143. MICRO-DETERMINATION OF COPPER IN BIOLOGICAL MATERIAL

By ALFRED EDEN and HENRY HAMILTON GREEN

From the Veterinary Laboratory, Ministry of Agriculture and Fisheries, Weybridge

(*Received 13 July 1940*)

In work on the physiology of Cu [Eden & Green, 1939; Eden, 1939; 1940, 1, 2,], involving unusually large numbers of determinations on blood, tissues, urine, faeces and animal foods, the Tompsett modification [1934, 1] of the method of McFarlane [1932], itself based on the diethyldithiocarbamate colour reaction of Callan & Henderson [1929], was at first explored. Apart from the somewhat laborious ashing or tissue extraction procedures, the technique for subsequent deionization of iron was not found universally satisfactory. Although results of the correct order of magnitude were generally obtained, and might therefore not be suspected at sight, the general drift was definitely high and the slightest imperfection in removal of iron from the sphere of reaction seriously affected percentage accuracy as real magnitudes of Cu diminished, so rendering the process uncertain for metabolism work in which an accurate balance sheet was required.

In the case of blood mere increase in the recommended quantity of deionizing pyrophosphate effected improvement, but since the same difficulty has been encountered by van Niekerk [1937] and by others (personal communications), and since the technique finally adopted at this laboratory, and subsequently used for several thousand determinations, not only guards against imperfect deionization of iron but also embodies simplifications of manipulation, enhanced speed, increased sensitivity and more compact apparatus, its recording should prove of service. The speed and simplicity are gained by combining a rapid wet combustion method with colour development in strongly alkaline medium, and subsequent shaking out with amyl alcohol, in the combustion tube itself. Sensitivity is increased by photometric measurement on reduced extraction volumes in micro-tubes providing adequate stratum thickness. A single operator can complete 30 determinations a day and, if necessary, real magnitudes as low as 0·3 μg. Cu, the quantity present in 0·5 ml. normal blood, can be read to the second significant figure, thus extending the method to work on small laboratory animals.

With regard to obviation of interference by formation of the coloured diethyldithiocarbamate compound of iron before extracting the corresponding compound of Cu, it is not always realized that deionization of iron with pyrophosphate [Warburg, 1927] is enormously enhanced by ensuring adequate concentration of ammonium salts in solution. The completely deionized compound is an iron ammonium pyrophosphate and not iron pyrophosphate itself; so that, provided that the concentrations of ammonium ions and pyrophosphate ions are made sufficiently high, the final colour development of the Cu compound can be conducted at any level of alkalinity at all. The tedious bringing to "slight alkalinity to litmus" and subsequent "heating to 80° for 15 min. and cooling" of MacFarlane and Tompsett is thus eliminated altogether. The same deionization behaviour holds when citrate is used to prevent precipitation of calcium phosphate. Ferric

ammonium citrate is completely unionized in presence of sufficient ammonium and citrate ions, so that it is unnecessary to use pyrophosphate for deionization when citrate is already present—a point apparently not realized either by Tompsett [1934, 2] or van Niekerk. Furthermore, manganese is deionized as completely as iron, both by citrate and by pyrophosphate in presence of sufficient excess of ammonium salts. The advantage of pyrophosphate over citrate is largely an aesthetic one; a colourless solution is obtained so that any colour subsequently formed with diethyldithiocarbamate is due to Cu and the completeness of its removal with amyl alcohol is apparent to the naked eye. Extraction from the slightly coloured citrate medium is equally complete although less obvious, but any alien colour, arising from possibly imperfect combustion, is masked.

With regard to colour development both McFarlane and Tompsett use 0·5 mg. 2% aqueous sodium diethyldithiocarbamate. Under the conditions described for deionization of the iron from 5 ml. blood with 5 ml. pyrophosphate interference can readily occur if the aqueous reagent is simply added without attention to "local excess factors" which dilute the deionizing pyrophosphate in regions of high concentration of diethyldithiocarbamate. van Niekerk evades the potential error by using 0·5 ml. of 0·5% concentration of reagent instead of 2·0%, but this reduction of total diethyldithiocarbamate is unnecessary if the concentrations of pyrophosphate and ammonium ions are raised and the reagent is added dropwise from a burette with constant shaking. In the present method dropwise addition of 2 ml. 0·5% reagent replaces uncontrolled addition of 0·5 ml. 2%.

With regard to destruction of organic matter McFarlane and van Niekerk used dry incineration. Tompsett either adopted extraction with trichloroacetic acid or an efficient but cumbersome wet combustion, involving evaporation to dryness with nitric acid on a water bath and subsequent completion of digestion with sulphuric and perchloric acids. In the technique adopted by us the main oxidation is arranged to occur at the expense of nitric and perchloric acids, sulphuric acid being merely used, in amount dictated by the final volume desired, to raise the temperature in the last stages of combustion and drive off most of the residual perchloric acid. The compact variation now described for destruction of anything up to 2 g. "dry matter" is clean, safe, rapid and of wide general application. The small combustion stand[1] with six guarded micro-burners allows accurate control of digestion and is also suitable for various micro-distillations.

Combustion. Quantities of the order of 5 ml. blood, 1–5 g. wet tissues or 1 g. dry food are introduced into an 8 × 1 in. pyrex test tube scratched at approximately 25 ml.; 1 ml. A.R. H_2SO_4, 3 ml. Cu-free $HClO_4$ and two glass beads are added, and the mixture gently heated over the lowered flame of a micro-burner. With blood a little initial frothing occurs but this is easily controlled. Water is driven off by gentle boiling and heating is continued until rings of darkened mixture begin to ascend the tube. The flame is then turned out, the tube allowed to cool for about a minute and 2–3 ml. A.R. HNO_3 carefully added from a teat pipette. If previous heating has been carried to the correct stage of "ascent of darkened rings" there is no undue frothing and controlled heating may be resumed at once. The dark brown liquid gradually lightens in colour, passing through orange to faint greenish-yellow or even colourless. Stronger heating is continued a few minutes past the pale final stage to drive off most of the residual $HClO_4$ and leave a final volume of about 1 ml., i.e. the quantity of H_2SO_4 originally added. The stages of combustion at the various boiling points of the three acids

[1] Made to order by Gallenkamp Ltd. and illustrated in catalogue, 11th edition, p. 1216.

go quietly and are readily distinguishable by the character of ebullition. If the HNO_3 is driven off too fast the liquid may change from orange to black as the boiling point rises to that of $HClO_4$, in which case the flame is turned down and a few more drops of HNO_3 added. It is essential to effect complete combustion of organic matter so as to obviate any alien tint in the subsequent alkalinization with NH_3 and extraction with amyl alcohol.

A complete single combustion of quantities of material of the order mentioned takes 30–45 min., but, since 6 tubes (1 stand) or 12 tubes (2 stands) can be supervised simultaneously, the average working time per determination can be reduced to 5 min.

For urine the combustion procedure has to be slightly modified because of the larger volume required to obtain sufficient Cu for subsequent determination. 50 ml. urine are measured into a 100 ml. pyrex Kjeldahl flask (of suitable neck diameter if the same clips of the micro-stand are to be used) and about 3 ml. HNO_3 added, with two glass beads to prevent bumping. Heating is carefully controlled to bring through the boiling point without undue frothing, and smooth boiling is continued over a low flame until the volume is reduced to about 2 ml. The flame is then turned out and, after cooling, the combustion procedure with 1 ml. H_2SO_4, 3 ml. $HClO_4$ and a few ml. HNO_3, follows exactly as above. In the final stages of evaporation of urine the thick mass in the combustion flask begins to splutter and a small harmless conflagration may occur with burning away of carbon. This makes no difference to the subsequent procedure. A considerable quantity of salts separates out in the final stages of digestion and after completion a few drops of water are added to the flask while still warm, to prevent solidification. The contents are then washed with about 10 ml. water into the usual 8×1 in. pyrex tube scratched at 25 ml.

Deionization. As a general procedure with materials of uncertain Ca and P content deionization of Fe follows with ammonium citrate, since this also prevents precipitation of calcium phosphate on subsequent alkalinization. The combustion residue in the tube is diluted with 5–10 ml. water and 2 ml. 50% ammonium citrate added, followed by 5 ml. ammonia sp. gr. 0·880. Of this ammonia about 3 ml. go to neutralization of the H_2SO_4 and formation of the ammonium sulphate desired to promote deionization, and the residual 2 ml. to rendering strongly alkaline. Water is then added to the 25 ml. mark. The solution is yellowish but clear, although slight opalescence does not matter. Unnecessarily long standing at this stage may cause slight opalescence if the amount of calcium phosphate present is high.

For material relatively low in Ca and P but high in Fe, such as blood or soft tissues, the pyrophosphate method of deionization is equally good, and preferable for reasons mentioned above. In place of 2 ml. 50% ammonium citrate 10 ml. 4% hydrated sodium pyrophosphate are used (i.e. large excess, twice the quantity of the McFarlane or Tompsett procedures), followed by 5 ml. ammonia sp. gr. 0·880 and making up to about 25 ml. as before. For some tissues comparatively rich in Ca and P, and for faeces, the colourless pyrophosphate method of deionization can also be used provided that the precipitate formed on alkalinization is boiled to render it granular. With urine, however, the citrate method should always be used since the fine precipitate first formed does not readily granulate and Cu may be occluded.

Colour development and extraction. 2 ml. of 0·5% recently filtered sodium diethyldithiocarbamate[1] are now added from a burette with constant shaking,

[1] Diluted from a stock 2% aqueous solution stored in the dark. Any deposit formed is filtered off before diluting aliquots for daily use.

followed by exactly 5 ml. amyl alcohol. The tube is then closed with a clean rubber bung and vigorously shaken for ½ min., a period sufficient to ensure extraction of the yellow copper compound. In the presence of the electrolytes in solution the amyl alcohol rapidly separates to the top. Sufficient is taken off with a teat pipette for colour measurement, and filtered through acid-extracted filter paper, or alternatively centrifuged, to remove suspended water. It is important to use extracted papers since amyl alcohol may acquire a faint yellowish tint in passing through some grades of ordinary filter paper. The quantity of amyl alcohol pipetted off is of course immaterial since the Cu content is measured by the intensity of colour conferred on the precisely measured 5 ml. amyl alcohol added to the approximately constant extraction volume of 25 ml. Extraction is practically complete with a single shaking and the solubility of amyl alcohol in water is slight, but both factors are allowed for by treating the standard Cu solutions in exactly the same way. A quantity of 3 ml. to 3·5 ml. filtrate is obtained without undue trouble, enough to fill the micro-cups of a Klett biocolorimeter and much more than required for the 1 ml. micro-tube of a Zeiss photometer. If the latter is available the quantity of amyl alcohol used for shaking out can be reduced to intensify the colour when unusually low Cu is encountered.

Colour measurement. Comparison may be made with narrow Nessler tubes, in colorimeter cups or, best of all, with a Zeiss "step photometer" measuring light extinction within a selected spectral range. In each case comparative standards are required, although in the case of the photometer they are only needed once in order to plot permanent graphs of extinction coefficients with different light filters. The stock Cu solution is prepared by dissolving 0·3928 g. $CuSO_4$, $5H_2O$ in 1 l. of water containing a drop of H_2SO_4, giving 0·1 mg. Cu per ml. This is diluted 1 in 10 as required for preparation of standards. Convenient matching ranges are made by transferring 0·5 ml., 1 ml. and 2 ml. of the diluted stock, corresponding to 0·005, 0·01 and 0·02 mg. Cu, to the 8" × 1 in. pyrex tubes, adding the same quantities of citrate or pyrophosphate and NH_3 as after combustion of unknowns, and extracting with 5 ml. amyl alcohol after dilution to 25 ml. and addition of carbamate reagent. All reagents[1] should of course be tested for freedom from Cu, and the magnitude of the "total blank" must be accurately determined and allowed for.

For photometric measurement a "total blank" is obtained by carrying through the whole process, including the combustion but omitting the organic matter, and using the amyl alcohol layer so obtained for the compensating cell of the instrument. In this case any traces of Cu in the reagents are automatically allowed for and the difference in extinction coefficients on the two sides corresponds exactly to the Cu in the material examined. Micro-tubes of 1 ml. capacity are used and since these give a stratum thickness of 50 mm. great sensitivity is obtained.

The yellow colour of copper diethyldithiocarbamate absorbs light over the whole visible spectrum, least in the red and most in the violet. Filters S 53, S 50 and S 47, corresponding to mean wave lengths of 528, 488 and 458 mμ are the most useful. S 53 may be used for quantities of Cu from 0·02 mg. down to 0·005 or even 0·002 mg., in the 5 ml. amyl alcohol used for extraction. Unexpectedly high ranges are dealt with by dilution with amyl alcohol. For low ranges S 50 and S 47 are preferable as giving greater extinction coefficients. In general the filter selected should show a conveniently readable coefficient of 0·1–0·5, corresponding to absorption of one-fifth to two-thirds of the total light, higher

[1] Copper-free $HClO_4$ is supplied by Hopkins and Williams Ltd.

and lower readings being inaccurate. If the Cu present gives a lower extinction coefficient than 0·12 the next higher filter is switched in. With a coefficient of 0·20, representing about one-third light absorption, the maximum reading error is within ±0·1 and by taking the mean of five rapid readings the error can easily be reduced to ±1%. The quantity of Cu is directly proportional to the extinction coefficient and graphs for varying concentrations are therefore straight lines passing through the origin. Each analyst should prepare his own graphs from his own standards, so equilibrating the personal equation of his total technique once and for all.

Table 1 shows the general relationships for filters S53, S50 and S47:

Table 1. *Extinction coefficients for Cu with various filters in 50 mm. stratum thickness*

mg. Cu present	S 53	S 50	S 47
0·0010	—	0·050	0·132
0·0020	0·067	0·143	0·302
0·0050	0·174	0·356	0·708
0·0075	0·259	0·559	1·101
0·0100	0·348	0·734	—
0·0150	0·531	1·089	—
0·0200	0·683	—	—

It will be noted that within the recommended reading range and the limits of error the values lie on straight lines, and that even for so small a magnitude as 0·001 mg. Cu the S47 filter gives the satisfactory reading of 0·132.

If a photometer is not available the ordinary Klett biocolorimeter, with micro-cups giving a convenient reading depth with the available 3–3·5 ml. amyl alcohol filtrate, is satisfactory, and quantities down to 0·001 mg. Cu per 5 ml. can be read with fair accuracy, using a standard sufficiently close to the unknown. The Cu colour is not strictly monochromatic and it is generally necessary to make up at least three standards (0·005, 0·01 and 0·02 mg. Cu) simultaneously with the unknown and set the most suitable one between 15 and 30 mm. With a standard of 0·01 mg. set at 20 mm. the reading with 0·02 mg. is about 10·7 mm. instead of the theoretical 10 mm., giving a result about 14% too low; similarly a real quantity of 0·003 mg. Cu read against an 0·01 standard set at 10 mm. reads about 28·2 mm., giving a result about 15% too high. In general, if it is desired to keep the error within ±5% it is necessary to discard readings farther apart than 15 and 30 mm. for a standard set at 20 mm. To attain the ±1% accuracy readily obtained with the photometer and permanent graphs, it is necessary to prepare a closer range of colorimeter standards and read within a few mm. of the unknown. Standards should be made up at the same time as the unknowns to obviate light fading and evaporation changes. Although the colour of the Cu compound in amyl alcohol is fairly stable and no appreciable change occurs in diffuse light in several hours, exposure to strong sunlight in presence of air effects slow fading.

For very low concentrations of Cu, such as 0·001 mg. in 5 ml. amyl alcohol, narrow Nessler tubes are preferable to the colorimeter since greater depth is available for colour differentiation. Simple tubes, such that a 2 ml. quantity of fluid provides a depth of about 16 cm., are convenient.

Accuracy of method. Numerous controls have been carried out with known quantities of Cu and Fe added to Cu-free organic matter before combustion, and by recovery of known quantities of Cu added to blood, liver tissue, urine and faeces. With real magnitudes of 0·01 mg. Cu or more there is no difficulty in

keeping the total error within ±2%, i.e. returning 0·0098–0·0102 mg., especially if the photometric finish is employed. As real magnitudes diminish the error increases and for quantities about 0·001 mg. an error of ±10% must be regarded as permissible with the procedure already described. By further refining the technique, however, reducing the dilution volume of combustion residue and the amount of amyl alcohol used for extraction, the higher accuracy can be restored and still smaller quantities of Cu satisfactorily estimated.

Further refinement of technique. It is obvious that the limiting factor in the sensitivity of the method is simply the intensity of the colour, which in turn depends upon the extent to which the volume of extracting amyl alcohol can be kept down, and the reading limits of the instrument. With the Zeiss photometer the smallest cell obtainable requires 1 ml. and to cover marginal losses this necessitates the use of a minimum of 1·5 ml. amyl alcohol for extraction. Taking 0·10–0·15 as a satisfactory coefficient for accurate reading with an S 47 light filter, the satisfactorily determinable quantity of copper comes down to 0·2–0·3 μg. Ability to determine such small magnitudes then brings the method within the scope of work in which other factors impose stricter limits upon the amount of material available, e.g. experiments upon the white rat from which 0·5 ml. blood is readily obtainable, although 5 ml. is not, or upon single plants in which the morphological distribution of Cu may be wanted.

The difficulties are purely mechanical, involving greater attention to levels of contamination immaterial with 3 μg. Cu but disastrous with 0·3 μg. For this reason the whole scale of operations is reduced, centrifugal separation of the aqueous haze in the amyl alcohol layer takes the place of filtration, and one or more controls with known traces of Cu are inserted in every series of combustions. The variation, as applicable to blood, is as follows.

0·5 ml. blood is measured into a specially cleansed 8 × 1 in. pyrex tube, followed by 0·3 ml. each of H_2SO_4 and $HClO_4$. At the same time a control tube omitting the blood is set up for use as blank in the compensating cell of the photometer, with a second control tube containing 0·3 μg. Cu from highly diluted stock standard. Digestion proceeds in the normal manner, about 0·3 ml. HNO_3 being used for each tube. After combustion is apparently complete a further two drops each of $HClO_4$ and HNO_3 are added and heating is repeated to ensure removal of the last trace of organic matter. If this is not done a faint yellowish colour may appear on subsequent alkalinizing, barely visible to the eye but nevertheless extractable by amyl alcohol and registering as Cu. To the final digestion residues, consisting mainly of the 0·3 ml. H_2SO_4 originally used, are added 2 ml. 4% sodium pyrophosphate (or 2 ml. 10% ammonium citrate if the material is high in Ca and P), followed by 2 ml. ammonia sp. gr. 0·880 and eight drops (0·4 ml.) of recently filtered 0·5% sodium diethyldithiocarbamate. The contents are then washed into 15 ml. centrifuge tubes with two successive portions of about 1 ml. water, bringing the total volumes to approximately 7 ml. in each case. Exactly 1·5 ml. amyl alcohol are added, the centrifuge tubes closed with clean well-fitting rubber bungs, vigorously shaken for ½ min., centrifuged at about 2500 r.p.m. for 10 min., removed, gently agitated to ensure coalescence of the main bulk of the amyl alcohol with that newly risen and centrifuged a further 5 min. Enough of the amyl alcohol layer is then carefully removed, with a teat pipette drawn to capillary point, to fill the micro-cell of the photometer, the "blank" going into the compensating cell. Filter S 47 is used for readings, calculations being made from charts previously constructed by the same analyst. The control tube containing the known 0·3 μg. suffices to check the accuracy of manipulation throughout.

Although scrupulous care is required for handling such minute real magnitudes there is no deviation from simplicity and, as shown in Table 2, results are nearly as good with 0·5 ml. as with 5 ml. of blood.

Table 2. *Comparison of results obtained on 0·5 and 5 ml. portions of blood using the technique described.*

Filter S 47 employed

No.	Quantity blood ml.	Extinction coefficient	Absolute quantity of Cu found mg.	Cu per 100 ml. mg.
1	5	0·460	0·0031	0·062
2	5	0·455	0·0031	0·062
3	5	0·450	0·0031	0·062
4	0·5	0·160	0·00032	0·064
5	0·5	0·155	0·00032	0·064
6	0·5	0·150	0·00031	0·062
7	0·5	0·170	0·00035	0·070

Summary

The diethyldithiocarbamate method for estimation of Cu in biological material is improved in simplicity, speed, accuracy, and sensitivity. Rapid wet digestion, with a combination of sulphuric, perchloric and nitric acids in 8 × 1 in. pyrex test tubes, is employed for destruction of organic matter, followed by deionization of iron with citrate or pyrophosphate in strongly alkaline medium. Subsequent colour formation and extraction with amyl alcohol are effected in the combustion tube itself. With quantities such as 5 ml. blood containing 3 μg Cu, the final reading is either colorimetric or photometric, but with limited material from small laboratory animals, involving determination of real amounts of Cu as low as 0·3 μg., photometric finish with a low wave-length light filter is essential. About 30 determinations per day can be completed by a single analyst.

REFERENCES

Callan & Henderson (1929). *Analyst*, **54**, 650.
Eden (1939). *J. Comp. Path. and Therap.* **52**, 249.
—— (1940, 1). *Nature, Lond.*, **145**, 36.
—— (1940, 2). *Nature, Lond.*, **145**, 628.
—— & Green (1939). *J. Comp. Path. and Therap.* **52**, 301.
McFarlane (1932). *Biochem. J.* **26**, 1022.
van Niekerk (1937). *Onderstepoort J. vet. Sci.* **9**, 623.
Tompsett (1934, 1). *Biochem. J.* **28**, 1544.
—— (1934, 2). *Biochem. J.* **28**, 2088.
Warburg (1927). *Biochem. Z.* **187**, 255.