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The Reactivity of Rheumatoid Factor with Human Gamma G Globulin

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Summary. The precipitation reaction between denatured human γG globulin and rheumatoid arthritic serum has been employed in quantitative form to provide further indication of the antibody nature of rheumatoid factor.

In addition, ultracentrifugal and optical rotation studies have revealed that the degree of reactivity of the γG globulin in such a system depends on its extent of denaturation irrespective of the type of denaturing agent (heat, alkali, detergent) employed.

Evidence is presented to suggest that denaturation processes responsible for effecting the precipitation reactivity of human γG globulin (with rheumatoid factor) bring about the rupture of inter-chain disulphide bonds in the 'fast' papain digestion (i.e. Fc) parts of the molecules and these take part in intermolecular bridging, leading to aggregate formation and probably to the exposure of new chemical groupings.

INTRODUCTION

Although the precipitation of aggregated human γG* globulin (previously termed 7Sγ) by rheumatoid arthritic sera is readily demonstrable, relatively little is known about the mechanism of the reaction involved. Are for instance the γ-globulin aggregates effective merely on account of their size, rendering visible the reaction between rheumatoid factor and the non-reactive (in the precipitation system) monomeric γG globulin (Edelman, Kunkel and Franklin, 1958)? Alternatively, does the aggregation of γG globulin lead to the unmasking of specific groups which are unavailable in the native protein for combination with rheumatoid factor (as is suggested by Glynn, Holborow and Johnson, 1957, and by others)? Moreover, is the antibody nature of rheumatoid factor now wholly acceptable, or can the counter claims of Aho (1961) (discussed in some detail by Glynn, 1963) be further substantiated?

The work to be described was undertaken in an attempt to provide further evidence in answer to these and other questions. By quantitative as well as qualitative investigations of the tertiary structural changes induced in the human γG globulin molecule, in rendering it precipitable by rheumatoid factor, it was hoped to learn more about the mode of combination of the reactants (and possibly, ultimately, more about the nature of the rheumatoid factor molecule itself).

The results of investigations of certain aspects of this work, involving a study of the reaction of rheumatoid factor with isolated polypeptide chains (in aggregate form) of

* The nomenclature adopted throughout is that of the *Bulletin of the World Health Organization* (1964) **30**, 447–450.

human γG globulin (Henney and Stanworth, 1964a) and with aggregated whole γG globulins of various animal species (Henney and Stanworth, 1964b) have been reported already.

The present report also includes the results of a quantitative comparison of the characteristics of the aggregated γG globulin–rheumatoid factor system with a conventional precipitating antigen–antibody (human γG globulin–homologous rabbit antibody) system.

MATERIALS AND METHODS

MATERIALS

1. *Human γG globulin.* Preparations were obtained from both individual and pooled normal human sera by 'batch' chromatography on diethylaminoethyl (DEAE) cellulose (Stanworth, 1961). The homogeneity of each sample was demonstrated by immunoelectrophoresis and gel diffusion precipitin analysis (employing a rabbit antiserum raised against whole human serum) and by analytical ultracentrifugation.
2. *Antisera* were raised in rabbits, according to the method described by Gell (1957).
3. *Rheumatoid arthritic sera.* Sera showing Rose-Waaler titres of not less than 1/1024 were separated from the blood of several patients with typical rheumatoid arthritis. The blood was incubated at 37° for 20 minutes, followed by storage for 2 hours at 4° to facilitate clot contraction. The separated sera were finally stored at −20° until used.
4. *Rheumatoid factor-rich preparations.* These were obtained by preliminary euglobulin precipitation followed by zone-centrifugation in a buffered sucrose gradient (as described by James, Felix-Davies and Stanworth, 1961).

METHODS

Denaturation of γG globulin

(a) *Denaturation by heat.* 1 g per cent (w/v) solutions of γG globulin (stored at −20° until use) were heated in a thermostatically controlled water bath at various temperatures (between 20° and 65°) for 20 minutes and then cooled rapidly back to room temperature.

(b) *Denaturation by NaOH.* Various amounts of 2 N NaOH solution were added to 1 g per cent (w/v) solutions of γG globulin to give final NaOH molarities ranging from 0·01–0·10 mmole/mg protein N. After incubation at 37° for 20 minutes the pH of the solutions was readjusted to 7·5 by dialysis (with stirring, at room temperature) against 0·05 M glycine–NaOH buffer.

(c) *Denaturation by sodium dodecyl sulphate.* Aliquots (2 ml) of 2 g per cent (w/v) γG globulin solution were added to equal volumes of sodium dodecyl sulphate (SDS) solution in 0·2 M phosphate buffer (pH 7·5) to give final detergent concentrations ranging from 0·0005 to 0·1000 M. The mixtures were incubated at 37° for 2 hours and then dialysed exhaustively at room temperature against 0·2 M phosphate buffer (pH 7·5).

Ultracentrifugal analysis

Measurements were carried out in a Spinco Model E machine, incorporating a Schlieren optical system. Both standard and wedge 12 mm cells were employed, runs being performed at 20° and 59,780 rev/min.

Optical rotatory dispersion measurements

The optical activity of approximately 1 g per cent (w/v) solutions of native and treated

γ-globulin solutions was measured over the range 300–600 mμ in a 0·5 dm cell, using a Bellingham and Stanley 'Pepol' 60 Spectropolarimeter. The measurements on the altered γG globulin samples were made immediately after completion of the treatment.

The specific optical rotation of the solutions at each wavelength $[\alpha]_\lambda$ was determined and the dispersion constant (λ_o) calculated by the method of Yang and Doty, i.e. from the slope of the curve obtained by plotting $[\alpha]_\lambda \lambda^2$ against $[\alpha]_\lambda$.

Estimation of sulphydryl groups

Sulphydryl groups were estimated by the colorimetric method of Jocelyn (1962) employing di-(5-carboxy-4-nitrophenyl) disulphide.

Precipitability by rheumatoid factor

Aliquots of 0·5 ml of 1 g per cent (w/v) altered γG globulin solutions were added to 0·3 ml portions of rheumatoid arthritic serum and the reaction volume adjusted to 5 ml with 0·15 M saline. After standing for 24 hours at 4°, the precipitate was separated by centrifugation, washed twice with cold 0·15 M saline, and dissolved in 1 ml 0·1 N NaOH solution. The protein content of the solution was estimated by a modified Folin procedure (Lowry, Rosebrough, Lewis Farr and Randall, 1951).

Measurement of antigen–antibody precipitation

A similar procedure to that described above was adopted. Quantitative precipitin titrations were performed by adding varying amounts of antigen (i.e. native human γG globulin) to a constant amount of antibody (i.e. 0·2 ml of specific rabbit antiserum to human γG globulin). After standing at 4° for 24 hours, the precipitate was separated by centrifugation, washed twice with cooled 0·15 M saline and dissolved in 1 ml 0·1 N NaOH solution. The protein content was again estimated by the modified Folin procedure of Lowry *et al.* (1951).

RESULTS

A. COMPARISON OF PRECIPITATION CHARACTERISTICS OF ALTERED HUMAN γG GLOBULIN–RHEUMATOID FACTOR AND NATIVE HUMAN γG GLOBULIN–HOMOLOGOUS RABBIT ANTIBODY SYSTEMS

The results of optimal proportion titration of the two systems are compared in Fig. 1. As will be observed, the quantitative precipitin curves are of very similar form. As in the conventional precipitin system (Fig. 1b), excess of 'antigen' (i.e. heat-treated γG globulin) leads to a decrease in the amount of precipitate obtained with rheumatoid factor (Fig. 1a).

In the conventional system this can be attributed to the formation of soluble complexes between human γG globulin and its rabbit antibody; in contrast, however, the comparable effect observed in the altered human γG globulin–rheumatoid factor system appears to be due to inhibition by unaltered γG globulin present in the system (Christian, 1958). Use of aggregated γG globulin which had been freed of native γG globulin by selective sodium sulphate precipitation gave the modified precipitin curve shown in Fig. 1(a). As will be seen, the curve flattened out at the higher 'antigen' concentrations but no decrease in precipitate formation was observed.

The effect of pH and time of incubation on the two precipitating systems was also examined. Antigen (0·2 ml) and antibody (0·3 ml) were mixed in the proportions shown (in Fig. 1) to produce maximum precipitation.

In one series, the pH of the mixture was adjusted to various values between pH 2 and pH 12. This was accomplished by addition of 4·5 ml of 0·15 M buffer of the appropriate

pH, followed by dialysis at 4° against about 500 ml of the same buffer. The amount of precipitate formed after keeping the mixtures at 4° for a further 24 hours were measured as already described. The variation of precipitation with pH in the two systems is compared in Fig. 2. The pH of optimum precipitation was similar, being in the region of 7–8.

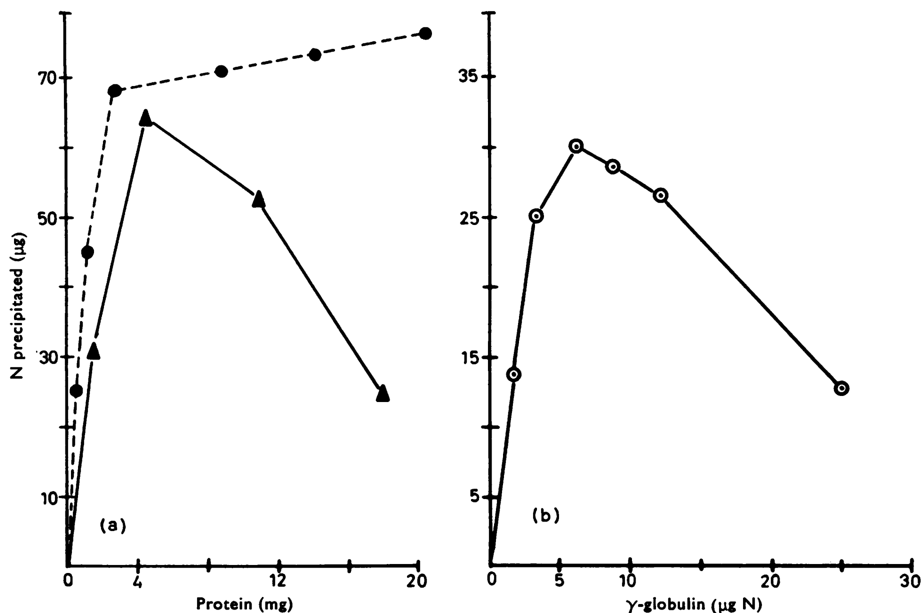

FIG. 1. The effect of antigen concentration on precipitate formation. (a) ▲, Whole heated (63° for 20 minutes) human γG globulin–rheumatoid serum; ●, heated γG globulin fraction (isolated by Na_2SO_4 precipitation)–rheumatoid serum. (b) Native human γG globulin–homologous rabbit antibody.

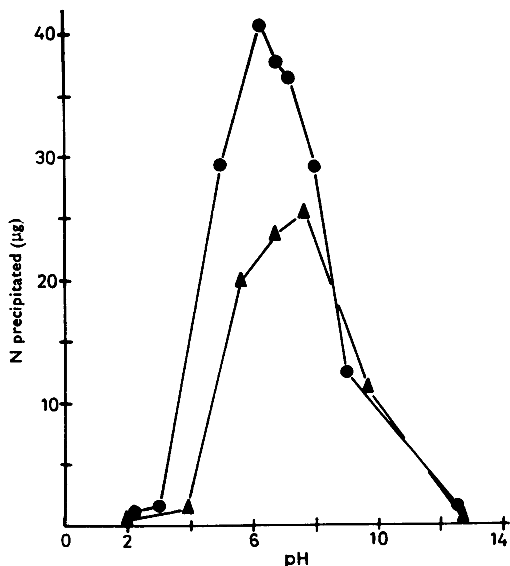

FIG. 2. The effect of pH on precipitate formation: ●, human γG globulin–homologous rabbit antibody; ▲, heat aggregated human γG globulin–rheumatoid serum.

In the other series, involving the variation of time of incubation at 37°, samples were withdrawn at various time intervals and the amount of precipitate measured. The results are illustrated in Fig. 3. As will be seen, the maximum precipitation was achieved in 20–30 minutes in both systems, again underlining their similarity.

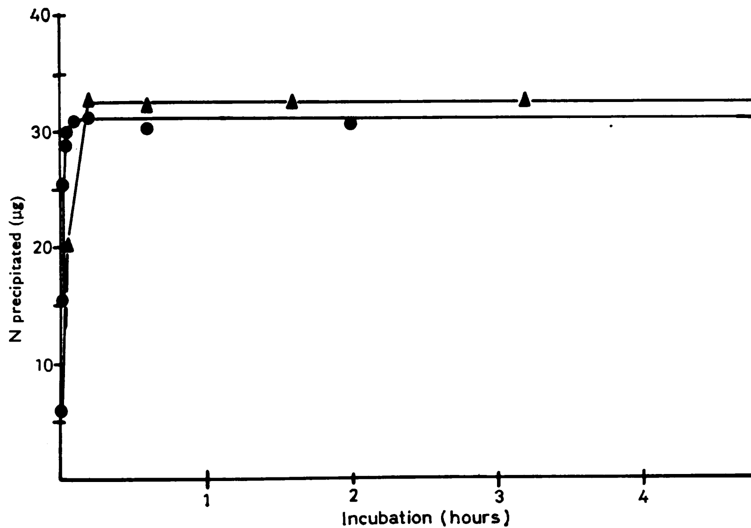

Fig. 3. Comparison of rate of formation of precipitate at 37° of: ●, human γG globulin–homologous rabbit antibody. ▲, heat aggregated human γG globulin–rheumatoid serum.

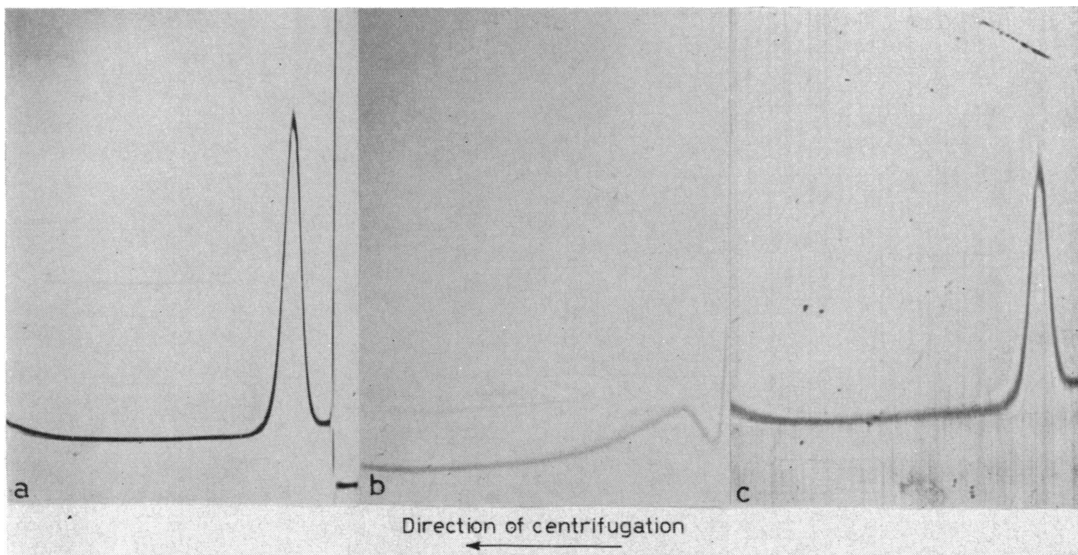

Fig. 4. The effect of parachloromercuribenzoate (PCMB) on aggregate formation of human γG globulin. (a) Native γG globulin; (b) γG globulin heated at 63° for 20 minutes; (c) γG globulin heated at 63° for 20 minutes in the presence of PCMB. Ultracentrifugation at 59,780 rev/min and 20° for (a) 24 minutes, (b) 10 minutes, and (c) 24 minutes.

B. NATURE OF ALTERATION OF HUMAN γG GLOBULIN IN RELATION TO ITS REACTIVITY WITH RHEUMATOID FACTOR

Ultracentrifugal analysis of human γG globulin which had been heated at 63° for 20 minutes demonstrated the formation of about 15 per cent of material sedimenting in the range of 20–40S (Fig. 4b). It was confirmed by selective sodium sulphate precipitation (Christian, 1958) and by zone centrifugation, that these aggregates were responsible for the precipitation of rheumatoid factor by the heat-treated γG globulin, the unchanged γG globulin failing to react.

The formation of aggregates by heat-treatment of γG globulin could be prevented by the presence of para-chloromercuribenzoate (PCMB) which is known to block sulphydryl groups (as is demonstrated by the ultracentrifugal patterns shown in Fig. 4). Conversely, it could be shown that SH groups were revealed as a result of the heat treatment in the absence of PCMB. The results obtained from measurement of free SH groups following the heating of γG globulin at various temperatures is shown in Table 1, where the reactivity with a standard volume (0·1 ml) of rheumatoid arthritic serum is included for comparison. As will be observed, an increase in the amount of free SH groups is accompanied by an increase in precipitability with rheumatoid factor. It was shown, however, that the SH groups *per se* were not essential for reactivity as treatment of the γ-globulin with SH blockers (e.g. PCMB, iodoacetate, iodoacetamide, N-ethylmaleimide, etc.) *after heating* had no effect on its ability to precipitate rheumatoid factor.

TABLE 1

Temperature of heating γ-globulin for 20 minutes	SH groups/mol γ-globulin (mol wt γ = 160,000)	μg N ppt. with std. 0·1 ml vol. rheumatoid serum
Room temp. 18°	Non-detectable	0·8
37°	Non-detectable	2·0
45°	0·1	2·8
52°	0·15	4·0
59°	0·30	6·8
63°	0·50	9·2

Changes induced in human γG globulin in rendering it reactive with rheumatoid factor were also followed by optical rotatory dispersion measurements. The effect of the various denaturation procedures on the optical rotatory dispersion of human γG globulin is shown in Fig. 5. As will be seen, irrespective of the method of denaturation the λ_0 value increased from approximately 200 mμ in the native state to approximately 220 mμ in the completely denatured state. The curves showed the same general form. Heat treatment (for 20 minutes) produced little change until the temperature rose above 45° (Fig. 5a), after which the λ_0 value rose steeply to a maximum at 63°. It was impossible to observe changes above this temperature on account of precipitation of the protein. Relatively small amounts of sodium hydroxide (Fig. 5b) and sodium dodecyl sulphate (Fig. 5c) effected comparable increases in λ_0.

The relationship between λ_0 and precipitability with rheumatoid factor is shown in Fig. 6. An approximately linear relationship was observed with both heat-treated (Fig. 6a) and alkali-treated (Fig. 6b) γG globulin. Preparations showing the highest λ_0 values (of

the order of 220 mμ) produced the largest amount of precipitate with a standard volume (0·3 ml) of rheumatoid arthritic serum. As in the case of the heat-treated γG globulin, alkali-treated preparations were shown by ultracentrifugation to contain aggregated material of similar sedimentation coefficient (i.e. 20–40S).

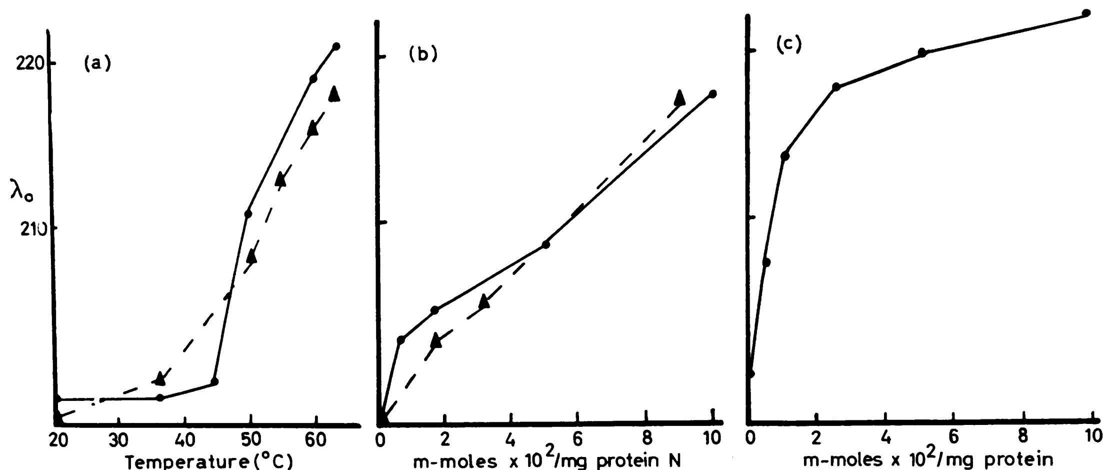

Fig. 5. The effect of various forms of denaturation on the rotatory dispersion constant (λ_o) of two preparations (▲ and ●) of human γG globulin. (a) The effect of incubation at various temperatures; (b) treatment with various concentrations of NaOH; (c) treatment with various concentrations of SDS.

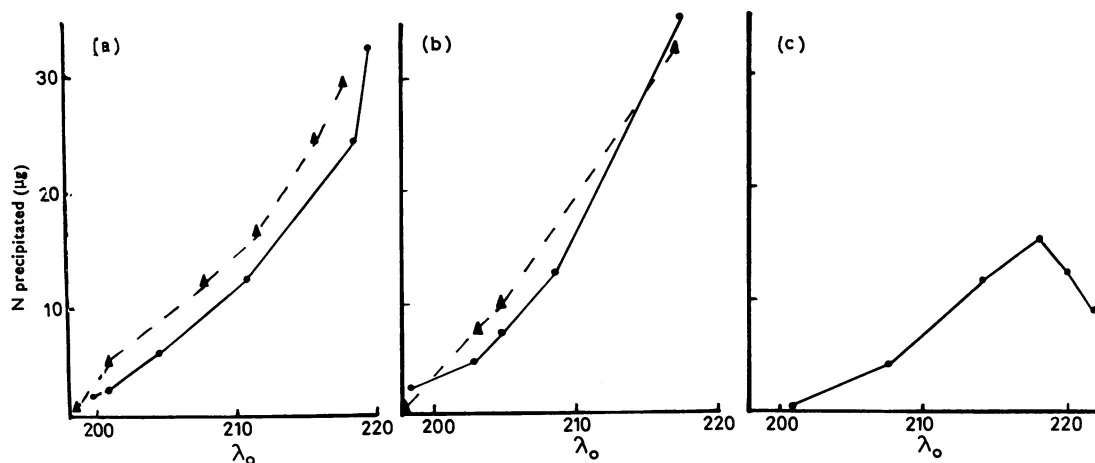

Fig. 6. The relationship between precipitability with rheumatoid factor and the optical dispersion constant (λ_o) of human γG globulin preparations denatured by: (a) heat treatment; (b) NaOH treatment; (c) SDS treatment, as shown in Fig. 5.

In contrast to the above results, the precipitation curve (Fig. 6c) obtained with detergent-treated human γG globulin preparations showed a maximum corresponding to a λ_o value of about 215 mμ. Further increases in λ_o produced a diminishing ability to precipitate rheumatoid factor.

Immunoelectrophoretic analysis demonstrated that the γG globulin in such preparations 'e' and 'f' (containing 500 and 1000 mols detergent/mol γG globulin respectively)

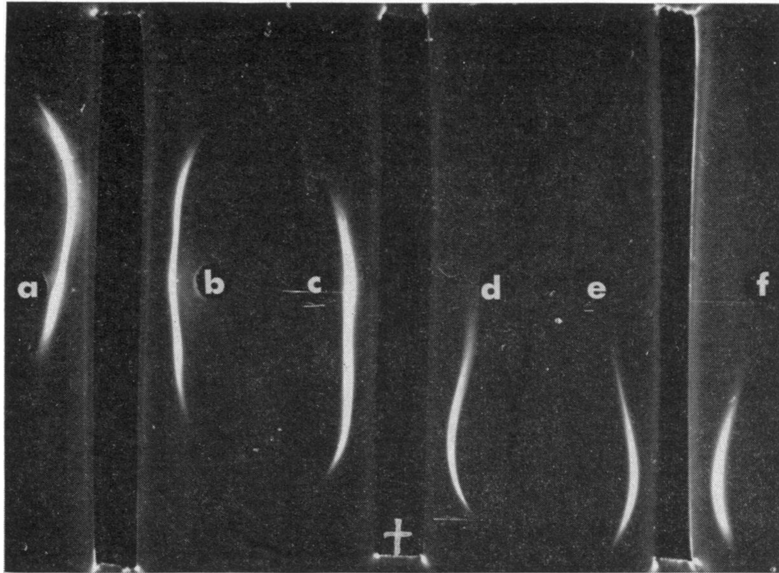

Fig. 7. Immunoelectrophoresis (0·06 M barbitone buffer, pH 8·6) of human γG globulin treated with various concentrations of sodium dodecyl sulphate. (a) Native γG globulin; (b) γG globulin + 0·005 M SDS; (c) γG globulin + 0·01 M SDS; (d) γG globulin + 0·025 M SDS; (e) γG globulin + 0·05 M SDS; (f) γG globulin + 0·1 M SDS.

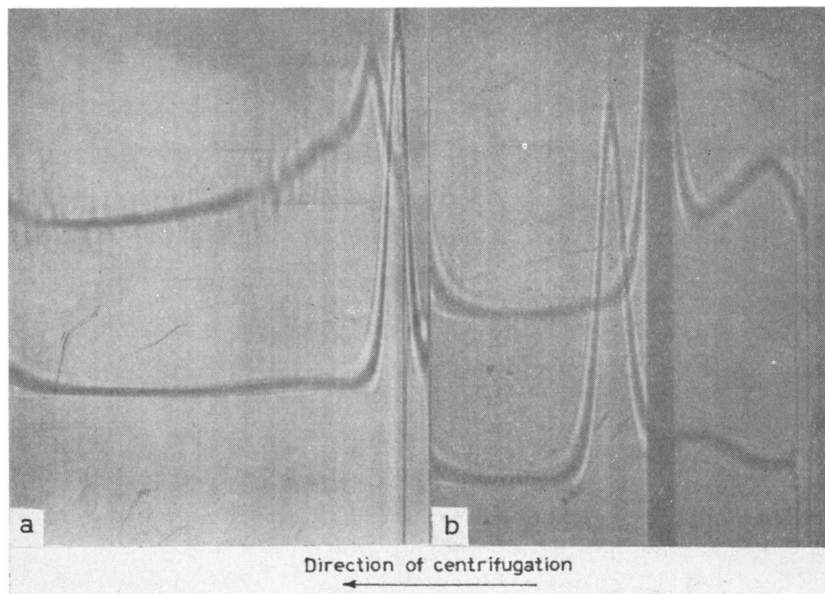

Fig. 8. The effect of sodium dodecyl sulphate (SDS) on the ultracentrifugal properties of human γG globulin. (a) Lower: native human γG globulin; upper: γG globulin + 0·005 M SDS showing the presence of aggregated material. (b) Lower: γG globulin + 0·05 M SDS; upper: γG globulin + 0·1 M SDS. In both cases the presence of slower material (of sedimentation coefficient 2–3S) is seen. Centrifugation at 59,780 rev/min and 20° for (a) 16 minutes, and (b) 104 minutes.

had a grossly enhanced electrophoretic mobility at pH 8·6 (see Fig. 7) as did the most active preparation 'd', containing 250 mols detergent/mol γG globulin. Presumably this effect is attributable to micelle formation between the γG globulin molecules and molecules of the highly charged detergent.

Ultracentrifugal analysis (see Fig. 8) revealed material sedimenting faster than the parent γG globulin in all but samples 'e' and 'f', in which, however, slower sedimenting material (of the order of 2–3S) was detectable. The charge effects resulting from the presence of relatively large amounts of detergent in these preparations complicated their ultracentrifugal analysis and the interpretation of the patterns obtained. It was not possible to detect the 20–40S aggregates observable in heat-treated 7S γ-globulin preparations after a short period of ultracentrifugation at 59,780 rev/min (Fig. 4b).

DISCUSSION

The results presented in section A provide further evidence in support of the antibody nature of rheumatoid factor. Under the varying conditions adopted, e.g. with respect to pH, temperature of incubation, etc., the aggregated γG globulin–rheumatoid serum system showed similar precipitation characteristics to a conventional precipitin system. As already mentioned however, use of *isolated* aggregated γG globulin as 'antigen' led to a flattening out of the optimal proportion titration curve once the point of maximal precipitation had been reached (shown in Fig. 1a). It is assumed that the unaggregated γG globulin present in the total heated γ-globulin preparation inhibits precipitate formation in the region of 'antigen' excess. This effect has been described by other investigators (Christian, 1958) who attributed it to a competition between the unaggregated γG globulin molecules and the aggregates for reaction with rheumatoid factor. The relative reactivities of the two forms could be explained on the basis of a hapten–antigen relationship, the unaggregated γ-globulin failing to precipitate the antibody (i.e. rheumatoid factor) but nevertheless being capable of inhibiting the antibody's reactivity with complete antigen (i.e. aggregated γ-globulin).

This raises the question as to the nature of the γ-globulin 'antigen' against which the rheumatoid factor is supposedly directed. The results of the physico-chemical studies described in section B clearly demonstrate that the degree of reactivity of γG globulin with rheumatoid factor in the precipitation system depends on the extent of denaturation of the γ-globulin irrespective of the agent employed in effecting this.

Ultracentrifugal analysis revealed aggregates in all heat-denatured or alkali-denatured γ-globulin samples. Moreover, both the size and concentration of these aggregates increased with an increase in the severity of the denaturation process employed. There was a parallel increase in precipitability with rheumatoid factor, as Fig. 6 (a–c) demonstrated. In contrast, Oreskes, Singer and Plotz (1963) reported that alkali-treated human γG globulin in which aggregates were not detectable (by O.D. 280/260 ratios) was capable of forming a precipitate with rheumatoid factor. It is quite possible, however, that these investigators would have found aggregates in their alkali-treated γG globulin preparations if ultracentrifugal analysis had been performed. Oreskes (1964, personal communication) subscribes to this suggestion.

The manner in which the structure of γG globulin is thought to be altered as a result of the heat (or alkali) denaturation treatment employed is outlined diagrammatically in Fig. 9. A previous investigation (James, Henney and Stanworth, 1964) showed that the

heating of human γG globulin at 37° for 16 hours led to 10S dimer formation between a proportion (up to 20 per cent) of the 7S molecules. It is supposed that multiple complexing of this type occurs when the γG globulin is heated at higher temperatures (up to 63°), resulting in the formation of polymers (20–40S) comprising several monomeric 7S units. The demonstration that this polymerization process was blocked when the γG globulin was heated in the presence of PCMB suggests that the complexing occurs through the formation of disulphide bonds between the γG molecules. In support of this idea is the demonstration that 10S γ-globulin can be converted back to the 7S form by treatment with 0·1 M mercaptoethanol (Turner, 1964).

As illustrated in the scheme outlined in Fig. 9, the complexing probably occurs between the Fc (previously termed 'fast') papain digestion pieces of the γG globulin molecules. It is possible that the rupture of a labile disulphide bond (or bonds) in this part of the molecule—analogous to that demonstrated recently by Palmer and Nisonoff (1964) in the Fc papain digestion fragment of rabbit γG globulin—provides the free SH groups necessary

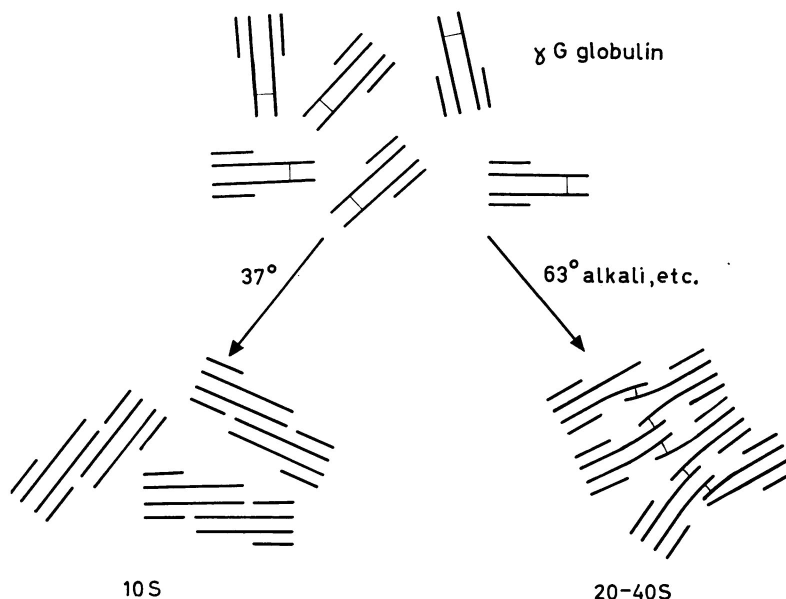

FIG. 9. Diagrammatic outline of proposed mode of alteration of γG globulin as a result of heating (at temperatures up to 63°) or other forms of denaturation treatment. It is supposed that ruptured inter-chain disulphide bonds in the Fc parts of the γG molecules take part in inter-molecular bridging, leading to aggregate formation and to the exposure of new chemical groupings.

for inter-molecular disulphide bridging. Ishizaka and Ishizaka (1964) have shown that aggregates prepared from the Fc papain digestion fragment of rabbit γ-globulin, following reduction and alkylation of such interchain disulphide bonds, develop the capacity to increase the permeability of the capillaries in guinea-pig skin. In this case, biological activity was induced by bridging inactive Fc papain pieces of rabbit γG globulin with bis-diazotized benzidine rather than by disulphide links, as is thought to occur following heat aggregation of native γG globulin or its Fc papain digestion piece.

Considerable evidence has been obtained from previous studies (Henney and Stanworth, 1964a) to indicate that the site of reaction of denatured human γG globulin with rheumatoid factor is also located in the Fc papain digestion fragment. This explains the

inability of other types of immunoglobulins (e.g. γA (previously γ_{1A} or β_{2A}) and γM (previously γ_{1M} or 19S γ))—in native or aggregated form—to precipitate rheumatoid factor, on account of their distinctive Fc papain digestion pieces (Franklin and Stanworth, 1961). Similarly, the γ-globulins of several species phylogenetically remote from man lack the ability to form precipitate with rheumatoid factor because the principal species specific antigenic determinants are also carried in the Fc papain digestion pieces (Henney and Stanworth, 1964b).

Hence, certain chemical and physical structural requirements are necessary for γG globulin to be capable of forming a precipitate with rheumatoid factor. The important question is whether structural alterations induced in the γG globulin molecule in achieving the requisite physical form lead also to essential chemical changes such as the unmasking of determinant groups which are unavailable (for reaction with rheumatoid factor) in the native γG globulin (as proposed by Glynn, 1963). Alternatively, it is possible that the aggregation process is merely a means of linking together 'univalent' antigen to form a 'multivalent' antigen, which is thereby capable of forming a precipitation lattice with the antibody (viz. rheumatoid factor).

The optical rotatory dispersion measurements performed in the present study provide strong evidence of changes in the tertiary structure of the γG globulin molecule as a result of the aggregation process. Such changes could result from the rupture of inter-chain disulphide bond(s) in the Fc papain digestion piece of the molecule in the manner already discussed. The work of Ishizaka and Campbell (1959) has shown that similar alterations to the Fc digestion fragment of antibody γG globulin can be achieved by combination with specific antigen. Only those soluble antigen–antibody complexes in which the antibody had undergone tertiary structural changes (as revealed by optical rotation measurement) were capable of inducing skin irritative reactions in guinea-pigs. It is of some significance to the present discussion, that such complexes (e.g. Ag_3Ab_2) were found to have the capacity to form precipitate with rheumatoid factor, whereas complexes comprising structurally unaltered antibody (e.g. Ag_2Ab) proved to be inactive in this respect. Moreover it has been recently shown that only the former type of complex (i.e. Ag_3Ab_2) —comprising rabbit antibody and BSA antigen—is capable of inducing specific antibody formation when injected into rabbits of similar allotype to the antibody donor, whereas the latter (Ag_2Ab) type of complex was ineffective (Henney, Stanworth and Gell, 1965).

As the reactive antigen–antibody complexes have been shown to possess sedimentation coefficients greater than 20S (Edelman *et al.*, 1958), i.e. of a similar order of magnitude to the sedimentation coefficients of active aggregates of heated human γG globulin it is possible, of course, that their size alone is the critical factor for precipitation with rheumatoid factor. Nevertheless, there is other independent evidence to show that the denaturation of γG globulin leads to the unmasking of new antigenic determinants. This is concluded for example from the work of Milgrom and Witebsky (1960) and McCluskey, Miller and Benacerraf (1962) who showed that denaturation of rabbit 7S γ-globulin led to the exposure of antigenic determinants undetectable in the native *rabbit* γ-globulin but present in *human* γG globulin.

It can be assumed that similar structural changes occurred in the human γG globulin, as a result of the controlled denaturation procedures employed in the present study. Their precise role in the precipitation reaction with rheumatoid factor, however, remains to be established. It seems probable that the exposure of such groupings is, at least, responsible for an increase of avidity of the γG globulin molecule for rheumatoid factor.

REFERENCES

Aho, K. (1961). 'The problem of the antibody nature of the rheumatoid factor.' *Ann. Med. exp. Fenn.*, **39**, supplement 7.

Christian, C. L. (1958). 'Characterization of the "reactant" (γ-globulin factor) in the F II precipitin reaction and the F II tanned sheep cell agglutination test.' *J. exp. Med.*, **108**, 139.

Edelman, G. M., Kunkel, H. G. and Franklin, E. C. (1958). 'Interaction of the rheumatoid factor with antigen-antibody complexes and aggregated γ-globulin.' *J. exp. Med.*, **108**, 105.

Franklin, E. C. and Stanworth, D. R. (1961). 'Antigenic relationship between immune globulins and certain related paraproteins in man.' *J. exp. Med.*, **114**, 521.

Gell, P. G. H. (1957). 'The estimation of the individual human serum proteins by an immunological method.' *J. clin. Path.*, **10**, 67.

Glynn, L. E. (1963). 'Rheumatoid arthritis and the rheumatoid factor.' *Clinical Aspects of Immunology* (Ed. by P. G. H. Gell and R. R. A. Coombs), p. 593. Blackwell Scientific Publications, Oxford.

Glynn, L. E., Holborow, E. J. and Johnson, G. D. (1957). 'The nature of the Rose-Waaler factor.' *Proc. roy. Soc. Med.*, **50**, 469.

Henney, C. S. and Stanworth, D. R. (1964a). 'Reaction of rheumatoid factor with the isolated polypeptide chains of human 7S γ-globulin.' *Nature (Lond.)*, **201**, 511.

Henney, C. S. and Stanworth, D. R. (1964b). 'The reactivity of rheumatoid factor with serum 7S γ-globulins of various species.' In *Protides of the Biological Fluids* (Ed. by H. Peeters), vol. 12, p. 155.

Henney, C. S., Stanworth, D. R. and Gell, P.G.H. (1965). 'Demonstration of the exposure of new antigenic determinants following antigen-antibody combination.' *Nature (Lond,.)* **205,** 1079.

Ishizaka, K. and Campbell, D. H. (1959). 'Biologic activity of soluble antigen-antibody complexes. V. Change of optical rotation by the formation of skin reactive complexes.' *J. Immunol.*, **83**, 318.

Ishizaka, K. and Ishizaka, T. (1964). 'Molecular basis of passive sensitization. II. The role of fragment III of γ-globulin and its disulphide bonds in passive sensitization and complement fixation.' *J. Immunol.*, **93**, 59.

James, K., Felix-Davies, D. and Stanworth, D. R. (1961). 'Studies on the isolation of rheumatoid factor.' *Ann. rheum. Dis.*, **20**, 369.

James, K., Henney, C. S. and Stanworth, D. R. (1964). 'Structural changes occurring in 7S γ-globulins.' *Nature (Lond.)*, **202**, 563.

Jocelyn, P. C. (1962). 'The effect of glutathione on protein sulphydryl groups in rat-liver homogenates.' *Biochem. J.*, **85**, 480.

Lowry, O. H., Rosebrough, N. J., Lewis Farr, A. and Randall, R. J. (1951). 'Protein measuremen, with the Folin phenol reagent.' *J. biol. Chem.*, **193** 265.

McCluskey, R. T., Miller, F. and Benacerraf, B. (1962). 'Sensitization to denatured autologous gamma globulin.' *J. exp. Med.*, **115**, 253.

Milgrom, F. and Witebsky, E. (1960). 'Studies on the rheumatoid and related serum factors. I. Autoimmunization of rabbits with γ-globulin.' *J. Amer. med. Ass.*, **174**, 56.

Oreskes, I., Singer, J. M. and Plotz, C. M. (1963). 'Reactivity of rheumatoid arthritis sera with altered and aggregated human γ-globulin.' *J. Immunol.*, **90**, 107.

Palmer, J. L. and Nisonoff, A. (1964). 'Dissociation of rabbit γ-globulin into half-molecules after reduction of one labile disulphide bond.' *Biochemistry*, **3**, 863.

Stanworth, D. R. (1961). 'A rapid method of preparing pure serum gamma globulin.' *Nature (Lond.)*, **188**, 156.

Turner, M. W. (1964). 'Studies on the antigenic and antibody characteristics of human immunoglobulins.' Ph.D. thesis, University of Birmingham.