CXL. SPECTROGRAPHIC STUDIES ON THE ANTIMONY TRICHLORIDE REACTION FOR VITAMIN A

II. INFLUENCE OF OXIDIZING AGENTS ON THE REACTION

BY OLAV NOTEVARP AND HARALD WILLIAM WEEDON

From the Norwegian Fisheries Research Station, Bergen, Norway

(Received 26 April 1938)

As mentioned in the first part of these studies [Notevarp & Weedon, 1936], the ratio between the two absorption bands of the antimony trichloride reaction for vitamin A is normally almost constant for potent preparations, but varies within wide limits for cod liver oils of usual potencies.

Mittelmann [1925; 1927], in publications reviewed by Bezssonoff [1929], claimed to have prepared cod liver oils giving no blue colour until some oxidation had taken place. Hawk [1929] obtained some increase in the blue value on exposure of cod liver oils to air. Lovern *et al.* [1931] were not able to prepare oils giving no reaction, but found a considerable rise in the 603 $m\mu$ band on oxidation of very fresh oils. The 572 $m\mu$ band remained approximately constant; this was confirmed by Heilbron *et al.* [1931]. We demonstrated earlier that very high concentrations of oil inhibited the 603 $m\mu$ band, but not the 572 $m\mu$ band, in fresh oils in which the 572 $m\mu$ band predominated.

In oils in which the maximum obtainable absorption has presumably been reached, the ratio of the 572 to the 603 $m\mu$ band is still far from constant. Morgan *et al.* [1935] found it to vary from 1.24 to 1.48 for 6 oils, Heilbron *et al.* [1931] from 1.09 to 1.77 for 15 oils. For concentrates of the same 6 oils Morgan *et al.* [1935] found from 1.72 to 2.06, while Carr & Jewell [1933] found 1.92 for vitamin A. We have found no published values for oils which approach the value for vitamin A.

As the 572 $m\mu$ absorption is little altered by the various factors that influence the reaction, it follows from the great variation of the ratio that the amount of inhibitor for the 603 $m\mu$ band must vary considerably. And as the ratio is always lower for oils than for concentrates, it may safely be assumed that the inhibition is never completely removed from reactions with oils by any of the means hitherto employed except saponification.

The very considerable inhibition shown by fresh oils is, as has been mentioned before, removed to a great extent by gentle oxidation, indicating that the inhibitor consists at least in part of an unsaturated substance. This may be the strongly inhibiting acid isolated by Emmerie [1932] in amounts of about 2.5 ml./l. from cod liver oil. Hydrogenation, resulting in total inactivation, required 4 atoms of hydrogen per mol.; calculated as oxygen this is about 7 mg./100 g. oil. According to Notevarp & Pillgram-Larsen [1933] this should give an increase in the Kreis value of 4-5 R.L.V., or a degree of oxidation which practically all commercial cod liver oils are subjected to during normal production operations. But of course it cannot be assumed *a priori* that the oxygen is absorbed by the inhibiting acid alone, although the unsaturation of the latter makes very easy oxidation probable.

In old or very rancid oils another kind of inhibition is seen, resulting in low or uncertain blue values for the oils, while their concentrates give clear reactions of normally high values.

The inhibition of the 603 $m\mu$ band would seem of considerable practical importance, in view of the great use made of the blue value commercially and scientifically for the determination of vitamin A. If no precautions are taken, very fresh or old oils will give values which lie far below those corresponding to the actual potency, and even after some oxidation has taken place the variations in the 603/572 $m\mu$ absorption ratio will indicate varying inhibition and therefore uncertain blue values. As will be shown later, the reagent may also provide a source of error, as some batches of it give higher values than usual with fresh oils.

These difficulties may presumably be overcome by carrying out the determinations on the unsaponifiable matter, as has been strongly advocated by a number of workers. For routine work, however, this is not practicable. In the time necessary for one determination on the unsaponifiable matter, it is possible to make a very large number of direct determinations. Also the preparation of the unsaponifiable fraction demands great skill and accuracy if the results are to be reliable, otherwise there is grave risk of increasing the error instead of reducing it.

The determination of the blue value of vitamin oils is today easily the most frequently used method of assaying vitamin A, and in view of the facts set out above, the accuracy and consistency of the values are surprisingly good. This must be due to the fact that it is chiefly the 603 $m\mu$ band that varies, and, as will be seen from Fig. 13 in the first part of this series, such variations influence the eye much less than variations near 572 $m\mu$, as long as the location of the absorption is unchanged.

Nevertheless, it would seem very desirable to be able to eliminate the variations in the 603 $m\mu$ absorption; this would mean a considerable increase in the reliability of the antimony trichloride reaction for vitamin A.

This study was started to investigate whether a definite maximum absorption at 603 $m\mu$ could be obtained by a definite amount of oxidation, by air or other means, so as to give better blue values without seriously increasing the complexity of the method.

EXPERIMENTAL

Determination of the degree of oxidation

To follow the degree of oxidation of the oils, we have used the Kreis test [cf. Taffel & Revis, 1931] as adapted to cod liver oils by Notevarg & Pillgram-Larsen [1932], which is shortly as follows.

The oil is shaken for 30 sec. with an equal volume of HCl, sp. gr. 1.19, in a small glass cylinder, internal diameter 20 mm.; 5 drops of phloroglucinol in alcohol, 50 g./l., are added, the mixture is shaken for a further 30 sec. and the resulting red colour is measured in a Lovibond Tintometer within 1 min. after the emulsion has separated. If the colour intensity is above 10 R.L.V. less oil is taken so as to get a colour below 10.

The other methods used have been described earlier.

Examination of oils at various stages of oxidation

For these experiments we used cod liver oils taken from the boilers immediately after steaming and placed in hermetically sealed bottles. Many of the oils are those described in Part I of these studies, so the same numbers are used.

First series. Quantities of cod liver oil (no. 5, Part I) were shaken with varying volumes of air during several days, and examined after some weeks' storage. The results are seen in Table I and Fig. 1.

Table I

Air/oil ratio	$E_{572\text{ m}\mu}^{10\text{ g./l.}}$	$E_{608\text{ m}\mu}^{10\text{ g./l.}}$	B.V. 18.18 g./l.	Curve in Fig. 1
0	0.54	0.49	5.8	I
0.2	0.59	0.78	7.6	II
0.7	0.58	0.80	—	—
1.3	0.63	1.08	—	III
4.0	0.65	1.07	11.0	IV
2 years later 0.2	0.66	1.09	10.0	—

Fig. 1. Influence of oxidation of oil no. 5 on the absorption spectrum of the blue solution. I-IV rising intensity of oxidation, see Table I.

Second series. Another sample of cod liver oil (no. 4) was evacuated, heated to remove water, and cooled in a stream of CO_2 . Quantities of this were again shaken with varying volumes of air. For the results see Table II.

Table II

Air/oil ratio	Kreis value	$E_{572\text{ m}\mu}$	$E_{608\text{ m}\mu}$	B.V. 18.18 g./l.
0	3.4	0.55	0.49	5.8
0.5	11	0.50	0.45	5.5
1	14	0.51	0.61	5.6
4	46	0.51	0.76	6.4
1½ years later 0.5	—	0.51	0.48	5.6
4	—	0.52	0.85	8.5

No definite maximum appeared to be reached even after considerable oxidation. The treatment of the oil might have influenced the result, so one more series was started.

Third series. Oil no. 3. Blue value and absorption rose, but the results do not allow any definite conclusions to be drawn (Table III). All three series show an

insignificant variation in the 572 m μ band, confirming what has been found by earlier workers. In the following the values for 572 m μ are therefore omitted.

Table III

Air/oil ratio	Kreis value	$E_{572\text{ m}\mu}$	$E_{603\text{ m}\mu}$	B.V. 18.18 g./l.
0	0.5	0.52	0.54	5.9
0 (heated)	3.0	0.53	0.63	6.2
0.25	—	0.54	0.75	7.6
0.5	—	0.55	0.76	7.9
2.0	14	0.57	0.90	8.3
(4.0 after 2 years)	—	0.42	0.65	6.8)

Fourth series. Another sample (oil no. 2) was shaken with about an equal part of air at room temperature, and samples were drawn from the main bulk at intervals. For the results see Table IV.

Table IV

Hours shaken	Kreis value	$E_{603\text{ m}\mu}$	B.V. 18.18 g./l.
0	0.6	0.30	3.2
41	—	0.30	3.7
70	1	0.52	5.2
100	2.5	0.64	6.7
200	4.5	0.70	7.2
Heated	200	0.60	5.4

Fifth series. Oil no. 1. The heating accelerated the oxidation more than was intended, so a fifth experiment was run, in which the Kreis value was raised by prolonged shaking with air at room temperature.

Table V

Kreis value	$E_{603\text{ m}\mu}$	B.V. 18.18 g./l.
0.6	0.36	6.0 (1a)
5.5	0.88	8.6 (1b)
30	0.88	8.8 (1c)

The last three series would seem to indicate that a degree of oxidation corresponding to a Kreis value somewhere about 10 is necessary to get the highest 603 m μ -absorption obtainable by direct oxidation with air. But the second series needed a Kreis value of nearly 50 before the maximum was reached. The only definite conclusion to be drawn is really that it would appear to be very difficult to find a useful analytical method based on oxidation with air.

Addition of oxidants to the reaction mixture

The work of Heilbron *et al.* [1931] already mentioned led us to investigate the possibility of obtaining maximum absorption by the addition of chemical oxidants before or after the reagent had been added. Chlorine and antimony pentachloride dissolved in carbon tetrachloride were chosen, and measurements of the blue value of oil no. 1, the one which was used in series 5, were made when oxidants in varying concentrations were added. The results are collected in Table VI. The concentrations given are those in the reaction mixture; SbCl₅ is calculated as active chlorine.

Table VI

Concentration of oxidant	B.V. 18.18 g./l.			
	Cl addition		SbCl ₅ addition	
	Before	After	Before	After
0.01	—	—	8.8	8.0
0.02	9.5	7.0	8.8	8.3
0.05	9.4	8.0	9.5	9.4
0.10	9.0	9.0*	9.5*	9.1*
0.20	9.0†	9.1*	Violet, turning red	

Unoxidized oil, ordinary reagent: B.V. = 6.0.

Max. obtainable with air B.V. = 8.8.

* Turning reddish very quickly.

† Rapidly diminishing intensity.

Addition of chlorine before the reagent gave the highest values and seemed promising, so spectrographic measurements were made with oils nos. 1, 2 and 4. See Table VII for the results.

Table VII

Oil no.	No addition of oxidant unoxidized oil		Max. obtainable with air		With Cl		
	$E_{603\text{ m}\mu}$	B.V.	$E_{603\text{ m}\mu}$	B.V.	Cl g./l.	$E_{603\text{ m}\mu}$	B.V.
1	0.36	6.0	0.88	8.8	0.02	0.70	8.1
					0.05	0.69	8.1
2	0.30	3.2	0.70	7.2	0.05	0.45	6.5
4	0.48	5.6	0.85	8.5	0.02	0.60	7.6

Both blue values and absorption rose, but the maximum values were definitely lower than those previously obtained by oxidation with air. This line of investigation was therefore not pursued further.

Addition of oxidants to the reagent

During work with series 3 it had been observed that some batches of reagent gave considerably higher 603 m μ absorption with fresh oils than normal. In determining the blue values of fresh oils we had often observed earlier that the values might vary with different batches of reagent. Attempts were made to determine the oxidizing properties of various batches by distillation with acid KI solution, and iodine was found to be liberated in amounts corresponding to 0.1–0.6 γ O₂/ml. reagent, i.e. about 0.5–3 mg. O₂/100 g. oil, in a reaction mixture of normal concentration (18.18 g./l.). This is of the same magnitude as the figure arrived at earlier, but the results were not consistent. At that time our major concern was to ascertain the influence of other oxidants, so in order to be certain of eliminating oxidation caused by the reagent, special care was taken to obtain reagents free from oxidizing substances. The antimony trichloride proved not to contain appreciable amounts of pentavalent antimony, so the oxidation must presumably have been due to decomposition of the chloroform.

These observations proved the possibility of oxidation by means of the reagent, but its spontaneous oxidizing properties could not be produced at will. The next step was therefore to try adding oxidizing substances to normal reagents. Various oxidants were tried: chlorine, which may safely be assumed to form pentachloride immediately, also antimony pentachloride as such, and bromine. Test tube experiments showed that a considerable amount of bromine can be added to the reagent before the brown colour persists, indicating the formation of mixed chloride-bromides of pentavalent antimony. Iodine does not

form mixed halogenides in this way, as there is no reduction in the depth of violet colour.

The use of chlorine, antimony pentachloride or bromine proved successful, as higher blue values were obtained than by any other means. Preliminary experiments were made to find the best oxidant and the most suitable concentration (see Fig. 2).

The maximum obtainable blue value was about the same for the three oxidants. Antimony pentachloride gave two slightly higher values, but these results have not since been confirmed, so they can probably be put down as experimental errors.

The results clearly showed that bromine gives a maximum blue value over a much wider range of concentration than chlorine. The minimum concentration necessary is about 0.02 g./l. or less, for all three oxidants.

Fig. 2.

Fig. 2. Blue values of oil no. 1 with varying concentrations of oxidants in the reagent. o Ordinary reagent. • SbCl₃ reagent. × Chlorine reagent. + Bromine reagent.

Fig. 3.

Fig. 3. Absorption curves of oil no. 1 unoxidized and oxidized by various means. I, unoxidized. II, chlorine added before reagent. III, maximum absorption after oxidation with air. IV, unoxidized oil with bromine reagent.

The rise in absorption was then measured spectrographically. In Table VIII are collected the results for three quite fresh oils using reagents containing different oxidants; in the case of one of the oils, the results at two stages of oxidation with air are given as well. For comparison are also given values for the unoxidized oils and for the oils after oxidation by the addition of chlorine and by air.

Table VIII

Oil no.	No addition		Cl before addition of reagent		Max. obtainable with air		Addition to reagent					
							Cl ₂		SbCl ₃		Br ₂	
	$E^{10 \text{ g./l.}}$ 603 mμ	B.V.	$E^{10 \text{ g./l.}}$ 603 mμ	B.V.	$E^{10 \text{ g./l.}}$ 603 mμ	B.V.	$E^{10 \text{ g./l.}}$ 603 mμ	B.V.	$E^{10 \text{ g./l.}}$ 603 mμ	B.V.	$E^{10 \text{ g./l.}}$ 603 mμ	B.V.
1a	0.36	6.0	0.70	8.1	0.88	8.8	1.23	13.0	1.25	14.0	1.35	13.8
1b	0.88	8.6	—	—	—	—	—	—	—	—	1.32	12.0
1c	0.88	8.8	—	—	—	—	1.05	10.3	—	—	1.10	10.5
2	0.30	3.2	0.45	6.5	0.70	7.2	—	—	0.93	9.4	0.94	9.4
4	0.48	5.6	0.60	7.6	0.85	8.5	—	—	—	—	1.23	10.8

The result is striking; this is shown clearly in Fig. 3 where the complete curves for oil no. 1 are shown.

Addition of oxidants to the reagent results in higher 603 $m\mu$ absorption values than can be obtained by any other means, and these values are obtained even with unoxidized oils, which with the ordinary reagent give higher absorption at 572 $m\mu$ than at 603 $m\mu$. One oil gave the same maximum with such reagents both unoxidized and after mild oxidation, which makes it probable that a definite maximum was reached. On the other hand, it appears that the 572 $m\mu$ absorption does not change more than can be ascribed to overlapping of the absorption bands.

Bromine gives slightly higher values than chlorine or antimony pentachloride, but the differences do not seem significant. Bromine, however, gives maximum blue value over far the greatest range of concentration, and is also the easiest to obtain and handle, so bromine has been chosen as the oxidizing reagent.

Optimum concentration of bromine. $E_{603\text{ }m\mu}$ was measured with three different concentrations of Br in the reagent. The results are seen in Table IX.

Table IX

Oil no.	Concentration of Br, g./l.			
	0	0.05	0.10	0.50
	$E_{603\text{ }m\mu}^{10\text{ g./l.}}$	$E_{603\text{ }m\mu}^{10\text{ g./l.}}$	$E_{603\text{ }m\mu}^{10\text{ g./l.}}$	$E_{603\text{ }m\mu}^{10\text{ g./l.}}$
15	0.21	0.30	0.32	0.32
3 fresh, Kreis 0.5	0.54	1.08	1.08	1.08
3 oxidized, 2 years' old	0.65	0.91	0.95	0.99
18 (old, oxidized)	17.8	19.0	18.3	19.0
14 Halibut liver oil	163	168	174	174
Kreis 600				

There seems to be a tendency towards rising values with rising concentrations for old and oxidized oils; for fresh oils no such tendency is apparent over the range tried.

The smallness of the rise in absorption with concentrations exceeding 0.1 g./l. suggests that higher concentrations are not necessary, so this concentration was chosen. For practical purposes it is unnecessary to measure the amount of bromine with any great accuracy, as variations in the concentration have no very marked effect. One drop of bromine to 1 l. of reagent corresponds to about 0.1 g./l.

*Relative intensities of the absorption bands with
bromine reagent*

In Table X we have collected values for the 572 and 603 $m\mu$ maxima obtainable with air, and with bromine reagent.

Table X

Oil no.	$E_{572\text{ }m\mu}$		$E_{603\text{ }m\mu}$		Ratio	
	Air	Br	Air	Br	Air	Br
15	0.14	0.19	0.21	0.32	1.50	1.69
2	0.45	0.50	0.70	0.94	1.55	1.88
19	—	0.60	—	1.06	—	1.77
3	0.55	0.60	0.96	1.08	1.74	1.80
4	0.52	0.65	0.85	1.23	1.63	1.90
1	0.60	0.70	0.88	1.35	1.47	1.93
20	—	0.85	—	1.60	—	1.88
21	—	1.0	—	1.80	—	1.80
Vitamin A (Carr & Jewell)	2600		5000		1.92	

No great accuracy is claimed for the determinations of the 572 m μ absorption, but the trend of the values seems unmistakable. The average ratio is considerably higher than that found by previous workers using the ordinary reagent, and approaches that of Carr & Jewell for vitamin A, which is included in Table X for comparison.

*Tintometer readings and spectral absorption
with bromine reagent*

Tintometer readings and E values obtained by the use of bromine reagent (0.1 g./l.) are given in Table XI, and plotted graphically in a bi-logarithmic co-ordinate system in Fig. 4 (cf. Table II and Fig. 5 in Part I of these studies, Notevarp & Weedon [1936]).

Table XI

Oil no.	$E_{503\text{ m}\mu}$	B.V.	Oil no.	$E_{503\text{ m}\mu}$	B.V.
1a	2.26	12.8	16	3.09	15.5
	1.13	8.0		1.62	10.5
1b	2.20	11.0		0.83	6.0
	1.10	6.5	17	3.63	14.5
1c	1.87	10.0		1.90	9.5
	0.98	6.3		0.97	6.2
2a	2.23	11.0	19	2.70	12.5
	1.61	9.0		1.95	10.0
	1.54	8.5		1.02	6.7
3a	1.76	10.8		1.49	9.1
	0.92	6.1		0.72	4.7
3b	1.55	8.8	20	2.61	14.0
4a	2.88	13.0		1.36	8.7
	1.37	10.7		0.78	5.3
	1.05	6.2		0.70	4.8
15	1.68	8.5	21	2.94	15.5
	1.17	6.5		1.54	9.4
	0.61	4.0		0.86	6.0
				0.79	5.8

Fig. 4. B.V. and E for cod liver oils with bromine reagent.

Fig. 4 shows that the function $B.V. = 6.5 (E_{603\text{ m}\mu})^{0.75}$ corresponds to the average of the points found. The spreading of the values is about the same as with the ordinary reagent. As has been said before, this spreading is probably chiefly due to the inherent inaccuracies of the determination of blue values.

DISCUSSION

The results obtained during these studies show that it is apparently very difficult to remove from fresh cod liver oils the factors inhibiting the 603 $\text{m}\mu$ band either by controlled oxidation with air or by addition of oxidants such as chlorine, antimony pentachloride or bromine before or after adding the reagent.

If, however, these oxidants are added to the reagent itself in minute quantities, a very definite increase in the 603 $\text{m}\mu$ band is obtained. The absorption maximum becomes constant and independent of the degree of oxidation up to the point at which partial destruction of the chromogen becomes highly probable; it is also independent of the concentration of oxidant in the reagent over a varying range. The absorption is also increased by such reagents in the case of old and oxidized oils. In all oils there is an increase in blue value and absorption beyond those which can be obtained with the ordinary reagent.

The ratio of the 603 $\text{m}\mu$ band to the 572 $\text{m}\mu$ band increases and approaches that found for pure vitamin A. The variation is probably no more than the experimental error, even for oils which with ordinary reagent show a predominance of the 572 $\text{m}\mu$ band.

These facts all indicate that the addition of oxidants to the reagent removes a very considerable portion of the inhibiting influences found in cod liver oil, even such inhibition as is not removed by gentle oxidation of the oil. The constancy of the maximum, independently of the degree of oxidation or of the concentration of oxidant, also makes it probable that the inhibition is completely or nearly completely removed. Whether this is the case can be ascertained by comparison of the absorption of oils with that of their concentrates, which will be discussed in Part III of these studies.

Bromine gives slightly higher values than the other oxidants that have been tried; it also gives maximum absorption over a much wider range of concentration. This and the accessibility of this substance make it natural to choose bromine as the standard oxidant for the new type of reagent.

The relation between E and B.V. follows a similar exponential curve to that for the ordinary reagent. The alteration of the 603/572 $\text{m}\mu$ ratio increases the constant by about one unit, and also makes the slope of the curve slightly steeper, corresponding to an exponent of about 0.75.

SUMMARY

A definite maximum of the 603 $\text{m}\mu$ band of the antimony trichloride reaction with cod liver oils could not be obtained either by controlled oxidation or by the addition of oxidants before or after adding the reagent.

Addition to the reagent of small quantities of oxidants such as chlorine, bromine or antimony pentachloride resulted in higher 603 $\text{m}\mu$ absorption values than were obtained by any other method for oils in all stages of oxidation ranging from quite fresh ones, with the 572 $\text{m}\mu$ band predominating when the ordinary reagent is used, to very old or rancid oils.

The ratio of $E_{603\text{ m}\mu}$ to $E_{572\text{ m}\mu}$ increases, becomes more constant and approaches that of pure vitamin A preparations, 1.92.

It is probable that the inhibition of the 603 m μ band is completely or nearly completely removed by the oxidizing reagent.

Bromine gives the highest absorption of the agents that have been tried; it is also the most generally accessible. 0.1 g./l. is a suitable concentration.

With this reagent the relation between B.V. and $E_{603\text{ m}\mu}$ follows the equation:

$$\text{B.V.} = 6.5 (E_{603\text{ m}\mu})^{0.75}.$$

REFERENCES

- Bezssonoff (1929). *Bull. Soc. Chim.* **11**, 1146.
Carr & Jewell (1933). *Nature, Lond.*, **131**, 92.
Emmerie (1932). *Acta brev. Neerl. Physiol.* **2**, 156.
Hawk (1929). *Science*, **69**, 200.
Heilbron, Gillam & Morton (1931). *Biochem. J.* **25**, 1352.
Lovern, Creed & Morton (1931). *Biochem. J.* **25**, 1341.
Mittelman (1925). *Nouvelles Inst. Agronom. Exp.* **3**, 1.
—— (1927). *Trans Int. Explor. North, Leningrad*, pp. 38, 39.
Morgan, Edisbury & Morton (1935). *Biochem. J.* **29**, 1645.
Notevarp & Pillgram-Larsen (1932). *Årsberetn. vedk. Norges Fiskerier*, 1931, No. 5, 31.
—— (1933). *Årsberetn. vedk. Norges Fiskerier*, 1932, no. 3, 39.
—— & Weedon (1936). *Biochem. J.* **30**, 1705.
Taffel & Revis (1931). *J. Soc. Chem. Ind., Lond.*, **50**, 87 T.