This paper deals with a new colorimetric reaction of vitamin A with glycerol 1,3-dichlorohydrin (which we henceforth will refer to as GDH) that appears to be suitable for the quantitative estimation of this vitamin.

The estimation of vitamin A in vitro has been the subject of many reviews in recent years (1-6). The two widely used methods at present are the ultraviolet absorption at 325 to 328 m\(\mu\), and the determination of the maximum absorption at 615 to 620 m\(\mu\) of the blue color formed on the addition of a solution of antimony trichloride in chloroform to the vitamin in the same solvent (Carr-Price reaction).

One disadvantage of the ultraviolet method is that there are substances in natural products other than vitamin A which absorb at around 328 m\(\mu\) (7). Another drawback of the method is the need for expensive equipment.

The Carr-Price reaction does not require expensive equipment and is more specific for vitamin A (8). Its disadvantages are the rapidity with which the maximum absorption must be read, owing to the fading of the color, and the instability of the reagent.

The new colorimetric reaction reported herein possesses the same advantages that the Carr-Price reaction has over the ultraviolet absorption method, and in addition possesses the following advantages over the Carr-Price reaction. (1) The stability of the color produced permits its measurement with ease any time from 2 to 10 minutes after the reagents are mixed. (We have even been able to measure the color in a visual colorimeter.) (2) The reagent employed is stable. It is not affected by traces of moisture and leaves no film which might interfere in the absorption of the colored solution. The disadvantage of the new reaction is that the extinction coefficient\(^1\) \((L_1^{1\%}, \lambda 550 \text{ m}\(\mu\)) of the color produced is about one-fourth that of the antimony trichloride blue color \((L_1^{1\%}, \lambda 615 \text{ m}\(\mu\)).

\(^{1}\) The term \(L_1^{1\%},\) introduced by Dann and Evelyn (9) for use in photoelectric colorimeters which employ a band of light of about 30 to 40 m\(\mu\) wide, has been used by many authors (10-12). The term is analogous to \(L_1^{1\%},\) the latter being used with those instruments which employ monochromatic light. In this paper, to the
The reagent used in the authors' reaction is the practical grade of GDH obtained from the Eastman Kodak Company. The keeping of this reagent requires no special precautions. One simply uses it as it comes from the bottle.

Upon the addition of this reagent to a solution of vitamin A in chloroform, an immediate blue color appears which rapidly changes into a more stable color resembling a dilute solution of potassium permanganate.

Fig. 1 shows the absorption curve of the immediate blue color. The absorption maximum is at 625 m\(\mu\). The shape of the curve is similar to the one obtained with antimony trichloride and vitamin A, but is not identical to it. The blue color formed with GDH appears to obey Beer's law up to 33 I.U. of vitamin A in 5.0 ml. of solution, but a complete study was not made of this point in view of the stability of the second color formed.

Fig. 2 shows the absorption spectrum of the violet color developed 2 minutes after the reagents were mixed. The maximum absorption occurs authors' knowledge, \(L_{10cm}^{1%}\), has been used for the first time with data obtained on a Coleman model 11 spectrophotometer because, although it is constructed with a diffraction grating rather than a light filter, it has a wave band of light 35 m\(\mu\) wide.
Fig. 2. Absorption curve of the violet color produced by vitamin A and glycerol 1,3-dichlorohydrin at the end of 2 minutes. The solution contained 16.92 I.U. of vitamin in 5.0 ml. of solution.

Fig. 3. Time curve of the changes in $L_{120}^{{\%}}$ at 550 m$\mu$ of the colors produced by 6.76 $\gamma$ of vitamin A and 60.08 $\gamma$ of carotene (90 per cent of $\beta$- and 10 per cent of $\alpha$-) with glycerol 1,3-dichlorohydrin at 25$^\circ$. The total volume of reaction mixture in each case was 5.0 ml.
at 550 m\(\mu\), and remains constant at 25° for from 2 to 10 minutes. After that time a slow deterioration of the color takes place, as can be seen in Fig. 3.

In Fig. 4 is presented the relationship between light absorption and concentration at 550 m\(\mu\). Beer's law is obeyed up to a concentration of 25 I.U. of vitamin A in 5.0 ml. of solution. Above this value the absorption is slightly less than that to be expected from Beer's law. Under the experimental conditions we employed, the blue color of antimony trichloride obeyed Beer's law up to a concentration of 23 I.U. in 4.0 ml. of solution.

The \(L_{1\%}^{1}\) at 550 m\(\mu\) for vitamin A in the concentrate employed was 1010. (In this calculation 1.00 I.U. equals 0.292 \(\gamma\) of vitamin A. The derivation of this factor is explained in the experimental portion of this paper.) The \(L_{1\%}^{1}\) of the immediate blue color at 625 m\(\mu\) with GDH was 1385. The \(L_{1\%}^{1}\) obtained at 615 m\(\mu\) with antimony trichloride was 3900. This compares with the \(L_{1\%}^{1}\) at 622 m\(\mu\) of 3880 for crystalline vitamin A (12).

To test the interference of substances found in fish liver oils upon the new colorimetric reaction, the vitamin A content of several fish liver oils was determined with GDH and antimony trichloride. The results are presented in Table I. The antimony trichloride method is one of the accepted procedures for the estimation of vitamin A in fish liver oils, and according to Oser et al. (8) the results are in closer agreement with those by the multiple level bioassay than with those obtained by the direct spectrophotometric method in the ultraviolet.
The agreement between the GDH and antimony trichloride methods is close. That the values obtained do not coincide may be ascribed to the differences in the interference upon the colors produced by the two reagents with vitamin A caused by the other components of the oil. A more exhaustive study will be undertaken in order to determine which method is more specific for vitamin A.

The \( I_{1}^{1%} \) values, \( \lambda 550 \) m\( \mu \), for vitamins D\(_2\) and D\(_3\), ergosterol, 7-dehydrocholesterol, and cholesterol, after 15 minutes, otherwise the conditions being the same as in the estimation of vitamin A, were 11.28, 5.96, 1.13, 0.08, and 0.00 respectively. These values (obtained from Sobel, Mayer, and Kramer (15)) at 2 minutes (the time recommended for the development of the color with vitamin A) are less than one-third of those at 15 minutes. Thus the interference of vitamin D and related sterols upon the GDH reaction is negligible.

The stability of the violet color produced by vitamin A and GDH suggested the possibility of employing the visual method in the estimation of the vitamin. Such a method would be useful in the field or in those laboratories where a spectrophotometer is not available. Solutions containing 1.26, 2.14, 3.54, and 4.04 I.U. of vitamin A in 1 ml. of reaction mixture were compared against a standard of 2.64 I.U. of vitamin A with the micro cups of the Bausch and Lomb visual colorimeter. The values calculated from the readings were 1.06, 2.12, 3.18, and 4.44 I.U. of vitamin A, respectively.

**TABLE I**

Comparison of Vitamin A Values Obtained on Fish Liver Oils by Glycerol 1,3-Dichlorohydrin and SbCl\(_3\) Methods

The values are expressed as international units of vitamin A per gm. of oil.

<table>
<thead>
<tr>
<th>Type of oil</th>
<th>Glycerol 1,3-dichlorohydrin</th>
<th>SbCl(_3)</th>
<th>([I_{1}^{1%}] \times [2000]) at 328 m( \mu )*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oil 1, whole</td>
<td>24,200</td>
<td>27,000</td>
<td>28,200</td>
</tr>
<tr>
<td>&quot; 1, unsaponifiable fraction†</td>
<td>24,000</td>
<td>25,000</td>
<td></td>
</tr>
<tr>
<td>&quot; 2, whole</td>
<td>8,070</td>
<td>8,240</td>
<td>10,200</td>
</tr>
<tr>
<td>&quot; 2, unsaponifiable fraction†</td>
<td>8,040</td>
<td>7,370</td>
<td></td>
</tr>
<tr>
<td>&quot; 3, whole</td>
<td>36,500</td>
<td>37,100</td>
<td>44,000</td>
</tr>
<tr>
<td>&quot; 5, &quot;</td>
<td>73,300</td>
<td>74,000</td>
<td>85,000</td>
</tr>
<tr>
<td>Concentrated Oil G†</td>
<td>1,285,000</td>
<td>1,204,000</td>
<td>1,240,000</td>
</tr>
<tr>
<td>&quot;</td>
<td>&quot;</td>
<td>1,013,000</td>
<td>1,035,000</td>
</tr>
<tr>
<td>&quot;</td>
<td>&quot;</td>
<td>1,802,000</td>
<td>1,705,000</td>
</tr>
<tr>
<td>&quot;</td>
<td>&quot;</td>
<td>1,841,000</td>
<td>1,710,000</td>
</tr>
</tbody>
</table>

* Determined by Dr. Oscar Gawron at the International Vitamin Corporation.
† Saponified according to the procedure of Oser, Melnick, and Pader (13).
‡ Unsaponifiable fraction obtained by the commercial saponification process of the International Vitamin Corporation (14).
In many natural products such as food and blood, carotene is often found with vitamin A and interferes in its colorimetric estimation. At present the most widely used method of evaluating this interference is that of Dann and Evelyn (9) which consists in reading the absorption of the yellow solution of carotene and vitamin A at 440 m\(\mu\) before the addition of antimony trichloride, thereby determining the amount of carotene present from a calibration curve, and subtracting from the maximum absorption obtained with antimony trichloride the absorption that the amount of carotene found would give with the reagent.

Another method has recently been proposed by Urban, Milder, and Carruthers (16) by which vitamin A and carotene are determined mutually and independently by means of a special photoelectric colorimeter that splits the beam of light emerging from a solution of vitamin A and antimony trichloride into two, one passing through a 589 m\(\mu\) filter measuring \(\beta\)-carotene at 0°, and the other passing through a 620 m\(\mu\) filter measuring vitamin A. No data are given to substantiate the claim.

The reaction of carotene with GDH was investigated under the same conditions by which the violet color with vitamin A was obtained. Upon the addition of the reagent to a mixture of \(\alpha\)- and \(\beta\)-carotene (1:9) in chloroform, a green color appears within 2 minutes. Fig. 5 shows the absorption of the color at the end of 6 minutes. Two maxima were observed, one at 475 m\(\mu\) and a second at 625 m\(\mu\), as well as one minimum at 550 m\(\mu\). This minimum coincides with the maximum for vitamin A, while the other maxima are almost at the minima for the vitamin.

Fig. 3 shows the relationship between the absorption of the green color at 550 m\(\mu\) and time. It is constant at from 6 to 15 minutes.

The relationship between the concentration of carotene and light absorption at 550 m\(\mu\) obeys Beer's law. This is shown in Fig. 6. This wavelength was chosen in order that we might be able to correct for the presence of carotene in the determination of vitamin A.

The next study made was a comparison of the interference of carotene upon the reaction of vitamin A with GDH and with antimony trichloride. 1 ml. of chloroform containing 18.53 I.U. of vitamin A and known increments of carotene was used in this study. The results are presented in Table II. 6 minutes were chosen in the GDH reaction because at that time both the vitamin A and carotene colors are stable (see Fig. 3). From the last column of Table II, it can be seen that in the antimony trichloride reaction the color due to 1 \(\gamma\) of carotene is equivalent to about 0.24 I.U. of vitamin A, whereas in the GDH reaction the color due to the same amount of carotene is equivalent to about 0.49 I.U. of vitamin A.

It seemed likely (see Fig. 3) that the interference of carotene in the GDH reaction would have been much less had a shorter period of time been
Fig. 5. Absorption curve of the green color produced by carotene (90 per cent of $\beta$- and 10 per cent of $\alpha$-) and glycerol 1,3-dichlorohydrin. The solution contained 122.6 $\gamma$ in 5.0 ml. of solution.

Fig. 6. Relationship between absorption at 550 $\mu\text{m}$ and the carotene (90 per cent $\beta$- and 10 per cent of $\alpha$-) concentration.
taken before the maximum absorption of the solution was read. Therefore, the interference of carotene upon the immediate blue color produced by the action of GDH on vitamin A was studied. Examination of Table II shows that in this reaction the color due to 1 γ of carotene is equal to about 0.17 I.U. of vitamin A.

The above studies suggest two methods of determining vitamin A (in solutions containing carotene) after the amount of carotene present is evaluated by the method of Dann and Evelyn (9) as described above. One method is to measure the absorption of the solution with GDH at 550 mμ at the end of 6 minutes. Subtract from the absorption obtained the increment due to carotene. This is read from a calibration chart previously prepared for various amounts of vitamin A and carotene by the method shown in Table II. This procedure possesses the advantages pointed out in this paper for the GDH reaction. Its disadvantage compared with the antimony trichloride method lies in the greater interference of carotene and in the need of an empirical correction chart.

A second method of evaluating carotene interference is to measure the absorption of the solution with GDH at the end of 5 seconds at 625 mμ. Subtract the increment due to carotene as described above. This procedure also possesses the advantages of the GDH reaction, except that the color must be read immediately owing to its rapid change to the violet
color. The carotene interference is about 30 per cent less than it is in the antimony trichloride reaction.

A more detailed study of the interference of carotene is now being undertaken and will be presented in a paper dealing with the application of the GDH reaction in the estimation of vitamin A in blood.

EXPERIMENTAL

Apparatus—All determinations were made in a Coleman universal spectrophotometer, model 11, with Filter PC-4 for the region of 400 to 700 mμ. This instrument employs a monochromatic band of light 35 mμ wide (which can be located with a precision of less than 2.0 mμ). For this reason, $L_{1%}^{cm}$ values are reported instead of $E_{1%}^{cm}$ values, which are used for pure monochromatic light. Direct readings of per cent transmission were made on the galvanometer scale by setting the blank at 100. The absorption path was 1.3 cm.

Reagents—

Practical glycerol 1,3-dichlorohydrin from the Eastman Kodak Company was stored in the ice box and brought to 25° before use. Each new batch should be standardized with known solutions of vitamin A.

GDH obtained from other firms possessed little or no chromogenic property with vitamin A. However, it was found that by distilling the inactive reagent, at 30 to 40 mm. pressure, in the presence of 1 to 2 per cent antimony trichloride a product was obtained possessing properties similar to the practical GDH from the Eastman Kodak Company.

Chloroform, analytical reagent grade, dried over anhydrous sodium sulfate, distilled, and kept over the same drying agent.

Antimony trichloride (reagent grade). A saturated solution in chloroform was prepared by shaking 90 gm. of the trichloride (from an unopened bottle) with 300 ml. of chloroform at room temperature. The saturated solution was filtered before using.

The standard vitamin A solution was prepared by dissolving a weighed amount of a distilled vitamin A concentrate from Distillation Products, Inc., Rochester, New York, (potency 500,000 i.u. per gm.) in a known volume of chloroform. This standard remained stable for considerable periods of time as shown by the constant $L_{1%}^{cm}$ values it gave with the GDH and antimony trichloride reagents with which it was frequently checked.

The conversion factor for converting international units of the concentrate into micrograms of vitamin A was 0.292. This was obtained by measuring the extinction coefficient of the concentrate in isopropanol at 328 mμ with a Beckman spectrophotometer. From this value, and by taking the $E_{1%}^{cm}$ equal to 1800 (calculated from the data presented in a
paper by Oser et al. (13) who have also shown that the \( E_{\text{1\%}}^{1\%} \) of distilled vitamin A esters is the same at 328 m\( \mu \) as the \( E_{\text{1\%}}^{1\%} \) of the saponified esters at 325 m\( \mu \), the weight of vitamin A per gm. of concentrate was calculated. It is noteworthy that using this conversion factor we obtained almost the same \( L_{\text{1\%}}^{1\%} \), that Baxter and Robeson (12) did for crystalline vitamin A alcohol. All such conversion factors are in doubt at present until proved by exact multiple level bioassays from different laboratories.

The standard carotene solution was prepared by dissolving a weighed amount of carotene (90 per cent of \( \beta \)- and 10 per cent of \( \alpha \)-) from the S. M. A. Corporation in a known volume of chloroform.

Color Development

Temperature—All colors were developed in a constant temperature bath at 25\( ^\circ \), and all reagents were preheated to this temperature.

Use of Practical Glycerol 1,3-Dichlorohydrin—1.0 ml. of chloroform containing vitamin A, carotene, or a mixture of both was pipetted into a 10 ml. glass-stoppered graduate. 4 ml. of reagent were added with a bulb pipette, and the graduate stoppered and inverted several times to insure homogeneous mixing. It was then placed in a 25\( ^\circ \) water bath, and at the end of the desired incubation period (we recommend 2 minutes for the determination of vitamin A), the solution was poured into a cuvette and its maximum absorption read.

To read the maximum absorption of the immediate blue color, the above procedure was revised as follows: The chloroformic solution containing vitamin A was measured with a pipette directly into the cuvette. Then, with the cuvette held in one hand, 4.0 ml. of the reagent were added in a rapid stream from a fast delivery pipette. The cuvette was vigorously tapped with the forefinger of the other hand several times, placed in the cuvette carrier, and the maximum absorption of the blue color was read. The time elapsed from the addition of the reagent to the reading of the maximum absorption should not exceed 5 seconds.

Use of Antimony Trichloride Reagent—To 1.0 ml. of chloroform containing the vitamin A, which was measured with a pipette directly into a cuvette, were added 3.0 ml. of antimony trichloride reagent with a fast delivery pipette. The maximum absorption was immediately read.

Visual Colorimetric Technique—The determination was carried out at room temperature with the violet color produced by the reaction of GDH with vitamin A. With each unknown a standard solution of vitamin A was run, the violet color in both being simultaneously developed. The unknowns (prepared from a known standard) were read against the standard at any time from 2 to 10 minutes after the reagents were mixed.

All the densities reported in this paper are the averages of duplicate determinations.
SUMMARY

A new calorimetric reagent, glycerol 1,3-dichlorohydrin, is proposed for estimating vitamin A. It possesses the advantage over the antimony trichloride reagent that the color developed with vitamin A is stable for from 2 to 10 minutes, which permits its absorption to be determined with ease. Other advantages are that the use of the reagent requires no special precautions and possesses good stability.

Spectrophotometric data are presented of the immediate transient blue color and the stable secondary violet color produced by reaction of the reagent with vitamin A, as well as the color formed by its reaction with carotene.

The interference of carotene upon the determination of vitamin A with the reagent has been studied.

The authors are indebted to Dr. Oscar Gawron, Research Laboratory, International Vitamin Division, American Home Products Corporation, New York, for the samples of fish liver oils which were used in this investigation.

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