CXXI. APPLICATION OF THE SODIUM DIETHYL-DITHIOCARBAMATE REACTION TO THE MICRO-COLORIMETRIC DETERMINATION OF COPPER IN ORGANIC SUBSTANCES.

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WHEN an aqueous solution of sodium diethyldithiocarbamate is added to a solution containing copper the normal copper salt of diethyldithiocarbamic acid, which has a golden-brown colour, is formed. Callan and Henderson [1929], who discovered this reaction, applied their method with satisfactory results to the determination of copper in inorganic substances, but were not concerned with biological material.

Of the colorimetric methods which have been proposed for estimating the copper content of biological material the Biazzo [1926] method as developed by Elvehjem and Lindow [1929] has been most used. Apart from the fact that the accuracy of this method has been recently questioned [see Ansbacher, Remington and Culp, 1931; Walker, 1930; Drabkin and Waggoner, 1930] the carbamate reaction has, at the outset, certain important advantages over it, since the latter is at least ten times more sensitive and gives the same colour with copper irrespective of the p_H of the solution.

The work described in this paper was undertaken because we believed that to study further the rôle of copper in metabolism, and to clarify our present knowledge in this regard, more accurate methods were required for the determination of copper in organic materials.

EXPERIMENTAL.

The normal copper salt of diethyldithiocarbamic acid has a rather limited solubility in aqueous solution. If the amount of copper in the solution to be tested exceeds 0.015 mg. in 10 cc. the coloured copper salt tends to precipitate slowly and colorimetry becomes impossible. We have found that the coloured copper salt is much more soluble in amyl alcohol and may be extracted from aqueous solution by this solvent. The reaction is thereby made applicable over a much wider range of copper concentration. The ionisation of the copper salt is depressed in the organic solvent, thus intensifying the colour and so giving the reaction greater sensitivity.

To ascertain whether the intensity of the observed colour is proportional to the concentration of copper in the solution a series of proportionality tests covering three ranges of copper concentration was made. The results are plotted in Fig. 1 from data obtained as follows. Varying amounts of a standard copper

Fig. 1. Showing the proportionality between the amount of copper present and the intensity of the colour developed with sodium diethyldithiocarbamate.

Note: The values plotted represent the colorimeter reading in mm. of that solution chosen as a standard when the other test solutions were set at 15 mm.

	Range of copper concentrations mg.	Copper content of standard solution mg.
Curve I	0.01 - 0.10	0.05
Curve II	0.005 - 0.04	0.02
Curve III	0.004 - 0.024	0.012

The diagonal lines represent the theoretical proportionality between copper present and colour produced.

solution were measured into 25 cc. glass-stoppered measuring cylinders and all diluted to the same volume (10 cc.)¹ with water. To each cylinder were added 1 drop 40 % NaOH, 5 cc. amyl alcohol (carefully measured) and finally 0.5 cc. of a 2 % aqueous solution of the carbamate reagent². The cylinders were stoppered and shaken, not too vigorously, for about one minute. The amyl alcohol layer, which separated very quickly and contained the whole of the coloured copper salt, was removed by means of a pipette and filtered into a clean dry test-tube through a small 2.5 cm. filter. This filtration, which removes the small amount of water suspended in the amyl alcohol, was adopted as a

¹ In all the experiments discussed in this paper special precautions were taken to preclude any possibility of copper contamination. For preparing the reagents, rinsing glassware, etc., only water redistilled from glass was used. All the reagents were tested for copper. NaOH prepared from sodium which contained no copper was always used. Merck's highest purity amyl alcohol was employed. The sodium salt of diethyldithiocarbamic acid was obtained from the Eastman Kodak Co. When incineration was employed to destroy organic matter only "vitreasil" dishes or porcelain dishes specially treated to ensure absence of copper [Elvehjem and Lindow, 1929] were used.

² An aqueous solution of sodium diethyldithiocarbamate keeps fairly well when stored in a dark-coloured bottle. A precipitate forms in a few weeks. After filtering the reagent may still be used.

time-saving measure. The amyl alcohol layer will become perfectly clear if allowed to stand for about 30 minutes. In the third series of proportionality tests the total volume of each solution was 5 cc. instead of 10 cc., and only 3 cc. of amyl alcohol were used for extraction. The colorimetry was carried out in a Duboscq colorimeter using micro-cups.

The depth of colour is directly proportional to the amount of the coloured copper salt present provided the difference between the amounts of copper in the standard solution and the test solution is not too great (Fig. 1). A standard containing 0.005 mg. copper was found to cover the range from 0.002 mg. to 0.010 mg. of copper very satisfactorily. The solutions are stable towards light for at least 12 hours. It is important that the amyl alcohol used should be of the highest purity. The colour fades slowly in the technical grade of solvent.

Extractions with chloroform and with carbon tetrachloride have been tried but were found to be unsatisfactory.

Callan and Henderson [1929] investigated the reaction of the carbamate reagent with the more common metals. Iron which gives a brown colour with the reagent is the only element present in the ash of biological material which would interfere with the determination of copper. Less than 0.05 mg. Fe gives no colour. The intensity of the colour given by 0.08 mg. Fe is approximately equal to that given by 0.005 mg. Cu. Because of the difference in colour the two solutions can only be very roughly matched.

According to Callan and Henderson [1929] iron can be completely removed as ferric hydroxide without loss of copper. This procedure has been found by Ansbacher *et al.* [1931] to give copper values which are too high. The present author [McFarlane *et al.*, 1930], in determining the copper content of hen's eggs, used double precipitation with NH_4OH to remove iron and found the filtrate to contain all the copper free from iron.

The estimation of copper in solutions containing relatively large amounts of iron when sodium pyrophosphate is present.

When sodium pyrophosphate is added to a solution containing copper and iron, $\text{Fe}_4(\text{P}_2\text{O}_7)_3$ and $\text{Cu}_2\text{P}_2\text{O}_7$ are formed. The former is an un-ionised compound and does not react with such ions as CNS^- [Warburg, 1927] nor does it react, as we have found, with the carbamate reagent. The pyrophosphate does not affect the reactivity of the copper. Drabkin and Waggoner [1930] recommend a 4 % solution of sodium pyrophosphate to remove the iron when determining copper by the Elvehjem and Lindow procedure. In a later paper Elvehjem and Hart [1931] claim that in using $\text{Na}_4\text{P}_2\text{O}_7$ for this purpose accurate results are obtained only when certain precautions as to p_{H} and temperature are taken.

To a series of tubes all containing the same amount of copper, but with increasing amounts of ferric chloride, varying amounts of 4 % $\text{Na}_4\text{P}_2\text{O}_7$ were added. The solutions were then made alkaline by adding 1 cc. conc. NH_4OH and heated in a water-bath at 80° for 10 minutes. No precipitate of ferric pyrophosphate was formed under these conditions. The colorimetry was com-

pleted as before, using one of the solutions as a standard. Against this the relative intensities of colour of the others were determined.

Table I. *The effect of sodium pyrophosphate on the recovery of copper from solutions containing 0.02 mg. Cu to which varying amounts of iron have been added.*

	Amount of iron added mg.	Amount of 4 % $\text{Na}_4\text{P}_2\text{O}_7$ added cc.	Colorimeter reading mm.*
1.	Nil	1.0	20.0
2.	Nil	3.0	20.8
3.	0.35	Nil	reddish-brown precipitate
4.	0.35	1.0	20.4
5.	0.70	1.0	21.0
6.	1.05	1.0	reddish-brown precipitate
7.	1.05	3.0	21.3
8.	1.40	3.0	21.8
9.	1.75	3.0	22.0

* The colorimeter readings are those of solution No. 1 as standard when adjusted to match the other test solutions which were always set at 20 mm.

We may thus conclude from Table I that copper can be accurately estimated in alkaline pyrophosphate solution in the presence of relatively large amounts of iron. The presence of traces of copper in the pyrophosphate and ferric chloride solutions was responsible for the variations in the colorimeter readings.

The determination of copper in crude glutamic acid¹.

Our first analyses were done on crude glutamic acid because of our interest in this product at the time. A qualitative examination had shown that it contained a very considerable amount of copper and iron.

Samples were incinerated in a muffle furnace at 380–400° for at least 12 hours. We have found that to recover copper quantitatively from the ash of organic materials the same careful control of the temperature of ashing as that recommended by Peters [1912] for the estimation of iron is necessary. The dishes were cooled and the ash was moistened with 1–2 cc. conc. HNO_3 . The HNO_3 was driven off by heating over a small flame and the dishes were replaced in the muffle for a short time. The ash should be perfectly white or, if much iron is present, reddish in colour. If unoxidised carbon is still present the wet incineration with HNO_3 should be repeated. The ash is completely soluble in hot 6*N* HCl.

The results of copper determinations on crude glutamic acid by various procedures are recorded in Table II. With the larger samples of glutamic acid suitable aliquots of the ash solution were taken for the analysis. Where $\text{Na}_4\text{P}_2\text{O}_7$ was used to remove the iron the procedure was the same as that already described. To remove iron as $\text{Fe}(\text{OH})_3$ an excess of concentrated NH_4OH was added to an aliquot of the ash solution in a 15 cc. centrifuge-tube. After heating on a boiling water-bath for 15 minutes the solution was centrifuged and the clear supernatant liquid decanted into a 25 cc. glass-stoppered cylinder.

¹ *d*-Glutamic acid (practical). Eastman Kodak Co.

Table II. *The copper content of crude glutamic acid as determined by different procedures.*

Wt. of glutamic acid	Copper added mg.	Method of destroying organic matter	Method of removing Fe	Colorimetric method used	% Cu in crude glutamic acid
10 g.		Dry ashing	Aliquot 1 $\text{Na}_4\text{P}_2\text{O}_7$	Carbamate reagent	0-0173
			" 2 "	" "	0-0167
			" 3 "	Elvehjem and Hart [1931]	0-013
10 g.		" "	Aliquot 1 NH_4OH	Carbamate reagent	0-0159
			" 2 "	" "	0-0168
			" 3 "	Elvehjem and Hart	0-011
2 g.	0-10	" "	Aliquot 1 $\text{Na}_4\text{P}_2\text{O}_7$	Carbamate reagent	0-0187*
			" 2 "	" "	0-0174
			" 3 "	Elvehjem and Hart	0-014
0-5 g.		" "	$\text{Na}_4\text{P}_2\text{O}_7$	Carbamate reagent	0-0174
0-5 g.	0-01	" "	" "	" "	0-0177*
0-5 g.		$\text{H}_2\text{SO}_4\text{-HClO}_4$ digestion	" "	" "	0-0185
0-5 g.		" "	" "	" "	0-0178
0-10 g.		Dry ashing	" "	" "	0-0201
					0-0179

* The amount of copper added was subtracted from the amount of copper found before calculating the copper content of the crude glutamic acid.

The $\text{Fe}(\text{OH})_3$ precipitate was dissolved in the minimum amount of 6N HCl (with the aid of heat) and $\text{Fe}(\text{OH})_3$ reprecipitated as before. The copper content of the combined supernatant liquids was then determined as described above.

Preparation of the standard copper solution. 0-3928 g. pure $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ was dissolved in water. A small amount of dilute H_2SO_4 was added to prevent the formation of basic cupric sulphate by hydrolysis. The solution was then diluted to 1 litre (1 cc. = 0-10 mg. Cu). In the majority of the determinations 1 cc. of a standard solution containing 0-01 mg. Cu per cc. was used. In preparing this standard the same reagents were added in the same amounts as those used in preparing the solution being analysed, *i.e.*, 6N HCl and $\text{Na}_4\text{P}_2\text{O}_7$ solution or concentrated NH_4OH .

The destruction of organic matter by wet incineration was carried out in a micro-Kjeldahl flask. 2 cc. concentrated H_2SO_4 and 1 cc. 60 % HClO_4 were added and the solution was heated over a micro-burner until only a faint yellow colour persisted and white fumes ceased to be evolved.

The values obtained for the copper content of crude glutamic acid (Table II) were in good agreement, whether the organic matter was destroyed by dry or by wet incineration. The removal of iron as $\text{Fe}(\text{OH})_3$ or as $\text{Fe}_4(\text{P}_2\text{O}_7)_3$ made no appreciable difference to the results. The values obtained, using the method of Elvehjem and Hart [1931], were considerably lower than those obtained when the same sample was analysed by means of the carbamate reagent.

The determination of copper in blood.

The copper content of blood or serum according to various authors is given in Table III. From the results of these analyses it appears that either the copper contents of the bloods of different species and of individuals within

the same species are subject to considerable variations or that the analytical methods are inaccurate.

Table III.

	Mg. copper per 100 cc.
Whole or defibrinated horse-blood [Elvehjem, Steenbock and Hart, 1929, 1].	0.058*
Three samples of " " " [Grendel, 1930].	0.147
Three samples of whole human blood [Schönheimer and Oshima, 1929].	0.113 to 0.114*
" " " [Grendel, 1930].	0.075
Horse-serum [Abderhalden and Möller, 1928].	0.19
Cow's serum [McHargue, Healy and Hill, 1928].	0.33
Ox-blood [Grendel, 1930].	0.076

* Same colorimetric method used.

Samples of blood (3–10 cc.) were evaporated to dryness on a water-bath, a small glass rod being used for stirring. The dried blood without removing the stirring rod was ashed at 400° as already described. The ash was dissolved in 2–5 cc. 6*N* HCl, with the aid of heat, the amount of HCl used depending upon the amount of blood taken. The solution of the ash was transferred to a 25 cc. glass-stoppered cylinder with the aid of a few cc. of water. 1–5 cc. saturated sodium pyrophosphate (about 5.4 g. Na₄P₂O₇, 10 H₂O in 100 cc. cold water) were added, the amount again depending upon the quantity of blood being analysed. For 5 cc. of blood 3 cc. saturated solution of Na₄P₂O₇ were used. Concentrated NH₄OH was added from a burette until the solution was just alkaline to litmus. The cylinders were placed in a water-bath (80°) for about 15 minutes and then cooled. With the larger samples of blood (*i.e.*, about 10 cc.) a slight white precipitate, presumably calcium phosphate and Mg₂P₂O₇, is formed but does not interfere with the colorimetric determination. The amount of copper present was finally determined, as already described, using the carbamate reagent and extracting with amyl alcohol. For 5 cc. of blood a suitable standard contained 0.01 mg. Cu to which had been added the same amount of Na₄P₂O₇ and NH₄OH and which had been diluted to the same volume as the test solution.

When the iron was removed as Fe(OH)₃ the above procedure was modified in the same way as that described for glutamic acid.

Results.

1. Whole ox-blood.

	Mg. copper in 100 cc. of blood
A. 5 cc. blood—ash oxidised with HNO ₃	
(1) Iron removed with NH ₄ OH	0.185
(2) " " " "	0.187
(3) " " " " Na ₄ P ₂ O ₇	0.187
B. 5 cc. blood—ash not oxidised with HNO ₃	
(1) Iron removed with NH ₄ OH	0.226
(2) " " " "	0.208
(3) " " " " Na ₄ P ₂ O ₇	0.191
C. 3 cc. blood—ash oxidised with HNO ₃	
(1) Iron removed with NH ₄ OH	0.172
(2) " " " " Na ₄ P ₂ O ₇	0.190

2. *Defibrinated ox-blood.*

Iron removed as $\text{Fe}_4(\text{P}_2\text{O}_7)_3$ and the ash oxidised with HNO_3 .

Amount of blood taken for analysis cc.	Amount of copper added mg.	Copper content of the blood calc. after subtracting the amount of copper added mg. per 100 cc.
2.0	0.004	0.221
2.0	0.008	0.214
5.0	Nil	0.202
5.0	0.006	0.194
5.0	0.010	0.210
3.0	Nil	0.190

3. *Whole pig-blood.*

10 cc. blood taken for analysis. Ash oxidised with conc. HNO_3	Mg. copper in 100 cc. blood
(1) Iron removed with NH_4OH	0.163
(2) " " " $\text{Na}_4\text{P}_2\text{O}_7$	0.180

4. *Whole human blood.*

Ash oxidised with HNO_3 . Iron removed with $\text{Na}_4\text{P}_2\text{O}_7$.

(1) 10 cc. blood from one individual	0.185
(2) 10 cc. " " another individual	0.170
(3) 5 cc. " (composite sample)	0.181
(4) 5 cc. " " "	0.210

Note. The composite samples were from oxalated blood received by this laboratory for routine analysis.

5. *Defibrinated ox-blood: sulphuric-perchloric acid digestion.*

To 3 cc. of blood in a 100 cc. Kjeldahl flask, 2 cc. conc. H_2SO_4 and 1 cc. 60 % HClO_4 were added and digestion continued until foaming ceased. Two further 1 cc. portions of 60 % HClO_4 were required to complete the incineration, when white fumes were no longer evolved and the light yellow-coloured sulphuric acid digest boiled very slowly. The copper contents of three samples of the same blood were determined with the following results. $\text{Na}_4\text{P}_2\text{O}_7$ was used to remove the iron.

(1) 0.215 mg. copper per 100 cc. blood
(2) 0.145 " " " " "
(3) 0.165 " " " " "

The results by this method of incineration while less accurate are of the same order of magnitude as those obtained by dry ashing. Difficulties in manipulation, at least so far as blood is concerned, would not warrant the substitution of acid incineration for dry ashing even if more consistent results were obtained after further study of the wet incineration method.

The copper content of blood can be accurately estimated by means of the carbamate reagent, particularly if the blood is ashed, the ash oxidised with HNO_3 and the iron removed as $\text{Fe}_4(\text{P}_2\text{O}_7)_3$.

The determination of copper in crystalline haemoglobin.

Elvehjem, Steenbock and Hart [1929, 1] have recorded the copper contents of different crystalline preparations of haemoglobin as 3.4 and 1.9 mg. Cu per 100 g. respectively for two samples from horse-blood, and 1.3 and 1.7 mg. Cu per 100 g. for two different preparations from rat-blood. These values appear to be very high, especially as, in the same paper, they record the copper content of the corpuscle fraction of horse-blood as only 0.043 mg. Cu per 100 cc. of blood. These analyses were made in an endeavour to answer the question "Is copper a normal constituent of the haemoglobin molecule?" Their conclusion, which was in the negative, was not based on their experimental findings but on the fact that a positive result was incompatible with the accepted molecular weight of haemoglobin. It is of interest in this connection to record here the copper content of a sample of crystalline haemoglobin, of German preparation but of unknown origin. This sample contained only 0.0057 mg. Cu per g. of haemoglobin as determined by our method of analyses.

The determination of copper in cow's milk.

It was with the realisation that there was a particular need for an accurate method of determining the minute amount of copper in cow's milk that we first undertook the investigations recorded in this paper. Perhaps the best evidence that existing methods for determining the copper content of milk are of doubtful accuracy is to be found in the numerous papers on the rôle of copper as an essential supplement to inorganic iron in curing nutritional (milk) anaemia in rats. We believe that much of the confusion which exists in this regard (see Elvehjem and Hart [1932] for brief discussion) is the inevitable result of carrying out such feeding experiments without an accurate knowledge of the copper content of the milk.

According to various authors the copper content of cow's milk, collected with special precautions, is:

	Mg. copper per litre
*Elvehjem, Steenbock and Hart [1929, 2]	0.15
*Drabkin and Waggoner [1930]	0.25
*Beard and Myers [1931]	0.44
**Supplee and Bellis [1922]	0.2 to 0.8
**Quam and Hellwig [1928]	0.26 to 0.52
Keil and Nelson [1931]	0.24

*Determined by the method of Elvehjem and Lindow [1929].

**Potassium ethyl xanthate method.

The value recorded by Keil and Nelson is "as determined by the sodium diethyldithiocarbamate method." Since these authors made no further comment it was to be assumed that the accuracy of this method had been established. We could find, in the literature, no earlier reference to the use of this reagent for determining the copper content of milk.

The estimation of copper in milk presents certain difficulties which are to some extent peculiar to this product. The minute amount of copper present

makes it necessary to use a large sample for the analyses. The solution of the milk-ash then contains a relatively large amount of calcium phosphate. The methods of removing iron, already discussed, are not applicable, as the copper cannot be quantitatively recovered from the voluminous precipitate of calcium phosphate which comes down in alkaline solution.

Precipitation of minute quantities of copper with hydrogen sulphide. The majority of the more recent determinations of the copper content of milk have been made by the method of Elvehjem and Lindow [1929]. By this procedure the copper (0.03 to 0.04 mg. from 250 cc. milk) is precipitated by H_2S in a total volume of 200 cc. 0.25*N* HCl. This large dilution of the milk-ash before H_2S precipitation appeared to us to involve an unnecessary danger of incomplete precipitation of cupric sulphide. These conditions of H_2S precipitation were probably adopted by Elvehjem and Lindow to avoid the simultaneous precipitation of much free sulphur which interfered with their final colorimetry. According to Ansbacher *et al.* [1931], the precipitation of small amounts of copper by H_2S is only quantitative when the concentration of acid is such that some sulphur is precipitated to adsorb and carry down the cupric sulphide. According to Walker [1930], the oxidation of cupric sulphide by nitric acid in the presence of filter-paper gives results for copper which are too high.

Our first experiments were conducted with an inorganic solution the composition of which approximated to that of a solution of milk-ash so far as the elements Fe, Cu, Ca and P were concerned. We first studied the influence of acidity and dilution on the recovery of the copper from this solution. The copper solutions prepared (see Table IV) were heated to 80° in 100 cc. glass-

Table IV.

	Amount of copper present mg.	Amount of 6 <i>N</i> HCl added cc.	Total volume at H_2S pre- cipitation cc.	Normality	Amount of copper recovered mg.
1.	0.02	5.0	100	0.25	0.011
2.	0.02	5.0	50	0.50	0.021
3.	0.02	5.0	10	2.5	0.020
4.	0.01	2.5	50	0.25	0.008
5.	0.01	2.5	10	1.25	0.012

stoppered Erlenmeyer flasks and while cooling were saturated with H_2S for 15 minutes. The flasks were stoppered and set aside for 24 hours. The solutions, without heating, were resaturated with H_2S for 15 minutes. To avoid the use of filter-paper, micro-Jena glass sintered filters set in test-tubes with a side-arm were used for filtration of the cupric sulphide under suction. The precipitate was washed on the filter with a few cc. of H_2S water which had just been used to rinse the Erlenmeyer flasks. After removing the clear filtrate the filter was replaced in the test-tube. 2 cc. 10 % H_2SO_4 and 1 cc. saturated bromine water were measured into the Erlenmeyer flask (to oxidise any CuS still remaining) and then transferred to the Jena filter and allowed to stand 15 minutes without

suction. A small glass rod was used to stir up the precipitate. By applying suction the solution was drawn into the test-tube. Four 1 cc. portions of water were used to rinse the Erlenmeyer flask and filter. A small amount of free sulphur is left on the filter. The CuS is completely oxidised and all the copper is in the filtrate.

The filter was removed and the excess bromine in the filtrate driven off by carefully bringing the contents of the test-tube to the boil over a small flame. After cooling, 0.75 cc. 40 % NaOH was added, *i.e.*, the amount requisite to make the solution just alkaline to phenolphthalein as previously determined by blank titration. The colorimetry with the carbamate reagent was carried out in 25 cc. glass-stoppered measuring cylinders as previously described.

The standard (usually containing 0.01 mg. copper) was prepared by measuring into a test-tube an aliquot of the standard copper solution. 2 cc. 10 % H_2SO_4 and 1 cc. saturated bromine water were added and the excess bromine was driven off by boiling. After transferring the solution to a 25 cc. glass-stoppered cylinder the preparation was completed in the same manner as described for the test solution. It was decided from these results (Table IV) that if the milk-ash were dissolved in 10 cc. 6*N* HCl and the solution diluted to not more than 50 cc. quantitative precipitation of copper by H_2S would be obtained.

The milk was obtained by milking directly into glass containers. Samples of 100–300 cc. were evaporated to dryness on a water-bath. The residue was ashed in a muffle furnace (for method of ashing see under glutamic acid). The ash was dissolved in 10 cc. 6*N* HCl and the solution transferred to a 100 cc. glass-stoppered Erlenmeyer flask with the aid of 40 cc. water. The precipitation of copper as cupric sulphide, the filtration and oxidation of this precipitate and the final colorimetric determination of copper by the carbamate reagent were carried out as described above. The three samples of milk analysed were collected over a period of two months.

Sample 1		Sample 2		Sample 3	
cc. milk analysed	Copper content mg. per litre	cc. milk analysed	Copper content mg. per litre	cc. milk analysed	Copper content mg. per litre
250	0.092	250	0.068	300	0.072
250	0.100	250	0.067	250	0.077
200	0.097	150	0.049	200	0.071
100	0.068	*300	0.120	**250	0.080
*300	0.144	—	—	*300	0.115

*. Determined by the method of Elvehjem and Lindow [1929].

** The CuS precipitate was oxidised on the Jena filter with 2 cc. hot concentrated HNO_3 instead of the $\text{Br}_2\text{-H}_2\text{SO}_4$ solution. The filtrate was neutralised with 3.15 cc. 40 % NaOH and the copper determination carried out with the carbamate reagent.

We can thus conclude from these results that the copper content of milk can be accurately determined by the method described provided the amount of milk taken for analyses is not less than 200 cc. In addition to the results recorded a number of analyses using 75–150 cc. of milk have given low results. It will

also be observed that with the same samples of milk the method of Elvehjem and Lindow gave considerably higher results than were obtained by the carbamate method. The oxidation of CuS in the cold by bromine in sulphuric acid solution has proved extremely satisfactory. Hot conc. HNO_3 gives the same results, provided the standard copper solution used for colorimetric comparison contains an equal amount of HNO_3 . The intensity of colour with the carbamate reagent is considerably less when HNO_3 has been used as the oxidising agent instead of the bromine-sulphuric acid solution.

The determination of copper in rat-livers.

Dr R. C. Hamilton of this laboratory in studying certain aspects of copper metabolism has analysed a large number of rat-livers for copper. With a few minor modifications the procedure recommended in this paper for estimating the copper content of blood was used. The samples of liver, about 1 g., were ashed and the ash was dissolved in 5 cc. 6N HCl with the aid of heat. 3 cc. saturated solution of $\text{Na}_4\text{P}_2\text{O}_7$ were used to remove the iron and 3 cc. amyl alcohol to extract the coloured copper salt.

	Weight of liver taken for analysis g.	mg. of copper per 100 g. of tissue— calculated on	
		A *Dry weight of liver	B Wet weight of liver
1. Normal adult rat (male)	1.6135	2.18	0.59
	1.3085	1.99	0.56
2. Normal adult rat (male)	1.4680	2.09	0.62
	1.8233	1.94	0.57
3. Normal adult rat (female)	1.2798	2.54	0.69
	1.0745	2.87	0.74
	0.9602	2.71	0.71

* The portion of liver was dried at 100° for 12 hours.

These results in the hands of another worker are a good confirmation of the consistency with which the copper content of biological materials can be determined by this method. Accurate copper determinations are possible using as little as 0.25 g. of liver.

SUMMARY.

1. An examination of the reaction between copper and sodium diethylthiocarbamate (Callan and Henderson) has revealed certain points of importance in the quantitative application of this reaction:

A. The golden-brown copper salt formed is much more soluble in amyl alcohol than it is in water. The coloured copper salt can be rapidly and quantitatively extracted from aqueous solution by amyl alcohol. The reaction may then be applied over a much wider range of copper concentration.

B. The golden-brown colour is intensified in the organic solvent. The sensitivity of the reaction is thus increased.

C. The depth of colour is directly proportional to the amount of copper present provided the range of copper concentrations is not too great.

D. In alkaline solution ferric pyrophosphate does not give the reaction but the reactivity of the copper is unimpaired. In alkaline pyrophosphate solution copper may be determined in the presence of iron.

E. The oxidation of cupric sulphide in the cold with saturated bromine water and 10 % sulphuric acid does not interfere with the subsequent application of the colour reaction.

2. These observations have been utilised in developing procedures by means of which copper may be accurately estimated in biological materials. Blood, milk, liver, crude glutamic acid and crystalline haemoglobin have been analysed for copper in the course of these investigations.

I desire in conclusion to express my thanks to Prof. G. Hunter for his constant advice and help in this work. I am also indebted to Dr R. C. Hamilton for permitting me to include the results of some of his copper determinations on rat-livers.

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