CIX. FEARON'S "PYROGALLOL" TEST AS A POSSIBLE BASIS FOR THE ESTIMATION OF VITAMIN A.

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WITH a view to finding a satisfactory basis for the quantitative trial of a colour test for vitamin A against parallel feeding experiments we have examined both the arsenic trichloride test [Rosenheim and Drummond, 1925] and Fearon's pyrogallol test [1925]. Having regard especially to the fact that in these tests the rôles of arsenic trichloride and trichloroacetic acid (not specially purified [Carr and Price, 1926]) are interchangeable we have come to the opinion that the two tests are not altogether independent. Since, *ceteris paribus*, the pyrogallol test is to be preferred to the arsenic trichloride test both on account of the permanence of its colour and of the safety of its reagents we have made it the subject of a detailed survey.

The Fearon test.

As described by its author this test consists in the treatment of a few drops of the given oil with crystals of pyrogallol and 5 cc. of a 12 % solution of trichloroacetic acid in light petroleum. A permanent pink colour is produced on boiling, on standing or on the addition of benzoyl peroxide, the depth of which was suggested as the basis of a colorimetric estimation.

Seeking to improve the test we employed wide ranges of phenols and condensing agents, but in the end only altered the phenol, finding resorcinol to produce a better pink than did pyrogallol. However, on applying the test, with this one modification, upon varying amounts of cod-liver oil results were obtained erratic both in depth and in shade. A further study of the test was necessary.

The effect of moisture.

Fearon has insisted that the solvent employed, whatever it be, must be rigorously dried. Our first care, therefore, was to examine the effect upon the colour of traces of water. When the latter was added in small quantity after the formation of the colour we found, instead of decoloration, a clear separation of the colour into the aqueous layer¹. Traces of water, therefore, did not seem objectionable except if causing cloudiness. But could such water prevent the formation of colour?

¹ This phenomenon was observed when the test was carried out as described by Fearon, using light petroleum as a solvent. Under other conditions decoloration did not always take place.

To answer this question a little oil was mixed with the trichloroacetic acid solution without the addition of resorcinol, the mixture at once being extracted with three drops of water. On testing with resorcinol the aqueous layer gave a deep carmine, the light petroleum layer only a faint pink. Water, therefore, does not prevent the formation of colour when sufficient acid is present. Its use, in fact, might provide a possible method for the isolation of the "vitamin" condensation products.

Both toluene and light petroleum, without special drying, proved efficient solvents, but owing to its lower volatility we adopted the former.

Oxidation and the effects of heating.

On standing in air, even after boiling, the colour developed both slowly and irregularly. Consequently benzoyl peroxide was used to hasten its formation. Too high concentrations of the latter, or heating even at low concentrations, caused a fading of the colour to brown, but by using 0.5 cc. of a saturated toluene solution of the peroxide in every test a regular colour development occurred in a reasonable time without any fading in the first few hours.

The stages of colour development deserve mention. On mixing cod-liver oil with the trichloroacetic acid solution there is a slight deepening in tint. On prolonged boiling a further deepening to sage green occurs, and a pink is no longer given with resorcinol. On the other hand, if resorcinol is added immediately on mixing the oil with the acid a faint pea-green usually results, which changes readily into the normal shade of pink.

Variations with resorcinol concentration.

In Fearon's technique the colouring agent, being almost insoluble in light petroleum, is added in the form of crystals. This, of course, ensures its ultimate supply in adequate quantity, but in solutions left to stand overnight we often noticed that such crystals had become more coloured than the bulk of the solution, pointing to imperfect colour development. As an improvement, therefore, we added our resorcinol in ethereal solution, and by so doing were able to make a quantitative study of the effect of this factor.

Table I.

Colours formed by eight drops of cod-liver oil treated with 5 cc. of 10 % trichloroacetic acid in toluene, 0.5 cc. of saturated benzoyl peroxide in toluene and one drop of ethereal resorcinol at each of the concentrations shown.

Time	Resorcinol concentration					
	0.25 %	0.5 %	1 %	2 %	4 %	8 %
Before adding peroxide	Faint pink	Faint pink	Faint green	Faint green	Faint green	Faint green
20 minutes later	Deep purple	Deep purple	Pink	Pink	Pale pink	Pale yellowish pink
2 hours later	Fading purple	Fading purple	Deep purple	Deep reddish purple	Carmine	Carmine

These results are of importance, especially since the colour change from purple to carmine was not merely one of depth, but a sharp transition from blue to red. Moreover, although without resorcinol no colour other than brown was obtained, the colour produced with but little resorcinol present was both denser and more rapid in formation than at higher concentrations. An adjustment of resorcinol concentration, therefore, was obviously essential to the proper working of the test.

The point at which the break occurred may be calculated at about 0.5 % of resorcinol on the amount of oil taken, and with pyrogallol a very similar phenomenon was observed with a break at about the same point. As an explanation of the formation of two distinct colours we might suggest, purely as an hypothesis, that while below 0.5 % two "vitamin" molecules combine per resorcinol molecule, above 0.5 % only one such molecule combines.

Quantitative technique.

Since the rapidity of colour formation varies in an inverse manner with the resorcinol concentration, it was necessary to employ such a concentration of the latter as to cause a full carmine coloration in a reasonable time without the formation of purple. The test was therefore carried out with resorcinol present to the extent of 0.5 % of the oil taken. The oil was first dropped in varying quantities into dry test-tubes. The appropriate quantity of ethereal resorcinol was added, and then, with thorough vertical shaking, 5 cc. of 10 % trichloroacetic acid in toluene and 0.5 cc. of benzoyl peroxide in the same solvent. After a period of 2 hours the colours were compared against a solution of magenta toned with methylene blue. Results are appended.

Table II.

	Drops of cod-liver oil			
	2	4	6	8
	Colour densities			
1st Exp. (T. M.)	5.6	9.8	15.2	19.0
2nd Exp. (T. M.)	5.6	9.8	15.7	21.7
(S. G. W.)	5.2	10.4	14.3	19.8
3rd Exp. (T. M.)	4.8	10.0	14.7	19.6
(S. G. W.)	4.5	9.4	14.2	18.4
Average of 2nd and 3rd Exps.	5.0	9.9	14.7	19.8

From these figures it will be seen that the colour developed was fairly accurately proportional to the amount of oil taken. However, in order to exclude the obvious criticism that the colour depths were determined not by the oil but by the resorcinol added, another experiment was carried out keeping the latter constant at one drop of 8 % solution in every case.

Table III.

Time (hours)	Drops of cod-liver oil			
	2	4	6	8
	Colour densities			
2	4.7	8.1	12.7	16.0
3	5.7	9.8	14.8	19.7
4	—	—	—	22.1
5	6.0	11.4	17.0	22.7
8	—	—	—	23.5 (brownish)

The linear agreement still holds good, but the colours are slow in reaching their maximum values. The best method of overcoming this difficulty is still to be decided, but in the meantime it is proposed to fix a definite time and temperature for colour development.

The specificity of the test.

In this work our primary purpose has been to place the test upon a quantitative basis, but from a qualitative standpoint we have also confirmed much of Fearon's evidence as to its specificity. Positive results were obtained with all cod-liver oils, with beef-suet, with butter-fat and with egg-yolk-fat; negative with olive oil, linseed oil, palm kernel oil and "ostelin" (a preparation rich in vitamin D). A sample of Newfoundland cod-liver oil was found to give a coloration about forty times as dense as that given by a sample of butter-fat.

SUMMARY.

- (1) Fearon's test for "vitamin A," suitably modified, is shown to be capable of quantitative application.
- (2) Unless the concentration of resorcinol (or pyrogallol) is suitably controlled a deep purple may develop in place of the usual carmine.
- (3) Under certain conditions it is possible to extract the "vitamin" condensation products with a little water.

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