A QUANTITATIVE METHOD FOR THE DETERMINATION OF BLOOD IN THE FECES OF SHEEP BY MEANS OF THE EVELYN PHOTOELECTRIC COLORIMETER*

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During the summer of 1938 one of us (J. S. A.), while engaged in experimental work with sheep infected with the stomach-worm, Haemonchus contortus, became interested in making a study of the nature of the anemia produced by these nematodes. The examination of the feces of sheep a few days after artificial infection with stomach-worm larvae revealed the presence of blood. Quantities of blood were also found in the contents of the fourth stomachs of severely infected animals at autopsy. The absence of other lesions in the digestive tract which could have been responsible for the hemorrhage observed showed that the blood came originally from lesions produced by the nematodes in the wall of the fourth stomach. Since these observations indicated that a considerable loss of blood occurred in sheep harboring heavy stomach-worm infections, it appeared to be of interest to make a quantitative study of this loss in an attempt to ascertain its relationship to the anemia observed. To do this it was first necessary to find a method for the quantitative determination of blood in the feces of sheep.

A search of the literature revealed that van Eck (1) had published a method for the quantitative determination of blood in human feces. This method, as originally described, was found to be unsatisfactory because of the large quantities of coloring matter and plant fiber present in the feces of sheep. A crude procedure was finally developed which involved the boiling of

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sheep feces containing blood with glacial acetic acid, the treatment of the diluted extract with commercial benzidine and hydrogen peroxide, and the comparison of the intensities of the properly diluted colored solutions with appropriate standards. This method made possible a rough estimate of the quantity of blood present, but it could not be relied upon to produce consistent results. Accordingly, at the first opportunity for continuing the investigation, the problem was again attacked in an attempt to find a more satisfactory technique.

Before the present work was begun, the attention of the authors was called to two important papers. The first, by Bing and Baker (2), described a technique for the use of a purified benzidine reagent, together with a 0.6 per cent solution of hydrogen peroxide, for the quantitative determination of hemoglobin in minute quantities of blood. The second, by Bing (3), gave an account of a method for the purification of commercial benzidine and stressed the importance of the purity of that compound in quantitative hemoglobin determinations.

Although the extraction of the hemoglobin or its derivatives from the feces and the dilution of the acetic acid extract as described in the present paper were carried out very much as in 1938, the benzidine reagent used was purified according to the technique of Bing, and the feces extract containing the acid hematin was treated with benzidine and hydrogen peroxide according to the technique of Bing and Baker. The relative color intensities of the resulting solutions were then measured by means of the Evelyn photoelectric colorimeter (4).

The method for the quantitative determination of blood in the feces of sheep as it is described in the following pages is presented simply as the result of an attempt on the part of the authors to solve a definite problem in connection with the study of the hemorrhage associated with stomach-worm infection in these animals. It is hoped that the information thus made available in the literature will be of some value to other workers interested in the quantitative study of gastrointestinal hemorrhage.

Materials and Reagents—The materials and reagents necessary for carrying out the procedure described are as follows:

1. Blood-free sheep feces.
2. 50 cc. round bottomed heavy glass centrifuge tubes.
4. Accurately calibrated pipettes of 1, 2, 4, 5, 10, 15, and 20 cc. capacity.
5. Glacial acetic acid.
6. 50 cc. volumetric flasks.
7. 10 cc. volumetric flasks.
8. Purified benzidine reagent of Ring.
10. 3 per cent hydrogen peroxide.
11. Paraffined corks to fit the colorimeter tubes.
12. No. 660 red filter for the colorimeter.

**Procedure**

The adequacy of the method as a whole was first tested on artificial mixtures containing known amounts of blood added to blood-free feces and a graph relating the quantity of blood to the colorimetric measurement was plotted (see Fig. 1). The operations necessary for obtaining the data for the curve are as follows:

1. Grind the fresh blood-free sheep feces in a mortar until the particles will pass through a screen having 10 meshes to the inch. Weigh thirty-five 1 gm. portions of the feces on an analytical balance and put each portion into a clean, dry, 50 cc. centrifuge tube.
2. Make a 1:10 dilution of a blood sample of known hemoglobin content with distilled water and mix thoroughly.
3. Number each centrifuge tube and carefully add the required amounts of diluted blood to each of the 1 gm. portions of feces, setting up the tubes in triplicate for each point on the curve to be determined. Mix the blood solution thoroughly with the feces, using individual glass stirring rods, and leave the rods in the tubes. Use the remaining two samples as duplicate blanks with blood omitted.
4. Add 10 cc. of distilled water to the blanks and proportionately less to the remaining tubes, so that the total volume of fluid added will be 10 cc. Mix thoroughly.
5. Add 15 cc. of glacial acetic acid to each tube and mix. Heat and remove each tube as soon as the contents begin to boil vigorously. Care must be taken to prevent the contents from boiling over. (The heating was facilitated by putting tubes in wire baskets and placing them on an electric hot-plate.)
6. Push particles adhering to the walls of the tubes back into
the solution, remove the stirring rods, and arrange them according to the number on the edge of a table with the acid-covered ends projecting so that none of the material on the rods may be lost. Centrifuge the tubes at approximately 2700 R.P.M. for 5 minutes.

7. Decant the supernatant fluid into properly numbered 50 cc. volumetric flasks. After transferring the contents of each tube, wash off the decanting rod with a small amount of glacial acetic acid.

8. Make three similar extractions, each with 10 cc. of glacial acetic acid, and bring the contents of the 50 cc. volumetric flasks up to volume with glacial acetic acid and mix thoroughly.

9. Put 1 cc. aliquots from the 50 cc. volumetric flasks into 10 cc. volumetric flasks and bring the contents of the 10 cc. volumetric flasks up to volume with glacial acetic acid and mix.

10. Put 2 cc. of the purified benzidine reagent into the bottom of each colorimeter tube and add 1 cc. aliquots from the 10 cc. volumetric flasks and mix.

11. Add 1 cc. of a 0.6 per cent hydrogen peroxide solution (3 per cent commercial solution freshly diluted with 4 parts of distilled water) and mix. Close with paraffined corks and allow the tubes to stand for 2 hours.

12. Dilute with 20 cc. of a 20 per cent solution of glacial acetic acid in distilled water, insert corks, and invert the tubes several times to mix the contents. Allow to stand for at least 8 minutes.

13. Adjust the colorimeter so that the blank reads 100 on the galvanometer scale when a No. 660 red filter is used. With this adjustment make the colorimetric readings on solutions derived from feces-blood mixtures and plot the average readings of triplicates against the volumes of blood employed.

Results

The results of a typical series of determinations made on sheep feces to which had been added the indicated quantities of calf blood containing 9 gm. of hemoglobin per 100 cc., together with the constant $K_1$ for each of the concentrations shown, are recorded in Table I.

The data in Table I show that the duplicate blanks checked perfectly, but that there was some variation in the readings obtained from the individual specimens making up each group of
triplicate samples containing a given amount of blood. In order to check further the reliability of galvanometer readings obtained from different samples of blood-free feces, a series of duplicate specimens from three healthy sheep on the same diet was tested at the same time and with the same reagents. The results of these tests are shown in Table II.

The data in Table II show that the standard deviation of the galvanometer readings obtained from the different samples of blood-free feces was ±0.38, a variation which may be considered negligible in view of the extreme sensitivity of the calorimeter used. The differences in these readings, as well as those observed between samples within each set of triplicates, were apparently due to errors introduced during the numerous pipetting and diluting operations necessary to carrying out the procedure outlined, for they tended to decrease as the worker became more expert.

### Table I

Data from Which Curve Shown in Fig. 1 Was Plotted, together with Constant $K_1$ for Each Point Determined

<table>
<thead>
<tr>
<th>Blood containing 9 gm. hemoglobin per 100 cc. added to 1 gm. sheep feces</th>
<th>Galvanometer readings*</th>
<th>Average galvanometer reading</th>
<th>Correction factor applied to average galvanometer readings</th>
<th>Corrected galvanometer readings</th>
<th>$K_1$</th>
</tr>
</thead>
<tbody>
<tr>
<td>cc.</td>
<td>A</td>
<td>B</td>
<td>C</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.00</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>0.05</td>
<td>92</td>
<td>91</td>
<td>94</td>
<td>0</td>
<td>92</td>
</tr>
<tr>
<td>0.10</td>
<td>83</td>
<td>81</td>
<td>84</td>
<td>0.05</td>
<td>83</td>
</tr>
<tr>
<td>0.20</td>
<td>62</td>
<td>63</td>
<td>66</td>
<td>0.05</td>
<td>64</td>
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<tr>
<td>0.30</td>
<td>50</td>
<td>48</td>
<td>49</td>
<td>0.1</td>
<td>49</td>
</tr>
<tr>
<td>0.40</td>
<td>41</td>
<td>38</td>
<td>37</td>
<td>0.1</td>
<td>39</td>
</tr>
<tr>
<td>0.50</td>
<td>31</td>
<td>29</td>
<td>27</td>
<td>0.15</td>
<td>29</td>
</tr>
<tr>
<td>0.60</td>
<td>20</td>
<td>22</td>
<td>21</td>
<td>0.15</td>
<td>22</td>
</tr>
<tr>
<td>0.70</td>
<td>14</td>
<td>15</td>
<td>17</td>
<td>0.15</td>
<td>15</td>
</tr>
<tr>
<td>0.80</td>
<td>11</td>
<td>11</td>
<td>10</td>
<td>0.15</td>
<td>11</td>
</tr>
<tr>
<td>0.90</td>
<td>7</td>
<td>6</td>
<td>7</td>
<td>0</td>
<td>7</td>
</tr>
<tr>
<td>1.00</td>
<td>3</td>
<td>3</td>
<td>4</td>
<td>0</td>
<td>3</td>
</tr>
</tbody>
</table>

Average .................................................... 141.97

Standard deviation ......................................... ±28.43

*Exponents refer to quarter units; i.e., 91.5 = 91.50.
Determination of Fecal Blood

As is also shown in Table II, the readings obtained from each series of triplicate samples were averaged, and the average corrected as indicated in the correction table accompanying the galvanometer. The corrected galvanometer readings were then plotted against blood volume as illustrated in Fig. 1.

When the colorimeter readings are linearly proportional to the transmitted light intensity, and the colored solution obeys Beer's and Lambert's laws, the relation \((a \log G)/C = K_1\) should apply, where \(G\) represents the galvanometer reading in per cent of that obtained from 100 per cent transmission, \(C\) represents the concentration, and \(K_1\) is a constant. In the calculation of \(K_1\) in Table I, \(C\) was expressed in mg. of hemoglobin in the colorimeter tube divided by 24, the volume of the colored solution in cc.

Although, as shown in Table I, the value of \(K_1\) over the whole curve had a standard deviation of about twice the permitted variation of 10 per cent, from 0.2 to 0.9 cc., the variation was found to be only 9.68 per cent. This finding showed that between these points, and within the 10 per cent allowable variation, the curve followed the laws of Beer and Lambert, and indicated that along this portion of the curve the concentration of hemoglobin extracted from the feces was for all practical purposes proportional to the corrected galvanometer readings obtained from the colorimeter. Below 0.2 and above 0.9 cc., however, the curve deviated markedly from this straight line relationship, owing probably in the first instance to the impossibility of extracting

### Table II

*Galvanometer Readings Obtained from Different Samples of Blood-Free Sheep Feces*

<table>
<thead>
<tr>
<th>Sheep No.</th>
<th>Sample No.</th>
<th>Galvanometer reading</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>100.0</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>99.5</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>100.0</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>99.5</td>
</tr>
<tr>
<td>3</td>
<td>1</td>
<td>99.5</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>99.0</td>
</tr>
<tr>
<td>Average</td>
<td></td>
<td>99.6</td>
</tr>
<tr>
<td>Standard deviation</td>
<td></td>
<td>±0.38</td>
</tr>
<tr>
<td>Average standard deviation</td>
<td></td>
<td>±0.15</td>
</tr>
</tbody>
</table>
all of the hemoglobin, and in the second to the extreme concentration of the acid hematin with respect to the amount of benzidine reagent present.

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

**Fig. 1.** Curve showing the corrected galvanometer readings obtained from the analysis of 1 gm. portions of sheep feces to which had been added the indicated volumes of calf blood containing 9 gm. of hemoglobin per 100 cc.

Any curve constructed on the basis of the foregoing procedure will be similar to the one illustrated, but will differ from it more or less on account of differences in the hemoglobin content of the blood sample and the purity of the benzidine reagent used.

**Determination of Unknown Quantities of Blood in Sheep Feces**

The procedure for determining the hemoglobin content of a sample of sheep feces containing an unknown quantity of blood
Determination of Fecal Blood

is the same as that just described with the following exceptions. (1) The total feces passed by the animal are weighed after screening; (2) additional blood is not added to the feces; (3) 10 cc. of distilled water are added to the blank and also to the feces containing the unknown quantity of blood; and (4) one blank and triplicate samples of the unknown are sufficient for the analysis.

As a practical test of the method, two sheep were drenched on several occasions with 200 to 400 cc. of blood from the same calf whose blood was used in plotting the curve in Fig. 1. The results of one of these tests following the administration of 400 cc. of blood mixed with 100 cc. of normal saline solution are recorded in Table III.

<table>
<thead>
<tr>
<th>Time after drenching</th>
<th>Weight of 24 hr. sample of feces</th>
<th>Average galvanometer readings</th>
<th>Blood indicated</th>
<th>Blood passed</th>
</tr>
</thead>
<tbody>
<tr>
<td>h. m.</td>
<td>gm.</td>
<td></td>
<td>cc.</td>
<td>cc.</td>
</tr>
<tr>
<td>24</td>
<td>158</td>
<td>72</td>
<td>0.140</td>
<td>22.12</td>
</tr>
<tr>
<td>48</td>
<td>314</td>
<td>67</td>
<td>0.175</td>
<td>54.95</td>
</tr>
<tr>
<td>72</td>
<td>365</td>
<td>76</td>
<td>0.125</td>
<td>45.63</td>
</tr>
<tr>
<td>96</td>
<td>372</td>
<td>100</td>
<td>0.000</td>
<td>0.00</td>
</tr>
<tr>
<td>Total blood passed</td>
<td></td>
<td></td>
<td></td>
<td>122.70</td>
</tr>
<tr>
<td>Percentage of volume administered</td>
<td></td>
<td></td>
<td></td>
<td>30.70</td>
</tr>
</tbody>
</table>

The data in Table III show that the 30.70 per cent of the ingested blood which was recovered from the feces of this sheep was passed within 72 hours of the time of its administration. In four similar tests with sheep drenched with 200 cc. of blood, the 25 to 50 per cent of the total volume administered which was recovered was passed within 48 hours after ingestion. While these percentages varied considerably in the different tests and were also relatively small, it must be remembered that the blood did not originate within the alimentary canal of the animal in question, and also that, although an effort was made to insure its immediate entrance into the fourth stomach, it was not always certain that the blood went directly into that organ. Accordingly, losses caused by bacterial decomposition and possibly others occurring in the process of rumination could have taken place during the time the
blood remained in the rumen. If, however, the blood had come from lesions within the digestive tract, as would have been the case in infections with stomach-worms or hookworms, it appears to the authors that a greater percentage of the blood lost by hemorrhage would be found in the feces, not only because the blood would enter the alimentary canal lower down and thus be subjected to less decomposition and absorption, but also because any stimulation of the intestine brought about by the presence of the infections would hasten its elimination. While the authors can offer no proof for this statement, it appears to them to be a reasonable hypothesis and to make the method described of more value in studying the quantity of blood lost in such cases.

Since the curve in Fig. 1 is plotted in terms of cc. of blood having a known hemoglobin content, it is apparent that, if the concentration of hemoglobin in the blood of the animal used in the tests is known, the number of cc. of blood represented by the hemoglobin present in the feces may be expressed in terms of the volume of blood of the animal in question and this volume in turn may be related to any anemia that may be present.

**DISCUSSION**

In order that this method may be used successfully, five important points must be emphasized.

(a) The same lot of purified benzidine reagent should be used for plotting the reference curve and for making the determinations on unknown quantities of hemoglobin in any one experimental series. In the course of the work leading up to the development of this method it was found that reference curves produced by different lots of purified benzidine were more or less parallel but were not identical. This finding indicated that, although decolorization of any two samples of benzidine is carried out in exactly the same way, there may still be differences in the amounts of impurities in the compounds, which would make them useless unless employed in connection with their own reference curves.

(b) All determinations should be made on fresh feces. It was found that when feces containing blood were allowed to remain at room temperature, or were dried at 50° and 100° for 24 hours, the amount of hemoglobin extracted was less than that obtainable from the same feces when fresh. This adsorption and fixation
of the hemoglobin was particularly noticeable in those samples of feces dried at the higher temperatures.

(c) All weighing, pipetting, and diluting must be done with meticulous care. On account of the sensitivity of the color reaction between the benzidine reagent and the hemoglobin derivatives present in the acetic acid extract the slightest error in carrying out any of the operations indicated in the procedure will cause large variations in the resulting color and in the consequent galvanometer readings. It is, therefore, of utmost importance that these operations be performed with the greatest accuracy.

(d) Always use fresh glacial acetic acid extracts for making colorimetric comparisons. The acid hematin present in the acid extracts apparently precipitates out within a few hours in the acid solution, so that the color obtained from extracts 24 hours old is much weaker than that obtained from the same extracts when fresh. Accordingly, unless fresh extracts are used, the resulting color will not represent the original quantity of hemoglobin extracted from the sample.

(e) Do not use a blank as a reference point for any other similar sample containing blood unless the two tubes have been diluted at the same time. All of the diluted solutions in the colorimeter tubes, including the blank, tend to darken on standing. The color intensity of any one tube within a group, however, will read the same in the colorimeter until precipitation takes place about 24 hours after the tubes are set up, provided the machine is reset as the blank darkens.

It will be noted that the actual amount of acid hematin introduced into the colorimeter tubes in step (10) of the procedure represents only 1/500th of the total quantity of blood added to the feces in step (3), and the question might well be raised as to why the determinations were made on such a small portion of the original material. The reason for this technique lies in the fact that benzidine reacts to some extent with other pigments in the feces, and only by great dilution of the extracts could check blanks be obtained from the same lot of blood-free feces.

There is no reason why the method described in this paper may not also be applied to the quantitative determination of blood in the feces of other animals provided that (1) suitable blanks are available, (2) a satisfactory reference curve can be constructed,
and (3) interfering substances that may react with the benzidine reagent are absent.

The method for the quantitative determination of blood in sheep feces described in the present paper has been found to give satisfactory results in tests for the determination of the quantity of fresh blood (hemoglobin) added artificially to 1 gm. portions of blood-free sheep feces.

The recovery of 25 to 50 per cent of the total volume of blood ingested by healthy sheep from the feces of these animals by the use of this method indicated that it may be of some value in the study of gastrointestinal hemorrhage in sheep.

SUMMARY

A method for the quantitative determination of blood in the feces of sheep is described. The method was tested on the feces of sheep which had been drenched with measured volumes of calf blood and by its use 25 to 50 per cent of the ingested blood was recovered. All of the blood recovered was passed within 72 hours of the time of ingestion. The limitations of the method and its further application to the feces of other animals are discussed.

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