Summary of Thesis.

"STUDIES IN IRON METABOLISM"

Submitted for the degree of Ph. D.

in the Faculty of Medicine.

by

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SUMMARY

An enzyme system incorporating inorganic iron into protoporphyrin has been demonstrated in human and rat liver, kidney, spleen and in human bone marrow and measured, using radioiron as a tracer. Studies on the enzyme content of the intracellular fractions of human and rat liver showed that while there was some activity in all fractions, the mitochondrial fraction was the most active.

Dialysis of tissue homogenates led to a loss of enzymic activity which could be restored by the addition of ascorbic acid, GSH, cysteine or DPNH. The effect of these substances was not entirely due to an effect of reduction of the ferric iron since potentiation of activity resulted when these substances were added to a system containing iron in the ferrous form.

Observations have also been made on the in vitro biosynthesis of haem from protoporphyrin and iron bound to siderophilin or to rat liver homogenate. Rat liver homogenate or a preparation from it of mitochondria served as the source of iron-incorporating enzyme. The reducing substances namely ascorbic acid, GSH, cysteine and DPNH, which were effective in increasing the incorporation into protoporphyrin of inorganic ferric iron also substantially increased the transfer of protein-bound iron for haem biosynthesis. ATP enhanced the transfer of iron bound to siderophilin for incorporation into protoporphyrin but had no effect on the transfer of iron bound to rat liver protein. From this it is clear that physiological reducing substances play an important role

in the transfer of iron from its protein-bound form for incorporation into protoporphyrin in the process of haem biosynthesis.

A technique has been established for the measurement of iron incorporating enzyme activity in haemolysates of rabbit bone marrow containing a known number of red cell precursors. The method has been applied to marrows of normal and bled rabbits. In the bled animal, the enzymic activity of a unit number of cells was greatly decreased as compared to normal but was increased by the addition of reducing substances. This would indicate that the marrow responds to blood loss by an increase in the production of cells rather than an increased capacity of each cell to form haemoglobin. The enzymic activity of liver homogenates was also measured in normal and bled rabbits. In this tissue there was no difference in the enzymic activity per gram of liver between the normal and the bled animals. There was, however, a marked decrease in iron incorporation by liver cells from animals which had been injected with 300 mgs. Imferon. In two rabbits made iron deficient and anaemic there was an increase in iron incorporation as compared to normal. From this it is clear that gross changes in the size of the iron pool will vitiate the correct interpretation of results on the measurement of haem synthesis using radio-iron as a tracer.

Nakao et al (1960) have suggested that plasma samples from a variety of haematological disorders possess a factor (or factors) stimulating haem synthesis as compared to normal plasma. The 'haem-stimulating

activity' was measured by an in-vitro technique in which radio-iron incorporation was used as a measure of haem synthesis.

This work has been repeated using the same technique and results show that any marked increase in haem formation, measured in this way, could be explained by the diminution of the residual iron-binding capacity of the plasma sample.

It is concluded that no definite evidence has yet been obtained for the presence of a specific haem-stimulating factor in human plasma.

A statistically significant difference has been shown between the absorption of radio-iron given along with a standard meal, between patients with acid present in their gastric juice and patients with a histamine-fast achlorhydria. Both of these groups of patients were anaemic and there was an absence of stainable iron in the bone marrow of both groups. The mean absorption from the meal of the patients with acid in their gastric juice was 57.5 per cent while the mean absorption of those patients with histamine-fast achlorhydria was 18.5 per cent.

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I N D E X

P R E F A C E

I wish to acknowledge my grateful thanks to Professor E.J. Wayne, in whose department this work was carried out, to Dr. A. Goldberg for his constant help and encouragement, to Dr. S. Kramer who performed the cell counts detailed in Section 3, to the British Empire Cancer Campaign for their grant in support of this research, to Miss C. Charteris who typed the manuscript and to my husband for his patience and help in the checking of it.

INTRODUCTION

INTRODUCTION

Of all the iron present in the human body seventy per cent is contained within the erythrocyte haemoglobin. The greater part of iron metabolism is concerned with the formation and breakdown of this protein. The work of the past fifteen years has resulted in a great increase in our understanding of the mechanism of haemoglobin biosynthesis. In 1946, Shemin and Rittenberg showed that ^{15}N glycine specifically labelled the haem of human haemoglobin and that the remaining carbon atoms of protoporphyrin were derived from acetate via a four-carbon unit. This unit was succinate, probably in the form of succinyl-co-enzyme A (the thiol ester) which could be derived from the Krebs tricarboxylic acid cycle, for example from α-oxo glutarate (Shemin and Kumin, 1952).

The union of glycine and succinate yields α amino-β keto-adipic acid, which loses CO_2 probably by a non-enzymic reaction to form δ-amino-laevulic acid (ALA) (Shemin and Russell, 1953). Brown (1958) has demonstrated stimulation of ALA formation by lipoic acid in an <u>in vitro</u> system when α-oxoglutarate is the substrate. Pyridoxal phosphate has also been implicated in this reaction (Schulman and Richert, 1956) and more highly refined systems have been shown to be both pyridoxal phosphate and co-enzyme A dependant (Laver and Neuberger, 1957, and Kikuchi, Shemin

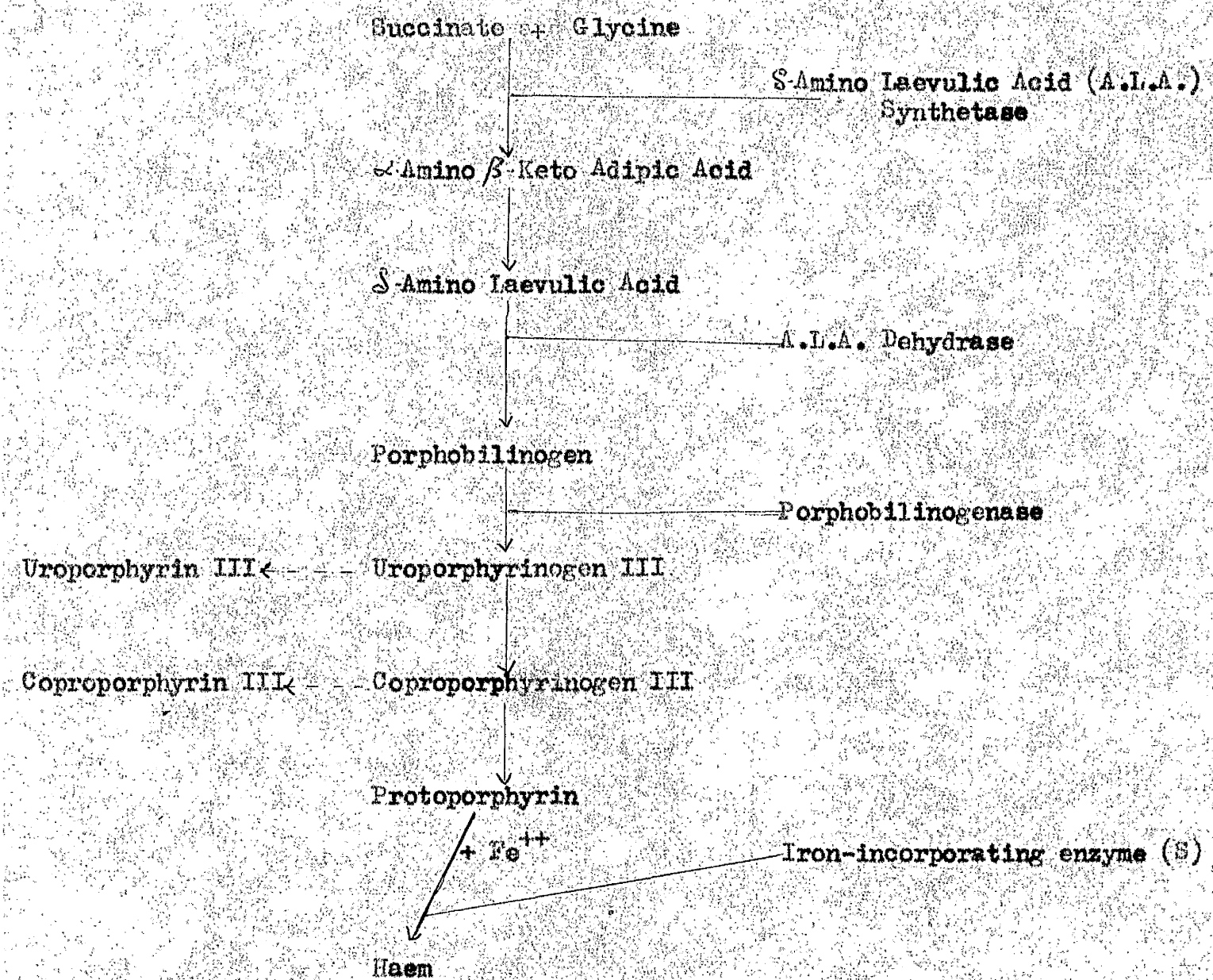


Figure 1. Scheme of Reaction for Haem Biosynthesis.

and Bachmann, 1958). Ferrous ions are also required for this reaction (Brown, 1959).

Two molecules of ALA then condense together, under the influence of a sulphydryl enzyme, δ -amino laevulic acid dehydrase, to form porphobilinogen. This enzyme is present in most of the tissues of the rabbit but is most active in liver, bone marrow, kidney and spleen (Gibson, Neuberger and Scott, 1955). It also occurs in yeast, bacteria and some plants (Gibson et al. 1955), as well as in the nucleated red cells of the chicken (Dresel and Falk, 1953) and of the duck (Schmid and Shemin, 1955). Within the liver cell, the enzyme is found in the soluble fraction, and is not attached to any particulate fraction (Gibson et al.,1955).

Porphobilinogen is further metabolised to uroporphyrin by an enzyme, porphobilinogenase, which produces under normal conditions the series III isomer, but if the enzyme solution is heated to 60°C yields uroporphyrin I (Lockwood and Rimington, 1957). Uroporphyrin is not readily utilised for haem biosynthesis (Dresel and Falk, 1956) but according to Neve, Labbe and Aldrich (1956) and Hoare and Heath (1958), suitably prepared uroporphyrinogen III is transformed to coproporphyrinogen III. From this it seems probable that only the reduced porphyrins lie on the direct biosynthetic pathway for haem formation. The free porphyrins which occur in tissues and excreta may be formed from these reduced

forms by auto-oxidation.

The side chain structures of coproporphyrinogen III (or coproporphyrin III) are further altered to yield protoporphyrin. Granick and Mauzerall (1958) have suggested that this conversion occurs in the particulate matter of chicken red cells via a porphyrin intermediate with three carboxylic acid side chain groups.

The final stage in haem formation is that of insertion of iron into the protoporphyrin structure. It is not known whether or not this occurs before haem is attached to the globin moiety. Eriksen (1955 and 1957) has suggested that a complex of protoporphyrin and globin is first formed and that iron is then inserted into the protoporphyrin molecule. Haem formation can occur non-enzymically if iron and protoporphyrin are heated together in warm glacial acetic acid but in chicken red cell homogenates (Goldberg, Ashenbrucker, Cartwright and Wintrobe, 1956) in preparations from duck erythrocytes (Kreuger, Melnick and Klein, 1956) and in rat liver homogenate (Minikami, 1958) the reaction has been shown to be enzymic. A system described by Perkoff, Schwartz and Tyler (1959) in which iron is incorporated into the haem of myoglobin is also enzymic.

Grinstein, Bannerman and Moore (1959) have shown that ^{14}C labelled protoporphyrin IX can be utilised for haem biosynthesis. The enzymic system which catalyses the incorporation of iron into

protoporphyrin has been fractionated and purified and recently Schwartz, Goudsmit, Hill, Cartwright and Wintrobe (1961) have demonstrated that the incorporation is facilitated by the presence of globin and have shown the end product of the reaction, as characterized by electrophoresis and chromatography, to be haemoglobin. This enzyme system responsible for the insertion of iron into protoporphyrin in chick red cell haemolysates has also been described by Goldberg (1959) and a "particle-free" preparation of the enzyme obtained. An ultra-filterable factor was found which potentiated the activity of the enzyme preparation. The activity of this factor was lost on oxidation. Ascorbic acid, reduced glutathione and ergothioneine, all physiological reducing substances, also increased the activity of the ultrafiltered enzyme preparation. The enzyme system described by Perkoff et al. (1959) for the incorporation of iron into myoglobin also required a dialysable factor. Addition of cysteine to the dialysed extract led to a partial restoration of its activity. Minikami (1958) also showed that cysteine potentiated the activity of his iron-incorporating enzyme preparation from rat liver.

In other stages of iron metabolism, reducing systems appear to play an important role. Thus at the stage of absorption from the gut, Moore, Dubach, Minnich and Roberts (1944) have shown that

ferrous iron is more readily absorbed than the ferric form. In a series of experiments using foodstuffs labelled with radio-iron, Moore (1955) showed that the addition of ascorbic acid resulted in a striking increase in the amount of iron absorbed from the diet. At pH values less than 5, iron is converted to a soluble ionic form and the reduction of ferric iron proceeds more readily. It seems possible therefore that the gastric acidity may influence the absorption of iron from the gut although Moore (1955) showed that non-anaemic subjects with achlorhydria do not show any reduced absorption of food iron and that absorption is not enhanced by the addition of hydrochloric acid. It is of importance that iron bound to protein, e.g. to ferritin or haemosiderin in iron storage depots, or to siderophilin, the iron-binding β-globulin in the plasma, is predominantly in the ferric (oxidised) form while iron in a state of transfer from proteins or through membranes must be in the ferrous (reduced) form. It would seem likely therefore that substances which can take part in oxidation-reduction systems might be involved in this shuttle service for iron.

The incorporation of iron into protoporphyrin appears to be deranged in several of the important anaemias - that of infection (Cartwright and Wintrobe, 1952), thalassaemia (Bannerman, Grinstein and Moore, 1959) lead poisoning (Goldberg, Ashenbrucker, Cartwright and Wintrobe, 1956; Eriksen, 1955), and the more recently described refractory normoblastic (sideroblastic) anaemias

(Dacie, Smith, White and Mollin, 1959; Heilmeyer, 1959). It would obviously be of value to establish a method for the determination of haem formation in the bone marrow and blood of patients with these and other anaemias in which haem formation may be impaired at the stage of iron incorporation. Previous attempts to compare the haem formation of the bone marrow or blood of one animal with another have shown considerable variation (Goldberg et al, 1956; Schwartz, Cartwright, Smith, and Wintrobe, 1959). Thomas (1955) compared different reference standards including fat free dry weight, nitrogen content, RNA and DNA content, total cell counts and differential cell counts and suggested that the best standard for comparison appeared to be the number of red cells.

Iron incorporation into protoporphyrin may also be affected by the humoral factors responsible for control of erythropoiesis. The study of humoral erythropoietic factors began with the much quoted paper by Carnot and Deflandre, (1906) describing the results of their work on rabbits, in which they bled a rabbit 30 ml. of blood on one day and on the next day bled the animal again and injected this serum into a normal rabbit. The red cell count in the recipient rose markedly within 24 hours. During the next forty years there were many attempts to repeat these findings but the results were conflicting and inconclusive.

Direct evidence for the existence of humoral factors controlling

red cell production was provided by Erslev (1953), Borsook, Graybiel, Keighley and Windsor (1953) and Gordon, Piliero, Kleinberg and Freedman (1954) and much confirmatory work has been done since that time. Most of the techniques used to measure the potency of erythropoietic factors require intact animals in which erythropoiesis has been partially inhibited before injection of the material for assay. For example, using hypophysectomised rats as recipients, Gurney, Jacobson and Goldwasser (1958) reported a high erythropoietin level in plasma and urine from patients with aplastic anaemia, acute leukaemia, and pernicious anaemia. Fried, Plzak, Jacobsen and Goldwasser (1957) showed that acute starvation slowed erythropoiesis and have used the starved animal as an assay subject. The same authors found that the inhibition of erythropoiesis caused by the repeated injection of red cells, causing a polycythaemia, could be reversed by the injection of plasma from anaemic donors. Linman, Bethell and Long (1958) have suggested that there may be two factors in plasma which control erythropoiesis, one which stimulates division of erythroblasts and one which enhances haemoglobin synthesis. Assay procedures using the intact animal are time-consuming and liable to error because of the variation between different animals and in factors other than those under test. Various attempts have therefore been made to demonstrate an erythropoietic factor, stimulating

haemoglobin formation, in an in vitro system. Normal rabbit serum has been shown to have a marked stimulatory effect on the rate of incorporation of ^{14}C labelled glycine into haem by rabbit bone marrow (Thomas, 1955). However, Goldberg et al. (1956) using an haemolysate of chicken blood as enzyme source and glycine as substrate, failed to show any difference in the uptake of radioiron into haem by deproteinised plasma of normal chickens and chickens made anaemic by either bleeding or by the administration of phenylhydrazine. Nakao and Hirashima (1960), using an haemolysate of chicken red cells as the source of enzyme and δ-amino-laevulic acid as substrate observed that addition of plasma from patients with polycythaemia, acute myeloid leukaemia and aplastic anaemia produced an increased incorporation of radio-iron into haem when compared to normal plasma. The discrepancy between these two studies is of interest since the method of measuring haem formation was similar; and because of the importance of establishing information on erythropoietic factors, it was necessary to determine the cause of differences.

1

INCORPORATION OF INORGANIC IRON INTO PROTOPORPHYRIN FOR HAEM FORMATION

Previous studies have shown that chicken erythrocytes and human reticulocytes contain the iron-incorporating enzyme or enzymes which incorporate iron into protoporphyrin to form haem (Goldberg, 1959). In the adult, the tissues predominantly concerned in haem synthesis are the bone marrow and liver. In the foetus, and in pathological states of extramedullary erythropoiesis in the adult, the liver and spleen play a major role in haem synthesis. Gibson, Neuberger and Scott (1955) have shown that the enzyme δ-amino-laevulic dehydrase is present not only in bone marrow and liver, but also in the kidney and spleen of the rabbit. The following experiments on human and rat tissues were undertaken to determine the sites - and their relative activity of the iron-incorporating enzyme system.

Bessis and Breton-Gorius (1957), have shown by electron microscopy that iron is preferentially located in the mitochondria of the haemoglobin-forming erythroblast. These histological studies have been a stimulus to determine which intracellular component is the richest source of the iron incorporating enzyme system. Ascorbic acid and reduced glutathione (GSH) potentiate this enzyme system when it is derived from chicken blood (Goldberg, 1959). Further investigations were therefore carried out to determine the effect of these and other substances on the enzyme system derived from human and rat tissues.

MATERIALS AND METHODS

Materials

Radio-iron, $^{59}FeCl_3$, specific activity 1 - 3 mc. per mg. iron and ^{59}ferrous iron (in ascorbic acid solution), was obtained from A.E.R.E., Harwell. Protoporphyrin methyl ester was prepared from blood by the following method.

The serum is removed from a specimen of time-expired human blood obtained from the Blood Bank. The haemoglobin is precipitated by the addition of 10 volumes of acetone, with continuous stirring and the mixture allowed to stand overnight. The acetone powder is filtered through a Buchner funnel and dried with absolute alcohol and ether. When dry it is transferred to centrifuge tubes and extracted two or three times with fresh aliquots of 10 per cent oxalic acid in redistilled methanol.

The iron is removed and the protoporphyrin converted to its dimethyl-ester by blowing hydrogen chloride gas very rapidly through 200 ml. portions of the haem solution in the presence of ferrous sulphate. When all the haem is converted to protoporphyrin dimethyl ester, confirmed by checking the spectrum, the solution is cooled, excess water is added and the protoporphyrin quickly and completely extracted into chloroform. The chloroform solution is washed twice with 2 volumes of water, then once with 2N ammonia. A precipitate results which is removed by filtration. Finally the chloroform is washed three times with water, filtered,

concentrated on a water bath and the protoporphyrin crystallised from hot chloroform/methanol. The absorption maxima in chloroform were 505 mμ, 545 mμ, 575 mμ and 630 mμ and in pyridine the maximum was at 408 mμ. One batch of protoporphyrin methyl ester was a gift from Professor Rimington, F.R.S. The ester was hydrolysed with 25% (per cent) HCl for 16 hours, dried in a dessicator and the free protoporphyrin re-suspended in M/7 sodium bicarbonate and the final solution adjusted to pH 7.4. Ascorbic acid, GSH, cysteine, as its hydrochloride (British Drug Houses), reduced diphosphopyridine nucleotide, (DPNH)(Sigma), were dissolved in water and the resultant solutions adjusted to pH 7.4 before use. The water used throughout these experiments was rendered ion-free by passage through an Elgastat de-ioniser. All glass-ware was washed first with chromic acid, then with 2N HCl and finally washed free from acid with water.

Methods

Preparation of tissue samples.

Rat tissues: Young female albino rats, Wistar strain, were anaesthetised with ether and killed by exsanguination. Rat intestine was washed free of its contents with repeated washes of 0.9 per cent sodium chloride. Each of the tissues was washed with water, dried on filter paper, weighed and homogenised with 3 or 7 times its volume of 0.25 M sucrose in an ice-cooled Potter glass homogeniser.

To the homogenate was added one-fourth of its volume of 0.45M

TRIS (tris 2-amino. 2-hydroxymethylpropane 1.3 diol)buffer pH 7.4. In the case of liver homogenates, intracellular constituents were fractionated (from a one in 8 homogenate) by the method of Schneider (1948) into nuclei, mitochondria and microsomes plus cell sap. Each fraction was suspended in 0.25M sucrose to the same volume as the original homogenate, and one fourth volume of 0.45M TRIS buffer pH 7.4 was added.

Human tissues: Samples of spleen and kidney removed from patients undergoing surgery for splenectomy or nephrectomy were treated similarly. Biopsies of normal liver were obtained from patients undergoing upper abdominal surgery. Bone marrow samples were obtained in the following way: a 10 ml. sample of mixed bone marrow and blood was withdrawn from the iliac crest by the method of Rheingold, Weifuse and Dameshek (1949) with a heparinised syringe and transferred to an ice-cooled heparinised measuring cylinder. The blood-bone marrow tissue was haemolysed by the addition of 20 ml. water and isotonicity restored with 5 ml. TRIS 0.45M, pH 7.4.

Incubation: The incubation was performed in 50 ml. Erlenmeyer flasks, each of which contained a portion of the material whose enzyme activity was to be measured, protoporphyrin, 1 µc $FeCl_3$, penicillin G and streptomycin sulphate, to prevent bacterial contamination, reducing substance (as required), 1 ml. TRIS buffer 0.45M and 0.9 per cent sodium chloride solution to a total volume of 10 ml. The effect of protoporphyrin concentration on haem formation by rat

liver homogenate is shown in Figure 2. A concentration of 2×10^{-5}M protoporphyrin was added in subsequent experiments.

The effect of changes in the pH of the incubation mixture on the ability of rat liver homogenate to incorporate radio-iron into protoporphyrin is shown in Figure 3.

The optimum pH is about 7.45 and is a fairly sharp peak. All the experiments were carried out therefore at pH 7.4. The pH of the flask contents rose during the course of the incubation by about 0.2 - 0.3 pH unit.

The effect of time of incubation on the incorporation of radio-iron into haem by rat liver homogenate is shown in Figure 4. The incubation time for subsequent experiments was 3 hours. The flasksfor incubation contained therefore the material for assay of enzyme activity (in 1 - 5 ml.), 1 μc $FeCl_3$, 2×10^{-5}M protoporphyrin, 1 ml. TRIS buffer 0.45M pH 7.4, ascorbic acid, GSH, cysteine or DPNH when used in a volume of 1 ml., 2 mgs. each of penicillin G. and streptomycin sulphate and 0.9 per cent sodium chloride solution to a total volume of 10 ml. After incubation at 37°C for 3 hours the reaction was stopped by the addition of 1 ml. M sodium cyanide.

Twenty-five ml. of carrier haemoglobin was added to each flask to facilitate isolation of haem (Goldberg et al.,1956). This carrier haemoglobin solution was prepared by haemolysing one volume of washed human red cells, with one volume of water. Isotonicity was restored by the addition of sodium chloride to a concentration of 0.9 per cent by weight.

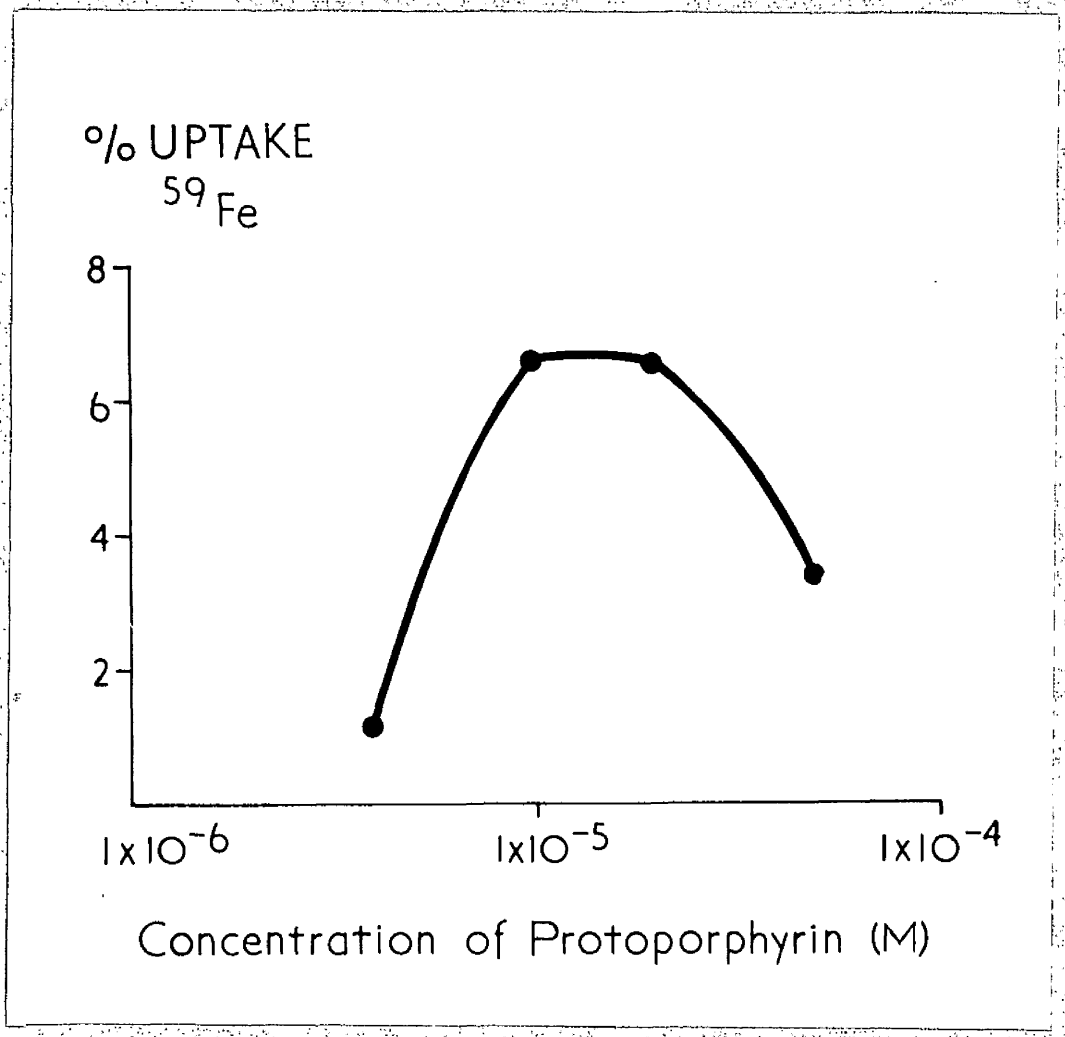


Figure 2. Effect of concentration of protoporphyrin on the iron-incorporating enzyme activity of rat liver homogenate.

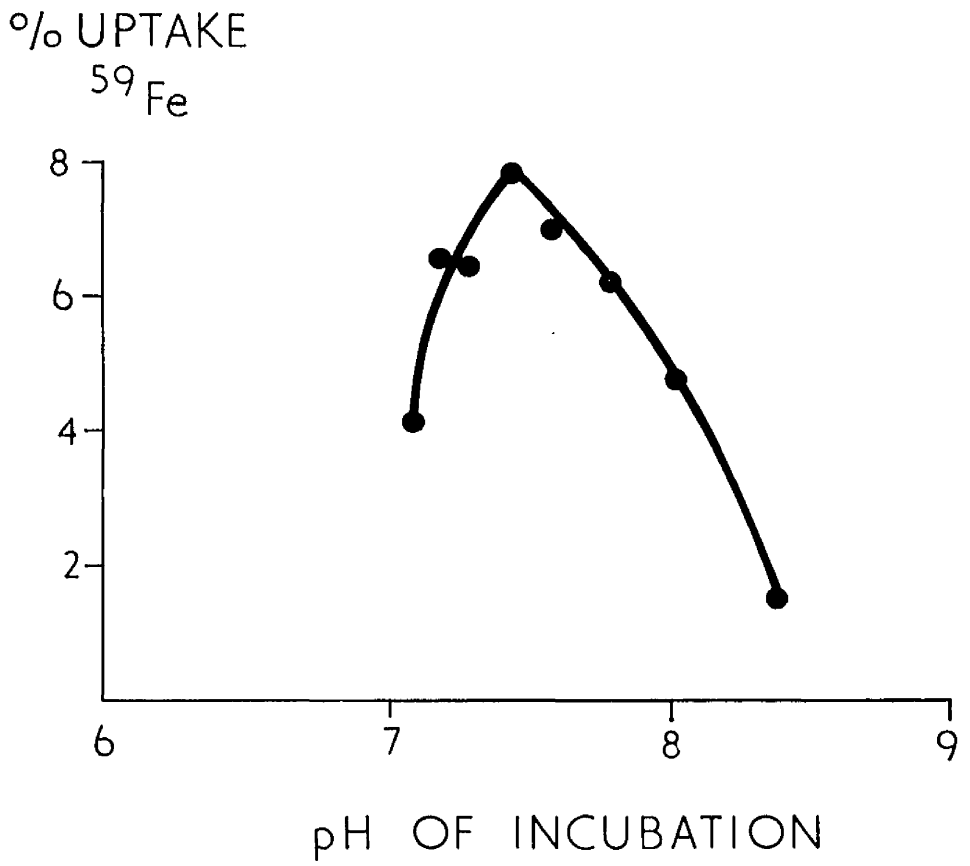


Figure 3. Effect of pH on the iron-incorporating enzyme activity of rat liver homogenate.

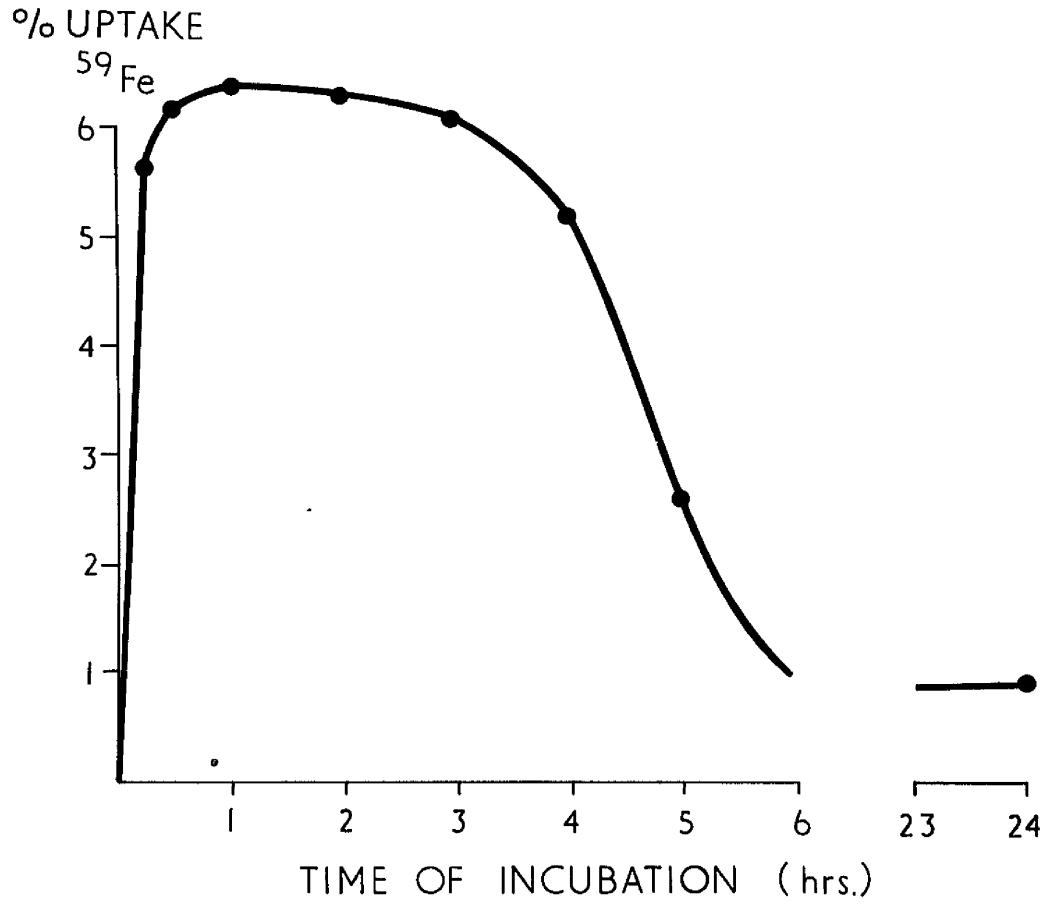


Figure 4. Effect of time of incubation on incorporation of iron into protoporphyrin by rat liver homogenate.

The flask contents were homogenised for 3 minutes in a Waring blender and centrifuged at 1600 g. for 20 minutes to remove tissue debris. Haem was isolated from the supernatant fluid by the method of Fischer (1941) and purified and recrystallised by the method of Shemin, London & Rittenberg (1950). Weighed samples of the isolated haem were counted in a well-type scintillation counter with a counting efficiency for iron of 22 per cent. The standard error of the counting procedure was ± 2 per cent and the reproducibility of the system as a whole ± 5 per cent.

Ascorbic acid and GSH contents of the rat liver homogenate and marrow-blood tissue were determined by the methods of Roe and Kuether (1943), and Woodward & Fry (1932) respectively. Haemoglobin concentration was measured by the alkaline haematin method of Clegg & King (1942).

RESULTS

All results are expressed as the percentage of added radio-iron which was incorporated into the haem. The total number of counts in the haem was obtained by multiplying the counts per milligram of haem by the weight of haem (mg.) in each flask after the addition of the carrier haemoglobin.

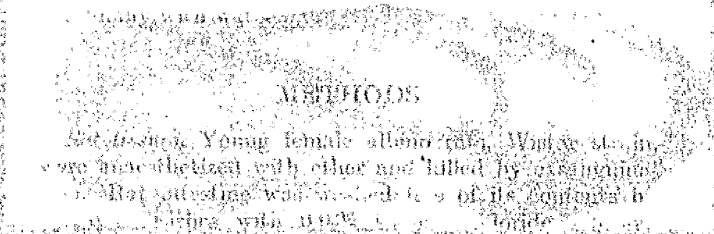
Rat tissues.

Liver: Ten rat liver homogenates gave an average uptake of ^{59}Fe of 4.8 per cent/g. of wet weight of tissue (S.D. $\pm$ 2.4, range 1.2 - 9.3 per cent). After dialysis against water at 4°C for 24 hours, the enzymic activity of the homogenate was decreased to about 15 per cent of the original value. The addition of ascorbic acid, GSH, cysteine or DPNH restored the dialysate to its former activity. The GSH and ascorbic acid contents of three liver homogenates before and after dialysis are shown in Table I. The effects of varying concentrations of ascorbic acid and reduced glutathione on dialysed rat liver preparations are shown in Figures 5 and 6 respectively. Maximum potentiation occurred at about 2×10^{-3}M ascorbic acid and 1.5×10^{-2}M GSH. Cysteine and DPNH also caused a potentiation in iron incorporation. The influence of these substances are shown in Figures 7 and 8 respectively. In order to see whether the effect produced by the addition of these physiological reducing substances could be entirely explained by an effect on the reduction of iron to its ferrous form, the following experiment was carried out.

TABLE 1.

GSH and ascorbic acid contents of three liver homogenates before and after dialysis against water at 4°C for 22 hr.

Ascorbic acid (μg./g. of tissue)		GSH (μg./g. of tissue)	
Before dialysis	After dialysis	Before dialysis	After dialysis
161	8	2200	400
177	6	2200	600
159	4	1800	300



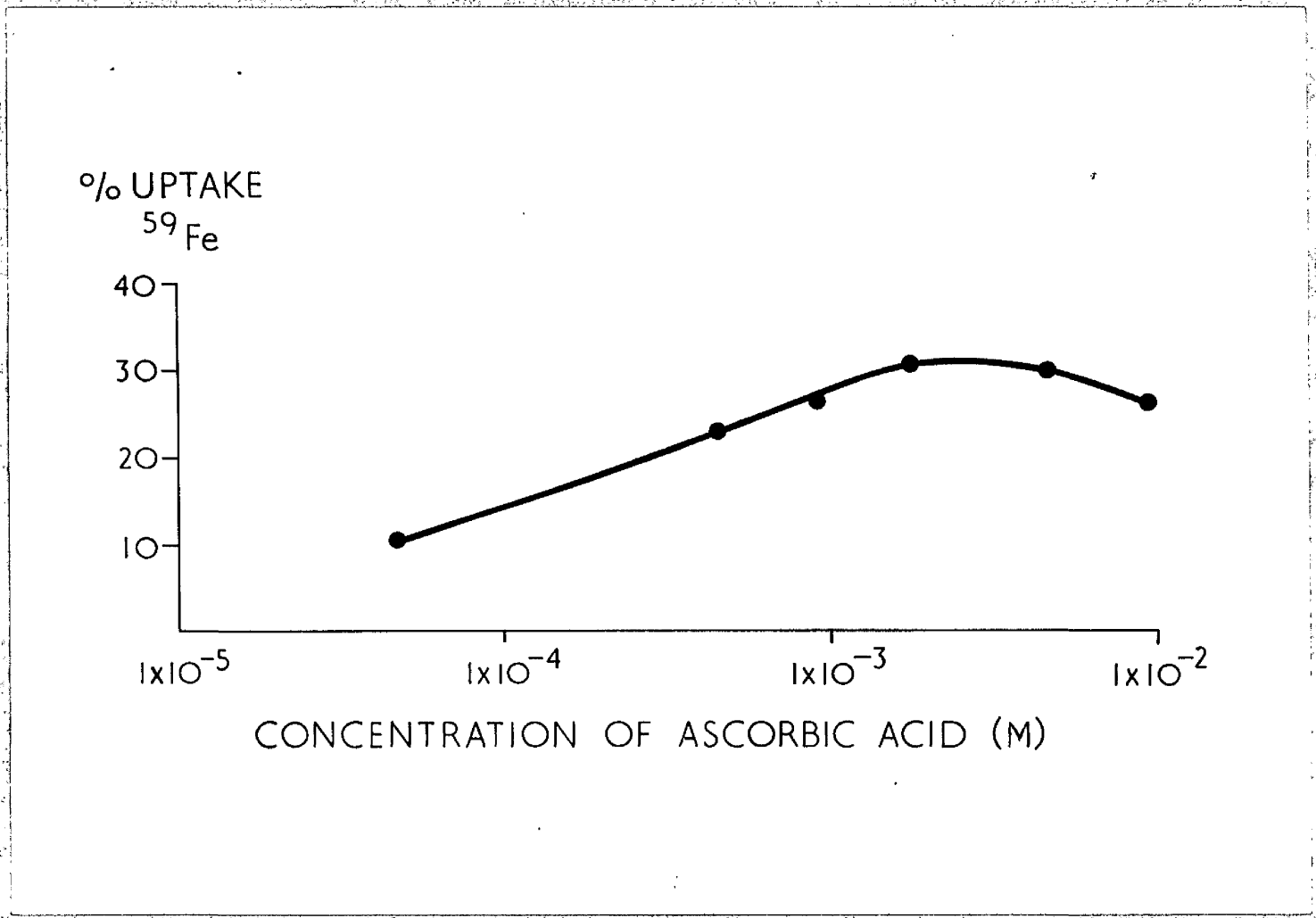


Figure 5. Effect of varying concentrations of ascorbic acid on the iron-incorporating enzyme activity of dialysed rat liver homogenate, at pH 7.4.

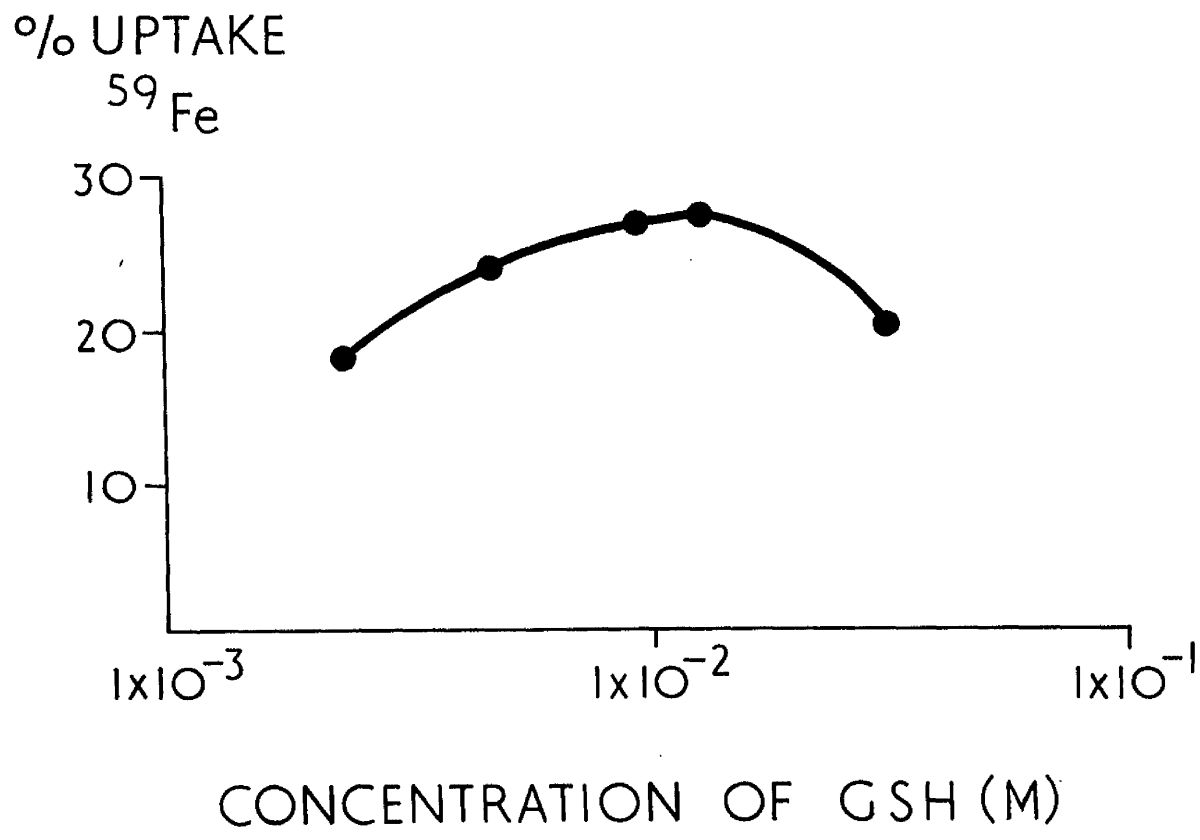


Figure 6. Effect of varying concentrations of reduced glutathione on the iron incorporating enzyme activity of dialysed rat liver homogenate at pH 7.4.

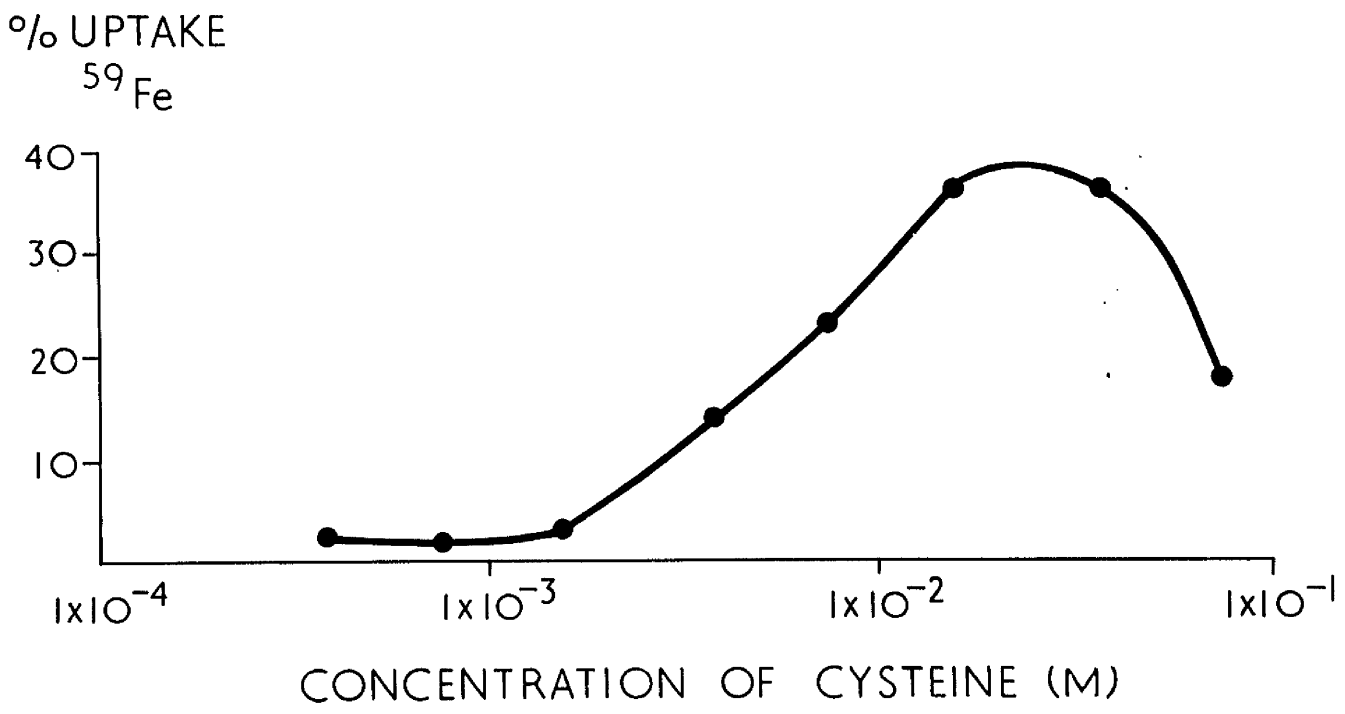


Figure 7. Effect of varying concentrations of cysteine on the iron-incorporating enzyme activity of dialysed rat liver homogenate at pH 7.4

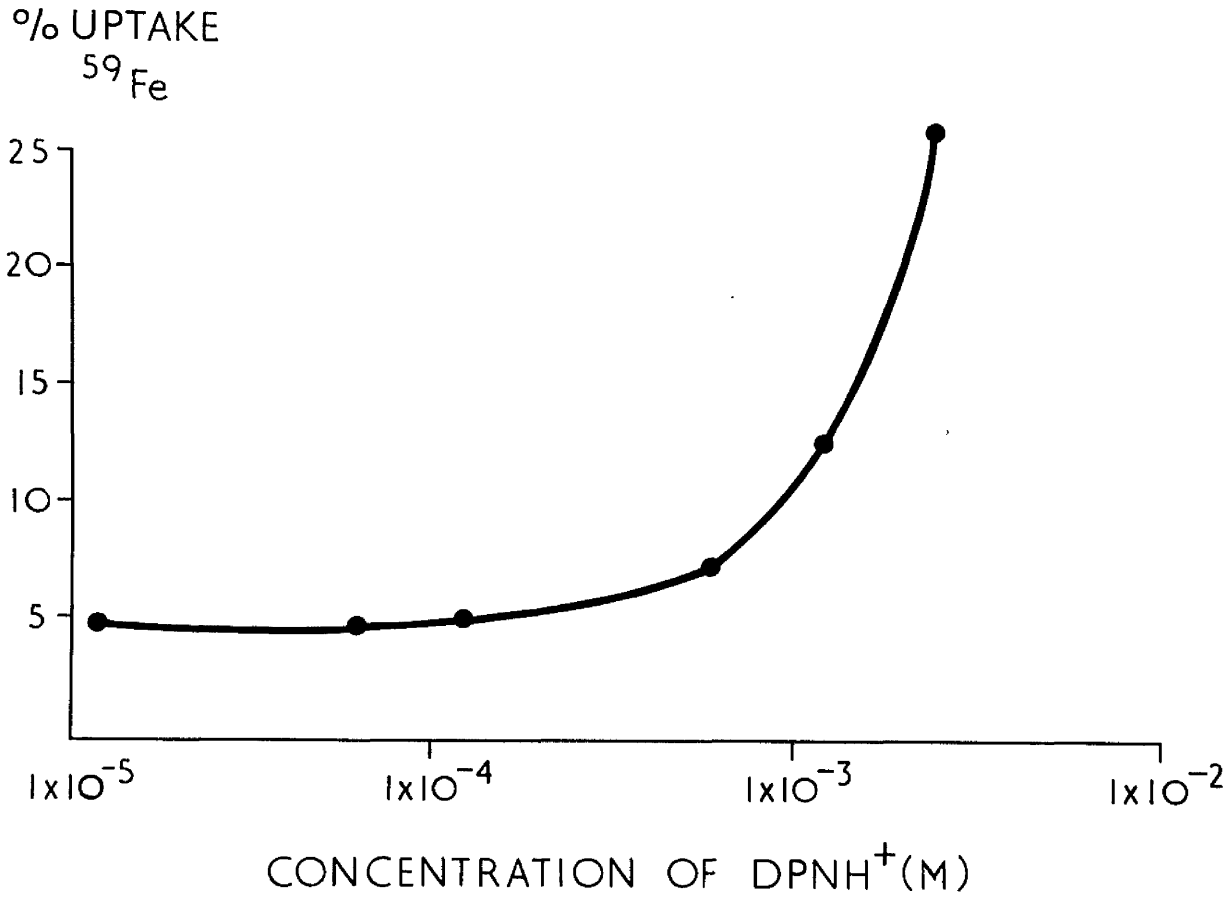


Figure 8. Effect of varying concentration of DPNH on the iron-incorporating enzyme activity of dialysed rat liver homogenate at pH 7.4.

Aliquots of dialysed rat liver homogenate were incubated with equal concentrations of radio-active ferrous iron, maintained in the ferrous state by the presence of ascorbic acid (ascorbic acid content per flask = 5×10^{-5}M) and with ferric chloride alone and in the presence of optimal concentrations of ascorbic acid, glutathione and cysteine (Table II). It is clear that ferrous iron is more readily incorporated than the ferric form and that even when the iron is in the reduced form, potentiation of iron incorporation occurs in the presence of ascorbic acid, GSH and cysteine.

The enzyme activities of the intracellular constituents of liver isolated from a sucrose homogenate are shown in Table III. Most of the activity is present in the mitochondrial fraction. Ascorbic acid and GSH increased the activity of all intracellular constituents.

Kidney and Spleen: Kidney homogenates showed an average uptake of ^{59}Fe of 0.8 per cent/g. wet weight in eight samples tested (S.D. $\pm$ 0.4, range 0.2 - 1.2 per cent) and spleen homogenates gave a similar incorporation with a mean value of 0.6 per cent in six samples (S.D. $\pm$ 0.3, range 0.3 - 1 per cent). Another tissue which showed marked enzyme activity was heart muscle which had an average uptake of 1.6 per cent (S.D. $\pm$ 0.5 per cent) in six samples. Small intestine, brain and leg muscle had very little enzymic activity (0.1 per cent incorporation). Heat treatment, i.e. placing in a boiling water bath for 4 minutes caused almost complete loss of activity in each of the tissue homogenates.

TABLE II.

Effect of ascorbic acid, reduced glutathione and cysteine on the incorporation of radio-iron in the ferrous and ferric forms into haem.

Addition	Per Cent Incorporation ^{59}Fe Per Gram Wet Weight	
	Ferric Iron	Ferrous Iron
None	1.6	11.9
Ascorbic acid 2×10^{-3} M	6.7	59.3
GSH 1.5×10^{-2} M	41.8	69.3
Cysteine 1×10^{-2} M	10.9	84.8

TABLE III.

Comparison of iron-incorporating enzyme activities of intracellular constituents of rat liver homogenate in sucrose and the effect of ascorbic acid and reduced glutathione on these activities.

Incubations were carried out under standard conditions at pH 7·4 (see Methods section) with 1 μc of $^{59}FeCl_3$ and 20 μM-protoporphyrin, with and without the addition of ascorbic acid (2 mM) or GSH (15 mM). Control flasks were made up in the same way as the test flasks but contained no enzyme source.

Expt. no.	Tissue source	Uptake of ^{59}Fe/g. of wet tissue (%)		
		No addition	With ascorbic acid	With GSH
1	Homogenate	6·3	8·7	8·4
	Nuclei	0·6	1·2	0·6
	Mitochondria	4·8	12·7	6·6
	Microsomes and cell sap	0·9	3·3	1·8
	Control	<0·1	0·4	<0·1
2	Homogenate	3·5	3·8	4·0
	Nuclei	0·5	2·2	1·9
	Mitochondria	2·5	6·2	4·2
	Microsomes and cell sap	0·3	1·8	1·5
	Control	—	0·2	<0·1

Human Tissues.

Bone Marrow: Bone marrow showed small but significant iron-incorporating enzyme activity in nine samples tested (Table IV) Normal human blood does not have significant iron-incorporating activity (Goldberg, 1959). In only one case (No. 6) did the addition of ascorbic acid or glutathione significantly increase the activity of the bone marrow samples. In this case (a patient with bronchial carcinoma and bone marrow metastasis) the marrow ascorbic acid and glutathione levels were very low. The marrow from a patient with scurvy and a patient with haemochromatosis showed values which were within the normal range.

Liver. A sucrose homogenate of liver contained significant iron-incorporating enzyme activity. The mitochondria contained most of the activity (Table V). The addition of ascorbic acid (1.4×10^{-4}M) and GSH (6.5×10^{-3}M) potentiated the activity of all the cellular constituents while heat treatment caused complete loss of activity, of the homogenate alone, and of the homogenate to which ascorbic acid (1.4×10^{-3}M) and GSH (6.5×10^{-3}M) had been added.

Spleen. Iron-incorporating activity was found in homogenates of two human spleens (Table VI) removed from patients with acquired haemolytic anaemia and myelofibrosis respectively. Dialysis of the homogenates against water for 24 hours reduced the activities to 10 per cent and 22 per cent respectively. Addition of ascorbic acid or GSH greatly potentiated the activity of the dialysate in both cases.

TABLE IV. **Iron-incorporating enzyme activity of iliac-crest 'bone-marrow-blood' tissue in normal and pathological states and the effect of ascorbic acid and reduced glutathione on this activity.**

Standard incubation procedure was followed with 1 μC of $^{59}FeCl_3$ and 20 μM-protoporphyrin with the addition where stated of 1·4 mM-ascorbic acid or 6·5 mM-GSH. Samples of the blood–bone-marrow tissue were analysed chemically for ascorbic acid, GSH and iron. Hb, Haemoglobin; P.C.V., packed-cell volume.

Patient no.	Diagnosis	Age (years)	Sex	Hb (g./100 ml.)	P.C.V. (%)	Uptake of ^{59}Fe/g. of wet tissue (%)			Marrow-blood analysis		
						Marrow alone	Marrow + ascorbic acid (1·4 mM)	Marrow + GSH (6·5 mM)	Ascorbic acid (mg./100 ml.)	GSH (mg./100 ml.)	Iron (μg./100 ml.)
1	Osteoarthritis	57	M	15·9	49·5	0·3	0·7	0·7	1·8	14	—
2	Rickets	14	M	13·0	44·5	0·5	0·5	0·5	1·2	23	108
3	Thyrotoxicosis	51	M	14·4	47·0	0·2	0·5	0·6	2·5	13	—
4	Cerebral thrombosis	57	M	15·9	47·5	0·2	0·2	—	1·2	—	83
5	Combined deficiency of vitamin B_{12} and folic acid	54	M	10·7	34·5	0·3	0·5	0·5	2·1	22	—
6	Bronchial carcinoma; deposits in bone marrow	62	F	13·9	42·5	0·3	1·4	1·5	0·5	6	—
7	Scurvy (treated)	58	M	8·4	25·0	0·3	0·4	0·3	0·97	19	100
8	Haemochromatosis	57	M	13·6	43·0	0·4	0·3	0·5	1·6	16	164
9	Thyrotoxicosis; iron-deficiency anaemia	59	F	11·1	39·0	0·1	0·3	—	0·96	—	70

TABLE V.

Comparison of iron-incorporating-enzyme activities of intracellular constituents of human liver homogenate in sucrose and the effect of ascorbic acid and reduced glutathione on these activities.

Standard procedure for incubation was followed at pH 7·4 with 1 μc of $^{59}FeCl_3$ and 20 μM-protoporphyrin. Ascorbic acid and GSH were added as indicated. Control flasks contained 0·9% sodium chloride solution in place of a source of enzyme.

Expt. no.	Tissue source	Uptake of ^{59}Fe/g. of wet tissue (%)		
		No addition	With L-ascorbic acid (0·14 mM)	With GSH (6·5 mM)
1	Homogenate	1·7	2·3*	44·4
	No enzyme source	<0·1	—	<0·1
2	Homogenate	1·2	3·5	10·9
	Mitochondria	1·0	1·0	2·8
	No enzyme source	<0·1	<0·1	—
3	Homogenate	3·4	6·8	15·3
	Nuclei	0·1	—	—
	Mitochondria	2·6	3·0	4·0
	Microsomes and cell sap	0·3	—	—
	No enzyme source	<0·1	<0·1	—

* D-Ascorbic acid gave similar incorporation.

TABLE VI.

Iron-incorporating enzyme activities of samples of spleen from a case of haemolytic anaemia and from a case of myelofibrosis and of samples from two kidneys; effect on the activity of these samples of dialysis and of the addition of ascorbic acid and reduced glutathione to dialysed and undialysed samples.

Tissue samples were homogenized in sucrose and incubated with 1 μC of $^{59}FeCl_3$ and 20 μM-protoporphyrin under standard conditions. Control flasks containing no enzyme source gave an uptake of ^{59}Fe into haem of less than 0·1 %.

Case	Tissue	Uptake of ^{59}Fe/g. of wet tissue (%)		
		No addition	With ascorbic acid (0·14 mM)	With GSH (6·5 mM)
Haemolytic anaemia	Spleen homogenate	1·3	1·8	2·7
	Dialysed spleen homogenate	0·1	2·0	1·2
Myelofibrosis	Spleen homogenate	5·4	12·8	15·1
	Dialysed spleen homogenate	1·2	19·6	31·1
Nephrectomy (renal calculus)	Kidney homogenate	0·7	5·7	42·2
Nephrectomy (renal neoplasm)	Kidney homogenate*	1·3	4·0	12·8

* Sample obtained from non-tumour-bearing area.

There was histological evidence of extramedullary erythropoiesis in the spleen from the patient with myelofibrosis, but not in the spleen from the patient with acquired haemolytic anaemia. The enzymic activity per unit weight of the former was about four times that of the latter.

Kidney: Two samples pf kidney showed iron-incorporating enzyme activities of 0.7 per cent and 1.3 per cent respectively. The addition of ascorbic acid and GSH potentiated these activities (Table VI).

DISCUSSION.

These experiments have demonstrated the presence of an iron-incorporating enzyme system in human and rat liver, spleen and kidney and in rat heart muscle as well as in human bone marrow. The enzyme activity is present in human tissues which synthesize haemoglobin in adult or foetal life or in pathological states of extramedullary erythropoiesis; the spleen in which this was present contained four times the enzyme activity per unit weight of that in which extramedullary erythropoiesis was absent. The location of the iron-incorporating enzyme system may also be explained by the formation of the cytochromes, catalase, and peroxidase by these tissues and can be related to the findings of Gibson et al. (1955) that δ-amino laevulic dehydrase is present in the bone marrow, liver, kidney and spleen of the rabbit. These results also confirm those of Minikami (1958) who, using a substantially different technique of enzyme measurement, was able to demonstrate iron-incorporating enzyme activity in rat liver, kidney, spleen, heart, brain and muscle.

The pH optimum found for rat liver homogenate as enzyme source agrees with the results of Nishida and Labbe (1959), although Minikami (1958), using a cholate extract of rat liver homogenate found a pH optimum around 7, and Rimington and Tooth (1961), using rat liver mitochondria as enzyme source and with the addition of 1×10^{-3}M ascorbic acid found maximum haem formation at pH 7.1.

Fractionation of the cellular contents of the human and rat liver

demonstrated that the mitochondria were the most active source of the iron-incorporating enzyme system. This finding is important in view of the work of Bessis and Breton-Gorius (1957), who showed that ferritin iron is mainly concentrated in the mitochondria of the erythroblast.

That the mitochondria are the richest source of the iron-incorporating enzyme system in the cell has also been confirmed by Miniknmi (1958) and Nishida and Labbe (1959). On the other hand, Rabinowitz and Olsen (1958) have suggested that in rabbit reticulocytes the microsomes are responsible for iron-incorporation into haemoglobin, and Schweet, Lamfrom and Allen (1958) have demonstrated the synthesis of haemoglobin in a cell-free system. These authors have found that non-cellular preparations containing no other particles than microsomes were able to synthesise the whole haemoglobin molecule provided iron and protoporphyrin were added to the system. Eriksen (1962), has suggested that these findings can be explained as being due to the presence of solubilised enzyme in the preparations of the above authors since Schwartz, Cartwright, Smith and Wintrobe (1959) have shown that although the enzyme in the chicken blood is associated with particles much heavier than microsomes, it can be easily separated from the particulate matter.

It is interesting to note also that the enzyme responsible for the formation of protoporphyrin from its immediate precursor, coproporphyrinogen, has been shown to be predominantly located in the

mitochondria of guinea pig liver (Sano and Granick, 1961).

Goldberg (1959) demonstrated that ascorbic acid and GSH in consentrations of the order present in human or chicken blood potentiate the iron-incorporating enzyme system when it is derived from chicken blood. It has now been shown that these physiological reducing substances potentiate iron incorporation in several human and rat tissues. Dialysis of tissue homogenates not only caused a marked reduction of their ascorbic acid and GSH contents (Table I) and presumably the content of other reducing substances, but also significantly decreased their enzymic activities, while addition of ascorbic acid, GSH, cysteine and DPNH to the dialysed homogenates often more than restored the activities. As has been shown, this is not entirely due to the effect of these substances in reduction of the ferric iron to ferrous form. Thus the initial loss of activity on dialysis was directly related to the loss of reducing substances. Mazur, Baez & Shorr (1955) have suggested that ascorbic acid and GSH and cysteine mobilise ferritin iron for haemoglobin synthesis and the present study has certainly confirmed the important bearing of ascorbic acid, GSH and cysteine on the mechanism of iron incorporation.

INCORPORATION OF PROTEIN-BOUND IRON INTO PROTOPORPHYRIN FOR HAEM FORMATION

About 75 per cent of the iron in the human body is bound to porphyrin molecules forming the prosthetic groups of haemoglobin, myoglobin and the tissue haem enzymes, catalase, peroxidase and the cytochromes. The remainder of the body iron is either stored throughout the reticulo-endothelial system in the proteins ferritin and haemosiderin or is attached to the plasma in siderophilin (transferrin), a β_1-globulin.

Haem biosynthesis has been shown to occur in many tissues of the body, (Minikami, 1958; Nishida and Labbe, 1959;) but the mechanism of the transfer of iron from ferritin, haemosiderin and siderophilin to protoporphyrin for haem formation is not yet clear.

Reduced glutathione, ascorbic acid and cysteine each potentiate the release of ferrous iron from ferritin (Mazur, Baez and Schorr, 1955). The enzyme xanthine oxidase also participates in the release of iron from hepatic ferritin in vitro (Green and Mazur, 1957) and in vivo (Mazur, Green, Saha and Carleton, 1958). The reverse reaction, that of incorporation of iron into ferritin has been shown to be mediated by ascorbic acid when the iron is in the inorganic ferric state (Loevus and Fineberg, 1957) and by ascorbic acid and adenosine triphosphate (ATP) when the iron is bound to plasma, (Mazur, Green and Carleton, 1960, and Mazur, Carleton and Carlsen, 1961).

Diphosphopyridine nucleotide (DPN) and triphosphopyridine nucleotide (TPN) and their reduced forms, DPNH and TPNH have been

shown by Nishida and Labbe (1959) to potentiate the incorporation of iron into haem, by rat liver homogenate. The mitochondria of rat liver are a rich source of the iron-incorporating enzyme system, the activity of which is greatly increased by the addition of ascorbic acid and GSH or cysteine (Minikami, 1958). GSH and cysteine have also been shown to potentiate the incprporation into haem of inorganic ferrous iron maintained in the reduced form by excess ascorbic acid. This suggests that these compounds are involved not only in the reduction of iron, but also in the mechanism of iron incorporation. Figure 9A indicates schematically these reactions.

The processes of iron exchange take place in vivo with iron in the protein-bound state. The present experiments were therefore undertaken to determine whether ascorbic acid, GSH and other physiological reducing substances render protein-bound iron available for haem biosynthesis.

METHODS

Radio-iron (^{59}Fe) was incorporated into rat liver protein and into human serum by the following methods.

Rat Liver.

Young female albino rats, Wistar strain, weighing 150-200 g., were each injected intraperitoneally with about 50 μc. of $^{59}FeCl_3$

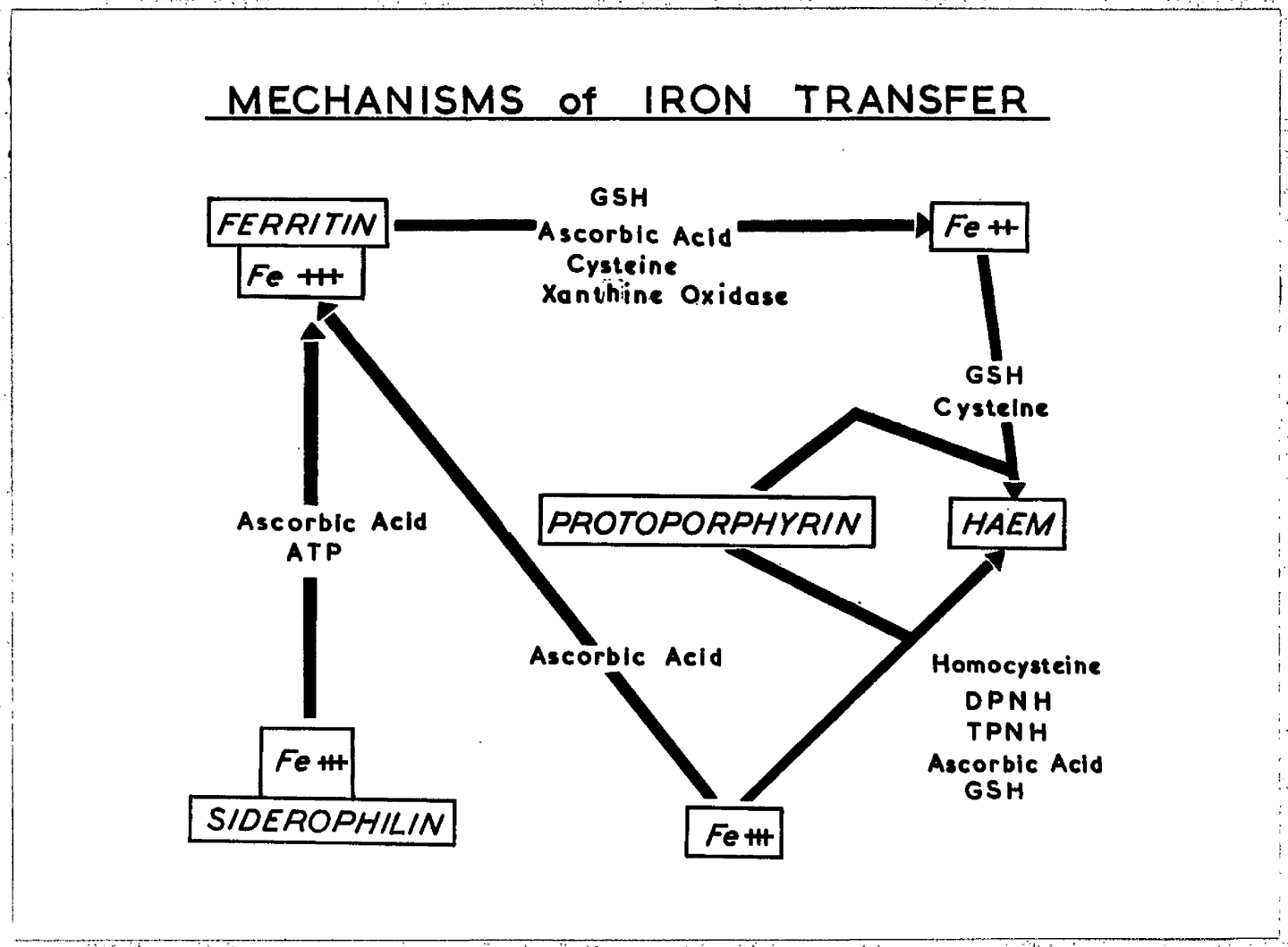


Figure 9A. Summary of previous studies on the mechanism of iron transfer to and from proteins and into protoporphyrin.

and 2,500 units of heparin. After 1-1½ hours the animals were anaesthetised with ether and killed by exsanguination. The liver was perfused through the portal vein with 20 ml. 0.9 per cent sodium chloride to remove trapped plasma. The organ was then excised and homogenised in ice-cold 0.25M sucrose to a 20 per cent homogenate. This was dialysed against ion-free water for about 20 hours at 4°C. in order to remove any unbound radioiron. The specific activity of the homogenate after dialysis was about 0.5 μc. per g. of tissue. One-fifth volume of TRIS buffer 0.45M pH 7.4 was then added to the homogenate.

Human serum.

Human serum was incubated with radio-iron for 1 hour at room temperature. The serum iron concentration and total iron-binding capacity had been determined (Ramsay, 1957) and enough $^{59}FeCl_3$ (specific activity 1-3 μc. per μg. of iron) was added to the serum sample to fully saturate the iron-binding protein.

Each sample of serum and dialysed liver homogenate was ultrafiltered (Goldberg, 1959) for 3-6 hours at 76 cm. Hg. pressure to ascertain that all the radio-iron was protein-bound. In no case was more than 0.5 per cent of the iron ultrafilterable.

Serum samples were subjected to paper electrophoresis for 16 hours at 1.2 milliamps current in a barbitone buffer pH 8.6 and the paper strip was scanned for radioactivity. The radioactivity resided

in the β-globulin ffaction, suggesting that the radio-iron was bound to siderophilin (Fig. 10). The method of estimation of haem formation was that previously described. A source of the iron-incorporating enzyme was incubated with protoporphyrin (2×10^{-5} M) and a source of protein-bound radio-iron for 3 hours at 37°C. When ^{59}Fe bound to liver protein was used, the liver homogenate served as both a source of ^{59}Fe and iron-incorporating enzyme. When ^{59}Fe bound to human serum was used, the source of iron-incorporating enzyme was rat liver mitochondria prepared by the method of Schneider (1948), and resuspended in 0.25M sucrose. One fifth volume of TRIS buffer 0.45 M, pH 7.4 was added to this suspension. The substances under test, ascorbic acid, GSH, cysteine, DPNH and ATP were dissolved in water and the pH of the solutions adjusted to 7.4 before use.

Haem was isolated, after addition of a carrier haemoglobin solution as previously described. Under these conditions the specific activity of the isolated haem gave an accurate measure of the transfer of protein-bound iron to protoporphyrin for haem formation.

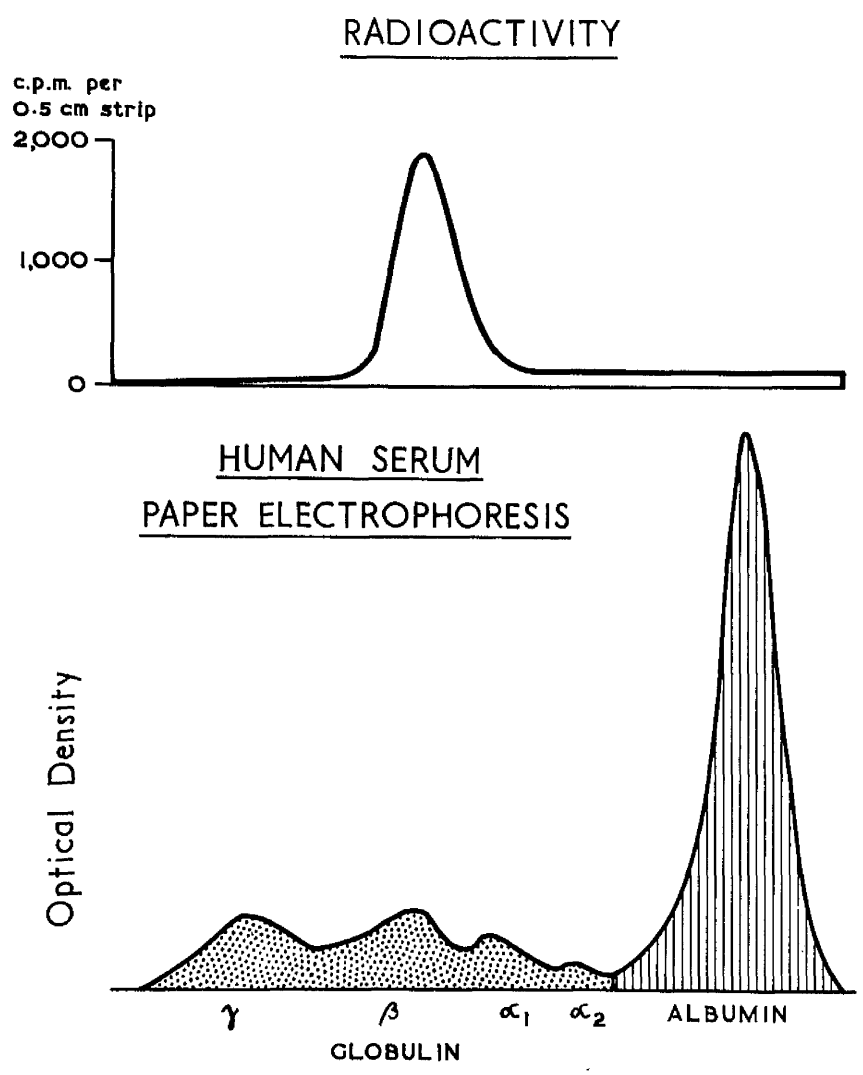


Figure 10. Paper electrophoresis of human serum bound to radio-iron. Result of scanning paper strip for radioactivity.

RESULTS

In this system, some haem formation from the incorporation of radio-iron bound to either serum or liver protein, into protoporphyrin occurred without the addition of other compounds. Haem formation was much increased by the addition of ascorbic acid, GSH, cysteine, DPNH and ATP.

The effect of varying concentrations of ascorbic acid on the incorporation into protoporphyrin of iron transferred from human siderophilin is shown in Figure 11 . Maximum potentiation of activity occurred at about 1×10^{-2} M ascorbic acid when the radio-iron was siderophilin-bound. When the radio-iron was bound to rat liver protein, maximum potentiation occurred at 3×10^{-3} M ascorbic acid (Figure 12). The effect of GSH on radio-iron release from rat liver protein and from human siderophilin for incorporation into haem was investigated. There was a marked increase in the amount of iron made available for incorporation as the concentration of GSH was increased. A maximum was obtained at a concentration of 1×10^{-2} M GSH for radio-iron transferred from rat liver protein (Figure 13) and at about 5×10^{-2} M GSH for radio-iron transferred from siderophilin (Figure 14).

In figure 15 is shown the effect of ascorbic acid and GSH separately and together on the transfer of radio-iron from human siderophilin into protoporphyrin. It is clear that there is an

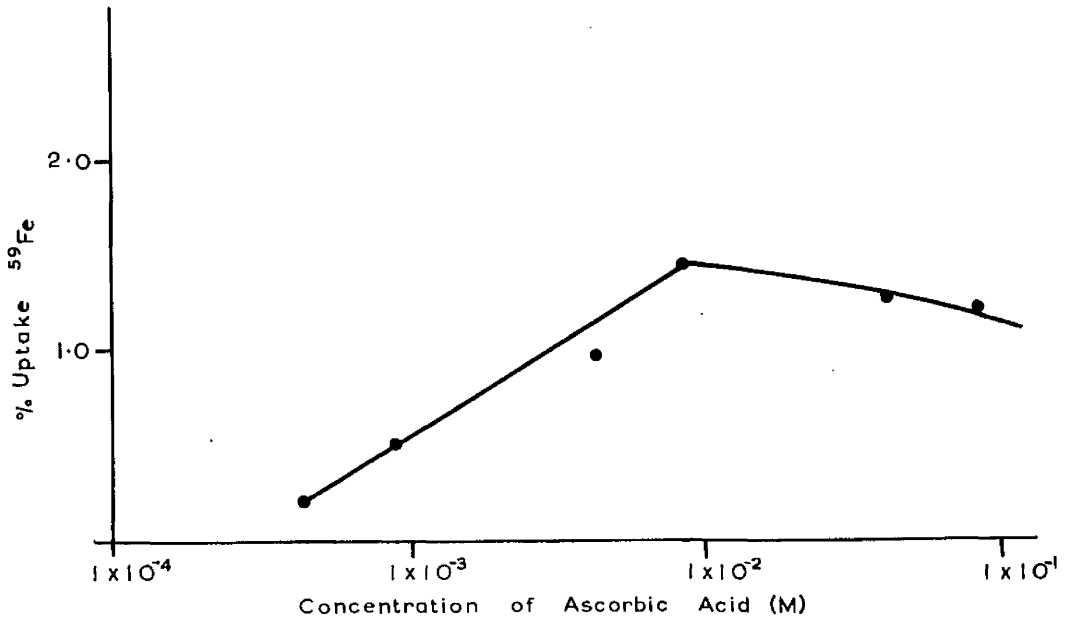


Figure 11. The effect of varying concentrations of ascorbic acid on the incorporation into protoporphyrin of iron bound to human siderophilin.

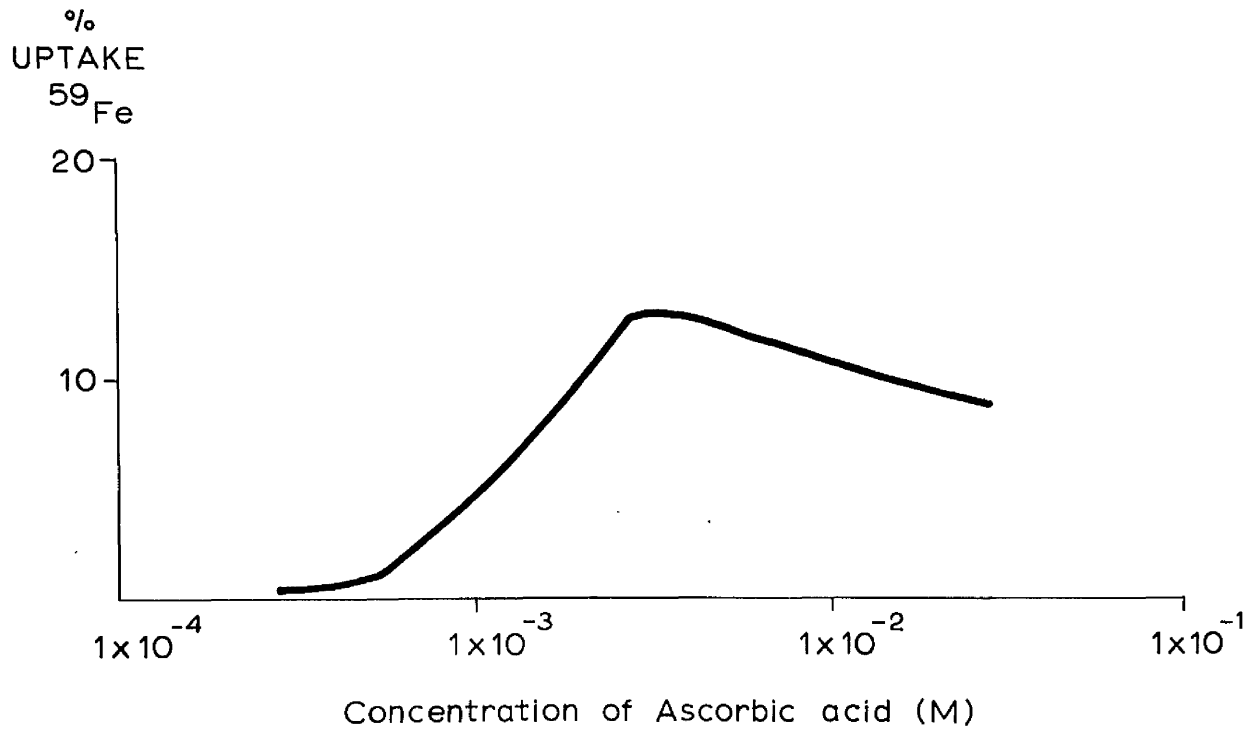


Figure 12. The effect of varying concentrations of ascorbic acid on the incorporation into protoporphyrin of iron bound to rat liver protein.

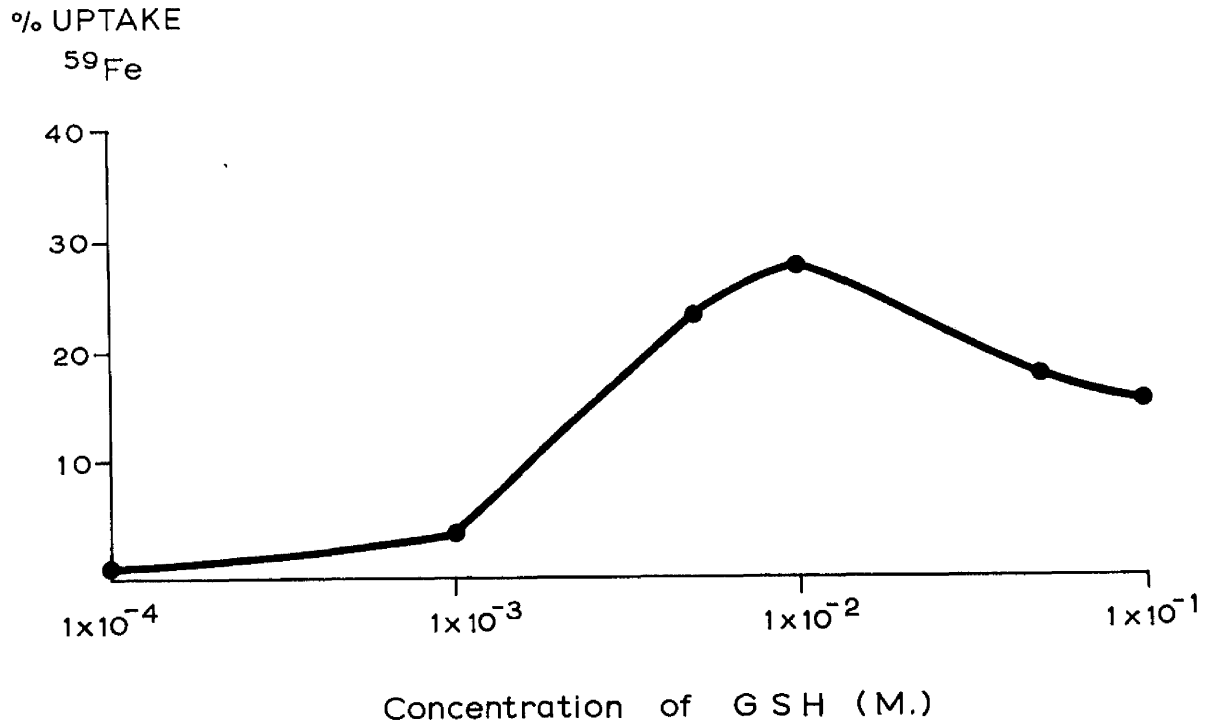


Figure 13. The effect of varying concentrations of reduced glutathione on the incorporation into protoporphyrin of iron bound to rat liver protein.

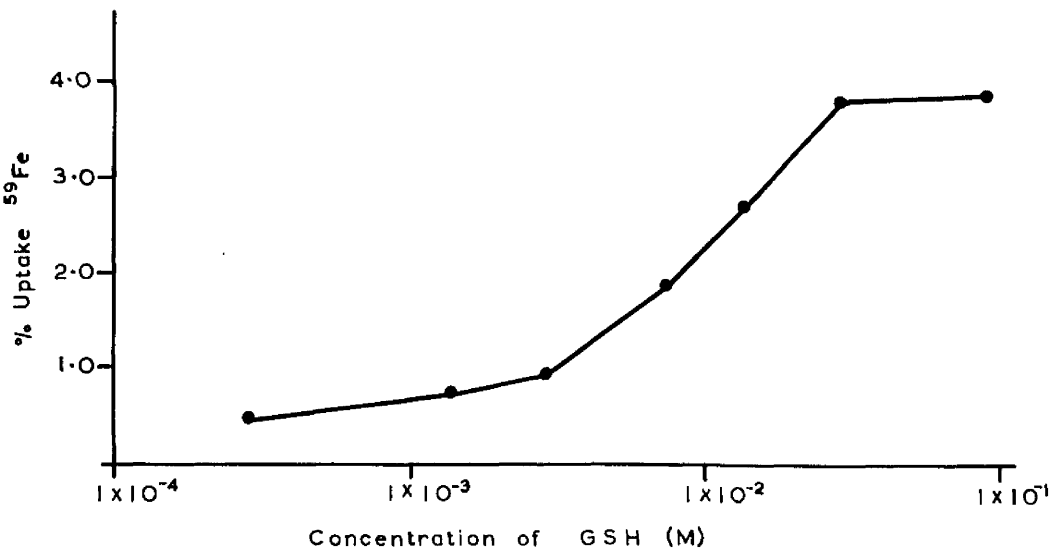


Figure 14. The effect of varying concentrations of reduced glutathione on the incorporation into protoporphyrin of iron bound to human siderophilin.

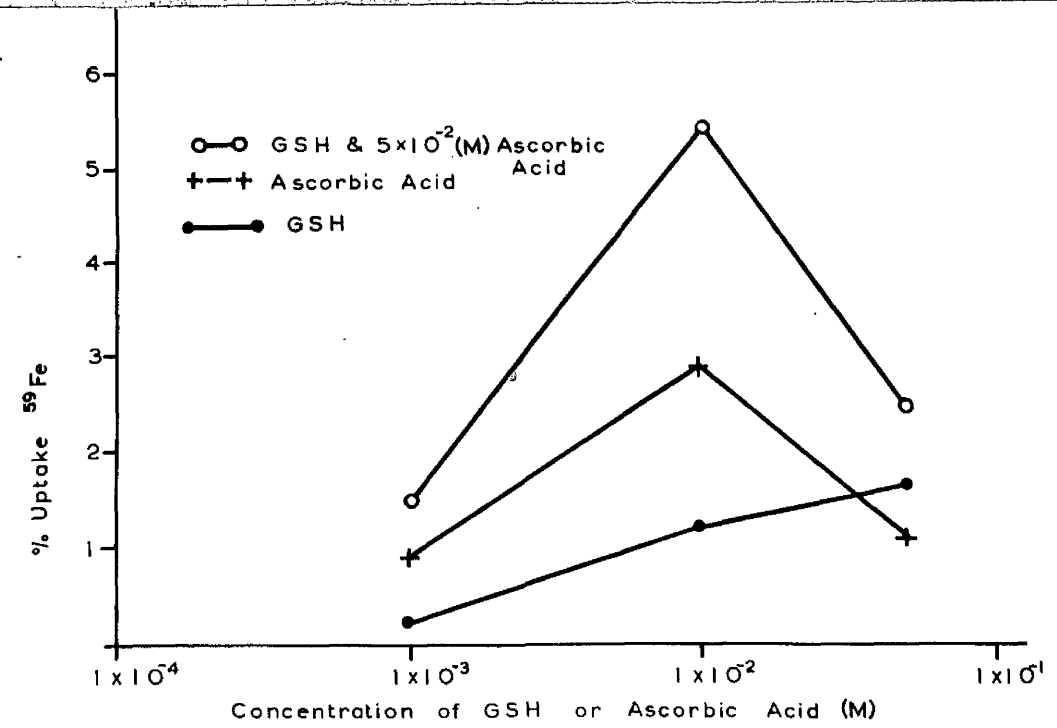


Figure 15. Effect of addition of varying concentrations of ascorbic acid alone, or reduced glutathione alone, and of reduced glutathione in the presence of ascorbic acid (5×10^{-2}M) on incorporation into protoporphyrin of iron bound to human siderophilin.

additive effect.

The addition of cysteine also increased the incorporation into haem of iron bound to siderophilin and to rat liver protein. Maximum iron release for haem synthesis occurred in both protein-bound radio-iron systems at a concentration of 1×10^{-2} M cysteine. In Figure 16 is shown the effect of cysteine on iron transfer from rat liver protein to protoporphyrin and in (Figure 17) the effect on iron transfer from human siderophilin.

DPN and DPNH were found by Nishida and Labbe (1959) to potentiate the conversion of protoporphyrin to haem. The possibility was tested that DPNH might have an effect on the release of iron from the protein-bound form for incorporation into haem. The effect of increasing concentration of DPNH on the incorporation of iron released from rat liver protein is shown in Figure 18 and from human siderophilin in Figure 19. There was an increase in the transfer of radio-iron as the concentration of DPNH was raised to 4×10^{-3} M. from both rat liver protein and siderophilin. The effect of the addition of ATP was tested on both systems. The results are shown in Table VII . There was an increase in incorporation of iron at the lower concentrations of ATP when siderophilin was the source of protein-bound iron. All concentrations of ATP appeared to inhibit the incorporation of iron from rat liver protein into protoporphyrin.

ATP 1.2×10^{-3} M was added to both systems in the presence of

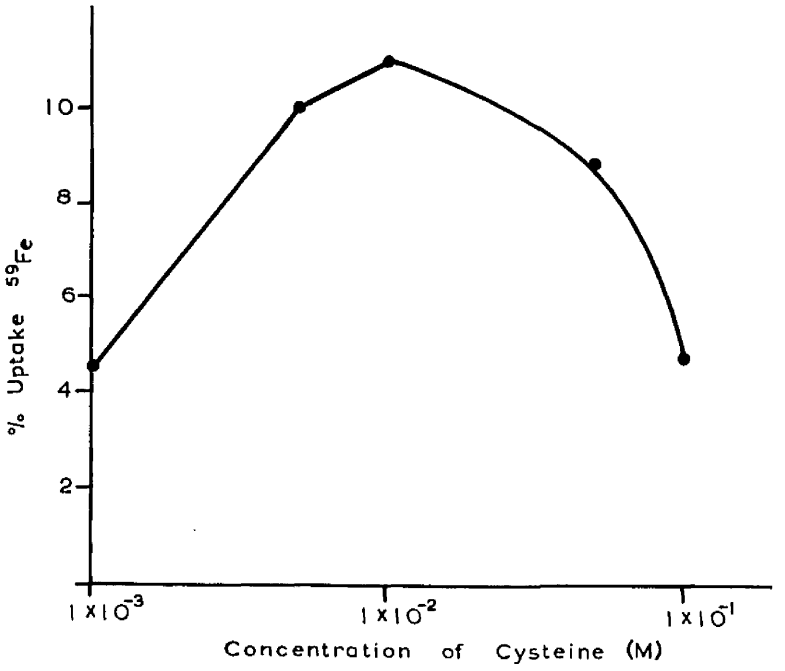


Figure 16. Effect of varying concentrations of cysteine on the incorporation into protoporphyrin of iron bound to rat liver protein.

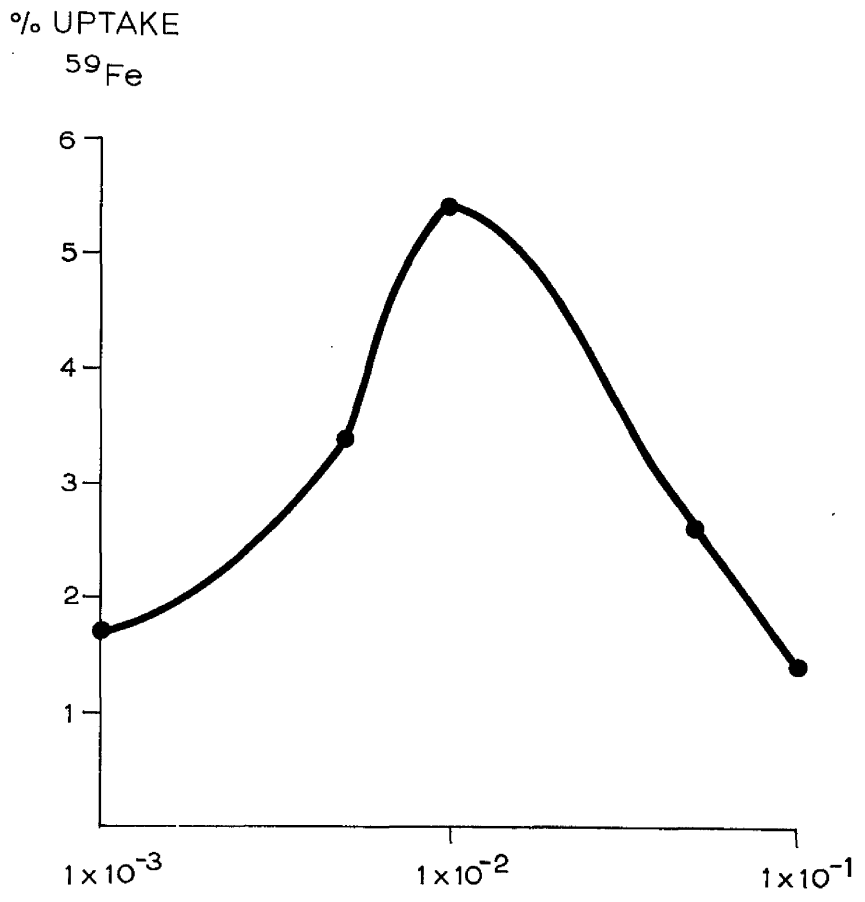


Figure 17. Effect of varying concentrations of cysteine on the incorporation into protoporphyrin of iron bound to human siderophilin.

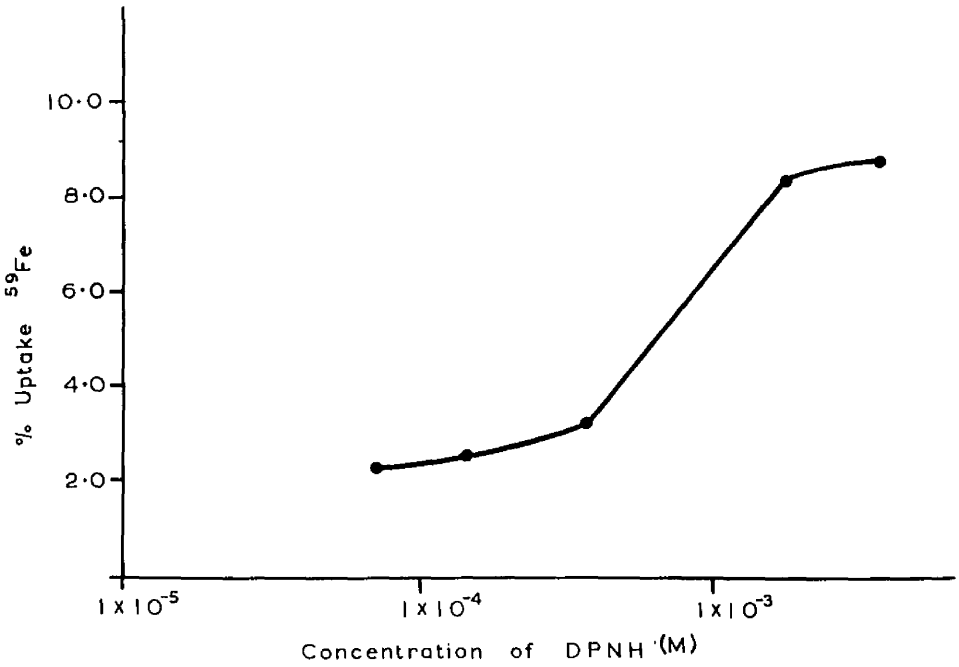


Figure 18. Effect of varying concentrations of reduced diphosphopyridine nucleotide on the incorporation into protoporphyrin of iron bound to rat liver protein.

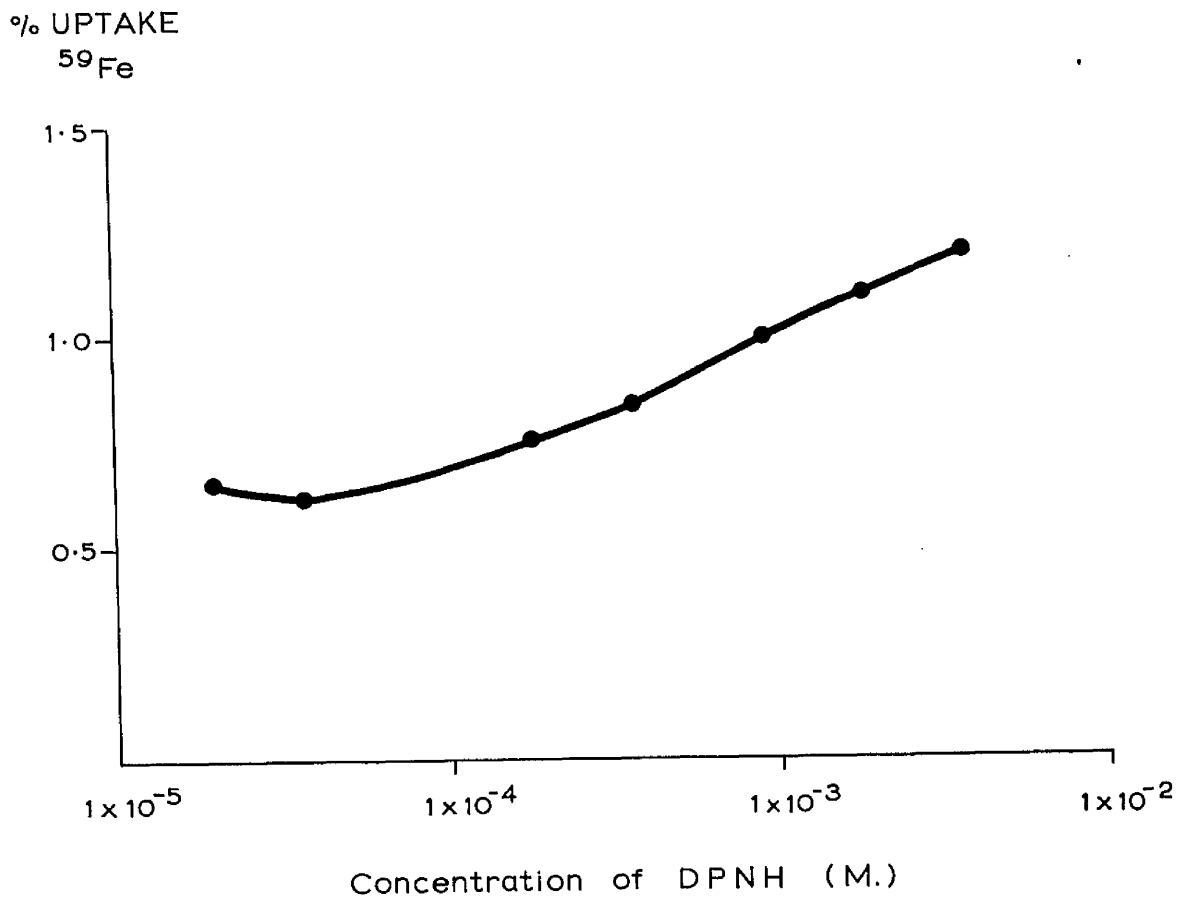


Figure 19. Effect of varying concentrations of reduced diphosphopyridine nucleotide on the incorporation into protoporphyrin of iron bound to human siderophilin.

TABLE VII.

Effect of increasing concentrations of adenosine triphosphate on the release of iron bound to rat liver protein and to siderophilin for incorporation into protoporphyrin.

Concentration of ATP (M.)	Uptake ^{59}Fe (%)	
	From rat liver protein	From human siderophilin
—		0·05
$5·9 \times 10^{-4}$		0·0
$1·2 \times 10^{-3}$	2·4	0·35
$1·8 \times 10^{-3}$	—	0·35
$5·9 \times 10^{-3}$	1·8	0·09
$8·3 \times 10^{-3}$		0·09
$1·2 \times 10^{-2}$	1·25	0·03

ascorbic acid, GSH, cysteine and DPNH (Table VIII). When iron was bound to siderophilin, ATP caused a further potentiation in activity in the presence of ascorbic acid, cysteine and DPNH, but not in the presence of GSH. When iron was bound to rat liver protein, ATP did not cause any further potentiation but rather a depression of activity.

TABLE VIII.

Effect of ascorbic acid, glutathione, cysteine and reduced diphosphopyridine nucleotide on the incorporation into protoporphyrin of iron bound to rat liver protein and to siderophilin, for incorporation into haem in the presence and absence of adenosine triphosphate.

Source of ^{59}Fe	Additions	ATP ($1 \cdot 2 \times 10^{-3}$M.)	^{59}Fe uptake (%)
Siderophilin	—	—	0·05
,,	Ascorbic acid 1×10^{-2}M.	—	2·9
,,	,,	+ ATP	4·6
,,	Glutathione $4 \cdot 8 \times 10^{-2}$M.	—	0·9
,,	,,	+ ATP	0·9
,,	Cysteine 1×10^{-2}M.	—	4·1
,,	,,	+ ATP	15·5
,,	DPNH $3 \cdot 6 \times 10^{-3}$M.	—	0·46
,,	,,	+ ATP	0·85
Rat liver homogenate	—	—	3·1
,,	Ascorbic acid 3×10^{-3}M.	—	6·9
,,	,,	+ ATP	5·8
,,	Glutathione 1×10^{-2}M.	—	10·4
,,	,,	+ ATP	9·7
,,	Cysteine 1×10^{-2}M.	—	14·8
,,	,,	+ ATP	13·2

DISCUSSION

These results show that in addition to facilitating the incorporation of iron into protoporphyrin, certain physiological reducing substances are also concerned in the release of iron when it is protein-bound. In this system a minimal release of iron from siderophilin and liver proteins occurred without the addition of reducing substances but this was greatly increased when some reducing substances were added. These compounds, ascorbic acid, GSH, cysteine and DPNH are found predominantly in their reduced form in all human living cells where each functions along with its oxidised form as an oxidation-reduction system. Mazur et al.(1955) have shown that GSH, ascorbic acid and cysteine potentiate the release of ferrous iron from ferritin as does the enzyme xanthine oxidase in vitro (Green and Mazur, 1957) and in vivo (Mazur et al. 1958). DPN and DPNH have been shown by Nishida and Labbe (1959) to stimulate the conversion of protoporphyrin to haem. There was a significant incorporation of protein-bound iron from siderophilin and rat liver protein, into haem, when DPNH was added to the system (Figures 18 and 19).

Reducing substances play an important role at all stages of iron metabolism. The iron, which is transported in siderophilin and is stored in ferritin and haemosiderin, is in the ferric state. However, Granick and Michaelis (1943) and Granick and Hahn (1944) failed to

detect direct incorporation of ferric iron into apoferritin but Beilig and Bayer (1955) have demonstrated the direct uptake in vitro of ferrous iron by apoferritin. Ascorbic acid has been found by Fineberg, Kasbekar and Loewus (1959) to be a highly specific factor for the uptake of inorganic ferric iron into apoferritin. No other physiological reducing substance mediated this incorporation (Loewus and Fineberg, 1957). The incorporation of plasma iron into hepatic ferritin requires ascorbic acid and ATP (Mazur et al. 1960, 1961).

In the present studies ATP potentiated the release of iron bound to siderophilin for haem biosynthesis when added to the system alone, and it increased the potentiation produced by the addition of ascorbic acid, DPNH and to a lesser extent, cysteine, (Table VIII). ATP did not, however, potentiate the release of iron bound to rat liver protein and when added to the system along with ascorbic acid, glutathione or cysteine, it caused a slight decrease in incorporation (Table VIII). Although ATP has an important role in the uptake of iron from plasma proteins into ferritin (Mazur et al. 1960, 1961) and into haem it does not appear to be concerned in the release of iron from ferritin for haem formation. Nishida and Labbe (1959) have further shown that ATP has no effect on the incorporation of inorganic ferric iron into haem. The other substances tested, ascorbic acid, GSH, cysteine, and DPNH were all effective in releasing iron from its

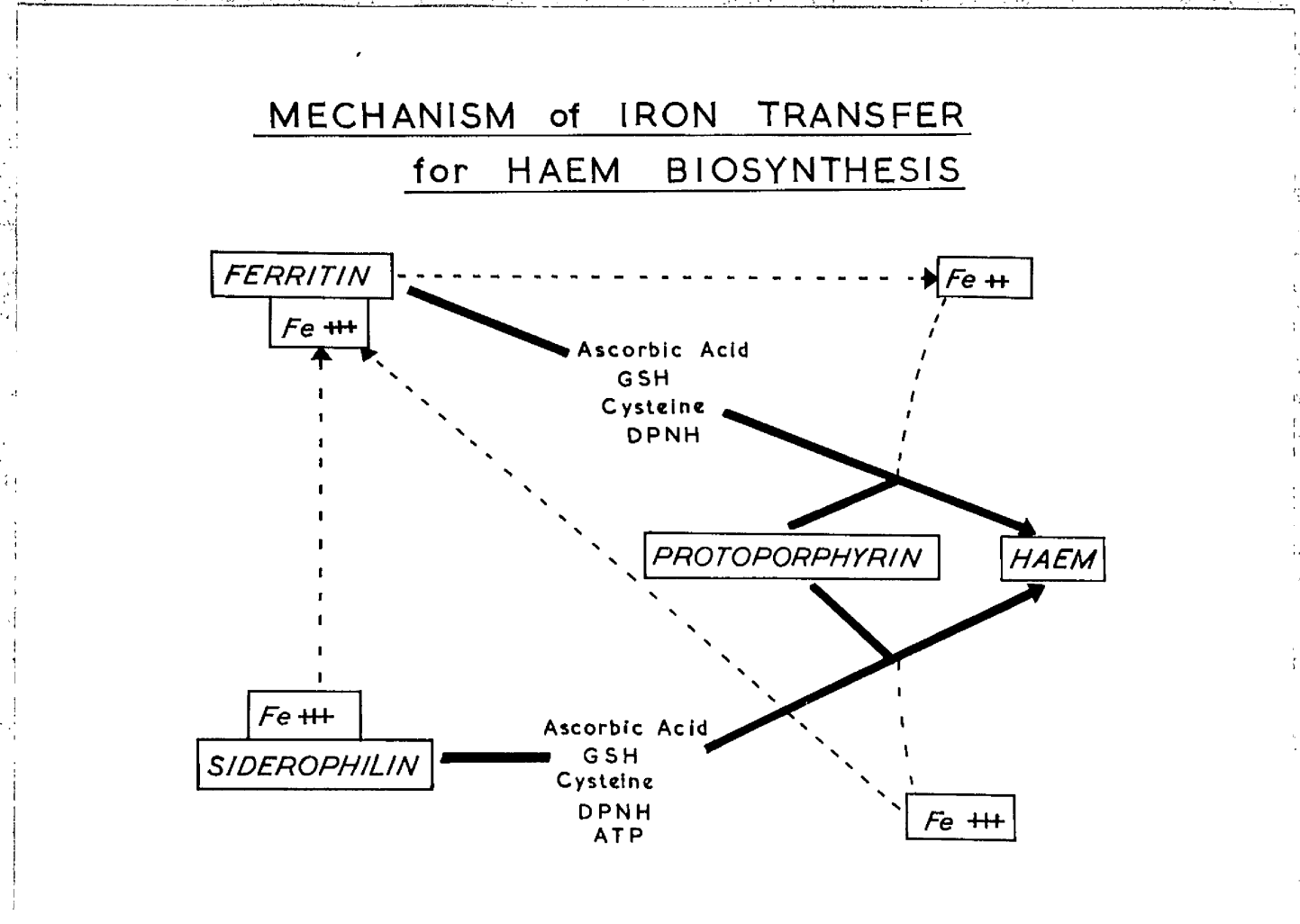


Figure 9 B. Summary of mechanisms of the transfer of iron from ferritin and siderophilin into protoporphyrin for haem biosynthesis.

protein-bound state for incorporation into haem.

These studies give some indication of the mechanisms involved in the transport and utilization of iron. It is clear that physiological reducing substances exert an important influence.

3

MEASUREMENT OF IRON INCORPORATION IN RELATION TO MARROW CELLS AND LIVER TISSUE IN THE RABBIT

The incorporation of iron into haemoglobin appears to be deranged in several of the anaemias - that of infection, rheumatoid arthritis, uraemia, thalassaemia, cancer, lead poisoning and the refractory normoblastic (sideroblastic) anaemias. There have been several studies in recent years on the *in vitro* incorporation of radioiron into protoporphyrin in the presence of blood, marrow and other tissues of man and experimental animals, (Goldberg, Ashenbrücker, Cartwright and Wintrobe, 1956; Krueger, Melnick and Klein, 1956; Schwartz, Cartwright, Smith and Wintrobe 1959; Kagawa, Minikami and Yoneyama, 1959;) and there is good evidence that this *in vitro* haem formation from iron and protoporphyrin is enzyme-dependent. Since a satisfactory assay method is already available, it seemed worthwhile to quantitate the iron-incorporating enzyme system in relation to a known number of erythrocyte precursors in the bone marrow. The mature mammalian erythrocyte is devoid of iron-incorporating enzyme activity; however, the reticulocyte (Walsh, Thomas, Chow, Fluharty and Finch, 1949; Goldberg et al. 1956) and the erythrocyte precursors in bone marrow are known to have such activity. In these studies, rabbit bone marrow was prepared so that both cell counts and measurement of enzyme activity could be carried out. The rabbits used were either normal or partially exsanguinated to mimic a haemorrhagic anaemia with marked marrow hyperplasia. In addition

to the enzyme activity of marrow cells that of hepatic cells was also measured in the normal and bled animals, in order to obtain a tissue control.

The aim of these studies in experimental animals is to provide the basis of a technique for the measurement of the iron-incorporating enzyme system in human marrow cells in normal and diseased states.

METHODS

All glass surfaces which came into contact with marrow or blood cells, with the exception of those of the syringe, were siliconised by the following method. 100 ml.of silicone fluid M 441 (Imperial Chemical Industries) was mixed with 900 ml. of petroleum ether. The glassware was immersed in the above mixture for 5 minutes; then washed with water and immersed for a further 5 minutes in a 2 per cent solution of ammonia. The glassware was then dried at 110°C for 2 hours. The glassware used was treated by this procedure three times initially and then once before each experiment. Syringes were not so treated because it was initially discovered that the process of siliconisation caused such corrosion of metal parts of syringes as to inhibit enzymic activity of blood and marrow tissue. The water used throughout was deionised by passage through an Elgastat deioniser.

Preparation of marrow cell suspension (Figure 20).

A suspension of marrow cells was prepared by a method similar to that used by Thomas (1955). Male rabbits of mixed type, average weight 2.40 kilos (S.D. $\pm$ 0.4) were killed by the intravenous injection of 40 ml. air and then exsanguinated. Tibiae, femori and humeri were rapidly removed, scraped free of flesh and placed on ice. The bones were cracked open and the marrow was rapidly removed and placed in 10 ml. of Hank's solution at 4°C. (Method 2 of Paul, 1960), after several marrow smears had been prepared for differential cell counting. The marrow was then forced 4 times through a stainless steel mesh (R.B. Turner and Co. Ltd. size 40; approximately 307 mm. square) situated at the head of a microfilter syringe. The resulting suspension was then centrifuged, at 4°C for 10 minutes at 200 g. The supernatant fat and Hank's solution were discarded and a further 10 ml. of Hank's solution added to the marrow cells. The marrow was then syringed six times under pressure through a stainless steel mesh (size 200; approximately 0.086 mm. square). In the resulting suspension the marrow cells were seen to be discrete or in clumps of two or three cells. Total cell counts, total nucleated cell counts and the percentage reticulocytes were estimated, using 0.4 - 0.6 ml. of this marrow suspension. The cell suspension was again spun at 200 g.

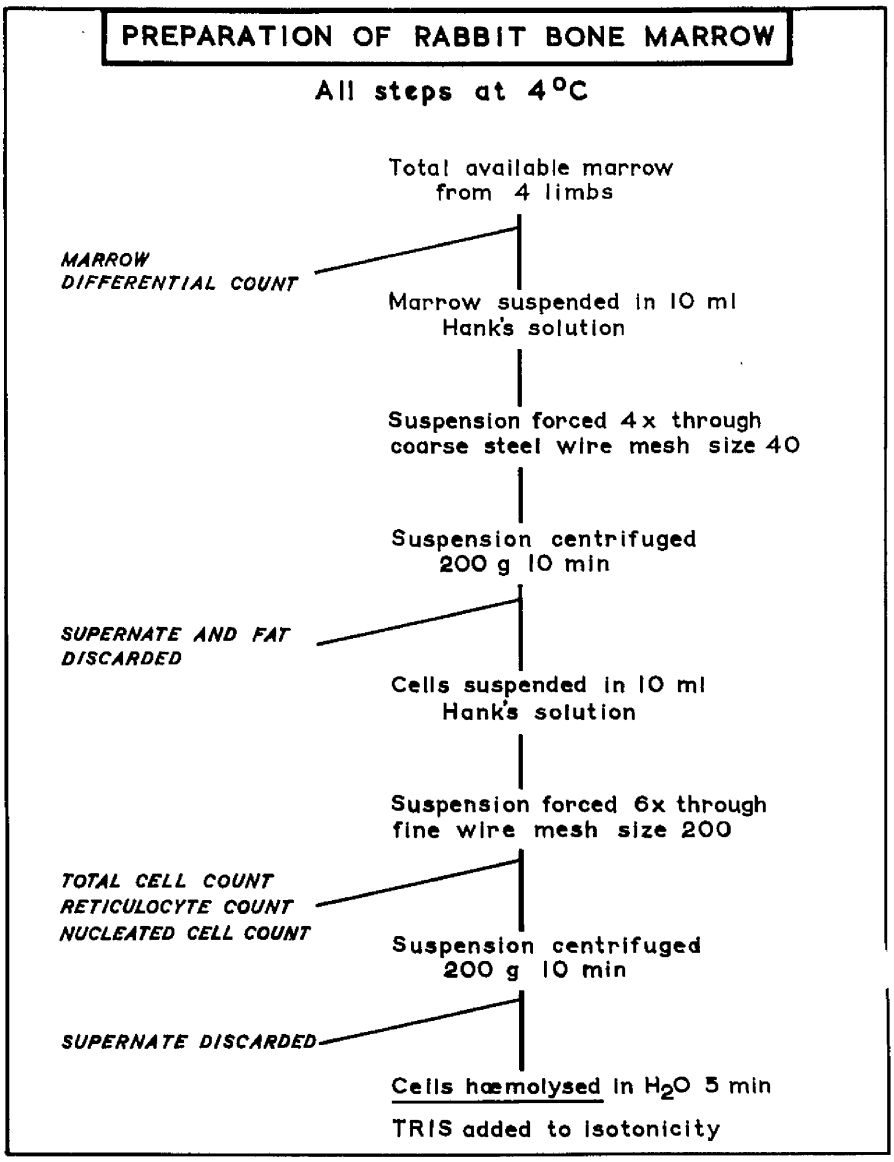


Figure 20. Preparation of marrow cell suspension.

for 10 minutes at 4°C. The supernatant solution was decanted and the cells haemolysed by the addition of an equal volume of water. After 5 minutes one fourth volume 0.45 M Tris buffer pH 7.4 was added to the haemolysate to restore isotonicity.

The composition of Hank's solution was as follows:-

	g. per litre
NaCl	8.00
KCl	0.40
$CaCl_2$	0.14
$MgSO_4 \cdot 7H_2O$	0.10
$Na_2HPO_4 \cdot 2H_2O$	0.06
KH_2PO_4	0.06
Glucose	1.00

Calcium and magnesium salts were dissolved in 100 ml. of water (solution 1). All other compounds were dissolved in 800 ml. of water (solution 2) and then solution 1 was added to solution 2. The resultant solution was filtered and 90 ml. aliquots were measured into containers which were sealed and sterilised by autoclaving. Sodium bicarbonate was made up separately. 0.35 g. was dissolved in 100 ml. of water and 10 ml. aliquots were sterilised by autoclaving.

Counting of Bone Marrow Cells.

Nucleated cells (N) were counted in duplicate after staining with N/10 HCl containing a few drops of methylene blue. The percentage

reticulocytes of marrow erythrocytes (R.M.E.) were determined by the method of Dacie (1956). A minimum of 500 red cells were counted. The marrow smears were stained with Leishman's stain and the percentage of normoblasts (P.N.) estimated in 500 to 1000 nucleated cells.

The numbers of marrow red cell precursors, that is normoblasts and reticulocytes were calculated in the following way:-

Number of normoblasts per ml. marrow suspension = per cent normoblasts (P.N) X total nucleated cells per ml.(N)

Number of reticulocytes per ml. marrow suspension = per cent reticulocytes (R.M.E.) X number of erythrocytes per ml.

= per cent reticulocytes (R.M.E.) X (total cell count per ml. - nucleated cell count per ml. (N)).

Preparation of Peripheral Blood.

Approximately 1 ml. of blood was added to 10 ml. of Hank's solution and the cells dispersed by shaking for 1 minute. The suspension was spun at 200 g. for 10 minutes at 4°C. The Hank's solution was decanted and the cells resuspended in 10 ml. of Hank's solution by shaking for 1 minute. 0.4 to 0.6 ml. of this suspension was used to estimate total cell counts and the percentage of reticulocytes. The cell suspension was again spun at 200 g. for 10 minutes at 4°C. An haemolysate prepared as for marrow cells was measured for its enzyme activity.

Quantitation of activity of iron incorporating enzyme.

Aliquots of marrow or peripheral blood haemolysates were added to flasks containing 1 μc ^{59} ferric chloride (specific activity about 2μc/μg iron), protoporphyrin and 1 ml. Tris buffer. Optimal concentrations of reducing substances (ascorbic acid; reduced glutathione, cysteine, (as cysteine hydrochloride), contained within 1 ml. were added to certain flasks. The volume of each flask was made up to 10 ml. with 0.9 per cent saline. The mixture was incubated with constant shaking at 37°C for 2½ hours and the reaction stopped by the addition of 1 ml. M potassium cyanide to each flask. Carrier haemoglobin was then added and haem isolated as previously described. The optimum pH for this system is in the range pH 7.0 - 7.5 and all experiments were carried out at pH 7.4 (Figure 21). The effect of increasing numbers of marrow cells on the per cent uptake of radio-iron into haem is shown in Figure 22. A linear relationship existed between cell counts and the percentage uptake of radio-iron into haem only up to 50 x 10^6 cells. Hence this number of cells was added in all succeeding experiments for both marrow and peripheral blood haemolysates.

A straight line relationship exists between the amount of protoporphyrin added to the system and the amount of haem synthesised as measured by ^{59}Fe incorporation into protoporphyrin up to a concentration

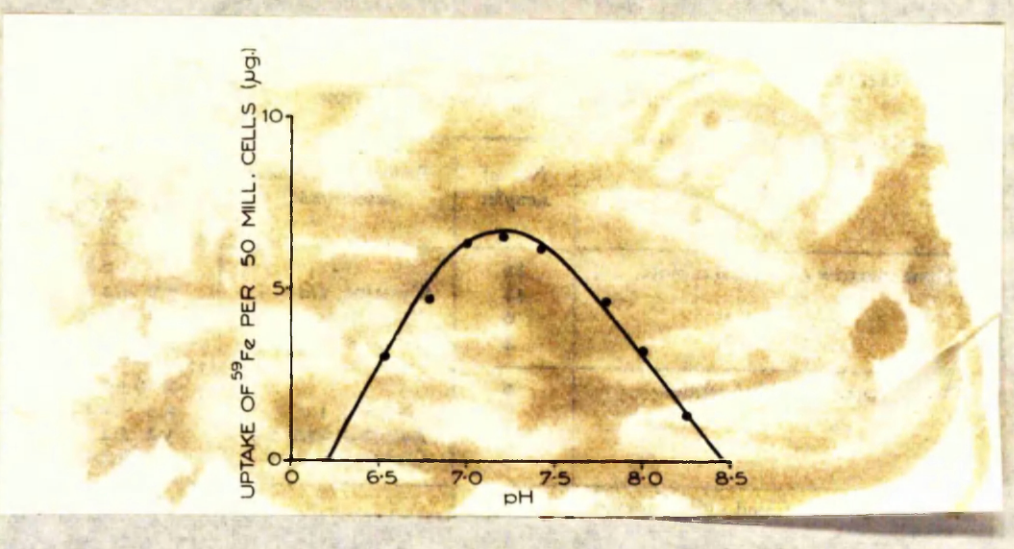


Figure 21. Effect of pH on the incorporation of radio-iron into haem by a haemolysate of 50 x 10^6 red cell precursors.

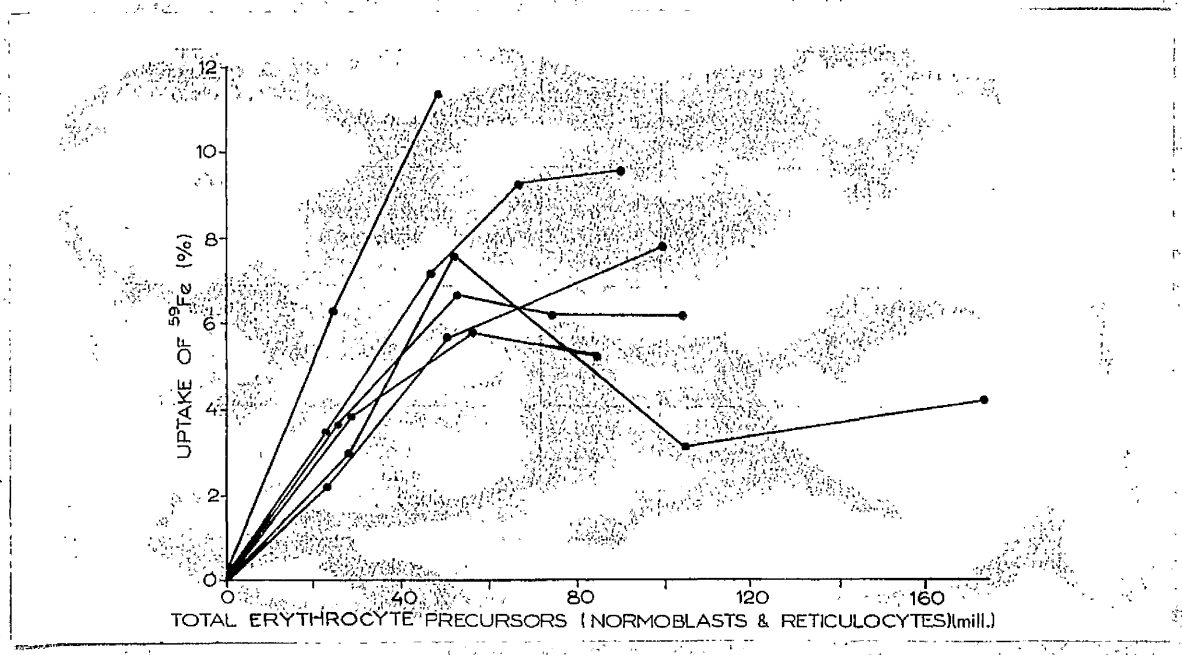


Figure 22. Effect of increasing numbers of red cell precursors on the incorporation of radio-iron into haem.

of 2×10^{-5} M protoporphyrin (Figure 23). This concentration was used in subsequent experiments.

If the marrow haemolysate is homogenised at 2,000 rpm. for 3 mins., in an ice-cooled Potter glass homogeniser the iron incorporating enzyme activity is reduced to about one-half (Table IX).

Ascorbic acid and GSH contents of the haemolysates were determined by the methods of Roe and Keuther (1943) and Woodward and Fry (1932) respectively, and expressed per 50×10^{-6} total marrow or peripheral blood cell counts.

Preparation of liver tissue.

Liver tissue was washed with water, dried on filter paper, weighed and homogenised with 3 times its volume of 0.25 M sucrose in an ice-cooled Potter glass homogenizer. To the homogenate was added one quarter of its volume of 0.45 M Tris buffer pH 7.4. The incubation mixture contained 5 ml. of the liver homogenate, protoporphyrin, 1 µc. of ^{59}ferric chloride, 1 ml. 0.45 M Tris, pH 7.4, and about 2 mg. each of penicillin G and streptomycin sulphate to prevent bacterial contamination. Ascorbic acid and cysteine when used were each contained in a volume of 1 ml. and the volume of each flask was made up to 10 ml. with 0.9 per cent sodium chloride. After incubation at 37°C for 2½ hours with mechanical shaking the reaction was stopped by the addition of 1 ml. of M potassium cyanide. The isolation of haem was as described previously. The effect of increasing

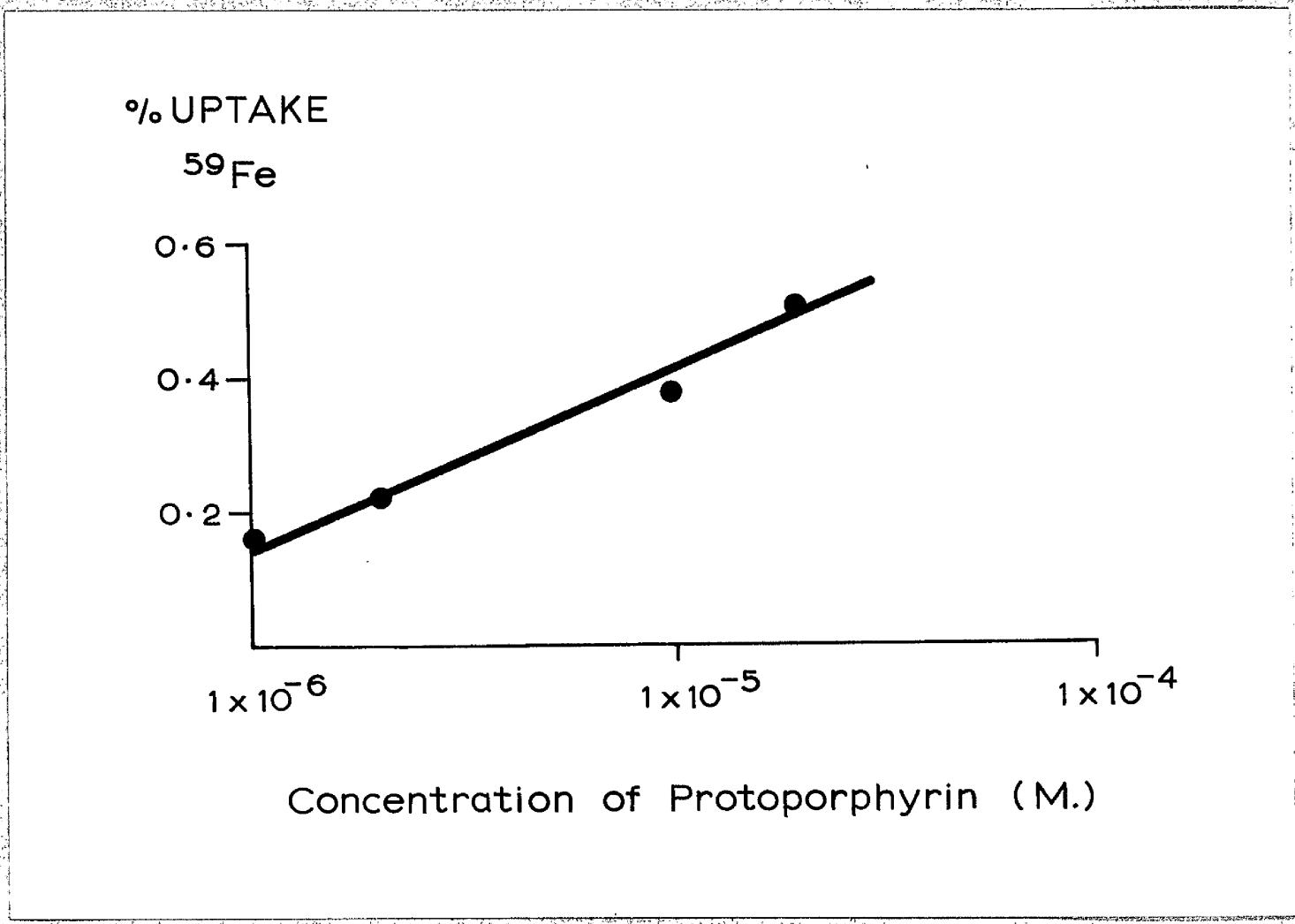


Figure 23. Effect of protoporphyrin concentrations on the incorporation of radio-iron into haem by a haemolysate of 50 x 10^6 cells of rabbit bone marrow.

TABLE IX.

Effect of homogenisation on the iron-incorporating enzyme activity of a haemolysate of red cell precursors of rabbit bone marrow.

No. of Cells x 10^6	^{59}Fe Per Cent Incorporation	
	Haemolysate	Homogenate
23.6	2.2	1.1
47.2	4.7	2.95
70.8	6.6	3.5
118.0	11.2	5.9

concentration of protoporphyrin on this system is shown in Figure 24. Haem formation is proportional to the amount of protoporphyrin added, up to 2×10^{-5} M protoporphyrin. This concentration was used in subsequent experiments. The results are expressed as the percentage incorporation into haem per gram wet weight of liver tissue.

Haematological values.

The volume of packed red cells (P.C.V.) was measured by spinning blood at 3,000 r.p.m. for 30 minutes. Reticulocytes in the peripheral blood were counted by a standard technique (Dacie, 1956). Haemoglobin content was measured by the alkaline haematin method of Clegg and King (1942).

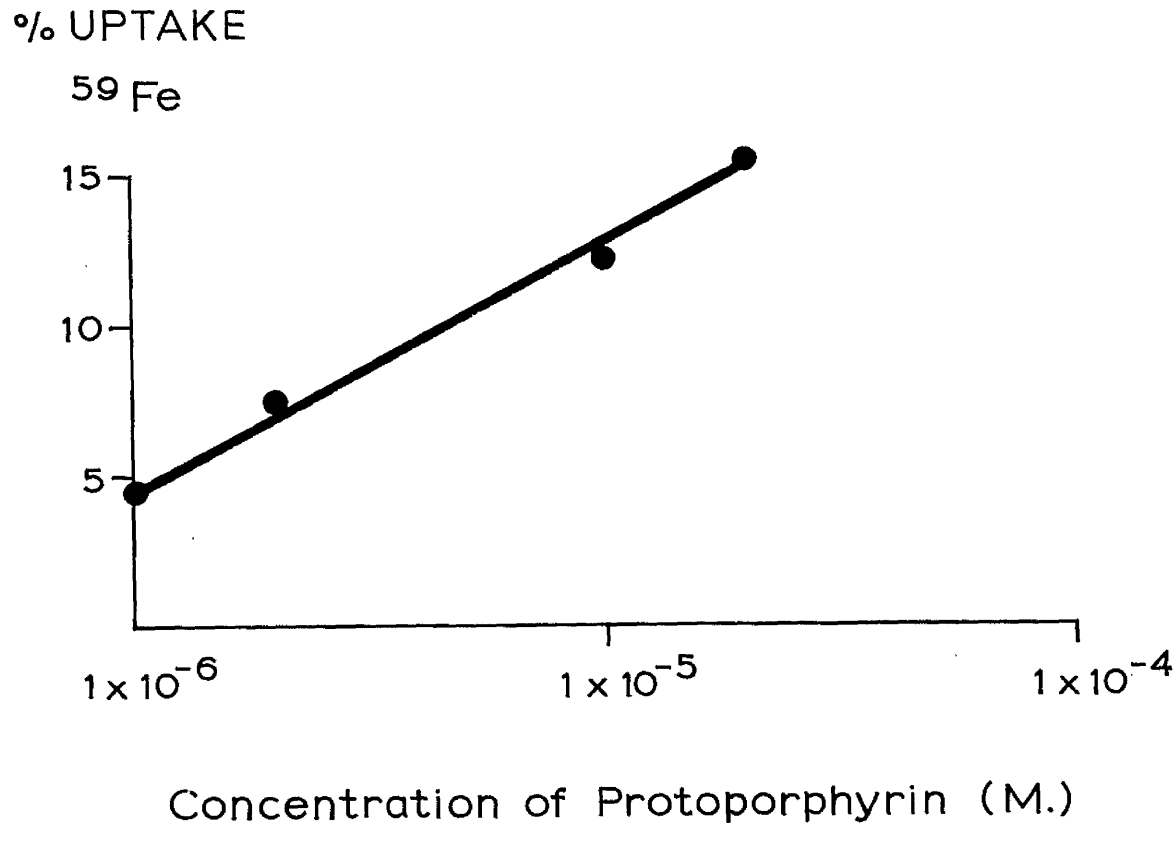


Figure 24. Effect of protoporphyrin concentration on the incorporation of radio-iron into haem by a homogenate of rabbit liver.

RESULTS

Marrow Counts.

Normal Rabbits. The results of marrow cell counts in 20 normal rabbits are summarised in Table X. All counts were done in duplicate. For total cell counts the mean difference between the two sets of counts was - 3.46 $\pm$ 3.94 where 3.94 is the standard error of differences. For nucleated cell counts the mean difference between the two sets of counts was - 6.35 $\pm$ 13.28 where 13.28 is the standard error of differences. There is thus no significant difference between the duplicate counts of either the total cell counts or nucleated cell counts.

Bled Rabbits. Six rabbits were bled 20 - 25 ml. daily for 4 days. The animals were then killed and their marrows examined on the 5th day. Compared to the normal rabbits the total number of nucleated red cell precursors in the marrows of the bled animals had doubled, while the reticulocyte pool in the marrow had decreased to one-fourth. At the same time there was a marked reticulocytosis in the circulating blood and these cells contained the heavy reticulum characteristic of the marrow reticulocyte.

Incorporation of radio-iron into haem by marrow cells.

Results are expressed as the percentage of added radio-iron which was incorporated into haem by 50 x 10^6 immature red cells. The effect was measured of varying concentrations of ascorbic acid, reduced glutathione and cysteine on the per cent uptake of radio-iron

TABLE X. Comparison of haematological values in normal bled rabbits and of the total number of normoblasts and reticulocytes recovered from the marrows of the bled animals. Results are expressed as the mean value ± standard deviation (where the standard deviation is of the form of S.D. $= \sqrt{\left[\frac{\Sigma x^2}{n} - \left[\frac{\Sigma x}{n}\right]^2\right]}$).

Rabbits		Weight (Kg.)	PCV (%)	Hb. (g/100ml)	Peripheral reticulocytes (%)	Weight of marrow (g)	Total No. of normoblasts recovered ($\times 10^6$)	Total No. of red cell precursors recovered ($\times 10^6$)
Group	No.							
Normal	20	2.40 ± 0.4	41.5 ± 2.2	13.5±0.7	3.0 ± 0.8	3.3 ± 3	509 ± 211	924 ± 329
Bled	6	2.26 ± 0.3	24.0 ± 2.9	8.1±1.4	19.7 ± 5.1	3.5 ± 1.2	895 ± 239	1104 ± 287

into haem using an haemolysate of 50 x 10^6 immature cells. The optimal concentrations of these reducing substances were 5 x 10^{-4}M ascorbic acid; 1 x 10^{-3}M cysteine and 7.5 x 10^{-3}M GSH. (See Figures 25, 26, 27). These optimal concentrations were used in this study.

The percentage uptake of radio-iron into haem by haemolysates of 50 x 10^6 immature red cells of the marrow from normal rabbits ranged from 0.5 to 2.9 per cent (mean 1.2 per cent, S.D. $\pm$ 0.8).

There was a substantial increase in the per cent uptake of radio-iron into haem when optimal amounts of reducing substances were added to this system (Table XI, Figure 28).

Bled Rabbits. The mean percentage uptake of radio-iron into haem by an haemolysate of 50 x 10^6 immature marrow cells of bled rabbits was minimal (mean 0.06 per cent $\pm$ 0.03). This is significantly different from the mean percentage uptake of normal bone marrow haemolysate ($p < 0.01$). Addition of reducing substances however, restored the enzymic activity to about 50 per cent of the values obtained when reducing substances were added to normal marrow cell haemolysates (Table XI, Figure 28). The mean percentage uptake of radio-iron into haem by 50 x 10^6 reticulocytes in the peripheral blood of bled rabbits was 0.2 per cent (S.D. $\pm$ 0.1, Table XI). The addition of reducing substances increased the enzymic activity to about 50 per cent of that found in 50 x 10^6 marrow cells of bled rabbits.

The GSH contents of the marrow haemolysates of 7 normal and 4 bled

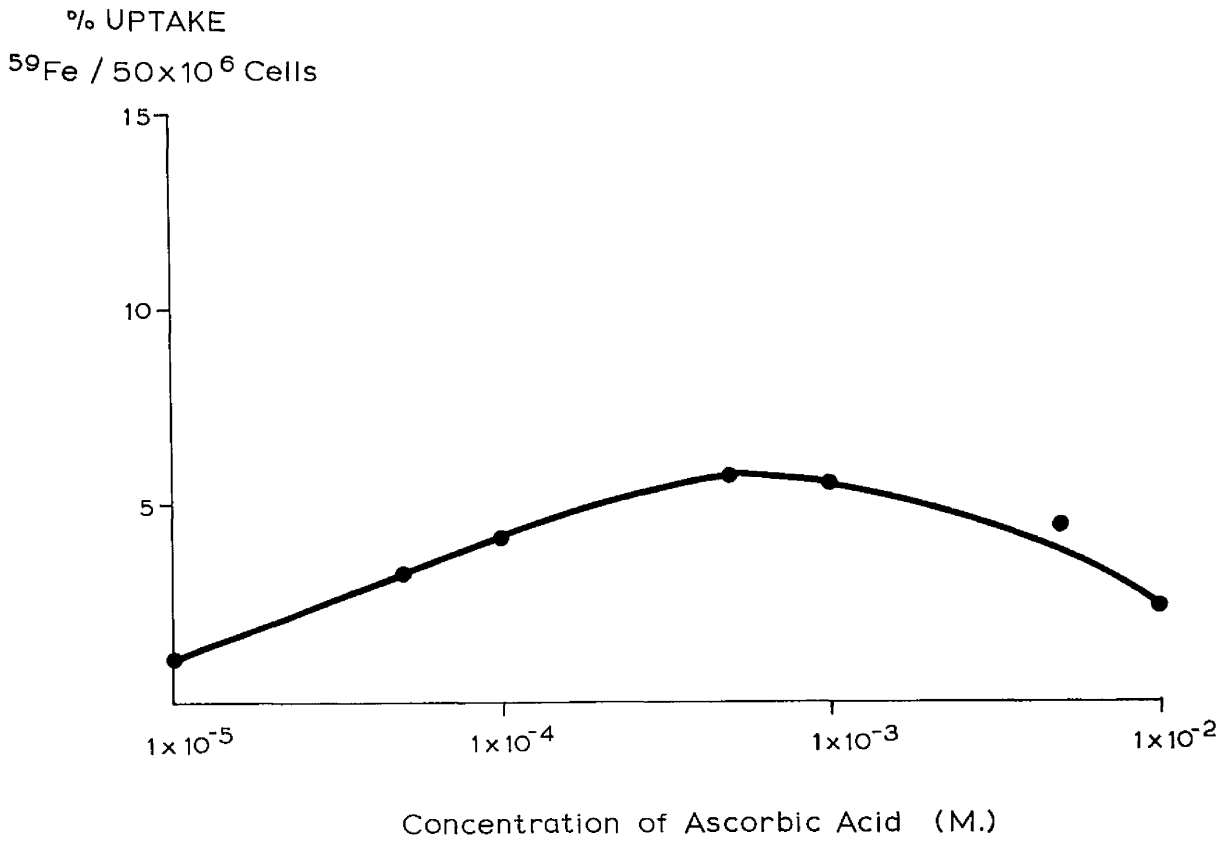


Figure 25. Effect of increasing concentration of ascorbic acid on the incorporation of radio-iron into haem by rabbit bone marrow haemolysate.

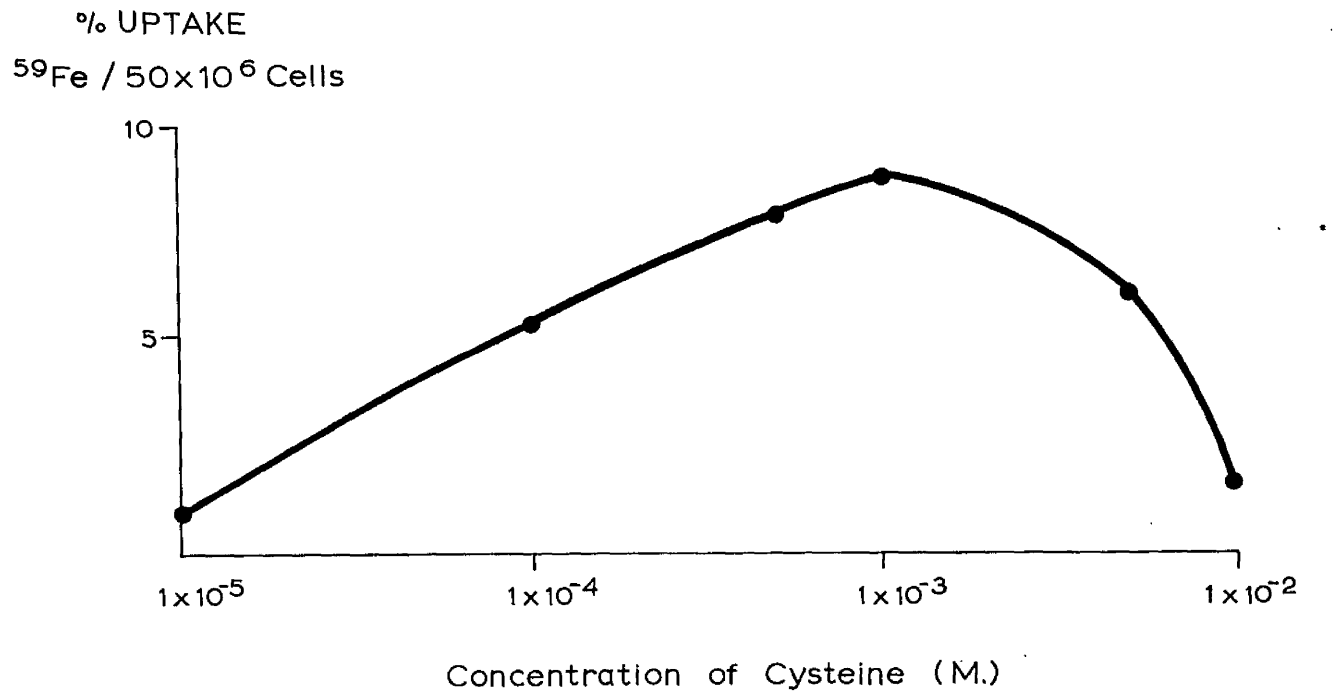


Figure 26. Effect of increasing concentration of cysteine on the incorporation of radio-iron into haem by rabbit bone marrow haemolysate.

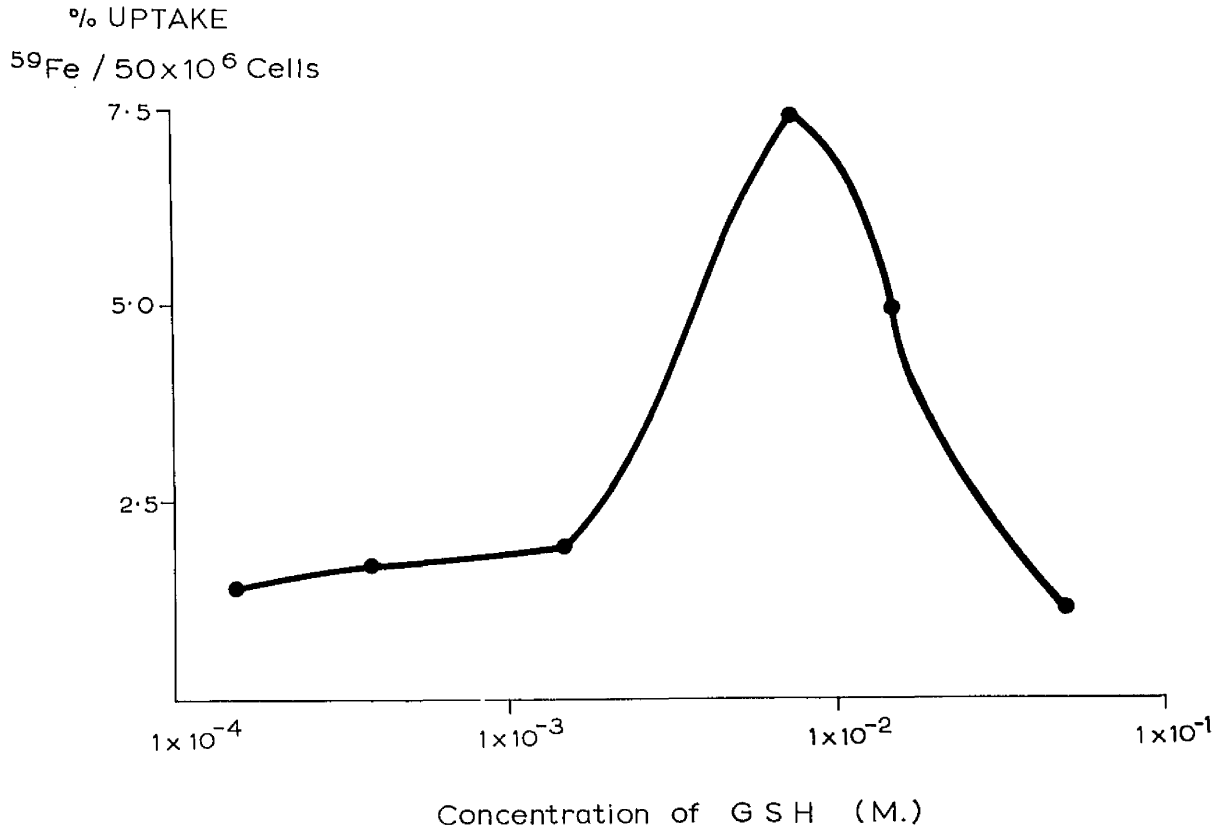


Figure 27. Effect of increasing concentration of reduced glutathione on the incorporation of radio-iron into haem by rabbit bone marrow haemolysate.

TABLE XI.

Incorporation of radio-iron into haem by haemolysates of bone marrow from normal and bled rabbits and peripheral reticulocytes of bled rabbits. Results are expressed as the mean value ± standard deviation.

Tissue	*No. of samples*	*Reducing substance added (mM)*	*Per cent incorporation of ^{59}Fe per 50 million cells*
Bone marrow of normal rabbits	12	–	1.2 ±0.8
	12	Cysteine, 1.0	5.7 ±2.6
	6	Ascorbic acid, 0.5	9.8 ±3.9
	4	Glutathione, 7.5	5.5 ±2.4
Bone marrow of bled rabbits	6	–	0.06±0.03
	6	Cysteine, 1.0	2.5 ±1.5
	6	Ascorbic acid, 0.5	5.2 ±2.4
	6	Glutathione, 7.5	1.9 ±1.1
Bone marrow of bled rabbits given ascorbic acid	3	–	0.17±0.07
	3	Cysteine, 1.0	4.5 ±1.4
	3	Ascorbic acid, 0.5	9.7 ±7.2
	3	Glutathione, 7.5	2.6 ±1.4
Bone marrow of bled rabbits given cysteine	3	–	0.15±0.06
	3	Cysteine, 1.0	0.8 ±0.6
	3	Ascorbic acid, 0.5	0.5 ±0.5
	3	Glutathione, 7.5	0.6 ±0.35
Reticulocytes of bled rabbits	6	–	0.2 ±0.1
	6	Cysteine, 1.0	1.2 ±0.9
	6	Ascorbic acid, 0.5	2.5 ±0.9
	6	Glutathione, 7.5	1.2 ±0.5
Reticulocytes of bled rabbits given ascorbic acid	3	–	0.26±0.07
	3	Cysteine, 1.0	1.9 ±0.22
	3	Ascorbic acid, 0.5	3.7 ±1.5
	3	Glutathione, 7.5	2.1 ±0.7
Reticulocytes of bled rabbits given cysteine	3	–	0.14±0.07
	3	Cysteine, 1.0	0.3 ±0.08
	3	Ascorbic acid, 0.5	0.36±0.09
	3	Glutathione, 7.5	0.3 ±0.09

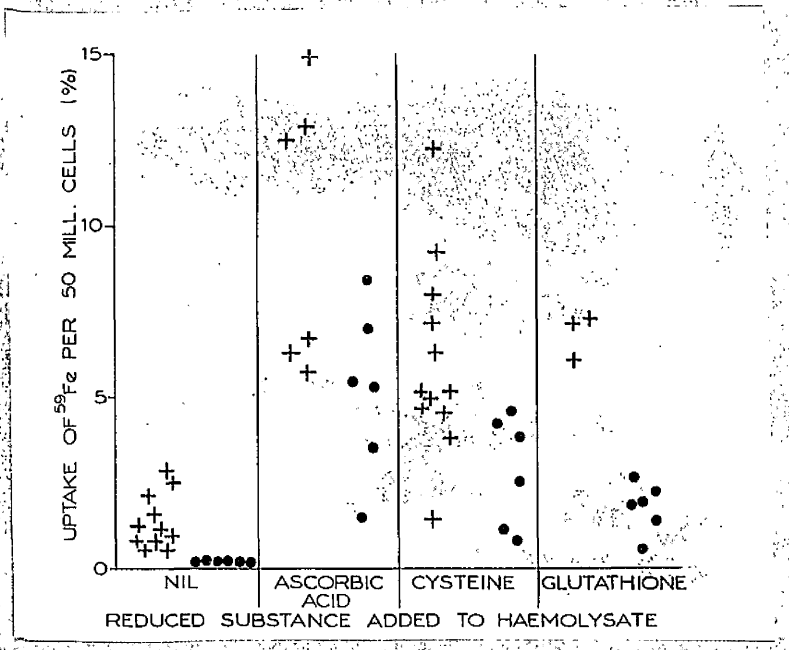


Figure 28. Effect of reducing substances on the incorporation of radio-iron into haem by haemolysates of red-cell precursors of normal and bled rabbits.

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...cnates gave an average uptake of ^{59}Fe with proto-
...nt per 25 mg. of liver ...rrogen. The addition of
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TABLE III

...LIVER FROM NORMAL, BLED AND IRON-DEFICIENT RABBITS AND RABBITS
...MATOLOGICAL VALUES FOR THE VARIOUS ... ANIMALS

...ipheral ...ocytes (%)	...Fe of liver ...amples	Reticulocyte substa... ...ab...	...

rabbits were respectively 27.7 $\pm$ 13 µg. per 50 x 10^6 cells and 5.3 $\pm$ 5.1µg./50 x 10^6 cells. The mean ascorbic acid content of marrow cells of 4 normal and 3 bled rabbits were respectively 0.6 $\pm$ 0.28 µg./50 x 10^6 cells and 0.8 $\pm$ 0.23 µg./50 x 10^6 cells (Figure 29). In view of the possibility that the apparent decrease in marrow cell enzyme activity of bled rabbits, was due to a loss of physiological reducing substances caused by blood loss, the following experiment was carried out. Six rabbits were bled 20 - 25 ml. of blood daily for 4 days. Three of these rabbits were given 500 mg. ascorbic acid intra-muscularly (I.M.) after each venesection and on the day of the experiment. The other three rabbits were given 100 mg. cysteine intravenously (I.V.) and 100 mg. I.M. after each venesection and 100 mg. I.V. on the day of the experiment. The results in these rabbits are recorded in Table XI.

Testing the significance of the difference of means between the incorporation of iron into protoporphyrin of bone marrow haemolysates of the bled animals given ascorbic acid, and those which did not receive ascorbic acid, gave $p > 0.4$. This shows that there is no significant difference between the two sets of results. Carrying out the same statistics on the mean iron incorporation into haem by bone marrow haemolysates of bled rabbits given cysteine, and by rabbits which did not receive cysteine, gave $p > 0.5$, which is not significant.

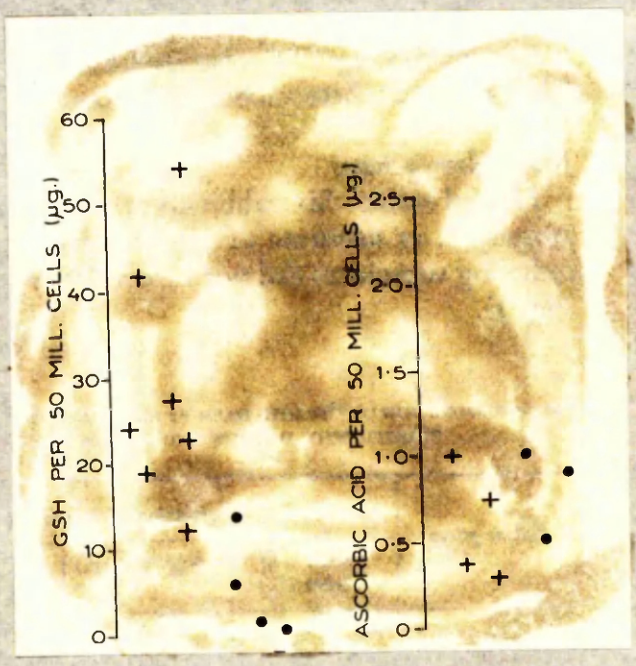

Figure 29. Concentration of reduced glutathione and ascorbic acid in marrow cells of normal and bled rabbits.

Liver Homogenates

Normal Rabbits. Six rabbit liver homogenates gave an average uptake of ^{59}Fe of 10.2 per cent $\pm$ 3.4 per gram wet weight of liver. The addition of reducing substances did not substantially increase the per cent uptake of radio-iron into haem (Table XII, Figure 30.).

Bled Rabbits. The per cent uptake of radio-iron into haem by liver homogenates from six rabbits each bled a total of 100 ml. over a period of 4 days, were in the same range as those found in normal liver tissue. The addition of reducing substances to these homogenates did not increase the uptake of radio-iron into haem (Table XII Figure 30). These values are not significantly different from those of normal livers.

Iron deficient rabbits. Two rabbits were repeatedly venesected (200 - 250 ml.) over 4 - 8 weeks to produce an iron deficiency state. In each, the per cent incorporation of radio-iron into haem was at the upper limit of normal. The addition of reducing substances to the liver homogenates enhanced the uptake of radio-iron into haem Table XII, Figure 30).

Rabbits given Imferon. Five rabbits were injected intramuscularly with 50 mg. Imferon and 3 rabbits with 300 mg. Imferon (Iron-dextran complex, Bengers). Two to four weeks later enzymic activity in the liver was studied. In the animals given 50 mg. Imferon the per cent uptake of radio-iron into haem by the liver homogenates was

TABLE XII. Comparison of the incorporation of radio-iron into haem by homogenates of liver from normal, bled and iron-deficient rabbits and rabbits treated with Imferon. Details of haematological values for variously treated animals. Results are expressed as the mean value ± standard deviation.

Rabbit Group	Haematological values			No. of liver samples	Reducing substances added	Incorporation of ^{59}Fe per gram wet weight
	P.C.V. (%)	Haemoglobin (g/100ml)	Peripheral reticulocytes (%)			
Normal	42.8 ± 2.8 (6)	13.6 ± 1.4 (6)	2.4 ± 1.4 (5)	6	-	10.2 ± 3.4
				6	Ascorbic acid 2×10^{-3} M	11.1 ± 4.6
				4	Cysteine 7.7×10^{-3} M	9.5 ± 4.4
Given 50 mg. Imferon	42.2 ± 3.1 (5)	13.4 ± 1.4 (5)	3.5 ± 1.0 (5)	5	-	7.2 ± 1.5
				4	Ascorbic acid 2×10^{-3} M	6.4 ± 2.4
				5	Cysteine 7.7×10^{-3} M	5.7 ± 0.6
Given 300 mg. Imferon	39.0 ± 0.8 (3)	12.7 ± 0.4 (3)	2.8 ± 0.3 (3)	3	-	2.4 ± 0.7
				3	Ascorbic acid 2×10^{-3} M	2.8 ± 0.4
				3	Cysteine 7.7×10^{-3} M	2.9 ± 0.1
Made iron deficient and anaemic	(1) 24.5 (2) 24.0	5.4 5.9	14.6 7.0	2	-	(1) 17.5 (2) 23.0
				2	Ascorbic acid 2×10^{-3} M	(1) 15.7 (2) 35.2
				2	Cysteine 7.7×10^{-3} M	(1) 20.0 (2) 35.2
Bled	20.9 ± 4.8 (4)	6.7 ± 1.2 (6)	20.7 ± 5.5 (3)	6	-	11.0 ± 4.8
				6	Ascorbic acid 2×10^{-3} M	12.4 ± 3.7
				4	Cysteine 7.7×10^{-3} M	13.4 ± 5.5

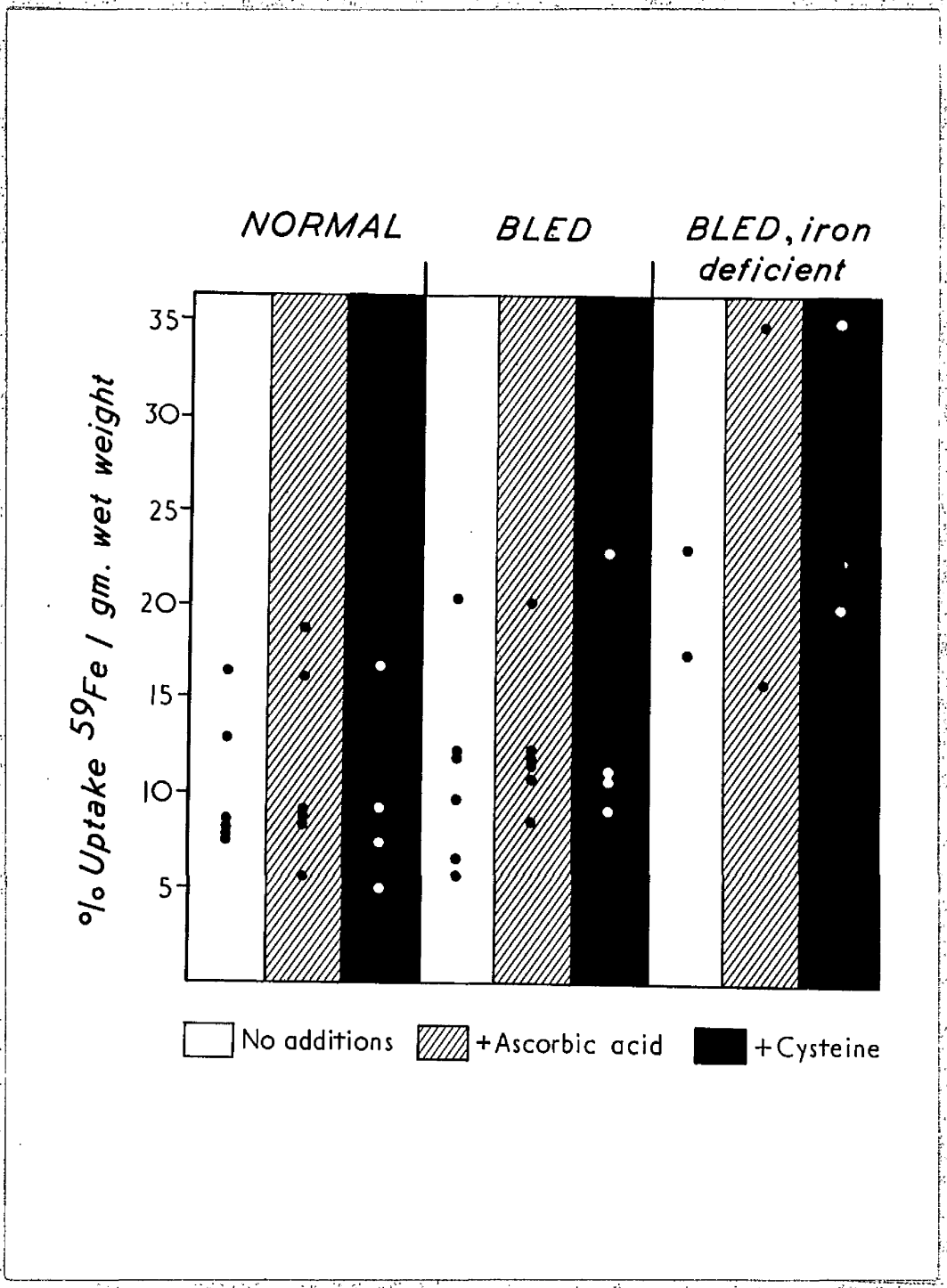

Figure 30. Effect of ascorbic acid (2×10^{-3}M) and cysteine (7.7×10^{-3}M) on the enzymic activity of liver homogenates from normal, bled and iron-deficient animals.

7.2 (S.D. $\pm$ 1.45), and in those given 300 mg. Imferon the per cent uptake was 2.4 per cent (S.D. $\pm$ 0.7)per gram wet weight of liver. Testing the significance of the difference of the means between the results for animals given 50 mg. of Imferon and the normal animals gave $p \leqslant 0.10$ while the same statistics on the results for animals given 300 mg. of Imferon gave $p \leqslant 0.01$. There was no increase in the per cent uptake of radio-iron into haem by these liver homogenates when reducing substances were added (Table XII Fig.31).

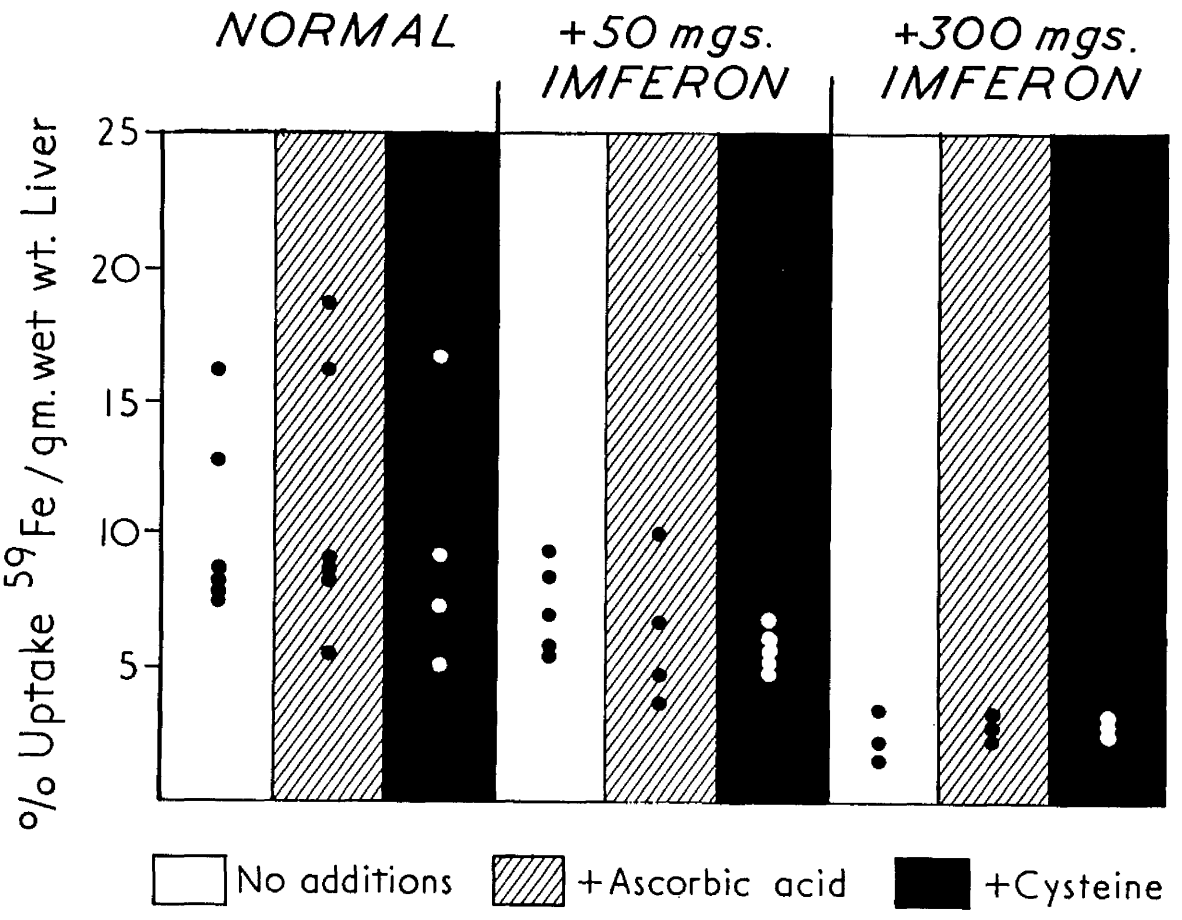

Figure 31. Effect of ascorbic acid (2×10^{-3} M) and cysteine (7.7×10^{-3} M) on the enzymic activity of liver homogenates from normal rabbits and rabbits dosed with 50 and 300 mgs. Imferon.

DISCUSSION

Previous attempts to compare the haem formation of the bone marrow or blood of one animal with that of another have shown considerable variation (Goldberg et al. 1956; Schwartz et al. 1959). Thomas (1955) compared different reference standards including fat free dry weight; nitrogen content; RNA and DNA content; cell counts and differential counts and suggested that the best standard for comparison appeared to be the number of red cells. The technique which has been described, clearly depends on accurate counting of cells and recognition of cell morphology. Total cell counts and nucleated cell counts were done in duplicate. There was no significant difference between the duplicates in either case. Since a quantitative determination of nucleated red cells was required the percentage of erythrocyte precursor cells in the total number of marrow cells was counted in a minimum of 500-1000 cells.

In this study the rate of haem synthesis was proportional to enzyme concentration when the number of immature red cells added to the system was not greater than 50×10^6. The variable uptake of radioiron when the number of cells added was greater than 50×10^6 may be explained by increase in the size of the iron pool with resulting dilution of the radioiron. When increasing amounts of $FeCl_3$ were added to a system containing 50×10^6 cells, there was a

progressive fall in the uptake of radioiron into haem (Fig. 32). It is also possible that when the number of cells added is greater than 50×10^6 the Fe^{59} will be firmly attached to cellular constituents (e.g. cell membrane receptors (Jandl, Inman, Simmons and Allen, 1959).

The per cent uptake of radioiron into haem was markedly increased by the addition of optimal amounts of reducing substances. It is of interest that the mean uptakes of radioiron into haem in the normal rabbit marrow specimens were similar with all three reducing substances (Table XI). In the individual specimens of marrow cells however, the increase in the uptake of radioiron into haem in the presence of reducing substances was variable. This is probably partly due to the variable content of reducing substances already present in marrow cells.

The per cent uptake of radioiron into haem by immature marrow cells from bled rabbits was markedly reduced. This reduction however, was not mainly due to any decrease in enzyme content per cell since the percentage uptake of radioiron into haem was substantially restored by addition of reducing substances. Morell, Savoie and London (1958) found that globin synthesis per unit number of immature erythroid cells from bled rabbits was not increased as compared with globin synthesis by immature red cells of normal rabbits. It would thus appear that the marrow responds to blood loss

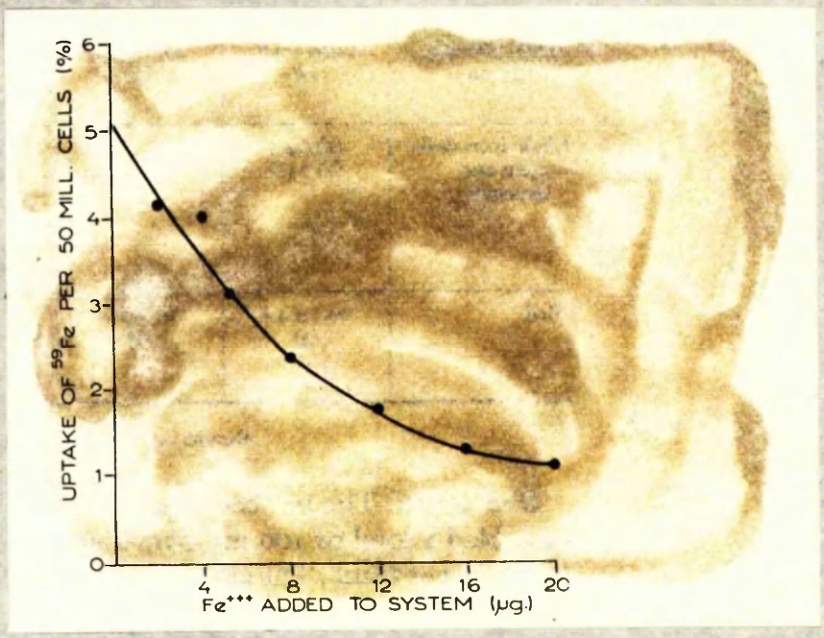

Figure 32. Effect of increasing amounts of non-radioactive iron on the incorporation of radio-iron into haem by a haemolysate of 50 x 10^6 red cell precursors of rabbit marrow.

by an increased production of cells rather than any increased capacity of the cell to form haemoglobin.

The GSH content of marrow cells from bled rabbits was depressed (Figure 29). The markedly reduced per cent uptake of radio-iron into haem by these marrow cells may be related to the loss of sulphydryl groups. From studies of inhibitors p-chloro-mercuriphenyl-sulphone and iodoacetamide (Schwartz et al. 1959) and also from studies of dialysis and ultrafiltration of enzyme preparations (Goldberg, 1959), it has been shown that active sulphydryl groups are required for enzyme activity. There was an apparent increase in the enzymic activity of marrow cells of bled rabbits which had been injected with cysteine as compared to the bled animals which had not been given cysteine, but this was not statistically significant. There was little or no potentiation in activity, when concentrations of reducing substances which caused potentiation in marrow preparations from normal and bled rabbits, were added to the marrow haemolysates from these cysteine-treated animals. Bled rabbits injected with ascorbic acid showed no statistically significant increase in marrow cell enzymic activity. In this case the addition of reducing substances to the haemolysates resulted in a potentiation of activity.

An alternative explanation for the depressed enzyme activity of marrow red cell precursors of bled rabbits is that the ^{59}Fe is firmly

bound by cell membrane receptors of immature red cells of bled rabbits. This possibility was excluded by the following experiment. Increasing amounts of non-radioactive ferric chloride were added to haemolysates of 50 x 10^6 immature marrow cells of bled rabbits to saturate these "receptors" prior to the addition of radioiron to the system. There was no increase in the per cent uptake of radioiron into haem as compared to a control (Figure 33).

The mean uptake of ^{59}Fe into haem by the nucleated cells of the marrow was approximately twice as great as the mean uptake of ^{59}Fe into haem by early reticulocytes. This corresponds to the ratio of 2/1 found in the study by Ersley and Hughes (1960). There is, however, a marked discrepancy between these findings using radio-iron and those reported by Morell and coworkers using (2 - ^{14}C) glycine. They found that the synthesis of haem by nucleated red cells was 8 to 48 times greater than the synthesis by early reticulocytes.

Most studies on the enzymic incorporation of iron into protoporphyrin in the formation of haem have used a system containing protoporphyrin, an enzymic source and ^{59}Fe. In a few studies 2 - ^{14}C labelled glycine has been used. When ^{14}C labelled protoporphyrin and ^{59}Fe have been used simultaneously haem synthesis as calculated on the basis of ^{59}Fe has fallen far short of that calculated on the basis of the utilisation of labelled protoporphyrin (Bannerman et al. 1959).

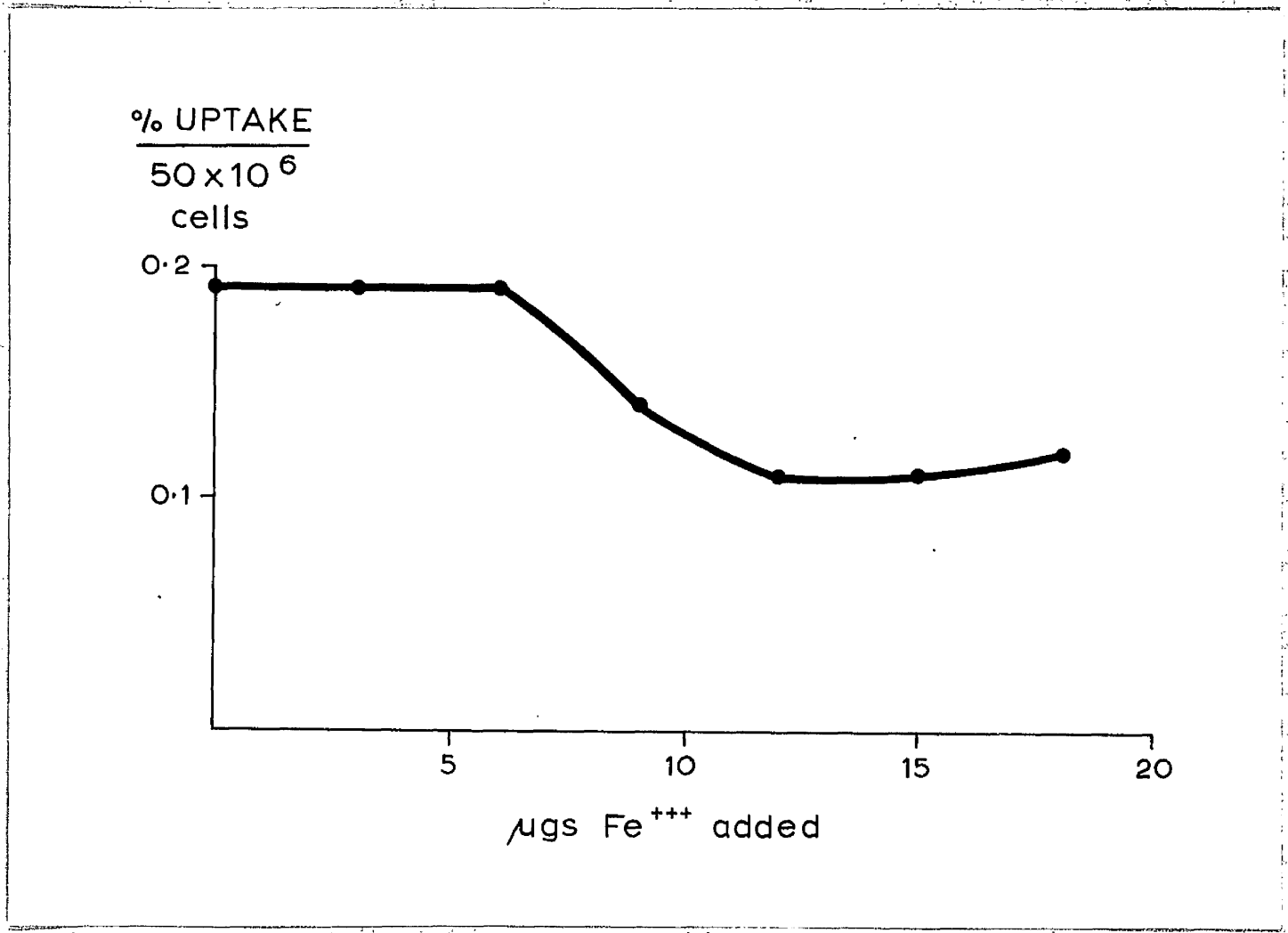

Figure 33. Effect of increasing amounts of non radio-active iron on the incorporation of radio-iron into haem by a haemolysate of 50 x 10^6 red cell precursors from the marrow of a bled rabbit.

These observations were explained by the dilution of the ^{59}Fe by the intracellular source of iron thereby reducing the amount of labelling of the newly synthesised haem. This is supported by the marked reduction in the uptake of radio-iron into haem by liver homogenates of rabbits which had previously been given 300 mg. Imferon as compared to the uptake by normal liver homogenate and liver homogenate of bled rabbits and iron deficient rabbits (Figure 31).

These findings are of importance since they demonstrate that the measurement of haem synthesis using radio-iron as a tracer may be liable to error, when there are wide variations in the sizes of the iron pool. In such cases the use of ^{14}C labelled protoporphyrin in addition to radio-iron would be of value in the accurate measurement of iron incorporating enzyme activity.

IS THERE A HAEM-STIMULATING FACTOR IN HUMAN PLASMA?

There have been many recent observations which strengthen the original studies of Carnot and Deflandre in experimental animals suggesting a humoral mechanism controlling erythrocyte production. The plasmas from patients with a variety of haematological disorders have also been found to stimulate erythropoiesis in experimental animals (Gurney et al. 1958). Recent observations by Linman et al. (1958) have suggested that there are probably two factors in plasma which exert control over erythropoiesis; one, a thermostable factor which stimulates division of erythroblasts and another factor which is only relatively thermostable, which enhances haemoglobin synthesis.

Various attempts have been made to demonstrate the haemoglobin stimulating factor in vitro. Normal rabbit serum has been shown to have a marked stimulating effect on the rate of incorporation of ^{14}C labelled glycine into haem by rabbit bone marrow (Thomas, 1955). This haem stimulating factor in serum was thermolabile. There are however, conflicting reports regarding the effect of plasma on haemoglobin formation as measured by the uptake of radio-iron into haem. Using an haemolysate of chicken red cells as the enzymic source and glycine as substrate, Goldberg et al. (1956) failed to show any difference in the uptake of radio-iron into haem between deproteinised plasmas of normal chickens and of chickens made anaemic by either bleeding or phenylhydrazine. The uptake of radio-iron into

haem was much greater when saline was substituted for any of these plasma extracts. In these experiments the iron content of each protein-free extract was determined and sufficient iron was added in order to keep constant the total iron content of every extract. Nakao et al. (1960), however, used radio-iron, with an haemolysate of chicken red cells as the enzymic source and δ-amino-laevulic acid as substrate and observed an increased incorporation of radio-iron into haem as compared to normal human plasma, when the plasmas from patients with polycythaemia, acute myeloid leukaemia and aplastic anaemia were each added to this system. The plasma from the patient with aplastic anaemia apparently stimulated the uptake of radio-iron into haem a hundred-fold. They consequently postulated that the plasma in these states had haem-stimulating activity. It should be noted however, that in the latter experiments a protein-free extract was not used except in the cases of two plasmas, in which no haem-stimulating activity was obtained. Although the above authors measured the iron content of each plasma sample, they did not measure the residual iron-binding capacity.

The discrepancy between these results of Goldberg et al.(1956) and Nakao et al.(1960), is of interest since the method of measuring haem formation was similar. The following study was undertaken to determine the cause of these differences. Two obvious factors were the varying size of the iron pools and the varying iron-binding

capacities of the plasma used in the studies of Nakao et al. The effect of these factors on iron-incorporation into haem were assessed by (1) the addition of human plasma of known total iron binding capacity obtained in a variety of haematological disorders and (2) by the addition of increasing amounts of iron to a standard aliquot of plasma which thus modified the residual iron-binding capacity as well as the total iron pool of the plasma.

METHOD

Five ml. chicken red cell haemolysate prepared by the method of Dresel and Falk (1954), δamino-laevulic acid (4.1×10^{-4}M) and 1 μc radio-iron ($^{59}FeCl_3$), specific activity about 2 μc/μg.) were incubated with 2 ml. of human plasma separated from heparinised blood. The pH was maintained at 7.4 by the addition of 0.45 M tris buffer (2-amino-2-hydroxymethylpropane 1 : 3 diol). The mixture was incubated with constant shaking at 37°C for 3 hours and the reaction stopped by the addition of 1 ml. M sodium cyanide. Carrier haemoglobin was then added and haem isolated by the method previously described. The total number of counts present in the haem was obtained by multiplying the counts per milligram of haem by the total number of milligrams of haem present in the original haemolysate. The results are expressed as the percentage of the added radio-iron which was incorporated into haem. There is some variation in the iron-incorporating activity of different chicken haemolysates

(Goldberg et al. 1956). For this reason 2 ml. saline was substituted for 2 ml. plasma in 2 control flasks in each experiment and the iron-incorporation into haem with each plasma sample was related to these saline controls in the following way:-

$$\text{Calculated iron-incorporation with plasma sample} = \text{Actual iron-incorporation with plasma} \times \frac{\text{Maximum iron-incorporation of all saline controls}}{\text{Iron-incorporation with respective saline control.}}$$

The serum iron concentration and total iron-binding capacity of plasma were measured by the method described by Ramsay (1957).

RESULTS

Comparison of the effects of different plasmas.

The iron-incorporation into haem was measured when 2 ml. samples of the plasmas of 16 patients were added to the system. The plasmas were obtained from six normal subjects, two patients with sideropaenia, one of whom was anaemic; one with haemochromatosis, one with an unexplained high serum iron, and seven with various disorders involving the haemopoietic system (Table XIII). There was a marked increase of iron-incorporation with the addition of the plasmas from 3 patients (Table XIII cases 14, 15, 16). in each of whom the level of plasma iron was markedly raised and the residual iron binding capacity correspondingly reduced.

The effect of addition of varying amounts of iron to normal plasma.

Increasing amounts of iron (4 - 8 µg.) as ferric ammonium sulphate were added to 2 ml. aliquots of a normal plasma so that there was a progressive increase in the saturation of the iron-binding capacity (Rath and Finch, (1949)). There was a sharp increase in iron-incorporation into haem as the iron-binding capacity approached the saturation level (Fig. 34). The addition of iron in excess of the total iron binding capacity of plasma did not, in the iron concentrations used, alter the percentage uptake of radio-iron into haem as compared with saturated plasma. Under all circumstances the percentage uptake of radio-iron into haem was less in the presence of plasma than in the presence of an equal volume of saline.

TABLE XIII.

Effect of plasmas of varying residual iron-binding capacities on the incorporation of radio-iron into haem.

Case no.	Diagnosis	Hemoglobin (G %)	Serum iron (μg%)	Total iron-binding capacity (μg%)	Residual iron-binding capacity (μg%)	Calculated % uptake of Fe^{59}
1.	Lead Poisoning	9.9	65	489	424	.28
2.	Secondary Polycythemia	16.4	10	336	326	.03
3.	*Idem.*	18.4	60	378	318	.02
4.	Myocardial Infarction	15	72	384	312	.26
5.	Idiopathic acquired hemolytic anemia	10.6	70	308	238	.04
6.	Mild Staphylococcal Infection	14.8	36	270	234	.07
7.	Essential Hypertension	15.7	20	254	234	.31
8.	Hematologically normal	14.8	98	326	228	.10
9.	Myocardial Infarction	13.9	78	300	222	.03
10.	Duodenal Ulcer	14.2	76	274	198	.08
11.	Cerebral Ischemia	15.7	60	222	162	.57
12.	Hypoplastic Anemia	8.3	130	288	158	.62
13.	Monocytic Leukemia	8.4	157	237	80	.045
14.	Aplastic Anemia	5.9	208	276	68	3.15
15.	Myocardial Ischemia	15.4	176	232	56	1.25
16.	Hemochromatosis	14.8	160	198	38	2.08

MAXIMUM UPTAKE OF RADIOIRON OF ALL SALINE CONTROLS = 12%

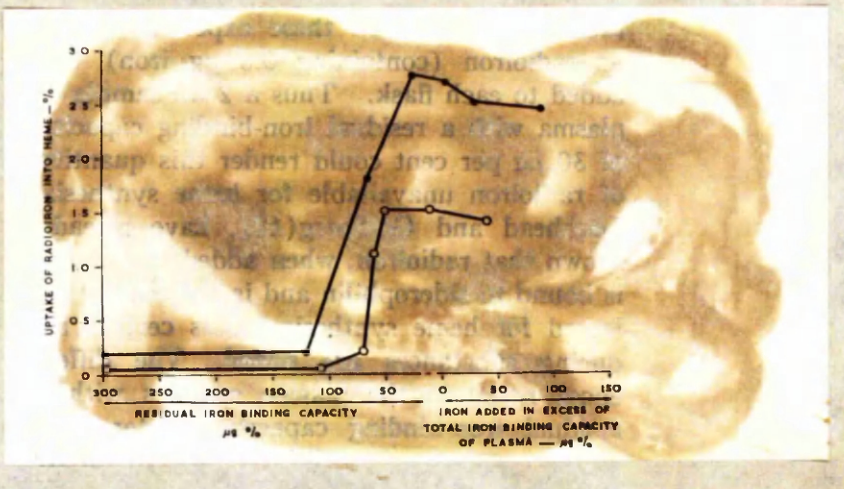

Figure 34. Effect of increasing amounts of added iron on haem synthesis of normal plasma.

DISCUSSION

There is at present ample evidence for the presence in human plasma of a humoral factor stimulating erythropoiesis. The evidence for a factor stimulating haem formation is still unconvincing and it is of importance that every report relating to this should receive a critical appraisal. London,(1962) has reported an increase in the activity of the enzyme amino-laevulic acid dehydrase in the bone marrow of rats, within twelve hours of administration of erythropoietin. Nakao et al. (1960) claimed that plasmas from a variety of haematological disorders had haem-stimulating activity using glycine or δ-ALA as substrate. In this study an identical technique has been used to assess this activity in the plasmas of 16 subjects. Increased haem formation occurred with only 3 of these plasmas, in each of which the residual iron-binding capacity was greatly diminished, 68 µg. per cent or less. In contrast to Nakao's results the plasmas from patients with secondary polycythaemia (Cases 2 and 3) and hypoplastic anaemia (Case 12) showed no evidence of increased haem formation but each of these patients had a residual iron-binding capacity greater than 158 µg. per cent. In the plasma of one patient with monocytic leukaemia (Case 13) in which the residual iron binding capacity was 80 µg. per cent, no increase in haem formation occurred.

These results suggested the importance of assessment of the residual iron-binding capacity of the plasma samples in a system using

radio-iron as a tracer for measuring haem synthesis. It was seen therefore, that the so-called haem-stimulating activity of the plasmas observed by Nakao et al. could be explained by a variability in their residual iron-binding capacities. This suggestion is corroborated by the results noted in Figure 34. There was an increased iron-incorporation into haem which occurred only on the addition of those plasmas in which the residual iron-binding capacity had been partially or completely saturated by non-radioactive iron. In these experiments 1 µc radio-iron (containing 0.6 µg. iron) was added to each flask. Thus a 2 ml. sample of plasma with a residual iron-binding capacity of 30 µg.% could render this quantity of radio-iron unavailable for haem synthesis. It has already been shown that radio-iron, when added to plasma, is bound to siderophilin and is not readily released for haem synthesis unless certain reducing substances are added. The differences noted in the plasma samples with a residual iron-binding capacity greater than 30 µg. per cent may well be accounted for by a varying level of physiological reducing substances present in each tissue system. These considerations have led to the conclusion that the apparent haem stimulating activity of the human plasmas noted by Nakao et al. could be explained by the varying residual iron-binding capacity of these plasmas.

5

HISTAMINE-FAST ACHLORHYDRIA AND IRON ABSORPTION

The association of gastric atrophy with iron deficiency anaemia has been recognised for fifty years (Faber, 1913). Two views are held; one, that the gastric mucosal changes are the result of the iron deficiency anaemia (Badenoch, Evans & Richards, 1957; Davidson and Markson, 1955); other observers believe that they prededé and are an aetiological factor in the development of the anaemia, (Meulengracht, 1932; Oliver and Wilkinson, 1933; Lees and Rosenthal, 1958; Rawson and Rosenthal, 1960). Achlorhydria, too, has for long been considered a fundamental feature of this anaemia, which was named by Witts in 1930 - simple achlorhydric anaemia. Badenoch et al. (1957) and Joske, Finckh and Wood (1955) found a good correlation between the severity of the gastric mucosal changes and the incidence of histamine-fast achlorhydria. They also showed that achlorhydria was twice as common in patients with iron deficiency anaemia as in normals, and four times as common in patients under 50 years.

There is doubt concerning the relationship of achlorhydria and iron absorption. Mettier and Minot (1931) and Minot and Heath (1932) suggested that acid gastric juice facilitated the absorption of iron. Heath and Patek (1937) suggested that the rate of the haemoglobin response to iron medication tended to be less marked in patients with iron deficiency anaemia who had achlorhydria, than in those with acid in the gastric juice. Williams (1959) found that 4

patients with achlorhydria had a diminished absorption of radio-iron (^{59}Fe) incorporated in bread. On the other hand, Brummer (1950) could not find any correlation between haemoglobin levels and gastric acidity in 100 women of 20 to 60 years. Moore (1955) could not increase the absorption of radioiron incorporated in cooked eggs given to patients with hypochlorhydria or schlorhydria by the addition of hydrochloric acid sufficient to reduce the pH of the food to 1.5. Pirzio-Biroli, Bothwell and Finch (1958) found no significant diminution in iron absorption in 4 subjects with histamine fast achlorhydria, all pernicious anaemia subjects in remission. It should be noted, however, that none of these 4 patients was anaemic or iron deficient. Biggs, Bannerman and Callendar (1961) have recently reported that patients with achlorhydria absorbed no less of a dose of radioiron given as ^{59}Fe-labelled haemoglobin, than comparable subjects, both iron defficient or deficient, with normal acid secretion; the addition of acid gastric juice to the test dose did not enhance iron absorption.

In view of these conflicting results, the absorption of iron has been compared in two groups of patients - one in which acid was present in the gastric juice, the other in which there was a histamine-fast achlorhydria. There is evidence that both iron deficiency and marrow

hyperplasia can stimulate the absorption of iron from the intestine (Pirzio-Biroli et al. 1958), and that steatorrhoea is associated with a depressed absorption of iron (Badenoch and Callander, 1954). For these reasons, the patients in both groups were matched in respect of their haematological states. All had iron deficiency; the two groups were equally anaemic; none of the patients had steatorrhoea. All of the patients were women.

METHODS

Clinical details of cases.

Fifteen female cases were investigated (Table XIV). All had iron deficiency associated with menorrhagia or with a previous history of menorrhagia and multiparity. One patient had haemorrhoids. Any patient with steatorrhoea was excluded from the series. Each of the patients had faecal fat estimations on collections taken from 3 to 6 days. The mean daily faecal fat was not greater than 5g. per day in any case. After an oral dose of xylose the 2 hour serum xylose level and the 5 hour urinary excretion of xylose was normal in every case (Chanarin and Bennett, 1962).

The diagnosis of iron deficiency was established in each case on the basis of blood findings, absence of stainable iron in the bone marrow and a characteristic serum iron and iron binding capacity (Table XIV). In case 2 neither the serum iron and iron binding capacity nor the stainable marrow iron was ascertained; the diagnosis of iron deficiency was, however, assured by the presence of a mean corpuscular haemoglobin concentration of 22 per cent and by the rise of the haemoglobin to normal levels after iron therapy, a result also obtained in all other patients.

Group A.

The 8 patients in this group had acid in their gastric juice, that is the pH of the juice was lower than 3.5, either before or after (Cases 5

TABLE XIV.

Clinical, biochemical and haematological details of patients with acid in their gastric juice and with histamine fast achlorhydria.

Case no.	Age (yr.)	Hæmoglobin (g. per 100 ml.)	Packed-cell volume (%)	Mean corpuscular hæmoglobin concentration (%)	Serum-iron (µg. per 100 ml.)	Total iron-binding capacity (µg. per 100 ml.)	Marrow iron	Fæcal fat (g. per day)	Xylose		Dietary iron (mg. per day)	Other causes of iron deficiency
									Serum (µg. per 100 ml.)	Urine (g.)		
Group A												
1	52	12·0	40	30	95	570	Absent	2·0	65	3·8	6	..
2	36	6·7	32	22	..	..	..	4·0	52	4·7	16	Menorrhagia Hæmorrhoids
3	28	7·6	32	25	10	416	Absent	2·0	39	3·1	12	..
4	34	7·0	28	27	20	510	Absent	3·3	46	6·9	17	Menorrhagia
5	62	7·3	27	27	16	455	Absent	2·0	66	7·1	11	..
6	42	8·1	32	28	30	400	Absent	1·5	31	4·0	5	Menorrhagia
7	24	10·6	39	28	20	500	Absent	2·5	50	4·6	10	Menorrhagia
8	37	8·0	28	25	25	510	Absent	1·5	43	6·5	6	..
Group B												
9	40	7·3	34	23	23	350	Absent	1·0	46	4·6	6	..
10	41	11·5	40	30	45	248	Absent	3·0	41	7·8	9	Menorrhagia
11	41	7·8	32	26	28	450	Absent	2·5	45	6·7	10	Menorrhagia
12	50	8·5	28	29	16	360	Absent	3·5	54	6·5	9	Menorrhagia
13	41	8·7	31	29	22	400	Absent	3·5	55	4·3	8	..
14	64	10·6	38	29	25	370	Absent	5·0	57	3·6	8	Multiple pregnancies (8)
15	42	8·0	32	27	45	380	Absent	2·0	36	4·0	11	Menorrhagia
Normal range	..	..	..	32–36	60–190	300–400	..	..	..	..	..	..

and 6) the administration of histamine in accordance with the augmented histamine test meal of Kay (1953).

Group B.

Six of the seven patients in this group had a histamine-fast achlorhydria after the augmented histamine test meal; the pH of the gastric juice did not fall below 6.4 either before or after the administration of histamine. Case 14 was intolerant of intubation. A Diagnex "tubeless" test meal was carried out on this patient immediately after the administration of histamine and an antihistamine in doses according to the test of Kay (1953). This showed that the gastric juice was achlorhydric. The Diagnex tubeless test correlates well with the histamine test meal (Bock and Witts, 1961). In cases 12 and 15 the Diagnex test was carried out in addition to the augmented histamine test meal and confirmed the finding of achlorhydria. The mean age and haemoglobin levels in Group A were 39.4 years and 8 g. per cent respectively as compared to 45.6 years and 8.9 g. per cent in Group B.

Test of iron absorption.

Iron absorption was measured by a technique modified from that of Pirzio-Biroli et al. (1958). The test meal consisted of 100 g. corned beef, 100 g. apple sauce, 200 ml. tomato juice, 3 g. cocoa and 13 g. of cream crackers. The total iron content of the meal was found to be 12.5mg. A tracer dose of 5 μc. $^{59}FeCl_3$ was administered half-way through

TABLE XV.

Comparison of ages and haemoglobin levels of patients in Group A (acid in gastric juice) and Group B (histamine fast achlorhydria).

Group	Age (yr.)			Hæmoglobin level at time of ^{59}Fe meal (g. per 100 ml.)		
	Range	Mean	Standard deviation	Range	Mean	Standard deviation
A	24–62	39·4	±11·6	6·7–12·2	8·0	±2
B	40–64	45·6	±8	7·3–11·5	8·9	±1·5

the meal. The utilisation for haemoglobin synthesis of absorbed ^{59}Fe was determined 14 days after the meal was administered by counting the radioactivity in a unit sample of blood and by measuring the blood volume. Ten μc. of $^{59}FeCl_3$ was then injected I.V. and the utilisation of this radioiron was then determined in the same way 14 days later. The per cent radioiron absorbed from the meal was calculated as follows.

$$\text{Per cent absorption of radioiron} = \frac{100}{\text{per cent radioiron utilisation after I.V. injection of } ^{59}Fe.} \times \text{Per cent utilisation of radioiron after the } ^{59}Fe \text{ meal.}$$

Blood volume was measured by the Evan's blue technique (Muldowney 1957). Haematological estimations were done by standard methods (Dacie, 1956).

RESULTS

The per cent absorption of radioiron from the meal in each case is noted in Table XVI and Fig. 35. The mean absorption of radioiron in Group A was 57.5 per cent and in Group B was 18.5 per cent. The difference in these two groups is statistically significant ($p. < 0.01$).

TABLE XVI.

Utilisation of radio-iron after meal containing 5 μC $^{59}Fe\ Cl_3$, and after an I.V. injection of 10 μC $^{59}Fe\ Cl_3$.

Case no.	^{59}Fe utilisation after meal (%)	^{59}Fe utilisation after intravenous radioiron (%)	Calculated absorption of ^{59}Fe from meal %
Group A			
1	15·7	69·0	22·8
2	13·3	78·0	17·0
3	57·3	73·4	78·0
4	55·5	100·0	55·5
5	50·2	74·0	67·8
6	56·2	87·0	64·6
7	60·4	91·5	66·0
8	57·6	65·1	88·5
			(mean 57·5)
Group B			
9	2·3	55·8	4·1
10	15·2	92·3	16·5
11	15·8	68·8	23·0
12	13·1	88·0	14·9
13	20·3	90·9	22·3
14	25·7	86·2	29·8
15	14·4	75·4	19·0
			(mean 18·5)

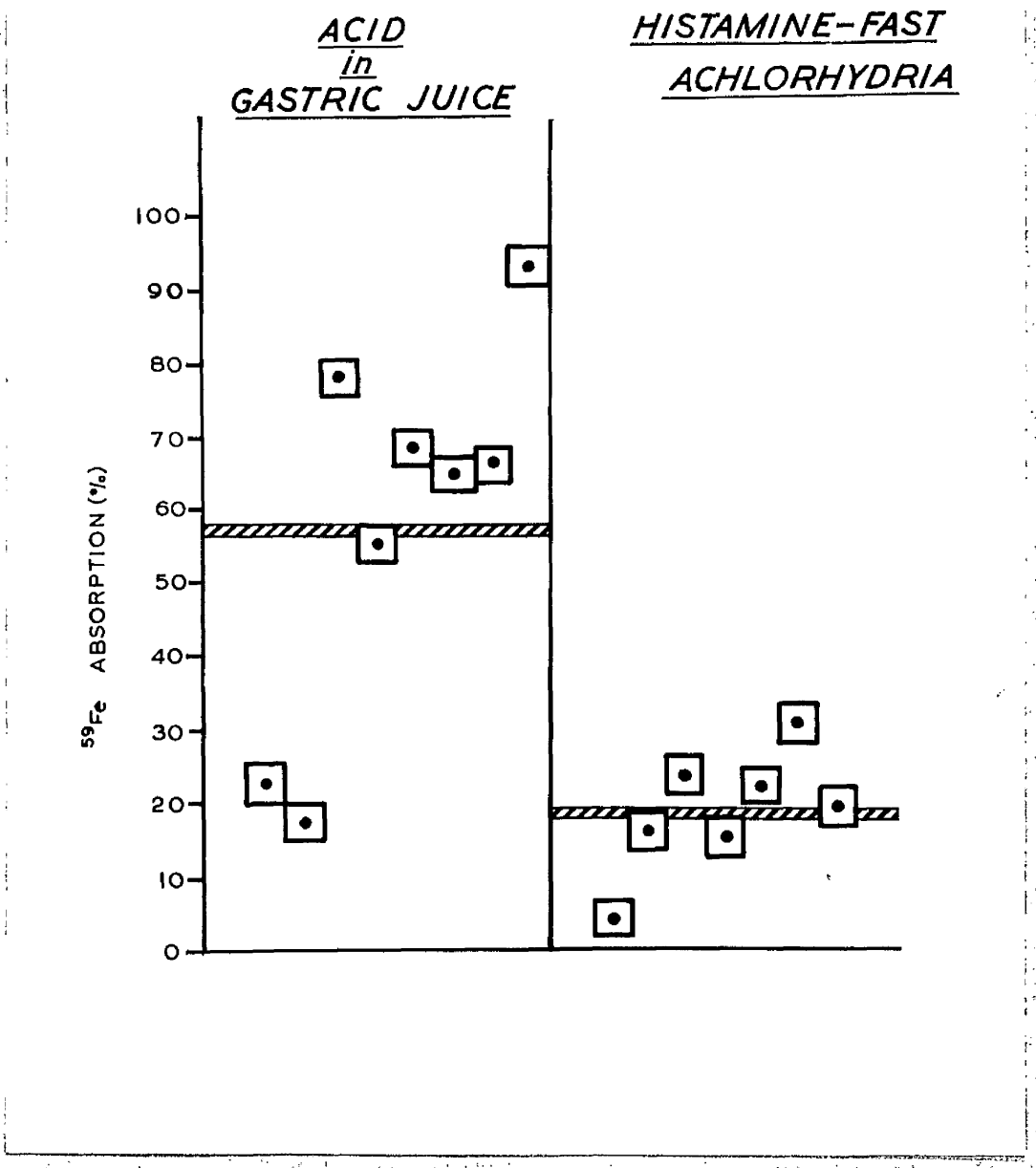

Figure 35. Per cent absorption of radio-iron taken along with a meal in 8 patients with acid in the gastric juice and 7 patients with a histamine fast achlorhydria.

DISCUSSION.

This study has shown that there is a significant impairment of iron absorption in patients with iron deficiency anaemia who have a histamine-fast achlorhydria as compared with a similar group of patients with the same degree of iron deficiency anaemia, who are able to secrete acid in their gastric juice.

These results are in agreement with Williams (1959) but contradict those of Biggs, Bannerman and Callander (1961). Although the latter authors used ^{59}Fe labelled rabbit haemoglobin, the radioiron was not given along with a meal. It is clear from previous work on iron absorption that it is important to give the tracer dose of radioiron along with a substantial meal. Thus Smith and Mallett (1957) using inorganic radioiron without a meal, failed to find a significant malabsorption of iron in anaemic patients with a partial gastrectomy, although Baird and Wilson (1958) and Stevens (1959) defined such a malabsorption in partial gastrectomy subjects, who were also anaemic. Baird and Wilson used organically bound radioiron with a meal and Stevens et al.(1959), as in the present studies, used inorganic radioiron along with a meal. Indeed Baird and Wilson failed to find a significant malabsorption of iron in partial gastrectomy subjects when a light meal was used, but did so when the radioiron was given in conjunction with a full meal. It is of interest that Williams (1959) administered radioiron added to bread. These facts may explain the

failure of Biggs et al. (1961) to detect any significant malabsorption of iron in their achlorhydric patients.

Deficiency in the iron stores has been shown to provoke an increased absorption of iron from the gut, (Pirzio-Biroli et al. 1958). The mechanism of this adaptive process is unknown, but the absorptive surfaces of the stomach, duodenum and small intestine must play some part in it. Thus the presence of a histamine-fast achlorhydria, like a partial gastrectomy, in some way prevents this enhancement of iron absorption and must be an important factor in the further development of iron deficiency anaemia, especially if the patient continues to lose blood and takes a diet poor in iron.

The actual role of the acid itself in iron absorption is dubious. From the work of Moore (1955), it seems unlikely that hydrochloric acid itself has any direct effect, although Brock and Taylor (1934) found that the in vitro dialysis of iron from iron ammonium citrate to serum was increased by the presence of 0.1N hydrochloric acid. Michaelides and Philis (1959) suggested that there is a reducing agent in normal gastric juice which is necessary for the assimilation of iron. It may be that the absorptive defect exists in the abnormal gastric mucosa associated with a histamine-fast achlorhydria. In pernicious anaemia, where the pathological changes in the stomach are similar, the acid secretion is clearly a coincidental, but not a directly related factor in the development of the macrocytic anaemia.

The results of the present studies have some relevance to the controversy as to whether the mucosal changes result from or precede and provoke the anaemia. There is no doubt that occasional improvement in the gastric mucosal pattern and return of acid to the gastric juice may occur after treatment of the anaemia (Badenoch et al. 1957). Nevertheless, there is more evidence that in established cases of gastric atrophy with or without complete achlorhydria, the pathological changes are irreversible.(Lees and Rosenthal, 1958).

Whatever effect the anaemia may have on the gastric mucosa, an abnormal mucosa, sufficient to cause a histamine-fast achlorhydria, is clearly a factor in the development of the anaemia. Furthermore, the increased incidence of achlorhydria with age (Vanzant et al. 1932) may account, in part at least, for the increased incidence of iron deficiency anaemia in the male over the age of 65 years (Kilpatrick,1961).

There is recent evidence which suggests that the gastric mucosal changes in pernicious anaemia such as lymphocyte and plasma cell infiltration as well as atrophy, may be due to a primary defect of immune tolerance (Irvine, Davies, Delamore and Williams,1962; Taylor Roitt, Doniach, Couchman and Shapland,1962). There is also some evidence that the gastric changes found in iron deficiency anaemia are associated with an auto-immune process (Markson and Moore, 1962). If, however, such a primary mechanism was responsible for the production of gastric atrophy of a degree insufficient to cause pernicious anaemia,

the changes induced might yet be sufficient to provoke the development of iron deficiency anaemia.

These considerations suggest that where iron deficiency anaemia is accompanied by achlorhydria, the gastric lesion is a primary factor in the development of the disease.

S U M M A R Y

SUMMARY

An enzyme system incorporating inorganic iron into protoporphyrin has been demonstrated in human and rat liver, kidney, spleen and in human bone marrow and measured using radioiron as a tracer. Studies on the enzyme content of the intracellular fractions of human and rat liver showed that while there was some activity in all fractions the mitochondrial fraction was the most active.

Dialysis of tissue homogenates led to a loss of enzymic activity which could be restored by the addition of ascorbic acid, GSH, cysteine and DPNH. The effect of these substances was not entirely due to an effect of reduction of the ferric iron since potentiation of activity resulted when these substances were added to a system containing iron in the ferrous form.

Observations have also been made on the *in vitro* biosynthesis of haem from protoporphyrin and iron bound to siderophilin or to rat liver homogenate. Rat liver homogenate or a preparation from it of mitochondria served as the source of iron-incorporating enzyme. The reducing substances namely ascorbic acid, GSH, cysteine and DPNH, which were effective in increasing the incorporation of inorganic ferric iron also substantially increased the transfer of protein-bound iron for haem biosynthesis. ATP enhanced the transfer of iron bound to siderophilin for incorporation into protoporphyrin but had no effect on iron bound to rat liver protein. From this it is clear that physiological

reducing substances play an important role in the transfer of iron from its protein-bound form for incorporation into protoporphyrin in the process of haem biosynthesis.

A technique has been established for the measurement of iron incorporating enzyme activity in haemolysates of rabbit bone marrow containing a known number of red cell precursors. The method has been applied to marrows of normal and bled rabbits. In the bled animal, the enzymic activity of a unit number of cells was greatly decreased as compared to normal but was increased by the addition of reducing substances. This would indicate that the marrow responds to blood loss by an increase in the production of cells rather than an increased capacity of each cell to form haemoglobin. The enzymic activity of liver homogenates was also measured in normal and bled rabbits. In this tissue there was no difference in the enzymic activity per gram of liver between the normal and the bled animals. There was, however, a marked decrease in iron incorporation by liver cells from animals which had been injected with 300 mg. Imferon. In two rabbits made iron deficient and anaemic there was an increase in iron incorporation as compared to normal. From this it is clear that gross changes in the size of the iron pool will vitiate the correct interpretation of results on the measurement of haem synthesis using radioiron as a tracer.

Nakao et al (1960) have suggested that plasma samples from

a variety of haematological disorders possess a factor (or factors) stimulating haem synthesis as compared to normal plasma. The 'haem stimulating activity' was measured by an *in vitro* technique in which radio-iron incorporation was used as a measure of haem synthesis. This work has been repeated using the same technique and results show that any marked increase in haem formation measured in this way, could be explained by the diminution of the residual iron-binding capacity of the plasma sample. It is concluded that no definite evidence has yet been obtained for the presence of a specific haem-stimulating factor in human plasma.

A statistically significant difference has been shown between the absorption of radioiron given along with a standard meal, between patients with acid present in their gastric juice and patients with a histamine-fast achlorhydria. The patients in both groups had iron deficiency. The mean absorption from the meal of the patients with acid in their gastric juice was 57.5 per cent while the mean absorption of those patients with histamine-fast achlorhydria was 18.5 per cent.

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APPENDIX

Appendix number		Reference pages in text
1.	Rat Tissues. Per cent uptake of radio-iron per gram wet weight	15 - 16
2.	Haematological and enzymic results from marrow cells of normal rabbits	40 - 41 Tables X, XI
3.	Haematological results and enzymic results from marrow cells and peripheral reticulocytes of variously treated rabbits	41 - 42 Tables X, XI
4.	Haematological values and results of enzymic studies on liver of rabbits given various treatment	43 - 44 Table XII

Rat Tissues.

Per cent uptake of radio-iron per gram wet weight.

Liver	Spleen	Kidney	Heart Muscle	Small Intestine	Brain	Leg Muscle
3.1	0.75	1.20	2.1	< 0.1	< 0.1	< 0.1
6.7	1.0	1.24	1.0	< 0.1	0.6	0.1
4.5	0.8	0.44	2.0	< 0.1	< 0.1	0.1
1.2	0.25	0.25	1.6	< 0.1	< 0.1	0.1
9.3	0.34	0.48	1.7	< 0.1	0.2	0.1
3.1	0.43	1.19	0.9	< 0.1	0.1	0.1
8.0		1.14				
3.3		0.28				
5.1						
3.9						

Treatment	Weight (Kg.)	PCV (per cent)	Haemoglobin (g. per cent)	Peripheral Reticulocytes (per cent)	Weight of Marrow (g.)	Total No. of normoblasts recovered ($\times 10^6$)	Total No. of red cell precursors recovered ($\times 10^6$)	Per Cent Uptake ^{59}Fe/50 $\times 10^6$ Cells.			
								No addition	+ Ascorbic Acid 5×10^{-4}M	+ Cysteine 1×10^{-3}M	+ GSH 7.5×10^{-3}M
	1.96	46	14.2	2.2	3.0	495	1020	0.65	15.0	5.3	6.0
	2.25	44	14.5	1.0	2.3	343	850	0.25	5.4	5.6	1.5
	2.175	-	13.0	3.0	3.0	376	986	0.68	13.2	4.9	-
	2.05	40	13.8	4.0	1.0	187	247	1.27	-	8.9	-
	2.35	41	13.2	3.2	4.1	530	898	1.30	-	3.8	-
	2.05	39	12.8	4.0	-	499	897	2.95	6.8	4.6	-
	3.225	42	13.4	1.3	4.3	558	965	-	-	4.8	-
	2.45	40	13.1	3.0	3.5	457	778	0.88	-	12.0	7.1
	1.975	46	15.1	-	-	567	848	0.28	-	1.1	-
	2.99	40.5	13.4	3.3	4.0	505	949	0.55	5.7	6.3	-
	2.35	41	12.9	4.0	-	553	1377	0.80	-	-	7.4
	2.98	38	12.9	3.0	5.6	948	1662	-	-	6.4	-
	2.25	42	12.9	3.0	-	829	1507	2.4	-	5.1	-
	2.275	39	13.4	4.2	1.5	492.5	645	2.0	12.4	-	-
	2.40	-	-	-	4.7	281	686	-	-	-	-
	2.625	43	14.3	3.4	4.1	479	837	-	-	-	-
	2.05	43	14.1	3.0	2.5	283	530	-	-	-	-
	2.285	38	12.3	2.5	3.7	636	971	-	-	-	-
	2.785	-	-	-	-	811	1286	-	-	-	-
	2.625	-	-	-	2.5	342	553	-	-	-	-

NORMAL

Haematological results and enzymic results from marrow cells of normal rabbits.

	Weight (Kg)	PCV (per cent)	Haemoglobin (g.per cent)	Peripheral reticulocytes (per cent)	Weight of marrow (g)	Total No. of normoblasts recovered ($\times 10^6$)	Total No. of red cell precursors recovered ($\times 10^6$)	Per Cent Uptake ^{59}Fe/50 x 10^6 Cells of Marrow: No addition	+ Ascorbic Acid 5×10^{-4}M	+ Cysteine 1×10^{-2}M	+ GSH 7.5×10^{-3}M	Per Cent Uptake ^{59}Fe/50 x 10^6 Peripheral Reticulocytes: No addition	Ascorbic Acid 5×10^{-4}M	Cysteine 1×10^{-3}M	GSH 7.5×10^{-3}M
	1.72	-	4.8	-	2.0	878	1225	0.10	8.6	4.35	2.75	0.5	1.85	1.35	1.4
	-	21.5	5.3	16.8	4.2	467	583	0.05	3.5	1.15	0.45	0.2	3.5	1.75	0.8
	2.48	22.5	5.3	-	3.9	930	1080	0.04	5.1	1.45	1.65	0.10	2.9	2.20	1.7
	2.20	-	5.1	26.0	3.5	796	1016	0.05	7.25	2.35	3.65	0.10	1.8	0.8	0.8
	2.48	28.0	7.0	23.0	-	1240	1544	0.03	1.43	1.38	0.85	0.05	2.4	0.8	0.6
	2.38	-	8.8	13.0	4.10	1060	1180	0.10	5.45	4.25	1.90	0.23	1.88	1.88	1.15
given Cysteine	2.58	-	7.0	31.0	5.6	1426	1651	0.19	1.3	1.7	1.1	0.17	0.5	0.4	0.4
	3.23	-	6.7	26.5	5.0	1278	1767	0.06	0.19	0.46	0.5	0.18	0.3	0.4	0.3
	3.01	-	6.0	43.0	-	1350	1526	0.21	0.12	0.23	0.27	0.06	0.3	0.2	0.2
given Ascorbic Acid	2.32	-	7.5	28.0	3.0	1093	1218	0.27	9.65	5.4	4.3	0.35	3.25	1.65	2.20
	2.05	25.5	6.7	11.0	4.1	902	1127	0.13	15.25	5.45	2.4	0.19	5.70	2.05	2.95
	2.0	-	7.0	21.0	-	746	1019	0.12	4.0	2.45	0.95	0.23	2.28	1.93	1.20

Haematological results and enzymic results from marrow cells and peripheral reticulocytes of rabbits given various treatment.

Treatment	PCV (per cent)	Haemoglobin (g. per cent)	Peripheral reticulocytes (per cent)	Per cent uptake ^{59}Fe ½ gram wet weight		
				No addition	+ Ascorbic Acid 2×10^{-3} M	+ Cysteine 7.7×10^{-3} M
Normal	41	13.3	4.0	8.6	5.6	5.0
	45	15.3	1.0	7.6	9.1	-
	40	13.0	4.0	12.8	16.1	9.1
	46	14.2	2.2	16.3	18.7	16.6
	44	14.5	1.0	7.8	8.7	7.3
	41	11.5	-	8.2	8.3	-
Given 50mg. Imferon	42	13.4	3.0	7.0	3.8	4.9
	48	15.8	2.0	9.3	-	6.0
	42	12.6	4.4	5.7	6.8	5.6
	39	12.4	4.8	8.3	10.0	6.6
	40	13.0	3.1	5.6	4.8	5.6
Given 300mg. Imferon	38	12.4	3.0	2.2	2.4	3.1
	40	13.3	3.0	3.3	3.3	2.8
	39	12.4	2.3	1.6	2.6	2.7
Bled	-	8.8	13.0	12.1	12.2	9.1
	28	7.0	23.0	20.3	20.1	22.8
	-	5.1	26.0	11.9	11.5	11.1
	22.5	5.3	-	6.5	11.6	10.7
	16.0	6.8	-	9.6	10.7	-
	17.0	7.1	-	5.7	8.5	-
Bled Iron Deficient	24.5	5.4	14.6	17.5	15.7	20.0
	24.0	5.9	7.0	23.0	35.2	35.2

Haematological values and results of enzymic studies on the liver of rabbits given various treatment.