A METHOD FOR THE DETERMINATION OF COPPER IN BLOOD SERUM*

BY GEORGE E. CARTWRIGHT, PATRICIA J. JONES, AND MAXWELL M. WINTROBE

(From the Department of Medicine, University of Utah School of Medicine, Salt Lake City)

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The demonstration that copper is transported in the serum (1) makes available a means of studying copper metabolism and gives rise to the necessity of having a simple and accurate method which can be applied to the measurement of a large number of samples. The purpose of this paper is to present such a method.

Sodium diethyldithiocarbamate has been found to be a satisfactory and sensitive reagent for the determination of copper. Callan and Henderson (2) discovered that when this substance was added to a solution of copper a golden brown color was produced. McFarlane (3) found that the colored copper salt could be rapidly and quantitatively extracted from aqueous solution by amyl alcohol and that the color was intensified in the organic solvent. The depth of color was reported to be directly proportional to the amount of copper present, provided the range of copper concentrations was not too great. The color complex is stable for at least 2 hours and the pH of the solution has little effect on the color intensity between pH 5.7 and 9.2. Iron gives a brown color with the reagent and is the only substance in biological ash which is known to interfere significantly. However, when sodium pyrophosphate is added, iron pyrophosphate is formed and this compound does not react with the carbamate, whereas the reactivity of the copper is unaffected (3, 4).

Locke, Main, and Rosbash (5) prepared a protein-free filtrate of serum by precipitating the proteins in the cold with trichloroacetic acid and determined the copper content of the filtrate with the carbamate reagent. Recoveries of added copper were not reported. Tompsett (6) demonstrated that the whole of the serum copper is present in such a filtrate and is present in such a form that it can be determined directly with carbamate. Using such a procedure, he obtained excellent recoveries and the results compared closely with those obtained by ashing. The observations of Yoshikawa, Hahn, and Bale are not in agreement (7). Using radioactive copper, they found that nearly all of the copper in plasma is bound in some manner to

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protein and that only about two-thirds is split off with trichloroacetic acid in the cold.

McFarlane (3) as well as Sachs, Levine, Anderson, and Schmit (4) has determined serum copper with the carbamate reagent by dry ashing first. However, ashing methods are fraught with many difficulties such as spattering, contamination from reagents, and volatilization of the copper if the temperature is too high. They are inconvenient, time-consuming, and require a muffle furnace or a digestion rack. Such methods do not lend themselves well to the determination of a large number of specimens. Therefore, it was felt that if a reliable method could be devised which avoided ashing and was relatively simple it would be well worth while.

EXPERIMENTAL

Standard copper solutions with amounts of copper varying from 1 to 10 \( \gamma \) per 10 ml. were treated according to the method of Locke, Main, and Rosbash (5) with 0.2 ml. of pyridine and 1 ml. of 1 per cent carbamate and extracted with 10 ml. of amyl alcohol. The alcoholic extracts were then read in the Evelyn photoelectric colorimeter with the 6 ml. aperture and Filter 440. The results are presented in Fig. 1. The extracts were cloudy and first had to be filtered, but even with this additional step results were not reproducible and a reliable standard curve could not be obtained. This can be explained by the fact that in order to read in the macro Evelyn colorimeter it was necessary to extract with at least 10 ml. of alcohol and as a result the final concentration of copper was not great. McFarlane (3) used larger concentrations of copper and smaller volumes of amyl alcohol, and measured the color intensity of a Duboscq colorimeter with micro cups. Locke, Main, and Rosbash (5) used only 2 ml. of alcohol and made crude colorimetric comparisons with a standard held in the sunlight. These differences probably account for the variability of the results of the amyl alcohol extractions. In any event, it was decided that this procedure was not suitable for use in the Evelyn colorimeter under these conditions.

In Fig. 2 the results of reading the color directly in the aqueous solution are shown. 1 ml. of a saturated solution of sodium pyrophosphate, 2 ml. of redistilled ammonium hydroxide, and 1 ml. of a 0.1 per cent solution of sodium diethyldithiocarbamate were added to 10 ml. of varying dilutions of a standard copper solution. The volume was then made up to 15 ml. with redistilled water and the solutions read in the Evelyn colorimeter with the 10 ml. aperture and Filter 440. As can be seen, the results were considerably more consistent than with the amyl alcohol extraction procedure. It was therefore decided to read the color directly in the aqueous solution and to avoid the additional as well as unnecessary and unreliable step of extracting with amyl alcohol.
To ascertain what portion of the total serum copper is present in a tri-
chloroacetic acid filtrate, samples of serum, to which known amounts
of copper were added, were pipetted into 15 ml. Pyrex centrifuge tubes and
3 ml. of redistilled water added. The tubes were then placed in a water
bath at boiling temperature until the solutions became opaque, were cooled,
and following the addition of 2 ml. of 20 per cent trichloroacetic acid were
placed in a water bath at 90-95° for 3 minutes with stirring. The tubes
were then spun at 2500 r.p.m. for 15 minutes. The supernatant solutions
were decanted and the copper content determined in the aqueous solution
with carbamate as described above. An average of 74 per cent of the added
copper was recovered, as can be seen in Table I. Following resuspension
of the original precipitate and a second extraction, it was possible to recover
a total of 89 per cent. Three extractions yielded an average recovery of 97
per cent.

Fig. 1. Showing the variability of amyl alcohol extractions of standard copper
solutions. L refers to photometric density. G is the galvanometer reading.
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FIG. 2. The standard curve obtained by adding sodium diethyldithiocarbamate directly to the aqueous copper solution. $L$ refers to photometric density. $G$ is the galvanometer reading.

### Table I

**Showing Average Recovery of Copper following One, Two, and Three Extractions of Trichloroacetic Acid Precipitate**

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>No. of trichloroacetic acid extractions</th>
<th>No. of determinations</th>
<th>Average recovery per cent</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>1</td>
<td>4</td>
<td>74</td>
</tr>
<tr>
<td>II</td>
<td>2</td>
<td>10</td>
<td>89</td>
</tr>
<tr>
<td>III</td>
<td>3</td>
<td>24</td>
<td>97</td>
</tr>
</tbody>
</table>

**Proposed Method**

1. Copper-free water. Redistil water in an all-glass distilling apparatus.
2. Trichloroacetic acid, 20 per cent in redistilled water. The trichloroacetic acid is first distilled in an all-glass apparatus to free it of copper.
3. Sodium pyrophosphate. Make up a saturated solution in redistilled water.
4. Ammonium hydroxide, approximately 28 per cent. Distil reagent grade ammonium hydroxide through an all-glass apparatus into redistilled water until the solution is saturated.

5. Sodium diethyldithiocarbamate (Eastman). Make up a 0.1 per cent solution with redistilled water.

6. Standard solution of copper. Dissolve 0.3928 gm. of CuSO₄·5H₂O in redistilled water. Dilute to 1 liter. (It is important to use clear crystals of copper sulfate that show no sign of efflorescence.) Make up working solutions from this.

All glassware used must be carefully cleaned and then rinsed three times with redistilled copper-free water.

Pipette 3 to 5 ml. samples, preferably 5 ml., of serum or plasma into ungraduated 15 ml. Pyrex centrifuge tubes. It is convenient to set up that number of tubes which can be centrifuged simultaneously. Add 1 ml. of glass-redistilled water to each, mix with glass rods, and place the tubes in boiling water. When the solutions become opaque, remove the tubes and cool. Add 2 ml. of 20 per cent trichloroacetic acid, stir thoroughly, and heat in the water bath at 90–95° for 5 minutes. Stir frequently while heating. Cool as before, remove the rods, and centrifuge the tubes at 3000 r.p.m. for 10 minutes. Decant the supernatant solutions into 15 ml. graduated centrifuge tubes.

To the original tubes add 1 ml. of trichloroacetic acid and 1 ml. of water, break up the precipitates well with the glass rods, and while stirring heat again in the water bath at 90–95° for 5 minutes. Remove, cool, and centrifuge as before. Decant the supernatant fluid into the tubes containing the first filtrate.

Again add 1 ml. of trichloroacetic acid and 1 ml. of water to the precipitates, treat, centrifuge, and decant as before.

To the graduated tubes containing the three filtrates add 1 ml. of a saturated solution of sodium pyrophosphate, 2 ml. of ammonium hydroxide, and 1 ml. of a 0.1 per cent solution of sodium diethyldithiocarbamate. Make the volume up to 15 ml. with redistilled water. Mix the solutions well by pouring back and forth between the centrifuge and colorimeter tubes. Read as soon as possible in the Evelyn photoelectric colorimeter, using Filter 440 and a reagent blank to set the galvanometer at 100.

Calculate the copper content of the solutions, using the formula

\[ L \times \frac{100}{\text{ml. serum used}} \times \frac{1}{K} = \text{copper, micrograms} \%
\]

\[ L \] refers to the photometric density and is calculated from the galvanometer reading \( G \) by the formula, \( L = 2 - \log G \). \( K \) is the calibration constant.
The value of $1/K$ as established on our instrument is 67.6.

Twelve representative recoveries with the method outlined above are presented in Table II. The method not only gives excellent recovery of added material but offers results which are consistently reproducible within $\pm 10$ per cent.

### Table II

**Results of Recovery of Copper following Addition of Known Amounts to Serum**

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Serum $\gamma$ per cent</th>
<th>Added $\gamma$ per cent</th>
<th>Total $\gamma$ per cent</th>
<th>Found $\gamma$ per cent</th>
<th>Recovery $\gamma$ per cent</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>88</td>
<td>43</td>
<td>131</td>
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<td>95</td>
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<td>103</td>
<td>47</td>
<td>150</td>
<td>149</td>
<td>99</td>
</tr>
</tbody>
</table>

**Average** ........................................................................................................ 97

### Table III

**Range of Serum Copper Values as Obtained by Various Authors**

<table>
<thead>
<tr>
<th>Author</th>
<th>Method</th>
<th>Serum copper $\gamma$ per cent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Locke et al.</td>
<td>Trichloroacetic acid</td>
<td>72-95</td>
</tr>
<tr>
<td>Tompsett</td>
<td>&quot;</td>
<td>183-245</td>
</tr>
<tr>
<td>Guillemet and Schell</td>
<td>Dry ashing</td>
<td>56-75</td>
</tr>
<tr>
<td>Warburg and Krebs</td>
<td>Catalytic</td>
<td>82</td>
</tr>
<tr>
<td>Sachs et al.</td>
<td>Dry ashing</td>
<td>82-132</td>
</tr>
<tr>
<td>Present method</td>
<td>Trichloroacetic acid</td>
<td>92-150</td>
</tr>
</tbody>
</table>

There is a small error incurred by the use of the Evelyn colorimeter with colored solutions of low intensity. Attempts were made to eliminate this error by concentrating the filtrates, by using smaller volumes of reagents, and by combining reagents. None of these methods proved practical. However, the excellent recoveries and reproducibility of results obtained with the method outlined justify this error.

In Table III the range of serum copper for normal adults as obtained by
various authors is summarized. Locke, Main, and Rosbash (5) do not mention filtering the cloudy amyl alcohol and used a crude comparator. They made twenty-eight determinations on "normal" adults but included only seventeen of the determinations in the average. The determinations were made following one trichloroacetic acid precipitation. The values reported by Tompsett (6) on eight individuals are extremely high. The cause for this is not apparent. His values for total blood copper are also out of line with those obtained by others (4). Guillemet and Schell (8) obtained low values for serum copper. Tompsett (6) states that the number of precipitations used in their method probably accounts for the low values obtained. Warburg and Krebs (9), using a method based on the fact that copper catalyzes the oxidation of cysteine to cystine, reported 0.082 mg. per 100 ml. as their average for ten normal adults. Sachs, Levine, Anderson, and Schmit (4) determined the copper content of the serum of ten adult males, using a dry ashing procedure followed by carbamate, and reported an average of 0.105 mg. of copper per 100 ml. of serum.

We have determined the copper content of the serum of twenty-five healthy adult males and twenty-five healthy adult females. The results are presented in Fig. 3. The average value for males was 116 γ per cent. The lowest value obtained was 92 γ per cent, and the highest 134 γ per cent. These values correspond well with those obtained by Sachs et al. (82 to 132 γ per cent) (4). For the females the average was somewhat higher, 131 γ per cent. The values ranged from 103 to 159 γ per cent.

![Fig. 3. The copper content of the serum of twenty-five normal males and twenty-five normal females.](http://www.jbc.org/DownloadedFrom)
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SUMMARY

1. Evidence has been presented that approximately 75 per cent of the copper contained in serum is present in the filtrates prepared by precipitation of the serum with trichloroacetic acid. Three warm extractions of the trichloroacetic acid precipitate have been shown to remove approximately 97 per cent of the copper.

2. A method for the determination of copper in the serum or plasma has been presented which is based on a triple warm extraction of a trichloroacetic acid precipitate followed by the colorimetric determination of copper with sodium diethyldithiocarbamate. The colored solutions were read in the Evelyn photoelectric colorimeter.

3. Values of serum copper for twenty-five normal males and twenty-five normal females are presented.

BIBLIOGRAPHY

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George E. Cartwright, Patricia J. Jones and Maxwell M. Wintrobe


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