

STUDIES ON IRON METABOLISM AND HAEMOGLOBIN

BIOCHEMISTRY IN THE DOMESTIC FOWL.

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**SUMMARY**

The cation exchange resin carboxymethyl-cellulose was routinely used for the purification and separation of the two hen haemoglobins I and II.

Spectral evidence is presented which indicates that the haemoglobins have the same prosthetic group namely protohaem IX  $\alpha$  .

By using mixtures of the iron-59 labelled and unlabelled haemoglobins I and II it has been shown that haem exchange only takes place to a small extent during the separation and isolation procedures employed.

In vivo studies have been made on the incorporation of iron-59 into the haemoglobins. No differences between the specific activities of I and II were found at any stage of the circulating avian red blood cell life span.

The incorporation of iron-59 and (2-carbon-14) glycine into the haemins has been studied during in vitro incubations of immature red cells. The specific activities of the haemins from the haemoglobins I and II were the same as each other for both iron-59 and carbon-14 at every stage of incubation from 15 minutes to 8 hours after the addition of the label. However, the haemin from the haemoglobin fraction which remained bound to the erythrocyte lysate sediment had in many experiments higher specific activities than the haemins from the haemoglobins I and II, particularly at the earlier stages of incubation.

Puromycin was found to inhibit the in vitro incorporation of iron-59 and (2-carbon-14) glycine into the haemins by 20-40% during 90 minute incubations.

The results of the studies on the incorporation of radioactive isotopes

into the haemins from the haemoglobins I and II are consistent either with the idea that haem is being drawn for haemoglobin formation from an intracellular haem pool at rates which are proportional to the relative amounts of the haemoglobins I and II, or with the idea that intracellular exchange of haem takes place between the haemoglobins I and II. Possible explanations of the higher incorporation of iron-59 and carbon-14 into the haemin from the membrane-bound haemoglobin are discussed.

Non-haem iron protein fractions have been found in fowl haemolysates and these can be separated from the haemoglobins I and II on carboxymethylcellulose and sephadex G-200 chromatography. These fractions became much more highly labelled than the haemoglobins I and II in studies on the incorporation of iron-59 both in vivo at early stages of the circulating erythrocyte life span and in vitro during incubations of immature red cells.

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CHAPTER 1 : General Introduction

### Multiple Haemoglobins

It has been known for a number of years now that in many animal species there exists in the erythrocyte more than one kind of haemoglobin capable of carrying oxygen. These heterogeneities which have been noted in species ranging from humans to cyclostomes (Gratzer and Allison (1960)) can be divided into several different classes, not necessarily in themselves mutually exclusive.

(a) Invariable heterogeneity : This occurs when all adult members of a given species exhibit two or more distinct haemoglobin components, the number of components being the same for all normal members of the species. An example of this is human haemoglobin with its well-defined heterogeneity of haemoglobin A<sub>1</sub>, consisting of about 95% of the total red blood cell haemoglobin and haemoglobin A<sub>2</sub>, consisting of about 1-3% of the total red blood cell haemoglobin (Marengo-Rowe (1965); Neerhout, Kimmel Wilson and Lahey (1966); Huisman and Dozy (1961)). The differences lie in their protein moities, the 4 polypeptide chains of haemoglobin A being  $\alpha_2 \beta_2$  and those of haemoglobin A<sub>2</sub> being  $\alpha_2 \delta_2$  with the  $\delta$ -chains having several amino acids different from the  $\beta$ -chains. Another animal with this type of heterogeneity is the horse (Bangham and Lehmann (1958)) which has two haemoglobins.

(b) Variable heterogeneity : This is found when the incidence of the haemoglobin in a given species varies according to the breed or strain of the animal concerned. An example of this is the sheep haemoglobins.

There are apparently three of these, two of them, haemoglobin A and haemoglobin B, being much more common than the third (called by various letters in the literature). Some breeds of sheep have haemoglobin A only (phenotype AA), some have haemoglobin B only (phenotype BB), and others again have both (phenotype AB), (Huisman, Van der Helm, Visser and Van Vliet (1959); Braend, Efremov and Helle (1964)). The third haemoglobin was first noted in sheep which were either naturally anaemic (Braend, Efremov and Helle (1964)), or were made so experimentally by massive bleeding (Van Vliet and Huisman (1964)). Braend and Efremov (1965) have found sheep of type ABN and AN where N is the third haemoglobin, and with one breed (Norwegian), they even obtained a majority of sheep with haemoglobin N and haemoglobin A. Amino acid analyses and electrophoretic data have supported the hypothesis that one pair of the polypeptide chains ( $\alpha$ -chains) of each of the three haemoglobins is identical while multiple structural differences exist among the other three pairs of chains (Huisman, Reynolds, Dozy and Wilson (1965)). Another example of this sort of intra-species genetic variation of haemoglobin type is cattle where five haemoglobins found over a wide range of breeds have been reported, all probably with the same  $\alpha$ -chains (Bangham (1957); Efremov and Braend (1965(a); 1965(b)).

(c) Foetal or Embryonic heterogeneity : This as the name implies is found in developing foetuses or embryos and in young animals. A foetal or embryonic haemoglobin coexists along with the adult haemoglobin, this foetal or embryonic component gradually disappearing from the blood of

the young animal as it matures. Apparently the foetuses of all mammals produce a foetal haemoglobin and in general the situation is similar to that of humans where the polypeptide structure of the foetal haemoglobin (haemoglobin F) chains is  $\alpha_2 \gamma_2$ , compared with the  $\alpha_2 \beta_2$  of haemoglobin A and  $\alpha_2 \delta_2$  of haemoglobin A<sub>2</sub>, the  $\gamma$ -chains being substantially different (17 differences) from the  $\beta$ -chains of haemoglobin A but the  $\alpha$ -chains being the same (Shroeder (1963)). Thus Muller (1961) has examined the foetal and adult haemoglobins of a number of animals including cattle, sheep, goats, rabbits and hares and has suggested from chromatographic and electrophoretic evidence that for any one of the species he uses, the adult haemoglobin(s) and the foetal haemoglobin all have  $\alpha$ -chains in common, the differences in the haemoglobins being due to the structure changes in the non- $\alpha$  chains.

(d) Abnormal heterogeneity :- This kind of heterogeneity, mainly looked at in humans, occurs when a haemoglobin is present which is not normally found, and which can sometimes give rise to a specific disease of the blood. This is a genetically controlled abnormality and very often the abnormal haemoglobin is present alongside the normal component, though this depends on the nature of the genetic defect. There are now scores of these abnormal haemoglobins, known (Schroeder (1963); Beale and Lehmann (1965)). The differences in properties between these abnormal haemoglobins and the normal haemoglobins, which are sometimes very great and can have far-reaching physiological results, seem very often to be due only to the substitution of one amino acid residue in

the normal haemoglobin by another. Since the different polypeptide chains are under separate genetic control, one can get different types and numbers of abnormal haemoglobins, depending on which chain is affected. Thus an individual heterozygous for an  $\alpha$ -chain mutation, will have four haemoglobins present in the blood, haemoglobin A, the abnormal variant of haemoglobin A, haemoglobin A<sub>2</sub> and the abnormal variant of haemoglobin A<sub>2</sub>. This is the case with, for example, the condition of haemoglobin I - trait, where haemoglobins A, I, A<sub>2</sub> and I<sub>2</sub> are found (Boulard, Cosset, Destaing, Duzer, Jonxis, Muller and Portier (1961)). On the other hand, an individual heterozygous for a  $\beta$ -chain mutation will only have three haemoglobins present as only haemoglobin A contains  $\beta$ -chains.

As far as birds are concerned, Manwell, Baker, Roslansky and Foght (1963) have reported what they call a "thalassaemic" pheasant. This was a wild pheasant (*Phasianus colchicus*) which had its major haemoglobin component almost entirely missing.

(e) Heterogeneity due to in vivo alteration of the haemoglobin molecule:-

This arises from the intracellular alteration of already preformed haemoglobin. It is not to be confused with the apparent haemoglobin multiplicity which can be observed due to faulty separation techniques or through the use of old or partially oxidised haemolysates (Huisman, Dozy, Horton and Nechtman (1966); Huisman (1966)). The type of haemoglobin change involved is not clear but again the human haemoglobins serve as a good illustration since more work has been done on humans than on other

animals.

In addition to haemoglobin A, haemoglobin A<sub>2</sub>, and small amounts of haemoglobin F, another haemoglobin fraction can be obtained from human haemolysates (Huisman and Meyering (1960)). This fraction, which is itself rather heterogeneous, is usually called haemoglobin A<sub>1</sub>. Meyering, Israels, Sebens, and Huisman (1960), by studying the in vivo incorporation of iron-59 into this haemoglobin A<sub>1</sub> fraction and into haemoglobin A, concluded that the haemoglobin A<sub>1</sub> component was formed in vivo as a result of changes in the haemoglobin A molecule, the amount of haemoglobin A<sub>1</sub> depending on the age of the circulating red blood cells. Muller (1961) obtained some evidence from the peptide patterns of tryptic digests which suggested that haemoglobin A<sub>1</sub> was glutathione bound haemoglobin A. Indeed he studied a whole series of animals and stated that this glutathione binding of haemoglobin was a fairly general phenomenon. Rosa and Labie (1962), from hybridisation experiments, suggested that haemoglobin A<sub>1</sub> is formed from haemoglobin A due to modification of the  $\beta$ -chain of haemoglobin A, and that this modification was possibly due to the combination of haemoglobin A with glutathione. However, Huisman and Dozy (1962) found that while haemoglobin A-glutathione complexes produced in vitro were very similar in many respects to the haemoglobin A<sub>1</sub> fraction, they were different from it in several properties. They were also unable to repeat consistently the results obtained by Muller (1961) on tryptic digests of haemoglobin A<sub>1</sub>. When they carried out hybridisation experiments, they could not distinguish between haemoglobin A<sub>1</sub> and haemoglobin A, but could show that in the

glutathione-haemoglobin A complex, glutathione was bound to the  $\beta$  - chains of haemoglobin A. Attasi (1964) has also produced evidence against haemoglobin A<sub>1</sub> being glutathione bound haemoglobin A. He found A and A<sub>1</sub> to have an identical amino acid composition and in their met, cyamet and oxy forms to be interconvertible. Thus it seems possible that the difference between haemoglobin A and A<sub>1</sub> is due to some type of change in the inter or intra-molecular arrangement of the haemoglobin molecule. As has been suggested by Huisman and Dozy (1962), observations such as those of Muller (1961) may be due to the oxidation of GSH in haemolysates of red blood cells upon ageing, and the subsequent formation of the haemoglobin A - GSSG complex, which behaves chromatographically and electrophoretically like haemoglobin A<sub>1</sub>.

An apparently similar kind of heterogeneity has now been reported in a number of animals (Huisman, Van de Brande, and Meyering (1960); Muller (1961); Rosa, Schapira and Dreyfus (1961)) and all the evidence suggests that it arises from the alteration of haemoglobin in the cell.

One is tempted at this stage to pose the question, "Why do animals have these several haemoglobins?" As yet, however, not enough is known for one to be able to answer this. Nevertheless, it is of interest to note the observations of Evans, Harris and Warren (1957) and Huisman, Van der Helm, Visser and Van Vliet (1959) on the two sheep haemoglobins A and B. They found that the haemoglobin with the greater affinity for oxygen, haemoglobin A, is more prevalent amongst highland sheep than the one with the lower affinity for oxygen, haemoglobin B,

which in its turn has a higher incidence of occurrence amongst lowland sheep. It has also been suggested by Perutz, Steinrauf, Stockell and Bangham (1959), that the heterogeneity of horse haemoglobin may be necessary because of its low solubility to prevent crystallisation of haemoglobin in the cell, since, by the phase rule, two compounds are more soluble in the presence of each other than either of them is separately.

Heterogeneity of the haemoglobin of the domestic

fowl (Gallus Domesticus)

(a) Adult hen haemoglobin :-

It is not clear at the moment into which of the above categories of haemoglobin heterogeneity hen haemoglobin can be placed. The discrepancies between the literature reports of different groups of workers may however be partly explicable in terms of genetic differences in the hens used as very often the strain or breed of bird is not stated.

The heterogeneity of hen haemoglobin was first observed by Johnson and Dunlap (1955) who obtained two components by paper electrophoresis. Since this time, dozens of workers using a large variety of techniques have separated hen haemoglobin into its fractions. The methods used have included paper electrophoresis (Johnson and Dunlap (1955); Rodnan

and Ebaugh (1956); Sydenstricker V.P., Oliver, Chandler, and Sydenstricker O. (1956)), cellulose acetate electrophoresis (Fraser (1964(a); Washburn (1966)), starch gel electrophoresis (Schall and Turba (1963); Wilt (1962)), alkali denaturation (Ams and Thorell (1960); Ramakrishnan and Barnabas (1962)), amberlite IRC-50 chromatography (Van der Helm and Huisman (1958); Saha (1959)), carboxymethyl-cellulose chromatography (Huisman, Martis and Dozy (1958); Muller (1961); Matsuda and Takei (1963); Allen (1969)), and immunoelectrophoresis (Wilt (1962); D'Amelio and Salvo (1964)). In general either two or three haemoglobin components have been obtained although there are two instances of five being separated (Schall and Turba (1963); Hashimoto and Wilt (1966)) both using starch-gel techniques. Also, if one compares the results of different groups of workers, using the same separation technique, there seems to be no correlation between the breed of hen and the number of haemoglobin fractions. Thus, using starch-gel electrophoresis and White Leghorns, two, three, and five haemoglobin fractions have been obtained (Wilt (1962); Manwell, Baker, Roslansky and Foght (1963); Hashimoto and Wilt (1966)).

Although genetic differences might explain some of these discrepancies, errors in technique cannot always be excluded. Thus, Manwell, Baker, Roslansky and Foght (1963) found that if they used haemoglobin samples which had been kept for several days at 4°C. they could get extra haemoglobin components. Huisman, Dozy, Horton and Nechtman (1966) and Huisman (1966) have observed similar phenomena with human haemoglobin. It is also possible that freezing and thawing of chicken haemoglobin

solutions. results in the formation of extra haemoglobin components (Manwell, Baker, Roslansky and Foght (1963); Sanders (1967)). Using hens, Huisman, Schillhorn Van Veen, Dozy and Nechtman (1964) obtained several minor haemoglobin bands during starch-gel electrophoresis, if the total haemoglobin had first been dialysed against phosphate buffers in the pH range 7.5 - 8.5, and showed that these bands were due to the binding of haemoglobin with varying amounts of phosphate.

There is, however, some indication that the number of components may be under genetic control. Thus Rodnan and Ebaugh (1957) using paper electrophoresis obtained three haemoglobin components from each of six New Hampshire chickens, but four out of five mongrel Leghorns used only gave two components under the same conditions. On the other hand, Manwell, Baker, Roslansky and Foght (1963) with starch-gel electrophoresis could find no consistent variation between the haemoglobins of the breeds they used, namely White Leghorns, New Hampshire and Columbian chickens. More recently, Washburn (1966), using cellulose acetate electrophoresis found that in his pedigreed population of birds, chickens could have either two or three components i.e. one major haemoglobin component and one or two minor ones. From the electrophoretic properties of the minor components from various sets of parents and progeny, he suggested that chickens with two minor haemoglobin components were heterozygous ( $A_1 A_2$ ) for a set of alleles controlling the production of the minor globin components, while those with one minor haemoglobin were homozygous either for one allele ( $A_1 A_1$ ), or for the remaining allele ( $A_2 A_2$ ). However, it is difficult to assess the quality of the work of

Washburn as full experimental details are not given.

In the present work, carboxymethyl-cellulose is used to separate the hen haemoglobins. This technique was first employed by Huisman, Martis, and Dozy (1958) who, using a pH gradient (6.8 - 8.0) obtained two haemoglobins, a minor fast moving component II and a major slow moving component I. The birds used were "stock for slaughter" and the Siamese jungle fowl. In their elution pattern, they also show further non-haemoglobin protein fractions, which are removed from their columns in front of the haemoglobin II. Muller (1961), using Leghorns and the same pH gradient as Huisman, Martis, and Dozy (1958), obtained the pre-haemoglobin protein fractions, haemoglobin II and haemoglobin I. He also found, however, that there was a further haemoglobin fraction in addition to I and II, which moved down his columns at the trailing edge of the major haemoglobin I. This he attributed to the glutathione binding of haemoglobin I. However, no other workers have observed this and in view of the similar lack of confirmation of the observations of Muller on the nature of human haemoglobin A, it seems unlikely that this further fraction is a genuine haemoglobin component. The elution patterns of hen haemolysates on CM-cellulose obtained by Matsuda and Takei (1963) can also be criticised. Using a gradient of increasing ionic strength they obtained three haemoglobin components from hens of an unspecified breed. Their third very minor haemoglobin component was eluted from their columns in front of the minor haemoglobin II. However, the pre-haemoglobin fractions observed by Huisman, Martis and Dozy (1958) and by Muller (1961) do not appear in their elution patterns. It is

possible, therefore, that since Matsuda and Takei started their gradient elution immediately after putting their lysates on the columns, their extra haemoglobin component may be merely a combination of the pre-haemoglobin fractions with some haemoglobin running down the column ahead of haemoglobin II. The work of Allen (1969) who used Light Sussex hens and Thornber hybrid 606 birds, gives more weight to this interpretation. He adsorbed lysates onto the tops of his columns and washed the columns with several bed volumes of firstly 0.01M phosphate pH 6.3 and then 0.01M phosphate pH 6.9 before commencing the gradient elution (0.01M phosphate pH 6.9 - 0.2M phosphate pH 6.9) of Matsuda and Takei (1963). With this procedure two non-haemoglobin protein fractions were removed from the columns before the application of the gradient and the gradient itself gave rise to only the two haemoglobins I and II. There was no sign whatsoever of any third haemoglobin.

Although on CM-cellulose chromatography there has been no unequivocal demonstration of three haemoglobin components, it seems that the adult domestic fowl can have either two or three haemoglobins. Two of these are apparently always present, the major haemoglobin I accounting for 70 - 80% of the total haemoglobin, and the minor haemoglobin II making up 20 - 30% of the total (Huisman and Schillhorn Van Veen (1964); Matsuda and Takei (1963)). The controversial third haemoglobin component when present amounts to less than 5% of the total (Matsuda and Takei (1963)).

(b) Embryonic haemoglobin :-

One aspect of the haemoglobin heterogeneity of the domestic fowl which has not been mentioned so far is the haemoglobin of the developing chick embryo. It seems likely that at early stages of embryo life there is at least one specific embryonic haemoglobin which disappears as the embryo matures (D'Amelio and Salvo (1961); Wilt (1962); Manwell, Baker, Roslansky and Foght (1963)) or shortly after the chicken has hatched (Huisman and Schillhorn Van Veen (1964)). It is also probable that in early embryonic life, the main haemoglobin component present is identical with the minor haemoglobin II of the adult fowl, this predominance of haemoglobin II decreasing in favour of haemoglobin I until the time of hatching (Fraser (1961); Wilt (1962)).

(c) Nature of "bound" haemoglobin :-

Avian erythrocytes are nucleated and the nuclei of the intact erythrocytes contain haemoglobin (O'Brien (1960); Davies (1961)). Depending on the lysis procedure employed, a substantial amount of haemoglobin very often remains bound to the nuclear sediment obtained after centrifugation of a red cell lysate (D'Amelio and Salvo (1959); Hammel and Bessman (1964); Kabat and Attardi (1967); Allen (1969)).

However, in fact it seems likely that the "bound" haemoglobin consists merely of the components I and II attached to the residue though in variable amounts according to the method of lysis used. When lysis is caused by saponin in isotonic sucrose, the sediment from the centrifuged lysate can be washed with the sucrose until no more haemoglobin is removed in the washings. Haemoglobin, however, still remains bound to the sediment and can be eluted with 1% saline. This fraction shows only one haemoglobin component on electrophoresis (D'Amelio and Salvo)) or on CM-cellulose chromatography (Allen (1969)) and in both cases behaves in similar fashion to haemoglobin I. Further confirmation of the similarity of the haemoglobin in this membrane-bound fraction and haemoglobin I has been provided by Allen (1969) who has carried out amino acid analyses of these haemoglobins and has found the analyses to give identical results within the limits of experimental error. One consequence of the binding of haemoglobin I and II will be that their relative amounts in a lysate supernatant will vary according to their degrees of attachment to the sediment, this in turn depending on the lysis procedure employed. Thus, a really accurate estimate of the ratio of haemoglobin I : haemoglobin II should take into account the "bound" haemoglobin which is why the percentages of Huisman and Schillhorn Van Veen, (1964) and of Matsuda and Takei (1963) can only be regarded as approximations.

(d) Nature of haemoglobins I and II :-

The two components I and II are typical haemoglobins, molecular weight about 68,000 (Huisman, Schillhorn Van Veen, Dozy and Nechtman (1964)) and each consisting of two pairs of polypeptide chains (Muller (1961); Matsuda, Maekawa and Otsubo (1965)). The amino acid analyses of the two haemoglobins have been carried out by several workers (Van der Helm and Huisman (1958); Saha (1964); Allen (1969)). There are some big discrepancies between the figures obtained by Van der Helm and Huisman (1958) on the one hand and Saha (1964) and Allen (1969) on the other. The results of Allen (1969) appear to be the most reliable as his methods of preparation ensured that the globins were pure before analysis. Nevertheless, all three sets of results indicate that haemoglobin II contains more of the acidic amino acids (Glu, Asp) and less of the basic amino acids (Lys, His, Arg) than does haemoglobin I.

All four pairs of polypeptide chains are said to be different as shown by starch-gel electrophoresis, amberlite-IRC-50 chromatography and peptide pattern analysis (Muller (1961); Saha (1964)). The amino acid analyses of the separated polypeptide chains have been carried out by Saha (1964) and he has found that all four pairs of chains from the two haemoglobins had substantially different amino acid contents. The N-terminal amino acids of the two different kinds of polypeptide chains from haemoglobin I are apparently val-leu and val-his; haemoglobin II has one pair of chains with masked N-termini and the other with val-his

as the N-terminal amino acids (Matsuda, Maekawa and Otsubo (1965)).

Huisman, Schillhorn Van Veen, Dozy and Nechtman (1964) have also found differences between the haemoglobins in their affinities for oxygen. In their work, the major haemoglobin I behaved like adult human haemoglobin with respect to its oxygen equilibrium in salt solutions of different molarities. As the ionic strength increased, there was a progressive rise in the  $P_{50}$  value (decrease in oxygen affinity). However, the minor haemoglobin II had a very low oxygen affinity in buffers of low ionic strength and this affinity increased rapidly with increase in salt concentration at constant pH. It is interesting that the oxygen affinity of a chicken red cell haemolysate was also found to increase with ionic strength and that the haemolysate had a decreased oxygen affinity compared with the isolated components, facts which suggested that there was some kind of interaction between the haemoglobins in the haemolysate.

The hen haemoglobins apparently exist together in the same cell. Using the fact that the two haemoglobins differ in their resistance to denaturation by alkali, Matioli and Thorell (1963) have followed the change caused by alkali in the absorbance of the haemoglobin in a single erythrocyte. They obtained alkali denaturation curves consistent with the idea that the different haemoglobin types are present together in the same red cell. Fraser (1964(b)) has also studied the haemoglobins in two morphologically distinct cell types found in the circulating blood of early chick embryos, and has argued that as the primitive erythroid line

changed to the definitive cell line during embryonic growth, the similarity of the haemoglobin electrophoresis patterns obtained from the two separated cell lines indicates the multiple haemoglobins were present in single cells of both lines.

Metabolic studies on the prosthetic groups of multiple haemoglobins.

In view of the fact that the erythrocytes of many species contain more than one kind of haemoglobin molecule, it is surprising that comparatively little work has been done on this intracellular compartmentalisation of haem. The red cell should be an especially good system for studying the synthesis of the haem moieties of two or more closely related, easily obtainable haemoproteins.

Faber, Falbe-Hansen, Keiding and Kornerup (1958) studied the in vivo incorporation of iron-59 into the haemoglobin of human patients with pernicious anaemia. They found that the alkali-resistant haemoglobin fraction (foetal haemoglobin) became much more highly labelled than the alkali-labile haemoglobin (main haemoglobin) and continued to be so even at periods after injection greater than the erythrocyte life span. However, it was later shown (Falbe-Hansen (1961); Falbe-Hansen and Jordal (1963)) that this peculiar finding was due to an error in technique. The method of Faber, Falbe-Hansen, Keiding and Kornerup (1958) for preparing the haemoglobin fractions had involved the treatment of a haemolysate with alkali and the subsequent precipitation of the denatured alkali-labile haemoglobin fraction with ammonium sulphate. The amount of the alkali-

resistant fraction had then been calculated by measuring the extinction of the remaining solution at 540 m $\mu$  assuming that only oxyhaemoglobin was contributing to the absorption. This was a wrong assumption. In fact denatured globin haemichromogen was contributing to the absorption at 540 m $\mu$  and so falsely high specific activities were obtained for the foetal haemoglobin fraction. When allowance had been made for the presence of the denatured globin haemichromogen, it was found that in fact the foetal haemoglobin fraction and the remainder of the haemoglobin had the same specific activities as each other at every stage of the erythrocyte life span.

Meyering, Israels, Sebens and Huisman (1960) have measured the uptake of iron-59 into the human haemoglobin fractions which can be separated by CM-cellulose chromatography, namely the haemoglobins A ( $\alpha_2 \beta_2$ ),  $A_2$  ( $\alpha_2 \delta_2$ ) and  $A_1$ . They took blood samples from a baby eight days and twenty one days after the injection of radioiron. At both stages the uptake of iron by haemoglobins A and  $A_2$  was very similar. However, as mentioned earlier, the haemoglobin  $A_1$  fraction gave somewhat different results. The red blood cells removed at eight days and at twenty one days were divided roughly into two fractions of young mean cell age (top layer) and old mean cell age (bottom layer) by centrifugation. At eight days, the haemoglobin  $A_1$  in the younger cells had an activity about 50% of that of haemoglobins A and  $A_2$  whereas there was hardly any activity detectable in the haemoglobin  $A_1$  fraction from the older cells. At twenty one days, the incorporation of iron-59 into the haemoglobin  $A_1$  component in the older cells was higher than that into

haemoglobins A and A<sub>2</sub>. This suggested the conversion of haemoglobin A to haemoglobin A<sub>1</sub> in the course of time and thus, as mentioned earlier, haemoglobin A<sub>1</sub> is an example of heterogeneity due to the alteration of a haemoglobin molecule in the mature erythrocyte.

Schapira, Labie, Rosa and Dreyfus (1962) have studied the incorporation of iron-59 into rabbit haemoglobin. Rabbits were injected with 60  $\mu$ c of iron-59 and samples of blood were taken, at various times after injection, from the sixteenth hour to the thirty eighth day. Using chromatography on amberlite IRC-50 resin, these workers separated the haemoglobin into three fractions arranged in order of elution :- (a) fast moving minor fraction ("old" haemoglobin); (b) major haemoglobin fraction; (c) "young" haemoglobin which was eluted from the columns at the tail of the major haemoglobin fraction by warming the columns from 4°C. to room temperature. The classification of the haemoglobin into "old" and "young" fractions was based on the specific activities obtained for the fractions over the period of study. Thus, on the first day and till the third day, the fraction (c) had a much higher specific activity than the fraction (b) which in turn was higher than (a). With time, the specific activity of the fraction (b) remained stable at a plateau level, a typical classical observation for a main haemoglobin fraction. However, that of the fast minor fraction (a) increased progressively until it became higher than that of (b). The tail haemoglobin (c) went from a higher specific activity than any to the lowest specific activity of all three fractions after the thirty fifth day. These observations of Schapira, Labie, Rosa and Dreyfus (1962), at any rate as far as the nature

of fraction (a) the "old" haemoglobin fraction was concerned, would seem to agree with those of Muller (1961) who separated rabbit haemoglobin on carboxymethyl-cellulose into a major haemoglobin fraction and a faster moving heterogeneous minor fraction which he believed was due to the glutathione binding of the former. On iron-59 incorporation studies, this glutathione-bound rabbit haemoglobin fraction would then be expected to behave in the manner obtained by Schapira, Labie, Rosa and Dreyfus, analagous to the observations on human haemoglobin A<sub>1</sub> made by Muller (1961) and Meyering, Israels, Sebens and Huisman (1960).

Huisman, Van der Helm, Visser and Van Vliet (1959) have studied the incorporation of iron-59 into the two sheep haemoglobins A and B in heterozygous sheep. Iron-59 was injected intravenously and blood samples were removed at different times from 3 to 100 days after the injection. The two haemoglobin components were separated from the stroma-free haemolysates of the washed red cells by their different solubilities in phosphate solutions and it was found that there was no significant difference between the specific activities of iron-59 in the haemoglobins at any stage of the red blood cell life.

The only other observation on the synthesis of the haem moieties of multiple haemoglobins seems to be that of Sheeler and Barber (1965) who used turtles for their investigations. These animals were first made anaemic by phenylhydrazine injection and the reticulocytes were then incubated with iron-59 labelled plasma. After three to five hours of incubation the two turtle haemoglobins were separated by paper electrophoresis and they found that the incorporated

iron was uniformly distributed in both haemoglobin components. This sort of observation, however, while not being completely unimportant does not convey as much information as do experiments of the type which Huisman, Van der Helm, Visser and Van Vliet (1959) performed. Serial measurements over a period of time are likely to give a more detailed understanding of the relationships between multiple haemoglobins.

#### Studies on the haem groups from the hen haemoglobins

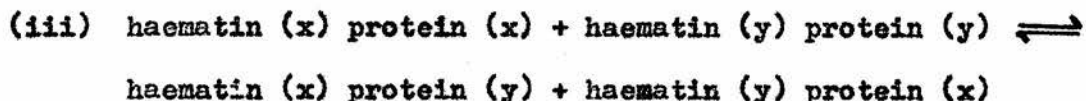
Several workers have studied the incorporation of radioactive amino acids into the two protein moieties (Saha and Ghosh (1960); Saha (1960); Matsuda and Ogata (1966); Kabat and Attardi (1967); Allen (1969)). Of these the most detailed examination has been carried out by Allen (1969) who has found that during in vitro incubations of immature chicken red cells, globin I incorporates radioactive amino acids to a greater extent than globin II. Now it appears likely that there is a close connection between haem synthesis and globin synthesis (London, Bruns and Karibian (1964); Grayzel, Hordhner, and London (1966)). It seemed important, therefore, in the present work, to study the relationship between the syntheses of the haem moieties of the two hen haemoglobins as a further step towards a more complete understanding of the formation and breakdown of the whole haemoglobins. For this purpose, both iron-59 and (2-carbon-14) glycine were used.

Hens are particularly suitable for work on a multiple haemoglobin system because their two haemoglobins are easily separable from small amounts of blood in sufficient quantities to make analysis fairly straightforward. They are also not too large to be kept in a laboratory and yet are a convenient size for in vivo work where a large number of serial samples is often required and in which case one does not wish to disturb the whole body system over much. Again, hens are fairly easily made anaemic if reticulocytes are required for in vitro work.

There is one important factor which must be taken into consideration in any study of haem synthesis in a multiple haemoglobin system. This is the possibility of haem exchange taking place between the haemoglobins during the experiment, both intracellularly and extracellularly in the purification and separation procedures. That haem is readily removed from haemoglobin at a low pH is well known. However, recently it has become apparent that even in neutral solution and in buffers of low ionic strength dissociation may occur. Rossi-Fanelli and Antonini (1960) have reported that in 0.1M phosphate pH 6.8 at 20-30°C. the following types of exchange can take place after several hours incubation :-

- (i) ferrihaemoprotein (x) + apohaemoprotein (y)  $\longrightarrow$  apohaemoprotein (x) + ferrihaemoprotein (y)
- (ii) haematin (x) protein + haematin (y)  $\longrightarrow$  haematin (y) protein + haematin (x)

Rosenqvist and Paul (1964) have observed a similar sort of exchange as (i). Bunn and Jandl (1966(a)); 1966(b); 1968) have produced a series of papers on exchange between whole haemoglobin molecules i.e. exchange of the type



They showed this by incubating mixtures of human ferrihaemoglobin F and ferrihaemoglobin A, in which one of the ferrihaemoglobins was labelled with iron-59 or carbon-14. Incubation was carried out under mild conditions (37°C; pH 7.2;  $\mu$  0.09) for periods of up to sixteen hours and it was found that there was complete randomisation of the haems at the end of this period. Only the methaemoglobins gave this haem exchange. Thus, after a five hour incubation the ferrihaemoglobins showed an exchange of 75% whereas the forms oxyhaemoglobin, deoxyhaemoglobin, ferrihaemoglobin cyanide and carboxyhaemoglobin, all had a percentage exchange in the range 3.1 - 7.5%.

These in vitro experiments suggest that intracellular exchange of haem in a multiple haemoglobin system is unlikely to be important unless there are significant amounts of methaemoglobin present or unless there is continual and rapid oscillation between the met- and oxy- forms of the various haemoglobin components. The amount of methaemoglobin in the avian erythrocyte is unknown. However in the human red cell only about 1% of

the total haemoglobin is in the methaemoglobin form (Bodansky (1951)). This level is apparently maintained by the balance of two opposing processes. These are the spontaneous oxidation of haemoglobin to methaemoglobin and the enzymic reduction of methaemoglobin to haemoglobin. In the erythrocyte there are also apparently two enzyme systems capable of reducing methaemoglobin, one linked to reduced NADP and the other to reduced NAD. However, under normal physiological conditions, the reduced NAD linked pathway appears to play the major role in the conversion of methaemoglobin (Gibson (1948)). Thus intracellular haem exchange, if it takes place, may be a function of the activity of this reduced NAD linked enzyme and the rate of oxidation of haemoglobin.

#### Aims of present study

The basic aims of the present study can be summarised as being to investigate by means of iron-59 and (2-carbon-14) glycine, the metabolic relationships between the haem moieties of the hen haemoglobins I and II both in vivo and in vitro, and to consider the effect of haem exchange on this relationship. It is considered that this study will give information leading towards a more complete understanding of the synthesis and degradation of whole haemoglobins in a multiple haemoglobin system.

CHAPTER 2 : Materials and Methods

Chemicals :- All chemicals used, unless otherwise stated, were of ANALAR grade.

Apparatus :- All apparatus used was washed in the following way so as to render it iron-free and also free from radioactive contamination: The glassware was first rinsed out several times with hot tap water and soaked overnight in a solution of Pyroneg detergent in water. It was then scrubbed in the detergent (except in the case of pipettes), rinsed out with tap water and again soaked overnight, this time in 25% (v/v) hydrochloric acid and rinsed out well with distilled water before drying.

Hens :- Both pure Light Sussex hens and Thornber hybrid 606 birds were used. No differences, as far as the haemoglobins were concerned, were observed between these two groups of birds. The hens were kept in individual cages and allowed free access to food and water. In general the required blood was removed from the wing veins of the hens. All procedures on the samples until the stage when the different haemoglobin components had been separated were carried out at 0 - 4°C. The blood was centrifuged (2,000 g; 10 min.) and the plasma taken off with a Pasteur pipette. The cells were then washed three or four times with ice-cold 1% saline (10 volumes) removing the supernatant with a Pasteur pipette each time after centrifugation.

Lysis of erythrocytes :- Three different methods of lysis were used, chosen as was most convenient for the purpose required. The same number of haemoglobin components was obtained from the lysate supernatants

with all three procedures.

(a) Lysis with water :- to one volume of the washed, packed red cells was added 6 volumes of ice-cold distilled water. The mixture was stirred up well and left for two hours at 0 - 4°C. After centrifugation (4,000 g; ½ hour), the supernatant haemoglobin solution (called hereafter "water lysate supernatant" was pipetted off from the red, jelly-like sediment.

(b) Lysis with water/toluene (Matsuda and Takei (1963)) :- 2 volumes of water and 1 volume toluene were added to 1 volume of washed packed red cells. The mixture was well shaken, left overnight at 0 - 4°C and centrifuged (4,000 g; ½ hour). The haemoglobin layer ("toluene lysate supernatant") was the central of the three layers obtained.

(c) Lysis with saponin (Hammel and Bessman (1964)) :- to 1 volume of washed, packed red blood cells was added 1 volume of a solution of 1% saponin in 0.25M sucrose - 0.003M CaCl<sub>2</sub> and the mixture allowed to stand for 5 minutes. The resultant lysate was then diluted with sucrose/CaCl<sub>2</sub> (5 volumes) and centrifuged (4,000 g; ½ hour). The supernatant haemoglobin was removed and the sedimented material washed with sucrose/CaCl<sub>2</sub> (10 volumes) until no more haemoglobin could be removed from it even although a substantial amount remained attached to the sediment. Starting with 3-4 ml blood, 2 washings of the sediment with sucrose/CaCl<sub>2</sub> were usually sufficient to remove most of the unbound haemoglobin. The

washings were added to the first supernatant ("saponin lysate supernatant"). Haemoglobin could then be removed almost completely from the sediment by washing it 2-3 times with 1% NaCl (5 volumes). This cell fraction is hereafter called the "membrane-bound haemoglobin". It accounts for 11% of the total red cell iron.

Carboxymethyl-cellulose :- The weak cation exchanger carboxymethyl-cellulose (Serva, capacity 0.69 m.eq/gm) was prepared for use in the following way as required :-

CM-cellulose (100 g) was shaken up with distilled water (1.5 l.) in a 2 litre stoppered measuring cylinder and allowed to settle. When the main body of the CM-cellulose had come down, the supernatant containing the fines was removed and discarded. This procedure of washing with water was repeated a further two times. After the third water wash, the CM-cellulose was then washed once with HCl (5% 1.5 l.), 5 further times with water (1.5 l.) (until the washings were only weakly acidic), once with NaOH ( $\frac{N}{2}$ ; 1.5 l.), and then with water until the washings were neutral to litmus (about six washings), discarding the supernatant liquid each time. Finally the CM-cellulose was washed twice with  $\text{KH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$  buffer (0.2M; 1 l.; pH 6.5) and kept at 3°C. in this buffer. When required for use, this slurry of CM-cellulose was poured into chromatography columns of the requisite size and the columns were then equilibrated with the starting buffer by washing them with at least 10 bed volumes of the starting buffer.

Sephadex :- Sephadex G-25 (coarse grade) was routinely used for equilibrating haemoglobin solutions with the starting buffer for CM-cellulose chromatography. In accordance with the manufacturers' instructions, the dry sephadex beads were first allowed to swell in water for at least two hours. The water was then decanted off and replaced by the required buffer. Chromatography columns (25 cm x 2 cm; bed volume 80 ml) of the sephadex were then poured and washed further with 3 bed volumes of buffer when they were ready for use. Protein solutions (haemoglobin) were applied to the tops of the columns and elution was carried out with the required buffer. When all the protein had been removed from the columns, the columns were made ready for further use by washing with three more bed volumes of buffer.

Iron Determinations:- The estimation of iron was the main method used for assaying haemin or haemoglobin solutions. It was also used for measuring the iron content of whole blood or of non-haem iron protein fractions. The method is that of Ramsay (1964) which involves the formation of the iron-dipyridyl complex. It was convenient to carry out the process in tubes graduated at 10 ml.

If the starting material contained haemoglobin it was first heated momentarily in a boiling water bath (until the red colour of the haemoglobin just turned to brown). This was done to prevent the catalytic destruction of the hydrogen peroxide which is used in the subsequent stages. If the starting material was a solution of haemin in 1% ammonia, most of the ammonia was first removed by heating on a boiling water bath

as otherwise troublesome yellow complexes tend to be formed. Hydrogen peroxide (3% in 1% acetic acid; 0.2 ml) was then added and the tubes heated in the boiling water bath until the solutions had turned a very pale yellow (about 5 minutes). Sodium sulphite (1.5M; 0.5 ml) was pipetted in, and the tubes shaken and allowed to stand for 5 minutes. 2,2'-dipyridyl (0.25% in 25% acetic acid; 1.0 ml) was then added. The volumes were made up to approximately 10 ml and the contents of tubes were well mixed. They were heated then for 30 minutes on the water bath to obtain full development of the pink ferrous-dipyridyl complex. After cooling, the volumes in the tubes were adjusted to exactly 10 ml and the tubes centrifuged if necessary. The optical densities of the solutions were read at 520 m $\mu$  against reagent blanks in a Unicam S.P. 600 spectrophotometer using 1 cm or 2 cm glass cells. The amount of iron in the samples was obtained by comparison with previously prepared standard graphs, 0 - 40  $\mu$ g Fe for 1 cm cells, and 0 - 20  $\mu$ g Fe for 2 cm cells.

Separation of haemoglobins :- Chromatography on CM-cellulose was used to separate the haemoglobins. The procedure employed was that of Matsuda and Takei (1963) as modified by Allen (1969), who made full investigation of all the stages of the separation.

(1) Complete elution patterns of lysate fractions :- CM-cellulose columns (9 cm x 1 cm) were set up and equilibrated with 0.01M phosphate pH 6.3. Lysate supernatants or membrane-bound cell fractions (2.0 - 6.0

ml; 30-250  $\mu$ g Fe) were added to the columns and washed on with a few ml. of the starting buffer. Under these conditions the haemoglobin is adsorbed to the CM-cellulose. The columns were washed with 0.01M phosphate pH 6.3 (5 bed volumes) and then with 0.01M phosphate pH 6.9 (5 bed volumes). No haemoglobin was removed in these buffers. A gradient elution was then carried out. A logarithmic concentration gradient was produced by allowing 0.2M phosphate pH 6.9 to flow into a constant volume mixing chamber containing initially 0.01M phosphate pH 6.9 (400 ml.). Fractions of the column eluates were collected and an equal volume of 0.01M phosphate pH 6.3 added to each (to bring the volume to approximately 3.5 ml.). The optical densities of the fractions were read at 415  $m\mu$  (Soret peak) and 280  $m\mu$  in a Unicam S.P. 500 spectrophotometer using 1 cm. silica cells. Figures 1(a) and 1(b) show the complete elution patterns of a saponin lysate supernatant and of the corresponding membrane-bound fraction. These were obtained at the same time from identical columns flowing at the same rate.

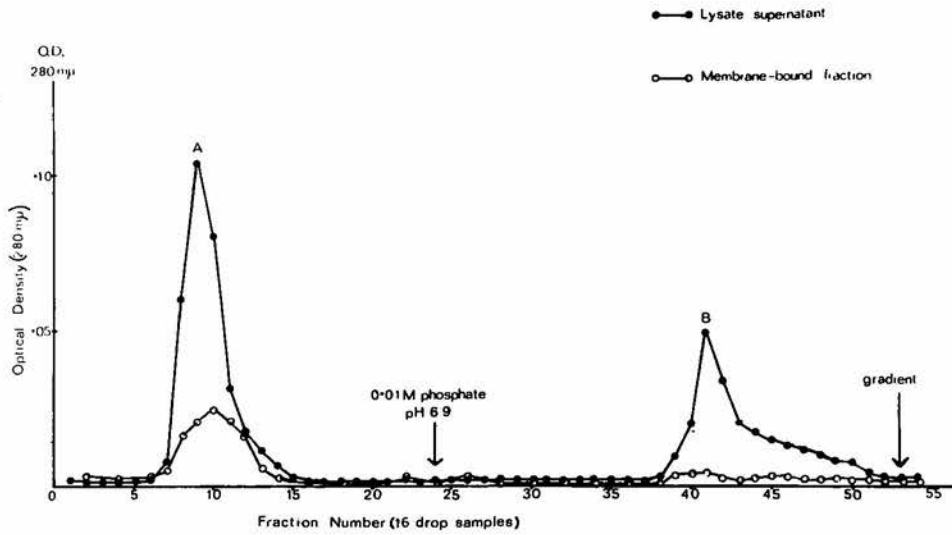


Figure 1 (a)

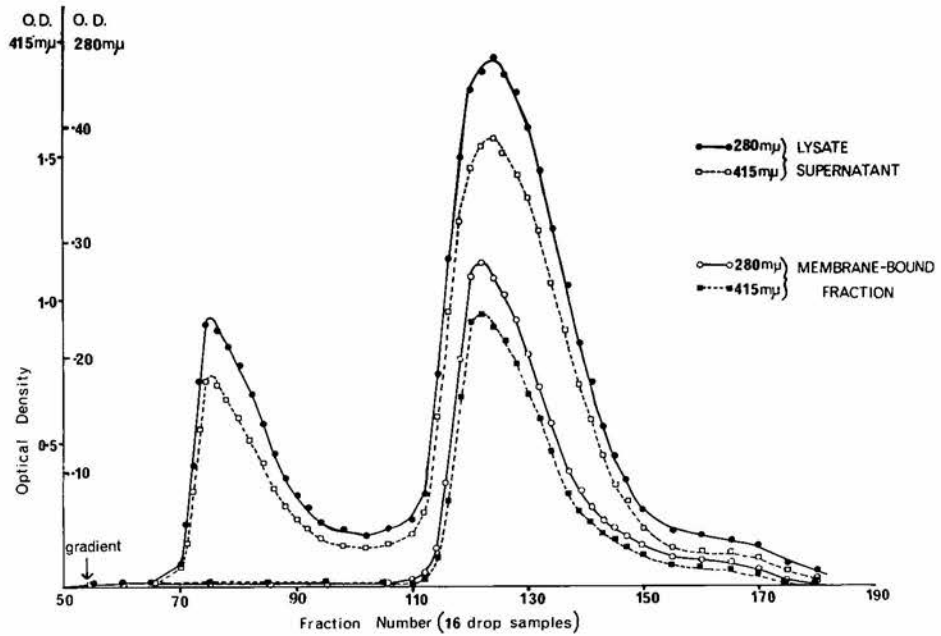


Figure 1 (b)

Figures 1(a) and 1(b)

Complete elution patterns of saponin lysate supernatant and membrane-bound haemoglobin fraction on CM-cellulose. Figure 1(a) shows the pre-haemoglobin protein fractions A and B. In Figure 1(b) are seen the haemoglobins I and II, haemoglobin II being the faster moving protein.

It can be seen that there are two protein peaks removed before the haemoglobin from the lysate supernatant, a peak A which comes off with 0.01M phosphate pH 6.3 and a peak B with 0.01M phosphate 6.9. Both these proteins are probably non-haem as shown by their very low Soret absorption. In the case of the membrane-bound haemoglobin fraction, only peak A was seen, peak B being absent. Using the gradient elution, two haemoglobins were obtained from the lysate supernatant fraction whereas with the membrane-bound fraction from a saponin lysate there is no trace whatsoever of any minor haemoglobin II (Allen 1969). Apparently all the membrane-bound haemoglobin has the same chromatographic mobility as the haemoglobin I of the lysate supernatant fraction.

The supernatants from the other lysis procedures gave elution patterns closely similar to those in Figures 1(a) and 1 (b).

(ii) Routine separation procedure :- In general the following technique was routinely employed for the separation of the hen haemoglobins :- The lysate supernatants (saponin, water or toluene) and/or membrane-bound haemoglobin fraction (saponin lysis only) were adsorbed onto separate CM-cellulose columns (9 cm x 1 cm for up to 250  $\mu$ g Fe, or 16 cm x 2 cm for up to 1000  $\mu$ g Fe) equilibrated with 0.01M phosphate pH 6.3. The columns were then washed successively with 0.01M phosphate pH 6.3 (5 bed volumes) and 0.01M phosphate pH 6.9 (5 bed volumes). Haemoglobin II was eluted from the lysate supernatant fractions using 0.039M phosphate pH 6.9.

Trials showed that this was a suitable molarity and pH for removing haemoglobin II without causing any noticeable movement of haemoglobin I down the columns. In the case of the membrane-bound fraction, no significant amount of haemoglobin II came off the columns with 0.039M phosphate pH 6.9. The remainder of the haemoglobin was then brought off the columns using 0.2M phosphate pH 6.9.

Preparation of the haemins :- In many experiments the haemins from the haemoglobins I and II (hereafter called haemin I and haemin II respectively\*) and the membrane-bound haemoglobin were required. The haemoglobins were obtained from the separation on CM-cellulose columns in a volume of 7-10 ml of buffer solution. This was too large a volume for the usual extraction of haemin from haemoglobin solution with acetone/HCl to be practicable. The haemoglobins were therefore first precipitated from solution by the addition of an equal volume of acetone. After the precipitates had been centrifuged down, they were taken up and well stirred with a glass rod in as small amount of water as was convenient (usually 0.3-0.5 ml but more if larger amounts of haemoglobin had been used). To one volume of this slurry was then added 10 volumes of acetone/HCl (1.0% HCl v/v) and using a glass rod to stir the mixture, as complete extraction of haemin as possible was obtained. The resultant globin precipitates though not absolutely white seemed to be substantially free of haem. After the proteins had been centrifuged down, 1.2 volumes of ammonium chloride solution (0.2%) were added to 1 volume of the acetone/HCl solutions to precipitate the haemins. It was found that this

\* Although evidence is adduced (pp. 39 and 40) that haemoglobin I and haemoglobin II have the same prosthetic group, it is convenient to speak of haemin I and haemin II instead of the haemin obtained from haemoglobin I and the haemin obtained from haemoglobin II.

precipitation was more nearly complete if it was allowed to take place for at least two hours. The haemins then were centrifuged down, washed once with water and dissolved in a convenient volume of 1% ammonia (usually 1.3 - 2.5 ml). These ammonia solutions were centrifuged and it was occasionally found that a very small amount of residue was obtained. After decanting the supernatants off, aliquots were used for plating and counting and iron determinations as required.

Check on the radiochemical purity of the haemins :- As mentioned in the previous section, when haemin was prepared from an acetone/HCl extract of precipitated haemoglobin, and dissolved in 1% ammonia, the final solution on centrifugation gave rise occasionally to a small amount of residue. Before more detailed studies on the incorporation of iron-59 and (2-carbon-14) glycine into the haemins were carried out, it was therefore thought that it would be advisable to test the effect on the haemin iron-59 and carbon-14 specific activities of the repeated precipitation of the haemins from ammonia solution with glacial acetic acid. In view of the work of Allen (1969) it was believed that the haemoglobins themselves were radiochemically pure. However, it was considered possible that some labelled globin was perhaps inadvertently being obtained in the haemin preparations. Blood was therefore incubated with iron-59 and (2-carbon-14) glycine in the manner described in Chapter 5. After 1½ hours the incubation was stopped and the three haemins prepared in solution in 1% ammonia. Aliquots of the haemins were analysed and their

specific activities determined as described later. To the remainder of the solutions was added slowly, dropwise, glacial acetic acid until the haemins precipitated. They were centrifuged down, washed with water and redissolved in 1% ammonia. The solutions were then centrifuged and decanted off into fresh tubes. Aliquots of these solutions were then also taken for analysis. The remaining haemin solutions were subjected to the same procedure of precipitation and redissolving another two times, samples being taken for analysis on each occasion. The specific activities of the haemins during these procedures were as shown in Table 1. It is apparent that the treatment used did not cause any alteration in the specific activities of the haemins.

Preparation of the globins :- In a few experiments, the globins from the haemoglobins were themselves required. To one volume of a haemoglobin solution was added 10 volumes of acetone/HCl (0.3% HCl v/v). The white globin precipitate was centrifuged down and the haemin supernatant discarded. As mentioned before, this latter solution was usually too dilute for precipitation of the haemin practicably when the starting materials were the haemoglobins separated from CM-cellulose columns. The globin was redissolved in water and again precipitated by the addition of 10 volumes of acetone/HCl. After centrifugation the precipitate was washed once with acetone and finally dissolved in water. Aliquots of this aqueous solution could be used for protein estimations and other analytical purposes.

**TABLE 1**

**Effect of repeated acetic acid precipitation on the apparent specific activity of haemin**

	Haemin	Precipitated from acetone -HCl with $\text{NH}_4\text{Cl}$ (0.2%)	Times precipitated from 1% ammonia with acetic acid		
			1	2	3
Carbon-14 r. specific activity (counts/min /ug Fe)	II	20.5	21.9	-	22.3
	I	22.2	23.3	23.8	22.8
	M	41.9	43.9	44.1	44.4
Iron -59 specific activity (counts/min /ug Fe)	II	29.5	32.9	-	31.4
	I	32.0	32.9	32.4	31.6
	M	52.0	52.7	54.5	53.5

Haemins II, I, M = Haemin from haemoglobin II, haemoglobin I, and the membrane - bound haemoglobin, respectively.

Protein determinations :- These were done by the method of Lowry, Rosebrough, Farr and Randall (1951) as modified by Eggstein and Kreutz (1955).

The reagents used were,

A 2%  $\text{Na}_2\text{CO}_3$  in 0.10N NaOH

B 0.5%  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  in 1% sodium citrate

C 1.0 ml of B + 50 ml of A made up every day as required.

D Folin-Ciocalteu reagent diluted with water so as to be 1N in acid. The dilution factor of this reagent was found by titration with NaOH using phenolphthalein.

For the determinations, solutions of protein (0.1 - 1.0 ml) were pipetted out into tubes, water to 1.0 ml added and then reagent C (5.0 ml). After at least 10 minutes, diluted Folin reagent (0.5 ml) was added very rapidly and the solutions mixed thoroughly immediately. The optical densities were measured after a further 30 minutes against reagent blanks at 500 m $\mu$ . The protein concentrations were then found by comparison with previously prepared standard graphs.

These calibration curves were obtained from the globins I and II prepared as described in the previous section. After the globins had been washed once with acetone, they were washed once with ether and finally dried overnight in a vacuum dessicator over  $\text{P}_2\text{O}_5$ . The dried globins were then weighed out accurately, dissolved in water and aliquots of these solutions used for the preparation of the calibration graphs for the range

0-650  $\mu$ g protein/sample. The two globins gave identical curves.

Radioactive assay :- Two methods of plating were used for measurement of radioactivity.

(a) Plating the ferrous dipyriddy mercuriodide complex :- This is the method of Ramsay and Fulton (1964) and was used when iron-59 only was being counted. It involves the conversion of the ferrous dipyriddy ion obtained during the method for iron determination into the insoluble ferrous dipyriddy mercuriodide complex.

Mercuric chloride (2.5 g) is dissolved in hot water (50 ml) and after cooling, sodium iodide (62.5 g) is dissolved in the solution. This final solution of sodium mercuriodide is then left for 48 hours before it becomes suitable for use as a precipitant. Measured aliquots (8 - 9 ml) of the ferrous dipyriddy solutions (containing about 3-40  $\mu$ g iron) obtained during iron determinations were pipetted into tubes which were then warmed to 41°C. in a water bath. After a few minutes, the sodium mercuriodide (0.1 ml) was added and the tubes well shaken. They were then incubated at 41°C. for 1½ - 2 hours during which time the insoluble ferrous dipyriddy mercuriodide complex was formed. The tubes were centrifuged for 20 minutes (2,500 g) and the now colourless supernatants then carefully decanted from the red precipitates. The mouths of the tubes were carefully dried with tissue paper and the tubes allowed to stand upside down so as to get maximum possible drainage of superfluous materials away from the precipitates. 2 - 3 drops of water were

then added to the precipitates and if necessary they could be left in this state for several days.

For the actual plating of the complex, planchets of copper ( $1\frac{3}{16}$ " in diameter) were used with their undersides previously coated with silicone (2 drops of silicone MS 1107, Hopkin and Williams, 2% in hexane) to prevent the creeping of the material over the edge during the plating process. The planchets were arranged on a metal tray placed on a thermostatically controlled hot plate and the aqueous suspensions of the precipitates were transferred with Pasteur pipettes to the planchets. Complete transfer of the ferrous dipyriddy mercuriodide complex from the tubes was made by using further alternate washes of water and acetone (in which the complex is soluble). After evaporation to dryness, the planchets were counted either in an I.D.L. Betamat counter with a Geiger Muller head (thin window; halogen-quenched tube, E.H.T. 620 volts; background 11 counts/minute), or in the same machine converted for use as a windowless scintillation counter with an anthracene crystal (1200 volts E.H.T.; 6 volts Disc. Bias; channel width 20; background 9 counts/minute). Generally all samples were counted until the standard error of counting was less than 2%. From a series of 40 duplicate measurements on haemin and haemoglobin it has been calculated that the standard deviation of the specific activity of any one sample was  $\pm 5.1\%$  (see Appendix). This standard deviation includes all the statistical errors inherent in the manipulations, including the iron determinations.

(b) Plating of haemin :-

(i) Plating conditions :- This method was used when carbon-14 and iron-59 were being counted together. Measured aliquots (0.1 - 0.5 ml) of the haemins to be assayed (in 1% ammonia) were pipetted onto flat-bottomed aluminium planchets (diameter 2.2 cm.). One drop of a very dilute solution of detergent (Nonidet P42, Shell Chemicals) in water and if necessary a few drops of 1% ammonia were added to the planchets so as to spread the haemin solutions over the surface. Evaporation to dryness was then carried out on the thermostatically-controlled hot plate. Counting was done on the I.D.L. Betamat converted for use as a windowless scintillation counter. The amounts of haemin plated were determined by measuring the iron content of further aliquots of the original solution.

(ii) Counting of iron-59 and carbon-14 in haemin simultaneously :- Conditions had to be found where one of the isotopes could be counted separately from the other in mixtures of the two. It is possible to find these because iron-59 is a medium  $\beta$  -emitter ( $\beta$  -rays 0.46, 0.27 MeV) whereas carbon-14 is only a weak  $\beta$  -emitter ( $\beta$  -rays 0.155 MeV). Using a mixture of (2-carbon-14) glycine and ferric-59 citrate and comparing the counts in this mixture with the counts obtained by the same amounts of the isotopes plated separately, one set of conditions (A) was found where the isotopes could be counted together, and another set (B) where iron-59 only was counted (Table 2). Thus with haemin which is doubly labelled one can count iron-59 separately, count both iron-59 and carbon-14 together and hence from these results calculate the counts due

TABLE 2

Conditions for counting iron - 59 and carbon - 14 in mixtures of the isotopes.

	A	B
E.H.T. voltage (volts)	1400	1200
Disc. Bias voltage (volts)	6	18
Efficiency of Fe <sup>59</sup> counts (%)	45	12
Efficiency of C <sup>14</sup> counts (%)	28	<.003

to carbon-14 only, provided one knows the ratio of the iron counts under the two sets of conditions. This ratio can be obtained by counting under both sets of conditions standard planchets plated with iron-59 only. It was necessary to have standard planchets because the set of conditions A was somewhat unstable. Small changes in either the E.H.T. or Disc. Bias voltages made comparatively large changes in the iron-59 counts. Routinely, three standard iron-59 planchets were counted with the haemin samples.

The standard deviations of the specific activities measured in this way on fifteen different duplicate haemin samples were :- for iron-59,  $\pm 2.5\%$ ; carbon-14,  $\pm 4.0\%$ .

(c) Self absorption :-

(i) No self absorption correction is necessary for the ferrous dipyriddy mercuriiodide method of plating iron-59 for amounts of iron up to 60  $\mu\text{g}$ . (Ramsay and Fulton (1964)). This is an amount greater than was ever used in the present work.

(ii) With the method involving the plating of haemin it was thought necessary to check for the self-absorption of either carbon-14 or iron-59. Haemin, doubly labelled with iron-59 and carbon-14 was prepared by incubating whole blood from an anaemic hen with ferric-59 citrate and 2-carbon-14 glycine (see Chapter 5). Aliquots of a solution of this haemin in 1% ammonia were plated for counting and further aliquots taken for iron determinations. As can be seen (Figure 2) there

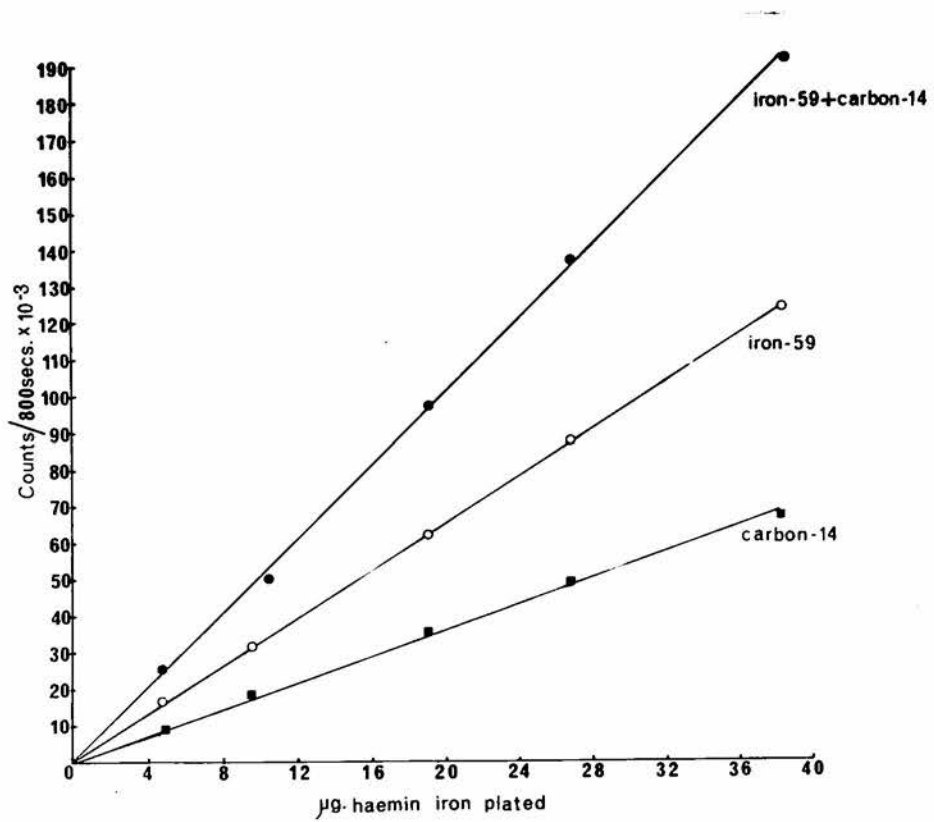


Figure 2

Test for self absorption. The effect on iron-59 and carbon-14 count rates of increasing the amount of haemin plated.

was no sign of any self absorption for either iron-59 or carbon-14 for up to an amount of haemin containing at least  $38 \mu\text{g}$  of iron (approximately  $0.46 \text{ mg}$  of haemin). In practice very seldom was more than  $20 \mu\text{g}$  of iron plated.

Examination of the haemins I and II :- Since most workers on multiple haemoglobins seem to have tacitly assumed that the haem moieties of the different haemoglobins are identical, it was thought that it would be worthwhile to check the similarity and purity of the haemins I and II. This was done by comparing the visible and ultraviolet spectra of the haemins and of their pyridine haemochromogens.

(a) Haemins :- The spectra of the haemins I ( $0.734 \mu\text{g Fe/ml}$ ) and II ( $0.647 \mu\text{g Fe/ml}$ ) in 1% ammonia were obtained, using a Unicam S.P. 500 spectrophotometer, in the range  $230 \text{ m}\mu - 680 \text{ m}\mu$  and are as shown (Figure 3). As can be seen they are very similar, both having a sharp absorption peak at  $386 \text{ m}\mu$  and a very flat one at  $605 - 610 \text{ m}\mu$ . When the optical densities per unit weight of iron were plotted, the two spectra were identical. They are typical spectra of haemin in alkaline solution (Maehly and Akeson (1958); Shack and Clark (1947)).

(b) Pyridine haemochromogens (Gallagher and Elliot (1965)) :- Solutions in 1% ammonia of haemin II ( $0.3 \text{ ml}$ ;  $6.47 \mu\text{g Fe}$ ) and haemin I ( $0.1 \text{ ml}$ ;  $7.34 \mu\text{g Fe}$ ) were pipetted into two  $10 \text{ ml}$  volumetric flasks. To each was added firstly  $2.0 \text{ ml}$  of pyridine and then a few grains of sodium dithionite. The mixtures were well shaken, made up to  $10.0 \text{ ml}$  with  $0.6\text{N}$  NaOH and allowed to stand for 1-2 minutes. The spectra were then

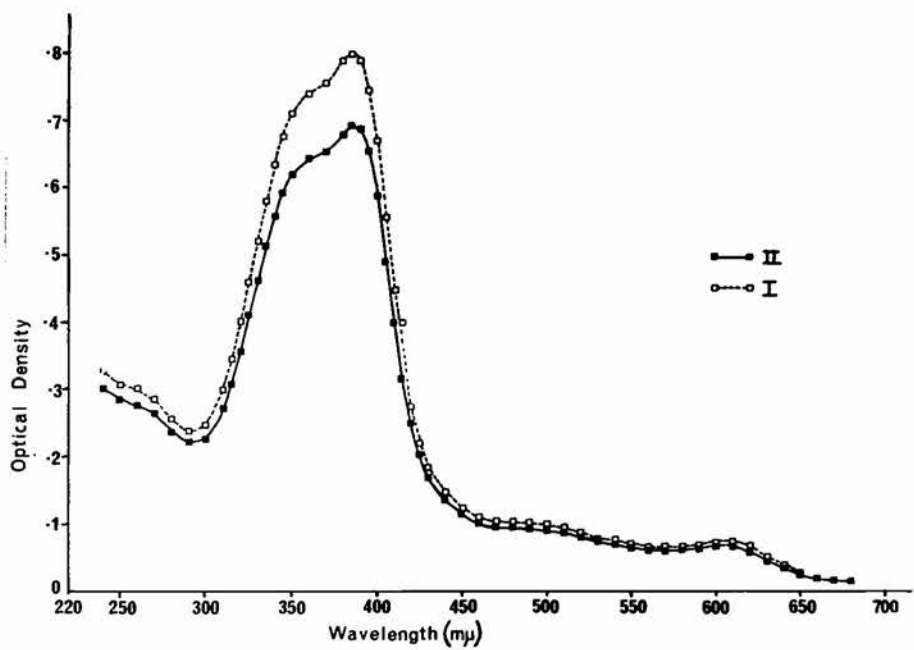


Figure 3

Visible and ultra violet spectra of haemins I and II in 1% ammonia solution.

read as quickly as possible (by hand using a Unicam S.P. 500 spectrophotometer) in the range 360-660 m/ $\mu$  and were as shown (Figure 4). Both have absorption peaks at 418 m/ $\mu$ , 521 m/ $\mu$  ( $\beta$  -band) and 554 m/ $\mu$  ( $\alpha$  -band) with no signs of any extraneous peaks in the Soret region.

Since the positions of the  $\beta$  and  $\alpha$  bands of a given pyridine haemochromogen are quite characteristic of that compound (Falk (1963)) it seems highly likely that the two pyridine haemochromogens here are identical. Gallagher and Elliot (1965) have examined the formation of pyridine haemochromogen in some detail. Depending on the concentrations and on the order of addition of reagents, they were able to obtain three forms of pyridine haemochromogen which they called I, II, III. Compound I was the classical pyridine haemochromogen which had absorption peaks (25°C) at 418.8 m/ $\mu$ , 526.5 m/ $\mu$  and 558 m/ $\mu$ . The actual experimental conditions in the present work were very similar to those of Gallagher and Elliot and should have favoured the formation of compound I. However, it seems possible that the compound III of Gallagher and Elliot has been formed. This compound was essentially an intermediate in Gallagher and Elliot's reaction sequence leading to the formation of compound I. It had absorption peaks (25°C) at 416 m/ $\mu$ , 523 m/ $\mu$  and 555 m/ $\mu$ . These are fairly close to the peak positions obtained in the present work.

The results of these experiments, therefore, suggest that haemoglobin I and haemoglobin II have the same prosthetic group, namely protohaem IX  $\alpha$ .

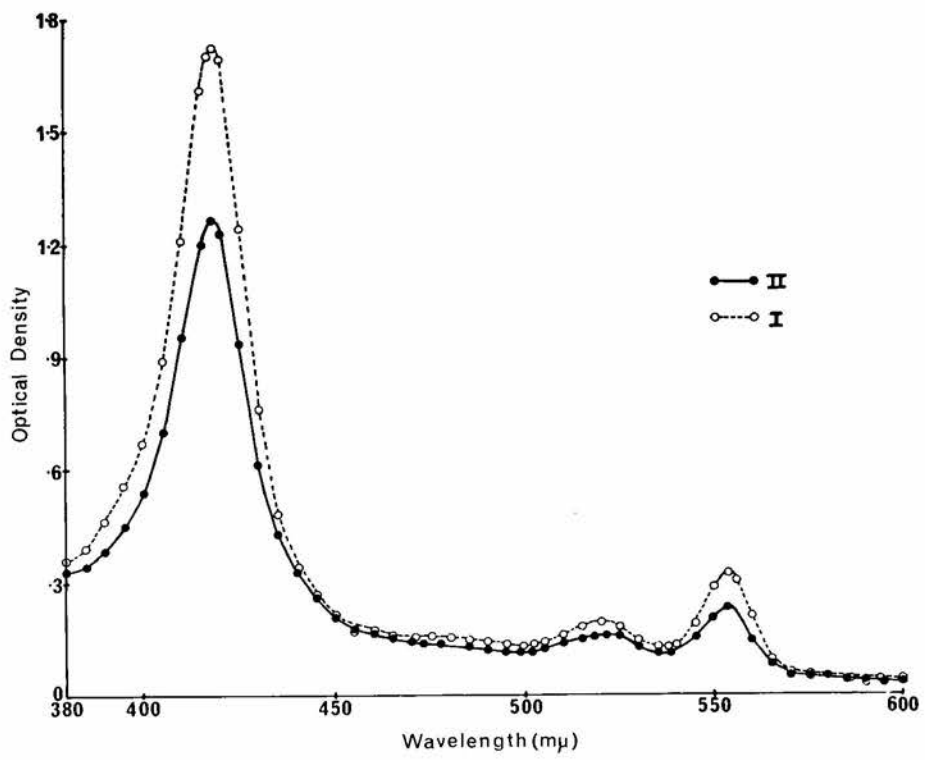


Figure 4

Spectra of the pyridine haemochromogens prepared from the haemins  
I and II.

CHAPTER 3 : Exchange Experiments

### Introduction

Because of the effect it would have on the results of studies on the incorporation of iron-59 and (2-carbon-14) glycine into the haemins from the haemoglobins I and II, it was thought important to ascertain whether there was haem exchange during the isolation procedures used. As mentioned in the General Introduction there are several indications that exchange can take place to a marked extent under very mild conditions in a matter of a few hours. In the present work it took up to 24 hours to perform all the necessary procedures on a lysate leading to the separated haemoglobins I and II. Experiments were therefore designed to find out whether haem exchange occurred during this period. If it could be shown that exchange was happening to a marked extent during the isolation of the haemoglobin from a lysate, then obviously interpretation of the results of studies on the incorporation of radioactive isotopes into the haemins I and II becomes extremely difficult.

### Experimental methods

The general pattern of the experiments was as follows : A hen was injected subcutaneously with  $8\mu\text{c}$  of iron-59 in the form of ferric citrate. Several days later, blood (10 ml) was removed from the wing vein and the two haemoglobin components I and II separated from it in the usual fashion on a large CM-cellulose column (19 x 2 cm). Blood was then also removed from another hen, one which had not been injected with radioiron. The washed red cells from this were lysed using the water/toluene procedure in order to give as concentrated a lysate as possible.

The principle behind the experiments was then to add iron-59-labelled haemoglobin I or II to the haemoglobin layer from the centrifuged lysate which contained both the haemoglobins I and II unlabelled. After being treated in various ways as described below, these mixtures were separated into their haemoglobin components and the specific activities of the different fractions measured.

Experiments Nos. 1, 3, 5 :- Fe<sup>59</sup>-labelled haemoglobin II (5.0 ml solution, containing 20 - 110  $\mu$ g Fe) was added to the cold toluene lysate supernatant (2.0 ml; 600 - 800  $\mu$ g Fe). The resultant mixtures were equilibrated with 0.01M phosphate pH 6.9 and left overnight at 2 - 4°C. The haemoglobins were then separated as usual and their specific activities measured.

Experiments Nos. 2, 4, 6 :- Fe<sup>59</sup>-labelled haemoglobin I (5.0 ml; 70-210  $\mu$ g Fe) was added to the cold toluene lysate supernatant (2.0 ml; 600-800  $\mu$ g Fe). The resultant mixtures were equilibrated with 0.01M phosphate pH 6.9 and left overnight at 2 - 4°C. The haemoglobins were then separated as usual and their specific activities measured.

Experiments Nos. 7, 8 :- These experiments were carried out in the same way as numbers 1 - 6 except that the Fe<sup>59</sup>-labelled haemoglobins I and II were subjected to re-chromatography on CM-cellulose before being added to their respective mixtures. This was so as to ensure that they were free of contamination one from the other. The mixtures were then equilibrated with 0.01M phosphate pH 6.9, left overnight at 2 - 4°C and the

haemoglobins separated as usual. However, before their specific activities were determined these separated haemoglobins were also each re-chromatographed on CM-cellulose.

Experiments Nos. 9, 10 :- Fe<sup>59</sup>-labelled haemoglobin II (2.0 ml; 23.8  $\mu$ g Fe) and Fe<sup>59</sup>-labelled haemoglobin I (2.0 ml; 77.6  $\mu$ g Fe) were added separately to two portions of a toluene lysate supernatant (1.0 ml; 346  $\mu$ g Fe). The mixtures were then equilibrated in 0.01M phosphate pH 6.3 and left overnight at 2 - 4°C before separation of the haemoglobins.

Experiments Nos. 11, 12 :- These were exactly the same as numbers 9 and 10 except that the mixtures were kept 24 hours longer in 0.01M phosphate pH 6.3 at 2-4°C before separation.

Experiments Nos. 13, 14 :- Fe<sup>59</sup>-labelled haemoglobin II (2.0 ml; 23.8  $\mu$ g Fe) and I (2.0 ml; 77.6  $\mu$ g Fe) were added to 2 portions of a cold toluene lysate supernatant (1.0 ml; 346  $\mu$ g Fe). The resultant mixtures were then incubated at 37°C for 3 hours. After equilibration in 0.01M phosphate pH 6.3, they were left overnight at 2-4°C before separation of the components.

Experiments Nos. 15, 16 :- Fe<sup>59</sup>-labelled haemoglobin II (2.0 ml; 23.8  $\mu$ g Fe) and I (2.0 ml; 77.6  $\mu$ g Fe) were added to two portions of a whole cold lysate (3.0 ml; 627  $\mu$ g Fe). This latter was simply an uncentrifuged lysate obtained by adding 6 volumes of water to 1 volume of washed red cells. The mixtures were then incubated at 37°C for 3 hours, centrifuged, and the supernatants equilibrated with 0.01M phosphate pH 6.3.

They were then left overnight at 2-4°C before separation of the components.

Experiments Nos. 17, 18 :- Fe<sup>59</sup>-labelled haemoglobins II (5.0 ml; 67.5 µg Fe) and I (5.0 ml; 15.2 µg Fe) were added to two portions of a cold toluene lysate supernatant (2.0 ml; 694 µg Fe). Prior to this the haemoglobins I and II had been separated from another portion of the unlabelled lysate. The globins I and II were prepared from this in a similar manner to that described by Rossi-Fanelli and Antonini (1958). This procedure is reported to give globin which is still capable of recombining with haematin to give a product which on reduction is indistinguishable from the original haemoglobin in several of its physicochemical properties (Rossi-Fanelli, Antonini and Caputo (1959); Rossi-Fanelli and Antonini (1959)).

20-30 volumes of acetone/hydrochloric acid (containing 3 ml 2N HCl per litre) previously cooled to 3°C were added to 1 volume of haemoglobin solution also at 3°C. The globin precipitates were as soon as possible centrifuged down at 0°C and dissolved in water. Measured portions of these solutions, of known measured protein concentration were then added to the two previously mentioned mixtures. To that containing Fe<sup>59</sup>-labelled haemoglobin II and unlabelled lysate supernatant was added globin I (1.5 ml; 2.16 mg globin) and to the other containing labelled haemoglobin I and unlabelled lysate supernatant was added globin II (0.5 ml; 2.95 mg globin). These mixtures were then left overnight at 2-4°C, equilibrated in 0.01M phosphate pH 6.9 and the components then separated as usual.

The results of these experiments 1-18 have been tabulated in Table 3. The table shows the specific activities (counts/min./ $\mu\text{g}$  Fe) of the added labelled haemoglobin, of the resultant mixture of this and the lysate, and of the two separated components from this mixture.

### Calculation

The calculations of % transfer of label and of % randomisation were made in the following manner :-

Let  $S_m$  = specific activity (counts/min./ $\mu\text{g}$  Fe) of the mixture to which the labelled haemoglobin has been added.

$S_2$  = specific activity (counts/min./ $\mu\text{g}$  Fe) of the haemoglobin II component separated from the mixture.

$S_1$  = specific activity (counts/min./ $\mu\text{g}$  Fe) of the haemoglobin I component separated from the mixture.

and  $h_1$  = amount of haemoglobin I ( $\mu\text{g}$  Fe) in mixture,

$h_2$  = amount of haemoglobin II ( $\mu\text{g}$  Fe) in mixture,

then  $S_m = \frac{c}{(h_1+h_2)}$  where  $c$  = total counts in mixture

$S_1 = \frac{c'}{h_1}$  where  $c'$  = counts in haemoglobin I

and  $S_2 = \frac{(c - c')}{h_2}$

substituting for  $c$  and  $c'$ , we obtain

$$S_2 = \frac{S_m(h_1+h_2) - S_1 h_1}{h_2} \qquad \frac{h_1}{h_2} = \frac{(S_2 - S_m)}{(S_m - S_1)}$$

TABLE 3

## EXCHANGE

Experiment Number	Fe <sup>59</sup> -labelled Hb added	s.a. of added Hb	Sm s.a. of resultant mixture	S2 s.a. of separated HB II from mixture	S1 s.a. of separated HbI from mixture	% transfer of label	% randomisation
1	II	43.4	1.19	4.30	0.08	5.0	6.7
2	I	43.4	2.45	0.19	3.06	1.6	7.7
3	II	69.0	11.4	28.8	0.26	1.4	2.3
4	I	72.0	19.3	0.37	23.9	0.4	1.9
5	II	57.0	3.76	10.03	0.11	1.8	2.9
6	I	59.8	8.91	0.16	11.90	0.5	1.8
7	II	48.9	0.972	2.76	0.088	6.1	9.0
8	I	48.9	2.29	0.19	3.29	2.7	8.3
9	II	84.9	5.85	17.7	0.52	6.1	8.9
10	I	86.0	15.8	0.69	20.9	1.1	4.4
11	II	84.9	5.85	17.7	0.77	10.0	13.1
12	I	86.0	15.8	0.82	20.8	1.3	5.2
13	II	84.9	5.80	17.9	0.72	8.7	12.4
14	I	86.0	14.8	1.39	20.3	2.7	9.4
15	II	84.9	3.91	13.0	0.51	10.4	13.0
16	I	86.0	10.2	0.81	15.8	3.0	7.9
17	II	50.2	4.42	11.0	0.22	3.0	5.0
18	I	45.1	0.980	0.069	1.378	2.1	7.0

Average % transfer II→I = 5.8%

Average % transfer I→II = 1.7%

Average % randomisation (all experiments) = 7.0%

(s.a. = specific activity in counts/min./ $\mu$ g Fe)

i.e. the final ratio of the amounts of haemoglobin I : II in the mixture

$$= \frac{S_2 - S_m}{S_m - S_1} : 1$$

Call this ratio r,

∴ proportion of iron-59 label in I : that in II = r . S<sub>1</sub> : 1 . S<sub>2</sub>

$$= \frac{r \cdot S_1}{S_2} : 1$$

Consider (a) the cases where haemoglobin II is added to mixtures,

then  $\frac{r \cdot S_1/S_2}{(1 + r \cdot S_1/S_2)} \times 100\%$  of the label in II has been transferred (% transfer).

Now for complete randomisation  $\frac{r}{(1+r)} \times 100\%$  would have been transferred.

∴ % randomisation =

$$\frac{r \cdot S_1/S_2}{(1 + r \cdot S_1/S_2)} \times \frac{(1+r)}{r} \times 100$$

substituting r =  $\frac{(S_2 - S_m)}{(S_m - S_1)}$ ,

this simplifies to % randomisation =  $\frac{S_1}{S_m} \times 100\%$

Similarly (b) when haemoglobin I is added to the mixtures,

$$\frac{1}{(1 + r \cdot S_1/S_2)} \times 100\% \text{ of the label in I has been transferred}$$

(% transfer).

Again, for complete randomisation  $\frac{1}{(r + 1)} \times 100\%$  would have been transferred.

$$\therefore \% \text{ randomisation} = \frac{(1 + r)}{(1 + r \cdot S_1/S_2)} \times 100\%$$

substituting  $r = \frac{(S_2 - S_m)}{(S_m - S_1)}$ ,

we get % randomisation =  $\frac{S_2}{S_m} \times 100\%$

Hence, knowing  $S_1$ ,  $S_2$  and  $S_m$  it is a very simple matter to work out the % transfer and the % randomisation for the two types of experiment.

### Discussion

It can be seen from Table 3 that the percentage randomisations are fairly low, the average for all experiments being 7%.

The experiments numbers 1 - 6 and 9 - 10 were "straightforward" measurements of exchange at pH 6.9 and 6.3 respectively, the haemoglobins in both groups of experiments being obtained and separated in the fashion used in general for metabolic experiments. In these experiments the "randomisations" were all less than 10%.

Experiments 7 and 8 were attempts to reduce this exchange by making sure that the separated haemoglobins assayed were pure and free of contamination one from the other. In fact, however, if anything the rechromatography slightly increased the exchange. This is probably because the extra procedures of necessity meant that the experiments 7 and 8 took considerably longer to perform than 1 - 6, and thus the chances of methaemoglobin being formed in the mixtures were increased. As mentioned earlier, Bunn and Jandl (1966(a); 1966(b); 1968) have shown that very little exchange of haems takes place between human oxyhaemoglobins A and F but that there is substantial haem transfer between the methaemoglobins. Thus the formation of small amounts of methaemoglobin may be the reason for the slight observed increase in experiments 7 and 8.

A similar explanation for the slightly increased exchanges may be made for the experiments where mixtures were left longer before separation (11, 12) and where the mixtures were warmed for three hours at 37°C (13 - 16) before separation. Both these procedures would be expected to increase the formation of methaemoglobin.

Experiments 15 and 16 were attempts to see whether the observed exchange could be increased by incubating the mixtures of haemoglobins in the presence of a whole water lysate i.e. whether the exchange was enzymic or not or whether there was some other intracellular factor which could cause exchange. However, as can be seen the percentage randomisations were the same as in experiments 11 - 14.

The final two experiments, 17 and 18, were also attempts to increase the exchange this time by the addition of free globin to the mixtures. This was done because it seemed reasonable to suppose that in the event of exchange between the two haemoglobins taking place, the free globins would be intermediates at some stage in the process. If this were so the addition of globin I to a mixture of labelled haemoglobin II and unlabelled haemoglobin I or the addition of globin II to a mixture of labelled haemoglobin I and unlabelled haemoglobin II, should have increased the percentage randomisations observed. This did not happen. An immediate obvious explanation might be that the globins added to the mixtures had been so denatured in their preparation that they were no longer able to combine with free haem to form haemoglobin. However, a few brief tests on the globin solutions prepared in the same way showed that this was not so :-

About 3 mg of crystalline haemin (prepared previously from ox blood by the method of Labbe and Nishida (1957)) was dissolved in as small a volume of 0.05N NaOH as possible and then the solution made up to 10 ml. with water. After centrifugation to remove any undissolved haemin, the haemin concentration in this solution was measured (solution A - 315  $\mu$ g haemin ml.). The following solutions were then made up :-

- B To globin II (3.0 ml; 2.07 mg globin) was added 0.1 ml haemin solution A (31.5  $\mu$ g haemin). The pH of this mixture was 3-4.
- C To solution B was added 1 drop of 1%  $\text{NH}_3$  to give a final pH of 8-9.
- D To solution C was added a further 0.05 ml of A (15.8  $\mu$ g haemin).

- E To globin I (3.0 ml; 4.92 mg globin) was added haemin solution A (0.1 ml; 31.5  $\mu$ g haemin). The pH of this solution was in the range 2-3.
- F To solution E was added 1 drop of 1%  $\text{NH}_3$ . This gave a final pH 8-9.
- G Haemin solution A (0.1 ml; 31.5  $\mu$ g haemin) was added to 3.0 ml of water. The pH of this solution was 7 - 7.5.
- H To solution G was added 1 drop of 1%  $\text{NH}_3$ . This gave a final pH of 8-9.

The spectra of these solutions B-H in the Soret absorption region were then read and were as shown in Figure 5. From these it would seem to be quite clear that the globins in the solutions are combining with the haemin. The typical haemin absorption peak is, on addition of the globins, very markedly shifted towards longer wavelengths and the absorption in this region is also increased in intensity. It is noteworthy that the final wavelength of maximum absorption (413 m $\mu$ ) was identical to that of solutions of the hen methaemoglobins at pH 8-9. These latter were prepared by oxidation of the oxyhaemoglobins I and II by 1%  $\text{K}_3\text{Fe}(\text{CN})_6$ , any excess  $\text{K}_3\text{Fe}(\text{CN})_6$  being removed by passage of the methaemoglobins down G-25 Sephadex columns.

Thus the fact that the "exchange" between the haemoglobins I and II was not increased by the addition of the globins to the mixtures was unlikely to be due simply to poor preparation of the globins. It is possible therefore that the exchange may take place by means of a

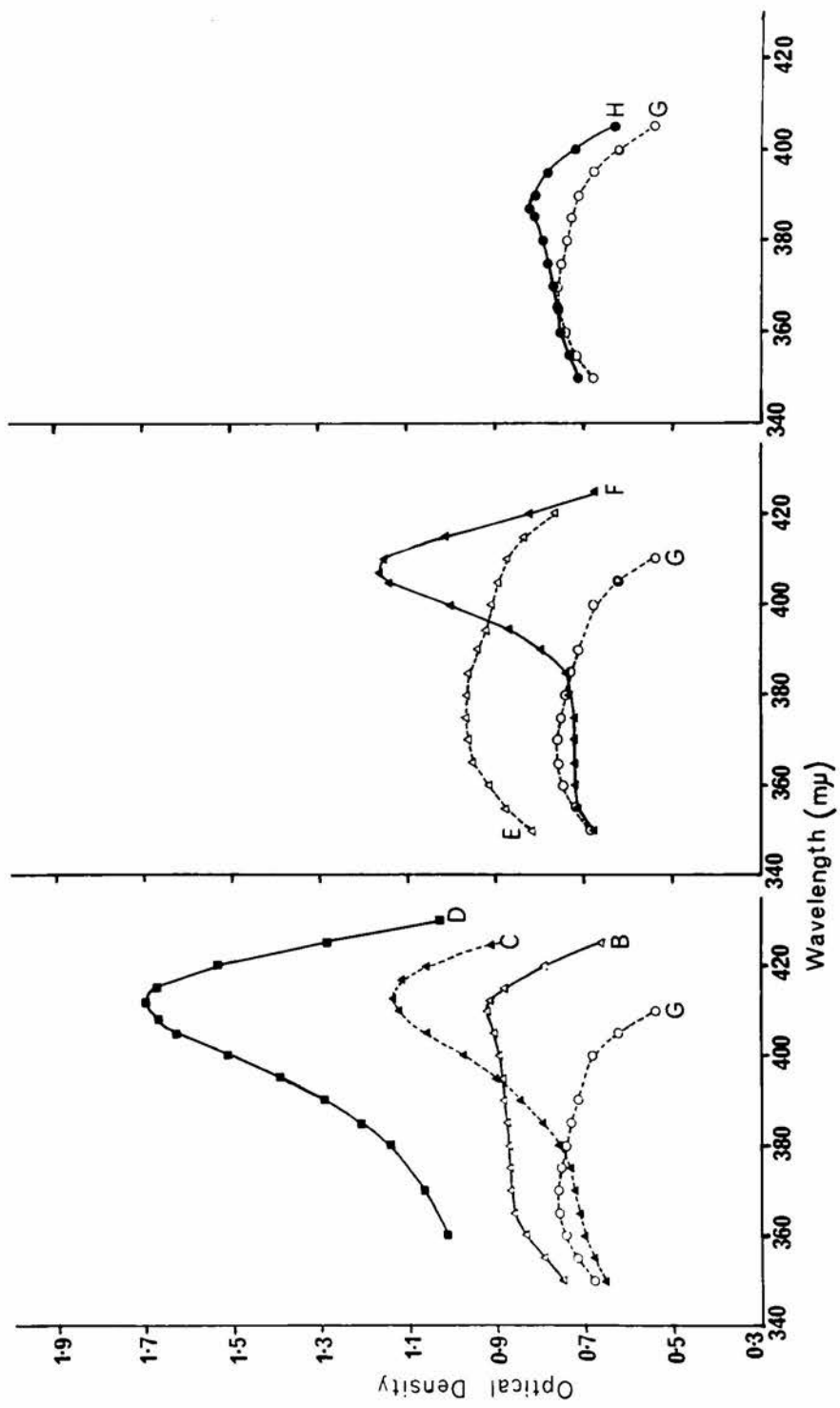


Figure 5

Alteration of the Soret region spectrum of haemin solution on the addition of globin I or globin II.

- B : Globin II (3.0 ml; 2.07 mg) plus haemin (0.1 ml; 31.5/ug); pH 3-4.  
C : As B; 1% NH<sub>3</sub> added to give pH 8-9.  
D : Globin II (3.0 ml; 2.07 mg) plus haemin (0.15 ml; 47.3/ug); 1% NH<sub>3</sub> added to give pH 8-9.  
E : Globin I (3.0 ml; 4.92 mg) plus haemin (0.1 ml; 31.5/ug); pH 2-3.  
F : As E; 1% NH<sub>3</sub> added to give pH 8-9.  
G : Haemin (0.1 ml; 31.5/ug) plus water (3.0 ml); pH 7-7.5.  
H : As G; 1% NH<sub>3</sub> added to give pH 8-9.

synchronous transfer of haems from one haemoglobin to the other without free globins as such ever being present as intermediates.

In experiments where one wanted to compare two haemoglobins, exchanges of the sort of order obtained here would make little difference to their specific activities. Thus suppose we have two haemoglobins A and B with specific activities  $x$  and  $y$  respectively. Suppose also  $a\%$  of the label A is then transferred to B, and  $b\%$  of the label in B transferred to A. Then the new specific activity of A

$$= \frac{(100 - a)x + a.y}{100} = \underline{x - \frac{a}{100} (x - y)}$$

and the new specific activity of B

$$= \frac{(100 - b)y + b.x}{100} = \underline{y - \frac{b}{100} (y - x)}$$

If the haemoglobins A and B are present in equal amounts, then  $a = b$ . More generally, however,  $a/b = \frac{B}{A}$  where B is the amount of haemoglobin B, and A is the amount of haemoglobin A. We can now test the effect of the transfer of label from one hen haemoglobin to the other by substituting values for  $a$ ,  $b$ ,  $x$  and  $y$  in the above equations.

Let haemoglobin A be I, haemoglobin B be II and if we take  $b$  to be say 10%, then since I : II is approximately 3 : 1,  $a$  will be around 3.3%. These values for  $a$  and  $b$  are in fact higher than the average of those obtained in the experiments as shown in Table 3 where the average for  $a$  was 1.7% and for  $b$ , 5.8%. If the specific activity of I ( $x$ ) is then say



10% higher than II (y), it can be seen that the haem exchange will cause only an increase of 1% in the specific activity of haemoglobin II, since the new specific activity will be

$$y - \frac{10}{100} \left( y - \left[ y + \frac{10}{100} \cdot y \right] \right) = y + \frac{y}{100}$$

The new specific activity of I will be

$$x - \frac{3.3}{100} \left( x - \left[ x - \frac{9.2}{100} \cdot x \right] \right) = x - \frac{3.03}{100} \cdot x$$

i.e. there will be a reduction of only 0.3% in this case.

Thus the exchanges obtained in these experiments will not materially affect the specific activities of the two haemoglobins I and II for differences of the sort of order tested.

Although it has been assumed so far that the results of these experiments are attributable to exchange, it is true that contamination of one haemoglobin by the other might contribute to the observed specific activities. The results of experiments 7 and 8, however, seem to make this unlikely, and in any case the experiments have set a satisfactorily low limit to the maximum possible combined effect of cross-contamination and exchange. It would certainly be good experimental practice to work as quickly as possible and under conditions likely to minimize the oxidation of haemoglobin to methaemoglobin.

CHAPTER 4 : In vivo incorporation of iron-59  
into the haemins I and II.

### Introduction

In these experiments only the incorporation of iron-59 into the haemoglobins was studied. The work was not extended to the use of carbon-14 mainly because of the high cost involved since large amounts of this isotope would be required for whole body studies. Also, measurements were only made on the haemoglobins I and II and not on the membrane-bound haemoglobin. This was because it was thought that by the stage of red cell life at which in vivo observations could be made, it was unlikely that there would be a difference in specific activity between this fraction and the others if indeed there ever was a difference (cf. in vitro experiments).

### General pattern of experiments

Birds were injected subcutaneously with 5-8  $\mu$ c of ferric-59 citrate in isotonic saline. Blood samples (3 ml) were removed from the wing veins at intervals up to 41 days after the injection of the radioiron. The washed erythrocytes were lysed by the addition of water and the haemoglobins I and II separated as described previously. Observations were made on 3 birds initially. With two of these (No. 1 and No. 2) the specific activities were measured by doing iron determinations on the whole haemoglobins and then plating the iron as the ferrous dipyriddy mercuriodide complex before counting. In the other experiment (No. 3), the haemins I and II were first separated from the haemoglobins before the specific activities were found by iron determinations and plating of ferrous

dipyridyl mercuriiodide. All measurements were done in triplicate or quadruplicate and were corrected for radioactive decay.

### Results and Discussion

The results of the experiments were as shown in Table 4. Those for birds 1 and 2 have also been plotted in graphical form in Figures 6 and 7.

The incorporation into the separated haemoglobins over the cell "life span" was similar in pattern to that obtained by Ramsay (1966) who studied the incorporation of iron-59 into the total unseparated haemoglobins i.e. in the first few days the observed radioactivity in the circulating erythrocytes was very low but after this initial lag there was a very rapid increase in the haemoglobin specific activity this approaching a maximum value at 8-12 days after injection. In the birds used here this level was maintained until about 26 days and then subsequently fell. The time of this fall is in reasonable agreement with the known "life span" of the hen erythrocyte of around 28 days (Ottesen (1948)).

It would also seem to be quite clear that there were no significant differences between the specific activities of the two haemoglobins at any stage of the red blood cell "life span". However, since it was considered that any difference between the specific activities of the two haemoglobins would most likely appear at early stages of the red blood cell life cycle, observations were made on another 6 hens which were injected with iron-59 as before and blood samples removed in all cases less

In vivo iron - 59 incorporation

Table 4

Bird No. 1

Days after injection	0	5	8	13	19	22	26	33	36	41
Specific Activity of II(c/min/ug Fe)	0	11.7	15.4	16.7	16.7	16.6	15.5	10.0	10.8	10.9
Specific Activity of I(c/min/ug Fe)	0	12.0	15.1	16.4	17.4	16.8	15.7	9.99	10.6	10.9

Bird No. 2

Days after injection	1	4	8	14	17	21	29	36
Specific Activity of II(c/min/ug Fe)	1.09	7.66	9.36	10.8	11.1	11.7	9.40	8.29
Specific Activity of I(c/min/ug Fe)	0.96	7.65	8.94	10.8	11.1	11.3	9.19	7.99

Bird No. 3

Days after injection	43 hours	10	16	25	34
Specific Activity of II(c/min/ug Fe)	1.09	84.2	89.5	70.1	29.3
Specific Activity of I (c/min/ug Fe)	1.04	83.2	84.3	67.8	29.7

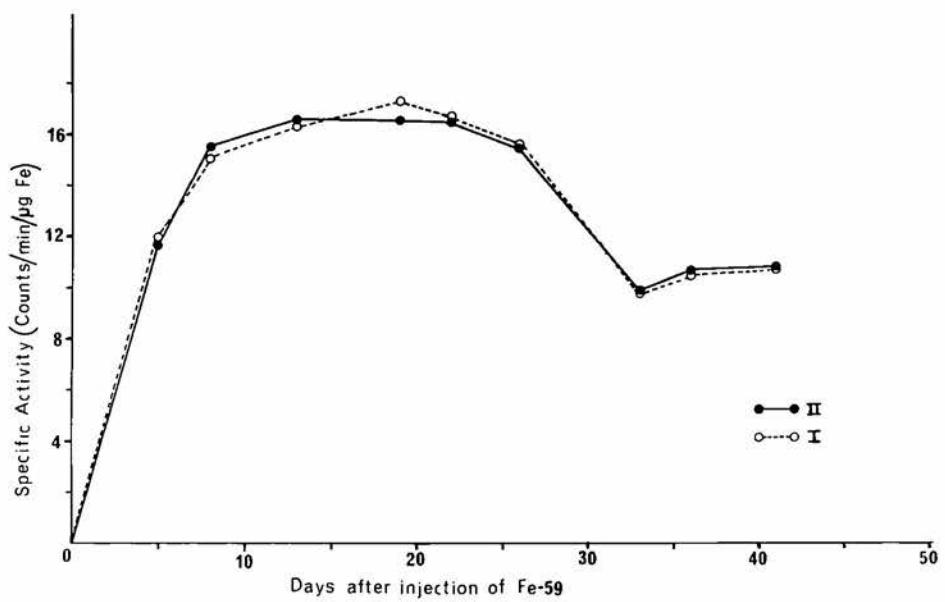


Figure 6

In vivo incorporation of iron-59 into the haemoglobins I and II (bird no. 1). The bird was injected subcutaneously with iron-59 (5 $\mu$ c). Samples of blood were removed from the wing veins at intervals after the injection. The haemoglobins I and II were then separated and their iron-59 specific activities determined.

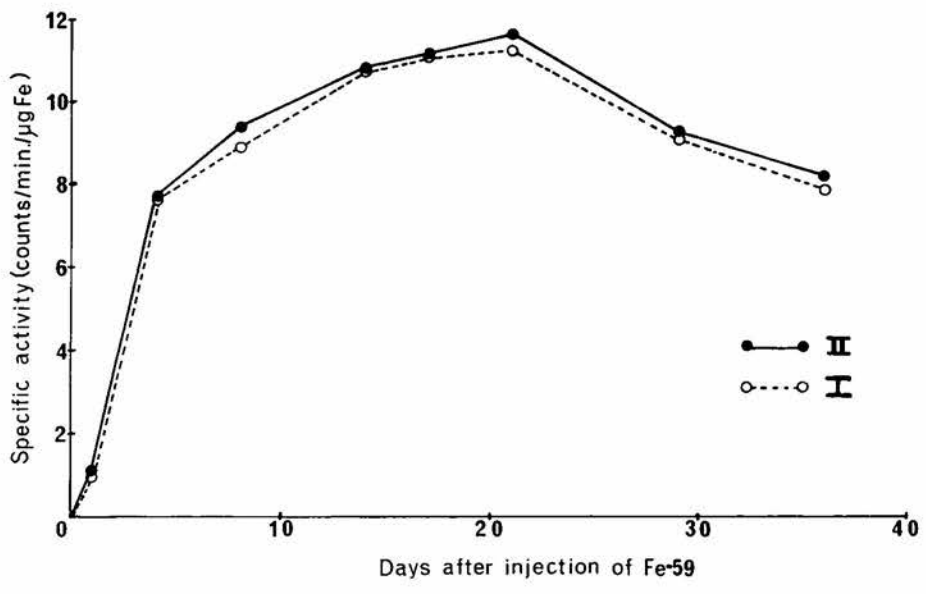


Figure 7

In vivo incorporation of iron-59 into the haemoglobins I and II (bird no. 2). The bird was injected subcutaneously with iron-59 (5 $\mu$ c). Samples of blood were taken from the wing veins at intervals after the injection. The haemoglobins I and II were then separated and their specific activities determined.

than 24 hours after the injections. The specific activities of either the haemins or the haemoglobins were measured and were as shown in Table 5. Again there seem to be no significant differences between I and II. Differences at this early stage might have been expected if say haemoglobins II and I were in different erythrocytes which were released into the circulation at differing stages of cell maturation, or, if in the same red cells as is more likely, they were synthesised in the marrow at different stages in the red cell line. Again, if the relative proportions of I and II were different in circulating erythrocytes of different ages i.e. if there was intravascular continuation of the synthesis of one of the haemoglobins, differences in specific activities should be found in in vivo observations made a few hours after injection.

The results obtained, therefore, would seem to be consistent with any of three main possibilities which are not necessarily mutually exclusive.

- (i) The two haemoglobins draw haem from the same "pool" at the same time and at relative rates proportional to the relative concentrations or rates of synthesis of the globins.
- (ii) There is exchange taking place between the haems of different haemoglobin molecules in the cell.
- (iii) There is exchange taking place between the iron atoms of different haemoglobin molecules in the cell.

Since it is generally believed that haem is synthesised in the

In vivo iron - 59 incorporation

TABLE 5

Bird No.	Hours after injection	Samples Analysed	Specific Activity of II(c/min/ug Fe)	Specific Activity of I(c/min/ug Fe)
4	6	haemins	0.71	0.78
5	6	haemins	1.53	1.69
6	18	haemoglobins	1.24	1.34
7	19	haemoglobins	1.32	1.28
8	17	haemins	0.11	0.12
9	18	haemoglobins	0.065	0.072

mitochondria (Goldberg and Lochhead (1960); Sano and Granick (1961)) while globin is synthesised elsewhere in the cell, (i) seems from the present results a fairly reasonable hypothesis. However, (ii) and (iii) cannot be excluded even although it has been shown already that haem exchange does not take place to a significant extent during the separation and isolation procedures. Those possibilities are considered more later.

CHAPTER 5 : In vitro incorporation of iron-59 and  
(2-carbon-14) glycine into the haemins  
I and II and into the haemin from the  
membrane-bound haemoglobin.

Section A : Incorporation of iron-59 and (2-carbon-14)  
glycine into haemin in incubations of immature  
avian red cells.

Introduction

Since erythrocytes only appear in the circulation at a comparatively late stage in their development, usually some three to four days after the uptake of iron-59 by the marrow in the hen, the use of immature red cells obtained from hens which had previously been subjected to massive venesection might give some information on the state of affairs at an earlier stage in erythrocyte development. Strictly speaking one has no choice other than to use blood from anaemic hens because although the circulating erythrocytes of the hen retain some ability to synthesise protoporphyrin from radioactive glycine (Dresel and Falk (1956(a)) the mature cells appear to be unable to take up iron from the plasma (Ramsay (1966)). It is only after preliminary bleeding that cells are obtained in the circulation which are able to take up iron. With in vitro work also, it now becomes practicable to study the incorporation of (2-carbon-14) glycine as well as iron-59.

Double-labelling experiments seemed potentially valuable from two points of view. Firstly, they might be able to distinguish between two of the possible conclusions made from the in vivo work, namely between whether there was exchange of whole haems between haemoglobin molecules or merely exchange of iron atoms. Secondly, information should be obtainable about the existence, relative sizes or relative rates of

utilisation of intracellular pools of glycine, protoporphyrin or iron.

### Experimental methods

General procedure :- Hens were made anaemic by removing blood from the jugular veins while the hens were under ether anaesthesia. It was found that if on each of days 1 and 2, 20-25 ml of blood were removed, then by day 4 the degree of reticulocytosis was such that the blood was very suitable for experimental studies. The blood for the incubations was removed from the wing veins, jugular veins or the heart in all cases without anaesthesia. Portions of the heparinised blood (generally 15-20 ml when two simultaneous incubations were being performed but up to 50 ml in other experiments where the whole blood sample was processed at the end of an incubation) were measured out into conical flasks of convenient size (25, 50 or 100 ml). If the incubations were to last longer than 2 hours, penicillin (0.02 mg/ml) and streptomycin (0.02 mg/ml) were added to the blood to stop bacterial growth. Flasks were then incubated in air at 37°C for 3-4 minutes on a water bath which was fitted with a shaking apparatus for keeping the blood mixed. To each flask was added an aliquot (usually 0.5 ml) of a radioactive mixture consisting of 2-carbon-14 glycine in 1% NaCl, ferric-59 citrate, and plasma from the same hen of which the blood was incubated. In general, the final amounts of radioactivity were 0.08  $\mu$ c iron-59 ( $1.1 \times 10^{-4}$  -  $6.8 \times 10^{-4}$   $\mu$  moles iron) per ml blood, and 0.57  $\mu$ c carbon-14 ( $1.8 \times 10^{-2}$  -  $2.6 \times 10^{-2}$   $\mu$  moles glycine) per ml blood. Incubation of the blood was then carried out in air at 37°C for periods of usually less than 2 hours but on occasion up to 8 hours. At

intervals as required, samples of blood (3.5 ml) were removed from the flasks. When two incubations were being carried out at one time all the additions and the taking of samples were arranged so that there was a constant time lag between the performance of the operations in the different flasks. The samples were immediately pipetted into ice-cold saline in centrifuge-tubes chilled in melting ice. Subsequent washing, lysis and separation procedures were carried out as described in the Materials and Methods section, Chapter 2. The lysis was performed using the saponin lysis procedure so that the three haemoglobin fractions I, II and the membrane-bound haemoglobin were obtained. The radioactive assay of the haemins from these haemoglobins was carried out using the double isotope assay method as already described.

From 4 duplicate incubations, each incubation involving the taking of 4 serial blood samples it has been calculated that the percentage standard deviation of a haemin specific activity from one incubation to another was  $\pm 5.1\%$  for iron-59 and  $\pm 5.2\%$  for carbon-14 counts. For the ratio of the carbon-14 counts : iron-59 counts in a haemin sample, the percentage standard deviation was  $\pm 5.9\%$ . A difference is considered significant if it is 3 times the relevant percentage standard deviation.

### Results

(1) General pattern of results :- Three different types of results were obtained from these in vitro incubations. In all three groups, except for very occasional completely random variations, there is no difference,

within the limits of the experimental error, between the specific activities of the haemins I and II for both carbon-14 and iron-59 counts at any stage of incubation from 15 minutes to eight hours after the addition of the radioactive tracers. The variation in the type of result was due to variations in the specific activities of the haemin from the membrane-bound haemoglobin (henceforth called haemin M) relative to those of the haemins I and II.

- (a) The specific activities of haemin M were the same (for both iron-59 and carbon-14) as the haemins I and II at all stages of the incubation.
- (b) The specific activities of the haemin M were greater (for both iron-59 and carbon-14) than those of the haemins I and II at early stages of incubation but were not significantly different at later stages.
- (c) The specific activities of haemin M were greater (for both iron-59 and carbon-14) than those of the haemins I and II at all stages of incubation.

Out of a total of 15 incubations in which serial sampling was used, 5 were of type (a), 6 of type (b) and 4 of type (c). All three classes have included examples of samples taken at periods ranging from 15 minutes to several hours after commencement of the incubations. However, one feature which all three types have in common is that the ratio of carbon-14 : iron-59 counts is the same within the limits of experimental error in all three haemins at each stage of incubation i.e. the difference in

carbon-14 specific activity between haemin M and the haemins I and II is paralleled by a similar proportionate difference in the iron-59 specific activities.

Examples of some typical results are shown in the Figures 8-10. Figures 8 and 9 are instances of iron-59 and carbon-14 incorporation into the haemins according to types (b) and (c) respectively, both over a 90 minute incubation period. The incorporation of iron-59 into haemins I and II usually rises linearly from zero time during this 90 minute time period whereas the incorporation of carbon-14 tends to lag initially before also rising linearly. The changes in the ratios of the carbon-14 : iron-59 counts of the haemins during the course of the 90 minute incubations are also shown. In every experiment except one, this ratio has increased with time.

In Figure 10 where the specific activities of haemin M were the same as those of haemins I and II at every stage of incubation, the immature cells are still capable of synthesising haemin even after 8 hours incubation. In the experiment shown, the carbon-14 : iron-59 ratio was still rising at this time. In several other experiments, however, it was found that this ratio levelled off after 2-4 hours.

(ii) Reincubation in unlabelled plasma:- The effect of incubating cells in labelled plasma for a short period and then reincubating the washed cells in unlabelled plasma obtained from the same bird has also been examined. Blood (20 ml) was incubated in the usual way for 15 minutes, after the

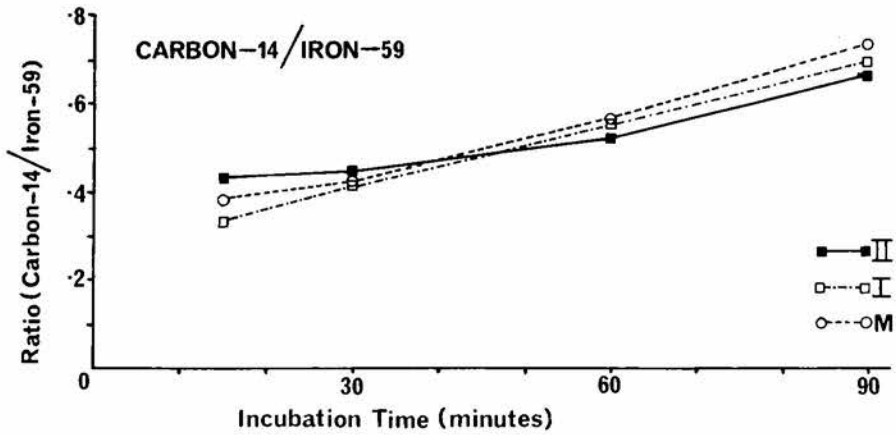
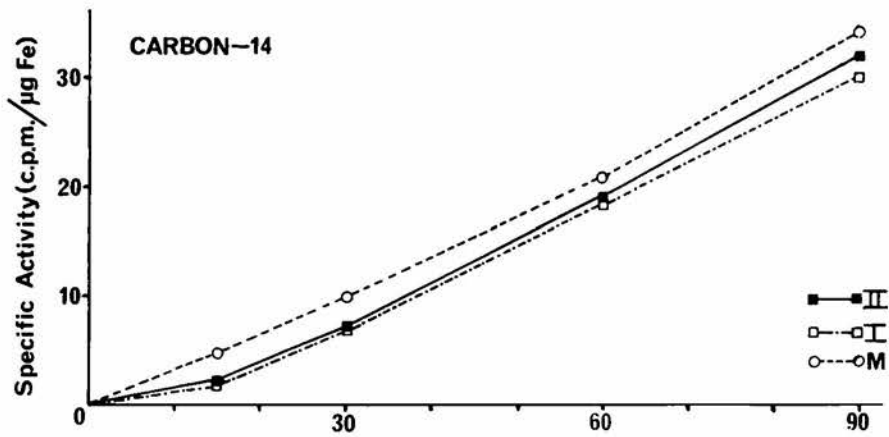
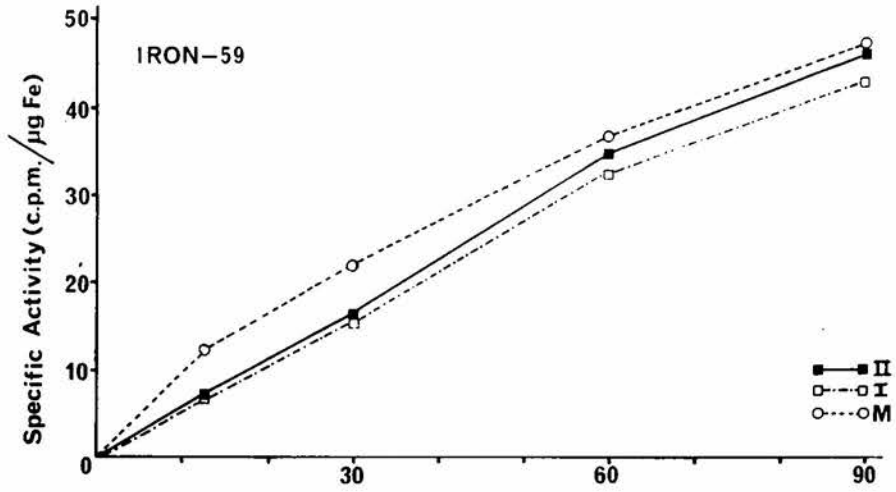


Figure 8

In vitro incorporation of iron-59 and 2-carbon-14 glycine into the haemins from haemoglobin II, haemoglobin I and the membrane-bound haemoglobin (Type (b); 90 minute incubation).

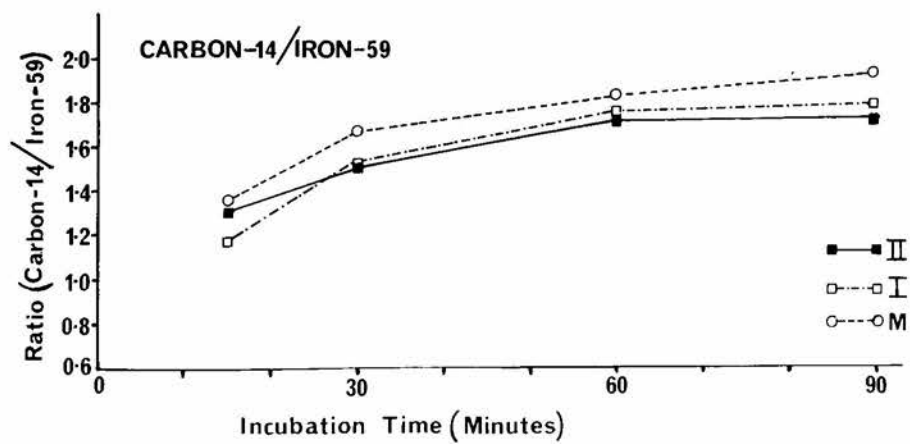
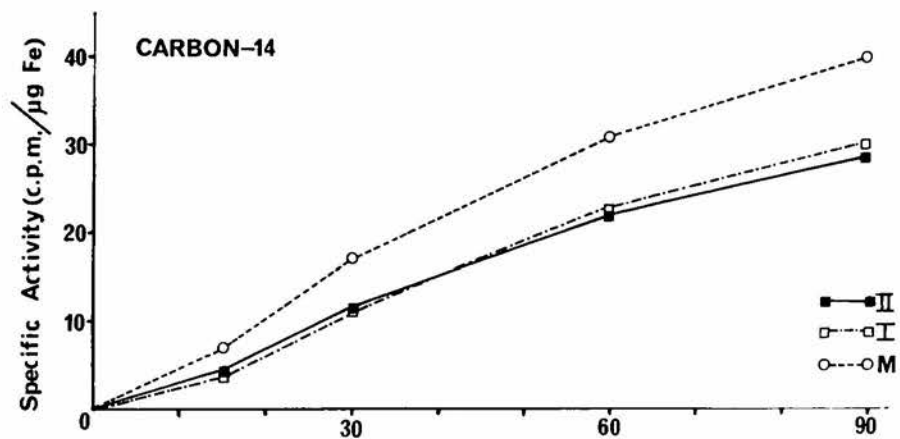
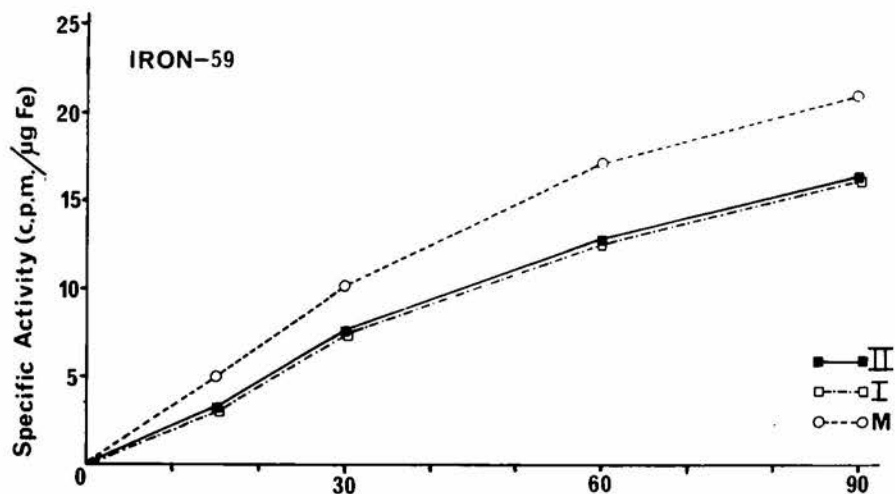


Figure 9

In vitro incorporation of iron-59 and 2-carbon-14 glycine into the haemins from haemoglobin II, haemoglobin I and the membrane-bound haemoglobin (Type (e); 90 minute incubation).

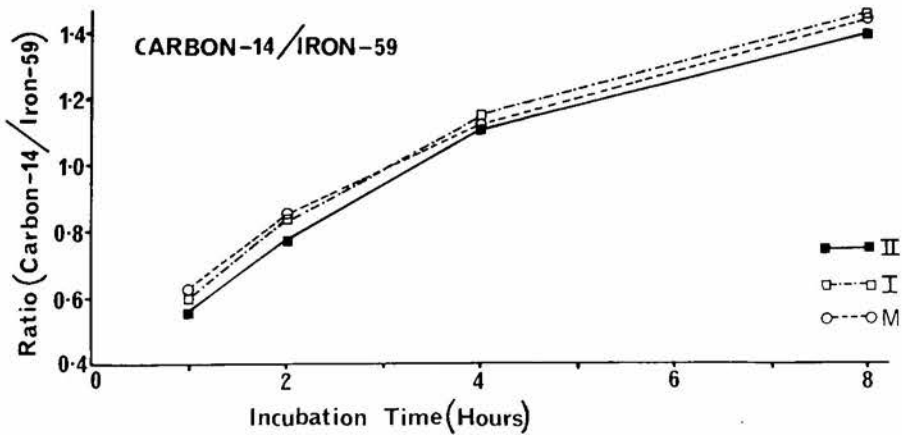
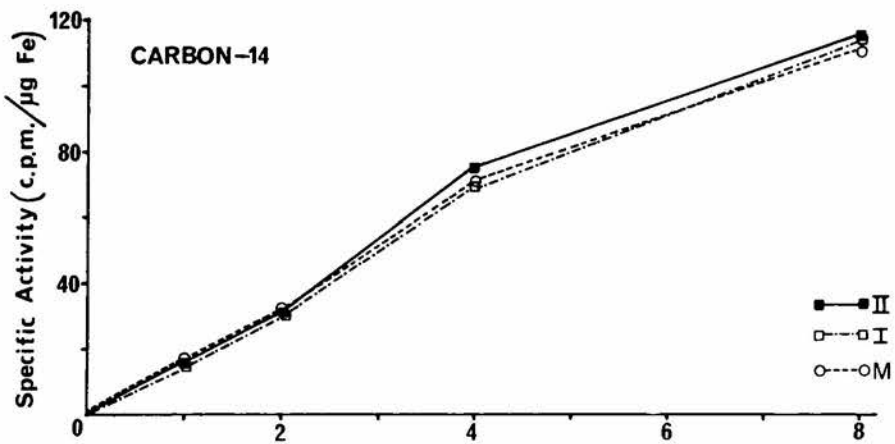
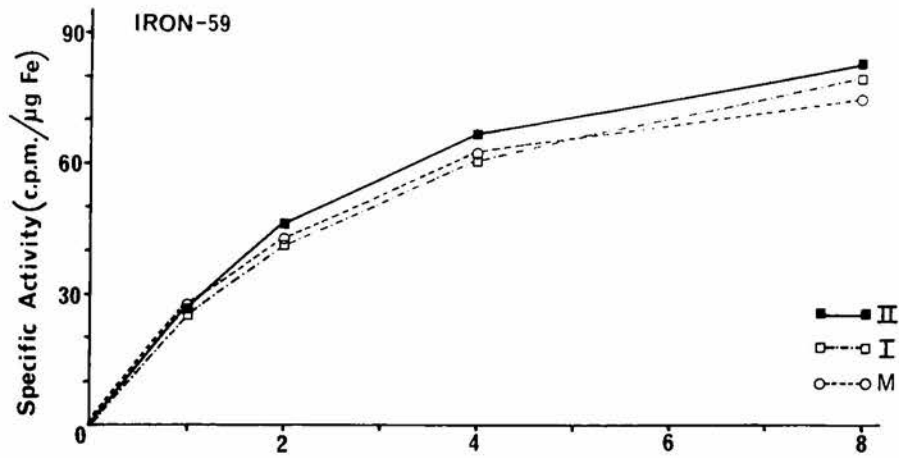


Figure 10

In vitro incorporation of iron-59 and 2-carbon-14 glycine into the haemins from haemoglobin II, haemoglobin I and the membrane-bound haemoglobin (Type (a)); 8 hour incubation).

addition of the iron-59 and carbon-14. A sample was taken at this time for analysis. The remainder of the blood was then as quickly as possible chilled and centrifuged to remove the plasma. The cells were washed once with ice-cold 1% saline and resuspended in the same volume of unlabelled plasma as the volume of labelled plasma originally removed. The operations of washing and resuspension, carried out at 4°C, took 25 minutes to perform. The cells in the unlabelled plasma were then reincubated in air at 37°C as usual and samples removed at 15, 30, 60 and 120 minutes after the recommencement of the incubation.

The results of this experiment are shown in Figure 11. Distinct differences on reincubation of the cells were found between the pattern of incorporation of iron-59 and carbon-14 into the haemins. The iron-59 specific activities of I and II rose on average by 42% during the 2 hour reincubation period, the activities approaching close to this maximum level after only 30 minutes. On the other hand, however, the carbon-14 specific activities of I and II increased by 300% during the 2 hour reincubation and were still in fact increasing at the end of this period.

### Discussion

The incorporation of iron-59 and carbon-14 into haemin M has tended to be higher than the incorporation into the haemins I and II. However, the haemins I and II, which constitute almost 90% of the total erythrocyte haemin, have consistently, except for the occasional random variation, had the same specific activities as each other for both iron-59 and carbon-14

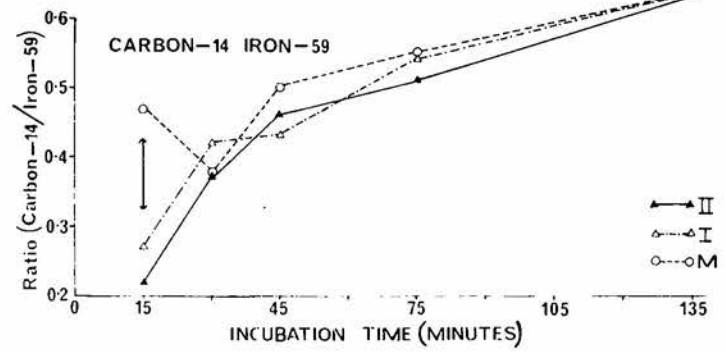
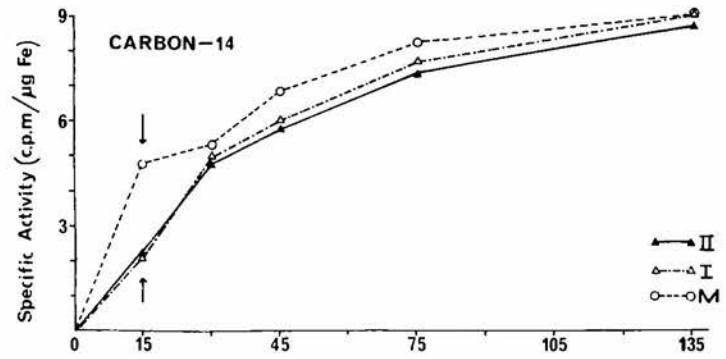
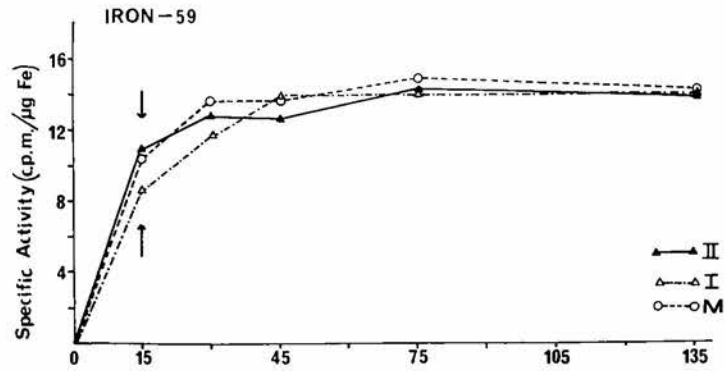


Figure 11

Incorporation of iron-59 and 2-carbon-14 glycine into the haemins II, I and M during the reincubation, in unlabelled plasma, of cells which had been previously incubated in labelled plasma. The arrows indicate the point at which the change from labelled to unlabelled plasma took place.

at every stage of incubation. At the outset of these in vitro experiments, as already stated, one of the objects had been to try and distinguish between two of the possible conclusions from the in vivo work, namely, that iron exchange or haem exchange was taking place between the haemoglobins I and II. The similarity of the carbon-14 specific activities of haemins I and II at each stage of in vitro incubation, leads to the conclusion that exchange of iron atoms alone is not the fundamental reason for the equality of the specific activities. Haem may be being drawn from a common haem pool for the formation of the haemoglobins I and II at rates proportional to their relative amounts (or rates of globin synthesis) but if this is so the present results give no information as to whether iron exchange or haem exchange is also taking place between the haemoglobins. If, on the other hand, haem is being incorporated into the haemoglobins at rates which are not proportional to their relative amounts, then the present results, where both the carbon-14 and iron-59 specific activities of haemins I and II were equal, indicate that there must be an exchange of intact haem molecules.

The observations on haemin M are parallel in some respects to those which have been made by Allen (1969) on the incorporation of radioactive amino acids into the corresponding globin. He incubated washed cells in an artificial medium and found that throughout his experimental period the globin from the membrane-bound haemoglobin had a higher specific

activity than the globins I and II. He did not obtain the variability which has been found in the specific activities of haemin M relative to haemins I and II in the present work.

The membrane-bound haemoglobin accounts for only about 11% of the red cell iron or 15% of the total quantity of haemoglobin I if it is right to regard the two as different pools of the same compound. One can try to account for the observations where haemin M has higher specific activities than haemins I and II always remembering that the globin from the membrane-bound haemoglobin is also more highly labelled than globins I and II. None of these hypotheses are fully satisfactory however, it being especially difficult to explain the variations from experiment to experiment in the present work.

One hypothesis could be based on the ideas of Hammel and Bessman (1964). These workers, using 0.25 M sucrose and saponin for lysis, and D'Amelio and Salvo (1959) using 0.25 M sucrose and freezing and thawing for lysis, believe that avian erythrocytes can be lysed to give a sediment containing essentially intact nuclei and that the haemoglobin extracted from this residue is haemoglobin normally present in the nuclei in the intact erythrocytes. Hammel and Bessman (1964) have studied the incorporation of amino acids into this haemoglobin in "nuclei" isolated both aqueously and non-aqueously from pigeon reticulocytes and from their results have concluded that in the avian red cell, haemoglobin is synthesised in the nucleus and then transferred to the cytoplasm. If their

ideas were true they might provide an explanation for the higher labelling of haemin M. However, in view of the work of Allen (1969) who was unable to reproduce the results of Hammel and Bessman (1964) on isolated nuclei, nuclear protein synthesis is not considered a very likely explanation of the present results.

Two further hypotheses can be put forward which depend on the idea that the adherence at any rate of the major part of the membrane-bound haemoglobin to the cell sediment is essentially an artifact of the haemolytic technique, with haemoglobin I being adsorbed in preference to haemoglobin II. The first of these is based on the fact that the lysate sediment will contain fragments of endoplasmic reticulum. It is possible that these fragments might have newly synthesised completed haemoglobin molecules attached to them. The membrane-bound haemoglobin fraction would therefore naturally on elution from the sediment be more highly labelled than haemoglobins I and II from the lysate supernatant. However, according to Felicetti, Colombo and Baglioni (1966), haem does not combine with globin chains while these are being synthesised on the polyosomes. Their results indicate rather that the association of haem and globin chains occurs after the peptide chains have been released.

The second makes the further assumption that newly synthesised haemoglobin is only able to mingle slowly with the main bulk of the haemoglobin in the cell and so remains in the vicinity of the site of synthesis. This haemoglobin might then be preferentially trapped by the cell fragments during lysis. On balance this would seem to be the most

likely hypothesis even if only because it is not easy to disprove it.

It is even more difficult to try and explain the variation in the specific activities of haemin M relative to haemins I and II in the present experiments. Duplicate incubations on the same samples of blood gave results which were in close agreement with one another, and, as far as possible, incubations were carried out under identical conditions. The variation, therefore, would seem to be due to some factor other than experimental technique. As it is believed that haem is synthesised in the mitochondria whereas globin is synthesised in a different part of the cell, the variability may be an expression of variation in exchange between free haem and haem in haemoglobin in the transfer of the former to the site of globin synthesis. It may also be an expression of variation in the rate of diffusion of newly synthesised haemoglobin I throughout the cell.

The rise in the ratio of carbon-14 : iron-59 counts during the course of an incubation is interesting. It is consistent with the idea that there exists a small pool of protoporphyrin in the cells (Schwartz and Wikoff (1952)). Another possibility is that the intracellular pool of glycine is larger relatively than that of iron. Labelled glycine entering the cell is being more diluted by intracellular glycine than the iron-59 is by intracellular iron. The intracellular ratio of the specific activity of glycine : specific activity of iron will increase with time and so therefore will the ratio of the carbon-14 counts : iron-59

counts in the haemins. There may of course also be small amounts of all the intermediates in the pathway of haem synthesis existing free in the cell. These too would have a delaying effect on the incorporation of the carbon-14 label into the haemins. However, it should be noted that the possibility is not excluded that iron is gaining access to the cells more rapidly than glycine and no account has been taken either of the relative amounts of iron and glycine in the plasma.

The result of the experiment involving the reincubation of washed cells in unlabelled plasma after their prior incubation in labelled plasma, where there was an increase of 300% in the carbon-14 specific activities of the haemins during the reincubation period in contrast to one of only 42% in the iron-59 specific activities, is a further indication that the organic precursors of haemin are found free in the cell. These must be present in amounts sufficient to cause a large and continuous rise in the haemin carbon-14 specific activities. The intracellular iron pool must be relatively much smaller because of the relative size of the increase in the haemin iron-59 specific activities.

Another interesting feature of the carbon-14 : iron-59 ratio is that this ratio is the same for haemin M as for the haemins I and II. This result on first sight is an indication that the haem M is coming from the same source in the cell and at the same time as haems I and II but is being withdrawn into the membrane-bound haemoglobin at a rate which is greater, in proportion to the amount of this haemoglobin, than is the rate of incorporation of haem into the haemoglobins I and II, relative to the

amounts of haemoglobins I and II. This idea is in conflict with two of the hypotheses put forward earlier to explain the nature of the membrane-bound haemoglobin, viz. that it is haemoglobin I trapped by the cell particles during lysis and preferentially containing some more newly synthesised haemoglobin than the lysate supernatant. The carbon-14 : iron-59 ratio of all the haemins increases during the incubations i.e. the more newly synthesised haem has a higher ratio. That there is no difference might possibly be taken as an indication that the globin I and the globin from the membrane-bound haemoglobin are separate entities withdrawing haem from a common intracellular haem pool at the same time but at different relative rates. However, it is quite possible that the limitations of the experimental method are such that any expected differences in the carbon-14 : iron-59 ratios will not be observed.

## Section B : The effect of puromycin on haem synthesis

### Introduction

In the literature there is evidence that haem inhibits its own synthesis. Thus, Burnham and Lascelles (1963), using both intact cells and purified enzyme preparations from *Rhodospseudomonas spheroides* have shown that haem inhibited porphyrin synthesis from glycine but not from  $\delta$ -aminolaevulinic acid (ALA), its effect being on the activity of the enzyme aminolaevulate synthetase. Similarly Karibian and London (1965),

studying incubations both of rabbit reticulocyte lysates and of the intact cells, have found in a large number of experiments that haemin ( $10^{-4}M$ ) added to their incubations inhibited the incorporation of (2-carbon- $^{14}$ ) glycine into haem by 48.6% ( $\pm 2.0\%$  s.e.) whereas the incorporation of ALA-4- $C^{14}$  was only inhibited by 12.2% ( $\pm 4.8\%$  s.e.). Thus it seems likely that free haem inhibits its own synthesis by means of a feedback effect on the synthesis of ALA from glycine. It was therefore thought that it might be interesting to test the effect of the suppression of globin synthesis on the incorporation of iron-59 and (2-carbon- $^{14}$ ) glycine into the haems in a multiple haemoglobin system. This type of experiment might possibly have given useful information on the metabolic relationship between the haemoglobins as it provides a means of separating haem and globin synthesis (Grayzel, Fuhr and London (1967)). In the present work globin synthesis is suppressed by the use of puromycin which should not have a direct effect on haem synthesis. However, a reduction or lack of globin available for combination with haem to form haemoglobin will lead to the intracellular accumulation of free haem. This in its turn should lead to the feedback inhibition of haem synthesis. In the present experiments it is the haemins prepared from the separated purified haemoglobins which are studied. Therefore, the observed effect of puromycin on the incorporation of iron-59 and (2-carbon- $^{14}$ ) glycine into the haemins will depend not only on the availability of preformed intracellular globin, but also on the extent to which the accumulated intracellular haem exchanges with haem in haemoglobin.

In both cases puromycin should cause a reduction in the incorporation of label into the haemins prepared from the whole haemoglobins.

### Methods and Results

Duplicate incubations were set up in the usual fashion. To one incubation flask a solution of puromycin in 1% NaCl (1.0 mM - 1.07 mM) was added to give a final concentration of 0.057 - 0.070  $\mu$  moles puromycin per ml blood. To the other was added an equivalent volume of 1% NaCl. Both these additions were made during a pre-incubation period (3 minutes) prior to the addition of the iron-59 and (2-carbon-14) glycine.

The effects of puromycin on the specific activities of the haemins during a 90 minute incubation are shown in Figures 12 (a), (b). The inhibition of incorporation of iron-59 and carbon-14 into all three haemins can be quite clearly seen. In Table 6 are shown the results of 5 experiments in which the incorporation of iron-59 and carbon-14, in incubations to which puromycin was added, were compared with controls. In these experiments the average inhibitions were in the range 22-45%.

In two of the above experiments, samples of globin I have been prepared from the control incubation and from the incubation to which puromycin had been added. Their carbon-14 specific activities were measured and it was found that the incorporation of carbon-14 had been inhibited

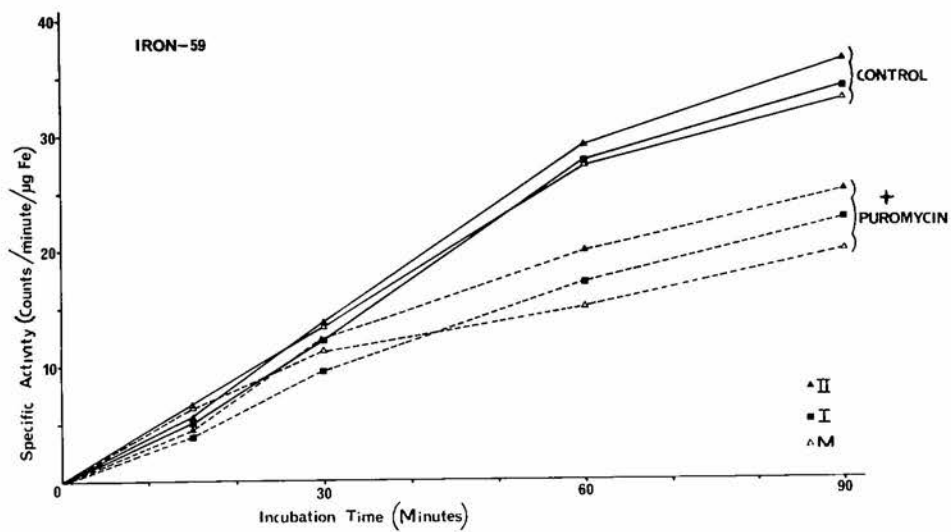


Figure 12 (a)

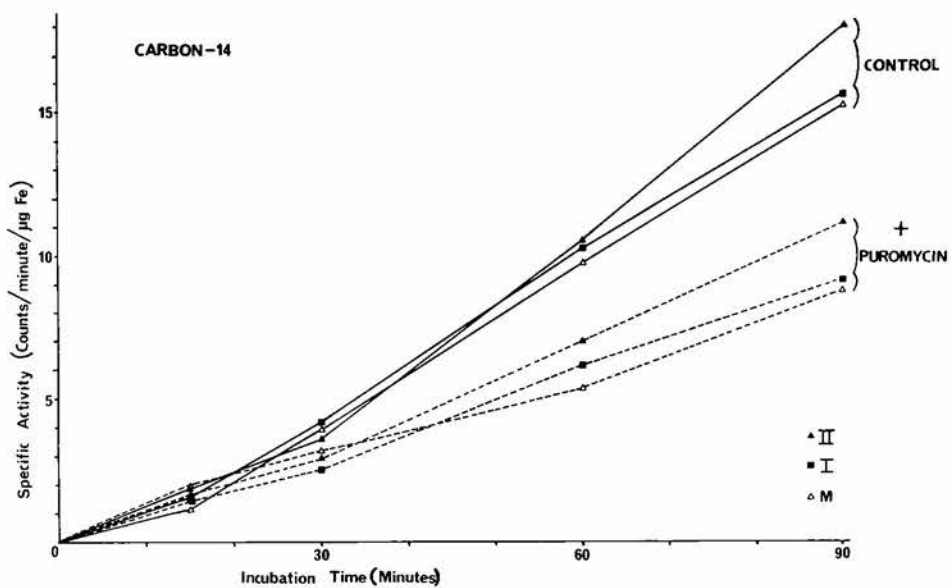


Figure 12 (b)

Figures 12(a) and 12(b)

Effect of puromycin on the incorporation of iron-59 and 2-carbon-14 glycine into the haemins II, I and M.

**TABLE 6****Inhibition of haem synthesis by puromycin**

Experiment No.	Incubation Time	% inhibition of $^{59}\text{Fe}$ incorporation			% inhibition of $\text{C}^{14}$ incorporation		
		M	II	I	M	II	I
1	90	25.4	29.5	34.9	26.6	39.8	44.7
2	90	40.0	30.8	33.7	43.0	38.1	41.8
3	75	8.6	28.2	33.4	16.6	29.2	46.4
4	120	12.7	16.5	24.8	1.8	24.4	28.0
5	90	25.3	40.5	51.0	25.0	45.6	60.1
Average		22.4	29.1	35.6	22.6	35.4	44.2

by 93% and 99% in the two incubations containing puromycin. This has confirmed the observations of Allen (1969) who used puromycin in the same concentration and found that it caused virtually complete and immediate cessation of the incorporation of radioactive amino acids into the globins I and II.

### Discussion

In these experiments puromycin has caused an almost complete inhibition of globin synthesis but only partial inhibition of the incorporation of iron-59 and carbon-14 into the haemins. On the average this inhibition is less than 50% during the incubation periods used. This is very similar to the result obtained by Grayzel, Fuhr and London (1967) who found that in the presence of puromycin or cycloheximide, both of which caused almost complete inhibition of protein synthesis, the incorporation of 2-carbon-14 glycine into haem in incubations of rabbit reticulocytes was inhibited by 50% in 4 hours. It should be emphasised, however, that these workers were measuring the incorporation into total cellular haem and not into the separated purified haemoglobins as in the present work.

The most likely explanation of these results is that in the absence of newly synthesised globin free haem tends to accumulate in the cell and inhibits haem synthesis by inhibiting the formation of ALA from glycine. Labelled haem can then only be incorporated into haemoglobin by combining with preformed globin or by exchanging with haem in haemoglobin.

It might be argued however, that puromycin because it stops the synthesis of protein is causing a reduction in the availability of one or more of the enzymes involved in the synthesis of haem. An objection to this is that, in view of the rapid effect of puromycin, it would presumably necessitate a fairly high turnover rate for the enzyme(s) concerned. Also even if this idea were true the fact remains that in the presence of puromycin, haem is still getting into haemoglobin to an extent only reduced by 30 - 50% of what it is in the absence of the antibiotic. This effect still must depend on the availability of preformed globin or on haem exchange.

It was thought worthwhile to check the effect of ALA on the puromycin inhibition with a view to gaining further evidence on the ideas put forward in this section. Samples of blood (3.5 ml) were incubated with iron-59 and (2-carbon-14) glycine as usual for 90 minutes, in the presence of puromycin (final concentration 0.061  $\mu$  moles/ml Blood) or ALA (made up by dissolving ALA hydrochloride in water and neutralising the solution with NaOH; final concentration  $0.83 \times 10^{-2}$  m.moles/ml blood) added as shown in Table 7. To control incubations

TABLE 7

Effect of ALA on the inhibition by puromycin of the incorporation of iron-59 and 2 carbon-14 glycine into the haemins M, II, I:

(a) Fe<sup>59</sup> incorporation

Incubation Number	Puromycin	ALA	Haemin specific activities(counts/min/ug Fe)		
			M	II	I
1,3	0	0	15.9(15.2,16.7)	9.59(9.09,10.09)	11.9(11.1,12.7)
2,4	+	0	11.9(11.0,12.8)	5.72(5.33, 6.10)	5.77(6.17,5.37)
5,7	0	+	24.8(26.9,22.7)	11.6(11.4,11.7)	14.6(14.3,14.8)
6,8	+	+	19.6(19.4,19.9)	7.84(7.25,8.42)	9.26(9.09,9.42)

(b) C<sup>14</sup> incorporation

Incubation Number	Puromycin	ALA	Haemin specific activities(counts/min/ug/Fe)		
			M	II	I
1,3	0	0	25.4(24.8,26.0)	14.2(13.5,14.9)	20.5(19.1,21.8)
2,4	+	0	19.1(18.1,20.0)	7.67(8.57,6.76)	8.15(8.17,8.12)
5,7	0	+	23.3(24.1,22.4)	9.22(8.91,9.53)	12.3(11.4,13.2)
6,8	+	+	14.1(14.9,13.3)	5.15(4.58,5.72)	6.01(5.77,6.24)

+ = present; 0 = absent

Figures shown are averages of duplicate incubations -

- duplicates shown in brackets (a, b).

were added equivalent volumes of 1% NaCl. It can be seen that the addition of ALA to an incubation carried out with or without the presence of puromycin causes an increase in the incorporation of iron-59 into all three haemins and a decrease in the observed carbon-14 incorporation. These results can be interpreted in the following way :- Unlabelled ALA is gaining access to the cells and is acting as a precursor for haem, causing dilution of the carbon-14 label in the haemins. The main site of feedback inhibition in the metabolic pathway leading to the synthesis of haem is being bypassed. Thus the addition of ALA to a puromycin incubation is causing an increase in haem formation and leading to a rise in the observed incorporation of iron-59 into the haemins, either because of the greater chances of haem-preformed globin combinations taking place or because of the increased possibilities of exchange between free haem and haem in haemoglobin. In an incubation in which no puromycin is present, the increased haem formation caused by the addition of ALA may also lead to a stimulation in the synthesis of globin (Bruns and London (1965); Grayzel, Horchner and London (1966)) and a consequent increase in haemoglobin formation as measured by the iron-59 incorporation. Thus although these experiments involving the addition of ALA to incubations do not provide much new information the results of them are not at variance with those already obtained.

It is instructive to calculate approximately the amount of haem synthesised in the course of a typical in vitro incubation, using iron-59 incorporation into haemoglobin as a measure of the haem formed. If one

assumes a plasma iron concentration in the anaemic hen blood of 50  $\mu\text{g}/$  100 ml plasma, a packed cell volume of 25% and an intracellular iron pool amounting to about 2% of the total erythrocyte iron, it can be shown that about 5  $\mu\text{g}$  of haem is synthesised per ml. of blood in the course of an incubation lasting 90 minutes. This is equivalent to 125  $\mu\text{g}$  of globin/ml blood or about 0.14% of the total cellular haemoglobin. It is not unreasonable to expect a preformed amount of this order since Winterhalter and Huehns (1963) by lysing human erythrocytes in the presence of iron-59 haemin have found 0.1% of their cell protein to be free globin. Thus it would seem that the amount of haem synthesised during a puromycin incubation does not of itself permit one to choose between haem exchange and the existence of preformed globin as being the sole explanation for the observed incorporation of iron-59 and carbon-14. However, an indication that exchange may not be taking place has been provided by Waxman, Freedman and Rabinovitz (1967). These workers added radioactive haemin (25  $\mu\text{M}$ ) to chilled rabbit reticulocytes and found that it was incorporated rapidly into haemoglobin in the cold ( $3 \times 10^{-5}$   $\mu$  moles haemin/mg haemoglobin). On increasing the haemin concentration to 50  $\mu\text{M}$  there was no rise in labelling of the haemoglobin. Furthermore there was also no increase in haemoglobin label if the cells were then incubated at 37°C in the presence of cycloheximide although there was if the cells were incubated under conditions where protein synthesis was allowed to take place. It would therefore seem that the haemin rapidly incorporated was due to the presence of free globin in the cells and that the lack of any subsequent increase in the label was an indication that intracellular exchange between haemin and haemoglobin does not take place.

The experiments 15 and 16 in the Chapter on in vitro haem exchange may also be a further indication that exchange does not take place between free intracellular haem and haemoglobin. In these experiments iron-59 labelled haemoglobin was added to an unlabelled whole lysate and the mixture incubated for 3 hours at 37°C. A haemolysate can continue to synthesise haem for several hours (Dresel and Falk (1956(b)) in the face of almost complete cessation of protein synthesis (Lamfrom and Knopf (1964)). It is possible therefore that labelled haemoglobin was being incubated in the presence of accumulating free haem. Thus the results of these experiments with lysates may be indicating that actual intracellular exchange does not take place.

CHAPTER 6 : Non-Haemoglobin Iron

## Introduction

There are several indications in the literature that avian erythrocytes contain significant amounts of non-haem iron. The first observation on this possibility came from Winegarden and Borsook (1933) who used a dialysis method to separate the non-haem iron and got a figure of 4-6% of the erythrocyte iron in the domestic fowl being non-haem. Schultze and Elvehjem (1934) found that haemoglobin values calculated from iron content of chicken erythrocytes were higher than the haemoglobin values obtained from the Newcomer method (acid haematin method) and from the difference got a figure of 12.7% for the non-haem iron. Burmester (1936-37) worked out the non-haem iron in a similar way from the difference between the total iron as determined by analysis and haemoglobin iron determined from oxygen capacity. He found that in the chicken the amount of non-haem iron increased as the percentage of immature cells increased, finding about 4.6% in non-anaemic birds and about 26.1% in very anaemic ones. In contrast to this, Rostorfer (1949) who, however, used ducks found with several different methods 0.8 - 6.0% of the iron in the duck erythrocyte to be non-haem, there being no significant difference in this amount between non-anaemic birds and those made anaemic by bleeding.

In view of these observations it was decided therefore to examine more closely the protein fractions A (pH 6.3 eluate) and B (pH 6.9 eluate) obtained from the CM-cellulose procedures described previously.

### Iron content of pre-haemoglobin fractions A and B

The fractions A and B were analysed for their iron contents. In ten experiments using a water lysate supernatant or a saponin lysate supernatant or a total cell haemoglobin fraction obtained by combining the saponin lysate supernatant with the membrane-bound haemoglobin fraction, it was found that both A and B contained iron (Table 8). As can be seen the average percentages of iron in A and B were 0.3% (range 0.0 - 0.6) and 1.2% (range 0.3 - 2.5) respectively. No iron was found (2 experiments) in the fraction A which is separated from the membrane-bound haemoglobin on CM-cellulose. The amount of iron in the fractions A and B was at least 5 times greater than the maximum amount which could be accounted to haemoglobin contamination as measured by the optical densities of A and B at the Soret absorption peak.

### Chromatography on G-200 sephadex

G-200 sephadex was allowed to swell in distilled water for 72 hours according to the manufacturers' instructions. A chromatography column (2 cm x 35 cm) of the sephadex in 0.01M phosphate pH 6.9 was set up and was completely equilibrated by washing with 3 bed volumes of the buffer. Blood was removed from a hen injected 7 days previously with 5 $\mu$ c of iron-59. The lysate supernatant was prepared as usual and added to the top of a CM-cellulose column. However, instead of adsorbing the haemoglobin to the column in the normal fashion, elution was performed at once with 0.039M phosphate pH 6.9 and the eluate collected. Thus the pre-

TABLE 8Non - haemoglobin Iron

Experiment Number	Erythrocyte fraction used	A (% of Fe put onto column)	B (% of Fe put onto column)
1	water lysate supernatant	0.3	0.7
2	water lysate supernatant	0.3	0.9
3	"	0.2	0.6
4	"	0.4	1.9
5	"	0.4	1.9
6	"	0.5	1.8
7	"	0.4	0.3
8	Saponin lysate supernatant	0.0	2.5
9	"	0.2	0.4
10	total cell haemoglobin	0.6	0.9

haemoglobin fractions A and B and haemoglobin II were obtained together. This was done for two reasons. Firstly, the amounts of the fractions A and B were so small compared with the amount of the total haemolysate that it was considered desirable to increase the relative proportions of A and B before C-200 sephadex chromatography. Secondly, haemoglobin II would act as a standard of known molecular weight and chromatographic behaviour for comparison with A and B.

The mixture of A, B and haemoglobin II (18 ml) was concentrated by dialysis against sucrose to give a final volume of 1.5 ml. 1.0 ml of this solution was applied to the top of the sephadex column and washed into the column with a little 0.01M phosphate pH 6.9. Elution was then carried out with this buffer, collecting 30 drop samples (2 ml approximately) by means of a fraction collector. The iron-59 activity of each fraction was then assayed by means of a well-type solid scintillation counter (NaI crystal  $\frac{1}{2}$ " x 2") and, after adding 1.0 ml of 0.01M phosphate pH 6.9 to each fraction, the optical densities were read at 274 m $\mu$  and 415 m $\mu$ . These are plotted in Figure 13.

It can be seen that there are two well-defined peaks of radioactivity, haemoglobin II (H) and a radioactive fraction (C) eluted from the column in front of the haemoglobin. The plot of the optical density at 274 m $\mu$  is interesting in that the pre-haemoglobin protein peak occurs earlier in the elution pattern (Fraction 13 as opposed to Fraction 15) than does the pre-haemoglobin iron-59 radioactivity peak. This would

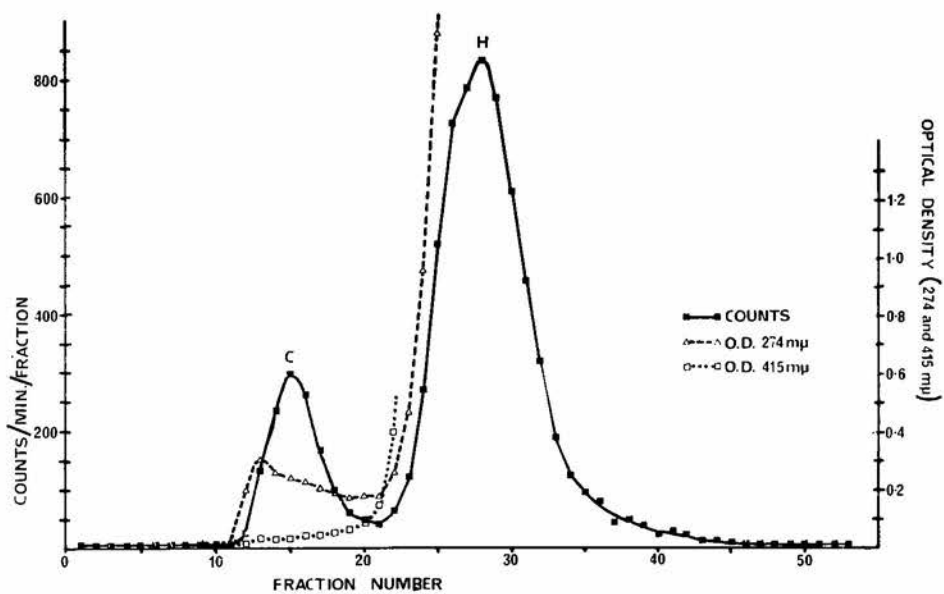


Figure 13

Separation of haemoglobin II (H) from non-haem iron (C) on G-200 sephadex. The column size was 35 cm x 2 cm and the eluting buffer 0.01M phosphate pH 6.9. 30 drop fractions were collected and assayed for iron-59 activity as described. Optical densities were measured after adding an equal volume (1.0 ml) of 0.01M phosphate pH 6.9 to each fraction.

seem to indicate that there is more than one protein fraction eluted from the column before the haemoglobin. The non-haem nature of the pre-haemoglobin material is shown by the spectrum of Fraction 14 in Figure 14. There is very little absorption in the Soret region and only one absorption peak in the spectrum at about 265 m $\mu$ .

#### Metabolic observations

In the course of the general metabolic work on the haemins, preliminary observations have been made on the fractions A and B both in vivo and in vitro.

(i) In vivo :- The specific activities of fractions A and B have been measured in 4 hens at 17 - 19 hours after the subcutaneous injection of iron-59 and in one of these birds measurement has also been made at 14 days. These specific activities are compared with those of the haemins or the haemoglobins I and II in Table 9. As can be seen, at early stages of the circulating erythrocyte life cycle, there are large differences between A and B and I and II. The specific activity of B, which was always larger than that of A, ranged from 4.8 to 26.5 times that of I and II, whereas A ranged from 1.4 to 7.2 times that of I and II. In the one observation made on a hen 14 days after the injection of radioiron the specific activities of A and B were very close to those of haemoglobins I and II.

(ii) In vitro :- Two in vitro incubations of blood have been

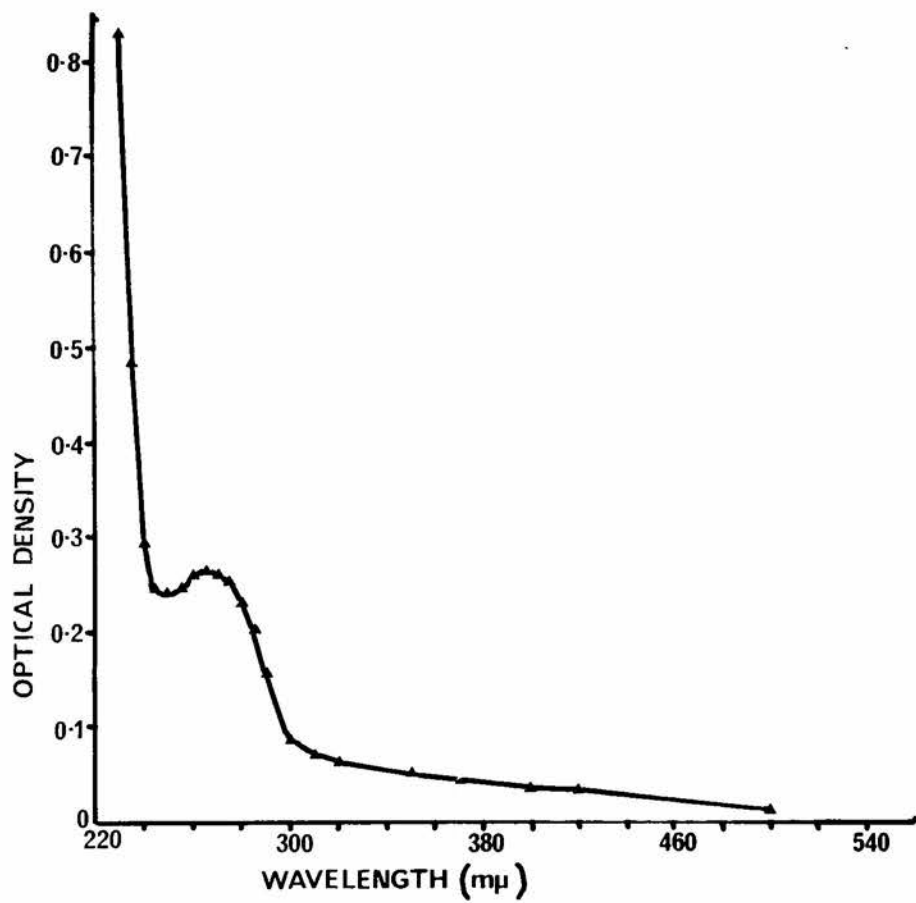


Figure 14

Spectrum of fraction 14 obtained from the G-200 sephadex chromatography shown in Figure 13. The spectrum (in 0.01M phosphate 6.9) was measured by hand using a Unicam S.P 500 spectrophotometer and 1 cm silica cells.

TABLE 9

In vivo incorporation of iron - 59 into protein fractions A and B  
and the haemins or haemoglobins I and II.

Bird No.	Time after injection	Specific Activities (Counts/min/ug Fe)			
		Haemin I or Hb I	Haemin II or Hb II	A	B
1	18 hours	1.24	1.34	6.3.	18.1
2	19 hours	1.32	1.28	1.9	6.4
3	17 hours	0.11	0.12	0.87	3.18
	14 days	35.8	34.4	29.7	36.0
4	18 hours	0.065	0.072	0.37	1.21

carried out in which observations on the iron-59 incorporation into A and B have been made. Figure 15 shows the specific activities of the fractions A and B during a 90 minute incubation period compared with the haemins. A and B both increased throughout the entire incubation, but, in contrast to the in vivo results, A was always higher than B. The specific activity of A was fairly constantly 20 - 24 times that of the haemins, that of B, 10 - 12 times that of the haemins.

In the second, experiment, immature red blood cells were incubated in iron-59 labelled plasma for 15 minutes. The blood was then centrifuged and the cells washed once with ice-cold saline. They were then reincubated in unlabelled plasma for a further 2 hours. Figure 16 shows how the specific activities of A and B changed relative to the haemins in this period. There was a very marked decline in the specific activity of A whereas that of B fell during the first hour but then rose slightly during the second hour of reincubation. Again the specific activities of both A and B were much higher than those of the haemins, B also being lower than A as in the previous experiment.

### Discussion

The present finding of non-haem iron (0.6 - 2.5% of the iron in the lysate fractions used) in avian red cells confirms the results of the earlier workers (Burmester (1936-37); Rostorfer (1949)). The amounts of these fractions are sufficiently large (up to 10% of the haemoglobin II iron) to make it important that they be separated from the haemoglobins

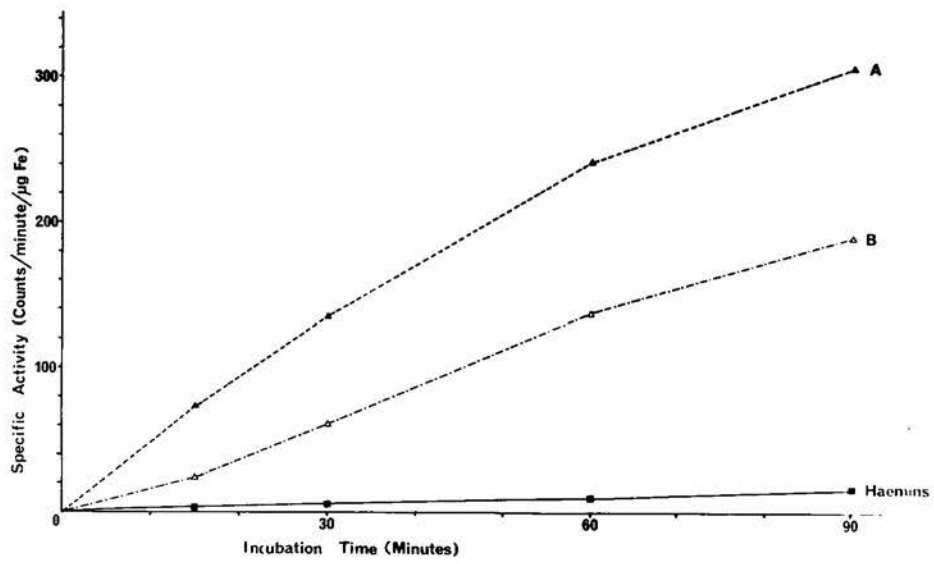


Figure 15

In vitro incorporation of iron-59 into the non-haem iron fractions A and B, and into the haemins during a 90 minute incubation.

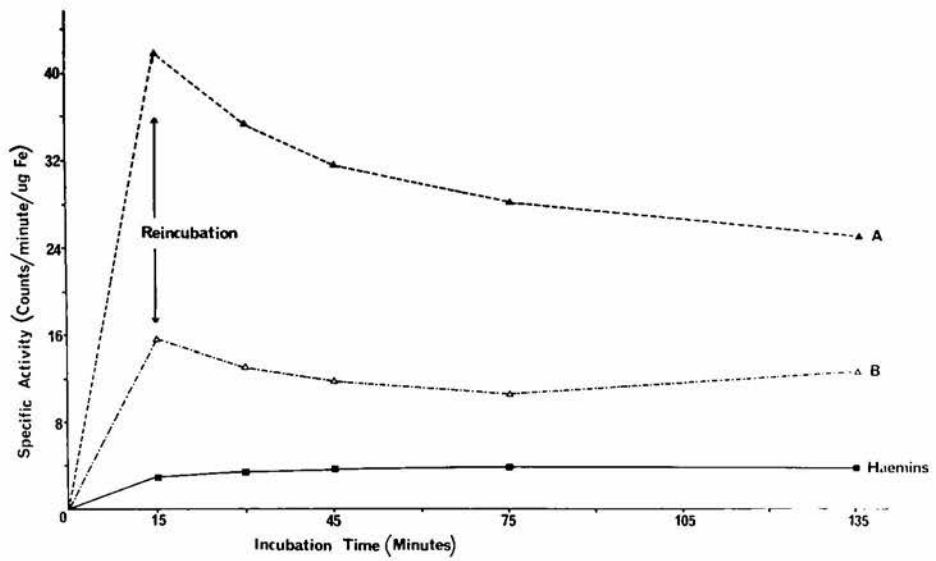


Figure 16.

The effect on the iron-59 specific activities of A, B and the haemins of reincubating, in unlabelled plasma, cells which had previously been incubated in labelled plasma.

in metabolic iron studies on the haemoglobins.

It is not possible as yet without further work to correlate completely the results of the CM-cellulose chromatography and the sephadex G-200 chromatography. However, the results of the latter would seem to indicate that at least one of the non-haem iron fractions obtained from the CM-cellulose separation had a molecular weight greater than that of haemoglobin (68,000). Furthermore, since G-200 sephadex excludes compounds of molecular weights greater than approximately 200,000, the fact that the protein peak appears earlier in the elution pattern than the radioactivity peak, is an indication that this non-haem iron fraction has a molecular weight less than 200,000. The molecular weight of ferritin is considerably greater than this, which would seem to exclude the possibility that the non-haem iron fraction was in fact ferritin.

The in vivo observations, indicating a much more rapid initial incorporation of iron-59 into A and B than into the haemins, agree with those of Ramsay (1966) who found that in hens the specific activity of the total erythrocyte iron was higher than that of the haemin iron 24 hours after the injection of radioiron, this difference being no longer found at 96 hours. Fractions A and B in the amounts and specific activities found in the present work would cause a significant difference between the specific activities of haemin iron and total iron at early stages of the red blood cell life cycle.

The in vitro experiments, as do the in vivo ones, show a much more

rapid incorporation of iron-59 into A and B than into the haemins. The reduction in the specific activity, particularly of fraction A, which is obtained on the reincubation of labelled cells in unlabelled plasma is very interesting. It would seem to indicate that fraction A is turning over fairly rapidly or possibly that the iron in A is being utilised for haem synthesis. However, the specific activities of the haemins rose by 40% during the reincubation period and it can be calculated that even if fraction A is 2-3% of the total lysate iron, then the fall in the specific activity of A during the reincubation period is sufficient to account for only half of the observed increase in the haemins. Presumably, therefore, iron must be present in the erythrocytes in forms other than haemoglobin and the fractions A and B. Much more work, however, is required before any real conclusions can be drawn about the relationships between A, B and the haemoglobins.

CONCLUSIONS

The main conclusions of the present work are :-

1. When the hen oxyhaemoglobins I and II are incubated together under a variety of mild conditions the percentage exchange of their haem groups is small (7.0%). This exchange is not significantly increased by the addition of fowl erythrocyte lysates or of free globins to the incubations.
2. Studies on the *in vivo* incorporation of iron-59 and the *in vitro* incorporation of iron-59 and (2-carbon-14) glycine into the haemins from the haemoglobins I and II, give results which indicate either that haem is drawn for haemoglobin formation from an intracellular source common to the two haemoglobins and at rates which are proportional to the relative amounts of the haemoglobins I and II, or that intracellular haem exchange occurs between the haemoglobins.
3. The *in vitro* incorporation of iron-59 and (2-carbon-14) glycine into the haemin from the haemoglobin fraction which remains bound to the red cell lysate sediment is found on occasion to be greater than the incorporation into the haemins from the haemoglobins I and II. It is suggested that this membrane-bound haemoglobin is haemoglobin I trapped by the cell fragments during lysis and containing preferentially a larger proportion of newly synthesised haemoglobin than does the unbound haemoglobin I.
4. A significant amount of non-haem iron has been found in fowl erythrocytes. This can be separated from haemoglobin by CM-cellulose or sephadex G-200 chromatography. During *in vivo* iron-59 incorporation studies the non-haem iron became more highly labelled than the haemoglobins I and II at early stages of the avian erythrocyte life span. This non-haem iron also was more highly labelled than the haemoglobins I and II during incubations of immature red cells in iron-59 labelled plasma and may be a precursor of haemoglobin iron.

APPENDIX

### Standard Deviation

The standard deviation of a method was calculated by a slight modification of the method of Copeland (1957). A series of duplicate measurements on different samples is made using the method of which the precision is required.

The standard deviation is then

$$\bar{s} = \sqrt{\frac{\sum (\text{differences between duplicates})^2}{2 (\text{number of pairs})}}$$
$$= \bar{s} \sqrt{\frac{\sum d^2}{2n}}$$

This formula is only useful as it stands if the different duplicates used in the calculation are of the same order of magnitude. It was therefore modified so that the difference between each pair of duplicates was expressed as the percentage of the mean of the duplicate i.e. the mean of each pair of duplicates was now in the form  $100 \bar{d}/2$

and the % standard deviation is

$$\bar{s} = \sqrt{\frac{\sum (d/m \times 100)^2}{2n}}$$

where  $m$  = observed mean of pair of duplicates.

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