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Effect of Straight Chain Aliphatic Amino Acids, Amines and Carboxylic Acids on the Aggregation of IgG on Freezing

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A preparation of IgG (1 g/100 ml) isolated from normal serum was used. Ultracentrifugal analysis showed that the ability of straight chain aliphatic α-amino acids with three or more carbon atoms to inhibit the aggregation of IgG on freezing decreased with increasing length of the carbon chain. Alanine did not only inhibit aggregate formation, but partly monomerized existing aggregates. While α-aminocaproic acid acted as a denaturant, α,ε-diaminocaproic acid acted as an inhibitor of denaturation. Substitution of hydrogen atoms in the amino group for methyl groups did not substantially affect the aggregation. Aggregates were formed when the carboxyl group in β-alanine was replaced by a sulphonate group. Aliphatic amines acted as denaturants. The sodium salts of the carboxylic acids were as effective as the amino acids — except the C_3 amino acids — in inhibiting aggregation.

It has been shown that the aggregation of immunoglobulin G (IgG) on freezing can be effectively prevented by addition of glycine at neutral pH.[1] It is also known that only traces of aggregates are formed in neutral globulin solutions containing at least 5 % NaCl.[1] Amino acids, such as proline, serine and arginine, and proteins as albumin and fibrinogen, retard the aggregation of IgG on freezing, but not as effectively as is the case with glycine.[1] Addition of iodoacetamide has no such effect.[1]

This paper concerns the effect of amino groups and carboxyl groups in substances added on the aggregation of immunoglubulin G on freezing. Data obtained on the variation in aggregate formation with added amino acid of varying carbon chain length and position of the amino groups are also presented.

MATERIAL AND METHODS

Serum. Frozen pooled sera from 50 registered blood donors was used.

Isolation of IgG. One part of serum was diluted with 3 parts of 0.2 M NaCl. The solution was made 1.84 M in respect of ammonium sulphate and pH adjusted to 7.

The precipitate formed was dissolved and fractionated on DEAE-cellulose and Sephadex G 200.[2]

The protein concentration of the IgG solution used was determined [3] to 1 g/100 ml.

Addition of various substances. Glycine, DL-α-alanine, β-alanine, DL-2-aminobutyric acid, 4-aminobutyric acid, DL-lysine monohydrochloride (α,ε-diamino caproic acid), propylamine, butylamine, betaine, sarcosine, and taurine were British Drug Houses Ltd. DL-Norleucine (α-aminocaproic acid) was E. Merck AG., Germany. Unless otherwise stated, the substances were added to IgG dissolved in 0.05 M phosphate buffer pH 7.0, 0.5 M in respect of NaCl. The solution was made 0.1 M in respect of the desired amino acid or added substance. When necessary the pH was adjusted to 7 with phosphoric acid or sodium hydroxide before the addition.

Aging. The sample was stored for 2 h at −20°C and afterwards allowed to thaw at room temperature. This was repeated 10 times, after which the sample was stored for more than three months at −20°C until analysed.

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

Determination of ratio between 7S molecules and aggregates was done in the way described previously.[3] Samples containing glycine, β-alanine, or γ-aminobutyric acid were analysed twice. The results of the analysis were given in different tables and showed the reproducibility of the method used.

RESULTS

The influence of straight chain aliphatic amino acids of different chain length on aggregation of immunoglobulin G by freezing is apparent from Table 1. The globulin used contained 5 % 10 S and 95 % 7 S molecules prior to addition of the amino acids. After the samples had been aged they were studied again in an analytical ultracentrifuge. Of the aged portion that contained no amino acid, 11 % of the molecules formed soluble aggregates and 18 % a precipitate. In the aged portion containing α- and β-alanine, only 7 S molecules could be demonstrated. Alanine not only inhibited aggregation, but also dissociated aggregates present in the original sample. Neither in the sample

Table 1. Ratio between aggregates and 7 S molecules in IgG frozen in the presence of amino acids with different carbon chain length and with different positions of amino groups.

Added subst.[a]	Distribution [b]			
	% 7 S	% 10 S	% 12 S	% precipitation
— [c]	71	11	trace	18
Glycine	92	6	1	0
α-Alanine	100	0	0	0
β-Alanine	100	0	0	0
α-Aminobutyric acid	93	7	0	0
γ-Aminobutyric acid	98	2	0	0
α-Aminocaproic acid	31	3	0	66
α,ε-Diaminocaproic acid	93	7	0	0

[a] The immunoglobulin contained 5 % 10 S and 95 % 7 S molecules before addition.
[b] Ultracentrifugal analysis after aging of the solutions.
[c] Dissolved in 0.05 M phosphate buffer pH 7.0, 0.5 M in respect of NaCl.

containing γ-aminobutyric acid nor in the sample containing glycine, α-aminobutyric acid or α,ε-diaminocaproic acid was aging accompanied by aggregation. α-Aminocaproic acid had, in contrast with α,ε-diaminocaproic acid, a denaturing effect. Of the molecules in the sample, 66 % precipitated on freezing.

Portions of the normal IgG aged in the presence of glycine, sarcosine, or betaine were examined by ultracentrifugal analysis. The soluble aggregates constituted 7 % in the solution containing glycine, 5 % of the sample containing sarcosine and 10 % of the one containing betaine. Thus substitution of the hydrogen atoms in the amino group of glycine with one or three methyl groups did not substantially affect aggregation by freezing.

Table 2. The aggregate formation of IgG when frozen in the presence of amino acids compared with when frozen in the presence of amines.

Added subst.	Distribution [a]				
	% 7 S	% 10 S	% 12 S	% > 12 S	% precipitation
β-Alanine	100	0	0	0	0
Propylamine	16	18	10	1	55
γ-Aminobutyric acid	98	2	0	0	0
Butylamine	21	17	6	1	55

[a] Ultracentrifugal analysis after aging of the solutions.

The aggregation of IgG when frozen in the presence of amino acids and amines is given in Table 2. No aggregates could be demonstrated in the aged solution containing β-alanine. Only 2 % of the molecules had formed aggregates in the solution containing γ-aminobutyric acid. The corresponding figure for the sample frozen without additional substances was 20 %; for the sample containing propylamine 84 %, and for the one containing butylamine 70 %.

β-Alanine and taurine were also compared regarding their inhibitory effect on aggregation during freezing of the samples. After aging in the presence of β-alanine the immunoglobulin contained only 7 S molecules, compared with 13 % when aged in the presence of taurine. 70 % of the aggregates were insoluble. The amount of aggregates increased when the carboxyl group in β-alanine was replaced by a sulphonate group.

The aggregation of IgG when frozen in the presence of amino acids, and 0.1 M aliphatic carboxylic acids neutralized with ammonia or sodium hydroxide is given in Table 3. Aggregation was about equal in the solution containing ammonium acetate or sodium acetate or glycine. Precipitation occurred in the sample containing sodium acetate. Precipitates also occurred in the sample containing ammonium phosphate. In this latter sample, 41 % of the molecules had aggregated. A tendency to precipitating was also noted

Table 3. The aggregate formation of IgG when frozen in amino acids compared with that of preparations frozen in the presence of aliphatic carboxylic acids neutralized with ammonia or sodium hydroxide before added to the globulin.

Added subst.[a]	Distribution [b]				
	% 7 S	% 10 S	% 12 S	% > 12 S	% precipitate
0.1 M Glycine	97	3	0	0	0
0.1 M $NH_4OH + H_3PO_4$ [c]	59	23	9	1	7
0.1 M NH_4OH + HAc [c]	96	4	0	0	0
0.1 M HAc + NH_4OH [c]	98	2	0	0	0
0.1 M HAc + NaOH [c]	96	2	0	0	2
0.1 M HCOOH + NH_4OH [c]	98	2	0	0	0
0.1 M HCOOH + NaOH [c]	98	0	0	0	2

[a] The immunoglobulin contained 5 % 10 S and 95 % 7 S molecules when the additions were done.
[b] Ultracentrifugal analysis after aging of the solutions.
[c] Substance used for adjusting pH to 7.

in the solution containing sodium formate, but not in the solution containing ammonium formate. The amounts of aggregates were, however, about the same. Formic acid or formate ions were as effective as inhibitors as acetic acid or acetate ions.

DISCUSSION

In this study it was shown that α,ε-diaminocaproic acid was as good an inhibitor of aggregation as glycine. It has been shown earlier that glycine is a better inhibitor of aggregation than arginine.[1] According to Levin,[4] the strength of ionic linkages in relation to the carboxylate side chain of glutamic acid or aspartic acid of proteins dissolved in neutral aqueous solutions could be larger for arginine than for α,ε-diaminocaproic acid. Carboxylate ions would thus probably not be accessible to external agents, when IgG is frozen.

The aggregation of IgG was more pronounced on freezing the samples in the presence of α-aminobutyric acid than with γ-aminobutyric acid. α-Aminocaproic acid acted as a denaturant whereas α,ε-diaminocaproic acid inhibited denaturation. The amount of aggregates was higher in the solution containing α-aminocaproic acid than in that containing α-aminobutyric acid. Raising of the temperature strengthens intramolecular hydrophobic bonds.[5] Hydrophobic bonds may be weakened on freezing of the sample. A further weakening may occur by the effect of non-polar parts of substances added. Aggregates formed on freezing of the sample dissociate, however, when the IgG solution is heated.[6] Hydrophobic bonds were probably not responsible for coupling the IgG molecules together when aggregation occurred.

The findings made in this study agree with those reported by workers in other parts of the protein field. Thus, Bull and Breese [7] found that fatty acids were denaturants of egg albumin and that their effectiveness increases with the length of the carbon chain. Smith [8] found that fatty acids are capable

of increasing the agglutination titers of hyperimmune rabbit anti-sheep erythrocyte serum, and that their ability increases with the length of the carbon chain.

Substitution of one or more hydrogens in the amino group of glycine by methyl groups did not substantially influence the aggregation of IgG on freezing. The forces giving the IgG molecule its native structure are very probably hydrophobic and/or electrostatic. In general, proteins in water solution have hydrophobic centers in the interior of the molecule. When the solutions were frozen, hydrophobic bonds might have been weakened. Simultaneously charged groups could become accessible. Unlike amino acids, amines acted as strong denaturants. The groups that became accessible would thus be mainly positively charged. In the native molecule they could have formed intramolecular side chain hydrogen bonds or ionic bonds.

β-Alanine was a better inhibitor of aggregation than was taurine. Replacement of the carboxyl group in β-alanine by a sulphonate group resulted in an increase of the aggregation. Electrostatic attraction between negatively charged carboxyl groups and positively charged groups in the protein were probably important for the coupling of the IgG molecules together on aggregation. This is in agreement with results obtained in a previous study [9] where it was shown that IgG preparations from normal sera and not containing the anodal part of the gammaglobulin on agarose gel electrophoresis at pH 8.6 seem to aggregate less than do sera containing the entire population of molecules.

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REFERENCES

1. Hansson, U.-B. *Acta Chem. Scand.* **22** (1968) 490.
2. Hansson, U.-B., Laurell, C.-B. and Bachmann, R. *Acta Med. Scand. Suppl.* **445** (1966) 89.
3. Hansson, U.-B. *Acta Chem. Scand.* **22** (1968) 483.
4. Levin, G. *Biochem. J.* **114** (1969) 83 p.
5. Kauzmann, W. *Advan. Protein Chem.* **14** (1959) 37.
6. Hansson, U.-B. *Acta Chem. Scand.* **22** (1968) 953.
7. Bull, H. B. and Breese, K. *Arch. Biochem. Biophys.* **120** (1967) 309.
8. Smith, J. K. *J. Immunol.* **100** (1968) 863.
9. Hansson, U.-B. *Acta Chem. Scand.* **23** (1969) 1828.

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