Physicochemical Studies on the Gelation of Hen's Egg Yolk

Delipidation of Yolk Plasma by Treatment with Phospholipase-C and Extraction with Solvents

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A method for the delipidation of egg yolk plasma using phospholipase-C, *n*-heptane, and 1-butanol has been described. An aggregating protein fraction and a soluble protein fraction were separated by the action of phospholipase-C. The aggregating protein fraction freed of most of the lipids by treatment with *n*-heptane and 1-butanol was shown to be the apolipoproteins of yolk plasma, whereas the soluble proteins were identified as the livetins. Carbohydrate and the N-terminal amino acid analysis of these protein fractions are reported. A comparison of these protein fractions with the corresponding fractions obtained by formic acid delipidation of yolk plasma has been made. The gelation of yolk plasma by the action of phospholipase-C has been interpreted as an aggregation of lipoproteins caused by ionic interactions. The role of lecithin in maintaining the structural integrity of lipoproteins has been discussed.

In an earlier report on the gelation of hen's egg yolk, it was shown that yolk plasma was capable of aggregation during freezing and thawing (Mahadevan *et al.*, 1969). Delipidation of yolk plasma by use of formic acid resulted in the separation of aggregating and nonaggregating protein components. Attempts to delipidate yolk plasma by enzymatic procedures indicated that phospholipase-C induced gelation at room temperature. Similar observations were reported by MacFarlane and Knight (1941) on whole egg yolk. Phospholipases have been used as tools for studying the nature of lipid–protein associations in various lipoproteins (Ashworth and Green, 1963; Burley and Kushner, 1963; Condrea *et al.*, 1962; Krumweide, 1958; Marinetti, 1961). In recent years, the role of lecithin in maintaining the biological activity of membrane-bound enzymes (Kielley and Meyerhof, 1950; Swanson *et al.*, 1964) and of thyroid gland (Macchia and Pastan, 1967) has been demonstrated by the use of phospholipase-C.

This paper describes the action of phospholipase-C on the lipoproteins of egg yolk plasma and the separation of the resulting aggregating and nonaggregating protein fractions. The chemical compositions of these protein fractions have been compared with those of aggregating and soluble proteins isolated by the formic acid delipidation procedure described earlier (Mahadevan *et al.*, 1969).

MATERIALS AND METHODS

Materials. One to 2-day-old unfertilized White Leghorn eggs were procured from a local market. Egg yolk fluids (liquid yolk) were obtained by the procedure of Powrie *et al.* (1963).

Separation of yolk granules from plasma (soluble phase) was acheived by high speed centrifugation, essentially according to the method of Schmidt *et al.* (1956). Liquid yolk was centrifuged at 300,000 × G for 1 hr in a preparative ultracentrifuge. The temperature during centrifugation was held at 25–30° C. The clear supernatant yolk plasma was carefully decanted.

The gel and soluble proteins obtained by formic acid delipidation of yolk plasma (designated FG and FS, respectively) were prepared as described earlier (Mahadevan *et al.*, 1969).

Phospholipase-C (EC. 3.1.4.3) from *Clostridium welchii* was a commercial sample of Sigma Chemical Co. (Lot 115B-1100). The crude, colored sample (2.8 units of activity per mg per min) was purified by chromatography on DEAE-cellulose. DEAE-cellulose (S&S 0.84 meq per g) was regenerated by soaking in 2M K_2HPO_4 overnight and washed exhaustively with distilled water until free of phosphate. Regenerated DEAE-cellulose was packed in a column of 28 × 1 cm and equilibrated with tris buffer pH 7.2, 0.05 M. Two hundred milligrams of crude phospholipase-C enzyme powder was dissolved in 5 ml of the same buffer, centrifuged to remove insoluble matter, and placed on the column. Elution was carried out using a linear gradient of sodium chloride, 0 to 0.2 M, in the same buffer. Three milliliter portions of the eluate were collected in an automatic fraction collector. All operations were carried out in a cold room (4–8 °C). Activity of each fraction was tested by incubating aliquots of each 3 ml fraction with tris buffer pH 7.3, 0.1 M containing 0.0001 M calcium chloride and using egg yolk lecithin (12 mg per assay) as substrate in a total volume of 1.5 ml. The reaction was stopped by the addition of 5 ml of ethanol:ether 1 to 2 solvent. The ether layer was discarded and 0.5 ml of the aqueous layer was used for assay of organic phosphorus. Phosphate estimation was carried out by the procedure of Marinetti (1962). Phospholipase-C activity was eluted between the sodium chloride concentrations of 0.03 and 0.075 M and was resolved into three separated peaks (Fractions I, II, and III). Fraction II was purified tenfold over the specific activity of the starting material. Similar resolution of phospholipase-C into three fractions has been reported by others on DEAE-cellulose chromatography of phospholipase-C from *C. perfringens* (Ispolatovskaya and Levdikova, 1962; Macchia and Pastan, 1967). No proteolytic activity was observed in these fractions when tested with casein as substrate. Each fraction was dialyzed and lyophilized. For most experiments reported in this paper, Fraction II of the purified phospholipase-C was used.

Analytical Methods. All analytical methods employed were the same as described earlier (Mahadevan *et al.*, 1969). *N*-terminal amino acid analyses of the protein samples were carried out by the FDNB method essentially according to the procedures described by Fraenkel-Conrat *et al.* (1955). The ether soluble DNP-amino acids were separated and identified by the conventional two-dimensional paper chromatographic

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procedures. The aqueous phase was analyzed for DNP-arginine by the method of Sampath Kumar *et al.* (1963). The identity of DNP-arginine was confirmed by its positive Sakaguchi reaction.

RESULTS

Delipidation of Yolk Plasma by Phospholipase-C Treatment and Solvent Extraction. PHOSPHOLIPASE-C TREATMENT. Twenty grams of yolk plasma were diluted with 40 ml of sterile water. Penicillin G and Streptomycin (700 units/mg) were added to give a final concentration of 0.02% w/v to prevent bacterial growth. The pH was adjusted to 7.2 with NaOH. Purified phospholipase-C 3.07 mg in 1.0 ml solution and 1.0 ml of 2% w/v solution of calcium chloride were added in four equal parts over a period of 2 hr to the diluted yolk plasma solution. The pH was maintained automatically in a pH-stat (Radiometer - Model TTT 1) by the addition of 0.91 *N* sodium hydroxide solution. At the end of 150 min, when the reaction had almost come to 0, the amount of alkali consumed accounted for about 95% of the total phospholipid present in the sample of yolk plasma used. During the reaction the solution of yolk plasma changed from an opalescent fluid to a viscous curdy liquid. The reaction mixture was left in the cold room overnight and then centrifuged in the cold at 15,000 rpm for 30 min in a Sorvall centrifuge. Two fractions, yellow insoluble cake (I) and a clear subnatant (II), were obtained. The subnatant (II) was carefully removed with a syringe, recentrifuged to remove any adhering insoluble material, and the clear liquid dialyzed against water in the cold and lyophilized. The total protein of this fraction, designated PLC-S (soluble proteins isolated by Phospholipase-C treatment), was found to be 0.45 g. The floating cake (I) was washed three times with cold water to remove any soluble protein and was suspended in water, dialyzed, and lyophilized. The weight of the lyophilized material was 7.6 g. The washings were pooled and the protein content was determined. The insoluble (I) and soluble (II) fractions contained 73% and 27%, respectively, of the total proteins of yolk plasma. Similar percentages of insoluble and soluble proteins were obtained in two more experiments.

The insoluble cake (I) contained free as well as bound lipid material. Phospholipase-C treatment removes only the phosphorylcholine of lecithin, leaving the diglyceride part associated with the proteins. To remove these diglycerides and other neutral lipids, a mild solvent extraction procedure using *n*-heptane was adopted. *n*-Heptane has been used for delipidation of lipoproteins (Gustafson, 1965).

Six grams of lyophilized (I) were suspended in 100 ml of chilled (−15° C) *n*-heptane and stirred on a magnetic stirrer for 30 min. The mixture was centrifuged at −15° C for 10 min at a speed of 2000 rpm in a MSE-Mistral centrifuge, and the heptane layer decanted off. This process of extraction was repeated four times. The heptane extracts, of which the last two were colorless, were combined and flash evaporated at 30° C. The residual lipid after drying *in vacuo* over P_2O_5 weighed 4.8 g, which accounted for about 90 to 95% of the total lipid of yolk plasma. The heptane-extracted (I) still retained a yellow color, indicating the presence of some lipids not extractable by heptane. Therefore, a more polar lipid solvent, 1-butanol, was used for removing these lipids. 1-Butanol has been used for removing lipids from membranes, mitochondria, and enzymes (Morton, 1950).

Heptane-extracted (I) was stirred with three 100-ml portions of butanol at −15° C for 30 min and after each extraction the mixture was centrifuged as before. The colorless sediment was suspended in cold ether (−15° C) and quickly filtered through a Büchner funnel under suction to remove traces of butanol. The dry powder, designated PLC-G (gel proteins isolated by Phospholipase-C treatment), was insoluble in several buffers, weak acid and alkaline solutions, and was soluble in 0.5% w/v sodium dodecyl sulfate solution. In these respects the PLC-G proteins resembled the gel proteins (FG) isolated by the formic acid treatment of yolk plasma (Mahadevan *et al.*, 1969). The butanol extracts were combined and flash-evaporated. The dried lipids, weighing 0.25 g, accounted for about 5% of total lipids. The heptane-extracted lipids contained 6% cholesterol and no lipid phosphorus, whereas the butanol-extracted lipids contained 10% cholesterol and 0.9% lipid phosphorus. Thin-layer chromatography indicated that the phospholipid in the butanol extract was chiefly phosphotidylethanolamine with traces of lecithin.

Table I. Analyses of Gel and Soluble Protein Fractions Obtained by Phospholipase-C Treatment of Yolk Plasma Followed by *n*-Hexane and 1-Butanol Extractions

Constituents Analyzed	Gel Proteins (PLC-G)	Soluble Proteins (PLC-S)
Lipids	% Dry Weight	
Total lipid	11.60	1.80
	% Total Lipid	
Total cholesterol	0.305	0
Lipid phosphorus	0.14	0
Carbohydrates[a]	% Dry Weight	
Hexoses	2.10	3.60
Sialic acid	0.47	1.42
Hexosamine	1.46	2.50

[a] Average values of duplicate samples analyzed.

Chemical Composition of PLC-G and PLC-S Proteins. The PLC-G and PLC-S proteins were analyzed for their lipid content by extracting with ethanol:ether solvent mixture. In Table I the lipid content and composition of lipids extracted from PLC-G and PLC-S proteins are given.

The soluble proteins (PLC-S) isolated by the procedure described in this paper contained negligible lipids, unlike the soluble proteins (FS) obtained by the formic acid delipidation procedure described earlier. The presence of 12% lipids in the PLC-G proteins containing 2.6% phospholipids and about 0.3% cholesterol is similar to the lipid composition of the gel proteins (FG) obtained by treatment with formic acid for 24 hr (Mahadevan *et al.*, 1969). This probably constitutes the tightly bound "core lipids" of the lipoproteins which are not easily extractable by these procedures.

The totally delipidated PLC-G and PLC-S proteins were analyzed for their carbohydrate contents (Table I). A comparison of the contents of hexoses, hexosamine, and sialic acid of the PLC-G fraction with the FG proteins indicates that they are similar except for the hexosamine content, which is slightly higher in the PLC-G proteins. However, the PLC-S proteins have much higher contents of hexoses, hexosamine, and sialic acids than the FS proteins. The absence of lipids in the PLC-S proteins shows that they are free of lipoproteins and may constitute only the pseudoglobulins of the yolk, the livetins. The high carbohydrate content of the PLC-S proteins supports this view, since β-livetin is known to be rich in these carbohydrates (Williams, 1962).

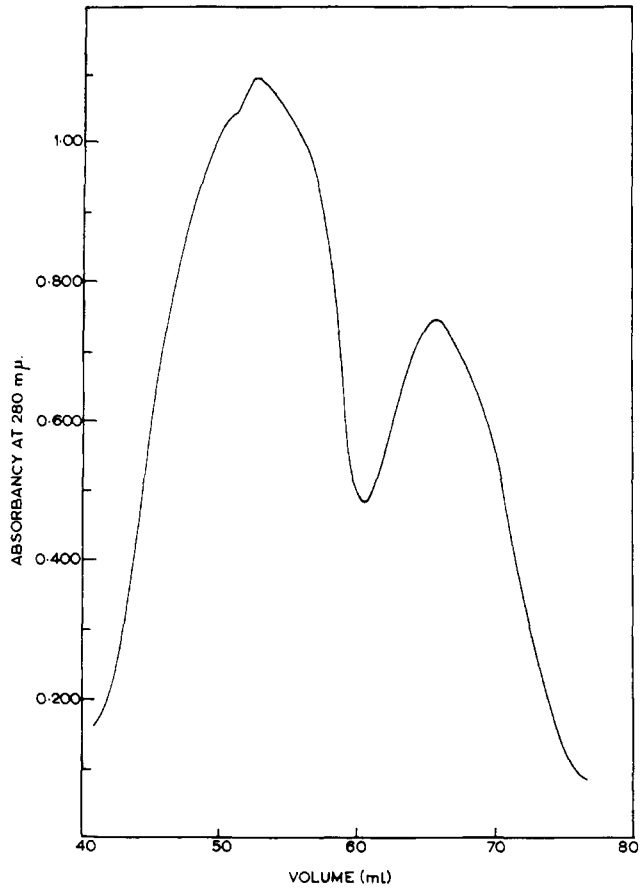

Figure 1. Molecular sieve filtration of PLC-G fraction on Biogel P-200. Void volume was 47 ml

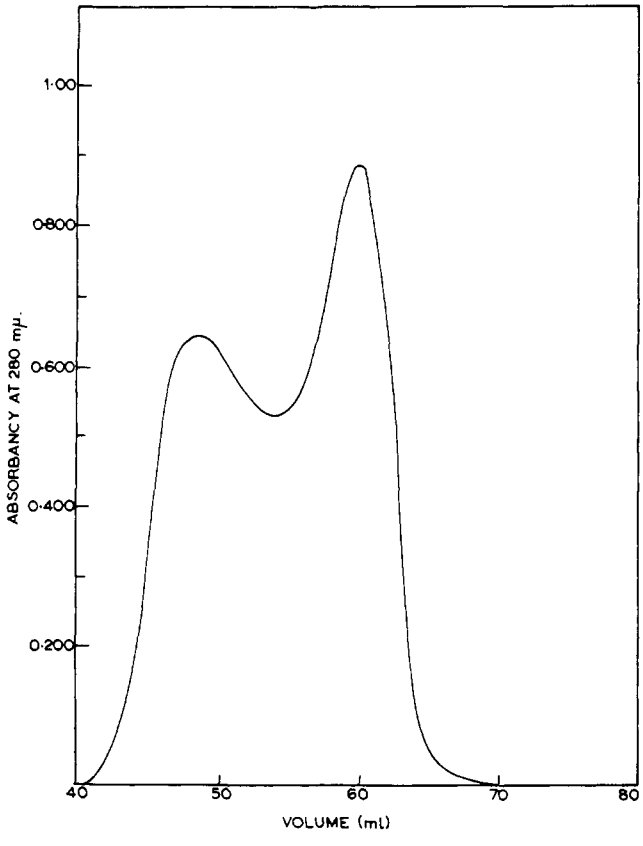

Figure 2. Molecular sieve filtration of PLC-S fraction on Biogel P-200. Void volume was 45 ml

Table II. *N*-Terminal Amino Acids of Egg Yolk Plasma Proteins

Protein Fractions	*N*-Terminal Amino Acids[a] Major	Minor
PLC-G	Lys, Arg	Ala, Ser
PLC-S	Ala, Arg, Lys	Ser
FG	Lys, Arg	Ala, Ser
FS	Lys, Arg	Ala, Ser

[a] Traces of aspartic and glutamic acids were noticed in all samples.

Table III. Percentage Composition of Protein Components in Biogel Fractionated PLC-G and PLC-S Proteins

Fraction	PLC-G %	PLC-S %
Fraction I	66	53
Fraction II	34	47

The *N*-terminal amino acids are given in Table II. All the proteins contained lysine and arginine as major *N*-terminal acids, except PLC-S, in which alanine was also a major *N*-terminal acid. These results are in agreement with those reported by Cook and Martin (1962) for low density lipoproteins and Cook (1961) for livetins.

Molecular Sieve Filtration on Biogel P-200. Gel filtration of PLC-G and PLC-S proteins through Biogel P-200 equilibrated with 0.5% w/v sodium dodecyl sulfate(SDS) was carried out as described earlier (Mahadevan *et al.*, 1969). One hundred milligrams of PLC-G and 50 mg of PLC-S proteins were separately dissolved in 10% w/v SDS solution, dialyzed against 0.5% w/v SDS solution, and separately chromatographed on Biogel columns of 2 cm diameter and 66 cm height. The elution patterns are given in Figures 1 and 2. With both PLC-G and PLC-S proteins, two components were separated, one eluting along with the void volume, and another which was slightly retarded. The relative amounts as measured by the areas under each peak are given in Table III. The elution patterns of PLC-G and PLC-S proteins on Biogel P-200 were different from those obtained for the FG and FS proteins reported earlier (Mahadevan *et al.*, 1969). The fraction that was excluded in the PLC-S proteins was less than the corresponding fraction in the FS proteins. This again substantiates the observation that whereas the PLC-S proteins were free of lipoproteins, the FS proteins contained partially delipidated lipoproteins.

Agar-Gel Electrophoresis of PLC-G and PLC-S Proteins. The PLC-G proteins had three components with mobilities similar to the corresponding bands of FG proteins. However, a component moving faster than reference BSA, which was present in FG proteins, was absent in PLC-G proteins. The PLC-S proteins had four components which were electrophoretically similar to the FS proteins (Figure 3).

Aggregating Properties of Yolk Plasma Proteins. Cleavage or alterations in lipid-protein bonds, either by formic acid treatment or by phospholipase-C action, resulted in the formation of gels. To determine the role of proteins in this phenomenon, gel and soluble proteins obtained by formic acid treatment (FG and FS proteins, respectively) and those obtained by phospholipase-C treatment (PLC-G and PLS-C proteins, respectively) were completely delipidated using chloroform:methanol (2:1, v/v) solvent extraction procedure. Such delipidated proteins dissolved in formic acid (98 to 100%) give water-clear solutions. However, when enough water was added to these solutions in formic acid, so as to bring the concentration of formic acid to 50% v/v, the gelling proteins

(FG and PLC-G) formed gels within a few hours, whereas the soluble proteins (FS) remained a viscous liquid. There was no change in the formic acid solution of PLC-S proteins. To study the role of solvents other than water in this process, dimethyl sulfoxide (DMSO) and dimethyl formamide (DMFA), which are known to substitute for water in certain hydration processes, were tested. When water was replaced either by DMSO or DMFA for diluting the formic acid solution in the above experiment, instantaneous gels were formed by FG, FS, and PLC-G proteins, whereas the PLC-S proteins remained in solution even after many hours. Several other proteins, as shown in Table IV, were tested similarly to determine whether this reaction is a general property of all proteins which are aggregating in nature. This phenomenon of instantaneous gelation under the conditions described above is not a property of proteins in general but appears to be a specific property of aggregating proteins such as γ-globulin, fibrinogen, etc.

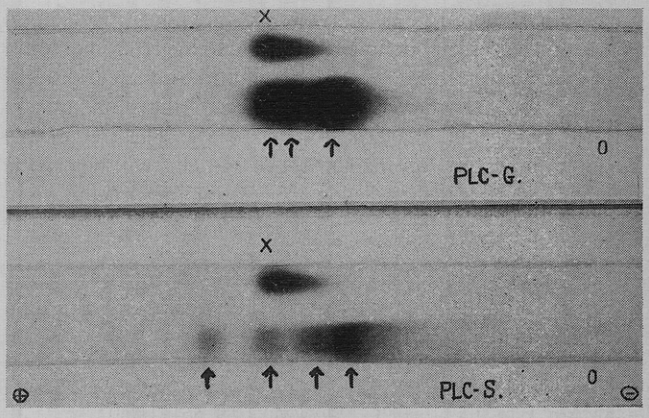

Figure 3. Agar electrophoresis of PLC-G and PLC-S proteins

O refers to origin, × refers to bovine serum albumin (BSA) reference spot, arrows indicate positions of component separated

DISCUSSION

Low density lipoproteins of egg yolk plasma were shown to aggregate, resulting in the formation of gels, when the lipids were removed (Mahadevan *et al.*, 1969). The investigations reported here suggest that the integrity of the structure of lipoproteins of the the yolk plasma is maintained by the phospholipids, since treatment with phospholipase-C induces gelation. Nearly 95% of the phospholipids present in yolk plasma was accessible to the action of the enzyme, suggesting that the phospholipids of the lipoproteins of the yolk plasma are situated on the surface of the molecule. Similar observations have been made by Burley and Kushner (1963). The fact that removal of the ionic part of lecithin molecule induced gelation suggests that the process of aggregation is primarily an ionic interaction. A similar conclusion drawn by Nowak *et al.* (1966) was based on dye-binding studies on the low density lipoproteins of egg yolk. Aggregation, however, may be aided by the association of nonpolar sites which were originally kept apart by the charged groups. The lipoproteins rendered insoluble by the action of phospholipase-C were solubilized in a solution of sodium dodecyl sulfate. Possibly the ionic part of the SDS molecule reacts with that area of the apolipoprotein molecule which was associated with the ionic part of the lecithin molecule in the native lipoprotein, thus preventing interactions between protein molecules. The solubility of low density lipoproteins in water has been attributed to the presence of polar groups of both protein and phospholipid moieties at the surface of micellar lipoproteins (Scanu, 1965). Observations reported in this paper are in conformity with this view. However, Augustiniak *et al.* (1964) believe that a certain proportion of both phospholipid and neutral lipid is required to maintain low-density lipoproteins in an orientation that confers aqueous solubility.

The insoluble fraction of the phospholipase-C treated yolk plasma upon delipidation by extraction with cold heptane and butanol was found to contain some lipids. These lipid moieties are probably the "core lipids" enmeshed in the protein chains and are extractable only by very drastic treatments using ethanol:ether solvent mixture which will denature the proteins.

The absence of lipids in PLC-S fraction indicates that the proteins in this fraction are the nonlipoprotein components of egg yolk plasma, *viz.*, the livetins. This conclusion is supported by the observation that the PLC-S proteins are rich in carbohydrates, which is characteristic of livetins, (Williams, 1962).

Table IV. Gelling Properties of Proteins

Proteins Tested[a]	Formation of Gels
Egg yolk plasma (low density lipoproteins)	+
Egg yolk granules[b] (high density lipoproteins)	+
Formic-gel fraction	+
PLC-G fraction	+
Formic-soluble fraction	+
PLC-soluble fraction	−
Bovine γ-globulin	+
Bovine fibrinogen	+
Bovine serum albumin	−
Bovine hemoglobin	−
Casein	−
Gelatin	−
Ovomucoid	−
Kaffirinin	−

[a] 40 mg of each protein was dissolved in 0.4 ml of formic acid (98–100%). 0.4 ml of DMSO was added, mixed quickly, and immersed in a cold water-bath 4° C. Gel formation was visually observed. [b] Egg yolk granules were prepared as described earlier (Mahadevan *et al.*, 1969).

The results shown in Table IV indicate that whereas the apolipoproteins derived from the low-density lipoproteins of yolk plasma have an intrinsic property of forming aggregates, the PLC-S proteins, which are the pseudoglobulins, do not have this property. The delipidated yolk granules, consisting chiefly of apolipoproteins of yolk, also form gels under these conditions. Possibly lipoproteins in general have the tendency to form insoluble aggregates upon delipidation. Such insolubility of apolipoproteins have been observed by many workers (Granda and Scanu, 1966; Martin *et al.*, 1959). Mahadevan *et al.* (1969) reported that yolk granules suspended in water do not form gels upon freezing and thawing. In the light of the above observations, it may be concluded that freezing and thawing does not cause sufficient rupture of lipoprotein bonds so as to induce gelation of granules of yolk. The gelation of FS proteins in this experiment again substantiates the observations that the FS proteins contain partially delipidated low density lipoproteins. The observation that PLC-S proteins do not gel under these conditions supports the view that they are free of lipoproteins.

The results of investigations reported in this paper and in the earlier paper (Mahadevan *et al.*, 1969), indicate that ag-

gregation of lipoproteins of yolk plasma takes place upon partial or complete delipidation. Such aggregation could yield either precipitates or gels, depending on the conditions. Gels are formed when large quantities of the fluid are entrapped between the aggregated particles. The pH of the medium and charge on the surface of proteins will determine whether the aggregation leads to gelation or precipitation.

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