VII. A NEW COLOUR TEST FOR THE DETERMINATION OF VITAMIN A.

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SINCE the paper of Carr and Price [1926] much attention has been directed towards the antimony trichloride reaction of vitamin A. Opinions as to the value of this test are not uniform; it is, however, generally emphasised that this colour reaction is not dependable enough for exact determinations; at best it can serve as an orientation within certain limits. In the determination of the vitamin A content of cod-liver oil this colour test yields uncertain readings. The colour of the reaction is not stable; at first the solution is blue but it soon becomes violet and finally red. For measuring the colour intensity of the Carr-Price reaction the Lovibond tintometer is generally adopted. Owing to the rapid colour change it is expedient to accomplish readings within a very short time—10 to 30 seconds. Moreover, the test is not specific for vitamin A, as other carotenoid substances show the same behaviour.

Owing to the drawbacks mentioned, several new tests have been proposed, none of which, however, is satisfactory.

We attempted therefore to devise a reaction characteristic of vitamin A and allowing its exact determination in cod-liver oil. We succeeded in finding an adequate test by a simple modification of the Carr-Price reaction: if the oil containing vitamin A is heated together with catechol and antimony trichloride, both dissolved in chloroform, the blue colour first produced soon turns into a violet-red. The following reagents are needed for the test:

1. Alcohol-free, absolute chloroform (lower alcohols and water impede the reaction).

2. 0.5 % solution of catechol in chloroform.

3. A cold saturated solution of antimony trichloride in chloroform.

The specimen of oil to be tested is first diluted with chloroform. To 1–2 ml. of this solution 1 ml. of the catechol solution and 2–3 ml. of the antimony trichloride solution are added. The test-tube containing the mixture is immediately transferred to a water-bath at 60° for 1–2 minutes. The blue colour first produced changes during this heating into an intense violet-red hue. In the cold this colour is not produced, as the change then takes the same course as without catechol. Subsequent heating of the solution which has undergone the change in the cold does not lead to the production of the violet-red colour; therefore heating must take place immediately after the reagents have been mixed.

Spectroscopic examination of the red fluid shows characteristic absorption bands. Four bands have been observed, two of which are pronounced enough to be measured with the diffraction spectroscope.

1. A broad band at the boundary of green and yellow, having indistinct limits; peak intensity at 552 $m\mu$.

2. A narrower, distinct band between green and blue; peak intensity at $476\text{ m}\mu$.

Besides those mentioned, two more dim bands were observed, one in the blue and the other in the violet.

Solutions of vitamin A irradiated and thus inactivated with ultraviolet light do not give the reaction described; the solution becomes pink at once, and changes into brownish red with a bluish tinge.

Irradiation by means of X-rays has a similar effect: the solution first becomes red, then reddish brown.

As mentioned, the reaction of Carr and Price is given not solely by vitamin A, but also by other carotenoid bodies. By the courtesy of Professor Zechmeister, to whom our thanks are due, we have been able to examine carotene, lycopin, zeaxanthin, capsanthin and physalin. None of these carotenoids gave our reaction; they gave the original blue colour of the Carr-Price test and no change of colour could be observed on heating. We were unable to purchase other carotenoids, which will be studied later.

Thus our reaction makes it possible to differentiate vitamin A from carotene. Moreover, vitamin A can be demonstrated simultaneously with carotene; the red of vitamin A and the blue of carotene blend into a vivid violet.

The same reaction carried out with ergosterol shows the colour change in an inverse succession, the solution first becoming coloured pink which changes slowly into blue. No catechol is needed for this reaction; the colour change is, however, greatly hastened by its presence. Heating does not enhance it, moreover this is disadvantageous; when heated, the solution becomes green, but on cooling the blue colour reappears.

With irradiated ergosterol (vitamin D, vigantol oil) no colour reaction is observed.

The blue colour produced with ergosterol shows an absorption band between yellow and green, having a peak intensity at $595\text{ m}\mu$; another band beginning at about $610\text{ m}\mu$ and showing an increasing intensity towards the red can also be observed.

The catechol reaction of vitamin A is also suitable for quantitative determinations. Under certain conditions the intensity of the violet-red colour is proportional to the concentration of vitamin A and can be measured colorimetrically. A 0.01 % solution of potassium permanganate is used for comparison. A specimen of oil containing vitamin A is dissolved in chloroform, yielding a solution of a known concentration. 1 ml. of this solution is employed, or a smaller quantity is diluted with chloroform exactly to 1 ml. Now 1 ml. of the catechol solution and 3 ml. of the antimony trichloride solution are added and the test-tube is put into the water-bath at 60° . After the solution has become violet-red, it is compared in the colorimeter with the standard solution of potassium permanganate.

The correct performance of this reaction depends on the following conditions.

1. The duration of heating. Too short heating produces a red hue with a bluish tinge, whereas prolonged heating yields a brick-red solution.

2. The concentration of the catechol solution. The colour is different if the solution contains more or less than 0.5 % of catechol. More dilute solutions tend to produce a bluish red tinge, higher concentrations a brown one. Catechol solutions should always be freshly prepared, as solutions older than 24 hours are liable to give bluish tinges.

Of cod-liver oil from 0.1 to 0.5 ml. should be taken. When heated the violet-red colour appears in as short a time as 10 to 15 seconds. The reading

should be accomplished as quickly as possible, for within a short time the colour fades into brown. This change is probably caused by resinification of the oil; not more than 0.5 ml. of oil should therefore be used for the test.

Of vitamin A concentrates we examined two brands, called "Essogen" and "Vogan," respectively. Chloroform dilutions were prepared, which had to be heated for 1 to 2 minutes. As these solutions were examined in high dilutions, their oil contents were very low and consequently their colours lasted much longer, averaging 5 to 10 minutes and even more.

The following are the results of several determinations:

1. Cod-liver oil. (The comparator cell of the Duboscq colorimeter stood at the mark 10.)

0.1 ml. of oil	reading	18.0
0.2 "	"	8.9
0.3 "	"	6.2
0.4 "	"	4.6
0.5 "	"	3.6

2. 0.5 ml. of "Vogan" diluted to 10 ml.

0.1 ml. of solution	reading	10.0
0.2 "	"	5.0
0.3 "	"	3.2
0.4 "	"	2.4
0.5 "	"	2.0

3. 0.1 ml. of "Essogen" diluted to 10 ml.

0.1 ml. of solution	reading	30.0
0.2 "	"	15.2
0.3 "	"	10.0
0.4 "	"	7.3
0.5 "	"	6.0

The values above indicate that with an increase of the quantity of vitamin A the readings proportionally decrease, thus the colour intensity is a linear function of the concentration. Comparison of these values showed that "Vogan" contained 36 times, "Essogen" 60 times as much vitamin A as the cod-liver oil examined.

In another experiment we measured the absorption of our solutions photo-electrically. We employed a cell of 2.9 mm. in diameter. The maximum intensity of the photoelectric current amounted to $83.5 \cdot 10^{-7}$ amperes. 0.1 to 1.0 ml. of a 1 : 20 solution of Vogan was used. The results are shown in the following table:

ml. of solution	Current intensity 10^{-7} amp.	Absorption $83.5/x$
0.1	47.5	1.8
0.2	39.5	2.1
0.3	28.5	2.9
0.4	22.5	3.7
0.5	18.0	4.6
0.6	13.5	6.2
0.7	10.5	7.9
0.8	7.0	11.8
0.9	5.5	15.2
1.0	2.5	33.4

Spectrophotometric examination of the colouring matter is being carried out. The relation of these data to the biological potency of the preparations examined is reserved for further study.

SUMMARY.

The reaction with catechol as described is an advance in two respects.

1. It makes possible differentiation of vitamin A from carotenoid substances.
2. The violet-red colour, similar to that of a dilute solution of potassium permanganate, is more stable than the blue colour obtained according to the Carr and Price method.

REFERENCE.

Carr and Price (1926). *Biochem. J.* **20**, 297.