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On the composition of IgG anti-IgG immune complexes

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Summary. The formation of different immune complexes of IgG was followed in a system comprising human IgG as antigen and rabbit IgG directed against the Fc portion of human IgG as antibody. Soluble IgG complexes were analysed by analytical zonal centrifugation. In antigen excess, 16S complexes predominated. 16S complexes are oligomers of IgG, mainly trimers and tetramers. In decreasing antigen excess larger and larger complexes were formed. It was, however, found consistently that oligomers were always formed in the largest amounts. The largest complexes detectable by this method consisted of about twenty IgG molecules. The solubility of different complexes in polyethylene glycol was also studied. Low concentrations of polyethylene glycol preferentially precipitate large complexes. Four and six per cent polyethylene glycol precipitated all types of IgG complexes although not completely. Polyethylene glycol was seemingly not bound directly to soluble immune complexes, but caused otherwise soluble complexes to precipitate by an indirect mechanism.

INTRODUCTION

Immune complexes consisting solely of IgG seem to play a role in the development of rheumatoid arthritis and other immune complex diseases (Kunkel, Müller-Eberhard, Fundeberg & Tomasi, 1961; review by Lambert & Casali, 1978). Complexed IgG is used also as a standard in several clinical methods for the quantification of circulating immune complexes (Lambert *et al.*, 1978). However, knowledge of the ways such complexes are formed and of the outcome of IgG–anti-IgG interactions in general is scarce.

The present paper contains an analysis of soluble complexes formed of human IgG and rabbit IgG directed against the Fc portion of human IgG. The system was standardized by difference turbidity. Complexes formed under defined conditions were analysed by zonal centrifugation. Precipitation of immune complexes with polyethylene glycol is frequently used in methods for detection of immune complexes. The solubility properties of IgG complexes in the presence of polyethylene glycol were therefore also studied, using zonal centrifugation.

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MATERIALS AND METHODS

Antigen and antibody

Purified human IgG was obtained from Miles (code No. 64–145, lot No. 37). It was iodinated with ^{125}I by the chloramine-T method (Hallaba & Drouet, 1971; Jacobsen & Steensgaard, 1979a). Rabbit IgG specific against the Fc portion of human IgG was obtained from Dakopatts (code No. A089, lot No. 028C). It was used without further purification.

Difference turbidimetric measurements

The method has been described previously in detail (Jacobsen & Steensgaard, 1979b). In short, turbidity was measured at 260 nm, using matched tandem quartz cuvettes (Hellma, 238) with unmixed antigen and antibody as blanks, thus eliminating interference due to self-absorption of antigen and antibody. To standardize the system, turbidity precipitin curves were recorded using an α-procedure. In the final reaction mixtures for difference turbidimetric measurements, the antibody concentration was 0·17 mg/ml total IgG. The antigen concentration in the final reaction mixtures was varied from 0·002 to 0·032 mg/ml. At maximum difference turbidity no free antigen was left in the supernatant upon centrifugation. One milligram of the antibody preparation was found to precipitate at least 0·06 mg antigen. All reactions took place in phosphate-buffered saline pH 7·4. $E^{1\%}_{280}$ of both human and rabbit IgG was taken as 14.

Analytical zonal ultracentrifugation

Zonal centrifugations were performed with a B-XIV titanium zonal rotor (Anderson, Waters, Fisher, Cline, Nunley, Elrod & Rankin, 1967) using Kontron and M.S.E. equipment. Gradients were produced by a Kontron gradient former (Steensgaard & Jacobsen, 1979). Sucrose gradients, 5–20% (w/w), in phosphate-buffered saline pH 7·4 were used. In this concentration range the sucrose gradient had isokinetic properties, and the initial gradient shelf could accept up to 100 mg protein (in 2 ml) as sample without showing signs of overloading (Steensgaard, Møller & Funding, 1975). The samples without polyethylene glycol contained less than 1 mg protein in total. One hundred millilitres of phosphate-buffered saline were used as overlay, and the rotor content was displaced by use of a 30% (w/w) sucrose solution. Centrifugations were carried out at 48,000 r.p.m. for 3 h corresponding to a force–time integral of $2{\cdot}9 \times 10^{11}$ rad^2/s. The rotor temperature during centrifugations was 8°.

Equivalent sedimentation coefficients ($S_{20\cdot w}$ values) were calculated by the principles of Martin & Ames (1961) by the computerized method (Steensgaard, Møller & Funding 1978). Seventy-two fractions of 10 ml were collected. Their volumes were determined as the differences between the weights of filled and empty fraction tubes divided by their densities, which in turn was calculated from their sucrose contents. Sucrose concentrations in the individual fractions were measured by refractometry. The calculated sedimentation coefficients corresponded to the last drop of each fraction. Upon calculation, the results were plotted as activity versus sedimentation coefficient. These calculations included correction for sectorial dilution in the zonal rotor during centrifugation. The areas below the sedimentation patterns shown in Figs 3 and 4 are thus directly proportional to the amount of substance included.

Samples for zonal centrifugation

Antigen excess samples were prepared by mixing a fixed amount of radioactive antigen with increasing amounts of antibody. All samples contained 170 ng human IgG as the antigen. On a weight basis the antigen/antibody ratios in the samples were 9·9, 6·7, 5·0, 3·4, 2·5, 2·0. The samples were incubated at 37° for 1 h and left overnight at 4°. The samples were mixed gently before being introduced into the rotor.

Polyethylene glycol precipitation

From pilot experiments a sample containing 5 ng human IgG (labelled with ^{125}I) and 34 ng rabbit anti-IgG was selected because of its rather large content of different soluble immune complexes. Polyethylene glycol (PEG, Fluka, 81300, mol wt approx 20,000) was added from a 25% solution to give final concentrations of 0, 2, 4 and 6%. PEG was added after incubation at 37°, but before incubation at 4°. Otherwise these samples were treated as described in the previous sections.

Calculations of complex size from their equivalent sedimentation coefficient

The sedimentation coefficient of a complex is a function of its size, its density (or its partial specific volume) and its shape. A graphical method for studying the impacts of these parameters has been given previously (Jacobsen & Steensgaard, 1979a). The present approach is based on the assumption that the partial specific volume of IgG and IgG complexes is 0·74. Thus

$$S_{20\cdot w} = M^{2/3}\left(\frac{1-\bar{v}\rho}{\sqrt[3]{\bar{v}}}\right) \Big/ \left[(\quad 6\pi\eta^3\sqrt{0{\cdot}75/(\quad\pi)}\,)\left(\frac{f}{f_0}\right)\right] \quad (1)$$

where $\bar{v}$ is the partial specific volume, ρ the density of the media, $\mathcal{N}$ Avogadro's number, f/f_0 the frictional ratio and η the viscosity (Tanford, 1971; Jacobsen & Steensgaard, 1979a). Insertion of constants lead to

$$S_{20\cdot w} = M^{2/3}\, 3{\cdot}43 \times 10^{-3} \Big/ \left(\frac{f}{f_0}\right) \quad (2)$$

We have assigned a molecular weight of 150,000 to both human and rabbit IgG allowing us to calculate approximate sedimentation coefficients for IgG complexes for values of f/f_0 from 1·3 to 1·7. The composition scales in Figs 3 and 4 are calculated directly from formula (2).

RESULTS

Characterization of reaction mixtures

Difference turbidity precipitin curves were recorded to titrate antigen against antibody. A difference turbidity precipitin curve is shown in Fig. 1. The curve reveals a clearly expressed zoning phenomenon. It should be noted that the maximum difference turbidity signal found here on a molar basis corresponds to what has been found previously for the reaction between human serum albumin and rabbit anti-human serum albumin IgG (Jacobsen & Steensgaard, 1979b).

Zonal centrifugations

From the difference turbidimetric measurements six samples were prepared with antigen/antibody ratios from 10 to 2. All samples contained the same amount of radioactively labelled antigen whereas the antibody concentration was varied accordingly. Figure 2 shows the directly measured effluent profile from the zonal rotor with all six experiments superimposed. The highest curve shows the mixture with the highest antigen/antibody ratio, and the curves below have decreasing antigen/antibody ratios. The first peak in all experiments contains unreacted antigen (labelled human IgG), whereas the second peak with its tailing end contains immune complexes. Two features are especially worthy of note. First, in decreasing antigen excess the total amount of soluble complexes seemed to decrease. Second, in the most extreme antigen excess example the complexes had a limited size. When the antigen/antibody ratio was lowered, however, the complexes became larger and sedimented further out in the gradient. The decrease in total amount of soluble complexes with decreasing antigen/antibody ratios with a constant initial amount of antigen reflected directly that more and more precipitate was formed as equivalence was approached. The figures show exclusively the complexes that retained their solubility when centrifuged.

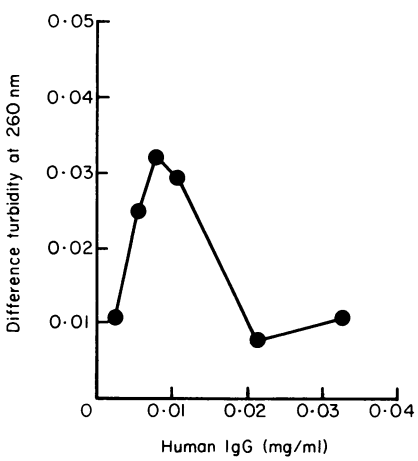

Figure 1. Difference turbidity precipitin curve of the reaction between human IgG and rabbit IgG against the Fc portion of human IgG. The antibody concentration was 0·17 mg/ml in the final mixtures. The antigen concentration is given on the abscissa.

In order to get an assessment of the molecular sizes of the complexes that were formed under the conditions described here, sedimentation coefficients were calculated and the results were converted to the plots shown in Fig. 3. These diagrams show the distribution of complexes along a sedimentation coefficient axis so that the height gives the relative amount of material corresponding to its size as defined by its sedimentation coefficient. Below each pair of figures a scale is given that allows an assessment of the composition of the complexes for a given sedimentation coefficient. In the highest antigen excess example, Fig. 3a, the complexes have sedimentation coefficients below 30S, and a substantial amount of unreacted antigen is present. Comparison with the composition scale indicates that most of these complexes are dimers, trimers and tetramers of IgG and larger complexes are formed only in very small amounts. In the next example (lower antigen excess, Fig. 3b) less free antigen is present and a characteristic development in the direction of formation of larger complexes can be seen. Complexes up to a size of 40S can now be detected. Still most of the complexes are likely to be oligomers of IgG, and with increasing size the amount of complexes decreases. With further decreasing antigen excess, Fig. 3c, the peak of free and unreacted antigen becomes lower than the highest complex peak. Complexes up to about 50S are detectable. Again the peak containing the smallest complexes dominates clearly and larger complexes are formed in decreasing amounts. In the last experiments, Fig. 3d, with the lowest antigen excess

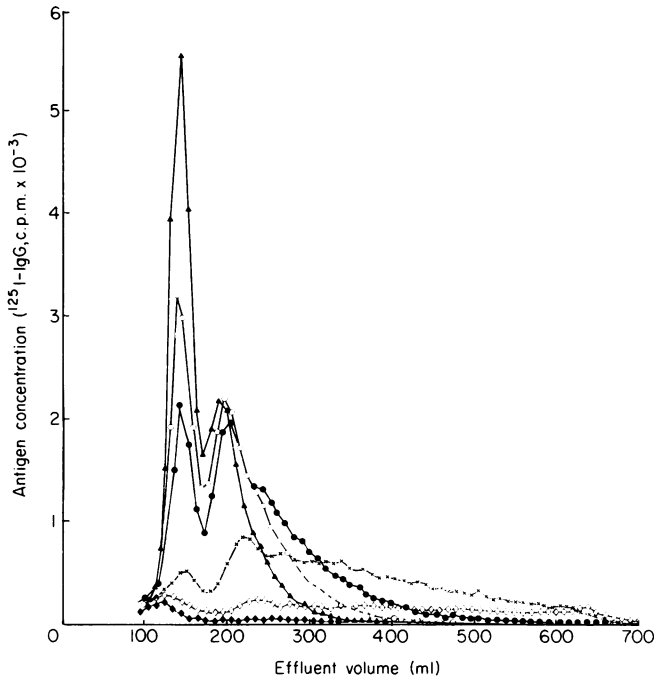

Figure 2. Direct effluent patterns of zonal centrifugation of six different mixtures of IgG complexes, using a constant amount of radioactively labelled antigen and increasing amounts of antibody. The total Ag/Ab ratios were ▲, 9·9; ○, 6·7; ●, 5·0; ×, 3·4; ◊, 2·5; and ♦, 2·0, respectively.

where this analysis is possible, complexes larger than 60S can be seen. Although the peak of the small complexes is still highest, it is clear that larger complexes dominate the complex pattern. The large complexes may reach a size that corresponds to a content of about 20 IgG molecules. It is a consistent finding, however, that the amounts of complexes decrease drastically with increasing complex size.

Polyethylene glycol precipitation of soluble IgG complexes

Polyethylene glycol is known to be able to precipitate certain soluble immune complexes (Zubler, Perrin, Creighton & Lambert, 1977) and PEG precipitation of soluble immune complexes is used in some methods for detection of immune complexes (Lambert *et al.*, 1978). To see which complexes are soluble in the presence of polyethylene glycol, increasing concentrations of polyethylene glycol were added to a standard immune complex sample and upon incubation at 4° overnight the entire samples were subjected to zonal centrifugation. The results are shown in Fig. 4. The composition of the standard sample is shown as the first part of Fig. 4a. The highest value in Fig. 4a was set to 100% and the other part figures are drawn relative to this. Increasing concentrations of polyethylene glycol clearly decrease the amount of soluble complexes. The largest complexes seem to precipitate nearly completely in the presence of PEG, but it is characteristic that a very large proportion of the smallest complexes also disappear. The amount of immune complexes precipitated by polyethylene glycol seems to be strongly correleted with the concentration of polyethylene glycol used.

DISCUSSION

The antigen–antibody reaction between human IgG and rabbit anti-Fc IgG was quantified by difference turbidimetry which we have used previously to characterize the reaction between another protein antigen (human serum albumin) and the corresponding rabbit

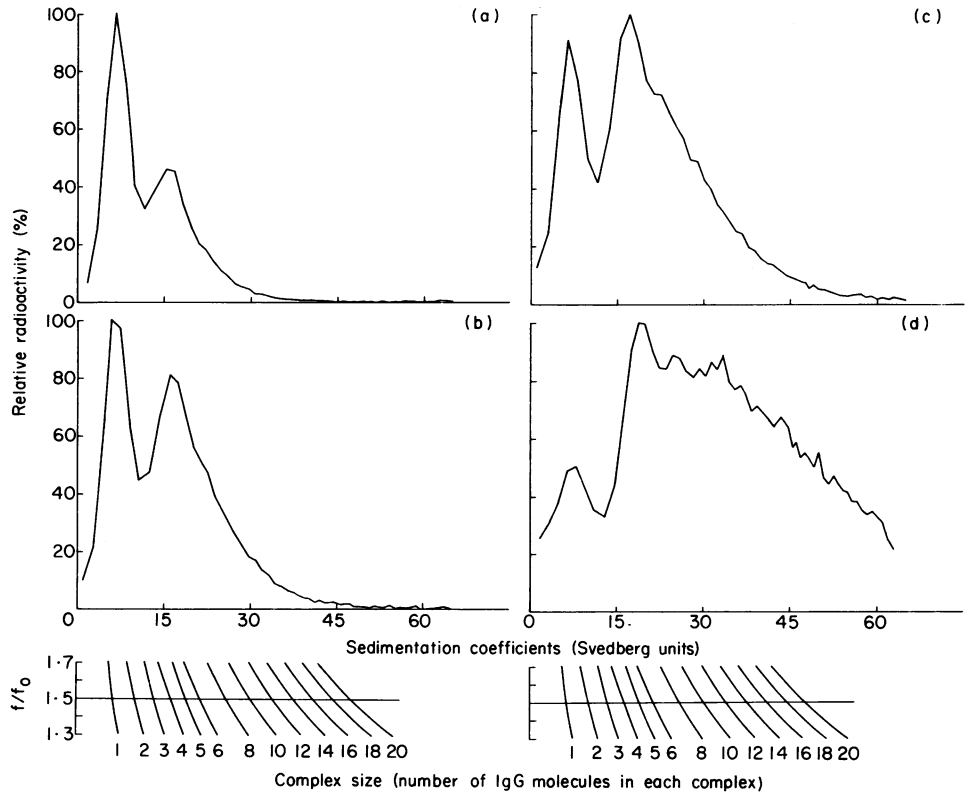

Figure 3. Computer-processed results of zonal centrifugation of antigen-excess IgG complexes. The abscissa gives the equivalent sedimentation coefficient ($S_{20,w}$ values in Svedberg units). The ordinate in each part figure gives the amount of antigen (determined from its radioactivity) as a percent of the maximum. The composition scale at the bottom gives the calculated position of complexes of a known size for different frictional ratios. (a) corresponds to the curve denoted with ▲ in Fig. 2; (b) corresponds to o in Fig. 2; (c) to ● in Fig. 2; and (d) to × in Fig. 2. The numbers on the composition scale are the number of IgG molecules in a complex.

antibodies. In the present case this method also provides a clear zoning phenomenon that is suitable for characterizing the system.

At maximum turbidity, 1 mg antibody precipitated at least 0·06 mg antigen suggesting that the preparation of rabbit IgG contains substantial amounts of IgG not directed against the present antigen. The antigen, in contrast, could be completely precipitated as seen in Fig. 2, and the whole amount is supposed to be reactive in the present system. It was therefore decided to use labelled antigen alone, as labelled antibody containing larger amounts of unreactive IgG molecules would obscure the outcome of the centrifugal experiments.

The general sedimentation pattern of immune complexes of IgG and IgG anti-IgG is illustrated in Fig. 2. The typical feature is that the smallest complexes are formed in highest antigen excess and lowering the antigen excess means that larger and larger complexes are formed. Yet it is also typical that larger complexes are formed only in the presence of even greater amounts of smaller complexes.

The computed composition scale in Figs 3 and 4 is based on the assumption that the partial specific volume of IgG complexes is 0·74 and that the particle density is independent of the complex composition. It is conceivable that the complex particle density increases with increasing size due to liberation of surface-bound water molecules in the processes of complex formation. This implies that the partial specific volumes would decrease, and that the complexes would sediment faster than predicted by the present

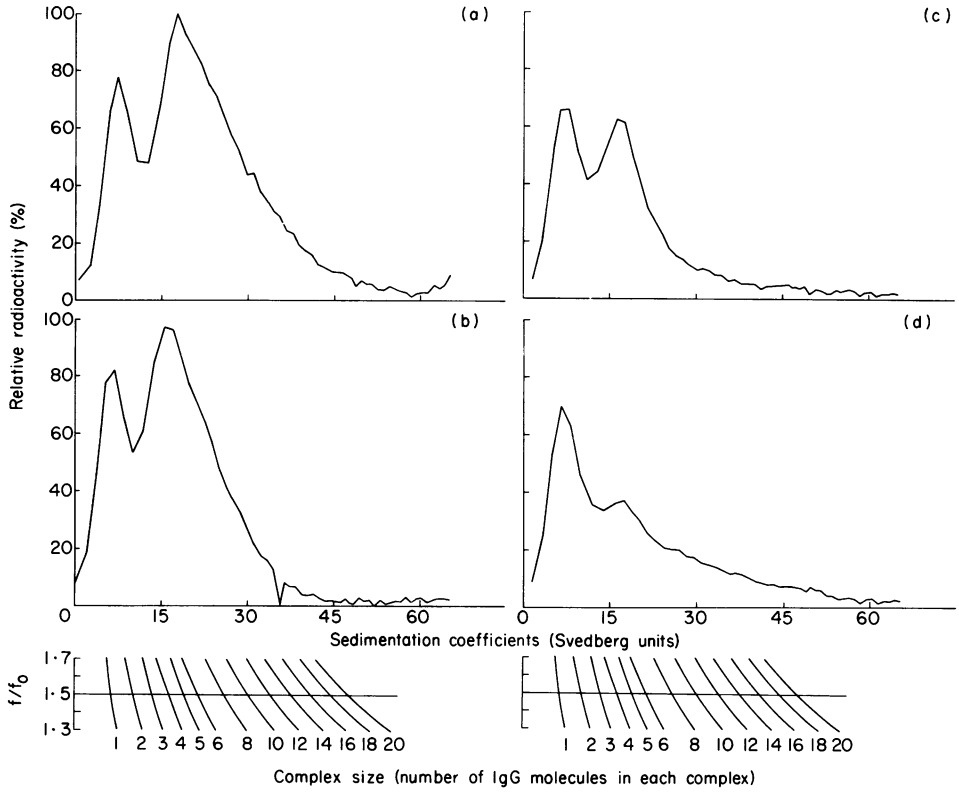

Figure 4. Computer-processed results of zonal centrifugations of IgG immune complexes after incubation with increasing concentrations of polyethylene glycol. (a) the result of zonal centrifugation of the standard sample of IgG complexes without addition of polyethylene glycol. (b) The result of zonal centrifugation of the sample after incubation with 2% polyethylene glycol. (c and d) The same after incubation with 4 and 6% polyethylene glycol, respectively. The highest value in (a) was set equal to 100% and other values (on the ordinate) in the other figures are relative to that.

theoretical treatment. Thus the composition scales shown below Figs 3 and 4 give the maximum sizes of complexes.

Figure 3 gives a rather clear-cut picture of the immune specific complex formation between human IgG and rabbit anti-human-IgG IgG. This system is thought of as a model of human IgG–anti–IgG reactions as seen in certain stages of rheumatoid arthritis and in other immune complex diseases. It appears that very small complexes are formed exclusively in extreme antigen excess, and that larger and larger complexes are formed when the antigen excess is lowered. The 16S peak dominating in all four examples is likely to consist mainly of IgG_3 ($=Ag_2Ab$) IgG_4 ($=Ag_2Ab_2$) and other small complexes. These small complexes are probably of the same composition as the so-called intermediate size immune complexes (smaller than 20S) originally described by Kunkel *et al.*,(1961) and recently analysed by zonal centrifugation by Møller, Kallerup & Linnet (1979). An interesting feature to note is that although such small complexes are present in all the examples presented here, they are present exclusively in the highest antigen excess example only (Fig. 3a). It is of special interest that dimeric IgG complexes appear only in small quantities in these experiments. In the studies of Pope, Teller & Mannik (1974; 1975) IgG_2 was the clearly dominating complex made of self-associating IgG rheumatoid factor. The present model system (employing rabbit IgG against human IgG) is not self-associating. The tendency to form dimeric complexes may therefore be a special feature of self-asso-

ciating IgGs, and the presence of dimers with sedimentation coefficients near 10S may be indicative of a self-associating system.

The analyses of complex mixtures in decreasing antigen excess show that very large complexes of human IgG and rabbit anti-IgG can be formed. The lowest antigen excess example revealed complexes which may contain more than 20 IgG molecules in appreciable amounts. Because they always exist together with smaller complexes and because they are formed in an antigen excess mixture, we think the largest complexes have a composition that can be described as Ag_8Ab_7 (IgG_{15}), Ag_9 Ab_8 (IgG_{17}), $Ag_{10}Ab_9$ (IgG_{19}) and so forth. Pure IgG complexes of this size have to our knowledge not been demonstrated in serum, but it is interesting that polymerized IgG used as standards for quantification of soluble immune complexes in some circumstances may contain such very large complexes (Johns & Stanworth, 1976; Stanworth & Johns, 1977).

Previous ultracentrifugal analyses of antigen–antibody complexes have been done mostly on complexes consisting of albumin and rabbit anti-albumin IgG (Singer, 1957; Steensgaard & Funding, 1974; Møller & Steensgaard, 1979; Jacobsen & Steensgaard, 1979a). Albumin has a lower molecular weight than has human IgG and the complexes studied previously have accordingly a smaller size. IgG complexes, however, are even somewhat larger than the corresponding albumin complexes on a compositional basis. Moreover, the clear shift in the direction of formation of large complexes as can be seen in Fig. 3, was not observed for the albumin–anti-albumin IgG system.

Polyethylene glycol is known to be able to precipitate certain immune complexes (Zubler *et al.*, 1977). To get an analysis of which of the many possible immune complexes are precipitated by polyethylene glycol, a standard sample was added increasing amounts of polyethylene glycol, and the remaining soluble complexes were analysed by the zonal centrifugation method. It appears that polyethylene glycol in low concentrations preferably precipitates the largest complexes. Larger concentrations of polyethylene glycol (4 and 6%) precipitate all types of complexes, although not completely. Polyethylene glycol precipitates small amounts of uncomplexed IgG, as judged from the peak of free and unreacted radioactive human IgG. It is interesting that addition of polyethylene glycol does not affect the average complex size suggesting that the soluble complexes have not absorbed significant amounts of polyethylene glycol themselves. Instead, we believe that polyethylene glycol indirectly favours an inherent tendency of the complexes to aggregate non-specifically, and that it creates a medium that favours the inherent tendency of immune complexes to precipitate by the same mechanisms as in precipitin reactions (Jacobsen & Steensgaard, 1979a; Steensgaard & Frich, 1979).

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REFERENCES

ANDERSON N.G., WATERS D.A., FISHER W.D., CLINE G.B., NUNLEY C.E., ELROD L.H. & RANKIN C.T., JR (1967) Analytical techniques for cell fractions. V. Characteristics of B-XIV and B-XV zonal centrifuge rotors. *Anal. Biochem.* **21,** 235.

HALLABA E. & DROUET J. (1971) ^{131}I-Labelling of human serum albumin by the iodine monochloride and chloramine-T methods. *Int. J. appl. Radiat. Isotopes,* **22,** 46.

JACOBSEN C. & STEENSGAARD J. (1979a) Evidence of a two stage nature of precipitin reactions. *Molecular Immunol.* **16,** 571.

JACOBSEN C. & STEENSGAARD J. (1979b) Measurements of precipitin reactions by difference turbidimetry: a new method. *Immunology,* **36,** 293.

JOHNS P. & STANWORTH D.R. (1976) A simple numerical method for the construction of isokinetic sucrose density gradients, and their application to the characterisation of immunoglobulin complexes. *J. immunol. Meth.* **10,** 231.

KUNKEL H.G., MÜLLER-EBERHARD H.J., FUDENBERG H.M. & TOMASI T.B. (1961) Gammaglobulin complexes in rheumatoid arthritis and certain other conditions. *J. clin. Invest.* **40,** 117.

LAMBERT P.-H. & CASALI P. (1978) Immune complexes and the rheumatic diseases. *Clin. rheumatic Dis.* **4,** 617.

LAMBERT P.-H. *et al.* (1978) A WHO collaborative study for the evaluation of eighteen methods for detecting immune complexes in serum. *J. clin. lab. Immunol.* **1,** 1.

MARTIN R.G. & AMES B.N. (1961 A method for determining the sedimentation behaviour of enzymes: application to protein mixtures. *J. biol. Chem.* **236,** 1372.

MØLLER N.P.H., KALLERUP H.E. & LINNET L. (1979) Demonstration and characterization of immune complexes formed *in vitro* and in patients with rheumatoid arthritis: sucrose density gradient ultracentrifugation. *XXVII. Annual Colloquium, Protides of the Biological Fluids* (Ed. by H. Peteers). (In press.)

MØLLER N.P.H. & STEENSGAARD J. (1979) Fc-mediated immune precipitation. II. Analysis of precipitating immune complexes by rate-zonal ultracentrifugation. *Immunology*. **38,** 641.

POPE R.M., TELLER D.C. & MANNIK M. (1974) The molecular basis of self-association of antibodies to IgG (rheumatoid-factors) in rheumatoid arthritis (IgG-rheumatoid factor/ultracentrifugal analysis). *Proc. nath. Acad. Sci.* (U.S.A.), **71,** 517.

POPE R.M., TELLER D.C. & MANNIK M. (1975) The molecular basis of self-association of IgG-rheumatoid factors. *J. Immunol.* **115,** 365.

SINGER S.J. (1957) Physical-chemical studies on the nature of antigen–antibody reactions. *J. Cell. comp. Physiol.* **50,** Suppl. 1, 51.

STANWORTH D.R. & JOHNS P. (1977) Ultracentrifugal methods of detecting soluble immune complexes. *Am. rheum. Dis.* Suppl. 1, 12.

STEENSGAARD J. & FUNDING L. (1974) On the formulation of a reaction scheme for the interaction between an antigen and its antibody. *Immunology*, **26,** 299.

STEENSGAARD J., MØLLER N.P.H. & FUNDING L. (1975) The effects of overloading in density-gradient centrifugation. *Europ. J. Biochem.* **51,** 483.

STEENSGAARD J., MØLLER N.P.H. & FUNDING L. (1978) Rate-zonal centrifugation: quantitative aspects. In *Centrifugal Separations in Molecular and Cell Biology* (Ed. by G. D. Birnie and D. Rickwood), p. 115. Butterworth, London.

STEENSGAARD J. & FRICH J.R. (1979) A theoretical approach to precipitin reactions. Insight from computer simulation. *Immunology*, **36,** 279.

STEENSGAARD J. & JACOBSEN C. (1979) A new gradient former and a simplified procedure for zonal centrifugation of immune complexes. *J. immunol. Meth.* (In press.)

TANFORD C. (1961) *Physical Chemistry of Macromolecules*, p. 317. John Wiley & Sons, New York.

ZUBLER R.H., PERRIN L.H., CREIGHTON W.D. & LAMBERT P.-H. (1977) The use of polyethylene glycol (PEG) to concentrate immune complexes from serum of plasma samples. *Ann. rheum. Dis.* **36,** Suppl. 1, 23.