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Heterogeneity of human haptoglobin *a* chains detected by two-dimensional gel electrophoresis

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Summary

The protein spots representing the haptoglobin α^{1F}, α^{1S} and α^{2} chains in two-dimensional gels of human plasma samples representative of the six common haptoglobin phenotypes were identified by comparing their position with those of purified haptoglobin and distinguished from other spots in the vicinity by comparison with plasma with undetectably low levels of haptoglobin. Silver staining indicated that the α^{1F} chain was represented by one spot in subtypes 1F-1F, 1F-1S and 1F-2, the α^{1S} chain by three spots in subtypes 1S-1S, 1F-1S and 1S-2 and the α^{2} chain by six spots in 1F-2, 1S-2 and 2-2.

1. Introduction

Plasma haptoglobin irreversibly binds haemoglobin released from erythrocytes and transports the resulting complex to the liver for degradation (Putnam, 1975). Human haptoglobin molecules consist of α and β polypeptide chains (Putnam, 1975). There are three forms of the α polypeptides. The two smaller α^{1F} (fast) and α^{1S} (slow) chains which are characterized by a lysine and a glutamic acid residue, respectively, at residue 53 (Bowman & Kurosky, 1982), are encoded by two alleles $Hp\alpha^{1S}$ and $Hp\alpha^{1F}$. The α^{2} chain which is almost twice the size of the α^{1} chain and has a lysine residue at residue 53 and a glutamic acid residue at residue 112 (Bowman & Kurosky, 1982), is encoded by the third common allele $Hp\alpha^{2FS}$ which is the product of a partial gene duplication probably resulting from an unequal crossover event in the chromosome of a heterozygote $Hp\alpha^{1F}/Hp\alpha^{1S}$ (Smithies, Connell & Dixon, 1962*b*). The three α chain allelic genes taken two at a time account for the presence of the six common haptoglobin phenotypes 1F-1F, 1S-1S, 1F-1S, 1F-2, 1S-2 and 2-2 (Smithies *et al.* 1962*b*). The genes encoding both the α and β chains are localized on chromosomes 16 (McGill *et al.* 1984).

Haptoglobin is resolved in 2D gels as four groups of spots corresponding to the α^{1F}, α^{1S}, α^{2} and β chains (Anderson & Anderson, 1977). The β chain can resolve into as many as 45 spots, the microheterogeneity being due to variable glycosylation, sialylation and proteolytic cleavage (Anderson & Anderson, 1979). In composite diagrams (Anderson & Anderson, 1977, 1984; Daufeldt & Harrison, 1984) the α^{1F} and α^{1S} chains are usually represented by one spot each and the α^{2} chains by four or five spots. Several unidentified spots of similar molecular weight migrate near the α^{1} and α^{2} spots and it cannot be ruled out that they represent modified forms of the α^{1} and α^{2} chains. Smithies *et al.* (1962*b*) predicted the existence of four types of $Hp\alpha^{2}$ alleles corresponding to the four possible chromosomal rearrangements resulting from crossing over within the haptoglobin locus in appropriate phenotype 2-1 heterozygotes and these may account for the multiple α^{2} spots. Teige, Olaisen & Pedersen (1985) reported that the products of two of the predicted $Hp\alpha^{2}$ alleles were represented by two different major Coomassie Blue-stained spots in 2D gels. However in some of their 2D gels additional spots of identical molecular weight were also detectable.

In the present investigation, Coomassie Blue staining and more sensitive silver staining were used to identify the spots representing the haptoglobin α^{1F}, α^{1S} and α^{2} chains in 2D gels of human plasma samples representative of the six common haptoglobin phenotypes.

2. Materials and methods

Blood samples were taken from male and female volunteers in S.E. Scotland and Manchester. The samples in heparinized or untreated tubes were incubated at room temperature for 2–4 h prior to centrifugation and removal of plasma or serum respectively which was aliquoted and stored at −70 °C. The haptoglobin phenotype was determined

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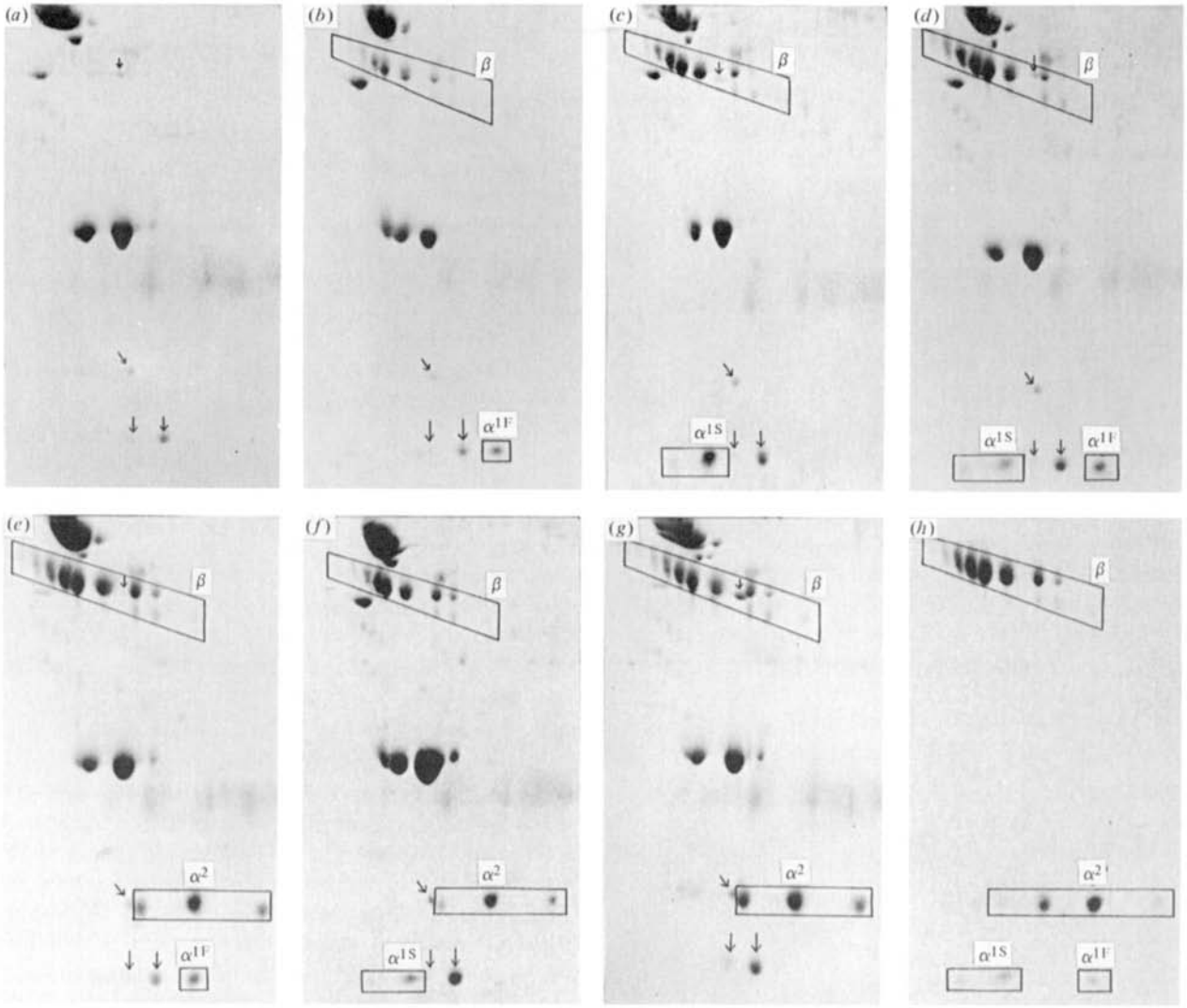

Fig. 1. 2D gels of human plasma proteins. (*a*) Plasma without haptoglobin. (*b–g*) Plasma samples with different haptoglobin phenotypes: (*b*) subtype 1F-1F; (*c*) subtype 1S-1S; (*d*) subtype 1F-1S; (*e*) subtype 1F-2; (*f*) subtype 1S-2; (*g*) phenotype 2-2; (*h*) purified human haptoglobin (Calbiochem) 10 μg. Gels were stained with Coomassie Brilliant Blue R. Abbreviations: β, haptoglobin β chains; α^{1F}, haptoglobin α^{1F} (fast) chain; α^{1S}, haptoglobin α^{1S} (slow) chain; α^2, haptoglobin α^2 chain. The commercial preparation of haptoglobin was purified from a mixture of common phenotypes and therefore contains both α^{1S} and α^{1F} chains in combination with α^2 chains. Stained with Coomassie Brilliant Blue R. The α^2 spots in Fig. 1 (e–g) correspond to the spots 3, 4 and 5 in Fig. 3. Arrows indicate proteins that are not related to haptoglobin. Acidic proteins to the left. Part of gel shown.

by mixing 4 μl plasma or serum with 8 μg haemoglobin A and fractionating the resulting complexes on non-denaturing 4–26% polyacrylamide gradient gels (Margolis & Kenrick, 1968) prior to staining with tetramethylbenzidine (see *Polyacrylamide Gel Electrophoresis, Laboratory Techniques*, Pharmacia Fine Chemicals, p. 55). The following samples were analysed: phenotype 1–1, 15 samples; phenotype 2–2, 7 samples; phenotype 2–1, 9 samples. Haptoglobin subtypes were determined by electrophoresis in formic acid-urea (Smithies *et al.* 1962*a*) using a 10% polyacrylamide slab gel.

Purified human haptoglobin (Calbiochem) was prepared from a mixture of the common phenotypes and was 98 and 99·3% pure according to immunological criteria and microzone electrophoresis, respectively. Contaminating traces of α_2 macroglobulin and $\alpha\beta$ glycoprotein would give subunits which have molecular weights greater than the haptoglobin β chain (Anderson & Anderson, 1977).

Plasma or serum was analysed by two-dimensional gel electrophoresis as previously described (O'Farrell, 1975; Anderson & Anderson, 1977; O'Farrell, Goodman & O'Farrell, 1977). 10 μl samples of plasma or serum or purified haptoglobin in physiological saline (Dulbecco B) were added to 40 μl 0·5% sodium dodecyl sulphate, 9·5 M urea, 0·5 mM dithiothreitol, 0·2% Ampholine (pH range 3–10) and incubated at 25 °C for 10 min prior to addition of 40 μl 9·5 M urea, 2% Nonidet P-40, 2% Ampholine (comprised of 1·6% pH range 5–7, 0·4% pH range 3–10). First-dimensional isoelectric focusing of this mixture was carried out as described by O'Farrell (1975). Proteins separated by isoelectric focusing were run in the second dimension

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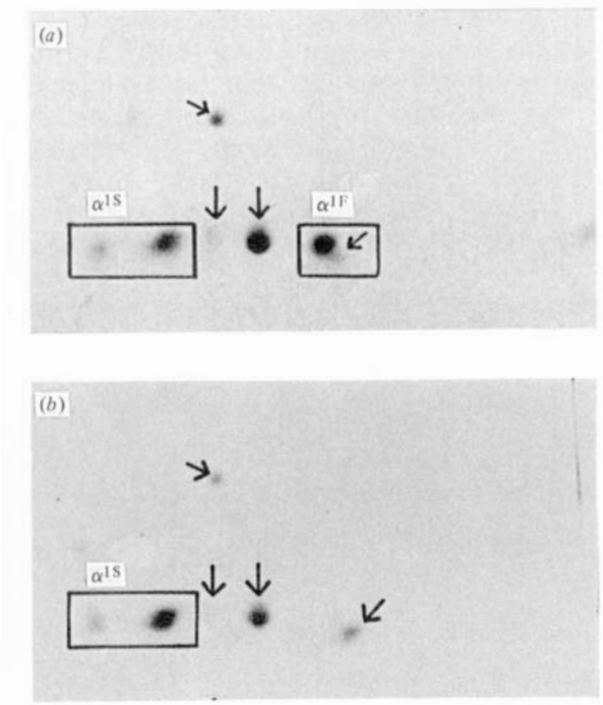

Fig. 2. 2D gels of human plasma proteins. (*a*) Subtype 1F-1S; (*b*) subtype 1S-1S. Stained with Coomassie Brilliant Blue R. See legend to Fig. 1 for explanation of abbreviations. Acidic proteins to the left. Part of gel shown.

on a discontinuous SDS polyacrylamide slab gel (Laemlli, 1970) with a 10% (w/v) acrylamide slab separating gel (300 × 150 × 1 mm) for 5 h at 30 mA and stained with Coomassie Brilliant Blue R as described previously (John & Purdom, 1984) or with silver (Sammons, Adams & Nishizawa, 1981).

3. Results

Haptoglobin subunit spots in 2D gels of human plasma and serum were identified by comparison with the spots of purified human haptoglobin. In each case coelectrophoresis of purified haptoglobin with plasma or serum indicated when spots comigrated. When 10 μg of purified haptoglobin were analysed on a 2D gel and stained with Coomassie Blue the α^{1F} chain was represented by one major spot, the α^{1S} chain by one major and one minor spot and the α^2 chain by two major and two minor spots (Figs. 1 *h*, 3 *a*). When the loading of haptoglobin was increased to 60 μg/10 μl, which is well above the average concentration in human plasma (13 μg/10 μl, 130 mgm/100 ml (Putnam, 1975)), the α^{1F} chain was still represented by one spot, but three spots were resolved from the α^{1S} chain and five spots from the α^2 chain (Figure 3b). An additional more basic α^2 spot could be detected in purified haptoglobin by more sensitive silver staining (Fig. 3*c*). Other silver-stained spots occurred near the α^{1S} spots of purified haptoglobin (arrowed in Fig. 3*c*) but they were assumed to be contaminant proteins because of their very low concentrations and different molecular weights.

The haptoglobin α^1 and α^2 chain spots in plasma were also distinguished from other protein spots nearby by comparison with the plasma of young boys with undetectably low levels of haptoglobin (Figs. 1*a*, 3*f*).

(i) *Phenotype 1-1*

Fifteen samples of this phenotype were analysed and were divided into three subtypes.

(*a*) Subtype 1F-1F (2 samples) showed one Coomassie Blue-stained spot (Fig. 1*b*) which was also found in subtypes 1F-1S and 1F-2 and in purified haptoglobin (Fig. 1*d*, *e*, *h* respectively) and was attributed to chain α^{1F}. Microheterogeneity of α^{1F} was suggested by the appearance of a Coomassie Blue-stained double spot in several samples (Fig. 2*a*). However, the presence of the same extra spot in a 1S-1S sample which does not contain the α^{1F} chain (Fig. 2*b*) and its absence in purified haptoglobin (Fig. 3*a*, *b*) indicates that it is a protein unrelated to haptoglobin. No further spots were detected in the α^{1F} chain region by silver staining (Fig 3*d–f*).

(*b*) Subtype 1S-1S (4 samples) showed one major Comassie Blue-stained spot (Fig. 1*c*) which was also found in 1F-1S and 1S-2 and in purified haptoglobin (Fig 1 (*d*, *f*, *h*) respectively) and was attributed to chain α^{1S}. Coomassie Blue staining only showed one of the more acidic α^{1S} spots in plasma (Fig. 1*c*, *d*, *f*) but silver staining showed three spots (Fig. 3*d*) which were also present in purified haptoglobin (Fig. 3*c*).

(*c*) Subtype 1F-1S (9 samples) showed the spots attributed to α^{1F} and α^{1S} (Figs. 1*d*, 3*d*).

The remaining plasma spots in the α^1 chain region were also found in haptoglobin-free plasma (arrowed in Figs. 1*a*, 3*f*) and in phenotype 2-2 (arrowed in Figs. 1*g*, 3*e*) but not in purified haptoglobin (Figs. 1*h*, 3*c*).

(ii) *Phenotype 2-2*

Seven samples of 2-2 phenotype showed four Coomassie Blue-stained spots in the α^2 chain region (Fig. 1*g*), but one of these was also present in the haptoglobin-free plasma (arrowed in Fig. 1*a*) and was not found in purified haptoglobin (Fig. 1*h*) showing it is not related to haptoglobin. Phenotype 2-2 plasma samples showed the same six silver-stained spots (Fig 3*e*) that were resolved from the α^2 chain of purified haptoglobin (Fig. 3*b*). Comparison with plasma samples with no detectable haptoglobin indicated that there were several proteins unrelated to haptoglobin in the α^2 region (arrowed in Fig. 3*f*).

(iii) *Phenotype 2-1*

Nine samples of 2-1 phenotype showed the same three Coomassie Blue-stained α^2 chain spots as phenotype

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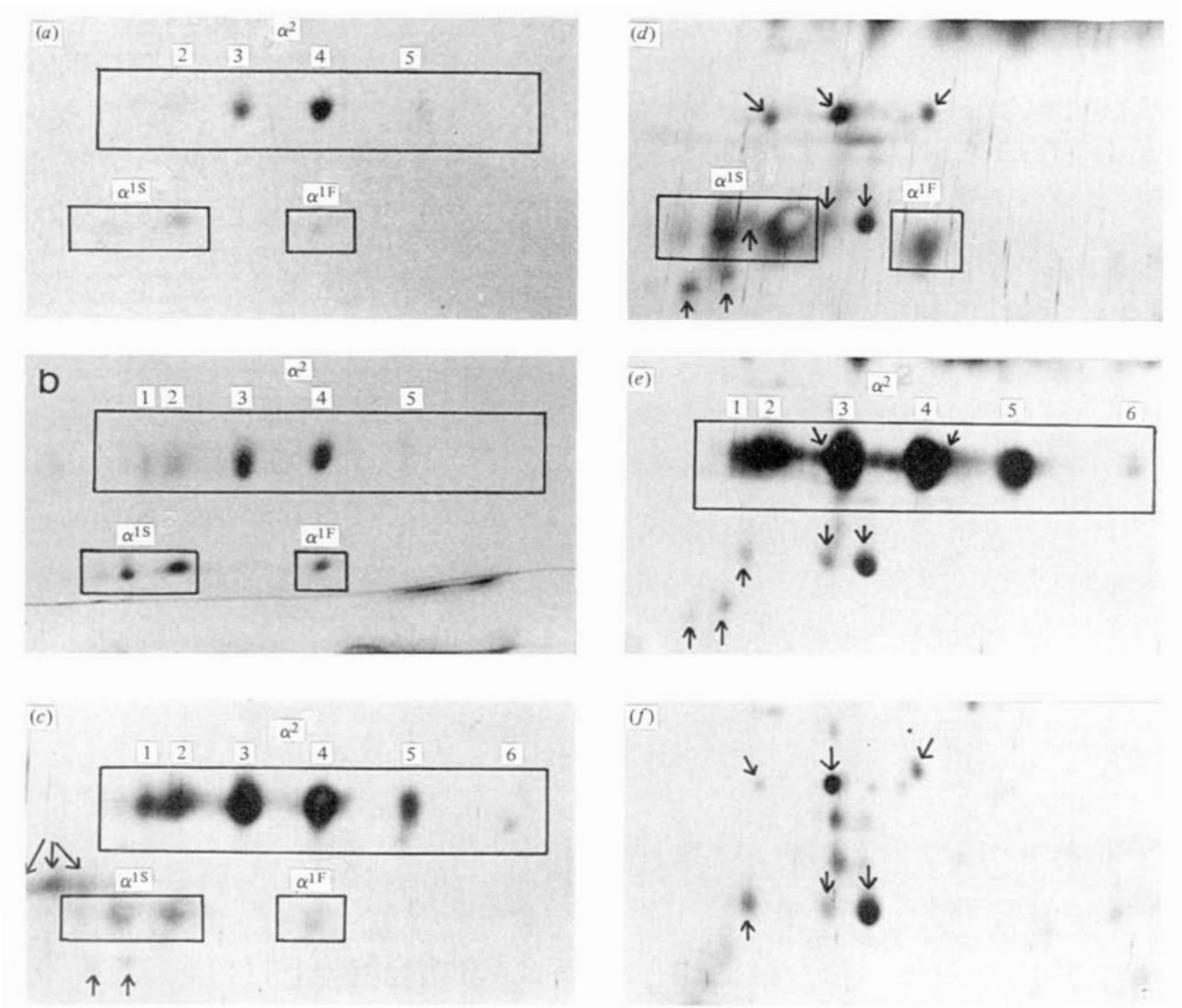

Fig. 3. 2D gels of human plasma proteins. (*a–c*) Purified haptoglobin: (*a*) 10 μg and (*b*) 60 μg stained with Coomassie Brilliant Blue R; (*c*) 10 μg stained with silver. (*d–f*) Plasma proteins stained with silver. (*d*) Subtype 1F-1S; (*e*) phenotype 2-2; (*f*) plasma without haptoglobin. See legend to Fig. 1 or text for explanation of abbreviations. Acidic proteins to the left. Part of gel shown.

2-2 but associated with either the α^{1F} chain in 5 samples of subtype 1F-2 (Fig. 1*e*) or with the α^{1S} chain in 4 samples of subtype 1S-2 (Fig. 1*f*). Silver staining indicated that six α^2 chain spots found in both these subtypes were identical to those in phenotype 2-2 (not shown).

4. Discussion

The haptoglobin α^{1F} and α^{1S} chains are usually represented by one spot each in 2D gels (Anderson & Anderson, 1977, 1984; Daufeldt & Harrison, 1984), the basic and acidic focusing positions presumably being due to the presence of lysine and glutamic acid respectively at residue 53 (Bowman & Kurosky, 1982). We found that the α^{1F} chain was represented by one spot in subtypes 1F-1F, 1F-1S and 1F-2. In contrast the α^{1S} chain was represented by one major and two minor spots in subtypes 1S-1S, 1F-1S and 1S-2. The two minor acidic α^{1S} chain spots were also found in purified haptoglobin. The 2D gels of Teige *et al.* (1985) also show the presence of an additional acidic spot in some samples containing the α^{1S} chain. The appearance of lateral additional spots in 2D gels is often due to variation in the carbohydrate content of a chain but there are no carbohydrate groups on the α chains of haptoglobin (Putnam, 1975). Yang & Przybylska (1973) found that one-dimensional isoelectric focusing separated the native protein of human haptoglobin 1S-1S subtype into at least eight distinct components.

The previously reported heterogeneity of the α^2 chains (Anderson & Anderson, 1977, 1984; Daufeldt & Harrison, 1984) may have been due to the presence of different alleles of *Hpα*². Smithies *et al.* (1962*b*) predicted the existence of four types of *Hpα*² alleles (designated $Hp\alpha^{2FS}$, $Hp\alpha^{2FF}$, $Hp\alpha^{2SF}$ and $Hp\alpha^{2SS}$) corresponding to the four possible chromosomal rearrangements resulting from crossing over within the haptoglobin locus in appropriate phenotype 2-1 heterozygotes. Two of the products of these *Hpα*²

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alleles α^{2FF} and α^{2SS} were distinguished from the other pair α^{2FS} and α^{2SF} by electrophoresis because they either had lysine residues or glutamic acid residues at both positions 53 and 112 in the α^2 chain (Nance & Smithies, 1963). It can be anticipated that such chains would show up as more basic or more acidic spots in 2D gels. Therefore, a possible explanation for the presence of the basic and acidic spots of the α^2 chain is that they represent the α^{2FF} and α^{2SS} chains respectively. Teige *et al.* (1985) analyzed the serum of an $Hp\alpha^{2FS}$ homozygote and found that the α^{2FS} chain was represented by one major basic Coomassie Blue-stained spot. However another minor more acidic spot was detectable in the gel illustrated. This minor spot was more prominent in the serum of one heterozygous individual with the $Hp\alpha^{2SS}$ allele who also showed an additional even more acidic spot. In contrast, we found the same three Coomassie Blue-stained spots in approximately similar proportions in 9 samples of 2-1 phenotype as well as in 7 samples of 2-2 phenotype. Furthermore, all these samples showed the same six silver-stained spots suggesting that the heterogeneity is not due to the presence of different alleles.

The isoelectric points of some of the α^2 chain spots (α^2 4, 5 and 6 in Fig. 3*c*) are more basic than the isoelectric points of either the α^{1F} or the α^{1S} spots. The crossover event which formed the $Hp\alpha^2$ allele resulted in the deletion of the DNA region of the *Hp* allele corresponding to residues 70–83 of the carboxyl terminus of the α chain from one allele and residues 1–10 on the region corresponding to the amino terminus of the allelic partner (Bowman and Kurosky, 1982). However, the overall balance of acidic and basic charges in the α^2 chain should remain the same.

Another possible explanation for the more basic α^2 chain spots is that substitution of more basic amino acids has occurred since the gene duplication event. However, recent data on the sequencing of cloned cDNA of α^{1S} and α^{2FS} mRNAs shows this is not so (Brune *et al.* 1984; Van der Straten *et al.* 1984). There must be some other as yet unidentified explanation for the apparent microheterogeneity of the α^2 chain.

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