CLXII. EFFECTS OF VARIOUS AGENTS ON COLOUR TESTS FOR VITAMIN A.

By STANLEY GORDON WILLIMOTT, THOMAS MOORE
AND FRANK WOKES.

*From the Biochemical Laboratory, Cambridge, and
the University of Liverpool.*

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IN a preliminary communication to the Biochemical Society [Moore and Willimott, 1926], as a result of further experience of Fearon's "pyrogallol" test, experimental evidence was brought forward to show that this is not specific for vitamin A. A more complete account of the grounds for this conclusion is now given.

Applied qualitatively, as found by Fearon [1925], and confirmed by Willimott and Moore [1926], the "pyrogallol" test appeared satisfactory. It was given by all cod-liver oils tested, by butter fat, beef suet, and by egg-yolk fat; it was not given by olive oil, linseed oil, palm kernel oil or by ostelin. The ratio obtained between a potent cod-liver oil and butter fat was reasonable (about 40), and varying the concentration of the oil caused corresponding alterations in the intensity of the colour produced. When, however, the "pyrogallol" test was applied to a series of cod-liver oils, two disconcerting facts emerged. First, the grading of the oils by the "pyrogallol" test did not agree with their reputed potencies. Secondly, oils when tested by our technique and by that of Carr and Price [1926] gave contradictory results. For example, on comparing a Newfoundland and a Norwegian oil, both of 1926 season, the "pyrogallol" test showed the Norwegian to be definitely more potent than the Newfoundland, whereas the antimony trichloride test of Carr and Price indicated that the Newfoundland oil was much more potent than the Norwegian. Rosenheim and Drummond [1925] have produced strong presumptive evidence for the specificity of the arsenic trichloride test. These facts which we had ascertained threw doubt on the belief that the Fearon test was essentially a modification of the arsenic trichloride and related tests. We had already shown that it was quite possible to carry out the tests with the reagents interchanged, using arsenic trichloride suitably diluted with toluene to obtain a pink colour, and using melted trichloroacetic acid to obtain a transient blue. Further experiments have shown the two tests to be quite distinct.

EXPERIMENTAL.

Treatment with sulphuric acid.

A sample of Newfoundland cod-liver oil was taken which had been found by previous feeding experiment to be a potent source of vitamin A. About 25 g. were put in a beaker, and concentrated sulphuric acid was gradually stirred in until no further purple colour developed, about 20 cc. being used. No precipitate was formed. An equal volume of water was added gradually, and after 10 minutes the mixture was transferred to a separating funnel, and the brown acid layer run off. The upper layer was washed many times with distilled water until freedom from acid was shown by the p_H of the washings being the same as that of the original oil (about 5.2). It was then extracted with ether, dried with anhydrous calcium chloride, and the ether distilled off under reduced pressure. The residue consisted of a clear oil only a trifle yellower than before treatment. This was tested with concentrated sulphuric acid, phosphorus pentoxide, arsenic trichloride, antimony trichloride in chloroform, and trichloroacetic acid with pyrogallol and with resorcinol. Negative results were obtained with all except Fearon's reagents, which gave as intense a carmine as before treatment. The Fearon's reagent was a 10 % solution in toluene of a sample of trichloroacetic acid of A.R. standard supplied by the British Drug Houses. This quite unexpected result was then further investigated with a view to confirmation.

It was found that a potent Norwegian cod-liver oil, of 1926 season, on treatment in the same manner with concentrated sulphuric acid gave the same results as had been obtained with the Newfoundland oil, so that the cause could not be attributed to the presence of some constituent peculiar to Newfoundland oil. Using fresh reagents, other samples of different origin again confirmed our findings. There still remained the possibility that the colours given by Fearon's test on cod-liver oils treated with concentrated sulphuric acid were due to sulphonated derivatives. To test this, olive oil was treated in exactly the same manner. No colour was obtained with Fearon's reagent either before or after treatment. The pink colour obtained with Fearon's reagent could not therefore be attributed to sulphonation.

The "sulphonated" oil was next saponified by heating for an hour with 10 % alcoholic potash on a water-bath, and the unsaponifiable matter was extracted with ether and evaporated. The residue gave negative results with Fearon's as well as with all other tests. The soaps obtained from the "sulphonated" oil were next tested, and gave negative results with all except Fearon's, which was faintly positive. Another experiment was performed in which the treated oil was thoroughly shaken with fuller's earth at 110°, but after this treatment a positive Fearon test was still obtained.

From these results a sharp differentiation was obtained. The "sulphonated" oil, according to Fearon's test, was still rich in vitamin A; according to all the other tests, it was devoid of that vitamin.

The "sulphonated" oil was now fed to two albino rats whose growth had been stopped by deficiency of vitamin A, vitamin D having been supplied by direct irradiation. 40 mg. daily doses were given. No growth response was shown, and the animals both developed severe xerophthalmia. Autopsies confirmed vitamin A deficiency. The same oil before treatment had promoted normal growth, and ensured complete protection from eye disease in doses of 16 mg.

Adsorption with phosphorus pentoxide.

Fearon found that when phosphorus pentoxide is mixed with cod-liver oil the sterols are removed by adsorption, and neither they nor the residual oil contain vitamin A, as shown both by feeding experiments and by colour tests. We therefore made use of this destructive action of phosphorus pentoxide on vitamin A in our further investigation of the colour tests.

20 g. of a potent Norwegian cod-liver oil were put in a glass mortar, and powdered phosphorus pentoxide was gradually ground into it. A deep purple colour slowly appeared after each addition of pentoxide, reaching its maximum in 7-10 minutes, and changing to brown on standing, a precipitate being thrown down. When about 10 g. had been added, the supernatant clear oil was tested. With concentrated sulphuric acid negative results were obtained; with phosphorus pentoxide, arsenic trichloride and antimony trichloride faintly positive; with trichloroacetic acid solution to which either resorcinol or pyrogallol had been added, strongly positive. As it appeared from these results that the vitamin had not been completely destroyed, the oil was treated with a further 10 g. of pentoxide, forming a thick paste. This was centrifuged and the supernatant clear oil tested. Negative results were obtained with concentrated sulphuric acid and phosphorus pentoxide; very faintly positive with arsenic trichloride and antimony trichloride; positive with Fearon's reagents.

The results of these experiments, which are summarised in Table I, prove the non-specificity of Fearon's "pyrogallol" test, and in addition show that the arsenic trichloride test of Rosenheim and Drummond and the antimony trichloride test of Carr and Price are more sensitive than the sulphuric acid and phosphorus pentoxide tests. We now sought for a method of vitamin destruction which could be more accurately controlled, and thus permit quantitative applications. Our first attempt was to destroy the vitamin by aeration at 100°. Unfortunately, we found that this produced a darkening in colour which prevented accurate measurement of the intensity given by any of the reactions. Another attempt in which cod-liver oil was heated in a thin film in the presence of air for many hours also proved unsuccessful.

Action of ultra-violet light.

Peacock [1926] has recently published results of an investigation in which cod-liver oil was exposed to the action of ultra-violet light, causing the destruction of vitamin A, as shown both by a negative arsenic trichloride test and by failure to protect from xerophthalmia or to produce growth in rats on a diet otherwise deficient in vitamin A. No details were given, however, as to the time of exposure or distance from the mercury vapour lamp. This method of destroying vitamin A seemed likely to obviate the difficulty of discoloration and, moreover, to permit strict quantitative control.

Table I. *Colour tests on cod-liver oil before and after treatment with agents which destroy vitamin A.*

				Conc. H_2SO_4	P_2O_5	AsCl_3	SbCl_3	CCl_3COOH + resorcinol	CCl_3COOH + pyrogallol
Untreated ...	...	...	...	++	++	++	++	++	++
"Sulphonated"—									
(a) Ether extract	...			—	—	—	—	+	+
(b) Ether extract (after shaking with fuller's earth)	...	...		—	—	—	—	+	+
(c) Ether extract (saponifiable fraction)	...			—	—	—	—	±	±
(d) Ether extract (unsaponifiable fraction)	...			—	—	—	—	—	—
"Depigmented" (with phosphorus pentoxide)—									
(a) Partially	...	...		—	±	+	+	++	++
(b) Completely	...	...		—	—	±	±	+	+

About 50 cc. of a potent Norwegian cod-liver oil were placed in a porcelain evaporating basin of such a size that the oil was not more than 1 cm. deep. It was then exposed at a distance of 7.5 cm. to the radiations from a Hewittic mercury vapour lamp known to be yielding large amounts of rays down to 200μ . The oil was frequently stirred with a thermometer. The temperature rose gradually to about 70° , at which it remained steady, and the natural odour of the oil was noticeably increased. No difference was observed in the colour. At intervals of 15 minutes 2 to 3 cc. of the oil were run off into dry bottles of non-actinic glass, which were kept away from the light and well stoppered. These samples of oil which had been irradiated for different periods of time under the same conditions were then tested for vitamin A with the different colour reactions. The results are recorded in Table II. It will be seen that concentrated sulphuric acid and phosphorus pentoxide both ceased to give positive tests after three-quarters of an hour's irradiation; arsenic trichloride and antimony trichloride did not become negative until after $1\frac{1}{2}$ hours; while Fearon's reaction persisted until the end of the experiment, when over $3\frac{1}{2}$ hours' exposure had been given. Peacock found that cod-liver oil when first exposed to ultra-violet light exhibits a fluorescence, which disappears gradually as the vitamin is destroyed. We have been unable to confirm this observation.

Table II. *Colour tests on cod-liver oil which has been exposed to ultra-violet light for different periods.*

Time exposed (minutes)	Temp. ° C.	Conc. H_2SO_4	P_2O_5	AsCl_3	SbCl_3	CCl_3COOH	CCl_3COOH
						+ resorcinol	+ pyrogallol
15	40	++	++	++	++	++	++
30	60	+	+	++	++	++	++
45	68	±	±	++	++	++	++
60	70	—	—	+	+	++	++
75	70	—	—	+	+	++	++
90	70	—	—	±	±	++	++
105	70	—	—	—	—	++	++
120	70	—	—	—	—	++	++
135	71	—	—	—	—	++	++
150	71	—	—	—	—	++	++
165	71	—	—	—	—	++	++
180	70	—	—	—	—	++	++
210	71	—	—	—	—	++	++

The experiment was repeated under the same conditions on another sample of the same oil, but withdrawing a few cc. every 5 minutes for the first hour, then every $\frac{1}{4}$ hour until negative arsenic and antimony trichloride tests were obtained. Exposure was continued until the end of $5\frac{1}{2}$ hours, when a positive Fearon test was still obtained.

The next day readings were taken in a Lovibond tintometer of the colour intensities produced on these irradiated oils by the arsenic trichloride and the antimony trichloride reagents. In the case of the first, 0.1 cc. of oil was added by means of a calibrated pipette to 2 cc. of pure arsenic trichloride in a dry test-tube, rapidly mixed by twirling the tube, transferred to a $\frac{1}{4}$ -inch cell already in position in the tintometer, and a reading taken as quickly as possible. On account of the transient nature of the colours produced, great difficulties were experienced in obtaining accurate readings, and readings were only approximate below the point where a third of the vitamin had been destroyed. On repeating with pure arsenic trichloride of another make the same results were obtained.

In the case of antimony trichloride, the directions of Carr and Price [1926] were followed, with certain modifications. As reagent we used a saturated solution of antimony trichloride in anhydrous chloroform, approximately 30 % (weight in volume). This was prepared by shaking excess of pure antimony trichloride, previously washed with chloroform, in a stoppered flask at intervals for 12 hours with anhydrous chloroform, and then filtering into a dry stoppered bottle of non-actinic glass. Although Carr and Price state that the solvent in their reagent does not need to be perfectly dry, our experience has been that when it is prepared with the ordinary B.P. chloroform according to their directions, this reagent tends to become cloudy on standing, thus preventing a clear colour being obtained for reading in the tintometer. We therefore dried our chloroform by shaking in a bottle of non-actinic glass for several days with anhydrous sodium sulphate, until it no longer gave a blue colour to anhydrous copper sulphate shaken with it, and a solution prepared from this anhydrous chloroform has given excellent results. A solution of the oil to be

tested, 20 % by volume, was made with this chloroform, and 0.2 cc. of this, equivalent to 0.04 cc. of the oil, was added to 2 cc. of the antimony trichloride reagent. The two were rapidly mixed by twirling, transferred to a $\frac{1}{2}$ -inch cell in the tintometer, and a reading taken immediately. The colours obtained, although still transient, lasted distinctly longer than those given by arsenic trichloride, and by taking readings within 15 to 20 seconds of mixing, it was possible to obtain results varying not more than 10 % from one another down to the point where over half the vitamin had been destroyed. Beyond this point the colours, though quite definite, vanished within a few seconds, so that an accurate measurement of their intensity could not be made. It was found that arsenic trichloride and antimony trichloride, when used in these concentrations, gave on the same oil practically the same tintometer readings. As it has been suggested by Rosenheim and Drummond [1925] and Takahashi *et al.* [1925] that the blue colour is indicative of vitamin content, we have used our readings of Lovibond blue units obtained with the arsenic and antimony trichloride reagents to construct the curve in Fig. 1, representing the effect of ultra-violet light on vitamin A in cod-liver oil.

Fig. 1. Effect of ultra-violet light on cod-liver oil, as measured by the arsenic trichloride and antimony trichloride reagents.

We have recently found that when this quantitative method was repeated on oils which had been kept in the dark for several weeks after irradiation, a drop in the reading was obtained, in one instance from 16 to 11 Lovibond units in 14 days. This would seem to indicate that exposure of the oil to ultra-violet light initiates destructive processes which continue for some time after the exposure has been terminated.

SUMMARY.

Cod-liver oil was exposed to the action of the following vitamin A-destroying agents: concentrated sulphuric acid, phosphorus pentoxide and ultra-violet light. The following results were obtained.

1. Fearon's "pyrogallol" test is non-specific. (This conclusion agrees with the findings of Rosenheim and Webster [1926], working on different lines.)
2. Concentrated sulphuric acid and phosphorus pentoxide are less sensitive tests for vitamin A than arsenic trichloride or antimony trichloride.
3. Using the last two tests as criteria of vitamin A content, parallel results were obtained when following the course of destruction of the vitamin by ultra-violet light. Antimony trichloride, however, seems more suitable for use in quantitative methods, since it gives colours which persist longer.
4. In view of the transient nature of the colours obtained with both these reagents, it is suggested that readings be taken not more than 30 seconds after mixing.

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REFERENCES.

- Carr and Price (1926). *Biochem. J.* **20**, 497.
Fearon (1925). *Biochem. J.* **19**, 888.
Moore and Willimott (1926). *J. Soc. Chem. Ind.* **45**, 759.
Peacock (1926). *Lancet*, ii, 328.
Rosenheim and Drummond (1925). *Biochem. J.* **19**, 753.
Rosenheim and Webster (1926). *Lancet*, ii, 806.
Takahashi, Nakamiya, Kawakami and Kitasato (1925). *Inst. Phys. Chem. Research, Tokyo*, **3**, 81.
Willimott and Moore (1926). *Biochem. J.* **20**, 869.