LXIV. COLOUR REACTIONS ATTRIBUTED TO VITAMIN A.

By FRANCIS HOWARD CARR and ERNEST ARTHUR PRICE.

From the Laboratories of The British Drug Houses, Ltd., Graham Street, City Road, N. 1.

(*Received March 17th, 1926.*)

Having regard to the wide variation in the vitamin A activity of different cod-liver oils, it is a matter of practical importance to find the most ready means of assessing their relative activity. The long period of time occupied in carrying out the biological test renders this method not only tedious and expensive, but impractical from the point of view of industrial establishments requiring to accept or reject oils under conditions allowing at most one or two weeks for a decision.

There is already good evidence that the well-known colour tests described by various workers, notably by Rosenheim and Drummond, may be expected ultimately to form a basis for a satisfactory chemical method of determining the vitamin value with great rapidity and approximate accuracy. All oils received in our laboratories for some time past have been submitted to these tests, in the first instance to the sulphuric acid test, and later to the trichloroacetic acid and arsenic trichloride tests of Rosenheim and Drummond. Biological tests have been applied to some of these oils.

We were naturally led, therefore, to make experiments with a view to improving the technique of the colour tests in order to find conditions whereby quantitative and strictly comparable readings may be obtained. This work has resulted in our utilising for the purpose yet another reagent of a similar character, namely, a solution of antimony trichloride in chloroform. By its use we have been enabled to determine standard conditions whereby direct readings of the intensity of the colour may be taken with the help of a tintometer. By following the conditions laid down observations made by different workers at different times show only unimportant differences.

While we do not claim to give proof that the colour observed is a direct measure of vitamin activity, so far as our results go they do afford strong support for this view, which was first put forward by Rosenheim and Drummond. Before such relationship can be proved a large number of oils must be tested by both the biological method and the colour test. We are only able to record the comparison of the methods on two samples, which Dr S. W. F. Underhill has submitted to biological test on rats, following the technique of Drummond and Watson [1922].

The present communication is made prematurely in the hope that it will induce other workers to join in the effort to prove or disprove the parallelism between the colour tests and vitamin A activity.

The principles we have had before us throughout the work are:

(1) that the test should be quantitative, that is to say:

(*a*) the quantity of oil taken must be accurately measured;

(*b*) the amount of reagent employed must be measured;

(*c*) the colour must be measured by comparison with a standard:

(2) that the colour produced should last for a sufficient length of time to enable an accurate observation to be made.

Judged from these points of view, arsenic trichloride is a fairly satisfactory agent, excepting that it cannot be diluted with chloroform or with any solvent we have been able to find, without a marked loss of sensitiveness. The great advantage of using a solvent is that the oil to be tested may be dissolved in it and delivered from a burette, and that the reagent may be similarly treated. The handling of liquid arsenic trichloride is, moreover, objectionable for reasons which will be obvious to all.

At first, the trichloroacetic acid test promised good results, but having occasion to change our laboratory stock of it for a supply of freshly manufactured material, we were puzzled to find that only the faintest colour was produced. Further enquiry showed that the colour-producing agent was present in the impure fractions of trichloroacetic acid and entirely absent from trichloroacetic acid which had been carefully fractionated and then recrystallised three times from benzene and three times from light petroleum. It is interesting to note that minute additions of phosgene and of dimethyl sulphate will render this pure trichloroacetic acid an effective reagent. We also observed that trichloroacetic acid is decomposed by heat into HCl, CO and $COCl_2$, thus accounting for the presence of $COCl_2$ in commercial samples of the acid; $CCl_3.COOH = COCl_2 + CO + HCl$. The colour given with impure trichloroacetic acid, however, rapidly changes, and we were therefore led to examine a number of other reagents, including those which have already been reported upon by other workers. The new ones of chief interest are the following.

(1) *Antimony trichloride in chloroform* gives a brilliant blue not undergoing perceptible change for three minutes.

(2) *Stannic chloride in chloroform* gives a deep blue colour rapidly changing to purple.

(3) *Anhydrous ferric chloride*, added to a solution of oil in chloroform, produces a fluorescent reddish violet colour. Owing to the difficulty of keeping it absolutely anhydrous, this reagent does not give uniform results.

(4) *Anhydrous aluminium chloride*, added in the form of fine powder to the oil, produces a red-violet colour fading to brown.

If the oil be dissolved in chloroform containing phosgene or dry hydrochloric acid gas, and a minute trace of aluminium chloride added, a fine

purple colour is produced very like that given by trichloroacetic acid, and of about the same duration. Without the $COCl_2$ the colour is red with very little blue, and fades more quickly.

(5) *Silicon tetrachloride*, added to cod-liver oil, gives a rose-pink colour; the reaction is not very sensitive but is not given with cholesterol.

(6) *Phosphorus oxychloride* added to the oil produces a transient blue rapidly fading to red.

The colour produced in all these reactions consists in the main of blue with varying proportions of red. Rosenheim and Drummond [1925] and Takahashi *et al.* [1925] regard the blue as indicative of vitamin content, and are disposed to ignore the red and yellow colours. From preliminary experiments on oils of more or less known activity we confirmed this view, but nevertheless set out to determine all the colour.

Since the amount of red, yellow and blue to be measured varies with different oils we did not find it very practicable accurately to compare the colour with that of standard liquids, such as solutions of aniline dyes or of more permanent inorganic compounds, for instance, carbonato-tetrammine cobaltic nitrate and ammoniacal copper sulphate, nor can we for the same reasons readily use a control cod-liver oil as a standard. We therefore employed a Lovibond tintometer in which the comparison is made with standard glasses, by which the colours may be exactly matched and numerically recorded. Of the reagents mentioned we found a 30 % solution of antimony trichloride in chloroform (weight in volume), decidedly the most suitable and convenient. Its advantages are:

(1) the oil and solvent do not need to be perfectly dry or entirely free from alcohol. (In many of the tests either water or alcohol interferes with the colour.)

(2) the colour is an intense blue, and slightly more intense and more permanent than that produced by arsenic trichloride. It is very much better in this respect than the colour produced by any other reagent we have tried.

(3) it may be depended on to produce the same intensity of colour with the same oil on every occasion.

(4) as compared with arsenic trichloride the reagent is innocuous; it is, however, somewhat corrosive to the skin.

The following are the conditions we have adopted.

The chloroform employed for dissolving the oil in the antimony trichloride is ordinary B.P. chloroform, that is, it contains 2 % of alcohol. Antimony trichloride is washed with a little chloroform, dried and weighed, then dissolved in chloroform to make a 30 % solution. It is allowed to stand and the clear solution decanted and used from a burette. The oil is dissolved in chloroform (we make a 20 % solution), and to 0·2 cc. of this solution, delivered from a 1 cc. burette, 2 cc. of the antimony trichloride solution are added. The liquid is at once transferred to a cell and the colour intensity measured against standard glasses in a Lovibond tintometer.

The following are the results obtained with four different oils, viewed through a depth of 8 mm.:

	Blue	Yellow
No. (1)	9·5	1·1
	9·5	1·1
	9·5	1·0
	9·5	1·1
No. (2)	3·3	0·6
	3·1	0·8
	2·9	0·6
	3·0	0·7
	3·0	0·6
No. (3)	4·9	0·7
	5·0	0·4
	5·0	0·8
No. (4)	3·7	0·9
	3·7	0·9
	3·7	0·9

Comparing the intensity of blue, the ratios of activity are:

Nos. (1) and (2): 1 to 3·3
Nos. (1) and (3): 1 to 2·0
Nos. (1) and (4): 1 to 2·6

Taking the ratio of yellow the figures are:

Nos. (1) and (2): 1 to 1·7
Nos. (1) and (3): 1 to 1·7
Nos. (1) and (4): 1 to 1·2

It should be observed that the amount of yellow rapidly increases and therefore must be quickly read.

The oils (1) and (2) have been tested biologically by Dr S. W. F. Underhill, who reports that he finds that No. (1) is at least three, possibly four or five, times as active as No. (2). It will be seen that the ratio of blue observed by the antimony chloride colour test, namely 3·3, is in close approximation to the result obtained by Dr Underhill, while the ratio of yellow, namely 1·7, is much less. Dr Underhill's report on the biological test of these oils is appended.

NOTE ON THE BIOLOGICAL TEST OF OILS (1) AND (2).

By SYDNEY WILLIAM FRANK UNDERHILL.

The technique adopted was that of Drummond and Watson; the oils were fed daily to the animals by means of graduated pipettes.

The results obtained are given in the following table:

	Oil	Daily dose (mg.)	Weeks of experiment	Growth in g. per week	
				Males	Females
Exp. No. 1	No. (1)	0·9	6	4·1	4·3
	,,	2·25	6	4·8	4·6
	,,	9·0	6	9·2	(3·9)
Exp. No. 2	No. (2)	3·0	6	2·0	3·5
	,,	6·5	5	7·6	7·2
Exp. No. 3	,,	45·0	4	13·4	11·5

Comparing equal doses of the two oils, it will be seen that 2·25 mg. of No. (1) gave a rate of growth which is two to three times greater than the rate given by 3·0 mg. of No. (2) oil in the case of the males, although the ratio between them is less in the case of the females. If an attempt be made to determine the respective doses which give equal rates of growth, it will be observed that 0·9 mg. of No. (1) corresponds to an amount of No. (2) which lies between 3·0 mg. and 6·5 mg.; that is, the former is at least three, possibly four or five times as active as the latter.

In view of the fact that deficiency of the anti-rachitic vitamin may be a limiting factor in growth experiments [Drummond, Coward and Handy, 1925], it is hoped to repeat some of these observations with the modified technique recently described by these authors. It may be mentioned, however, that estimation of the calcium content of the bones of the experimental animals has shown the presence of the anti-rachitic factor in the oils fed. Scrutiny of the growth curves gives no indication that the growth observed was due to "activation" of vitamin A present in the body under the influence of the anti-rachitic factor; moreover, in Exps. 1 and 2 certain of the animals (not included in the table) were given daily doses of irradiated oils [Drummond, Rosenheim and Coward, 1925]; no extra growth occurred in response to this supplement.

The above experiments were not commenced in the first instance with a view to comparing the oils, and it is realised that a more accurate estimate of their relative strengths would be obtained by making the comparison on similar groups of rats under the same conditions. Experiments with this object have been begun, but the results are not yet available.

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