Studies on the Biochemistry of Lead, Iron and Copper

A Thesis presented by,

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Preface

The work that has been described in this thesis has been done during the period 1934 until the present time. All of the estimations have been done by the writer himself. The animals were under the sole charge of the writer who possesses an Animal License, Certificates A and B. Much of the work has been published in the form of papers, a list of which follows. Reprints of those which are available are enclosed under separate cover.

1. 'Thiolacetic acid as a reagent for the determination of the inorganic iron content of certain biological materials'
   Biochemical Journal (1934) 28, 1536 - 1543
2. 'The copper content of blood'
   Biochemical Journal (1934) 28, 1544 - 1549
3. 'Studies of the complexes of iron with various biological materials'
   Biochemical Journal (1934) 28, 1802 - 1804
4. 'The excretion of copper in urine and faeces and its relati to the copper content of the diet'
   Biochemical Journal (1934) 28, 2088 - 2091
5. 'The copper and 'inorganic' iron contents of human tissues'
   Biochemical Journal (1935) 29, 480 - 486
6. 'The copper content of the blood in pregnancy'
   British Journal of Experimental Pathology (1935) 16, 67
   (with D. F. Anderson)
7. 'The lead content of human tissues and excreta'
   Biochemical Journal (1935) 29, 1851 - 1864
   (with A. B. Anderson)

8. 'The distribution of lead in human bones'
Biochemical Journal (1936) 30, 345 - 346

9. 'Lead poisoning'
Lancet (1939) 1, 559
(with A. B. Anderson)

10. 'The determination of lead in biological materials'
Biochemical Journal (1939) 33, 1231 - 1236

11. 'The influence of certain constituents of the diet upon the absorption of lead from the alimentary tract'
Biochemical Journal (1939) 33, 1237 - 1240

12. 'Studies in lead mobilisation'
British Journal of Experimental Pathology (1939) 20, 408 - 416
(with J. N. M. Chalmers)

13. 'Further studies on the absorption, mobilisation and excretion of lead'
British Journal of Experimental Pathology (1939) 20, 512 - 516

14. 'A study of factors influencing the absorption of iron and copper from the alimentary tract'
Biochemical Journal (1940) in preparation

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LEAD

Introduction

Lead is one of the commonest elements in the earth's crust. Lead and its salts are of biological importance because of their toxicity.

Lead salts precipitate proteins and act as local astringents. Acute toxicity is generally low but cumulative effects tend to be produced. Acute lead poisoning is rare and is usually produced by the acetate. Chronic lead poisoning is commoner and possesses characteristic symptons. These are as follows:

1. lead line at the margin of the gums,
2. anaemia,
3. paroxysmal colic and constipation,
4. 'drop' wrist and other peripheral motor paralyses,
5. tremors and convulsions,
6. psychic changes (encephalopathia etc.)

There are three possible modes of entrance of lead into the body; 1. alimentary tract, 2. respiratory tract, 3. skin. Absorption of lead from the alimentary tract has been recognised from time immemorial. Absorption of lead from the respiratory tract would result from the inhalation of dust and vapour containing lead or its compounds. This mode of absorption has been held in dispute but Aub et alia (1926) have shown that this does occur. It is debatable whether or not lead or its compounds are absorbed through the intact skin, but it is agreed that absorption can occur through the broken skin. Agreement

also exists that lead tetra-ethyl, a constituent of 'anti-knock' petrols, can be absorbed through the intact skin but this is due to its fat soluble properties.

The toxic effects of lead have been recognised from time immemorial. Hippocrates, about 370 B. C., recognised lead as being responsible for attacks of colic in a man who was described as an extractor of metals. Nicardo, who lived in the 2nd century B. C., noted the relationship between lead and constipation, abdominal pain and pallor and mentioned lead palsy for the first time. Discoides is quoted by Alderson (1852) as saying that 'the drinking of litharge causes oppression to the stomach, belly and intestines, with intense wringing pains, sometimes it even wounds the intestines by its pressure, it suppresses the urine, while the body swells and acquires an unsightedly blue hue'. He also mentioned paralysis and delirium as being produced by lead. About 1000 A. D., Avicenna recommended the use of violent purgatives to relieve a form of colic which was probably due to lead. Creato, who lived about 1600 A. D., attributed the colic which was prevalent in Moravia at that time and was known to end in paralysis to the use of 'falsified wine'. A similar conclusion was reached by Citois, a physician of Poitiers, regarding the colic which was prevalent in his district. It appears to have been the practice on the Continent at this period to treat sour wine with litharge or red lead in order to remove the acidity and so make it palatable. In 1656 Stockhusen

published a treatise in which he recognised that lead poisoning could result from the inhalation of dust containing lead. In 1745 Hexham described a condition appearing in Devonshire which had symptons similar to those of lead colic. Later this was shown by Sir John Baker (1767) to be lead coli and was due to the lead utensils used in the preparation and the storage of cider. A similar type of colic of simila aetiology was described by Luzuricaga in Spain in 1796. At the beginning of the 19th century several brilliant clinical descriptions of lead poisoning appeared. Grisolle (1836, 18 described lead colic, line and encepholopathy very accurately. Three years later, Tanquerel des Planches published his epoch making book in which he described the clinical aspects of the disease. He laid particular emphasi on the lead line, this becoming known as the Burtonian line, because of the description published by Burton in 1840. Sinc the time of Tanquerel, little appears to have been added to t clinical knowledge of the disease except perhaps by Déjérine-Klumpfe (1889) who wrote an excellent treatise on lead palsies. Experimental studies on lead poisoning appear to have been started by Orfila in 1814.

An important clinical sign in lead poisoning is the lead line. Typically it occurs in the gingiva near the border of the teeth, usually near decaying teeth, either in the gum or in the mucosa of the lip or cheek opposite these. Rapid caries seems to develop whenever a lead line appears near teeth apparently good. The true lead line is situated

within the tissues and is peculiar to lead poisoning except possibly in bismuth poisoning. Microscopical examination shows that the line is composed of irregular amorphous granul which are entirely sub-epithelial. It is presumed that it i produced by the interaction of dissolved lead and hydrogen sulphide to form insoluble lead sulphide. In experimental work on animals, Aub et alia (1926) found that lead line coul be produced in carnivorous animals e.g. cat, but not in herbivorous animals e.g. rabbit. They believed that the lead line is produced from hydrogen sulphide derived from the putrefaction of food debris left in the mouth. In suppo of this theory it has been shown that the incidence of lead line is related to dental hygeine. Of cases of suspected lead poisoning admitted to the Glasgow Royal Infirmary within recent years, often no lead line could be found even when the diagnosis was confirmed. This appears to be due to the fact that on examination, the patients were found to be edentulous. The extraction of remaining teeth appears to be often advised as part of the treatment of early symptons.

Extensive clinical and pathological studies have been made of lead palsy, colic and encephalopathy, and of the relationship of lead to constipation. These have been reviewed by Aub et alia (1926) in their monograph. Lead colic is generally believed to be produced by the action of lead on the smooth muscle of the intestine.

Encephalopathy, the most severe of the toxic episodes of lead poisoning is now fortunately rare owing to more

stringent public health measures. The pathology of these conditions, in spite of the extensive work done, is still in a debatable state.

Chronic lead poisoning is always accompanied by an anaemia. A marked feature of the blood picture is the large number of stippled cells present. Although stippled cells are not specific to lead poisoning but appear in other conditions, they are most constant in lead poisoning. Blood examinations for haemoglobin content and for the presence of stippled cells form part of the usual routine clinical examination of lead workers. In the view of a large number of clinicians, these examinations give the most exact picture of the patients' condition. An enormous amount of work has been done on the effect of lead upon blood and its formation. This has been reviewed by Aub et alia (1926).

It has been shown that lead has a marked effect on the germ cell. Lead poisoning in the mother and even in the father has been shown to result often in still-births. The mortality rate of the offspring of such parents is also high, particularly during the first three years of life [Paul (1860), Porak (1894), Lewin (1904), Oui (1907), Legge and Goadby (1912)]. Numerous cases have been reported of the use of lead, chiefly as lead diachylon, to produce abortion [Ransom (1900), Scott (1902), Hall (1905), Hall and Ransom (1906)]. The reaction appears to be due to the effect of lead on the smooth muscle of

the uterus in the same way as it acts on the smooth muscle of the intestine during colic.

In severe lead poisoning the liver is often affecte The effects of lead on the liver have occupied much of the attention of German and Italian workers within recent years [Vigliani (1933), Legeder (1934), Vigliani and Angeleri (1935, 1936), Fellinger (1936), Carrie (1936), Vigliani (1936), Mertens (1937), Vigliani and Waldenström (1937), Vigliani and Libowitzky (1937)] .

Exposure to unusual amounts of lead has resulted from,

1. the contamination of foodstuffs and drinking water with lead,

2. the extensive use of lead and its compounds in industr

Contamination of foodstuffs with lead must have bee extremely common at one time. With more stringent public health measures this has now been much reduced, but cases do still occur. In modern times contamination of foodstuffs with lead occurs usually during processing. The most notable exception has been in the case of fruit. It has become prevalent, especially in the U. S. A., for lead arsenate to be used as an insectide, resulting in fruit and vegetables becoming contaminated. A large amount of work has been done in the U. S. A., on the toxicity of lead arsenate [Talbort and Taylor (1933), Greene (1936, 1937), Shields, Mitchell and Ruth (1939)] . Contamination is often due to the use of utensils made of

lead, containing lead solder or glazed with lead compounds. This is particularly true when the material has an acid reaction e.g. fruit juices and fermented liquids. Numerous cases of lead poisoning due to this have been reported; wines [Campbell (1886), Alden (1889), Arnaud (1936), Yang (1936), Yang, Chang and Liu (1937)] ; beer [Sutton (1938)] ; fruit juices [Duy (1935)] . Tinned sardines hav recently been found to be heavily contaminated with lead, due to preparation over a lead containing grill [Lampitt and Rooke (1933)] . An unusual case, resulting in an epidemic of lead poisoning, has been reported from Austria [Berger, Studeney and Rosigger (1932)] . Lead was used to counterbalance a grinding wheel in a flour mill with the result that the flour was impregnated with fine particles of metallic lead.

Drinking water, contaminated with lead, has been held responsible for outbreaks of lead poisoning on many occasions. Natural waters are usually almost completely free of lead. The contaminations have always been caused by the use of lead pipes. Lead pipes for the transport of water are still used extensively in some localities. The plumbosolvency of natural waters is extremely variable. Soft waters, especially those containing much dissolved oxygen and carbon dioxide are particularly plumbosolvent. Hard waters are considered to have a low plumbosolvency and it is considered safe to transport such waters through lead pipes as they become coated with a layer of insoluble calcium salts and are thus protected from corrosion.

Waters from peaty areas, containing large amounts of organic acids are very plumbosolvent. In this country no legal limit exists as to the maximum amount of lead permitted in drinking water. Various authorities have on occasions expressed their opinion. Within recent years outbreaks of lead poisoning due to contaminated water supplies have occurred in Scotland [Univ. of Aberdeen Report (1933)], France [Thouvenet (1931)] and Germany [Kruse and Fischer (1931)]. The subject of lead in drinking water has been studied by the Water Pollution Research Board [(1934), Ingleson (1938)].

Poisoning produced by the consumption of foodstuffs and drinking water contaminated with lead is due entirely to alimentary absorption.

A large amount of lead poisoning exists in industry With the rise of industry and the more widespread use of lead, this has assumed great importance. In industry absorption can take place from the respiratory tract as well as from the alimentary tract. Many authorities consider that the former is the more important entrance.

The number of industrial processes using lead or its derivatives is legion. The following represents a short summary:

1. lead mining
2. lead smelting
3. handling and fabrication of lead
    a. manufacture of lead articles
    b. handling metallic lead in hot processes e.g.

lead burning, soldering, tempering and plumbing

c. brass and other founding in which lead is used

d. buffing and polishing metallic surfaces

4. manufacture of lead salts and compounds, lead pigments and organic lead compounds

5. manufacturing processes in which lead compounds are used

storage battery, paint, glass, rubber and chemical industries

6. application and removal of lead containing paints, enamels and glazes

painting, spray painting, vitreous enamelling, pottery dipping

sandpapering, scraping and chipping painted surfaces

flame cutting of painted metal

7. tree spraying of lead containing insectides

8. typographic trades

type founding, electro-typing, stereotyping

In lead mining, the incidence of poisoning appears to be much higher in the new countries e.g. U. S. A., than in the older countries e. g. those in Europe. This is due apparently to differences in the type of ore mined. In the older countries the mines are deep and the ore consists chiefly of sulphide which is poorly soluble. In the newer mines, which are shallow, the ore consists chiefly of oxidised forms of lead e.g. carbonate, sulphate and oxides, which are much more soluble. It is to be expected that as these mines become deeper, the yield of sulphide will

increase, with a consequent decrease in the incidence of lead poisoning.

The manufacture and handling of lead tetra-ethyl is particularly dangerous owing to the ease with which it is absorbed through the skin.

At times unusual cases of lead poisoning are repor One of the strangest cases was the occurrence in a large number of families in Baltimore and Nashville, Tenessee, produced by the use of discarded storage battery cases as fuel. Several cases of encephalopathy were reported [Williams et alia (1933), Crutcher (1933)]. Cases of lead poisoning have been reported from the use of lead opiu pipes [Yang, Chang and Liu (1937)], theatrical grease paint containing lead pigments [Bartleman and Dukes (1936) and from shrapnel and bullets left in wounds [Londres (193 Leschke (1934), Haenisch (1936)]. The use of cosmetics containing lead compounds has resulted in a large number of cases of lead poisoning amongst women and suckling children in Japan [Kato (1932)]. Many children have been shown to have suffered from lead poisoning in Queensland, Austral [Nye (1933)]. It was concluded that the source of lead was from the paint used on the verandas and railings where the children played. Under the action of the tropic sun, this paint powdered readily and as a result was in a form readily assimilated by the children. The Queensland Government have prohibited the use of lead paint containing more than 5% soluble lead.

Lead poisoning, due to eating and sucking toys etc. containing lead, have been reported in children [Aub et al (1926), Taylor and Schram (1936)].

Extensive lead poisoning has been reported amongst wild ducks in certain well hunted districts in the U. S. A. [Torrey, Thorpe and Graham (1934)]. This was caused by the birds swallowing lead shot which was retained in the bulbo-ventriculus. Dowdell and Green (1937) have recommend the use of shot made of lead magnesium alloy as this disintegrates rapidly on contact with water.

The incidence of lead poisoning as a result of ingestion of contaminated foodstuffs and drinking water is l The occurrence of lead contamination in sardines in this country was met effectively by a meeting of Port Medical Officers of Health at the Ministry of Health where it was decided to condemn any consignment which contained more than 20 parts of lead per million. At a later meeting it was decided to reduce the limit to 6 parts per million.

Limits of lead content are not applied to foodstuff in general in this country, but only in particular cases when evidence of gross contamination has been brought to the notice of the Ministry of Health as in the case cited above.

The incidence of lead poisoning in industry has decreased very much. The measures adopted to prevent lead poisoning in industry are chiefly concerned with personal hygeine and the prevention of fumes and dust being inhaled. A great deal of work in this field has been done by

Sir Thomas Oliver (1913 - 1921) and Legge and Goadby (1912) in Britain, Meilliére (1902, 1903) in France, Teleky (1909 - 1921) in Germany and Hamilton (1911 - 1921) in the U. S. A.

Lanza (1935) in a recent review considers that in spite of the preventative measures taken, a considerable amount of mild lead poisoning exists, much of which escapes diagnosis. This view warrants a much more extensive investigation into the biochemistry of lead than hitherto has taken place in an attempt to discover more delicate methods of diagnosis.

The Estimation of Lead in Animal Tissues and Excreta

The major problem which had to be solved before any investigations could be contemplated, was to devise a method for the estimation of the small amounts of lead that occur in animal tissues and excreta. The requirements of such a method were accuracy, speed and sensitivity in order that large quantities of material were not necessary as the estimation of the lead content of the blood of living persons was contemplated.

Biological materials of the nature of tissues and excreta consist of a heterogeneous mixture of organic compounds and mineral salts of which lead, if present, forms a very small part. As a result, any method resolves itself into three essential stages; 1. destruction of the organic matter, 2. separation of the lead, 3. estimation of the lead. Almost all methods that have been devised, consist of these three stages. Up to 1934, almost all methods have employed reactions which are used in macro analytical chemistry and as a result the data obtained have been very unsatisfactory.

To destroy organic matter, two methods have been employed; 1. ignition, 2. digestion with sulphuric acid and some oxidant e.g. nitric acid.

To separate lead, the following reactions, singly or in combination have been used;

1. precipitation as the sulphide,
2. precipitation as the sulphate,

3. precipitation as the chromate,

4. deposition in the metallic form or as the peroxide by electrolysis.

As a general rule, the first reaction has been used as a preliminary to the others. The objection to precipitation methods for separating small amounts of a substance is obvious. In the first place, the precipitate is usually colloidal in nature and difficult to filter. In the second place, all 'insoluble' substances have definite solubilities which although small become appreciable when small quantities are involved. Electrolytic methods usually fail in the presence of large amounts of iron [Francis et alia (1929)].

A large number of methods have been devised to estimate lead after separation. These are as follows;

1. nephelometrically as the sulphide,
2. nephelometrically as the sulphite [Cooksey and Walton (1929)],
3. colorimetrically by the blue colour produced when lead peroxide and tetramethyldiaminophenylmethane interact,
4. colorimetrically by the colour produced when lead chromate and semicarbazide interact [Fairhall (1924)],
5. by titrating the iodine liberated when potassium iodide is added to lead chromate in the presence of sulphuric acid [Fairhall (1924)].

None of the above reactions are specific for lead. Reaction 3 to 5 are to be condemned because they are not concerned

with lead but with the ion to which it is attached. The sulphide test is probably the oldest of the above but it lacks sensitivity. A real objection may be laid against the tetramethyldiaminodiphenylmethane test as it involves the electrolytic deposition of lead as the peroxide and manganese, a constant constituent of tissues and excreta, tends to be deposited as the dioxide at the same time and this reacts with the reagent.

Fairhall (1924) described a method in which the lead was precipitated as sulphide and then as chromate. The lead chromate was then determined either 1. colorimetrically with semicarbazide or 2. by titration with sodium thiosulphate after the addition of potassium iodide and sulphuric acid. This method was used by Aub et alia (1926) in their investigations. Kehoe et alia (1926, 1933) used a modification of this method. Later they (1935) acknowledged a loss of 0.07 mg. Pb per sample by this method. In an examination of the lead content of urine, Cooksey and Walton (1929) made a preliminary separation of the lead by electrolysis and estimated it nephelometrically as sulphite. Francis, Harvey and Buchan (1929) described a process involving the precipitation of lead as sulphide, followed by electrolysis and precipitation as sulphate. The lead was estimated nephelometrically as the sulphide. Weyrauch and Muller (1933) and Litzner and Weyrauch (1932, 1933) investigating the distribution of lead in man, separated the lead as

sulphide and then as the peroxide by electrolysis. They estimated the lead colorimetrically with tetramethyldiaminodiphenylmethane. An estimation by any of these methods would take about 3 days to complete.

The spectrograph has been used to identify and estimate lead in biological materials. The results obtained have been very conflicting.

The Author's Method

The method consists of 3 stages; 1. destruction of the organic matter, 2. separation of the lead, 3. estimation of the lead. It will be considered under these headings in order for convenience, although this was not the order of development.

Destruction of organic matter

Two methods are available; 1. digestion with sulphuric acid and an oxidant e.g. nitric acid, perchloric acid, 2. ignition.

The objection to the first is that often large quantities of sulphuric acid and oxidant are required and consequently a large quantity of alkali is necessary for neutralisation. This leads to a high blank even when the purest reagents are used.

Destruction by ignition, which avoids this high blank, has been criticised on the grounds that lead may be lost by volatilisation. The writer has found that materi containing a large amount of ash, consisting chiefly of phosphate, may be ignited in a silica dish over a bunsen

burner without loss of lead. When materials of low ash content are treated similarly, a loss of lead may occur. For this reason, the ash content of such materials has been increased by the addition of sodium phosphate (Na_2HPO_4). Biological materials have therefore been divided into two classes;

1. high ash content - ignited without the addition of sodium phosphate e.g. urine, faeces, milk and bone,
2. low ash content - sodium phosphate added prior to ignition e.g. blood and soft tissues.

This ignition method has been found to be quite satisfactory under the above conditions. Towards the end, ignition may be assisted by allowing the ash to cool, adding a little concentrated nitric acid and re-heating.

Separation of the lead

Separation of lead by precipitation or by electrolysi was not considered for reasons stated already. A method suggested by Allport and Skrimshire (1932) for separating lead from solutions of the ash of dyestuffs appeared at first to have possibilities. An alkaline solution of the ash was shaken with a chloroform solution of diphenylthiocarbazone (dithizone). Lead was extracted by the chloroform as a lead-dithizone complex. Iron was not extracted and other metals were not extracted if cyanide was present. The writers recognised certain difficulties. The aqueous solutions must be perfectly clear, the slightest turbidity due to iron or phosphates interfering with the separation. As the extractions must

be made in alkaline solution, this is difficult, for even when citrates have been added, a solution may be perfectly clear and yet iron or phosphates may be precipitated in colloidal form and so prevent complete extraction of lead. The pH of the solution needs careful adjustment, which is difficult when some classes of material are used. If the organic matter has been destroyed by wet oxidation, the nature of the oxidant used has a marked influence. Allport and Skrimshire (1932) found that if nitric acid had been used, extraction of the lead was generally incomplete. The writer has found the method to give erratic results and so was abandoned. Lynch, Slater and Osler (1934) used this method of separation in an examination of the lead content of some human tissues. The lead was estimated by the sulphide reaction.

In a search for a more satisfactory method, the writer found that sodium diethyldithiocarbamate offered possibilities. This substance was described by Callan and Henderson (1929) as being suitable for the estimation of small amounts of copper and since then it has been used extensively for this purpose. As a preliminary, the writer carried out an investigation of the general properties of this substance.

Sodium diethyldithiocarbamate, a white substance, is easily soluble in water. It reacts with metals to form organic complexes, many of which are soluble in organic solvents e.g. ether. The nature of these complexes was not

studied. Of the metals that occur in biological materials and react thus are iron, copper, lead, zinc, manganese and cobalt. These complexes have low solubilities in water but are easily soluble in ether.

The lead complex is white and dissolves in ether to form a colourless solution. With the exception of zinc, the complexes of the other metals are coloured and form coloured solutions in ether. The lead complex is formed in acid and alkaline solution and in alkaline solution its formation is not inhibited by the presence of citrate, pyrophosphate or cyanide. The complexes of the other metals are also formed in both acid and alkaline solution but in alkaline solution they are not formed if cyanide is present. In alkaline solution the iron complex is not formed if citrate or pyrophosphate is present.

The effects of other organic solvents was not investigated as ether was found to be quite satisfactory as an extractant.

Before lead can be estimated by the process to be described in the next stage, it is necessary to obtain it free from iron and copper. This may be done by the use of sodium diethyldithiocarbamate. The separation may be carried out by adding to a solution of the salts of the metals, sodium citrate, ammonia to make alkaline and cyanide. On the addition of sodium diethyldithiocarbamate, the lead complex alone is formed, and may be extracted with

ether. The presence of citrate is essential as it prevents the precipitation of phosphates and metallic hydroxides in alkaline solution.

This technique has been found to be quite satisfactory for urine, soft tissues and blood. In the case of milk, faeces and bone, certain modifications have been found to be advantageous. The ash of this class of material contains a considerable amount of calcium phosphate, which in spite of the presence of citrate, tends to precipitate when the solution is made alkaline. This is liable to prevent a quantitative separation of lead. For this reason, a twofold extraction has been emplo

Soldium diethyldithiocarbamate is added to an acid solution of the ash. All the metallic complexes are formed and all are extracted by ether. The extracted metals are converted into the inorganic state and the process of extraction repeated but in alkaline solution in the presence of citrate and cyanide. Under these conditions the lead complex alone is formed and this alone is extracted with ether.

Estimation of lead

Fischer and Leopoldi (1934) described a very sensitive colorimetric method for the estimation of lead. An alkaline solution of the lead salt was shaken with a carbon tetrachloride solution of dithizone. The lead formed a complex with dithizone which was extracted by the carbon tetrachloride to produce a pink coloured

solution. It was decided that this reaction merited investigation.

Dithizone, an organic substance, dissolves in carbon tetrachloride and chloroform to produce green coloured solutions. It is soluble in water only if alkalin in reaction, the solution being coloured brown. When an alkaline solution of a lead salt is shaken with carbon tetrachloride and dithizone, pink lead-dithizone is formed which is extracted by the carbon tetrachloride. If excess dithizone has been used, the colour of the lead complex will be masked by the green colour of unchanged dithizone. A pure extract of the lead complex may be obtained by separating the carbon tetrachloride and extracting it repeatedly with dilute potassium cyanide solution. Unchanged dithizone but not the lead-dithizone is removed by this process.

The reaction was studied quantitatively. It is necessary to have the lead present in a suitable medium. As a result of separation, the lead will be present as an organic complex. To convert this into an inorganic form, digestion with sulphuric acid and an oxidant is most suitable. This leaves a residue containing lead sulphate which is insoluble in water. To ensure solution, water, acetic acid and sufficient ammonia was added to make the final reaction alkaline. In such a solution, lead sulphate is easily soluble and by using a suitable amount of ammonia, the correct reaction at which lead and

dithizone react may be obtained. A series of lead solution concentrations ranging from 0.005 to 0.8 mg. Pb, were prepared in a medium of the above composition. Using 10 ml carbon tetrachloride, the lead complex was prepared. These were compared one against another in a colorimeter and it was found that the depth of colour was proportional to the concentration of lead.

Fischer and Leopoldi, prior to colorimetric comparison, treated carbon tetrachloride extracts of the lead complex with mineral acid, the colour changing from pink to green. The writer has found that no advantages are derived from this. The writer has found that for colorimet comparison, the best depth of colour is that containing about 0.01 - 0.02 mg. Pb.

Excess dithizone must be used to produce a quantitative result but too great an excess must be avoided as under such conditions the formation of the lead complex is inhibited.

Dithizone is very susceptible to oxidation to produce a substance which dissolves in carbon tetrachloride to produce a yellow coloured solution, and cannot be removed by cyanide extraction. This substance is produced from dithizone in the presence of iron and copper salts and by bright sunlight. The absence of iron and copper salts is effected by the separation described above. In dealing with biological materials, the effect of copper is practically negligable owing to the low concentrations

present. Bright sunlight, but not diffuse light such as present in a laboratory, produces this oxidation product rapidly. The writer believes that it is the ultra-violet component of bright sunlight which is responsible for this reaction. In diffuse light, a carbon tetrachloride solution of the lead complex will remain unchanged for a very long period.

The specificity of the reaction was then investigated. A large number of metals were examined and none of these interfered with the estimation of lead with dithizone under the conditions stated above. The only apparent exception was bismuth. Bismuth, even in the presence of cyanide, was found to produce a complex with dithizone which was extracted by carbon tetrachloride to form an orange coloured solution. The bismuth complex is unstable, for when a carbon tetrachloride solution is extracted repeatedly with dilute cyanide solution it is removed. More extractions with cyanide are usually required to remove bismuth-dithizone complex than free dithizone. Although bismuth does not occur naturally in human tissues and excreta, it is liable to be present as bismuth is used in therapeutics. The amounts of bismuth that would occur in tissues and urine under such conditions would not interfere with the estimation of lead. If present, bismuth will be separated along with lead as its complex with sodium diethyldithiocarbamate has the same properties. Its presence will be detected at the commencement of the

colorimetric estimation of lead. In actual experiment it was found that 0.01 mg. Pb could be accurately estimated in the presence of 0.1. mg. bismuth. As a result of medication with 'stomach powders' containing bismuth salts, faeces can at times contain considerable amounts of bismuth. Under such conditions, an estimation of lead is impracticable and it is advisable to stop the medication and to collect the specimen when the alimentary tract is free of bismuth.

During the course of this work, bismuth was found in three samples of faeces, none in any other types of material.

Apparatus and reagents

All glassware was Pyrex, ignitions were carried out in silica dishes and glass distilled water was used. Filter papers were washed with dilute acid and then with distilled water.

All chemicals, even the purest, contain traces of lead and these if used in any quantity produce quite a large blank. It is possible to reduce this by further purification but it was wished to avoid this as it was hoped to produce a method which would be suitable for routine purposes. For this reason, the amounts of reagents have been kept as low as possible except in a few cases. Fairly large quantities of sodium phosphate and citrate are required but simple methods of purification have been devised. Commercial dithizone contains a yellow oxidation product which dissolves in carbon tetrachloride to form a

yellow solution and it cannot be removed by cyanide extraction. Purification is thus essential. It has not been found practicable to keep purified solutions, hence it is purified just before use.

1. Concentrated hydrochloric acid - analar reagent
2. Concentrated sulphuric acid - analar reagent.
3. Concentrated nitric acid - analar reagent
4. Glacial acetic acid - analar reagent
5. Perchloric acid (20%) - analar reagent
6. Ammonia (sp. gr. 0.88) - analar reagent
7. Potassium cyanide (10%) - PbT (B. D. H.)
8. Potassium cyanide (1%) -
   Reagent 7 diluted 1 in 10
9. Ether - analar reagent
10. Sodium diethyldithiocarbamate - 2% in water
11. Carbon tetrachloride - analar reagent
12. Sodium citrate - 20% (Lead free)
    To 1 litre of a 20% solution of sodium citrate are added 100 ml. of 0.1% dithizone in chloroform and the mixture shaken well. As required, a portion is separated and filtered to remove suspended particles of chloroform.
13. Sodium phosphate ($Na_2HPO_4.12H_2O$) - 10% (lead free)
    This solution is added to soft tissues and blood prior to ignition.

    As required, the necessary volume of a stock solution is placed in a separating funnel, ether and sodium diethyldithiocarbamate added and the mixture shaken.

After allowing to settle, the lead free aqueous layer is run off.

14. Standard solution of lead acetate

0.1831 g. of lead acetate $Pb(C_2H_3O_2)_2.3H_2O$, is dissolved in distilled water containing 5 ml. of glacial acetic acid. The volume is made up to 1 litre with water. 1 ml. of this solution is equivalent to 0.1 mg. Pb. This solution is diluted as required so that 1 ml. is equivalent to 0.01 mg. Pb.

15. Dithizone reagent

A stock solution of 0.1% commercial dithizone in carbon tetrachloride is kept. When required, an aliquot is shaken with an equal volume of 0.5% ammonia. After allowing to settle, the aqueous layer containing pure dithizone is separated and used.

In the following, details of the method as applied to various types of materials are described. The final colorimetric estimation is the same in each case.

Urine

500 ml. of urine are evaporated to dryness in a silica dish and then ashed by ignition.

The ash is dissolved in 100 ml. of water containing 5 ml. of concentrated hydrochloric acid. The solution is transferred to a separating funnel, 50 ml. of 20% sodium citrate added and the mixture made alkaline by the addition of ammonia, sp. gr. 0.88. 5 ml. of 10% potassium

cyanide are added. 5 ml. of 2% sodium diethyldithiocarbamate are added and the mixture extracted three times with ether, 25 ml. being used on each occasion. The ether extracts which are washed separately, are transferred to a hard glass round bottomed flask.

The ether is evaporated off and the residue digested with 1 ml. concentrated sulphuric acid and 1 ml. perchloric acid to destroy organic matter.

The residue is diluted with water, 1 ml. glacial acetic acid and 5 ml. ammonia, sp. gr. 0.88, added and the mixture diluted to 25 ml. with water.

Soft Tissues

To 100 ml. of lead free 10% sodium phosphate in a silica dish are added 100 g. fresh tissue. After drying, the mixture is ashed by ignition.

The ash is dissolved in 100 ml. of water containing 10 ml. of concentrated hydrochloric acid. The procedure is then as for urine.

Blood

To 100 ml. of lead free 10% sodium phosphate in a silica dish are added 20 ml. blood. After drying the mixture is ashed by ignition.

The ash is dissolved in about 50 ml. of water containing 5 ml. of concentrated hydrochloric acid. The solution is transferred to a separating funnel, 5 ml. of 20% sodium citrate added and the mixture made alkaline by the addition of ammonia, sp. gr. 0.88. 5 ml. of 10% potassium

cyanide are added. 2 ml. of 2% sodium diethyldithiocarbamate are added and the mixture extracted twice with ether, 20 ml. being used on each occasion. The ether extracts which are washed separately are collected in a hard glass round bottomed flask.

The ether is evaporated off and organic matter destroyed by digestion with 0.2 ml. concentrated sulphuric acid and 0.5 ml. perchloric acid.

To the digest are added, 3.5 ml. water, 0.2 ml. glacial acetic acid and 1.5 ml. ammonia, sp. gr. 0.88.

Faeces

10 g. of dried faeces are ashed by ignition in a silica dish. The ash is dissolved in 100 ml. of water containing 10 ml. concentrated hydrochloric acid. The solution is diluted to 200 ml. with water.

50 ml. of the ash solution are introduced into a separating funnel and 10 ml. of 2% sodium. diethyldithiocarbamate added. The mixture is extracted three times with ether, 25 ml. being used on each occasion. The ether extracts are collected in a hard glass round bottomed flask and the ether evaporated off. The residue is digested with 1 ml. concentrated sulphuric acid and 1 ml. perchloric acid.

The residue is diluted with water, 1 ml. concentrated hydrochloric acid added and the mixture heated. The solution is transferred to a separating funnel and diluted to about 50 ml. with water, 5 ml. of 20% sodium citrate

added and the mixture made alkaline by the addition of ammonia, sp. gr. 0.88. 5 ml. of 10% potassium cyanide are then added, followed by 5 ml. of 2% sodium diethyldithiocarbamate. The mixture is then extracted three times with ether, 25 ml. being used on each occasion. The ether extracts are collected in a hard glass round bottomed flask.

The ether is evaporated off and the residue digested with 1 ml. concentrated sulphuric acid and 1 ml. perchloric acid. To the residue are added, water, 1 ml. glacial acetic acid, and 5 ml. ammonia, sp. gr. 0.88. The mixture is diluted to 25 ml. with water.

Bone

20 g. of bone are ashed by ignition in a silica dish. The ash is dissolved in water containing hydrochloric acid and the solution diluted to 200 ml. with water. 50 ml. of this solution are taken and proceeded with as in the case of faeces.

Milk

500 ml. of milk are evaporated to dryness in a silica dish and then ashed. The ash is dissolved in water containing hydrochloric acid and proceeded with as in the case of faeces.

The colorimetric estimation of lead

Preparation of the standard ;

The following mixture is prepared. To 1 ml. of concentrated sulphuric are added water, 1 ml. glacial acetic

and 5 ml. ammonia, sp. gr. 0.88. The mixture is diluted to 25 ml. with water.

A known amount of lead is added to 5 ml. of this solution. To this are added 5 ml. of 1% potassium cyanide and 10 ml. carbon tetrachloride. An ammoniacal solution of dithizone is added drop by drop, with constant shaking until excess has been added. Too great an excess must be avoided. Sufficient excess is indicated when the carbon tetrachloride layer has reached its maximum intensity of redness and the aqueous layer in tinged brown. The aqueous layer is then separated and discarded. The carbon tetrachloride layer, containing the red coloured lead complex, is shaken repeatedly with aliquots of 5 ml. of 1% potassium cyanide until excess dithizone has been removed as shown by the aqueous layer being no longer coloured. The carbon tetrachloride extract is then passed through a filter paper to remove droplets of water and is then ready for comparison.

A range of standards may be prepared but the writer prefers to use a standard containing 0.02 mg. Pb and using a volume of unknown to conform to this. This standard may be prepared by using 2 ml. of a standard solution of lead acetate containing 0.01 mg. Pb per ml.

Preparation of the unknown ;

Urine, faeces, soft tissues, bone and milk ;

The lead is contained as lead sulphate in a solution of ammoniacal ammonium acetate with a volume of

25 ml. To 5 ml. of this solution are added 5 ml. of 1% potassium cyanide and 10 ml. carbon tetrachloride. The colour is developed in the same way as that of the standard.

In the event of the lead content of the unknown being low, 10 ml. of the solution is used, the amounts of the other reagents being the same. If the lead content of the unknown is high, a smaller volume than 5 ml. is used. In this case the solution is diluted to 5 ml. by the addition of an ammoniacal ammonium acetate solution having the same composition as that used to prepare the standard. The amounts of the other reagents are the same.

Blood ;

In the case of blood, the whole of the lead containing solution is used. To the mixture, containing the lead in the flask used for digestion, are added 5 ml. of 1% potassium cyanide and 10 ml. of carbon tetrachloride and the colour developed as above.

Blank ;

A blank should always be done on a new set of reagents. In estimating the blank, the complete process is carried out. The blank is small and is thus difficult to estimate accurately. As a result, the following method has been adopted. Before the estimation, 0.02 mg. Pb is added to the blank. This after development of the colour with dithizone is compared with a standard containing 0.02 mg. Pb. The blank is then calculated from the difference.

With the exception of blood, the development of the colour is best carried out in glass stoppered tubes.

A series of recovery experiments were carried out and the results are shown in Table 1. It will be seen that added lead could be estimated accurately.

The method that has been described is specific for lead and gives accurate results. An estimation can be completed in a comparatively short time. Excluding drying and ignition, an estimation may be completed in 1 hour.

Since this method has been published, new methods for estimating lead in biological materials have been published by other workers ; spectrographic [Blumberg and Scott (1935), Cholak (1935)]; polarigraphic [Teisinger (1936)]; colorimetric dithizone methods [Hubbard (1937), Willoughby and Wilkins (1938)].

Table I. *The recovery of added Pb*

	Initial Pb content mg.	Pb added mg.	Total Pb found mg.	Pb recovered mg.
Urine (500 ml.)	0·120	0·050	0·163	0·043
	0·120	0·100	0·215	0·095
	0·120	0·200	0·308	0·188
	0·120	0·250	0·362	0·242
	0·120	0·500	0·625	0·505
	0·042	0·020	0·079	0·037
	0·042	0·050	0·096	0·054
	0·042	0·100	0·140	0·098
	0·155	0·200	0·360	0·205
	0·155	0·400	0·565	0·410
Faeces (10 g.)	0·030	0·200	0·235	0·205
	0·030	0·500	0·549	0·519
	0·030	1·000	1·036	1·006
	0·042	0·030	0·079	0·037
	0·042	0·050	0·096	0·054
	0·042	0·100	0·140	0·098
	0·031	0·040	0·072	0·041
	0·031	0·080	0·115	0·084
	0·031	0·120	0·154	0·123
Milk (500 ml.)	0·050	0·050	0·105	0·055
	0·050	0·100	0·145	0·095
	0·050	0·200	0·248	0·198
	0·050	0·500	0·560	0·510
Liver (100 g. fresh)	0·243	0·050	0·299	0·056
	0·243	0·100	0·350	0·107
	0·243	0·200	0·440	0·197
	0·243	0·400	0·640	0·397
	μg.	μg.	μg.	μg.
Blood (20 ml.)	16	10	27	11
	16	20	37	21
	16	40	58	42
	19	10	28	9
	19	20	39	20
	19	40	58	39
	12	10	22	10
	12	20	34	22
	12	40	53	41

The Distribution of Lead in Human Tissues

The available data concerning the presence or otherwise of lead in 'normal' human tissues has been in a very conflicting state.

Meilliére (1903) stated that he could detect lead in the organs of nearly all the subjects examined by him. Aub et alia (1926) state that the lead retained by an apparently normal individual is held almost exclusively by the skeleton. Later work illustrated the presence of lead in 'normal' bones but there was considerable variation. Barth (1931) found 0.01 - 0.06 mg. Pb per g. ash or approximately 5 - 30 mg. Pb per kg. fresh bone, while Lynch et alia (1934) found 14 - 146 mg. Pb per kg. fresh bone. Weyrauch and Muller (1933) found no appreciable amoun of lead in liver, spleen or brain. Sheldon and Ramage (1931 using a spectrographic method, found lead occurring spasmodically in 'normal' human tissues while Boyd and De (1933), also using a spectrographic method, found lead well marked in the liver and present in all organs examined except the brain. Lynch et alia (1934), in an examination of a few organs, obtained values of the order of 1.5 mg. Pb per kg. fresh tissues in some livers and none in others. Kehoe et alia (1933) found appreciable amounts of lead in the tissues of two cases apparently normal shortly before death. The conflicting results reported are undoubtedly due to inaccurate methods.

Tissues were obtained post-mortem from persons with

no history of exposure to lead other than the 'normal' hazard, and the lead content determined. The results are shown in Table 2a.

In a later investigation, the lead contents of different types of bone viz. rib, vertebra, femur and tibia were examined. The results are shown in Table 2b.

The lead content of normal blood was then determined. This was taken from living persons and was undertaken as it was considered that such an estimation might prove of value in the diagnosis of lead poisoning. The blood was collected in an all glass syringe fitted with a stainless steel needle, transferred to a Pyrex test tube and measured as quickly as possible. About 20 ml. were used. The results are shown in Table 2c.

In an examination of the tissues of persons with no history of exposure to lead other than the 'normal' hazard, lead was found in every one examined viz. bone, liver, spleen, kidney, lung and brain. The highest concentrations were present in bone. Bones such as femur and tibia were found to contain much higher concentrations of lead than rib or vertebra. Of the soft tissues, the highest concentrations were found in the liver. It would appear that the lead content of such bones as femur and tibia increases with age. The lead contents of r vertebra and soft tissues are unaffected by age.

Lead was found to be invariably present in circulating blood. In 40 cases, the figures ranged from

40 - 70 µg. Pb per 100 ml., with an average of 55 µg. Pb per 100 ml. blood.

Since this work was completed and published, investigators in various parts of the world have recorded the lead content of 'normal' human blood. These confirm the figures reported here [Blumberg and Scott (1935) - spectrographic method ; Kehoe, Thamann and Cholak (1935) - spectrographic method ; Teisinger (1936) - micropolarigraph method ; Taeger and Schmitt (1937) - dithizone method ; Willoughby and Wilkins (1938) - dithizone method].

TABLE 2A.

THE LEAD CONTENT OF HUMAN TISSUES.

	Age	Sex	Occupation	Diagnosis	Liver		Kidney		Spleen		Brain		Rib	Vertebra	Right lung
					mg. per kg.	Total mg.	mg. per kg.	Total mg.	mg. per kg.	Total mg.	mg. per kg.	Total mg.	mg. per kg.	mg. per kg.	Total mg.
				(*a*) Cases with no known occupational exposure to lead.											
1	61	M	None	Carcinoma colon	1·50	1·65	0·80	0·19	—	—	—	—	10·72	4·47	—
2	35	M	Timekeeper	T.B. meningitis	0·85	1·22	1·26	0·44	—	—	0·36	0·50	9·76	8·93	—
3	59	F	Housewife	Diabetes mellitus	1·10	1·96	2·60	0·83	—	—	—	—	8·43	5·58	—
4	69	M	None	Carcinoma rectum	2·50	2·99	1·50	0·41	—	—	—	—	6·90	5·35	—
5	50	F	Housewife	Chronic nephritis	1·20	1·37	—	—	0·63	0·09	0·64	0·73	7·80	5·45	—
6	2	M	—	Bronchopneumonia	1·53	—	0·74	—	—	—	0·32	0·42	9·53	8·23	—
7	35	M	Cinema proprietor	Cerebral aneurism	1·26	2·39	1·43	0·56	—	—	0·55	0·72	12·9	14·7	—
8	40	F	Housewife	Haemorrhage	0·95	0·93	1·73	0·45	—	—	—	—	5·26	6·18	—
9	57	M	Ex-soldier	Syphilitic aortitis	1·57	2·04	0·72	0·22	0·77	0·17	—	—	8·15	6·61	—
10	41	M	Iron dresser	Lobar pneumonia	2·56	3·69	2·04	0·69	5·90	0·83	—	—	7·47	8·75	—
11	64	M	Machinist	Valvular disease	0·99	1·55	1·21	0·51	1·49	0·28	—	—	11·89	7·17	—
12	40	F	Housewife	Myocarditis	2·10	3·82	0·98	0·31	0·95	0·34	0·72	0·86	10·00	10·95	0·65
13	37	M	Storekeeper	Bact. endocarditis	1·35	1·80	0·91	0·29	0·64	0·15	—	—	7·59	5·96	0·28
14	39	F	Housewife	Acute pancreatitis	0·98	1·18	0·87	0·22	0·74	0·05	—	—	10·23	9·75	0·26
15	19	F	Typist	Obstruction	4·63	4·42	1·13	0·23	0·69	0·08	—	—	5·43	7·51	0·88
16	25	F	—	Carcinoma rectum	2·14	3·42	0·87	0·33	0·72	0·19	—	—	5·00	5·03	0·82
17	59	M	Warehouseman	Carcinoma oesophagus	2·13	1·89	0·74	0·16	3·39	0·23	—	—	5·85	4·21	0·28
18	23	F	Shop assistant	Diabetes mellitus	2·00	3·50	0·83	0·31	3·84	0·42	0·43	0·64	11·00	4·28	0·38
19	54	F	Housewife	Diabetes mellitus	1·49	3·73	0·85	0·31	0·37	0·25	—	—	8·55	5·70	0·42
20	6 weeks	M	—	Subarachnoid haemorrhage	1·95	0·39	3·55	0·14	3·08	0·07	0·24	0·07	1·57	2·60	0·079 (both)
	Mean, excluding No. 20				1·73	2·42	1·35	0·38	1·68	0·26	0·50	0·645	8·55	7·095	0·50

TABLE 2B

The results are expressed in mg. Pb per kg. fresh bone.

	Age	Occupation	Diagnosis	Rib	Vertebrae	Femur	Tibia
			Males.				
1	25	Quarryman	Nephritis	5·9	4·6	33·9	27·8
2	28	Newsagent	Aspirin poisoning	9·8	6·4	25·6	27·0
3	29	Clerk	Ulcers of small intestine	4·2	4·6	21·1	20·6
4	35	Boiler fireman	Carc. of stomach	13·3	7·3	48·2	28·6
5	50	Hairdresser	Carc. of stomach	7·9	4·6	48·1	42·1
6	58	Labourer	Nephritis	8·9	8·6	29·0	26·5
7	59	Engineer	Uraemia	12·8	11·6	70·0	62·7
8	66	Iron moulder	Carc. of pelvic colon	8·6	6·5	42·3	48·6
9	67	Retired	Cerebral haemorrhage	9·1	8·2	55·9	43·7
10	67	Retired	Fractured skull	11·1	12·4	108·3	96·5
11	68	Blacksmith	Carc. of bronchus	11·3	7·6	74·1	43·4
12	74	Restaurant proprietor	Coronary thrombosis	13·4	12·6	82·2	81·6
			Females.				
13	12	—	Malignant thymoma	4·0	3·4	19·1	17·9
14	19	Shop assistant	Subacute bacterial endocarditis	16·5	10·6	74·8	60·0
15	22	Clerk	Pernicious anaemia	7·2	6·2	18·2	15·3
16	30	Housewife	Burns	9·5	8·4	34·1	29·8
17	39	Housewife	Subacute bacterial endocarditis	9·8	10·6	44·5	46·8
18	49	Housewife	Toxic goitre	8·7	9·0	33·3	27·8
19	61	Housewife	Abscesses of the kidneys	17·5	16·5	61·7	61·5

Table 2c

The lead content of the blood of 'normal' men and women

μg. Pb per 100 ml.

Blood lead	No. of cases
40	5
45	7
50	10
55	5
60	8
65	4
70	1
Average 55	Total 40

The Lead Content of the Tissues of Human Foetuses

The tissues of four stillborn human foetuses were obtained and their lead content estimated. The results are shown in Table 3.

Appreciable amounts of lead were found in these tissues but the concentrations were much lower than in tha adult. It must be assumed that lead is transferred by the mother to the foetus through the placental circulation.

TABLE 3

THE LEAD CONTENT OF THE TISSUES OF HUMAN FOETUSES

	Months gestation	Sex	Weight g.	Liver mg. per kilo	Liver Total mg.	Kidney mg. per kilo	Kidney Total mg.	Brain mg. per kilo	Brain Total mg.	Femur mg. per kilo
1	8	M	2400	0·33	0·04	0·66	0·01	0·12	0·03	1·49
2	twin	F	2100	0·83	0·07	0·63	0·01	0·21	0·04	2·66
3	8·5	M	—	0·63	0·06	0·63	0·01	0·18	0·07	1·47
4	7	M	1600	0·95	0·06	0·67	0·01	0·16	0·04	1·30
			Mean	0·68	0·06	0·65	0·01	0·17	0·045	1·73

Lead in Milk

As lead could be detected and estimated in all tissues and excreta examined, one would naturally expect it to be secreted in milk and an excellent opportunity arose to investigate this.

On a farm in a neighbouring county, water was supplied through a lead pipe. This water was very plumbosolvent and was found to contain 6 mg. Pb per litre. This water was consumed by the cows. Samples of milk were obtained from various cows and the lead content estimated. The results are shown in Table 4.

Lead in appreciable amounts was present in all of the samples, values of 0.069 - 0.212 mg. Pb per litre being obtained.

Table 4

The Lead Content of Milk

	mg. Pb per litre
1.	0.094
2.	0.069
3.	0.093
4.	0.083
5.	0.170
6.	0.212
7.	0.143

The Lead Content of Human Excreta.

Originally it was the practice for a sample of urine or faeces to be sent to the laboratory to be tested for the presence of lead if plumbism was suspected. A positive result was taken to support the diagnosis. Later it was realised that lead occurred in urine and faeces under normal conditions, although the amounts were small. Several investigators then attempted to determine the normal range, especially that in urine [Cooksey and Walton (1929), Francis et alia (1929), Kehoe et alia (1933)]. These investigations were undertaken in order to determine a standard upon which a diagnosis of plumbism might be made in a suspected case. No particular precautions were taken as to diet etc. The results were expressed in mg. Pb per litre in the case of urine and in mg.Pb. per 100 g. dried faeces or in mg. Pb per g. faecal ash. The mean values for urinary lead were much the same but the ranges showed great variation.

It has been the experience of the writer that often in plumbism, especially in the mild type, the excretion of lead does not greatly exceed that in the normal. According to Kehoe et alia (1933), removal from exposure to large amounts of lead, results in a rapid fall in excreted lead until values just above the normal/

normal are reached, these levels being then maintained for a long period. It is the mild type that is liable to defeat the clinician and it is in this type of case that the biochemist's assistance is most needed.

The writer considers that results should be expressed in terms of mg. Pb per day, as this would eliminate many variable factors. In addition the results should have reference to some standard diet, the lead content of which is known, in fact one should attempt to do a lead balance.

For a number of years it has been our practice in the Glasgow Royal Infirmary to do this. The patient is placed on a standard diet, the lead content of which is known, and the urine and faeces collected over a period of 3 days. The lead content is then estimated.

A number of normal patients were examined thus. The diet contained 0.22 mg. Pb per diem. The results are shown in Table 5 and indicate that for all practical purposes they were in balance. At the head of the Table are shown the lead contents of the urine and faeces of 3 persons whose diets were uncontrolled. These are much higher.

TABLE 5.

THE LEAD CONTENT OF HUMAN EXCRETA.

	Sex	Days collection	Occupation	Urine mg. *per diem*	Faeces mg. *per diem*
			(*a*) Normals.		
1	M	3	Laboratory worker	0·16	0·40
2	M	3	Do.	0·03	0·40
3	M	1	Do.	0·085	0·39
			(*b*) Hospital patients.		
4	M	3	Warehouseman	0·04	0·20
5	F	3	Housewife	0·025	0·23
6	M	3	Miner	0·06	0·26
7	M	2	Packer	0·055	0·23
8	F	3	Housewife	0·07	0·24
9	F	3	Do.	0·05	0·20
10	M	2	Miner	0·06	0·22
11	M	2	Engineer	0·05	0·24
12	M	3	Brass moulder	0·04	0·20
13	M	2	Packer	0·06	0·20
			Mean for patients	0·05	0·22

Origin of the Lead present in Normal Tissues and Excreta.

The lead present in the tissues and excreta of 'normal' human beings appears to be of twofold origin; 1. lead in foodstuffs and drinking water, 2. lead present in the atmosphere.

A large amount of the food that we consume contains traces of lead. This subject has been reviewed by Monier-Williams (1938). This lead is not due entirely to the manufacturing processes through which much of the food of a civilised community passes, for lead is distributed very widely throughout this planet, even normal soil containing traces. Kehoe et alia (1933) found lead present in the urine, faeces and foodstuffs of a community of Indians living under primitive conditions. The figures were lower than those of a civilised community. Drinking water is responsible for a certain amount of lead for although natural waters do not as a rule contain appreciable amounts of lead, the use of lead pipes and storage tanks is still common. The writer has estimated the lead content of the drinking water used in various part of Glasgow. The results are shown in Table 6 indicating that appreciable amounts are present.

Another source of lead is that present in dust, especially in industrial regions. This lead would be absorbed from the respiratory tract. A number of investigations/

investigations of the lead content of both the air and dust in industrial towns has been made. In Bradford, Leeds and Huddersfield, Manley (1937) found that the dust contained 0.053 - 1.234% lead.

Table 6.

The Lead Content of the Drinking Water in the City of Glasgow.

The results are expressed in mg. per litre.

		Remarks.
1.	0.03	iron pipes.
2.	0.09	lead pipes only.
3.	0.06	"
4.	0.08	"
5.	0.43	lead pipes and storage tank.
6.	0.57	"
7.	0.55	"
8.	0.34	"
9.	0.27	"

Factors Influencing the Absorption of Lead.

Lead may be absorbed from the respiratory tract, alimentary tract and skin. In industrial lead poisoning absorption from the respiratory tract is considered to be the important factor. In the 'normal' hazard and in lead poisoning due to contamination of foodstuffs and drinking water, absorption is from the alimentary tract. Absorption from the alimentary tract is undoubtedly influenced by a number of factors.

Aub et alia (1926) compared the degree of absorption of lead in two groups of cats, one group being on a milk diet and the other on a milk free diet. They could detect no marked difference and concluded that the amount of calcium in the diet had no influence upon the amount of lead absorbed from the alimentary tract. Shelling (1932) produced evidence which seemed to suggest that diets high in calcium render lead more toxic than diets high in phosphorus. Weyrauch and Necke (1933) found that the simultaneous oral administration of milk or mucilage and white lead to rabbits did not decrease to any appreciable extent the amount of lead absorbed. On the other hand they found that oil and margarine increased the amount of lead absorbed by more than 10 times. Sobel et alia (1938) in a study of experimental lead poisoning in/

in rats, found that about twice as much lead was absorbed on a diet containing viosterol as without it.

Adult male mice were the animals used throughout this work. Two basic diets were used, namely a high calcium diet and a low calcium diet of the following compositions;

Low calcium diet

Shelling (1932)

	g.
Whole wheat flour	400
Casein.	100
Corn starch.	325
Wheat gluten	50
Olive Oil.	40
Sodium chloride.	20
Potassium chloride.	15
Butter	omitted.

High calcium diet.

	g.
Whole wheat flour	700
Whole milk powder.	300
Marmite	50

In the first experiments the effect of high and low calcium diet upon the absorption of lead was studied.

The mice were fed with the specified diets (2.5 g. per/

per mouse per day) to which were added supplements of lead in the form of lead acetate. Each mouse was housed in a separate glass jar, floored with sawdust. No restriction was made upon the amount of water drunk. The amount of diet supplied was constant per mouse per day and was made up into a thick paste with water and placed in a porcelain dish, this minimising loss by spillage. No difficulty was experienced in obtaining complete consumption of the daily rations. The experimental period was 14 days. At the end of this period the jars were cleaned out and all the animals placed on the high calcium diet for 4 days to remove unabsorbed lead from their alimentary tracts. The animals were then killed. The lead content of each whole animal was then determined. The lead content of control animals and also of unsupplemented diets were also estimated. The results are shown in Table 7.

It will be seen that absorption of lead was high on a low calcium diet and low on a high calcium diet. In view of this, experiments were carried out in which mice were fed on the low calcium diet containing added supplements of lead to which had been added calcium glycerophosphate (0.5 g. per mouse per day). This in effect converted the low calcium diet into a high calcium diet/

diet. Here again the effect of the high calcium diet was to produce only a small absorption of lead (Table 7).

The effect of fat and vitamin D upon the absorption of lead was then studied. It was considered that fat might hinder the absorption of lead by the formation of insoluble lead soaps. In this series of experiments the mice were placed upon the low calcium diet, containing supplements of added lead, to which was added olive oil at the rate of 1 ml. per mouse per day. No specific influence of fat upon the absorption of lead could be detected (Table 7). It was considered that vitamin D might aid the absorption of lead in the same way that it aids the absorption of calcium. In this series of experiments mice were placed on a) the high calcium diet, b) the low calcium diet, containing supplements of added lead to which was added cod liver oil at the rate of 3 drops per mouse per day. The experimental results obtained did not indicate any marked influence by vitamin D upon the absorption of lead (Table 7).

It is interesting to speculate as to the reasons for the influence of calcium upon the absorption of lead from the alimentary tract. According to Shields et alia (1939) absorption of lead takes place not in the stomach but rapidly from the small intestine. Lead in diets will be/

be in insoluble forms but will pass into solution rapidly in the stomach owing to the action of the acid of the gastric juice. Upon entering the small intestine, the stomach contents will meet the alkaline pancreatic fluid and bile, as a result the now intestinal contents will tend to become neutral and possibly alkaline in reaction. This process will tend to precipitate the lead. It is generally agreed that lead to be absorbed must be in solution. As a result the degree of lead absorption will depend upon the rate at which the stomach contents are neutralised in the small intestine. It is suggested that upon a low calcium diet, owing to the low base content, the stomach contents are more acid than usual and as a result neutralisation in the small intestine will take longer while on high calcium diet, owing to the high base content, the opposite takes place.

Although large amounts of lead were absorbed on a low calcium diet, the absorption by animals receiving the same quantities of lead in different experiments showed marked variation. Other important factors must therefore be involved such as the amount of water consumed etc. Under comparable conditions however, the amount of lead absorbed is related to the amount of lead consumed.

Telfer (1939) has shown that the absorption of calcium and magnesium from the alimentary tract is increased by the oral administration of hydrochloric acid. It is probable/

probable that lead would behave similarly. The following experiment was carried out. A number of mice were fed with high calcium diet containing 1 mg. Pb (as lead acetate) and 0.5 ml. N. hydrochloric acid per mouse per day for a period of 14 days. At the end of this period they were placed on the high calcium diet alone for 4 days to remove unabsorbed lead from their alimentary tracts and then killed. The total lead content of each animal was then estimated. The results are shown in Table 8. It will be seen that lead is more easily absorbed under these conditions. The results of this experiment lend support to the theory advanced above, and indicate the importance of the degree of gastric acidity on the absorption of lead.

TABLE 7.

EXPERIMENTAL PERIOD - 14 DAYS.

The results are expressed as (A) mg. total Pb, (B) mg. Pb per 100 g. body wt.

Supplement of Pb (mg. Pb per mouse per day)	0·05		0·10		0·50		1·00	
	A	B	A	B	A	B	A	B
				Low Ca diet				
Exp. 1	0·046	0·224	0·159	0·795	0·254	1·270	0·445	2·42
2	0·143	0·681	0·211	1·055	0·510	2·55	0·909	5·05
3	0·057	0·259	0·149	0·709	0·200	0·87	0·385	1·68
4	0·133	0·665	0·182	0·867	0·450	2·37	0·666	1·33
Average		**0·457**		**0·856**		**1·76**		**2·63**
				High Ca diet				
Exp. 5	0·033	0·173	0·042	0·210	0·055	0·282	0·057	0·317
6	0·042	0·221	0·054	0·284	0·042	0·200	0·050	0·217
Average		**0·197**		**0·247**		**0·241**		**0·267**
				Low Ca diet + Ca glycerophosphate (high Ca diet)				
Exp. 7	0·027	0·142	0·028	0·155	0·029	0·129	0·028	0·143
8	0·026	0·108	0·027	0·117	0·030	0·115	0·036	0·180
Average		**0·125**		**0·136**		**0·122**		**0·162**
				Low Ca diet + olive oil				
Exp. 9	0·133	0·633	0·182	0·860	0·250	1·25	0·366	1·74
10	0·125	0·571	0·252	1·200	0·714	3·10	0·833	3·97
Average		**0·602**		**1·030**		**2·18**		**2·86**
				Low Ca diet + cod liver oil				
Exp. 11	0·128	0·609	0·222	0·925	0·714	3·10	0·800	4·21
12	0·133	0·665	0·159	0·757	0·360	2·00	0·500	2·63
Average		**0·637**		**0·841**		**2·55**		**3·42**
				High Ca diet + cod liver oil				
Exp. 13	0·029	**0·132**	0·040	**0·167**	0·046	**0·230**	0·054	**0·135**

Pb content of control animals = 0·020 ± 0·003 mg. Pb.
Pb content of the high Ca diet = 0·002 mg. Pb per mouse per day.
Pb content of the low Ca diet = 0·002 mg. Pb per mouse per day.

TABLE 8.

Adult Male Mice. High Calcium Diet + 1 mg. Pb (lead acetate) + 0·5 ml. N HCl per mouse per day. Period, 14 days.

	Weight. (g.)	Total lead. (mg.)	Lead. (mg. Pb per 100 g.)
1	28	1·429	5·10
2	19	0·909	4·78
3	21	1·111	5·29
4	21	1·176	5·60
5	23	1·000	4·35
6	17·5	0·667	3·81
7	21·5	0·909	4·23
8	31·5	1·111	3·53
		Average	4·58

The Lead Content of the Blood and Excreta in Suspected Plumbism.

The lead content of the blood and excreta in a number of cases of suspected plumbism has been examined.

The excreta were collected over periods of 3 days while the patients were on the standard diet (see page 57). In three cases the excreta were collected when the patients were on other diets ; Case No.19 was on low calcium diet + ammonium chloride during one period and on high calcium diet during another period; Case No.29 was on the standard diet during one period and on high calcium diet during another period; Case No.30 was on the standard diet during one period and on the standard diet and ammonium chloride during another period. In every case, the blood lead was estimated once or several times but the excreta were not examined in every case. The results are shown in Tables 9a and 9b. In Table 9a are included those cases in which the blood lead did not exceed 100 µg. Pb per 100 ml. Table 9b includes those cases in which the blood lead at some period exceeded 100 µg. Pb per 100 ml. The cases included in Table 9b were all diagnosed as plumbism while of the cases included in Table 9a, only Case No.19 was confirmed as such.

In the cases of lead poisoning diagnosed as such (Table 9b), the blood lead was at some period increased. In some/

some of these cases, the excretion of lead was increased greatly but in others it did not greatly exceed the normal. In Case No. 24 it will be noted that the amount of lead excreted decreased to a level not greatly exceeding the normal as the time of removal from exposure to lead increased. This is in agreement with the findings of Kehoe et alia (1933).

Of the cases shown in Table 9a, only No.19 was finally diagnosed as plumbism as although the blood lead was normal, the excretion of lead was high. In the other cases, the blood lead and lead content of the excreta were normal or raised only slightly.

TABLE 9A.

Case	Occupation	Symptoms	Final diagnosis	Blood-lead (μg./100 ml.	Average of 3 days' excretion in mg. per day			Remarks
					Urine	Fæces	Total	
1	Policeman	Loss of power in the arms	Progressive muscular atrophy	55	0·05	0·19	0·24	—
2	Carter	Numbness of hands and feet	Polyneuritis	65	0·06	0·19	0·25	—
3	Printer	Pain in the back and sides	Neoplasm of cord	56	0·06	—	—	—
4	Plumber	Abdominal pain	—	42	0·06	—	—	—
5	Painter	Colic	Spastic colitis	56	0·06	0·21	0·27	—
6	Stereotyper	Pain in the chest	Coronary disease	52	0·065	—	—	—
7	Metal-worker	Drop-wrist followed by facial paralysis	Cerebral softening	91	0·095	—	—	Died. Autopsy
8	Housewife	Wrist- and foot-drop (bilateral)	Alcoholic neuritis	46	—	—	—	—
9	Acetylene-burner	Diarrhœa, and abdominal pain	—	70	—	—	—	—
10	Painter	Headache, vomiting, loss of power in right arm	—	49	—	—	—	Outpatient
11	Plumber	Headache	—	51	0·05	0·59	0·64	—
12	Painter	Weakness of legs, headache	Arteriosclerosis	90	—	—	—	—
13	Plumber	Vomiting and abdominal pain	Appendicitis	60	—	—	—	Operation
14	Painter	Bad taste in the mouth and abdominal pain	Appendicitis	64	0·17	0·34	0·51	—
15	Printer	—	—	54	—	—	—	Outpatient
16	Printer	Numbness and tingling of hands and feet (pigmentation of mouth)	? Addison's disease	48	—	—	—	—
17	—	Pain in lumbar region	—	33	—	—	—	Outpatient
18	Painter	Weakness and wasting of hands	—	63	—	—	—	—
19	Explosives factory worker	Colic and constipation ; weakness of hands	Chronic plumbism	75	0·11	1·16	1·27	Ordinary diet
					0·17	1·14	1·31	Low-calcium diet and NH_4Cl
					0·11	0·68	0·79	High-calcium diet

TABLE 9B.

Case	Days after first specimen	Occupation	Symptoms	Blood-lead (μg.100 ml.)	Average of 3 days' excretion in mg. per day			Remarks
					Urine	Fæces	Total	
21	0	Red-lead painter	Cramps in legs and abdominal pain	109	0·13	0·40	0·53	—
22	0	Ditto	Admitted in coma ; fits	137	0·07	0·32	0·39	—
23	0	Scrap-lead refiner	Abdominal pain, cramps in legs ; readmission ; pain in legs	123	0·17	0·473	0·643	Blue line, punctate basophilia
	65			132	0·06	—	—	
24	0	Ship-breaker (oxyacetylene)	Colic and constipation	207	0·29	2·53	2·82	Blue line
	20			200	0·16	1·56	1·72	
	41			55	0·06	0·36	0·42	Well ; no pain
	77			92	—	—	—	
	130			61	—	—	—	
25	0	Red-lead painter	Colic and vomiting	80	—	—	—	Punctate basophilia absent
	150		Readmission for return of pain with melæna	322	0·18	0·31	0·49	Definite punctate basophilia
	159			260	—	—	—	
	167			126	—	—	—	
26	0	Hydraulic-press worker	Colic and pain in right leg	113	0·20	0·80	1·0	—
27	0	Painter's labourer	Pains in abdomen, drop-foot, and cramps in hands	350	—	—	—	—
	20			230	0·06	0·17	0·23	
	58			55	—	—	—	
	307			48	—	—	—	
28	0	Red-lead painter	Bad taste in mouth, abdominal discomfort, chronic constipation	348	—	—	—	Blue line
	82			207	—	—	—	
	90			—	0·51	0·14	0·65	
	95			140	—	—	—	
	104			83	—	—	—	
	111			90	—	—	—	
29	0	Bath enameller	Pain in chest (intercostal neuritis)	400	—	—	—	—
	5			340	0·21	0·92	1·13	Ordinary diet
	9			—	0·20	0·9	1·1	High-calcium diet
30	0	Accumulator repairer	Pains in chest	200	—	—	—	—
	25			33	—	—	—	
	32			—	0·15	0·27	0·42	Ordinary diet and NH_4Cl
	38			—	0·09	0·39	0·48	Ordinary diet
	44			80	—	—	—	

The Mobilisation of Lead.

Aub and his co-workers (1926) believed that lead is stored in the bones and as such exerts no toxic action. They believed that the lead present in the soft tissues is solely responsible for the toxic episodes that occur in plumbism. As a consequence, the condition of a person who has absorbed large quantities of lead will depend upon the distribution of lead between the soft tissues and the skeleton. Aub et alia (1926) have shown clinically that the toxic episodes of plumbism are relieved quickly by high calcium therapy and that the clinical improvement is accompanied by a decrease in the amount of lead excreted. This would suggest that lead is being laid down in the skeleton. The clinical side of this work has been confirmed by Belknap (1929).

Two different views are held regarding the treatment of plumbism ; in one a high calcium intake is recommended in order to keep lead stored in the bones; the other favours a process of de-leading as it is considered that a large store of lead in the bones constitutes a potential danger as in metabolic disturbances it is liable to be mobilised and pass into the soft tissues. Aub et alia (1926) support the second view.

Aub et alia (1926) carried out an investigation of/

of agents likely to produce mobilisation of lead. They found that acidosis producing substances e.g. ammonium chloride and mineral acids, potassium iodide, sodium bicarbonate and low calcium intake have this property. Similar results were obtained with parathormone in conjunction with a low calcium intake by Hunter and Aub (1937). In this work the efficiency of the agent was assessed by its ability to increase the total excretion of lead. Litzner, Weyrauch and Barth (1931) could confirm the efficiency of sodium bicarbonate but not of acidosis producing substances, their conclusions being based upon the effect of the agent upon the urinary excretion of lead.

Apart from effects upon excretion, no worker has shown experimentally that these agents do actually produce a liberation of lead from the skeleton and their effect upon the blood lead has not been studied. The following investigations were concerned with these.

A These investigations were carried out on animals, adult male mice being used.

The lead content of a number of mice was increased by feeding them with a low calcium diet containing lead (as lead acetate) at the rate of 1 mg. Pb per mouse per day for a period of 14 days.

In the first experiment, the animals were fed on/

on the low calcium diet and lead as described above. At the end of the period, one third of the animals were kept on low calcium diet, but without lead for 4 days and then killed (a). The remainder were placed on a high calcium diet for 7 days and then half of their number were killed (b). The remainder were placed on the low calcium diet, and then killed at the end of 7 days (c). In each mouse, the soft tissues were separated from the skeleton and their lead contents estimated. The percentage of the total lead present in the soft tissues and skeleton was calculated. The results are shown in Table 10.

It will be seen that following absorption of lead on a low calcium diet, lead predominated in the soft tissues. The effect of high calcium diet was to cause lead to predominate in the skeleton but it should be noted that the amount of lead in the soft tissues is not negligible and is probably quite capable of producing damage. The transference of the mice from high calcium to low calcium diet resulted in an increased percentage of lead in the soft tissues. These results indicate that the distribution of lead between the soft tissues and skeleton is influenced by the calcium content of the diet.

In the following experiments the effect of other agents were studied. These were,

1. Potassium iodide with low calcium intake,

2/

2. Sodium bicarbonate with low calcium intake.

3. Ammonium chloride with low calcium intake.

4. Potassium iodide with high calcium intake.

5. Sodium bicarbonate with high calcium intake.

6. Ammonium chloride with high calcium intake.

The lead content of adult male mice was increased as described above. They were then placed on a high calcium diet for 7 days in order to produce a predominance of lead in the skeleton. The animals were then placed on treatment for 7 days, after which they were killed and the lead content of their soft tissues and skeletons estimated. The percentage of the total lead present in soft tissues and skeleton was then calculated. The results are shown in Table 11.

It will be seen that these substances produced mobilisation of lead on both high and low calcium intake. That the substance was producing an effect on low calcium intake, which itself has a mobilising effect, is indicated by the higher percentage of lead in the soft tissues.

Several important facts emerge from this investigation. Acidosis producing substances, which are usually used in conjunction with a low calcium intake to produce mobilisation, are effective on a high calcium intake. Acidosis from metabolic causes should therefore be avoided in plumbism as a high calcium intake cannot neutralise/

neutralise its effect. Potassium iodide and sodium bicarbonate should not be administered to persons with an excess of lead unless mobilisation of lead is desired. 'Stomach' powders, containing sodium bicarbonate, are common offenders in this respect.

Separation of the soft tissues and bones of mice.

The animal was skinned and the carcase heated with a small quantity of distilled water in a silica dish on a steam bath. At the end of 30 minutes, the bones could be separated quite easily from the soft tissues. It was considered that under such conditions, possible solution of lead present in the bones would be a minimum. The skin was included with the soft tissues.

TABLE 10

The results are expressed in mg. Pb.

A. *On low calcium diet alone.*

	Mouse No. 1.	Mouse No. 2.	Mouse No. 3.
Lead content of soft tissues	0·154	0·168	0·150
Lead content of skeleton	0·105	0·125	0·100
Total lead	0·259	0·293	0·250
Percentage of lead in skeleton	40·6	42·8	40
Percentage of lead in soft tissues	59·4	57·2	60

B. *Low calcium diet followed by high calcium diet.*

	Mouse No. 4.	Mouse No. 5.	Mouse No. 6.
Lead content of soft tissues	0·077	0·089	0·080
Lead content of skeleton	0·250	0·312	0·282
Total lead	0·327	0·401	0·362
Percentage of lead in skeleton	76·4	77·8	77·9
Percentage of lead in soft tissues	23·6	22·2	22·1

C. *Low calcium diet followed by high calcium diet, followed by low calcium diet.*

	Mouse No. 7.	Mouse No. 8.	Mouse No. 9.
Lead content of soft tissues	0·077	0·087	0·090
Lead content of skeleton	0·143	0·151	0·178
Total lead	0·220	0·238	0·268
Percentage of lead in skeleton	65	64	62
Percentage of lead in soft tissues	35	36	38

TABLE II.

-Mobilization of Lead by Addition of 50 mg. per Mouse per Day of Ammonium Chloride, Potassium Iodide and Sodium Bicarbonate to Low and High Calcium Diets.

Low Calcium Diet.

	No addition.			+ ammonium chloride.			+ potassium iodide.			+ sodium bicarbonate.		
Mouse No.	1.	2.	3.	4.	5.	6.	7.	8.	9.	10.	11.	12.
Lead content of soft tissues (mg.)	0·050	0·050	0·066	0·110	0·136	0·104	0·091	0·146	0·121	0·110	0·100	0·108
Lead content of skeleton (mg.)	0·100	0·091	0·132	0·140	0·148	0·110	0·099	0·154	0·146	0·800	0·140	0·140
Total lead (mg.)	0·150	0·141	0·198	0·250	0·284	0·214	0·190	0·300	0·267	0·190	0·240	0·248
Per cent. lead in soft tissues	33·4	35·5	33·6	44	48	48·6	48	48·7	45·3	57·9	41·6	44
Per cent. lead in skeleton	66·6	64·5	66·4	56	52	51·4	52	51·3	54·7	42·1	58·4	56

High Calcium Diet.

	No addition.			+ ammonium chloride.			+ potassium iodide.			+ sodium bicarbonate.		
Mouse No.	13.	14.	15.	16.	17.	18.	19.	20.	21.	22.	23.	24.
Lead content of soft tissues (mg.)	0·049	0·039	0·066	0·124	0·081	0·104	0·082	0·090	0·100	0·080	0·090	0·104
Lead content of skeleton (mg.)	0·144	0·145	0·233	0·125	0·098	0·106	0·077	0·040	0·120	0·160	0·090	0·111
Total lead (mg.)	0·193	0·184	0·299	0·249	0·179	0·210	0·159	0·130	0·220	0·240	0·180	0·215
Per cent. lead in soft tissues	25·4	26·7	23	50	45·2	49·5	51·6	69·2	45·4	33·3	50	48·3
Per cent. lead in skeleton	74·6	73·3	77	50	54·8	50·5	48·4	30·8	54·6	66·7	50	51·7

B. In this investigation, the effect of certain agents upon the lead content of the blood in plumbism was investigated. In three of the cases, the lead content of the excreta was determined. The results are shown in Charts 1 - 11.

The effect of variation in the calcium content of the diet upon lead mobilisation as shown by the amount of lead in the blood is illustrated by Cases 4 - 11, a high calcium intake being followed by a marked fall in blood lead. As the treatment in some cases lasted over a period of weeks, this may have been due in part to loss of lead by excretion. That the calcium content of the diet does exert a powerful influence is shown by Case 7, who was placed alternately on high and low calcium intake. On high calcium intake, the lead content of the blood fell while on low calcium intake it rose.

The effect of an acidosis producing substance viz. ammonium chloride in conjunction with a low calcium diet was studied in Cases 1 - 3. In Cases 1 and 3, the effect was to produce marked increases in blood lead, the levels being very much higher than with low calcium diet alone. In Case 1, in which the rate of lead excretion was investigated, the total amount of lead excreted was increased but not to anything like the same level as the increase/

increase in blood lead. In Case 3, high calcium intake and acidosis therapy were alternated several times. On each occasion responses were obtained as evidenced by changes in the blood lead. In Case 2, the blood lead was hardly affected by ammonium chloride treatment although the rate of lead excretion was increased. This may be explained by the fact that this subject had been exposed to lead for only a short period and consequently his store o lead would be comparatively low. Case 11 illustrates the effect of potassium iodide upon the blood lead. In this case also, the increase in blood lead was not accompanied by a similar increase in lead excreted.

Changes in the excretion of lead as the result of mobilisation were most marked in the faeces, the urine being hardly affected.

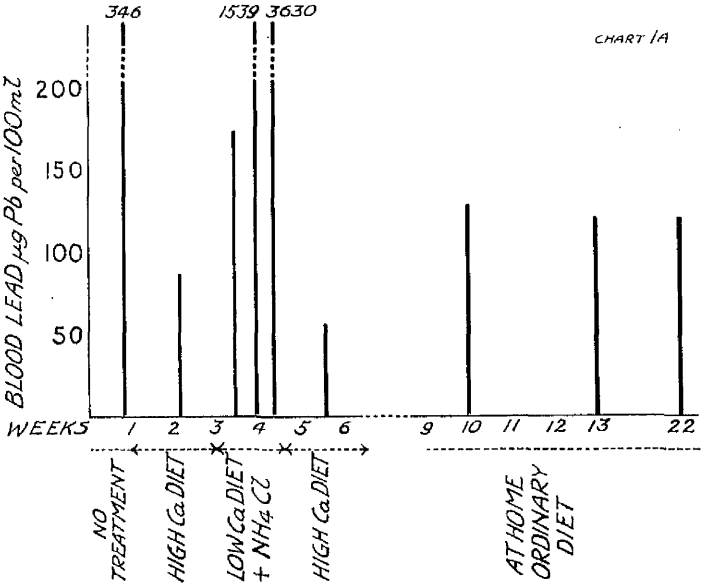
Case 1.

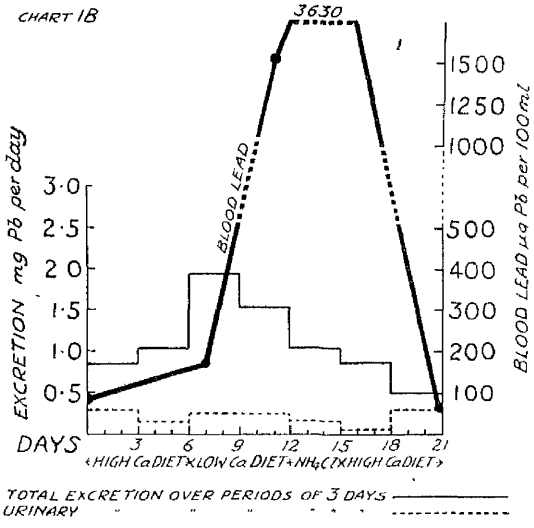
Lead burner (battery works)

Male - 29 years.

Lead poisoning.

The investigations were carried out over an extended period. Following high calcium intake for 14 days, the patient was placed on low calcium diet and ammonium chloride for 9 days. Following this, high calcium diet was maintained. During the beginning of the last period, injections of calcium gluconate were given. During the period of medication with ammonium chloride and for 6 days before and after it, the excreta were collected in periods of 3 days and the lead content estimated. The lead content of the blood was estimated at intervals. The results are shown in Charts 1a and 1b.





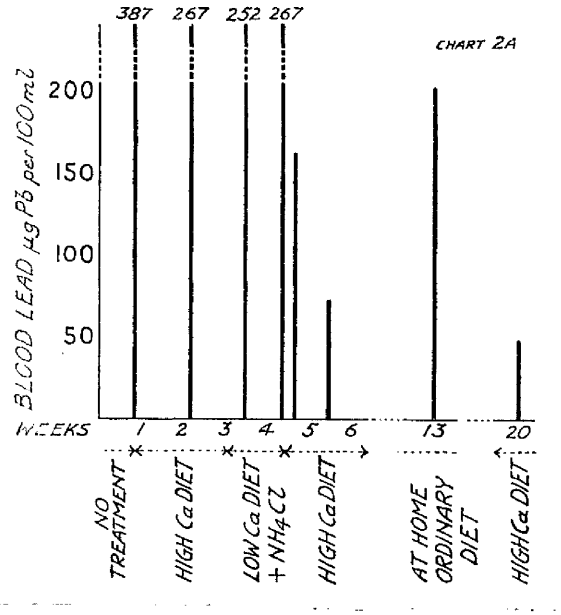
Case 2.

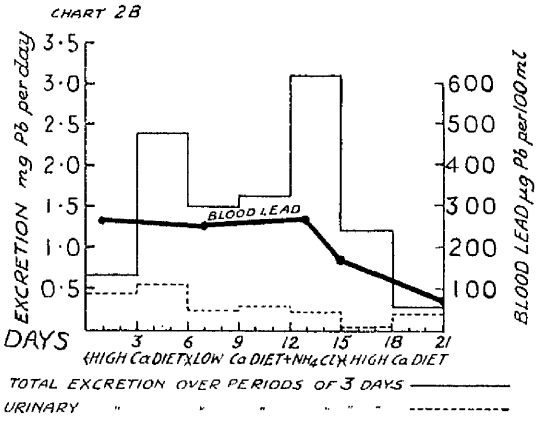
Red lead worker.

Male - 32 years.

Lead poisoning.

This patient was treated similarly to Case 1. The results are shown in Charts 2a and 2b.





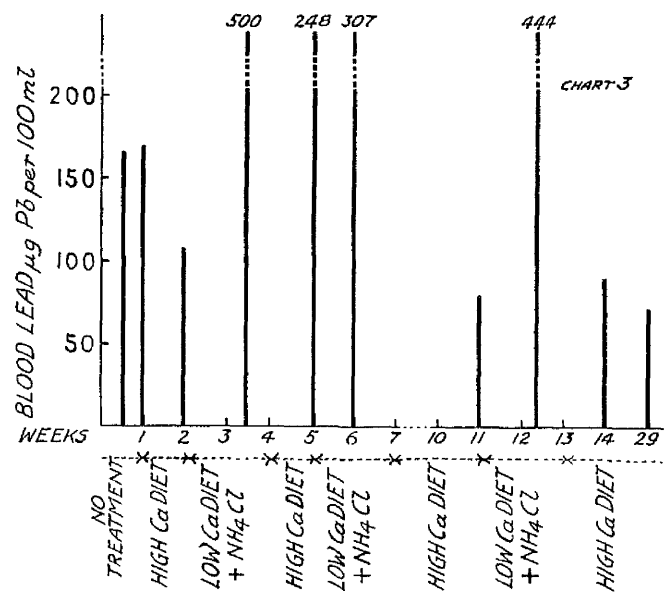
Case 3.

White lead worker.

Male - 66 years.

Lead poisoning.

This patient was placed on alternate periods of high calcium intake and low calcium intake and ammonium chloride. Finally he was maintained on high calcium intake. The lead content of the blood was estimated at intervals. The results are shown in Chart 3.



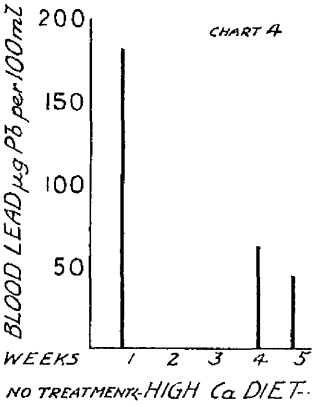
Cases 4, 5 and 6.

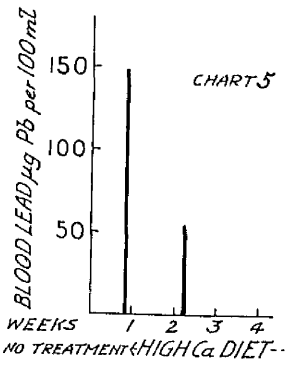
Lead workers with vague symptoms.

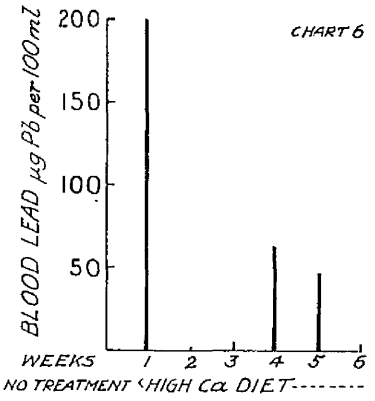
Males - 23, 33 and 56 years.

Increased lead absorption

The lead content of the blood of these patients was determined initially and then after a period of high calcium intake. The results are shown in Charts 4, 5, and 6.







CASE 7.

Lead poisoning due to a lead contaminated drinking water.

Female - 27 years.

The effect of high and low calcium intake upon the lead content of the blood was investigated. The results are shown in Chart 7.

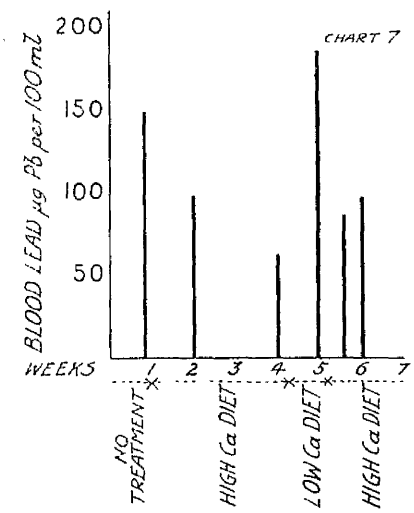
Cases 8, 9 and 10.

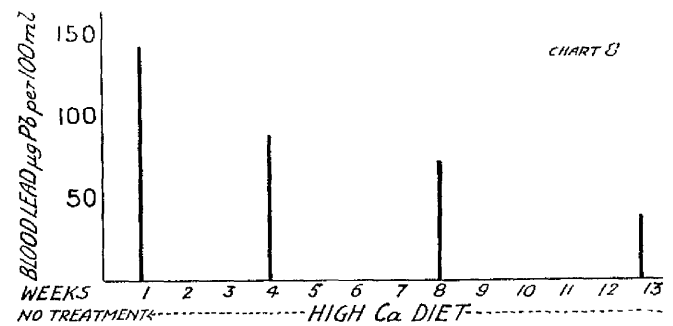
Increased lead absorption due to a lead contaminated drinking water.

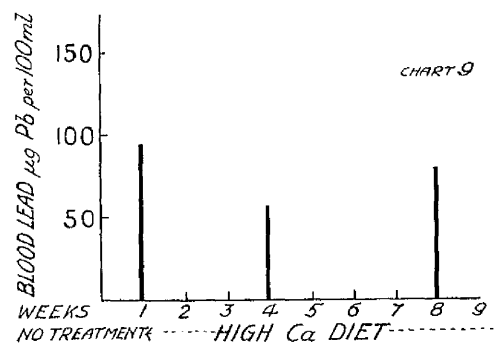
Males - 34 and 7 years.

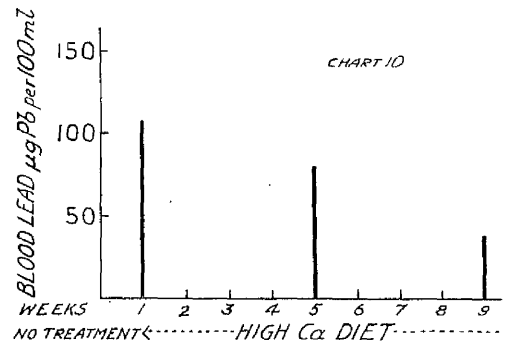
Female - 9 years.

The lead content of the blood was determined initially and then after a period of high calcium intake. The results are shown in Charts 8, 9 and 10.









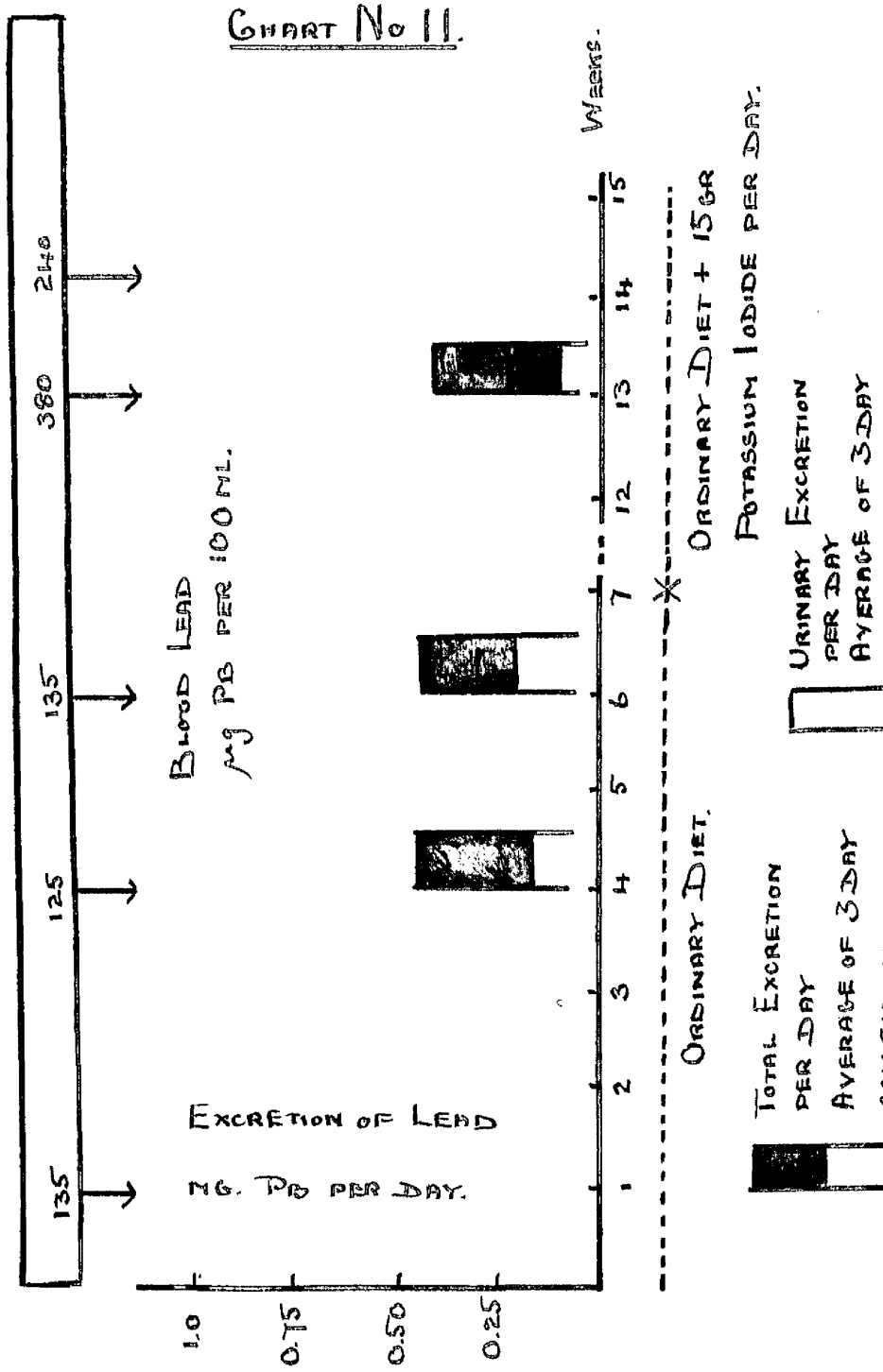
Case 11

Solder maker

Male

Lead poisoning

The effect of potassium iodide upon the blood lead and the amount of lead excreted was investigated. The result are shown in Chart No. 11.



The Lead Content of the Tissues in Increased Lead Absorption.

Tissues were obtained from 4 cases with histories of abnormal exposure to lead and their lead contents estimated. The results are shown in Table 12.

The lead content of the tissues of the second case are within normal limits except rib which shows a slight increase. The exposure was probably only slight.

In the other cases, all the soft tissues show marked increases. In bone, the most marked increases are in rib and vertebra .

From an examination of 'normal' tissues, one concludes that the long bones have the greatest affinity for lead. During life there appears to be an accumulation of small quantities of lead, most of which is laid down in such bones as femur and tibia but not in bones of the structure of rib and vertebra. The probability is that such lead is difficult to mobilise. When comparatively large quantities of lead are being absorbed over a short period, it appears to be laid down in such bones as rib and vertebra rather than in tibia and femur. This is probably due to their more vascular nature, and it would appear that such lead is comparatively easy to mobilise. If it were possible to compare the total lead content of an old person with no history of abnormal exposure/

exposure to lead with that of a young person with a history of abnormal exposure to lead and with active symptons of plumbism, one might find little difference, the difference between the two conditions being due to differences in distribution, chiefly amongst the bones.

In a chemical examination of the tissues of a case of suspected plumbism we have in the past been inclined to lay stress on the results obtained from the long bones. The results obtained here indicate that the data obtained from rib and vertebra are more important.

TABLE 12.

The results are expressed in mg. Pb per kg. fresh tissues.

	1.	2.	3.	4.
Sex.	M.	M.	M.	F.
Age.	41	60	60	26.
Liver	4.50	2.40	5.40	7.14
Kidney	1.00	2.88	2.00	4.62
Brain	1.00	-	1.36	3.08
Rib	119.4	22.0	50.6	52.1
Vertebra	18.8	8.5	13.3	-
Femur	-	-	50.6	52.1
Tibia	-	-	78.9	53.3
Remarks	Painter	Printer	Metal worker.	Lead contaminat drinking water.

The Excretion of Lead.

Kehoe et alia (1933) have shown that after removal from exposure to lead, excretion falls rapidly until levels just exceeding the normal are reached. The writer considered that it would be of interest to determine the rate at which recently absorbed lead could be eliminated.

The lead content of adult male mice was increased by the method described already. The animals were then placed on high calcium diet for 7 days to remove unabsorbed lead from their alimentary tracts and to produce a transference of lead from soft tissues to skeleton. They were then placed in separate glass jars, floored with filter paper. Half of the mice were placed on high calcium diet and the remainder on low calcium diet for a period of 14 days. At the end of this period the animals were killed and their total lead contents estimated. The lead content of the material in the jars was then estimated. After allowing for the lead content of the filter paper and the diet consumed during the experimental period, the amount of lead excreted was calculated. From these figures, the percentage of the original total lead that had been eliminated, was calculated. The results are shown in Table 13.

It will be seen that recently absorbed lead may be eliminated very rapidly. On low calcium diet, about 60% was excreted while on high calcium diet about 25% was excret

TABLE 13.

-Lead Excreted in 14 days on Low and High Calcium Diets.

Low Calcium Diet.

	Mouse No. 1.	2.	3.	4.
Total lead (mg.)	0·105	0·150	0·141	0·099
Excreted lead (mg.)	0·175	0·225	0·220	0·198
Original total lead (mg.)	0·280	0·375	0·361	0·297
Per cent. original total lead excreted	62·5	60	60·9	66·7

High Calcium Diet.

	Mouse No. 5.	6.	7.	8.
Total lead (mg.)	0·193	0·184	0·289	0·320
Excreted lead (mg.)	0·060	0·049	0·068	0·082
Original total lead (mg.)	0·253	0·231	0·357	0·402
Per cent. original total lead excreted	23·7	21·2	19	20·4

Discussion.

A method has been described for the estimation of lead in biological materials. It is specific for lead and gives accurate results. An estimation may be carried out quickly and as no complex apparatus is required, it is very suitable for routine use. In the hands of other workers it has proved successful. It cannot be over emphasised that in work of this nature, reliable data cannot be obtained unless the method of analysis is satisfactory. Much of the earlier work on the biochemistry of lead suffered from this defect.

It has been shown quite conclusively that lead occurs in the tissues and excreta of persons with no history of exposure to lead other than the 'normal' hazard. The origin of this lead has been discussed viz. food and lead containing dust. The distribution of lead throughout the body is however uneven. The highest concentrations appear to be situated in the skeleton but even here, the distribution is uneven, for such bones as femur and tibia contain higher concentrations than rib and vertebra. Of the soft tissues examined, liver was found to have the highest concentration. The concentration of lead in the long bones appears to increase with age whereas that of the other tissues is unaffected. There appears to be a very slight retentio of/

of lead throughout life, most of which is laid down or stored in the long bones. All lead absorbed from the alimentary tract must pass through the liver, where much of it is trapped and excreted, thus protecting the other tissues Lead absorbed from the respiratory tract does not pass directly through the liver and hence absorption from this site is more dangerous. The results indicate that as for other metals e.g. iron and copper, liver tissue has a marked affinity for lead.

Lead is a constant constituent of blood. In the normal, the range is narrow and similar ranges have been obtained by workers in various parts of the World.

Lead occurs in the organs of foetuses, being derived from the mother, presumably through the placental circulation. This indicates that at no time is man free of lead.

Lead has been found to occur in the excreta. This is to be expected as it has been shown that foodstuffs, drinking water and even the atmosphere contain traces of lead. It has been stated that the excretion of metals such as lead can occur through the intestine as well as the kidney. This statement is sometimes difficult to prove owing to the difficulty in distinguishing between unabsorbed and excreted metal in the faeces. The writer, however, has produced evidence to show that lead can be excreted through the intestine and that the excretion by this route may/

may exceed that by the kidney. The kidney appears to have no power of concentrating lead, as the lead content of urine is much smaller than that of blood. Similar results have been obtained with iron and copper (later sections).

Evidence has been presented to show that the absorption of lead from the alimentary tract is influenced by the composition of the diet. Absorption is high on a low calcium diet and low on a high calcium diet. Reasons have been put forward to account for this. Fat and vitamin D appeared to have no influence. Absorption of lead was increased very much when mineral acid was added to the diet. This indicates the importance of the gastric acidity in lead absorption.

The effect of various agents upon the distribution of lead between the soft tissues and skeleton has been studied. Low calcium intake, ammonium chloride, sodium bicarbonate and potassium iodide produce mobilisation of lead, as evidenced by the high percentage of the total lead present in the soft tissues of mice after treatment with these. High calcium intake has the reverse effect. The effect of sodium bicarbonate, ammonium chloride and potassium iodide was shown to be independent of the calcium content of the diet. On high calcium intake, the quantity of lead present in the soft tissues could not be regarded as being negligable and probably was quite capable of producing/

producing damage. The effect of the above agents upon the lead content of the blood in men with histories of increased exposure to lead was studied. Mobilising agents produced marked increases in blood lead while high calcium intake produced a marked fall.

In man it was found that mobilising agents produced much more marked changes in the blood than in the excreta. The question of the wisdom of de-leading arises s mobilisation may result in an influx of lead into the soft tissues without a compensatory increase in excretion. On the other hand, high calcium therapy may leave non-negligable amounts of lead in the soft tissues. The obvious answer, is more stringent preventative measures. It is suggested that de-leading should never be attempted without adequate laboratory control. This should include frequent estimations of the lead content of the blood and excreta.

It has been shown that in mice, after removal fr exposure to lead, absorbed lead may be eliminated very rapidly. Frequently persons with suspected lead poisoning are not admitted to hospital for examination until some time has elapsed since the last exposure and as a result the amount of lead excreted may not greatly exceed the normal. Under such conditions, it cannot be assumed that a comparatively low lead excretion suggests a mild exposur

References/

References to individual susceptibility to lead are often made. The writer believes that much can be explained by the biochemical knowledge that has accumulated. Absorption of lead from the alimentary tract is influenced by the composition of the diet and by the degree of gastric acidity, both very variable factors. The clinical condition is influenced not by the total amount of absorbed lead but by its distribution between the soft tissues and skeleton. Low calcium intake, acidosis from metabolic disorders and the indiscriminate use of such drugs as sodium bicarbonate and potassium iodide will produce mobilisation. Such factors must be taken into consideration when the question of susceptibility arises.

Various procedures have been used in the diagnosis of plumbism ; 1. clinical, 2. haematological, 3. radiological, 4. chemical. Of these, the chemical examinati produces the only evidence of increased lead absorption. Chemical examinations have usually been carried out on isolated samples of urine and/or faeces. The writer conside that chemical examinations should be much fuller. The examination of the lead content of the excreta often gives important information, but the collection should be made while the patient is on a diet of known lead content. This and the interpretations to be derived therefrom have been discussed already. Of particular value, is the estimation of the lead content of the blood. This gives information as/

as to whether the lead is confined mainly to the skeleton or is present in high concentration in the soft tissues. In a case of plumbism the blood lead could be normal due to conditions of diet etc. It is suggested that in a case of suspected plumbism, in the event of the blood lead being normal, the estimation should be repeated after the patient has been under one of the lead mobilisation procedures. An examination of the lead content of the excreta at the same time would give confirmatory evidence. Lanza (1935) has suggested that a considerable amount of mild lead poisoning occurs, much of which escapes diagnosis. In view of this, the above technique appears to be justified

There is no justification in making a diagnosis of lead poisoning on evidence of increased lead absorption alone, in fact the final diagnosis must remain with the clinician. Clinical symptoms are often obscure. Satisfactory conclusions can only be made through the closest co-operation between clinician and biochemist.

Under natural conditions, lead occurs in food-stuffs and animal tissues. The question must remain open as to whether these minute amounts of lead are accidental or have some physiological function as in the case of copper and cobalt.

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Iron and Copper

INTRODUCTION

(a) Iron.

The amount of iron in the body is small but its functions are of the highest importance. It is estimated that the iron content of an adult man or woman is about 3 g., most of which is in the form of haemoglobin. There is no considerab reserve of relatively inactive iron in the body, hence if the intake fails to equal the output, anaemia soon develops. Investigations of iron metabolism have as a result been largely concerned with the study of haemoglobin formation and anaemia. In addition to respiratory function, there is evidence that iron compounds are concerned with a large number of other biological processes.

It has long been known that iron is essential to the nutrition of both animals and plants and that it is present in the ash of such. A few decades ago it was assumed that iron existed in the food as oxide or phosphate and that haemoglobin was formed by the combination of iron and protein. Results obtained during the latter half of the 19th century threw doubt upon the utilisation of inorganic iron for the productio of haemoglobin. It was believed that iron salts when injected acted as poisons and when given by mouth were almost quantit: :atively eliminated in the faeces. In spite of this, inorganic iron salts were used and found efficient in the treatment of anaemia. To harmonise these views with clinical experience, it was suggested that inorganic iron might act by absorbing

the hydrogen sulphide of the intestine and thus protecting the food iron. Another view was that inorganic iron salts might act by stimulation of the absorbing tissues [Bunge (1884), von Hösslin (1882), Gottlieb (1891), Socin (1891), Woltering (1895), Tartakowsky (1903, 1904)].

Bunge examined the iron present in a number of foodstuffs and could detect but traces of inorganic iron. From egg yolk he isolated a compound containing iron which he called 'haematogen' and regarded it as the precursor of haemoglobin.

Since the World War the question of iron absorption has become very much clearer. It has been shown that inorganic iron salts are absorbed and converted into haemoglobin whereas iron compounds of the nature of haematin are not [Hart et alia (1928), Elvehjem (1932)].

Elvehjem and his co-workers found that the efficiency of a foodstuff to cure nutritional anaemia in rats was dependent on its inorganic iron content. They estimated the inorganic iron content of various foodstuffs directly, by the use of $\alpha\alpha'$-dipyridyl [Elvehjem et alia (1933), Elvehjem et alia (1934), Sherman et alia (1934)]. They found that as a rule, a large part of the total iron of foodstuffs was in the inorganic form.

Following this work a large amount of research has been concerned with the absorption of iron from the alimentary tract, humans being used as experimental subjects. These have indicated that upon a large intake of inorganic iron, the body was capable of absorbing large amounts and that it was not subsequently excreted [Hutchison (1937), Brock (1937),

Widdowson and McCance (1937)]. In anaemia it has been suggested that only a small amount of this absorbed iron is converted into haemoglobin.

It is generally agreed that inorganic iron to be absorbed must be in a dialysable form and that absorption takes place in the upper part of the small intestine. Lintzel (1931) and Heubner (1926) state that ferric but not ferrous iron forms non-ionisable complexes with proteins. As a result they believe that ferrous iron is absorbed easily but that ferric iron must be reduced in the intestine prior to absorption. Whipple and Robcheit-Robbins (1936), Hahn (1937) and Brock and Hunter believe that ferric iron can be absorbed as such.

The gastric acidity has been recognised as being a very important factor in the assimilation of iron, especially the food iron and it has been recognised that a lack of secretion of hydrochloric acid in the stomach is responsible fo certain forms of hypochromic anaemia [Lintzel (1931), Starkenstein (1934), Strauss (1934), Kellog and Mettier (1936), Barer and Fowler (1937)].

The question of the iron content of the human diet has received attention. Studies have failed to show any correlation between the total iron content of the diet, the concentration of other constituents and the incidence of anaemi

(b) Copper.

Although copper as a metal has been known for centuries, the importance of its compounds in biological

activities in other than certain invertebrates has been known only recently.

It has been known for a long time that copper is a constituent of plants, Meissner (1817) being probably the first worker to note this. Sarzeau (1830) noted the presence of copper in plants and made quantitative estimations. Deschamps (1848) showed that a relationship existed between the copper in plants and that in soil and Chevreul (1868) suggested that copper was distributed widely in organic matter Later work showed the universal distribution of copper throughout the plant world and the suggestion was made that it was essential in plant metabolism as the highest concentrations were found present in the more active parts e.g. young shoots, leaves etc. [Maquenne and Demoussy (1920), Guérithault (1920), Fleurant and Levi (1920), Bertrand (1920)].

It has been known for a long period that certain marine animals contain considerable amounts of copper. Harless (1847) detected copper in Eledone and Helix pomatia and showed that it did not exist as a salt. Fredericq (1878) obtained a copper containing protein from the blood of Octupus vulgaris and although he was the first to give this compound a definite physiological function, earlier workers had suggested the importance of a compound of this nature in the blood of invertebrates [Blasius (1866), Bert (1867)]. Since this period a considerable amount of investigation has been carried out into the chemical nature and physiological properties of the haemocyanins [Parsons and Parsons (1923), Begemann (1924),

Redfeld et alia (1926), Stedman and Stedman (1926), Hogben and Pinhey (1926, 1927)]. The occurrence of different haemocyanin has been established. The chemistry of the haemocyanins has received attention but no copper compound of the nature of haematin has been isolated [Dhéré and Burdell (1919), Dhéré (1920), Conant, Dersch and Mydam (1934)].

An interesting copper compound is turacin, a pigment found in the feathers of the South African bird, Turaco, and was first described by Church (1869). It is believed to be a copper porphyrin. Laidlow (1904) showed that haematoporphyrin would combine with iron and copper, the compound with the latte resembling turacin. Fischer and Hilger (1923) have shown that turacin porphyrin is probably uroporphyrin, a pigment excreted in the urine of man in conditions known as congenital porphyrinuria.

The distribution of copper in the tissues of higher animals has only received serious attention within recent years [Bodansky (1921), McHargue (1925, 1926), Cunningham (1931)].

The first definite evidence that copper is essential in the metabolism of the higher animals was obtained by the Wisconsin workers who showed that it is necessary for the formation of haemoglobin. They fed young animals (rabbits, rats etc.) with cows' milk, a diet which as well as being deficient in iron has a low copper content, and eventually an anaemia developed. The administration of purified iron salts produced no response until a copper salt was included also [Hart et alia (1925, 1927, 1928), Waddell et alia (1929)].

This work was confirmed by McHargue et alia (1928), Krauss (1929), Titus et alia (1929) and Cunningham (1931).

Some workers have attempted to show that purified iron salts were effective in curing this type of nutritional anaemia [Beard and Myers (1931), Keil and Nelson (1931)], while others have suggested that elements other than copper have a similar effect [Titus et alia (1929), Myers and Beard (1929, 1931)]. All of this work has since been discounted.

A considerable amount of work has been done on the relation of copper in the treatment of certain types of anaemia in man.

A number of workers have presented evidence to show that iron in combination with copper is more effective than iron alone in the treatment of nutritional anaemia in children [Lewis (1931), Josephs (1931), Bloxsom (1932), Caldwell and Dennett (1932), Cason (1934), Hutchison (1937)]. These results are not surprising as it has been shown that the copper content of ordinary infant dietaries does not greatly exceed the supposed requirement. Ramage, Sheldon and Sheldon (1933) found reduced copper contents in the livers of infants during the nursing period and increased values as soon as they were placed on a mixed diet. Parsons and Hawksley (1933) and Mackay (1933) are inclined to believe that copper deficiency does not play any important part in common nutritional anaemias in babies in England.

It has been reported that better results were obtained when certain types of anaemia in adults were treated

with iron in combination with copper than with iron alone [Dwyer (1930), Mills (1930, 1931), Waugh (1931), Adamson and Smith (1931), Gross (1932), Dameshek (1933), Wintrobe and Biebe (1933), Machold (1934)]. Other workers have concluded that in the treatment of the usual cases of anaemia in adults, no advantage is gained by the inclusion of copper in therapeuti preparations of iron [Heath (1933), Bethell et alia (1934), Davidson and Leitch (1934)]. It is generally accepted that iron and copper are ineffective in pernicious anaemia.

Workers have found that iron in combination with copper is effective in combating nutritional anaemia in sucklin pigs [Hart et alia (1930), Hunt and Carroll (1933)].

In higher animals it has been shown that copper has no influence upon the absorption of iron but influences its conversion into haemoglobin [Cunningham (1931), Josephs (1932), Elvehjem and Sherman (1932)].

The quantity of copper in foodstuffs has received considerable attention. No foodstuff has been found free of copper but great variations have been found, the figures ranging from 0.1 mg. per k.g. fresh celery to 44.1 mg. per kg. fresh calf liver.

Copper catalyses the oxidation of sulphydril compounds. Factors affecting this reaction have been studied and their relation to biological activities discussed [Matthews and Walker (1909), Harrison (1927), Warburg (1927), Elvehjem (1930), Elliott (1930), Summer and Poland (1933), Hellerman et alia (1933), Bersin and Legemann (1933), Michaelis and Runström

(1934)].

The value of copper in the growth of higher plants has been reviewed by Hoagland (1932). Felix (1927) and Allison (1927) have improved the production of a large variety of plants on peat soils by the addition of copper. It has been shown that the production of cytochrome in yeast necessitates the presence of copper as well as iron [Elvehjem (1931), Parsons and Hickmans (1933)].

The Determination of Iron in Biological Materials.

For the estimation of small amounts of iron such as are present in biological materials, several reagents and methods are available. These may be enumerated as follows:

1. Potassium thiocyanate - colorimetric.
2. Thiolacetic acid - colorimetric [Lyons (1927)].
3. $\alpha\alpha'$dipyridyl - colorimetric [Hill (1931)].

Of these methods, the reaction between ferric iron and potassium thiocyanate is probably the oldest. No reaction occurs with ferrous iron. Various methods employing this reaction are in existence. Some workers prefer to compare the colours in aqueous solution while others extract the ferric thiocyanate with amyl alcohol prior to colorimetric comparison, Certain difficulties may be experienced. Pyrophosphates depress the reaction partially or completely. When it is necessary to ash organic materials containing much phosphorus, a wet digestion method in which an excess of sulphuric acid is present at the end of the reaction is essential. Elvehjem (1930) has employed ignition to destroy organic matter but heated the ash with alkali to convert pyrophosphates into orthophosphates. Kennedy (1927) and Elvehjem and Hart (1926) state that pyrophosphates are not formed during sulphuric-percholoric acid digestion, but the writer has noted that unless excess of sulphuric acid is present at the end of the reaction, they are liable to be formed. McFarlane (1932) has shown that the depth of colour produced is influenced by the

concentrations of thiocyanate and sulphuric acid present.

The reaction between iron and thiolacetic acid was first described by Andreasch (1879). Lyons (1927) showed that the reaction could be employed to estimate small amounts of iron. When thiolacetic acid is added to a solution of an iron salt, ferric or ferrous, and alkali added, a purple colour is produced. Lyons believed that when thiolacetic acid is added to a solution of a ferric salt, a substance $Fe\left[S.CH_2.COOH\right]_3$ is formed which is immediately reduced to $Fe\left[S.CH_2.COOH\right]_2$ and this in alkaline solution gives an intensely coloured ion - $Fe\left[S.CH_2.COO\right]^{=}$. The presence of oxidising agents prevents the formation of the colour due to the formation of dithioglycollic acid which does not give the reaction. Lyons found that strong bases produced rapid colour fading and that ammonia was the most satisfactory to use.

Lyons showed that the reaction was uninfluenced by the presence of a large number of substances, both inorganic and organic. Hanzal (1933) and Burmester (1934) have applied the reaction to the estimation of iron in biological materials.

Hill (1931) has used $\alpha\alpha'$dipyridyl to estimate iron in biological materials. This substance reacts with ferrous iron but not ferric iron to produce a red colour. Ferric salts will react if a reducing agent e.g. sodium hydrosulphite is added.

The writer wished to make direct determinations, if possible, of non-haematin iron in biological materials and it

was considered that thiolacetic acid might serve this purpose. The range of the reaction was studied. A standard solution of iron was prepared containing 0.0855 g. of iron alum in 1 litre of 1% sulphuric acid (1 ml. ≡ 0.01 mg. Fe). Into a series of tubes were measured, 0.001, 0.002, 0.003, 0.004, 0.005, 0.01, 0.02, 0.03, 0.04, and 0.05 mg. Fe. These were diluted to 5 ml. with water, 2 drops thiolacetic acid added and then 1 ml. ammonia, sp. gr. 0.88. It was found that in the tubes containing 0.005 - 0.05 mg. Fe, the depths of colour were strong enough to permit comparison in a colorimeter. On comparison it was found that the depth of colour was proportiona to the concentration. Although distinct differences in depth of colour could be distinguished in the tubes containing 0.001 - 0.005 mg. Fe, they were too weak to compare in a colorimeter. To estimate amounts of iron of this order, it has been found best to use direct comparison with a series of standards in standard tubes. The reaction has been found to be uninfluenced by the presence of such substances as amino acids, pyrophosphat :es, citrates etc.

A number of estimations of total iron have been made in biological materials. The majority of these have been made on mice and rat tissues. In these, thiolacetic acid has been used. Since the colour is developed in a medium with an alkaline reaction interference from calcium phosphate, if present, appeared probable. It has been found that precipitate of calcium phosphate may be removed by centrigulisation and accurate results may be obtained.

In general, organic matter was destroyed by ignition in a silica dish over a bunsen burner under the same conditions as that used in lead estimations.

A few estimations of the iron content of urine and egg have been made. In these, the thiocyanate reaction was used, as certain technical difficulties made the use of thiolacetic acid impracticable.

$\alpha\alpha'$-dipyridyl has been used as a test for ferrous iron in certain sections of this thesis. It has been stated that it is effective over a range pH 3.5 - 8.5, this has been confirmed.

Non-haematin Iron.

It is generally recognised that iron exists in biological materials in at least two forms (a) haematin iron, (b) non-haematin iron. Non-haematin iron has received considerable attention within recent years.

In the hens' egg, nearly all the iron is located in the yolk and many believe that it exists as an organic complex with protein like properties. This complex was first studied by Bunge (1884) who named it 'haematogen', and regarded it as the precursor of haemoglobin. Hugounenq and Morel (1905) found that decomposition of 'haematogen' by acids produced a black pigment, containing 2.6% iron, which they called 'haematovin'.

Hill (1931) has submitted evidence to show that all the iron in egg yolk is present as ferric iron, probably as colloidal ferric hydroxide and that no organic complex exists. He found that when $\alpha\alpha'$-dipyridyl was added to a suspension of egg yolk in an acid acetate buffer, no colour developed, but on the addition of sodium hydrosulphite a red colour was produced and determined the amount of iron present by comparison with a standard. He found that the iron content of egg yolk as determined by this simple procedure agreed with the iron content estimated after ashing.

McFarlane (1932) found that lecitho-vitellin prepared from egg yolk contained a fair percentage of iron which appeared to be present in very stable combination. He concluded that part at least of the iron of the egg is present as an organic complex.

Additional evidence appeared to be necessary to decide this point.

The contents of one egg were well mixed, treated with an equal volume of 20% trichloroacetic acid and filtered. To 5 ml. of the filtrate were added 2 drops of thiolacetic acid followed by 1 ml. ammonia, sp. gr. 0.88. No colour was produced. A known volume of standard iron solution was added to another portion of the filtrate and the test repeated. The depth of colour produced corresponded to the amount of iron added, indicating that none of the constituents of the filtrate interfered with the reaction.

To the contents of another egg some iron alum solution was added and the proteins precipitated with trichloroacetic acid as before. The filtrate gave a negative reaction for iron.

The following experiment was then carried out. 5 ml. of thiolacetic acid were added to the contents of one egg and the proteins precipitated by the addition of an equal volume of 20% trichloroacetic acid. 1 ml. of ammonia, sp.gr. 0.88, was added to 5 ml. of the filtrate; a positive reaction for iron was obtained.

A series of experiments was then made in which the iron content as estimated directly on the protein free filtrate was compared with the total iron content estimated after ashing

The contents of an egg were diluted to 100 ml. with water, 10 ml. of the mixture were taken, 2 ml. of thiolacetic

acid added followed by 15 ml. of 20% trichloroacetic acid and the volume made up to 30 ml. with water. The mixture was then filtered. To 5 ml. of the filtrate was added 1 ml. of ammonia, sp.gr. 0.88 and then compared with a standard, prepared as follows. To 2 ml. of standard iron solution (2 ml. = 0.02 mg. Fe) were added 0.33 ml. thiolacetic acid and 2.5 ml. of 20% trichloroacetic acid and the volume made up to 5 ml. with water. 1 ml. of ammonia, sp. gr. 0.88, was then added. The results obtained by this method were compared with those determined after ashing.

The results are shown in Table 14. At the same time the recovery of added iron was studied, using direct determinations in a protein free extract. The results are shown in Table 15. It will be seen that the iron content of the egg as estimated by the two methods is the same within the limits of experimental error and that added iron could be estimated quantitatively.

From the above investigations it would appear that the iron of the hens' egg is the part of some organic molecule. Added ferric iron behaves in exactly the same way therefore one must conclude that the iron of the hens' egg is in a similar state to ferric iron added to an organic mixture of the nature of egg yolk. Thiolacetic acid has a reducing action and one would assume that ferrous iron does not form such strong combinations as ferric iron with organic compounds such as are present in egg yolk. There seems to be little evidence to suggest that the iron of the hens' egg exists as the

hydroxide.

Investigations were then carried out to determine whether substances other than thiolacetic acid were capable of liberating iron from egg yolk.

The yolk of one egg was diluted to 100 ml. with water. Four 10 ml. samples were treated as follows:

1. control - diluted to 15 ml. with water.
2. 2 ml. thiolacetic acid added and the volume made up to 15 ml. with water.
3. 5 ml. of 4% sodium pyrophosphate added and the volume made up to 15 ml. with water.
4. 2.5 ml. of 5% sodium hydrosulphite added and the volume made up to 15 ml. with water.

After standing for 5 minutes, 15 ml. of 20% trichloroacetic acid were added and the mixture filtered. To 5 ml. of the filtrate were added 2 drops of thiolacetic acid followed by 1 ml. ammonia, sp. gr. 0.88. The depths of colour produced were compared with a standard (0.015 mg. Fe), prepared similarly. The results are shown in Table 16.

Sodium pyrophosphate and hydrosulphite were both capable of liberating the iron of egg yolk quantitatively. The latter is effective because of its reducing action while the former has the property of forming non-ionised compounds with iron. Experiments were carried out in which sodium glycerophosphate and citrate and dithioglycollic acid were used. The filtrates gave negative reactions for iron.

Investigations were then carried out to determine

the nature of the substances with which ferric iron forms such stable complexes.

Iron alum solutions were added to milk, solutions of sodium caseinate, gelatin and edestin and suspensions of egg white and lecithin and samples treated in the same way as the egg yolk suspensions described above.

Within the limits of the amounts used, milk, sodium caseinate and egg white gave negative reactions for iron. Lecithin, gelatin and edestin contained appreciable amounts of iron which were allowed for. The lecithin suspension was prepared by pouring an ethereal solution into boiling water.

The results are shown in Table 17. Iron was not present in the control filtrates of milk, lecithin or sodium caseinate but was recovered quantitatively in the control filtrates of gelatin and edestin. Iron was present in control filtrates of egg white but recovery was incomplete, this being probably due to traces of lecithin. Milk, sodium caseinate and lecithin reacted similar to egg yolk. Although ferric iron forms complexes with simple proteins, it must be concluded from these results that compounds with phosphatides and phosphoproteins are much more stable.

Table 14

The iron content of the hen's egg

	mg. Fe per whole egg	
	Thiocyanate method after ashing	Direct determination in trichloroacetic acid extract
Whole egg		
1.	0.92	0.90
2.	1.26	1.25
3.	1.00	0.96
4.	1.27	1.21
5.	1.25	1.17
6.	1.11	1.18
7.	1.27	1.19

Table 15

The recovery of iron added to egg and determined by the thiolacetic acid method in trichloroacetic acid extracts

	Initial iron content mg.	Iron added mg.	Total iron content mg.	Iron recovered mg.
1.	0.067	0.088	0.153	0.086
2.	0.097	0.070	0.159	0.062
3.	0.072	0.070	0.136	0.064
4.	0.087	0.088	0.174	0.087
5.	0.088	0.088	0.172	0.084
6.	0.079	0.088	0.160	0.081

TABLE 16

THE IRON CONTENT OF EGG YOLK.

MG. FE PER EGG.

	Thiolacetic acid	Sodium pyrophosphate	Sodium hydrosulphite	Control
1	1·28	1·24	1·22	Nil
2	1·24	1·18	1·26	Nil
3	0·96	0·99	1·00	Nil
4	1·10	1·12	1·17	Nil
5	1·16	1·12	1·10	Nil

TABLE 17

RECOVERY OF IRON ADDED TO VARIOUS BIOLOGICAL MATERIALS.

	Iron added mg.	Ferric iron. Iron recovered (mg.) Control	Thiolacetic acid	Sodium pyro-phosphate	Sodium hydro-sulphite
1 % lecithin suspension	0·10	Nil	0·099	0·097	0·096
	0·05	Nil	0·052	0·054	0·051
2 % sodium caseinogenate	0·10	Nil	0·096	0·102	0·105
	0·05	Nil	0·052	0·053	0·051
Milk	0·10	Nil	0·106	0·105	0·107
	0·05	Nil	0·053	0·047	0·048
Egg-white	0·10	0·091	—	—	—
	0·10	0·081	0·102	0·106	0·098
	0·15	0·124	—	—	—
	0·10	0·078	0·106	0·107	0·103
2 % edestin	0·10	0·099	—	—	—
	0·15	0·145	—	—	—
	0·20	0·193	—	—	—
2 % gelatin	0·10	0·094	—	—	—
	0·15	0·144	—	—	—
	0·20	0·198	—	—	—

The Dialysis of Iron Salts

The object of the present experiments was to determin some of the factors which might influence the dialysis of inorganic iron from organic mixtures and their possible relationship to alimentary absorption.

In a previous section, the writer has shown that ferr iron forms very stable complexes with substances of the natur of phosphatides and phosphoproteins. Such stable complexes are not produced with protein. It was considered that the formation of such complexes might influence the rate at which inorganic iron dialyses.

Parchment thimbles (Whatman) were used as dialysing membranes. In each case 5 ml. of mixture, containing an added iron salt (1 mg. Fe) was dialysed against 15 ml. of distilled water for 4 hours. At the end of this period, the iron content of the external fluid was estimated.

Egg yolk and egg white suspensions, containing added iron (ferrous sulphate, iron ammonium citrate, iron alum) were dialysed. Ferrous iron dialysed readily whereas the dialysis of ferric iron was nil or negligable.

Under normal conditions, absorption of iron probably takes place from the small intestine from a mixture of digest protein. The reaction of such contents is probably acid. Dialysis was investigated under similar conditions. Suspensi of egg white and egg yolk were digested with pepsin and hydrochloric acid. These peptic digests, to which iron (ferrous sulphate, iron ammonium citrate, iron alum) had been added, were dialysed against distilled water. Ferrous sulpha

dialysed readily. Ferric iron dialysed readily from peptic digests of egg white but not from peptic digests of egg yolk. Acid peptic digests of casein had the same effect as egg yolk As these digests were acid in reaction, inability of ferric iron to dialyse could not be due to precipitation as phosphat The inability of ferric iron to dialyse is undoubtedly due to the formation of strongly combined undissociated compounds between ferric iron and such substances as phosphatides and phosphoproteins or their degradation products.

Peptic digests of egg white and egg yolk were neutralised by the addition of sodium bicarbonate and the dialysis experiments, described above, repeated. Dialysis of ferric and ferrous iron from egg white digests were little affected by neutralisation. The dialysis of ferrous iron from egg yolk digests was reduced very markedly by neutralisation.

In a previous section it has been shown that pyrophosphates are capable of liberating ferric iron from its complexes with phosphatides etc. As a result, experiments were carried out in order to determine whether pyrophosphates were capable of increasing the rate of dialysis of ferric iro The pyrophosphate was added as the sodium salt. In acid peptic digests of egg yolk, pyrophosphate had little effect. This could be due to the rapidity with which it is hydrolysed to orthophosphate by mineral acids. Pyrophosphates increased to a marked extent the dialysis of ferric iron from neutralised digests of egg yolk and from undigested egg yolk and egg white.

Dialysis of ferric iron from suspensions of undigested egg white was increased by the addition of acid.

Examples of the results obtained from these dialysis experiments are shown in Table 18a.

Similar dialysis experiments were carried out, using lead acetate and copper sulphate (1 mg. Pb, 1 mg. Cu). The results indicated that these metals did not form strongly combined complexes with phosphatides and phosphoproteins or their degradation products. As a result one must conclude that these substances should have no specific inhibiting effect upon the absorption of these metals from the alimentary tract. Typical results are shown in Table 18b.

Methods of Analysis

Iron - directly with thiolacetic acid and ammonia

Copper - directly with sodium diethyldithiocarbamate (see later section)

Lead - with dithizone after digestion with sulphuric and perchloric acids

Table 18 a.

Dialysis of Iron.

Quantity of iron subjected to dialysis - 1mg.

The figures refer to mg. of Fe that have dialysed in 4 hours.

Undigested egg white	
Ferrous sulphate	0.56
Iron alum	0.00
Iron ammonium citrate	0.01
Iron alum + sodium pyrophosphate	0.28
Egg white digested with Pepsin pH - 4.0	
Ferrous sulphate	0.41
Iron alum	0.41
Iron ammonium citrate	0.38
Peptic digest of Egg White neutralised with sodium bicarbonate	
Ferrous sulphate	0.36
Iron alum	0.20
Iron ammonium citrate	0.19
Iron alum + sodium pyrophosphate	0.19
Undigested Egg Yolk	
Ferrous sulphate	0.52
Iron alum	0.00
Iron ammonium citrate	0.00
Iron alum + sodium pyrophosphate	0.26

Egg Yolk digested with Pepsin

pH - 4.1

Ferrous sulphate	0.56
Iron alum	0.03
Iron ammonium citrate	0.03
Iron alum + sodium pyrophosphate	0.03

Peptic digest of Egg Yolk neutralised with sodium bicarbonate

Ferrous sulphate	0.03
Iron alum	0.00
Iron alum + sodium pyrophosphate	0.20

Casein digested with Pepsin

pH - 4.2

Ferrous sulphate	0.39
Iron alum	0.06
Iron ammonium citrate	0.01

Acidified undigested Egg White

pH - 4.0

Iron alum	0.23

Table 18 b.

Dialysis of Copper.

Quantity of copper subjected to dialysis - 1mg.

The figures refer to mg. of Cu that have dialysed in 4 hours.

Undigested egg white	0.00
Peptic digest of egg white (pH - 4.2)	0.38
Peptic digest of egg white neutralised with sodium bicarbonate	0.05
Undigested egg yolk	0.04
Peptic digest of egg yolk (pH - 4.5)	0.38
Peptic digest of egg yolk neutralised with sodium bicarbonate	0.04
Acidified undigested egg white (pH - 4.6)	0.22

Dialysis of Lead

Quantity of lead subjected to dialysis - 1mg.

The figures refer to mg. of Pb that have dialysed in 4 hours.

Undigested egg white	0.00
Peptic digest of egg white (pH - 4.2)	0.45
Peptic digest of egg white neutralised with sodium bicarbonate	0.03
Undigested egg yolk	0.03
Peptic digest of egg yolk (pH - 4.5)	0.48
Peptic digest of egg yolk neutralised with sodium bicarbonate	0.02
Acidified undigested egg white (pH - 4.6)	0.26

Factors influencing the Absorption of Iron from the Alimentary Tract

Little work appears to have been done regarding this important problem. Results obtained from dialysis and other experiments have indicated the probability that materials containing phosphatides and phosphoproteins are likely to interfere with the absorption of iron from the alimentary trac This is of great importance as certain foodstuffs, ranking high nutritionally, are rich in these e.g. egg, milk. The writer has shown that lead absorption is dependent on the calcium content of the diet. The probability is that the absorption of iron is influenced in a similar manner.

In the first instant, the effect of phosphatides and phosphoproteins upon the absorption of iron was studied. In these experiments, young female mice (aged 3 weeks) were used. In general, the experiments were carried out under similar conditions to those with lead. The diets, which were of low calcium content, had the following compositions ;

Diet A

	g.
Whole wheat flour	300
Casein	200
Corn starch	325
Wheat gluten	50
Olive oil	40
Sodium chloride	20
Potassium chloride	15
Butter	15

Diet B

	g.
Whole wheat flour	400
Corn starch	325
Wheat gluten	50
Olive oil	40
Sodium chloride	20
Potassium chloride	15
Butter	15

The diets were fed at the rate of 2.5 g. per mouse per day and contained supplements of iron alum (0.8 mg. Fe per mouse p day). The duration of the experiments was 14 days. At the end of this period the animals were placed on the diets less added iron for 4 days to remove unabsorbed iron from their alimentary tracts, killed and their total iron contents estimated.

The animals were placed on the following diets,

1. Diet A,

2. Diet B + 1 ml. egg white per mouse per day,

3. Diet B + 1 ml. egg yolk per mouse per day.

Control animals were killed at the commencement of the experiment and their total iron contents estimated.

The results are shown in Table 19a and it will be see: that the presence of phosphatides and phosphoproteins in the diet does inhibit the absorption of ferric iron from the alimentary tract.

The experiments were repeated using iron pyrophosphat The results, which are not reported in detail, agreed with th obtained with iron alum. This was due probably to the hydrol of the pyrophosphate by the acid of the gastric juice.

The experiments were repeated using ferrous sulphate as the source of iron. These results are shown in Table 19b and agree with those obtained with iron alum.

The influence of the calcium content of the diet upon the absorption of iron has been examined. The diets used had the following compositions ;

<u>High calcium diet</u>

	g.
Whole wheat flour	700
Whole milk powder	300
Marmite	50

Low calcium diet

	g.
Whole wheat flour	400
Casein	100
Corn starch	325
Wheat gluten	50
Olive oil	40
Sodium chloride	20
Potassium chloride	15
Butter	15

The diets were fed at the rate of 2.5 g. per mouse per day.

Adult male mice were used in the following experiment The animals were placed on a) high calcium diet, b) low calci diet, to which was added iron (iron ammonium citrate) at the of 2 mg. Fe per mouse per day for a period of 21 days. The animals were then placed on the diets less added iron for 4 days to remove unabsorbed iron from their alimentary tracts killed and their total iron contents estimated. A number of animals of the same group were killed before the commencement of the experiment and their total iron contents estimated. The results are shown in Table 19c.

In this experiment, female mice, aged 3 weeks, were used. These animals were placed on a) high calcium diet, b) low calcium diet, to which had been added iron as iron ammonium citrate at the rate of 0.8 mg. Fe per mouse per day. At the end of 14 days, the animals were placed on the diets l iron for 4 days to remove unabsorbed iron from their alimentary tracts, killed and their total iron contents

estimated. A number of animals of the same group were killed before the commencement of the experiment and their total iron contents estimated. The results are shown in Table 19c.

The results obtained indicate that the absorption of iron from the alimentary tract is influenced by the calcium content of the diet in the same way as lead. Absorption of iron is high on a low calcium diet and low on a high calcium diet. The writer has shown that the addition of hydrochloric acid to the diet increases the absorption of lead. It seemed probable that iron would behave in a similar manner. The following experiment was therefore carried out.

Female mice, aged 3 weeks, were placed on the high calcium diet, containing 0.8 mg. Fe (as iron ammonium citrate) and 0.5 ml. of N. hydrochloric acid per mouse per day for a period of 14 days. The animals were then placed on the high calcium diet alone for 4 days to remove unabsorbed iron from their alimentary tracts, killed and their total iron contents estimated. The results are shown in Table 19c and indicate that iron is absorbed more easily when mineral acid is added to the diet. This experiment indicates the importance of the gastric acidity in the absorption of iron.

In animal experiments, the subject usually consumes a diet, every mouthful of which has the same composition. In man, this does not occur. This point is often neglected by workers in nutrition. In man the composition of the diet should vary throughout the day. In view of the fact that the absorption of essential constituents of the diet may be subjected to mutual inhibition it seems apparent that the dail

diet should be so arranged into meals that mutual inhibition is reduced to a minimum. To attempt to simulate this in animals, young female mice were fed on the high and the low calcium diets on alternate days together with added iron for 14 days. At the end of this period, they were placed on high calcium diet alone for 4 days to remove unabsorbed iron from their alimentary tracts, killed and their total iron contents estimated. The results are shown in Table 19d. It will be seen that under such conditions, iron absorption was good and in addition, their growth was much better than in the oth experiments.

Method of Analysis

The animal was ignited in a silica dish over a bunsen burner. The ash was dissolved in water containing 5 ml. concentrated hydrochloric acid and the volume made up to 50 ml. The iron content was estimated in an aliquot with thiolacetic acid and ammonia.

Table 19a

Young female mice (aged 3 weeks)

Added iron - 0.8 mg. Fe (iron alum)

Time - 14 days

	Weight g.	Total iron mg.	Iron mg. Fe per 100 g. mouse
Controls			
1.	9.5	0.59	6.02
2.	11.0	0.67	6.09
3.	9.0	0.59	6.55
4.	10.5	0.71	6.76
5.	10.5	0.71	6.76
6.	10.5	0.71	6.76
7.	9.5	0.53	5.57
8.	9.0	0.45	5.00
	Average	0.62	6.25
Diet A			
9.	14.0	0.63	4.50
10.	18.0	0.70	3.90
11.	19.5	0.71	3.64
12.	17.5	0.78	4.47
13.	16.0	0.78	4.38
14.	12.0	0.64	5.23
	Average	0.71	4.36

	Weight g.	Total iron mg.	Iron mg. Fe per 100 g. mouse
Diet B + Egg yolk			
15.	19.0	0.68	3.58
16.	19.0	0.67	3.52
17.	18.5	0.79	4.27
18.	20.5	0.82	4.00
19.	20.0	0.78	3.90
20.	15.5	0.73	4.71
21.	17.5	0.65	3.72
22.	21.5	0.73	3.39
23.	18.5	0.70	3.79
24.	17.5	0.59	3.37
25.	17.0	0.71	4.18
26.	20.5	0.78	3.80
	Average	0.71	3.85
Diet B + Egg White			
27.	17.0	0.90	5.88
28.	17.5	1.06	6.06
29.	14.5	1.00	6.96
30.	14.5	1.06	7.31
31.	13.5	0.91	6.70
32.	14.5	0.89	6.10
33.	14.5	0.93	6.41
34.	15.5	1.06	6.84
35.	12.5	0.91	7.24
36.	14.0	0.98	7.00

	Weight g.	Total iron mg.	Iron mg. Fe per 100 g. mouse
37.	16.5	1.14	7.00
38.	12.0	1.10	6.92
	Average	1.00	6.80

Table 19 b

Female mice (aged 3 weeks)

Added iron - 0.8 mg. Fe (ferrous sulphate) per day

Time - 14 days.

	Weight g.		Total iron mg.	Iron mg. per 100 g. mouse
Controls				
1.	8.5		0.44	5.17
2.	7.0		0.53	7.57
3.	9.0		0.43	4.78
4.	9.5		0.53	5.58
5.	9.0		0.45	5.00
6.	10.0		0.62	6.20
Diet B + Egg white		Average	0.50	5.72
7.	16.0		1.20	7.50
8.	17.5		1.15	6.56
9.	15.5		1.03	6.64
10.	13.0		0.98	7.54
11.	11.5		0.80	6.94
12.	12.5		0.86	6.88
		Average	1.00	7.01

	Weight g.	Total iron mg.	Iron mg. per 100 g. mouse
Diet B + Egg Yolk			
13.	17.5	0.73	4.17
14.	13.0	0.65	5.00
15.	15.5	0.65	4.19
16.	16.0	0.63	3.94
17.	13.5	0.73	5.41
18.	15.5	0.74	4.77
	Average	0.69	4.58

Table 19 c

Adult male mice

Added iron - 2 mg. Fe (Iron Ammonium Citrate) per mouse per day

Time 21 days.

	Weight g.	Total iron mg.	Iron mg. per 100 g. mouse
Controls			
1.	30	1.80	6.00
2.	24.5	1.67	6.54
3.	21	1.26	6.00
4.	24.5	1.56	6.54
5.	27	1.80	6.67
		Average	6.35
High calcium diet			
6.	22	1.45	6.60
7.	19.5	1.14	5.90
8.	23	1.34	5.83
9.	18	1.54	6.56
		Average	6.22
Low calcium diet			
10.	23	2.03	8.83
11.	23.5	1.96	8.34
12.	24.5	2.03	8.29
13.	23	2.35	10.22
		Average	8.92

Female mice (aged 3 weeks)

Added iron - 0.8 mg. Fe (Iron Ammonium Citrate) per mouse per day.

Time - 14 days

	Weight g.	Total iron mg.	Iron mg. per 100 g. mouse
Controls			
1.	10.5	0.53	5.05
2.	10.5	0.54	5.14
3.	10.0	0.68	6.80
4.	10.5	0.54	5.14
5.	11.5	0.58	5.04
6.	9	0.51	5.67
7.	8	0.50	6.25
8.	8	0.41	5.12
	Average	0.51	5.03
High calcium diet			
9.	11.0	0.75	6.82
10.	14.0	0.81	5.78
11.	9.0	0.70	7.77
12.	8.5	0.63	7.53
13.	9.5	0.71	7.47
14.	11.0	0.83	7.55
15.	11.0	0.83	7.55
16.	9.0	0.69	7.66
	Average	0.74	7.27

	Weight g.	Total iron mg.	Iron mg. per 100 g. mouse
Low calcium diet			
17.	13	1.11	8.54
18.	12	1.09	9.08
19.	10.5	1.09	10.38
20.	8	1.38	17.25
21.	8.5	0.97	11.41
22.	8.5	1.17	13.76
23.	9	1.09	12.11
24.	7.5	0.90	12.00
	Average	1.10	12.04
High calcium diet and hydrochloric acid			
25.	11.0	1.09	9.91
26.	11.5	1.01	8.75
27.	10.0	1.10	11.00
28.	10.5	1.00	9.52
29.	9	1.05	11.66
30.	8.5	1.08	12.71
31.	8.5	0.98	11.53
32.	8	1.06	13.25
	Average	1.04	11.04

Table 19 d.

Female Mice (aged 3 weeks)

Added iron - 0.8 mg.Fe (Iron Ammonium Citrate)

Time - 14 days

	Weight g.	Total iron mg.	Iron mg. per 100 g. mouse
High and low calcium diet on alternate days			
33.	22	1.10	5.00
34.	16	0.70	4.38
35.	19	1.03	5.42
36.	22	1.15	5.23
37.	17	1.00	5.99
38.	19	0.88	4.62
39.	17.5	0.88	5.00
40.	14	0.92	5.67
	Average	0.96	5.17

The Reduction of Ferric Iron in the Alimentary Tract and its Significance in Absorption

Many workers believe that ferric iron cannot be absorbed as such but must be reduced to the ferrous state pri to absorption. This reduction is said to occur in the small intestine [Lintzel (1931), Starkenstein and Weden (1930), Reimann and Fritsch (1930), Moore et alia (1939)]. Other workers believe that ferric iron can be absorbed as such [Whipple and Robscheit-Robbins (1936), McCance and Widdowso (1937, 1938), Brock and Hunter (1937)]. The evidence presented by both sides is conflicting. The former base thei views on the following ; 1. ferric but not ferrous iron forms non-dissociated compounds with proteins, 2. in the treatment of anaemia, ferrous salts often produce a better response than ferric salts. The latter base their views on the following ; 1. in iron balance experiments, ferric iron has been found to be absorbed as easily as ferrous iron, 2. in the treatment of anaemia, ferric iron often produces as good a response as ferrous iron. Those who hold the first vie have in no case indicated that reduction of ferric iron does take place in any part of the alimentary tract. To examine th the following experiments were carried out.

The effect of various foodstuffs, used in animal diets upon ferric iron was examined. 1 mg. Fe^{+++} (iron alum) was added to about 1 g. of the foodstuff suspended in about 5 ml. of water and a little $\alpha\alpha'$-dipyridyl added. $\alpha\alpha'$-dipyridyl reac with ferrous iron to produce a red colour but not with ferric iron. In neutral solution, no reaction occurred but in acid

solution (pH 4 - 5) a number of substances produced a marked reduction of ferric iron as evidenced by the production of a red colour with $\alpha\alpha'$-dipyridyl. The substances capable of reducing ferric iron in acid solution were,

whole wheat flour,
whole milk powder,
egg white (boiled and unboiled),
wheat gluten,
cows' milk (boiled and unboiled),
commercial egg albumin .

Casein, glucose, lactose, egg yolk (boiled and unboiled) were without effect. Peptic digests of egg white and egg yolk were then examined. An acid solution of egg white (pH - 4.2) produced rapid reduction of ferric iron whereas after neutralisation with sodium bicarbonate it had no effect. Acid and neutralised peptic digests of egg yolk had no effect.

The effect of those substances which had no reducing action on ferric iron, upon ferrous iron was then examined. With the exception of egg yolk no effect was observed. Ferrous iron allowed to stand in contact with egg yolk became oxidised to the ferric state. This was shown by the fact that when 1 mg. Fe^{++} (as ferrous sulphate) was added to 5 ml. of egg yolk the mixture would give no reaction for ferrous iron with $\alpha\alpha'$-dipyridyl at the end of 1 hour. This had been suspected since ferrous iron added to egg yolk suspensions could not be recovered quantitatively in trichloroacetic acid extracts as would have been expected. The writer considers that the phenomenon is due to autoxidation, the phosphatides being

responsible.

The reducing effect of certain foodstuffs should now be considered. The effect was most marked with egg white, egg albumin and gluten, proteins which contain a high percentage of cysteine. The writer considers that the reducing action is in all probability due to the cysteine molecule. In view of this, the reducing action of whole whea flour should be due to gluten and that of milk to lactalbumin The inability of casein to reduce ferric iron is probably due to its low cysteine content.

Ferric iron was found to dialyse readily from acid an neutralised peptic digests of egg white and from acidified but not neutral egg white. The ready dialysis of ferric iron from such acid mixtures is undoubtedly due to reduction i.e. ferrous iron passed through the membrane. Dialysis of ferric iron from neutralised egg white digests was found to be due to the development of an acid reaction during dialysis. Ferr iron was observed to dialyse from egg yolk and from acid pept digests of egg yolk. That this was possible is due to the fa that autoxidation takes time. The writer believes that the inability of ferrous iron to dialyse from neutralised peptic digests of egg yolk was due primarily to its precipitation as phosphate.

Similar factors may be responsible for the fact that added ferric iron may be recovered quantitatively in trichloroacetic acid extracts of egg white etc but not from egg yolk etc.

The absorption of ferric iron as such would be hindere

in fact one might say completely prevented by the presence of phosphatides etc in the diet. It is probable that all protein act in a similar manner unless reduction takes place. As to the fate of the phosphatides in the alimentary tract, very little is known, they probably have a long survival. In addit bile will have a similar action owing to its lecithin content. As a result the writer considers that under normal conditions of diet, absorption of ferric iron as such is either nil or negligable.

Ferrous iron should be absorbed easily. The writer ha produced evidence to show that ferric iron can be reduced in t alimentary tract, not in the small intestine as others have suggested, but in the <u>stomach</u>. This has been confirmed in viv Two rats were fed on a) boiled egg white + iron alum, b) boiled egg yolk + iron alum, respectively and at the end of two hours killed and their stomach and intestinal contents examined for the presence of ferrous iron with $\alpha\alpha'$- dipyridyl. The animal that had been fed on egg white, showed the presence of ferrous iron in both stomach and intestine very markedly whereas in the animal that had been fed on egg yolk, no trace of ferrous iron could be found in either stomach or intestine.

Ferric iron must be reduced to the ferrous state prior to absorption, and as indicated, this can occur in the stomach. This is influenced by two factors, 1) the gastric acidity, 2) the composition of the diet. The diet should have a reducin effect towards iron i.e. it should contain substances of the nature of albumin. gluten etc. The gastric acidity is of importance since the reducing power of the diet only becomes

effective at acid reactions.

When iron is administered along with food its fate depends to a large extent on the diet. If the diet is predominately reducing then it will be immaterial whether the iron is administered in the ferric or ferrous state since in both cases an equilibrium mixture, predominating in ferrous iron, of the same composition, will result in both cases. If autoxidisable properties predominate then whether the iron is administered in the ferric or ferrous state, the final state of the iron will be ferric. If the diet is 'inert', then ferrous and ferric iron will remain unchanged, the former being absorbed and the latter not.

One is now able to obtain a clearer insight into the results obtained from the animal experiments reported in the previous section..

The low absorption of iron by mice on a diet containing egg yolk was no doubt due to the predominating autoxidation properties of the diet. On egg white, iron absorption was much higher, this would be due to the predominating reducing properties of the diet. Iron absorption was poor on a diet containing a large amount of casein, this probably being due to the comparative inertness of the diet. It also becomes apparent why similar results were obtained when ferrous iron and ferric iron were fed on diets containing added egg yolk and egg white.

Rose et alia (1934) and Sherman, Elvehjem and Hart (1934) found that egg yolk produced very little regeneration in nutritional anaemia in rats. They believed that copper deficiency was the primary factor although the former workers

considered that there were other factors e.g. the form inwhich the iron was present in the egg yolk.

The question naturally arises as to whether the iron of the egg is available at all, that is whether the effect of white predominates over the yolk or vice-versa. Experiments were carried out to determine this. Peptic digests of unboile and boiled whole egg were prepared. At pH 4 - 5. a reducing effect was observed. This was confirmed in vivo on a rat, as described earlier. These results indicate that the effect the white does predominate over that of the yolk.

The Non-Haematin Iron of Human Serum and Plasma

Many writers have estimated what they describe as the non-haematin iron content of serum and plasma. The following results have been reported.

	mg. Fe per 100 m
Starkenstein and Weden (1928)	0.57 - 0.79
Riecker and Winters (1930)	0.9 - 1.4
Locke et alia (1932)	0.06 - 0.12
Marlow and Taylor (1934)	0.4 - 0.7
Moore (1937)	0.06 - 0.17

In these investigations the serum or plasma was ashed prior to the estimation of iron.

The writer has found that, as in the case of egg yolk, trichloroacetic acid extracts of serum or plasma gave negative reactions for iron and added inorganic iron could not be recovered in such extracts. As a result the following technic was adopted.

To 5 ml. of serum or plasma were added 8 drops of thiolacetic acid and 5 ml. of 20% trichloroacetic acid and the mixture centrifuged. 1 ml. of ammonia, sp. gr. 0.88, was added to 5 ml. of the supernatant fluid. Under such condition a definite reaction for iron was obtained and added inorganic iron could be recovered quantitatively. As the depth of colou was too faint to compare with a standard in a colorimeter, the iron content was estimated by direct comparison with a series of standards.

From the results shown in Table 20, it will be seen that added inorganic iron could be estimated quantitatively

by this method. The iron content of 10 normal sera was estimated and the results are shown in Table 21, the range being 0.12 - 0.20 mg. Fe per 100 ml.

It would appear convenient to refer to the serum iron estimated above as 'inorganic' iron. After this work had bee published, Fowweather (1934) published a paper in which he reported the total iron content of human plasma, shown to be free of haemoglobin. His range for total iron agrees with th writer's range for 'inorganic' iron. One is forced to conclu that the high figures reported by some writers must have been due to the use of serum and plasma in which there had been considerable haemolysis.

The effect of plasma upon the dialysis of iron salts (iron alum, iron ammonium citrate, ferrous sulphate) has been studied. These were carried out in a similar manner to those described in an earlier section. The results are shown in Table 22 and indicate that whereas ferrous iron dialysed easily, ferric iron was almost non-dialysable.

To a sample of plasma, $\alpha\alpha'$-dipyridyl was added. A red colour did not develop until after the addition of sodium hydrosulphite, this indicating that in the sample, the iron was in the ferric state. A sample of serum (5 ml.) to which had been added 1 mg. ferric iron, as iron alum, and a little $\alpha\alpha'$-dipyridyl was acidified to give pH 4 - 5. A red colour developed, indicating that on the acid side, the serum protei are capable of reducing ferric iron to the ferrous state. Th was rather to be expected since the plasma proteins contain a high content of sulphur containing amino acids. Barkan

(1927) showed that plasma iron was not dialysable but became so after incubation with 0.4% hydrochloric acid at 37° C for 24 hours. He used this as the basis of a method for the estimation of plasma iron. He estimated the iron content of an ultrafiltrate prepared from plasma that had been subjected to the above procedure. He obtained values of 0.08 - 0.17 mg. Fe per 100 ml., which are in agreement with those obtained by the writer. This phenomenon observed by Barkan can be explained by the results obtained by the writer.

The 'inorganic' iron content of sera from cases of pernicious anaemia and anaemia following haemorrhage has been estimated. The results are shown in Table 23. It will be seen that the values are increased in the former and decreased in the latter condition.

Table 20

Recovery of ferric iron added to serum

mg. Fe

	Initial	Iron added	Total iron	Iron recovered
1.	0.004	0.01	0.013	0.009
2.	0.004	0.01	0.013	0.009
3.	0.005	0.015	0.020	0.015
4.	0.004	0.01	0.014	0.01
5.	0.005	0.015	0.019	0.014

Table 21

The 'inorganic' iron content of normal sera

	mg. Fe per 100 ml.		
1.	0.20	6.	0.20
2.	0.18	7.	0.14
3.	0.14	8.	0.16
4.	0.14	9.	0.20
5.	0.18	10.	0.12

Table 22

Dialysis of Iron from Serum.

Quantity of iron subjected to dialysis - 1 mg.

The figures refer to mg. Fe that have dialysed in 4 hours.

1 mg Fe (ferrous sulphate)	0.30
1 mg Fe (iron alum)	0.01
1 mg.Fe (Iron ammonium citrate)	0.01
1 mg.Fe (iron alum) and sodium pyrophosphate	0.30

Table 23

The 'inorganic' iron content of human serum.

mg. Fe per 100 ml.

Pernicious Anaemia.

	Hb. %	Serum Iron
1.	36	0.26
2.	60	0.21
3.	30	0.23
4.	51	0.32

Anaemia following Haemorrhage

	Hb. %	Serum Iron
1.	30	0.05
2.	73	0.09
3.	45	0.06
4.	43	0.06
5.	33	0.05
6.	40	0.06
7.	38	0.04
8.	35	0.04
9.	41	0.06

The Non-Haematin Iron of Whole Blood

a) 'Inorganic' Iron

Trichloroacetic acid extracts of whole blood were prepared and it was found that they gave positive reactions for inorganic iron but added inorganic iron could not be recovered quantitatively. To estimate the 'inorganic' iron content of whole blood, the following technique was used.

To 5 ml. of whole blood were added a) 5 ml. of wate and 8 drops of thiolacetic acid or b) 5 ml. of 4% sodium pyrophosphate. 5 ml. of 20% trichloroacetic acid were then added and the mixture filtered. Iron was estimated directl in the filtrate by the addition of thiolacetic acid and ammonia. Inorganic iron, added as iron alum, could be recovered quantitatively as shown by the results in Table 2

It has been found that thiolacetic acid attacks haemoglobin slowly to produce 'inorganic' iron. The proces is slow and the filtrations in the above method are exceedingly rapid. Comparison was made between the results obtained by the use of a) thiolacetic acid, b) sodium pyrophosphate, to liberate 'inorganic' iron in whole blood. The results as shown in Table 25, indicate that no appreciable differences exist.

The 'inorganic' iron content of whole blood from a number of men has been estimated. The results are shown in Table 26, and range from 0.93 to 1.50 mg. Fe per 100 ml. It will be obvious that these amounts of 'inorganic' iron are present mainly in the red corpuscles.

Using the above method, Shorlands and Wall (1936)

obtained similar results to those of the writer.

To a trichloroacetic acid extract of whole blood in which sodium pyrophosphate had been used to liberate 'inorganic' iron, $\alpha\alpha'$ dipyridyl was added and an immediate red colour was produced. This could indicate that at least part of the 'inorganic' iron of the red blood corpuscles is in the ferrous state or it could have been the result of the reducing action of the plasma when the trichloroacetic acid was added. This result must be held in doubt although one might expect that part at least of the iron in the R. B. C. is in the ferrous state since they contain such reducing substances as glutathione etc.

b) 'Easily split Iron

Barkan (1927 - 1938) has estimated what he terms the 'easily split iron' in blood. He regards 'easily split iron' as the precursor of bilirubin. 'Easily split iron' has been estimated by a number of writers using various methods ;

1. treatment of blood with hydrochloric acid at 37° C follow by ultrafiltration and estimation of the total iron content of the ultrafiltrate (Barkan),
2. heating blood with 5N sulphuric acid and estimation of the total iron content of a trichloroacetic acid extract [Starkenstein and Weden (1928)],
3. heating blood with normal acid and estimating the total iron content of a trichloroacetic acid extract [Moore (1937

'Easily split iron' values are considerably higher than the 'inorganic' iron values obtained by the writer. 'Easily split iron' values appear to depend upon the method

used e.g. lower values are obtained when hydrochloric acid is used than when sulphuric or nitric acid is used [Moore (1937)].

The writer is of the opinion that much of the iron that is included under the heading of 'easily split iron' is derived by decomposition processes from haemoglobin and as such is of no physiological or pathological significance. More work is necessary before our ideas on this subject are clarified.

Table 24

The recovery of iron added to whole blood.

mg.

Initial	Iron added	Total iron	Recovery.
0.020	0.015	0.033	0.013
0.020	0.020	0.042	0.022
0.020	0.010	0.030	0.010
0.026	0.010	0.037	0.011
0.026	0.015	0.043	0.017
0.026	0.020	0.045	0.019

Table 25

The results are expressed as mg.

Fe per 100 ml.

blood.

A. - thiolacetic acid

B. - sodium pyrophosphate

	A.	B.
1.	1.30	1.29
2.	1.34	1.33
3.	1.14	1.11
4.	1.40	1.38

Table 26

The 'inorganic' iron content of whole blood.
The results are expressed in mg. Fe per 100 ml. blood.

1.	1.14	5.	0.96
2.	1.06	6.	1.08
3.	0.93	7.	1.40
4.	1.36	8.	1.56

The Iron content of Urine.

Earlier writers have reported the presence of iron in normal urine but more recent work has thrown doubt on this.

Lintzel (1929) and Lanyar et alia (1933) state that the iron content of normal urine does not exceed 0.01 mg. per litre. Henriques and Roland (1928) obtained values of 0.08 to 0.32 mg. Fe per litre while Coons (1932) reported values of 0.06 to 0.48 mg. Fe. per litre in the urine of pregnant women. Later Marlow and Taylor (1934) have obtained values of 0.03 to 0.8 mg. Fe per litre in the urine of normal men.

To estimate the small amounts of iron that may be present in urine, special precautions must be taken. In the present study, urine from normal males was used and was collected in paraffin wax coated bottles. It was found that a thiocyanate colorimetric method was the most suitable to use and was carried out as follows.

A mixture of 50 ml. urine and 5 mil. concentrated nitric acid was evaporated almost to dryness in a pyrex flask. 5 ml. of concentrated sulphuric acid were added and the mixture heated to remove nitric acid. 5 ml. of perchloric acid were then added and the mixture heated until all the organic matter was destroyed. A blank was carried out at the same time. After cooling, the residue was diluted to 20 ml. with water, 4 ml. of 40% potassium thiocyanate added and the red ferric thiocyanate extracted with 5 ml. of amyl alcohol. The amyl alcohol layer was separated and compared with a standard

prepared as follows. The standard containing 0.005 mg. Fe and 1 ml. concentrated sulphuric acid was diluted to 10 ml. with water. 2 ml. of 40% potassium thiocyanate were added and the red ferric thiocyanate extracted with 5 ml. of amyl alcohol.

Although the purest chemicals that could be obtained, were used, the blank was appreciable. Urine from 6 normal males was examined. The colorimeter readings of the blanks and the urines were almost the same, the iron contents of these urines in no case exceeding 0.01 mg. per litre. The results of Lintzel and Lanyar et alia were thus confirmed. The probability is that the other investigators neglected to take blanks into account.

Lanyar et alia found that no increase in the iron content of urine resulted from the oral administration of iron salts. This was repeated, iron ammonium citrate being administered in three cases. The results are shown in Table 27 and it will be seen that the iron content of the urine was increased.

Table 27

The iron content of urine before and after the administration of iron ammonium citrate (90 gr. per day). The results are expressed in mg. Fe per litre.

Case 1	
Before iron treatment (average of 3 days)	< 0.01
During iron treatment (average of 5 days)	0.52
Case 2	
Before iron treatment (average of 3 days)	< 0.01
During iron treatment (average of 2 days)	0.52
Case 3	
Before iron treatment (average of 3 days)	< 0.01
During iron treatment (average of 3 days)	0.42

The 'Inorganic' Iron Content of Human Tissues.

The following method was adopted:

Some broken glass and 10 g. of finely cut up 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 for 15 minutes after which 20 ml. of 20% trichloroacetic acid were added and the mixture ground up. After a further 15 minutes the mixture was filtered 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 about 0.03 mg. Fe, was diluted to 5 ml. with water, 2 drops of thiolacetic acid added and then 1 ml. of ammonia, sp. gr. 0.88. This was compared with a standard prepared similarly.

At the same time, the total iron content of rib and vertebra was estimated. This was carried out on a solution of the ash in hydrochloric acid.

The results are shown in Table 28. Of the soft tissues, liver and spleen had the highest concentrations of 'inorganic' iron, which is to be expected. The 'inorganic' iron content of the spleen was fairly constant but that of the liver showed great variations. This is to be expected as the liver is regarded as being the chief store for iron.

The 'inorganic' iron contents of the other soft tissues were low but comparatively constant. The iron content of such bones as rib and vertebra was high. These bones are very vascular and much of the iron could have been present in other than bone tissue e.g. bone marrow.

The distribution of iron throughout a number of rats was determined. The results are shown in Table 29, and indicate that less than 14% of the total iron was present in the skeleton. These figures are probably on the high side as it was impossible to separate bone marrow. The skeleton appears to have little affinity for iron and therefore one must conclude that the reserve iron must be held mainly in the soft tissues.

TABLE 28

THE 'INORGANIC' IRON CONTENT OF HUMAN TISSUES

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

Table 29

The distribution of iron in the rat (mg.)

	1.	2.	3.	4.
Skin	1.05	1.16	1.08	1.00
Liver	0.87	0.92	1.17	1.06
Kidneys	0.21	0.18	0.15	0.17
Spleen	0.41	0.71	0.35	0.53
Bones	0.93	0.82	0.80	0.87
Remainder	3.25	3.68	2.88	3.60
Total	6.72	7.47	6.38	7.23
% Total iron in the skeleton	13.8	10.9	12.6	12.0

THE DETERMINATION OF COPPER IN BIOLOGICAL MATERIALS.

A large number of methods have been devised and used for the estimation of the small amounts of copper present in biological materials. Some writers have used macro-chemical methods but owing to the large quantities of material required, are generally unsatisfactory. A number of colorimetric methods have been devised and are as follows:-

1. potassium ethyl xanthate method,
2. Biazzo method (1926),
3. diethyldithiocarbamate method [Callan and Henderson (1929)].

Potassium ethyl xanthate reacts with copper to produce a yellow colour. In the Biazzo method, copper is allowed to react with pyridine and potassium thiocyanate, a green colour being produced. In the third method, copper is allowed to react with sodium diethyldithiocarbamate, a golden brown colour being produced. This is a very sensitive reaction, it being possible to detect one part of copper in ten million parts of solution.

The accuracy of the Biazzo method has been questioned by a number of writers [Ansbacher et alia (1931), Walker

(1930), Drabkin and Waggoner (1930)]. A serious disadvantag of the method is interference by iron ; this necessitates a preliminary separation of the copper. This method has been used by the Wisconsin workers in their investigations into the metabolism of copper.

McFarlane (1932) appears to have been the first to use sodium diethyldithiocarbamate for the estimation of copper in biological materials. He showed that the sensitivity of the reaction was increased by extracting the pigment with amyl alcohol. The reaction is independent of pH but iron interferes. McFarlane (1932) showed that interference from iron could be prevented by carrying out the reaction in an alkaline solution containing sodium pyrophospnate.

The writer has applied the reaction to the estimation of copper in various types of biological materials but in some cases modifications were found necessary or advantageous.

Blood

As a preliminary a slight modification of McFarlane's method for blood was used.

5 ml. of blood was digested with 5 ml. of

concentrated nitric acid in a pyrex test tube in a boiling water bath. When the contents were almost dry, 1 ml. of concentrated sulphuric acid was added and the mixture heated. Perchloric acid was added and the heating continued until all the organic matter was destroyed. The residue was diluted to 10 ml. with water, 5 ml. of 4% sodium pyrophosphat added and the mixture made alkaline to litmus by the addition of ammonia. 5 ml. of amyl alcohol were added followed by 0.5 ml. of 2% sodium diethyldithiocarbamate and the mixture shaken vigorously. The amyl alcohol layer, containing the golden brown copper complex, was separated and compared in a colorimeter with a standard containing 0.01 mg. Cu, prepared similarly.

The amyl alcohol extracts contained suspended particles of water which were removed by filtration or by centrifuging. Satisfactory results were obtained.

Locke et alia (1932) attempted to estimate copper in human serum by applying sodium diethyldithiocarbamate directly to trichloroacetic acid extracts. The possibility of estimating copper directly in trichloroacetic acid extracts of blood was investigated.

As a preliminary, the effect of various substances, many of which occur in protein free extracts of blood was

investigated. These substances, the nature and amounts used being shown below, were added to 0.01 mg. Cu (as copper sulphate) and the reaction developed as described above.

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

With the exception of potassium cyanide, none of the above substances prevented the quantitative development of the reaction. No colour developed in the presence of cyanide. In the case of glutathione and thiolacetic acid, on the addition of the reagent no colour developed until the contents of the tubes were shaken vigorously after which the amyl alcohol extracts were found to match the control quantitatively. This was due probably to the reduction of the copper to the cuprous state, which does not react with the reagent, this being reversed on shaking with air.

1 volume of blood, plasma or serum was diluted with 3 volumes of water and 1 volume of 20% trichloroacetic acid added. The mixture was filtered. To 25 ml. of the filtrate were added 2 ml. of 4% sodium pyrophosphate and the mixture made alkaline to litmus by the addition of ammonia. 5 ml. of amyl alcohol were added, followed by 0.5 ml. of 2% sodium diethyldithiocarbamate and the mixture shaken vigorously. The amyl alcohol layer was separated and compared with a standard, containing 0.01 mg. Cu, prepared similarly.

Results obtained by this method were compared with those obtained after destruction of the organic matter. The results are shown in Table 30.

It will be seen that copper present in the trichloroacetic acid filtrates reacts directly with sodium diethyldithiocarbamate. The results obtained by the two methods are the same within the limits of experimental error. This indicates that the copper present in the blood is in a comparatively simple form. There were indications that the copper present in the corpuscles is present, at least in part, in the cuprous state.

The results shown in Table 31 indicate that added copper could be estimated quantitatively by the second

method.

Urine.

Urine contains a large amount of calcium phosphate which tends to precipitate in alkaline solution. To prevent this, sodium citrate was added. It was found that interference from iron was prevented in alkaline solution.

50 - 100 ml. of urine were evaporated almost to dryness with 5 ml. of concentrated nitric acid in a Kjeldahl flask. 2 ml. of concentrated sulphuric acid were added and the mixture heated until the nitric acid was driven off. The remainder of the organic matter was destroyed by the addition of perchloric acid and further heating. The digest was diluted to a known volume with water. To an aliquot, containing about 0.01 mg. Cu, were added 5 ml. of 20% sodium citrate and the mixture made alkaline by the addition of ammonia. 5 ml. of amyl alcohol were added, followed by 0.5 ml. of 2% sodium diethyldithiocarbamate and the mixture shaken vigorously. The amyl alcohol layer was separated and compared in a colorimeter with a standard, prepared similarly.

Faeces.

For similar reasons, the technique used for faeces

was similar to that used for urine.

1 g. of dried faeces was (a) ignited in a silica basin over a bunsen burner, or (b) digested with 5 ml. of concentrated sulphuric acid and 15 - 25 ml. of perchloric acid to destroy organic matter. The residue was dissolved in distilled water, containing hydrochloric acid, and diluted to a known volume. An aliquot, containing about 0.01 mg. Cu, was taken for analysis which was carried out in the same way as urine.

Diets

To estimate the copper content of diets the following method was employed.

A half day sample of the diet was dried and ground up. An aliquot of this was transferred to a silica basin and 100 ml. of 10% sodium phosphate added. The mixture was dried and ignited over a bunsen burner. The ash was dissolved in water containing hydrochloric acid and made up to a known volume. An aliquot, containing about 0.01 mg. Cu, was taken for analysis which was carried out as for urine and faeces.

To destroy organic matter, an ignition method was adopted because a large amount of material had to be used as the ash content was low. When samples of diet were ashed in silica dishes, low results and poor recoveries of

added copper were obtained. Urine and faeces could be ignite under similar conditions to give accurate results. These materials usually gave a large ash residue whereas in the case of diets it was small. When diets were ashed, it was found that invariably the silica of the basin was attacked. These difficulties were overcome by mixing the diet with sodium phosphate prior to ignition. Under such conditions consistent results and quantitative recoveries of added coppe were obtained.

From the results shown in Table 32, it will be seen that copper added to urine, faeces and diets could be estimated quantitatively.

Soft Tissues.

Owing to the low ash content of soft tissues, the method adopted was similar to that for diets.

It was decided to see whether copper could be estimated directly in trichloroacetic acid extracts of soft tissues as in the case of blood. The following method was used.

10 g. of finely cut tissue was ground up with some broken glass in a mortar. 40 ml. of 10% trichloroacetic acid were added and the grinding continued. The supernatant

fluid was filtered 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 tissues were made up to 50 ml. The final estimation was made directly, using 20 ml. of extract, as described for blood.

The results obtained by this method were compared with those obtained after ashing. The results are shown in Table 33. It will be seen that copper may be estimated directly in trichloroacetic acid extracts of soft tissues. The results obtained by the two methods are the same within the limits of experimental error.

Bone.

Bone offers special difficulties owing to its very high calcium phosphate content. A preliminary separation of the copper is necessary. Many writers have attempted to separate copper as the sulphide but this is time consuming and liable to lead to difficulties. The following method has been found to give rapid and accurate separations of copper.

A sample of bone (30 - 40 g.) was ashed in a silica basin over a bunsen burner. The ash was dissolved in water

containing hydrochloric acid and the volume made up to 250 ml. 50 ml. of the solution were introduced into a separating funnel, sodium diethyldithiocarbamate added and the mixture extracted 3 times with ether, 25 ml. being used on each occasion. The combined ether extracts were collected in a digestion flask and the ether evaporated off. The residue was digested with 1 ml. of concentrated sulphuric acid and 1 ml. of perchloric acid to destroy organic matter. The residue was diluted to 25 ml. with water and the copper determined in an aliquot.

Milk.

For similar reasons, the copper content of milk was astimated by the same method as that used for bone.

From the results shown in Table 34, it will be seen that copper added to milk or solutions of bone ash were estimated quantitatively.

The method, as described, is specific for copper, in so much that none of the metals that occur normally in animal tissues and excreta and plant tissues interfere. Bismuth does interfere. Bismuth does not occur in animal tissues and excreta under normal conditions but the metal and its salts are used therapeutically. Bismuth forms a lemon yellow complex which is soluble in amyl alcohol under

the same conditions as copper. To be certain that the colour in the amyl alcohol layer is due to copper, after colorimetric comparison, the extract should be shaken with a solution of potassium cyanide. If due to copper, the colour will disappear, if due to bismuth, it will remain. It is obvious that an estimation of the copper content of faeces should never be made after oral administration of bismuth carbonate. Throughout this work, the writer has not been troubled with bismuth.

It has been found that the depths of colour produced by amounts of copper of the order of 0.01 mg. Cu, proved very satisfactory for colorimetric analysis. As a result, throughout this work, a standard containing 0.01 mg. Cu and amounts of unknown to conform to this have been used.

TABLE 30

The copper content of blood and serum

(mg. Cu per 100 ml.)

A Complete destruction of the organic matter.

B Direct determination on trichloroacetic acid filtrate.

Whole blood

A	B
0.174	0.186
0.169	0.174
0.157	0.171
0.165	0.156
0.200	0.182
0.193	0.199

Serum

A	B
0.256	0.263
0.204	0.205
0.238	0.235
0.220	0.229
0.196	0.204

TABLE 31.

Recovery of copper added to whole blood or serum

(mg. Cu per 100 ml.)

A Initial copper content

B Copper added

C Total copper estimated

D Copper recovered

Whole blood

A	B	C	D
0.156	0.143	0.289	0.133
0.156	0.286	0.435	0.279
0.156	0.429	0.683	0.427
0.223	0.143	0.378	0.155
0.223	0.286	0.500	0.277

Serum

A	B	C	D
0.245	0.143	0.378	0.133
0.245	0.286	0.526	0.281
0.217	0.143	0.351	0.134
0.217	0.143	0.356	0.139
0.217	0.286	0.490	0.273

TABLE 32

RECOVERY OF COPPER ADDED TO URINE, FAECES AND DIET.

		Initial copper content mg.		Copper added mg.	Total copper mg.	Copper recovered mg.
Urine	1	0·216	(*a*)	0·1	0·301	0·085
			(*b*)	0·2	0·400	0·184
			(*c*)	0·3	0·530	0·314
	2	0·160	(*a*)	0·1	0·262	0·102
			(*b*)	0·2	0·368	0·208
			(*c*)	0·3	0·454	0·294
Faeces	1	0·104		0·2	0·300	0·196
	2	0·109		0·1	0·211	0·102
	3	0·083		0·1	0·179	0·096
	4	0·081		0·2	0·296	0·215
	5	0·091		0·2	0·310	0·219
	6	0·088		0·2	0·292	0·204
Diet	1	0·086		0·10	0·180	0·094
	2	0·102	(*a*)	0·10	0·210	0·108
			(*b*)	0·20	0·300	0·198
			(*c*)	0·40	0·506	0·404
	3	0·096	(*a*)	0·20	0·288	0·192
			(*b*)	0·50	0·595	0·499

TABLE 33.

THE COPPER CONTENT OF TISSUES.

(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

TABLE 34.

Recovery of added copper

A Initial copper content

B Copper added

C Total copper content

D Copper recovered

Bone (mg.)

A	B	C	D
0.051	0.050	0.106	0.055
0.051	0.075	0.127	0.076
0.051	0.100	0.159	0.108
0.051	0.200	0.259	0.208
0.051	0.300	0.353	0.302

Milk (mg. per litre)

A	B	C	D
0.140	0.100	0.246	0.106
0.140	0.200	0.342	0.242
0.140	0.300	0.448	0.308
0.140	0.400	0.541	0.401
0.140	0.500	0.648	0.508

The Copper Content of Blood.

The copper content of the blood has been estimated on many occasions. The following results have been reported.

Human whole blood.

0.113 - 0.114 mg.per 100 ml. - Schönheimer and Oshima (1929)

0.185 - 0.210 mg.per 100 ml. - McFarlane (1932)

Human serum or plasma.

0.124 mg. per 100 ml. - Warburg and Krebs (1927)

0.056 - 0.075 mg.per 100 ml. - Guillemet (1931)

0.08 - 0.095 mg. per 100 ml. - Locke et alia (1932)

Horse whole blood.

0.058 mg. per 100 ml. - Elvehjem et alia (1929)

Horse serum.

0.19 mg. per 100 ml. - Abderhalden and Møller (1928)

0.09 mg. per 100 ml. - Locke et alia (1932)

Ox whole blood

0.14 mg. per 100 ml. - McHargue (1925)

0.185 - 0.226 mg. per 100 ml. - McFarlane (1932)

Ox serum.

0.058 - 0.082 mg. per 100 ml. - Guillemet (1931)

Pig serum.

0.194 mg. per 100 ml. - Warburg and Krebs (1927)

0.067 - 0.118 mg. per 100 ml. - Guillemet (1931)

Rabbit serum.

0.104 mg. per 100 ml. - Warburg and Krebs (1927).

0.05 - 0.072 mg. per 100 ml. - Locke et alia (1932)

It would appear that the copper content of the blood of different species and even of individuals within the same species must vary widely or most of the methods are inaccurate.

Guillemet used a method in which the blood was ashed and the copper precipitated first as the hydroxide and then as the metal by electrolysis. Then the copper was dissolved in phosphomolybdic acid, a blue solution resulting and the copper estimated by titrating this solution with potassium permanganate until the blue colour disappeared. This writer estimated amounts of copper of the order of 0.1 mg. by this method. Warburg (1927) utilised the fact that copper catalyses the oxidation of cysteine to cystine to estimate the copper content of serum. Using large quantities of blood, McHargue (1925) and Abderhalden and Møller (1929) employed macro chemical methods. Elvehjem et alia (1929) used the Biazzo method while Locke et alia (1932) and McFarlane (1932) used sodium diethyldithiocarbamate to estimate copper.

The present writer has estimated the copper content of the blood of 21 normal males and 10 normal females. The estimations were made directly on trichloroacetic acid filtrates as described earlier. The

results are shown in Table 35. The range, 0.185 - 0.229 mg. Cu per 100 ml., is very narrow. These results agree with those obtained by McFarlane. Since this work was published, Sachs et alia (1935) have determined the copper content of normal human blood but obtained a slightly lower range.

The question arises as to the distribution of copper between the corpuscles and the plasma. Guillemet (1932) found much higher concentrations of copper in the plasma than in the whole blood in the ox, pig and dog. Schindel (1931) obtained higher values for the copper content of the plasma than the cells in man. Elvehjem et alia (1929) found more copper in the corpuscles than in the plasma in defribinated horse blood. McHargue et alia (1928) found more copper in the plasma than the corpuscles in the ox. The values obtained by Elvehjem et alia and Guillemet were much lower than those obtained by the writer. The figures obtained by Schindel were of the same order as those obtained by the writer.

Serum from clotted blood, plasma from citrated blood and whole blood were examined. The results are shown in Table 36. It will be seen that plasma contains slightly higher concentrations of copper than corpuscles.

The copper content of whole blood and plasma from

a number of animals of different species has been determined. From the results shown in Table 37, it will be seen that the ranges are of the same order as that in man.

TABLE 35.

The copper content of normal Human blood.

mg. Cu per 100 ml.

Males

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

Females

1.	0.208	5.	0.196	9.	0.196
2.	0.228	6.	0.188	10.	0.216
3.	0.198	7.	0.196		
4.	0.216	8.	0.214		

Table 36

Distribution of copper between corpuscles and plasma (human)

mg. Cu per 100 ml.

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

TABLE 37

The copper content of the whole blood and plasma of various species

mg. Cu 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	0.187
	2.	0.187	0.199
	3.	0.208	0.208
	4.	0.205	0.217

		Whole blood
Rabbit	1.	0.143
	2.	0.147
	3.	0.144
	4.	0.155
	5.	0.139
Guinea Pig.	1.	0.179
	2.	0.192
	3.	0.186
	4.	0.184

The Copper Content of Urine and Faeces.

So far only one investigation into the copper content of urine appears to have been made. Rabinowitch (1933) obtained values of a trace to 0.41 mg. Cu per litre of urine. He found that the addition of copper to the diet produced an increase in the copper content of the urine.

In the present investigation the copper content of the faeces and daily diet was estimated as well as that of the urine. The results are shown in Table 38.

With the exception of Case No.17, there was a balance between the intake and output of copper within the limits of experimental error. In Case No.17 the intake of copper was low and there was a negative balance. From this case one might assume that the minimum amount of copper necessary to preserve equilibrium is of the order 0.7 mg. Cu per day.

The copper content of a number of urines is shown in Table 39. These figures agree closely with those obtained by Rabinowitch.

TABLE 38.

Case	No. of days of collection	Av. daily excretion of copper in the urine mg.	Av. daily excretion of copper in the faeces mg.	Total daily excretion of copper mg.	Av. daily content of copper in diet mg.
1. Normal	6	0·29	2·60	2·89	2·72
2. Fracture	15	0·36	1·98	2·34	2·44
3. Fracture	13	0·52	1·88	2·40	2·44
4. Fracture	21	0·45	2·08	2·53	2·42
5. Fracture	21	0·50	2·15	2·65	2·42
6. Fracture	20	0·36	2·08	2·44	2·42
7. Normal	7	0·38	2·18	2·56	2·41
8. Normal	10	0·33	2·07	2·40	2·41
9. Normal	9	0·29	2·00	2·29	2·41
10. Normal	14	0·30	1·93	2·23	2·34
11. Fracture	16	0·44	1·70	2·14	2·29
12. Fracture	20	0·22	1·83	2·05	2·22
13. Normal	12	0·21	1·67	1·88	2·12
14. Amputation of leg	18	0·18	1·56	1·70	1·89
15. Nephritis	8	0·32	1·24	1·56	1·43
16. Nephritis	8	0·16	1·00	1·16	1·21
17. Carcinoma of stomach	8	0·11	0·52	0·63	0·21

TABLE 39.

THE COPPER CONTENT OF URINE.

	No. of cases	Minimum	Maximum	Average
1. Copper excretion in mg. per litre	16	0·08	0·48	0·18
2. Copper excretion in mg. *per diem*	24	0·12	0·52	0·28

The Copper Content of Human Tissues.

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. Cu per kg. dry tissue and ranged in descending order of concentration his results may be summarised as follows: liver, kidney, brain, pancreas and spleen. Other writers have obtained similar results [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 on pathological subjects. In certain pathological conditions it has been shown that the copper content of the liver is very much increased - pigmented and non-pigmented cirrhosis of the liver, yellow atrophy of the liver, and haemochromatosis [Schönheimer and Oshima (1929), Cherbuliez and Ansbacher (1930), Kleinmann and Klinke (1930), Herkel (1930)]. It

has been shown that the copper content of foetal organs are very much higher than in the adult [Kleinmann and Klinke (1930), Cunningham (1931), Sheldon and Ramage (1931)].

The question of bone seems to have been neglected. Of the above writers, Herkel and Sheldon and Ramage alone have examined this tissue. The former, as a result of two analyses, obtained values of 3.7 and 4.03 mg. per kg., while Sheldon and Ramage state that bone contains only a trace of copper.

Tissues were obtained from a number of cases post mortem, and their copper contents estimated. The results are shown in Table 40 and agree with those obtained by other workers. Rib and vertebra contained comparatively high concentrations of copper but these results are probably high due to their vascular nature and the inability to separate bone marrow.

The distribution of copper between the soft tissues and skeleton of 4 adult mice was determined. The results are shown in Table 41 and indicate that no more than 15% of the total copper was present in the skeleton. These results are probably high due to the inability to separate bone marrow. The skeleton must have little affinity for copper and reserves of copper must be stored in the soft tissues.

Tissues were obtained from 4 foetuses and their copper contents estimated. The results are shown in Table 42 and indicate that the copper contents of the livers were much increased.

TABLE 40

THE COPPER CONTENT OF HUMAN TISSUES

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

Table 41

Distribution of copper in the mouse

	1	2	3	4
Weight (g.)	32	33.5	29.5	35
Soft tissues (mg.)	0.085	0.078	0.068	0.073
Skeleton (mg.)	0.015	0.012	0.008	0.010
Total copper (mg.)	0.100	0.090	0.076	0.083
% Total copper in Skeleton	15.0	12.5	10.5	11.9

Table 42

The copper content of the tissues of human foetuses

mg. Cu per kg. fresh tissue

	1	2	3	4
Brain	1.25	1.06	0.90	1.16
Liver	55.6	48.6	47.4	40.3
Kidney	7.6	3.26	-	-
Femur	1.06	1.26	1.96	2.06

Factors influencing the Absorption of Copper, from the Alimentary Tract

The writer has shown that the absorption of both iron and lead from the alimentary tract is influenced by the calcium content of the diet. It has been shown also that the addition of acid to the diet increases the absorption of these elements. It seemed highly probable that the absorption of copper from the alimentary tract is governed by similar factors. The purpose of the following experiments was to confirm this. Dialysis experiments have indicated that the absorption of copper should not be specially inhibited by the presence of phosphoproteins or phosphatides as in the case of ferric iron.

The following basic diets, fed at the rate of 2.5 g. per mouse per day, were used throughout these experiments and had the following compositions:

Low calcium diet

	g.
Whole wheat flour	400
Casein	100
Corn starch	325
Wheat gluten	50
Olive oil	40
Sodium chloride	15
Potassium chloride	15
Butter	15

High calcium diet.

	g.
Whole wheat flour	700
Whole milk powder	300
Marmite	50

In the first experiment, adult male mice were placed on (a) low calcium diet, (b) high calcium diet, (c) high calcium diet containing 0.5 ml. of N. hydrochloric acid per mouse per day, to which had been added copper (as copper sulphate at the rate of 2 mg. Cu per mouse per day for a period of 21 days. At the end of this period, the mice were placed on the high calcium diet alone for 4 days to remove unabsorbed copper from their alimentary tracts, killed and their total copper contents estimated.

In the second experiment, young female mice (aged 3 weeks) were placed on (a) low calcium diet, (b) high calcium diet, (c) high calcium diet containing 0.5 ml. of N. hydrochloric acid per mouse per day, to which had been added copper (as copper sulphate) at the rate of 0.8 mg. Cu per mouse per day for a period of 14 days. At the end of this period the animals were placed on high calcium diet alone to remove unabsorbed copper from their alimentary tracts, killed and their total copper contents estimated.

The results are shown in Table 43, and it will be seen that they are very similar to those obtained with lead. They indicate that absorption of copper is highest on a low calcium diet and is influenced by the gastric acidity. Even under apparently optimum conditions, one must conclude that either the absorption of copper is low or else the retaining power of the tissues is low. Under similar conditions, lead appears to be much easier absorbed and retained.

Table 43

A

Adult male mice - 2 mg. Cu per mouse per day

Period - 21 days.

	Weight g	Total copper mg	Copper mg.per 100 g. mouse.
Controls			
1.	19	0.031	0.16
2.	25	0.031	0.12
3.	20	0.033	0.17
4.	21	0.029	0.14
5.	19	0.031	0.16
6.	24	0.023	0.10
7.	16	0.025	0.16
8.	25	0.025	0.10
		Average	0.14
Low calcium diet			
9.	21	0.062	0.29
10.	19	0.040	0.21
11.	21	0.052	0.25
12.	19	0.048	0.25
13.	15	0.046	0.31
14.	20.5	0.050	0.24
15.	21	0.054	0.26
16.	18	0.062	0.33
		Average	0.27

	Weight g.	Total copper mg.	Copper mg. per 100 g. mouse
High calcium diet			
17.	19.5	0.032	0.16
18.	21	0.038	0.18
19.	18.5	0.028	0.15
20.	23	0.039	0.17
21.	22.5	0.033	0.14
22.	18.5	0.029	0.14
23.	20	0.034	0.17
		Average	0.16
High calcium diet and hydrochloric acid.			
24.	24	0.065	0.28
25.	23	0.062	0.27
26.	23.5	0.071	0.30
27.	22.5	0.050	0.22
28.	25.5	0.081	0.32
29.	23	0.048	0.21
30.	23	0.057	0.24
31.	21.5	0.081	0.37
		Average	0.28

B

Female mice (3 weeks) - 0.8 mg. Cu per mouse per day

Period - 14 days.

	Weight g.	Total copper mg.	Copper mg. per 100 g. mouse
Controls			
1.	9.5	0.027	0.28
2.	9	0.025	0.26
3.	8.5	0.025	0.29
4.	8.5	0.020	0.24
5.	7.5	0.016	0.21
	Average	0.023	0.25
Low calcium diet			
6.	16	0.063	0.39
7.	14	0.043	0.31
8.	10.5	0.036	0.34
9.	13	0.042	0.32
10.	14	0.042	0.30
11.	14.5	0.100	0.69
12.	13.5	0.046	0.34
13.	13.5	0.048	0.36
	Average	0.053	0.38

	Weight g.	Total copper mg.	Copper mg. per 100 g. mouse
High calcium diet			
14.	14.5	0.028	0.19
15.	14.5	0.031	0.21
16.	13.5	0.029	0.22
17.	16	0.030	0.19
18.	17	0.031	0.18
19.	12.5	0.025	0.20
	Average	0.029	0.22
High calcium diet and hydrochloric acid.			
20.	14	0.051	0.36
21.	12.5	0.053	0.42
22.	14.5	0.048	0.33
23.	12.5	0.052	0.42
24.	13.5	0.040	0.30
25.	13.5	0.048	0.36
26.	13	0.042	0.32
	Average	0.048	0.36

The Copper Content of the Blood in Certain Conditions.

Increased copper content of the blood has been reported in pregnancy [Warburg and Krebs (1927), Krebs (1928) Locke et alia (1932)]. The normal values obtained by these workers were very much lower than those obtained by the writer. The copper content of blood from women in various stages of pregnancy was estimated and the results are shown in Table 44.

It will be seen that the copper content of the blood is increased in pregnancy, the increase occurring chiefly in the last three months. Dieckmann and Wegner (1936) have shown that in pregnancy, although there is a slight decrease in the haemoglobin %, there is a large increase in blood volume. The copper content of the organs, especially the liver is much increased in the foetus. Either or both of these phenomena are probably connected with the increased blood copper in pregnancy.

Warburg and Krebs (1927) have reported increases in the copper content of the sera of pigeons with anaemia due to haemorrhage. Sheldon and Ramage (1931), using a spectrographic method, noted in an examination of 28 human

bloods that the strongest lines due to copper were in a case of carcinoma and a case of anaemia due to haemorrhage. Locke et alia (1932) reported increases in the copper content of the serum in carcinoma but decreases in anaemias.

The copper content of blood from cases of anaemia has been estimated and the results shown in Table 45.

A marked increase in blood copper was observed in many of those conditions associated with an anaemia; obstructive jaundice, acholuric jaundice, hyperthyroidism, leukaemia, after haemorrhage. There were no increases in pernicious anaemia. Where anaemia was associated with carcinoma, there were increases with the exception of one case which showed a sub-normal value. This case showed by balance experiment that there was a daily loss of copper (Case 17 - page 192). In a series of cases of anaemia associated with nephritis, the blood copper was increased, normal or decreased. These increases of blood copper are undoubtedly associated with increased regeneration of blood. This large influx of copper into the blood stream could be derived from other tissues or by an increased retention, probably the former. Sub-normal values are probably due to a negative copper balance and would suggest that the administration of copper salts would be beneficial.

At the conclusion of this work, a paper by Sachs et alia (1935) appeared which confirmed all the results reported here. These writers could not offer any explanation for the low blood copper values in anaemia associated with nephritis.

The copper content of the blood was estimated during treatment in 3 cases of post haemorrhagic anaemia. From the results shown in Table 46, it will be seen that there was a fall towards normality.

In obstructive jaundice, cirrhosis of the liver, hyperthyroidism, myxoedema, pneumonia, diphtheria, diabetes mellitus, carcinoma and nephritis, when there was no anaemia, the blood copper was normal.

Table 44.

The copper content of the blood in pregnancy

mg. Cu per 100 ml.

4 months' pregnancy.		
	1.	0.200
	2.	0.257
5 - 6 months' pregnancy.		
	3.	0.216
	4.	0.228
	5.	0.228
	6.	0.232
	7.	0.246
	8.	0.255
	9.	0.284
	10.	0.363
7 months' pregnancy.		
	11.	0.204
	12.	0.255
	13.	0.286
	14.	0.286

8 months' pregnancy.

15.	0.250
16.	0.367

at term.

17.	0.229
18.	0.244
19.	0.250
20.	0.250
21.	0.250
22.	0.258
23.	0.259
24.	0.259
25.	0.280
26.	0.286
27.	0.290
28.	0.294
29.	0.321
30.	0.333
31.	0.359
32.	0.359
33.	0.365
34.	0.366
35.	0.380

Table 45.

The copper content of blood

in anaemias

mg. Cu per 100 ml.

		Hb %
Acholuric jaundice		
1.	0.301	48
Myeloid leukaemia		
2.	0.311	42
3.	0.305	32
Lymphatic leukaemia		
4.	0.273	23
Carcinoma of the Breast		
5.	0.245	75
6.	0.294	70
7.	0.290	40
8.	0.290	38
Carcinoma of the Colon		
9.	0.290	55
Carcinoma of the Prostate		
10.	0.289	52
Carcinoma of the Stomach		
11.	0.158	22

		Hb %
Obstructive jaundice		
12.	0.333	48
13.	0.253	45
After haemorrhage		
14.	0.267	73
15.	0.330	59
16.	0.288	44
17.	0.320	43
18.	0.385	42
19.	0.400	42
20.	0.294	40
21.	0.400	38
22.	0.420	38
23.	0.340	38
24.	0.360	35
25.	0.370	33
26.	0.320	30
Nephritis		
27.	0.246	68
28.	0.177	68
29.	0.177	65
30.	0.200	65
31.	0.240	65

Nephritis			Hb %
	32.	0.275	65
	33.	0.196	64
	34.	0.176	64
	35.	0.147	60
	36.	0.275	55
	37.	0.227	45
	38.	0.167	45
	39.	0.215	45
	40.	0.218	45
	41.	0.325	45
	42.	0.152	42
	43.	0.309	35
	44.	0.136	28
	45.	0.133	28
Pernicious Anaemia			
	46.	0.212	45
	47.	0.215	45
	48.	0.223	44
	49.	0.208	54
	50.	0.223	50
	51.	0.227	48

Table 46

Case 1

Hb %	33	40	42	45	50	78
Blood copper	0.36	0.29	0.21	0.22	0.21	0.19

Case 2

Hb %	67	67	68	75
Blood copper	0.29	0.22	0.20	0.18

Case 3

Hb %	40	57	61	74
Blood copper	0.39	0.31	0.18	0.19

Discussion

The writer has shown that ferric iron but not ferrous iron forms very stable compounds with substances of the nature of phosphatides and phosphoproteins. Whether such stable compounds are formed with simple proteins is debatable. Failure to realise this in the past, has resulted in the isolation of iron containing compounds, many of which have been designated as haemoglobin precursors. Iron which can be extracted with trichloroacetic acid with or without the aid of thiolacetic acid, sodium hydrosulphite or sodium pyrophosphate and capable of direct estimation with thiolaceti acid in such extracts has been designated as 'inorganic' iron to distinguish it from haematin iron. It has been considered that this is appropriate since inorganic iron salts added to biological materials react in a similar manner. A simple method for estimating the 'inorganic' iron content of biologic materials has been described.

Evidence has been produced to show that ferric iron as such is not absorbed from the alimentary tract and that all iron that is absorbed is in the ferrous state. It has been shown that reduction of ferric iron can occur in the stomach.

The following factors have been shown to influence the absorption of iron ;

1. gastric acidity,
2. the calcium content of the diet,
3. the oxidation - reduction potential of the stomach contents as determined by the diet.

The gastric acidity is important in two respects,

1) it aids solution of the iron, 2) it is necessary for the reducing action of the diet, if any, to take effect. Lack of gastric acidity has been held responsible for the hypochromic anaemia which often occurs in achlorhydria. Ferric iron would not be absorbed under such conditions but ferrous iron should be absorbed if the diet does not contain substances that produce autoxidation and substances e.g. phosphates, that are liable tp precipitate iron.

The calcium content of the diet has a marked effect. The effect is similar to that produced with lead and is probably of similar cause. During the first few months of man's separate existence, his diet consists solely of milk. Milk is deficient in iron and as a result, a store of iron is laid down in the foetus, chiefly in the liver, to suffice for this period. Milk is not the best medium for iron to be absorbed from, owing to its high calcium content. The writer suggests that Nature in her wisdom realises this and instead of secreting milk of a high iron content to the detriment of the mother and much of which would be wasted, has laid down a store in the foetal liver.

It has been shown that babies fed on cows' milk, develop an anaemia which is more severe and develops earlier than those on human milk [Mackay (1931), Fullarton (1937)]. It has been suggested that human milk contains a higher iron content than cows' milk and that it is more easily absorbed. The writer considers that this may be explained. Human milk differs from cows' milk in two respects, 1) lower calcium content, 2) higher lactalbumin content. Both of these factors

should produce a better absorption of iron, the former by producing a lower buffering action and the latter a more reducing medium.

Attempts have been made to determine the minimum amount of iron necessary in the daily diet [Sherman (1933)] and the relationship between diet and the incidence of anaemia [Davidson et alia (1933)]. All have failed. The writer considers that it is impossible to state any particular quantity of iron as being a minimum as the degree of iron absorption depends on the other constituents of the diet. The same applies to the other investigation.

Workers in nutrition usually consider human requirements in terms of the daily intake. The writer has shown that absorption of essential substances may result in mutual inhibition. As a result, a daily diet should be plann into meals in such a way that mutual inhibition is reduced to a mimimum. One could even go a stage further and plan a meal into courses since it is well known that food tends to layer in the stomach in such a way that digestion and absorption of one course is independent of another. This is of particular importance in the case of growing children in which the requirements are large.

A large number of iron salts, both ferric and ferrous have been used in the treatment of hypochromic anaemias. The may be administered on an empty stomach or after a meal. The advantage of the latter is that their passage through the intestine will be delayed and absorption should be greater. When administered after a meal, it should be immaterial

whether ferric or ferrous salts are used, provided that the diet contains iron reducing substances and hydrochloric acid secretion is normal. The latter can be remedied if defective by the administration of hydrochloric acid. It is an obvious advantage that the diet should have predominating reducing properties.

Although ferric iron appears to be capable of dialysi readily in the presence of pyrophosphates, this does not appe to be of any physiological importance.

'Inorganic' iron has been shown to be distributed throughout the tissues. The iron content of the skeleton is low and therefore its capacity as a reserve for iron is negligable. The highest concentrations were found in spleen and liver. In the former, the iron is derived probably from haemoglobin of broken down R. B. C. while in the latter the iron will be derived from this source and from absorbed iron. The 'inorganic' iron content of other tissues were found to be low but fairly constant. The liver is usually regarded as holding the greater part of the iron reserves but the fact should not be neglected that no inconsiderable amount may be held in the soft tissues at low concentrations ; the importan factor is quantity, a point often neglected. The 'inorganic' iron content of plasma is low and is considered to represent transport iron. The 'inorganic' iron content of the R. B. C is higher. Its function is obscure.

The tissues, especially the liver, are said to have a great affinity and retaining power for iron in that iron from the haemoglobin of broken down R. B. C. is not excreted

or only excreted with difficulty. McCance and Widdowson (19
have stated that recently absorbed iron is only excreted with
difficulty and then in the urine and not by the intestine.
Thet go so far as to suggest that other metals are not excret
by the intestine. With regard to iron, the writer has no
results to contradict this but considers that their statement
is probably incorrect since it has been irrefutably shown
that both lead and copper are excreted by the intestine, in
fact it appears to be the more important path of excretion.
The great affinity of the tissues for iron is due probably
to the fact that much of the iron is in the ferric state
and that phosphatides are distributed widely throughout the
body.

A simple method for the estimation of copper in
biological materials has been described. It has been shown
that copper is extracted quantitatively from tissues by
trichloroacetic acid and in such extracts reacts directly wit
sodium diethyldithiocarbamate. Copper occurs in all tissues
including blood. The quantity of copper in the skeleton
is small and therefore the capacity of this tissue to act as
a reserve of copper is negligable. The highest concentration
of copper were found in the liver ; this organ is usually
regarded as holding the greater part of the copper reserves o
the body.

It has been shown that the absorption of copper from
the alimentary tract is influenced by 1) the degree of gastri
acidity, 2) the calcium content of the diet. During the firs
few months of man's separate existence, his diet consists

solely of milk. Milk is deficient in copper and as a result, a store of copper is laid down in the foetus, chiefly in the liver, to suffice for this period. Milk is not the best medi for copper to be absorbed from, owing to its high calcium content. The writer suggests that Nature in her wisdom realises this and instead of secreting milk of high copper content, to the detriment of the mother and much of which would be wasted, has laid down a store in the foetal liver. After weaning, the diet should contain a much higher copper content. Undoubtedly during this period positive copper balances are necessary. Absorption of copper depends on the composition of the diet. Remarks have been made as to the importance of arranging the daily diet into meals in such a way as to obtain maximum absorption and minimal mutual inhibition. Similar remarks may be applied to copper.

During periods of increased haemoglobin formation there will be increased demands for 'inorganic' iron. This will result in a flow of iron from the reserves to the site of haemoglobin formation. Such iron must be carried by the blood stream, the plasma probably being the chief carrying agent. The plasma iron appears to be in the ferric state and as such non-dialysable. It seems probable that at some period it becomes dialysable i.e. it becomes reduced to the ferrous state, before it can be utilised. It is however useless to speculate on such problems without experimental evidence. Thi aspect of iron metabolism has been sadly neglected and the writer considers that research along these lines may produce very fruitful results.

The iron content of urine under normal conditions is low and is probably connected with the low plasma iron and the fact that it appears to be in the ferric state. The iron content of the urine is increased after oral ingestion of iron salts. The copper content of urine is comparatively high This is probably connected with a comparatively high plasma copper and the fact that copper is more easily dialysable than iron.

It has been proved definitely that copper catalyses the production of haemoglobin from 'inorganic' iron. The exact rôle of copper is however unknown. It has been suggeste that an intermediate compound between copper and a porphyrin like substance is formed but there is no definite evidence. At the present time there is no experimental evidence to indicate how copper acts. We have however the fact that in hypochromic anaemias and in the later stages of pregnancy, periods of increased haemoglobin formation, the blood copper is in general increased very much. As a result, the writer is of the opinion that the copper effect probably takes place in the blood itself.

There nas been much controversy as to whether the administration of copper is essential in anaemias in man. None of these studies appear to have included any estimations of blood copper. The writer considers that such estimations might throw some light on the problem. The prevalence of low blood coppers in anaemias associated with nephritis requires explanation. The writer considers that this indicate a copper insufficiency, this being due probably to the nature

of the diets used in the treatment of nephritis.

Very little is known as to the fate of haemoglobin except that the iron is held tenaciously by the tissues and the porphyrin part of the molecule is excreted in the bile as bilirubin. Numerous workers [Barkan (1925 - 1937), Starkenstein and Weden (1930), Moore et alia (1939)] have concerned themselves with what they describe as the 'easily split' iron of the blood. It is located in the corpuscles. Barkan and Scnales (1937) believe that this is derived from so called pseudo-haemoglobins which they regard as intermediates in the decomposition of haemoglobin. The writer is inclined to the view that it is an artifact. Estimations of 'easily split' iron involve drastic treatment of blood, a most complex mixture, with acid at high temperature. No worker has shown that haemoglobin is not attacked under such circumstances. The writer has been working on the problem recently and although the work has not been completed, has obtained sufficient evidence to show that haemoglobin does decompose under such conditions.

Haemochromatosis, although a rare condition, has received considerable attention. Of particular interest, is the origin of the large deposits of iron in the soft tissues, especially the liver. A considerable amount of work has been done on the subject and has been reviewed by Sheldon (1934). It is now considered that these large deposits of iron are due to a positive iron balance over a long period. The tissues, especially the liver, generally show an increased copper content. Mallory and his co-workers (1925, 1931) have

attempted to show a relationship between copper poisoning and haemochromatosis. This view is not accepted.

The many riddles with which we are confronted are due to our very incomplete knowledge of the fundamentals of iron metabolism. It is strange that although the importance of iron in respiration has been known for a long period, so littl is known about the physiological processes in which this element is involved. This lack of knowledge is probably responsible for our inability to understand the rôle of copper Methods of approach to these subjects have been very similar. It appears to the writer that different and more variable methods of approach are required.

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Lead, Iron and Copper

Contrasts and Comparisons

Iron, copper and lead are representatives of the heavy metals and although the interest in the two former is chiefly physiological and in the latter chiefly toxicological, it is of interest to compare and contrast them biochemically in so far as our knowledge permits.

All three exist in two forms of different valencies iron in the divalent and trivalent states, copper in the monovalent and divalent states and lead in the divalent and tetravalent states. We know that the two forms of iron may be found in animal tissues and diets and in the case of copper we have evidence that although the divalent form is more general, the monovalent form may be found in certain situations e.g. the R. B. C. There is no evidence to sugges that lead exists in biological materials in anything but the divalent state. The possible differences in physiological action of two forms of a metal with different valencies should not be overlooked.

Under normal conditions, the human diet contains considerably more iron than copper and considerably less lead than copper. Marked differences may be observed in the capacity of the body to absorb and retain these. Lead appears to be most easily absorbed and retained and because of its toxic properties, it is just as well that it exists in normal diets in small quantities. The absorption of thes three metals appears to be dependent upon the calcium content of the diet and the gastric acidity in the same way. The absorption of iron is complicated by the fact that ferrous iron is absorbed but not the ferric form. This

necessitates a reducing mechanism in the alimentary tract. Lead appears to be most easily retained. This is probably connected with the fact that whereas the skeleton has a great affinity for lead, it has little affinity for iron or copper. The liver has a marked affinity for all three metals.

Lead and copper probably exist in animal tissues in comparatively simple forms but iron exists in various forms, some simple and others complex e.g. haemoglobin, ferric complexes.

Lead and copper appear to be fairly easily excreted the intestine being important in this respect. Iron on the other hand does not appear to be as easily excreted. This is probably due to two reasons, 1. much of the body iron is present as haemoglobin, 2. the distinctive properti of ferric organic complexes.

The kidney is usually regarded as a concentrating organ. Although iron, copper and lead are present in urine their concentrations are much lower than in plasma. Two causes probably contribute to this. In the first place, these metals are probably circulating as metallic protein complexes which are poorly ionised and as a result the concentration of metal in the glomerular filtrate will be representative of the ionised part only and in the second place no concentration occurs during passage through the tubules.

The mobilisation of lead under the influence of various agents is unique and is connected with its depositi

in the skeleton. Im this respect it resembles calcium.

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