THE SPECTROPHOTOMETRIC DETERMINATION OF VITAMINS D₂ AND D₃*

BY CYRIL H. NIELD, WALTER C. RUSSELL, AND A. ZIMMERLI

(From the Department of Agricultural Biochemistry, New Jersey Agricultural Experiment Station and Rutgers University, New Brunswick)

(Received for publication, July 10, 1940)

Many color reactions have been reported for vitamin D, but few (1–5) have been developed for the quantitative determination of this factor. Of the colorimetric methods which had been reported at the time this investigation was begun, the Brockmann-Chen (4) method, which depends on the formation of a yellow color by reaction of the vitamin with a chloroform solution of antimony trichloride, appeared to be the most promising. Although the preparation of an antimony trichloride reagent for the determination of vitamin A involves few difficulties, great care must be taken in its preparation when it is to be used for the determination of vitamin D. Brockmann and Chen (4), Wolff (6), Emmerie and van Eekelen (7), and Ritsert (8) state that the reagent is unstable, its sensitivity changing with time, and that it must be free from alcohol and moisture. Ritsert has found the method exceedingly delicate and claims that reliable results can be obtained by the investigator only after long experience in the preparation and use of the reagent. He reports, furthermore, that difficulty is experienced in preparing reagents of identical sensitivity and that the sensitivity must be redetermined periodically because of the instability of the reagent. Our experience with this reagent fully substantiated Ritsert’s claims, and consequently a study of the reaction between antimony trichloride and vitamin D was undertaken in this laboratory with the purpose of developing a method that would give easily reproducible results.

It was thought that the variation in sensitivity might be due to decomposition of the reagent with the formation of acid. It

* Journal series paper of the New Jersey Agricultural Experiment Station, Department of Agricultural Biochemistry.

73
Determination of Vitamins $D_2$ and $D_3$

was found that saturating the antimony trichloride solution with dry hydrogen chloride increased the sensitivity, but the resulting reagent was extremely hygroscopic and difficult to handle. Addition of acetic anhydride eliminated this difficulty but it was effective only over narrow limits of concentration.

Substitution of acetyl chloride for the hydrogen chloride and acetic anhydride eliminated the above disadvantages and produced a reagent which had all the favorable features of the reagent proposed by Brockmann and Chen, exhibited none of its inconsistencies, and was 3 times as sensitive. No difficulty was experienced in reproducing reagents of identical properties. The reagent may be used $\frac{1}{2}$ hour after preparation and is stable for at least 9 weeks. Consequently no periodic control is necessary. A method has been developed with this reagent by means of which it is possible to determine 0.2 $\gamma$ of vitamin $D_2$ or $D_3$.

EXPERIMENTAL

Preparation of Reagent—Merck's reagent chloroform was washed seven times with equal portions of distilled water. The chloroform was shaken with an excess of phosphorus pentoxide and then run rapidly through filter paper. The chloroform was fractionated and the first cloudy portion and the last 10 per cent were discarded. Then 15 to 22 gm. of Merck's reagent antimony trichloride were dissolved in 100 ml. of purified chloroform and the mixture warmed to 35–45° to effect rapid solution of the salt. The solution was filtered, and to every 100 ml. of the filtrate 2.0 ml. of Merck's redistilled acetyl chloride were added.

Spectrophotometric Procedure—Solutions of crystalline vitamins $D_2$ and $D_3$ in Merck's reagent chloroform were used in these studies. 0.10 to 1.00 ml. of the test solution containing 2 to 20 $\gamma$ of vitamin was run from a microburette into a glass-stoppered graduated cylinder, containing enough reagent to make a total volume of 25.00 ml. The optical density of the solution at 500 m$\mu$ was determined within 4 minutes in a Bausch and Lomb universal spectrophotometer. The standard cell of the instrument has a capacity of slightly over 23.0 ml.; thus an excess of 2.00 ml. is left for convenience in filling the cell.

In another series of experiments an absorption cell of approxi-

---

1 Crystalline vitamins $D_2$ and $D_3$ were generously donated by the Winthrop Chemical Company, Inc., Rensselaer, New York.
Nield, Russell, and Zimmerli

approximately 2.3 ml. capacity was used. It was made by cementing a piece of small bore glass tubing into a 10 cm. polariscope tube. The use of this cell did not impair the accuracy of the method and it permitted the determination of as little as 0.2 \( \gamma \) of vitamin D.

The yellowish pink-colored compound produced by the reagent with vitamin \( D_2 \) or \( D_3 \) absorbs none of the incident light at 550 m\( \mu \) and shows maximum absorption at 500 m\( \mu \) (Fig. 1). This fact was utilized in determining the optical density of the test solution. The blank cell was eliminated, and the optical density was determined at 500 and 550 m\( \mu \). The difference between the two densities represented the absorption due to the reaction product of the vitamin with the reagent. This eliminated errors which might have arisen from differences in general absorption by the blank cell and that containing the test solution.

Absorption Characteristics of Colored Solution—The yellowish pink color reaches its maximum intensity within 30 seconds and

![Absorption curve of the color produced by vitamins \( D_2 \) and \( D_3 \) with the reagent. The solutions contained 8 \( \gamma \) of vitamin in a total volume of 25 ml.](http://www.jbc.org/)

Fig. 1.
is stable for from 4 to 5 minutes. The average of several readings taken during this period gives an accurate determination of the

![Graph](image)

**Fig. 2.** Time curve of the change of absorption at 500 m\(\mu\) of the color produced by the reagent with vitamin D\(_2\). The solution contained 8 \(\gamma\) of vitamin in a total volume of 25 ml.

![Graph](image)

**Fig. 3.** Relationship between absorption at 500 m\(\mu\) and the vitamin concentration.

optical density. The color fades slowly, so that after 10 minutes it has lost 7 to 10 per cent of its initial intensity (Fig. 2).
The $E_{1\%}^{1\%}$ values for crystalline vitamins D$_2$ and D$_3$, calculated from the density readings at 500 m$\mu$ for 8 $\gamma$ of vitamin D in a 25 ml. volume (Fig. 1), are of the same magnitude, approximately 1800. Fig. 3 shows that the optical density of the colored solution is proportional to the vitamin concentration. Included in Fig. 3 are three points determined with the small cell of 2.3 ml. capacity, which has been described previously.

![Graph showing the effect of acetyl chloride concentration on sensitivity.](http://www.jbc.org/)

**Fig. 4.** The effect of acetyl chloride concentration on the sensitivity of the reagent. Points between 0 and 1 per cent were not determined. The solution contained 8 $\gamma$ of vitamin D$_3$ in a 25 ml. volume.

**Effective Range of Acetyl Chloride Concentration**—The limits of acetyl chloride concentration that will yield a reagent of optimum sensitivity are conveniently wide. Fig. 4 shows that reagents containing 1 to 4 per cent acetyl chloride possess the same sensitivity, whereas 6 per cent acetyl chloride diminishes the sensitivity approximately 10 per cent below the optimum, and this concentration is consequently too high.
78 Determination of Vitamins D₂ and D₃

Effect of Antimony Trichloride Concentration—The concentration of the antimony trichloride may be varied to a considerable extent without effecting any change in the quality of the reagent. The sensitivity remained essentially constant when the antimony trichloride concentration was varied between 15 and 30 gm. of the salt per 100 ml. of chloroform (Table I). When 10 gm. per 100 ml. of chloroform were used, the sensitivity of the reagent was appreciably below optimum and the coloration faded faster than usual, but after standing 1 week the reagent exhibited optimum sensitivity. A concentration of 22 gm. of salt per 100 ml. of chloroform was used in these investigations.

Effect of Alcohol—Ethyl alcohol in a concentration of 0.3 per cent or less does not interfere with the reaction; 0.7 per cent alcohol interferes slightly, whereas 1.4 per cent alcohol markedly lowers the sensitivity of the reagent. Merck's chloroform, which contains 0.7 per cent ethyl alcohol, cannot be used for the preparation of the reagent without the purification described earlier, since its use results in a sensitivity 5 to 7 per cent below optimum. Indications are that this interference is not merely due to the alcohol content of the chloroform.

Attention is now being given to the determination of vitamin D in milk, poultry feeds, and other products.

**Table I**

Effect of Antimony Trichloride Concentration on Extinction Coefficient of Vitamin D₂

<table>
<thead>
<tr>
<th>Age of reagent</th>
<th>30</th>
<th>20</th>
<th>15</th>
<th>10</th>
<th>7.5</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>days</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>1841</td>
<td>1800</td>
<td>1797</td>
<td>1657</td>
<td>1231</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>1781</td>
<td>1797</td>
<td>1834</td>
<td>1800</td>
<td>1700</td>
<td>1441</td>
</tr>
</tbody>
</table>

* The figures refer to the number of gm. of antimony trichloride added to 100 ml. of chloroform.

Summary

1. A new reagent for the determination of vitamins D₂ and D₃, consisting of a solution of antimony trichloride and acetyl chloride in chloroform, has been described.
2. The limits of concentration of antimony trichloride and acetyl chloride, within which the sensitivity of the reagent is constant, have been determined.

3. The reagent produces a yellowish pink color with vitamins D₂ and D₃ which reaches its maximum intensity within 30 seconds and is stable for from 4 to 5 minutes.

4. The absorption curves of the reaction product of the reagent with vitamins D₂ and D₃ have been determined in a Bausch and Lomb spectrophotometer. The two curves are identical, having a maximum at 500 m.μ.

5. The $E_{100}^{1.0}$ values at 500 m.μ for vitamins D₂ and D₃ are identical and are approximately 1800, which is about 3 times the value given by the reagent proposed by Brockmann and Chen.

6. The optical density, as determined by the difference in absorption at 500 and 550 m.μ, is directly proportional to the vitamin concentration.

7. The lower limit of the amount of vitamin that can be accurately determined by the method described is approximately 0.2 γ.

BIBLIOGRAPHY

THE SPECTROPHOTOMETRIC DETERMINATION OF VITAMINS D$_2$ AND D$_3$

Cyril H. Nield, Walter C. Russell and A. Zimmerli