COMPARATIVE STUDY OF METHODOLOGIES FOR ESTIMATION OF HEMOGLOBIN-F
ASSESSMENT OF THE QUANTITATIVE RADIAL IMMUNODIFFUSION METHOD FOR HEMOGLOBIN-F ESTIMATION

BY

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Thesis

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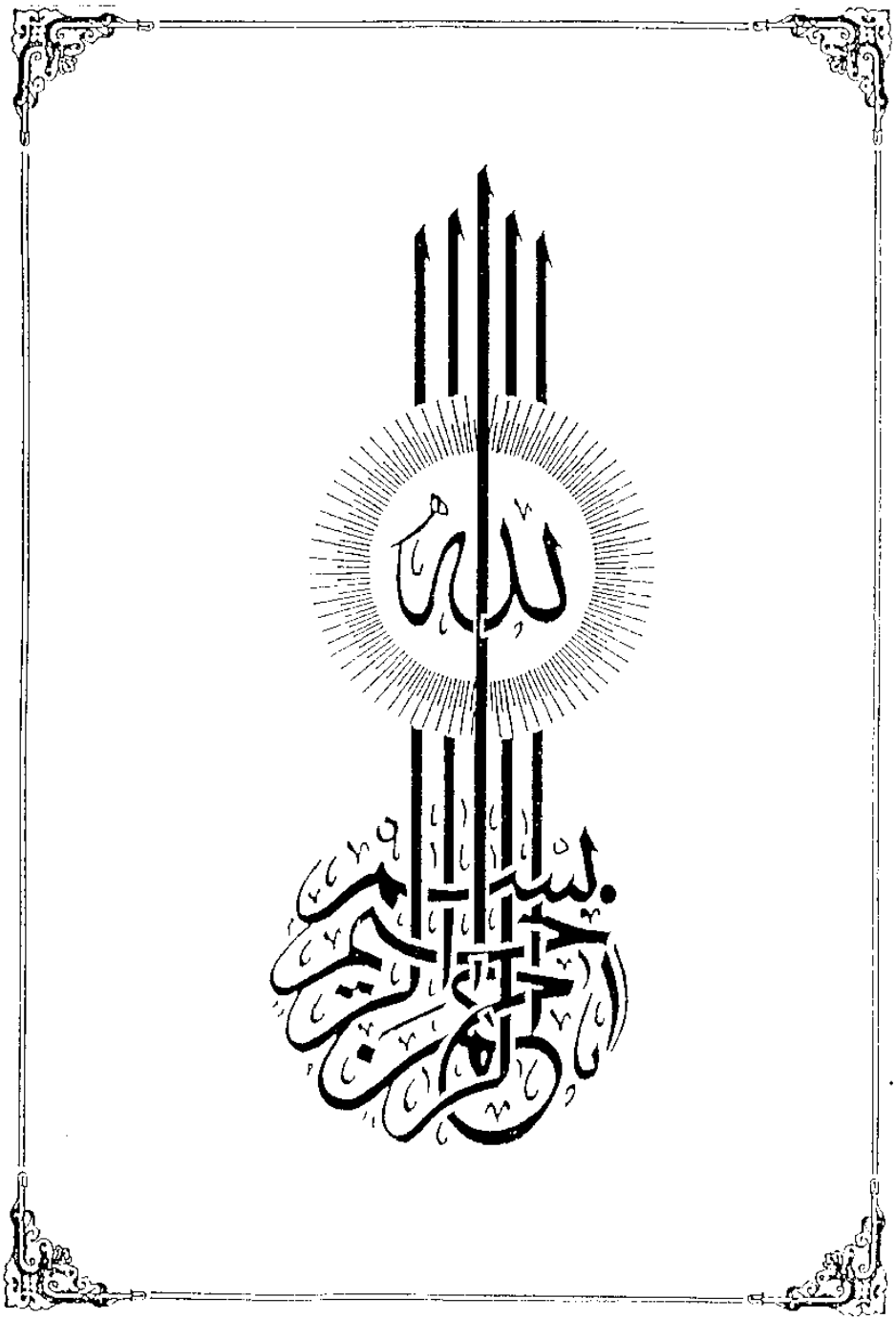

DEDICATION

"I would like to admit my mother whose encouragement and continuous prayers have motivated me to complete my study. She has always told me that 'when there is a will, there is a way' which I have already put in mind and tried hard to apply it in my life."

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INTRODUCTION AND AIM OF WORK

Hemoglobin F (Hb-F) is the major respiratory pigment of the intrauterine life (Cooper and Hoagland,1972). The perinatal switch from Hb-F to adult hemoglobin (Hb-A) is never fully completed (Wood et al., 1977a). Residual Hb-F is produced throughout life within a minority of red cells called "F-cells" (Boyer et al., 1975a). In normal adults Hb-F constitutes less than 1% of the total hemoglobin (Weatherall et al., 1979).

Elevated levels of Hb-F are reported in various inherited and acquired hematologic disorders, certain malignancies and chronic renal disease (Bertles, 1974; Wood et al.,1975; Abraham et al.,1977, Papayannopoulou et al., 1980). The postnatal reactivation of Hb-F synthesis is attributed to increased production of F-cells, or increased production of Hb-F/F-cells (Dover et al., 1978a,b). Since only slight elevations of Hb-F occur in most of these conditions, accurate determination of Hb-F is needed to differentiate between normal and elevated levels.

The standard routine methods of estimating Hb-F based on alkali denaturation originally described by Singer et al. (1951b) were of low sensitivity and not very

reliable in the low ranges (Konn and Payne, 1972). A modified and more sensitive method was introduced by Pembrey et al. (1972). Until relatively recently no routine immunological method was developed for measuring Hb-F, though a precipitin tube method was described by Chernoff (1953b). In 1974, Chudwin and Rucknagel introduced an alternative method of Hb-F estimation based on radial immunodiffusion (RID) which measured specifically Hb-F in whole blood lysate and proposed its use as an accurate method for screening for thalassemia.

Aim Of The Work

This study is carried out to evaluate the radial immunodiffusion method (Chudwin and Rucknagel, 1974) and to compare it with the currently used method of Pembrey et al. (1972) based on the alkali denaturation principle. The reproducibility and the accuracy of the method along with its diagnostic utility are to be assessed in our laboratory.

Review of Literature

REVIEW OF LITERATURE

Hemoglobin Structure and Function

Hemoglobin (Hb) is an iron-containing protein complex which, in all vertebrates, is enclosed in a specialized cell whose main function is the transport of oxygen from the lung to the tissues. (Cooper and Hoagland, 1972).

Hb constitutes approximately ninety-five percent of the erythrocyte protein. Most of the remaining erythrocyte protein consists of the various enzymes that protect the Hb and sustain the viability of the erythrocyte. (Harris and Kellermeyer, 1970).

Human Hbs are tetramers with a molar mass of 64456g/mol. The tetrameric molecule measures 6.4 by 5.5 by 5.0 mm and consists of 4 subunits, each having one polypeptide chain and one heme group. The ferrous ion atoms of the heme group have the special property of being able to combine reversibly with oxygen without being oxidized to the ferric form. This is possible as the polypeptide chains fold around the heme groups enclosing them by hydrophobic pockets. The four polypeptide chains normally consist of two pairs, whose amino acid sequence differs with the

type of Hb (Perutz, 1969; 1978). However, the heme is identical in all types.

The Hb tetramer exists in one of two alternative forms, according to the interrelation of the 4 subunits, and dependant on the state of oxygenation of the molecule. Deoxygenated Ho has a T (tense) structure , the subunits being tightly held together by salt bridges. This form has a low affinity for oxygen. However, when oxygen binds to each heme group, the salt bridges are broken until a critical point is reached, and the Hb structure "clicks" over to the R (Relaxed) structure, where the subunits are less tightly held together. This form has a high affinity for oxygen (Perutz, 1970).

The polypeptide chains of human Hbs are basically six, designated by the Greek letters: alpha (α), Beta (β) gamma (γ), delta (δ), epsilon (ϵ) and Zeta (ζ). The amino acid sequences of α β γ and δ chains are already known (Braunitzer et al., 1961). α-Chain consists of 141 amino acids, while β γ δ chains contain 146 amino-acids (Konigsberg and Hill, 1962).

δ-chains are similar to β-chains in amino acid sequences but differ only in 10 places, while γ-chains show 39

differences in sequence from β chains (Huehns and Beaven, 1971; Kleihauer, 1977).

ε-chains differ from β chains in 24 amino acids. This accounts for a 30% divergency from β chains. When compared to γ chains, ε chains show difference in 18 amino acids, accounting for about 24% divergency.

Still there are portions of the primary structure (at sites 31-40, 60-66, 83-87, and 145-146) that are common in β, δ, γ and ε chains (Gale et al., 1979).

The structure of ζ chain is determined for about 90% and found to diverge from α chain for about 35% of its sequence (Kamuzora and Lehmann, 1975). Of the 26 sites responsible for subunit contacts, 21 are aligned in both α and in ζ; similarly, of the 15 Hb contacts, 12 can be aligned i.e. the ζ and α chains still have some limited features in common (Fantoni et al., 1981).

Recently, it is concluded that ζ and ε chains are the embryonic counterparts of the adult α and β, δ or γ chains respectively (Clegg, 1981). During development, ε and γ chains synthesis is turned off while the β and δ chains are activated (figure 1). At each stage α - chain, synthesis is synchronized with the other globin chains,

so that no large excess of any subunit exists. This occurs through a mechanism which controls the synthetic rate of individual peptide chains (Nienhuis, 1978).

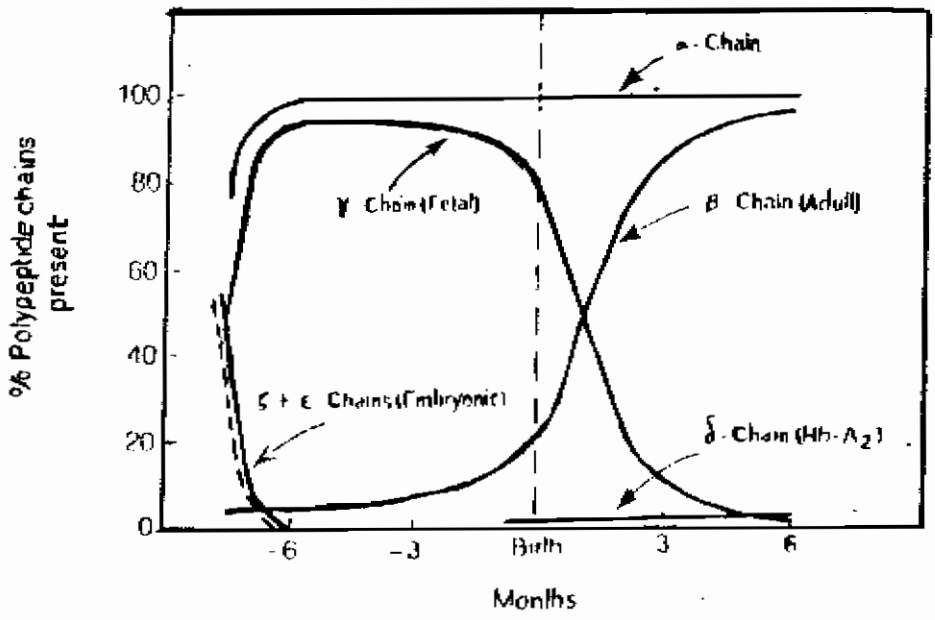

Figure 1. Developmental changes in human hemoglobin chains (cited from Wimberley, 1982).

Normal Human Hemoglobins (Table 1)

These comprise adult, fetal and embryonic Hbs.

A. Adult Hemoglobins:

Many studies have revealed the heterogeneity of the Hb in red blood cells of normal adult (Huisman and Meyering, 1960; Schneck and Schroeder, 1961; Jones and Schroeder, 1963). Adult Hb is composed of a major fraction, Hb-A and a minor fraction, Hb-A_2.

I. Major Adult Hemoglobin or HbA:

It comprises 96 to 98 percent of the Hb of normal adults and has a structural formula of $\alpha_2 \beta_2$ (Rhinesmith et al., 1957).

Its synthesis is activated early in fetal life, and it forms about 5-10% of the Hb in normal fetus from six weeks onwards to reach a significant level at birth (Weatherall et al., 1974; Terrenato et al., 1981). After birth there is a rapid increase in Hb A level so that by nine to twelve months of age it reaches the normal adult level (Lewis, 1974).

By column chromatography, the heterogeneous nature of HbA can be demonstrated (Allen et al., 1958; Huisman

and Meyering, 1960).Thus, three minor components are splitted and designated HbA_{Ia}, HbA_{Ib} and HbA_{Ic}. These minor components have lower isoelectric points and faster chromatographic mobilities than the main band of Hb A and are collectively referred to as fast Hbs. On zone electrophoresis at alkaline pH, these components appear as smear (Called HbA_3) running anodal to the main component. They have carbohydrate residues attached to their β-chains and so referred to as glycosylated Hbs (Bookchin and Gallop, 1968). HbA_{Ic} has glucose attached, while HbA_{Ia} forms a complex with fructose and HbA_{Ib} may have G6pD attached. $Hb\text{-}A_{Ia+b}$ and $Hb\text{-}A_{Ic}$ are present in decreased amounts in patients with hemolytic anemia while they are found in increased quantities in uncontrolled diabetic patients and those with myelocytic leukemia. $Hb\text{-}A_{Ia+b}$ is increased in some patients with iron deficiency anemia and perhaps in polycythemia vera. The $Hb\text{-}A_{Ic}$ fraction decreases in some patients with iron deficiency (Horton and Huisman, 1965) and may show two fold increase in uncontrolled diabetic patients whether they are insulin or non insulin - dependant (Rahbar, 1968, Paulson, 1973). $Hb\text{-}A_{Ic}$ estimation reflects the overall level of hyperglycemia in diabetic patients during the preceding one-two months and is thus used as an index of