Human Haemoglobins

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I—Structure of Haemoglobin II—The Genetic Control of Haemoglobin Synthesis III—The Biosynthesis of Haemoglobin IV—Clinical Features of Abnormalities in Haemoglobin Synthesis

During the past 15 years the study of the normal and abnormal human haemoglobins has advanced our understanding of the genetic control of protein synthesis and also provided an example of the various ways in which abnormalities of protein synthesis can cause disease. There have been a number of reviews on this subject (Itano, Bergren, and Sturgeon, 1956; Itano, 1957; Beaven and Gratzer, 1959; Lehmann and Ager, 1960; Bannerman, 1961; Hill, 1961; Ingram, 1961; Rucknagel and Neel, 1961; Baglioni, 1963a; Jonxis, 1963), and this review mentions only the more important early advances, but deals in more detail with those of present interest.

I: STRUCTURE OF HAEMOGLOBIN

Haemoglobins are large, complex, protein molecules, the function of which is to transport oxygen from the lungs to the tissues. Recent work by Perutz and his collaborators (Perutz, Rossman, Cullis, Muirhead, Will, and North, 1960; Cullis, Muirhead, Perutz, Rossman, and North, 1962) has revealed the over-all arrangement of the molecules, and the model these workers have built on the basis of their x-ray crystallographic work is shown in Fig. 1 and 2. In over-all shape, the molecule resembles a spheroid 64 Å × 55 Å × 50 Å. It can be seen that each molecule consists of four polypeptide chains and that a haem group is attached to each chain. Each molecule consists of two pairs of identical chains, called α- and β-chains, so that its structure can be written α₂β₂. In Fig. 1 and 2, the two α-chains are in white, while the two β-chains are in black. The internal arrangement of the two types of chains is very similar, the main difference being that the β-chain is slightly longer, and this is confirmed by the amino acid sequence studies described later. The model further shows that there is relatively little contact between pairs of like chains, whereas there is striking complementarity between adjacent surfaces of α- and β-chains. The haem groups are not particularly close to one another, and their arrangement offers no clue to the influence they exert on each other in the oxygenation reaction.

Recently, however, Muirhead and Perutz (1963) and Perutz (1964) have found that the arrangement of the subunits alters during oxygenation. It appears that the β-chains move closer together on oxygenation, decreasing the distance between their haem groups by 7 Å.

The haem groups lie in four separate pockets on the surface of the molecule, each pocket being formed by the folds of one of the four polypeptide chains. Each haem group is attached to its polypeptide chain by a co-ordinate linkage from the iron of the haem group to a specific histidine residue at position 87 in the amino acid sequence in the α-chain and at position 92 in the β-chain. Another histidine occurs in each polypeptide chain on the opposite side of the haem group (58α and 63β) and is linked to the iron of the haem...
group through either an oxygen molecule in oxyhaemoglobin or a water molecule in reduced haemoglobin.

**Normal Human Haemoglobins**

There are several normal human haemoglobins at different stages of human development: adult, foetal, and embryonic.

**Haemoglobins Found in Adult Life.**

HAEMOGLOBIN A. As explained above, Hb-A consists of two $\alpha^A$-chains joined to two $\beta^A$-chains. The formula for Hb-A can thus be written $\alpha^A_2\beta^A_2$ (superscripts, as, for example, $\alpha^A$, designate the source of each chain, while subscripts denote the number of each type of chain present (Schroeder, 1959)). The amino acid sequences of each chain are given in Fig. 3. When the sequences of the two chains are compared, it is found that the $\beta$-chain has 5 amino acids more than the $\alpha$-chain and, although many parts of the amino acid sequence are the same, there are approximately 84 differences between the two chains. The molecular weight of the haemoglobin molecule calculated from the above structure is 64,500.

HAEMOGLOBIN A2. Hb-A2 occurs in all normal haemolysates after the postnatal period and amounts to 1.5-3% of total haemoglobin (Kunkel, Ceppellini, Müller-Eberhard, and Wolf, 1957). It is distinguished from Hb-A by its slower electrophoretic migration towards the anode and can be separated by all the conventional systems. It can also be separated by chromatography on carboxymethylcellulose (CMC) (Huisman, Martis, and Dozy, 1958), diethylaminoethylcellulose (DEAE) (Huisman and Dozy, 1962) and Amberlite IRC 50 (Schnek and Schroeder, 1961). A convenient two-step preparative method has also been described (Huehns and Shooter, 1961b). In its alkali denaturation rate and absorption spectrum, Hb-A2 resembles Hb-A.
Hb-A₂, like other haemoglobins, consists of four polypeptide chains, two of which are identical to the α⁺-chains (Muller and Jonxis, 1960; Huehns and Shooter, 1961a, b; Ingram and Stretton, 1961; 1962a, b). The other two are very similar to the β-chains but differ by ten amino acid substitutions (Table I) (Ingram and Stretton, 1961; 1962a, b; Hill and Kraus, 1963; Jones, 1964). These non-α-chains have been called γ-chains, and the structure of Hb-A₂ is therefore α₂β₄γ₂δ₂.

**TABLE I**

<table>
<thead>
<tr>
<th>Position in Amino Acid Sequence</th>
<th>Residue in β⁺-chain</th>
<th>Residue in δ⁺-chain</th>
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<tr>
<td>9</td>
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<tr>
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<td>117</td>
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<td>125 (or 124)</td>
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<td>GluNH₂</td>
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<tr>
<td>126</td>
<td>Val</td>
<td>Met</td>
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</table>

**Other Minor Adult Haemoglobins.** Several other minor haemoglobin components are found in adult haemolysates. Hb-A₃, which forms approximately 10% of total haemoglobin (Kunkel and Wallenius, 1955), appears to be derived from Hb-A during *in vivo* ageing of the red cells (Kunkel and Bearn, 1957; Meyering, Israels, Sebens, and Huisman, 1960). Hybridization of Hb-A₃ shows that it differs from Hb-A in the β-chains (Rosa and Labie, 1962). More recent work indicates
that Hb-A₂ is a mixture of haemoglobins (Schnek and Schroeder, 1961; Huisman and Meyerling, 1960). Schnek and Schroeder (1961) have detected several minor components in normal haemolysates by chromatography on IRC 50 and have called these Hb-A₁₃, Hb₁₁, Hb₁₂, Hb₁₃. The largest of these, Hb-A₁₂, amounting to approximately 5% of total haemoglobin, has the structure α²β²γ₃δ₃, the β₃-chain differing from the β₂-chain by the substitution of the terminal NH₂ group (Holmqvist and Schroeder, 1964). It has also been suggested that part of the Hb-A₂ fraction is composed of a complex of oxidized glutathione (GSGS) with Hb-A (Muller, 1961; Huisman and Dozy, 1962). However, it is not known whether this complex is present in vivo. Hybridization experiments show that the GSGS is attached to the β-chains.

A small quantity of foetal haemoglobin is also present in adult red cells (Chernoff, 1953b), but this does not amount to more than 0.4% (Beaven, Ellis, and White, 1960b).

**Haemoglobins in Foetal Life.**

**FOETAL HAEMOGLOBIN.** Hb-F is the major pigment found in the red cells during foetal life and differs in several respects from Hb-A. It was first detected in 1866 by Körber, who noticed that this pigment was denatured more slowly by alkali than that found in adult red cells.

Foetal haemoglobin, like Hb-A, consists of four polypeptide chains. Two of these are identical to the α¹-chains (Schroeder and Matsuda, 1958; Hunt, 1959; Schroeder, 1963), while the other two chains have the same number of amino acids as the β-chains but differ from them in 39 amino acid residues and are called γ-chains. The structure of Hb-F is thus α²β²γ₄. The amino acid sequence of the γ-chains is given in Fig. 4.

Hb-F differs from Hb-A by its greater resistance to alkali denaturation (Körber, 1866), and this property is used to measure the amount of Hb-F in various haemolysates (Singer, Chernoff, and Singer, 1951; Beaven, Ellis, and White, 1960a; Betke, Marti, and Schlicht, 1959). The haem absorption spectrum of Hb-F is identical to that of Hb-A. However, there is a distinct difference in the protein absorption at about 290 mμ. In Hb-F the tryptophan fine structure band is resolved at 2,896 Å, while in Hb-A it is unresolved at 2,910 Å (Jope, 1949; Beaven et al., 1960a), the difference arising from the altered tryptophan/tyrosine ratios of the two haemoglobins.

Hb-F can be separated from Hb-A by electrophoresis in agar gel, using a sodium citrate buffer at pH 6.2 (Robinson, Robson, Harrison, and Zuelzer, 1957), or in starch gel with a tris-ED.T.A.-borate buffer at pH 8.6 (O. Smithies, unpublished). In other electrophoretic systems, consistent separation of Hb-A and Hb-F is difficult to achieve. Chromatographically, Hb-F is most conveniently separated on IRC 50 resin, using a sodium phosphate buffer at approximately pH 7.0 (Allen, Schroeder, and Balog, 1958). This method is useful for the preparation of pure Hb-F.

**MINOR FOETAL HAEMOGLOBINS.** Several minor haemoglobins are present in foetal blood. The largest of these is Hb-F₁ (Allen et al., 1958), which amounts to approximately 10% of total haemoglobin. This has the structure α²βγ₃δ, the γ₃-chain differing from the γ₁-chain by the substitution of the terminal NH₂ group with an acetyl group (Schroeder, Cua, Matsuda, and Fenninger, 1962). A small amount of Hb-γ₄ is also detected by starch gel electrophoresis (Karakhis and Fessas, 1963) and amounts to approximately 0.3% of total haemoglobin in normal cord bloods (Huehns, Dance, Beaven, Hecht, and Motulsky, 1964b).

After birth, the proportion of Hb-F found in haemolysates rapidly falls (Fig. 5), only traces being present after the first year of life (White and Beaven, 1959).

**Embryonic Haemoglobins.** The possibility of the existence of a human embryonic haemoglobin was first suggested by Drescher and Künzer (1954). Recently, it has been shown by starch gel
electrophoresis (Fig. 6) that there are two embryonic haemoglobins, Hb-Gower 1 and Hb-Gower 2 (Huehns, Flynn, Butler, and Beaven, 1961a; Huehns, Dance, Beaven, Keil, Hecht, and Motulsky, 1964a).

Hb-Gower 2 consists of four polypeptide chains. Again, two \( \alpha \)-chains are present, while the other two chains differ from the \( \beta \)-, \( \gamma \)-, and \( \delta \)-chains by more than one amino acid substitution and have been called \( \epsilon \)-chains (Huehns et al., 1964a; Huehns, Hecht, Keil, and Motulsky, 1964c).

Hb-Gower 2 has an alkali denaturation rate intermediate to Hb-A and Hb-F, and the ultraviolet absorption spectrum resembles that of Hb-F rather than that of Hb-A. It has not yet been possible to study Hb-Gower 1 in detail, but it has been suggested that it consists solely of \( \epsilon \)-chains.

In the youngest embryo studied, which had a crown-rump (C.R.) measurement of 25 mm, the haemolysate contained mainly Hb-F, 24\% Hb-Gower 1, 13\% Hb-Gower 2, approximately 5\% Hb-A, and 1\% Hb-\( \gamma \). The amount of these...
embryonic haemoglobins falls rapidly as development proceeds and they are not normally found after the 100 mm. C.R. stage of development (Fig. 5).

The Abnormal Haemoglobins

The discovery by Pauling, Itano, Singer, and Wells (1949) that the haemoglobin in sickle cell disease differed from normal haemoglobin in its rate of migration on electrophoresis showed for the first time that an abnormal protein molecule could be the primary cause of a severe disease. Since then, a large number of haemoglobins have been discovered. Some of these have been found through the study of patients with various haematological disorders, but the majority have been discovered during surveys carried out in various parts of the world. These have been distinguished from each other by differences in behaviour on electrophoresis. Some of the abnormal haemoglobins with similar electrophoretic mobilities have been further differentiated by other methods, for example, by chromatography on IRC 50 (Huisman and Prins, 1955). The properties of the various abnormal haemoglobins have been reviewed by Beaven and Gratzer (1959), Lehmann (1960), and Lehmann and Ager (1960). The relative electrophoretic mobilities of various abnormal haemoglobins are shown in Figs. 7 and 8. More recently, the structure of a number of abnormal haemoglobins has been determined (as outlined in the next section) and on this basis the existence of definitive species has been firmly established (Table II).

The next discovery in the study of the abnormal haemoglobins was made by Ingram (1956, 1958, 1959) and Hunt and Ingram (1958), who showed that in sickle cell haemoglobin, or Hb-S, only the \( \beta \)-chains were abnormal, while the \( \alpha \)-chains were identical to those found in Hb-A. The structure of Hb-S could then be written as \( \alpha^A \beta^S \). Ingram also showed that in each \( \beta^* \)-chain only one amino
Acid out of I46 was different, the glutamyl residue which is found in the βA-chain at position 6 from the N-terminal end being replaced by a valyl residue. A shorthand for this structure gives the number of the amino acid residue substituted and the new amino acid as a superscript to the affected chains: Hb-S would then be αSβA

Fig. 8. Relative electrophoretic mobilities on starch gel electrophoresis at pH 8.6 of human haemoglobin variants.

A similar situation has now been found for almost all other abnormal haemoglobins so far analysed (except those mentioned later), one amino acid residue being substituted in either the β- or the...
mutations
findings
class
are
Hb-C, lysine
corresponding places
at
haemoglobins
lead
to
methaemoglobinaemia
to
chain

parts
amino acid
Lehmann,
and
types of
me
be
(Baglioni
(24x582)
chemically
previously thought
be
I964). However,
its
be
Lehmann,
and
I963),
and
GE5A

Haemoglobin B variants. Hb-B, which was the first Hb-A variant
discovered (Ceppellini, 1959; Huisman and Meyer-
ing, 1960), occurs in approximately 1% of American Negros. On electrophoresis it moves more
slowly than Hb-A4 (Fig. 8). Chemical studies indicate that it has abnormal δ-chains (Horton,
Payne, Bridges, and Huisman, 1961) and that the amino acid substitution is glycine → arginine at

Hb-Flatbush is another Hb-A variant with
abnormal δ-chains (Rannie, Jacobs, Bradley, and
Cordova, 1963). This haemoglobin migrates on
electrophoresis with Hb-S (Fig. 9).

Hb-Sphaki is probably another variant of
Hb-A with abnormal δ-chains (C. Krimbas,
N. A. Barnicot, and R. B. MacConnell, unpub-
lished). It was found in a single family in Greece.
On electrophoresis it migrates between Hb-A2 and
Hb-B2.

Haemoglobin F Variants. Hb-F Roma
was found in an Italian neonate (Silvestroni and Bianco,
1963). These workers showed that this was a
γ-chain variant of Hb-F by hybridization, spectral
and alkali denaturation studies. On electrophoresis,
Hb-F Roma migrates more rapidly than either
Hb-F or Hb-A towards the anode. Hb-Texas
(Schneider, Arat, and Haggard, 1964a) is another
γ-chain variant. On electrophoresis, this haemoglo-
bolin migrates slightly slower (cathodally) than
Hb-C. Chemical studies indicate that a glutamyl
residue is replaced by a lysyl residue in tryptic
peptide γTPF either at position 5 or position 6
(R. Schneider and R. T. Jones, personal com-
munication). This substitution is the same as
that found in Hb-C (β6Glu → Lys) or
Hb-G
Georgetown (β7Glu → Lys) and, similarly,
occur near the N-terminal of the polypeptide
chain involved. Other γ-chain variants of Hb-F
may be Hb-Alexandra (Fessas, Mastrokalo, and
Fostinopoulos, 1959; Vella, Ager, and Lehmann,
1959) and Hb-Aegena (Fessas, Karaklis, and
Gnafakis, 1961). On electrophoresis Hb-Alexandra
migrates with Hb-O, Hb-Aegena slightly faster
than Hb-A (Fig. 8).

α-chain variants of Hb-A4 and Hb-F are found
in all subjects who synthesize abnormal α-chains;
thus, for example, Hb-G4 (α95Glu) is accompanied
by its own Hb-A variant, called Hb-G4 (α95Glu)
(Huehns and Shooter, 1961a) and during foetal life
the corresponding α-chain variant of Hb-F,
TABLE II
CHEMICAL DIFFERENCES BETWEEN ABNORMAL HAEMOGLOBINS AND HAEMOGLOBIN A

<table>
<thead>
<tr>
<th>Abnormal Haemoglobin</th>
<th>Amino Acid Residue Position</th>
<th>Residue in Hb-A</th>
<th>Residue in Abnormal Haemoglobin</th>
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<td><strong>α-CHAIN ABNORMALITIES</strong></td>
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<tr>
<td>Philadelphia</td>
<td>68</td>
<td>Asg</td>
<td>Lys</td>
<td>Baglioni and Ingram, 1961</td>
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<td>Bristol</td>
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<td></td>
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<td>Dance, Huehns, and Shooter, 1964</td>
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<td>Gazankoke</td>
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<td>Weatherall, Sigler, and Baglioni, 1962</td>
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<td>D-Washington</td>
<td></td>
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<td></td>
<td></td>
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<tr>
<td>I</td>
<td>16</td>
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<td>Asp</td>
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<td>Medellin</td>
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<td>Tyr</td>
<td>Gerald and Efron, 1961</td>
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<td></td>
<td></td>
<td>Miyaji et al., 1963</td>
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<td>M-Kankakee</td>
<td>87</td>
<td>His</td>
<td>Tyr</td>
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<td>Indonesia</td>
<td>116</td>
<td>Glu</td>
<td>Lys</td>
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<td>Arg</td>
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In the following haemoglobins only the abnormal tryptic peptide has been determined

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In the following haemoglobins only the abnormal polypeptide chains have been determined

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<td>Russ</td>
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**β-CHAIN ABNORMALITIES**

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<td>S</td>
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<td>Glu</td>
<td>Lys</td>
<td>Baglioni and Lehmann, 1962</td>
</tr>
<tr>
<td>Zurich</td>
<td>63</td>
<td>His</td>
<td>Arg</td>
<td>Muller and Kingma, 1961</td>
</tr>
<tr>
<td>G. Galveston</td>
<td>43</td>
<td>Glu</td>
<td>Ala</td>
<td>Bowman et al., 1964</td>
</tr>
<tr>
<td>G. Texas</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hikari</td>
<td>61</td>
<td>Lys</td>
<td>Asp</td>
<td>Shibata, Miyaji, Iuchi, Ueda, and Takeda, 1964</td>
</tr>
<tr>
<td>G. Acera</td>
<td>79</td>
<td>Asp</td>
<td>Asp</td>
<td>Lehmann, Beale, and Boi-Doku, 1964</td>
</tr>
<tr>
<td>G. Coumbata</td>
<td>22 or 26 (βTpIII)</td>
<td>Glu</td>
<td>Ala</td>
<td>Schneider et al., 1964b</td>
</tr>
<tr>
<td>Seattle</td>
<td>70 or 76 (βTpIX)</td>
<td>Ala</td>
<td>Glu</td>
<td>Huehns, Hartmann, Hecht, and Motulsky, unpublished</td>
</tr>
<tr>
<td>Rambam</td>
<td>69 or 74 (βTpIX)</td>
<td>? Gly</td>
<td>? Asp</td>
<td>Salomon et al., 1964</td>
</tr>
<tr>
<td>Kenwood</td>
<td>143</td>
<td>His</td>
<td>Glu or Asp</td>
<td>Bayrakci, Josephson, Singer, Heller, and Coleman, 1964</td>
</tr>
</tbody>
</table>

*In these haemoglobins only the abnormal tryptic peptide has been determined.*

| Köln                  | 'Core'                    | Hutchinson et al., 1964 |
| St. Mary's            | 'Core'                    | Buchanan et al. (unpublished) |
| R (Durham 1)          | βTpI                      | Chernoff and Liu, 1961 |
| Dβ                   | βTpIII                    | Benzer et al., 1958 |

*In these haemoglobins only the abnormal polypeptide chain has been determined.*

| M. Milwaukee 2       |                           | Gerald and Efron, 1961 |
| Kβ                   |                           | O’Gorman, Allsopp, Lehmann, and Sukumaran, 1963 |
| Lβ                   |                           | Gammack et al., 1961 |
| P                    |                           | Silvestroni et al., 1963 |

Hb-G F (α2γδ2), is found (Minnich, Cordonnier, Williams, and Moore, 1962; see also later for further references).

### Haemoglobins with Unusual Structures.

(a) **Haemoglobins Without α-Chains.** Three haemoglobins without α-chains have been described, corresponding to the normal haemoglobins A, F, and A2. All these haemoglobins migrate on electrophoresis more rapidly towards the anode than their α-chain-containing parent species, and this difference in electrophoretic mobility is more marked near neutral pH. Hb-H, the first of these to be discovered (Rigas, Koler, and Osgood, 1955, 1956; Gouttas, Fessas, Tsevrenis, and Xefteri, 1955), consists of four βA-chains: Hb-βA4 (Jones, Schroeder, Balog, and Vinograd, 1959). Fast foetal haemoglobin (Fessas and Papsyrou, 1957) or Hb-Bart’s (Ager and Lehmann, 1958) consists of four γF-chains, Hb-γ4 (Hunt and Lehmann, 1959; Kekwick and Lehmann, 1960), and Hb-δ consists solely of δ-chains (Dance and Huehns, 1962). Hb-Gower I may be a fourth haemoglobin without α-chains, possibly consisting solely of ε-chains (Huehns et al., 1964a). Huisman (1960) reports the occurrence of a haemoglobin consisting solely of β chains, Hb-Augusta I, and another with only βC-chains, Hb-Augusta II.
Fig. 9. The amino acid substitutions in the haemoglobin variants (modified from Baglioni, 1963a).
(b) **Haemoglobins Consisting Solely of α-Chains.** Two haemoglobins of this type have been described: Hb-α^A^ (Huehns, Shooter, Dance, Beaven, and Shooter, 1961b) and Hb-α^G^ (Huehns, Dance, Shooter, and Beaven, 1962b). These workers prepared the α-chain haemoglobins by acid dissociation of Hb-A or Hb-F. Since then, Hb-α^A^ has been detected by starch gel electrophoresis in small amounts in haemolysates from patients with α-thalassaemia major, particularly following splenectomy (Fessas and Loukopulos, 1964), and from patients with unstable haemoglobins (Huehns and Shooter, unpublished). Hb-α^G^ cannot be detected in normal haemolysates prepared either from normal adult blood or cord blood by starch gel electrophoresis, though 0-02% of added Hb-α^A^ can be detected. Chernoff (1964) has isolated trace quantities (0-05-0-3%) of Hb-α^A^ from normal haemolysates by DEAE column chromatography. However, the finding that when Hb-A, or Hb-F are present more Hb-α^A^ can be detected on re-chromatography of the DEAE column eluate raises the possibility that DEAE promotes the dissociation of some haemoglobins.

(c) **LEPORE Haemoglobin.** Hb-Lepore was first described by Gerald and Diamond (1958b) in association with the clinical picture of thalassaemia. On starch block electrophoresis Hb-Lepore migrates with Hb-S and amounts to approximately 10-15% of total haemoglobin in the trait form. There are at least two forms of Hb-Lepore. Baglioni (1962b) has shown that these haemoglobins consist of the N-terminal part of the δ-chain joined to the C-terminal part of the β-chain, the total number of amino acids in the new δ-β-chain being the same as in the β-chain. There are several types of Hb-Lepore, which differ in the proportion of δ- and β-chain present. In Hb-Lepore_Boston_, obtained from a subject of Italian origin (Gerald and Diamond, 1958b; Baglioni, 1962b), tryptic peptides 1-5 are like those in the δ-chain, and 6-11 could be either from the δ- or β-chain, while 12-15 are β-like. Hb-Lepore_Hollandia_ (Neeb, Beiboer, Jonxis, Kaars Sijpsteijn, and Muller, 1961; Barnabas and Muller, 1962; Baglioni, 1962b) has tryptic peptides 1-3 from the δ-chain and 4-15 from the β-chain (Fig. 10). Several other haemoglobins are probably of the Lepore type: Hb-Pylos (Fessas, Stamatoyannopoulos, and Karaklis, 1962) and Hb-G (Silvestroni and Bianco, 1958). A Hb-Lepore found in a subject of Italian origin has a similar structure to that of Hb-Lepore_Boston_ (Barkhan, Stevenson, Pinker, Dance, and Shooter, 1964).

Baglioni (1962b) has discussed the genetic event that could have led to the formation of a δ-β-polypeptide chain. Two possibilities are mentioned, both of which require the Hb_δ_ and Hb_β_ loci to be closely linked (see later). The first possibility is a deletion of part of the δ and β loci with the formation of a new δ-β gene. On this hypothesis it is difficult to understand why the δ-β-chain synthesized is the same length as the β- or δ-chain. The second, and apparently more likely, situation, which accounts for the
Methods for the Identification of the Abnormal Haemoglobin

(i) Electrophoresis. This is the primary method for the detection of any haemoglobin variant. Originally, moving boundary electrophoresis was used, but this was soon replaced by paper electrophoresis, and this method is the most widely used technique. However, a number of the haemoglobins that are associated with disease, such as Hb-Lepore, Hb-St. Mary's, Hb-H, and Hb-γ4, are not easily detected by this procedure, particularly as they are often present in low concentrations, while variants of Hb-A2 cannot be detected at all. Starch gel electrophoresis has a much greater sensitivity and is now the method of choice. Suitable buffer systems have been previously described (Gammack, Huehns, Shooter, and Gerald, 1960), but the greatest over-all sensitivity can be achieved using a tris-E.D.T.A.-borate buffer system (O. Smithies, unpublished). This is sensitive both to the rapidly migrating haemoglobins such as the non-α-chain haemoglobins, Hb-β4 and Hb-γ4, as well as to "slow" minor components. The usual pH used is 8.6 (109 g. tris [2-amino-2-(hydroxy-methyl) propane-1:3 diol] 5.84 g. disodium E.D.T.A., 30.9 g. boric acid/l., diluted 1 to 20 for making the gel and 1 to 7 for the buffer vessels). One of the advantages of this system is that concentrated haemolysates can be applied without overloading the gel, thus facilitating the detection of minor components. The sensitivity can be further increased by staining the gel with benzidine or o-tolidine. Another advantage is that 1% of Hb-F can be detected. In order to detect the presence of Hb-α4, which remains on the origin in this system at pH 8.6, the gel can be made at pH 8.3, using 69.9 g. tris/litre instead of 109 g.

The relative concentration of Hb-A2, which is of some importance in the diagnosis of thalassaemia trait, is best estimated by starch block electrophoresis (Kunkel, 1954). It can also be estimated by column chromatography on DEAE (Huisman and Dozy, 1962). In practice, these methods are time-consuming and open to error if not carried out carefully. An estimate to determine whether Hb-A2 is raised can be obtained by careful comparison of the Hb-A2 zones obtained on starch gel electrophoresis from normal haemolysates with those from suspected thalassaemia minor after adjustment of the haemoglobin concentration in both.

Agar gel is also a good supporting medium for electrophoresis of haemoglobins (Yakulis, Helliwell, Josephson, and Singer, 1960; Shibata and Iuchi, 1962) and gives some special separations when used at acid pH (Robinson et al., 1957).

(ii) Chromatography. This method is mainly used for the purification of haemoglobins before chemical analysis and has been carried out on IRC50 (Allen et al., 1958; Clegg and Schroeder, 1959; Schneek and Schroeder, 1961; Jones and Schroeder, 1963), carboxymethylcellulose (CMC; Huisman et al., 1958) or diethylaminoethylcellulose (DEAE) (Huisman and Dozy, 1962). Huisman and Prins (1955) have described a qualitative method which has often been used for the identification of haemoglobins (see Lehmann, 1960).

(iii) Hybridization. This method, first devised by Itano and his co-workers (Itano and Singer, 1958; Itano, Singer, and Robinson, 1959), is used in the identification of the abnormal chains of a haemoglobin variant. A suitable micro-adaptation of the original method, using starch gel for the analysis of the haemoglobins formed, has been described (Gammack et al., 1960). More recently, the formation of hybrid haemoglobins between human and canine haemoglobins has been used (see Fig. 12) (Itano and Robinson, 1959; Robinson and Itano, 1960; Huehns, Shooter, and Beaven, 1962a; Shibata, Iuchi, Ueda, Miyaji, and Takeda, 1962b).

(iv) 'Fingerprinting'. This method is used to identify the abnormal tryptic peptide and is fully described by Ingram (1958) and Baglioni (1961). The globin of the purified abnormal haemoglobin is prepared (Anson and Mirsky, 1930; Ross-
Fanelli, Antonini, and Caputo, 1958; Teale, 1959) and digested with trypsin. The resulting peptide mixture is then analysed by high-voltage electrophoresis, followed by chromatography at right angles. The resulting peptide pattern or 'fingerprint' is compared with that prepared from normal haemoglobin (Fig. 13). The abnormal peptide is then eluted and the amino acid sequence determined. One of the drawbacks of this method is that some of the peptides formed are insoluble, the so-called 'core'. Recently, Jones (1964) has aminoethylated the cysteinyl residues in haemoglobin by treatment of the globins with ethylenimine at pH 8.6 in 8M urea. The S-(2-aminoethyl)-cysteinyl ('pseudolysine') residues formed lead to several more sites in the 'core' of the molecule, which are cleaved by trypsin, and thus no insoluble peptides are formed. The peptide mixture formed can then be conveniently separated by automatic column chromatography.

One of the questions in the examination of the abnormal haemoglobin is how far the investigation of each separate sample containing a haemoglobin variant should be taken. From the clinical point of view, only a limited amount of work is necessary. The ability of the red cells to sickle on reduction with metabisulphite would distinguish between the presence of Hb-D and Hb-S. The identification of some of the other abnormal haemoglobins, such as Hb-Lepore, Hb-H, Hb-M, and the unstable haemoglobins, is suggested by the associated clinical picture. For the remaining haemoglobins, the identification is often presumptive, depending on careful comparison with known markers. The relative proportion of the abnormal haemoglobin found in the heterozygote and the origin of the patient are both taken into consideration. From the biochemical geneticist's point of view, no identification is complete until the abnormal amino acid residue has been located. Even haemoglobins apparently identical by electrophoresis and chromatography as well as by hybridization are found to be different by fingerprinting, for example, the various types of Hb-D and Hb-J mentioned earlier. On the other hand, an identical fingerprint is found in Hb-J_{Rambam} and Hb-Seattle. One of these has a glycine residue replaced by aspartic acid in tryptic peptide IX, while in the other an alanine is replaced by glutamic acid in the same peptide. In practice, rare variants found in ethnically different populations probably merit detailed study.
(v) Separation of the Polypeptide Chains of Hb-A. The peptide analyses described above are very much simplified if the α- and β-chains are first separated. This can be achieved by column chromatography on Amberlite IRC 50 in a urea gradient at pH 2 (Wilson and Smith, 1959) or by countercurrent distribution (Hill and Craig, 1959). Hayashi (1961) has separated the chains by differential precipitation. The globin chains can also be obtained by starch gel electrophoresis (Muller, 1960). It is much more difficult to separate the polypeptide chains with their haem groups attached, and chromatography at acid pH gives only small yields (Huehns et al., 1961b). More recently, striking separations of the α- and β-chains of Hb-A with their haem groups still attached have been obtained after reacting the haemoglobin with an excess of sodium para-(Chloromercuri) benzoate (PCMB) in the presence of 0.2M-NaCl at pH 6.0 (R. Bucci and C. Frontacelli, unpublished).

Other Biochemical Studies

(i) Immunological Studies of Human Haemoglobins. Immunological studies on haemoglobin have been reported, and antibodies to Hb-A, Hb-F, and Hb-A₂ have been prepared (Chernoff, 1953a, Westendorp Boerma, Huisman, and Mandema, 1960; Heller, Yakulis, and Josephson, 1962a; Schneider and Arat, 1964; Askonas and Smyth, 1964). Rucknagel and Chernoff (1955) have used anti-Hb-F to measure small amounts of Hb-F in normal adults and during pregnancy.

Recently, Westendorp Boerma and Huisman (1964) have produced antisera in rabbits which, after suitable absorption procedures, can differentiate between Hb-S and Hb-C, a remarkable degree of specificity when it is remembered that these haemoglobins only differ by one amino acid on each of the β-chains. Another interesting observation is that there is an immunological difference between horse oxy- and deoxyhaemoglobin (Reichlin, Bucci, Antonini, Wyman, and Rossi-Fanelli, 1964). This observation provides further evidence that oxyhaemoglobin has a different conformation from deoxyhaemoglobin, as has already been shown by the studies of Muirhead and Perutz (1963) referred to above.

(ii) The Site of Chromium Attachment to Haemoglobin. Radioactive chromium (⁵¹Cr) is commonly used to label red cells for in vivo red cell survival studies. It is known that it is attached to the haemoglobin molecule (Gray and Sterling, 1950). Experiments by Chernoff (1961) suggest that the site of attachment might be on the α-chains. However, the work of several other investigators (Pearson and Vertrees, 1961; Heisterkamp and Ebaugh, 1962; Malcolm, Ranney and Jacobs, 1963) makes it more likely that it is mainly attached to the β-chain. Perhaps the method of labelling red cells with ⁵¹Cr is critical.

II: The Genetic Control of Haemoglobin Synthesis

Structural Loci

Adult Haemoglobin. In 1949, Pauling et al. showed that the haemoglobin of patients with sickle cell disease differed from that of normal persons and their studies indicated that,

'two normal genes lead to the production of only normal haemoglobin; one normal and one abnormal gene lead to the production of both normal and sickle haemoglobin and two abnormal genes lead to the production of only sickle haemoglobin'.

At about the same time, the family studies of Beet (1949) and of Neel (1949), using the sickle
test, also demonstrated the inheritance of the disease. Shortly after this, a number of abnormal haemoglobin (Hb-C, Hb-D, Hb-E, etc.) were discovered which showed similar inheritance, and Itano (1953) suggested that because individuals with both Hb-C and Hb-S carried no Hb-A, the genes responsible for these two haemoglobins were alleles. This was shortly afterwards confirmed by the family studies of Ranney (1954). A similar allelism was proposed for Hb-D, Hb-E, and Hb-G (Neel, 1956). This rests mainly on the finding that double heterozygotes carry no Hb-A, rather than on classical genetic analysis. The discovery by Smith and Torbert (1958) of a family where two abnormal haemoglobins, Hb-S and Hb-Hopkins 2, were segregating independently, and the finding that one had abnormal \( \alpha \)-chains while the other had abnormal \( \beta \)-chains, led to the present concept of the genetic control of haemoglobin synthesis (Itano et al., 1959).

Because the abnormal haemoglobins have either abnormal \( \alpha \)-chains or abnormal \( \beta \)-chains, it was suggested that these were controlled by separate genetic loci (Itano and Robinson, 1959; Ingram, 1959). Studies of several families (see Fig. 14) (Smith and Torbert, 1958; Itano et al., 1959; Atwater, Schwartz, and Tocantins, 1960b; Baglioni and Ingram, 1961; Raper, Gammack, Huehns, and Shooter, 1960) in which \( \alpha \)-chain and \( \beta \)-chain variants were segregating and in which some individuals had inherited both abnormalities showed that the locus controlling the synthesis of the \( \alpha \)-chains, the so-called Hb\(_{\alpha}\) locus, was not linked to that controlling the \( \beta \)-chain, the so-called Hb\(_{\beta}\) locus. Furthermore, those individuals that had inherited both abnormalities carried not only both abnormal haemoglobins but also Hb-A and a fourth haemoglobin with both abnormal \( \alpha \)- and \( \beta \)-chains (Raper et al., 1960; Baglioni and Ingram, 1961). On the basis of all these findings, a hypothesis for the genetic control of synthesis of Hb-A was developed (Itano and Robinson, 1960; Raper et al., 1960; Baglioni and Ingram, 1961).

The synthesis of the \( \alpha \)- and \( \beta \)-chains of Hb-A is controlled by pairs of genes at different genetic loci, and the genotype can conveniently be written \( Hb^a A Hb^a A Hb^b B Hb^b B \) (the superscript \( A \) indicating that the gene is controlling the synthesis of a normal polypeptide chain). Each gene at each locus indirectly determines the synthesis of a particular type of polypeptide chain. These then associate to form the haemoglobins found in vivo (Fig. 15).

Those individuals who inherit one abnormal gene will make two haemoglobins (Fig. 16).

If a person inherits two abnormal genes, three different situations can arise.

(i) A person can inherit two abnormal genes of the same type, as, for example, in the genotype \( Hb^a A Hb^a A Hb^c B Hb^c B \). In these subjects, only \( \alpha^2 \) and \( \beta^2 \) chains are formed, and only Hb-C (\( \alpha^2 \beta^2 \)) is found in the red cells. The inheritance of two genes of the same type usually leads to a

Fig. 14. Pedigree of 'Hopkins 2 pedigree' showing independent segregation of the genes for Hb-S and Hb-Hopkins 2 (from Smith and Torbert, 1958; and Bradley et al., 1961).
mild haemolytic disease, such as Hb-C disease, Hb-E disease, etc.

(ii) When two different abnormal genes occur at the same locus, as, for example, in the genotype $Hb_{\alpha}^{A}/Hb_{\alpha}^{A}$ $Hb_\beta^{G}/Hb_\beta^{C}$, $\alpha^A$, $\beta^G$, and $\beta^C$-chains are made, leading to the formation of only Hb-S and Hb-C.

(iii) Finally, if two different mutations occur, one at each locus, as, for example, in the individuals with four adult haemoglobins mentioned earlier, four different polypeptide chains are synthesized, leading to the formation of four haemoglobin species (Fig. 17).

In this connexion, it is of interest that individuals with clinically severe sickle cell anaemia have been reported who carry two abnormal haemoglobins.
Human Haemoglobins

Haemoglobin genotype

Hb_A / Hb_α

Hb_A1 / Hb_α1

Hb_A2 / Hb_α2

Hb_F / Hb_F

Hb_Gower 1 / Hb_Gower 2

Polypeptide chains synthesized

β^A

δ^A

α^A

γ^F

ε^ε

Subunits formed

α^A β^A

α^A δ^A

α^A γ^F

α^A ε

ε^ε

Haemoglobins found in vivo

Hb-A

Hb-A1

Hb-A2

Hb-F

Hb-Gower 1

Hb-Gower 2

Adult

Foetal

Embryonic

Fig. 18. The genetic control of the synthesis of normal adult haemoglobins, Hb-A and Hb-A1, foetal haemoglobin, and the embryonic haemoglobins, Hb-Gower 1 and Hb-Gower 2 (from Huehns and Shooter, 1964a).

(Pugh, Monical, and Minnich, 1964; Hall-Craggs, Marsden, Raper, Lehmann, and Beale, 1964). In these individuals there is homozygosity at the Hb_β locus for the β^A gene but heterozygosity at the Hb_α locus, giving the genotype Hb_β^A Hb_β^α Hb_α^δ Hb_α^ε. Clearly, two haemoglobins, Hb-S (α^A δ^A) and Hb-Gower S (α^A β^α) will be made, and, as both contain β^A-chains, the patient clinically has sickle cell disease.

Haemoglobins A^A and F. The genetic control of synthesis of Hb-A and Hb-F follows the same pattern as that of Hb-B. As the α-chains of the normal human haemoglobin are all identical, it has been suggested that these arise from a common metabolic pool and thus are controlled by the same genetic locus (Huehns and Shooter, 1961a). The non-α-chains of Hb-A and Hb-F, the δ- and γ-chains respectively, are controlled by their own genetic loci, the Hb_δ and Hb_γ loci. Although no genetic studies on the embryonic haemoglobins have been possible, it is postulated that there is a separate genetic locus for the ε-chain. The outline for the control of synthesis of the normal haemoglobins would then be as in Fig. 18. The above hypothesis is supported by the discovery of variants of δ- and γ-chains mentioned earlier. The idea that the α-chains of Hb-F, Hb-A, and Hb-A are formed from a common metabolic pool implies that if abnormal α-chains are being synthesized, not only will a Hb-A variant be found but also the corresponding variants of Hb-F and Hb-A1 with abnormal α-chains (Fig. 19).

Studies of several people synthesizing abnormal α-chains have, in fact, shown that they carry not only the corresponding Hb-B variant but also the corresponding variant of Hb-A: Hb-Gower (Shooter, Skinner, Garlick, and Barnicot, 1960; Huehns and Shooter, 1961a, b), Hb-I (Atwater, Huehns, and Shooter, 1961; Bourd, Cosset, Destaing, Ducer, Jonxis, Muller, and Portier, 1961), Hb-Q (Dornandy, Lock, and Lehmann, 1961), Hb-No (Baglioni, 1962b), Hb-D and Hb-G (Weatherall and Boyer, 1962) and Hb-F: Hb-G and Hb-D F (Minnich et al., 1962; Weatherall and Boyer, 1962; Weatherall and Baglioni, 1962), Hb-I (Raney, dO'Brien, and Jacobs, 1962).

‘Controller Genes’ in Haemoglobin Synthesis

During development, the synthesis of embryonic haemoglobin is succeeded by the synthesis of foetal haemoglobin which, in turn, is replaced by the adult haemoglobins, Hb-A and Hb-A1, shortly after birth. This can be explained in terms of regulatory genes which control the rates of synthesis of various polypeptide chains, a concept derived from work in bacterial genetics (Jacob and Monod, 1961). A mutation at one of these controller loci might result in changes in the rate of synthesis of a polypeptide chain without any alteration in its structure, and it has been suggested that ‘the hereditary persistence
of foetal haemoglobin' and 'thalassaemia' are of this type (Neel, 1961; Motulsky, 1962; Sturgeon, Schroeder, Jones, and Bergren, 1963; Zucker-kandl, 1964).

### Linkage Relationships of the Loci Controlling the Synthesis of Haemoglobin

(i) **Hb\(\alpha\)** and **Hb\(\beta\)** Loci. Studies of several families in which both \(\alpha\)- and \(\beta\)-chain variants of Hb-A are segregating (quoted above) show that the locus controlling the synthesis of the \(\alpha\)-chains is not linked to that of the \(\beta\)-chains. It has also been shown that neither loci are closely linked to genetic loci controlling certain blood or serum groups (Bradley, Brawner, and Conley, 1961).

(ii) **Hb\(\beta\)** and **Hb\(\delta\)** Loci. There are several families in which a Hb-A\(\alpha\) variant with abnormal \(\delta\)-chains segregates with a \(\beta\)-chain variant and no cross-overs have been detected, though this could have taken place in 45 instances (Ceppellini, 1959; Horton et al., 1961; Ranney et al., 1963; Boyer Rucknagel, Weatherall, and Watson-Williams, 1963; Huisman, 1963). These family studies thus indicate that the locus controlling the synthesis of the \(\delta\)-chain is closely linked to that for the \(\beta\)-chain. The family study of Huisman, Punt, and Schaad (1961) showed that the locus for \(\delta\)-chains was linked to that for \(\beta\)-thalassaemia, and as the latter was linked or allelic to that controlling the synthesis of \(\beta\)-chains (see below), it also strengthened the finding of \(\delta\) to \(\beta\) linkage.

(iii) **Hb\(\gamma\)** and **Hb\(\gamma\)** Loci. At the present time, no studies of families in which variants of both the \(\gamma\)- and \(\beta\)-chains occur have been reported, and the question of whether the locus controlling the synthesis of the \(\gamma\)-chains is linked to the locus controlling the synthesis of \(\beta\)- and \(\delta\)-chains cannot be definitely answered. However, studies of families in which the \(\beta\)-chain variants Hb-S or Hb-C are segregating with a gene causing persistence of foetal haemoglobin, the so-called 'high-F' gene, indicate that the 'high-F' gene is closely linked (or allelic) to the \(\beta\)-chain locus (Rucknagel and Neel, 1961; Conley, Weatherall, Richardson, Shepard, and Charache, 1963). At present, it seems reasonable to assume that the 'high-F' gene might be at, or closely linked to, the locus for the \(\gamma\)-chains, and this leads to the conclusion that Hb-\(\gamma\) and Hb-\(\delta\) loci are closely linked.

(iv) **Linkage Relationships of the Thalassaemia Loci.** The results of studies of families in which both \(\beta\)-thalassaemia (see later) and haemoglobins S, C, or B\(\beta\) are segregating have been reviewed by Rucknagel and Neel (1961) and Motulsky (1964b) and indicate that the gene causing \(\beta\)-thalassaemia is closely linked or allelic to the \(\beta\) and \(\delta\) structural loci. There appears to be only one clear instance of a cross-over between the \(\beta\)-thalassaemia gene and that for an abnormal haemoglobin, in this case, Hb-B\(\delta\) (Moore and Pearson, 1964). Motulsky (1964b) tentatively interprets this finding as evidence in support of the idea that the locus for \(\beta\)-thalassaemia is outside the \(\beta\delta\)-structural
locus but closely linked to it. Clearly, further family studies to resolve this point are needed.

The α-thalassaemia locus, like the Hbα structural locus, is not linked to the Hbβ structural locus (Tuchinda, Rucknagel, Minnich, Boonyaprakob, Balankura, and Suvatee, 1964). There are no data yet to show whether α-thalassaemia trait is linked to the Hbα structural locus. The biochemical interaction between α-thalassaemia and α-chain variants of haemoglobin is similar to that between β-thalassaemia and the various β-chain haemoglobin variants, and this suggests that the genetical situation might be analogous to that for β-thalassaemia.

The Problem of the Inheritance of Haemoglobin M

Although, in several families, Hb-M follows the semi-dominant pattern of Mendelian inheritance seen with other abnormal haemoglobins, in several instances the disease is not found in either of the parents of one of the affected individuals. Three families (Sahawi, Hunger, and Betke, 1962; Josephson, Weinstein, Yakulis, Singer, and Heller, 1962; Heller, Weinstein, Yakulis, and Rosenthal, 1962b) like this have now been studied in detail, and the determination of a number of blood group characters in various members of the families is consistent with the stated parentage (Sahawi et al., 1962; Josephson et al., 1962). This could be explained if the formation of methaemoglobin in vivo in these individuals only occurred by the interaction of the haemoglobin abnormality with some other, as yet undiscovered, factor. This explanation is ruled out by the absence of any haemoglobin abnormality in detailed studies of the unaffected family members. It would then appear that the occurrence of Hb-M in these individuals is due to new mutations.

Haemoglobins and Chromosomal Abnormalities

It appears firmly established, as outlined in the preceding section, that haemoglobin synthesis is controlled by a number of genetic loci, one structural locus for each of the different polypeptide chains. There are probably also a number of regulatory loci. One of the most intriguing questions is on which of the 46 chromosomes these loci are situated. The inheritance of the abnormal haemoglobins indicates that they are not X-linked. Recently, the haemoglobins from patients with the various chromosomal triplications, the so-called trisomies, have been investigated. Huehns, Hecht, Keil, and Motulsky (1964c) and Huehns et al. (1964a, b) report that, in trisomy 13–15, trace amounts of the embryonic haemoglobin Hb-Gower 2 persist until birth and that Hb-γ4 is present in slightly increased amounts. They also found that in six older children with the same trisomy, Hb-F was increased. The data on the Hb-F in these individuals are still very scanty, but it appears that its disappearance is delayed as compared to that of normal individuals. Powars, Rohde, and Graves (1964) have also found persistence of Hb-F in their patient with trisomy 13–15. These results can be interpreted in several ways, but raise the possibility that some of the loci controlling haemoglobin synthesis are located on the chromosome triplicated in this syndrome. However, another view is that these findings represent a more generalized immaturity in biochemical development. The haemoglobins found in trisomy 17–18 and trisomy 21–22 (Down’s syndrome) have also been studied. Huehns et al. (1964c) report no difference from normal in both conditions (one patient with trisomy 17–18 and six with trisomy 21–22). D. L. Rucknagel (personal communication) has found that in 10% of Negroes with Down’s syndrome, Hb-F is increased (90 cases studied).

Further information will be obtained if similar patients can be found who also have an abnormal haemoglobin, since the ratio of normal to abnormal haemoglobin might differ if the triplicated chromosome carries one or more of the loci controlling haemoglobin synthesis. D. L. Rucknagel (personal communication) has studied the Hb-A to Hb-S ratios in patients with trisomy 21–22 and found them to be normal. Of course, if a patient with one of the trisomies carries, say, Hb-S, Hb-C, and Hb-A, he must then have inherited three different Hbβ structural genes, one on each of the triplicated chromosomes. Although the combination of a mating of a patient with Hb-S-C disease with a normal person giving rise to an infant with one of the trisomies must be extremely rare, the search for and detailed study of such patients may lead to further advances in this interesting field.

The Abnormal Haemoglobins and the Genetic Code

In the genetic control of protein synthesis, it is postulated that the amino acid sequence of any protein is represented on the chromosome by a definitive sequence of bases in the DNA. Crick, Barnett, Brenner, and Watts-Tobin (1961), from their genetic experiments in bacteria, have suggested that each amino acid is coded for by three (or perhaps a multiple of three) bases and that each
‘code word’ is specific for one amino acid. The different code words are non-overlapping and follow directly one after another. It appears that the code is ‘degenerate’, that is, there may be more than one code word for any particular amino acid. In order that synthesis of protein can take place, the DNA code is transcribed on to the so-called messenger RNA (mRNA) which is thought to form the template on the ribosomes on which the protein is synthesized. Each amino acid is added at the right place because of the complementary fit of the amino acid transfer RNA. This hypothesis implies that the amino acid sequence of a protein is co-linear with the nucleotide sequence in the corresponding gene (Sarabhai, Stretton, Brenner, and Bolle, 1964; Yanofsky, Carlton, Guest, Helinski, and Henning, 1964). The triplet code letters which probably code for each amino acid on the mRNA have been the subject of much investigation (Nirenberg, Jones, Leder, Clark, Sly, and Pestka, 1963). A current summary of the proposed RNA code words is given in Table III. It can be

seen that more than one ‘code word’ has been proposed for some amino acids, in keeping with the suggested ‘degeneracy’ of the genetic code. It has been postulated that a point mutation is a change of a single nucleotide base in a three-letter code word and all the known amino acid substitutions in the abnormal haemoglobins are consistent with this hypothesis. For example, the two substitutions at position 6 of the \( \beta^A \)-chain, glutamic acid \( \rightarrow \) valine in Hb-S and glutamic acid \( \rightarrow \) lysine in Hb-C fit in as shown below.

\[
\begin{align*}
(\text{Hb-}\text{A}) & \quad \text{Glu} \\
& \quad \text{UUG} \\
(\text{Hb-}\text{C}) & \quad \text{Lys} \\
& \quad \text{UAA}
\end{align*}
\]

### III: THE BIOSYNTHESIS OF HAEMOGLOBIN

It has been shown that the synthesis of the \( \alpha \)- and \( \beta \)-chains of Hb-A are controlled by separate, unlinked genes, and that in individuals carrying both abnormal \( \alpha \)-chains and abnormal \( \beta \)-chains four types of haemoglobins are found (Fig. 17). It can thus be assumed that the \( \alpha \)-chains are synthesized separately from the \( \beta \)-chains. From the work of Bishop, Leahy, and Schweet (1960) and Dintzis (1961), it is known that synthesis of the polypeptide chains begins at their N-terminals and the amino acids are added sequentially until the chains are completed. The haem groups are separately synthesized (see Rimington, 1959). Following synthesis, the polypeptide chains and haem groups are assembled into the tetramer haemoglobin found in the red cell. At present, it is not known at which stage the haem groups are attached, but there is some knowledge of the order in which the polypeptide chains assemble. This comes from recent experiments on the dissociation of haemoglobin.

#### The Dissociation of Haemoglobin

It is well known that haemoglobin dissociates at acid pH and that in mixtures of suitable haemoglobins exchange of chains takes place with the formation of hybrid haemoglobins. A good illustration is the formation of hybrids between human and canine haemoglobins (Robinson and Itano, 1960; Huehns et al., 1962a) (Fig. 12). The composition of the hybrids formed is given by the following over-all reaction:

\[
\frac{\alpha^A \beta^A}{2} + \frac{\alpha^C \beta^C}{2} \rightarrow \frac{\alpha^A \beta^C}{2} + \frac{\alpha^C \beta^A}{2}.
\]

Some of each of the parent species are also reformed. Although this result may be explained most readily by postulating that each haemoglobin dissociates into \( \alpha \) and \( \beta \) subunits and that these subunits then exchange, there are a number of observations...
that do not fit into this scheme. For example, it is known that both Hb-A and canine haemoglobin dissociate into subunits immediately the pH is lowered below 5.0. Yet it is necessary to hold the two haemoglobins at these pH values for many hours before exchange of subunits and formation of hybrids occur (Labie, Rosa, Dreyfus, and Shapira, 1962; Huehns, Shooter, and Beaven, 1964d). Clearly if the subunits were α and β, there would be no reason for this delay in exchange. However, it can be accounted for if it is assumed that the initial rapid dissociation is into αβ subunits, that these further dissociate at a slower rate into single α- and β-chains, and that the exchange takes place at the single chain level (Vinograd and Hutchinson, 1960). Supporting evidence for this mechanism comes from the finding that the isolated αA-chain subunits from Hb-A do, in fact, exist in solution as stable monomers rather than αβ dimers (Huehns et al., 1961b). The question then arises as to why only like αβ subunits unite, since species with unlike α- or unlike β-chains have not been observed. Moreover, attempts to produce such species by a number of different experimental approaches have failed (Huehns and Shooter, 1964b). For example, globins with the structure αβ have been prepared which reform tetramer haemoglobins on the addition of haem (Winterhalter and Huehns, 1964). When a mixture of globin C is mixed with globin A and haem added, three haemoglobin species could form, as follows.

However, on starch gel analysis of the reacted product, only Hb-A and Hb-C were detected. What then prevents an αAβA subunit combining with an αAβC subunit to form the hybrid αAβAβC? One explanation (Vinograd and Hutchinson, 1960) is that recombination is specific and that only like αβ subunits can combine with one another. This explanation seems unlikely, since it is very difficult to see how a single amino acid substitution on the surface of the coiled polypeptide chain could prevent association of an αAβA subunit with an αAβC subunit. Moreover, all the single amino acid substitutions in the many abnormal haemoglobins would have to obey the same restriction. Recently Guidotti, Konigsberg, and Craig (1963) have put forward a plausible explanation. They suggest that such mixed hybrids with unlike α- and unlike β-chains do form, and that there is no specificity in the recombination of αβ subunits. They argue that the reason why these species are not observed is because even at neutral pH haemoglobin is dissociated (Drabkin and Wise, 1962; Huehns, 1962; Huehns, Beaven, and Stevens, 1964f) and is in equilibrium with αβ dimers, i.e. αβ = 2αβ (Huehns, 1962). It follows that in any separation process the species αβαβ, for example, will dissociate into αAβA and αAβC subunits and that these will then predominantly form the species αβαβ and αβαβ. This hypothesis eliminates the need to postulate steric factors to prevent unlike αβ subunits combining.

There are two haemoglobin species in which unlike chains occur, Hb-F (αAβXγF) (Schroeder et al., 1962) and Hb-A(αAβXαF) (Holmquist and Schroeder, 1964). In Hb-F1, the N-terminal of one of the γ-chains is blocked by an acetyl group, while in Hb-A(αAβXαF) the N-terminal of one of the β-chains is blocked by an unidentified grouping. If the above explanations prove correct, then some special explanation for the ability to isolate these two mixed species must be found and this is discussed elsewhere (Huehns and Shooter, 1964b).

It does not necessarily follow that the assembly of the haemoglobin molecule will maintain the same course as its dissociation, but the properties of the isolated subunits, in particular, the stability of the monomer αA-chain subunit, as well as the close contact between α- and β-chain subunits demonstrated by the x-ray work, make this the most plausible mechanism.

Since, in the normal adult, equal numbers of α- and β-chains are produced, there is presumably a mechanism adjusting the rate of synthesis of one of the chains to that of the other. In deficiency of α-chain synthesis—α-thalassaemia—Hb-β, is found in quantity (see later), while in the severest deficiencies of β-chain synthesis—β-thalassaemia major—only trace amounts of free α-chains are found (Fessas and Loukopoulos, 1964). These findings have led to the suggestion that completed β-chains are necessary for the free release of α-chains from their site of synthesis (Huehns and Shooter, 1962). In this way, the effective rate of α-chain synthesis...
is adjusted to that of β-chains. In release of the completed chains, an αβ subunit is formed, and finally two of these combine to form the tetramer haemoglobin molecules found in the red cell. The haem group is separately synthesized. It appears that the rate of haem synthesis is linked to that of globin synthesis by a feedback mechanism, any excess haem formed depressing the activity of δ-aminolaevulic acid (δ-ALA) synthetase (Lascelles, 1960; Burnham and Lascelles, 1963).

It is not clear yet at which stage the haem group combines with the globin chains. The finding that there is a small amount of free globin in the red cells (Winterhalter and Huehns, 1963) leads to the speculation that the αβ intermediate postulated above is globin (which is known to have the structure αβ) and that the addition of haem brings about the association of two αβ subunits.

IV: CLINICAL FEATURES OF ABNORMALITIES IN HAEMOGLOBIN SYNTHESIS

The various ways in which abnormalities of protein synthesis might cause disease are well demonstrated by the abnormalities in haemoglobin synthesis. It can be envisaged that disease can be caused in several ways:

(i) the production of an abnormal protein molecule—the haemoglobinopathies;

(ii) the reduction in the amount of normal protein synthesized—the thalassaemias;

(iii) developmental anomalies—the persistence of foetal haemoglobins.

The Haemoglobinopathies

The haemoglobinopathies can be divided into several groups. Clinically, the most important of these is sickle cell disease and its variants, and this disease process is brought about by a specific new property of the protein consequent to the amino acid substitution. The haemoglobin M diseases form another group, in which the ability to carry oxygen is due to the in vivo formation of abnormal methaemoglobins. Other haemoglobinopathies are characterized by the presence of haemolytic anaemia associated with the presence of unstable abnormal haemoglobins in the heterozygous state. Finally, there are the mild diseases that occur when some abnormal haemoglobin, such as Hb-C, Hb-D, or Hb-E, is found in the homozygous state.

(j) Sickle Cell Disease. The clinical features of this disease and the milder syndromes caused by the interaction with other haemoglobin syndromes are already well described (Wintrobe, 1961). However, in recent years, the way in which the substitution of a single amino acid (glutamic to valine) in each of the β-chains leads to this severe disease has now been delineated by Murayama (1962b, 1964). He has suggested that in Hb-S the substitution of a glutamic residue at position 6 by a valyl residue leads to the formation of a ring of amino acids at the N-terminus of each of the β-chains, the N-terminal valine forming a non-covalent (hydrophobic) bond with the genetically introduced valine at position 6. These cyclic structures on each of the β-chains are situated in such a position that when the haemoglobin molecule is reduced, they interlock with the α-chains of the next haemoglobin molecule, forming long chains. These aggregates are presumably birefringent spindle-shaped bodies, 1–15 μ in length, the so-called tactoids (Sherman, 1940), which characteristically occur in sickle cells. The fact that the in vitro and in vivo formation of sickle cells depends on the oxygen tension is well known (Hahn and Gillespie, 1927; Lange, Minnich, and Moore, 1951; Harris, Brewster, Ham, and Castle, 1956) and fits in well with Murayama’s ideas. Muirhead and Perutz (1963) have shown that the β-chains of haemoglobin are 7 A further apart than in the oxygenated form, while the α-chains show no such change in position. Clearly, if a ‘key’ on each of the β-chains can fit into the α-chains of the next molecule in the reduced state, this fit will not be possible in the oxygenated state when the β-chains in the molecule are closer together.

The sickling of the erythrocyte causes two significant changes. First, it causes an increase in the mechanical fragility (Diggs and Bibb, 1939; Lange et al., 1951; Harris et al., 1956) and this leads to the random destruction of a proportion of red cells before the process is reversed by re-oxygenation of the haemoglobin in the lungs. The second change is an increase in the viscosity of the blood (Ham and Castle, 1940; Harris et al., 1956), which, in certain circumstances, leads to stasis of the red cells, with consequent further loss of oxygen and increased sickling. The sickled cells then form thrombi which give rise to the well-known thrombotic symptoms of the disease. These trapped red cells are then lysed, adding further haemolysis to the random destruction of red cells mentioned above. The lessened severity of the different variants of sickle cell disease is well known and appears to be due to the relative resistance to sick-
ling of these cells when compared with sickle cell anaemia cells (Griggs and Harris, 1955). It appears that lower oxygen tensions are necessary for the production of sickling in these cells, as well as for the increase in mechanical fragility and blood viscosity. In sickle cell trait, sickling does not commence until the oxygen tension drops to less than 10 mm. Hg, which is lower than that normally found in the tissues. This finding is, of course, in agreement with the clinical observation that the trait condition only leads to pathology under special circumstances (Levin, 1958). In Hb-SC disease and sickle-cell thalassaemia, the increase in mechanical fragility of the red cells begins at an oxygen tension of 50 mm. Hg. These two diseases are, therefore, almost as severe as sickle cell disease. In sickle-cell persistent Hb-F disease, the increase in mechanical fragility does not begin until the oxygen tension drops to below 30 mm. Hg, and the clinical condition is relatively mild.

In this connexion, it is of interest that in vitro sickling of erythrocytes has been reported in association with other abnormal haemoglobins besides Hb-S, namely, Hb-γ, (Lie Injo Luan Eng, 1961), Hb-I (Atwater, Schwartz, Erslev, Montgomery, and Tocantins, 1960a) and Hb-C Georgetown (Pierce, Rath, and McCoy, 1963). Murayama (1964) has examined the sickling phenomena in red cells from the subject with Hb-C Georgetown and found that the mechanism differs from that causing sickling in red cells containing Hb-S.

(ii) Haemoglobin M Disease. In this group of haemoglobinopathies, the ability to carry oxygen is impaired due to the formation of an unusually stable methaemoglobin derivative. This leads in turn to cyanosis which is present at birth or commences shortly afterwards, but is not associated with the presence of any serious physical disability. The lack of disability distinguishes ‘biochemical’ methaemoglobinemia from ‘physiological’ methaemoglobinemia (due to congenital heart disease). The biochemical methaemoglobinemia fall into two groups: those due to deficiency in the methaemoglobin reductase enzyme system (Gibson, 1948; Scott, 1960; Townes and Morrison, 1962) and those due to abnormal haemoglobins; the latter are the Hb-M diseases.

Hörlein and Weber (1948, 1951) were the first to show that primary abnormality in their cases of methaemoglobinemia lay in the globin part of haemoglobin, while the haem group was normal. The disease is characterized by the appearance of cyanosis at birth or shortly afterwards, without any of the disabilities associated with cyanosis due to congenital heart disease. The prognosis is good. The disease can be distinguished from the other ‘biochemical’ methaemoglobinemia due to enzyme abnormalities by definitive differences in the spectrum of the acid methaemoglobin derivative and by the presence of the abnormal haemoglobin. The spectral difference seen is in the region of 550–650 mμ and consists of a shift of the normal absorption maximum 632 to a lower wavelength at approximately 600 mμ (Fig. 20). The degree of these changes varies in the different types of Hb-M described. Minor changes in the spectrum or the presence of Hb-M in whole haemolysates may only lead to filling in of the normal minimum at 600 mμ.

The electrophoretic demonstration of the abnormal haemoglobin may be difficult using conventional paper or starch gel electrophoresis, and analysis of the methaemoglobin derivatives at pH 7.0 is recommended (Gerald, 1958).

A large number of patients with Hb-M disease have been described and are reviewed in detail
by Jaffe and Heller (1964). The various haemoglobins M which have been chemically studied are listed in Table II, and it can be seen that several haemoglobins originally described separately are identical.

The stability of the methaemoglobin form and the spectral abnormality can readily be explained on the basis of the amino acid substitution found in these haemoglobins (Gerald and Efron, 1961; Baglioni, 1963a). In two of the Hb-Ms, the substitution is at the histidine residue distal to the haem group which, as has already been noted, is bonded in Hb-A to a water molecule on the sixth co-ordination position of the iron atom. In Hb-M_{Boston}, the histidine in the α-chains is replaced by tyrosine as is the corresponding histidine (position 63) in the β-chain in Hb-M_{Saskatoon}. The reason why the methaemoglobin form of these two haemoglobins resists reduction to the physiologically useful form of haemoglobin is explained by this substitution, the tyrosine anion being in a position to form a stable complex with the ferric iron of the haem group. Just how critical the location of this residue for the formation of such a complex is, is illustrated by the substitution in the variant Hb-Norfolk. Here, the next amino acid to the distal histidine residue in the α-chain is involved, an aspartic acid residue replacing the glycine (position 51), but no stable methaemoglobin results. This section of the polypeptide chain is an α-helix and therefore the glycine residue points in a direction which is at an angle of approximately 100° to the direction of the histidine residue. The side chain of the fourth residue from the distal histidine, on the other hand, points in roughly the same direction as the histidine, and a substitution here, glutamic acid replacing valine (position 567), produces a stable methaemoglobin, namely Hb-M_{Milwaukee}.

In Hb-M_{Watery}, the so-called proximal histidine at position 87 in the α-chain is replaced by a tyrosine residue (Miyaji, Iuchi, Shibata, Takeda, and Tamura, 1963a; Jones, Coleman, and Heller, 1964). In normal haemoglobin, this histidine is linked to the iron of the haem group. Presumably, in Hb-M_{Watery}, the haem group is held in position by co-ordination to the histidine at position 58 on the opposite side of the haem group, and the new tyrosine residue forms a similar, stable complex with the ferric iron as the tyrosine at position 58 in Hb-M_{Boston}, leading to the usual spectral abnormality.

Another haemoglobin variant that may be related to the haemoglobins M has been reported (Reissmann, Ruth, and Nomura, 1961). This haemoglobin causes cyanosis due to a lowered oxygen affinity and impaired haem-haem interactions.

Some of the unstable haemoglobins referred to in the next section have their abnormality in the same region of the polypeptide chain, near the haem group. These haemoglobins are associated with haemolytic disease and more rapid methaemoglobin formation in the red cell. Some of them also show differences in the absorption spectrum of the acid methaemoglobin derivative in the region 550–650 μm, similar to those seen in the Hb-Ms. Conversely, it might be expected that some of the M-type haemoglobins would show some instability with consequent haemolytic disease. A shortened 51Cr-labelled red cell survival and increase in reticulocytes have been reported in association with Hb-M_{Chicago (Saskatoon)} (Josephson et al., 1962). In several other instances a raised reticulocyte count has been reported (Heck and Wolf, 1958) (3·3%), Hb-M_{Milwaukee 1} (2·3%) (Pisciotta, Ebbe, and Hinz, 1959), Hb-M_{Milwaukee 2} (4%) (Pisciotta et al., 1959). There was definitive haemolysis in the patient who carried both Hb-C trait and Hb-M_{Milwaukee 2}, whereas in Hb-C trait the reticulocyte count was normal. It thus appears that some haemolysis is present in a number of patients with Hb-M disease.

(iii) The ‘Unstable Haemoglobin’ Diseases

A number of abnormal haemoglobin variants has recently been discovered in which an amino acid substitution produces a molecule that is less stable than the normal form, and a number of haemoglobinopathies due to such haemoglobins have now been described.

HAEMOGLOBIN ZÜRICH DISEASE. This is a familial haemoglobinopathy (Frick, Hitzig, and Betke, 1962; Bachmann and Marti, 1962) characterized by severe haemolytic crises precipitated by oral sulphonamides. During the crises, the haemoglobin drops to 3–6 g. per 100 ml. with a high (40%) reticulocytosis. The red cells in the stained smear show marked anisocytosis and fragmentation, with the presence of many large inclusion bodies, thought to be precipitated haemoglobin. During the quiescent stage of the disease there is very little anaemia, but haemolysis is still present, as shown by a raised reticulocyte count and red cell survival.

The abnormal haemoglobin is easily detected by electrophoresis at pH 8·6, migrating on paper or starch gel with Hb-S. The amino acid substitution is in the β-chains, the so-called distal histidine at position 63 being replaced by arginine.
(Muller and Kingma, 1961). The rate of methaemoglobin formation in the red cells incubated in vitro at 37° C. is significantly increased when compared to normal controls. No spectral abnormalities of this haemoglobin have been reported.

**Haemoglobin Ube-1 Disease.** This disease has been reported in one person from Japan (Shibata, Iuchi, Miyaiji, Ueda, and Takeda, 1963). His parents did not have any abnormality. It is a severe haemolytic disorder associated with Heinz body formation. The condition of the patient was considerably improved by splenectomy.

The abnormal haemoglobin is easily separated by electrophoresis, amounting to approximately 10% of total haemoglobin. It migrates between Hb-S and Hb-C. Structural studies indicate that this abnormality is in the insoluble tryptic peptides (X-XII) of the \( \beta \)-chain with loss of reactivity of the sulphydryl at position 92.

**Haemoglobin Seattle Disease.** This disease is a mild non-spherocytic haemolytic anaemia with a haemoglobin of approximately 11 g. % and 2-4% reticulocytes (Huehns, E. R., Hartmann, J., Hecht, F., and Motulsky, A. G., unpublished). In the affected family, three heterozygous carriers of the abnormal haemoglobin in two generations have the disease, making it likely that the haemoglobin is directly related to the disease.

The haemoglobin is distinctive in its electrophoretic properties. It does not separate from Hb-A by paper or starch block electrophoresis in the usual barbiturate buffers at pH 8-6, though the amount of Hb-A, appears somewhat increased. It is also not separable on starch gel electrophoresis in the tris-citrate/borate discontinuous system of Poulis (1957). Thus, this abnormal haemoglobin cannot be detected by the usual electrophoretic systems. Hb-Seattle can, however, be easily separated by starch gel electrophoresis, using a sodium phosphate buffer at pH 7.4 or a tris-E.D.T.A./borate buffer at pH 8.6. It amounts to 40% of total haemoglobin in the red cells. The abnormal haemoglobin is less stable than Hb-A to heat denaturation.

Though there are no inclusion bodies or methaemoglobin in fresh red cells, both appear more rapidly than in normal cells on in vitro incubation. The absorption spectrum of the acid methaemoglobin derivative of Hb-Seattle is slightly abnormal in the region of 550-650 m\( \mu \), showing some filling in of the normal minimum at 600 m\( \mu \). Structural studies indicate that the abnormality is in the \( \beta \)-chains, an alanine residue being replaced by glutamic acid at position 70 or 76 in peptide \( \beta TpIX \).

**Haemoglobin St. Mary's Disease.** Hb-St. Mary's disease (Buchanan, A., Barkhan, P., Crome, P. E., Mollison, P. L., and Huehns, E. R., unpublished) is a well-compensated haemolytic disease with a shortened red cell life-span and 10% reticulocytes. The patient had a number of haemolytic crises with moderately severe anaemia. The disease is distinguished from the other haemolytic anaemias due to abnormal haemoglobins in that the abnormal haemoglobin cannot be resolved by paper or starch block electrophoresis in barbiturate buffer, pH 8.6, or by starch gel electrophoresis in tris-citrate/borate buffer, pH 8.6, or phosphate buffer, pH 7.4. However, Hb-St. Mary's can be separated by starch gel electrophoresis in a tris-E.D.T.A./borate buffer, pH 8.6, when it migrates close to Hb-S. It amounts to 11% of total haemoglobin. Structural studies of the isolated haemoglobin indicate that the abnormality is probably in the insoluble (core) tryptic peptides of the \( \beta \)-chains. The possibility that the abnormality is close to the haem group is suggested by the finding that the absorption spectrum of the acid methaemoglobin derivative is abnormal in the region 550-650 m\( \mu \). The haemoglobin is unstable and is more readily denatured by heat than is normal haemoglobin (Fig. 21).

Both Hb-Seattle and Hb-St. Mary's are haemoglobins which are more unstable in vitro than Hb-A, and this instability can be shown in various ways, one of which is heat denaturation. However, studies by Sephadex chromatography show that the instability of the molecule leads to both increased dissociation of the molecule as well as polymer formation at neutral pH*. This tendency is shown to a significantly greater degree by Hb-St. Mary's than Hb-Seattle, which is consistent with the in vivo pathological effect of the two haemoglobins. It is also of interest that Hb-\( \alpha A \) is found in the haemolsysate containing these haemoglobins, indicating that the \( \beta \)-chains—the abnormal chains—of these haemoglobins are more unstable than the complementary normal \( \alpha \)-chains.

**Haemoglobin Köln Disease.** Pribilla (1962) has described a family in which some members have a mild haemolytic disease associated with an abnormal haemoglobin and the formation of

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* In vitro polymer formation has been noted in one human haemoglobin without any associated disease in the heterozygous or homozygous states (Tondo, Salzano, and Rucknagel, 1963).
Heinz bodies. The abnormal haemoglobin amounted to 5% of total haemoglobin and on electrophoresis it migrated close to Hb-S. More recently, a second family, with the same abnormal haemoglobin, has been described in Scotland (Hutchison, Pinkerton, Waters, Douglas, Lehmann, and Beale, 1964). In this family, the haemoglobin abnormality was found in three generations, and each affected person showed haemolytic anaemia with Heinz body formation. There was some improvement of the disease on splenectomy. Structural studies show that the haemoglobin has abnormal β-chains with the abnormality in the insoluble (core) tryptic peptides. As with Hb-Seattle and Hb-St. Mary’s, free α-chain haemoglobin was found in the haemolysate (Huehns, E. R., unpublished). The electrophoretic properties of Hb-Köln distinguish it from Hb-St. Mary’s and Hb-Seattle.

The ‘heat-unstable haemoglobin’ diseases.

Two families with this type of unstable haemoglobin have been described (Grimes and Meisler, 1962; Grimes, Meisler, and Dacie, 1964; Dacie, Grimes, Meisler, Steingold, Hemsted, Beaver, and White, 1964). The first patient (Grimes et al., 1964) had a severe haemolytic anaemia with a haemoglobin of 6–8 g. per 100 ml., and 22–47% reticulocytes. Following splenectomy there was some clinical improvement, and the red cells were found to contain many Heinz bodies. No abnormal haemoglobin could be detected by electrophoresis on paper or starch gel of fresh haemolysates. However, a proportion (approximately 35%) of the haemoglobin was relatively unstable and denatured readily on heating to 50° C. Both parents of this child are alive and well and have normal blood counts.

In the second family (Dacie et al., 1964) a mild haemolytic anaemia was found in four generations of the same family. The haemoglobin values ranged from 10–14 g. per 100 ml. with reticulocyte counts of 10%. Heinz bodies were found only in the red cells of one patient who had previously been splenectomized. However, Heinz bodies could be detected in the red cells of the other affected individuals after in vitro incubation of the red cells.

On heating the haemoglobin solution to 50° C. for one hour, a precipitate of haemoglobin developed, indicating the presence of some heat-unstable haemoglobin. On paper electrophoresis, only some trailing pigment behind Hb-A was seen. On starch gel electrophoresis in a tris-citrate/borate buffer system at pH 8.6, three abnormal zones could be seen besides Hb-A, Hb-A2, and Hb-A3. Two of these migrated between Hb-A and Hb-A2, and one just slower than Hb-A3. The total amount of abnormal pigment was approximately 15%. The three abnormal zones seen on starch gel electrophoresis may represent aggregates of haemoglobin with different molecular weights being
retarded to differing extents by the pores in the gel. At pH 6.9, in phosphate buffer, a small zone migrating more rapidly toward the cathode was seen. This extra zone was not seen after the addition of cyanide. Further study shows that this largely consists of free haem (G. H. Beaven, personal communication), and this finding may indicate that the haem-protein linkage in this haemoglobin is less stable than in normal haemoglobin.

There are a number of other reports of haemolytic anaemias in association with inclusion bodies (Lange and Akeroyd, 1958; Schmid, Brecher, and Clemens, 1959; Scott, Haut, Cartwright, and Wintrobe, 1960), and it appears likely that at least some of these will eventually be shown to be associated with an abnormal haemoglobin.

From the diseases described in this section, it is clear that a proportion of the non-spherocytic haemolytic anaemias is due to the presence of abnormal haemoglobins in the heterozygous state and that the haemolytic process is directly related to the instability of the haemoglobin. This direct relation between the presence of an abnormal unstable haemoglobin and disease seems certain in those studies where several members of a family in more than one generation are affected. In other reports, where only an isolated case has been studied, the relation is not at all clear. In these instances, the abnormal haemoglobin may be a chance finding or, alternatively, only part of the story, the disease only being manifest when the abnormal haemoglobin is accompanied by some unidentified interacting factor. A detailed study of other red cell components is clearly indicated in all cases. The difficulty of detecting some of the haemoglobin abnormalities suggests that other cases of unexplained haemolytic anaemia may also be due to the presence of an abnormal haemoglobin and that this may be detected only by the most careful studies. The properties of the various unstable haemoglobins described suggest that, in screening patients for this type of haemoglobin abnormality, electrophoresis of both the oxy- and the methaemoglobin derivatives in starch gel, using a number of different buffer systems at several pH values, should be carried out. The finding of Hb-\alphabeta in the haemolysate or the presence of a heat-unstable fraction may be the first indication that one is dealing with a haemoglobin abnormality.

The reasons why these haemoglobins are less stable than Hb-A are not clear. It can be postulated that the presence of a different amino acid in the critical region near the haem group causes a strain in the molecule leading to some conformational changes, thus producing a less stable molecule.

(iv) **Haemoglobinopathies Associated with the Homozygous State.** These have been well described and comprise Hb-C disease (Spaet, Alway, and Ward, 1953), Hb-E disease (Lehmann, Story, and Thein, 1956; Na-Nakorn and Minnich, 1957), and Hb-D disease (Bird and Lehmann, 1956).

There is mild haemolysis which is not fully compensated, leading to haemoglobin levels of 10–12 g. per 100 ml. The stained blood film shows some hypochromia and target cells. These diseases are found in subjects who are homozygous for any one of the above abnormal haemoglobins, the heterozygous condition being benign. The cause of the haemolysis in these syndromes is not clear but may arise because these haemoglobins show a slight instability which is not enough to cause disease when present together with Hb-A. It is also puzzling that in these cases the mild haemolysis is not fully compensated by increased red cell production, though more severe haemolysis, as, for example, in Hb-St. Mary's disease, shows so much better compensation.

**The Thalassaemias**

The characteristic abnormality in thalassaemia is the reduction in the amount of haemoglobin in the red cells due to diminished haemoglobin synthesis. As haemoglobin consists of the prothetid haem group joined to the protein moiety, globin, impairment of haemoglobin synthesis could be due either to diminished synthesis of haem or globin.

The finding of Bannerman, Grinstein, and Moore (1959) of impaired incorporation of 14C-glycine into haem and some haem precursors in *in vitro* bone marrow cultures prepared from patients with thalassaemia appeared to support the idea that the prime abnormality of this disease was an abnormality of haem synthesis. On the other hand, the well-documented clinical observation that infants with thalassaemia major are normal at birth, developing the disease at about 3–6 months of age, is opposed to the idea that the primary abnormality is a defect in haem synthesis. This is because the haem groups of foetal haemoglobin and adult haemoglobin are identical, namely, ferrous protoporphyrin IX, and hence impairment of its synthesis should lead to anemia during foetal as well as adult life. On the other hand, the protein part of these molecules differs, containing
γ-chains in foetal haemoglobin (αγγ) and β-chains in adult haemoglobin (αββ). Recent work has shown that haem exerts a negative feedback on the synthesis of δ-amino-laevulinic acid (ALA) in red cell precursors (Lascelles, 1960; Burnham and Lascelles, 1963; London, 1964; Steiner, Baldini, and Dameshek, 1964). The reduced rate of haem synthesis in thalassaemia can thus be explained by 'feedback' inhibition of the synthesis of δ-ALA by the un-utilized haem formed in the thalassaemic red cell. Finally, Marks and Burka (1964) have shown that the synthesis of β-chains is impaired in the 'cell-free system' prepared with ribosomes from β-thalassaemic red cell precursors. From these considerations it appears that thalassaemia is an abnormality of globin synthesis.

It has already been noted that the α- and β-chains of haemoglobin are synthesized independently and are under separate genetic control. This implies that, just as there are two main groups of abnormal haemoglobins, α-chain variants and β-chain variants, there would also be two groups of thalassaemias, one affecting the synthesis of α-chains and one affecting the synthesis of β-chains, and these have been called α-thalassaemia and β-thalassaemia respectively (Ingram and Stretton, 1959). As there are also δ- and γ-chain haemoglobin variants, it might be expected that δ- and γ-thalassaemia also occur. One person without any Hb-A4 has been reported, and he may be an example of δ-thalassaemia (Fessas and Stamatoyannopoulos, 1962). The existence of γ-thalassaemia has also been claimed (Hamilton, Sheets, and Brousseau, 1962). However, the only two established groups are α- and β-thalassaemia.

The genes causing thalassaemia can, of course, occur in the heterozygous or homozygous state. Furthermore, it might be envisaged that there are a number of different mutations leading to varying degrees of impairment of polypeptide chain synthesis. Some mutations would completely, or almost completely, inhibit the synthesis of a particular polypeptide chain, while others would only partially inhibit it. This hypothesis implies that there is a whole range of mutations causing varying degrees of inhibition in the synthesis of either the α- or β-chains. In the simple heterozygote it is difficult to measure the degree of inhibition of any particular thalassaemia gene. However, an approximate measure of the degree of inhibition caused by a given thalassaemia gene is shown by the proportion of Hb-A found in subjects who carry an abnormal haemoglobin. This is illustrated in mixed heterozygotes for β-thalassaemia and Hb-S or Hb-C. If no Hb-A is found, it may be inferred that complete suppression of βA-chains has been caused by the thalassaemia gene. In others, various amounts of Hb-A are found, due to different degrees of inhibition of βA-chain synthesis. The same argument applies to α-thalassaemia interacting with haemoglobins with abnormal α-chains. A full review of the thalassaemias has recently been published by Motulsky (1964b).

**Classification.** On the basis of the above hypothesis, the various thalassaemic syndromes that have been described can be classified as shown below. It should be pointed out that though this classification does not embrace every described variant of thalassaemia, it does provide a useful way of categorizing these syndromes.

(i) β-thalassaemia

(a) β-thalassaemia Trait. This is a mild anaemia with the morphological changes in the blood smear typical of thalassaemia as well as a raised Hb-A (Gerald and Diamond, 1958a). The increase in the proportion of Hb-A₂ is thought to be due to the continued synthesis of Hb-A₄ at a normal rate while that of Hb-A is impaired. In a proportion of these subjects, the foetal haemoglobin is also raised 2-5% above the normal maximum level of 0.4%. In those who carry a β-thalassaemia gene, which only partially inhibits the synthesis of Hb-A, the proportion of Hb-A₂ might be only slightly increased. Several authors (Zuelzer, Robinson, and Booker, 1961; Malamos, Fessas, and Stamatoyannopoulos, 1962; Fessas, 1964) have described a form of thalassaemia trait affecting the synthesis of β-chains in which Hb-A₄ is present in normal concentration but Hb-F is increased, and it is possible that this is due to a mutation suppressing both β- and δ-chain synthesis (βδ-thalassaemia) (Motulsky, 1964b).

(b) β-thalassaemia Major. This is the classical thalassaemia major. Family studies have shown that these persons have inherited two β-thalassaemia genes (Gerald and Diamond, 1958b). Because β-chain synthesis is almost completely inhibited, severe disease results which begins at the time in development when γ-chain synthesis normally ceases and β-chain synthesis commences. The severe anaemia produces a stress situation in the erythroid marrow, which, by a mechanism not yet understood, leads to the continuation of Hb-F synthesis but at a rate far below that necessary for complete compensation. It appears that the Hb-F produced by such a situation is unevenly distributed among the red cells (cf., sickle cell...
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disease—Singer and Fisher, 1952). This results in the production of red cells containing varying amounts of haemoglobin. Those cells that contain very little haemoglobin are destroyed in the marrow, the so-called 'ineffective erythropoiesis' which is a prominent feature of the disease. The remaining (smaller) proportion reaches the circulation.

One of the puzzling features of β-thalassaemia is that though the proportion of Hb-A₂ is raised in β-thalassaemia trait, it is normal or low in the homozygous state. If δ-chain synthesis were proceeding at a normal rate, it might be expected that Hb-A₁ would be much higher in the homozygous condition than in the trait form. A possible explanation is that the continued synthesis of γ-chains suppresses that of the δ-chains by some kind of 'feedback' mechanism.

(c) β-thalassaemia Intermedia. As the name implies, this disease is of intermediate severity to β-thalassaemia major and minor. It is characterized by a haemoglobin of 6–10 g. per 100 ml. with the typical blood picture of thalassaemia. Usually, there is gross splenic enlargement as well as the bone changes seen in thalassaemia major. Hb-F is raised from 20–80 % and the proportion of Hb-A₂ is often greater than normal. Family studies often do not show the β-thalassaemia trait in both parents, and it is presumed that genetically this condition is due to the interaction of two β-thalassaemia genes, one of which completely inhibits β-chain synthesis, while the other causes only partial inhibition. This clinical syndrome is also found in patients suffering from Hb-Lepore-thalassaemia disease (Gerald and Diamond, 1958b; Fessas et al., 1962; Neeb et al., 1961; Pearson, Gerald, and Diamond, 1959; Beaven, Gratzer, Stevens, Shooter, Ellis, White, and Gillespie, 1964) or homozygous Lepore disease (Neeb et al., 1961; Fessas et al., 1962). In both these conditions, a large proportion of the haemoglobin formed in the red cells is Hb-F. Milder clinical syndromes of this type are the well-documented interactions of abnormal haemoglobins such as Hb-C, Hb-E, or Hb-D with β-thalassaemia described below.

(d) Interaction of β-thalassaemia with a β-chain Variant of Hb-A. Hb-S thalassaemia disease is a clinical variant of sickle cell disease (Singer, Singer, and Goldberg, 1955). Hb-C-thalassaemia disease (Singer, Kraus, Singer, Rubinstein, and Goldberg, 1954; Zuelzer and Kaplan, 1954), Hb-D-thalassaemia disease (Hynes and Lehmann, 1956), and Hb-E-thalassaemia disease (Na-Nakorn and Minnich, 1957; Kochhar and Kathpalia, 1963) are some interactions of this type. Clinically, these are variants of thalassaemia of intermediate to mild severity. The diagnosis is made from the clinical picture and the electrophoretic analysis of the haemolysates. Often the abnormal haemoglobin and Hb-A will be found, but the proportion of Hb-A is lower than 50 %, whereas in the simple abnormal haemoglobin trait Hb-A usually exceeds the abnormal haemoglobins in amount. In some patients, no Hb-A is synthesized, and the differentiation from the homozygous haemoglobinopathies rests on family studies. Hb-Lepore-thalassaemia disease (Gerald and Diamond, 1958b) is a much more severe disease closely resembling thalassaemia major, with a haemoglobin level of approximately 8 g. per 100 ml. The main pigment in the red cells is Hb-F (65–70 %) with 5–7 % Hb-Lepore and a low level of Hb-A₂.

(ii) Interaction of α- with β-THALASSAEMIA (Fessas, 1962). Clinically, this disease is a form of thalassaemia minor. The diagnosis can only be established by family studies.

(iii) α-THALASSAEMIA. As the α-chains of Hb-A, Hb-F, and Hb-A₂ are identical, α-thalassaemia affects haemoglobin production both before birth as well as later in life, and it appears that the effect is clinically more severe during foetal life than later.

(a) α-thalassaemia Trait. This trait is somewhat milder than the β-thalassaemia trait, presumably because there is good compensation by the remaining normal Hb-α gene. The clinical features of the trait in persons in whom one Hb-α gene is completely inhibited have been outlined by Malamos et al. (1962). At birth, these people carry 5–10 % Hb-γ, and this disappears during the neonatal period together with Hb-F. Later in life, the patient has a slight anaemia, with a blood film suggesting thalassaemia. The Hb-A₂ level is normal. There are no clinical signs. The diagnosis can be confirmed by finding occasional cells with inclusion bodies after incubation with brilliant cresyl blue. In a few patients, a trace of Hb-γ₄ is found in the haemolysate.

Subjects who inherit an α-thalassaemia gene which only partially suppresses the synthesis of α-chains have no detectable abnormality later in life but at birth have a slight increase of Hb-γ₄ in the range of 1–2 %.

(b) α-thalassaemia Major. This is due to the inheritance of two α-thalassaemia genes causing severe α-chain suppression (see above). In these subjects, no α-chains are synthesized, and death in utero apparently occurs at approximately 32
weeks’ gestation and the foetus shows severe hydrops foetalis, anaemia, and erythroblastosis. (Lie-Injo Luan Eng and Jo Biwan Hie, 1960; Lie-Injo Luan Eng and Lie Hong Gie, 1961; Lie-Injo Luan Eng, 1962).

(c) \(\alpha\)-thalassaemia Intermedia or Haemoglobin H Disease. Recent work on Hb-H disease leaves little doubt that it is caused solely by a deficiency of \(\alpha\)-chain synthesis. Since the \(\alpha\)-chains for the three normal haemoglobins, Hb-A, Hb-A\(_2\), and Hb-F, are derived from a common metabolic pool, \(\alpha\)-chain deficiency should lead not only to the formation of Hb-\(\beta_4\), but also in the same person to the formation of haemoglobins consisting solely of \(\gamma\)- and \(\delta\)-chains, Hb-\(\gamma_4\) and Hb-\(\delta\), respectively. The fact that this occurs has recently been demonstrated in four patients with Hb-H disease (Dance, Huehns, and Beaven, 1963). The finding that some of the \(\delta\)-chains synthesized are used in the formation of Hb-\(\delta\) accounts for the low levels of Hb-A\(_4\) found consistently in the disease. Secondly, the occurrence of Hb-\(\gamma_4\) without Hb-\(\beta_4\) in some of the individuals who are synthesizing both \(\beta\)- and \(\gamma\)-chains must be explained. It has previously been suggested that the available \(\alpha\)-chains are used preferentially for the formation of Hb-A rather than Hb-F (Huehns, Flynn, Butler and Shooter, 1960), and this has been confirmed by \textit{in vitro} recombination experiments using the isolated chain subunits (Huehns, Beaven, and Stevens, 1964e). When the published pedigrees of families with Hb-H disease are studied, it appears likely that the disease is caused by the interaction of two abnormal genes. The occurrence of detectable \(\alpha\)-thalassaemia trait in some of the relatives is well documented. Huehns \textit{et al.} (1960) have suggested that Hb-H disease is due to two \(\alpha\)-thalassaemia genes. The work of Lie Injo Luan Eng already referred to indicates that the inheritance of two severe \(\alpha\)-thalassaemia genes leads to intrauterine death. It is therefore suggested that Hb-H disease is caused by the interaction of a severe \(\alpha\)-thalassaemia gene (causing complete suppression of \(\alpha\)-chain synthesis) with a milder \(\alpha\)-thalassaemia gene (causing partial suppression of \(\alpha\)-chain synthesis) (Huehns, 1962). As the incidence of the severe type of \(\alpha\)-thalassaemia genes is known (Malamos \textit{et al.}, 1962), the occurrence of families in which Hb-H appears in several generations (Gouttas \textit{et al.}, 1955; Bingle, Huehns, and Pranker, 1958; Ramot, Sheba, Fisher, Ager, and Lehmann, 1959; Minnich, Na-Nakorn, Tuchinda, Pravit, and Moore, 1956) indicates that the mild \(\alpha\)-thalassaemia gene is relatively common, occurring in approximately 20% of the population.

The published family data of Hb-H disease are consistent with this hypothesis (E. R. Huehns and A. G. Motulsky, unpublished; Wasi, Na-Nakorn, and Suinghamrong, 1964). Confirmation of this explanation for Hb-H disease could be obtained if it is shown that all newborns from parents with the disease carry some Hb-\(\gamma_4\), and also if the incidence in the affected population of the mild type of \(\alpha\)-thalassaemia gene postulated above could be shown to be approximately 20%.

To date five newborns from patients with Hb-H disease have been examined and four of these carried increased amounts of Hb-\(\gamma_4\) (Fessas 1960; E. R. Huehns, unpublished). It is, however, not known whether the methods used would have detected the small increases postulated to occur in some of these cases.

(d) \textit{The Interaction of \(\alpha\)-thalassaemia With an \(\alpha\)-chain Variant of Haemoglobin A.} \(\beta\)-Q-\(\alpha\)-thalassaemia disease clinically resembles Hb-H disease, except that instead of Hb-A, Hb-Q is found (Vella, Wells, Ager, and Lehmann, 1958; Dormandy \textit{et al.}, 1961; Lie-Injo Luan Eng and Hart, 1963). Because no Hb-A is present in these cases, the \(\alpha\)-thalassaemia would be of the severe type. One patient with Hb-I-\(\alpha\)-thalassaemia disease has been described (Atwater \textit{et al.}, 1963). Clinically, the picture was that of thalassaemia trait. The patient’s haemolysate showed approximately 80% Hb-I and 20% Hb-A; Hb-A\(_2\) and Hb-H were also present (Atwater \textit{et al.}, 1961). In this case, the \(\alpha\)-thalassaemia allowed the formation of significant amounts of Hb-A and was therefore of the mild type.

The Genetic Abnormality in Thalassaemia. During the past few years, consideration has been given to the type of mutation that gives rise to thalassaemia. Studies of offspring in families in which \(\beta\)-thalassaemia and abnormal haemoglobins occur indicate that the genetic loci controlling the synthesis of the \(\beta\)-chains and those causing \(\beta\)-thalassaemia are either allelic or closely linked (see above). Several hypotheses have been advanced.

(a) The rate of haemoglobin synthesis is diminished because the ‘Hb-A’ synthesized in these persons is not normal Hb-A but contains a ‘silent’ amino acid substitution (Itano, 1957; Ingram and Stretton, 1959). The finding (Guidotti, G., quoted by Baglioni, 1963a) that the amino acid composition of isolated peptides from two such samples of ‘Hb-A’ was identical to normal Hb-A argues against this idea but does not altogether exclude it.
(b) The alternative view that the reduced rate of synthesis is due to a mutation of a closely-linked rate-controlling locus, such as the operator locus postulated in the control of bacterial protein synthesis by Jacob and Monod (1961), has been discussed by a number of workers (Neel, 1961; Motulsky, 1962; Sturgeon et al., 1963; Zuckermandl, 1964). However, these hypotheses do not explain all the findings in thalassaemia.

(c) Recently, Itano (1964) has advanced a third hypothesis. This depends on the idea that the genetic code is degenerate, that is, that two different triplet code words (see above) can code for the same amino acid. In normal persons, the normal triplet code word is present, but in thalassaemic persons, the normal code triplet is replaced by a different one which codes for the same amino acid. One of the factors that limits the rate of addition of any particular amino acid to the growing end of a polypeptide chain during synthesis is the availability of amino acid transfer-RNA fitting any particular coding triplet, and it is argued that there is plenty of transfer-RNA to fit the normal coding triplet but a deficiency of transfer-RNA for the abnormal triplet. This deficiency of availability of a particular amino acid leads to a block in the synthesis of the affected polypeptide chain and the decreased normal globin production seen in thalassaemia.

(d) Ingram (1964) has put forward still another hypothesis. This proposes that in thalassaemia the messenger RNA for the affected chain is abnormal and blocks some of the ribosomal sites of protein synthesis so that they are not available for haemoglobin synthesis.

(e) Another possibility is that the messenger RNA formed, if abnormal, would be less stable than normal in mRNA. Each mRNA would then only be available for the synthesis of a smaller number of polypeptide chains than its normal counterpart.

The multiplicity of hypotheses put forward to explain the findings in thalassaemia indicates that at the present time the data on which a sound theory can be based are not yet available, and it is possible that there is more than one cause of thalassaemia.

The Hereditary Persistence of Foetal Haemoglobin

A number of investigators have described a benign condition in Negroes in which the production of Hb-F continues into adult life, 20–30% of total haemoglobin being foetal in type, the remainder being Hb-A and Hb-A1, (Edington and Lehmann, 1955a, b; Jacob and Raper, 1958; Went and MacIver, 1958; Herman and Conley, 1960; Bradley et al., 1961; Kraus, Koch, and Burkett, 1961; Thompson, Mitchener, and Huisman, 1961; Conley et al., 1963). It occurs in about 1% of American Negroes. The distribution in Africa is not known in detail, but about 1% of the population is affected in West Africa (Conley et al., 1963). Several studies have indicated that the foetal haemoglobin in these persons is the same as that found in normal cord blood (Thompson et al., 1961; Schroeder, Sturgeon, and Bergren, 1962; Baglioni, 1963b). The foetal haemoglobin is uniformly distributed in the red cells (Thompson et al., 1961; Bradley et al., 1961). This contrasts with the finding of uneven Hb-F distribution in the red cells in other conditions, such as sickle cell anaemia (Singer and Fisher, 1952) or thalassaemia major (Shepard, Weatherall, and Conley, 1962), where the persistence of Hb-F is not the direct result of the genetic abnormality but secondary to a stress situation in the bone marrow.

When a person inherits this condition as well as a gene causing the production of a haemoglobin variant with abnormal β-chains, no Hb-A is produced*. When such persons are mated to normal persons, their children carry either the 'high F' gene or the abnormal haemoglobin (Thompson and Lehmann, 1962; Conley et al., 1963; see also Rucknagel and Neel, 1961), indicating that the 'high F' gene is closely linked or allelic to the Hbβ locus. Whether the γ-chain structural locus is linked to that at which the 'high F' gene is situated is not known. Data on this point will come from the study of families in which both the 'high F' gene and a γ-chain variant of Hb-F are found.

Besides the interaction with abnormal haemoglobins, interaction with β-thalassaemia has been reported. These patients have approximately 65–70% Hb-F and the clinical picture of thalassaemia intermedia (Kraus et al., 1961; Wheeler and Krevans, 1961; Barkham and Adinolfi, 1962).

One person probably homozygous for the 'high F' gene has been reported (Wheeler and Krevans, 1961). He had only slight haematological abnormality; he carried only Hb-F, and no Hb-A or Hb-A1 could be detected.

* These people carry these traits in repulsion. If the 'high F' gene occurred in coupling to an abnormal Hbg gene, the production of the corresponding abnormal haemoglobin would be suppressed and the phenotype could not be distinguished from the simple trait. The same applies to the progeny of the matings mentioned, and the only cross-over that could be distinguished would be one that leads to a normal person.
Several hypotheses to explain these findings have been advanced. Wheeler and Krevans (1961) suggest that it might be due to a deletion of the Hb\(_s\) and Hb\(_s\) loci. The deletion must also lead to the continued production of Hb-F in sufficient amounts to compensate for the lack of Hb-A production. Other workers (Neel, 1961; Motulsky, 1962; Sturgeon et al., 1963; Zuckerandl, 1964) have postulated that it is due to a mutation affecting the regulatory mechanism controlling the Hb\(_s\) and Hb\(_s\) loci, leading to failure to turn on the synthesis of these chains and at the same time failure to turn off \(\gamma\)-chain production.

Two further types of inherited persistence of foetal haemoglobin have been described, which differ from the 'high F gene' described above in that the amount of Hb-F found in the heterozygotes is significantly lower. The first occurs in 0.25% of Greeks (Fessa and Stamatoyannopoulos, 1964). In these individuals, Hb-F levels vary from 11–18%, and Hb-A\(_s\) is within the lower range of normal. There are no haematological abnormalities, and Hb-F is evenly distributed in all red cells. This trait has also been described in association with \(\beta\)-thalassaemia; clinically, this is a form of \(\beta\)-thalassaemia intermedia, with 20–40% Hb-F and a raised Hb-A\(_s\). The other type occurs in about 1% of the population of Southern Switzerland. It is not associated with any clinical or haematological abnormality (Marti and Bütler, 1961; Marti, 1963).

### The Occurrence of Haemoglobin Abnormalities in Other Diseases

**Haemoglobin A\(_s\).** Variation between normal people in the level of Hb-A\(_s\) is small, and there is also little variation in disease besides that occurring in thalassaemia. There is one report that Hb-A\(_s\) is raised in pernicious anaemia and in iron-deficiency anaemia (Josephson, Masri, Singer, Dworkin, and Singer, 1958). Another report suggests that Hb-A\(_s\) is raised after bone marrow transplantation (Bridges, Neill, and Lehmann, 1961). From the small amount of data available, it is not possible to determine whether the above changes are present in all patients.

**Haemoglobin F** (for review, see Beaven et al., 1960b). Delayed disappearance of Hb-F after birth is associated with a number of conditions such as prematurity (Jonxis, 1951), congenital spherocytosis, and other congenital haemolytic anaemias. The foetal haemoglobin levels in these infants eventually reach normal levels. There is also a delay in the disappearance of Hb-F in trisomy 13–15 and in a proportion of Negroes with Down's syndrome (trisomy 21–22) already referred to.

In acute leukaemia in young children, high Hb-F levels are often found. In adult patients with leukaemia, small increases in Hb-F are occasionally found, particularly in patients with erythroleukaemia. A raised Hb-F has also been reported in aplastic anaemia (Jones, 1961; Jonxis, 1961). In pernicious anaemia, Hb-F is present in small amounts (1–2%) in approximately half the patients examined. It is of interest that the proportion of this pigment often rises shortly after the commencement of treatment with vitamin B\(_12\) before the maximum reticuloocyte response has been reached (Beaven et al., 1960b).

Small amounts of foetal haemoglobin have also been found in approximately 10% of women during the second trimester of pregnancy by a sensitive immunological technique. These slightly raised levels fall to normal adult levels in the last trimester (Rucknagel and Chernoff, 1955). The occasional finding of high Hb-F levels in late pregnancy may indicate transplacental foetal haemorrhage. Bromberg, Salzberger, and Abrahamov (1957) report a significantly raised Hb-F in four patients with molar pregnancy.

Iron deficiency, acquired haemolytic anaemia, and congenital cyanotic heart disease are more associated with a raised Hb-F.

**Haemoglobin H.** A number of patients with Hb-H in association with erythroleukaemia have been described (Bergren and Sturgeon, 1960; White, Ellis, Coleman, Beaven, Gratzler, Shooter, and Skinner, 1960; Beaven, Stevens, Dance, and White, 1963; C. A. Finch, A. G. Motulsky, unpublished). The red cells in these patients show hypochromia and some target cells and resemble those seen in the congenital form of Hb-H disease. As these patients come from populations in which Hb-H disease has not been reported, it appears that the appearance of this abnormal haemoglobin is due to the leukaemia. In two of them, small amounts of Hb-\(\gamma\) were also present (E. R. Huehns, unpublished). Another report of an unstable haemoglobin associated with leukaemia comes from India (Mathur and Misra, 1962).

**Sickle Cell Trait.** There is one report of a variation in the proportion of Hb-A to Hb-S in megaloblastic anaemia (Heller, Yakulis, Epstein, and Friedland, 1963). It was found that the proportion of Hb-S present was unusually low when the
patient was first seen. After recovery from the anaemia, the proportion of HB-S increased significantly.

**Animal Haemoglobins.** Van Vliet and Huisman (1964) have studied experimental anaemia in sheep and shown definitive changes in the relative proportion of types of haemoglobin present in the red cells. If the experimental animals were allowed to recover, the haemoglobin pattern returned to that at the beginning of the study.

**Distribution of Abnormalities of Haemoglobin Synthesis**

Since the discovery of the abnormal haemoglobin, it has been shown that some, such as HB-S, HB-C, HB-D, and HB-E, occur quite frequently in some parts of the world, while in others they are only found occasionally. It has also been found that the thalassaemias only have a high incidence in certain areas. The harmful, and often lethal, effects of these inherited abnormalities in the homozygous state raise the problem of why these deleterious genes are not rapidly eliminated from the population. Theoretically, this can be prevented if the heterozygous state confers some compensating advantage (heterosis) and is fitter in the Darwinian sense than either the normal or abnormal homozygote. At certain frequencies the harmful effects of the abnormal gene in a given population will be exactly balanced by the conferred advantage. Such an equilibrium is stable and is known as a balanced polymorphism (Stern, 1960). In other words, if homozygotes die prematurely or are so infertile that they seldom reproduce (and hence do not transmit the abnormal gene), their Darwinian fitness is said to be near zero. The gene can, nevertheless, be maintained in the population if the heterozygotes are fitter than the normal homozygotes in this sense. This increased fitness may be due to a relative immunity to some disease which can cause premature mortality or impairs fertility. Clearly, such a disease must be prevalent in the areas in which the relevant red cell traits are found and much effort has been directed towards identifying such factors. Haldane (1949) suggested that thalassaemia protected against malaria. Allison (1954) drew attention to the possible role of sickle cell trait in protecting against malignant tertian malaria and provided the first data in support of this hypothesis. He also pointed out that in a hyperendemic region the effect should be most marked during the first years of life, before immunity to malaria is acquired. There is now a great deal of support for the hypothesis that the sickling trait protects against malaria, and in particular against plasmodium falciparum, and this hypothesis is now widely accepted. The importance of malaria in maintaining the frequency of thalassaemia is not yet fully documented. In relation to the other common haemoglobin traits, only scanty data are available. This interesting aspect of the haemoglobin story is discussed in more detail by a number of authors (Allison, 1961; Motulsky, 1964a; Rucknagel and Neel, 1961; Livingstone, 1958; Allison, 1964).

**The Distribution of Sickle Cell Trait.** The distribution of the sickle cell gene is shown in Fig. 22. It can be seen that sickling occurs in tropical Africa and also in the Mediterranean region, southern Arabia, and India. Besides occurring in these areas, it is, of course, also found in emigré populations, such as the American and West Indian Negroes. The incidence of the gene varies enormously from place to place; the highest frequencies are found in certain peoples of East Africa, reaching 40% in Amba on the slopes of Mount Ruwenzori.

The distribution of the sickling gene, in general, corresponds well to that of falciparum malaria (Fig. 23). There are, however, areas where falciparum malaria is common and the sickling trait is not found, such as South-east Asia. The reasons for this are not entirely clear. It may be that the trait has never been introduced into these areas either by mutation or immigration. Alternatively, protection against malaria may be afforded by one of the other red cell traits. For example, in South-east Asia HB-E, thalassaemia, and glucose 6-phosphate dehydrogenase (G6PD) deficiency all occur, and all are thought to give protection against malaria, the relatively more lethal sickle gene being excluded. This problem has been studied in Greece, where the sickle cell trait and thalassaemia are found. It was found that in regions where the sickle gene frequency is high, the frequency of thalassaemia is low and vice versa (Barnicot, Allison, Blumberg, Deliyannis, Krimbas, and Ballas, 1963). The theoretical aspects of this situation have been discussed by Penrose, Smith, and Sprott (1956).

It would be expected that considerable protection against death from malaria would be needed to balance the loss of sickle cell genes in regions where its frequency is high. The demonstration of this is one of the most vital links in the malaria hypothesis. In general, death rates from malaria are difficult to obtain, because it is often difficult to be sure that malaria, if present, is not just an incidental finding.
The results of these studies have been summarized by Motulsky (1964a) and are given in Table IV. This shows that whereas 23 out of the 100 patients dying from cerebral malaria would have been expected to carry the sickling trait, only one such patient was found, and this gives strong support to the hypothesis that the high incidence of sickling trait is due to its protection against death from falciparum malaria.

The Distribution of Haemoglobin C. High frequencies of Hb-C are only found in certain regions of West Africa, the trait reaching a frequency of almost 30%. Besides this, sporadic cases are found elsewhere in the African continent. There is some evidence that in those areas where both Hb-S and Hb-C are found in the population, their frequencies are inversely related (Rucknagel and Neel, 1961). As might be expected, Hb-C is also found in the Negro populations of America and the West Indies. The high frequency of Hb-C is also thought to be due to its protective effect against malaria, and this is supported, but by no means proved, by the few studies that have been carried out (Motulsky, 1964a). Hb-C disease is much milder than sickle cell disease and an equilibrium frequency of Hb-C
TABLE IV
MALARIAL MORTALITY IN Hb-S TRAIT (AS) (From Motulsky, 1964a)

<table>
<thead>
<tr>
<th>Location</th>
<th>% AS in Population</th>
<th>No. Dead From Malaria</th>
<th>Observed No. AS Dead From Malaria</th>
<th>Expected No. AS Dead From Malaria</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leopoldville, Congo</td>
<td>26</td>
<td>23</td>
<td>0</td>
<td>6</td>
</tr>
<tr>
<td>Lulusburg, Congo</td>
<td>20</td>
<td>21</td>
<td>1</td>
<td>6-1</td>
</tr>
<tr>
<td>Ibadan, Nigeria</td>
<td>24</td>
<td>27</td>
<td>0</td>
<td>6-5</td>
</tr>
<tr>
<td>Accra, Ghana</td>
<td>8</td>
<td>13</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Kampala, Uganda</td>
<td>19</td>
<td>16</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>100</td>
<td>1*</td>
<td>22-6</td>
</tr>
</tbody>
</table>

*$^2 = 20.5; p < 0.001.$

could be maintained by a much smaller selective advantage of the heterozygote than that conferred by the sickle cell trait, making it correspondingly more difficult to demonstrate.

**The Distribution of Haemoglobin D.** Hb-D is found sporadically in many parts of the world. It occurs somewhat more commonly in certain regions of India but even there the maximum incidence of the trait is 2% in Sikhs of the Punjab (Bird and Lehmann, 1956). As has already been mentioned, detailed studies of samples of Hb-D have shown that there are several chemically distinct forms.

**The Distribution of Haemoglobin E.** This is shown in Fig. 24. It can be seen that Hb-E is found almost exclusively in South-east Asia, although cases have been reported to occur as far away as Turkey (Aksoy and Lehmann, 1957). The distribution of the abnormal haemoglobins in South-east Asia has recently been reviewed by Lie Injo Luan Eng (1964). The reasons for the frequency of Hb-E in this region are not clear, but again protection against malaria has been suggested (Kruatrachue, Na-Nakorn, Charoenlarp, and Suwanakul, 1961; see Allison, 1961; Motulsky, 1964a).

**The Distribution of Thalassaemia.** The map shown in Fig. 25 does not differentiate between the distribution of $\alpha$- and $\beta$-thalassaemia, but mainly represents that of the latter. $\alpha$-thalassaemia is, however, known to occur in at least some of the areas in which $\beta$-thalassaemia is present and has been reported from Italy, Greece, Israel, and other Eastern Mediterranean countries, South-east Asia, and West Africa (see Motulsky, 1964b). Again, it has been suggested that protection against falc-
param malaria maintains the frequency of the thalassaemia traits, but the evidence for this is not nearly so convincing as in the case of the sickle cell trait.

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