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CHARACTERIZATION OF A HETEROGENEOUS CHICKEN PLASMA PROTEIN, HEF, BY ANALYTICAL ISOTACHOPHORESIS IN AGAROSE GEL

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SUMMARY

Chicken plasma contains proteins that associate with immunoglobulin. One of these proteins enhances the titre of haemagglutinating alloantibodies, and it was therefore named HEF, haemagglutination enhancing factor. A purified HEF preparation mixed with ampholytes splits into four bands in analytical agarose isotachophoresis. One of the HEF bands can be separated from two others with beta-alanine as discrete spacer. The separated HEF populations differ in molecular size and in their ability to enhance agglutination.

INTRODUCTION

The titre of haemagglutinating chicken antibodies can be enhanced if normal chicken serum is added to the sensitized erythrocytes during titration[1]. This effect is due to an immunoglobulin binding protein, HEF (haemagglutination enhancing factor), present in normal chicken serum[2]. HEF is not an immunoglobulin, since it is found in equal amounts in serum from normal and bursectomized chickens[2]. HEF is not the first complement-factor, Clq, because chicken Clq has been purified, and shown to be very similar in structure and function to the mammalian homologue[4]. Chicken Clq was isolated from whole plasma by selective unstacking using discrete spacer and granulated gel in preparative flat-bed isotachophoresis (ITP)[3]. In these ITP experiments, HEF gave a different distribution pattern, dependent on which spacers were used. The preparative ITP experiments were performed with preparations containing immunoglobulin, and it was suggested that the observed pattern might reflect aggregates of HEF and immunoglobulin. To test this hypothesis, analytical ITP was performed on purified HEF in an agarose gel. This medium was chosen because the elution volume of HEF on gel filtration corresponds to a molecular weight higher than 500,000.

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MATERIALS AND METHODS

Protein samples

Chicken plasma was obtained from inbred lines of American White Leghorn. Citrated cock plasma with low lipoprotein content was preferred as starting material for purification of HEF[5]. In brief, the major part of fibronectin was removed by absorption on glutaraldehyde-fixed gelatine (Oxoid, London, U.K.) before precipitation with polyethylene glycol 6000 (Fluka, Buchs, Switzerland). The precipitate formed at 5% w/v was dissolved and chromatographed successively on DEAE-Sephacel, Heparin-Sepharose CL 6B and gelatine coupled to cyanogen bromide-activated Sepharose 4B (Pharmacia, Uppsala, Sweden). The final preparation was dialysed against 146 m*M* sodium chloride, 5 m*M* sodium barbitone (pH 7.6) and stored at −80°C. The protein concentration was 0.74 mg/ml, as determined by the method of Lowry *et al.*[6].

Crossed immunoelectrophoresis (CIE) and crossed immunoisotachophoresis (CIITP)

CIE with zone electrophoresis as the first dimension was performed as described by Axelsen *et al.*[7]. CIITP[8] was performed with ITP agarose gels in the first dimension. The ITP gels were very carefully transferred to 10 × 10 cm glass plates and sealed with agarose type HSA (Litex, Glostrup, Denmark). The gels were made *ca.* 2 mm thick with the use of a brass bar as barrier. The brass bar was cut free, and antibody-containing gel was cast[7]. Electrophoresis in the second dimension was performed at 4 V/cm for 30 min, followed by 2 V/cm overnight. The antisera used were produced by immunizing rabbits with either whole chicken serum or purified HEF.

Polyacrylamide gel electrophoresis (PAGE)

Gradient PAGE was performed in 2–16% gels, PAA 2/16 (Pharmacia), preequilibrated for 45 min at 120 V in an electrophoresis buffer: 0.09 *M* Tris [tris(hydroxymethyl)aminomethane], 0.08 *M* boric acid, 0.0025 *M* Na_2EDTA (pH 8.4).

After sample application the gel was electrophorised at 70 V for 45 min followed by 120 V for 21 h. Gels were fixed for 30 min in 10% sulphosalicylic acid, stained with 0.1% Coomassie Brilliant Blue R-250 in 8% acetic acid, 25% ethanol, and destained in acetic acid–ethanol.

Isoelectric focusing (IEF)

IEF was run on a LKB ampholine PAG plate, pH range 3.5–9.5 (LKB, Bromma, Sweden) with 1 *M* phosphoric acid at the anode and 1 *M* sodium hydroxide at the cathode. Samples were applied on filter paper 1 cm from the cathode. Focusing was carried out with 25-W constant power. The filter papers were removed from the gel after 0.5 h, and electrophoresis was continued for 1.5 h. After focusing, one strip of the gel was cut in pieces of 1 cm and eluted with water for pH measurement. The rest of the gel was fixed in 3.5% sulphosalicylic acid, 11.5% trichloric acid for 30 min, and stained and destained as described for gradient PAGE.

Isotachophoresis

Electrophoresis was performed on a flat-bed apparatus, FBE 3000 (Pharmacia) with a 25 × 25 cm cooling plate thermostated at 4°C. The buffer system consisted of 0.060 *M* hydrochloric acid, 0.182 *M* Tris (pH 8.3) as anode buffer and 0.230 *M* EACA (epsilon-aminocaproic acid), 0.012 *M* Tris (pH 8.9) as cathode buffer[9]. The separation gel was prepared by casting 15 ml of 0.8% agarose-type HSIF (Litex) dissolved in anode buffer on a pre-heated levelled glass plate (25.5 × 6.0 cm), equipped with a 1-cm wide, 1.5-mm thick silicone rubber gasket, cut in one piece and clamped along the edges of the glass plate as casting frame. After gelation the gasket and adhering gel were cut away with a scalpel, leaving a separation gel of 23.5 × 4.0 cm, *ca.* 1.5 mm thick. The glass plate with gel was placed on the cooling plate with glycerol in between. Ten filter papers (Whatman No. 1) soaked in the respective electrode buffers were used as wicks, and a glass plate, covering the gel area, was placed on top of the wicks. A pre-run was carried out at 1 mA for 5 h to get the borderline between the leading and the terminating electrolyte into the gel. With the buffer system used a yellow line indicates this boundary. The sample was applied in the leading zone absorbed on filter paper, 5 × 10 mm. From 50 μl up to 350 μl were soaked into as many filters as needed, and the filter papers placed on top of each other in either one or two rows. The application area did not exceed 1 × 2 cm. No pretreatment of sample was necessary. Ampholine (LKB), amino acids (Sigma) and one drop of bromphenol blue were added just before application. Electrophoresis was continued at 1 mA overnight (12–20 h), after which the application filter paper was removed. The current was gradually increased to 4–5 mA over a period of 9–11 h, and the electrophoresis was stopped, when the bromphenol blue front was 1 cm from the anode. The first 10 cm of the gel from the blue front was cut in half and the two equal parts further analysed. One part was transferred to another glass plate for immunoelectrophoresis, the counterpart of the ITP gel was fixed for 5 min in 30% methanol containing 3.5% sulphosalicylic acid and 11.5% trichloracetic acid, washed twice in 95% ethanol for 10-min periods, pressed, dried and stained as for immunoelectrophoresis.

Haemagglutination

One drop of a 2.5% suspension of chicken erythrocytes, sensitized with a subagglutinating amount of allo-antibody and one drop of the fraction to be tested, were mixed on a glass plate. Agglutination was read macroscopically after 10 min[2].

RESULTS

Characterization of HEF

CIE. The purity of the HEF preparation was tested in CIE. Only one precipitate occurs against rabbit-anti-chicken serum (Fig. 1).

IEF. Samples were dialysed for 60 min at room temperature against 1% glycine before IEF. Prolonged dialysis increased precipitation in the dialysis bag, and precipitated protein was usually seen at the application site. IEF resolved purified HEF into three weak distorted bands and a diffuse zone in the pH range 6.05–6.15 (Fig. 2).

ITP. CIITP of chicken plasma shows that the plasma proteins are separated

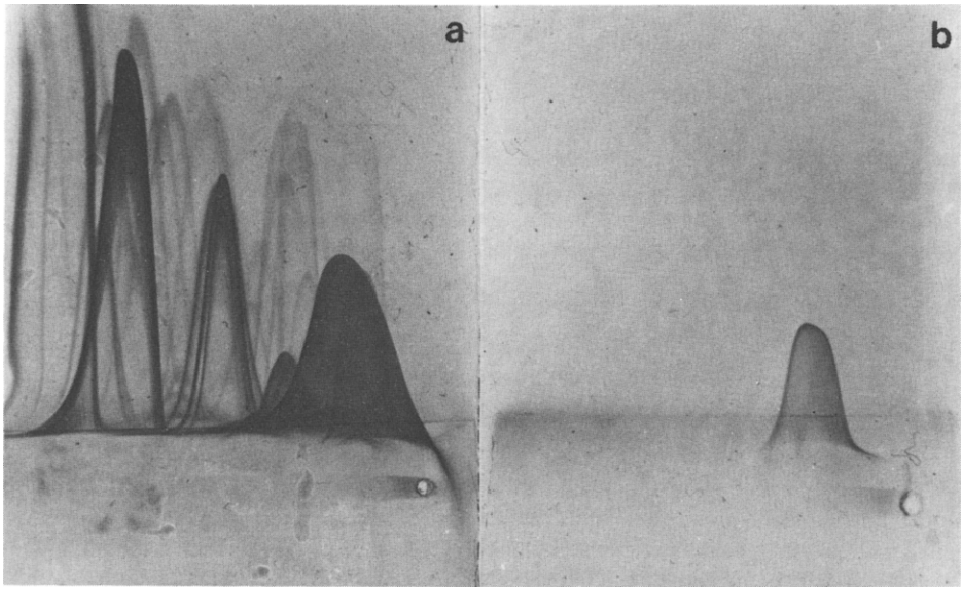

Fig. 1. CIE with 2 μl of chicken plasma (a) and 10 μl of purified HEF (b) as antigen. Rabbit-anti-chicken serum (200 μl) was included in the second dimension gels. The anode is to the left in the first dimension, and at the top in the second dimension.

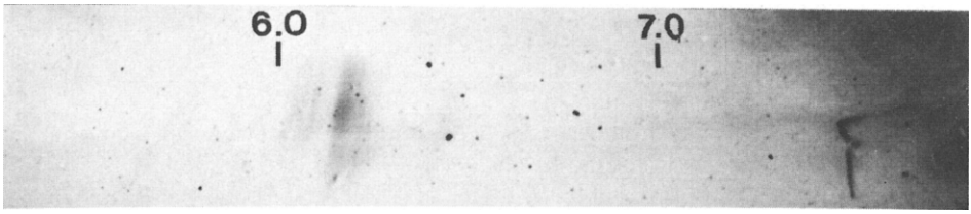

Fig. 2. IEF of 40 μl of purified HEF in pH range 3.5–9.5.

into zones, according to their mobility in the pH gradient of Ampholine and beta-alanine spacing, and that HEF migrates in the gamma-globulin region (Fig. 3).

ITP of the purified HEF with different spacings are shown on Figs. 4–7. Both the immunoelectrophoresis and the protein stain of the ITP gels are shown. With Ampholine pH 8–9.5 as spacer (Fig. 4), four closely spaced bands are seen, when the ITP gel is stained for protein. The corresponding immunoprecipitate formed by rabbit-anti-chicken serum covers the area of the four bands and forms a continuous line for antigen identity. To analyse the four bands further, ITP was performed with discrete spacers (those that had previously distributed HEF in different positions in preparative ITP of partly purified material[3]). Comparison of the ITP patterns (Figs. 5–7) shows that the molecular HEF species are located in mixed zones. This confirms earlier observations[3] and indicates that the distribution is a property of HEF and is independent of the presence of immunoglobulin.

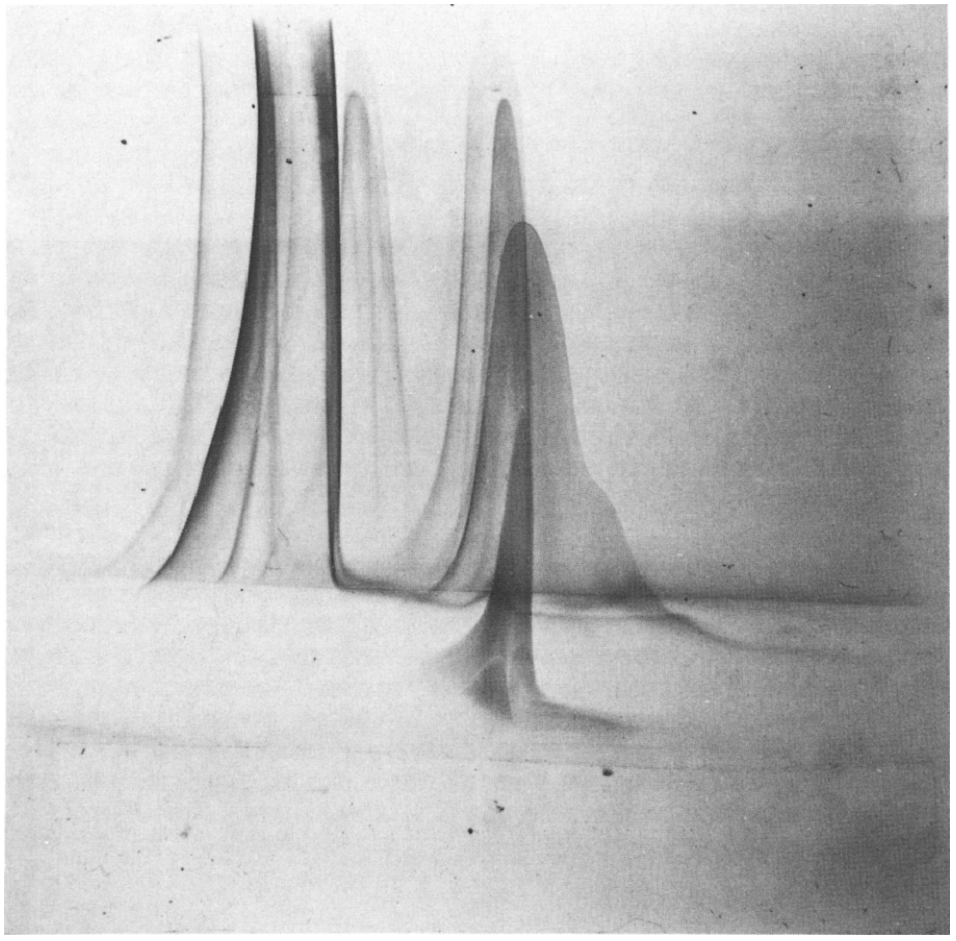

Fig. 3. CIITP of 15 μl of cock plasma mixed with 30 μl of Ampholine (pH 8–9.5) and 30 μl of beta-alanine (100 mg/ml). The ITP strip was combined with a gel containing initially 100 μl of monospecific rabbit-anti-HEF serum (1.5 cm gel) and in the rest of the gel (6.5 cm) 1 ml of rabbit-anti-chicken serum. The anode is to the left in the first dimension, and at the top in the second dimension.

Characterization of ITP bands

ITP with beta-alanine spacing was repeated with larger amounts of protein and increased spacer concentration. The extra spacer increased the distance from the blue front to the terminating zone boundary by 5 cm. Bands were seen directly in the gel, and it was not necessary to stain part of the gel for a reference pattern. Three pieces, A, B and C, were cut out of the gel, as indicated on Fig 7, and eluted by diffusion overnight in 246 m*M* sodium chloride, 5 m*M* sodium barbitone (pH 7.6).

For CIE, eluates were run in parallel on the same first-dimension gel. After immunoelectrophoresis in the second dimension, the superimposed plates (Fig. 8c) show that A and B eluates run with different mobility. This was to be expected from ITP, but it may also explain why the immune precipitate of purified HEF is very broad (Fig. 1b), and on some plates even occurs as a double peak.

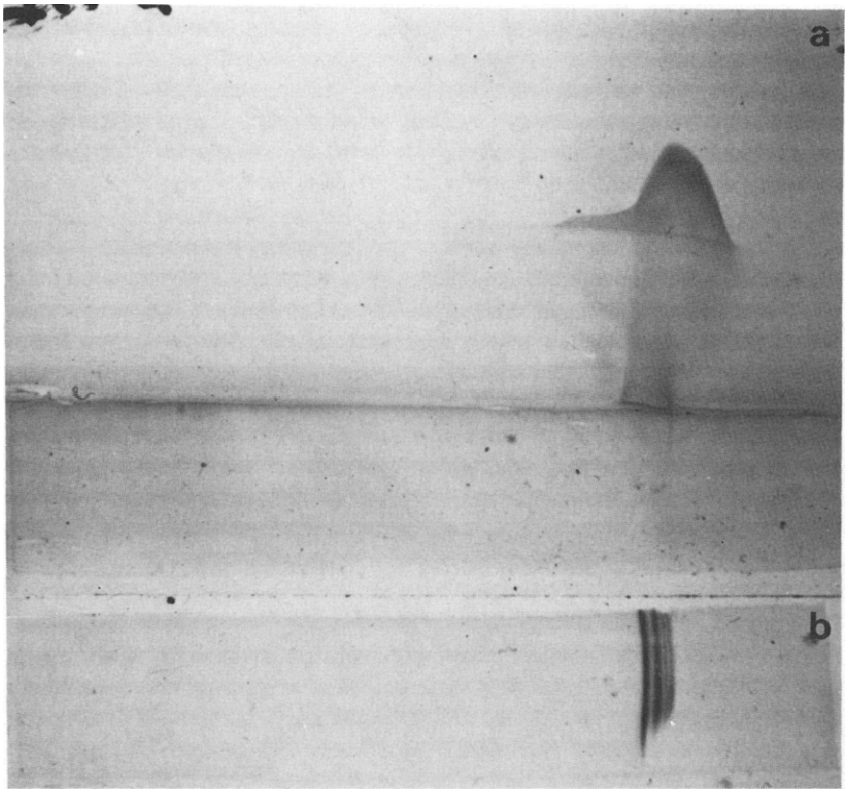

Fig. 4. CIITP (a) and total protein pattern (b) of 80 μl of purified HEF mixed with 30 μl of Ampholine (pH 8–9.5). The anode is to the left in the first dimension, and at the top in the second dimension.

The eluate from C did not react with rabbit-anti-chicken serum, nor did it show any bands after protein stain of gradient PAGE. It is probably a low-molecular weight contaminant from the affinity columns, or it might be an impurity from the buffers used.

The analysis of the A and B eluates on gradient PAGE is shown in Fig. 9. By comparison with the pattern of the purified HEF preparation, which was the sample applied in ITP (row a, Fig. 9), and estimating the molecular weight from the markers by linear extrapolation, it is seen that the gel piece A contains HEF protein corresponding to 930,000, while the B eluate is mainly higher molecular forms, 1,400,000 and 1,700,000. When the eluates were tested in haemagglutination, only B was able to cause agglutination.

DISCUSSION

ITP has a resolving power equivalent to IEF, but ITP can be performed at an ionic strength and at a pH where the protein of interest carries a net charge, and thus problems with precipitation are avoided. For characterization of macromolecules, a

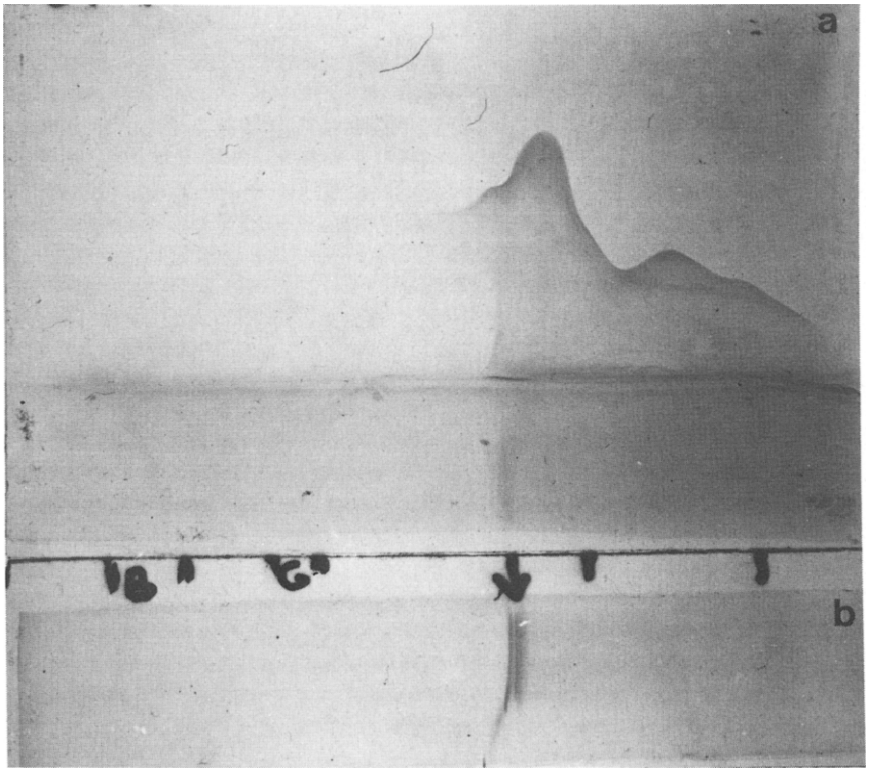

Fig. 5. CIITP (a) and total protein pattern (b) of 80 μl of purified HEF mixed with 30 μl of glycine (100 mg/ml). The anode is to the left in the first dimension, and at the top in the second dimension.

separation gel with non-sieving properties is essential, and this was obtained by the use of agarose. This medium has the advantage over polyacrylamide of being easily combined with immunoelectrophoresis[8,10] and of being preferable for the elution of protein after electrophoresis. The disadvantage of agarose is its low mechanical strength, which causes problems especially in the cathodic part of the gel at the start of electrophoresis. Owing to the low solubility of HEF, a buffer system of high ionic strength was chosen on purpose. With this buffer system, a current greater than 1 mA will cause an increased heat production, and the increased water evaporation tends to collapse the cathodic, terminating part of the gel. Because the main purpose of this study was to characterize a specific protein, the experimental conditions were not systematically optimized. One possibility, which was not tried, is to copolymerize agarose gel with acrylamide; this might even give an additional separation parameter[11]. Technical improvements, such as enhanced cooling, the prevention of evaporation, and the development of completely uncharged agarose, are needed before agarose-ITP could be a routine method.

In analytical agarose-ITP the purified HEF is resolved in four closely spaced bands when mixed with Ampholine. One might represent impurities in the reagents used or a minor contaminant from the purification procedure. When beta-alanine is

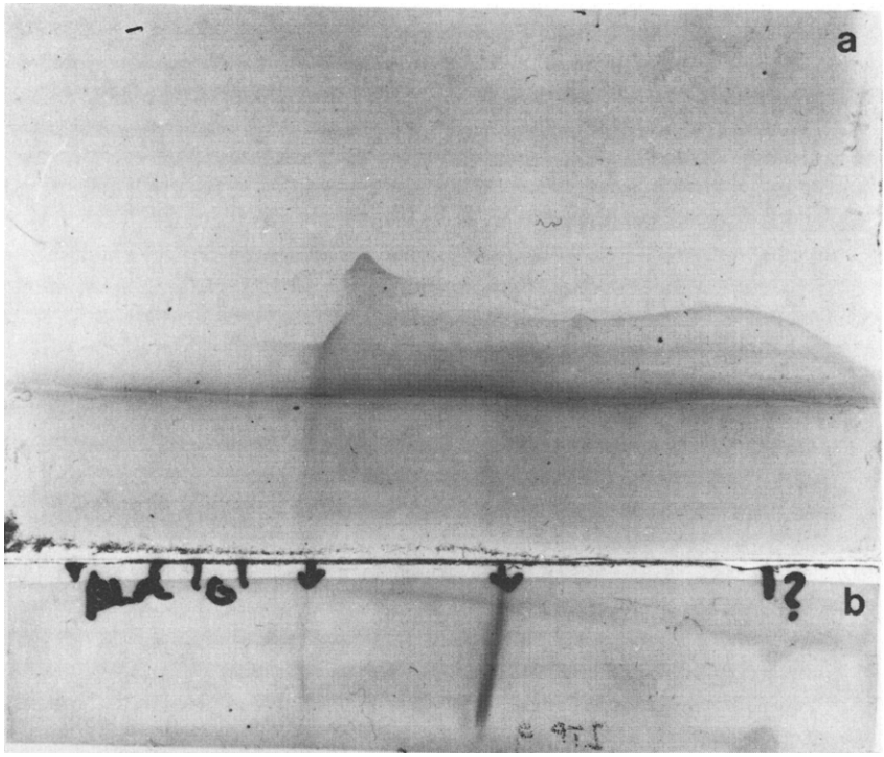

Fig. 6. CIITP (a) and total protein pattern (b) of 80 μl of purified HEF mixed with 30 μl of D-alanine (100 mg/ml). The anode is to the left in the first dimension, and at the top in the second dimension.

used as spacer, the most anodic HEF band can be separated from the two other HEF bands. The separated HEF populations are found to represent different polymeric forms.

The analytical ITP results thus confirm the heterogeneity observed earlier in preparative ITP. In addition they show that the aggregates are polymeric HEF rather than immunoglobulin-associated HEF. The formation of aggregates probably also accounts for the heterogeneity observed during purification, no matter which parameter the separation was based on: size, charge or carbohydrate differences[5], and they might also explain the enhanced effect in agglutination of frozen and thawed serum compared with fresh serum[1].

The HEF monomer is supposed to have a molecular weight of 500,000, based on gradient PAGE in 8 *M* urea[5]. This implies that the most anodic of the ITP bands is dimeric HEF, and that the agglutinating fraction consists of trimeric and tetrameric forms. That only highly polymeric forms enhance agglutination could be due to a trivial concentration difference, but it is tempting to speculate that only polymers higher than dimers have the geometrical arrangement that favours agglutination: in other words, that the distance between two erythrocytes requires more than two HEF molecules in extension to link two antibody molecules on different erythrocytes.

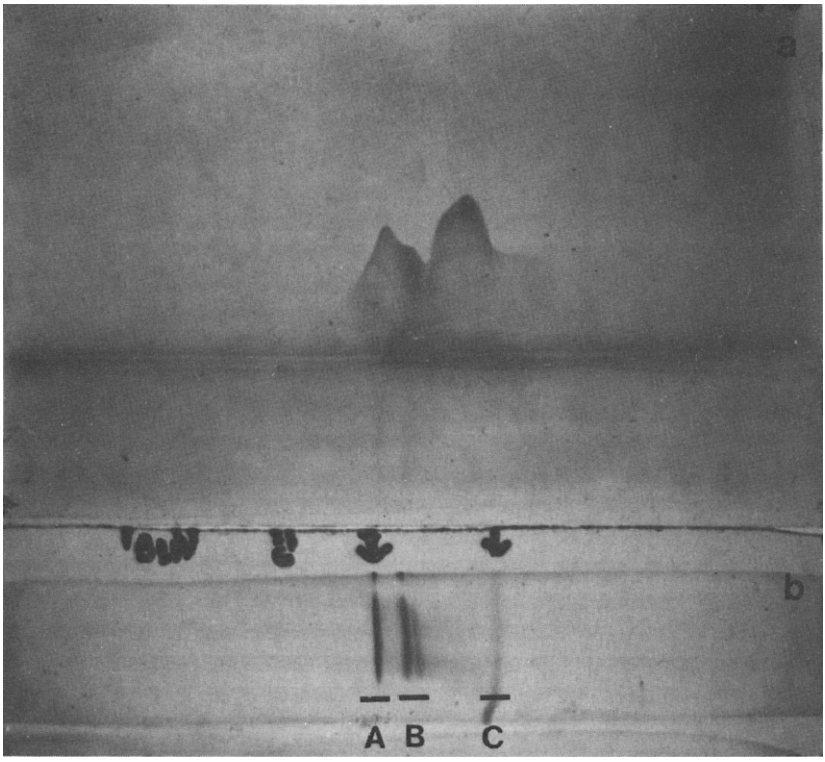

Fig. 7. CIITP (a) and total protein pattern (b) of 80 μl of purified HEF mixed with 30 μl of beta-alanine (100 mg/ml). The anode is to the left in the first dimension, and at the top in the second dimension.

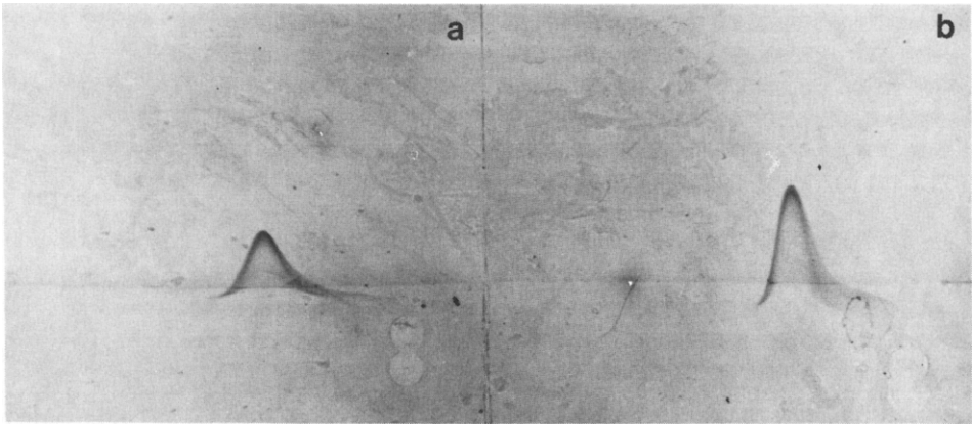

Fig. 8.

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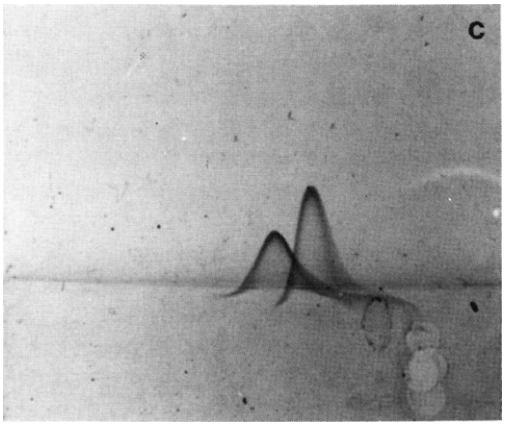

Fig. 8. CIE of eluates from ITP of 350 μl of HEF mixed with 10 mg of beta-alanine. The ITP gel pieces were excised as indicated in Fig. 7 and eluted overnight in 250 μl of 246 m*M* sodium chloride, 5 m*M* sodium barbitone (pH 7.6). Then, 25 μl of A-eluate (a) and 25 μl of B-eluate (b) were run on the same first-dimension gel. The second-dimension gels contained 150 μl of rabbit-anti-chicken serum; (c) represents (a) and (b) superimposed. The anode is to the left in the first dimension, and at the top in the second dimension.

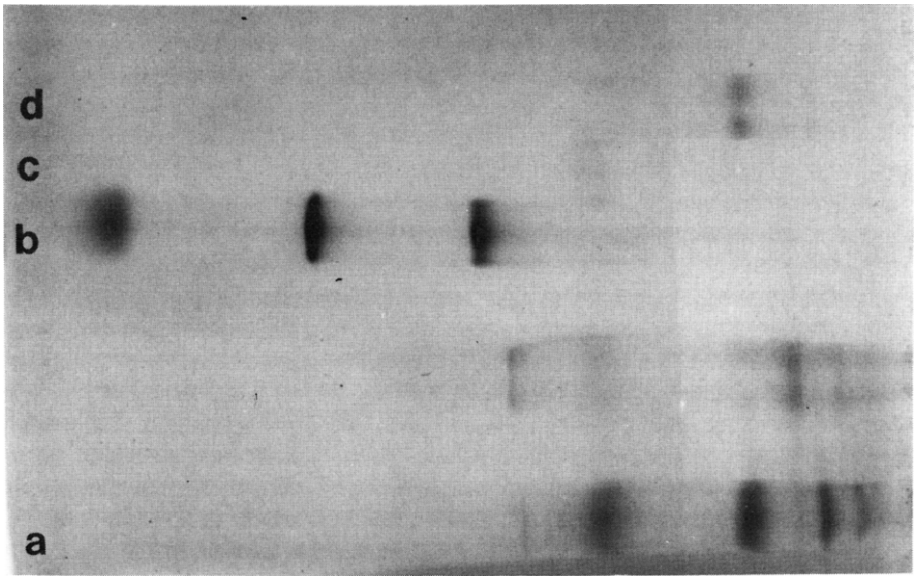

Fig. 9. Gradient PAGE, 2–16%, of the same eluates as analysed in Fig. 8: namely, 80 μl of A-eluate (c), 80 μl of B-eluate (d) and 40 μl of purified HEF (a). The markers (b) are thyroglobulin (669,000), ferritin (440,000) and catalase 232,000 (HMW Calibration Kit, Pharmacia). The anode is to the left.

Although this *in vitro* function might be the result of an artefact, produced either by freezing or by purification, it has been possible to use the haemagglutination test as a screening method for isolation of a single protein, to which no mammalian homologue has been found yet. Preliminary results[5] indicate that a human protein

of similar structure exists, and that the main target protein for its binding is not immunoglobulin. This protein should be identified, and its connection with the immune system examined.

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