SOME ASPECTS OF IRON
METABOLISM IN BIRDS

by

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SUMMARY

Plasma iron in laying hens is about five times higher than in non-laying birds and mammals. About half the total iron has properties different from those of transferrin-bound iron. It has been suggested by some workers that the non-transferrin iron is bound to conalbumin.

In the present work studies were made on plasma containing the native non-radioactive iron and on plasma to which radioactive iron (^{59}Fe) had been added in vivo or in vitro. Several types of separation techniques (gel filtration on Sephadex, adsorption on magnesium carbonate, chromatography on DEAE-cellulose) were employed for the fractionation of the plasma and the identification of the iron-binding components. Studies were also made on cockerels where a plasma iron picture similar to that found normally in laying hens could be induced by the administration of oestrogen. The results of all these experiments indicate that the non-transferrin iron is associated with phosphoprotein and not with conalbumin, although an attempt to isolate ^{59}Fe-labelled phosphoprotein quantitatively was only partially successful. The findings of other workers who have used various electrophoretic techniques are discussed and found to be explicable on the basis of this hypothesis.

Specific radioactivity measurements made after the incubation of ^{59}Fe-transferrin (in normal cockerel plasma) with the phosphoprotein iron in plasma from laying hens show that

there is no exchange between the two iron fractions in vitro. Similar measurements made after the intravenous injection of ^{59}Fe-transferrin show that in laying hens and oestrogen-treated cockerels transferrin iron serves as a precursor of the phosphoprotein iron. Thus the phosphoprotein iron is presumably derived from transferrin iron in vivo by some process other than simple transfer in the plasma. It is proposed that the iron is taken from transferrin by the newly synthesized phosphoprotein which is leaving the liver cells to be released in the blood plasma, and that the possible function of the phosphoprotein iron is to serve as a source of iron for the eggs.

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INTRODUCTION

1. ROLE OF PLASMA IRON IN IRON METABOLISM

Iron is the most versatile of all biochemically active metals and can be found associated with many compounds of physiological importance such as haemoglobin, myoglobin, ferritin, haemosiderin and the cytochromes. The presence of iron in the blood plasma and its physiological importance were first conclusively demonstrated in 1925 by Fontès and Thivolle. This finding led to a great deal of research on the nature of plasma iron and its role in iron metabolism in man and various animal species. The results of these studies have been reviewed by Laurell (1947), Drabkin (1951), Laurell (1952), Ramsay (1958), Moore (1959-60), Underwood (1962), and Bothwell & Finch (1962).

It is now well known that in all animals the blood plasma contains iron. In normal mammals, male birds and non-laying female birds the concentration is usually 100-200 µg./100 ml. plasma. All this iron is chemically combined with a β_1-globulin which has been named transferrin (Holmberg & Laurell, 1947) or siderophilin (Schade, Reinhart & Levy, 1949). This iron-binding protein is present in the plasma of all mammals and birds; and its role in iron metabolism, its variations in physiological and pathological conditions, and its properties have been reviewed by Laurell (1952), Ramsay (1958), Moore (1959-60), Underwood (1962), Bothwell & Finch (1962), Giblett (1962), Putnam (1965), and Bearn & Parker (1966).

The more recent studies show that the molecular weight of transferrin is significantly lower than the previously accepted value of 90000. Thus Charlwood (1963) found the molecular weights of human, monkey and rat transferrins to be close to 68000, while Roberts, Makey & Seal (1966) reported a molecular weight of 73200-76000 for human transferrin, and Leibman & Aisen (1967) found pig transferrin to have a molecular weight of 81000-82000.

Each molecule of transferrin can bind two atoms of ferric iron (Ehrenberg & Laurell, 1955). The exact mechanism of the binding of iron to transferrin is not known but it has been proposed by Windle, Wiersema, Clark & Feeney (1963) that Fe^{3+} is octahedrally coordinated to three tyrosyl oxygen atoms, two nitrogen atoms (from imidazole groups of histidine or guanidyl groups of arginine), and one bicarbonate ion. In most mammals and non-laying birds the plasma iron usually saturates 30-40% of the total iron-binding capacity of transferrin (Underwood, 1962).

The main features of the biochemical pathways of iron metabolism in mammals are now quite clear. In comparison with the vast amount of work on iron metabolism in mammals very little seems to have been done on birds. The results of studies on birds by Ramsay & Campbell (1954), Halkett, Peters & Ross (1958), Ramsay (1966), and Davis, Norris & Kratzer (1968) suggest that the basic processes of iron metabolism in mammals and birds are similar, apart from the changes associated with pregnancy in mammals and egg laying in birds.

Many of the investigations on iron metabolism have been facilitated by the use of radioactive isotopes of iron. Isotope experiments with radioactive ^{59}Fe show that in normal mammals and non-laying birds:

1) Most of the iron in the plasma is travelling to the bone marrow where it is utilized for haemoglobin synthesis. This indicates the predominance of haemoglobin synthesis in the iron metabolism of non-egg-laying animals. When the red cells are destroyed most of the iron released is transported as transferrin-iron in plasma from the sites where the red cells are destroyed to the sites where new cells are formed and reutilized for haemoglobin synthesis. Thus the main part of the plasma iron forms a link in a narrow cyclic process (haemoglobin ⟶ plasma iron ⟶ haemoglobin).

2) While the total amount of iron in the circulating plasma of normal man is only 3-4 mg. or less than 0·1% of the body iron, the turnover rate is so rapid that from 25 to 40 mg. are transported each day. The importance of this turnover rate can be appreciated when it is realized that each day enough haemoglobin is normally broken down and resynthesized to turn over 20-25 mg. of iron.

3) Normally there is very little exchange between the

plasma iron and iron stored in the liver, spleen, bone marrow, etc.

4) Iron absorbed from the intestine makes only a small contribution to the plasma iron and absorption in the normal subject probably more or less balances excretion. However, iron absorption is increased in cases of excessive demand for iron e.g. in iron deficiency, during growth, in the latter half of pregnancy in mammals and probably during egg production in birds.

5) About 75% of the plasma iron is derived from the catabolism of haemoglobin while the remaining 25% is derived from the catabolism of myoglobin, storage iron, absorbed iron, etc.

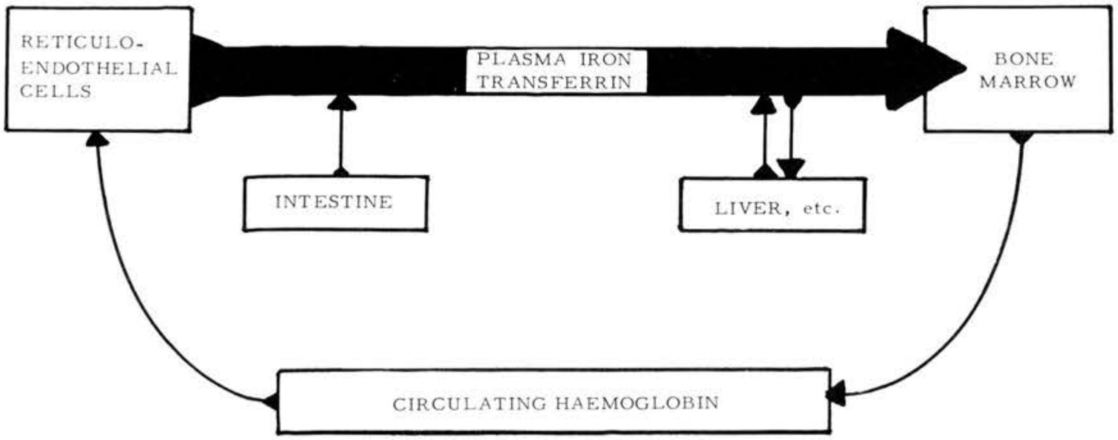
6) The kinetics of plasma radioiron studied during 14 days in normal human subjects can be expressed as the sum of two exponential components. Such a curve involves the existence of a noncirculating iron pool which exchanges slowly with the plasma iron pool (Pollycove & Mortimer, 1961; Najean, Dresch, Ardaillou & Bernard, 1967). According to Pollycove & Mortimer (1961) and Pollycove & Maqsood (1962) a labile iron pool exists on the membranes of immature erythrons and this iron is derived from plasma transferrin-bound iron and transfers back to plasma as well as transferring irreversibly into erythrons for haem synthesis. However, Najean et al. (1967) found this pool to be diffuse in the whole organism (marrow, liver, spleen, intestine) and suggested that the second exponential component is due to exchange with a

metabolic compartment of various forms of nonhaem iron e.g. "non-storage form of ferritin iron", non-ferritin protein-bound iron in the intestine and non-ferritin iron in erythroblasts. Also according to Najean et al. (1967) 5-10% of transferrin-bound ^{59}Fe injected intravenously interchange immediately with a lymphatic-hepatic iron pool. Even when, however, further work has clarified the situation, it will remain true that the major part of the plasma iron is destined for irreversible uptake by haemopoietic tissue.

The role of the plasma iron in the major processes of iron metabolism in mammals and non-laying birds is outlined diagrammatically in Fig. 1.

The role of plasma iron in iron metabolism in laying birds is even more important than in non-laying birds. Thus Halkett, Peters & Ross (1958) showed that in laying hens both egg iron (about 1 mg. per egg) and haemoglobin iron (about 0·5 mg. per day) are derived from the plasma iron pool.

Fig. 1. MAJOR PATHWAYS OF IRON METABOLISM IN MAMMALS AND NON-LAYING BIRDS.



2. THE PRESENT PROBLEM

Non-transferrin iron in the plasma of the laying hen

Plasma iron in the laying hen is about five times higher than the 100-200 µg./100 ml. usually found in the plasma of male birds, non-laying female birds and most mammals (Ramsay & Campbell, 1954; Planas & de Castro, 1960; Planas, de Castro & Recio, 1961).

Representative values from the literature are presented in Table 1 with characteristic human figures for comparison.

More than half the total iron in the plasma of the laying hen appears not to be transferrin-bound because it is removed by treatment of the plasma with magnesium carbonate (Planas & de Castro, 1960; Planas, de Castro & Recio, 1961) which does not affect transferrin-bound iron (Ramsay, 1957 b); and electrophoresis under the conditions of Poulik (1957) of laying hen plasma labelled in vivo with ^{59}Fe gives two widely separated iron fractions and less than half the radioactivity corresponds to the transferrin while the remainder corresponds to an unidentified fast migrating component (Lush & Ramsay, cited by Lush, 1964).

Planas and his associates (Rodriguez & Planas, 1964; Martin-Mateo & Planas, 1965; Martin-Mateo, Sebastian & Planas, 1965; Planas, 1967) suggested that conalbumin, the iron-binding protein characteristically found in egg white, is responsible for the binding of the non-transferrin iron and that the iron removed

Table 1

Plasma iron in the domestic fowl and the human adult

Species and condition	No. of cases	Plasma iron (µg./100 ml.)	Reference
Domestic Fowl			
Laying hens	5	768 (670-942)	(a)
Laying hens	16	500±42 (σ : 166)	(b)
Non-laying hens	23	129±10 (σ : 48)	(b)
Male birds	23	103±5 (σ : 22)	(b)
Human Adult			
Males	35	127 (67-191)	(c)
Females	35	113 (63-202)	(c)
Pregnant (3rd trimester)	106	94 (22-185)	(c)

(a) Ramsay, W.N.M. and Campbell, E.A. (1954). Biochem. J. 58, 313.

(b) Planas, J. and de Castro, S. (1960). Revta esp. Fisiol. 16, 197.

(c) Morgan, E.H. and Carter, G. (1960). Australasian Ann. Med. 9, 209.

by magnesium carbonate is conalbumin-bound. This explanation is highly unlikely since conalbumin and transferrin are almost identical in structure (Williams, 1962), and the mechanisms by which iron is bound to the two proteins are believed to be identical (Windle, Wiersema, Clark & Feeney, 1963; Aasa, Malmström, Saltman & Vänngard, 1963; Aisen, Leibman & Reich, 1966). Indeed, conalbumin and transferrin are so similar in structure and iron-binding properties (for reviews see Putnam, 1965; Bearn & Parker, 1966) that Williams (1968) suggested that the name 'Ovotransferrin' (Komatsu & Feeney, 1967; Azari & Baugh, 1967) should be used for the egg white protein instead of the classical name 'conalbumin'.

This thesis reports investigations into the nature of the plasma iron in the laying hen with particular reference to the identification of the non-transferrin iron and its metabolic relation to transferrin iron.

It has already been noted that the iron content of the plasma is much higher in the laying hen than in the non-laying bird. This is only one of many changes in composition induced by the onset of laying and maintained as long as eggs were regularly produced. Thus the blood plasma of the laying hen has a total calcium level two or three times that observed in the male or sexually immature female (e.g. Winget & Smith, 1958; Hertelendy & Taylor, 1961); a total lipid level that is several times higher than in males and non-laying females (e.g. Lorenz, Entenman & Chaikoff, 1938; Walker, Taylor & Russell, 1951; Heald & Badman, 1963); and contains a phosphoprotein fraction that is virtually absent from the plasma of non-laying hens and male birds (Laskowski, 1935 a, b; Roepke & Hughes, 1935). All these changes are considered to be controlled by the endogenous oestrogens and can be induced in non-laying hens or male birds by treatment with oestrogens (see Lorenz, 1954; and Sturkie, 1965, for reviews). A plasma iron level similar to that found normally in laying hens was induced in immature pullets (Ramsay & Campbell, 1956) and cockerels (Greengard, Mendelsohn & Gordon, 1965) by the administration of oestrogens and the latter workers suggested without direct evidence that the non-transferrin iron in the plasma might be bound to the phosphoprotein.

The phosphoprotein phosvitin has been isolated from the plasma of the oestrogenized laying hen (Common & Mok, 1959; Mok, Martin & Common, 1961), the untreated laying hen (Heald & McLachlan, 1963 a), the oestrogenized immature pullet (Heald & McLachlan, 1963 b; Heald & McLachlan, 1964), and the oestrogenized cockerel (Greengard, Sentenac & Acs, 1965). The above various workers showed that the phosphoprotein isolated from the various plasma was chemically identical with the phosphoprotein phosvitin which had been isolated from egg yolk by Mecham & Olcott (1949). Heald & McLachlan (1963 a) consider that phosvitin is the only phosphoprotein present in the plasma of the laying hen. Since the plasma and egg phosvitins are chemically identical, and since the phosphoprotein is synthesized in the liver (Flickinger & Rounds, 1956; Vanstone, Dale, Oliver & Common, 1957; Heald & McLachlan, 1965; Greengard, Sentenac & Acs, 1965), it appears likely that the phosphoprotein in the plasma of the laying hen is phosvitin on its way to the egg yolk.

Mecham & Olcott (1949) found egg phosvitin to have a molecular weight of about 21000 but this work has been criticized by Joubert & Cook (1958) who reported a molecular weight of 30000. However, it was discovered later (see Mok, Martin & Common, 1961) that there was a computation error in Joubert & Cooks' paper and that the correct calculation gave a value of 36000. More recently Mok, Martin & Common (1961) found that phosvitin prepared from hen's serum or hen's egg yolk has a molecular weight of 42000 as indicated by ultracentrifugal analysis and 40000 as calculated from tryptophan content. Allerton & Perlman (1965) showed that the amino acid composition of phosvitin is consistent with a molecular weight of 40000 to 45000.

The phosphoprotein exists in the plasma in complex with other plasma constituents such as calcium (Schjeide & Urist, 1956;

Urist, Schjeide & Mclean, 1958; Benowitz & Terepka, 1968) and lipoproteins (McIndoe, 1959; McCully & Common, 1961). Metallic cations such as Ca^{2+} and Mg^{2+} have an aggregating effect on phosvitin molecules, possibly by forming a bridge between phosphate groups on adjacent molecules, (Mecham & Olcott, 1949; Joubert & Cook, 1958). In view of the aggregating effect of metallic cations and the presence of phosvitin in complex with lipoproteins, the particle in which phosvitin exists in the plasma must have a weight much higher than 42000. Schjeide & Urist (1959) by employing ultracentrifugal analysis (in 20% NaCl) found a molecular weight of 154000 for a phosphoprotein-containing fraction in oestrogenized cockerels plasma, while Shaw & Clegg (1966) by using gel filtration on Sephadex G-200 found the molecular weight of component 1 (phosphoprotein-containing fraction) of the blood serum of diethylstilbestrol treated cockerels to be 195000. However, the high concentration of sodium chloride used by Schjeide & Urist is likely to cause dissociation of the native phosphoprotein complex, and the borate buffer pH 8·6 used by Shaw & Clegg has been shown by McCully & Common (1961) to favour dissociation of the phosphoprotein from the lipoproteins of the serum. Thus the above values for the apparent molecular weight may be low, and probably do not reflect the true size of the complex particle in which phosvitin is present in the plasma. It appears that this phosphoprotein with its lipoprotein and mineral complex is transferred via the blood plasma to the ovary where it is deposited in the developing egg yolk (McIndoe, 1959; Schjeide & Urist, 1959; Schjeide & Urist, 1960).

Phosvitin contains 10% phosphorus (Mok, Martin & Common, 1961; Heald & McLachlan, 1963 a) which is mainly if not solely present as phosphorylserine (Heald, 1962). The presence of iron in egg phosvitin has been known for a long time (Mecham & Olcott, 1949). Taborsky (1963) showed that egg phosvitin promotes a rapid oxidation of ferrous ion and binds ferric ion strongly. He suggested that of the six coordination positions of the ferric ion four are filled by phosphate oxygen atoms and the remaining two positions are probably occupied by water or some anion present in the solution e.g. chloride. When phosvitin is saturated with iron it would have an iron content of 8·85% and a molar phosphorus-iron ratio of 1·92 (Taborsky, 1963). Considering that saturated transferrin contains about 0·125% iron (Surgenor, Koechlin & Strong, 1949), the iron-binding capacity of 1 mg. phosvitin will be about seventy times higher than that of 1 mg. transferrin.

Since most, if not all, of the phosphoprotein in the plasma of the laying hen is chemically identical to egg phosvitin, or is actually phosvitin on its way to the egg yolk, and since in our experience laying hens may have an average plasma phosphoprotein level of 120 mg./100 ml., the amount of phosphoprotein in the plasma of the laying hen would be far more than adequate for the binding of all the non-transferrin iron in the plasma as well as complexing with other cations such as calcium. The presence of this iron-binding phosphoprotein in the plasma of the laying hen and its possible association with the non-transferrin iron have been overlooked by Planas and his associates.

In the present investigations into the nature of the non-transferrin iron several types of separation techniques have been employed separately and in combination. These techniques included gel filtration on Sephadex G-25, adsorption on magnesium carbonate, gel filtration on Sephadex G-200 and ion-exchange chromatography on DEAE-cellulose. The techniques were chosen for reasons that would become clear in the coming pages. In the experiments that involved column work the material to be fractionated was labelled with ^{59}Fe in vivo or in vitro.

Rat plasma was used for the study of the behaviour of transferrin-bound iron since in the rat all the plasma iron is transferrin bound, and iron added to the plasma in vitro or in vivo is combined very rapidly (within minutes) with transferrin so long as the quantity of iron added is not more than the unsaturated iron-binding capacity of transferrin. Since Planas and his associates claim that conalbumin-bound iron is removed by magnesium carbonate under conditions which do not affect transferrin-bound iron, some of the properties of the iron bound to conalbumin were investigated by subjecting iron bound in vitro to conalbumin in egg white to some of the above fractionation procedures. Cockerel plasma, which normally contains no phosphoprotein, was used for some control experiments. Since both the non-transferrin iron and the phosphoprotein appear to be present in the plasma of the laying hen as a result of secretion of oestrogen by the ovary and can be produced in the plasma of cockerels by treating the birds with oestrogen the present studies were extended to oestrogen-treated cockerels for further investigations into the relation between the non-transferrin iron and the phosphoprotein.

In the investigations into the metabolic relation between transferrin iron and the non-transferrin iron, the transferrin in normal cockerel plasma was labelled with ^{59}Fe and was then incubated with plasma from laying hens or injected intravenously into laying hens and oestrogen-treated cockerels and the two iron fractions were separated using magnesium carbonate and their relations studied.

In the light of the present investigations and those of others it has been possible to speculate on the function of the non-transferrin iron and to propose a model for the major pathways of iron metabolism in the laying hen.

MATERIALS AND METHODS

1. GENERAL

All chemicals were of the A.R. quality whenever possible.

The apparatus used throughout this work was radiochemically free of iron. This was achieved by allowing the apparatus to soak overnight in 20% citric acid in dilute Nonidet P42 detergent (Shell Chemicals), scrubbing, allowing to soak overnight in 20% HCl, and then washing thoroughly with distilled water.

The birds used were Light Sussex (or Light Sussex Cross) hens and about three months old cockerels. They were kept in large, single battery cages under artificial lighting (18 hr. of light in each 24 hr.) and were allowed free access to water and commercial layers' mash. Blood specimens were drawn from the wing veins into heparinised syringes. Plasma was obtained by centrifuging the blood immediately. Fresh plasma was used throughout this work and the anticoagulant employed was always solid heparin (Sigma).

The white of fresh hen's eggs was used as a source of conalbumin.

Radioactive iron (^{59}Fe) in the form of ferric chloride (in 0·1 N HCl) or ferric citrate (Radiochemical Center, Amersham) was diluted with 1% NaCl and the acidity of ferric chloride solutions was neutralised with sodium bicarbonate where necessary.

In in vivo experiments the ^{59}Fe was injected subcutaneously (s/c) or intravenously (i/v). In in vitro experiments the ^{59}Fe was incubated with the plasma at 37°C (in an oven or water bath) with mixing at intervals.

Sephadex was obtained from Pharmacia (Uppsala, Sweden) and gel filtration was carried out according to Flodin (1962) and as described in the more recent Pharmacia literature.

Diethylaminoethyl cellulose (DEAE-cellulose) was obtained from Serva, Heidelberg, and prepared for chromatography according to Peterson & Sober (1962).

Magnesium carbonate (hydrated, basic, light) was obtained from the British Drug Houses Ltd.

2. SEPARATION METHODS

2.1. Gel filtration on Sephadex G-25

Gel filtration on Sephadex G-25 was used to find out if the non-transferrin iron is associated with high molecular weight substances or is present in ionic form because the magnesium carbonate which removes more than half the iron from the plasma of the laying hen has been shown by Ramsay (1957 b) to adsorb ionic iron.

Procedure

The dry sephadex was suspended overnight in enough distilled water so that, after sedimentation, the excess volume was about ten times that of the swollen gel. It was then washed with the eluant (0·5% KCl) and packed into a cylindrical glass column (7 x 2 cm.). The packed column was washed with about five bed volumes of the eluant. 0·5 ml. plasma containing a tracer quantity of ^{59}Fe was placed on top of the column and eluted with 0·5% KCl at a flow rate of about 30 ml./hr. The eluate was collected in 1·5 ml. fractions (Locarte fraction collector, drop counting).

2.2. Magnesium carbonate adsorption

Ramsay (1957 b) showed that finely divided magnesium carbonate, which is insoluble, adsorbs ferric ion from solution but not iron which is bound to transferrin and used this as a basis for his method for the determination of total iron-binding capacity (TIBC) (Ramsay, 1957 b) where excess iron is added to serum or heparinised plasma and the remaining iron after transferrin is saturated is removed by shaking with magnesium carbonate. In the course of applying Ramsay's method to laying hen serum, Planas, de Castro and Recio (1961) found that magnesium carbonate adsorbs all the added iron as well as about half the iron originally present in the serum. The latter workers also showed that simple dilution of 1·0 ml. laying hen serum to 3·0 ml. with distilled water followed by shaking with 200 mg. magnesium carbonate for 30 minutes separated the iron into two roughly equal fractions, one adsorbed on magnesium carbonate.

Thus magnesium carbonate adsorption provides a quick method of separating the plasma iron in the laying hen into two fractions. However, the adsorptive properties of magnesium carbonate may vary from one preparation to another (Ramsay, 1957 b). The quantity of magnesium carbonate required for separation of transferrin iron from non-transferrin iron was therefore checked by comparing the values obtained by magnesium carbonate separation with those obtained by chromatographic separation on DEAE-cellulose (p.20) and the technique was modified accordingly. One way of doing this was to use laying hen plasma labelled in vivo with ^{59}Fe and compare the counts obtained after magnesium carbonate treatment with the counts (transferrin-^{59}Fe) found in the fractions eluted with 0·1 M NaCl in 0·005 M tris-HCl buffer pH 8 from a DEAE-cellulose column equilibrated with the same solution. In three different experiments the values for radioactivity (counts/min./ml. plasma) obtained after magnesium carbonate treatment were 13168, 21561, 37886 and the corresponding values obtained by DEAE-cellulose chromatography were 13014, 21189 and 37608.

The trace amount of iron present in magnesium carbonate did not interfere with the measurements except perhaps by an increase of a few micrograms per 100 ml. plasma where unsaturated transferrin was present.

Procedure

The procedure generally adopted was as follows:

200 mg. magnesium carbonate were suspended in 1·0 ml. heparinised plasma plus 3·0 ml. distilled water in a test tube. The tube contents were shaken frequently and thoroughly for 30 minutes and then centrifuged at 3000 rpm. for ten minutes. 2·0 ml. of the clear supernatant fluid were then analysed for radioactivity, iron or phosphoprotein as required.

In cases where the plasma iron level was expected to be high and where only small volumes of blood were to be withdrawn so as not to upset the physiological state of the bird (e.g. in experiments on oestrogen-treated cockerels), 0·5 ml. plasma was treated with magnesium carbonate. The 0·5 ml. plasma was diluted to 3·0 ml. with distilled water and after shaking with 200 mg. magnesium carbonate for 30 minutes 1·5 ml. of the supernatant fluid was used for analysis. Even at this level the procedure was fairly reproducible. The coefficient of variation of ten values for iron (determined by Ramsay's method, see p. 23) in magnesium carbonate treated plasma was 4·1% (mean: 268 μg./100 ml., standard deviation (s.d.): 11).

The quantities adsorbed on magnesium carbonate were obtained by subtracting the values after magnesium carbonate treatment from the values in equivalent volumes of plasma that have not been treated with magnesium carbonate.

2.3. Gel filtration on Sephadex G-200

The dry gel was allowed to swell in excess water and left to stand for at least three days with intermittent stirring and decantation. It was then washed with the eluant and packed into the column (size 18 x 1", volume 205 ml., Pharmacia). The column was equilibrated with the eluant by washing with several column volumes. The sample (1·0 ml.) was applied from the bottom of the column and elution carried out with upward flow using an upward flow adaptor (Pharmacia). Upward flow has the advantage over downward flow in that it minimizes compression of the gel bed and allows for finer control of the flow rate.

The eluate used was NaCl in tris-HCl buffer. Preliminary experiments showed that high ionic strength (1 M NaCl in 0·1 M tris-HCl buffer, pH 8·0) provided good separation of the plasma proteins and full recovery of transferrin iron but rather poor recovery of the non-transferrin iron. Lower ionic strength (0·15 M NaCl in 0·005 M tris-HCl buffer, pH 7·4) was therefore used in some experiments where the good separation was sacrificed for a full recovery of the iron. The eluate was always collected in 2 ml. fractions by drop counting (Locarte fraction collector). The elution flow rates were 10-20 ml./hr. Fractionation was carried out at 2-4°C.

2.4. Ion-exchange column chromatography on DEAE-cellulose

Connelly & Taborsky (1961) showed that DEAE-cellulose equilibrated with 0·005 M tris-HCl buffer, pH 8·0, containing 0·1 M NaCl adsorbs egg-yolk phosvitin in a ratio of at least 1:4 (protein to exchanger) and increasing the salt concentration to 0·4 M NaCl leads to complete desorption of the protein. This was confirmed by Heald & McLachlan (1963 a) who also showed that the phosphoprotein isolated from the plasma of the laying hen behaves like egg phosvitin.

DEAE-cellulose has been widely employed for the fractionation of the plasma proteins and it can be predicted from the work of Sober, Gutter, Wyckoff & Peterson (1956), Sober & Peterson (1958), and Block, Keller & Miller (1959) that transferrin can be eluted from DEAE-cellulose columns with relatively low salt concentrations.

Preliminary experiments in the present investigations in which plasma samples from rats and laying hens were subjected to chromatography on DEAE-cellulose showed that transferrin with its bound iron are not adsorbed on a DEAE-cellulose equilibrated with 0·1 M NaCl in 0·005 M tris-HCl buffer, pH 8·0, whereas the non-transferrin iron and the phosphoprotein in the plasma of the laying hen are adsorbed to the column and can be eluted more or less completely by increasing the salt concentration to about 0·4 M NaCl. 2 to 3 g. exchanger were found to be more than sufficient for adsorbing the phosphoprotein present in 1 to 2 ml. plasma.

Procedure

The following procedure was employed, (although in some experiments slightly different conditions were used).

DEAE-cellulose columns (size 5·5 x 2 cm., containing 2-3 g. cellulose) were prepared according to Peterson & Sober (1962) and equilibrated with the starting buffer (0·1 M NaCl in 0·005 M tris-HCl buffer, pH 8·0). 1 to 2 ml. plasma (containing ^{59}Fe) were loaded on the column and mixed with several volumes of the starting buffer at the top of the column. Elution was carried out with the starting buffer till there was no radioactivity in the eluate and then with a NaCl gradient in 0·005 M tris-HCl buffer, pH 8·0. The mixing chamber contained 200 ml. of the starting buffer (0·1 M NaCl in 0·005 M tris-HCl buffer, pH 8·0) while the reservoir contained 1·0 M NaCl in 0·005 M tris-HCl buffer, pH 8·0. Continuous mixing was attained by means of a magnetic stirrer. The eluate was collected in fractions of 3 ml. or 5 ml. each (Locarte fraction collector) at a flow rate of 25-40 ml./hr.

Theoretical gradient curves were calculated according to Alm, Williams & Tiselius (1952) and Svensson (1958), and the expected curves confirmed by estimating the concentration of NaCl in the eluate.

3. ANALYTICAL METHODS

3.1. Iron

Unless otherwise stated the determination of iron in the plasma was made photometrically according to a method described by Ramsay (1957 a). This method depends on the property of iron of forming a pink complex with 2,2'-dipyridyl in the presence of a reducing agent. At the same time the proteins are precipitated by heat at slightly acid reaction followed by shaking with chloroform.

Procedure

Equal volumes of plasma, 0·1 M sodium sulphite (freshly made) and 0·1% 2,2'-dipyridyl in 3% (v/v) acetic acid plus a suitable volume of distilled water (to bring the total volume to 4-6 ml.) were mixed in a tube with a ground-glass neck. The mixture was heated in a boiling water bath for 5 minutes and then allowed to cool. 2 ml. chloroform were added and the tube was stoppered and shaken vigorously for 30-60 seconds. The stopper was removed and the tube was centrifuged at 3000 rpm for 5-10 minutes. The optical density of the clear supernatant fluid was measured at 520 mμ against a blank and the iron content calculated from a standard graph for iron determination.

Two volumes of sulphite and dipyridyl per volume of plasma were used when the plasma iron was expected to be high e.g. when determining the concentration of iron in plasma from laying hens or oestrogen-treated cockerels, or when excess iron had been added to plasma as in total iron-binding capacity determinations.

1·0 ml. plasma was used for iron determination in most of this work, but in some cases when dealing with limited volumes of plasma from laying hens or oestrogen-treated cockerels 0·5 ml. or even 0·25 ml. plasma were used for analysis. When 0·5 or 1·0 ml. plasma were analysed according to the above procedure and the final volume was 5 or 6 ml., the coefficient of variation of eleven duplicate analyses ranging in values from 186 to 1097 µg./100 ml. was 0·81% (Appendix I). When 0·25 ml. plasma was used for analysis and the total volume was 4 ml., the coefficient of variation of ten replicate analyses of a single sample was 0·9% (mean: 669 µg./100 ml., s.d.: 6).

3.2. Total iron-binding capacity (TIBC)

(Ramsay, 1957 b)

This method actually measures the level of transferrin in plasma where transferrin is the only iron-binding protein e.g. in mammalian or cockerel plasma (Ramsay, 1958). An excess of iron is added to serum or heparinised plasma and after transferrin is saturated the remaining iron is removed by adsorption on magnesium carbonate. The iron content of an aliquot of the supernatant is determined as for plasma iron and the total iron-binding capacity or transferrin level is expressed as μg. iron per 100 ml. plasma.

Procedure

The procedure adopted in this work was as follows:

2 ml. of a solution of ferric chloride (solution in 0·00 N HCl and contains 5 μg. iron/ml.) were mixed with 1·0 ml. plasma plus 1·0 ml. distilled water in a test tube. After 5 minutes 200 mg. magnesium carbonate were added and the tube contents were agitated frequently and thoroughly for 30-60 minutes. This was followed by centrifugation at 3000 rpm for about 10 minutes and determination of the iron content of 2·0 ml. of the clear supernatant using Ramsay's dipyridyl method.

3.5. Radioactivity

The radioactivity of ^{59}Fe was determined by measuring its γ-radiation using a well-type solid scintillation counter (sodium iodide crystal, $\frac{3}{4}$ x 2"). In a series of measurements the specimens counted had the same (or about the same) volume. Distilled water was used for adjusting the volumes where necessary. The volume of material counted did not exceed 5 ml.

The standard error of counting never exceeded 3% except when the total counting rate was of about the same order as background. The recorded counts were corrected for background and decay.

3.4. Total protein

The concentration of proteins in the fractions was estimated by measuring the optical density at 280 mμ (1 cm. cells, UNICAM SP 500). Distilled water was used for diluting the fractions obtained from Sephadex G-25 to 4·5 ml., and those obtained from Sephadex G-200 to 3 ml. The fractions from DEAE-cellulose columns were undiluted.

3.5. Phosphoprotein

The procedure followed was based on Schmidt & Thannhausers' method (1945) for the estimation of phosphoproteins in animal tissues, Delory's technique (1938) for the precipitation of phosphate in the presence of interfering substances, and Allen's method (1940) for the estimation of phosphorus. Phosphoprotein phosphorus was used as a measure of the amount of phosphoprotein. Other sources of phosphorus were therefore removed and the inorganic phosphate liberated as a result of alkaline hydrolysis was then estimated. It was necessary to precipitate the phosphate by Delory's technique because otherwise there was interference caused by the hydrolysis products from the protein.

Procedure

0·2 - 0·5 ml. plasma (or fractions from plasma) were used for analysis. The material was washed twice with perchloric acid (2 ml., 5% w/v), followed by extracting twice by heating to boiling in a warm water bath with 5 ml. of a mixture of ethanol-ether (3:1, v/v) and extracting similarly with a mixture of methanol-chloroform (5 ml., 1:1 v/v). The residue was then dried thoroughly by heating in a boiling water bath and hydrolysed by incubation with 2 ml. 1N KOH for 18 hr. at 37°C. The tube contents were then mixed with 6 ml. perchloric acid (18%, v/v), and after chilling for 30 minutes (Heald, 1962), centrifuged and the supernatant fluid transferred to another tube. The supernatant fluid was rendered alkaline to phenolphthalein by adding concentrated ammonia solution. 1·0 ml. calcium chloride solution (2·5%, w/v) and 1·0 ml. suspension of magnesium carbonate in water (0·5%, w/v) were added, mixed and after allowing to stand for 30 minutes, mixed again and centrifuged. The supernatant fluid was poured off and the precipitate was dissolved in 0·8 ml. 60% perchloric acid. About 4 ml. distilled water and 0·4 ml. ammonium molybdate (8·3%, w/v) were then added. Any traces of insoluble material were centrifuged down. The supernatant was transferred to a 10·0 ml. graduated tube and mixed with 0·8 ml. amidol reagent (1 g. amidol and 20 g. sodium bisulphite in 100 ml. distilled water, made weekly). The volume was adjusted to 10·0 ml. by adding distilled water. The optical density of the blue colour was read against a blank at 700 mμ after 5 minutes and not more than 30 minutes.

The amount of phosphorus in the sample was calculated from a standard graph for phosphorus estimations. Since phosphorus comprises 10% of the phosphoprotein phosvitin (Mok, Martin & Common, 1961; Heald & McLachlan, 1963 a), the phosphorus values were multiplied by ten when the results were expressed as phosphoprotein instead of phosphoprotein phosphorus.

The use of perchloric acid in the above procedure gave results similar to those obtained by using trichloroacetic acid as in the original method. Thus the values for phosphoprotein level obtained when two different samples of laying hen plasma were treated with perchloric acid were 105 and 143 mg./100 ml. while the corresponding values obtained when trichloroacetic acid was used were 109 and 143 mg./100 ml. The above procedure has the advantage over that of Schmidt & Thannhauser in that the sample is treated with a small volume of perchloric acid in the initial step instead of the 20 volumes of 7% trichloroacetic acid used in the original method, and centrifugation is used instead of filtration. Determination of the phosphoprotein phosphorus of 0·5 ml. laying hen plasma by the above procedure gave the value 12·5 mg./100 ml. while determination of the total phosphorus (Allen, 1940) content of the residue before alkali hydrolysis obtained from an equivalent volume of plasma gave the value 11·8 mg./100 ml., thus confirming that treatment with perchloric acid, ethanol-ether, and methanol-chloroform removed all sources of phosphorus other than phosphoprotein phosphorus which was subsequently liberated completely by alkali hydrolysis.

3.6. Sodium

The concentration of sodium or sodium chloride was determined by means of an EEL flame photometer. An aliquot of the fraction (0·1 ml. in case of the fractions obtained from chromatography on DEAE-cellulose) was diluted to a suitable concentration and the sodium content estimated by comparing the photometer reading to that of standard solutions readings plotted in a graph.

3.7. Lipids

Lipids were extracted by treating the material (whole plasma, aliquots of fractions, or freeze-dried material from fractions) with chloroform methanol (2:1, v/v) (Folch, Lees & Sloane Stanley, 1957).* The solvent was then evaporated off by heating in a warm water bath. The residue was dissolved in a small volume of chloroform and an aliquot spotted on a silica gel plate activated by heating in an oven at 100°C for a few minutes. The spot was allowed to dry and was then sprayed with phosphomolybdic acid in methanol (5%, w/v). The sprayed plate was heated for a few minutes in an oven at 100°C. A positive test is indicated by the appearance of distinct blue spots on a light green background.

Semiquantitative estimations were carried out directly on the plate by comparing the intensity of the blue colour of the test sample to that of known standards.

Separation and identification of the components of a lipid mixture was made by subjecting the sample and standards to thin layer chromatography on silica gel. The lipid was made salt-free by shaking the chloroform-methanol extract with a little volume of distilled water.* The solvent system used for chromatography was:

Petroleum ether: 90 volumes, ether: 10 volumes, acetic acid: 1 volume.

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* Lipids were extracted by treating the material with 20 volumes of chloroform-methanol and the extract was made salt-free by shaking with 0.2 volume of distilled water.

After about 45 minutes the plate was dried and stained for lipids as described before.

EXPERIMENTAL AND RESULTS

1. NATURE OF THE NON-TRANSFERRIN IRON

1.1. Gel filtration on Sephadex G-25

5 µc ^{59}Fe (ferric chloride) were injected intravenously into a laying hen. Blood was collected after an hour. 0·5 ml. plasma was subjected to gel filtration on Sephadex G-25 in 0·5% KCl (Methods, p. 16) and the fractions were analysed for radioactivity, protein and sodium in that order.

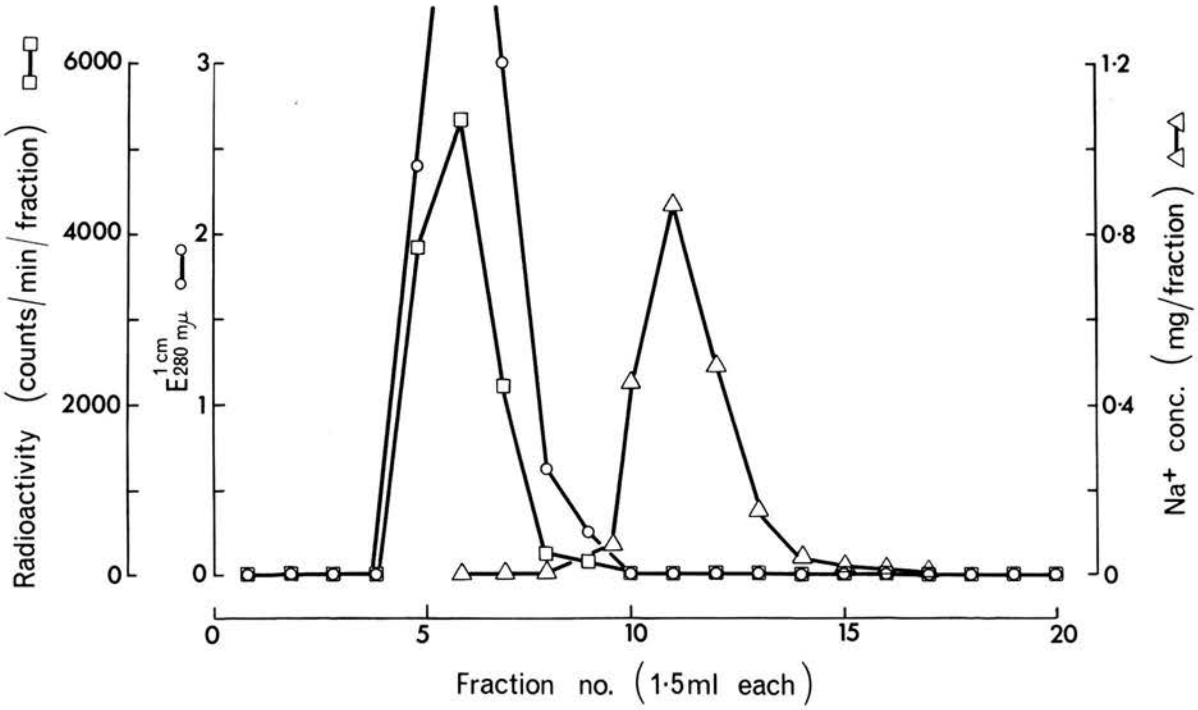
Fig. 2 shows that gel filtration on Sephadex G-25 separated the high molecular weight substances (proteins) from the diffusible ions (Na^+) and it can be seen that no radioactivity appeared in the fractions containing sodium. All the radioactivity applied to the column was recovered in the fractions containing proteins, indicating that the non-transferrin iron in the plasma of the laying hen is associated with high molecular weight substances, possibly proteins.

1.2. Magnesium carbonate adsorption

1.2.1. Effect of $MgCO_3$ treatment on transferrin-bound iron.

Plasma from rats and non-oestrogenized cockerels was used as a source of transferrin-bound iron. The plasma (1·0 ml.) was either treated with $MgCO_3$ as described in "Methods" and the iron

Fig. 2. Gel filtration on Sephadex G-25 of laying hen plasma labelled *in vivo* with ^{59}Fe ($FeCl_3$, i/v)



concentration determined before and after $MgCO_3$ treatment, or a tracer quantity of ^{59}Fe (ferric chloride) was added to the plasma and the radioactivity determined before and after $MgCO_3$. The results obtained by both methods confirm that $MgCO_3$ treatment does not affect transferrin-bound iron. Thus, determinations of the concentration of iron in the plasma from six rats gave a mean value of 186 µg./100 ml. before $MgCO_3$ and 188 µg./100 ml. after $MgCO_3$. Similarly, the mean plasma iron values in five cockerels were 134 µg./100 ml. in the untreated plasma and 135 µg./100 ml. in the $MgCO_3$ treated plasma.

1.2.2. Effect of $MgCO_3$ treatment on conalbumin-bound iron

Egg white from a fresh hen's egg was filtered (Whatman No.1 filter paper) and the filtrate was mixed with a tracer quantity of ^{59}Fe (ferric chloride) and allowed to stand at laboratory temperature for 20 minutes. When an aliquot of this material (1·0 ml.) was subjected to the $MgCO_3$ procedure described in "Methods" and the radioactivity measured before and after the treatment the values obtained (counts/min./ml.) were: 185046 before the treatment and 185077 after the treatment.

Thus conalbumin-bound iron, like transferrin-bound iron, is not affected by the $MgCO_3$ adsorption procedure used in this investigation.

1.2.3. Effect of $MgCO_3$ treatment on plasma iron in the laying hen

Determination of the plasma iron in 27 laying hens before and after $MgCO_3$ treatment showed that before $MgCO_3$ treatment the mean value was 729 µg./100 ml. (range 411-1142) but after the treatment the mean value was 303 µg./100 ml. (range 216-448). Thus more than half the plasma iron was removed by $MgCO_3$ treatment. This is in agreement with the results of Planas et al. (1961).

Table 2 shows that $MgCO_3$ treatment also removed phosphoprotein from the plasma of the laying hen.

Since $MgCO_3$ treatment does not affect the iron bound to transferrin and conalbumin, and since gel filtration shows that the plasma iron in the laying hen is associated with high molecular weight substances, it seems possible that the iron removed by $MgCO_3$ is phosphoprotein iron. The very definite relation between the phosphoprotein and the iron removed by $MgCO_3$ can be seen from Fig. 3 where the values for phosphoprotein level in 16 laying hens are plotted against the corresponding values for the iron removed by $MgCO_3$. The regression line relating iron removed by magnesium carbonate (y) to phosphoprotein level (x) can be expressed as

$$y = bx + a$$

where y is the expected value as distinct from the observed value of iron removed by magnesium carbonate, b is the slope of the line and a is the intercept. The calculations were carried out

Table 2

Plasma iron and plasma phosphoprotein in six laying hens before and after $MgCO_3$ treatment.

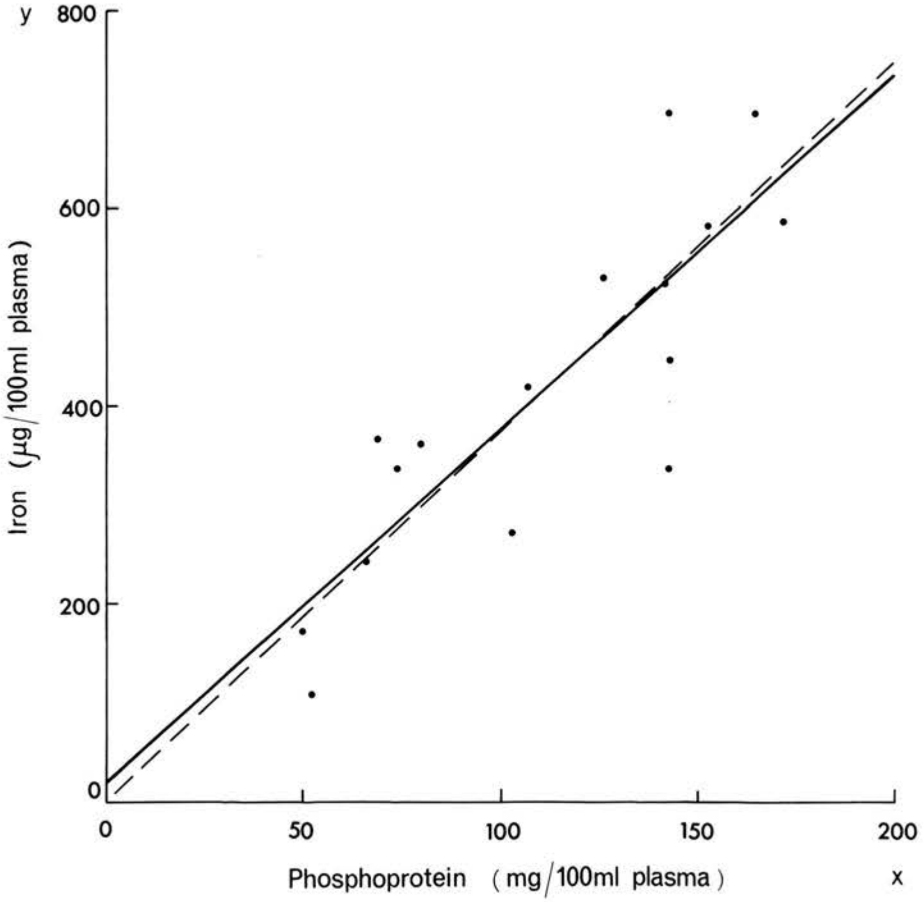
Plasma Iron

	Before $MgCO_3$	After $MgCO_3$
Mean	654 µg./100 ml.	255 µg./100 ml.
Range	411 - 963	216 - 314

Plasma phosphoprotein

	Before $MgCO_3$	After $MgCO_3$
Mean	97 mg./100 ml.	0·57 mg./100 ml.
Range	50 - 143	0·4 - 0·9

Fig. 3. Relation between the iron removed by $MgCO_3$ treatment and the phosphoprotein level in the plasma of sixteen laying hens.
———, Regression line of y on x.
– – – –, Best fit straight line through the origin.



as described by Moore & Edwards (1965). The value of b is 3·54 ± 0·59 µg. iron/mg. phosphoprotein and that of a is 23·0 ± 70·6 µg. iron/100 ml. plasma. The value of b is highly significantly different from zero ($P < 0{\cdot}001$) whereas a is not significantly different from zero ($0{\cdot}7 < P < 0{\cdot}8$). Since the intercept is both statistically and quantitatively insignificant these findings are consistent with the hypothesis that the iron removed by magnesium carbonate is phosphoprotein-bound iron, and the amount of iron per unit of phosphoprotein phosphorus can best be estimated by computing the slope of the best fit line through the origin. Computation of the best fit straight line through the origin ($y' = b'x$, Moore & Edwards, 1965) gives a value of b' (slope) of 3·73 µg. iron/mg. phosphoprotein, which corresponds to an average of 0·021 atoms of iron per atom of phosphoprotein phosphorus. In phosphoprotein saturated with iron, one expects about 0·5 atoms of iron per atom of phosphorus (Taborsky, 1963). Thus the quantity of 'phosphoprotein iron' in the plasma comprises about 4·2% of the total iron-binding capacity of the phosphoprotein.

Other statistical comparisons on the same sixteen samples showed that there was no relation between phosphoprotein level and transferrin iron level, or between the concentration of the iron removed by $MgCO_3$ and that of transferrin iron.

1.3. Gel filtration on Sephadex G-200

Fig. 4 shows the behaviour of transferrin-bound iron when rat plasma (labelled with ^{59}Fe) was subjected to gel filtration on Sephadex G-200 using 0·1 M tris-HCl buffer, pH 8·0, containing 1·0 M NaCl. The plasma proteins were separated into the usual three groups (cf. Gelotte, Flodin & Killander, 1962; Dolezalova, Brada & Kocent, 1965) and all the radioactivity applied to the column was recovered in the fractions where transferrin is expected to appear. Similar results were obtained when cockerel plasma labelled in vivo with ^{59}Fe (ferric chloride, i/v) was fractionated in the same manner (Fig. 5).

On the fractionation of laying hen plasma labelled in vivo with ^{59}Fe (14 μc ^{59}Fe, ferric citrate, in 2·4 μg. iron were injected subcutaneously and blood was collected after 45 minutes) using 0·1 M tris-HCl buffer, pH 8·0, containing 1·0 M NaCl, about 90% of the radioactivity was recovered in the fractions containing proteins. The recovered iron was separated into two fractions of which one corresponded to transferrin while the other corresponded to the phosphoprotein-containing fractions (Fig. 6). The appearance of phosphoprotein in the fractions containing the very high molecular weight substances is consistent with the presence of phosphoprotein in the plasma as part of a large particle. Results similar to the above were obtained when laying hen plasma labelled in vivo with $^{59}FeCl_3$ was fractionated using 0·1 M tris-HCl

Fig. 4. Gel filtration on Sephadex G-200 of rat plasma labelled *in vivo* with ^{59}Fe (ferric citrate, s/c)

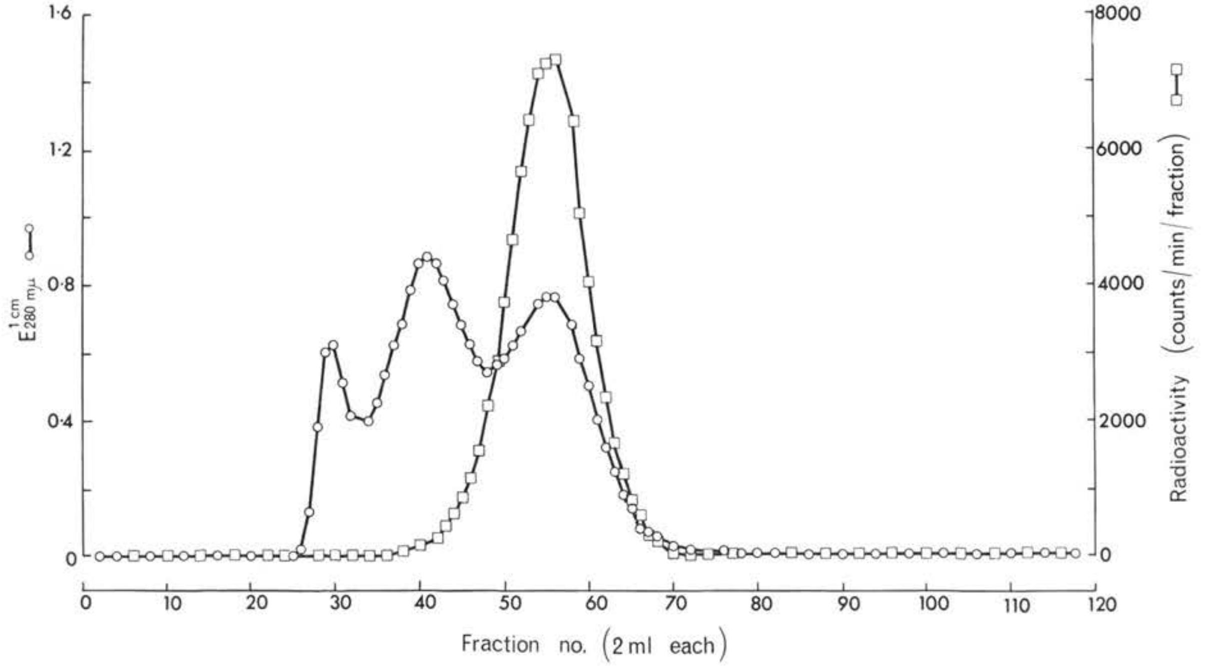


Fig. 5. Gel filtration on Sephadex G-200 of cockerel plasma labelled *in vivo* with ^{59}Fe (FeCl$_3$, i/v)

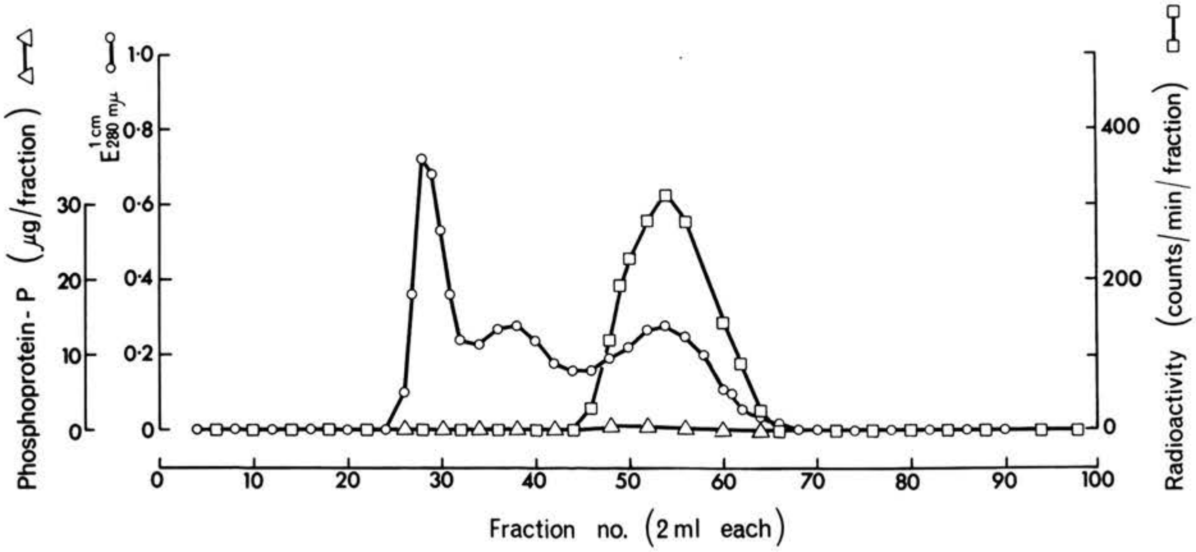
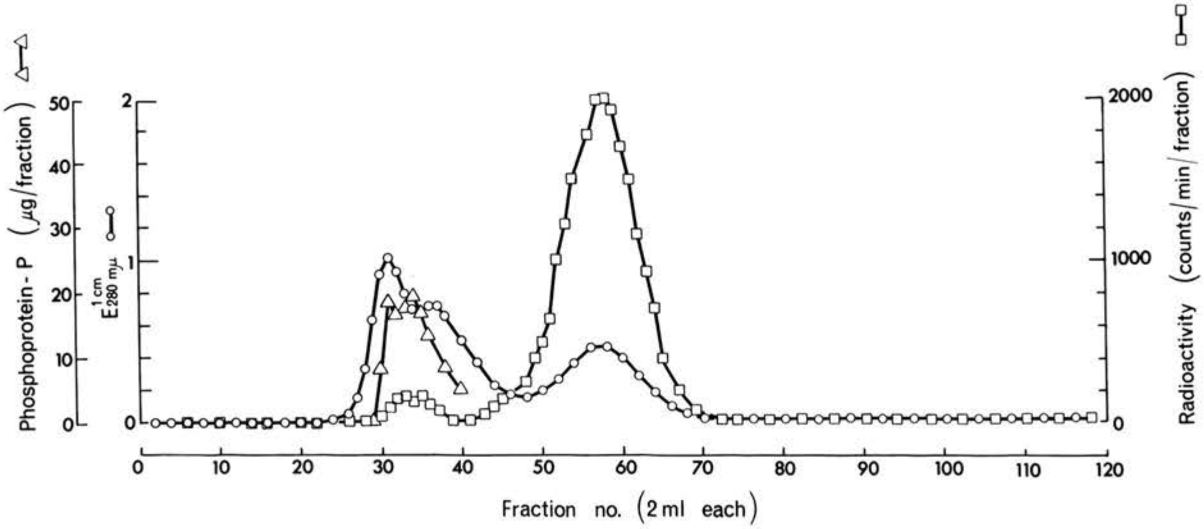


Fig. 6. Gel filtration on Sephadex G-200 of laying hen plasma labelled *in vivo* with ^{59}Fe (ferric citrate, s/c)



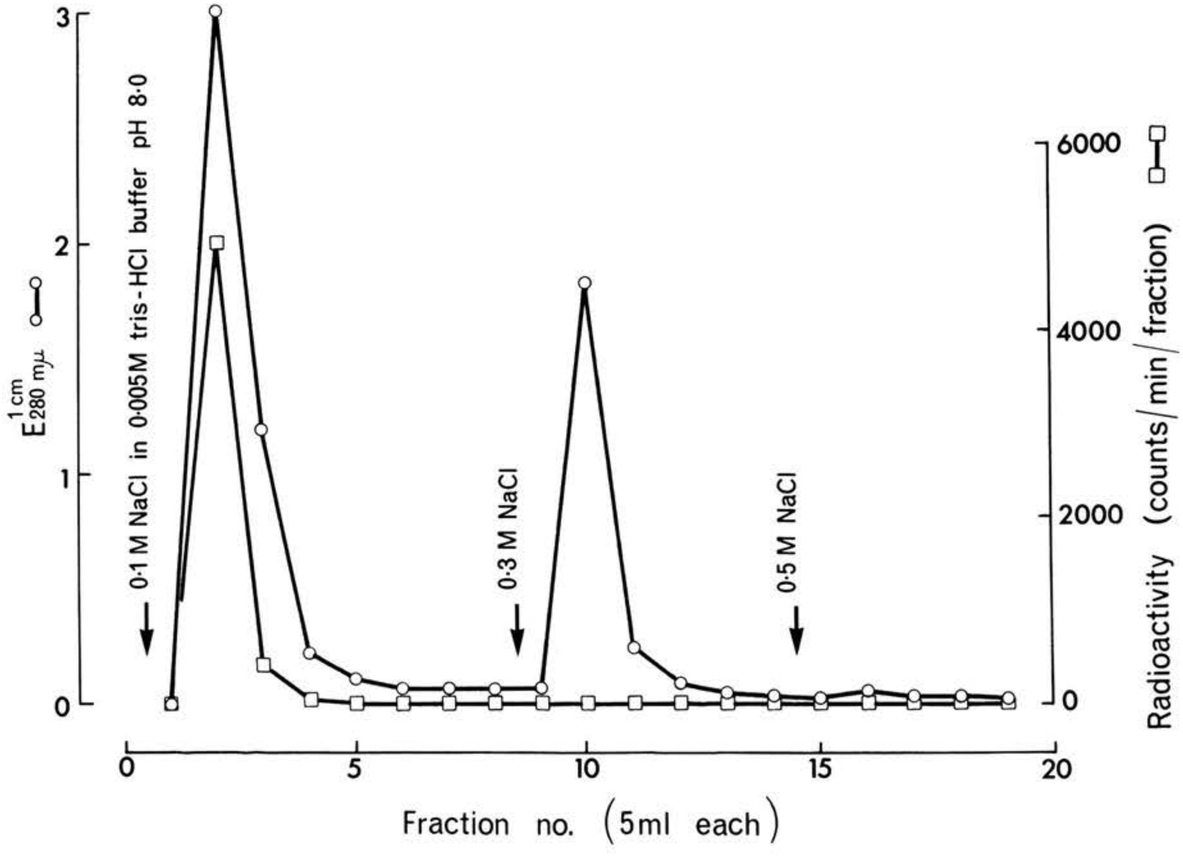
buffer, pH 8·0, containing 0·15 M NaCl. Complete recovery of the radioactivity in the protein-containing fractions could be obtained by using 0·15 M NaCl in 0·005 M tris-HCl buffer pH 7·4 and the extra recovered iron corresponded to the phosphoprotein.

1.4. Chromatography on DEAE-cellulose

1.4.1. Behaviour of transferrin-bound iron

4 μc ^{59}Fe (ferric citrate, in 0·7 μg. iron) were injected subcutaneously into a rat. Blood was collected after an hour (cardiac puncture) into a heparinised syringe. 1·0 ml. plasma was loaded on a DEAE-cellulose column equilibrated with 0·1 M NaCl in 0·005 M tris-HCl buffer, pH 8·0. Elution was carried out with 0·1 M NaCl in 0·005 M tris-HCl buffer, pH 8·0 and with a stepwise increase in NaCl concentration to 0·3 M and 0·5 M as indicated by the arrows in Fig. 7. All the radioactivity applied to the column (transferrin-bound ^{59}Fe) was recovered in the fractions eluted with 0·1 M NaCl in 0·005 M tris-HCl buffer pH 8·0 (Fig. 7).

Fig. 7. Chromatography on DEAE-cellulose of rat plasma labelled *in vivo* with ^{59}Fe



1.4.2. Behaviour of conalbumin-bound iron

0·5 ml. egg white labelled in vitro with ^{59}Fe was applied to a DEAE-cellulose column (containing about 2·5 g. cellulose) equilibrated with 0·1 M NaCl in 0·005 M tris-HCl buffer, pH 8·0, and elution was carried out with a NaCl gradient (Methods, p.22). The arrow (Fig. 8) indicates the start of the gradient.

It can be seen from Fig. 8 that conalbumin-bound iron behaves exactly like transferrin-bound iron.

1.4.3. Chromatography on DEAE-cellulose of laying hen plasma labelled in vivo with ^{59}Fe

14 μc ^{59}Fe (ferric citrate, in about 2·4 μg. iron) were injected subcutaneously into a laying hen. Blood was collected after 45 minutes. 1·0 ml. Plasma was applied to a DEAE-cellulose column equilibrated with 0·02 M NaCl in 0·005 M tris-HCl buffer, pH 8·0. Elution was carried out with a gradient increase in NaCl concentration. The mixing chamber contained 200 ml. of 0·02 M NaCl in 0·005 tris-HCl buffer, pH 8·0, while the reservoir contained 1·0 M NaCl in 0·005 M tris-HCl buffer, pH 8·0. Fractions (3 ml. each) were collected at a flow rate of about 20 ml./hour. The fractions were analysed for radioactivity, protein, NaCl and phosphoprotein phosphorus in that order. The results are shown in Fig. 9.

It can be seen from Fig. 9 that the iron was separated into

Fig. 8. Chromatography on DEAE-cellulose of egg white labelled *in vitro* with ^{59}Fe

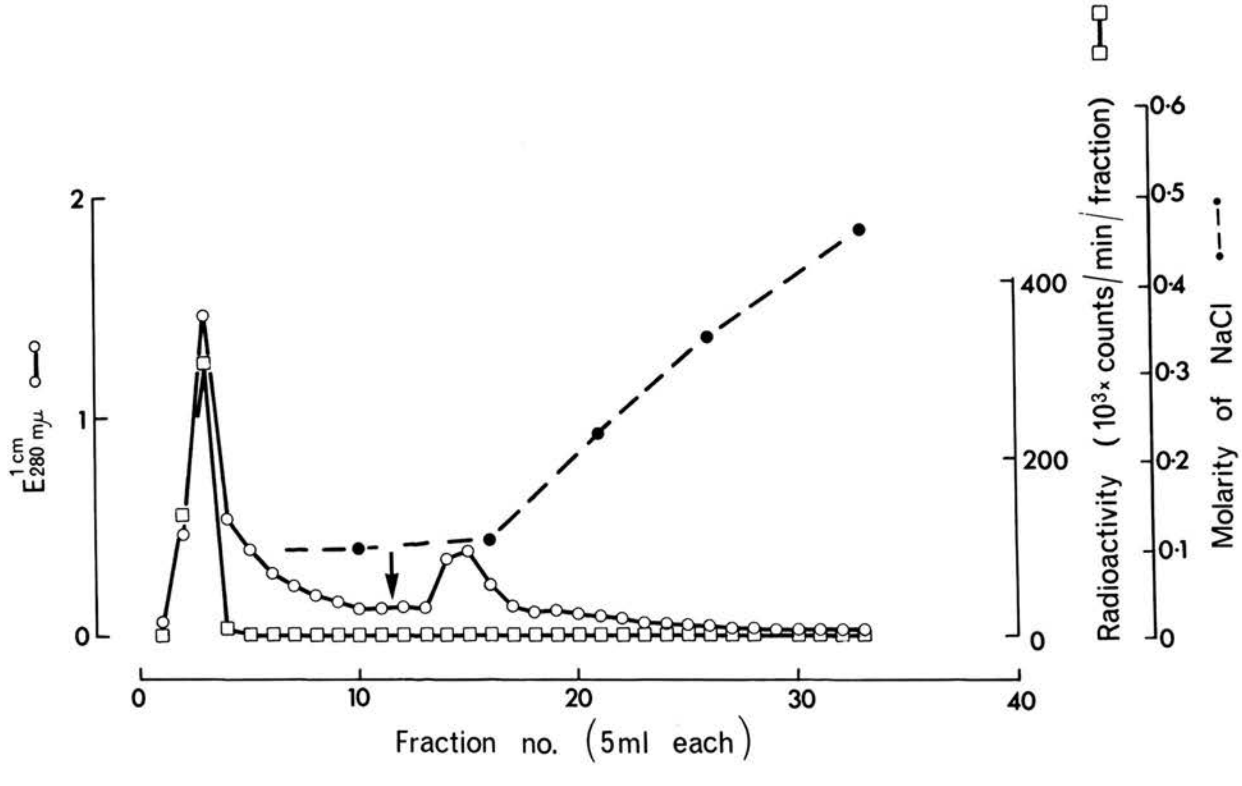
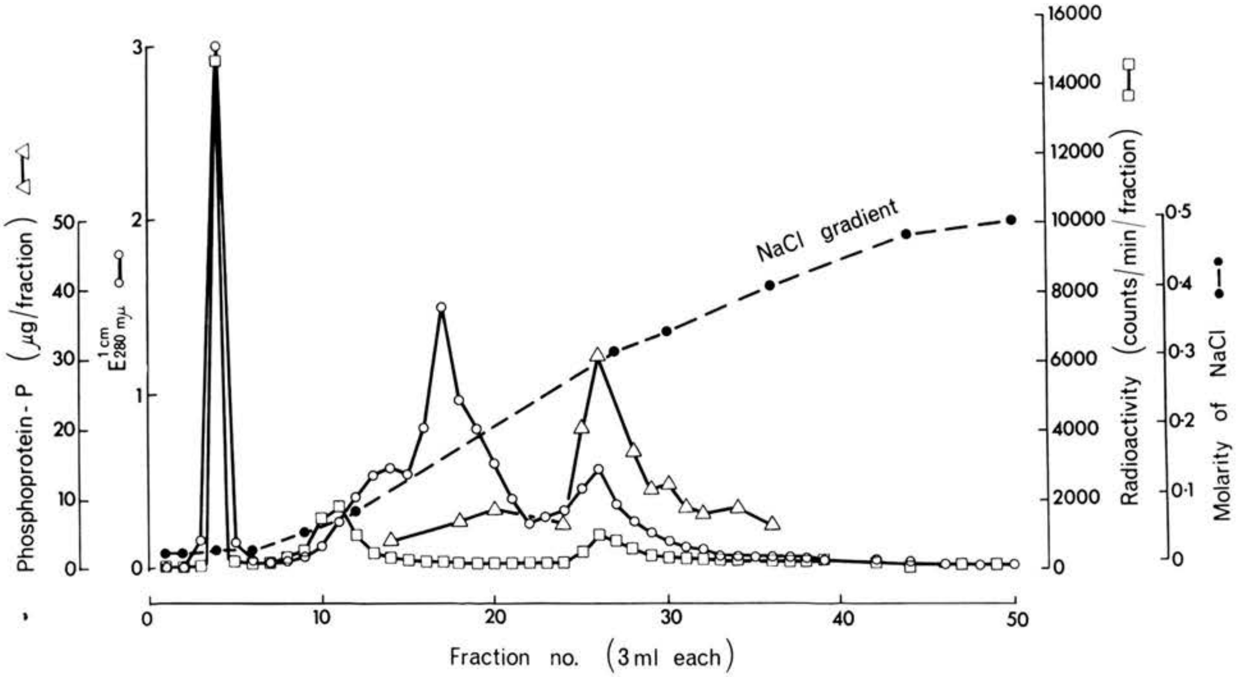


Fig. 9. Chromatography on DEAE-cellulose of laying hen plasma labelled *in vivo* with ^{59}Fe (ferric citrate, s/c)



three fractions, two of which were eluted with 0·1 M NaCl which corresponds to mammalian transferrin, whereas the third fraction started to be eluted as the molarity of NaCl approached 0·3 M and was eluted with about 0·4 M NaCl which is known to elute the phosphoprotein (Connelly & Taborsky, 1961; Heald & McLachlan, 1963 a; Greengard, Sentenac & Acs, 1965); and the distribution of ^{59}Fe in this fraction corresponds to that of the phosphoprotein phosphorus. 94% of the total radioactivity in the plasma was recovered in the fractions, 78% of the radioactivity appeared in the transferrin fractions and 16% appeared in the phosphoprotein fractions. $MgCO_3$ treatment showed that 79% of the radioactivity corresponded to transferrin whereas only 32% of the plasma non-radioactive iron corresponded to transferrin. Thus, the distribution of radioactivity in the plasma after 45 minutes of the administration of tracer ^{59}Fe subcutaneously does not reflect the true distribution of the native iron in the plasma.

When a sample of plasma was collected from the hen used in this experiment after 34 days, $MgCO_3$ treatment showed that the ^{59}Fe in the plasma (^{59}Fe released from destroyed red cells) followed the same distribution as that of the non-radioactive iron and half of each was removed by $MgCO_3$.

1.4.4. Chromatography on DEAE-cellulose of cockerel plasma labelled in vivo with ^{59}Fe

Plasma was labelled by injecting ^{59}Fe (ferric chloride, 2 μc in 0·25 μg. iron) in the wing vein of a three months old cockerel and collecting blood after 20 minutes. 1·0 ml. plasma was subjected to chromatography on DEAE-cellulose (Methods, p. 22). The arrow in the figure (Fig. 10) indicates the start of the gradient.

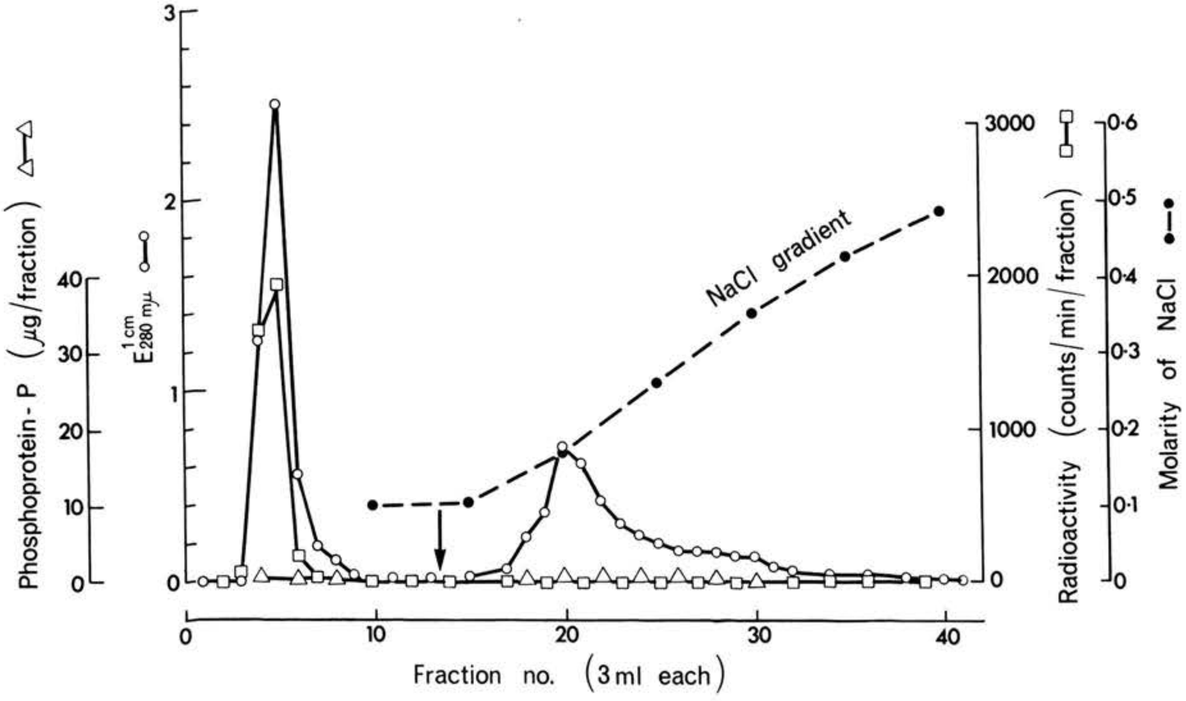
It can be seen from Fig. 10 that in cockerel plasma where phosphoprotein is absent all the radioactivity appeared in the fractions eluted with 0·1 M NaCl. Recovery of radioactivity was 100%.

In view of the full recovery of ^{59}Fe in this experiment and the close agreement between the values for transferrin ^{59}Fe obtained by adsorption on magnesium carbonate and chromatography on DEAE-cellulose in the previous experiment, it appears that the unrecovered iron in the previous experiment belonged to the non-transferrin fraction.

1.5. Combination of Techniques

0·5 ml. of a solution of ^{59}Fe (ferric chloride, containing 2 μc ^{59}Fe and about 0·2 μg. iron) was injected intravenously into a laying hen. Blood was collected after 20 minutes. 1·0 ml. of the fresh heparinised plasma was subjected to gel filtration on Sephadex G-200 using 0·15 M NaCl in 0·005 M tris-HCl buffer pH 7·4. The fractions (2 ml. each) were analysed for

Fig. 10. Chromatography on DEAE-cellulose of cockerel plasma labelled *in vivo* with ^{59}Fe ($FeCl_3$, i/v)



radioactivity and were then diluted to 3 ml. with distilled water and analysed for protein (E 280 mμ). The results are presented in Fig. 11.

It can be seen from Fig. 11 that the iron is separated into two fractions, of which one corresponds to transferrin (peak II) whereas the other (peak I) migrates with the proteins of much higher molecular weight. All the radioactivity applied to the column was recovered in the protein-containing fractions.

1.5.1. Chromatography on DEAE-cellulose of 'Sephadex G-200' Peak I fractions

The fractions containing the first peak of radioactivity (fractions 29-47) were pooled together in a beaker and 0·1 M NaCl in 0·005 M tris-HCl buffer was added with stirring (magnetic stirrer) till the pH of the pooled fractions was brought to 8·0. The material was then loaded on a DEAE-cellulose column equilibrated with 0·1 M NaCl in 0·005 M tris-HCl buffer pH 8·0 (starting buffer). Elution was carried out with the starting buffer till the optical density of the eluate at 280 mμ was 0·0 and then with a NaCl gradient as described on page 22. The arrow in Fig. 12 indicates the start of the gradient. The eluate was collected in fractions (5 ml. each) at a flow rate of 25 ml./hour. The fractions were analysed for radioactivity, protein, NaCl and phosphoprotein phosphorus in that order.

Fig. 11. Gel filtration on Sephadex G-200 of laying hen plasma labelled <u>in vivo</u> with ^{59}Fe (FeCl$_3$, i/v)

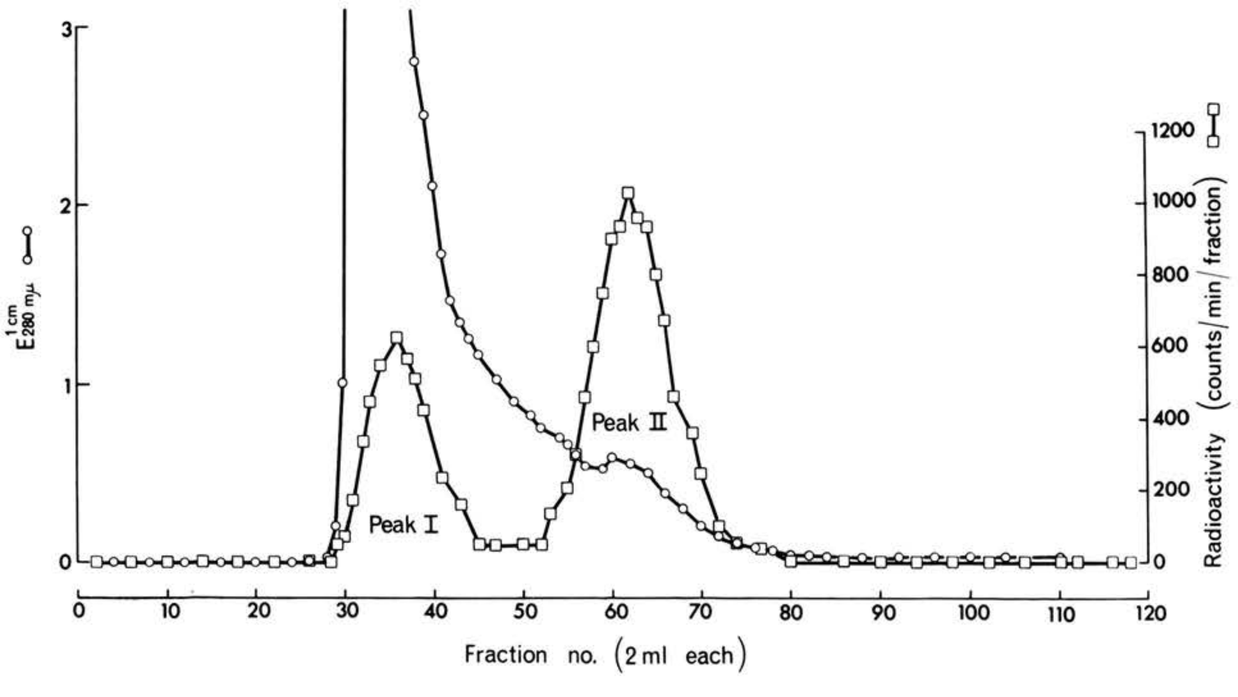
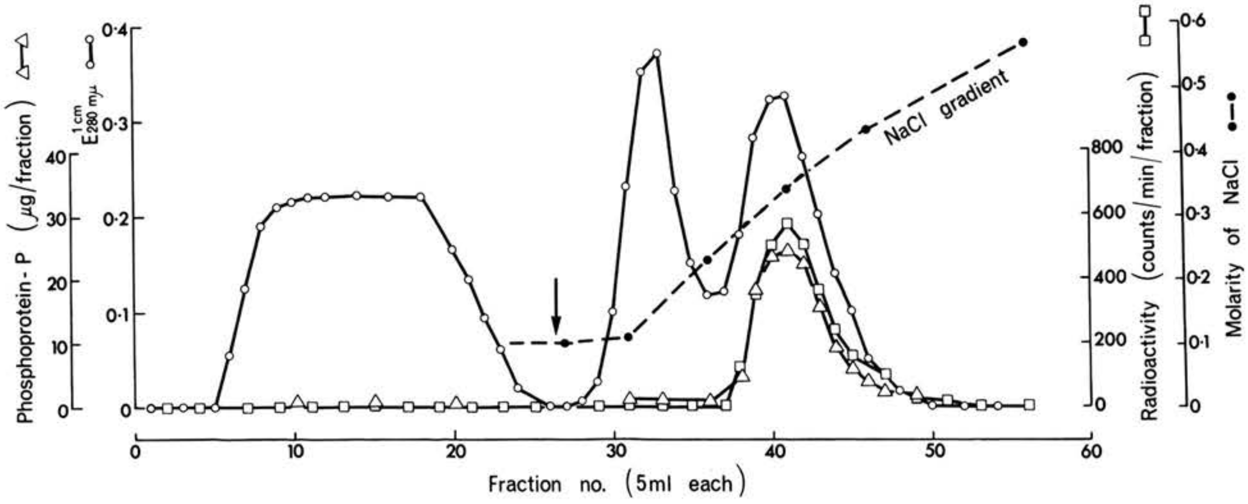


Fig. 12. Chromatography on DEAE-cellulose of Sephadex G-200 Peak I (Fig. 11) fractions.



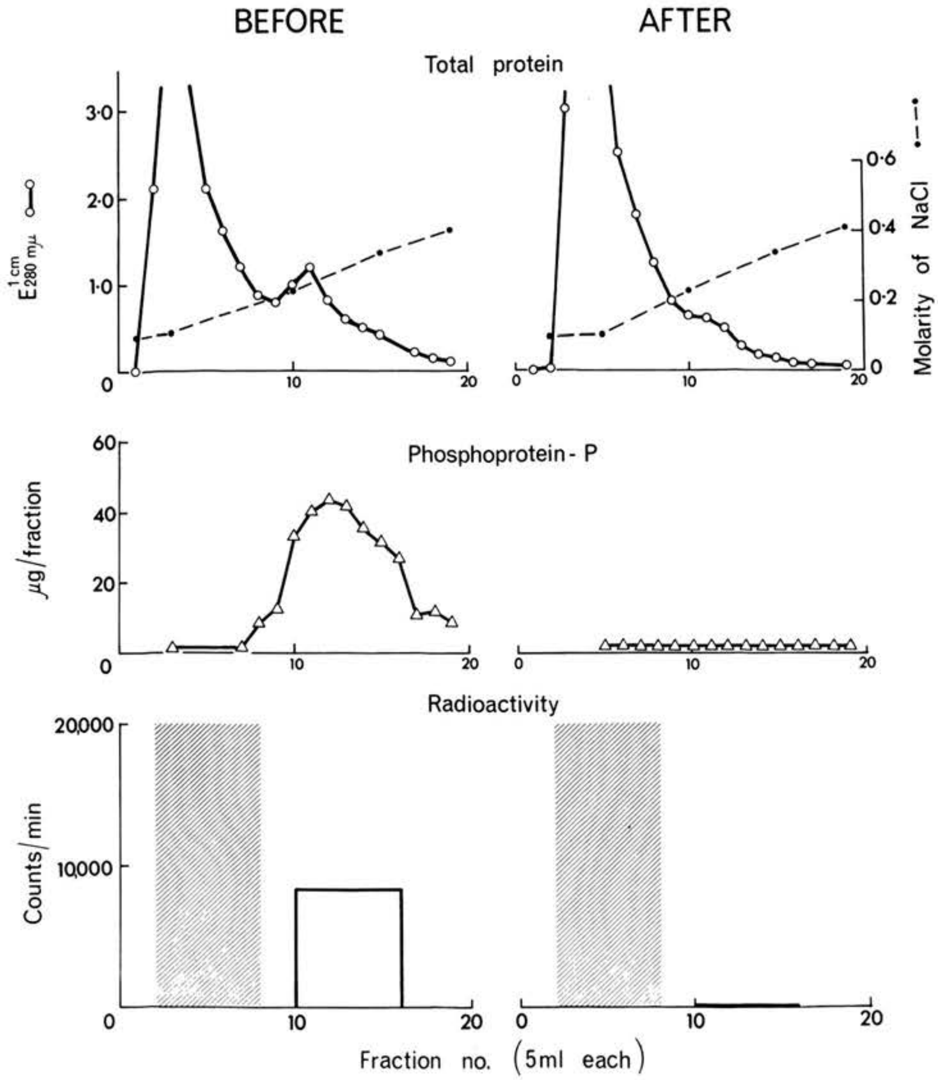
It can be seen from the results presented in Fig. 12 that none of the ^{59}Fe was eluted with 0·1 M NaCl, indicating the absence of transferrin and conalbumin, but one can see the close association between the phosphoprotein phosphorus and the ^{59}Fe eluted with about 0·4 M NaCl. Moreover, the ratio of the number of counts per minute to phosphoprotein phosphorus in fractions 39 to 43 was 20, 21, 23, 22, 22; which is consistent with a specific binding of the iron to the phosphoprotein phosphorus.

1.5.2. Chromatography on DEAE-cellulose before and after $MgCO_3$ treatment

2·0 ml. of the ^{59}Fe-labelled laying hen plasma used in the above experiment were treated with $MgCO_3$ and an aliquot of the supernatant (equivalent to 1·0 ml. plasma) was chromatographed on DEAE-cellulose simultaneously with 2·0 ml. untreated plasma. The columns were equilibrated with 0·1 M NaCl in 0·005 M tris-HCl buffer, pH 8·0, and elution was carried out with a NaCl gradient (Methods, P.22) immediately after the application of the sample. Fractions (5 ml. each) were collected at a flow rate of 20-25 ml./hour. The fractions were analysed for radioactivity, protein, NaCl and phosphoprotein phosphorus in that sequence.

The results are presented in Fig. 13 where correction is made for the difference in volumes of the plasma chromatographed.

Fig. 13. Chromatography on DEAE-cellulose of laying hen plasma labelled <u>in vivo</u> with ^{59}Fe ($FeCl_3$, i/v) before and after treatment with $MgCO_3$.



The radioactivity is presented in histogram form for the sake of the quantitative comparison of the distribution of ^{59}Fe before and after $MgCO_3$. The shaded rectangle represents the total counts eluted with 0·1 M NaCl (transferrin ^{59}Fe) while the unshaded one represents the counts eluted with about 0·4 M NaCl.

As can be seen from Fig. 13 in the untreated plasma the iron was separated into two fractions of which one migrated with transferrin, while the other migrated with phosphoprotein phosphorus, but in the $MgCO_3$ treated plasma both the phosphoprotein phosphorus and the iron migrating with it were removed whereas transferrin iron remained unaffected.

Thus, the combination of techniques provides further evidence that the non-transferrin iron is likely to be bound to phosphoprotein and not conalbumin.

1.6. In vitro binding of iron by laying hen plasma

Experiments in which tracer amounts of $^{59}FeCl_3$ were added to laying hen plasma ($< 0{\cdot}05$ μg.Fe^{3+}/ml. plasma, incubated at 37°C for an hour) and the plasma was fractionated using $MgCO_3$, gel filtration on Sephadex G-200 and chromatography on DEAE-cellulose showed that all the iron added was found to correspond to transferrin and none of the radioactivity appeared in the phosphoprotein-containing fractions. Since transferrin is known to bind ferric ions very rapidly it appears that the small amounts of added iron were bound to the transferrin before the phosphoprotein could bind any of the iron. It, therefore, seemed interesting to see what happens when large amounts of iron (i.e. large enough to saturate transferrin and leave an excess of iron) are added. Consequently, the following experiments were carried out.

^{59}Fe solutions (ferric chloride, 0·6 ml. in about 0·02 N HCl) were added to heparinised plasma (4 ml.) from a laying hen to give:

Flask A] 0·05 µg. Fe^{3+} (containing 0·2 µc $^{59}Fe^{3+}$) per ml. plasma.

Flask B] 10 µg.Fe^{3+} (containing 0·2 µc $^{59}Fe^{3+}$) per ml. plasma.

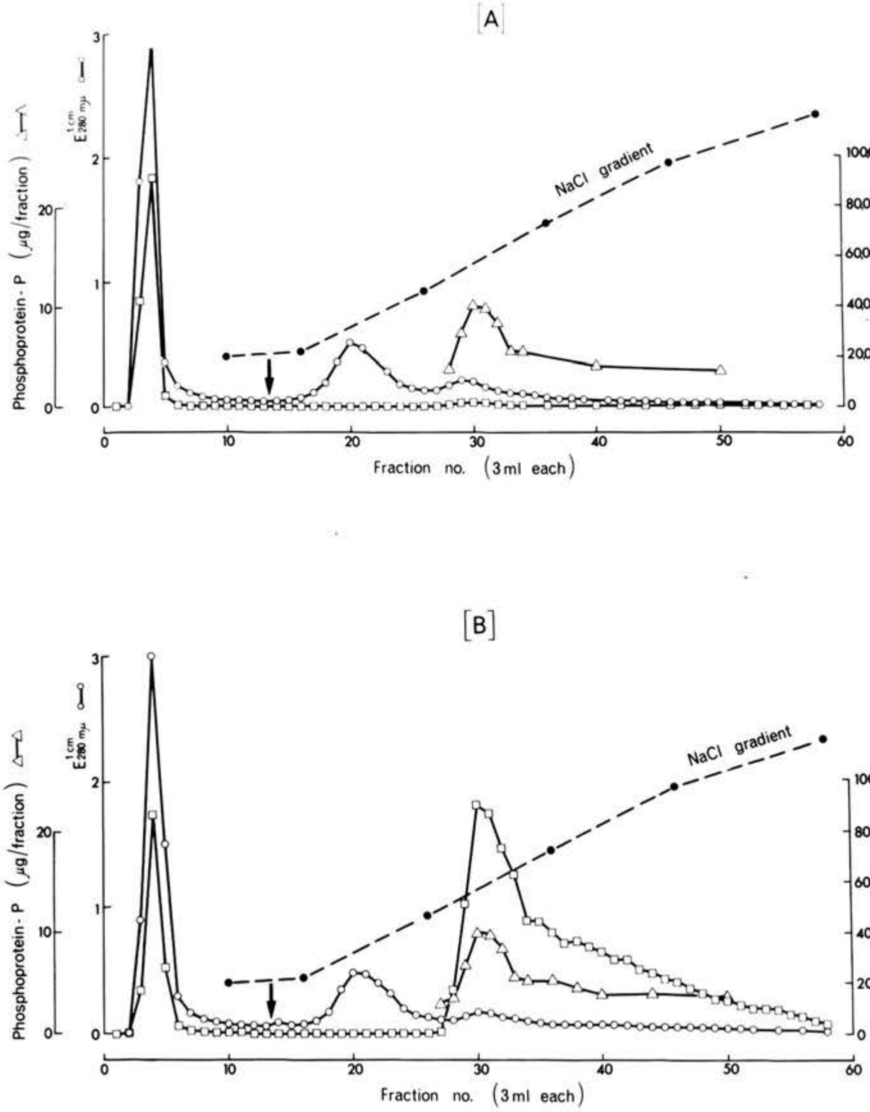
After one hour incubation at 37°C 1·0 ml. from each flask was subjected to chromatography on DEAE-cellulose (Methods, p.22). The eluate was collected in fractions of 3 ml. each. The first 13 fractions were eluted with 0·1 M NaCl in 0·005 M tris-HCl buffer, pH 8·0, and then the NaCl gradient was set up. The fractions were analysed for radioactivity, protein, NaCl and phosphoprotein phosphorus. The results are given in Fig. 14.

It can be seen from the results that when a small quantity of iron was added to the plasma (A) most of the radioactivity (85%) appeared in the transferrin fractions and only a small proportion (about 9%) appeared in the phosphoprotein-containing fractions; but when a large quantity of iron was added

Fig. 14. Chromatography on DEAE-cellulose of laying hen plasma incubated (1 hr.) with:

[A] 0·05 μg Fe^{3+} (containing 0·2 μc $^{59}Fe^{3+}$) per ml. plasma

[B] 10 μg Fe^{3+} (containing 0·2 μc $^{59}Fe^{3+}$) per ml. plasma



(B) most of the radioactivity (> 73%) appeared in the phosphoprotein-containing fractions and about 10·8% appeared in the transferrin fractions. The iron appearing in the phosphoprotein fractions is likely to be bound to the phosphoprotein since preliminary experiments showed that ferric ion in solution or added in excess to non-oestrogenized cockerel plasma was retained in the column under the fractionation conditions employed.

$MgCO_3$ treatment of aliquots of the plasma showed that 85·9% of the radioactivity corresponded to transferrin in the case of A and 10·9% in the case of B, in good agreement with the results of DEAE-cellulose chromatography.

In another experiment in which the addition of excess iron (labelled with ^{59}Fe) to laying hen plasma (0·1 ml. ferric chloride solution [in 0·02 N HCl] containing 0·04 μc $^{59}Fe^{3+}$ and 10 μg.Fe^{3+} per ml. plasma) was followed by $MgCO_3$ treatment after 5 to 60 minutes, 97·5 to 97·7% of the added iron was removed by $MgCO_3$. When 0·1 ml. of the above ferric chloride solution was shaken with 200 mg. $MgCO_3$ suspended in 2·9 ml. distilled water for 30 minutes (to adsorb the iron on $MgCO_3$) and then shaken again with 1·0 ml. plasma for 30 minutes, 99·4% of the iron remained adsorbed in $MgCO_3$.

Determination of the plasma iron level in the above two experiments and in the plasma from five other laying hens together with the determination of the iron concentration after $MgCO_3$

treatment with and without prior addition of iron (10 μg.Fe^{3+}/ml., $MgCO_3$ treatment after 5 minutes) gave the results shown in Table 3 where plasma iron after $MgCO_3$ treatment without prior addition of iron represents transferrin-bound iron while that after $MgCO_3$ with prior addition of iron represents transferrin level.

It can be seen from Table 3 that the values for plasma iron after $MgCO_3$ treatment are about the same irrespective of whether iron was added to the plasma or not. This is in agreement with the findings of Planas et al. (1961). Thus, the transferrin in the plasma of the laying hen appears to be saturated or almost saturated. The apparent uptake of iron by transferrin in the experiments in which only a tracer of high specific activity labelled iron (in acid solution) was added may be due to a local acidification effect resulting in the replacement of some of the non-radioactive iron originally bound to transferrin by the newly added radioactive iron. On the other hand, it is possible that experiments of the type first described by Planas et al. (1961) would fail to detect an unsaturated iron-binding capacity of a few micrograms.

It also appears from Table 3 that the level of transferrin in the plasma of the laying hen is similar to that in non-laying birds and mammals.

It can also be seen from Table 3 that $MgCO_3$ treatment

Table 3

Plasma iron in seven laying hens after treatment with magnesium carbonate with and without prior addition of iron to the plasma

	Mean (µg./100 ml.)	Range
Total plasma iron	607	411 - 769
Plasma iron after $MgCO_3$ treatment without prior addition of iron	275	224 - 403
Plasma iron after $MgCO_3$ treatment with prior addition of iron	272	217 - 403

removed all the added iron and more than half the plasma iron. This would be the expected result if the added iron was bound to phosphoprotein or was still in ionic form but not if it was bound to conalbumin. However, chromatography on DEAE-cellulose indicates that most of the added iron appeared in the phosphoprotein-containing fractions. Thus, $MgCO_3$ treatment might have removed the phosphoprotein with its originally bound iron as well as its newly bound iron.

1.7. Plasma iron in the oestrogen-treated cockerel

Two cockerels (weighing about 3 Kg. each) were injected subcutaneously with 1·0 mg./100 g. body wt. of stilbestrol dipropionate (May & Baker Ltd., preparation in oil containing 10 mg./ml.). Blood specimens (3-4 ml. each) were drawn immediately after the injection and at the times indicated in column 1 in Table 4. The fresh heparinised plasma was analysed for phosphoprotein and iron before and after treatment with $MgCO_3$. 0·2 ml. aliquots of plasma were used for phosphoprotein estimation. The volumes of plasma used for iron determination and $MgCO_3$ treatment were varied according to the expected plasma iron level as judged from preliminary experiments. At low plasma iron

Table 4

Concentrations of iron and phosphoprotein in the plasma of oestrogen-treated cockerels.

Time (days after Stilbestrol)	Plasma iron (μg./100 ml.)		Plasma iron after $MgCO_3$ treatment		Plasma iron after $MgCO_3$ with prior addition of excess iron to plasma		Plasma phosphoprotein (mg./100 ml.)		Plasma phosphoprotein after $MgCO_3$ treatment	
	A	B	A	B	A	B	A	B	A	B
0	124	219	124	224	249	333	0·0	0·0	-	-
1	179	214	159	214	488	388	0·87	0·0	0·0	0·0
2	647	413	408	338	418	338	53·1	36·1	0·0	0·0
4	866	856	269	269	279	289	150·5	146·2	4·8	1·7
6	756	617	219	239	219	249	127·0	74·0	0·35	0·0
8	348	149	239	149	249	388	13·1	1·7	0·0	0·0
10	154	154	154	154	209	209	0·4	0·0	-	-

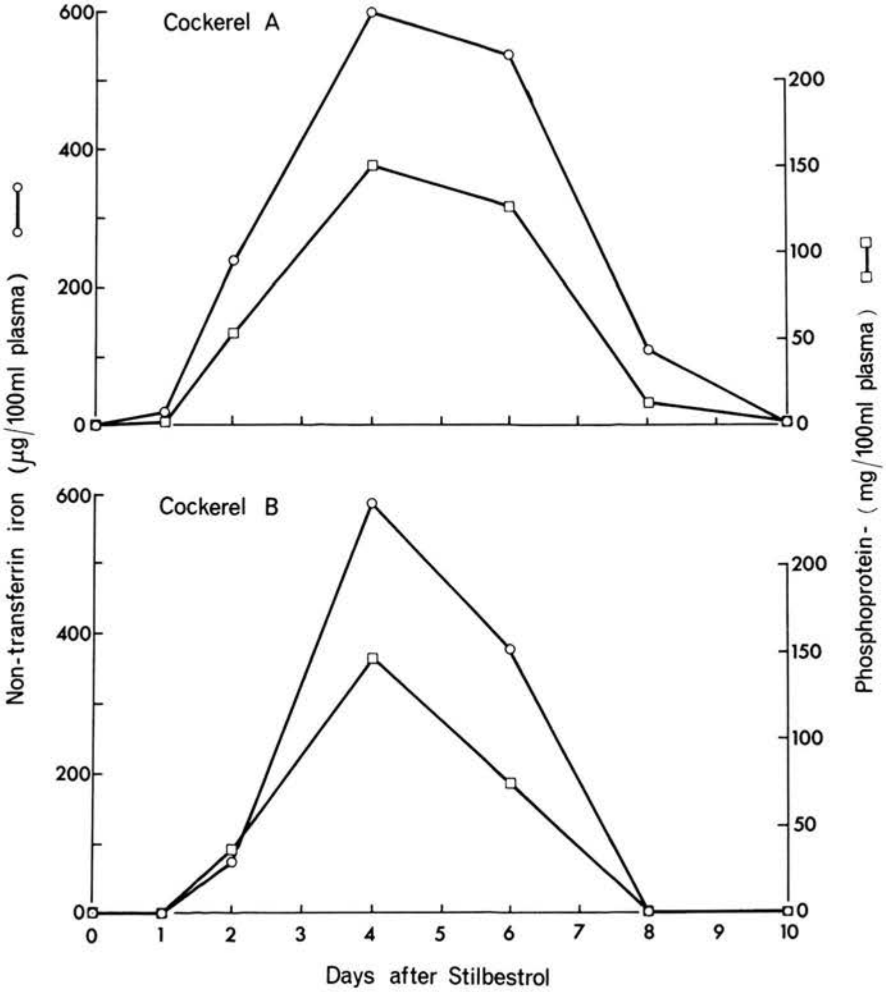
levels (Table 4) 0·5 ml. plasma was used for plasma iron determinations and 1·0 ml. plasma was used for $MgCO_3$ treatment, but at high levels half these volumes were used. When excess iron was added to plasma prior to $MgCO_3$ treatment ferric chloride solution (5 μg.Fe^{3+}/ml., solution in 0·005 N HCl) was added to give two volumes of ferric chloride solution per one volume of plasma.

The results are shown in Table 4.

It can be seen from Table 4 that the appearance of phosphoprotein in the plasma coincided with the rise in plasma iron level and the appearance of the non-transferrin iron (non-transferrin iron = total plasma iron minus plasma iron after $MgCO_3$ treatment without prior addition of iron), and when the phosphoprotein concentration reached levels similar to those found in laying hens the plasma iron levels were also similar to those found in laying hens and transferrin was saturated with iron. The drop in plasma iron levels occurred simultaneously with the drop in phosphoprotein level.

It can also be seen from Table 4 that $MgCO_3$ treatment which removed the non-transferrin iron and the phosphoprotein from laying hens plasma had the same effect on oestrogen-treated cockerels plasma. Furthermore, in the oestrogen-treated cockerel, as in the laying hen, the variations in the non-transferrin iron were closely related to those of the phosphoprotein (Fig. 15).

Fig. 15. Variations in the non-transferrin iron and the phosphoprotein in the plasma of oestrogen-treated cockerels



Thus, the plasma iron picture in oestrogen-treated cockerels is very similar to that found in normal hens with the onset of laying, during the laying season and at the cessation of egg production.

All these findings indicate that the non-transferrin iron may be associated with phosphoprotein but certainly not with conalbumin.

1.8. Isolation of phosphoprotein-bound iron from the plasma of the laying hen

All the evidence thus far supports the view that the non-transferrin iron in the plasma of the laying hen is bound to the phosphoprotein. The absolute proof would be to isolate the pure phosphoprotein with the iron still bound to it.

There are two kinds of difficulty about this isolation. In the first place, the amount of iron involved is small, and since the phosphoprotein has been shown by Taborsky (1963) to bind iron, any small amount of iron found in the final product might be acquired from the reagents or other sources during the isolation. This problem can be solved by labelling the non-transferrin iron in vivo before attempting the isolation.

The second difficulty is more serious, and arises from the fact (see pages 9 to 11) that phosphoprotein is present in the plasma not free but in some type of complex with lipoproteins. This complex appears to be held together by calcium ions since chelation or removal of calcium ions leads to dissociation of the phosphoprotein from the lipoproteins (McCully & Common, 1961). Moreover, procedures for the isolation of phosphoprotein from the plasma involve working at low pH or using chelating agents or both. Thus, Mok, Martin & Common (1961) adjusted the pH of serum to 5·3 by adding 80 ml. 0·1 N HCl to 200 ml. serum, and Heald & McLachlan (1963 a) used EDTA and sodium acetate solution pH 4·0 in the course of isolation of phosvitin. Under these conditions both calcium and iron are likely to be dissociated from proteins. Furthermore, the procedure of Mok, Martin & Common failed to isolate phosvitin from the plasma of unoestrogenized laying hens, and that of Heald & McLachlan gave an overall yield of 39-67% of the total protein phosphorus.

Attempts in this investigation to purify the phosphoprotein by using a shallower NaCl gradient for DEAE-cellulose chromatography (either by having no NaCl in the tris-buffer in the mixing chamber or by using 0·5 M NaCl in the tris-buffer in the reservoir) led to poor recovery of the non-transferrin iron and even after re-chromatographing the phosphoprotein-containing fractions contained lipids which were shown by thin layer chromatography to be similar to the lipids present in the lipophosphoprotein complex (cf. McIndoe, 1959).

When laying hen plasma labelled in vivo with ^{59}Fe (6 μc $^{59}FeCl_3$ [in about 1·5 μg. iron] injected intravenously, blood collected after an hour) was treated with the chelating agent sodium citrate (10 mg./ml. plasma, allowed to stand for an hour) and then fractionated on DEAE-cellulose (Methods, p.22), about 48% of the non-transferrin ^{59}Fe (i.e. about 48% of the ^{59}Fe removed by $MgCO_3$ from a sample of plasma untreated with citrate) appeared in the fractions which usually contain the phosphoprotein (Fig. 16). In plasma untreated with citrate fractionated under similar conditions there was close agreement between the amount of iron removed by $MgCO_3$ and that appearing in the fractions containing phosphoprotein.

Fractions 24 to 31 (Fig. 16) were pooled and the salt concentration was brought down to 0·1 M NaCl by the addition of 0·005 M tris-HCl buffer, pH 8·0. The material was then loaded on a DEAE-cellulose column equilibrated with 0·1 M NaCl in 0·005 M tris-HCl buffer, pH 8·0. Fractions were eluted with a NaCl gradient (Methods, p.22). The results are shown in Fig. 17. The arrow in the figure indicates the start of the gradient.

It can be seen from Fig. 17 that the chromatographic behaviour of the phosphoprotein and the iron recovered in the fractions and the close parallelism in the distribution of the

Fig. 16. Chromatography on DEAE-cellulose of sodium citrate treated laying hen plasma

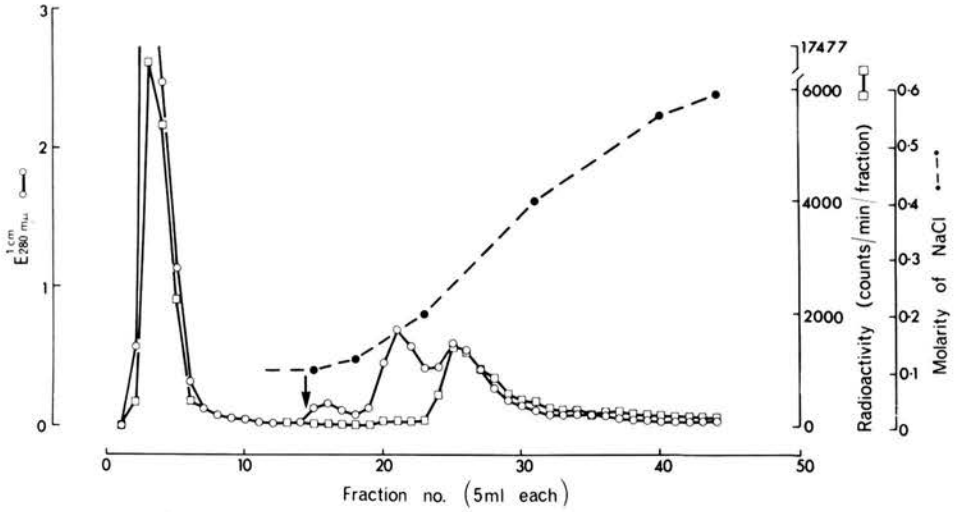
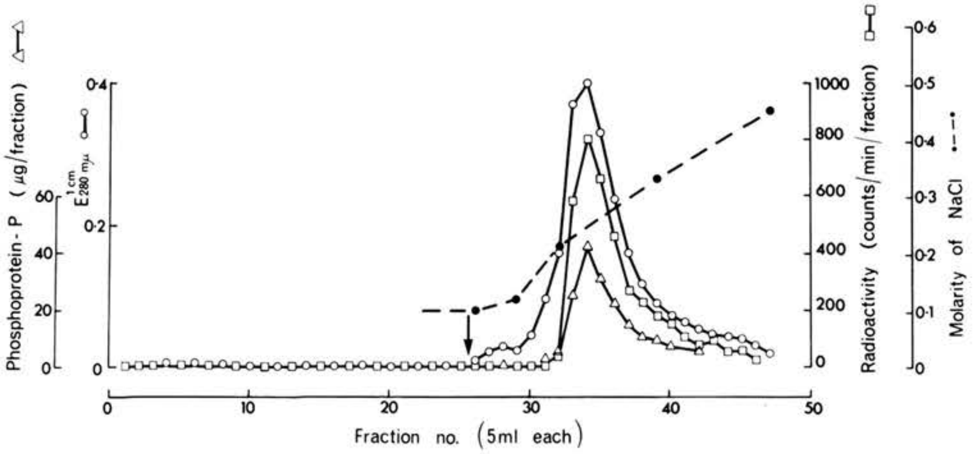


Fig. 17. Rechromatography on DEAE-cellulose of fractions 24-31 (Fig. 16)



phosphoprotein phosphorus and the ^{59}Fe suggest that the iron is bound to the phosphoprotein. This is supported by the fact that the Fe/P ratio (counts/min./fraction ÷ µg. phosphoprotein P /fraction) was almost constant in the eight fractions covering the bulk of the elution peak. The Fe/P ratio obtained for fractions 33 to 40 (Fig. 17) were: 23, 19, 21, 21, 19, 21, 20, 21. The phosphoprotein-containing material obtained by rechromatography of fractions 24 to 31 from the preliminary experiment (Fig. 16) contained about 75% of the plasma phosphoprotein, 19% of the non-transferrin ^{59}Fe, and 2% of the plasma lipids.

Although this experiment goes some distance towards confirming that the non-transferrin iron is bound to phosphoprotein, the isolation can only be regarded as partially successful, the more so because the phosphorus content of the protein in the iron-binding fractions was not determined. Since even a mild chelating agent like citrate caused the loss of so much of the iron, no further attempts at isolation were made.

2. METABOLIC RELATION BETWEEN TRANSFERRIN IRON AND THE NON-TRANSFERRIN IRON

2.1. In vitro studies

Fresh heparinised cockerel plasma was used as a source of transferrin. The concentration of iron in the plasma and the total iron-binding capacity (TIBC) were determined, the unsaturated iron-binding capacity (UIBC) of transferrin calculated (UIBC = TIBC less plasma iron) and then ^{59}Fe (ferric chloride) was incubated at 37°C for about 30 minutes with sufficient quantity of plasma to ensure binding of all the iron to transferrin. In the experiments in which saturated transferrin was used ^{59}Fe was incubated with plasma as before and then transferrin was saturated by adding the exact amount of non-radioactive iron (ferric chloride) required for saturation of transferrin and incubating for 30 minutes.

A measured volume of the plasma containing ^{59}Fe bound to transferrin (^{59}Fe-transferrin) was mixed in a flask with an equal volume of fresh heparinised plasma from a laying hen and the mixture was gently rocked for 4 hours in a 37°C water bath. Specific activities of transferrin iron and non-transferrin iron were measured at time zero and after 4 hours of incubation.

The iron fractions were separated by treating aliquots of the mixture (1 ml. each) with magnesium carbonate (Methods, p.18). The amounts of radioactive and non-radioactive iron were determined in magnesium carbonate treated plasma and in similar aliquots that had not been treated with magnesium carbonate. The radioactivity was determined first by measuring the γ radiation of ^{59}Fe using the well-type scintillation counter and then the material was used for the determination of iron according to Ramsay's dipyridyl method. Values for non-transferrin iron (or Fe^{59}) were obtained by subtracting the amounts obtained after magnesium carbonate treatment from the amounts found in the untreated material. The specific activity was obtained by dividing the number of counts per minute per unit volume by the amount of iron found by chemical analysis in the same material.

Tables 5 and 6 show the results of two experiments in which ^{59}Fe bound to transferrin which was 46% saturated (Table 5) or fully saturated (Table 6) with iron was incubated with plasma from laying hens.

It can be seen from the results in Tables 5 and 6 that in both cases the non-transferrin iron did not become labelled, nor was there any fall in the specific activity of transferrin iron. Thus, in vitro, transferrin iron does not serve as a source of the non-transferrin iron and there is no exchange between the two iron fractions.

Table 5

Specific activities of transferrin iron and non-transferrin iron following the incubation (37°C) of ^{59}Fe-transferrin (unsaturated) with laying hen plasma.

Iron: µg./ml. incubation mixture

Radioactivity (^{59}Fe): counts/min./ml. incubation mixture.

Specific activity (S.A.): counts/min./µg. iron.

Time	Total iron	Total ^{59}Fe (x)	Trans-ferrin iron	Trans-ferrin ^{59}Fe (y)	S.A. of trans-ferrin iron	Non-trans-ferrin iron	Non-trans-ferrin ^{59}Fe (x-y)	S.A. of Non-trans-ferrin iron
0	3·96	130372	2·39	130400	54560	1·57	-28*	0
4 hr.	3·96	130622	2·39	130526	54613	1·57	96*	0

* Taking into consideration a counting error of 0·33% and the fact that the counts were made on different pipettings, these differences can be ignored.

Table 6

Specific activities of transferrin iron and non-transferrin iron following the incubation (37°C) of ^{59}Fe-transferrin (saturated) with laying hen plasma.

Iron: µg./ml. incubation mixture.

Radioactivity (^{59}Fe): Counts/min./ml. incubation mixture.

Specific activity (S.A.): Counts/min./µg. iron.

Time	Total iron	Total ^{59}Fe (x)	Transferrin iron	Transferrin ^{59}Fe (y)	S.A. of transferrin iron	Non-ferrin iron	Non-ferrin ^{59}Fe (x-y)	S.A. of non-transferrin iron
0	5·41	37886	2·24	37964	16948	3·17	-78*	0
4 hr.	5·41	38642	2·24	38586	17226	3·17	56*	0

* Taking into consideration a counting error of 0·56% and the fact that the counts were made on different pipettings, the values for (x-y) can be ignored.

2.2. In vivo studies

Tracer amounts of radioactive iron (^{59}Fe, ferric chloride in 0·1 N HCl) were incubated at 37°C for about 30 minutes, with sufficient quantity of fresh plasma from a normal cockerel to ensure binding of all the iron to transferrin. The acidity of ^{59}Fe solution was neutralised by adding to the plasma a suitable quantity of 0·1 M sodium bicarbonate in 0·95% sodium chloride immediately before the addition of the iron. After incubation, a measured volume (1 ml.) of the material containing ^{59}Fe-transferrin was injected in the wing vein of a laying hen or an oestrogen-treated cockerel, or (for control purposes) a non-laying hen. The birds used in these experiments weighed about 2 Kg. each. In experiments with oestrogen-treated cockerels each bird received a single subcutaneous injection of stilbestrol dipropionate (1 mg./100 g. body wt.) and the ^{59}Fe-transferrin was injected two days after the administration of oestrogen when the non-transferrin iron and the phosphoprotein are found in the blood plasma, and the effect of oestrogen is still maintained (Table 4). After the injection of ^{59}Fe-transferrin blood specimens were collected at intervals for several hours from the wing vein that had not been used for the injection of ^{59}Fe-transferrin and from the jugular vein if necessary. Great care was taken to make the sizes of the specimens as small as possible so as not to upset the physiological state of the bird. Small heparinised needles were used for collecting the blood into heparinised syringes. The blood specimen (about 1-1·5 ml.) was transferred from the syringe to a small iron-free tube having a small diameter, centrifuged immediately,and the maximum possible volume of plasma obtained by using a very fine Pasteur pipette.

Transferrin iron was separated from the non-transferrin iron by treating the plasma with magnesium carbonate. Aliquots of the plasma were analysed for radioactivity and iron before and after magnesium carbonate treatment and the values for the non-transferrin iron (or ^{59}Fe) obtained by subtracting the values for plasma iron (or ^{59}Fe) after magnesium carbonate treatment from the corresponding values for the untreated plasma. Specific activities are expressed as counts per minute per μg. iron.

In experiments on oestrogen-treated cockerels 0·5 ml. aliquots of the plasma were treated with magnesium carbonate (Methods, p.19). Aliquots of magnesium carbonate supernatant and 0·25 ml. aliquots of untreated plasma were analysed first for radioactivity (well counter) and then the iron content was determined using Ramsay's dipyridyl method. In experiments on laying and non-laying hens 0·4 ml. samples of plasma were treated with magnesium carbonate. The 0·4 ml. plasma was shaken for 30 minutes with 1·0 ml. distilled water and 40 mg. magnesium carbonate, centrifuged, and 0·7 ml. of the clear supernatant fluid was analysed simultaneously with 0·2 ml. untreated plasma. The radioactivity was measured first using the well counter, and then the iron content of the material was determined by a method developed in the course of the present work (Appendix II) while attempting to design a simple sensitive method that combines the chemical determination of iron with the measurement of the radioactivity of ^{59}Fe.

Table 7 shows the results of a control experiment in which ^{59}Fe-transferrin was injected into a non-laying hen and the plasma ^{59}Fe was determined before and after magnesium carbonate treatment. The differences between the counts before and after magnesium carbonate treatment can be accounted for by the experimental errors involved in pipetting and counting. Thus, as expected, in the plasma of the non-laying hen the ^{59}Fe was always bound to transferrin.

Five experiments were performed on laying hens and three experiments were performed on oestrogen-treated cockerels. The results of a typical experiment on a laying hen are shown in Table 8 and those of a typical experiment on an oestrogen-treated cockerel are shown in Table 9. The specific radioactivities of transferrin iron and non-transferrin iron presented in Tables 8 and 9 are plotted against the time after the injection of ^{59}Fe-transferrin (Figures 18 and 19). It can be seen from Figures 18 and 19 that the curves obtained for specific activity-time relationships fulfil all the criteria described by Zilversmit,

Table 7

Radioactivity (before and after $MgCO_3$ treatment) in plasma samples from a non-laying hen injected intravenously with ^{59}Fe-transferrin.

Time after the injection	Radioactivity (counts/min./0·2 ml. plasma)	
	Before	After
5 min.	5549	5639
0·5 hr.	2059	1984
1 hr.	619	613
1·5 hr.	291	276
2·5 hr.	84	75

Table 8

Distribution of radioactive and non-radioactive iron in the plasma of a laying hen injected intravenously with ^{59}Fe-transferrin.

Iron: µg./0·2 ml. plasma.

Radioactivity (^{59}Fe): Counts/min./0·2 ml. plasma.

Specific activity (S.A.): Counts/min./µg. iron.

Time	Total plasma iron	Total plasma ^{59}Fe	Transferrin iron	Transferrin ^{59}Fe	S.A. of transferrin iron	Non-transferrin iron	Non-transferrin ^{59}Fe	S.A. of non-transferrin iron
5 min.	1·764	9087	0·876	8107	9255	0·888	980	1104
0·5 hr.	1·94	7534	0·84	4426	5269	1·100	3108	2825
1 hr.	1·82	6935	0·788	3027	3841	1·032	3908	3787
1·5 hr.	1·644	5169	0·756	1920	2540	0·888	3249	3659
2·5 hr.	1·642	4078	0·62	1260	2032	1·022	2818	2757
4 hr.	1·58	3000	0·708	934	1319	0·872	2066	2369
6 hr.	1·464	1985	0·564	596	1057	0·900	1389	1543

Table 9

Distribution of radioactive and non-radioactive iron in the plasma of an oestrogen-treated cockerel injected intravenously with ^{59}Fe-transferrin.

Iron: µg./0·25 ml. plasma.

Radioactivity (^{59}Fe): Counts/min./0·25 ml. plasma.

Specific activity (S.A.): Counts/min./µg.iron.

Time	Total plasma iron	Total plasma ^{59}Fe	Transferrin iron	Transferrin ^{59}Fe	S.A. of transferrin iron	Non-transferrin iron	Non-transferrin ^{59}Fe	S.A. of non-transferrin ^{59}Fe
5 min.	1·344	13804	0·796	13563	17039	0·548	241	440
20 min.	1·194	10227	0·672	9443	14052	0·522	784	1502
40 min.	1·194	8400	0·622	5594	8994	0·572	2806	4906
1 hr.	1·12	6879	0·547	3653	6678	0·573	3226	5630
1·5 hr.	1·12	5876	0·549	2862	5213	0·571	3014	5278
2·5 hr.	1·194	4383	0·647	1480	2287	0·547	2903	5307
4 hr.	1·269	3282	0·622	820	1318	0·647	2462	3805
6 hr.	1·244	2430	0·622	494	794	0·622	1936	3113

Fig. 18. 'Specific activity-time' relations of transferrin iron and non-transferrin iron in laying hen plasma following the injection (i/v) of ^{59}Fe-transferrin

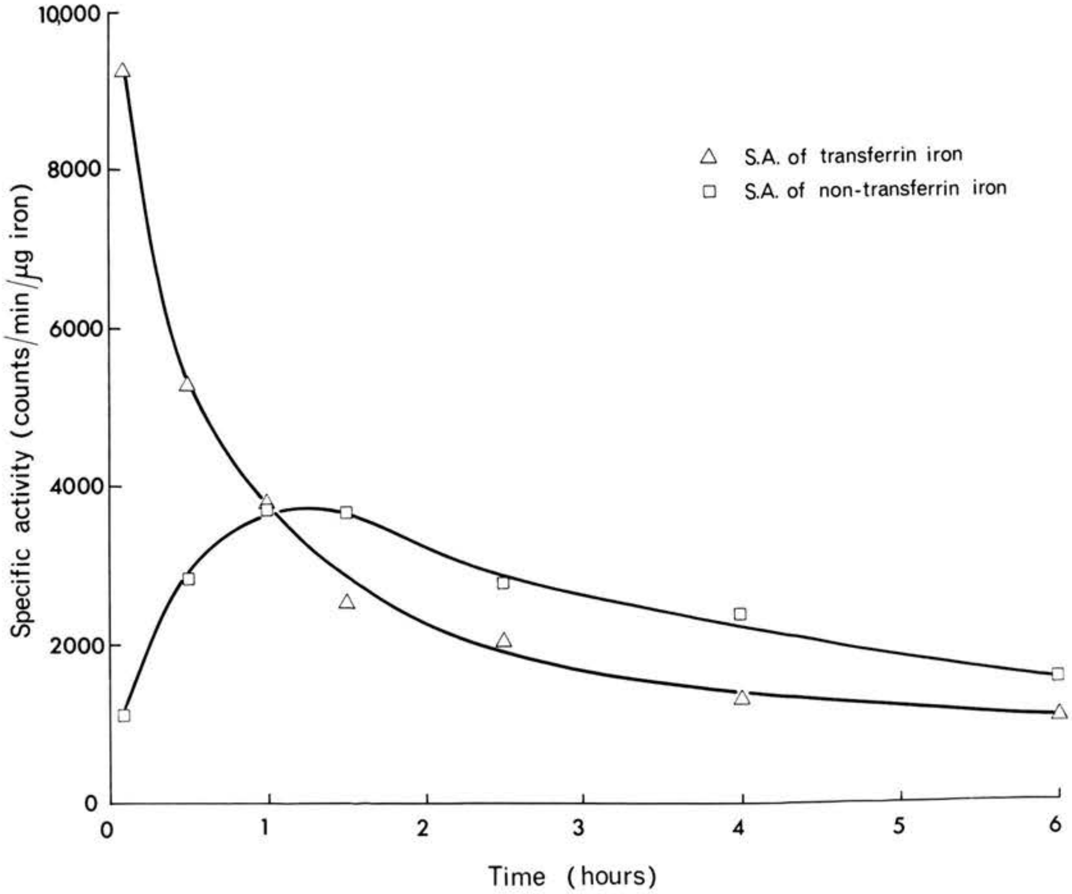
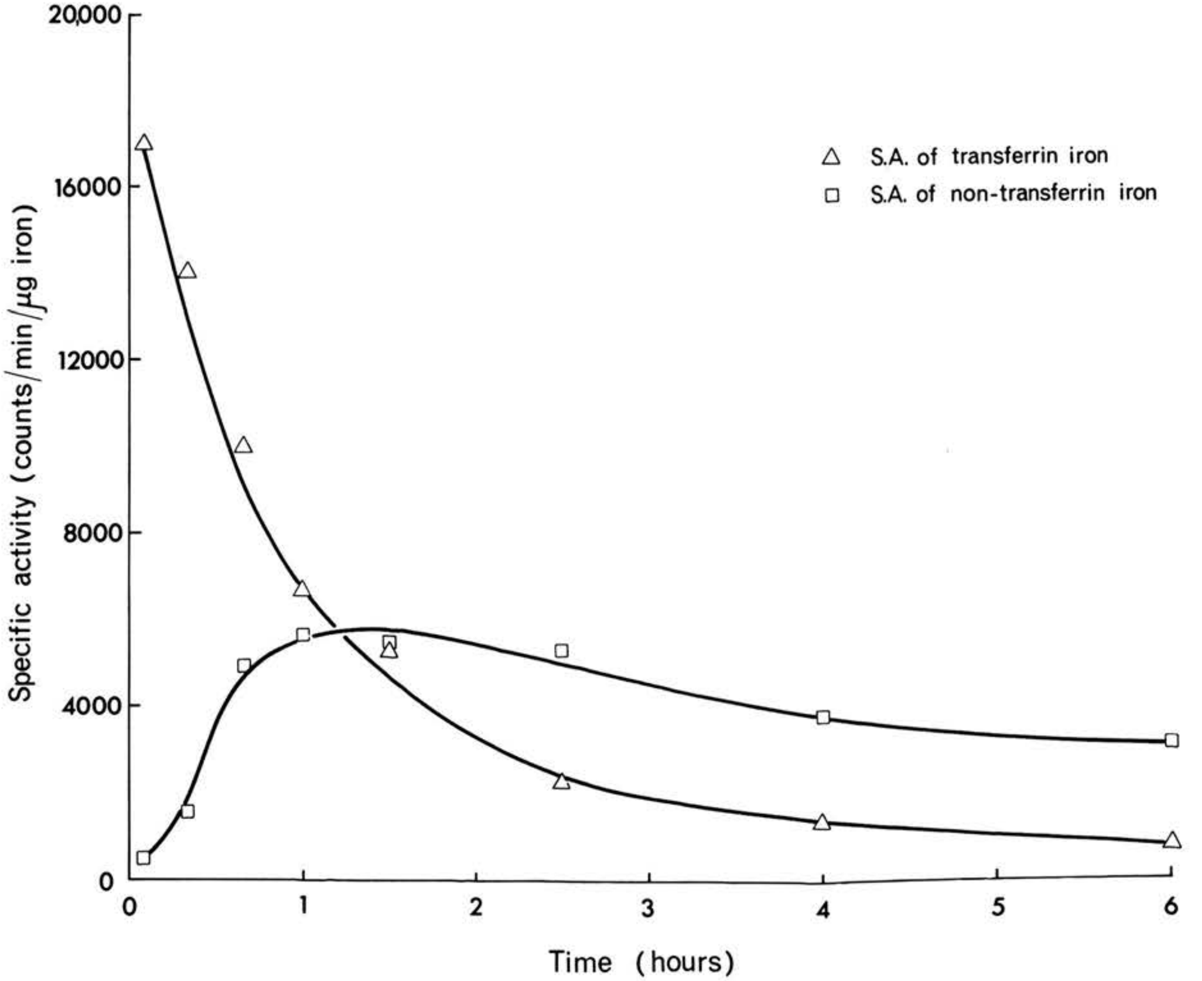


Fig. 19. 'Specific activity-time' relations of transferrin iron and non-transferrin iron in oestrogen-treated cockerel plasma following the injection (i/v) of ^{59}Fe-transferrin



Entenman and Fishler (1943) for a direct precursor-product relationship. Thus, the specific activity-time curve of transferrin iron was initially higher than that of the non-transferrin iron, crossed the latter at its peak and then fell below it. This indicates that in vivo transferrin iron serves as a direct precursor of the non-transferrin iron in the plasma of the laying hen and the oestrogen-treated cockerel.

DISCUSSION

1. Nature of the non-transferrin iron

The results of the present investigations into the nature of the non-transferrin iron in the plasma of the laying hen suggest strongly that the non-transferrin iron is combined with phosphoprotein and not with conalbumin. Thus, the application of separation techniques which depend on quite different properties of proteins indicates that this iron fraction is associated with phosphoprotein. Furthermore, a very definite relation between the phosphoprotein and the iron removed by magnesium carbonate could be established. The results of the experiments in which iron was added to laying hen plasma in vitro also indicate that the iron-binding components in laying hen plasma are likely to be transferrin and phosphoprotein. Moreover, the changes that occur in the plasma iron picture in hens as a result of secretion of oestrogen by the ovary could be induced by treatment of cockerels with oestrogen; and in the oestrogen-treated cockerel results similar to those found in the laying hen were obtained.

Interpretation of the findings of other workers in the light of the results of this study lend further support to the view that the non-transferrin iron is combined with phosphoprotein. Thus the fast migrating iron-binding component detected by Lush & Ramsay (cited by Lush, 1964) on electrophoresis under the conditions of Poulik (1957) of laying hen plasma labelled in vivo with ^{59}Fe could be phosphoprotein since it can be seen from the work of McCully & Common (1961) that this electrophoretic behaviour is expected of the phosphoprotein once it is dissociated from the lipoproteins by the citrate and the borate in the buffers. This iron-binding component could not be detected by Williams (1962) who used similar electrophoresis conditions because he added a tracer amount of ^{59}Fe to laying hen plasma in vitro and apparently the added iron was preferentially taken up by transferrin. This may also explain why all the radioactivity appeared in the β-globulin fraction only when Barber & Sheeler (1961) added ^{59}Fe to adult chicken serum in vitro (serum iron values indicate that the serum used was laying hen serum) before subjecting the serum to starch gel electrophoresis. Martin-Mateo, Sebastian & Planas (1965) who used paper electrophoresis in veronal buffer (pH 8·6) for the fractionation of laying hen plasma labelled with ^{59}Fe in vivo or in vitro could not have detected the fast migrating iron-binding component because under these electrophoresis conditions the phosphoprotein migrates with the lipoproteins (see McCully & Common, 1961).

The problems of isolating pure phosphoprotein still chelated with cations such as calcium have been discussed already (pp. 49, 50). It has been confirmed that in order to isolate the pure phosphoprotein one needs to dissociate it from the lipoproteins by the use of methods which are likely to remove calcium ions, and it appears that procedures that are successful in doing this also remove the iron. However, all the circumstantial evidence which has been accumulated suggests that the phosphoprotein binds all the non-transferrin iron of the plasma and not merely the fraction isolated in these experiments.

2. Metabolic relation between transferrin iron and the non-transferrin iron

The results of the experiments in which ^{59}Fe-transferrin was incubated with plasma from laying hens show that *in vitro* transferrin iron does not serve as a precursor of the non-transferrin iron and there is no exchange between the fractions. However, when ^{59}Fe-transferrin was injected into laying hens and oestrogen-treated cockerels, the results show that *in vivo* transferrin iron serves as a direct precursor of the non-transferrin iron according to the criteria of Zilversmit et al. (1943) for a direct precursor-product relationship. Thus, the non-transferrin iron is presumably derived from transferrin iron in vivo by some process other than simple transfer in the plasma. Since the evidence indicates that the non-transferrin iron is phosphoprotein-bound it seems possible that this process takes place in the liver where the phosphoprotein is synthesized. The direct precursor-product relationship and the rapidity of the process suggest that the iron that appears in the plasma associated with the phosphoprotein is not involved with the storage iron pool. It thus seems possible that the iron is taken from transferrin by the newly synthesized phosphoprotein which is leaving the liver cells to be released in the blood plasma. However, the present state of knowledge does not allow one to speculate further on how this process takes place.

3. Function of the non-transferrin iron

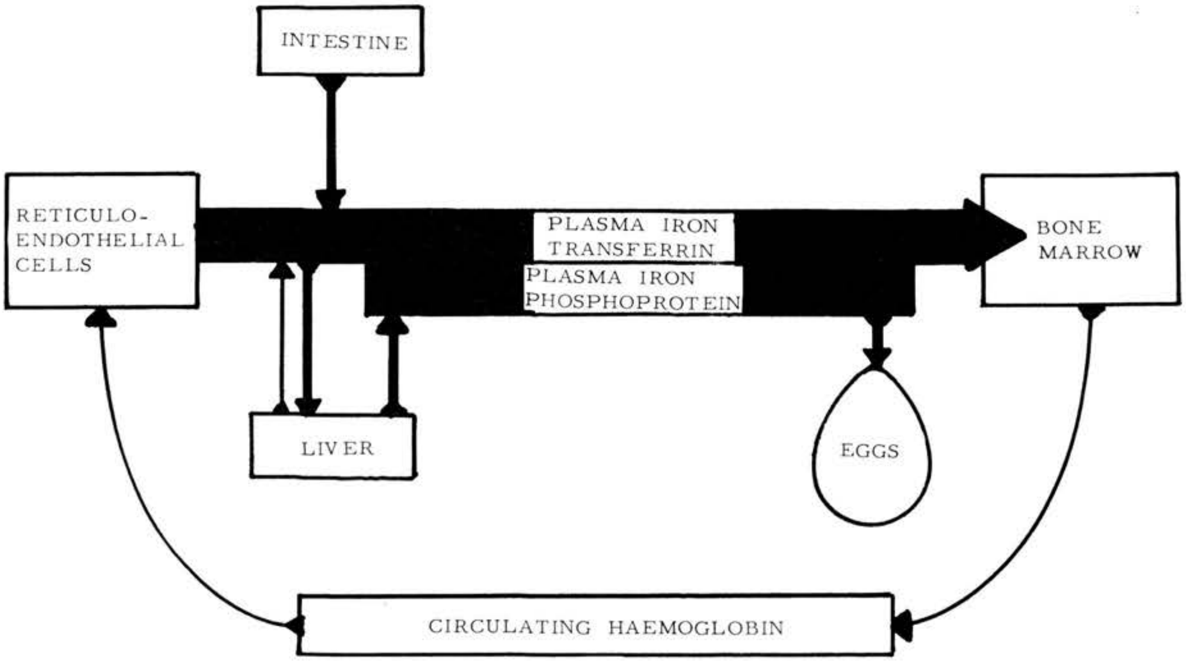
It has already been pointed out (pp. 9 - 11) that the phosphoprotein of egg yolk is synthesized in the liver and is then transferred via the blood plasma (in complex form with other substances such as calcium and lipoproteins) to the ovary where it is deposited in the developing egg yolk. Thus it seems possible that after the iron is combined with the phosphoprotein in the liver, the phosphoprotein with its bound iron is released in the blood plasma to be deposited in the developing yolk. This is supported by the results of Halkett, Peters & Ross (1958) who injected ^{59}Fe into laying hens and showed that egg yolk iron was phosphoprotein-bound and that the iron was laid down in the yolk more or less directly from the plasma. Furthermore, Ramsay and Campbell (1954) determined egg and plasma iron in fifteen hens over a whole season and showed that there was a high correlation between the mean plasma iron and mean egg iron.

The iron in the egg is found in the yolk except for minute traces in the "albumen" and the shell (Romanoff & Romanoff, 1949). The total iron content of the egg yolk can be accounted for approximately by the iron content of the egg yolk phosphoprotein, phosvitin (Greengard, Sentenac & Mendelsohn, 1964). A rather wide range of values have been reported for the iron content of egg phosvitin. Thus Mecham & Olcott (1949) reported the iron content of phosvitin to be 0·4%, while some other authors reported the following values: 0·1-1·0% (Allgen & Norberg, 1959), 0·3% (Connelly & Taborsky, 1961), 0·4-0·6% (Greengard et al. 1964) and 0·29% (Allerton & Perlman, 1965). It is interesting to note that it was found in the present work that when the amounts of plasma iron corresponding to phosphoprotein (i.e. amounts of iron removed by $MgCO_3$) in seventeen laying hens were expressed as percentages of the plasma phosphoprotein level the mean value obtained was 0·38 (range 0·21-0·53) which is within the range of values reported for the iron content of the egg phosphoprotein. This is consistent with the view that the plasma phosphoprotein with its bound iron is deposited in the developing egg yolk.

It thus seems possible that the function of the phosphoprotein iron present in the plasma of the laying hen is to serve as a source of iron for the eggs. Iron required for haemoglobin synthesis, cytochromes, etc. is probably transported by transferrin as in non-laying birds and mammals.

Possible roles of plasma iron in the major processes of iron metabolism in the laying hen are outlined diagrammatically in Fig.20.

Fig. 20. MAJOR PATHWAYS OF IRON METABOLISM IN THE LAYING HEN



CONCLUSIONS

It can be concluded from the results of the investigations presented in this thesis that:

1. It is most probable that the non-transferrin iron in the plasma of the laying hen is bound to phosphoprotein;
2. The phosphoprotein iron is derived from transferrin iron in vivo;
3. The possible function of the phosphoprotein iron is to serve as a source of iron for the eggs.

APPENDICES

APPENDIX I

Calculation of the coefficient of variation from a series of duplicate analyses

The procedure employed for calculating the coefficient of variation (C.V.) of sets of duplicate measurements was based on the method of Copeland (1957) for calculating the standard deviation of duplicate analyses. The differences between the duplicate measurements were expressed as percentages of the means, these percentages were squared, and the sum of these squares was divided by double the number of pairs of measurements. The square root of this value represents the coefficient of variation.

The formula used was:

$$\text{C.V.} = \sqrt{\frac{\sum\left(\frac{(a - b)\ 200}{(a + b)}\right)^2}{2N}}$$

where a and b represent the two duplicate measurements and N represents the number of pairs.

APPENDIX II

The determination of iron and assay of the radioactivity of ^{59}Fe in the same sample of blood plasma

Some iron kinetics experiments in small animals (e.g. hens) necessitate the administration of ^{59}Fe into the animal and the determination of the concentration of iron and assay of the radioactivity in a large number of samples of blood plasma collected at intervals after the administration of ^{59}Fe. The volume of the blood collected should not upset the physiological state of the animal any more than is unavoidable. This means that one may need to analyse a very small volume of blood plasma. Experiments of this nature would therefore be greatly facilitated by the availability of a simple sensitive technique that combines the radioactivity assay with the iron determination. In the hope of achieving this the following technique was designed.

Principle

Since an unusually sensitive method is needed, an unusually sensitive iron coordinating reagent must be used, the final volume of solution must be as small as possible and the solution must be suitable for subsequent radioactivity assay by a sensitive method. 4,7-diphenyl-1,10-phenanthroline (bathophenanthroline) is one of the most sensitive reagents that form a stable coloured complex with iron and this reagent is much more sensitive than dipyridyl or 1,10-phenanthroline (Peterson, 1953). Bathophenanthroline and its ferrous complex are not soluble in water but they are soluble in organic solvents such as isoamylalcohol (Peterson, 1953) or n-butanol or n-hexanol (Ramsay, 1957 a). Thus the iron (or ^{59}Fe)-bathophenanthroline complex can be extracted in a small volume of an organic solvent. This gives ^{59}Fe in solution in a solvent suitable for liquid scintillation counting of β radiation. ^{59}Fe can be counted with a high efficiency and low background by means of liquid scintillation (Leffingwell, Melville & Riess, 1959; Dern & Hart, 1961; Lockner, 1965; Eakins & Brown, 1966; Graber, McKee & Heyssell, 1967). However, the methods that have been proposed by the above authors are rather tedious and none of them seems to have gained favour in any laboratory besides that of the originators.

The use of bathophenanthroline together with liquid scintillation provide the basis of a simple sensitive method that combines the chemical determination of iron with the measurement of radioactivity. However, before the iron is extracted in complex with bathophenanthroline it is necessary to avoid the interference of the normal yellow pigments of plasma and to reduce the iron and librate it from its combination with proteins. It was found possible to remove interfering substances by treating the plasma with ethanol or acetone, when the iron was precipitated with the proteins while the interfering substances were extracted. The use of an aqueous ascorbic acid reagent was found suitable for making the iron available for complexing with bathophenanthroline but it allowed interference by haemoglobin. However, it is easy to take blood from fowls without haemolysis. Extraction of water into the organic solvent was minimized by incorporating a high concentration of sodium chloride in the ascorbic acid solution.

In the preparation of the samples for liquid scintillation counting it was found necessary to destroy the pink colour of iron-bathophenanthroline complex and to make sure that water and other quenching agents were removed. The colour was destroyed by treatment with H_2O_2 and subsequent heating both destroyed excess of this and removed residual water from the mixture.

The procedure finally adopted involved:

1) precipitation of proteins together with iron by addition of ethanol;

2) suspension of precipitate in ascorbic acid reagent followed by brief heating to ensure reduction.

3) shaking with measured volume of n-butanol containing bathophenanthroline.

4) measurement of the pink colour of iron-bathophenanthroline complex.

5) destruction of the pink colour of iron-bathophenanthroline agents, mixing with CIBA scintillator BBOT, and counting at 0 to 4°C.

The high sensitivity of the method calls for exceedingly clean working conditions. Glassware, water, and reagents used must be free from iron. One way of making the glassware free from radioactive and non-radioactive iron is to soak overnight in citric acid in detergent, heat to boiling, scrub, wash with water, soak in 20% HCl overnight, and then wash extensively with distilled water.

Experimental Procedure

1. Determination of iron

Reagents

Redistilled ethanol.

Redistilled n-butanol.

Ascorbic acid reagent. A 2% solution of L-ascorbic acid (BDH) in water containing 0·04 N perchloric acid and 4 M sodium chloride is equilibrated with n-butanol (ratio of aqueous to organic phase = 2:1·5) by shaking the mixture vigorously in a separating funnel for about 10 minutes. The mixture is allowed to stand and the two layers are separated.

Bathophenanthroline reagent. Bathophenanthroline (4,7-Diphenyl-1,10-Phenanthroline, Sigma) is dissolved in the n-butanol that has already been shaken with the ascorbic acid reagent to give 0·5 mg. bathophenanthroline per ml. butanol.

Iron-free ascorbic acid reagent. The ascorbic acid reagent mentioned above is made iron-free by shaking with suitable volumes of the bathophenanthroline reagent in a separating funnel till there is no red colour in the butanol phase and finally shaking twice with a suitable volume of distilled butanol to remove any bathophenanthroline that may be present in the aqueous phase. This reagent can be used for about a week.

Standard iron solutions. Suitable standard solutions can be prepared by diluting a stock solution of ferric ammonium sulphate (in 0·2 N H_2SO_4) containing 100 µg. Fe^{3+}/ml.

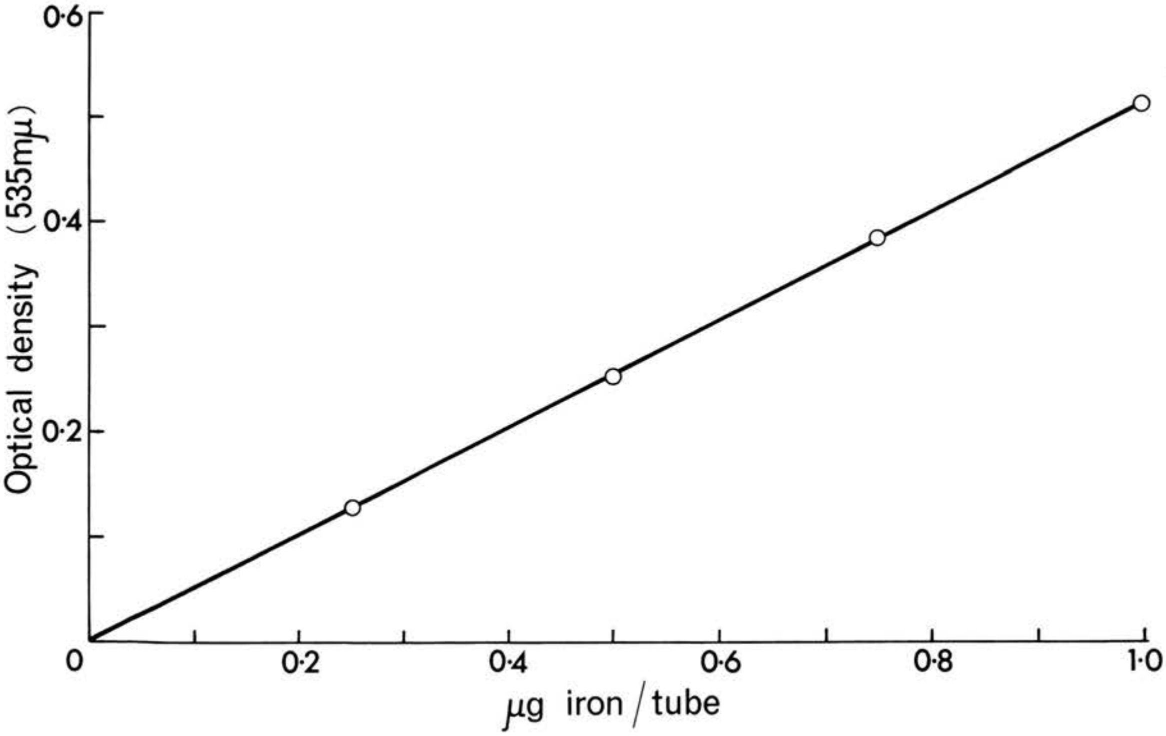
Procedure

0·1 to 0·2 ml.plasma are added to 0·4 to 0·3 ml. distilled water in a glass-stoppered tube and mixed. 6 ml. redistilled ethanol are then poured in from a wide mouthed pipette graduated to the tip and the tube allowed to stand for 1-2 minutes. The tube is then centrifuged for about 2 minutes at 3000 rpm. The supernatant fluid is poured off and the tube allowed to drain over tissue paper for about 2 minutes. The precipitate is suspended in 1·0 ml. distilled water and 1·0 ml. distilled water is pipetted in another tube to serve as a blank. 1·0 ml. of the iron-free ascorbic acid reagent is added to each tube, mixed, and the tubes are heated for about 4 minutes in 85-90°C water bath with mixing at intervals. The tubes are removed from the water bath, allowed to cool, 1·5 ml. bathophenanthroline reagent added to each, stoppered, and each shaken vigorously for 3-5 minutes. The tubes are then centrifuged (4 min., 3000 rpm) and the optical density of the red colour of ferrous-bathophenanthroline in butanol measured at 535 mμ against a blank making sure that the solutions are clear. 20 mm. microcells and Unicam SP 500 were used in the work presented in this thesis.

The concentration of iron in the sample was obtained from standard calibration curves as follows:

Measured amounts of a standard iron solution were pipetted into glass-stoppered tubes and the volume in each tube was brought to 1·0 ml. with distilled water. The tubes were treated exactly as for plasma iron determination except that the ethanol treatment was omitted. The concentrations of iron were plotted against the corresponding optical densities (Fig. (i)) and the slope of the line (slope = $\frac{\text{optical density}}{\mu\text{g. iron/tube}}$) was calculated. The concentration of iron in the sample can then be obtained by multiplying the optical density of the sample by $\frac{1}{\text{slope}}$.

Fig. (i). Calibration curve for iron determination using the Bathophenanthroline method



Calibration curves were prepared whenever ascorbic acid and bathophenanthroline reagents were made.

The plasma iron values obtained by analysing 0·1 to 0·2 ml. plasma using the bathophenanthroline method were in reasonable agreement with those found in 1·0 ml. plasma samples analysed by Ramsay's dipyridyl method. Representative values where the plasma samples were analysed using both methods simultaneously are shown in Table (i).

When the bathophenanthroline method was employed for ten simultaneous determinations on 0·2 ml. portions of the same plasma (laying hen plasma), the mean iron concentration was 718 μg./100 ml. (s.d. 11·2) with a coefficient of variation of 1·56%.

When various amounts of $^{59}FeCl_3$ in 1·0 ml. samples of ferric chloride solution (1 μg.Fe^{3+}/ml.) or in 0·1 ml. samples of plasma from laying hens (10 different samples) was subjected to the bathophenanthroline procedure and the radioactivity of the aqueous and the butanol phases was measured using the well type solid scintillation counter, recovery of ^{59}Fe in the butanol phase was 95-99%.

Table (i)

Plasma iron values obtained by the bathophenanthroline method and Ramsay's dipyridyl method.

Specimens (heparinised plasma from laying hens)	Plasma iron (µg./100 ml.)	
	Dipyridyl method	Bathophenanthroline method
1	478	486
2	511	496
3	541	526
4	579	567
5	926	944
6	1014	987
7	1030	1028

2. Assay of radioactivity by liquid scintillation counting

A suitable volume (1·0 ml.) of ^{59}Fe-bathophenanthroline in butanol is transferred to a counting vial. 0·5 ml. butanol and 0·2 ml. hydrogen peroxide (1·5%, v/v, in butanol) are added and mixed. The vial contents are heated carefully (at a temperature just below the boiling point of butanol) for about five times the time required for decolourization with hydrogen peroxide to destroy excess of hydrogen peroxide and to remove traces of water that might be in the butanol. This step seems to govern the reproducibility of the method. 4·0 ml. of a solution of CIBA BBOT scintillator (1%, w/v, in toluene) are added and the vial is covered with a polythene cap and refrigerated. Some turbidity may appear on the addition of the scintillator but it usually disappears on refrigeration. The β-radiation of ^{59}Fe can then be counted.

A Panax liquid scintillation counter was used in the present work and counting was carried out at 0 to 4°C (E.H.T. Volts 1100, Disc. Bias 10 mV). A 'blank' vial was prepared for counting in the same way as the sample, except that the 1·0 ml. butanol extract was replaced by 1·0 ml. bathophenanthroline reagent, and counting was carried out in the same manner as that used for the sample.

Comparison of this method of counting with that of measuring γ radiation using the well-type solid scintillation counter showed that the liquid scintillation method gave more than double the net counts (ratio 2·17:1) obtained by the well-counter (efficiency of the well counter about 30%) with an average background (47 counts/min.) less than one fifth of that of the well counter (average background: 300 counts/min.)

However, the use of the liquid scintillation procedure may be limited by its rather poor reproducibility. Thus, when ^{59}Fe was injected into a laying hen and blood was collected after six hours, determination of the radioactivity of the butanol extracts of eight portions (0·2 ml. each) of the same plasma gave a mean value of 2273 counts/minute, with a coefficient of variation of 6·4%. So many counts were recorded (> 20,000) that the statistical counting error made only a negligible contribution to the total variability.

In view of the rather poor reproducibility of the liquid scintillation procedure the radioactivity was determined in the present work by measuring the γ radiation of ^{59}Fe using the well-type counter and the lower efficiency of the well counter was compensated for by using larger amounts of ^{59}Fe and counting for longer periods if necessary.

APPENDIX III

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Phosphoprotein-bound Iron in the Plasma of the Laying Hen

By K. E. Ali and W. N. M. Ramsay. (*Veterinary Unit, Department of Biochemistry, University of Edinburgh*)

Plasma iron in the laying hen and the oestrogenized non-laying bird is 200–500% higher than in the non-laying bird (Ramsay & Campbell, 1954, 1956). About half the total appears to be transferrin-bound. Rodriguez & Planas (1964) suggested that the remainder is bound to conalbumin, the iron-binding protein characteristically found in egg-white, but their evidence overlooks the similarity in properties between conalbumin and transferrin. The phosphoprotein phosvitin has iron-binding properties (Taborsky, 1963), and Greengard, Mendelsohn & Gordon (1965) suggested without direct evidence that the non-transferrin iron in the plasma of oestrogenized cockerels might be bound to phosphoprotein.

Magnesium carbonate fails to remove transferrin-bound iron from plasma (Ramsay, 1957), but it has now been found to remove all the phosphoprotein phosphorus and about half the total iron from the plasma of laying hens and oestrogenized cockerels. Gel filtration on Sephadex G-200 in 0·15 M NaCl–5 mM-tris–HCl buffer, pH 7·4, and ion-exchange fractionation on DEAE-cellulose in 5 mM-tris–HCl buffer, pH 8·0, with an NaCl gradient (0·1–0·5 M) both separate the iron into two fractions, of which one migrates with transferrin and the other with phosphoprotein. Combinations of these techniques lend further support to the view that the non-transferrin iron is associated with phosphoprotein and not with conalbumin.

When ^{59}Fe-labelled plasma from a normal cockerel is mixed with plasma from a laying hen, the non-transferrin iron in the latter does not become labelled in 4 hr. at 37°, nor is there any fall in the specific radioactivity of the transferrin iron. There is thus no exchange between the fractions *in vitro*. On the other hand, specific radioactivity measurements made at intervals for several hours after the intravenous injection of [^{59}Fe]-transferrin (normal cockerel plasma labelled *in vitro*) show that in the laying hen and the oestrogenized cockerel transferrin iron serves as a precursor of phosphoprotein iron (Zilversmit, Entenman & Fishler, 1943), presumably by some process other than simple transfer in the plasma.

Greengard, O., Mendelsohn, N. & Gordon, M. (1965). *Science*, **147**, 1571.
Ramsay, W. N. M. (1957). *Clin. chim. Acta*, **2**, 221.
Ramsay, W. N. M. & Campbell, E. A. (1954). *Biochem. J.* **58**, 313.
Ramsay, W. N. M. & Campbell, E. A. (1956). *Quart. J. exp. Physiol.* **41**, 271.
Rodriguez, R. & Planas, J. (1964). *Rev. esp. Fisiol.* **20**, 83.
Taborsky, G. (1963). *Biochemistry*, **2**, 266.
Zilversmit, D. B., Entenman, C. & Fishler, M. C. (1943). *J. gen. Physiol.* **26**, 325.

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REFERENCES

Aasa, R., Malmström, B.G., Saltman, P. & Vänngard, T. (1963). Biochim. biophys. Acta 75, 203.

Aisen, P., Leibman, A. & Reich, H.A. (1966). J. biol. Chem. 241, 1666.

Allgen, L.-G., & Norberg, B. (1959). Biochim. biophys. Acta 32, 514.

Alm, R.S., Williams, R.J.P. & Tiselius, A. (1952). Acta chem. scand. 6, 826.

Allen, R.J.L. (1940). Biochem. J. 34^2, 858.

Allerton, S.E. & Perlman, G.E. (1965). J. biol. Chem. 240, 3892.

Azari, P. & Baugh, R.F. (1967). Archs Biochem. Biophys. 118, 138.

Barber, A.A. & Sheeler, P. (1961). Comp. Biochem. Physiol. 2, 233.

Bearn, A.G. & Parker, W.C. (1966). In "Glycoproteins", p. 413. Ed. by Gottschalk, A. Amsterdam: Elsevier Publishing Co.

Benowitz, N.L. & Terepka, A.R. (1968). Proc. Soc. exp. Biol. Med. 29, 46.

Block, R.J., Keller, S. & Miller, D.W. (1959). Archs Biochem. Biophys. 83, 427.

Bothwell, T.H. & Finch, C.A. (1962). Iron Metabolism. Boston: Little, Brown & Co.

Charlwood, P.A. (1963). Biochem. J. 88, 394.

Common, R.H. & Mok, C.C. (1959). Nature, Lond., 183, 1811.
Connelly, C. & Taborsky, G. (1961). J. biol. Chem. 236, 1364.
Copeland, B.E. (1957). Am. J. clin. Path. 27, 551.

Davis, P.N., Norris, L.C. & Kratzer, F.H. (1968). J. Nutr. 94, 407.
Delory, G.E. (1938). Biochem. J. 32^1, 1161.
Dern, R.J. & Hart, W.L. (1961). J. Lab. clin. Med. 57, 322.
Dolezalova, V., Brada, Z. & Kocent, A. (1965). Biochim. biophys. Acta 107, 294.
Drabkin, D.L. (1951). Physiol. Rev. 31, 345.

Eakins, J.D. & Brown, D.A. (1966). Int. J. appl. Radiat. Isotopes 17, 391.
Ehrenberg, A. & Laurell, C.B. (1955). Acta chem. scand. 9, 68.

Flickinger, R.A. & Rounds, D.E. (1956). Biochim. biophys. Acta 22, 38.
Flodin, P. (1962). Dextran Gels and their Application in Gel Filtration. Pharmacia, Uppsala.
Folch, J., Lees, M. & Sloane Stanley, G.H. (1957). J. biol. Chem. 226, 497.
Fontès, G. & Thivolle, L. (1925). Compt. rend. Soc. Biol. 93, 687.

Gelotte, B., Flodin, P. & Killander, J. (1962). Archs Biochem. Biophys., Suppl. 1, 319.

Giblett, E.R. (1962). Progr. med. Genet. 2, 34.

Graber, S.E., McKee, L.C. & Heyssel. (1967). J. Lab. clin. Med. 69, 170.

Greengard, O., Sentenac, A. & Mendelsohn, N. (1964). Biochim. biophys. Acta 90, 406.

Greengard, O., Mendelsohn, N. & Gordon, M. (1965). Science 147, 1571.

Greengard, O., Sentenac, A. & Acs, G. (1965). J. biol. Chem. 240, 1687.

Halkett, J.A.E., Peters, T. & Ross, J.F. (1958). J. biol. Chem. 231, 187.

Heald, P.J. (1962). Biochem. J. 83, 212.

Heald, P.J. & McLachlan, P.M. (1963 a). Biochem. J. 87, 571.

Heald, P.J. & McLachlan, P.M. (1963 b). Nature, Lond., 199, 487.

Heald, P.J. & Badman, H.G. (1963). Biochim. biophys. Acta 70, 381.

Heald, P.J. & McLachlan, P.M. (1964). Biochem. J. 92, 51.

Heald, P.J. & McLachlan, P.M. (1965). Biochem. J. 94, 32.

Hertelendy, F. & Taylor, T.G. (1961). Poult. Sci. 40, 108.

Holmberg, C.-G. & Laurell, C.B. (1947). Acta chem. scand. 1, 944.

Joubert, F.J. & Cook, W.H. (1958). Can. J. Biochem. Physiol. 36, 399.

Komatsu, S.K. & Feeney, R.E. (1967). Biochemistry (Wash.) 6, 1136.

Laskowski, M. (1935 a). Biochem. Z. 275, 293.

Laskowski, M. (1935 b). Biochem. Z. 278, 345.

Laurell, C.B. (1947). Acta physiol scand. 14, Suppl. 46.

Laurell, C.B. (1952). Pharmacol. Rev. 4, 371.

Laurell, C.B. (1960). In "The Plasma Proteins", vol. 1, p. 349. Ed. by Putnam, F.W. New York: Academic Press.

Leffingwell, T.P., Melville, G.S. & Riess, R.W. (1959). J. Lab. clin. Med. 53, 622.

Leibman, A. & Aisen, P. (1967). Archs Biochem. Biophys. 121, 717.

Lockner, D. (1965). Scand. J. clin. Lab. Invest. 17, 247.

Lorenz, F.W., Entenman, C. & Chaikoff, I.L. (1938). J. biol. Chem. 122, 619.

Lorenz, F.W. (1954). Vitams Horm. 12, 235.

Lush, I.E. (1964). Ph.D. Thesis. Edinburgh University.

McCully, K.A. & Common, R.H. (1961). Can. J. Biochem. Physiol. 39, 1451.

McIndoe, W.M. (1959). Biochem. J. 72, 153.

Martin-Mateo, M.C. & Planas, J. (1965). Revta esp. Fisiol. 21, 1.

Martin-Mateo, M.C., Sebastian, M.F. & Planas, J. (1965). Revta esp. Fisiol. 21, 179.

Mecham, D.K. & Olcott, H.S. (1949). J. Am. chem. Soc. 71, 3670.

Mok, C.C., Martin, W.G. & Common, R.H. (1961). Can. J. Biochem. Physiol. 39, 109.

Moore, C.V. (1959-60). Harvey Lectures Ser. 55, 67.

Moore, P.G. & Edwards, D.E. (1965). Standard Statistical Calculations, p. 48. London: Sir Isaac Pitman & Sons, Ltd.

Morgan, E.H. & Carter, G. (1960). Australasian Ann. Med. 9, 209.

Najean, Y., Dresch, C., Ardaillou, N. & Bernard, J. (1967). Am. J. Physiol. 213, 533.

Peterson, E.A. & Sober, H.A. (1962). In "Methods in Enzymology", vol. 5, p. 3. Ed. by Colowick, S.P. & Kaplan, N.O. New York: Academic Press Inc.

Peterson, R.E. (1953). Analyt. Chem. 25, 1337.

Planas, J. & de Castro, S. (1960). Revta esp. Fisiol. 16, 197.

Planas, J., de Castro, S. & Recio, J.M. (1961). Nature, Lond., 189, 668.

Planas, J. (1967). Nature, Lond., 215, 289.

Pollycove, M. & Mortimer, R. (1961). J. clin. Invest. 40, 753.

Pollycove, M. & Maqsood, M. (1962). Nature, Lond., 194, 152.

Poulik, M.D. (1957). Nature, Lond., 180, 1477.

Putnam, F.W. (1965). In "The Proteins", vol. 3, 2nd ed., p.211. Ed. by Neurath, H. New York: Academic Press.

Ramsay, W.N.M. & Campbell, E.A. (1954). Biochem, J. 58, 313.

Ramsay, W.N.M. & Campbell, E.A. (1956). Quart. J. exp. Physiol. 41, 271.

Ramsay, W.N.M. (1957 a). Clin. chim. Acta 2, 214.

Ramsay, W.N.M. (1957 b). Clin. chim. Acta 2, 221.

Ramsay, W.N.M. (1958). In "Advances in Clinical Chemistry", vol. 1, p. 1. Ed. by Sabotka, H. & Stewart, C.P. New York: Academic Press.

Ramsay, W.N.M. (1966). Quart. J. exp. Physiol. 51, 221.

Roberts, R.C., Makey, D.G. & Seal, U.S. (1966). J. biol. Chem. 241, 4907.

Rodriguez, R. & Planas, J. (1964). Revta esp. Fisiol. 20, 83.

Roepke, R.R. & Hughes, J.S. (1935). J. biol. Chem. 108, 79.

Romanoff, A.L. & Romanoff, A.J. (1949). The Avian Egg p. 353. New York: John Wiley & Sons, Inc.

Schade, A.L., Reinhart, R.W. & Levy, H. (1949). Archs Biochem. 20, 170.

Schjeide, O.A. & Urist, M.R. (1956). Science 124, 1242.

Schjeide, O.A. & Urist, M.R. (1959). Expl. Cell. Res. 17, 84.

Schjeide, O.A. & Urist, M.R. (1960). Nature, Lond., 188, 291.

Schmidt, G. & Thannhauser, S.J. (1945). J. biol. Chem. 161, 83.

Shaw, J.S.C. & Clegg, R.E. (1966). Poult. Sci. 45, 600.

Sober, H.A., Gutter, F.J., Wyckoff, M.M. & Peterson, E.A. (1956). J. Am. chem. Soc. 78, 756.

Sober, H.A. & Peterson, E.A. (1958). Federation Proc. 17, 1116.

Sturkie, P.D. (1965). Avian Physiology, 2nd ed., p. 578. London: Bailliere, Tindall & Cassell.

Surgenor, D.M., Koechlin, B.A. & Strong, L.E. (1949). J. clin. Invest. 28, 73.

Svensson, H. (1958). Science Tools 5, 37.

Taborsky, G. (1963). Biochemistry (Wash.) 2, 266.

Underwood, E.J. (1962). Trace Elements in Human and Animal Nutrition, 2nd ed., p. 10. New York: Academic Press Inc.

Urist, M.R., Schjeide, O.A. & Mclean, F.C. (1958). Endocrinology 63, 570.

Vanstone, W.E., Dale, D.G., Oliver, W.F. & Common, R.H. (1957). Can. J. Biochem. Physiol. 35, 659.

Walker, H.A., Taylor, M.W. & Russell, W.C. (1951). Poult. Sci. 30, 525.

Williams, J. (1962). Biochem. J. 83, 355.

Williams, J. (1968). Biochem. J. 108, 57.

Windle, J.J., Wiersema, A.K., Clark, J.R. & Feeney, R.E. (1963). Biochemistry (Wash.) 2, 1341.

Winget, C.M. & Smith, A.H. (1958). Poult. Sci. 37, 509.

Zilversmit, D.B., Entenman, C. & Fishler, M.C. (1943). J. gen. Physiol. 26, 325.