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I

STUDIES on HUMAN IgD

MOLECULAR WEIGHT and SEDIMENTATION COEFFICIENT

II

STUDIES on HUMAN IgM

CHARACTERISTICS of PRODUCTS of MILD REDUCTIVE CLEAVAGE

Final Technical Report

by

D. S. Rowe, H. Isliker, F. Dolder and H. D. Welscher

October 1968

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W.H.O. International Reference Centre for Immunoglobulins

Institut de Biochimie, Université de Lausanne
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SECTION II - Studies on Human IgM

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INTRODUCTION

Immunoglobulin D constitutes a class of human immunoglobulins which is antigenically and functionally distinct from the other classes currently recognized: IgG, IgA, IgM and IgE. The antigenic distinctiveness of IgD lies in its heavy polypeptide chains. IgD is present in non-myeloma sera in a relatively small amount but is not sufficiently different in its chromatographic and other characteristics to permit its isolation by physicochemical methods from IgA, IgE and IgG. ~~(1).~~ The most convenient source of IgD for physicochemical studies consists of D-myeloma proteins occurring in serum of rare patients with multiple myeloma. This paper describes the isolation of D-myeloma proteins from two such sera and their sedimentation constants and molecular weights.

MATERIALS and METHODS

Pathological sera. Sera from six patients were studied. D-myeloma proteins isolated from the sera of two of these individuals proved suitable for molecular weight studies. The first was from patient Mi who has previously been described as patient B.M. (2). The second was from patient Na and was kindly provided by Dr. S. Barandun. Serum Mi had been stored at -20°C for two years before analysis; serum Na had been stored for approximately one month.

Fractionation procedure. Twelve ml of serum were dialyzed for several hours against 0.01 M phosphate buffer, pH 8.0, at 4°C. The serum was then applied to a column of DEAE-cellulose Whatman "DE-32. microgranular" 2.5 x 25 cm and eluted with phosphate buffer, pH 8.0, in a gradient from 0.01 M to 0.3 M using a Varigrad as previously described (1). The optical density of the fractions was read at 280 mμ and fractions containing IgD were identified by Ouchterlony analysis using antisera specific to this protein. Appropriate fractions were pooled and concentrated to approximately 3 ml by ultrafiltration at 4°C. The concentrated fractions were then applied to the bottom of a column of Sephadex G-200, 2.5 x 90 cm, in 0.05 M Tris buffer, pH 8.0, containing 0.2 M NaCl. Fractions were eluted by upward flow with the same buffer pumped at a flow rate of approximately 2 ml per cm^2 per hour. Optical densities were read at 280 mμ or at 254 mμ in a "Uvicord" recording spectrophotometer. IgD containing fractions were pooled and concentrated by ultrafiltration at 4°C. The concentrated protein was stored at -70°C.

Preliminary studies showed that the D-myeloma protein in one serum (Mi) was stable when separated under these conditions. D-myeloma proteins from four other pathological sera were unstable and were recovered chiefly as Fc-like fragments eluted after IgG from the G-200 column. In a series of experiments on one of these sera it was found that the D-myeloma protein became split during the stage of chromatography on DEAE-cellulose. A much greater yield of unsplit material was obtained when all fractionation procedures were carried out at 4°C and in the presence of epsilon-amino caproic acid (EACA). Serum Na was fractionated in this way: 0.001 M EACA was added to the initial dialysing buffer and to the buffer used to equilibrate and elute the DEAE-cellulose column and sufficient EACA was added to the fraction collector tubes before fractionation to render each fraction 0.01 M. The Sephadex G-200 column was equilibrated with buffer containing 0.01 M EACA.

Preparation of IgG. IgG was prepared by chromatography of pooled normal human serum on DEAE-cellulose. The serum, dialyzed against 0.01 M pH 8.0 phosphate buffer was applied to the column and IgG was eluted as a single peak using the same buffer. This IgG was passed on a Sephadex G-200 column equilibrated with 0.05 M Tris pH 8.0 containing 0.2 M NaCl. Fractions from the top of the peak were used for ultracentrifugal analyses.

Ouchterlony and immunoelectrophoretic analyses were carried out using standard techniques. Antisera to IgD were prepared to D-myeloma proteins S.J., and Mi in sheep and rabbits, and were rendered specific by absorption with IgG or with normal human serum deficient in IgD (1). Antisera to other immunoglobulins were prepared in sheep and rabbits and rendered specific by absorption as previously described (3). Horse polyvalent antihuman serum was obtained from the Institut Pasteur (batch No. 223+419) and from Sevac, Prague (batch No. EL 15).

Quantitative radial diffusion tests for IgG, IgA and IgM were carried out by a modification of the method of Mancini et al. The provisional standard used for calibration has previously been described (3).

Ultracentrifugal analyses were performed in 0.05 M Tris-HCl buffer, pH 8.0. containing 0.20 M NaCl at rotor speeds of 44,770 rpm in a Spinco model E ultracentrifuge. Molecular weights were determined by the approach to sedimentation equilibrium method of Archibald (4) on proteins previously equilibrated against the same buffer.

Protein concentrations of the isolated D-myeloma proteins were calculated from optical density at 280 mμ

$$\left\{ E_{280}^{1cm\ 1\%} = 13.5 \right\}$$

RESULTS

Isolation of D-myeloma proteins. Two preparations (A and B) of D-myeloma protein were isolated from two samples of serum Mi. Figure 1-1 shows the chromatographic separation on DEAE-cellulose for subsequent purification. Figure 1-2 shows the separation of this material on Sephadex G-200. Fractions of the first part of the second peak were found to be free from IgG and other plasma proteins and were pooled for the subsequent analyses. Immunoelectrophoretic analysis (Fig. 1-3) shows that after chromatography on DEAE-cellulose, the D-myeloma protein of preparation Mi(A) was of identical mobility to the protein of unfractionated serum. However, after fractionation on Sephadex G-200 and subsequent concentration the myeloma protein was more positively charged than the myeloma protein in the unfractionated serum (Fig. 1-4). Similar analyses of preparation B showed no change of charge after isolation. The reason for the difference between the two preparations is not clear. However, the various stages of isolation were carried out more rapidly in the case of preparation B.

One preparation of D-myeloma protein was isolated from serum Na and the chromatographic separations are shown in Figures 2-1 and 2-2. This preparation appeared to be of identical electrophoretic mobility with the D-myeloma protein in the unfractionated serum (Fig. 2-3).

Quantitative radial diffusion analyses of the isolated proteins showed only trace contamination with IgG and IgA (Table 1). Immunoelectrophoretic analyses of these preparations at a concentration of 11 mg/ml using polyvalent antisera to human serum proteins showed either no precipitation lines or a faint line identical in electrophoretic mobility with that produced by an antiserum to IgD.

Determination of sedimentation constants. The isolated proteins sedimented as single homogeneous peaks (Figs. 3-1 and 3-2). The concentration dependence of the sedimentation constants of D-myeloma proteins Mi(B) and Na, and of IgG prepared from normal human serum are shown in Figure 3-3. Sedimentation constants at infinite dilution were obtained by extrapolation of the calculated regression lines and are shown in Table 2.

Molecular weight determinations. The three D-myeloma and the IgG preparations were studied by the approach to sedimentation equilibrium method. The results are presented in Table 2. The values determined at the meniscus and bottom of the cell agreed within 4% of the average values, which is a good indication of the molecular homogeneity of these preparations.

Table 1

Protein and immunoglobulin concentrations in myeloma sera and isolated D-myeloma proteins

	Total protein*) mg/ml	IgG mg/ml	IgA mg/ml	IgM mg/ml
Serum Mi		2.8	0.16	0.04
MP Mi(Λ)	8.0	0.04	0.03	$<$ 0.006
Serum Na		3.2	0.24	0.06
MP Na	11.0	0.05	0.04	$<$ 0.006

*) Total protein from optical density at 280 mμ. MP Mi(Λ), MP Na are myeloma proteins of figures 1-2 and 2-2, respectively.

Table 2

Sedimentation constants and molecular weights of D-myeloma proteins and of IgG

	D-myeloma proteins			IgG
	Mi(A)	Mi(B)	Na	
$s^{\circ}_{20,w}$	{6.06 / 6.12}*)	6.14	6.19	6.64
MW_m	179,000	182,000	184,000	161,000
MW_b	193,000	185,000	182,000	162,000
average	186,000	183,500	183,000	161,000

Molecular weights MW_m and MW_b obtained by sedimentation equilibrium from measurements at the meniscus and the bottom of the cell, respectively.

*) Values obtained in two measurements at $E^{1cm}_{280} = 5.0$

DISCUSSION

IgD is difficult to isolate from normal human serum. It is present in small amount (median concentration 0.03 mg/ml) and it is difficult to separate from IgG and IgA (1). Myeloma proteins, therefore, provide the most suitable material for physicochemical and other analyses.

D-myeloma proteins, as well as the IgD of normal serum, are frequently found to be unstable on storage.(3, 6, 7). In the present work instability was chiefly apparent during fractionation and took the form of splitting into low molecular weight fragments, which occurred especially during chromatography on DEAE-cellulose. The fragment carrying the specific determinants of IgD migrated further toward the anode than the intact protein on immunoelectrophoretic analysis. This fragmentation did not occur in all sera. When it did occur it could be largely prevented by performing all fractionations at 4°C in the presence of epsilon-amino caproic acid. Splitting of this type has been noted also in unfractionated sera in our collection. It occurred in serum SJ (8) and in other serum samples. A second change, which was observed to occur during fractionation, was that of increased positive charge of preparation A of D-myeloma Mi. (Fig. 1-4). Since the molecular weight of this preparation was not demonstrably different from that of preparation B of D-myeloma Mi, which showed no change of mobility, this change could not be due to loss of a large fragment from the IgD molecule. This type of change has not been observed to occur in stored serum or during digestion by papain or pepsin.

The sedimentation constants of the two D-myeloma proteins, extrapolated to zero concentration, were 6.14 and 6.19. The concentrated dependence of sedimentation constants of each of the proteins was also virtually identical (Fig. 3-3). These values are based on analyses performed at pH 8.0 in Tris HCl buffer I = 0.25. At this relatively high pH a charge effect might occur, which could lead to a reduction of sedimentation rate. However, IgG from normal serum run under the same conditions was found to extrapolate to 6.64S. Lower values for the extrapolated sedimentation constant of some D-myeloma proteins compared with IgG, have been reported by Hansson et al (9). However, simmlarity of the two proteins Mi and Na contrasts with the differences of sedimentation constants (6.19, 6.54 and 6.67 S°_{20w}) of three D-myeloma proteins reported by these authors. Rowe and Fahey previously reported a sedimentation constant of 7.04 for D-myeloma SJ (8).

The reason for the differences of sedimentation constants reported for these proteins is not clear. They may arise either from dif-

ferences between individual D-myeloma proteins or from differences in the methods of preparation leading to changes in the molecules.

The molecular weights of the two D-myeloma proteins average 184,000. The two proteins gave almost identical molecular weight values. IgG from normal serum under the same conditions had a molecular weight of 164,000. The high molecular weight of IgD compared with IgG is consistent with its earlier elution from Sephadex G-200 columns. It contrasts with the lower values for the sedimentation constants of IgD compared with IgG and indicates that IgD may be a more compact molecule than IgG. The reason for the higher molecular weight of IgD is unknown. Delta heavy chains may be heavier than gamma chains or the molecule may include significant carbohydrate or other prosthetic groups.

The role of IgD in the immune response is as yet unclear. IgD comprises only a minor component of the total immunoglobulins. Increased plasma IgD is found occasionally in chronic infections and frequently in kwashiorkor, and in plasma from African children. No specific antibody has yet been demonstrated within the IgD class but in view of the heavy and light chain structure of this protein and of its synthesis in plasma cells it is likely that antibodies do occur (10). Knowledge of such biological characteristics of the class as tissue binding and the capacity of aggregates to fix complement, and alter vascular permeability is, therefore, important in a study of this class. Such studies are now in progress on the isolated proteins. In addition, the predominance of type L chains among D-myeloma proteins (7) and in plasma cells synthesising delta chains (11) is unique among the immunoglobulin classes and indicates a special relationship of delta to lambda compared with kappa chains. This relationship may be due to a preferential binding by delta chains of lambda rather than kappa chains. Recombination studies with isolated chains are, therefore, planned.

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Immunology 1969 (in press)

Fig. 1-1. DEAE-cellulose chromatography of myeloma serum Mi(A) Fractions 55-61 as indicated by the horizontal lines, were pooled. Ouchterlony analyses for transferrin (Tf), IgD and α_2-macroglobulin (α_2-M) gave positive precipitin lines in the indicated fractions.

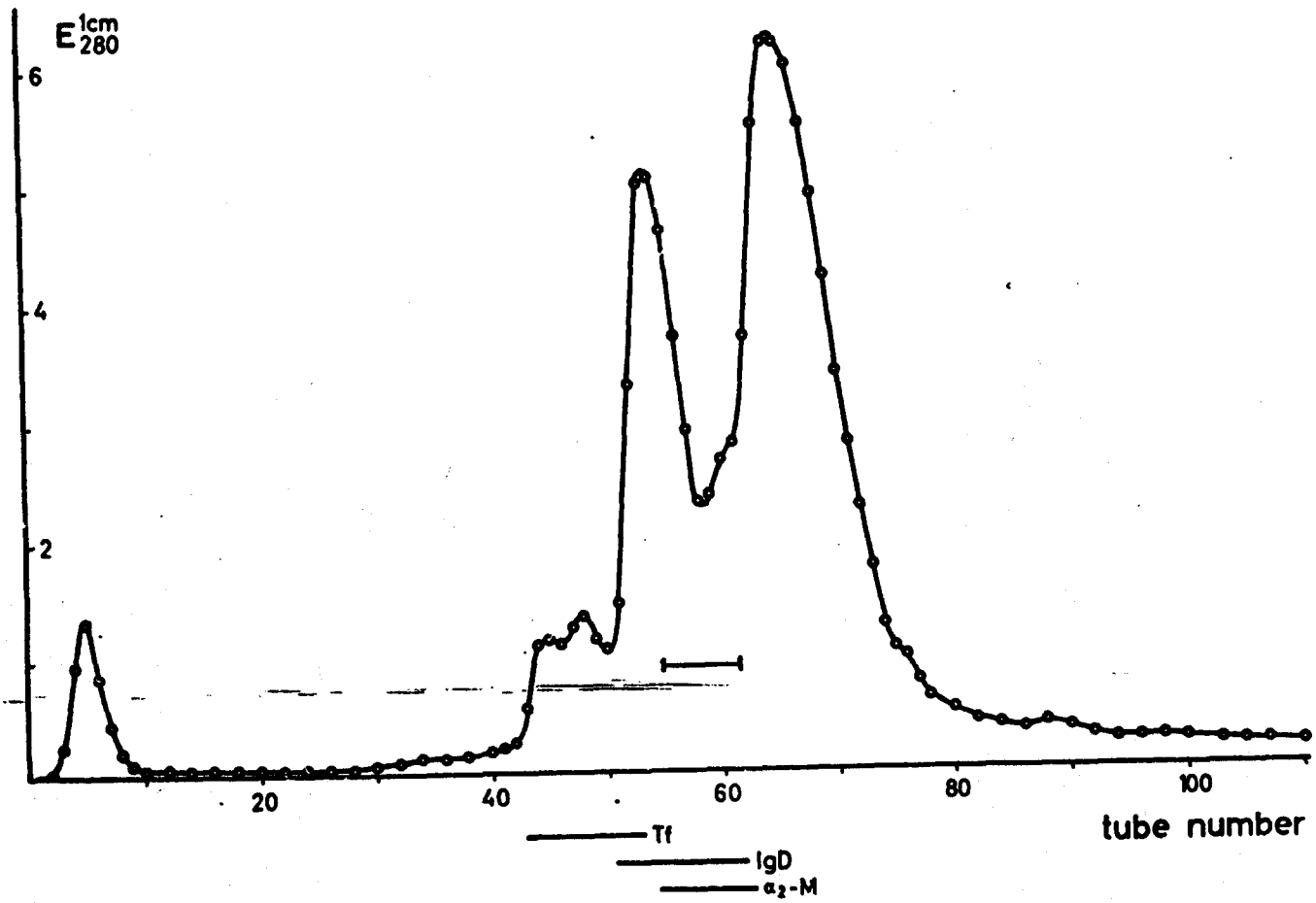


Fig. 1-2. Sephadex G-200 gel filtration of IgD containing fractions from Fig. 1-1. Fractions 48-52, as indicated by the horiztonal line, were pooled and used for physicochemical analysis.

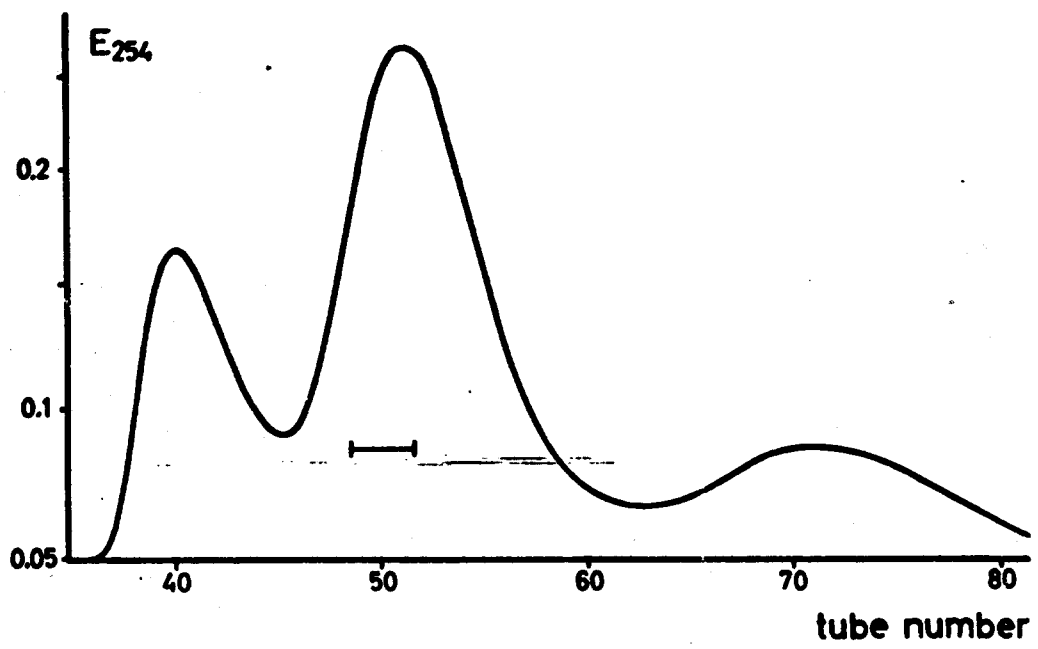


Fig. 1-3. Immunoelectrophoresis of myeloma protein Mi(A) after DEAE-cellulose chromatography. The IgD fraction of Fig. 1-1 at 10 mg/ml as compared with unfractionated serum Mi(A). NS is normal human serum, MS is the myeloma serum, MP the partially purified myeloma protein, a-NS is antiserum to human serum proteins, a-IgD is specific anti-IgD serum.

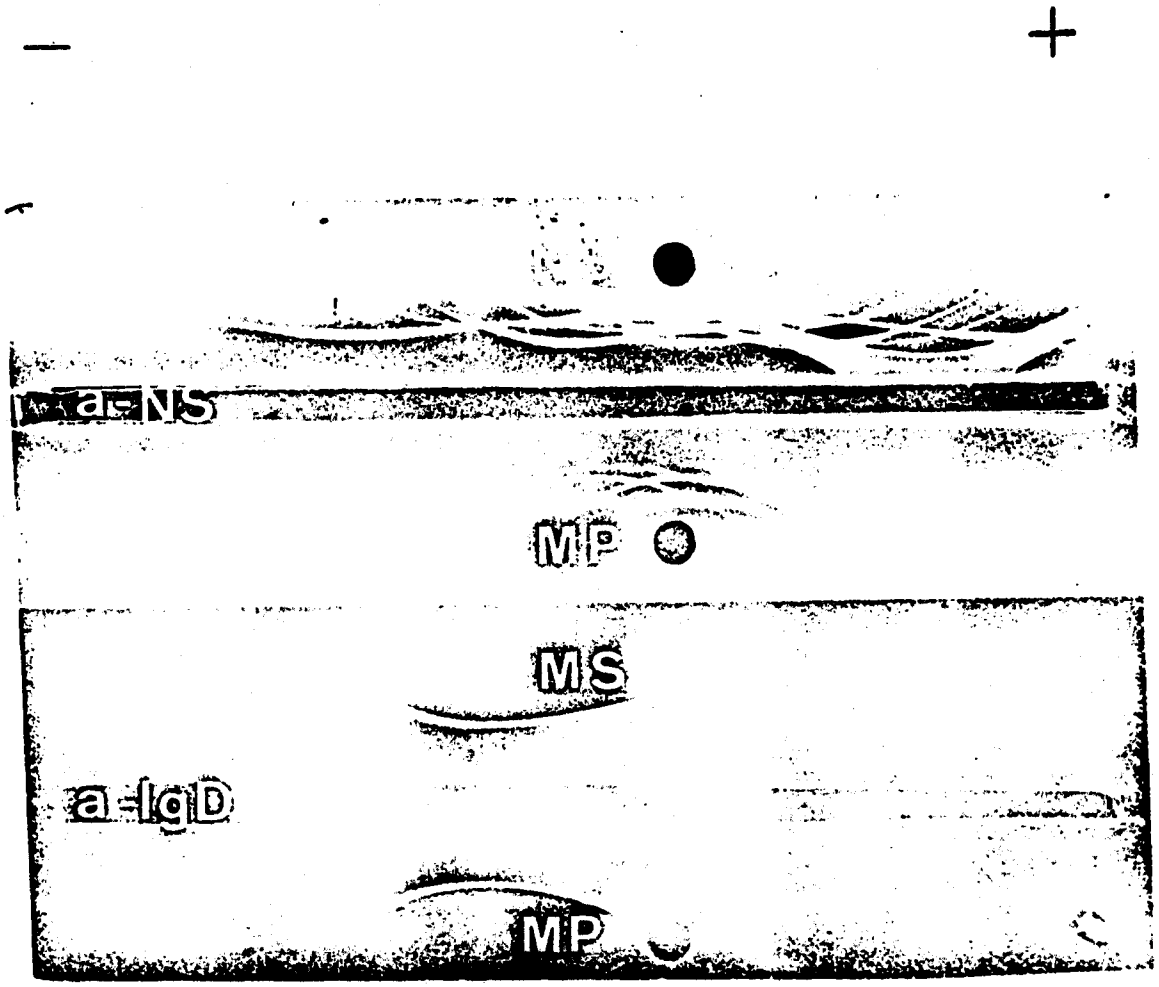


Fig. 1-4. Immunoelectrophoresis of myeloma protein Mi(A) after Sephadex G-200 gel filtration. The IgD fraction of Fig. 1-2 at 8 mg/ml as compared with unfractionated serum Mi(A). NS is normal human serum, MS is the myeloma serum, MP the purified myeloma protein, a-NS is antiserum to human serum proteins. a-IgD is specific anti-IgD serum.

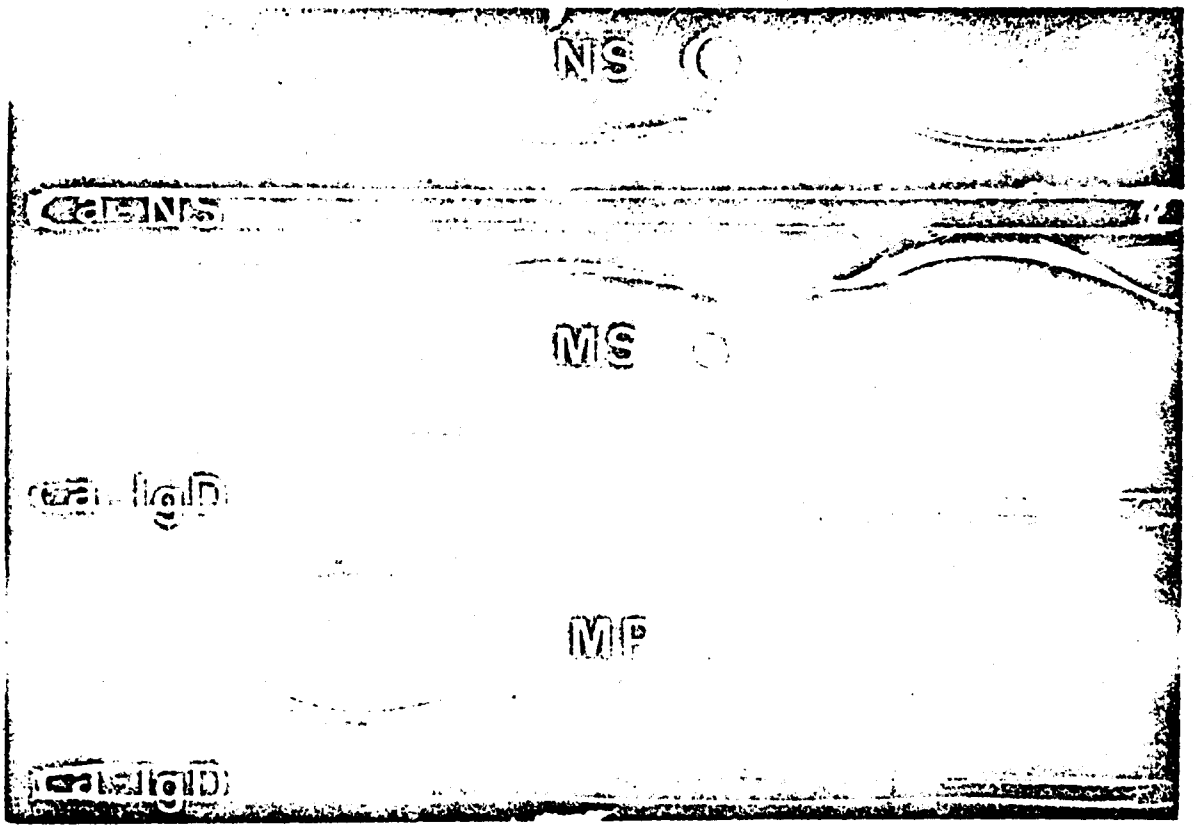


Fig. 2-1. DEAE-cellulose chromatography of myeloma serum Na. Fractions 35-42 were pooled.

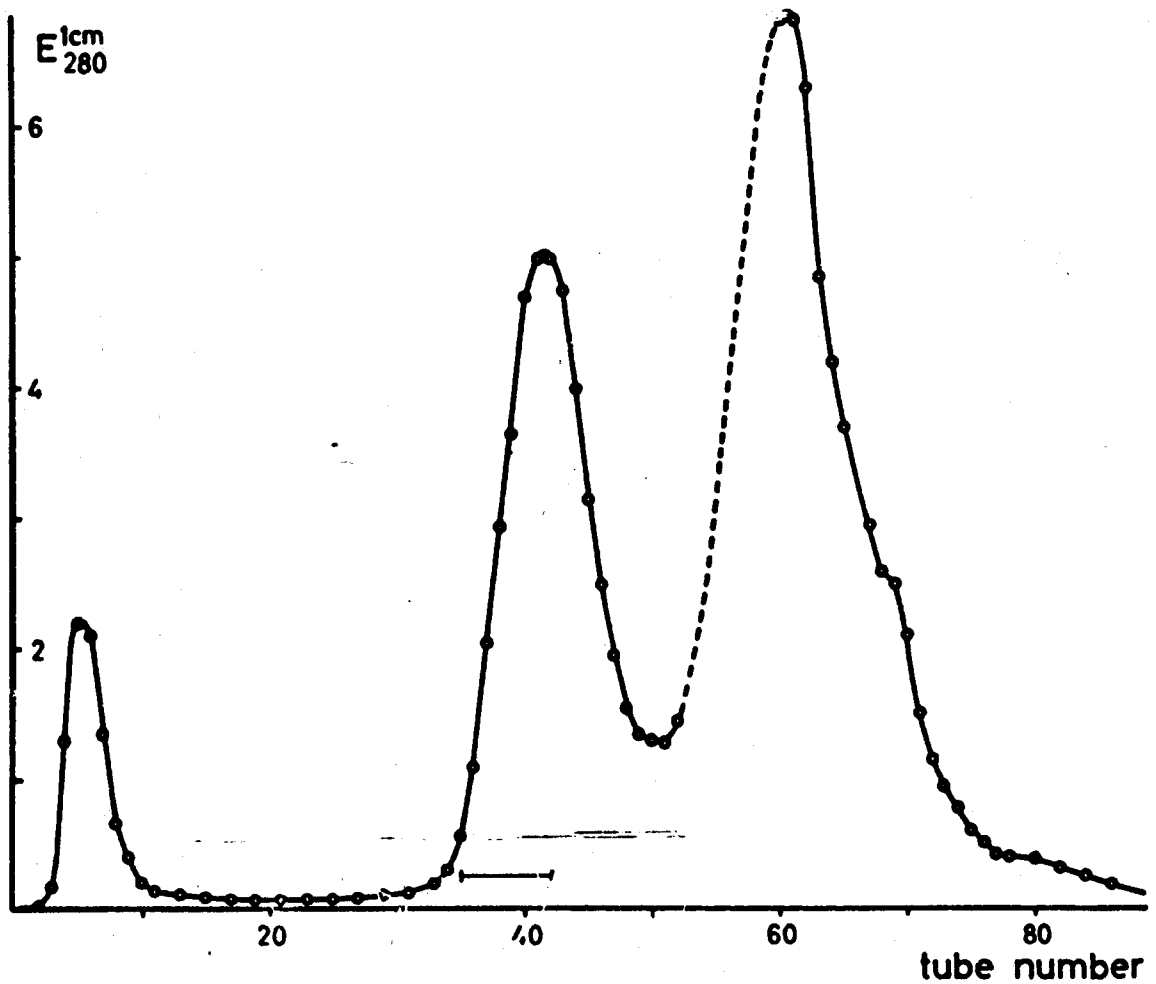


Fig. 2-2 Sephadex G-200 gel filtration of IgD containing fractions from Fig. 2-1. Fractions 43-46 were pooled.

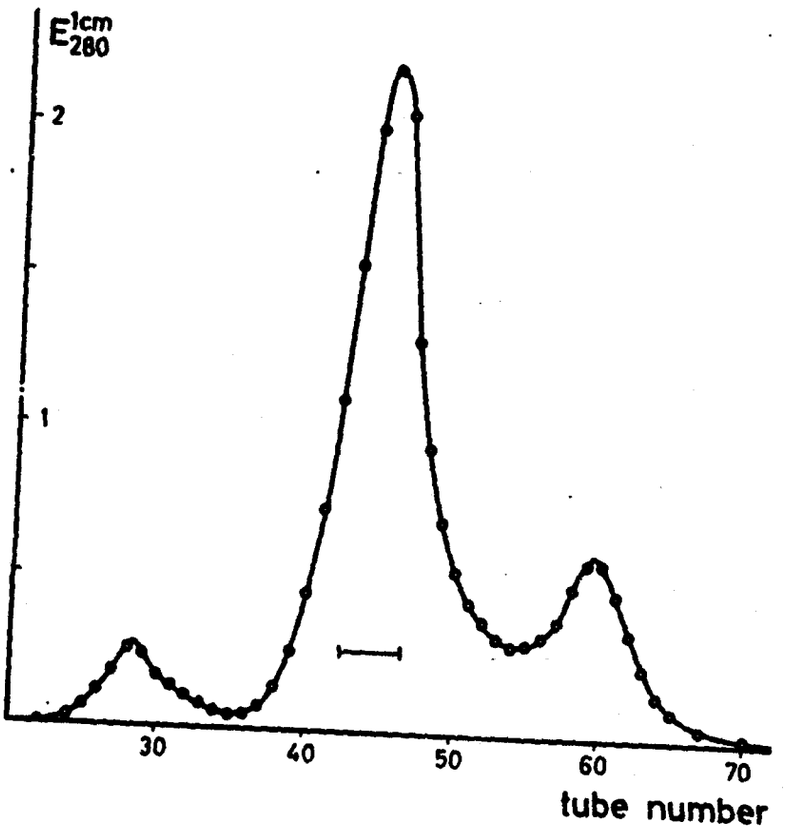


Fig. 2-3. Immunoelectrophoresis of unfractionated and fractionated serum Na. MS is myeloma serum Na, MP is myeloma protein Na after Sephadex G-200 gel filtration (IgD fraction of Fig. 2-2 at 11 mg/ml), a-IgD is specific anti-IgD serum.

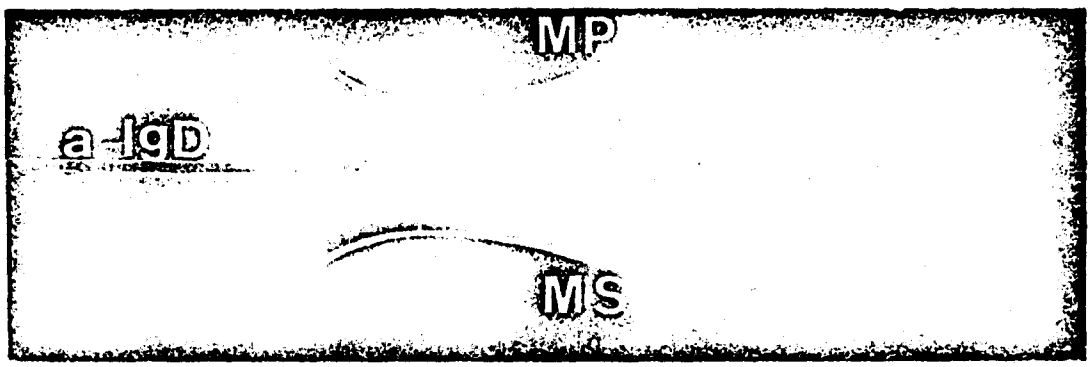


Fig. 3-1. Sedimentation pattern of myeloma protein Mi(B). The analysis was carried out at 44,770 rpm and at 20°C in 0.05 M Tris HCl buffer, pH 8.0, containing 0.20 M NaCl. The protein concentration was 12 mg/ml. The photograph was taken 65 minutes after reaching speed; sedimentation is to the right.

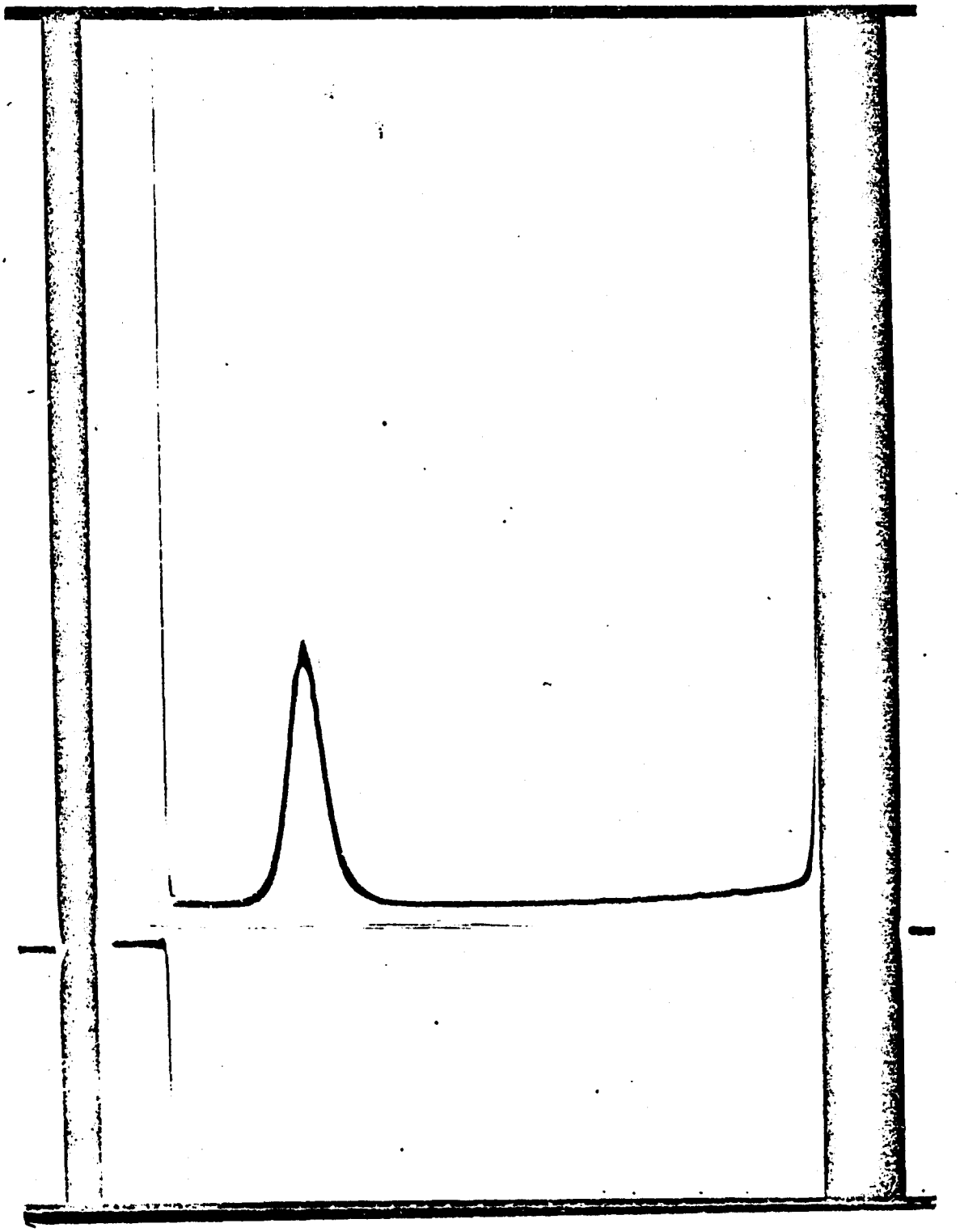


Fig. 3-2. Sedimentation pattern of myeloma protein Na. The conditions are the same as for protein Mi(B) in Fig. 3-1. The protein concentration was 8.2 mg/ml and the photograph was taken 90 minutes after reaching speed; sedimentation is to the right.

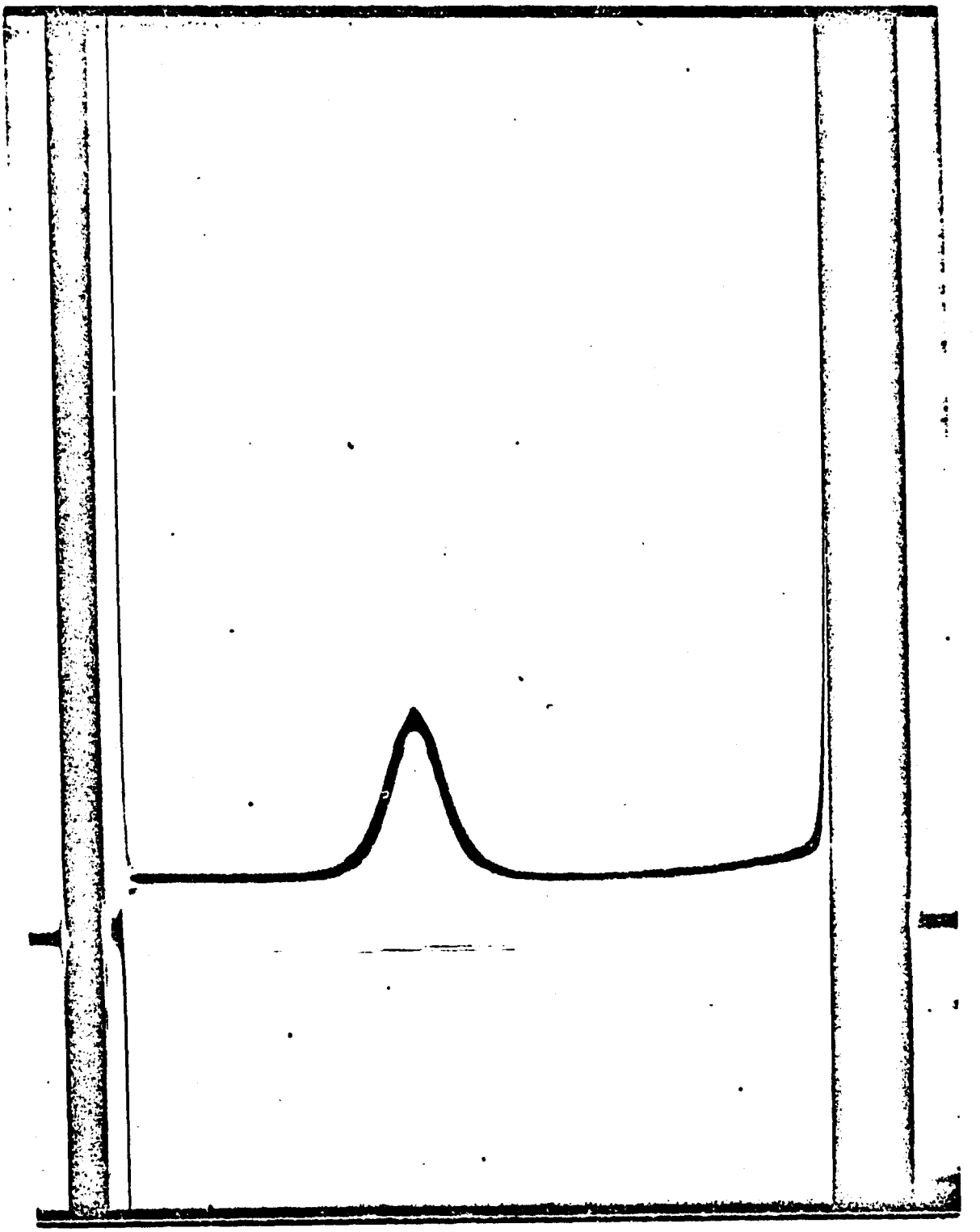
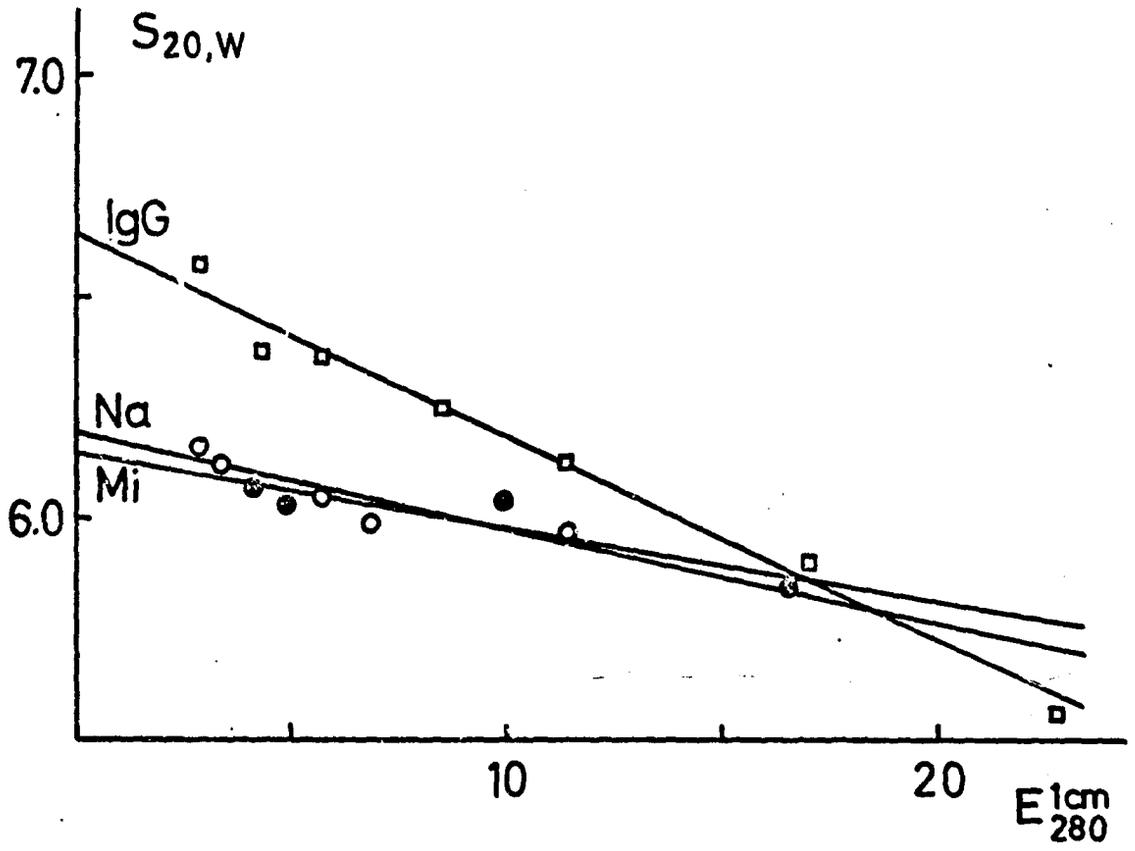


Fig. 3-3. Concentration dependence of the sedimentation constants of B-myeloma proteins and IgG. Pooled human IgG (□---□---□---), myeloma protein Na (o---o---o---) and myeloma protein Mi(B) (•---•---•---). All experiments were performed in 0.05 M Tris HCl buffer, pH 8,0, containing 0.20 M NaCl, the viscosity correction factor being calculated as for 0.25 M NaCl.



PROJECT ON IgM

INTRODUCTION

The full understanding of the role of human IgM antibodies in the immune response is dependent inter alia, on a knowledge of how this protein is assembled from its constituant polypeptide chains into subunits, of molecular weight about 160,000, and from five subunits into the 19 S IgM molecule of molecular weight 8 to 900,000. It is also relevant to inquire which functions of the 19 S molecule are retained in the subunits and which functions require association of subunits for their activity. This latter question is the more important since circulating subunits have been demonstrated in certain diseases, including recently in trypanosomiasis, which is a disease of special interest to this laboratory.

The long term aim of the study of IgM in this laboratory is to define function in terms of structure for a variety of IgM antibodies. The work which is reported here is a necessary preliminary to the biological studies. Thus, although a number of biological studies have previously been made of IgM subunits, little attempt has been made to assess the degree of denaturation (due to reduction of intrasubunit disulphide bonds) of the subunits, which can be produced by reductive cleavage. Research is, therefore, concentrated on the investigation of mild reducing agents for the production of subunits and on the characterization of the subunits so produced in terms of the numbers of cleaved disulphide bonds. This study will be of importance a. with respect to the study of the biological properties of minimally denatured subunits, b. with respect to the characterization of the number and site of the intersubunit bonds present in the 19 S molecule. The study has been carried out on three examples of Waldenström macroglobulins, which can be obtained as homogeneous proteins of high purity.

Previous studies of human Waldenström macroglobulins by Suzuki and Deutsch (1) and Chaplin et al (2) have shown that one molecule of 19 S IgM contains 65 to 82 SS-bridges. Under the usual conditions of reduction of the SS-bridges by appropriate concentrations of some aliphatic thiols, it is possible to cleave out of the set of about 70 SS-bridges an average number of 24 to 25. This number is sufficient not only to obtain five subunits of a molecular weight of 160,000 but also to separate all of the different polypeptide chains (heavy or μ chains, light K or λ chains) of the molecule without any further procedure involving reduction. It can, therefore, be assumed that these 24 SS-bridges account for all of the interchain bonding in the IgM molecule.

On the basis of these findings Miller and Metzger (3) proposed a number of different models for the arrangement of polypeptide chains in the IgM molecule (Fig. 1) and some additional information has been obtained by electron microscopy performed by Svehag (4). These latter data suggest that the circular models (model D, E, F) are more probable than the linear arrangements (model A, B, C).

In recent years particular interest has been focussed in developing reduction methods which affect less than the usual 24 SS-bonds per 19 S molecule and which result in the formation of the above mentioned five subunits of molecular weight 160,000. Theoretically, this could be achieved by cleaving 5 or 10 intersubunit SS-bonds in the circular models or by cleaving 4 or 8 SS-bonds in the linear arrangements (see Fig. 1).

In terms of biological activities such a mild reduction should produce a subunit of approximately 160,000 molecular weight considerably different in conformation from the usually produced subunits. Such "native" subunits would be optimal for the study of biological properties such as complement fixation activity, combination with antigen, etc.

Not many promising results, however, have been presented in this field up to the present time: Miller and Metzger used cysteine as reducing agent and found that a maximum of 24 free sulphhydryls per IgM molecule were formed during the reaction. However, the number of moles of SH varied considerably and appeared to decrease with increasing time of exposure to reducing agent. Although the isolated subunit scarcely dissociated at all into heavy and light chains when gel filtered with propionic acid as solvent, the number of cleaved disulphide bonds was not conclusively determined. The method had another technical drawback since it did not allow analysis of the reduction products by means of amino acid analysis, since disulphide interchange and formation of mixed disulphides involving the reducing cysteine could considerably affect the results of the cysteine analysis. This method has been investigated further by Miss Shirley Miekka, who in turn found a minimal number of 4 sulph-hydryls being freed during the reaction per subunit. This finding would point rather to model D or F being the appropriate one (8).

Another method has been recently presented by Harboe (5) involving dialysis at low temperature against a solution of mercaptoethanol. However, no statement as to the number of disulphides cleaved during this reaction has been presented, the only evidence for the intrasubunit disulphides remaining intact being again the stability of the reduced products on gel filtration in propionic acid. This method has been checked in this laboratory but its specificity for intersubunit disulphides has not been confirmed (see Results).

Almost simultaneously with our work, Morris and Inman (6) reported on a method using mercaptoethylamine as a reducing agent. Analysis of their subunits indicated 2 to 3 SH cleaved and alkylated per subunit under their conditions. Some comments on these results will be presented below.

MATERIALS

Preparations of IgM. Waldenström IgM was prepared from three different sera (Ma, El, La) in the following way: the crude serum was diluted with a sixfold volume of distilled water for euglobulin precipitation; alternatively, the serum was dialysed against distilled water. The precipitate was redissolved in 0.25 M Tris/NaCl pH 8.0 and this procedure repeated usually three times. Proteins El and La were passed through a Sephadex G-200 column. If subsequently, the pure 19 S component was required, the native IgM preparation was passed through a column of Sepharose-4 B. All gel filtration procedures were done on gels previously equilibrated against Tris/NaCl buffer of an ionic strength of 0.25 and a pH of 8.0. Concentration of the fractions were routinely performed by pervaporation of the preparation in an appropriate piece of "Visking" dialysis tubing.

Reduction methods. If not otherwise stated, the conditions were as follows: the appropriate concentrations of thiols were added to a 1% protein solution in 0.25 M Tris/NaCl buffer and the reaction allowed to continue at room temperature, care being taken that there was no access of air during the whole process. For determining the rate of formation of 7 S material, samples of 10 ml of the reaction mixture were taken at different stages of the process and alkylated as indicated below.

Alkylation procedure. Three different methods were used for alkylation of the SH groups freed during reduction: 1. if no previous separation of the excess reducing thiol was required, alkylation was performed by adding iodoacetamide in a 10% excess over the molarity of the reducing thiol, e.g. giving a concentration of iodoacetamide of 0.22 M in the case of the reduction with 0.2 M 2-mercaptopyridine (see below). The pH was adjusted at 8.0 by adding solid Tris and the reaction allowed to continue for 15 minutes; 2. 1 ml of the reaction mixture (10 mg of protein) was dialysed against 2 liters of a solution of 0.001 M thiol for two hours, giving a concentration of the thiol at equilibrium of 1,1 X 10^{-3} molar. Alkylation was then performed by adding 0.1 ml of a 0.016 M solution of iodoacetamide in Tris/NaCl buffer to give a final concentration of 0.0016 M iodoacetamide, the pH being adjusted at 8.0 by adding solid Tris as mentioned already above. Alkylation was allowed to continue for 90 minutes at room temperature; 3. 1 ml of the reaction mixture (10 mg of protein) was passed through a Sephadex G-25 column, which had been previously equilibrated with 0.001 M solution of the corresponding thiol. The flow rate was adjusted at 120 ml per hour and the protein containing fractions was obtained after 20 minutes on the column. The protein fractions were concentrated by pervaporation, this procedure taking again two to three hours. Alkylation was performed as indicated above under (2). In all three alternative cases of the alkylation procedure, samples were dialysed subsequently against 0.25 M Tris/NaCl buffer for 24 hours. The two latter procedures

were investigated with the object of avoiding the use of high concentrations of alkylating agent, which would be inconvenient when using radioactive alkylating materials as planned in future experiments.

Rate of the formation of 7 S material. The percentage of reduced subunits produced during the reduction was estimated on analytical ultracentrifuge runs performed on a Spinco Model E ultracentrifuge at 44,770 rpm. Usually, 0.5 ml of the alkylated sample was centrifuged and the relative amounts of 19 S compared to 7 S material was calculated.

Kinetics of the formation of free SH groups was done following the procedure of Miller and Metzger (3). 0.5 cc of the reaction mixture (5 mg of protein) was precipitated and washed five times with an equal amount of trichloroacetic acid. After a final centrifugation at 15,000 rpm for 30 minutes, the precipitate was resolubilized in 5 M guanidin. The protein concentration of this solution was determined from the optical density at 280 mμ. Subsequently, the sample was mixed with an equal volume of a solution containing 5×10^{-3} M dithiobisnitrobenzoic acid (DTNB, Ellman's reagent) and the optical density read at 412 mμ. The amount of SH equivalents was calculated on the basis of an extinction coefficient of the reduced form of DTNB of 13,600 at 412 mμ, and the SH equivalent referred to the actual yield of 7 S material present in the final stage of the reduction, usually 35% in protein Ma.

Dissociation of the protein into μ (heavy) and light polypeptide chains. The different reduced and alkylated subunits were dialysed overnight against formate buffer pH 3.5 of an ionic strength of 0.02 and passed subsequently on a Sephadex G-100 column equilibrated against the same buffer. The different fractions were tested for μ and light chain determinants by Ouchterlony double-diffusion analysis using a gel system containing 1% agarose in 0.1 M barbitol (pH 8.2) and appropriate antisera specific to μ or light chain determinants.

Ultracentrifugal analysis: determination of S_{20w} values were performed routinely on 7 S material previously dialysed against 0.25 M Tris/NaCl buffer pH in a Spinco Model E ultracentrifuge at 44,770 rpm. For extrapolation to infinite dilution protein concentrations were determined by measurements of the optical density at 280 mμ. An extinction coefficient of 12.0 for a 1% protein solution was used (Miller and Metzger (3)).

Optical rotatory dispersion of some of the different subunits was measured between 215 and 315 mμ on a Perkin-Elmer P 22 spectropolarimeter, using cells of 0.2 and 0.02 cm length. Values lower than at 215 mμ were not obtained since the high extinction of the sample did not allow measurements with reasonable accuracy. (These measurements were kindly performed by Bodenseewerk Perkin-Elmer).

The observed rotations, $\alpha\lambda$, were converted to specific rotations $[\alpha]_\lambda$, and then into $[R']_\lambda$ by the formulae:

$$[\alpha]_\lambda = \frac{\alpha_\lambda \cdot 100}{c\ (g/100ml)} \qquad [R']_\lambda = \frac{3}{n^2+2}\ \frac{M}{100}\ [\alpha]_\lambda$$

where the refractive index, n, was taken as 1.33 from 350 to 260 mu and as 1.37 below 260 mu (9). A mean residue weight, M, of 108 (10) was used for all reduced subunits of IgM.

Peptide mapping and diagonal electrophoresis of cystein peptides. Since we are planning to characterize the intersubunit as bonds in terms of their surrounding peptides, preliminary experiments have been staged on tryptic digests of the native IgM preparations as well as on the different subunits. Usually, digestion was continued for 24 hours at 37°C using twice crystallized trypsin (Difco) in a final protein enzyme ratio of 50:1. If not otherwise stated, half of the amount of the enzyme required was added for the initial 6 hours, the final amount being added only after these 6 hours. Under these conditions no trypsin autolysis products were detectable. In some cases, especially for native IgM preparations, a preliminary treatment with performic acid and trichloroacetic acid is advisable.

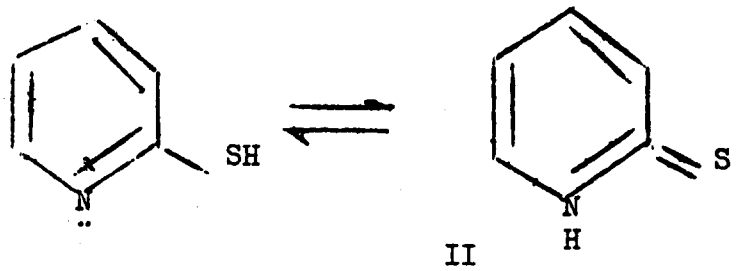
For obtaining the usual two-dimensional peptide maps chromatography on Whatman 3MM paper was performed using butanol/acetic acid/water 3:3:1 as solvent system. The subsequent electrophoresis step was carried on at pH 3.5 in pyridine/acetic acid, using a voltage of 3 kV for 55 minutes.

Further studies on the cystein containing peptides were performed using the method indicated by Brown and Hartley (7). Usually, both electrophoresis steps were performed in a pyridine/acetic acid system at pH 6.5 using a voltage of 1 kV for 4½ hours.

We are planning to further extend these investigations by using C14 labelled peptides and characterizing the active carboxymethylcystein containing peptides by means of autoradiography.

RESULTS

Subunits of different size have been isolated and characterized by Miller and Metzger (3) and Suzuki and Deutsch (1). In our attempts to develop a mild reduction method that leaves the intra-subunit SS-bonds intact, we tried a number of thiols as reducing agents in which the SS-group was linked to a heterocyclic pyridine or pyrimidine ring system. These substances promised a rather mild reduction, since similar thiols had been shown to display rather modest redox potentials: thiohistidin - 0.009, 4-methylthiouracil + 0.01, - 0.2 V as compared to cystein used by Miller and Metzger -0.50 V. In addition to this feature, in heterocyclic thiols with the SH adjacent to nitrogen a tautomerism effect was expected to render the reducing properties of the SH-group even more feeble, as e.g. in 2-mercaptopyridine:



These theoretical considerations could be partly confirmed (see below) but it turned out to be rather difficult to find appropriate thiols of the above mentioned structure which had good water solubility. After experiments with dimercapto-pyrimidine, thiouracil and others, the most apprerpiate reducing agent of that class of thiols was found to be 2-mercaptopyridine, which is soluble in saline solutions to a limit of around 0.3 M.

Rate of formation of subunits. Since it was important to distinguish the cleavage of intersubunit disulphide bonds from that of intrasubunit disulphide bonds, efforts were made to determine the minimum concentrations of the heterocyclic thiols which would effect cleavage of 19 S components into their reduced subunits. The minimum concentration of 2-mercaptopyridine required to dissociate the native IgM with a reasonable yield at pH 8.0 and 23° was found to be 0.2 M.

Rate of formation of 7 S and the rate of disappearance of the 19 S component of three different IgM preparations treated in this way, was studied. (No components sedimentation between 19 S and 7 S were observed after alkylation). The data of Table 1 and Figures 2 and 5 show that when the logarithm of the residual 19 S component is plotted against time of reduction a straight line is obtained for the reduction with 0.2 M 2-mercaptopyridine of all three Waldenström macroglobulins. This indicates that the formation of subunits is a first order re-

action (for protein Ma at least during the first 24 hours of the reduction). The rate constants were calculated to be of the order of 3.23×10^{-6} sec^{-1} for Ma, 8.80×10^{-6} for La and 1.65×10^{-5} for El protein at 25°C. It is a striking feature that these values are by a factor of 10^3 smaller than the rate constants calculated for reductions with mercaptoethanol or similar aliphatic thiols. (Suzuki and Deutsch (1)). Thus the observation of Suzuki and Deutsch that the reduction of IgM to form subunits is a relatively fast reaction, must be limited to reduction using strong aliphatic thiols as reducing agents.

Rate of formation of free -SH. It was found that the mild reduction using 2-mercaptopyridine produced an amount of SH equivalents, which was at the limit of detection of the DTNB method. However, some semi-quantitative results were obtained by the comparison of the titration of SH-groups in subunits treated with dithiothreitol (known to cleave 24 SS-bonds) and subunits treated with 2-mercaptopyridine. Whereas dithiothreitol produced a total of around 10 free SH per IgM subunit, 2-mercaptopyridine yielded only much lower amounts (values lower than 2 -SH per IgM subunit, SH- concentrations of around 5×10^{-6} equivalents per ml of reaction mixture). (Figs. 6 and 7). It should be pointed out, however, that these findings were based exclusively on protein Ma and that they need confirmation either by analysis of carboxymethylcystein or by investigation of the resistance of the subunit to acid treatment (see below).

Physicochemical analysis of IgM subunits prepared with 2-mercaptopyridine. The data of Table 2 indicate that all the subunits prepared with one of the three "mild" reduction methods display rather high sedimentation rates as compared to the subunits isolated recently by Morris and Inmann (6) and described as having only intersubunits disulphides cleaved. Compared with the subunits of IgM prepared by Suzuki and Deutsch (1) our subunits would be of the order of 8 S rather than of the order of 7 S (using the terminology of Suzuki and Deutsch). Whether this can be accounted for by the postulation of Suzuki and Deutsch of one additional light polypeptide chain per subunit is still not known. At the present time, we have no evidence of the existence of such an additional light chain in any of our three IgM preparations.

Resistance of IgM subunits to acid treatment. The separated 7 S material from protein Ma was dialysed exhaustively againt 0.02 μ formate buffer of pH 3.25. It was found that this was a critical ionic strength which gave best chain separations. The dialysed material was passed on a Sephadex G-100 column equilibrated against the same buffer and the different fractions were characterized by antisera. The dithiothreitol treated material eluted as two peaks, the ratio between the peaks being of the order of 4:1. The first peak was found to contain μ

chain determinants only, the second peak K chain determinants only (Ma protein was of light chain type K).. The acid treated subunits prepared with 2-mercaptopyridine were run on the identical column which had been used for the DTT treated material. It was found that the isolated 7 S peak from 0.2 M 2-mercaptopyridine eluted as one single peak on G-100. This peak contained both μ and K chain determinants. This was good evidence of the fact that some intrasubunit SS -bonds were not cleaved under these conditions and that, in fact, a more native subunit was obtained than with some other reduction methods (Fig. 9).

Conformational analysis of the IgM subunits by means of Optical Rotatory Dispersion (ORD). ORD spectra were obtained for comparing the differently prepared IgM subunits in terms of secondary structure. No qualitative changes, however, could be observed with .02 cystein treated and non-selectively reduced, subunits (.001 DTT, .2-mercaptoethanol). The only striking feature was the extraordinarily high mean residual rotation [R'] of the cystein treated and supposedly mildly reduced subunit, which attained a value of around -2300° at the wavelength 233 mμ. On the other hand, supposedly non-selectively reduced subunits, achieved values of only 1350° and 1250° respectively. Whether this difference can be positively interpreted as the mildly reduced subunit having retained a relatively higher amount of α-helix as compared to the non-selectively reduced subunits, is not known at the present time. It can be noted, however, that the values of 1350° and 1250° of the DTT reduced subunits compare well with the maximum mean residue rotation of normal human IgG at the wavelength of 233 mμ, this latter being around 1600° (5).

On the other hand, all different IgM subunits showed a slight Cotton-effect with a maximum at the wavelength of 285 mμ. Although this effect has been attributed to the rotation of SS-bonds, no change was noted between cystein treated and DTT treated subunits. It must be borne in mind, however, that this effect was relatively feeble and difficult to measure, and that no difference between the three kinds of subunits could be noted. It is interesting still to notice that this second Cotton-effect occurs at a considerably lower wavelength in normal human IgG (245 mμ),

Table 1

Rate of formation of IgM subunits by reduction with 0.2 M 2-mercaptopyridine

The numbers indicated are in arbitrary units and represent the area of the ultracentrifugal peaks of 19 S and 7 S components respectively.

PROTEIN Ma

Alkylation with 10% excess of iodoacetamide

Time	Amount of 19 S	Amount of 7 S	Yield of 7 S component %	
30 min.	182	5	2.67	
1 hr.	172	5	2.97	
	134	3	2.22	
2 hrs.	218	10	4.47	
4 "	138	11	7.4	
8 "	110	13	10.5	
9 "	163	21	11.4	
12 "	108	19	15.0	
16 "	73	15	17.0	
20 "	123	26	17.5	
24 "	107	28	20.7	
	125	31	19.9	
	127	40	24.0	
	56	14	20.0	
96 "	43	26	37.6	
	45	20	31.0	
Alkylation with dialysis of excess thiol				
60 hrs.	45	19	29	30.0
	47	22	31	
84 "	31	15	32	33.5
	29	16	35	
108 "	72	40	36	34.3
	66	33	33	
	63	33	34	
132 "	38	28	43	43.0
	38	29	43	
Alkylation using G-25 filtrations				
60 hrs.	28	14	33	34.2
	28	14	33	
	28	16	36	
108 "	43	28	39	40.0
	41	26	41	

Table 1
(cont.)

PROTEIN E1

Alkylation with dialysis of excess thiol

Time	Amount of 19 S	Amount of 7 S	Yield of 7 S component %	
21 hrs.	23	55	70.6	
	24	47	66.3	69.7
	23	58	71.6	
	10	24	70.5	71.6
	8	24	75.0	73.5
24 "	10	31	75.6	
	10	36	78.3	
	12	41	77.4	
	12	39	76.5	
	11	41	78.8	
	10	32	76.3	77.2
	22	76	77.6	75.9
	27	70	72.2	
	18	56	75.5	
	25	79	76.0	
	23	58	71.7	74.6

PROTEIN La

14 hrs.	95	75	44.1	44.1
	97	76	44.0	
108	6	177	96.7	96.7
	6	171	96.7	
86	10	178	94.7	93.5
	15	181	92.4	

Table 2

Sedimentation properties of IgM subunits

(a) Experimental values	Alkylation procedure (see page 13) (1) 110%	(2) dialysis	(3) Sephadex G-25
.2 M 2-MPyridine	6.82_1	7.36_4	6.89_4
.02 M Cystein	6.28_5	7.30_4	6.90_4
.1 M mercaptoethanol at 4°C	6.89_2	6.81_4	7.09_4
mercaptoethylamine (Morris and Inmann)	6.45 to 5.35		

All experiments were staged on 1% protein solutions at pH 8.0. Morris and Inmann worked with protein concentrations of 4 mg/ml.

The subscript to the right of the sedimentation rate indicates the number of runs on which the calculations are based.

(b) Calculated sedimentation properties at infinite dilution

1. Present data(*)			
0.2 M 2-MPyridine	7.62	8.16	7.69
.02 M Cystein	7.08	8.10	7.70
.1 M mercaptoethanol at 4°C	7.69	7.61	7.89
2. Morris and Inmann (6)(**) mercaptoethylamine	5.75 to 6.85		

(*) For infinite dilutions one has to add an average increment of +.70 to .90 to the experiemntal values obtained at 1% protein concentrations. (1 and own calculations).

(**) In this case, where the protein concentration was 4 mg/ml, an increment of .35 has been added.

DISCUSSION

Amongst the different reduction methods of Waldenström macroglobulins published to the present time, the reduction with 2-mercaptopyridine offers some unusual characteristics: whereas all other reductions involving thiols as reducing agents (1, 3, 5, 6) were found to have rate constants of the order of $k = 10^{-3}$ sec^{-1}, our method has a particularly low rate constant of the order of $k = 10^{-6}$ sec^{-1}, i.e. lower by a factor of 10^3. For the first time it can be pointed out, therefore, that not all reductions with thiols are necessarily fast reactions, as has been stated, e.g. by Suzuki and Deutsch (1). But, while the drawback of our method in producing low yields after only short time periods can be overcome by an appropriate increase of the reaction time, the small k value can, on the other hand, be interpreted as a sign of particularly mild conditions, which in turn are appropriate for retaining conformational and biological properties of the native protein. While any other of the "mälder" reduction methods (Miller and Metzger, Harbo, Morris and Inmann, Miekka) use thiols with relatively high redox potentials and are, therefore able to produce a statistical cleavage of perhaps all SS-bonds, our method has been designed especially for selecting a subpopulation of the most reactive and labile SS-bonds, which are still able to be reduced by the low redox potential of our reducing agent.

A comparison of the "mildly" reduced subunits of IgM in terms of molecular size and SS-bridges actually involved in the reduction procedure is especially difficult since the different authors active in this field do not use identical criteria for characterizing their products. Miller and Metzger (3) referred their calculations of freed sulph-hydryls to a molecular weight of 908,000 for the entire IgM and paid little attention to the molecular size of the subunits actually formed in their attemps at "selective" reduction. No assay of SH-content has been presented for the method involving dialysis at low temperature (Harbo (5). No information on the molecular size could be obtained at the present time for the investigation with cystein performed by Miekka (8). On the other hand, Morris and Inmann (6) accepted a value of 160,000 molecular weight for their subunits making them calculate a value of 2,3 sulph-hydryls per subunit. It seems, however, that the subunits of these authors could differ considerably in molecular weight from the subunits found after a reduction with 2-mercaptopyridine (see Table 2), which makes it difficult to judge conclusively if the two methods are consistent in terms of reaction products.

We are planning, therefore, 1. to use additional methods for assaying SH-groups in our procedure, 2. to refer these values to an experimentally determined molecular weight of the subunit actually produced in our procedure, 3. to define the cystine peptides of our subunits with the objective of locating them in the

primary structure of the molecule. We are bearing in mind that for a final discussion of the differences of several methods, attention should be paid to the fact that individual IgM macroglobulins can differ quite considerably from each other (as has been shown above for the rate constants k) and that, therefore, experiments should be staged on a number of different IgM preparations.

Nevertheless, we are able to state already at the present time, that the reduction of protein SS-bonds with aromatically substituted thiols can be considered an especially mild procedure which should therefore, be an appropriate attempt for the studies of biological properties of the cleavage products.

These studies of the nature of disulphide cleavage required to produce IgM subunits and of the subunits produced by minimal cleavage should permit two further lines of study on the IgM class. Firstly, the production of subunits which are minimally or not at all denatured by the rupture of internal disulphide bonds permits a more satisfactory characterization of the functional characteristics of these subunits than has been possible with previously obtained products. Such studies will include antigen binding, complement fixation, etc. The relationship of these subunits to naturally occurring subunits will also be studied. In this respect the IgM found in the serum of cases of trypanosomiasis is of especial interest since appreciable quantities of a low molecular weight of these subunits have been described.

Secondly, minimal cleavage of SS-bonds may result in cleavage of these bonds which are <u>exclusively</u> intersubunit. In this case it should be possible to demonstrate the location of these bonds in the (heavy) chains of IgM.

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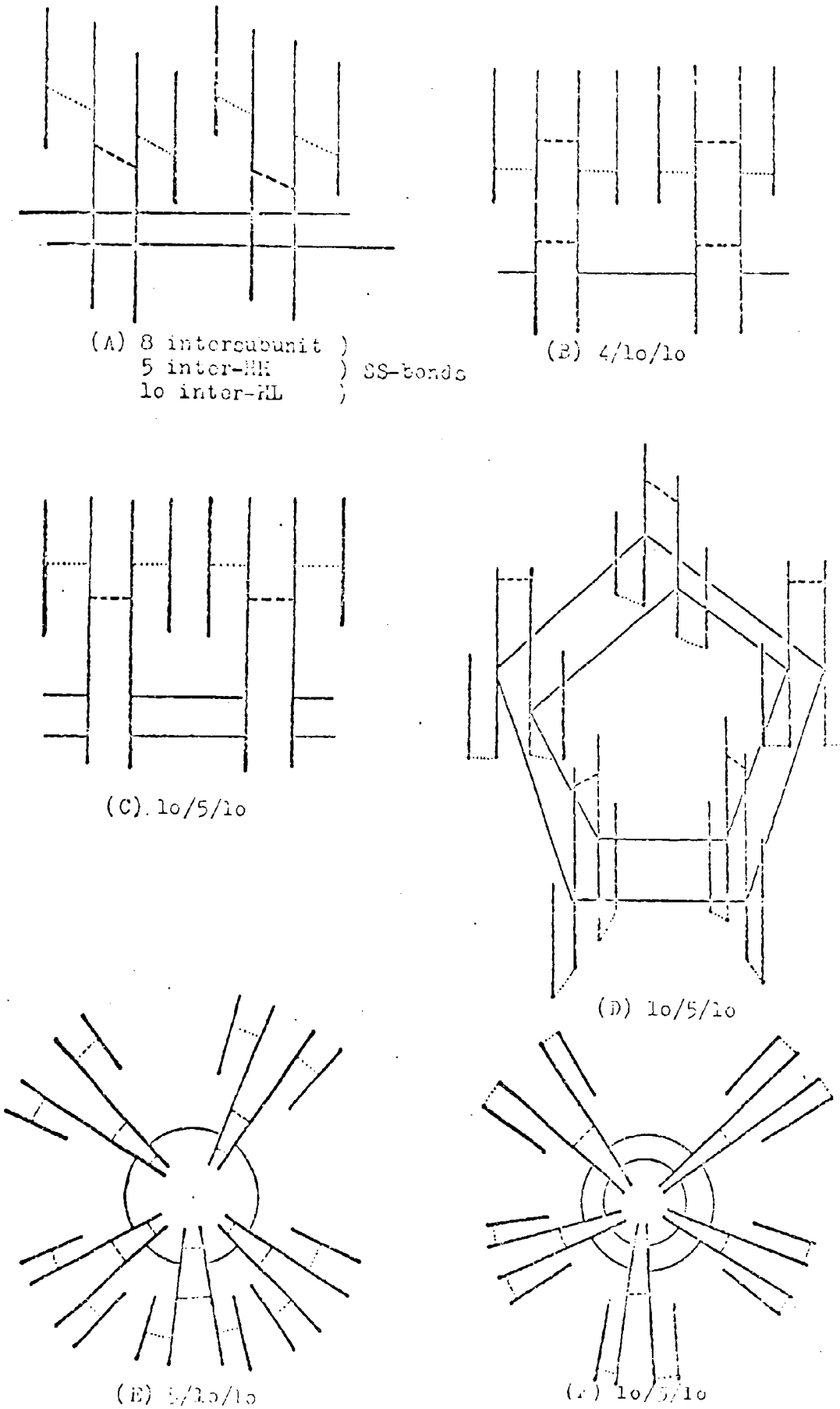


Fig. 1. Different arrangements of polypeptide chains in IgG models proposed [illegible]

Fig. 2 Reduction of IgG Ma with .2 M Mercaptopyridine
Kinetics of Formation of 7 S Subunits

The differently marked experimental values are taken from different experiments.

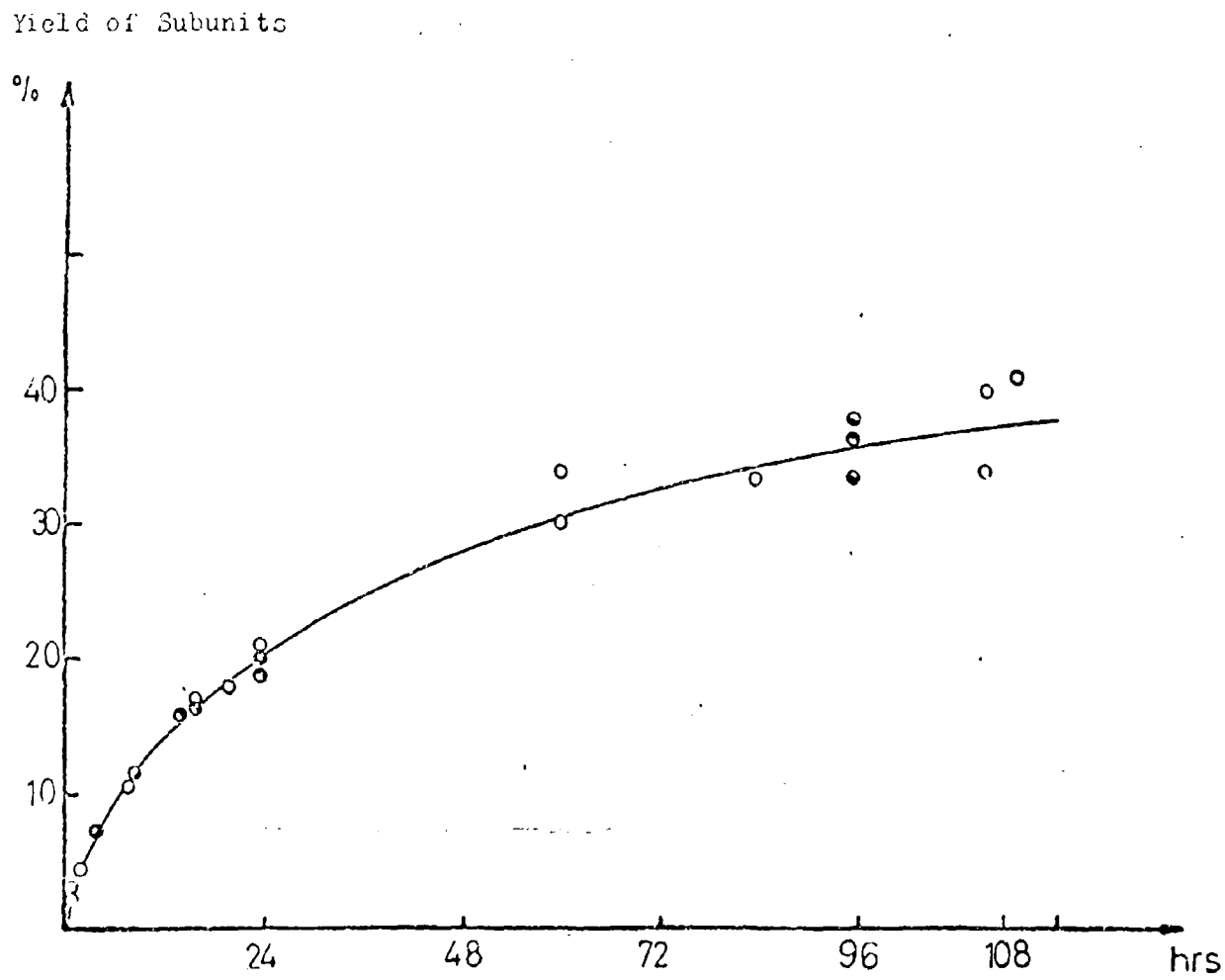


Fig. 3 Reduction of IgM El and Ln with .2 M Mercaptopyridine
Kinetics of Formation of 7 S Subunits
The differently marked experimental values are taken from different experiments.

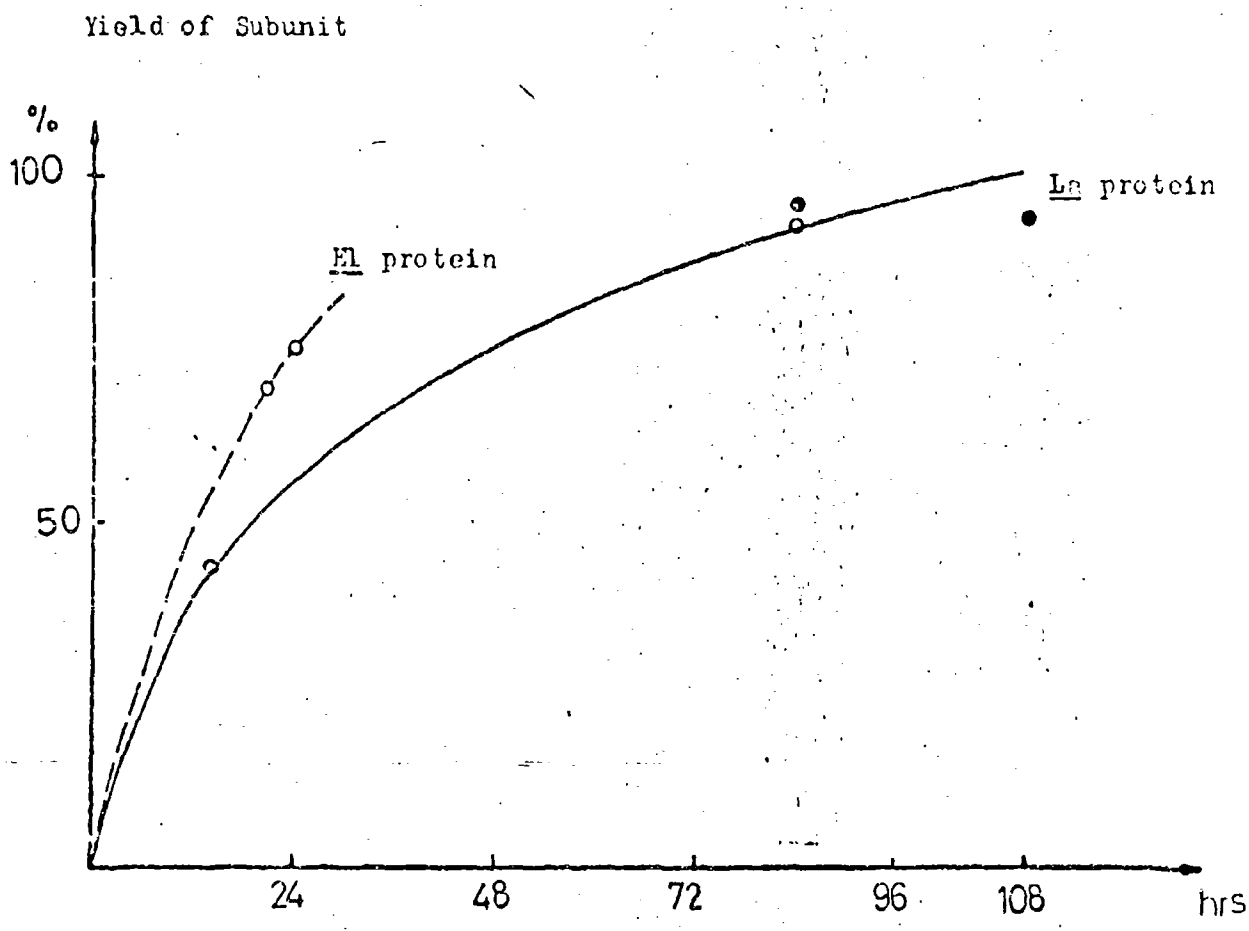


Fig. 4 Reduction of 1 mM Hg with .2 M 2-Mercaptopyridine
Rate of Disappearance of 19 S Material
$k = 3.23 \times 10^{-6}$ sec^{-1}
Data from the same experiments as in fig. 3.

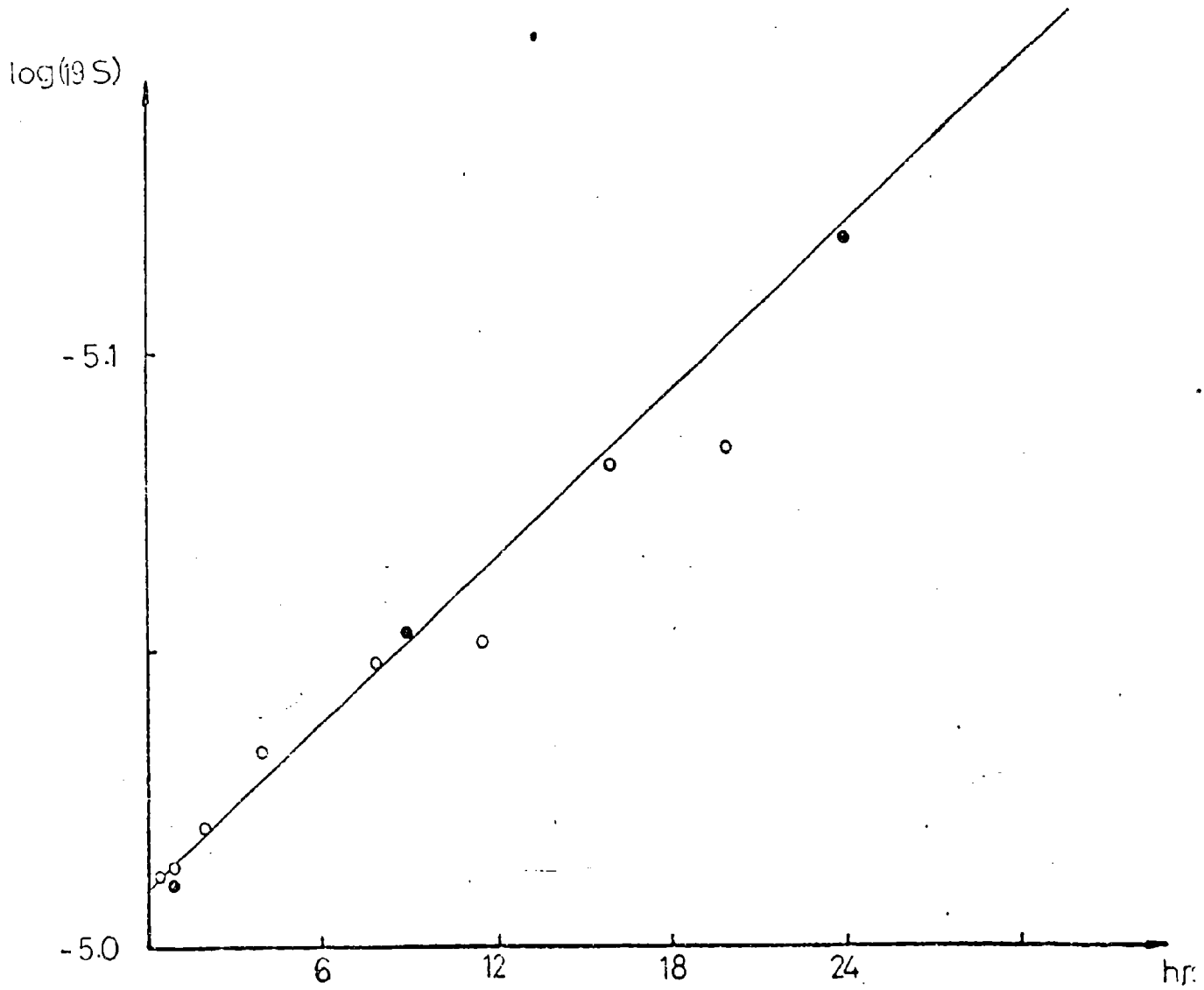


Fig. 5 Reduction of IgM Ma, El, and La with .2 M Mercaptopyridine
Rate of Disappearance of 19 S Material

k values: Ma 3.23×10^{-6} sec^{-1}
La 8.80×10^{-6}
El 1.65×10^{-5}

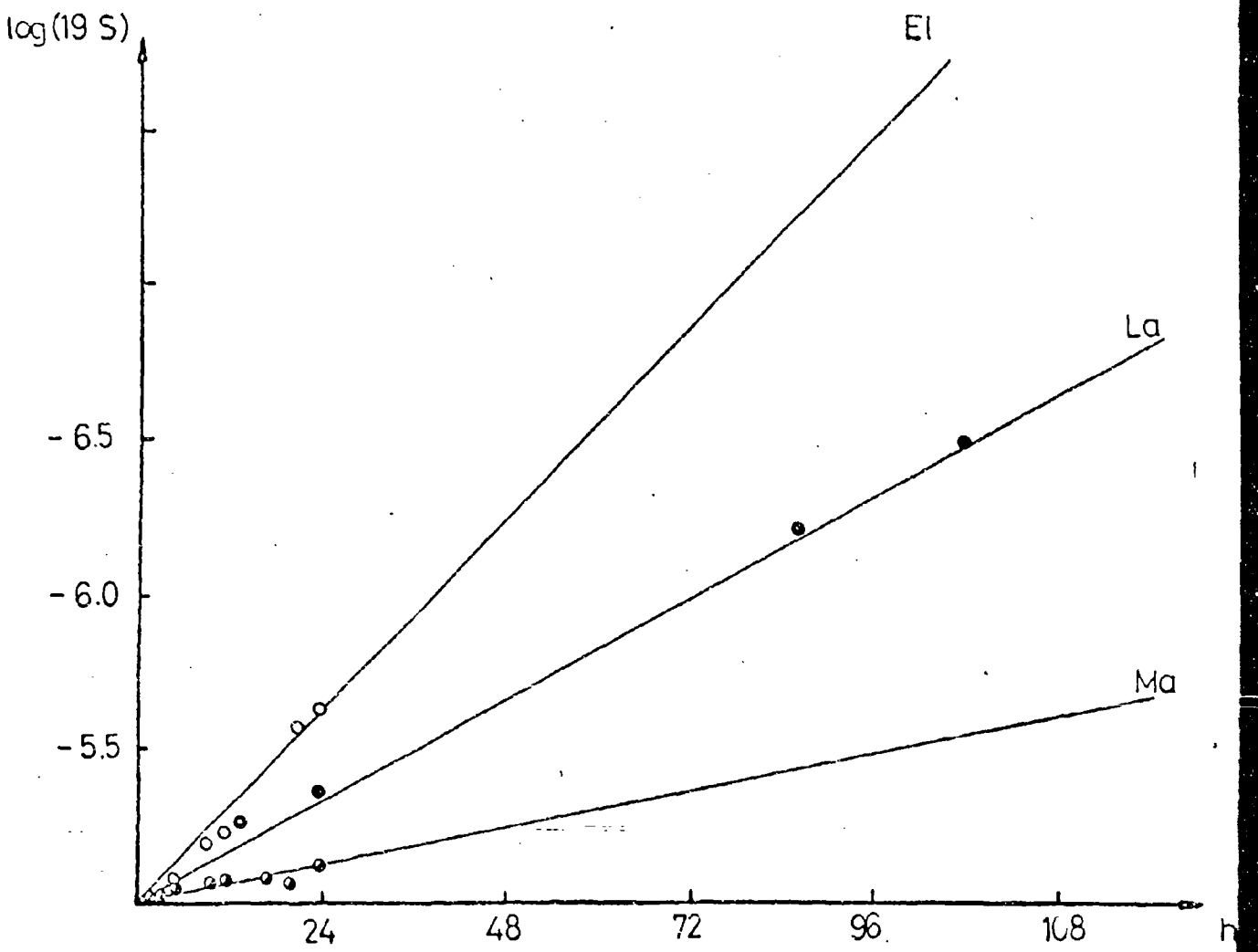


Fig. 6 Reduction of 1gM Hb with .2 M 2-Mercaptopyridine

Titration of -SH-Equivalents freed during Reaction (Experiments performed with PTKB)

No significance is attributed to the shape of the curve given by the mercaptopyridine treated material, since technical difficulties did not allow precise measurements (indicated as o---o)

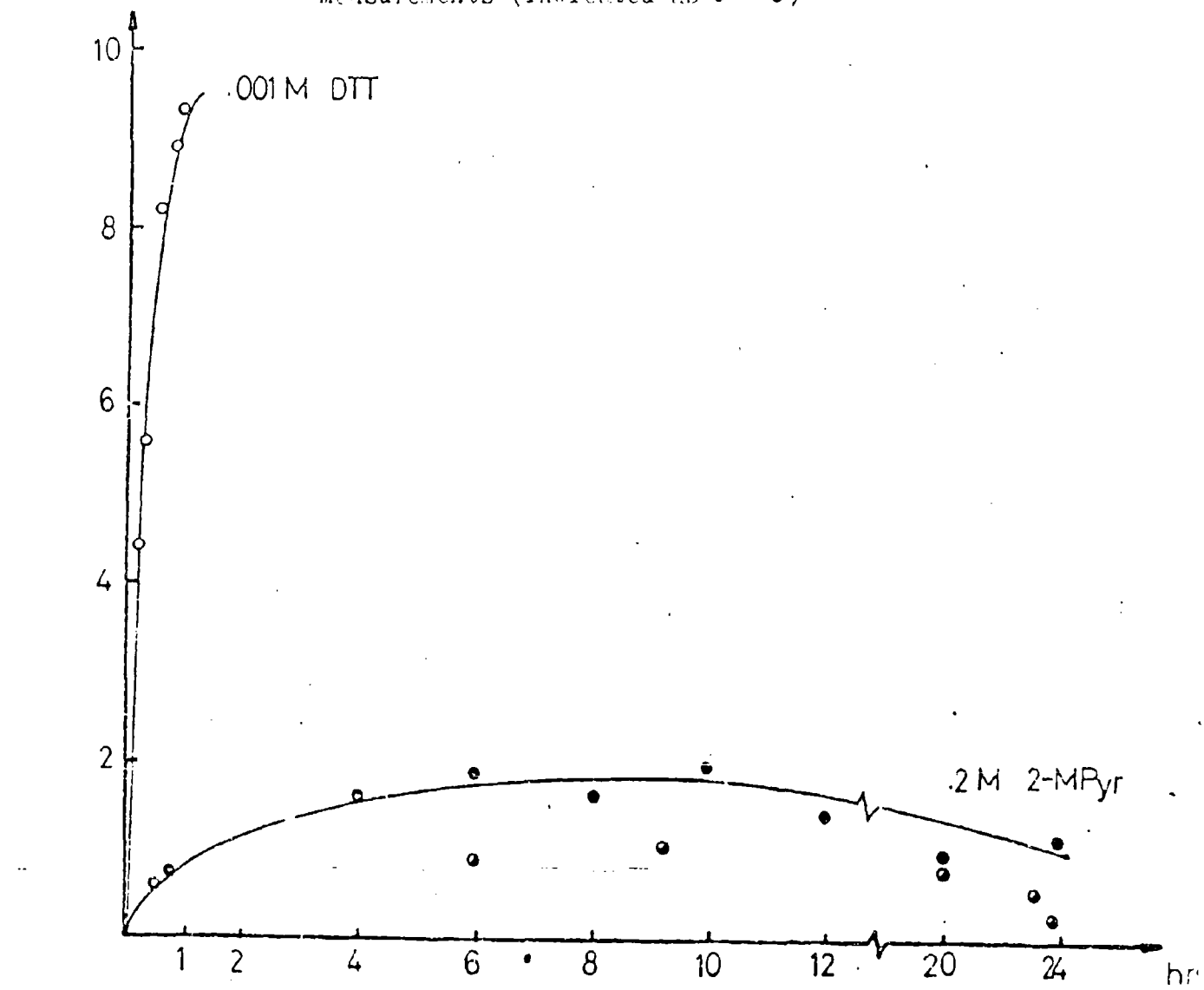
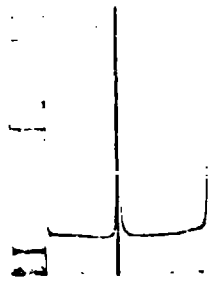


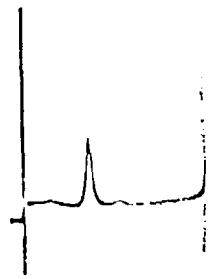
Fig. 7 Reduction of 1% Ma with .2 M 2-Mercaptopyridine

Ultracentrifugal Analysis of the rate of Formation of 7 S Material

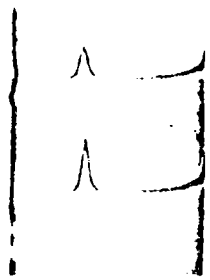
The runs were performed at 44,770 rpm at 20°C, pictures being taken after 48 min. after reaching speed. Sedimentation is towards the right.



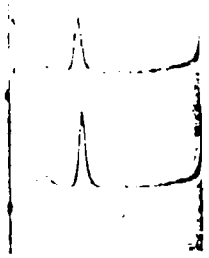
(a) Starting material
19 S



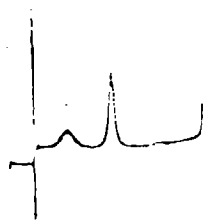
(b) 8 hours reduction
10.5 % yield



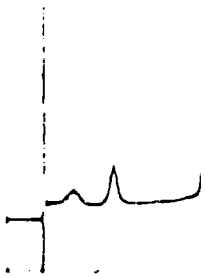
(c) 12 hrs.(lower)
16 hrs. (upper)
15 resp. 17 %



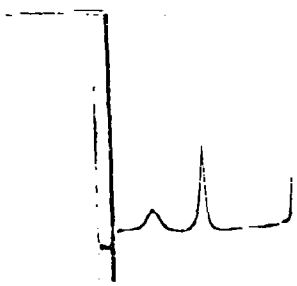
(d) 20 hrs. (lower)
24 hrs.(upper)
17.5 resp. 20.7 %



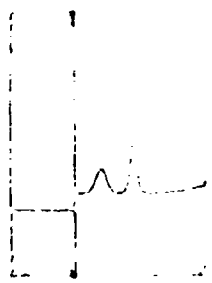
(e) 60 hrs.
30.0 %



(f) 84 hrs.
33.5 %



(g) 108 hrs.
34.3 %



(h) 132 hrs.
43 %

Fig. 8 Optical Rotatory Dispersion of several IgM Subunits

Na Protein in .25 M Tris/NaCl buffer, path length .02 cm (215 to 260 mu), .2 cm (260 to 350 mu).

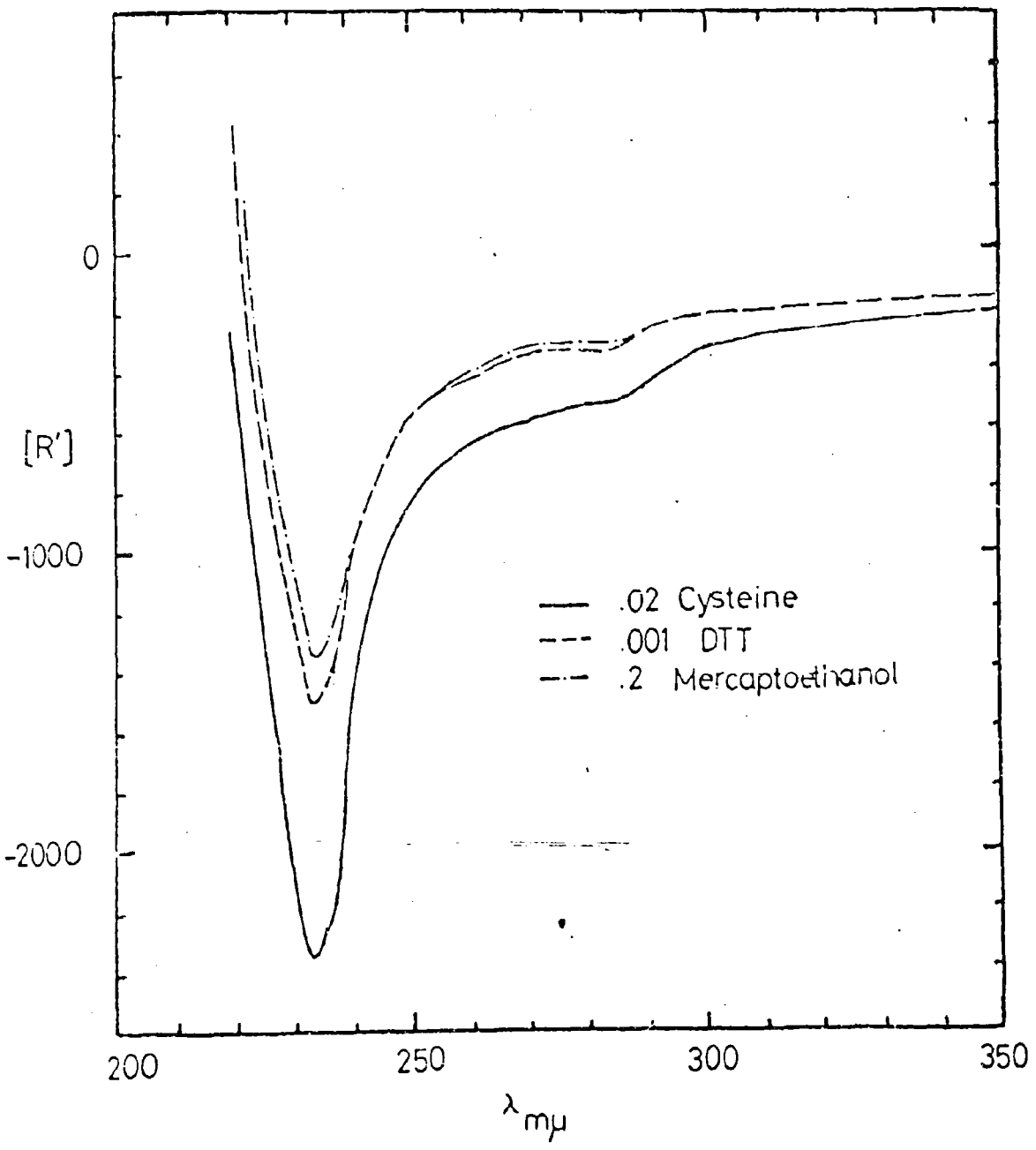
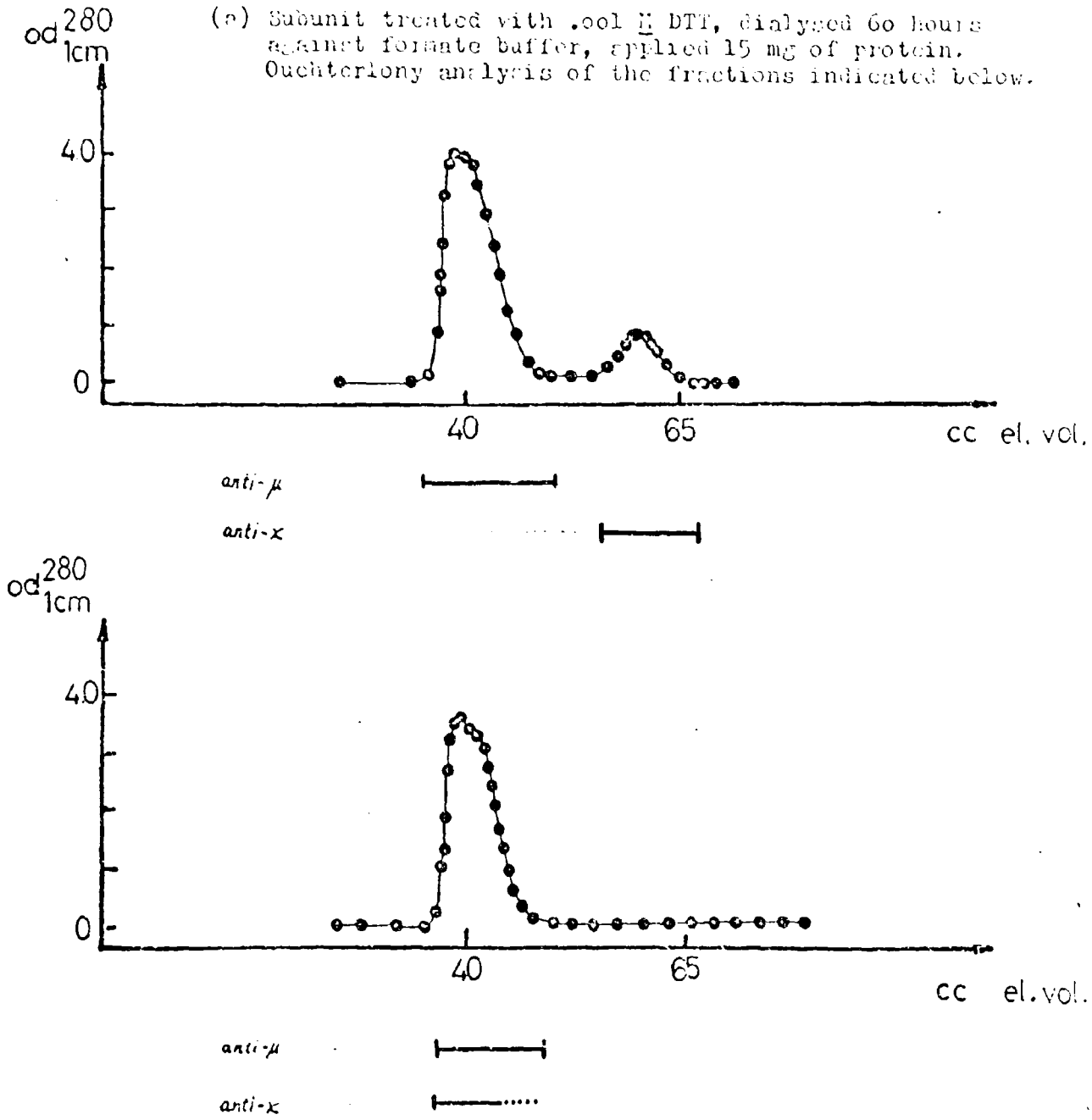


Fig. 9 Resistance of IgM Subunits against Acid Treatment. Elution pattern on Sephadex G-100 column, equilibrated against .02 μ formate Buffer pH 3.25.



(a) Subunit treated with .001 M DTT, dialysed 60 hours against formate buffer, applied 15 mg of protein. Ouchterlony analysis of the fractions indicated below.

(b) Subunit treated with .2 M 2-Mercaptopyridine Dialysed 72 hours against formate buffer, applied 11 mg of protein. Ouchterlony analysis of the fractions indicated.

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13. ABSTRACT
Studies of the significance of immunoglobulin M and immunoglobulin D in the immune response of humans are a long term project of this laboratory. Our recent studies in Africa have demonstrated a relationship of plasma IgM to malarial parasitaemia and malarial antibodies and have confirmed increased plasma IgM in trypanosomiasis. The IgM molecule is comprised of five subunits, the study of which is necessary for the understanding of the function of the intact molecule, especially since subunits are found in the plasma in conditions associated with elevated IgM, notably trypanosomiasis. Methods of treatment of intact IgM to produce minimally denatured subunits were investigated. 2-mercapto-pyridine was found to be a promising reducing agent yielding subunits by a reduction of a minimum number of SS-bonds. The kinetics of the reaction and some characteristics of the subunits were described. Studies in Africa have also shown increased plasma IgD levels in kwashiorkor and in apparently healthy African children. Laboratory studies have been concerned with the isolation of IgD (myeloma protein) from serum. Conditions were found to prevent the cleavage of this protein during isolation. Immunochemical and physicochemical tests demonstrated the homogeneity of the isolated protein which was found to have a molecular weight of 185,000. The isolated proteins were, therefore, suitable for studies of complement fixation and of other biological properties which are now in progress.

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