Helmholz, L., and Levine, R. (1942), *J. Am. Chem. Soc. 64*, 354.
Jaffe, H. H., Freedman, L. D., and Doak, G. O. (1953), *J. Am. Chem. Soc. 75*, 2209.
Kreiter, V. P., and Pressman, D. (1963), *Biochemistry 2*, 97.
Landsteiner, K. (1945), The Specificity of Serological Reactions, revised ed., Cambridge, Mass., Harvard University Press.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193*, 265.
Nisonoff, A., and Pressman, D. (1958), *J. Immunol. 80*, 417.
Pauling, L., and Pressman, D. (1945), *J. Am. Chem. Soc. 67*, 1003.
Pauling, L., Pressman, D., and Grossberg, A. L. (1944), *J. Am. Chem. Soc. 66*, 784.
Pressman, D., and Brown, D. H. (1943), *J. Am. Chem. Soc. 65*, 540.
Pressman, D., Pardee, A. B., and Pauling, L. (1945), *J. Am. Chem. Soc. 67*, 1602.
Pressman, D., and Siegel, M. (1953), *J. Am. Chem. Soc. 75*, 686.
Pressman, D., Single, S. M., Grossberg, A. L., and Pauling, L. (1944), *J. Am. Chem. Soc. 66*, 1731.
West, J. (1930), *Z. Krist. 74*, 306.

# The Polypeptide Chains of Rabbit γ-Globulin and Its Papain-cleaved Fragments*

ERIC MARLER, CHARLES A. NELSON, AND CHARLES TANFORD

*From the Department of Biochemistry, Duke University Medical Center, Durham, N. C.*

*Received September 19, 1963*

It is shown by sedimentation equilibrium measurements that rabbit γ-globulin consists of four polypeptide chains, two with a molecular weight of close to 50,000, and two with a molecular weight of close to 25,000. Similar measurements show that each of the three fragments of γ-globulin, which are obtained by the action of papain, has a molecular weight of close to 50,000. Each fragment consists of two polypeptide chains of molecular weight close to 25,000 apiece. From these data it can be concluded that the action of papain on the parent molecule has been to split the two heavier polypeptide chains into approximately equal halves. All of the foregoing data confirm the model of the gross structure of γ-globulin which has been proposed by Porter (1962).

It has recently been suggested by Porter (1962) that rabbit γ-globulin probably consists of four polypeptide chains, two A chains having a molecular weight of about 50,000 each, and two B chains having a molecular weight of about 25,000 each. A similar structure has been suggested by Edelman and Benacerraf (1962) as one of several possibilities for human γ-globulin. The work presented in this paper was initiated before Porter's and Edelman's results were available. It set out to determine the number of polypeptide chains in rabbit γ-globulin, and their molecular weights, by a method which would not require separation of the chains. The result is an unequivocal demonstration of the correctness of Porter's conclusions.

In addition to determining the nature of the polypeptide chains of rabbit γ-globulin itself, we have made similar studies on the fragments of the molecule which are obtained by the action of papain. Each molecule yields three fragments by this procedure: fragment III, which is crystalline and plays no part in conferring specificity on those γ-globulin molecules which are specific antibodies, and two fragments which are the carriers of antibody specificity. These latter fragments are designated fragments I or II, depending on their ease of elution from a carboxymethyl-cellulose column at *p*H 5.5. We have studied fragments of type I only, but there is every reason to believe that the gross structure of fragments of type II is the same as that of fragments of type I (Palmer *et al.*, 1962).

* This work has been supported by a research grant (AM-04576) from the National Institute of Arthritis and Metabolic Diseases, U. S. Public Health Service, and by a research grant (G-17477) from the National Science Foundation. The authors are supported, respectively, by a Public Health Service training grant (2A-5074), by a Public Health Service post-doctoral fellowship, and by a Public Health Service research career program award.

## MATERIALS AND METHODS

Rabbit γ-globulin has been prepared in this laboratory by a variety of methods. Physical and chemical studies which will be published at a later date indicate that most of these methods yield proteins which do not differ appreciably from each other, except that they possess somewhat different titration curves. The sedimentation velocity data reported in this paper were obtained from several such different preparations.

The quantitative measurement of the molecular weights of the polypeptide chains of γ-globulin (Table II), which constitutes the most important result reported, was carried out on a sample of rabbit γ-globulin prepared by a modification of the procedure of Gorodskaya (1950), using the reagent Rivanol (2-ethoxy-6,9-diamino-acridine lactate) for the initial precipitation of the acidic proteins of rabbit serum. Excess Rivanol was removed by precipitation with approximately 1 M KI, and the crude globulin was then purified by chromatography on DEAE-cellulose (0.05 M potassium phosphate buffer, *p*H 7.0), precipitation with $(NH_4)_2SO_4$, and a second column chromatography, this time on DEAE-Sephadex. The final product was dialyzed against 0.1 M KCl and stored as a lyophilized powder. This protein proved virtually homogeneous by sedimentation velocity, including little or none of the heavy component ($s_{20,w} \sim 10$) which we have found present in most preparations of γ-globulin and of specific antibodies.

The fragments of γ-globulin were prepared by the method of Porter (1959), except that mercaptoethanol was used as reducing agent in place of cysteine. The reaction was stopped by addition of iodoacetamide.

In order to reduce the proteins to their polypeptide chains they were dissolved in 6–7 M guanidine hydrochloride and then allowed to stand at room temperature for at least 12 hours in 0.1 M mercaptoethanol

at *p*H 8. When protection of the liberated sulfhydryl groups was desired, they were treated with iodoacetamide to produce the *S*-carboxamidomethyl derivatives.

Other methods of making polypeptide chains (used in preliminary experiments only) were *S*-sulfonation (Swan, 1957; Pechère *et al.*, 1958), using guanidine hydrochloride in place of urea as dispersing agent, and the method of oxidation with performic acid (Hirs, 1956). The performic acid reaction was terminated by use of bisulfite ions.

Guanidine hydrochloride was prepared by acidification of guanidine carbonate, which was precipitated from a saturated aqueous solution by addition of ethanol (Anson, 1941).

The principal experimental method used in this study was the method of sedimentation equilibrium. The Beckman-Spinco analytical ultracentrifuge was used, with either schlieren or interference optics. Protein solutions were usually dialyzed against solvent, which consisted of 6 M guanidine hydrochloride, plus excess mercaptoethanol in those runs in which free SH groups were present on the protein molecules. Double-sectored cells were used, containing protein on one side and solvent on the other.

When schlieren optics were used, the vertical displacement of the phase-plate image was taken to be proportional to $dc/dr = \Sigma dc_i/dr$, where $c$ is the total protein concentration at a distance $r$ from the center of rotation, and individual types of polypeptide chains are designated by subscripts $i$. The quantity ln $[(dc/dr)/r]$ was plotted versus $r^2$. Essentially linear plots were obtained, the slope of which, in the middle region of the solution column, would give the $z$-average molecular weight if the protein solution were ideal (van Holde and Baldwin, 1958; Yphantis, 1960). Since solutions in guanidine hydrochloride are far from ideal, an apparent molecular weight is obtained, which yields the true $z$-average molecular weight only on extrapolation to zero protein concentration.[1,2]

The nonideality in 6 M guanidine solution is so great that experimental molecular weights at the higher concentration used in schlieren optics experiments were reduced as much as 50% below the extrapolated molecular weight. Under these conditions the actual value of the molecular weight depends to some extent (a few per cent) on the method of extrapolation to zero protein concentration. If the protein solution contained a single sedimenting species, the equilibrium state of which could be described by the relation

$$\frac{1}{rc}\cdot\frac{dc}{dr} = \frac{(1 - \bar{v}\rho)\omega^2}{RT}\cdot\frac{M}{1 + 2BMc} \qquad (1)$$

where $B$ is the second virial coefficient (in the equation for chemical potential) in units of (cc/g)(g/mole)$^{-1}$, then

$$\frac{d \ln [(dc/dr)/r]}{dr^2} \equiv \frac{M_{app}(1 - \bar{v}\rho)\omega^2}{2RT} = \frac{M(1 - \bar{v}\rho)\omega^2}{(1 + 2BMc)^2 2RT} \qquad (2)$$

and the correct extrapolation would be a plot of $1/M_{app}^{1/2}$ versus $c$, which would yield straight lines with $1/M^{1/2}$ as intercept and $2BM^{1/2}$ as slope. There is no rigorous justification for applying equation (2) to mixtures containing more than one sedimenting component, but we have found that the plot based on equation (2) gives straight lines for our data, and have thus used it for all extrapolations of schlieren optics data.

When interference optics were used for sedimentation equilibrium studies, interference fringes were assumed to be a measure of concentration $c$ at any point $r$, relative to the concentration $c_a$ at the upper meniscus. High speeds and/or column heights were used, so that values of $c_a$ would be very small. It was possible to use very low concentrations of protein, so that nonideality could often be ignored. When nonideality corrections were necessary, they were made by means of a simple procedure based on equation (1) with a single value of the factor $2BMc$ for all protein components. An integrated form of equation (1) was used to convert observed concentrations to what they would have been in an ideal solution. Values of $\bar{M}_w$ in the upper part of the cell were estimated from plots of ln $(c - c_a)$ versus $r^2$, assuming $c_a = 0$. A calculated value for $c_a$ was then obtained from this value of $\bar{M}_w$, and this was used to yield a plot of ln $c$ versus $r^2$ for final analysis. Number, weight, and $z$-average molecular weights for the original solution were then obtained by procedures given by Wales *et al.* (1951), Lansing and Kraemer (1935), and Svedberg and Pedersen (1940).[3]

The partial specific volume of γ-globulin, measured after dialysis against 6 M guanidine hydrochloride according to the procedure of Casassa and Eisenberg (1961), was 0.72 cc/g. When this procedure is used, 6 M guanidine hydrochloride can be treated as a single diffusible component in the analysis of sedimentation equilibrium data. It should be noted that the partial specific volume so measured is about 0.02 cc/g smaller than the corresponding figure for dilute salt solutions. A similar effect of guanidine hydrochloride on $\bar{v}$ has been observed by Kielley and Harrington (1960) for myosin.

It was assumed that the value of $\bar{v}$ found for γ-globulin applies also to the polypeptide chains and the fragments.

## Results

*Molecular Weights of γ-Globulin and Fragments.*—Cammack (1962) has recently reported a molecular weight of 137,000 for rabbit γ-globulin. Molecular weights of the protein are currently being determined in this laboratory by several methods. The preliminary results obtained so far suggest that Cammack's figure is too low, though they do point to a molecular weight considerably lower than the value of 160,000 reported some years ago by Nichol and Deutsch (1948),

[1] It may be noted that the quantity $d \ln [(dc/dr)/r]/dr^2$ used here for molecular weight determination is exactly one-half the quantity $d[(dc/dr)/r]/dZ$ where $Z = \int_a^r (dc/dr)/dr$, where $a$ is the upper meniscus. This relation is true even if $c$ represents refractive index rather than concentration, i.e., if no assumption is made about the relation between the vertical displacement of the phase-plate image and $dc/dr$. The data obtained are thus the same as would be obtained using method II of van Holde and Baldwin (1958).

[2] Alternative treatments of the data, using the auxiliary determination of the excess refractive index of solution over solvent in a synthetic-boundary cell, were avoided because very small differences in guanidine concentration between protein solution and dialysate could produce relatively large contributions to the excess refractive index, which is a useful quantity only if it is a direct measure of protein content.

[3] In applying these procedures we benefited greatly from several conversations with Dr. David Yphantis of the Rockefeller Institute, New York, New York. Parts of our method of using interference optics are based entirely on techniques developed by Yphantis.

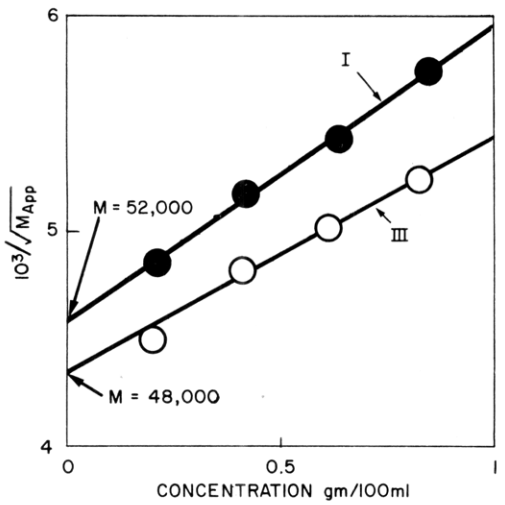


FIG. 1.—Apparent molecular weights of fragments I and III, determined by sedimentation equilibrium, using schlieren optics, in 6 M guanidine hydrochloride, *p*H 4, at 25°. The rotor speed was 20,410 rpm.

and the value of 187,000 reported by Charlwood (1959). Our best current estimate is 145,000 ± 5000.

The molecular weights of papain fragments I and III were determined as part of the present study. Sedimentation equilibrium (schlieren optics) was used, the solvent being 6 M guanidine hydrochloride at *p*H 4. The results, shown in Figure 1, lead to molecular weights of 52,000 and 48,000, respectively, for fragments I and III, with a probable reliability of about ± 4000. The value for fragment I is in good accord with a value determined by Charlwood (1959), but our result for fragment III is far below Charlwood's figure of 80,000. (Two fragments I plus fragment III should correspond to the weight of a molecule of γ-globulin. The molecular weight of γ-globulin obtained in this way from our data for the fragments is 152,000).

*Polypeptide Chains: Preliminary Results.*—The method used in this study was to subject the protein first to the action of a dispersing agent, which should break all noncovalent linkages and unfold the protein as completely as permitted by the disulfide bonds. Second, the unfolded protein was subjected to reduction or oxidation to break all disulfide bonds. The final product should consist of a mixture of the separated polypeptide chains of the protein, regardless of whether these chains were initially held together by disulfide bonds or by noncovalent linkages. Detailed studies of the denaturation of γ-globulin and its subunits, which are in progress in this laboratory, have shown that 8 M aqueous urea, which is a commonly used dispersing agent, does not produce complete unfolding of the molecule. Complete unfolding can be produced, however, by 6 M guanidine hydrochloride. In this solvent the optical rotatory properties become those typical of unfolded proteins, and further increase in the guanidine concentration leads to no additional increase in levorotation. Thus 6 M guanidine hydrochloride was used as the dispersing agent.

Reduction or oxidation was effected by standard methods described in the experimental part of this paper. Sedimentation velocity experiments (see next paragraph) showed that essentially similar patterns were obtained from the products of two reductive and one oxidative method of cleavage, which was taken as an indication of complete cleavage of disulfide bonds, since it is unlikely that such different methods would produce identical extents of cleavage if the

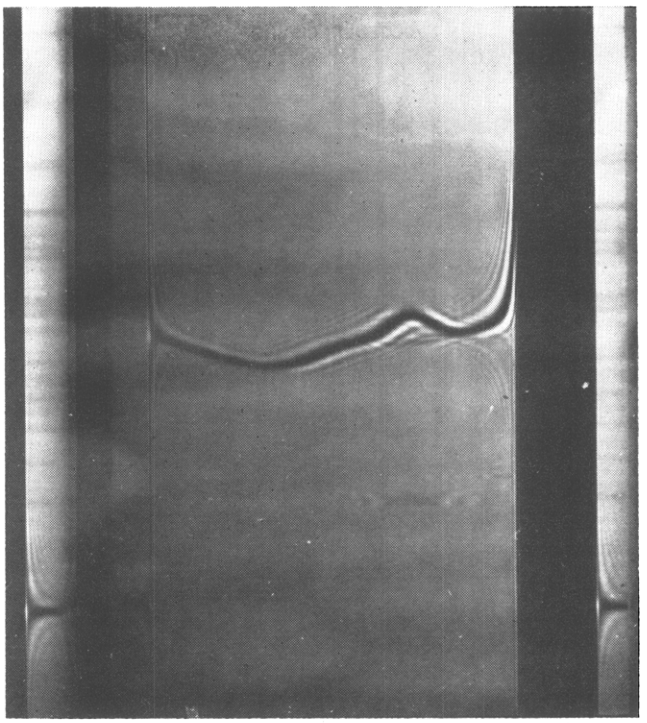

FIG. 2.—Sedimentation pattern of reduced and alkylated polypeptide chains of γ-globulin in 2.5 M guanidine hydrochloride. The rotor speed was 67,770 rpm.

TABLE I

SEDIMENTATION COEFFICIENTS IN 6 M GUANIDINE HYDROCHLORIDE AT 25°[a]

| Protein | Method of Dissociation | $s \times 10^{13}$ |
|---|---|---|
| γ-Globulin | Untreated | 1.29 |
| γ-Globulin | Mercaptoethanol + iodoacetamide | 0.59 |
| γ-Globulin | Sulfonated | 0.59 |
| γ-Globulin | Performic acid | 0.62 |
| Fragment I | Mercaptoethanol + iodoacetamide | 0.48 |
| Fragment I | Performic acid | 0.49 |
| Fragment III | Mercaptoethanol + iodoacetamide | 0.52 |

[a] Sedimentation coefficients were measured at 59,780 rpm at a concentration of 0.5 g/100 cc, and they are uncorrected. The values of $s_{20,w}$ which would correspond to $s \times 10^{13} = 0.6$ and 0.5 are, respectively, $s_{20,w} \times 10^{13} = 1.7$ and 1.4.

cleavage were incomplete. At least one sample of each of the parent molecules (γ-globulin and fragments I and III), after reduction with mercaptoethanol and carboxamidomethylation, was hydrolyzed and subjected to amino acid analysis. Essentially all the original cystine was found as carboxymethylcystine, and no free half-cystine was observed, showing that disulfide bond rupture was certainly complete when mercaptoethanol reduction was used.[4]

Preliminary examination of the polypeptide chains was made by sedimentation velocity in 6 M guanidine hydrochloride. In each case a single broad peak was observed, with the sedimentation coefficients given in Table I. It should be noted that the reason for the broad peak lies in the high density of the solvent (ρ

[4] Complete rupture of all disulfide bonds is of course not necessary to get complete separation into polypeptide chains. Porter (1962), in fact, uses much milder conditions than we have used to achieve chain separations.

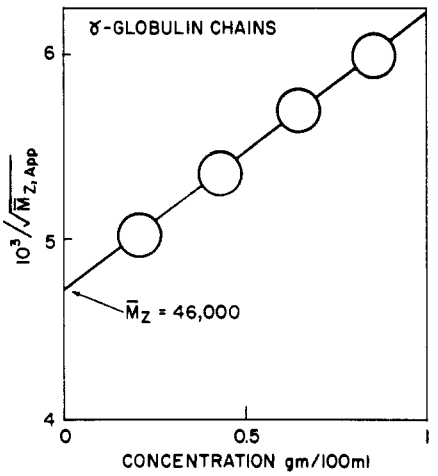


FIG. 3.—Apparent *z*-average molecular weights of the polypeptide chains of rabbit γ-globulin, in 6 M guanidine hydrochloride containing 0.1 M mercaptoethanol, at *p*H 7.8 and 25°. Schlieren optics were used and the rotor speed was 20,410 rpm.

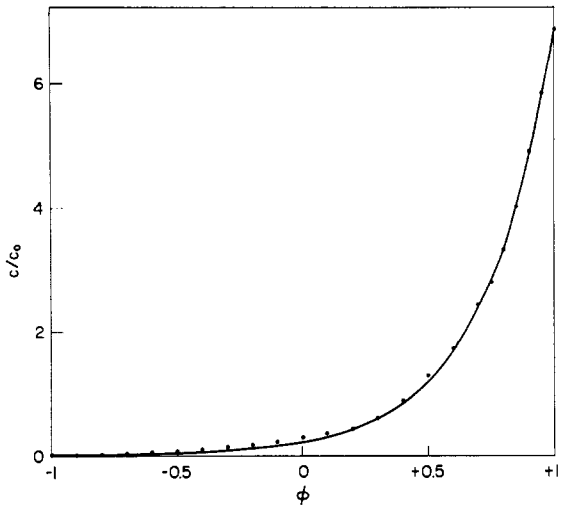


FIG. 4.—Solute distribution as a function of the reduced radial distance ($\phi$) in sedimentation equilibrium of the polypeptide chains of rabbit γ-globulin, in 6 M guanidine hydrochloride, containing 0.1 M mercaptoethanol, at *p*H 7.6 and 25°. Interference optics were used, and the rotor speed was 27,690 rpm. Protein concentration was 0.02 g/100 cc, and the solution column was 4.3 mm. The points are experimental, and the curve is calculated for the theoretical solute distribution of an equimolar mixture of components of molecular weights 50,000 and 25,000, respectively. A nonideality correction, based on a second virial coefficient of $5 \times 10^{-4}$ (cc/g)(g/mole)$^{-1}$ has been included. The slight irregularities seen in the observed data are due to cell distortion; a blank run to correct for cell distortion was not made. (The parameter $\phi$ is defined as $[r^2 - r_c^2]/[r_b^2 - r_c^2]$, where $r_b$ and $r_c$ represent, respectively, the positions of the lower meniscus and the center of the solution column.)

= 1.15 g/cc), which decreases the sedimentation velocity without an accompanying diminution in the rate of diffusion. (The viscosity of the solvent is also higher than that of water, but this reduces both sedimentation and diffusion rates, so that it does not lead to an appreciable increase in boundary spreading during a sedimentation run.) A consequence of the excessive boundary spreading is that it makes it invalid to equate a single peak in the sedimentation diagram with the presence of a single sedimenting component. For this reason the data of Table I must be regarded as reflecting the average properties of whatever polypeptide chains are formed from γ-globulin, fragment I, and fragment III, without giving information as to whether the chains from any one molecule are identical or different in size.

The data of Table I are similar to those reported originally by Edelman and Poulik (1961), and indicate that both γ-globulin and its papain-cleaved fragments are indeed dissociated into smaller subunits by cleavage of all disulfide bonds. Since all polypeptide chains without residual disulfide bonds are unbranched flexible polymer chains, they should obey the general relations for sedimentation of flexible polymers (Tanford, 1961). Assuming that 6 M guanidine hydrochloride is a good solvent, the sedimentation coefficient should vary with molecular weight as $M^{0.45}$. The sedimentation coefficients of roughly $0.6 \times 10^{-13}$ and $0.5 \times 10^{-13}$, observed for the polypeptide chains of the parent γ-globulin and of the papain fragments, respectively, thus indicate that the average size of the chains from the parent molecule is about 50% larger than the average size of the chains from either of the fragments.[5]

The sedimentation equilibrium data reported below indicate that the polypeptide chains obtained from γ-globulin (but not those from the fragments) are of two kinds of appreciably different size. It seemed desirable to confirm this by sedimentation velocity, and the reduced and alkylated γ-globulin was therefore reexamined under conditions which would diminish the boundary spreading. A higher speed (67,770 rpm) was used, and the concentration of guanidine hydrochloride was reduced to 2.5 M ($\rho$ = 1.07 g/cc). Although the original γ-globulin is only partially unfolded at this concentration of guanidine, it is reasonable to expect more complete unfolding after all disulfide bonds are broken. In any event, complete unfolding is not essential; only an absence of association between the chains is necessary, and 2.5 M guanidine should suffice for this purpose. The result of this experiment is shown in Figure 2.[6] Two peaks are clearly observed, with sedimentation coefficients of approximately 1.1 and $1.7 \times 10^{-13}$, respectively. The corresponding values of $s_{20,w}$ are 1.4 and $2.2 \times 10^{-13}$, respectively. Again assuming that these sedimentation coefficients vary as $M^{0.45}$, we conclude that the faster-moving peak corresponds to a chain which is about 2.5 times as heavy as the slower-moving chain.

Because sedimentation equilibrium is capable of giving much more precise information about molecular weights than can be obtained from diffuse sedimentation patterns similar to that given in Figure 2, the sedimentation velocity studies were not pursued further. It is to be noted however that the data are entirely consistent with those reported here from sedimentation equilibrium studies.

*Sedimentation Equilibrium Results.*—The polypeptide chains of γ-globulin were examined by sedimentation equilibrium, using both schlieren and interference optics. For these experiments, the protein was re-

[5] The "average" measured is approximately the weight-average of $M^{0.45}$.

[6] The data in Fig. 2 were actually obtained with a sample of human rather than rabbit γ-globulin. Both human and rabbit proteins were used in a series of experiments in which the guanidine concentration was varied. The two proteins behaved essentially similarly, but it was difficult to obtain really good resolution of the two peaks because too high a guanidine concentration led to increased boundary spreading, whereas too low a concentration led to aggregation between chains and a decrease in the area under the sedimentation diagram peaks. The clearest picture, that shown in Fig. 2, happened to be obtained with human γ-globulin.

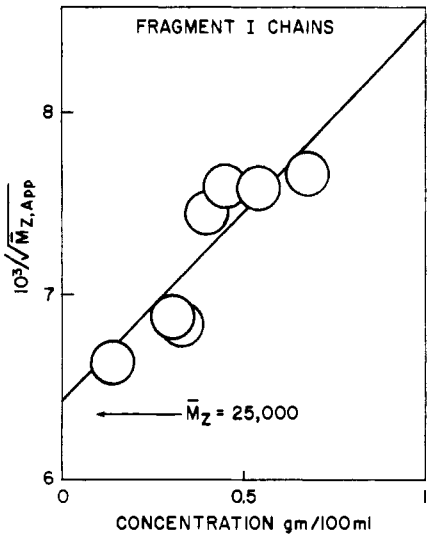


FIG. 5.—Apparent $z$-average molecular weights of reduced and alkylated polypeptide chains of fragment I, in 6 M guanidine hydrochloride, at $p$H 4 and 25°. Schlieren optics were used, and different rotor speeds, ranging from 20,410 to 31,410 rpm, were employed.

TABLE II
MOLECULAR WEIGHTS OF THE POLYPEPTIDE CHAINS OF RABBIT γ-GLOBULIN

| | $\bar{M}_n$ | $\bar{M}_w$ | $\bar{M}_z$ |
|---|---|---|---|
| *Experimental* | | | |
| Interference Optics | 37,500 | 41,000 | 45,400 |
| Schlieren Optics | — | — | 46,000 |
| *Calculated*, for equimolar Mixtures[a] of chains with molecular weights of: | | | |
| 50,000 and 25,000 | 37,500 | 41,700 | 45,000 |
| 50,000 and 20,000 | 35,000 | 41,400 | 45,900 |
| 55,000 and 20,000 | 37,500 | 45,700 | 50,900 |
| 50,000, 20,000, and 5,000 | 25,000 | 39,000 | 45,500 |

[a] It is assumed that the γ-globulin molecule consists of two identical halves. See text.

duced in 0.1 M mercaptoethanol in 6 M guanidine hydrochloride, and was kept in that solvent throughout dialysis and the sedimentation run. The results using schlieren optics are shown in Figure 3. They lead to a $z$-average molecular weight of 46,000. The solute distribution observed using interference optics is shown in Figure 4. These data yield values for number, weight and $z$-average molecular weights. All the molecular weight data are summarized in Table II.

Table II also contains calculated values of $\bar{M}_n$, $\bar{M}_w$, and $\bar{M}_z$, based on different assumptions about the number of chains actually present and their molecular weights. The sum of the molecular weights of all chains must be equal to the molecular weight of γ-globulin itself, i.e., it must lie between 140,000 and 150,000. However, it was pointed out earlier that the two fragments I obtained from any one molecule by the action of papain are essentially identical. Moreover there is strong evidence, which will be cited, that fragment III consists of two identical polypeptide chains. It therefore appears probable that γ-globulin consists of two identical halves, and that all polypeptide chains must be present in duplicate. The calculations have been based on this premise.

Comparison between the observed and calculated results of Table II shows that our data are compatible

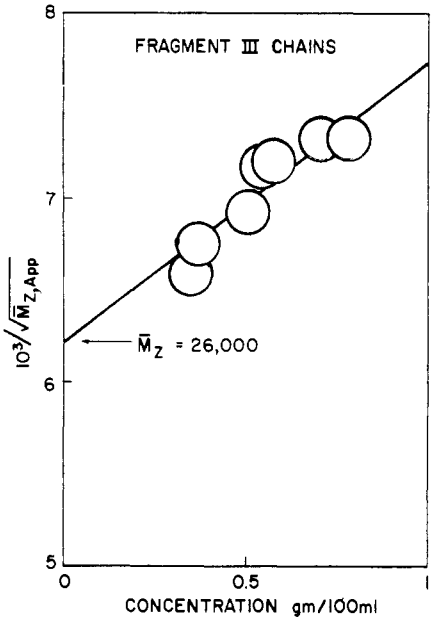


FIG. 6.—Apparent $z$-average molecular weights of reduced and alkylated polypeptide chains of fragment III, in 6 M guanidine hydrochloride, at $p$H 4 and 25°. Schlieren optics were used, and different rotor speeds, ranging from 20,410 to 42,040 rpm, were employed.

with the presence of four chains in the γ-globulin molecule, two with molecular weight 20,000–25,000 and two with molecular weight close to 50,000. Little deviation in the assumed molecular weight of the heavy chain is possible because the value of $\bar{M}_z$ is heavily weighted by the molecular weight of the heaviest component. As a further test of the compatibility of our data with the presence of two light and two heavy chains we have calculated the theoretical solute distribution for molecular weights of 25,000 and 50,000 for the two kinds of chains. This theoretical curve is shown in Figure 4, and is seen to agree closely with the experimental plot through the entire solution column.

The last entry in Table II shows calculated molecular weights based on the presence of six rather than four polypeptide chains. Its most important feature is the low calculated value of $\bar{M}_n$. Other assumed molecular weights for a six-chain (or five-chain) model would lead to a similar result. The experimental data would therefore seem to exclude the possibility that more than four polypeptide chains are present. This conclusion, however, is invalid, because the experiment on which the observed value of $\bar{M}_n$ is based was designed to yield a density of interference fringes optimal for determining the distribution of protein in the molecular weight range 20,000 to 50,000, in the range of concentration of the original protein solution. Polypeptide chains of molecular weight less than 10,000 would be present in smaller concentrations, and would also make much smaller contributions to the fringe displacement per unit concentration. Their presence would thus make a negligible contribution to the fringe pattern from which $\bar{M}_n$ was calculated. In order to test for the presence of small chains it is necessary to use higher rotor speeds and/or longer solution columns (such that heavier species would be forced to the bottom of the solution column, and the optimal concentration distribution in the bulk of the solution column would be obtained with molecular weights in the range 3000–10,000) and also to use higher concentrations because each γ-globulin molecule would

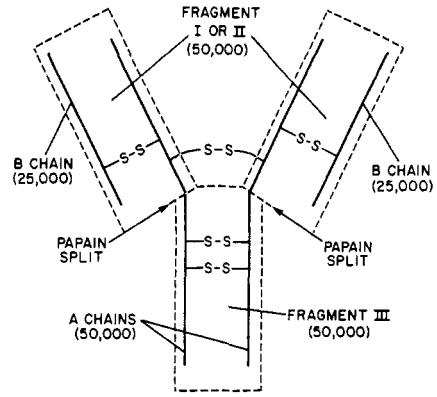


FIG. 7.—The gross structure of the γ-globulin molecule. The figures represent approximate molecular weights. The locations of the five interchain disulfide bonds are taken from Fleischman et al. (1963). According to Porter, there are 16 additional intrachain disulfide bonds.

yield only small amounts of small chains. In addition, the possibility that small chains leak out of the dialysis bag during the dialysis preceding sedimentation equilibrium must be considered.

Additional experiments to test for these possibilities were therefore carried out, and proved entirely negative. A sedimentation equilibrium run was carried out at high speed and high protein concentration on a solution which had not been dialyzed against solvent. Interference optics were used, and no fringe displacement whatever was observed in the upper parts of the solution column. The first detectable displacement occurred where it would be expected for particles with molecular weights in the 25,000–50,000 range. Moreover, prolonged dialysis of a similar solution, with equal volumes of liquid inside and outside the dialysis bag, did not lead to the appearance outside the bag of an appreciable amount of material absorbing ultraviolet light.

Sedimentation equilibrium studies of the polypeptide chains of fragments I and III were carried out with schlieren optics only. Reduced and carboxamidomethylated chains were used. The results are shown in Figures 5 and 6, and yield $z$-average molecular weights of about 25,000 for each fragment. The simplest interpretation of the data is that each fragment consists of two polypeptide chains of molecular weight about 25,000 each. Because of the fact that the $z$-average molecular weight predominantly reflects the properties of the heaviest component of a mixture, the existence of at least one polypeptide chain with molecular weight near 25,000 is established, but the results do not exclude the possibility that fragment I may consist of one chain of molecular weight 25,000–30,000 plus smaller chains. In the case of fragment III such a possibility would seem to be excluded by studies of the tryptic peptides of the fragment which we have carried out in cooperation with Dr. R. L. Hill (Nelson *et al.*, 1963). These studies strongly indicate that fragment III consists of two identical halves. This conclusion, together with the molecular weight of 48,000 for fragment III itself, and the observed $z$-average molecular weight for the polypeptide chains, cannot be compatible with the presence of more than two polypeptide chains.

## DISCUSSION

The conclusions of this paper may be summarized in terms of the gross structure for rabbit γ-globulin and the papain fragments shown in Figure 7. This structure is identical to the structure proposed by Porter (1962) for rabbit γ-globulin and to one of the possible structures suggested by Edelman and Benacerraf (1962) for human γ-globulin. A possibility not excluded by Porter's and Edelman's procedure, that one or two relatively small polypeptide chains might have escaped notice, is eliminated by our work.

The actual molecular weight values we have reported must be considered subject to some uncertainty, partly because of the assumption that γ-globulin, with disulfide bonds intact, and the polypeptide chains, either with free sulfhydryl groups or with carboxamidomethylated sulfhydryl groups, all have the same effective partial specific volume in 6 M guanidine hydrochloride. While it is unlikely that the variation in $\bar{v}$ can be great, it must be noted that a change in $\bar{v}$ of 0.005 cc/g would alter the molecular weights calculated from sedimentation equilibrium data by 3%.

Since this paper was written, two studies, covering essentially the same subject, have been reported by Pain (1963) and by Small *et al.* (1963). Their results agree closely with ours, the only significant discrepancy being in the molecular weight of fragment I, for which Pain obtains a value of 40,700, compared to our value of 52,000 ± 4000. It is noteworthy that the molecular weights of the polypeptide chains were determined by Pain and by Small *et al.* on preparations of the chains which had been separated from each other before ultracentrifugation, whereas the results of this paper were obtained directly from studies of the mixture of chains obtained by reduction of γ-globulin or its fragments.

## REFERENCES

Anson, M. L. (1941), *J. Gen. Physiol. 24*, 399.
Cammack, K. A. (1962), *Nature 194*, 745.
Casassa, E. F., and Eisenberg, H. (1961), *J. Phys. Chem. 65*, 427.
Charlwood, P. A. (1959), *Biochem. J. 73*, 126.
Edelman, G. M., and Benacerraf, B. (1962), *Proc. Nat. Acad. Sci. U. S. 48*, 1035.
Edelman, G. M., and Poulik, M. D. (1961), *J. Exptl. Med. 113*, 861.
Fleischman, J. B., Porter, R. R., and Press, E. M. (1963), *Biochem. J. 88*, 220.
Gorodskaya, O. S. (1950), *Biokhimiya 15*, 507.
Hirs, C. H. W. (1956), *J. Biol. Chem. 219*, 611.
Kielley, W. W., and Harrington, W. F. (1960), *Biochim. Biophys. Acta 41*, 401.
Lansing, W. D., and Kraemer, E. O. (1935), *J. Am. Chem. Soc. 57*, 1369.
Nelson, C. A., Noelken, M. E., Buckley, C. E., III, Tanford, C., and Hill, R. L. (1963), *Fed. Proc. 22*, 657.
Nichol, J. C., and Deutsch, H. F. (1948), *J. Am. Chem. Soc. 70*, 80.
Pain, R. H. (1963), *Biochem. J. 88*, 234.
Palmer, L. F., Mandy, W. J., and Nisonoff, A. (1962), *Proc. Nat. Acad. Sci. U. S. 48*, 49.
Pechère, J. F., Dixon, G. H., Maybury, R. H., and Neurath, H. (1958), *J. Biol. Chem. 233*, 1364.
Porter, R. R. (1959), *Biochem. J. 73*, 119.
Porter, R. R. (1962), *in* Basic Problems in Neoplastic Disease, New York, Columbia University Press, p. 177.
Small, P. A., Jr., Kehn, J. E., and Lamm, M. E. (1963), *Science 142*, 393.
Svedberg, T., and Pedersen, K. O. (1940), *in* The Ultracentrifuge, New York, Oxford University Press, pp. 8–9.
Swan, J. M. (1957), *Nature 180*, 643.
Tanford, C. (1961), Physical Chemistry of Macromolecules, New York, Wiley, p. 382.
van Holde, K. E., and Baldwin, R. L. (1958), *J. Phys. Chem. 62*, 734.
Wales, M., Adler, F. T., and van Holde, K. E. (1951), *J. Phys. Chem. 55*, 145.
Yphantis, D. A. (1960), *Ann. N. Y. Acad. Sci. 88*, 586.