A CRITICAL STUDY OF THE ANTIMONY TRICHLORIDE COLOR TEST FOR VITAMIN A

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The recent criticism of the antimony trichloride color reaction as a specific test for vitamin A, by Hawk (1), and Jones et al. (2), while others still accept the test as at least qualitative is responsible, in part at least, for this study of the antimony trichloride color test. In 1925, Rosenheim and Drummond (3) showed that arsenic trichloride, dimethyl sulfate, trichloroacetic acid, acetyl chloride, and some inorganic dehydrating agents such as sulfuric acid and phosphorus trichloride give a deep blue color with cod liver oil. In 1926, Carr and Price (4) suggested a reaction based on the use of antimony trichloride, but failed to show any quantitative relation between the color test and biological assay. Wokes and Willimott (5) suggested the use of a saturated solution of antimony trichloride, and also that the solution of the oil should be made the same day as it was to be used.

Concerning the specificity of the test the following references will give some idea of the variance of opinion with regard to its accuracy. Jones, Briod, Arzoomanian, and Christiansen (2) compared the biological test with the Wokes method and found the results incompatible. Norris and Danielson (6) claim that the color test checks within reasonable limits with the biological assay. Von Euler, Rydboom, and Hellstrom (7) modified the method by determining the greatest dilution at which the blue color was just visible. With this method, results closely agreeing with the animal testing methods were obtained. Ahmad and Drummond (8) found that results of animal tests agree within reasonable limits with those of the colorimetric method, although they make no claim that the color reaction is specific. Evers (9) found that the quantity of oil taken influenced the final color.
By adding an inactive oil, *e.g.* peanut oil, he found his results to be more uniform. Hawk (1) claims that cod liver oil exposed to air or other similar treatments showed a deeper blue color with antimony trichloride than oil kept in the dark. This and other observations caused Hawk to question the validity of the antimony trichloride test for vitamin A. Drummond (10) could not, on the other hand, confirm this observation of Hawk. Norris and Church (11) have shown that the test must be controlled with respect to the temperature, time, and concentration of the reagents.

Von Euler and Rydbom (12) have defined the unit quantity of vitamin A as a substance sustaining growth promotion for a period (8 weeks) on a minimal dosage of 0.05 mg. per day. Drummond (13) has pointed out that in feeding tests a test is really made to determine whether all the deficiencies of the diet are made good by the supplement, and hence there must be considerable possible error and variation in these biological assays.

Wokes (14) (with one of us (W. R. B.)) pointed out that with cod liver oil and antimony trichloride, two bands are produced, one between 475 and 482 *mµ* and the other between 535 and 550 *mµ*. There is also a sharply defined band at about 614 *mµ* which fades and after several minutes a second band is produced at 528 *mµ*. Concomitant with this fading there is a change in color of the solution from blue to red.

There has apparently been no systematic study of this color reaction in which the absorption spectra of the solutions were determined, the original investigators and most of the later workers using the Lovibond tintometer or some form of colorimeter rather than a spectrophotometer. In the present work a Bausch and Lomb spectrophotometer was used. This instrument had attached to it a modified Duboscq colorimeter which permitted the rapid introduction of solutions into the optical path, the rapid change of cell thickness, and the use of a convenient all glass cell. The construction of this spectrophotometer permitted either a qualitative examination over a wide portion of the spectrum to determine the position of the band or a quantitative determination, within a narrow portion of the spectrum, of the intensity of the band. Observations on a number of solutions showed that the blue solutions produced a band in the red
portion of the spectrum which was not always in the same place but varied between two different wave-length positions—one at 578 m\(\mu\) and the other at 610 m\(\mu\). The separate identity of these bands could be shown in some solutions in which both bands could be observed at the same time, while in other solutions either one or the other appeared. The maxima of both of these bands were obtained in about the same time interval, 20 to 40 seconds at 25°. However, since readings could be started within 5 seconds after the solutions were mixed together and continued at 5 second intervals thereafter, no difficulty was experienced in observing the maxima and in actual practice greater time intervals than 5 seconds were used.

Examination of the faded or red solutions showed the presence of two bands, one at 532 m\(\mu\) and the other at 472 m\(\mu\). On comparison of these results with those obtained from the blue solutions it was found that solutions exhibiting the 578 m\(\mu\) band gave on fading the 472 m\(\mu\) band and the solutions exhibiting the 608 m\(\mu\) band gave on fading the 532 m\(\mu\) band. Solutions showing both the 578 and 608 m\(\mu\) bands gave both the 472 and 532 m\(\mu\) bands. The earlier workers, using colorimetric methods, rather than spectrophotometric methods of observation were unable to distinguish other than slight differences in hue between these two solutions. Two possible explanations for the existence of these two series of bands are that two different substances are present, or that the conditions of concentration, temperature, or other substances present may cause a change in the molecular configuration of the color-forming substance. The available evidence seems to favor both of these explanations. By changing the concentration of solutions, changing the temperature, or by the introduction of certain chemicals an oil may be made to give either of the two bands, providing, of course, that some of the color-forming material is present. On the other hand carotene, the much discussed supposed precursor of vitamin A, gives with the antimony trichloride reagent a sharp band at 590 m\(\mu\) which fades as do the cod liver oil bands to yield a band at 488 m\(\mu\).\(^1\)

From these latter results and from a number of observations on other oils it seemed that of the two possible bands which might

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\(^1\) We are indebted to Doctor James H. C. Smith of the Carnegie Institution of Washington for a sample of carotene for these experiments.
be used to indicate the concentration of the vitamin A, the 608 $m\mu$
band was to be preferred.

The optimum conditions of concentration for the production

![Graph](image)

**Fig. 1.** Change of extinction coefficient values (532 $m\mu$ band) with time for various concentrations of cod liver oil. The concentration of SbCl$_3$ in these solutions was 14 per cent (unsaturated). The extinction coefficient values have been reduced to equivalent oil concentrations.

of the 608 $m\mu$ band were determined by a series of tests on a few commercial samples of cod liver oil.

It was found that the best results were obtained by using a saturated solution of antimony trichloride in anhydrous chloro-
form. With a less than saturated solution of antimony trichloride, the absorption readings were not proportional to the amount of oil taken, without making a correction. The effect of concentration of SbCl₃ reagent can be seen in the accompanying graphs. In Fig. 1, a 14 per cent solution of SbCl₃ is used, and the spectro-

![Diagram](http://www.jbc.org/)

**Fig. 2.** Same as Fig. 1, except that the extinction coefficient values have been corrected by subtracting 0.40 before being reduced to equivalent oil concentrations.

photometric readings are changed to what they would be if a 10 per cent solution of oil had been used, and a cell thickness of 4 cm. In Fig. 2, a correction (empirical) has been made by subtracting 0.40 from the spectrophotometric readings before changing the concentrations to 10 per cent. With this correction,
the values check very closely with each other, except for solutions containing 6 per cent or less of oil. With a saturated solution of SbCl₃, the readings are proportional to the concentration of the oil. These readings were made on the 532 mµ band. Figs. 3 and 4 show the effect of varying the concentration of the SbCl₃ reagent on the 608 mµ absorption band. Again, with saturated solutions of SbCl₃, the values of the extinction coefficient, with the exception of those below 6 per cent, are proportional to the concentration of the oil used. With less than saturated solutions of SbCl₃, the readings are not proportional to the concentration of the oil.

The saturated solution of antimony trichloride in chloroform was prepared in the following manner. Anhydrous antimony
Anhydrous chloroform was prepared by washing the c. p. product with water to remove the alcohol, dried over calcium chloride, and finally dried with sodium. The chloroform was poured off from the sodium and distilled (b.p. 61.5°). The solution of the reagent was prepared by adding the anhydrous chloroform to the anhydrous antimony trichloride, and refluxed until the antimony trichloride was all melted (m.p. 73°). The mixture was shaken gently and then allowed to cool, whereby the excess antimony trichloride crystallized out. By this method, a saturated solution

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**Fig. 4.** Change of extinction coefficient values (608 mμ band) with time for various concentrations of cod liver oil. The concentration of SbCl₃ in these solutions was 16 per cent (unsaturated). The extinction coefficient values have been reduced to equivalent oil concentrations.
of antimony trichloride in chloroform (about 18.5 per cent by weight) could be quickly made. This solution becomes yellow on standing and should therefore be renewed frequently.

The concentration of the oil used determines whether the spectroscopic readings are proportional to the amount of oil taken. It was found that by using a saturated solution of antimony trichloride and a solution of cod liver oil of such a strength that only the 608 m\(\mu\) band is produced that the concentration of the oil is proportional to the reading of the 608 m\(\mu\) band 20 seconds after mixing. The time, 20 seconds, was chosen because the maximum absorption generally occurs then. If a more concentrated solution of oil is used, the 578 m\(\mu\) band is produced. In the particular oils tested, the 608 m\(\mu\) and 532 m\(\mu\) readings for a 6 per cent or less solution of oil are at variance with the expected values.

The procedure adopted for the analysis of oils is as follows: Place 5 cc. of saturated antimony trichloride solution in a spectrophotometric cell. Add 1 drop of acetic anhydride (to react with hydrochloric acid, water, etc., present). Add 0.5 cc. of a chloroform solution of the oil to be tested, so that it forms a layer on top of the antimony trichloride reagent. Mix by shaking, and at

### TABLE I

<table>
<thead>
<tr>
<th>SbCl(_3) per cent</th>
<th>Cod liver oil per cent</th>
<th>Cod liver oil solution cc.</th>
<th>Band produced</th>
</tr>
</thead>
<tbody>
<tr>
<td>9.5 20</td>
<td>0.1-1.2</td>
<td>Present</td>
<td></td>
</tr>
<tr>
<td>9.5 40, 60</td>
<td>1.0</td>
<td>&quot;</td>
<td></td>
</tr>
<tr>
<td>13.0 20</td>
<td>0.1-0.5</td>
<td>Present</td>
<td></td>
</tr>
<tr>
<td>16.5 1</td>
<td>0.1-0.5, 4</td>
<td>Present</td>
<td></td>
</tr>
<tr>
<td>16.5 2</td>
<td>1, 2</td>
<td>Present</td>
<td></td>
</tr>
<tr>
<td>16.5 2</td>
<td>5</td>
<td>&quot;</td>
<td></td>
</tr>
<tr>
<td>16.5 10, 20, 30</td>
<td>0.5</td>
<td>Present</td>
<td></td>
</tr>
<tr>
<td>16.5 40, 50</td>
<td>0.5</td>
<td>&quot;</td>
<td></td>
</tr>
<tr>
<td>16.5 60, 70, 80, 90, 100</td>
<td>0.5</td>
<td>Present</td>
<td>Present</td>
</tr>
<tr>
<td>Saturated 2-30</td>
<td>0.5</td>
<td>&quot;</td>
<td></td>
</tr>
<tr>
<td>40, 50</td>
<td>0.5</td>
<td>&quot;</td>
<td></td>
</tr>
<tr>
<td>50-100</td>
<td>0.5</td>
<td>&quot;</td>
<td></td>
</tr>
</tbody>
</table>
exactly 20 seconds after mixing, the intensity of the 608 mp band is observed. Then a reading is made at 578 mp, to note if there is a band there. If a band is present at 578 mp, the oil solution is too concentrated, and a more dilute solution should be used.

### Table II

**Relationship between Bands at 608 mp and 532 mp**

<table>
<thead>
<tr>
<th>Observation No.</th>
<th>Cod liver oil (per cent)</th>
<th>Extinction coefficient</th>
<th>Ratio</th>
<th>Mean</th>
<th>Probable error</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>608 mp (20 sec.)</td>
<td>532 mp (5 min.)</td>
<td>608 mp/532 mp</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>10</td>
<td>1.50</td>
<td>0.48</td>
<td>3.12</td>
<td>2.84 ± 0.09</td>
</tr>
<tr>
<td>2</td>
<td>10</td>
<td>1.75</td>
<td>0.70</td>
<td>2.50</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>10</td>
<td>1.65</td>
<td>0.55</td>
<td>3.00</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>10</td>
<td>0.95</td>
<td>0.45</td>
<td>2.11</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>10</td>
<td>1.12</td>
<td>0.36</td>
<td>3.11</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>10</td>
<td>1.27</td>
<td>0.39</td>
<td>3.28</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>10</td>
<td>1.25</td>
<td>0.55</td>
<td>2.28</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>10</td>
<td>1.76</td>
<td>0.62</td>
<td>2.84</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>7</td>
<td>1.59</td>
<td>0.50</td>
<td>3.18</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>13</td>
<td>1.67</td>
<td>0.57</td>
<td>2.93</td>
<td></td>
</tr>
</tbody>
</table>

### Table III

**Relationship between Bands at 578 mp and 472 mp**

<table>
<thead>
<tr>
<th>Observation No.</th>
<th>Cod liver oil (per cent)</th>
<th>Extinction coefficient</th>
<th>Ratio</th>
<th>Mean</th>
<th>Probable error</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>578 mp (maximum)</td>
<td>472 mp (5 min.)</td>
<td>578 mp/472 mp</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>40</td>
<td>1.82</td>
<td>1.65</td>
<td>1.10</td>
<td>1.26 ± 0.03</td>
</tr>
<tr>
<td>12</td>
<td>50</td>
<td>0.90</td>
<td>0.64</td>
<td>1.41</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>60</td>
<td>0.94</td>
<td>0.77</td>
<td>1.22</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>70</td>
<td>1.32</td>
<td>1.10</td>
<td>1.20</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>80</td>
<td>1.10</td>
<td>0.76</td>
<td>1.44</td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>90</td>
<td>1.62</td>
<td>1.35</td>
<td>1.20</td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>100</td>
<td>1.85</td>
<td>1.48</td>
<td>1.25</td>
<td></td>
</tr>
</tbody>
</table>

In order to check the results, another solution of oil of a different concentration is made and observed. The readings of the 608 mp band should be proportional to the amount of oil used. Since a 10 per cent solution of oil usually gives good results, it is customary to try the 10 per cent first. If the extinction coefficient
of the 608 \( m_\mu \) band for 2 cm. cell thickness is less than 1.50 to 1.75, the check solution may be 20 per cent. If the reading is greater than 1.75, but less than 2.00 to 2.50, a 15 per cent solution is usually correct for the check. If greater than 2.00 to 2.50, the check solution should be 7 or 8 per cent. If the 10 per cent solution gives a 578 \( m_\mu \) band, weaker solutions must be used.

The effect of changing the concentration of the reagent and of the oil is shown in Table I. It was found that either, or both, the 578 \( m_\mu \) or 608 \( m_\mu \) bands could be produced.

When the 608 \( m_\mu \) band fades, the 532 \( m_\mu \) band appears. When the 578 \( m_\mu \) band fades, the 472 \( m_\mu \) band appears. Tables II and III show that some relation exists between these two sets of bands. A similar relationship can be shown between the 578 \( m_\mu \) band and the 472 \( m_\mu \) band.

The probable error is calculated from the formula

\[
\text{Probable error}^* = \frac{0.8453 \Sigma (+ v)}{n \sqrt{n - 1}}
\]

where \( \Sigma (+ v) \) is the sum of all the deviations from the mean without regard to sign, and \( n \) is the number of values.

For low temperature work, the saturated solution of antimony trichloride had to be diluted to 0.5 concentrated, and a little extra acetic anhydride added to keep the antimony trichloride in solution.

The following experiments were tried.

\textit{Experiment a}—5 cc. of saturated solution (18.5 per cent) of SbCl\(_3\), 5 cc. of CHCl\(_3\), 4 drops of acetic anhydride, and 1 cc. of 6 per cent cod liver oil.

This mixture at 0° gave bands at 578 and 608 \( m_\mu \). These bands were narrow at first, and then they spread out. The 578 \( m_\mu \) band faded much more rapidly than the 608, and the solution was still blue at the end of 15 minutes. The solution gradually became colorless and after 60 minutes it began to turn pink (at room temperature this occurred in about 8 minutes).

\textit{Experiment b}—With 1 cc. of 3 per cent oil, weak bands were produced at 578 and 608 \( m_\mu \) at 0°.

Experiment c—With 1 cc. of 5 per cent oil at 0°, a rather broad weak band is produced at 578, and a strong narrow band at 608 μm.

Experiment d—With 1 cc. of 5 per cent oil at −5°, there were no bands at first, in 2 minutes one at 608 developed, and in 2½ to 3 minutes a 578 μm band developed. The 608 μm band became more intense than the 578. The band at 608 μm lasted about 35 minutes, and the one at 578 about 10 minutes.

Experiment e—These solutions (Experiments a, b, c, and d) had to be taken out of the cold brine to be read, thus causing them to warm up somewhat and hastening the fading. Another sample was kept in ice for 1½ hours and then read. The 608 μm band was still fairly strong, but the 578 μm band was absent.

SUMMARY

It has been shown that the SbCl₃ color solutions in the test for vitamin A may have two different absorption bands, one at 578 μm and the other at 608 μm. Both of these bands fade and the respective solutions develop new bands at 472 and 532 μm.

Conditions of concentration have been determined by which only one of the two bands is produced (608 μm). Cod liver oils when observed under these conditions yield extinction coefficient values of the 608 μm band which are proportional to the concentration of the oil. There appears to be a definite relation between the extinction coefficient values of the blue solution (608 and 578 μm bands) and the faded or red solutions (532 and 472 μm bands).

Data will be presented in a subsequent paper on the comparisons of results obtained by this method with those obtained by biological assay.

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