XL. THE IRON AND COPPER CONTENTS AND THE HAEMOPOIETIC ACTIVITIES OF STOMACH AND LIVER PREPARATIONS.

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THE modern treatment of pernicious anaemia with preparations of liver or hog's stomach has led to much speculation as to the precise cause of the success of the treatment, and many laborious fractionation experiments have been carried out with the object of isolating and studying the properties of the active anti-anaemic substances presumed to be present in these organs. These active principles have not yet been isolated in pure form; only concentrated preparations or fractions are known whose haemopoietic activity can be determined solely by the clinical method of observing their effects in cases of pernicious anaemia.

The investigations of Cohn *et al.* [1928; 1930] suggest that the active principle in liver is a nitrogenous base, possibly an ω-amino- or imino-acid. Further, the work of two of us [Wilkinson and Klein, 1932; 1933; 1934; Klein and Wilkinson, 1933; 1934] has shown that the anti-anaemic factor in hog's stomach, haemopoietin, is not identical with the liver principle but is probably an enzyme, which, reacting with a substrate present in the normal diet, produces a substance which from its chemical and therapeutic properties appears to be identical with the so-called active liver principle; this liver principle is utilised for the formation of red blood cells by some mechanism at present unknown.

The liver therapy had its origin in the classical experiments of Whipple *et al.* [1920], who, selecting liver owing to its relatively high iron content, showed that it was the most potent foodstuff in causing blood regeneration in dogs which had been kept at a constant low haemoglobin level by bleeding. This work on post-haemorrhagic anaemia induced Minot and Murphy [1926] to try the effect of liver feeding on cases of pernicious anaemia. The success they had with this diet was later paralleled by the even more striking results that followed the treatment of pernicious anaemia with preparations of hog's stomach [Wilkinson, 1930; 1931].

The preparations of liver and stomach used in the treatment of pernicious anaemia contain small amounts of iron and copper and it has been suggested in many quarters [*cf.* for example Schultze, 1932] that they owe their value to the presence of these mineral constituents. Iron, of course, is known to be essential for haemoglobin formation and has been used with great success in the treatment of the hypochromic microcytic anaemias.

The possibility that copper may also be an active blood-regenerating agent or may stimulate haemoglobin formation has been suggested by many workers [*cf.* McHargue *et al.*, 1928; Flinn and Inouye, 1929; Hart *et al.*, 1928; Waddell *et al.*, 1929, 1, 2; Elvehjem, 1932]. According to Warburg and Krebs [1927],

Schönheimer and Oshima [1929], Guillemet [1931], McFarlane [1932], Locke *et al.* [1932] and Tompsett [1934, 1], human blood contains small amounts of copper, the amount being of the order of 0·1–0·2 mg. Cu per 100 ml. McHargue [1925] suggests that it may also perform important functions in the absorption and transfer of oxygen in the respiration of mammals. Indeed, copper is an essential constituent of haemocyanin, the respiratory pigment of certain molluscs and crustacea. Beard and Myers [1928], working on nutritional anaemia of rats, found that traces of copper added to iron brought about regeneration of haemoglobin in less time than the same dose of iron alone. Mills [1930], among others, reports the successful use of copper and iron in idiopathic anaemia of adults.

The experimental evidence so far accumulated appears to suggest that iron and copper may play an important part in certain forms of anaemia, though whether they do so in pernicious anaemia is open to question. The object of the present work was to determine whether the quantities of iron and copper present in various liver and stomach preparations and fractions used in the treatment of pernicious anaemia could be correlated with the haemopoietic activities of the fractions as judged by clinical trial on cases of this disease.

Experimental.

In view of the relatively small quantities of iron and copper present in the preparations used, it was decided to employ colorimetric methods of estimation using a Klett colorimeter, and throughout the work, distilled water and analytical reagents shown to be free from iron and copper were used. In view of the fact that there appears to be some vagueness in the literature as to the exact procedure in these methods, it has been thought advisable to describe our experimental technique at some length.

Ashing of preparations. The following dry ashing method was found to be the most satisfactory. Porcelain crucibles (2 in. in diameter) of the best Berlin make were soaked in alcoholic sodium acetate and then evaporated to dryness several times with concentrated hydrochloric acid in order to remove any iron and copper from the glaze [*cf.* Elvehjem and Lindow, 1929]. In the case of stomach preparations, 5 g. samples were generally used, but, owing to scarcity of material, 1 g. or even less of some of the liver fractions was used. The crucible was heated over a very low flame until visible charring commenced, when concentrated sulphuric acid (about 10–15 drops per 5 g. sample) was carefully added. Heat was then gently applied until a carbonaceous mass was obtained. A further similar quantity of acid was then added and heating continued for approximately an hour, when ashing was usually complete. If this were not so, the crucible was allowed partially to cool, one drop of acid added to any portion where carbonaceous material remained and the crucible reheated for a short period.

Extraction of ash. This was carried out by heating for a short time almost to boiling point with concentrated hydrochloric acid (5–10 ml.) over a microburner. The acid solution was then measured into a volumetric flask (50 ml. for a 5 g. sample, 20 ml. for smaller samples) and further extractions were made with similar quantities of water. The solution was made up to volume after cooling, filtered through a sintered glass filter and was then ready for the iron and copper estimations.

Preparation of standard iron and copper solutions. Iron standards were prepared as follows:

(*a*) Ferric alum A.R. (0·8630 g.) was dissolved in 50 ml. water, 20 ml. of 10 % hydrochloric acid were added to prevent hydrolysis and the solution was then

diluted to 1 litre (1 ml. = 0·1 mg. iron). The purity of the salt was confirmed by gravimetric analysis.

(*b*) Pure iron wire (0·0639 g.) was dissolved in 50 ml. of 10 % hydrochloric acid, oxidised with 5 ml. hydrogen peroxide (20 vols.) and the solution boiled and made up to 500 ml. This solution should contain 0·128 mg. iron per ml., and colorimetric estimation by the thiocyanate method (following the details given below), taking the ferric alum solution as standard, gave a value of 1 ml. = 0·129 mg. iron.

The standard copper solution contained 0·3928 g. copper sulphate A.R. ($CuSO_4$, $5H_2O$) per litre (1 ml. = 0·1 mg. copper).

Determination of iron by the thiocyanate method. The intense wine-red coloration produced by the action of thiocyanates on ferric salts (sensitivity 1 in 1,000,000) has been the basis of the common methods in use for the determination of iron in biological material. Many workers recommend extraction of the colouring matter with amyl alcohol before colorimetric comparison [*cf.* Kennedy, 1927; Elvehjem, Kemmerer *et al.*, 1929; McFarlane, 1932], whilst others consider this procedure to be unnecessary [*cf.* Brown, 1922; Wong, 1923; Rees, 1933]. We find, however, that very poor colour matches are obtained if readings are made directly without extraction. On the other hand, with the extraction method, it is sometimes necessary to dry the amyl alcoholic extracts with sodium sulphate owing to their turbidity.

An accurately measured aliquot portion (usually 5 or 10 ml.) of the unknown solution was measured into a 100 ml. cylindrical separating funnel, the iron oxidised to the ferric condition by addition of 2 drops of concentrated nitric acid and water added from a burette to a volume of 20 ml. This was followed by an exact volume of amyl alcohol (usually 20 ml. but varying according to the colour developed) and 5 ml. of 20 % potassium thiocyanate solution. Extraction was then carried out immediately, the amyl alcoholic extract dried if necessary, by filtration through a thin layer of anhydrous sodium sulphate (about 1 g.) on a sintered glass filter, and the depth of colour compared with that of a standard iron solution freshly made up in an identical manner.

The minimum concentration of iron which gave a sufficiently intense colour for convenient reading (standard at 20 mm. depth) on the colorimeter was $2{\cdot}5 \times 10^{-6}$ mg. iron per ml. amyl alcohol solution (*i.e.* $\equiv 0{\cdot}5$ ml. of standard extracted with 20 ml. amyl alcohol).

Determination of iron by the thiolacetic acid method. Lyons [1927] has shown that ferric or ferrous salts give a purple coloration with thiolacetic acid (sensitivity 1 in 5,000,000) which can be utilised for the quantitative estimation of iron, and Hanzal [1933], Burmester [1934] and Tompsett [1934, 2] have applied the test to the determination of iron in biological material. The main objections to the use of this reaction as a basis for a colorimetric method of analysis are (*a*) the instability of the coloration which fades in the course of a few hours, owing apparently to the presence of copper, and (*b*) the formation of a fine precipitate of alkaline earth hydroxides and phosphates in the ammoniacal solution which makes readings difficult. We find, however, that when the solutions are allowed to stand for 15 minutes and then filtered through a sintered glass filter, the results are in good agreement with those obtained by the thiocyanate method.

An aliquot portion of the unknown solution was diluted with water to 20 ml., 1 drop of thiolacetic acid added, followed by 1 ml. of ammonia (sp. gr. 0·88). It was occasionally necessary to add 2–3 ml. of ammonia when the aliquot portion of the unknown solution was large. The depth of colour was then compared with that of 1 ml. of the standard iron solution treated similarly.

The minimum concentration of iron which gave a sufficiently intense colour for convenient reading (standard at 30 mm.) on the colorimeter was $4{\cdot}0 \times 10^{-6}$ mg. iron per ml. solution (*i.e.* $\equiv$ 1 ml. of standard solution made up to a total volume of 25 ml.).

Estimation of copper in the presence of iron. The most delicate colour test for copper is the reaction with sodium diethyldithiocarbamate [Callan and Henderson, 1929], which gives a golden-yellow coloration with dilute solutions of copper salts (sensitivity 1 in 100,000,000). Since iron also gives a coloration with this reagent, a modification of the original method first suggested by Haddock and Evers [1932] has been used. It was found, however, that carbon tetrachloride as used by the latter authors was unsatisfactory as an extractant since several extractions were necessary for the complete removal of the colour developed and, moreover, the solutions in this solvent were not very stable. The use of amyl alcohol, introduced by McFarlane [1932] in place of carbon tetrachloride, had none of these objections and has recently been recommended by Tompsett [1934, 1].

The unknown solution (25 ml. or an aliquot portion made up to 25 ml. with water) was introduced into a 100 ml. cylindrical separating funnel and 2 drops of concentrated nitric acid were added. This was followed by 1 g. of citric acid and, after solution had taken place, 4 ml. of ammonia (sp. gr. 0·88) were added, giving a p_H greater than 9·0. The total volume was then made up to 40 ml. with water from a burette. A known volume of amyl alcohol (usually 20 ml.) followed by 5 ml. of a 0·1 % solution of sodium diethyldithiocarbamate was now added and the whole vigorously shaken for a short time. After separation had taken place, the aqueous layer was removed. It was very occasionally found necessary to dry the amyl alcohol layer as in the iron estimation described above. Comparison was then made with a standard copper solution (0·5 ml. or 1 ml.) treated in the same way as the unknown.

In many cases where the quantity of available material was small (0·35–0·5 g.), the hydrochloric acid solution of the ash was made up to 25 ml., and 20 ml. were subsequently extracted with 10 ml. of amyl alcohol.

The minimum concentration of copper giving a sufficiently intense colour for convenient reading (standard at 30 mm.) was $2{\cdot}5 \times 10^{-6}$ mg. copper per ml. of amyl alcohol solution (*i.e.* $\equiv$ 0·5 ml. of standard extracted with 20 ml. amyl alcohol).

Discussion of results.

The results of our determinations of iron and copper in various products derived from hog's stomach and from various livers are shown in Tables I–III. Estimations were carried out in duplicate except in the cases of one or two human liver fractions where the amount available did not permit of duplicate ashing.

All the preparations have been tested for haemopoietic activity on specially controlled cases of pernicious anaemia, the stomach and ox liver preparations being administered orally and the human liver fractions by intramuscular injection. Haemopoietically active products produced a "peak" in the number of reticulocytes in the circulating blood in the course of a few days, followed by a fall to normal values, and this was accompanied by an increase in the number of red blood cells and in the percentage of haemoglobin [*cf.* Wilkinson, 1932; 1933]. The clinical material used for this purpose conformed to certain rigid criteria already laid down by one of us, and the clinical details have already been published elsewhere [Wilkinson, 1932; Klein and Wilkinson, 1933; 1934; Wilkinson and Klein, 1934].

In considering the determinations on the desiccated stomach preparations (Table I) it will be noted that the copper content was very small compared with the amount of iron present and showed quite a reasonable constancy, varying only between 1·3 and 2·0 mg. per 100 g. The iron content of these products was very much higher and, moreover, showed variations between wider limits. One of the preparations (A) gave a much higher figure for iron than the others. The data presented appear to show conclusively that there is but little difference in the metallic contents of the haemopoietically active and inactive preparations, the inactive preparation F, for example, containing practically the same amount of iron and copper as the active preparation H. The haemopoietic inactivity of certain desiccated stomach preparations is due then not to any deficiency or excess of iron or copper but to the methods of treatment during their manufacture, which causes inactivation of the sensitive gastric enzyme, haemopoietin [Wilkinson, 1932]; this is further manifest from the fact that gentle heating destroys haemopoietin but has no influence on the iron and copper contents of the preparations.

Table I.

Preparation	Haemopoietic activity	Mean values mg. per 100 g. preparation: Iron	Copper
Commercial desiccated hog's stomach			
A	Active	33·2	1·7
B	Inactive	17·5	1·7
C	Active	10·5	1·8
D	Active	12·4	1·9
E	Active	16·4	1·3
F	Inactive	11·5	1·2
G	Inactive	8·5	2·0
H	Active	11·5	1·5
Fractions from hog's stomach press juice			
Fraction P5	Active	95·8	3·3
Fraction P5 (i)	Inactive	97·3	5·9
Fraction P5 (ii)	Active	33·2	7·0

Table I also shows the iron and copper contents of three stomach fractions prepared by two of us from the expressed juice of hog's stomach [Wilkinson and Klein, 1932; Klein and Wilkinson, 1933].

Fraction P5 was obtained by precipitating the press juice directly with alcohol. Fraction P5 (i) represented the precipitate rich in pepsin obtained by dissolving fraction P5 in $N/10$ hydrochloric acid and bringing to p_H 4·2 by addition of alkali. Fraction P5 (ii) was the pepsin-free precipitate obtained by adding alcohol to the filtrate from the preparation of fraction P5 (i).

Two of these fractions (P5 and P5 (i)) had very high iron contents, though one was active and the other was not. Moreover, the active fraction P5 (ii) had only one-third of the amount of iron present in the inactive fraction P5 (i), which provides further evidence of lack of any relationship between haemopoietic activity and metallic content. In point of fact, most of the iron present in these fractions was probably derived from the metal press used in the preparation of the press juice.

Table II gives the results obtained with an ox liver extract and an extract prepared by digesting ox liver with hog's stomach—both preparations were for oral use and were clinically very active, the latter preparation being more active than the former. In one of these fractions, the quantity of copper was in

Table II. *Preparations from ox liver and hog's stomach.*

Preparation	Haemopoietic activity	Mean values mg. per 100 g. preparation	
		Iron	Copper
Liver extract S.P.I. (7·8 g. ≡ ½ lb. liver)	Active	23·6	43·9
Liver—hog's stomach L.S.I. (6·4 g. ≡ ½ lb. liver)	Active	12·3	9·6

excess of the iron content whereas in the other the converse held. Here again there appears to be no evidence of any parallelism between the haemopoietic activities and the contents of iron and copper.

The results obtained with liver fractions prepared for intramuscular injection from various normal and abnormal human livers as described elsewhere [Wilkinson and Klein, 1934] are shown in Table III.

Table III. *Fractions from human livers.*

Clinical source of liver	Fraction	Haemopoietic activity	Mean values mg. per 100 g. fraction	
			Iron	Copper
Untreated pernicious anaemia	L.I. 3	Inactive	<1·0	8·6
	L.I. 19	Inactive	4·3	9·1
Partially treated pernicious anaemia	L.I. 1	Slight	<1·0	15·7
Remitting pernicious anaemia	L.I. 4	Active	—	4·3
	L.I. 13	Active	4·9	15·7
Normal	L.I. 7	Active	4·0	2·9
	L.I. 12	Active	18·9	5·1
Haemolytic anaemia	L.I. 22	Active	5·8	11·3
Polycythaemia rubra	L.I. 14	Very active	<1·0	7·9

It will be seen that there is a very wide variation in the copper and iron contents of the different liver fractions and that no correlation exists between their clinical potencies and contents of these metals.

Thus, in the haemopoietically active fractions iron varied from <1·0 to 18·9 mg. while the copper was between 2·9 and 15·7 mg.; the inactive or slightly active ones had ranges of <1·0 to 4·3 mg. for iron and 8·6 to 15·7 mg. for copper.

It is clear, therefore, that the haemopoietic activities of these liver fractions are quite independent of the iron and copper they contain, but it should be appreciated that these values do not represent the metallic contents of the original fresh tissues, which values do not concern the main objects of this paper.

In passing it may be noted, however, that Meyer and Eggert [1932] found that only part of the iron and copper in livers from various mammals could be extracted with water. These metals tended to accumulate in the fraction which was precipitated by 67 % alcohol, a fraction which Whipple *et al.* [1930] found effective in microcytic hypochromic anaemias. On the other hand, the fraction subsequently obtained by precipitation in 95 % alcoholic solution (corresponding with the fraction G shown by Cohn *et al.* [1928] to be effective in pernicious anaemia) contained less copper and very little iron. These authors concluded that the substance in liver effective in pernicious anaemia has no relation to the iron and copper content of the liver. Moreover, Cohn *et al.* [1930] state that they have succeeded in preparing from liver a fraction containing no iron which is very effective in the treatment of pernicious anaemia.

The evidence, then, provided by our own data and those of these other workers goes to show that the iron and copper contents of the preparations of liver and stomach used in the treatment of pernicious anaemia bear no relationship to their haemopoietic potencies, and there is no support for the view that the anti-(pernicious) anaemic factors owe their activities to the presence or absence of these metals. Further, the total amounts of iron and copper in these preparations are infinitesimal in comparison with the quantities ingested in the ordinary diet and, indeed, in comparison with the normal therapeutic dose used in the treatment of other anaemias.

SUMMARY.

1. Determinations have been made of the quantities of iron and copper in various clinically tested preparations of stomach and liver used in the treatment of pernicious anaemia.

2. Iron was determined colorimetrically by slight modifications of existing methods involving the use of potassium thiocyanate and thiolacetic acid respectively.

3. Copper was estimated colorimetrically by a modification of the sodium diethyldithiocarbamate method.

4. The results do not indicate any obvious relationship between the iron and copper contents and the haemopoietic potencies as judged by clinical trial on cases of pernicious anaemia.

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