LXI. A STUDY OF THE EFFECT OF HEAT AND OXIDATION ON COD-LIVER OIL AS MEASURED BY COLOUR TESTS.

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In a previous communication [Willimott, Moore and Wokes, 1926], the destruction of vitamin A in cod-liver oil by concentrated sulphuric acid, by phosphorus pentoxide and by ultra-violet light was followed by means of the four principal colour tests put forward for the detection of this vitamin, viz.: concentrated sulphuric acid [Drummond and Watson, 1922]; powdered phosphorus pentoxide [Fearon, 1925], arsenic trichloride [Rosenheim and Drummond, 1925] and antimony trichloride [Carr and Price, 1926].

It was found that while these reagents all gave comparable results, the last two were much more sensitive and were in agreement when applied quantitatively, a Lovibond tintometer being used to measure the intensity of blue colour, which is now regarded by many as an index of the vitamin content [Rosenheim and Drummond, 1925; Takahashi *et al.*, 1925]. It was also attempted to follow the destruction of the vitamin when the oil was aerated at 100°, but a difficulty was encountered in the darkening in colour of the oil produced by heating which interfered with the colour tests. This difficulty has since been overcome, and a detailed study has been made of the effect of aeration at different temperatures.

Most of the previous work has been carried out on butter fat as the source of vitamin A, and conflicting results have been obtained. It seems probable that these discrepancies have been increased by the fact of the wide variation in vitamin content of different samples of butter and by the fact that the fat-soluble vitamins had not then been differentiated. With the exception of Osborne and Mendel's studies [1915, 1920], the earlier work in America [Steenbock, Boutwell and Kent, 1918] and also in this country [Drummond, 1919] had suggested that the destruction of vitamin A was due to heat alone and not to oxidation. Hopkins' investigations, however, carried out in 1919 [1920, 1] gave the first clear explanation when he showed conclusively that aeration as well as heat was an essential factor in the destruction of vitamin A in butter fat [1920, 2].

Hopkins [1920, 2] found that after one hour's aeration at 120° the butter still maintained a steady though subnormal growth throughout the experimental period (11 weeks), and that even after 2 hours' aeration the vitamin

was not completely destroyed. Drummond and Coward [1920] found 3 hours' aeration at 96° sufficient for complete destruction and if this process is a chemical one, a rise of 24° might be expected to reduce the time below 2 hours. Moreover, Rosenheim and Drummond [1925] found that one hour's aeration of cod-liver oil at 100° completely destroyed its vitamin A. In view of these conflicting results we decided to investigate the effect of aeration at different temperatures above and below 100°, using colour tests to measure the content of vitamin A. Although the specificity of the latter is not yet conclusively established, our experience leads us to think that, by the adoption of certain precautions, it is possible to make them reliable with a much smaller experimental error than is the case with animal experiments. We have also found that, in any given sample of oil, partial destruction of the vitamin either by irradiation [Willimott, Moore and Wokes, 1926; Willimott and Wokes, 1927] or by aeration (unpublished results) appears to render the remainder of vitamin A present in the oil unstable, so that it continues decomposing, and nearly half may be lost during the time required to carry out an animal experiment. For these reasons we have relied mainly upon colour tests.

Experimental.

A sample of Norwegian cod-liver oil, known by feeding experiments to be potent in vitamin A, was used in this investigation. About 50 cc. were placed in a boiling-tube and heated in a water-bath until the temperature of the oil became steady at 97–98°. A brisk current of air was then drawn through at the rate of 4 to 6 bubbles per second. At the end of every 5 minutes after aeration started, a few cc. of the oil were withdrawn by means of a pipette, and transferred to tightly-stoppered bottles of non-actinic glass. During withdrawal of the samples the air current was discontinued for 15 to 20 seconds. These samples of oil which had been aerated at 98° for known periods of time were at once examined for their content of vitamin A by the four colour tests. The results, which are given in Table I, show that under the conditions of our experiment, the vitamin A in cod-liver oil is destroyed by about 75 minutes' aeration at 98°. It will be noticed that, as in our previous work on the destruction of vitamin A by irradiation, concentrated sulphuric acid and phosphorus pentoxide were found less sensitive reagents than arsenic trichloride and antimony trichloride. With these last two reagents, the initial blue colour fades gradually to green, the time taken decreasing as more of the vitamin is destroyed. The end-point was taken as the time when the change from blue to green was practically instantaneous, and was probably correct to within 5 minutes.

We next attempted to measure the vitamin content of each of these samples of aerated cod-liver oil by means of the arsenic trichloride and antimony trichloride reagents, using a Lovibond tintometer. Since publishing our previous communication [Willimott, Moore and Wokes, 1926], we have

made a thorough study of the antimony trichloride test for vitamin A, and reached further conclusions of some importance, which may be summarised as follows [Wokes and Willimott, 1927].

Table I. *Colour tests on cod-liver oil which has been aerated at* 98° *for different periods.*

Time aerated (minutes)	Conc. H_2SO_4	P_2O_5	$AsCl_3$	$SbCl_3$	Starch iodide test
0	+ +	+ +	+ +	+ +	−
5	+ +	+ +	+ +	+ +	+
10	+	+	+ +	+ +	+
15	+	+	+ +	+ +	+
20	+	+	+ +	+ +	+
25	+	+	+ +	+ +	+ +
30	±	±	+	+	+ +
35	−	−	+	+	+ +
40	−	−	+	+	+ +
45	−	−	+	+	+ +
50	−	−	+	+	+ + +
55	−	−	+	+	+ + +
60	−	−	+	+	+ + +
65	−	−	+	+	+ + +
70	−	−	±	±	+ + + +
75	−	−	−	−	+ + + +

NOTE. The starch iodide test was applied by putting a drop of aerated oil on a piece of filter paper previously soaked in an aqueous solution of soluble starch and potassium iodide (about 1 % of each) and then dried. If a yellow colour could be observed within 5 seconds, the test was considered to be + + + +, between ¼ and 1 minute + + +, between 2 and 5 minutes + +, more than 10 minutes +.

The reaction consists of a series of colour changes—blue—yellow—red, with intermediate shades. It can be retarded by using anhydrous solvents, and working at a low temperature. In practice 15° or 16° appears to be the most suitable temperature, and it is suggested that correction be made for any wide deviation from that figure. (The temperature coefficient of the reaction is approximately 2.) The reaction is accelerated when the vitamin is rendered unstable by irradiation or aeration. The tintometer reading should be taken exactly 30 seconds after mixing, and as the colour is continually changing, the changes originating at the top of the liquid where there is exposure to air, it is necessary to mix the liquid continually with a small glass rod to ensure a uniform blend of colour. When about two-thirds of the vitamin has been destroyed, the blue colour has practically all disappeared within 30 seconds after mixing, and it is therefore impossible to obtain a tintometer reading, although the colour tests are still quite definitely positive.

Destruction of the vitamin appears to produce substances which give an *immediate* yellow colour with the reagent. We find that when working with irradiated or aerated oils it is necessary to put in more yellow units for the initial reading in order to secure an accurate colour match at the 30 second interval. If, however, a test is made on a sample of oil in which the vitamin has been completely destroyed, the colour immediately produced is red-brown. This same colour is finally reached in any test, although in a stable oil it may not be attained for over an hour.

When the concentration of cod-liver oil in the reaction mixture is plotted against blue colour produced by either arsenic trichloride or antimony trichloride, we find in the case of all the oils so far examined that the results cease to be a linear function above a concentration of 2 to 3 %. We therefore suggest that the amounts taken be arranged so that the readings of Lovibond blue units, using a half-inch cell, are not higher than 15 to 20.

The experimental findings which we have described above show the necessity of adopting various precautions when using either arsenic trichloride or antimony trichloride as reagent for vitamin A. We have now carried out some hundreds of observations on oils from Norway, Newfoundland and Iceland, and have formed the opinion that, when these precautions are observed, the experimental error on the results obtained is probably less than 10 %.

We next estimated by means of the antimony trichloride reagent the vitamin A content of each of the samples of cod-liver oil which had been aerated at 98° for different periods of time, taking in each case 0·2 cc. of a 20 % V/V solution of the oil, and mixing in a half-inch cell with 2 cc. of a solution of antimony trichloride in anhydrous chloroform, about 26 % W/V. In doing so we adopted all the precautions suggested by our previous experience. At least three readings were taken on each sample, and found to agree within 10 %. A number of readings were also obtained with arsenic trichloride, using the technique previously described [Willimott, Moore and Wokes, 1926], and were found to agree with those obtained with antimony trichloride within the limits of experimental error. The mean results are plotted in Fig. 1, in which the curve marked 98° represents the effect on vitamin A, as measured by colour tests, of aeration at 98° for different periods of time, under the conditions of our experiment. The temperature of the oil was taken every minute, and was found not to vary more than a degree either way, with an average value of 98°. The rate of aeration we had adopted was approximately the same as that of Hopkins [1920, 2]. In order to test the effect of increasing the rate of aeration, a second experiment was carried out in which the air was drawn through at a maximum speed of 10 to 12 bubbles per second (practically a continuous stream), the temperature being maintained at 98°, and results estimated as before. The results of these two experiments showed the variation to be less than the experimental error.

Further experiments were then carried out at 88°, 108°, 118°, and 125°, using a paraffin-bath in the last three cases, and adopting the same precautions. The results are plotted in continuous lines in Fig. 1. In order to estimate how much of the destruction of the vitamin was due to heat *per se*, in each experiment a blank was run by putting in the bath a test-tube of oil loosely plugged with cotton-wool, from which samples were withdrawn at 15 minute intervals. The vitamin content of each of these samples was also estimated in the same manner, and the results for each temperature are shown by the broken lines.

Turning to the effect of aeration on vitamin A, we found that the time required for complete destruction was 105 minutes at 88°, 75 minutes at 98°,

50 minutes at 108°, 35 minutes at 118°, and 30 minutes at 125°. These results agree closely with those obtained by previous workers, using both animal experiments and colour tests.

The temperature coefficient for the destruction of vitamin A by aeration, calculated from our data for each 10 degrees rise between 88° and 125°, would seem to be between 1·4 and 1·5. This is similar to the temperature coefficient of 1·5 obtained by Delf [1918] for the destruction of vitamin C by heat.

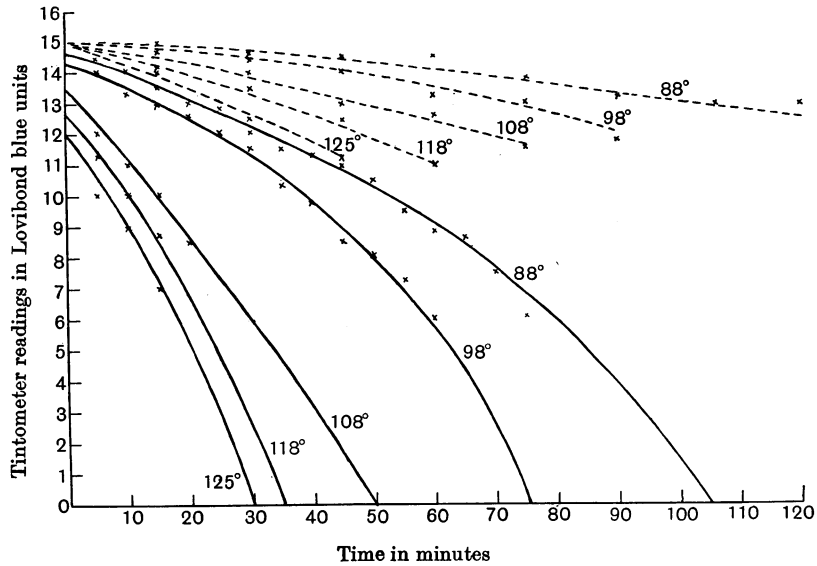

Fig. 1. Effect of heat and oxidation on vitamin A.

Similar results were obtained in the case of the oil heated without aeration at the same series of temperatures. Since there was contact with air at the small surface in the tube, the rate of destruction, although much less than that of the vitamin in the oil aerated at the same temperature, was greater than has been observed by previous workers in the case where air was strictly excluded.

In view of the opinion that the destruction of the vitamin is due to the oxygen of the air, we thought it of interest to examine the aerated oils for oxidising substances. Zilva [1920, 1922] has shown that the destructive action of ultra-violet rays is due to the ozone which they have produced in the surrounding air. Rosenheim and Webster [1926] obtained evidence of the presence of peroxides in cod-liver oil in which the vitamin A had been destroyed by exposure to air and sunlight at room temperature. In our own experiments on the destruction of vitamin A in cod-liver oil by ultra-violet light, we have been able to detect ozone in the samples of irradiated oils, after removal from the vicinity of the mercury vapour lamp, in amounts which

steadily increased as the destructive action proceeded. In the case of the destruction of the vitamin by aeration, we do not think that ozone is an active agent. Schönbein [1858] certainly suggested that ozone may be produced in the slow oxidation of various oils containing unsaturated hydrocarbons, but this suggestion was refuted by Engler [1898, 1904] who stated that the tests obtained were due, not to free ozone, but to unstable peroxides. For instance, turpentine after shaking with air will colour starch iodide paper blue, decolorise methylene blue, etc. We therefore applied, both to the aerated oils immediately after collection, and to the air which had been drawn through them, a number of tests considered characteristic of ozone, of hydrogen peroxide, and of organic and other peroxides. The results, which are summarised in Table II, would seem to indicate that the destruction of vitamin A by aeration is probably due to volatile organic peroxides. This tentative conclusion is of interest in connection with our previous suggestion [Willimott and Wokes, 1926, 2] that the distribution of vitamin A in the *Citrus* fruits may possibly be related to the presence of peroxidases.

Table II. *Tests for oxidising substances in cod-liver oil after aeration.*

Test applied to	Starch iodide	Acid $KMnO_4$	Tetra-methyl base	$Ti(SO_4)_2$	Guaiacum and peroxidase
Oil before aeration	−	−	−	−	−
Oil after aeration	+	+	−	−	?
Air before passage through aerated oil	−	−	−	−	−
Air after passage through aerated oil	+	+	−	−	?
Ozone	+	−	+	−	−
Hydrogen peroxide	+	+	−	+	+
Organic peroxides	+	+	−	−	±

NOTES.

When the air, after passage through the oil, was bubbled through strong chromic acid before passing into starch iodide, a negative result was obtained. This method is suggested by Mellor [1922] for differentiating between ozone (gives positive) and peroxide (gives negative).

As a source of peroxidase free from oxygenase we used an aqueous extract of the colourless residue obtained by exhausting the rind of lemons with 90 % alcohol, according to the method of Onslow [1919]. We have previously shown this to contain peroxidase only [Willimott and Wokes, 1926, 1]. The tincture of guaiacum had been boiled with charcoal to remove peroxides.

As sources of organic peroxides we used various autoxidised oils, such as turpentine and orange rind oil, heated to 98°.

SUMMARY.

Cod-liver oil was aerated at different temperatures between 88° and 125°, and colour tests were made at frequent intervals for vitamin A. Qualitative agreement was obtained with the four tests, concentrated sulphuric acid, phosphorus pentoxide, arsenic trichloride and antimony trichloride, and the last two gave the same end-point for complete destruction of the vitamin.

Arsenic trichloride and antimony trichloride were then applied quantitatively to estimate the vitamin. Curves were obtained representing the course of destruction of the vitamin at 88°, 98°, 108°, 118° and 125°. The temperature coefficient for 10° was found to be about 1·4 to 1·5. These results were in

agreement with observations made by other workers, using animal experiments.

The results of a preliminary series of tests applied to the oils after aeration indicated that the destruction of the vitamin may have been due to volatile organic peroxides.

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REFERENCES.

Carr and Price (1926). *Biochem. J.* **20**, 497.
Delf (1918). *Biochem. J.* **12**, 416.
Drummond (1919). *Biochem. J.* **13**, 81.
Drummond and Coward (1920). *Biochem. J.* **14**, 734.
Drummond and Watson (1922). *Analyst*, **47**, 341.
Engler (1898). *Ber. deutsch. chem. Ges.* **31**, 3046.
—— (1904). Kritische Studien über die Vorgänge der Autoxidation (Braunschweig).
Fearon (1925). *Biochem. J.* **19**, 888.
Hopkins (1920, 1). *Brit. Med. J.* ii, 147.
—— (1920, 2). *Biochem. J.* **14**, 725.
Mellor (1922). A Comprehensive Treatise on Inorganic and Theoretical Chemistry (London) **1**, 951.
Onslow (1919). *Biochem. J.* **13**, 1.
Osborne and Mendel (1915). *J. Biol. Chem.* **20**, 379.
—— —— (1920). *J. Biol. Chem.* **41**, 549.
Rosenheim and Drummond (1925). *Biochem. J.* **19**, 753.
Rosenheim and Webster (1926). *Lancet*, ii, 806.
Schönbein (1858). *Ann. Chim. Phys.* (3), **52**, 221.
Steenbock, Boutwell and Kent (1918). *J. Biol. Chem.* **35**, 517.
Takahashi, Nakamiya, Kawakami and Kitasato (1925). *Inst. Phys. Chem. Research, Tokyo*, **3**, 81.
Willimott, Moore and Wokes (1926). *Biochem. J.* **20**, 1292.
Willimott and Wokes (1926, 1). *Biochem. J.* **20**, 1008.
—— —— (1926, 2). *Biochem. J.* **20**, 1299.
—— —— (1927). *Pharm. J.* **118**, 217.
Wokes and Willimott (1927). *Analyst.* (In the press.)
Zilva (1920). *Biochem. J.* **14**, 740.
—— (1922). *Biochem. J.* **16**, 42.