PART-I

CHARACTERIZATION OF HEMOGLOBIN FROM THE REPTILE UROMASTIX HARDWICKII

PART-II

PROTEIN PATTERNS OF HUMAN CATARACTOUS LENS AND PLASMA

THESIS SUBMITTED TO THE UNIVERSITY OF KARACHI IN FULFILMENT OF THE REQUIREMENT FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

by

RUKHSANA LALANI
M.Sc.(Kar.) M.S. (N.Carolina)

H.E.J. RESEARCH INSTITUTE OF CHEMISTRY
UNIVERSITY OF KARACHI

OCTOBER 1984

DEDICATED TO
MY PARENTS

ACKNOWLEDGEMENT

It is a great pleasure for me to express the deepest sense of gratitude to Professor Dr. Zafar H. Zaidi for his expert guidance, tremendous efforts and gracious co-operation throughout this project.

Special thanks are due to Professor Dr. Salimuzzaman Siddiqui, F.R.S., Director of Institute for providing the facilities to persue this work.

My sincere appreciation are due to Dr. M.Mukhtar Ahmed of Liaquat National Hospital, Karachi; Dr. A. Razzak Lakhani of Charania Hospital, Karachi for providing human cataract lenses and plasma from cataractous patients; and Dr.V.Hirani of J.P.M.C. Karachi for normal human lenses.

My indebtness are also due to Dr. M.Ata-ur-Rahman, Professor of Biochemistry, J.P.M.C., Karachi for his enlightening discussions, and Dr.Shahida Zaidi for her helpful suggestions.

I gratefully acknowledge the financial assistance by University Grants Commission, Islamabad.

On this occasion, I would like to remember all my friends and colleagues who have very graciously extended all the needed support and help.

Indeed, this study would have not been possible, without the constant and continuous encouragement and inspiration from my family. I shall always remain grateful to them.

Last but not least, I would like to thank Mr.Maqsood Alam for typing this manuscript so cautiously and neatly.

CONTENTS

PART-II: PROTEIN PATTERNS OF HUMAN CATARACTOUS LENS AND PLASMA

PART-I

CHARACTERIZATION OF HEMOGLOBIN FROM THE REPTILE UROMASTIX HARDWICKII

ABSTRACT

The present study on hemoglobin of Uromastix hardwickii has been undertaken to separate and characterize hemoglobin components. The separation of hemoglobin components was achieved by subjecting it to Sephadex G-75 column chromatography, ion-exchange chromatography using different resins and variety of buffers. The separation on molecular weight basis gave four peaks but when their homogeneity was checked, it was found that all the four peaks were not pure components. The separation on charge basis resolved into number of peaks. Checking the purity of these peaks by triton electrophoresis and N-terminal analysis showed only two of them to be pure. The purity of the same were checked on HPLC showing them to be homogeneous. However, the amino acid sequence using DABITC method could be achieved only to a limited number.

INTRODUCTION

1.1. HEMOGLOBIN

Hemoglobin has been most extensively studied of proteins. A great deal of progress has been made since 1864, when Hoppe-Seyler, first introduced the term "Hemoglobin" to describe the pigment of blood. It was the first protein which was crystallized (Reichert, 1849), its physiological function established (Pfluger, 1875), its immunological (Nuttale 1904), and the crystallographic studies performed (Reichert and Brown 1909) and the structural specificity assigned for each species. It was purified to the extent that its molecular weight was determined (Adair 1924) and much later its primary structure was elucidated (Braunitzer etal. 1961) and X-ray structure analyzed (Perutz etal, 1960, 1968).

1.2. FUNCTION OF HEMOGLOBIN

In the blood stream, there are about 5 million erythrocytes per millimeter, each erythrocyte contains 280 million molecules of hemoglobin and each hemoglobin molecule has four heme groups.

Hemoglobin is the oxygen transport protein in many species. Its role is to bind molecular oxygen to its heme irons in the lungs and to deliver it to the muscles. It brings back to the lungs the by-product of this oxidation i.e. the carbon dioxide; thus preventing the accumulation of acid in the muscles. This double function is achieved through the reversible change of the structure which was demonstrated by Haurowitz (1938).

1.2.1. Bohr effect

Hemoglobin constitutes about 90% of the total protein in erythrocytes; this is concentrated in the cytoplasm. Because of the ability of hemoglobin to bind oxygen, the whole blood can absorb about 21 ml of gaseous oxygen per 100 ml, whereas blood plasma alone can absorb only about 0.3 ml of oxygen by physical solution. A plot of percent saturation of hemoglobin against oxygen partial pressure is sigmoidal. The hemoglobin has a relatively low affinity for binding the first one or two molecules of oxygen, but once they are bound the binding of subsequent oxygen molecules is greatly enhanced. The position of hemoglobin-oxygen equilibrium is also effected by the pH as is shown by the following equation:

$$HHb^{+} + O_2 \rightleftharpoons HbO_2 + H^{+}$$

where HHb^{+} is a protonated species of deoxyhemoglobin molecule.

Since this reaction is reversible, increasing the H^{+}ion concentration will cause the equilibrium to shift to the left towards the decreased saturation, whereas decreasing the H^{+}ion concentration will cause the equilibrium to shift to the right, towards increased saturation. This effect of pH on the oxygen-hemoglobin equilibrium is called the Bohr effect.

Many factors affect the Bohr effect. The hemoglobin molecule can be considered as an allosteric molecule even though the property of allostery is usually ascribed to enzyme, which hemoglobin is not. This theory of allostery has been proposed by Monad etal. (1965) to explain the behaviour of hemoglobins. Its

substrate is oxygen and the allosteric effectors are proton, chloride ion and 2-3, diphosphoglycerate (DPG). All these allosteric agents have different roles in different animals. Organic phosphate effectors enhance the alkaline Bohr-effect and decreases the oxygen affinity by stabilizing the deoxy-structure. The primary organic phosphate allosteric effectors are adenosine triphosphate (ATP) and guanosine triphosphate (GTP) in elasmobranchs and bony fishes, whereas amphibians and mammals utilize ATP and 2,3-diphosphoglycerate (DPG), birds and turtles utilize inositol phosphate (IP) and reptile utilizes ATP (Bartlett, 1980).

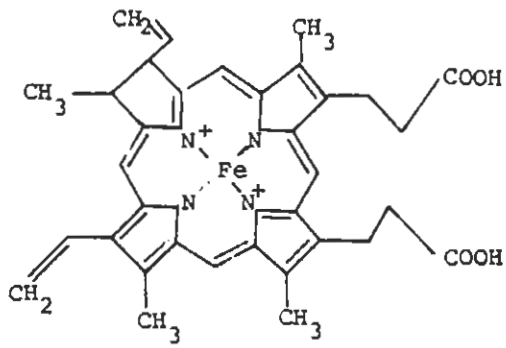
According to the studies of Perutz etal. (1980) about 50% of the alkaline bohr effect in human deoxyhemoglobin depends on a hydrogen bond between the COOH terminal histidine and the aspartate at the β-93 position of the same chain, and 25% of the alkaline bohr effect results from the salt bridges between the α-NH_2 groups of α-chain, a 10% from arginines of the neighbouring α-chain, another 10% of the Bohr effect is contributed by α-122 histidine (Nishikura, 1978) for which the cause is not known. In the mutant hemoglobin chains the bohr effect also depends on β-143 histidine and β-144 Lys; when these are replaced by other amino acids, the Bohr effect is decreased. Thus a very minor change in Bohr effect can bring about a fairly large amount of functional differences in closely related hemoglobins, as required by the organism:

Very recently Irina etal.(1984) have used high resolution proton magnetic resonance spectroscopy at 300 and 600 MHz to investigate the contacts in hemoglobin and also the Bohr effect. Studies on

the minor component hemoglobin A_2 ($\alpha_2\delta_2$) of human blood revealed that the replacements of β-chains by the δ-chains in HbA_2 conserves the $\alpha_1\delta_2$ interface but slightly disturbs the $\alpha_1\delta_1$ interface. In addition, the studies also show that in both the forms of HbA_2 i.e. deoxy and carbonmonoxy, one histidine have local confirmation and/or different electrostatic environments than normal adult hemoglobin. In human normal adult Hb both β 166 and β 177 histidine residues were found to be titratable in carbonmonoxy and deoxy forms contributing towards the "Bohr effect". These studies also suggested that in sickle hemoglobin the property does not originate due to β-6 alteration alone bu: involves additional amino acid residues which are different in β-and δ-chains.

1.3. STRUCTURE OF HEMOGLOBIN

Hemoglobin consists of two moieties, the protein part named globin and the prosthetic part heme which is an iron chelating porphyrin system. The porphyrin consists of four pyrole rings, being linked by methene bridges (Kuster 1912) with an iron present in the core. The iron content of the hemoglobin was found to be 0.35%



(Engelhert, 1825) which was later confirmed by Drabkin in 1957. From the measurements of Adair (1924) and the known iron content of the molecule, it was concluded that in a molecule with

molecular weight of 68,000, there are four heme groups. The chemical derivative of heme (Haurowitz, 1928) strongly suggested that the prosthetic groups of all hemoglobins were identical. Thus, it is apparent that, the difference in the hemeglobins of various species were due to the globin portion of the molecule.

The first end group analysis on hemoglobins were performed by Porter and Sanger (1948). Human hemoglobin and a number of other mammalian hemoglobins were examined by fluorodinitrobenzene method, which showed valine to be the terminal amino group in adult human and in horse hemoglobins. In cow, sheep and goat hemoglobins the terminal amino groups were valine and methionine.

Rhinesmith etal. (1958) suggested that the two polypeptide chains of human hemoglobin be designated as α and β chains, and this nomenclature has been extended to the hemoglobins of other species. According to this image the hemoglobin molecule being a tetramer in most species can be defined chemically as $\alpha_2\beta_2$. In a few animals the monomeric and dimeric forms are also present.

1.3.1. Perutz model

The low resolution model of horse methemoglobin was presented by Perutz in 1960. The X-ray studies by Bragg and Perutz (1952a,b, 1954) and Perutz etal. (1960) suggested that hemoglobin molecule is spherical with approx. 64x55x50 A.

The phase angles of crystalline hemoglobin were determined by preparing a number of isomorphous derivatives, mercury and various mercury derivatives which reacted with the sulfhydryl groups in the

hemoglobin molecules. The heme groups tend to be located at the surface of the molecule in four widely separated pockets embedded in the peptide chains. The iron atoms located at the corners of an irregular tetrahedron and separated by a distance of 25 to 30 A. This distance is not enough to permit direct interactions between them (Cullis etal. 1962).

The packing of chains in the hemoglobin molecule allows close contact between unlike subunits and a little contact between α and α or β and β. The αβ contacts are of two types: (1) the $\alpha_1\beta_1$ or $\alpha_2\beta_2$ contact involving B,G and H helices and GH corner called packing contacts because they represent subunit's packing that is unchanged when the hemoglobin molecule goes from its deoxy to oxy configuration, and (2) the $\alpha_1\beta_2$ or $\alpha_2\beta_1$ contact involving mainly C and G helices and FG termed the sliding contact, since they undergo the contact when there is a change in ligation state of the heme. The sliding is of the order of 2.7 residues in oxy and deoxy hemoglobin.

1.4. PRIMARY STRUCTURE OF VERTEBRATE HEMOGLOBIN

Vertebrate hemoglobins are tetrameric proteins. The 2 pair chains of globin are different and are designated as α and β-chains. The main component of normal human hemoglobin is HbA (Rhinesmith etal. 1958). The molecule possess a doubly symmetrical structure (Perutz, 1951). In contrast to human hemoglobin, other mammals usually consist of more than one main component differing in the sequence of one or both chains (Perutz etal. 1959).

Some of the animal hemoglobins have been reviewed by Gratzer and Allison (1960) which are monomeric and dimeric in nature (Hunt etal. 1978).

The globin sequences of vertebrates showed at least 60 different α-family (α,δ), and 66 different β-family (β,γ,δ and ε). Each of the two families of globin chains has its own particular pattern of similarities. A comparative study of the known sequences of vertebrates shows that all the 60 known α-hemoglobin chains family have 23 identical amino acids in 141 positions. In 66 known β hemoglobin chains family have 20 identical residues in 146 positions. This shows a higher mutation rate in the β-chain as compared to that of α-chain (Dickerson and Gies, 1983).

1.5. REPTILE HEMOGLOBIN

1.5.1. Classification of reptiles

Reptiles occupy an important position in animal kingdom and constitute about 70% of the vertebrates present on earth and have as many as 5,000 species. Reptiles are usually classified into following four groups:

i) the chelonia or turtles
ii) the squamata or snakes and lizards
iii) the Rhynchocephalia
iv) the crocodilia

The order squamata is divided into ophidia and lacertilia suborders. There are about 2500 species of lizards distributed throughout the tropical and semi-tropical parts of the world in

suborder lacertilia. The members of this group are widely spread into various ecological conditions like aquatic, terrestrial and desert.

Amongst the lizards Uromastix is an important genus. The species in this genus are reported to be eleven by Smith (1935) and seven by Ditmars (1953).

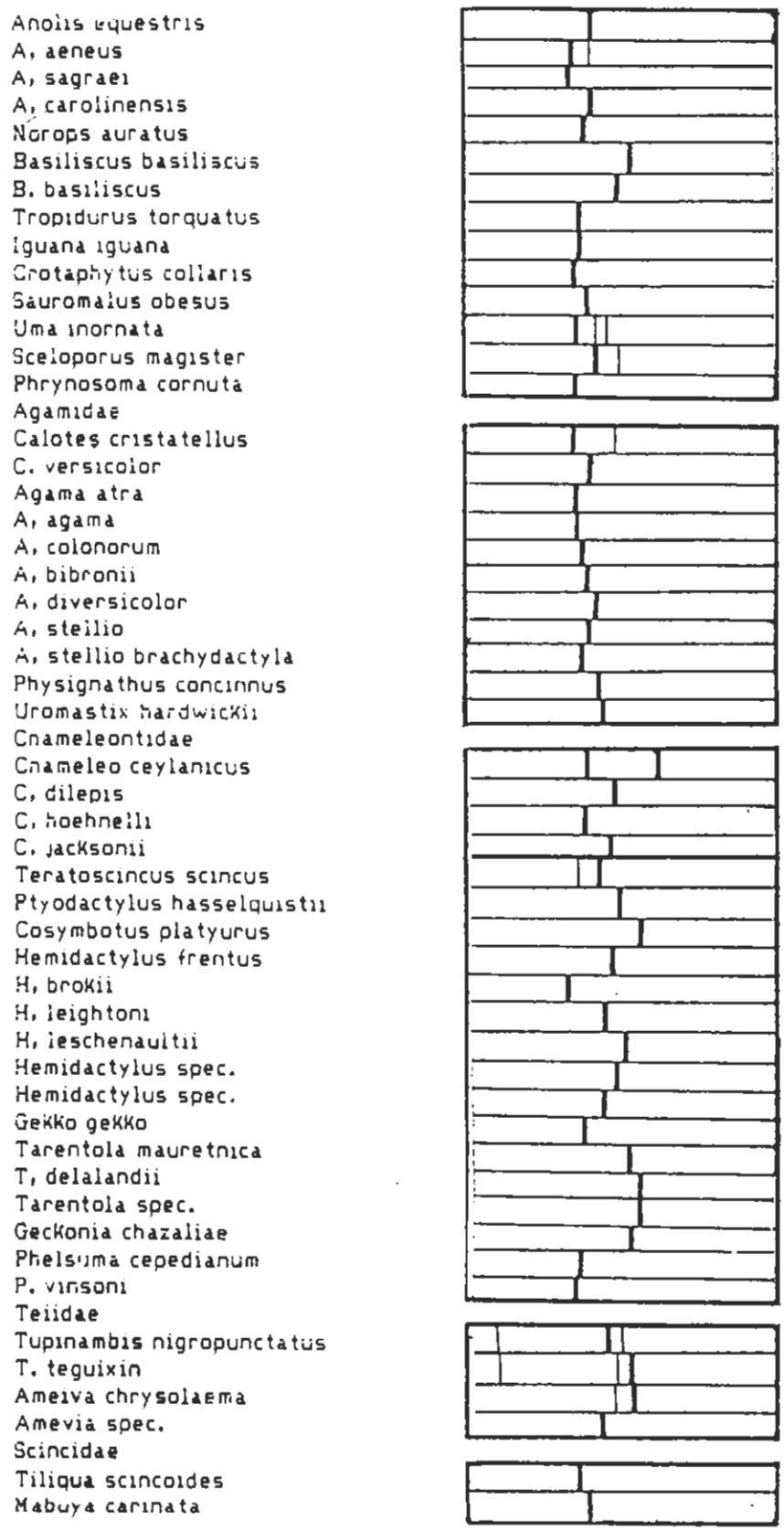
1.5.2. Hemoglobin

Reptiles have nucleated erythrocytes which are slowly replaced (Brace and Altland 1955; Altland and Brace, 1962). Standard hematological parameters such as blood volume, hemoglobin content, hematocrit value vary widely in reptiles. These are known to be a function of altitude (Hadley and Burns, 1968, Vinegar and Hillyard, 1972), Season (Semple etal. 1970) and diet (Musacchia and Sievers, 1962).

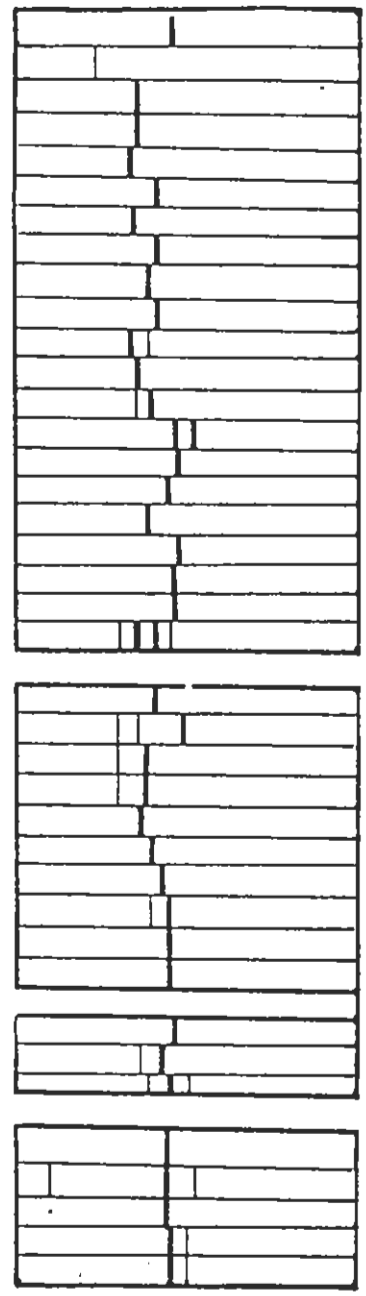
1.5.3. Electrophoretic studies

Most reptiles have multiple hemoglobins. Dessauer etal. (1957) have reported the presence of 1 to 5 components in turtles and lizards. Smaller number of components present in snakes, aligators and crocodiles. Riggs etal. (1964) and Sullivan and Riggs (1964,1967a) reported polymorphism in reptilian hemoglobin to be due to disulfide bond formation. The presence of polymeric hemoglobin in reptiles was reported as early as 1934 by Svedberg and Hedenius. More than 50 chelonian species were examined by Sullivan and Riggs (1976b) using the starch gel electrophoresis. All the species studied showed 2 major and 4 to 5 minor bands in hemoglobin.

Fig.1.1:ELECTROPHORETIC PATTERN OF THE HEMOGLOBIN FROM LIZARDS



M. multifasciata
M. quinquetaeniata
M. capensis
M. spec.
Riopa sundevallii
Eumeces schneideri
Chalcides ocellatus
C. viridanus
C. chalcides
C. mionecton
Lacerta ocellata
L. viridis
L. agilis
L. vivipara
L. muralis
L. sicula campestris
L. melisellensis
L. hispanica
L. galloti
Latastia longicaudata
Acanthodactylus scutellatus
A. pardalis
Cordylidae
Gerrhosaurus major
G. flavigularis
Cordylus giganteus
C. jonesii
C. vittifer
Pseudocordylus subviridis
Platysaurus guttatus
P. intermedius
P. minor
Anguidae
Gerrhonotus multicarinatus
Ophisaurus apodus
Anguis fragilis
Varanidae
Varanus griseus
V. exanthematicus
V. bengalensis
V. salvator
V. varius



Dessauer etal. (1957) & Dessauer and Fox (1964) studied a number of hemoglobins from lizards and showed the presence of multiple hemoglobins. Dessauer etal. (1957) examined hemolyzates of 40 species of Snakes showing that many species had run towards cathode indicating high iso-electric points. The electrophoretic studies of 24 species of Snakes (Bovidae, Viperidae and Colubridae) were performed by Schwantes (1972); showing the similar results as Dessauer etal. (1957). These results are represented in Figure 1.1.

1.5.4. Primary structure of reptile hemoglobin

A few studies are reported on the primary structure of reptile hemoglobin. Duguet etal. (1974) has elucidated the primary structure of the α-chain of Vipera aspis. This sequence showed 46 out of 141 residues to be invarient when compared to the human α-chain. The amino acid substitutions in Viper are not evenly distributed along the poly-peptide chain. About 50% of the changes were noted to be present in the first 32 residues from N-terminal.

The other sequences reported are of the three species of crocodiles; namely Caimen Crocodylus; Crocodylus Niloticus and the Alligator Mississipiensis (Leclercq etal; 1981). Each species showed the presence of a single dimeric globin having one α and β-chain. Recently, Naqvi (1984) has reported the sequence of Uromastix Hardwickii, a species of lizards. The sequence is based on alignment of tryptic peptides with sequence of human globin. The sequence study reported by them is shown in Figure 1.2.

1.6. EVOLUTION AND PHYLOGENY

Various groups of workers have carried out the investigations

```
1                                   10                                  20
Val Leu Thr Asp Asp Asp Lys Asn His Val Arg Ala Ile Trp Gly His Val Ser Asn Asn Pro Glu Ala Phe Gly

                                                                        Val Gly Pro His Leu Asp Asp Tyr Gly

                    30                                  40                                  50
Ala Glu Ala Leu Tyr Arg Leu Phe Thr Ala His Pro Ala Ser Lys Thr Tyr Phe Ser His Phe Asp Leu His Glu

Gly Glu Ala Leu His Arg Asn Phe Glu Val Tyr Pro Gln Thr Lys Thr Tyr Phe Pro His Phe Asp Ala Ser Ala

                                    60                                  70
Asn Ser Ala Gln Ile Arg             Lys Val Val Asp Ala Leu Thr Gln Ala Val Asn Asn Leu Asp Asp

Gly Ser Asn Gln Leu Lys

                    80                                  90
Leu Ser Gly Ala Ile Ser Lys Leu Ser Asp Leu His Ala Glu Lys
```

Figure 1.2a: Amino acid sequence of the α-chain of Uromastix hardwickii.

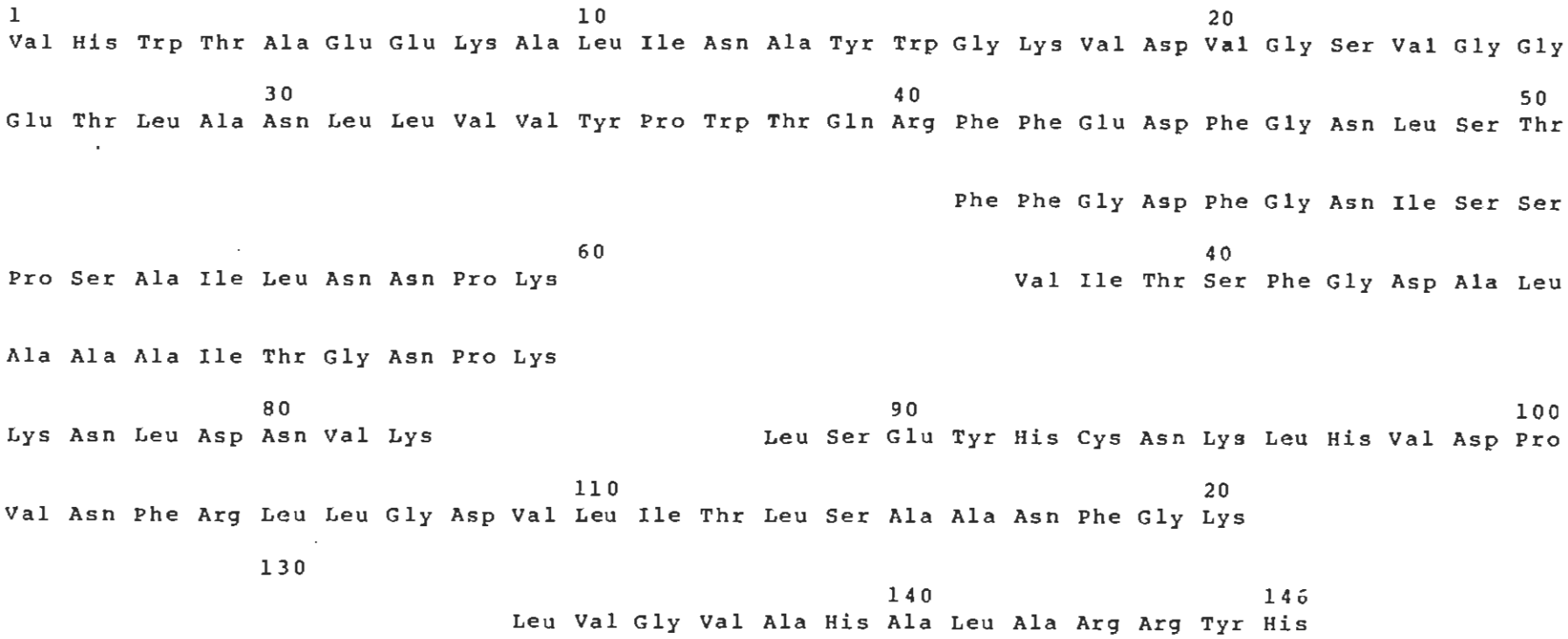
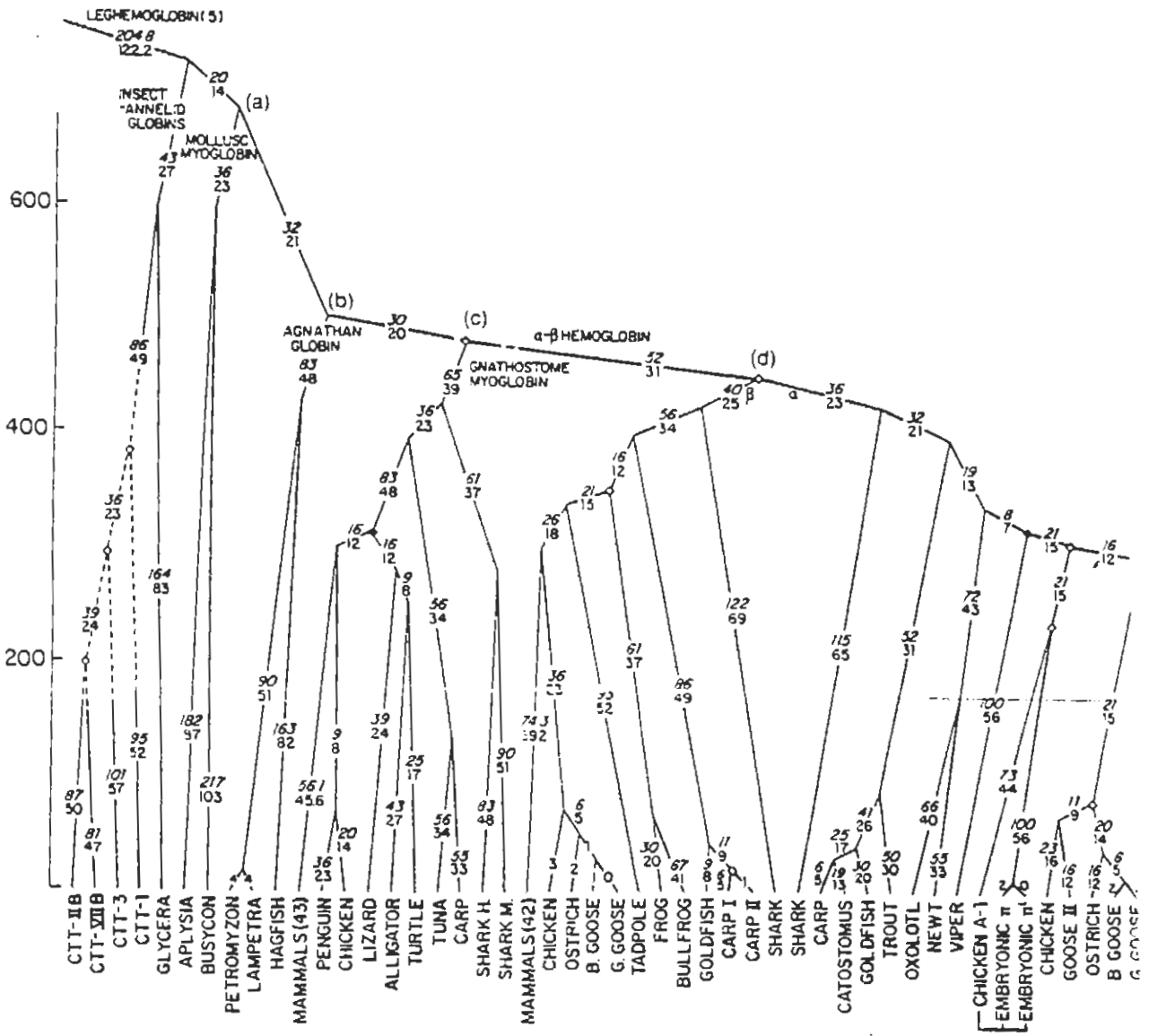


Figure 1.2b: Amino acid sequence of the β-chain of Uromastix hardwickii.

on the phylogeny of hemoglobin molecule by its amino acid sequence of related groups of animals (Braunitzer, 1972; Dayhoff etal. (1969) Zucker Kandl, 1965 and Goodman, 1973). In evolution two processes may be possible i.e. convergence or the divergence. In the convergence process, non-homologous genes are assumed as precursors which have again reached the present degree of evolution. In divergence a common precursor gene has to be assumed of which the successor genes have evolved in the course of evolution through the process of gene duplication, mutation and selection into its present/recent constitution and multiplicity. The study of primary structures of hemoglobin is necessary in order to understand the evolution of hemoglobin as it gives an exact information about the degree of relationship.

Goodman etal. (1975) constructed a geneological tree of the globin gene from the available data on sequences by maximum parsimony method assuming that the best geneology has the lowest nucleotide substitution or replacements. Goodman etal. have concluded that the rate of hemoglobin evolution have been non-uniform. They suggested that the sequence positions with these amino acids residues involved in the $\alpha_1\beta_2$ contacts showed many more replacement at the time of the emergence of jawed vertebrates when the tetrameric form of hemoglobin was emerging. After the early evolution in the $\alpha_1\beta_1$ contact region these sequence positions have undergone fewer substitutions compared with the residues on the exterior of the protein during the vertebrate evolution.

A comparable pattern of fast rates in early cold-blooded vertebrates is observed in the globin phylogeny. The rate variations


LEGHEMOGLOBIN(5)
INSECT
ANNELID
GLOBINS
MOLLUSC
MYOGLOBIN
(a)
(b)
AGNATHAN
GLOBIN
(c)
α-β HEMOGLOBIN
GNATHOSTOME
MYOGLOBIN
(d)
600
400
200
CTT-IIB
CTT-VIIIB
CTT-3
CTT-1
GLYCERA
APLYSIA
BUSYCON
PETROMYZON
LAMPETRA
HAGFISH
MAMMALS(43)
PENGUIN
CHICKEN
LIZARD
ALLIGATOR
TURTLE
TUNA
CARP
SHARK H.
SHARK M.
MAMMALS(42)
CHICKEN
OSTRICH
B. GOOSE
G. GOOSE
TADPOLE
FROG
BULLFROG
GOLDFISH
CARP I
CARP II
SHARK
SHARK
CARP
CATOSTOMUS
GOLDFISH
TROUT
OXOLOTL
NEWT
VIPER
CHICKEN A-1
EMBRYONIC π
EMBRYONIC π'
CHICKEN
GOOSE II
OSTRICH
B GOOSE

observed using the parsimony approach (Goodman etal. 1975) are supported by Holmquist etal. (1976) and Matsuda (1978). Accelerated α and β-evolution occurred in lineages of cold-blooded vertebrates, marsupials and the early placental mammals. The decelerated evolution occurred in two lineages leading to birds and mammals and was specially pronounced in higher primates. The rate of evolutions were much higher in the reptilian and amphibian than in the lineages of warm-blooded vertebrates. These accelerated rates occurred during the evolutionary transition of hemoglobin from a monomeric protein to a sophisticated allosteric form of tetramer. During this transition, the sites of the globin sequence which evolved at the most accelerated rate were precisely the sites responsible for the advance in function whereas, after the transition these sites hardly changed at all in the hemoglobin of warm-blooded vertebrates. Therefore it can be concluded that the initial fast rates were due to positive natural selection and the subsequent slow rates to stabilizing selection.

Hemoglobin often occurs in multiple forms within a single species. The gene multiplicity has been extensively studied in the human hemoglobin and as many as six chains (α,β,γ,δ,ε,τ) have been characterized. The divergence of these is depicted in the phylogenetic tree of the globin family.

The hemoglobin gene differentiates into α and β during the evolution of fish approximately 450-500 million years ago (Dickerson 1971). The α subdivides into α and δ some times before the reptilian ancestors of mammals and birds diverged. β-differentiates into β and fetal γ during the rise of mammals. The γ gene later produces both γ and ε and the β gene in primates separated into β and δ genes.

1.7. OBJECTIVES OF THE STUDY

A study on hemoglobin of Uromastix hardwickii, a lizard, has been undertaken to ascertain the number of components, isolate them, determine their amino acid composition, and to undertake their sequences.

It has been seen that though reptiles form a major portion of life on this planet, the hemoglobin of only a few species has been studied in detail. The inadequacy has left a gap in the phylogenetic tree of hemoglobin. This study on the hemoglobin of Uromastix hardwickii which has not been studied previously is an attempt to bridge this gap.

MATERIALS AND METHODS

2.1. BLOOD FROM UROMASTIX

Uromastix hardwickii, a species of lizards, were caught near Thatta in the province of Sind. These were identified and blood was let by cutting the animal gently near the neck. It was collected in 3.8 percent sodium citrate solution that acted as an anticoagulant.

2.2. EXTRACTION OF HEMOGLOBIN

Hemoglobin from fresh blood of Uromastix was extracted using the procedure of Drabkin (1964). The anticoagulated blood was centrifuged using Labofuge Centrifuge - model 1 (Karlkolb, West Germany) at a speed of 4,800. The settled red cells were washed several times with physiological saline (0.9% sodium chloride). The cells were ruptured by leaving these overnight in deionized distilled water with a slight layer of toluene. The cell debris were separated by centrifugation and the clear supernatant containing hemoglobin solution was lyophilyzed using freeze dryer, Eyela model FD-1 (Tokyo, Japan).

2.3. PHOTOMETRIC ESTIMATION OF HEMOGLOBIN CONCENTRATION

The standard method for determining the hemoglobin concentration is the photometric measurement of oxidized and cyano-ligand hemoglobin according to Cannan (1958).

The transformation solution was prepared by mixing 0.005M $K_3(Fe(CN)_6)$ and 0.025M KCN in distilled water (Betke and Savelsberg, 1950). A 20 µl of the hemoglobin solution was treated with 4 ml of the transformation solution. After 15 minutes of reaction, the absroption of the solution was recorded at 540nm using the transformation

solution as blank. The hemoglobin concentration was calculated using the following formula:

$$C_{Hb} = \frac{A_{540} \times \text{amt. of Sample}}{\varepsilon_{540} \times \text{area of Cuvette}}$$

where A_{540} = Absorbance at 540nm

ε_{540} = Extinction Co-efficient

2.4. SEPARATION OF HEME

The separation of heme from hemoglobin was carried out according to the procedure of Anson and Mirsky (1930). Pre-cooled solutions of hemoglobin and 2 percent HCl in acetone were prepared. The mixing was carried out by dropwise addition of hemoglobin solution in acetone-HCl mixture. The precipitated globin was centrifuged, washed thrice with cooled acetone and freeze dried.

2.5. GEL FILTRATION

The hemoglobin was subjected to gel filtration chromatography using sephadex gel. Sephadex is an anhydroglucose polymer cross-linked to epichlorohydrin which separates on the basis of different molecular weights. Sephadex gels of various pore sizes were used. Lower grades i.e. G-10 and G-15 were mostly employed for desalting, G-25 for the separation of small peptides and G-50, G-75, G-100 and G-150 for the separation of large peptides and proteins. Sephadex G-75 was found to be the most suitable gel for the separation of hemoglobin component and separation was carried out on this gel.

Swelling of Sephadex gel:

Swelling of the gel was carried out using eluant buffer. The required amount of gel powder was weighed, calculated according to

the swelling capacity, poured in an excess volume of eluant and left normally for overnight.

Packing of the column:

The excess buffer from the gel was decanted and the fresh buffer was added in such a volume so as it made a fairly thick slurry. The slurry was deareated under vacuum. Columns of various dimensions were prepared and mounted vertically on the stand. About one third volume of the column was filled with the buffer to avoid any trapping of air bubbles. The column outlet was closed and the slurry was then poured through the sides of the column. The column outlet was opened to allow slow flow. After the gel settled the equilibration of the column was carried out by running at least two bed volumes of buffer with the desired flow rate. The void volume of the column was determined using dextran blue (Serva Heidelberg).

Sample application:

A known quantity of hemoglobin was dissolved in a known volume of eluant buffer loaded on the column by gently layering it on the top taking care not to disturb the surface, and allowed to adsorb completely.

Elution of sample:

The sample was eluted with eluant buffer (1.8M acetic acid in case of sephadex G-75) with a constant flow rate 18 ml/hr. The fractions of 3 ml each were collected on LKB Ultro-Rack 7000, connected with an Ultra-Violet monitor UV-1. The absorbance was recorded at 280nm. The chromatography was performed at 20^{o}C, and in

certain cases at 4°C, using a thermostatically controlled circulating water bath.

2.6. ION-EXCHANGE CHROMATOGRAPHY

Ion-exchange chromatography was used for the separation of components in the hemoglobin. The separation on ion-exchange is achieved on the basis of charges on the molecules. An ion-exchanger consists of an insoluble matrix to which groups have been covalently bound. The charged groups are associated with mobile counter-ions. These counter-ions can be reversibly exchange with other ions of the same charge without altering the matrix. Several attempts were made using various types of ion-exchangers to separate hemoglobin components.

2.6.1. Separation on DEAE-Sephadex (with pH and Salt Gradient)

Diethyl-aminoethyl (DEAE)-Sephadex are weakly basic anion exchangers, and works over a wide range of pH (2-10).

Separation of hemoglobin components was carried out on DEAE-Sephadex A-50 (Pharmacia Fine Chemicals). The resin was suspended in the starting buffer containing 0.01M potassium phosphate, 100mg/lit of potassium cyanide and 2 ml/lit of thiodiglycol, and pH adjusted to 9.0, using phosphoric acid. The column of the dimension 20 x 1.5cms was packed and equilibrated as stated earlier in Section (2.5). A uniform flow rate of 20 ml/hr was maintained using LKB peristaltic pump. A known quantity of the sample was dissolved in the equilibration buffer and subjected to the column. After complete adsorption of the sample the elution was performed applying a linear gradient of both the pH and salt.

2.6.2. Separation on Sp-Sephadex

Strongly acidic cation exchange resin sulphopropyl (SP) - Sephadex was swollen in the starting buffer and a column (20 x 1.5cm) was packed and equilibrated. The buffer used was 0.05M potassium phosphate of pH 5.5. The sample after dissolving in the buffer was applied on the column. The desorption of the sample was carried out by gradient elution of both pH and salt (KCl) from 5.5 - 10pH and 0-0.5M respectively.

2.6.3. Separation on CM-Cellulose

Carboxy methyl-cellulose (Whatman, Microgranular CM52, preswollen) was used for the separation of hemoglobin components. Several analytical runs were performed varying the conditions of pH and concentrations. The following conditions were found to be most suitable:

A column of 30 x 0.8cms was packed with the pre-swollen CM-cellulose resin as described in the earlier section equilibrated with the initial buffer of 0.01M sodium phosphate at pH 2.9. The flow rate of the column was set at 12 ml/hr using an LKB peristaltic pump. The major component separated out as the first peak on sephadex G-75 was applied to the column in the same buffer with a slightly lower pH around 2.5, allowed to adsorb completely on the column under gravity. The separation was carried out with stepwise salt-gradient. The gradients used were 0.01M, 0.1M and 0.2M sodium phosphate buffers. The pH was kept constant at 2.9 throughout the experiment. Fractions of 3 ml each were collected and the absorbance recorded at 280nm as stated earlier.

2.7. PURITY ON HPLC

2.7.1. The purity of the components were determined using reverse phase high performance liquid chromatography where the separation is achieved according to the solubility of the sample. The HPLC system used was Jasco Model Twincle HPLC with GP-A40 Gradient Programmer and Recorder RC-250 (Tokyo, Japan). Reverse phase column of C_{18} μ-Bondapack (15x0.46cm) was used with isocratic solvent system of 50/50 (v/v) mixture of acetonitrile and water. Absorbance was recorded at 280nm.

2.7.2. The components were also checked for their purity using the HPLC System, Alltech Ltd. LDC Model III. The column of RP-18, ODS-11-Spherisorb (5μ particle size) with the dimension 15x0.46cm was used. The elution was carried out with 50/50 (v/v) mixture of methanol and water. The detection of the purity was performed using the refractive index which has a specific value for each substance.

2.8. ELECTROPHORESIS

2.8.1. Disc gel electrophoresis

The hemoglobin samples were analyzed by disc-gel electrophoresis, using discontinuous system of Ornstein (1964) and Davis (1964). The following solutions were used for the preparation of polyacrylamide gels:

Solution A: The solution was prepared by dissolving 36.3gms of tris-(hydroxymethyl)-aminomethane (Fluka, Switzerland), in 48 ml of 1N HCl and 0.46 ml N,N,N,N-tetramethyl ethylene diamine (Merck, Darmstadt), made upto 100 ml with distilled water, pH 8.9.

Solution B: Acrylamide solution was made by mixing 25.0gm acrylamide and 0.4gm N,N-methylene bis acrylamide (Serva, Fein-biochemica, Heidelberg) in distilled water. The volume was made upto 100 ml.

Solution C: A freshly prepared solution of 0.14gm of ammonium per sulfate dissolved in the distilled water to make 50 ml of solution.

Preparation of Gels:

Gels of various percentages were prepared by mixing different ratios of the above solutions as represented in Table 2.1.

TABLE 2.1

MIXING RATIOS OF THE GELS WITH 1.57 CROSS LINKING

Gel solution	5%	10%	12%	15%
Solution A	1 ml	2 ml	2.4 ml	3 ml
Solution B	3.2 ml	6.4 ml	7.68 ml	9.6 ml
Solution C	2 ml	2 ml	2 ml	2 ml
Distilled water	9.8 ml	5.6 ml	3.92 ml	1.4 ml
Total volume	16 ml	16 ml	16 ml	16 ml

The lower ends of the gel were blocked using parafilm and gel tubes (0.4 x 8cms) were filled with this solution, leaving about 1.5cm space and left for polymerization for approximately 30 minutes. A layer of water was also added carefully to the gel to produce a smooth upper surface.

Sample preparation:

A protein sample weighing a known quantity was dissolved in the upper electrode buffer. A drop of saturated solution of

bromophenol blue was also added in the sample solution as tracking dye.

Sample application:

The water layer from the top of gel was replaced by a layer of saturated sucrose solution and a known quantity of the sample was applied on top of the gel for electrophoresis. The space in the tube was filled with the upper electrode buffer avoiding mixing and air bubbles in the system.

Composition of electrode buffers:

The following electrode buffers were prepared:
Upper electrode buffer: Tris-(hydroxymethyl)-amino-methane 7.68 gm; glycine (Merck, Darmstadt) 3.84gm, pH 8.9. The volume made upto one litre with distilled water.

Lower electrode buffer: Tris-(hydroxymethyl)-amino-methane 14.48gm; pH adjusted to 8.0 using HCl and solution made upto one litre with distilled water.

Staining solution:

Two percent amido black 10-B in 7% acetic acid was prepared according to the method of Ritchie etal. (1966).

Coomassie brilliant blue R-250 0.25% in 10% trichloroacetic acid and 30% methanol, following the procedure of St.Groth etal. (1963).

Destaining solution:

Seven percent acetic acid in case of amido black 10-B,

and Ethanol: Acetic acid: Water (3:1:6), in case of Coomassie brilliant blue R-250 (Steel jr., 1978).

Electrophoretic conditions:

After applying the sample and filling the upper and lower chambers with appropriate buffers, the electrodes were connected to the power supply Model-SAE 2761 (Shandon, England). An electric current of 5mA per tube was applied initially for 10 minutes and later increased to 7mA per tube for a period of 2½ hours.

2.8.2. SDS-Polyacrylamide electrophoresis

This was used for determining the molecular weight of protein. The preparation of the gel solutions and electrode buffers were similar as stated in section for disc-gel electrophoresis except that 0.1% SDS was added in the gel and buffers.

Sample preparation:

The sample approximately 1mg was dissolved in upper electrode buffer and 25µl of β-mercaptoethanol was added. The sample was heated for 10 minutes in water bath at 100°C and subjected to electrophoresis immediately.

2.8.3. Iso-electric focusing

Iso-electric focusing was carried out using the procedure of Wrigley (1969). The following stock solutions were used for the preparation of gels:

Solution A: 30gm acrylamide, 1.0gm N,N-methylene-bis-acrylamide, volume made upto 100 ml with deionized distilled water.

Solution B: 40 percent solution of ampholyte in deionized distilled water. Pharmalyte (pH range 3-10) was obtained from pharmacia fine chemicals (Uppsala, Sweden).

Solution C: One percent potassium per sulfate, 0.1gm/10ml.

Solution D: N,N,N,N-Tetra methyl ethylene diamine (Merck, Darmstadt), 5µl.

The mixing ratios of above solutions were: Sol.A 4ml, Sol.B 1ml, Sol.C 1ml, Sol.D 5µl and water 14ml. The mixture was degassed quickly and poured in the gel tubes 0.4 x 12cms and the gels were prepared as described earlier.

Cathode electrode buffer: 0.4% ethanol-amine

Anode electrode buffer: 0.4% (H_2SO_4) Sulfuric acid

Both these buffers were prepared in deionized double distilled water.

Sample application:

The sample was applied as stated in the section (2.8.1), a layer of saturated solution of sucrose followed by 25µl of sample, and 25µl of 40% ampholyte solution was added. Finally the space in the tube was carefully filled with upper electrode buffer avoiding mixing and the trapping of air bubbles.

After the application of the samples on the gels, a current of 2mA per tube was applied with increasing voltage upto 350 volts for a period of 3 hours.

Fixing solution: This was prepared by mixing 40ml of ethanol and 10ml trichloro-acetic acid. The volume of the solution was made upto 100ml with distilled water. Staining and destaining of the IEF gels were carried out using Coomassie Brilliant-blue R-250, and the solutions were prepared as described in disc electrophoresis section (2.8.1)

2.8.4. Triton-electrophoresis

The method introduced by Rovera etal. (1978) was used for the separation of hemoglobin chains on electrophoresis. Polyacrylamide gels containing urea, acetic acid and Triton X-100, in different ratios as described below, were used to resolve microgram quantities of hemoglobin subunit polypeptides. The following stock solutions were prepared:

Solution A: 30% acrylamide and 0.4% N,N-methylene bis acrylamide in distilled water.
Solution B: 20% Triton X-100 in distilled water.
Solution C: Ammonium per sulphate 2%.

To make 20ml of the gel solution, the quantities mixed were: Sol.A 8ml, Sol.B 2ml, Sol.C 0.4ml, urea 9.6gm, TEMED 0.1ml, acetic acid 1ml. The solutions mixed and degassed quickly under vacuum. After pouring in the tubes the gels were carefully overlaid with distilled water and it was left overnight for polymerization.

Electrophoresis buffer:

A 5% solution of acetic acid was used as the running buffer for Triton electrophoresis.

Sample buffer: This was prepared by dissolving 48.5gm of urea in 10ml acetic acid and 10ml of β-mercaptoethanol. The volume was made upto 100ml with deionized distilled water.

Electrophoretic conditions:

The gels before applying the samples were subjected to the process of pre-electrophoresis at a constant voltage of 200 volts with anode on top using the electrophoresis buffer. After one hour of pre-electrophoresis the buffers in both the chambers were changed.

The protein samples were dissolved in the sample buffer, and applied on the gels very carefully using a micro-syringe. The electrophoresis was carried out at constant voltage of 140 volts for 4 hours. The gels were stained for half an hour with 2% amido black 10-B in 7% acetic acid. The destaining was carried out by diffusion with repeated changes of 7% acetic acid solution.

2.9. OXIDATION WITH PERFORMIC ACID

Prior to hydrolysis, the protein samples were oxidized using performic acid, following the procedure of Hirs (1967). For the preparation of performic acid, pre-cooled ($0^{\circ}C$) formic acid and hydrogen peroxide were mixed in the ratio of 9:1 (v/v) at $0^{\circ}C$ for 4 hours. A known quantity of the protein sample was mixed with performic acid in a ratio of 1:40 (w/v). This mixture was then left overnight at $0^{\circ}C$. The treated material was dried under vacuum by leaving the sample in the desiccator over KOH/H_2SO_4.

2.10. AMINO ACID ANALYSIS

The amino acid analysis of the protein was performed on Biotronik amino acid analyzer (Biotronik, West Germany). The amino

acid analyzer system involves, cation exchange resins of definite specification based on the pioneering work by Moore and Stein (1948), Speckman etal.(1958), Hamilton and Anderson (1959) and Peterson and Sober (1959).

2.10.1. Amino acid analyzer Biotronik LC6001

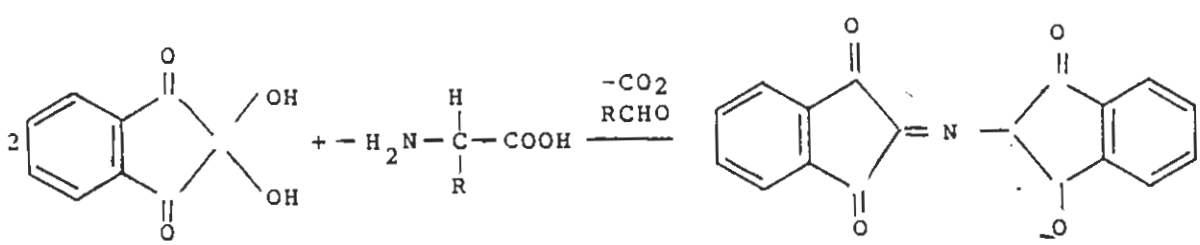
Amino acid analyzer is an automatic system with a sample injector and computing integrator for peaks. The system involves photometric detection of ninhydrin reacted amino acids. The photometer is attached to the recorder which inturn is attached to the computing integrator. Schematic representation of the separation programme on LC6001 is shown here in the Flow diagram, and Table 2.2 shows the composition of the buffers used.

2.10.2. Preparation of ninhydrin reagent

Twenty grams of ninhydrin and 1g of Stannous chloride were dissolved in 750ml of methyl cellusolve, in the presence of nitrogen stream. 250ml of 4M sodium acetate buffer, pH 5.5 was added and N_2 passed for 20 minutes at a pressure of 0.1 bar to remove oxygen.

2.10.3. Detection

The ninhydrin reagent is pumped to the mixing block where it mixes with the eluate, and reaches the reaction bath. The following reaction takes place according to Moore and Stein (1948).



(Rheumann's purple)

SCHEMATIC REPRESENTATION OF AMINO ACID ANALYZER LC 6001

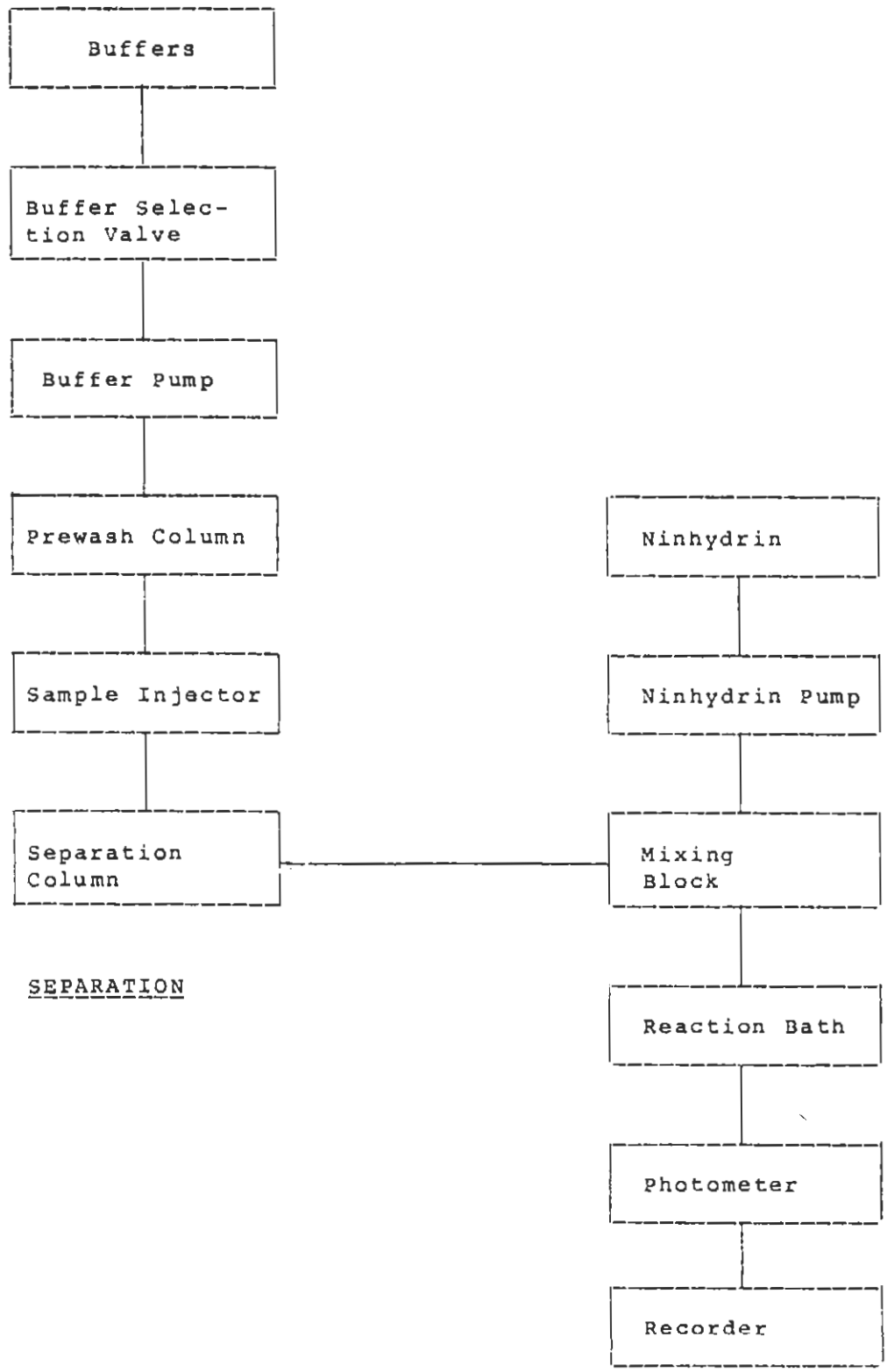


TABLE 2.2

	Buffer A	Buffer B	Buffer C	Buffer D	Regeneration Solution	Sample Diluting Buffer
pH-Value	3.50	4.25	5.35	10.20	-	2.20
Sodium-Concentration	0.12n	0.18n	0.18n	0.2n	0.2n	0.12n
Citrate-Concentration	0.08m	0.06m	0.06m	0.033	-	0.08m
Sodium hydroxide	9.6g	14.4g	14.4g	-	16g	4.8g
Citrate acid	33.6g	25.2g	25.2g	-	-	16.8g
Hydrogen chloride 37%	6.0ml	14ml	5ml		-	8ml
Methylcellusolve	200.0ml	-	-		-	-
Brij 30% solution	4ml	4ml	4ml	4ml	4ml	-
Phenol liquified	2ml	2ml	2ml	-	-	1ml
Thiodiethanol 25% in H_2O	-	-	-	-	-	20ml
Boric acid	2g	2	-	-	-	-
Sodium citrate	-	-	-	19.6g	-	-
Sodium tetraborate	-	-	-	38.1g	-	-
FINAL VOLUME	2 ℓ	2 ℓ	2 ℓ	2 ℓ	2 ℓ	1 ℓ

INSTRUMENT DATA FOR THE STANDARD HYDROLYZATE PROGRAM

Separation Column	0,4 x 25 cm	Buffer Flow	20 ml/h
Resin Type	BTC 2710	Reagent Flow	20 ml/h
Resin Bed Height	21 cm		
Prewash Column	0,6 x 8 cm	Temperature T_1	47.5°C
Resin Type	BTC F	Temperature T_2	61.5°C
Resin Bed Height	3 cm		

The reaction product flows through the photometer, and the absorbance of the reacted material is recorded on a built in recorder at 570nm and 440nm.

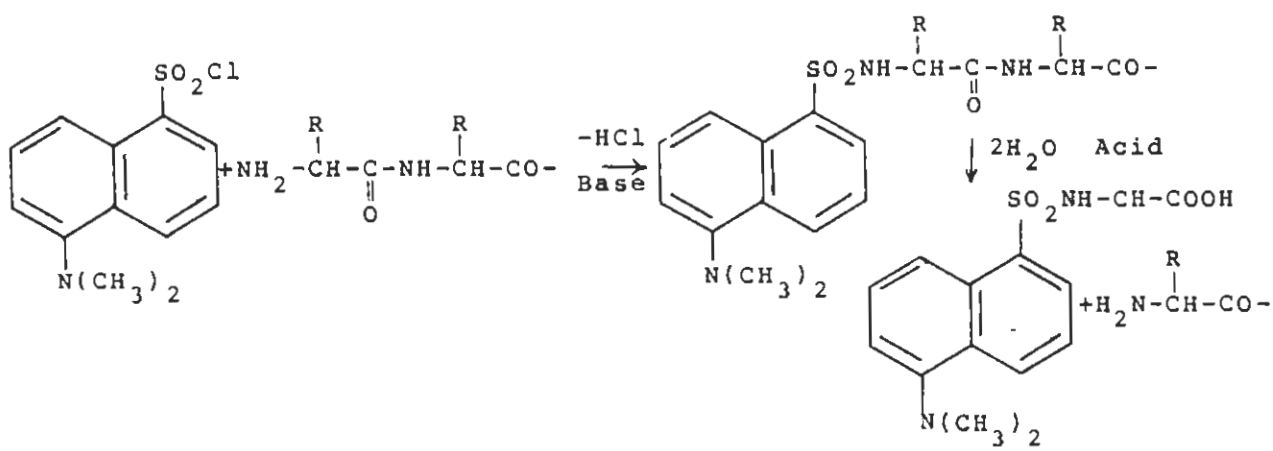
2.10.4. Sample preparation for Amino Acid Analysis

About 1mg of the protein was taken in 5.7N HCl in a test tube. The tube was sealed under vacuum and heated in an oven at 110°C for a period of 20 hours. The contents of the tube were then cooled to room temperature and the acid from the hydrolysed sample was removed on a rotary evaporator. The dried hydrolyzate was dissolved in 0.2M sodium citrate buffer pH 2.2 and subjected to amino acid analysis.

2.11. N-TERMINAL SEQUENCE ANALYSIS

2.11.1. Dansylation

The method of Gray and Hartley (1963) was followed for the determination of N-terminal amino acid. The reaction involves the coupling of the terminal amino group with 1-dimethyl amino naphthalene-5-sulphonyl chloride (DNS-Cl or Dansyl Chloride). This results in a dansylated derivative having a yellowish green fluorescence which can be identified under an ultraviolet lamp at 254nm.



A known quantity of protein sample was dissolved in 0.2M sodium bicarbonate solution and adding an equal volume of 0.25% solution of dansyl chloride in acetone. The pH was maintained at 8.5. The tube properly stoppered was allowed to stand in dark for one hour at 37°C and its contents were dried in vacuum. The dried sample was hydrolysed with 5.7N HCl in a sealed tube for 20 hours at 110°C. HCl from the hydrolyzed samples was removed under vacuum and the extraction of the DNS-amino acid derivative was carried out with ethyl acetate. The ethyl acetate extract containing the dansylated amino acid derivative was subjected to thin layer chromatography using Silica gel $60F_{254}$ pre-coated plates from E-Merck; (Darmstadt, West Germany). The solvent system used were Benzene:Pyridine:Acetic acid (4:1:0.2), according to Cole etal. (1965). The Fluorescent Spots were identified under an ultraviolet lamp at 254nm. The Rf values were compared with those of the standard DNS-amino acids, applied on the side of tlc plate.

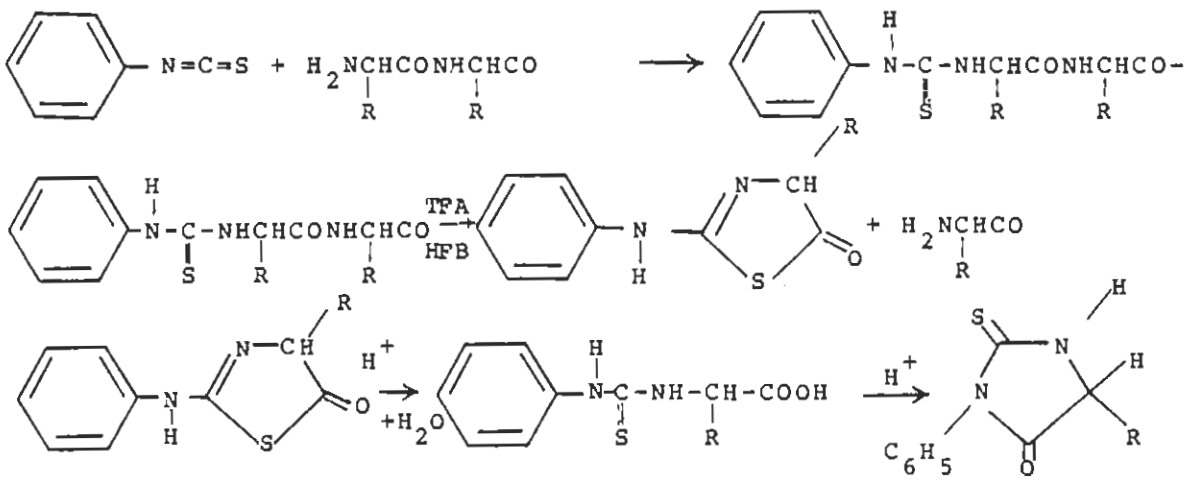
2.11.2. Edman degradation

The N-terminal amino acid sequence analysis was carried out by phenyl isothiocyanate (PITC) method according to Edman and Begg (1967) The following series of reactions forms the basis of PITC degradation.

Coupling: The formation of a phenyl thio-carbamyl (PTC)-derivative of the peptide by coupling the free α-amino group with PITC.

Cleavage: This reaction involves the cleavage of PTC-peptide at the peptide bond nearest to the PTC-substituent. This requires a strongly acid medium and leads to the formation of a peptide with one amino acid less than that of the original.

Conversion: This reaction takes place in an aqueous acid medium and consists in fact of two reactions i.e. hydrolysis of thiozolinone to the PTC-amino acid and the cyclization of the later to corresponding PTH-amino acid.



The same procedure was adopted for the sequence determination of amino acids with certain modifications. A 0.1μ mole of a peptide or a protein was dissolved in 0.2ml of 50% aqueous pyridine in a conical test tube. A 0.1ml of 5% solution of phenyl isothiocyanate (PITC) in pyridine was added and the mixture was left at 37°C for a period of 2 hours and 30 minutes. The solution was then evaporated to dryness under vacuum. The excess reagent was removed by washing the residue thrice with benzene. Few drops of water were added to increase the volume of aqueous layer. After vigorous stirring, the two phases were separated by centrifugation, and the organic phase was discarded. The aqueous phase was then dried under vaccum. The resulting PTC - peptide was treated with anhydrous trifluoroacetic acid (0.2ml) for one hour at 25°C. The acid was removed by leaving it under vacuum in a desiccator.

2.11.3. DABITC method

DABITC method was carried out for the mannual amino acid sequence analysis referring the work of Chang etal. (1978) and Von Bahr-Lindstrom etal. (1982). These methods employ a double coupling technique i.e. a first coupling with DABITC and a second coupling with PITC to achieve a quantitative coupling and conversion reaction. After the acid cleavage and conversion reaction only the colored DABITC-amino acids are identified by thin layer chromatography (TLC). The procedure adopted was as follows:

Preparation of crystalline DABITC:

About 30mg of 4N-N-dimethyl amino azobenzene-4'-isothiocyanate (DABITC) was recrystallize from 30ml of boiling acetone.

Preparation of standard:

a) 30µl Diethylamine + 300µl 50% pyridine + 15µl DABITC solution.

b) 60µl ethanol amine + 300µl 50% pyridine + 300µl DABITC solution.

The above solutions were heated for one hour at 50°C in water bath and dissolved in 400µl of ethanol.

DABITC solution was prepared by dissolving 0.8mg of DABITC in 450µl of pyridine.

The following experimental procedure was adopted:

The protein or a peptide was dissolved in 80µl of 50% pyridine and adding 40µl of the DABITC solution (in pyridine), the contents were flushed with N_2 and left in water bath at 50°C for a period of 45 minutes. After 45 minutes of heating the addition of 10µl PITC was followed by another 30 minutes of heating at 50°C. The amino acid

derivative was extracted thrice with 500µl (each) of heptane:ethyl-acetate (2:1), with a vigorous shaking. The upper layer was discarded after centrifugation and the lower, dried under vacuum. After complete drying, 50µl of TFA was added and the contents were heated for 15 minutes at 50°C, followed by drying under vacuum. The residue was dissolved in 30µl water and extracted twice with 50µl (each) of butyl-acetate. The two phases were separated by centrifugation. The lower aqueous phase dried and used for next cycle, and the butyl acetate layer (i.e. upper layer) was dried under the stream of nitrogen. To the residue, 30µl of 50% TFA was added and heated for another 50 minutes at 50°C. The contents dried under vacuum for a little longer time, to remove any traces of TFA and the residue dissolved in 5µl of ethanol for chromatography on polyamide 3 x 3cm plates (Chin Chang; Taiwan), using an internal standard. The plates were run in two directions, using the following two different solvent systems:

i) 33% acetic acid

ii) Toluene: hexane:acetic acid (6:4:3)

The plates were dried completely after each run. The plate was developed with HCl vapours and amino acids were identified according to their specific colours.

RESULTS

3.1. ELECTROPHORESIS

The disc-gel electrophoresis of crude hemoglobin showed the presence of six bands of different mobilities from cathode to anode (Figure 3.1a). SDS-electrophoresis showed the presence of four proteins (Figure 3.1b). Iso-electric focusing carried out on polyacrylamide gels with a gradient of 3-10 pH showed as many as fourteen bands (Figure 3.1c). The triton electrophoresis carried out in urea-acrylamide gels revealed the presence of six chains in crude hemoglobin (Figure ?.1d).

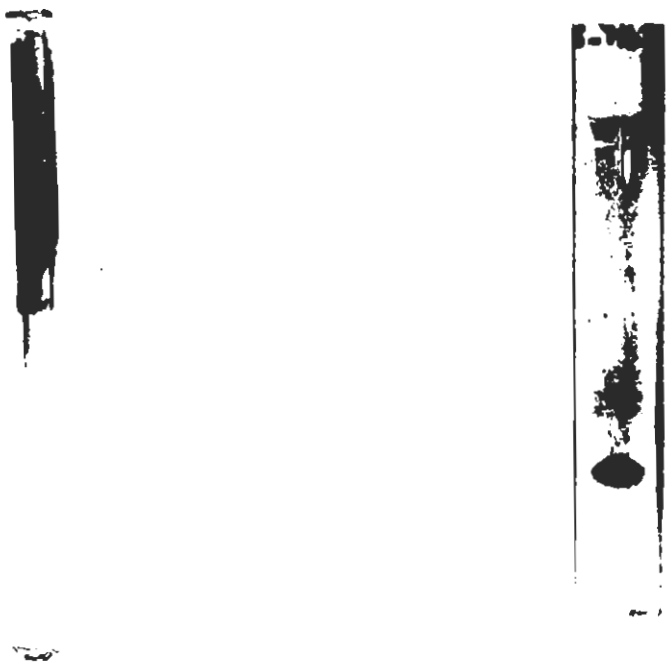
3.2. SEPHADEX CHROMATOGRAPHY

3.2.1. Separation on Sephadex G-50

Crude hemoglobin was separated on Sephadex G-50 (100 x 0.8cm) in 30% acetic acid. The elution was carried out with the same solution at a flow rate of 15ml/hr. Fractions of 2ml each were collected. Figure 3.2 shows the elution pattern. The peaks eluted at 20ml (void volume 20ml), 34ml, 46ml and 56ml respectively.

3.2.2. Separation on Sephadex G-75

The elution pattern of Sephadex G-75 is represented in Figure 3.3. The first peak eluted at 74ml, a little earlier than the void volume (void volume 90ml). The second and third peaks appeared at 104ml and 164ml respectively and the last peak eluted at 210ml. Fractions from each peak were pooled, freeze dried and their homogeneity determined by electrophoresis as well as terminal analysis.



a b

Fig.3.1: Electrophoretic patterns of crude hemoglobin from Uromastix hardwickii on polyacrylamide.

a) Without SDS
b) With SDS

c

d

Fig. 3.1-c: Iso-electric focusing of crude hemoglobin on polyacrylamide gel (0.4x12cm). Ampholyte pH 3-10.

Fig. 3.1-d: Electrophoresis of crude hemoglobin on polyacrylamide-triton gel (12%, 0.5x7.5cm).

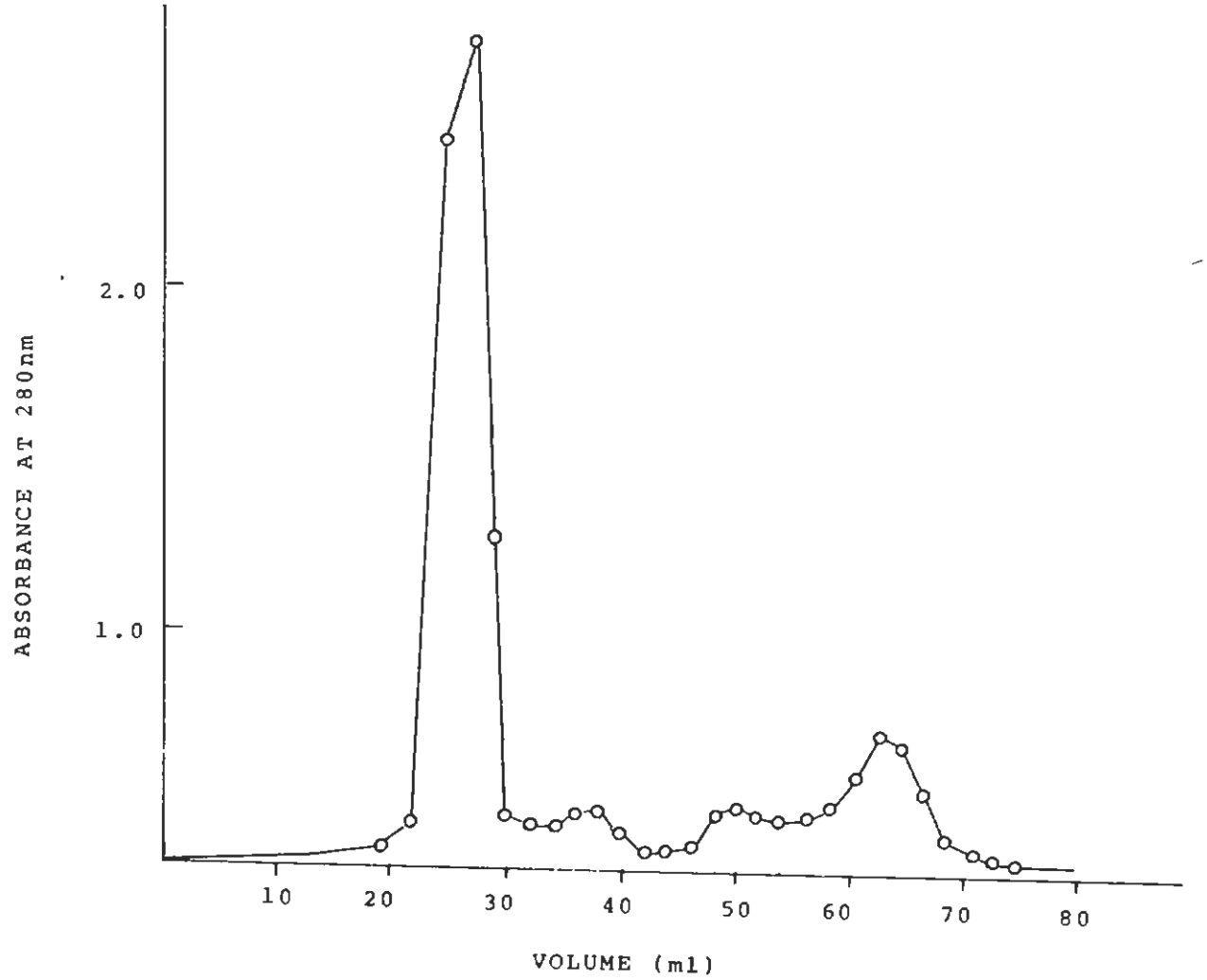


Fig. 3.2: Elution pattern of crude globin on Sephadex G-50 column (100x0.8cm). Eluent, 30% acetic acid; Flow rate; 15ml/hr., Fraction volume, 2ml/tube.

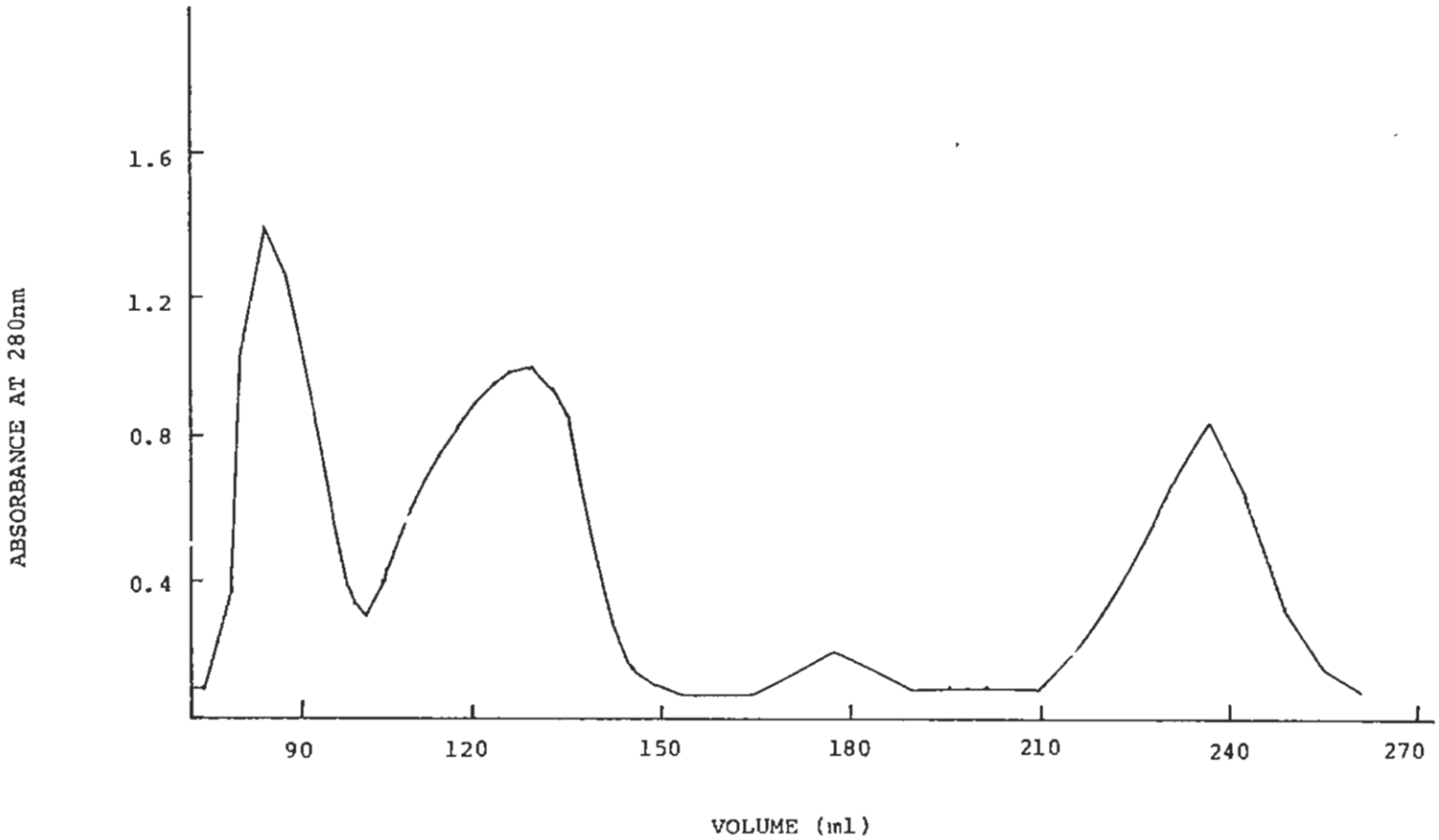


Fig.3.3: Elution pattern of crude globin on Sephadex G-75 column chromatography (130x1.5cm). Elution with 1.8M acetic acid with a flow rate of 18ml/hr. Fraction volume 3ml/tube. Absorbance noted at 280nm.

3.2.3. Separation on Sephadex G-100

A completely soluble sample of Uromastix hemoglobin (approx. 10mg) was subjected to a column (20 x 0.9cm) of Sephadex G-100. The sample was eluted with 10% acetic acid. The elution profile of hemoglobin is represented in Figure 3.4. The peaks eluted at 12ml (void volume 12ml), 21ml and 39ml respectively.

3.3. ELECTROPHORESIS OF SEPHADEX PEAKS

The peaks eluted from Sephadex G-75 chromatography were checked for their homogeneity by disc-gel electrophoresis, SDS-electrophoresis, Iso-electric focusing and triton electrophoresis. Figure 3.5 shows the results and found to contain more than single band.

3.4. N-TERMINAL ANALYSIS OF THE SEPHADEX PEAKS

The N-terminal amino group of the sephadex peaks were analysed by using dansyl chloride method. All the peaks showed several spots on tlc plate.

3.5. ION-EXCHANGE CHROMATOGRAPHY

3.5.1. Elution profile on Sp-Sephadex

The first peak eluted from Sephadex G-75 column was further separated on Sp-Sephadex C-50. The sample elution was carried out in 0.05M phosphate buffer by using a pH gradient from 5.5 - 10pH and also salt gradient of KCl (0.0 to 0.05M). No separation were achieved in this system.

3.5.2. Elution profile on DEAE-Sephadex

The separation of the Sephadex component was subjected to DEAE-Sephadex using Tris-HCl pH 8.0, the peaks were unresolved. When

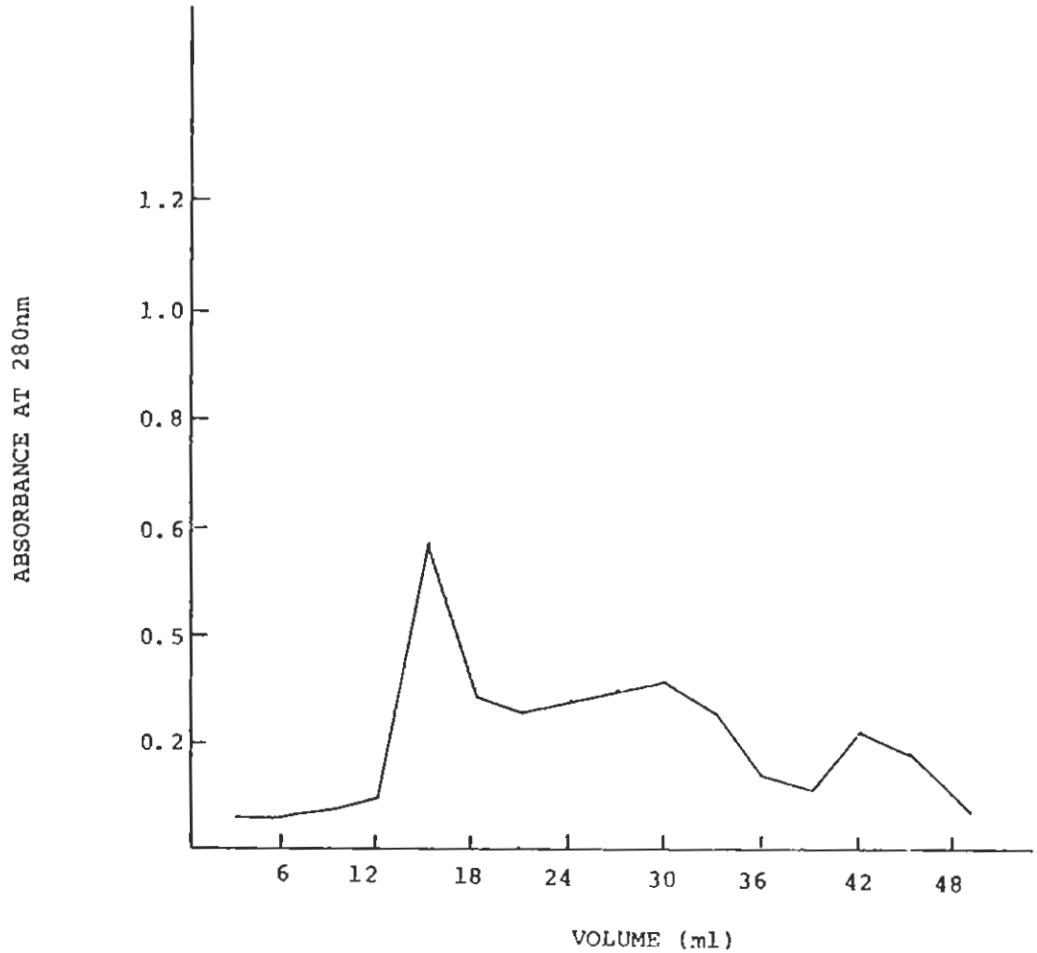


Fig.3.4: Elution pattern of crude globin on Sephadex G-100 column (20x0.9cm). Elution with 10% acetic acid with a flow rate of 10ml/hr. Fraction volume 3ml/tube.

S_1 S_2

Fig.3.5-a: Iso-electric focusing of Sephadex peaks S_1 and S_2.

S_1: First peak from Sephadex G-75

S_2: Second peak from Sephadex G-75

S_1 S_2

Fig.3.5-b: Triton electrophoresis of Sephadex peaks S_1 and S_2.
S_1: First peak from Sephadex G-75
S_2: Second peak from Sephadex G-75

the separation was carried out with 0.01M potassium phosphate buffer containing KCN and thiodiglycol (pH 9.0), there was no separation and precipitation occured on the column.

3.5.3. Elution profile on CM-Cellulose

The first peak from Sephadex G-75, having six components of hemoglobin, was subjected to CM-Cellulose column chromatography and elution was carried out with phosphate buffer pH 2.9. The stepwise sodium phosphate molarity gradient of 0.01M, 0.1M and 0.2M was employed for elution. Figure 3.6 shows the elution pattern of the hemoglobin components present in the first peak of Sephadex G-75.

3.6. ELECTROPHORESIS OF CM-CELLULOSE PEAKS

The peaks eluted from CM-Cellulose were subjected to electrophoresis. Figure 3.7 shows the electrophoretic patterns of these peaks indicating the purity of two of the peaks S_1CM_b and S_1CM_c. The peaks S_1CM_a contains mixture of different components.

3.7. N-TERMINAL ASSAY OF PEAKS FROM CM-CELLULOSE

The purity of the peaks were determined by N-terminal amino acid analysis using DNS & Edman degradation method. The pure components S_1CM_b and S_1CM_c both were analysed for N-terminal amino acid and found to contain a single spot corresponding to DNS-Val in both the peaks.

3.8. PURITY OF PEAKS ON HPLC

The peaks separated on ion-exchange column of CM-Cellulose were further analysed for their homogeneity using high performance liquid chromatography. Figure 3.8 shows the single absorbing component,

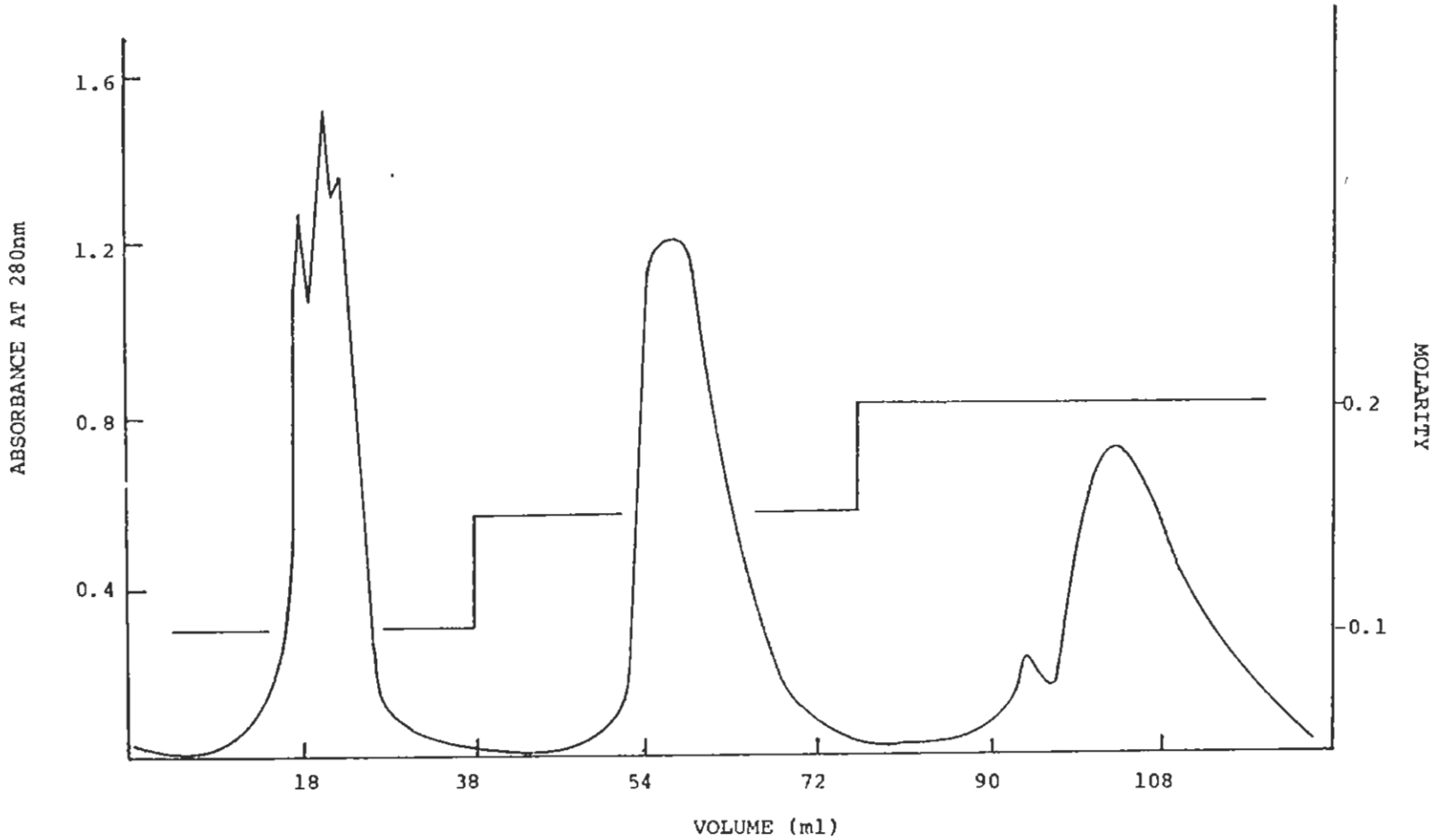
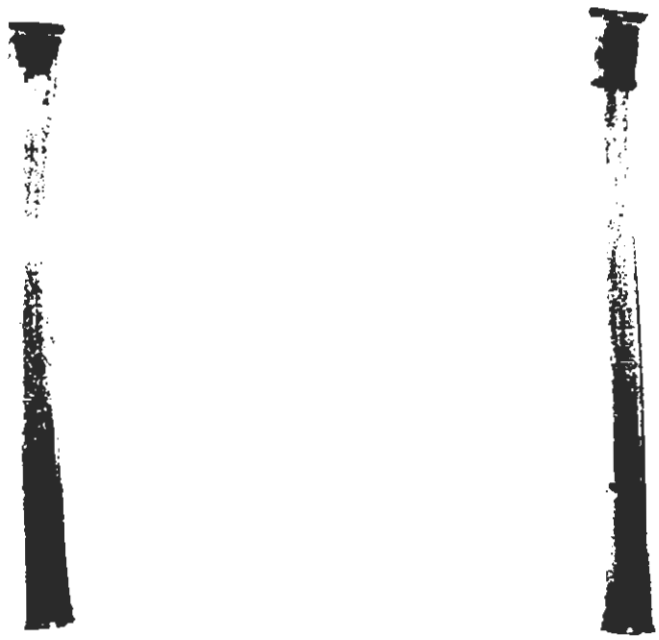


Fig.3.6: Elution pattern of peak S_1 on CM-Cellulose column (30x0.8cm). Equilibration buffer, 0.01M sodium phosphate pH 2.9; Stepwise gradient elution with increasing molarity of 0.01, 0.1 and 0.2M; Flow rate 12ml/hr.



S_1CM_b S_1CM_c

Fig. 3.7-a: Electrophoresis of CM-Cellulose peaks S_1CM_b and S_1CM_c on charge basis:

S_1CM_b S_1CM_c

Fig.3.7-b: Triton electrophoresis of CM-Cellulose peaks S_1CM_b and S_1CM_c.

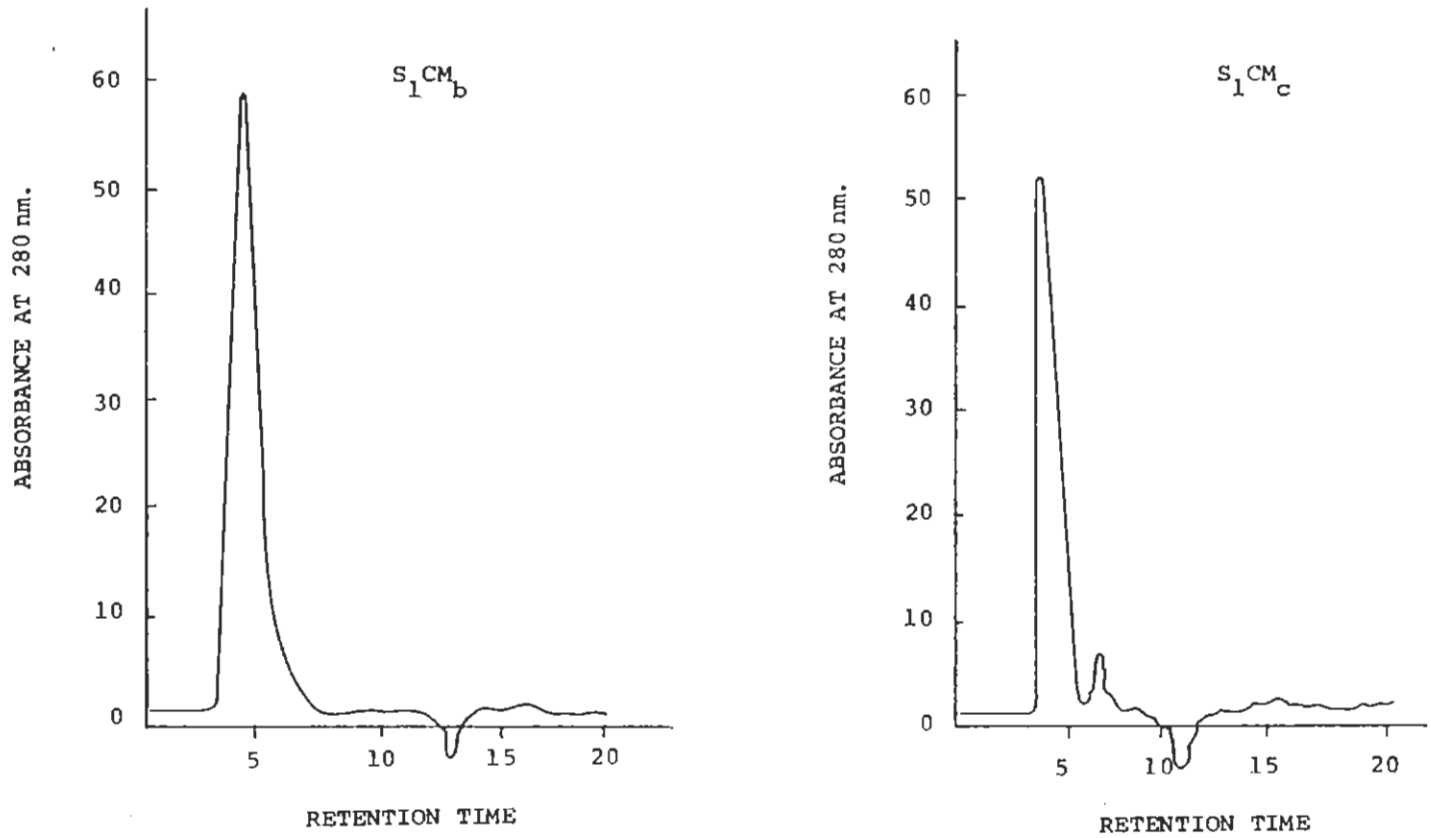


Fig.3.8: Elution pattern on Reverse phase HPLC of CM-Cellulose peaks S_1CM_b and S_1CM_c on μ-Bondapak C_{18} column (15x0.46cm). Elution with 50/50 (v/v) of acetonitrile:water.

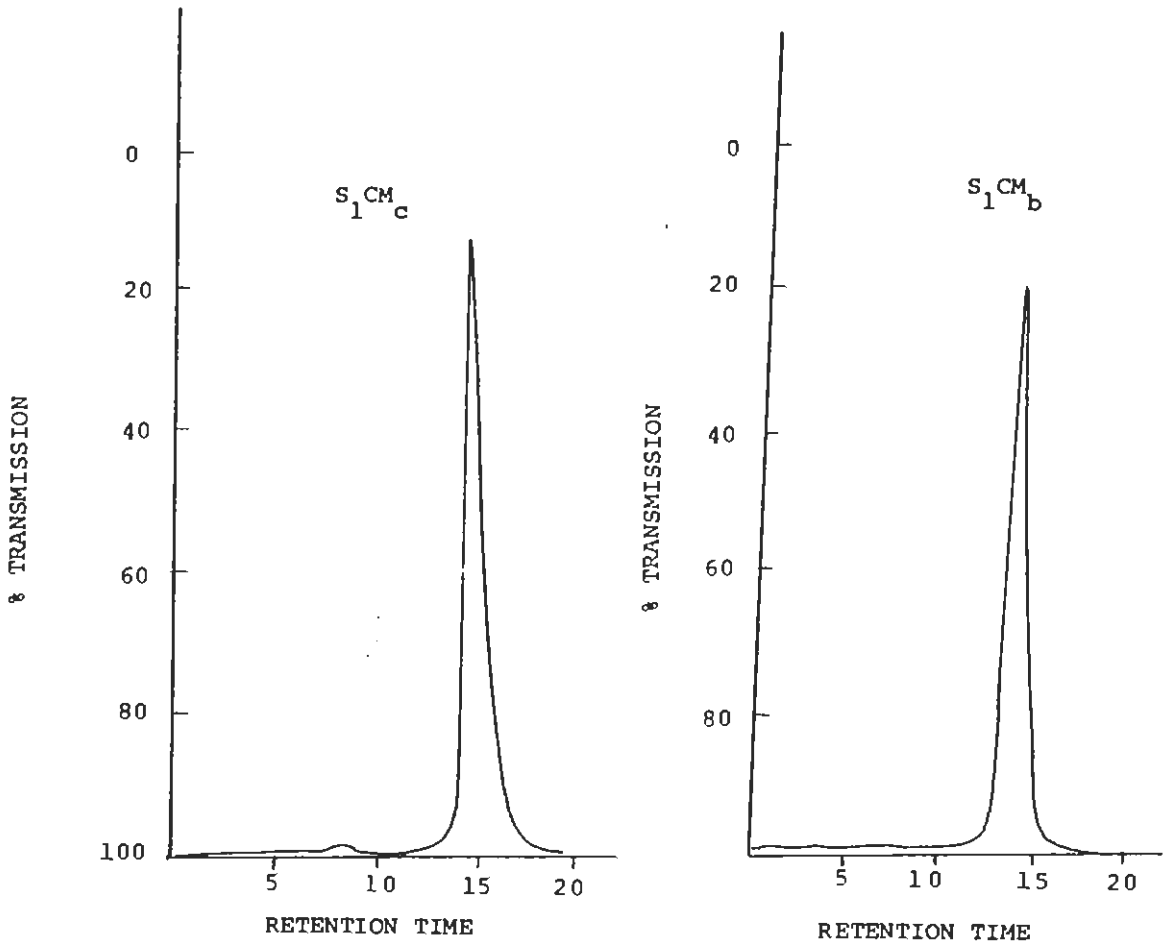


Fig.3.9: Elution pattern on HPLC of CM-Cellulose peaks S_1CM_b and S_1CM_c on Rp-18, ODS-11 Spherisorb column (5μ particle size). Elution with 50% (v/v) of methanol:water.

TABLE 3.1

AMINO ACID COMPOSITION (RESIDUE/1000)

Amino Acids	Hemoglobin Crude	S_1CM_b	S_1CM_c
Aspartic acid	146.63	116.44	123.28
Threonine	34.11	6.85	13.69
Serine	70.22	6.85	6.85
Glutamic acid	77.22	6.85	34.24
Proline	27.73	41.09	44.95
Glycine	181.01	95.89	82.12
Alanine	86.92	143.03	116.44
*Cystein	+	+	+
Valine	52.24	102.74	87.04
*Methionine	+	6.85	13.69
Isoleucine	22.99	54.79	47.90
Leucine	65.53	116.44	116.44
Tyrosine	35.89	13.70	20.54
Phenylalanine	8.53	54.79	54.79
Histidine	77.61	69.31	61.49
Lysine	68.42	95.89	75.34
Arginine	44.95	68.49	101.20
Tryptophan	nd	nd	nd

*Determined after oxidation
nd = not dertermined
+ = Present in traces

and Figure 3.9 shows a single peak of refractive index (RI) for the components S_1CM_b and S_1CM_c.

3.9. AMINO ACID COMPOSITION

The amino acid analysis of hydrolysed crude hemoglobin and peaks obtained from chromatographic separations were performed on Biotronik Amino Acid Analyzer. Table 3.1 shows the amino acid composition of these fractions.

3.10. SEQUENCE ANALYSIS OF S_1CM_b AND S_1CM_c

Amino acid sequence of S_1CM_b and S_1CM_c components were analyzed by manual degradations upto four residues using DABITC method. The results of the first step revealed valine as the main spot in both the components. In the second step glycine was the major spot in S_1CM_b whereas tyrosine in S_1CM_c and in the third step tyrosine and serine were the terminal residues in S_1CM_b and S_1CM_c respectively. At the fourth step of sequence analysis, it was difficult to distinguish the major amino acid spots.

S_1CM_b: $-NH_2$-Val-Gly-Tyr-

S_1CM_c: $-NH_2$-Val-Tyr-Ser-

DISCUSSION

The present study on Uromastix hardwickii was undertaken to separate hemoglobin components, study their amino acid sequences and thus establish its evolutionary pattern in reptiles. Experiments designed to achieve these goals were electrophoresis, gel filtration, ion-exchange chromatography, N-terminal analysis, HPLC and manual degradation by DABITC.

The disc gel electrophoresis on polyacrylamide gels resulted in six proteins. This finding contradicts the results reported earlier by De Smet (1978) that Uromastix hardwickii has a single protein.

SDS-electrophoresis of crude hemoglobin showed 4 protein bands indicating the presence of 4 hemoglobins with different molecular weights suggesting the probability of monomers, dimers, tetramers and also some aggregates of these. The molecular weights of proteins present in the Uromastix hardwickii were found to be in the range of 16,000 to 80,000 daltons.

Iso-electric focusing yielded 14 to 15 protein bands with different intensities showing their different concentrations. The major number of bands appeared in the region between pH 4.8 and 9.0 indicating that the proteins have a wide range of iso-electric points. This multiplicity of protein bands may be due to the polymorphic nature of reptile hemoglobin.

The separation of globin chains carried out on polyacrylamide gels in the presence of urea and triton X-100 revealed the presence of six bands suggesting six chains in Uromastix hardwickii.

The separation of hemoglobin on Sephadex G-75 gave 4 peaks which were consistent with the 4 protein bands in SDS electrophoresis. The first peak emerged before the first void volume indicating the presence of high molecular weight of protein since the exclusion limit of Sephadex G-75 is about 75,000 daltons. The elution of second peak from G-75 was about 10 to 15ml after the first void volume suggesting the molecular weight of this protein to be somewhere around 70,000 daltons. Subsequently, the third and the fourth peaks were eluted in the second and the third void volumes respectively corresponding to the lower molecular weights. This elution pattern suggests that the Uromastix hemoglobin is found in aggregate form. This finding was also depicted on the separation of hemoglobin on Sephadex G-50 and G-100.

The purity of the separated G-75 peaks determined by different types of electrophoresis did not provide any definite results regarding their homogeneity. The N-terminal analysis also suggested a heterogeneous nature of these peaks.

Further resolution of the Sephadex first peak i.e. S_1 was carried out on ion-exchangers like DEAE-Sephadex, Sp-Sephadex, CM-Sephadex and CM-Cellulose. The separation on ion-exchange columns was not satisfactory with the exception of that performed on CM-Cellulose. The peak S_1 separated on the latter using the stepwise gradient of phosphate buffer molarity (0.01M, 0.1M and 0.2M) at constant pH of 2.9 revealed the presence of six components; among those, two of the components eluted in 0.1M and 0.2M concentration of phosphate buffers showed single peaks. The homogeneity determined by disc electrophoresis showed single band and the

N-terminal analysis also confirmed the results revealing a single N-terminal amino acid for both the components. However, other spots of very low intensity were also found.

The purity of these samples checked intensively on the analytical high performance liquid chromatography revealed the presence of a single component.

The two peaks from CM-cellulose, when subjected to manual Edman degradation using a modified technique DABITC showed that the peaks are not homogeneous. The sequencing could be carried out only to a limited number of amino acids which is inexplicable, as all procedures for checking purity of peptide chains, such as triton and IEF confirmed the purity of the chains. It is possible that a procedure not usually adopted for the separation would need to be developed for this purpose.

REFERENCES

Adair, G.S. (1924) *Proc.Cambridge Philos.Soc.*, 1, 75.

Anson, M.L. and Mirsky, A.F. (1930) *J.Gen.Physiol.*, 13, 469.

Atland, P.D. and Brace, D.C. (1962) *Amer.J.Physiol.*, 203, 1188.

Bartlett, G.R. (1980) *Amer.Zool.*, 20, 103.

Betke, K. and Sevelsberg, W. (1950) *Biochem.Z.*, 320, 431.

Brace, K.G. and Atland, P.D. (1955) *Amer.J.Physiol.*, 183, 91.

Bragg, W.L. and Perutz, M.F. (1952a) *Acta Cryst.*, 5, 277.

Bragg, W.L. and Perutz, M.F. (1952b) *Acta Cryst.*, 5, 323.

Bragg, W.L. and Perutz, M.F. (1954) *Proc.Roy.Soc.*, A225, 315.

Braunitzer, G. (1972) *Scientia*, 107, 189.

Braunitzer, G., Gehring-Muller, R., Hilschmann, N., Hilse, K., Hobom, G., Rudloff, V. and Wittman-Liebold, E. (1961) *Hoppe-Seyler's Z. Physiol.Chem.*, 325, 283.

Cannan, R.K. (1958) *Science*, 127, 1376.

Chang, J.Y., Brauer, D. and Wittman-Liebold, E. (1978) *FEBS LETT.*, 93, 205.

Cole, M., Fletcher, J.C. and Robson, A. (1965) *J.Chromat.*, 20, 616.

Cullis, A.F., Muirhead, H., Perutz, M.F., Rossman, M.G. and North, A.C.T. (1962) *Proc.Roy.Soc.*, A265, 161.

Davis, B.J. (1964) *Annals N.Y.Acad.Sci.*, 121, 404.

Dayhoff, M.O., Eck, R.U. and Park, C.M. (1969) Atlas of Protein Sequence and Structure (Dayhoff, M.O. ed.) Nat.Biomed.Res.Found. Silver Spring, pp.75.

DeSmet, W.H.O., (1978) *Acta Zoologica. Et. Pathologica Antverpiensia* 70, 119.

Dessauer, H.C. and Fox, W. (1964) Taxonomic Biochemistry and Serology (C.A. Leone, ed.) pp.625 Ronald Press, New York.

Dessauer, H.C., Fox, W. and Ramirez, J.R. (1957) *Arch.Biochem. Biophys.*, 71, 11.

Dickerson, R.E. (1971) *J.Mol.Evol.*, 1, 26.

Dickerson, R.E. and Gies, I. (1983) "Hemoglobin-Structure, Function and Evolution", W.A.Benjamin, Inc.Menlo Park, California.

Ditmars, R.L. (1953) Reptiles of the World, McMillan and Co., New York. 11th Print.

Drabkin, D.L. (1957) *Fed.Proc.*, 16, 740.

Drabkin, D.L. (1964) *J.Biol.Chem.*, 164, 703.

Duguet, M., Chauveol, J.P. and Acher, R. (1974) *Febs Lett.*, 47, 333.

Edman, P. and Begg, G. (1967) *Eur.J.Biochem.*, 1, 80.

Engelhert, J.F. (1825) "Commentatio de Verma materiae Sanguinis Purpurem Colorem impertientis nature" Thesis, Gottingen.

Goodman, M., (1973) *Symp.Zool.Soc.(London)*, 33, 339.

Goodman, M., Moore, G.W. and Matsuda, G. (1975) *Nature*, 253, 603.

Gratzer, W.B., and Allison, A.C. (1960) *Biol.Rev.Cambridge Phil.Soc.*, 35, 459.

Gray, W.R. and Hartley, B.S. (1963) *Biochem.J.*, 89, 59.

Hadley, N.F. and Burns, T.A. (1968) *Copeia*, 4, 737.

Hamilton, P.E. and Anderson, R.A. (1959) *Anal.Chem.*, 31, 1564.

Haurowitz, F. (1928) *Hoppe-Seyler's Z.Physiol.Chem.*, 173, 118.

Haurowitz, F. (1938) *Hoppe-Seyler's Z.Physiol.Chem.*, 254, 266.

Hirs, C.H.W. (1967) Methods in Enzymology 11, 197, Academic Press, New York.

Holmquist, R., Jukes, T.H.Moise, H., Goodman, M. and Moore, G.W. (1976) *J.Mol.Biol.*, 105, 39.

Hoppe Seyler (1864) *Virchows Arch.*, 29, 233.

Hunt, L.T., Hurst-Calderone, S. and Dayhoff, M.O. (1978) Atlas of Protein Sequence and Structure, Vol.5, Suppl. 3, Nat.Biomed.Res. Found., Washington, pp.229.

Irina, M.R., Allison, K.L.C., Lin, Susan Ferro-Dosch and Chien Ho. (1984) *Biochim.et Biophysic.Acta*, 785, 123.

Kuster, W. (1912) *Z.Physiol.Chem.*, 82, 463.

Leclercq, F., Schnek, A.G., Braunitzer, G., Stangl, A. and Schrank, B. (1981) *Hoppe-Seyler's Z.Physiol.Chem.*, 362, 1151.

Matsuda, G. (1978) Evolution of Protein Molecules, Japan Scientific Societies Press, Centre for Academic Publications, Tokyo, pp.175.

Monad, J., Wyman, J. and Changeux, J.P. (1965) *J.Mol.Biol.*, 12, 88.

Moore, S. and Stein, W.H. (1948) *Annals N.Y.Acad.Sci.*, 49, 265.

Musacchia, X.J. and Sievers, M.L. (1962) *Trans.Amer-Microsc.Soc.*, 81.198.

Naqvi, S. (1984) "Amino acid sequence of the hemoglobin from the reptile Uromastix hardwickii". Ph.D. Thesis, Univ.of Karachi.

Nuttale, G.H.F. (1904) Blood immunity and Blood Relationship, Cambridge Univ. Press, London.

Nishikura, K. (1978) *Biochem.J.*, 173, 671.

Ornstein, L. (1964) *Annals.N.Y.Acad.Sci.*, 121, 321.

Perutz, M.F. (1951) *Nature*, 167, 1053.

Perutz, M.F. (1960) *Brookhaeven Symp.Biol.*, 13, 165.

Perutz, M.F., Kilmartin, J.V.Nishikura, K., Fogg, J.H., Butler, P.J.G. and Rollema, H.S. (1980) *J.Mol.Biol.*, 138, 649.

Perutz, M.F., Muirhead, H., Cox, J.M. and Goaman, L.C.G. (1968) *Nature (London)*, 219, 131.

Perutz, M.F. Rossmann, M.G., Cullis, A.F., Muirhead, H., Will, G. and Worth, A.C.T. (1960) *Nature (London)*, 185, 416.

Perutz, M.F.Steinranf, L.K., Stockell, A. and Benghem, A.D. (1959) *J.Mol.Biol.*, 1, 402.

Peterson, E.A., Sober, H.A. (1959) *Anal.Chem.*, 31, 857.

Pfluger, E. (1875) *Pfluegers Arch.Gesamte Physiol.Menschen Tiere*,10,215.

Porter, R.R. and Sanger, F. (1948) *Biochem.J.*, 42, 287.

Reichert, K.B. (1849) *Arch.Anal.Physiol.Wiss.Med.*, 1849, 197.

Reichert, E.T. and Brown, A.P. (1909). The Crystallography of the Hemoglobins, Carnegie Institution, Washington.

Rhinesmith, H.S., Schroeder W.A., and Martin, N. (1958) *J.Amer.Chem. Soc.*, 80, 3358.

Ritchie, R.F., Harter, J.G., and Bayles, T.B. (1966) *J.Lab.Clin.Med.*, 68, 842.

Riggs, A., Sullivan, B. and Agee, J.R. (1964) *Proc.Nat.Acad.Sci.US*, 51, 1127.

Rovera, G., Magarian, C. and Borus, T.W. (1978) *Anal.Biochem.*, 85, 506.

Schwantes, A.R. (1972) Ph.D. thesis, "Hemoglobinas e heptoglobinas em Seypentes (Squamata, Reptilia)", Universidale Federal do Rio Grande do Sol.

Semple, R.E., Sigsworth, D. and Stitt, J.T. (1970) *Can.J.Physiol. Pharmacol.*, 48, 282.

Smith, M.A. (1935) Fauna of British India, Reptiles and Amphibia Vol.II Sauria Taylor and Francis, London.

Speckman, D.M., Stein, W.H., Moore, S. (1958) *Anal.Chem.*, 30, 1185.

St.Groth, F.S., De Webster, R.G. and Datyner, A. (1963) *Biochem. Biophys.Acta*, 71, 377.

Steel, Jr. J.C.H., Nielson thB (1978) *Anal.Biochem.*, 84, 218.

Sullivan, B. and Riggs, A. (1964) *Nature (London)*, 204, 1098.

Sullivan, B. and Riggs, A. (1967a) *Comp.Biochem.Physiol.*, 23, 437.

Sullivan, B. and Riggs, A. (1967b) *Comp.Biochem.Physiol.*, 23, 499.

Svedberg, T. and Hedenius, A. (1934) *Biol.Bull.*, 66, 191.

Vinegar, A. and Hillyard, S.D. (1972) *Comp.Biochem.Physiol.*, A43,317.

Von Bahr-Lindstrom, H., Hempel, J. and Jornvall, H. (1982) *J.Prot. Chem.*, 1, 257.

Wrigley, C.W. (1969) *Sci.Tools*, 15, 17.

Zuckerkandl, E. (1965) *Sci:Amer.*, 212(5), 110.

PART-II

PROTEIN PATTERNS OF HUMAN CATARACTOUS LENS AND PLASMA

ABSTRACT

The present study shows the comparison of protein patterns of lens and plasma of cataractous subjects with their respective normals using electrophoresis and amino acid analysis. In PAG electrophoresis, iso-electric focusing, and SDS-electrophoresis, one, four and two bands were missing respectively in the cataractous lens. The amino acid analysis also showed differences. There was abundance of urea in cataractous lens but the subjects studied had no history of renal failure. Comparing the protein patterns of plasma from normal and cataractous subjects revealed lesser number of protein bands. In PAG electrophoresis one/two bands were less than the normal and in IEF, six protein bands were present against ten in normals. The amino acid studies carried out on plasma of these patients also showed some changes on comparing with normals.

INTRODUCTION

L E N S

1.1. DISTRIBUTION AND ETIOLOGY OF CATARACT

Cataract formation is a major cause of visual disability and blindness throughout the world (Spector, 1974). It is responsible for blindness of nearly 5 million people in Asia and Africa and for about 75% of the visual disabilities in Pakistan (Kirmani, 1984).

The term 'cataract' describes the optical heterogeneity of the lens, producing discontinuity of the refractive index and increased light scattering, i.e. lens opacity.

The exact biochemical conditions which causes perceivable dispersion of light passing through the lens, are poorly understood. The following factors may affect the transparency of the lens:

i) Aggregation of lens proteins to a heavier group having larger molecular weight and a greater refractive index (Benedek, 1971).

ii) Retention of liquid between the fibres of the lens (Miller and Benedek, 1973).

iii) Formation of some complex and disordered fibres from the fibre member of the lens (Dilley etal. 1976).

iv) Deposition of calcium oxalate/calcium orthophosphate in subcapsular connective tissue (Bron and Habgood, 1976; Van Heyningen, 1972.

v) A high level of urea in the lens in patients with uremia or in those with diarrhoea (Harding and Rixon 1981). This increased urea level if transmitted to lens could have a trifold effect, an osmotic effect, an effect in perturbing protein and the conversion of urea to cynate and subsequent carbamylation of lens proteins.

1.2. SENILE CATARACT

Senile cataract is the most common form in man. It is an affection of old age. Both the eyes are involved, but generally one in advance of the other. The time required for full development varies greatly. It may ripen completely in a few months, may require years to develop, or may become stationary at any stage of its maturation.

The senile cataract is pathologically divided into cortical and nuclear cataract. In cortical cataract the opacity may be subcapsular and/or cortical in location and range from lens vacuoles and water clefts to peripheral spoke opacities. Lower wet weights and low protein content have been reported in this form of cataract (Maraini and Mangili, 1973; Van Heyningen, 1976). In the nuclear variety, opacification of the lens starts in the part immediately surrounding the nucleus and liquification of the lenticular matter takes place leaving the small nucleus intact. Brown and black cataracts are the advanced forms of nuclear cataracts. Morgagnian cataract is the hyper mature stage of the same. Protein contents are found to be same as in normal lens. The proteins aggregate mainly in the nucleus of the lens which is a characteristic feature of senile nuclear cataract (Dilley and Pirie, 1974).

1.3. DEVELOPMENT OF NORMAL LENS

The crystalline lens first appears as a lens plate which is developed from the surface ectoderm and is in contact with the optic outgrowth. The lens plate thickens on ectoderm surface for 3 weeks and lens vesicle forms in 4th week and subsequently gets separated.

The lens is a hollow sphere of epithelial cells, which grow by elongation of the posterior cells into lens fibre. These fibres soon fill the lens vesicle and forms embryonic nucleus hy the end of 12th week of foetal life. With the process of elongation of epithelial cells, the synthesis of β- and γ-crystallins starts (Papaconstantinou, 1965; 1967; Schubert etal. 1970; McAvoy, 1978). The lens assumes final shape at 4 months and goes on developing (Kirmani etal. 1982) throughout the span of life. The lens loses its direct blood supply in its early embryonic development (Smelser, 1965) and then gets nutrition from the surrounding structures.

1.4. PHYSICAL CHARACTERISTICS OF THE NORMAL LENS

The lens is almost spherical at birth. Later in life, it grows eleptical as fibres added to the periphery, make it flatter. Thus produces a greater refractive power which helps to compensate for the short anterio-posterior diameter of the eye. The consistency of the lens changes throughout life. At birth it is soft and may be compared with soft plastic whereas in old age it is hard like glass accounting for greater resistance to change of shape.

1.5. FUNCTION OF LENS

The function of the lens is to focus the luminous rays so that they form a perfect image on the retina. To accomplish this, the refractive power of the lens must change with the distance of the object, relative to the light rays whether these are parallel or divergent. This alteration in the refractive power of the lens is known as accommodation and is produced by a change in shape effectively, mainly its anterior curvature, brought about by the relaxation of the

suspensory ligament, caused by the contraction of the cillary muscle.

1.6. PROTEIN CONSTITUENTS OF LENS

The lens is very rich in protein which comprises as much as 35% of the wet weight of the adult lens (Lerman and Zigman, 1965; Van Heyningen, 1972). The major portion of the proteins are water soluble crystallins which have been characterized as alpha (α), beta (β) and gamma (γ) crystallins. Detailed studies on the lens crystallin fractions i.e. α,β and γ from cow (Lawson etal. 1981; Harding, 1982; Berbers etal. 1984; Bours, 1984); mice (Russel, 1981; Inana etal. 1982) and chick (Mishima & Ikeda 1981) have been undertaken. More recently much work has been directed towards the human lens proteins (Harding, 1972; Spector etal. 1973; Dilley and Pirie, 1974; Ringens etal. 1978; Zigler etal. 1980; 1981; Kabasawa etal. 1982; Stanojevica-paovic etal. 1983).

The α-crystallins are of higher molecular weights. The α- and β-crystallins exists as aggregates while the γ-crystallins occurs as monomers (McAvoy, 1978).

The following table shows the results of soluble proteins i.e. α, β and γ-crystallins from bovine lens characterized by gel filteration, ion-exchange chromatography, electrophoresis and iso-electric focusing.

SOLUBLE LENS PROTEINS OF BOVINE*

Crystallins	Molecular weight	Molecular form	Range of PI
α	Over $5x10^5$	Aggregates	pH 4.8-5.0
β	4 to $20x10^4$	Aggregates	pH 5.7-7.0
γ	About $2x10^4$	Monomers	pH 7.1-8.1

*Harding, 1982.

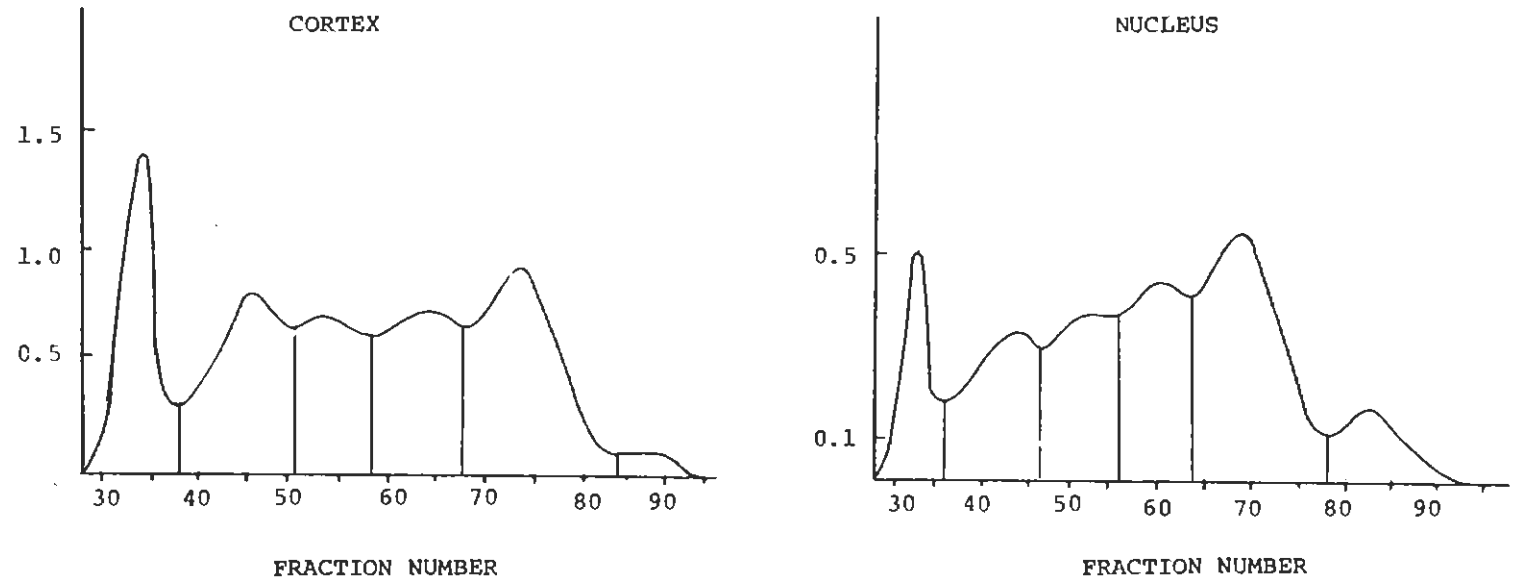


Fig.1.1: Elution pattern obtained by gel chromatography on Sephadex G-200 of soluble lens proteins from cortical and nuclear parts of normal lens.

Spector etal. (1973) have characterized normal human lens protein by DEAE-cellulose chromatography. Ringens etal. (1978), studied normal human lens by gel filtration on G-200 (Fig.1.1) and separated the soluble lens protein into 5 peaks which were labelled as HM+α, β_1, β_2, β_3 and γ-crystallins. The quantitative distribution of these fractions is shown as follows:

RELATIVE PERCENTAGE OF HUMAN EYE LENS FROM G-200

NORMAL LENS	HM+α	β_1	β_2	β_3	β total	γ
Cortex	13.4	25.4	15.1	17.1	57.6	29.0
Nucleus	8.7	18.2	20.1	20.8	59.1	32.2

1.7. AMINO ACID COMPOSITION OF LENS

Lens crystallins have been studied for their amino acids. The individual lens crystallins and their sub-fractions have been studied for the amino acids by several workers (Bjork, 1961; 1964; Spector 1965; Hoenders etal. 1968; Mok and Waley, 1968; Dickenson etal. 1968; Croft, 1972; Zigler and Sidbury 1973; Herbrink and Bloemendal, 1974; Herbrink etal. 1975; Horwitz, 1976; Roy and Spector 1976; Zigler and Sidbury, 1976a; 1976b; 1976c; Kabasawa etal. 1977; Kramps etal. 1977; De Jong etal. 1977; Inana etal. 1982; Berbers etal. 1984).

1.8. CHANGES IN CATARACT LENS

1.8.1. Protein changes

Irregular distribution of protein results in opacification (Phillipson, 1969a; 1969b). The lens protein aggregate and become insoluble with a concomitant decrease in total protein and water soluble protein content (Mach, 1963; Spector etal. 1973; Harding, 1979). Many workers noted a decrease proportion of proteins with lowest

molecular weight and electrophoretic mobility, assumed to be γ-crystallin in human cataracts (Harding and Dilley, 1976; Kramps etal. 1976; Ringens etal. 1978).

Kramps etal. (1976) fractionated cataract and normal lenses into water soluble, urea soluble and urea insoluble fractions. A decrease in water-soluble and urea-soluble proteins were observed in cataract lens. The polypeptide chain composition in water soluble fractions were found to be similar in normal and cataractous lenses (Kramps, 1977; Maraini and Mangili, 1973).

An increase in high molecular weight proteins with age has been observed in Juvenile cataract lens (Golenda, 1970; Roy and Spector 1976; Augusteyn, 1977; Bushell & Duncan 1978, Judith,1980). In normal lenses, 5% of the total soluble proteins has a molecular weight $150x10^6$, whereas in cataractous lens, larger amounts upto 10-15% of the total soluble proteins were found. High molecular weight proteins increased by aggregation of α, β and γ-crystallins which correspondingly decreased, the decrease being especially marked in the γ-crystallin (Wada etal. 1981). According to the Fransworth etal. (1981), a 43,000 dalton polypeptide in human lens was found in normal, but missing in cataractous lens.

In the cataract lenses, aggregation of proteins due to disulfide linkages was observed (Buckingham, 1971; Spector, 1972; Harding, 1973). Garner etal. (1981) reported γ-crystallin, to be a major polypeptide disulphide linked to membrane protein in human cataract. Wada etal. (1981) reported that the aggregation of the β-crystallin occurred much earlier in cataractous than in normal lens.

Loss of low molecular weight (LMW) protein in the senile cataract lens were observed by Charlton & Van Heyningen 1968 & Francois etal. 1969· Ringens etal. (1978) reported that there is no qualitative change in the lens crystallins during the formation of cataracts.

1.8.2. Electrophoretic studies

The electrophoretic and chromatographic studies on the cataractous lens showed the decrease content of low molecular weight crystallins (Topaszto, 1962; Mach, 1963; Malik etal. 1969; Francois etal. 1969; Clark etal. 1969; Kramps etal. 1976; Kramps, 1977)., which on immuno-electrophoresis, found to be heterogenous showing 3 different precipitin lines (Francois etal. 1965). Witmer and Buhler (1960) carried out immuno electrophoretic studies on cataractous and normal lens proteins demonstrating the α-crystallins to be the species specific antigen.

Agar-gel electrophoresis of normal human lens soluble proteins revealed 6 major fractions and 10 sub-fractions whereas, in the cataractous lenses a few sub-fractions were missing (Malik etal. 1969).

Kimura etal. (1973) studied the human lens crystallins individually, by SDS-gel electrophoresis demonstrating the presence of 3 major protein bands in α-crystallin with corresponding molecular weights of 27,)00; 29,000 and 34,000 daltons, β-crystallins were shown to have 2 main bands having molecular weights of 27,000 and 29,000 daltons and the γ-crystallins showed 2 main bands with the molecular weights 25,000 and 27,000 and several minor bands.

The heterogeneity of γ-crystallin from normal and cataractous human lenses were studied by PAGE, with and without SDS, showing that a band at molecular weight 24,000 in SDS-PAGE was separated into six sub-fractions (Kabasawa etal. 1977; Kabasawa, 1980). The larger quantities of sub-fractions γ-5 and γ-6 were found in cataractous lenses compared to the normal ones (Kabasawa etal. 1982).

Similar studies were performed on bovine γ-crystallins (Bjork, 1961;1964; Kabasawa etal. 1977; Kibbelaar and Bloemendal, 1976; Zigler etal. 1981) and mice (Russel, 1981; Doria etal. 1984) showing their homogeneity in terms of size, but heterogeneity electrophoretically. The heterogeneity were similarly elucidated by IEF (Ringens etal. 1978; Zigler etal. 1981).

Van Haard etal. (1979) performed IEF in the presence of urea and SDS-gel electrophoresis, showed very diffuse bands on old human lens, and indicating narrow range of heterogeneity in charge and molecular weight.

The PI range of α-crystallin is pH 4.23 to 5.43, of β-crystallin pH 4.23-8.86 and of γ-crystallin in the range of pH 4.60-5.85 (Mishima & Ikeda 1981). Recent studies of Zigman etal. (1983) using IEF, demonstrated that the PI of crystallins of rats, dog, fish, calf and human differ within a range of pH 8.87-6.80. In each specie, one γ-crystallin component with an iso-electric point of 7.5 is present but in different concentrations.

1.8.3. Amino acid studies

The amino acid compositions of lens proteins have been

undertaken to understand the process of cataractogenesis, and special attention has been paid to some amino acids like cystein, tryptophane and histidine. The amino acid compositions has been reported by many workers but none of these are comparable (Schaeffer and Murray, 1950; Zacharias and Helena, 1951; Anima and Piare, 1966; Bernat etal. 1966; Graeber, 1966; Bernat & Bombicki 1968; Barber, 1968; Clark etal. 1969; Jawadi and Selim, 1971; Takemoto and Azari 1976; Goldschmidt, 1917; Agarwal etal.1979; Agarwal etal. 1980; Garner and Spector, 1980; Koch etal. 1982; Khurana etal. 1982; Rao etal. 1982).

P L A S M A

1.9. GENERAL DESCRIPTION

Plasma is frequently analysed for diagnostic purposes. Its composition reflects the activity of the other organs of the body and is not distorted by its own metabolic activity.

Plasma is a pale yellow colloidal fluid in which the cellular components of the blood i.e. erythrocytes, leukocytes and platelets are suspended. It represents 50 to 60% of the blood volume. The solid contents of the plasma is 8 to 9%. It is very dense and viscous having specific gravity of 1.026 and viscosity of 1.7 to 2.0. The molal concentration of plasma is 0.30 and the osmotic pressure at $37^{o}C$ is 7.6 atmospheres. The average pH of plasma is 7.4 and it ranges from 7.33 to 7.51. The quantitative difference between plasma and serum is that the former contains fibrinogen, a blood clotting protein, while the later does not include it (West etal. 1966).

undertaken to understand the process of cataractogenesis, and special attention has been paid to some amino acids like cystein, tryptophane and histidine. The amino acid compositions has been reported by many workers but none of these are comparable (Schaeffer and Murray, 1950; Zacharias and Helena, 1951; Anima and Piare, 1966; Bernat etal. 1966; Graeber, 1966; Bernat & Bombicki 1968; Barber, 1968; Clark etal. 1969; Jawadi and Selim, 1971; Takemoto and Azari 1976; Goldschmidt, 1917; Agarwal etal.1979; Agarwal etal. 1980; Garner and Spector, 1980; Koch etal. 1982; Khurana etal. 1982; Rao etal. 1982).

P L A S M A

1.9. GENERAL DESCRIPTION

Plasma is frequently analysed for diagnostic purposes. Its composition reflects the activity of the other organs of the body and is not distorted by its own metabolic activity.

Plasma is a pale yellow colloidal fluid in which the cellular components of the blood i.e. erythrocytes, leukocytes and platelets are suspended. It represents 50 to 60% of the blood volume. The solid contents of the plasma is 8 to 9%. It is very dense and viscous having specific gravity of 1.026 and viscosity of 1.7 to 2.0. The molal concentration of plasma is 0.30 and the osmotic pressure at $37^{o}C$ is 7.6 atmospheres. The average pH of plasma is 7.4 and it ranges from 7.33 to 7.51. The quantitative difference between plasma and serum is that the former contains fibrinogen, a blood clotting protein, while the later does not include it (West etal. 1966).

110. AMINO ACIDS OF THE PLASMA

The amino acids commonly occur in proteins are present in free form in plasma (or blood). Normal human plasma has been analyzed for its free amino acid content by many workers (Adams, 1974; Dickenson etal. 1965; Perry and Hansen, 1969; Aoki etal. 1974; Lewis etal. 1980). The amino acid composition of normal human plasma is tabulated in table 1.1.

111. PLASMA PROTEINS

Plasma proteins represents an exceedingly complex mixture at a concentration of about 70g/litre representing one third mass of the blood proteins. These includes simple proteins, lipoproteins, glycoproteins, traces of large number of proteins that function as immune bodies (antibodies), enzymes and hormones, plasma is known to contain more than 100 proteins, many of which have not been characterized for their functions, beside hormones, tissue-derived enzymes and erythrocyte derived proteins (Putnam, 1976).

The characteristics of the plasma proteins may be acidic, basic or neutral and the molecular weight ranges from $1x10^4$ daltons to greater than $1x10^6$ daltons (Schwick etal. 1977). These are generally referred to as "Albumins", "Globulins" and "Fibrinogens", these designations include the complexity of proteins. Albumins are soluble in water where as globulins and fibrinogen are insoluble in water but soluble in dilute salt solutions.

A large number of plasma proteins belong to descrete functions, such as immunoglobulins, complement system, acute phase

TABLE 2.1

AMINO ACID COMPOSITION OF NORMAL HUMAN PLASMA (μ mol/lit)

Amino Acid	Reference 1	Reference 2
Alanine	336	360±16
Valine	213	225± 9
Glycine	237	234±10
Isoleucine	63	60± 1
Leucine	11	115± 5
Proline	194	185±11
Threonine	129	138± 7
Serine	115	99± 4
Aspartic acid	7	1
Asparagin	c	56± 3
Methionine	23	21± 1
Phenylalanine	53	48± 2
Glutamic acid	58	24± 3
Glutamine	53[c]	640±13
Tyrosine	72	54± 3
Ornithine	60	58± 3
Lysine	153	186± 8

c: Asparagin and Glutamine are not resolved. Total value given as Glutamine.

1: Dickenson et al., 1965.

2: Perry and Hansen, 1969.

reactants and blood clotting factors (Austin and Rizza, 1974; Putnam, 1975;1977; Geisow and Gorden, 1978; Anderson and Lunden, 1979). More than seventy plasma proteins have been individually isolated and characterized (Schwick and Heide, 1977; Anderson and Lunden, 1979). Some of the important proteins from human plasma are shown in table 1.2.

The immunoglobulins constitute a unique group of proteins present in plasma which are responsible for removing extraneous molecular species against it. Any species occurring naturally or synthetic which causes the production of antibodies is called an antigen. All proteins which function as antibodies are immunoglobulins (Table 1.3).

In addition to the immunoglobulins, plasma contains the complement system which comprises of 20 proteins in the blood and collectively represents about 4% of the total serum proteins. The components are either enzymes or enzyme inhibitors (Muller-Eberhard, 1975; Porter and Ried, 1979) and are involved in several aspects of the inflammatory response (Alper& Davis, 1980). Each component of the complement system has a different function to perform and if any of these components is missing, its specific biological function will also be lacking. Two pathways for the activation of the complement system have been proposed, the classical pathway and the alternate pathway. In the classical pathway, the activation of component is initiated by the formatior of antigen-antibody complexes. The alternate pathway can also be activated by antibody-antigen complexes but through a different route, or directly by foreign cells (Schultz, 1976; Porter, 1979; Porter and Reid, 1979) Table 1.4.

TABLE 1.2

IMPORTANT PROTEIN COMPONENTS OF HUMAN PLASMA

Protein	M.W.	Subunit M.W.	pI	Conc. mg/100 ml	Carbohydrate %
Blood coagulation proteins					
Fibronectin	440 000	2 x 220 000		25-40	
Factor V, Proaccelrin	410 000			1	
C1g	350 000			33	3.64
Factor I, fibrinogen	340 000	α 2 x 65 000 β 2 x 55 000 λ 2 x 47 000	5.5	200	7
Factor XIII, Fibrin stabilizing factor	320 000			1	4.9
Factor XI, Haemophilic factor C	160 000			1	
Factor VIII, Antihaemophilic globulin	1100 00			1	5.8
Plasminogen	93 000		6.4-8.5	20	17.1
Factor XII, Hageman factor	90 000		7.9	10	
Factor II, Prothrombin	72 000			15	9
Factor IX, Haemophylic factor B	67 000		4.1-4.5		17.1
Factor VII, Proconvertin	63 000		5.6	0.1	50
Factor X, Stuart factor C	55 000			5	15
Protease inhibitors					
α_2-Macroglobulin	820 000		5.4	240; 290	9.43

Protein	M.W.	Subunit M.W.	Pi	Conc. mg/100 ml	Carbohydrate %
α_2-Plasmin inhibitor (antiplasmin)	700 000	Single			11.7
Inter-α-trypsin inhibitor	160 000		5.2	400-700	8.4
C_1-Esterase inhibitor	104 000				34.7
α_1-Antichymotrypsin	68 000	Single	3.75-4.0	45	24.6
Antithrombin III	62 000			29	13.4
α_1-Antitrypsin	54 000	Single	4.0	290	11.44
α_1-Proteinase inhibitor	52 000	Single		130	12.5
Transport proteins					
β-Lipoprotein	2 000 000			400	
α-Lipoprotein	170 000			300	
Ceruloplasmin	134 000		4.4	22	8
Transferrin	76 500		6.0	230	5.9
Albumin	67 000		5.85	4000	Non glycoprotein
Prealbumin	50 000		4.7	25	0.4

References: Anderson and Lunden, 1979; Putnam 1975· Schwick etal. 1977; Schwick and Haupt, 1980).

TABLE 1.3

IMMUNOGLOBULINS

Protein	M.W.	Subunit M.W.	PI	Conc. mg/100 ml	Carbohydrate %
IgM	970 000	2 x 72 000 2 x 23 000		150	10
IgE	188 000	2 x 72 000 2 x 23 000		0.03	13
IgD	172 000	2 x 60 000 2 x 23 000		3	9
IgG3	165 000	2 x 50 500 2 x 23 000		100	
IgA1	160 000	2 x 58 000 2 x 23 000		300	7
IgA2	160 000	2 x 56 000 2 x 23 000		50	11.7
IgG1	146 000	2 x 50 000 2 x 23 000	5.8-7.3	900	3
IgG2	146 000	2 x 50 000 2 x 23 000		300	
IgG4	146 000	2 x 50 000 2 x 23 000		50	

References: Putnam (1975); Wasserman and Capra (1977).

TABLE 1.4

PROTEINS OF THE COMPLEMENT SYSTEM

Protein	M.W.	Subunit M.W.	pI	Conc. mg/100 ml	Carbohydrate %
Classical Pathway					
C1q	410 000	6α 24 000 6β 23 000 6α 22 000		15	8
C4	206 000	α 93 000 3β 78 000 γ 33 000		40	4
C3	180 000	2_{β}^{α} 105 000 75 000	6.1-6.8	125	3
C5	180 000	2_{β}^{α} 105 000 75 000		8	
C8	174 000	α 77 000 3β 63 000 γ 13 700		8	
C1r	166 000	2 x 83 000		5	
C6	128 000	single		7.5	
C7	121 000	single		5.5	
C2	110 000	single		1.5	
C1S	83 000	single		5	

Contd..

Protein	M.W.	Subunit M.W.	pI	Conc. mg/100 ml	Carbohydrate %
Alternative Pathway					
Properdin	216 000	four identical chains of 54 000	<9.5	2.5	9.8
C3	180 000	α105 000 β 75 000	6.1-6.8	125	3
βlH	150 000	single	5.8-6.2	50.0	
C3b inactivator	97 000	α 55 000 β 42 000	5.8-6.1	5.0	
Factor B	93 000	single	6.6-6.8	25.0	
D	23 500	single	7.2-7.5	0.2	

References: Muller-Eberhard (1975); Hugli (1979); Schwick and Haupt (1980).

TABLE 1.5

ACUTE PHASE REACTANT PROTEINS OF HUMAN PLASMA

Protein	M.W.	Subunit M.W.	pI	Conc. mg/100 ml	Carbohydrate %
Fibrinogen	340 000	α 2 x 65 000 β 2 x 55 000 λ 2 x 47 000	5.5	200	7
Complement component C3	180 000	α 105 000 β 75 000	6.1-6.8	125	3
Ceruloplasmin	132 000	single	4.4	35	7
C-Reactive protein	118 000	5 x 21 500		0.1	Non-glycoprotei
Haptoglobin 1-1	100 000		4.1	170-235	16
α_1-Antichymotrypsin	68 000	single	3.75-4.0	45	27
α_1-Antitrypsin	54 000	single	4.0	290	12
α_1-Acidglycoprotein	44 000	single	2.7	90	37.0

References: Koj (1974); Schwick etal. (1977); Schwick and Haupt (1980).

The proteins designated as acute phase reactants are those plasma proteins which increase or decrease in concentration after trauma or during acute infections (Koj, 1974). These include haptaglobin, fibrinogen, orosomucoid (α_1-acidglycoprotein), complement component C_3, α_1-antichymotrypsin, ceruloplasmin, α_1-antitrypsin and C-reactive protein. Excluding C-reactive protein, these are all glycoproteins (Schwick etal. 1977) (Table 1.5).

1.12 ELECTROPHORETIC STUDIES

Over the past decades, electrophoresis has developed into one of the most important methods for the investigation of biological materials and is probably the most efficient procedure for the analysis of proteins. The literature survey has shown the extensive work on plasma protein electrophoresis. Several investigators studied normal human plasma using paper electrophoresis (Armstrong etal. 1947; Jencks etal. 1956; Owen, 1958, Ogryzlo etal. 1959), cellulose acetate strip electrophoresis (Kohn, 1957;1976; Johansson, 1972) and polyacrylamide gel electrophoresis (Mogi, 1965a; Felgenhauer etal. 1967; Margolis and Kenrich, 1969; Trinth et al. 1977; Manabe etal. 1978).

The electrophoretic separation of protein from normal human plasma shows 6 distinct zones. These are albumin, α_1 and α_2-globulins, β-globulins, fibrinogen and γ-globulin, in the decreasing order of mobility. Armstrong etal. 1947 reported the quantitative composition of normal plasma. Similarly, Mitsuo (1961) studied plasma of different vertebrates. The study showed that the composition differs from specie to specie. Table 1.6 shows the plasma composition of some vertebrates.

TABLE 1.6

PLASMA PROTEIN COMPOSITION OF VERTEBRATES

	Albumin	α_1-globulin	α_2-globulin	β-globulin	γ-globulin	Fibrinoge
HUMAN*	52.0	5.3	8.7	13.4	6.5	11.0
MONKEY	53.6	7.0	8.1	12.6	10.2	8.5
HORSE	33.3	7.4	14.4	16.4	12.6	15.8
OX	30.3	5.6	16.5	23.1	12.3	12.2
PIG	44.2	5.8	15.4	21.0	6.8	6.8
GOAT	43.7	7.4	7.7	10.2	18.5	12.6
SHEEP	51.6	7.0	12.6	7.8	12.0	9.2
DOG	37.2	8.9	9.6	11.3	13.0	20.0
CAT	41.5	6.0	20.4	8.5	14.2	9.4
RABBIT	61.8	5.8	6.4	9.8	7.3	8.9
GUINEA PIG	57.5	8.9	6.2	13.0	6.5	7.9
RAT	60.6	4.1	6.6	15.6	8.8	4.3
MOUSE	46.6	5.2	9.6	10.8	12.0	18.0
CHICKEN	29.7	3.9	6.9	10.3	19.2	25.3

Reference: Mitsuo 1961.

*The values for Human are as presented by Armstrong et al., 1947.

1.13. PLASMA PROTEIN CHANGES IN DISEASED SUBJECTS

1.13.1. Electrophoretic studies

The levels of certain plasma proteins fluctuate in pathological conditions. The separation by electrophoresis acts as a quick diagnostic tool (Jencks etal. 1956; Mogi, 1965b; Tarnoky, 1968 and Tarnoky and Dowding, 1967). Routh and Paul (1961) showed a decrease in the plasma albumin and an increase in globulin components occurs in the disease conditions like carcinoma, liver disease, arthritis and heart disease.

The blood an accessible tissue, has been used extensively in the diagnosis of various diseases affecting different organs of the body. However very little work has so far been carried out on the blood/plasma for studying the pathogenesis of cataract formation (Ruddemann and Schneider 1947; Baron etal. 1955; Samedova, 1962).

Baron etal. (1955) have shown an increase in α_1 and α_2-globulin fractions in the plasma of patients with cataract. Samedova (1962) has reported an increase of γ-globulin in addition to α_2-globulin and a simultaneous decrease of albumin in the serum of cataract patients. The report also states the tendency of normal total protein level towards hypo-proteinemia. In the vacuolar lens an increase in β-globulin fraction of serum and in the snowflake opacities, an increase of γ-globulin fraction has been reported (Ruddemman and Schneider, 1947).

1.13.2. Amino acid studies

Analysis of amino acid in plasma and other body fluids for various pathological conditions of the body (Wahren etal., 1976;

Clarke etal. 1978; Waterhouse etal., 1979) have been performed in detail. There are a few reports on the amino acid studies in plasma of the cataract patients.

The role of some amino acids like cysteine, tryptophan and histidine of lens proteins in the process of cataract has been subject of many investigations.

Tryptophan is an unusually distinct plasma amino acid involved in neurotransmission and unlike other amino acids it is bound to serum albumin (Leklem, 1971) and has been studied the most (Allegri and Angi, 1981; Cotlier and Sharma, 1980; Cotlier etal. 1981; Chadwick etal. 1981).

Allegri and Angi (1981) reported that there was no difference in serum free and total tryptophan levels between patients with senile cataract and controls, whereas the contradictory reports by Chadwick etal. (1981) and Cotlier etal. (1981) showed higher level of the same in the cataract patients.

1.14. OBJECT OF THE PRESENT STUDY

The studies on human cataract lenses have mostly been carried out in the western world. Harding and Rixon (1980;1981) used Pakistani cataracts along with the cataracts from India to study the possible factors for the prevalence of cataractogenesis in the tropical countries. Other studies in Pakistan involve enzyme changes during the maturation stages in cataract (Siddiqui, 1975; Ahmed, 1979; Siddiqui and Rehman, 1980). The electrophoretic patterns of proteins from cataract lenses has not been the subject of any of these studies.

The present study was therefore, undertaken to achieve the following purposes:

i) To investigate the electrophoretic protein patterns of the cataract lens and compare it with that of the normal lens.

ii) To study the changes, if any, in the plasma protein patterns of the subjects with cataract and those of the controls, and to correlate them with the changes in the lens proteins.

iii) To perform the amino acid analysis of cataractous lens, plasma from cataractous patients and compare them with the normal.

MATERIAL AND METHODS

2.1. SAMPLE COLLECTION

2.1.1. Lens

Human cataract lenses obtained immediately after the cataract surgery were taken in normal saline (0.9% sodium chloride solution), and stored at 4°C. Thirty-five cataractous lenses were collected which were identified as senile cataracts and were analysed individually.

Only two normal* lenses could be obtained from the eye bank and were stored in the same way as the cataractous lenses.

2.1.2. Blood

The venous blood samples of the cataractous subjects were collected in a pre-sterlized vials with anticoagulants. The anticoagulant used was ethylene diamine tetra acetic acid (EDTA). The plasma was separated within half an hour and stored at -20°C until used.

The plasma for the purpose of control was obtained from seven healthy persons between forty to seventy years of age.

2.2. PREPARATION OF THE SAMPLES FOR ANALYSIS

2.2.1. Saline extracts of lens

The lenses both cataractous and normal were crushed and homogenized in saline solution individually using a glass homogenizer. The homogenate were centrifuged for about 10 minutes at 3,000 RPM. The residue containing the cell membranes was discarded and supernatant, the saline extract was stored at -20°C until further use.

---

*Clear lenses with no opacification.

2.2.2. Plasma

The plasma was separated by centrifugation at 3,000 RPM and stored at -20°C until use. Sodium azide was added as a preservative to prevent microbial growth. The residue containing the cells was discarded.

2.3. EXPERIMENTAL ANALYSIS

2.3.1. Electrophoresis

A) Membrane electrophoresis

i) Membrane electrophoresis of lens proteins:

Electrophoresis of the lens proteins was carried out using cellulose-acetate membrane/strip (7.5cm x 15 cm) from Shandon, England.

Composition of the electrode buffer:

The electrode buffer for the membrane electrophoresis was prepared by taking Tris-(hydroxymethyl)-aminomethane 16.1gm; ethylene diamine tetra acetic acid 1.52gm; boric acid 0.92gm; potassium cyanide 65mg; dissolved in distilled water and the volume was made upto 1 litre with distilled water. The pH of the buffer was 8.9.

Saturation of the membrane/strip:

Cellulose acetate strips (7.5 x 15cm) were saturated with the electrode buffer by putting gently the membrane in a tray filled with the buffer, avoiding any air bubble in between, and taking care not to leave any part of the strip dry. The strip was carefully and uniformly soaked and saturated for a period of two and a half hours. After saturation, the strip was taken out and pressed between the filter papers, to remove excess of buffer from the strip and samples to be analyzed were applied immediately.

Sample application:

A known quantity of the samples of lens extracts were applied by using jet drawn capillaries. Six or seven samples were applied on a pre-saturated strip, as a line of 2mm each. The point of sample application was about 2.5cm away from the cathode end of the strip.

Electrophoretic conditions:

After setting the strip on the electrophoretic chamber, and connecting it to the electrodes, the electrophoresis was carried out for thirty minutes at constant voltage of 350 volts.

Staining of strip:

At the end of electrophoresis, the strip was stained with one percent Amido Black 10B in 5% acetic acid for 5 minutes.

Destaining of strip:

The destaining of the strip was carried out with 5% acetic acid solution until the strip became clear.

Fixing of protein bands:

The protein bands on the cellulose acetate strip were fixed by leaving it in methanol: water (1:9) solution for 10 minutes and the strip later air-dried.

ii) Membrane electrophoresis of plasma proteins:

Electrophoresis of plasma was performed on cellulose acetate membrane. This was carried out in following two different buffer systems:

(a) Tris:EDTA: Boric acid (TEB) buffer, which was prepared by dissolving Tris-(hydroxymethyl)-aminomethane, 10.2gm; ethylene diamine tetra acetic acid 3.2gm and boric acid 0.6gm in distilled water. The volume of the buffer was made upto one litre and the pH adjusted to 8.4.

(b) Sodium barbital/verenol buffer with the concentration of 0.075M; which was prepared by diluting the contents of concentrated verenol buffer (Beckman B-2) to one litre with distilled water.

In both procedures, the membrane was first saturated with the electrode buffer for a period of 20 minutes. Electrophoresis was then performed as described earlier [2.3.1.A(i)].

B) Polyacrylamide gel electrophoresis

i) Continuous disc-gel electrophoresis:

The lens and plasma proteins were subjected to polyacrylamide gel electrophoresis. The polyacrylamide gels were prepared as described earlier (Part A, Section 2.8.1).

The continuous gel electrophoresis was performed according to Raymond and Weintraub (1959). The electrode buffer used was Tris:EDTA:Boric acid buffer, containing 0.0025M potassium cyanide. The pH of the buffer was adjusted to 8.9. After applying the samples on the gels as described, (Part A, Section 2.8.1), the electrophoresis was performed applying a constant current of 8mA/tube with a corresponding starting voltage of approximately 50 volts. The electrophoresis was carried out for two hours.

ii) Discontinuous disc-gel electrophoresis:

Discontinuous disc-gel electrophoresis of lens and plasma proteins was performed according to Ornstein (1964) and Davis (1964) procedure. 12% polyacrylamide gels were used for separating media and two different buffers were Tris-glycine pH 8.9 as upper electrode buffer, and Tris-HCl pH 8.0 as the lower electrode buffer. The compositions for these buffers is described in part A, section 2.8.1. The electrophoresis was carried out as described in above section for continuous disc-gel electrophoresis.

C) SDS-polyacrylamide electrophoresis

i) Discontinuous:

The SDS-electrophoresis was used to determine the molecular weights of proteins. The procedure adopted was similar as stated in part A, section 2.8.2.

ii) Continuous:

In this system the electrophoresis was performed in the presence of 0.1% SDS in gel solutions as well as the electrode buffers.

D) Iso-electric focusing

Iso-electric focusing of the lens and plasma proteins were performed according to Wrigley (1969), details have been given earlier in part A, section 2.8.3.

2.3.2. Amino acid analysis

This was carried out on Biotronik amino acid analyzer LC 6001. The lens extracts and plasma proteins were analyzed using

following two different programmes:

i) Free amino acid analysis by physiological separating programme:

Preparation of samples for free amino acid analysis:

The samples were deproteinized soon after the collection by protein precipitation method (Biotronik procedure). 200µl of sulphosalicylic acid solution (10%) was added to 800µl of sample and mixed thoroughly. The mixture was left at 4°C for about 30 minutes and precipitated proteins were removed by centrifugation at 3,000 RPM for 10 minutes. The clear supernatant named as free amino acid extracts were frozen at -20°C until subjected to amino acid analysis. The analysis performed using the buffer systems as presented in Table 2.1.

ii) Amino acid analysis of protein hydrolyzates by hydrolyzate programme

Preparation of protein hydrolyzates:

The hydrolysis of the proteins was carried out by taking a mg protein in 1ml of 5.7 N HCl solution in a sample vial. The vial was then sealed under vacuum and heated in an oven at 110°C for a period of 20 hours. The contents of the vial were cooled to room temperature and excess acid was removed on a rotary evaporator. The dried sample was taken in 0.2M sodium citrate buffer, pH 2.2 and subjected to amino acid analysis using the buffer systems as described in part A, section (2.10.1).

TABLE 2.1

	Buffer A	Buffer B	Buffer C	Buffer D	Buffer E	Regeneration solution	Sample diluting buffer
pH Value	2.68	3.05	3.50	4.05	3.30	-	2.20
Lithium concentration	0.12n	0.13	0.20n	0.30n	1.40	0.3n	0.12n
Citrate concentration	0.07m	0.07m	0.07m	0.07m	0.07m	-	0.07m
Lithium hydroxide x H_2O	10.1g	10.9g	16.8g	16.8g	16.8g	25.2g	5.0g
Citric acid	29.4g	29.4g	29.4g	29.4g	29.4g	-	14.7g
Lithium chloride	-	-	-	8.5g	101.7g	-	-
Hydrochloric acid 37%	14.0ml	13.0ml	18.0ml	8.0ml	16.0ml	-	9ml
Brij 30% solution	4ml	4ml	4ml	4ml	4ml	4ml	-
Phenol liquified	2ml	2ml	2ml	2ml	2ml	-	1ml
Thiodiethanol 25% in H_2O		-	-	-	-	-	20ml
Methylcellusolve	140ml	120ml	-	-	-	-	-
FINAL VOLUME	2 ℓ	2 ℓ	2 ℓ	2 ℓ	2 ℓ	2 ℓ	1 ℓ

INSTRUMENT DATA FOR THE SEPARATION PROGRAM FOR PHYSIOLOGICAL FLUIDS

Separation Column Size	0,4 x 25 cm	Buffer Flow	20 ml/h
Resin Type	BTC 2710	Reagent Flow	20 ml/h
Resin Bed Height	14 cm		
Prewash Column Size	0,6 x 8 cm	Temperature T_1	38,5 °C
Resin Type	BTC F	Temperature T_2	57 °C
Resin Bed Height	3 cm		

RESULTS

3.1. ELECTROPHORESIS

3.1.1. Membrane/strip electrophoresis

Lens: The electrophoresis of cataractous lens protein carried out on cellulose acetate strips using TEB buffer revealed the presence of six protein bands. The normal lens electrophoresis under same conditions showed seven protein bands (Fig.3.1).

Plasma: The separation of plasma proteins was performed on the strips using two different buffer systems. Figure 3.2a shows the separation pattern in the TEB buffer which shows five and four bands in thirty and five cataractous plasma respectively. The control plasma separated into six protein bands. In verenol buffer system, it separated into four bands in the plasma from cataract patient and five bands in plasma from control (Fig.3.2b).

3.1.2. Continuous disc-gel electrophoresis

Lens: The lens proteins disc-gel electrophoresis carried out in the continuous buffer (TEB with KCN) showed seven/six and seven bands (Fig.3.3a) in cataractous and normal lens respectively.

Plasma: The continuous disc-electrophoresis of the plasma proteins separated into eight/nine fractions in case of plasma from cataractous patient and nine fractions in the plasma from control (Fig.3.3b).

3.1.3. Discontinuous disc-gel electrophoresis

Lens: The separation of normal lens on discontinuous disc-gel electrophoresis showed seven diffuse bands and the cataractous lens showed six bands (Fig.3.4a).

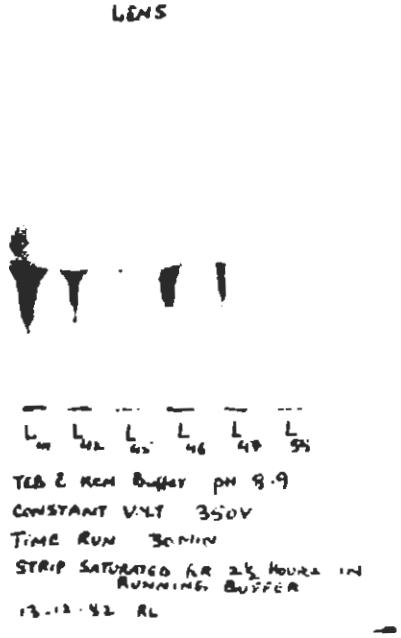
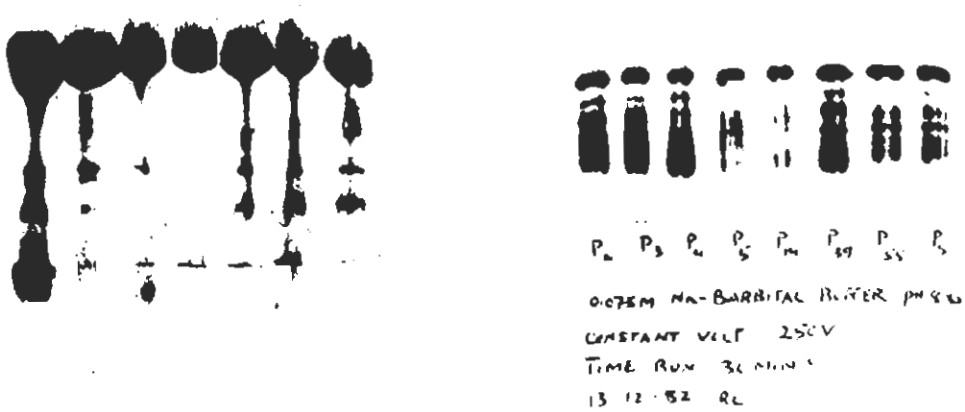


Fig.3.1: Cellulose acetate strip electrophoresis of lens proteins using Tris:EDTA:Boric acid (16.1:1.52:0.92) buffer with 0.0025M KCN, pH 8.9.



a b

Fig.3.2: Cellulose acetate strip electrophoresis of plasma proteins:
a)Tris:EDTA:Boric acid (10.2:3.2:0.6) buffer, pH 8.4.
b)Sodium barbital buffer (0.075M), pH 8.65.

a b

Fig.3.3: Disc-gel electrophoresis using polyacrylamide gels and the continuous buffer system of Tris:EDTA:Boric acid.

a) Lens
b) Plasma

a b

Fig.3.4: Disc-gel electrophoresis in the discontinuous buffer system of Tris-glycine and Tris-HCl using polyacrylamide gels.

a) Lens
b) Plasma

Plasma: The plasma proteins when applied on PAGE, using discontinuous buffer system, revealed the presence of eleven protein bands in case of control plasma and ten/eleven bands (Fig.3.4b) in the plasma from cataract patient.

3.2. SDS-PAGE

3.2.1. Discontinuous SDS-PAGE

Lens: The normal lens, separated on SDS-PAGE, showed the presence of four protein bands and the cataractous lens showed only two bands as shown in Figure 3.5a.

Plasma: The separation of plasma proteins on the same system revealed the presence of four and seven bands in the plasma from cataract patient and control plasma respectively (Fig.3.5b).

3.2.2. Continuous SDS-PAGE

Lens: In the continuous buffer system on SDS-PAGE, the protein from normal lens, revealed the presence of four bands and the cataractous proteins showed two bands (Fig.3.6a).

Plasma: Figure 3.6b shows the results of SDS-PAGE in the continuous buffer system revealing eight/nine and seven bands in the normal and plasma from cataract patient respectively.

3.3. ISO-ELECTRIC FOCUSING

Lens: Iso-electric focusing of cataractous and normal lens proteins, carried out on polyacrylamide gels, revealed the presence of only six protein bands with different iso-electric points and ten bands with different intensities were observed in cataract and normal lens respectively (Fig.3.7a).

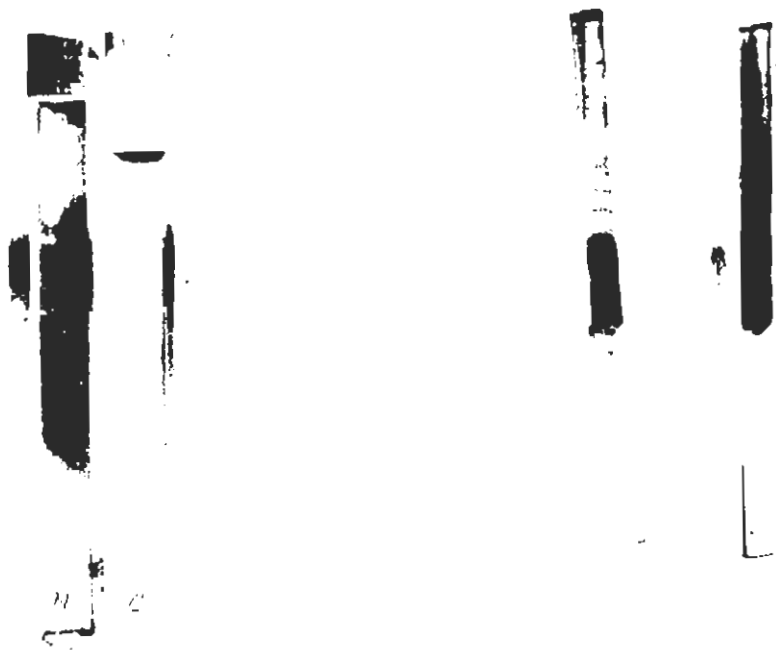
Fig.3.5: Polyacrylamide gel electrophoresis in the presence of SDS, using discontinuous buffer system of Tris-glycine and Tris-HCl.

a) Lens
b) Plasma

a b

Fig.3.6: Polyacrylamide gel electrophoresis in the presence of SDS using continuous buffer system of Tris:EDTA:Boric acid with KCN.

a) Lens
b) Plasma



a b

Fig.3.7: Iso-electric focusing of lens and plasma proteins in polyacrylamide gels; Ampholine pH 3-10 was used (bottom to top).

a) Lens
b) Plasma

TABLE 3.1

FREE AMINO ACIDS OF LENS

Amino Acids	Normal Lens*	Cataractous Lens**	P-Value
Alanine	69.44±5.83	67.60± 2.85	N.S.
Aspartic acid	0.38±0.10	-	-
Cysteine	121.34± 8.57	70.61±16.08	N.S.
Glutamic acid	22.80± 2.19	148.41±26.67	N.S.
Glutamine	44.21± 3.15	105.27± 3.38	$P < 0.001$
Glycine	98.54± 3.49	168.93±54.87	N.S.
Isoleucine	169.44± 9.71	53.80±11.76	$P < 0.001$
Leucine	199.90± 2.27	22.65± 1.11	$P < 0.001$
Lysine	16.06± 2.46	21.94± 1.50	N.S.
Methionine	33.42± 1.30	73.10± 4.56	$P < 0.001$
Ornithine	104.38± 7.52	44.90± 8.52	$P < 0.001$
Phosphoserine	5.17± 0.48	40.13±14.71	N.S.
Serine	62.90± 3.77	-	-
Taurine	2.74± 0.71	59.65± 8.84	$P < 0.001$
Threonine	16.76± 2.40	-	-
Urea	2.74± 0.34	223.19±45.26	$P < 0.001$

*Mean value of 2 samples

**Mean value of 35 samples

The values are presented as mean ± S.E.
N.S. Statistically not significant.
P-Values < 0.01 - < 0.001 are taken as significant.

TABLE 3.2

FREE AMINO ACIDS OF PLASMA

Amino Acids	Normal Plasma*	Cataractous Plasma**	P-Value
Alanine	366.68±20.36	185.56±40.73	$P < 0.001$
α-Aminoadipic acid	45.92±12.61	-	-
Asparagin	21.54± 6.26	-	-
Aspartic acid	15.18± 6.18	25.16± 3.36	N.S.
Citrulline	10.39± 1.60	13.98± 2.06	N.S.
Cystine	190.16±42.19	151.47± 9.41	N.S.
Glutamic acid	90.11±11.93	88.94±10.27	N.S.
Glutamine	550.89±71.28	338.47±47.17	N.S.
Glycine	311.49±70.69	365.00±64.95	N.S.
Histidine	75.42±10.76	68.81±11.63	N.S.
Isoleucine	62.63±10.19	163.73±22.22	$P < 0.001$
Leucine	146.44± 4.59	291.81±28.45	$P < 0.001$
Lysine	102.04±23.83	94.92±20.92	N.S.
Methionine	18.11± 8.54	46.69±14.73	$P < 0.001$
Ornithine	32.50±10.87	152.01±11.20	$P < 0.001$
Phenylalanine	195.49±20.51	221.16±13.08	N.S.
Phosphoserine	6.75± 1.99	18.47± 6.34	N.S.
Serine	130.72±31.44	174.35±18.90	N.S.
Taurine	63.45±18.24	118.30±29.33	N.S.
Threonine	114.62±19.76	79.54±12.98	N.S.
Tyrosine	67.86±10.73	102.75± 7.94	$P < 0.001$
Valine	58.85± 4.62	117.84± 7.25	N.S.
Urea	283.77±10.52	227.48±46.83	N.S.

*Mean value of 7 samples

**Mean value of 35 samples

The values are presented as mean ± S.E.
N.S. Statistically not significant.
P-Values < 0.01 - < 0.001 are taken as significant.

TABLE 3.3

AMINO ACIDS OF LENS (TOTAL PROTEIN HYDROLYZATES)

Amino Acids	Normal Lens*	Cataractous Lens**	P-Value
Alanine	52.66± 6.62	50.00±0.96	N.S.
Arginine	81.50±16.73	89.22±8.90	N.S.
Aspartic acid	95.68± 6.56	99.54±4.90	N.S.
Glutamic acid	157.18± 6.75	152.61±6.07	N.S.
Glycine	91.95± 7.96	84.00±0.63	N.S.
Histidine	42.74± 8.20	44.29±2.33	N.S.
Isoleucine	41.45± 0.98	34.77±2.79	N.S.
Leucine	67.47± 5.35	73.11±3.85	N.S.
Lysine	48.40± 1.60	44.82±5.88	N.S.
Methionine	9.73± 3.56	7.01±0.94	N.S.
Phenylalanine	67.36± 3.95	62.26±0.94	N.S.
Proline	45.25± 1.89	51.16±3.42	N.S.
Serine	85.44± 3.05	99.97±5.45	N.S.
Threonine	31.21± 2.80	37.51±1.99	N.S.
Tyrosine	51.23± 5.78	41.11±1.31	N.S.
Valine	30.77± 3.27	28.67±1.43	N.S.

* Mean value of 2 samples

** Mean value of 35 samples

The values are presented as mean ± S.E.
N.S. Statistically not significant.
P-Values < 0.01 - < 0.001 are taken as significant.

TABLE 3.4

AMINO ACIDS OF PLASMA (TOTAL PROTEIN HYDROLYZATES)

Amino Acids	Normal Plasma*	Cataractous Plasma**	P-Value
Alanine	607.10±116.67	767.50±87.57	N.S.
Arginine	262.90± 43.56	366.00±38.84	N.S.
Aspartic acid	767.80±114.83	542.50±99.93	N.S.
Glutamic acid	1024.35±217.72	1298.50±138.97	N.S.
Glycine	339.60± 48.93	519.00± 66.02	N.S.
Histidine	349.55± 57.77	741.00± 83.10	N.S.
Isoleucine	158.55± 19.73	378.00± 45.78	N.S.
Leucine	631.25±113.77	1227.00± 10.74	$P < 0.001$
Lysine	641.85±150.54	749.50± 76.36	N.S.
Methionine	125.00± 3.54	338.00± 40.98	$P < 0.01$
Phenylalanine	345.45± 90.16	689.50± 49.51	$P < 0.01$
Proline	400.20± 66.89	629.50± 87.33	N.S.
Serine	468.75±108.96	624.50± 60.26	N.S.
Threonine	419.85± 95.81	560.50± 63.06	N.S.
Tyrosine	180.20±140.57	501.75± 93.48	N.S.
Valine	318.15± 58.62	798.00± 57.70	$P < 0.001$

*Mean value of 7 samples

**Mean value of 35 samples

The values are presented as mean ± S.E.
N.S. Statistically not significant.
P-Values < 0.01 - < 0.001 are taken as significant.

Plasma: The normal human plasma on iso-electric focusing showed thirteen protein bands, and in the plasma from cataract patient only nine bands were visible (Fig.3.7b).

3.4. AMINO ACID ANALYSIS

Free amino acids: The free amino acid analysis of the lens and the plasma proteins was carried out on Biotronik amino acid analyzer. Table 3.1 shows the quantitative pattern of the free amino acids in the lens. Table 3.2 shows the results of this analysis on plasma.

Protein hydrolyzate: The amino acids from protein hydrolyzates of the lenses and plasma were also analysed. Table 3.3 shows the quantitative pattern of amino acids from lens protein hydrolyzates and Table 3.4 presents the same from the hydrolyzates of plasma proteins.

DISCUSSION

The etiology of senile cataract is not known. Any agent which sharply changes the refractive index of the lens, producing scattering of light, or increases the light absorbing molecules results in opacification of the lens. Harding and Rixon (1981) have noticed a high incidence of cataract in the subcontinent of India and Pakistan, and also that the onset occurs about 20 years earlier. They have suggested that frequent episodes of diarrhoea may be a major cause of this high and early incidence. Diarrhoea may produce malnutrition, acidemia, dehydration and uraemia; all of which may directly or indirectly produce cataract. The high levels of urea in the blood produced by diarrhoea, or even as the result of renal failure, may be transmitted to the lens, where urea could be converted to cynate with subsequent carbamylation of lens protein. This change is probably responsible for the opacification of the lens.

Harding and Rixon (1980,1981) have also compared the senile cataractous lenses from Pakistani patients with those from Oxford, and have found that extensive carbamylation of lens protein had occurred in Pakistani lens but not in English ones, nor in the normal human lens. In the present study the amount of urea in cataractous lenses was many times more than that found in the controls. This may be responsible for the unfolding of lens proteins leading to cataract formation. However, our patients were not in renal failure and their blood urea levels were normal.

Malik etal. (1969) have performed electrophoresis of normal as well as cataractous lens proteins demonstrating fewer

number of protein bands in the cataractous lens. This finding has been confirmed in the present study by electrophoresis on cellulose acetate strip. Also, the loss of one or more protein bands was observed on other forms of electrophoresis e.g. SDS-gel electrophoresis showed loss of 2 bands, PAGE showed loss of one band and IEF showed loss of 4 bands. The iso-electric points of the missing bands were 6.95, 6.90, 6.60 and 6.25.

This observation that the protein bands are lacking in the cataractous lenses indicates that these proteins have either become insoluble or have ceased to be synthesized. However, the other workers (Francois etal. 1954; Clark etal. 1969; York etal. 1972; Maraini and Mangili, 1973; Kramps, 1977 and Ringens etal. 1978) have failed to observe a reduction in the number of bands though they have noted a quantitative difference.

The physiological changes in the body are reflected in the body fluids like plasma, urine, cerebrospinal fluid etc. In cataract formation the alterations are likely to be reflected in the fluids which are closest to the eye i.e. aqueous and vitreous humours. These fluids are however, difficult to obtain in the living subject. Therefore, plasma which is easily accessible was obtained from the patients with cataract and from controls from a similar age group and examined for any possible change in protein pattern or amino acid composition.

Electrophoresis of plasma from controls and cataractous subjects showed six and five/four proteins respectively. The band(s) missing from the plasma of cataractous patients were the third from the origin towards anode and in some cases the second and third both. Similar differences were noted in electrophoresis with SDS and in IEF. The proteins noted to be missing in the plasma from cataractous patients had the following iso-electric points: 6.90, 6.95, 7.0 and 7.1. The amino acid composition showed both qualitative as well as quantitative changes.

This is the first study undertaken in Pakistan on the proteins of lens and plasma of patients with cataract and in normal controls. The results show missing protein bands in cataractous lens and plasma and an accumulation of urea in the cataractous lens. However, the metabolic pathways responsible for these changes are not fully understood.

REFERENCES

Adams, R.F. (1974) *J.Chromat.*, 95, 189.

Agarwal, V.B., Agarwal, S.C. and Ram, N. (1979) *Indian J.Exp.Biol.*, 17(9), 882.
Agarwal, V.B., Agarwal, S.C. and Ram, N. (1980) *Indian J.Exp.Biol.*, 18(10), 1200.
Ahmed, S.S. (1979) Ph.D. Thesis, Department of Biochem. J.P.M.C., University of Karachi.

Allegri, G. and Angi, M.R. (1981) *Lancet*, 2(8238), 157.

Alper, C.A. and Davis III, A.E. (1980) *Ann.Rev.Clin.Biochem.*, (David, M.G. ed.) John Wiley and Sons Inc.Vol.I, pp.357.

Anderson, L.O. and Lunden, R. (1979) in "The Plasma Proteins" (Blomback, B. and Hanson, L.A. eds.) John Wiley and Sons, Chichester, pp.17.

Anima, D. and Piare, L.R. (1966) *Current Sci.(Ind.)*, 35(1), 13.

Aoki, T.T.Brennan, M.F., Muller, W.A. and Cahill, Jr. G.F. (1974) *Adv.Enzyme Regul.*, 14, 157.

Armstrong, S.H. Jr., Budka, M.J.E. and Morrison, K.G. (1947) *J.Am. Chem.Soc.*, 69, 416.

Augusteyn, R.C. (1977) *Pap.Congr.-Aust.N.Z.Assoc.Adv.Sci.*, 46(7), 388.

Austin and Rizza (1974) in "Structure and Function of Plasma Proteins" (Allison, A.C., ed.) Vol.1, pp.169.

Barber, G.W. (1968) *Invest.Ophthalmol.*, 7(5), 564.

Baron, J.B., Guillon, S. and Peyrau, M. (1955) *Ann.Bilol.Clin.*, (Paris), 13, 104.

Benedek, G.B. (1971) *Appl.Optics*, 10, 459.

Berbers, G.A.M., Hoekman, W.A., Bloemendal, H., Jong, W.W.De., Kleinschmidt, T. and Braunitzer, G. (1984) *Eur.J.Biochem.*, 139, 467.

Bernat, R. and Bombicki, K. (1968) *Acta Physiol.Pol.*, 19(2), 205.

Bernat, R. Hryniewiecki, L. and Straburzynski, G. (1966) *Acta Physiol.Polon.*, 17(2), 225.

Bjork, I. (1961) *Exp.Eye Res.*, 1, 145.

Bjork, I. (1964) *Exp.Eye Res.*, 3, 254.

Bours, J. (1984) *Curr.Eye Res.*, 3(5), 691.

Bron, A.J. and Habgood, J.O. (1976) *Trans.Ophth.Soc.U.K.*, 96, 265.

Buckingham, R.H. (1971) *Biochem.J.*, 124(5), 54.

Bushell, A.R. and Duncan, G. (1978) *Interdiscip.Top-Gerontol.*, 13, 247.

Chadwick, C., Phipps, D.A. & Powell, C. (1981) *Lancet*, 2(8246), 583.

Charlton, J.M. and Van Heyningen, R. (1968) *Exp.Eye Res.*, 7(1), 47.

Clark, R. Zigman, S. and Lerman, S. (1969) *Exp.Eye Res.*, 8, 172.

Clarke, E.F., Lewis, A.M. and Waterhouse, C. (1978) *Cancer*, 42, 2909.

Cotlier, E. and Sharma, Y.R. (1980) *The Lancet, March 15*, 607.

Cotlier, E., Yog Raj Sharma, J.Z., James Pucklin, B.T. and John, I. (1981) *Exp.Eye Res.*, 33, 247.

Croft, L.R. (1972) *Biochem.J.*, 128, 961.

Davis, B.J. (1964) *Annals.N.Y.Acad.Sci.*, 121, 404.

De Jong, W.W., Gleaves, J.T., Boulter, D. (1977) *J.Mol.Evol.*, 10(2), 123.

Dickenson, J.C., Durham, D.G., Hamilton, P.B. (1968) *Invest. Ophthalmol.*, 7(5), 551.

Dickenson, J.C., Rosenblum, M. and Hamilton, P.B. (1965) *Pediatrics*, 36, 2.

Dilley, K.J., Bron, A.J. and Habgood, J.O. (1976) *Exp. Eye Res.*, 22, 155.

Dilley, K.J. and Pirie, A. (1974) *Exp. Eye Res.*, 19, 59.

Doria, R., Grown, J. and Maier, K. (1984) *Curr.Eye Res.*, 3(5), 723.

Felgenhauer, K., Bach, S. and Stammler, A. (1967) *Klin.Wschr.*, 45, 371.

Francois, J., Rabaey, M. and Boyen-Rikkers, L. (1969) *Exp.Eye Res.*, 8, 157.

Lawson, E.Q., Carl, F.S., Randolph, V.L. and Middaugh, C.R. (1981) *J.Biochim.*, 256(3), 6523.

Leklem, J.E. (1971) *Am.J.Clin.Nutr.*, 24, 659.

Lerman, S. and Zigman, S. (1965) *Invest.Ophthalmol*, 4, 643.

Lewis, A.M., Waterhouse, C. and Jacobs, L.S. (1980) *Clin.Chem.*, 26(2), 271.

Mach, H. (1963) *Altersstar.Klin.Mbi.Augenheilk.*, 143, 689.

Malik, S.R.K., Gupta, A.K., Chattergi, S. and Agarwal, P.S. (1969) *Exp.Eye Res.*, 8, 93.

Manabe, T., Tachi, K., Kojima, K.& Obuyama, T. (1978) *Leibutsu Butsuri Kagaku*, 22(2), 171.

Maraini, G. and Mangili, R. (1973) *Exp.Eye Res.*, 16, 123.

Margolis, J. and Kenrich, K.G. (1969) *Nature*, 221(5185), 1056.

McAvoy, J.W. (1978) *J.Embryol.Exp.Morph.*, 44, 149.

Miller, D. and Benedek, G. (1973) in "Intraoculer Light Scattering" (Thomas ed.) Springfield.

Mishima, N. and Ikeda, A. (1981) *Exp.Eye Res.*, 32(4), 368.

Mitsuo, A. (1961) *Tokyo Jikrikai Ikadaijaku Zasshi*, 76(11), 2597.

Mogi, G. (1965a) *Seibutsu-Butsuri-Kagaku (Japan)*, 11, 99.

Mogi, G. (1965b) *Seibutsu-Butsuri-Kagaku (Japan)*, 11, 171.

Mok, C.C. and Waley, S.G. (1968) *Exp.Eye Res.*, 7, 148.

Muller-Eberhard, H.J. (1975) *Ann.Rev.Biochem.*, 44, 697.

Ogryzlo, M.A., Maclachlan, M., Dauphinee, J.A. and Fletcher, A.A. (1959) *Am.J.Med.*, 27, 596.

Ornstein, L. (1964) *Annals.N.Y.Acad.Sci.*, 121, 321.

Owen, J.A. (1958) *Adv.Clin.Chem.*, 1, 238.

Papaconstantinou, J. (1965) *Biochim.Biophys.Acta*, 107, 81.

Papaconstantinou, J. (1967) *Science*, 156, 388.

Perry, T. and Hansen, S. (1969) *Clin.Chim.Acta*, 25, 53.

Phillipson, B. (1969a) *Invest.Ophthalmol.*, 8, 271.

Phillipson, B. (1969b) *Invest.Ophthalmol.*, 8, 281.

Porter, R.R. (1979) in "The Chemistry and Physiology of the Human Plasma Proteins" (Bing, D.H., ed.) ; Pergamon Press, p.281.

Porter, R.R. and Reid, K.B.M. (1979) in "Advances in Protein Chemistry" (Anfinsen, C.B., Edsall, J.T. and Richards, F.M. eds.) Academic Press New York and London, vol.33, pp.1.

Putnam, F.W. (1975) in "The Plasma Proteins" (Putnam, F.W. ed.) Academic Press, New York London, vol.1, pp1.

Putnam, F.W. (1976) in "Trace Components of Plasma" (G.A.Jamieson and Tibor, J. Greenwalt, eds) Alan R.Liss, Inc. New York.

Putnam, F.W. (1977) in "The Plasma Proteins" (Putnam, F.W., ed.) Academic Press, New York London, vol.3, pp.1

Rao, G.N., Raj, N.D. and Sadasivudu, B. (1982) *IRCS Med.Sci.Libr. Compend.*, 10(8), 598.

Raymond, S. and Weintraub, L. (1959) *Science*, 130, 711.

Ringens, P.J., Liem - The, K.N., Hoenders, H.J. and Wollensak, J. (1978) *Interdis.Top.Gerontol.*, 13, 199.

Routh, J.I. and Paul, W.D. (1961) *Proc.Iowa Acad.Sci.*, 68, 229.

Roy, D. and Spector, A. (1976) *Biochemistry*, 15(5), 1180.

Ruddemann, A.D. and Schneider, R.W. (1947) *Trans.Am.Ophthalmol.Soc.*, 45, 319.

Russel, P. (1981) in "New Dir.Ophthalmic Res." (Sears, Marvin Lloyd ed.) Yale University Press, New Heaven, pp.109.

Samedova, U.N. (1962) *Azerb.Med.Zh.*, 5, 39.

Schaeffer, A.J. and Murray, J.D. (1950) *Arch.Ophthalmol.(Chicago)*, 43, 202.

Schubert, E.E., Trevithick, J.R. and Hollenberg, M.J. (1970) *Can.J. Ophthalmol.*, 5, 353.

Schultz, D.R. (1976) *TIBS*, 1, 81.

Schwick, H.G. and Heide, K. (1977) *TIBS*, 2, 125.

Schwick, H.G., Heidi, K. and Haupt, H. (1977) in "The Glycocongugates" (Horwitz, M.I. and Pigmans, W., eds.) Academic Press, London and New York. Vol.1, pp.261.

Schwick, H.G. and Haupt, H. (1980) *Angew.Chem.Int.Ed.Eng.*, 19, 87.

Siddiqui, M.A. (1975) Ph.D. Thesis, Dept. of Biochem., J.P.M.C., University of Karachi.

Siddiqui, M.A. and Rehman, M.A. (1980) *Exp.Eye Res.*, 31, 463.

Smelser, G.K. (1965) *Invest.Ophthalmol.*, 4, 398.

Spector, A. (1965) *Invest.Ophthalmol.*, 4, 579.

Spector, A. (1972) *Isr.J.Med.Sci.*, 8, 1577.

Spector, A. (1974) *Invest.Ophthalmol.*, 13, 325.

Spector, A., Stauffer, J. and Sigelman, J. (1973) *Cib.Found.Symp.*, 19.

Stanojevicapaovic, A., Cuperlovic, M. and Movessijan, M. (1983) *Periodicum Biologorum.*, 85(3), 73.

Takemoto, L.J. and Azari, P. (1976) *Exp.Eye Res.*, 23(1), 1.

Tarnoky, A.L. (1968) in "Chromatogr. and Electroph. Techn.", (Smith, I., ed.) W.Heinemann Med. Books, London, Vol.II.

Tarnoky, A.L. and Dowding, B. (1967) *Clin.Chem.*, 1, 48.

Topaszto, I. (1962) *Acta Ophthalmol.*, 40, 77.

Trinth, H.C., Ngo, T.P., Vu, T.B. (1977) *Rev.Med.(Hanoi)*, 104.

Van Heard, P.M.M., Martens, G.J.M. Mestrom, J.J.L., Hoenders, H.J. and Wollensak, J. (1979) *Ophthalmic Res.*, 11(5-6), 433.

Van Heyningen, R. (1972) *Exp.Eye Res.*, 13, 136.

Van Heyningen, R. (1976) *Exp.Eye Res.*, 15, 685.

Wada, E., Sugiura, T., Nakamura, H. and Tsumita, T. (1981) *Biochim. Biophys.Acta*, 667(2), 251.

Wahren, J., Felig, P. and Hagenfeldt, L. (1976) *J.Clin.Invest.*, 57, 987.

Wasserman, R.L. and Capra, J.D. (1977) in "The Glycocongugates" (Horowitz, M. and Pigman, W. eds.) Vol.1, pp.323.

Waterhouse, C., Clarke, E.F. and Heinig, R.E. (1979) *Am.J.Clin.Nutr.*, 32, 2423.

West, E.S., Todd, W.R., Mason, H.S. and Van Bruggen, J.T. (1966) in "Text Book of Biochemistry" Chap.15, pp.554.

Witmer, R. and Buhler, E. (1960) *Arch.Ophthalmol.Graefe's*, 162, 193.

Wrigley, C.W. (1969) *Sci.Tools*, 15, 17.

York, A.T., Martinez, C. and Manski, W. (1972) *Ophthalmic Res.*, 3, 183.

Zacharias, D. and Helena, Z. (1951) *Am.J.Ophthalmol.*, 34(5), pt.II. 104.

Zigler, J.S.Jr., Horwitz, J. and Kinoshita, J.H. (1980) *Exp.Eye Res.*, 31, 41.

Zigler, J.S. Jr., Horwitz, J. and Kinoshita, J.H. (1981) *Exp.Eye Res.*, 32, 21.

Zigler, J.S. and Sidbury, J.B. (1973) *Exp. Eye Res.*, 16, 207.

Zigler, J.S. and Sidbury, J.B. (1976a) *Invest.Ophthalmol.*, 15(8), 673.

Zigler, J.S. and Sidbury, J.S. (1976b) *Comp.Biochem.Physiol.*, 53B, 349.

Zigler, J.S. and Sidbury, J.S. (1976c) *Comp.Biochem.Physiol.*, 55B, 19.

Zigman, S., Schultz, J. and Yulo, T. (1983) *Comp.Biochem.Physiol.*, 75B(3), 425.