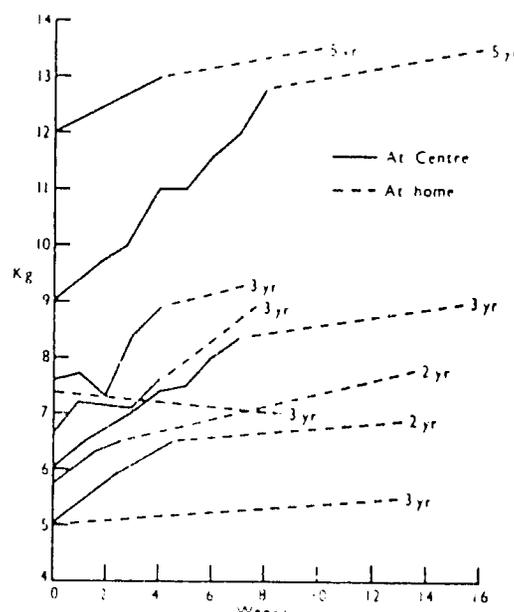


FIG. 4. Weight gain at Nutrition Centre and at home.

induced or exacerbated by a failure to synthesise RBP owing to lack of protein in the food. If the liver is not supplied with the necessary amino acids it is unable to make RBP and, even if vitamin A is supplied by injection or orally, it will not be released (1, 2, 5). Sixteen Thai children with PCM and clinical vitamin A deficiency showed no change in serum RBP values twenty-four hours after injection of 100,000 IU of vitamin A (5). This result is more striking than that reported here but the two studies basically agree that malnourished children who are also clinically vitamin A deficient do not have a sufficient store of RBP in their livers to bring the serum RBP up to normal within twenty-four hours after vitamin A injection, as might have been ex-

TABLE 2  
Effect of treatment on xerophthalmia and vision

Diagnosis	No. eyes	Result at discharge or follow-up
X1B <sup>a</sup>	2	Normal
X2 <sup>b</sup>	9	7 normal, 2 slight opacities not impeding sight
X3A <sup>c</sup>	11	5 small opacities allowing sight, 4 serious opacities, 2 blind.
X3B <sup>d</sup>	2	2 clearing at 2.6 weeks, end result unknown.

<sup>a</sup> Conjunctival xerosis plus Bitot spot. <sup>b</sup> Corneal xerosis. <sup>c</sup> Corneal xerosis plus ulceration. <sup>d</sup> Keratomalacia.

pected from experiments on rats deficient solely in vitamin A. In the doubly deficient child good food is necessary and some time must elapse before the biosynthesis of RBP can be raised to a level capable of supporting a reasonably steady state supply of RBP to the plasma. It has been previously noticed in relatively short-term experiments on protein-deficient rats, that at least 24 hr was required for the liver to recover sufficient protein synthetic capacity to sustain a normal plasma RBP level (17).

The concentration of RBP and of PA in the serum of children at the Madurai NRC rose to normal after about a week. During the first twenty-four hours of feeding the holo-RBP responded proportionally more than total RBP presumably because the injected vitamin A initiated the release of native holo-RBP from the liver into the blood, whereas the apo-RBP already in circulation became gradually metabolized.

The locally available, cheap, vegetarian food given the children produced a satisfactory rise in weight of 1.2 kg in the first month. This is almost the same as that reported in Thai children fed 4 g protein from full cream milk powder and 175 kcal/kg per day (18). A still faster catch-up growth has been achieved by feeding a diet based on milk protein ad libitum to Jamaican children in hospital (19).

Too few children with severe corneal xerophthalmia remained long enough for a complete assessment to be made of what the NRC treatment achieved in saving their sight. Many problems remain before corneal xerophthalmia can be adequately treated. There are organizational problems of follow-up and the continuation of the diet at home. Research problems lie in determining the benefits or dangers of large doses of vitamin A by injection or orally to severely malnourished children and, similarly, the benefits or dangers of feeding a high protein, high energy diet ad libitum from the start. Demonstration to the parents of benefit to their child is the mainstay of any progress. 

The authors are grateful to the Royal Commonwealth Society for the Blind for generous support. A. P. wishes to thank both the Wellcome Trust and the World Health Organization for research grants. We acknowledge our debt to the staff of the NRC and in

particular to Mr. P. Subbiah and Miss Gowri. Dr. J. Mann of the Department of Social and Community Medicine, Oxford University, kindly helped with statistics.

## References

1. GOODMAN, DE W. S. Vitamin A transport and retinol-binding protein metabolism. *Vit. Horm.* 32: 167, 1974.
2. PETERSON, P. A., S. F. NISSON, I. OSTBERG, I. RASK AND A. VAHLQUIST. Aspects of the metabolism of retinol-binding protein and retinol. *Vit. Horm.* 32: 181, 1974.
3. GLOVER, J., C. JAY AND G. J. WHITE. Distribution of Retinol binding protein in tissues. *Vit. Horm.* 32: 215, 1974.
4. SMITH, F. R., DE W. S. GOODMAN, M. S. ZAFAR, M. K. GABR, S. MARGALY AND V. J. PATWARDHAN. Serum vitamin A, retinol-binding protein and prealbumin concentrations in protein-calorie malnutrition. I. A functional defect in hepatic retinol release. *Am. J. Clin. Nutr.* 24: 973, 1973.
5. SMITH, F. R., R. SUSEKIDU, O. THANANGKUL, C. LEITZMANN, DE W. S. GOODMAN AND R. J. OLSOS. Plasma Vitamin A, retinol binding protein and prealbumin concentrations in protein-calorie malnutrition III. Response to varying dietary treatments. *Am. J. Clin. Nutr.* 28: 732, 1975.
6. ESCHBIECK, Y., H. G. VAN DEN SCHRIEK, P. DENAYER AND M. DEVISSCHER. The role of retinol-binding protein in protein-calorie malnutrition. *Metabolism* 24: 633, 1975.
7. MUTO, Y., J. E. SMITH, P. O. MITCHELL AND DE W. S. GOODMAN. Regulation of retinol-binding protein by vitamin A status in the rat. *J. Biol. Chem.* 247: 2542, 1972.
8. ARROYAVE, G. Interrelations between protein and vitamin A metabolism. *Am. J. Clin. Nutr.* 22: 1119, 1969.
9. VENKATASWAMY, G., K. A. KRISHNAMURTHY, P. CHANDRA, S. A. KABIR AND A. PIRI. A nutrition rehabilitation centre for children with xerophthalmia. *Lancet* i: 1120, 1976.
10. W.H.O. Tech. Rep. Series 890 Vitamin A deficiency and xerophthalmia. Geneva 1976.
11. MASCINI, G., A. O. CARBONARA AND J. F. HEREMANS. Immunochemical quantitation of antigens by single radial immunodiffusion. *Immunochemistry* 2: 235, 1965.
12. GLOVER, J., L. MOXLEY, H. MUMMAL AND S. M. WASTON. Micro-methods for fluorometric assay of retinol-binding in blood plasma. *Clin. Chim. Acta* 50: 371, 1974.
13. SIGEL, S. *Non-parametric Statistics for the Behavioral Sciences*. New York, McGraw Hill, 1956.
14. SMITH, F. R., B. UNDERWOOD, C. R. DUNNING, A. VARMA AND DE W. S. GOODMAN. Depressed plasma retinol-binding protein in cystic fibrosis. *J. Lab. Clin. Med.* 80: 423, 1972.
15. GOMEZ, E., R. R. GALVAN, S. FRESK, J. GRAVIOLS, R. CHAVIZ AND J. VAZQUEZ. Mortality in second and third degree malnutrition. *J. Trop. Pediatr.* 2: 77, 1956.

16. ISGENBEEK, Y., M. DE VESCHER AND P. DE NIJER. Measurement of prealbumin as index of protein-calorie malnutrition. *Lancet* 2: 106, 1972.
17. MURRAY, H., AND J. GLOVER. Effects of dietary deficiencies of protein and retinol on the plasma level of retinol-binding protein in the rat. *Brit. J. Nutr.* 32: 549, 1974.
18. OLSON, R. E. The effect of variations in protein and calorie intake on the rate of recovery and selected physiological responses in Thai children with protein-calorie malnutrition. In: *Protein-Calorie Malnutrition*, edited by R. E. Olson. New York: Academic Press, 1975, p. 275.
19. ASHWORTH, A. Ad lib feeding during recovery from malnutrition. *Brit. J. Nutr.* 31: 109, 1974.

**V.**  
**Carotenoid Analysis**

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## DETERMINATION OF THE CAROTENOID PHYTOENE IN BLOOD BY HIGH-PRESSURE LIQUID CHROMATOGRAPHY

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### SUMMARY

A sensitive and specific high-pressure liquid chromatographic assay was developed for the determination of phytoene in blood with an overall recovery of 86 ± 6.0% and a limit of detection of 50–100 ng per ml of blood. This method provides for rapid and simple quantitation of phytoene using 1 ml or less of blood.

The assay was used in the determination of phytoene blood levels in the dog following intravenous and oral administration of 10-mg/kg doses.

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### INTRODUCTION

Phytoene, a naturally occurring carotenoid<sup>1</sup>, is an endogenous precursor in the biosynthesis of  $\beta$ -carotene<sup>2</sup>, and is reported to be under investigation as an oral sunscreen<sup>3</sup>.  $\beta$ -Carotene has been used successfully in patients with erythropoietic protoporphyria to reduce the photosensitivity associated with this disease<sup>4,5</sup>. Studies with  $\beta$ -carotene to determine its effects on a normal individual's response to sunburn radiation (UV-B, 280–315 nm) indicate it is somewhat effective against sunburn radiation. These findings prompted the investigation of phytoene as an oral sunscreen<sup>6</sup>.

The major structural differences between phytoene and  $\beta$ -carotene are the presence of three conjugated double bonds in phytoene compared to the nine conjugated double bonds of  $\beta$ -carotene and the cyclization of the terminal ends of phytoene to form the  $\beta$ -ionone rings in  $\beta$ -carotene (Fig. 1). The biosynthesis of Vitamin A from  $\beta$ -carotene is known to take place mainly in the intestinal mucosa during absorption<sup>7</sup> in an *in vivo* reaction sequence involving (a) cleavage of  $\beta$ -carotene into retinal (aldehyde), followed by (b) reduction of retinal to retinol (alcohol). Retinoic acid is formed by oxidation of the aldehyde (Fig. 1). These conversions can be effected either by symmetric or asymmetric fission or by terminal oxidation<sup>8</sup>.

In order to assess the pharmacokinetics and oral absorption characteristics of phytoene, it was necessary to develop an assay capable of determining phytoene in the presence of  $\beta$ -carotene in blood. The classical column chromatographic-spectrophotometric assays for the determination of carotenoids are time-consuming<sup>7</sup>, while

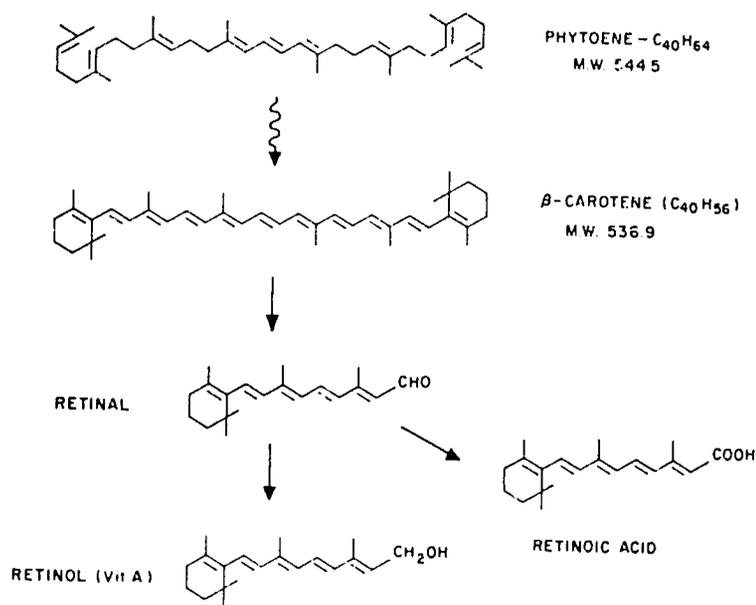


Fig. 1. Biosynthetic pathway for the conversion of phytoene to retinol (Vitamin A).

the spectrofluorometric methods are non-specific and prone to errors due to endogenous interferences<sup>9-11</sup>. Gas chromatographic analysis has been used following derivatization with silylating reagents, but this method is prone to error due to thermal instability of the derivatives<sup>12,13</sup>. High-pressure liquid chromatography (HPLC) appeared to be the method of choice, since these compounds can be analyzed at ambient temperatures without chemical derivatization and exploiting their differential spectrophotometric properties for quantitation. This technique has been successfully used in the analysis of vitamin A in bulk chemical products and pharmaceutical formulations<sup>12</sup>.

The method presented here quantitates phytoene using its ultraviolet (UV) absorbance at 280 nm to advantage, since neither  $\beta$ -carotene ( $\lambda_{\text{max.}} = 445$  nm) nor vitamin A ( $\lambda_{\text{max.}} = 325$  nm) absorb significantly at 280 nm. Furthermore, they are all chromatographically resolved, thus imparting further specificity to the assay.

## EXPERIMENTAL

### HPLC analysis of phytoene in blood

**Column.** The column used was a 0.5 m  $\times$  2 mm I.D. stainless-steel column containing 1% ODS Permaphase chemically bonded on Zipax (DuPont, Wilmington, Del., U.S.A.).

**Instrumental parameters.** A DuPont Model 830 high-pressure liquid chromatograph equipped with a Model 836 multi-wavelength UV and fluorescence detector (operated in the UV mode at 280 nm) was used. The isocratic mobile phase used was a mixture of water-methanol (5:95) at a head pressure of 500 p.s.i. and a flow-rate of 0.8 ml/min. The column was operated at ambient temperature. Under these con-

ditions the retention time of phytoene was 2.5 min and that of  $\beta$ -carotene was 4.9 min. Typical chromatograms are shown in Fig. 2. The detector sensitivity was  $4 \times 10^{-2}$  a.u.f.s., and the chart speed on the 1.0-mV Honeywell recorder (Model No. 194) was 30 in./h. Under these conditions 100 ng of phytoene per  $10 \mu\text{l}$  injected gives nearly full-scale pen response. The minimum detectable amount of phytoene is 50-100 ng/ml of blood.

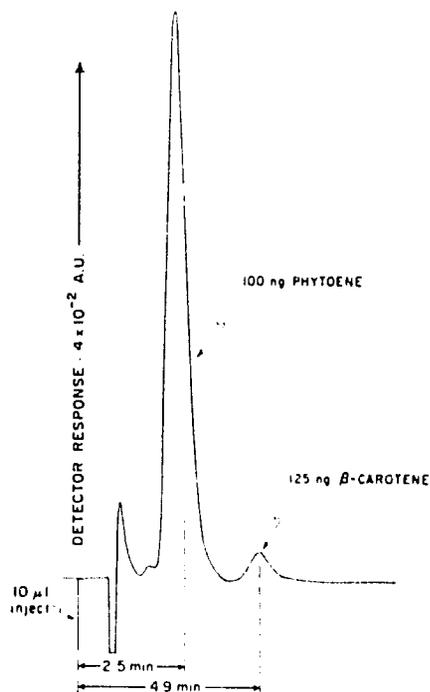


Fig. 2. Relative sensitivity of phytoene and  $\beta$ -carotene at 280 nm.

*Calibration of phytoene by HPLC.* A calibration (external standard) curve of the peak area of phytoene *versus* concentration in the range of 25-100 ng per  $10 \mu\text{l}$  of standard solution is prepared. The linear range of quantitation is from 10-1000 ng of compound. A fresh calibration curve of the external standards and of the recovered internal standards are prepared for each day of analysis to establish the reproducibility of the HPLC system.

#### *Assay in blood*

*Preparation of standard solutions.* Weigh out 10 mg of phytoene and dissolve in 10 ml of isopropanol to yield a stock solution (A) containing 1 mg/ml. Make serial dilutions of solution A to yield working solutions B<sub>1</sub>-B<sub>4</sub> containing the following concentrations of phytoene (per 0.1 ml of isopropanol): B<sub>1</sub>, 250 ng; B<sub>2</sub>, 500 ng; B<sub>3</sub>, 750 ng and B<sub>4</sub>, 1000 ng. Aliquots ( $10 \mu\text{l}$ ) of these solutions are injected as external standards for establishing the liquid chromatographic parameters. Aliquots (0.1 ml) of the same solutions are added to blood as the internal standard calibration curve

for the determination of the concentration in the unknowns and for the determination of percent recovery.

**Reagents.** All reagents must be of analytical reagent grade ( $> 99\%$  purity). Normal saline is prepared by dissolving 0.9 g of NaCl in 100 ml of deionized distilled water (0.9% solution). Absolute ethanol is used to deproteinize the blood, and a mixture of *n*-hexane (Fisher, H-301) and isopropanol (ACS grade) (95:5) is used as the solvent for extraction. A water-methanol (5:95) mixture is used as the mobile phase for HPLC analysis.

**Procedure.** The flow diagram of the extraction procedure is shown in Fig. 3.

Into a 50-ml centrifuge tube (PTFE No. 16, stoppered), place 1.0 ml of whole blood, 2.0 ml of normal saline, and mix well on a vortex mixer. Add 2.5 ml of absolute ethanol, mix occasionally, and wait for complete deproteinization (approx. 5 min). Extract the entire mixture with 10 ml of *n*-hexane-isopropanol (95:5) by shaking for 10 min on a reciprocating shaker (Eberbach, Ann Arbor, Mich., U.S.A.)

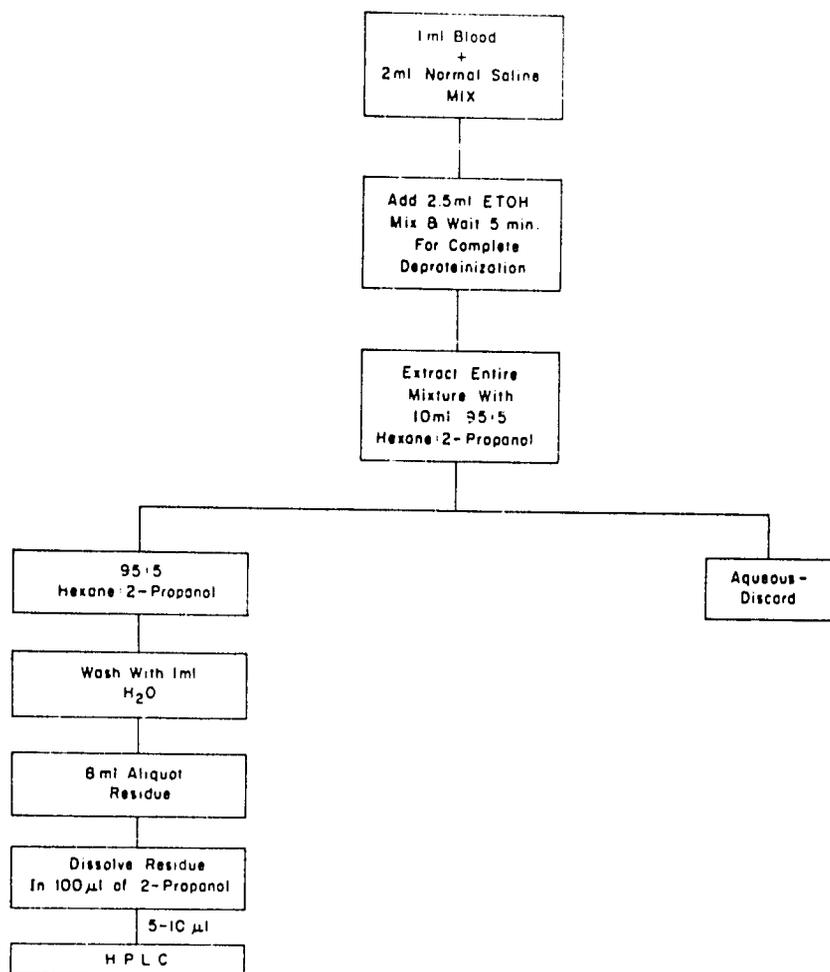


Fig. 3. Flow diagram of extraction procedure.

at 80–100 strokes/min. Along with the samples run a specimen of control blood and four 1.0-ml specimens containing 0.1 ml of standard solutions B<sub>1</sub>, B<sub>2</sub>, B<sub>3</sub>, or B<sub>4</sub>. Centrifuge the samples at 2500 rpm (1500 g) in a refrigerated centrifuge (Model PR-J, rotor No. 253; Damon, IEC, Needham, Mass., U.S.A.) at 5° and transfer a 9.0-ml aliquot into a 15-ml conical centrifuge tube. Wash this aliquot with 1.0 ml of deionized, distilled water by shaking on a reciprocating shaker for 5 min and centrifuging at 2500 rpm (1500 g) in a refrigerated centrifuge (5°) for 5 min. Remove and discard the lower aqueous layer with a hypodermic syringe fitted with a 20-gauge 6-in. cannula (Becton Dickinson, Rutherford, N.J., U.S.A.). If a heavy lipid layer remains at the interphase, a second 1.0-ml water wash is necessary. Transfer an 8.0-ml aliquot into a 15-ml conical centrifuge tube and evaporate to dryness at 60° in a

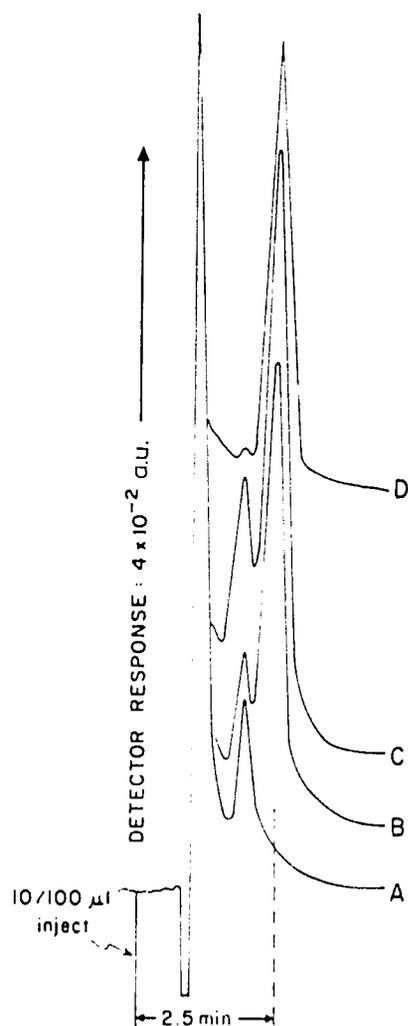


Fig. 4. Chromatograms of HPLC analysis of dog blood hexane-2-propanol (95:5) extracts of (A) control blood, (B) control blood containing added authentic standard, (C) dog blood post 10-mg/kg oral dose of phytoene, and (D) authentic standard.

N-EVAP evaporator (Organomation Assoc., Worcester, Mass., U.S.A.) under a stream of clean, dry nitrogen. Dissolve the residues in 100  $\mu$ l of isopropanol and inject a 5–10- $\mu$ l aliquot. Typical chromatograms of blood extracts are shown in Fig. 4.

*Calculations.* The concentration of phytoene in the unknowns is determined by interpolation from the calibration curve of the internal standards processed along with the unknowns, using the direct calibration (peak area *versus* concentration) technique. The percent recovery of the internal standards is determined by comparing the slope value (cm<sup>2</sup> peak area per ng of compound) of the internal standard curve to that of the external standard curve.

## RESULTS AND DISCUSSION

A sensitive and specific HPLC assay was developed for the determination of phytoene in blood using the principle of reverse-phase partition chromatography. The extraction procedure used is a modification of a previously published assay for phytofluene and retinol determined by spectrofluorometry<sup>9</sup>, and provides for rapid

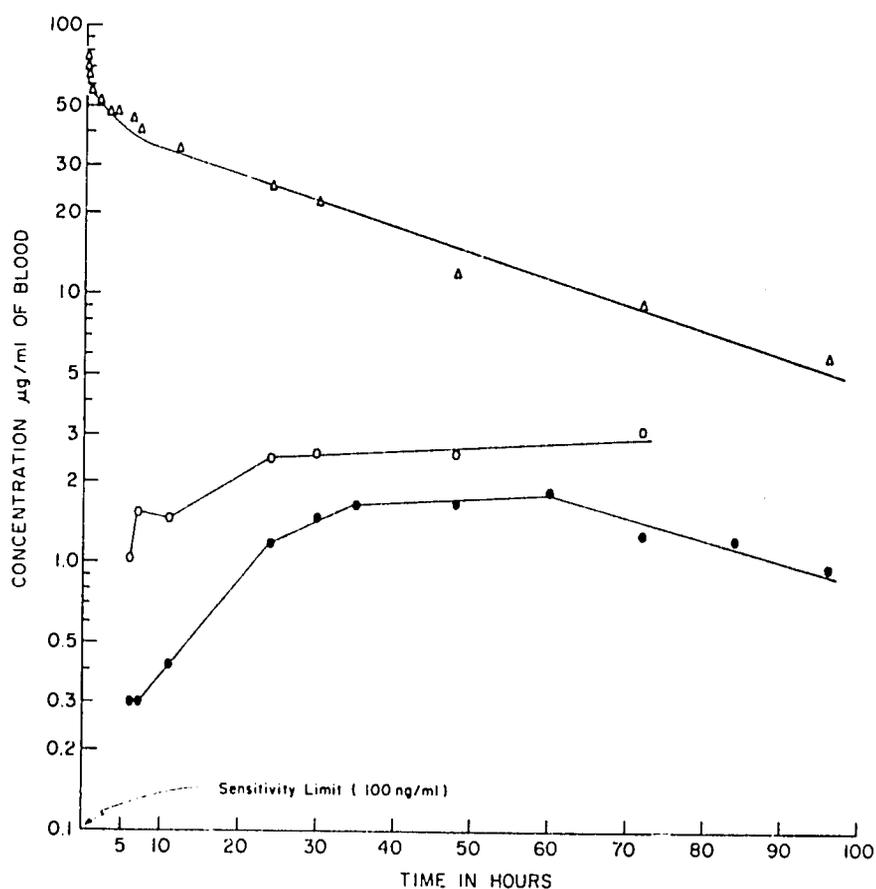


Fig. 5. Blood levels of phytoene in a dog following 10-mg/kg doses by intravenous ( $\Delta$ ) and oral ( $\circ$ , encapsulation;  $\bullet$ , suspended) routes.

and simple quantitation of phytoene using 1 ml or less of blood. A typical chromatogram of phytoene in dog blood after a 10-mg/kg oral dose is shown in Fig. 4 and a typical chromatogram of authentic phytoene and  $\beta$ -carotene (Fig. 2) shows baseline resolution between the two compounds. The difference in sensitivity at 280 nm between the two is approximately 20:1. Therefore,  $\beta$ -carotene will not interfere in the quantitation of phytoene, even if present in very large amounts. Under these conditions Vitamin A ( $\lambda_{max} = 325$  nm) will not be detected at all, thus imparting further specificity to the assay. Studies are in progress to investigate the use of multi-wavelength detection systems and differential extraction procedures for the selective determination of  $\beta$ -carotene and retinol produced endogenously following the administration of phytoene as a biosynthetic precursor<sup>14</sup>. No endogenous phytoene is measurable in control blood (Fig. 4A).

#### *Recovery and sensitivity limits of HPLC assay*

The overall recovery of phytoene is of the order of  $86 \pm 6.0\%$  (S.D.) from blood. The sensitivity limits of detection are 50–100 ng per ml of blood. These limits can be increased by either extracting larger volumes of blood or by increasing the detector sensitivity. If greater sensitivity is required, extra solvent washes must be carried out in order to effect a better cleanup of the sample so that biological impurities are minimized.

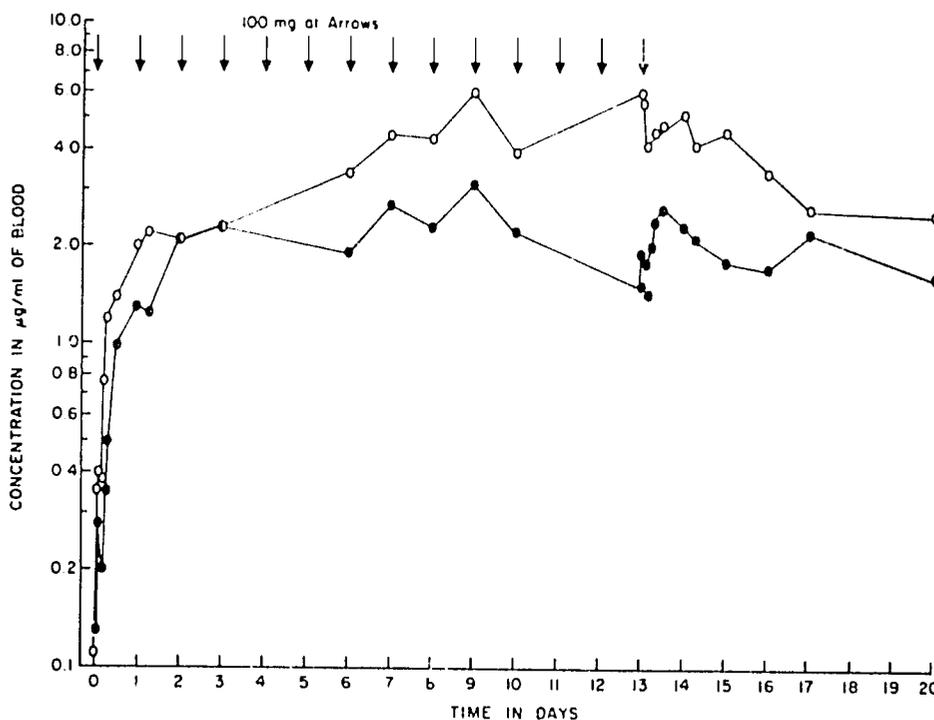


Fig. 6. Blood levels in dogs following multiple oral administration of 100-mg doses (at arrows) of an experimental formulation of phytoene. ○, Dog No. 1; ●, dog No. 2.

*Application of the method to biological specimens*

Blood levels of phytoene were determined in a pilot study in a single dog following the administration of 10-mg/kg doses by both intravenous and oral routes. Following the intravenous dose, a biphasic blood level curve was observed over the 96-h sampling interval (Fig. 5). The blood level measured at the 96-h time point (6  $\mu\text{g/ml}$ ) was about 2 orders of magnitude over the sensitivity limit of the assay, indicating sufficient assay sensitivity to measure blood levels over another 100-h interval. Following two different oral doses, the blood level curves indicated slow and prolonged absorption.

Studies following chronic oral dosing were carried out in which two dogs received 100 mg orally per day for 14 days of an experimental formulation. Blood samples were collected daily for 20 days. The resultant blood level curves (Fig. 6) suggest that the drug appears to have reached a plateau ranging between 2 and 4  $\mu\text{g/ml}$ . These studies confirm oral absorption of phytoene and allow for the investigation for its intended clinical application as an oral sunscreen<sup>6</sup>.

## REFERENCES

- 1 J. B. Davis, L. M. Jackman, P. T. Siddes and B. C. L. Weedon, *J. Chem. Soc., C*, (1966) 2154.
- 2 T. W. Goodwin, in Otto Isler (Editor) *The Carotenoids*, Birkhauser, Basle, 1971, pp. 577-636.
- 3 M. M. Mathews-Roth and M. A. Pathak, *Photochem. Photobiol.*, 21 (1975) 261.
- 4 M. M. Mathews-Roth, M. A. Pathak, T. B. Fitzpatrick, I. C. Harber and E. H. Kass, *N. Engl. J. Med.*, 282 (1970) 1231.
- 5 M. M. Mathews-Roth, M. A. Pathak, T. B. Fitzpatrick, I. C. Harber and E. H. Kass, *J. Amer. Med. Ass.*, 228 (1974) 1004.
- 6 M. M. Mathews-Roth, M. A. Pathak, J. Parrish, T. B. Fitzpatrick, E. H. Kass, K. Foda and W. Clemans, *J. Invest. Derm.*, 59 (1972) 349.
- 7 H. Thommen, in Otto Isler (Editor), *The Carotenoids*, Birkhauser, Basle, 1971, pp. 637-668.
- 8 B. M. Davies and T. W. Goodwin, *Chemistry and Biochemistry of Plant Pigments*, Academic Press, New York, 1965, p. 489.
- 9 J. N. Thompson, P. Erdody and W. B. Maxwell, R. Brien and T. K. Murray, *Biochem. Med.*, 5 (1971) 67.
- 10 J. N. Thompson, P. Erdody and W. B. Maxwell, *Biochem. Med.*, 8 (1973) 403.
- 11 F. A. Bubb and G. M. Murphy, *Clin. Chim. Acta.*, 48 (1973) 329.
- 12 M. Vecchi, J. Vesely and G. Oesterhelt, *J. Chromatogr.*, 83 (1973) 447.
- 13 R. F. Taylor and B. H. Davies, *J. Chromatogr.*, 103 (1975) 327.
- 14 J. C. Bauernfeind, *Agr. Food Chem.*, 20 (1972) 456.

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## USE OF REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSIS FOR THE DETERMINATION OF PROVITAMIN A CAROTENES IN TOMATOES\*

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### SUMMARY

The usual methods for provitamin A evaluation of foods convert the total pigment amount, determined spectrophotometrically, into vitamin A units. Since the totally inactive lycopene is the major carotenoid in the tomato, such readings result in erroneously high provitamin A values.

In view of the recent development of chemically bonded, reversed-phase, microparticulate packings and their use in high-performance liquid chromatography which combines highly accurate and reproducible resolution with the speed and ease of operation, a new method using such a system was developed to isolate carotenoid pigments from tomato samples. A 15-min column separation was thus achieved, dramatically decreasing the analysis time of the classical open column chromatographic procedures, which often result in unresolved and altered fractions due to long-term exposure to oxygen, light, solvents and sometimes adsorbent.

$\beta$ -Carotene and lycopene were determined and quantitated in six tomato samples.  $\beta$ -Carotene, 100% vitamin A-active, was expressed in International Units of vitamin A. The newly developed method gives a more reliable evaluation of the fruit potency in vitamin A than the methods of the Association of Official Analytical Chemists currently used for food composition tables.

### INTRODUCTION

Differences in the biopotency of carotenoids as vitamin A precursors result from their individual structures. The  $\beta$ -ring present in retinol is essential for their

\* R.I. Agricultural Experiment Station Contribution No. 1839.

activity.  $\beta$ -Carotene, having two such rings, is considered 100% vitamin A-active.  $\alpha$ -Carotene is only one half as potent while acyclic carotenoids such as lycopene, also naturally present in foods, are totally inactive (Fig. 1). The determination of provitamin A levels in foods hence requires the isolation and accurate quantitation of those carotenoids with biological significance. Few of the analytical methods available are suitable for this purpose: open-column chromatography, besides being time-consuming and allowing long-term exposure of carotenoids to oxygen, light, adsorbents and solvents, often fails in resolving the most potent vitamin A precursor, all-*trans*- $\beta$ -carotene, from its less active geometrical isomer,  $\alpha$ -carotene (1); both this method and thin-layer chromatography lack in reproducibility and accurate quantitation<sup>1,2</sup>; gas chromatography provides the latter advantages but cannot be used with the thermally labile carotenoids<sup>3,4</sup>.

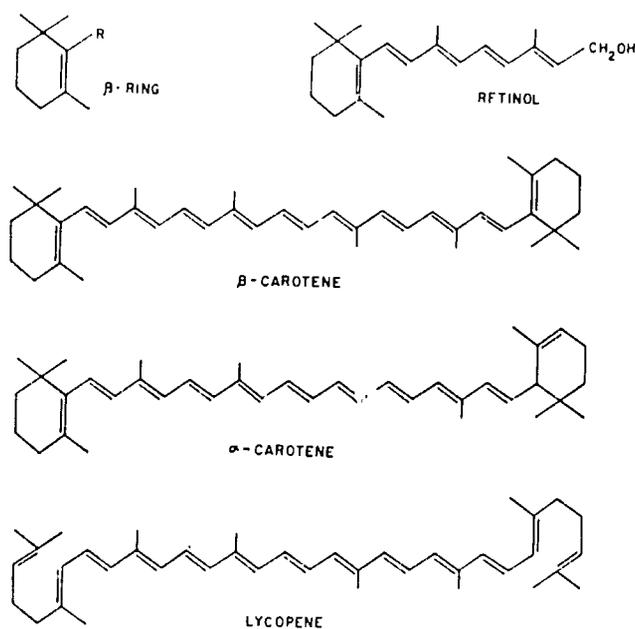


Fig. 1. Importance of  $\beta$ -ring in carotenoid activity as vitamin A precursors.

The advent of high-performance liquid chromatography (HPLC), which made available rapid, reproducible, quantitative and accurate analyses, opened up new possibilities for the study of carotenoids. The use of this technique together with the recently developed microparticle packings has resulted in faster solute distributions between the mobile and stationary phases, hence sharper elution profiles for individual compounds and the increased resolution capacity for a given separation<sup>5</sup>.

Although some carotenoids could not be separated by HPLC on silica<sup>6,7</sup>, a carotenoid mixture was resolved on both magnesium oxide<sup>1</sup> and alumina columns<sup>8</sup> by gradient elution; however, long re-equilibration periods at initial conditions were necessary for subsequent analysis<sup>8</sup>. The isolation of some isoprenoids and polyprenols was also successful on chemically-bonded, reversed-phase packings<sup>9,10</sup>. These non-

polar stationary phases present several advantages over the normal-mode polar adsorbents: they are neutral to the sample and unaffected by the presence of water or changes in the mobile phase<sup>9,11</sup>. This inherent stability renders them most suitable for routine sample analysis. The separation of carotenes was thus attempted by the use of organic eluting solvents in reversed-phase, and the resolution of  $\beta$ -carotene from other carotenoids was sought for estimation of provitamin A values in tomato samples.

## MATERIALS AND METHODS

### *Standard solutions*

All standards were purchased from Sigma (St. Louis, Mo., U.S.A.). Crystalline  $\alpha$ -carotene was dissolved in petroleum ether; the resulting solution contained 333.75  $\mu\text{g/ml}$  (0.5216 mmoles/l). Crystalline  $\beta$ -carotene was dissolved in petroleum ether (b.p., 40–60°) the resulting solution contained 154  $\mu\text{g/ml}$  (0.2869 mmoles/l). The concentration of lycopene dissolved in dichloromethane was 166.66  $\mu\text{g/ml}$  (0.3104 mmoles/l, solution I); 1 ml of this solution in 25 ml dichloromethane constituted solution II (0.0124 mmoles/l).

The retention times of lycopene,  $\alpha$ - and  $\beta$ -carotene averaged 7.82, 12.4 and 13.2 min, respectively. The peak areas were measured for quantitation.

### *Liquid chromatograph*

A Waters Model 6000A Solvent delivery system (Waters Assoc., Milford, Mass., U.S.A.) was used. This is a reciprocating plunger pump which permits digital selection of a constant flow-rate. Waters U6K injector system allowed loading of the sample at atmospheric pressure and assured accurate injections (no sample loss due to back pressure on the system). A Waters Model 450 Variable Wavelength Detector was set at 470 nm. This wavelength was found most suitable for the simultaneous detection of  $\alpha$ -carotene,  $\beta$ -carotene and lycopene.

A SF 770 Spectroflow Monitor variable-wavelength detector equipped with SFA 339 Wavelength drive and MM 700 Memory Module, all from Kratos Inc., Schoeffel Instrument Division (Westwood, N.J., U.S.A.), was used for obtaining stopped-flow visible spectra.

Chromatographic peaks were recorded on a Houston Omniscrite recorder. The following chromatographic columns were used in the course of this study: stainless-steel (30 cm  $\times$  3.9 mm I.D.)  $\mu$ -Porasil (Waters); stainless-steel (25 cm  $\times$  4.6 mm I.D.) packed with LiChrosorb RP-8 (Brownlee, Santa Clara, Calif., U.S.A.); stainless-steel (30 cm  $\times$  3.2 mm I.D.)  $\mu$ Bondapak  $\text{C}_{18}$  (Waters); stainless-steel (25 cm  $\times$  4.6 mm I.D.) Partisil-PXS-10.25ODS-2 (Whatman, Clifton, N.J., U.S.A.); stainless-steel (25 cm  $\times$  4.6 mm I.D.) Partisil-PXS-5/ODS (Whatman).

### *Solvents*

Isooctane, chloroform and acetonitrile (Burdick & Jackson Labs., Muskegon, Mich., U.S.A.), diethyl ether (Mallinckrodt, St. Louis, Mo., U.S.A.) and methanol (Matheson, Coldeman & Bell, Norwood, Ohio, U.S.A.) were all residue-free and suitable for chromatography and spectrophotometry. Solvents were filtered through a 0.5- $\mu\text{m}$  glassfiber filter (Gelman, Ann Arbor, Mich., U.S.A.) and degassed under vacuum prior to use.

### *Preparation of food samples*

*Extraction procedure.* Six tomato samples (red-ripe Massachusetts greenhouse tomatoes), each weighing *ca.* 160 g, were individually cut into small pieces and homogenized under a stream of nitrogen in acetone for 1–2 min in a blender. The initial homogenate was filtered through a sintered glass funnel, pore size 20–30  $\mu\text{m}$  (Whatman), under reduced pressure and the residue recovered for extraction. The procedure was repeated until complete extraction of all pigments was achieved. The acetone extract was then added to an equal volume of freshly distilled, peroxide-free, petroleum ether (PE) in a separatory funnel, mixed and diluted with water. Upon formation of two layers, the lower aqueous phase was re-extracted once with PE and the bulked PE solutions washed three times with water to remove acetone<sup>3,4</sup>.

### *Saponification*

Saponification is generally necessary in carotenoid analysis to remove unwanted lipid material which could interfere with the chromatography of compounds of interest.

Extracts were evaporated to dryness using a rotary evaporator, and a solution of 15% KOH in methanol was added to the round bottom flask. The alkaline mixtures were left in the dark for 14 h at room temperature. They were afterwards gradually added to freshly distilled petroleum ether in a separatory funnel. Water was slowly poured into the funnel so as not to form an emulsion. When two phases appeared, the lower aqueous phase was drawn off and extracted three times with fresh volumes of PE. The ethereal solutions were then bulked in a separatory funnel and washed free from alkali by repeated additions of water, followed by discarding the resultant aqueous layers. Each saponified extract was then concentrated to 100 ml in a rotary evaporator and stored under nitrogen in a volumetric flask<sup>3,4</sup>.

### *Removal of sterols*

The different samples were kept in the freezer overnight, at  $-10^{\circ}\text{C}$ , and the sterols precipitated to the bottom of the containers<sup>3,4</sup>.

### *Aliquots for HPLC*

Ten ml from each flask were filtered through a 0.5- $\mu\text{m}$  glass-fiber filter before injection. Care was always taken not to expose any of the samples to light. All samples were stored under nitrogen in the freezer. Both 50- $\mu\text{l}$  and 10- $\mu\text{l}$  samples in petroleum ether were injected, the former to determine mainly  $\beta$ -carotene amounts and the latter, lycopene. Concentrations of each compound were determined from the slope of the calibration plots in which peak area was plotted against amount injected (nmoles  $\beta$ -carotene or lycopene). The detector responses were found to be linear over the entire working range.

### *Identification of peaks in HPLC eluents*

Initial peak identification was based on retention times and comparison with the standards as well as co-chromatography with the standards. Since retention times alone are not sufficient for positive identification, stopped-flow visible spectra of the chromatographic peaks were also obtained. The Schoeffel variable wavelength detector is equipped with a memory module which automatically stores the spectral background

caused by changes in the optical properties of solvents, flow-cell light path and monochromator, and later subtracts it from the scans of the compounds. In order to obtain a stopped-flow scan, the flow is arrested at the top of each peak and the corrected spectrum scanned over the desired wavelength range.

## RESULTS AND DISCUSSION

*Optimization of chromatographic conditions*

A Waters  $\mu$ Porasil (10- $\mu$ m silica particles) was first tried. Pure isooctane, used as a mobile phase, resulted in long retention times (large  $k'$  values) and did not resolve  $\alpha$ - and  $\beta$ -carotene (*cf.*, ref. 7); with addition of chloroform to isooctane both  $\beta$ -carotene and lycopene were eluted with the same retention time. Gradients were found to be impractical since the column re-equilibration necessitated more than 3 h under any set of conditions.

All other columns used had non-polar packings and were operated under reversed-phase conditions. The Brownlee column packed with LiChrosorb RP-8 ( $C_{18}$  chemically bonded to silica, 10- $\mu$ m particle size) resulted in too rapid elutions.

The increased "thickness" of the stationary phase in the  $C_{18}$ - over the  $C_8$ -coated packings is expected to increase the  $k'$  values<sup>12</sup>. This was indeed found to be true with Waters  $\mu$ Bondapak  $C_{18}$  (10- $\mu$ m particle size) and Whatman Partisil-PXS-10, 25ODS-2 (also 10- $\mu$ m particle size). The latter column and a mixture of 8.3% chloroform in acetonitrile as a mobile phase gave the best separation for a mixture of  $\alpha$ - and  $\beta$ -carotene standards, although resolution ( $R_s = 0.63$ ) was still inadequate

TABLE I  
INJECTIONS OF  $\beta$ -CAROTENE,  $\alpha$ -CAROTENE AND LYCOPENE STANDARDS ON PARTISIL-10 ODS-2 AND PARTISIL-5 ODS

	<i>Partisil-10 ODS-2</i>		<i>Partisil-5 ODS</i>		
	<i>Chloroform: in acetonitrile</i>		<i>Chloroform: in acetonitrile</i>		
	<i>11.6% (1 ml/min)</i>	<i>8.5% (1 ml/min)</i>	<i>11.6% (2 ml/min)</i>	<i>8.5% (2 ml/min)</i>	<i>8.0% (2 ml/min)</i>
$k'$					
$\beta$ -Carotene	6.0	8.48	5.84	7.90	8.84
$\alpha$ -Carotene	5.57	7.62	5.56	7.43	7.92
lycopene	3.23	4.63	3.21	4.31	4.84
$\alpha$					
$k'_{\beta-car}$					
$k'_{\alpha-car}$	1.07	1.11	1.05	1.06	1.11
$k'_{\alpha-car}$					
$k'_{lyc}$	1.72	1.64	1.73	1.72	1.63
$k'_{\beta-car}$					
$k'_{lyc}$	1.85	1.83	1.82	1.83	1.82
$R_s = \frac{2(V_{\beta-car} - V_{\alpha-car})}{(W_{\beta-car} + W_{\alpha-car})}$		0.63	1.0	1.2	$\geq 1.5$

Increasing the efficiency of the column by using 5- $\mu\text{m}$  particles<sup>13</sup> in Whatman Partisil-PXS-5/ODS improved the resolution of the  $\alpha$ - and  $\beta$ -carotene standards to a value of 1.2 (Table I). Samples were then chromatographed using this column and a mobile phase containing 8% chloroform in acetonitrile. Fig. 2 shows a chromatogram resulting from injecting the three standards simultaneously: the geometric isomers,  $\alpha$ - and  $\beta$ -carotene, were resolved.

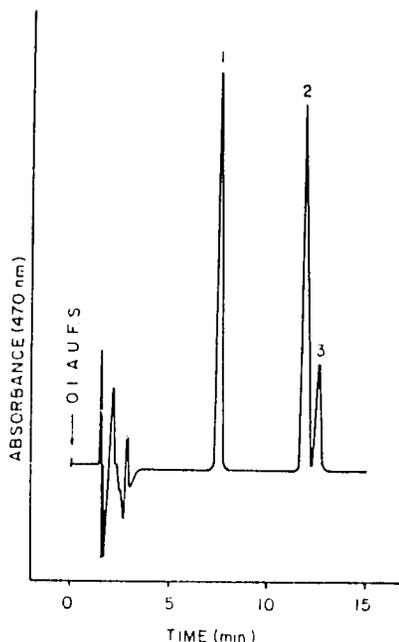


Fig. 2. Separation of standards: 1 lycopene; 2  $\alpha$ -carotene; 3  $\beta$ -carotene (*R*, for peaks no. 2 and 3, 1.46). Chromatographic conditions: column, Partisil-5/ODS, 5  $\mu\text{m}$ ; eluent, 8.0% chloroform in acetonitrile; flow-rate, 2.0 ml/min; temperature, ambient; detection, 470 nm; sensitivity, 0.1 A.U.F.S.

#### Peak identification and quantitation

Under the chromatographic conditions used, the lower detection limits for lycopene,  $\alpha$ -carotene and  $\beta$ -carotene standards were found to be 0.00395, 0.0372 and 0.0285 nmoles, respectively.

Chromatograms of tomato extracts run under the conditions described in the experiment are shown in Fig. 3. In reversed-phase chromatography, more polar compounds elute first<sup>14</sup>. This, together with classical literature on tomato pigments<sup>15,17</sup>, indicates that the early eluted peak, X is probably a xanthophyll whereas peak Y, which elutes between lycopene and  $\beta$ -carotene, might be  $\gamma$ -carotene.  $\alpha$ -Carotene is not prominent in any sample. As expected<sup>15</sup>, the major components of tomato extracts, lycopene and  $\beta$ -carotene, were found to be in a *ca.* 9:1 ratio. Identification of these was done by comparing both the retention times and the visible spectra (380–600 nm) of the peaks from the extracts and the standard solutions. Close agreement between electronic absorption spectra (stopped-flow scanning method) confirms this identifi-

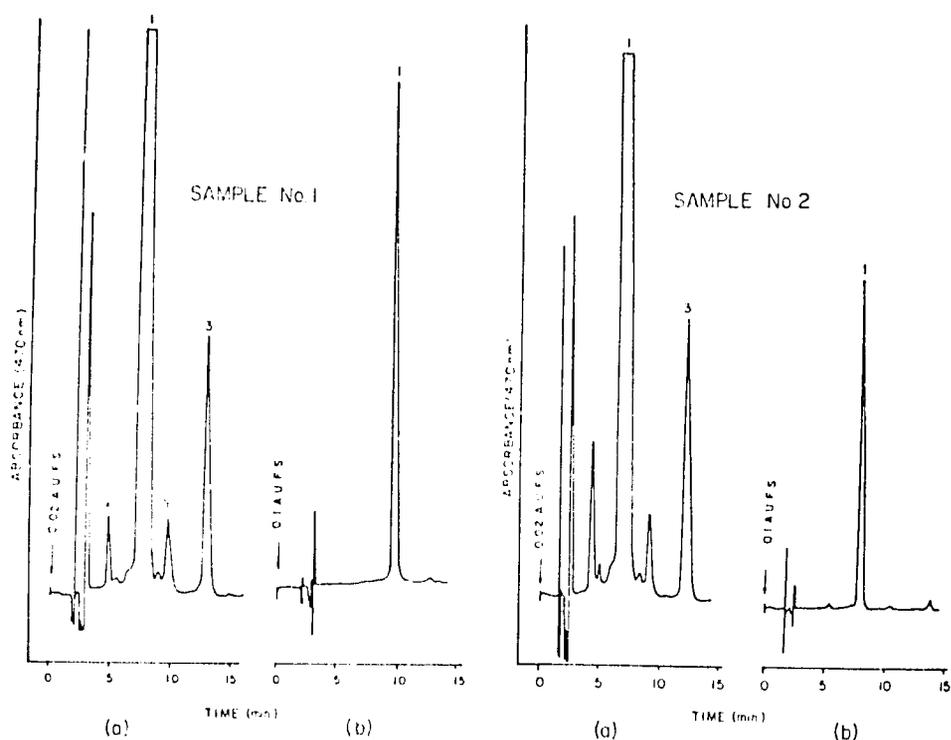


Fig. 3. Chromatograms of two tomato extracts: (a) 50  $\mu$ l injected; (b) 10  $\mu$ l injected. Chromatographic conditions as in Fig. 2. Peaks 1 and 3 are lycopene and  $\beta$ -carotene, respectively.

cation since the absorbance is a function of the chromophore<sup>3</sup>, thus a characteristic of each carotenoid (Fig. 4).

The chromatographic peaks of  $\beta$ -carotene and lycopene were quantitated using the external calibration method; the values obtained for the tomato samples studied were shown in Table II.

A report from the National Academy of Sciences estimates that only one sixth of the  $\beta$ -carotene from a diet is actually converted to retinol in humans<sup>18</sup>. The average  $\beta$ -carotene value of five samples, excluding sample No. 5, amounted to 1.218  $\mu$ g/g or 1.218/6 = 0.2031  $\mu$ g/g retinol equivalents. Since 0.3  $\mu$ g retinol is by definition equivalent to 1 International Unit (I.U.) of vitamin A, the tomatoes studied contained 0.2031/0.3 = 0.6771 I.U./g.

The actual potency of the sample may be a little underestimated since  $\gamma$ -carotene, also a vitamin A precursor<sup>16</sup>, was not accounted for. The resulting differences, however, should not be too important since  $\gamma$ -carotene is known to occur only in very small quantities in tomatoes<sup>15,17</sup> and has only one half the bioactivity of  $\beta$ -carotene<sup>16</sup>.

On the other hand, including inactive pigments such as lycopene, which is present in large amounts in the tomato fruit, would overrate the provitamin A content of the sample. The method of the Association of Official Analytical Chemists (AOAC) which includes this carotenoid as well as others<sup>8</sup>, results in erroneously high values (Table III).

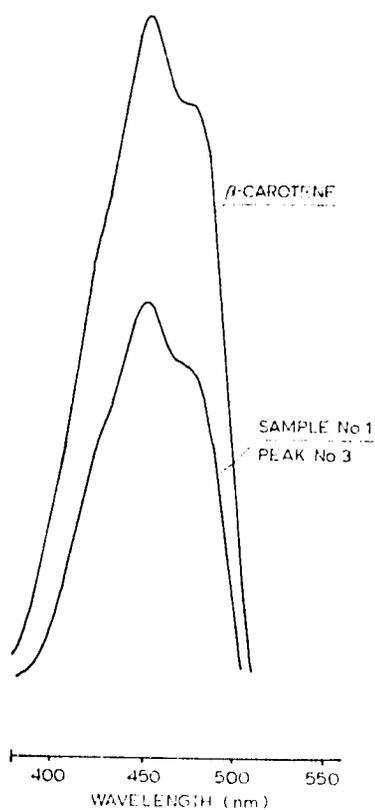


Fig. 4. Comparison of visible absorption spectra of  $\beta$ -carotene standard and peak no. 3 in sample no. 1. Scanning rate, 100  $\mu\text{m}/\text{min}$ ; sensitivity, 0.02 A.U.F.S.

TABLE II

QUANTITATION OF LYCOPENE AND  $\beta$ -CAROTENE IN TOMATO SAMPLES

Sample No.	Lycopene ( $\mu\text{g}/\text{g}$ sample)	$\beta$ -Carotene ( $\mu\text{g}/\text{g}$ sample)
1	3.840	1.196
2	9.926	1.209
3	8.981	1.270
4	9.985	1.205
5	9.295	0.805
6	10.72	1.212

TABLE III

MEASUREMENT OF PROVITAMIN A VALUES IN TOMATOES: COMPARISON OF THE HPLC AND AOAC METHODS

In each case it is assumed that one sixth of the measured pigment(s) is converted to retinol.

Methodology	Av. value ( $\mu\text{g}/\text{g}$ )	Retinol equivalent ( $\mu\text{g}/\text{g}$ )	I.U./g
$\beta$ -Carotene as obtained by HPLC	1.218	0.203	0.677
$\beta$ -Carotene and lycopene obtained by HPLC	11.001	1.833	6.111
AOAC method	18.063	3.010	10.035

It is therefore of interest to quantitate vitamin A precursors only and to improve literature values which were obtained by using classical chromatographic and spectrophotometric techniques.

In summary, a new HPLC reversed-phase method was developed for the separation of carotenoids using a 5- $\mu$ m particle column. The analysis of the saponified and washed extract is performed isocratically in less than 15 min. With the mobile phase of 8% chloroform in acetonitrile, the column pressure was found to be 2000 p.s.i.; it however, increased beyond usable range over period of time. The newly developed 5- $\mu$ m packings, although more efficient than the regular 10- $\mu$ m particles, have a shorter column life.

Repeated injections of standards and sample No. 1 were used to demonstrate the reproducibility of peak areas and retention times. The reversed-phase packings possess great stability and the ability to separate isocratically compounds of a wide polarity range (the acyclic lycopene being different from the bicyclic double-bond positional isomers  $\alpha$ - and  $\beta$ -carotene).

In addition, nanogram quantities of compounds under study can be detected and since the analysis time is short the pigment decomposition and formation of artifacts are minimized<sup>1</sup>. All this makes the described HPLC system suitable for routine assays of provitamin A content in natural products. This is only a preliminary study and more work is necessary to insure the significance of quantitative data. This method can easily be applied to the analyses of various other vegetables and fruits, in order to determine the best natural sources of provitamin A, encourage their growth and promote their daily consumption.

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#### REFERENCES

- 1 I. Stewart, *J. Agr. Food Chem.*, 25 (1977) 1132.
- 2 T. Hiyarna, M. Nishimura and B. Chance, *Anal. Chem.*, 29 (1969) 339.
- 3 B. H. Davies, in E. W. Goodwin (Editor), *Chemistry and Biochemistry of Plant Pigments*, Vol. 2, Academic Press, New York, 2nd ed., 1976, pp. 38-165.
- 4 L. C. Raymundo, A. E. Griffiths and K. L. Simpson, *Phytochemistry*, 6 (1967) 1527.
- 5 C. A. Frolik, T. E. Tavela and M. B. Sporn, *J. Lipid Res.*, 19 (1978) 32.
- 6 I. Stewart, *J. Assoc. Offic. Anal. Chem.*, 60 (1977) 132.
- 7 A. Fiksdahl, J. T. Mortensen and S. Liaaen-Jensen, *J. Chromatogr.*, 157 (1978) 111.
- 8 S. K. Reeder and G. L. Park, *J. Assoc. Offic. Anal. Chem.*, 58 (1975) 595.
- 9 P. C. Donnahey and E. N. Heming, *Biochem. Soc. Trans. 557th Meeting, Liverpool, 1975*, Vol. 3, pp. 775-776.
- 10 T. Chojnaeki, W. Jankowski, T. Mankowski and W. Sasak, *Anal. Biochem.*, 69 (1975) 114.
- 11 K. Eskins, C. R. Scholfield and H. J. Dutton, *J. Chromatogr.*, 135 (1977) 217.
- 12 A. Nakae and G. Muto, *J. Chromatogr.*, 120 (1976) 47.
- 13 S. K. Hajibrahim, P. J. C. Tibbets, C. D. Watts, J. R. Maxwell, G. Eglinton, H. Colin and G. Guiochon, *Anal. Chem.*, 50 (1978) 549.
- 14 C. G. Simpson, *Practical High Performance Liquid Chromatography*, Hyden & Son, New York, 1976, pp. 8-12.
- 15 J. C. Bauernfeind, *Encyclopedia of Food Technology and Food Science Series*, 3 (1977) 113.
- 16 B. Borenstein and R. H. Bunnell, *Advan. Food Res.*, 15 (1967) 195.
- 17 J. L. Fiasson, N. Arpin, P. Lebreton and P. Bouchez, *Chim. Anal. (Paris)*, 51 (1969) 227.
- 18 *Recommended Dietary Allowances*, National Academy of Sciences, Washington, D.C., 8th ed., 1974, pp. 50-54.

## ANALYTICAL METHODS - CAROTENOIDS by B.H. Davies

In T.W. Goodwin (ed.) "Chemistry and Biochemistry of Plant Pigments,"  
Volume 2, Second Edition, Academic Press, London (1976), pp. 150-153.

TABLE XXVII

 $E_{1\text{ cm}}^{1\%}$  values for carotenoids

Carotenoid	$E_{1\text{ cm}}^{1\%}$	$\lambda$ (nm)	Solvent	Reference
Aleuriaxanthin	2600	460	Light petroleum	Liaaen-Jensen (1965b)
Aleuriaxanthin	2440	463	Acetone	Arpin <i>et al.</i> (1973)
Anhydroeschscholtzanthin	3018	499	Hexane	Zechmeister and Wallcave (1953)
$\beta$ -Apo-2'-carotenal	2730	498	Light petroleum	Rüegg <i>et al.</i> (1959)
$\beta$ -Apo-8'-carotenal	2640	457	Light petroleum	Rüegg <i>et al.</i> (1959)
$\beta$ -Apo-10'-carotenal	2190	435	Light petroleum	Rüegg <i>et al.</i> (1959)
$\beta$ -Apo-12'-carotenal	2160	414	Light petroleum	Rüegg <i>et al.</i> (1959)
Astacene	1690	498	Pyridine	Isler and Schudel (1963)
Aurochrome	2012	409	Benzene	Barber <i>et al.</i> (1960)
Auroxanthin	1850	402	Ethanol	Karrer and Rutschmann (1942)
Azafrin	2200	409	Light petroleum	Davies (1965)
Bisdehydro- $\beta$ -carotene	2400	471	Light petroleum	Isler <i>et al.</i> (1956b)
Bixin	4200	456	Light petroleum	Davies (1965)
Canthaxanthin	2200	466	Light petroleum	Isler and Schudel (1963)
Canthaxanthin	2200	469	Cyclohexane	Surmatis <i>et al.</i> (1970)
Canthaxanthin	2092	480	Benzene	Warren and Weedon (1958b)
Capsanthin	2072	483	Benzene	Warren and Weedon (1958a)
Capsorubin	2200	489	Benzene	Warren and Weedon (1958a)
$\alpha$ -Carotene	2800	444	Light petroleum	Schwieter <i>et al.</i> (1965)
$\alpha$ -Carotene	2180	477	Carbon disulphide	Goodwin (1955)
$\beta$ -Carotene	2592	453	Light petroleum	Schwieter <i>et al.</i> (1965)
$\beta$ -Carotene	2592	453	Hexane	Isler <i>et al.</i> (1956a)
$\beta$ -Carotene	2620	453	Ethanol	Isler <i>et al.</i> (1956a)
$\beta$ -Carotene	2505	457	Cyclohexane	Isler <i>et al.</i> (1956a)
$\beta$ -Carotene	2337	465	Benzene	Isler <i>et al.</i> (1956a)
$\beta$ -Carotene	2396	465	Chloroform	Isler <i>et al.</i> (1956a)

TABLE XXVII—continued

Carotenoid	$E_{1\text{ cm}}^{1\%}$	$\lambda$ (nm)	Solvent	Reference
$\beta$ -Carotene	2008	484	Carbon disulphide	Isler <i>et al.</i> (1956a)
$\beta$ -Carotene-5,6,5',6'-diepoxide	2394	451	Benzene	Barber <i>et al.</i> (1960)
$\gamma$ -Carotene	2760	462	Hexane	Bindl <i>et al.</i> (1970)
$\gamma$ -Carotene	3100	462	Light petroleum	Schwieter <i>et al.</i> (1965)
$\delta$ -Carotene	3290	456	Light petroleum	Schwieter <i>et al.</i> (1965)
$\epsilon$ -Carotene	3120	440	Light petroleum	Schwieter <i>et al.</i> (1965)
$\epsilon$ -Carotene	2890	444	Cyclohexane	Isler and Schudel (1963)
$\zeta$ -Carotene	2555	490	Hexane	Davis <i>et al.</i> (1966)
$\beta$ -Carotenone	1626	490	Chloroform	Yokoyama and White (1968)
Chrysanthemaxanthin	2100	421	Ethanol	Karrer and Jucker (1943)
Citranaxanthin	2145	463	Light petroleum	Yokoyama and White (1965a)
Crocetin	4320	450	Light petroleum	Isler and Schudel (1963)
Crocetindial	3970	445	Benzene	Eugster <i>et al.</i> (1969)
Cryptocapsin	1972	486	Benzene	Cholnoky <i>et al.</i> (1963)
$\alpha$ -Cryptoxanthin	2636	446	Hexane	Cholnoky <i>et al.</i> (1958)
$\alpha$ -Cryptoxanthin	2355	457	Benzene	Cholnoky <i>et al.</i> (1958)
$\beta$ -Cryptoxanthin	2386	452	Light petroleum	Isler <i>et al.</i> (1957)
Deepoxyneoxanthin	2350	445	Ethanol	Krinsky and Levine (1964)
3,4-Dehydro- $\beta$ -carotene	2330	461	Light petroleum	Isler and Schudel (1963)
3,4-Dehydrolycopene	3000	492	Light petroleum	Liaaen-Jensen (1965b)
2,2'-Dihydroxy- $\beta$ -carotene	2060	452	Acetone	Kj $\ddot{a}$ rsen <i>et al.</i> (1972)
Echinenone	2158	458	Light petroleum	Davies (1965)
Echinenone	2110	461	Cyclohexane	Surmatis <i>et al.</i> (1970)
Echinenone	2091	472	Benzene	Warren and Weedon (1958b)
Eschscholtzanthin	3269	472	Hexane	Entschel and Karrer (1957)
Flavoxanthin	2550	432	Benzene	Kuhn and Brockmann (1932)
Fucoxanthin	2036	478	Carbon disulphide	Bonnett <i>et al.</i> (1969)
Fucoxanthinol	1453	452	Ethanol	Bonnett <i>et al.</i> (1969)
Gazaniaxanthin	2580	462	Acetone	Arpin and Liaaen-Jensen (1969)
Helenien	1394	445	Hexane	Cholnoky <i>et al.</i> (1958)
Helenien	1254	457	Benzene	Cholnoky <i>et al.</i> (1958)
2-Hydroxy- $\alpha$ -carotene	2330	447	Acetone	Kj $\ddot{a}$ rsen <i>et al.</i> (1972)
2-Hydroxy- $\beta$ -carotene	2290	452	Acetone	Kj $\ddot{a}$ rsen <i>et al.</i> (1972)

4-Hydroxyechinenone	2250	454	Light petroleum	Liaaen-Jensen (1965a)
2-Hydroxyplectanixanthin	2445	476	Acetone	Liu <i>et al.</i> (1973)
Isofucoxanthin	1600	453	Light petroleum	Jensen (1966)
Isozeaxanthin	2400	451	Light petroleum	Isler and Schudel (1963)
Lutein	2550	445	Ethanol	Strain (1938)
Lutein	2236	458	Benzene	Cholnoky <i>et al.</i> (1967)
Lutein	2160	475	Carbon disulphide	Kuhn and Smakula (1931)
Lycopene	3450	472	Light petroleum	Schwieter <i>et al.</i> (1965)
Lycopene	3370	487	Benzene	Surmatis and Ofner (1963)
Lycophyll	3240	508	Acetone	Markham and Liaaen-Jensen (1968)
Lycoxanthin	3080	474	Acetone	Kjøsen and Liaaen-Jensen (1972)
Methyl apo-6'-lycopenoate	2600	471	Light petroleum	Kjøsen and Liaaen-Jensen (1969)
Mutatochrome	2260	428	Diethyl ether	Hertzberg and Liaaen-Jensen (1967a)
Mutatochrome	1989	437	Benzene	Barber <i>et al.</i> (1960)
Myxoxanthophyll	2160	478	Acetone	Hertzberg and Liaaen-Jensen (1969a)
Neochrome	2270	424	Ethanol	Cholnoky <i>et al.</i> (1966)
Neoxanthin	2243	439	Ethanol	Cholnoky <i>et al.</i> (1966)
Neurosporaxanthin	1715	477	Light petroleum	Aasen and Liaaen-Jensen (1965)
Neurosporaxanthin	2210	486	Benzene	Zalokar (1957)
Neurosporene	2918	440	Hexane	Davis <i>et al.</i> (1966)
Oscillaxanthin	750	490	10% Pyridine/MeOH	Hertzberg and Liaaen-Jensen (1969b)
Philosamiaxanthin	2555	446	Ethanol	Chino <i>et al.</i> (1969)
Physalien	1340	452	Light petroleum	Isler <i>et al.</i> (1956d)
Physoxanthin	2400	445	Hexane	Bodea and Nicoară (1957)
Phytoene (15- <i>cis</i> )	757	286	Hexane	Davis <i>et al.</i> (1966)
Phytoene (all- <i>trans</i> )	915	286	Hexane	Davis <i>et al.</i> (1966)
Phytofluene	1577	347	Hexane	Davis <i>et al.</i> (1966)
Plectanixanthin	2505	474	Acetone	Liu <i>et al.</i> (1973)

TABLE XXVII—continued

Carotenoid	$E_{1\%}^{1\text{cm}}$	$\lambda$ (nm)	Solvent	Reference
Prolycopene	1920	434	Light petroleum	Goodwin (1955)
Pyrenoxanthin	1763	454	Chloroform	Yamamoto <i>et al.</i> (1969)
Retrodehydro- $\beta$ -carotene	3205	472	Light petroleum	Isler and Schudel (1963)
Retrodehydro- $\beta$ -carotene	3130	473	Cyclohexane	Surmatis <i>et al.</i> (1970)
Rhodoxanthin	2500	490	Hexane	Isler and Schudel (1963)
Rhodoxanthin	2386	491	Cyclohexane	Surmatis <i>et al.</i> (1970)
Rubixanthin	2909	462	Acetone	Arpin and Liaen-Jensen (1969)
Semi- $\alpha$ -carotenone	1431	509	Chloroform	Yokoyama and Guerrero (1970)
Semi- $\beta$ -carotenone	1850	467	Hexane	Yokoyama and White (1968)
Sintaxanthin	2588	462	Benzene	Yokoyama and White (1965b)
Tarachrome	2045	432	Benzene	Eugster and Karrer (1957)
Taraxanthin	2800	442	Ethanol	Kuhn and Lederer (1931b)
Taraxanthin	2373	455	Benzene	Eugster and Karrer (1957)
7,8,11,12-Tetrahydro- $\gamma$ -carotene	1800	378	Light petroleum	Davies and Rees (1973)
7,8,11,12-Tetrahydrolycopene	2519	395	Hexane	Davis <i>et al.</i> (1966)
Torularhodin	2040	507	Light petroleum	Isler and Schudel (1963)
Torularhodin	1932	515	Chloroform	Simpson <i>et al.</i> (1964b)
Torularhodinaldehyde	2840	514	Light petroleum	Schwieter <i>et al.</i> (1966)
Torularhodin methyl ester	2950	497	Light petroleum	Isler <i>et al.</i> (1959)
Torulene	3240	480	Light petroleum	Rüegg <i>et al.</i> (1961)
Triphasiaxanthin	1654	480	Chloroform	Yokoyama <i>et al.</i> (1970)
Violaxanthin	2550	443	Ethanol	Karrer and Jucker (1943)
Violaxanthin	2240	454	Benzene	Eugster and Karrer (1957)
$\alpha$ -Zeaxarotene	1850	421	Hexane	Petzold <i>et al.</i> (1959)
$\alpha$ -Zeaxarotene	2450	421	Light petroleum	Davies (1965)
$\beta$ -Zeaxarotene	1940	427	Hexane	Petzold <i>et al.</i> (1959)
$\beta$ -Zeaxarotene	2520	428	Light petroleum	Rüegg <i>et al.</i> (1961)
Zeaxanthin	2350	452	Light petroleum	Isler and Schudel (1963)
Zeaxanthin	2340	452	Acetone	Aasen and Liaen-Jensen (1966)
Zeaxanthin	2540	450	Ethanol	Strain (1938)

**VI.**  
**Factors Influencing Analytical Results**

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## FACTORS AFFECTING THE LABORATORY MANAGEMENT OF HUMAN SERUM AND LIVER VITAMIN A ANALYSES

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### SUMMARY

A number of factors influencing the accuracy of human serum and foetal liver vitamin A and carotenoid analyses have been investigated.

Serum vitamin A, when estimated by the Carr-Price antimony trichloride reaction, apparently increases after frozen storage and the serum carotenoids decrease. At 4° serum vitamin A and carotenoids were stable for 6 days and at room temperature for 3 days. Liver vitamin A was found to be stable during storage at -20° for at least 24 weeks.

Exposure of serum to artificial light did not decrease vitamin A levels to as great an extent as sunlight.

Neither haemolysis nor heparinisation of blood appreciably affected the vitamin A levels. Carotenoid levels were decreased in the presence of haemolysis.

Precautions are suggested in order to obtain reproducible results with the antimony trichloride estimation procedure.

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### INTRODUCTION.\*

In experimental and industrial research involving analysis of vitamin A in tissues or foodstuffs, it is relatively easy to keep specimens under optimal conditions. In clinical research circumstances are not always so favourable, since blood samples often have to be transported or stored before investigation and thus interfering factors, such as small volume, haemolysis, exposure to light, various storage temperatures, etc., are frequently encountered. Valuable or irreplaceable samples are often discarded because it is not known whether there has been any vitamin A deterioration in them.

The effects of some of these factors have been investigated in various studies<sup>1-9</sup>, in which differing methods of vitamin A estimation have been used or compared, but the quantitative evaluation of some important factors has been overlooked.

The Carr-Price antimony trichloride method of vitamin A estimation is still widely used in research laboratories in this country and is also the recommended method for the determination of vitamin A in foodstuffs<sup>9</sup>. It has been found com-

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parable with other procedures<sup>3-5,9,10</sup>, and is therefore used by many laboratories with no access to expensive spectrophotometric equipment.

This study was undertaken to evaluate the conditions possibly affecting human serum and foetal liver vitamin A results when estimated by the antimony trichloride method.

#### METHODS

All chemicals used were of A.R. quality or spectroscopic grade. Only freshly made reagents, kept in amber glass containers, were used since optical transmission of solvents used for vitamin A analysis can vary with age and purity<sup>5,7</sup>. Crystalline vitamin A acetate and  $\beta$  carotene (Roche Products Limited) were used for the calibration curves which were calculated in  $\mu\text{g/ml}$ . Optical transmission was measured with a Unicam SP. 1300 colorimeter.

#### *Estimation procedure*

The serum procedure used was based on Yudkin's<sup>2</sup> modification of Kimble's<sup>1</sup> method. The standard procedure now generally adopted is that described by Moore<sup>11</sup> and Sharman<sup>12</sup>, which we further modified by the addition of a reagent blank sample to the extraction and by measuring the blue colour produced when extracted vitamin A aliquots are added to antimony trichloride at a standardised time interval of 10 sec.\*

The method used for liver was based on that of Tosic and Moore<sup>13</sup> with the addition of using a reagent blank.

The experimental error encountered in the serum estimation covers a wide range<sup>2,3,5</sup>. In this study, variations greater than  $\pm 10\%$  have been considered to be due to factors other than experimental error for both serum and liver estimations. All estimations were carried out in duplicate.

Description of the experimental methods will be included together with the results for each of the factors studied.

#### *Storage of serum at different temperatures*

Pooled human serum was stored in the dark at various temperatures and the vitamin A and carotenoids estimated after different intervals.

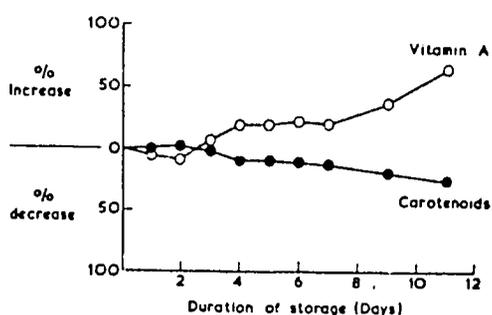


Fig. 1.

\* Precise details of the estimation and calculation procedures are available on request.

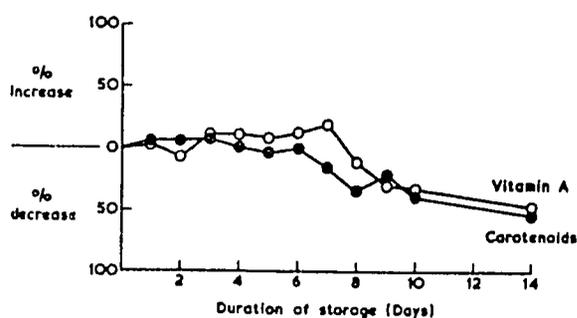


Fig. 2.

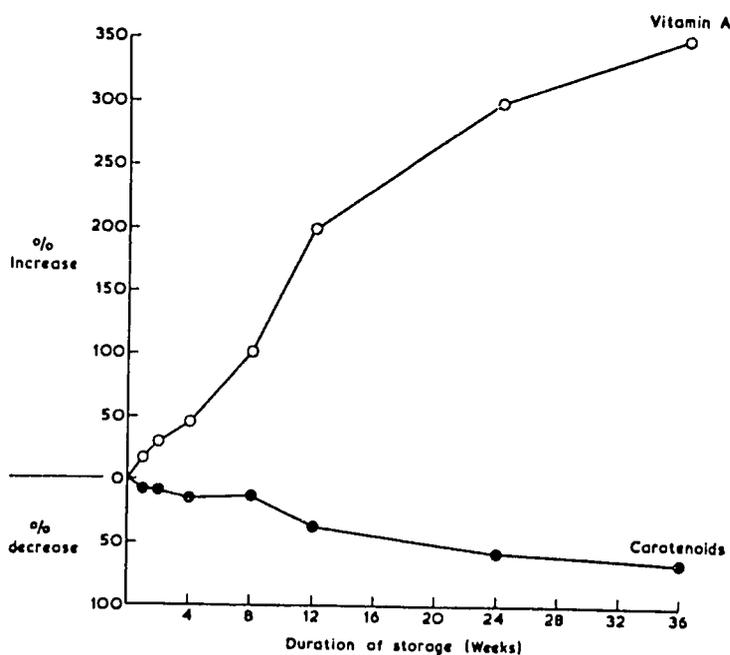


Fig. 3.

At room temperature ( $24^{\circ}$ – $28^{\circ}$ ) signs of deterioration were observed after only 4 days storage (Fig. 1).

With chilled samples (at  $4^{\circ}$ ) a decrease in both vitamin A and carotenoids was noticeable after 8–9 days (Fig. 2).

Deep frozen samples (at  $-20^{\circ}$ ) were estimated after 1, 2, 8, 12, 24 and 36 weeks of storage. Increases in the vitamin A content and decreases in the carotenoids were observed after only one week (Fig. 3). Variations in vitamin A and carotenoids in Figs 1–3 are expressed as percentages of the initial readings.

#### *Different lighting conditions and exposure to air*

Pooled human serum samples were exposed to sunlight, artificial light (plus normal daylight) or kept in total darkness for periods of 2 and 4 h, and changes in vitamin A and carotenoid concentrations recorded. The effect of air on the same samples was additionally ascertained (Table I).

TABLE I

CHANGES OCCURRING IN SERUM VITAMIN A AND CAROTENOIDS ON EXPOSURE TO VARIOUS LIGHTING CONDITIONS AND TO THE AIR

Lighting condition and length of exposure	Vitamin A		Carotenoids	
	Covered	Exposed to air	Covered	Exposed to air
Sunlight				
2 h	18.1% decrease	24.3% decrease	0% <sub>0</sub>	3.0% <sub>0</sub> increase
4 h	55.5% decrease	72.0% decrease	5% <sub>0</sub> increase	13.2% <sub>0</sub> increase
Artificial light				
2 h	0% <sub>0</sub>	2.0% <sub>0</sub> decrease	0% <sub>0</sub>	2.4% <sub>0</sub> decrease
4 h	6.5% <sub>0</sub> decrease	12.0% <sub>0</sub> decrease	4.2% <sub>0</sub> increase	1.9% <sub>0</sub> increase
Total darkness				
2 h	3.0% <sub>0</sub> increase	4.7% <sub>0</sub> decrease	5.2% <sub>0</sub> increase	3.0% <sub>0</sub> decrease
4 h	1.5% <sub>0</sub> decrease	8.9% <sub>0</sub> increase	1.7% <sub>0</sub> increase	1.7% <sub>0</sub> increase

*Haemolysis*

Blood from one source was divided into 2 equal samples, one was shaken to cause haemolysis, whilst the other was separated immediately. The vitamin A and carotenoid concentrations of both serum samples were determined and compared, and there was found to be no difference in vitamin A content between haemolysed and non-haemolysed serum, but an 18.7% decrease in carotenoids in the presence of haemolysis.

*Anti-coagulants*

We investigated the effects of treatment with lithium heparin on vitamin A and carotenoid levels since the possibility exists that plasma and serum vitamin A and carotenoids might need to be compared. There was no significant difference between them.

*Volume of serum*

In order to test whether different volumes of serum give interchangeable results, the vitamin A and carotenoid levels of 1, 2, 3, 4, 5 and 6 ml of pooled serum were analysed.

As shown in Table II, volumes of between 4 and 6 ml gave reproducible results and 3 ml was of borderline accuracy. In the absence of a reagent blank the excessively high values obtained with 1 and 2 ml samples were even more exaggerated.

We also tried to modify the procedure to a micro-scale by reducing the volume of

TABLE II

VARIATIONS IN VITAMIN A AND CAROTENOID VALUES OBTAINED WITH CHANGES IN SERUM VOLUME

Volume of serum	Vitamin A	Carotenoids
6 ml	Standard	Standard
5 ml	4.5% <sub>0</sub> decrease	1.6% <sub>0</sub> decrease
4 ml	1.1% <sub>0</sub> decrease	7.0% <sub>0</sub> decrease
3 ml	11.6% <sub>0</sub> increase	5.9% <sub>0</sub> decrease
2 ml	38.0% <sub>0</sub> increase	39.8% <sub>0</sub> decrease
1 ml	38.3% <sub>0</sub> increase	33.1% <sub>0</sub> decrease

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antimony trichloride to 0.5 ml and using a micro-cell, but the results were not satisfactory.

#### *Dilution of serum*

Some workers dilute small serum samples with distilled water in order to achieve the recommended volume of 6 ml. In a similar series of experiments to the previous one the vitamin A and carotenoid contents were analysed in 1, 2, 3, 4, 5 and 6 ml samples of pooled serum diluted to give starting volumes of 6 ml in each case.

TABLE III  
EFFECTS OF THE DILUTION OF SERUM ON THE VITAMIN A AND CAROTENOID VALUES

Volume	Vitamin A (decrease)	Carotenoids (decrease)
5 ml serum + 1 ml water	1.3%	8.3%
4 ml serum + 2 ml water	10.8%	24.0%
3 ml serum + 3 ml water	68.5%	80.6%
2 ml serum + 4 ml water	78.5%	97.5%
1 ml serum + 5 ml water	78.8%	100.0%

This was found to affect both the vitamin A and carotenoid values with serum volumes of 4 ml and less (Table III). In the presence of additional water, the serum protein would not completely precipitate on addition of ethanol. It would appear, therefore, that the vitamin A remained protein bound and was thus not completely extracted.

#### *Storage of livers at different temperatures*

Homogenised human foetal livers were stored in the dark, in sterile containers, at 4° and -20°. Those stored at 4° were either freshly obtained or had been previously stored at -20°. After a week's storage at 4°, there was a mean decrease of 7.6% in the vitamin A content of fresh livers and a mean decrease of 0.4% in vitamin A content of the livers previously stored at -20°. At -20° there were no significant changes in vitamin A content even after 24 weeks storage (Fig. 4).

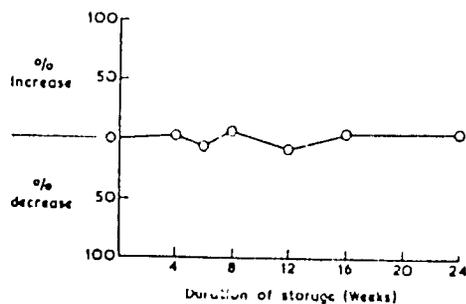


Fig. 4.

#### *Sampling technique for foetal livers*

The vitamin A concentration in the human foetal liver is reported to have a considerable lobar variation which is closely related to gestational age<sup>14</sup>.

The vitamin A concentration was therefore compared between right and left

liver lobes of 13 human foetal livers between 18 and 28 weeks of gestation, and 26 livers of over 28 weeks. There was no significant lobar variation between 18 and 28 weeks but the concentration was significantly higher ( $p < 0.01$ ) in the right lobe after 28 weeks of gestation. This factor could affect results if a standardised sampling technique is not used.

#### DISCUSSION

Reports on the stability of serum vitamin A on storage have been based mainly on other methods of estimation of the vitamin. With fluorometric<sup>8</sup> and spectrophotometric<sup>3</sup> techniques, it is apparently stable for up to 4½ months at  $-20^{\circ}$ . During our own investigations at  $-20^{\circ}$  there was a progressive increase in apparent vitamin A content of the serum with length of storage, accompanied by a decrease in carotenoids. Sera showing large increases in vitamin A content gave a slate blue colouration in the antimony trichloride reaction.

The degradation products, isoanhydrovitamin A and retroanhydrovitamin A, which are formed by dehydration of vitamin A<sup>15</sup>, both give a violet colour in the antimony trichloride reaction<sup>16</sup>. Hickman<sup>17</sup> described these isomers as being "formed whenever the vitamin is mistreated". It would seem that the most probable explanation for the erroneously high vitamin A values observed by us after storage is that these products are interfering in the antimony trichloride reaction<sup>18</sup>.

Of the temperatures tested, chilled storage can be recommended as being the most suitable, as serum vitamin A and carotenoids were found to be stable at  $4^{\circ}$  for at least a week. At room temperature, we found the vitamin to be stable for only 3 days and the carotenoids for 6 days.

It has been suggested<sup>15,18</sup> that compounds chemically or otherwise related to vitamin A may either inhibit or potentiate the blue colour produced in the antimony trichloride reaction. We have found that synthetic cholesterol, ethinyloestradiol and norethisterone acetate when added to serum, did not affect the reaction<sup>19</sup>.

It has been known for many years that vitamin A is sensitive to light<sup>20,21</sup>, and is readily oxidised. In our experiments, the destruction of serum vitamin A when exposed to bright sunlight was as expected, as was its potentiation when additionally exposed to air. The effects of artificial light were, however, minimal (Table I).

Haemolysis of serum samples has been reported not to affect the vitamin A level when estimated by the antimony trichloride method<sup>1,7</sup>. The ultra-violet absorption method is reported to give erroneously high vitamin A<sup>2</sup> and carotenoid<sup>4,5</sup> values in the presence of haemolysis. We found that haemolysed serum can be used when estimating vitamin A by the antimony trichloride method, provided that one is not using the carotenoid value for anything other than correction of the vitamin A reading. It is a consistent finding of our laboratory that the greater the degree of haemolysis, the lower is the carotenoid value.

One of the main disadvantages of the antimony trichloride method is that comparatively large volumes of serum are required for extraction. Kimble<sup>1</sup> recommends the use of 3.5–5 ml plasma; Yudkin<sup>2</sup> used 3 ml; Moore<sup>12</sup> 1 ml to 6 ml and Sharman<sup>13</sup> suggests 3 ml to 6 ml. As large samples of blood are not always obtainable, valuable specimens have been discarded because they are not of the stipulated volume.

Since we have found the method employed applicable for volumes of 3 ml or

more we use, in practice, a standard volume of 4 ml for all serum analyses and avoid any dilution of the samples.

Since the effects of saponification of serum samples before extraction of vitamin A have already been thoroughly investigated<sup>1,2,4,8,10,15,23-24</sup> this factor was not included as part of our study.

There seems to be no problem as regards the storage of liver specimens,  $-20^{\circ}$  being the temperature of choice. Our results also indicate that samples taken from the deep freeze should be thawed and estimated immediately as those placed first at  $4^{\circ}$  showed considerable loss of vitamin A.

As our findings regarding the distribution of vitamin A in the foetal liver further confirm those previously reported we wish to stress the importance of standardising the sampling method or utilising whole liver homogenates in human foetal liver studies and also suggest that the pattern of vitamin A distribution in the adult liver, and that of other species, is also clarified.

In view of our findings it would be advisable to pay attention to the factors discussed when using the antimony trichloride method for analysis of human tissues or serum. We would particularly stress the need to standardise the storage period, or if this is not possible, to consider applying correction factors. In addition, amber, or silverfoil covered bottles should be provided for the collection of specimens. During clinical trials, involving the determination of serum vitamin A, it is essential that control and index samples be kept under the same conditions and a standardised procedure used for all analyses.

#### ACKNOWLEDGEMENTS

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#### REFERENCES

- 1 M. S. KIMBLE, *J. Lab. Clin. Med.*, 24 (1939) 1055.
- 2 S. YUDKIN, *Biochem. J.*, 35 (1941) 551.
- 3 O. A. BESSEY, O. H. LOWRY, M. J. BROCK AND J. A. LOPEZ, *J. Biol. Chem.*, 166 (1946) 177.
- 4 A. E. SOBEL AND S. D. SNOW, *J. Biol. Chem.*, 171 (1947) 617.
- 5 J. G. BIERI AND M. O. SCHULTZE, *Arch. Biochem. Biophys.*, 34 (1951) 273.
- 6 W. O. CASTER AND O. MICKELSON, *Amer. J. Clin. Nutr.*, 3 (1955) 409.
- 7 M. H. UTLEY, E. R. BRODOVSKY AND W. N. PEARSON, *J. Nutr.*, 66 (1958) 205.
- 8 J. KAHAN, *Scand. J. Clin. Lab. Invest.*, 18 (1966) 679.
- 9 Report of the Vitamins (Fat Soluble) Panel, *Analyst*, 89 (1964) 9.
- 10 J. B. NEELD AND W. N. PEARSON, *J. Nutr.*, 79 (1963) 454.
- 11 T. MOORE, *Vitamin A*, Elsevier, Amsterdam, 1957, p. 587.
- 12 I. M. SHARMAN, Procedure adopted at the Dunn Nutritional Laboratory, Cambridge (1966).
- 13 P. TOSIC AND T. MOORE, *Biochem. J.*, 39 (1945) 498.
- 14 I. GAL, I. M. SHARMAN AND J. PRYSE-DAVIES, in D. H. M. WOOLAM (Ed.), *Advances in Teratology*, 5, Logos Press, London (in press).

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- 15 M. KOFLER AND S. H. RUBIN, *Vitamins Hormones*, 18 (1961) 315.
  - 16 K. TSUKIDA, M. ITO AND F. IKEDA, *Int. J. Vit. Nutr. Res.*, 41 (1971) 158.
  - 17 K. HICKMAN, *Ann. Rev. Biochem.*, 12 (1943) 363.
  - 18 W. J. DANN, *Biol. Symp.*, 12 (1947) 13.
  - 19 I. GAL, C. PARKINSON AND I. CRAFT, *Brit. Med. J.*, ii (1971) 436.
  - 20 P. R. PEACOCK, *Lancet*, ii (1926) 328.
  - 21 E. L. SMITH, F. A. ROBINSON, B. E. STERN, F. E. YOUNG, *Biochem. J.*, 33 (1939) 207.
  - 22 T. LINDQVIST, *Acta Med. Scand.*, suppl. 97 (1938).
  - 23 L. B. PETT AND G. A. LE PAGE, *J. Biol. Chem.*, 132 (1940) 585.
  - 24 J. I. M. JONES AND R. T. HAINES, *Analyst*, 68 (1943) 8.
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## HEMOLYSIS AND REAGENT PURITY AS FACTORS CAUSING ERRATIC RESULTS IN THE ESTIMATION OF VITAMIN A AND CAROTENE IN SERUM BY THE BESSEY-LOWRY METHOD<sup>1</sup>

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### INTRODUCTION

The micro-method of Bessey et al. ('46) for the determination of vitamin A and carotene in small quantities of blood serum has been extensively used in nutritional surveys. Laboratories using this method have frequently experienced erratic results, yet its convenience has resulted in wide adoption for such studies.

Several reports are concerned with the reproducibility of results obtained with this method and some have recommended precautions necessary to obtain valid data (Sobel and Snow, '47; Bieri and Schultze, '51; Caster and Mickelsen, '55; Clayton et al., '54; Karmarkar and Rajagopal, '52). The effect of hemolysis has been mentioned by two groups of workers (Sobel and Snow, '47; Bieri and Schultze, '51), although no detailed study of it appears to have been made. Sobel and Snow ('47) stated that "hemolyzed blood has been found to give higher carotene values, and due to the high blanks after irradiation, the results tend to be unreliable." In the

<sup>1</sup>This research was supported by a grant from the Tennessee Valley Authority and augmented by the Williams-Waterman Fund.

presence of hemolysis they noted an increase in carotene values ranging from 23 to 117%. Although the increase in vitamin A caused by hemolysis was not mentioned, as much as 52% increase was recorded in their tabulated data.

Bieri and Schultze (51) reported that the observation of certain precautions led to valid results. Each serum was examined against a white background and discarded if there was evidence of hemolysis. According to them "hemolyzed serums gave consistently high values for vitamin A, since the products of hemolysis are partially destroyed by ultraviolet irradiation."

The conditions of collecting, transporting and storing specimens in nutrition surveys often favor hemolysis; hence detailed understanding of the influence of hemolysis on this procedure is desirable.

In this laboratory, data obtained in nutrition surveys appeared to indicate that vitamin A levels of hemolyzed sera were consistently higher than those of non-hemolyzed sera. The present study confirms this observation, defines some factors responsible, and leads to the recommendation of precautions which avoid this source of error.

#### EXPERIMENTAL PROCEDURE

From each of 5 laboratory personnel, approximately 30 ml of blood were obtained and allowed to clot. After centrifugation, an aliquot (approximately 3 ml) of serum was removed. The remaining blood was vigorously stirred for several minutes and recentrifuged, so that slightly hemolyzed serum was obtained. More vigorous stirring for longer periods yielded further hemolysis; and freezing for several minutes was used to obtain extreme hemolysis. Varying degrees of hemolysis were thus obtained in the same serum sample and were coded "1" for the non-hemolyzed sample and "2", "3" and "4" for the samples with different degrees of hemolysis. The sera were analyzed for hemoglobin using the Bing and Baker method as modified by Ham (50). Vitamin A and carotene determinations were performed on all serum aliquots by the

Carr-Price (antimony trichloride) method (Carr and Price, '26; Dann and Evelyn, '38) as well as by the micro-method of Bessey et al. ('46). The data, summarized in table I, indicate a non-linear increase in apparent serum vitamin A corresponding to the increase in serum hemoglobin when the Bessey-Lowry method was used. Hemolysis did not have a consistent influence on the vitamin A levels as measured by

TABLE I

*Vitamin A and carotene values of sera exhibiting varying degrees of hemolysis*

DONOR		LEVELS OF HEMOLYSIS OF EACH DONOR'S BLOOD			
		1	2	3	4
M	Serum hemoglobin <sup>1</sup>	0.00	0.02	0.08	0.28
	Vitamin A, micro <sup>2</sup>	216	277	312	366
	Vitamin A, Carr-Price <sup>2</sup>	145	154	153	140
	Carotene, micro <sup>3</sup>	190	190	190	188
	Carotene, Carr-Price <sup>3</sup>	169	181	188	178
F	Serum hemoglobin	0.00	0.07	0.24	0.43
	Vitamin A, micro	310	422	404	441
	Vitamin A, Carr-Price	164	229	187	190
	Carotene, micro	110	108	111	114
	Carotene, Carr-Price	97	108	97	94
T	Serum hemoglobin	0.00	—	0.22	0.59
	Vitamin A, micro	293	—	368	450
	Vitamin A, Carr-Price	213	—	217	217
	Carotene, micro	204	—	209	209
	Carotene, Carr-Price	188	—	206	204
K	Serum hemoglobin	—	0.01	0.22	0.78
	Vitamin A, micro	—	263	343	502
	Vitamin A, Carr-Price	—	192	174	168
	Carotene, micro	—	163	159	186
	Carotene, Carr-Price	—	151	164	145
H	Serum hemoglobin	0.00	0.03	0.10	0.27
	Vitamin A, micro	286	310	366	418
	Vitamin A, Carr-Price	149	—	158	142
	Carotene, micro	303	303	302	268
	Carotene, Carr-Price	255	271	286	308

<sup>1</sup> Serum hemoglobin, gm/100 ml serum.

<sup>2</sup> Vitamin A, I.U./100 ml serum.

<sup>3</sup> Carotene,  $\mu$ g/100 ml serum.

the Carr-Price reaction. Even in the absence of hemolysis, the vitamin A values obtained by the micro-method were higher in each instance than were those found by use of the Carr-Price procedure. Carotene values were unaffected by hemolysis, and the two methods of analysis were in relatively good agreement.

Washed erythrocytes were also analyzed by both the Carr-Price method and the micro-method. Blood samples from two laboratory workers were allowed to clot, and the sera separated by centrifugation. The red cells were then washed by centrifuging several times with 0.9% saline. Distilled water was added in an amount estimated to replace the serum and the mixture was stirred vigorously. Aliquots of this red cell hemolysate, as well as the collected sera, were analyzed by the two methods previously indicated. The results are recorded in table 2. The values obtained by the Carr-Price method were near zero for both vitamin A and carotene, but the micro procedure indicated very high apparent vitamin A and carotene levels. The Carr-Price and micro vitamin A and carotene analyses on serum agreed well when there was no hemolysis, but in the presence of slight hemolysis the vitamin A by micro-analysis was nearly three times greater, and the corresponding carotene was half that obtained by the macro-analysis.

In order to establish firmly that hemoglobin was the material responsible for the higher values obtained by the micro method, solutions of purified hemin and hemoglobin were studied. Equimolar solutions (approximately 0.0001 molar) of hemin<sup>2</sup> and hemoglobin<sup>3</sup> were used. Hemoglobin went into solution readily; the hemin was dissolved by the addition of dilute ammonia. This 0.0001 molar hemoglobin is equivalent to 0.67 gm per 100 ml which is a level that might be found in hemolyzed serum. It is obvious from table 3 that dilute solutions of both hemin and hemoglobin give vitamin A values that are falsely high when analyzed by the micro-method.

<sup>2</sup> Recrystallized, Eastman Organic Chemicals.

<sup>3</sup> Pure scales, Pfanstiehl.

Values obtained by the Carr-Price method are consistently low.

TABLE 2  
*Vitamin A and carotene values of washed red cells and serum*

SAMPLE	VITAMIN A		CAROTENE	
	Carr-Price	Micro	Carr-Price	Micro
	<i>I.U./100 ml</i>		<i>µg/100 ml</i>	
I Red cell hemolysate	15	962	3	252
Serum <sup>1</sup>	137	380	88	42
II Red cell hemolysate	18	1658	9	318
Serum	109	104	161	161

<sup>1</sup>Slightly hemolyzed.

TABLE 3  
*Vitamin A and carotene values of 0.0001 M solutions of hemin and hemoglobin*

SAMPLE	VITAMIN A		CAROTENE	
	Carr-Price	Micro	Carr-Price	Micro
	<i>I.U./100 ml</i>		<i>µg/100 ml</i>	
0.0001 M Hemin	16	88	1	5
0.0001 M Hemoglobin	13	119	5	8

Correspondence with Dr. Guillermo Arroyave<sup>4</sup> revealed that hemolyzed samples analyzed by the Bessey-Lowry method at INCAP gave no indication of increased vitamin A value. Therefore, the possibility was examined that reagent differences might explain the discrepancy.

The reagents used in the micro-method are absolute ethyl alcohol (redistilled, stored in a ground-glass stoppered reagent bottle), 11 N KOH (kept in either a glass bottle with a rubber stopper or in a polyethylene bottle), and a 1:1 mixture of kerosene<sup>5</sup> and xylene,<sup>6</sup> stored in a reagent bottle with ground-glass stopper and not protected from light. When this

<sup>4</sup>Institute of Nutrition of Central America and Panama (INCAP), Guatemala City, Guatemala, C. A.

<sup>5</sup>Fisher odorless.

<sup>6</sup>Merck reagent.

study was undertaken, KOH and kerosene-xylene mixtures 6 months old were available; alcohol which had been redistilled 10½ months previously was also available. In addition to these "aged" solutions, fresh reagents were prepared. Kerosene and xylene were used without special purification and newly opened reagent grade alcohol was used without redistilling. All possible combinations of these three reagents (8 combinations, coded 1 to 8, described in table 4) were employed in the analyses of hemolyzed and non-hemolyzed aliquots of

TABLE 4  
*The effect of aged reagents on vitamin A and carotene values of serum before and after hemolysis*

REAGENT COMBINATION			VITAMIN A		CAROTENE	
			No hemolysis	Hemolysis	No hemolysis	Hemolysis
<i>Alcohol</i>	<i>KOH</i>	<i>Kerosene-xylene</i>	<i>I.U./100 ml</i>	<i>I.U./100 ml</i>	<i>µg/100 ml</i>	<i>µg/100 ml</i>
1. aged	aged	aged	186	586	120	6
2. fresh	fresh	fresh	174	230	122	120
3. aged	fresh	aged	169	955	119	6
4. aged	fresh	fresh	186	235	124	120
5. aged	aged	fresh	202	284	124	120
6. fresh	aged	aged	211	772	124	4
7. fresh	aged	fresh	197	279	125	124
8. fresh	fresh	aged	178	762	122	6

one serum sample with the results which appear in table 4. Even with all freshly prepared reagents (combination 2), the vitamin A value of serum from hemolyzed blood was somewhat higher than the corresponding non-hemolyzed sample. However, in 4 cases (reagent combinations 1, 3, 6 and 8) vitamin A values were three to 5 times higher when the serum was hemolyzed; the carotene was reduced nearly to zero. This marked reduction in carotene is in contrast to the agreement on hemolyzed and non-hemolyzed samples shown in table 1. This discrepancy in carotene values is due to differences in the kerosene-xylene mixtures; the aged mixture described above was not the same batch as was used in obtaining the data in

table 1. It is evident that the high vitamin A and low carotene values on hemolyzed samples are due to the aged kerosene-xylene mixture, but that aged KOH (combinations 5 and 7) also gives a high vitamin A value.

To obtain further information concerning the effect of the ageing of kerosene-xylene mixtures on values for hemolyzed serum, data were obtained on hemolyzed and non-hemolyzed aliquots of the same serum using different kerosene-xylene mixtures which varied in age from freshly prepared to 38 weeks old. No purification of reagents was employed. The results are presented in table 5. In the presence of hemolysis

TABLE 5

*The effect of the ageing of kerosene-xylene on vitamin A and carotene values of serum before and after hemolysis*

AGE OF KEROSENE XYLENE	VITAMIN A		CAROTENE	
	No hemolysis	Hemolysis	No hemolysis	Hemolysis
	<i>I.U./100 ml</i>	<i>I.U./100 ml</i>	$\mu\text{g}/100\text{ ml}$	$\mu\text{g}/100\text{ ml}$
<i>weeks</i>				
38	115	826	140	92
26	110	833	142	47
25	197	810	143	94
23	138	812	144	144
4	146	279	146	178
0	171	296	148	170

the older (23 to 38 weeks) kerosene-xylene mixtures caused a striking increase in apparent vitamin A content and in three of 4 cases an apparent reduction in carotene. A 4-week-old mixture gave similar results as did a freshly prepared one in non-hemolyzed blood, but both led to a nearly two-fold increase in apparent vitamin A content and slightly elevated carotene levels in hemolyzed samples. Since even a freshly prepared kerosene-xylene mixture proved unsuitable for use in the presence of hemolysis, purification of these reagents was studied. Changes in the spectral absorption characteristics of this reagent have been recorded by Bieri et al. (51) who stated that the absorption of kerosene-xylene in the region of 310 to 400 m $\mu$  gradually increases after standing for about two

months in a glass-stoppered bottle unprotected from light. They suggest that errors due to changes in this solvent can be avoided by storing the kerosene-xylene mixture in the dark and by frequent checks of its absorption in the region of 310 to 400 m $\mu$ . Karmarkar et al. (52) advise that the kerosene-xylene be refluxed over metallic sodium for several hours, followed by distillation of the xylene at 138°C and the kerosene at 180 to 195°C to eliminate impurities which interfere with the accuracy of the method. Arroyave<sup>7</sup> (56) purifies the kerosene by shaking with activated charcoal and filtering through a fine sintered glass filter. The absorption spectrum of xylene as compared with water is tested when a new bottle is opened and it is redistilled when necessary.

A study of the influence of purification of xylene-kerosene on the phenomenon following hemolysis was made as follows: (1) Xylene was redistilled in an all glass still and the 134 to 137°C fraction collected; (2) Kerosene was shaken with a 5% solution of sodium bicarbonate, washed with distilled water, and stored overnight over 4-mesh calcium chloride. The kerosene (100 ml) was then shaken with approximately 3 gm of activated charcoal<sup>8</sup> and filtered through Whatman #1 filter paper; (3) The kerosene and xylene were mixed just before using and stored in a dark place. The purified kerosene and xylene were not stored for any length of time before mixing.

A venous blood sample was divided into two portions; one was allowed to clot before centrifugation and the serum removed and the second placed immediately in a freezer at -25°C for three hours. The hemoglobin concentration of the latter was determined and aliquots were added to known volumes of the non-hemolyzed serum to obtain increasing concentrations of hemoglobin up to 1.0 gm per 100 ml. These samples, along with a "completely" hemolyzed sample, were analyzed for vitamin A, using three batches of kerosene-xylene reagent as detailed in table 6. This experiment was repeated

<sup>7</sup> Arroyave, G., private communication.

<sup>8</sup> Dareso G-60, activated charcoal obtained from Atlas Powder Company, Wilmington, Delaware.

TABLE 6

The effect of purification of kerosene-xylene on the reliability of data obtained on non-hemolyzed and hemolyzed aliquots of serum

		TRIALS													
		I <sup>1</sup>							II						
Reagents	Hemoglobin <sup>2</sup>	0.00	0.11	—	0.57	—	—	14.3	0.00	0.12	—	0.48	0.73	1.21	12.1
A															
Kerosene-xylene, freshly prepared and especially purified	Vitamin A <sup>3</sup>	129	140	—	119	—	—	432	134	154	—	186	157	138	401
	Carotene <sup>4</sup>	136	138	—	144	—	—	298	118	118	—	115	116	112	262
B															
Kerosene-xylene, 7 months old, not purified	Vitamin A	228	230	—	289	—	—	594	144	188	—	280	289	328	666
	Carotene	140	142	—	154	—	—	292	125	132	—	140	150	152	438
C															
Kerosene-xylene, 20 months old, not purified	Vitamin A	420	468	—	486	—	—	1060	138	478	—	417	512	552	1621
	Carotene	140	26	—	22	—	—	182	120	27	—	22	29	25	236
		III							IV						
Reagents	Hemoglobin <sup>2</sup>	0.00	0.13	0.26	0.52	0.78	1.20	12.9	0.00	0.12	0.24	0.48	0.72	1.17	11.7
A															
Kerosene-xylene, freshly prepared and especially purified	Vitamin A <sup>3</sup>	112	149	168	188	228	268	442	125	153	151	146	151	149	280
	Carotene <sup>4</sup>	115	116	123	122	136	159	294	118	118	116	116	113	111	182
B															
Kerosene-xylene, 7 months old, not purified	Vitamin A	182	206	234	250	219	254	754	168	188	219	258	241	219	508
	Carotene	124	128	118	123	126	126	346	124	124	122	119	121	121	251
C															
Kerosene-xylene, 20 months old, not purified	Vitamin A	99	635	522	523	537	526	1314	127	701	876	—	679	723	1411
	Carotene	121	28	12	14	17	12	188	121	68	23	12	12	12	210

<sup>1</sup>I, II, III and IV represent replicate experiments on serum samples from the same donor, but drawn on different days and analyzed at different times. Increasing degrees of hemolysis of each is indicated by the hemoglobin value.

<sup>2</sup>Hemoglobin, gm per 100 ml serum.

<sup>3</sup>Vitamin A, I.U. per 100 ml serum.

<sup>4</sup>Carotene,  $\mu$ g per 100 ml serum.

4 times on serum obtained from the same donor on different days. The kerosene used in reagent A was again treated with activated charcoal just prior to trial IV (this had not been done since approximately a week prior to trial I) but this apparently did not alter the results. The data summarized in table 6 show the varied results which may be obtained unless extreme care is taken in the purification of reagents. Considering trial IV, one may conclude that if the kerosene-xylene is especially purified and freshly prepared, the carotene value will not be affected by moderate hemolysis. Even the use of specially purified and freshly prepared kerosene-xylene mixture does not prevent a small increase in apparent vitamin A content in slightly hemolyzed samples. In the presence of a very old, unpurified kerosene-xylene mixture, the false increase in vitamin A content resulting from hemolysis is striking, and the carotene is reduced to a level approximating zero. It is also evident that unreliable data will result from non-hemolyzed samples if old or unpurified reagents are used.

The practical implications of this study are obvious since nutrition surveys which employ the micro-method of Bessey and Lowry are numerous. Under survey conditions, the samples must often be transported from field to laboratory as whole blood, and some hemolysis is not uncommon. If such hemolyzed samples are analyzed without the precaution of using a carefully purified kerosene-xylene mixture, false results will be obtained. The kerosene and xylene should be mixed immediately prior to using, and if it is necessary to keep this solvent for short periods of time, it must be protected from light.

#### SUMMARY

Hemolysis causes a false increase in serum vitamin A values when the micro-method of Bessey and Lowry is used. An especially purified and freshly prepared kerosene-xylene mixture minimizes the false increase in vitamin A caused by hemolysis and avoids errors in the carotene measurements. An unpurified kerosene-xylene mixture permitted to stand at

room temperature unprotected from light may cause as much as a 10-fold increase in the apparent vitamin A content of a hemolyzed blood sample and a definite diminution in the apparent content of carotene. In the absence of hemolysis, an aged, unpurified kerosene-xylene mixture resulted in a 4-fold increase in apparent vitamin A content but no change in the carotene level. Only freshly purified and mixed kerosene-xylene should be used in the Bessey-Lowry procedure for determining serum carotene and vitamin A. Serum samples showing evidence of more than a trace of hemolysis should be discarded. The Carr-Price reaction is relatively unaffected by hemolysis and is therefore the method of choice in circumstances where sufficient blood can be obtained.

## LITERATURE CITED

- BESSEY, O. A., O. H. LOWRY, M. J. BROCK AND J. A. LOPEZ 1946 The determination of vitamin A and carotene in small quantities of blood serum. *J. Biol. Chem.*, *166*: 177.
- BIERI, J. G., AND M. O. SCHULTZE 1951 Microspectrophotometric determination of vitamin A and carotene in blood. *Arch. Biochem. Biophys.*, *31*: 273.
- CARR, F. H., AND E. A. PRICE 1926 Colour reactions attributed to vitamin A. *Biochem. J.*, *20*: 497.
- CASTER, W. O., AND O. MICKELSEN 1955 Serum vitamin A level: A critique of methods and significance. *Am. J. Clin. Nutrition*, *3*: 409.
- CLAYTON, M. M., M. J. BARCOCK, W. D. FOSTER, S. STREGEVSKY, R. E. TUCKER, A. W. WELLS AND H. H. WILLIAMS 1954 A referee blood experiment involving the use of microchemical methods. *J. Nutrition*, *52*: 383.
- DANN, W. J., AND K. A. EVELYN 1938 The determination of vitamin A with the photoelectric colorimeter. *Biochem. J.*, *52*: 1008.
- HAM, T. H., Ed. 1950 *A Syllabus of Laboratory Examinations in Clinical Diagnosis*. Harvard University Press.
- KARMARKAR, G., AND K. RAJAGOPAL 1952 Assay of vitamin A and carotene in blood serum. *Current Sci.*, (India), *21*: 193.
- SOBEL, A. E., AND S. D. SNOW 1947 The estimation of serum vitamin A with activated glycerol dichlorohydrin. *J. Biol. Chem.*, *171*: 617.

## IMMUNOCHEMICAL QUANTITATION OF ANTIGENS BY SINGLE RADIAL IMMUNODIFFUSION\*

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**Abstract**—When an unknown amount of antigen is allowed to diffuse radially from a well in a uniformly thin layer of antibody-containing agar for a sufficient time to allow all antigen to combine, the final area reached by the precipitate is directly proportional to the amount of antigen employed, and inversely proportional to the concentration of antibody. It is also shown that the temperature at which the plates are incubated has no perceptible influence upon the results. By standardizing the technical conditions of the experiment it is possible to use this principle for the immunochemical determination of antigens. In the experimental albumin-antialbumin system here described, the lower limit of the method was found to correspond to 0.0025  $\mu\text{g}$  of antigen, and to an antigen concentration of 1.25  $\mu\text{g}$  per ml. The standard deviation of the antigen determinations was less than 2 per cent of the mean.

### INTRODUCTION

AMONG the various immunochemical precipitin methods employing diffusion in agar gels, the technique of single radial immunodiffusion has been the least studied. By definition the single-diffusion type of precipitin reaction is performed by incorporating one of the two partners of the reaction, usually the antibody, into the agar gel, at a uniform concentration, whereas the other reactant, usually the antigen, is introduced into a well from which it is allowed to diffuse into the gel where it will react with the 'internal reactant'. The system is termed 'linear' if such diffusion takes place in one dimension, as is the case in the technique of Oudin,<sup>(1)</sup> in which narrow glass tubes are employed. The term 'radial' immunodiffusion is applied to systems in which the gel is spread out on a surface, with diffusion taking place radially, starting from a circular well.

Single radial immunodiffusion has apparently first been employed by Petrie<sup>(2)</sup> in his studies on the growth of bacterial colonies on gelified media containing specific antisera. He observed that such cultures became surrounded by one or more ring-shaped precipitates, which might eventually coalesce with similar precipitates formed around neighbouring cultures of the same strain of organisms.

Ouchterlony<sup>(3)</sup> was the first to exploit single radial immunodiffusion for semi-quantitative purposes, in his studies on the toxin-producing ability of different strains of *Corynebacterium diphtheriae*. His method was based on the observation that there existed a relationship between the width of the precipitate and the amount of antigen produced by the cultures, and an inverse relationship between the size of the precipitate and the concentration of the antiserum employed. Unfortunately these studies were not further pursued. Feinberg<sup>(4)</sup>, Hayward and

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Augustin,<sup>(6)</sup> and Crowle<sup>(6)</sup> have described a semi-quantitative modification of the single radial immunodiffusion method, in which use was made of the end-point principle. Serial dilutions of the same antigen solution were tested in the same antiserum plate, and the highest dilution still giving rise to a visible ring of precipitate around the antigen well was taken as the end-point. The latter technique has also been carried out on cellulose acetate foil soaked in antiserum.<sup>(7,8)</sup>

In a preliminary report from this laboratory<sup>(9)</sup> it was shown that a linear relation existed between the area of the precipitate and the concentration of the antigen, provided that diffusion was allowed to proceed until all antigen had been combined. The single radial diffusion method is therefore suitable for very accurate quantitative determinations, without any resort to end-point methods and the interpolations they require.

The present paper is concerned with the technique as well the quantitative aspects of single radial immunodiffusion.

## MATERIAL AND METHODS

### 1. *Preparation of the agar*

To 100 ml of barbiturate buffer of pH 8.6 and ionic strength 0.1 (made by dissolving 9 g of sodium diethylbarbiturate, 65 ml of 1.10 N HCl and, as a preservative, 0.5 g of sodium azide, in distilled water, and adjusting the volume to 1 l.) is added 3 g of Special Agar-Noble (Difco). This suspension is placed in a boiling water-bath and stirred until all the agar has dissolved. Distilled water is added to replace losses due to evaporation. The stock solution of agar is distributed over a number of wellstoppered test-tubes and stored at +4°C.

### 2. *Preparation of the agar-antiserum mixture*

The required amount of solidified 3 per cent agar-gel is melted on a boiling water-bath and allowed to cool down to 60°C. The antiserum, or a suitable dilution of it made in barbiturate buffer, is brought to 55°C, after which equal volumes of both solutions are mixed, as thoroughly as possible but avoiding bubbling, with the aid of a pipette pre-heated to 60°C in the water-bath. The antiserum-agar mixture is poured, without delay, into the mold hereafter described, using the same heated pipette.

### 3. *Preparation of the antiserum-agar plates*

In order to obtain antiserum-agar layers of strictly uniform thickness, use is made of a mold. The base plate which will have to support the gel consists of a carefully cleaned photographic glass plate, 1 mm thick, measuring 10 × 7 cm. Upon this is placed a U-shaped frame (Fig. 1) made of 1 mm thick brass, whose three branches measure 8 mm in width, their lengths corresponding to the dimensions of the base plate. The frame is covered by another glass plate of similar dimensions, whose lower surface is siliconized. The base plate should *not* be siliconized. The three pieces of the mold are tightly held together by means of clamps (Fig. 1).

The frame is now held in a slightly slanting position and the tip of the heated pipette containing the antiserum-agar mixture is applied to the lower corner of the

slit. Filling the mold in this way prevents the inclusion of air bubbles. After solidification (10–15 min), the clamps are removed and the siliconized top plate is carefully slid off from the gel, after which the brass frame is in turn removed. The bottom plate is left to support the gel.

#### 4. Application of the antigen samples

Circular wells are punched out in the gel, using a needle of 2 mm bore. The small cylinders of gel cut out by the needle are removed by suction. Each of the

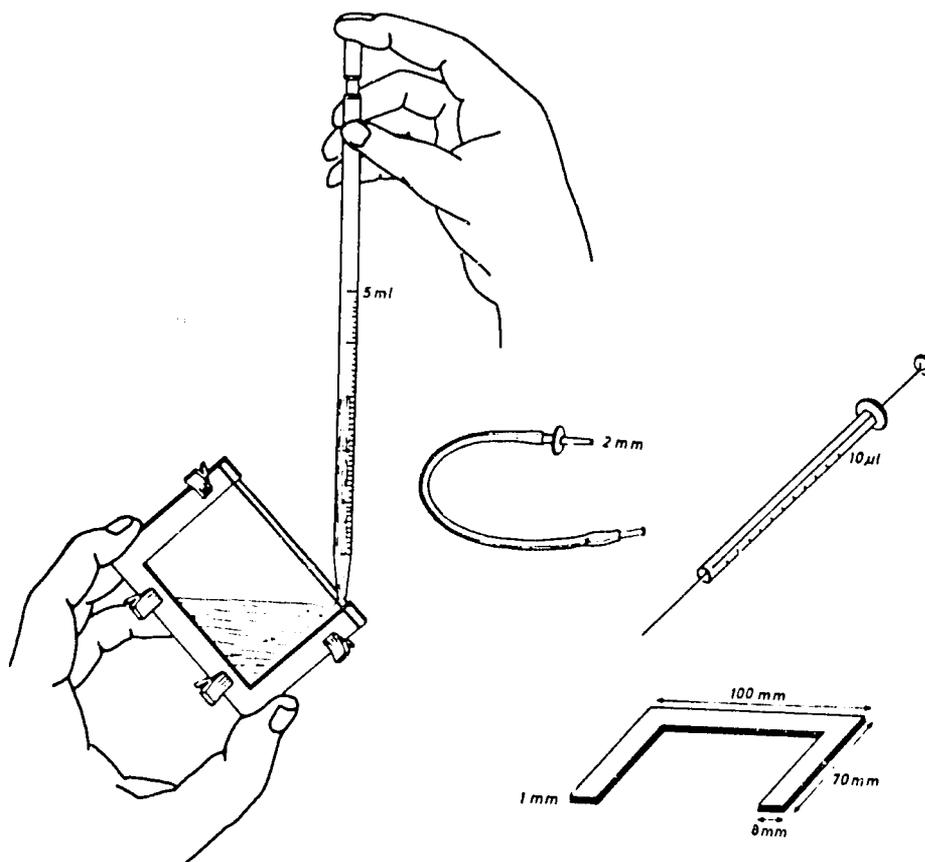


FIG. 1. Preparation of an immunodiffusion plate.

wells receives  $2 \mu\text{l}$  of antigen solution, which is delivered by means of a needle mounted on a microsyringe (Microliter Syringe  $\pm$  701-N, Hamilton Co., Whittier, Calif.). The plate is now kept, in a strictly horizontal position, in a moist box, until the antigen solutions have been soaked up by the gel (10–30 min). To prevent drying, the antiserum-agar plates are stored under a 2 cm thick layer of paraffin oil. A few crystals of thymol are dissolved in the oil to prevent the growth of anaerobic micro-organisms. It has been found practical, though not indispensable, to incubate the oil-covered plates at  $37^\circ\text{C}$ .

### 5. *Measurement of the size of the precipitates*

As will be described under Results, the ring-shaped precipitates which form around the antigen wells grow in size during a few days, after which no further increase in dimensions is observed. A typical plate with seven antigen wells is illustrated in Fig. 2. Final records are made when the precipitates may be assumed to have reached their final size. After removing the oil by a short rinse in petroleum ether, the magnified silhouettes of the precipitate rings are projected on strong paper and their contours are pencilled out. The corresponding antigen wells are similarly recorded. The circles are cut out and weighed. This method of recording surfaces has been found advantageous because the form of the precipitates may occasionally depart from the ideal circular shape. Merely recording the diameters may give erroneous results.

Weak precipitates of insufficient contrast can be brought out by staining. This is also advantageous when it is desired to store the preparations for later study. For this purpose the plates are washed with normal saline, in Petri dishes, for two to three days, with several daily changes of the washing fluid. The preparations are then soaked for one day in distilled water (renewed 3 times), in order to remove the salt. After drying at room temperature or at 37 C, the plates are immersed for 30 min in an aqueous solution containing 1 g of Amido Black, 4.1 g of sodium acetate and 30 ml of acetic acid per liter. The background color is removed by three successive baths of an aqueous solution containing 50 ml of acetic acid and 5 ml of glycerine per liter.

## RESULTS

All values for precipitate areas mentioned in the present article include the areas of the corresponding antigen wells. They are expressed in mg of paper weight, as obtained by cutting out and weighing the disks drawn in pencil on cardboard paper after projection of the magnified silhouettes of the original precipitates.

### A. *Fundamental quantitative relations*

1. *Growth of the precipitates as a function of time.* The area of the precipitate, including the antigen well, produced by the diffusion of 10 $\mu$ g of human serum albumin in an agar plate containing a twelve-fold dilution of rabbit antiserum\*, was measured repeatedly during incubation at 37 C (Fig. 3). The size of the precipitate ceased to increase after an initial period of rapid growth. As with the linear single diffusion of Oudin, the increase of the precipitate was roughly proportional to the square root of time during the earliest period of diffusion (Fig. 4).

This type of growth curve was found with all antigen-antibody systems tested, but marked variation was found as to the time required by the precipitates to reach their final size, as will be discussed in the following sections.

2. *Relation between the antigen concentration and the final size of the precipitates.* Serial dilutions of a solution containing 18 mg/ml of human albumin were allowed to diffuse in a plate containing a twelve-fold dilution of rabbit antiserum. The areas

\* This antiserum precipitated all the antigen from an equal volume of an 0.1 per cent solution of human serum albumin.

of the different precipitates were measured repeatedly and the values were plotted as a function of the antigen concentration (Fig. 5). It was thus found that there existed a direct relation between the time required for the attainment of the final size of a precipitate and the corresponding concentration of antigen. The most striking finding, however, was that of a linear relation between the antigen con-

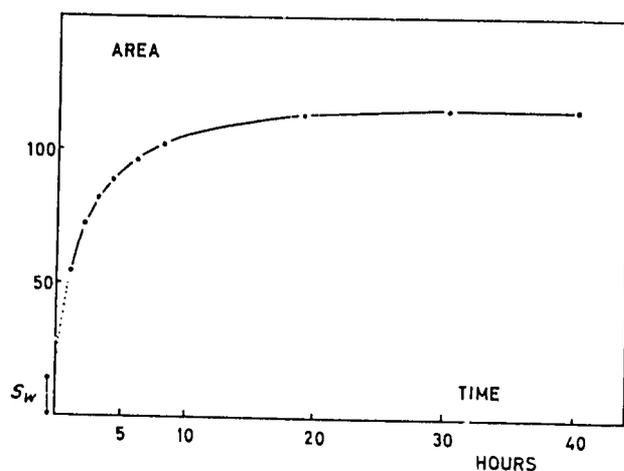


FIG. 3. Growth of the area of a precipitate as a function of time.

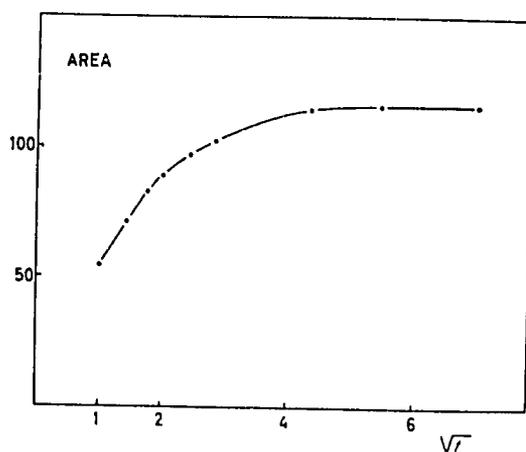


FIG. 4. Growth of the area of a precipitate as a function of the square root of time.

centration and the area of the precipitate at the end of the diffusion. In the case illustrated in Fig. 5 this relation was explored over a concentration range of antigen extending from 1.125 to 18.00 mg/ml., but larger concentrations of antigen were found to order themselves on the straight line if longer times of incubation were employed.

Statistical analysis confirmed the absence of any significant non-linear terms in

B

the regression line relating the terminal size of the precipitates ( $S$ ) to the amount of antigen ( $Q_{ag}$ )

$$S = S_0 + K \cdot Q_{ag} \quad (1)$$

The significance of the intercept  $S_0$  obtained by extrapolating the curve back to zero concentration, and of the slope,  $K$ , of the line, will be examined later.

3. *Relation between the concentration of the antiserum, the size of the antigen well, and the final size of the precipitate.* Rabbit antiserum\* against human serum-albumin was diluted resp. to 49, 35 and 16 per cent of its original concentration, and each of these dilutions was employed to coat three diffusion plates. In each of these nine plates, twelve antigen wells were punched out. Each set of twelve holes

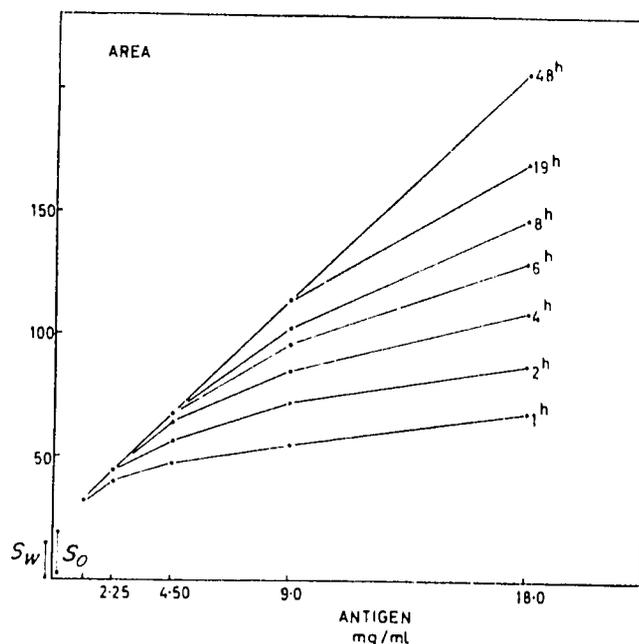


FIG. 5. Relation between area of precipitate and amount of antigen after different times of diffusion.

was composed of three series of four, whose respective diameters were 1.5, 2.2 and 3.8 mm. Each series of four wells received respectively 18.00, 9.00, 4.50 and 2.25  $\mu$ g of human albumin dissolved in a standard volume of 2  $\mu$ l. In summary, each set of antigen dilutions was tested in three types of wells, against three concentrations of antiserum, all tests being carried out in triplicate.

The nine regression lines calculated from the data charted in Table 1 are shown in Fig. 6. The origin of the variation of the regression coefficient  $K$  in these nine experiments is analyzed in Table 2, whereas Table 3 is concerned with a similar analysis of the intercepts  $S_0$ .

Statistical analysis of the variance of the slope value  $K$  demonstrates that this parameter is independent of the diameter of the antigen well (Table 2).

\* This antiserum precipitated all the antigen from an equal volume of an 0.1 per cent solution of human serum-albumin.

In fact, aside from the small experimental error, the entire variation of  $K$  is ascribable to the effect of the antiserum concentration,  $C_{ab}$ . The data indicate that the slope  $K$  varies inversely with the concentration of antibody, and this relationship is well brought out by plotting  $K$  against the reciprocal of  $C_{ab}$ , as shown in

TABLE I. INFLUENCE OF ANTIBODY CONCENTRATION AND SIZE OF ANTIGEN WELL UPON THE SIZE OF THE PRECIPITATE AREAS AT TERMINATION OF DIFFUSION

Anti-serum conc. (%)	Diameter of antigen well (mm)	Antigen concentration (mg ml)				Linear regression	
		9	4.5	2.25	1.125	S	$S_0 + K C_{ag}$
16	1.5	216	112	60	35	S	$10.12 + 25.40 C_{ag}$
		215	108	62	36		
		211	110	61	36		
		217	115	68	42		
	2.2	214	115	66	42	S	$17.09 + 24.90 C_{ag}$
		216	120	66	42		
		235	137	84	58		
		242	135	86	61		
	3.8	234	135	86	60	S	$34.58 + 25.31 C_{ag}$
		87	48	29	20		
		90	50	31	21		
		85	49	31	21		
35	2.2	90	54	36	26	S	$17.67 + 9.28 C_{ag}$
		93	56	35	27		
		92	54	37	27		
	3.8	108	71	51	43	S	$33.90 + 9.50 C_{ag}$
		109	73	53	44		
		112	74	52	44		
49	1.5	63	37	24	18	S	$10.82 + 6.47 C_{ag}$
		62	37	23	17		
		63	36	23	16		
	2.2	70	42	30	23	S	$16.25 + 6.64 C_{ag}$
		71	43	31	24		
		68	44	29	22		
		89	63	49	41		
	3.8	89	61	47	42	S	$34.53 + 6.77 C_{ag}$
		87	64	47	40		

Fig. 7. The effect of the antibody concentration on the slope of the straight line relating the size of the precipitate to the concentration of the antigen may therefore be written as:

$$K = m + n \frac{1}{C_{ab}} \quad (2)$$

Statistical analysis of the variance of the intercept,  $S_0$ , demonstrates that this parameter is independent of the antibody concentration,  $C_{ab}$ , and that its variation is entirely ascribable to the effect of the cross-sectional area of the antigen well,  $S_w$

(Table 3). The linear character of the latter relation is demonstrated by Fig. 8, in which  $S_0$  is plotted as a function of  $S_w$ . Therefore:

$$S_0 = p + q \cdot S_w \quad (3)$$

### B. Reproducibility of the method

1. *Variation due to the use of different plates and different positions within a single plate (Experimental error).* A volume of 5.2 ml of rabbit antiserum against human serum-albumin\* was mixed with 5.8 ml of barbiturate buffer. This solution was heated to 55 C in a water-bath, and thoroughly mixed with 11.0 ml of a 3 per cent molten agar solution made with the same buffer. Four immunodiffusion plates, each

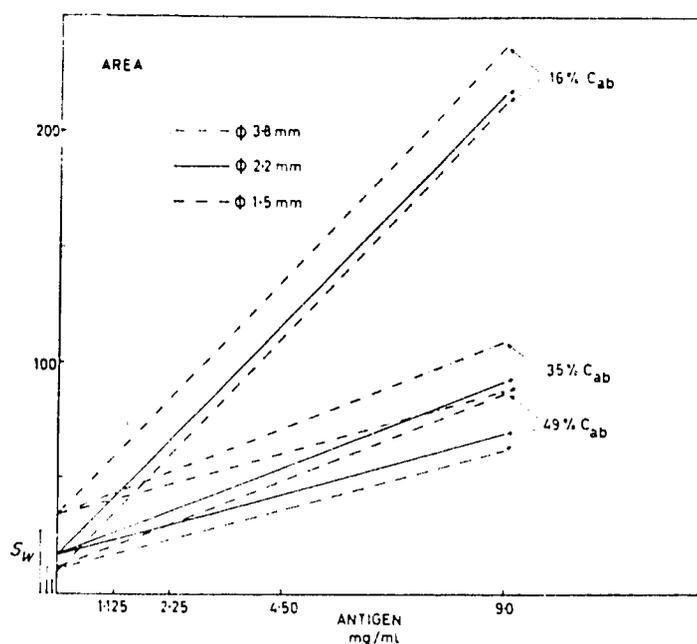


FIG. 6. Influence of concentration of antibody and size of the antigen well upon the final size of the precipitate.

1 mm thick, were prepared from this agar-antibody solution. A different brass frame was employed for each plate, so as to provide a test on the reproducibility in function of possible variations in the thickness of the gel layers.

Sixteen antigen wells of 2.2 mm diameter were punched out in each plate, using the same instrument. The pattern of arrangement of the wells was the same for all plates. Each antigen well received 2  $\mu$ l of a 0.911 per cent solution<sup>†</sup> of crystalline human serum-albumin. After 6 days of diffusion, i.e. at a time well after termination of the diffusion for this particular antigen-antibody system, the areas of the precipitates were recorded from the unstained preparations.

\* This antiserum precipitated all the antigen from an equal volume of an 0.1 per cent solution of human serum-albumin.

<sup>†</sup> Determined by the biuret method.

TABLE 2. ANALYSIS OF THE SLOPE VALUE  $K$  AS A FUNCTION OF THE ANTIBODY CONCENTRATION AND THE SIZE OF THE ANTIGEN WELL

Concentration of antiserum (%)	Diameter of antigen well (mm)			
	1.5	2.2	3.8	
16	25.40	24.90	25.31	75.61
35	9.51	9.58	9.50	28.59
49	6.47	6.64	6.77	19.88
Total	41.38	41.12	41.58	124.08

## Analysis of variance

Item	Sum of squares	Degrees of freedom	Variance	Variance ratio ( $F$ )
Antiserum concentration	599.1752	2	299.5876	7701.4807
Diameter of antigen well	0.0354	2	0.0177	0.4550
Error	0.1558	4	0.0389	
Total	599.3664	8		

In this randomized-block experiment the slope value,  $K$ , proves to be independent from the diameter of the antigen well ( $F$  non-significant), whereas the term corresponding to the antibody concentration is highly significant at the 0.1 per cent level.

TABLE 3. ANALYSIS OF THE INTERCEPT VALUE  $S_0$  AS A FUNCTION OF THE ANTIBODY CONCENTRATION AND THE SIZE OF THE ANTIGEN WELL

Concentration of antiserum (%)	Diameter of antigen well (mm)			
	1.5	2.2	3.8	
16	10.12	17.09	34.58	61.79
35	11.17	17.67	33.90	62.74
49	10.82	16.25	34.53	61.60
Total	32.11	51.01	103.01	186.13

## Analysis of variance

Item	Sum of squares	Degrees of freedom	Variance	Variance ratio ( $F$ )
Antiserum concentration	0.2486	2	0.1243	0.3051
Diameter of antigen well	898.6688	2	449.3344	1102.9307
Error	1.6299	4	0.4074	
Total	900.5473	8		

The term corresponding to the antibody concentration is non-significant, whereas the one corresponding to the diameter of the antigen well is highly significant at the 0.1 per cent level.

The results are presented in Table 4. For each plate the data are grouped in blocks of four, corresponding to the four quadrants of the immunodiffusion plates (Fig. 9). This method of presentation was intended to provide a test on the influence of the position of the wells within the plates.

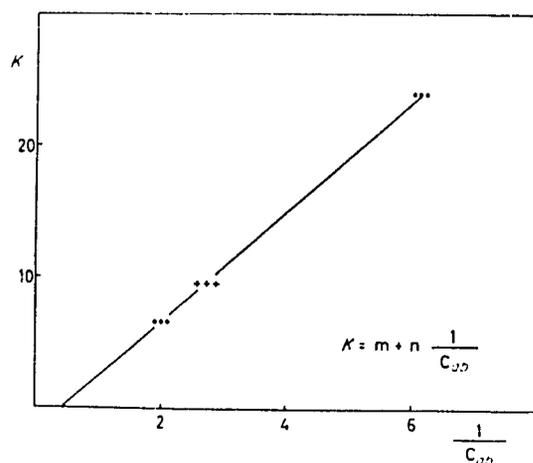


FIG. 7. Change of the slope of the regression line as a function of the antibody concentration.

The statistical analysis of the data is presented in Table 5. The results indicate that: (1) there was a perceptible variation of the results from one plate to another, obviously because of small differences in the thickness of the frames employed; (2) the amount of variation due to the position occupied by the antigen wells in the

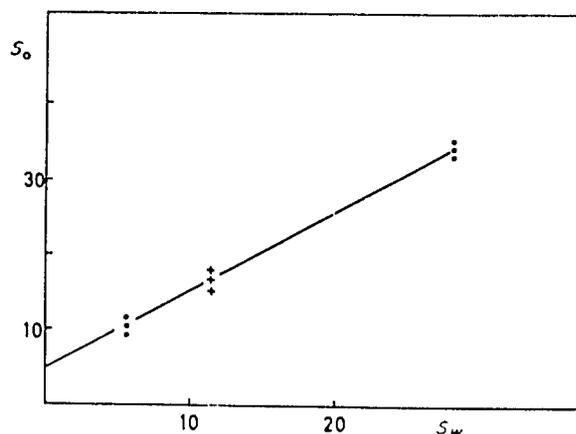


FIG. 8. Change of the intercept of the regression line as a function of the size of the antigen well.

plates (quadrants and interaction between plates and quadrants) was insignificant; (3) the residual variation, which may be termed the experimental error, amounted to 1.7 per cent, when expressed as the standard deviation in per cent of the mean. If the total variance of all 64 observations was used to calculate the standard deviation, the latter became 2.0 per cent of the mean.

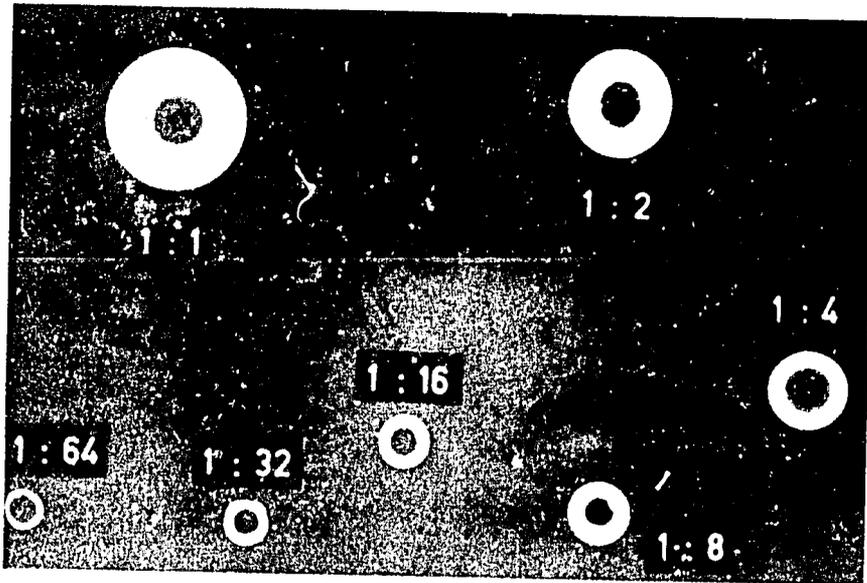


FIG. 2. Unstained immunodiffusion plate. The agar contained rabbit anti-serum against human serum-albumin. The antigen wells were filled with serial dilutions of a 1 per cent albumin solution, as indicated on the photograph. The plate was photographed on a dark background, using indirect light. This picture was taken on the 5th day of the experiment, when diffusion had ceased.

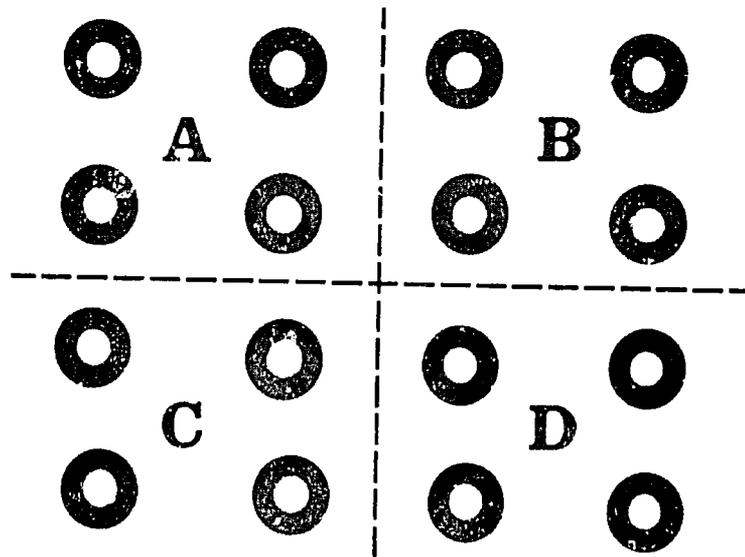


FIG. 9. Block-wise arrangement of sixteen antigen wells in a diffusion plate. Photograph of a plate stained after termination of the diffusion.

(Facing p. 244)

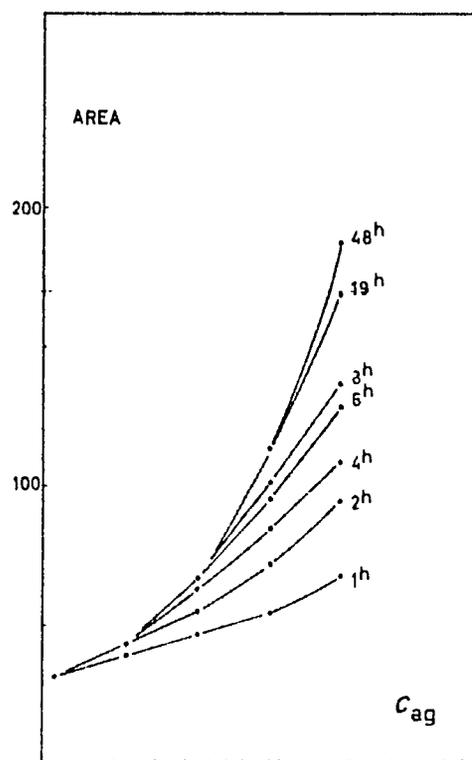


FIG. 13. Relation between the area of the precipitates and the logarithm of the antigen concentration. This figure represents a rearrangement of the data from Fig. 5.

TABLE 4. INTER-PLATE AND INTRA-PLATE VARIATION

Quadrants	Plates				
	I	II	III	IV	
A	135.7	137.0	136.6	139.3	
	141.0	137.0	139.0	140.5	
	133.4	133.8	138.5	139.0	
	134.2	135.2	138.3	140.5	
	544.3	543.0	552.4	559.3	2199.0
B	141.8	135.7	137.4	141.4	
	137.8	142.0	135.7	136.0	
	143.0	139.0	138.4	140.5	
	136.7	136.0	137.5	139.5	
	559.3	552.7	549.0	557.4	2218.4
C	138.5	134.4	138.5	141.0	
	137.4	132.4	142.5	132.0	
	134.0	135.0	137.0	137.6	
	135.3	134.5	139.6	138.4	
	545.2	536.3	557.6	549.0	2188.1
D	137.0	132.3	136.1	137.3	
	132.3	137.9	137.4	139.2	
	136.4	132.0	137.8	140.5	
	134.4	139.0	138.3	142.4	
	540.1	541.2	549.6	559.4	2190.3
	2188.9	2175.2	2208.6	2225.1	8795.8

TABLE 5. STATISTICAL ANALYSIS OF THE DATA FROM TABLE 4

Item	Degrees of freedom (N)	Sum of squares	Variance	Ratio variance (F)	P
Plates	3	96.3131	32.1044	5.7489	0.01-0.001
Quadrants	3	35.6781	11.8927	2.1296	0-10
Interaction between plates and quadrants	9	81.9182	9.1020	1.6298	0.20-0.10
Error	48	268.0550	5.5844*		
Total	63	481.9644	7.6502†		

\* Standard deviation:  $\sqrt{5.5844}$  = 2.363 (i.e. 1.7194 per cent of the mean).  
 † Standard deviation:  $\sqrt{7.6502}$  = 2.765 (i.e. 2.0112 per cent of the mean).

2. *Variation due to differences in volume of a solution containing constant amounts of antigen.* A volume of 0.6 ml of rabbit antiserum against human serum-albumin\* was mixed with 1.9 ml of buffer, and after heating, with 2.5 ml of a 3 per cent agar solution. One diffusion plate, 1 mm thick, was set up with the mixture, and sixteen antigen wells of 4.5 mm diameter were punched out after solidification of the gel. The wells were arranged in rows of four, with each row receiving respectively 5.800, 2.900, 1.450 and 0.725  $\mu\text{g}$  of crystalline serum albumin. These amounts of antigen were applied in a volume of 2  $\mu\text{l}$  of barbiturate buffer for the 4 wells of the first row, in 4  $\mu\text{l}$  for the second row, 8  $\mu\text{l}$  for the third row, and 16  $\mu\text{l}$  for the fourth row.

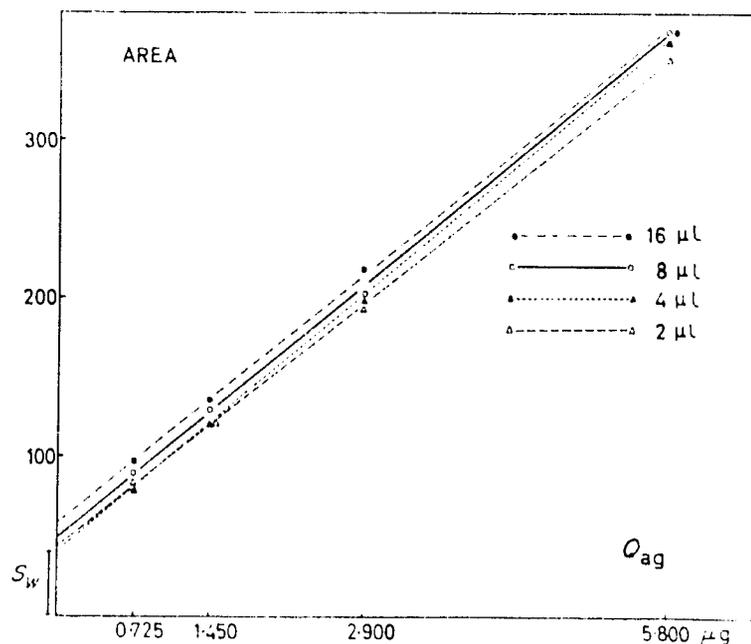


FIG. 10. Effect of increasing volumes of solvent upon the area of the precipitates given by constant amounts of antigen.

The purpose of this experiment was to test whether the amount of fluid introduced together with the antigen would affect the area of the precipitate.

The results and their statistical analysis are indicated in Table 6. It is obvious that: (1) the data from each experiment align themselves on a linear regression, as expected; (2) the slopes of the four regression lines may be regarded as identical; (3) the four regression lines are not congruent but run at some distance alongside each other. There is a distinct tendency for larger volumes of solvent to produce larger areas of precipitate with identical amounts of antigen. The latter conclusion is also apparent from an inspection of Fig. 10.

3. *The influence of changes in temperature upon the final areas of the precipitates.* Three immunodiffusion plates were set up with one and the same antibody-agar mixture, and incubated at different temperatures. A volume of 0.30 ml of rabbit

\* Same antiserum as in the preceding experiment.

antiserum against human serum-albumin\* was mixed with 7.2 ml of buffer and 7.5 ml of hot 3 per cent agar. Three diffusion plates, 1 mm thick, were poured, and in each plate five antigen wells of 2.2 mm diameter were punched out. The five antigen wells in each plate received respectively 0.625, 1.250, 2.500, 5.000, and 10.000  $\mu\text{g}$  of crystalline human albumin, all samples being applied in 2  $\mu\text{l}$  of solvent buffer. The first plate was incubated at 37°C, the second plate at 21°C and the third plate at 4°C.

The sizes of the precipitates were estimated repeatedly during the incubation. It was observed that the precipitate corresponding to the largest amount of antigen

TABLE 6. INFLUENCE OF THE VOLUME OF SOLVENT UPON THE AREA OF THE PRECIPITATE

Amount of antigen*	Volume of solvent			
	2 $\mu\text{l}$	4 $\mu\text{l}$	8 $\mu\text{l}$	16 $\mu\text{l}$
1	84	81	89	96
2	119	118	130	135
4	193	199	202	219
8	352	363	369	369

*Individual regression lines*

For 2  $\mu\text{l}$  S 42.87 38.43  $Q_{\text{ag}}$ ,

For 4  $\mu\text{l}$  S 38.52 40.46  $Q_{\text{ag}}$ ,

For 8  $\mu\text{l}$  S 47.96 39.88  $Q_{\text{ag}}$ ,

For 16  $\mu\text{l}$  S 58.30 39.05  $Q_{\text{ag}}$ .

*Comparison of the four individual regressions*

Mean square of differences

between regression coefficients  
 $\frac{\quad}{\text{Error mean square}} = 1.8408 \quad P = 0.20$

Mean square of differences

between means  
 $\frac{\quad}{\text{Error mean square}} = 20.0926 \quad P = 0.001$

\* For the purpose of simplifying the statistical computations, the smallest amount of antigen (0.725  $\mu\text{g}$ ) is given the arbitrary value 1.

attained its final size after approximately 8 days in the 37°C plate, 10 days in the 21°C plate, and 14 days in the 4°C plate. All results were recorded together on the 15th day, and these data are listed in Table 7.

The results clearly indicate that the three regression lines may be regarded as indistinguishable, both with respect to their slopes and to their positions. In other words differences in temperature had absolutely no perceptible effect on the final size of the precipitates. This conclusion is also illustrated by Fig. 11.

*C. Sensitivity of the method*

The following experiment was undertaken in order to assess the lower limit of the amount of human serum albumin which can accurately be estimated by the single radial immunodiffusion method.

\* Same antiserum as in the preceding experiments.

Rabbit antiserum against human serum-albumin\* was diluted respectively 5, 25, and 50 times, by means of barbiturate buffer, and from each dilution 2.5 ml were mixed with 2.5 ml of hot 3 per cent agar. Each dilution was used to prepare one immunodiffusion plate. The final antiserum concentrations in the three plates thus corresponded respectively to 1/10, 1/50 and 1/100 times the strength of the undiluted antiserum. Eight antigen wells were punched out in each plate, and filled with respectively 0.01, 0.02, 0.04, 0.08, 0.16, 0.32, 0.64 and 1.28  $\mu\text{g}$  of human serum-albumin (in 2  $\mu\text{l}$  of buffer) for the first plate, with 0.005, 0.01, 0.02, 0.04, 0.08, 0.16, 0.32 and 0.64  $\mu\text{g}$  for the second plate, and with 0.0025, 0.005, 0.01, 0.02, 0.04, 0.08, 0.16 and 0.32  $\mu\text{g}$  of antigen for the third plate.

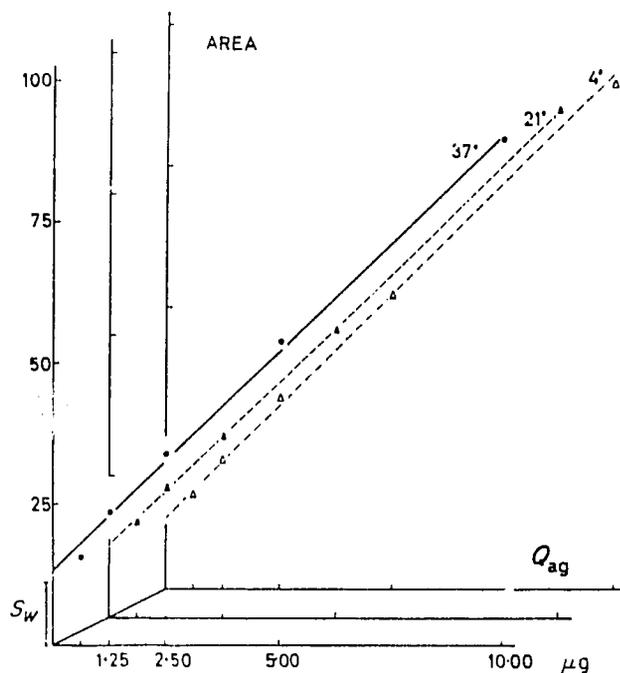


FIG. 11. Effect of temperature on the size of the precipitates given by constant amounts of antigen.

It was observed that the precipitates in the plate containing the highest dilution of the antiserum were so faint as to be unreadable, even after staining with Amido Black. Since previous experience had indicated that an appreciable fraction of the precipitate obtained in this method consisted of non-specifically co-precipitated proteins from the antiserum, it was attempted to increase the strength of the weakest precipitates by incorporating serum from a non-immune rabbit into the agar-antibody mixture. Practically, the barbiturate buffer employed to dilute the anti-serum was replaced by non-immune rabbit serum which had been shown, by a previous test, not to react with human serum-albumin. By using this artifice the precipitin areas of the third plate now became perfectly recordable after staining with Amido Black.

\* Same antiserum as in the preceding experiments.

The results obtained by this modified method are indicated in Table 8 and the corresponding regression lines are illustrated in Fig. 12.

Considering first the plate with the strongest antibody mixture (1 : 10), it was observed that the precipitates given by the lowest amounts of antigen (0.01 and 0.02  $\mu\text{g}$ ) hardly extended beyond the rim of the antigen well. Since it was felt that their areas could not be estimated with the desired accuracy, it was thought preferable to exclude them from the computation of the corresponding regression line. However, after this line had been calculated from the remaining six points, it was found that the value obtained for 0.02  $\mu\text{g}$  did not significantly depart ( $P$ : 0.10-0.20) from the theoretical figure, which was 23.36 mg of paper weight. The value

TABLE 7. INFLUENCE OF THE TEMPERATURE UPON THE AREA OF THE PRECIPITATE

Amount of antigen*	Temperature		
	37 C (Plate I)	21 C (Plate II)	4 C (Plate III)
1	16	17	17
2	24	23	23
4	34	32	34
8	54	52	52
16	90	90	92

*Individual regression lines*

$$\text{For } 37 \text{ C } S = 13.58 + 4.84 Q_{\text{ag}},$$

$$\text{For } 21 \text{ C } S = 12.79 + 4.84 Q_{\text{ag}},$$

$$\text{For } 4 \text{ C } S = 12.96 + 4.94 Q_{\text{ag}}.$$

*Comparison of the three individual regressions*

$$\frac{\text{Mean square of differences between regression coefficients}}{\text{Error mean square}} = 0.3033 \quad P > 0.20$$

$$\frac{\text{Mean square of differences between means}}{\text{Error mean square}} = 0.8315 \quad P > 0.20$$

\* For the purpose of simplifying the statistical computations, the smallest amount of antigen (0.625  $\mu\text{g}$ ) is given the arbitrary value 1.

for 0.01  $\mu\text{g}$ , in contrast, was significantly different ( $P$  : 0.01-0.02) from the expected value of 24.49. Judging from this set of data it would appear that the lowest amount of antigen which can accurately be estimated by this method would lie between 0.01 and 0.02  $\mu\text{g}$ .

However, this limit can be lowered by using a more diluted preparation of antiserum, since it was shown before that for a given amount of antigen the size of the precipitate increased in function of the reciprocal of the antibody concentration. Indeed, the 0.01  $\mu\text{g}$  point, which was not readable with the 1 : 10 diluted antiserum, perfectly fell on the regression line when the antiserum was diluted 1 : 50 and 1 : 100. With the latter dilution, the point corresponding to 0.005  $\mu\text{g}$  fell exactly on the corresponding regression line, but the 0.0025  $\mu\text{g}$  point seemed to be somewhat

erratic and was therefore discarded from the computation. However, statistical analysis proved that even this experimental figure could have been trusted, since its departure from the expected value (24.11) was not significant ( $P$  0.10-0.20).

The lower limit of sensitivity, as inferred from the results with the 1 : 100 anti-serum dilution, should therefore lie at about 0.0025  $\mu\text{g}$  of antigen.

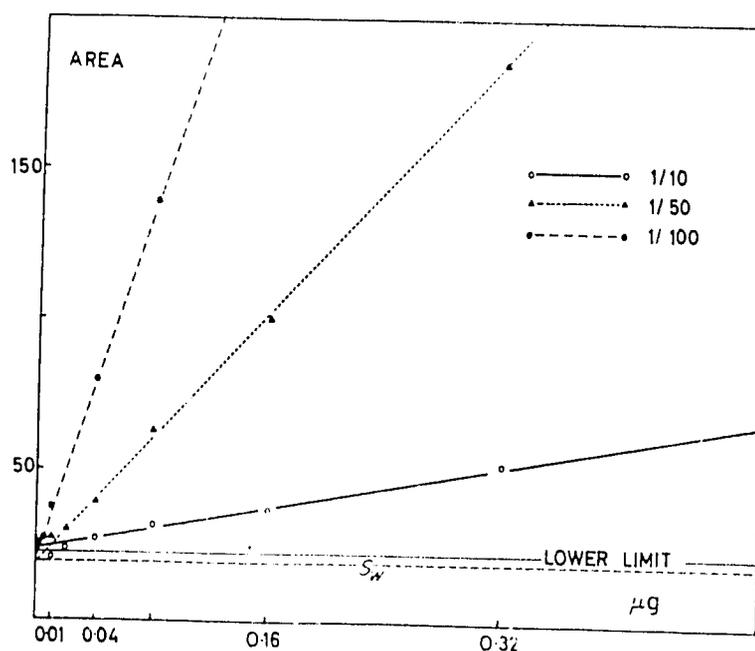


FIG. 12. Increase in sensitivity obtained by diluting the antiserum.  $S_w$ : area of the antigen well (of 2.2 mm diameter).

Lower limit: critical size of precipitate below which accurate measurements are impossible.

Regression lines were calculated by the method of the least squares as explained in the text. The points corresponding to 1.28 and 0.64  $\mu\text{g}$  for the 1 : 10 dilution, and the point corresponding to 0.16  $\mu\text{g}$  for the 1 : 100 dilution, were included in the computation but have not been featured on the graph.

## DISCUSSION

### A. Principle of the method

Oudin<sup>(1)</sup> discovered that when an antigen was allowed to diffuse along one dimension in a column of agar mixed with antiserum, the migration rate of the 'leading edge' of the precipitate through the gel was (1) proportional to the square root of time for any antigen-antibody system tested; (2) proportional to the logarithm of the concentration of antigen; and (3) proportional to the logarithm of the reciprocal of the antibody concentration.

In the two-dimensional system here studied, the rate of growth of the precipitate area failed to display any of the three relations described by Oudin, except during the very first stages of the diffusion. For the antigen-antibody system illustrated in Fig. 4 the linearity of the growth of  $S$  as a function of  $t^{1/2}$  was abandoned after 2 hr. Similarly, when the data from the experiment shown in Fig. 5 were re-arranged on semi-logarithmic graph paper (Fig. 13), the points of the curve

obtained at 1 hr fell on a straight line, except the one corresponding to the highest antigen concentration. At later times this relation was progressively abandoned until at the termination of diffusion the straight-line relation illustrated in Fig. 5 was obtained.

It is significant that each precipitate ceased to grow as soon as it had reached the size which was directly proportional to the corresponding amount of antigen. This end-point of growth was not attained simultaneously for the different precipitates given by serial dilution of the same antigen. In fact, the time required for each precipitate to complete its growth increased as a function of the final size to be achieved (Fig. 5). In one particular experiment a plate was stored at 37 C for 4 months; at the end of this time the precipitates had retained the size which they displayed at the moment when the straight-line relation had been reached.

It would also seem that the time required for a precipitate to achieve its final size increases as a function of the molecular weight of the antigen. This aspect of the question was not studied in detail, but it was noted that disk sizes of about 1 cm in diameter were achieved in less than 24 hr by Bence-Jones proteins, in 3 days by albumin, in 1 week by  $\gamma$ G-immunoglobulins, and in 10 days by  $\gamma$ M-immunoglobulins.

The linear character of the relation between the amount of antigen and the final precipitate size on one hand, and the temperature-independence of the system on the other hand, indicate that the final precipitate size in single radial immunodiffusion is not primarily determined by the diffusion rate of the antigen. In this respect the method here described sharply contrasts with that of Oudin, in which the concentration of the antigen is evaluated from the rate of migration of the leading edge of the precipitate. The fact that diffusion should rapidly come to a stop in the radial method is of course due to the great disproportion between the small amount of antigen used and the practically unlimited antibody space available to it. It would seem that the single radial diffusion method, insofar as it is based on the measurement of final precipitate areas, is more related to the classical immunological precipitin methods in which the amount of precipitate formed by the combination of an unknown quantity of antigen and a limitless supply of antibody is measured. What is evaluated in the immunodiffusion method here described is essentially the volume of antibody-containing agar gel which is engaged in combination with the sample of antigen, after all antigen has been consumed. The moment at which the latter situation is achieved is heralded by the cessation of the growth of the precipitin area. At this moment the system has reached the state in which no freely diffusible antigen, or possibly small antigen-antibody complexes, are left available to engage additional antibody. One may assume that at such time the composition of the precipitate resembles that of an antibody-antigen precipitate formed at equivalence.

In agreement with the explanation given above, it is found that the area of the precipitate at termination of growth is not so much related to the amount of antigen *per se* as to the ratio of the amount of antigen to the concentration of antibody imbibing the plate. Indeed, by combining equations (1) and (2) one obtains:

$$S = S_0 + m \cdot Q_{ag} + n \frac{Q_{ag}}{C_{ab}} \quad (4)$$

### B. *Applicability and reproducibility*

The applicability of the single radial immunodiffusion system here described to the quantitation of proteins in mixtures such as serum, has been verified for a large number of serum proteins, viz. prealbumin, albumin,  $\alpha_1$ -antitrypsin,  $\alpha_1$ -acid-glycoprotein (orosomucoid), haptoglobin,  $\alpha_2$ -macroglobulin, transferrin,  $\beta_{1A}$ -globulin, and the three immunoglobulins,  $\gamma G$ ,  $\gamma A$  and  $\gamma M$ , as well as to Bence-Jones proteins and isolated light chains from immunoglobulins. Other species of serum proteins have been determined with this method by Störiko and Augener,<sup>(11)</sup> whereas Rümke<sup>(12)</sup> has applied it to the quantitation of a prealbumin component in the serum and urines of mice and rats. A more or less similar procedure has also been used by Tomasi and Zigelbaum<sup>(13)</sup> for the quantitation of  $\gamma A$ -immunoglobulins in various human secretions.

It should be stressed that in order to obtain strict linear dependency upon the amount of antigen, readings should be postponed until there is no further growth of the precipitate areas. Of course, if only semi-quantitative results are desired, the precipitate sizes can be recorded at any time after the diffusion has started, provided use is made of a sufficient number of standards in order to allow for the non-linearity of the  $S$  vs.  $Q_{a2}$  curve. It must also be pointed out that, even if readings are made from the final straight line, a mere application of the slide rule for the purpose of interpolation is not justified. Indeed, only in rare cases will the curve, when extrapolated to zero concentration of antigen, intersect the  $S$  axis at the value  $S_w$ . In other words, the curve does not usually start from the point corresponding to the rim of the antigen well, but from some point situated outside this rim. The value of the intercept will in fact depend upon a number of variables, among which the size of the antigen wells (equation 3) and the volume of fluid introduced into the well (Table 6).

When the 64 data from the experiment described in Table 4 were summarized over four different immunodiffusion plates made with the same antigen-antibody mixture, the total standard deviation barely exceeded 2 per cent of the mean. Naturally, the error due to the use of different plates will depend upon the care with which the different frames are constructed and adjusted to uniform thickness. When large series of determinations impose the simultaneous use of different frames, it is advisable to carry out a test for homogeneity in the manner here described.

When comparisons are restricted to the data obtained in a single immunodiffusion plate, the experimental error of the method is decreased to 1.7 per cent, when expressed as the standard deviation in per cent of the mean. This accuracy is of the order expected from reliable chemical quantitation methods, and is certainly one of the attractive features of this technique.

As to the influence of the position of the antigen wells within a given plate, this item did not significantly contribute to the variation observed in the present series of experiments. Of course, a test such as the quadrant analysis here employed, would be useful if doubt existed as to the regularity of the thickness of the frames.

It is further shown that the results are not influenced by the temperature at which the incubation is carried out. This feature completely distinguishes the method here described from the single linear immunodiffusion of Oudin<sup>(4)</sup> One of the advantages of this independence from temperature is that the diffusion plates

need not necessarily be incubated at a constant temperature. For instance, there is no inconvenience in storing the plates, without any special thermal insulation, at room temperature, even if the latter varies markedly during the day. The only restriction to be made is that sudden important changes in temperature are liable to cause the formation of concentric striae in the precipitin disks. This was observed more frequently with proteins of small molecular weight, i.e. high diffusion coefficient. If pronounced, the phenomenon may give the erroneous impression that more than one antigen-antibody system is involved, but this difficulty is rather theoretical when monospecific antisera are employed.

TABLE 8. LOWER LIMIT OF THE METHOD

Amount of antigen $Q_{ag}$ (in $\mu\text{g}$ )	Size of precipitates ( $\pm S$ )		
	1 : 10 Dilution of antiserum*	1 : 50 Dilution of antiserum*	1 : 100 Dilution of antiserum*
1.28	136	not tested	not tested
0.64	77	indistinct	not tested
0.32	52	185	indistinct
0.16	37	100	260
0.08	32	63	139
0.04	27	39	80
0.02	24	30	49
0.01	21	27	38
0.005	not tested	not visible	27
0.0025	not tested	not tested	26
Size of antigen well	19	19	19

\* Final dilution in the antibody-agar gel

*Regression lines*

1 : 10 Plate  $S \dagger = 23.62 + 87.01 Q_{ag}$ ,

1 : 50 Plate  $S \ddagger = 20.19 + 512.44 Q_{ag}$ ,

1 : 100 Plate  $S \ddagger = 20.38 + 1494.39 Q_{ag}$ .

† Computed from the six highest figures.

‡ Computed from the six figures indicated in this table.

### C. Sensitivity

As shown in the present paper, the lower limit of the amount of antigen which can accurately be measured with this method approaches 0.0025  $\mu\text{g}$  if highly diluted antiserum is employed. Since this amount was contained in 2  $\mu\text{l}$  of solvent, the lowest measurable concentration of antigen would be 1.25  $\mu\text{g}/\text{ml}$ . Although this conclusion was reached from experiments with human serum-albumin, there is no reason why it should not apply to other antigens as well. Indeed, the principle which determines the lower limit of sensitivity is a quite general one: (1) a precipitin disk ceases to be accurately readable either when it becomes too faint to be observed or (2) when its dimensions do not sufficiently extend beyond the rim

of the antigen well. The lower limit of dimension which can safely be accepted has been indicated in Fig. 12, and should theoretically be the same for any antigen-antibody system. As to the density of the precipitate, this parameter depends on the concentration of active antibody incorporated in the gel, i.e. on a parameter which can be changed at will, irrespective of the nature of the antigen against which this antibody is directed.

The critical dimension of the precipitate is not liable to improvement by artificial means, but the density of the precipitin disk can be enhanced, both by staining and by the addition to the system of some non-specific co-precipitating agent such as non-immune serum. Both artifices were employed in the present study and helped to bring down the lower limit of sensitivity to as little as 0.0025  $\mu$ g of antigen.

Further improvements upon the sensitivity can probably be obtained by increasing the volume of antigen solution introduced into the wells. If the use of larger antigen wells and or a more complete filling of such wells as a means of increasing the sensitivity of the method are contemplated, two important precautions should be kept in mind. One is that all the samples to be compared to a given reference solution of antigen, as well as the standard solution itself, must be applied in identical volumes of solvent (Fig. 10).

The other restriction is that all samples, including the reference solutions, should also be applied in antigen wells of identical dimensions.

#### REFERENCES

- <sup>1</sup> OUDIN J., *C.R. Hebd. Séanc. Acad. Sci. Paris*, **228**, 1890 (1949).
- <sup>2</sup> PETRIE G. F., *Br. J. Exp. Path.* **13**, 380 (1932).
- <sup>3</sup> OUCHTERLONY Ö., *Acta Pathol. Microbiol. Scand.* **26** 516 (1949).
- <sup>4</sup> FEINBERG J. G., *Int. Archs Allergy* **11**, 129 (1957).
- <sup>5</sup> HAYWARD B. J. and AUGUSTIN R., *Int. Archs Allergy* **11**, 192 (1957).
- <sup>6</sup> CROWLE A. J., *J. Lab. Clin. Med.* **55**, 593 (1960).
- <sup>7</sup> FEINBERG J. G., *Nature, Lond.* **194**, 307 (1962).
- <sup>8</sup> FEINBERG J. G., *J. Clin. Path.* **16**, 282 (1963).
- <sup>9</sup> MANCINI G., VAERMAN J. P., CARBONARA A. O. and HEREMANS J. F., *Protides Biol. Fluids* **11**, 370 (1964).
- <sup>10</sup> OUCHTERLONY Ö., *Prog. Allergy* **6**, 30 (1962).
- <sup>11</sup> AUGENER W., *Protides Biol. Fluids* **12**, 363 (1964).
- <sup>12</sup> RÜMKE PIC. and WÜHNG P. J., *Acta Endocrinol.* **47**, 156 (1964).
- <sup>13</sup> TOMASI T. B. and ZIGELBAUM S. D., *J. Clin. Invest.* **42**, 1552 (1963).