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Aggregation of Human Immunoglobulin G upon Freezing

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Investigation of the aggregation of normal and myeloma immunoglobulins when frozen revealed a correlation between the percentage of formed aggregates and storage period in the cold. No correlation was found between the percentage of formed aggregate and protein concentration in the interval 0.2 to 1.4 g/100 ml. The amount of aggregates formed varied between 26 and 42 % for four different normal IgG's. The amount of aggregate of the order of 10 S varied between 22 and 33 % and the amount of aggregate of the order of 12 S between 4 and 10 %. Traces of precipitate occurred in three preparations. The amount of aggregate formed varied between 13 and 42 % for seven myeloma IgG. The amount of aggregate with the order of 10 S varied between 13 and 32 % and the amount of aggregate with an order of 12 S between 0 and 10 %. Traces of precipitate occurred in three preparations.

A certain correlation was found between the electrophoretic mobility of the myeloma IgG and the percentage of formed aggregate but not between the percentage of formed aggregate and type of light chain, Gm-type, or sedimentation constant.

7 S molecules separated from formed aggregates could form aggregates to the same extent as the original population of IgG molecules.

Changes in the antigenic properties of frozen and thawed or otherwise denatured rabbit immunoglobulin G (IgG) have been reported[1–3] without any description of the sedimentation properties of these immunoglobulins. Human IgG, by exposure to about 60°C, has been used in the study of the anticomplementary effect of aggregated IgG. The aggregates formed on such denaturation of the immunoglobulin have *s*-values of at least 9.5.[4] When normal IgG is heated, 15 to 20 % of it aggregates. The corresponding range for various myeloma IgG is 0—100 %.[5–6] The presence of immunoglobulin aggregates is believed to be one of the causes of the side effects of intravenous administration of gamma-globulin.[7] Pure IgG preparations are often stored at −20°C before they are used.

This paper is concerned with the degree of aggregation of IgG when frozen and thawed. The sedimentation properties of normal immunoglobulins and myeloma immunoglobulins before and after freezing were noted and compared with other properties of the IgG molecules.

MATERIALS AND METHODS

Sera. Fresh sera from apparently healthy persons (A, B, C, D, and E), frozen sera with M-components of type IgG stored at −20°C for one or more years, and fresh serum with M-component of type IgG (L) were used. In the designations of the immunoglobulins the capital denotes the person from whose serum the IgG was prepared. The numeral after the capital denotes the order in which the serum was removed and the IgG was prepared.

Isolation of IgG from normal and from myeloma sera. The serum was diluted with three parts of 0.2 M NaCl. The solution was made 1.84 M in respect of ammonium sulphate and the pH adjusted to 7. The precipitate formed was dissolved and fractionated as previously described,[8] on DEAE-cellulose and Sephadex G 200.

Phosphate-NaCl buffer. 0.05 M phosphate buffer, 0.5 M in respect of NaCl at pH 7.0.

The protein concentration was calculated from light absorption at 280 nm in phosphate-NaCl buffer. An extinction coefficient ($E^{1\,cm}_{1\,\%}$ 280) of 12.7 was used for calculating the protein concentration in mg per ml. The error of the method was less than ± 3 %.

Agar gel electrophoresis was run in agarose (1 %) in diemal buffer at pH 8.6.

Immunoelectrophoresis was performed according to Scheidegger.[9] As antiserum goat antihuman total serum was used.

Storage in the cold. The sample was stored at −20°C.

Freezing and thawing. The sample was stored for 2 h at −20°C and then allowed to thaw until it reached room temperature. This was repeated 10 times.

Ultracentrifugation. A Spinco Analytical Ultracentrifuge Model E with Schlieren optics was used.

Determination of s-values of non-aggregated IgG and calculation of the *s*-values at infinite dilution in water at 20°C ($s_{20\,w}°$) were done as previously described.[8] Calculation of the *s*-values in water at 20°C ($s_{20\,w}$) for the various components in the aggregated IgG preparations was done in the same way. The components given as 10 S had *s*-values between 9.5 and 10.5. The components given as 12 S had *s*-values between 11.5 and 12.5.

Determination of the ratio between 7 S molecules and aggregates. During acceleration of the centrifuge it was checked that the sample did not contain components that migrated to the bottom of the cell before it reached an rpm of 59 780. Photographs were taken at as favourable angles as possible and the curves were copied after ten-fold enlargement. The surfaces under the various "peaks" were measured with a planimeter. Only photographs where the various components were well separated from one another were used. As a rule, two measurements were made of each sample. The measuring error expressed as maximum deviation from the mean was less than 2 % for components in a concentration of more than 0.3 g/100 ml, less than 5 % for concentrations of 0.05 to 0.29 g/100 ml, less than 10 % for concentrations of 0.01 to 0.049 g/100 ml, and less than 100 % for concentrations below 0.01 g/100 ml. The measuring error was so dominant that all other errors could be ignored.

Separation of 7 S molecules from aggregates. The IgG preparations containing aggregate were dialysed against 0.05 M phosphate buffer pH 7.0 and filtered through a column of gel containing Sephadex G 200, equilibrated with the same buffer. The elution diagram showed only one peak. The early eluates contained aggregated IgG; the middle and final fractions, only 7 S molecules.

RESULTS

The IgG preparations from normal sera revealed no inhomogeneity on ultracentrifugation. Myeloma IgG occurred in serum in relatively large amounts (Table 3) and proved to be homogeneous on analysis in the ultracentrifuge. All the IgG preparations used on immunoelectrophoresis produced only one line at the site of the IgG.

The amount of aggregates increased with the number of times that the preparations were frozen and thawed. The increase was greatest in the interval 0—10 and then less obvious.

Table 1. Results of ultracentrifugal analysis of IgG preparations prepared from the serum of one individual.[a]

IgG prep.	Conc. g/100 ml [b]	$s_{20\,w}°$ [c]	Time at −20°C, months	Distribution 7 S %	10 S %	12 S %	Precipitation [d]
A 1	0.2	6.89	17	53	34	13	traces
A 2	0.2	6.90	13	64	30	6	»
A 3	1.4	6.83	13	68	30	3	»
A 4	0.4	6.90	11	71	25	4	»
A 5	0.4	6.90	11	71	26	3	»

[a] Each preparation had been stored in the cold and then frozen and thawed 10 times.
[b] Of non-aggregated preparation.
[c] Sedimentation constant at infinite dilution in water at + 20°C on non-aggregated preparation.
[d] Traces = opalescent.

Table 1 gives the amount of aggregates formed in various IgG preparations produced from serum from one and the same person. The preparations were dissolved in phosphate-NaCl buffer and had been stored in the cold before they were frozen and thawed. A 1 had been stored for the longest and A 5 for the shortest time at the abovementioned temperature. The total amount of aggregates formed were 47 % in A 1, 36 % in A 2, and 33 % in A 3. A 1 and A 2 contained the same concentration of IgG. The difference in concentration between A 2 and A 3 was large. The amount of aggregates formed in A 4 and in A 5 was 29 %. Both had the same protein concentration and both had been kept for the same time in the cold. The s-values at infinite dilution in water at 20°C were all about 6.90.

Some IgG preparations from healthy persons were stored in the cold after they had been frozen and thawed. They were examined one month

Table 2. Results of ultracentrifugal analysis of some individual normal IgG's.[a]

IgG prep.	Conc.[b] g/100 ml	$s_{20w}°$ [c]	Distribution 7 S %	10 S %	12 S %	Precipitation [d]
B 1	0.6	6.79	57	32	10	traces
C 1	0.7	6.74	68	26	5	»
D 1	0.8	6.52	62	33	5	0
E 1	0.7	6.87	74	22	4	traces

[a] All preparations had been frozen and thawed and then stored in the cold for one month.
[b] Of non-aggregated preparation.
[c] Sedimentation constant at infinite dilution in water at + 20°C on non-aggregated preparation.
[d] Traces = opalescent.

later. The results are given in Table 2. B 1 contained 42 % aggregates, C 1 31 %, D 1 38 %, and E 1 26 %. The protein concentration varied between 0.6 and 0.8 g/100 ml. The *s*-values at infinite dilution in water at 20°C varied between 6.52 and 6.87.

The result of an examination of the composition of various frozen myeloma IgG's is given in Table 3. The preparations had been stored at −20°C before they were frozen and thawed. The series included two IgG's of light chains of type L (F 1 and H 1). H 1 was Gm (1−4+5−). F 1 had not been grouped. The others were Gm (1−4−5−). F 1 formed the largest percentage of aggregates. It had the highest *s*-value (6.95) and was electrophoretically situated in the gamma$_2$ field. G 1, H 1, J 1, and K 1 contained about 25 % aggregates and all had a higher anodal mobility than F 1. The *s*-values in the group varied between 6.46 and 6.91. L 1, which contained 0.6 g/100 ml protein, formed 17 % aggregate of the order of 10 S; and L 2 from the same person contained 0.8 g/100 ml protein and formed 16 % aggregate of the same order of magnitude. Also L had a higher anodal mobility than F 1. The *s*-values for L 1 and L 2 were equal. M 1 formed the smallest amount of aggregate and had a higher cathodal mobility than F 1. The *s*-value for M 1 was 6.77.

The tendency of persistent 7 S molecules to aggregate after the aggregates had been removed was studied both for the IgG E 2, which contained 81 % of 7 S-, 18 % of 10 S-, and 1 % of 12 S molecules and the IgG L 1, which contained 83 % of 7 S- and 17 % of 10 S molecules. The aggregates were separated from the 7 S molecules by gelfiltration on Sephadex G 200. When the gelfiltration was done in phosphate-NaCl buffer the aggregated molecules occurred in all fractions, which was not the case when a buffer of low ionic strength was used. The fractions containing only 7 S molecules were dialysed against phosphate-NaCl buffer. After the dialysis the solutions were frozen and thawed and stored in the cold for one month. The 7 S fraction from E 2 now contained 57 % of 7 S-, 38 % of 10 S-, and 5 % of 12 S molecules, while the corresponding fraction from L 1 contained 85 % of 7 S- and 15 % of 10 S molecules. The IgG preparations used in the abovementioned investigation were re-examined after they had been stored in the cold for a further three months. It was found that E 2 contained 29 % aggregates, *i.e.* 10 % more than before gelfiltration, while L 1 contained 17 %, *i.e.* the same percentage as when the gelfiltration was started.

DISCUSSION

In vivo aggregated myeloma protein of type IgG,[10] dissociated when the IgG was diluted to a concentration below 0.8 g/100 ml. Our two IgG preparations produced from serum from the same person with different protein concentrations (0.2 and 1.4 g/100 ml) formed the same percentage of aggregates. Thus, human IgG, dissolved in phosphate-NaCl buffer, did not show the dependence on concentration exhibited by *in vivo* aggregated myeloma IgG. Putnam [11] reported that, as a rule, it was difficult to control the aggregation process of proteins and that the process among other things was sensitive to the extent of heating. Thus, we found in these two IgG preparations that had been prepared from serum from one person, dissolved in the same buffer

with the same protein concentration, frozen and thawed an equal number of times, and stored for an unequally long period in the cold, different amounts of aggregates. The IgG that had been stored in the cold for 17 months contained a larger amount of aggregates than the one that had been stored at the same temperature for 11 months. It thus appeared probable that the percentage of aggregates varied with duration of storage in the cold.

The IgG preparations obtained from four normals and frozen, thawed and stored in the cold for one month formed aggregates in an amount varying between 26 and 42 %. The differences could not be ascribed to differences in the concentration or to the error of the method. It was thus probable that the differences were due to molecular differences between the individual IgG preparations. These differences were not reflected in differences in sedimentation properties of the molecules since no relation between *s*-value and percentage of formed aggregate could be demonstrated.

The percentage of aggregate formed in seven myeloma IgG's varied between 13 and 42 % and the variation could not be ascribed to the errors of the method. No correlation was found between the percentage of formed aggregates and type of light chain, Gm-type, or sedimentation constant. Neither could the differences be ascribed, to any appreciable extent, to the small differences in the period of storage in the cold. For the difference in the percentage of formed aggregate was 7 % for two IgG preparations prepared from serum from the same individual and treated in exactly the same way in every respect except that one had been stored in the cold for 11 months and the other for 13 months.

A certain relation was, however, found between aggregate formation and the electrophoretic mobility of the myeloma IgG's. Those myeloma IgG's whose molecules had the same electrophoretic mobility as the bulk of the molecules of normal IgG formed the largest amount of aggregates, while those molecules that had a higher anodal or cathodal mobility than these formed a smaller amount of aggregates. No differences could be demonstrated in the content of more anodally and cathodally charged molecules of the various normal IgG preparations. Morse [6] found a relation to exist between the aggregation of heated human myeloma IgG and its electrophoretic mobility in starch gel but pointed out that the aggregation could not be correlated directly with the electrophoretic mobility because the correlation did not hold for all of the myeloma IgG's studied. The discrepancy between this result and those noted for frozen aggregated myeloma IgG could be explained by the assumption that 7 S molecules are not denatured in the same way by heating and by freezing. Another explanation might be that separation on agar gel occurs according to charge, while separation on starch gel occurs according to charge and molecular size.

The 7 S molecules from a normal IgG that had not formed aggregates when first frozen formed a larger percentage of aggregates after the aggregates formed in association with the initial treatment had been removed. The 7 S molecules from a myeloma IgG that had been freed from aggregates, on the other hand, formed an equally large percentage of aggregates as the original population of IgG molecules. The difference in reaction between the two was to a certain extent understandable when it was found that the original popula-

Table 3. Properties of some individual myeloma IgG's.

IgG prep.	Conc. in serum g/100 ml	Conc.in prep.[a] g/100 ml	s_{20w} ° [b]	El. phor. mob.	Light chain type	Gm	Time at −20°C months	Distribution [c]			
								7 S	10 S	12 S	Precipitation [d]
F 1	2.0	0.5	6.95	γ_2	L	—	11	57	32	10	0
G 1	2.1	0.6	6.72	$\beta_2-\gamma_1$	K	1−4−5−	11	75	22	2	traces
H 1	2.0	1.1	6.91	γ_1	L	1−4+5−	11	76	21	2	0
J 1	2.0	0.7	6.46	$\gamma_1-\gamma_2$	K	1−4−5−	11	76	22	3	traces
K 1	1.0	0.5	6.81	$\gamma_1-\gamma_2$	K	1−4−5−	13	77	21	2	0
L 1	8.5	0.6	6.82	γ_1	K	1−4−5−	12	83	17	0	0
L 2	8.5	0.8	6.78	γ_1	K	1−4−5−	12	84	16	0	0
M 1	2.3	0.5	6.77	γ_3	K	1−4−5−	13	87	13	0	traces

[a] Of non-aggregated preparation.
[b] Sedimentation constant at infinite dilution in water at + 20°C on non-aggregated preparation.
[c] In ultracentrifuge after the preparation has been stored in the cold and frozen and thawed.
[d] Traces = opalescent.
—not performed.

tion of 7 S molecules in the former IgG formed more aggregates when the preparation was frozen for a longer period whereas the original population of 7 S molecules in the lastmentioned immunoglobulin did not form more aggregates when the immunoglobulin was frozen further.

Myeloma immunoglobulins are chemically more homogeneous than immunoglobulin preparations from normals. If it be assumed that the myeloma protein used is a homogeneous fraction, the reaction of the myeloma protein, on the one hand, in the examination accounted for in Table 3, and, on the other hand in the above-mentioned experiment suggests that all the IgG molecules can form aggregates but that the ratio between the amount of 7 S molecules and the amount of aggregates is determined by an "equilibrium constant". With regard to the normal IgG, the 7 S molecules separated from aggregates formed more aggregates after a shorter period of freezing than did the original population of molecules after a longer period of freezing. This might suggest that the molecules were affected by the primary treatment and therefore more readily formed intermolecular bonds on subsequent freezing.

Conell and Painter [12] found that human IgG stored at + 2°C formed fragments resembling those obtained when immunoglobulins were treated with plasmin or papain. When the composition of three IgG preparations (B 1, C 1, and D 1), stored at + 4°C for one month, was studied in the ultracentrifuge it was found that none of the preparations contained either fragments or aggregates. One explanation for this might be that IgG's are broken down more quickly at the high concentrations used by Conell and Painter; another might be that proteolytic enzymes occurred in various amounts.

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REFERENCES

1. Milgrom, F. and Witebsky, E. *JAMA* **174** (1960) 56.
2. McCluskey, R. I., Miller, F. and Baruj, B. *J. Exptl. Med.* **115** (1962) 253.
3. Williams, R. C. and Kunkel, H. *Proc. Soc. Exptl. Biol. Med.* **112** (1963) 555.
4. Frommhagen, L. H. and Fudenberg, H. *J. Immunol.* **89** (1962) 336.
5. Laurell, A.-B. and Nilsson, U. *Acta Pathol. Microbiol. Scand.* **59** (1963) 79.
6. Morse, J. H. *J. Immunol.* **95** (1966) 722.
7. Barandun, S., Kistler, P., Jeunet, F. and Isliker, H. *Vox Sanguinis* **7** (1962) 157.
8. Hansson, U.-B., Laurell, C.-B. and Bachmann, R. *Acta Med. Scand. Suppl.* **445** (1966) 89.
9. Scheidegger, J. J. *Intern. Arch. Allergy Appl. Immunol.* **7** (1955) 103.
10. Kochwa, S., Smith, E., Brownell, M. and Wasserman, L. R. *Biochemistry* **5** (1966) 277.
11. Putnam, F. W. *The Proteins* I, Part B (1953) 807.
12. Conell, G. E. and Painter, R. H. *Can. J. Biochem.* **44** (1966) 371.

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