CXXIX. A NEW METHOD OF DETERMINING THE VITAMIN A CONTENT OF BLOOD.

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THE first observations relating to the amount of vitamin A in blood are due to Euler and Steffenburg [1928], who stated that the growth of rats kept on a diet devoid of vitamin A was enhanced by adding blood or serum to their ration. Euler also demonstrated that fat extracted from blood serum gives the well-known blue colour reaction with antimony trichloride. The method used for quantitative determination of vitamin A in blood has been published by Rösio [1929] and consists in a multiple extraction with ether of 25–100 ml. of blood serum, evaporation *in vacuo* of the combined ethereal extracts after drying with sodium sulphate and treatment of the residue with antimony trichloride as described by Carr and Price.

This simple procedure however could not yield accurate results, since not only vitamin A but also carotene is present in variable quantities in blood serum; moreover carotene is extracted by ether, and gives a blue colour with antimony trichloride. Though the growth-stimulating action of carotene upon rats kept on a diet devoid of vitamin A is similar to that of vitamin A, and though in the study of certain questions it may be permissible to determine the quantities of vitamin A and carotene together, it would be fallacious to attribute the results obtained with this method to vitamin A alone, especially since the blue colours produced by allied compounds are of different intensities.

Another method devised by van Eekelen [1931] allows for the presence of carotene by subtraction from the total intensity of the blue colour of that part which is due to carotene. The method of van Eekelen, published later by Menken [1932] and utilised also by Wendt [1935], consists in precipitating 20 ml. of serum with 100 ml. of 96 % alcohol and centrifuging. The supernatant alcoholic fraction is extracted with light petroleum. The slightly yellowish extract is then concentrated *in vacuo* to 1 ml. and utilised for the determination of carotene and later of vitamin A. According to van Eekelen the yellow tinge of this petroleum extract is due solely to carotene, the concentration of which can be determined by colorimetric measurement. The difference between the total blue colour obtained by treatment of the concentrated extract according to Carr and Price and the colour accounted for by the colorimetrically determined carotene is taken to represent vitamin A.

This method of determination seems to be objectionable from several points of view. When determining the carotene content of the blood, Connor [1928] precipitates the serum with 95 % alcohol. After centrifuging, the yellowish alcoholic fraction is discarded on account of its xanthophyll content, whilst the precipitate is extracted with benzene and the carotene content of the serum is determined by colorimetric measurement of this latter extract. Thus the carotene content of the alcoholic fraction and the blue value of carotene to be subtracted finally from the colour of the Carr-Price reaction will vary according to the method of precipitation, the shaking of the tube containing the precipitate and the speed of centrifuging. It must also be remembered that all carotenoids,

including vitamin A, are easily adsorbed by different media and it is well-nigh impossible to estimate the amount adsorbed by the protein precipitate. Nor is the yellow colour of the petroleum extract attributable solely to carotene, so that the conversion of the yellow colour into blue units is somewhat uncertain; moreover the reading of the final blue colour must be accomplished within 30 seconds, because of its quick fading. This urgency of reading entails personal error apart from the practical uncertainty of the method, the results of which therefore give at best indirect, and probably often incorrect, information.

These difficulties are overcome by the new colour test of Rosenthal and Erdélyi [1934, 1], which is specific for vitamin A and in which the colour is much more stable, rendering hurried reading unnecessary. The new colour reaction consists in warming the vitamin-containing material with a chloroform solution of catechol or guaiacol [Rosenthal and Erdélyi, 1934, 2] and antimony trichloride, when the initial blue colour of the solution soon turns into a purplish red. This hue remains unchanged for 5 to 10 minutes in the case of catechol and for periods of hours in that of guaiacol, depending on the purity of the material tested. The intensity of the colour obtained is proportional to the concentration of vitamin A and can be measured by means of colorimetric estimation, the colour being identical in tint with that of a dilute solution of potassium permanganate. Since the colour of the permanganate solution changes within a couple of days and the extinction of this changed standard solution does not correspond to that of the coloured solution obtained in the reaction, colorimetric comparison is possible only by constantly adjusting the tube containing potassium permanganate solution to the same point of the scale (10). This difficulty can be overcome by the use of the "absolute colorimeter" of Leitz, which permits colorimetric estimation against a special "grey solution" in monochromatic light, at the wave-length of the maximum absorption of the new test. Concerning the details of the colorimetric estimation we refer to Rosenthal and Erdélyi [1934, 1, 2]; here it may suffice to mention that this new method makes it possible to express the vitamin A content in "grey units."

This new method has been employed for direct determination of vitamin A in blood. At first we proceeded by extracting the serum or plasma with light petroleum. This extract, containing both carotene and vitamin A, was shaken five times in a separating funnel with 86 % methyl alcohol, after which treatment carotene had passed into the top layer (petroleum) whilst vitamin A was contained in the bottom layer (methyl alcohol). The collected and united alcoholic extracts were then diluted with an equal part of distilled water and extracted again with light petroleum, which this time removed the vitamin A. The petroleum extracts were washed with water, dried and evaporated at a low temperature *in vacuo*, and the residues were dissolved in absolute chloroform and used for determination of carotene and vitamin A, respectively.

Later the method has been simplified by the observation that the blue colour given by carotene with antimony trichloride disappears on warming with guaiacol; small amounts of carotene, such as occur in blood, do not therefore interfere with the direct determination of vitamin A.

The final method for determination of vitamin A is thus as follows. The carotene content of the blood to be taken should not exceed the threshold value mentioned (5 γ in 1 ml. extract). 10–30 ml. of blood are extracted four times in a separating funnel with 50 ml. of light petroleum (B.P. < 55°). The greater the quantity and the lower the specific gravity of the petroleum, the more easily can emulsification be avoided. Should emulsification nevertheless occur, the fluid may be treated with a few ml. of petroleum and centrifuged at low speed for 3–4 minutes.

Addition of one drop of 96 % alcohol to the emulsion may also be of assistance. If alcohol has been employed, the petroleum extract must be thoroughly washed twice with distilled water. The collected petroleum extracts are dried over a minimum amount (0.01–0.02 g.) of anhydrous sodium sulphate for 2 hours, repeatedly filtered through dry filters and evaporated *in vacuo* at 40°. In order to remove the last traces of water the residue is dried once more for 20–30 minutes *in vacuo*.

The residue is tested as follows. Since such small quantities of vitamin may produce a very faint colour the final volume of the solution is reduced to 1 ml. (instead of 5 ml.) and the determination is made with the micro-cell of the Leitz absolute colorimeter. To the dry residue of the extract 0.25 ml. of absolute chloroform, 0.25 ml. of 5 % guaiacol in absolute chloroform and 0.5 ml. of a saturated solution of SbCl_3 are added. The mixture is put into a water-bath at 55° for 1½ minutes exactly. During this time the initial blue colour changes into purplish red. Both temperature and duration of warming should be adhered to exactly in order to keep volatilisation of chloroform within constant limits.

It would be most desirable to use a solution of pure vitamin A of known concentration as a standard. As we had no such preparation at our disposal, we had to express the quantity of vitamin A in another way.

A very simple method of comparison was given by the close similarity of the final colour of our reaction to that of 0.001 % potassium permanganate solution. Reading can take place in white or in monochromatic light corresponding to the maximum absorption of the colour in question. In this latter case we used light filter No. 7 (greenish yellow) of the Leitz absolute colorimeter.

The following data will show the results of several determinations (reduced to 1 ml. of serum):

Blood samples	Colorimeter readings with permanganate	The same values expressed in "grey units", calculated to 100 ml. of serum
1	2.71	5.4
2	3.84	7.6
3	2.85	5.5
4	4.05	8.3
5	3.12	6.2

In another method of colorimetric examination we used the "grey solution". In this case we fixed the cell containing 1 ml. of "grey solution" at 10 mm. and varied the layer of the solution to be tested. From the reading of the unknown and from the volume of the serum tested, the value of "grey units" corresponding to the vitamin A content of 1 ml. of serum may be calculated. The following equation serves for conversion into "grey units":

$$\text{Grey units} = \frac{\text{Reading of standard}}{\text{Reading of unknown} \times \text{ml. of serum}}.$$

If we could work with pure vitamin A, the intensity of the colour produced would be proportional to the absolute amount of vitamin A.

It cannot be foreseen what results of clinical and pathological importance may arise from the systematic examination of vitamin A in the blood, but, at any rate, it seems that the method described will furnish interesting and reliable data.

SUMMARY.

1. The new method of determining vitamin A in blood makes it unnecessary to remove carotene, since this substance does not react in the guaiacol test.
2. The new reaction thus permits direct determination of vitamin A in blood.

3. A dilute solution of potassium permanganate or the "grey solution" of the Leitz absolute colorimeter may be used as standards for comparison.

4. Until it becomes possible to determine the absolute amount of vitamin A with the aid of this new colour test, its expression in "grey units" seems suitable for research purposes.

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