A COLORIMETRIC METHOD FOR THE ESTIMATION OF FREE FORMALDEHYDE AND HEXAMETHYLENAMINE.

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It has previously been ascertained¹ that the phloroglucinol or Jorissen test is among the most sensitive color tests for free formaldehyde. The advantages claimed for it are: (1) simplicity; (2) great sensitivity; (3) it reacts with free or liberated formaldehyde only, and does not itself liberate formaldehyde from such compounds as hexamethylenamine; (4) finally, with the phloroglucinol test there is a gradation of colors, from pink to deep red, depending on the concentration of formaldehyde, which suggested to us the possibility of its application in a quantitative manner. However, the colors are not permanent. Their maximum intensity is reached within 3 minutes and then they gradually change to a violet and finally disappear altogether. This is particularly true with the high dilutions. Therefore we sought for a substance, or mixture of substances, which would give us the same quality of color as in the phloroglucinol test, and yet be permanent when used in making a set of standards.

After considerable experimentation with metallic salts and different dyes, a mixture of Congo red (0.025 per cent in water containing 5 per cent alcohol) and methyl orange (0.01 per cent in water) was found to be satisfactory. A mixture of these in the proper proportions is only necessary in matching the reds of phloroglucinol in the lower concentrations of formaldehyde. For the higher concentrations Congo red alone suffices.

¹ Hanzlik, P. J., and Collins, R. J., Arch. Int. Med., 1913, xii, 578. The reagent consists of phloroglucinol (reagent-Merck) 0.1 gm. dissolved in 10 cc. of 10 per cent sodium hydroxide.
It is important to note that samples of Congo red, even of the same manufacture, are apt to differ considerably in point of quantity, but apparently not in quality, of color. Owing to this it is necessary to standardize any particular specimen of Congo against a known standard made from an inorganic salt before it can be used to prepare the permanent Congo standard for the dilutions. We believe that we have successfully achieved this with potassium bichromate and sulfuric acid. The following procedure for standardizing Congos has been found to work satisfactorily.

1.7616 gm. of $K_2Cr_2O_7$ by titration against alkali (roughly about 30 cc. of a 5 per cent solution) and 11.5537 gm. of absolute $H_2SO_4$ (about 7 cc. concentrated) are mixed and diluted to the mark in a 50 cc. Nessler tube with a column of 12 cm. This is equivalent to 50 cc. of 1:100,000 absolute formaldehyde, or 50 cc. of a Congo standard 1:100,000, which contains 0.000625 gm. of the original dry Congo red, or 2.5 cc. of 0.025 per cent. It is only necessary, therefore, to prepare a proper mixture of $K_2Cr_2O_7$ and $H_2SO_4$, and when an unknown Congo solution is standardized against this, the quantity used will contain 0.000625 gm. of Congo. The strong solution can then be diluted or made accordingly, the standard dilutions from this to be equivalent to the different concentrations of formaldehyde.

The proportions of Congo red and methyl orange which have been worked out for the different concentrations of absolute formaldehyde are presented in Table I. The basic solutions for making the standards are not mixed until ready for use. We have kept both solutions for 2 to 3 months without any demonstrable change. However, after the dyes are mixed and diluted to the proper volume with water, deterioration occurs with the weaker colors within a week, the stronger colors remaining permanent for at least 2 weeks. It is advisable, therefore, to prepare a set of dilutions each day. The technique of making standards consists simply in measuring the quantity of each solution necessary with an accurately graduated pipette into 50 cc. Nessler tubes of the short variety with columns of fluid 12 cm. high. These are then diluted with water to the mark, gently agitated, and are ready for use.

For the estimation of formaldehyde in clear aqueous solutions
containing formaldehyde, the technique is as follows: An aliquot portion, 1, 5, or 10 cc., of the solution are measured into the Nessler tube, phloroglucinol reagent (1 to 2 cc.) is added, and the whole is diluted to the 50 cc. mark and gently agitated. After standing 3 minutes the tube is matched against a series of tubes containing the standard colors just described. The results can be expressed in percentage or gm. of formaldehyde. In calculation allowance must be made for dilution in the Nessler tube.

**TABLE I.**

<table>
<thead>
<tr>
<th>Concentration of formaldehyde.*</th>
<th>Percentage concentration of formaldehyde.</th>
<th>Congo red** 0.025 per cent.</th>
<th>Methyl orange** 0.01 per cent.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1: 20,000</td>
<td>0.005</td>
<td>20.0</td>
<td>0</td>
</tr>
<tr>
<td>1: 30,000</td>
<td>0.0033</td>
<td>11.0</td>
<td>0</td>
</tr>
<tr>
<td>1: 40,000</td>
<td>0.0025</td>
<td>9.0</td>
<td>0</td>
</tr>
<tr>
<td>1: 50,000</td>
<td>0.002</td>
<td>8.0</td>
<td>0</td>
</tr>
<tr>
<td>1: 60,000</td>
<td>0.0016</td>
<td>5.0</td>
<td>0</td>
</tr>
<tr>
<td>1: 80,000</td>
<td>0.00125</td>
<td>4.0</td>
<td>0</td>
</tr>
<tr>
<td>1: 100,000</td>
<td>0.0010</td>
<td>2.5</td>
<td>0</td>
</tr>
<tr>
<td>1: 200,000</td>
<td>0.0005</td>
<td>0.85</td>
<td>0.40</td>
</tr>
<tr>
<td>1: 250,000</td>
<td>0.0004</td>
<td>0.65</td>
<td>0.35</td>
</tr>
<tr>
<td>1: 500,000</td>
<td>0.0002</td>
<td>0.25</td>
<td>0.18</td>
</tr>
<tr>
<td>1: 750,000</td>
<td>0.00014</td>
<td>0.20</td>
<td>0.15</td>
</tr>
<tr>
<td>1: 1,000,000</td>
<td>0.00010</td>
<td>0.13</td>
<td>0.10</td>
</tr>
</tbody>
</table>

* Total volume of solution = 50 cc. in a Nessler tube with a 12 cm. column.

** The quantities of Congo red and methyl orange here indicated are mixed and diluted with water to the mark in a 50 cc. Nessler tube with a 12 cm. column.

For instance, if 5 cc. of the formaldehyde solution were used and diluted to 50 cc., then the percentage concentration to which the matched color corresponds is multiplied by 10. This gives the percentage concentration of the formaldehyde solution. From this the absolute weight in gm. of formaldehyde can, of course, be easily calculated.

For urine the procedure must be slightly modified because of the presence of phosphates which, when precipitated by the alkali of the phloroglucinol reagent, interfere with the reading of
Formaldehyde and Hexamethylenamine

the color. These are removed by adding to an aliquot portion of the urine a few drops of concentrated (50 per cent) sodium hydroxide and filtering, then washing with a little water to the original volume.

If the urine is concentrated or deeply colored, an equal volume of the same or some other urine of about the same color must be added to the standards containing the mixtures of Congo red and methyl orange. The estimation is then carried out in exactly the same way as for aqueous solutions.

We have compared the colorimetric method with the Romijn or iodine method,² the U.S.P. peroxide method with heat,³ and the sodium hydroxide heat pressure method.⁴ Distillates from known quantities of hexamethylenamine were used. Results illustrative of the data obtained are indicated in Table II. The following mean percentage recoveries of the theoretical yield of formaldehyde were obtained with the different methods: colorimetric, 99 per cent; Romijn, 88 per cent; U.S.P., extremely variable (none to 96 per cent); hydroxide and pressure, extremely variable (none to 90 per cent). In order to be able to obtain these percentage recoveries with the other methods usually ten times the quantity of the distillate was necessary for single estimations, as compared with the colorimetric. It was also found that the colorimetric method is more accurate with higher dilutions of formaldehyde, because it is more difficult to read small differences between more intense than between weaker colors.

For direct application to urine the other methods with which the colorimetric was compared are not suitable, since urine itself consumes iodine, hydroxide, and peroxide. Distillation of urine containing such an easily decomposable formaldehyde compound as hexamethylenamine is not permissible if an idea of the formaldehyde liberated during its passage through the body is to be obtained. Here the only choice is the colorimetric method.

Salkowski⁵ has recently practiced a modification of the Leach ferric chloride test as a colorimetric method for the estimation of formaldehyde.

³ U. S. Pharm., 8th revision, 1900, 266.
⁴ Smith, C. E., Am. J. Pharm., 1898, lxx, 86.
⁵ Salkowski, E., Biochem. Z., 1915, lxvii, 337; 1915, lxxi, 365.
In this hydrochloric acid is used, and this precludes its use for the estimation of free formaldehyde in the presence of easily decomposable formaldehyde compounds, such as hexamethylenamine. We have not practiced the phenylhydrazine-nitro-prusside test for quantitative purposes because of difficulties with the test which were pointed out in a previous paper. It has been used by Dunning.\(^6\)

**TABLE II.**

*Estimation of Formaldehyde in Hexamethylenamine.*

<table>
<thead>
<tr>
<th>Amount of hexamethylenamine (gm.)</th>
<th>Equivalent in HCHO (gm.)</th>
<th>Formaldehyde recovered.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Romijn (per cent)</td>
</tr>
<tr>
<td>1.0</td>
<td>1.28</td>
<td>51 (4)*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>75 (40)</td>
</tr>
<tr>
<td>0.5</td>
<td>0.64</td>
<td>53 (7.8)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>76 (78)</td>
</tr>
<tr>
<td>0.05</td>
<td>0.064</td>
<td>82 (25)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>96 (100)</td>
</tr>
<tr>
<td>0.005</td>
<td>0.0064</td>
<td>None (22)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>96 (220)</td>
</tr>
</tbody>
</table>

**Romijn** per cent:

- 51 (4)
- 75 (40)
- 53 (7.8)
- 76 (78)
- 82 (25)
- 96 (100)
- None (22)
- 96 (220)

**U. S. P.** per cent:

- None (4)
- 32 (7.8)
- 86 (78)
- 90 (78)
- None (22)
- None (100)

**Alkali** per cent:

- None (4)
- 41 (7.8)
- 90 (78)
- None (22)
- None (100)

**Colorimetric** per cent:

- 98 (4)
- 100 (7.8)
- 96 (8.2)
- 98 (8)
- None (22)
- None (100)
- 100 (7.8)
- 100 (22)
- 102 (15)

Hexamethylenamine in each case above was decomposed with the aid of weak acid (three drops of 85 per cent phosphoric).

<table>
<thead>
<tr>
<th>Amount of hexamethylenamine (gm.)</th>
<th>Equivalent in HCHO (gm.)</th>
<th>Formaldehyde recovered.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0</td>
<td>1.28</td>
<td>None (1) 78 (10)</td>
</tr>
<tr>
<td>0.5</td>
<td>0.64</td>
<td>None (1) 74.25</td>
</tr>
<tr>
<td>0.05</td>
<td>0.064</td>
<td>None (25) 74.7 (25)</td>
</tr>
<tr>
<td>0.005</td>
<td>0.0064</td>
<td>None (25) 74.7 (25)</td>
</tr>
</tbody>
</table>

**Romijn** per cent:

- None (1) 78 (10)
- None (1) 74.25
- None (25) 74.7 (25)
- None (25) 48 (50)

**U. S. P.** per cent:

- None (1) 82 (10)
- None (1) 69 (20)
- None (25) 74 (100)
- None (25) 48 (50)

**Alkali** per cent:

- None (1) 82 (10)
- None (1) 69 (20)
- None (25) 74 (100)
- None (25) 48 (50)

**Colorimetric** per cent:

- 78.1 (1)
- 78.1 (1)
- 78.1 (25)
- 78.1 (25)

Hexamethylenamine in each case was decomposed with the aid of strong acid (20 cc.), sometimes phosphoric, sometimes sulfuric.

* The figures in brackets denote the number of cc. of distillate used for estimation. The distillate in each case measured 1,000 cc.

For this it is only necessary to distil an aliquot portion of the solution or urine containing hexamethylenamine, and then apply the colorimetric procedure described above for free formaldehyde to the distillate (distilled or diluted to a definite volume). Distillation is not facilitated by the addition of acid. On the contrary, there seems to be a loss when large or moderate quantities of phosphoric or sulfuric acid are used. Only 78 per cent was recovered (see Table II), as compared with 96 to 100 per cent when only a trace or no acid was used. For calculating the amount of hexamethylenamine recovered the formaldehyde determined is divided by 1.28, since 1 gm. of hexamethylenetetramine yields 1.28 gm. of formaldehyde.

In order to arrive at the proper amount of undecomposed hexamethylenamine due allowance must be made for free formaldehyde if any is present, by subtracting this from the total amount of formaldehyde recovered in the distillate. This is of importance in connection with urines and old standing solutions of hexamethylenamine.

That a good recovery is obtained with this method is indicated by the results presented in Table II, which when converted into hexamethylenamine will indicate the same percentage recovery as the formaldehyde.

The administration of 2 gm. of hexamethylenamine by mouth, on two different occasions, to the same individual, resulted in a recovery of 38 per cent in 6 hours on one occasion, and 71 per cent on another occasion when the excretion was completed. During both experiments the water intake was maintained constant. 1 gm. of the drug taken on two different occasions by the same individual gave recoveries of 70 and 76 per cent, respectively. In these two experiments no attention was paid to the fluid intake; and in all cases (except the 6 hour experiment) the urine was collected until it failed to give positive tests with bromine water and the phloroglucinol reagent. Similar results were recently reported by Falk and Sugiura, using their iodine method for the quantitative estimation of hexamethylenamine. Owing to

the few observations which we have made, no explanation for these low excretory results can as yet be offered. Further observations on this and other features of hexamethylenamine excretion and decomposition will be reported later.

CONCLUSIONS.

1. A method for the colorimetric estimation of free formaldehyde and hexamethylenamine is described, in which the phloroglucinol reagent is used.

2. Permanent color standards which exactly match the phloroglucinol in different concentrations of formaldehyde can be made from mixtures of 0.025 per cent Congo red and 0.01 per cent methyl orange.

3. These mixtures have been experimentally determined for a number of concentrations of formaldehyde between 1:1,000,000 and 1:20,000.

4. The colorimetric method is more accurate than the Romijn, U.S.P., and hydroxide pressure methods.

5. It is more accurate for high than for low dilutions of formaldehyde.

6. The colorimetric method possesses an important advantage over other methods in that it is directly applicable to urine for the determination of free formaldehyde.
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