CXLVIII. SPECIFICITY IN TESTS FOR VITAMIN A. A NEW CONCEPTION OF THE CHROMOGENIC CONSTITUENTS OF FRESH AND AGED LIVER OILS.

By ISIDOR MORRIS HEILBRON, ALBERT EDWARD GILLAM and RICHARD ALAN MORTON.

From the Department of Chemistry, The University of Liverpool.

(*Received July 3rd, 1931.*)

Evidence is accumulating to the effect that the vitamin A of liver oils is a colourless, or almost colourless substance, characterised by a broad continuous band of selective absorption in the ultra-violet, with a maximum near 328 $m\mu$. The vitamin acts as a chromogen in the sense that with antimony trichloride it gives rise to one and possibly more than one absorption band in the visible.

In the earlier stages of the reaction, the colour arises to a considerable extent from a pronounced absorption band in the yellow-green, the maximum being near 583 $m\mu$ but suffering a variable displacement towards 565–575 $m\mu$ in the presence of certain little-understood, unsaturated substances present in liver oils. The evidence indicates that associated with vitamin A is a substance, which, unlike the vitamin, is highly transparent to light of wave-lengths near 328 $m\mu$ but shares with it the property of acting as a chromogen towards antimony trichloride, giving rise in this case to a coloured compound absorbing maximally in the orange at 620 $m\mu$, the band again undergoing a displacement (to 600–610 $m\mu$) in the presence of certain unsaturated substances of unknown constitution normally present in fish-liver oils.

The two chromogens at present appear to be distinct and separate entities, but their occurrence together, and in quantities not widely different as regards order of magnitude makes it necessary to investigate the possibility of kinship in origin or function.

It is thus clear that the colour test with antimony trichloride occupies a position of cardinal importance in the present phase of development in our knowledge of vitamin A. It is therefore worth while to note the characteristics of the ideal colour test so that the antimony trichloride test may be seen in proper perspective.

The simplest case, in which subjective or objective colorimetry reaches its highest accuracy, is that of solutions containing a single absorbing solute which rigidly conforms with the Lambert-Beer absorption law[1]. The solutions

[1] In any two solutions exhibiting identical absorption of light $c_1 = c_2 d_2/d_1$, where c_1, c_2 are concentrations and d_1, d_2 are thicknesses.

to be compared should not differ in respect of the proportion of any colourless constituents able to depress or enhance the visible absorption of the coloured solute.

The two solutions under comparison should thus exhibit identical spectral absorption curves when the thicknesses are so adjusted that the number of molecules absorbing visible rays is the same in both light paths. Under these circumstances the matching of both solutions separately against coloured glasses is not open to serious objection.

Some of the characteristics of the antimony trichloride colour test, especially as applied to sources of vitamin A may now be noted.

(i) Visible colorations varying from red to blue are given with a wide variety of substances ranging from carotenoids to sterols, and including besides vitamin A, highly unsaturated polyene acids such as the crocetins. There is therefore, in a fundamental sense, nothing very specific about the colour test.

(ii) The reagent itself is a source of difficulty. Ordinary B.P. chloroform contains alcohol, which tends to inhibit the production of the maximum intensity of colour. Specially purified chloroform[1] dissolves appreciably less anhydrous antimony trichloride than B.P. chloroform, and relatively small changes in concentration of antimony trichloride appreciably affect the absolute intensity of the blue colour. The reagent as sometimes used is unstable towards light and moisture, and if kept for a few weeks, even in dark brown bottles, tends to deposit a heavy oily liquid.

(iii) As a criterion of vitamin A, the colour test is applied to a wide variety of substances, including mammalian-liver fats, butter extracts, crude and refined oils from fish-livers of many different species, and non-saponifiable extracts at various stages of concentration and diluted in various ways. The "environment" of vitamin A may therefore include sterols, carotenoids and many different saturated and unsaturated substances. It is rarely possible to feel confident that the colour test is free from interference arising either as a result of the presence of chromogens other than vitamin A or as a result of some degree of inhibition. Even restricting attention to one source such as "cod-liver oil," the variations in ordinary physical and chemical properties between individual samples are often very great, quite apart from variations in vitamin A potency. The conditions are so adverse in the sense of a lack of constancy in the environment of vitamin A that the only surprise which need be felt is that the colour test responds as well as it is in fact found to do.

(iv) The colour is transient; not only does it fade, but the rate of fading, the succession of colour changes and the more or less final colour are all subject to considerable variations. There is no doubt that, as at present available (*e.g.* in concentrates), vitamin A itself does not form a stable compound with antimony trichloride and that a succession of secondary actions occurs. Thus the two chromogens which at present appear to be of greatest interest

[1] *Cf.* Report of Cod-Liver Oil Colour Test Sub-Committee, March, 1931, Pharmacopoeia Commission, General Medical Council.

give coloured substances with initial maxima varying from 565 to 585 $m\mu$ and 600 to 624 $m\mu$ depending on the environment. Maxima at 535, 495 and 465 $m\mu$ have all been recorded as the solutions faded. Evidence of other chromogens is provided by the appearance in certain cases of bands at 635, 645, 656, 680 and 690 $m\mu$. All these maxima have been found in fish-liver oils and concentrates. The chromogenic properties of substances in the carotenoid and sterol groups are equally complicated.

(v) The colour test is very sensitive to mass action. In most cases the use of half-strength reagent effects an enormous reduction in the intensity of the blue colour. Even this is not a simple case of mass action, for as we shall show later, it is evident from the spectroscopic data that different chromogens are affected differently by a change in concentration of the reagent.

(vi) It has recently been shown (*cf.* preceding papers) not only that there are two chief chromogens in liver oils, but that the relative proportions may sometimes differ so widely as to effect a gross qualitative change in the shape of the spectral absorption curves characterising the solutions. Thus the 572 $m\mu$ maximum is sometimes vastly stronger than the 606 $m\mu$ maximum and *vice versa*.

In view of the complexity of the phenomena associated with the colour test, the experimental methods must be as searching as possible. Colorimetry, using standard glasses, is of distinct value as a simple and convenient way of expressing colour intensities with some approach to accuracy. For research purposes colorimetry is however of limited value unless frequent parallel spectroscopic determinations are carried out.

If the spectral absorption curves for the blue solutions were always the same, it is indisputable that objective colorimetry using photoelectric cells and recording instruments would provide a highly accurate method for measuring intensities, especially if used in conjunction with a monochromator or a filter capable of eliminating all but a narrow strip of the spectrum. Photoelectric spectrophotometry is however less useful than visual methods for examining the spectra of the blue solutions with which we are concerned, because of the need for rapid detection of variable maxima in the absorption curves. With the Hilger-Nutting spectrophotometer a blue solution can be seen at once to be either normal or abnormal, the wave-length of maximal absorption can be selected, and the intensity of absorption determined, all in a few seconds. The photoelectric technique is unsuitable for exploring spectra, its usefulness is for measuring the intensity of absorption at a given wave-length quantitatively. Unfortunately, each blue solution must be examined visually before the wave-length suitable for the photoelectric method can be selected.

Technique. The following is a description of the technique used in these laboratories.

The reagent (a saturated solution of antimony trichloride in purified chloroform) is kept in the apparatus shown in Fig. 1. The oil or concentrate

is dissolved in pure chloroform, the concentrations chosen being 20 % for an average cod-liver oil and 0·2 % for a concentrate about 100 times as rich as an oil. The solution is placed in the modified pipette shown in Fig. 2, so that

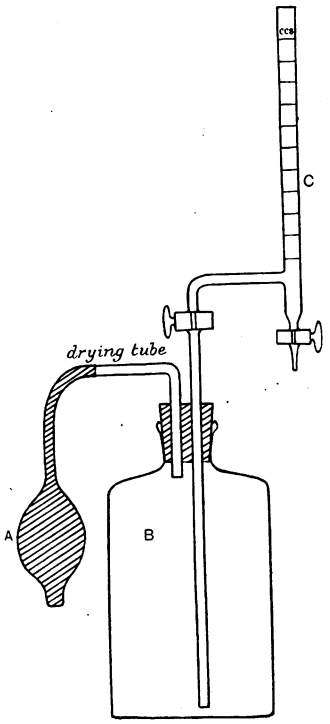


Fig. 1.

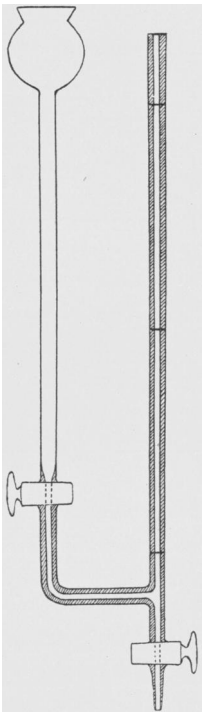


Fig. 2.

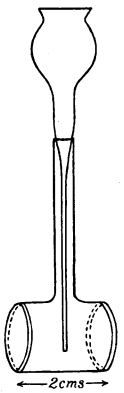


Fig. 3.

Fig. 1. Type of apparatus used for storage of antimony chloride in chloroform.
A. Rubber bulb with valve. B. Stock bottle of solution. C. 10 cc. burette.
A drying-tube can be placed between the bulb and the air inlet if the reagent is to be kept in this apparatus.

Fig. 2. Form of pipette used for measurement of chloroform solutions of cod-liver oil (2 cc. graduated in tenths).

Fig. 3. Type of fused quartz cell used for spectroscopic examination of antimony chloride-vitamin A blue colours.

frequent pipetting of chloroform is avoided. We are indebted to Mr H. Rogerson for these simple and efficient methods.

The volume of solution used is 0·3 to 0·6 cc., generally 0·5 cc. This is run into a test-tube, and 5 cc. of antimony trichloride are added. The mixture is at once poured into a 2 cm. fused quartz cell with plane parallel fused-on end plates (*cf.* Fig. 3) which is already mounted in position in the Hilger-Nutting spectrometer. The positions of absorption maxima are first ascertained. The cell is then emptied and the attachment at the spectrometer eye-piece adjusted so that a narrow strip of the spectrum on either side of the maximum is isolated. A fresh mixture is then prepared and the intensity of absorption at the maximum is determined. A number of readings are always made on

fresh mixtures, usually by two observers, and the average values reduced to a basis of 1 %, 1 cm. The ultra-violet absorption spectra are determined in the ordinary way, the results being expressed in terms of log I_0/I (E) at 328 $m\mu$ for a 1 % solution and a 1 cm. cell, no corrections being used unless the persistence of the band is abnormally small, in which case the fact of correction is noted. The numerical values of $E^{1\%}_{1\,cm.}$ 572 and $E^{1\%}_{1\,cm.}$ 328 $m\mu$ are in most cases identical within experimental error. We have therefore adopted $E^{1\%}_{1\,cm.}$ 1·0 as a spectroscopic unit. This value is on the average equal to a blue value (Carr-Price) of 12·5 Lovibond.

It has been shown [Morton, Heilbron and Thompson, 1931] that the blue solutions with liver oils or concentrates may exhibit very marked qualitative differences in absorption spectra, the maximum being liable to occur at 572 $m\mu$ or 606 $m\mu$. Gillam and Morton [1931] on the basis of the comparison of ultra-violet absorption and spectroscopic data on the colour test have shown that an almost exact linear relationship obtains between the intensity of the 328 $m\mu$ maximum and the 572 $m\mu$ chromogen, whereas the relationship between the 328 $m\mu$ band and the 606 $m\mu$ band exhibits a sufficient number of serious discrepancies from linearity to impugn the hypothesis of a common origin. It has also been shown [Coward, Dyer, Morton and Gaddum, 1931], on the basis of the latest and fullest comparison of biological assays with physico-chemical data, that the relative intensities of the 328 $m\mu$ and 572 $m\mu$ maxima provide trustworthy criteria of potency, whereas the values based on the 606 $m\mu$ maximum and on the Lovibond tintometer readings exhibit many anomalies. Further, Lovern and Morton [1931] in studying the oil from the livers of the monk or angler fish observed instances in which the concentration of the 606 $m\mu$ chromogen relatively to the 572 $m\mu$ chromogen was abnormally low, but in which the intensity of the 328 $m\mu$ ultra-violet band corresponded very closely with the intensity of absorption at 572 $m\mu$. The same authors also encountered cases of codling- and cod-liver oils in which similarly low concentrations of the 606 $m\mu$ chromogen were found, although the 572 $m\mu$ chromogen occurred strongly and in amount agreeing with the intensity of the ultra-violet band. We now desire to submit fresh evidence pointing still more definitely to the existence of two distinct chromogens in liver oils.

The claims made by Mittelmann [*cf.* Lovern, Creed and Morton, 1931] seemed to be of special interest as soon as misgivings arose as to the validity of a strict correlation between the unresolved blue colour test and the ultra-violet selective absorption. It became evident in testing the Mittelmann process that the particular oils studied showed an increased chromogen content on standing. The most natural hypothesis to advance as an explanation of such a spontaneous change was that of oxidation. It seemed therefore desirable to ascertain whether the process could be accelerated. Accordingly the following methods were tried.

(i) A current of ozonised oxygen was passed through the oil in the cold for a short time.

(ii) The oil was shaken with about one-fifth its volume of a concentrated solution of hydrogen peroxide for 10–15 minutes, the excess of peroxide washed away with water and the oil dried with anhydrous sodium sulphate and filtered.

(iii) The oil was dissolved in chloroform, a solution of benzoyl peroxide added and the mixture left to stand in the cold for 10–15 minutes. A small quantity of this solution was then used for the antimony trichloride colour test.

(iv) The oil was treated with barium peroxide.

(v) The oil was treated with sodium peroxide.

Method (iv) proved useless and method (v) ineffective in the absence of water, but very striking results were obtained with the other three reagents. The oils investigated were remarkable for the abnormally high $572 m\mu/606 m\mu$ intensity ratio, absorption at the latter point being quite feeble. As will be seen from Table I, the treatment with ozone results in a very large increase in the measurable concentration of the $606 m\mu$ chromogen, the $572 m\mu$ chromogen and the ultra-violet absorption being but little changed (*cf.* Fig. 4).

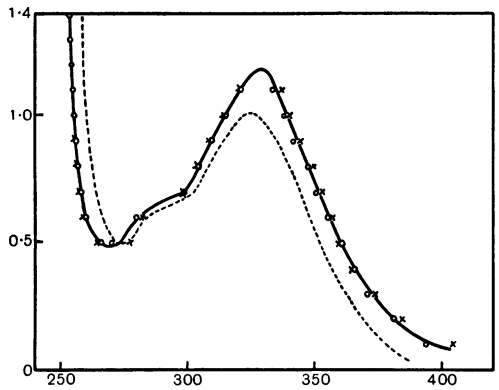


Fig. 4.

—o—o— Steamed C.L.O. 1·5 % (1 cm.).
×—×—× Steamed C.L.O. ozonised 1·5 % (1 cm.).
- - - - - - - Mittelmannised oil from 10 tins 1·5 % (1 cm.).

The enhanced concentration of the $606 m\mu$ chromogen is well shown in the case of the monk-liver oil. This oil had been obtained a very short time previously by ether-extraction of the livers and had been stored in a glass bottle under ordinary laboratory conditions. The codling oils were obtained by the Mittelmann process, the fresh livers having been sealed in evacuated tins and autoclaved at 120° for 2 hours. The oils were examined within 2–3 days of the fish being caught. Here again the $572 m\mu$ chromogen predominated to a marked extent over the $606 m\mu$ chromogen, but the ultra-violet intensity was in good agreement with the higher value. Ozonisation or treatment with peroxides resulted in a very considerable increase in the $606 m\mu$ chromogen. These experiments were repeated, fresh tins being opened for the purpose. The results proved to be reproducible (*cf.* Table I).

Table I. *The action of O_3, H_2O_2*, etc., *on very fresh liver oils.*

Oil	Remarks	Ultra-violet test $E^{1\%}_{1\,cm.}$ 328 $m\mu$	$SbCl_3$ colour test $E^{1\%}_{1\,cm.}$ 572 $m\mu$	$SbCl_3$ colour test $E^{1\%}_{1\,cm.}$ 606 $m\mu$
Monk-liver oil (pigmented)	As received	0·82	0·825	0·25
	Treated ozone	0·95	0·69	0·96
	Treated H_2O_2	—	0·82	0·96
	+ benzoyl peroxide	—	0·83	1·00
Codling-liver oil (small livers)	As received (liver in tins)	0·12	0·12	0·06
	Same. Next day	0·16	0·12	0·176
Codling-liver oil (larger livers)	As received	0·22	0·21	0·13
	+ O_3. 5 minutes	—	0·19	0·32
	+ O_3. 10 ,,	—	0·215	0·29
	+ O_3. 20 ,,	—	0·18	0·31
	+ O_2. 20 ,,	—	0·19	0·13
Codling-liver oil (larger livers)	As received	0·19	0·18	0·125
	+ O_3. 1 minute	—	0·17	0·14
	+ O_3. 3 minutes	—	0·175	0·20
	+ O_3. 5 ,,	—	0·175	0·25
	+ O_3. 10 ,,	0·19	0·17	0·27
	Treated H_2O_2	0·21	0·175	0·32
Cod-liver oil	As received	0·73	0·76	0·355
	+ O_3. 20 minutes	0·73	0·59	0·93
Cod-liver oil	As received	0·69	0·72	0·39
	+ O_3. 30 minutes	0·73	0·72	0·93
	After 11 days	0·75	0·75	1·00

Later tests showed that the unusual preponderance of the 572 $m\mu$ chromogen was not due to the Mittelmann process *per se* (*cf.* Lovern, Creed and Morton, 1931), since, using the same livers, the 572 $m\mu$ chromogen predominated in the fresh steamed oil and in the oil from newly opened tins. It is significant however that a threefold increase in the 606 $m\mu$ chromogen was obtained on treatment with ozone, hydrogen peroxide or benzoyl peroxide.

On examining our records for cod-liver oils which when first tested had shown the 572 $m\mu$ chromogen preponderating over the 606 $m\mu$ chromogen, a number of these were discovered, and all were re-examined for ultra-violet absorption and by the antimony trichloride colour test. It was found that in each of these, after the oil had stood for several months, a significant change had occurred, the concentration of the 606 $m\mu$ chromogen having increased considerably. The ultra-violet absorption exhibited a very much smaller increase in intensity, whilst the 572 $m\mu$ band was also but little changed. Each oil was then subjected to treatment with ozone or hydrogen peroxide, and in some cases it was found that the "ageing" process could be carried still further. In other cases it was found that the ageing process had reached a maximum, and that further treatment with ozone or hydrogen peroxide merely resulted in the gradual destruction of both chromogens as evidenced by lessened absorption both at 572 and 606 $m\mu$ in the colour test and at 328 $m\mu$ in the ultra-violet absorption (Table II).

We have thus demonstrated for eight cod-liver oils, two codling-liver oils and at least one monk-liver oil that two chromogens can be detected in the oils. Spontaneous or induced changes, which may be oxidations, result in a

Table II. *Spectroscopic data on the spontaneous and the accelerated ageing of cod-liver oils.*

		Ultra-violet test		Colour test	
Oil	Remarks	$E^{1\%}_{1\,cm.}$ 328 $m\mu$	$E^{1\%}_{1\,cm.}$ min.*	$E^{1\%}_{1\,cm.}$ 572 $m\mu$	$E^{1\%}_{1\,cm.}$ 606 $m\mu$
1	As received	1·37	0·90	1·40	1·22
	2 months later	1·42	0·95	1·33	1·20
	Treated O_3	1·40	—	1·41	2·12
	Treated H_2O_2	—	—	1·52	2·42
2	As received	1·86	1·66	1·74	1·53
	5 months later	2·0	1·73	1·90	2·55
	Treated H_2O_2	—	—	1·90	2·83
3	As received	1·87	1·26	2·32	1·98
	7 months later	2·25	1·53	2·01	2·75
	Treated H_2O_2 (overnight)	—	—	1·75	2·50
4	As received	1·90	0·67	1·86	1·62
	5 months later	2·03	0·80	1·95	2·33
	Treated H_2O_2	—	—	2·09	2·70
5	As received	1·52	0·72	1·62	1·51
	5 months later	1·60	0·95	1·52	2·02
	Treated H_2O_2	—	—	1·41	2·05
6	As received	1·07	—	1·07	0·72
	7 months later	1·17	—	1·14	1·75
7	As received	1·23	0·90	1·35	1·52
	Treated H_2O_2	—	—	1·32	1·90

* This is the intensity of absorption at the minimum but the wave-length is variable.

very considerable increase in one chromogen before any apparent change occurs in the quantity of the other chromogen. On the other hand, the ultra-violet absorption is not appreciably affected by the spontaneous or induced enhancement of the 606 $m\mu$ chromogenic value. It is however undesirable to generalise from these results, for we have examined the question whether all absolutely fresh oils contain less of the 606 $m\mu$ chromogen than of the 572 $m\mu$ chromogen, but by some spontaneous or induced change can be enriched in the 606 $m\mu$ chromogen. Definite evidence has been obtained to the effect that in a number of quite fresh oils the 606 $m\mu$ band not only predominates slightly over the 572 $m\mu$, but is present at its full intensity, ozonisation effecting no increase.

In our experience, an oil in which the 572 $m\mu$ chromogen predominates over the 606 $m\mu$ chromogen is somewhat unusual. The two chromogens are so far not demonstrably related and there is thus no reason to assume a constant ratio either for the 572 and the 606 $m\mu$ intensities or the 572 and the potential 606 $m\mu$ intensity. The lack of constancy in the ratios (Table III) further emphasises the independence of the two chromogens, although the differences are not so great as to preclude kinship.

In evaluating the characteristics of concentrates we have repeatedly noticed that the "blue value" increases much more rapidly than the intensity of absorption in the ultra-violet. This is probably analogous to an observation made by Smith and Hazley [1930].

Table III.

$E^{1\%}_{1\,cm.}$ 572 $m\mu$	$E^{1\%}_{1\,cm.}$ 606 $m\mu$ (maximum attainable)	Ratio 606 : 572 $m\mu$
1·4	2·4	1·71 : 1
1·75	2·85	1·63 : 1
2·3	2·5	1·09 : 1
1·86	2·7	1·45 : 1
1·62	2·05	1·27 : 1
1·07	1·75	1·64 : 1
1·35	1·90	1·41 : 1
0·82	1·0	1·22 : 1
0·21	0·32	1·52 : 1
0·18	0·32	1·77 : 1
0·76	0·93	1·22 : 1
0·72	1·0	1·37 : 1

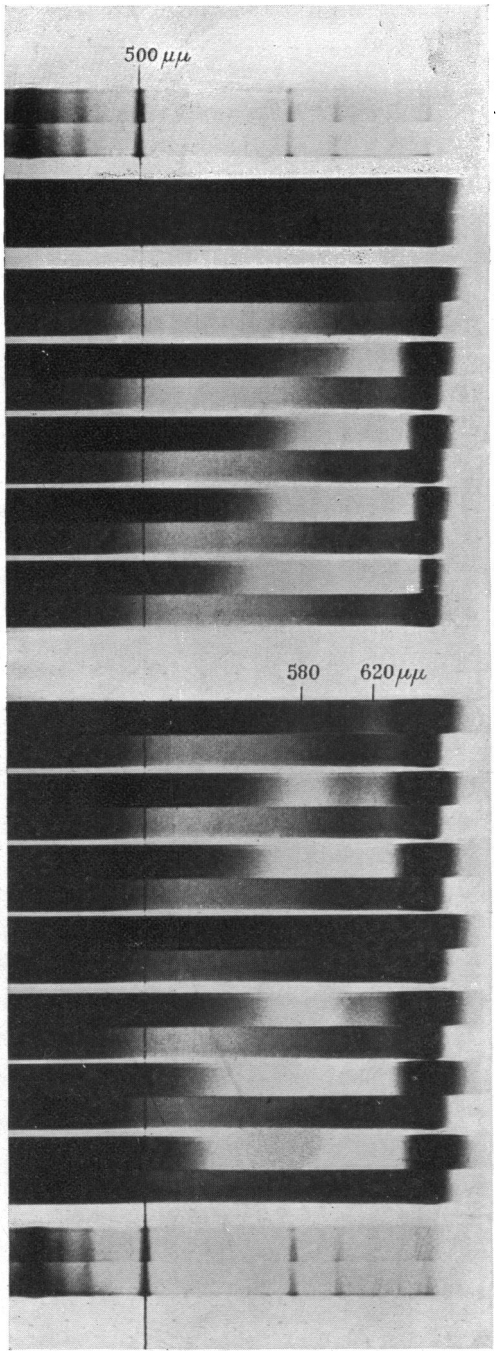
During the process of saponification the 572 $m\mu$ chromogen or its environment is changed so as to displace the maximum to 583 $m\mu$, but for this chromogen the increase in the intensity of absorption given by the colour test is exactly proportional to the degree of concentration effected. On the other hand, if the oil is such that the 606 $m\mu$ band fails to reach the maximum intensity possible for the oil, then for this second chromogen the increase in absorption surpasses that to be expected from the degree of concentration. In addition, a displacement to 620 $m\mu$ occurs. When concentrates are dissolved in either seal oil or whale oil the two revert to 606 and 572 $m\mu$, but in arachis oil no such reversion takes place. Clearly therefore the displacement of the maxima is somehow connected with the nature of the medium.

The results with concentrates are of importance in connection with the views of Norris and Church [1930, 1, 2]; *cf.* Smith and Hazley [1930]. In carrying out quantitative studies concerning the validity of the colorimetric test these authors were led to conclude that liver oils contain a saponifiable substance which tends to inhibit the production of the blue colour. Norris and Church [1930, 2] found that addition of oleic acid to concentrates produced the same inhibitory effect.

Separation of the two absorption maxima (colour test).

Spectroscopic examination of the blue solution obtained by the interaction of the antimony trichloride reagent and the majority of liver oils and concentrates shows the absorption curve to be unsymmetrical, with the sharper decrease of absorption occurring on the long-wave side of the maximum, Fig. 5 (*b*). With the anomalous oils, however, the curve is still unsymmetrical but in the opposite sense, Fig. 5 (*a*), the steep descent being now on the side of shorter wave-lengths. Now in most cases, starting from an oil giving higher absorption at 572 than at 606 $m\mu$, ozonisation or treatment with hydrogen peroxide effects so great an increase in the absorption at 606 $m\mu$ that the curve becomes of the type shown in Fig. 5 (*b*).

As we have seen, the interpretation advanced in this paper involves the view that an inflexion indicates the presence of a partially masked absorption



Aluminium spark.

Tungsten spark under water (control).

Cell	log I_0/I	Halibut oil cc.	$SbCl_3$ reagent cc.
2 cm.	0·8	0·05	5
,,	,,	0·10	5
,,	,,	0·15	5
,,	,,	0·20	5
,,	,,	0·25	5

Cell = 2 cm. log I_0/I = 0·8.

Halibut oil cc.	$SbCl_3$ reagent cc.	Interval min.	$SbCl_3$ reagent cc.
0·10	0·3	1	4·7
0·15	0·3	1	4·7
0·20	0·3	1	4·7
0·10	0·3	2	4·7
0·20	0·3	2	4·7
0·30	0·3	2	4·7
0·40	0·3	2	4·7

Aluminium spark.

(*a*) illustrates the appearance of one band in the colour test;
(*b*) ,, ,, ,, two bands ,, ,,

band. It would however be an advantage if the real existence of the 572 $m\mu$ band in normal oils could be demonstrated even more clearly. We have found this to be possible by following the procedure described below.

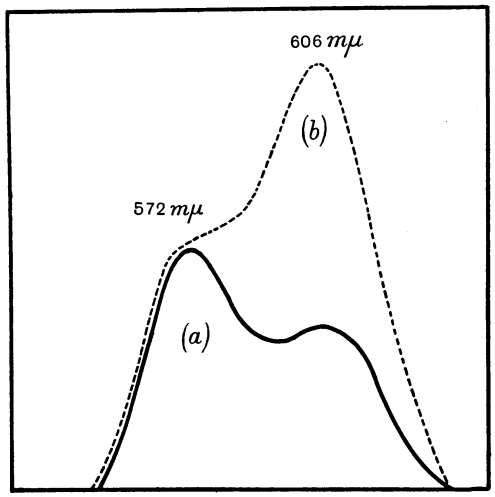


Fig. 5. Diagrammatic representation of the absorption spectra of the blue solutions produced with (*a*) certain fresh oils, (*b*) the same after spontaneous or induced ageing.

The usual quantity (0·5 cc.) of a solution of liver oil in chloroform is run into a test-tube and a small quantity (0·3 cc.) of the antimony reagent added. The solution is then allowed to stand for 1 minute and the remainder (4·7 cc.) of the reagent is then added and the solution examined spectroscopically. In nearly every case two separate absorption bands with maxima at 565–575 and 600–620 $m\mu$ can be seen very clearly. The intensity of the former band is practically the same as when the whole of the reagent is added at once, but the latter band is very much reduced in intensity.

It has been possible to photograph solutions treated in this way and the results are reproduced in Plate VI, whilst Fig. 6 shows a schematic representation of the absorption curves of the various coloured substances.

Table IV illustrates the results obtained by the alternative procedures.

Table IV.

		$E^{1\%}_{1\,cm.}$ 600–612 $m\mu$	$E^{1\%}_{1\,cm.}$ 572–575 $m\mu$
Halibut oil 1.	Procedure (*a*)*	61·2	35·7
	,, (*b*)	27·3	36·4
Halibut oil 2.	,, (*a*)	101	58·5
	,, (*b*)	62	50
Cod-liver oil 1.	,, (*a*)	1·05	0·9
	,, (*b*)	0·80	0·85
Cod-liver oil 2.	,, (*a*)	2·75	2·2
	,, (*b*)	1·87	2·1
Cod-liver oil 3.	,, (*a*)	0·85	0·72
	,, (*b*)	0·52	0·70

* (*a*) Normal procedure. (*b*) Preliminary addition of a small quantity of $SbCl_3$ reagent.

The striking phenomenon about the procedure involving the preliminary addition of a small quantity of reagent is that the reduction in intensity of

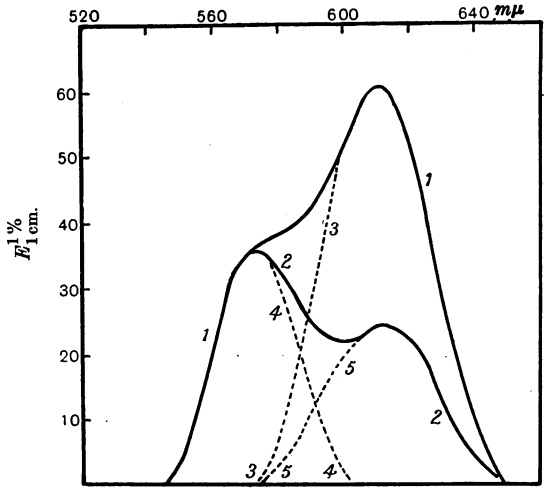


Fig. 6.

1. Schematic representation of absorption curves of the antimony trichloride blue colour with a halibut oil, measured on the freshly mixed solution, the reaction being carried out in the normal way (*a*).
2. Ditto, the reaction being carried out adding a small quantity of $SbCl_3$ reagent 1 minute before adding the bulk of the reagent (*b*).
3. Ditto, the 606 $m\mu$ band in case (*a*).
4. Ditto, the 572 $m\mu$ band in cases (*a*) and (*b*).
5. Ditto, the 606 $m\mu$ band in case (*b*).

1. Summation of 3 and 4. 2. Summation of 4 and 5.

the blue colour is substantially confined to the 606 $m\mu$ band, while the 572 $m\mu$ band is obtained at almost its full intensity.

Another but less satisfactory method whereby the two absorption bands can be manifested is to vary the concentration of antimony trichloride in the reagent.

Table V indicates the values obtained with a rich cod-liver oil.

Table V.

	$E^{1\%}_{1\,cm.}$ 606 $m\mu$	$E^{1\%}_{1\,cm.}$ 572 $m\mu$
Saturated solution of antimony trichloride (in B.P. chloroform previously treated with anhydrous calcium chloride)	5·23	4·05
Two-thirds saturated reagent	3·9	3·6
Half saturated reagent	3·2	3·1

With the full strength reagent the 606 $m\mu$ maximum is shown very clearly and an inflexion is seen at 572 $m\mu$, but with the diluted reagents two bands are visible separately, although neither is present at its full intensity.

The reality of two absorption bands in the coloured solutions is thus established beyond question and the variations in the relative intensities of the

two bands under different conditions go a long way towards accounting for the non-linear colour-concentration curves which have been recorded by many investigators.

Discussion.

Without prejudicing the issue concerning vitamin A, the physical data lead us to postulate the following entities.

(i) A substance X, which gives rise to the ultra-violet absorption with its maximum at $328 m\mu$.

(ii) The chromogenic substance X' in oils which gives rise to the $572 m\mu$ maximum.

(iii) The chromogenic substance X'' present in concentrates giving rise to the $583 m\mu$ maximum.

(iv) The chromogenic substance Y' in oils responsible for the $606 m\mu$ maximum.

(v) The chromogenic substance Y'' of concentrates causing the band at $620 m\mu$ in the colour test.

(vi) A substance giving rise to an absorption band with a maximum near $280 m\mu$.

(vii) The substance or substances which react with antimony trichloride to give coloured compounds with maxima at 640–650 $m\mu$ and 690–700 $m\mu$.

Confining our attention for the present to the first five, the comparison of intensities of absorption at 328 and $572 m\mu$ for oils leads to the view that X and X' are identical. Similar comparisons using concentrates indicate that X and X'' are identical. This leads to the possibility that X' is an ester and X'' the alcohol derived from it on saponification. This suggestion does not however provide an explanation of the variability of the maximum in the colour test, because (*a*) in liver oils (*e.g.* halibut oils) which have much higher blue values than cod-liver oils there is a progressive displacement from 570 to $582 m\mu$, the very rich oils being chromogenically very like concentrates, and (*b*) when a concentrate is dissolved in seal oil or whale oil the maximum for the solution appears at 570, but if arachis oil is used it remains at $583 m\mu$.

Exactly similar considerations lead to the view that the $606 m\mu$ chromogen Y' is identical with the $620 m\mu$ chromogen Y'', the differences being due entirely to the displacing action of unsaturated substances. This does not however account for the apparent increase in the 606–620 $m\mu$ chromogen in certain oils on ozonisation or treatment with hydrogen peroxide. The following three possible explanations of the phenomena suggest themselves, but the task of ascertaining which is the true one promises to be unusually difficult.

(1) Vitamin A, which we may regard as the material responsible for the $328 m\mu$ maximum, is the chromogen responsible for both the 572–583 $m\mu$ band and the 606–620 $m\mu$ maximum. Antimony trichloride is known to form the double compounds:

$$C_6H_4(CH_3)_2, SbCl_3 \qquad C_6H_4(CH_3)_2, 2SbCl_3 \textit{ etc.},$$

so that there is nothing inherently impossible in the compounds $SbCl_3$, vitamin A; $2SbCl_3$, vitamin A.

(2) There are two independent chromogens *X* and *Y* present in the oil. The absolute amount of *Y* present is not increased by ozonisation *etc.*, but the reaction

$$Y + SbCl_3 \rightarrow SbCl_3, Y \text{ (blue substance)}$$

only occurs to its full extent in the absence of certain "inhibitors" which are eliminated by treatment with ozone or hydrogen peroxide.

(3) The quantity of *Y* present in an oil undergoes a real increase on ozonisation by virtue of the presence of a precursor substance, which is converted into *Y* either by spontaneous ageing or accelerated oxidation. Such a precursor, if it exists, must be chromogenically inert, or relatively so, and will absorb rays of wave-lengths near $328 m\mu$ comparatively feebly.

The explanation given under (1) involves that the "chromogens" are not capable of separation, it accounts at once for the somewhat startling fact that an animal depleted of vitamin A but supplied with carotene accumulates both "chromogens" in the liver, but it demands for the ozonisation experiments the removal of "inhibitor" substances, which before ozonisation can form complexes with antimony trichloride to such an extent as to modify profoundly the equilibria:

$$\text{Vitamin A} + SbCl_3 \rightleftharpoons SbCl_3, \text{ vitamin A.}$$
$$SbCl_3, \text{ vitamin A} + SbCl_3 \rightleftharpoons 2SbCl_3 \text{ vitamin A.}$$

The inhibitor substances must however remove antimony trichloride from the sphere of action of vitamin A without themselves forming coloured substances. The fact that changes in the concentration of the reagent markedly affect the relative intensities of the 572 and $606 m\mu$ bands is consistent with an explanation attributing a dominant rôle to mass action. The idea that both bands arise from vitamin A is attractive because of its avoidance of the complication of a new chromogen, but its weaknesses are patent in view of the very hypothetical nature of the essential "inhibitors" and the difficulty of reconciling it with the results of a preliminary addition of the antimony trichloride reagent. Finally, it would seem reasonable to expect the full development of the $606 m\mu$ band to be at the expense of the $572 m\mu$ band, and this is not realised.

The explanation given under (2) enables us to retain temporarily the validity of the $606 m\mu$ band as a possible criterion of vitamin A (although probably forcing us to abandon the $328 m\mu$ band) without postulating a threefold increase in potency on ozonisation. It again however compels us to attribute a very important rôle to the inhibitors about which so little is known. It minimises the possibility of a simple correlation between ultra-violet absorption or feeding tests and the colour value and gives no rational account of the apparent correlation between the 328 and $572 m\mu$ bands.

Explanation (3) seems on the whole to fit the facts best, but its consequences are serious. It involves the view that tintometric methods of assay can only be regarded as good approximations. It requires two new substances, a chromogen and a chromogen precursor, the absorption spectra of which in the ultra-violet have so far eluded our attempts to discover them. It fits in extremely well with the best biological assays to which we have access, but it raises the question of the function of the new chromogen and its precursor.

From whatever angle the new data are regarded a plurality of working hypotheses seems inevitable. Whilst we have frankly envisaged the difficulties, it is necessary to define our attitude. The most plausible interpretation leads us to suggest the following physico-chemical criteria for vitamin A.

(i) A colourless or pale yellow substance exhibiting selective ultra-violet absorption free from fine structure and absorbing maximally at or near 328 $m\mu$, and (ii) giving with antimony trichloride a coloured substance characterised by a maximum in the yellow between 580 and 590 $m\mu$, whilst the molecular extinction coefficients at 328 and 580–590 $m\mu$ expressed in terms of vitamin concentration should be approximately equal.

These criteria are not free from a measure of uncertainty from the strictly numerical standpoint, because the change in environment involved in the preparation of rich concentrates displaces the chromogen from 572 to 583 $m\mu$, the vitamin itself remaining a minor constituent of even the richest concentrates so far obtained. Further concentration might result in a continuation of this shift in wave-length.

Again, antimony trichloride produces coloured substances with a wide range of sterol and carotenoid derivatives, and in many cases the absorption maxima are found in the yellow-red region of the spectrum. It would be by no means surprising if the 583 $m\mu$ maximum were found to be by itself non-specific for vitamin A. Again, an absorption maximum near 320–330 $m\mu$ in the ultra-violet has never been claimed as a specific test for vitamin A, it merely indicates a property of vitamin A. There would be nothing very unexpected in the discovery of a sterol or carotenoid derivative exhibiting both a maximum near 580-590 $m\mu$ with antimony trichloride and an absorption band very like that observed in liver oils, the substance being still distinct from vitamin A. It would however be much less probable to find the same relative intensities of absorption at 328 and 583 $m\mu$. The nearest approach to satisfying these criteria has been obtained with "dihydrocarotene" prepared by the method of Smith [1931]. This product, which is undoubtedly a mixture, gives selective absorption in the near ultra-violet and with antimony trichloride a blue solution exhibiting maxima at 583 and 648 $m\mu$ [Heilbron and Morton, 1931]. The interpretation of these results must await the examination of the separate constituents present in the mixture, both by biological and physico-chemical methods [*cf.* Drummond and Ahmad, 1931; Karrer, Euler and Hellström, 1931].

SUMMARY.

1. The ideal conditions for accurate colorimetric determinations are considered, and the actual characteristics of antimony trichloride as a colour-producing reagent are reviewed in detail.

2. In a considerable number of oils characterised in the colour test by predominance of the 572 $m\mu$ band over the 606 $m\mu$ band, a large increase in the intensity of the latter band can be obtained by treating the oil beforehand with ozonised oxygen, hydrogen peroxide or benzoyl peroxide.

Oils which initially showed an excess of the 572 $m\mu$ chromogen over the 606 $m\mu$ chromogen undergo a slow spontaneous ageing which results in a marked increase in the intensity of the 606 $m\mu$ band. Increases in the 606 $m\mu$ absorption are not at the expense of the 572 $m\mu$ chromogen and are not accompanied by similar increases in the latter, and the absorption at 328 $m\mu$ remains practically constant throughout.

3. The reality of the two chromogens and the possibility of recording the 572–583 $m\mu$ band even in oils and concentrates in which the 606–620 $m\mu$ chromogen predominates can be demonstrated visually and photographically by a modification of the ordinary procedure in the colour test, which suppresses or eliminates some of the 606 $m\mu$ chromogen.

4. The bearing of the data on the vitamin A problem is discussed in detail, and the most plausible criteria for the vitamin are stated.

5. The two main chromogens behave as if they were separate and distinct entities but a close genetic relationship seems highly probable.

We are indebted to the Department of Scientific and Industrial Research for a grant which has enabled Mr R. H. Creed to be of much service in the laboratory work.

REFERENCES.

Coward, Dyer, Morton and Gaddum (1931). *Biochem. J.* **25**, 1102.
Drummond and Ahmad (1931). *J. Soc. Chem. Ind.* **50**, 183 T.
Gillam and Morton (1931). *Biochem. J.* **25**, 1346.
Heilbron and Morton (1931). *J. Soc. Chem. Ind.* **50**, 183 T.
Karrer, Euler and Hellström (1931). *Svensk Kemisk Tidskrift*, No. 5.
Lovern and Morton (1931). *Biochem. J.* **25**, 1336.
—— Creed and Morton (1931). *Biochem. J.* **25**, 1341.
Morton, Heilbron and Thompson (1931). *Biochem. J.* **25**, 20.
Norris and Church (1930, 1). *J. Biol. Chem.* **85**, 477.
—— —— (1930, 2). *J. Biol. Chem*, **87**, 139.
Smith (1931). *J. Biol. Chem.* **90**, 597.
—— and Hazley (1930). *Biochem. J.* **24**, 1942.