METALS IN MEDICINE

by

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PART C THE URINARY EXCRETION OF IRON IN HEALTH AND DISEASE

PART D THE EFFECT OF IRON ON FERRITIN SYNTHESIS IN RAT INTESTINAL MUCOSA

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ABBREVIATIONS

The following abbreviations have been used in the text of this study.

ALA	δ aminolaevulic acid
CP	coproporphyrin
RNA	ribonucleic acid

PART A. TREATMENT OF LEAD POISONING WITH ORAL PENICILLAMINE

1. History of lead poisoning

The discovery of lead was not recorded but it is mentioned in the Old Testament and was known to the Assyrians and early Egyptians. Recorded history credits the Romans with the first extensive use of lead, mostly for water pipes, and thus to the beginning of its history as an occupational disease. The clinical features of lead intoxication have long been recognised. Hippocrates first described severe abdominal colic although it was not until 1572 that the term 'colic' was coined by Citois and became generally used to describe the symptoms of lead poisoning. At this time, an extensive epidemic, characterised by severe abdominal colic, occurred in France in the province of Poitou. This was later identified as lead poisoning, the origin of the poisoning being traced to vintners who had 'sweetened' sour wine by the addition of litharge (lead oxide) to the wine so that sugar of lead (lead acetate) was formed. In 1656, Stockhausen recognised that lead poisoning was an occupational disease. More than one hundred years later in 1759, Huxham made his classical description of 'Devonshire colic', later diagnosed by Baker (1772) as being caused by the consumption of cider which had been adulterated by storage in lead glazed pottery. Huxham reported

vomiting and constipation, delirium, languor, limb paralysis and epilepsy. In 1814, Orfila carried out experimental investigations on lead toxicology, the first investigation of its kind since lead poisoning had been recognised as an occupational disease, over 150 years previously. Later, in 1839, a classical description of the symptoms of lead intoxication, which differs little from that of the present day, was elucidated by Tanqueral des Planches. In 1840, at St. Thomas's Hospital, London, Burton noted that patients on lead oxide therapy, produced a 'blue line' or Burtonian line on the gums, one of the recognised diagnostic signs of lead poisoning today.

2. Signs and symptoms of lead poisoning

Lead poisoning (plumbism or saturnism) is almost exclusively an occupational disease (although it is a hazard in childhood) which is normally chronic in nature. It may resemble many disorders, rendering diagnosis difficult and may be initially asymptomatic or present as a non-specific illness. The poison affects the whole body but especially the nervous system, the gastrointestinal tract and the blood-forming tissues. Its victims become pallid, digestion is deranged and anorexia and painful colic with constipation results. The loss of power

is generally shown first in the fingers, hands and wrists and the condition known as 'wrist drop' soon follows, rendering the victim useless for work. The palsy will extend to the shoulder and shortly after to the legs also. Impairment of renal function can occur and the sight can be weakened or even lost. One of the early signs of lead poisoning is the pallor of the skin giving rise to pale lips and an ashen face. The cause of the pallor is not known. It occurs before any marked change in blood and cannot be explained on the basis of anaemia (Goodman and Gillman, 1955). In patients having their own teeth, a lead line may develop along the gums, but similar pigmentation can occur after the absorption of mercury, bromine, silver, tellurium and iron. "Premature ageing" is often associated with advanced lead poisoning.

3. Chemical Diagnosis of Lead Poisoning

Lead exposure is said to occur when the level of lead in blood and urine exceeds the upper normal limit. Most authors do not state this limit but prefer instead to state a critical value beyond which the diagnosis of lead poisoning must be made. Kehoe (1961, Lec. 3) recognised this limit as occurring when the lead concentration in blood reached 80 µg./100 g. whole blood

and when the urinary excretion of lead exceeded a value as low as 150 µg./litre or as high as 240 µg./litre dependent on the urine sample. The Committee on Lead Poisoning (1943) recognised that the diagnosis of lead poisoning should be made when the urinary excretion of lead exceeded 150 µg./litre and that lead concentrations in excess of 70 µg./100 ml. whole blood were indicative of recent lead exposure.

4. "Physiology" of Lead

Absorption of Lead into the Body.

Lead can enter the body mainly by way of the respiratory tract, the alimentary tract and the skin.

In normal metabolism, most of the lead ingested in food and beverages passes along the alimentary tract and is excreted, mainly in faeces, with little or no absorption. Kehoe (1961) has shown that the introduction of lead compounds in solution into the alimentary tract, along with food and beverages, results in a limited degree of absorption of lead (8-12%) but that the inhalation of lead on a schedule corresponding to that of industry causes a prompt increase in the lead content of the tissue, blood and urine. The pulmonary absorption of lead is

fairly efficient (30-50%).

The absorption of inorganic lead compounds through the skin is of little practical significance in the development of lead poisoning (Aub et al, 1926) but organic lead compounds, such as tetraethyl lead, can rapidly penetrate intact skin. Abrasion of the cutaneous epithelium may allow the passage of lead into the systemic circulation and lead on the hands may often be transferred to the mouth.

Transportation of Lead and its influence on Metabolism.

Lead is transported by the blood, reducing the erythrocyte life span by some specific mechanism which is not yet known. At least 90% of the lead is bound to red blood cells (Bambach et al, 1942). Young red blood cells are affected, probably in the marrow, before their formation is complete. The inhibition of maturation of young red blood cells can be reflected by the rise in the percentage of basophilic stippled cells (Basophilic stippling of erythrocytes in peripheral blood is not specifically related to the absorption of abnormal quantities of lead since it occurs in many blood diseases).

As early as 1936, Watson suggested that porphyrinuria might

be a manifestation of an effect of lead on the synthesis of haemoglobin. He found that in the urine of patients suffering from lead intoxication, there were abnormally high concentrations of coproporphyrin, uroporphyrin and δ aminolaevulic acid. These substances are precursors in the synthesis of haem (Table 1) and can be measured quantitatively in the presence of urinary lead. Circulating lead is able to interfere in the early stages of porphyrin metabolism, probably due to its inhibitory action on -SH containing enzymes (Haeger-Aronsen, 1960). This causes the urinary excretion of abnormal quantities of porphyrins.

The toxic effect of lead on young red blood cells and on the synthesis of haem, usually causes a secondary anaemia to develop.

Deposition of Lead.

The preliminary distribution of lead occurs via the general circulation to the kidneys, liver, lungs, spleen and muscles. But within a few days, the metal is redistributed and the major part is deposited in an inactive form in the flat bones (Aub et al, 1926). Lead can be removed from circulation and stored in bones due to deposition along with calcium when

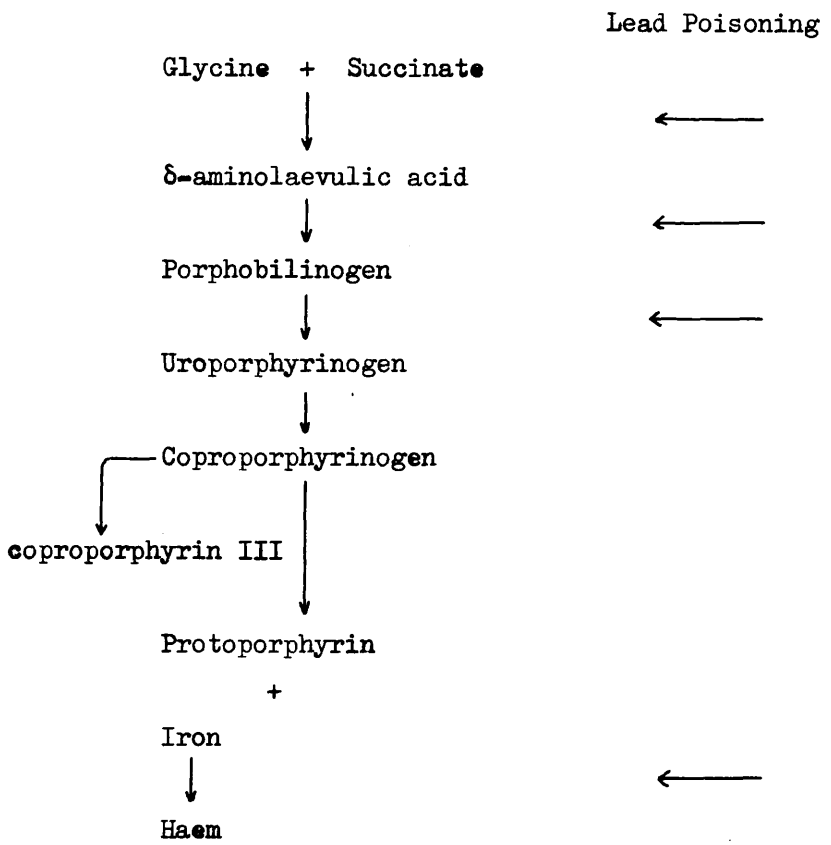


TABLE 1

Possible sites of disturbed haem biosynthesis in lead poisoning.

a high calcium diet (e.g. milk) is maintained. In prolonged lead exposure there is generally a greater concentration of lead in the long bones (Kehoe, 1961, Lec 2).

Excretion of Lead.

Lead is eliminated from the body via the intestines and kidney, the faecal excretion being far greater than that of urine. The major portion of faecal lead represents unabsorbed lead. Under normal conditions, during lead balance, the urinary excretion of inorganic lead is approximately 9% of the amount ingested (Kehoe, 1961, Lec 2). The cessation of exposure to lead is followed by a gradual excretory loss of lead from the body until, after a time appreciably longer than the period of exposure, the lead concentrations of excreta and blood fall back to normal levels.

Mobilisation of Body Lead.

In prolonged lead poisoning much lead is stored in the bones and this may be mobilised during metabolic stress, in acidosis due to lowering of blood pH (Aub et al, 1925) and inadvertently as a result of the administration of drugs, e.g. parathyroid extract (Hunter and Aub, 1927). This unexpected

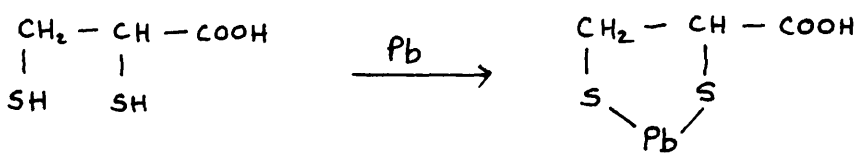
release of lead may cause grave, even fatal illness. In prolonged renal failure, lead is also mobilised from the bones and released into the soft tissues where it is accessible to chelation.

5. Chelation

The principle of chelation has found application in the treatment of lead poisoning. The role of chelating agents in this regard is to form with the metal a diffusable complex which is readily excreted from the body. A given chelating agent may have a strong affinity for a metal but be useless as a detoxifying agent for reasons of rapid metabolism in vivo, or toxicity to the patient requiring treatment. A number of chelating agents have been used to accelerate the excretion of lead from the body and one of the first to be used was B.A.L.

B.A.L.

B.A.L. (British Anti-Lewisite, dimercaprol), a dithiol derivative of glycerol, came into use in 1945.



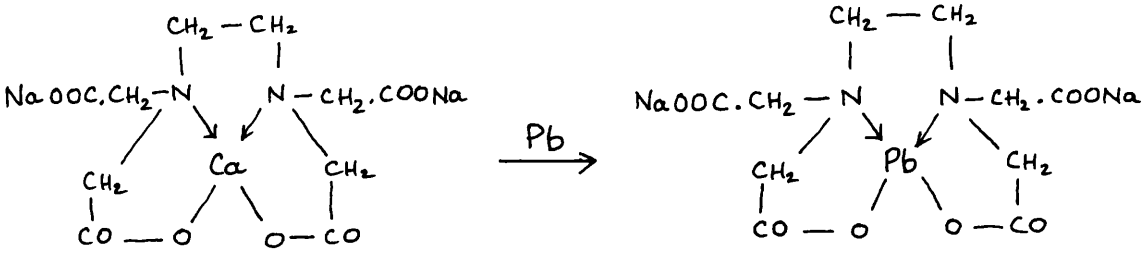
It was used as a complexing agent for both lead and copper.

However, not only is the intramuscular injection of the viscous liquid painful but the resulting lead complex is not very stable. After eight days of treatment in doses not greater than 2.5 mg./kg. body weight at one time, 4-6 times daily, 2 days rest is required. After a few years, it became evident that dimercaprol was not particularly suitable as an antidote to plumbism, since it is rapidly metabolised in vivo and also causes the development of skin rashes, fever and even coma in some patients. B.A.L. initially increases urinary lead excretion quite markedly but the effect wanes rapidly and the amount of lead mobilised is negligible. Apparently only lead in blood can be chelated by B.A.L., lead in bone and soft tissue being too firmly bound to be mobilised (Ryder and Kehoe, 1947). As the interest in B.A.L. waned, so the first favourable reports on E.D.T.A. were published (Bessman et al, 1952).

E.D.T.A.

E.D.T.A. (calcium disodium ethylenediaminetetraacetate, calcium disodium versenate) forms a water soluble, stable, non-toxic complex with lead in blood and soft tissue and once bound, the chelate passes freely through most of the membranes of the

body, being excreted rapidly by the kidney. E.D.T.A. is not metabolised in the body.



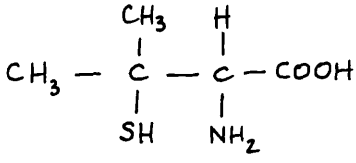
In experiments with rats, 95-98% of labelled ^{14}C versenate appeared in the urine six hours after administration (Foreman et al, 1953). However in patients with poor renal function, there is a prolonged period of increased lead excretion. This keeps the versenate circulating in the body for a much longer period causing elevated blood lead levels (Rieders et al, 1955; Bessman and Layne, 1955). The administration of intravenous E.D.T.A. produces a maximal excretion of urinary lead (Byers and Maloof, 1954) in contrast to that of intramuscular E.D.T.A. Thus the former is used mainly in the treatment of acute lead poisoning.

E.D.T.A. has however proved disappointing since severe cases of renal damage, some of them fatal, occurred after excessive dosage (Dudley et al, 1955; Foreman et al, 1956). It is not usual to exceed a dose of 1g. infused slowly at twelve hour intervals. Courses of treatment must not last for more than five days but may

be repeated after one week.

Penicillamine

Penicillamine ($\beta\,\beta$ dimethyl cysteine) is so named because it was the first crystalline substance, a primary amine, to be identified among the products of the hydrolysis of penicillin (Abraham et al, 1943).



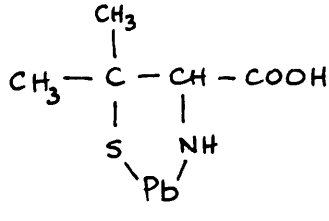
The asymmetric carbon atom means that penicillamine can exist in the D,L or DL form. The D stereoisomer is used in the preparation of D penicillamine hydrochloride which is usually given orally in capsule form, each capsule containing 150 mg. Penicillamine can also be given intravenously.

Penicillamine was first used by Walshe (1956) to chelete copper in the treatment of Wilson's Disease. The copper-penicillamine chelate was very stable and greatly increased the urinary excretion of copper. In 1957, Boulding and Baker published a preliminary communication on the treatment of metal poisoning with penicillamine and stated that high doses (900 mg/day) of penicillamine may apparently be given for a long period with safety although no long-

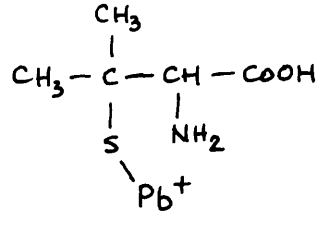
term trials were quoted in the article. They showed that 900 mg. of penicillamine per day given to a lead smelter with a urinary lead excretion of 510 µg./litre, caused the urinary output to increase to 2000 µg. lead/litre. A female worker, exposed to lead vapours, with a urinary excretion of 530 µg. lead/litre after one month of E.D.T.A. therapy, was given 900 mg. penicillamine per day. On the 4th and 13th day of treatment, 1530 and 2000 µg. lead/litre urine were excreted respectively.

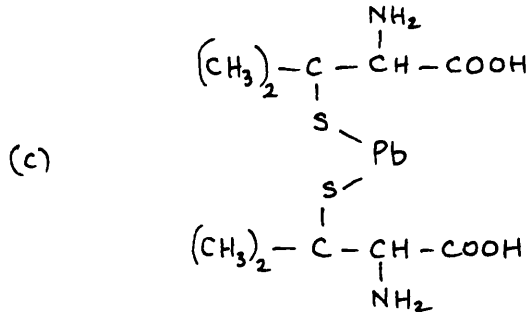
Oral pencillamine is rapidly absorbed from the small intestine. The effectiveness of penicillamine appears to be due in part to its relative stability to metabolic degradation, unlike its non-methylated parent compound cysteine (Aposhian, 1961). Penicillamine is able to form a firm, extremely soluble non-toxic complex with lead which is rapidly excreted by the kidney. The chelation of lead by penicillamine depends on the affinity of lead for the sulphydryl group and the complex may be one of the following:-

(a)



(b)





(c)

In 1959, Fellers and Shahidi reported the onset of nephrotic syndrome in a 16 years old boy after eleven months continuous penicillamine treatment for Wilson's Disease. In the same year, Seignette and his co-workers published a paper comparing versenate and penicillamine in the treatment of one patient with Wilson's Disease and one with lead poisoning. The patient with lead poisoning worked in an accumulator factory and had an average basal urinary lead excretion of 250 µg./24 hours. Daily 900 mg. doses of oral penicillamine given on four consecutive days raised the urinary excretion of lead to 2400, 2400, 1800 and 1800 µg./24 hours respectively. After a two day rest, 1000 mg. of intravenous E.D.T.A. per 24 hours was given daily for four consecutive days resulting in an excretion of 3500, 3500, 2000 and 2000 µg. of lead/24 hours respectively. The authors stated that one patient on continuous penicillamine therapy for eight months for the treatment of Wilson's Disease, showed no untoward reactions.

6. Purpose of the Study

At the start of this work, penicillamine was not being generally used in the treatment of lead poisoning although its value in the treatment of Wilson's Disease was recognised and Walshe (1956) had recommended its use for the treatment of gold and mercury poisoning. The following study was carried out with the object of 1) determining the optimal dose of penicillamine required for the excretion of absorbed lead in cases of lead exposure and lead poisoning and 2) observing any side effects of penicillamine therapy if any, before 3) investigating whether a small prophylactic dose of penicillamine could usefully be given at regular intervals of time to those working under conditions of lead exposure, to discourage the accumulation of lead in the body.

7. Incidence of Lead Poisoning.

The incidence of lead poisoning is high in the following industries:-

a) In the manufacture of storage batteries, lead plates are made and covered in a paste of litharge, PbO. When the plates are dipped in dilute sulphuric acid, litharge is converted to lead sulphate.

b) In the building and breaking yards of ships and cars, lead is present in pure or alloy form and as red lead, Pb_3O_4. The use of oxyacetylene burners for cutting metals, often painted with red lead, and the use of high temperatures for welding, cause the formation of particulate lead and the volatilisation of lead compounds.

c) In the potteries and glass industries, red lead, litharge and white lead, $2PbCO_3.Pb(OH)_2$ are used in making flint glasses and other forms of glass and also in making glazes for pottery.

8. Outline of Study

A short term study was carried out on nine patients with chronic or subacute lead intoxication to determine the effect of oral D-penicillamine, in varying doses, on the excretion of urinary lead, coproporphyrin (CP) and δ aminolaevulic acid (ALA) as well as the clinical manifestations of the disease. The urinary excretion of CP (Chisholm and Harrison, 1956) and of ALA (Haeger-Aronsen, 1960) provide sensitive biochemical indices of 'metabolically active' and presumably toxic lead. Blood lead, haemoglobin, reticulocyte count and punctate basophilia were also determined.

A long term study to determine the effect of long term oral penicillamine therapy was carried out for a period of 10 weeks on four oxyacetylene metal burners working in a shipbuilding yard and for a period of 16 weeks on one worker from an accumulator factory. All five subjects showed clinical and biochemical evidence of lead intoxication.

Section AII METHODS AND RESULTS

1. Haematological Methods.

Determination of urinary coproporphyrin and δ aminolaevulic acid

Haemoglobin was measured by the alkaline haematin method. Other haematological values were obtained by standard methods (Dacie 1956):-

Reticulocytes.

Non-nucleated young red cells (or reticulocytes) are an intermediate form between the nucleated normoblast and normal red cell. They contain strands of nuclear material and stain with brilliant cresyl blue, showing a basket-like or reticulated appearance. In normal metabolism, a blood smear shows concentrations of 0.5 - 2% of reticulated cells.

Basophilic stippled cells

Punctate basophilia are red cells which show a stippled or dotted appearance. They may appear in blood as a result of lead exposure or due to certain blood disorders, but they are not present in normal blood metabolism. Stippled cells stain with Loffler's methylene blue and are counted under oil immersion lens, several fields being scanned.

Urinary coproporphyrin

This was determined using the ether and hydrochloric acid extraction method of Rimington (1961).

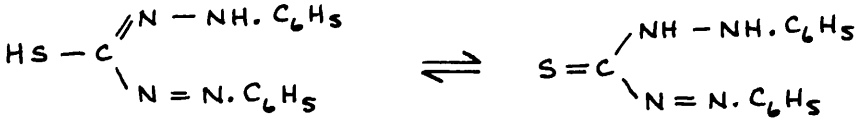
Urinary δ aminolaevulic acid

This was determined using the ion exchange resin technique of Mauzerall and Granick (1956).

2. Lead

Lead can be estimated quantitatively by the formation of its dithizonate complex or by its precipitation as the sulphide or chromate. Due to its extreme sensitivity, dithizone is the reagent of choice for the assay of microgram quantities of lead.

Dithizone (diphenylthiocarbazone), soluble in chloroform and carbon tetrachloride, forms a red dithizonate complex with lead. Up to pH 12, dithizone acts as an acid with the H on the S removable and exists in equilibrium in both forms.

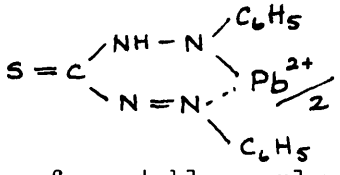


I ⇌ II

If dithizone in form II is written as H_2Dz, then the reaction with lead can be written as follows,

$$Pb^{2+} + 2H_2Dz \rightleftharpoons Pb(HDz)_2 + 2H^+$$

the lead dithizonate complex having the form



Dithizone can form stable complexes with many metals, e.g. Pb, Bi, Sn^{II}, Tl^{I}, Pd, Hg, Ag, Cu, Au and Zn, under alkaline or acid conditions. Each of these metals forms a dithizonate at a specific pH or pH range but if several of these metals are present in any one solution, more than one may be extracted into the dithizone-solvent layer.

Mechanism of Lead Extraction.

It is sometimes difficult to separate one metal in the presence of others and, to improve the separation factor, masking or sequestering agents are used. The masking agent usually forms water soluble complexes with the interfering metals in competition with the extracting agent. Masking agents (e.g. cyanide, citrate) form sufficiently strong complexes with interfering metals so that

the latter are prevented from reacting with the extracting agent either altogether or at least until the pH is much higher than the value needed for quantitative extraction of the metal of interest. Very often the metal of interest also forms a complex with the masking agent with the result that a somewhat higher pH range is required for its extraction.

At pH 8-10 in the presence of cyanide, lead is quantitatively extracted by dithizone, dissolved in chloroform (Dithizone-metal complexes are more soluble in chloroform than carbon tetrachloride). Under such conditions, stannous tin, thallium and bismuth are also extracted and calcium and magnesium phosphates can interfere. These phosphates can be masked by the addition of citric acid (tartrate is less effective) before final adjustment of pH. No attempt was made at preliminary extraction of Sn^{2+} Tl^{+} or Bi^{2+} since these elements are not normally present in the body in appreciable amounts.

Determination of lead in urine, faeces and blood

Lead was estimated by the dithizone method of Gonzales et al (1954) as summarised below. This was later modified.

For blood, urine and tissue digests Gonzales et al recommended a wet digest, to which was added 5 ml. of distilled water and 5 ml. of saturated sodium chloride (NaCl) in 5% hydrochloric acid (HCl) for each 5 ml. of 36 N sulphuric acid used in the digestion process. (The NaCl - HCl solution dissolved any insoluble lead sulphate present). Any insoluble precipitate was filtered off and the filter paper washed with 5 ml. of distilled water. After the addition of 2 drops of phenol red indicator, the filtrate was made alkaline with 14 N ammonium hydroxide. 5 ml. of 10% citric acid was added (to dissolve precipitated phosphates and prevent their reprecipitation on adjustment of pH) and the pH adjusted to 7.6 with ammonium hydroxide. 2 ml. of 10% potassium cyanide (KCN) was added and the solution transferred to a 250 ml. separating funnel. The aqueous solution was shaken with small portions of dithizone solution (15 mg./l chloroform, $CHCl_3$) and the $CHCl_3$ layers combined and shaken with two 10 ml. portions of water, discarding the washings. The dithizone solution was then shaken with 20 ml. of stripping reagent (5 ml. 14 N ammonium hydroxide + 10 ml. 10% KCN + 85 ml. water) and again washed with two 10 ml. volumes of water. The $CHCl_3$ layer was run into a 50 ml. volumetric flask and the volume made up with $CHCl_3$. The optical density of the red lead

dithizonate in $CHCl_3$ was read against a $CHCl_3$ blank in a spectrophotometer at a wavelength of approximately 510 mμ.

Preparation of lead-free glassware

Glassware was freed from lead by washing in the normal manner, immersing in 8 N nitric acid overnight and rinsing thoroughly with deionised water.

Extraction of lead from simple chemical solution

Initial steps were taken to draw up a calibration graph, relating the concentration of lead to the optical density.

Lead solutions in 250 ml conical flasks were adjusted to pH 7.6 with 14 N ammonium hydroxide(NH_4OH) using 1% w/v phenol red indicator and 2 ml 10% KCN w/v was added. After transfer, with water washes, to a 250 ml. Quickfit separating funnel, the dark green dithizone solution (15 mg/l. $CHCl_3$) was added from a burette and the separating funnel shaken vigorously for 30 seconds. If the $CHCl_3$ layer was bright red (indicating saturation of existing dithizone), further dithizone was added and the funnel again shaken. A dull red $CHCl_3$ layer with a dark tinge indicated the extraction of the major portion of lead. The $CHCl_3$ layer was run off into a second separating funnel and 2-3 0.5 ml portions

of $CHCl_3$ were added to the 1st separating funnel and drawn off into the second separating funnel to wash all traces of lead dithizonate from the outlet tube (This was done at every subsequent $CHCl_3$ separation). The aqueous layer was shaken vigorously with a further 3 ml of dithizone solution for 30 seconds. When the phases had separated, the deep blue-green $CHCl_3$ extract (indicating the presence of excess unchelated dithizone) was run off and combined with the first $CHCl_3$ extract. The aqueous layer was discarded. 15 ml of stripping reagent (100 ml. water + 5 ml 14 N. NH_4OH + 10 ml 10% KCN) were added and the separating funnel shaken. If the aqueous layer was an orange brown shade (indicating backwashing of large quantities of dithizone), the first separating funnel was rinsed out with deionised water and 1-2 ml of $CHCl_3$ run through the outlet tube (so that no water remained) before the $CHCl_3$ layer was run into this separating funnel. This $CHCl_3$ extract was shaken with a further 10 ml of stripping reagent when the aqueous layer should be pale yellow (discard) and the $CHCl_3$ extract a clear bright red. After allowing the layers to separate and having checked that the outlet tube was dry (water disperses in $CHCl_3$ to cause cloudiness),

the $CHCl_3$ extract was run off into a 50 ml. volumetric flask with the aid of a $1\frac{1}{2}$" filter funnel. The solution was made up to the mark with $CHCl_3$ and gently mixed before reading on the Hilger uvispek.

It was found that all standard stopcock greases - vaseline, apiezon, Steven's rubber grease and silicone grease - were attacked by chloroform. For a short time, chloroform itself or a fluorohydrocarbon grease were used as lubricants. At room temperature, the fluorohydrocarbon grease was fairly hard and did not allow stopcocks to be turned easily, although it was otherwise satisfactory. These in turn were superseded by glycerol. Since glycerol mixes readily with water, stopcock glycerol must be renewed after each complete lead extraction. At all possible stages in the extraction of lead, aqueous solutions were not left in contact with stopcock glycerol, chloroform being added where required to prevent dissolution.

The stability of the lead dithizonate complex in chloroform with time was confirmed by checking the optical density over a two hour period, using optical cells fitted with ground glass stoppers to prevent evaporation of chloroform. If protected from light and evaporation, the lead dithizonate complex in chloroform is stable

for at least a few days. The absorption maximum of lead dithizonate in chloroform was determined on the Hilger uvispek and found to be at 515-516 mμ (Fig. 1) which is in agreement with the findings of Marmet (1958).

Calibration Graph.

A calibration graph was determined, using working solutions prepared from a standard lead acetate trihydrate solution of 200 μg.lead/ml. The concentrations of lead as lead dithizonate in μg./50 ml. $CHCl_3$ were plotted against the optical density reading obtained on the Hilger uvispek at wavelength 516 mμ and slit width 0.05 mm. Since dithizone and lead dithizonate in chloroform obey Beer's Law if monochromatic light is used, the calibration graph (Fig. 2) was a straight line passing through the origin (tangent 0.00595).

3. Collection of urine, faecal and blood samples.

Urine

Lead-free polythene containers (cleaned as under glassware) were used for 24 hour urine collection. All collections contained thymol crystals as preservative and were kept cool and protected from light to avoid accelerating the breakdown of coproporphyrin

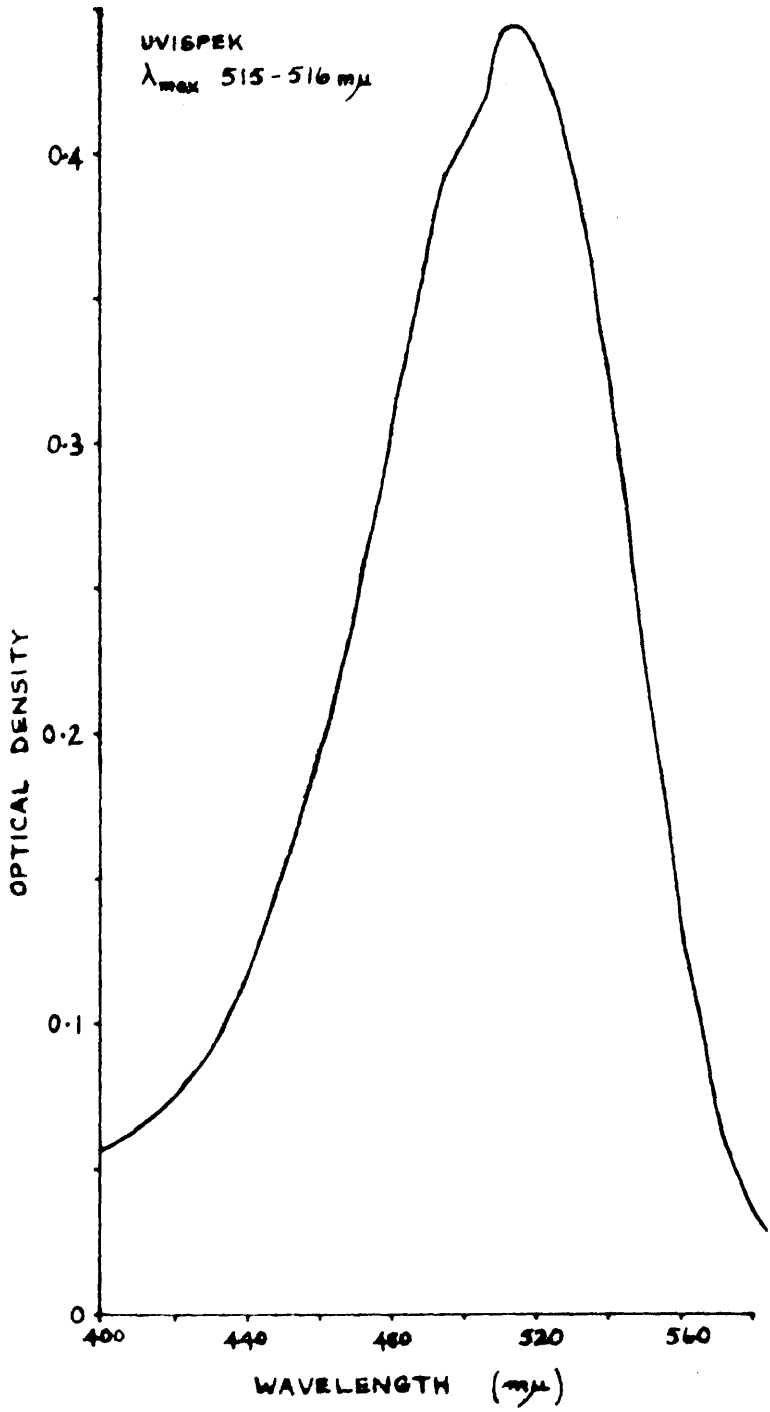


Figure 1. Determination of the absorption maximum of lead dithizonate in chloroform on the Hilger uvispek.

and δ amindaevulic acid. 30-100 ml. of urine were used for lead estimation.

Faeces.

Faecal samples were collected in lead free polythene containers and homogenised in glass homogenisers by means of a 'Kenmix.' 30-40 g. of the homogenate, for lead estimation, was weighed directly into a 50 ml. Kjeldahl digestion flask.

Blood.

Approximately 20 ml. of venous blood was withdrawn for lead estimation. The blood was collected in a heparinised bottle and weighed directly into a 50 ml. digestion flask. (Blood was measured by volume initially. Taking the mean specific gravity of blood was 1.06 (Wintrobe, 1961) all weights were converted to volumes of blood).

4. Concentration of Urine

In order to digest 100 ml. urine samples, initial concentration had to be effected. Lead can be precipitated from alkaline urine by addition of ammonium oxalate solution, lead oxalate being entrained along with calcium oxalate. However, complexed lead (e.g. lead penicillamine complex) would not be precipitated with inorganic lead.

As much as 40% of the urinary lead can escape determination if the method of coprecipitation is used (Dinischiotu et al, 1960). Other methods of concentration were tried.

Evaporation.

The most common method of concentrating large volumes of urine (up to 500 ml.) is by evaporation from an evaporating basin placed on a source of heat. This method was rejected as loss of sample through splashing and frothing was unavoidable.

Freeze Drying.

100 ml. of urine was placed in a 500 ml. round-bottomed flask, resting in a mixture of solid carbon dioxide and alcohol, and the flask spun so that a thin layer of urine was frozen over its entire inside surface. When this flask was connected to the freeze drying apparatus and a vacuum applied, the rate of loss of water was very slow, two 100 ml. samples being reduced to ca. 20 ml. in 7 hours. When a 5-10 ml. urine concentrate was obtained, it was difficult to remove the dried material completely from the inside of the flask with dilute acid and still keep the overall volume small. If a smaller flask (e.g. a 250 ml. round-bottomed flask) containing 100 ml. of urine was used, the layer of frozen urine was so thick

that frothing occurred on connecting to the vacuum, resulting in loss of material into the main frozen compartment of the freeze drying apparatus.

This method was rejected not only because it was time consuming but also because of transfer difficulties after concentration.

Evaporation under controlled conditions.

100 ml. of urine was pipetted into a 250 ml. conical flask and an anti-bumping device added, i.e. a bubble stick, which can also be used as a glass rod to facilitate liquid transfer. On placing on the hotplate, most urine samples frothed violently, becoming cloudy in the process. After experimentation with anti-foam reagents (e.g. capryl alcohol and anti-foam A), it was found that the addition of 5 ml. of concentrated nitric acid caused the urine aliquot to clarify and boil without frothing. The increased acidity not only caused the oxidation and breakdown of some organic compounds (turning the urine black in the process) but helped to keep other substances in solution until, as the colour of the urine steadily lightened, the volume was reduced to 10-15 ml. The concentrate was poured into a 50 ml. kjeldahl digestion flask and

the conical flask rinsed once with ion-free water. The washing was added to the kjeldahl flask. Two ml. 16N nitric acid were heated in the conical flask until nitric acid refluxed to the top of the flask (to remove any remaining lead) and the acid added to the kjeldahl. The conical flask was rinsed out four times with small portions of ion free water and the washings added to the kjeldahl flask.

5. Digestion

A wet digestion technique, using sulphuric and nitric acids, was employed in preference to incineration. The latter requires a higher temperature and may lead to loss of lead due to volatilisation.

Urine

The kjeldahl flask containing urine was placed in an electric heating mantle and the volume rapidly reduced to ca. 5 ml. when $2\frac{1}{2}$ ml. 36N sulphuric acid (H_2SO_4) were added. (Failure to add H_2SO_4 to urine and faeces before dryness is reached results in the rapid emission of a column of dense white fumes, proceeded by spontaneous ignition, with consequent loss of the sample inside the kjeldahl.) Boiling was continued until the oxides of nitrogen

were given off and charring occurred. 11N nitric acid was added dropwise as necessary to oxidise organic material. Digestion was complete only when on continued boiling, further charring did not occur. Small volumes of ion-free water were added to the kjeldahl and allowed to boil off to eliminate the last traces of nitric acid. Urine digests are colourless.

Faeces

The 30-40 g. homogenate was warmed in the presence of 3 ml. 16N nitric acid and digested as above. Faecal digests are colourless.

Blood

A weighed sample was warmed in the presence of 3 ml. 16N nitric acid and stirred frequently, by bubble stick, to stop foam rising into the neck of the kjeldahl flask. Once boiling had commenced, when the solution turned a greenish colour, $2\frac{1}{2}$ ml. of 36N H_2SO_4 were cautiously added. If H_2SO_4 is not present before the oxides of nitrogen are given off, a very violent reaction occurs resulting in almost complete ejection of the sample from the kjeldahl. A further 1 ml. of H_2SO_4 may be required during digestion. The digestion process was as before. Blood digests

are straw yellow and have a trace of pale coloured precipitate present. (A light brown precipitate, the colour of which may be difficult to discharge is evidence of an incomplete digestion.)

Preparation of Digests for Extraction of Lead

While blood, faecal and urine digests were still quite warm, 5 ml of ion-free water were cautiously added to each and the contents of each kjeldahl vigorously swirled. For every 1 ml of 36 N. H_2SO_4 used in the digestion process, 2.5 ml of a solution of equal volumes of saturated sodium chloride and 5% v/v HCl were added. (This concentration of solution was sufficient to convert any insoluble lead sulphate into its soluble chloride form.) All digests were left to stand overnight to allow complete precipitation of insoluble substances. A finely divided dense white precipitate formed in large amounts in faecal digests and in trace amounts, or not at all, in urine digests. A small quantity of this white solid was amassed by pooling precipitates collected after filtration of faecal digests. On cationic and anionic analysis of the precipitate, calcium and sulphate ions were identified. The presence of calcium sulphate in digests of ~~bio~~ biological material was confirmed by Sandell (1959 p 552).

6. Extraction of Lead from Blood, Urine and Faecal Digests

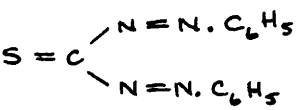
The contents of each kjeldahl plus several washes with ion-free water, were transferred to a 250 ml conical flask, filtering off any heavy calcium sulphate precipitate with No 40 filter paper. Two drops of phenol red indicator were added to the conical flask and the solution made alkaline with 14 $\underline{N}.NH_4OH$. $2\frac{1}{2}$, 5, $12\frac{1}{2}$ ml 20% citric acid (w/v) were added to the conical flasks containing prepared digests of blood, urine and faeces respectively. The pH was readjusted to 7.6 with NH_4OH and 2, 2 and 3 ml 10% KCN (w/v) were added for blood, urine and faecal samples respectively. Each solution was transferred with four water washes to a 250 ml Quickfit separating funnel which already contained 5-8 ml of dithizone (30 mg/litre $CHCl_3$). Contact of glycerol, on the stopcocks, with aqueous solutions was avoided as much as possible, due to the solubility of glycerol in water. For 30 seconds, the two phases were shaken vigorously. The layers were separated and the aqueous phase extracted with a further 3 ml portion of dithizone. The combined $CHCl_3$ extracts were shaken with 15 ml and 10 ml volumes respectively of stripping reagent (5 ml $14\underline{N}.NH_4OH$ + 10 ml 10% KCN (w/v) + 100 ml water). If the concentration of lead dithizonate in the $CHCl_3$

extract was small, the extract was run off into a 25 ml volumetric flask in order to conserve $CHCl_3$ and to give as high a reading for optical density as possible. 50 ml volumetric flasks were used only for high concentrations of extracted lead. A blank sample, containing the same amount of acid used in the digestion process, was treated in exactly the same way as the samples. The optical density of the lead solutions were read against chloroform on the uvispek at 516 mμ and all readings adjusted to a theoretical 50 ml volume (linear graph). After subtraction of the blank, the lead concentrations were read directly from the calibration graph.

Formation of diphenylthiocarbodiazone

Some blood samples, digested in the usual manner, gave a yellow chloroform layer on shaking with dithizone. This solution did not extract lead.

Dithizone can be oxidised under mild conditions to diphenylthiocarbodiazone,



a compound which is insoluble in alkaline or acidic solutions but which dissolves in chloroform giving a yellow or brown solution.

Since extraction of urine digests using the same dithizone solution gave no yellow chloroform extract, an oxidising agent was present in blood digests. No strong oxidising agent was present since the last traces of nitric acid had been removed. Iron was the only element known to be present in large quantities (on addition of NaCl-HCl solution, the yellow colour of ferric chloride was observed) and it was complexed as a ferricyanide. If the latter were able to oxidise dithizone, the removal of iron from the digest should result in normal extraction of lead.

The yellow chloroform extract and aqueous layer were transferred to a 250 ml conical flask and 2 ml of 10% KCN added. On acidifying with 1-2 ml of 16N nitric acid, the solution immediately turned green blue and a suspension of prussian blue (potassium ferric ferrocyanide, $KFe\left[Fe(CN)_6\right]$) formed on heating. This was filtered off when the volume had reduced by half. The filtrate was boiled until the volume was ca. 5 ml and no nitric acid remained. The solution was again filtered to remove the final traces of $KFe\left[Fe(CN)_6\right]$ before extracting with dithizone in the usual manner, when only lead dithizonate was formed. The

lead content of a corresponding blank was estimated and subtracted.

This interference of iron was observed at the terminal stage of the study when blood lead values for normal subjects and those of a cross-section of workers in a ship-breaking yard (listed in Part B) were being determined. Fourteen samples in all were involved. Further blood samples were obtained for 7 of the 8 workers from the ship breaking yard and these gave the normal coloured, chloroform extract due to lead dithizonate. Of the remaining 6, iron was removed (as prussian blue) in 4 of the samples, when a normal extraction of lead followed.

It would appear that the presence of a small concentration of a reducing agent during dithizone extraction may be of advantage.

7. Time required for digestion and extraction of lead

12-15 100 ml urine samples can be concentrated and transferred to kjeldahl flasks in 1-1½ hours. Once the charring stage of the digestion is reached (about 20 mins for urine and warmed blood samples and 1 hour for faecal samples), it takes almost one hour to complete the digestion of, for example 35 g of a faecal aliquot or 22 ml of blood. The time taken for complete

digestion of urine samples after reaching the charring stage may vary from 10 minutes upwards depending on the initial urine concentration. Twelve digestions can be supervised at one time. If the concentration of other urine samples is to be effected at the same time, digestions can be made to proceed more slowly by reduction of mantle temperature.

The digests were grouped on the basis of the volume of nitric acid used in the digestion process (nitric acid being added to some where required), appropriate blanks were prepared and excess nitric acid was boiled off (30-45 minutes). Lead can be extracted from four solutions and the optical density of each read within one hour.

On an average, 12-15 samples can be concentrated, digested and their lead content estimated in two normal working days.

8. Precision of the method of lead assay

Six urine and six blood samples were analysed for lead and good agreement found (Table 2).

	LEAD µg/100 ml	
No.	URINE	BLOOD
1	84.2	63.4
2	82.1	64.5
3	84.7	63.9
4	81.7	65.4
5	84.4	63.0
6	84.8	64.7
MEAN	83.6	64.1
S.D.	$\pm$1.2	$\pm$1.0

Table 2 Precision of determination of lead in urine and blood.

Since the concentration of lead in faeces was generally low, liquification of faeces was carried out using the minimum volume of water. Faecal lead samples were always analysed in duplicate and the average result taken. The standard error calculated from double determinations on 26 faecal samples with lead concentrations between 6.9 and 411. µg per 100g of homogenate (mean 96.8 µg) was 7.7 µg per 100g, compared to a S.E. for 12 urine samples with lead concentrations between 3.9 and 83.0 µg/100ml (mean 30.2 µg) of 0.7 µg/100ml.

The accuracy of the method was tested by adding known quantities of lead to 100 ml aliquots of urine. The results, given in Table 3, show that the method is useful for the determination of lead in biological material, 83 to 90% of the total lead being recovered using two extractions with dithizone.

LEAD (µg)			
PRESENT	ADDED	FOUND	RECOVERED
83.1	10.0	93.6	10.5
83.1	20.0	101.0	17.9
83.1	40.0	119.0	35.9
4.47	10.0	12.70	8.23
4.47	20.0	21.43	16.96
4.47	40.0	38.00	33.53

Table 3 Recovery of lead added to 100 ml. aliquots of urine.

9. Recovery of radioactive lead

^{210}Pb ($t_{\frac{1}{2}}$ 19.4 years) was added to 100 ml urine samples which were concentrated, digested and extracted for lead. The activity of lead dithizonate in chloroform was determined by counting a 10 ml aliquot in a Geiger Muller tube. The activity of lead recovered however was only a fraction of that added. As ^{210}Pb decays, a daughter product bismuth is formed ($t_{\frac{1}{2}}$ ^{210}Bi 2.6 x 10^6 years). Lead 210 emits weak β -rays and some X-rays. Bismuth 210 however emits very strong β rays. Since an equilibrium mixture of ^{210}Pb had been taken, ^{210}Bi was also present but, contrary to expectation, ^{210}Bi had not extracted as a dithizonate along with lead.

The ^{210}Pb solution was extracted with dithizone and 10 ml of radioactive lead dithizonate in $CHCl_3$ (2953 cpm) added to a 100 ml urine sample. After concentration, digestion and extraction, the total activity extracted from the urine two days later was 1663 cpm, uncorrected for decay. Since chemical recoveries were greater than this, no further attempts were made to recover radioactive lead. It may be that counting ^{210}Pb as a liquid sample on a β counter was at fault. ^{210}Pb as a precipitate is best counted on a windowless counter e.g. a tritium counter or an X-ray spectrometer, none of which were available.

10. Normal levels of lead, coproporphyrin, and δ aminolaevulic acid

The levels of lead in blood and urine and of CP and ALA in urine were determined for a group of normal subjects (Table 4) and are summarised in Table 5. Some normal subjects did not wish to give blood.

The levels of blood lead and urine lead established in normal subjects (Table 5) are in agreement with those quoted by Goodman & Gillman (1955) (mean blood lead 30 µg/100g, mean urinary lead 27 µg/litre) and Kaplan & McDonald (1938) (mean blood lead 31 µg/100g). The levels of urinary CP and ALA in normal subjects (Table 5) are also in agreement with those quoted by Zieve et al (1953) (mean for men and women 166 $\pm$ 45 and 134 $\pm$ 42 µg/day respectively) and Haeger-Aronsen (1960) (mean 0.29 $\pm$ 0.14 mg/100 ml) respectively.

Kehoe (1961 Lec. 1) stated that the lead content of random faecal samples obtained from two populations of normal subjects were

a) mean 0.232 mg, S.D. $\pm$ 0.288 (447 subjects)

b) mean 0.398 mg, S.D. $\pm$ 0.310 (102 subjects)

In ten normal subjects, he found the mean daily faecal excretion of lead to be 0.22 $\pm$ 0.20 mg.

No.	Sex	Age	Blood Lead µg/100 ml	Lead µg/day	CP µg/day	ALA mg/day
1	M	52	49.1	84.8	255	2.86
2	M	30	37.6	71.5	383	4.16
3	M	22	-	32.3	202	3.91
4	F	25	26.0	26.6	250	2.48
5	F	56	-	35.3	106	1.55
6	F	52	25.8	24.1	100	0.38
7	M	13	21.6	25.8	90.5	2.24
8	M	42	16.1	5.4	24.1	0.54
9	M	35	14.3	14.7	63.3	2.30
10	F	20	14.7	16.7	46.4	3.43
11	M	44	11.7	11.0	199	4.07
12	M	37	14.0	47.8	205	5.38
13	M	38	26.9	59.9	133	5.10
14	M	27	17.1	24.5	262	4.20
15	M	13	24.7	17.3	129	2.35
16	M	22	-	38.7	120	2.99
17	M	22	-	31.4	82	2.40
18	M	22	-	49.9	122	3.30
19	M	22	-	40.4	87	3.66
20	M	22	-	45.4	108	2.58
21	F	20	21.2	-	-	-
22	F	24	20.1	-	-	-
23	F	22	25.0	-	-	-
24	M	28	23.6	-	-	-
25	M	39	22.9	-	-	-

Table 4 Blood lead and urinary excretion of lead, coproporphyrin and δ aminolaevulic acid in 25 normal subjects.

	No. of subjects	Mean	S.D.	Range
Blood lead	18	22.9 µg./100 ml	8.2	11.7 - 49.1
Urinary lead	20	35.2 µg./day	19.9	5.4 - 84.8
		23.3 µg./litre	12.5	7.0 - 65.2
Urinary coproporphyrin	20	148.3 µg./day	79.2	24.1 - 383.1
		93.2 µg./litre	43.9	47.3 - 196.4
Urinary δ-aminolaevulic acid	20	2.99 mg./day	0.44	0.38 - 5.38
		0.20 mg./100 ml.	0.08	0.02 - 0.35

TABLE 5

Normal values of blood lead, and urinary lead, coproporphyrin and δ-aminolaevulic acid.

11. Clinical Cases

Cases 1-7 are oxyacetylene metal-burners who have worked in a ship-breaking yard from 8 to 35 years. All of these subjects had been found to have increased coproporphyrin in their urine on routine testing.

Case 1. - Aged 42. Admitted to hospital two years previously with acute abdominal colic. He still had intermittent abdominal pain. Hb 14 g./100 ml., but punctate basophilia was present in the peripheral blood and bone-marrow.

Case 2. - Aged 55. Admitted to hospital in 1946, 1948, and 1957 for treatment of lead-poisoning. On the present admission he complained of intermittent abdominal pain. Hb 13 g/100 ml.

Case 3. - Aged 42. In the past year he had complained of weakness of both hands and wrists. Hb 12.5 g/100 ml.

Case 4. - Aged 52. Admitted to hospital in 1953, 1958, and 1960 for treatment of lead-poisoning. He had no symptoms on his present admission, but his haemoglobin level was 10g/100 ml and the reticulocyte count 4%.

Case 5. - Aged 60. No symptoms on admission. Hb 13 g/100 ml.

Case 6. - Aged 50. Admitted in 1948, 1952, 1953, and 1960 for treatment of lead-poisoning. On his present admission he complained of intermittent epigastric and lower abdominal pain. Hb 15 g/100 ml.

Case 7. - Aged 60. Admitted on two previous occasions (1948, 1954) for treatment of lead-poisoning. HB 12 g/100 ml. He had no symptoms on admission.

Case 8. - A 48-year-old foreman of an accumulator factory had been at this employment for 15 years. One year previously he had been treated with sodium calciumedetate because of right wrist and finger drop. He was readmitted on January 24, 1962, because of a recurrence of this paralysis. He felt reasonably well, though his haemoglobin level was 10 g/100 ml. In 1952 he had a skin rash after a penicillin injection.

Case 9. - A man aged 38 had been making lead ingots for only six weeks. He presented with abdominal and lumbar pain, vomiting, and constipation. On examination he had a "blue line" at the gum-tooth margin. Hb 11 g/100 ml. Reticulocyte count 3%. Punctate basophilia was noted in bone-marrow smears but not in his peripheral blood.

12. Scheme of therapeutic investigation: Short-term trial of penicillamine

This was carried out in hospital in a metabolic ward. All patients had a pretreatment period of 7 to 12 days for the measurement of the daily output of urinary and/or faecal lead, urinary CP and ALA. Haematological and some blood lead estimations were made. Cases 1 and 2 had seven day courses of penicillamine of 900 mg/day. Case 9 had two separate courses of penicillamine of 900 mg/day, each course lasting seven days. Cases 3 and 4 had 900 mg of penicillamine daily for 4 days, followed by 1500 mg daily for four days. Cases 5, 6 and 7 had 1500 mg daily for seven days. These details are listed in Table 6.

Case	Penicillamine (mg./day)		Mean Lead (μg./day)		Mean Coproporphyrin (μg./day)		Mean δ-Aminolaevulic Acid (mg./day)	
	Dose	Duration	Before	During	Before	During	Before	During
1	900	7 days	398	939	975	377	40·0	10·8
2	900	7 ,,	218	855	588	211	25·5	17·1
3	900	4 ,,	192	796	1,269	983	31·5	25·8
	1,500	4 ,,		1,136		133		12·8
4	900	4 ,,	141	738	786	606	28·8	19·7
	1,500	4 ,,		946		198		12·2
5	1,500	7 ,,	202	1,033	722	224	22·6	11·9
6	1,500	7 ,,	149	521	612	159	23·2	5·1
7	1,500	7 ,,	200	746	499	158	12·9	6·0
8	150–1,200	23 ,,	242	756	878	276	43·8	11·7
9	900	7 ,,	—	—	1,502	851	64·9	36·4
	900	7 ,,	876	1,261	632	400	29·5	16·5

Table 6 Short-term trial. Summary of the mean urinary lead, coproporphyrin and δ-aminolaevulic acid in Cases 1-9 before and during treatment.

13. Results of the Short-term Trial

In every patient prior to treatment with penicillamine the values of urinary lead, CP and ALA, were greatly in excess of normal levels. On the other hand, the faecal lead values prior to treatment fell (Case 1) or lay (Cases 2-7) within the normal range (Kehoe 1961 Lec 1). This is explained by the fact that faecal lead excretion is not a satisfactory index of lead intoxication, where the exposure is mainly due to the vapours of lead, and that the faecal lead values approach normal within a few days after the termination of such exposure. (Goodman & Gillman, 1955)

Clinical Course

There was clinical improvement during treatment in each of the patients who had symptoms. In Cases 1, 2, and 6 there was an absence of abdominal pain. Case 3 claimed that there was a return of strength to his hands. In Case 8 there was improvement in muscle power after two weeks of treatment. Complete return of power occurred after a longer course of penicillamine. In Case 9 there was a rapid cessation of abdominal pain, vomiting, and constipation within three days of the beginning of penicillamine treatment.

Urine

The highest basal urinary lead excretion recorded amongst these nine patients was that of the patient suffering from acute lead intoxication (Case 9). He was admitted to hospital prior to the lead study and was given penicillamine therapy for two periods, each of seven days, the results of the second period of which are shown in Fig. 3. The lead excretion was determined on the remaining available urine samples and a marked rise in lead excretion was noted on commencement of oral penicillamine. Day 14 marked the end of the first course of penicillamine and from day 15, the urinary excretion of ALA steadily increased until its value was doubled. CP, on the other hand, rose immediately to a high level on discontinuation of penicillamine therapy. On resumption of penicillamine treatment, the excretion of ALA decreased slowly while that of CP showed an immediate pronounced fall. On the 30th day, the urinary excretion of CP had fallen within the normal range while the urinary excretion of both ALA and lead were still 4 times the upper normal value.

When 900 mg of penicillamine was given daily to Cases 1 and 2, there was a significant rise in the urinary lead excretion, which declined slowly after the initial high output (Figs. 4 and 5).

Case 9

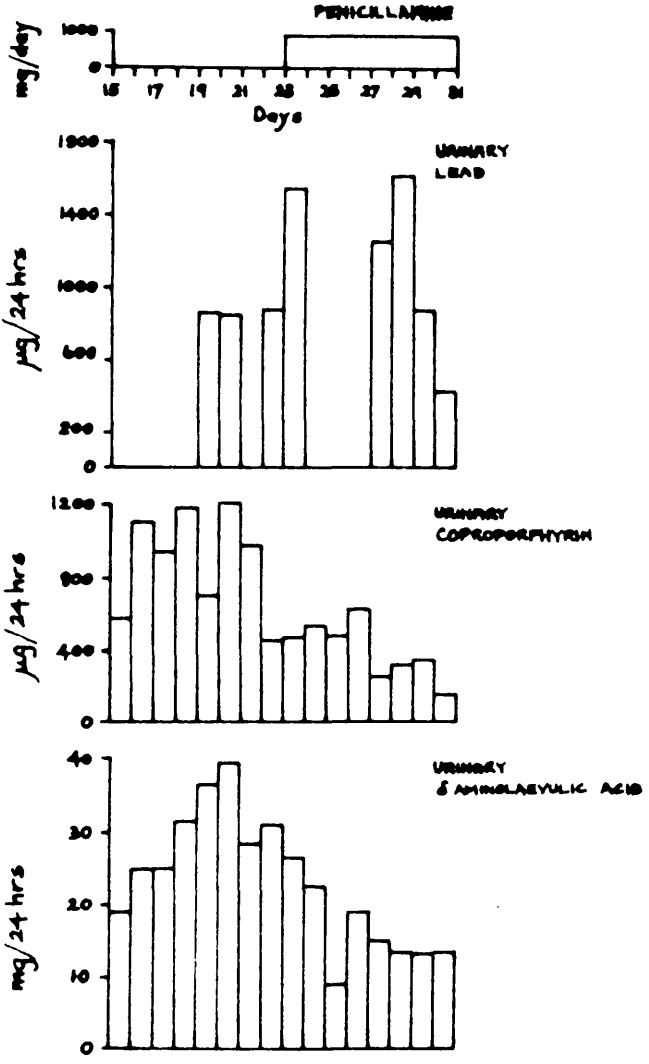


Figure 3. Short-term Trial. Excretion of urinary lead, coproporphyrin and δ-aminolaevulic acid in Case 9 before and during treatment with oral penicillamine, 900mg daily for eight days (second course).

Case 1

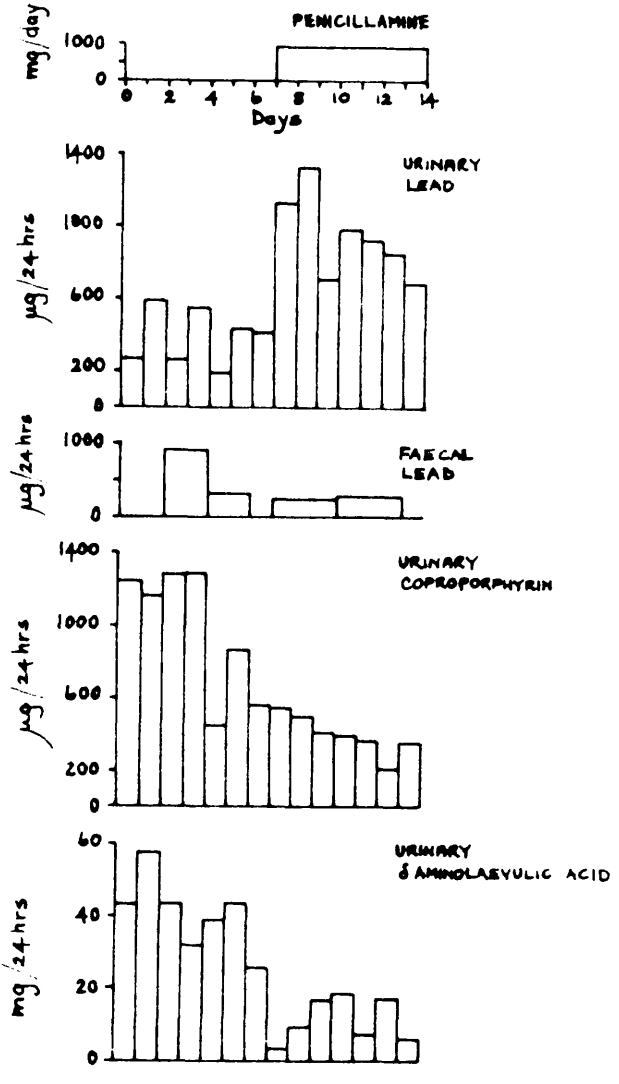


Figure 4. Short-term Trial. Excretion of faecal lead and urinary lead, coproporphyrin and δ-aminolaevulic acid in Case 1 before and during treatment with oral penicillamine, 900mg daily for seven days.

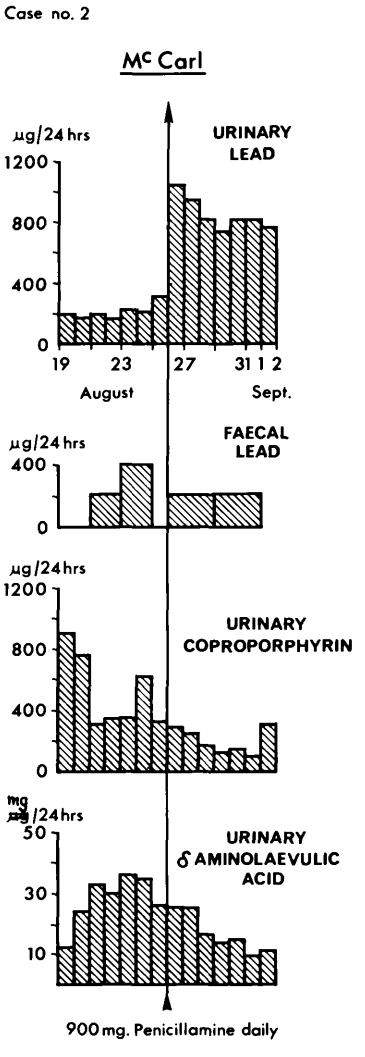


Figure 5. Short-term Trial. Excretion of faecal lead and urinary lead, coproporphyrin and δ-aminolaevulic acid in Case 2 before and during treatment with oral penicillamine, 900mg daily for seven days.

On admittance to hospital, the urinary excretions of CP were high but during the 7 pre-treatment days, they fell of their own accord due to termination of lead exposure. The excretion of ALA however remained fairly high during this period. During the period of increased lead excretion, urinary excretion of CP and ALA decreased markedly, tending towards but not reaching normal levels.

In Cases 3 and 4, the administration of 1500 mg of penicillamine daily after an initial 4 day course of 900 mg of penicillamine daily caused a further increase in lead excretion, with a more pronounced decline thereafter than that recorded with 900 mg (Figs. 6 and 7). In Case 3, the excretion of CP decreased dramatically with the 1500 mg dose of penicillamine. Once more, the excretion of ALA decreased at a slower pace than that of CP under the influence of increased lead excretion.

On the second day of treatment with 1500 mg of penicillamine per day, a peak excretion of urinary lead was recorded in Cases 5, 6 and 7 (Figs. 8, 9 and 10). The decline in lead excretion following this peak output was not as marked as it had been in Cases 3 and 4 (Figs. 6 and 7). Over the seven days of penicillamine therapy, the excretion of urinary CP and ALA decreased, attaining normal values in all three cases on the final day.

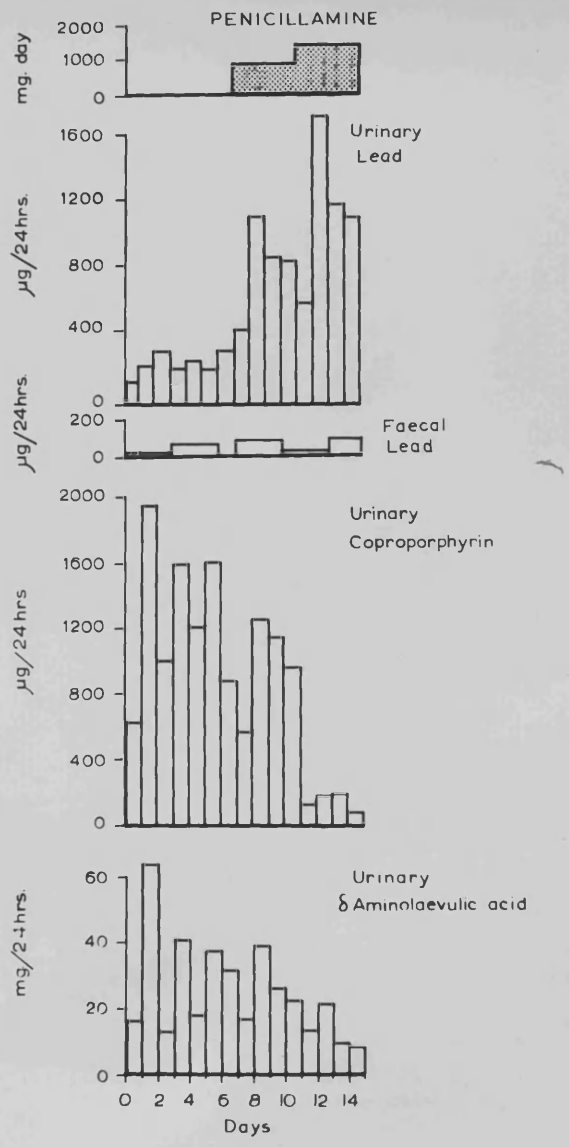


Figure 6. Short-term Trial. Excretion of faecal lead and urinary lead, coproporphyrin and δ-aminolaevulic acid in Case 3 before and during treatment with oral penicillamine, 900mg daily for four days and 1500mg daily for four days.

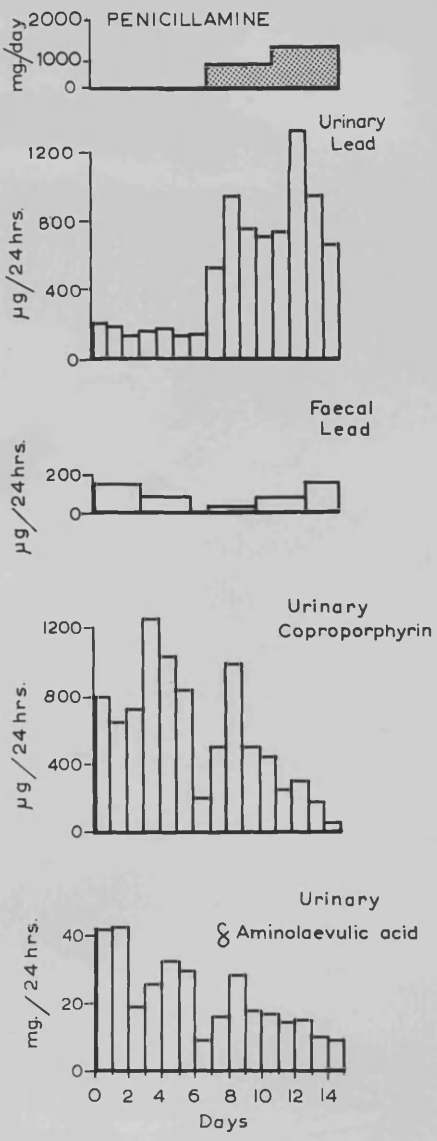


Figure 7. Short-term Trial. Excretion of faecal lead and urinary lead, coproporphyrin and δ-aminolaevulic acid in Case 4 before and during treatment with oral penicillamine, 900mg daily for four days and 1500mg daily for four days.

Case 5

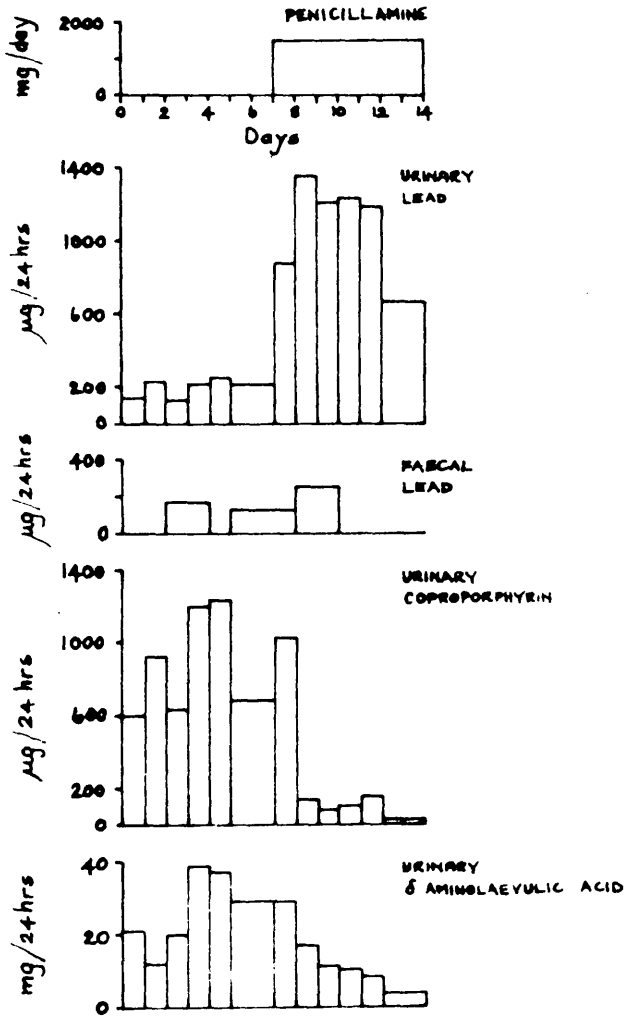


Figure 8. Short-term Trial. Excretion of faecal lead and urinary lead, coproporphyrin and δ-aminolaevulic acid in Case 5 before and during treatment with oral penicillamine, 1500mg daily for seven days.

Case 6

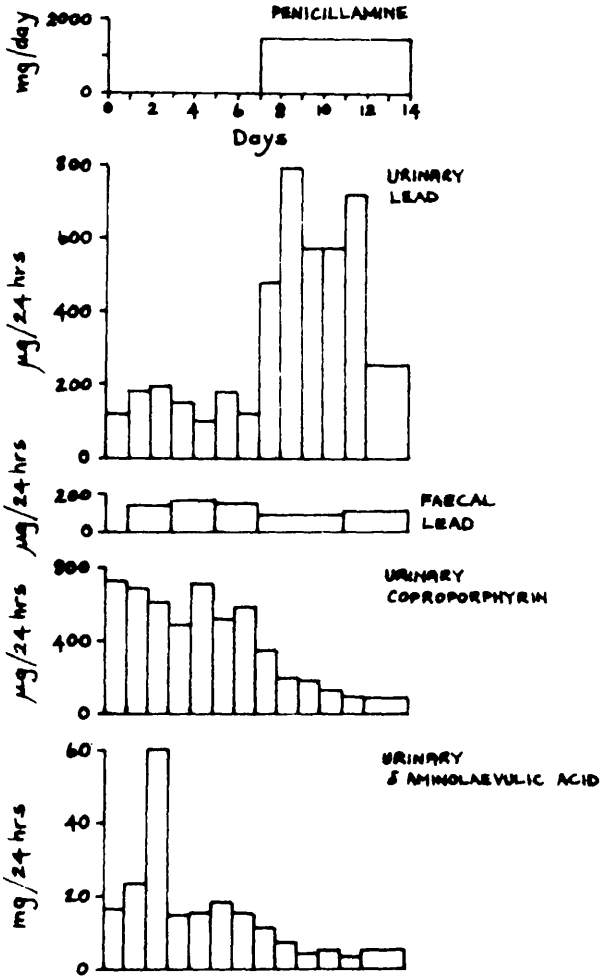


Figure 9. Short-term Trial. Excretion of faecal lead and urinary lead, coproporphyrin and δ-aminolaevulic acid in Case 6 before and during treatment with oral penicillamine, 1500mg daily for seven days.

Case 7

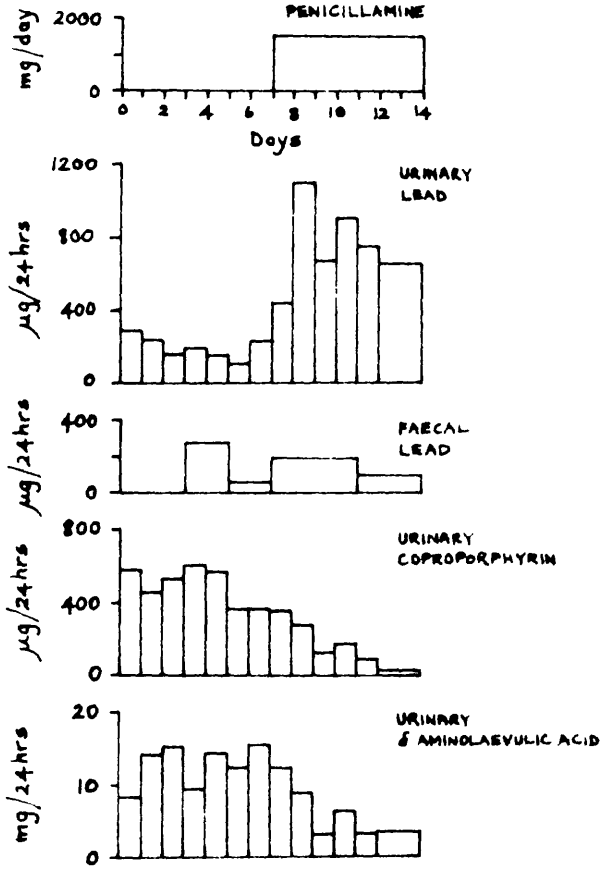


Figure 10. Short-term Trial. Excretion of faecal lead and urinary lead, coproporphyrin and δ-aminolaevulic acid in Case 7 before and during treatment with oral penicillamine, 1500 mg. daily for seven days.

In view of the previous history of penicillin sensitivity in Case 8, penicillamine was given in gradually ascending dosage from 150 to 1200 mg/day. After a minimum single dose of 150 mg, penicillamine was given in divided dosage thrice daily. As low a dose as 300 mg/day of penicillamine caused an immediate rise in the urinary excretion of lead (Fig. 11). Each increase in the daily dose of penicillamine brought about a corresponding increment in urinary lead excretion until, at 900 mg of penicillamine per day, the maximum level of excretion of lead was reached, doses of 1050 and 1200 mg of penicillamine inducing no further increase in lead excretion. It is notable that excessive lead was still being excreted in the urine when the levels of urinary CP and ALA had returned to normal. During the pretreatment period, the urinary excretion of both CP and ALA declined slightly, due to the patient no longer being exposed to lead. The level of urinary lead excretion, as usual, did not fall during this period of non-exposure.

In Cases 1-8, the patients with sub-acute and chronic lead poisoning, 7 of them presented with an average basal urinary excretion of lead of about 200 µg/day. Case 4 had an average basal urinary lead excretion of twice this value. The patient

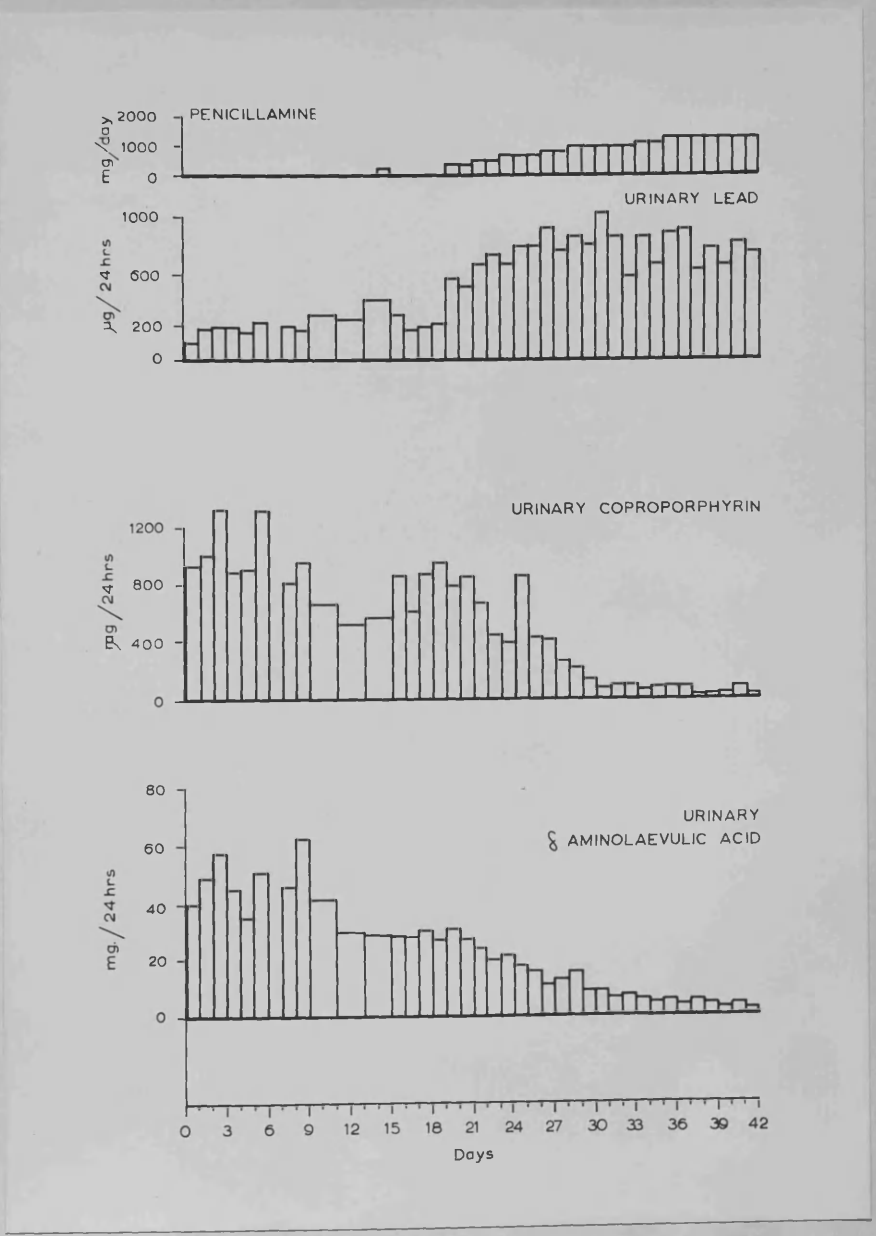


Figure 11. Short-term Trial. Excretion of urinary lead, coproporphyrin and δ-aminolaevulic acid in Case 8 before and during treatment with increasing doses of oral penicillamine.

with acute lead intoxication, Case 9, excreted an average of 876 µg Pb/24 hour urine during a non-treatment period.

Faeces

There was no significant rise in faecal lead excretion after the administration of penicillamine in Cases 1-7 (Table 7, Figs. 4-10). All faecal values, with the exception of Case 1, lay within the normal range on admittance to hospital, but after 4 days in hospital and of non-exposure to lead, the faecal lead excretion of Case 1 also fell to normal values. During the period of study, there was a decline in most cases in faecal lead excretion comparing the pretreatment and treatment values as seen in Table 7.

Blood

i) Blood lead

There was a fall in blood lead in Cases 5, 6, 7 and 8 (Figs. 12 and 13) comparing the levels before and during penicillamine treatment (Table 8).

Cases 3 and 4 had blood lead values of 84 and 77 µg/100 ml respectively on the final day of penicillamine therapy. No pre-treatment values were obtained.

Case No.	BEFORE TREATMENT		DURING TREATMENT	
	No. of days collected	Mean output µg/day	No. of days collected	Mean output µg/day
1	4	603	6	243
2	4	316	6	218
3	3	59	8	66
4	6	102	8	78
5	6	153	7	93
6	5	149	2	254
7	4	171	7	145

Table 7 Short-term trial. Faecal excretion of lead in Cases 1-7 before and during treatment with penicillamine.

Case No.	BEFORE TREATMENT		DURING TREATMENT	
	No. of samples	Mean blood lead (µg/100 ml)	No. of samples	Mean blood lead (µg/100 ml)
5	5	67.6	8	57.7
6	2	82.7	5	77.0
7	5	83.4	8	76.0
8	2	151.7	3	89.9

Table 8 Short-term trial. Mean blood lead in Cases 5-8 before and during treatment with penicillamine.

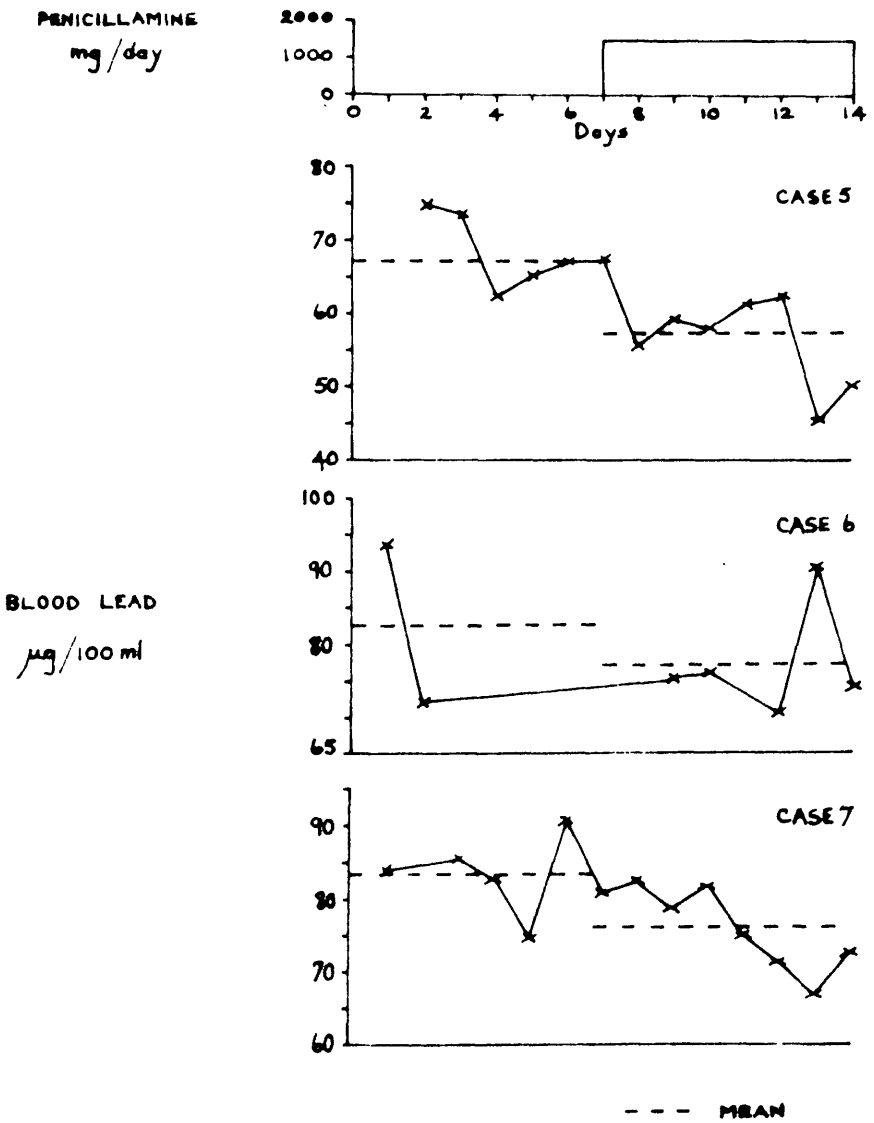


Figure 12. Blood lead in Cases 5, 6 and 7 before and during treatment with oral penicillamine, 1500 mg. daily for seven days.

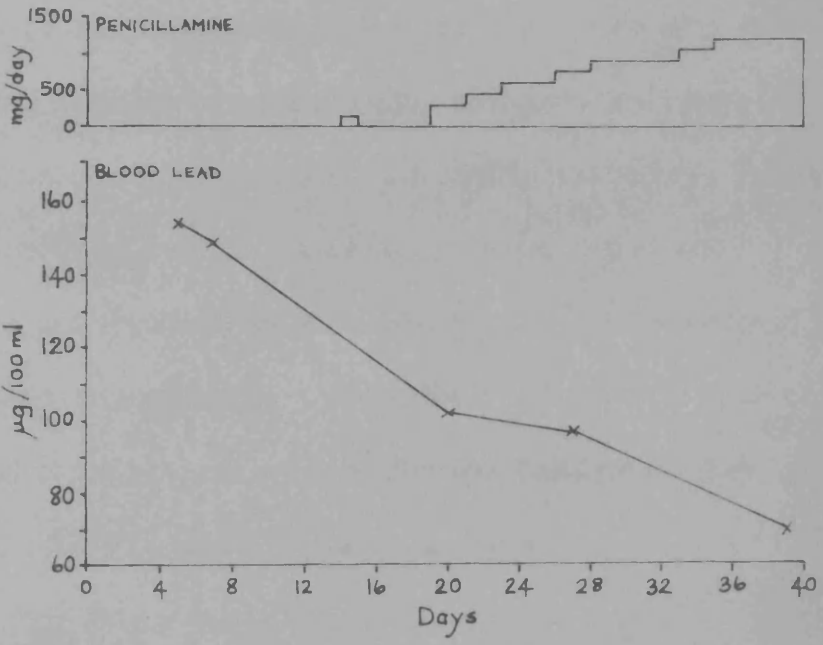


Figure 13. Blood lead in Case 8 before and during treatment with increasing doses of oral penicillamine.

ii) Lead in plasma or erythrocytes?

Blood samples were obtained from Case 5 and another patient, neither of whom were being given penicillamine. After determination of P.C.V., the blood was centrifuged, the plasma drawn off and its volume measured. The red blood cells were gently mixed with a volume of normal saline to remove any traces of plasma. After centrifuging and discarding the saline, the r.b.c's were measured in a 10 ml measuring cylinder. The lead content of plasma and erythrocytes were determined. In both cases, there was no lead in the plasma. Using the P.C.V., the total lead per 100 ml of whole blood was calculated and found to be 73.8 and 54.3 μg for Case 5 and the other patient respectively.

iii) Haemoglobin

Haemoglobin values determined on admission to hospital and stated under clinical cases, were low in Cases 4, 8 and 9.

iv) Punctate basophilia

Stippled cells were present in the peripheral blood of Cases 4 and 8, in bone marrow only in Case 9, and in peripheral blood and bone marrow in Case 1. (Cases 1 and 9 had high levels of basal urinary lead excretion. Case 8 suffered from wrist drop and Case 4, like Case 8, had marked anaemia.)

v) Reticulocyte Count

On admission to hospital, the reticulocyte count was determined for each patient. Cases 2, 3, 6 and 7 had a reticulocyte count of less than 2%. Cases 1, 4, 5, 8, 9 had a reticulocyte count of 2.5, 4, 2, 6 and 3% respectively.

Cases 1, 4, 8, and 9 had values greater than normal.

14. Scheme of Therapeutic Investigation: Long-term Trial of Penicillamine

A long-term trial of penicillamine lasting ten weeks was carried out on Cases 1, 2, 3, and 4 while they were at work in the ship-breaking yard. This long-term trial was begun three months after the end of the short-term trial in Cases 1 and 2 and 10 days after the end of the short-term trial in Cases 3 and 4. Before treatment was begun all of these men were anaemic, with haemoglobin levels of 11.4, 10.2, 10.8, and 8.9 g/100 ml respectively. In addition all had an increased excretion of lead in the urine. Cases 1 and 2 had intermittent abdominal pain. Case 3 complained of weakness of his hands and wrists. Case 4 had no symptoms, although he was in fact the most anaemic of the group. Penicillamine was given in a dosage of 300 mg daily for three weeks and then 600 mg daily for the remaining seven weeks. The capsules, each containing 150

mg of penicillamine, were presented each morning to the men by a medical orderly and taken in divided dosage twice daily. On Friday sufficient capsules were given to suffice over the week-end.

Case 8 remained anaemic after his initial treatment. He had developed paresis of his right hand on two occasions within one year and yet was determined to return to his work as a foreman in an accumulator factory. For these reasons he was given a course of penicillamine, 600 mg/day for a period of four months while he was at work.

In Cases 1, 2, 3, and 4, overnight urine specimens (volume ca. 500 ml) were collected twice weekly and tested for lead, coproporphyrin, and δ-aminolaevulic acid. In Case 8 urine specimens were tested every four weeks. In all five cases haemoglobin levels and reticulocytes were assessed every four weeks.

Throughout these trials the subjects continued to use a protective mask in the same way as they had previously done.

15. Results of the Long-term Trial

In Cases 1, 2, 3 and 4 there was no significant response to 300 mg of penicillamine daily (Figs. 14-17). It must be repeated that this trial was carried out while the subjects were at work and exposed to further lead intoxication. Although the excretion of

Case 1

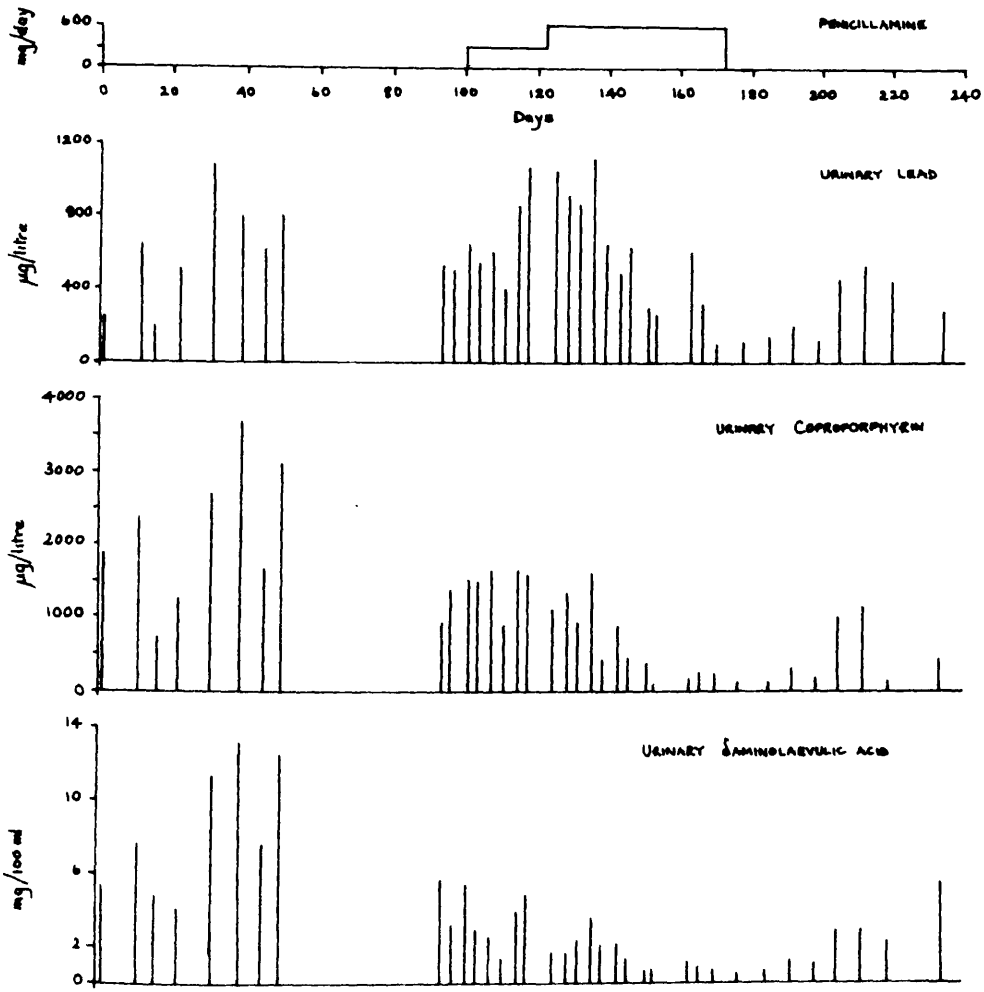


Figure 14. Long-term Trial. Excretion of urinary lead, coproporphyrin and δ-aminolaevulic acid in Case 1 before, during and after treatment with oral penicillamine, 300 mg. daily for three weeks and 600 mg. daily for seven weeks.

Case 2

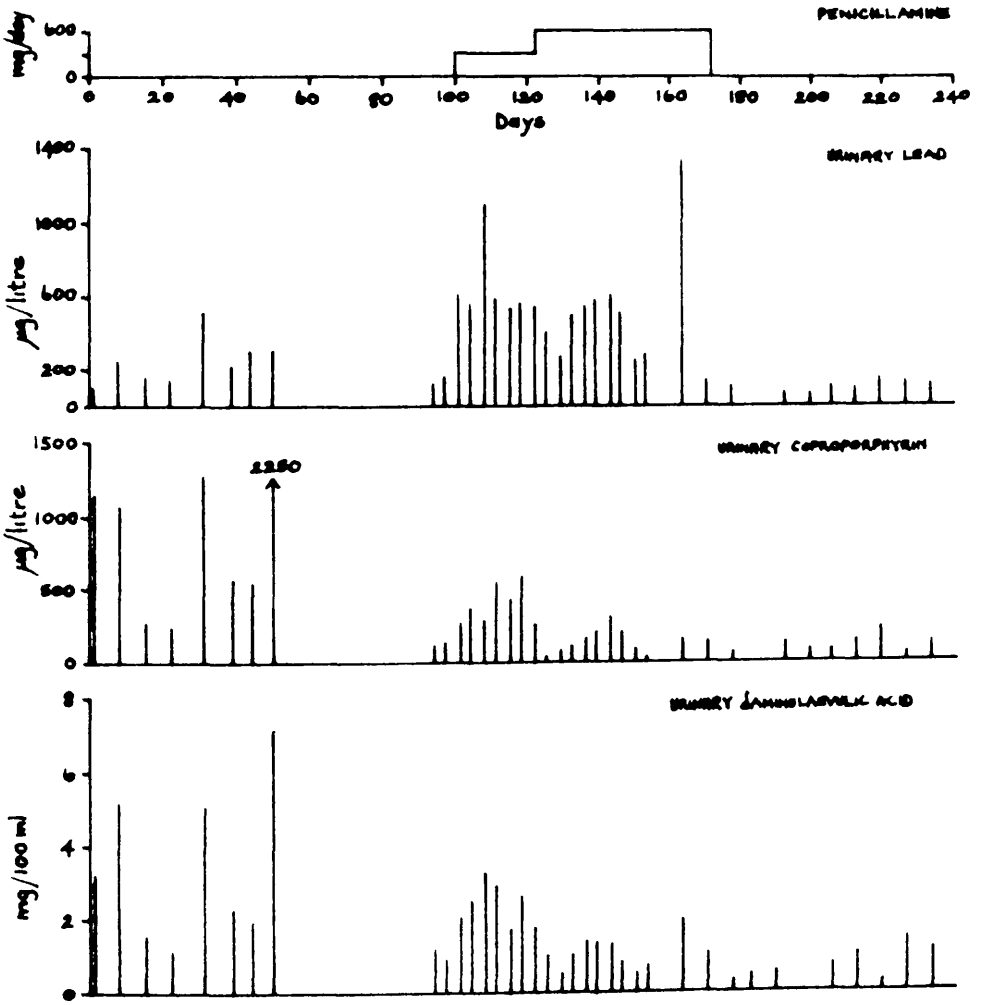


Figure 15. Long-term Trial. Excretion of urinary lead, coproporphyrin and δ-aminolaevulic acid in Case 2 before, during and after treatment with oral penicillamine, 300 mg. daily for three weeks and 600 mg. daily for seven weeks.

Case 3

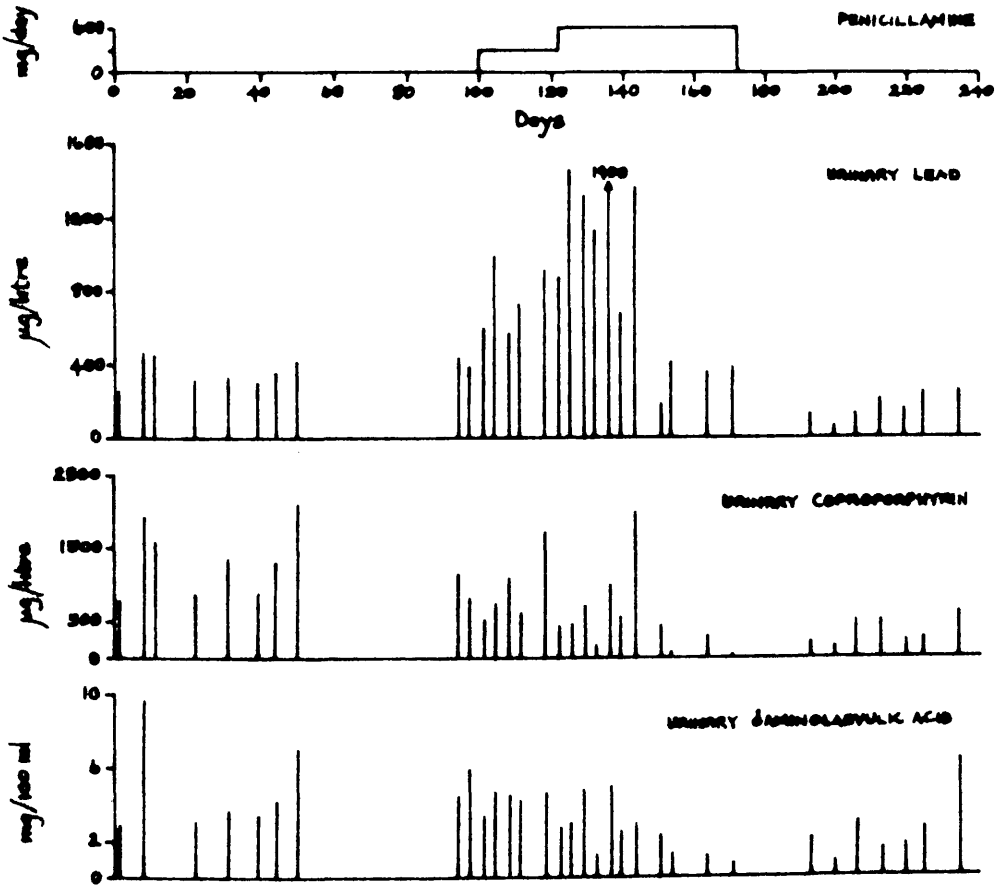


Figure 16. Long-term Trial. Excretion of urinary lead, coproporphyrin and δ-aminolaevulic acid in Case 3 before, during and after treatment with oral penicillamine, 300 mg. daily for three weeks and 600 mg. daily for seven weeks.

Case 4

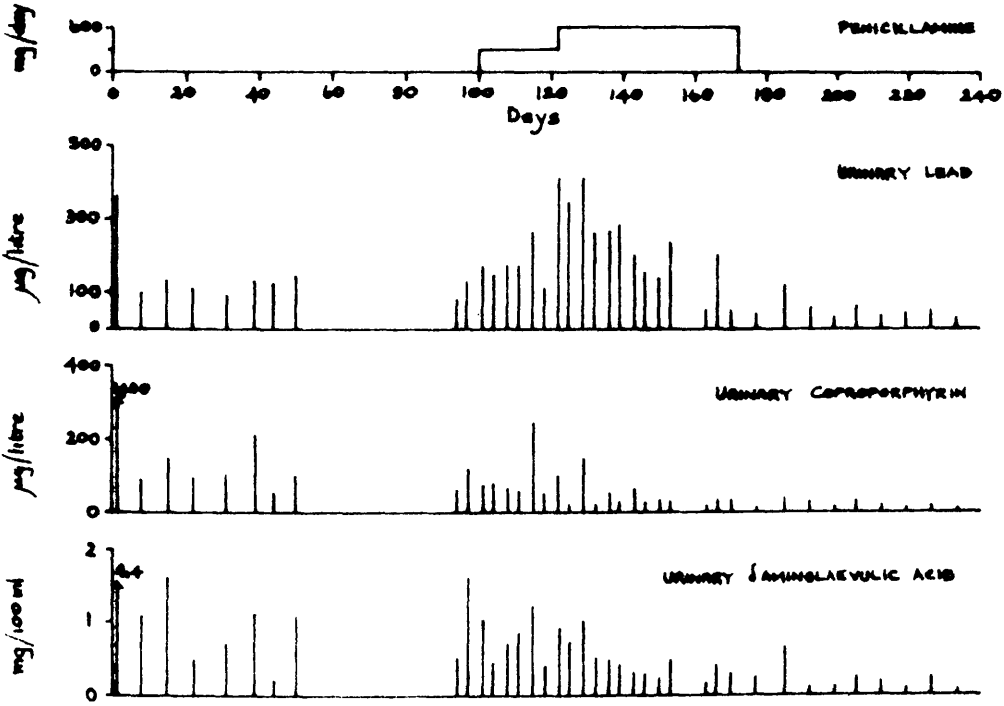


Figure 17. Long-term Trial. Excretion of urinary lead, coproporphyrin and δ-aminolaevulic acid in Case 4 before, during and after treatment with oral penicillamine, 300 mg. daily for three weeks and 600 mg. daily for seven weeks.

urinary lead rose sharply in Case 2 and to a lesser extent in Case 3 on commencement of treatment with 300 mg of penicillamine per day, there was no significant change in the excretion of CP and ALA in all four cases. After three weeks, the dose of penicillamine was raised to 600 mg/day. Case 1 showed a significant rise in urinary lead excretion. This began to decline at day 140 simultaneously with a decrease in the urinary excretion of CP and ALA. In Case 2, the urinary lead excretion, which had shown a significant increase on 300 mg of penicillamine therapy, did not alter when the dose was raised to 600 mg/day. The excretion of CP and ALA however showed a marked decrease on commencement of 600 mg of penicillamine daily. On increasing the dose of penicillamine, the urinary lead excretion of Case 3 rose for the second time. Once again, the excretion of CP and ALA fell only during the period when 600 mg of penicillamine was being given. In Case 4, there was a significant rise in urinary lead excretion only with 600 mg of penicillamine daily. After the initial high output, the urinary lead excretion began to decrease, accompanied by a corresponding decrease in the excretion of ALA.

In all 4 cases, 600 mg of penicillamine was required to cause a decrease in the urinary excretion of CP and ALA although

smaller doses (e.g. 300 mg) may still cause a significant increase in the excretion of urinary lead.

At the end of the seven week course of 600 mg of penicillamine daily, the urinary excretion of CP and ALA in Cases 1-4 had fallen to normal levels. In all cases, the urinary lead excretion after penicillamine therapy was, as expected, slightly lower than that before penicillamine therapy but within 4-6 weeks of discontinuation of penicillamine, there was a gradual rise in the urinary excretion of lead, CP and ALA in Cases 1, 2 and 3.

After treatment with penicillamine, the haemoglobin levels in Cases 1, 2, 3 and 4 rose to 14.5, 13.3, 13.2, and 11.8 g/100 ml as compared with 11.4, 10.2, 10.8, and 8.9 g/100 ml respectively before treatment (Fig. 15). Cases 1 and 2 claimed that their abdominal pain had ceased, and Case 3 stated that his wrists and hands had regained their former strength. Throughout these months of treatment with penicillamine none of these four subjects had any untoward effect of drug therapy; in particular, the urine was free of protein at the end of the treatment period.

Case 3, after the initial short-term treatment, had only slight residual weakness of his right hand, but he was still anaemic (Hb 10.4 g/100 ml). After four months of treatment with 600 mg

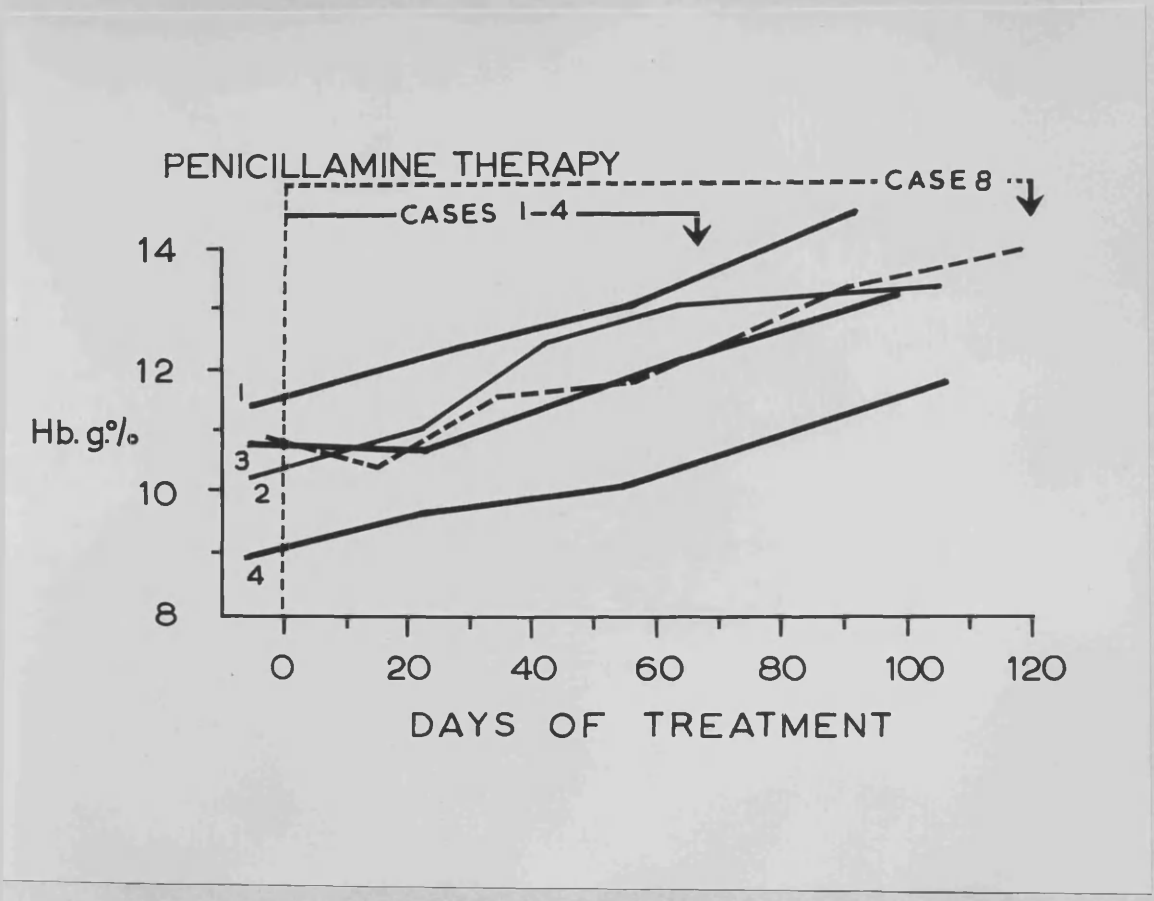


Figure 18. Haemoglobin levels in Cases 1, 2, 3, 4 and 8 before, during and after long-term trials of penicillamine.

penicillamine/day he had normal power in his hand and his haemoglobin level had risen to 13.8 g/100 ml. Throughout this period, the urinary lead excretion remained fairly high (Table 9). Nine weeks after leaving hospital (i.e. day 111), the urinary excretion of CP reverted to abnormal values. On day 42, the final day in hospital, Case 8 had a normal urinary excretion of 0.18 mg ALA/100 ml. On day 54, while on penicillamine therapy of 600 mg per day, the excretion of ALA had risen to the abnormal value of 0.58 mg/100 ml urine. At the end of the four month period, the blood lead had not been lowered (73.5 µg/100 ml on day 161 compared to 70.1 µg/100 ml on day 39, i.e. 3 days before the start of the 4 month penicillamine course). Although without symptoms at the end of the 4 months' treatment this patient was found to have protein in his urine (0.4 g/100 ml). After a few days, this fell to 0.1 g/100 ml but two months after the cessation of penicillamine treatment, he continued to excrete about 0.1% protein in his urine. He has maintained the power of his right hand and his haemoglobin level has remained normal. His exposure to lead has been discontinued.

	Day				
	54	89	111	138	161
Blood Lead µg/100 ml	-	-	-	-	73.5
Urinary Lead µg/litre	255	635	-	272	-
Urinary Copro. µg/litre	129	50	805	492	-
Urinary ALA mg/100 ml	0.58	-	1.72	1.60	-

Table 9 Long-term trial. Blood lead and urinary excretion of lead, coproporphyrin and δ aminolaevulic acid in Case 8 during treatment with oral penicillamine, 600 mg daily for four months.

Section A.III

Discussion

These studies have shown that penicillamine, given orally, causes a prompt and marked excretion of urinary lead in patients with lead intoxication. A dose of 300 mg/day is sufficient to effect a significant lead excretion, provided the patient is no longer exposed to further lead intoxication, but marked excretion was induced by daily doses of 900 to 1,500 mg. (Fig.19) The increased lead excretion was not effected through the faecal route. There was a fall in the blood-lead levels with treatment and also a marked fall in the urinary levels of coproporphyrin and of δ-aminolaevulic acid. The urinary excretions of these substances are thought to be sensitive indices of metabolically active lead (Haeger-Aronsen, 1960). For this reason it is of considerable interest that the continued administration of penicillamine in Case 8 yielded a marked excretion of lead at a time when the urinary levels of copro-

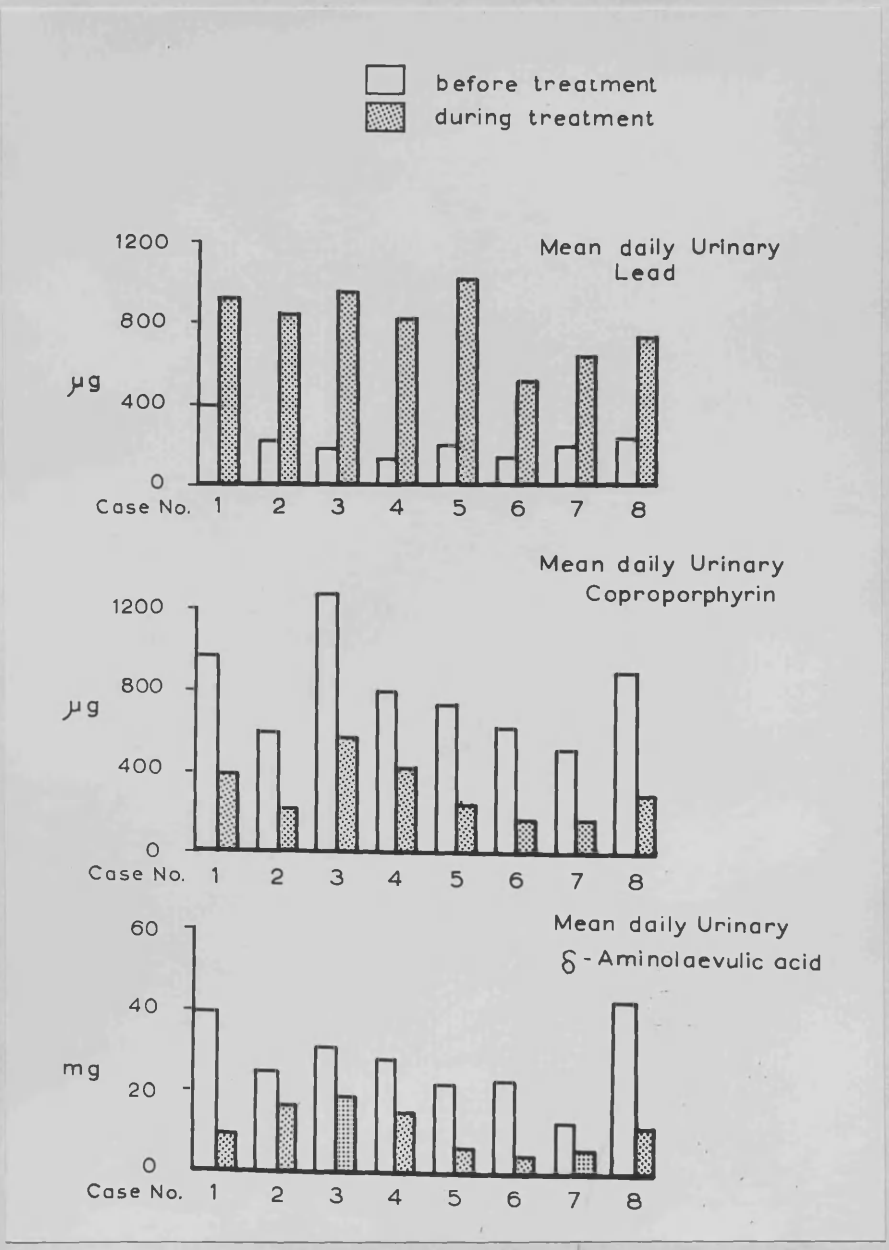


Figure 19. Comparison of the excretion of urinary lead, coproporphyrin and δ-aminolaevulic acid in Cases 1-8 before and during treatment with oral penicillamine.

porphyrin and δ-aminolaevulic acid were normal. This suggests that penicillamine can mobilize stored lead for excretion.

There was a substantial increase in the haemoglobin levels in Cases 1, 2, 3, 4, and 8 after long-term treatment from two to four months, even though these patients were still engaged in a lead-exposed occupation. The anaemia of lead-poisoning is due to two main factors. Firstly, an inhibition of haem synthesis, at several stages in the formation of haem and especially in the final phase of iron incorporation into the haem or haemoglobin molecules (Goldberg et al., 1956). There is in addition a haemolytic factor (Wintrobe, 1961); a significant reduction in the erythrocyte life-span has been demonstrated, returning to normal after treatment (Goldberg, 1960). Both of these effects are probably caused by a direct effect of lead on the biosynthesis of haemoglobin in the marrow and on the erythrocyte membrane respectively, and it is therefore not surprising that the simple removal of body

lead should prompt an increase in the haemoglobin level. It is notable, however, that the relief of the anaemia was a gradual process over a period of two to four months. There was, in addition, a complete return of motor power to the right hand of Case 8 after a prolonged course of treatment, as well as a rapid cessation of abdominal pain, vomiting, and constipation in Case 9.

The pathogenesis of the neurological and abdominal symptoms in lead-poisoning is unknown. It is possible that the mechanism of production of these symptoms has a common biochemical pathway with that of acute intermittent porphyria (Goldberg et al., 1962). In both diseases there is a marked increase in the excretion of δ-aminolaevulic acid. In lead-poisoning these symptoms are relieved by the removal of lead from the body by chelators of lead, such as sodium calciumedetate or penicillamine. Peters et al. (1957) have suggested that in their experience the symptoms of acute intermittent porphyria may also be relieved by chelating agents. There is, however, insufficient confirmatory evidence on this point.

The studies on the long-term administration of penicillamine are of practical importance to the prophylaxis of lead-poisoning. The best prophylaxis is, of course, prevention of entrance of the metal into the body by the established methods of industrial hygiene. Nevertheless, it must be accepted that some lead-workers find it impossible to wear a mask constantly; nor is the general standard of industrial hygienic equipment of such efficiency as to allow us to ignore the drug prophylaxis of lead intoxication. Penicillamine has now been used in the long-term treatment of Wilson's disease for six years; Walshe (1962), whose experience in this field is extensive, has found this drug to be particularly non-toxic. There was no evidence, in his experience, of renal damage with the D isomer of penicillamine, which is that used in this country. Scheinberg (1962), who has also had considerable experience in the treatment of Wilson's disease with penicillamine, has not found proteinuria in any of his patients which could be attributed to the drug. Fellers and Shahidi (1959) noted the onset of frank nephrotic syndrome in a male patient of 16 years who had been given 2 g of penicillamine a day for nine months. The clinical manifestations, including the proteinuria, disappeared after the withdrawal of the drug. Our experience of Case 8 has shown that, in lead-poisoning,

penicillamine given over a period of four months can be nephrotoxic.

In the present studies it has been shown that 600 mg of penicillamine a day orally is an effective dose. When given over a period of two to four months to lead-workers it has induced a marked lead excretion, a return to normal levels of urinary coproporphyrin and δ-aminolaevulic acid, and a rise of the haemoglobin level. Case 8 had a history of skin reaction following a penicillin injection 10 years previously. With a gradually increasing dosage of penicillamine, no skin reaction occurred, but at the end of this period he developed protein in his urine, with other evidence of renal damage. This evidence must, at present, contraindicate the possible use of penicillamine on a long-term basis in lead-poisoning; nevertheless, penicillamine is a useful drug in short-term courses of not more than four weeks. Subjects showing early clinical evidence of lead intoxication - for example, with mild anaemia or occasional gastro-intestinal symptoms - might receive oral penicillamine while at home or after being transferred to work not involving the risk of exposure to lead. The urine should be tested for protein at least once weekly. Any worker with a more severe degree of lead-poisoning should be treated in

hospital. It should again be emphasized that the established protective measures form the best prophylaxis. In addition to these industrial indications for penicillamine the drug has obvious advantages over a parenteral lead chelator in the treatment of lead intoxication in children.

Summary

D-Penicillamine, given orally in doses of 600 to 1,500 mg/day, induced a marked excretion of urinary lead in nine patients with subacute or chronic lead intoxication. There was a concurrent fall in the urinary coproporphyrin and δ-aminolaevulic acid levels and in the level of lead in the blood. The route of excretion was entirely by way of the urine.

Five lead-exposed subjects, while working in a shipbreaking yard or in an accumulator factory, were given oral penicillamine for periods of from two to four months. There was a persistent excretion of urinary lead, a fall in the urinary coproporphyrin and δ-aminolaevulic acid to normal levels, a rise in the haemoglobin levels, and a relief of neurological or abdominal symptoms. The effective dose of penicillamine for this trial was 600 mg/day. One patient developed proteinuria after four months of penicillamine

treatment. For this reason routine testing for proteinuria should be carried out in patients during treatment; the duration of a single course of penicillamine should not exceed four weeks; patients during treatment should be removed from a lead-exposed environment.

It is suggested that, with these stipulations, oral penicillamine is a useful drug in the treatment of lead-poisoning and may be of value in the treatment of lead-poisoning in children.

PART B. SURVEY OF INDUSTRIAL LEAD EXPOSURE

Section B.I INTRODUCTION

In Britain, most cases of lead poisoning originate in lead industries such as ship-building and ship-breaking and in factories where batteries and accumulators are made. On such industrial sites or in such factories, the exposure to lead dust and fumes is not necessarily confined to those actively working with lead. Varying degrees of exposure are suffered by lead workers and their co-workers in industry. A survey was carried out in the same ship-breaking yard which had employed 7 of the patients studied in the previous section. Haematological tests, blood lead and urinary lead, coproporphyrin and δ-aminolaevulic acid were determined in all of the selected subjects where possible.

1. Purpose of the Study

The purpose of the study was to determine:- 1) relative sensitivity of the above tests in detecting industrial lead exposure; 2) the relative dangers of lead exposure to different groups of workers on the one site; 3) if a relationship existed between the clinical manifestations of the disease and its chemistry, i.e. whether a particular symptom appeared when a certain threshold value of one of the above tests was reached.

2. Relative sensitivity of some tests for lead poisoning

Of the tests carried out, the relative sensitivity of some have already been assessed. In an article published in 1961, Shrand drew up a table of diagnostic aids to lead poisoning. A part of this table only is reproduced below since the remainder is applicable particularly to children.

Diagnostic Aid	Probability of a +ve Result
Coproporphyrinuria (ether + HCl extraction)	100
Urinary ALA	100
Basophilic stippling	18

Urinary ALA is cited as a sensitive indice of exposure to lead due to the increase in excretion of urinary ALA varying directly with the degree of exposure to lead. The increase in excretion of ALA is said usually to precede that of CP (Haeger Aronsen, 1960).

There has been doubt as to the value of the test for punctate basophilia which is one of the routine tests for suspected lead poisoning. This test was included in the survey. If there proved to be as low a probability of a +ve result as 18%, then such a test would not be suited to routine industrial inspection.

3. Routine screening of workers

In the ship-breaking yard, routine medical inspection of each burner was performed, on an average of once per fortnight, by a visiting doctor. This screening may take the form of a simple checkover and a blood sample and/or spot urine sample may be taken for haemoglobin and CP estimation respectively. The physical appearance (e.g. pallor) of the worker may well give an indication of lead exposure if the worker has been known to the doctor for a period of time.

All burners in this ship-breaking yard were supplied by the firm with protective masks, the filter of which, at the nose-mouth area, was renewed freqeuntly. The filter gave protection only against dust. Although the majority of burners found it impossible to wear the mast constantly, especially in warm weather, all agreed that masks were worn for certain tasks involving a greater degree of lead exposure and as much as possible at all other times. The men co-operated well in having their filters changed regularly and only one burner was on record as being constantly reprimanded for his apathy.

Section B.II METHODS, ORGANISATION OF THE SURVEY AND RESULTS

1. Methods

The biochemical data for all tests was obtained using the methods listed in Section A.II.

2. Organisation of the Survey

From a ship-breaking yard employing 142 people, a total of 48 were chosen for the survey. A cross-section of all workers on the site were selected. Within each particular group, the duration of exposure to lead of each individual was as varied as possible. With the co-operation of the management, lead-free containers were distributed to groups of workers the evening before each visit, so that overnight urine specimens could be collected. Such samples were less likely to be contaminated that those collected during the day. When urine samples were collected, blood samples were also taken. Each employee stated his duration of lead exposure, whether a protective mask was worn and for what percentage of his working time. Blood samples were analysed for lead and the presence or absence of punctate basophilia was noted. Haemoglobin and packed cell volume were determined. Urine samples were analysed for lead, coproporphyrin and δ-aminolaevulic acid. This information is listed in Table 10.

No.	OCCUPATION	SEX	AGE (YEARS)	DURATION OF EXPOSURE (YEARS)	DISTANCE FROM LEAD BURNING (YARDS)	% MASK PROTECTION	BLOOD PUNCTATE BASOPHILIA	BLOOD HAEMO-GLOBIN g.%	BLOOD PACKED CELL VOLUME	BLOOD LEAD ug./100 ml.	URINE LEAD ug./litre	URINE CP ug./litre	URINE ALA mg./100 ml
1	FOREMAN	M	59	38	0	-	-	-	-	-	77.3	157	1.23
2	BURNER	M	36	12	0	50	NIL	14.2	44	60.8	444	1493	4.70
3	"	M	42	15	0	10	NIL	13.0	41	65.4	125	412	1.97
4	"	M	39	12	0	75	NIL	14.8	42	72.5	209	275	1.92
5	"	M	48	16	0	50	NIL	14.7	44	79.6	130	186	1.94
6	"	M	51	16	0	75	NIL	12.5	40	71.5	130	325	1.17
7	"	M	30	13	0	60	NIL	15.2	46	306 ◆	168	135	2.54
8	"	M	24	2.5	0	70	NIL	15.2	46	60.5	85.5	59.7	0.53
9	"	M	50	17	0	5	NIL	13.4	42	75.0	129	1314	1.92
10	"	M	43	14	0	80	NIL	13.8	42	95.3	75.3	21.7	0.72
11	"	M	49	10	0	30	NIL	15.7	44	107	237	139	2.32
12*	"	M	61	35	0	50	NIL	12.0	37	97.6	101	43.1	0.26
13	"	M	48	10	0	30	-	14.9	43	57.2	214	493	1.26
14	"	M	32	1.5	0	30	NIL	13.0	42	41.5	245	517	0.62
15	"	M	33	10	0	30	NIL	15.7	46	47.3	115	541	1.20
16	"	M	30	3	0	30	NIL	12.3	40	54.2	68	22.7	0.22
17	"	M	48	2.5	0	55	NIL	13.5	40	78.0	298	182	5.16
18*	"	M	51	12	0	100	NIL	14.4	44	36.4	220	153	0.90
19	"	M	41	6	0	10	NIL	14.1	39	27.1	250	905	1.64
20	"	M	32	7	0	75	NIL	14.2	41	85.4	195	517	1.80
21*	"	M	52	27	0	70	1/500	11.9	38	186 ◆	35.9	1.7	0.02
22*	"	M	41	16	0	50	-	14.2	42	38.0	164	608	6.40
23*	"	M	56	22	0	75	NIL	14.1	43	54.6	143	143	1.08
24*	"	M	43	10	0	70	NIL	14.2	41	98.3	279	472	5.68
25	BRASS BURNER	M	50	16	200	-	NIL	14.5	42	49.8	77.5	27.0	0.23
26	STRIPPER	M	50	4	3	-	NIL	14.9	46	64.8	42.0	40.4	0.08
27	"	M	60	16	3	-	NIL	16.2	47	12.1	73.8	68.6	0.26
28	"	M	40	10	3	-	NIL	13.4	-	31.4	28.8	6.0	0.08
29	"	M	37	3	3	-	NIL	16.2	46	47.5	25.0	61.2	0.33
30	"	M	47	3	3	-	NIL	14.8	42	42.1	39.5	39.2	0.27
31	"	M	45	3	3	-	NIL	15.6	47	52.5	59.5	54.0	0.26
32	SLINGER	M	28	7	3	-	NIL	14.2	40	51.8	113	110	0.94
33	"	M	34	6	3	-	NIL	17.3	49	59.6	49.8	73.8	0.27
34	CRANE OPERATOR	M	51	14	9	-	NIL	16.0	45	68.0	62.3	46.6	0.29
35	"	M	62	22	9	-	NIL	15.9	45	43.7	61.8	37.1	0.15
36*	GENERAL LABOURER AND FIREMAN	M	58	16	0	-	-	14.3	40	67.9	118	134	2.24
37	GENERAL YARD LABOURER	M	59	4	25	-	NIL	14.6	44	37.0	35.3	50.5	0.26
38	LOCO DRIVER	M	57	25	30	-	NIL	16.5	45	38.0	35.8	62.5	0.18
39	STORAGE	M	29	4	30	-	NIL	15.8	44	41.2	75.5	97.3	0.29
40	"	M	58	14	30	-	NIL	15.0	43	36.3	35.0	59.3	0.14
41	"	M	55	11	30	-	NIL	13.8	41	30.0	8.3	22.8	0.12
42	PLANT ATTENDANT	M	72	5	25	-	NIL	15.0	46	36.3	35.3	24.3	0.25
43	ELECTRICIAN	M	43	16	30	-	NIL	16.4	47	35.9	30.3	122	0.23
44	ENGINEER	M	25	5	30	-	NIL	-	-	17.1	41.0	135	0.18
45	GATEMAN	M	73	6	100	-	NIL	15.8	44	16.5	14.5	42.2	0.11
46	BOOKKEEPER	F	45	16	30	-	NIL	14.7	42	24.2	14.5	55.4	0.12
47	GENERAL MANAGER	M	59	28	98	-	NIL	17.6	47	20.4	15.0	5.4	0.04
48	SHIP MANAGER	M	58	29	25	-	NIL	17.0	5	33.3	36.0	59.8	0.33
49	CRANE OPERATOR	M	38	14	9	-	NIL	15.4	45	75.1	168	275	0.52
50	BURNER	M	54	1	0		NIL	13.0	40	86.2	95.7	217	-
51	"	M	48		0		-	13.5	-	81.4	-	1420	-
52	"	M	19		0		-	18.4	-	36.4	-	4184	-
53	"	M	37		0		NIL	13.0	43	85.7	-	88	-
54	"	M	62		0		NIL	15.2	-	105	-	669	-
55	"	M	36	4	0		[illegible]	14.2	43	89.8	-	-	-
56	LEAD SMELTER	M	39	1	0		[illegible]	12.3	40	324	-	856	-
57	BATTERY WORKER	M	48	15	0		[illegible]	11.4	37	146	156	795	3.00

* Nos. 21-24 finished a 10 week course of penicillamine 10 weeks previously.
Nos. 12, 18, 36 had a 7 day course of penicillamine 8 months previously.

◆ In case of accidental contamination a second blood sample was taken one week later and analysed for lead. A similar result was obtained.

TABLE 10. Survey of industrial lead exposure. General clinical data on all workers.

Employees 1-48 worked in a ship-breaking yard on Clydeside. Employees 49-55 worked in this or another subsidiary ship-breaking yard of the same firm. Employee 56 worked in a metal foundry as a lead smelter. Employee 57 was a foreman in a battery factory, listed in Part A as Case 8. Nos. 49-57 all attended outpatient clinics, presenting with various symptoms of subacute or chronic lead intoxication. The results for nos. 49-57 listed in Table 10 are those before treatment was given. Urine results are incomplete due to spot samples being small. These latter 9 lead workers with symptoms may serve as a basis for comparison with the group of burners, the majority of whom described symptoms of a milder nature - constipation, occasional abdominal pain - or none at all.

3. Results

A summary of results is given in Table 10.

a) Working distance from site of lead burning

The distance in yards which each employee in the ship-breaking yard worked from the site of lead burning was measured. This distance was plotted against blood lead and urinary lead, CP and ALA for each worker respectively. The upper normal limits for lead, CP and ALA, derived from results in Section A.II, are shown in Table 11.

	Mean + 2 S.D.
Blood lead µg./100 ml.	39.3
Urinary lead µg./litre	48.3
Urinary CP µg./litre	181.0
Urinary ALA mg./100 ml.	0.36

TABLE 11

Upper limit (mean + two standard deviations) of blood lead and urinary lead, coproporphyrin and δ-aminolaevulic acid in 20 normal subjects.

The relationship between blood lead and distance is shown in Fig. 20. Blood lead values above normal were recorded for burners (22 out of 24), strippers[x] (4 out of 6), slingers[+] (2 total) and crane operators (3 total), working 0, 3, 3 and 9 yards respectively from the site of lead burning. Those working at 25 and 30 yards from the lead site had blood lead values about the upper normal limit. At 50 yards, normal blood lead levels were recorded.

When the urinary lead excretion was plotted against distance, values above the upper normal limit were noted for burners (23 out of 24), strippers (2 out of 6), slingers (2 total) and crane operators (3 total), working 0, 3, 3 and 9 yards respectively from the site of lead burning. At 25 and 30 yards, 3 out of 9 workers had abnormal urinary lead excretion.

About half of the values for the urinary excretion of CP by burners fell within the normal range. All other groups of workers showed normal levels of CP excretion.

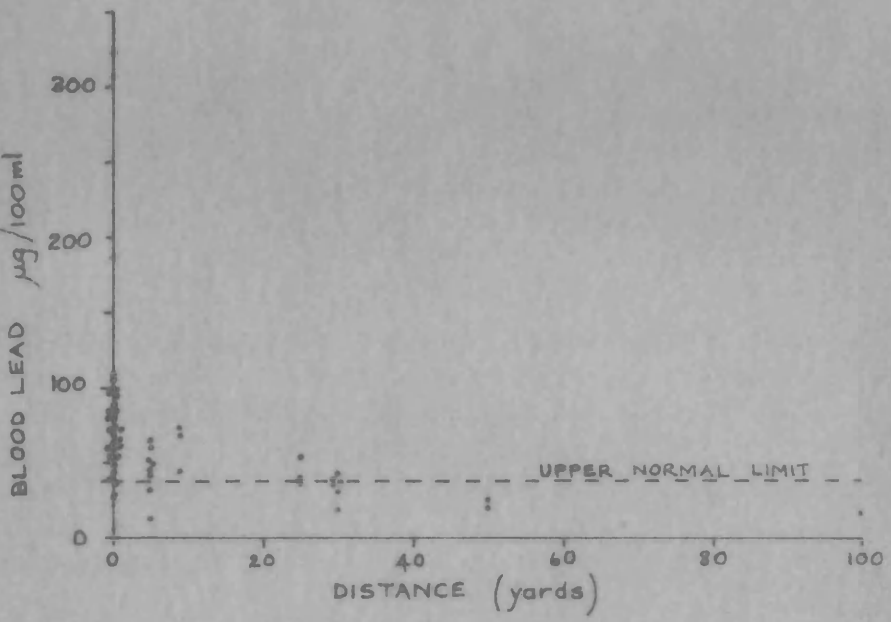
By contrast the urinary excretion of ALA by burners was mostly abnormal, only 3 normal values being recorded. All other workers, with the exception of one slinger and one crane operator (working

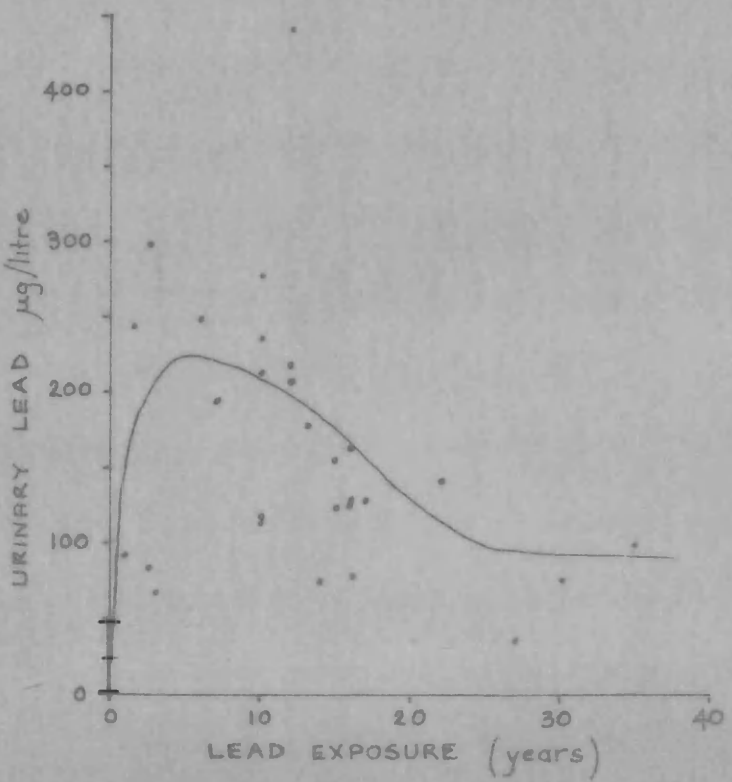
x Strippers strip and separate metal scrap left by burners.

+ Slingers load metal scrap into wagons.

Figure 20. Relationship between blood lead and working distance from the site of lead burning for Nos. 1-48.

Figure 21. Relationship between years of exposure to lead and the excretion of urinary lead for Nos. 1-24. The curve is drawn through the mean of five points listed in Table 12. The mean urinary lead ± two standard deviations in normal subjects is shown.


BLOOD LEAD µg/100ml
200
200
100
0
UPPER NORMAL LIMIT
0
20
40
60
80
100
DISTANCE (yards)


URINARY LEAD µg/litre
400
300
200
100
0
0
10
20
30
40
LEAD EXPOSURE (years)

3 and 9 yards respectively from the site of lead burning) had levels of ALA within the normal range.

b) Duration of exposure to lead (Burners Only)

The number of years that each burner had been actively working with lead-containing metals, i.e. his duration of exposure to lead, was obtained and related to his excretion of urinary lead, CP and ALA and blood lead respectively.

The duration of lead exposure for the group of 24 burners has been divided into five periods as listed in Table 12, and the mean value of lead, CP and ALA for each period stated. The following paragraph refers to mean values of lead, CP and ALA at all times.

During the first 3 years of lead exposure, there was a sharp rise in the excretion of urinary lead (Fig. 21), a steady rise in blood lead and the urinary excretion of ALA, and a gentler rise in the excretion of urinary CP. Between 4 and 7 years, the excretion of urinary CP increased with that of urinary lead while the blood lead and the excretion of urinary ALA tended to remain constant. With continued exposure to lead of 8-11 years, the urinary lead tended to remain constant, while the blood lead began to rise associated with a rise in the excretion of ALA and a fall in the

	Duration of Exposure to lead (years)				
	0 - 3	4 - 7	8 - 11	12 - 16	17 - 35
Mean blood lead µg./100 ml.	59 (4)	56 (2)	68 (4)	92 (9)	103 (4)
Mean urine lead µg./litre	174 (4)	223 (2)	211 (4)	185 (9)	97 (5)
Mean urine CP µg./litre	195 (4)	711 (2)	411 (4)	402 (9)	332 (5)
Mean urine ALA mg./100 ml.	1.63 (4)	1.72 (2)	2.61 (4)	2.47 (9)	0.90 (5)

TABLE 12

Duration of exposure to lead. The thirtyfive year period is divided into five subperiods. The number of subjects and the mean value of blood lead and urinary lead, coproporphyrin and δ-amindaevulic acid is listed for each subperiod.

excretion of CP. Between 12 and 16 years, the excretion of urinary lead began to decline while that of blood lead and urinary CP and ALA remained constant. From 17-35 years, the urinary excretion of lead, CP and ALA decreased while that of blood lead remained constant. This decrease in the later years was probably due to burners being put on to lighter work because of advancing years.

c) Mask Protection (Burners Only)

Since each burner was asked to assess, as a percentage, the length of time he wore his mask daily, these figures may well be subject to large errors.

Inspection of the graphs relating % Mask Protection to urinary lead, CP and ALA respectively, showed a very wide scatter of points with a tendency for the excretion of these three substances to increase with diminishing mask protection.

Since the majority of blood lead values lie in the 50-100 µg range, the average blood lead did not vary with varying mask protection.

d) Haemoglobin

The relationship of haemoglobin with blood lead and urinary lead, CP and ALA respectively was determined by calculating the

correlation coefficient (Table 13). None was significant but it must be remembered that about 50% of the employees showed little or no chemical evidence of lead exposure. The correlation between haemoglobin and blood lead is shown (Fig. 22). The decrease of haemoglobin was associated with an increasing blood lead (and urinary lead, CP and ALA respectively).

e) Blood lead. Relationship with urinary lead, CP and ALA.

The correlation between blood lead and urinary lead, CP and ALA respectively was not significant (Table 13). This is confirmed in part by the studies of Perales & Gonzales (1954) who obtained a correlation coefficient for blood and urine lead of + 0.20. Fig. 23 shows the plot of blood lead versus urine lead.

f) Urinary lead. Relationship with urinary CP and ALA.

There was a highly significant correlation between urinary lead and urinary ALA (Table 13). As the urinary excretion of lead increased initially, that of CP and ALA remained within normal limits.

At approximately 75 and 100 µg of lead/litre urine, the urinary excretion of ALA and CP respectively began to diverge from normal values (Figs. 24, 25).

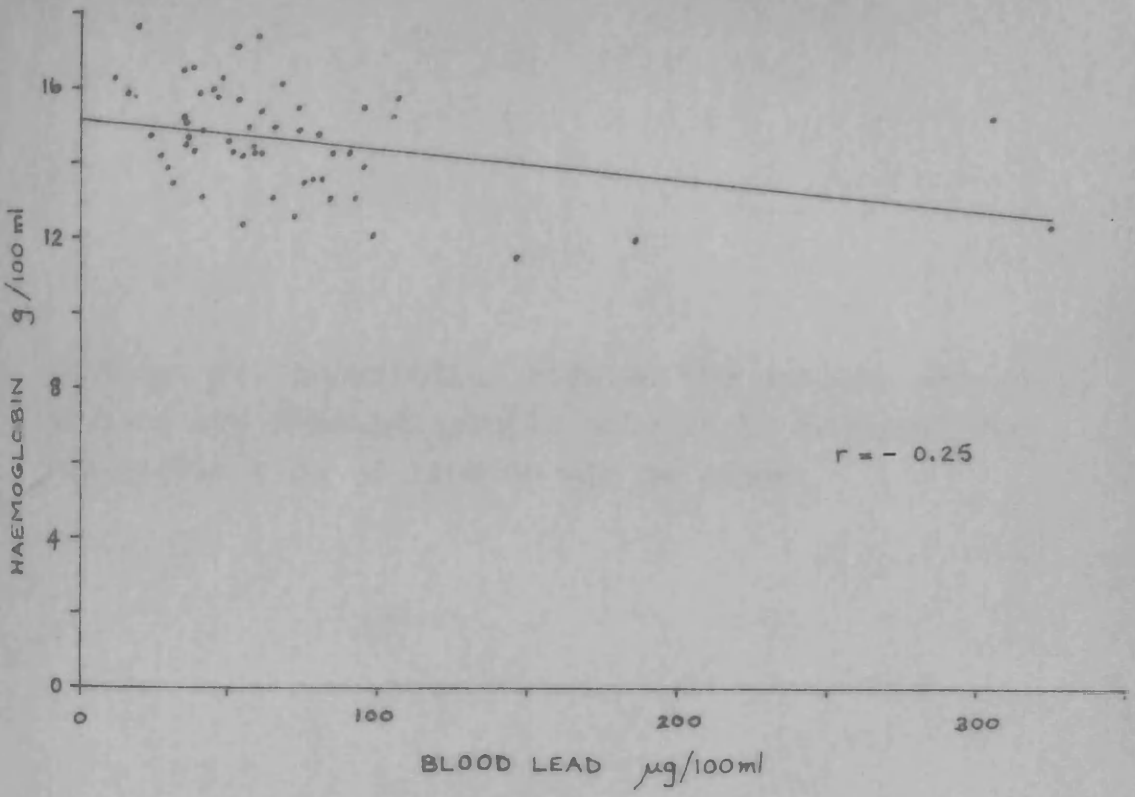
x		y	n	r	p	Haeger-Aronsen (1960)
Lead µg./100 ml.	blood	Haemoglobin g.%	55	-0.250	>0.05	
Lead µg./litre	urine	"	49	-0.210	>0.05	
CP µg./litre	urine	"	54	-0.218	>0.05	
ALA mg./100 ml.	urine	"	48	-0.245	>0.05	
Lead µg./litre	urine	Lead µg./100 ml.blood	50	+0.210	>0.05	
CP µg./litre	urine	"	55	+0.168	>0.05	
ALA mg./100 ml.	urine	"	49	+0.232	>0.05	
ALA mg./100 ml.	urine	CP µg./litre urine	50	+0.579	<0.001	
CP µg./litre	urine	Lead µg./litre urine	51	+0.695	<0.001	r = +0.68 (n = 85) p <0.001
ALA mg./100 ml.	urine	"	50	+0.744	<0.001	r = +0.92 (n =160) p < 0.001

TABLE 13

Significance of the correlation coefficient r for n samples of x and y.

Figure 22. Correlation between haemoglobin and blood lead in 55 workers. The regression line of haemoglobin on blood lead is drawn.

Figure 23. Correlation between blood and urinary lead in 50 workers. The regression line of blood lead on urinary lead is drawn.


HAEMOGLOBIN g/100 ml
16
12
8
4
0
r = - 0.25
0
100
200
300
BLOOD LEAD µg/100ml

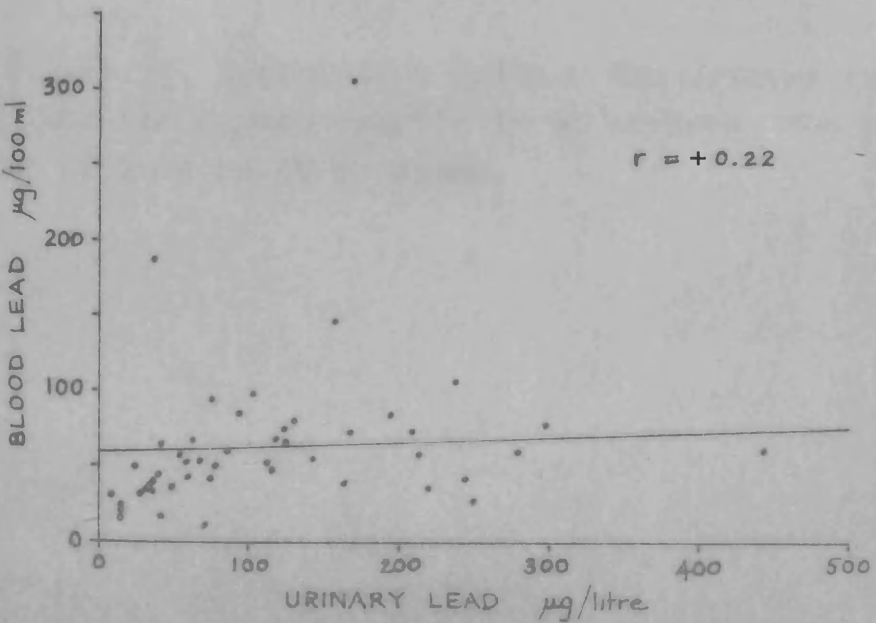
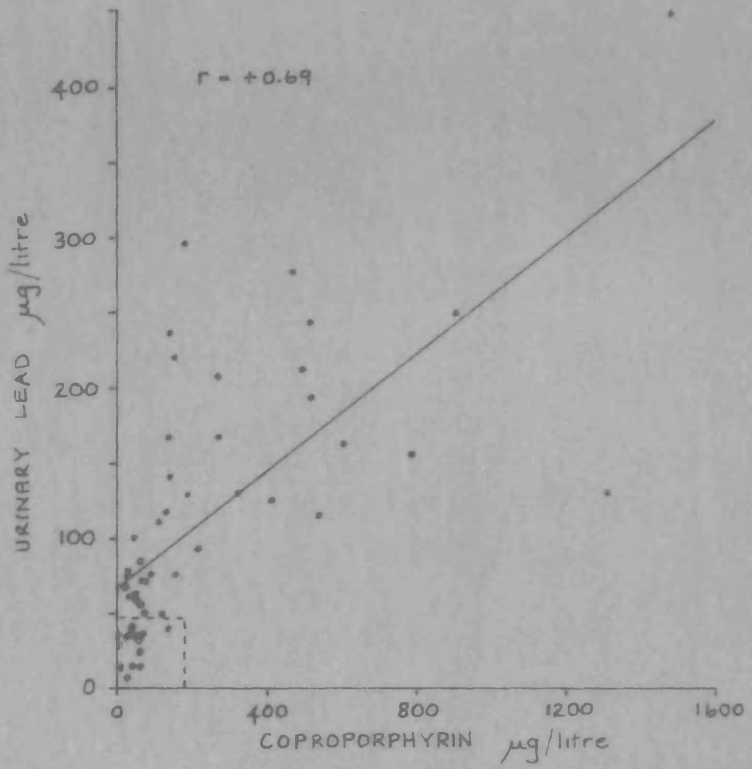
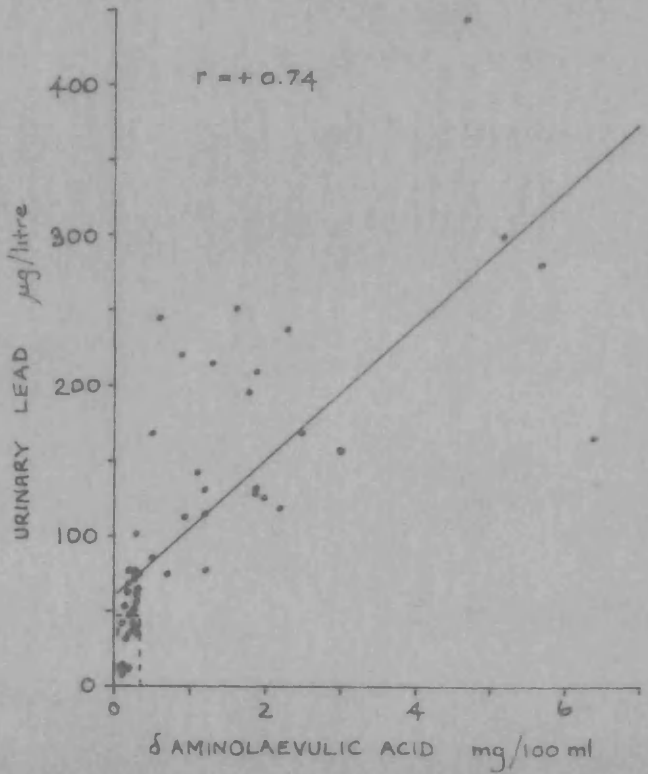

BLOOD LEAD µg/100 ml
300
200
100
0
r = + 0.22
0
100
200
300
400
500
URINARY LEAD µg/litre

Figure 24. Correlation between the urinary excretion of lead and δ-aminolaevulic acid in 50 workers. The regression line of lead on ALA is drawn.

Figure 25. Correlation between the urinary excretion of lead and coproporphyrin in 51 workers. The regression line of lead on CP is drawn.


r = +0.69
URINARY LEAD µg/litre
400
300
200
100
0
0
400
800
1200
1600
COPROPORPHYRIN µg/litre


r = +0.74
URINARY LEAD µg/litre
400
300
200
100
0
0
2
4
6
δ AMINOLAEVULIC ACID mg/100 ml

g) Urinary CP. Relationship with urinary ALA.

The correlation between urinary CP and urinary ALA was highly significant. CP and ALA initially tended to increase together, but well beyond the upper normal limits of both, the scatter of points became greater, apparently due to the variability of CP values (i.e. low CP values with high ALA values).

h) Blood

i) Haemoglobin and packed cell volume (P.C.V.). Anaemia is a secondary symptom in lead intoxication. Where the haemoglobin is low, the P.C.V. should be correspondingly lowered.

Among nos. 1-48, only two subjects, nos. 12 and 21, had a haemoglobin of 12 or less. No. 12 had a fairly high blood lead, 98 µg/100 ml, although his urinary CP and ALA excretion were normal. No. 21 had finished an extended course of penicillamine ten weeks previously and although his urinary lead, CP and ALA were normal, his blood lead was high, 186 µg/100 ml.

ii) Punctate basophilia. Among nos. 1-48, only No. 21 showed the presence of stippled cells in peripheral blood. Three of the nine outpatients showed basophilic stippling of erythrocytes. Nos. 21, 55, 56 and 57 had blood lead levels of 186, 90, 324 and

146 µg/100 ml respectively and haemoglobin values of 11.9, 14.2, 12.3 and 11.4 g/100 ml respectively.

4. Conclusions

1. The chemical tests for blood lead, urinary lead, CP and ALA were shown to be sensitive enough to detect industrial lead exposure at its outset.

2. Of 23 potentially lead exposed subjects (excluding burners), 14 had lead in blood levels above the normal upper limit stated in Table 11. These were:- brass burner (1), strippers (4/6), slingers (2/2), crane operators (2/2), general labourer and fireman (1) and ship manager (1).

3. No relationship was observed between the clinical manifestations of lead poisoning and its chemistry, e.g. one burner with a blood lead of 306 µg/100 ml did not complain of any specific symptoms. His haemoglobin was 15.2 g %; another burner with a blood lead of 186 µg/100 ml and a haemoglobin of 11.9 g % also felt quite well; a battery worker with a blood lead of 146 µg/100 ml and a haemoglobin of 11.4 g % had wrist drop.

Section B.III

Discussion

At one time, the sole screening method used by many industrial concerns for the diagnosis of lead exposure was that of the presence of stippling of young red cells. This was regarded as an early sign of lead poisoning. Such cells however are not peculiar to lead absorption. On the other hand, in many cases of undoubted lead poisoning and in chronic cases, the presence of basophilic stippling is not demonstrable (Waldman & Seideman, 1950). Maloof (1950) has stated that not too much emphasis should be placed on the absence of stippled cells. As shown in Table 10, the almost total absence of stippling gives weight to this view.

Although the determination of lead in blood can give an insight into the length and intensity of lead exposure, changes in the urinary lead concentration occur more promptly and are more marked than those of blood concentration (Kehoe 1935). In 1961 (Lec 3) Kehoe stated that the critical level above which lead intoxication must be diagnosed, is a little above or a little below 80 µg/ 100 g whole blood. The lead in urine concentration which corresponds to the critical value of blood is a range rather than a

single value because of the physiological factors which result in an appreciable variability in the rate of the urinary excretion of lead. The value may be as low as 150 µg lead/litre or as high as 240 µg lead/litre.

Many workers state that lead in blood values greater than 50 µg/100 ml or 50 µg/100 g should not be considered normal (Momcilo et al, 1955; Bradley et al, 1956 respectively). This is in agreement with the normal values established in Part A of this study for blood lead, the highest of which was 49.1 µg/100 ml. Using the normal lead values obtained earlier, the mean + 2 standard deviations (39.3 µg/100 ml blood, 48.3 µg/litre urine) however has been taken arbitrarily as the limit above which lead exposure was diagnosed in this study. The following paragraphs are based on this assumption. No dividing line was drawn between lead exposure and lead intoxication.

A falling haemoglobin associated with a rising blood lead appeared at the later stages of lead exposure, i.e. when a haemoglobin level of 12% or less was detected, this appeared to be associated with a fairly lengthy exposure to lead since high lead in blood levels ($>$ 100 µg/100 ml) were also present.

When results of the tests for subjects 1-48 were examined, the greatest degree of lead exposure was experienced by burners (Group 1), followed by crane operators and slingers (Group 2). Strippers (Group 3) were the next most exposed, followed by all others working 25-30 yards from the site of burning (Group 4). It is of interest to note in Group 2 that because the crane operators worked almost directly above the site of lead burning, they ran the same risk of exposure to lead as slingers who worked much closer to the site of lead burning.

Among the 24 burners in Group 1, 21 excreted abnormal amounts of urinary lead and ALA. 18 of the 21 had abnormal blood lead levels, but only 14 of the 21 showed abnormal excretion of CP. On the other hand, the excretion of abnormal quantities of CP was always associated with an abnormal urinary lead and ALA and (except for one case) with an abnormal blood lead. In Group 2, all four subjects showed abnormal lead in blood and lead in urine values. Only one ALA value was abnormal. All CP levels were normal. In Groups 3 and 4, the excretion of CP and ALA was normal, although many had abnormal urine and/or blood lead values.

The above evidence suggested that the excretion of CP attained abnormal levels after that of ALA, rather than both attaining normal values together. Haeger Aronsen (1960 p. 102) has also suggested this. Assuming this to be the case, the development of lead exposure in the majority of subjects follows the general pattern of an increase, above the normal limit, of 1) urinary lead 2) blood lead 3) urinary ALA and 4) urinary CP.

Stages 3 and 4, representing the biochemical change in the enzyme system for the porphyrins, may be regarded as one of the earliest biological changes produced by lead (de Langen and ten Berg, 1948; Maloof, 1950). Following the increased excretion of CP, basophilic stippled cells may appear (de Langen and ten Berg, 1948) later confirmed by Maloof (1950). As the exposure becomes greater lead anaemia is likely to be detected (p. 73) and this may be followed by the various symptoms of lead poisoning including colic and palsy. Maloof (1950) stated that the excretion of CP was greatly in excess of normal long before any symptoms appeared.

For routine screening of workers and early diagnosis of lead exposure, the determination of urinary ALA and CP would be

of value, especially since it has been established that the latter two are related in a highly significant manner to that of urinary lead (Table 13). As yet no simple semi-quantitative test is known for the determination of ALA in urine but one is available for CP (Waldman & Seideman, 1950; Harrold et al, 1952). This test is based on the fact that porphyrins are pigments which fluoresce in UV light. If a small aliquot of acidified urine is shaken with ether, the presence or absence of porphyrins, extracted into the ether layer, can be seen under UV light. A positive test, which may be graded into at least 3 categories occurs when the lead in urine value is greater than 150 µg lead per litre (Waldman & Seideman, 1950; Harrold et al, 1952).

Of the 4 main tests reviewed i.e. blood lead, urinary lead CP and ALA, all are sensitive indices of lead exposure. Only that of CP however has been adopted, in simplified form, as a rapid screening method for the detection of industrial lead exposure, and it is recognised as the most simple and most useful indication of the degree of exposure to lead (Horiuchi, 1955).

Routine screening should be extended to subjects working within a 10 yard range of the perimeter of the site of lead burning, since they are likely to be exposed to lead fumes and airborne particles.

In agreement with the findings of Perales et al (1954), there was no significant correlation between the symptoms of lead poisoning and the levels of blood lead and urinary lead, coproporphyrin and δ aminolaevulic acid respectively. It appears that the tolerance level of absorped lead varies from person to person.

Summary

A survey was carried out in a ship-breaking yard to determine the sensitivity of the following tests - blood lead, urinary lead, coproporphyrin and δ-aminolaevulic acid - as screening methods in the detection of lead exposure. The urinary excretions of coproporphyrin and δ-aminolaevulic acid respectively have been shown to be related to the urinary excretion of lead in a highly significant manner ($p < 0.001$). The excretion of abnormal quantities of coproporphyrin appeared to follow that of δ-aminolaevulic acid. Since there is as

yet no simplified test for δ-aminolaevulic acid, that of coproporphyrin (available in simplified form) is recommended for screening. Basophilic stippling of red cells occurred only in a minority of cases. Subjects working within a 10 yard range of the perimeter of the site of lead burning should be routinely screened. There was no significant correlation between the symptoms of lead poisoning and blood lead, urinary lead, coproporphyrin and δ-aminolaevulic acid respectively.

PART C. THE URINARY EXCRETION OF IRON IN HEALTH AND DISEASE.

Section C.I INTRODUCTION

1. Outline of iron metabolism

In contrast to lead, iron is an essential element of the body. The normal adult body contains 3 - 5 g. of iron which is almost exclusively complexed to protein (eg. transferrin, ferritin) or present in haem (eg. haemoglobin, myoglobin, haem enzymes). About 66% of the total iron is present in circulating haemoglobin. Approximately 25% is present as reserve iron, stored mainly in liver, spleen, kidney and bone marrow in the form of ferritin and haemosiderin. Myoglobin contains about 3% of the total iron and about 5% is accounted for in the parenchyma of other organs. The remaining 1% is distributed among the various haem-containing enzymes and transferrin (Moore, 1958).

Ingested iron must be reduced to the ferrous form in stomach and intestine before it can be absorbed (Moore et al., 1944). Although small amounts of iron may be absorbed by the stomach, the absorption of iron is greatest in the upper part of the small intestine and progressively less in the more distal segments (Wintrobe, 1956). The diminishing absorption of iron from the jejunum and ileum may well be a result of the gradual conversion of iron to less soluble complexes on its passage down the gastrointestinal tract (Fig. 26).

It has been found that patients with iron deficiency absorb iron more efficiently than do normal subjects (Hahn et al., 1939; Dubach et al., 1948) and that ferritin is synthesised in the

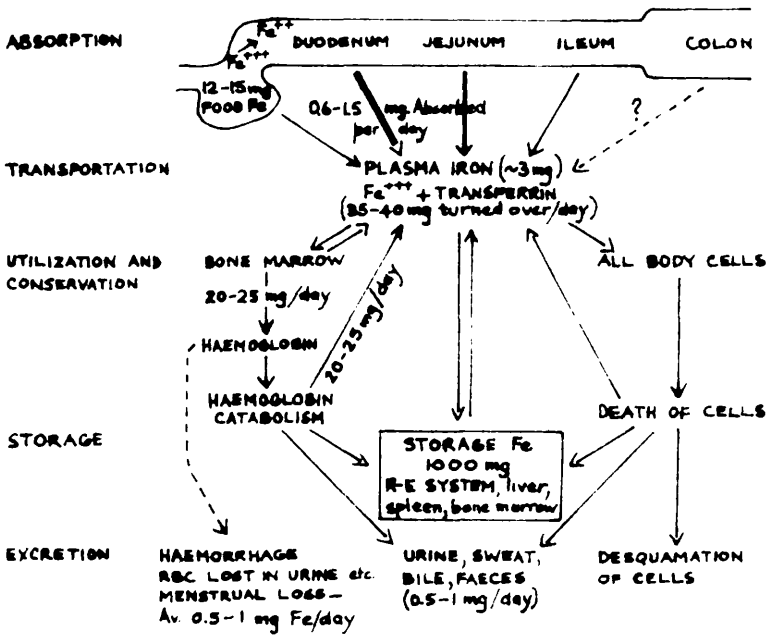


Figure 26. Schematic outline of iron metabolism in the adult.

intestinal mucosa of guinea-pigs as a direct result of the administration of large quantities of iron (Granick, 1946a). There is no doubt that the intestinal mucosa serves to some extent as a regulator of iron excretion and that the presence or absence of ferritin appears to be of some importance in this process (Crosby, 1963) as discussed in Part D. There is also evidence that the absorption of iron may be increased when erythropoiesis is stimulated (Bothwell et al., 1956).

When iron passes into the plasma, it is oxidised to the ferric state and transported by transferrin, a β_1 globulin. Although the amount of iron in plasma is small - about 50 - 180 μg.% - its turnover is rapid, averaging approximately 560 μg. per kg. of body weight per day (Bush et al., 1956). This quantity of plasma iron in a man weighing 70 kg. is roughly 35 - 40 mg. per day and of this, 20 - 25 mg. are used by the bone marrow each day for haemoglobin synthesis (Moore, 1958). A small amount is taken up by other cells of the body for the formation of myoglobin and of cellular enzymes. Iron transported in the plasma can be deposited in the storage organs where it may be used or excreted. The quantity of iron stores in the tissues of a healthy adult is approximately 1 g., the liver and spleen being the chief storage sites. Storage iron is found intracellularly in a protein complex as ferritin (which is water soluble) and as haemosiderin (which is water insoluble) (Shoden et al., 1953). Both compounds are capable of being mobilised by the body for

haemoglobin synthesis when the need arises.

One very important characteristic of iron metabolism is the efficient way the body conserves the metal. When erythrocytes are destroyed, almost all of the iron released from haemoglobin is reused. Iron released from other cells that die is similarly conserved. Small but significant amounts are excreted. Whenever erythrocytes are lost by haemorrhage or in urine or whenever cells are desquamated from the surfaces of the body or from body orifices, the iron they contain must be considered lost.

Whenever iron loss exceeds iron absorption, the blood haemoglobin falls and anaemia develops. In both sideropenia and iron deficiency anaemia, there is an absence of stainable iron in the bone marrow and in both states, the serum iron is decreased (normal mean $\pm$ 2 S.D. is 134 $\pm$ 51 µg.%. Dreyfus and Shapira, 1964) while the total serum iron binding capacity is increased (normal range 300 - 350 µg.%. Dreyfus and Shapira, 1964).

The only important use of iron in modern medicine is in the treatment of iron deficiency anaemia. The administration of iron to patients with normal blood values serves only to increase the reserve iron of the body and not to increase the red blood cells or haemoglobin above their normal physiological limits. On the other hand, a large increase in the reserve iron of the body over a period of years, may be brought about by a defect in metabolism. Such is the case in primary idiopathic haemochromatosis where the process of accumulation of iron within the body is so slow that a great number

of years may pass before the disease is detected. This disease is now regarded as a congenital one and it must be assumed that the onset of accumulation of iron dates back to childhood. Over such a lengthy period, the daily accumulation of small quantities of iron may be due to the plasma-storage iron exchange (which is normally very small) becoming the major component of plasma iron turnover.

Iron excess in the body results in the deposition of iron in the parenchyma of many organ systems, as in idiopathic haemochromatosis, and may lead to eventual tissue damage, manifested by altered organ function and fibrosis. This tends to increase with the passage of time. Appreciation of this problem has led to therapeutic efforts to remove iron from the body in an attempt to arrest or reverse the course of these iron storage diseases. Repeated phlebotomy has been demonstrated to be the best effective method of iron removal, and is now accepted as the treatment of choice in cases of idiopathic haemochromatosis. However, in patients with haemochromatosis following repeated transfusions for various types of refractory anaemia, venesection is not feasible. In focal iron overload eg. idiopathic pulmonary haemosiderosis, excess iron can only be removed by the action of chelating agents.

2. Chelating agents

The most recently introduced chelating agent for iron is desferrioxamine B methane sulphonate (desferrioxamine B mesylate). The Ciba trade name for this substance is Desferal. The ferrioxamines are members of a class of naturally occurring substances, the so-called siderochromes. These combine with ferric iron to form

brownish-red complexes and they can be divided into two groups, the sideromycins and the sideramines. The sideromycins are iron-containing antibiotics. Sideramines are substances which on the one hand promote the growth of certain micro-organisms and on the other may antagonise the antibacterial effects of the sideromycins.

Ferrioxamine B which contains one molecule of chelated ferric iron, belongs to the sideramines and is isolated as a metabolite from Streptomyces pilosus. Ferrioxamine B was first used in the treatment of anaemia associated with iron deficiency but trials were not successful due to the compound being excreted rapidly by the kidney without significant release of iron. The use of the corresponding iron-free compound, desferrioxamine B, as an iron chelator in vivo was then investigated. Desferrioxamine B, a trihyroxamic acid derivative forms an octahedral complex with ferric iron called ferrioxamine B (Fig. 27). Since the complex has a low molecular weight and hydrophilic properties, it is easily excreted by the kidney. When given orally, Desferal is poorly absorbed from the small intestine. After parenteral administration in animals, it is widely distributed in the tissues. The mobilisation of tissue iron is indicated by a rise in serum iron due to the presence of the complex. Clinical trials have confirmed that Desferal is well tolerated and non-toxic. No side reactions have been described. Of great importance is the fact that Desferal is a selective chelator of iron. Although the stability constant of ferrioxamine is 10^{31} (Bickel et al., 1960),

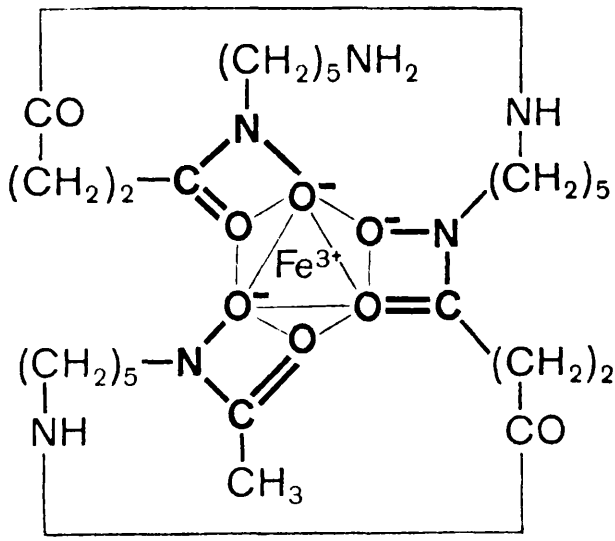


Figure 27. Ferrioxamine B.

Desferal has a minimal effect on the urinary excretion of sodium, potassium, zinc, cobalt, cadmium, copper, magnesium, aluminium, manganese and chromium (Tripod and Keberle, 1962; Bickel et al., 1960, 1962).

Desferal can chelate ionic iron and ferritin iron (Wohler, 1964b) but it cannot chelate iron bound to transferrin, or haemoglobin iron in washed or haemolysed erythrocytes (Moeschlin and Schnider, 1963). There is conflicting evidence as to whether it can remove iron from haemosiderin (Mann and Maier, 1962; Wohler, 1964b).

Before the advent of Desferal, two other chelating agents were used to complex iron in patients with iron storage disease. These were calcium disodium ethylenediaminetetraacetate (E.D.T.A.) and calcium trisodium diethylenetriaminepentaacetate (D.T.P.A). Brick and Rath (1964) found that dose for dose, the administration of D.T.P.A. caused a far greater excretion of iron than that obtained with E.D.T.A. However, these versenate derivatives enhance the excretion of essential trace elements like copper, zinc and magnesium (Moeschlin and Schnider, 1964). It is of interest that although at pH 7.25 the Fe(III) - Desferal complex is more stable than the corresponding E.D.T.A. and D.T.P.A. complexes, the position is reversed at pH 4 (Schubert, 1964).

About 1962, small quantities of Desferal, made available for research purposes, stimulated interest in the urinary excretion of iron (Bannerman et al., 1962; Smith, 1962; Wohler, 1964a).

Under normal conditions, the loss of iron in the urine is apparently insignificant (Lintzel, 1931). In ten normal male subjects, Cartwright et al., (1954) found the mean urinary iron excretion to be 48 µg./24 hours; in no instance was a value higher than 64 µg./24 hours obtained. This view was confirmed by the radio-isotopic studies of Dubach et al., (1955) which showed negligible amounts of radio-activity in the urine in the first two days following intravenous injection of radio-iron.

It has already been shown that the administration of Desferal is useful in the detection of states of generalised iron overload (Bannerman et al., 1962). In such states, Desferal greatly increased the urinary excretion of iron. If, in patients with iron deficiency and iron overload, and in normal subjects, a correlation could be established between the body iron stores and the urinary output of iron during Desferal treatment, then such a test would be of use as a diagnostic aid. In states of iron deficiency especially, such a test could provide a measure of tissue iron depletion and hence avoid the necessity of marrow aspiration.

3. Purpose of the study

The first part of the study deals with the effect of Desferal on the urinary iron excretion of normal subjects and of patients with sideropenia and iron deficiency anaemia. Studies with Desferal were carried out in diseases with focal iron overload (eg. the renal haemosiderosis associated with paroxysmal nocturnal haemoglobinuria and idiopathic pulmonary haemosiderosis) and in

states of generalised iron overload (idiopathic haemochromatosis).

In 1954, Cartwright and his co-workers demonstrated that increased urinary excretion of iron took place in patients with gross proteinuria. Further studies to determine the mechanism of this excretion were carried out and are described in the second part of this study.

In 1965, Fielding noted that the urinary excretion of iron in patients with a haemolytic condition was somewhat greater than in healthy controls. In the same year, in collaboration with Dr.A. Goldberg, Keberle carried out experiments on two guinea-pigs, administering oral phenylhydrazine to induce haemolysis. Both animals were given ^{59}Fe as ferrous sulphate by gastric tube on Day 1 and on Day 14. On Day 40, guinea-pig A was given phenylhydrazine (30 mg./kg.) daily for four days, as well as Desferal (100 mg./kg.) daily for five days. Control guinea-pig B was given only Desferal for five days. The radioactivity of ^{59}Fe, excreted in urine and faeces, was determined daily during this period in A and B and is shown in Fig. 28. On Day 41, the urinary excretion of ^{59}Fe in A rose immediately in response to the combined Desferal and phenylhydrazine therapy, showing a maximum eightfold rise over control B on Day 44. The excretion of faecal ^{59}Fe in A however, did not rise significantly until Day 44 (ie. the fourth day of combined treatment) when a fourfold increase over control B was recorded. In an earlier experiment, using the same animals and same doses, a single dose of phenylhydrazine had been given during a course of Desferal treatment. Only a slight increase in the elimination of urinary and faecal ^{59}Fe, over the control,

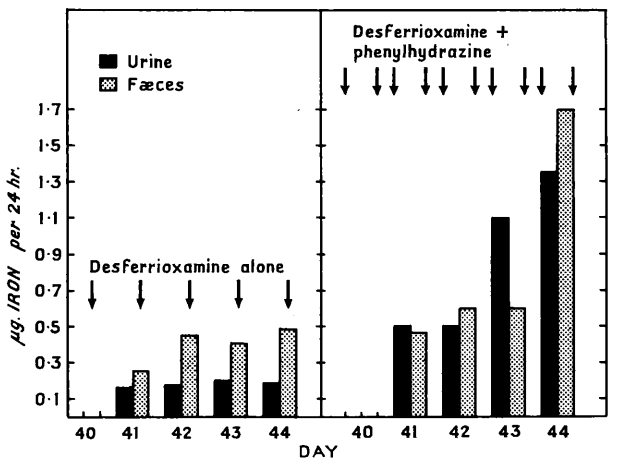


Figure 28. Urinary and faecal iron excretion in guinea-pigs. Animal A (on the right) was given combined Desferal and phenylhydrazine treatment. Control animal B (on the left) was given only Desferal.

was found.

From the above experiments, it can be seen that the faecal and urinary excretion of iron during Desferal treatment was only significantly increased when phenylhydrazine was given for more than one day. The advantages and disadvantages of administering phenylhydrazine in combination with Desferal to patients with iron overload were considered.

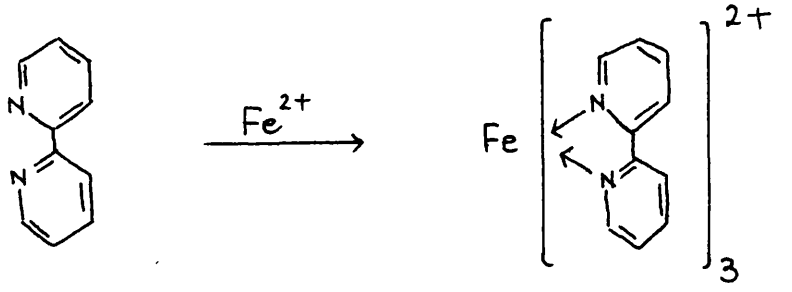
In primary idiopathic haemochromatosis, long term treatment with Desferal has been of limited value since it generally removes no more than 20 mg. of iron per day (Moeschlin and Schnider, 1963). Since phlebotomy is the treatment of choice for primary idiopathic haemochromatosis, this figure compares unfavourably with the venesection of one pint of blood which effectively removes approximately 230 mg. of iron. If, in patients with primary idiopathic haemochromatosis, the excretion of iron during combined phenylhydrazine and Desferal treatment was enhanced to over 200 mg. of iron per week, then this treatment could serve as an alternative to that of phlebotomy. However, because phenylhydrazine is such an effective haemolytic agent when introduced into the body, careful consideration was given to the dose administered. Phenylhydrazine has been little used medicinally in the past forty years except in the treatment of patients with polycythaemia where daily doses of 100 mg. have been extensively employed without any untoward effect (Wintrobe, 1961).

The third part of the study deals with the effect of the haemolysis provoked by varying doses of phenylhydrazine on the urinary iron excretion during Desferal treatment in patients with various forms of iron overload.

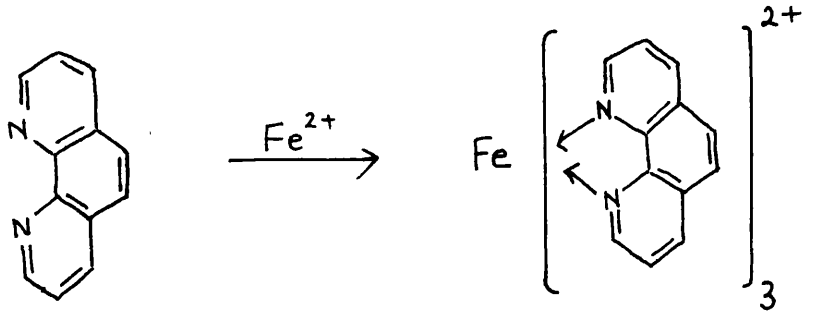
Section C.II METHODS AND RESULTS

1. Iron estimation

2:2[1] dipyridyl and 1:10 phenanthroline are sensitive reagents for the quantitative colorimetric determination of traces of iron, giving red and orange-red complexes respectively with ferrous iron in aqueous solution. Three molecules of each bidentate ligand react with one molecule of ferrous iron respectively to give coordination complexes in which the arrangement of nitrogen atoms around the iron molecule is octahedral.



2:2[4] dipyridyl



1:10 phenanthroline

2:2[1] dipyridyl and o-phenanthroline can complex ferrous iron in acid or alkaline solution. In biological materials, however, ferrous iron is best complexed under acidic conditions to avoid the precipitation of hydroxides and phosphates of interfering metals. The concentration of iron in urine was determined initially using the o-phenanthroline method and latterly using the dipyridyl method.

Concentration and digestion of 100 ml. urine samples were carried out as before (Section A.II). On addition of 2 ml. of deionised water to the urine digest contained in 2 ml. of 36N sulphuric acid, calcium sulphate was precipitated. Although calcium sulphate dissolves in a saturated solution of ammonium sulphate by forming the double salt $CaSO_4 (NH_4)_2SO_4 . H_2O$, the solution obtained after pH adjustment was not perfectly clear. Rather than buffer the existing salt to make it completely soluble, it was decided to eliminate calcium sulphate by filtration. On filtration, loss of iron, through precipitation with calcium sulphate, was avoided by the addition of 2 ml. 5N hydrochloric acid to the digest to convert iron present as ferric sulphate to the more stable chloride compound. The yellow colour of ferric chloride in solution was only visible when 40 µg. or more of iron were present. The digest was filtered into a volumetric flask, using washed No. 44 (7cm.) filter paper, and the Kjeldahl flask rinsed out with 5 - 6 small portions of water. After trials with various methods of pH adjustment and colour development, using hydroquinone as reducing agent and o-phenanthroline as complexing agent, there was still inadequate duplication of results. It was not known whether the method for colour development was

inadequate or whether iron had been lost during digestion and/or during filtration. The possible loss of iron during digestion and during transfer to the volumetric flask was determined using ^{59}Fe.

One ml. of a solution of radioactive ferric chloride was added to two 100 ml. urine samples from a normal subject and to a Kjeldahl flask (for the reagent blank) in the following manner:- The activity of 1 ml. of ^{59}Fe solution contained in a test-tube was determined using a scintillation counter. The radioactive solution was poured into a 100 ml. urine aliquot and the activity of the ^{59}Fe remaining in the test-tube determined. The difference in activity was that added to the urine sample. The urine samples were concentrated and digested as before. 2 ml. of water and 2 ml. 5N hydrochloric acid were added to the digest. This was heated gently for 2 - 3 minutes to eliminate the last traces of nitric acid. (When less than 40 µg. of iron were present (ie. no yellow colour due to ferric chloride) traces of nitric acid in hydrochloric acid were visible as the yellow colour of aqua regia. On gentle heating and swirling, nitric acid was evolved and the solution became colourless). The digest was allowed to stand overnight to allow complete precipitation of calcium sulphate and filtered into a 25 ml. volumetric flask. The pH was adjusted and hydroquinone and o-phenanthroline were added. After making up to volume, the activity was determined and correction made for decay.

The recovery of ^{59}Fe from urine as shown in Table 14 was good but the colorimetric determination of iron, estimated at the same time was not, thus providing that the method of colour development required modification.

Sample	^{59}Fe added c.p.m.	^{59}Fe found c.p.m.	% ^{59}Fe recovered
100 ml. urine	3049	2923	96
100 ml. urine	3316	3283	99
Blank	3078	2879	94

TABLE 14

Recovery of ^{59}Fe added to 100 ml. aliquots of urine.

Optimal pH

o-phenanthroline was used to complex ferrous iron at pH 2 - 4.5 (Possible range 2 - 9). In this pH range, the precipitation of hydroxides and phosphates of interfering metals are prevented. At pH 5, copper may interfere due to the reduction of Cu(II) to Cu(I) which forms a coloured complex with o-phenanthroline (Sandell, 1959 p. 539). Since the concentration of iron in normal urine was likely to be in the range 2 - 10 µg.% (Cartwright et al., 1954), it was not feasible to divide the filtrate into two parts and use 1 part to determine the pH adjustment required.

The pH was initially adjusted to pH 4.5 by the addition of 8 ml. of 6M ammonium acetate for every 1 ml. of sulphuric acid (H_2SO_4) used in the digestion process. When 2 ml of H_2SO_4 was used (the minimum volume required for the digestion of 100 ml. of urine) ammonium acetate had to be used to wash out the Kjeldahl flask during transfer of the digest to the 25 ml volumetric flask. Such an alkaline wash may cause the remaining traces of iron in the Kjeldahl flask to be precipitated as ferric phosphate (Although phosphate is destroyed by H_2SO_4 digestion, pyrophosphate may be formed. On the addition of water, this is converted to phosphate). Due to some H_2SO_4 being used up in salt formation during digestion, the volume of 6M ammonium acetate added may be in excess giving pH values above 5. The use of ammonium acetate was discontinued.

p-nitrophenol was the only suitable indicator for the adjustment of pH since it is colourless in acid solution and yellow in alkaline solution, the yellow end-point being visible at pH 5.6. It was intended to add ammonium hydroxide to the iron-containing filtrate until the

yellow end-point of p-nitrophenol was reached and, by the addition of a slight excess of acid, ensure a colourless solution and a pH below 5. The excess acid was added as H_2SO_4 rather than as hydrochloric acid since this may intensify the yellow colour of ferric chloride, when iron is present in large quantities.

Two drops of 1% w/v p-nitrophenol in 10% v/v ethyl alcohol were added to the iron-containing filtrate in a 20 ml. volumetric flask. Using 14N ammonium hydroxide, the pH was adjusted to the yellow end-point, precipitation of insoluble hydroxides occurring only in a few instances. 1 - 3 small drops of $12\underline{N}.H_2SO_4$ were added until the yellow colour just disappeared and no cloudiness was observed. At this stage, the pH of the majority of samples was 3.0 - 3.3. Above pH 4, there was a tendency for traces of insoluble material to remain. While the solution was still warm (about $40^{o}C$) with the heat of neutralisation of H_2SO_4, 1.5 ml. 2% w/v aqueous hydroquinone and 0.5 ml. 1:10 phenanthroline hydrate (2% w/v alcoholic solution) were added. The solution was allowed to stand for 2 hours for complete colour development (Bandemar and Schaible, 1944) and made up to volume. The optical density of the solution was read at 508 mμ on the Hilger uvispek. Estimations were carried out in duplicate.

It was found that the reducing agent and complexing agent had to be added to a warm iron solution otherwise full colour development did not occur within two hours. At higher concentrations than given above, hydroquinone tended to precipitate out of solution on standing for two hours. Hydroquinone was made up daily.

The absorption maximum of the ferrous-phenanthroline complex in aqueous solution was found to be 507 - 508 mμ. on the Hilger uvispek

(Fig.29). This is in agreement with the findings of Sandell (1959, p. 525).

A stock solution of iron (0.1 mg./ml.) was prepared by dissolving 863 mg. of analar ferric ammonium sulphate $FeNH_4(SO_4)_2.12H_2O$ in ion-free water containing 10 ml of 11N hydrochloric acid and making up to 1 litre. The optical density of 20 ml. solutions of the ferrous-phenanthroline complex for concentrations of iron up to 100 µg. were determined. The calibration graph was linear, obeying Beer's Law. The tangent was 0.01018.

An alternative method for estimating iron using sodium sulphite $Na_2SO_3.7H_2O$ as reducing agent and $2:2^1$ dipyridyl as complexing agent was also developed.

Solutions of sodium sulphite are alkaline. $2:2^1$ dipyridyl is insoluble in water and is best dissolved in glacial acetic acid before dilution with water. Initial experiments with varying concentrations of sodium sulphite and $2:2^1$ dipyridyl were made difficult due to pH requirements of the iron complex (so that copper did not interfere) and due to the maximum allowable volume of iron reagents in the 20 ml. volumetric flask being about $2\frac{1}{2}$ - 3 ml. 1 ml. of 2% (w/v) $2:2^1$ dipyridyl in 60% v/v acetic acid and 1 ml. of 0.75M sodium sulphite were added to an iron-containing solution (prepared as before) at pH 3.0 - 3.3. (Addition of sulphite before dipyridyl should be avoided since this causes the pH to rise to about 9 and brings about heavy precipitation). An aliquot of the solution in a graduated test-tube was placed in a waterbath at 100°C

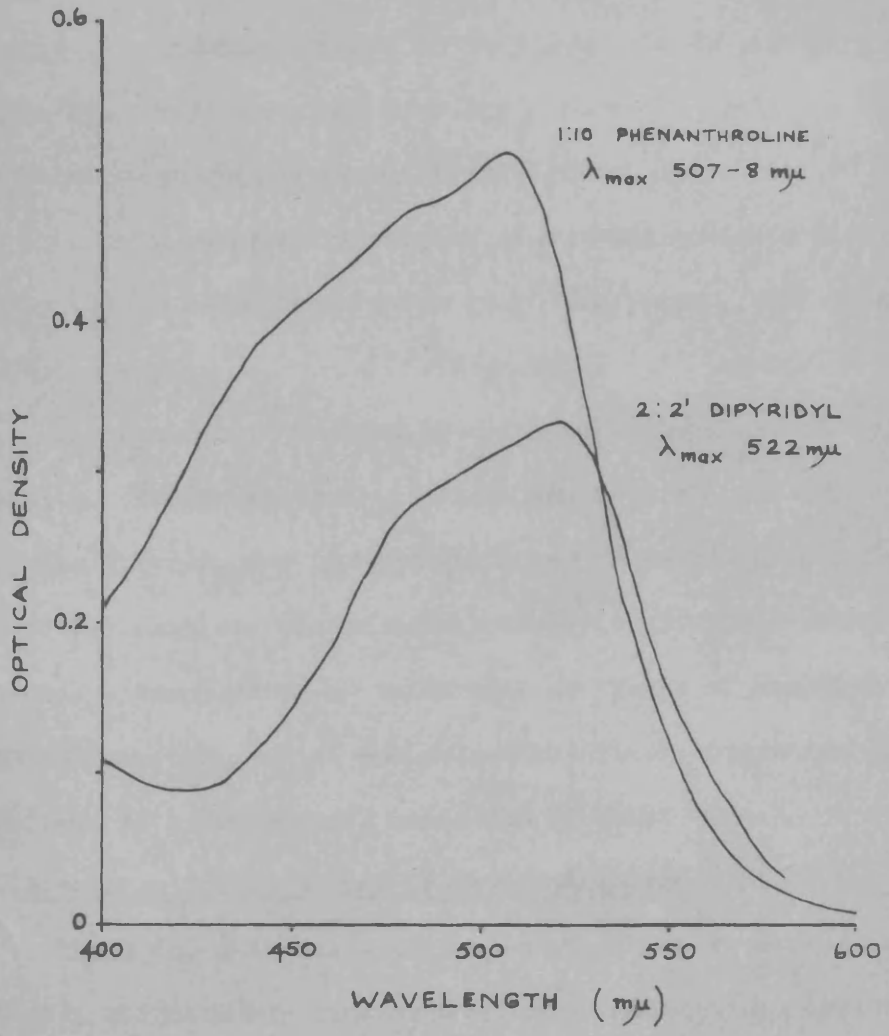


Figure 29. Absorption maximum of the ferrous complex of 1:10 phenanthroline and 2:2' dipyridyl.

for 15 minutes to attain full colour development. After cooling and making up to volume, the optical density of the solution was read at 522 mμ. (The absorption maximum of a solution of the ferrous-dipyridyl complex was found to be 522 mμ. on the Hilger uvispek (Fig. 29), confirming the findings of Sandell (1959, p. 525)). A calibration graph was drawn of the optical density of 20 ml. solutions of the ferrous-dipyridyl complex at varying concentrations of iron up to 100 μg. The calibration graph was linear. The tangent was 0.008.

The estimation of iron in urine using both methods gave the same results. More attention to detail was required for the o-phenanthroline method: firstly, the iron reagents had to be added to a warm solution, otherwise complete colour development did not occur within two hours; secondly, care had to be taken that no traces of ammonium hydroxide remained in the neck of the volumetric flask, otherwise on the addition of hydroquinone, oxidation to brown quinone occurred.

2. Precision of the method of iron estimation

Using the o-phenanthroline method, the iron content of six 100 ml. aliquots of urine from a single patient was estimated. The results were 11.3, 11.6, 10.9, 11.0, 10.9 and 10.9 μg. of iron per 100 ml. urine, mean 11.1, standard deviation $\pm$ 0.26. The standard error calculated from double determinations on 100 urine samples with iron concentrations between 1.5 and 27.0 μg. per 100 ml. of urine (mean 17.2 μg.) was 0.4 μg. per 100 ml.

Using the o-phenanthroline and dipyridyl methods, the recovery

of known quantities of iron added to aliquots of urine were determined. 92 - 112% of the iron added was recovered. (Table 15).

3. Spot test for iron

A rough quantitative test was developed to determine the presence of large quantities of iron in urine, ie. 0.1 to 2.0 mg. per 100 ml. To four ml. of urine in a test-tube, were added 0.5 ml. 2% w/v 2:2[1] dipyridyl in 60% v/v acetic acid and 0.5 ml. thioglycollic acid. After heating for a few minutes in a beaker of water at 100°C, the colour developed was compared with a series of standard iron solutions, of concentration 0.1 to 2.0 mg. per 100 ml. prepared in exactly the same manner. The colour of the ferrous-dipyridyl complex developed in the comparison standards is stable indefinitely.

Thioglycollic acid is a much stronger reducing agent than sodium sulphite, which does not reduce ferric iron under the conditions of the test. Although thioglycollic acid itself may react with iron in alkaline solution to give a red coloured complex, under the acidic conditions of the spot test it acts only as a reducing agent.

This test has only been used to facilitate the selection of an iron in urine concentration within the range of determination of the methods previously described. The spot test, performed on fifty urine samples with iron concentrations from 0.1 to 1.8 mg./100 ml., gave the following results; in 33,14 and 3 tests, the iron concentration lay within 10, 20 and 30% respectively of the true concentration.

4. Iron-free glassware

Glassware was freed from iron by immersing in 6N hydrochloric acid overnight and rinsing thoroughly with deionised water.

1:10 phenanthroline

Urine Aliquot	IRON (μg.) Present	Added	Found	Recovered
100 ml.	7.3	10	16.5	9.2
	7.3	10	18.3	11.0
	7.3	20	27.5	20.2
	7.3	20	27.8	20.5
100 ml.	34.1	10	44.7	10.6
	34.1	10	44.2	10.1
	34.1	20	54.9	20.8
	34.1	20	54.9	20.8
20 ml.	53.2	10	62.7	9.5
	53.2	10	63.0	9.8
	53.2	20	72.2	19.0
	53.2	20	73.0	19.8
2:2' dipyridyl				
25 ml.	41.8	10	51.8	10.0
	41.8	10	53.0	11.2
	41.8	20	61.1	19.3
	41.8	20	63.0	21.2

TABLE 15

Recovery of iron added to various urine aliquots, using the 1:10 phenanthroline and 2:2' dipyridyl methods.

Polythene winchesters, for urine collection, were freed from iron in the same manner.

5. Urinary haemoglobin, ferritin and haemosiderin

The urinary output of iron as haemoglobin, ferritin and haemosiderin was estimated in two patients with paroxysmal nocturnal haemoglobinuria. The urinary pH was adjusted to 7 with disodium hydrogen orthophosphate, and an equal volume of saturated (iron-free) ammonium sulphate added. After 2 hours at $0^{\circ}C$, the urine was centrifuged; an iron estimation on the supernate represented iron in haemoglobin. The precipitate was then extracted with water to dissolve ferritin; iron estimations on the supernate and precipitate measured iron in ferritin and iron in haemosiderin respectively (Drysdale and Ramsay, 1965).

6. Urinary protein

Urinary protein was estimated by the method of Lowry et al., (1951) using Folin and Ciocalteau reagent. To 1 ml. of protein solution was added 5 ml. of the following solution, prepared freshly each day (1 ml. 1% w/v copper sulphate + 1 ml. 2% w/v sodium tartrate + 100 ml 2% w/v sodium carbonate in 0.1N sodium hydroxide). After 10 minutes, 0.5 ml. of 50% v/v Folin and Ciocalteau reagent was added and mixed immediately. A blank sample containing 1 ml. water was treated in the same manner. Thirty minutes later, the optical density of the blue protein complex was read on the Hilger uvispek at wavelength 750 mμ. and slit width 0.05 mm.

Using standard solutions of crystalline bovine albumin, a calibration graph was drawn of optical density versus protein content

(Fig. 30). Urine aliquots were diluted 10- to 50-fold (to give a protein content of 20 mg.% or less) and two 1 ml. volumes taken for duplicate protein estimation. All samples were read against a reagent blank. Protein standards were included in each batch determination.

Electrophoresis of urinary proteins.

A short length of 22/32" visking tubing containing 25 - 35 ml. of urine was placed in a 6" x 1" test-tube containing polyethylene glycol, molecular weight 20,000. After concentration to 5 - 10 ml., the dialysis tube containing the urine sample was suspended in normal saline for a few hours to dialyse out phosphates and other salts. The urine concentrate was then ready for electrophoresis.

In patients with proteinuria, concentrated dialysed specimens of urine can be separated into four fractions - albumin and α_1 α_2 and β globulins - by electrophoresis in barbitone acetate buffer at pH 8.6 (ie. sodium barbitone 20 g., sodium acetate 13 g., 135.6 ml. 0.1N hydrochloric acid plus water to give a final volume of 2 litres). Six 34 cm. strips of Whatman No. 3 chromatography paper 5 cm. broad were immersed in buffer, blotted firmly and placed in position in the E.E.L. horizontal electrophoresis tank. Two small volumes of prepared urine (usually 0.05 and 0.10 ml.) were carefully applied in a thin band on a pencil line drawn 13 cm. from the end of each strip. The tank was connected to a Volkam power pack and a constant current of 2 ma. per strip passed for approximately 24 hours. The strips were removed, dried and placed in an air oven at 105°C for 15 minutes to denature the protein bands. The protein bands

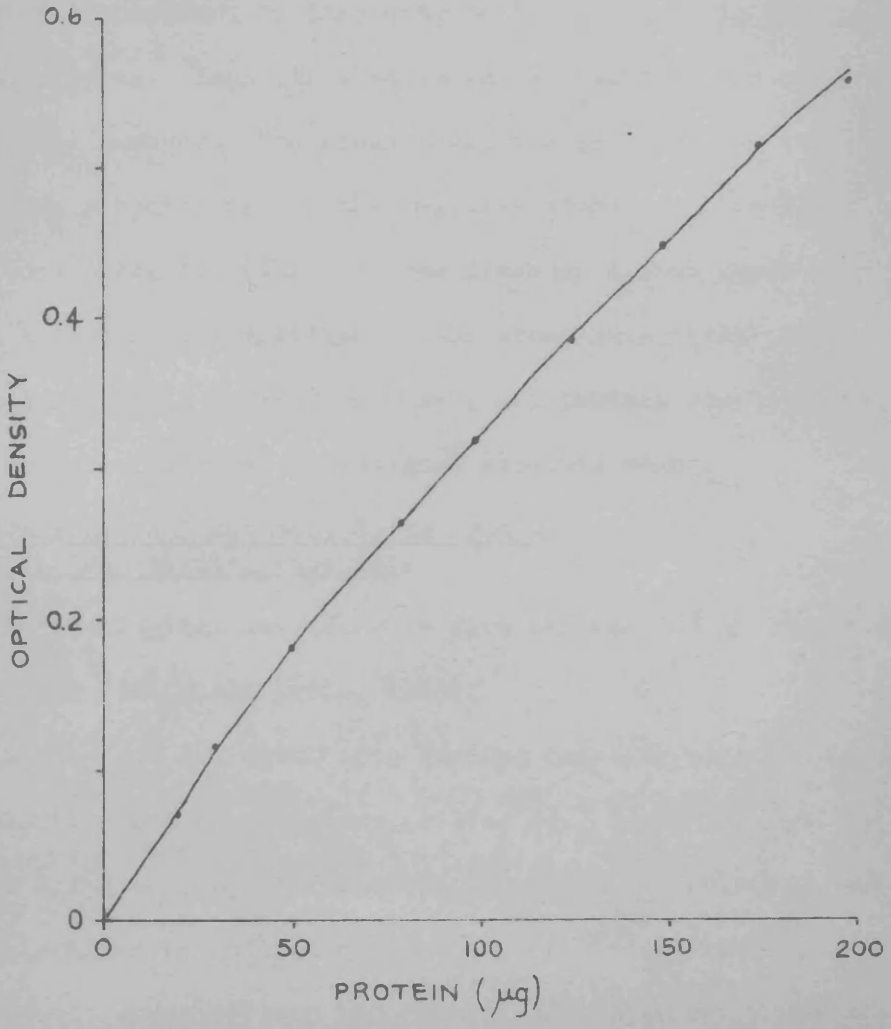


Figure 30. Calibration graph of crystalline bovine albumin versus optical density at 750 mμ on the Hilger uvispek.

were stained and fixed by immersing the strips for 5 minutes in a solution of 1% w/v bromophenol blue in 95% alcohol saturated with mercuric chloride. The background dye was washed away under running water until a white background was obtained. The strips were allowed to dry, placed in an oven at 100°C for 5 minutes and rendered transparent by immersing in liquid paraffin at 110°C for 30 minutes. Each strip was mounted between glass and placed in the E.E.L. Scanner. The areas under the peaks of the resulting scan were proportional to the relative amounts of the protein fractions present. (The scan was drawn on 1 inch graph paper to facilitate the determination of the areas under each peak). When the total protein content is known, all protein fractions separated by electrophoresis can be assigned absolute values.

7. Haematological methods. Serum iron and total iron binding capacity.

Haematological measurements were carried out by standard techniques (Dacie and Lewis, 1963).

Serum iron and total iron binding capacity were estimated by the modified method of Ramsay (1957a, b). Serum iron was determined by the sulphite-dipyridyl method. The total iron binding capacity was determined by saturation of the iron binding capcity with a calculated excess of iron and removal of the excess unbound iron with magnesium carbonate before estimating the iron content of the supernate as above.

8. The effect of Desferal on the urinary excretion of iron in normal subjects and in patients with sideropenia, iron deficiency anaemia and iron overload.

Clinical data

The haematologically normal subjects had haemoglobin levels of 14. 6 g./100 ml. or above, a normal percentage saturation of the total iron binding capacity and normal amounts of stainable iron in the bone marrow. The subjects described as sideropenic had haemoglobin values between 12 and 13 g./100 ml., a percentage saturation of the total iron binding capacity below 15% and an absence of stainable iron in bone marrow sections. The patients with iron deficiency anaemia had haemoglobin levels below 12.0 g./100 ml., an absence of stainable marrow iron, and a saturation of the total iron binding capacity below 10%. The iron-deficient subjects had a complete response in haemoglobin solely to iron therapy.

The two patients with paroxysmal nocturnal haemoglobinuria had a chronic haemolytic anaemia associated with haemoglobinuria and haemosiderinuria. Both had hypochromia of the red cells associated with an absence of stainable iron in the bone marrow.

One patient with idiopathic pulmonary haemosiderosis was studied. He was a young man of 22 years with recurrent severe iron deficiency anaemia. The diagnosis was confirmed by chest x-ray and lung biopsy which showed macrophages heavily laden with iron.

Desferal test

Twenty-four hour collections of urine were made in iron-free polythene containers to provide a basal value for urinary iron output. 600 mg. of Desferal was then injected intramuscularly and the urine

again collected during the following 24 hours. (This urine collection has been called that "during Desferal administration" in the Results section). A third 24 hour urine collection (called that "after Desferal administration") was made in most patients. The urinary iron excretion was determined in all instances.

Since a standard dose of desferrioxamine was given to all subjects, it was considered important in the sideropenic and iron deficiency anaemia patients to relate the subsequent urinary iron output to body weight. This is not necessary in patients with iron overload since there are large differences between these cases and normal subjects. The results in the iron deficient subjects were expressed as µg. urinary iron in 24 hour per kg. of lean body mass and also of whole body weight. A better separation of results was obtained between the two iron deficient groups and the normal group when lean body mass rather than whole body weight was used.

The estimate of lean body mass was obtained as follows: the red cell volume (RCV) was estimated from height and weight (Nadler, et al., 1962). The lean body mass (LBM) was then derived from the following equation:

$$LBM = \frac{RCV - 155.17}{32.74}$$ (Muldowney, 1957)

Results

Desferal test in normal subjects and patients with iron deficiency.

In normal subjects, the basal urinary excretion of iron was about 100 µg. per day or less in both male and female subjects.

The iron content of the urine collected during the administration of a 600 mg. dose of Desferal reached a higher level in male subjects (range 473 - 1,233 µg./day, mean 838) than in female subjects (range 415 - 822 µg./day, mean 631). On the day after Desferal administration the urinary iron excretion was higher than the basal value, but much lower than that during Desferal administration (Table 16).

In the sideropenic subjects, the basal urinary excretion of iron lay below 100 µg. per day. The urinary iron output during a single 600 mg. dose of Desferal in both male and female subjects (range 365 - 478 µg./day, mean 424 and range 351 - 814 µg./day, mean 454 respectively) tended to be lower than in normal subjects (Table 17).

In subjects with iron deficiency anaemia, the basal urinary excretion of iron was about 100 µg. per day or less in both male and female subjects. The urinary iron output during Desferal administration tended to be similar or slightly lower than in subjects with sideropenia, ranging from 70 to 376 µg. per day (mean 238) in male subjects and from 85 to 475 µg. per day (mean 320) in female subjects (Table 18).

For reasons given earlier, it was considered important in these three groups - normal, sideropenic and iron deficiency anaemic - to relate the urinary iron excretion, during the administration of a standard dose of Desferal to lean body mass.

The urinary iron excretion of normal, sideropenic and iron

		Urinary iron µg./24 hrs.		
	No.	Before Desferal	During Desferal	After Desferal
Normal Males	1	43.7	1233	306
	2	41.6	852	158
	3	38.7	594	155
	4	63.0	593	199
	5	84.0	473	-
	6	86.7	867	-
	7	91.8	1144	-
	8	55.5	945	-
Normal Females	1	104.0	584	146
	2	79.2	415	-
	3	66.3	654	136
	4	96.0	459	-
	5	56.4	720	-
	6	54.0	730	164
	7	67.3	645	-
	8	62.7	663	-
	9	75.1	617	-
	10	44.2	822	-

TABLE 16

Desferal test in normal subjects:urinary excretion of iron before, during and after one 600 mg. dose of desferrioxamine B mesylate.

		Urinary iron µg./24 hrs.		
	No.	Before Desferal	During Desferal	After Desferal
Iron Deficiency Anaemia Males	1	94.0	327	-
	2	74.0	69.4	-
	3	52.5	111	-
	4	83.5	308	153
	5	68.7	376	-
Iron Deficiency Anaemia Females	1	92.9	311	115
	2	28.3	275	130
	3	13.2	354	191
	4	97.0	581	209
	5	64.2	370	97.6
	6	92.0	318	188
	7	-	255	122
	8	93.1	284	75.5
	9	73.5	197	65.8
	10	56.0	361	86.7
	11	31.8	85.0	67.0
	12	42.7	118	-
	13	94.0	320	-
	14	109.0	437	-
	15	38.0	254	-
	16	38.5	357	106
	17	89.2	276	108
	18	55.9	387	74.4
	19	48.5	475	475
	20	89.6	326	-
	21	40.6	244	-
	22	40.2	379	119
	23	48.0	391	-

TABLE 18

Desferal test in patients with iron deficiency anaemia; urinary excretion of iron before, during and after one 600 mg. dose of desferrioxamine B mesylate.

deficiency anaemic subjects during the administration of 600 mg. of Desferal was expressed as μg. iron in 24 hours/kg. lean body mass (Tables 19, 20 and 21 respectively) and are shown in Fig. 31. The mean value of urinary iron, expressed as μg. in 24 hours/kg. lean body mass, and the standard deviation for each group is given in Table 22.

In both male and female subjects, the urinary iron excretion (expressed as μg. iron in 24 hours/kg. lean body mass) of the sideropenic group lay between those of the normal and the iron deficient group. In male subjects, there was no overlap in the urinary excretion of iron (expressed as μg. iron in 24 hours/kg. lean body mass) between any two of the three groups under study. In female subjects, there was an overlap in the urinary iron output (expressed as μg. iron in 24 hours/kg. lean body mass) in all three groups (Fig. 31).

Using the Student's t test, the mean urinary iron excretion (expressed as μg. iron in 24 hours/kg. lean body mass) between any two of these groups, both male and female, were found to be significantly different from each other ($p < 0.01$) (Table 23).

The Desferal test was also carried out in one patient with sideroblastic anaemia and in two with cutaneous hepatic porphyria (Table 24).

In all three cases, the basal urinary excretion of iron was less than 100 μg./day. The female patient with sideroblastic anaemia gave a response to Desferal above the normal range shown in Table 16, proving that previous iron therapy to raise the haemoglobin level,

	No.	Height (inches)	Weight (kg)	Lean Body Mass (L.B.M.)	Iron µg./24 hrs.	µg. iron in 24 hours	
						kg.	L.B.M.
Normal Males	1	66	72	51.3	1233		24.0
	2	61	56.6	40.0	852		21.3
	3	65	64.0	46.9	594		13.2
	4	64	63.0	45.9	593		12.9
	5	64	52.2	41.2	473		11.5
	6	68	77.3	57.9	867		15.0
	7	70	70.0	56.2	1144		20.3
	8	65	58.6	43.8	945		21.6
Normal Females	1	64	57.0	38.4	584		15.2
	2	61	45.5	31.4	415		13.2
	3	62	50.0	33.6	654		19.5
	4	56	53.6	30.2	459		15.2
	5	58	49.6	30.8	720		23.4
	6	62	59.4	37.6	730		19.4
	7	67	65.7	44.3	645		14.6
	8	62	54.3	35.7	663		18.6
	9	60	73.3	41.4	817		20.1
	10	60	50.0	32.4	822		25.3

TABLE 19

Urinary iron excretion, expressed as µg. in 24 hours / kg. lean body mass, in normal subjects given 600 mg. of desferrioxamine B mesylate.

	No.	Height (inches)	Weight (kg)	Lean Body Mass (L.B.M.)	Iron µg./24 hrs.	µg. iron in 24 hrs. kg.	µg. iron in 24 hrs. L.B.M.
Sideropenic Males	1	66	68.6	49.6	459		9.3
	2	65	67.0	48.7	365		7.5
	3	66	81.8	54.8	478		8.7
	4	64	60.0	44.3	446		10.1
	5	70	48.0	45.1	370		8.2
Sideropenic Females	1	64	47.0	33.8	368		10.8
	2	62	57.8	37.6	392		10.4
	3	64	66.0	42.0	814		19.4
	4	58	67.5	38.1	403		10.6
	5	64	53.6	37.5	534		14.2
	6	62	55.9	36.0	556		15.4
	7	60	55.0	34.4	366		10.7
	8	60	50.9	33.9	351		10.4
	9	64	60.0	39.3	397		10.1
	10	62	71.0	42.0	360		8.6

TABLE 20

Urinary iron excretion, expressed as µg. in 24 hours / kg. lean body mass, in patients with sideropenia given 600 mg. of desferrioxamine B mesylate.

	No.	Height (inches)	Weight (kg.)	Lean Body Mass (L.B.M.)	Iron µg./24 hrs.	µg. iron in 24 hrs. Kg.	µg. iron in 24 hrs. L.B.M.
Iron Deficiency Anaemia Males	1.	68	65.5	50.7	327		6.4
	2	66	53.6	40.6	69.4		1.7
	3	64	46.4	38.9	111		2.9
	4	67	68.2	50.6	308		6.1
	5	74	67.0	58.0	376		6.5
Iron Deficiency Anaemia Females	1	62	47.0	32.5	311		9.6
	2	57	48.0	29.9	275		9.2
	3	64	58.5	39.2	354		9.0
	4	61	60.0	36.8	581		15.7
	5	64	42.0	32.0	370		11.5
	6	64	66.4	41.9	318		7.6
	7	62	58.7	37.6	255		6.8
	8	66	54.0	41.0	284		6.9
	9	62	54.0	35.8	197		5.5
	10	60	63.0	37.8	361		9.6
	11	62	73.2	41.4	85		2.1
	12	67	80.0	50.1	118		2.4
	13	61	44.0	35.6	320		8.9
	14	62	59.8	42.2	437		10.4
	15	63	56.8	42.5	254		5.9
	16	62	57.0	41.6	357		8.6
	17	64	66.0	46.8	276		5.9
	18	62	45.5	32.1	387		12.0
	19	61	57.3	35.9	475		13.2
	20	62	64.0	39.3	326		8.3
	21	62	40.0	30.4	244		8.1
	22	60	45.0	30.3	379		12.2
	23	60	74.0	41.4	391		9.4

TABLE 21

Urinary iron excretion, expressed as µg. in 24 hours / kg. lean body mass, in patients with iron deficiency anaemia given 600 mg. of desferrioxamine B mesylate.

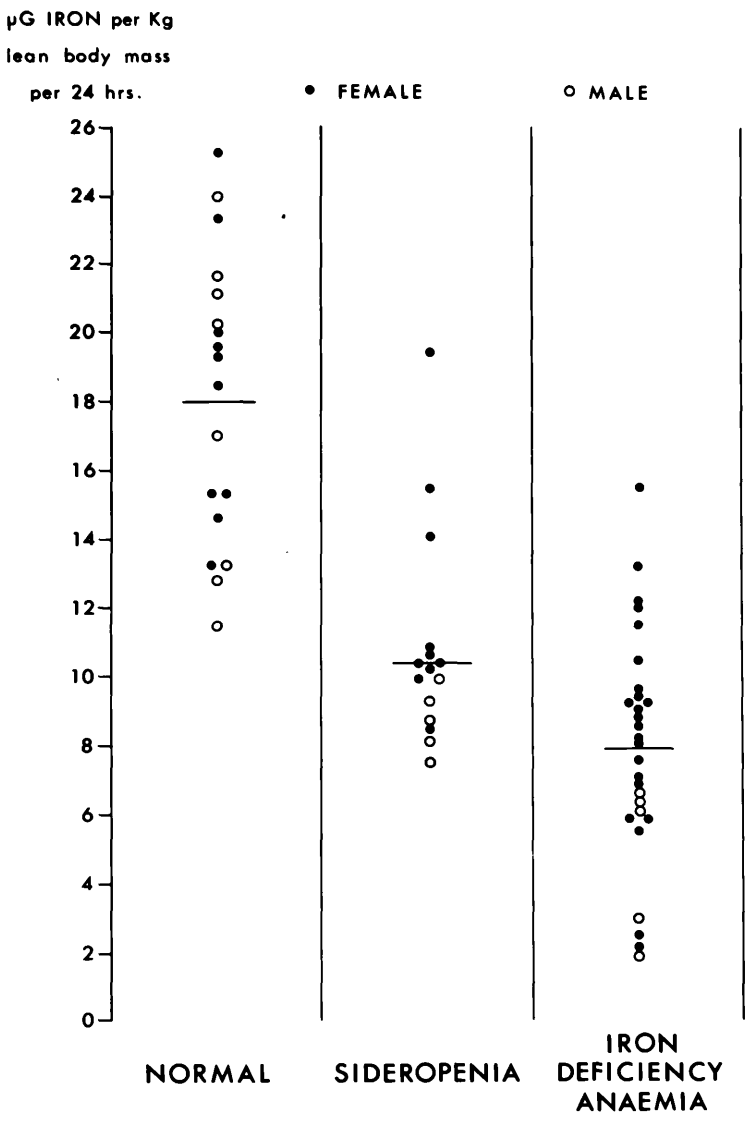


Figure 31. Desferal Test in normal subjects (a), and in patients with sideropenia (b) and iron deficiency anaemia (c).

	M A L E S			F E M A L E S		
	Normal	Sideropenic	Iron Deficiency Anaemic	Normal	Sideropenic	Iron Deficiency Anaemic
No. of cases	8	5	5	10	10	23
Mean excretion	17.5	8.8	4.7	18.4	12.1	8.6
Standard deviation	±4.8	±.3	±2.2	±3.9	±3.3	±3.2

TABLE 22

Mean urinary excretion of iron and standard deviation, expressed as μg. iron in 24 hours / kg. lean body mass, in normal, sideropenic and iron deficiency anaemic males and females.

	Groups	t	p
Male	Normal and Sideropenia	3.91	<0.01
	Sideropenia and iron deficiency anaemia	3.67	<0.01
	Normal and iron deficiency anaemia	5.48	<0.001
Female	Normal and Sideropenia	3.94	<0.001
	Sideropenia and iron deficiency anaemia	2.83	<0.01
	Normal and iron deficiency anaemia	7.60	<0.001

TABLE 23

The significance of a difference in the mean urinary iron excretion (expressed as μg. iron in 24 hours / kg. lean body mass) between any two of the following groups - normal, sideropenic and iron deficiency anaemic - in both male and female subjects was calculated using the Student's t test.

	Urinary iron µg./24 hours	
	Before Desferal	During Desferal
Sideroblastic anaemia	73.5	1061
Cutaneous hepatic porphyria	66.2	1223
" " "	75.1	532

TABLE 24

Desferal test: urinary excretion of iron before and during one 600 mg. dose of desferrioxamine B mesylate.

had served only to increase the storage iron above normal. (In sideroblastic anaemia, due to a defect in protein metabolism, the synthesis of haem synthetase is depressed and this limits the production of haemoglobin). In cutaneous hepatic porphyria, the urinary response to Desferal of 1223 and 532 μg. of iron per day fell within the normal range found for male and female subjects respectively.

Desferal test in states of iron overload

The results of the Desferal test in patients with iron overload are given in Table 25. Except for two patients with paroxysmal nocturnal haemoglobinuria, the basal urinary iron excretion ranged from 30 to 146 μg. per day. The mean basal urinary iron excretion in normal subjects and in patients with sideropenia, iron deficiency anaemia and iron overload are shown in Table 26.

a) Primary idiopathic haemochromatosis

Cases 1, 2 and 3 were untreated. Case 4 was partially treated. Case 5 had received repeated venesections totalling 50 pints of blood, over a period of two years. In cases 1 - 4, the urinary iron excretion rose from very low basal levels to over 3,000 μg. per 24 hours during Desferal administration; in the fifth (treated) case, the urinary iron output during Desferal administration was normal (Table 25).

b) Focal iron overload

i) Paroxysmal nocturnal haemoglobinuria

In this condition, there is heavy deposition of iron in the kidney (renal haemosiderosis) (Crosby, 1953). Both patients

	Urinary iron excretion µg./24hrs.	
	Basal	During 600 mg. Desferal
Normal (mean of eighteen subjects)	70 ± 19	719 ± 233
Primary idiopathic haemochromatosis (untreated), Case 1.	84	4,800
Primary idiopathic haemochromatosis (untreated), Case 2.	146	8,800
Primary idiopathic haemochromatosis (untreated), Case 3.	88	10,600
Primary idiopathic haemochromatosis (partially treated), Case 4.	78	3,600
Primary idiopathic haemochromatosis (treated), Case 5.	30	631
Paroxysmal nocturnal haemoglobinuria, Case 1.	4,000	9,000
Paroxysmal nocturnal haemoglobinuria, Case 2.	16,100	22,800
Idiopathic pulmonary haemosiderosis	75	1,600

TABLE 25

Desferal test in states of iron overload.

No.	Subjects	Mean urinary iron output (μg 24 hr)	Standard deviation
9	Normal males	69	±19·2
13	Normal females	74	±22·6
6	Sideropenic males	62	±7·6
12	Sideropenic females	72	±23·3
6	Iron deficiency anaemia – males	76	±15·7
25	Iron deficiency anaemia – females	67	±33·2
5	Idiopathic haemochromatosis	85	±37
1	Sideroblastic anaemia	74	-
2	Cutaneous hepatic porphyria	71	±4
1	Idiopathic pulmonary haemosiderosis	75	-

TABLE 26

Mean urinary iron excretion in normal subjects and in patients with sideropenia, iron deficiency anaemia and iron overload.

were excreting large quantities of iron without any provocative test (Table 25). After the administration of Desferal, there was a considerable increase in the daily urinary iron output, from 4,000 to 9,000 ug. and from 16,100 to 22,800 ug. respectively.

Haemoglobin, ferritin and haemosiderin iron was estimated in the urine of both these patients (Table 27). (Case 1 had been given Desferal; Case 2 was given no Desferal). In both cases, the major part of the iron was excreted as haemosiderin. The presence of large amounts of urinary haemosiderin was confirmed by staining the urinary deposit with acidified potassium ferricyanide (Prussian blue reaction).

	Urinary iron (ug./24 hr.)	
	Case 1	Case 2
Iron in haemoglobin	2,100	600
Iron in haemosiderin	5,400	10,000
Iron in ferritin	4,300	5,500
Total iron	8,800	16,100

TABLE 27

Urinary iron excretion in two patients with paroxysmal nocturnal haemoglobinuria.

ii) Idiopathic pulmonary haemosiderosis

The Desferal test (Table 25) showed a rise in the urinary iron excretion from its insignificant basal level to 1,600 ug./24 hours.

Long-term Desferal therapy. In idiopathic pulmonary haemosiderosis, the accumulation of iron in the lungs may lead to massive fibrosis. For this reason, the patient was given intermittent Desferal treatment over a period of 256 days. (Figs. 32 a and b). Initially the patient was given Desferal therapy in hospital over a 60 day period, with a 10 day rest after 31 days. A dose of 600 mg.

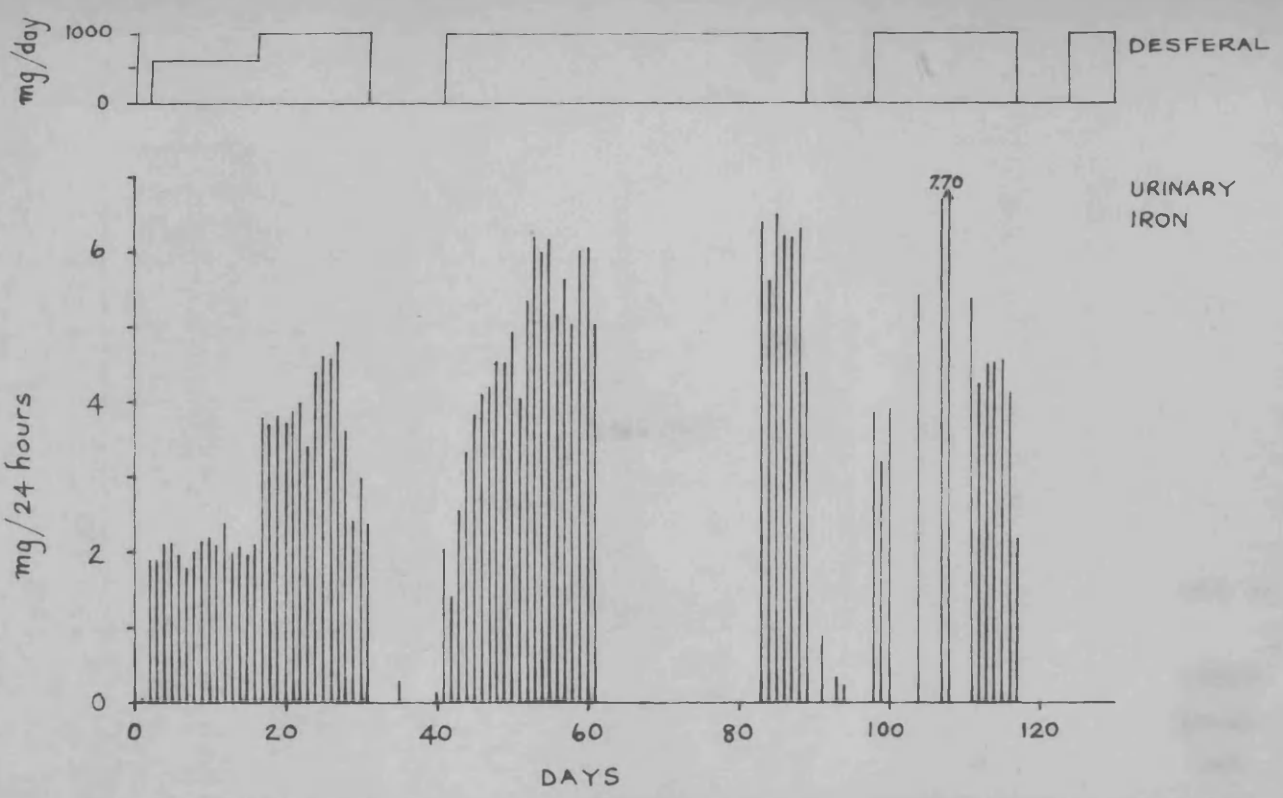


Figure 32a. Urinary excretion of iron in a patient with idiopathic pulmonary haemosiderosis. Doses of 600 mg. or 1000 mg. of Desferal daily were given intermittently as indicated.

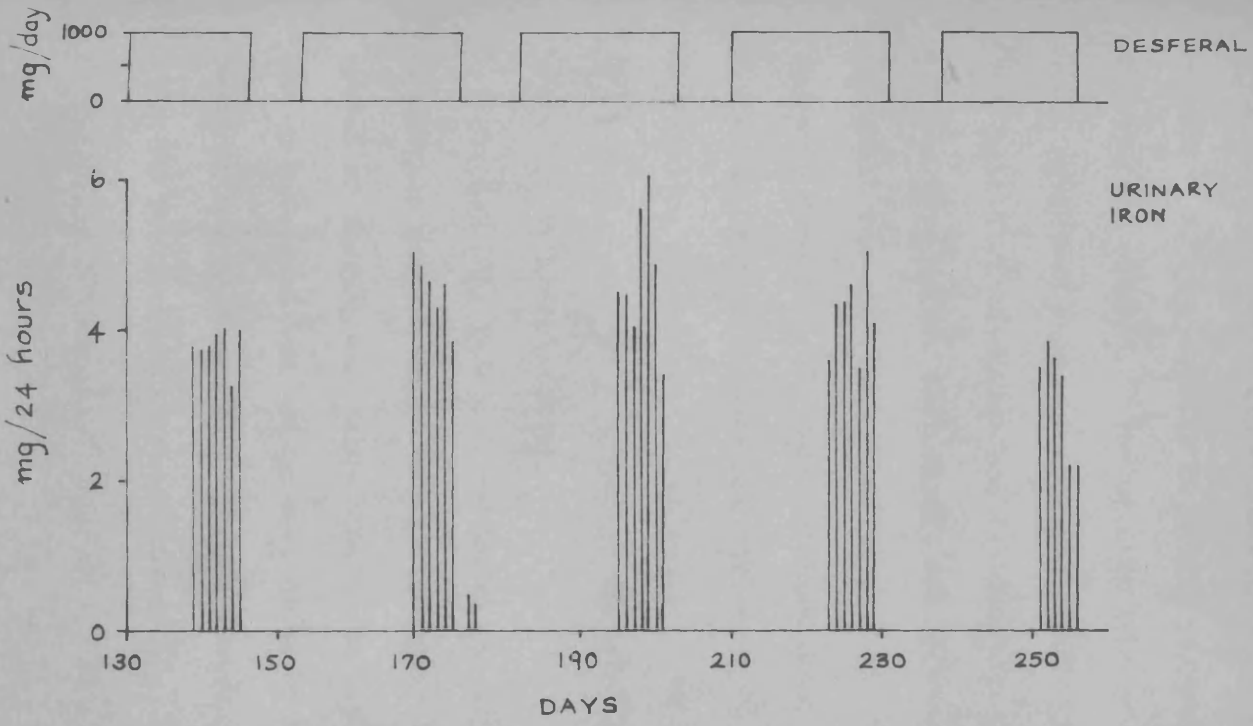


Figure 32b. Urinary excretion of iron in a patient with idiopathic pulmonary haemosiderosis. Daily doses of 1000 mg. of Desferal were given intermittently as indicated.

of Desferal produced a significant sustained increase in the urinary iron excretion. When the dose of Desferal was increased to 1,000 mg. per day (given as 500 mg. twice daily), the urinary excretion of iron doubled in value. After a 10 day rest, Desferal therapy was resumed. The urinary iron excretion steadily increased until more than 6 mg. of iron was being eliminated. Following this period of treatment and observation, during which no ill effects were noted, the patient was allowed home. Desferal treatment was continued, administered by district nurse, and it appeared from Fig. 32a that the high level of iron excretion (6mg. daily) was sustained over the following 30 days. From day 90 to day 256 the patient was given Desferal daily for three weeks in every four. He was admitted to hospital for one of these three weeks for urine collection.

On day 256, Desferal treatment was terminated due to a mild recurrence of iron deficiency. The total iron removed during the period of therapy was calculated to be 0.86 g. Where the patient was on treatment but not on collection of urine, the calculation was based on the iron output of previous seven days. No figure was included for periods of non-treatment.

9. Urinary excretion of protein and iron in patients with proteinuria.

Clinical cases

Ten of the twelve patients with proteinuria were suffering from the nephrotic syndrome. In Case 12, the disease was in partial remission (Table 28).

Case	Age (yr)	Diagnosis	Urinary iron (μg 24 hr)	Urinary protein (g 24 hr)	Urinary albumin (g 24 hr)	Urinary β-globulin (g 24 hr)
1	19	Nephrotic syndrome (renal amyloidosis)	596	4·1	3·2	0·38
2	22	Nephrotic syndrome	267	10·2	9·3	0·9
3	15	Nephrotic syndrome	272	10·2	9·41	0·79
			301	9·5	8·64	0·87
4	10	Nephrotic syndrome	143	3·02	2·44	0·58
5	19	Nephrotic syndrome	994	10·0	7·98	1·26
6	18	Nephrotic syndrome	480	8·6	8·04	0·56
7	40	Nephrotic syndrome	400	9·56	8·57	0·99
8	42	Nephrotic syndrome	188	5·7	5·20	0·50
9	42	Nephrotic syndrome	86·9	6·85	6·50	0·35
10	51	Chronic glomerulonephritis	78·3	5·18	5·18	0
11	68	Dysproteinaemia	68·6	1·4	1·4	0
12	9	Nephrotic syndrome (treated)	77·6	2·75	2·75	0

TABLE 28

The urinary excretion of iron and protein in twelve patients with proteinuria.

Plan of study

24 hour collections of urine were made in iron-free containers. The total (mixed) protein was determined by the method of Lowry et al., (1951). Horizontal paper strip electrophoresis of dialysed concentrated samples of urine were carried out to determine the relative amounts of albumin and α_1, α_2, and β globulins present. Iron was estimated by the method described earlier except that initially, during concentration of urine, additional 11N nitric acid was required to convert heat coagulated proteins to more soluble substances.

Results

Fig. 33 shows the separation of urinary proteins in Case 5 by paper strip electrophoresis. Albumin is the fastest running component and is followed by α_1, α_2, and β globulins respectively. Albumin was the major protein fraction present as can be seen from the intensity of the band and from the area under the peak of the scan. α_1 and α_2 globulins were present although it was not possible to estimate either separately. Only one other patient had α_1 and α_2 globulins present in urine (Case 1). Since the β globulin fraction was not completely separated from the $\alpha_1 \alpha_2$ globulins, the lowest point on the scan between the two fractions was taken as the dividing line. The ratio albumin: $\alpha_1 \alpha_2$ globulin: β globulin was found to be 202:19:32 by determination of the area under the corresponding peaks of the scan. Since the sum of the three areas is equal to the total protein, 10.0 g. / 24 hour urine, the absolute

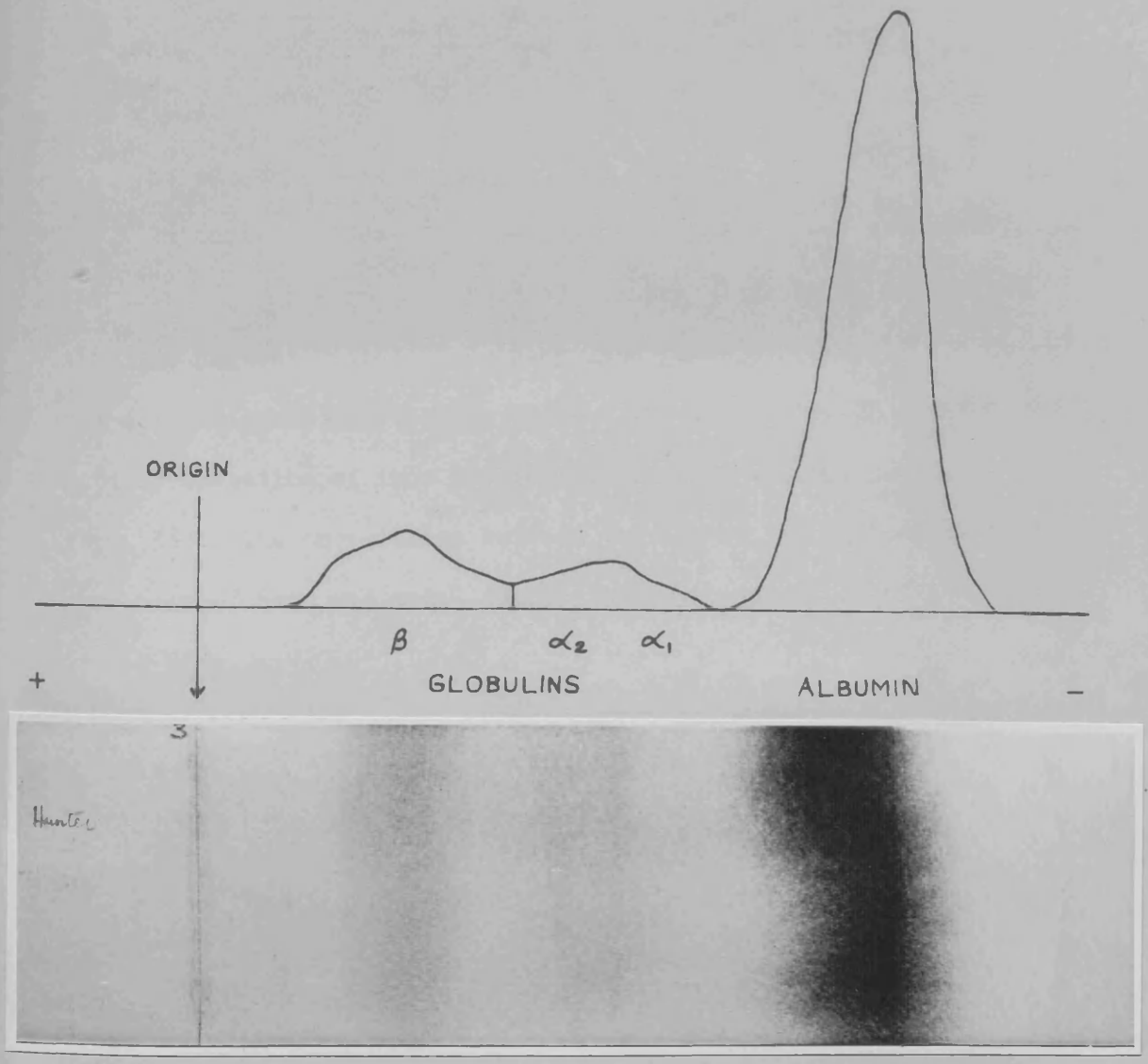


Figure 33. Separation of urinary proteins in Case 5 by paper strip electrophoresis. The photograph of the stained protein fractions and the corresponding scan, drawn using the E.E.L. Scanner, are shown.

value of each protein component can be calculated.

The iron and protein content of urine in Cases 1 - 12 are listed in Table 28. The absolute quantities of albumin and β globulin, calculated from the relative quantities found, are also given.

The urinary excretion of iron in Cases 9 - 12 lay below 100 µg./day. In three of these four cases, no β globulin was detected in the urine. The highest output of urinary β globulin co-incided with the highest output of urinary iron (Case 5) (Table 28). There was a significant correlation between the log of the 24 hour urinary excretion of iron and β globulin ($r = + 0.80$, $p < 0.01$) (Fig. 34). The correlation between the log of the 24 hour urinary excretion of iron and total urinary protein was not significant ($r = + 0.64$, $p < 0.05$).

10. The effect of a phenylhydrazine-induced haemolysis on the urinary excretion of iron during Desferal administration.

Clinical data

Cases 1 - 10 all had forms of iron overload. Seven of the patients had primary idiopathic haemochromatosis, six untreated and one (Case 7) treated. (The latter patient was included in the previous study as Case 5). The remaining three patients had cutaneous hepatic porphyria with haemosiderosis, sideroblastic anaemia and idiopathic pulmonary haemosiderosis respectively (Table 29). (The last patient was included in the previous study).

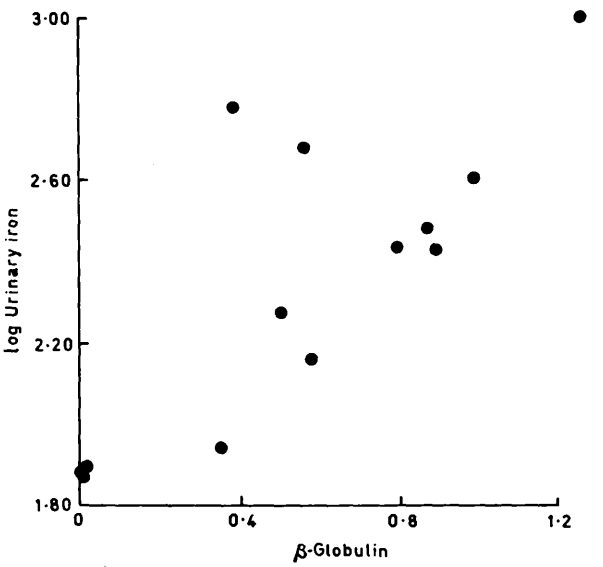


Figure 34. Correlation of iron and β globulin in the urine of twelve patients with proteinuria. Correlation coefficient $r = +0.80$, $p < 0.01$.

Case No.	Diagnosis	Urinary iron µg./24 hrs. A	B
1	Primary idiopathic haemochromatosis (PIH)	41	13,650
2	P.I.H.	124	10,525
3	Cutaneous hepatic porphyria with haemosiderosis	193	12,078
4	P.I.H.	40	2,002
5	P.I.H.	452	16,038
6	Idiopathic pulmonary haemosiderosis	75	3,439
7	P.I.H. (treated)	28	570
8	Sideroblastic anaemia	164	3,773
9	P.I.H.	298	8,785
10	P.I.H.	647	20,728

A (before) and B (during) administration of 1,000 mg. Desferal.

TABLE 29

Desferal test in ten patients with iron storage disease.

Phenylhydrazine was used as the haemolytic agent in a dose of 100 mg. per day in Cases 1 - 8. This dose was used initially since it has been extensively employed in the past without any untoward effect for the treatment of patients with polycythaemia vera (Wintrobe, 1961). In Cases 9 and 10, phenylhydrazine was given in a daily dose of 75 mg. A second course of phenylhydrazine treatment of 50 mg./day was administered to Cases 2 and 5.

Phenylhydrazine was given orally in 25 mg. tablets, two, three and four times per day dependent on dose. In all cases, Desferal was given in twice daily intramuscular injections of 500 mg. each.

Plan of study

In each case, 24 hour specimens of urine, for iron estimation, were collected before and during the period of treatment. Haemoglobin, reticulocyte count and serum bilirubin were measured before and after treatment as detailed below.

Cases 1 - 8 were given 1,000 mg. of Desferal daily for two days, 100 mg. of phenylhydrazine daily for two days and combined therapy of 1,000 mg. of Desferal and 100 mg. of phenylhydrazine daily for three days. Using the same plan of study, a further investigation using a smaller dose of phenylhydrazine (50 mg./day) was carried out in Case 2 and Case 5. In Cases 9 and 10, two days of Desferal therapy (1,000 mg./day) were followed by combined Desferal (1,000 mg./day) and phenylhydrazine (75 mg./day) therapy

for six days.

Results

The urinary iron excretion before and during Desferal administration in Cases 1 - 10 is given in Table 29. In the previous topic, the maximum basal urinary iron output in 6 patients with states of iron overload was 146 µg./day. From Table 29, only five of the ten patients have values below 146 µg. iron per day. It is apparent that until information is available from a greater number of patients, no generalisation with regards to the basal urinary iron output in states of overload should be made. However, it is of interest to note that the two patients with the highest basal urinary iron excretion, 452 and 647 µg. per day, recorded the highest urinary iron output during Desferal treatment, 16,038 and 20,728 µg. per day respectively. Compared to the latter two figures, the basal excretion of iron in both cases are insignificant.

100 mg. dose of phenylhydrazine

The effect on the urinary iron excretion of a 100 mg. dose of phenylhydrazine administered during Desferal treatment in Cases 1 - 8 is shown in Table 30. In all cases, after two days of Desferal treatment, the administration of phenylhydrazine alone for two days did not maintain the high urinary iron output. It dropped markedly on the first and again on the second day of phenylhydrazine treatment (see Table 30). When Desferal was given in combination with phenylhydrazine (100 mg./day) the urinary iron output in all cases on the first day of treatment exceeded the urinary iron output on the first day of Desferal (only) treatment. The mean urinary iron

	24 hour urinary iron excretion (μg.) in patients on:						
Case No.	Control Day	Desferal (1000 mg./day)		Phenylhydrazine (100 mg./day)		Desferal (1000mg./day) and Phenylhydrazine (100 mg./day)	
1	41	13,650	7,269	1,648	686	20,219	21,986	20,300
2	124	10,525	11,430	2,009	921	12,436	15,390	19,957
3	193	12,078	11,031	1,894	769	16,677	19,845	24,090
4	40	2,002	2,191	604	251	4,579	6,205	5,445
5	452	16,038	10,989	840	1,026	26,065	30,340	27,800
6	75	3,439	4,625	-	-	5,429	3,830	3,899
7	28	570	631	158	84	668	681	672
8	164	3,773	5,932	1,583	491	-	4,549	5,456

TABLE 30

24-hour urinary iron excretion in Cases 1 - 8 during treatment with desferrioxamine B mesylate alone and in combination with phenylhydrazine (100 mg./day).

excretion during Desferal treatment and the mean during the administration of both Desferal and phenylhydrazine for Cases 1 - 8 are shown in Table 31.

In patients with untreated primary idiopathic haemochromatosis, the results in Table 31 show that when phenylhydrazine is given during Desferal therapy, a further 42 - 178% increase in the urinary iron excretion is recorded. In the three patients with treated primary idiopathic haemochromatosis, idiopathic pulmonary haemosiderosis and sideroblastic anaemia, there was no significant increase in the urinary iron output when phenylhydrazine was given during Desferal therapy (Table 31).

In all patients given 100 mg. of phenylhydrazine daily, the haemoglobin level did not fall below 12.5 g.% except in the patient with sideroblastic anaemia whose haemoglobin remained at 11 g.% even after phenylhydrazine treatment.

75 mg. dose of phenylhydrazine

The urinary iron output in Cases 9 and 10 on treatment with Desferal alone and in combination with phenylhydrazine (75 mg./day) are shown in Table 32. In Case 10, the urinary iron excretion increased steadily from 18 mg. on the first day to 38 mg. on the sixth day of combined therapy. In both cases, there was a further significant increase (mean 44 and 61%) in the urinary iron output when phenylhydrazine was given during Desferal therapy.

50 mg. dose of phenylhydrazine

In Cases 2 and 5, the effect, on the urinary excretion of iron, of a 50 mg. dose of phenylhydrazine, alone and in combination with

Case No.	Diagnosis	24 hour urinary iron excretion (μg.) in patients on:- Control Day	Desferal* (mean of 2 days)	Phenylhydrazine + (mean of 2 days)	Desferal* and phenylhydrazine+ (mean of 3 days)	% increase on combined therapy compared with Desferal alone.
1	P.I.H.	41	10,459	1,167	20,853	99
2	P.I.H.	124	10,977	1,465	15,627	42
3	Cutaneous hepatic porphyria	193	11,554	1,331	20,204	75
4	P.I.H.	40	2,096	477	5,825	178
5	P.I.H.	452	13,513	933	28,055	108
6	Idiopathic Pulmonary haemosiderosis	75	3,852	-	4,385	14
7	P.I.H. (treated)	28	600	121	673	12
8	Sideroblastic anaemia	164	4,852	1,037	5,003	3

* 1,000 mg./day

+ 100 mg./day

TABLE 31

Mean 24-hour urinary iron excretion in Cases 1 - 8 during treatment with desferrioxamine B mesylate alone and in combination with phenylhydrazine (100 mg./day).

Treatment	Day	Urinary iron µg./ 24 hrs. Case 9	Case 10
-	1	298	647
Desferal (1,000 mg./day)	2	8,785	20,728
	3	18,219	17,000
Combined therapy of Desferal (1,000 mg./day) and phenylhydrazine (75 mg./day)	4	16,700	18,400
	5	21,113	21,960
	6	21,512	24,754
	7	25,410	27,666
	8	24,480	31,824
	9	23,484	37,920

Case No.	24 hour urinary iron excretion (µg.) on:- Control Day	Desferal+ (mean of 2 days)	Desferal and phenylhydrazine* (mean of 6 days)	% increase on combined therapy compared with Desferal alone
9	298	13,502	21,685	61
10	647	18,864	27,071	44

+ 1,000 mg./day

* 75 mg./day

TABLE 32

24-hour urinary iron excretion in Cases 9 and 10 during treatment with desferrioxamine B mesylate alone and in combination with phenylhydrazine (75 mg./day).

Desferal, is shown in Table 33. In Case 2, during combined Desferal and phenylhydrazine therapy, the urinary iron excretion rose steadily over the four days of treatment, the mean daily excretion being 14,874 µg. compared to a mean, during Desferal administration alone, of 10,319 µg. ie. a 44% increase on combined therapy. In Case 5, the rise in the urinary iron output on combined therapy was not sustained over the four day treatment period. There was only a 6% increase in the urinary iron excretion over that found during Desferal administration alone.

In Case 2, 50 mg. and 100 mg. of phenylhydrazine given during Desferal treatment resulted in further increase of 44% and 42% respectively in the urinary iron output. On the other hand, in Case 5, 50 mg. and 100 mg. of phenylhydrazine given during Desferal treatment resulted in further increases of 6% and 108% in the urinary iron output (Tables 31 and 33).

The initial reticulocyte count in all patients was less than 2%. After phenylhydrazine treatment it was raised in four cases to 2 - 4%. The serum bilirubin was increased in all but one patient but in no instance was the value greater than 3.5 mg./100 ml. (Normal range 0.05 - 0.24 mg./100 ml., mean 0.1. Documenta Geigy, 1962).

Section C.III DISCUSSION

The present studies of the basal urinary iron output confirm those of Cartwright et al., (1954) and contradict those where higher values have been obtained (Tepe, 1953). Such high values may be accounted for by external contamination with iron during collection

24 hour urinary iron excretion (µg.) in patients on:-

Case No.	Control Day	Desferal (1,000 mg./day)			Phenylhydrazine (50 mg./day)		Desferal (100 mg./day) and phenylhydrazine (50 mg./day).			
2	124	11,746	7,679	11,532	2,243	467	11,640	15,984	15,360	16,515
5	452	14,283	14,283	-	-	-	19,390	14,940	9,059	17,106

24 hour urinary excretion (µg.) on:-

Case No.	Control Day	Desferal* (mean of 3 days)	Desferal* and phenylhydrazine+ (mean of 4 days)	% increase on combined therapy compared with Desferal alone.
2	124	10,319	14,874	44
5	452	14,283	15,124	6

* 1,000 mg./day

+ 50 mg./day.

TABLE 33

24-hour urinary iron excretion in Cases 2 and 5 during treatment with desferrioxamine B mesylate alone and in combination with phenylhydrazine (50 mg./day).

or laboratory procedures (Bothwell and Finch, 1962, p. 121). The urinary losses of iron in normal, sideropenic and iron deficient subjects appear to be uninfluenced by the body iron content, being approximately 100 µg. or less daily. In states of iron overload the urinary losses may vary, apparently to some extent dependent on the tissue iron stores. Eleven of the fourteen patients excreted less than 200 µg. of iron daily, 647 µg. being the highest daily excretion.

The nature of the physiological urinary iron losses is uncertain. In normal subjects, 0 - 90 mg. of protein in 24 hours may fail to be reabsorbed from the glomerular filtrate and it is possible that small urinary losses of transferrin may thus occur. Finch (1964) has found as much as 1 mg. daily of transferrin in the urine of normal persons. Other possible sources of urinary iron are desquamated urinary tract cells, and the physiological urinary loss of erythrocytes; accepting a normal excretion of not more than 200,000 erythrocytes per hour (Gadeholt, 1964), the daily excretion of iron from this source is approximately 0. 5 µg.

Increased urinary losses of iron were found in patients with proteinuria, confirming the findings of Cartwright et al., (1954) and Rifkind et al., (1961). Whereas the relation between urinary iron and total urinary protein was not significant, the relation between iron and β globulin was significant. This confirms the findings of Rifkind et al., (1961) and suggests that the renal leak of transferrin is responsible for the increased urinary iron excretion. The urinary losses of iron and transferrin account for

the low concentrations of these substances found in the serum of severely proteinuric patients, and the iron deficiency state resulting from such losses may contribute to the anaemia of nephrosis. It is of interest that one of the patients studied had an absence of stainable iron in the marrow; she made a partial response to oral iron therapy, the haemoglobin level rising from 6.7 to 10.3 g.% in two months.

The two patients with paroxysmal nocturnal haemoglobinuria excreted large quantities of iron daily in the urine. Both patients had an absence of stainable iron in the bone marrow and a hypochromic blood picture, although this latter finding has been stated by Crosby (1953) to be rare.

The urinary iron excretion during Desferal in both sideropenic and iron deficiency anaemic patients was reduced when compared with normal subjects. The urinary iron output appears therefore to reflect the tissue iron depletion found in these states. There was however some overlap between normal and iron deficient subjects in the urinary iron excretion during Desferal. This was also found by Fielding (1965) using a differential ferrioxamine test. It has been assumed that before anaemia develops in states of iron deficiency, there must be complete depletion of the tissue iron stores (Bothwell and Finch, 1962, p. 281). The increased iron output during Desferal in the iron deficient patients suggests that some ferritin, which does not take up marrow iron stain, is still present.

In the patients with untreated primary idiopathic haemochromatosis, large quantities of iron were excreted following a single dose of

Desferal. It is of interest that the urinary iron output in the treated case was normal. Desferal is of limited value in the treatment of primary idiopathic haemochromatosis but the Desferal test is useful not only in confirming the diagnosis, but also in assessing the effectiveness of iron removal by venesection. It may also be of use in detecting iron overload in relatives of patients (Bannerman et al., 1962).

The two conditions associated with focal iron overload are characterised by heavy deposition of iron in specific organs, i.e. in the kidneys in paroxysmal nocturnal haemoglobinuria and in the lungs in idiopathic pulmonary haemosiderosis. In both instances, there co-exists generalised iron depletion leading, especially in pulmonary haemosiderosis, to severe iron deficiency anaemia. The two patients with paroxysmal nocturnal haemoglobinuria excreted even more iron in the urine when Desferal was given; presumably this additional iron had been mobilised from the kidneys. Desferal also induced an increase in iron excretion in the patient with idiopathic pulmonary haemosiderosis. This increase was much greater than would be expected in a severely iron deficient patient or even a normal subject. It is of interest that the iron deposits in the lungs, which are not available to the body for haemoglobin synthesis, were able to be mobilised by Desferal. Renal haemosiderosis has not been reported to cause renal insufficiency (Crosby, 1953) and there is thus no indication for Desferal therapy. In idiopathic pulmonary haemosiderosis, on the other hand massive lung fibrosis may occur with fatal results. For this reason, treatment with Desferal in this patient was continued for over nine months in an attempt to mobilise

iron from the lungs. This resulted in 0.86 g. of iron being removed.

In seven patients with untreated haemochromatosis or haemosiderosis, the effect of a mild phenylhydrazine-induced haemolysis on the urinary iron excretion during Desferal treatment caused a further significant increase in the iron excretion of 42 - 178%. The precise mechanism by which more iron is made available for chelation is not known. Chelation of iron by Desferal may be furthered at one or more of the following stages: (1) when iron is released from the protoporphyrin linkage; (2) when the stimulation of erythropoiesis by haemolysis provokes the mobilisation of iron from the depot stores of haemosiderin. It has not yet been established whether, as a result of haemolysis, the level of plasma ferritin is raised. If proven, such an increase would make available a form of iron which is readily chelatable by Desferal.

An insignificant rise in the urinary iron excretion was recorded when three patients with idiopathic pulmonary haemosiderosis, treated idiopathic haemochromatosis and sideroblastic anaemia were given phenylhydrazine during Desferal therapy. In idiopathic pulmonary haemosiderosis, the iron deposited in the lungs is not available for haemopoiesis and this may explain the failure of phenylhydrazine, in combination with Desferal, to further increase the urinary iron excretion. The male patient with treated primary idiopathic haemochromatosis had depleted iron stores as a result of repeated venesections. In sideroblastic anaemia, there is usually some degree of haemolysis present and this would reduce the effect of phenylhydrazine

on the urinary iron output during Desferal therapy.

In animal experiments, Keberle and Goldberg (1965) have demonstrated an increase in both faecal and urinary iron excretion on combined Desferal and phenylhydrazine therapy. The increase in the faecal excretion of iron may be related to the induced haemolysis, since it has been shown in dogs that a phenylhydrazine-induced haemolysis can provoke a striking increase in the biliary iron excretion (Dubach et al., 1955). Enhanced faecal excretion of iron was demonstrated when patients with transfusional siderosis were given Desferal (Gevirtz et al., 1965). Further studies are in progress to investigate the faecal excretion of iron, in patients with iron overload, during treatment with Desferal alone and in combination with phenylhydrazine.

Although the value of Desferal as a specific chelator of iron is widely recognised, the therapeutic relevance of its use in combination with phenylhydrazine cannot yet be defined. Not only is it necessary to assess the total daily iron excretion in both faeces and urine during combined therapy, but the frequency and the degree of haemolysis which may be safely undertaken, using phenylhydrazine, must be determined. If the faecal and urinary excretion of iron exceeds 200 mg. per week and if repeated courses of mild haemolysis may be safely undertaken, then combined Desferal and phenylhydrazine therapy may provide an alternative to venesection in the treatment of iron storage diseases.

Summary

The 24-hour urinary excretion of iron has been measured in twenty-two normal subjects, in eighteen patients with sideropenia, in thirty-one patients with iron deficiency anaemia and in fourteen patients with various forms of iron overload. In states of iron overload, eleven of the fourteen patients excreted less than 200 μg. of iron daily in the urine (Maximum found 647 μg./day). In all other patients and in normal subjects, 100 μg. of iron or less was excreted daily.

Patients with proteinuria and paroxysmal nocturnal haemoglobinuria may excrete iron much in excess of this. In twelve patients with proteinuria, up to 1 mg. of iron was lost each day in the urine; a good correlation was found between the urinary output of iron and of β globulin, confirming the view that excessive losses of transferrin are responsible for the increased urinary iron excretion in such patients.

Two patients with paroxysmal nocturnal haemoglobinuria excreted daily up to 5 and 20 mg. of iron respectively in the urine. The major portion of this iron was lost as haemosiderin, ferritin and haemoglobin accounting for the remainder; these losses were associated with an iron deficiency state in both patients.

The 24-hour urinary excretion of iron following a single intramuscular injection of 600 mg. of Desferal was measured in normal subjects and in patients with iron overload and iron depletion. Patients with untreated or partially treated

haemochromatosis had considerably greater urinary iron excretion following Desferal than normal subjects. The urinary iron output following Desferal in sideropenia and iron deficiency anaemia was reduced, reflecting the tissue iron depletion found in these states. It was notable that the mean iron output in the sideropenic group was less than in the normal subjects, but greater than in the patients with frank iron deficiency anaemia.

Two patients with paroxysmal nocturnal haemoglobinuria and one with idiopathic pulmonary haemosiderosis excreted excessive increments of urinary iron following Desferal, although each of these three patients had iron deficiency anaemia.

The long-term use of Desferal for the treatment of patients with iron storage disease has hitherto been of limited value because it generally removes, in the urine, no more than 20 mg. of iron per day. The effect of a mild phenylhydrazine-induced haemolysis on the urinary excretion of iron has been studied in ten of these patients.

There was a 42 - 178% increase in the urinary iron excretion when seven patients with untreated iron-storage disease (six with primary idiopathic haemochromatosis and one with cutaneous hepatic porphyria and haemosiderosis) were given phenylhydrazine during Desferal treatment. There was no significant increase in the urinary iron excretion when phenylhydrazine was given to three patients with treated primary idiopathic haemochromatosis, sideroblastic anaemia and idiopathic pulmonary haemosiderosis on Desferal therapy.

The doses of Desferal and phenylhydrazine administered were 1,000 mg. and 100 mg. respectively per day. There was a transient

haemolytic anaemia in one patient given the latter dose for fourteen days, so further studies were carried out using smaller doses. Daily 75 mg. doses of phenylhydrazine given during Desferal therapy, produced a significant increase in the urinary iron excretion ($p < 0.01$).

It is not yet possible to define the therapeutic applications of these observations to the long-term management of iron-storage disease. It is necessary to assess the combined faecal and urinary iron output as well as the frequency and degree of haemolysis which may be safely undertaken.

PART D. THE EFFECT OF IRON ON FERRITIN SYNTHESIS IN RAT INTESTINAL MUCOSA

Section D.I INTRODUCTION

The isolation from mammalian spleen, of a brown iron-containing compound by Laufberger (1934), occurred during a period of intense interest and fruitful research into the problems of the absorption, excretion and transportion of iron by the body. Laufberger named this iron storage compound ferritin.

In the 1930's it was shown that approximately 20-25% of iron in the body was in a storage form and was present mainly in liver, spleen and bone marrow (Hahn, 1937). It was appreciated that the storage iron did not appear to participate in active metabolic processes but represented a reserve, which varied from person to person, mobilisable for haemoglobin synthesis when required (Hahn, 1937).

Storage iron is found in two forms, ferritin and haemosiderin. Ferritin is a water soluble protein, stable at 80°C in near neutral solution. It is precipitated by half saturation with ammonium sulphate and may be crystallised as the cadmium salt (Laufberger, 1935, 1937; Granick, 1942). Ferritin consists of a homogeneous protein, apoferritin with a molecular weight of 465,000 (Rothen, 1944) and an iron containing fraction, present in the form of

inorganic micelles. The latter appears to be made up of ferric hydroxide-phosphate complexes, with the empirical formula $(FeOOH)_8$ $(FeO: PO_3H_2)$ (Granick & Hahn, 1944). The presence of electron dense clusters of iron atoms in the ferritin molecule has been demonstrated by electron microscopy, the clusters being arranged in regular well defined patterns, apparently based on a structure common to all the molecules (Farrant, 1954; Kerr & Muir, 1960). The structure of ferritin is that of a protein shell (apoferritin) covering an iron-containing core (Hofman & Harrison, 1963). Ferrous iron can be incorporated directly into apoferritin (Bielig & Bayer, 1955) and the resulting ferritin may contain up to 23% of its dry weight as iron (Granick, 1946 b).

Haemosiderin has long been recognised, its granular particles being demonstrable in tissue preparations by the Prussian blue reaction (Perls, 1888). It was so named by histologists who believed that the granules were a result of excessive haemoglobin catabolism (Cook, 1929). The structure of haemosiderin is not defined. Many authors have reported the presence of ferritin and proteins in purified haemosiderin

(Shoden et al, 1953; Richter, 1960; McKay and Fineberg, 1958). In 1961, Shoden & Sturgeon stated that haemosiderin and ferritin were not only chemically but also structurally dissimilar and suggested that haemosiderin consisted of an amorphous condensation of iron particles (mainly ferric hydroxide) in an essentially protein-free aggregate. Since the fundamental property which distinguishes ferritin from haemosiderin is the solubility of ferritin in water (Laufberger, 1934), they stated that until the nature of haemosiderin is established, all non-ferritin (i.e. water insoluble) storage iron should be classed as haemosiderin for convenience.

Seventy years ago, the presence of Prussian blue staining granular deposits in the macrophages of the intestinal villi and in the epithelial cells of the small intestine were reported in animals given large doses of iron by mouth (Lipski, 1893; McCallum, 1894; Hochhous & Quincke, 1896). Early workers assumed these to be haemosiderin. (The Prussian blue stain is now known to detect not only haemosiderin but also ferritin, if present in high concentrations. The stain, however, is much less intense (Shoden & Richter, 1960).) Histological demons-

tration of iron in the wall of the intestine did not give information as to whether the iron was being absorbed or excreted (McGowan, 1930) and for many years there was controversy as to whether the intestine was able to excrete as well as absorb iron. During these years, most of the problems of iron therapy which troubled physicians were fundamentally traceable to their ignorance of the mechanism by which iron was absorbed from the intestinal tract. Clinical observations had shown that simple ferrous salts were apparently utilised to a greater extent for haemoglobin formation than were complex ferric salts, such as ferric ammonium citrate (Heubner, 1924, 1926; Wiechowski, 1927; Witt, 1936) but this had not been demonstrated experimentally. There was no dependable direct method of measuring the immediate absorption of iron into the blood stream from the gastrointestinal tract. After Starkenstein & Harvalik (1933) found that ferric iron in blood is transported combined with globulin, it was demonstrated by Moore et al (1936, 1937) and Heilmeyer & Plotner (1937) that plasma iron is of metabolic importance, serving as the medium of iron transportation in the mammalian organism. An index of the amount of iron being absorbed from the gastro-

intestinal tract can be gained by measuring increases in plasma iron (Moore et al, 1939 b).

By 1939, Moore and his co-workers (1939 b) were able, from their own studies and by reviewing available literature, to put forward the following picture of absorption of oral iron. On reaching the stomach, ingested iron is affected by the free hydrochloric acid normally present. This tends to ionise and dissolve iron not already present in solution. On reaching the duodenum, iron is subject to the action of alkaline intestinal juices and to the action of reducing agents. The latter tend to reduce any trivalent iron to the ferrous form before the change to non-ionisable salts occurs. Absorption of iron, in the ferrous form, takes place largely in the upper portion of the small intestine. When iron is absorbed, it passes directly into blood plasma.

At an earlier period, the work of Lintzel (1931) on iron metabolism under various conditions tended to show that the body could assimilate or reject oral iron depending on the needs for iron, a consideration which the work of Fontes & Thivolle (1932) on dogs seemed to corroborate. These authors believed that

the process of iron metabolism was controlled by selective intestinal absorption. It was later assumed that iron metabolism was regulated by absorption and not by excretion (Heilmeyer & Plotner, 1937; Moore et al, 1939 a). At this time, McCance & Widdowson (1937) had come to the conclusion that there was no evidence that iron was excreted by the bowel and that iron metabolism was regulated by controlled absorption.

Hahn and his co-workers (1943) were the first to show the importance of ferritin in the absorption of iron from the gastrointestinal tract. They found that when oral iron was given to dogs 1-6 hours before radio-iron, the absorption of radio-iron was much depressed. These results led to the concept of a 'mucosa block' in which it was suggested that the mucosal cells had the capacity to prevent the excessive uptake of iron. Hahn and his co-workers considered that the ability of the intestine to reject iron was a result of saturation of a physiological receptor such as apoferritin. As early as 1937, Hahn had stated that the form in which iron is ingested appeared not so important as the unknown intestinal factor or factors involved in limiting the absorption and hence the utilization of iron.

Granick (1946 a) showed that when large amounts of iron salts were given to guinea-pigs, ferritin accumulated in the mucosal cells of the gastrointestinal tract. He used a semi-quantitative method for the measurement of the accumulation of ferritin in the mucosa which depended on the counting of ferritin crystals under a 'high-dry' or oil immersion lens. Granick considered that ferritin played two roles - one facilitating the absorption of iron and the other blocking the further absorption of iron, i.e. when minimal amounts of iron were available from the lumen of the gastrointestinal tract, individual ferrous ions could pass through the mucosal cells and into the bloodstream. With increasing amounts of iron available, apoferritin was synthesised and ferritin was formed in the mucosa of the small intestine. If much ferritin was formed, it blocked the passage of iron through the mucosa ("mucosal block"), tending to stop further absorption of iron.

From the evidence available, it would appear that normally there is little or no apoferritin in the intestinal mucosa but that on the administration of oral iron, apoferritin is synthesised rapidly in the epithelial cells (Granick, 1946 a; Gabrio & Salomon, 1950; Heilmeyer, 1958; Charlton et al, 1963). The

parenteral administration of iron has been shown to induce increased ferritin synthesis in liver (Fineberg & Greenberg, 1955 a; Drysdale & Munro, 1966). This suggests that iron may stimulate the synthesis of a specific protein in a manner analagous to that in which an injected antigen provokes the production of antibody protein. That iron and apoferritin seem intimately related is further illustrated by the findings that the levels of apoferritin decrease when iron is mobilised from the tissues by bleeding (Granick, 1943). Heilmeyer (1958) has shown that ferritin serves as a mediator of iron absorption in the gastrointestinal tract but believes that an as yet unknown regulator of iron uptake exists. Keiderling & Wohler (1954) have shown, in guinea-pigs, that at the time of maximal ferritin content in the duodenum, on the tenth day of iron feeding, iron absorption was still clearly going on, so that a total mucosal block was certainly not present. This was confirmed by the results of Heilmeyer (1958). He found that on feeding guinea-pigs iron daily, there was a good absorption of iron until the 14th day after which there was no increase of iron in the organs. He stated that this may be interpreted to mean saturation of the organs coincidental with

decreasing absorption for at the same time, there was a decrease in the ferritin content of the small intestine. Haemosiderin, which was at first only chemically demonstrable, was formed along with ferritin in the intestinal mucosa. As the absorption of iron increased, histochemically demonstrable haemosiderin was formed in the interstitial tissue rather than in the epithelium. On the basis of his studies and those of Keiderling & Wohler, Heilmeyer (1958) proposed a new hypothesis modifying that of Granick (1946 a):- When iron enters the mucosal cell, it may be stored as ferritin or haemosiderin. By means of a reducing substance, ferritin is broken down and the ferrous ions released leave the cell and pass into the bloodstream where they are transported by transferrin. Whether haemosiderin or ferritin is formed depends on the amount of iron administered and the duration of the cellular iron uptake. With large doses, iron is stored briefly as haemosiderin and later changed to ferritin. With slow administration, ferritin is formed immediately. The relatively large amounts of absorbed intraluminary iron is diverted to storage in the liver and the intestinal wall, so that the plasma is protected from iron in excess of its binding capacity.

Crosby & Conrad (1963) have shown, by parenteral administration of ^{59}Fe, how the intestine is able to excrete iron. By autoradiography, labelled iron was found initially at the base of the villi in the intestinal mucosa. The radio-iron was incorporated into newly formed columnar epithelial cells, which migrate over a two day period for rats, from the crypts to the tips of the villi. There they, and the iron they contained, were lost as the cells were desquamated at the end of their life cycle into the intestinal lumen. Radio-iron was also found in the epithelial cells of the intestinal villi when oral ^{59}Fe was given. Conrad & Crosby (1963) suggested that the iron sequestered in the intestinal epithelial cells serves "not as an intermediate stage in the absorptive process, but as a means of frustrating the absorption of iron when it is not needed". This and other work led Crosby (1963) into a general hypothesis on the control of iron balance by the intestinal mucosa. He suggested that iron incorporated into the epithelial ferritin cannot be released and therefore is lost at the end of the three days life cycle of the mucosal cell. He noted that when the iron stores were replete the newly forming epithelial cells could synthesise apoferritin, thus preventing the

entry of dietary iron into the body, but that in iron deficiency anaemia, the mucosal cells contained relatively little ferritin. He postulated that in iron deficiency these absorptive cells were constructed with 'a relative lack of ferritin' apparatus so that dietary iron can pass freely into the cell and then freely into the body. Inherent in this hypothesis is the suggestion that there are two types of mucosal cell, one containing "ferritin apparatus" in iron-replete subjects and another with a relative lack of "ferritin apparatus" in states of iron deficiency. Crosby tried to explain the three day lag in iron absorption following acute bleeding, by the development of a different type of mucosal absorptive cell within that period, a cell which would not contain "ferritin apparatus" and thus allow an increased entry of iron through it. It would perhaps seem more reasonable to assume that the cell in each of these states was similar but that its ability to synthesise apoferritin was influenced by some factor, for example iron itself. If in fact iron given parenterally or even enterally provoked apoferritin synthesis in the intestinal mucosa, this could explain many of Crosby's observations. Hitherto, the effect of parenteral iron on

ferritin synthesis in the intestinal mucosa has not been elucidated, although Fineberg & Greenberg (1955 a) and Drysdale & Munro (1966) have shown that parenteral iron stimulates the ^{14}C amino acid incorporation into liver ferritin.

In the present study, iron was given orally and parenterally to rats and the effect of this was observed on the incorporation of ^{14}C labelled leucine into the ferritin of the gastrointestinal mucosa. Small scale isolation of mucosal ferritin was achieved by using a specific antiserum prepared by injection of horse spleen ferritin into rabbits.

Section D.II MATERIAL, METHODS AND RESULTS

1. Experimental Animals

a) Rabbits. Five Californian rabbits, weight 2.5-3.5 kg., were used for the production of antiserum. They were given injections of iron sorbitol citric acid at intervals, dependent on the volume of blood removed, to safeguard against possible iron deficiency.

b) Rats. Rats were obtained from a standard colony and were delivered by rail. To ensure standard conditions, they were kept for one week after delivery before use.

2. Experimental Data

The details of iron and radioactive tracer given to rats in each group are given later.

After the rat was killed, its small intestine (from the beginning of the duodenum to the beginning of the caecum) was removed, washed through with 20-25 ml of 0.9% w/v sodium chloride and gently squeezed to expel the remaining saline. The intestine was then slit along its length, cut into short strips and laid on a stainless steel sheet. The mucosa was scraped off with

a microscope slide (Munro & Goldberg, 1964) and weighed. 2.5-4.5 g of mucosa were obtained when the animals were given a normal diet up to the time of the experiment. Four volumes (w/v) of water were used to homogenise the mucosa in a Potter Elvehjem type of homogeniser using a Teflon (polytetrafluoroethylene) piston. The homogenate was heated with constant stirring to 80°C (Laufberger, 1937) in a water bath. The heat-coagulated protein was centrifuged down and the "heat supernatant fraction", containing heat-stable mucosal ferritin was decanted, filtered through No. 1 filter paper, centrifuged and decanted to ensure that no particles of heat coagulum remained. The method now follows that devised by Drysdale & Munro (1965) for the isolation of liver ferritin. After noting the volume (up to 15 ml), 0.5 ml ferritin antiserum (prepared as noted later) was mixed with the "heat supernatant fraction". (All precipitations were carried out in the presence of excess antibody.) After incubation for one hour at 37°C and standing overnight at 4°C (Mazur et al, 1960), the brown ferritin precipitate was collected by centrifugation and washed twice with 3 ml of 0.9% saline. The precipitate was dissolved in 0.1 ml 0.1N.NaOH and made up to 10 ml with deionised water. One ml samples were removed for duplicate protein estimation and

iron was determined in the remaining 8 ml. The volume used for the spectrophotometric reading of the iron estimation was recovered and combined with the rest before addition of perchloric acid (PCA) to give a concentration of 0.35N for protein precipitation. After standing for two hours at 4°C, the precipitate was spun down, washed twice with 3 ml 0.2N.PCA, finally dissolved in 0.1N.NaOH and mounted on a stainless steel planchette. The activity of ^{14}C labelled ferritin was calculated after counting on a gas-flow Geiger Counter. (Livers were removed and homogenised as above but normally ferritin was isolated from only 2 ml of the "heat supernatant fraction" of liver homogenate.)

The specific activity (c.p.m./mg protein) of the heat coagulable non-ferritin protein was determined by dissolving a small amount in 0.1N.NaOH and adding water to a suitable volume before estimating protein concentration per ml and activity per ml by PCA precipitation as above (Drysdale, 1965).

3. Determination of Protein

Protein was estimated by the method of Lowry et al (1951) as detailed in Section C.II.

4. Estimation of Ferritin Iron

Ferritin iron was estimated by the addition of 1 ml 0.5% w/v 2:2' dipyridyl in 60% v/v acetic acid and 0.5 ml 1.5M sodium sulphite to the sample in a conical graduated centrifuge tube. This mixture was heated for one hour in a boiling water bath to ensure complete iron extraction. The extinction was read at 520 mμ after centrifugation of any protein precipitate formed (Drysdale & Munro, 1965).

5. Determination of the Activity of ^{14}C labelled Ferritin

Protein precipitates were dissolved in 0.1N.NaOH and mounted on discs of lens tissue in order to standardise self absorption (Garrow & Piper, 1955). Normally 80-750 μg of ^{14}C labelled protein were mounted and counted on a gas-flow Geiger Counter to a standard error of 4% or less.

6. Preparation of Ferritin

a) Isolation and crystallisation

Crystalline ferritin was prepared by the method of Granick (1946 b) and Mazur & Shorr (1948, 1950) from fresh horse spleen. The spleen was minced, homogenised with 1½ volumes of water (w/v)

and heated to 80°C in a water bath. After centrifugation, the dark brown supernate containing heat stable ferritin was decanted. (The precipitate may be extracted with a small volume of water to remove residual ferritin, before discarding.) The solution was adjusted to pH 4.6 with 50% (v/v) acetic acid to precipitate any denatured ferritin and other proteins (Agner, 1943). The precipitate was discarded. An equal volume of saturated ammonium sulphate was added to the solution and it was allowed to stand for two hours at 4°C. After centrifugation, the supernate was discarded and the precipitate extracted with water to dissolve ferritin. The ferritin solution was half saturated with ammonium sulphate as before and the precipitate was taken up in the minimum volume of water. A quarter volume of cadmium sulphate was added to the ferritin solution and the ferritin allowed to crystallise (0.25 ml of 5-20% w/v cadmium sulphate was added to 1 ml test solutions of ferritin to determine the optimal cadmium concentration required for crystallisation). After a few days, the ferritin crystals were collected by centrifugation and dissolved in the minimum volume of 2% w/v ammonium sulphate. Ferritin was crystallised four times by this process. Finally

the ferritin solution was dialysed against running water to remove the bulk of the cadmium ions. Ferritin was precipitated out of solution by the addition of an equal volume of saturated ammonium sulphate and washed twice with further volumes of saturated ammonium sulphate to further reduce the cadmium concentration. The ammonium sulphate was removed by dialysis against 0.9% saline.

b) Gel filtration

Ferritin was passed through G.200 Sephadex in an attempt to remove any remaining toxic cadmium ions (Drysdale, 1965). It has since been shown that non-ferritin antigens are present in crystalline horse spleen ferritin but that they can be removed by chromatography on G.200 Sephadex (Paterson et al, 1965).

Sephadex is a modified dextran in the form of minute beads which swell considerably in aqueous solution. Molecules larger than the largest pores of the swollen Sephadex cannot penetrate the gel particles and pass through the bed in the liquid phase outside the particles. Smaller molecules however, penetrate the gel to a varying extent dependent on their size and shape.

G.200 Sephadex was made up in 0.9% saline. Ferritin, molecular weight 465,000 (Rothen, 1944) is completely excluded

from the gel particles. No marker dye was added to the ferritin solution since the passage of the brown solution through the gel is easily visible. Columns, 6" long and $\frac{5}{8}$" diameter, were $\frac{2}{3}$ rds filled with a suspension of the gel. A circle of filter paper placed on top of the gel enabled ferritin to be layered on to the gel without disturbance. Saline was added when ferritin had drained through the surface. Ferritin eluted from the column was precipitated by half saturation with ammonium sulphate, dissolved in 2% ammonium sulphate and dialysed against 0.9% saline.

c) Gel electrophoresis

The purity of ferritin was checked by polyacrylamide gel electrophoresis using Tris(hydroxymethyl)aminomethane(Tris) buffer. The latter two compounds are available commercially under the trade names cyanogum 41 and Trizma Base respectively.

6.25 ml 1M citric acid and 25 ml 1M Trizma Base were made up to 1 litre and the pH adjusted to 6.6 with citric acid. (Buffer solution, pH 6.6). The gel buffer was prepared by adding 4 drops each of 40% w/v ammonium persulphate and N:N:N':N' tetramethyl-ethylenediamine to a 4.5% w/v solution of cyanogum 41 in buffer solution, pH 6.6. (The first two reagents catalyse the setting

of the gel.) After air had been extracted by suction pump, the gel buffer was poured into a perspex tray, 13 cm by 5 cm. The lid was placed in position, excluding all air bubbles, and weighted to keep air out. Three perspex squares attached to the underside of the lid caused three slits to be formed in the gel on solidification (3 hours). The gel was placed on the central compartment of the electrophoresis bath and connected to the vessel buffer (0.25M Trizma Base) by wicks of double thickness chromatography paper, overlapping each end of the gel by 0.5 cm. Ferritin was applied into one or more of the slits and a perspex slide placed on top of the gel. A constant current of 200 V was passed until the yellow band of ferritin was seen to have moved 0.5 cm or more from the origin (about 7 hours). After electrophoresis, the surface layer was sliced off, using a gel cutter, in order to eliminate surface smears. The gel was stained for protein and iron simultaneously in a solution of Ponceau S (0.05% w/v) and potassium ferricyanide (0.2% w/v) in 3% w/v trichloroacetic acid and washed with 5% v/v acetic acid.

Protein impurities which stain red if present, migrate in front of the ferritin bands. The ferritin bands stain blue since Prussian blue overstains the red protein stain.

Results of gel electrophoresis

No protein impurities were seen in any of the ferritin preparations which had been passed through G.200 Sephadex. In every preparation, the electrophoresis pattern of ferritin showed not one band but three bands, i.e. 1 major band, followed by 2 minor bands. The presence of several ferritin bands agrees with the findings of Richter (1964) who demonstrated at least three electrophoretically distinct, but antigenically identical components in crystalline horse spleen ferritin, the major component comprising 75-85% of the total.

7. Ferritin Antiserum

An antiserum to horse spleen ferritin was prepared in rabbits by the method of Mazur & Shorr (1948). This antiserum has been shown to quantitatively precipitate rat ferritin under suitable conditions (in experimental data) (Mazur et al, 1960). Apoferritin is also precipitated by the antiserum since the antibody produced in response to immunisation with ferritin is directed towards the protein moiety of ferritin (Mazur & Shorr, 1950).

a) Preparation

Crystalline horse spleen ferritin was emulsified with complete Freund's Adjuvant, which increases the antibody production, and injected subcutaneously into the hind legs of Californian rabbits on four occasions at weekly intervals. Each rabbit received a total of 30 mg of ferritin injected over one month, each dose being approximately double the previous one. The rabbits were bled (40 ml each) ten days after the final injection and as required. A booster dose of ferritin was injected at approximately six monthly intervals to maintain the antibody titre.

The antiserum was pooled and merthiolate (1 part in 10,000) added as preservative (Mazur & Shorr, 1950). The antisera was divided into small aliquots and deep frozen.

b) Immunoelectrophoresis

The specifity of the ferritin antiserum was checked by immuno-electrophoresis using a solution of ferritin which had not been passed through G.200 Sephadex to remove non-ferritin antigens.

A 1-1.5 mm thick layer of 0.8% w/v ionagar in 0.05M barbitone buffer, pH 8.6, on a siliconed glass sheet, 20 x 10 cm, was allowed to set (30 minutes) and wells and troughs, for antigens and anti-

bodies respectively, cut out. The agar gel was placed on the central compartment of the electrophoresis bath and paper wicks, set in the agar on long side of the glass plate, dipped into 0.05M barbitone buffer. A few drops of ferritin solution, which had not been passed through G.200 Sephadex, were placed in the antigen well and a constant voltage, 200 V, applied for approximately four hours. (No dye marker is needed since the brown colour of ferritin is easily visible.) The current was switched off and the paper wicks removed. The trough was filled with ferritin antiserum, prepared as above, and the plate left in a damp chamber for two days after which the precipitin line was clearly visible. The plate was immersed in a 5% dilution of 40% w/v formalin in 0.05M barbitone buffer for 30 minutes to fix precipitated protein. The agar gel was washed in saline for one day and in water for one day. The plate was stained for protein with an aqueous solution of Azo black (0.2 g% w/v) in ethylene glycol (25% v/v) and was washed with water.

Results of immunoelectrophoresis

The immunoelectrophoretic pattern obtained showed only one precipitin line, demonstrating the presence of one ferritin antibody in the rabbit antiserum. (Fig. 35)

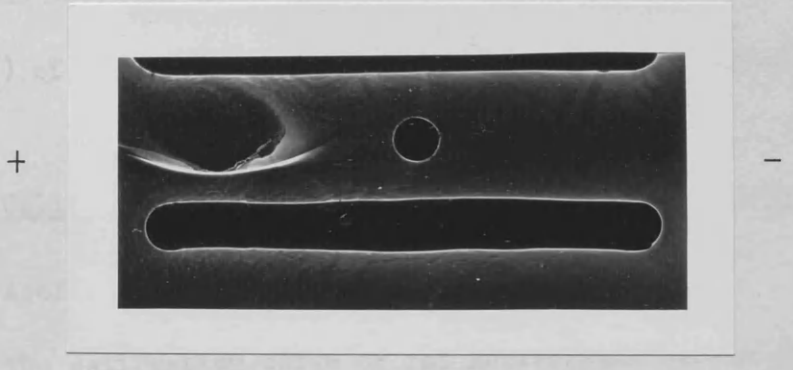


Figure 35. Immunoelectrophoretic pattern of the ferritin fraction before purification by G.200 Sephadex gel filtration, with a specific antiserum to crystalline horse spleen ferritin (in the trough).

c) Standardisation of ferritin antiserum

Standardisation of ferritin antiserum was carried out using the "heat supernatant fraction" of liver homogenate as the source of ferritin antigen. 0.01 ml to 1 ml of the antiserum was added to aliquots of liver ferritin. Ferritin was precipitated and washed, as detailed earlier, and the iron and protein content determined. The precipitin curves obtained are shown in Fig. 36. Normally 0.5 ml antiserum was found to be sufficient to precipitate all the ferritin in either intestinal mucosa or a small aliquot (2 ml) of the "heat supernatant fraction" of liver homogenate.

8. Calibration Graphs: Apoferritin and mixed protein

a) Apoferritin

The calibration curve of rat apoferritin versus optical density was obtained from work carried out on a related project by Drysdale (1965).

b) Mixed protein

For the determination of the protein content of heat-coagulated tissue, a calibration graph of mixed protein (i.e. all proteins) versus optical density was required. It has

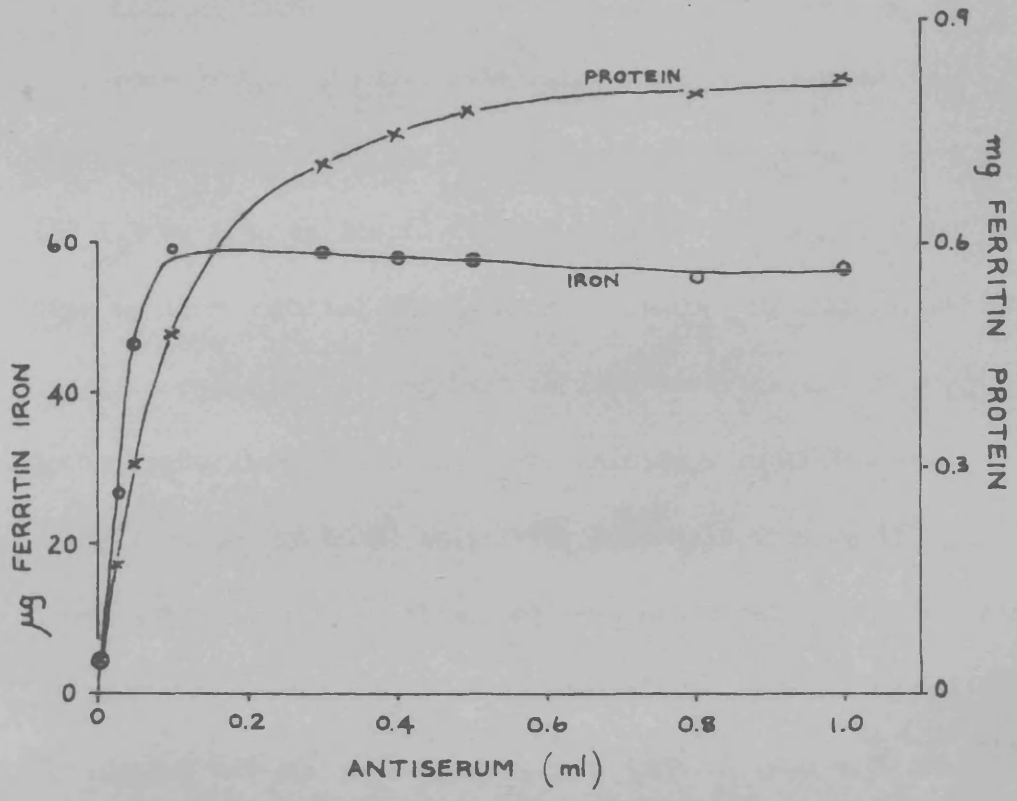


Figure 36. Precipitin curves for ferritin protein and ferritin iron, in a solution obtained from liver homogenate, using rabbit anti-horse spleen ferritin serum.

been assumed that the mixed protein concentration is given by the calibration curve of crystalline bovine albumin versus optical density, shown earlier in Fig. 30.

9. Plan of Study

Four groups of adult male rats were given respectively (A) 0.6 mg iron as ferric ammonium citrate by gastric tube, (B) 1.5 mg iron as ferric citrate by gastric tube, (C) 1.0 mg iron as iron sorbitol citric acid (Jectofer) by intramuscular injection, and (D) 2.5 mg iron as iron sorbitol citric acid by intramuscular injection. The animals were killed (by a sharp blow on the head) at varying intervals from $2\frac{1}{2}$ to 19 hours after iron administration; two hours before the sacrifice of each rat, it was injected intraperitoneally with 5 μc 1-^{14}C-DL-leucine per 100 g. body weight. Control rats were given saline instead of the iron preparation, either by gastric tube or by intramuscular injection (Table 34).

The two hour period mentioned above was chosen because Fineberg and Greenberg (1955 b) have shown that, in guinea pigs, parenteral iron stimulates the maximal incorporation of ^{14}C glycine into liver ferritin between $1\frac{1}{2}$ and 3 hours. This

Group	Total No. of rats	Weight (g) Mean ± S.D.	Iron per rat Oral	Parenteral	Times of killing after iron (hours)
A	22	185 ± 20	0.6 mg. iron as ferric ammonium citrate	-	2(control), 2½, 3, 4, 5, 6.
B	24	163 ± 5	1.5 mg. iron as ferric citrate	-	2(control), 2½, 3, 4, 5, 6.
C	20	144 ± 7	-	1 mg. iron as iron sorbitol citric acid	2(control), 3, 5, 8, 19.
D	34	147 ± 13	-	2.5 mg.iron as iron sorbitol citric acid	2(control), 3, 5, 8, 19.
	28*	144 ± 7	-		

* In only 28 rats out of a total of 34 was the liver analysed.

TABLE 34

Details of animal experiments. Each rat was given 5 μc l - ^{14}C - DL - leucine /100 g. body weight 2 hours before death. Control rats were given saline in place of iron.

two hour period is a small enough fraction of the turnover time of ferritin to ensure estimation of initial rates of incorporation. At the same time, in such a short period, the incorporation data are unlikely to be seriously affected by the loss of ^{14}C label due to any concurrent ferritin breakdown.

The oral preparations of iron used for Groups A & B were adjusted to pH 7.4 before administration. 1-^{14}C-DL-leucine was dissolved in sterile saline (0.9% w/v), divided into 1 ml aliquots of 100 μc/ml and stored in the deep-freeze. The aliquots were diluted to 20 μc/ml before use.

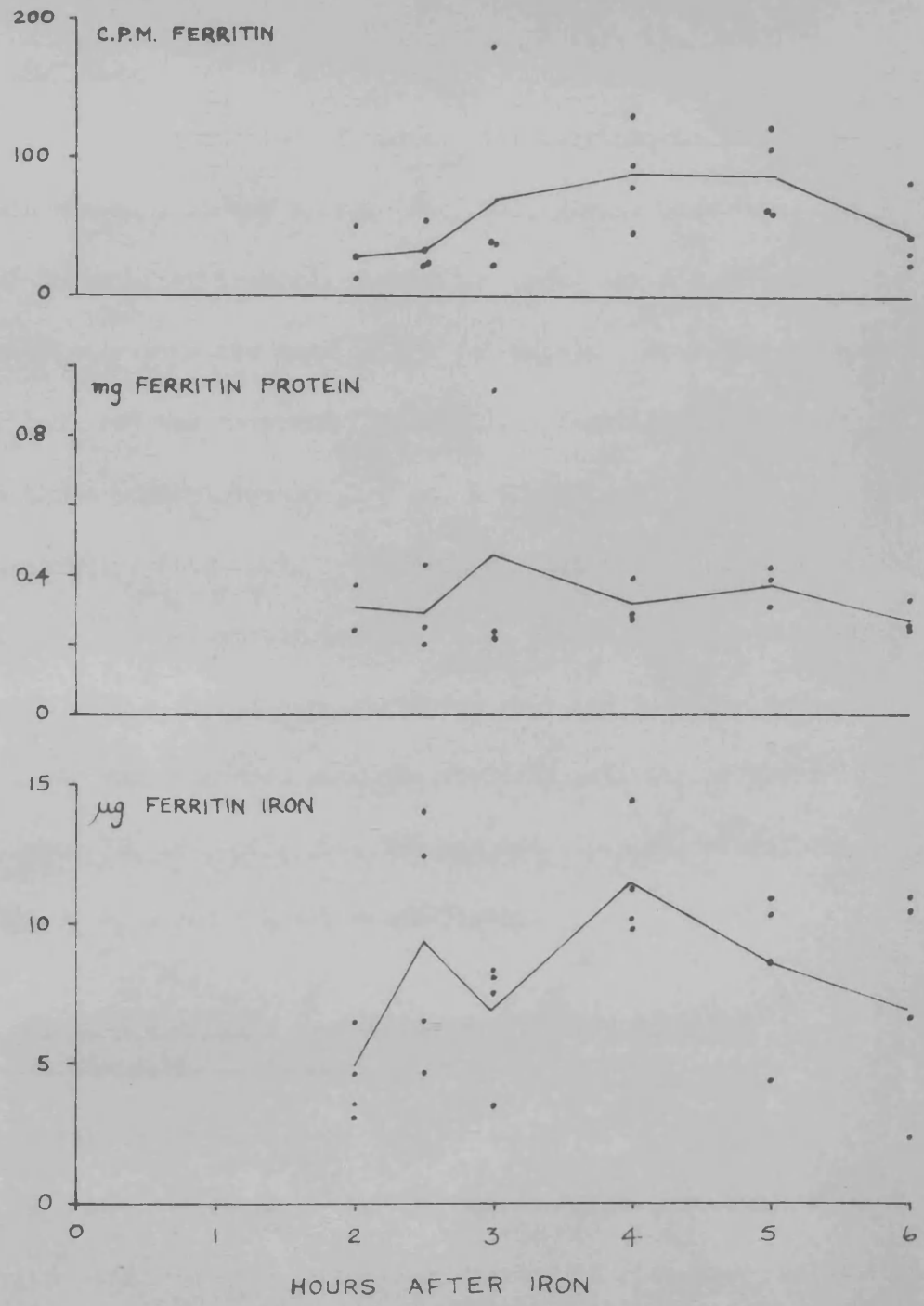
10. Results for Group A. Oral ferric ammonium citrate (0.6 mg iron per rat)

A mean threefold increase over the mean of control animals in the synthesis of labelled ferritin in the intestinal mucosa occurred 4-5 hours after administration of iron, falling to control levels at 6 hours (Fig. 37). Although the mean ferritin iron had doubled $2\frac{1}{2}$ hours after iron administration and remained high thereafter (Fig. 39), ferritin protein remained more or less at the control value throughout the experiment (Fig. 38).

Figure 37. Induction of ^{14}C labelled ferritin in intestinal mucosa at time intervals after 0.6 mg. oral iron per rat. The graph through the mean values is shown. Control rats, plotted at 2 hours, were given saline in place of iron. Each rat was given 5 μc. 1-^{14}C-DL-leucine intraperitoneally two hours before death.

Figure 38. Total ferritin protein in rat intestinal mucosa at time intervals after 0.6 mg. oral iron per rat. The graph through the mean values is shown. Control rats, plotted at 2 hours, were given saline in place of iron.

Figure 39. Total ferritin iron in rat intestinal mucosa at time intervals after 0.6 mg. oral iron per rat. The graph through the mean values is shown. Control rats, plotted at two hours, were given saline in place of iron.


C.P.M. FERRITIN
200
100
0
mg FERRITIN PROTEIN
0.8
0.4
0
μg FERRITIN IRON
15
10
5
0
0
1
2
3
4
5
6
HOURS AFTER IRON

11. Results for Group B. Oral ferric citrate (1.5 mg iron per rat)

The mean production of radioactive ferritin in intestinal mucosa showed a saddle effect (Fig. 40), giving peak values at 3 and 5 hours after iron administration (mean six and five fold rise respectively over the mean of control rats). At 3 hours, maximum ferritin iron was recorded (Table 35). Ferritin protein only rose above control levels at 3 and 6 hours (mean 25% and 70% rise respectively) (Fig. 42). The specific activity (c.p.m./mg protein) of non-ferritin protein (i.e. heated coagulated protein) did not show a significant variation over the 2-6 hour period (Fig. 41) when compared with the specific activity of ferritin, i.e. mean 72, 189, 313, 264, 286 and 104 c.p.m./mg protein at 2, $2\frac{1}{2}$, 3, 4, 5 and 6 hours respectively.

12. Results for Group C. Intramuscular iron sorbitol citric acid (1 mg iron per rat)

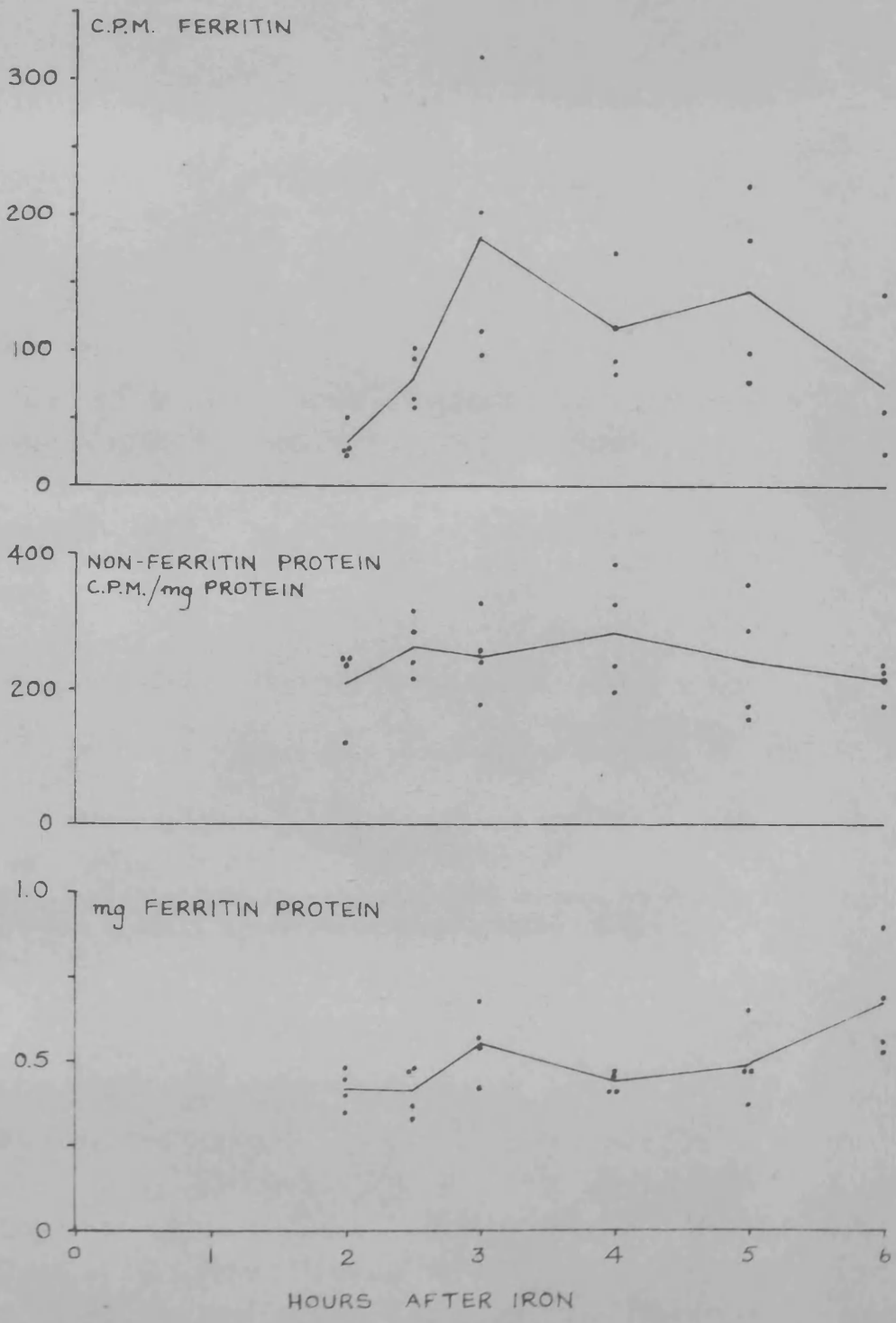
a) Intestinal Mucosa

Maximum incorporation of ^{14}C leucine into intestinal mucosal ferritin occurred at 3 hours (mean five fold rise over the mean control), falling to twice the control level at 5 hours and to the control level at 19 hours (Fig. 43). The specific activity

Figure 40. Induction of ^{14}C labelled ferritin in intestinal mucosa at time intervals after 1.5 mg. oral iron per rat. The graph through the mean values is shown. Control rats, plotted at 2 hours, were given saline in place of iron. Each rat was given 5 μc. 1-^{14}C-DL-leucine intraperitoneally two hours before death.

Figure 41. Specific activity of non-ferritin protein in intestinal mucosa at time intervals after 1.5 mg. oral iron per rat. The graph through the mean values is shown. Control rats, plotted at 2 hours, were given saline in place of iron. Each rat was given 5 μc. 1-^{14}C-DL-leucine intraperitoneally two hours before death.

Figure 42. Total ferritin protein in rat intestinal mucosa at time intervals after 1.5 mg. oral iron per rat. The graph through the mean values is shown. Control rats, plotted at 2 hours, were given saline in place of iron.


C.P.M. FERRITIN
300
200
100
0
400
NON-FERRITIN PROTEIN
C.P.M./mg PROTEIN
200
0
1.0
mg FERRITIN PROTEIN
0.5
0
0
1
2
3
4
5
6
HOURS AFTER IRON

	Hours after iron administration					
	2*	2½	3	4	5	6
Ferritin Iron (µg.)	22.3	15.1	27.4	18.0	19.6	20.1
	10.7	9.9	13.9	17.8	13.8	24.7
	16.0	15.2	23.4	12.3	11.2	13.0
	4.1	22.0	20.7	19.6	12.5	13.5
MEAN	13.3	15.5	21.3	17.0	14.3	17.8

* Control rats. These were given saline in place of iron.

TABLE 35

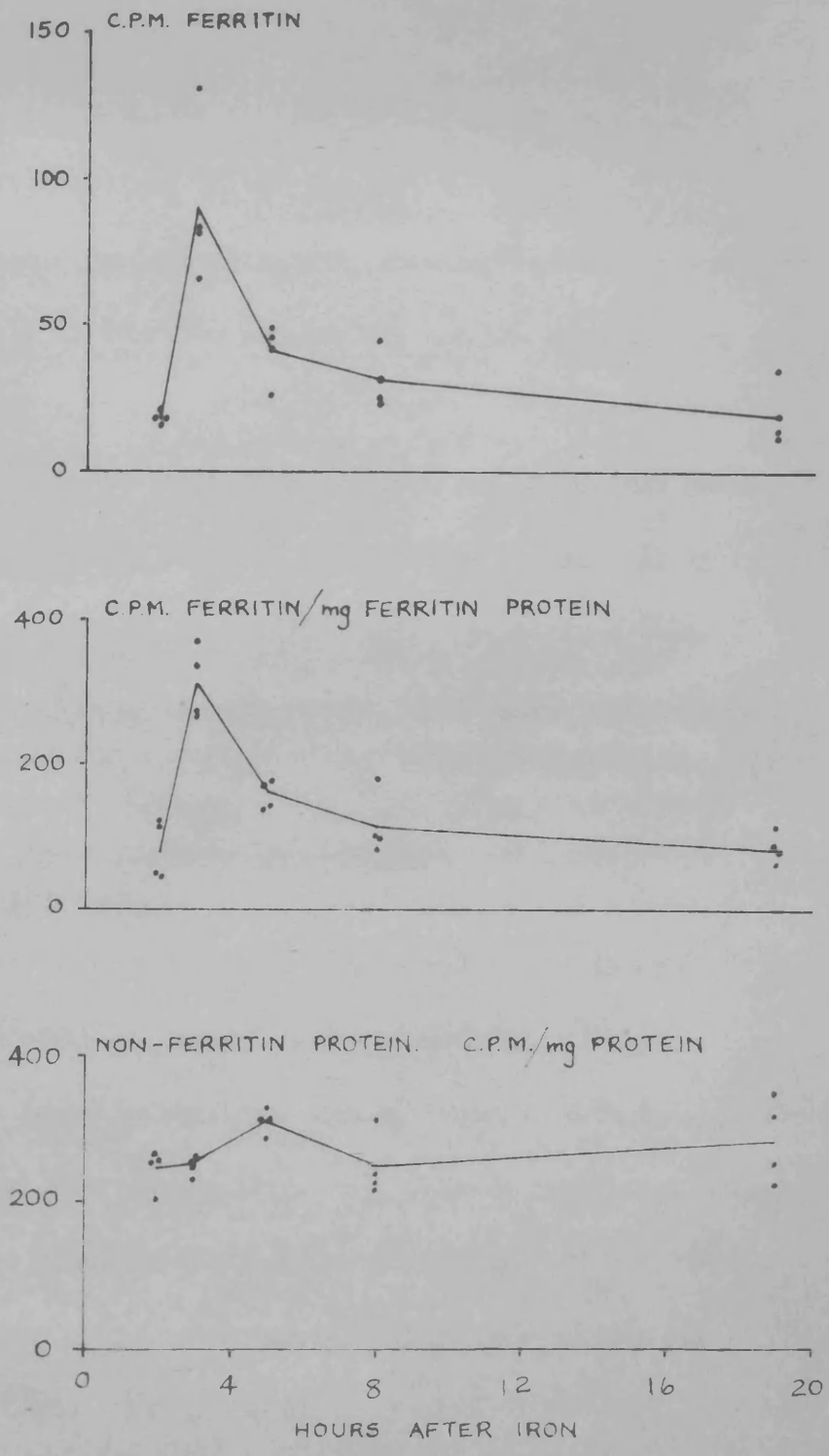
Total ferritin iron in rat intestinal mucosa at 2½, 3, 4, 5 and 6 hours after the administration of 1.5 mg. oral iron.

Figure 43. Induction of ^{14}C labelled ferritin in intestinal mucosa at time intervals after 1 mg. parenteral iron per rat. The graph through the mean values is shown. Control rats, plotted at 2 hours, were given saline in place of iron. Each rat was given 5 μc. 1-^{14}C-DL-leucine intraperitoneally two hours before death.

Figure 44. Specific activity of ferritin in intestinal mucosa at time intervals after 1 mg. parenteral iron per rat. The graph through the mean values is shown. Control rats, plotted at 2 hours, were given saline in place of iron. Each rat was given 5 μc. 1-^{14}C-DL-leucine intraperitoneally two hours before death.

Figure 45. Specific activity of non-ferritin protein in intestinal mucosa at time intervals after 1 mg. parenteral iron per rat. The graph through the mean values is shown. Control rats, plotted at 2 hours, were given saline in place of iron. Each rat was given 5 μc. 1-^{14}C-DL-leucine intraperitoneally two hours before death.

INTESTINAL MUCOSA



of ferritin (Fig. 44) is similar to that of the activity of ferritin (Fig. 43) but quite different to the specific activity of non-ferritin protein (Fig. 45), showing that iron stimulates the synthesis of ferritin protein but not the synthesis of other proteins.

At 3 hours, the mean ferritin iron was double the mean of control rats, and remained high until 8 hours. At 19 hours, it approached control level (Fig. 46). Ferritin protein levels remained more or less constant throughout the experiment (Fig. 47).

b) Liver

In liver, a mean seven fold increased synthesis of ^{14}C labelled ferritin occurred 5 hours after iron injection. The activity of ferritin remained high at 8 hours, returning to the control level at 19 hours (Fig. 48). As in intestinal mucosa, the specific activity of liver ferritin (Fig. 49) was similar to that of the activity of ferritin (Fig. 48) but was quite different to the specific activity of mixed liver protein (Fig. 50). There was a significant increase in liver ferritin protein, maximal 8 hours after injection of iron, representing

Figures 46 and 47. Total ferritin iron and ferritin protein respectively in intestinal mucosa at time intervals after 1 mg. parenteral iron per rat. The graph through the mean values is shown. Control rats, plotted at 2 hours, were given saline in place of iron.

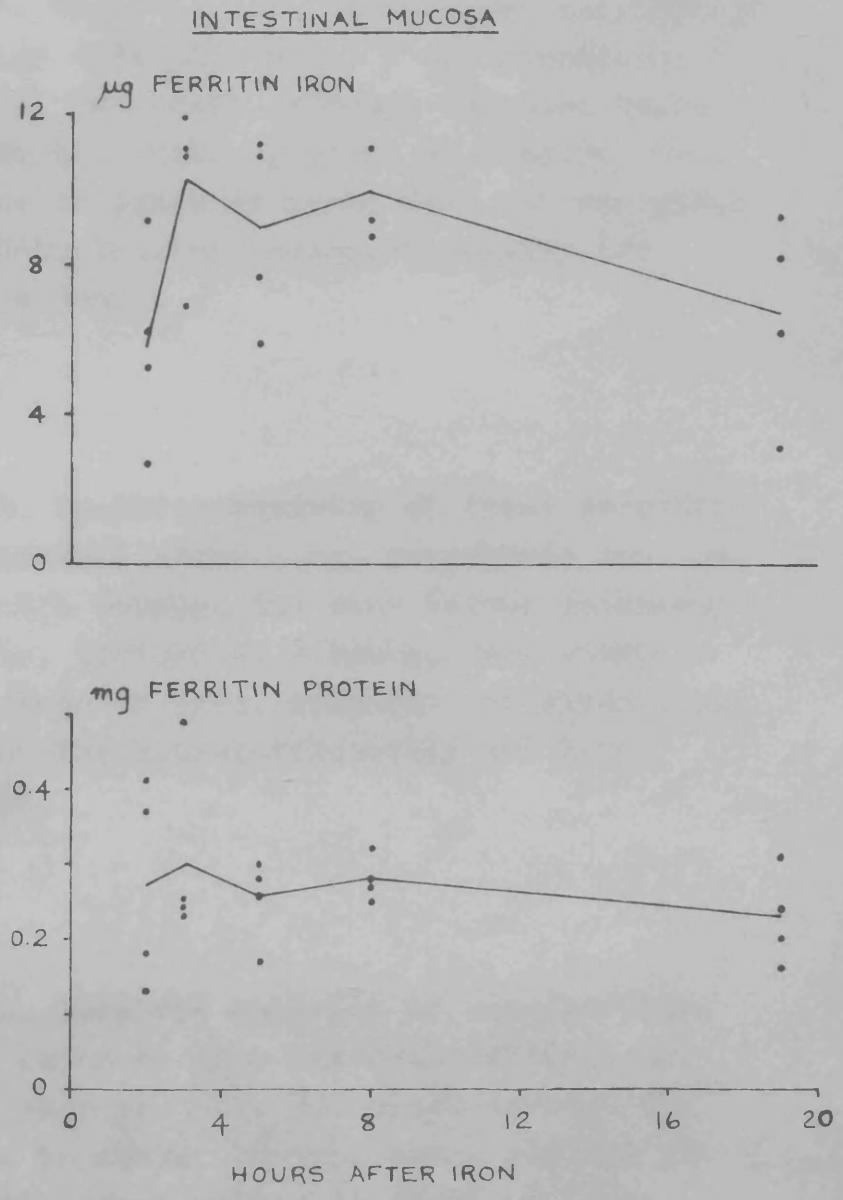
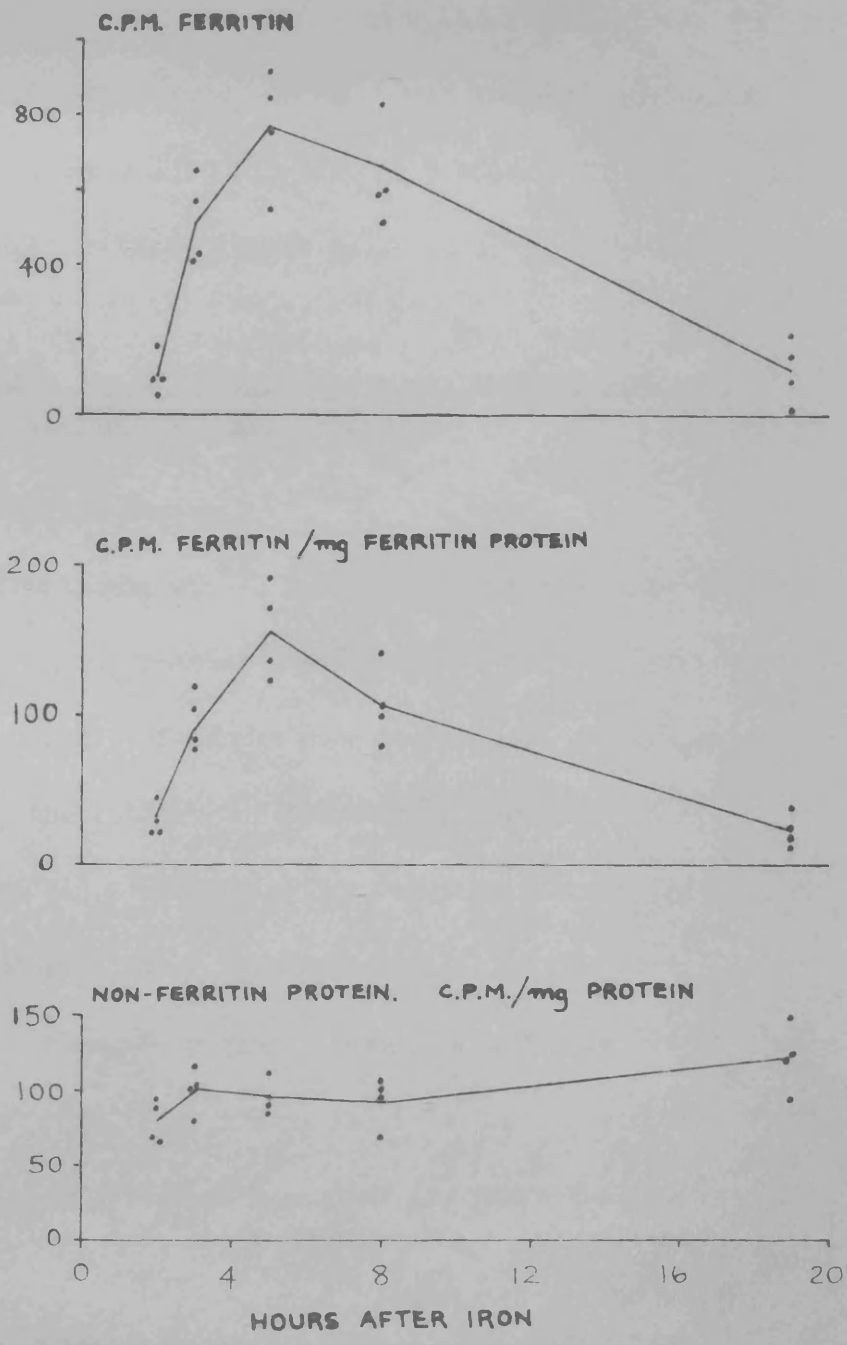

INTESTINAL MUCOSA
µg FERRITIN IRON
12
8
4
0
mg FERRITIN PROTEIN
0.4
0.2
0
0
4
8
12
16
20
HOURS AFTER IRON

Figure 48. Induction of ^{14}C labelled ferritin in liver at time intervals after 1 mg. parenteral iron per rat. The graph through the mean values is shown. Control rats, plotted at 2 hours, were given saline in place of iron. Each rat was given 5 μc. 1-^{14}C-DL-leucine intraperitoneally two hours before death.

Figure 49. Specific activity of liver ferritin at time intervals after 1 mg. parenteral iron per rat. The graph through the mean values is shown. Control rats, plotted at 2 hours, were given saline in place of iron. Each rat was given 5 μc. 1-^{14}C-DL-leucine intraperitoneally two hours before death.

Figure 50. Specific activity of non-ferritin protein in liver at time intervals after 1 mg. parenteral iron per rat. The graph through the mean values is shown. Control rats, plotted at 2 hours, were given saline in place of iron. Each rat was given 5 μc. 1-^{14}C-DL-leucine intraperitoneally two hours before death.

LIVER


C.P.M. FERRITIN
800
400
0
C.P.M. FERRITIN /mg FERRITIN PROTEIN
200
100
0
NON-FERRITIN PROTEIN. C.P.M./mg PROTEIN
150
100
50
0
0
4
8
12
16
20
HOURS AFTER IRON

a mean two fold rise over the mean control (Table 36). Ferritin iron showed a mean two fold rise over the mean control at 3 hours. Although it had fallen slightly at 5 hours, this level was maintained until 19 hours (Table 36).

13. Results for Group D. Intramuscular iron sorbitol citric acid (2.5 mg iron per rat)

a) Intestinal Mucosa

The synthesis of ^{14}C labelled ferritin in intestinal mucosa, high at 3 hours, reached a maximum at 5 hours after iron injection, showing a mean tenfold rise over the mean of control rats. At 19 hours, the activity of ferritin approached that of the control rats (Fig. 51). Ferritin iron, high at 3 hours, reached a plateau 5 hours after iron injection. At 19 hours, the value remained unchanged, giving a mean fourfold rise over the mean of control rats (Fig. 52). Ferritin protein rose by 60% at three hours and reached a maximum ($2\frac{1}{2}$ times the mean control value) at 5-8 hours. 19 hours after iron injection, ferritin protein was still double the control value (Fig. 53).

	Hours after iron injection				
	2*	3	5	8	19
Liver	128	169	182	174	33
Ferritin	46	171	127	304	341
Iron	143	341	182	191	133
(µg.)	201	416	414	253	400
MEAN	130	274	226	231	252
Liver	3.07	5.37	4.38	5.78	3.54
Ferritin	3.94	5.43	6.14	5.14	5.47
Protein	3.91	4.95	5.32	5.58	1.45
(mg.)	2.27	5.18	3.96	7.57	7.53
MEAN	3.30	5.23	4.95	6.02	4.65

* Control rats. These were given saline in place of iron

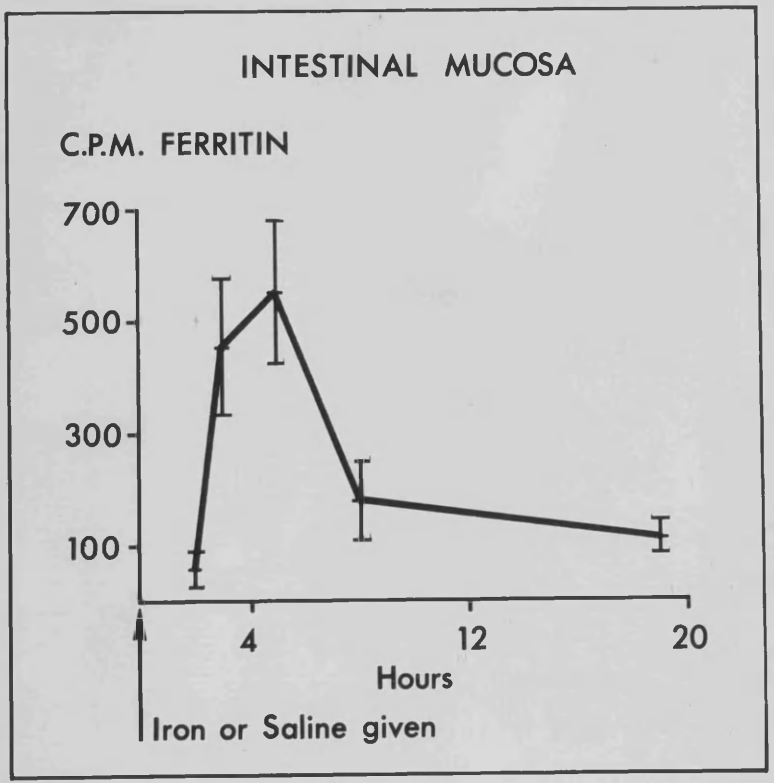
TABLE 36

Total ferritin iron and ferritin protein in rat liver at 3, 5, 8 and 19 hours after the administration of 1 mg. of parenteral iron.

b) Liver

In the livers of these rats, there was an enhanced synthesis of ^{14}C labelled ferritin which was maximal 8 hours after iron injection (mean twentyfold rise over the mean control value) and fell to near control levels at 19 hours (Fig. 51). Liver ferritin protein was still rising 19 hours after iron injection. The mean value at 19 hours was thrice that of the mean of control rats (Fig. 53). Liver ferritin iron (Fig. 52) followed a similar pattern to liver ferritin protein (Fig. 53), rising more swiftly initially but still increasing at 19 hours after iron injection when the mean value was three times that of mean value for control animals.

In both intestinal mucosa and liver, the specific activity of non-ferritin protein was similar to that in Group C, showing no significant variation. The fact that the specific activity of the non-ferritin protein remains constant emphasises the specificity of action of iron on ferritin synthesis, rather than on protein synthesis in general.



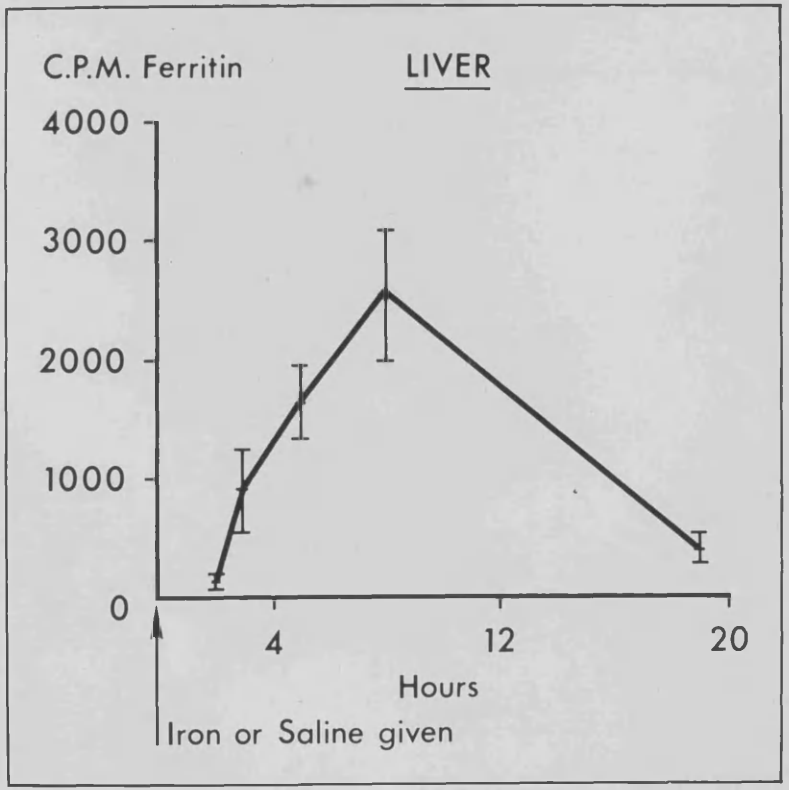
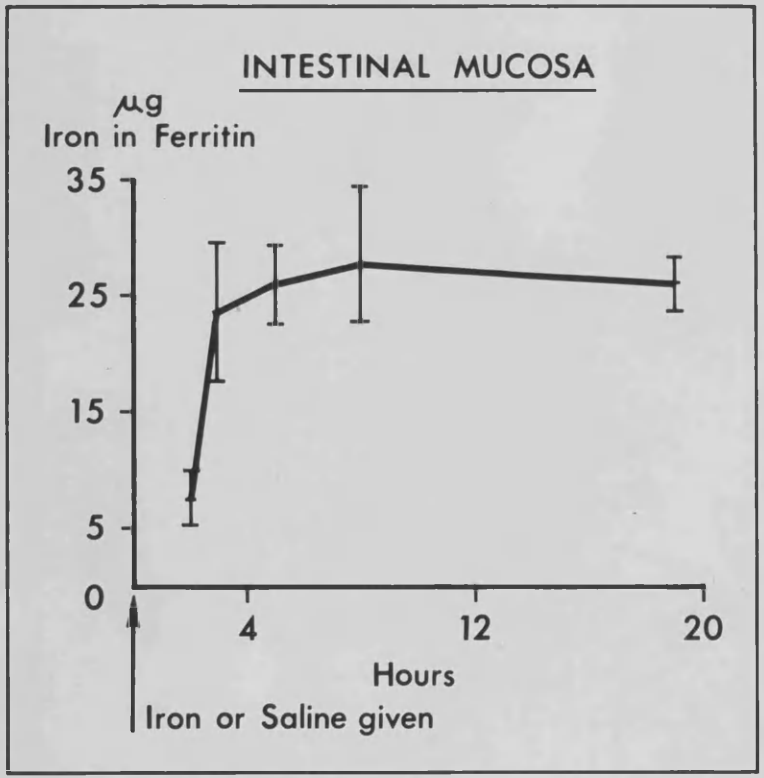


Figure 51. Induction of ^{14}C labelled ferritin in intestinal mucosa and liver at time intervals after 2.5 mg. parenteral iron per rat. Control rats, plotted at 2 hours, were given saline in place of iron. Each point represents the mean $\pm$ standard deviation of 6-8 rats (intestinal mucosa) and 5-7 rats (liver).



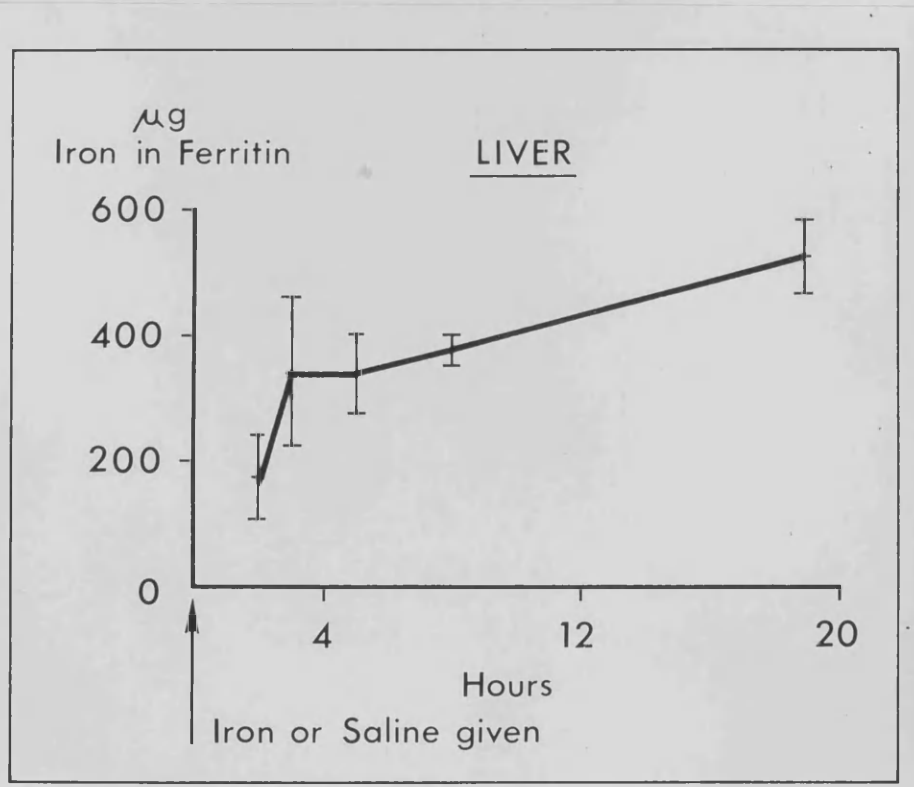
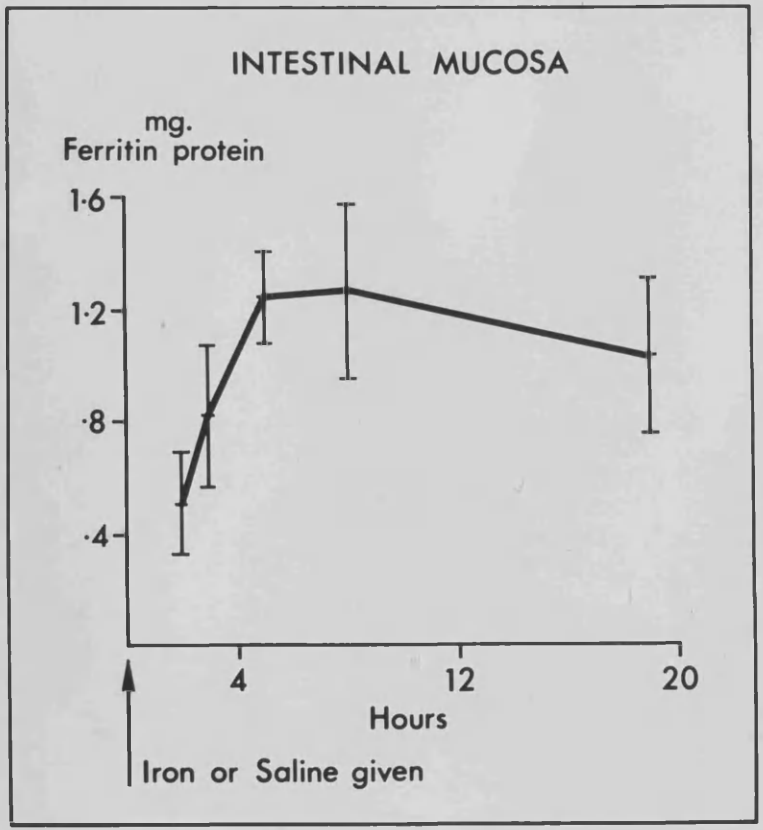


Figure 52. Total ferritin iron in rat intestinal mucosa and liver at time intervals after 2.5 mg. parenteral iron per rat. Control rats, plotted at 2 hours, were given saline in place of iron. Each point represents the mean $\pm$ standard deviation of 6-8 rats (intestinal mucosa) and 5-7 rats (liver).



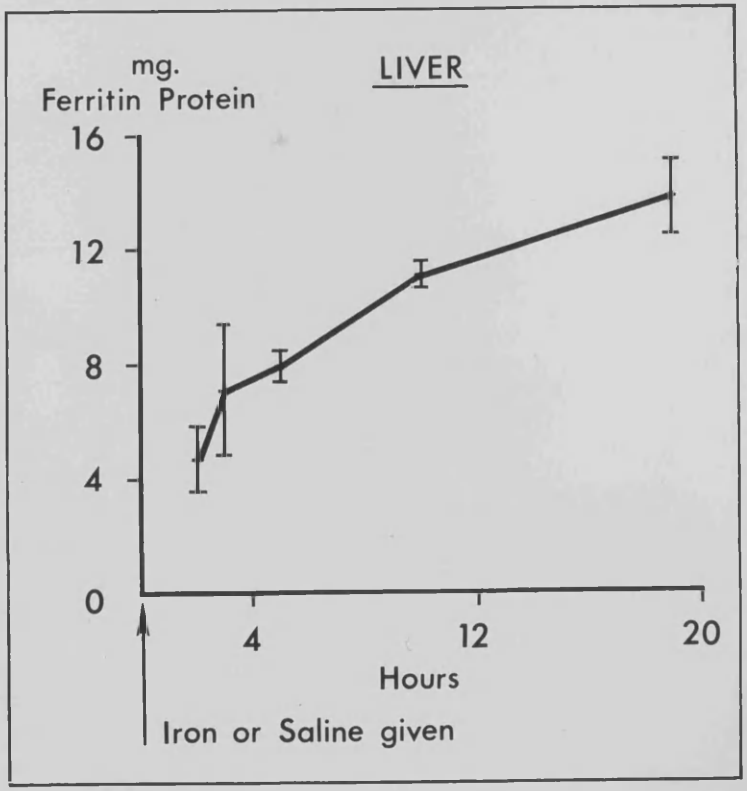


Figure 53. Total ferritin protein in rat intestinal mucosa and liver at time intervals after 2.5 mg. parenteral iron per rat. Control rats, plotted at 2 hours, were given saline in place of iron. Each point represents the mean $\pm$ standard deviation of 6-8 rats (intestinal mucosa) and 5-7 rats (liver).

Section D.III

Discussion

These experiments have demonstrated that the intramuscular injection of 1 or 2.5 mg of iron as iron sorbitol citric acid provokes a 5 - 10 fold increase of ferritin synthesis in the intestinal mucosa of the rat, as shown by the incorporation of ^{14}C labelled leucine into ferritin. The rise in ferritin synthesis was maximal about 3 to 5 hours after the injection of iron and gradually declined after about 8 hours. At the same time, with 2.5 mg of iron sorbitol citric acid, there was an increasing content of mucosal ferritin protein and ferritin iron; these increments were maximal at about 5 to 8 hours and remained high until 19 hours after injection. There was a 3 and 6 fold stimulation of ferritin synthesis in the rat intestinal mucosa when oral iron was given in a dose of 0.6 mg and 1.5 mg respectively to each rat. There has also been confirmation of the findings of other authors (Fineberg and Greenberg, 1955 a, and Drysdale and Munro, 1966) of the effect of parenteral iron on hepatic ferritin synthesis. The increased rate of ferritin synthesis in the liver was however

twice that which occurred in the intestinal mucosa, that is a 20-fold rise as against a 10-fold rise in the intestinal mucosa after 2.5 mg of iron in iron sorbitol. It can be argued that these doses of iron are grossly in excess of a comparable dose given weight for weight to man or a comparable amount of iron which might reach the intestinal mucosa. On the other hand over 30% of the iron in iron sorbitol is excreted in the kidney (Pringle et al, 1962) and only a proportion of the iron will in fact reach the intestinal mucosa after the parenteral route. Although the level of iron is greatly in excess of that which is achieved by an ordinary meal containing iron, it is not unusual for a dose of 200 - 400 mg of iron to be presented to the human intestinal mucosa in treatment of iron deficiency anaemia, a dose which is comparable with that given to the rats in the present experiments.

How can these results be interpreted in the light of Crosby's concept of iron absorption and excretion by the intestinal mucosal cells? From the present studies it would seem reasonable to suggest that the presence of ferritin in

the mucosal cell is influenced by the amount of iron which passes into it and that in states of iron deficiency, where there is little iron in any of the cells of the body, there is consequently very little ferritin or apoferritin in these mucosal cells. This absence of the "ferritin curtain" allows the unobstructed passage of iron through the cell. On the other hand in iron loaded states a considerable amount of iron present within the cell will provoke the synthesis of apoferritin to trap incoming iron, which is then excreted as ferritin when the cell is sloughed off. This will therefore diminish iron absorption in the iron loaded state (Fig. 54). These results substantiate Crosby's hypothesis and answer a question which this hypothesis has presented, namely why there is little mucosal ferritin in iron deficiency, but ample in states of iron repletion. They also help to explain how iron absorption and excretion may be controlled by the iron content of the body.

Although the exact mechanism of increased ferritin synthesis by iron in the intestinal mucosa has not been studied in the present experiments, such a mechanism may be surmised

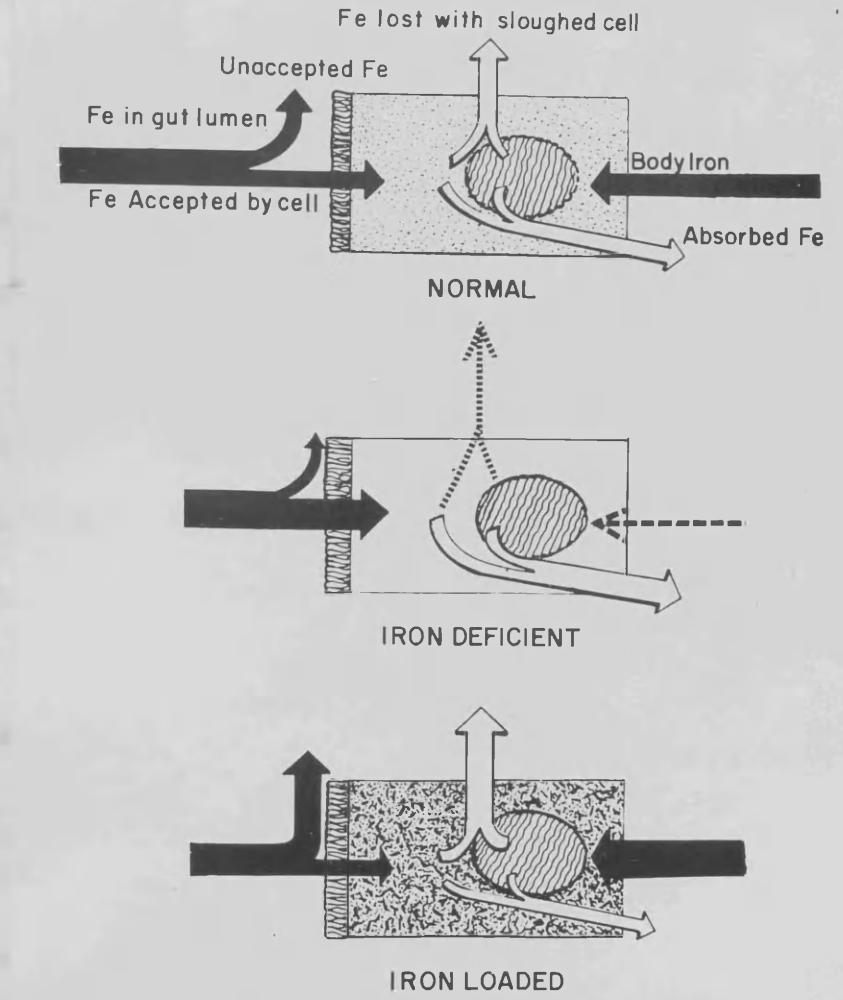


Figure 54. Control of iron absorption by the intestinal mucosa. In iron overload, there is much ferritin present in the mucosal cell and this tends to trap incoming iron, thus preventing absorption. In iron deficiency, there is little or no ferritin present, allowing iron to pass freely through the cell to be absorbed into the bloodstream.

from the work of Drysdale and Munro, 1966, on the mechanism of increased ferritin synthesis in rat liver after parenteral iron injection. The latter authors showed that the increased ferritin synthesis did not depend upon an increased production of messenger RNA since the stimulation was unaffected by actinomycin D, which blocks the synthesis of messenger - RNA. They concluded that iron causes an apparent induction of ferritin by stabilising apoferritin. Studies are in progress to challenge the possibility that this mechanism also occurs in the intestinal mucosa of the rat.

Summary

It has been suggested that the iron incorporated into ferritin of intestinal mucosa provides a means for the excretion of unrequired iron. In iron deficiency there is little mucosal ferritin but in states of iron repletion there is ample.

In the present study oral and parenteral administration of iron in the rat caused a 3 to 10 fold increase respectively in ferritin synthesis in intestinal mucosa,

assayed by the rate of incorporation of 1-^{14}C-DL-leucine into ferritin. Small scale isolation of mucosal ferritin was achieved by using a specific antiserum prepared by injection of horse spleen ferritin into rabbits.

These results help to explain how iron absorption and excretion may be controlled by the iron content of the body.

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