The Organ-Specificity of Ferritin in Human and Horse Liver and Spleen

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1. Ferritin was isolated from human and horse spleen and liver, and apoferritin prepared therefrom. 2. The electrophoretic mobilities of the four apoferritins were determined on polyacrylamide gels and on cellulose acetate strips, and all found to be equal. 3. Homologous ferritins share reactions of identity in immunodiffusion experiments, whereas heterologous ferritins show only partial identity. 4. The subunit molecular weight of each of the apoferritins was determined by polyacrylamide-gel electrophoresis in sodium dodecyl sulphate and by chromatography on agarose columns in 6M-guanidine-HCl. A value of approx. 18500 was found in all cases. The proteins all had sedimentation coefficients of 17–18S. It thus seems that they have identical quaternary structures. 5. The amino acid compositions of the proteins revealed distinct differences both between organs and between species. This was confirmed by analysis of the tryptic peptide patterns, where it was found that about one-third of the peptides were common to the four proteins and the other two-thirds varied from protein to protein. 6. It is concluded that the apoferritins present in the liver and spleen of human and horse are both organ- and species-specific. 7. The apoferritin isolated from the liver of a patient with idiopathic haemochromatosis was identical with normal human liver apoferritin by the criteria described above.

Ferritin, together with haemosiderin, is the principal storage form of iron in mammals, where it is found chiefly in the liver, spleen and bone marrow (Granick, 1943). It is also found in the mucosal cells of the small intestine, in reticulocytes, in placenta, testis, kidney, heart and skeletal muscle. The iron of ferritin can be removed by reduction to give an iron-free protein, apoferritin (Granick & Michaelis, 1943), which has the same electrophoretic mobility as ferritin (Mazur & Shorr, 1950). Horse spleen apoferritin has a molecular weight of 440000 (R. R. Crichton, R. Eason, A. Barclay & C. F. A. Bryce, unpublished work; Rothen, 1944; Harrison, 1959; Richter & Walker, 1967; Björk & Fish, 1971) and consists of 24 chemically identical subunits of molecular weight 18500 (Crichton, 1972a; Crichton & Bryce, 1970; Bryce & Crichton, 1971a,b). The structure, synthesis and function of ferritin have been reviewed (Crichton, 1971, 1972b).

It has been shown, on the basis of differences in electrophoretic mobilities, that different ferritins occur in a number of different organs within the same species (Allfrey *et al.*, 1967; Gabuzda & Gardner, 1967). Three types of ferritin were identified in human spleen, liver and reticulocytes respectively. In human bone marrow two types of ferritin were found, one corresponding in mobility to spleen ferritin and the other to reticulocyte ferritin (Allfrey *et al.*, 1967). The

same phenomenon was described in rabbits (Gabuzda & Gardner, 1967), and it was shown that iron administered as transferrin was predominantly incorporated into anabolic erythroblast ferritin whereas iron administered as heat-damaged reticulocytes was found mostly in the catabolic reticuloendothelial ferritin (Gabuzda & Pearson, 1969). The presence of distinctive ferritins in liver and kidney of rats, and of two ferritins in rat heart, has also been reported (Linder-Horowitz *et al.*, 1970). These claims have been disputed by Lee & Richter (1971), who found the electrophoretic mobilities of ferritins from human liver, spleen and bone marrow to be equal, and the same to be true of ferritins from adult, foetal and regenerating rat liver, rat spleen and rat kidney, and for the ferritins from liver, spleen and bone marrow of the rabbit.

In view of possible functional differences between ferritins of different organs, it is important to ascertain whether or not tissue-specific isoferritins do exist. We present here a report on the isolation of ferritin from human and horse liver and spleen and, for the first time, a detailed analysis of the apoferritins from these four tissues in terms of their electrophoretic behaviour, immunological cross-reactivity and chemical composition. The polypeptide molecular weight, the sedimentation coefficients of the intact proteins, the amino acid compositions and tryptic peptide

patterns have been determined, and the organ-specificity of the isoferritins has been established. A preliminary account of this work has been published (Crichton *et al.*, 1970).

Experimental

Materials

Horse spleen ferritin was purchased from Schwartz/Mann, Orangeburg, N.Y., U.S.A. Trypsin (1-chloro-4-phenyl-3-toluene-*p*-sulphonamidobutan-2-one-treated) was from Worthington Biochemicals Corp., Freehold, N.J., U.S.A. The sources of reference proteins used as molecular-weight markers have been given previously (Crichton & Bryce, 1970; Bryce & Crichton, 1971a). Guanidine hydrochloride was from British Drug Houses Ltd., Poole, Dorset, U.K., and was purified as described by Bryce & Crichton (1971a). Acrylamide and bisacrylamide were from Serva, Heidelberg, Germany. All other reagents were of analytical grade or of the purest grade available.

Methods

Isolation of ferritin. Fresh human liver and spleen samples were obtained *post mortem* from the Departments of Pathology of Glasgow Western Infirmary and Stobhill General Hospital, Glasgow, and the Institut für Pathologie, Klinikum Steglitz der Freien Universität, Berlin. Horse liver and spleen were obtained from freshly slaughtered animals. The tissues were frozen and stored at -10°C and the ferritin was usually prepared within a few days, by the procedure given in Scheme 1. The methods are essentially those of Granick (1943) and Penders *et al.* (1968), but with several modifications. After the homogenization of the tissue, treatment at $70\text{--}75^{\circ}\text{C}$ for 10 min in a water bath with continuous stirring was done. The introduction of a filtration step after centrifugation of the heat-treated homogenate eliminated traces of the pellet, which tended to be slightly disrupted by decantation of the supernatant. Whatman no. 1 paper was used. After ammonium sulphate precipitation the supernatant was usually yellow in colour and was discarded. Sometimes it was darker brown in colour, and a second ammonium sulphate precipitation was then done with 50% ammonium sulphate, and the precipitates were combined. With the ultracentrifugation method of Penders *et al.* (1968) the sedimentation of ferritin in the first centrifugation could be greatly improved by first dialysing the protein solution overnight against water to remove most of the ammonium sulphate. Between high-speed centrifugations a centrifugation at 23 000g was used to clarify the protein solution and also to remove any bacterial contaminants. In the alternative procedure the protein solution was concentrated

by ultrafiltration at a pressure of $2.8\text{--}3.5\text{ kN/m}^2$ ($40\text{--}50\text{ lbf/in}^2$) with a Diaflo ultrafiltration apparatus and a UM 10 filter (Amicon Ltd., High Wycombe, Bucks., U.K.), and the concentrated solution was then passed through an MF Millipore filter of pore size $0.45\text{ }\mu\text{m}$ (Millipore U.K., Ltd., Wembley, Middx., U.K.) to remove bacterial contaminants. This solution was then applied to a column of Sephadex G-200 ($100\text{ cm}\times 2.5\text{ cm}$), which was eluted under gravity with 0.1 M -sodium phosphate buffer, pH 7.0, and the ferritin-containing fraction that eluted with the void volume was collected. Crystallization of ferritin solutions, which had been extensively dialysed against water, was done with $5\text{--}10\%$ (w/v) cadmium sulphate, either added as a $10\text{--}20\%$ (w/v) solution in the cold, or added gradually in solid form to a final concentration of $5\text{--}10\%$ (w/v).

Apo ferritin from haemochromatotic liver. Professor R. Williams, King's College Hospital, London, generously provided a post-mortem sample of human liver from a patient with primary (idiopathic) haemochromatosis, and we isolated ferritin from it. The clinical details supplied were: 'The patient was a 70-year-old male who presented on 5.8.70 with a 2-year history of ascites, ankle swelling, increased breathlessness, loss of weight and skin pigmentation. Investigation revealed a diabetic glucose-tolerance curve, and a higher-than-normal urinary iron excretion after administration of desferrioxamine. The results of liver function tests were consistent with hepatic cirrhosis, and liver biopsy showed fibrosis with chronic inflammatory cells and large quantities of iron. Chest X-ray and skin biopsy were also consistent with a diagnosis of idiopathic haemochromatosis. Before death the patient was being treated by venesection of 250 ml/week.'

Apo ferritin was prepared from ferritin by reduction and dialysis either with sodium dithionite and $\alpha\alpha'$ -bipyridyl or with thioglycolic acid as described previously (Crichton, 1969, 1972b).

Electrophoresis. Discontinuous electrophoresis of both apo ferritin and ferritin was done on polyacrylamide gels at pH 8.65 and pH 3.0. Samples were dialysed overnight at 4°C against the electrode buffer in the former case. For electrophoresis at low pH the samples were first dissociated into subunits by treatment with 67% (v/v) acetic acid at 0°C for 1 h and then dialysed overnight into 0.01 M -glycine-HCl buffer, pH 3.0, at 4°C (Harrison & Gregory, 1968). Under these conditions the apo ferritin remains dissociated.

For the electrophoresis at pH 8.65, a concentration of 7.5% (w/v) acrylamide was used with 0.32% cross-linking. The electrode buffer stock solution contained 85 mM-Tris, 3.5 mM-EDTA and 16 mM-boric acid adjusted to pH 9.2 with HCl and was diluted 1:10 (v/v) for use. The gels were prepared as described by Braun *et al.* (1968) in 37 mM-Tris-HCl, pH 8.65. Samples containing 25–50 μg of each protein were

Scheme 1. Isolation procedures employed for preparation of ferritin

Details are presented in the text.

applied. The electrophoresis was carried out at a constant current of 2mA/gel for 50–60min.

The gels for electrophoresis at pH3.0 contained 10% acrylamide and 0.24% cross-linker, and were prepared as described by Shepherd & Gurley (1966). However, 0.01M-glycine-HCl buffer, pH3.0, was used as electrode buffer. The same amount of sample was used as for the alkaline gels. Electrophoresis was for 30–40min at 2.5mA/gel.

After extrusion of the gels, staining was carried out for 10min with 0.5% Amido Black in 5% (v/v) acetic acid. Destaining was in 1% (v/v) acetic acid.

Electrophoresis on cellulose acetate strips (6cm × 15cm) was done with samples that had been dialysed against 0.075M-sodium barbital buffer, pH8.6. This was also used as electrode buffer. The protein solutions (5mg/ml) were applied to the strips with a Beckman micro-applicator and the electrophoresis was performed in a Beckman microelectrophoresis apparatus (Beckman Instruments, Palo Alto, Calif., U.S.A.) at 14V/cm for 150min. The strips were stained with Amido Black (0.5% in methanol-acetic acid, 9:1, v/v) for 20min and de-stained with methanol-acetic acid-glycerol (87:12:10, by vol.).

Immunodiffusion. Antiserum was raised in rabbits by intramuscular injections of graded doses of commercial horse spleen ferritin after dialysis against 0.15M-NaCl at 2°C. The doses were 2, 4, 8 and 16mg. Each dose was administered in an equal volume of complete Freund's adjuvant. Immunodiffusions were carried out in 2% agar gels, buffered at pH8.2 in 0.01M-sodium phosphate buffer (Ouchterlony, 1948) for 48h. The ferritin concentration in the wells was 0.25mg/ml, irrespective of the source of the ferritin. This value was established by prior analysis of horse spleen ferritin (1mg/ml) in serial dilutions against rabbit anti-(horse spleen ferritin) antiserum. The gels were washed with 0.15M-NaCl for 72h and the precipitin lines fixed with 0.3M-trichloroacetic acid. Staining was not necessary.

Subunit molecular-weight determinations. Polyacrylamide-gel electrophoresis in sodium dodecyl sulphate was done as previously described (Crichton & Bryce, 1970; Bryce & Crichton, 1971a). The mobility of each of the apoferritins relative to a Bromophenol Blue marker was determined, and from a plot of the logarithm of the molecular weight of a number of protein markers against their electrophoretic mobilities the polypeptide chain molecular weights of the proteins were determined. The molecular-weight markers used were bovine serum albumin, γ -globulin (light and heavy chain), leucine aminopeptidase, ovalbumin, chymotrypsinogen A, trypsin, myoglobin, lysozyme, ribonuclease, chymotrypsin (light and heavy chain) and cytochrome *c*.

The subunit molecular weights of the apoferritins were also determined by chromatography on calibrated columns of Sepharose 6B (Pharmacia U.K.,

London W.5, U.K.) eluted with 6M-guanidine-HCl, pH5.0 (Bryce & Crichton, 1971a,b). The same molecular-weight markers as for the electrophoretic determination of molecular weight were used with the exception of lysozyme, leucine aminopeptidase, trypsin, ribonuclease and chymotrypsin.

Determination of sedimentation coefficients. Sedimentation-velocity analyses were done in a Beckman-Spinco model E analytical ultracentrifuge (Beckman Instruments, Palo Alto, Calif., U.S.A.) equipped with regulated speed control, temperature control and u.v. scanner Multiplex assembly. An An-D rotor was used with double-sector cells at a speed of 40000rev./min and at 15°C. Protein samples (0.4–0.8mg/ml) in 0.1M-sodium borate buffer, pH7.4, were dialysed against this buffer for 24h before analysis. Sedimentation coefficients were calculated by the method of Schachmann (1959) and reduced to the standard state $s_{20,w}$ after correction for buffer and temperature. No correction was made for dependence of the *s* value on protein concentration.

Amino acid analysis. Protein samples (0.2–1.0mg) were hydrolysed at 110°C *in vacuo* with 6M-HCl containing 0.02% (v/v) mercaptoethanol. Analyses were carried out on a Locarte amino acid analyser (Locarte Co., London W.1, U.K.) and on BioCal analysers (BioCal Instruments, München-Gräfelfing, Germany) equipped with automatic sample loading and by using a single-column system with stepwise elution. Cysteine was determined after oxidation to cysteic acid (Hirs, 1956). Tryptophan was determined by the method of Edelhoch (1967) and by conversion into the 2-nitrophenylsulphenyl derivative (Boccu *et al.*, 1970).

Trypsin digestion and peptide 'maps'. Trypsin digestion of apoferritins was done at 37°C for 16h in 0.06M-NaHCO₃, pH8.0, with an enzyme to protein ratio of 1:20. Peptide 'maps' were prepared as described by Crichton (1969) and the numbering of the peptides there proposed for horse spleen apoferritin has been used in this paper.

Results and Discussion

Isolation of ferritin

We have made several modifications to the procedure of Granick (1943) because of problems encountered with the isolation of ferritins from human tissues. Very poor yields of human liver and spleen ferritin were obtained by crystallization from cadmium sulphate, and the crystals were semi-amorphous with rounded edges, as reported by Granick (1943). Although better crystals were obtained from horse liver, the yields were also poor. This was observed by Laufberger (1937), who was able to isolate the iron-storage protein from horse spleen but not liver. We found that human ferritin isolated by the Granick

(1943) procedure gave inconsistent amino acid analyses and peptide 'fingerprints', presumably through contamination with small amounts of other proteins or peptides. Several serum proteins can adsorb to ferritin with the production of insoluble complexes (H. Yamada & T. G. Gabuzda, personal communication). When extensive haemolysis had occurred before death we often found large amounts of haemoglobin in the supernatant fraction after heat treatment of spleen homogenates, and this material remained after the ammonium sulphate precipitation step with the ferritin. In such cases it was desirable either to wash the tissue with cold saline before homogenization, or to use the method of Penders *et al.* (1968) since the haemoglobin was easily separated from the ferritin by ultracentrifugation. With tissues from patients who had had bacterial infections, bacterial contamination was removed by ultrafiltration. No differences were found in the chemical properties of ferritins prepared by the two methods outlined in Scheme 1. However, the ultracentrifugation method was much more satisfactory in cases where the amount of tissue available was small.

Electrophoresis of ferritins and apoferritins

Ferritin and apoferritin behave identically on electrophoresis (Mazur & Shorr, 1950). Plate 1(a) shows the electrophoretic analysis of apoferritins from the four tissues by discontinuous polyacrylamide-gel electrophoresis at pH 8.65. Each of the proteins gives one major monomer band (α -band), and in some of the gels smaller amounts of dimers and trimers can be seen. Gel (g) in Plate 1(a) shows oligomer bands obtained with 150 μ g of horse spleen ferritin extending to octomer. However, other than this well-established aggregation (Suran & Tarver, 1965; Harrison & Gregory, 1965; Kopp *et al.*, 1966), there was no evidence for any other protein components. This was also found at pH 3.0, on cellulose acetate electrophoresis and on polyacrylamide gels in sodium dodecyl sulphate. When any two apoferritins

were mixed and analysed (Plate 1a: gels c and f) only one monomer band was observed. This confirms the findings of Lee & Richter (1971), who found only one band in human liver, spleen and bone marrow ferritins each with the same mobility. We also were unable to detect any differences between these apoferritins, either in polyacrylamide gels at pH 3.0 with protein that had been dissociated into subunits or on cellulose acetate strips at pH 8.6. It seems that reports claiming separation of human liver and spleen ferritins were incorrect (Allfrey *et al.*, 1967), probably because the proteins were never subjected to co-electrophoresis. It is clear from an inspection of Plate 1(a) that, although the six apoferritin samples were subjected to electrophoresis under the same conditions at the same time, there are differences in the absolute distances to which each of the proteins have migrated. However in the gels (c) and (f) of Plate 1(a), where two proteins were present, no separation can be seen.

Interestingly, the four proteins appear to have the same net charge as judged by our inability to separate them on electrophoresis. This conservation of surface charge may reflect the ability of the oligomer to form aggregates (see gel g of Plate 1a). The first step in the formation of haemosiderin from ferritin is thought to be such an aggregation (Sturgeon & Shoden, 1964), although little is known about this process. In a ferritin preparation from the lung of a patient with pulmonary haemosiderosis the aggregate forms of ferritin normally seen on starch-gel electrophoresis of ferritin were not found (Bungert *et al.*, 1971). However, the protein was not compared in terms of its chemical composition with normal human lung ferritin, although it had a quite similar electrophoretic mobility to that of the normal human α -band.

Immunodiffusion

Plate 1(b) shows the result of testing the four ferritins against rabbit anti-(horse spleen ferritin) antiserum by gel diffusion. The rabbit antiserum is known

Table 1. *Subunit molecular weights and sedimentation coefficients of apoferritins*

The subunit molecular weights and sedimentation coefficients were determined as described in the text. S.E.M. values are given for subunit molecular weights as determined by both methods. The sedimentation coefficients corrected for buffer and temperature to the standard $s_{20,w}$ are the mean of four independent determinations.

Source	Subunit mol.wt.		Sedimentation coefficient $s_{20,w}$ (S)
	Polyacrylamide-gel electrophoresis in sodium dodecyl sulphate	Chromatography in 6M-guanidine-HCl	
Horse liver	18400 $\pm$ 400	18800 $\pm$ 600	16.9
Horse spleen	18300 $\pm$ 300	18800 $\pm$ 400	16.8
Human liver	18600 $\pm$ 500	18700 $\pm$ 500	17.3
Human spleen	18300 $\pm$ 400	18500 $\pm$ 500	16.8

Table 2. *Amino acid composition of horse and human apoferritin*

The results are presented as mol/100mol and as residues of each amino acid/subunit based on the molecular weights determined for the subunit (Table 1). In all cases the results are the means of at least ten determinations on four or more different preparations. S.E.M. values are given for the human spleen and liver and for the horse liver proteins; the numbers of determinations are given in parentheses. The values for tryptophan were the mean of the values obtained by the two different methods. The results for horse spleen are taken from Bryce & Crichton (1971a).

Amino acid	Mol/100mol				Residues/subunit (18 500 daltons)			
	Horse		Human		Horse		Human	
	Spleen	Liver	Spleen	Liver	Spleen	Liver (14)	Spleen (10)	Liver (12)
Cys	1.78	1.58	1.04	0.93	2.9	2.6 ± 0.25	1.7 ± 0.31	1.5 ± 0.30
Asx	10.73	11.08	11.95	11.85	17.3	17.9 ± 0.32	19.3 ± 0.34	19.2 ± 0.43
Thr	3.42	3.48	3.79	3.83	5.5	5.6 ± 0.31	6.1 ± 0.27	6.2 ± 0.22
Ser	5.59	5.53	4.78	5.76	9.0	8.9 ± 0.46	7.7 ± 0.43	9.3 ± 0.30
Glx	14.83	15.74	13.83	14.38	23.9	25.3 ± 0.15	22.3 ± 0.21	23.9 ± 0.17
Pro	1.73	1.92	2.36	1.81	2.8	3.1 ± 0.53	3.8 ± 0.37	2.9 ± 0.60
Gly	6.15	6.47	7.22	6.33	9.9	10.2 ± 0.13	10.8 ± 0.15	10.1 ± 0.22
Ala	8.68	8.33	8.55	8.49	14.0	13.4 ± 0.24	13.8 ± 0.21	13.7 ± 0.30
Val	4.28	4.28	3.73	3.89	6.9	6.9 ± 0.38	6.0 ± 0.45	6.3 ± 0.53
Met	1.74	1.55	1.68	1.83	2.8	2.5 ± 0.19	2.7 ± 0.25	2.9 ± 0.27
Ile	2.17	2.24	2.36	1.55	3.5	3.6 ± 0.34	3.8 ± 0.23	2.5 ± 0.28
Leu	15.50	15.10	14.46	14.52	25.0	24.2 ± 0.42	23.3 ± 0.56	23.4 ± 0.60
Tyr	3.11	2.55	2.73	3.75	5.0	4.1 ± 0.38	4.4 ± 0.35	6.0 ± 0.52
Phe	4.53	4.78	4.35	4.30	7.3	7.7 ± 0.30	7.0 ± 0.15	6.9 ± 0.21
His	3.59	4.03	4.28	3.36	5.8	6.5 ± 0.33	6.9 ± 0.28	5.4 ± 0.32
Lys	5.42	5.46	6.69	6.47	8.7	8.8 ± 0.28	10.4 ± 0.36	10.4 ± 0.36
Arg	5.90	5.59	5.78	5.21	9.5	9.0 ± 0.43	9.3 ± 0.41	8.4 ± 0.35
Trp	1.31	1.31	1.37	1.37	2.1	2.1 ± 0.14	2.2 ± 0.17	2.2 ± 0.15

to cross-react with ferritin from other species, including man (Mazur & Shorr, 1948). Although homologous ferritins (i.e. those from the same species) show reactions of identity, a reaction of partial identity only was seen between heterologous ferritins, in agreement with observations of Richter (1967) and Han *et al.* (1968). The latter reaction is expected in view of the known cross-reactivity of the serum (Mazur & Shorr, 1948) and, since it implies the presence of common antigenic determinants, is in accord with the finding (below) that there are a number of tryptic peptides common to human and horse ferritins.

However, the identical reaction of homologous ferritins does not necessarily imply antigenic or structural identity, since some proteins appearing to be similar by Ouchterlony immunodiffusion can be distinguished by more sophisticated immunological techniques such as micro-complement fixation (Levine, 1967).

Molecular-weight and sedimentation studies

Table 1 presents the results of determination of the polypeptide molecular weights and of the sedimenta-

tion coefficients of the four apoferritins. Horse spleen apoferritin is composed of 24 chemically identical subunits (Crichton, 1972a). The oligomer has a molecular weight of 443 000 (R. R. Crichton, R. Eason, A. Barclay & C. F. A. Bryce, unpublished work) whereas the subunit molecular weight is 18 500 (Crichton & Bryce, 1970; Bryce & Crichton, 1971a). There is good agreement between the subunit molecular weights found by the two methods used, and in all cases the subunit molecular weights are closely similar to those previously found for horse spleen apoferritin. It seems likely that all four proteins have an identical quaternary structure, since the sedimentation coefficients are all around 17S.

Amino acid composition

The amino acid compositions are given in Table 2. The agreement from preparation to preparation was good, as can be seen from the standard errors in the table. The proteins differ significantly from one another; clearly the two horse proteins are more similar to one another than to either of the two heterologous proteins, and the same applies to the two human proteins. For the human proteins the differences between

EXPLANATION OF PLATE 1(a)

Analysis of apoferritin at pH8.65 by discontinuous polyacrylamide-gel electrophoresis

The samples are as follows: (a) Horse spleen apoferritin; (b) horse liver apoferritin; (c) horse spleen apoferritin plus horse liver apoferritin; (d) human spleen apoferritin; (e) human liver apoferritin; (f) human spleen apoferritin plus human liver apoferritin; (g) horse spleen ferritin at concentration fivefold that used in gels (a)–(f). The bands at the bottom of the gels are an artifact of the buffer system.

EXPLANATION OF PLATE 1(b)

Immunodiffusion of human and horse apoferritin against rabbit anti-(horse spleen ferritin) antiserum

The central well in each case contained the antiserum. The samples in each well were as follows: 1, human liver apoferritin; 2, human spleen apoferritin; 3, horse liver apoferritin; 4, horse spleen apoferritin; 5, human liver apoferritin; 6, horse liver apoferritin; 7, horse spleen apoferritin; 8, human spleen apoferritin; 9, horse spleen ferritin; 10, horse spleen ferritin.

EXPLANATION OF PLATE 2

Tryptic peptide 'map' obtained with 1.5 mg of horse spleen apoferritin, digested with 30 μ g of trypsin as described in the text

Electrophoresis was at 6 V/cm for 16 h in pyridine–acetic acid – water (1:10:989, by vol.), pH 4.5; chromatography was in butanol – acetic acid – water (4:1:5, by vol.).

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Fig. 1. Tryptic peptide patterns of (a) horse spleen apoferritin, (b) horse liver apoferritin, (c) human spleen apoferritin and (d) human liver apoferritin

The numbering system is as described in the text. Only spots of comparably strong intensity were included (cf. Plate 2 and Fig. 1a).

these two apoferritins are greater than between the two equine proteins.

Tryptic 'maps'

Plate 2 shows a typical tryptic 'map' of horse spleen apoferritin. There are 25 major tryptic peptides, numbered (Crichton, 1969) as shown in Fig. 1(a). Fig. 1(b), 1(c) and 1(d) show the tryptic 'maps' of horse liver, human spleen and human liver apo-

ferritins, and the peptides that do not occur in horse spleen apoferritin are numbered from 26 on. Thus there are eight peptides present in horse liver apoferritin (26-33), seven in human spleen apoferritin (34-40) and five in human liver apoferritin that do not occur in the horse spleen protein.

Fig. 2 presents a composite peptide 'map' in which the peptide spots common to all four apoferritins are shown as solid areas, and the others identified as being common to any three proteins, to any two and

lastly those which are unique to any one apoferritin.

The proteins are uniquely different from one another (Fig. 1). Thus the horse proteins are much more similar than the two human proteins. About one-third of the tryptic peptides are common to the four apoferritins whereas the other two-thirds are present in at least one protein (Figs. 1 and 2). The agreement between the number of tryptic peptides and the number of lysine plus arginine residues for both horse proteins is poor, and in each case there are considerably more peptides than would be expected. Several of the tryptic peptides from the horse spleen protein (R. R. Crichton & M. D. P. Boyle, unpublished work) are the products of incomplete digestion, and, in one case, of non-tryptic digestion. The agreement between the number of tryptic peptides and the lysine plus arginine content is not always reliable and can often be misleading (Harris & Hindley, 1965).

These results are in marked contrast to those reported by Richter *et al.* (1970), who found only one tryptic peptide in digests of human liver apoferritin that was not present in horse spleen apoferritin, and conversely one peptide in the horse protein that was absent from the human protein. Hanging curtain electrophoresis at low voltage on 3MM paper may result in a better separation of the peptides than that achieved on thin layers of microcrystalline cellulose as used by Richter *et al.* (1970). Also, the conditions of tryptic digestion differed markedly, Richter *et al.* (1970) using heat-denatured apoferritin and digestion for 2 h at room temperature with an enzyme/protein ratio of 1:70 whereas we used native apoferritin and digestion for 16 h at 37°C with an enzyme/protein ratio of 1:20.

Apoferritin from haemochromatotic liver

Primary haemochromatosis is a genetically determined disorder of iron metabolism characterized by widespread accumulation of iron as ferritin and haemosiderin in both reticuloendothelial and parenchymal cells, with impairment especially of hepatic, pancreatic and cardiac structure and function (Polycove, 1966). The common findings, as in the above case, are a characteristic pigmentation, hepatic cirrhosis and diabetes mellitus. There is considerable evidence that haemosiderin, which normally comprises almost one-half of the total iron store (Kaldor, 1958; Shoden *et al.*, 1953), is derived from ferritin since it contains about 25–33% iron associated with denatured ferritin in a water-insoluble iron-protein complex (Sturgeon & Shoden, 1964). The atomic structure of the haemosiderin iron core cannot be distinguished from that of ferritin by X-ray-diffraction and Mössbauer spectroscopy (Fischbach *et al.*, 1971).

Primary haemochromatosis may be caused by an

Fig. 2. Composite peptide pattern for all four apoferritins

The spots in solid black represent peptides occurring in all four proteins, the stippled spots those that occur in any three of the proteins, the spots with diagonal lines those that occur in any two of the proteins and the outlined spots peptides unique to only one of the apoferritins.

alteration in the structure of apoferritin that results in abnormal absorption and storage of iron. Saadi (1962) showed by starch-gel electrophoresis, ultracentrifugal analysis and 'fingerprinting' of the tryptic peptides that ferritin from a patient with haemochromatosis was identical with normal liver ferritin. We have found the same result with apoferritin from haemochromatotic liver as judged by the criteria outlined above. This reinforces the view that whatever the genetic lesion in idiopathic haemochromatosis it does not appear to be related to an alteration in the structure of apoferritin.

Origins and significance of organ-specificity of ferritins

We have established that liver and spleen apoferritins from both human and horse have similar quaternary structures, most probably consisting of 24 identical polypeptide chains of molecular weight 18 500. They appear to have the same surface charge, since no differences can be detected in their electrophoretic mobilities either on polyacrylamide gels or on cellulose acetate, and the dissociated subunits cannot be separated at low pH. Differences can, however, be detected between species by immunodiffusion, and between all four proteins by amino acid analysis and by 'fingerprinting' of their tryptic peptides. It

thus seems that apoferritin is organ-specific. Our data are consistent with the existence of separate genes for the isoferritins, rather than other possibilities such as post-synthetic modification within each organ of a basic subunit structure.

The two polypeptide chains associated with the isoenzymes of lactate dehydrogenase are synthesized under separate genetic control (Cahn *et al.*, 1962). In addition the structural differences between lactate dehydrogenases have a definite functional significance (Dawson *et al.*, 1964). As pointed out above, it has been proposed that the two ferritins found in bone marrow serve different functions, namely an anabolic ferritin involved in erythropoiesis and a catabolic ferritin involved in storage of iron derived from the breakdown of haemoglobin (Gabuzda & Pearson, 1969). It has yet to be established whether the structural differences between the isoferritins of spleen and liver also reflect functional differences. However, they may be related to the respective roles of the liver and spleen in iron metabolism, namely storage and erythropoiesis.

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References

- Allfrey, C. P., Jr., Lynch, E. C. & Whitley, C. E. (1967) *J. Lab. Clin. Med.* **70**, 419–428.
- Björk, I. & Fish, W. W. (1971) *Biochemistry* **10**, 2844–2848.
- Boccu, E., Veronese, F. M., Fontana, A. & Benassi, C. A. (1970) *Eur. J. Biochem.* **13**, 188–192.
- Braun, V., Crichton, R. R. & Braunitzer, G. (1968) *Hoppe-Seyler's Z. Physiol. Chem.* **349**, 197–210.
- Bryce, C. F. A. & Crichton, R. R. (1971a) *J. Biol. Chem.* **246**, 4198–4205.
- Bryce, C. F. A. & Crichton, R. R. (1971b) *J. Chromatogr.* **63**, 267–280.
- Bungert, H. J., Leonhardt, H. & Macha, H. N. (1971) *Clin. Chim. Acta* **32**, 385–389.
- Cahn, R., Kaplan, N. O., Levine, L. & Zwilling, E. (1962) *Science* **136**, 962–969.
- Crichton, R. R. (1969) *Biochim. Biophys. Acta* **194**, 34–42.
- Crichton, R. R. (1971) *N. Engl. J. Med.* **284**, 1413–1422.
- Crichton, R. R. (1972a) *Biochem. J.* **126**, 761–764.
- Crichton, R. R. (1972b) *Angew. Chem.* in the press.
- Crichton, R. R. & Bryce, C. F. A. (1970) *FEBS Lett.* **6**, 121–124.
- Crichton, R. R., Millar, J. A. & Cumming, R. L. C. (1970) *Biochem. J.* **117**, 35p.
- Dawson, D. M., Goodfried, T. L. & Kaplan, N. O. (1964) *Science* **143**, 929–933.
- Edelhoch, H. (1967) *Biochemistry* **6**, 1948–1954.
- Fischbach, F. A., Gregory, D. W., Harrison, P. M., Hoy, T. G. & Williams, J. M. (1971) *J. Ultrastruct. Res.* **37**, 495–503.
- Gabuzda, T. G. & Gardner, F. H. (1967) *Blood* **29**, 770–779.
- Gabuzda, T. G. & Pearson, J. (1969) *Biochim. Biophys. Acta* **194**, 50–54.
- Granick, S. (1943) *J. Biol. Chem.* **149**, 157–167.
- Granick, S. & Michaelis, L. (1943) *J. Biol. Chem.* **147**, 91–97.
- Han, S. S., Han, I. H. & Johnson, A. G. (1968) *Exp. Haematol.* **17**, 17–20.
- Harris, J. I. & Hindley, J. (1965) *J. Mol. Biol.* **13**, 894–913.
- Harrison, P. M. (1959) *J. Mol. Biol.* **1**, 69–80.
- Harrison, P. M. & Gregory, D. W. (1965) *J. Mol. Biol.* **14**, 626–629.
- Harrison, P. M. & Gregory, D. W. (1968) *Nature (London)* **220**, 578–580.
- Hirs, C. H. W. (1956) *J. Biol. Chem.* **219**, 611–621.
- Kaldor, I. (1958) *Aust. J. Exp. Biol. Med. Sci.* **36**, 173–176.
- Kopp, R., Vogt, A. & Sund, H. (1966) *Abh. Deut. Akad. Wiss. Berlin, Kl. Med.* **223–232**.
- Laufberger, V. (1937) *Bull. Soc. Chim. Biol.* **19**, 1575–1582.
- Lee, J. C. K. & Richter, G. W. (1971) *Comp. Biochem. Physiol.* **39B**, 325–333.
- Levine, L. (1967) in *Handbook of Experimental Immunology* (Weir, D. M., ed.), pp. 707–719, Blackwell, Oxford.
- Linder-Horowitz, M., Reuttinger, R. T. & Munro, H. N. (1970) *Biochim. Biophys. Acta* **200**, 442–448.
- Mazur, A. & Shorr, E. (1948) *J. Biol. Chem.* **176**, 771–784.
- Mazur, A. & Shorr, E. (1950) *J. Biol. Chem.* **182**, 607–627.
- Ouchterlony, O. (1948) *Ark. Kemi Mineral. Geol.* **26B**, 1–22.
- Penders, T. J., De Rooij-Dijk, H. H. & Leijnse, B. (1968) *Biochim. Biophys. Acta* **168**, 588–590.
- Pollycove, M. (1966) *Metab. Basis Inherited Dis.* 2nd edn., 780–810.
- Richter, G. W. (1967) *Exp. Mol. Pathol.* **6**, 96–105.
- Richter, G. W. & Walker, G. F. (1967) *Biochemistry* **6**, 2871–2880.
- Richter, G. W., Moppert, G. A. & Lee, J. C. K. (1970) *Comp. Biochem. Physiol.* **32B**, 451–458.
- Rothen, A. (1944) *J. Biol. Chem.* **152**, 679–693.
- Saadi, R. (1962) *Rev. Fr. Etud. Clin. Biol.* **7**, 408–410.
- Schachmann, H. K. (1959) *Ultracentrifugation in Biochemistry*, pp. 75–90, Academic Press, New York.
- Shepherd, G. R. & Gurley, L. P. (1966) *Anal. Biochem.* **14**, 356–363.
- Shoden, A., Gabrio, B. W. & Finch, C. A. (1953) *J. Biol. Chem.* **204**, 823–830.
- Sturgeon, P. & Shoden, A. (1964) in *Iron Metabolism* (Gross, F., ed.), pp. 121–146, Springer-Verlag, Berlin.
- Suran, A. A. & Tarver, H. (1965) *Arch. Biochem. Biophys.* **111**, 399–406.