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Polypeptide Chain Structure of Rabbit Immunoglobulins. II. γM-Immunoglobulin*

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ABSTRACT: γM-Immunoglobulin was isolated from endotoxin-stimulated rabbits by means of ultracentrifugation, electrophoresis, gel filtration, and sedimentation through a density gradient. After reduction, alkylation, and column chromatography, all in guanidine hydrochloride, the molecule was found to contain about 78% heavy chains and 22% light chains. The hexose and hexosamine content of the heavy chain was determined.

The molecular weights of intact γM-immunoglobulin, heavy chain, and light chain were found to be 850,000–900,000, ~70,000, and 22,000–23,000 respectively. Evidence for a subunit of ~180,000 molecular weight was also obtained. These results suggest that γM-immunoglobulin is composed of five subunits, each of which contains two heavy and two light chains. Comparison of heavy chains from γM- and γG-immunoglobulin from the same rabbit showed gross differences in amino acid and carbohydrate composition, fingerprints, and molecular weight. On the other hand, the light chains had the same molecular weight and very similar fingerprints.

γM Immunoglobulins,[1] whose reported molecular weights vary from 0.75 to 1.3 × 10^6 (Kabat, 1939; Pedersen, 1945; Caputo and Appella, 1960; Kovacs and Daune, 1961; Franěk, 1962; Miller and Metzger, 1965), comprise 5–10% of the total serum immunoglobulins (Kunkel, 1960). They are not dissociated by 6.6 M urea, wide variations in temperature or pH, or ultrasonic vibrations (Putnam, 1959). Deutsch and Morton (1958) have shown that these macromolecules can be reduced by mercaptans, yielding heterogeneous subunits with sedimentation coefficients in the 6–7S range and molecular weights in the neighborhood of 180,000. These subunits contain the same percentage of carbohydrate as the parent molecules, and hence are different from γG-immunoglobulins (Kunkel, 1960).

Cohen (1963) has demonstrated that human γM-immunoglobulins are composed of two types of polypeptide chains in roughly the same proportion as the two types of chains in γG-immunoglobulin molecules. Furthermore, he and Carbonara and Heremans (1963) were able to show by immunological and electrophoretic criteria that the light chains are similar or identical in all three classes (γG, γA, and γM) of immunoglobulins, but that the heavy chains are different. Recent work on the amino acid and carbohydrate composition of the heavy and light chains of human γM- and γG-immunoglobulins has confirmed these conclusions (Chaplin *et al.*, 1965).

In this study we have isolated the heavy and light polypeptide chains of extensively reduced rabbit γM-immunoglobulin and have characterized them with respect to mass. This has enabled us to propose a tentative model for γM-immunoglobulin. In addition, we have compared the chains of γM molecules with those of γG.

Methods

Preparation and Analysis of Protein Fractions. Adult New Zealand rabbits (5–6 kg) were given ten successive daily intravenous injections of 0.25 μg of purified *S. enteritidis* endotoxin, which is almost wholly polysaccharide. They were then bled several times during the next few days by cardiac puncture. KBr was added

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[1] We have followed the recommendations of the World Health Organization Committee on Nomenclature for Human Immunoglobulins, whereby γM is synonymous with 19Sγ, γ_1M, or β_2M, and γG with 7Sγ, γ_2, or γ_{SS}((1964), *Bull. World Health Organ. 30*, 447).

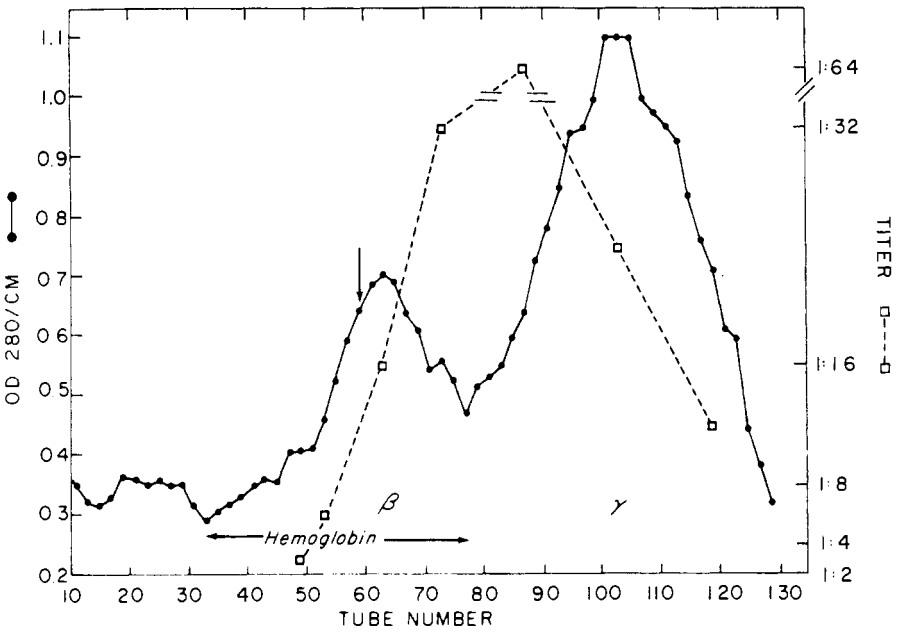

FIGURE 1: Representative elution pattern from the preparative electrophoresis column. Albumin was removed by countercurrent elution during the actual electrophoresis and is not shown. All protein to the right of the arrow was pooled and used for the next step in the isolation procedure.

to the serum to make the density 1.1–1.2, following which it was centrifuged in the cold for 5 hr at 39,000 rpm in the No. 40 rotor of the Spinco Model L ultracentrifuge. The top one-third of each tube, containing the lipid, was removed by means of a tube slicer. The remainder was dialyzed against 0.05 M Veronal, pH 8.6, and electrophoresed[2] in an LKB 5800 A, size 60, Porath preparative electrophoresis column, with formalated ethanolyzed cellulose as the stabilizing medium, in order to separate $\alpha 2$ macroglobulin from the β- and γ-globulins. The duration of electrophoresis was 25–50 hr at 200–500 v and 150–600 mamps. After elution with buffer most of the β and all of the γ fractions were pooled, made 0.5 M in NaCl, and concentrated by ultrafiltration to 20–40 mg/ml. To aid in determining which fractions should be used as the source of γM-immunoglobulin, antibody titers against *S. enteritidis* were determined in selected tubes by the bentonite flocculation procedure (Wolff *et al.*, 1963).

The concentrated protein was then passed through a 120 × 5.7 cm column of 3.5% polyacrylamide gel preequilibrated with 0.35 M NaCl which had been prepared according to the procedure of Hjertén (1962) using 5% cross-linker. The flow in the column was against gravity and loading was facilitated by making the density of the sample greater than that of the solvent with which the column had been equilibrated and by following the sample with 50 ml of 1 M NaCl. The initial peak in the eluate, after being concentrated by ultrafiltration, proved to be heterogeneous when examined by sedimentation velocity. Therefore, 1 ml containing 10 mg of the mixture of proteins was centrifuged in a linear density gradient of 28 ml of 5–28% sucrose, essentially as described by Martin and Ames (1961). Centrifugation was performed in the SW 25.1 rotor of the Spinco Model L ultracentrifuge at 23,000 rpm for 26 hr at about −5°, after which the tubes were punctured and 35 drop fractions were collected. The OD of the individual fractions or dilutions thereof was determined in the Beckman DU spectrophotometer at 280 mμ. Appropriate fractions were concentrated by pressure dialysis against 0.35 M NaCl.

γG-Immunoglobulin was obtained by concentrating the front half of the second peak from the polyacrylamide column and passing the protein through DEAE-cellulose as described by Levy and Sober (1960).

Sedimentation velocity studies were performed in the Spinco Model E ultracentrifuge at 39,460 rpm and 21°.

Reductive Studies and Separation of Polypeptide Chains. Extensive reduction of immunoglobulins and alkylation and separation of the resulting polypeptide chains, all in guanidine, were performed as described in the previous paper (Small and Lamm, 1966) except that the protein concentration during reduction of γM-immunoglobulin ranged from 0.1 to 1%. Partial reduction and alkylation were carried out according to Deutsch (1963).

Molecular Weights. Molecular weights were determined by the high speed sedimentation equilibrium method of Yphantis (1964) using procedures discussed in the companion paper (Small and Lamm, 1966). For centrifugation of macroglobulins the AN-J rotor was used. Proteins were dissolved in the following media:

[2] Performed by H. Kimel.

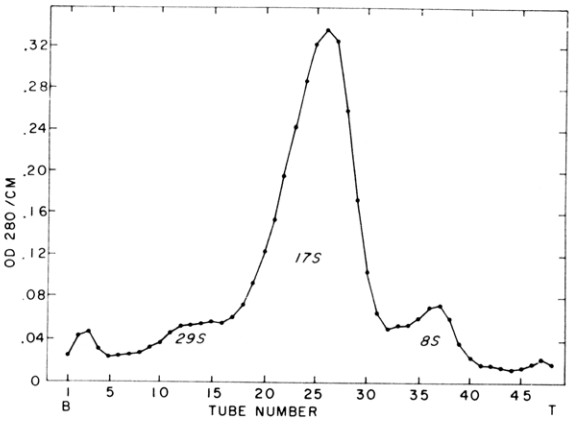

FIGURE 2: Separation of proteins in a sucrose density gradient. The indicated sedimentation coefficients were determined in the analytical ultracentrifuge. B and T refer to the bottom and top respectively of the liquid column.

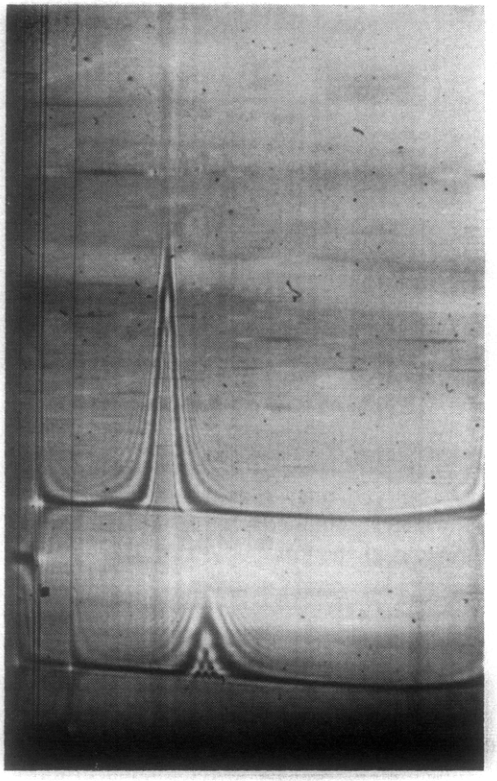

FIGURE 3: Schlieren patterns of purified γM-immunoglobulin. Bottom pattern at 1.3 mg/ml; top pattern (wedged window) at 3.7 mg/ml. Phase plate angle, 60°. Solvent: 0.35 M NaCl.

macroglobulins in either 0.35 M NaCl or 5 M guanidine hydrochloride; the products of partial reduction in 0.1 M Tris–saline, pH 8.2; and the products of extensive reduction in 5 M guanidine hydrochloride. Partial specific volumes of 0.722 and 0.703 were used for experiments in saline and guanidine, respectively (see Discussion).

Protein Fingerprints. Fingerprinting was performed by the method of Katz *et al.* (1959). Extensively reduced and alkylated polypeptide chains, dissolved in 5 M guanidine hydrochloride, were exhaustively dialyzed against distilled water, and then lyophilized. Samples (2 mg) were suspended in 4 ml of 1% NH_4HCO_3, and 2 μl of 1% trichloroacetic acid precipitated trypsin (Worthington Biochemical Corp., lot 591, previously shown (Small *et al.*, 1965) to be low in chymotryptic activity) in 0.001 N HCl was added. Eight hours later an additional 2 μl of trypsin was added, and digestion was allowed to proceed for a total of 32 hr, at which time the solutions were clear. Previous work on light chains of γG-immunoglobulins had shown that essentially all peptide bonds involving lysine and arginine are broken under these conditions (Small *et al.*, 1965). The hydrolysates were lyophilized, dissolved in water, and chromatographed on Whatman No. 3 paper in 1-butanol–acetic acid–water (3.4:1:5) for 24 hr. After drying, the peptides were electrophoresed in a Model D electrophorator (Gilson Medical Electronics) in pyridine–acetic acid–water (1:10:289) for 1 hr at 3200 v. The papers were then dried and stained with ninhydrin solution, 0.11% ninhydrin in ethanol–acetic acid–collidine (7.5:2.5:1).

Chemical Composition. Amino acid analysis was performed as previously described (Small and Lamm, 1966). Hexose was measured by the method of Winzler (1955) using mannose as a standard and reading at 505 mμ. Hexosamine was determined by Schloss' modification (1951) of the Elson–Morgan procedure, adapted to permit analysis of amounts of hexosamine in the 10–20 μg range.

Immunoelectrophoresis was performed according to Scheidegger (1955). Allotypes were determined by double diffusion in agar. Antibody estimations, allotype determinations, amino acid analyses, and fingerprints were all performed on protein obtained from the same sample of serum from a single rabbit.

Results

Figure 1 shows a representative elution pattern after preparative electrophoresis. The location of antibodies to *S. enteritidis* and of hemoglobin proved helpful in deciding how to divide the fractions. The γ and β_2 globulins (Figure 1) were filtered through the column of polyacrylamide gel and were resolved into a small, symmetrical initial peak followed by a much larger peak containing γG-immunoglobulin. The first peak, however, when evaluated by sedimentation velocity, was found to contain about 70% 17S, 20% 8S, and 10% 29S proteins. Refiltration of the protein of this initial peak did not lead to resolution of the three components.

The 17S, 8S, and 29S components could be resolved, however, by density gradient centrifugation, and a typical result is shown in Figure 2. There was variation

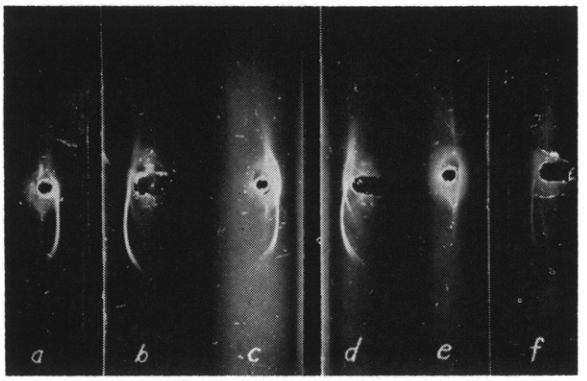

FIGURE 4: Immunoelectrophoresis of γM- (wells a, b, c, and d) and 29S (wells e and f) immunoglobulins. Wells b, d, and f were enlarged to accommodate more antigen. The troughs contained a goat anti-rabbit serum antiserum, which was diluted twofold in the middle trough. The anode is at the top.

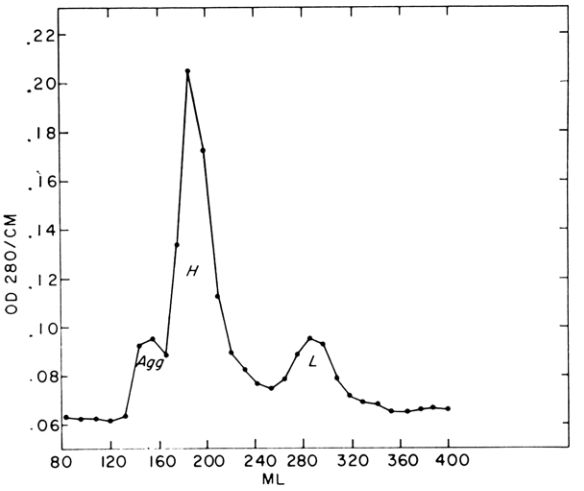

FIGURE 5: Elution pattern from Sephadex G-200 column after reduction and alkylation of γM-immunoglobulin. The fourth peak, which contains small reagent molecules, is not shown. H and L refer to the two kinds of component polypeptide chains, and "Agg" refers to the material in the first peak, thought to represent an aggregate or subunit (see text).

in the relative amounts in the three peaks depending on the particular serum and/or preparation. Protein from the middle peak was homogeneous when examined by sedimentation velocity in the analytical ultracentrifuge (Figure 3). The main boundary was always symmetrical throughout its migration, and no other peaks were noted. The yield of γM-immunoglobulin was about 10–15 mg from 100 ml of serum.

By immunoelectrophoretic analysis the final preparation of γM-immunoglobulin contained a small amount of unidentified contaminant in the β_1 region. This contaminant was not apparent when the immunoelectrophoresis was performed in the usual manner (Figure 4). The contaminant was barely visible when the central well was enlarged to accommodate about three times the usual volume, but was distinct when the antiserum was diluted twofold. It did not take a lipid stain. The 29S protein line was similar in position and shape to that of the γM-immunoglobulin when analyzed by immunoelectrophoresis (Figure 4).

Antibody activity against *S. enteritidis* was measured in the different immunoglobulin fractions from an individual rabbit serum. The titers of 1 mg/ml solutions of γM-, γG-, and 8S immunoglobulins were 1:768, 1:8, and 1:16, respectively. The predominance of this antibody activity in the γM-immunoglobulin is consistent with the work of others (see, for example, Weidanz *et al.*, 1964).

The *b* locus allotypic specificity was found to be type Ab4 for both the purified γM- and γG-immunoglobulins from the one rabbit studied. On the other hand, the *a* locus allotypic specificity was detected only in the γG-immunoglobulin and found to be type Aa1 when analyzed by double diffusion in Ouchterlony plates with both immunoglobulins at similar concentrations. With double diffusion in Preer tubes (a more sensitive technique) the *a* locus allotype was still readily apparent in the γG-immunoglobulin; however, in the case of the γM-immunoglobulin, a faint line appeared only after several days. When the antiserum for the allotype was absorbed with γG-immunoglobulin, no line could be detected with the γM-immunoglobulin in the Preer tube. Our results are difficult to interpret. If γM-immunoglobulin does indeed have *a* locus allotypic sites, it may be that only a small proportion of the molecules contain them and/or that these allotypic sites, though present in γM-immunoglobulins, are buried. Todd (1963) has previously reported finding the same allotypes for both *a* and *b* loci in γM and γG fractions.

After reduction and alkylation in guanidine hydrochloride and passage through Sephadex G-200 equilibrated in guanidine four peaks emerged, the first three of which contained protein (Figure 5). The last peak emerged at the end of one column volume and contained some of the reagents of low molecular weight used for reduction and alkylation. In a total of three different experiments, the first peak varied in amount from 12 to 41% of the total protein coming off the column (Table I). For reasons to be discussed later, the first peak is thought possibly to contain a subunit composed of several polypeptide chains. The second peak contains heavy polypeptide chains, and the third peak light polypeptide chains. In spite of the variation in the per cent of protein in the initial peak, the ratio of heavy to light chains was constant (Table I) at 78:22, based on ultraviolet absorption.

Single amino acid analyses of 24-hr hydrolysates indicated marked differences between the heavy chains of γM- and γG-immunoglobulin. The most striking differences occurred in serine, alanine, and phenyl-

TABLE I: Sephadex G-200 Gel Filtration of Reduced, Alkylated γM-Immunoglobulin.[a]

Protein (mg)	Elution Volume (ml) of H Chain	Elution Volume of L Chain	H/(H + L)[b]	Elution Volume of Aggregate[c]	Eluted Protein in Aggregate (%)	Recovery (%)[d]
6	190	285	77%	150	12	89
18	185	280	78%	140	38	88
4	190	285	78%	140	41	92

[a] All gel filtration experiments were performed on the same column. [b] The ratio of the amount of H chain eluted to the sum of the H and L chains eluted, assuming equal extinction coefficients for the H and L chains. [c] "Aggregate" refers to protein that comes off the column prior to the H chain. Intact γM-immunoglobulin was eluted at 140 ml in a separate experiment. [d] The ratio of the total amount of eluted protein to the amount of protein originally reduced.

alanine, which were more abundant on a percentage basis in γM heavy chains, and in aspartic acid and lysine, which were more prevalent in γG heavy chains. In addition, the heavy chain of γM-immunoglobulin was found to contain 9.0% hexose and 3.3% hexosamine. By comparison, the heavy chain of γG-immunoglobulin contained 1.4% hexose and 1.5% hexosamine. In more detailed work on human immunoglobulins, Chaplin *et al.* (1965) previously reported differences in amino acid and carbohydrate composition between the heavy chains of γM- and γG-immunoglobulin.

Fingerprints of heavy chains from γM- and γG-immunoglobulins are illustrated in Figure 6. Significant differences are readily apparent. By comparing the two maps with each other and with the aid of a map of a mixture of the two types of heavy chain (not shown), we were able to find only about five similar spots (designated by arrows). In our experience, the bottom three of these five are invariably present in the fingerprints of extensively reduced and alkylated heavy and light chains, and may represent free amino acids. The yellow spot (circled) in the upper left-hand corner of each map is significant since in γG heavy chains it has been related to *a* locus allotypic specificity (P. A. Small, R. A. Reisfeld, and S. Dray, in preparation). It is noteworthy that these spots are different since they are represented separately in the map of the mixture.

Unfortunately, only enough light chains were available for one experiment, and those fingerprints were technically somewhat unsatisfactory. Nevertheless, the peptide maps of the light chains of γG- and γM-immunoglobulins are shown in Figure 7, A and B. For purposes of comparison, a γG light chain fingerprint from a rabbit of the same genotype, obtained in another study (Small *et al.*, 1965), is presented in Figure 7C. It is clear that spots 19 and 25 (arrows), which are characteristic of the *b*4 allotypic specificity (Small *et al.*, 1965), are present in all three maps. In addition there are a number of other similar spots and no significant differences that we can be sure of.

Results of the molecular weight studies are given in Figures 8, 9, and 10 and Table II. The preceding paper discusses evaluation of the molecular weight data, methods of calculation, and criteria used to select "best values." In addition it should be noted from the ln *c vs.* r^2 plots (Figure 8) that for γM in guanidine (Figure 8, A and B) and γM "aggregate" (Figure 8, E and F) there is definite nonideal behavior, which is more obvious at higher speeds and concentrations (*cf.* Figure 8A with 8B and 8E with 8F). The plot for γM heavy chain (Figure 8G) indicates homogeneity and some nonideality, which was more apparent at higher concentrations and speed (not shown). Since the weight-average molecular weights of γM in guanidine, γM "aggregate," and γM heavy chain do not differ greatly from their *z*-average molecular weights (Table II) there is not much heterogeneity by this criterion. Hence, for these samples an extrapolation to weight-average molecular weights at infinite dilution is probably valid (Figures 9 and 10). γM in saline is heterogeneous as evidenced by the difference between its weight- and *z*-average molecular weights (Table II). γM partially reduced in saline is markedly heterogeneous as seen in Figure 8D and by a comparison of its weight- and *z*-average molecular weights (Table II). It is probable that the slope of its molecular weight *vs.* concentration (Figures 9 and 10) is at least partially due to heterogeneity.

From considerations such as these, our "best" molecular weights are 850,000–900,000 for γM-immunoglobulin and about 70,000 and 22,000–23,000 for its heavy and light chains, respectively (Table II). The molecular weight of the heavy chain of γM-immunoglobulin is therefore about one-third greater than for the heavy chain of γG. This is consistent with the fact that the heavy chain of γM emerged at about 190 ml from the Sephadex column (Table I), before the heavy chain of γG, which emerged at about 220 ml. In contrast, the molecular weights of the light chains from γM- and γG-immunoglobulins are the same (*cf.* Small and Lamm, 1966). The molecular weight of the "aggregate" in the first peak eluted from the Sephadex column following extensive reduction was about 180,000, in the same range as the heterogeneous fragments resulting from partial reduction (Table II). In

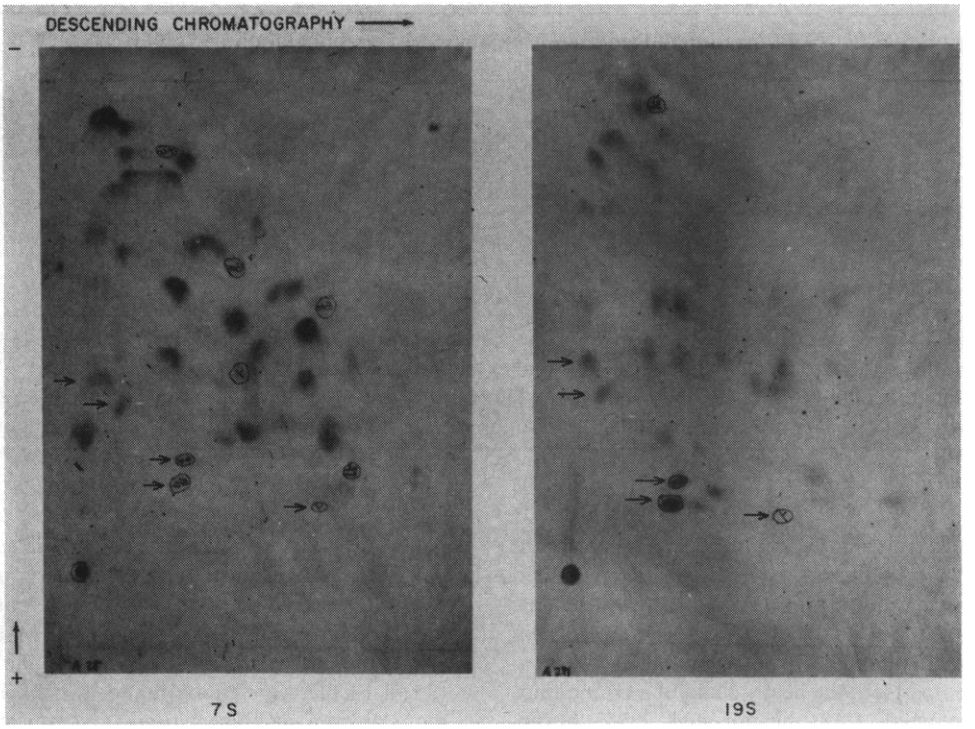

FIGURE 6: Fingerprints of γG- (7S) and γM- (19S) immunoglobulin heavy chains. Sample was applied as a spot in the lower left-hand corner. Non-blue spots were circled.

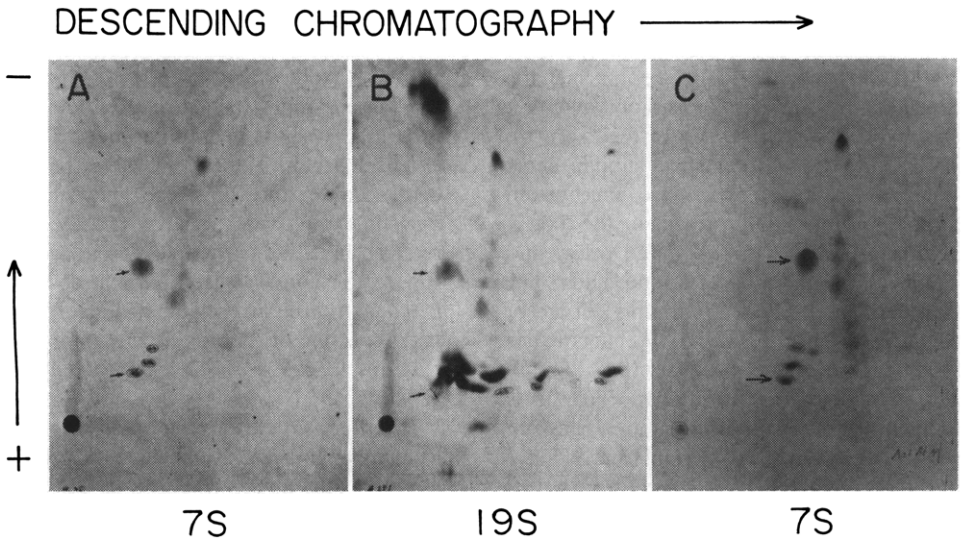

FIGURE 7: Fingerprints of light chains from γG- (7S) and γM- (19S) immunoglobulins. A and B were obtained in this study and C in another study (Small *et al.*, 1965). The arrows indicate spots characteristic of the *b*4 allotypic specificity.

addition, the 29S molecule was found to have a molecular weight of approximately 1.8×10^6 in saline and 0.9×10^6 in guanidine, thus suggesting it to be a non-covalently linked dimer of the γM-immunoglobulin. Franklin (1960) has previously shown that human 29S immunoglobulin can be dissociated by acid into 19S molecules.

Discussion

The γM-immunoglobulin was isolated by a number of procedures employing density, charge, and size differences to separate it from other proteins. The purified material was homogeneous by sedimentation velocity but was shown by immunoelectrophoresis to be

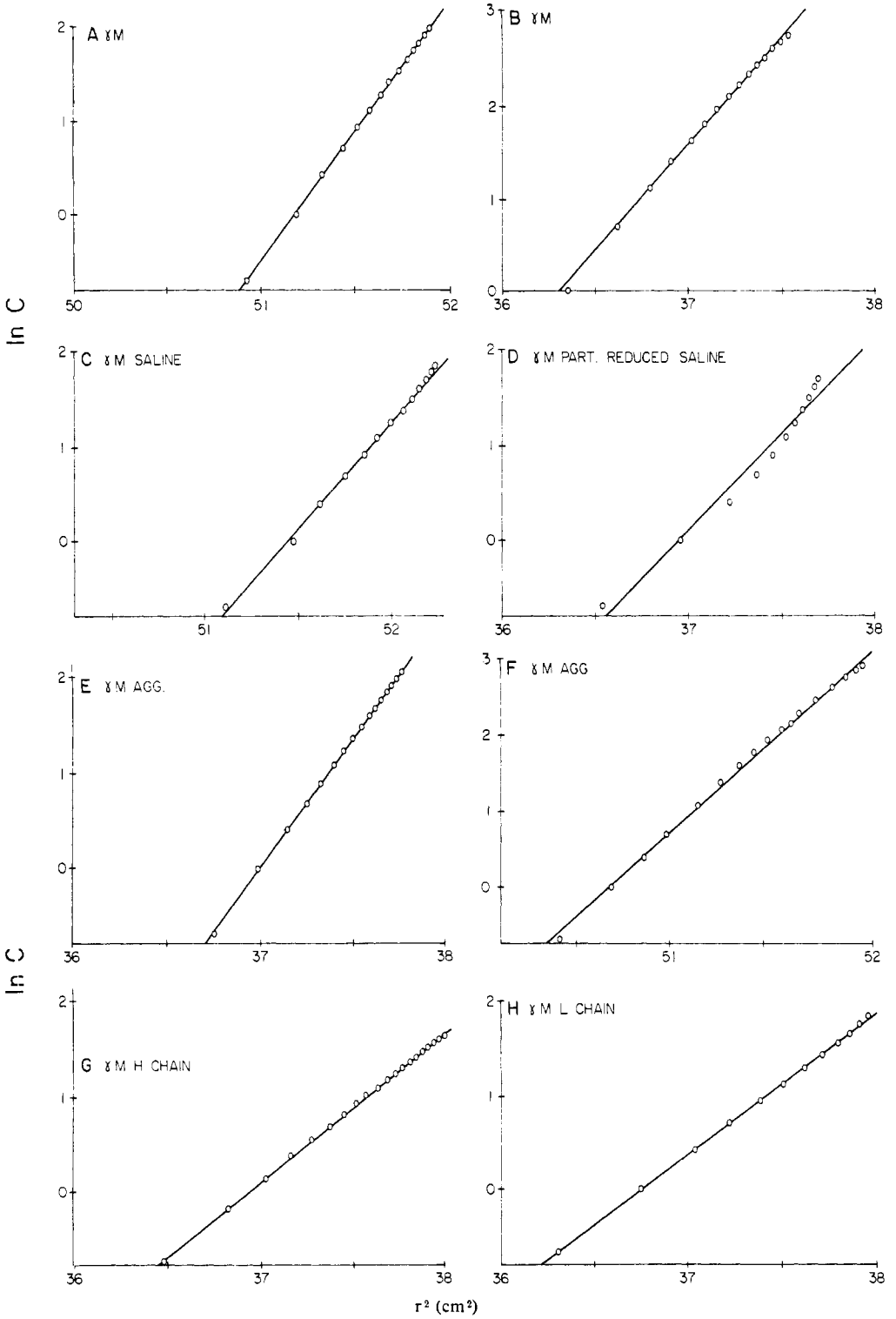

FIGURE 8: Representative plots of the logarithm of the concentration (in fringe numbers) *vs.* the radial distance squared. The lines are least-squares fits of the data. When not specified, the solvent is 5 M guanidine hydrochloride.

	Protein	Concn (mg/ml)	Speed (rpm)
A	γM-immunoglobulin	0.25	8,225
B	γM-immunoglobulin	1.0	11,272
C	γM-immunoglobulin (saline)	0.25	6,166
D	γM subunit from partial reduction (saline)	0.4	13,410
E	γM "aggregate" from extensive reduction	0.4	17,980
F	γM "aggregate" from extensive reduction	1.2	17,980
G	γM heavy chain	0.4	24,630
H	γM light chain	0.4	35,600

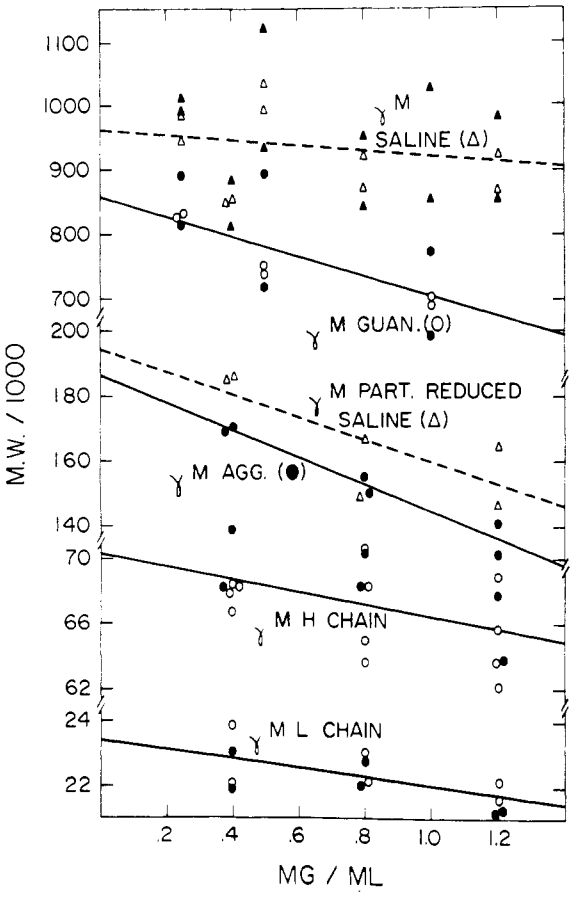

FIGURE 9: Weight-average molecular weights. Open symbols represent molecular weight determinations performed at lower speeds while closed symbols represent higher speed determinations (usually 1.5 to 2 times the force of gravity of the lower speed determination). The lines are least-square fits of the experimental points. All determinations were performed in 5 M guanidine hydrochloride except the γM saline and the γM partially reduced saline which were done in saline solutions and are represented by triangles. γM, γM partially reduced, γM Agg, γM H chain, and γM L chain refer to γM-immunoglobulin, γM subunit from partial reduction, γM aggregate (or subunit, see text) from extensive reduction, γM heavy chain, and γM light chain, respectively.

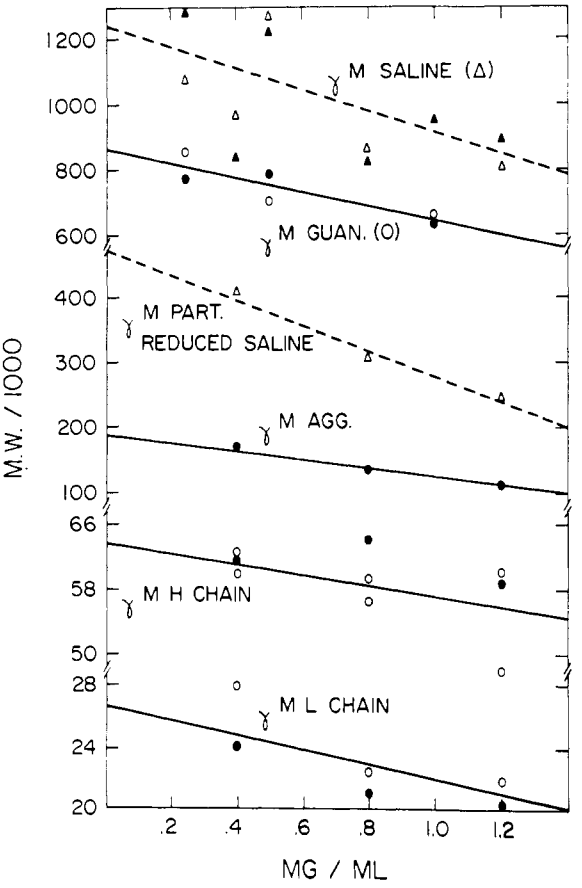

FIGURE 10: z-Average molecular weights. Otherwise the same as Figure 9.

slightly contaminated. We feel that the unidentified substance is very probably present in small amounts since it was not evident when immunoelectrophoresis was carried out in the usual manner but only when the amount of antigen was increased and/or the antiserum was diluted.

Fingerprint studies revealed marked differences between the heavy chains of γM- and γG-immunoglobulins, indicative of gross differences in amino acid sequence. Similar peptides were so few as to suggest that, if these chains have a common evolutionary precursor, they have undergone many changes since their divergence. Differences in the amino acid composition of the heavy chains of γM and γG were directly demonstrated by amino acid analysis. Furthermore, carbohydrate determinations indicated that γM heavy chains contain much more carbohydrate than γG heavy chains. Nevertheless, the difference in size of the heavy chains from the two classes of immunoglobulins probably cannot be attributed solely to carbohydrate content. In contrast, the light chains of γM- and γG-immunoglobulin were found to be similar with regard to size, allotypic determinants, and fingerprints. Thus our experiments on heavy and light chains from γM- and γG-immunoglobulins support the previous conclusions of others (Carbonara and Heremans, 1963; Cohen, 1963; Chaplin *et al.*, 1965) that the light chains are very similar or identical, whereas the heavy chains are different.

Molecular weights calculated for whole γM-immunoglobulin and its component polypeptide chains will depend on the values chosen for the partial specific volume, $\bar{v}$. Values of $\bar{v}$ reported in the literature for the whole molecule include 0.715 for horse (Kabat, 1939), 0.733 for human (Pedersen, 1945), and 0.722 for human (Miller and Metzger, 1965). We have assumed a $\bar{v}$ = 0.722 for molecular weight calculations of γM-

TABLE II: Molecular Weights $\times 10^{-3}$.[a,b]

	Weight Average	z Average	Best Value
γM-Immunoglobulin	960 ± 38*	1240 ± 100*	~900*
	860 ± 33	850 ± 42	850
Heavy chain	70.3 ± 1.5	64.0 ± 3.7	70
Light chain	23.5 ± 0.5	26.6 ± 1.4	22–23
"Aggregate"	186 ± 4	190 ± 8	180
Partially reduced γM-immunoglobulin	194 ± 12*	475 ± 25*	~180*

[a] All molecular weights were measured in 5 M guanidine hydrochloride except those indicated by an asterisk, which were measured in saline. [b] Speeds used (rpm): γM in saline, 6166, 6569, and 8225; γM in guanidine hydrochloride, 8225 and 11,272; heavy chain, 24,630 and 35,600; light chain, 35,600 and 39,460; "aggregate," 17,980; partially reduced γM, 13,410.

immunoglobulin in saline. The corresponding value of $\bar{v}$ in 5 M guanidine hydrochloride should probably be less, by analogy with other proteins (Kielley and Harrington, 1960). For γM-immunoglobulin and its component chains in guanidine we have used a value of 0.703, which is our measured value for γG-immunoglobulin light chains in guanidine (Small and Lamm, 1966) and therefore should correspond to the value for γM-immunoglobulin light chains. We do not know the correct value of $\bar{v}$ for γM-immunoglobulin or its heavy chain in guanidine. In other species the $\bar{v}$ of γM is less than that of γG, probably due to the higher carbohydrate content of γM. Therefore, the $\bar{v}$ of rabbit γM is probably less than that of γG, found to be 0.711 in guanidine (Small and Lamm, 1966). It should be noted that a 1% error in $\bar{v}$ would lead to a 3.9% error in molecular weight if determined in 5 M guanidine (2.7% if in saline).

Although the "aggregate" eluted in the first peak from the Sephadex column in different experiments contained a variable percentage of the total protein, the ratio of protein in the second peak (heavy chain) to that in the third peak (light chain) was constant (Table I and Figure 5). This suggests that the "aggregate" is composed of heavy and light chains in the same proportion as is present in the intact molecule. The "aggregate" was homogeneous by sedimentation velocity, by the characteristics of the ln c *vs.* r^2 plots in the calculations of molecular weight for runs at different initial concentrations, and by the agreement between its weight- and z-average molecular weights. Since the molecular weight was about 180,000, a composition of two heavy and two light chains is suggested for the "aggregate."

The polypeptide chains in the "aggregate" are almost certainly not linked by disulfide bonds since an attempted re-reduction failed to change its size. At present we do not know how to eliminate it. It may be pertinent that, in the original procedure for separating extensively reduced polypeptide chains of γG-immunoglobulin in guanidine (Small *et al.*, 1963), a small amount of aggregate was usually produced. The size of this aggregate also was unaffected by attempted re-reduction. The aggregate from γG-immunoglobulin could be eliminated, however, by modifying the reduction procedure (Small and Lamm, 1966). Furthermore, since the molecular weight of the "aggregate" from γM-immunoglobulin is about 180,000, if it were a single polypeptide chain indigenous to native γM-immunoglobulins it would have to comprise at least 20% of the molecule. This is inconsistent with the one experiment in which the "aggregate" accounted for only 12% of the eluted protein (Table I) and with the findings of Miller and Metzger (1965).

When γM-immunoglobulin was partially reduced, a heterogeneous mixture resulted, but most of the fragments appeared to have a molecular weight in the neighborhood of 180,000. The similarity in the molecular weights of the fragments produced by partial reduction and the "aggregate" obtained after extensive reduction suggests that rabbit γM-immunoglobulin contains subunits composed of several polypeptide chains. This conclusion is in agreement with the findings of Deutsch (1963), who described a 190,000 molecular weight subunit from a "γM-like" human protein, and of Miller and Metzger (1965), who have obtained 185,000 molecular weight subunits from human γM-immunoglobulin following mild reduction.

Some years ago, Deutsch and Morton (1958) reported that 6–7S subunits result from the incomplete reduction of macroglobulins. Since the molecular weights of γG- and γM-immunoglobulins were thought to be about 160,000 and 1,000,000 respectively, many workers have considered the γM proteins to be composed of six subunits. However, since the heavy chain of γM-immunoglobulin has a molecular weight of 70,000 and since the subunit appears to have a molecular weight near 180,000 [(2 × 70,000) + (2 × 22,500) = 185,000] it appears likely that γM-immunoglobulin molecules are composed of only five subunits (875,000/

180,000 = 4.9). Intact γM-immunoglobulin should therefore contain ten heavy and ten light chains. Miller and Metzger (1965) have come to a similar conclusion for human γM-immunoglobulin.

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