57. ESTIMATION OF VITAMIN A AND CAROTENE IN HUMAN BLOOD

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DURING the past few years numerous investigations have been carried out on the vitamin A and carotene contents of human blood in health and various diseases. Unfortunately, the method of estimation varies from author to author and in many cases the results are given in other than international units [Dost, 1937; Kimble, 1939; May *et al.* 1940], so that comparison is impossible. The following investigation was undertaken with a view to discovering the relative efficacies of the various methods of estimation. A preliminary series of normal blood values is also given.

Discussion of methods

The extraction is usually done on serum or plasma. Many authors saponify the serum with alcoholic KOH, some in the cold to eliminate the effect of haemolysis [Willstaedt & With, 1938], others on a boiling water bath for longer [Lindquist, 1938] or shorter [Pett & Le Page, 1940] periods. Clausen & McCoord [1938] and Kimble [1939] extract directly from serum precipitated with alcohol. The extraction is done either with ether or light petroleum and the resulting extract evaporated under nitrogen or carbon dioxide or in air.

The estimation of the vitamin A is usually carried out by means of the blue reaction of Carr and Price, but the absorption at $328\text{ m}\mu$ is sometimes estimated directly [Chevallier & Choron, 1935]. The depths of the yellow colour of the carotene and the blue colour of the vitamin A reaction have been estimated in the following ways.

1. *Comparison in colorimeter with standard solutions* (potassium dichromate for carotene and either copper sulphate or a mixture of copper sulphate and cobalt nitrate [Schneider & Widmann, 1935] for the blue colour). The main criticism of this method is the fact that the colours of standard and unknown are not spectrographically the same. (Indeed 10 % copper sulphate solution may be used as part of the filter system in photoelectric colorimetry of the blue colour.) Besides this the rapid fading of the blue colour makes matching difficult.

2. *Lovibond tintometry*. The same criticisms apply (see Notevarp & Weedon [1936] for more detailed discussion of these points).

3. *The Zeiss step-photometer* is more accurate and fairly satisfactory but again colour matching is difficult because of fading.

4. *Spectrophotometry* at $328\text{ m}\mu$ is accurate but needs complicated apparatus and there are several interfering factors at this wave-length.

5. *Photoelectric colorimetry*. This method is at present the most satisfactory. It eliminates the personal factor of colour matching and makes it easy to estimate the density of the blue colour at its real maximum. It should, however, be remembered that the solutions do not necessarily follow Beer's Law [Koehn & Sherman, 1940], so that a calibration curve or table must be constructed. The

best method of estimation would be photoelectric colorimetry with monochromatic light (a 'monochromator') but there is, as far as I know, no such instrument available.

For the present investigation a simple photoelectric colorimeter was therefore used, with combinations of filters with maximum transmission at $440\text{ m}\mu$ for the carotene and $620\text{ m}\mu$ for the blue colour of the Carr-Price reaction (Fig. 1).

Fig. 1. Simple photoelectric colorimeter.

A. Simple circuit to give coarse and fine adjustment of illumination.

B. Source of illumination. 6 V. 3 W. Mazda lamp with small U-filament, run off four 2 V. cells to give increased proportion of blue light for the $440\text{ m}\mu$ filter. Ordinary hemispherical torch reflector behind.

C. Space for filters. Filter $620\text{ m}\mu$ maximum; Wratten 26 red gelatin filter combined with 1 mm. thickness of 'calorex' glass and 1 mm. thickness of 'signal green' glass, manufactured by Messrs Chance. Filter $440\text{ m}\mu$ maximum; Wratten gelatin filters 35 and 2A combined with 2 mm. thickness of 'signal green' glass.

D. Space for card to cut off all light falling on photocell when not in use. Thin glass plate between this and filters.

E. Glass cell holding test solutions. Volume about 4 ml. depth 10 mm. optically ground. Marked on edge at 0.3 ml. and 3.3 ml.

F. Photocell. Selenium barrier cell manufactured by Mr Wender. Size 1 cm. $\times$ 2 cm. Thin glass plate in front.

Galvanometer; mirror type; sensitivity 12 cm. deflexion at 1 m. per microamp. (Messrs Pye Ltd.).

A smaller glass cell and smaller photoelectric cell may be used. The volumes used in these experiments are the most suitable for the sizes given.

Calibration

Carotene. A solution of carotene in arachis oil¹ containing 200 international units ($120\text{ }\mu\text{g.}$) per g. was used. A weighed quantity was dissolved in light petroleum and aliquots made up to the 3.3 ml. mark in the standard cell. The deflexions on the galvanometer from the 'centre position' were noted and the calibration table constructed (Table 1).

Vitamin A. A solution was made of about 10 mg., accurately weighed, of pure vitamin A 2-naphthoate¹ in 25 ml. pure arachis oil containing a trace of quinol. 5 ml. of this solution were made up to 50 ml. with pure dried chloroform and aliquots of this were used for the Carr-Price reaction. (The actual volumes were aliquots made up to 0.3 ml. and 3 ml. of reagent added, but these depend on the size of the cell in the colorimeter) (Table 2).

Correction for blue colour produced by carotene. A solution of a weighed quantity of carotene was made in chloroform. Aliquots were made up to 0.3 ml.

¹ Kindly supplied by British Drug Houses Ltd., London.

and 3 ml. of antimony trichloride reagent were added in the colorimeter with the 620 m μ filter in place. The readings were noted and the calibration table constructed (Table 3).

Table 1. *Calibration table for pure β -carotene*

Vol. of aliquot in ml.	Galvanometer reading mm.	Filter 440 m μ		
		Log I_0/I	Total μ g. in solution	Log I_0/I per μ g.
0.05	92.5	0.0339	0.70	0.0487
0.1	85.5	0.0680	1.40	0.0485
0.2	74	0.1308	2.79	0.0468
0.3	64.5	0.1904	4.17	0.0454
0.4	57.5	0.2403	5.58	0.0431
0.5	51	0.2924	6.98	0.0419
0.6	45	0.3468	8.37	0.0414
0.7	41	0.3872	9.77	0.0397
0.8	38	0.4202	11.16	0.0377
0.9	35.5	0.4498	12.56	0.0367
1.0	33	0.4815	13.95	0.0345

Table 2. *Calibration table for vitamin A 2-naphthoate*

Vol. of aliquot in ml.	Galvanometer reading mm.	Filter 620 m μ		
		Log I_0/I	Total inter- national units in solution	Log I_0/I per international unit
0.025	87	0.0648	2.20	0.0295
0.05	75.5	0.1264	4.40	0.0292
0.075	65	0.1914	6.61	0.0290
0.10	56	0.2561	8.81	0.0290
0.125	49	0.3141	11.01	0.0285
0.15	44	0.3608	13.21	0.0274
0.2	35.5	0.4553	17.62	0.0260
0.25	29.5	0.5360	22.02	0.0244
0.3	24.5	0.6151	26.43	0.0234

Vitamin A 2-naphthoate contains 2,225,000 international units vitamin A per g. [Mead *et al.* 1939].

Table 3. *Correction curve for blue colour produced by β -carotene*

Vol. of aliquot in ml.	Galvanometer reading mm.	Filter 620 m μ		
		Log I_0/I	Total μ g. in solution	Log I_0/I per μ g.
0.025	98	0.0079	0.92	0.00873
0.05	96.25	0.0168	1.84	0.00920
0.075	94.5	0.0246	2.76	0.00894
0.10	93.5	0.0339	3.68	0.00920
0.125	91.0	0.0410	4.60	0.00893
0.15	89	0.0504	5.52	0.00913
0.20	86	0.0655	7.36	0.00892
0.25	82.5	0.0835	9.20	0.00908
0.30	79.5	0.0996	11.04	0.00903

Comparison of methods for extraction from blood

Multiple samples of serum or plasma were extracted by each of the following four methods:

1. Preliminary saponification with alcoholic KOH on a boiling water bath followed by extraction with ether ('alkali digestion method') [De Haas & Meulemans, 1938; Lanzing, 1938; Van Eekelen & Emmerie, 1934]. (For details see Lindquist [1938].)

2. Preliminary saponification with alcoholic KOH on a boiling water bath followed by extraction with light petroleum [Pett & Le Page, 1940].

3. Extraction three times with ether after precipitation with alcohol; no saponification.

4. Extraction by light petroleum, after precipitation with alcohol; no saponification [Kimble, 1939; May *et al.* 1940; Clausen & McCoord, 1938]. (For details see later.)

The results are given in Table 4.

Table 4

Extraction of serum or plasma by different methods; results in $\mu\text{g.}$ per 100 ml. for carotene and international units per 100 ml. for vitamin A.

Subject	Date	Saponification followed by				Simple extraction with			
		Extraction with ether		Extraction with light petroleum		Ether after precipitation with alcohol		Light petroleum after precipitation with alcohol	
		Carotene	Vitamin A	Carotene	Vitamin A	Carotene	Vitamin A	Carotene	Vitamin A
V. K. C.	9. i. 41	149	99	108	78	149	102	153	102
A. C.	11. i. 41	135	115	71	50	135	115	135	139
S. L.	22. i. 41	102	157	73	144	102	157	102	157
I. L.	3. ii. 41	118	180	76	132	118	152	116	169
J. D.	11. ii. 41	154	129	140	121	140	154	154	155
M. S.	14. ii. 41	151	150	106	152	113	161	140	161
J. S.	21. ii. 41	116	150	65	130	140	145	121	134
A. B.	24. ii. 41	124	140	40	84	94	140	134	138
B. C.	5. iii. 41	104	125	67	98	134	122	134	130
B. W.	5. iii. 41	50	96	27	90	50	118	66	128
C. D.	21. iii. 41	50	96	34	50	50	115	59	101
Pooled serum	10. ii. 41	170	381	76	234	170	370	160	375

It can be seen from Table 4 that saponification followed by extraction with light petroleum gives consistently lower results than any other method. (Incidentally I could not confirm that saponification for a longer time than 5 min. (see Pett & Le Page [1940]) had any effect on the final result.) Simple extraction either with ether or petroleum after precipitation gives results as good as or better than those obtainable by Lindquist's more complicated method. As the simple light petroleum extraction is the easiest of all it is used for the estimation in normal sera.

Procedure for estimation in blood

3 ml. of serum or plasma are put into a narrow-necked tube. 3 ml. of 95 % alcohol are added slowly with shaking, followed by 6 ml. of pure light petroleum (B.P. 40–60°) and the tube tightly corked. It is then shaken by hand for 10 min. The tube is centrifuged for 1 min. at low speed to separate the layers. The light petroleum layer is removed into another tube and a 5 ml. aliquot taken. This is evaporated in a gentle stream of carbon dioxide or nitrogen in a water bath at 30–40° and then at 70° for a few seconds. The residue is taken up in light petroleum and made up to 3.3 ml. in the standard cell. The deflexion of the galvanometer from the 'centre position' with the 440 μm filter in place is noted and the result expressed in $\mu\text{g.}$ of carotene per 100 ml. of serum. The solution is evaporated to dryness under carbon dioxide or nitrogen and the residue dissolved in chloroform and made up to the 0.3 ml. mark. The 620 μm filter is put in and the light altered to the 'centre position'; the cell is put in and 3 ml. of antimony trichloride reagent added all at once. The reading at the point of temporary stability is noted and after correction for the amount of blue colour produced

by the carotene, the vitamin A is expressed in international units per 100 ml. of serum.

Notes. 1. The 'centre position' is found as follows. The cell containing pure petroleum up to 3.3 ml. is put in with the 440 $m\mu$ filter. The light is altered to give a reading of 100. The reading when the cell is removed is the 'centre position' for the 440 $m\mu$ filter. Similarly 0.3 ml. of chloroform and 3 ml. of antimony trichloride reagent are used with the 620 $m\mu$ filter.

2. The alcohol must be added slowly and the mixture shaken while adding. Also vigorous shaking by hand must be continued for at least 10 min. Estimations of the extract after different periods of shaking show that with the alcohol added slowly almost complete extraction occurs after 5 min., but when it is added all at once a tough precipitate forms which does not yield the maximum readings until shaking for 20 min. has been carried out. Mechanical shaking on a machine also gives low readings. Evaporation of the extract with air destroys some of the vitamin A but leaves the carotene unharmed.

3. The yellow pigment measured in serum extracts is not all carotene. The proportion of the different pigments, however, is not easy to discover and it varies from serum to serum. The correction for carotene is applied on the assumption that all the yellow colour is due to carotene. Even if only half is carotene the difference would usually be only about 10 or 15 units of vitamin A.

To check the efficiency of the method several further tests were made.

1. The residue after removal of the light petroleum was re-extracted with a further 6 ml. of light petroleum. The results were never higher than 5 $\mu g.$ of carotene and 6 units of vitamin A per 100 ml. serum.

2. Duplicate samples never gave a difference of more than 2 in the galvanometer readings. This corresponds to an error of about $\pm 3\%$.

3. Extractions of different volumes of the same serum gave the same final results.

4. Slight degrees of haemolysis had no effect on the results.

5. Storage of the serum for times up to 48 hr. in a refrigerator at 2° had no effect and the results were the same as the estimations on fresh serum.

Table 5. *Normal values*

Subject	Date	Carotene in $\mu g.$ per 100 ml. serum or plasma	Vitamin A in international units per 100 ml. serum or plasma
1	12. vii. 40	151	132
2	22. vii. 40	150	112
3	27. vii. 40	220	157
4	27. vii. 40	182	136
5	27. vii. 40	241	122
6	29. vii. 40	153	135
7	29. vii. 40	161	100
8	15. viii. 40	117	112
9	12. viii. 40	117	136
10	28. viii. 40	130	107
11	6. ix. 40	117	84
12	20. ii. 41	90	110
13	20. ii. 41	104	102
14	19. iii. 41	118	151
15	19. iii. 41	58	94
16	19. iii. 41	98	103
17	19. iii. 41	67	120
18	20. iii. 41	63	100
19	20. iii. 41	82	72
20	20. iii. 41	90	121
21	25. iii. 41	94	97
22	25. iii. 41	104	86
23	25. iii. 41	50	103

The recovery from serum of added vitamin A and carotene is not admissible as evidence of accuracy as there is no relation between the form of vitamin A and carotene already present in the serum and that in which it is added.

There follows a series of values on normal healthy volunteers between 18 and 30 years of age (Table 5). They were all tested on the dark adaptation tester described by the author [Yudkin, 1941] and only those normal by this test were used.

The number of cases is too small to draw any definite conclusions though it is of interest to note that those estimated in February and March are in general lower than those done in the autumn, a possible seasonal effect. The range in general is similar to that of Lindquist [1938] and Kimble [1939].

SUMMARY

The methods of estimating vitamin A and carotene in human plasma are discussed. The reasons for using a photoelectric colorimeter for the measurement of the colours are given.

A parallel series of results obtained by the use of four different methods of extraction shows that the simple extraction from serum or plasma precipitated with alcohol is the most satisfactory.

A preliminary series of normal readings is given.

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