### Title
Mild “Unstable Haemoglobin Haemolytic Anaemia” caused by Haemoglobin Shepherds Bush (B74 (E18) Gly→Asp)




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When the C-terminal sequence of the human haptoglobin β chain is compared with the C-termini of other proteins, it is interesting that it is slightly similar to the Fc fragment (heavy chain) of IgG (ref. 10) and very similar to the C-termini of chymotrypsinogen[11] and trypsinogen[12] (Table 2). Sequential homology between the haptoglobin α chain and the κ and λ chains of Bence Jones proteins has been observed previously[4]. The striking similarity of the C-termini of the haptoglobin β chain, chymotrypsinogen and trypsinogen is surprising, and may possibly indicate a relationship of haptoglobin with the serine protease family.

Table 2. CARBOXYL TERMINAL SEQUENCE COMPARISON OF HUMAN Hp β, CHYMOTRYPSINOGEN A, TRYPSINOGEN AND HUMAN IgG H CHAIN

| | |
|---|---|
| Hp β chain | -Val- Glx- Lys- Thr- Ile- Ala- Glu- Asn- COOH |
| Chymotrypsinogen A* | -*Val-* *Gln-* Gln- *Thr-* Leu- *Ala-* Ala- *Asn-* COOH |
| Trypsinogen* | -Ile- Lys- Glu- *Thr-* *Ile-* *Ala-* Ser- *Asn-* COOH |
| Human IgG H chain† | -Thr- *Gln-* *Lys-* Ser- Leu- Ser- Leu- Ser- Pro- Gly- COOH‡ |

* Bovine.

† Human myeloma γG1 and γG2.

‡ Two terminal gaps are introduced in the first three protein sequences to maximize homologies with IgG H chain.

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## Mild "Unstable Haemoglobin Haemolytic Anaemia" caused by Haemoglobin Shepherds Bush (B74 (E18) Gly→Asp)

A 30 YEAR old South African woman of British extraction was investigated because of a persistent reticulocytosis. As a child she had been chronically anaemic, and at the age of six her spleen had been removed. After that time she had been well, but on one occasion she became "ill" after a course of sulphonamide. She was found to have a well compensated haemolytic anaemia; the haemoglobin level was 13 g/100 ml. of blood, and 5–8 per cent of her red cells were reticulocytes. Though of normal size the red cells showed target cell formation, basophilic stippling, and 75–80 per cent contained Heinz bodies. The test for abnormally heat labile haemoglobin[1] was positive. The patient's mother was found to carry the same haemoglobin abnormality but was not thought to suffer from severe haemolysis. She did, however, experience an episode of severe haemoglobinuria following sulphonamides.

The haemolysate contained 2·1 per cent Hb F as determined by alkali denaturation[2]. Paper[3] and starch gel[4] electrophoreses were used to demonstrate a higher than normal Hb $A_2$ fraction (3·5 per cent), a small amount (0·8 per cent) of a haemoglobin with the mobility of free α chains and a haemoglobin fraction moving more anodically than Hb A. Its separation from Hb A was incomplete by these methods, but chromatography on a 'DEAE–Sephadex' column using a *p*H gradient[5] yielded a well separated abnormal fraction amounting to 24 per cent which was subsequently used for the determination of the amino-acid substitution. On electrophoresis of the total globin in starch gel with 6·5 M urea at *p*H 8·6 (ref. 6), bands were found in the position of normal α and β chains, and in addition one band, amounting to about a quarter of the total globin, was seen which moved anodically to the β chain, indicating that the abnormal haemoglobin had an additional negative charge in the β chain. A quantitative separation of the globin chains obtained from the whole haemoglobin was made on a carboxymethyl–cellulose column in the presence of 8 M urea[7] and showed a new β chain peak, amounting to 24 per cent of the non α chains.

An unstable haemoglobin found previously at Hammersmith Hospital had already been named Hb Hammersmith[8], and the new variant has therefore been named Hb Shepherds Bush.

The purified abnormal haemoglobin fraction was converted into globin and digested with trypsin for 2 h at *p*H 8·6. The fingerprint[9,10] of the abnormal haemoglobin (Fig. 1) differed from that of Hb A by the absence of the spot representing $\beta^{A}$TpIX (residues 67–82 of the 146 of the β chain) and the presence of a new spot which stained for histidine[11]. It was displaced towards the anode relative to $\beta^{A}$TpIX and had a lower chromatographic mobility. All the other spots normally found on the fingerprint of Hb A were present in their usual positions and gave the expected specific staining reactions[11]. The insoluble "core" which remained after tryptic digestion was examined by peptic digestion and fingerprinting, but no evidence for any further abnormalities was obtained.

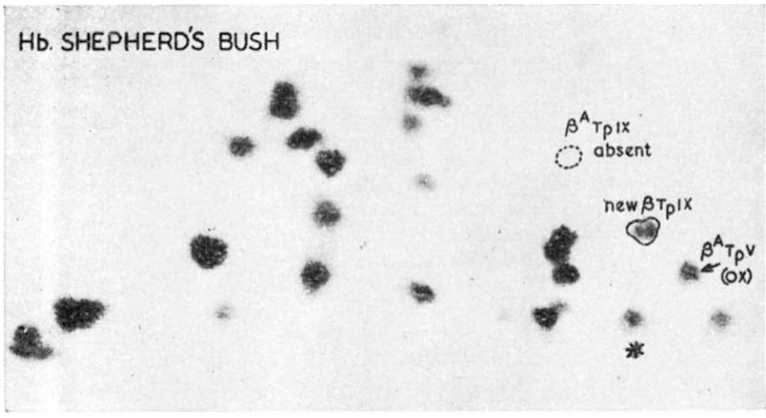
Table 1. AMINO-ACID COMPOSITION OF βTpIX (β67–82) OF Hb SHEPHERDS BUSH

| Amino-acid | Hb SB | | | Hb A | Expected for $\beta^{A}$TpIX |
|---|---|---|---|---|---|
| Asp | 3·4 | 3·6 | 3·7 | 3·0 | 3 |
| Ser | 0·9 | 1·5 | 1·25 | 1·0 | 1 |
| Gly | 1·0 | 1·3 | 1·3 | 2·0 | 2 |
| Ala | 1·9 | 2·2 | 2·1 | 2·1 | 2 |
| Val | 0·8 | 1·0 | 1·0 | 0·9 | 1 |
| Leu | 3·8 | 3·6 | 3·7 | 4·0 | 4 |
| Phe | 0·7 | 0·9 | 0·9 | 1·0 | 1 |
| Lys | 1·0 | 1* | 1* | 1* | 1 |
| His | 1·2 | 1* | 1* | 1* | 1 |
| Yield (μmoles) | 0·024 | 0·021 | 0·021 | 0·017 | |

* A lysyl is presumed to be present because the C terminal residues of tryptic peptides would either be lysyl or arginyl and the presence of arginine could be excluded by a negative staining reaction. A positive test for histidine was taken to indicate the presence of histidyl.

The peptide was isolated by preparative fingerprinting; the electrophoretic separation (1·5 h) was more prolonged than usual to ensure adequate separation from another nearby peptide (βXIIIa 121–130). Table 1 shows that the new peptide resembled $\beta^{A}$TpIX in composition except that it contained only one residue of glycine instead of two and approximately four residues of aspartic acid instead of three; this suggested a Gly→Asp substitution. Although asparagine also produces aspartic acid on hydrolysis, a Gly→Asn mutation would not alter the charge of βTpIX, and furthermore this substitution is not compatible with a single base change of the codons for glycine[13].

There are two glycine residues in $\beta^{A}$TpIX at positions 69 and 74; in Hb J Cambridge, β69 Gly–Asp[9], which is not unstable, the first of these is substituted. It therefore seemed likely that the new haemoglobin was β74 Gly→Asp, and in order to confirm this the mutant peptide was further degraded with thermolysin into smaller peptides. Thermolysin is a bacterial protease which hydrolyses peptide bonds on the amino side of amino-acids with bulky hydrophobic side chains[14–16]—for example, phenylalanine, leucine, valine and so on—but is inhibited by the




Electrophoresis *p*H 6·4

Fig. 1. Fingerprint of Hb Shepherds Bush. For details see text. An asterisk indicates the point of application.

immediate proximity of a charged amino group. Fig. 2 shows the amino-acid sequence of βATpIX and the expected points of thermolysin hydrolysis. Glycine 74 is in an acidic peptide which would become more acidic on substitution of glycine by aspartic acid.

further hydrolysis. The new unstable Hb Shepherds Bush can therefore be described as $\alpha_2\beta_2$ 74 (E18) Gly→Asp.

The glycine at position β74 is the eighteenth residue of the E helix[17,18] and is at an internal site which forms part of the hydrophobic lining of the haem pocket, but is not in direct contact with the haem group[19]. Position E18 is occupied by glycine in all the mammalian β chains so far tested[20] and in the δ chain of human Hb $A_2$, and by alanine in the γ chain of human Hb F. The equivalent position in the α chain (α69) is occupied by alanine in human and animal α chains. Position E18 corresponds to position 75 in myoglobin and this is occupied by isoleucine in human, whale, dolphin and seal myoglobins[21,22]. In all cases the amino-acid has a nonpolar side chain. Aspartic acid is an "antihelical" residue[23]; β73 (E17) is aspartic acid in normal haemoglobin and the presence of an additional β74 (E18) aspartic acid side chain can be expected to weaken the E helix further by interfering with the carbonyl part of the preceding β70 (E14) alanine residue.

| Thermolysin peptides | 1 | | | | | | | 2 | | | | | 3 | | | | 4 | | | 5 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| βATpIX | + Val | Leu | Gly | Ala | Phe | Ser | − Asp | GLY | Leu | 3a Ala | + His | Leu | − Asp | Asn | Leu | + − Lys | | | | | |
| Residue No. | 67 | 68 | 69 | 70 | 71 | 72 | 73 | 74 | 75 | 76 | 77 | 78 | 79 | 80 | 81 | 82 | | | | | |
| Helical notation | E11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | EF1 | 2 | 3 | 4 | 5 | 6 | | | | | |
| βSBTpIX | + Val | Leu | Gly | Ala | Phe | Ser | − Asp | − ASP | Leu | Ala | + His | Leu | − Asp | Asn | Leu | + − Lys | | | | | |

Fig. 2. Sequences of normal and abnormal βTpIX and expected thermolysin peptides 1–5. Full arrows represent main sites of thermolysin hydrolysis; dotted arrows, other sites of hydrolysis. Positive and negative signs denote electric charges at *p*H 6·4.

The abnormal βTpIX and normal βATpIX were eluted from preparative fingerprints and digested with thermolysin for 3–5 h at 37° C in 0·2 M ammonium acetate buffer (*p*H 7·4) containing 5 mM barium acetate. The barium was removed by precipitation as the sulphate and centrifugation, and the digests were evaporated to dryness *in vacuo*. Diagnostic and preparative fingerprints of the thermolysin digests were made in the same way as for tryptic digests, and the peptides were eluted, hydrolysed and analysed.

Fig. 3 is a drawing of a fingerprint of a thermolysin digest of βATpIX. In the corresponding fingerprint of the abnormal βTpIX, spot 2 was absent and two new spots X and Y were present. Amino-acid analysis of X (Table 2) showed that it corresponded to the expected product of a β74 Gly→Asp substitution. Peptide Y contained chiefly serine and aspartic acid and could be derived from X by

Table 2. AMINO-ACID COMPOSITION OF THERMOLYSIN PEPTIDES: NO. 2 FROM A, AND X AND Y FROM Hb SHEPHERDS BUSH IN FIG. 3

| Amino-acids | Hb A Peptide 2 β71–74 | Hb Shepherds Bush Peptide X β71–74 | Hb Shepherds Bush Peptide Y β72–73 |
|---|---|---|---|
| Asp | 1·0 (1) | 2·0 (2) | 1·0 (1) |
| Ser | 0·8 (1) | 1·2 (1) | 0·8 (1) |
| Glu | | *0·3* | *0·4* |
| Gly | 1·0 (1) | *0·3* (0) | *0·4* |
| Ala | | | *0·2* |
| Phe | 0·75* (1) | 0·6* (1) | |
| Yield (μmoles) | 0·079 | 0·017 | 0·017 |

* Phenylalanine is the N-terminal residue and is partly destroyed by ninhydrin staining.

Figures in brackets are the expected numbers of residues; those in italics are considered to represent contamination.

Hb Shepherds Bush represents another instance where a charged amino-acid is introduced into the haem pocket and one has to explain why this does not cause complete disruption of the structure. Examination of a scale model shows that the β carboxyl group of the aspartic acid might be drawn out of the haem pocket towards the surface of the molecule possibly to form an ionic link with histidine β77 (EF1). Similar explanations have been suggested in the cases of Hb Zürich β63 (E7) His→Arg[24] and Hb Borås β88 (F4) Leu→Arg[25]. Using his model of horse haemoglobin[26] Dr M. F. Perutz showed us why a β74 (E18) Gly→Asp mutation would be expected to make the molecule unstable: the part of the haemoglobin where E18 is situated is tightly packed and the E and F

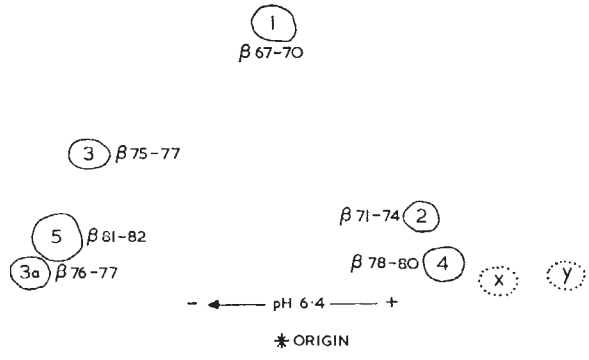



Fig. 3. Fingerprint of the thermolysin digest of the tryptic peptide βIX (β67–82) from Hb A. In the corresponding preparation from Hb Shepherds Bush, spot 2 is missing and spots X and Y are found instead.

helices are lying closely opposite each other. Although it can just accommodate an alanine instead of a glycine as in the α and γ chains, the insertion of an aspartic acid side chain at E18 in the β chain would interfere with the residues of threonine β84 (EF8) and phenylalanine β85 (F1) lying opposite and move the E and F helices apart, thereby weakening the hydrophobic forces which hold the haem pocket together.

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## Allotype Polymorphism of Low Density β-Lipoproteins in Pig Serum (LDLpp 1, LDLpp 2)

A great variety of serum antigens have been found in a number of mammalian species[1,2].

A young pig 'Chester 16-4' was injected five times at 14 day intervals with 5 ml. pooled sera of five 'Hampshire' pigs to which 1·5 ml. Freund's complete adjuvant had been added. The Ouchterlony double-diffusion technique as modified by Rapacz *et al.*[3] was used to demonstrate isoprecipitins against serum antigens. The immune serum, C16-4, gave positive reactions with three of the donor sera. The precipitation lines in the agar were clearly visible, crescent shaped and curved towards the peripheral well as shown by the band between the centre well (*A*) and peripheral well 1 (Fig. 1). This indicates that the antigen is of relatively high molecular weight. Normal sera from other pigs were tested against the antiserum, and a second band was observed in several cases, for example, the band between the centre well (*A*) and well 2 (Fig. 1). None of the three donor sera mentioned here showed this specificity. Pigs of three breeds, 397 of the Hampshire, 431 of Chester and 305 of Poland China breeds (from the University of Wisconsin Experimental swine herd), were tested. Only in the Hampshire breed were antigenic specificities found, with the frequency of 37 per cent.

The following techniques were used in an effort to identify the antigens: (1) gel chromatography separation, (2) gel immunoelectrophoresis, (3) staining with protein and lipid dyes, (4) paper electrophoresis, and (5) ultracentrifugation. Normal sera of phenotypes Lpp 1, Lpp 2 and Lpp 1,2 (Fig. 1) were fractionated on a 'Sephadex *G*-200' column. Antigen activities were restricted to the first peak supporting previous observation that the antigens are of high molecular weight. When the sera were tested by immunoelectrophoresis, the antigens were found to migrate to the α-globulin position. The stronger precipitin line stained weakly with protein reagent 'Amido Black 10*B*' and strongly with lipid stain 'Oil Red O'. The second precipitate (the one closer to the central well) stained like the first, but much less intensely. The antigens were found in the β-globulin and the β-lipoprotein position when serum samples were run on paper electrophoresis.

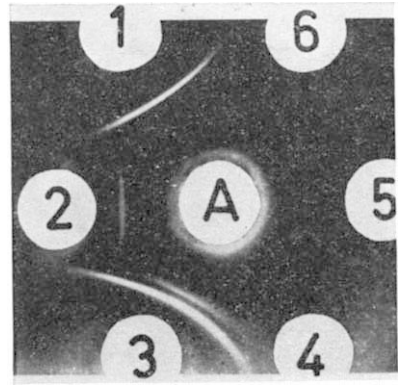


Fig. 1. Double diffusion precipitation test in agar gel. Precipitation reactions observed in this study between the immune serum placed in the centre well *A* and the normal sera placed in the peripheral wells 1–6 represent four phenotypes: Lpp 1 (reaction between *A* and 1); Lpp 2 (reaction between *A* and 2); Lpp 1,2 (reaction between *A* and 3); and Lpp 0 (lack of precipitate between *A* and 4, 5, 6).

Further identification of the lipoprotein allotypes was attempted according to the Cohen technique, using the preparative ultracentrifuge, by flotation based on selective solvent density. The antiserum reacted only with the 1·019–1·063 density material which indicates that the antigens are low density β-lipoproteins. Allotypic polymorphism of low density β-lipoproteins has been found in humans[4–8]. Absorptions were carried out to determine the relation between the antigens and to explain the unexpected presence of the second specificity in some sera. Lpp 1, Lpp 2, Lpp 1,2 and Lpp 0 are the symbols proposed for the four phenotypes observed in Fig. 1.

Absorption with all sera of Lpp 2 type removed only precipitins directed against the weaker antigen, leaving the antibodies for the stronger specificity. The Lpp 1,2 type serum absorbed all precipitating antibodies as did each Lpp 1 type serum used.

The ability of Lpp 1 serum to remove the antibodies for both types is consistent with its antigenic ability to