A Micro-method for the Estimation of Vitamin B₁

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A micro-method for the estimation of vitamin B₁ has been described by Atkin, Schultz & Frey [1939]. It is based on the measurement of the rate of fermentation of sugar by baker's yeast, using a Warburg or similar apparatus, and is sensitive to 0.005 μg. of vitamin B₁. The method described below is based on the thiochrome technique and is of similar sensitivity. It was developed during work on the distribution of vitamin B₁ in the wheat grain and has been applied successfully to small quantities (1–50 mg.) of cereal products [Hinton, 1942].

EXPERIMENTAL

The method is based upon the optical design of the Spekker Photoelectric Fluorimeter. In this instrument, an image of the mercury discharge tube is brought to a focus at a point opposite the photo-cell, the focused beam normally passing through the test solution contained in a large glass cell. In the micro-method here described, the glass cell is replaced by a suitable holder by means of which a capillary tube containing the test solution is placed in the image, which measures 2 x 20 mm. approximately in the focal plane, and thus receives the full irradiation transmitted by the system (Fig. 1).

The Spekker Fluorimeter was used in conjunction with a Cambridge Short-Period Galvanometer of sensitivity 3000 mm./μA., and resistance 196 ohms, which was used at full sensitivity.

Procedure

The strength of ferricyanide to produce maximum fluorescence in the test solution is first ascertained by the procedure summarized below. With our cereal extracts this was found by testing a range of strengths from 0.05 to 0.5 %, in steps of 0.05 %. For standard aneurin solutions the best concentration was 0.005 %.

The pipette to be used for the test solution is first washed out with the solution, in readiness. 0.1 ml. of 30 % NaOH is measured into a 2 × 3 in. tube, 0.02 ml. of the appropriate ferricyanide solution run in on top and left undisturbed. 0.1 ml. of the test solution is then measured out and, immediately before this is added to the tube, the NaOH and ferricyanide are mixed by shaking. The test solution is run in and the whole again mixed by shaking. Oxidation is carried out in this way with each tube in a series which, in order to check the calibration curve, includes three standard aneurin solutions of different strengths. 0.2 ml. of water-saturated isobutanol is then added to each tube, and extraction carried out by stirring for 30 sec. The tubes are allowed to stand for 10–15 min. to allow separation of the layers, those containing cereal extracts being centrifuged for 3–4 min. at 1500 r.p.m. to improve the separation. The isobutanol layer is drawn up directly into the capillary tube for measurement in the fluorimeter. A no. 39 Wratten filter was used in front of the right-hand photo-cell (Fig. 1).

The calibration curve was prepared by the same procedure, using solutions containing from 0.001–0.2 μg. aneurin/0.1 ml.
Examination of the procedure

Accuracy of the method. Standard aneurin solutions in N/20 HCl were found to be stable until cloudiness and growth developed. In most cases they were stable for several months. Cereal extracts were made with N/5 HCl [Nicholls, Booth,Kent-Jones, Amos & Ward, 1942] applied on a micro scale.

In the case of solutions of pure aneurin the extreme variations between replicates were ± 20% at concentration of aneurin in the region of 0-001 µg., decreasing to ± 5% at 0-05 µg.

It should be noted that attention was paid to the possibility of decomposition of thiocarbanthoin by light during the preparation of the thiocarbanthoin solution, especially in the case of the smallest quantities. Readings obtained when the operations were carried out near a window in the full daylight of a clouded sky did not, however, differ significantly from those carried out in very weak artificial light (1–2 candle power). The only precaution taken, therefore, was to shade the operations from sunlight or from very bright sky light.

The capillary test-cell and holder. Greatest sensitivity was obtained with a glass tube of oval section and 1 × 3 mm. bore. For good results certain details required attention: the holder (Fig. 2) was made so that the angle which the oval section made with the light beam could be adjusted to the optimum and the tube adjusted squarely in the beam; fluorescence from the glass wall was masked off; adjustments were made to the lamp-house, sliding carrier, etc. of the fluorimeter to make the whole as stable as possible; additional shielding of the photo-cells was provided and great changes in illumination of them during a period of use was avoided.

The length of the tube was 8 cm. To fit it a rubber 'policeman', slipped right down over the end of the tube, is slid carefully upwards whilst the point dips below the surface. The contents are ejected by the reverse movement. On each occasion before the tube is placed in position for measurement the outside should be cleaned by moistening with ethanol and polishing with a cloth. A considerable amount of isobutanol remains on the inside of the tube after it is emptied and the tube should therefore be rinsed with two lots of the solution to be measured. This is done by drawing up a drop of the solution and running it along the tube. The same tube was used for measurement of blank, standard and unknown, and it should be marked and always placed in the holder the same way up.

Standard of fluorescence. As a permanent standard, a crystal of fluorite was used, which was mounted in a holder and masked by a diaphragm to give a fluorescence intensity of the same order as a solution of quinine sulphate of 0-5 µg./ml.

Expression of results. The figures given in the text to illustrate each point are expressed in a relative form. They were expressed in terms of aneurin found on the basis of the fluorescence reading, and then recalculated for each series, the highest yield in the series being taken as 100.

Optimum volumes of reagents. To attain maximum sensitivity, the amount of isobutanol was kept at a minimum. The tube required 0-12 ml., and a total of 0-2 ml. provided enough excess to give the necessary two rinsings. Iso- butanol saturated with water was used. 0-1 ml. was chosen as a convenient quantity of test solution, and 0-1 ml. of NaOH gave a total volume most suitable for the subsequent extraction. The smallest quantity of potassium ferricyanide solution which could be handled with confidence is 0-02 ml., and this amount was used in all cases. It was found most satisfactory to keep the volume constant in each case and to adjust the concentration of the solutions as necessary. The reactions were carried out in test-tubes 2 × ⁵/₈ in.

Handling of these small volumes was facilitated by constructing special pipettes to hold the quantities required, the capillary tube being drawn off slightly and the tip bent round and ground (Fig. 3). The solutions are blown out on to the side of the tube and can be placed just where required.

Fig. 2. Holder made from sheet metal with $\frac{1}{4}$" brass base to fit the cell carrier of the Spekker Photoelectric Fluorimeter and hold the capillary-tube test cell at the correct angle and position.

Methanol was omitted, as, under these conditions, it seriously affected the partition of the thiocarbanthoin in the two phases. Jansen [1936] used methanol because of its protective action towards thiocarbanthoin, which is thus made less sensitive to excess ferricyanide. The methanol, however, increases the proportion of thiocarbanthoin remaining in the aqueous phase; this effect is not serious when the volume of isobutanol is many times greater than the aqueous phase. In the present case, the volumes of the two phases are equal and the serious effect of the methanol on the final fluorescence is seen from the following figures:

<table>
<thead>
<tr>
<th>Methanol (ml)</th>
<th>Nil</th>
<th>0.01</th>
<th>0.04</th>
<th>0.08</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reading</td>
<td>100-0</td>
<td>95-0</td>
<td>81-5</td>
<td>54-0</td>
</tr>
</tbody>
</table>

These results were obtained by carrying out the oxidation in the absence of methanol, which was added finally with the isobutanol. 0-08 ml., which corresponds to about 30% of the reaction mixture, causes an excessive reduction in sensitivity. The aim was to attain maximum sensitivity, but some reduction could be accepted if tolerance of the thiocarbanthoin towards excess ferricyanide was thereby obtained. (Tolerance in this paper is defined as the range of concentration of ferricyanide over which the same fluorescence reading is obtained for a fixed amount of aneurin.) The following figures illustrate the tolerance obtained, with 0-2 µg. standard aneurin solutions, by the addition to the
reaction mixture of 0.02 ml. of methanol, which produces a permissible drop in sensitivity:

Strength of ferricyanide (%)  0-003  0-006  0-012  
Reading  89-0  100-0  100-0  
Strength of ferricyanide (%)  0-025  0-05  0-1  
Reading  100-0  97-0  93-5  

There is a tolerance range of about 8 times. Without methanol, the tolerance range is reduced to twice:

Strength of  0-0006  0-0012  0-0025  0-005  0-01  
Ferricyanide (%)  Reading  47-5  82-0  99-0  100-0  95-0  

As 50 times more ferricyanide is required for a cereal extract than for the standard aneurin solution, the slight increase in tolerance noted above was not worth considering. In Jansen’s experiments he used about 60% methanol in the reaction mixture and reported a range of 500 times.

It is noted from the two series of figures that the optimum strength of ferricyanide is 10 times greater when methanol is present. This is because the methanol enters into the reaction, inactivating the ferricyanide. With 0.1% ferricyanide the following figures were obtained:

<table>
<thead>
<tr>
<th>Methanol (ml.)</th>
<th>0-01</th>
<th>0-04</th>
<th>0-08</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reading</td>
<td>100-0</td>
<td>88-7</td>
<td>6-8</td>
</tr>
</tbody>
</table>

This effect is much greater than the effect on the thiochrome plate. With 0.5% ferricyanide solution, the figures were:

<table>
<thead>
<tr>
<th>Methanol (ml.)</th>
<th>0-01</th>
<th>0-04</th>
<th>0-08</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reading</td>
<td>89-5</td>
<td>100-0</td>
<td>77-0</td>
</tr>
</tbody>
</table>

In light of the effect of the methanol on partition, this indicates a reversed effect which is due to reduction of the ferricyanide concentration towards the optimum by the action of the methanol.

The inactivation of the ferricyanide proceeds slowly, and the above results were obtained by mixing the NaOH, ferricyanide and methanol together and allowing the mixture to stand some minutes before adding the aneurin solution. The time effect is illustrated by the data obtained with 0.05% ferricyanide:

| Aneurin (0.02µg.) added immediately on mixing | 100-0 |
| Aneurin (0.02µg.) added after 1 hr. | 50-0 |

Aneurin (0.15µg.) added after time intervals as shown:

<table>
<thead>
<tr>
<th>Min.</th>
<th>3</th>
<th>8</th>
<th>13</th>
<th>18</th>
<th>23</th>
<th>28</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reading</td>
<td>100-0</td>
<td>98-5</td>
<td>96-8</td>
<td>91-5</td>
<td>88-0</td>
<td>78-0</td>
</tr>
</tbody>
</table>

Again, if a stronger ferricyanide solution is used, the effect is first to increase the fluorescence, which is due to reduction in ferricyanide towards the optimum. The following data were obtained with 0.1% ferricyanide:

| Aneurin (0.2µg.) added immediately on mixing | 89-5 |
| Aneurin (0.2µg.) added after 45 min. | 100-0 |

The strength of the NaOH solution has an effect on the speed of this reaction, e.g. with 0.1% ferricyanide and 0.04 ml. methanol, and after the same time of standing these figures were obtained with 0.15µg. aneurin:

| NaOH (%) | 30 | 40 | 60 |
| Reading  | 100-0 | 54-0 | 10-7 |

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Again, with a stronger solution of ferricyanide and the same amount of methanol, the effect is reversed:

Reading with 0.5% ferricyanide  76-0  89-0  100-0

Optimum strength of ferricyanide. Since it is not practicable to add sufficient methanol to affect the tolerance appreciably, the strength of ferricyanide needs adjusting for each type of material tested, to obtain maximum fluorescence. For standard aneurin solutions the optimum strength is 0.005%.

<table>
<thead>
<tr>
<th>Aneurin (µg.)</th>
<th>0-005</th>
<th>0-01</th>
<th>0-002</th>
<th>0-003</th>
<th>0-004</th>
<th>0-005</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reading</td>
<td>100-0</td>
<td>100-0</td>
<td>97-0</td>
<td>95-5</td>
<td>97-5</td>
<td>100-0</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Ferricyanide (%)</th>
<th>0-15</th>
<th>76-0</th>
<th>77-8</th>
<th>91-2</th>
<th>95-5</th>
<th>97-5</th>
<th>100-0</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-01</td>
<td>61-6</td>
<td>66-0</td>
<td>88-0</td>
<td></td>
<td></td>
<td></td>
<td>100-0</td>
</tr>
<tr>
<td>0-005</td>
<td>80-0</td>
<td>75-0</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>100-0</td>
</tr>
</tbody>
</table>

The optimum strength for various cereal extracts is shown below:

<table>
<thead>
<tr>
<th>Ferricyanide (%)</th>
<th>0-05</th>
<th>0-10</th>
<th>0-15</th>
<th>0-20</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. 1 extract</td>
<td>63-0</td>
<td>90-5</td>
<td>100-0</td>
<td>97-0</td>
</tr>
<tr>
<td>No. 2 extract</td>
<td>63-0</td>
<td>90-5</td>
<td>99-5</td>
<td>100-0</td>
</tr>
<tr>
<td>No. 3 extract</td>
<td>61-2</td>
<td>67-0</td>
<td>91-0</td>
<td>94-0</td>
</tr>
</tbody>
</table>

A ferricyanide solution of 0.005% is unstable if exposed to strong light, decomposing in 30 min. or so. Protected from the light, it has been kept for 1 week and may be stable for longer. It was preferred to make a fresh solution by diluting a stock solution of 0.5% each day, and to keep it shaded from daylight.

Optimum strength of NaOH. Solutions varying from 7 to 60% have been tested with varying strengths of ferricyanide. The following figures illustrate the results and indicate that 30% is the most satisfactory:

<table>
<thead>
<tr>
<th>NaOH (%)</th>
<th>20</th>
<th>25</th>
<th>30</th>
<th>35</th>
<th>40</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reading</td>
<td>89-0</td>
<td>96-2</td>
<td>100-0</td>
<td>96-2</td>
<td>89-0</td>
</tr>
</tbody>
</table>

Order of mixing the reagents. The following figures indicate the degree of destruction of 0.2µg. of aneurin when it is mixed with the NaOH and allowed to stand varying times before the ferricyanide is added:

<table>
<thead>
<tr>
<th>Ferricyanide added</th>
<th>0</th>
<th>3</th>
<th>6</th>
<th>9</th>
<th>12</th>
<th>15</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reading (after min.)</td>
<td>100-0</td>
<td>70-0</td>
<td>47-5</td>
<td>34-0</td>
<td>25-4</td>
<td>14-3</td>
</tr>
</tbody>
</table>

Jansen mixed the aneurin solution and ferricyanide first, adding the NaOH immediately afterwards. Greater fluorescence is obtained, however, by adding the aneurin solution to the mixture of NaOH and ferricyanide:

| 0-2µg. of aneurin added to ferricyanide | 83-5 | 81-3 | 77-2 |
| 0-2µg. of aneurin added to mixture | 100-0 | 100-0 | 100-0 |

| NaOH and ferricyanide | 38 |
The mixture with NaOH is unstable, when the ferri-
cyanide concentration is of the order of 0.005%. The
following method of mixing the reagents was therefore
adopted: 0.1 ml. of NaOH is placed first in the bottom of
the tube. 0.02 ml. of ferriyanide is then pipetted on to
the side of the tube just above the surface of the NaOH,
so that it runs down and floats on the surface of the solu-
tion. This partition is not disturbed until the test solution
is measured out and is ready for adding. With the pipette
in one hand, the NaOH and ferriyanide are mixed by
shaking the tube, the test solution immediately run in and
the whole shaken again. All the reagents are thus mixed
together within 30 sec. The times shown in the results
below are times elapsing between floating the ferriyanide
on the NaOH, and mixing with addition of aneurin solution:

<table>
<thead>
<tr>
<th>Time elapsing (sec.)</th>
<th>20</th>
<th>120</th>
<th>180</th>
<th>240</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reading</td>
<td>99.7</td>
<td>99.7</td>
<td>98.0</td>
<td>100.0</td>
</tr>
</tbody>
</table>

Once this stage has been reached, the addition of, and
extraction with, isobutanol can be carried out within any
reasonable time. In the following series the stated times
elapsing between carrying out the oxidation and extraction
with isobutanol.

<table>
<thead>
<tr>
<th>Time elapsing (min.)</th>
<th>0</th>
<th>5</th>
<th>10</th>
<th>15</th>
<th>20</th>
<th>25</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reading</td>
<td>95.3</td>
<td>100.0</td>
<td>95.3</td>
<td>95.5</td>
<td>95.3</td>
<td>99.0</td>
</tr>
</tbody>
</table>

*Extraction with isobutanol.* With these small volumes,
bubbling a gas through the liquid is not a successful method of
mixing the two phases. Stirring was found to be quite
satisfactory. A small glass stirrer made from glass rod
drawn out to a diameter of about 1 mm. and shaped into
an elongated loop 1 cm. long was fastened directly in the
chuck of a small laboratory motor. A frictional brake gave
the smooth speed control necessary. With standard aneurin
solutions, high speeds, 1000 r.p.m. or more, were necessary
to break up the phases completely, but with cereal extracts
much slower speeds, 200 or 300 r.p.m., were sufficient and
essential, since high speeds produced a fine emulsion which
could not be broken down by simple methods. Extracts
from some products, e.g. wheat germ, emulsified more
readily than others, e.g. endosperm; the lowest speed which,
sufficed to break up the phases was never exceeded. The
following experiment, in which the solution was oxidized
in bulk and portions taken for extraction, shows that time
of mixing is not critical:

<table>
<thead>
<tr>
<th>Time of mixing (sec.)</th>
<th>5</th>
<th>10</th>
<th>20</th>
<th>40</th>
<th>60</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reading</td>
<td>95.7</td>
<td>99.5</td>
<td>97.5</td>
<td>100.0</td>
<td>99.0</td>
</tr>
</tbody>
</table>

As a measure of safety, 30 sec. was adopted as standard.
The two layers are left in the original tubes, the isobutanol
being drawn off directly into the capillary test cell. With
cereal extracts the isobutanol layer was cleared by centri-
fuging at about 1500 r.p.m.; this was not necessary with
standard aneurin solutions. Within the time elapsing
between extraction and measurement in the fluorimeter
is not important:

<table>
<thead>
<tr>
<th>Measured after (min.)</th>
<th>0</th>
<th>5</th>
<th>10</th>
<th>15</th>
<th>20</th>
<th>25</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reading</td>
<td>95.0</td>
<td>97.5</td>
<td>99.0</td>
<td>99.0</td>
<td>100.0</td>
<td>97.0</td>
</tr>
</tbody>
</table>

*Calibration curves.* Calibration curves have been pre-
pared with different filters in front of the right-hand photo-
cell; three of these are reproduced in Fig. 4. The shape of
the curve is dependent upon the transmission of the filter
and the quality of the light emitted by the fluorescent
standard. With Wratten filter no. 47 (curve not given) the
maximum transmission is in the region a little above the
thiochrome fluorescence. For this reason, the photo-cell
will respond much below 0.005 µg. The Hilger no. 6,
which has a similar but broader transmission, produces a
similar shaped curve, A in Fig. 4, and as it is much less
dense, allows the photo-cell to respond to 0.001 µg. The
lowest measurements, however, come on an insensitive
part of the drum, and with this filter a group of calibration
curves shows considerable spread below 0.01 µg. The
Wratten no. 39, though of overall density greater even
than that of no. 47, transmits well in the region of thio-
chrome fluorescence and allows response at 0.001 µg. The
shape of the curve, B in the figure, is closely similar to the
shape of the unfiltered curve C. This filter also shifts the
lowest reading slightly on the drum and a group of curves
showed no serious spread over the whole range. On these
grounds, no. 39 was judged to be the most suitable filter
so far used.

Each curve shown in Fig. 4 is the average of a group of
six to eight complete curves. It is desirable to deal with
groups of curves in this way as, in common with other
photo-electric colorimeters, the Spekker may exhibit a
slight shift in the whole curve from day to day. This is
particularly the case when it is used as described here, and
for this reason it is advisable to put one or two aneurin
standards in with each run of estimations. When the
capillary test cell containing the thiochrome solution has
been placed in position, the photo-cells must be balanced
without delay, as the thiochrome is then unstable and the
readings fall continuously, especially with solutions of
0.1 µg. of aneurin and above. There is ample time, however,
to carry out the balancing precisely, as no significant change
in the reading occurs in 30 sec.

**SUMMARY**

1. A micro-technique for the estimation of vita-
min B<sub>1</sub> has been developed. The method is based
on the determination of thiochrome with the
Spekker Fluorimeter. A special cell is used and a
precise plan must be followed with the different
reagents normally used in the conversion to thiochrome.

2. In solutions of pure aneurin (or in cereal extracts) the method is accurate to ±20% at concentrations in the region of 0.001 µg, the accuracy increasing to ±3% at 0.05 µg.

REFERENCES


Application of the Millon Reaction to the Determination of Chlorophenols in Body Fluids and Tissues

BY B. ZONDEK, B. SHAPIRO AND S. HESTRIN, Gynaecological-Obstetrical Department, Rothschild Hadassah-University Hospital and the Hormone Research Laboratory, Hebrew University, Jerusalem, Palestine

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The versatile antibacterial usefulness of halogenated phenols makes valuable a specific micro-method for their determination in body fluids and tissues [Zondek, 1942]. The present paper describes a semimicro-method for p-chloro-m-xylanol (referred to as CX) and p-chloro-m-cresol (referred to as CC) in the presence of phenol, e.g. in urine. Patients given CX may excrete this substance in concentrations up to 50 mg./100 ml. The halogenated phenol is allowed to react with Millon reagent at room temperature, the coloured substance so formed being extracted with ether and thus obtained in crystalline clear solution suitable for photometric measurement. Phenol itself under these conditions gives a coloured substance which is only sparingly soluble in ether. The method is therefore highly specific.

Several investigators have used the Millon reaction for the determination of tyrosine, e.g. Folin & Ciocalteu [1927]. The present successful extension of such a method to at least two selected chlorophenols suggests that further investigation would render possible a more general application of the Millon reaction to analytical methods, in which the conventional procedures for the estimation of phenols are often inadequate.

EXPERIMENTAL

Reagents. Reagent 1: A mixture of 50 ml. of a 20% solution of mercuric acetate and 30 ml. of 25% (v/v) HNO₃ (d = 1.42).

Reagent 2: A 1:4 diluted solution of 100 g. of mercury in 200 g. of HNO₃ (Millon reagent).

Standard solutions: 1 g. of CX or CC, twice recrystallized from ethanol and dried in vacuo, is dissolved in 5 ml. of NaOH and diluted with H₂O to 1 l. In amber bottles the solutions are constant for at least 1 month.

A. Procedure with aqueous solutions

Determination of CX. To 15 ml. of test solution in a test-tube of c. 30 ml. capacity are added 2 ml. of reagent 1 and then, if no precipitate has formed, 1 ml. of reagent 2. If a precipitate forms, filter and carry out the test on a measured portion. The mixture is kept at room temperature for 30 min., cooled in ice-water, and 5 ml. of chilled ether added. The colour is brought into the ether phase by shaking, and the test-tube immediately stoppered. Within the next hour the ether extract is transferred to a 0.5 cm. cuvette covered with a glass slide. The extinction value (E) is determined in a Pulfrich photometer with filter S 43. The concentration of the unknown is read by reference to a standard curve prepared by plotting E for known solutions similarly treated. This curve should be renewed for each fresh batch of reagent and should be checked every 2 weeks.

Determination of CC. CC is determined by a somewhat modified procedure. 1 ml. of reagent 1 and 1 ml. of reagent 2 are added to 10 ml. of the test solution. The mixture is incubated for 2 hr. at 37°C, cooled in ice-water for 5 min., and shaken with 5 ml. of cold ether. Photometry is carried out within 1 hr. (filter S 50 and 0.5 cm. cuvette).

Results. The colour formed with CX is dirty red in the water phase, but clear yellow in the ethereal solution. With CC the colour is red in water and in ether. Typical standard curves are given in Fig. 1. Within a wide range the relation between E and the concentration of the halogenated phenol is nearly linear. Concentrations of CX or CC greater than 0.5 mg./100 ml. were determined with an error of 5%. Lower concentrations may be determined.