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

I. THE RELATION BETWEEN TINTOMETER READINGS AND SPECTRAL ABSORPTION OF THE BLUE SOLUTION.

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According to our present knowledge there seems no doubt that there exists an intimate connexion between vitamin A and the blue colour produced with a number of dehydrating agents, especially antimony trichloride in chloroform. The colour follows the vitamin even into the most potent concentrates, approaching 100% purity, and no really serious discrepancies are on record that cannot find a rational explanation. The reaction has gained a firm footing, both in science and commerce, on account of its simplicity, even though a number of sources of error adhere to it. Very much has been written about this reaction, but it appears to the present writers that several of the fundamental facts have not yet been fully explained.

No really satisfactory explanation has yet been found for the relation between concentration and blue value, as measured in the Rosenheim-Schuster Lovibond Tintometer, within the range of reasonably accurate measurement, i.e. B.V. about 4–12.

For potent oils and concentrates giving a blue value of 6 for concentrations of about 1 g./l. and lower most authors agree that the relation is linear, within the usual range of measurement [Norris & Church, 1929; 1930; Smith & Hazley, 1930]. But at least two of the graphs of Norris & Church contradict this, and a number of the straight lines of Smith & Hazley might equally well have been drawn as curves. Evers [1929], on the other hand, finds lower values the higher the quantity taken, especially of oils with a high vitamin content, e.g. ox liver fat.

Norris & Church [1930] show curves for a number of oils; they do not find proportionality even over a small range, and find no mathematical relation which satisfies all their curves. It seems that they exaggerate the accuracy of tintometer readings, according to our experience. Drummond & Hilditch [1930] allege that the colour intensity is a linear function up to "10% concentration"; at higher concentrations this does not hold ("10%" corresponds to 9·09 g./l.). The fact that several of the straight lines of these authors do not pass through the origin seems difficult to explain. Coward *et al.* [1931] say that it is a well-known fact that the relation between intensity of colour and concentration of an oil is not linear, but is represented by a line which falls away at higher concentrations to a curvilinear function. Gillam & Morton [1931] recommend some "caution in using the Lovibond technique as a quantitative method of assay...".

Notevarp & Hjorth-Hansen [1931] in a publication from this Institute state that the colour/concentration relation for oils follows an exponential function

$$B_c = b_1 . c^{0.7}$$

where b = the colour obtained by the concentration 1, B = the colour obtained by the dilution c.

The relation has also been mentioned in another publication [Notevarp, 1935].

Apart from slight discrepancies in the accurate wave-lengths concerned, it is agreed that the colour given by potent oils and concentrates is due to absorption bands at 617–620 $m\mu$ and 580–585 $m\mu$, the former predominating in the relation of about 10:5 to 10:6.

In less potent oils, such as ordinary cod liver oil, the bands are displaced to 600–606 $m\mu$ and 570–575 $m\mu$ respectively. Their relative intensities may vary within wide limits, either may be the strongest although the 606 $m\mu$ band usually predominates.

The shift of the bands is probably due to the change of solvent effected by the oil; that bands shift when the solvent is changed is well known [v. e.g. Scheibe, 1925]. The other changes in the bands will be treated in another publication which we hope will be forthcoming shortly.

In this publication are given the wave-lengths found by means of an empirical scale on the instrument at our disposal, 572 $m\mu$ and 603 $m\mu$ for oils, 580 $m\mu$ and 618 $m\mu$ for concentrates. We do not consider the exact situation of the bands to be of major importance.

The qualities of the reagent, such as concentration, temperature, contents of moisture and impurities, age etc. are factors which influence the result. It has been shown by several authors that the intensity of blue increases with the concentration of antimony trichloride [Wokes & Willimott, 1927, 1, 2; Norris & Church, 1930; Brode & Magill, 1931, and others]. This has been confirmed in detail at the Fisheries Research Station; it is hoped that it will be possible to publish these results later, and it should only be mentioned here that the colour/concentration curve appears to approach asymptotically to a maximum value. This cannot actually be reached, but this means that small variations in reagent concentration are insignificant when the solution is nearly saturated. For practical application, however, 220 g./l. is now universally accepted as the standard concentration, so this question need not be discussed here. The same applies to temperature, 18° being now universally employed. Water and alcohol, impurities both likely to occur, lower the results; they should be entirely removed from the chloroform and kept away from the reagent. Light alters the reagent in the course of time, probably by the formation of decomposition products of the solvent. This indicates the necessity for avoiding unnecessary exposure of the chloroform to light and air during purification, otherwise the result may be a reagent which does not give the maximum values from the outset.

Experimental.

All reactions with antimony trichloride reagent have been carried out at 18° ± 0·5°; reagent concentration 220–225 g./l. throughout. Every care has been taken in preparing the reagent; the chloroform has been washed, dried with freshly heated potassium carbonate and distilled over a small amount of calcium hydride. For the last batch the distillation was carried out in a partial vacuum, at about 30°, as difficulties were encountered in getting the reagent to give maximum values. A whole sample of trichloride was taken for each lot of reagent, and

poured straight from the newly-opened bottle into the chloroform. After dissolving by warming to about 25°, the concentration was determined, chloroform added if necessary, and the reagent transferred to the stock bottle by sucking it over into the evacuated bottle through a U-bent glass tube with a stopcock. In this way contact with moist air was reduced to a minimum, and sediments in the solution were left in the first flask.

The reagent was measured out by means of a very simple burette designed first by the late R. Engström, the design being improved by one of the authors (H. W. W.) to that shown in Fig. 1. The burette is filled either by compressing the air in the bottle or by suction through the top. Ordinary corks have been used, they are fitted in the empty apparatus after softening with steam and dried in place. The burette is calibrated by comparing the weight of reagent measured out with the weight of the contents of an ordinary pipette. This is necessary because the tip below the cock, which should be as small as possible, retains an amount which differs for fluids of varying viscosity and capillarity.

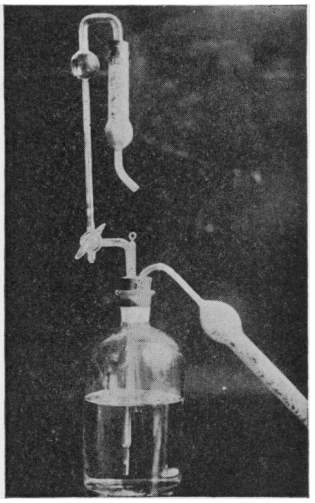

Fig. 1.

The oil solution was run into the cell from a 1 ml. pipette graduated to 0·01 ml. and the reagent added. Immediately afterwards the mixture was stirred by a current of dry air blown in through a thin glass tube with a capillary opening. This gives a very satisfactory stirring. The amount of chloroform vapour carried off reduces the volume less than 0·2 %. Nitrogen was first employed, but it was found later that the oxygen in the air does not affect the reaction to any noticeable extent. Apparently the reaction is much less sensitive to disturbances after the reagent has been added.

Oils and concentrates were diluted for the antimony trichloride reaction with the same chloroform as that used for making the reagent, after this had been evacuated to remove dissolved air and saturated with CO_2. The solutions were examined within a few hours of preparation, most of them within an hour.

A tintometer of the British Drug Houses' design has been used for the determination of blue values.

Spectrographic measurements have been carried out on a Zeiss "Spektrograf fur Chemiker" by the rotating sector method, with photographic recording. For the yellow and orange bands several panchromatic materials have been found satisfactory. The best material we have found are "Agfa Rot Hart" plates. Where possible 5 sec. have been used as standard time of exposure, varying the aperture so that the product of this and the sector opening is constant.

All the oils examined have been of wholly known origin, either produced by the Fisheries Research Station or received from the producer direct. What is said applies therefore only to pure cod liver oil, which has undergone no refining or other process which might alter its chemical or physical properties. The concentrates have been prepared with every possible care in the accepted way.

Expression of results. All concentrations are expressed in accordance with the Paris Convention for the Expression of Analysis of Foodstuffs.

Concentrations in connexion with the antimony trichloride reaction are expressed as g./l. or mg./l. chromogenic substance in the reaction mixture. The

small variation in $SbCl_3$-concentration resulting from using varying amounts of chromogen solution (0·05 to 0·5 ml.) does not appreciably influence the results. Concentrations of concentrate solutions are expressed as the corresponding concentrations of the oil, to make comparison possible.

Intensity of spectral absorption is expressed by the extinction coefficient E for a 10 g./l. solution, as defined by Bunsen & Roscoe, see International Critical Tables. This, by the way, does not contain the unnecessary reference to a 1 cm. cell so frequently met in publications on spectrography.

Results.

Change in absorption with time.

The blue colour changes with the time after the reaction is started; the absorption maximum changes and the shape of the absorption curve is altered. To be certain of observing the maximum of absorption, and also to study the shape of the curve, a number of observations were carried out at intervals after mixing on almost every blue solution measured.

The equipment allows the first exposure to begin 5–7 sec. after the reagent has been added. For concentrates it appears that the maximum is reached at or before this time; exposures from 20 to 25 sec. already show an appreciable fading. The 618 $m\mu$ band is always the strongest except in the case of partially destroyed concentrates. Both bands rise and fall simultaneously.

The alteration with time of the absorption curve for oils varies. Normal oils, with the 603 $m\mu$ band predominating, give the 572 $m\mu$ band maximum, masked by the rising 603 $m\mu$ band; almost immediately this band then fades. The 603 $m\mu$ band reaches its maximum after about 30 sec., remains nearly constant for about 25 sec. and then fades slowly (Fig. 2). In the tintometer this will be seen by the

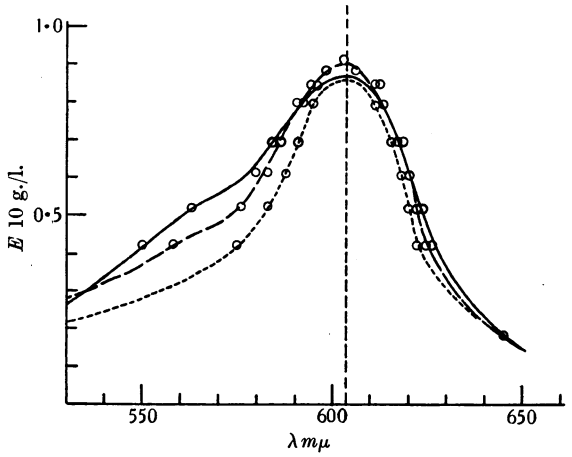

Fig. 2. —— 10–15 sec.; – – – 35–40 sec.; - - - - 85–90 sec.

colour being at first of a purple hue, turning gradually more greenish. As the complementary colour of 572 $m\mu$ is a colour at the beginning of the purple line, whereas the complementary of 603 $m\mu$ is at 495 $m\mu$, an aquamarine colour, this is easily explained.

The opposite is the case with oils with the greatest absorption at 572 $m\mu$. Here the 603 $m\mu$ band reaches its maximum in 10 sec. or less and fades very

quickly, whilst the 572 $m\mu$ band develops its maximum in the course of 60–90 sec. and remains stable for a comparatively long time. In the tintometer this is seen as a very transient aquamarine colour, changing to a more and more purplish blue. In the case of very high oil concentrations the intensity of the 603 $m\mu$ band is greatly reduced, and the development of the 572 band is delayed: it also becomes narrower, the maximum, however, being the same. The concentrations in question are far above (2–3 times) those in practical use.

The curves of Fig. 2 have been drawn so that the marking decreases with time, to attempt to give a stereoscopic effect. Intermediate values have been omitted for clarity; they do not contribute anything particularly noteworthy.

The antimony trichloride reaction and Beer's law.

The results published by Brode & Magill [1931] indicate that the absorption is proportional to the oil concentration. On the other hand, a number of authors allege that the oil inhibits the reaction in higher concentrations. We have therefore measured a number of oils, especially weak oils in high concentrations, to find out whether such inhibition takes place. We have also measured more normal oils and concentrates, although if the weak oils follow Beer's law there is little reason why stronger oils or concentrates should not.

Some of our results are put together in Table I, and shown graphically in Fig. 3. The normal concentration, 0·04 g. in 2·20 ml. = 18·18 g./l., is drawn in as a vertical line.

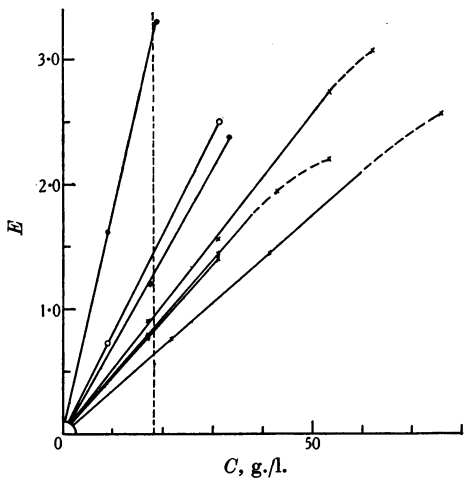

Fig. 3. E and concentration. o—o 618 $m\mu$; •—• 603 $m\mu$; ×—× 572 $m\mu$.

It will be seen that the absorption maximum is a linear function of the concentration in all cases, falling off only when the volume of oil solution effects a considerable dilution of the reagent. There is nothing to indicate that the oil has any disturbing influence in the reaction, or rather, any disturbance is proportional to the amount of oil taken; the blue solution follows Beer's law.

The relation between tintometer readings and spectral absorption.

Blue values over the range of the tintometer and the corresponding maximum densities have been determined for a number of oils and concentrates (Table II).

Table I.

Oil no.	Maximum at $m\mu$	Concentration g./l.	Layer cm.	ml. oil solution	E_{max}
2*a*	572	21·8	1	0·1	0·76
		41·5	1	0·2	1·45
		76·3	0·5	0·4	2·56
4*c*	572	17·0	1	0·2	0·76
		31·2	1	0·4	1·41
4*a*	572	17·0	1	0·2	0·80
		31·2	1	0·4	1·44
		43·1	0·5	0·6	1·94
		53·5	0·5	0·8	2·20
4*b*	572	17·0	1	0·2	0·90
		31·2	1	0·4	1·56
		53·5	0·5	0·8	2·73
		62·4	0·5	1·0	3·06
2*b*	603	17·5	0·5	0·1	1·20
		33·4	0·5	0·2	2·38
7	603	8·90	1	0·1	1·62
		18·8	0·5	0·2	3·30
2*b* conc.	618	8·94	1	0·1	0·73
		31·4	0·5	0·4	2·52

Table II.

	Max. 527 $m\mu$			Max. 603 $m\mu$			Max. 618 $m\mu$	
	E 572 $m\mu$	B.V.		E 603 $m\mu$	B.V.		E 618 $m\mu$	B.V.
1*a*	0·44	3·5	1*b*	0·77	4·4	1	0·54	6·0
	0·84	5·5		1·47	8·3		0·55	6·5
	0·84	6·0	1*c*	0·77	4·7		1·04	10·0
	1·53	8·0		1·47	8·5		1·04	10·5
2	0·55	3·3	2	0·61	4·0		2·08	16·0
4	0·81	5·5		1·02	5·0	2	0·70	7·6
5	0·89	5·5		1·08	6·2		0·73	7·4
6	0·76	5·0		1·20	7·0		0·76	7·6
				1·21	7·1		1·07	9·6
				2·33	10·5		1·29	10·7
			3	1·06	5·5	3	2·20	12·0
				1·24	6·8		2·30	12·5
				1·28	7·3	4	0·37	4·3
				1·50	7·6		0·71	7·6
				1·51	7·4		1·36	12·0
				1·61	7·2	7	1·12	8·0
			4	0·85	4·8		2·15	12·0
				1·01	5·2		2·23	14·0
				1·28	6·1	8	0·59	5·0
				1·43	8·0		0·88	9·5
			5*a*	1·34	7·3		1·15	8·5
				1·48	7·9		1·15	10·0
			5*c*	1·80	9·5	10	1·15	8·6
				1·82	8·5		1·64	12·0
			7	1·60	7·2	11	0·86	6·5
				3·20	12·5	12	0·53	5·0
			8	0·84	8·0		0·80	9·0
			10	3·00	11·0		1·00	9·0
			13	0·37	2·4	14	0·35	3·3
				0·73	4·1		0·36	3·3
				0·72	4·3		0·51	4·6
				1·05	6·0		0·70	5·6
				1·43	7·3		0·72	5·8
				1·37	7·4		0·67	5·9
				1·46	7·6		0·96	7·4
				2·10	9·6		1·02	8·1
				2·86	11·3		1·40	9·7
				2·73	11·7		1·33	10·4
							2·04	13·4

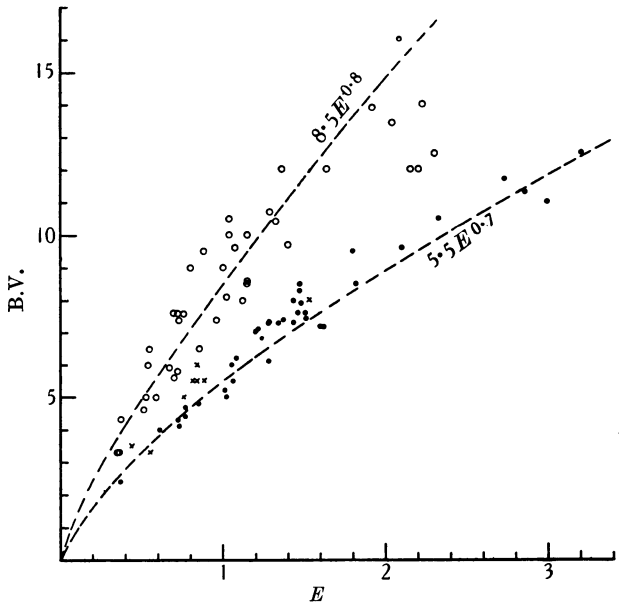

Fig. 4. *E* and B.V. o—o 618 $m\mu$; •—• 603 $m\mu$; ×—× 572 $m\mu$.

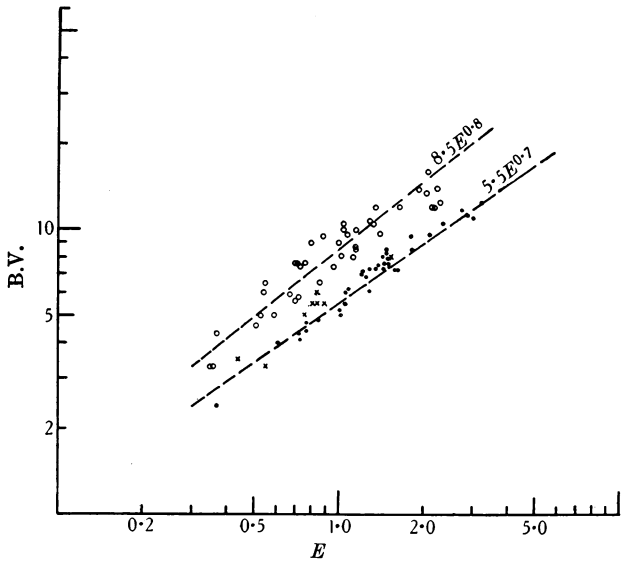

Fig. 5. *E* and B.V. on logarithmic scale.
o—o 618 $m\mu$; •—• 603 $m\mu$; ×—× 572 $m\mu$.

As might be expected they do not show a linear relationship, either for oils or for concentrates; this appears clearly from Fig. 4. In Fig. 5 the values are plotted in a bi-logarithmic coordinate system. Here they will be seen to group around nearly parallel straight lines, one for each predominating absorption band. Straight lines in such coordinate systems are exponential functions. The line formed by the values for solutions with the maximum at $618 m\mu$ has a slope corresponding to an exponent of about 0·8, those for $603 m\mu$ of about 0·7. The lie of the lines give the two functions:

$$\text{B.V.} = 5{\cdot}5 . E^{0{\cdot}7} \text{ for oils, max. } 603 m\mu.$$
$$\text{B.V.} = 8{\cdot}5 . E^{0{\cdot}8} \text{ for concentrates, max. } 618 m\mu.$$

Solutions with maximum at $572 m\mu$ give intermediate values, but the number of determinations does not allow any definite function to be derived. The functions above are to be regarded as average values, and do not claim great accuracy.

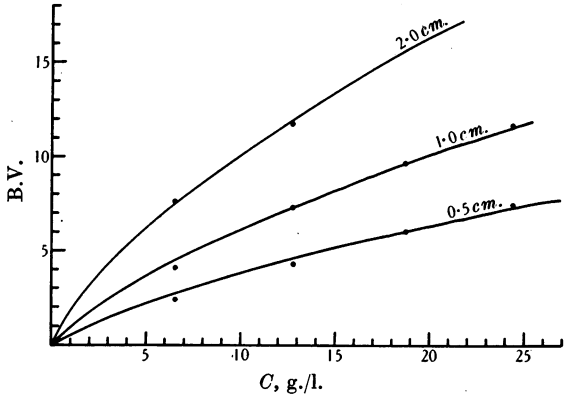

Fig. 6. B.V. and concentration. Cod liver oil.

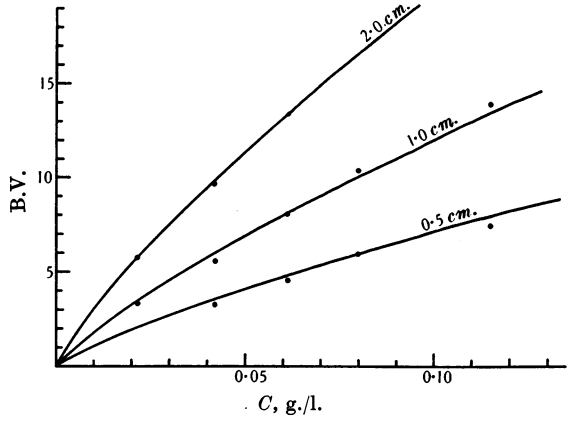

Fig. 7. B.V. and concentration. Halibut liver oil.

This is mainly because tintometer readings, even when carried out with the greatest care by experienced workers, are not very reliable. Variations in lighting compensation and also individual differences, or even differences in the same observer from day to day, all go to making the readings uncertain. It might be

mentioned that the observers in question have had from three to five years' experience with the tintometer.

As the density is proportional to the concentration, the dilution curves for single oils or concentrates would naturally be expected to conform with exponential curves with the same exponents. In Figs. 6 and 7 values for various concentrations and thicknesses of layer for a medicinal cod liver oil and a strong halibut liver oil are drawn together with the corresponding exponential curves; the observed values will be seen to conform satisfactorily with these curves.

Blue value and thickness of layer.

As the blue solution follows Beer's law, it follows from the fact that the blue readings are not proportional to concentration and consequently not to density, that the blue reading cannot be proportional to the thickness of layer, but must follow a relation similar to that between blue reading and concentration or density. We have measured one ordinary and one potent oil in 0·5, 1 and 2 cm. cells; the measurements were made by three experienced observers. The resulting average readings are given in Table III. Graphically, in an ordinary coordinate

Table III.

Cod liver oil			Halibut liver oil		
Layer cm.	Concentration	B.V.	Layer cm.	Concentration	B.V.
0·5	6·55	2·4	0·5	0·0418	3·3
	12·8	4·3		0·0613	4·6
	18·75	6·0		0·080	5·9
	24·4	7·4		0·115	7·4
1	6·55	4·1	1	0·0214	3·3
	12·8	7·3		0·0418	5·6
	18·75	9·6		0·613	8·1
	24·4	11·7		0·080	10·4
2·0	6·55	7·6		0·115	13·9
	12·8	11·3	2·0	0·0214	5·8
				0·0418	9·7
				0·0613	13·4

system (Figs. 8 and 9) it appears clearly that the readings are not proportional. Curves for exponential functions with the exponent 0·8 for the potent oil, 0·7 for the normal oil, have been drawn in, the values will be seen to agree well with these curves. In a bi-logarithmic system they will be seen (Figs. 10 and 11) to lie approximately on straight lines, with slopes corresponding to the exponents mentioned.

In Fig. 10 the lowest values do not agree well with the curve drawn which corresponds to the exponent 0·7. In fact it appears that very low values do not agree well with the exponential function, probably because the density becomes more influenced by the neutral caused by surface reflection, and less by the colour. This, however, is of little practical interest. Such low readings should be avoided anyhow as they are uncertain.

Why is the colour/concentration relation not linear?

The fact that the blue solution follows Beer's law, even in oil concentrations approaching three times those usually employed, and that the colour is not proportional to the layer of identical solutions, makes it obvious that the non-linear relation cannot be attributed to any "inhibitory" effect of the oil. The explanation must be purely physical, and must lie in the nature of the colours that are matched so as to appear alike to the eye.

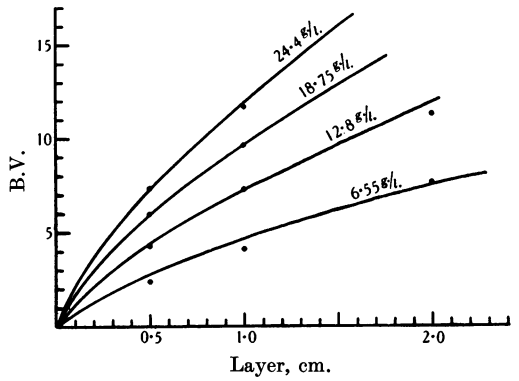

Fig. 8. B.V. and thickness. Cod liver oil.

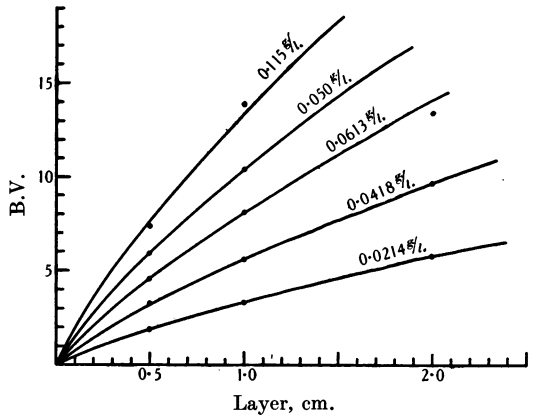

Fig. 9. B.V. and thickness. Halibut liver oil.

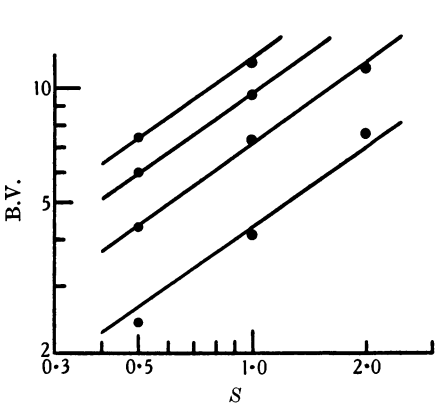

Fig. 10. B.V. and thickness on bi-logarithmic coordinates. Cod liver oil.

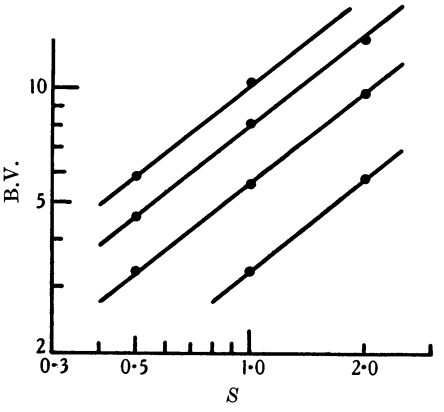

Fig. 11. B.V. and thickness on bi-logarithmic coordinates. Halibut liver oil.

For two colours to appear alike to the eye, it is necessary that two factors are more or less identical. First the colour quality must be the same. (Any given colour quality may be produced in innumerable ways by mixing three colours, provided that none of these can be produced by mixing the two others. Thus the tintometer can theoretically produce all known colour qualities.) If the colour quality is the same, to appear alike the two colours must transmit equal percentages of the incident light, reduced wave-length by wave-length to the sensitivity of the human eye. Such measurements of transmission may be made by computation, as has been done for the tintometer glasses by Gibson & Harris [1922], or more conveniently by means of a special photo-electric cell which has the same sensitivity curve as the eye. We have made photo-electric measurements of the transmission of the glasses of one of our tintometers, and for oil blue and concentrate blue solutions. The results should give straight lines in a linear/logarithmic coordinate system, and from Fig. 12 will be seen that they practically

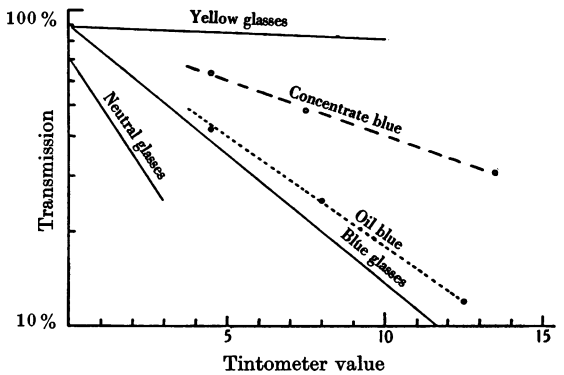

Fig. 12. Tintometer value and transmission.

do so. The points on the glass lines have been omitted as they lie practically on the line. It will be seen that the oil blue is only slightly more transparent than the glasses, bearing out the fact that ordinary oil blues need only yellow and little or no neutral for compensation. The yellow glasses practically only change the colour quality; they reduce the brightness very little.

Concentrate blues are much more transparent than the corresponding glass combination, so they need substantial reduction in brightness by neutral glasses.

The great physical difference of the colours is shown in a striking manner if the complete absorption curves of the 10 blue glass and oil and concentrate blue solutions which would need 10 blue to match are drawn together. In Fig. 13 the broken lines are the unreduced curves, the whole lines are the curves after they have been reduced to the sensitivity curve of the eye. The glass curve, measured on one of our tintometers, corresponds closely with that of Gibson & Harris [1922].

Discussion.

As has been shown, the reason why the blue readings of antimony trichloride blue solutions are not proportional to concentration is purely physical (for a relation of a similar nature see Ferguson [1935]). It should therefore be possible to find the exact relation between density and blue reading through exact mathematical calculation, based on the laws of physics and physiological optics. This we shall not attempt here. For practical purposes the relations established

earlier in this paper agree with experiment well within the limits of error of the methods, and the nature of the laws of light absorption makes it probable that the exact relation would have a similar form.

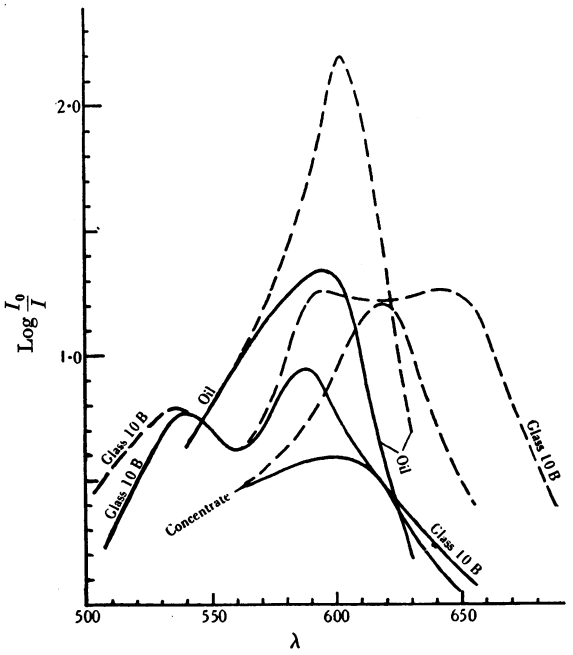

Fig. 13. Absorption curves for oil, concentrate and glass of tintometer value 10 blue.
– – – – Measured curves; —— Reduced curves.

The fact that the dilution curve follows an exponential function, together with the fact that variations in the amount of chromogen solution from 0·05 to 0·4 ml. do not affect the result means a considerable simplification of the determination of the blue value.

It is easy to understand why it has been necessary to measure the blue value at or near a given value, usually 6 blue. The "blue value" of a chromogenic substance must in reality be based on the concentration necessary to give an agreed reading (6) in the tintometer. This divided into the standard concentration, 18·18 g./l., and multiplied by the standard reading, 6, gives the blue value. Now, what has been said makes it possible to determine this concentration without diluting backwards and forwards to obtain a solution which gives 6 blue with 0·2 ml. + 2 ml. reagent. Any reading, at least between 4–5 and 10–12 blue, obtained with any amount between 0·05 and 0·4 ml. chromogen solution may be used for finding the "6-concentration".

If the concentration C gives a blue value B, the concentration c_6 which will give a blue reading 6 can be found by the equation

$$c_6 = C_B \cdot \left(\frac{B}{6}\right)^{\frac{1}{0\cdot7}}$$

or 0·8 for concentrates.

This means that it will be possible to get readings on solutions from less than half as strong to six or eight times stronger than that which would give 6 blue with 0·2 ml.

The equation is, of course, easily worked out on a slide-rule, but if several are to be worked out a graph like Fig. 14 will be a great help. Here the reading 6 corresponds to a relative concentration k. A reading B will be found to correspond to a relative concentration C_B the value of which may be read from the graph, and the concentration which will give 6 is $\frac{1}{C}$ of the concentration which gave B. The graph is even easier to construct on bi-logarithmic paper (for instance Schleicher & Schull, Düren, paper no. 365½) where exponential curves, as has been said before, are straight lines.

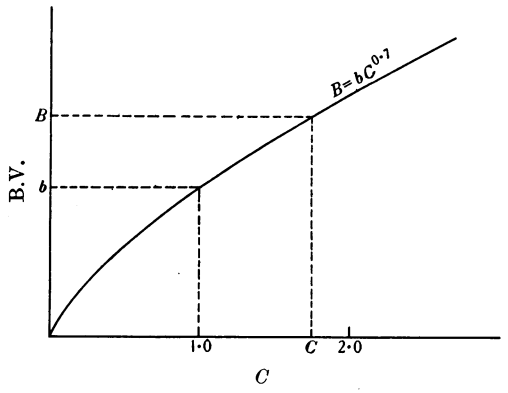

Fig. 14.

The relation between density and tintometer reading throws light on the relation between the blue value and the concentration of the substance, very probably vitamin A, which gives rise to the 618 and 603 $m\mu$ bands. If the extinction coefficient of the chromogen itself is unaltered when the band shifts, then it will be apparent from Figs. 4, 5 and 13 that a 603 $m\mu$ blue solution will contain nearly twice as much chromogen as a 618 $m\mu$ blue solution giving the same reading. Or, on the other hand, a solution with a given maximum density at 603 $m\mu$ will give a much lower reading in the tintometer than a solution with the same density at 618 $m\mu$.

Here again purely physical phenomena throw light on a question concerning the antimony trichloride reaction which has not so far found a satisfactory explanation, the relation between the blue value of oils and their concentrates. If the extinction coefficient, calculated for the oil, is unchanged by the preparation of the concentrate, then a blue value perhaps nearly twice that of the oil should be expected. Actually still higher values are found [v. e.g. Morgan & Pritchard, 1936]; this is because the extinction coefficient increases if no chromogen is lost, as the fatty matter inhibits the reaction. This side of the question will be dealt with in a paper which we hope will appear shortly.

Summary.

Complete absorption spectra of the blue colours concerned in the antimony trichloride reaction have been taken.

The blue solutions, both of oils and concentrates, follow Beer's law, far beyond the concentrations encountered in practice. Only when amounts of oil solution above 0·5 ml. are used is a slight falling off observed, which is due to dilution of

the reagent and not to chemical inhibition by the oil. The amount of oil solution may safely be varied from 0·05 to 0·4 ml.

The well-known fact that the blue readings are not proportional to concentration is confirmed spectrographically. As the solutions follow Beer's law, the lack of proportionality is a purely physical phenomenon.

The following relations are found between E and blue reading:

$$\text{B.V.} = 5{\cdot}5 . E^{0{\cdot}7} \text{ for max. } 603 m\mu.$$
$$\text{B.V.} = 8{\cdot}5 . E^{0{\cdot}8} \text{ for max. } 618 m\mu.$$

Between a blue reading B obtained by a concentration C and a reading b obtained by a reading c there exists the relation

$$b = B . \left(\frac{c}{C}\right)^{0{\cdot}7} \text{ (0·8 for concentrates).}$$

The blue value, as read in the tintometer, of a solution with a given absorption maximum is 40–60 % higher when the band lies at $618 m\mu$ than when it lies at $603 m\mu$. Even if the reaction were not inhibited by the oil, a concentrate should therefore always give a blue value nearly twice that of the corresponding oil if they are measured at the same reading.

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