The Separation of Ferritin and Haemosiderin for Studies in the Metabolism of Iron

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1. Ferritin and haemosiderin in diluted tissue cannot be quantitatively separated by centrifugation unless the pH is brought below 6. 2. These iron-storage substances can be separated from one another and from haemoglobin by ion-exchange chromatography on CM-cellulose, when good recoveries of total tissue iron are obtained. 3. The ferritin fraction has been identified by its solubility, its appearance under the electron microscope, crystallization with Cd^{2+} and the fact that it corresponds quantitatively to the heat-stable portion of the tissue non-haem iron.

Iron is believed to be stored in mammalian tissues in two forms, ferritin and haemosiderin, which account for almost all the non-haem iron in storage organs like liver and spleen. Ferritin is soluble in water and dilute salt solutions over a wide range of pH, and remains soluble after heating to 80° near the neutral point. It is precipitated by half-saturation with ammonium sulphate and may be crystallized in the presence of Cd^{2+} (Laufberger, 1935, 1937; Granick, 1946). Ferritin consists of a protein, apoferritin, combined with a variable amount of a basic ferric phosphate (Granick, 1946). Studies with the electron microscope (Farrant, 1954; Richter, 1957; Kerr & Muir, 1960) have shown that the electron-dense clusters of iron atoms in ferritin molecules form a series of well-defined patterns apparently based on a structure common to all the molecules.

Haemosiderin is usually regarded as a granular substance responsible for the staining of tissues rich in iron by the Prussian blue reaction (Neumann, 1888). Difficulties in purification have led to the expression of widely varying opinions on its structure (Cook, 1929; Behrens & Asher, 1933; Ludewig, 1957, 1959; McKay & Fineberg, 1958; Shoden & Sturgeon, 1960; Wöhler, 1960). It has been suggested that haemosiderin is merely an aggregate of ferritin molecules (Shoden, Gabrio & Finch, 1953) or that it at least contains some ferritin (Richter, 1960). Ludewig (1959), however, could find by immunochemical methods only a very small proportion of apoferritin in his preparations, and Shoden & Sturgeon (1961) have emphasized the importance of ensuring that haemosiderin granules are prepared from tissue previously freed from ferritin. The same

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workers (Shoden & Sturgeon, 1960, 1961) considered that ferritin and haemosiderin are physicochemically distinct substances. It is clear that until the nature of haemosiderin is established the belief that all non-ferritin storage iron is to be classed as haemosiderin must be regarded simply as a convenient working hypothesis.

The physiological importance of iron storage has stimulated a number of attempts to separate ferritin from haemosiderin and from the haemoglobin that is a constant contaminant except in animals killed by viviperfusion. Other iron compounds, including haem pigments of the cytochrome group, are present in sufficiently low concentration to be neglected (Drabkin, 1951), except possibly in tracer studies or when iron stores are very low.

Most procedures begin with heat coagulation (Hampton & Kahn, 1953; Fulton & Ramsay, 1960) or an attempt at differential centrifugation (Kaldor, 1958) or a combination of the two (Gabrio, Shoden & Finch, 1953). Heat coagulation causes precipitation of haemoglobin to a degree that varies with pH and ionic strength. The bulky coagulum is difficult to wash, but Hampton & Kahn (1953) used the iron content of the precipitate as an estimate of haemosiderin. Fulton & Ramsay (1960) calculated haemosiderin as the difference between the total non-haem iron of the tissue and the ferritin iron, which they precipitated with ammonium sulphate from the supernatant fraction obtained after heat coagulation.

Gabrio *et al.* (1953) found the results of centrifugal separations to vary with the force applied. They made an arbitrary choice of conditions because they believed that there would be a continuous gradation in size and properties between physicochemically identifiable ferritin molecules and histologically

visible haemosiderin granules. Kaldor (1958), using much greater dilutions of tissue, found that the apparent amount of haemosiderin was unchanged between such wide limits of centrifugal force as 500g and 10000g. Some variability in behaviour is to be expected; haemosiderin granules obviously vary in size, and, although the largest may reach 6μ in diameter (Richter, 1960; Shoden & Sturgeon, 1960), no lower limit has been set. On the other hand, the apparent density of ferritin varies with the amount of iron enclosed in the apoferritin shell (Rothen, 1944; Mazur, Litt & Shorr, 1950; Fineberg & Greenberg, 1955). Nevertheless, it would be expected that the force required to sediment ferritin would be in a higher range than that required for haemosiderin, unless the latter substance shows much more heterogeneity in size than has hitherto been accepted. Kaldor (1958) does not say whether all the non-haem iron in his supernatant fractions showed the properties of ferritin.

The present paper describes a reinvestigation of the problem of the centrifugal separation of ferritin and haemosiderin with special reference to the effect of pH and ionic strength. It also gives an account of a fractionation of the major components of the tissue iron by ion-exchange chromatography on CM-cellulose. That this might be possible was suggested by previous work on the electrophoretic properties of ferritin and haemosiderin (Mazur & Shorr, 1950; Keiderling & Wöhler, 1954). The method is comparatively simple and appears to be very suitable for routine analysis.

METHODS

Most of the experiments were conducted with homogenates prepared from the livers and spleens of normal albino rats of varying strain, sex and age. When other species were used, their tissues and organs were handled in the same way.

Tissue homogenates. Portions (0.5–2.0 g.) of deep-frozen tissue (-20°) were chopped as finely as possible with a stainless-steel razor blade from which the manufacturers' wax coating had not been removed. In control experiments the iron content of liver prepared for analysis by homogenization only was compared with that of liver that had been previously chopped. No significant difference was found. A weighed quantity of chopped tissue was transferred to a chilled homogenizer tube (Potter & Elvehjem, 1936) and homogenized in ice-cold water with a polytetrafluoroethylene pestle. For different purposes the tissue was diluted from fivefold to 20-fold in the homogenization. The homogenate was used as soon as possible and was always kept below 5° . No homogenate was used after its colour had changed from pink to brown, but, when well-chopped tissue was rapidly and briefly homogenized with the least possible rise in temperature, such a change did not develop for several hours.

Carboxymethylcellulose. At first, CM-cellulose was prepared in the laboratory (Peterson & Sober, 1956), but later

a commercial product (Serva) proved satisfactory. After the preliminary treatment recommended by the manufacturer the material was thoroughly washed with the chosen starting buffer and suspended in a sufficient quantity to give a slurry that could be easily poured. A column corresponding to about 0.6 g. of air-dried CM-cellulose was poured in a glass tube (diam. 12 mm.) with a flat perforated base on which the adsorbent was supported by a small piece of cellulose tissue. The 'hold-up' volume was 3.5–4 ml. It was usual to operate six to eight columns simultaneously from reservoirs placed to give a hydrostatic pressure equivalent to about 20 cm. H_2O .

Heat coagulation. When a diluted homogenate is heated to 80° without the addition of buffers or other salts, the amount of iron coagulated depends on the degree of dilution. Laufberger (1937) and Granick (1946) diluted minced tissue with twice its volume of water in the course of the preparation of ferritin. Such mixtures are not conveniently handled in small-scale quantitative analyses, and at much greater dilutions some non-ferritin non-haem iron escapes coagulation. Consistent results, however, are obtained with fresh tissue diluted with 4–20 vol. of water, provided that the ionic strength is brought up to at least 0.03 by the addition of NaCl. The liquid is then heated to 80° in a water bath, cooled and centrifuged to remove coagulated material. Additional NaCl, up to an ionic strength of at least 0.3, has no further effect on the results.

Radioactive experiments. [^{59}Fe]Ferric citrate solution supplied by The Radiochemical Centre, Amersham, Bucks., was diluted with water or 1% NaCl. Rats were given a single subcutaneous injection of 1–3 μ Ci in a volume of 0.2–0.5 ml. The total quantity of iron was about 1 μ g. When radioactive transferrin was required, the animals were bled from the heart about 30 min. after the injection. They were killed after 3 days for the preparation of radioactive haemoglobin or ferritin.

^{59}Fe was assayed by the β -counting procedure of Ramsay & Fulton (1964), in which the ferrous bipyridyl complex is precipitated as a mercuri-iodide and transferred quantitatively to planchets. When the counting rate exceeded 300 counts/min. the standard error of counting was $\pm 1\%$; below that it was $\pm 3\%$.

Analytical procedures. (a) Iron determinations. These were made by the 2,2'-bipyridyl method (Hill, 1931) after appropriate preliminary treatments. Solutions that did not contain haemoglobin, or in which it was intended to minimize the participation of haem iron in the reaction, were heated in a boiling-water bath (15 min.) with HCl (final concn. 0.1 N). Iron was liberated from haem pigments, including haemoglobin, by treatment with hydrogen peroxide (Peters, 1947; Ramsay, 1964). If present, the iron of ferritin and haemosiderin also reacts after treatment with peroxide, so that this method effectively gives an estimate of total iron (Ramsay & Campbell, 1954). After the chosen pretreatment the solution or suspension (usually 8–10 ml.) in a tube graduated at 10 ml. was treated with bipyridyl (0.5% in 60% acetic acid; 1 ml.) and sodium sulphite (1.5 M; 0.5 ml.) and heated in a boiling-water bath for 30 min. The tube was cooled, the volume adjusted to 10 ml. and the extinction measured at 520 m μ after centrifuging. Any persistent turbidity could usually be removed by centrifuging again after vigorous shaking with chloroform.

(b) Total non-haem iron. When a diluted tissue homogenate is treated with an equal volume of saturated ammo-

nium sulphate solution, all the storage iron is precipitated along with variable but usually small quantities of haemoglobin and other haem pigments. Only a small proportion of the precipitated haem pigment reacts with bipyridyl in the presence of sulphite unless the peroxide treatment is included. It seems therefore justifiable to describe as 'total non-haem iron' that quantity of iron in a fresh homogenate that is precipitated by half-saturation with ammonium sulphate and that, after being heated with HCl, gives a pink colour when heated with bipyridyl and sulphite. This conclusion was supported by the analysis of livers perfused *in situ* with Ringer solution by both the hepatic artery and the portal vein. Total iron measured after wet-ashing was closely similar to that found by the procedure described above. When blood containing radioactive haemoglobin was added to homogenates of liver or spleen before analysis, less than 5% of the radioactivity was recovered in the ferrous bipyridyl complex.

EXPERIMENTAL AND RESULTS

Separation of ferritin from haemosiderin by centrifugation. In preliminary experiments diluted spleen homogenates (1 g. of tissue plus 19 ml. of water) were spun for 30 min. in a Spinco model L ultracentrifuge at centrifugal forces up to 57 000g. The non-haem iron remaining in the supernatant fluid in each case was subtracted from the total non-haem iron content of the original homogenate to give the amount of non-haem iron in the sediment. As Fig. 1 shows, the sedimentation of non-haem iron continues smoothly far above the centrifugal force of 1400g for 30 min. used by Kaldor (1958). Reference to Fig. 2, as well as to the work of Rothen (1944) and Mazur & Shorr (1948), shows that very little ferritin is sedimented at such low speeds, so that the continuous change in the amount of iron sedimented must have been due to differences in the amount of haemosiderin sedimented. The observations were repeated with a similarly diluted homogenate on which determina-

tions had been made of heat-stable iron precipitable by half-saturation with ammonium sulphate as well as of total non-haem iron. The former was presumed to correspond to ferritin and the difference between the two to haemosiderin. The same analyses were performed on the supernatant fractions, after centrifuging samples of the homogenate for 30 min. at forces ranging from 2000 to 75 000g. Subtraction of the values from the corresponding ones for the original homogenate gave estimates of the iron present in the various sediments as ferritin and haemosiderin. The analyses can only be considered approximate, but the possible margin of error is not large enough to make a marked difference to the results. These, calculated as percentages of the amounts present in the homogenate, are plotted in Fig. 2 against the centrifugal force employed. It is evident that, even when the force is sufficient to sediment a substantial proportion of the ferritin present, some iron with the properties of haemosiderin remains in the supernatant fluid.

The separation of ferritin from haemosiderin by the simple centrifugation of a diluted homogenate is thus impossible, but if the pH is lowered more promising results are obtained. Acetic acid and sodium chloride were added in the course of dilution of a spleen homogenate to give specimens at each of three ionic strengths (0.025, 0.10 and 0.4) and seven pH values between 6.9 and 4.1. The tubes were refrigerated for 1 hr. and centrifuged for 15 min. at 1400g. The precipitate was treated with hydrochloric acid, bipyridyl and sulphite. The iron detected was presumably derived from haemosiderin,

Fig. 1. Sedimentation of iron from diluted spleen homogenate.

Fig. 2. Sedimentation of non-haem iron fractions from diluted spleen homogenate. ○, Heat-stable; ●, heat-coagulable.

Table 1. *Sedimentation of haemosiderin at different values of pH and ionic strength*

Values are given as percentages of the amount of heat-coagulable non-haem iron precipitated by $(\text{NH}_4)_2\text{SO}_4$ in the original homogenate.

pH	Percentage of heat-coagulable non-haem Fe precipitated		
	I 0.025	I 0.10	I 0.40
4.1	98	100	97
4.5	102	101	99
5.0	102	100	97
5.4	101	96	86
6.0	96	72	24
6.4	68	28	13
6.9	23	19	11

because no ferritin is sedimented at this low centrifugal force, and in the analysis the peroxide treatment for haem pigments was omitted. The results (Table 1) show that at all three ionic strengths lowering the pH facilitates the sedimentation of haemosiderin, and that the effect is greater at lower ionic strengths. The maximum amount of iron sedimented was equal to the amount of heat-coagulable non-haem iron in the original homogenate, so that dilution of a homogenate and lowering the pH below 6 seem to enable the complete separation of ferritin from haemosiderin to be made at low centrifugal forces. These experiments appear to show that some haemosiderin granules may be much smaller than has hitherto been suspected.

Ion-exchange chromatography. The remarkable effect of pH on the centrifugal separation of ferritin and haemosiderin (Table 1) and the electrophoretic differences reported by Keiderling & Wöhler (1954) suggested that ion-exchange chromatography might form the basis of a useful procedure.

DEAE-cellulose. In preliminary experiments haemosiderin was readily separated from haemoglobin and ferritin, but good conditions for the separation of the latter two substances were not found.

Fractionation on a column of CM-cellulose. If the pH is below 5 and the ionic strength less than about 0.015 (the exact limits depend on the nature of the buffer anion) all the major iron compounds in tissues remain on the column. Ferritin loses iron in solutions more acid than pH 4.6, especially in citrate buffers. It is therefore necessary to adjust the pH of the starting buffer carefully. As long as interest centres on the iron content of the fractions, some latitude in both pH and ionic strength of the eluting buffers is permissible. Both acetate and citrate buffers have been used, but citrate is preferred

because in experiments on the effect of the addition of radioactive transferrin it diminished the contamination of some of the fractions.

A column prepared as described above is equilibrated with the starting buffer, 6mM-sodium citrate-4mM-citric acid solution, pH 4.95. A fresh homogenate is brought to approx. pH 4.9 with 0.5N-acetic acid (0.15m-equiv./g. of spleen; 0.125m-equiv./g. of liver) and the dilution adjusted to 1:9 with water. A suitable volume (1-2ml.; the column will not deal satisfactorily with the equivalent of more than 300mg. of tissue) is pipetted on to the surface of the CM-cellulose and gently stirred into the uppermost 1 cm. layer. This ensures a satisfactory rate of flow. The starting buffer (10ml.) is then run through the column and the eluate discarded. It is followed by 19mM-sodium citrate-6mM-citric acid buffer, pH 5.5. The eluate contains a yellow to brown iron-rich substance (fraction I) that usually leaves the column in less than 20ml. The buffer is then changed to 42mM-sodium citrate-8mM-citric acid-0.3M-sodium chloride solution, pH 5.7. A second iron-rich substance (fraction II) now leaves the column, but this may require as much as 60ml. of buffer for complete elution. Another iron-rich substance (fraction III) can be slowly eluted with slightly alkaline solutions, but is only removed completely with 0.1N-sodium hydroxide (20ml.), which clears the column of all protein.

A hand spectroscope showed fraction II to be rich in oxyhaemoglobin, but gave no evidence for haemoglobin derivatives in fractions I and III. When the fractions were half-saturated with ammonium sulphate, all the iron in fractions I and III, but none of that in fraction II, was precipitated. This suggests that fraction II consists largely of oxyhaemoglobin. Fractions I and III were dialysed against water and centrifuged briefly at about 1400g. In two experiments the supernatant solution from fraction I contained 91 and 94% of the iron and that from fraction III only 1.4 and 0.1%. Similar results were obtained when the dialysis was against acetate buffer, pH 4.8 and I 0.013. Fraction I was dialysed, concentrated by the addition of dry Sephadex G-75 and treated with cadmium sulphate to make the final concentration 3%. Brown crystals similar to those described by Granick (1943) were obtained. Fraction III yielded no crystals, but it was not possible to perform an exactly parallel experiment because the iron-rich substance was precipitated on dialysis.

Other preparations were dialysed against 0.1M-ammonium acetate (in which both fractions were somewhat soluble) and sprayed on carbon grids for electron microscopy. Dr A. R. Muir has reported to us that 'in fraction I all the electron-dense images have the size and shape consistent with those

observed in similar examination of pure, crystalline ferritin. In fraction III the electron-dense images show a variety of shapes and sizes. A proportion of the images are identical with those obtained from pure ferritin, but many could not be produced by any orientation of ferritin molecules'. In fact, the number of images in fraction III that resembled those from ferritin was a very small proportion of the total. In a control experiment a preparation of fraction I was treated with 0.1N-sodium hydroxide before dialysis against ammonium acetate. Since this preparation also showed ferritin under the electron microscope, the irregular appearance of fraction III was not caused by the action of alkali on ferritin. Thus, though fraction I contains ferritin, the iron in fraction III is present in a different and much less soluble form.

Quantitative studies of tissue iron. By using six different spleens comparisons were made of the iron content of the three ion-exchange fractions with the total iron, the total non-haem iron and the heat-stable iron precipitable by half-saturation with ammonium sulphate (Table 2). The sum of the iron contents of the three fractions corresponds closely to the total iron of the homogenate, and the sum of fractions I and III agrees with the total non-haem iron. Further, the amount of iron in fraction I is similar to the amount precipitated by ammonium sulphate from the supernatant fluid after heat-coagulation. These analyses support the conclusion that fractions I and III contain the non-haem storage iron of the tissue, and that the ferritin is in fraction II. Fraction III must therefore contain the haemosiderin or at least the iron derived from it. Similar analyses conducted on liver also gave good correspondence between the different methods, but the results are not given in detail because the small amount of iron normally found in fraction III makes the comparison less precise.

Recovery experiments with ^{59}Fe -labelled haemoglobin and ferritin. The above conclusions received additional support from the following experiments. Lysed washed erythrocytes from a rat

previously treated with ^{59}Fe ferric citrate were added to a spleen homogenate before fractionation. Fraction II contained 95% of the added radioactivity, fraction I 0.5% and fraction III 2.3%. In another experiment a spleen homogenate from a rat treated with ^{59}Fe ferric citrate was heat-coagulated in the usual way. The supernatant was half-saturated with ammonium sulphate and the precipitate was dialysed and reprecipitated twice more. The ferritin-rich solution was added to a non-radioactive homogenate before chromatography. Of the radioactivity, 97.3% was recovered in fraction I, 1.9% in fraction II and 1.1% in fraction III.

When serum (0.5 ml.) containing radioactive transferrin-bound iron was added to tissue (1 g.) during homogenization, fraction I was heavily contaminated. The degree of contamination was less if the volume of starting buffer run through the column before elution was increased. In one experiment with citrate buffers, 59% of the added radioactivity (2480 counts/min.) was found in the starting buffer (25 ml.), 36% in fraction I, and 0.4 and 0.6% in fractions II and III. When acetate buffers were used, fractions II and III each contained up to 6% of the radioactivity, and there was no improvement in the recovery of label in fraction I. These results are probably not directly attributable to the behaviour of transferrin on the column, because the combination of iron with transferrin is a reversible process linked with the dissociation of an acid group

Table 3. *Iron present as ferritin and haemosiderin in the liver and spleen of normal adult male rats*

Results are expressed as mg. of Fe/100 g. wet wt. and are given as means $\pm$ S.D.

	No. of Animals	Ferritin	Haemosiderin
Liver	46	8.4 $\pm$ 1.5	2.4 $\pm$ 0.6
Spleen	46	13.7 $\pm$ 3.5	36.4 $\pm$ 10.1

Table 2. *Fractionation of iron in fresh rat spleen by different methods*

All values given in mg. of Fe/100 g. of tissue. CM-cellulose fractions, obtained as described in the text, are designated I, II and III.

Spleen ...	A	B	C	D	E	F
Total iron	37.6	40.0	40.3	43.3	61.1	112.0
I + II + III	37.1	38.4	39.0	43.6	61.3	114.5
Non-haem iron	6.9	7.7	12.0	24.4	34.9	97.0
I + III	6.6	9.0	11.9	24.1	36.6	99.1
Heat-stable iron pptd. by $(\text{NH}_4)_2\text{SO}_4$	1.9	3.4	4.4	9.7	10.2	29.0
I	2.1	3.4	4.3	9.8	10.6	25.1

of pK approx. 5.7 (Surgenor, Koechlin & Strong, 1949).

Analytical reproducibility. Eight rat spleens with non-haem iron contents ranging from 7 to 300 mg./100 g. were separately homogenized and six fractionations were made on each homogenate. The statistical variability of the results depended on the quantity of iron present, and the coefficient of variation was 2.2% for fraction I, 3.4% for fraction II and 2.3% for fraction III. Rechromatography of fraction I after dialysis against the starting buffer gave about 90% recovery in the corresponding fraction and 10% in fraction III. Rechromatography of fraction III gave 98% recovery, but the experiment is of limited significance because the eluent for this fraction removes all adherent material from the column.

Illustrative results. Table 3 gives the ferritin and haemosiderin iron concentrations found in the liver and spleen of mature male rats (265–419 g.) on an adequate diet (Bethard, Wissler, Thompson, Schroeder & Robson, 1958) that provided a normal daily intake of iron (about 3 mg. per rat). Ferritin accounted for 79% of the storage iron in liver, but only 27% of that in spleen. Kaldor (1958), using a simple centrifugal separation, found similar values for the livers of rats of the same age and sex, but found that ferritin appeared to account for 67% of the storage iron in the spleen. The discrepancy is explained by the experiments on the sedimentation of ferritin and haemosiderin described in the present paper.

Only a few analyses have been made on tissues from other species. Rabbit tissues can be fractionated with the buffer systems used above, but tissues from man, the domestic fowl and the sea trout (*Salmo trutta*) require buffers of slightly different composition. The fact that the fractions desired are all coloured is of great assistance in developing any modifications that may be necessary.

DISCUSSION

The ion-exchange procedure separates ferritin quantitatively from haemoglobin and from the remainder of the tissue non-haem iron, which is presumably derived from haemosiderin. The properties of the fraction in which it is found suggest that ferritin comes through the analysis largely unchanged. Haemosiderin, on the other hand, is altered, since it is apparently made soluble by the treatment with dilute alkali. Centrifugal experiments have shown that the size and weight of the iron-containing particles in the alkaline solution are of the same order as those of ferritin molecules. This, however, does not indicate a mere disaggregation or depolymerization to ferritin, as is clearly shown by the electron-microscopic observations.

The nature and extent of the change wrought by alkali are unknown. If the pH is lowered to 5 the material is precipitated, but it can be redissolved in alkali or rechromatographed, when its behaviour on the column is unchanged.

The experiments with radioactive ferritin and haemoglobin show that errors involving cross-contamination would be unlikely to arise from these substances if the technique is used in metabolic studies with radioactive iron. No similar test has been made with haemosiderin, because it is considered that the large granules obtained by the usual methods of preparation may not be truly representative, but it seems unlikely that this substance would give rise to error. On the other hand, the presence in the tissue of radioactive transferrin-bound iron causes considerable contamination, particularly of ferritin. The concentration of iron in plasma is so low, however, that there is only likely to be a significant error during the first few hours of a labelling experiment.

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