Spectrophotometric Determination of Serum Copper with Biscyclohexanoneoxalyldihydrazone

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Standard copper colorimetric methods, which commonly use diethyl dithiocarbamate, give a molar absorbance index of about 8000, in aqueous solution. This low sensitivity makes impractical a reliable determination of copper on trichloroacetic filtrates of human sera. Nilsson (*3*) showed that the compound biscyclohexanoneoxalyldihydrazone reacts with the cupric ion in alkaline solution to give a blue color. This copper complex has a molar absorbance index of 16,000 at 600 mμ. It gives a clear, stable, blue-colored solution with constant absorbance with the cupric ion over a pH range of 7.0 to 9.0. The biscyclohexanoneoxalyldihydrazone does not give a color with any other cations or anions commonly encountered in biological materials.

THE very low concentrations of copper present in human sera require the use of very sensitive reagents for its determination. The use of diethyl dithiocarbamate or dithizone has not proved entirely satisfactory. Both of these reagents, in addition to being inadequately sensitive, lack specificity. The former reagent forms a yellow-colored colloidal suspension with microgram quantities of copper, and this complex may then be extracted with various organic solvents. However, interfering colors form with iron, cobalt, nickel, and bismuth. Also the yellow-colored complex fails to follow Beer's law over a wide range of concentrations when certain spectrophotometers or filter photometers with poor quality monochromatic light are used. Dithizone itself is colored, reacts with many metals, and in order to achieve selectivity requires complex extraction procedures in a narrow pH range. The reagent "cuproine" (2,2′-biquinoline) is specific when applied to the spectrophotometric determination of copper, but lacks the desired sensitivity (*2*). Recently, Smith has described three new copper specific reagents —2,9-dimethyl-1,10-phenanthroline (*4*), 4,7-diphenyl-1,10-phenanthroline (*5*), and 2,9-dimethyl-4,7-diphenyl-1,10-phenanthroline (*6*). All of these reagents are specific under the described conditions.

All of these chromogenic reagents for copper lack the desired sensitivity when applied to the determination of copper in serum. Nilsson (*3*), in a study of the condensation products of oxalhydrazide with aldehydes and ketones noted that many of the hydrazones formed gave a blue color with microgram quantities of copper salts. Nilsson found that the hydrazone formed by the reaction of 1 mole of oxalhydrazide with 2 moles of cyclohexanone gave a very intense blue color with copper. This reagent, biscyclohexanoneoxalyldihydrazone (Figure 1), was later applied to a quantitative spectrophotometric procedure for the determination of copper in paper pulp products by Wetlesen (*7*). Table I compares the sensitivity of this compound to other reagents frequently used for the determination of copper.

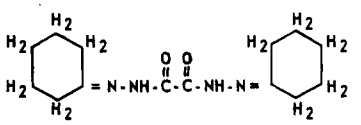

Figure 1. Biscyclohexanoneoxalyldihydrazone

This paper describes the application of this reagent to a procedure for the determination of human serum copper.

REAGENTS

Trichloroacetic Acid, 20%. Add 20 grams of reagent grade or redistilled trichloroacetic acid to redistilled water and make to volume of 100 ml.

Hydrochloric Acid, 2*N*.

Tribasic Potassium Phosphate (Merck). Make a saturated aqueous solution. The solution is hygroscopic and should be checked for complete saturation.

Phenolphthalein, 0.1% aqueous.

Biscyclohexanoneoxalyldihydrazone. Obtained from G. Frederick Smith Chemical Co. Saturated solution in 50% ethyl-alcohol. Dissolve with gentle heating.

Standard Copper Solutions. Dissolve 0.3928 gram of copper sulfate pentahydrate in redistilled water and make to 1000 ml. From this stock solution prepare dilute standards containing 1 to 5 γ per ml.

The procedure used for the release of the copper from the serum proteins is an adaptation of the method described by Gubler and others (*1*).

PROCEDURE

Add 1 ml. of serum or plasma (heparinized) to a 10-ml. rounded test tube, 16 × 100 mm.

Add 0.7 ml. of 2*N* hydrochloric acid and let stand at room temperature 5 to 10 minutes.

Add 1 ml. of 20% trichloroacetic acid, mix with thin stirring rod, and centrifuge at 2500 r.p.m. for 30 minutes.

Table I. Relative Sensitivities of Various Chromogenic Reagents Used for Determination of Copper

Chromogenic Agent	Wave Length Maximum Absorption, mμ	Molar Absorbancy Index
Diethyldithiocarbamate (aqueous)	440	8,000
Diethyldithiocarbamate (amyl alcohol)	440	12,700
2,2′-Biquinoline	540	5,900
2,9-Dimethyl-1,10-phenanthroline	454	8,000
4,7-Diphenyl-1,10-phenanthroline	420	12,000
2,9-Dimethyl-4,7-diphenyl-1,10-phenanthroline	480	14,100
Biscyclohexanoneoxalyldihydrazone	600	16,000

Pipet 2.0 ml. of supernatant into graduated tube, or 19-mm. diameter Coleman cuvette, graduated to 3.5 ml.

Add one drop of phenolphthalein indicator to tubes, and mix.

Add saturated tribasic potassium phosphate dropwise with mixing (1.0 to 1.5 ml.) until solution turns just pink (pH 8.0 to 8.2).

Add 2*N* hydrochloric acid dropwise with mixing until 1 drop makes the solution colorless (1 to 3 drops usually needed). This should bring the pH of the solution within the range of 7.5 to 7.9.

Add 0.2 ml. of the biscyclohexanoneoxalyldihydrazone solution to each tube. Make to volume of 3.5 ml. with redistilled water. Mix and let stand at least 5 minutes, but read within 60 minutes.

Carry reagent blanks and standards containing 2 γ of copper through the procedure with the unknown sera. It is also advisable to carry a standard serum through with each set of unknowns.

Read at a wave length of 600 mμ in Coleman Jr. spectrophotometer (or other suitable spectrophotometer). The 3.5-ml. volume in the 19-mm. Coleman cuvette is read by placing a flat cork support approximately 8 mm. high in the bottom of the standard cuvette carrier or adapter.

EXPERIMENTAL

Sensitivity and Conformity to Beer's Law. The colored cupric biscyclohexanoneoxalyldihydrazone complex in aqueous solution

has a molar absorbance index of 16,000 at its point of maximum absorption, 600 mμ (Figure 2). This corresponds to a sensitivity of approximately 0.03 p.p.m. The cupric complex in aqueous solution conforms to Beer's law at 600 mμ in concentrations up to 4 p.p.m.

Optimum pH. Figure 3 portrays the optimum pH for color development. The optimum varies slightly with time of standing, and after 15 minutes a range of pH 7.0 to 9.0 gives a constant and maximum color, whereas if the solutions are read immediately after mixing, a slightly narrower maximum range is obtained.

Stability of Color Complex with Respect to Time and Temperature. The maximum color development is present within 5 minutes after addition of the biscyclohexanoneoxalyldihydrazone. The stability of the blue color complex is related to the concentration of phosphate buffer. However, not until 60 minutes after mixing does a gradual fading of the color begin to occur. Thereafter, this represents about 1% per hour with the concentrations of phosphate used in the procedure. With concentrations of phosphate less than one tenth of those described for use in the serum method, the blue complex in diffuse light is stable for 12 hours. At 24 hours, there is approximately 10% color fading. In the dark, the color is stable for 3 days. The color is more stable at 4° C. than at room temperature (25° C.).

Effect of Reagent Concentration. If enough reagent is present to complex all of the copper, a further increase in the concentration of the reagent will not produce a significant change in absorbance at 600 mμ. At optimum pH, experiments in which varying amounts of reagent were added to a constant amount of copper indicate that 8 moles of reagent are required to give maximum color with 1 mole of copper.

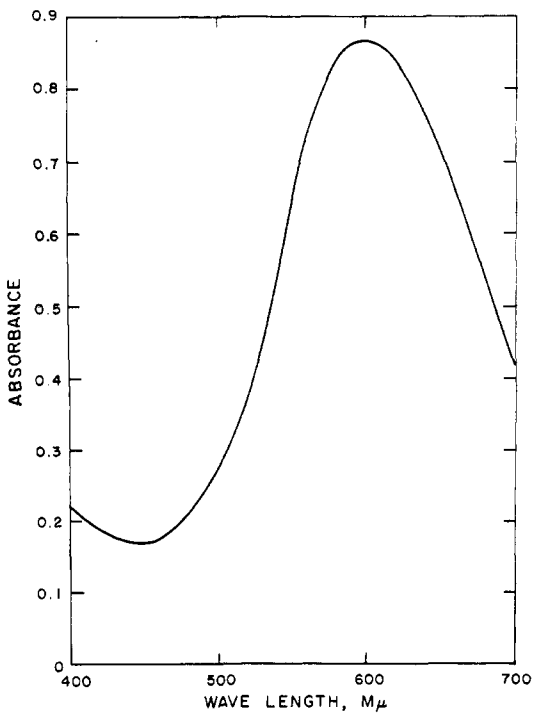

Figure 2. Spectral absorption curve of cupric biscyclohexanoneoxalyldihydrazone at pH 7.5

Table II. Effect of Various Anions and Cations on Cupric Biscyclohexanoneoxalyldihydrazone Color Complex

Ion	Added as	No Interference, P.P.M.	Ion	Added as	No Interference, P.P.M.
$Cr_2O_7^{--}$	$K_2Cr_2O_7$	1000	NH_4^+	$(NH_4)_2SO_4$	1000
$Br_4O_7^{--}$	$Na_2Br_4O_7$	1000	Na^+	NaCl	1000
NO_3^-	$NaNO_3$	1000	K^+	KCl	1000
NO_2^-	$NaNO_2$	1000	Li^+	LiCl	500
SCN^-	KSCN	1000	$P_2O_7^{--}$	$Na_4P_2O_7$	500
Br^-	KBr	1000	$S_2O_3^{--}$	$Na_2S_2O_3$	100
Cl^-	NaCl	1000	CrO_4	Na_2CrO_4	50
Fl^-	NaF	1000	Ba^{++}	$Ba(C_2H_3O_2)_2.H_2O$	50
I^-	NaI	1000	Ca^{++}	$CaCl_2.2H_2O$	25
SO_3^{--}	Na_2SO_3	1000	Mg^{++}	$Mg(C_2H_3O_2)_2.4H_2O$	10
SO_4^{--}	Na_2SO_4	1000	Sn^{++}	$SnCl_2.2H_2O$	10
$C_2H_3O_2^-$	$NaC_2H_3O_2$	1000	Mn^{++}	$MnCl_2$	10
$C_6H_5O_7^{---}$	$Na_3C_6H_5O_7.2H_2O$	1000	Sr^{++}	$SrCl_2$	10
$C_2O_4^{--}$	$K_2C_2O_4.H_2O$	1000	Ce^{+++}	$Ce(SO_4)_3.8H_2O$	10
$C_4H_4O_6^{--}$	$Na_2C_4H_4O_6.2H_2O$	1000	Cd^{++}	$CdCl_2.2^1/_2H_2O$	1
CO_3^{--}	Na_2CO_3	1000	Hg^+	$HgNO_3.H_2O$	1
HCO_3^-	$NaHCO_3$	1000	Hg^{++}	$Hg(NO_3)_2.H_2O$	1
MoO_4^{--}	$Na_2MoO_4.2H_2O$	1000	Cr^{+++}	$CrCl_3$	1
Wo_4^{---}	$Na_3Wo_4.2H_2O$	1000	Co^{++}	$Co(C_2H_3O_2)_2.4H_2O$	1
AsO_4	$NaHAsO_4.7H_2O$	1000	Fe^{+++}	$FeCl_3$	1
PO_4^{---}	$Na_3PO_4.12H_2O$	1000	Al^{+++}	$AlCl_3$	1
Ag^+	$AgNO_3$	1000	Versene	Ca Versenate	0.5

Effect of Presence of Foreign Ions. The method employed for examining these effects was the same as in the outlined procedure, except that the foreign ion was added in solution to a small volume of 2*N* hydrochloric acid containing microgram quantities of copper. If a precipitate formed after color development, it was removed by centrifuging. Table II lists the effects of some of the more common ions. An interference is defined as an alteration of more than ±2% in the absorbance of an aqueous solution at pH 7.0 to 9.0 containing 1 p.p.m. of cupric ion.

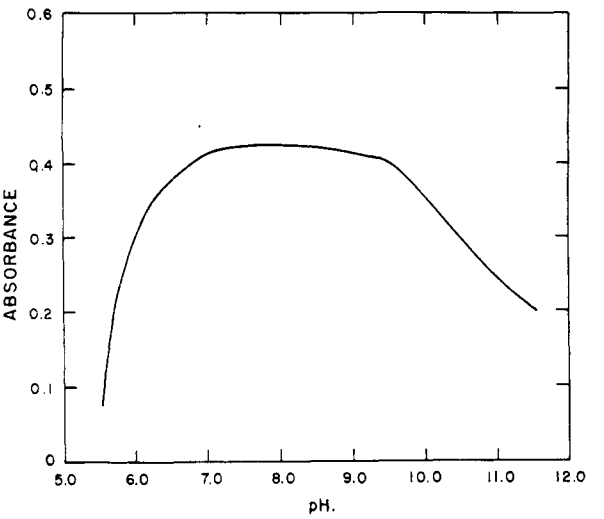

Figure 3. Curve of absorbance of cupric biscyclohexanoneoxalyldihydrazone complex versus pH at 600 mμ

Evaluation of concentrations above 1000 p.p.m. were made in only a few instances. Many of the cations interfered at a low concentration because of the turbidity developed at the pH 7.5 used for color development. Lead, zinc, and nickel interfered at concentrations of 0.5 p.p.m. with formation of a precipitate. Cyanide prevents color development at concentrations of less than 0.1 p.p.m. All of the interferences noted were of a negative character, and none of the 48 ions tested gave any color with this reagent. With certain anions, color development was accelerated, and with some cations full color development was delayed. Many of the cations that interfere by formation of a precipitate

Table III. Recovery and Precision of Copper Added to Serum, Expressed in Absorbance

1 Ml. of Serum	1 γ of Cu	1 Ml. of Serum + 1 γ of Cu		
		Found	Calculated	Deviation, γ
0.088	0.072	0.162	0.160	+0.03
0.089	0.072	0.162	0.161	+0.01
0.086	0.072	0.163	0.158	+0.06
0.091	0.070	0.160	0.161	−0.02
0.089	0.071	0.160	0.160	0

of the metal hydroxides or hydrated oxides at alkaline pH can be prevented by addition of citrate. At 3000 p.p.m. citrate does not interfere with color, but at 6000 p.p.m. an inhibition of color occurs. Iron may be present in concentrations up to more than 10 p.p.m. if citrate is present.

Recovery and Precision. Table III lists the recovery and degree of precision obtained when copper is added to normal pooled serum in the range of concentrations commonly found in serum. Presumably this method measures total serum copper, since comparable results are obtained with this reagent when applied both to trichloroacetic acid filtrates of sera and wet ashed digests of sera. Also, when a trace of radioactive copper-64 was added to human serum and the specific activity of the copper in the trichloroacetic acid filtrate compared with the specific activity of the copper in a wet ash digest, identical values were obtained.

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A Colorimetric Coulometer

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A new type of coulometer has been investigated, operating on the principle of the measurement of a color change produced by the electrode reaction. The most sensitive coulometer was obtained by having the electrode reaction change the pH of the solution and measuring this pH with an acid-base indicator. This coulometer could be used in the range 0.01 to 1.00 coulomb. Other electrode reactions described could be used for measurements up to 10 coulombs.

IN THE coulometric investigation of processes at small solid electrodes it was necessary to use a coulometer that could measure one coulomb and less. The common coulometers, such as the silver (*3*) and oxyhydrogen coulometer (*2*), require a high degree of precision in the measurement of quantities of electricity this small. Most of the electrical devices (*1*) were prohibitive in cost. Therefore, the possibility of using a simple laboratory colorimeter as a coulometer was investigated. The colorimeter could measure the number of coulombs by measuring the change in color in a solution caused by an electrode reaction in the solution.

Any electrode reaction which could of itself produce or destroy a color, or a reaction, which produced products, which in turn, could cause some type of indicator to change color, could be used in this colorimetric coulometer.

EQUIPMENT AND PROCEDURE

The coulometer was designed around a 12-mm. sample tube of a clinical-type Klett-Summerson colorimeter. To prevent the anode and cathode reactions from interfering with each other the two half cells were separated by a salt bridge. A rotating electrode and stirrer were combined for the coulometer half cell as shown in Figure 1. Rotary motion to the electric stirrer was supplied by an inverted magnetic stirrer. This type of drive was used to aid in removal of the electrode, since, before each colorimeter reading was taken, the electrode-stirrer was removed to prevent any error from absorption and reflection of light by the wire.

In the investigation of the various reactions, the number of coulombs was measured by timing the passage of a constant current through a galvanometer shunt system which had been previously calibrated as a microammeter. The constant current source was obtained by operating a 45-volt battery into a heavy load.

RESULTS

Three classes of color producing reactions were investigated—a reaction in which a colored substance is removed or produced by direct reaction at the electrode; a reaction in which the solute reacts at the electrode and the products of this reaction subse-

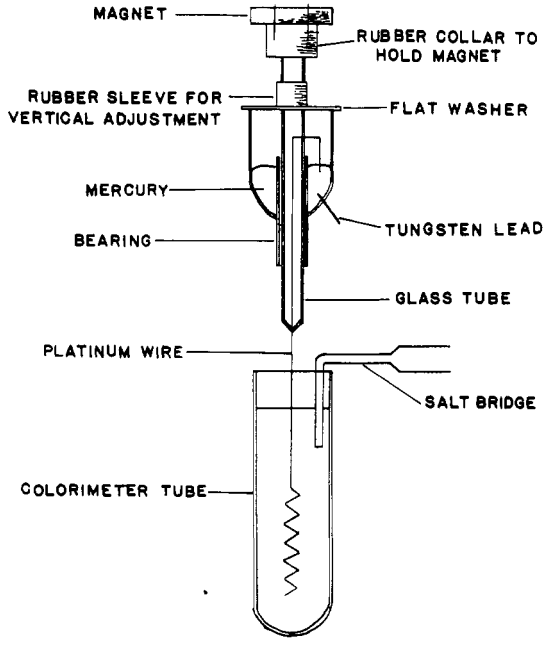

Figure 1. Coulometer half cell

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