CXLVII. THE ANTIMONY TRICHLORIDE COLOUR TEST AND THE ULTRA-VIOLET ABSORPTION OF LIVER OILS AND CONCENTRATES.

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In a recent paper [Morton, Heilbron and Thompson, 1931] it was stated that the blue solution obtained by the interaction of liver oils and antimony trichloride was liable to exhibit an absorption maximum at $572m\mu$, instead of, or in addition to, the maximum at $606m\mu$ which has hitherto been studied [Morton and Heilbron, 1928; Drummond and Morton, 1929]. It was also recorded that with concentrates a maximum at $583m\mu$ occasionally made its appearance with or without the simultaneous appearance of the more usual band at $620m\mu$. No wholly satisfactory explanation of these observations being forthcoming, it was decided to examine a large number of oils and concentrates as completely as possible with a view to re-testing the correlation between ultra-violet absorption [Morton and Heilbron, 1928] and the spectroscopic data on the colour test. For this purpose, the intensities of absorption at $572m\mu$ and $606m\mu$ were determined for the blue solutions, irrespective of whether one or two maxima were visually apparent in the eye-piece of the spectroscope.

From the results of Drummond and Morton [1929] it is clear that the blue colour with antimony trichloride and the ultra-violet band at $328m\mu$ both vary in intensity exactly as would be expected if both were due to vitamin A. The experimental error of the biological assay is however so great that although the correlation of these properties with the vitamin is highly probable, it is not proved in a rigid quantitative sense.

The ultra-violet band is however on a different footing from the blue colour. It provides a mechanism for the photochemical destruction of vitamin A and itself disappears on irradiation. The band moreover appears in the liver-fat of rats fed on a vitamin A-deficient diet with carotene as a growth-promoting supplement, such fat being itself potent in vitamin A. The band is however absent from the liver-fat of rats maintained on a vitamin A-free diet without added carotene [Moore, 1930; Drummond, Ahmad and Morton, 1930]. Similar considerations indicate that absence of chromogen coincides with absence of vitamin, and presence of chromogen coincides with presence of vitamin. This state of affairs would be not unsatisfactory were it evident that the chromogenic property of liver oils is due to a single, well-defined entity. The colour-

producing mechanism is however complicated and difficult to describe unambiguously. The displacement from 606 to 620 $m\mu$ shown by concentrates is troublesome in the sense that it lessens the precision of the description of the blue colour. It raises the question as to whether purer preparations of vitamin A would give rise to an enhanced displacement. Moreover the appearance of bands at 572 or 583 $m\mu$ in certain oils and concentrates can only mean an unusual variability of the colour substance or complex, or else the existence of a new chromogen in certain oils and concentrates. If a new chromogen can appear in a few oils, the possibility cannot be evaded that all oils may contain varying proportions of two chromogens.

As a first step in investigating this matter, attention may be drawn to the fact that if a series of oils is tabulated in order of increasing intensity of absorption at 328 $m\mu$, the absorption at 606 $m\mu$ should increase steadily down the table. Examination of Table I, which shows the data for 52 liver oils in

Table I. *Cod- and other fish-liver oils in which the value of $E_{1\text{ cm.}}^{1\%}$ 328 $m\mu$ is less than the value of $E_{1\text{ cm.}}^{1\%}$ 606 $m\mu$.*

328 $m\mu$	606 $m\mu$	328 $m\mu$	606 $m\mu$
0.14	0.15	1.38	1.59
0.5	0.54	1.40	1.60
0.5	0.84	1.50	1.51
0.53	0.62	1.55	1.80
0.55	0.76	1.55	1.91
0.62	1.0	1.64	1.75
0.66	0.88	1.70	1.9
0.8	1.22	1.87	1.98
0.8	1.07	1.95	2.05
0.85	0.88	2.62	3.70
0.96	1.38	3.67	5.1
0.97	1.5	4.83	8.5
1.0	1.2	5.0	12.0
1.05	1.7	5.2	9.5
1.05	1.35	5.2	6.84
1.1	1.68	5.25	8.5
1.1	1.18	5.3	7.5
1.13	1.55	5.4	7.8
1.13	1.65	6.0	8.91
1.15	1.75	6.0	7.5
1.15	1.47	6.0	7.72
1.20	1.35	6.25	9.2
1.20	1.82	7.0	9.0
1.25	1.45	10.0	21.5
1.26	1.65	10.0	15.0
1.30	1.38	11.0	18.8

all of which the 606 $m\mu$ band is well shown, discloses the same general trend over the table as a whole, but many minor discrepancies. In Table II are given the data for 19 oils in which the intensity of the 606 $m\mu$ band is unusually low in relation to the ultra-violet intensity of absorption.

If now a selection from both Tables is made of a number of oils practically indistinguishable as regards ultra-violet absorption, it will be seen how very far from real constancy the blue colour intensities can be.

The conclusion is inevitable that either the over-all experimental error of the physical assay is 250 %, or the 606 $m\mu$ chromogen and the substance

Table II. *Cod-liver oils in which the value of $E_{1\text{cm.}}^{1\%}$ 328m μ is greater than the value of $E_{1\text{cm.}}^{1\%}$ 606m μ .*

328m μ	606m μ	328m μ	606m μ
0.14	0.05	1.15	1.0
0.50	0.50	1.15	0.72
0.57	0.50	1.22	1.0
0.58	0.58	1.35	1.25
0.62	0.35	1.37	1.22
0.75	0.57	1.38	1.25
0.75	0.45	1.8	1.78
0.85	0.81	1.86	1.53
0.85	0.66	1.89	1.62
1.07	0.72		

Table III. *Cod-liver oils closely alike as regards ultra-violet absorption but differing in blue value.*

$E_{1\text{cm.}}^{1\%}$ 328m μ	$E_{1\text{cm.}}^{1\%}$ 606m μ	$E_{1\text{cm.}}^{1\%}$ 328m μ	$E_{1\text{cm.}}^{1\%}$ 606m μ
1.07	0.72	1.15	0.72
1.1	1.18	1.15	1.00
1.05	1.75	1.15	1.47
1.05	1.70	1.15	1.75
1.1	1.68		

responsible for the 328m μ maximum are not simply related. The discrepancy is vastly greater than any experimental error of which we are aware in the physical measurements.

It must however be emphasised that these data embody the results of a very large number of tests on materials ranging from the purest medicinal oil to very crude fish-liver oils of the cattle feeding variety.

A careful inspection of our data on concentrates reveals a similar state of affairs.

Table IV. *To illustrate the more rapid increase in the 620m μ band than in the 328m μ as richer and richer concentrates are compared.*

$E_{1\text{cm.}}^{1\%}$ 328m μ	$E_{1\text{cm.}}^{1\%}$ 620m μ	$E_{1\text{cm.}}^{1\%}$ 328m μ	$E_{1\text{cm.}}^{1\%}$ 620m μ
13	22.4	267	650
22	33	275	400
85	174	357	772
112	274	370	950
113	200	500	1140
115	213		

Judging from the mass of data it is impossible to regard these two sets of figures on the blue colour and the ultra-violet absorption respectively as measuring the concentration of the same substance.

We may now consider the data for oils and concentrates in which the intensities of absorption in the colour test have been measured both at 572 and 606m μ (583 and 620m μ for concentrates).

From Table V it will be seen that there is a much closer parallelism between the intensities at 572 and 328m μ than is to be found between those of the 606

Table V.

Material	Ultra-violet absorption		Colour test	
	$E_{1\text{ cm.}}^{1\%}$ 328 $m\mu$	$E_{1\text{ cm.}}^{1\%}$ min.	$E_{1\text{ cm.}}^{1\%}$ 572 $m\mu$	$E_{1\text{ cm.}}^{1\%}$ 606 $m\mu$
Codling-liver oil (a)	0.125	0.11	0.12	0.06
„ (b)	0.22	0.20	0.20	0.13
Cod-liver oil 1	0.38	0.38	0.33	0.33
„ 2	0.55	0.45	0.54	0.54
„ 3	0.55	0.52	0.54	0.62
„ 4	0.55	0.55	0.37	0.47
„ 5	0.57	0.50	0.50	0.50
„ 6	0.57	0.50	0.50	0.58
„ 7	0.67	0.57	0.50	0.61
„ 8	0.75	0.65	0.57	0.57
„ 9	0.82	—	0.83	0.25
„ 10	0.80	—	0.70	0.92
„ 11	0.85	0.55	0.68	0.85
„ 12	0.85	—	0.70	0.88
„ 13	0.95	—	0.92	1.14
„ 14	0.95	0.75	0.81	1.20
„ 15	1.03	0.80	1.02	1.35
„ 16	1.1	—	1.0	1.18
„ 17	1.15	0.95	1.25	1.35
„ 18	1.27	1.05	1.41	1.45
„ 19	1.30	—	1.28	1.50
„ 20	1.37	0.9	1.40	1.22
„ 21	1.40	—	1.37	1.25
„ 22	1.50	0.7	1.65	1.51
„ 23	1.60	—	1.20	1.65
„ 24	1.70	—	1.85	1.90
„ 25	1.65	0.55	1.45	1.92
„ 26	1.80	—	1.43	1.78
„ 27	1.86	1.60	1.74	1.53
„ 28	1.87	—	2.32	1.98
„ 29	1.89	0.50	1.86	1.62
„ 30	1.95	1.0	1.80	2.05
„ 31	4.3	—	3.81	4.76
Fish-liver oil 1	6.2	2.5	5.83	8.91
„ 2	3.67	2.0	3.4	5.1
Halibut-liver oil	11.5	4.0	23	28
Liver oil	4.83	—	4.4	8.5
Concentrate	57.5	—	59.4	101.2
„	267.0	170	286	650

and 328 $m\mu$ bands. This fact is fully borne out in Table VI, which is merely a representative selection of the data which have been accumulated.

Table VI. *The trend of spectroscopic data with increasing concentration of vitamin A.*

	$E_{1\text{ cm.}}^{1\%}$ 328 $m\mu$	$E_{1\text{ cm.}}^{1\%}$ 572 to 583 $m\mu$	$E_{1\text{ cm.}}^{1\%}$ 606 to 630 $m\mu$
Cod-liver oils	1.0	1.0	1.35
Rich fish-liver oils	6.25	5.8	9.0
Concentrates 1	55	60	100
„ 2	267	286	650
„ 3	370	400-500	950

The results of a comparison of biological assays made in London with similar spectroscopic data, support the view which is emerging from this work, namely that the 572 $m\mu$ (583 $m\mu$) chromogen is identical with the substance

responsible for the $328m\mu$ band and is in fact vitamin A, whereas the $606m\mu$ ($620m\mu$) band has been recorded at from $\frac{1}{3}$ to 3 times the intensity of the $328m\mu$ band, a total variation of the order 10:1, whilst the gross variation for the comparison of the $572m\mu$ ($583m\mu$) intensity at the $328m\mu$ band is less than 2:1.

The use of the $328m\mu$ band as a quantitative criterion of vitamin A involves the measurement of the gross absorption at $328m\mu$, a magnitude theoretically including:

- (i) the true contribution of the $328m\mu$ band, *plus*
- (ii) the contribution of the materials always present in cod-liver oils *etc.*, *i.e.* the normal fatty glycerides and the cholesterol, *plus*
- (iii) the contribution of the variable and occasional non-vitamin A constituents, *e.g.* free acids and other impurities.

We have hitherto [Morton and Heilbron, 1928; Drummond and Morton, 1929] used a correction term of 0.13 to 0.22 ($\log I_0/I$ for a 1 % solution and a 0.1 cm. cell) for the joint effect of (ii) and (iii) in average medicinal cod-liver oils. Further experience on a wider range of materials has convinced us that the absorption of the fatty glycerides and the cholesterol is negligibly small, and that the excessively high values for the absorption at $328m\mu$ arise from the presence of impurities which vary from sample to sample. The correction of the $328m\mu$ intensity has therefore been abandoned as too uncertain in its operation. On the whole there is very striking agreement, considering the adverse conditions, between the intensity of absorption at 328 and $572m\mu$ ($583m\mu$). This implies that the molecular extinction coefficient of the $328m\mu$ absorption band found in alcoholic solutions is approximately the same as that of the blue pigment band at $572m\mu$ resulting from the interaction of antimony trichloride with the chromogen. It is not uncommon in the study of the absorption spectra of related organic compounds to meet cases of bodily wave-length displacement of an absorption band without great change in extinction coefficients as we pass from one substance to others. That such a result is found with liver oils perhaps gives independent support to the view that the ultra-violet band and the $572m\mu$ band in the colour test are properties of vitamin A and a derivative respectively.

It may be stated that we have carefully explored the possibility that the intensity of absorption at $328m\mu$ is a summation of two factors, one the absorption arising from the $572m\mu$ chromogen and the other that due to the $606m\mu$ chromogen. This enquiry indicates that the substance responsible for the $606m\mu$ band with antimony trichloride is very transparent to light of the wave-lengths near 320–330 $m\mu$.

The views we have advanced necessitate some caution in using the Lovibond technique as a quantitative method of assay. The blue colour is complex, arising from at least two apparently independent absorption bands; in oils we find with antimony trichloride a band in the yellow-green ($572m\mu$), and a band in the orange ($606m\mu$), whilst with concentrates the bands are in the

yellow ($583m\mu$) and the orange-red ($620m\mu$). Complementary to these bands we have colours which may be roughly described as ranging from violet-blue to greenish-blue. Matching such colours against glasses coloured blue with cobalt gives only a very approximate evaluation of intensities because the procedure rests upon the subjective matching of physically distinct phenomena. Moreover the two absorption bands both count in the matching, so that the colorimetric procedure results in a rough evaluation of the oils as regards the combined quantities of two chromogens present. In actual practice the Lovibond technique gives better results than would be anticipated from this argument, owing to the fact that in a fair percentage of cod-liver oils the relative intensities of the $606m\mu$ and $572m\mu$ absorption bands are near 1.35:1. A prolonged search has however, as we have shown, made clear that this ratio is by no means to be relied upon in all cases.

We have further obtained definite evidence [Heilbron, Gillam and Morton, 1931] that the concentration of the $606m\mu$ chromogen can increase spontaneously without any accompanying change in the $572m\mu$ chromogen or the $328m\mu$ absorption intensity. Finally we have been able to achieve this increase in the laboratory, so that the existence of two independent chromogens in liver oils seems to be established.

CONCLUSIONS.

Liver oils contain two chromogens which, with antimony trichloride, give coloured substances with absorption maxima at $606m\mu$ and $572m\mu$ respectively. In concentrates these maxima are displaced to $620m\mu$ and $583m\mu$. A comparison of ultra-violet absorption spectra with spectroscopic data on the colour test discloses (i) that the parallelism between the intensity of the $606m\mu$ band and the intensity of the $328m\mu$ band breaks down so seriously in extreme cases as to render it improbable that the $606m\mu$ chromogen is vitamin A; (ii) that the $572m\mu$ chromogen and the substance responsible for the $328m\mu$ band are probably identical, (iii) that the blue colour for rich oils and concentrates is often much deeper than would be expected on the basis of correlation between blue colour and ultra-violet absorption. Hence it is concluded that the matching of blue colours with Lovibond glasses, though it may act as a rough guide to vitamin A potency is theoretically unsound.

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