CXLI. THE USE OF 7-METHYLINDOLE IN THE ANTIMONY TRICHLORIDE COLOUR TEST FOR VITAMIN A.

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It is now generally agreed that the blue colour which results when antimony trichloride is added to chloroform solutions of liver oils and concentrates owes its origin, in part at least, to vitamin A. As a quantitative criterion of potency, the test, however, suffers from considerable disadvantages, among which the following may be mentioned.

(a) The chromogenic power of the substances present in liver oils is smaller than would be expected from the values obtained with the unsaponifiable extracts [Smith and Hazley, 1930; Smith, 1931; Coward, Dyer, Morton and Gaddum, 1931] owing to the occurrence of inhibitors in the saponifiable portion [*cf.* Norris and Church, 1930, 1, 2]. This effect is highly variable and militates powerfully against accuracy in the colorimetric assay [Lovern, Creed and Morton, 1931; Gillam and Morton, 1931].

(b) The coloured solutions exhibit complex and qualitatively variable absorption spectra [Morton, Heilbron and Thompson, 1931; Heilbron, Gillam and Morton, 1931]. Maxima in the spectral absorption curves have been recorded at 572–583, 606–620, 643 and 693 $m\mu$, and there is considerable evidence of complexity in the chromogenic constituents of liver oils.

(c) The intensity of absorption at a given wave-length or the absolute depth of blue colour varies considerably with the time elapsing between mixing and recording intensities, and with concentrations in the system: CHCl_3 — SbCl_3 —vitamin A—non-chromogenic substances.

It has been shown [Heilbron, Gillam and Morton, 1931] that with many oils the spectral absorption curve shown after reaction with antimony trichloride has occurred consists of a summation of two distinct absorption bands with maxima at 572 and 606 $m\mu$ respectively. Sometimes one, sometimes the other, is the more intense. In all cases the 606 $m\mu$ band eventually becomes the stronger, either by spontaneous ageing or treatment with ozone. Increases in the 606 $m\mu$ band are never at the expense of a reduction in the intensity of the 572 $m\mu$ band, which remains practically constant, as also does the 328 $m\mu$ ultra-violet maximum characteristic of vitamin A. In concentrates, the 606 $m\mu$ band is shifted to 617 $m\mu$ and is always much stronger than the other band. Heilbron, Gillam and Morton [1931] were able to show

that in all cases the shorter wave maximum is present in concentrates at $583m\mu$ but that its existence is usually indicated only by an inflexion in the absorption curve. By employing a slight modification of the ordinary procedure in carrying out the colour test they were able to demonstrate the real existence of this maximum. The preliminary addition of a very small quantity of antimony trichloride reagent to the chloroform solution of the concentrate, one minute before carrying out the ordinary test, effects a striking reduction in the intensity of the blue colour. This reduction is largely confined to the absorption band at $617m\mu$ so that the $583m\mu$ maximum, which is masked in the solution obtained by the ordinary procedure, is now fully revealed.

In discussing the results, the authors envisaged two main possibilities, namely that the two chromogenic mechanisms denoted either two separate chromogens or two distinct reactions between vitamin A and antimony trichloride.

Brode and Magill [1931] also observed the existence of two variable absorption bands in the colour tests, and recorded maxima at 608 and $578m\mu$. The solutions were unstable and gradually became red, exhibiting maxima at 532 and $472m\mu$. These authors also correlated the 608 and $532m\mu$ maxima and considered the latter to arise from a spontaneous decomposition of the substance causing the 608 $m\mu$ band. Similarly the 578 and $472m\mu$ maxima were found to be connected in some way. In this laboratory the 532 and $472m\mu$ maxima have not been regularly noticed and little evidence of any significant correlation has been obtained. It is, however, important that Brode and Magill obtained valuable evidence of a complex chromogenic mechanism. Like Heilbron, Gillam and Morton [1931] they were however unable to decide whether liver oils contain two separate chromogens or whether the two bands indicate alternative or successive reactions between vitamin A and antimony trichloride.

Another valuable piece of confirmatory evidence was supplied by Emmerie, Eekelen and Wolff [1931]. These authors state: "By treating a vitamin A preparation...with some drops of furan, methylfuran, pyrrol, indol, or skatol (all substances of related chemical structure), and then adding antimony trichloride, the mixture turns purple instead of blue, and in the spectroscope the $610m\mu$ band is no longer seen, while the $572m\mu$ band remains unaltered. The physiological activity is unimpaired and the preparation (without antimony trichloride) shows the $328m\mu$ band just as the original substance did."

Eekelen *et al.* [1931] extended these observations and recorded data which tend to increase the difficulties of interpretation. Apparently, the low values in the colour test for oils are due to the presence of traces of inhibiting substances, but the results, whilst far from conclusive, point rather to heterogeneity of chromogens than to different stages in the reaction between antimony trichloride and vitamin A.

The work of the Dutch investigators seemed to indicate a more convenient method for separating the chromogenic effects than the original one emanating

from these laboratories. Accordingly the statements made were tested and fully substantiated.

A large number of pure substances of the type suggested were tried, including 2-, 3- and 7-methylindole. The most convenient substance was found to be 7-methylindole.

In Table I the fact is demonstrated that the $617m\mu$ maximum suffers considerable inhibition before the $583m\mu$ maximum is appreciably affected. In

Table I. *Effect of 7-methylindole on the antimony trichloride colour test applied to materials very rich in vitamin A.*

	Wt. of concentrate used mg.	Wt. of 7-methyl- indole used mg.	$E_{1\text{ cm.}}^{1\%} 617m\mu$	$E_{1\text{ cm.}}^{1\%} 583m\mu$	Ratio wt. 7-methyl- indole/wt. concentrate
1	0.0562	0.375	660	1070	6.5
	"	0.300	750	1100	5.2
	"	0.225	910	1180	3.9
	"	0.150	1250	1250	2.6
	"	0.075	1525	1310	1.3
	"	0	2350	1310	—
	"	Trace only	—	1300	—
2	0.0522	0.375	690	965	7.18
	"	0.3	790	960	5.76
	"	0.225	1206	1200	4.3
	"	0.150	1300	1210	2.88
	"	0.075	1870	1260	1.44
	"	0	2160	1350	—
3	0.05	0	3300	1600	—
	"	0.2	1600	1550	4
4	0.04	0	4200	2135	—
	"	0.16	2000	1980	4

Table II the last column indicates the intensity at $583m\mu$ after just sufficient 7-methylindole has been added to render the $617m\mu$ maximum definitely weaker than the $583m\mu$ maximum. The main results may be summarised as follows.

(1) 7-Methylindole, at the concentrations used, is practically transparent in the region $290\text{--}350m\mu$.

(2) The absorption spectrum of a solution containing vitamin A concentrate and 7-methylindole in chloroform agrees quantitatively with the summation of the separate curves for the concentrate and the 7-methylindole, so that there is no evidence of chemical interaction before the addition of antimony trichloride.

(3) In all cases, from cod-liver oils to the richest concentrate, the incorporation of a trace of 7-methylindole effects an inhibition of the capacity to give the blue colour.

(4) The inhibition can be regulated by adding greater or smaller quantities of 7-methylindole.

(5) The masking effect of the 617 band on the $583m\mu$ maximum can be eliminated by adding a carefully regulated quantity of inhibitor so that the

Table II.

Material	$E_{1\text{ cm.}}^{1\%}$ 328 $m\mu$ Ultra-violet absorption	$E_{1\text{ cm.}}^{1\%}$ 617 $m\mu$ Antimony trichloride colour test without added 7-methylindole	$E_{1\text{ cm.}}^{1\%}$ 583 $m\mu$ colour test	$E_{1\text{ cm.}}^{1\%}$ 583 $m\mu$ (colour test) intensity when just enough 7-methylindole has been added to make 617 $m\mu$ band weaker than that at 583 $m\mu$
Halibut-liver oil non-sap. (average)	1000 1154	2350 3030	1350 1450	1300 1300
Pig-liver concentrate	525	1200	840	650
Cod-liver oil concentrate	270	541	360	315
Mammalian concentrate	130	320	230	200
	*(290, 190)			
Cod-liver non-sap.	115	221	124	110
" "	57.5	96	65	60
Pig-liver oil (a)	30	63	41	34
" (b)	14.4	36.8	17.5	14.8
Pig-liver oil (store pigs) (a)	—	0.99	0.92	0.9
" " (b)	—	1.6	1.1	1.0
Cod-liver oil crude non-sap.	16	34.4	18	16
	*(290, 14)			
Sheep-liver oil	19.7	30	28	28
	*(290, 25.7)			
Cod-liver oil	0.73	0.76	0.59	0.5
"	5.5	15.2	9.0	8.2

* Occasionally in fish-liver oils and concentrates, and more often in materials from mammalian livers, we have observed an absorption band (max. 290 $m\mu$) superposed on the 328 $m\mu$ band of vitamin A. Dr T. Moore and Dr N. S. Capper have also observed this effect (private communication). At first sight this band would seem to be due to the decomposition product with a maximum at 290 $m\mu$. This explanation is not wholly satisfactory because the materials exhibiting a maximum at 290 $m\mu$ without other evidence of decomposition, exhibit high values in the colour test for the 583 $m\mu$ band. A substance closely related to vitamin A and not derived from it by decomposition may be responsible. In this connection it is well to emphasise a point which merits further investigation: as has been shown by Kuhn and Brockmann [1931], α -carotene and β -carotene, containing α - and β -ionone rings respectively, are both capable of inducing growth. Presumably each is converted *in vivo* into a vitamin A-like substance. There is thus the possibility of two isomeric vitamin A compounds, one with and one without the ethenoid linkage of the ring conjugated to the side chain. The properties of such isomerides cannot be predicted but it is quite possible that the 290 and 328 $m\mu$ bands may respectively be due to the α - and β -isomerides.

617 and 583 $m\mu$ bands can be seen side by side, at approximately equal intensities, before any appreciable inhibition of the 583 $m\mu$ band has occurred.

(6) Using the richest vitamin A concentrates (Carr-Price blue value > 50,000), the 617 $m\mu$ band is almost exactly twice as intense as the 583 $m\mu$ maximum without addition of inhibitor, but if 7-methylindole is added before the antimony trichloride reagent, in the ratio 7-methylindole/concentrate = 4/1, the two bands appear with roughly equal intensity, the inhibition being confined to the longer wave maximum.

(7) The addition of larger quantities of 7-methylindole effects a slow inhibition of the 583 $m\mu$ band, but this is always very much smaller than the inhibition of the 617 $m\mu$ maximum.

(8) In carrying out the antimony trichloride colour test as a quantitative measure of vitamin A potency, serious inaccuracies arise when the test is carried out on oils owing to the variable action of inhibitors, but these are greatly reduced when observations are carried out on the non-saponifiable

matter [Smith and Hazley, 1930; Coward *et al.*, 1931]. In all cases, much more accurate results can be obtained if attention is concentrated on the $583m\mu$ maximum, using just sufficient 7-methylindole to allow the 617 and $583m\mu$ bands to be seen in the spectroscope. Under these circumstances, the estimates of potency are in close agreement with the values deduced from the intensity of the $328m\mu$ band.

(9) Accepting the molecular weight of vitamin A as near 300 (7-methylindole, 133) and making use of the fact that four parts by weight of inhibitor to one of the richest vitamin preparations are necessary for optimum results, this is equivalent to approximately 10 molecules of 7-methylindole to 1 mol. vitamin.

(10) Inhibition by means of 7-methylindole does not readily occur with the $693m\mu$ band recorded by Morton, Heilbron and Thompson [1931], and it does not appear to be possible to establish any rigid correlation between this band and the $583m\mu$ maximum.

(11) In view of the fact that inhibition occurs with complete success when the colour tests on the richest concentrates of vitamin A are carried out in the presence of 7-methylindole, such concentrates cannot be accepted as homogeneous without a satisfactory explanation of the apparent independence of the 617 and $583m\mu$ bands. Vitamin A may well react in two stages with antimony trichloride, but there is no direct evidence on this point. The ultra-violet absorption spectrum discloses definite information of only one absorbing entity, the colour test reveals two chromogenic mechanisms. No decision is yet possible as to whether the richest concentrates are mixtures of extremely closely related substances, or whether one and the same molecule can give rise to the complex colour reaction.

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