STUDIES ON THE FRAGMENTATION OF HUMAN IMMUNOGLOBULIN M BY CYANOGEN BROMIDE

BY

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A B S T R A C T

Human IgM proteins have been isolated from several pathological sera by euglobulin precipitation followed by gel filtration. The purity of the isolated IgM proteins was determined by cellulose acetate electrophoresis. By reacting with anti-human IgM serum and anti-human Kappa (K) chain serum the immunological properties were determined. The polymeric IgM molecule (Mol-wt ≃ 950,000) was converted into pentameric Fcμ fragments using trypsin, and into monomer subunits (IgM_s) using cysteine, and the purities and immunological characteristics of these products were studied. Their molecular weights were also estimated by gel filtration chromatography and by SDS polyacrylamide gel electrophoresis.

Intact IgM, its pentameric Fcμ fragments and the IgM_s subunits were partially denatured by 0.05 Molar hydrochloric acid (HCl), and cyanogen bromide was allowed to react with the proteins in these acidic conditions. Protein digests were subjected to analysis using cellulose acetate electrophoresis and polyacrylamide gel disc electrophoresis in denaturing buffers. The digests were fractionated by column gel chromatography in a denaturing buffer. The molecular weights, electrophoretic mobilities, amino acid compositions, and immunological properties of the isolated fragments were established.

The results show that satisfactory partial cleavage of IgM and IgM_s can be effected using cyanogen bromide in 0.05M HCl. $(Fc\mu)_5$ and Fabμ fragment suitable for further characterisation are obtained in high yield.

ABBREVIATIONS

CNBr - Cyanogen bromide

Gu-HCl- Guanidine hydrochloride

Mol-Wt- Molecular weight

RF - Rheumatiod factors

T.L.G.- Thin Layer Gel chromatography

Tris - Tris(hydroxymethlamin methan)

TEMED- NNN'N'-tetramethylethylenediamine

W.H.O.-World Health Organisation

SDS - Sodium Dodcyl Sulphate

I.U.I.-International Union of Immunological Science

γ- Gamma ; contd,continued

μ- mu ; Log.Mol.Wt.,Log_{10}.Molecular weight.

ε- epsilen ; O, Origin.

δ- Dalta

α- Alpha

K - Kappa

λ- Lambda

Abbreviation of the amino acid residues are as follows:-

Ala - Alanine

Arg - Arginine

Asp - Aspartic acid

Cys - Cystine calculated as cyseic acid

Glu - Glutamic acid

Gly - Glycine

His - Histidine

Ile - Isoleucine

Leu - Leucine

Lys-- Lysine

Met - Methionine

Phe - Phenylalanine

Pro - Proline

Ser - Serine

Thr - Thrionine

Tyr - Tyrosine

Val - Valine

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CHAPTER I

IMMUNOGLOBULIN

GENERAL FEATURES

1.1. INTRODUCTION :

Immunoglobulin molecules are specific products of cellular metabolism containing characteristic polypeptide chains which may be identified by immunological, chemical and physical means. In addition to antibody molecules, myeloma proteins, Bence jones proteins, Waldenstrom macroglobulin proteins, cryoglobulin proteins, light polypeptide chains found in normal serum and urine, and the portion of heavy chain found in heavy chain disease are considered to be immunoglobulins.

The development of the electrophoretic method of protein analysis by Tiselius (1937) enabled the distinction of five major protein fractions in serum, one of which presented itself as a heterogeneous fraction: the so called γ- globulins. Antibody activity appears to reside in this fraction (Tiselius and Kabat, 1938). Heidelberger and Pederson (1937) showed that the rabbit antibody Pneumococcal polysaccharide had a molecular weight of about 150,000 (S_{20} =7S) while the horse antibody of the same specificity had a molecular weight of about 10^6 (S_{20} = 19S).

Antibodies appear to be heterogeneous in nature with respect to size and electrophoretic behaviour (Tiselius and Kabat, 1938 ; Smithies,etal., 1955 ; Poulik,etal., 1958) and to specificity (Grabar and Williams, 1953 ; Laurell, 1966 ; Oudin, 1946 ; Ouchterlony, 1948). In 1960 Heremans proposed the term "Immunoglobulin", and later, the World Health Organization (W.H.O.) published "Nomenclature for Human

"Immunoglobulin", since then it has been in general use, and the Ig is frequently used to indicate the immunoglobulin.

In 1847, Henry B. Jones observed in a patient's serum and urine an abnormal protein which tended to precipitate between 50°C and 60°C, but redissolved at 100°C and reprecipitated on cooling. This protein had immunological features and was called Bence jones protein. Korngold and Lipari (1955) showed that there are two types of Bence jones proteins called Kappa (K) and Lambda (L) and that there could be distinguished by their antigenic determinants.

Edelman and Gally (1962) showed that a Bence jones protein and the light chain of the myeloma protein of a patient are usually identical. The immunological relationship between Bence jones protein and myeloma proteins, normal immunoglobulins and Waldenstrom macroglobulins showed that Kappa (K) and Lambda (L) are antigenically distinguishable classes of light chains, common to all immunoglobulin molecules.

Each immunoglobulin molecule has either the Kappa (K) type or the Lambda (L) type light chains but not both (Deutsch etal., 1955 ; Franklin etal, 1964 ; Korngold and Lipari, 1955 ; Mannik,etal., 1962, 1963 ; Migit,etal., 1963). Studies (Gally,etal., 1964, 1965 ; Edelman etal., 1968 ; Solomon, 1969 ; Porter, 1973 ; Caggiono etal., 1969 ; Putnam,etal., 1967, 1973 ; Watanabe,etal., 1973 ; and VictorLiu,etal.,1976) of the amino acid sequence of Bence jones proteins, myeloma proteins and Waldenstrom macroglobulins, have established the basic primary structure of both light and heavy chains, each of which is composed of a variable terminal portion (V) and a constant (C) terminal portion, and most of the amino acid differences between immunoglobulin chains of a single group (e.g. Kappa) are found in the variable region. It is generally accepted that at least two genes are involved in the synthesis of each immunoglobulin polypeptide chain, one gene coding

for the variable (V) region and the other for the constant (C) region of each chain (Dreyer and Bennett, 1965 ; Wang,etal, 1970).

Hydrolysis of immunoglobulins with papain, pepsin and carboxypeptidase resulted in the reduction of their molecular size to a smaller fragments (Porter, 1950).

Papain breaks IgG molecules in the presence of cysteine into three fragments, two Fab fragments and one Fc fragment. The two Fab fragments (Fragment antigenic binding) are identical in amino acid content, antibody activity and having a molecular weight of about 45,000. The Fc fragment (Fragment crystallized) has a molecular weight of about 52,000 and differs from Fab fragment in its chemical composition and behaviour (Porter, 1959 ; Noelken,etal., 1965).

Meanwhile Edelman (1959) showed that treatment of IgG with mild reducing agents dissociated it into heavy chains of molecular weight of about 50,000 and light chains of molecular weight of about 23,000. In 1960, Nisonoff etal repeated Porter's experiment using another proteolytic enzyme, pepsin. A fragment with a molecular weight of about 100,000 was obtained. This fragment could be split into two identical subunits, each of which was essentially similar to Porter's Fab. This peptic fragment was called $(Fab)_2$. The Fc portion was cleaved into a series of small peptides by pepsin.

On treating Fab fragment with suphydryl reducing agents a whole light chain and a portion of the heavy chain called Fd were obtained. The Fc fragment similarly treated dissociated into two monomer components (Edelman, 1959).

Cohen and Porter (1964) showed that the light chains separated from immunoglobulins of several species exist in some 8 to 10 electrophoretically distinct bands on 8 Molar urea-starch gel electrophoresis at pH 8; three out of the ten move towards the cathode

and the rest move towards the anode. The heavy chain showed a diffuse broad band under these conditions. Studies, (Edelman,etal, 1959, 1962 ; Fleischmann,etal, 1963 ; Hilschmann and Craig, 1965 , and Edelman,etal, 1961) of the chemical and antigenic relationship between Bence jones proteins, myeloma immunoglobulin light chains, led to their division into classes and subclasses.

Five classes of immunoglobulin molecules are known to exist in a human serum, namely IgG, IgA, IgM, IgE and IgD (W.H.O. 1964, 1969 , I.U.I., 1975). Each molecule consists of heavy and light polypeptide chains linked by disulphide bonds and noncovalent bonds. This classification is based on the antigenic and sequence analysis of the constant regions (CH) of the immunoglobulin heavy chains which are class specific, in contrast to the light chain which is type specific. Some of these classes are further subdivided into subclasses, reflecting the variations in their heavy chain amino acid sequence. For example, IgG is divided into four subclasses called IgG_1, IgG_2, IgG_3 and IgG_4 which are characterized by possessing γ_1, γ_2, γ_3 and γ_4 heavy chains respectively (Edelman,etal, 1969.; Schur, 1972.; Natvig,etal, 1973.; Grey,etal, 1964). Two immunoglobulin A subclasses known as IgA_1 and IgA_2 were found (Kunkel and Prendergast, 1966 ; Terry and Fahey, 1966 ; Feinstein and Franklin, 1966 ; Torano and Putnam, 1978 ; Vaerman and Heremans, 1966), and possibly two subclasses of immunoglobulin M have been recognized (Deutsch and Mackenzie, 1964 ; Harboe,etal, 1965 ; Mackenzie,etal, 1969).

It is believed that immunoglobulins belonging to the same class or subclass have identical amino acid sequences in the constant regions of their heavy chains (C_H), whereas in the variable region (V) of each monoclonal immunoglobulin polypeptide chains has a unique amino acid sequence (Dreyer,etal, 1965 ; Putnam, 1959 ; Wang , etal, 1974 ; Gally ,

etal, 1972 and Porter, 1973). Similarities in the amino acid sequences of the variable regions of light and heavy chains of the immunoglobulin molecules, permitted the structural definition of four subgroups for Kappa (V_K), four or five subgroups for Lambda (V_λ) chains and at least three variant regions for heavy chains (V_H) (Kohler,etal, 1970 ; Hilschman,etal, 1972 ; Stoop,etal, 1969).

Each constant regions of the heavy chains of IgM and IgE were divided into four homologous regions called domains designated (C_{H1}, C_{H2}, C_{H3} and C_{H4}) (Watanabe,etal, 1973 ; Putnam,etal, 1973 ; Solomon, 1976) and three homologous domains have been identified for the heavy chains of IgG and IgA (Edelman, etal.,1969 ; Victorliu,etal, 1976 ; Torano and Putnam, 1978). Each domain consists of (107 - 120) amino acid residues and is stabilized by one intrachain disulphide bridge. The variable and constant domains of both heavy and light chains are folded together in compact structures, thus those intrachain disulphide bridges are periodically distributed along heavy and light chains. The domain hypothesis in itself has an important consequence with respect to the evolution of the immunoglobulin genes and enables the evolution of the structural basis of their different functions. It is suggested that there was a primitive precursor gene specifying about 100 amino acid residues (Edelman, etal, 1969).

Since this work is mainly concerned with human IgM Table 1. summarized its main characteristic along with those of the other four major human immunoglobulin classes.

T A B L E 1

Physicochemical properties of the major human immunoglobulin classes.

Class Properties	Short Name	
	IgG	IgA
H-chain class	γ gamma	α alpha
H-chain subclass	$4(\gamma_1, \gamma_2, \gamma_3, \gamma_4)$	α_1, α_2
Light chain	K, λ	K, λ
Nomenation	IgG(K)/IgG(λ)	IgA(K)/IgA(λ)
Molecular formula	$\gamma_2, K_2/\gamma_2, \lambda_2$	$(\alpha_2, K_2)_n/(\alpha_2, \lambda_2)_n$ n=1,2,3..etc.
Molecular Weight	150,000	170,000-500,000
Carbohydrate Content (percent)	about 3	7
Concentration in normal serum (mg/dl)	800-1600	50-250
Per cent of serum Immunoglobulin	75%	15%
Sed-coefficient	6.5 - 7 S	7 S (9,11,13,15,17 etc.)
H-chain variable region subclasses (V_H)	V_{H1} - 4	V_{H1} - 4
L-chain variable region subclasses (VL)	V_{K1} - 4 V_{λ_1} - 5	V_{K1} - 4 V_{λ_1} - 5
Electrophoretic Mobility at pH 8.6 (10^{-5} cm^2 volt^{-1}sec^{-1})	-0.6 to +3.0	+ 1.2 to + 3.6

References: Nisonoff, etal., (1975), Harkness, D.R. (1970), Edelman, etal., (1969) and Cohen (1966).

(contd) TABLE 1

IgM	IgD	IgE
μmu	δ dalta	ε epsilon
Several	-	-
K, λ	K, λ	K, λ
IgM(K)/IgM(λ)	IgD(K)/IgD(λ)	IgE(K)/IgE(λ)
$(\mu_2,K_2)_n/(\mu_2\ 2)_n$ n=1,2,3...etc.	$\delta_2,K_2/\delta_2,\lambda_2$	$\varepsilon_2,K_2/\varepsilon_2,\lambda_2$
850,000 - 10^6(?) and polymers.	180,000	200,000
12	-	10.7
40-120	0.5-3	0.01-0.04
7%	0.2%	0.002%
18-20 S and polymers	7 S	8 S
V_{H1-4}	V_{H1-4}	V_{H1-4}
V_{K1-4}	V_{K1-4}	V_{K1-4}
$V_{\lambda 1-5}$	$V_{\lambda 1-5}$	$V_{\lambda 1-5}$
+ 2.0	-	-

1.2. ABNORMAL GLOBULIN PROTEIN

1.2.A. Multiple myeloma : This disease, myelomatosis, is characterized by a large increase in the number of plasma cells, which are probably of an abnormal type (Rundle,etal, 1950). Associated with the plasma cells proliferation is a moderate to large increase in the globulin fraction of serum.

Von Rustizky (1873) first described the presence of unusual urinary protein and called this pathological condition "multiple myeloma".

Longsworth, Shedlovsky and Mac-Innes (1939) were first to make electrophoretic studies in cases of multiple myeloma. They pointed out the existence of unusual patterns in this condition. Two of their cases exhibited a tall narrow spike representing an abnormal protein component which migrated with the mobility of a beta globulin while the third had a normal pattern.

Shortly after, Kekwick (1940) confirmed the presence of this abnormal serum component in myelomatosis but noted that its mobility was variable, migrating as a gammaglobulin in four cases and as a betaglobulin in a fifth. Later Gutman,etal., (1941) pointed out that while this component had the mobility of a beta or gammaglobulin, it occasionally had an intermediate mobility. They designated this component, which appeared in the electrophoretic pattern as a sharp peak between β and γ globulins, an 'M' component. However, the mobility of this abnormal protein varies from one case to another, migrating with alpha, beta or gamma in some cases, and with intermediate mobility in others (Adams,etal., 1949). Harold and Gerald (1954) attributed the presence of these abnormal electrophoretic patterns which are characterized by tall narrow, sharply defined peaks to the presence of a large amount of a relatively homogeneous abnormal protein and present only in a small

proportion in the family of normal proteins. However, these paraproteins are thought to be produced by a single colony of plasma cells called neoplastic cells (Osserman, 1965 ; Stiehm,etal.,1973; Solomon,1976).

The extensive physico chemical and immuno chemical studies of myeloma proteins carried out in the recent years (Dorner,etal., 1969 ; Grey,etal, 1964 ; Migita,etal., 1963) have established the close relationship of these proteins to normal immunoglobulins and at the same time have helped to elucidate the structure and polypeptide composition of the normal immunoglobulins.

In multiple myeloma, the total globulin level at times reaches values of 10 to 13gm/100 ml of the patient serum (Adams,etal., 1961 ; Briere,etal., 1964). Studies by (Walter,etal, 1974 ; Osserman, 1965 ; Caggiano,etal, 1967) have shown that IgG representing about 55%, IgA about 23% IgD about 1 per cent and IgE which is extremely rear about 0.01% the cases grouped under the symptoms of multiple myeloma. About 20 per cent of myeloma cases were accompanied by the excretion of Bence jones protein. When it is only IgM the condition is called Waldenstrom's macroglobulinemia (Waldenstrom, 1944), and when it is only a heavy chain the condition is termed heavy chain disease (Franklin, etal, 1964 ; Walter,etal, 1974). When multiple myeloma is associated with IgG, it contains a heavy chain belonging to only one of the four subclasses and when it is associated with light chain, it contains either Kappa or Lambda type (Stiehm and Fulginiti, 1973). Bence jones protein may exist in monomer form of molecular weight of about 23,000 (Gally and Edlman, 1964) or in dimeric form of molecular weight of about 45,000 (Gally and Edelman, 1964.; Solomon, 1969 , 1976 ; Van Eijk and Monfoort, 1964). Polymeric forms of higher molecular weight "(tetramer)", of Bence jones proteins have been detected in a patient with multiple myeloma; these forms have been attributed to noncovalent association

of disulphide - linked dimers (Grey etal., 1968 ; Caggiano etal., 1969 ; Parr etal., 1971).

However, the isolation of IgM from normal serum is very difficult because of its low concentration (about 0.01% ml of the patient serum); owing to its high concentration in such sera (macroglobulinemia) the IgM can readily be isolated (Putnam etal., 1967).

1.2.B. CRYOGLOBULINEMIA

Another pathological condition concerning the presence of high level of abnormal globulin proteins which have a tendency to form a precipitate or gels upon cooling was called cryoglobulinemia (Lerner etal, 1947) or cryoimmunoglobulinemia (Grey and Kohler, 1973). This phenomenon was first observed by Wintrob and Bull (1933) in the serum from a patient suffering from myeloma. Cryoglobulin may occur as monoclonal IgG (Meltzer and Franklin, 1966 ; Saha,etal., 1970) or IgA (Wanger etal., 1968 ; Taked,etal., 1974 ; Grey,etal, 1973) or Bence jones protein (Alper, 1966 ; Grey,etal, 1973) or a mixture of IgG with either IgM (Wang etal, 1974 ; Klein,etal., 1972 , Grey,etal, 1973) or IgA (Wanger,etal., 1968 ; Taked,etal, 1974) or both (Klein,etal, 1972 ; Grey and Koher, 1973).

However, Cryoglobulins are not uniquely associated with any one particular heavy chain class or subclass or light chain type.

Grey and Kohler (1973) reported that many normal sera contain a small amount of cryoglobulin (upto 80 µg/ml); whereas in pathological cryoglobulinemia the cryoglobulin may constitute over 70% of the total protein.

Several recent studies indicated that the unusual solubility of monoclonal cryoimmunoglobulins may be due to the formation of antigen - antibody type complex (Grey,etal, 1968 ; Deutsch, 1969). A high

molecular weight form of IgG found in the serum of a patient with multiple myeloma could be dissociated into 7 S IgG molecules by lowering the pH below 4,0. This suggested that the high molecular weight species were formed by a self association of 7 S IgG molecules with non-covalent bonds (Kohwa,etal., 1966 ; Pope,etal., 1974).

However, in a monoclonal cryoglobulinemia which is usually IgG-anti-IgG or IgM-anti-IgG (Lospalluto,etal, 1962 ; Grey,etal., 1968., Deutsch, 1969) the anti-IgG is usually IgM but may also occur as IgA (Whitsed and Penny, 1971 ; Klein,etal., 1972).

Mackenzie etal.,(1968) reported that some of the IgM antiglobulin reacts only with the native human IgG, whereas others react, in addition, with the aggregated human IgG or rabbit IgG or both.

Zinneman,etal.,(1973) reported the biochemical properties of a cryoglobulin. The μ heavy chain of the IgMK (MCE) they studied had a low content of tyrosine and methionine (6 moles of tyrosine and 3 moles of methionine per μ chain). However, the work of Wang,etal., (1974) concerning the relationship of the amino acid differences between cryoglobulin and normal IgM proteins they studied showed that the content of tyrosine in these proteins did not differ appreciably from that in other human immunoglobulins. Instead, the content of serine appears to be lower in the light chains of cryoglobulin than in other myeloma light chains.

1.2.C. RHEUMATOID FACTORS AND COLD AGGLUTININS

Rheumatoid factors provide another example of recognition of one immuoglobulin molecule by another unrelated immunoglobulin molecule. Most often, the rheumatoid factors are IgM immunoglobulins which display reactivity toward restricted portions of IgG molecules (Grey,etal., 1968).

These antiglobulins, termed rheumatoid factors (RF), may also be of the IgG or IgA class (Nisonoff,etal, 1975 ; Litman, 1975).

The presence of RF in the Sera of rheumatoid patients was first recognized by Waaler, E (1940) on the basis of the capacity of the serum to agglutinate sheep red blood cells sensitized with rabbit antibody.

RF generally, although not always, reacts with the Fc portion of IgG. A given RF may react with from one to four of the human IgG subclasses (Mackenzie,etal, 1968 ; Natvig,etal., 1973) reported that, a number of RF's have been found to react with IgG_1, IgG_2 and IgG_4, but not with IgG_3. The IgM antiglobulin can be dissociated from the IgG in the precipitate by gel filtration at low pH (Nisonoff,etal, 1975). Most patients with rheumatoid arthritis posses heterogeneous antiglobulin, although monoclonal factors sometimes appear after prolonged illness (Nisonoff,etal, 1975 ; Litman, 1975). The first unequivocal demonstration of monoclonal IgM with anti-IgG activity appears to be that of Kritzman, etal.,(1961). The serum of their pateint had an extremely high level of IgM with restricted electrophoretic heterogeneity. The protein precipitated strongly with aggregated IgG from pooled human serum; the optional pH for reaction, however, was 5.5 and no precipitation occured at pH 8.0.

However, many monoclonal IgM proteins which have anti-IgG activity are cryoglobulins; that is, they fail to precipitate with IgG at 37°C but do form precipitate at lower temperature (Nisonoff,etal, 1975).

An interesting property of rheumatoid factors is that they are predominantly of the K light chain type (Capra, etal, 1971). These Kappa chains most often have asparagine and serine at position 30 and 31 respectively in the first hyper variable region (VK_1), suggesting

that these residues are involved in defining the anti-gamma globulin binding site of rheumatoid factor (Capra,etal, 1971). Such antiglobulins are found also in other chronic inflammatory disorders. In cases of chronic cold agglutinine disease, these antiglobulins are termed cold agglutinins (Schubothe, 1966 ; Nisonoff,etal, 1975). The agglutinins are nearly always of the IgM classes (Nisonoff,etal, 1975) although examples of IgG and IgA molecules displaying cold agglutinins - like activity exist.

Sometimes serum agglutinins are auto reactive with the patient's erythrocytes (Schubothe, 1966). These antibodies are active at low temperature, but in-active at 37°C, and are directed toward the blood group specificities i, I or Pr antigen (Nisonoff,etal, 1975 ; Williams, etal, 1968 ; Feizi,etal, 1971).

The vast majority of cold agglutinins have Kappa-type light chains (Harboe,etal.,1965). Cold agglutinins can readily be isolated by taking advantage of their thermal properties. They are absorbed by red blood cells or stroma at low temperature (0 – 20°C); after centrifugation to separate the cells from the plasma, the agglutinins can be eluted by raising the temperature to 37°C. The purified agglutinins retain antibody activity and are capable of agglutinating red blood cells at low temperature or lysing the cells in the presence of complement (Williams,etal, 1968 ; Nisonoff,etal, 1975). Studies by Williams,etal, (1968) and Cooper (1968) showed the isolated IgM anti-I cold agglutinins proteins, migrated, upon electrophoresis on cellulose acetate, as a sharp, narrow band with the mobility of a β or γ -globulin. Light chains isolated from the protein gave 3-4 bands.

Studies by Cooper,etal, 1970 ; Wang,etal, 1973 and Gergely,etal,

1973 of the amino acid sequences of the light chains and heavy chains isolated from IgM anti-I cold agglutinins and IgM anti-Pr specificity showed a great similarity in hypervariable regions than in other segments of the polypeptide chain.

IMMUNOGLOBULIN G

1.3.

The immunuglobulin IgG molecule has a molecular weight of about 150,000 and consists of two identical heavy polypeptide chains termed (γ), each having a molecular weight of about 50,000 and two identical light polypeptide chains type (K or L), each having a molecular weight of about 23,000.

These chains are held together by interchain disuphide bonds and non-covalent bonds (Edelman etal, 1969,) and can be separated by the use of sulphydryl reducing agents followed by alkylation and fractionation in a dissociating solvent (Deutsch,etal, 1961 ; Small,etal, 1963 ; Cebra, 1964 ; Cohen and Press, 1964 ; Edelman, 1959 ; Fleischman,etal, 1962, 196 ; Utsumi,etal, 1966 ; Pink and Milstein, 1967).

Two types of disulphide bonds are present in all immunoglobulins. These are the interchain and intrachain disulphide bonds. In all immunoglobulin molecules one interchain disulphide bond links one heavy chain and one light chain, and the rest of the interchain disulphide bonds hold the two equivalent heavy chains together. However, in IgG molecule the number of interheavy chain disulphide bonds varies from one subclass to another. For example IgG_1 and IgG_4 each has two, IgG_2 has four and IgG_3 has probably five interheavy chain disulphide bonds (Frangione,etal, 1968 ; Michaelen,etal, 1973 ; Milstein,etal, 1970 ; Pink and Milstein, 1967.,). The role of the disulphide bonds of immunoglobulins is to stablise the secondary, tertiary and quaternary structures of the immunoglobulin molecules (Isenman,etal, 1975). Immunoglobulin G constitutes about 75% of the immunoglobulin in normal human serum. The percentage composition with respect to subclasses is

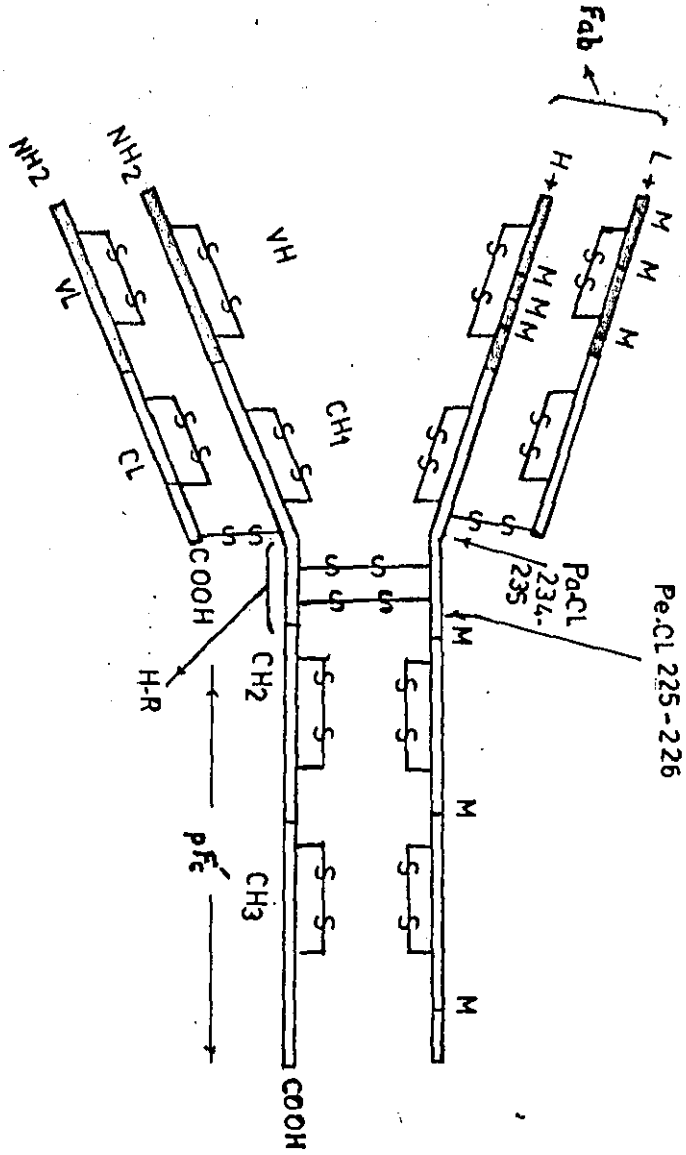


Fig-I- Schematicdigram of the Human IgGI EU(Edelman et al 1969) showing the heavy(H) and the light(L) chains, the variable and the constant regions, the disulphide bonds and the proteolytic fragments Fc and Fab.Papin(Pa-CL) and Pepsin(Pe-CL)cleave the molecule at positions indicated and the positions of methionine(M) residues.H-R(hinge region)

IgG_1 (about 70%), IgG_2 (about 20%), IgG_3 (about 6%), and IgG_4 (about 4%). Since all the immunoglobulins seem to possess the same structural unit, the structure of IgG_1 would be a model for all IgG molecules subclasses Fig. (I-IgG).

1.3.1. ENZYMATIC AND CHEMICAL CLEAVAGE OF IgG

Apart from the Fab and Fc fragments which may be obtained by treatment of IgG with papain at neutral pH or with pepsin at pH 4 - 4.5 results in a fragment known as $(Fab)_2$ fragment together with other subfragments of the heavy chain such as pFc (mol-wt-25,000), the cleavage taking place, on the C-terminal side of heavy - heavy chain disulphide bonds. However, the susceptibility of IgG subclasses to proteolysis by papain or pepsin varies, some of them requiring the presence of cysteine (Putnam, etal., 1962 ; Kanamaru, etal., 1977) and others not. Trypsin cleaves IgG molecules int 3.5S fragments and other small peptides derived from the Fc portion; the 3.5S fragments were indistinguishable in their sedimentation rate and antigenic determinants from the 3.5S fragments obtained from IgG digested with activated papain (Putnam, etal, 1962 ; Edelman etal, 1968 ; Schrohenloher, 1963). In the model of the IgG molecule it was assumed the non-covalent interaction between the two half molecules are confined, to a large extent, the two C-terminal half of the heavy chain, i.e. the Fc moiety (Nisonoff, etal, 1960 ; Charlwood and Utsumi, 1969). Chemical reagents which selectively cleave peptide bonds adjacent to specific amino acids have been helpful in recent years in structural studies of proteins. Cleavage of methionyl bonds with cyanogen bromide has been the method of perference, as practically no side reaction takes place (Gross and Witkop, 1961). The denaturation of globulin at low pH values increases the extent of the reaction.

Cyanogen bromide in 0.05M HCl (Lahav,etal., 1965) or in 0.3 - 0.6M HCl (Cahnmann,etal, 1966, 1965) cleaves the human and rabbit IgG molecules at the methionine residues near the hinge region, about half of the methionyl peptide bonds being broken. A major fragment of 5S with molecular weight of about 95,000 can be recovered. It has a higher mobility than the native molecule on starch gel electrophoresis at pH 3.5. The 5S fragment has antigenic properties similar to the native molecule denoted by $(Fab)_2$, and strongly aggregated at neutral pH in the absence of dissociating solvent, and can be split into 3.3S Fab fragment by sulphydryl reducing agents (Cahnmann,etal., 1966 ; Lahav, etal., 1966). It has been shown that on treatment of rabbit A_{12} IgG with cyanogen bromide in 0.3M HCl it cleaves to a 5S fragment, whereas similar treatment of rabbit A_{11} IgG produced an additional cleavage at methionine residue adjacent to the cysteine participating in the inter-heavy chain disulphide bond to yeild 3.5S fragment (Kindt,etal., 1970). The additional methionine residue participated in the Fd - Fc region (i.e. at position 215) of the γ heavy chain of rabbit A_{11} IgG was shown to be N-terminal of the Fc fragment obtained on papain digestion (Prahl, etal., 1969), and it is replaced by a threonine residue in allotype A_{12} (Prahl,etal, 1968, 1969). However, reaction of IgG or its heavy and light chains with cyanogen bromide in 70% formic acid results in the cleavage of all their methionyl peptide bonds (Porter, 1967 ; Waxdal, etal., 1968, Bourgous,etal., 1970 ; Tracey,etal., 1976 ; Poljak,etal., 1977 ; Piggot and Press, 1967 ; Sjoguist, 1966). However, studies on the primary structure of 101 Bence jones proteins of both type Kappa and Lambda (Quattrocchi,etal, 1969), reveal that no two have identical amino acid compositions. Theoretically, the number of peptide bonds splitting in the protein by cyanogen bromide in 70% formic acid is equal to the sum of methionine residues present in the molecule. The heavy chain of a human pathological IgG (Daw) (Piggot and Press,

1967) contains four methionine residues and thus should be split by CNBr in 70% formic acid into five fragments, while its light chain (type L) contains no methionine and is therefore not cleaved by cyanogen bromide. In human IgG_1 (EU) (Edelman,etal, 1969), its heavy chain contains six methionine residues and its light chain (type K) contains three methionine residues, thus the whole molecule should be split by cyanogen bromide in 70% formic acid into eleven fragments.

1.3.2. THE PRIMARY STRUCTURE OF HUMAN IMMUNOGLOBULIN G

The primary structure of human immunoglobulins have mostly been studied using myeloma and Bence jones proteins because these proteins are easily obtained in a homogeneous form, and in large quantities. Early detailed studies of the amino acid sequences of the light chain (K or L) were reported by Hilschman and Craig (1965), Putnam etal.,(1966, 1967). However, Edelman etal.,(1969) published the entire covalent structure of an IgG_1 (EU) immunoglobulin. The heavy chain of IgG_1 (Edelman etal., 1969) consists of four homologous domains, three comprising the constant region (CH) designated (C_{H1}, C_{H2} and C_{H3}) and one comprising the variable region termed (V_H), in contrast to the heavy chain, the light chain has two domains termed (V_L) for the variable region and (C_L) for the constant region. These domains each consist of (109 - 118) amino acid residues stabilized by one intrachain disulphide bridge. These intrachain disulphide bridges are thus periodically distributed along heavy and light chains at positions 22 - 96, 144 -200, 261 - 321, 367 - 425 for heavy chains and at positions 23 -88, 134 -194 for light chains. The interheavy disulphide bonds located at positions 226 and 229, while the heavy-light chain bond connects residue 220 on the heavy chain with the light chain at position 214. The methionine residues of γ_1 chain are located at positions 48, 54, 81, 252, 357 and 428, while on the light chain (Edelman, 1971) are located at positions 4, 48 and 97. The heavy (γ_1) chain of IgG_1 (EU)(Edelman etal, 1969) contains 446 amino acid residues and its light chain (type K) contains 214 amino acid residues.

IMMUNOGLOBULIN M

1.4.1. INTRODUCTION: PHYSICOCHEMICAL PROPERTIES

An antibody molecule with a molecular weight of about 10^6 and a sedimentation coefficient of about 19-20S was first recognized in the sera of cattle and horses by Heidleberger and Pederson (1937). Later on in 1944 Jan Waldenstrom reported that in the serum of a patient with myelomatosis a high level of an abnormal globulin protein (S_{20} = 19 - 20S, molecular weight of about 10^6), with free boundary electrophoretic mobility corresponding to γ - β globulin fraction could be detected. This protein tended to aggregate in serum at temperature less than 7°C and on dialysis or dilution with pure distilled water but redissolved in neutral saline, at room temperature.

This protein is now designated immunoglobulin M (IgM) (often referred to as 19S IgM), and the pathological condition is known as Waldenstrom's macroglobulinemia.

IgM constitutes about 7 per cent of the immunoglobulin in normal human serum. However, in spite of its low concentration in normal sera, IgM plays an important role in various autoimmune diseases. Low molecular weight of IgM molecules with sedimentation coefficients of 7S and 11S were recognized in sera from patients with a variety of blood disease such as macroglobulinemia, multiple myeloma, and systemic erythematosus (Petermann, et al, 1941 ; Stobo and Tomasi, 1967 ; Soloman, 1969 ; Parr etal., 1974).

The human immunoglobulin M (22S macroglobulin) molecule and rabbit immunoglobulin M (29S macroglobulin) molecules were possibly dimers of 19S IgM molecules linked together by non-covalent bonds (Suzuki and

Deutsch, 1966 ; Uki, etal.,1974). Larger IgM molecules with a sedimentation coefficient rate of Ca. 36 S and 44 S. or higher values up to 150 S (Schultze etal, 1962) are known. They are also assumed to be polymeric forms of the 19 S IgM molecules joined together by non-covalent bonds. The IgM proteins most often are euglobulins and can be purified by a variety of techniques, the most common procedure employing an initial precipitation step at low ionic strength. This euglobulin usually contains from 1 to 5% of γG globulin (Deutsch and Morton, 1958), which may readily be removed gel filtration.

Significant differences in the molecular weights of the IgM globulins have been observed. For example; Filitti-Wurmer etal (1968, 1970) reported that the molecular weight of IgM molecules obtained from individual macroglobulinemia sera are 620,000 - 1,180,000. However, most of the recent studies have yield molecular weights between 850,000 and 10^6 (S_{20} = 18 - 20S) for human Waldenstrom macroglobulins (Miller and Metzger, 1965 ; Suzuki etal., 1967 ; Johnson and Miller, 1970 ; Putnam etal., 1973 ; Zikan etal, 1973 ; Watanabe etal., 1973).

Putnam (1959) observed that various abnormal macroglobulins obtained from individual patients have electrophoretic mobility in the range of γ and α_2 globulin fraction in vernal buffer pH 8.6, ionic strength 0.1 Molar estimated by free boundaries electrophoresis, and that they show various solubilities in the pH range 3.5 - 8.6. A similar conclusion was obtained by Deutsch and Morton (1958) who reported that various macroglobulins showed marked differences in their electrophoretic mobility at pH 8.6, some of them migrated as α_2 globulins, some as γ_3 globulins and some have an intermediate mobility. The differences in the electrophoretic mobility may be related to the differences in the carbohydrate contents and the isoelectric point of those macroglobulins (Virella

etal, 1976). Studies of human IgM have been greatly facilitated by the occurence of large amounts of protein in sera of patient with Waldenstrom's macroglobulinemia; owing to its high concentration in such sera the IgM can readily be isolated (Putnam etal, 1967).

However, the isolation of pure IgM from normal serum is a rather formidable task because of its low concentration in the blood.

1.4.2. REDUCTION OF IgM TO IgM_S, H AND L CHAINS.

Under certain condition, the native (intact) IgM (19S) molecule may be dissociated with sulphydryl reagents to yield the monomer 7 S subunits (IgM_S) only or its individual heavy and light chains. Deutsch and Morton (1957) were able to reduce the 19 S (IgM) molecule to 7 S subunits (IgM_S) using 0.1M β-Mercaptoethanol. Extensive characterization of the physical and chemical properties of a single IgM antibody molecule was carried out by Miller and Metzger (1956). They showed that on reduction of the 19 S (IgM) molecule with the mercaptoethanol or cysteine in 6 molar Gu-HCl. 190-SH groups were released per mole of IgM (19 S), while in aqueous solution, only 50-SH groups were released per mole of IgM (19S) and that 20-SH groups out of these 50-SH groups were heavy-light chain, (H-L) interchain disulphide bridges. They also reported that on reduction of IgM molecules with cysteine, both inter and intra subunits disulphide bonds were cleaved. The monomer 7 S subunits (IgM_S) were stable in propionic acid, but tended to aggregate on incubation in neutral solution. Each IgM_S subunit may further be dissociated under appropriate conditions into its heavy and light chains.

Miller and Metzger (1965b) proposed that the 19 S (IgM) molecule consists of five identical monomer subunits (IgM_S) disulphide bonded, perhaps in a circular structure, and that each IgM_S subunit was composed of two identical μ heavy chains and two identical light chains linked together by non-covalent bonds and disulphide bonds in a symmetrical tetrapolypeptide chain structure like IgG. (Fig. 2).

In addition to the light chains and the heavy chains, a third polypeptide chain named 'J' chain is present in polymeric IgA and IgM

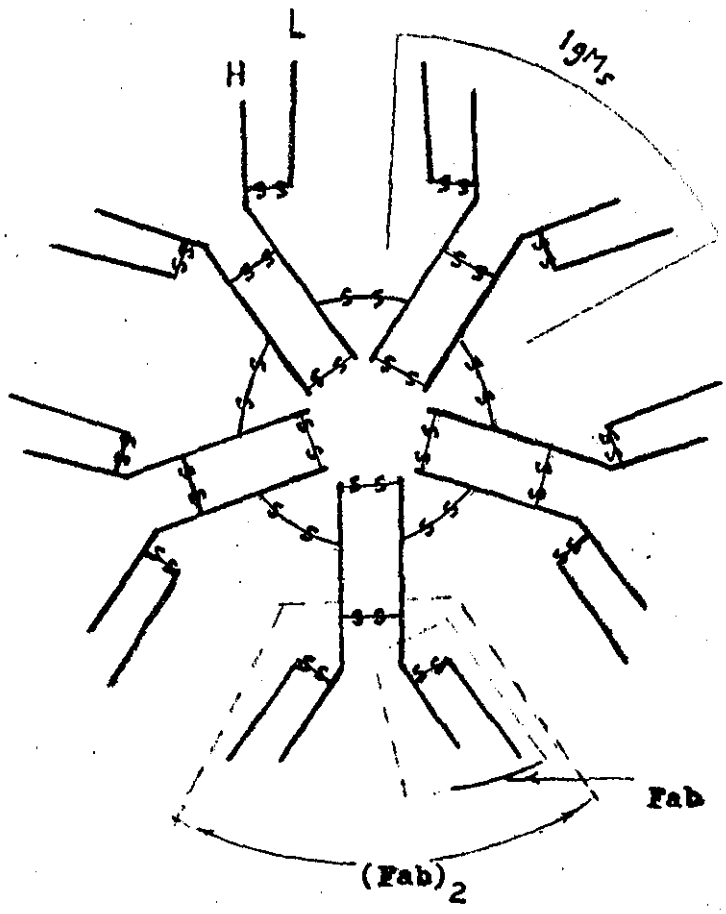


Fig-2-

The proposed model for pentameric IgM showing the (IgMs) subunit, the heavy(H) and the light(L)chain as well as the Fab,(Fab)2 and the interchain disulphide bonds.

molecules, but not in monomeric IgM and IgA (Mestecky etal, 1971 ; Halpern and Koshland,1970). 'J' chain has a molecular weight of about 15,000 (Ricardo,etal, 1973 ; Wilde,etal, 1973 ; Schrohenloher,etal, 1973). It has been shown that 'J' chain is associated with the Fc-region of the heavy chain (Inman etal, 1974 ; Mestecky etal, 1974 ; Lebreton etal, 1976). Based on the molecular weight, amino acid content and immunological properties it was shown that the 'J' chains associated with IgM molecules were indistinguishable from the 'J' chains associated with IgA molecules. (Morrison etal, 1972 ; Mestecky, 1971).

Recombination experiments suggest that the 'J' chain may play a role in the structural polymerization of the polymeric immunoglobulin molecules and it is thought to be attached by disulphide bonds, most probably, to the Fc portions of these immunoglobulins (Mestecky etal, 1972 , Dellacorte etal, 1973 ; Parkhouse, 1973 ; Chapuis etal, 1973). Reduction of IgM molecules or their subunits (IgM_S) with sulphydryl reagents in denaturation solvents followed by alkylation, yielded heavy and light chains. Suzuki and Deutsch (1967) suggested that the 19 S IgM molecule might have five 8 S subunits each of which consisted of two heavy chain and three light chains, two of which could be readily removed to give 7 S subunits. Their inference has been obtained by calculating multiples of the low molecular weight light chains which have been reproducible compared to the variable molecular weights obtained for heavy chains. Unfortunately their explanation for the mechanism of the dissociation of the IgM molecule is considered to be hypothetical rather than proven. The molecular weight of the resulting monomer 7 S subunits (IgM_S) has been reported to be within 150,000 - 200,000 (Miller and Metzger, 1965 ; Suzuki and Deutsch, 1967 ;

Feinstein etal.,1969; Johnson and Miller,1970 ;Putnam etal.,1973 ; Zikan and Bennett,1971). Filitti-Wurmser etal(1970) obtained IgM_s subunits molecular weight of 190,000, 164,000 and 144,000 from three different Waldenstrom macroglobulins. Investigation on the number of interchain disulphide bonds of the IgM molecule showed that one interchain disulphide bond links one heavy chain and one light chain. However, the number of inter heavy chain disulphide bonds and inter subunit disulphide bonds has been variously erported. For example (Miller and Metzger,1965 ;Chaplin etal.,1965) reported one or two inter subunit disulphide bond(s), but Nisonoff etal.,1975 ;Putnam etal.,1973 ;Morris and Inman,1968; Mukkur and Inman,1970, reported one inter subunit disulphide bond and two intra subunit disulphide bonds.

The arrangement of the inter chain disulphide bridges described by Bealeand Feinstein,1969 ;Beale and Buttress,1969,1972. showed that each light chain linked with one heavy chain by a single interchain disulphide bond formed in the Fabμ (Fdμ) region and that the two inter heavy chain disulphide bonds were formed in the Fcμ region, one near to the C-terminus of the μ chain and the other involved in the intersubunit disulphide bond located in the Cμ3 domain (i.e.near to the N-terminal of the Fcμ region) obtained on the hot trypsin digestion.

Zikan and Bennett(1971) showed that oxidation and sulfitolysis of the IgM molecule followed by gel filtration in 5M Gu-HCl, resulted in a high yields of heavy and light chains. Each heavy chain contained five intrachain disulphide bonds and each light chain contained two intrachain disulphide bonds and was linked with the heavy chain by a single interchain disulphide bridge. The 7S subunit contained Ca-18 disulphide bonds.

The μ chainsdiffer from the γchains in several respects such as

antigenic properties (Cohen, 1963 ; Miller and Metzger, 1965 ; Chaplin, etal, 1965 ; Metzger, 1970) and carbohydrate content (Chaplin,etal, 1965 ; Beale and Buttress, 1969, 1972 ; Putnam,etal, 1967).

The molecular weight of the μ chain has been variously reported to be 67,000 (Suzuki and Deutsch, 1967 ; Beale and Buttress, 1972) 70,000 (Miller and Metzger, 1965 ; Metzger, 1970 ; Johnson and Miller, 1970) 75,000 (Bennett, 1969) 80,000 (Virella,etal, 1976).

The molecular weights ranging from 49,000 to 72,000 have been determined on μ chains from three Waldenstrom macroglobulins (Filitti-Wurmser,etal.,1970). In contrast to the molecular weight of the heavy chain, the light chains (K or L) type associated with with IgM molecules are the same size (22,000 - 23,000) and similar to the other light chains (K or L) associated with the other immunoglobulins classes and subclasses (Metzger, 1970). However, on the basis of amino acid content the light chains (L) type have no methionine residues, whereas the K type have 1 - 3 methiones (Cohen,etal, 1967 ; Van Eijk and Monfoort, 1965). The heavy chain of human IgM contains five oligosacchloride sites, one located in the Fab_{μ} ($Fd\mu$) region, one in the hinge region and three in the Fc region of the molecule. It has been suggested that they play an important role in biological and electrophoretic properties of the IgM (Beale and Feinstein, 1969 ; Beale and Buttress, 1972 ; Hurst,etal, 1973 ; Shimizu,etal, 1971).

DEGRADATION OF IMMUNOGLOBULIN M (IgM) WITH PROTEOLYTIC ENZYMES

1.4.3.

Early reports by Petermann and Pappenheimer (1941) suggest that macroglobulins might be cleaved by pepsin into smaller molecular weight fragments. Porter, R. R. (1959) reported that three fragments of approximately the same size formed by papain digestion of rabbit IgG. IgM fragments such as Fab, Fc and $(Fab)_2$ may be obtained through various enzymatic cleavages, similar as in IgG.

Incubation of rabbit or human IgM (19 S) with papain in the presence of substrate cysteine yields Fab_μ fragments (of molecular weight 45,000) of slow cathodic mobility in starch gel electrophoresis and an Fc fragment of faster anodic mobility (Mihaesco and Seligmann, 1968; Ungar-Waron and Michael, 1967 ; Putnam, etal, 1966).

Short term incubation of IgM molecules at room temperature with papain in the absence of cysteine yields a Fab_μ fragment (S_{20} = 3.5 S) and an $(Fc\mu)_5$ fragment (S_{20} = 10.6 S, molecular weight of about 320,000) which upon reduction and alkylation produced 3.2 S fragments (Onoue, etal, 1968). Suzuki (1969) showed that treatment of a 19 S Waldenstrom macroglobulin (IgM) molecule with pepsin and papain produced three types of fragments. The heaviest fragments (12 S) had a molecular weight of 539,000 and consisted of four different types of subunits (S_{20} = 7 S, 4.5 S, 3.5 S and 2.8 S). The intermediate 6 S fragment had a molecular weight of 127,000 and consisted of the complete 'L' chain and a part of the heavy chain. Reduction and alkylation of these fragments (6 S) produced a monomer subunit (4 S). The lightest fragments (3.5 S) had a molecular weight of 55,000 and consisted of a light chain and a Fd-like fragment. They also reported that degition

of their 8 S subunits (IgMs) in an identical manner yielded 6 S, 4 S and 3.5 S fragments. The 6 S fragment resembled the 6 S fragment obtained from IgM (19 S) globulins in physicochemical and immunological properties. The 4 S fragments (of molecular weight 72,000) were indistinguishable from the reduced and alkylated products of the 6 S fragments and are considered to be a monomers of the 6 S fragments. The 3.5 S fragments were found to be analogous to the lightest (3.5 S) fragment obtained from 19 S (IgM). Dorrington and Mihaesco 1970 reported that papain and pepsin split D IgM (S_{20} = 19 S, molecular weight 890,000)into three pricipal regions. The first region corresponding to the papain Fab_{μ} (of molecular weight 45,000) and pepsin Fab'_{μ} (of molecular weight 48,000) consists of light chain disulphide bonded to the N-terminal portion of the u chain (Fdμ). The second region, destroyed by papain but not by pepsin under mild conditions, is present in the peptic $(Fab\mu'')_2$ (of molecular weight 119,000) fragment and corresponds to the hinge region of the μ chain.

The third region is represented by a large papain $(Fc\mu)_5$ fragment of molecular weight 315,000) which on reduction is 6 M Gu-HCl forms a fragment of molecular weight 31,500.

From the above experiment, it seems that both of the pepsin and papain are capable of breaking the IgM molecule at different sites on the μ chain at the hinge region which is lacking the interheavy chain disulphide bond to produce Fab'_{μ} and Fab_{μ} repectivwly and that the Fab'_{μ} of a molecular weight slightly higher than the molecular weight of Fab_{μ}.

However, digestion of a Waldenstrom macroglobulin IgM (19 S) with chymotrypsin at 37°C yields a Fab_{μ} fragment (of molecular weight 70,000),

an $(Fab_{\mu})_2$ fragment(of molecular weight 135,000) and another peptide derived from Fc region (Chen,etal, 1969).

Long term incubation of 19 S IgM with trypsin at 37°C (pH 8.0) yields Fab_{μ} fragment (S_{20} = 4.4 S, molecular weight of about 47,000) an $(Fab_{\mu})_2$ fragment (S_{20} = 6.14 S, molecular weight 114,000) and only small peptides from the Fc region (Miller and Metzger, 1966). When the immunoglobulin M (IgM) molecule is incubated with trypsin at higher temperature (60 - 65°C), it yields Fab_{μ} fragments (S_{20} = 3.5 S, molecular weight 41,000) an $(Fc_{\mu})_5$ fragment (S_{20} = 10.8 S, molecular weight 342,000) and small peptides related to u chains. Mild reduction of this $(Fc_{\mu})_5$ dissociates it into 3.4 S Fc fragment (of molecular weight 67,300) (Plaut,etal, 1970).

Cleavage of the IgM molecule with trypsin is temperature dependant such that at 70°C and for a period of 30 minutes trypsin denaturates the IgM molecule, while at 56°C and for the same period (i.e. 30 minutes) trypsin cleaves the IgM molecule into Fab_{μ} (S_{20} = 3.7 S, molecular weight 41,000), a $(Fab_{\mu})_2$ fragments (S_{20} = 6 S, molecular weight 95,000), and a $(Fc_{\mu})_5$ fragments (S_{20} = 10.8 S, molecular weight 342,000) (Plaut,etal, 1970). The $(Fc_{\mu})_5$ derived from a L type IgM molecule when treated with trypsin at 65°C for a period of eight minutes yielded an additional fragment with electrophoretic mobility intermediate between that of $(Fc_{\mu})_5$ and Fab_{μ} fragment. This fragment related to the light chain immunologically, has a molecular weight of about 20,000, and has been designated as intermediate light chain (Int-L) (Plaut,etal, 1972).

Shimizu,etal.,(1974) reported that by partial denaturation of IgM molecules in 4-5 molar urea at 25°C for 4-24 hours followed by digestion

with trypsin at 25°C for two hours or longer, Fab_{μ} and $(Fc_{\mu})_5$ fragment were obtained. With a higher concentration of urea (6-8 molar) no precipitation lines were detected using immunoelectrophoresis in agar gel, suggesting that the IgM molecule or its fragments were either completely digested to peptides or denaturated and lost antigenicity. With lower concentration of urea (i.e. less than 2 molar) or shorter times of pre-incubation or digestion the cleavage of IgM to form Fabu and $(Fc\mu)_5$ was not complete. No $(Fc_{\mu})_5$ was obtained with less than 2 molar urea, suggesting that the conformation of IgM molecule at high temperature and in certain concentrations of urea may be similar. In urea or at higher temperature the middle part or $C\mu_2$ region (i.e. hinge region) of the IgM molecule changes to a form more susceptible to trypsin digestion.

Reduction of pentameric Fc fragments by reaction with sulphydryl compounds results in dissociation into smaller fragments. The molecular weight of the resulting monomer Fc fragments has been reported as 67,000 (Plaut and Tomassi, 1970), 60,000 (Hester etal., 1973), 43,000 (Chen, etal., 1974), and between 31,000 - 35,000 (Hester,etal., 1973, 1975, Plaut,etal., 1972 ; Conradie and Visser, 1973).

THE PRIMARY STRUCTURE OF THE IgM MOLECULE

1.4.4.

The primary structures of human immunoglobulins M (IgM) have mostly been obtained using Waldenstrom macroglobulins and myeloma proteins because these proteins are easily obtained in a homogeneous form, and in large quantities. Studies on the amino acid composition of the three immunoglobulins (IgM, IgA and IgG) (Heimburger, etal., 1964 ; Putnam,etal., 1967) revealed that no two of them were identical. Similar studies of the peptide maps of 25 Waldenstrom macroglobulins (Franklin and Frangione, 1968) also indicated that none of them appeared to have identical amino acid composition. The evidence from peptide maps of the μ chains of these proteins suggests the existence of both a variable and a common sequence, and that the variation may be limited to a relatively small portion of the chain. Bennett (1969) studied the molecular weight and the peptide maps of four heavy chains from individual IgM proteins (Kappa type) and indicated a strong similarity in their amino acid composition.

However, from this comparative study, it appears certain that the heavy chains of Waldenstrom macroglobulin will differ individually in primary structure.

The complete amino acid sequence of the μ chains of two types K human IgM (Ou and Gal) molecules were established by Watanabe,etal, 1973 ; and Putnam,etal., 1973.

Watanabe,etal.,(1973) established the complete amino acid sequence of Gal heavy (μ) chain and showed that it has five homologous domains.

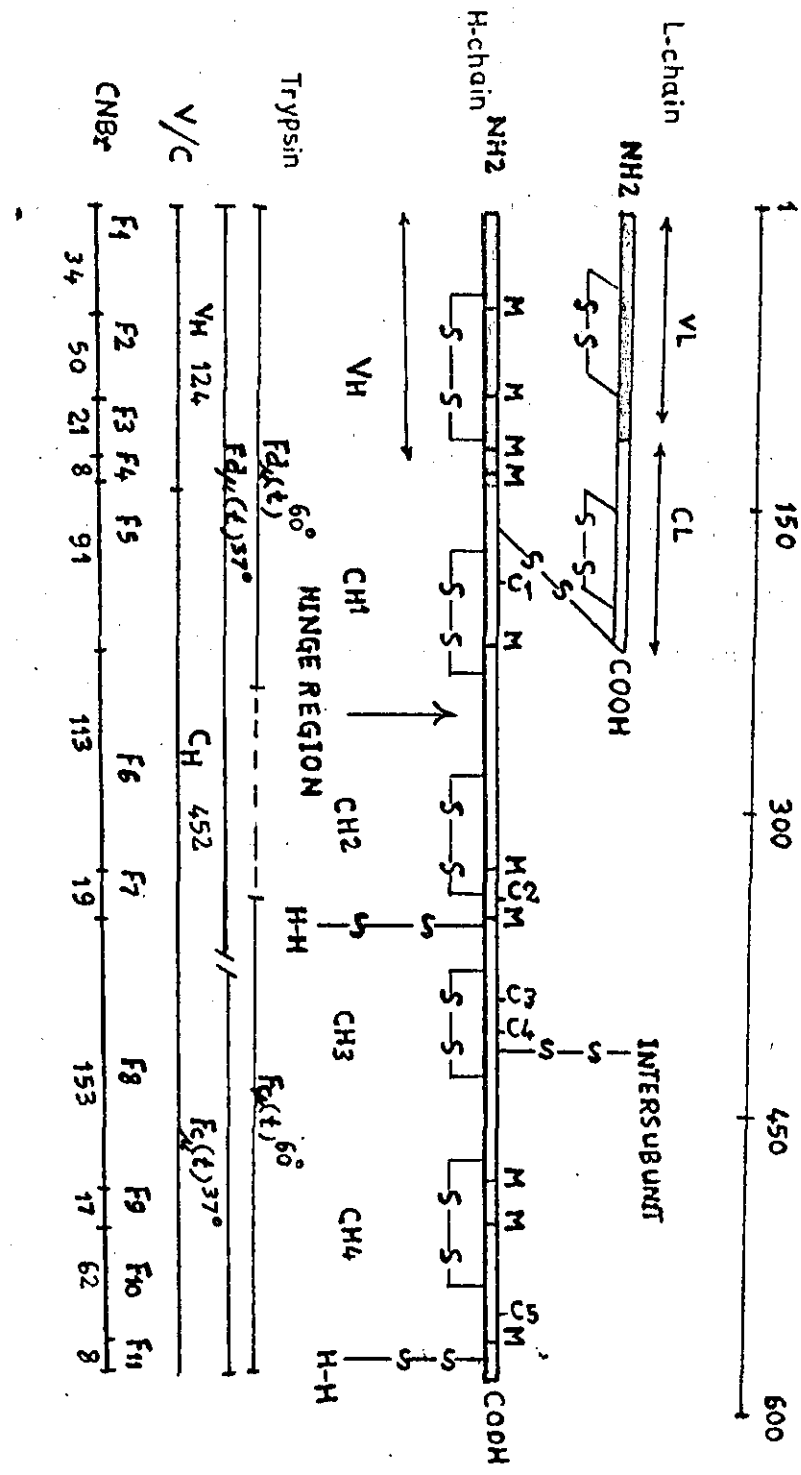


Fig-3- Schematic structure of the mu heavy chain and the K light chain of the IgM OU (Putnam et al,1973) showing: I- the inter and intrachain disulphide bridges, 2/ the two homology regions of the L chain (V_K and C_K) and, 3/the five homology regions of the mu heavy chain(V_H and $C_{H1}-C_{H4}$), the positions of the oligosaccharides(C1-C5) and also the positions of the methionine(M) residues, the positions cleavage by CNBr (F1-F11) and trypsin at 37 and 65C , and the position of the hinge region.

One in the VH region comprises 119 amino acid residues and the other four domains are designated C_{H1}, C_{H2}, C_{H3} and C_{H4}. The μ chain (Gal) is composed of 571 amino acid residues and the methlonine residues occur at position 34, 85, 197, 312, 331, 484, 501 and 563. The interheavy light chain disulphide bond is formed at position 133 (Fd_{μ}) region, the interheavy - heavy chain disulphide bonds are located at position 331 and 570 of the Fc region. While the inter subunit disulphide bond is located at position 408 of the Fc region. In 1973 Putnam etal also published the complete sequence of Ou heavy chain and showed that it has five homology regions or domains, each containing some 110 to 120 amino acid residues with one intrachain disulphide loop of about 60 residues. (Fig. 3). Two of the domains (VH and C_{H1}) are in the Fd region, one (C_{H2}) is in the hinge region, and two (C_{H3} and C_{H4}) are in the Fc region. The C_{H4} domains is sometimes called the extra domain.

The sequence of the μ chain of protein Ou comprises 578 amino acid residues. The V_H region comprises 1-124 amino acid residues and the C_H region comprises 125-578 amino acid residues.

The methionine residues are located at positions 34, 84, 105, 113, 204, 318, 336, 489, 506 and 568. The inter subunit disulphide bond is at position 414 of the Fc region. While the inter heavy - heavy chain disulphide bond formed in positions 337 and 575. The inter heavy - light chain disulphide bond formed at position 140 (Fd_{μ} region) and the intraheavy chain disulphide bonds linked position 22-97, 153-212, 259-320, 367-426, 476-536. A segment rich in proline, around residues 230 in Ou protein is considered to be the hinge region although it lacks the characteristic interchain disulphide bond of that region in

IgG (Paul,etal, 1971). The carbohydrate sites in Ou heavy chain (μ) and Gal heavy chain (μ) are located at position 170, 332, 359, 402, and 563 for Ou molecule and at position 163, 328, 389, 369 and 558 for the Gal IgM molecule (Watanabe,etal, 1973 ; Putnam,etal, 1973). These two proteins had different V_H sequences, but very similar C_H sequences. In terms of size and amino acid composition, Ou heavy chain had a larger size and more methionine residues than the μ heavy chain of Gal IgM and most of the differences in the methionine residues are located in the V_H regions of the two proteins. Proteins Ou and Gal both have K chains belonging to the V_{K1} subgroup. The V_H regions of the two proteins are in the V_{H11} and V_{H111} subgroups, respectively.

The molecular weight of protein Ou, Calculated from the known amino acid sequence and estimated carbohydrate content, is 956,000 or 971,000 including the 'J' chain (Putnam,etal, 1973). The molecular weight of the μ chain of protein Ou is 72,000 daltons and its 7 S subunit had a molecular weight of 190,000 daltons.

REASSOCIATION OF IgMs SUBUNITS AND 'J' CHAIN

1.4.5.

If IgM is reduced to IgMs without subsequent alkylation, and reducing agent is removed by dialysis or gel filtration, reassembly occurs to varying degrees and polymers with sedimentation coefficient of 10 to 19 Sas well as larger aggregates have been reported.

Kownatzki (1973) reduced a human 19-S IgM and removed the 'J' chain by gel filtration in dithiothreitol solution. The 7 S subunits polymerised in the absence of 'J' chain but there was appreciable formation of material sedimenting faster than 19 S. The results suggested that 'J' chain might have some controlling influence on polymerisation. However, Feinstein (1973) reduced and reversibly blocked a pathological 19 S IgM with dithionitrobenzoic acid. After removing 'J' chain by gel filtration and unblocking the subunits it was found that they polymerised equally well with or without the presence of 'J' chain. Della Cort and Parkhouse (1973) have found that stoichoimetric amounts of 'J' chain and a disulphide interchange enzyme are required to effect intracellular polymerization. It therefore appears that 'J' chain might causes a conformational change and/or rearrangement of bridges which allows polymerization. The results published by Beale (1974) after reduction and alkylation of Porcine 12 S Fc pentamer that had no 'J' chain could be reduced and repolymerized as efficiently as 19 S IgM. This indicated that, the 'J' chain could not be an essential requirement for non-covalent binding between subunits.

Reassembled IgM isolated in the presence of IM propionic acid did not dissociate in 5M guanidium hydrochloride (Ricardo, etal., 1974). These observations suggested that there was some weak non-covalent

interplay in the reassembled IgM was neglected by gel filtration in acid. However, reassociation of IgM from IgM_S or $(Fc\mu)_5$ from monomer Fc_μ is not always affected by the presence or the absence of 'J' chain or covalent or non-covalent bonds.

More, recently, Eskeland, (1977), Paus and Eskeland (1978) showed that repolymerisation of IgM from IgM_S and $(Fc\mu)_5$ from monomer Fc_μ occur in the presence of Zn or Zn and Cu ions. He suggested that the metal ions and the protein sulphydryl groups are involved in intramolecular bridges at places where the disulphide bridges are located.

Smyth and Utsumi, 1967 ; Payne, 1969 showed that the carbohydrate have a controlling function in the proteolysis of IgG. The hinge peptide released by papain digestion contained twice as much carbohydrate in bovine than in human or rabbit IgG and the same was true for the peptide $(Fab)_2$ fragment of their proteins; and the rate of the fragmentation of bovine material into the Fab and Fc was slower than that of the human and rabbit protein (Payne, 1969). Awdeh, etal., (1970) demonstrated that electrophoretic heterogeneity developed in newly synethesized immunoglobulin during incubation with serum and suggested that this arose either out of charged carbohydrate groups as a result of deamidation of amide groups in the protein.

Minta, etal., (1972) reported that the electrophoretic heterogeneity in the Fc fragments prepared from human IgG_1 by plasmin digestion, was due to their differences in carbohydrate contents. They found no differences in the ability of these fragments to fix complement or to precipitate cutaneous anaphylaxis in guinea-pigs. The differences in their hexose and sialic acid might interact with the borate ions in the gel buffer to produce charge differences between the individual components

which results in electrophoretic heterogeneity. Shimizu,etal.,(1971) had indicated that five oligosaccharides designated (C_1 - C_5) were located at homologous sites in the Ou and Di μ heavy chains. Three of these (C_1 - C_3) are of complex carbohydrate composition; and contains fucose, mannose, galactose, glucosamine, and sialic acid. The two "simple" oligosaccharide (C_4 and C_5) contain only mannose and glucosamine. The first carbohydrate C_1 is in the Fd region of the μ chain and is attached to Asx - 170. C_2 is in the hinge region, and C_3, C_4 and C_5 are all on the Fc_μ fragment.

Virella,etal.,(1976) have shown that the polymeric paraprotein IgM K type heterogeneity seen on cellulose acetate electrophoresis (pH 8.6) is associated with differences in carbohydrate concentration.

The polymerizing role of carbohydrate groups in glycoproteins has been demonstrated (Kaverzneva, etal., 1975 ; Klein, etal., 1975) on the example of the reassembly of human pentameric immunoglobulin M (IgM) from the free chains and subunits obtained after reduction splitting of IgM. According to these authors the time course of self-assembly is shorter in the presence of carbohydrate (between 15 minutes to 24 hours), while in the absence of carbohydrate, the self - assembly goes as far as the re-association of the chains to half IgM_s and stop almost completely in the stage of subunits of immunoglobulin (IgM_s).

VALENCE OF IgM

1.4.6.

The IgG classes have two binding sites per molecule shared between the heavy and the light chain, the IgM molecule (19 S) has ten binding sites, two per subunit (IgM_s), two per $(Fab_\mu)_2$ and one per Fab_μ, but only five of these ten binding sites are sometimes available (Adberg,etal., 1972 ; Chavin,etal., 1969 ; Ashman and Metzger, 1969 ; Goldstein, 1975). The Fc region of IgM molecule is responsible for several biological functions of this molecule. Both the sites that bind complement and sites by which cytophilic antibody attaches to cells are located in this region (Plaut,etal., 1972 ; Conradie and Bubb, 1977). The relative binding capacity increased with increasing state of aggregation of IgM (Nisonoff,etal., 1975), the 19 S IgM molecule was 15 times as effective, per unit weight, as the IgM_s. A 35 S aggregate of IgM had approximately four times as much binding capacity per weight as 19 S IgM; and the IgM_s subunit was about as effective as IgG. A similar conclusion for the $Fc_{\mu 5}$ fragment prepared by hot trypsin digestion of IgM (19 S) type L light chain has been reported by Plaut, etal.,(1972). This $(Fc_\mu)_5$ had the complement fixation 31 times as effectively as intact IgM on a weight basis, and 19 times on a molecular basis, where as the $(Fc_\mu)_5$ obtained from two individual IgM type K light chain under the same condition were found to be 7 and 11 as effective in complement fixation as the parent molecule on the molar basis. The reduced monomers Fc_μ of two different $(Fc_\mu)_5$ (i.e. from K and L IgM type) preparation have a complement fixing capacity identical (on a weight basis) to that of unreduced preparations.

THE THREE DIMENSIONAL STRUCTURE OF IMMUNOGLOBULINS

1.4.7.

IgG_1 immunoglobulin, its Fab and Fc fragments, as well as Bence Jones proteins, IgM and IgA were studied by X-ray crystallography, electron microscope, circular dichroism and optical rotatory dispersion. However, the structural view of an intact IgG molecule (Valentine etal., 1967 ; Svehag,etal., 1969 ; Feinstein and Rowe, 1965) was pictured like a Y-shape (or possibly a T-shape), whose arms were the Fab fragments and whose axis the Fc fragment (Fig. I.IgG).

These three fragments are joined together in a flexible region called "hinge region", the average dimensions of each arm (i.e. Fab fragment) were 65 x 35A°, and 50 x 40A° for the Fc fragment (Feinstein etal., 1969, 1971 ; Poljak etal., 1972, 1973 ; Sarma etal.,1971).

However, both of the monomeric forms of IgM and IgA were pictured like a Y-shape (Green etal., 1969 ; Feinstein etal., 1969). The serum IgA dimers are shaped like "double Y" in which the two Y-shaped (or possibly T-shaped) IgA monomers are joined end to end through their Fc parts via a link that may have significant flexibility. The estimated length of each free arm (i.e. Fab) is 70 A° and 30 A° width and over all the length of the two linked arms is 125 A° and 30 A° wide (Feinstein etal., 1971). The structure of secretory IgA, on the other hand, has been interpreted as more compact with the two IgA monomers super imposed on each other and the secretory component presumably inserted between the Fc parts of the α chain (Bloth,etal., 1971; Bjok, etal., 1974)

Mild reduction of colostral IgA yielded Y-shaped 7 S monomer

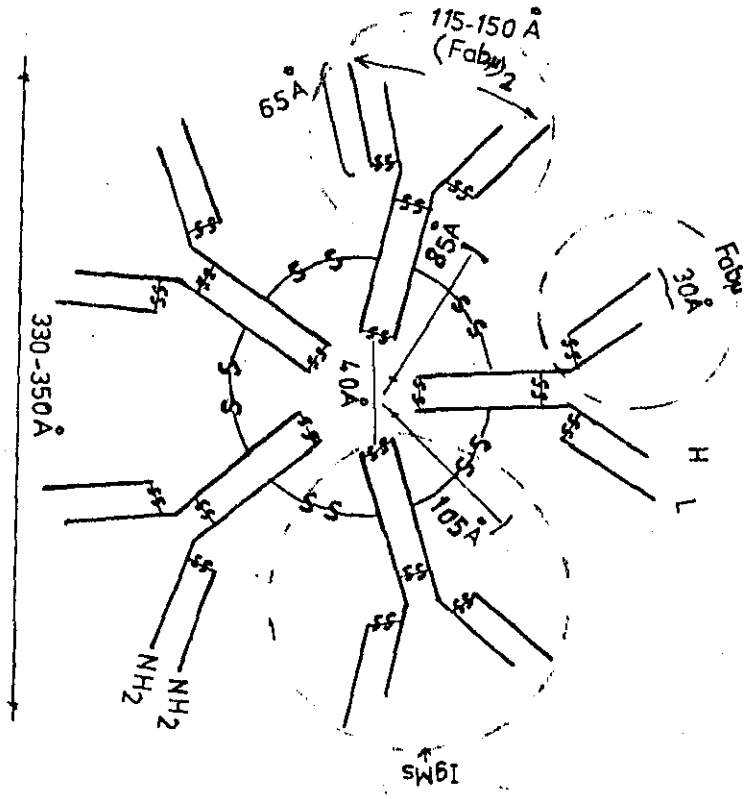


Fig-4- Schematic representation of pentameric IgM (Metzger,I970), showing: the dimension of the whole molecule,its Fabμ, $(Fab\mu)_2$,its $(Fc\mu)_5$fragment and IgM_s subunit,H and L chains and interchain disulphide bonds .

with the dimensions, Fab, 35 x 70 A°, and Fc, 40 x 70 A° (Bloth etal., 1971). The structural studies (Miller etal., 1965, 1966 ; Metzger, 1970) suggests that intact polymer IgM molecules (S_{20} = 19 S) consists of 5 identical IgG like subunits linked by μ heavy chain disulphide bond, possibly near the C-terminal ends of the Fc fragment (Abel and Gray, 1967). These five subunits are arranged to form a five-arm star like structure, whose arms are $(Fab_{\mu})_2$ fragments and whose centre is the $(Fc_{\mu})_5$ fragment. After papain digestion (Mihaesco and Seligmann, 1968), the central ring comprises the $(Fc\mu)_5$ and has an outer diameter 85 A°, inner diameter 40 A° and the protrusions are 20-30 A°.

Each subunit (i.e. 7 S IgM_S) contains quantitively two Fab_{μ} fragments and one Fc fragment (i.e. 10 Fab and 10 monomer Fc fragments per 19.S IgM molecule). The two Fab_{μ} fragments liked with the Fc part at the "hinge region", each of which has an average dimension of 65 x 35 A°. The end to end distance between two neighbouring Fab units in the same subunit has been reported to be 115-150 A°. The total span of the whole intact IgM molecule to be 300-350 A°. Fig. 4. IgM (Feinstein etal., 1971 ; Metzger, 1970).

Further support to the ultrastructural view that suggests a pentameric structure was reported by Ashman and Metzger (1969), who were able to determine that a homogeneous Waldenstrom's macroglobulin (19 S) with binding affinity for nitrophenyl ligands had 10 binding sites in the contact IgM, 2 in the 7 S subunit IgM_S, 2 in the $(Fab\mu)_2$ peptic unit, and one in the Fab_{μ} unit.

CNBr REACTION

1.4.8.

Enzymatic digestion of immunoglobulin molecules has proved a useful method for studies correlating the biological function of such molecules with their chemical structure. Most of the recent progress in understanding the molecular structure of IgM molecules is due to physicochemical and immunological studies of their subunits obtained by "longitudinal" cleavage (interchain disulphide and non-covalent bond cleavage) and by "transverse" cleavage (limited proteolysis) of the native molecule. Reagents that are capable of selectively splitting peptide bonds adjacent to specific amino acids in the molecule are required.

Cyanogen bromide reacts with sulphur in the thioether side chain of methionine to yield a mixture of homoserine and homoserine lactone plus methyl thiocyanate. If this reaction occurs when the methionine is in the peptide linkage, the bond involving the carbxyl group of methionine is cleaved (Gross and Witkop, 1961). All fragments produced by the action of CNBr will contain C-terminal homoserine or its lactone (unless methionine is C-terminal). If the methionyl side chains in a protein or peptide become oxidized to the sulfoxide or sulfone, CNBr cleavage will not occur. The reaction is performed in acidic media which are usually denaturing solvents for proteins and peptides. Originally, 0.1 N HCl was employed (Gross and Witkop, 1962), or more recently, 70% aqueous formic acid (Steers etal., 1965) or aqueous trifluoro acetic acid have been used (Drapeau and Yanofsky, 1967., Schroeder etal, 1969 ; Titani etal.,1972). A difference in the extent of cleavage has been noted when using 70% formic acid or 0.1 N HCl.

In the case of B - galactosidase, 80% of the susceptible bonds were cleaved in 0.1 N HCl, while 96% cleavage was achieved in 70% formic acid (Steers etal, 1965). This concentration of formic acid has also been found to be optimal for the fragmentation of high molecular weight proteins. For example in immunoglobulin IgG (Waxdal etal, 1968) more than 96% of methionyl bonds were cleaved, while only 52% of these methionyl bonds were cleaved in 0.3 N HCl (Cahnmann etal, 1966). However, under certain conditions cyanogen bromide reacts not only with methionyl bonds, but also reacts with methionyl derivatives, for example S-methyl cysteine (Gross and Morel, 1965, 1974). At alkaline pH cyanogen bromide reacts with the basic groups of mono and diamino acids (Schreiber and Witkop, 1964) of proteins. However, the choice of pH is governed by the necessity to denature the protein to expose the side chains of methionine to attack by cyanogen bromide.

A study of the reaction of CNBr in 0.3 N HCl with the common amino acids showed that, besides Methionine, only cysteine (Swan, 1958) but not cystine, tyrosine, or tryptophan reacted. Cyanogen bromide cleavage has been employed with excellent results in studies related to the elucidation of the primary structure of several proteins, e.g. ribonucleus (Gross and Witkop, 1961, 1962), myoglobin (Edmundson, 1963), and IgA (Mestecky etal, 1974).

CNBr in 0.05 M HCl or 0.3 M HCl cleaved the human and rabbit IgG molecules into an $(Fab)_2$ and Fc fragment, about half of their methionine peptide bonds being cleaved (Lahav etal, 1966; Cahnmann etal, 1965). But in 70% formic acid cleavage of all the methionyl peptide bonds occured (Waxdal etal, 1968; Sjoquist 1966; Piggot and Press, 1967).

Comparison with $(Fab)_2$ and Fab fragments produced by proteolytic

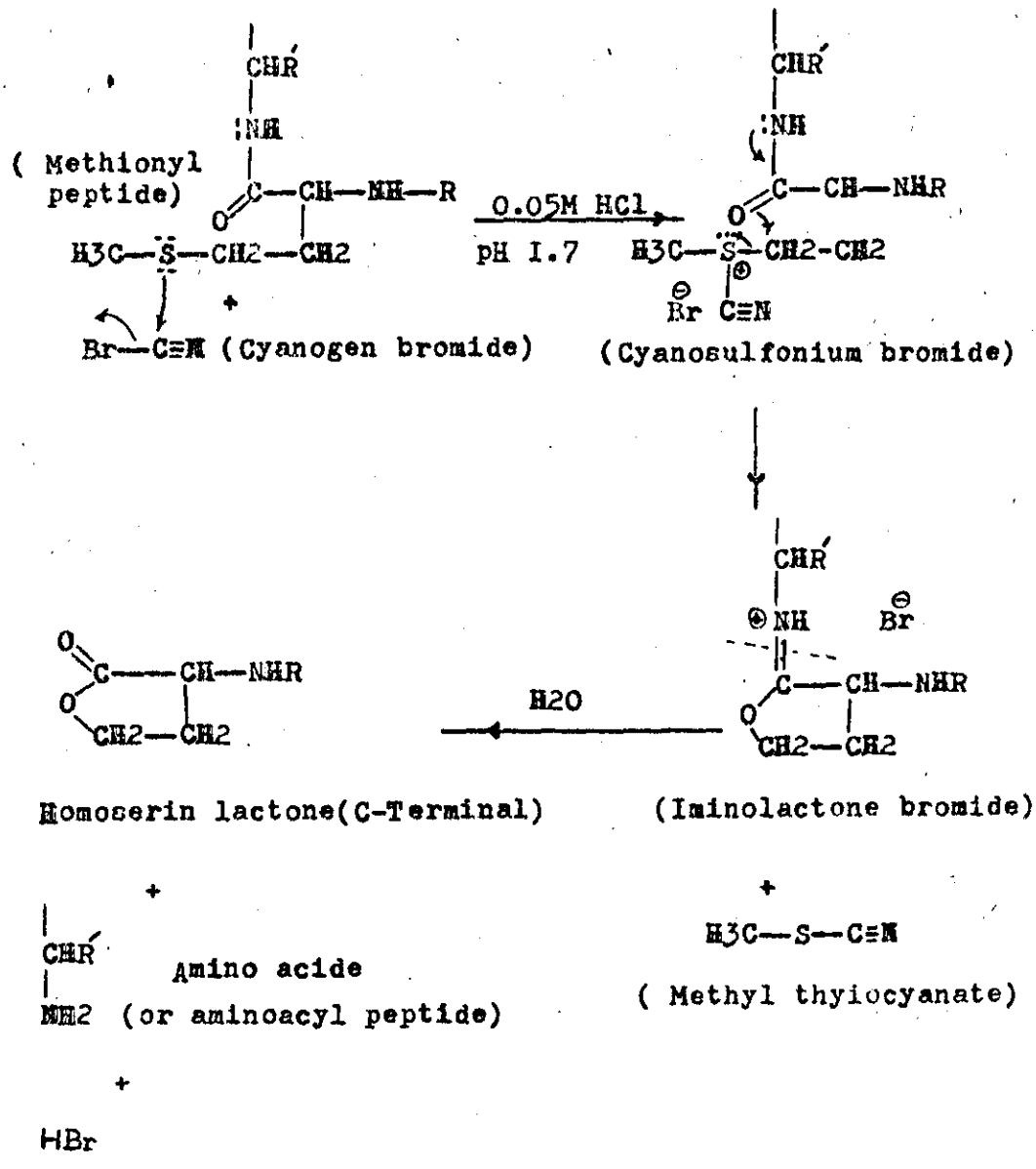
enzymatic cleavage indicated that the cleavage was at a different point on the immunoglobulin molecule (Cahnmann etal, 1965 , 1966). Results similar to the CNBr digestion of IgG were obtained for IgM molecule when treated in an identical manner. Limited cleavage of methionine at a certain position in proteins has also proved useful. For instance, the finding that only methionine 15 in native collagen is cleaved by CNBr (Bornstein etal, 1966) permits the conclusion that this sequence is part of the non helical portion of the continuing collagen peptide chain. This increases the probability of obtaining large fragments which provide valuable information, important either for the correct positioning of peptide derived from the enzymic digests or for the confirmation of previously established sequences.

In 70% formic acid a total degradation of the IgM molecule occured (Putnam etal, 1973 ; Zikan and Bennett, 1973 ; Hurst etal, 1973).

Zikan etal, 1973 showed that treatment of IgM molecule with CNBr in 70% formic acid produced a Fab_{μ} fragment (Mol.Wt.54,900), an $(Fc\mu)_5$ fragment and other peptides.

In 0.05 M HCl, partial degradation is known to occur, producing Fab_{μ} fragment (of molecular weight about 50,000 to 60,000) and a large fragment with a molecular weight possibly of about 800,000 antigenically identical to $(Fc\mu)_5$ fragment (Miller, 1968). This fragment was designated CF_1. After dialysis against 5 M Gu-HCl, CF_1 showed two components in the ultracentrifuge, whose sedimentation coefficients were calculated 5.6 S and 2.4 S (Miller, 1968). However, the method has some disadvantages for example: certain peptides and proteins have been observed to be particularly susceptible to solvolysis in dilute acid, and their possible cleavage under the mild acidic conditions of

Fig-5- Mechanism of Cyanogen Bromide reaction



the cyanogen bromide treatment constitutes an undesirable side reaction. Some proteins are insoluble or unstable in strong acid conditions or in concentrated organic solutions and the cyanogen bromide products may be insoluble or tend to aggregate in the neutral solutions (Cahnmann etal., 1966). Cyangen bromide is a poisonous substance and difficult to use.

THE AIM OF THIS WORK MAY BE SUMMARISED AS FOLLOWS :

1.5.

1. To obtain a high yield of fragments by treating IgM molecule and its subunits (IgM_s) with cyanogen bromide in 0.05 M HCl.

2. To obtain the accurate molecular weight values for the fragments produced from CNBr digestion under the above condition.

3. To characterize as fully as possible these fragments including their immunological and electrophoretic properties.

4. To compare the fragments produced with analogous proteolytic fragments obtained from the same immunoglobulin molecules.

CHAPTER 2

MATERIALS AND METHODS

2.1.

2.1.1. MATERIALS

Sephadex G200 fine, G200 superfine, G25 fine, sepharose 4B and DEAE sephadex A 50 were obtained from pharmacia fine chemicals, Uppsala, Sweden, Coommassie brilliant blue R250, Poneaus, Nigrosine, Bromophenol blue, Folin and ciocalteu phenol reagent, Urea, 2 mercapto ethanol, NN-methylene bisacrylamide, NNNN-tetra-methyl 1,2-diamino-ethane, aristar-HCl, Dl-nor lencine (chromatographically homogeneous) were all obtained from BDH chemical company Ltd., Poole - Great Britain, using "Analar" grade whenever possible. Molecular weight markers with the molecular weight range 14,300 - 71,000 and 53,000 - 265,000 for polyacrylamide gel electrophoresis were obtained from BDH Ltd.. Acrylamide, iodoacetic acid were obtained from Fisons Scientific Apparatus Ltd., Loughborough, Leicestershire, England, using "Analar" grade whenever possible. L-cysteine hydrochloride and guanidine hydrochloride were obtained from the Sigma Chemical Company (Poole, Dorset, Great Britain).

Trypsin (bovine Pancreas, 2 x crystallized), pepsin, thyroglobulin (bovine), albumin (bovine) cytochrome-C (practical grade), egg albumin bovine globulins (IgG) 99% pure, were all supplied by Sigma.

Ninhydrin, thiodiglycol (pure), 2-methoxy ethanol and BRIJ were obtained from East Anglia chemicals and Hopkin and Williams, Great Britain. Amino acid standard A-A-5 grade "A", was obtained from Calbiochem Ltd., England. "Cellogel" strips were obtained from whatman labsales, Springfield Mill, Maidstone, Kent-England. Chromatography

filter papers No. 1 size 25 x 25 cm, thickness 0.16 mm, medium flow rate were obtained from Whatman Ltd., England.

Agarose (indubiose) was obtained from (L'industrie Biologique Française S. A. France). Human transferrin, Human-Albumin (electrophoresis purity 100%) were obtained from Behring Werke AG-Marbury-lahn, Germany.

Soybean trypsin inhibitor, was obtained from Koch-light laboratories Ltd., England. Cyanogen bromide, stated to be 97% pure, was obtained from Ralph N Emanuel Ltd., England. Dialysis tubing (visking tubing, inflated diameter 8/32" and 18/32") was obtained from the Scientific Instrument Centre Ltd., London, England.

Sodium dodecyl sulphate (SDS) was obtained from Cambrian Chemical Ltd., England. Other chemicals were obtained from BDH Ltd., and from Fisons Ltd., using "Analar" grade whenever possible.

PREPARATION OF GELS

2.1.2.

The choice of gels depended on the molecular weights of the proteins to be separated (Table 2).

Gel chromatography column: The chromatographic tubes, 40-70 cm in length, the internal diameter 2.6 cm supplied by Pharmacia Fine Chemical, Uppsala, Sweden were fitted with an outlet tube at the bottom end, the packing being carried out with gels preswollen as described by the manufacturers.

2.1.3. APPLICATION OF SAMPLE TO THE GEL COLUMN

The prepared gel columns were washed before use with at least twice bed volume of eluant. The mixture of protein sample in the buffer solution was layered onto the top of the gel surface of the column as described by the supplies. The sample was allowed to pass down throughthe gel column. The column was eluted with the same eluant at a flow rate of 10 mls per hour. The operating pressure of 20-25 cm for the sephadex columns (size 40-70 x 2.6 cm) and 10 cm of water for the sepharose columns (size 55-70 x 2.6 cm). The void volumes of the sephadex and sepharose columns were determined using blue dextran 2000 (pharmicia) monitered in glass cells of 1 cm path length, at 630 mm (Mann etal, 1969, 1972) using a pyeunicam s.p. 600 spectrophotometer.

In all gel filtration experiments, the concentration of the protein samples was about 2-3 % of serum protein concentration and their

TABLE 2

GEL TYPE AND PROPERTIES

Gel type	Gel volume ml/g dry-wt	Fractionation range (mol-wt)	Type of chromatography	Comments
Sephadex G 200 fine	30-40	5,000-800,000	Columns 70x2.6cm 40x2.6cm	Separation of $(Fc\mu)_5^T$, IgM_S subunits and isolation of IgM.
Sephadex G 200 Superfine	20-25	5,000-250,000	Thin layer glass plates size 20x20cm gel thickness 0.6mm	Determination of molecular weights of proteins and their fragments.
Sephadex G 25 fine	4-6	1,000-5,000	Columns 40x2.6cm	Quick filtration to remove small molecules, mainly reagents used in reactions with proteins.
Sepharose 4 B	(Agarose concentration 4%).	10^5-20x10^6	Columns 70x2.6cm	Separation of the CNBr digestion of IgM, $(Fc\mu)_5^T$, and IgM_S subunits.
DEAE-Sephadex A 50	(An ion exchanger)	30,000-200,000	Columns 40x2.6cm	Separation of cyanogen bromide products.

volumes did not exceed 5% of the bed volumes of the gel columns and all the experiments were run at room temperature (Ca 20-25°C).

COLLECTION AND MONITORING OF FRACTIONS

2.1.4.

Eluted protein fractions, Ca 2.8 ml in volume, were normally collected in an automatic fraction collector, and their absorbances were monitored at 280 nm using a Pye Unicam s.p. 600 spectrophotometer. Selected fractions were stored at 4°C for further purification and characterization.

2.2.1. ELECTROPHORESIS

2.2.2. Principle:

Zone electrophoresis is distinguished from moving boundary electrophoresis principally in that the former involves the use of a support on which the material moves by solvent flow, but in the latter ions move freely in solution. Typical supports employed in zone electrophoresis include paper, cellulose, starch gel or blocks of polyurethane foam, and polyacrylamid gels. In zone electrophoresis, the material to be examined is initially applied to the support in a narrow bond approximately mid-way between the buffer reservoirs in which the electrode is immersed. As the electrophoresis proceeds, each component migrates towards the cathode or the anode, depending on its charge, at its characteristic rate. Thus at the termination of a run, each component is, ideally, clearly separated from others. The support may then be divided into sections, and the individual components may

be eluted separately. Thus, zone electroresis is useful both for the analysis of multi-component mixtures and for the analysis of purified proteins from such mixtures. The choice of support in zone electrophoresis is largely dictated by the purpose of experiment. In 1959, Kohn introduced cellulose acetate membrane as an alternative to paper. The media offer a number of valuable features superior to those of paper: 1. cellulose acetate membrane has a homogeneous microporous structure with relatively few non-acetylated hydroxyl groups, making it virtually non-absorptive. 2. Proteinscan be studied above and below their isoelectric points. 3. Short running time (2hrs - 20mins). 4. Sharp resolution, staining and destaining very satisfactory, easily to handle, and only small sample loading <1ul.

CELLULOSE ACETATE ELECTROPHORESIS

2.2.3.

This was carried out on "Cellogel" strips size 14 x 5.7 cm with electrophoresis apparatus (supplied by Gelman Instruments Co., Michigan, U.S.A.) to follow the process of protein fractionation and for testing the purity or homogeneity of protein samples obtained from gel filtration experiments. The Cellogel strip was soaked in 0.07 M Tris-barbital buffer pH 8.8, supplied by Gelman Instruments Company for 15 minutes prior to use, following the procedure of sargent (1965). The strip was blotted between filter paper to remove the excess buffer, and the protein sample was immediately applied to the middle of the cellogel strip using a semi-micro sample applicator (1.9 ul, 9 mm) (Reeve Angel). The strip was positioned between the electrode vessel in the electrophoresis tank (Gelman electrophoresis apparatus model 51170-1) containing the same tris-barbital buffer, pH 8.8. Electrophoresis was allowed to proceed for 2 hours at a constant voltage (150 volts). After the electrophoresis

the strip was stained with ponceaus for one hour, then with nigrosine for further one hour, then destained with successive changes of 5% acetic acid until clearly stained protein bond was shown on a white background. The strip was stored in the destaining solution in a closed glass box for photography.

ISOLATION AND CHARACTERIZATION OF A HUMAN MACROGLOBULIN IgM PROTEIN

2.3.1.

2.3.2. Introduction:

In order to determine the primary structure of a protein it is necessary to obtain reasonable quantities of it in a pure state. In the case of IgM, the protein has to be obtained free from the other proteins of serum, predominantly other immunoglobulins (IgG, IgA, IgD, IgE), lipoproteins, fibrinogen and haptoglobulins. The high molecular weight of the IgM molecule causes it to be complete excluded from the gel beads of a sephadex G 200 (Flodin and Killander, 1962). Since most IgM proteins are euglobulins i.e. they are precipitated by diluting the serum or plasma with distilled water. This preliminary separation technique was employed.

2.3.3. Isolation of IgM:

This was carried out following the procedure described by Putnam etal.,(1967), for the preparation of IgM based on euglobulin precipitation. Pathological human sera obtained from patients with diagnosis of Waldenstrom macroglobulinemia were thawed at room temperature, then diluted ten times with distilled water. Euglobulin precipitation was

allowed to proceed at 4°C for 24 hours; the mixture was then centrifuged at 3 x 10^3 rev/sec for 15 minutes, and the supernatant discarded. The precipitated proteins were washed three times with cooled redistilled water, recollected by centrifugation at 3 x 10^3 rev/sec for 10 minutes, and then redissolved in as a small a volume as possible of 0.1 M Tris-HCl 0.3 M NaCl buffer, pH 8.2 containing 0.01% sodium azide. The sample was applied to column of sephadex G 200 (size 40 x 2.6 cm) (which was previously washed and equilibrated with the same tris-HCl buffer), allowed to pass down a gel filtration column followed by eluant. 3 ml fractions were collected at aflow rate of 10 ml per hour using a fraction collector. The absorbance of each fraction was monitored at 280 nm on an s.p. 600 spectrophotometer. The fractions which cluted at the void volume of the column were collected, Pooled and concentrated using a minicon concentrator type B 15. (Amicon Corporation Massachusetls U.S.A.). A typical elution pattern is shown in Figuers,6,7,and 8(see results,chapter 3.).

IMMUNOCHEMICAL TECHNIQUES

2.4.1.

2.4.2.A.

I. Double Immunodiffusion (Ouchterlony method).

Principle:

Elek and Ouchterlony, 1948; and Ouchterlony, 1953, devised a double immunodiffusion technique, in which both the antigen and the antibody molecules were allowed to diffuse towards each other from separate wells punched in a gel layer, so that a precipitin was formed. In this technique, a central well in which the antigen was applied is symmetrically surrounded by a number of wells in which the antibodies

are placed, both the antigen and the antibodies diffuse readily into the gel from their application wells. Under such circumstance, the antigen - antibody precipitates will occur when the antigen and the antibody are present at the equivalent ratio.

2.4.2.A.

II. Ouchterlony Technique

Procedure:

Clean microscopic slides 7.5 x 5 cm were placed on a horizontal surface and coated with 5 ml of molten 1.5% agarose in barbital buffer, pH 8.6, ionic strength 0.045, containing 0.1% sodium azide as preservative. The gels were allowed to solidify at room temperature for about 15 minutes, and then the sample wells were made by means of an Ouchterlony punch.

The gel inside the wells was removed by suction. 5 µl human macroglobulin IgM solution of about 1% concentration was placed in the outer wells and then antisera (5ul of anti-human lightchain K type, prepared in goat and 5 ul of anti-human IgM (µ chain specific), prepared in coat (supplied by Miles-Yeda Ltd., U.S.A.)) were applied in the central well individually using a Hamilton syringe (purchased from Hamilton micromeasure N.V. Haque, Holland). The diffusion was allowed to proceed in a humid box for 48-72 hours until the precipitin bands formed, the slides were carefully washed with 0.9% NaCl solution for 48 hours with several changes of saline to remove non-precipitated protein, then stained with 0.25% ($\frac{W}{V}$) Coomassie blue R 250 dissolved in (ethanol 96%, acetic acid and water, 9:2:9 respectively) for one hour. The destaining solution was ethanol 96%, acetic acid and water 9:2:9 respectively. Several changes of destaining solution were found to be necessary to

remove the excess of dye and to distinguish the precipitin arcs from the gel background. Finally the slides were dried at room temperature and the precipitin arcs were identified.

2.4.2.B. IMMUNOELECTROPHORESIS

I. Principle:

Immunoelectrophoresis as described by Grabar and Williams (1953) is a combination of two stages of electrophoresis and radial immunodiffusion in gels. In the electrophoresis stage, the protein mixture is forced to move and fractionated by an electricfield in a carrier medium is similar to that in a liquid medium. In the radial immunodiffusion stage, the separated proteins were allowed to react with a specific antisera applied in a trough, cut parallel to the direction of the electrophoretic migration. Both the separated proteins and the applied antiserum were allowed to diffuse through the carrier medium by incubation in a moist chamber for a certain period of times. Under such circumstances, the reaction between the fractionated proteins and the antiserum takes place.

2.4.2.B.

II. Procedure (Grabar and Williams Technique):

This technique followed the procedure of J. Clausen, (1972). Clean microscopic slides size 7.5 x 2.5 CM were placed on a horizontal level surface and coat with 2.5 ml of 1.5% $(\frac{W}{V})$ agarose in barbital buffer, pH 8.6, ionic strength 0.045, containing 0.1% sodium azide as preservative, and 1 mM calcium lactate. The gel was left to solidify at room temperature for about 15 minutes, then the sample well is cut

at a position of the middle of the gel plate by using a gel puncher of 3 mm diameter, then the gel were removed from the well by suction. The gel plate was transferred and arranged on the electrode vessel by means of filter paper Wicks. The electrode vessel were filled to the same level with the same barbital buffer, pH 8.6, ionic strength 0.045, then 5 ul of 1% human IgM solution was applied to the sample well followed by 5 ul of 0.1% $\frac{W}{V}$ Bromophenol blue dissolved in redistilled water as a marker for electrophoretic migration. The electrophoresis was allowed to proceed at a constant voltage (100 volts) for about one hour. At the end of electrophoresis, the gel plate was carefully removed from the electrophoresis tank, the antiserum trough (about 4 x 0.2 cm) was cut parallel to the direction of electrophoretic migration by means of a template device and a razor blade, then the gels were gently removed from the trough by suction. About 25 ul of mono specific antisera (anti-human IgM μ chain specific) was applied to the antiserum trough, the gel plate was left in a moist chamber for about 72 hours, and then washed with 0.9% saline (NaCl) for 24 hours with several changes of saline to remove unreacted protein. It was then stained in 0.25% $\frac{W}{V}$ coommassie blue R 250 dissolved in ethanol, acetic acid and water (9:2:9 respectively), for about one hour, and destained as above. Finally the gel plate was dried at room temperature in a plastic box and the precipitin lines were identified.

2.5. PURITY OF THE ISOLATED MACROGLOBULINS (IgM)

The purity of the isolated macroglobulins (IgM) were checked by electrophoresis on "cellogel" strips in tris-barbital buffer (pH 8.8), by Ouchterlony immunodiffusion and by SDS gel electrophoresis. The isolated IgM proteins appeared to be homogeneous, each containing Kappa type light chains.

2.6. PREPARATION AND PURIFICATION OF 7 S SUBUNITS (IgM_s) FROM THE ISOLATED PATHOLOGICAL "Har" IgM

2.6.1. Introduction:

The conversion of 19 S IgM to 7 S subunits by reduction with sulphydryl reagents such as mercaptoethanol was first demonstrated in 1957 (Deutsch and Morton, 1957). Since then it has been well established that the reduction products reaggregate slowly when sulphydryl reducing agents are removed, but are stabilized by treatment with alkylating agents such as iodoacetamide or iodoacetoacetic acid. It was concluded that the reaggregated products formed from reduced IgM associated through non-covalent interaction and that the reducing agents had reduced the disulphide bonds linking the 7 S subunits and the alkylating agents prevented such linkage from reforming by alkylating the resulting sulphydryl groups. (Metzger, 1970).

2.6.2. Preparation of IgM_s Subunits:

In the present work, the subunits of (Har) IgM macroglobulin were prepared and isolated using the method of Miller and Metzger (1965).

10 mg/ml of IgM in 0.1 M Tris-HCl/0.3M NaCl buffer, pH 8.6, containing 1 mM sodium azide was reduced with 0.02 M cysteine (Sigme) at 25°C for one hour, the solution was alkylated by addition of a two fold molar excess of iodoacetamide and allowed to stand for 30 minutes at room temperature. The reaction mixture was subsequently fractionated on sephadex G 200 column (70 x 2.6cm) in 0.1M Tris-HCl/0.3M NaCl buffer, pH 8.6. An elution pattern obtained during the preparation of IgM_s subunits is shown in Fig. 11 (see results chapter 3).

Fractions containing IgM_s subunits were pooled and concentrated using aminicon concentrator, type B_{15} and stored at 4°C.

2.6.3. Purity of IgM_s Subunits:

Purification was effected by reapplication of the IgM_s materials pooled from the previous gel filtration experiment. Elution was carried out from sephadex G 200 column (70 x 2.6 cm) equilibrated with 1 Molar propionic acid. Elution was carried out by the same buffer (i.e. 1 M propionic acid) to reduce the aggregation phenomenon (Miller and Metzger, 1965). 3 ml samples were collected and the absorbance of each fraction was determined as described above. Fractions with high absorbance readings were pooled and stored at 4°C for further characterization.

2.7. TREATMENT OF HUMAN MACROGLOBULIN IgM WITH TRYPSIN

2.7.1. Action of trypsin:

Trypsin exhibits the highest degree of substrate specificity known for endopeptidases, only those bonds involving the carboxyl groups of

lysine and arginine residues are hydrolysed. This permits the estimation of the number of tryptic peptides expected from a protein if the amino acid composition is known. Theoretically the number of peptide bonds split in the protein by trypsin is equal to the sum of lysine and arginine residues present in the molecule plus a C-terminal peptide. However, the relative rates of cleavage of susceptible peptide bonds are influnced by the chemical nature of the side chains in the immediate vicinity. Generally, the presence of polar groups close to the susceptible bond decrease the rate of hydrolysis. Side chains with a net negative charge (e.g. aspartic acid, glutamic acid, cystic acid) which are adjacent to a lysine or arginine residues tend to reduce significantly the rate of hydrolysis. The number of tryptic peptides and the lysine plus arginine residues is not always equal and reliable and can be misleading (Harris and Hindley, 1965).

2.7.2. PREPARATION AND PURIFICATION OF $(Fc\mu)_5$ FRAGMENTS

I. Methods:

Hydrolysis of IgM with trypsin at 65°C was carried out following the procedure described by Plaut etal.,(1972). The IgM (Har) was dissolved in 0.05 M Tris-HCl/0.15 M NaCl buffer (pH 8.1) containing 0.01 M Calcium chloride at a protein concentration of 20 mg/ml. The protein solution (Ca-40mg of IgM) was heated to 65°C prior to the addition of trypsin at an enzyme to protein ratio 1:20 $\frac{W}{W}$ respectively. The digestion was carried out at 65°C for 8 minutes and was terminated by the addition of soybean trypsin inhibitor (1 mg inhibitor to 1 mg trypsin). The IgM digest was fractionated on a sephadex G 200 column (67 x 2.6 cm), equilibrated with 0.05 M Tris-HCl/0.15M NaCl buffer (pH 8.1)

containing 0.01% sodium azide. 3 ml fractions were collected at a flow rate 12 ml/hr. Each fraction was monitored at 280nm. The selected protein fractions were examined by immunodiffusion with a mixture of antisera IgM (μ chains specific) and antisera K chains. The fractions were pooled according to their reaction with those antisera and concentrated. An elution pattern obtain during the preparation of $(Fc\mu)_5$ is shown in Fig. 24. The elution material containing $(Fc\mu)_5$ fragments were further purified by rechromatography on a sephadex G 200 gel filtration column (65 x 2.6 cm) eluted with tris-HCl buffer (pH 8.1) as above. Fractions with the highest optical density readings were pooled and stored at 4°C for further studies.

II. Dissociation of $(Fc\mu)_5$ Fragment:

The $(Fc\mu)_5$ fragments obtained from "Har" IgM were suspended in tris-buffer saline, pH 8.2 containing 6M Gu-HCl at a concentration of 10mg/1ml (Ca-10 mg) and dissociated by the addition 2 - mercapto ethanol to give a final concentration of 0.1 M (Fleischman, J.B., Porter, R.R., and Press, E.M., 1963). After standing at room temperature for 2 hours, the solution was alkylated by addition of a 100 per cent molar excess of iodoacetamide and allowed to stand for 15 minutes at room temperature. The reaction mixture was subsequently fractionated on sephadex G200 Column (70 x 2.6 cm) in 0.1M Tris-HCl/0.3M NaCl buffer, pH 4.5 containing 6 M guanidine hydrochloride (Gu-HCl).

2.8. TREATMENT OF IgM WITH CYANOGEN BROMIDE

2.8.1.

A lyophilized Waldenstrom IgM (50 mg) was dissolved in 5 ml of 0.1 M tris-HCl/0.3 M NaCl buffer, pH 8.2, then transferred to a dialysis

tube and then hydrolysis process was allowed to proceed by dialysing against 0.05 M HCl solution (pH 1.7) at room temperature for 24 hours. Acid denatured (0.05 M HCl) IgM (about 50 mg) was placed in a glass bottle and to this solution, cyanogen bromide (0.25 gms) was added and immediately the glass bottle stoppered. The mixture was gently shaken until the cyanogen bromide dissolved and was then allowed to stand in a sealed bottle at room temperature for 20 hours. The reaction mixture was then lyophilized to remove unreacted cyanogen bromide, and other volatile products. The freeze dried digest of IgM was examined by "cellogel" strip electrophoresis in Tris-barbital buffer, pH 8.8 containing 6 M urea.

2.8.2. FRACTIONATION OF IgM TREATED WITH CYANOGEN BROMIDE

2.8.2.I. Gel filtration:

The lyophilized product (IgM/CNBr) was dissolved in 5 ml guanidine hydrochloride (Gu-HCl). The separation of fragments from the CNBr digestion of IgM was achieved by fractionation on sepharose 4B. The gel filtration column (size 70 x 2.6 cm) was packed with preswollen sepharose 4B and equilibrated with 6 M guanidine hydrochloride (Gu-HCl), pH 4.5. The freeze dried product as dissolved in the equilibration solvent (5ml of 6M Gu-HCl) was applied onto the top of the gel filtration column, sepharose 4B (67 x 2.6 cm) and allowed to pass through it a flow rate of 5 ml per hour. The eluting buffer was 6 M Gu-HCl, pH 4.5 3 ml samples were collected with the aid of automatic fraction collector. The absorbance (optical density) for each fraction was measured in Silica cells of 1 cm path length at 280 nm. Selected fractions were stored at 4°C for further characterization.

The fractionation profile of the CNBr digest on sepharose 4B was shown in figures 15 and 16 (see results chapter 3).

2.9. CYANOGEN BROMIDE CLEAVAGE OF IgM_s SUBUNITS AND $(Fc\mu)_5$ FRAGMENTS

2.9.1.

Lyophilized IgM_s subunits or $(Fc\mu)_5$ fragments were suspended in 0.1 M Tris-HCl/0.3 M NaCl buffer, pH 8.2, hydrolysed by dialysing against 0.05 M solution hydrochloric acid at room temperature overnight, and were then transfered into separate glass bottles. Solid cyanogen bromide was added to each sample to give protein to cyanogen bromide weight ratio 1:5. Digestion was carried out in sealed bottles for 20 hours at room temperature, terminated by the addition of 5 volumes of ice-cold distilled water, and immediately lyophilized. The freeze-dried digest of $(Fc\mu)_5$ and IgM_s subunit "Har" were examined by "cellogel" strips electrophoresis in pH 8.8. Tris barbital. Sodium barbital buffer containing 6 Molar urea and by S D S polyacrylamide gel electrophoresis system.

2.9.2. FRACTIONATION OF CNBr DIGEST OF IgM_s SUBUNITS:

The freeze-dried digest of IgM_s subunits "Har" was dissolved in 6 Molar guanidine hydrochloride (Gu-HCl) buffer (pH 4.5) (Ca-20mg in 5 ml Gu-HCl) and were fractionated by gel filtration in 6M Gu-HCl (pH 4.5) on 70 x 2.6 cm of sepharose 4B. The eluant was freeze-dried and dissolved in small volume of 6 M Gu-HCl, and then chromatographed on sephadex G 200 column (size 65 x 2.6cm) equilibrated with 6 molar guanidine hydrochloride, pH 4.5. The fractionation profile of the cyanogen bromide digest on sephadex G 200 was shown in Fig. 30..

2.10. DISC-ELECTROPHORESIS TECHNIQUE

2.10.1. I.

Polyacrylamide gels were made by polymerization of acrylamide monomer in the presence of methylene-bis-acrylamide, Davis (1964). The gels were prepared in siliconized glass tubes (Size 11.8 x 0.6 cm), following the procedure of Weber and Osborn (1972).

2.10.1. II. Procedure:

5% and/or 7% acrylamide gels used in this experiment were prepared as described by Weber and Osborn (1972).

The gel solution was carefully added to the electrophoresis tubes (size 11.8 x 0.6 cm) maintained in a vertical position. The bottoms of the tubes were sealed with plastic serum caps, and the tubes filled with gel solution to within 1 cm of the top; a layer of distilled water was then added so as to exclude air. Gelling was normally complete in about 15 minutes and was evident by the appearance of a sharp division between the upper water layer and the gel below. Carefully the plastic caps and the water were removed, then the tubes were immediately arranged in the disc-electrophoresis apparatus (Shandon Disc-electrophoresis kit). The cathode was the upper electrode and the anode was the bottom electrode. The protein samples and the markers protein were dissolved in the electrophoresis buffer (Ca 0.5mg/ml) individually. Approximately 25 µl of the protein solution was applied to the surface of each gel and carefully layer with one drop of 0.05% Bromophenol blue in distilled water (about 5 µl), then one drop of glycerol followed by the electrophoresis buffer. Each buffer compartment was filled with electrophoresis buffer. The sample being run in

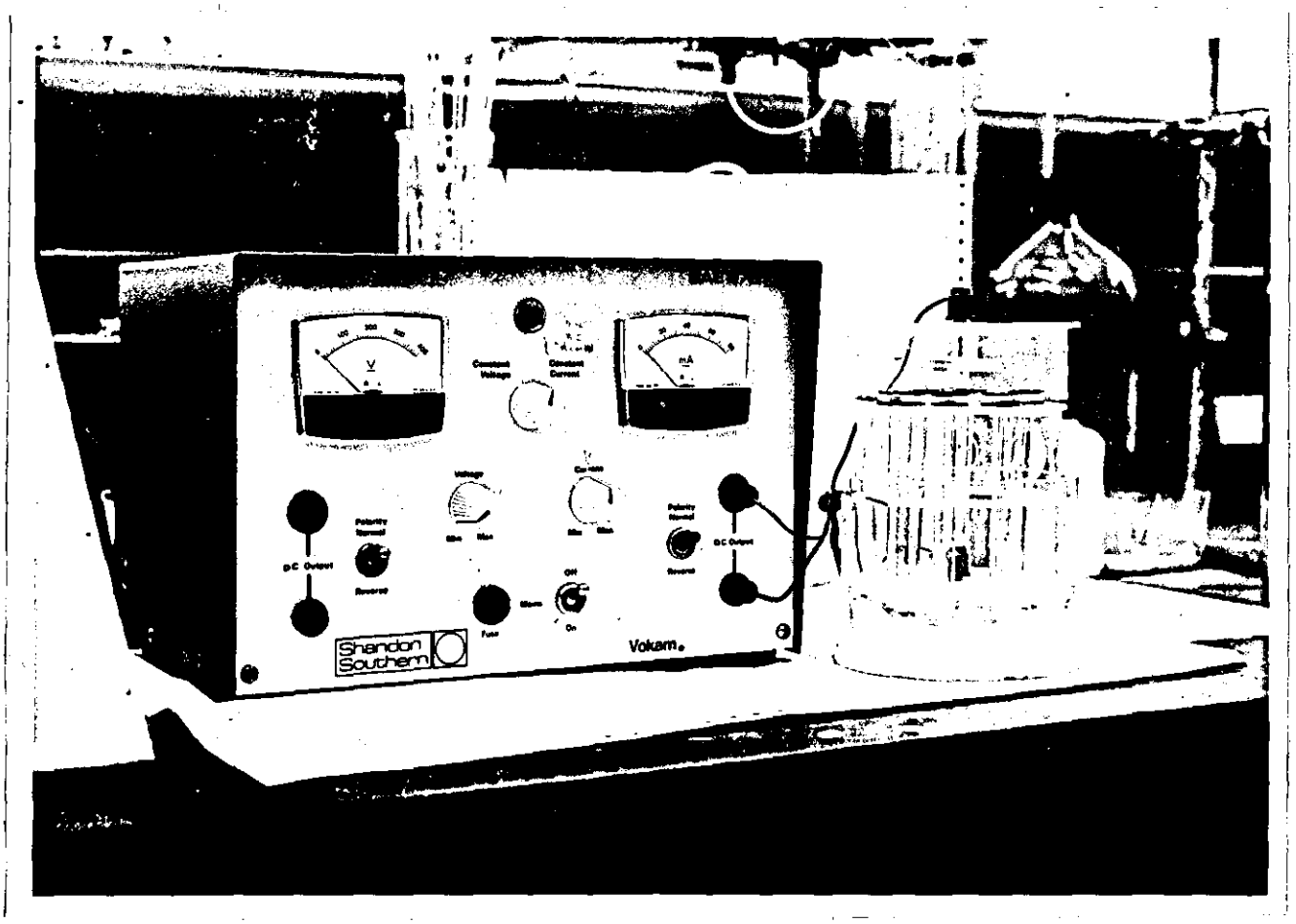


Plate No.1. Disc electrophoresis apparatus for molecular weight determinations.

duplicate. Electrophoresis was carried out for 4 hours, at constant current (50m A per 8 tubes). After the run the gels were removed from the tubes by inserting a fine hypodermic needle along the sides of the tubes and injecting a jet of water until the gel came away from the side of the tube. The gel was removed by running water from syringe with a fine hypodermic needle till the gel slips out of the tube. Each isolated gel is immersed in the test-tube filled with 0.25% coommassie brilliant blue R 250 dissolved in (methanol, acetic acid and water, 5:1:9) for 60 minutes and then after staining the gels were carefully washed in running tap water for few minutes, and then left in 7% ($\frac{V}{V}$) acetic acid. Blue protein bands were developed on a clear background in about two days. Finally the gel was stored in stoppered test-tube filled with 5% acetic acid.

2.10.2. DETERMINATION OF MOLECULAR WEIGHTS BY GEL FILTRATION CHROMATOGRAPHY

2.10.2. I. Thin-layer gel filtration

2.10.2.Ia. Principle

In T.L.G. a layer of swollen gel (e.g. Cross-linked dextran gel, or agarose gel) is spread onto a glass plate. The gel beads, which adhere to the plate without the addition of a fixative and form the stationary phase, and the interstitial fluid forms the mobile phase. The plate is tilted at about 10-20^{o} to the horizontal and becomes stabilized during the transport of mobile phase through the layer. Descending flow is produced by the difference in the height between the reservoirs. After equilibration samples are applied as spots or bonds and the plate is developed by tilting it to the same angle as used for equilibration. Protein spots can be detected on chromatogram

before or after removal of the mobile phase and solvent within the gel or they can be transfered and printed on filter paper.

2.10.2b. DETERMINATION OF MOLECULAR WEIGHT BY THIN-LAYER GEL FILTRATION

Method:

A thin layer gel of sephadex G 200 superfine was prepared on glass plate (size 20 x 20 cm) following to the procedure of Klaus etal (1972). The prepared plate was left at 10°C at room temperature on T.L.G. apparatus marked by pharmacia (plate I) and washed overnight with 6 M Gu-HCl buffer, pH 4.5 with plate angle 10°C at room temperature. Solution of pure IgM and its CN Br fragments and hot trypsin fragments and also the standard proteins were prepared at concentration of 5mg/ml in the same Gu-HCl buffer, pH 4.5. 5ul of each sample was placed on each thin layer plate. The samples were then allowed to run for 4 hours with a plate of angle of 10°C at room temperature, then after running time the spots were transferred onto a filter paper Whatman No 1 by placing the filter paper on the glass plate for about 5 minutes, the dried, staining with 0.25% coommassie blue R 250 dissolved in (methanol in acetic acid, with ratio 9:1 respectively) for 30 minutes, then destaining with destaining solution made from, methanol: acetic acid: water, with ratio 5:1:5 ($\frac{V}{V}$) respectively. Several changes of destaining solution was found to be necessary. Finally, the developed filter paper was dried, using a hair drier and the protein spots were examined. The average of migration distance (Rm) from the origin of application to the centre of each stain spot, was measured. A standard curve was obtained by plotting the (Rm) values of standard

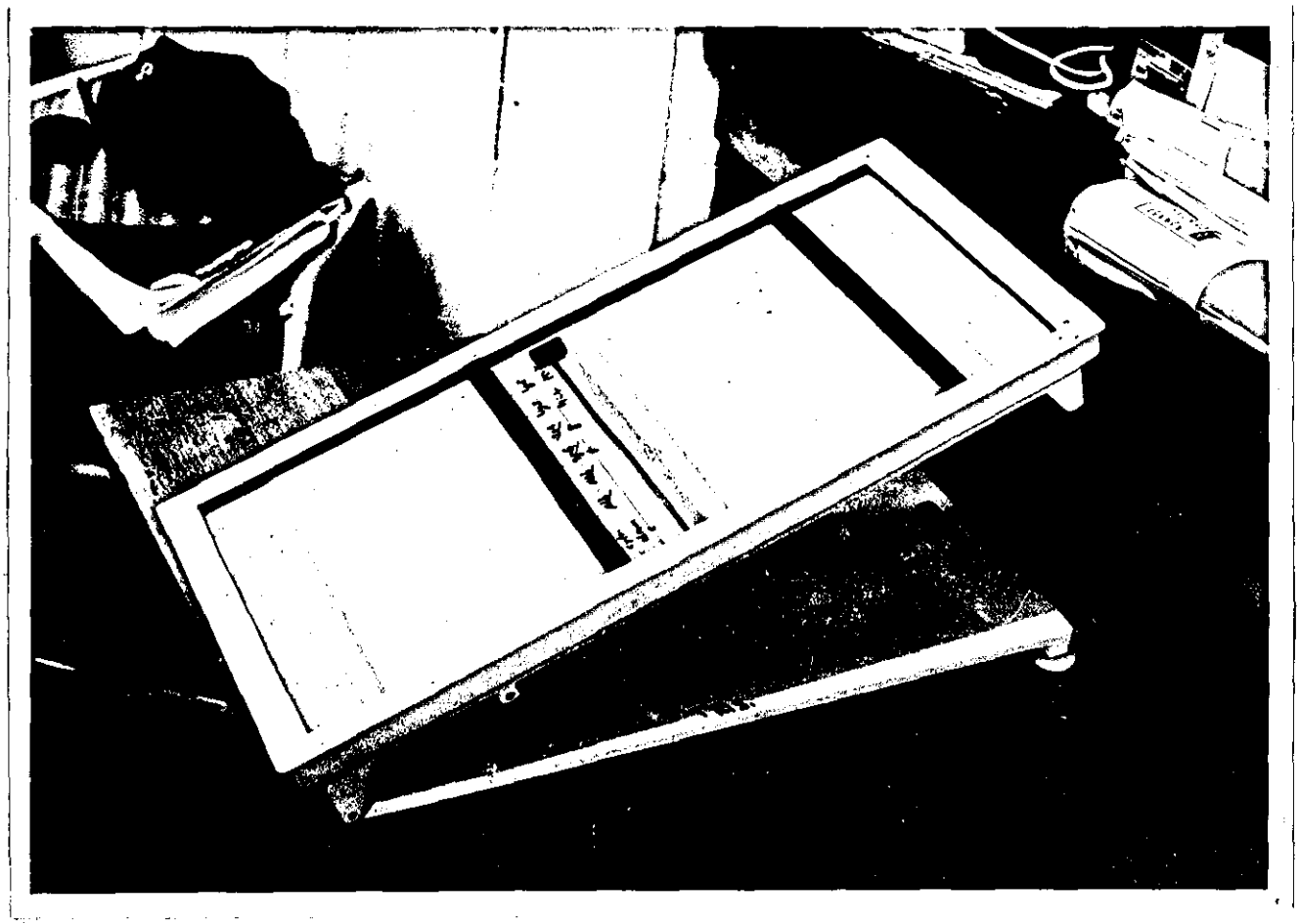


Plate No.2 . Pharmacia T.L.G. apparatus for the molecular weight determinations.

samples against natural logarithms of their molecular weight using cytochromec as an internal standard (i.e. the Rm equal to the ratio of the distance travelled by protein divided by the distance travelled by cytochromec or, Rmp/Rmc, where Rmp = the migration of protein and Rmc = the migration of cytochromec). The molecular weights of the fragments were obtained from the standard curve byusing their Rm values.

2.10.3. MISCELLANEOUS

2.10.3.I. Determination of protein concentration:

Protein concentrationswere estimated by measuring the absorbance at 280 nm in 1 cm cuvettes, with a Unicam s.p. 600 spectrophotometer fitted with a deuterium lamp. For IgM, IgM_S and IgM fragments, an extinction coefficient of 12 was used for 1% protein solution (Miller and Metzger, 1965a). However, for specimens used for the amino acid analysis, the protein concentrations were determined using the spectrophotometric method of Lowry etal (1951).

2.11. AMINO ACID ANALYSIS

2.11.1. Introduction:

The qualitative and guantitative estimation of each of the alpha amino acids which compose a mixture, e.g. protein hydrolysate, may be achieved through the use of chemical, physicochemical, microbiological, biochemical, or chromatographic techniques. The chemical approach, which was employed virtually exclusively during this century, consists of the preliminary separation of each of the component amino acids and its subsequent estimation either gravimetrically or colorimetrically. Reagents which are utilized for the colorimetric determination

of amino acids may be classified as either specific or general. Specific color reagents are those which give color formation by either interaction with a particular moiety in the side chain of a given amino acid e.g., the guanidine group of arginine, the phenolic ring of tryrosine, the imidazole nucleus of histidine, or sulfhydryl function of cysteine. General color reagents, on the other hand, induce reaction which depend upon the presence of an α-amino function, on carboxyl group and/or an α-hydrogen atom, and hence are common to the entire class of α-amino acids. The most important of these reagents is tri keto hydrindene hydrate, the trade name which is ninhydrin which on heating with amino acids facilitate a colorimetric determination (Rehuman, 1910, 1911 ; Moore and Stein, 1949). However, the interaction of aqueous solution of α-imino acids proline and hydroxyproline with excess ninhydrin led to the formation of a yellow coloured compound (Grassmann etal, 1934 ; Johnson and McCalid, 1957, 1958).

The measurement of the color density is effected at 570 nm for all amino acids with the exception of proline and hydroxyproline, which are measured at 440 nm. In 1949 Stein and Moore designed a chromatographic separation of the amino acids which allowed the guantitation of all the amino acids in yields frequently exceeding 99% of the total amino acid content. In the last few years this method has been further improved and refined in such a manner that the amino acid analysis of the protein hydrolysate can be performed automatically.

2.11.2. PRINCIPLE OF THE AMINO ACID TECHNIQUE (MOORE AND STEIN,1958):

The complete hydrolysis of protein may be accomplished by the use of strong acid, strong base, or proteiolytic enzymes as catalysts.

The use of constant boiling HCl 6N at 110°C for 12-96 hours, the peptide bonds are quantitatively hydrolysed (relatively long periods of hydrolysis are required for the complete liberation of valine, leucine and isoleucine) (Smith etal., 1954 ; Harris etal.,1956). Yielding the amino acid hydrochlorides. Tryptophan is completely destroyed in this process and small losses of cysteine, serine and threonine (Rees, 1946 ; Hirs etal., 1954 ; Noltmann etal., 1965). Following the complete hydrolysis of protein, the analysis is complete by obtaining a quantitative analysis for each of the amino acids. The separation of amino acids is most readily accomplished by employing ion exchange chromatography. The amino acid mixture is placed on the column at pH 2.2 - 3, when the individual amino acids are positively charged. The column is then developed by gradually increasing the pH and the ionic strength of the buffers with which the column is washed.

These conditions gradually cause neutralization of the positive charges on the amino acids and, in addition, weaken salt linkages. Thus, as the column is developed the acidic amino acids, such as glutamic and aspartic amino acids, are removed readily from the resin, followed by the neutral amino acids, and finally, basic amino acids. The amino acids are visualized by reaction with ninhydrin. This reagent is mixed with the eluant stream in the mixing chamber. The colour is developed by passing the mixture of reagent and eluant through a reaction coil immersed in a boiling water bath (or a hot silicon fluid). The absorbance of the resulting solution is measured continuously at 570 and 440 nm as it flows through cylindrical glass cells of 2 mm bore. The output from these cells is plotted directly with a two channel, point-plot recorder. The amino acids are identified by their retention time with a precision of 100 $\pm$ 3%.

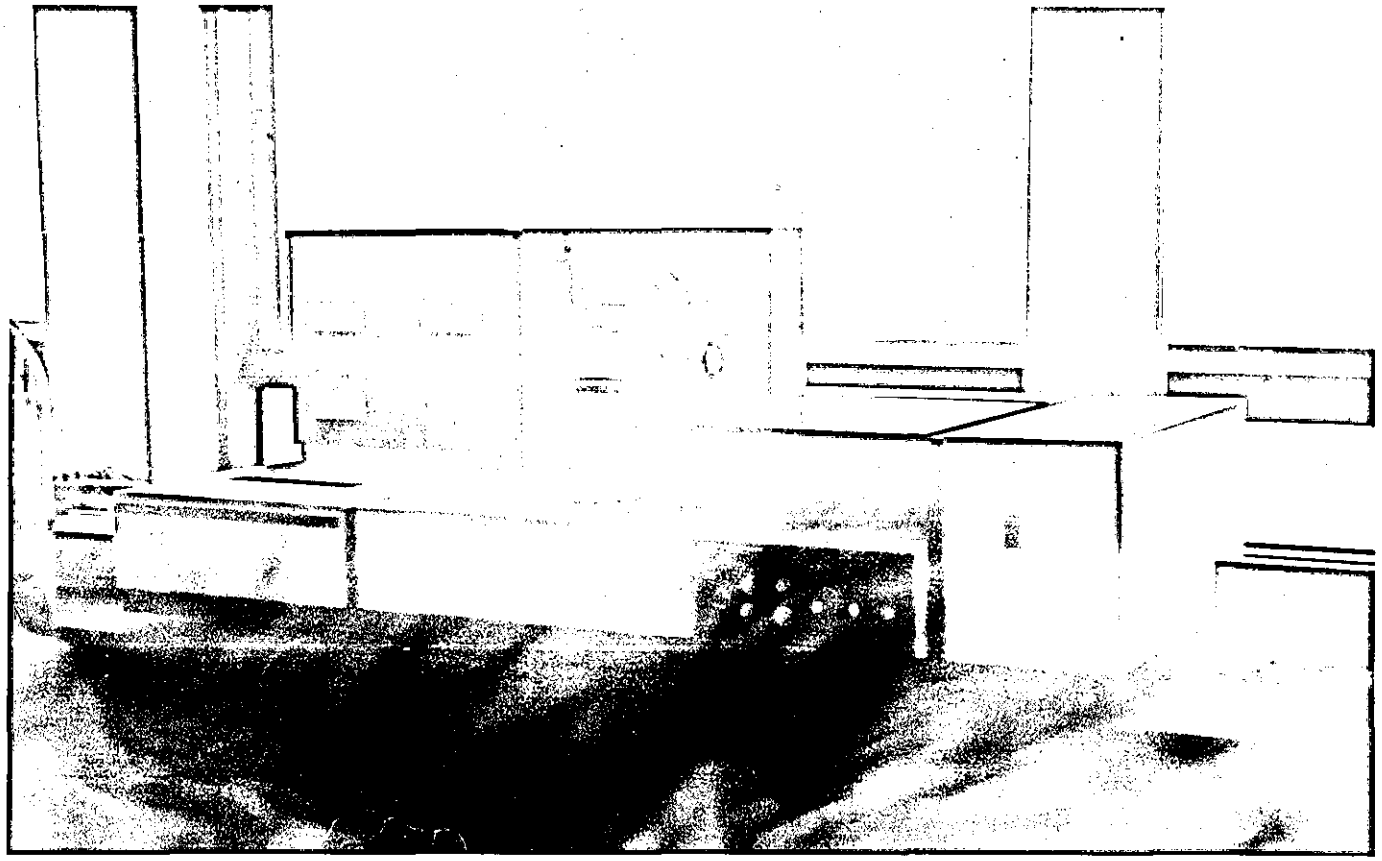


Plate No. 3. J 180 (Rank Hilger) Amino acid analyser for the determination of the amino acid compositions (acid, neutral and basic).

2.11.3. Method:

Amino acid analysis was performed on a chromaspek ion-exchanger chromatograph J 180 amino acid analyser (plate 3), (Rank Hilger Westood. Margate, Kent - England), with a single column (3 5 x 0.3 cm) for the determination of acidic, neutral and basic amino acids.

Protein or peptide (1 mg) was weighed accurately in a clean ampoule and to it was added approximately 1.5 ml 1:1 Aristar HCl: water containing 5% ($\frac{V}{V}$) of 0.1 M thioglycolic acid. In certain cases, 2 u moles of l - norleucine were also added. The solution was degassed, flushed with nitrogen, and the ampoule sealed in vacuo. Hydrolysis was performed at 110°C for 24 hours after which the ampoule was opened, and the acid removed in vacuo as described by Hirs (1967). The dried material (i.e. hydrolysate) was dissolved in a suitable volumes of 0.2 N sodium citrate buffer, pH 2.2 and the amino acids residues were determined in the hydrolysate, using the amino acid analyser.

2.11.4. Reaction of protein with performic acid:

Cystine and cysteine were both determined as cysteic acid, after treatment of the protein (or peptide) with performic acid. Performic acid was freshly prepared, as described by Hirs (1967). Protein (1 mg) was oxidized, in a hydrolysis ampoule, with performic acid (1 ml), at 0°C for four hours. After this time the solution was reduced to dryness under a vacuum with a bath temperature of 40-45°C, and the excess acid was removed by repeated evaporation after the addition of triply-distilled water. 6 normal hydrochloric acid (1.5 ml) "Aristar"-HCl (BDH Ltd.) was added to the residue (in certain cases 2 u moles of l-norleucine were also added as an internal standard), and the ampoule sealed in vacuo. Hydrolysis was at 110°C for 24 hours, after this time the ampoule

was opened and the hydrolysate was dried at 40°C in vacuo ina rotatory evaporator. The dried material (i.e. the hydrolysate) was dissolved in suitable volumes of 0.2 N sodium citrate buffer, pH 2.2 and cysteic acid was determined in the hydrolysate, using the J 180 amino acid analyser (Rank Hilger Ltd.).

CHAPTER 3.

RESULTS

Section A: 3-A-1. Characterization of the purified Waldenstrom macroglobulins (IgM) and their monomer 7S subunits (IgM_s).

All the immunoglobulins M (IgM) investigated in this work were obtained from serum or plasma samples taken from patients suffering from Waldenstrom's macroglobulinemia or a similar disorder. All the samples contained a large homogeneous immunoglobulin instead of normal heterogeneous immunoglobulin (Reisner and Franklin, 1961). The gel filtration patterns (Fig. 6,7 and 8) show that all the three purified pathological macroglobulins (IgM), show a single asymmetrical peak coming out of the gel columns at the void volume. The cellulose acetate electrophoresis of these purified macroglobulins (IgM), shows a single narrow band migrating towards the anode (+ve) in 0.05M Tris-barbital buffer at pH 8.8.

Examination of the electrophoretic patterns shows distinct differences in electrophoretic mobilities. For example, IgM "Har" showed a faster electrophorectic mobility towards the anode than the IgM "Kat", and a similar result was obtained from the macroglobulin IgM "Man". (Figures 9 - 10). A similar observation was made by Deutsch and Morton (1958), who reported the isolation of pathological IgM globulins by a two stage process, i.e. by euglobulin precipitation followed by gel filtration on sephadex G200, have marked differences in their electrophoretic mobilities on free boundary electrophoresis at pH 8.6. These results may be explained on the basis of differenes in their isoëlectric points (Deutsch, etal, 1958 ; Putnam, 1959) and in their carbohydrate contents (Virella, etal, 1976). However, Chaplin

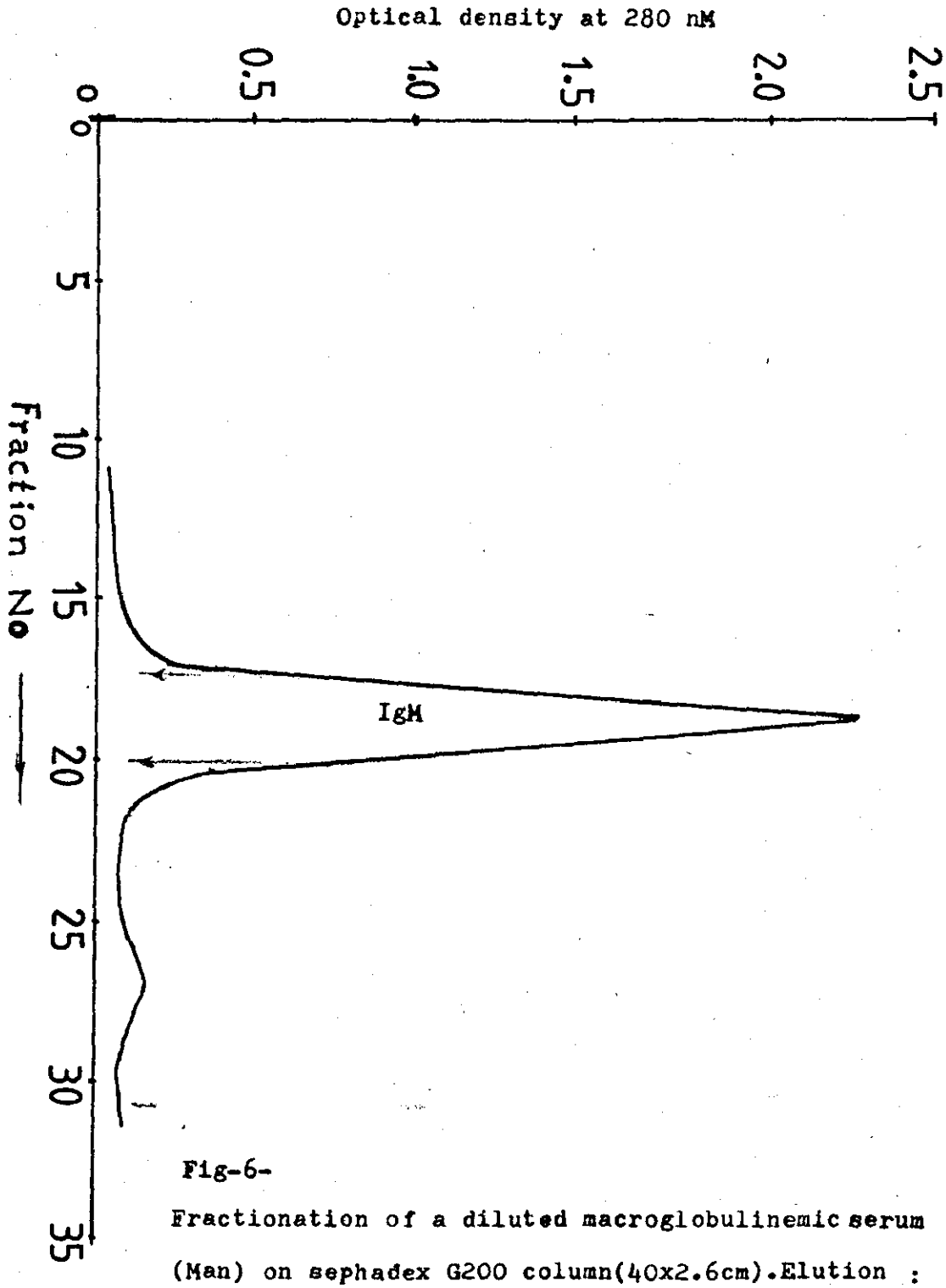


Fig-6-

Fractionation of a diluted macroglobulinemic serum (Man) on sephadex G200 column(40x2.6cm).Elution : 0.1M Tris-HCl/0.3M NaCl buffer(pH8.2).Fractions between the arrows were retained.

Fraction volume= 3ml/ tube.

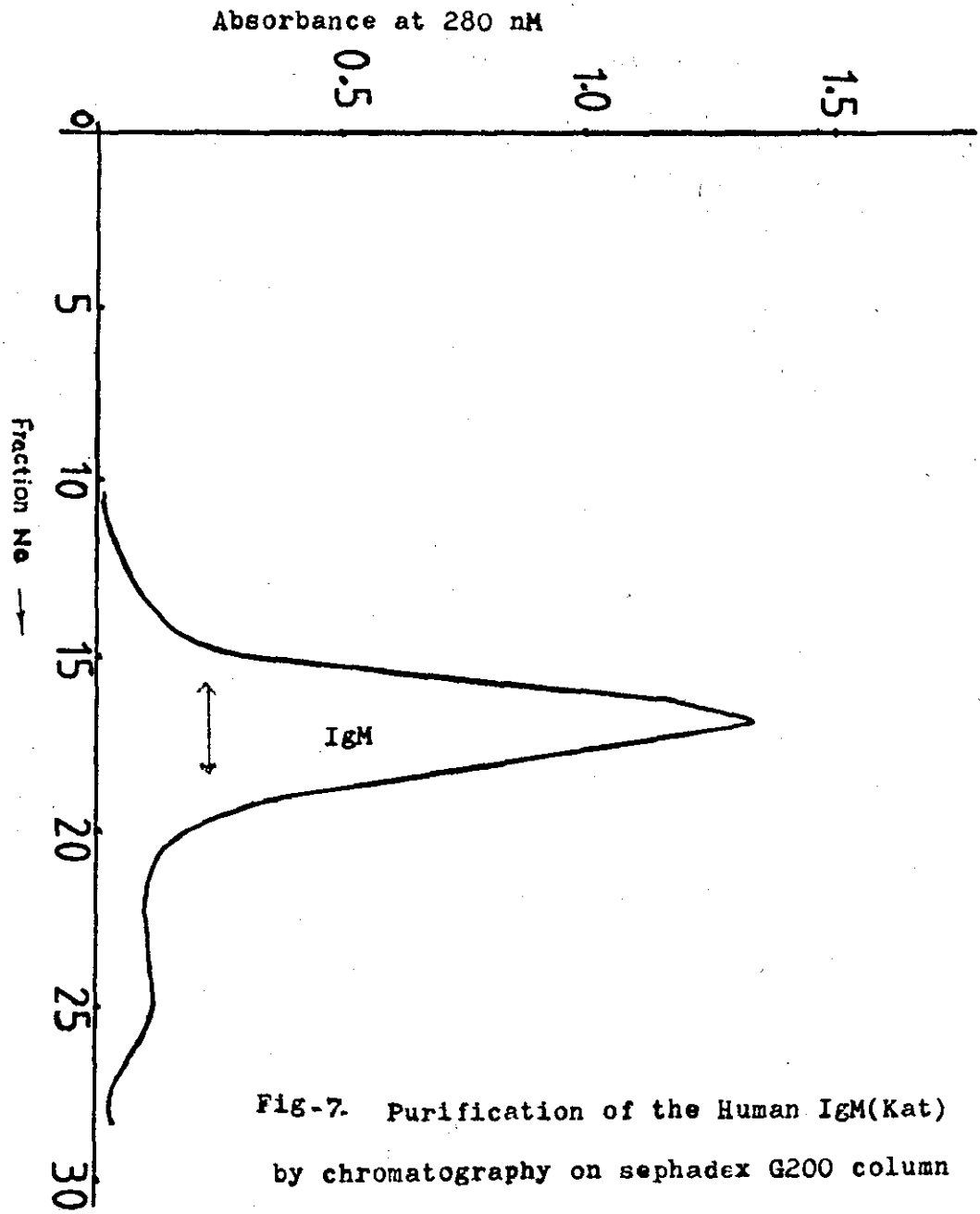


Fig-7. Purification of the Human IgM(Kat) by chromatography on sephadex G200 column (35x2.6cm) in 0.1MTris-HCl/0.15M NaCl buffer pH 8.2. Fraction volume = 3ml/tube

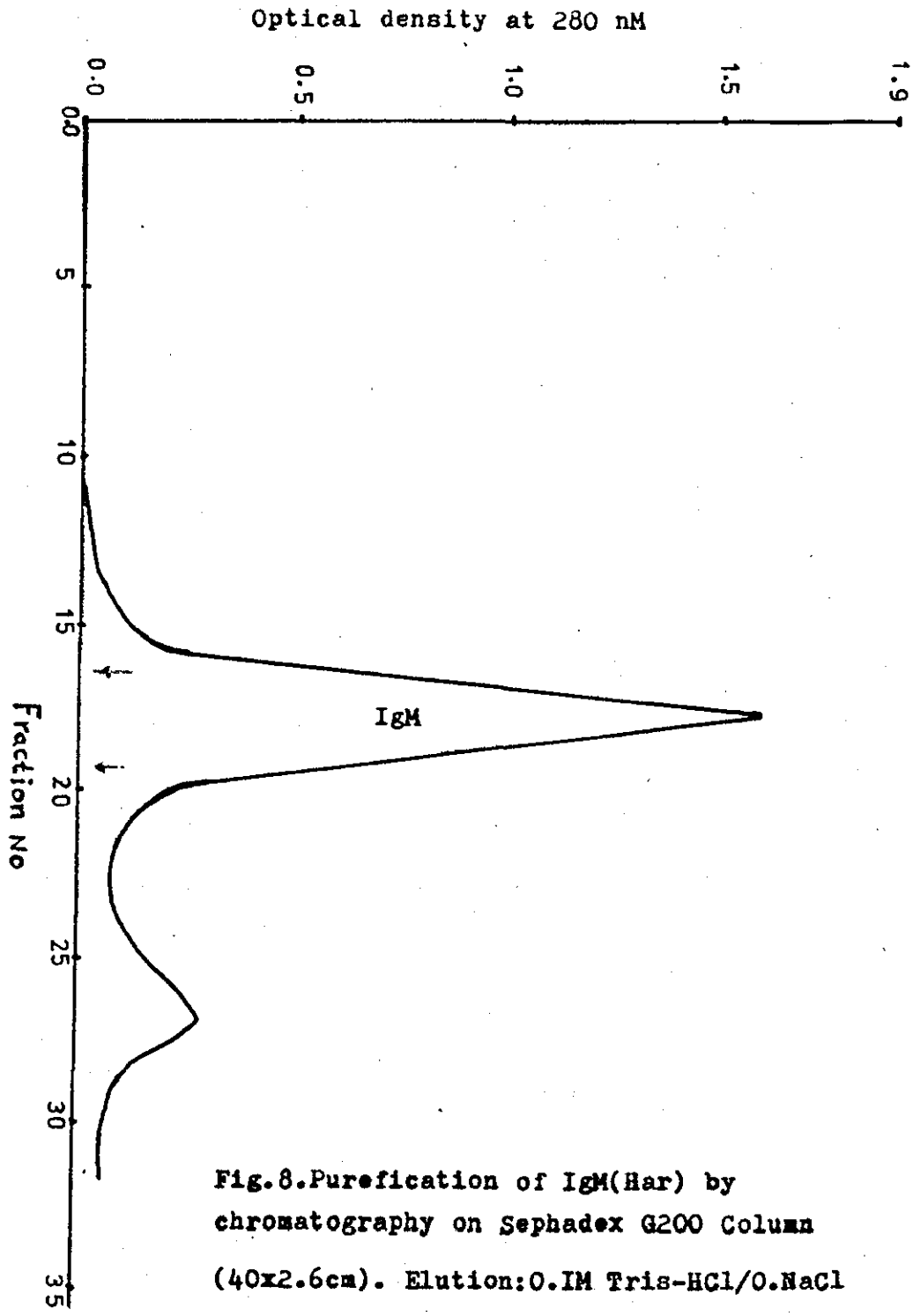


Fig.8.Purefication of IgM(Har) by chromatography on Sephadex G200 Column (40x2.6cm). Elution:0.IM Tris-HCl/0.NaCl buffer,pH 8.2.Fraction volume=3ml/tube . Fractions between the arrows were pooled and examined on Cellogel acetate membrane electrophoresis and immunodiffusion .

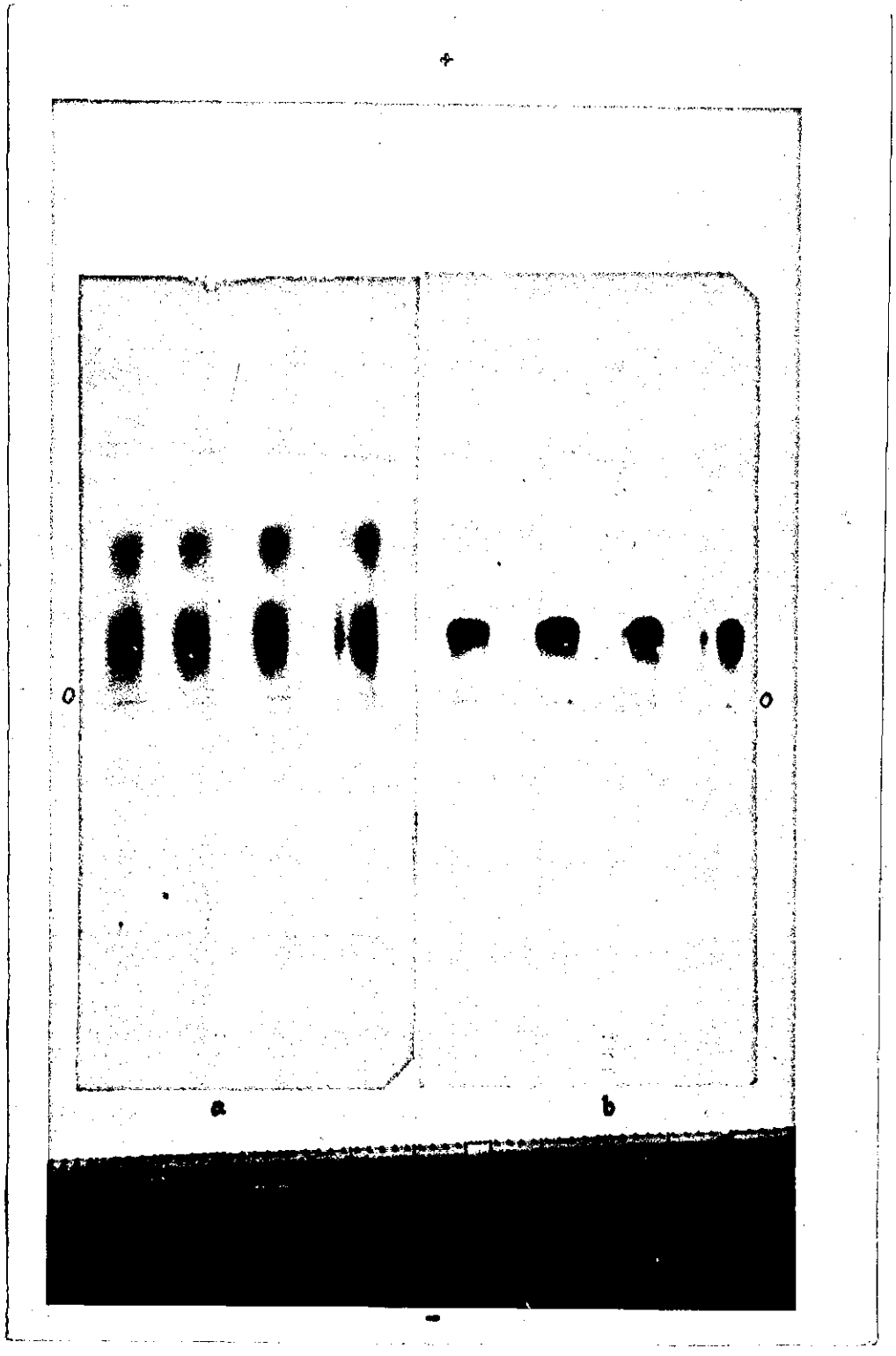


Fig. 9. Cellulose acetate electrophoresis patterns showing: (a) "Man" IgM and CNBr, and (b) "Man" IgM only.

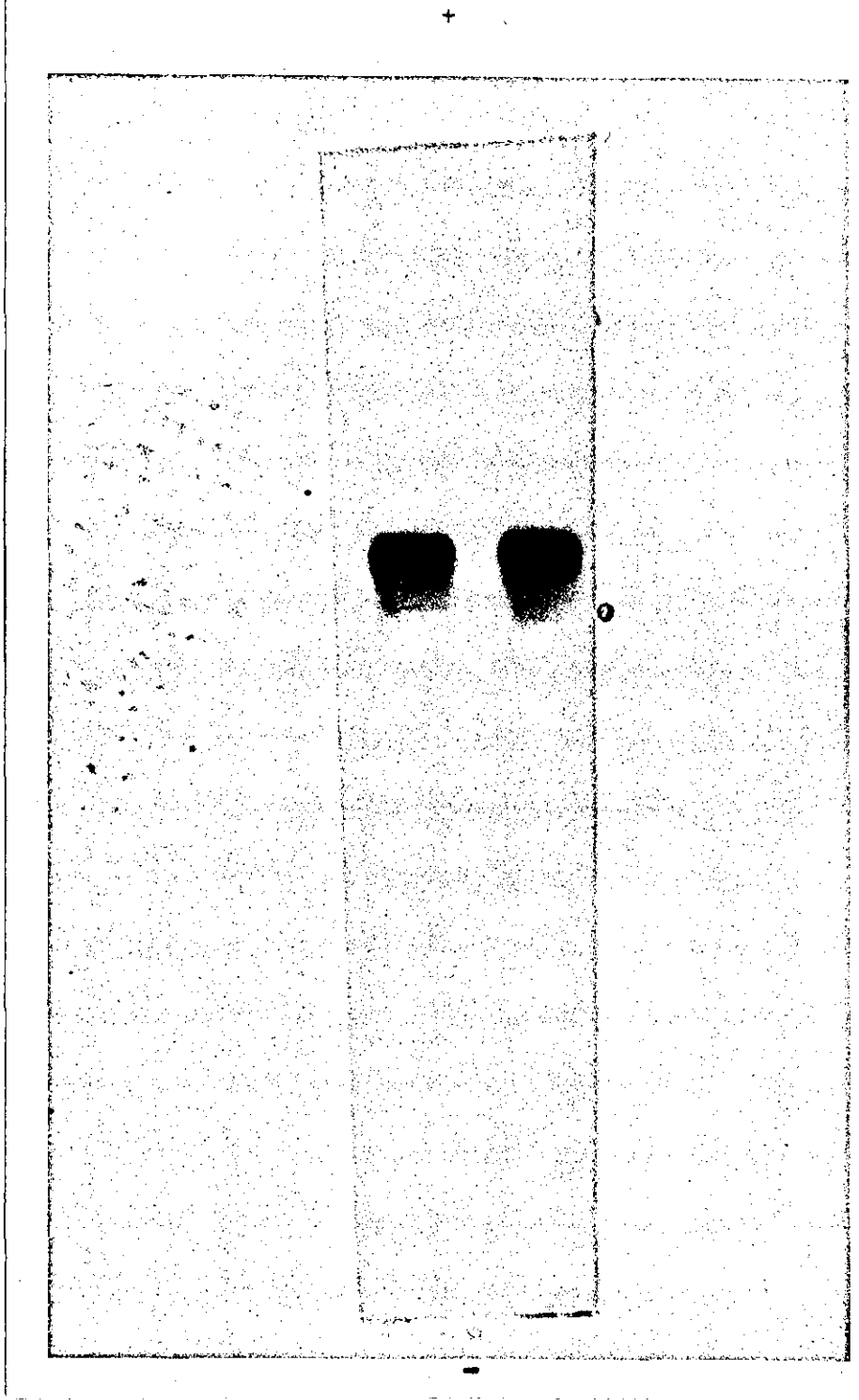


Fig.10. Cellulose acetate electrophoresis pattern of a "Har"IgM.

etal.,(1965) and Floden etal.,(1962) reported that, the isolated IgM proteins by gel filtration on Sephadex G200 contain α- β lipoproteins, haptoglobins and α_2 - macroglobulin and may also contain IgA and IgG which usually elute out within the void volume.

Anyhow , the great sensitivity of the immunoelectrophoresis and SDS polyacrylamide gel electrophoresis methods, showed that the isolated IgM proteins are completely pure i.e. no contamination with either IgA, IgG or lipoproteins. All are type K light chains.

It has been reported that the IgM proteins isolated by the two stage method (Morris and Inman, 1968., Putnam,etal., 1967) are completely pure by the criteria of immunoelectrophoresis and ultra centrifugation, even though some contamination by lipoprotein could be expected. This is an indication of the failure of lipoproteins to precipitate during the initial euglobulin fractionation (Chaplin, etal., 1965 ; Putnam,etal., 1967 ; Suzuki and Deutsch, 1967). In this work no attempt was made to isolate the 19S IgM component. In fact Deutsch and Morton (1958) reported that the purified pathological macroglobulins IgM contained about 85% of 19 S IgM.

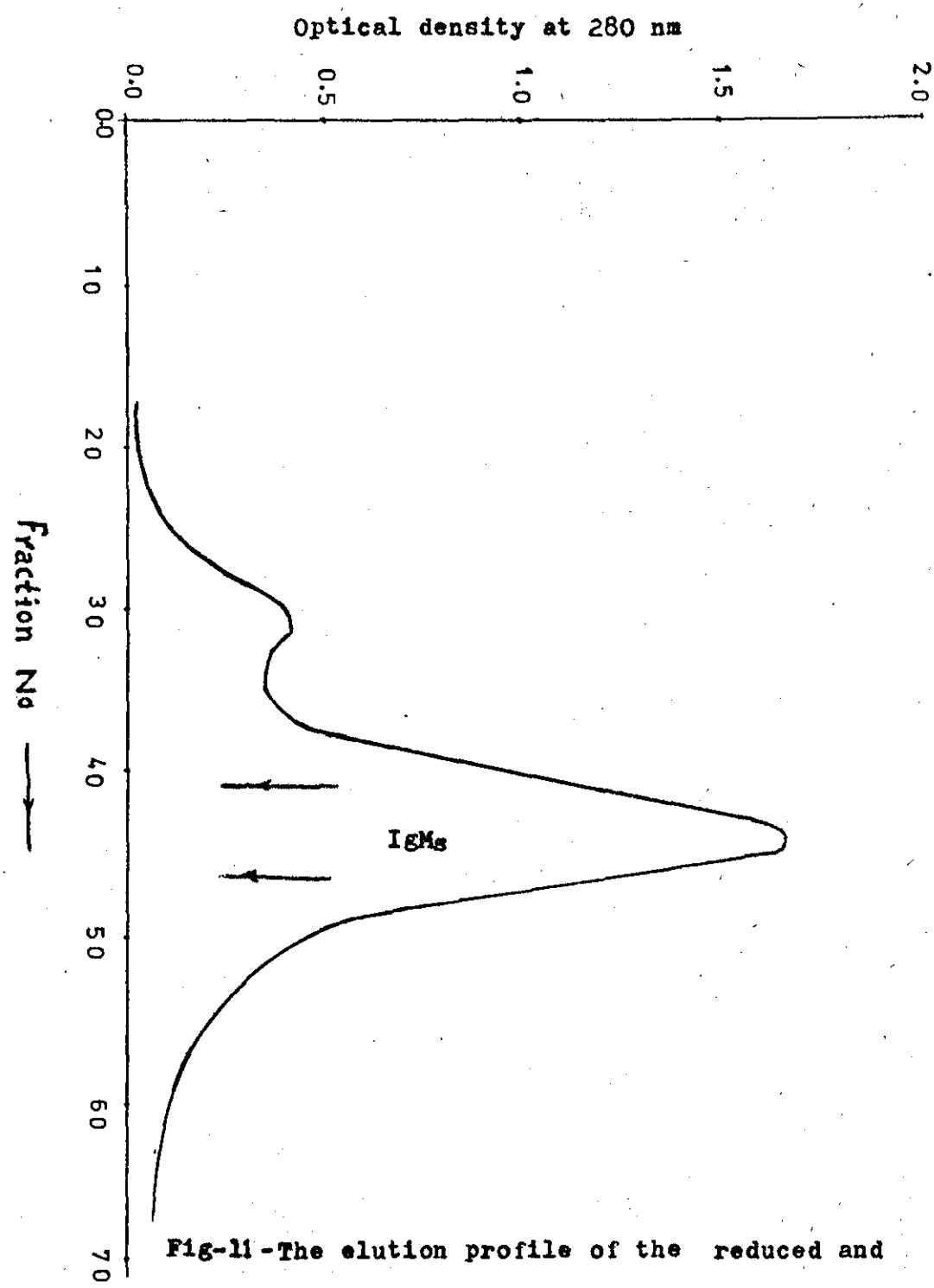


Fig-11 -The elution profile of the reduced and alkylated "Har"IgM on Sephadex G200 column (67X2.6cm) in 0.05M Tris-HCl/0.15M NaCl buffer, pH 8.6.The first peak contains incomplete reduced IgM, the second peak contains IgM_s . Fraction volume=2.8ml/tube .

3-A-2.

Purification and characterization of 7 S subunits (IgM_s).

When macroglobulin IgM is reduced in an aqueous solution with cysteine (Miller and Metzger, 1965) or with Dithiothreitol (Beale and Feinstein 1969) or with 2 - mercaptoethanol (Morris and Inman, 1968) 7 S subunits (IgM_s) are formed. According to these authors, the subunits fail to dissociate when chromatographed in the presence of 1 M propionic acid. Among these reducing agents, cysteine appeared to be the most selective one. Miller and Metzger (1965) and Beale and Feinstein (1969) reported that mild reduction of 19 S IgM with low concentrations (e.g. 20mM) of cysteine yielded solely IgM_s and the remaining intact IgM. They concluded that IgM_s thus obtained maintains its interchain disulphide bond integrity.

In this work, the 7 S subunits were prepared by using the method of Miller and Metzger (1965) as described in methods (Cahpter 2). The monomer 7 S subunits were purified from the reduced and alkylated IgM by successive passage over columns of sephadex in Tris-HCl and 1 Molar propionic acid, removing the unreduced IgM and the aggregated material, respectively.

In the first gel filtration experiment, the prepared 7 S subunits show a large single peak combined with another small peak which seems to be unreduced IgM (Fig. 11). The regel filtration of these 7 S subunits in 1 M propionic acid gave a symmetrical single peak (Fig. 12). Cellogel acetate electrophoresis at pH 8.8, of the purified IgM_s subunit shows a single bond with electrophoretic mobility nearly similar to the parent molecule. In fact, by cellulose acetate electrophoresis, it is not possible to distinguish between different protein

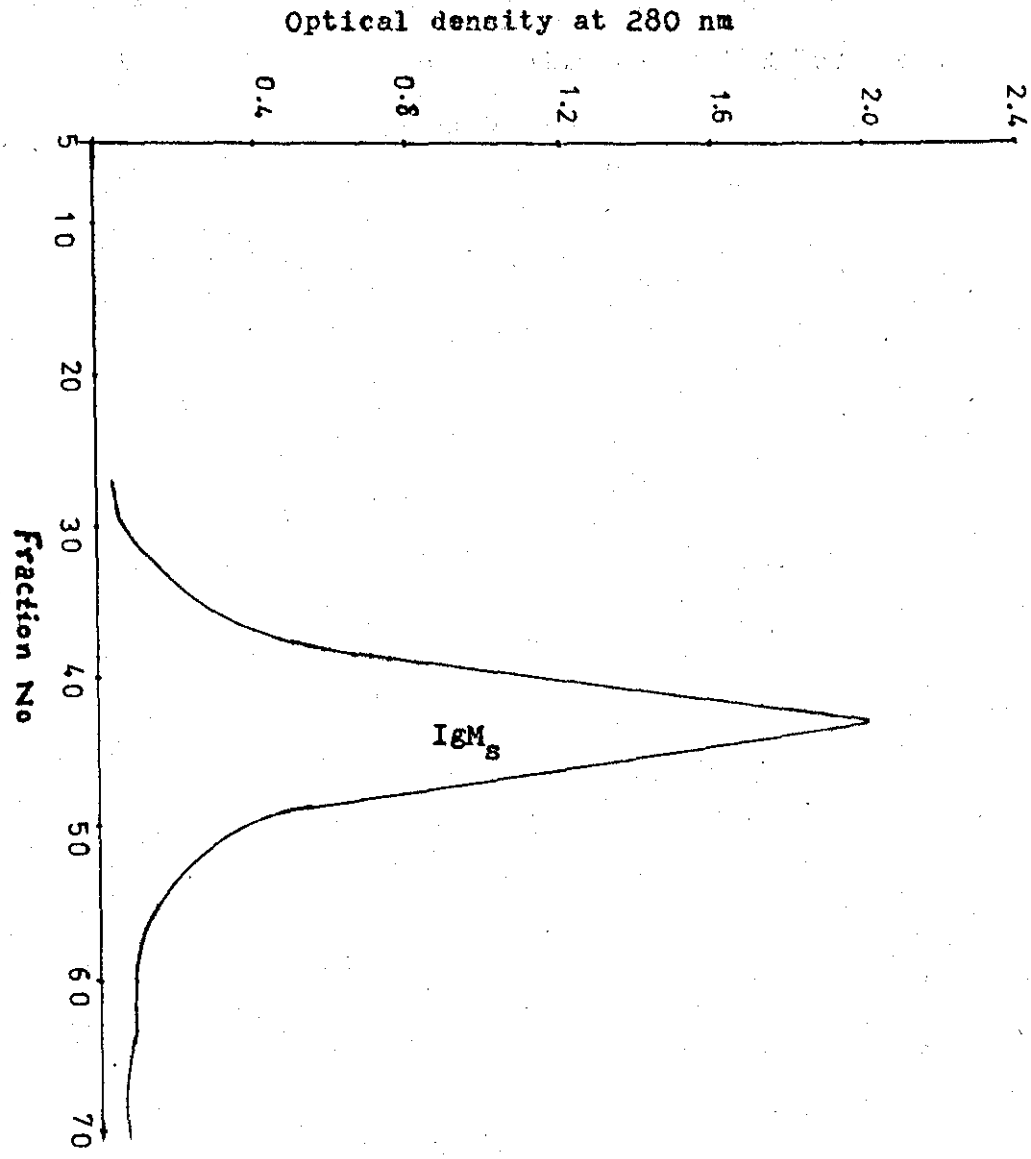


Fig-12. Regel filtration of IgM$_s$ subunit on Sephadex G200 column(65X2.6cm) in I M Propionic acid. Fraction volume=2.8ml.

components on the basis of their molecular weights, because this technique is based on the differences in electrophoretic mobility rather than on the molecular weights. By immunodiffusion, the isolated 7 S subunits (IgM_s) reacted with anti-IgM (μ chain specific) and anti-K antisera identically to the parent IgM. It can be deduced that, these 7 S subunits (IgM_s) contain both the intact heavy (μ) and light chains.

Cooper (1967) reported that, by immuno-electrophoresis, the 7 S subunit had a longer precipitin line which extended further toward the anode and diffued much faster than the 19 S IgM. in addition it extended further toward the antiserum through than the purified 19 S IgM. This indicates that the 7 S subunit has a smaller size than the 19 S IgM.

3-A-3.

Determination of the molecular weight of IgM and its 7 S subunit (IgM_s).

1. By gel chromatography.

The determination of the molecular weight of proteins by means of gel filtration has been reported by a very large number of workers (e.g. Whitaker, 1963 ; Morris, 1964 ; Andrews, 1964 ; Leach and O'Shea, 1965). It is widely recognized that the gel filtration through sephadex is a powerful method for the fractionation of proteins according to their molecular weights, since fractions emerge from the column in order of decreasing molecular size. However, it is clear that the same anomalies that characterize the gel filtration behaviour of certain undenatured proteins in column experiments also apply to thin-layer gel filtration (Morris, 1964 ; Determan and Michel, 1965 ; Radola, 1968). These problems appear to result from absorptive effects (Whitaker, 1963), variable degrees of dissociation or aggregation (Andrews, 1964 ; Chao and Einstein, 1969), and/or differences in overall molecular shape from the standard proteins used for calibration (Tomasi and Kornguth, 1967 ; Chao and Einstein, 1969). The abnormal behaviour of some proteins remains inexplicable e.g. IgG- globulins (Andrews, 1965 ; Radola, 1968). These anomalies, together with the fact that not all proteins are readily soluble in dilute aqueous buffers, prompted an investigation of the gel filtration behaviour of proteins in denaturing solvents (Tanford, etal, 1967 ; Davison, 1968 ; Chao and Einstein, 1969 ; Fish, etal, 1969, 1970). Fish, etal (1969) introduced gel filtration chromatography in 6 Molar guanidine hydrochloride (Gu-HCl) for the fractionation and estimation of the molecular weights of proteins or protein polypeptide chains and concluded that the linearity between the distribution coefficient or the elution volume against the log-molecular weight

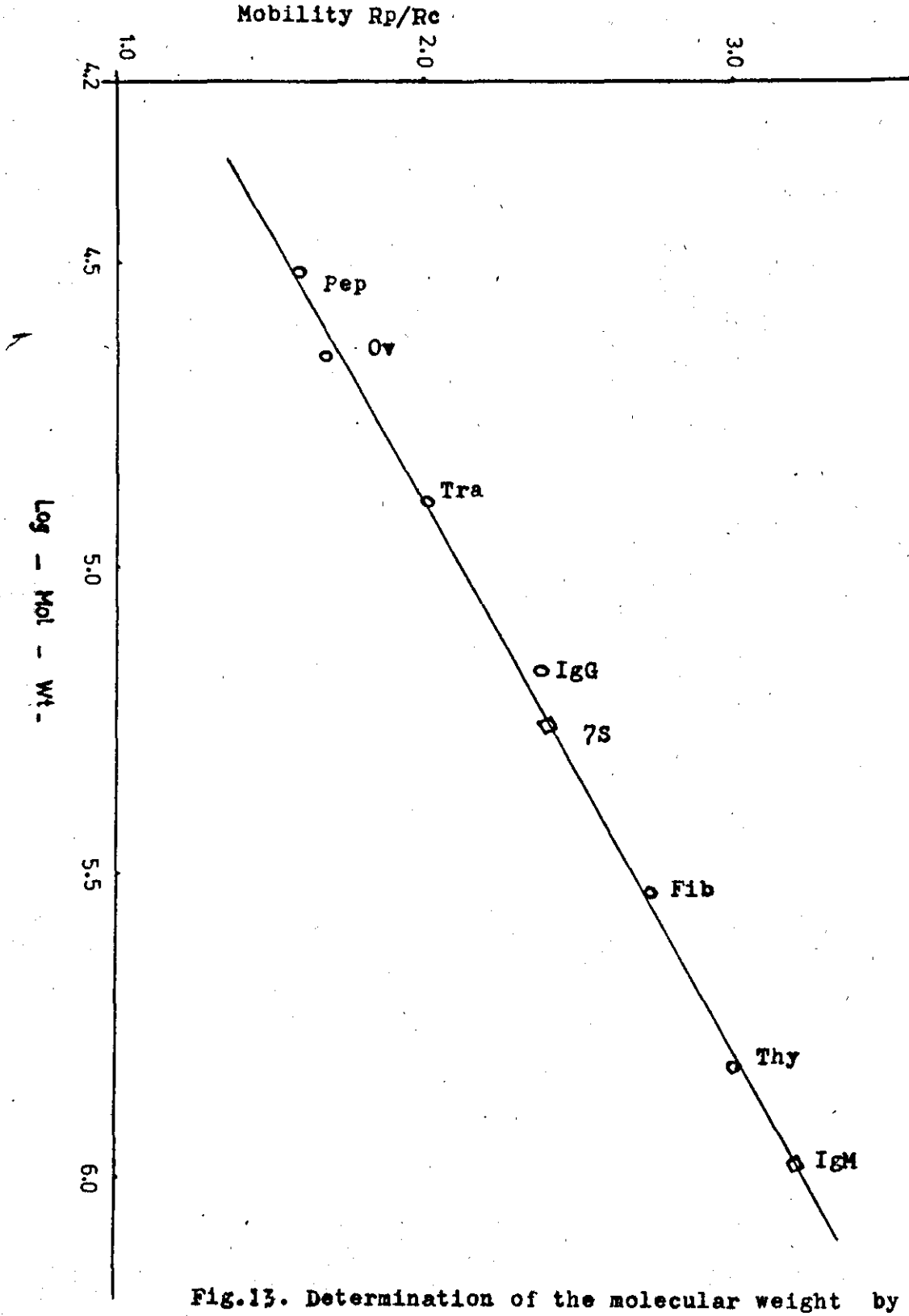


Fig.13. Determination of the molecular weight by T.L.G. The diagram represents the Human Transferren(Tra),Human IgG(IgG),Pepsin(Pep),Fibrinogen(Fib),Thyroglobulin(Thy), IgM_S Subunit(7S),"Har"IgM,Ovalbumin(Ov),Rc and Rp represent the mobility of the Cytochrome-C and the mobility of the protein respectively.

still exist. Guanidine hydrochloride (Gu-HCl) has proved to be the most powerful among commonly used protein denaturants and solubilizing agents, because of its property of abolishing non-covalent interactions of polypeptide chains. The high ionic strength of this solution (i.e. Gu-HCl) also prevents electrostatic interaction between basic proteins a sephadex. Thus, the gel migration rate is a simple function of the polymer length of the molecule and anomalies due to differences in native conformation disappear. However, proteins with all the disulphide bonds reduced in concentrated guanidine hydrochloride (Gu-HCl) solutions behave as randomly coiled polymers (Tanford, 1967). The absence of non-covalent interactions between proteins in this medium (i.e. Gu-HCl) ensure that aggregation of polypeptide chains does not take place, except in-so-far as disulphide bonds might prevent complete unfolding of certain parts of molecule (Tanford, 1968, 1969). To eliminate these problems, it may be essential to reduce the disulphide bonds by the addition of sulphydryl reducing agents, in addition to use of an internal standard whose molecular weight in known.

The details of the T.L.G. methods used for the determination of the molecular weights were described in chapter 2. The molecular weights obtained for IgM "Har" and its monomer 7 S subunits compared with the standard proteins used as shown in table 3 and figure 13.

T A B L E 3.

Molecular weight determination by thin-layer gel filtration on sephadex G200 in 0.1 M Tris-HCl/0.3 M NaCl buffer (pH 4.5) containing 6 M Gu-HCl. The following proteins were used to obtain the calibration curve for determination the molecular weights of "Har" IgM, and its 7 S subunit using T.L.G.. The experimental details are given in chapter 2.

Protein	Molecular weight	Log-Molecular weight
Cytochrome - C	12,400	4.0934
Pepsin	35,000	4.5440
Ovalbumin	43,000	4.6334
Human Transferrin	76,600	4.8842
γ - globulin(IgG)	150,000	5.1760
Thyroglobulin	670,000	5.8260
Fibrinogen	340,000	5.53140
"Har" IgM	Estimated 933,254	5.97
"Har" 7 S subunit(IgM_s)	" 186,209	5.27

3-A-4.

Determination of the molecular weight of 7 S subunit (IgM_s).

1. By Disc-electrophoresis.

Although both column and thin-layer gel filtration techniques yield reasonably accurate molecular weight estimates for many proteins, neither procedure is universally applicable to all proteins. The sensitivity of Disc-electrophoresis in sodium dodecyl sulphate (SDS) appears to be somewhat better than that of thin-layer gel filtration and gel filtration in columns (Shapiro,etal, 1967 ; Weber and Osborn, 1969 ; Wright,etal, 1971).

3-A-4-1-1.

Disc-electrophoresis in the presence of SDS.

A technique has been devised (Davis, 1964 ; Shapiro,etal, 1966, 1967 ; Shapiro and Maizel, 1969) for the estimation of molecular weight of proteins and polypeptide chains by empirical approach based on the determination of the rate of migration on poly acrylamide gel electrophoresis in the presence of sodium dodecyl sulphate (SDS). The reliability of the method has been demonstrated by Weber and Osborn (1969) who studied forty (40) proteins with well characterized polypeptide chains, and showed that in no case the technique gave results which were in disagreement with generally accepted values obtained by other methods.

All proteins when treated with sodium dodecyl sulphate (SDS), are converted to a complex having strongly negative charges. The mobilities of these complexes in free solution, are almost identical. Electrophoresis carried out in gels with pores small enough to restrict mobility results in a pattern of bands in which the observed mobility

is almost linear to the molecular size of the protein and is unaffected by its original charge. Shapiro, etal.,(1967) were the first to take advantage of these facts as a basis for the estimation of molecular weight. Using a number of proteins of known molecular weights they were able to demonstrate the existence of a linear relationship between molecular weight or log-molecular weight and relative mobility of the protein. These observations have been confirmed and extended by many workers using continuous (Weber,etal., 1972 ; Payne, 1973 ; Dunker,etal., 1969 ; Shapiro,etal., 1969) and discontinuous (Ames, 1974 ; Laemmli, 1970 ; Neville, 1971) buffer systems. In practice therefore, the molecular weight of a protein may be obtained from its mobility, using a plot of mobilities of marker (standard) proteins against the logarithm of their known molecular weights.

Glycoproteins show a decreased SDS binding ratio, that generally leads to decreased electrophoretic mobility and erroneous weight determinations (Pitt-Rivers,etal., 1968 ; Segrest,etal.,1971 ; Bretscher 1971 ; Schubert, 1970 ; Glorsmann,etal., 1971 ; Voyles,etal., 1974 ; Banker,etal., 1972 ; Anderson, etal.,1974). The presence of disulphide bonds limits unfolding of polypeptide chains and may lead to decreased SDS binding with consequent lower mobilities (Pitt-Rivers and Impiombato, 1968 ; Reynolds and Tanford, 1970 ; Griffith, 1972). Pitt-Rivers and Impiombato (1968), showed that, much less SDS is bound by proteins containing disulphide (S-S) bonds, and this reduced the migration of the protein in the gels, which affected the estimation of its molecular weight. Complete binding between such a protein and SDS can be acheived after the reduction of the disulphide bonds by mercapto-ethanol or dithiothreitol.

Dunker, etal,(1970) reported that the apparent molecular weights

generally fell within 5-6% of literature values. Reynolds and Tanford (1970) found that SDS monomer concentration higher than 8 x 10^{-4}M, 1.4 gm SDS would bind to 1 gm of protein, resulting in a complex of rod-like shape, the length of which varies uniquely with the molecular weight of the protein moiety. Because of the high level of binding of SDS, the charge contribution of the protein will rarely be more than 10% of the charge introduced by the detergent binding. However, protein molecules with exceptionally high net charge are expected to show deviations from the typical molecular weights versus mobility relationship. Thus histones with their very high positive net charge show the usual SDS binding (Reynolds and Tanford, 1970), but typically low electrophoretic mobilities are found at pH 7.2. However, an increasing number of proteins have been shown to behave anomalously on SDS-polyacrylamide gel. Examples of such anomalies have been described by Banker, etal., (1972) ; Anderson, etal., (1974) and Rayim, etal., (1971).

However, the estimated molecular weights of Kat IgM and its 7 S subunit (IgM_s) using T.L.G. and SDS polyacrylamide gel (5%) repectively has given us a molecular weight of 900,000 and 180,000 for IgM and 7 S subunit repectively.

Small polypeptide chains of molecular weights less than 15,000, behave abnormally on SDS gels (Fish, etal., 1970).

Estimation of the molecular weight of the purified 7 S subunit (IgM_s) was carried out on a 5% polyacrylamide gel in sodium dodecyl sulphate without reducing agent - essentially as described by Weber, etal., (1972). The results obtained together with protein markers used in this experiment are summarized in table 4, and figure 14. The apparent molecular weight of IgM_s was 187,000 $\pm$ 8,000.

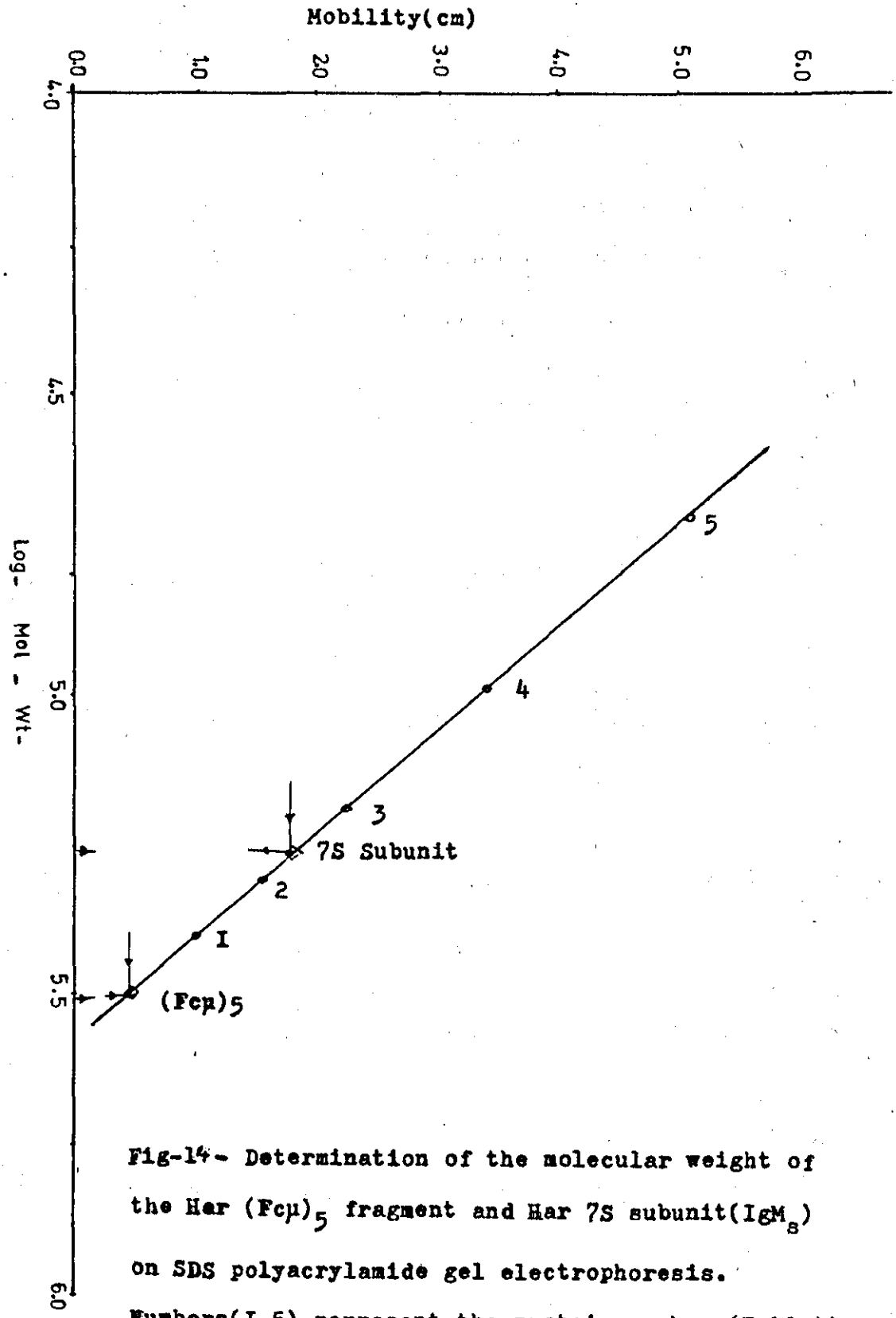


Fig-14- Determination of the molecular weight of the Har $(Fc\mu)_5$ fragment and Har 7S subunit(IgM_s) on SDS polyacrylamide gel electrophoresis. Numbers(I-5) represent the protein markers(Table4).

TABLE 4.

The following protein markers (B.D.H. Ltd.) were used to obtain the calibration curve for determination the molecular weights of IgM fragments produced by chemical and enzymatic digestion on SDS-polyacrylamide gel electrophoresis.

Protein Markers	Molecular weight	log-molecular weight
Monomer	53,000	4.7243
Dimer	106,000	5.0253
Trimer	159,000	5.2014
Tetramer	212,000	5.3263
Pentamer	265,000	5.4232
Hexamer	318,000	5.5024
Har $(Fc_{\mu})_5$ (trypsin 65°C	Estimated 323,594	5.51
Har 7 S subunit (IgM_S)	Estimated 186,209	5.27

Discussion: The method of electrophoresis in SDS has been used by many investigators.

Control experiments confirm that the electrophoretic process does not normally introduce artifacts, but accurately reflects the state of the sample, whether prepared from soluble molecular weight standards (Shapiro, etal, 1967; Weber and Osborn, 1969; Dunker and Rueckert, 1969 ; Smith, 1976 ; Payne, 1976) or dissolved membranes (Strauss, etal, 1968; Keihn and Holland, 1968; and Berg, 1969). There are minor reservations attached to this conclusion. Overloading the gel results in broadened bands that distort the pattern. Such overloaded bands increase the mobilities of minor components running just ahead of them and, in some cases, entrap material at their hyper sharp leading edges. Another reservation derives from the possibility that some artifacts due to intermolecular association may occur; these have not been excluded in every case. As suggested by Allison and Humphrey (1952) and Smithies (1962), electropheresis can be useful tool for the approximation of molecular weight. Sodium dodecyl sulphate (SDS) minimizes the native charge differences in the proteins and that all proteins migrate as anions as the result of complex formation with SDS. The extensive disruption of hydrogen, hydrophobic, and disulphide linkages by SDS and mercaptoethanol results in the quantitative solubilization of many relatively insoluble proteins. These factors and the ease of polyacrylamide technique strongly recommend it as the electrophoretic method of choice.

Early investigations by Deutsch and Morton (1958) and Putnam (1959) have indicated that pathological macroglobulins from different sera have different sedimentation coefficients, electrophoretic mobilities as well as isoelectric points. Therefore their molecular weights and

chemical compositions may differ significantly. The values obtained here are in good agreement with other recent determinations. Putnam, etal.,(1973) found a value of 950,000 for IgM ou and 190,000 for its monomer 7 S subunit (IgM_s),and Seon and Pressman (1976) reported a value of 950,000 for the macroglobulin IgM "Wider" and its 7 S subunits (IgM_s) had a molecular weight of 185,000. Miller and Metzger (1965) obtained a figure of 890,000 for macroglobulin IgM (R.E.W) and its monomer 7 S subunit had a molecular weight of 185,000, in each case for the pathological IgM sample. Macroglobulin IgM "Man" used in this work had a molecular weight of about 950,000 (Miller, 1968).

The molecular weight for the pathological IgM "Har" and its 7 S subunit (IgM_s) investigated in this work were quite close to $9\text{-}9.5 \times 10^5$ and $18\text{-}19 \times 10^4$, repectively. However other workers have provided evidence for a wider range of values (620,000 - 1,200,000) for intact IgM and reductive subunit IgM_s (Filitti-Wurmser, etal., 1968, 1969). We can not provide an adequate explaination for these discrepancies at this this juncture. While these differences might be due to the presence of the known subclasses of μ heavy chains (Mackenzie, etal.,1969 and Franklin and Frangione, 1967, 1969) or the subgroups of IgM with different carbohydrate content (Davie and Osterland, 1968) or may be due to the presence of monomeric, dimeric and polymeric forms of macroglobulin IgM molecules (Stobo,etal, 1967 ; Solomon, 1969 ; Parr,etal., 1974), they seem to be outside the anticipated variation for which such differeces would account.

In summary, the data presented in this section provides an additional support for a five - subunit model of IgM, and is most compatible with the theory that the IgM subunit is potentially bivalent /i.e. formed from heavy (μ) and light (K or L) chains/ as well as the

native IgM molecule. This implies that the parent macroglobulin IgM molecule has ten potential combining sites, and offers some evidence regarding the distribution of disulphide linking the chains to each other. The molecular weight of the whole IgM molecule being about 950,000 and that there are only five subunits, witheach subunit (IgM_s) having a molecular weight of about 185,000 significantly higher than that of normal IgG. However, the presence of other polypeptide chains (e.g. J chain) cannot be ignored in calculating molecular weight as their existence in the structure has not been investigated in our work.

Section B: 3-B-1. Cyanogen bromide digestion of IgM.

3-B-1-1.

Results:

The CNBr cleavage of IgM was carried out in 0.05 M HCl for 24 hours as described in materials and methods (Chapter 2). The lyophilized product was passed through a column of sepharose 4B, in order to remove all by-product materials, the column was washed with 6 M Gu-HCl as described in methods. The eluant was freeze-dried and dissolved in a small volume of 6 M Gu-HCl (pH 4.5), and then the solution was re-chromatographed on sepharose 4B. The fraction profile of the cyanogen bromide digestion on sepharose 4B is shown in figures 15 and 16. Three peaks can be clearly distinguished, and are marked A, B and C. The first two peaks (i.e. A and B) were found to be homogeneous by thin-layer gel filtration.

The proteins present in fraction A and B appear to have similar electrophoretic mobilities on cellulose acetate electrophoresis in 0.05 M Tris-barbital - sodium barbital buffer containing 6 Molar urea at pH 8.8. The results are shown in Figures 9 ,17 and 18.Futher characterization of these fractions was carried out as follows. Inspection of the electrophoretic mobilities: Fraction A showed a faster electrophoretic mobility towards the anode than fraction B.

On incubation in aqueous solution at neutral pH, fragment A showed a strong aggregation and this phenomenon cannot be avoided by the addition of 1% SDS prior to disc-electrophoresis. On the other hand fragment B showed only a slight aggregation at pH 7.0. By cellulose acetate electrophoresis, the treated IgM (i.e. IgM/CNBr) showed two well separated bands (Fig.9,17& 18) instead of a single band as shown by the native untreated IgM proteins. This indicates that the cleavage

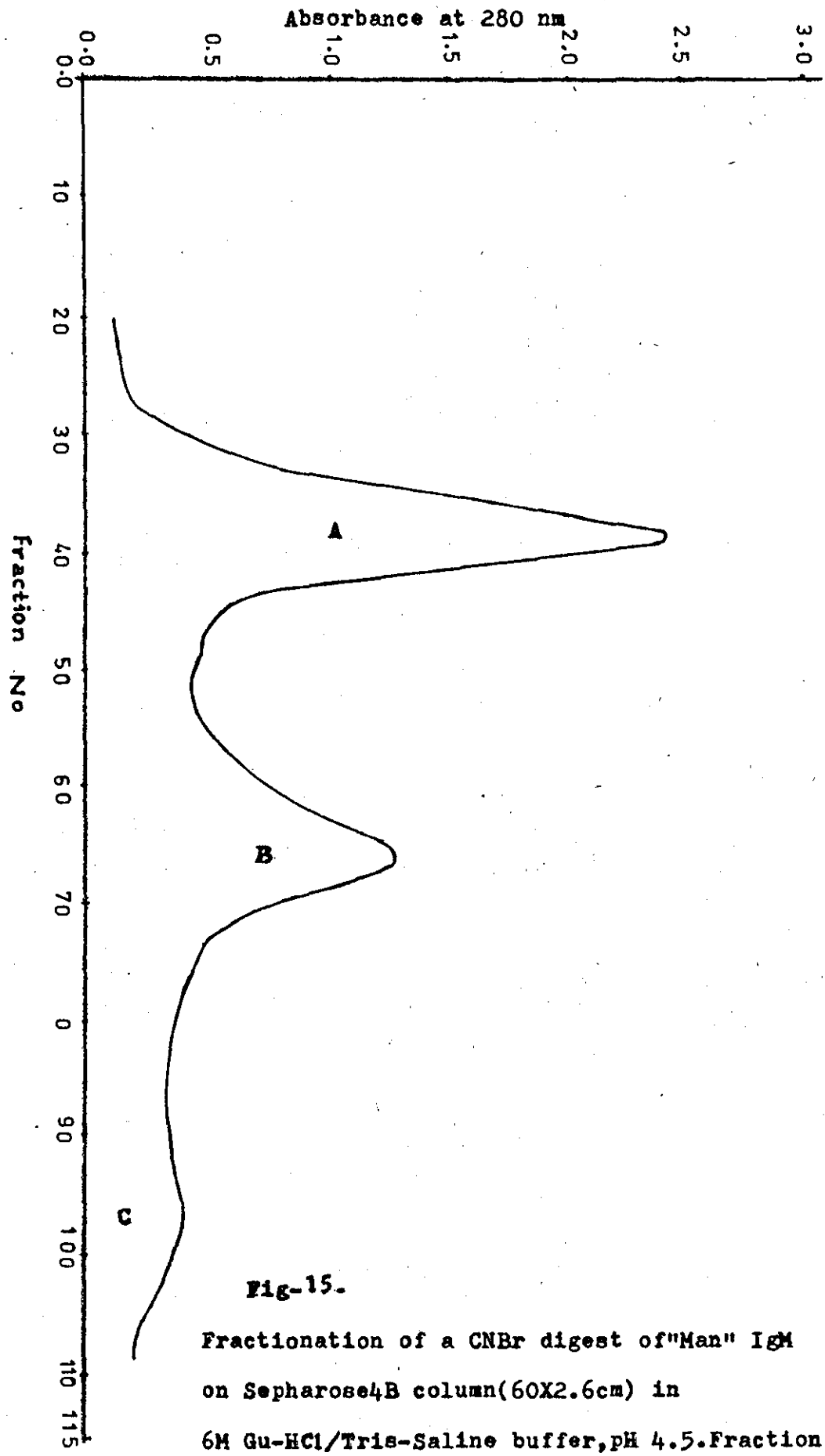


Fig-15.

Fractionation of a CNBr digest of"Man" IgM on Sepharose4B column(60X2.6cm) in 6M Gu-HCl/Tris-Saline buffer,pH 4.5.Fraction volume= 3ml/tube .

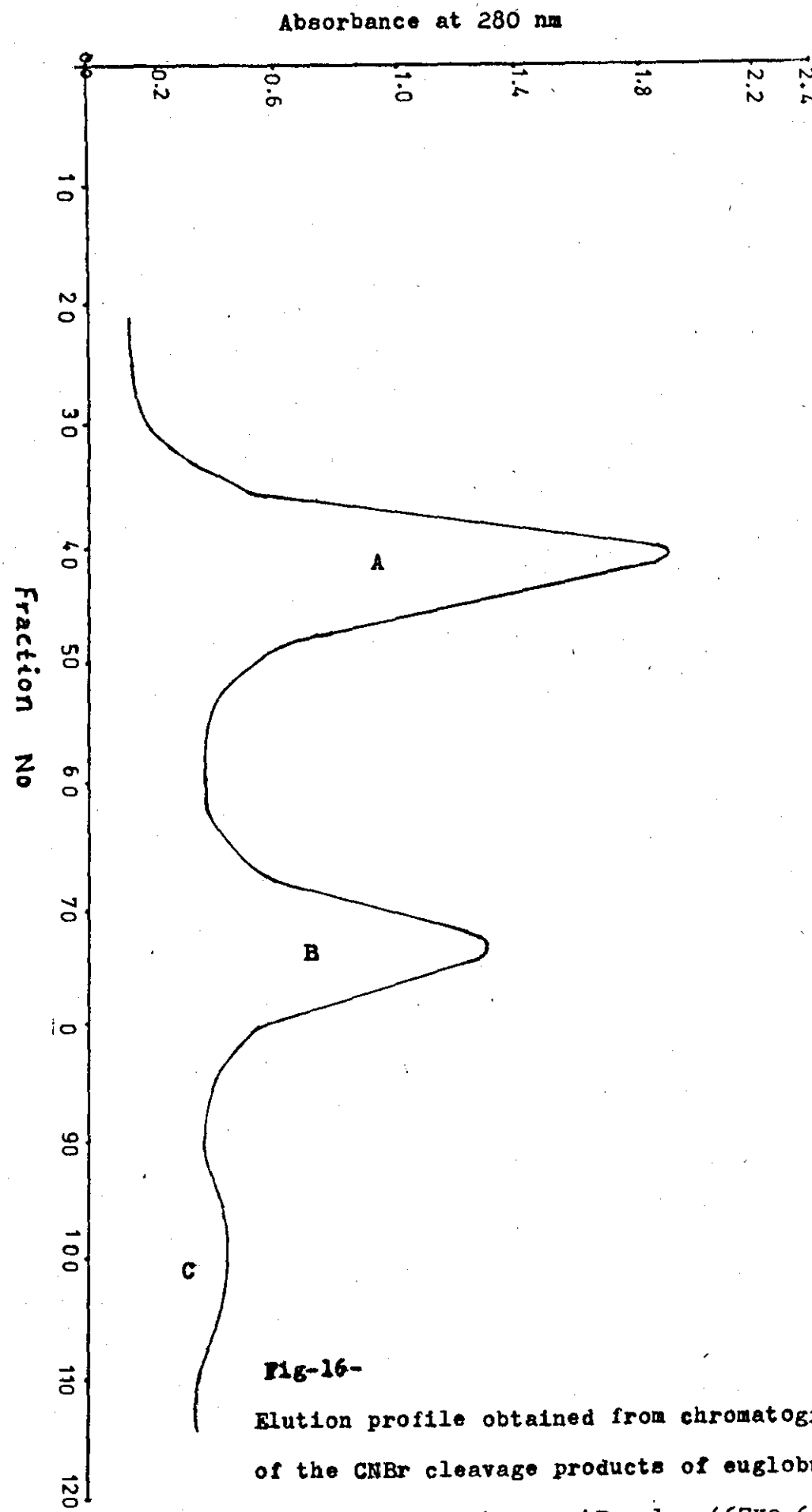


Fig-16-

Elution profile obtained from chromatography of the CNBr cleavage products of euglobulin "Har" IgM on Sepharose 4B column(67X2.6cm) in 6M Gu-HCl/Tris-saline buffer, pH 4.5. Fraction volume= 3ml/tube .

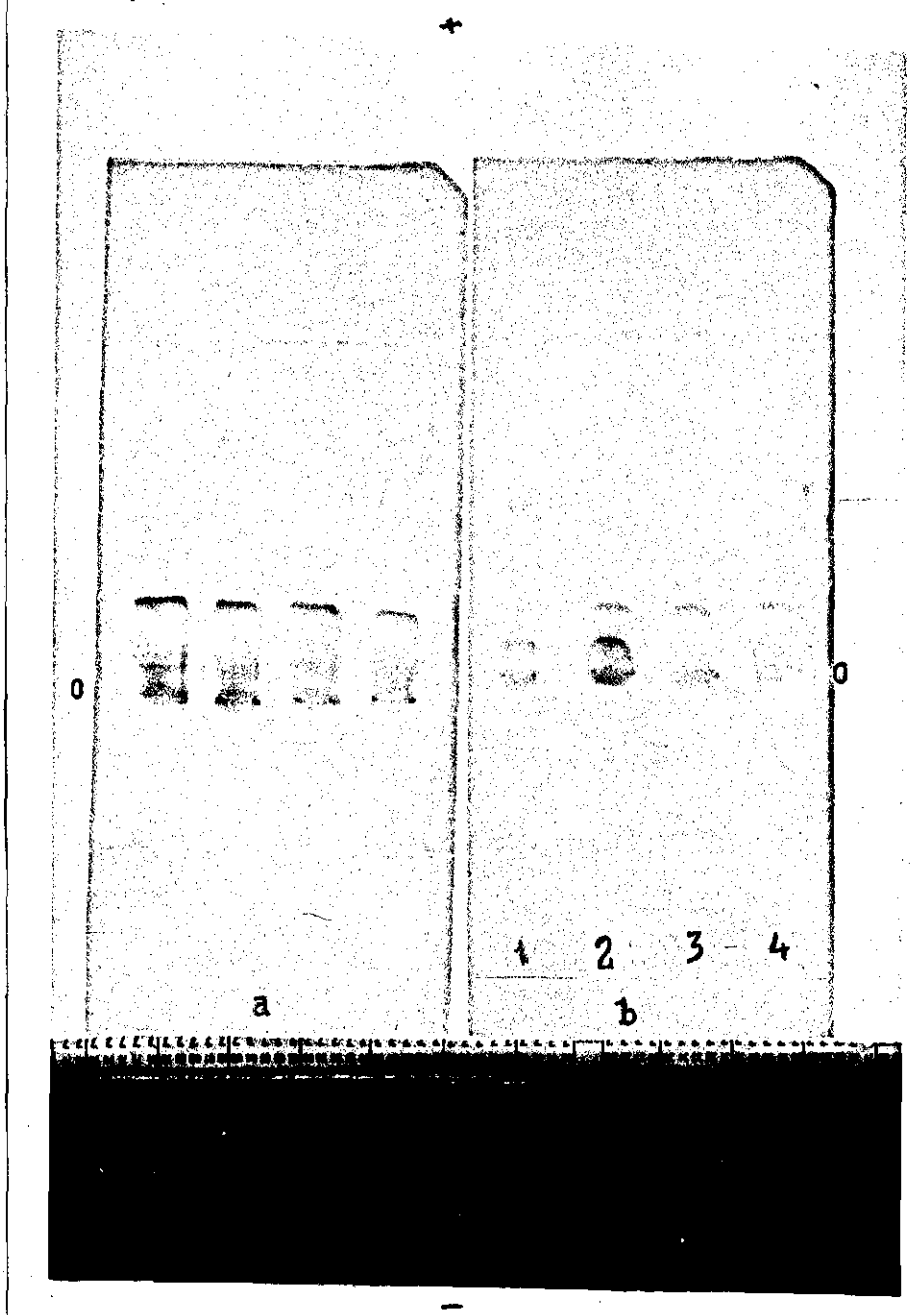


Fig.17. Cellulose acetate electrophoresis pattern comparison of:

(a) "Har" IgM and CNBr.

(b) 1. "B" fragment, 2 "Har" IgM and CNBr, 3 and 4 "A" fragment.

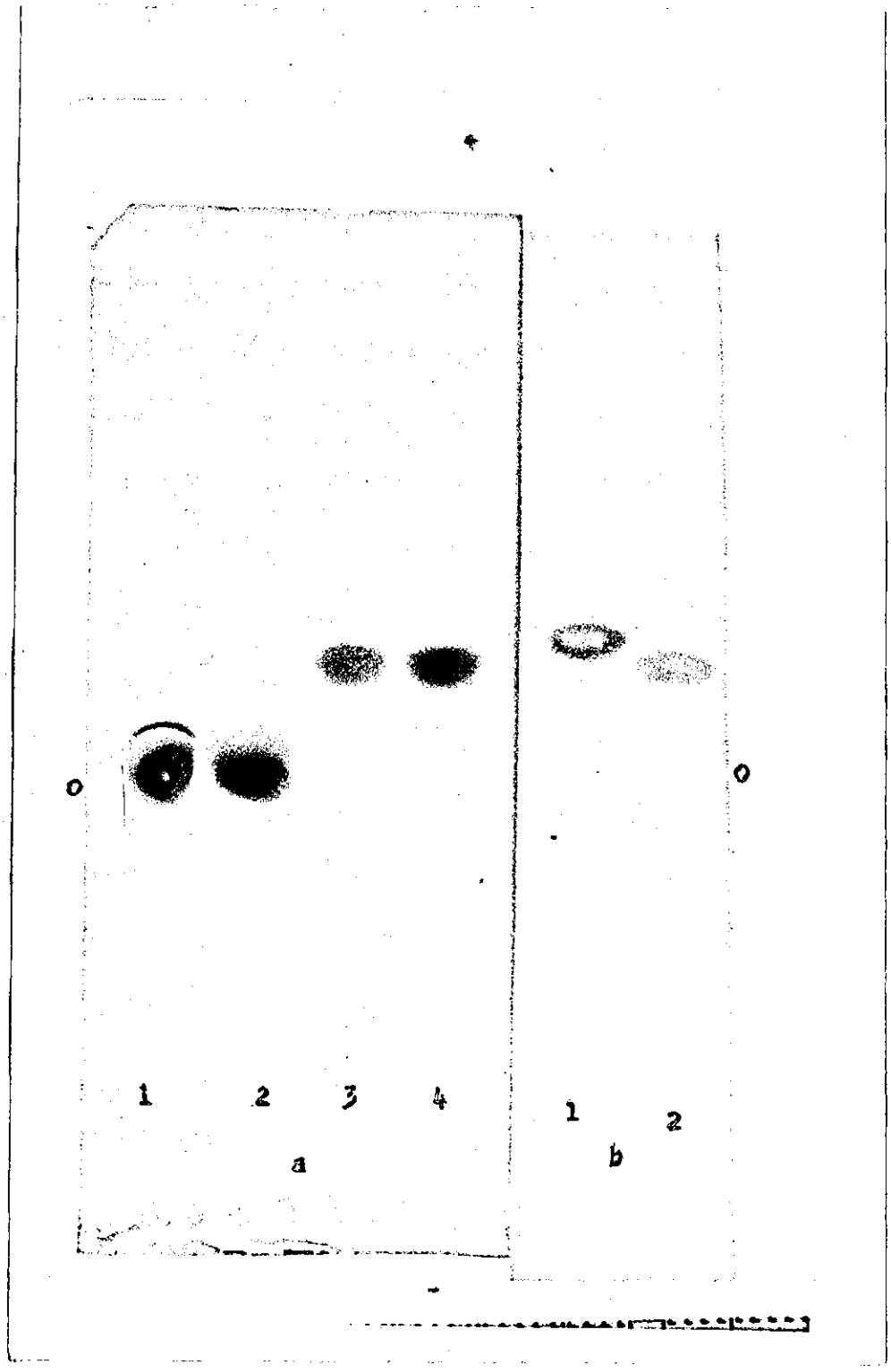


Fig.18 . Cellulose acetate electrophoresis : pattern comparison of:

(a) 1.2 "B" fragment, 3 - 4 "A" fragment

(b) 1 "Har" IgM, 2 "Har" IgM_s

of IgM with CNBr actually took place.

3-B -1-2.

Immunological properties of the CNBr - produced fragments.

In each instance selected fractions were taken for immunological testing against anti sera-IgM (μ chain specific) and K chains. Fractions A, B and C from IgM "Har", initially dissolved in 6 M guanidine hydrochloride, were tested with antisera to IgM (μ chain specific) and to K-type light chains, with the results shown in fig.19 .

Fraction B precipitated with the anti-K-Serum but not with the anti-IgM (μ chain specific). Fraction A gave a single precipitin line on reaction with anti-IgM serum (μ chain specific), but showed no apparent reaction with anti-sera K chains by Ouchterlony method fig.19 , A was antigenically similar to $(Fcu)_5$ (trypsin 65°C) fragment, deficient compared with the parent macroglobulin IgM molecule. Similar results were obtained from fragments A and B produced by cyanogen bromide digestion of IgM "Man" when similarly reacted with antisera IgM (μ chain specific) and K chains i.e. Man A precipitated with anti-serum to the μ heavy chains, but not with antiserum to K light chains, and Man B precipitated with antisera K chains, but not with antiserum to μ heavy chains. Similar results were obtained from fragment A and B produced by CNBr digestion of IgM "Kat" when similarly reacted with antiserum to μ heavy chains and to K light chains. In the first step of gel fractionation of CNBr digestion of IgM "Man", fraction B showed a strong reaction with antiserum to K chains and a faint reaction with anti μ chain. The latter failed to show any reaction with anti μ chain after the regel filtration of B fraction (i.e. fraction B didnot react with anti μ chain), indicating that in the first gel filtration step fraction B may be contaminated with fraction A which is completely removed

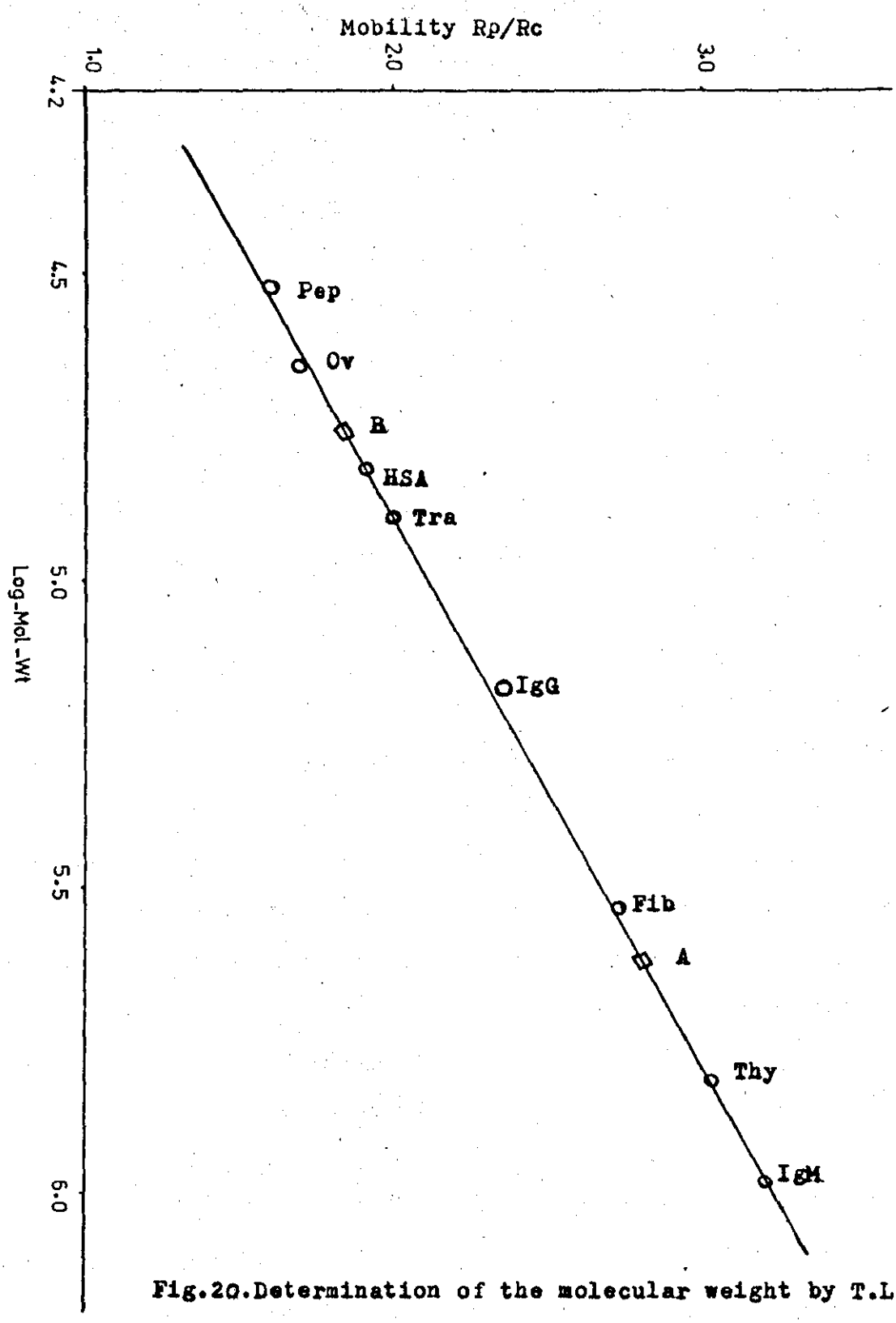


Fig.20.Determination of the molecular weight by T.L.G. The diagram represents the Human serum albumin(HSA), Pepsin(Pep),Human Transferren(Tra),Human IgG(IgG), Ovalbumin(Ov),Fibrinogen(Fib),Thyroglobulin(Thy),"Har" IgM(IgM),B and A fragments. Rp and Rc represent the mobility of the protein and the mobility of Cytochrome-C respectively.

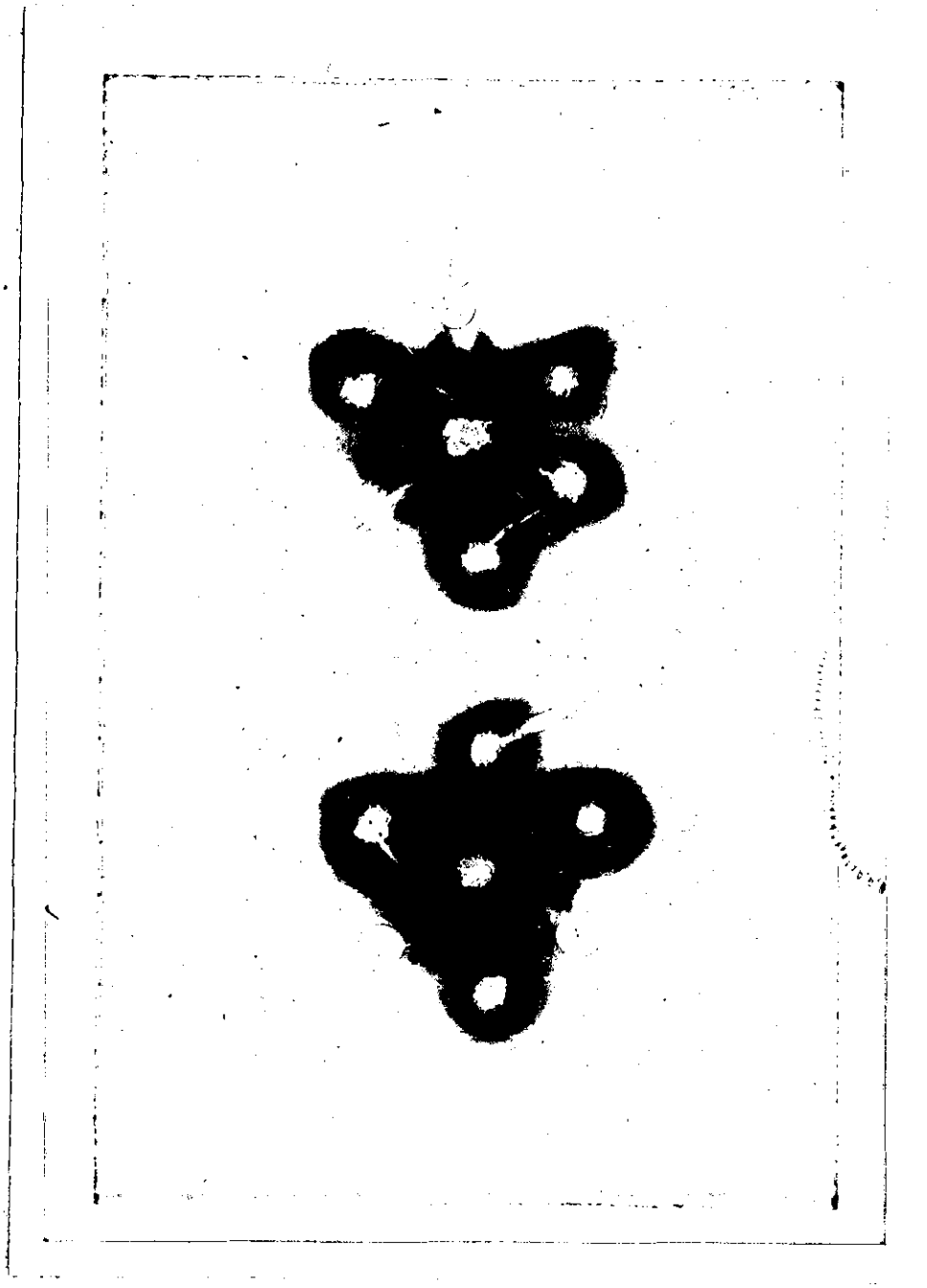


Fig.19. Immunodiffusion study of the IgM(Har) and its cyanogen bromide producing.

Top. Center well contains antiserum K chain: Clockwise, from top, "A" fragment, Native IgM (Har), "B" fragment, "Har" IgM_s, "C" fragment, and "Har" IgM and CNBr.

Bottom. Clockwise beginning at the top, "A", IgM, B, IgM_s, C, and IgM/CNBr respectively and the central well contains antiserum μ chain.

Note: The gel wells break on drying.

on the regel filtration of B. However, the "Man" C, "Har" C and "Kat" C fractions showed no reaction with either anti μ chains or anti K chains sera, indicating that C may be dialyzable fragments or may be traces of cyanogen bromide with a small absorbance.

The molecular weights of the proteins (A and B) were determined, by SDS-polyacrylamide gel (5 and 7%) electrophoresis at pH 7.1 without a reducing agent. The protein was treated with 1% SDS, essentially as described by Weber etal (1972), and then subjected to eletrophoresis in the presence of 0.2% SDS. Fragment B showed a single protein bond with a mobility equivalent ot a molecular weight of 52-54,000. On the other hand fragment A was too large to enter the polyacrylamide matrix (i.e. completely aggregated on the top of the polyacrylamide gel inside the tube). Thus the molecular weight of A could not be determined by this method. The molecular weights of A and B were also determined using thin-layer gel filtration on sephadex G200 in 0.1M Tris-HCl buffer, pH 4.5 containing 6 Molar guanidine hydrochloride, in order to eliminate the aggregation phenomenon (Elakovich etal.,1978 ; Hester etal., 1974). Both fractions A and B were suspended in 6 M Gu-HCl, pH 4.5 and then applied to the T.L.G. plate with the standard proteins. Fraction A migrated as a single protein band to the position corresponding to an apparent molecular weight of about 4×10^5 daltons, and B migrates as one protein band to the position corresponding to molecular weight 55,000 (fig. 20).

3-B-1-3.

Reduction of Fragment A:

Freeze-dried preparations of fragment A were dissolved in 0.1M Tris-HCl/0.3, NaCl buffer, pH 8.2 containing 6 Molar Gu-HCl at a concentration of 10mg/ml. 0.1M 2-mercaptoethanol (Small and Lamm, 1966) was added to this solution. Reduction was allowed to proceed at room

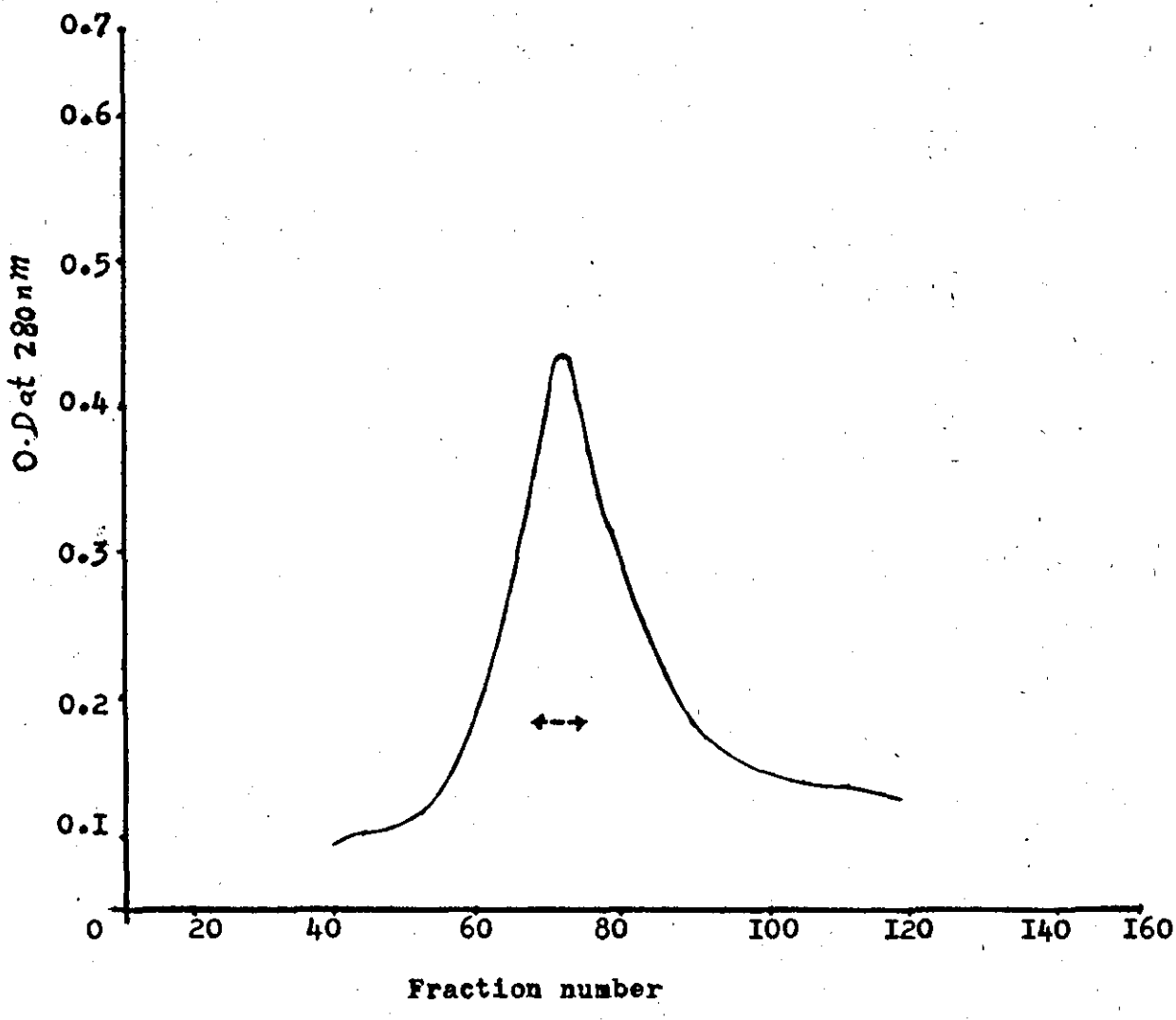


Fig-21-

Gel filtration of reduced and alkylated fragment A on sepharose 4B in 6 M Gu-HCl-Tris/saline buffer pH4.5, column size(60x2.6cm).Fraction volume 2.8ml. Fractionsbetween the arrows were retained.

O.D means optical density.

temperature for 2 hours under nitrogen. Alkylation was accomplished by reaction of the reduced material with a 100 per cent molar excess of iodoacetamide at room temperature for 15 minutes. After this time, the alkylated sample was fractionated by filtration through a sepharose 4B (70 x 2.6cm) column in 0.1 M Tris-HCl/0.3 M NaCl buffer, pH 4.5 containing 6 M Gu-HCl. 3 ml samples were collected at a flow rate of 10 ml per hour and monitored at 280 nm. The elution pattern of dissociated A fragment is shown in fig. 21. The molecular weights of reduced-alkylated A fragments were determined by polyacrylamide gel electrophoresis in 0.1% SDS as described earlier (chapter 2). There was a linear relationship between electrophoretic mobility and log (molecular weight) of the protein markers. The results obtained are summarised in table 5 and figure 22 and 23.

TABLE 5.

Protein	Molecular weight	log-molecular weight
Protein Markers:		
Monomer	53,000	4.7243
Dimer	106,000	5.0253
Trimer	159,000	5.2014
Tetramer	212,000	5.3263
Pentamer	265,000	5.4232
Hexamer	318,000	5.5024

(contd) TABLE 5.

Protein	Molecular weight	log-molecular weight
A(reduced and alkylated)		
Main bonds:	Estimated	
1 -	58,884-60,256	4.78
2 -	41,686	4.62
3 -	29,512	4.47
Minor bonds:		
1	17,782	4.25
2	11,220	4.05
3	4,073	3.61

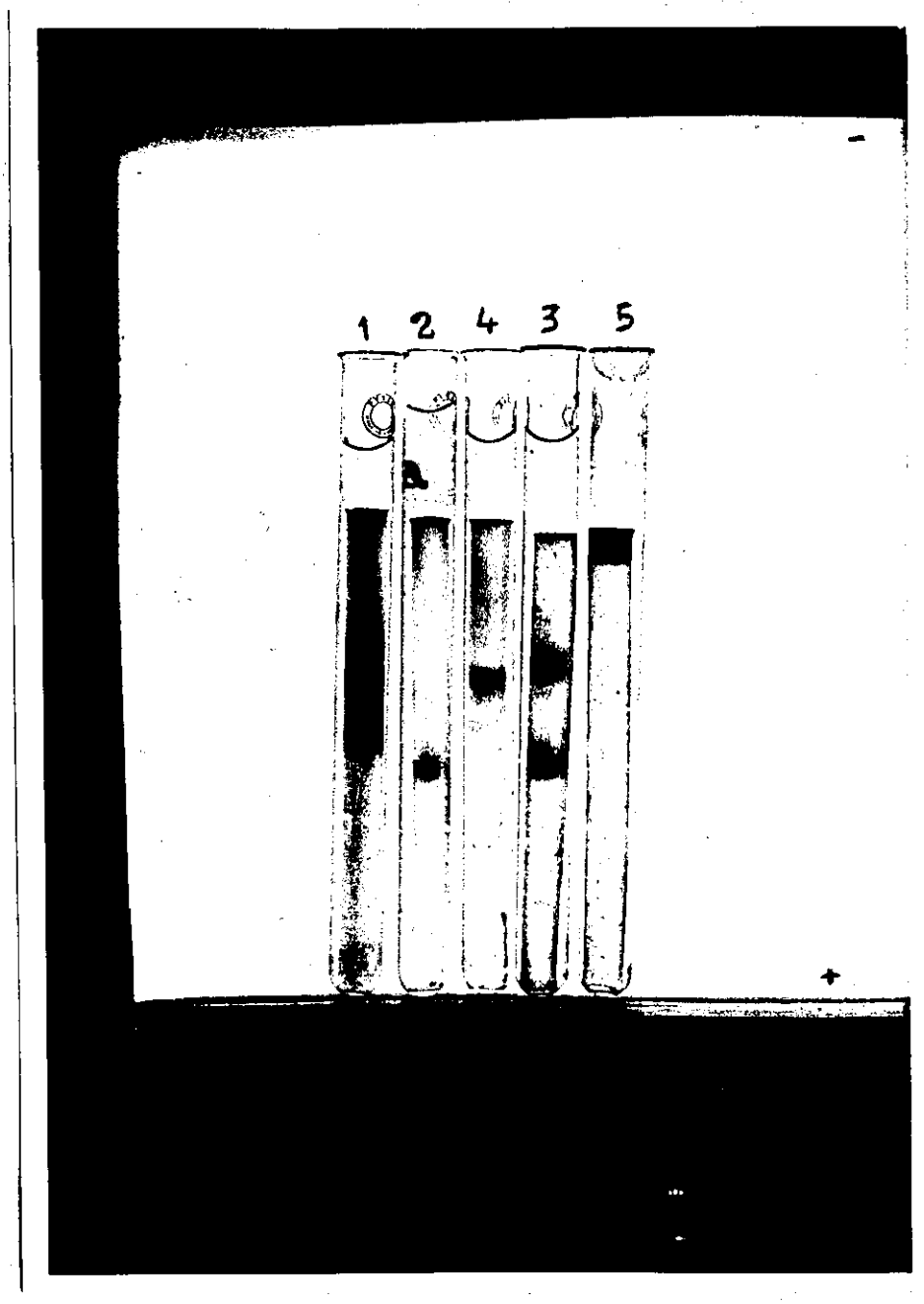


Fig.22. SDS gel electrophoresis pattern comparison of CNBr producing fragments of Har IgM

1.(A) reduced and alkylated

2.(A) regel filtrated after reduction and alkylation

3.(B) plus (A) regel filtrated after reduction and alkylation

4.(B) fragment.

5.(A) fragment.

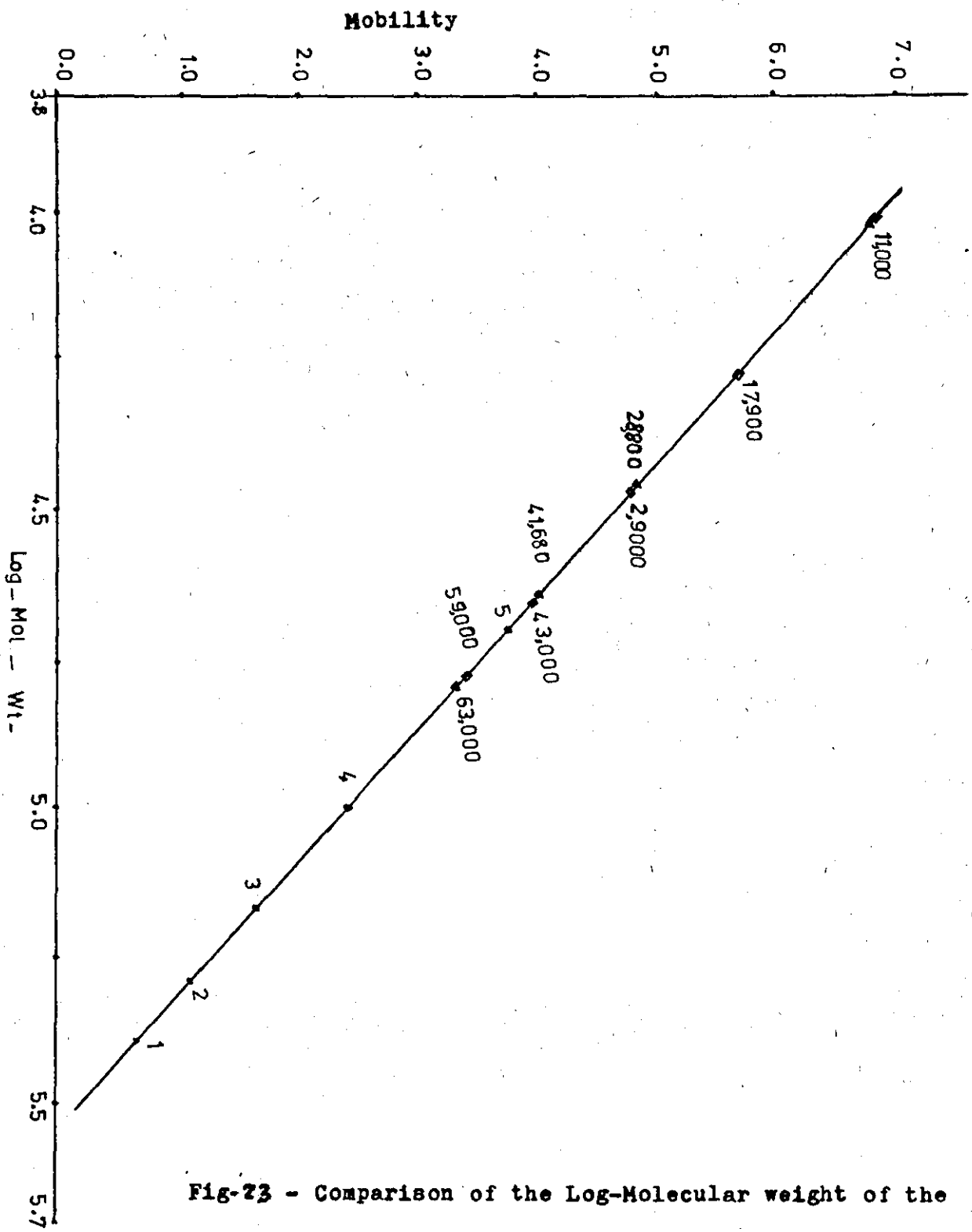


Fig-23 - Comparison of the Log-Molecular weight of the standard marker proteins(1-5) listed in Table 4 against their electrophoretic mobilities on SDS-polyacrylamide gel(5%).(determination of the molecular weight of the reduced and alkylated "A" and $(Fc\mu)_5$-CNBr products).

3-B-2.

Properties of trypsin products

3-B-2-1 Results:

The trypsin hydrolysate of pathological IgM "Har" at 65°C was resolved into three distinct peaks on a sephadex G200 gel filtration column (fig.24). The material of the first peak, which eluted at the void volume of the column, rechromatographed on a sephadex G200 column (65 X 2.5 cm) in Tris-HCl/NaCl buffer (pH 8.1), had a molecular weight of about 320,000 ± 20,000 determined by thin-layer gel filtration (fig.25) and by SDS-gel electrophoresis (fig.26). It showed a positive reaction with anti-μ chain antiserum, but no reaction was observed with anti-K antiserum by the Ouchterlony method. The other two peaks represent Fabμ fragments and small peptides (Plaut and Tomasi, 1970 ; Chen etal., 1974). The first fragment (fig.24) showed a single band on cellugel electrophoresis in 0.05M Tris-barbital - Sodium barbital buffer (pH 8.8). The combination of the above results (i.e. the physicochemical and the immunological data), indicates that the material of the first peak (fig.24) could be considered as $(Fc\mu)_5$ fragment.

Cyanogen bromide digestion of $(Fc\mu)_5$ 65°C fragment.

3-B-2-2.

The acid (0.05M HCl) denatured $(Fc\mu)_5$ 65°C fragment was treated with cyanogen bromide under the same conditions as the native IgM. The reaction mixture gave a single broad band on cellogel electrophoresis in 0.05M Tris-barbital - sodium barbital buffer (pH 8.8) containing 6 M urea. However, when this component (i.e. $(Fc\mu)_5$ 65°C - CNBr product)

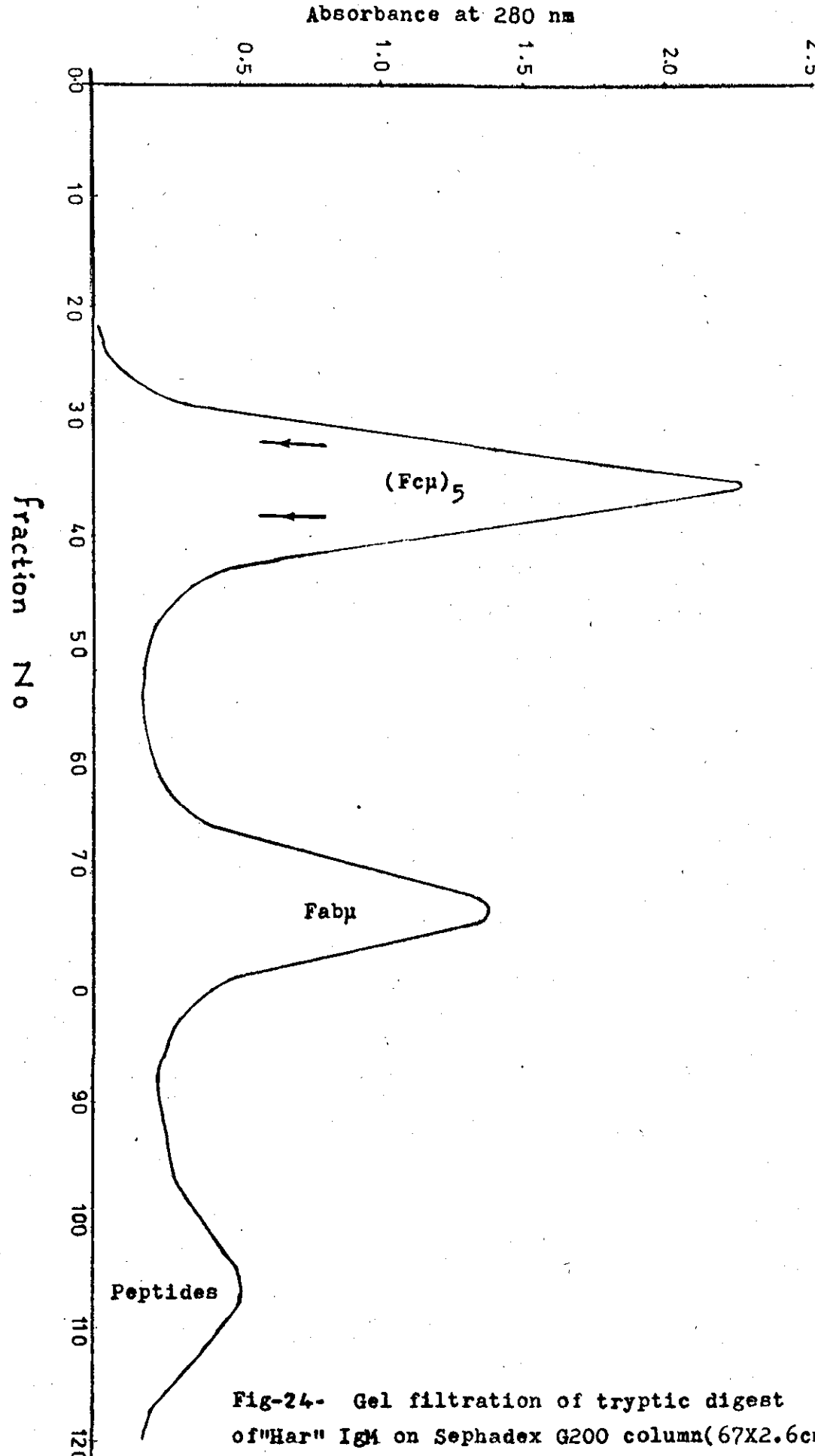


Fig-24- Gel filtration of tryptic digest of "Har" IgM on Sephadex G200 column (67X2.6cm) in 0.05M Tris-HCl/0.15M NaCl buffer, pH 8.I. The first peak contains $(Fc\mu)_5$ fragment.

Fraction volume=3ml/tube.

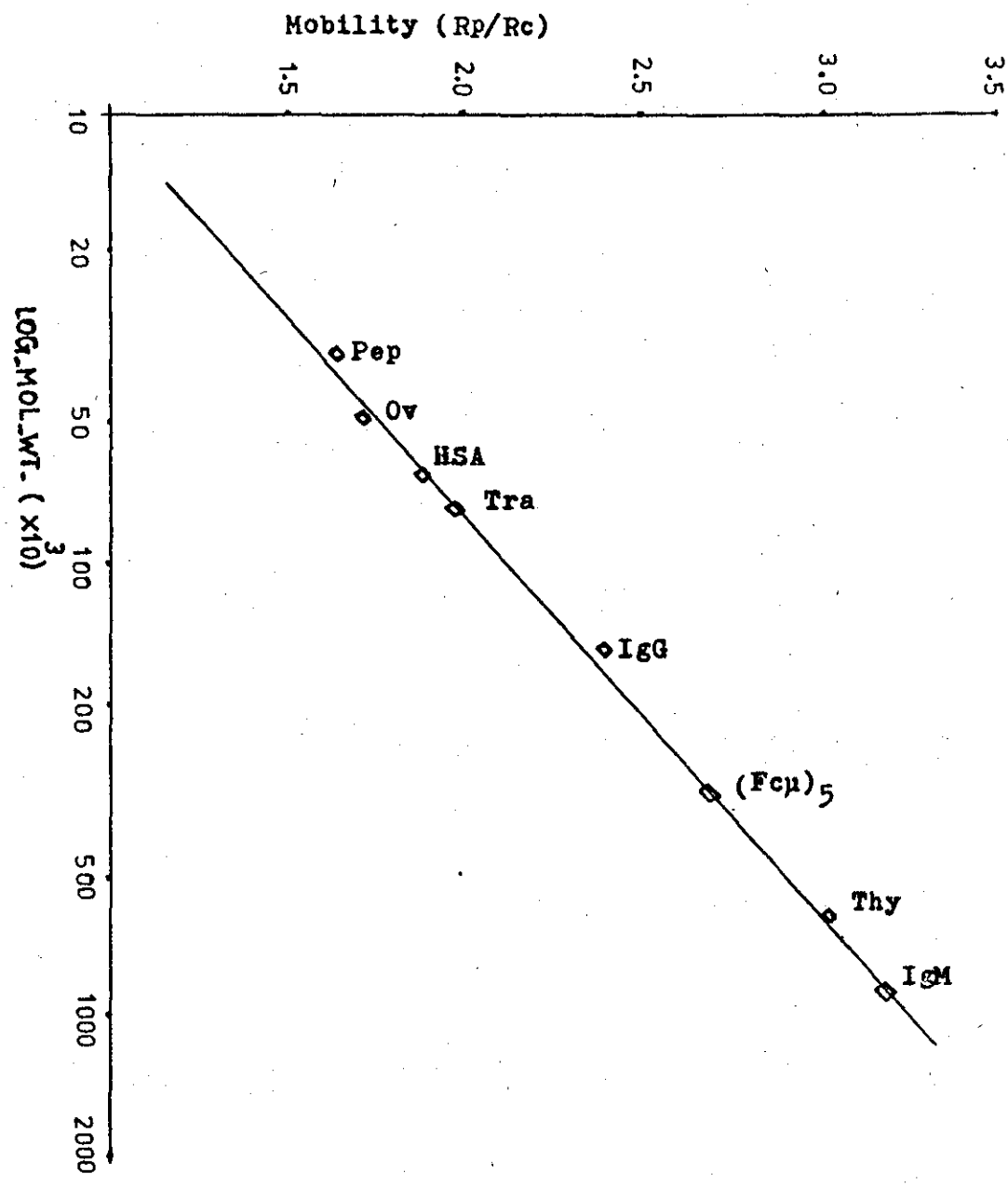


Fig.25. Plot of Rp/Rc against molecular weight on semilograthm paper .

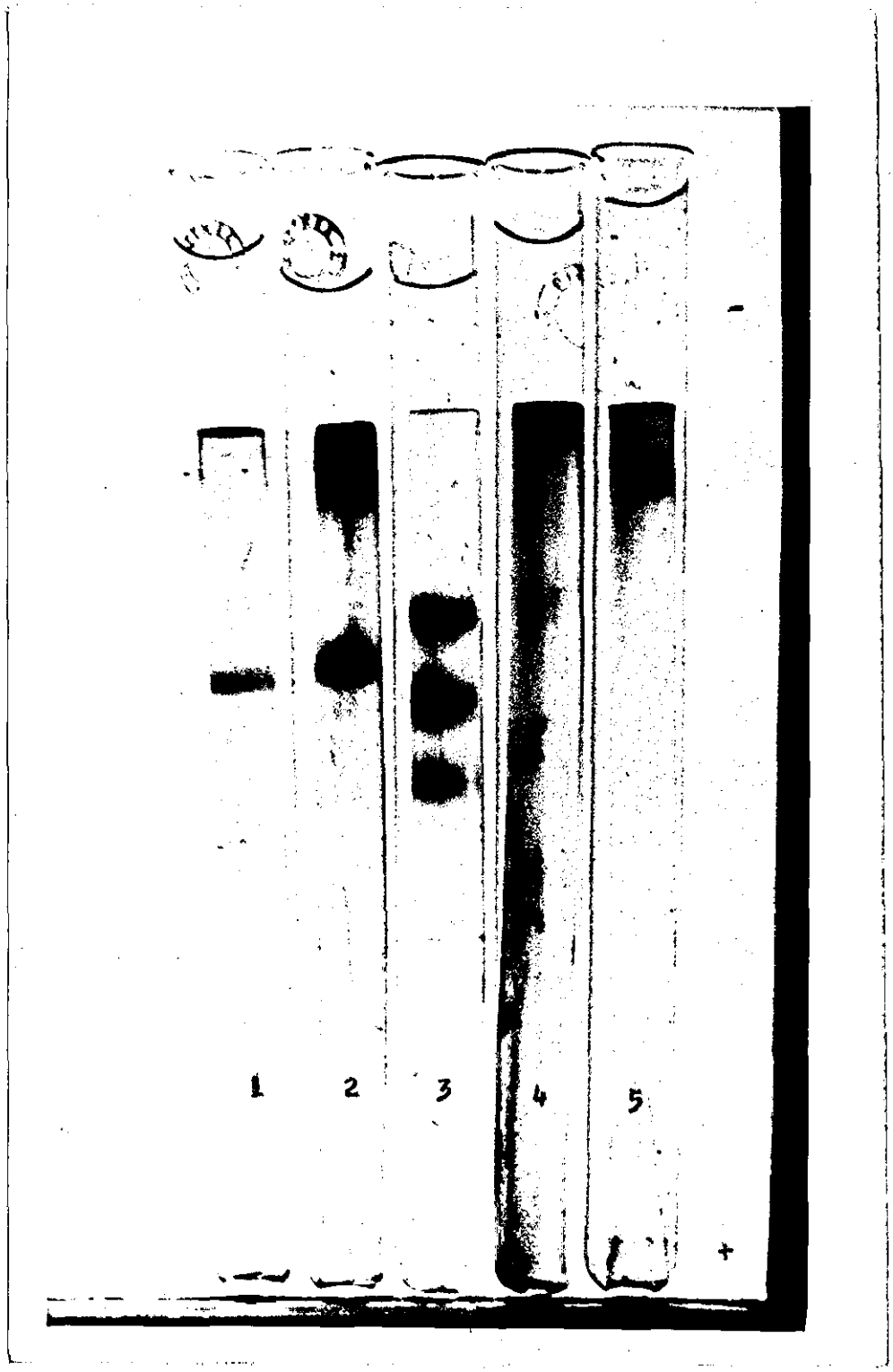


Fig. 26. SDS gel electrophoresis pattern comparison:

1. "Har" B fragment
2. "Har" IgM/CNBr
3. $(Fc\mu)_5$/CNBr reduced and alkylated
4. Har IgM, and .5.$(Fc\mu)_5$ $65^{\circ}C$

was reduced with 2 mercaptoethanol in 6 Molar Gu-HCl for 2 hours, followed by alkylation with iodoacetic acid for 15 minutes (Lamm and Small, 1966) it was resolved into three main bands on SDS-polyacrylamide gel electrophoresis, similar to those obtained from the reduced and alkylated A fragment of the whole IgM molecule, but not identical in their molecular weights. Fig. 22. shows the reduced and alkylated "Har" $(Fc_\mu)_5$ 65°C fragment after the cyanogen bromide digestion.

Polyacrylamide gel electrophoresis.

3-B-2-3.

A standard curve for molecular weight determinations (fig. 22) was constructed from standard protein markers (Table 5) and the molecular weight of the reduced and alkylated $(Fc_\mu)_5$ 65°C fragments after CNBr digestion was estimated from it. The three main fragments had electrophoretic mobilities equivalent to molecular weights of 63,000, 43,000 and 28,800 repectively. Normal $(Fc_\mu)_5$ 65°C (i.e. from trypsin digestion) yielded a single major component (molecular weight 34,000) after reduction and alkylation.

3-B-2-4.

Discussion:

The molecular weight for B (52,000) compares satisfactorily with those (i.e. Fabμ fragments) reported in literature. Table 6 shows the comparisons of the molecular weight of B fragment obtained from the CNBr digestion of IgM "Har", with other Fabμ fragments obtained from IgM by digestion with various enzymes.

T A B L E 6.

Fragment	Molecular weight	Enzyme	References
Fabμ	41-48,000	Trypsin	Miller and Metzger,1966 ; Beale and Buttress,1969 ; Plaut and Tomasi,1970
	52,000	Trypsin	Chen etal, 1974
	50-55,000	Papain	Onoue etal, 1967 ; Unger-Waron,1967 ; Suzuki, 1969.
	37-45,000	Papain	Mihaesco etal,1968 ; Chen etal, 1979 ;Seven-Erik etal, 1969 ; Dorrington and Mihaesco,1970.
	40,000	Chymotrypsin	Chen etal,1969.
Fabμ	48-55,000	Pepsin	Suzuki,1969 ; Dorrington and Mihaesco, 1970.
Fabμ	55,000	CNBr (70% formic acid)	Zikan and Bennett,1973
B	52-55,000	CNBr (0.05M HCl)	Present work

These data suggest that B fragment may consist of disulphide bonded light chain and Fdμ (V_H, $C_{\mu 1}$and $C_{\mu 2}$). The alternative explanation is that the B represent Fabμ fragment.

On the other hand the molecular weight determined for A(400,000) is comparable to that reported in literature (Table 7).

TABLE 7.

Comparison of the Mol-wt data for A & $(Fc\mu)_5$ 65° with the papain and tryptic $(Fc\mu)_5$ reported in literature.

Fragment	Molecular weight	Enzyme	References
$(Fc\mu)_5$	300,000-320,000	Papain	Onoue etal,1968 ; Seven-Erik etal,1969 ; Dorington and Mihaesco, 1970
	320-342,000	Trypsin	Plaut and Tomasi,1970, 1972 ; Paus etal,1978
	380-400,000	Trypsin	Hester etal,1973,1975 ; Eriklindh etal,1976
	270,000	Trypsin	Florent etal,1974
"Har"$(Fc\mu)_5$	65° 320-340,000	Trypsin	Present Work
"Har" A	400,000	CNBr	Present Work

These observations (i.e. Tables 6 and 7) indicated that IgM can also be represented by the pentameric (5 X IgM_s) model i.e. 10 Fabμ fragments and one $(Fc\mu)_5$ fragment initially proposed by Miller and Metzger (1965) and subsequently confirmed by Plaut and Tomasi (1970) and Dorrington and Mihaesco (1970), and that these general structural features are probably shared by all IgM immunoglobulins, including 19 S IgM - globulins; and that both the enzymatic and chemicals listed above (Tables 6 and 7) cleaved the macroglobulin IgM molecule mainly at or near the hinge region, but at different positions.

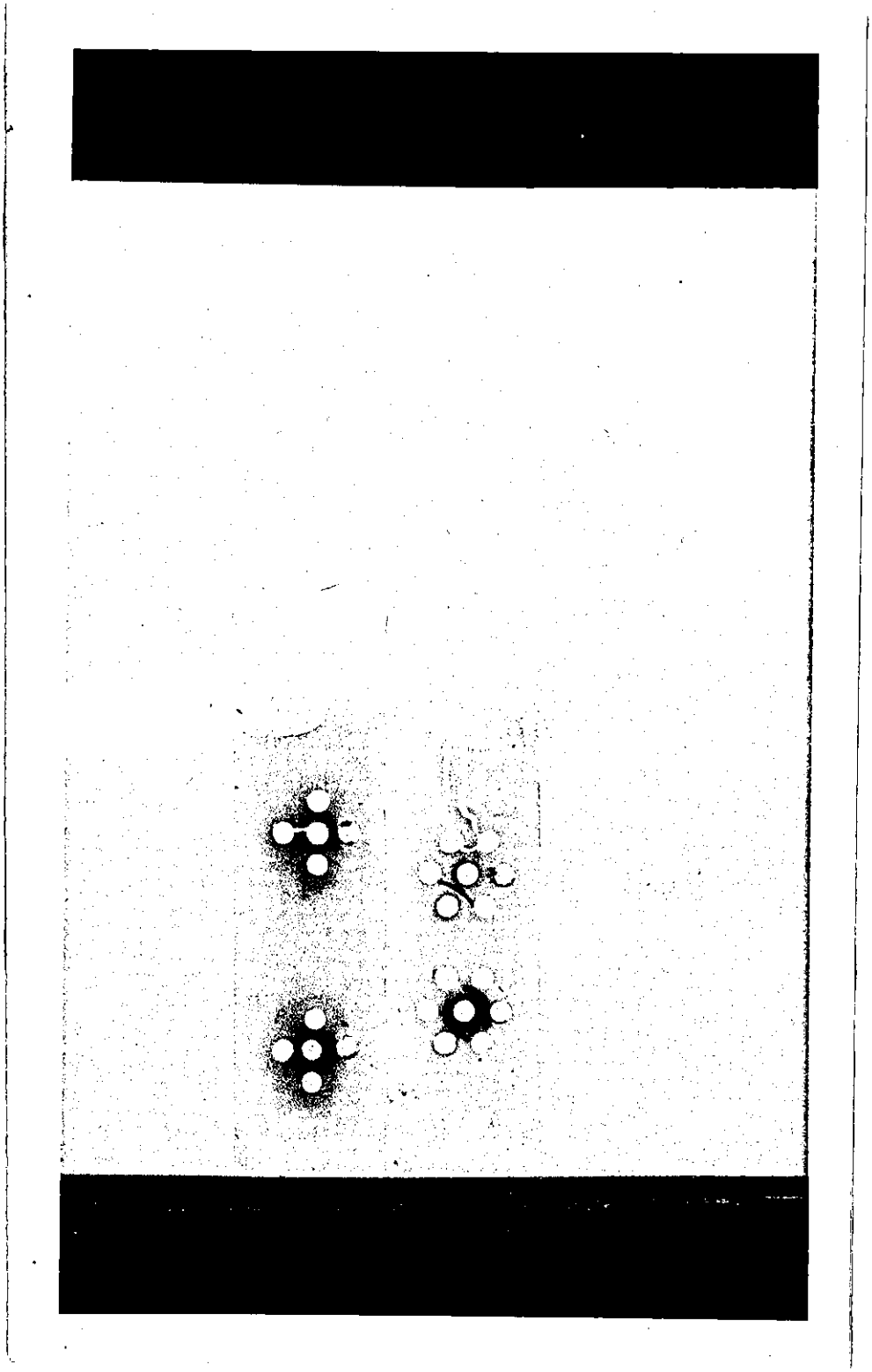


Fig.27. Immunodiffusion analysis of IgM and its CNBr produced fragments:

LEFT top: Central well contains K chain. Clockwise, beginning at the top, IgM, B, IgM_S and IgM/CNBr.

bottom: Central well contains antiserum μ chain. Clockwise from the top the same order.

RIGHT top: Central well contains K chain. Clockwise, beginning at the top, A, $(Fc\mu)_5$, C, IgM, blank, IgM_S.

bottom: Central well contains μ chain. Clockwise, beginning at the top, A, $(Fc\mu)_5$, C, IgM, IgM_S, blank.

On reduction the molecular weight of the Har $(Fc\mu)_5$ 65° fell to 34,000 in 6M Gu-HCl, indicated that the polymeric $(Fc\mu)_5$ 65° is made up to ten monomer Fcμ disulphide bonded units. The A and $(Fc\mu)_5$ 65° fragments, split from the macroglobulin IgM "Har" by CNBr and by hot trypsin respectively, are chemically very similar but not identical, although their antigenic identity was supported by immunodiffusion (fig. 27). Their chemical non-identity is indicated by the greater molecular weight of A fragment compared with $(Fc\mu)_5$ 65°. SDS-gel electrophoresis showed that the reduced and alkylated A fragment separated into three main bands (Table 5) with electrophoretic mobilities equivalent to molecular weights of 59,000, 41,800 and 29,000. Similar results were obtained with the reduced and alkylated $(Fc\mu)_5$ - CNBr component: the major bands corresponded to molecular weights 63,000, 43,000 and 28,800. This indicates that components produced on reduction and alkylation of A and $(Fc\mu)_5$ - CNBr are similar, and the high molecular weight components (i.e. 63,000, 59,000, 41,800 and 43,000) may be due to incomplete reduction or incomplete chemical digestion. However, both reduced and alkylated A and $(Fc\mu)_5$ - CNBr showed minor bands, on SDS-gel electrophoresis, of molecular weights 17,500, 11,000 and about 4,000. This may be explained by the production of $C\mu_3$ or J chain, $C\mu_4$ and a C-terminal portion of the μ chain (i.e. F_8, F_{10}, and F_{11} respectively - Putnam etal, 1973).

3-B-3. Cyanogen bromide digestion of "Har" 7 S subunit (IgM_S).

3-B-3-1.

In the preceding section it has been shown that the limited cleavage of IgM by cyanogen bromide gave rise to two distinct major fragments. However, it becomes difficult to interpret the results in terms of the whole structure of IgM molecules, particularly because of the complexity of the largest fragment (A). Therefore, the 7 S subunit (IgM_S) was subjected to cyanogen bromide digestion in an attempt to obtain further information about the structure of the IgM.

3-B-3-2. Action of CNBr:

Acid denatured (0.05M HCl) 7 S subunit (IgM_S) was treated with CNBr in the same way as the native IgM molecule (Chapter 2). The products of treated 7 S subunit proteins obtained from the freeze-drier were redissolved and tested by SDS-gel electrophoresis in phosphate buffer (pH 7.1) (fig. 28), by cellogel electrophoresis in 0.05M Tris-barbital sodium barbital buffer (pH 8.8) containing 6M urea (fig. 29), and by gel filtration on columns of sepharose 4B and sephadex G200 (fig 30).

3-B-3-2-1.

Results:

The appearance of two well defined peaks in the elution pattern from this particular gel filtration (fig. 30) was confirmed by disc-electrophoresis. These fractions were designated (a) and (b). The molecular weights of the proteins (a) and (b) were determined, using SDS-gel electrophoresis. The protein was treated with 1% SDS, and then subjected to disc-gel electrophoresis in the presence of 0.2% SDS, as described earlier. Two major protein bands result, with mobilities equivalent to molecular

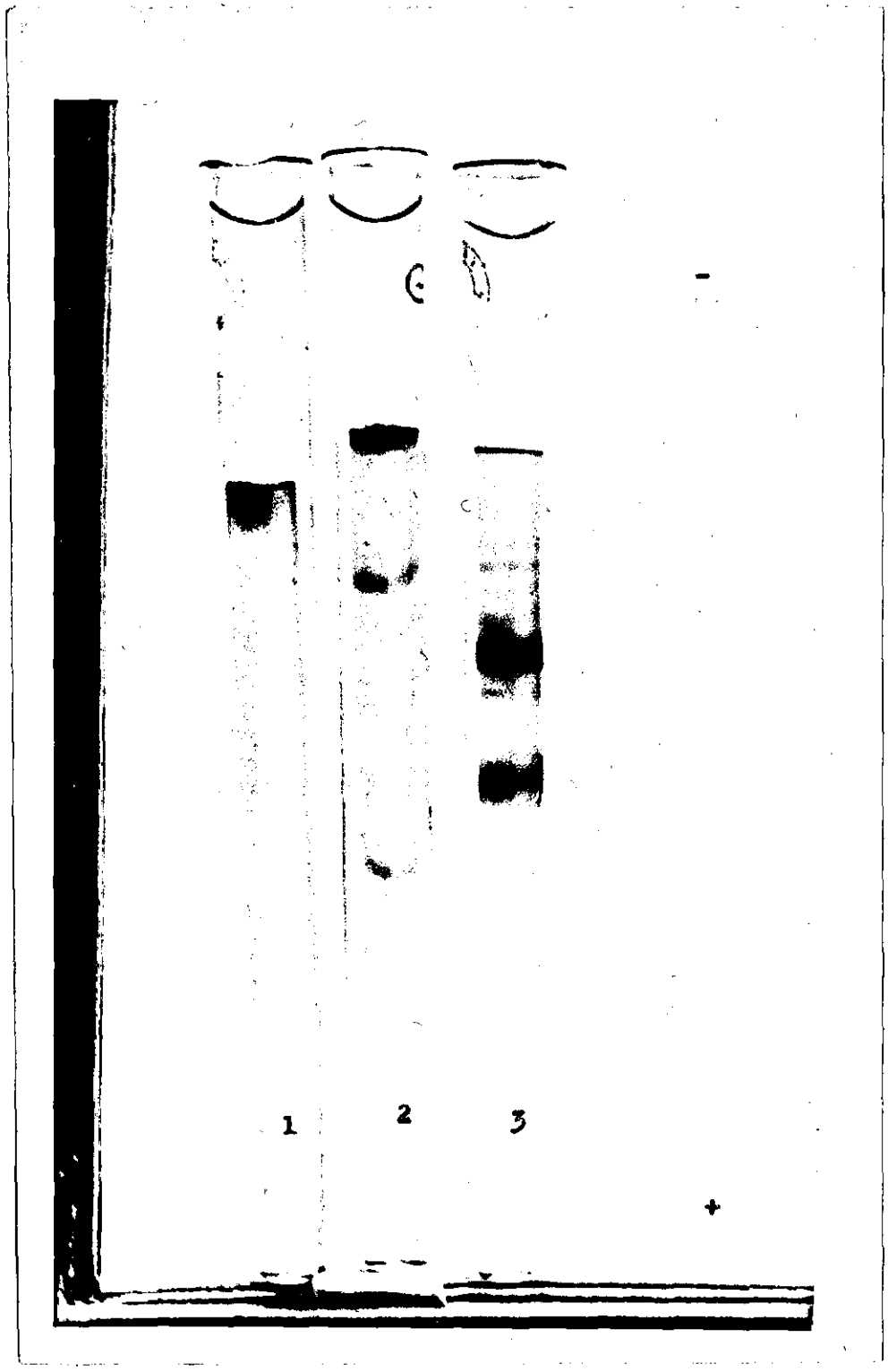


Fig. 28. SDS gel electrophoresis pattern:

1. "Har" IgM, 2 "Har" IgM_S, 3 IgM_S after CNBr digestion.

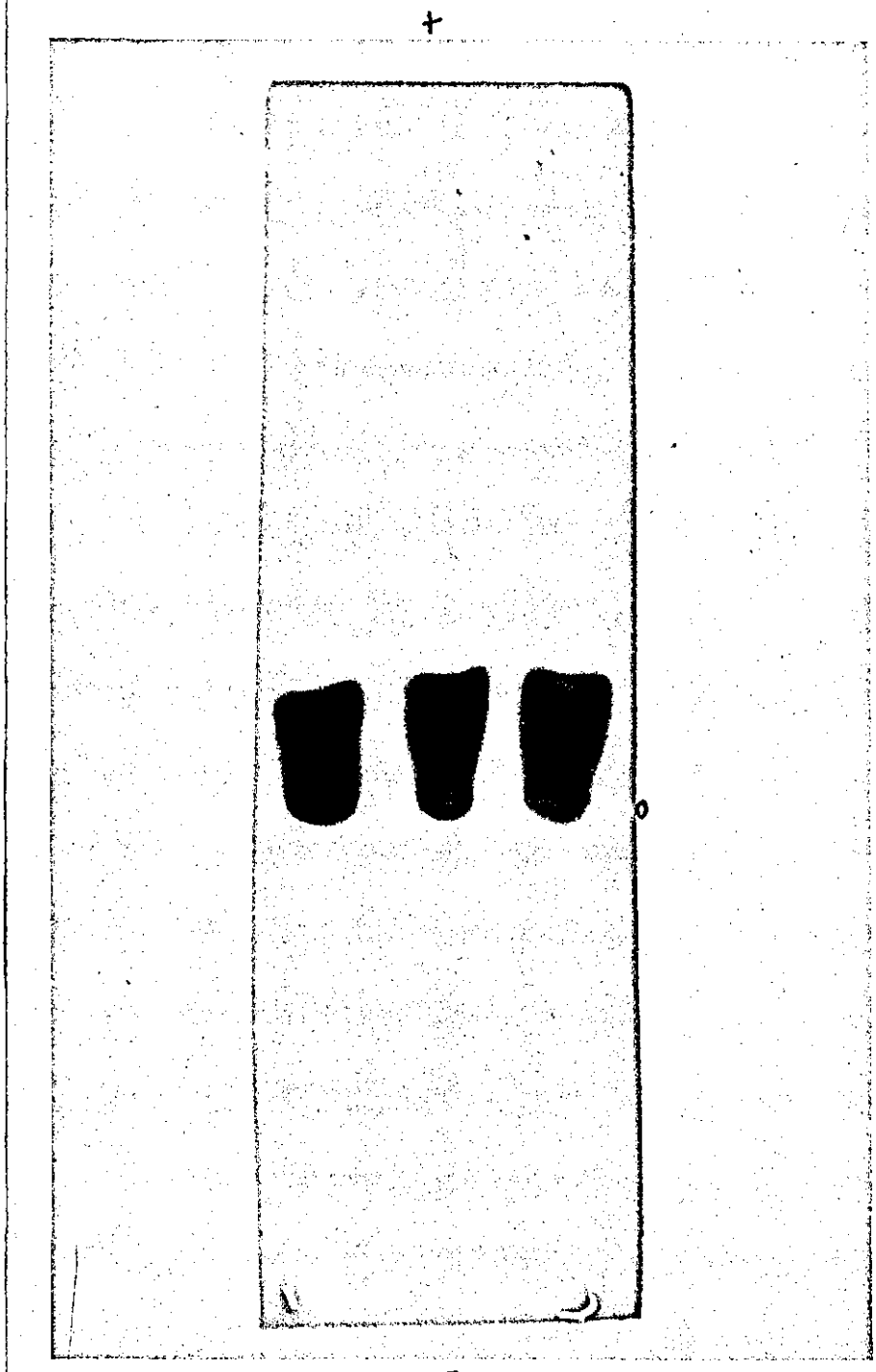


Fig.29.Cellulose acetate electrophoresis pattern of a "Har"IgM_g/CNBr.

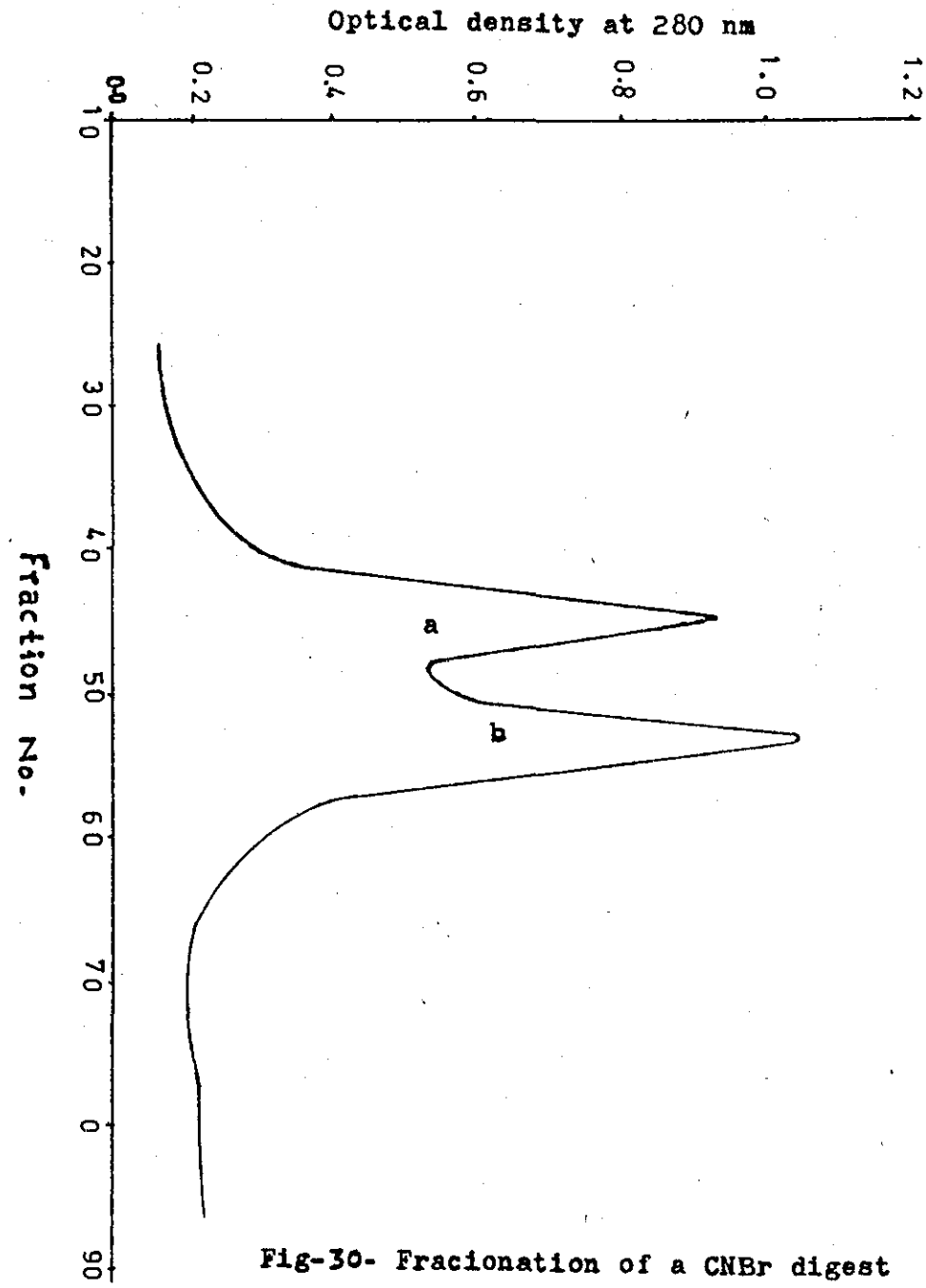


Fig-30- Fracionation of a CNBr digest of IgM_s subunits on Sephadex G200 column (65X2.6cm) in 6M Gu-HCl/Tris-saline buffer, pH 4.5. Fraction volume= 3ml .

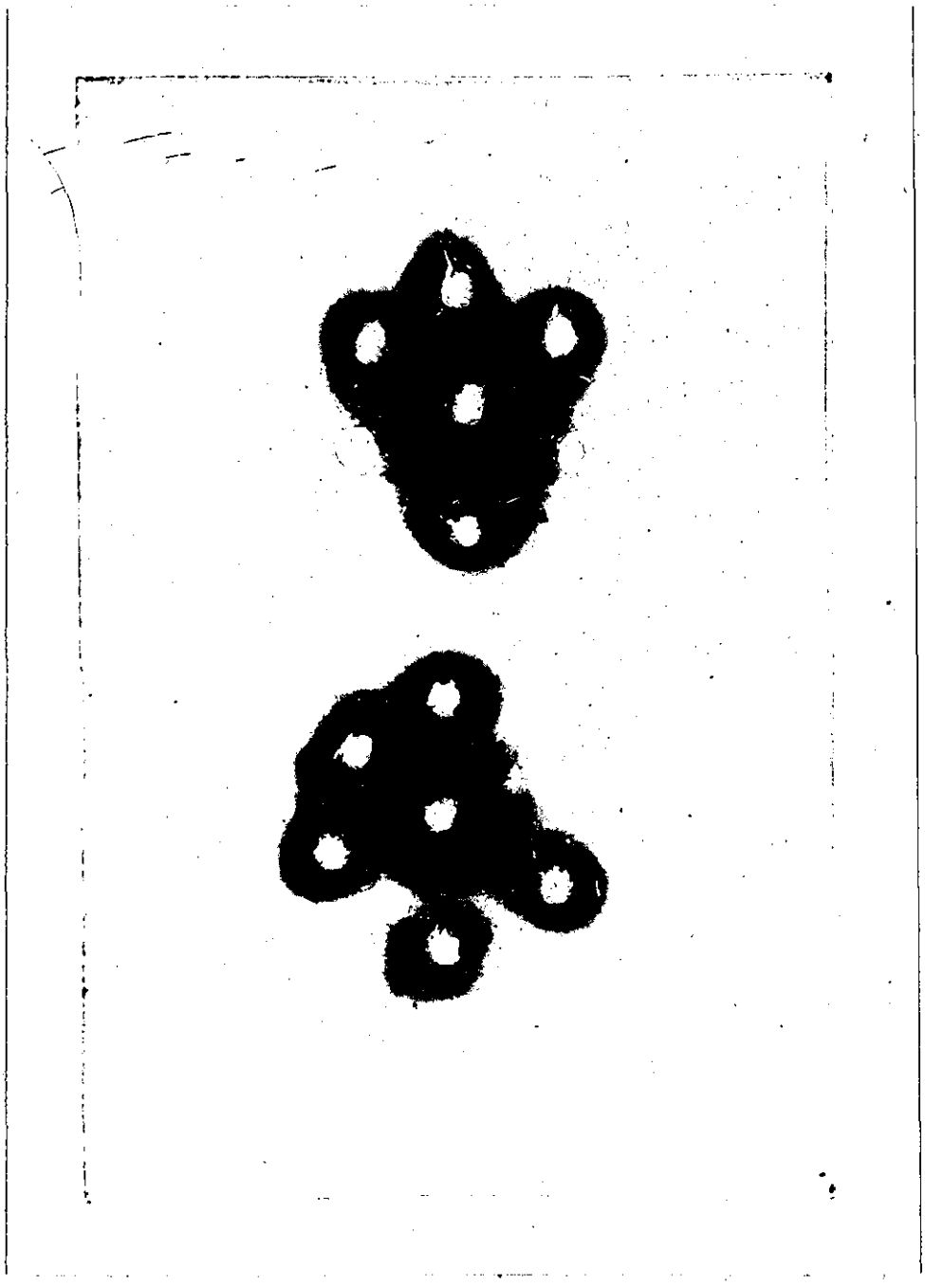


Fig.31. Immunodiffusion analysis of "Har" IgM and its CNBr produced fragments.

TOP : Central well contains K chain. Clockwise, beginning at the top, Har IgM/CNBr, (b) fragment, A fragment, "Har" IgM, (a) fragment, Har IgM_S.

BOTTOM : As above, but the central well contains antiserum μ chain.

weights of 75-78,000 and 52-54,000 repectively.

3-B-3-2-2.

Immunological properties of a and b fragments.

On reaction with anti-μ chain and anti-K chain antisera, fragment (b)gave a precipitin line with the latter, but not the former (Fig.31). On the other hand fragment(a)gave a faint precipitin line with the anti-μ chain antiserum, but failed to react with antisera K chains. This indicates that fragment(b)is a Fabμ - like fragment(similar to B of the whole IgM molecule) and that(a)could be dimer of Fcμ disulphide bonded fragment (i.e. Fc_{μ}-Fc_{μ}).

Section C: 3-C-1. Chemical analysis.

3-C-2: Amino acid composition of IgM (Har) and its CNBr produced fragments.

Samples for amino acid analysis were chromatographed on sephadex G 25 columns, in order to remove the small peptides and the other insoluble material. Selected fractions were freeze-dried, and then the protein concentration was determined by using Lowry method (1951). Duplicate samples containing 1 mg of protein and 0.2 μ mol of norleucine standard were hydrolysed in 6M HCl under a nitrogen atmosphere for 24 hours in sealed tubes at 110°C. Two analyses were performed on each sample by a single column (35 X 0.3 cm) of chromaspex ion-exchange chromatograph resin (Rank Hilger Ltd) for the determination of acidic, neutral and basic amino acids on the amino acid analyser J 180 (Rank Hilger). Corrections were made for loss of serine and threonine during hydrolysis and for incomplete liberation of valine and isoleucine (Rees, 1946., Hirst,etal, 1954). Another sample was treated with performic acid before hydrolysis to oxidise the sulphur-containing amino acids and was used to calculate the cysteine and cystine as cysteic acid. The amino acid was expressed as moles per mole.

TABLE 8

Amino acid compositions of Har IgM compared with other Waldenstrom macroglobulins values are given in moles per mole of IgM.

Amino acid residue	Suzuki, etal., 1967 Native IgM 19S (10^6) P.I	A.U	V.I	Chaplin, etal., 1965 19S IgM (10^6)	Himburger, etal., (1964) 19S IgM(10^6)
Asp	714	596	520	539	619
Thr	757	702	576	556	716
Ser	930	1005	800	614	799
Glu	883	755	616	660	825
Pro	534	470	400	429	544
Gly	518	490	421	410	528
Ala	512	496	382	370	493
½ Cystine	141	-	130	183	155
Val	654	609	514	523	655
Met	78	65	58	69	89
Ile	256	211	199	215	239
Leu	574	508	487	470	560
Tyr	289	229	217	227	258
Phe	324	283	232	233	269
Trp	149	176	150	130	152
Lys	405	365	309	332	381
His	150	136	86	126	133
Arg	387	287	250	278	324
Total	8255	7383	6347	6364	7739

n.d. = not determined.

½ Cystine = cysteine.

(contd) TABLE 8

Present data		Zinneman, etal, 1973.			Amino acid residue
moles/mole Har IgM(95×10^4)	Har IgM_s 185,000	Li. IgM_s 185,000	Co. IgM_s 185,000	Scha IgM_s 185,000	
630	125	116	116	116	Asp
740	147	124	130	154	Thr
905	180	188	192	208	Ser
740	148	126	134	138	Glu
515	102	130	83	156	Pro
545	108	202	220	198	Gly
490	96	126	157	160	Ala
190	36	32	30	32	½Cystine
595	118	156	140	138	Val
90	18	12	14	14	Met
250	50	36	46	50	Ile
570	114	110	112	110	Leu
250	48	42	36	40	Tyr
275	55	38	44	48	Phe
n.d	n.d	-	-	-	Trp
375	74	86	70	64	Lys
125	24	22	20	18	His
320	63	36	52	48	Arg
7510	1488	1582	1596	1692	Total

n.d=not determined.

½Cystine=cysteine.

TABLE 9

Amino acid composition of Har IgM and its 7 S subunit before and after cyanogen bromide digestion.

Values are given in moles per mole of protein.

Amino acid residue	Native IgM(Har) Mol-wt 950,000	IgM/CNBr Mol-wt 950,000	Har 7S subunit Mol-wt 185,000	7S subunit/ CNBr Mol-wt 185,000
Asp	630	630	125	125
Thr	740	740	147	146
Ser	905	905	180	180
Glu	740	740	148	148
Pro	515	515	102	102
Gly	545	545	108	107
Ala	490	490	96	96
Cys	95	95	18	18
Val	595	595	118	117
Met	90	46	18	8
Ile	250	250	50	50
Leu	570	570	114	114
Tyr	250	250	48	48
Phe	275	275	55	55
Trp	n.d.	n.d.	n.d.	n.d.
Lys	375	375	74	74
His	125	125	24	24
Arg	320	320	63	62
Total	7510	7472	1488	1475

Cys=cystine calculated as cysteic acid , n.d. = not determined.

3-C-3: Discussion:

The amino acid composition of the native IgM and its monomer 7 S subunit (IgM_S) were established on the basis of their molecular weight. Summation of the amino acid composition of five monomer subunit (5 X IgM_S) plus 5 disulphide bonds was in close agreement with that of the intact IgM molecule. In summary, the amino acid compositions of IgM molecule and IgMs subunit are in fairly good agreement with the previously published results. The majority of the differences reflect expected variation from one IgM protein to another. The most important information that can be obtained from the amino acid analysis concerns the values for methionine and cystine. The sum of five times the residues (cystine and methionine) was identical to the total values of cystine and methionine of the intact IgM molecule with the exception of 5 cystine (intersubunit disulphide bonds), indicating that the native IgM molecule consists of 5 disulphide bonded subunits. On the other hand analysis of both IgM and IgM_S after CNBr digestion showed that about 48.8% and 55% of their methionine residues had been attacked by CNBr. This indicates that these conditions provide partial digestion (C.F. 70% formic acid) *i.e.* that some of the methionine residues were more available to attack by CNBr than others. Florent, etal., (1974) have described studies on the primary structure of the V region and the switch point in a series of pathological human IgM protein and showed that there was a varying number and distribution of the methionine residues in the V regions of μ chains.

TABLE 10

Amino acid compositions of IgM fragments produced by hot trypsin and cyanogen bromide, and compared with OU $(Fc\mu)_5$ (sequence 336-578, Putnam,etal., 1973). The amino acid composition are expressed in terms of moles per mole protein.

Amino Acid Residue	Theory(336-578) $(Fc\mu)_5$ Putnam, etal., 1973.	Present Data Har $(Fc\mu)_5$ 65°C Mol-wt $32X10^4$	Present Data Har $(Fc_\mu)_5$ CN Br Mol-wt $32X10^3$
Asp	190	207	207
Thr	267	295	295
Ser	200	210	210
Glu	220	240	240
Pro	190	185	185
Gly	120	130	130
Ala	150	170	170
Cys	35	30	30
Val	200	198	198
Met	30	30	20
Ile	85	98	98
Leu	170	175	175
Tyr	70	70	70
Phe	80	86	86
Trp	40	n.d	n.d
Lys	70	70	70
His	60	65	65
Arg	110	120	120
Total	2287	2379	2369

n.d = not determined

Cys = Cystine calculated as cysteic acid

(contd) TABLE 10

Present Data		Theory (331-571) $(Fe_{II})_5$ Watanbe. etal, 1973.	Amino Acid Residue
Har A Fragment Mol-wt $4X10^5$	Har B Fragment Mol-wt $52X10^3$		
220	36	190	Asp
310	37	300	Thr
230	52	210	Ser
270	42	240	Glu
190	28	190	Pro
134	32	120	Gly
190	29	170	Ala
32	5	35	Cys
205	32	200	Val
22	2	30	Met
100	13	90	Ile
180	34	170	Leu
73	13	70	Tyr
92	16	80	Phe
n.d	n.d	40	Trp
73	23	80	Lys
67	6	60	His
125	16	100	Arg
2513	416	2385	Total

n.d=not determined.

Cys=Cystine calculated as cysteic acid.

3-C-4.

Amino acid compositions of A and $(Fc_\mu)_5$ fragment.

3-C-5. Discussion:

The two (A and $(Fc_\mu)_5$) fragments show a general similarity in amino acid composition. However, amounts of cystine determined after hydrolysis for 24 hours were much less than those expected. As a result, cystine was determined as cysteic acid after oxidation of the protein. Unfortunately, cystine could not be determined from these analysis since poor recoveries were obtained even after hydrolysis for 48 hours. The reason for this low recovery is probably related in some way to the structure of A and $(Fc_\mu)_5$ proteins. Cystine was therefore determined as cysteic acid. The measured amount of cysteic acid not corrected as suggested by Moore (1963) since the recovery of cysteic acid may not be the same for all proteins; the results quoted therefore represent minimum values. On the other hand fragments B and b have the same molecular weight (about 52,000) and are similar in their amino acid contents, indicating that CNBr cleaves the IgM producing two large fragments (C.F. hot trypsin) one corresponding to Fab-like fragment and the other containing $(Fc_\mu)_5$ fragment.

TABLE 11

The amino acid compositions of 7 S subunit and its CNBr produced fragments.

The values for the different fractions are expressed as moles per mole of protein.

Amino acid residue	Har 7S subunit Mol-wt $185X10^3$	Har 7S subunit CNBr Mol-wt $185X10^3$	a Mol-wt $75X10^3$	b Mol-wt $52X10^3$	2b+a Mol-wt $179X10^3$
Asp	125	125	45	37	119
Thr	147	146	56	38	132
Ser	180	180	60	50	160
Glu	148	148	50	42	134
Pro	102	102	40	27	84
Gly	108	107	30	32	94
Ala	96	96	35	28	91
Cys	18	18	5	5	15
Val	118	117	52	30	112
Met	18	8	4	2	8
Ile	50	50	18	14	46
Leu	114	114	35	35	105
Tyr	48	48	15	13	41
Phe	55	55	18	17	52
Trp	n.d	n.d	n.d	n.d	-
Lys	74	74	21	23	67
His	24	24	10	6	22
Arg	63	62	20	16	52
Total	1488	1475	514	415	1334

n.d. = not determined

Cys = cystine calculated as cysteic acid

3-C-6 Amino acid compositions of B, b and a fragments

The compositions of B and b are generally similar. There are however, some not worthy differences. For instance, the B fragment contains significantly more serine and valine and much less threonine than b. However both B and b show the same values for cystine and methionine contents, indicating that, the CNBr cleaved the native IgM molecule and its 7 S subunit at the $Fc\mu$ region in the same position produced Fab_{μ} - like fragments (i.e. B and b fragments respectively); and these provides a further evidence that macroglobulin IgM contains 10 Fab_{μ} fragments and one $(Fc\mu)_5$ and that its 7 S subunit contains two Fab_{μ} fragments and one dimer $Fc\mu$ (i.e. $Fc\mu$ - $Fc\mu$) linked together at a flexible region called hinge region.

The sum of the amino acid compositions of 2 B and one a fragment gave close agreement with that of the intact 7 S subunit (IgM_S) molecule with the exception of three disulphide bonds (2 at the VH region and one at the C-terminal of the μ chain - Putnam etal, 1973).

CHAPTER 4

GENERAL DISCUSSION

The present studies, based on physicochemical and immunological data, confirm that the molecular weight of IgM is close to 950,000, and it consists of five IgM_S subunits each of molecular weight Ca 185,000. IgM_S has been reported to comprise two μ chains and two light chains (Miller and Metzger, 1965 ; Lamm and Small, 1966 ; Putnam,etal, 1967 ; Johnson and Miller, 1970). On the basis of chemical determinations, one light chain is bound through a single disulphide bond to one μ chain (Chaplin,etal, 1965 ; Putnam,etal,1967 ; Beale and Buttress, 1972). The IgM_S subunits appear to be linked with one another by a single disulphide bond located in position 414 of the μ chain (Morris and Inman, 1968 ; Beale and Buttress, 1972 ; Putnam,etal, 1973).

It is well known that either papain or pepsin cleaves the particular peptide bond in the H-chains of IgG molecules to produce two Fab and an Fc, or an $(Fab)_2$ fragment respectively. Digestion of IgM or IgM_S with papain (Onoue,etal, 1967, 1968 ; Suzuki, 1969), pepsin (Kishimoto, etal, 1968 ; Suzuki, 1969) or trypsin (Miller and Metzger, 1966), and fragments corresponding to $Fc\mu$, $(Fab\mu)_2$ and $Fab\mu$ have been identified. The region of IgM_S subunit (Fc_μ) corresponding to Fc from IgG seemed particularly susceptible to enzymatic digestion (Onoue etal, 1967 ; Ungar-Waron,etal, 1967 ; Kishimoto,etal, 1968). However, incubation with trypsin at 60°C cleaves the macroglobulin IgM pentameric molecule into ten monomer $Fab\mu$ fragments and a decameric Fc_μ portion of the μ chain (Plaut and Tomasi, 1970). Trypsin cleavage occurs in the "hinge" region just prior to the first interheavy (μ - μ) chain disulphide bridge (Paul,etal, 1971). Although the Fc_μ fragment is homogeneous in the sequence and by other technique, the $Fab\mu$ fragment appears heterogeneous

at COOH-terminal end of the Fdμ portion ($C\mu_2$ domain) of the μ chain where an undetermined number of peptides may be removed. Sequenator analysis established the identity of the N-terminal region of the Fc_μ fragments obtained by trypsin cleavage at 60°C and showed that the missing part of the μ chain by hot trypsin digestion is inbetween 214 to 325 amino acid residues of the μ chain which cleaved into small peptides (Putnam,etal., 1971, 1972, 1973 ; Shimizu,etal., 1971, 1974 ; Flornt,etal., 1974). In the condition used in this work, CNBr and trypsin generate similar fragments of IgM, trypsin (60°C) preferntially attacking the Fc-like portions of the molecule yielding $Fab\mu$ and $(Fc_\mu)_5$ fragments (Plaut,etal., 1970), and CNBr removing at least some of the Fabμ-like fragments. The similarity, in certain conditions, of the action of these two substances (i.e. trypsin and CNBr) on IgG is coincidental (see above chapter 1), and depends on the presence of exposed methionine residues in the Fc portion of the molecule, near the "hinge" region. Edelman,etal.,(1969)in their domain hypothesis, postulate that both H and L-chains are folded in compact domains which are stabilized by intrachain disulphide loops and connected to neighbouring domains by less tightly folded stretches of polypeptide chains. The amino acid sequence of human IgM has been completed by Putnam etal (1971, 1972, 1973). In several respects, the IgM molecule has a unique structure compared with IgG (Paul,etal., 1971 ; Shimizu,etal., 1971 ; Wang,etal., 1973 ; Low,etal., 1976). The most obvious difference is that the μ chain has about 130 more amino acid residues than the γ chain; this is equivalent to one extra homology region or domain. It has been suggested that the extra domain of the μ chain is in the middle of the molecule (Shimizu,etal., 1974) i.e. the segment or domain designated $C\mu_2$ (Putnam,etal., 1973). It may be that the loosely folded regions of the μ chain are susceptible to CNBr (as well as to hot trypsin) digestion and depends on the exposed methionine residues in the Fdμ

portion of the molecule.

Both the digestion methods described in this work (i.e. trypsin and CNBr digestion of IgM) have been shown to be capable of producing well defined fragments of the IgM molecule. The CNBr digestion of IgM led to the formation of two distinct (if not more) fragments which are designated A and B as they are eluted respectively from a Sepharose 4B gel filtration column eluted with 6M Gu-HCl buffer, pH 4.5. The IgM_s subunits were digested with CNBr in an identical manner to give rise to two distinct fragments which were similarly designated a and b.

The A fragment which tended to precipitate in distilled water or in pure phosphate or Tris-HCl buffers at pH 7.0, could also be converted into smaller subfragments by reduction and alkylation in 6 Molar guanidine hydrochloride at pH 4.5. The molecular weight value calculated from Disc-gel electrophoresis and T.L.G. depended on assumptions concerning the mobility of the molecule, and a number of uncertainties may arise when the molecular weights are determined by these methods (see chapter 2). The B fragment was estimated to have a molecular weight of Ca 52-55,000. The B and b fragments obtained from IgM and IgM_s subunit repectively, both contained light chain determinants and may thus have come from the part of the IgM molecule analogous to the Fab portion of IgG. Suzuki (1969) has isolated a very similar fragment by digestion of human IgM samples with papain and with pepsin. Their Fab_μ - like fragment has a molecular weight of Ca 52,000. This molecular weight is apparently analogous to the Fab fragment of IgG, since it contained the L-chain determinants produced by the action of the trypsin on human IgM (Miller and Metzger, 1966), and of a porcine estrase on human IgM (Chen, etal., 1967). On the other hand the immunological characteristics and the estimated molecular weight of A fragment (Ca 400,000) are similar to $(Fc\mu)_5$ fragment (Plaut, etal, 1970 ; Hester, etal, 1973, 1975) suggesting

that the A fragment may consist of several subfragments disulphide bonded rather than a single $(Fc\mu)_5$ as reported before (Plaut etal, 1970). Recently, Chen etal.,(1974) reported that digestion of IgM with trypsin at 65°C resulted in conversion of this molecule to Fabμ fragments and a mixture of materials derived from the Fcμ region of the molecule. It appears that $(Fc\mu)_5$ fragment is composed of Fc$\acute{\mu}$ subfragment and a residual $(Fc\mu)_5$ fragment held together by noncovalent bonds and that the trypsin at 65°C causes further digestion of $(Fc\mu)_5$ fragment to produce Fc$\acute{\mu}$. This Fc$\acute{\mu}$ subfragment apparently has a tendency to aggregate and hence elutes ahead of $(Fc\mu)_5$ upon sepharose 6 B gel filtration (Chen,etal, 1974 ; Elakovich,etal, 1978). SDS gel electrophoresis and sequence analysis of this Fc$\acute{\mu}$ subfragment indicated that it has a molecular weight of 10-13,000 dalton and is derive from the Cμ4 homology region (Chen,etal.,1974 ; Hester,etal, 1975 ; Elakovich,etal, 1978).

Zikan,etal.,(1973) reported that digestion of human IgM (Dau) with CNBr in 70% formic acid resulted in the cleavage of all the methionine residues and Fab$_\mu$-like fragments of molecular weight 54,900 designated MB,and MA fragment related to an $(Fc\mu)_5$ fragment, and small peptides were obtained. They also reported that digestion of $(Fc\mu)_5$ fragment with CNBr in 70% formic acid degradated it into the respective subfragments, FcA, FcB, FcC and FcD. Zikan and Bennett (1973) have found that the aggregated material in the first peak from sephadex G200 chromatography eluted with 1% NH_4HCO_3 pH 8 is related to Fcμ portion of tryptic digestions of the IgM molecule.

These studies suggest that CNBr digestion of human IgM in milder conditions (i.e. 0.05M HCl) may causes further fragmentation of Fcμ portions as the same as hot trypsin digestion yielding several covalent bonded subfragments.

Tables 6 and 7 (see above chapter 3) summarize some of the physico-chemical properties of the various fragments obtained in the present studies in comparison with those which have been reported in literature. Although it should be borne in mind that the data listed in Table 6 and 7 obtained from pathological IgM-globulins of different sources, similarties in molecular size among the corresponding fragments can be clearly seen. However, according to Sargent and George (1975) the SDS-polyacrylamide gel systems allows the molecular weights of proteins to be determined to within 10%. Thus the apparent molecular weight of one major band (i.e. 29-30,000) in the reduced and alkylated subunit of A and $(Fc\mu)_5$ 65° rather than the expected 32,000, it is not yet possible to ascertain whether this band represents Fcμ fragment or Fcμ́ (Hester, etal, 1974) subfragment derived from further fragmentation of $(Fc\mu)_5$ fragment. Further structural studies should be carried out to clarify these points.

Interpretation of the amino acid analysis of IgM_s and relative yields of a and b fragments after CNBr digestion, showed that the sum of the amino acid residues of a plus 2b pieces is very close to the amino acid composition of undigested IgM_s subunit with the exception of the methionine residues. This indicated that the IgM_s is composed of these two pieces (i.e. a plus 2b). The minor differences in the amino acid composition of IgM_s and a plus 2b pieces suggest the presence of some small peptides as a result of cyanogen bromide (CNBr) digestion of IgM_s molecule. These could be removed by gel filtration columns during the fractionation process. However, further studies should be carried out to identify and to clarify these points.

It is now generally recognized that many peptide bonds involving certain amino acids e.g. isoleucine and valine, are difficult to

hydrolyse. These amino acids may of course, be coupled to any of the other amino acids present in the protein and such residues will be incompletely recovered when the hydrolysis is incomplete.

TABLE 12

The amino acid composition of "Har" IgM, its IgM_s, $(Fc\mu)_5$ and their CNBr produced fragments (A,B,a,b,), and the sum of composition of a plus 2b fragments. The values are given in moles per mole of protein.

Amino acid residues	Har IgM Mol-wt 95X10^4	IgM/CNBr Mol-wt 95X10^4	IgM_s Mol-wt 185,000	IgM_s/CNBr Mol-wt 185,000	A Mol-wt 4X10^5	$(Fc\mu)_5$ 65° Mol-wt 32X10^4	$(Fc\mu)_5$CNBr Mol-wt 320X10^3
Asp	630	63	125	125	220	207	207
Thr	740	740	147	146	310	295	295
Ser	905	905	180	180	230	210	210
Glu	740	740	148	148	270	240	240
Pro	515	515	102	102	190	185	185
Gly	545	545	108	107	134	130	130
Ala	490	490	96	96	190	170	170
Cys	95	95	18	18	32	30	30
Val	595	595	118	117	205	198	198
Met	90	46	18	8	26	30	20
Ile	250	250	50	50	100	98	98
Leu	570	570	114	114	180	175	175
Tyr	250	250	48	48	73	70	70
Phe	275	275	55	55	92	86	86
Trp	n.d	n.d	n.d	n.d	n.d	n.d	n.d
Lys	375	375	74	74	73	70	70
His	125	125	24	24	67	65	65
Arg	320	320	63	62	125	120	120
Total	7510	7468	1488	1473	2517	2379	2369

Cys = cystine calculated as cysteic acid

n.d = not determined

(contd) TABLE 12.

Amino acid residues	B Mol-wt $52X10^3$	a Mol-wt $75X10^3$	b Mol-wt $52X10^3$	a + 2b Mol-wt $179X10^3$
Asp	36	45	37	119
Thr	37	56	38	132
Ser	52	60	50	160
Glu	42	50	42	134
Pro	28	40	27	84
Gly	32	30	32	94
Ala	29	53	28	91
Cys	5	5	5	15
Val	32	52	30	112
Met	2	4	2	8
Ile	13	18	14	46
Leu	34	35	35	105
Tyr	13	15	13	41
Phe	16	18	17	52
Trp	n.d	n.d	n.d	n.d
Lys	23	21	23	67
His	6	10	6	22
Arg	16	20	16	52
Total	416	514	415	1334

n.d= not determined.

Cys=cystine calculated as cysteic acid

The amino acid composition of the whole IgM (Har) molecule (Table 8) differs considerably from that of the normal IgM (Chaplin, etal, 1965) and also from that of Waldenstrom's macroglobulins with a similar light chain specificities (i.e. K type) (Suzuki, etal, 1967) particularly in the content of cystine, methionine and trypsin. The amino acid composition data indicate significantly higher amounts of cystine, methionine, and tyrosin in IgM (Har) than in other reported IgM globulins (Himburger, etal, 1964 ; Chaplin, etal, 1965 ; Suzuki, etal, 1967). The tyrosin content of a given class of immunoglobulin has been remarkably stable in the amino acid analysis of the hydrolysates, whereas the amounts of such amino acids as lysine, threonine, serine, valine, etc. are more variable. The present investigation has also presented an interesting aspect of protein biosynthesis and gene regulation. The peculiar physicochemical properties of A and B fragments (see above chapter 3) produced by CNBr digestion of this macroglobulin (i.e. "Har" IgM) may be attributed to mutation of amino acid sequence arround the strategic points which are responsible for structural stability. However, whether or not the observed differences (few basic, neutral and acidic amino acid residues) in "Har" IgM can be directly correlated with the diagnoses will require a careful examination because of the following reason: Amino acid compositions presented in Table 8, 9 and 10 are calculated on the basis of the estimated molecular weights and on the assumption that all light chains Kappa type (Mol-wt 23,000) and all μ chains consist of identical number of amino acid residues (214) and (578) respectively. The latter may not be true, since all of the IgM proteins except normal human IgM are the products of malignant disease process and thus may have some deletion or addition in their sequences. Even if μ chains examined have neither deletion nor addition and consist of identical number of amino acids, their

V_H subgroups may differ from each other. Furthermore, at present we do not know if the constant portions of these μ chains have any variations in their amino acid sequences, only the sequential study of amino acid arrangement will permit an understanding of the changes occuring in molecular conformation and the reason for their structural itergrity. Results of the amino acid analysis appear to indicate the marked differences in the amino acid composition between IgM_s subunit associated with "Har" IgM and those IgM_s subunits associated with Waldenstrom IgM globulins (Zinneman,etal., 1973). Both myeloma IgM_s (Zinneman,etal., 1973) showed less cystine, methionine and tyrosin than "Har" IgM_s but more glycin and serine residues (See above chapter 3 Table 9). Furthermore, our conclusion that the constitution of the human IgM is not always the same for all the individuals is consistant with the analytical work of Putnam,etal.,(1967) and Bennett (1969). Putnam,etal.,(1967) determined the amino acid composition of H and L-chains of five monoclonal IgM, type Kappa. From peptide maps of four H chains one can see that some peptides are shared by all the H chains, while others are contained in three out offour. Other peptides were detected in only one or two of the H-chains. The peptide patterns and end group data presented by Putnam,etal.,(1967) give the first chemical evidence for structural difference in the H-chains of IgM globulins from different patients with Waldenstrom's macroglobulinemia. (Putnam,etal.,(1967) and Bennett (1969)). These authors showed that the μ chains obtained from individual IgM globulins have different amount in their methionine (5-7 residues) and tyrosin (6-22 residues) contents.

The nomenclature of the CNBr fragment corresponds to that used for IgM OU (Putnam,etal, 1973). Although the number of CNBr fragments expected from the μ chain of a pathological IgM may vary from 6 to 11

depending on the methionine content of the V region, the number obtained from the Fcμregion should be constant in the absence of isotypic substitutions of methionine. However, in addition to comparison of amino acid composition in terms of single amino acids or groups of amino acid (e.g. hydrophilic, hydrophobic) a series of parameters thought to reflect several macromolecular properties were calculated from the composition data contained in tables 8-12 (see chapter 3). A fragment was similar to the proposed $(Fc_{\mu})_5$ (Putnam,etal, 1973) i.e. methionine residue 336 through 578 amino acid residues or to the $(Fc\mu)_5$ (Watanabe,etal, 1973) i.e. residue 331 through 571 amino acid residues and was similar to the molecular size of the $(Fc_{\mu})_5$ (Hester,etal, 1975 ; Zikan and Bennett, 1973). After reduction and alkylation of the A fragment, it resolved into smaller subunits of molecular weights (Ca 59,000, 41,800, 29,000, 17,700, 11,200 and about 4,000) (See chapter 3) as identified by gel electrophoresis in sodium dodecyl sulphate. By means of chromatography on sepharose 4B in 6M Gu-HCl buffer, pH 4.5 a fragment of molecular weight (Ca 29-30,000) was separated. The ten chains in the $(Fc\mu)_5$ fragment are tightly held together in symmetrical array by two interchain disulphide bridges between each chain in each of the five pairs, and each pair is joined to two other pairs by an intersubunit bridge. Thus from these studies it was concluded that the CNBr cleavage may take place at other methionine sites in the Fcμregion as occurred at methionines 489, 506 and 568 in Ou Fcμ(Putnam,etal, 1973) and in normal Fcμ(Florent,etal, 1974). These bands are in the Cμ 4 domain and at the C-terminal of the μ chain (Putnam,etal, 1973) and thus are probably some-what exposed. A therefore, probably contains inter and intrachain disulphide bonds responsible for keeping the subunit together (designated 6, 7, 8 and 9 fig. 32.). On the basis of current model for IgM it is likely that at least one of these interchain disulphide bonds connects

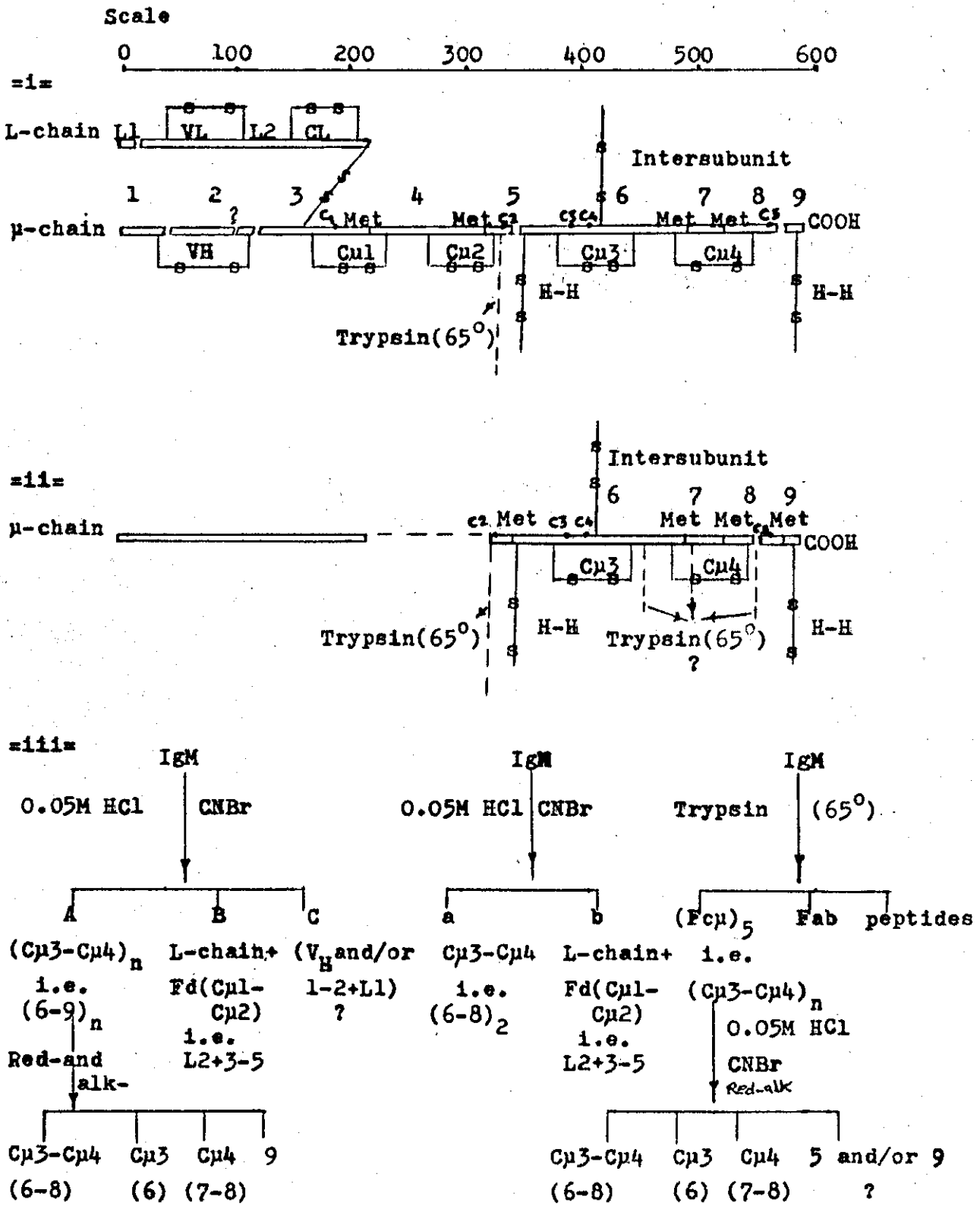
Fig.32. Diagram of a monomeric subunit of human IgM(Har) protein showing:

i / position of CNBr subfragment. Adapted from the IgM structural model proposed by Putnam,et al(1973).

ii / The proposed tryptic cleavage of Har Fcμ region

iii / Isolation of IgM(Har) fragment .Flow sheet summarizes the various procedures to isolate and derive the relative order of the CNBr fragments of IgM(Har) . The numbersrefer to the placement order from the N-terminal of the μ chain.

Fig.32(i,ii,and iii)



n=10 ; Red,reduction;alk,alkylation .

individual subunits, and a second one connects μ chains within one subunit. The structural relationship between Fcμ (Chen,etal, 1974) and J chain (Mestecky,etal, 1971 ; Inman,etal, 1974) found in the reduction and alkylation products of the IgM cannot at present be easily determined.

On the other hand the B fragment containing low cystine (5 residues) compared to the proposed Ou Fabμ (6 cystine residues) Putnam,etal, 1973) fragment (see chapter 1 Fig.3). At present we do not know whether the missing single disulphide bond could be resolved from V_H or $C_{\mu}2$ region or some decomposition of cystine or cysteine during the preparation of hydrolysate. Further experimentation is needed to clarify this point. On the basis of the molecular weight, immunological determination and amino acid composition of B fragment, it appears that it consists of L chain and a disulphide bonded Fd_{μ} portion (designated L_2, 3, 4 and 5 fig. 32.). Studies by Huster,etal., (1973) on the location and the arrangement of the five oligosaccharides (C_1 - C_5) on the μ chain molecules derived from several macroglobulin IgM proteins suggest that none of them were identical, indicating thereby that there is a possible single amino acid substitution. At this juncture and by analogy (Putnam,etal.,1973) we do not know the location and the number of methionine sites in the V_H region or in the hypervariable region of the "Har" μ chain or the V_H subgroup of the "Har" μ chain or the number of methionine sites in the "Har" L-chain, but we are assuming that the C_H region of the "Har" μ chain has the same number of the methionine residues and at the same sites on the C_H region of Ou μ chain (Putnam,etal., 1973).

When comparing the partial structure of "Har" $(Fc\mu)_5$ with our order of CNBr pieces of IgM several additional relationship between A and the prepared "Har" $(Fc_{\mu})_5$ were found. The similarity of the

molecular weights of the subunits derived from the reduction and alkylation of the A fragment with those subunits derived from the reduced and alkylated $(Fc\mu)_5$ (Har) after CNBr digestion, suggest that the CNBr cleavage occurred at the same place of the Fc_μ region (fig 32-). The fact that the cystine and methionine contents of Har $(Fc_\mu)_5$ obtained from the chromatogram are slightly less than that proposed by Putnam, etal.,(1973) suggests that the tryptic cleavage took place at other sites in the Fc_μ region. This is supported by the work of Florent, etal.,(1974) who found the cleavage after lysine 445 in Dau Fc_μ and also in normal $Fc\mu$. Elakovich, etal., 1978 ; Hester, etal, 1975 ; and Chen, etal., 1974 had also found the cleavage at sites 468, 491, 515 and 546. These cleaved bonds are midway between $C\mu_3$ and $C_{\mu 4}$ domain and in the $C_{\mu 4}$ itself. It would appear from these facts that these bonds are probably exposed and can be cleaved by trypsin.

Since the Fc portion of IgG probably has a different primary structure from that of the analogous region of IgM (Low, etal., 1976) such a residue may not occur in the latter molecule, and it is not surprising that CNBr may does not attack the IgM molecule in the same region. It seems thus that the action of CNBr on the IgM globulin under the described condition (i.e. 0.05M HCl) led to the cleavage of about 50% of the methionine residues of the IgM molecule similar to those in partial cleavage of IgG with CNBr (Cahnmann, etal, 1965, 1966 ; Lahav, etal, 1967). Fragments produced by CNBr cleavage of IgG (Cahnmann, etal, 1965, 1966 ; Lahav, etal, 1967 ; Prahl, etal, 1968, 1969 ; Waxdal, etal, 1968 ; Kindt etal., 1970) were similar to the fragments obtained in the present work (i.e. A and B fragments) in the following respects: behaviour in neutral pH and on SDS-gel electrophoresis, antigenic determinant, and alkaline urea electrophoresis. However, from the results discussed,

there is an indication that only two fragments were obtained from the reaction of IgM with CNBr. Under such conditions it is evident that this method provides a satisfactory way of obtaining good yields of Fc_{μ} and Fab_{μ} pieces of IgM molecule. One possible mechanism for their production is shown in the proposed model (fig. 32).

CHAPTER 5

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APPENDIX

SPECIAL REAGENTS AND BUFFERS

1. Guanidine hydrochloride :

The commercial guanidine hydrochloride (Gu-HCl) salt was repurified and crystallized following the procedure of Nozki, (1972). 6 Molar Gu-HCl solution prepared in this way had an optical density (1 cm, 280 nm) of less than 0.12.

2.A. 0.1M Tris-HCl /0.3M NaCl, pH 8.2 :

This was prepared by dissolving 24.228 gms of tris (hydroxy-methyl) -methylamide in 1 litre of thrice-distilled water. A concentrated hydrochloric acid was added drop-wise until the pH 8.2. 0.3M sodium chloride (36.064 gms) was added and the solution was made up to two litres with thrice-distilled water.

2.B. 0.05M Tris-HCl/ 0.15M NaCl, pH 8.1 :

This was made by dilution the above tris-HCl-saline buffer(2.A) with distilled water with a ratio 1:1, and the pH was adjusted to pH 8.1 by addition drops of 5M HCl.

3.A. 0.05M Tris-barbital-sodium barbital buffer, pH 8.8 (Gelman High Resolution):

This was prepared by dissolving: Tris 9.6 gms, 4.1 gms barbital, and 16.3 gms barbital sodium in two litres of triply-distilled water. This buffer had a pH 8.8. This solution stored at 4°C and discard after 4 weeks.

3.B. 0.05M Tris-barbital-sodium barbital buffer,pH 8.8, containing 6M urea:

This was prepared as above (3.A.) and 6 Molars (360 gms) Urea was added to this solution before it was made up to two litres.

4. Buffers for Disc-polyacrylamide gel electrophoresis

Gel buffer : This was prepared as described in booklet BDH.Ltd., (1976). 8.82 gms dihydrogen orthophosphate, dihydrate, 20.4 gms di-sodium hydrogen orthophosphate, 2.0 gms sodium dodecyl sulphate (SDS), triply-distilled water to one litre.

Acrylamide solution : 22.2 gms acrylamide, 0.6 gms methylenebis acrylamide, thrice-distilled water to 100 ml.

Ammonium persulphate solution : (15 mg/ml), freshly prepared. This was prepared by dissolving 75 mg of $(NH_4)_2 SO_4$ in 5 ml distilled water.

5. Electrophoresis stains :

5.A. For cellulose acetate strips (work and work, 1972)

i. Poneau S 5.5 gms poneau S was dissolved in 200 mls of 5% trichloro acetic acid prepared in thrice-distilled water.

ii. Nigrosin 10 mgs were dissolved in 200 mls of 2% glacial acetic acid in distilled water.

5.B. For polyacrylamide gel and immunodiffusion techniques (Dako, 1975)

Coomassie brilliant blue R250 5 gms

Ethanol 96% .. .. .. 450 mls

Acetic acid .. .. .. 100 mls

Thrice-distilled water .. 450 mls

Coomassie dye is added to the ethanol/acetic acid mixture and left at room temperature for overnight. The solution is filtrated before the water is added.

5.C. For thin layer gel chromatography (work and work, 1972; Pharmacia, 1973)

C.a. Bromophenol blue: 1% Bromophenol blue in ethanol saturated with mercuric chloride.

C.b. Coomassie Brilliant blue R250, 0.25% in methanol-glacial acetic acid (9 : 1 $\frac{V}{V}$)

6. Preparation of buffer solutions for amino acid analysis (Renk Helger, 1978)

6.A. Acid buffer I (pH 2.2)

10.5 gm Citric acid (AR)

11.7 gm Sodium Chloride (AR)

2.5 ml Thiodiglycol (LR). See note 1

3.5 ml 10% Brij - 35. See note 2

Make up to 1 litre with deionised water.

Note 1. 25% $\frac{V}{V}$ Thiodiglycol in water.

Note 2. 10% Brij - 35; dissolve 100 gm in deionised water with warming, add 50 ml Methanol (AR) and dilute to 1 litre with water.

6.B. Acid buffer II (pH 2.2)

10.5 gm Citric acid (AR)

11.7 gm Sodium Chloride (AR)

2.5 ml Thiodiglycol (LR). See note 1 above

100 ml Methyl alcohol (AR)

3.5 ml 10% Brij - 35. See note 2 above

Make up to 1 litre with deionised water.

6.C. Basic buffer - Citrate - phosphate (pH 11.5 approx)

14.7 gm Tri-sodium citrate (AR)

6.46 gm Tri-sodium Orthophosphate (AR)

12.5 ml 4% E.D.T.A. (AR). See note 3

3.5 ml 10% Brij - 35. See note 2 above

Make up to 1 litre with deionised water

Note 3. Ethylene diamine tetra-acetic acid (disodium salt)

Dissolve 40 gm in deionised water with stirring and dilute to

1 litre with water.

6.D. Ninhydrin (pH 5.5 approx.)

10 gm Ninhydrin (AR)

216 gm Sodium Acetate Tri-hydrate (AR)

100 Glacial Acetic Acid (AR)

400ml 2-Methozyethanol (Chromatographically tested)

10 ml 10% Brij - 35. See note 2(on the previous page).

Make up to 1 litre with deionised water.

Dissolve the Ninhydrin in 2.0 Methoxyethanol. When dissolved, add 300 ml water, the Sodium Acetate and Acetic acid. When this has dissolved, add the Brij - 35 and dilute to 1 litre with water.

6.E. Wash Solution

400 ml 2 methoxyethanol (chromatographically tested)

10 ml 10% Brij - 35. See note 2 (on the previous page).

Make up to 1 litre with deionised water.

6.F. Cyanide (Reducing agent)

1% stock solution :

To 1 gm of Sodium Cyanide (AR) add 4 gm Sodium Carbonate and dilute to 100 ml with deionised water.

Working Solution :

Dilute 1.0 ml of stock solution, 2.0 ml of 4 Molar Sodium hydroxide and 10 ml 10% Brij - 35 (see Note 2 on the previous page) to 1 litre with deionised water.

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