LV. THE COPPER AND "INORGANIC" IRON CONTENTS OF HUMAN TISSUES.

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THE present paper is concerned with the concentration of copper and "inorganic" iron in various tissues and bones of the human body and also with suitable methods for their determination.

COPPER.

Several writers have determined the distribution of copper in human tissues. Herkel [1930] obtained values of 2·88–12·9 mg. Cu per kg. fresh liver and ranged in descending order of concentration his results may be summarised as follows: liver, kidney, spleen, pancreas and bone. Cunningham [1931] found that the average concentration of copper in three human livers was 24·9 mg. per kg. of dry tissue and ranged in descending order of concentration his results may be summarised as follows: liver, kidney, brain, pancreas and spleen. Others have published analyses of single organs, especially the liver, which agree more or less with the analyses of the above two writers [Schönheimer and Oshima, 1929; Kleinmann and Klinke, 1930; Cherbuliez and Ansbacher, 1930; Gordon and Rabinowitch, 1933].

The majority of distribution studies such as these must almost necessarily be done with pathological subjects. In certain pathological conditions of the liver in the adult, there appears to be a very marked increase in the concentration of copper. This occurs often in both pigmented and non-pigmented cirrhosis of the liver [Schönheimer and Oshima, 1929; Cherbuliez and Ansbacher, 1930; Kleinmann and Klinke, 1930; Herkel, 1930]. Gordon and Rabinowitch [1933] in one case of yellow atrophy of the liver obtained the extraordinarily high value of 179·3 mg. copper per kg. fresh tissue. Herkel [1930] states that in haemochromatosis the concentration of copper is increased in all the organs, with the exception of the kidney and bone. The copper content of foetal organs appears also to be higher than in adults [Kleinmann and Klinke, 1930; Cunningham, 1931; Sheldon and Ramage, 1931]. Sheldon and Ramage [1931] report that five foetal livers examined by them contained 5–7 times the concentration of copper present in adult organs. In a survey such as is intended here, it will be necessary then to exclude foetal organs and organs from cases of liver disease.

The question of bone seems to have been neglected. Of the above-mentioned writers only Herkel [1930] and Sheldon and Ramage [1931] appear to have examined this material. The former, as a result of two analyses, obtained values of 3·7 and 4·03 mg. per kg. fresh material. Sheldon and Ramage state that bone contains only a trace of copper.

The various colorimetric methods for the determination of copper in biological material have been discussed elsewhere [Tompsett, 1934, 2]. With the exception of Sheldon and Ramage, who used a spectrographic method, the above-mentioned analyses were obtained by modifications of the Biazzo method. The writer has found the method of Callan and Henderson [1929] as modified by

McFarlane [1932] to be the most sensitive and accurate. In this method sodium diethyldithiocarbamate is added to the solution under test, a yellow complex with copper is formed and this is extracted by shaking with amyl alcohol. The extract is compared in a colorimeter with a standard prepared similarly. This reaction, although independent of p_H, is carried out in alkaline solution in the presence of pyrophosphate to prevent interference by iron.

Methods.

Tissues. The following method is based on that used for blood and reported previously [Tompsett, 1934, 2].

A porcelain pestle and mortar and some broken glass were treated several times with hot dilute hydrochloric acid to render them copper-free. The tissue to be examined was cut up into small pieces and 10 g. ground up with the broken glass. About 40 ml. of 10 % trichloroacetic acid were added and the grinding continued. The supernatant fluid was filtered through an acid-washed filter-paper and the residue washed with 10 % trichloroacetic acid until the requisite volume of filtrate was obtained. Extracts of liver tissue were made up to 100 ml., while extracts of other organs were made up to 50 ml. The final estimation was made directly, using 20 ml. of extract with a standard containing 0·01 mg. copper as described for blood.

The results obtained by this method were compared with those obtained after ashing either with sulphuric and perchloric acids or by ignition in a silica basin. For the ignition method the tissues (10–20 g.) were mixed with 5 g. of copper-free sodium phosphate for reasons that have been stated in a previous paper in connection with the analyses of samples of diet [Tompsett, 1934, 4].

From the figures shown in Table I it will be seen that the copper contents of tissues as determined by the two methods are the same within the limits of experimental error, that is copper may be extracted completely from tissues by trichloroacetic acid and reacts directly with sodium diethyldithiocarbamate.

Table I. *Copper content of fresh tissue.*

(mg. Cu per 1000 g.)

Tissue		A Determined directly in trichloroacetic acid extract	B Determined after ashing tissue
Liver	1	22·24	22·80
	2	6·20	6·16
	3	5·69	5·74
	4	4·94	5·06
	5	5·26	5·10
Kidney	6	3·33	3·52
	7	3·63	3·45
	8	3·01	2·84
Brain	9	6·96	7·14
	10	2·27	2·20
	11	4·57	4·61
Spleen	12	1·40	1·38
	13	1·16	1·16
	14	2·27	2·18
Pancreas	15	4·00	3·79
	16	1·96	2·06
	17	2·22	2·20

Bone. Bone offers special difficulties in the determination of copper, owing to its high content of calcium phosphate. Since the final determination is carried out at alkaline reaction, precipitation of calcium phosphate would be so great as to interfere with the result. The same difficulties are met with in milk, owing to its relatively high concentration of calcium phosphate and low concentration of copper. In the case of faeces, urine and samples of diet, materials containing relatively much lower concentrations of calcium phosphate, copper may be determined directly in solutions of their ash by addition of sodium citrate to prevent precipitation of the phosphates [Tompsett, 1934, 4]. With bone or milk, however, there is no alternative but to make a preliminary separation of copper.

Many authors have separated copper as the sulphide but this is time-consuming and liable to lead to difficulties. The following method has been found to give accurate and rapid separations of copper.

The sample of bone (30–40 g.) was ashed in a silica basin. The final traces of carbon were destroyed by the addition of 10 ml. concentrated nitric acid and further heating. The ash was dissolved in distilled water containing 15 ml. of concentrated hydrochloric acid filtered through an acid-washed filter-paper and diluted to 250 ml. A sample of 50 ml. was measured into a 750 ml. separating funnel and diluted to about 400 ml. with distilled water. To the solution 100 ml. of 20 % sodium citrate were added and the whole made slightly alkaline to litmus by the addition of ammonia. After the addition of 5 ml. of 2 % aqueous sodium diethyldithiocarbamate, the yellow copper complex which was formed was extracted with ether. 25 ml. of ether were added and the whole vigorously shaken. A further 25 ml. of ether were added and the vigorous shaking repeated. The ether layer containing the copper complex separated very quickly. The aqueous layer was run off and the ether washed twice with about 50 ml. of distilled water. The ether extract was then run off into a 300 ml. Kjeldahl flask and the separating funnel rinsed with 25 ml. of ether which was transferred to the Kjeldahl flask. The aqueous layer and washings were re-extracted with 25 ml. ether which was washed and added to the other ether extracts. The ether was evaporated off on a steam-bath and the organic matter destroyed by heating with 1 ml. concentrated sulphuric acid and 1 ml. of perchloric acid. The contents of the flask were diluted to 25 ml. with distilled water and the copper content determined in an aliquot (about 5 ml.) using 0·01 mg. Cu as standard.

From the results shown in Table II it will be seen that copper added to solutions of bone ash could be recovered quantitatively by this technique.

Table II. *The recovery of copper added to an acid solution of bone ash.*

	Initial copper content mg.	Copper added mg.	Total copper content mg.	Copper recovered mg.
1	0·046	0·050	0·095	0·049
2	0·046	0·075	0·118	0·072
3	0·046	0·100	0·152	0·106
4	0·064	0·050	0·111	0·047
5	0·064	0·075	0·144	0·080
6	0·064	0·100	0·157	0·093

Table III gives the copper content of cows' milk as determined by the above procedure. No special precautions were taken in the collection of this milk which was unpasteurised and delivered in iron cans. These figures agree very closely with those obtained in America by Lindow *et al.* [1929] who obtained an average value of 0·15 mg. Cu per litre.

Table III. *The copper content of cows' milk.*

(mg. Cu per litre.)

1	0·11	3	0·14	5	0·11	7	0·10
2	0·13	4	0·12	6	0·16	8	0·14

Average—0·13.

"INORGANIC" IRON.

No figures for the "inorganic" iron content of human tissues appear to be available. Copper may be extracted from biological materials with trichloroacetic acid but "inorganic" iron is extracted either partially or not at all [Tompsett, 1934, 1, 3].

It has been shown that the whole of the iron of egg yolk is in the "inorganic" form, which confirms the earlier work of Hill [1931]. When a suspension of egg yolk was treated with trichloroacetic acid, no iron could be detected in the filtrate, but if, prior to the precipitation of the proteins, thiolacetic acid, sodium hydrosulphite or sodium pyrophosphate were added, the whole of the iron was present in the filtrate. This work was extended further when it was shown that this phenomenon observed with egg yolk was due to the presence of indiffusible phosphorus compounds, *e.g.* phospholipins and phosphoproteins. When solutions of iron salts were added to a suspension of lecithin or solutions of caseinogen or to milk and the protein or phospholipin precipitated with trichloroacetic acid, no iron could be detected in the filtrate, but if thiolacetic acid, sodium hydrosulphite or sodium pyrophosphate were added prior to the addition of trichloroacetic acid, the iron could be estimated quantitatively in the filtrates. On the other hand, when mixtures of ferric salts and either egg white or solutions of edestin were treated with trichloroacetic acid, the iron could be estimated quantitatively in the filtrates. It has been suggested that ferric but not ferrous iron forms complexes with these indiffusible phosphorus compounds, and that upon reduction of the ferric iron by sodium hydrosulphite or thiolacetic acid these complexes are destroyed. The complex also appears to be unstable in the presence of sodium pyrophosphate which is probably due to the property of iron of forming non-ionised compounds with pyrophosphates.

The iron was detected and determined with thiolacetic acid, which gives a purple colour with iron salts on the addition of ammonia. The reaction is very delicate, is quantitative and is not affected by the presence of organic substances such as may be present in filtrates like the above. Both ferric and ferrous salts give the reaction since the former are reduced to the ferrous state by thiolacetic acid.

In his investigations Hill [1931] used $\alpha\alpha'$-dipyridyl. This substance gives a red colour with ferrous salts and no reaction with ferric salts. He found that when egg yolk was suspended in an acid acetate buffer and $\alpha\alpha'$-dipyridyl added, no colour developed, whereas when a reducing substance such as sodium hydrosulphite was added, a red colour developed. When this colour was compared with standards, he noted that it accounted for all the iron of the yolk.

McFarlane [1934] has studied the iron of the rat's liver. He found that trichloroacetic acid extracts gave only faint reactions for iron with the thiocyanate reagent, whereas the "inorganic" iron as determined by Hill's $\alpha\alpha'$-dipyridyl method was very much higher.

Method.

For the complete extraction of "inorganic" iron by trichloroacetic acid, the complexes must be broken down first. For this type of extraction it was considered that sodium pyrophosphate would answer this purpose better than a reducing agent such as thiolacetic acid or sodium hydrosulphite. The extraction and estimation were carried out as follows:

Some broken glass and 10 g. of finely cut tissue were ground up in a porcelain mortar. 20 ml. of 4 % sodium pyrophosphate were added and the grinding continued. The mixture was allowed to stand 15 minutes after which 20 ml. of 20 % trichloroacetic acid were added and the mixture ground. After a further 15 minutes the mixture was filtered through an acid-washed filter-paper and the residue washed with 10 % trichloroacetic acid until the volume of the extract was either 100 ml. (liver and spleen) or 50 ml. (other tissues). An aliquot portion of the filtrate, containing no more than 0·03 mg. Fe, was diluted to 5 ml. with distilled water and 6 drops of thiolacetic acid were added, followed by 1 ml. of ammonia (sp. gr. 0·88). This was compared in a colorimeter with a standard similarly prepared and containing 0·005, 0·010 or 0·020 mg. Fe.

If copper is being estimated in the same tissue as "inorganic" iron, it may be determined in the extract prepared for the determination of "inorganic" iron, so that only one extraction is necessary.

Table IV.

A. *Copper.*

The figures are expressed in mg. Cu per kg. fresh tissue (1) and in mg. Cu per organ (2).

	Liver		Kidney		Brain		Spleen		Pancreas	Vertebra	Rib
	(1)	(2)	(1)	(2)	(1)	(2)	(1)	(2)	(1)	(1)	(1)
1	3·09	3·40	2·27	0·54	—	—	—	—	2·10	2·83	6·40
2	9·09	12·99	2·36	0·83	2·27	3·18	—	—	1·96	1·63	32·06
3	6·59	15·82	3·82	1·91	4·57	6·40	—	—	2·20	2·84	8·95
4	5·43	8·14	3·57	1·25	3·16	4·42	—	—	—	2·13	47·70
5	4·86	8·75	2·56	0·82	—	—	1·16	0·20	2·46	4·88	10·21
6	3·74	5·24	2·12	0·44	—	—	2·27	0·36	2·36	3·40	3·71
7	2·96	3·26	—	—	2·16	2·81	1·96	0·28	2·06	1·81	4·02
8	5·46	9·83	2·91	1·02	3·96	5·44	2·04	0·28	2·22	2·96	9·81
9	7·94	11·81	3·42	1·02	4·84	6·54	1·96	0·20	2·86	3·04	21·62
10	3·16	5·21	2·16	0·69	2·22	2·84	1·84	0·20	2·54	2·91	14·61
11	4·44	6·04	2·84	1·11	3·04	3·65	2·41	0·28	2·16	1·84	9·45
12	6·12	11·97	3·01	0·84	3·24	3·56	1·92	0·23	2·04	4·16	8·61

B. *"Inorganic" iron.*

The figures are expressed in mg. Fe per kg. fresh tissue (1) and in mg. Fe per organ (2).

	Liver		Kidney		Brain		Spleen		Pancreas	Vertebra	Rib
	(1)	(2)	(1)	(2)	(1)	(2)	(1)	(2)	(1)	(1)	(1)
1	162·4	178·6	10·1	2·4	—	—	93·6	9·4	18·2	128·6	103·4
2	36·6	51·2	9·6	3·4	6·2	8·7	—	—	10·8	123·1	147·6
3	69·2	166·0	3·3	1·6	14·8	20·7	—	—	7·0	142·9	119·5
4	50·6	75·9	9·2	3·0	7·6	10·6	—	—	—	134·0	111·8
5	80·7	144·3	5·1	1·6	4·0	5·2	169·4	29·6	4·2	133·2	114·6
6	27·7	38·8	7·4	1·6	—	—	100·3	10·0	13·4	167·4	161·4
7	64·6	71·1	7·9	1·5	8·6	11·2	96·4	14·5	8·4	146·1	151·8
8	71·4	128·5	4·6	1·4	11·1	15·5	164·6	23·0	6·7	126·0	111·0
9	45·6	68·4	5·4	1·6	14·6	19·7	84·5	8·9	8·4	111·6	109·2
10	84·2	138·9	8·4	2·7	8·4	10·8	126·3	13·9	9·3	136·4	146·1
11	91·6	124·6	7·6	3·0	11·2	13·4	116·4	15·1	8·4	154·3	138·4
12	39·4	76·4	6·9	1·9	6·6	7·3	84·6	10·2	7·1	121·4	109·6

The copper and "inorganic" iron contents of tissues.

In Table IV are shown the concentrations of copper and "inorganic" iron in the tissues of 12 cases. These cases had no organic disease of the liver. The bones examined were the rib and the vertebra. Figures are given for the iron content of these bones. These were determined in the solutions of their ashes by the thiolacetic acid method as used for the tissue extracts. Although these figures cannot be classed as "inorganic" iron yet it is more than likely that the greater part of the iron in bones is in the inorganic form. A precipitate of calcium phosphate always appears during the estimation of iron in bone but this may be removed by centrifuging without interfering with the results.

Discussion.

In a previous paper [1934, 2] the writer has reported that when the proteins of blood are precipitated with trichloroacetic acid, the whole of the copper is present in the filtrate and reacts directly with sodium diethyldithiocarbamate. It has been shown in this paper that the copper of tissues such as liver, kidney, brain, pancreas and spleen may be completely extracted with trichloroacetic acid and the copper may be determined directly in the extracts with sodium diethyldithiocarbamate in the same way as blood. During the progress of this work McFarlane [1934] published a paper on similar lines. He extracted rat livers with trichloroacetic acid, found that these extracts gave a direct reaction for copper with sodium diethyldithiocarbamate and that direct estimations using these extracts agreed with those obtained after ashing.

A method has been described for the extraction of copper from solutions of bone and milk ash by means of sodium diethyldithiocarbamate and ether. This extraction is independent of p_H so that it is as efficient in acid as in alkaline solution. It is carried out in alkaline solution in the presence of pyrophosphate to prevent the extraction of iron. If the extraction be carried out as soon as the solution of the ash is obtained it is not necessary to add pyrophosphate since sufficient of this substance is formed during the ignition process. Since the reagent itself is insoluble in ether, the ethereal extracts contain only minimum amounts of organic material. Chloroform was tried as a solvent but it was found that the solubility of the copper complex in this solvent is very poor, nor does chloroform separate very easily.

The values obtained for the copper content of various tissues with the exception of bone agree with those obtained by other workers. Vertebra contains a very low concentration of copper which is fairly constant throughout the series. Rib on the other hand appears to contain very varied amounts of copper, exceeding that in the liver in many cases even when differences of water concentration are taken into consideration. It appears possible that the bones may act as stores for copper as well as the liver.

As would be expected, liver and spleen contain the highest concentrations of "inorganic" iron. Tissues such as brain, kidney or pancreas contain on an average less than a tenth of the concentration of "inorganic" iron of the liver or spleen. The values obtained for the spleen are more constant than those for the liver but from the size of the latter organ it must be the principal store of "inorganic" iron. Vertebra and rib contain fairly high concentrations of iron and, in contrast to copper, the concentrations are almost identical in the two types of bone.

The term "inorganic" iron is probably incorrect since this iron will be in some form of organic combination. It may be justified since ferric salts added

to egg yolk *etc.* react in the same way and require the same methods for their separation.

All tissues such as have been examined contain considerable amounts of haematin iron. It would appear, however, that the whole or almost the whole of the iron present in plasma is in the "inorganic" form. The writer [1934, 1] has estimated the "inorganic" iron content of normal sera and obtained values of 0·12–0·22 mg. per 100 ml. Fowweather [1934] in a recent paper has estimated the total iron of plasma, obtaining values of 0·06–0·18 mg. per 100 ml. He noted that precipitation of the proteins with trichloroacetic acid removed a considerable portion of the iron.

SUMMARY.

1. Copper may be determined directly with sodium diethyldithiocarbamate in trichloroacetic extracts of liver, kidney, spleen, brain and pancreas.

2. A method has been described for the determination of copper in bone and milk.

3. The copper and "inorganic" iron contents of a series of tissues including bone have been determined.

4. The values obtained for the copper contents of liver, spleen, kidney, pancreas and brain agree with those obtained by other writers. Vertebra contains very low concentrations of copper while the concentrations in rib are extremely variable.

5. Liver and spleen contain high concentrations of "inorganic" iron whereas kidney, brain and pancreas contain very low concentrations.

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