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THE EFFECT OF AMPHOLYTES ON FERRITIN ISOELECTRIC FOCUSSING PATTERNS

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SUMMARY

Ferritin was subjected to isoelectric focussing (IEF) on agarose gels containing different commercial carrier ampholytes. In two gels protein staining revealed banded patterns which differed from one another, while a third gel yielded zones rather than discrete bands, indicating that the bands may be artefacts.

The differences between banded patterns were studied by isolating bands from an IEF gel and refocussing these on gels containing either the original ampholyte or a different ampholyte preparation. Striking differences were noted.

Chromatofocussing of ferritin resulted in the elution of broad peaks between the same pH limits as indicated by IEF patterns.

INTRODUCTION

The microheterogeneity of ferritin was first demonstrated [1] by isoelectric focussing (IEF) more than a decade ago and this technique has subsequently been widely used for the characterisation of ferritin from various tissues. The multiple bands (isoferritins) in these IEF patterns differ according to the tissue from which the ferritin was isolated [2]. These differences may be explained by a model [3] in which the 24-subunit protein shell of ferritin contains different proportions of two distinct types of subunit, designated H and L, following the general formula L_nH_{24-n}, where n may assume values between 0 and 24.

While the validity of the IEF patterns of ferritin has been challenged [4] experimental evidence that the multiple bands may be artefacts has only recently been published [5]. This evidence is based on the appearance of a striated pattern which appeared with a modified iron stain on an acrylamide gel in which IEF had been carried out. The striations were independent of the presence of protein. When ferritin was present a high degree of correspondence

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was noted between protein bands staining for iron and dark striations in the gel.

In this paper we present evidence which shows that the IEF pattern of ferritin varies with the ampholyte used and thus represents a technique artefact. This is supported by the chromatographic characterisation of ferritin using chromatofocussing, a recently introduced chromatographic technique.

MATERIALS AND METHODS

Ferritin was isolated from human heart and liver, obtained post mortem, as previously described [6]. Samples of ferritin (10—15 μg) were subjected to isoelectric focussing in thin layers (80 mm × 115 mm × 1 mm) of 1% agarose gel (Pharmacia, Uppsala, Sweden, Agarose IEF) containing 12% sorbitol (Merck, Darmstadt, G.F.R., extra pure for microbiology) and 2.5% ampholytes of one of the following types: Ampholine pH 4—6 (LKB, Stockholm, Sweden, Batch No 44); Pharmalyte pH 4—6.5 (Pharmacia, Lot EL 13994); Bio-Lyte pH 4—6 (Bio-Rad Labs., Richmond, CA, U.S.A., Lot No. 21211). Calibration proteins (Pharmacia, low p*I* calibration kit) were run on each gel in addition to the ferritin samples.

Focussing was carried out on an LKB Multiphor 2113 apparatus and constant power (6 W per gel) was supplied by an LKB 2103 power supply. Coolant (10°C) was circulated through the Multiphor apparatus by a Lauda refrigerator/pump. The minimum duration of each run was 2 h during which time the voltage had risen to an essentially constant value. In one trial samples were applied at both the anodal and cathodal ends of a gel containing Pharmalyte carrier ampholytes, and in a further trial the concentration of these ampholytes was increased to a level of 5%. At the end of each run the gels were fixed (10% trichloroacetic acid, 5% sulphosalicylic acid), washed and stained with Coomassie blue R250.

Refocussing of heart ferritin

Semi-preparative scale IEF of heart ferritin was carried out on a slab of agarose containing Biolyte as described above. At the end of the focussing run, portions of the gel containing prominent bands were excised and the ferritin contained in those gel slices was concentrated and freed of ampholyte in an electrophoretic concentrating device [7]. The ferritin fractions thus isolated were refocussed on gels containing Ampholine and Bio-Lyte, respectively. The gels were fixed and stained as before and scanned at 570 nm in a Varian 635 spectrophotometer.

Chromatofocussing

Liver and heart ferritin were chromatofocussed on PBE 94 exchanger with Polybuffer 74 (Pharmacia) according to the methods prescribed by the manufacturers. A glass chromatography column (20 × 0.9 cm) was packed with PBE 94 exchanger and equilibrated with 0.025 *M* histidine·HCl buffer, pH 6.2. Liver or heart ferritin which had been dialysed against this buffer was applied to the column and eluted with Polybuffer 74, diluted with boiled distilled water, and adjusted to pH 4 with hydrochloric acid. The final dilu-

tion was 1:8. All buffers were degassed before use. The column effluent was monitored at 280 nm and the pH of each 3-ml fraction was measured.

RESULTS

Analytical IEF

In each of the three gels, as shown in Fig. 1, the calibration proteins (in order from the cathode: bovine carbonic anhydrase A, p*I* 5.85; β-lactoglobulin A, p*I* 5.20; soybean trypsin inhibitor, p*I* 4.55) focussed into sharp bands. In the gels containing LKB Ampholine (Fig. 1a) and Bio-Lyte (Fig. 1c), respectively, heart ferritin focussed into discrete bands between pH 4.6 and 5.6, while liver ferritin focussed in the range pH 5.0—5.6. The focussing patterns were however different. By contrast, no discrete bands could be seen for either heart or liver ferritin in the gel containing Pharmalyte although the marker proteins focussed at the correct p*I* values (Fig. 1b). However, the pH limits of the zones formed by heart and liver ferritins were similar to the pH limits found for these ferritins on gels with either LKB Ampholine or Bio-Lyte.

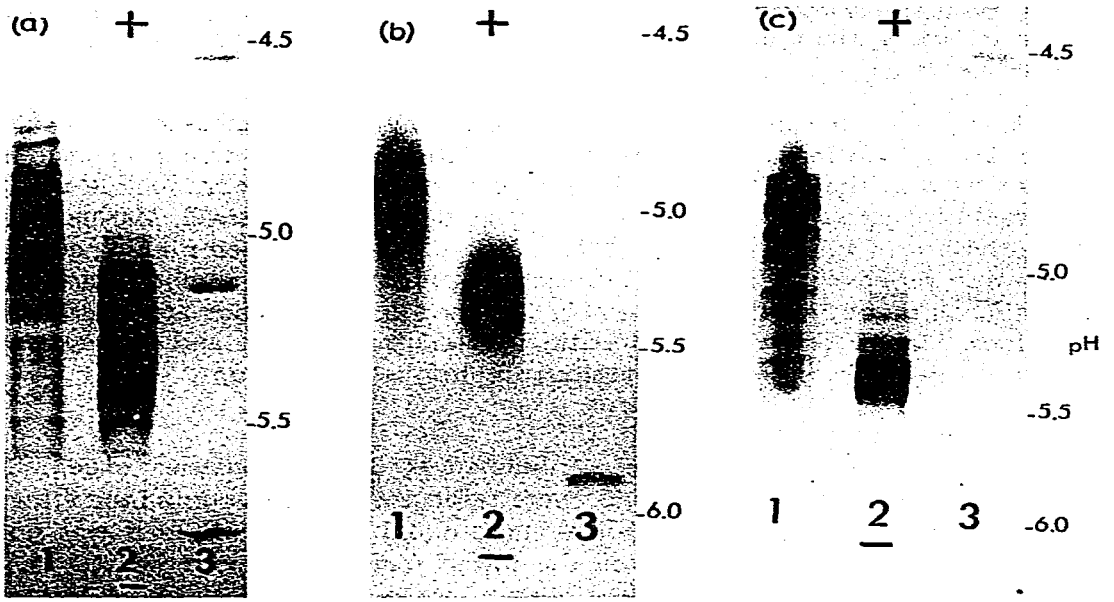

Fig. 1. IEF patterns in gels containing (a) LKB Ampholine ampholyte; (b) Pharmalyte ampholyte; (c) Bio-Lyte ampholyte. Channels 1 were loaded with heart ferritin, channels 2 with liver ferritin and channels 3 with calibration proteins (see text).

Fig. 2 demonstrates that when ferritin samples are applied at both anodal and cathodal ends of a Pharmalyte-containing gel and focussed as described above, the equilibrium position of either liver or heart ferritin is independent of point of application, indicating that equilibrium is attained under the focussing conditions described and that ferritin is not restricted by the gel matrix.

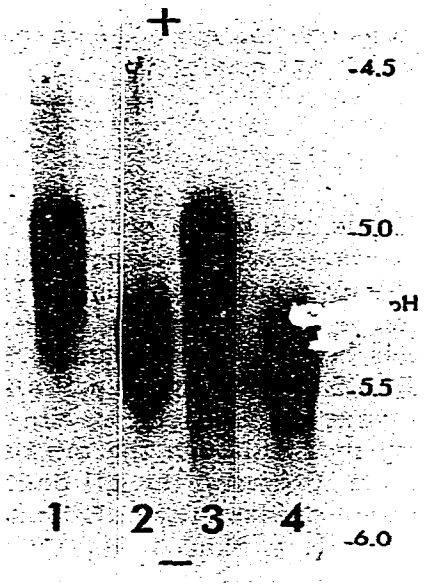

Fig. 2. Demonstration of focussing equilibrium. Samples of heart and liver ferritin were applied to both anodal and cathodal ends of a gel containing Pharmalyte and focussed as described in the text.

A trial in which the concentration of ampholyte was increased to 5% did not influence the ferritin pattern and the differences in focussing behaviour of ferritin with the different ampholyte preparations were reproducible when an alternative agarose of electrofocussing grade was used (Isogel, Marine Colloids Division of F.M.C., Marcus Hooke, PA, U.S.A.) and when running conditions were varied with regard to duration of run, and power applied. In these runs the calibration proteins invariably focussed as sharp bands at the correct p*I* values. Similar behaviour of ferritin isolated from spleen and kidney was observed when focussed with the various ampholyte preparations.

Refocussing of heart ferritin

When fractions of heart ferritin, obtained by semi-preparative scale IEF on agarose gels containing Bio-Lyte, were refocussed on gels containing either Bio-Lyte or Ampholine striking differences could be seen. While it was not possible to isolate a single band in the initial focussing step, it will be seen that in general a single major band with minor contaminants was isolated as revealed by refocussing on Bio-Lyte agarose gels. However, the same fraction on Ampholine-containing gels could show either greater complexity (Fig. 3a and b) or a reduced number of bands (Fig. 3c, d).

Chromatofocussing

Liver ferritin was resolved into two poorly differentiated peaks by this technique (Fig. 4a) while no distinct peaks are to be seen in the elution pattern of heart ferritin (Fig. 4b). However, it will be seen that both proteins were eluted over a wide pH range which corresponds to the characteristic range of p*I* values of the two ferritin types.

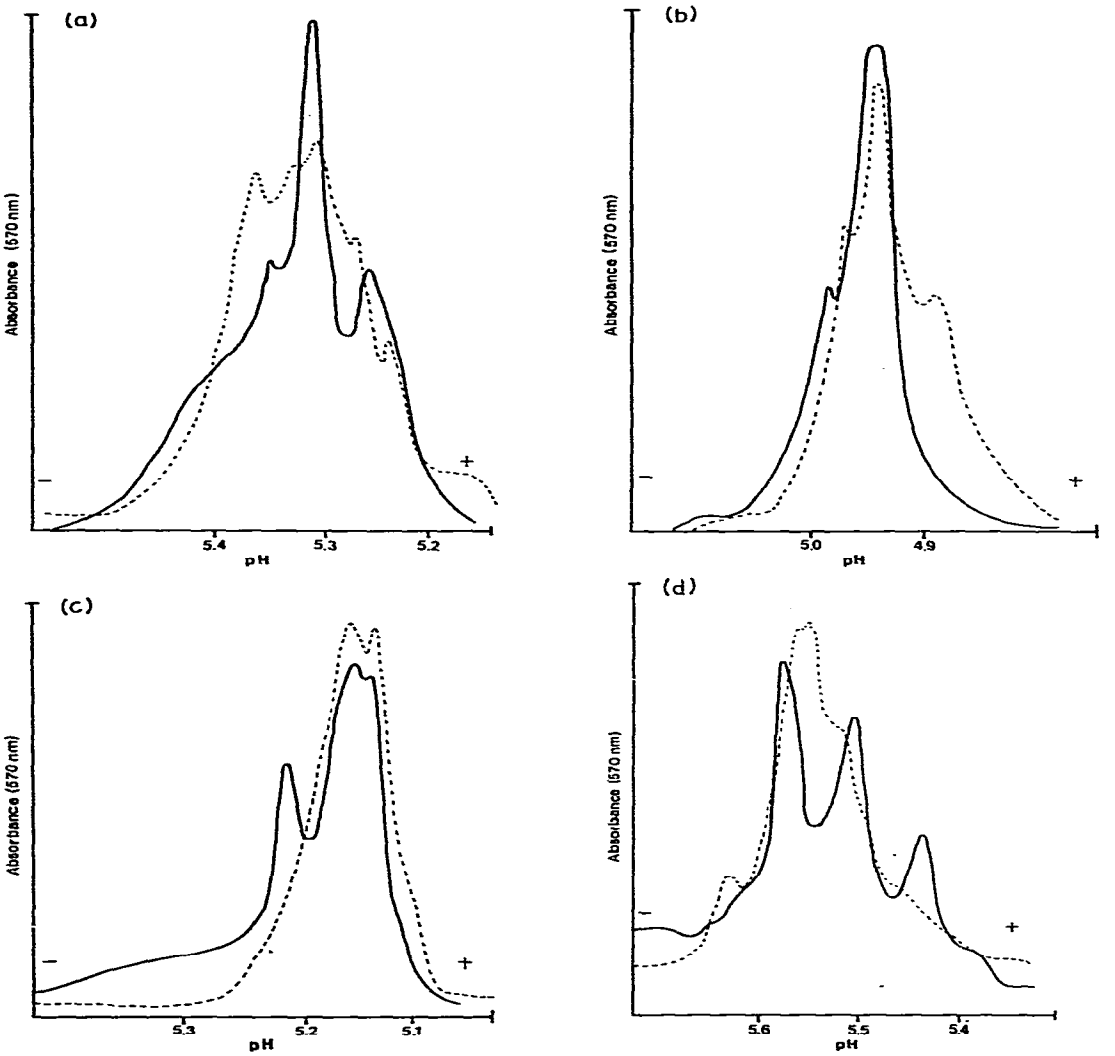

Fig. 3. Densitometric scans of IEF patterns of heart ferritin fractions obtained from a Bio-Lyte IEF separation refocussed on gels containing either Bio-Lyte (——) or Ampholine (- - - -). Scans are superimposed for comparison. (a) and (b) represent scans in which Ampholine gave a greater number of bands than Bio-Lyte; (c) and (d) represent less complex patterns than Bio-Lyte.

DISCUSSION

The evidence presented here clearly demonstrates that the IEF profile of ferritin is a product of the ampholyte preparation. Whereas the patterns seen with LKB Ampholine resemble those previously reported [2], the Pharmalyte ampholytes gave a picture which shows no discrete banding at all, merely zones or areas of localisation between pH limits which are common to all three ampholytes. The fact that marker proteins focussed as discrete sharp bands, and the demonstration that focussing equilibrium was achieved and that the movement of ferritin was not impeded by the agarose gel used, counter

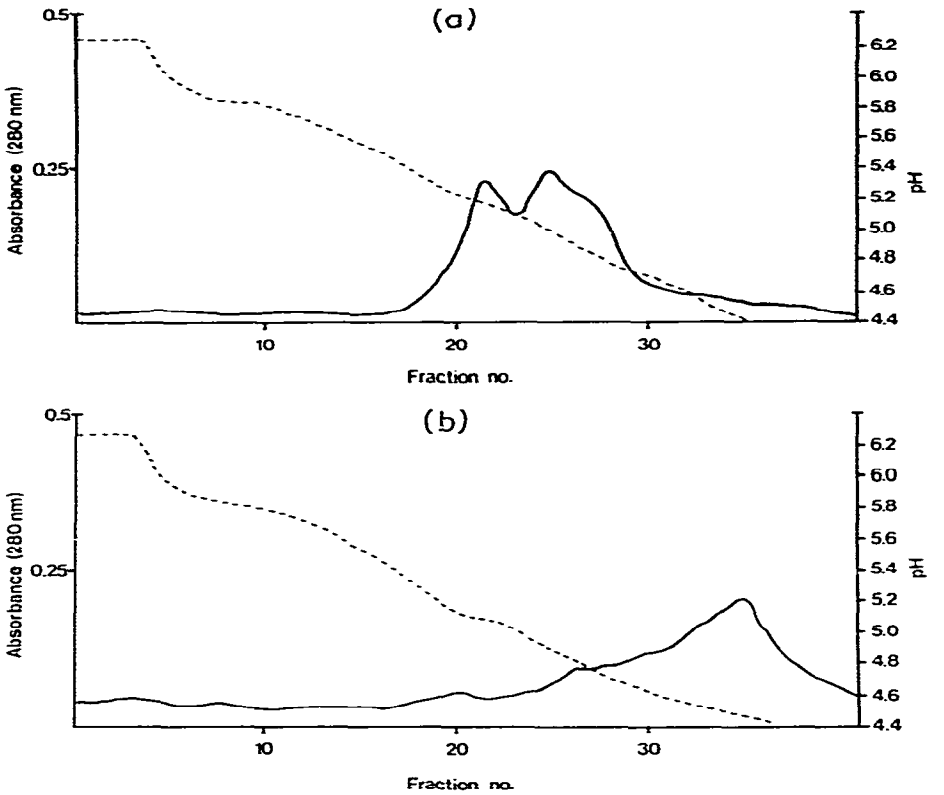

Fig. 4. Elution patterns obtained on chromatofocussing (a) liver ferritin and (b) heart ferritin. ——, Absorbance at 280 nm; — — —, pH.

the interpretation of these zones as technique failure. Ampholytes also apparently differ by batch preparation: with the batch preparation of Pharmalyte used in this study no discrete bands were formed, while in the Pharmalyte preparation used by other workers [5] focussing into discrete bands was observed.

The fact that distinctly different patterns resulted from refocussing ferritin fractions on an alternative ampholyte is strong support for the view that the discrete bands are artefacts rather than the characterisations of distinct biological entities.

The reason for the variation in ferritin IEF profiles with different ampholyte preparations is unknown. Although it has been suggested that micro-steps in the pH gradient are responsible [5], the present study goes no further than demonstrating that marker proteins of known p*I* value invariably focussed appropriately, irrespective of the ampholyte used. It follows from these observations that the IEF profile of ferritins has only relative relevance inasmuch as it should be related to a specific preparation of ampholytes.

While IEF depends on the establishment of a pH gradient by migration of amphoteric substances in an electric field, the recently introduced technique of chromatofocussing achieves the same effect independently of an electrical field. The elution pattern of liver and heart ferritin with this technique con-

firms that ferritin exhibits heterogeneity with regard to isoelectric point, but the lack of distinct peaks again suggests that the discrete bands seen in some IEF patterns are artefacts.

That ferritin exhibits heterogeneity with regard to p*I* is not in dispute. On IEF the ferritins localised between the pH limits characteristic for their tissues of origin, and eluted on chromatofocussing between approximately the same pH limits. For a given ampholyte the IEF patterns were consistently reproducible. Thus the heterogeneity of ferritin is confirmed, and may, perhaps, be greater than formerly thought. We therefore believe that IEF remains a useful technique for the characterisation of ferritin, provided it is used on a comparative basis and the interpretation of patterns is strictly qualified in terms of ampholyte used. Where possible, supporting physical characterisation by other methods is clearly indicated.

ACKNOWLEDGEMENT

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REFERENCES

1 Y. Mákino and K. Konno, J. Biochem. (Tokyo), 65 (1969) 471.
2 L.W. Powell, E. Alpert, J.W. Drysdale and K.J. Isselbacher, Brit. J. Haematol., 30 (1975) 47.
3 J.W. Drysdale, P. Arosio, T.G. Adelman, J.T. Hazard and D. Brooks, in R.R. Crichton (Editor), Proteins of Iron Storage and Transport in Biochemistry and Medicine, North-Holland, Amsterdam, 1975, p. 359.
4 S.M. Russell, P.M. Harrison and S. Shinjo, Brit. J. Haematol., 38 (1978) 296.
5 S. Shinjo and P.M. Harrison, FEBS Lett., 105 (1979) 353.
6 W.M. Deppe, S.M. Joubert and P. Naidoo, J. Clin. Path., 31 (1978) 872.
7 D. Stathakos, in P.G. Righetti (Editor), Progress in Isoelectric Focusing and Isotachophoresis, Elsevier/North-Holland Biomedical Press, Amsterdam, 1975, p. 65.