CCV. THE COPPER CONTENT OF BLOOD.

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FROM experiments on suckling rats it has been shown that copper has an important influence in the formation of haemoglobin and the red blood cell. The Wisconsin workers [Hart *et al.*, 1927; 1928; Waddell *et al.*, 1929; Elvehjem and Sherman, 1932] have shown that new-born rats fed on a diet of cow's milk after weaning develop an anaemia. Addition of purified iron salts to the diet causes no abatement of the anaemia although iron is stored in the liver. The addition of copper however produces a marked reticulocyte response and haemoglobin increase. These results have been confirmed by others [Cunningham, 1931; Josephs, 1932; Muntwyler and Hanzal, 1933]. In addition Cunningham and Josephs have shown that copper feeding reduces the iron in the liver and tissues and increases that in the blood.

In view of the importance of this work it appears that a knowledge of the variations of the copper content of blood in various conditions would be of considerable value. The copper content of blood or serum as determined by various writers is shown in Table I. It appears that the copper content of the bloods of different species and of individuals within the same species must vary widely, or most of the methods are inaccurate.

Table I.

Author	Species	Type of blood	Result mg. per 100 ml.
1. Elvehjem <i>et al.</i> [1929]	Horse	Whole blood	0.058
2. Schönheimer and Oshima [1929]	Human	Whole blood	0.113-0.114
3. Abderhalden and Møller [1928]	Horse	Serum	0.19
4. McFarlane [1932]	Human	Whole blood	0.185-0.210
"	Pig	"	0.163-0.180
"	Ox	"	0.185-0.226
5. Locke <i>et al.</i> [1932]	Human	Serum	0.08-0.095
"	Rabbit	"	0.05-0.072
"	Horse	"	0.09
6. Warburg and Krebs [1927]	Human	"	0.124
"	Rabbit	"	0.104
"	Pig	"	0.194
7. McHargue [1925]	Ox	Whole blood	0.14
8. Guillemet [1931]	Cow	Serum	0.058-0.082
"	Pig	"	0.067-0.118
"	Human	Plasma	0.056-0.075

Guillemet and Schell [1931] used a method involving the dry ashing of blood, precipitation of copper as hydroxide, followed by filtration, dissolution in acid and deposition on platinum by electrolysis. The copper was then dissolved in "reactive phosphomolybdic acid" to form a blue solution and the copper

determined by titration with standard permanganate solution until the blue colour disappeared. These writers determined amounts of copper of the order of 0.1 mg. by this method. The number of precipitations renders this method unsatisfactory and probably accounts for the low values obtained by this method.

Warburg [1927] has devised an ingenious method for the determination of copper in serum utilising the fact that copper catalyses the oxidation of cysteine to cystine. To prevent the interference of iron, sodium pyrophosphate is added. Warburg found that copper could be determined directly in serum without the necessity of ashing.

Some writers analysing large samples of blood have used macro-chemical methods. Among those may be mentioned McHargue [1925] and Abderhalden and Møller [1928].

Among the colorimetric methods used for the determination of the copper content of biological material are:

1. The potassium ethyl xanthate method.
2. The Biazzo method [1926] as modified by Elvehjem and Lindow [1929].
3. The diethyldithiocarbamate reaction of Callan and Henderson [1929] as modified by McFarlane [1932].

Recently the accuracy of the Biazzo method has been questioned [*cf.* Ansbacher *et al.*, 1931; Walker, 1930; Drabkin and Waggoner, 1930]. A serious disadvantage of the reaction is the interference by iron, which involves the separation of copper as sulphide at a carefully adjusted p_H .

In the third reaction an aqueous solution of sodium diethyldithiocarbamate is added to a solution of copper to produce a golden brown colour. Callan and Henderson [1929], who discovered the reaction, obtained satisfactory results when it was applied to the determination of copper in inorganic mixtures. McFarlane [1932] found that the brown colour could be extracted with amyl alcohol thereby increasing the sensitivity of the reaction. At the present time this is the most sensitive reaction for copper—it being possible to detect one part of copper in ten million parts of solution—and is also independent of p_H . Iron interferes by producing a dirty brown colour but this may be prevented by the addition of sodium pyrophosphate and carrying out the reaction in a slightly alkaline medium.

As a preliminary a slight modification of McFarlane's [1932] method for the determination of copper in blood was used. This was carried out as follows:

5 ml. of blood were measured into an 8 × 1 inch pyrex tube followed by 5 ml. of concentrated nitric acid. This was allowed to digest in a boiling water-bath until the contents of the tube were almost dry. Then the remaining organic matter was destroyed by heating with 1 ml. of concentrated sulphuric acid and perchloric acid (about 3 ml.). The contents of the tube were diluted to 10 ml. with distilled water and 5 ml. of 4 % sodium pyrophosphate solution added. The solution was treated with ammonia until slightly alkaline to litmus and then placed in a water-bath at 80° for 15 minutes. After cooling 5 ml. of amyl alcohol were added followed by 0.5 ml. of 2 % aqueous sodium diethyldithiocarbamate, and the whole was well shaken. A standard containing 0.01 mg. of copper was prepared in exactly the same way. After separating, the amyl alcohol layers were removed and filtered through filter-paper to remove suspended water and compared in a colorimeter.

Any glass apparatus that was used was cleaned first with nitric acid and then with glass-distilled water and chemicals of A.R. standard were used. Filter-papers were washed with dilute acid and distilled water and dried. The standard solution contained 0.01 mg. copper per ml. This was prepared by diluting a stock solution containing 0.3928 g. $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ in 1 litre (1 ml = 0.1 mg. Cu). This solution contained a little sulphuric acid to prevent formation of insoluble basic copper sulphate.

Satisfactory results were obtained by this method of the order obtained by McFarlane [1932].

Locke *et al.* [1932] attempted to determine the copper content of human serum by applying the diethyldithiocarbamate reagent directly to trichloroacetic acid filtrates. They obtained values of 0.08–0.095 mg. per 100 ml. serum, *i.e.* about half of those obtained by McFarlane and the present writer for whole blood (see Table III, first column). The possibility of estimating copper in protein-free filtrates without the necessity of ashing was therefore studied.

As a preliminary various substances, many of which are normally present in protein-free extracts of blood or sera, were added to a series of tubes each containing 0.01 mg. Cu. The amounts of these substances added are shown in Table II. To each of these tubes were added 2 ml.

Table II.

Substance	Amount used mg.	Substance	Amount used mg.
Glucose	5	Thiolacetic acid	5
Tyrosine	1	Urea	5
Alanine	5	Glycine	5
Glutathione	5	Potassium cyanide	10
Glutathione	10		

of 4 % sodium pyrophosphate and then ammonia until faintly alkaline to litmus. 5 ml. of amyl alcohol were added to each followed by 0.5 ml. of 2 % sodium diethyldithiocarbamate solution and the mixture well shaken. The only substance found to prevent the reaction was potassium cyanide. In all the other cases the same colour was produced as in the control. In the cases of thiolacetic acid and glutathione, on the addition of the reagent no colour was produced until the contents of the tubes were vigorously shaken when the amyl alcohol layers were found to match the control. This was probably due to the reduction of the copper to the cuprous condition by the free SH groups, this being reversed on shaking with air.

Trichloroacetic acid filtrates were prepared as follows. The trichloroacetic acid was distilled *in vacuo* and then made up to 20 % with distilled water.

1 volume of blood, plasma or serum was diluted with 3 volumes of distilled water and then 1 volume of 20 % trichloroacetic acid added. After shaking the mixture was filtered. To 20–25 ml. of filtrate were added 2 ml. of 4 % sodium pyrophosphate and the whole made faintly alkaline to litmus with ammonia. 5 ml. of amyl alcohol were then added followed by 0.5 ml. of diethyldithiocarbamate reagent and the whole vigorously shaken. A standard containing 0.01 mg. copper was similarly prepared.

Results obtained by this method were compared with those obtained after destruction of the organic matter by the method described earlier in this paper and are shown in Table III.

It will be seen that the whole of the copper is present in the trichloroacetic acid filtrate in such a form as to react directly with the reagent.

Table III. *Copper in mg. per 100 ml. whole blood or serum.*

	A. Complete destruction of organic matter	B. Direct determination on trichloroacetic acid filtrate
Whole blood:		
1	0.174	0.186
2	0.169	0.174
3	0.157	0.171
4	0.165	0.156
5	0.200	0.192
6	0.192	0.199
Serum:		
7	0.256	0.263
8	0.204	0.205
9	0.238	0.235
10	0.220	0.229
11	0.196	0.204

Further results shown in Table IV indicate that copper added to whole blood or serum could be recovered quantitatively.

Table IV. *Recovery of copper added to whole blood or serum.*

	Copper content of blood etc. mg. Cu per 100 ml.	Copper added mg. Cu per 100 ml.	Total copper determined mg. Cu per 100 ml.	Copper recovered mg. Cu per 100 ml.
Whole blood:				
1	0.156	0.143	0.289	0.133
2	0.156	0.286	0.435	0.279
3	0.156	0.429	0.683	0.427
4	0.223	0.143	0.378	0.155
5	0.223	0.286	0.500	0.277
Serum:				
1	0.245	0.143	0.378	0.133
2	0.245	0.286	0.526	0.281
3	0.217	0.143	0.351	0.134
4	0.217	0.143	0.356	0.139
5	0.217	0.286	0.490	0.273

The question naturally arises as to the distribution of copper between the corpuscles and plasma. Guillemet [1932] found much higher concentrations of copper in the plasma than in the whole blood of the ox, pig and dog. Schindel [1931] obtained higher values for the copper content of the plasma than the cells in man. Elvehjem *et al.* [1929] however obtained more copper in the corpuscles than the plasma in defibrinated horse blood. McHargue *et al.* [1928] in the case of one cow found the plasma to contain more copper than the corpuscles. This animal does not appear to have been normal. The values obtained by Elvehjem *et al.* and Guillemet are very much lower than those obtained by the writer, Schindel's figures however are of the same order.

For the study of the distribution of copper in the blood of man, serum from clotted blood and plasma from citrated blood were used. It was considered that the use of oxalate to prevent clotting might possibly cause a loss of copper from the plasma.

Table V. *Distribution of copper between plasma and corpuscles of human blood.*

	Whole blood mg. Cu per 100 ml.	Serum mg. Cu per 100 ml.		Whole blood mg. Cu per 100 ml.	Plasma from citrated blood mg. Cu per 100 ml.
1	0.184	0.197	9	0.225	0.238
2	0.225	0.245	10	0.227	0.227
3	0.174	0.183	11	0.208	0.227
4	0.192	0.209	12	0.238	0.227
5	0.195	0.197	13	0.220	0.236
6	0.210	0.217	14	0.207	0.220
7	0.238	0.205			
8	0.202	0.220			

From the results shown in Table V it will be seen that copper is more or less equally distributed between the cells and the plasma. In Table VI are shown the copper contents of the bloods of 22 normal males and 10 normal females. It will be seen that no apparent difference exists between the sexes—the values ranging from 0.185 to 0.229 mg. per 100 ml.

Four samples of cerebrospinal fluid were examined for copper. The copper content ranged from 0.032 to 0.067 mg. per 100 ml.

Table VI. *The copper content of normal human blood (expressed in mg. per 100 ml. blood).*

Male					Female				
1	0.187	9	0.198	16	0.194	1	0.208	6	0.198
2	0.197	10	0.222	17	0.186	2	0.228	7	0.196
3	0.220	11	0.189	18	0.218	3	0.198	8	0.214
4	0.229	12	0.219	19	0.208	4	0.216	9	0.196
5	0.220	13	0.194	20	0.194	5	0.196	10	0.216
6	0.202	14	0.192	21	0.216				
7	0.185	15	0.207	21	0.189				
8	0.194								

Owing to the widely different values reported for the copper contents of the blood and plasma (or serum) of different species, analyses of the whole blood and plasma (from citrated blood) from various animals were made. From the results shown in Table VII it will be seen that the values are of the same order as for the human being in the case of the ox, sheep, pig, horse and guinea-pig, while in the case of the rabbit they are slightly lower. McFarlane [1932], who analysed the blood of the ox and of the pig, obtained similar results to those of the writer.

Table VII. *The copper content of the whole blood and plasma of various species expressed in mg. per 100 ml.*

		Whole blood	Plasma
Sheep:	1	0.163	0.172
	2	0.167	0.156
	3	0.172	0.172
	4	0.180	0.183
	5	0.156	0.161
Ox:	1	0.223	0.208
	2	0.200	0.208
	3	0.190	0.180
	4	0.180	0.190
	5	0.192	0.192
Pig:	1	0.181	0.191
	2	0.165	0.161
	3	0.168	—
	4	0.185	0.200
	5	0.167	0.185
Horse:	1	0.179	0.167
	2	0.187	0.199
	3	0.208	0.208
	4	0.205	0.217
Rabbit:	1	0.143	—
	2	0.147	—
	3	0.144	—
	4	0.155	—
	5	0.139	—
Guinea-pig:	1	0.192	—
	2	0.186	—
	3	0.179	—
	4	0.184	—

DISCUSSION.

It has been shown that the diethyldithiocarbamate reagent offers a simple and reliable method for the determination of copper in blood. Since the whole of the copper is present in the filtrate after the precipitation of the proteins with trichloroacetic acid and is in such form that it may be determined directly—ashing is not necessary. It does not appear from these findings that any copper

complex analogous to haemoglobin exists in the blood. This is to be expected since even the haemocyanins are unstable. Roche [1930] has shown that it is possible to separate copper from haemocyanin by adjusting the solution to p_H 2.5 and then dialysing. Pirie [1931, 2], in a study of the catalytic oxidation of sulphhydryl compounds by iron and copper, found that although the haemocyanins were themselves catalytically inactive, they were decomposed at p_H 2.1, yielding catalytically active copper.

Pirie [1931, 1] has shown that the addition of copper sulphate to sulphhydryl compounds results in the formation of cuprous derivatives. It is to be expected therefore that part at least of the copper present inside the red blood cell will be in the cuprous condition owing to the presence of glutathione. This is also suggested by the fact that the full colour is not obtained with whole blood filtrates unless they are vigorously shaken. Owing to the absence of free SH groups from plasma the copper is present in the cupric form.

SUMMARY.

1. Normal human blood contains from 0.185 to 0.229 mg. Cu per 100 ml. which is more or less equally distributed between plasma and corpuscles.

2. The copper contents of sheep, ox, pig, horse and guinea-pig bloods are of the same order as that of human blood. Rabbit blood however shows slightly lower values.

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