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Veterinary Biochemical Studies.

I.—A Rapid Method for the Determination of Copper in Biological Material.

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Of the micro-methods which have been proposed for the determination of copper, the method of Biazzo (1926) as modified by Elvehjem and Lindow (1929) has been found to be the most practicable. Until recently McFarlane, (1932), the accuracy of this method has never been questioned. At this laboratory the method was found to give fairly satisfactory results when applied to biological material. In the cases of spleen and blood where the presence of excess iron, due to the large aliquots necessary, interfered with the colour development, the copper had to be separated from the iron at a carefully adjusted pH.

The discovery by Callan and Henderson (1929) that when an aqueous solution of sodium-diethyldithiocarbamate is added to a solution containing copper, a golden brown colour is developed with the formation of a normal copper salt of diethyldithiocarbamic acid. This colour reaction forms the basis for the method discussed here and has lately found much favour in the colorimetric determination of copper.

MacFarlane (1932) extracted the colour quantitatively with amyl alcohol, thereby intensifying the colour and increasing the sensitivity of the method.

It was found necessary, on account of the great number of analyses to be done here, that a rapid and accurate method was essential, and for this purpose the methods of MacFarlane (1932) and Tompsett (1934) were modified and applied as described here.

A large number of copper determinations was done by this method in conjunction with certain copper experiments at present in progress at Onderstepoort. These determinations were done on both normal and pathological post-mortem material as well as on several hundred grass and shrub samples.

These results will be incorporated in later publications from this Institute, in a study on the rôle of copper in certain stock diseases.

Reagents.

1. *Copper Standard.*—Dissolve one gram of purest electrolytic copper in sufficient concentrated nitric acid, dilute and make up to 100 c.c. with distilled water. Dilute 10 c.c. of this solution to one litre which gives a stock solution containing ·1 mgm. Cu per c.c. Dilute this again to obtain a working standard of ·01 mgm. Cu per c.c.

2. *Magnesium Oxidizing Mixture.*—Shake up a 20 per cent. aqueous solution of magnesium nitrate with magnesium carbonate until saturation, filter and bottle.

3. Make a ·5 per cent. aqueous solution of sodium-diethyldithiocarbamate, filter if necessary, and keep in a dark coloured flask.

4. Saturated solution of sodium citrate.

5. 4 per cent. solution of sodium pyrophosphate.

6. 20 per cent. solution of ammonium hydroxide.

It is essential to use only the purest chemicals and in all events do blank tests upon them.

All porcelain or silica dishes should be periodically cleaned according to Elvehjem and Lindow (1929) as follows:—

Put one gram of sodium acetate into each basin and dissolve in sufficient alcohol, evaporate and ignite. The contents are then extracted with hydrochloric acid 1:1 for several days.

Material.

Use 10 grams of liver and 20 grams of spleen, kidney, lung and 20 c.c. blood, respectively, for each determination. In the case of faeces and food use 5-20 grams according to the amount of copper expected to be present.

Method.

In all cases weigh the material into 50 c.c. silica dishes and add 5 c.c. of the magnesium oxidizing mixture. If this is omitted it is found that on ignition even at fairly low temperatures a certain amount of slagging of the copper with the silica of the dishes takes place, consequently recovery of copper in the determination is low as can be seen from the following data:—

In this case liver was finely minced and well mixed. Known amounts of copper were added to the weighed out quantities of liver

prior to ignition. The copper content of the liver was determined with the addition of magnesium mixture and the average result of five determinations taken. In the cases where no magnesium mixture was added the slagging was visible to the naked eye and results were low.

The average copper content of the liver was found to be 0·326 mgm. See Table I.

TABLE I.

No.	C.c. of magnesium mixture added.	Mgm. copper in liver.	Mgm. copper added.	Mgm. copper recovered.	Mgm. copper not accounted for.
1	0	0·326	0·0	0·164	— 0·162
2	0	0·326	0·5	0·525	— 0·301
3	0	0·326	1·0	1·020	— 0·306
4	0	0·326	2·0	2·084	— 0·242
5	0	0·326	3·0	3·150	— 0·176
6	5	0·326	0·1	0·448	+ 0·022
7	5	0·326	0·5	0·832	+ 0·006
8	5	0·326	1·5	1·766	— 0·060
9	5	0·326	2·0	2·226	— 0·100
10	5	0·326	3·0	3·256	— 0·070

The time taken for the complete ignition of the samples without the addition of magnesium nitrate mixture was approximately twice that of the last five samples in Table I. It is thus clear that the complete ignition of biological material, without an adequate oxidizing agent is very time-consuming and unsatisfactory. The effectiveness of this oxidizing agent is especially noticeable in the cases of livers of stock that have been dosed with copper salts or where copper has been added to the sample. In such cases the green copper salt can be seen to be concentrated largely at one spot lying on top of the magnesium salt, without making contact with the surface of the basin.

The ignition can be carried out at fairly high temperatures, without the loss of copper either due to slagging or conversion into insoluble compounds. In fact, an added advantage of a fairly high temperature is that a large per cent. of iron is rendered insoluble.

After incineration is complete, extract the contents of the dishes for about 30 minutes with 5 c.c. concentrated nitric acid. This can be accomplished without heating on account of the readily soluble form in which the copper is present. Dilute the contents with 5 c.c. water, filter carefully through Whatman No. 40 paper into 50 c.c. volumetric flasks. Wash thoroughly with distilled water up to volume.

Take 0·5-5 c.c. aliquots of liver extract, 5-10 c.c. of kidney and 20 c.c. of lung, spleen and blood extracts, respectively.

Pipette these aliquots into 50 c.c. test tubes and add water till about 20 c.c. volume. To these add 4 c.c. of 4 per cent. sodium pyrophosphate solution. According to McFarlane (1929) this is added in order to suppress the ionisation of the iron compounds. It was, however, found that it also improves the separation of the iso-amyl alcohol from the solution containing the copper.

For each 5 c.c. aliquot used now add 2·5 c.c. of saturated sodium citrate solution, e.g. 10 c.c. per 20 c.c. spleen extract. This prevents the precipitation of magnesium and calcium salts, which would absorb some of the colour formed later on in the procedure and consequently too low values would be obtained as can be seen from Table II.

TABLE II.

C.c. sodium citrate added.	Mgm. copper added.	Mgm. copper recovered.	Mgm. copper not accounted for.
0	0·08	0·065	— 0·015
0	0·16	0·128	— 0·032
0	0·16	0·133	— 0·027
5	0·07	0·070	0·0
5	0·08	0·078	— 0·002
5	0·16	0·163	+ 0·003

Add 20 per cent. ammonium hydroxide until alkaline to litmus. Add exactly 10 c.c. of iso-amyl alcohol and ·5 c.c. of the ·5 per cent. solution of sodium-diethyldithiocarbamate reagent. Swirl the tube and close with the hand or stopper to shake the contents vigorously for half a minute. Treat several standards containing ·01, ·02, ·03, and ·05 mgm. Cu respectively exactly in the same manner. Pipette about 8 c.c. of the amyl alcohol into 10 c.c. centrifuging tubes and centrifuge out any water present at approximately 3,000 r.p.m. for 5-10 minutes. Decant the supernatant alcoholic layer into colorimeter cups and read against the standard most closely corresponding in colour. The use of ·5 c.c. of a ·5 per cent. solution of the carbamate reagent differs from the recommendation of McFarlane (1932) who uses ·5 c.c. of 2 per cent. strength. It was, however, found that ·5 c.c. of ·5 per cent. strength is sufficient to precipitate 0·3 mgm. copper, i.e. approximately fifteen times the amount of copper usually taken for a determination.

The main object for this change is that when more of the reagent is used a precipitation of iron tends to occur which does not happen when the lesser amount is employed. This interference of iron may be eliminated, as McFarlane stated, by the addition of one drop of a 40 per cent. sodium hydroxide solution and heating for fifteen minutes at 80° C. in water. It was found in cases of organic extracts containing relatively much iron, as well as in blank determinations to which iron had been added, that this boiling with the addition of alkali could be omitted without giving too high values as a result of the coloured iron precipitate formed when more of the reagent is used. This can be seen from Tables III and IIIA.

TABLE III.

Blank Determinations.

Mgm. copper added.	Mgm. Fe added.	Treatment.	Mgm. copper recovered.	Percentage copper recovered.	Remarks.
·02	0·0	Heated at 80° C. + alkali + ½-c.c. of 2 per cent. reagent	·020	100	
·02	0·0	,, ,,	·019	95	
·02	1·0	,, ,,	·018	90	
·02	2·0	,, ,,	·018	90	
·02	3·0	,, ,,	·019	95	
·02	4·0	,, ,,	·020	100	
·02	5·0	,, ,,	·020	100	
·02	0·0	Used ½-c.c. of 2 per cent. reagent without boiling or alkali	·020	100	
·02	0·0	,, ,,	·021	105	
·02	1·0	,, ,,	·020	100	
·02	2·0	,, ,,	·020	100	
·02	3·0	,, ,,	·021	105	Iron did not interfere.
·02	4·0	,, ,,	·021	105	
·02	5·0	,, ,,	·024	120	Iron definitely interfered.
·02	10·0	,, ,,	·025	125	
·02	1·0	Used ½-c.c. of ·5 per cent. reagent without boiling or alkali	·020	100	
·02	2·0	,, ,,	·020	100	
·02	3·0	,, ,,	·019	95	
·02	4·0	,, ,,	·020	100	
·02	5·0	,, ,,	·019	95	
·02	10·0	,, ,,	·023	115	Iron interfered.

TABLE IIIA.

Determination on Spleen Extracts.

Amount.	Treatment.	Mgm. copper found.
20 c.c.	Heated at 80° C., + alkali + ½-c.c. of 2 per cent. reagent	0·023
20 c.c.	,, ,, ,, ,,	0·025
20 c.c.	,, ,, ,, ,,	0·023
20 c.c.	Without heating or adding alkali, + ½-c.c. of ·5 per cent. reagent	0·025
20 c.c.	,, ,, ,, ,,	0·024
20 c.c.	,, ,, ,, ,,	0·022
20 c.c.	,, ,, ,, ,,	0·022

PLANT MATERIAL.

Although these analyses were made on filtrates obtained by the method of extraction of Louw (1934), it was nevertheless found that the plant material could be treated and extracted in the same manner as the other biological material as described herein. The results obtained by these two different ways of extraction compared favourably.

SUMMARY.

1. An accurate and rapid method, which is a modification of the methods of McFarlane (1932) and Tompsett (1934) for the determination of copper, has been presented.

2. An oxidizing agent in the form of magnesium nitrate and carbonate is used to accelerate the ignition and render the copper easily soluble.

3. The quantity of sodium di-ethyldithiocarbamate is decreased to eliminate the interference of iron compounds usually found in organic material.

4. The addition of sodium citrate solution to render the calcium and magnesium salts soluble in the ammoniacal solution is discussed.

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