

HAEMOPEXIN METABOLISM IN THE RABBIT

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by

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ABSTRACT

Haemopexin is a haem-binding glycoprotein found in plasma. Its main known function is the elimination of haem liberated into the plasma as a result of intravascular haemolysis. Earlier investigations have suggested that the metabolic fates of haemopexin and the haem-haemopexin complex are different, but there are no recorded quantitative measurements of haemopexin turnover. For this reason, the metabolism of ^{125}I -haemopexin has been studied in normal, haem-loaded and phenylhydrazine-treated rabbits.

Haemoglobin structure and the metabolic pathways for the elimination of this molecule and its intermediate metabolites from the plasma are reviewed and there follows a detailed account of the chemistry and function of haemopexin and its distribution in normal and disease states.

Present experiments showed that, in normal rabbits, labelled haemopexin was eliminated from the plasma with a half-clearance time of 20-30 hours. When haemopexin was haem-saturated in vivo, 90% of the labelled haemopexin was eliminated from the plasma with a half-clearance time of 1.5-2.0 hours. Rates of fractional and absolute catabolism, intravascular/extravascular haemopexin distribution and haemopexin-haem binding capacity were determined in each experimental group of rabbits.

In haem-loaded and phenylhydrazine-treated rabbits, the rapid turnover of haemopexin is best explained by the prompt elimination of the haem-haemopexin complex from the plasma, followed by rapid catabolism of the complex. Since haemopexin is catabolised as a consequence of its haem clearance function, it may be regarded as a 'suicidal' protein and placed in the same group as haptoglobin. It is likely that the haemopexin synthesis rate is capable of a marked increase in response to rising levels of plasma haem.

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HISTORICAL

In 1968, Neale, Aber and Northam added haemoglobin or haematin to human serum and showed that these substances were bound by certain plasma proteins. These complexes were separated by paper electrophoresis, the positions of the haem containing proteins being determined by benzidine staining. Serum containing 1.2 mg haemoglobin/ml showed staining in the α_2 , β_1 and albumin regions. At a concentration of 2 μ g haematin/ml serum, the β_1 and albumin fractions contained haem.

De Vaux St-Cyr, Courcon and Grabar, in 1958, showed that this haemoglobin- or haem-binding β_1 globulin was present in the perchloric acid soluble fraction of human plasma; and Biserte *et al.*, (1960), considered that the protein bound haemoglobin, myoglobin and cytochrome C. However, Nyman (1960), demonstrated that the protein bound haem and that globin was not included in the complex. Haem-binding globulin was characterised as the β_{1B} globulin of human serum by immunoelectrophoresis (Grabar, de Vaux St-Cyr and Cleve, 1960) and these workers called the protein haemopexin because of its haem binding property.

Haemopexin was purified from the perchloric acid soluble fraction of human serum by Schultze, Heide and Haupt (1960) and the physicochemical characteristics of the molecule were described by Heimburger *et al* (1964).

INTRODUCTION

In humans, normal daily red cell destruction in the reticulo-endothelial system involves the catabolism of 4-6 g haemoglobin (Freeman, 1964). In haemolytic states, the rate of red cell destruction is increased and haemoglobin may be liberated into the plasma. Haemoglobin and its intermediate metabolites are rapidly removed from the plasma, the plasma proteins, haptoglobin, haemopexin and albumin being involved in this process.

An account is given of haemoglobin structure and metabolism, the function of haptoglobin in the removal of haemoglobin from the plasma and the role of haemopexin in the elimination of plasma haem. The structure of the haemopexin molecule and its ability to bind haem from plasma and methaemalbumin are described in detail.

1. HAEMOGLOBIN STRUCTURE AND METABOLISM

Normal adult haemoglobin is a globular protein of MW64,000; it can be seen from Figure 1 that the molecule is composed of identical halves, each half consisting of 1 α and 1 β polypeptide globin chain, with a haem group contained in a fold of each chain. The haem molecule contains 1 atom of ferrous iron which is hexacovalent, binding with 4 nitrogen atoms of a tetrapyrrole ring, 1 nitrogen atom of an imidazole group of a histidine residue in the globin chain, the sixth valence being free to form a reversible attachment with oxygen. The structure of the haem molecule binding to oxygen and globin is shown in Figure 2.

The biosynthesis of haem

In the developing red blood cell, δ amino-laevulinic acid is formed from condensation of succinyl CoA and glycine by the mitochondrial bound enzyme aminolaevulinic acid synthetase. Two molecules of δ aminolaevulinic acid are converted to porphobilinogen as shown in Figure 3.

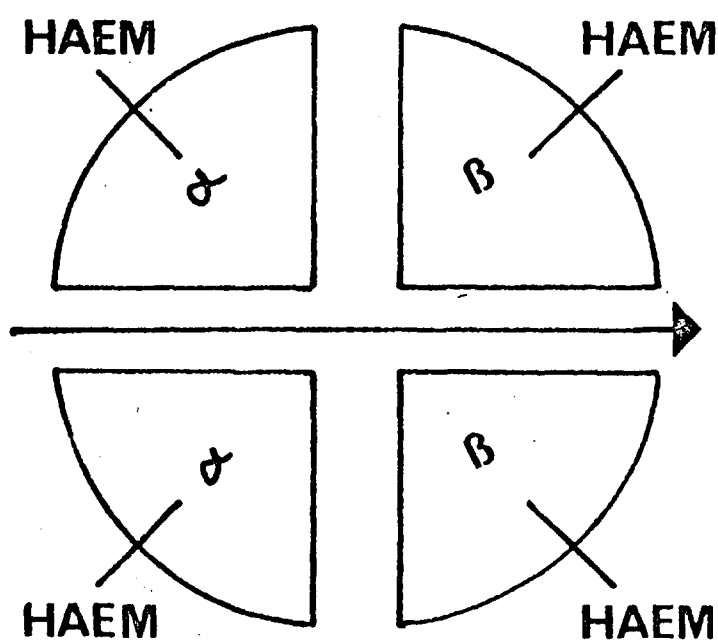


Figure 1. The haemoglobin molecule.

This diagram of the haemoglobin molecule shows the 2 α and 2 β globin chains with associated haem groups. The arrow indicates how cleavage into 2 $(\alpha\beta)$ subunits occurs (adapted from Wintrobe 5th Ed. 1961).

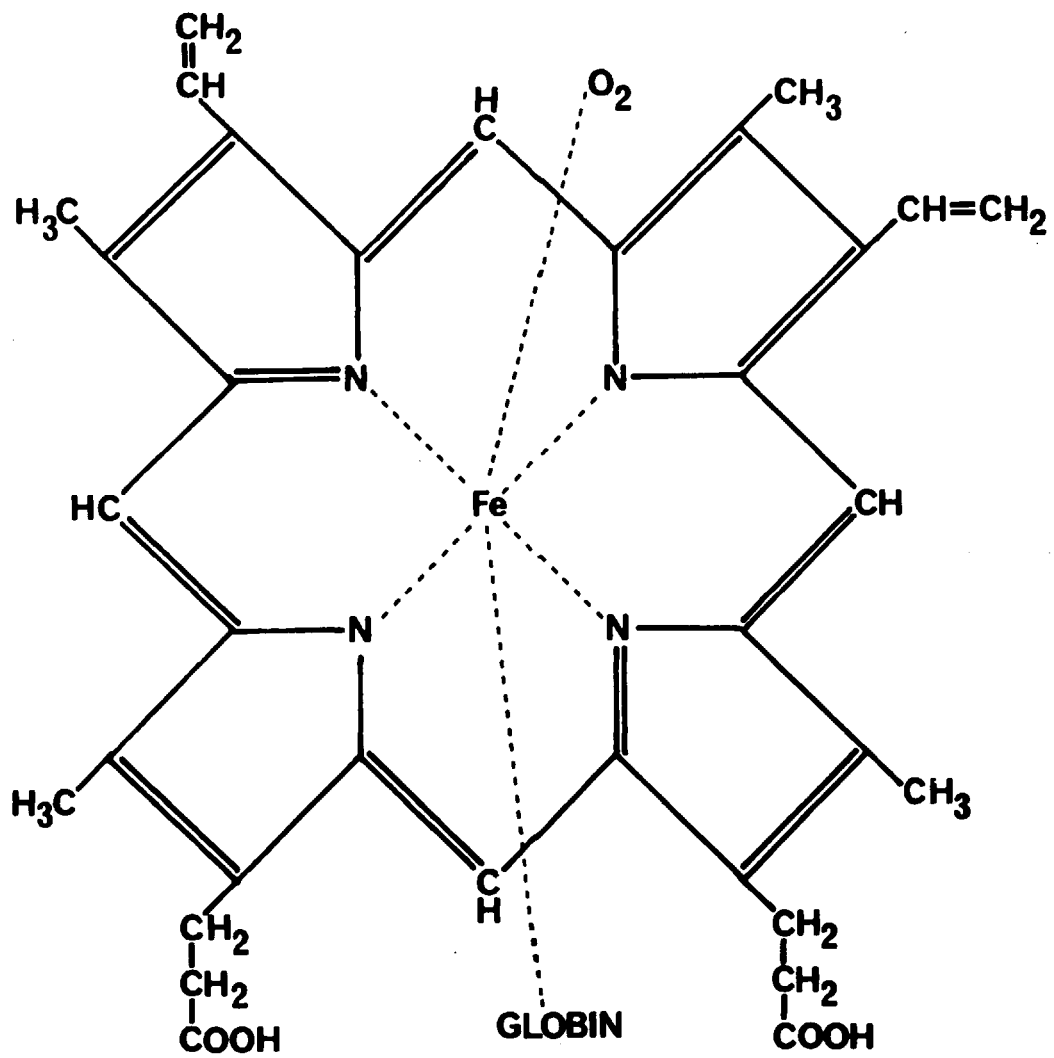


Figure 2. The haem molecule showing binding to globin and oxygen.

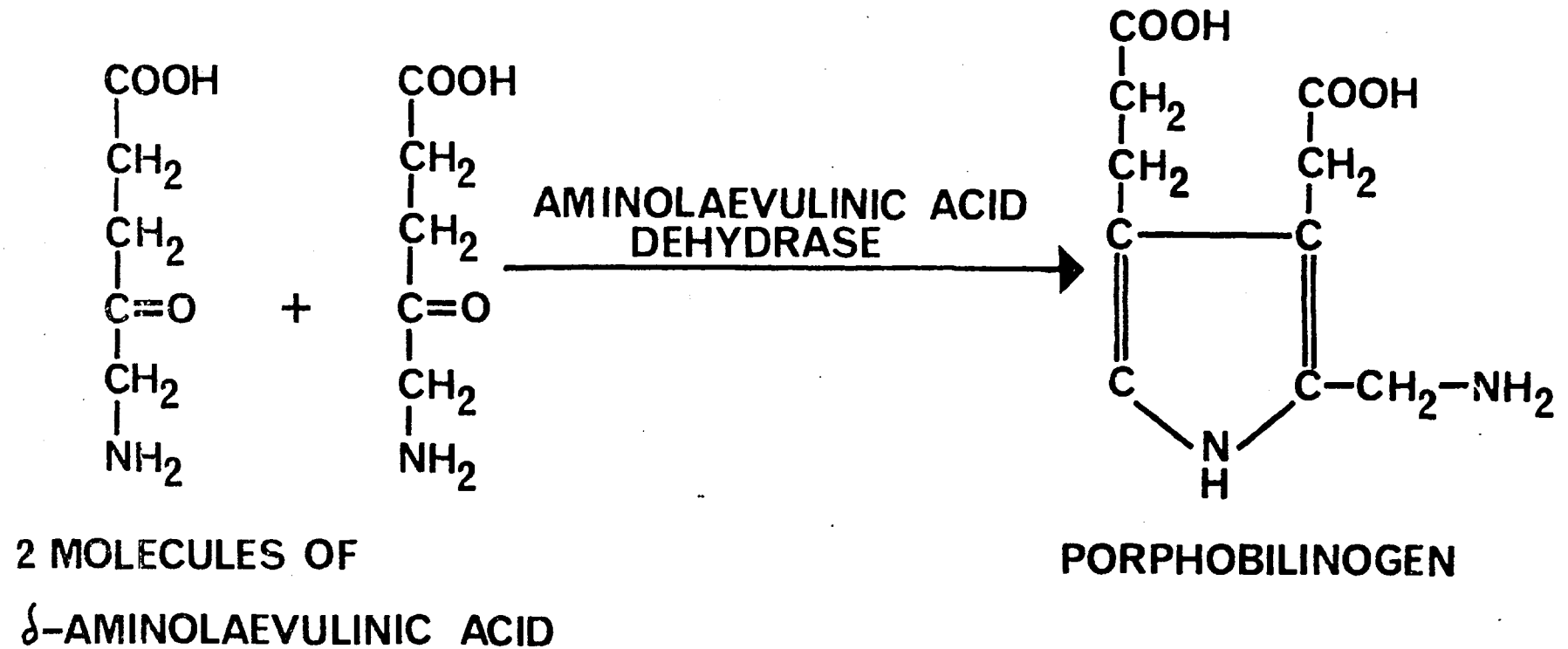


Figure 3. Conversion of δ -aminolaevulinic acid to porphobilinogen.

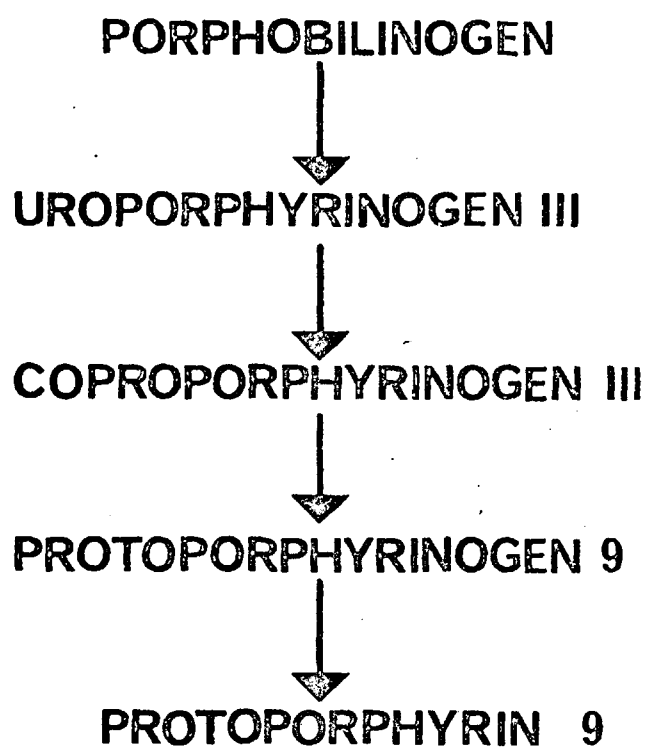


Figure 4. Conversion of porphobilinogen to protoporphyrin 9.

The conversion of coproporphyrinogen III to protoporphyrinogen 9 is effected by a mitochondrial bound enzyme (Harris and Kellermeyer 1970).

4 molecules of porphobilinogen, catalysed by porphobilinogenase, form a tetrapyrrole ring compound, uroporphyrinogen, which is ultimately converted to protoporphyrin 9. These stages in the formation of haem are shown in Figure 4.

At physiological pH, in the presence of reduced glutathione, cysteine and globin, mitochondrial haem synthetase converts protoporphyrin 9 to haem by the insertion of iron into the molecule.

Formation of haemoglobin

Haemoglobin synthesis occurs in the immature red blood cell. Haemoglobin is formed by α globin chains combining with β globin chains on the ribosomes to form $\alpha\beta$ subunits, which are then freed into the cytoplasm and combine with haem. Two $\alpha\beta$ subunits unite to form the $\alpha_2\beta_2$ haemoglobin molecule. This process is reversible; it has been demonstrated that, in solution, the haemoglobin molecule splits into dimers and monomers.

$$\alpha_2\beta_2 = 2(\alpha\beta) = 2\alpha + 2\beta \text{ (Guidotti, 1967)}$$

Maintenance of ferrous haemoglobin in the red cell

It is essential that the iron in haemoglobin is maintained in the ferrous state since oxidation to ferric methaemoglobin prevents binding of oxygen. In the red cell, the ferric iron in methaemoglobin is reduced to the ferrous form by the mechanisms shown in Figure 5. The enzyme methaemoglobin reductase catalyses the reduction of ferric iron by NADH (reduced nicotinamide adenine dinucleotide) and NADPH (reduced nicotinamide adenine dinucleotide phosphate) while reduced glutathione and ascorbate reduce ferric iron directly.

Destruction of non-viable red cells is predominantly an extravascular process occurring in the reticuloendothelial system of spleen, liver and bone marrow (Keene and Jandl, 1965). If free haemoglobin is liberated into the plasma during this process, the iron of the haemoglobin molecule

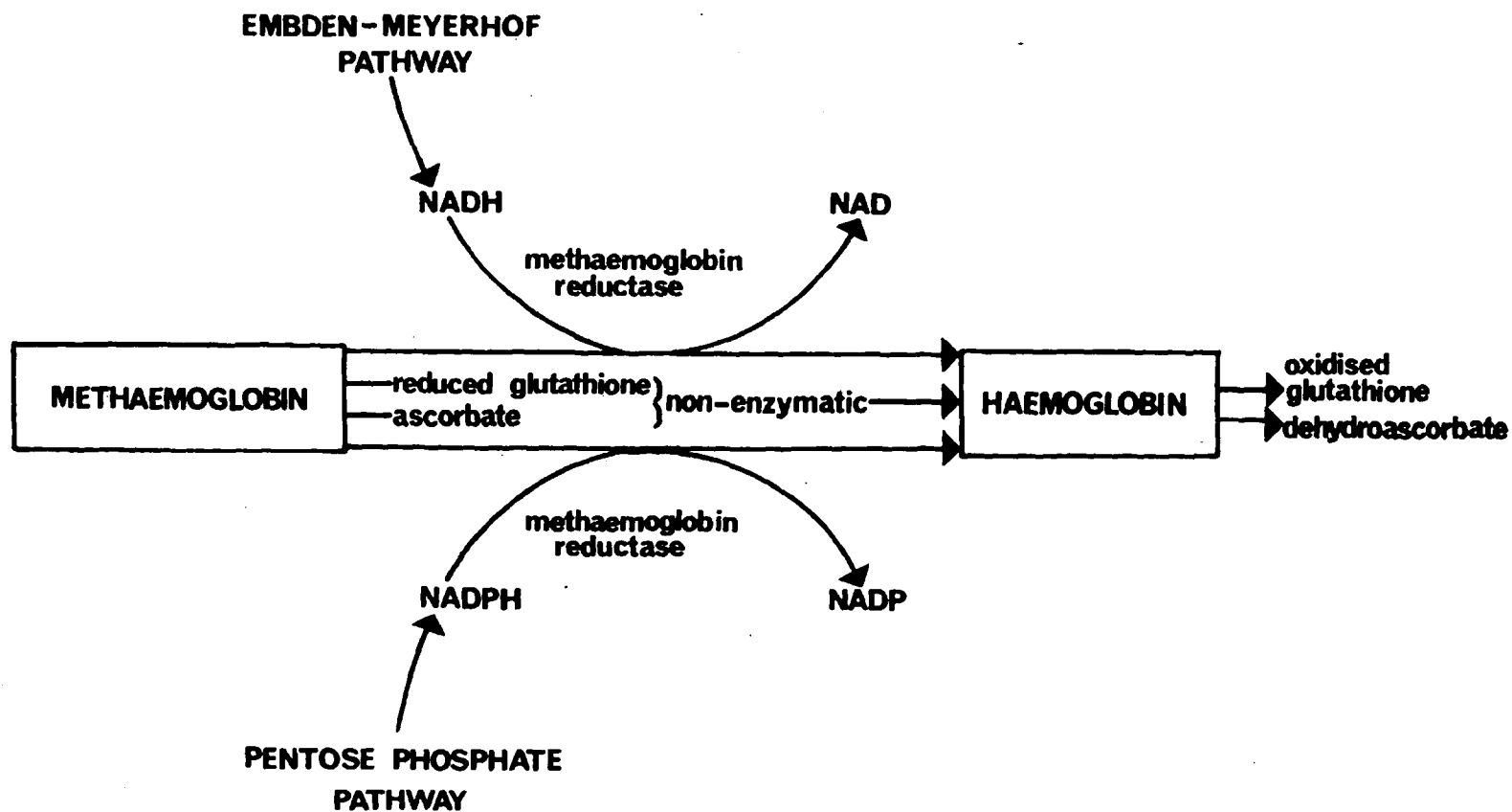


Figure 5. Mechanisms for the maintenance of ferrous haemoglobin in the red cell.

NADH reduced nicotinamide adenine dinucleotide.

NAD oxidised " " "

NADPH reduced " " " phosphate.

NADP oxidised " " " "

is rapidly oxidised, forming methaemoglobin. The binding capacity of the β chains of haemoglobin for ferric haem is lower than that for ferrous haem, so that haem dissociates from methaemoglobin into the plasma (Bunn and Jandl, 1968).

Haemoglobin catabolism

Following the sequestration and breakdown of red cells in the reticuloendothelial system, haemoglobin is catabolised. Globin is degraded and recycled in the amino acid pool. 65-80% of the haem groups are converted to bilirubin (Ostrow, Jandl and Schmid, 1962), biliverdin being an intermediate in this process.

Conversion of haem to biliverdin occurs in microsomes of the reticuloendothelial system in liver, spleen and bone marrow and in hepatic parenchymal cells and renal tubular epithelium (Tenhunen, Harver and Schmid, 1970; Tenhunen, 1972). Microsomal haem oxygenase oxidises the α methene group of haem to carbon monoxide, iron is released and biliverdin is formed. This process requires NADH (reduced nicotinamide adenine dinucleotide) and molecular oxygen and uses cytochrome P450 as the terminal oxidase. Biliverdin is reduced to bilirubin by biliverdin reductase.

Very little iron released from haem is lost in the urine, the pathways for haemoglobin breakdown being highly efficient in iron conservation and the iron is reutilised in new haemoglobin synthesis.

2. THE FATE OF FREE HAEMOGLOBIN IN THE PLASMA

The mechanisms for the removal of free haemoglobin from the plasma are shown in Figure 6 and it can be seen that haptoglobin, haemopexin and albumin form important metabolic pathways for the elimination of haemoglobin and haem from the plasma. The extent to which these mechanisms are involved is dependent on the rate of liberation of free haemoglobin into the plasma.

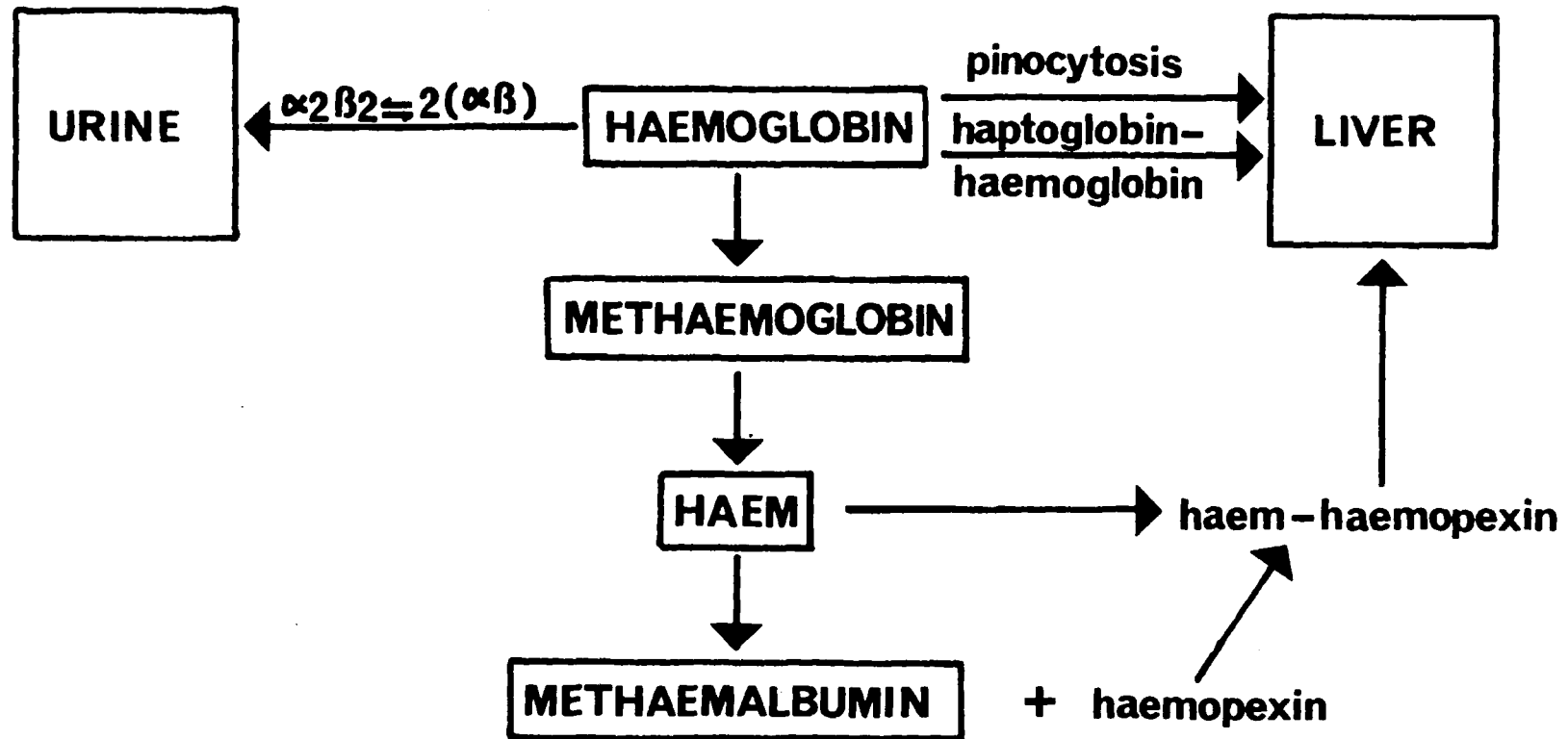


Figure 6. Mechanisms for the removal of free haemoglobin from the plasma.

a) Normal state

Normal human plasma contains approximately 0.3 mg haemoglobin/100 ml (Bunn, 1972). Amounts of this order are bound and cleared as the haptoglobin-haemoglobin complex.

Haptoglobin

Haptoglobin is an α_2 globulin found in plasma which has a haemoglobin binding capacity of 50-150 mg/100 ml plasma (Bunn, 1972). Approximately one-third of the total haemoglobin catabolised per day is eliminated as the haptoglobin-haemoglobin complex (Freeman, 1964).

Haptoglobin is a glycoprotein consisting of 2 pairs of α and β polypeptide chains; it forms stable complexes with haemoglobin and with globin (Pintera, 1971). The globin fraction of haemoglobin is bound by globin binding sites on the β chains of haptoglobin (Shim, Lee and Kang, 1965). Haptoglobin probably binds haemoglobin on an equimolar basis (Freeman, 1964); however, an intermediate complex consisting of haptoglobin and haem-globin dimer ($\alpha\beta$) has been demonstrated when haptoglobin is in excess (Shim, Lee and Kang, 1965). Free α globin chains readily combine with haptoglobin and their presence is required for the binding of β globin chains by haptoglobin. It is likely that the complex is formed by separate binding of $\alpha\beta$ units through the α chain (Nagel and Gibson, 1971). Nyoglobin, a monomeric protein resembling the β chain of haemoglobin, is not bound by haptoglobin.

Haptoglobin is synthesised in the liver (Alper et al., 1965). It was believed that the haptoglobin-haemoglobin complex was catabolised by the reticuloendothelial system in the liver (Peters and Alper, 1966; Pintera, 1971) but recent experiments have demonstrated uptake of the haptoglobin-haemoglobin complex by hepatic parenchymal cells (Hershko, Cook and Finch, 1972). The haptoglobin-haemoglobin complex is not excreted in the urine and binding of haemoglobin by haptoglobin is an efficient method of conserving haemoglobin iron in the body (Giblett, 1968).

The half-life of haptoglobin in man is 3-4 days (Freeman, 1964; Giblett, 1968). On injection of 3 mg haptoglobin as the haptoglobin-haemoglobin complex into a normal man, Freeman (1964) found that the complex was cleared from the plasma with a half-clearance time of 9 minutes. Laurell and Nyman (1957) found that following the injection of haptoglobin-saturating amounts of haemoglobin into normal subjects, the haptoglobin-haemoglobin complex was eliminated from the plasma with a half-life of 5-6 hours. Haptoglobin synthesis does not rapidly compensate for the loss of the haptoglobin-haemoglobin complex when intravascular haemolysis is increased above normal levels and plasma haptoglobin concentration becomes depleted (Giblett, 1968).

Assuming equimolar binding, the amount of haptoglobin catabolised per day is approximately one-third the amount of haemoglobin degraded during normal red cell destruction (Freeman, 1964). However, the actual ratio of haptoglobin: haemoglobin removed in vivo may be greater than that based on equimolar binding if the haptoglobin-haemoglobin dimer ($\alpha\beta$) complex is involved (Giblett, 1968).

If haemoglobin liberated into the plasma is in excess of haptoglobin binding capacity, other pathways become involved in haemoglobin removal. Haemoglobin splits into haem-globin dimers, MW32000, which may be excreted into the urine (Bunn, Estman and Bull, 1969) or be reabsorbed by the renal tubules with conversion of iron to haemosiderin in the tubular epithelium. Remaining haemoglobin is oxidised to methaemoglobin and dissociated haem is eliminated from the plasma by pathways involving haemopexin and albumin.

b) Acute haemolytic episodes

In some cases, for example in paroxysmal nocturnal haemoglobinuria, paroxysmal cold haemoglobinuria and haemolytic transfusion reactions, a sudden episode of increased red cell destruction may release large quantities of haemoglobin into the plasma. Under these circumstances, haptoglobin is rapidly depleted and other pathways are involved in the elimination of free plasma haemoglobin. Depending on the rate and magnitude of haemoglobin release, varying amounts of haem-globin dimer will be excreted in the urine, since the absorptive capacity of the renal tubules for haem-globin dimer is likely to be exceeded.

Goldfischer et al. (1970) demonstrated pinocytosis of free haemoglobin by hepatic parenchymal cells in rats receiving large doses of haemoglobin (40-400 mg/kg) but this process has yet to be satisfactorily demonstrated in man.

Circulating plasma haemoglobin is oxidised to methaemoglobin, from which the haem moiety dissociates. Free plasma haem is rapidly bound by haemopexin and albumin, which together constitute the pathways for elimination of haem to the hepatic parenchymal cells (Müller-Eberhard, Bosman and Liem, 1970).

Both during and immediately after an acute haemolytic episode, plasma levels of haptoglobin and haemopexin may be reduced to zero, the extent of this reduction depending on the amount of free plasma haemoglobin. Once haemoglobin and intermediate metabolites have been eliminated from the plasma, the concentration of haemopexin returns to normal within 24-48 hours (Müller-Eberhard et al., 1969; Sears, 1969) whilst there may be a delay of up to 12 days before normal plasma levels of haptoglobin are restored (Sears, 1968).

g) Chronic haemolytic states.

In these conditions there is an increased rate of red cell destruction extending over a long period and plasma levels of haptoglobin and haemopexin will be reduced according to the severity of the haemolysis. The rate of haemoglobin release may not be sufficient to produce haemoglobinuria, but renal excretion of haem-globin dimer occurs, followed by absorption of this molecule by the renal tubules, where haemosiderin is formed. Haemosiderinuria results from desquamation of the renal tubular epithelium.

3. HAEMOPEXIN

Haemopexin is involved in the elimination of haem from the plasma in both acute and chronic haemolytic states.

The protein was purified by Schultze, Heide and Haupt in 1960 from the perchloric acid soluble fraction of human plasma and was isolated as the β_{1B} globulin of human plasma by immunoelectrophoresis by Grabar, de Vaux St-Cyr and Cleve, 1960.

Physicochemical properties of haemopexin

Haemopexin is a sialoprotein and the molecule contains approximately 20% carbohydrate. The amino acid and carbohydrate constituents of haemopexin are listed in Table 1.

Human and rabbit haemopexins have been estimated to have a molecular weight of 70,000 and 63,000 respectively (Müller-Eberhard *et al.*, 1969; Müller-Eberhard, 1970) and later values obtained by sedimentation equilibrium gave a value of $57,000 \pm 3,000$ for both human and rabbit haemopexins (Seery, Hathaway and Müller-Eberhard, 1972).

At present, only one N terminal amino acid has been found (Müller-Eberhard, 1970) and reduction alkylation experiments suggest that the molecule does not contain a subunit structure (Birkol and Müller-Eberhard, 1971).

TABLE 1 AMINO ACID AND CARBOHYDRATE COMPONENTS
OF HUMAN HAEMOPEXIN

	%	moles/mole protein
lysine	4.16	26
histidine	3.25	19
ammonia	1.24	62
arginine	4.42	23
asparagine	6.49	45
threonine	3.46	27
serine	3.59	33
glutamine	7.74	48
proline	4.62	38
glycine	3.18	45
alanine	2.80	32
$\frac{1}{2}$ cysteine	1.44	11
valine	3.45	28
methionine	1.14	7
isoleucine	1.59	11
leucine	5.94	42
tyrosine	3.74	18
phenylalanine	3.96	21
tryptophane	4.30	18
Total	70.51	554
hexose	9.00	40
acetylhexosamine	7.40	27
sialic acid	5.00	13
fucose	0.40	2
TOTAL	92.31	636

Data from Heimbürger et al. (1964)
using a MW of 80,000 for haemopexin

Purification

Haemopexin is now purified by the perchloric acid method of Schultze, Heide and Haupt, followed by DEAE chromatography and preparative zone electrophoresis. Purified haemopexin consists of two fractions, haemopexin monomer and a polymerised form which can be converted to haemopexin monomer by treatment with 5-7 M urea (Müller-Eberhard and English, 1967).

Haem binding

On addition of haem to haemopexin, there is a shift in absorption maximum from 395 nm to 412 nm due to the formation of the haem-haemopexin complex. Haem binding is equimolar (Heide *et al.*, 1964). Evidence for change in molecular conformation after haem binding is obtained from measurements of circular dichroism and optical rotatory dispersion (Müller-Eberhard and Grizzuti, 1971) and there is reduced antigenicity of haemopexin when complexed with haem (Müller-Eberhard, 1970). Apart from binding haem, haemopexin also binds coproporphyrins and uroporphyrins (Koskela, Toivonen and Rintola, 1969) but bilirubin is not bound.

Chelation of haem from haem containing molecules

In vitro experiments have shown that rabbit and human haemopexins can chelate the haem prosthetic group from cytochrome P450, with consequent reduction of biological activity of the cytochrome (Müller-Eberhard *et al.*, 1969). Transfer of haem from tissue bound proteins may occur in vivo and it is thought that certain types of cytochrome may serve as tissue receptor sites for circulating plasma haem. More importantly, affinity of haemopexin for haem has been shown in vitro and in vivo to be greater than that of albumin for haem (Müller-Eberhard and Liem, 1968; Müller-Eberhard *et al.*, 1969). The role of albumin as a clearance pathway in haemoglobin metabolism is therefore of significance, since it appears to act as a large reservoir for haem when plasma haem concentration exceeds the binding capacity of endogenous haemopexin. The exact fate of haem bound to albumin is not completely understood, but it is likely that elimination of haem from methaemalbumin to hepatic parenchymal cells occurs by a transfer

process involving haemopexin until the haem binding capacity of haemopexin approaches saturation (Müller-Eberhard *et al.*, 1969; Müller-Eberhard, Bosman and Liem, 1970). It is not known whether haem can be eliminated directly from methaemalbumin: experimental evidence suggests that this is unlikely. Rapid *in vivo* transfer of haem from methaemalbumin to haemopexin in the rabbit was demonstrated by Müller-Eberhard *et al.*, (1969). These workers also showed that albumin and methaemalbumin were cleared from the plasma at the same rate, with a half-clearance time of 4.5-5.5 days. They concluded that most, if not all, circulating haem was removed from the plasma as the haem-haemopexin complex.

Distribution

Haemopexin has been isolated from the plasma of the human, rabbit, rat, guinea pig and mouse, but only human, rabbit and mouse haemopexins have been partially characterised (Müller-Eberhard and English, 1967; Hrkal and Müller-Eberhard, 1971; Witz and Gross, 1965). Genetic polymorphism has been described in the rabbit (Grunter, 1966).

Serum haemopexin in health and disease

a) Normal levels

Normal human serum haemopexin levels are shown in Table 2.

Haemopexin has been found in the serum of a 10½ week old human foetus (Citlin and Biasucci, 1969). Foetal origin is likely as it has been shown in the rabbit that the amount of haemopexin passing from the maternal circulation to the foetal circulation is very small (Ross, Liem and Müller-Eberhard, 1971).

TABLE 2 NORMAL SERUM HAEMOPEXIN LEVELS IN THE HUMAN

Age	Haemopexin concentration range (mg%)	Mean haemopexin concentration (mg%)
foetus 13 weeks	-	3
neonates	6.5-25	18
premature infants		
cord serum		
1-9 months	10-70	-
1-12 years	40-70	60
20-40 years	66-100	77
pregnant females	44-100	75
20-40 years		
50-70 years		
	50-80	65

Data compiled from Hanstein and Müller-Eberhard (1968)

Mean haemopexin concentration in the rabbit is 60 mg/100 ml serum with a range of 40-80 mg/100 ml serum (Müller-Eberhard et al., 1969).

It has been shown in the rabbit that serum haemopexin concentration is not subject to circadian fluctuations (Smibert, Licm and Müller-Eberhard, 1972).

b) Levels in disease

In general terms, increased breakdown of red blood cells may be associated with a low serum haemopexin level if the degree of intravascular haemolysis releases more haemoglobin than can be cleared by haptoglobin. Low serum haemopexin levels are almost invariably associated with reduced or absent haptoglobin (Hanstein and Müller-Eberhard, 1963; Müller-Eberhard and Cleve, 1963; Müller-Eberhard et al., 1968; Sears, 1968).

If increased red cell destruction is associated with a haptoglobinaemia and haemopexin deficiency, high levels of methaemoglobin are found in the plasma, methaemalbumin acting as a reservoir for plasma haem in excess of the haemopexin binding capacity. Thus the plasma level of haem is the decisive factor determining plasma haemopexin concentration: plasma haem levels greater than 6.0 µg/ml (equivalent to 150 µg/ml haemoglobin) result in decreased plasma haemopexin concentration (Müller-Eberhard et al., 1968).

Disorders with an increased rate of intravascular red cell destruction are shown in Tables 3 and 4. The conditions in Table 3 are those in which the most marked degree of intravascular haemolysis is likely to occur. When red cell destruction is persistently severe, both haptoglobin and haemopexin levels are zero or approaching zero. In conditions where there is episodic but massive intravascular haemolysis, plasma haptoglobin and haemopexin concentrations fluctuate between zero and normal levels, depending on whether the concentration was determined at the time of the haemolytic crisis, or between crises.

TABLE 3 SERUM HAEMOPEXIN AND HAPTOGLOBIN LEVELS
IN DISEASE

Condition	Haemopexin concentration range (mg%)	Haptoglobin concentration range (mg%)
thalassaemia major	0-5 0, 5*	0-50 5, 10*
G6PD deficiency + primaquine	0*	0*
mechanical haemolysis	0	0
malaria	0*	0*
P.N.H.	40-60 0-65 0*	15-35 0-50 0*
S cell anaemia	0-40 15*	0-25 10*
S cell haemoglobin C	10-40 0-40	15-70 0-25
transfusion reaction anti-D, anti-C, anti-E	0	0
thalassaemia haemoglobin E	15*	5*
pyruvate kinase deficiency + severe haemolytic anaemia	10*	10*

* denotes single determination only

Data compiled from Müller-Eberhard *et al.* (1968); Müller-Eberhard (1970) and Sears (1968). Determination of haptoglobin concentration was by haemoglobin binding and radial immunodiffusion. Haemopexin was measured by haem binding and radial immunodiffusion.

**TABLE 4. SERUM HEMOPEXIN AND HAPTOGLOBIN LEVELS
IN DISEASE**

Condition	Hemopexin concentration range (mg%)	Haptoglobin concentration range (mg%)
thalassaemia trait	40-95	2-120
S cell trait	35-70	35-80
spherocytosis	40-65	60-65
autoimmune haemolytic anaemia	30-85 20-100	5-40 0-30
pernicious anaemia	30-70	10-30

Legend as for Table 3

Table 4 lists several haematological disorders in which there is decreased red cell survival. Again, depending on the severity of the haemolytic process, plasma levels of haptoglobin and haemopexin may be reduced, but in the conditions listed, reduction is significantly less common than in the conditions given in Table 3.

Sears (1968) found in 6 patients with thalassaemia major that all had anaptoglobinaemia associated with haemopexin deficiency. Whilst low serum haemopexin levels are invariably associated with reduced or absent haptoglobin, Müller-Eberhard and Cleve (1963) described haemopexin deficiency without reduction of haptoglobin in several children with thalassaemia major, but had to postulate that a haem compound might be present which depleted haemopexin without affecting haptoglobin. In haemorrhagic pancreatitis, a haem compound may be liberated into the circulation which reduces the haemopexin level without altering the concentration of haptoglobin in the plasma (Sears, 1968).

Diagnostic importance of serum haemopexin levels

Low levels of serum haemopexin have also been described in protein-losing nephropathies and progressive liver disease (Müller-Eberhard, 1970). Raised levels have been reported in some malignant diseases, infections, rheumatoid arthritis and diabetes mellitus. Following stress, the concentration of serum haemopexin does not increase above twice the normal level and haemopexin is not considered to be an acute phase protein (Kushner *et al.*, 1972).

It has been suggested that once serum haptoglobin is decreased in haemolytic disorders, measurement of serum haemopexin levels may be of use in determining the severity of the haemolytic process. Measurement of haemopexin: albumin ratio in amniotic fluid during gestation has been used to assess foetal red cell destruction in Rhesus incompatibility (Müller-Eberhard and Bashore, 1970). The results have shown a degree of accuracy described as greater than that obtained by determination of bilirubin in amniotic fluid.

Haemopexin metabolism

At the time of commencing this investigation, no direct measurements of haemopexin synthesis or catabolism had been reported. Müller-Eberhard *et al.*, (1969) showed that within 5 hours of haem injection, plasma haemopexin concentration fell steeply, but this decrease was rapidly corrected once the haem was eliminated from the plasma. Injection of ^{125}I -haemopexin- ^{59}Fe -haem showed that both the ^{125}I and ^{59}Fe rapidly accumulated in the liver, other organs (kidney, spleen, lung) showing negligible activity (Müller-Eberhard, Bosman and Lien, 1970). The activity localised in the liver was demonstrated autoradiographically to be in the parenchymal cells and it was concluded that the haem-haemopexin complex is rapidly eliminated from the plasma and that the consequent reduction in plasma haemopexin concentration reflects an increased rate of catabolism of haem complexed haemopexin. The localisation of the haemopexin label in hepatic parenchymal cells suggests that these cells, or their immediate environment, constitute an important site of catabolism of the protein. The increase in plasma haemopexin concentration observed after the removal of the haem-haemopexin complex from the plasma was thought to be due to an increased rate of haemopexin synthesis (Müller-Eberhard *et al.*, 1969).

Certain essential aspects of haemopexin metabolism have not been directly investigated, namely, the fractional and absolute catabolic rates, intravascular/extravascular distribution and the effects of haem on the catabolism and distribution of haemopexin. Neither total haem binding capacity nor the extent to which this may change in response to raised levels of plasma haem is known at present. It has not been ascertained whether haemopexin is recycled in the plasma after transfer of haem to the hepatic parenchymal cells, or whether the complex is catabolised immediately, thus placing haemopexin in an analogous situation to that of haptoglobin, where binding of haemoglobin results in rapid elimination and catabolism of the complex.

At present, it is assumed that the major site of haemopexin synthesis is the liver. In the absence of any direct measurements of haemopexin synthesis rate, the absolute response to injection of haem is not known. Following haem injection, increase in plasma haemopexin level to approximately twice the normal level has been described by Müller-Eberhard et al. (1969). A more striking increase in plasma haemopexin concentration follows the injection of the porphyrogenic or carcinogenic chemicals allylisopropylacetamide, 3-methylcholanthrene and lead acetate (Ross and Müller-Eberhard, (1970). This increase in plasma haemopexin is due to increased synthesis since the response can be blocked by ethionine.

Objectives of the investigation

The main purpose of this investigation was to carry out the first complete quantitative study of ^{125}I -haemopexin turnover in normal and haem-loaded rabbits, to permit determination of fractional and absolute catabolic rates, intravascular/extravascular distribution of haemopexin and the effect of haem upon haemopexin turnover and distribution.

Plasma haemopexin concentration was measured to obtain accurate determinations of total body haemopexin haem binding capacity, and its variation in response to change in the amount of haem injected was examined. For this purpose several experiments were completed in which ^{59}Fe -haem was used to permit simultaneous determination of plasma clearance of haemopexin and haem.

The relative importance of haemopexin in haemoglobin catabolism is not known, therefore plasma ^{125}I -haemopexin turnover has been measured following the injection into rabbits of graded doses of haem and in animals with phenylhydrazine-induced haemolytic anaemia.

A critical appraisal has been made of the sources of haemopexin used in these experiments and the extent to which the study of plasma clearance of haemopexin in haem-loaded rabbits can be used to assess haemopexin synthesis rate.

MATERIALS AND METHODS

1. Rabbits

New Zealand White Rabbits* were used throughout and were maintained on Short and Ganuge 1 diet (Oxoid)**

2. Rabbit haemopexin

Pure rabbit haemopexin was prepared from rabbit serum by Dr U Müller-Eberhard*** by the following method. Either pooled normal rabbit serum was used or pooled serum from rabbits with increased haemopexin levels induced by injection of 3-methylcholanthrene (1 intraperitoneal injection of 4 mg 3-methylcholanthrene/Kg body weight) or lead acetate (1 sub-cutaneous injection of 150 mg lead acetate/Kg body weight). Rabbits were bled by cardiac puncture 48 hours after injection when serum haemopexin concentrations had risen to at least 3 times base line values.

Pooled rabbit serum was treated with perchloric acid by the method of Schultze *et al.* (1961; 1963), by adding 1 part serum to 3 parts 0.15 M sodium chloride and 0.5 parts perchloric acid at -2°C . Exposure to perchloric acid was kept to the minimum and the mixture was centrifuged. By this precipitation method only perchloric acid soluble proteins are retained in the supernatant. The supernatant was decanted and neutralised with 0.1 M sodium hydroxide, concentrated by ultrafiltration and reprecipitated with 2.0 M ammonium sulphate at pH 5.0. The supernatant was separated by centrifugation and dialysed against sodium phosphate buffer pH 7.2 $T/2 = 0.025$ prior to chromatographic separation as described by Müller-Eberhard and English (1967).

* Obtained from A. E. Moss Ltd., White Cloud Farm, Tring, Herts.

** Oxoid, Southwark Bridge Road, London SE1.

*** Department of Biochemistry, Scripps Clinic and Research Foundation, La Jolla, California.

A 7.4 cm x 60 cm column of DEAE cellulose was equilibrated with sodium phosphate buffer pH 7.2 $\Gamma/2 = 0.025$. 16-18 g protein were applied and after layering of the protein fraction, the column was washed with 4000 mls starting buffer at a flow rate of 10 mls per minute. Haemopexin was eluted when the ionic strength of the buffer was raised to $\Gamma/2 = 0.05$. 20 ml fractions were collected; protein concentration was determined by absorbance at 280 nm and haemopexin was identified by immunoelectrophoresis.

According to the method of Müller-Eberhard and English, further purification was carried out by two consecutive preparative zone electrophoreses on Geon 427 (polyvinylchloride resin). For the first electrophoresis, 400-500 mg protein were applied to Geon blocks 56 cm long, 1 cm high and 30.5 cm wide; electrophoresis was continued for 30 hours at a voltage of 7.1 volts per cm in 0.04 M citric acid and 0.03 M dibasic sodium phosphate, pH 3.5.

A second electrophoresis of 20-30 mg protein on blocks 56 cm long, 1 cm high and 7.5 cm wide for 30 hours at 5.4 volts per cm in the same buffer resulted in the removal of all contaminants.

Haemopexin was eluted from the blocks by 5-10 mls sodium barbital buffer pH 8.6 $\Gamma/2 = 0.05$. Following the second electrophoresis, treatment of haemopexin with 5-7 M urea resulted in conversion of polymerised haemopexin to the monomeric form. After removal of urea by dialysis against several changes 0.05 M phosphate buffer pH 7.0 for 18 hours, haemopexin remained in the monomeric form.

¹²⁵I-labelling of haemopexin

Haemopexin monomer was labelled with ¹²⁵I by the method of McConahey and Dixon (1966).

A 30 ml beaker holding 1-5 mg haemopexin in 4 ml 0.05 M phosphate buffer pH 7.0 and a 2.5 cm polyethylene stirring rod was placed in a crystallizing dish containing ice on a magnetic stirrer. 500 μ Cl ^{125}I in reductant free 0.1 M sodium hydroxide per mg haemopexin were added, followed by 100 μ g chloramine T. Five minutes later, addition of 100 μ g sodium metabisulphite neutralised any remaining oxidising reagent. Chloramine T and sodium metabisulphite solutions were freshly prepared in distilled water at a concentration of 200 μ g/ml. Non-protein-bound ^{125}I was removed by dialysis against several changes of 0.15 M phosphate buffer, pH 7.4, over an 18 hour period. Following dialysis, more than 97% of the total ^{125}I activity was precipitable by 3.3 M ammonium sulphate. Iodinated haemopexin was centrifuged at 105000 g for 60 minutes to remove trace amounts of protein aggregates.

40-90% uptake of ^{125}I was obtained; assuming a MW of 58000 for rabbit haemopexin, the molecular ratio varied between 1 atom I:100 molecules haemopexin and 1 atom I:50 molecules haemopexin. The specific activity of the haemopexin batches ranged from 2.9×10^8 dpm/mg to 1×10^9 dpm/mg.

Purity of the haemopexin

Purity of the labelled haemopexin was assessed by polyacrylamide gel electrophoresis and by haem binding studies.

Polyacrylamide gel electrophoresis

Presence of haemopexin aggregates and purity of ^{125}I -haemopexin were determined by polyacrylamide gel electrophoresis using the method and apparatus described by Davis (1964). The reservoir buffer was 0.05 M Tris and 0.384 M glycine, pH 8.3, diluted 1 in 10 with distilled water for use. 200 μ g protein were electrophoresed in gel tubes 63 mm x 5 mm for 30 minutes at 5 m Amps per tube. Gels were stained with 1% amido Schwartz B in 7% acetic acid and washing solution for removal of excess stain was 7% acetic acid.

No contaminants or haemopexin aggregates were present on polyacrylamide gel electrophoresis.

Haem binding

Haem binding by ^{125}I -haemopexin was determined by spectrophotometric titration at 413.5 nm. The optical density ceased to increase when the ratio of haem to haemopexin was equimolar. This method was described by Heide *et al.* (1964).

Haemopexin was shown to polymerise after 30-60 minutes incubation at 60°C (Müller-Eberhard, 1970) and for the experiments, rabbit ^{125}I -haemopexin was stabilised with unlabelled purified rabbit transferrin. Rabbit transferrin inhibited polymerisation and the samples were stored at -20°C prior to use.

3. Haem

Unlabelled crystalline haem was obtained from Koch-Light Laboratories Ltd*.

Biologically labelled ^{59}Fe haem was prepared by injecting a rabbit (3 Kg) with 60 μCi $^{59}\text{FeCl}_3$ in isotonic citrate-saline. The blood was collected by cardiac puncture 14 days later and placed in heparinised containers.

^{59}Fe -haem was isolated by the method of Labbe and Nishida (1957).

1 volume blood was added with stirring to 12 volumes freshly prepared extraction solvent containing 2% strontium chloride hexahydrate in glacial acetic acid and acetone (1:3v/v). This mixture was allowed to stand with occasional stirring for 20-30 minutes at room temperature. After filtration through coarse filter paper, washing the residue twice with 1 volume extraction solvent, the mixture was transferred to a flask and heated to 100°C. Care was taken to prevent the temperature exceeding 102°C.

* Koch-Light Laboratories Ltd, Colnbrook, Bucks.

The solution was allowed to cool and crystallization of haem was complete on cooling to room temperature. Haem was centrifuged and washed twice with 50% ethanol in water and once with ethanol/ether mixture.

^{59}Fe activity in weighed samples was determined and the specific activity was estimated to be 1.1×10^4 cpm/mg. For injection, haem was dissolved in 0.15 M sodium carbonate.

4. ^{59}Fe -haemoglobin

^{59}Fe -haemoglobin was prepared by injecting 60 μCi $^{59}\text{FeCl}_3$ in isotonic citrate-saline into a 3 Kg rabbit. 14 days later, the blood was collected by cardiac puncture and heparinised. The blood was lysed by addition of water and centrifuged for 1 hour at 120,000 g. The supernatant was diluted 1:2 (v/v) with isotonic saline and centrifuged again for 1 hour at 120,000 g. The haemoglobin solution was concentrated by ultrafiltration against isotonic saline and the solution for injection contained 122 mg haemoglobin/ml. Specific activity of the ^{59}Fe -haemoglobin was 5.9×10^3 cpm/mg.

5. Phenylhydrazine

Phenylhydrazine HCl was obtained from Koch-Light Laboratories Ltd. For injection, a sterile solution of 0.04% phenylhydrazine HCl in isotonic saline containing 0.1% chlorocresol was prepared.

6. Starch gel electrophoresis

Plasma samples from rabbits receiving haem and haemopexin were studied by starch gel electrophoresis. Starch gel apparatus and electrostarch were obtained from Otto Hiller Co*. The bridge buffer contained 0.3 M boric acid and 0.06 M sodium hydroxide, pH 8.2, gel buffer consisted of 1 volume bridge buffer + 9 volumes distilled water. For the gel, 50 g electrostarch were made up in 500 mls gel buffer. 50 μl plasma samples were applied to the gel and electrophoresed vertically at 2.5 volts/cm for 16 hours at 4°C.

* Otto Hiller Co., Madison, Wisconsin.

The completed gel was sliced and stained for haem with the benzidine mixture of Crosby and Firth (1956) consisting of 1% benzidine in 90% acetic acid and 5% hydrogen peroxide (1:1 V/V); and for protein with 1% amido Schwartz B in 10% acetic acid. Benzidine was obtained from Koch-Light Laboratories Ltd. Excess protein stain was removed by solution containing 4.5 parts ethanol, 0.5 parts glacial acetic acid and 5 parts distilled water. Haemopexin bands were excised from the gel and their radioactivity was determined.

7. "Iobeads" (inorganic iodine resin)* were used for the estimation of non-protein-bound ^{125}I in plasma samples, as the high solubility of haemopexin in acid solution prevented its complete precipitation from plasma by trichloroacetic acid or phosphotungstic acid.

THE DETERMINATION OF PLASMA HAEMOPEXIN HALF-CLEARANCE TIME

In all experiments, rabbits were weighed each day and maintained on a normal diet. Beginning four days prior to ^{125}I -haemopexin injection, Lugol's iodine solution was added to the drinking water to a concentration of approximately 0.05% and maintained for the duration of the experiment.

1. Normal Rabbits

Plasma haemopexin half-clearance time was studied in 18 normal rabbits. Table 5 lists rabbit group, weight and source of haemopexin for normal rabbits. 100 μg ^{125}I -haemopexin were administered by intravenous injection as described below.

* Technicon Chemical Co Inc, Ardsley (Chauncey), New York.

TABLE 5 NORMAL RABBITS

	R	Weight (Kg)	Source of haemopexin
Group 1	2	3.12	normal serum
	2a	3.32	
	3	3.49	
	4	2.55	
	5	2.48	
	9	2.57	serum from rabbits injected with 3-methylcholanthrene
	10	2.14	
	11	2.39	
	12	2.60	serum from rabbits injected with 3-methylcholanthrene
	13	2.75	
	14	2.27	
	17	1.93	
Group 2	1	2.65	serum from rabbits injected with 3-methylcholanthrene
	2	2.20	
	2a	2.37	
	3	2.88	
	4	2.14	
	5	1.75	

Injection and sample collection

^{125}I -haemopexin was diluted with isotonic saline to give a protein concentration of 50 $\mu\text{g}/\text{ml}$ and a ^{125}I -haemopexin standard was prepared. At zero time, 100 μg ^{125}I -haemopexin were injected into the marginal vein of the left ear using a Butterfly ^(R) 23 infusion unit* and were washed through with 2 mls isotonic saline. After 3 minutes, a 0.5-1.0 ml blood sample was collected from the opposite ear into a sequestrene container and further venous samples were taken at intervals during an 80 hour period, by which time plasma ^{125}I activity had dropped to less than 5% of the injected dose.

* Abbott Laboratories, North Chicago, Illinois.

2. Haem-loaded rabbits

Table 6 lists animals injected with 25 mg unlabelled haem followed by 100 μ g ^{125}I -haemopexin and control animals receiving 100 μ g ^{125}I -haemopexin alone. The same animals and a single batch of haemopexin were used for both experiments: after measurement of ^{125}I -haemopexin turnover, control animals were rested for 3 weeks before the haem-loading experiment.

TABLE 6 25 MG HAEM RABBITS AND CONTROLS

Group 3	R	Weight (Kg)	Source of haemopexin
25 mg haem	1	2.22	serum from rabbits injected with lead acetate
	2	2.40	
	3	2.63	
	4	2.52	
	5	2.38	
	6	2.65	
controls	1	2.05	
	2	2.05	
	3	2.11	
	4	2.03	
	5	1.87	
	6	2.18	

TABLE 7 ANIMALS RECEIVING VARYING DOSES OF ^{59}Fe HAEM + 100 μg ^{125}I -HAEMOPEXIN AND CONTROL ANIMALS RECEIVING 100 μg HAEMOPEXIN ALONE

	^{59}Fe haem (mg)	R	Weight (Kg)	Source of haemopexin
Group 4	25 mg	1 2 3 4 5	2.55 3.36 3.19 2.15 1.85	serum from rabbits injected with lead acetate (2 batches)
Group 5	1.75 mg	1	1.92	
		2	1.95	
		3	2.40	
	2.0 mg	4	3.33	
	4.0 mg	5	2.18	
		6	3.13	
controls		1	3.42	
		2	2.20	

Haem-loaded rabbits: Injection and sample collection

Haem samples were dissolved in 0.15 M sodium carbonate for injection and the left ear was cannulated by the above method. Haem was washed into the ear vein by 2 mls isotonic saline, followed 5 minutes later by 100 μg ^{125}I -haemopexin, again washed in with 2 mls isotonic saline. After injection of ^{125}I -haemopexin at zero time, blood samples were taken from the opposite ear at 3 minutes, 5 times during the first 14 hours and at intervals for the remainder of the experiment, which was continued for 80-96 hours after zero time.

3. Rabbits with intravascular haemolysis

a) Phenylhydrazine-treated rabbits

2 rabbits received 2.5 mg phenylhydrazine HCl/Kg body weight by intravenous injection on alternate days for 3 weeks prior to the injection of 100 μ g ^{125}I -haemopexin. On the day following phenylhydrazine administration, blood samples were taken into sequestrene containers with great care to avoid lysis: samples taken from normal rabbits under the same conditions showed no lysis. Packed cell volume (PCV), reticulocytes, whole blood haemoglobin and plasma haemoglobin were estimated and plasma samples were submitted to starch gel electrophoresis and examined for the presence of methaemalbumin by benzidine staining.

Packed cell volume (PCV) was measured by the microhaematocrit method and Merrett tubes were used for the reticulocyte count. Whole blood haemoglobin was estimated by the cyanmethaemoglobin method (Dacie and Lewis, 1968) and plasma haemoglobin was measured spectrophotometrically by the method of Cripps (1968). The absorption peaks at 560 and 592 nm were subtracted from the peak at 576 nm and the results were read off a standardisation graph.

After injection of 100 μ g ^{125}I -haemopexin at zero time, sampling was carried out as in haem-loaded rabbits.

b) ^{59}Fe -haemoglobin loaded rabbit

600 mg ^{59}Fe -haemoglobin were injected into a 4.25 Kg rabbit and plasma clearance of ^{59}Fe -haemoglobin was followed. Plasma samples were studied by starch gel electrophoresis and urine and faeces were collected for estimation of ^{59}Fe -activity.

Collection of urine and faeces

Urine and faeces were collected for 5 days following ^{125}I -haemopexin injection and pooled. In one rabbit receiving 25 mg haem followed by 100 μg ^{125}I -haemopexin, urine samples were obtained after inserting a self-retaining catheter into the bladder. This procedure, carried out 3 hours before injection, permitted collection of more exact information.

Measurement of non-protein-bound ^{125}I

Non-protein-bound ^{125}I was estimated in all plasma samples of rabbits in group 2 and in the 24 hour samples of rabbits in groups 3, 4 and 5.

0.2 ml plasma was made up to 1 ml with distilled water and a small quantity of preswollen Iobeads was added. 0.2 ml supernatant was taken and ^{125}I activity determined. Results were expressed as percentage free iodide in the plasma.

In a control experiment, ^{125}I was added to plasma before addition of Iobeads. Retention of ^{125}I by the Iobeads was greater than 99%.

Quantitation of plasma haemopexin

To quantitate results found on starch gel electrophoresis, selected plasma samples were sent to Dr U Müller-Eberhard for quantitation by the radial immunodiffusion method of Mancini *et al.*, (1965) according to the modification of Smibert *et al.*, (1972).

Measurement of radioactivities

a) Plasma samples

Blood samples were centrifuged and 0.2 ml plasma was counted on a Packard Autogamma Spectrometer. In ^{59}Fe -haem/ ^{125}I -haemopexin experiments, counts attributable to the ^{59}Fe and ^{125}I labels were determined simultaneously. Settings were 25% gain 300-900 (^{59}Fe) and

50% gain 100-115 (^{125}I). Cross counts were negligible and counting error was within 1% of the total counts measured.

Calculation of injected dose: PCV was measured in all 3 minute blood samples and the injected dose for each animal was calculated from the 3 minute plasma counts and total plasma volume. Blood volume was taken to be 7% body weight (Wiseman and Irving, 1963).

b) Urine and faeces

2 mls samples were taken for estimation of activity and were counted as above. Total excreted ^{125}I activity was expressed as a percentage of the injected dose.

^{125}I -haemopexin and ^{59}Fe -haem standards were counted at all times to allow correction for radioactive decay.

Graphic resolution and analysis of plasma activity curve

For graphic resolution and analysis of experimental data, the procedure and nomenclature of Matthews (1957) were used throughout. Plasma activity was expressed as a fraction of the 3 minute plasma counts and plotted against time on semi-logarithmic paper. From the graph and by computer analysis by the method of least squares, the plasma curve was resolved into 2 or more components described by exponential terms according to the general formula $y = C_1 e^{-b_1 t} + C_2 e^{-b_2 t} + \dots C_n e^{-b_n t}$. Slope (b), y-axis intercept (C) and half clearance time were determined for each component. Slope, $b = \frac{.693}{\text{half clearance time in days}}$

Experimental model

The experimental model of Matthews (1957) shown in Figure 7 for a system containing four compartments was chosen.

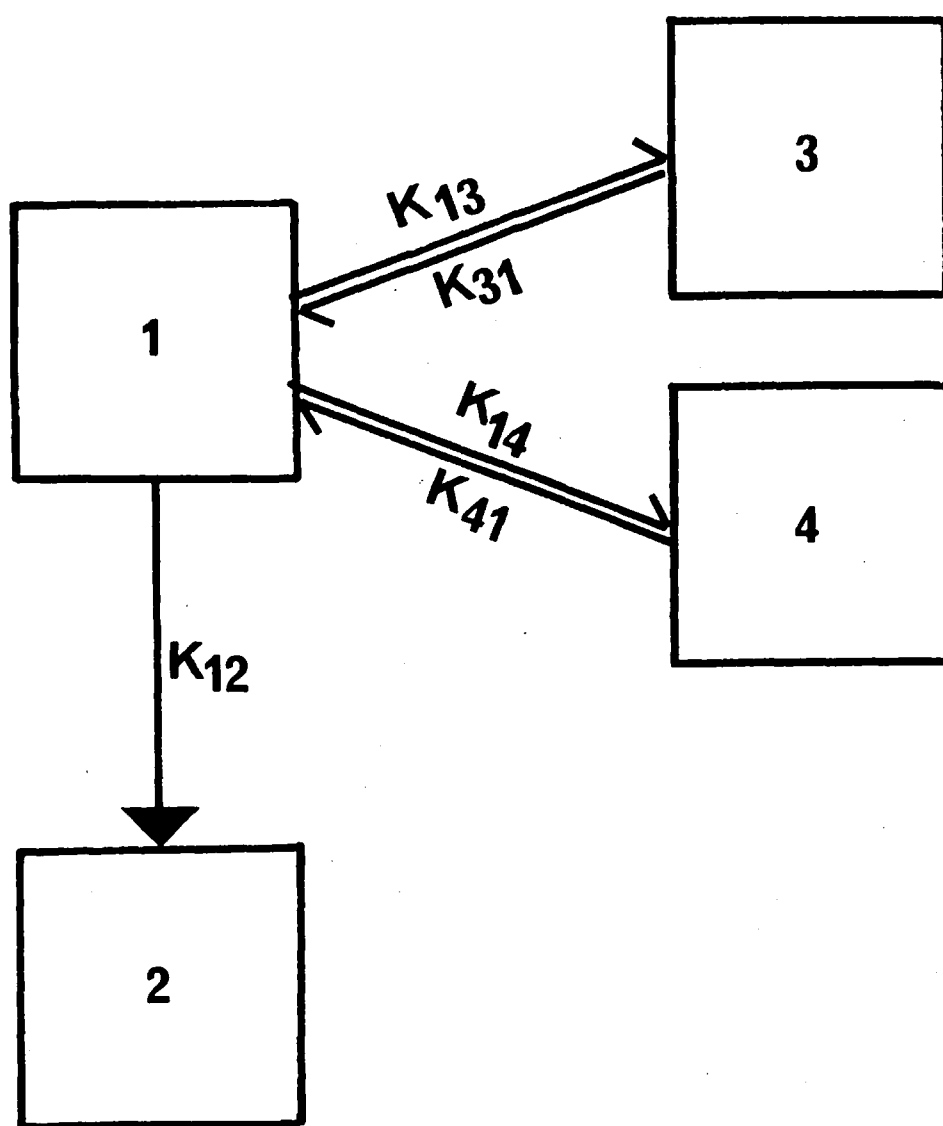


Figure 7. A 4-compartment form of model used for the evaluation of plasma ^{125}I -haemopexin clearance (after Matthews, 1957).

Compartment 1 = intravascular protein

Compartment 2 = urine and faeces

Compartments 3 and 4 = extravascular protein

The K s are rate constants.

The label was injected into compartment 1, equilibrated with compartments 3 and 4 and was assumed to have irreversible exit from compartment 1 to urine and faeces in compartment 2.

The intravascular compartment is considered to be the central compartment (compartment 1) while compartments 3 and 4, connected reversibly to compartment 1, represent the protein in the extravascular spaces. Compartment 2 represents urine and faeces and is connected to compartment 1 in the direction 1→2 only. It is assumed that the system is in dynamic equilibrium, total protein in each compartment being constant, that rate of breakdown is equal to rate of synthesis and that rates of transfer of protein from one compartment to another are constant.

Calculation of fractional catabolic rate

The catabolic rate, K_{12} was calculated from the formula:

$$K_{12} = \frac{1}{C_1/b_1 + C_2/b_2 + C_3/b_3} \quad 1$$

expressed as a fraction of the total intravascular haemopexin pool per day.

Calculation of extravascular haemopexin as a fraction of intravascular haemopexin

The capillary permeabilities, K_{13} and K_{14} , were derived from:

$$K_{31} = \frac{1}{2} [C_1(b_3 - b_1) + C_2(b_3 - b_2) + b_1 + b_2 + \sqrt{\{C_1(b_3 - b_1) + C_2(b_3 - b_2) + b_1 - b_2\}^2 + 4C_2(b_2 - b_1)(b_3 - b_2)}] \quad 2$$

$$K_{41} = \frac{1}{2} [C_1(b_3 - b_1) + C_2(b_3 - b_2) + b_1 + b_2 - \sqrt{\{C_1(b_3 - b_1) + C_2(b_3 - b_2) + b_1 - b_2\}^2 + 4C_2(b_2 - b_1)(b_3 - b_2)}] \quad 3$$

$$K_{14} = \frac{(K_{41} - b_1)(K_{41} - b_2)(K_{41} - b_3)}{K_{41}(K_{31} - K_{41})} \quad 4$$

$$K_{13} = \frac{(K_{31} - b_1)(K_{31} - b_2)(K_{31} - b_3)}{K_{31}(K_{41} - K_{31})} \quad 5$$

Ratio of sizes of compartments 3 and 4 to size of compartment 1
 $= K_{13}/K_{31}$ and K_{14}/K_{41} respectively, since compartmental sizes are constant.

In experiments where the plasma activity curve resolved into
 2 ^{Components} ~~compartments~~ on analysis, the presence of only 1 extravascular compartment
 was assumed and $\bar{Y}$ (extravascular protein) was derived from:

$$\bar{Y} = \frac{C_1 C_2 (b_2 - b_1)^2}{(C_1 b_2 + C_2 b_1)^2} \quad 6$$

expressed as a fraction of the intravascular haemopexin pool, $\bar{X}$.

Calculation of absolute catabolic rate and extravascular haemopexin mass

Total intravascular haemopexin was calculated for each rabbit
 using a haemopexin concentration of 44.2 mg/100 mls plasma (mean of 12
 values obtained in normal rabbits by Dr U Müller-Eberhard using the radial
 immunodiffusion method of Mancini et al., (1965), according to the
 modification of Smibert et al., (1972), SD = 15.4), a PCV of 40% and
 taking blood volume to be 7% body weight (Wiseman and Irving, 1963).

Absolute catabolic rate = fractional catabolic rate x $\bar{X}$ (mg)
 (mg/24 hours)

+

Extravascular protein mass (mg) = $\bar{Y} \times \bar{X}$ (mg)

Calculation of haem binding capacity

To calculate haem binding by total haemopexin, the molecular weight
 of haem was taken to be 617 and that of haemopexin 63,000 (Müller-Eberhard
et al., 1969) and equimolar binding of haem by haemopexin was assumed
 (Heide et al., 1964).

RESULTS

Polyacrylamide gel electrophoresis, spectrophotometric assay and haem binding estimations indicated that the labelled haemopexin was pure, haem-free and biochemically active in monomeric form. The monomeric state was well preserved when haemopexin was stored in homologous transferrin solution. In addition, during the metabolic experiments, the rabbits all remained healthy and gained in weight.

DETERMINATION OF PLASMA HAEMOPEXIN HALF-CLEARANCE TIME

1. Normal rabbits (Groups 1 and 2)

A plasma haemopexin clearance curve characteristic of the series is shown in Figure 8. By computer analysis the curve was resolved into two components, each described by an exponential function with y-axis intercepts C_1 and C_2 and slopes b_1 and b_2 respectively.

Equilibration of ^{125}I -haemopexin between the intravascular and extravascular compartments was complete 20-24 hours after zero time, when the rate of plasma haemopexin clearance became constant. Points lying along the component $C_1 e^{-b_1 t}$ and calculated points along the component $C_2 e^{-b_2 t}$ in the 18 rabbits studied were used to obtain the correlation coefficient for each line. Results for the correlation coefficient were in the range 0.965-0.996, indicating that the points were a close fit to a straight line.

Tables 8:1 and 8:2 summarise the results of graphic resolution of the ^{125}I -haemopexin clearance curve in normal rabbits. Half-clearance times for components $C_1 e^{-b_1 t}$ and $C_2 e^{-b_2 t}$, values for y-axis intercepts C_1 and C_2 and rate constants b_1 and b_2 are given.

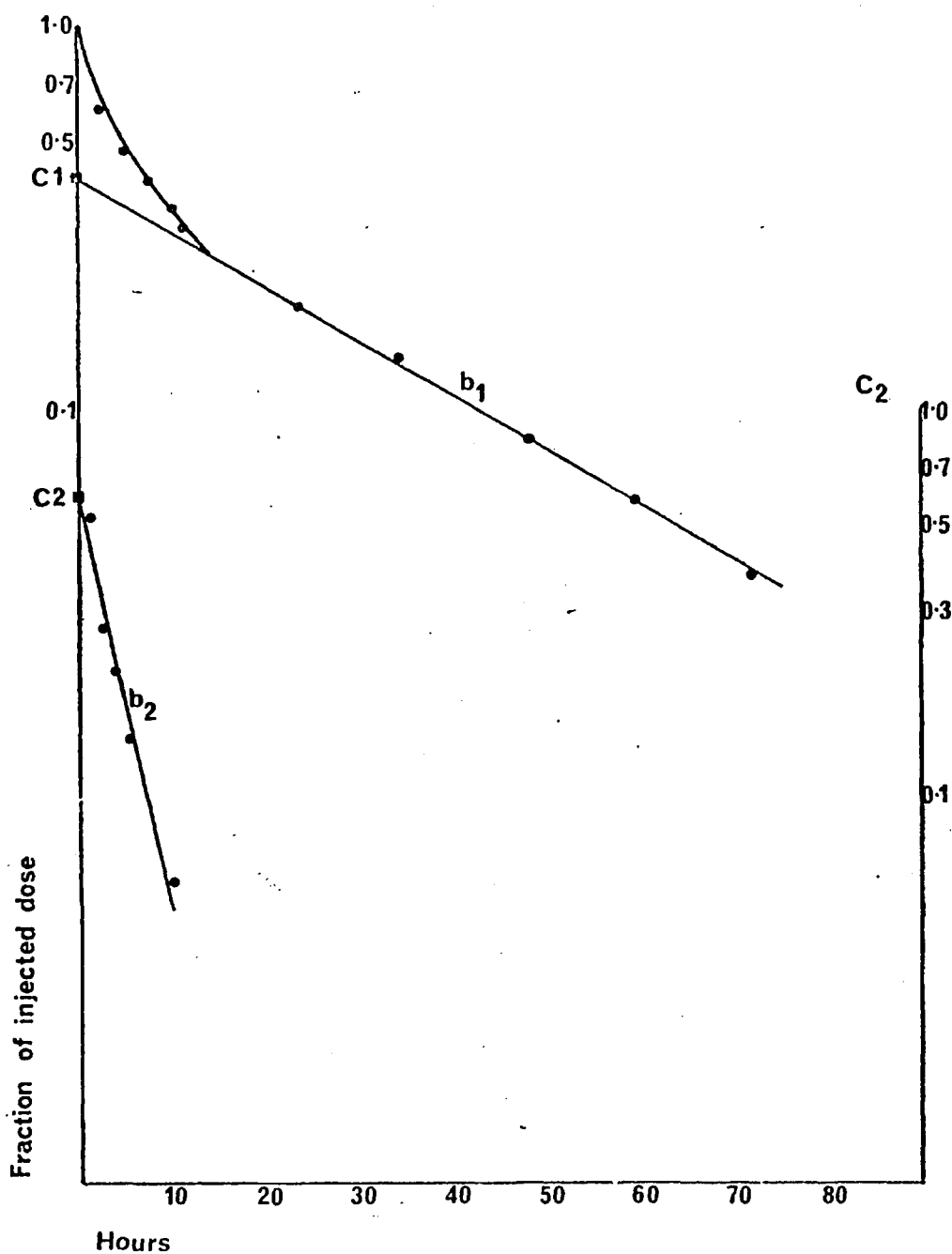


Figure 8. Plasma clearance of ^{125}I -haemopexin in a normal rabbit from group 1. The curve is described by the exponential equation $y = C_1 e^{-b_1 t} + C_2 e^{-b_2 t}$ where C_1 , C_2 are the y-axis intercepts and b_1 , b_2 the respective rate constants.

$$C_1 e^{-b_1 t} = 21.4 \text{ hours}$$

$$C_1 = 0.4$$

$$C_2 e^{-b_2 t} = 2.66 \text{ hours}$$

$$C_2 = 0.6$$

Exponential 1 (slope b_1) is subtracted from curve 1 to give exponential 2 (slope b_2).

GROUP 1 RABBITS INJECTED WITH 100 μ g ^{125}I -HAEMOPEXIN

TABLE 3:1 ANALYSIS OF PLASMA ACTIVITY CURVE

R	Wt (Kg)	half-clearance time (hours)		y-axis intercept (fraction of injected dose)		rate constant	
		$C_1 e^{-b_1 t}$	$C_2 e^{-b_2 t}$	C_1	C_2	b_1	b_2
2	3.12	21.40	1.84	0.426	0.574	0.775	9.03
2a	3.32	21.40	2.66	0.400	0.600	0.775	6.25
3	3.49	20.02	1.69	0.546	0.454	0.830	9.82
4	2.55	20.02	2.53	0.478	0.522	0.830	6.60
5	2.48	18.82	3.46	0.465	0.535	0.881	4.80
$\bar{x}$	2.99	20.33	2.44	0.463	0.537	0.817	7.30
SD	0.45	1.09	0.71	0.056	0.056	0.046	2.07
9	2.57	27.40	3.07	0.400	0.600	0.608	5.42
10	2.14	20.02	2.98	0.291	0.709	0.830	5.53
11	2.39	20.02	2.92	0.357	0.643	0.830	5.68
$\bar{x}$	2.37	22.48	2.99	0.349	0.650	0.756	5.56
SD	0.22	4.26	0.08	0.055	0.055	0.128	0.13
12	2.60	18.82	2.90	0.392	0.608	0.881	5.77
13	2.75	18.82	3.31	0.350	0.650	0.881	5.02
14	2.27	20.02	5.18	0.493	0.507	0.830	3.21
17	1.93	17.70	1.99	0.480	0.520	0.942	8.35
$\bar{x}$	2.39	18.84	3.35	0.429	0.571	0.884	5.59
SD	0.37	0.95	1.34	0.069	0.069	0.046	2.13

Source of haemopexin:-

R 2- 5 normal serum

R 9-11 serum of rabbits injected with 3-methylcholanthrene

R12-17 serum of rabbits injected with lead acetate

GROUP 2 RABBITS INJECTED WITH 100 μg ^{125}I -HAEMOPEXIN

TABLE 8:2 ANALYSIS OF PLASMA ACTIVITY CURVE

R	Wt (Kg)	half-clearance time (hours)		y-axis intercept (fraction of injected dose)		rate constant	
		$C_1 e^{-b_1 t}$	$C_2 e^{-b_2 t}$	C_1	C_2	B_1	b_2
1	2.65	30.1	4.23	0.344	0.656	0.554	5.90
2	2.20	30.1	3.61	0.303	0.697	0.554	4.62
2a	2.37	33.4	5.46	0.463	0.537	0.498	3.06
3	2.88	27.2	4.11	0.300	0.700	0.612	4.05
4	2.14	30.1	2.52	0.399	0.601	0.554	6.60
5	1.75	30.1	4.11	0.318	0.682	0.554	4.05
$\bar{x}$	2.33	30.17	4.01	0.355	0.646	0.554	4.71
SD	0.40	1.96	0.954	0.065	0.065	0.036	1.31

Source of haemopexin: serum of rabbits injected with 3-methylcholanthrene

Mean plasma haemopexin half-clearance time for component $C_1 e^{-b_1 t}$ for the whole series was 23.64 hours (SD 5.26), while half-clearance time values for component $C_2 e^{-b_2 t}$ ranged from 1.69-5.46 hours. Mean value for y-axis intercept C_1 was 0.4 (SD 0.075) of injected activity and C_2 was found by subtracting C_1 from unity.

One rabbit excluded from the series developed a respiratory infection during the experiment and two distinct rates of haemopexin clearance were observed with half-clearance times of 18.8 hours and 70.05 hours respectively.

For evaluation of the plasma activity curve a model of three compartments was chosen; a model of this type is shown in figure 9. When calculating catabolic rate, K_{12} , it was assumed that labelled haemopexin was in a state of reversible equilibrium between the intravascular and extravascular compartments with irreversible exit from the former.

Tables 8:3 and 8:4 show results of compartmental analysis, intravascular and extravascular protein mass and total haemopexin haem binding capacity.

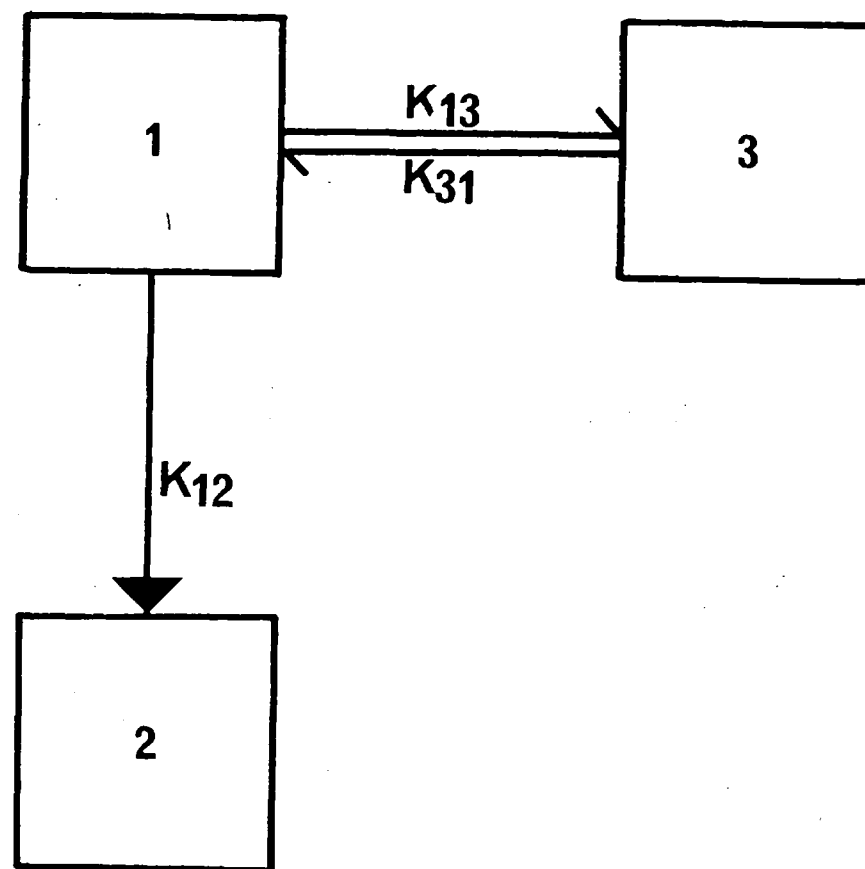


Figure 9. A 3-compartment form of model used for the evaluation of plasma ^{125}I -haemopexin clearance (after Matthews, 1957).

Compartment 1 = intravascular protein

Compartment 2 = urine and faeces

Compartment 3 = extravascular protein

The K s are rate constants.

The label was injected into compartment 1, equilibrated with compartment 3 and was assumed to have irreversible exit from compartment 1 to urine and faeces in compartment 2.

GROUP 1 RABBITS INJECTED WITH 100 μg ^{125}I -HAEMOPEXIN

TABLE 8:3 COMPARTMENTAL ANALYSIS

R	catabolic rate K_{12}	EV/IV haemo- pexin $\bar{Y}/\bar{X}$	IV haemo- pexin $\bar{X}$ (mg)	EV haemo- pexin $\bar{Y}$ (mg)	Total haemo- pexin (mg)	Total haem binding capacity (mg)
2	1.63	0.915	57.8	53.0	110.8	1.085
2a	1.63	0.637	61.4	39.1	100.5	0.984
3	1.43	0.612	65.0	39.8	104.8	1.026
44	1.50	0.648	47.3	30.6	77.9	0.762
5	1.56	0.516	46.0	23.7	69.7	0.682
$\bar{x}$	1.55	0.666	55.5	37.24	92.74	0.908
SD	0.086	0.149	8.48	11.02	17.91	0.176
9	1.30	0.873	47.7	41.6	89.3	0.875
10	2.10	0.940	39.7	37.3	77.0	0.751
11	1.34	0.825	44.2	36.4	80.6	0.789
$\bar{x}$	1.75	0.879	43.87	38.4	82.3	0.805
SD	0.408	0.058	4.01	2.78	6.32	0.064
12	1.82	0.725	48.2	34.9	83.1	0.814
13	1.90	0.720	50.8	36.5	87.3	0.855
14	1.33	0.355	42.0	14.9	56.9	0.557
17	1.74	0.686	35.8	24.5	60.3	0.591
$\bar{x}$	1.72	0.621	44.2	27.7	71.9	0.704
SD	0.30	0.179	6.71	10.06	15.5	0.152

Legend as for Table 8:1

EV = extravascular

IV = intravascular

GROUP 2 RABBITS INJECTED WITH 100 μg ^{125}I -HAEMOPEXIN

TABLE 8:4 COMPARTMENTAL ANALYSIS

R	catabolic rate K_{12}	EV/IV haemo- pexin $\bar{Y}/\bar{X}$	IV haemo- pexin $\bar{X}$ (mg)	EV haemo- pexin $\bar{Y}$ (mg)	Total haemo- pexin	Total haem binding capacity (mg)
1	1.35	1.07	49.5	53.0	102.5	1.00
2	1.43	1.07	41.0	43.9	84.9	0.831
2a	0.90	0.57	44.2	25.2	69.4	0.680
3	1.51	0.92	62.3	57.2	119.5	1.170
4	1.23	1.00	39.7	39.7	79.4	0.778
5	1.35	0.856	32.6	27.9	60.5	0.593
$\bar{x}$	1.295	0.914	44.88	41.15	86.03	0.842
SD	0.215	0.188	10.18	12.94	21.74	0.212

Legend as for Table 8:2

Mean catabolic rate, K_{12} , for the whole series was 1.53 (SD 0.28) times the intravascular haemopexin pool per day and extravascular haemopexin, $\bar{Y}$, was 0.77 (SD 0.2) times the mass of intravascular haemopexin. Mean total haemopexin haem binding capacity was 0.824 mg (SD 0.175). For a 2.5 Kg rabbit, blood volume 175 mls (7% body weight) and PCV 40%, the following values can be calculated.

1. Intravascular haemopexin = 46.4 mg
2. Absolute haemopexin catabolism per day = 71 mg
3. Extravascular haemopexin = 35.7 mg
4. Total haemopexin = 82.1 mg
5. Catabolic rate as a fraction of the total haemopexin pool per day = 0.865
6. Total haemopexin haem binding capacity = 0.804 mg haem.

^{125}I activity in pooled urine and faeces ranged from 87% to 92% of the injected dose.

Non-protein-bound ^{125}I , measured in all plasma samples of rabbits in group 2, reached maximum level approximately 24 hours after injection, when a mean value of 2.4% free ^{125}I (SD 0.42) was obtained. This showed that loss of the haemopexin label occurred only as a result of normal haemopexin catabolism.

2. Rabbits receiving 25 mg haem (Group 3)

Figure 10 shows the plasma haemopexin clearance curve obtained from a rabbit injected with 25 mg haem. In this instance, 80% of injected ^{125}I -haemopexin was cleared from the plasma in 5 hours. The plasma clearance curve in animals loaded with 25 mg haem contained two components of which $C_1e^{-b_1t}$ represented the clearance of a residual 5% of injected activity. Results for correlation coefficient for points along $C_1e^{-b_1t}$ and $C_2e^{-b_2t}$ ranged from 0.976 to 0.993.

GROUP 3 RABBITS INJECTED WITH 25 mg HAEM + 100 μg ^{125}I -HAEMOPEXIN
TABLE 9:1 ANALYSIS OF PLASMA ACTIVITY CURVE

R	Wt (Kg)	half-clearance time (hours)		y-axis intercept (fraction of injected dose)		rate constant	
		$C_1e^{-b_1t}$	$C_2e^{-b_2t}$	C_1	C_2	b_1	b_2
1	2.22	23.10	1.65	0.079	0.921	0.721	10.08
2	2.40	21.40	2.08	0.140	0.860	0.778	8.32
3	2.63	25.00	1.60	0.060	0.940	0.666	10.39
4	2.52	27.30	1.23	0.034	0.966	0.610	13.59
5	2.38	25.00	1.60	0.055	0.945	0.666	10.39
6	2.65	23.10	1.55	0.059	0.941	0.721	10.70
$\bar{x}$	2.47	24.15	1.618	0.071	0.929	0.694	10.58
SD	0.17	2.055	0.272	0.037	0.037	0.059	1.703
CONTROLS*							
1	2.05	20.02	5.77	0.343	0.657	0.884	2.80
2	2.05	21.40	4.25	0.336	0.664	0.778	3.90
3	2.11	20.02	4.35	0.280	0.720	0.834	3.83
4	2.03	20.02	4.05	0.259	0.741	0.834	4.10
5	1.87	21.40	4.77	0.344	0.656	0.778	3.48
6	2.18	18.82	4.29	0.292	0.708	0.885	3.87
$\bar{x}$	2.05	20.28	4.58	0.309	0.691	0.832	3.66
SD	0.10	0.98	0.629	0.037	0.037	0.048	0.468

* All control rabbits received 100 μg ^{125}I -haemopexin

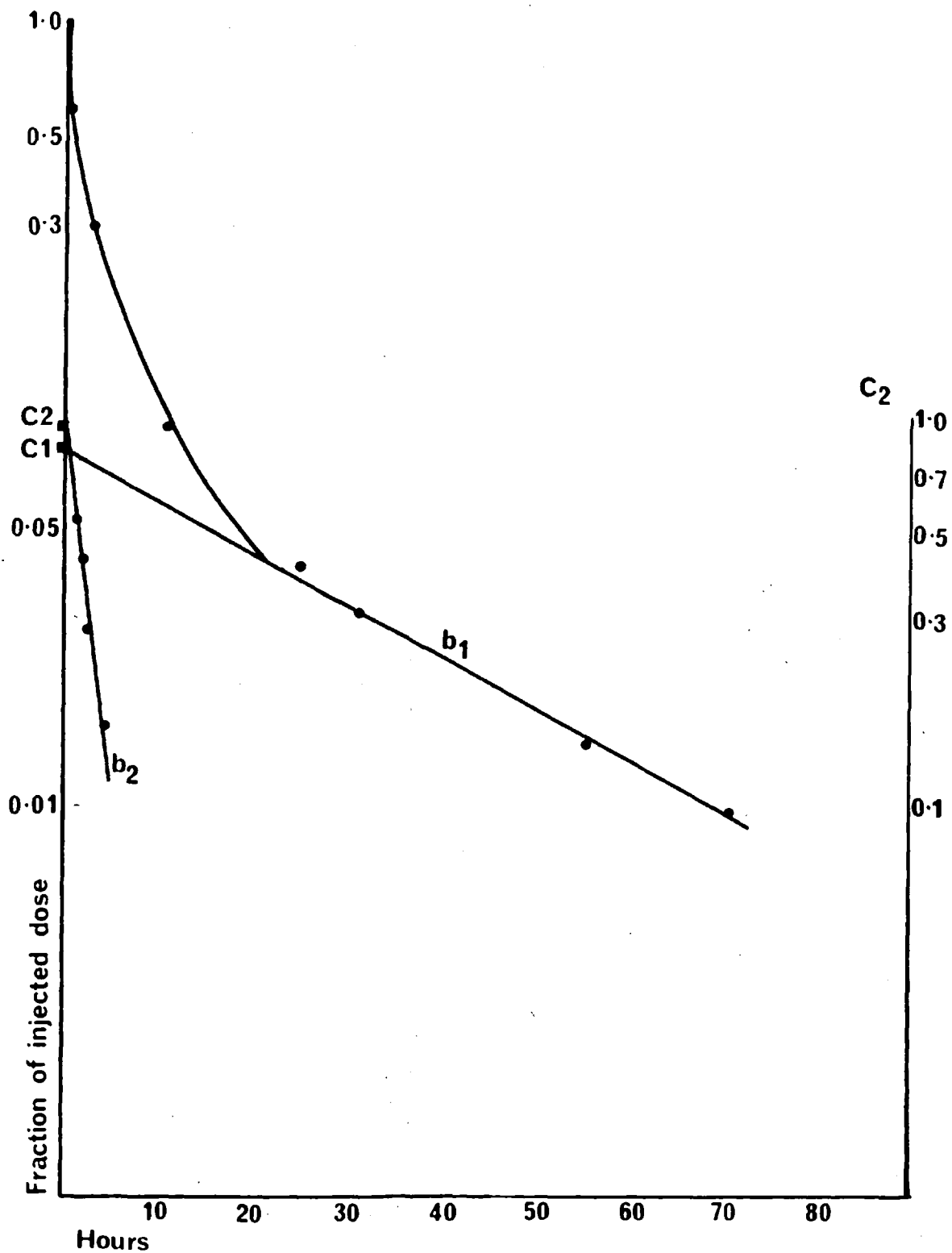


Figure 10. Plasma clearance curve of ^{125}I -haemopexin in a haem-loaded rabbit receiving 25 mg haem. The curve is described by the exponential equation $y = C_1 e^{-b_1 t} + C_2 e^{-b_2 t}$

$$C_1 e^{-b_1 t} = 23.1 \text{ hours}$$

$$C_1 = 0.079$$

$$C_2 e^{-b_2 t} = 1.65 \text{ hours}$$

$$C_2 = 0.921$$

The results of graphic resolution in haem-loaded and control rabbits are shown in table 9:1. It can be seen that in haem-loaded rabbits, 90-95% of injected activity was cleared from the plasma with a mean half-life of 1.6 hours (SD 0.27). Y-axis intercept C_1 was 0.071 (SD 0.037) compared to 0.31 (SD 0.037) in control animals.

GROUP 3 RABBITS INJECTED WITH 25 mg HAEM + 100 μ g ^{125} I-HAEMOPEXIN
TABLE 9:2 COMPARTMENTAL ANALYSIS

R	catabolic rate K_{12}	EV/IV haemo- pexin Y/X	IV haemo- pexin X (mg)	EV haemo- pexin Y (mg)	Total haemo- pexin (mg)	Total haem binding capacity (mg)
1	4.97	2.99	41.0	122.5	163.5	1.600
2	5.46	2.05	44.6	91.5	136.1	1.333
3	5.54	3.41	48.5	165.5	214.0	2.096
4	7.70	5.18	46.8	243.0	289.8	2.838
5	5.66	2.43	44.2	151.5	195.7	1.917
6	5.70	3.19	49.0	156.2	205.2	2.010
$\bar{x}$	5.84	3.375	45.68	155.03	200.72	1.966
SD	0.949	1.02	3.02	50.90	52.38	0.513
CONTROLS*						
1	1.57	0.426	38.0	16.7	54.7	0.536
2	1.70	0.648	38.0	24.6	62.6	0.613
3	1.90	0.653	39.3	25.7	65.0	0.637
4	2.03	0.726	37.6	27.3	64.9	0.636
5	1.59	0.562	34.9	19.6	54.5	0.534
6	1.95	0.602	40.2	24.2	64.4	0.631
$\bar{x}$	1.79	0.603	38.00	23.02	61.02	0.598
SD	0.196	0.103	1.806	4.026	5.045	0.049

* All control rabbits received 100 μ g ^{125} I-haemopexin

Table 9:2 shows results of compartmental analysis in Group 3 animals. Catabolic rate, K_{12} , for haem-loaded rabbits was 5.84 (SD 0.949) times the intravascular haemopexin pool per day; extravascular haemopexin, $\bar{Y}$, was 3.38 (SD 1.02) times the mass of intravascular haemopexin and total haemopexin haem binding capacity was 1.966 mg haem (SD 0.513). Control results for catabolic rate, extravascular haemopexin and total haemopexin haem binding capacity were 1.79 (SD 0.196), 0.603 (SD 0.103) and 0.598 mg (SD 0.049) respectively. These results fall within the range of results obtained for Groups 1 and 2.

In haem-loaded animals, pooled urine and faeces contained 89% (SD 4.38) of the injected activity. In the catheterised rabbit, 85% of injected ^{125}I -haemopexin was eliminated from the plasma with a half-clearance time of 2.1 hours; 8 hours after zero time 55% of injected activity had been recovered in the urine as non-protein-bound ^{125}I , and after 24 hours, 89% of injected activity was found in the urine. Total excreted activity in control rabbits for the 5 days of the experiment was 88% (SD 2.30) of the injected dose.

Plasma non-protein-bound ^{125}I did not exceed 4.9% of the plasma activity in the 24 hour plasma samples in haem-loaded rabbits and 4.3% of the plasma activity in the 24 hour samples in control animals.

3. Rabbits receiving 25 mg ^{59}Fe -haem (Group 4)

In these animals plasma clearance of ^{125}I -haemopexin and ^{59}Fe -haem was studied.

a) ^{59}Fe -haem clearance

The half-clearance times of injected ^{59}Fe -haem are shown in Table 10:1. The mean value was 2.85 hours (SD 0.882). In all rabbits receiving 25 mg haem, 90% of injected ^{59}Fe -haem activity was eliminated from the plasma between 8-17.5 hours after zero time.

GROUP 4 RABBITS INJECTED WITH 25 mg ⁵⁹Fe-HAEM + 100 µg ¹²⁵I-HAEMOPEXIN
TABLE 10:1 CLEARANCE OF ⁵⁹Fe-HAEM FROM THE PLASMA

R	Half-clearance time (hours)
1	3.75
2	3.0
3	3.5
4	2.0
5	2.0
$\bar{x}$	2.85
SD	0.322

In 3 animals, the plasma ⁵⁹Fe-haem activity curve was resolved into 2 components and Figure 11 shows the ⁵⁹Fe-haem clearance curve for 1 of these rabbits. Correlation coefficients for points lying along components $C_1e^{-b_1t}$ and $C_2e^{-b_2t}$ ranged from 0.976-0.996.

GROUP 4 RABBITS INJECTED WITH 25 mg ⁵⁹Fe-HAEM + 100 µg ¹²⁵I-HAEMOPEXIN
TABLE 10:2 ⁵⁹Fe-HAEM CLEARANCE: COMPARTMENTAL ANALYSIS

R	half-clearance time (hours)		y-axis intercept (fraction of injected dose)		rate constant		catabolic rate
	$C_1e^{-b_1t}$	$C_2e^{-b_2t}$	C_1	C_2	b_1	b_2	K_{12}
1	6.80	0.387	0.622	0.378	2.449	43.3	3.8
2	6.27	0.500	0.646	0.354	2.655	33.0	3.95
3	5.20	0.580	0.673	0.327	3.194	28.88	4.3
$\bar{x}$	6.09	0.489	0.647	0.353	2.766	35.06	4.017
SD	0.815	0.097	0.026	0.026	0.385	7.43	0.257

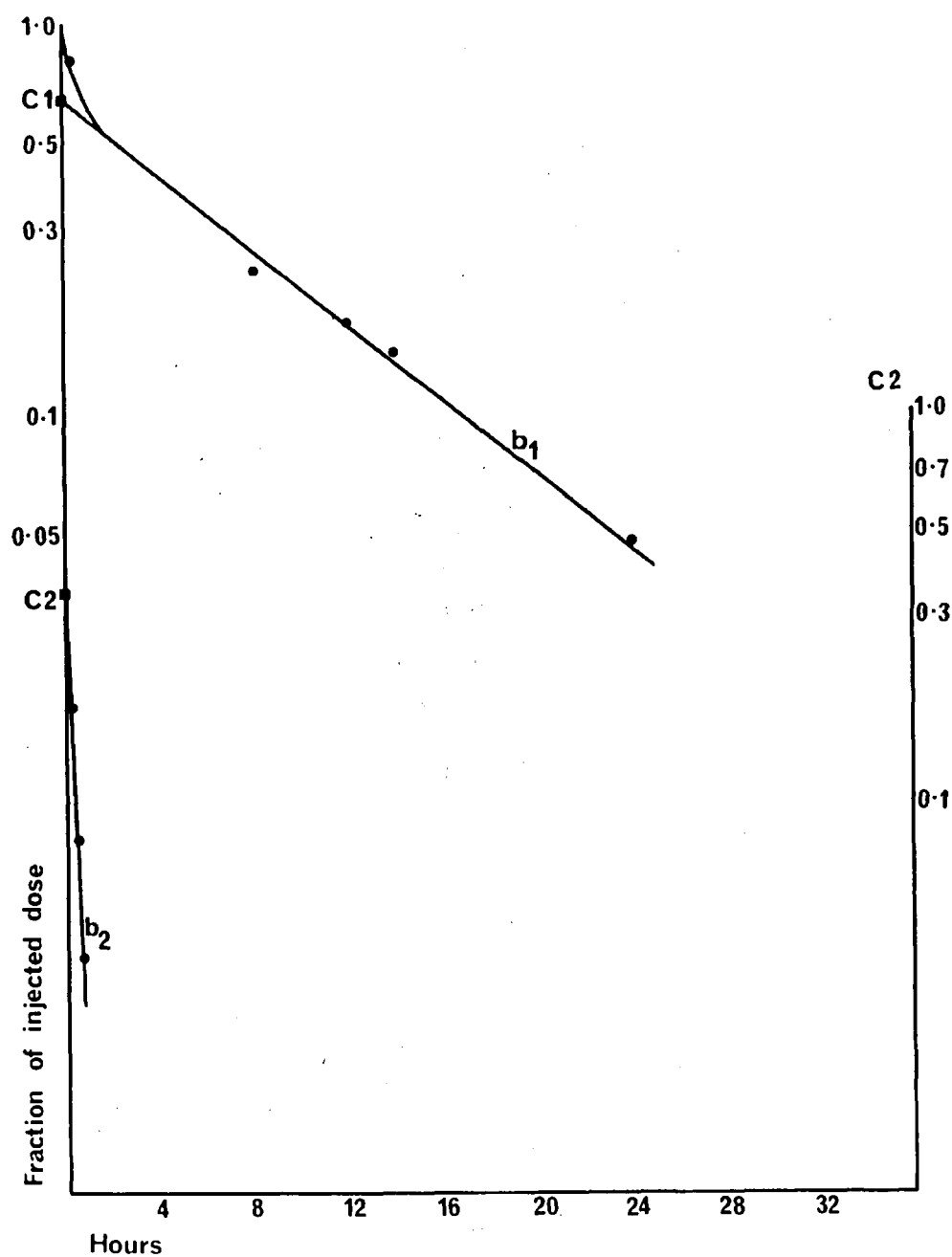


Figure 11. Plasma clearance of ^{59}Fe -haem in a high dose haem rabbit receiving 25 mg haem. In this example the plasma clearance curve was described by the exponential equation $y = C_1 e^{-b_1 t} + C_2 e^{-b_2 t}$

$$C_1 e^{-b_1 t} = 6.27 \text{ hours}$$

$$C_1 = 0.646$$

$$C_2 e^{-b_2 t} = 0.5 \text{ hours}$$

$$C_2 = 0.354$$

Results of graphic analysis and values for catabolic rate, K_{12} , are given in Table 10:2. Mean half-clearance time for component $C_1e^{-b_1t}$ in the 3 rabbits studied was 6.09 hours (SD 0.815), while mean half-clearance time for component $C_2e^{-b_2t}$ was 0.489 hours (SD 0.097). Mean catabolic rate was 4.017 (SD 0.257) times the intravascular pool per day.

b) ^{125}I -haemopexin clearance

Tables 11:1 and 11:2 show results of graphic resolution and compartmental analysis obtained from the plasma ^{125}I -haemopexin clearance curves of Group 4 rabbits.

GROUP 4 RABBITS INJECTED WITH 25 mg ^{59}Fe -HAEM + 100 μg ^{125}I -HAEMOPEXIN
TABLE 11:1 ^{125}I -HAEMOPEXIN CLEARANCE: ANALYSIS OF PLASMA ACTIVITY CURVE

R	Wt (Kg)	half-clearance time (hours)		y-axis intercept (fraction of injected dose)		rate constant	
		$C_1e^{-b_1t}$	$C_2e^{-b_2t}$	C_1	C_2	b_1	b_2
1	2.55	33.4	1.8	0.073	0.927	0.499	9.24
2	3.36	43.0	1.6	0.101	0.899	0.387	10.34
3	3.19	37.6	2.0	0.091	0.909	0.441	8.35
4	2.15	33.4	2.2	0.101	0.899	0.499	7.53
5	1.85	33.4	1.6	0.178	0.822	0.499	10.34
$\bar{x}$	2.62	36.16	1.84	0.109	0.891	0.465	9.16
SD	0.65	4.23	0.26	0.040	0.040	0.050	1.24
controls*							
1	3.42	36.8	3.4	0.320	0.680	0.440	4.90
2	2.20	30.2	3.1	0.300	0.700	0.550	5.36

* All control rabbits received 100 μg ^{125}I -haemopexin

GROUP 4 RABBITS INJECTED WITH 25 mg ^{59}Fe -HAEM + 100 μg ^{125}I -HAEMOPEXIN
TABLE 11:2 ^{125}I -HAEMOPEXIN CLEARANCE: COMPARTMENTAL ANALYSIS

R	catabolic rate K_{12}	EV/IV haemo- pexin $\bar{Y}/\bar{X}$	IV haemo- pexin $\bar{X}$ (mg)	EV haemo- pexin $\bar{Y}$ (mg)	Total haemo- pexin (mg)	Total haem binding capacity (mg)
1	4.07	4.01	47.3	189.0	236.3	2.314
2	2.87	4.67	62.3	291.0	353.3	3.460
3	3.18	3.84	59.2	228.0	287.0	2.811
4	3.12	3.07	40.2	123.5	163.7	1.603
5	2.29	2.79	34.5	96.3	130.8	1.281
$\bar{x}$	3.11	3.68	48.7	185.56	234.2	2.294
SD	0.644	0.755	11.95	78.66	90.33	0.885
CONTROLS*						
1	1.15	1.24	63.1	78.2	141.3	1.384
2	1.49	1.21	40.8	49.5	90.3	0.883

* All control rabbits received 100 μg ^{125}I -haemopexin

As in haem-loaded rabbits in Group 3 90-95% of injected activity was eliminated from the plasma with a mean half-clearance time of 1.84 hours (SD 0.26). Component $C_1 e^{-b_1 t}$, representing the clearance of approximately 10% of injected activity, had a mean half-clearance time of 36.16 hours (SD 4.23). Mean y-axis intercept C_1 was 0.109 (SD 0.04) compared with control values for C_1 of 0.32 and 0.30. Mean catabolic rate, K_{12} , was 3.11 (SD 0.644) times the intravascular haemopexin pool per day; mean extravascular haemopexin was 3.68 (SD 0.755) times the mass of intravascular haemopexin and mean total haem binding capacity was 2.294 mg (SD 0.885).

Control values in the 2 animals studied fitted well with data already obtained, except that the half-clearance times of component $C_1 e^{-b_1 t}$ were 36.8 hours and 30.2 hours.

^{125}I activity in pooled urine and faeces in Group 4 rabbits was 92.5% (SD 6.18) of the injected activity for haem-loaded animals and 93% of the injected dose was recovered in both control rabbits.

Non-protein-bound ^{125}I in 24 hour plasma samples did not exceed 4.5% of plasma activity in haem-loaded animals and 3.7% in controls.

4. Rabbits receiving 1.75-4.0 mg ⁵⁹Fe-haem (Group 5)

a) ⁵⁹Fe-haem clearance

In 5 out of the 6 rabbits, the plasma activity curve was described by a single exponential function of time with a mean half-clearance time of 0.608 hours (SD 0.124). A typical example of the graph of plasma ⁵⁹Fe-haem activity, plotted against time is shown in Figure 12. Correlation coefficients for points along the lines were in the range 0.952-0.991. Half-clearance times for ⁵⁹Fe-haem are shown in Table 12.

GROUP 5 RABBITS INJECTED WITH 1.75-4.0 mg ⁵⁹Fe-HAEM + 100 µg ¹²⁵I-HAEMOPEXIN

TABLE 12 CLEARANCE OF ⁵⁹Fe-HAEM FROM THE PLASMA

Haem dose (mg)	R	Half-clearance time of injected ⁵⁹ Fe dose (hours)
1.75	1	0.45
1.75	2	0.65
1.75	3	0.65
2.00	4	0.80
4.00	5	0.60
4.00	6	0.50
	$\bar{x}$	0.608
	SD	0.124

b) ¹²⁵I-haemopexin clearance

Plasma haemopexin half-clearance time was studied in 6 rabbits receiving 1.75-4.0 mg haem. A typical example of the ¹²⁵I-haemopexin clearance curve is shown in Figure 13. The plasma activity curve was resolved into 3 components and Table 13:1 shows the half-clearance times of components $C_1e^{-b_1t}$, $C_2e^{-b_2t}$ and $C_3e^{-b_3t}$, values for C_1 , C_2 , C_3 and the respective rate constants b_1 , b_2 and b_3 . Results for correlation coefficient for points along components $C_1e^{-b_1t}$, $C_2e^{-b_2t}$ and $C_3e^{-b_3t}$ for all rabbits were in the range 0.981-0.996.

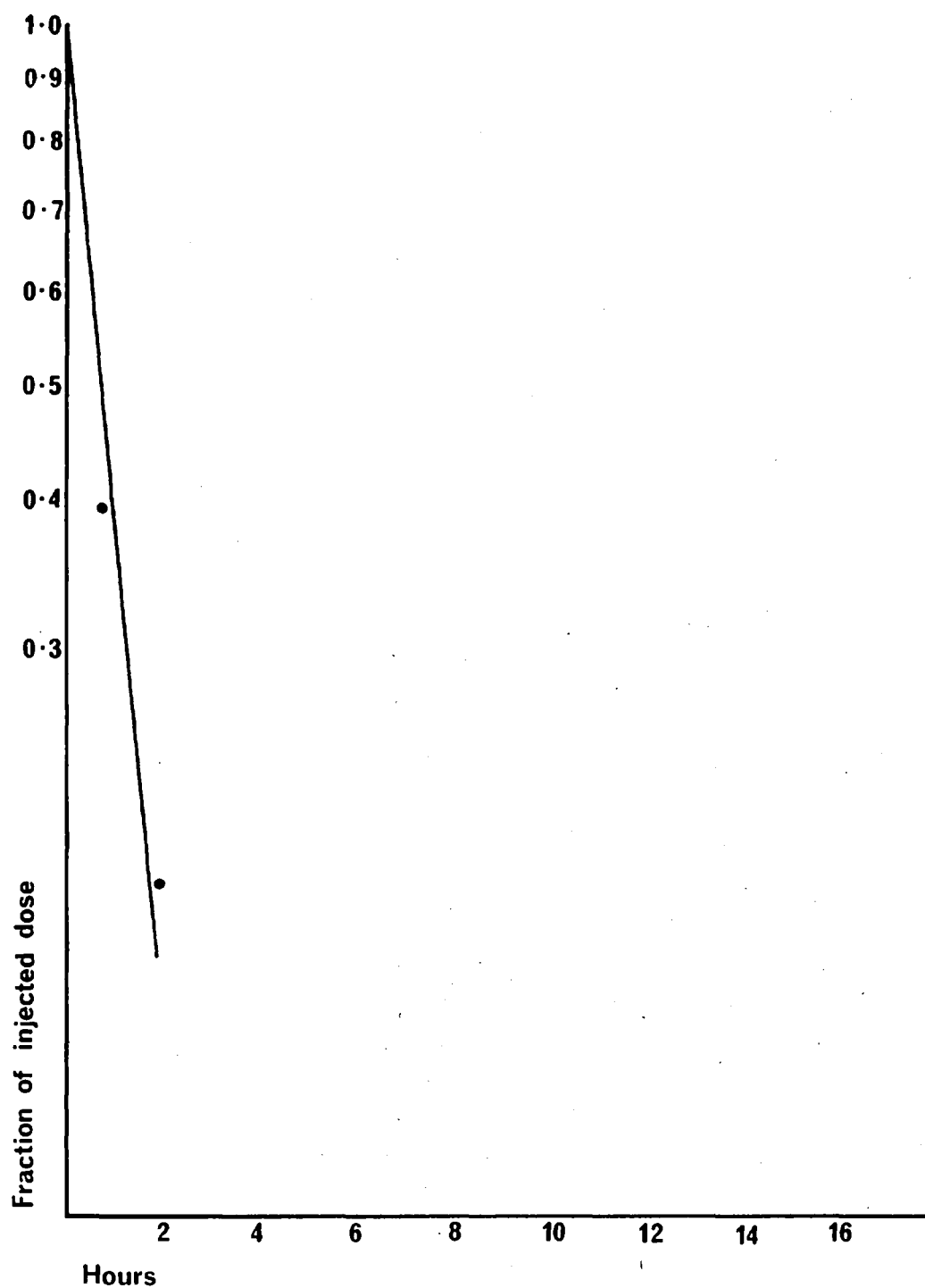


Figure 12. Plasma clearance of ^{59}Fe -haem in a low dose haem rabbit receiving 1.75 mg haem. Clearance was described by a single exponential function of time. Half-clearance time in the example shown was 0.65 hours.

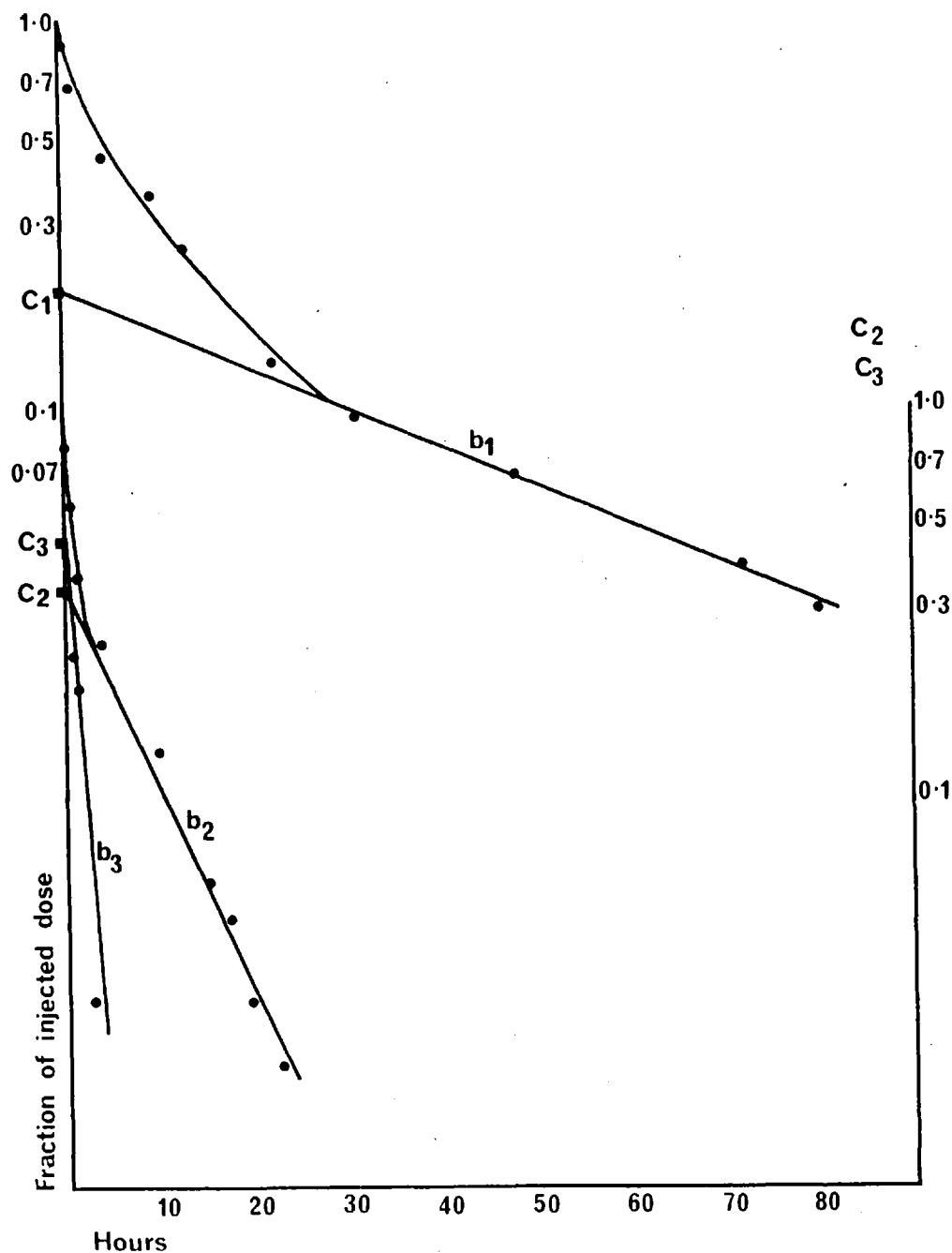


Figure 15. Plasma clearance of ^{125}I -haemopexin in a low dose haem rabbit receiving 4 mg haem. The curve is described by the exponential equation $y = C_1 e^{-b_1 t} + C_2 e^{-b_2 t} + C_3 e^{-b_3 t}$

$$C_1 e^{-b_1 t} = 30.1 \text{ hours} \quad C_1 = 0.20$$

$$C_2 e^{-b_2 t} = 6.2 \text{ hours} \quad C_2 = 0.34$$

$$C_3 e^{-b_3 t} = 1.0 \text{ hours} \quad C_3 = 0.46$$

Curve 1 - exponential 1 (slope b_1) = curve 2

Curve 2 - exponential 2 (slope b_2) = exponential 3 (slope b_3)

GROUP 5 RABBITS INJECTED WITH 1.75-4.0 mg ^{59}Fe -HAEM + 100 μg ^{125}I -HAEMOPEXIN

TABLE 13:1 ^{125}I -HAEMOPEXIN CLEARANCE: ANALYSIS OF PLASMA ACTIVITY CURVE

^{59}Fe -haem (mg)	R	Wt (Kg)	half clearance time (hours)			y-axis intercept (fraction of injected dose)			rate constant		
			$C_1 e^{-b_1 t}$	$C_2 e^{-b_2 t}$	$C_3 e^{-b_3 t}$	C_1	C_2	C_3	b_1	b_2	b_3
1.75	1	1.92	43.0	3.1	1.0	0.35	0.31	0.34	0.39	5.36	16.7
1.75	2	1.95	30.1	5.6	0.5	0.29	0.40	0.31	0.55	2.97	33.3
1.75	3	2.40	30.1	4.7	0.6	0.27	0.36	0.47	0.55	3.54	27.7
2.00	4	3.33	43.0	5.9	1.4	0.12	0.39	0.49	0.39	2.70	11.9
4.00	5	2.18	30.1	6.2	1.0	0.20	0.34	0.46	0.55	2.68	16.7
4.00	6	3.13	37.5	4.2	1.0	0.11	0.46	0.43	0.45	3.96	16.7
	$\bar{x}$	2.49	35.63	4.95	0.92	0.223	0.377	0.417	0.48	3.535	20.5
	SD	0.61	6.39	1.18	0.33	0.097	0.052	0.074	0.08	1.026	8.16
Controls*	1	3.42	36.8	3.4		0.32	0.68		0.44	4.9	
	2	2.20	30.2	3.1		0.30	0.70		0.55	5.36	

* All control rabbits received 100 μg ^{125}I -haemopexin

The mean half-clearance time of component $C_1 e^{-b_1 t}$ in rabbits receiving ^{59}Fe -haem was 35.6 hours and mean value for y-axis intercept C_1 was 0.223 (SD 0.097) compared with control values for C_1 of 0.32 and 0.30 in the 2 rabbits studied. Values for C_1 varied between 0.11 and 0.35, the latter being within the range of the control group, and it can be seen from Table 13:1 that the highest values for C_1 were associated with the lowest doses of haem. Control results fell within the range of results already obtained, except that the half-clearance times of component $C_1 e^{-b_1 t}$ were 36.8 and 30.2 hours in the 2 rabbits studied.

Tables 13:2 and 13:3 show calculated results for permeability constants, extravascular protein mass, catabolic rate and total haemopexin haem binding capacity in animals receiving ^{59}Fe -haem. The experimental model chosen for the calculation of these results is shown in Figure 7.

GROUP 5 RABBITS INJECTED WITH 1.75-4.0 mg ^{59}Fe -HAEM + 100 μg ^{125}I -HAEMOPEXIN

TABLE 13:2 ^{125}I -HAEMOPEXIN CLEARANCE: COMPARTMENTAL ANALYSIS

Haem (mg)	R	Permeabilities				Ratio of extravascular to intravascular protein masses		
		K_{13}	K_{31}	K_{14}	K_{41}	compt 3	compt 4	compts 3+4
1.75	1	3.182	12.177	3.269	2.797	0.261	1.169	1.430
1.75	2	8.830	23.630	1.326	1.530	0.374	0.867	1.241
1.75	3	8.040	18.444	1.884	1.794	0.436	1.050	1.486
2.00	4	3.148	7.200	1.746	0.854	0.437	2.044	2.481
4.00	5	5.354	9.945	1.421	1.285	0.539	1.111	1.650
4.00	6	3.854	11.019	2.599	1.039	0.350	2.501	2.851
	$\bar{x}$	5.401	13.736	2.041	1.550	0.400	1.457	1.857
	SD	2.495	6.115	0.752	0.697	0.094	0.656	0.651

GROUP 5 RABBITS INJECTED WITH 1.75-4.0 mg ^{59}Fe -HAEM + 100 μg ^{125}I -HAEMOPEXIN

TABLE 13:3 ^{125}I -HAEMOPEXIN CLEARANCE: COMPARTMENTAL ANALYSIS

Haem (mg)	R	catabolic rate K_{12}	EV/IV haemopexin $\bar{Y}/\bar{X}$	IV haemopexin $\bar{X}$ (mg)	EV haemopexin $\bar{Y}$ (mg)	Total haemopexin (mg)	Total haem binding capacity (mg)
1.75	1	1.01	1.430	35.4	50.6	86.0	0.842
1.75	2	1.52	1.241	36.2	45.0	81.2	0.795
1.75	3	1.65	1.486	44.6	66.3	110.9	1.086
2.00	4	1.98	2.481	61.8	153.0	214.8	2.104
4.00	5	1.95	1.650	40.5	67.7	108.2	1.060
4.00	6	2.57	2.851	58.3	166.0	224.3	2.197
	$\bar{x}$	1.78	1.857	46.133	91.433	137.567	1.347
	SD	0.524	0.651	11.326	53.608	64.649	0.633

In rabbits receiving ^{59}Fe -haem, mean catabolic rate was 1.78 (SD 0.524) times the intravascular haemopexin pool per day; mean extravascular protein was 1.857 (SD 0.651) times the mass of intravascular haemopexin and mean total haemopexin haem binding capacity was 1.347 mg haem (SD 0.633).

Recovery of ^{125}I in the urine and faeces was 85% (SD 4.7) of the injected dose in animals receiving haem and 93% of the injected activity in both controls.

Non-protein-bound ^{125}I ranged from 2.1-4.1% of the plasma activity in the 24 hour plasma samples of both haem-loaded and control rabbits.

Summary of ^{125}I -haemopexin clearance data in normal and haem-loaded rabbits

Table 14:1 summarises results obtained in the 26 normal rabbits, 11 high dose haem rabbits and 6 low dose haem rabbits. Mean values are given in each case.

TABLE 14:11

	controls	high dose haem	low dose haem
$C_1 e^{-b_1 t}$	23.62 (SD 5.50)	29.61 (SD 6.97)	35.62 (SD 6.40)
$C_2 e^{-b_2 t}$	3.55 (SD 1.07)	1.72 (SD 0.277)	4.95 (SD 1.18)
C_1	0.372 (SD 0.077)	0.088 (SD 0.041)	0.223 (SD 0.089)
K_{12}	1.57 (SD 0.28)	4.60 (SD 1.629)	1.78 (SD 0.522)
$\bar{Y}/\bar{X}$	0.769 (SD 0.229)	3.51 (SD 0.879)	1.857 (SD 0.651)

Mean y-axis intercept C_1 in controls was 0.372 (SD 0.077) compared with the high dose haem result of 0.088 (SD 0.041) and the low dose haem value of 0.223 (SD 0.089). The result of a t-test carried out between control and high dose haem values was $t = 11.44$, $n = 36$, $p = <0.001$. A t-test on control and low dose haem values for C_1 gave the result $t = 4.064$, $n = 31$, $p = <0.001$.

Results for catabolic rate, controls 1.57 (SD 0.28), high dose haem rabbits 4.60 (SD 1.629) and low dose haem animals 1.78 (SD 0.522) were treated similarly. The result of a t-test between control and high dose haem values was $t = 9.3$, $n = 36$, $p = <0.001$. The result for the test on control and low dose haem values was not significant ($t = 1.34$, $n = 31$, $p = 0.2$) but if only the last three values for K_{12} were taken, the result was $t = 3.32$, $n = 28$, $p = 0.001-0.005$.

Extravascular protein, $\bar{Y}$, was 0.769 (SD 0.229) times the mass of intravascular haemopexin in control animals, 3.51 (SD 0.879) in high dose haem rabbits and 1.857 (SD 0.651) in low dose haem animals. Results of t-tests were, for high dose haem rabbits and controls, $t = 15.00$, $n = 36$, $p = <0.001$ and for low dose haem animals and controls, $t = 7.102$, $n = 31$, $p = <0.001$.

The values in Table 14:2 for a 2.5 Kg rabbit, PCV 40% and blood volume 175 mls were calculated for a normal rabbit and for rabbits receiving high doses (25 mg) and low doses (1.75-4.0 mg) of haem.

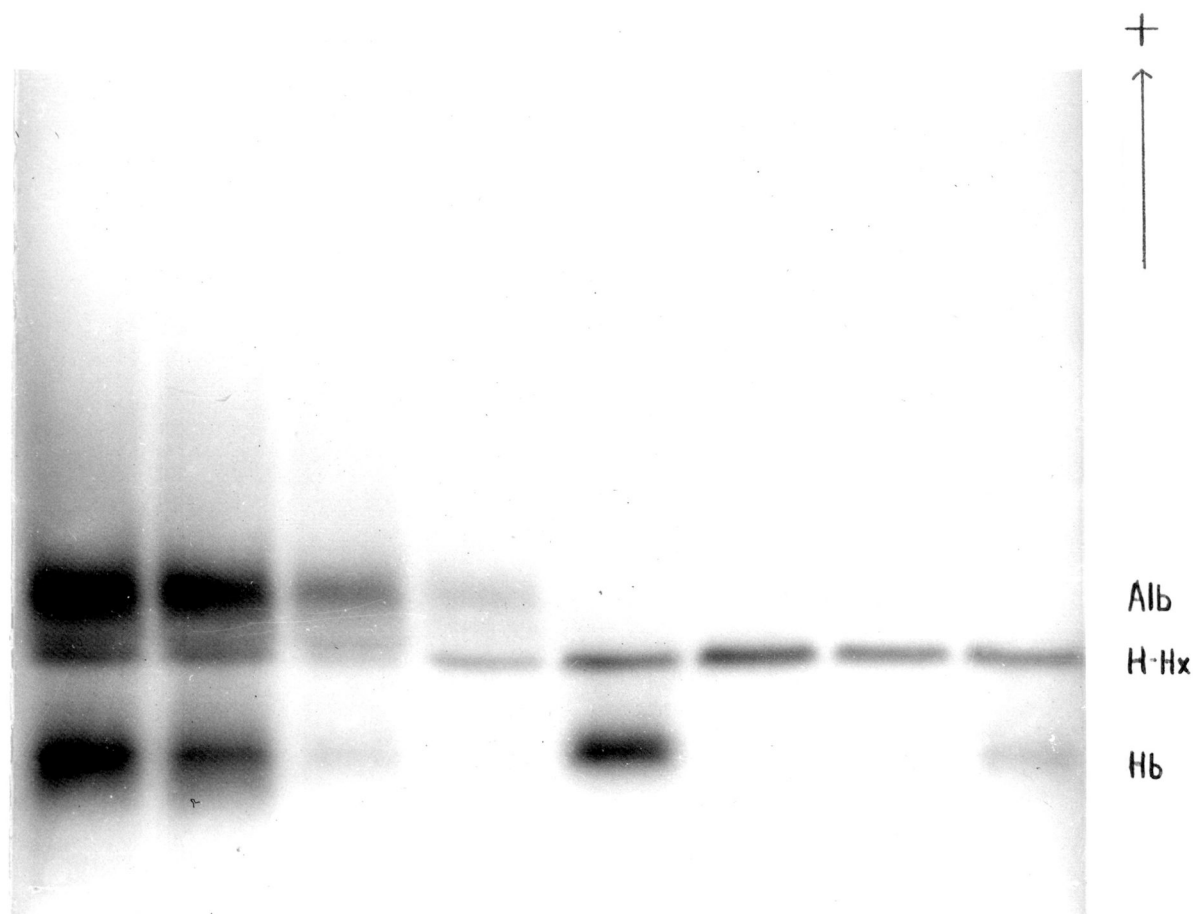
TABLE 14:2

	control	high dose haem	low dose haem
Haemopexin catabolised per day (mg)	72.84	213.44	82.59
Fraction of total haemo- pexin pool catabolised per day	0.887	1.02	0.623
Extravascular haemopexin (mg)	35.68	162.86	86.15
Total haemopexin (mg)	82.08	209.26	132.55
Total haemopexin haem binding capacity (mg)	0.804	2.049	1.298

Results of starch gel electrophoresis and immunoquantitation

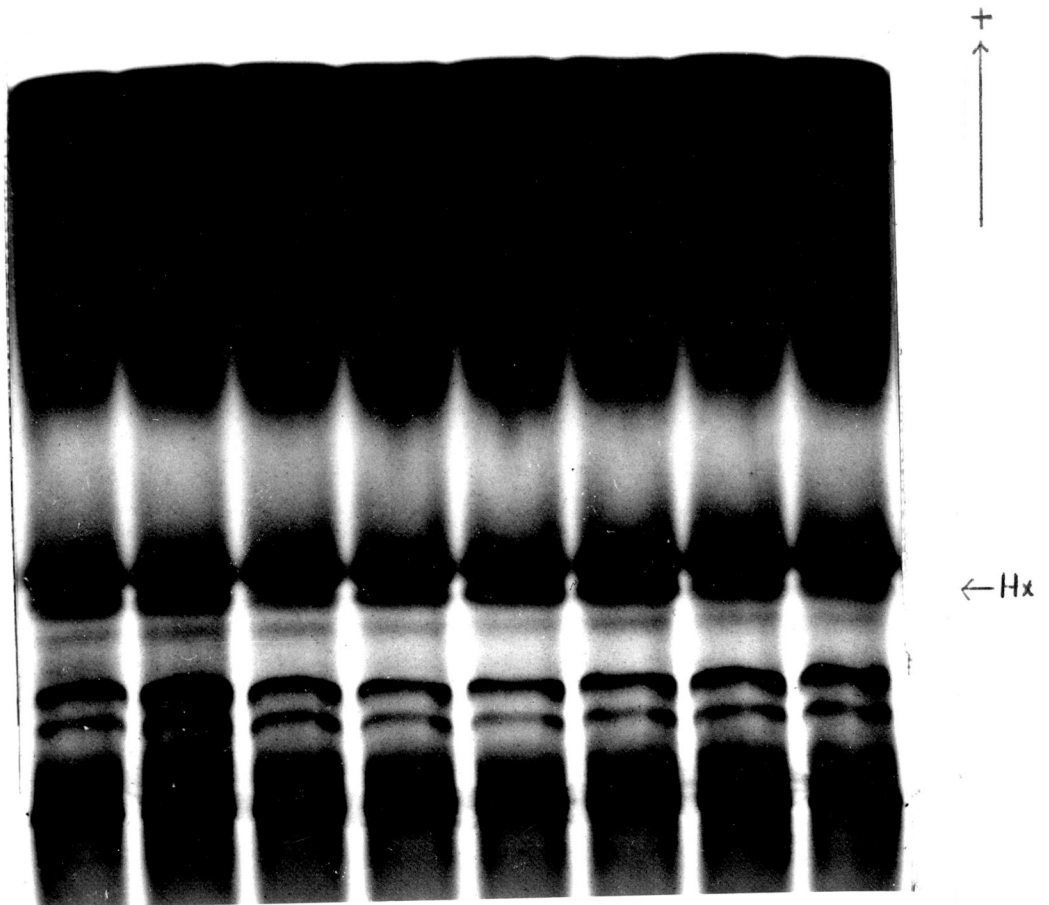
Rabbits receiving 25 mg haem

Starch gel electrophoresis of plasma samples obtained from a rabbit receiving 25 mg haem is shown in Figures 14a and 14b. Figure 14a shows a gel slice in which haem is stained. The haem-haemopexin and methaemalbumin bands are marked and free haemoglobin in the plasma may be disregarded. The 3 minute sample shows that injected haem was rapidly bound by haemopexin and albumin. Haem bound to haemopexin quickly fell in concentration to reach a minimum after 3 hours. After this time, haem associated with haemopexin increased in concentration, reaching a peak in the 24 hour sample.



Alb = methaemalbumin
 H-Hx = haem-haemopexin
 Hb = haemoglobin

Figure 14a. Starch gel electrophoresis of plasma samples from a rabbit receiving 25 mg haem. The gel is stained with benzidine/hydrogen peroxide solution to show haem. From left to right, plasma samples are; 3 minutes, 30 minutes, 3, 8, 14, 24, 48 and 72 hours after the injection of haem and labelled haemopexin. The volume of plasma in each insert was 50 μ l.



Hx = haemopexin

Figure 14b. A second slice of gel taken from the electrophoresis described in figure 14a was stained with Amido-Schwartz B in 10% acetic acid to demonstrate plasma proteins. The plasma samples are the same as in figure 14a. The haemopexin band is marked and shows a well defined peak level of concentration in the 24 hour plasma sample.

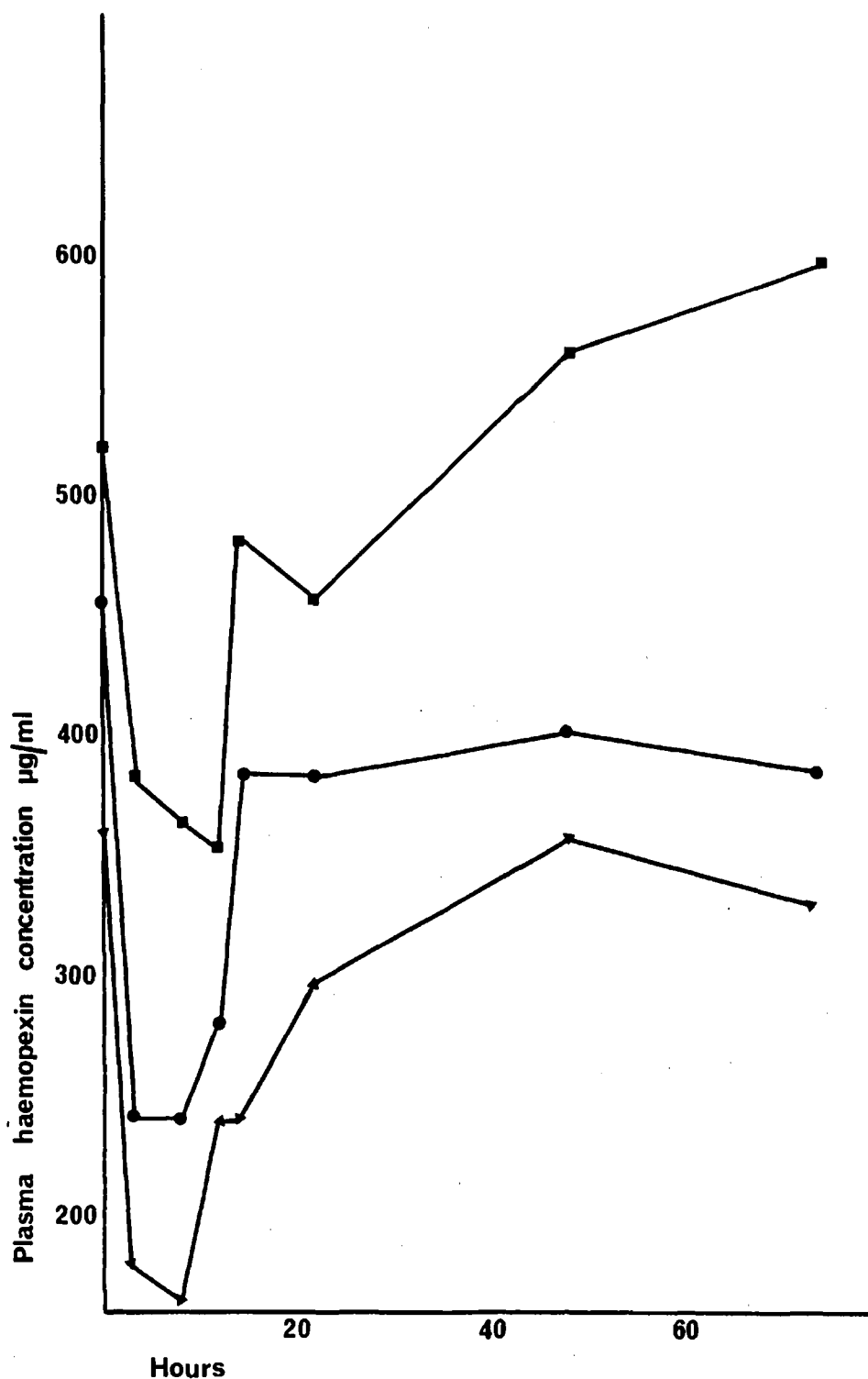


Figure 15. Plasma concentration of haemopexin in 3 rabbits receiving 25 mg haem. Following injection of haem, there was a rapid fall in haemopexin concentration which remained low during the period in which defibrinogen was present in the plasma. After elimination of haem from the plasma, haemopexin levels increased and approached resting levels after 24 hours.

Figure 14b shows a gel slice in which the plasma proteins are stained and the haemopexin band is indicated. It can be seen that, following injection of haem, plasma haemopexin concentration fell, reaching the lowest value in the 3 hour sample. After 3 hours, plasma haemopexin level increased to reach a peak in the 24 hour sample.

Radioactivity in the excised haemopexin bands decreased to 10% of the initial level within 8 hours and activity in later samples was undetectable.

This general pattern of results was observed in other rabbits receiving 25 mg haem, although there was slight variation in the extent of the increase in haemopexin concentration and the time interval after which the increase occurred.

Staining patterns in Figures 14a and 14b correlate with immunoquantitation results shown in Figure 15. Following haem injection, plasma haemopexin concentration fell rapidly by 27-50%. Minimum levels of plasma haemopexin were found after 3 hours and plasma haemopexin concentration increased after 8-12 hours.

Rabbits receiving 1.75-4.0 mg haem

The results of starch gel electrophoresis show that the doses of ^{59}Fe -haem were bound to haemopexin in the 3 minute plasma samples and methaemalbumin was undetectable by the methods used. Generally, the results were similar in other respects to those shown in Figures 14a and 14b.

Clearance of ^{59}Fe -haem from albumin

Plasma samples of one rabbit receiving 25 mg ^{59}Fe -haem were subjected to starch gel electrophoresis. The methaemalbumin bands were excised, the radioactivity in each band determined and expressed as a fraction of the activity of the methaemalbumin band in the 3 minute sample. The half-clearance time of ^{59}Fe -haem from albumin was 3.3 hours.

Clearance of ^{125}I -haemopexin from the plasma of rabbits with intravascular haemolysis

a) Injection of haemoglobin

To investigate the effect of a single haemolytic episode on plasma haemopexin concentration, 600 mg ^{59}Fe -haemoglobin (equivalent to 24 mg haem) were injected into a 4.25 Kg rabbit.

The plasma clearance curve of ^{59}Fe -haemoglobin is shown in Figure 16. Clearance was described by a single exponential function of time and the correlation coefficient for points lying along the line was 0.9995. ^{59}Fe -haemoglobin was eliminated from the plasma with a half-clearance time of 1.2 hours.

14.8% of the injected activity was recovered in the urine after 24 hours, 14.1% being excreted in the 2 hours following ^{59}Fe -haemoglobin injection.

Plasma samples were electrophoresed in starch gel and the results are shown in Figure 17. Methaemalbumin was undetectable by the methods used. The haem-haemopexin band is clearly defined and shows no decrease in haem associated with haemopexin until clearance of haemoglobin from the plasma is almost complete.

b) Phenylhydrazine-treated rabbits

As an alternative to haemoglobin injection, phenylhydrazine was administered to induce intravascular haemolysis. Table 15 shows results of reticulocyte count, plasma and whole blood haemoglobin, PCV and starch gel electrophoresis during the 3 weeks of phenylhydrazine treatment prior to ^{125}I -haemopexin injection.

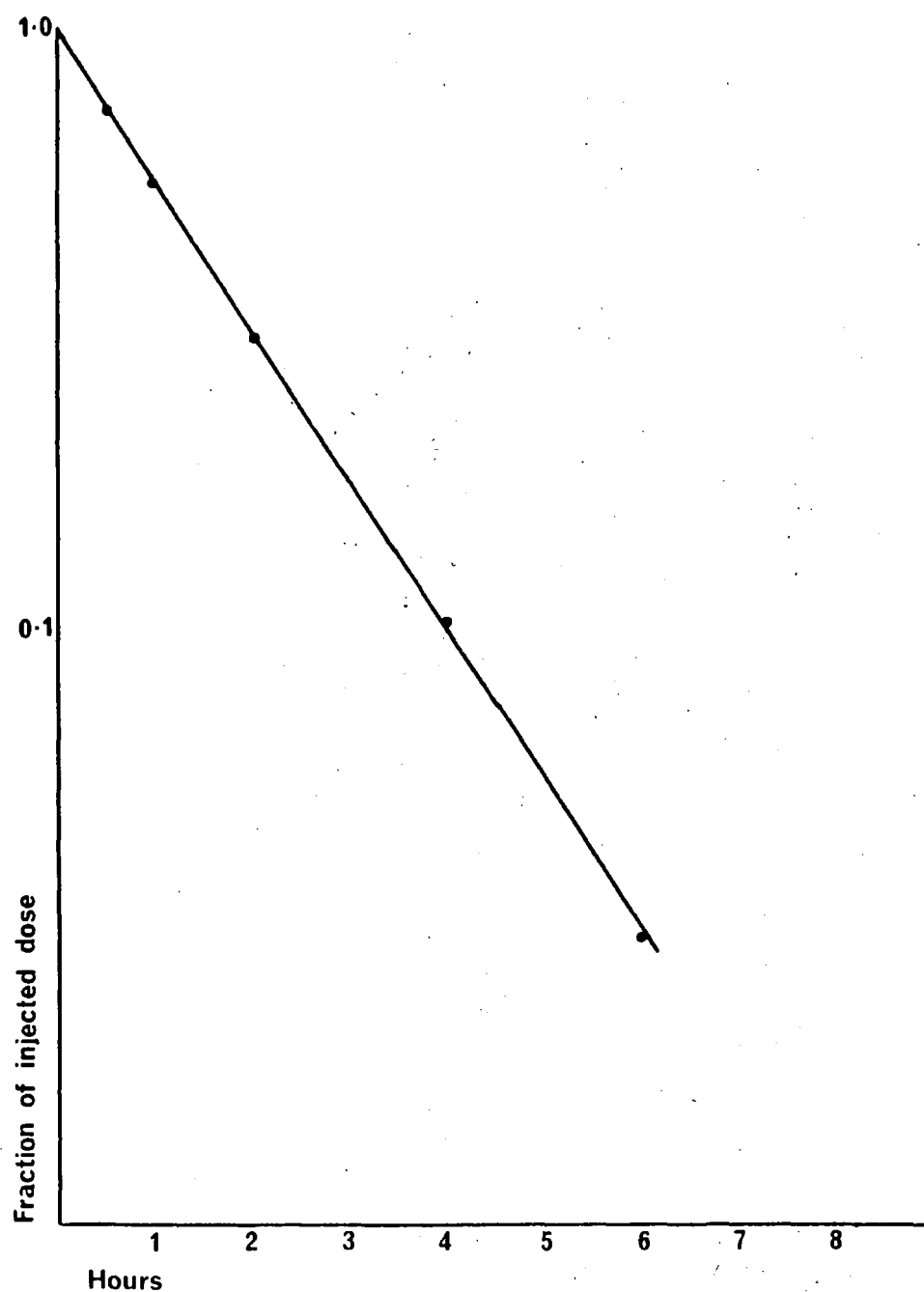
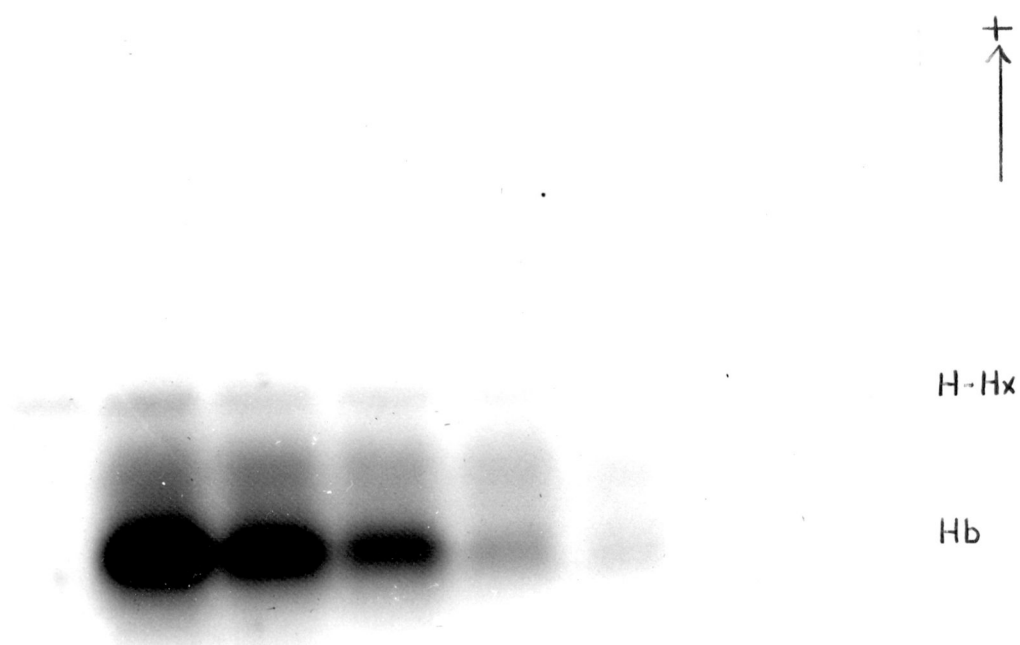


Figure 16. Plasma clearance of ^{59}Fe -haemoglobin in a 4.25 Kg rabbit receiving 600 mg ^{59}Fe -haemoglobin. Clearance was described by a single exponential function of time. Half-clearance time was 1.2 hours.



H-Hx = haem-haemopexin

Hb = haemoglobin

Figure 17. Haemoglobin rabbit: Starch gel electrophoresis of plasma samples. Left to right: Sample prior to haemoglobin injection and samples at 3 minutes, 30 minutes, 1, 2, 4 and 6 hours after haemoglobin injection. Haem-haemopexin and haemoglobin bands are marked.

TABLE 15 PHENYLHYDRAZINE-TREATED RABBITS

R	Day	Reticulo- cytes %	Plasma Hb mg/100 mls	Whole blood Hb g/100 mls	PCV %	Methoemalbumin on starch gel
1	1	5.5	11.5	13.6	38	-
	3	7.0	12.6	12.0	37	-
	8	10.4	20.8	11.6	38	-
	10	13.1	24.8	11.4	38	faint
	15	14.2	21.4	11.0	39	distinct
	17	14.2	26.8	11.2	39	distinct
	21	14.4	30.4	11.4	39	distinct
2	1	4.8	16.0	13.8	39	-
	3	6.8	11.2	12.6	38	-
	8	11.2	18.2	12.0	38	-
	10	13.3	21.4	11.8	38	faint
	15	14.5	23.0	11.4	38	distinct
	17	14.3	28.2	11.4	38	distinct
	21	14.7	33.4	11.2	38	distinct

Day 1 = day following 1st phenylhydrazine injection

On day 21, 100 µg ¹²⁵I-haemopexin were injected into the marginal ear vein

The plasma haemopexin clearance curves for both phenylhydrazine-treated animals were similar to those obtained in low dose haem rabbits. An example of the plasma haemopexin clearance curve in low dose haem rabbits is shown in Figure 13. The clearance curve was resolved into 3 components; $C_1e^{-b_1t}$, $C_2e^{-b_2t}$ and $C_3e^{-b_3t}$ and Tables 16:1, 16:2 and 16:3 show results of graphic resolution and compartmental analysis for phenylhydrazine-treated rabbits. The experimental model used is shown in Figure 7.

It can be seen from Table 16:1 that y-axis intercept C_1 values of 0.147 and 0.171 of the injected activity were obtained in the 2 rabbits studied. Results in Table 16:3 for catabolic rate were 2.14 and 2.04 times the intravascular haemopexin pool per day and values for extravascular protein were 1.971 and 1.61 times the mass of intravascular protein. These values were similar to those obtained in rabbits receiving 2-4 mg haem.

PHENYLHYDRAZINE-TREATED RABBITS INJECTED WITH 100 μg ^{125}I -HAEMOPEXIN

TABLE 16:1 ANALYSIS OF PLASMA ACTIVITY CURVE

R	Wt (kg)	half clearance time (hours)			axis intercept (fraction of injected dose)			rate constants		
		$C_1 e^{-b_1 t}$	$C_2 e^{-b_2 t}$	$C_3 e^{-b_3 t}$	C_1	C_2	C_3	b_1	b_2	b_3
1	3.17	33.4	4.76	0.6	0.147	0.541	0.469	0.499	3.5	27.7
2	3.50	30.1	5.08	1.1	0.171	0.480	0.520	0.555	3.26	15.3

PHENYLHYDRAZINE-TREATED RABBITS INJECTED WITH 100 μ g ^{125}I -HAEMOPEXIN

TABLE 16:2 COMPARTMENTAL ANALYSIS

R	Permeabilities				Ratio of extravascular to intravascular protein masses		
	K_{13}	K_{31}	K_{14}	K_{41}	compt 3	compt 4	compts 3+4
1	6.614	19.945	1.877	1.145	0.532	1.639	1.971
2	3.311	10.883	1.623	1.233	0.300	1.310	1.610

TABLE 16:3 COMPARTMENTAL ANALYSIS

R	catabolic rate K_{12}	EV/IV haemopexin $\bar{X}$	IV haemopexin $\bar{X}$ (mg)	EV haemopexin (mg)	Total haemopexin (mg)	Total haem binding capacity (mg)
1	2.14	1.971	58.8	116.0	174.8	1.712
2	2.04	1.610	65.0	105.0	170.8	1.665

DISCUSSION

This investigation has been concerned with the fate of haemoglobin liberated into the plasma as a result of intravascular red cell destruction. The plasma proteins haptoglobin, haemopexin and albumin are involved in the elimination of free haemoglobin and haem from the circulation: haptoglobin binds haemoglobin whereas haemopexin and albumin bind haem. These mechanisms for haemoglobin and haem clearance result in the catabolism of haem in hepatic parenchymal cells with retention of iron for haemoglobin synthesis.

The extent to which haptoglobin, haemopexin and albumin are involved in haemoglobin and haem clearance depends on the rate of intravascular haemolysis. In normal conditions, free plasma haemoglobin is bound and removed by haptoglobin. However, when the rate of intravascular red cell destruction is increased, haptoglobin is depleted as a result of rapid catabolism of the haptoglobin-haemoglobin complex (Freeman, 1964). As replacement of haptoglobin by synthesis does not rapidly compensate for the loss of the haptoglobin-haemoglobin complex, plasma haptoglobin concentration may fall to zero. Consequently, the haemoglobin level in the plasma increases and renal excretion of haem-globin dimer ($\alpha\beta$ subunit) may occur if haemolysis is severe. Free circulating haemoglobin is oxidised to methaemoglobin from which the haem moiety dissociates and is bound by haemopexin and albumin.

The objectives of the present investigation were: to make an accurate quantitative study of haemopexin turnover in normal and haem-loaded rabbits; to investigate the effect of haem on haemopexin distribution and to study the relationship between total body haemopexin haem binding capacity and the amount of haem cleared from the plasma. In order to discover the extent of haemopexin involvement in the plasma clearance of haemoglobin and its metabolites, haemopexin turnover was measured in animals with increased red cell destruction due to administration of phenylhydrazine.

HAEMOPEXIN TURNOVER

The labelled haemopexin used in these experiments was pure, haem-free, undenatured and in the monomeric form. The protein was shown to be biochemically active and it was assumed to form, in vivo, a homogeneous pool with endogenous haemopexin. The use of perchloric acid in the preliminary stages of haemopexin purification provides a protein which is readily iodinated and aggregation of the purified protein is minimised, probably because oxidation of labile sulphydryl groups occurs. The protein aggregation in haemopexin preparations purified by methods avoiding the use of perchloric acid makes the material entirely unsuitable for measurement of fractional catabolic rate.

For evaluation of ^{125}I -haemopexin clearance, the procedure and nomenclature of Matthews (1957) were used. Matthews described a model with 3 or 4 compartments, as shown in Figure 7, for the analysis of gamma globulin and albumin turnover in the rabbit. Labelled protein, injected into the intravascular compartment (compartment 1), equilibrates with protein in the extravascular spaces (compartments 3 and 4). Breakdown of labelled protein to the urine (compartment 2) is considered to take place from the intravascular compartment. It is assumed that the system is in dynamic equilibrium, total protein in each compartment being constant, that rate of breakdown is equal to the rate of synthesis and that rates of transfer from one compartment to another are constant.

In general, following the intravenous injection of ^{125}I -labelled protein, there is a rapid fall in plasma activity caused mainly by exchange of labelled molecules with unlabelled molecules in the extravascular space: catabolism of labelled protein also contributes to this initial fall in activity. Once equilibration is complete, the fall in plasma activity becomes constant in rate.

¹²⁵I-haemopexin clearance, studied in normal and haem-loaded rabbits, showed that haem increased haemopexin catabolism. In normal rabbits, haemopexin was cleared from the plasma with a half-clearance time of approximately one day. Experiments in haem-loaded animals showed that the haem-haemopexin complex is rapidly catabolised, the extent of haemopexin catabolism being dependent on the amount of haem injected.

Normal haemopexin turnover

Normal human plasma contains approximately 0.3 mg haemoglobin per 100 mls plasma: amounts of haemoglobin of this order are bound and cleared by haptoglobin, which has a haemoglobin binding capacity of 50-150 mg per 100 mls plasma (Bunn, 1972). Thus, in normal animals, haemopexin was assumed to be cleared from the plasma in the haem-free state.

In normal rabbits, clearance of labelled haemopexin from the plasma was consistent with fractional catabolism from a homogeneous plasma pool and non-protein-bound ¹²⁵I in the plasma could be attributed to catabolism of normal haemopexin.

43% of total haemopexin was extravascular, mean extravascular haemopexin being 0.77 times the mass of intravascular haemopexin. Fractional catabolic rate was 1.53 times the intravascular haemopexin pool per day, compared with values of 0.3-0.4 for ¹³¹I-fibrinogen (McFarlane, 1963) and 0.161-0.21 for ¹³¹I-albumin (Matthews, 1957).

It was of interest that the plasma half-clearance times in the two groups investigated were significantly different: mean values were 20.4 hours and 30.17 hours respectively and a t-test carried out on the half-clearance times gave the result $n = 17$, $t = 8.46$, $p < 0.001$. This may be due to a batch defect in the haemopexin or in the animals used for the experiments, although obvious factors were excluded. The rabbits were of similar strain and weight, experimental procedure was carefully

controlled and preparative methods for haemopexin were common to all samples. Source of rabbit serum was also excluded, as animals in Groups 1 and 2 both received haemopexin purified from the serum of rabbits injected with 3-methylcholanthrene. The difference in plasma half-clearance times was approximately 30% and from a biological viewpoint this could be less significant than statistical analysis indicated.

Haemopexin turnover in haem-loaded rabbits

In rabbits injected with 25 mg haem, total haemopexin haem binding capacity was calculated to be 0.7-1.6 mg haem; ^{therefore,} 25 mg haem saturated endogenous and labelled haemopexin, excess haem being rapidly bound by albumin. The 3 minute plasma samples, electrophoresed in starch gel, showed methaemalbumin in the plasma.

Comparison of plasma ¹²⁵I-haemopexin clearance curves in normal and haem-loaded rabbits showed that the haem-haemopexin complex was rapidly eliminated from the plasma. In haem-loaded animals 90% of the labelled protein was cleared from the plasma within 8 hours of injection.

Following injection of 25 mg haem, immunoquantitation showed that plasma haemopexin concentration fell by 27-50% within 8 hours. Over the same period, ¹²⁵I-activity in the starch gel haemopexin zone decreased by 90% and continued to decline, indicating that the rise in plasma haemopexin concentration after 8-12 hours was caused by newly synthesised haemopexin. Low plasma haemopexin levels coincided with methaemalbuminaemia.

The results indicated that rapid elimination of the haem-haemopexin complex from the plasma was promptly followed by catabolism of the complex. Excretion data obtained in a catheterised rabbit showed that 55% of injected activity was present in the urine as non-protein-bound ¹²⁵I within 8 hours of ¹²⁵I-haemopexin injection. Evidence for increased haemopexin catabolism was also obtained from compartmental analysis of the plasma activity curve: mean catabolic rate was 3-4 times higher than in normal animals.

Injection of haem also affected haemopexin distribution: in rabbits receiving 25 mg haem a larger fraction of total haemopexin was located in the extravascular pool. Extravascular haemopexin, expressed as a fraction of the intravascular haemopexin mass, increased by 3-4 times.

In animals receiving 1.75-4.0 mg haem, because the haem was injected prior to ^{125}I -haemopexin, the haem was bound by endogenous haemopexin leaving a variable amount of excess haem available for binding by labelled haemopexin. The amount of haem-haemopexin complex formed depended on the haem binding capacity of endogenous haemopexin in relation to the dose of injected haem.

In animals injected with 1.75 mg haem, endogenous haemopexin cleared the injected haem before administration of labelled haemopexin and these animals gave results which fell within the normal range. The initial rapid fall in plasma activity in animals receiving 2 mg and 4 mg haem was caused mainly by elimination of the haem-haemopexin complex from the plasma: equilibration of labelled haemopexin and haem-haemopexin and catabolism of residual haem-free haemopexin also contributed to the initial decrease in plasma activity. Catabolic rate in these animals was approximately 1.5 times higher than normal and extravascular haemopexin, expressed as a fraction of the intravascular haemopexin mass, increased by 1.5-2 times.

Haemopexin turnover in rabbits with intravascular haemolysis

To investigate the effect of a single haemolytic episode on plasma haemopexin turnover, ^{59}Fe -haemoglobin equivalent to 24 mg haem was injected intravenously into a rabbit. Following haemoglobin injection, absence of methaemalbumin in the plasma was demonstrated by starch gel electrophoresis of plasma samples. Haem-haemopexin was present in starch gel, but this band did not show the fall in concentration which was observed 3 hours after injection of 25 mg haem. Thus, there did not appear to be a significant increase in haemopexin catabolism following a single

injection of haemoglobin. After haptoglobin saturation, plasma clearance of haem-globin dimer by the kidneys occurred and when the absorptive capacity of the renal tubules was exceeded, activity appeared in the urine.

As an alternative to haemoglobin injection, phenylhydrazine was administered to induce intravascular haemolysis. Prior to ^{125}I -haemopexin injection, plasma samples of phenylhydrazine-treated rabbits were electrophoresed in starch gel; haemoglobin and methaemalbumin were present in the plasma.

Phenylhydrazine-treated rabbits gave similar results to rabbits receiving 2 mg and 4 mg haem: catabolic rate was approximately 1.5 times higher than in normal animals and extravascular haemopexin mass increased by 1.5-2.0 times. Thus, in rabbits with phenylhydrazine-induced haemolytic anaemia and consequent liberation of haemoglobin into the plasma, haemopexin plays an important part in the elimination of haem from the circulation.

CLEARANCE OF ^{59}Fe -HAEM FROM THE PLASMA

The results confirmed that rate of elimination of haem depended on the amount of haem injected (Müller-Eberhard *et al.*, 1969). Small doses of haem were cleared from the plasma at a faster rate than large amounts of haem.

When the dose of ^{59}Fe -haem did not exceed the binding capacity of endogenous haemopexin, clearance was consistent with removal of haem from a homogeneous plasma haem pool of haem-haemopexin: clearance of the ^{59}Fe label was described by a single exponential function with a mean half-clearance time of 0.6 hours. The similarity in rate of haem clearance and the initial component of haemopexin clearance in the same rabbits was in accordance with removal of haem from the plasma as a complex with haemopexin.

In animals receiving 25 mg haem, the plasma ^{59}Fe clearance curve was evaluated into two exponential functions, indicating the presence of two or more haem pools, of which haem-haemopexin and methaemalbumin were the most important. 90% of the injected activity was eliminated from the plasma between 8 and 17.5 hours after zero time, with a half-clearance time of 2.0-3.75 hours. Catabolic rate was approximately four times the intravascular haem pool per day. As an intravascular haem pool is not present in normal rabbits this may suggest that four times the injected dose can be eliminated per day.

Rapid elimination of haem from the plasma as a complex with haemopexin has two physiologically important functions: iron loss from the body is prevented and rapid removal of haem from the plasma may have bacteriostatic significance. Iron is essential for bacterial growth and there is evidence that acquisition of iron from the host is a fundamental feature of bacterial pathogenicity. Unsaturated transferrins in serum and milk prevent bacterial growth (Bullen, Rogers, and Griffiths, 1972) and bacteriostasis is reversed by saturating the proteins with iron. Clinically, increased susceptibility to infection has been noted in various haemolytic disorders and Kaye, Gill and Cook (1967) demonstrated increased susceptibility to salmonellosis in mice treated with phenylhydrazine.

THE ROLE OF HAEMOPEXIN IN PLASMA HAEM CLEARANCE

Haemopexin is catabolised in the process of transporting haem to the liver and can be placed with haptoglobin in the group of "suicidal" proteins (Freeman, 1964). Since haem-haemopexin is rapidly catabolised, the amount of haem removed by endogenous haemopexin would not exceed the total haemopexin binding capacity of 0.5-2.0 mg haem. Thus, removal of 25 mg haem from the plasma by haemopexin would require increased haemopexin synthesis to accommodate the haem load.

It is not known if haem is transferred to the liver by methaemalbumin: present evidence suggests that this is unlikely. Müller-Eberhard *et al.*, (1969) demonstrated rapid *in vivo* transfer of haem from methaemalbumin to haemopexin in the rabbit and showed that ^{131}I -labelled albumin and methaemalbumin were cleared from the plasma at the same rate, with a half-clearance time of 4.5-5.5 days. They concluded that most, if not all, circulating haem is removed from the plasma in the form of the haem-haemopexin complex.

The 3-4 fold increase in haemopexin turnover observed in rabbits receiving 25 mg haem is insufficient to account for the entire elimination of 25 mg haem within 12 hours of injection. In the presence of methaemalbumin, a large proportion of newly synthesised haemopexin would bind haem and be rapidly catabolised. It is likely that, as hepatic parenchymal cells are probably the site of haemopexin synthesis and catabolism, rapid transfer of haem from methaemalbumin to haemopexin could occur within the liver in close proximity to hepatic parenchymal cells. The haem-haemopexin complex would then be very rapidly eliminated and most of the haem transfer process would be beyond the reach of the methods of investigation used. In animals receiving haem, it is probable that the rise in catabolic rate understated the extent by which synthesis rate was increased and plasma haemopexin concentration gave a poor indication of the extent of increase in synthesis rate and the size of the total haemopexin pool.

Thus it is possible that haem transfer to hepatic parenchymal cells is a specific function of haemopexin. However, in subjects made haemopexin deficient by haem injection or chronic intravascular haemolysis, only methaemalbumin is present in the plasma and yet haem continues to be eliminated, although at a slower rate than in normal subjects (Sears, 1970). Sears injected approximately 1 mg ^{59}Fe -haem per Kg body weight into normal and haemopexin deficient human subjects. Clearance of haem from the plasma of normal subjects was described by a single exponential

function of half-clearance time 7.8 hours, whereas, in haemopexin deficient plasma, haem clearance was described by two exponential functions of mean half-clearance times of 3.9 hours and 22.2 hours respectively. ^{59}Fe -haem clearance in the rabbit in the present investigation was consistent with these results. In haemopexin deficient subjects, transfer of haem from methaemalbumin to newly synthesised haemopexin could take place in the liver in the vicinity of the parenchymal cells. If this occurred, the apparent clearance rate of haem from methaemalbumin would reflect the rate of production of newly synthesised haemopexin by hepatic parenchymal cells.

In conclusion, the present experiments showed that labelled haemopexin was eliminated from the plasma of normal rabbits with a half-clearance time of 20-30 hours, compared with values of 2.5-3.0 days for ^{131}I -fibrinogen (McFarlane, 1963), 5.0-8.4 days for ^{131}I -albumin and 5.3-7.0 days for ^{131}I -gamma globulin (Schultze and Heremans, 1966). Clearance was consistent with catabolism of normal, haem-free, haemopexin.

In haem-loaded and phenylhydrazine-treated rabbits, the increased turnover of haemopexin is explained by the rapid elimination of the haem-haemopexin complex from the plasma followed by catabolism of the complex. As haemopexin is catabolised as a result of its haem clearance function it may be regarded as "suicidal" and placed in the same group as haptoglobin (Freeman, 1964).

The role of methaemalbumin in plasma haem clearance has yet to be determined. However, it is probable that methaemalbumin acts as a plasma storage reservoir for haem when haem levels exceed the haemopexin binding capacity and transfer of haem to hepatic parenchymal cells may be a specific function of haemopexin. It is likely that the haemopexin synthesis rate is capable of a marked increase in response to rising levels of plasma haem.

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HEMOPEXIN METABOLISM IN THE RABBIT

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Hemopexin metabolism in the rabbit

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Plasma clearance of homologous ^{125}I -hemopexin has been studied in 18 rabbits; from the experimental data the following mean values were determined: (1) plasma half-clearance time, 22.7 hours; (2) catabolic rate (fraction of the intravascular pool per day), 1.57; (3) extravascular hemopexin, (fraction of intravascular hemopexin), 0.8; and (4) absolute total hemopexin catabolized per day, 66 mg. Data for the clearance of ^{125}I -hemopexin from the plasma was shown by computer analysis to fit a pattern described by a double exponential equation of the type, $y = C_1e^{-b_1t} + C_2e^{-b_2t}$ where C_1 , C_2 , and b_1 , b_2 were the y axis intercepts and rate constants of the slow and fast components of clearance, respectively. After intercompartmental equilibration was complete, the exponential manner of ^{125}I -hemopexin plasma clearance was consistent with fractional catabolism from a homogeneous pool. The methods for purification of hemopexin and their possible effect on the metabolism of this protein are discussed.

The physiologic role of hemopexin as a specific heme-transporting protein has recently been reviewed⁸ and no published information about the metabolism of this protein is currently available.

Accordingly, plasma clearance of ^{125}I -labelled, purified homologous hemopexin has been studied in the rabbit. The results are described here and attention is drawn to certain problems connected with this work which may be of importance in the planning of future metabolic experiments with hemopexin.

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Table I. The data of the 4 experimental groups of rabbits are shown. Also shown for each group are the mean values for y-axis intercept, $T_{1/2}$, and rate constants of components *A* and *B*; values for catabolic rate and extravascular hemopexin were derived from compartmental analysis of the plasma clearance curve

Group	No. in group	Source of plasma for hemopexin	Mean weight (kg.)	y-axis intercept: fraction of injected dose		Half clearance time (hours)		Rate constant		Catabolic rate K_1	Extra vascular hemopexin $\bar{Y}/\bar{X}$
				C_1	C_2	<i>A</i>	<i>B</i>	b_1	b_2		
1	5	Normal serum	2.99 (0.48)	0.453 (0.058)	0.533 (0.046)	20.7 (1.12)	2.40 (0.72)	0.804	6.93	1.56	0.71
2	3	Induced 3-MC	2.33	0.349	0.644	22.5	2.23	0.74	7.74	1.79	1.06
3	4	Induced lead acetate	2.39 (0.63)	0.429 (0.068)	0.569 (0.069)	18.8 (0.24)	3.31 (1.65)	0.884	5.02	1.68	0.60
4	6	Induced 3-MC	2.32	0.348 (0.055)	0.620 (0.050)	28.8 (2.09)	3.94 (0.55)	0.58	4.5	1.25	0.83

Materials

New Zealand white rabbits (1.8 to 3.2 kilograms) were used in all experiments and were maintained on a standard diet.

Preparation of hemopexin. Rabbit hemopexin was purified from pooled serum of normal rabbits or from rabbits in which raised serum hemopexin levels and been induced with lead acetate⁹ or with 3-methylcholanthrene (3-MC).¹⁰ Hemopexin was purified from serum by the perchloric acid method¹¹ which was then followed by the purification technique of Muller-Eberhard and English.⁶ Each batch of purified hemopexin was labeled with ¹²⁵Iodine monochloride (New England Nuclear Corp., Boston, Mass.) according to the method of McConeahy and Dixon⁴ and through the courtesy of Dr. F. J. Dixon, Jr. By this method, 0.1 to 0.2 atoms of iodine were bound to each molecule of hemopexin. Specific activity of the labeled hemopexin samples was determined and found to vary between 1.59×10^7 and 1×10^8 d.p.m. per milligram of hemopexin.

Labeled, purified hemopexin was shown to bind heme in normal equimolar proportions. Purity of hemopexin and the presence of hemopexin aggregates was assessed by electrophoresis in polyacrylamide gel.¹ Serum hemopexin concentration was determined by quantitative radial immunodiffusion.² Since purified hemopexin in solution tends to undergo aggregation, after iodination, the labeled protein was added to a purified homologous transferrin solution of 0.5 to 2.0 mg. per milliliter of protein concentration. In this form, hemopexin underwent negligible aggregation.

Methods

The determination of hemopexin plasma half-clearance time. Eighteen rabbits comprised the 4 groups studied, each group receiving one batch of hemopexin (see Table I). The experiments were carried out as follows: Rabbits were housed in metabolic cages and weighed each day; 4 days prior to the experiment, iodine was added to the drinking water at a concentration of approximately 0.05 per cent and maintained for the duration of the experiment.

¹²⁵I-hemopexin was diluted with isotonic saline to give a protein concentration of 50 μ g per milliliter; a weighed sample and standard were then prepared. The marginal vein of the

left ear was cannulated and a small blood sample taken for estimation of hematocrit. At zero time, 100 μ g of ^{125}I -hemopexin were injected and washed through the cannula with 2 ml. of isotonic saline. After 3 minutes, a 0.5 ml. blood sample was taken from the opposite ear and further venous samples were taken at intervals during an 80 hour period, by which time plasma ^{125}I activity had dropped to less than 5 per cent of the injected dose. Radioactivity and hematocrit were determined on each blood sample and radioactivity was expressed as count. minute⁻¹. ml.⁻¹ plasma. Appropriate, weighed standards of ^{125}I -hemopexin were also counted. All ^{125}I activity was determined in a well-type scintillation counter and under the conditions used, the counting error was within 1 per cent of the total counts measured. Plasma volume was calculated on the basis of body weight.

In Group 4 experiments, besides the estimation of radioactivity and hematocrit, the percentage of nonprotein-bound ^{125}I in all plasma samples was estimated. For this purpose ion-exchange resin was used (Iobeads: inorganic iodine resin for protein-bound-iodine estimation, Technicon Chemical Company Inc., Tarrytown, N. Y.) because of the high solubility of hemopexin in acid solution prevented its complete precipitation from plasma with either trichloroacetic acid or phosphotungstic acid.

For the analysis and graphic resolution of experimental data and calculation of metabolic rate, the procedure and nomenclature adopted by Matthews³ was used throughout. Plasma ^{125}I levels were calculated, then expressed as a fraction of the injected dose and plotted against time on semilogarithm paper, permitting graphic resolution of a plasma curve with two components *A* and *B*. From the graph and by computer analysis by the method of least squares, the slope, y-axis intercept, and half-clearance time of each component was determined.

Because plasma hemopexin was rapidly catabolized, experiments were concluded early on the fourth day, and it proved difficult to make accurate 24 hour collections of rabbit urine and feces for determination of excreted ^{125}I activity. Instead, a total collection was made over a 5-day period commencing at the time of hemopexin injection. The total excreted ^{125}I activity was estimated and expressed as a percentage of the injected dose.

Results

Electrophoresis, spectrophotometric assay, and heme-binding estimations indicated that the labelled hemopexin was pure, heme-free, and biochemically active protein in monomeric form. As explained, the monomeric state was well preserved when hemopexin was stored in homologous, purified transferrin solution.

During the experiments recorded here, all the rabbits remained healthy and increased in weight. Two rabbits were excluded from the series because they developed respiratory infections which affected the metabolism of hemopexin.

In 6 experiments of Group 4, non-protein-bound ^{125}I in plasma reached its maximum level approximately 24 hours after injection, when a mean value of 2.4 per cent free ^{125}I (S.D. 0.42) was determined. This showed that in vivo loss of hemopexin label occurred only as a result of normal hemopexin catabolism.

In all experiments, recovery of ^{125}I in the pooled urine and feces from a 5-day collection was estimated and found to be between 87 and 92 per cent of the injected dose.

Hemopexin plasma clearance curves were drawn, the example shown in Fig. 1 being characteristic of the series. The curve was resolved into components *A* and *B*, each described by an exponential function and having y-axis intercepts, C_1 and C_2 and slopes b_1 and b_2 , respectively. The half-clearance time ($T_{1/2}$) of each component was determined. After approximately 20 hours, the plasma clearance curve became linear (Component *A*) and remained linear until ^{125}I -

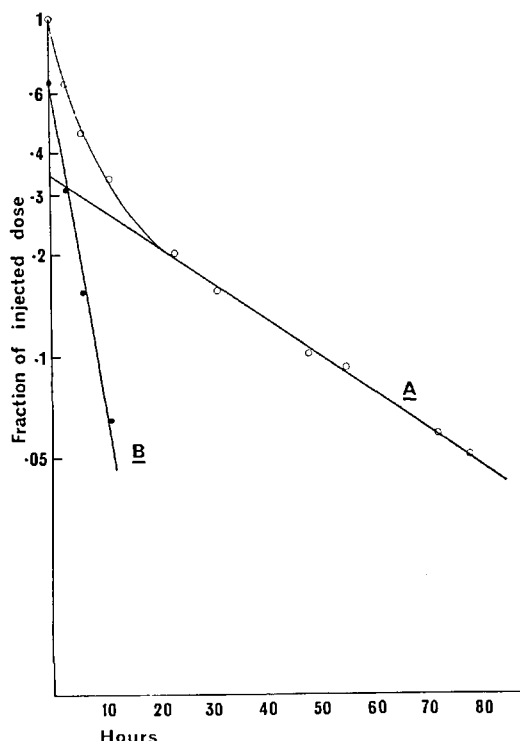


Fig. 1. The pattern of plasma clearance of homologous ^{125}I -labelled hemopexin in the rabbit is shown. The curve is described by the double exponential equation $y = C_1e^{-b_1t} + C_2e^{-b_2t}$ C_1 and C_2 being the y-axis intercepts, and b_1 , b_2 the rate constants of components A and B, respectively, of the plasma clearance curve. The $T_{1/2}$ of components A and B was 27.5 and 3.1 hours, respectively.

hemopexin activity in plasma was less than 5 per cent of the injected dose, at which point experiments were terminated. Points lying along component A in the 18 examples studied were used to calculate the correlation coefficient for each line. Individual results for the correlation coefficient were within the range 0.965 to 0.996 indicating that, in each case, the points were a close fit to a straight line described by the exponential term $C_1e^{-b_1t}$.

In Table I are shown: (1) The mean values from each Group for C_1 and C_2 , the $T_{1/2}$ (hours), and rate constants b_1 and b_2 of components A and B, respectively. Standard deviations, where calculated, are shown in brackets. (2) Catabolic rate K_1 calculated from:

$$K_1 = \frac{1}{C_1/b_1 + C_2/b_2}$$

expressed as a fraction of the intravascular hemopexin pool per day. (3) Extravascular hemopexin ($\bar{Y}$) derived from:

$$\bar{Y} = \frac{C_1C_2(b_2 - b_1)^2}{(C_1b_2 + C_2b_1)^2}$$

expressed as a fraction of the intravascular hemopexin pool ($\bar{X}$).

Plasma hemopexin $T_{1/2}$ for the whole series was approximately 1 day: expressed as a fraction of reported values for $T_{1/2}$ rabbit albumin and IgG¹² and rabbit fibrinogen,⁵ values of 0.1 to 0.2 and 0.3 to 0.5, respectively, are obtained. Mean $T_{1/2}$ values for component *B* were between 2.23 and 3.94 hours. Equilibrium of injected hemopexin between the intravascular and extravascular compartments was complete within approximately 20 hours, that is, approaching the plasma $T_{1/2}$ of hemopexin.

Y-axis intercept values C_1 and C_2 were 0.35 to 0.45 and 0.53 to 0.64, respectively, and compared favorably with the results of similar investigations on homologous albumin and IgG in rabbits.³ These results confirmed that the injected hemopexin contained only insignificant amounts of denatured or aggregated protein.

Catabolic rate K_1 , expressed as a fraction of the intravascular hemopexin pool catabolized per day, was determined from compartmental analysis to be 1.57. Extravascular hemopexin was calculated to be 0.75 times the intravascular hemopexin pool. Mean plasma hemopexin concentration in 12 rabbits was 44.2 mg. per 100 ml. (S.D., 15.4) compared with other published values of 60 mg. per 100 ml. with a range of 40 to 82 mg. per 100 ml.⁷ Assuming a rabbit weighing 2.5 kilograms, to have a blood volume of 175 ml. (7 per cent of the body weight) and a hematocrit of 40, total intravascular hemopexin would be 44.4 mg.

The following values for rabbit hemopexin can be calculated: (1) Catabolic rate (fraction of total hemopexin pool per day) equals 0.84; (2) extravascular hemopexin ($\bar{Y}$) equals 35 mg.; (3) absolute hemopexin catabolism per day equals 66 mg.; and (4) total hemopexin-heme binding capacity equals 0.44 mg.

To calculate total heme binding by hemopexin, the molecular weight of heme was taken to be 617 and of hemopexin 63,000⁷: equimolar binding of heme with hemopexin was assumed.

Discussion

Data from the clearance of ¹²⁵I-hemopexin from plasma was shown by computer analysis to fit a pattern described by the double exponential equation

$$y = C_1e^{-b_1t} + C_2e^{-b_2t}$$

where C_1 , C_2 and b_1 , b_2 are the y-axis intercepts and rate constants of the plasma clearance components *A* and *B*, respectively. After intercompartmental equilibrium was complete, hemopexin clearance was consistent with fractional catabolism from a homogenous pool. In the steady state, the pattern of clearance was also consistent with hemopexin, having a reversible equilibrium between plasma and the extravascular compartment with irreversible exit from one of these compartments. Since it was probable that hemopexin was catabolized in a heme-free state from a homogenous plasma pool, the same form of compartmental analysis used in metabolic experiments with rabbit albumin and IgG³ was applied to the analysis of hemopexin: it assumed that hemopexin catabolism occurred within the intravascular compartment.

Clearance data also indicated that hemopexin used in these experiments was pure, undenatured, and not aggregated: values for C_1 and C_2 and the rate of decrease in plasma ¹²⁵I activity during the early phase of intercompartmental

equilibration were within acceptable limits; values for the level of nonprotein-bound ^{125}I in plasma could be ascribed solely to normal hemopexin catabolism. These values are affected if the injected sample contains significant amounts of nonviable protein.

Thus it was significant that the hemopexin plasma $\text{T}_{1/2}$ of Groups 1 to 3 should differ from that of Group 4 ($t = 7.24$; $N = 16$; $P 0.001$), since a discrepancy of this type might suggest a batch defect either in the hemopexin or in the rabbits used in the experiments. Obvious factors were excluded: the rabbits were of similar strain, weight, and source of supply; experimental procedure was carefully controlled, and preparative methods for hemopexin were common to all samples. The source of hemopexin, from induced rabbit serum, was also excluded as a causal factor since Groups 2 and 4 both received hemopexin obtained from 3-MC-induced rabbit serum. From a biologic viewpoint the difference in plasma hemopexin $\text{T}_{1/2}$ between Groups 1 to 3 and 4 was approximately 30 per cent and could be less significant than statistical analysis indicated.

The desirability of using perchloric acid in the preliminary stages of hemopexin isolation from serum is questionable when the pure hemopexin is required for metabolic experiments. Hemopexin purified in this way has been successfully used for the determination of its physicochemical characteristics,⁶ but this does not exclude the possibility that hemopexin prepared in this way is altered enough to affect its metabolism when compared with native hemopexin. The hemopexin used in these experiments was shown to be biochemically active and it was assumed that, *in vivo*, it formed an homogenous pool with the endogenous hemopexin. A comparison with hemopexin prepared by less stringent methods is desirable, but at present not possible: The perchloric acid stage in hemopexin preparation inhibits protein aggregation once the hemopexin is purified, probably by oxidation of one or more labile sulfhydryl groups on the molecule. While the extent of this oxidation may influence hemopexin metabolism, the aggregation of protein which occurs in other hemopexin preparations presents an even greater hazard and such material is entirely unsuitable for measurement of fractional catabolic rate.

R. S. Lane thanks Dr. A. S. McFarlane for his advice.

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Plasma Clearance of ^{125}I -labelled Haemopexin in Normal and Haem-loaded Rabbits

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SUMMARY. Plasma clearance of ^{125}I -labelled rabbit haemopexin has been studied in six normal control rabbits and in a test group of rabbits haem-loaded with 25 mg of haem.

Following intravenous injection, equilibration of ^{125}I -haemopexin between intravascular and extravascular compartments appeared to be complete within 2.4 hr and the decline in plasma activity then became constant in rate. The plasma activity curve was resolved into two exponential functions of time and from the data, fractional catabolic rate, intravascular:extravascular distribution of haemopexin and total haem binding capacity were calculated.

Control group. Mean plasma half-clearance time of haemopexin was 20.3 hr; fractional catabolic rate was 1.8 (SD 0.19) times the intravascular haemopexin pool per day. Extravascular haemopexin was 0.6 (SD 0.11) times the mass of haemopexin in the intravascular pool.

Test group. 90–95% of injected ^{125}I -activity was eliminated from the plasma with a mean half-clearance time of 1.57 hr and was associated with a significant urinary excretion of labelled iodine. Fractional catabolic rate was 5.84 (SD 0.3) times the intravascular haemopexin pool per day and extravascular haemopexin was 3.38 (SD 1.03) times the mass of haemopexin in the intravascular pool.

Haemopexin concentration in normal rabbit plasma was 31–52 mg/100 ml; intravascular plus extravascular haemopexin was 54.5 mg (mean value) and total haem binding capacity of haemopexin was 0.53 mg (mean value).

Intravascular haem rapidly complexed with haemopexin and the data suggested that this complex was promptly eliminated from the plasma and the haemopexin catabolized.

The glycoprotein haemopexin binds haem (Aber & Rowe, 1966) and is involved in its elimination from the plasma (Sears, 1968, 1970; Muller-Eberhard *et al*, 1969). The plasma concentration of haemopexin decreases after administration of haem to humans and rabbits

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(Sears, 1968, 1969) and there is evidence that, after binding haem, haemopexin is eliminated from the plasma as the haem-haemopexin complex (Muller-Eberhard *et al.*, 1970). Sears (1969) observed that depletion of plasma haemopexin levels after haematin injections could result either from inhibition of synthesis, or, more probably, by enhancement of clearance of haemopexin from the plasma.

Plasma clearance of labelled haemopexin has been studied in normal rabbits (Lane *et al.*, 1972) and the effect of intravascular haem on the catabolism of haemopexin has now been investigated. These results are presented in this communication.

MATERIALS AND METHODS

The preparation of materials and the methods used for the study of plasma haemopexin clearance in normal rabbits have been fully described elsewhere (Lane *et al.*, 1972) and the present experiments were carried out in a similar manner. New Zealand white rabbits weighing 1.9–2.7 kg were used.

Experiments were performed with a single batch of purified rabbit haemopexin which was labelled with ^{125}I according to the method described by McConahey & Dixon (1966) using I in 0.125 N reductant-free sodium hydroxide. Specific activity of the labelled haemopexin was 6×10^8 dpm per mg haemopexin. Crystalline haem was purchased from Koch-Light Laboratories Ltd: for use, haem samples were dissolved in 0.15 M sodium carbonate solution. Plasma samples and appropriate ^{125}I -haemopexin standards were counted on a Packard Autogamma Spectrometer; counting error was within 1% of the total counts measured. Blood volume in the rabbits was taken to be 7% of body weight (Wiseman & Irving, 1963).

Starch gel electrophoresis. Plasma samples from rabbits receiving haem and haemopexin were studied by starch gel electrophoresis. The bridge buffer was prepared from 0.3 M boric acid and 0.06 M sodium hydroxide, pH 8.2; for the gel buffer, 1 vol of bridge buffer was diluted with 9 vol of distilled water. Samples of plasma (50 μl) were applied to the gel and were electrophoresed vertically at 2.5 V/cm for 16 hr at 4°C. The completed gel was sliced and stained to show haem with a mixture of equal volumes of 1% benzidine in 90% acetic acid and 3% hydrogen peroxide. After staining, the haemopexin bands were cut from the gel and their ^{125}I -activity determined.

Selected samples of rabbit plasma were used for the estimation of haemopexin concentration by quantitative radial immunodiffusion (Mancini *et al.*, 1965) according to the modification of Smibert *et al.* (1972).

The study of haemopexin clearance from the plasma of normal and haem-loaded rabbits was carried out in two groups of animals. Controls were six normal rabbits which each received an intravenous injection of 100 μg ^{125}I -haemopexin in isotonic saline. After completing this experiment, these rabbits were rested for 3 weeks and then used again in the test group, to which an extra rabbit was added making a total of seven. Test rabbits had an intravenous catheter passed into the lateral vein of the left ear: through this catheter, 25 mg haem in 2 ml of sodium carbonate solution was slowly injected and washed in with 2 ml of isotonic saline; this was followed 5 min later by 100 μg ^{125}I -haemopexin, also washed in with 2 ml of isotonic saline.

After the injection of ^{125}I -haemopexin at zero time, 1 ml blood samples were taken at intervals during a period of 96 hr. Aliquots of plasma were taken for determination of radio-activity, plasma haemopexin concentration, starch gel electrophoresis and non-protein-bound ^{125}I activity. Urine and faeces were collected for a total of 5 days and the ^{125}I content determined and expressed as a percentage of the injected dose. In the extra rabbit added to the haem-loaded test animals, urine samples were obtained after inserting a self-retaining catheter into the bladder. This procedure, carried out 3 hr before the injection of haem, permitted more exact information to be obtained on the urinary excretion of ^{125}I .

RESULTS

The labelled, purified haemopexin was shown to be haem-free and biochemically active protein in monomeric form by carrying out polyacrylamide gel electrophoresis, spectrophotometric analysis and haem-binding estimations as described by Hrkal & Muller-Eberhard (1971). In addition, during the metabolic experiments, the rabbits remained healthy and all increased in weight.

Control Rabbits

The methods and terminology of Matthews (1957) were used for the analysis of plasma clearance data: by computation with the curve stripping procedure the plasma activity curve was shown to be described by an exponential equation of the form $y = C_1 e^{-b_1 t} + C_2 e^{-b_2 t}$ where coefficients C_1 , C_2 are the y -axis intercepts and b_1 , b_2 the respective rate constants. The plasma clearance curve from a Control rabbit is seen in Fig 1(a): two exponentials are shown with respective rate constants b_1 and b_2 ; equilibration of ^{125}I -haemopexin between intravascular and extravascular compartments was apparently complete 2.4 hr after zero time and the rate of plasma haemopexin clearance then became constant. Computed values for the correlation coefficient of points lying along exponential $C_1 e^{-b_1 t}$ in the six controls varied between 0.982 and 0.994 indicating that, in each case, these points were a close fit to a straight line. For evaluation of the plasma activity curve, a three compartment form of mammillary model was used as shown in Fig 2. When calculating catabolic rate K_{12} it was assumed that labelled haemopexin was in a state of reversible equilibrium between intravascular and extravascular compartments with irreversible exit from the former.

Catabolic rate was expressed as the fraction of the intravascular pool catabolized per day:

$$K_{12} = \frac{1}{C_1/b_1 + C_2/b_2}$$

Extravascular haemopexin $\bar{Y}$ expressed as a fraction of the intravascular haemopexin pool $\bar{X}$ was calculated from the following equation:

$$\bar{Y} = \frac{K_{13}}{K_{31}} \times \bar{X} = \frac{C_1 C_2 (b_2 - b_1)^2}{(C_1 b_2 + C_2 b_1)^2} \bar{X}$$

$\bar{X}$ was determined for a rabbit weighing 2 kg with an assumed plasma volume of 4.2% of body weight using the mean value for plasma haemopexin concentration obtained from 12 estimations.

In control rabbits, plasma half-clearance time of haemopexin was 20.3 hr (SD 0.92).

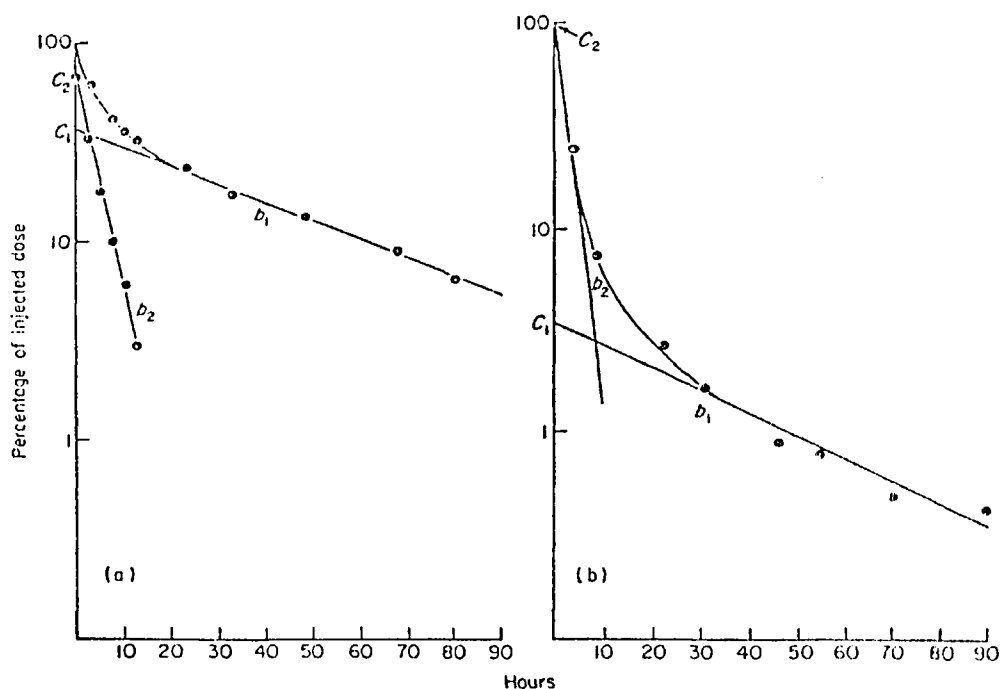


FIG 1. (a) Plasma clearance of ^{125}I -labelled rabbit haemopexin in a normal control rabbit from Group 1. The curve is described by the exponential equation $y = C_1 e^{-b_1 t} + C_2 e^{-b_2 t}$ where C_1 , C_2 are the y-axis intercepts and b_1 , b_2 are the respective rate constants. Mean values for $t_{1/2}$ of exponentials $C_1 e^{-b_1 t}$ were 20.3 and 3.37 hr respectively. (b) Plasma clearance curve of ^{125}I -haemopexin in a haem-loaded rabbit from Group 2. In the example shown, 87% of the injected activity was cleared from the plasma with a half-clearance time of 1.95 hr. The initial rapid plasma clearance of ^{125}I is ascribed to the rapid elimination of haem- ^{125}I -haemopexin from the plasma.

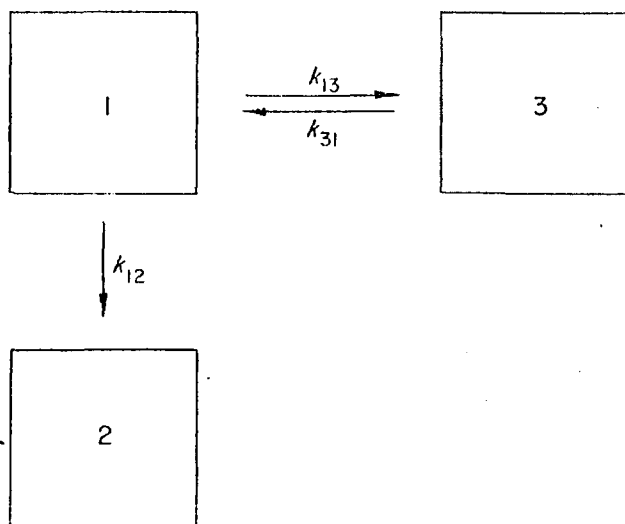


FIG 2. A three-compartment form of mammillary model used for the evaluation of plasma ^{125}I -haemopexin clearance (after Matthews, 1957). Compartment 1 = intravascular protein; compartment 2 = urine + faeces; compartment 3 = extravascular protein. The label was injected into compartment 1 equilibrated with compartment 3 and was assumed to have irreversible exit from compartment 1 to urine and faeces in compartment 2.



FIG 3. Starch gel electrophoresis of plasma samples from a rabbit in Group 2: this rabbit was injected with 25 mg of haem and then with 100 μ g ^{125} I-haemopexin. The gel is stained with a solution of benzidine in hydrogen peroxide to show haem. Haem bound to haemopexin and to albumin are marked while the free plasma haemoglobin is marked, but can be disregarded. From left to right, plasma samples taken at 3 min, 30 min, 3, 8, 14, 24, 48 and 72 hr after the injection of haem and labelled haemopexin. The volume of plasma in each insert was 50 μ l.

Coefficient C_1 expressed as a fraction of the injected ^{125}I -activity was 0.31 (SD 0.03); coefficient $C_2 = 1 - C_1$. Rate constants b_1 and b_2 were 0.81 (SD 0.09) and 4.94 (SD 0.25) respectively. Catabolic rate K_{12} was 1.8 (SD 0.19) times the intravascular haemopexin pool per day; plasma haemopexin concentration was 31–52 mg/100 ml in 12 estimations and extravascular haemopexin $\bar{Y}$ was 0.6 (SD 0.11) times the mass of intravascular haemopexin X .

^{125}I -activity in the pooled urine and faeces was 88% (SD 2.3) of the injected dose. In the plasma tested, plasma activity in the form of non-protein-bound ^{125}I did not exceed 4.3%.

Haem-loaded Rabbits

Fig 1(b) shows the plasma haemopexin clearance curve obtained from a rabbit intravenously loaded with 25 mg haem. In this instance, 87% of the injected ^{125}I -activity was cleared from the plasma in 5 hr. The plasma activity curve in haem-loaded rabbits also contained two exponentials of which $C_1 e^{-b_1 t}$ merely represented the plasma clearance of a residual 5% of the injected activity.

90–95% of injected activity was eliminated from the plasma with a mean half-clearance time of 1.57 hr. Coefficient C_1 was 0.071 (SD 0.037) % of the injected activity; rate constants b_1 and b_2 were 0.695 (SD 0.126) and 10.6 (SD 1.73) respectively. Catabolic rate K_{12} was 5.84 (SD 0.3) times the intravascular haemopexin pool per day and extravascular haemopexin $\bar{Y}$ was 3.38 (SD 1.03) times the mass of intravascular haemopexin.

Pooled urine and faeces contained 84–92% of the injected activity: in the catheterized rabbit, 85% of the injected ^{125}I -haemopexin was eliminated from the plasma with a half-clearance time of 2.1 hr and after 8 hr 42% of the injected activity had been recovered in the urine as non-protein-bound ^{125}I . Plasma non-protein-bound ^{125}I did not exceed 4.9% of the plasma activity during the initial 24 hr of the experiment.

Starch gel electrophoresis of plasma samples obtained from a haem-loaded rabbit is shown in Fig 3: haem-haemopexin and haem-albumin bands are marked and the free haemoglobin in the plasma can be disregarded. The 3 min plasma sample shows that the injected haem was rapidly bound to haemopexin and albumin. Haem bound to haemopexin quickly decreased in concentration to reach a minimum at 3 hr after zero time; in the presence of haem-albumin, this loss of haem-staining reflected a fall in haemopexin concentration. After 3 hr, haem-staining increased in intensity in the region of the haemopexin band and reached a peak approximately 24 hr after zero time.

Immunoquantitation data on haemopexin concentration in plasma samples from three haem-loaded rabbits are shown in Fig 4. The results are complimentary to the electrophoretic pattern described above. Following the injection of haem, there was a rapid fall of 32–48% in the resting plasma haemopexin concentration; minimum levels of plasma haemopexin were reached after 3 hr, but after 8–12 hr haemopexin concentration increased and returned to resting levels 24–48 hr after zero time.

Combining the results of starch gel electrophoresis and immunoquantitation, it can be seen that the period of depressed plasma haemopexin levels coincided with the presence of haem-albumin in the plasma. While total plasma haemopexin concentration fell by up to 48% of resting levels within 8–12 hr after haem injection, ^{125}I -activity in haemopexin bands excised from the starch gel, decreased by more than 90% indicating that the increased levels of plasma haemopexin that followed comprised only endogenous haemopexin.

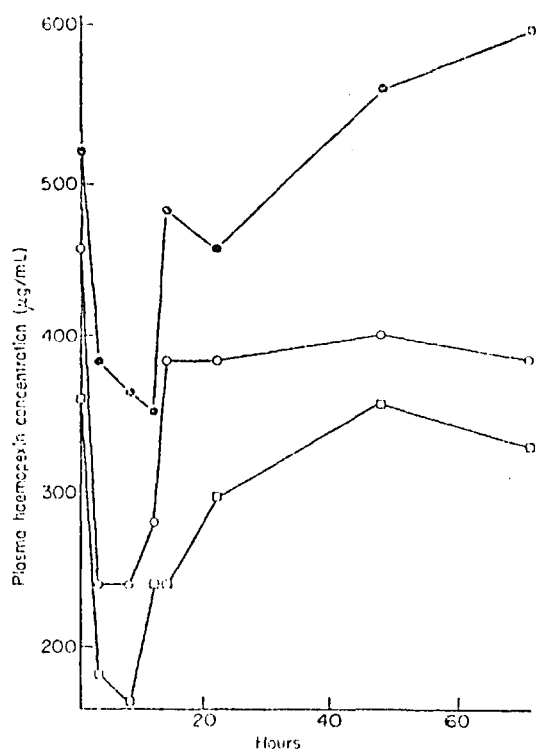


FIG. 4. Plasma concentration of haemopexin in three haem-loaded rabbits from Group 2: these results are typical of results from the entire group. Following the injection of haem, there was a rapid fall in haemopexin concentration which remained low during the period in which haem-albumin was demonstrable in the plasma. Once haem was eliminated from the plasma, haemopexin levels increased, approaching resting values after 24 hr.

DISCUSSION

The results obtained from the six control rabbits in the present experiments were in close agreement with those of an earlier study in 18 normal rabbits (Lane *et al.*, 1972).

Many variables were removed by using the same six rabbits and the same batch of labelled haemopexin in both control and haem-loaded groups. The approximate haem-binding capacity of endogenous haemopexin is 0.5 mg; therefore, 25 mg of haem injected into test rabbits saturated the endogenous and labelled haemopexin and the excess haem was rapidly bound by albumin. It was assumed that the *in vivo* haem-binding affinity of endogenous and labelled haemopexin were the same.

Combining the experimental data from haem-loaded rabbits it is clear that, following the intravenous injections of haem and labelled haemopexin, haem rapidly complexed with endogenous and labelled haemopexin and with albumin. A marked decrease in both plasma activity and plasma haemopexin concentration was observed during the initial 3 hr but, after this time, plasma levels of haemopexin started to increase while the decline in plasma

activity continued. Within the initial 8-10 hr after haem injection, a significant fraction of injected activity was excreted in the urine as non-precipitable labelled iodine, also, approximately 90% of the injected haem was eliminated from the plasma.

These results were best explained by the prompt removal of haem-haemopexin complex from the plasma, followed by rapid catabolism of the haemopexin. *In vivo*, haemopexin binds haem from haem-albumin (Muller-Eberhard *et al*, 1969) and, after injection, haem-haemopexin complex can be localized within the hepatic parenchymal cells (Muller-Eberhard *et al*, 1970). It is probable that albumin acts as a plasma storage reservoir for haem, when haem levels exceed the binding capacity of haemopexin. Rapid catabolism of haem-haemopexin would mean that the total haemopexin pool could remove approximately 0.5 mg haem: for 25 mg of haem to be eliminated from the plasma in the form of haem-haemopexin complex, fractional catabolic rate of haemopexin would have to increase considerably with the corresponding demand on synthesis rate. In fact, values for K_{12} in haem-loaded rabbits were approximately 3 times greater than in normal rabbits but this degree of increased haemopexin turnover would be insufficient to account for the entire elimination of 25 mg haem in 12 hr.

In haem-loaded rabbits, the initial fall in plasma haemopexin concentration was quickly restored. In addition, calculation showed that a much larger fraction of the total haemopexin pool was located in the extravascular compartment in haem-loaded rabbits. These results were taken as indirect evidence of greatly increased rates of haemopexin synthesis. However, in the presence of plasma haem-albumin, much of the newly synthesized haemopexin would bind haem from the haem-albumin and then be catabolized. In haem-loaded animals, therefore, plasma haemopexin concentration gives a poor indication of the extent of increase in synthesis rate and the size of the total haemopexin pool.

It is interesting to consider that, if hepatocytes are the centre both of haemopexin synthesis and catabolism, rapid transfer of haem from haem-albumin to newly synthesized haemopexin could occur within the liver near the surface of the cells. The haem-haemopexin complex so formed would be very rapidly eliminated, thus a large part of the haem-transfer process between haem-proteins would be beyond the reach of the methods of investigation currently in use. Haem transfer to hepatocytes may be a specific function of haemopexin and it seems possible that haemopexin turnover can increase sufficiently for the elimination of a significant haem-load. However, in subjects made haemopexin deficient by haem injections or chronic intravascular haemolysis, only haem-albumin is present in the plasma yet haem continues to be eliminated although at a slower rate than is observed when haemopexin is present (Sears, 1970). In haemopexin deficient subjects, it is possible that the apparent haem-clearance from haem-albumin is limited by the rate of haemopexin synthesis; in this situation, haem-clearance would reflect the rate of haemopexin production.

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