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The microdetermination of biological copper with oxalyldihydrazide

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SYNOPSIS Methods are described for the determination of microgram quantitites of biological copper in aqueous medium as the intensely coloured oxalyldihydrazide-acetaldehyde complex (molar extinction coefficient 23,000 to 23,500).

The methods are applicable on a routine basis to any biological material, in particular to serum, urine, or tissues, such as liver, brain, or kidney. In the case of urine a simple semi-quantitative screening method is also described. For quantitative work copper is liberated from serum by acid extraction and protein precipitation, and from urine and tissues by a rapid wet-ashing procedure. Recoveries of added copper from urine are quantitative. Precision is high although day-to-day control with standards and blanks is desirable. The methods are applied in an investigation of the diurnal variations in a normal man and in two cases of Wilson's disease.

Various workers have described the use of oxalyldihydrazide and acetaldehyde for the colorimetric determination of microgram quantities of biological copper. In 1956 Gran showed that in aqueous ammoniacal solution these reagents gave an intense blue-red colour with cupric ion, with a relative light absorption about three times that of the copper-diethyldithiocarbamate complex in organic solvents. Since then oxalyldihydrazide and acetaldehyde have been used, for example, in investigations of sea water (Bowles and Nicks, 1961), enzymes (Stark and Dawson, 1958), and serum (Summers, 1960). The sensitivities of the various methods described differ, the maximal molar extinction coefficients of the coloured complex ranging from 22,000 to 29,500, with great variation in the recommended time for colour development (20 minutes to 18 hours).

In this publication we present methods which were established for clinical purposes and which apply to any biological material, in particular, serum, urine, and such tissues as liver, kidney, and brain. We have worked out conditions for maximum sensitivity and for colour development under simplified and reproducible conditions. The methods are considered suitable for routine or occasional application in the average clinical laboratory.

REAGENTS AND APPARATUS

All reagents consist of, or are made from, best quality materials of analytical grade. Ion-free water is used throughout. Constant boiling hydrochloric acid is made by distillation of diluted acid. Trichloracetic acid (boiling point ~196°C.) is purified if necessary by distillation at normal pressures in all-glass apparatus fitted with an air condenser. Nitric acid S.G. 1·42 may be similarly purified if necessary, but with a water-cooled condenser. All glassware is hydrochloric acid-washed. In addition, flasks used in the ashing process are pretreated with boiling ashing mixture. The vessel for the dilute copper standard is treated with cetyltrimethylammonium bromide (Cetavlon) to prevent adsorption of copper ions and then washed with water.

2M HYDROCHLORIC ACID Dilute 164 ml. of constant boiling azeotrope (760 mm. pressure) to 1 litre with water and mix.

TRICHLORACETIC ACID 10%

AMMONIA S.G. 0·880

OXALYLDIHYDRAZIDE 1% IN 0·5M HYDROCHLORIC ACID Store at 5°C. This keeps for at least a month. The solid is easily prepared by Gran's method. Dissolve 7·3 g. of ethyl oxalate (B.D.H. reagent grade) in 40 ml. of ethanol (95 to 100%) and 2·5 g. of hydrazine monohydrate in 15 ml. of ethanol. Warm the solutions and mix. Place in the refrigerator for an hour. Filter the crystals on a fritted glass suction funnel and wash with a little cold ethanol. Recrystallize them from boiling water.

ACETALDEHYDE 40% Dilute 40 ml. ice-cold acetaldehyde (very volatile) to 100 ml. with ice-cold water. Store at

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5°C. This reagent is stable apart from slight losses of acetaldehyde.

2M AMMONIUM CHLORIDE IN 9M AMMONIA Dissolve 107 g. of ammonium chloride in water and make up to 500 ml. with that solvent; add 500 ml. of ammonia S.G. 0·880. Mix and store at 5°C.

PERCHLORIC ACID 70%

COPPER STANDARD (5 μg. Cu/ml.) Make a 500 μg. Cu/ml. standard by dissolving 0·1964 g. copper sulphate pentahydrate in 0·1N sulphuric acid and diluting to 100 ml. with the acid. Dilute this solution 1 in 100 with water for the working standard.

NITRIC ACID, CONCENTRATED, S.G. 1·42

SULPHURIC ACID, CONCENTRATED, S.G. 1·84

METHOD FOR SERUM

To 1 ml. of serum add 1 ml. of 2M hydrochloric acid, with shaking; mix well and allow to stand at room temperature for 10 minutes. Then add 1 ml. of 10% trichloracetic acid likewise and let stand for three minutes. Centrifuge. Take 2 ml. of supernatant and mix with 0·5 ml. ammonia buffer. Add 0·2 ml. of oxalyldihydrazide reagent and mix, then 0·3 ml. of acetaldehyde reagent and mix again. Incubate for 11 minutes at 60°C. and immediately cool the tubes in cold running water. For the copper standards take 0·2 ml. and 0·4 ml. of the 5 μg./ml. working standard and dilute each to 1 ml. with water. Then proceed as for serum, but omitting centrifugation; take a 2 ml. portion as for serum. Make a blank by using 1 ml. of water in place of 1 ml. of serum and then proceed as for the standard or the serum.

Colorimeter readings are made against the blank at 542 mμ or with an appropriate filter such as the Ilford 625, yellow-green. The calculation or the serum level is obvious.

METHOD FOR URINE

PRELIMINARY SEMI-QUANTITATIVE SCREENING To 10 ml. of slightly acid urine add 1 ml. of concentrated hydrochloric acid, and after 10 minutes at room temperature add 0·3 ml. of 1% oxalyldihydrazide, 0·5 ml. of 40% acetaldehyde, and 2 ml. of ammonia buffer. Make up a blank as for the test but omit the addition of oxalyldihydrazide. For an internal standard add 4 μg. copper to 10 ml. of urine, and then proceed as before. Incubate all tubes at 60°C. for 11 minutes. Cool, centrifuge or filter, and observe the colours.

Normal urines show no visible increase over that in the blank. In Wilson's disease, particularly in untreated cases, marked colour is evident in the test and the amount of copper may be estimated semi-quantitatively by means of the internal standard. In nephrosis or cirrhosis of the liver some increase may be seen.

QUANTITATIVE ANALYSIS For analysis of a normal urine 30 ml. is required. In cases of Wilson's disease, cirrhosis of the liver, or nephrosis, 10 ml. of urine is usually sufficient. In either case, evaporate the urine to low bulk with 0·5 ml. concentrated nitric acid in a 100 to 200 ml. round-bottomed, long-necked flask of good quality borosilicate glass. Use a glass bead to prevent bumping and heat until all the liquid has evaporated and only a partly charred mass remains. Allow the flask to cool somewhat, then for 30 ml. urine add in order, 2 ml. of concentrated nitric acid, 0·6 ml. of concentrated sulphuric acid, and finally 0·4 ml. of perchloric acid. For 10 ml. of urine, use half these quantities.

Next heat moderately until the bulk of the nitric acid has disappeared, and then fairly strongly until the charred residue is oxidized. It is seldom necessary to add more nitric acid and perchloric acids. Continue heating until the bulk of the nitric and chlorine oxides have disappeared but not so strongly that the sulphuric acid is lost as sulphur trioxide. Then allow the flask to cool, and rotate it so that the bead does not become enmeshed eventually in a solid lump of the clear phosphate-sulphate glass. Practically all ashings proceed quickly and smoothly. Next add 4·5 ml. water down the walls of the flask and warm it to dissolve as much residue as possible. Cool and add 3 ml. ammonia-ammonium chloride. The solution should then be strongly alkaline. Add 0·3 ml. of oxalyldihydrazide reagent and 0·5 ml. of acetaldehyde reagent, mixing after each addition. Put the flask in a 60°C. water bath for 11 minutes and then cool in running water. If preferred, the contents of the flasks may be transferred to copper-free test-tubes for incubation. Centrifuge or filter the contents to remove phosphates and other insoluble substances.

Prepare two blanks. For a 'method' blank evaporate appropriate amounts of nitric, sulphuric, and perchloric acids to low bulk and then continue as for the ashed urine, and for a 'standard' blank take 0·5 ml. of concentrated sulphuric acid and then proceed as for the ashed urine. Prepare a standard by measuring into a tube 0·8 ml. of dilute copper reagent (4 μg. Cu), 0·5 ml. of concentrated sulphuric acid, and 3·7 ml. of water, and then proceed as for the aqueous solution of the ashed urine. Read the tests and standard, all in duplicate preferably, against their appropriate blanks as for the determination of serum copper.

EXPERIMENTAL

PREPARATION OF SPECIMENS FOR CHELATE FORMATION There is no difficulty in obtaining ionic copper from serum, since Gubler, Lahey, Ashenbrucker, Cartwright, and Wintrobe (1952) showed that merely standing the serum at room temperature with suitable acids will liberate all the copper. Heating was contraindicated.

Liberation of copper from urine components proved a more difficult matter. We made numerous attempts to extract the copper with dithizone or oxine using a variety of solvents and different *p*H values. Recoveries of added copper were invariably poor. Total wet ashing using a highly oxidizing medium as described under 'Method' was eventually found to be quite satisfactory. Smooth oxidation of carbonaceous matter occurred almost invariably and usually no further additions of oxidant were necessary.

Small portions of wet tissue were dealt with in the same way as urine, without the necessity for grinding or mincing.

NATURE AND CONCENTRATION OF REACTANTS We investigated various alkalis, aldehydes, and ketones in order to assess the sensitivity and speed of reaction in forming the coloured copper chelate. Ammonia was the only satisfactory alkali, particularly in combination with ammonium chloride, to give some degree of buffering. Oxalyldihydrazide is sparingly soluble in common solvents (0·3% in water) and has previously been used at low concentrations. However, it dissolves more readily in dilute acid to give a reasonably stable reagent. The higher concentration leads to faster chelate formation. Acetaldehyde is the most satisfactory of the carbonyl compounds. An excess is essential for formation of the red-blue colour. With low concentrations of aldehyde a different blue colour forms, changing to the usual blue-red on addition of excess acetaldehyde. The blue complex has a much lower molar extinction coefficient than that of the red-blue one at 542 μ.

TIME AND TEMPERATURE OF REACTION When we developed the colour at room temperature (apart from the warming which occurred on mixing the reagents), complex was still forming after 30 minutes. In further experiments with incubation at 37°C. for 25 minutes followed by cooling, the colour continued to increase slowly for the next 21 hours. Incubation at 60°C. for varying periods was next tried. Although this is above the boiling point of acetaldehyde, its solubility in water and the formation of acetaldehyde-ammonia prevent much loss. Under such conditions colour rises to a maximum in 10 to 12 minutes. Continued heating tends to break down the complex. After 15 minutes 97% of maximum colour remains, dropping to 93·5% after 25 minutes.

RESULTS AND DISCUSSION

MOLAR EXTINCTION COEFFICIENTS AND LINEARITY For the serum method we found a mean molar extinction coefficient of about 23,500 and for urinary copper one of about 23,000. This is in reasonably close agreement with the results of Stark and Dawson (ϵ_{mol} = 22,000) but considerably lower than Gran's original value of 29,500. The amount of colour was found to be linear with copper concentration up to the limits of reliability of any particular reading instrument.

REPRODUCIBILITY In earlier work a set of octuplicate standards gave readings of 0·181 ± 0·005 optical density units or less than ± 3% extreme variation. Later, when the methods were improved somewhat, quadruplicate standards read 0·206 ± 0·003 units for the urine method and 0·412 ± 0·004 for the serum method.

Although the reproducibility on any one day is thus satisfactory, we found that both the blanks and the absolute amount of colour per unit of copper vary slightly from week to week and for this reason we include standards with each determination rather than set up a calibration curve.

RECOVERIES OF COPPER FROM SERUM AND URINE Gubler *et al.* (1952) showed that recoveries of copper from plasma by acid extraction and precipitation were excellent, in fact slightly better by 3% than those obtained by wet ashing. We have assumed, therefore, that for serum the same findings would hold. On the other hand, information on recoveries from urine seems on less sure grounds. Consequently, we investigated urines from three normal men and from one man with Wilson's disease (Table I).

TABLE I

RECOVERIES OF COPPER ADDED TO URINE

Cu in Aliquot (μg.)	*Cu in Aliquot + Addition* (μg.)	*Cu Recovered* (μg.)	*Cu Added* (μg.)	*Recovery* (%)
0·48	4·48	4·00	4·00	100
0·41	4·48	4·07	4·00	102
0·38	4·35	3·97	4·00	99
12·90	17·55	4·65	5·00	93

Recoveries of added copper were quantitative for total amounts up to about 5 μg. In the fourth case we ashed a deliberately large amount of urine with a high copper content, but nevertheless a reasonably good recovery of added copper ensued. Since total ashing is used, these results indicate that all of original urinary copper is being determined. Furthermore, it is evident that urinary phosphates do not interfere at any stage. None of the cations found in urine would be expected to interfere in the amounts in which they usually occur (Stark and Dawson, 1958).

NORMAL VALUES AND DIURNAL VARIATION No extensive investigation of normal values has been made in the present study. Four normal men were examined and their urinary outputs were 21, 21, 27 and 42 μg. per 24 hours, in keeping with the statement by Varley (1958) that normal values have been tending towards lower levels as methodology has improved.

One of the normal men referred to above was also investigated for possible variation in his copper excretion over a 24-hour period (Table II). Considerable fluctuations were evident, the rate of output for the overnight period being about three times that of the afternoon to evening period, with other rates

TABLE II

DIURNAL VARIATIONS IN URINARY COPPER OUTPUT BY A NORMAL MAN

Period	*No. of Hours*	*Urine Volume (ml.)*	*Total Cu (μg.)*	*Cu Concentration (μg./l.)*	*Output Rate (μg./hr.)*
7.10 a.m. to 9.10 a.m.	2·0	99	1·70	17·2	0·85
9.10 a.m. to 1.42 p.m.	4·53	212	2·93	13·8	0·65
1.42 p.m. to 7.36 p.m.	5·9	230	2·48	10·8	0·42
7.36 p.m. to 10.10 p.m.	2·57	166	2·24	13·5	0·87
10.10 p.m. to 7.10 a.m.	9·0	368	11·96	32·5	1·33

falling between these extremes. Obviously, 24-hour collections are necessary, particularly if diseases other than hepatolenticular degeneration are being considered.

INVESTIGATIONS IN WILSON'S DISEASE Two cases were followed. The first was that of a man of 19 in whom the disease was already advanced and who showed the Kayser-Fleischer rings, marked mental deterioration accompanied by euphoria, slurred speech, and rigidity and spasm. There was marked cirrhosis with a grossly abnormal distribution of serum proteins on electrophoresis. The albumin-globulin ratio was 0·8:1. The serum copper level was normal on one occasion at 70 μg.% and high on two others at 133 and 150 μg.% because of intercurrent infection. Serum copper oxidase activity was normal on the one occasion on which it was estimated. The urinary copper output was 950 μg./day on one occasion and 1,024 μg./litre on another (incontinence prevented the collection of a 24-hour specimen). The patient subsequently died and some of the organs were examined for copper content. The amounts of copper found per gram of wet tissue were as follows: liver, 225 μg.; brain, 54 μg.; kidney, 83 μg.; brain (parietal cortex), 49 μg.; skin, 2 μg. The value for kidney was 377 μg./g. when based on dry weight of tissue. In normal man the approximate mean copper contents per gram dry weight are: for liver, 25 μg.; for brain, 18 μg.; for kidney, 18 μg.

The second case was that of a man of 27 who showed less advanced symptoms; there was no apparent mental deterioration but Kayser-Fleischer rings were clearly present. The urinary output was determined before treatment began. On the first occasion (day 1) it was 2,110 μg. Cu/24 hours and on the second (day 15), 2,310 μg./day. Intermittent treatment with B.A.L. and potassium sulphide followed. By day 22 there was a marked drop in output to 463 μg./day; successive determinations were 458, 890, 800, and 1,100 μg. for days 33, 41, 44, and 47. Therapy is at present continuing.

It is evident that determinations of urinary copper are considerably more useful than those for serum, since, in the former case, the distinction between normals and abnormals is so much greater. Furthermore, the urinary output seems to be relatively little affected by complications such as intercurrent infections which, if unsuspected, can lead to misinterpretation of serum copper analyses.

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