Physico-Chemical Studies on the Gelation of Hen's Egg Yolk

Separation of Gelling Protein Components from Yolk Plasma

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A study of the component(s) in egg yolk responsible for gelation of yolk on freezing and thawing has shown that granule-free yolk plasma, obtained by high-speed centrifugation of yolk, has the capacity to gel. As with the whole yolk, gelation of yolk plasma on freezing and thawing could be inhibited by additives such as sugars, sodium chloride, proteolytic enzymes, and phospholipase-A. Phospholipase-C, which induces gelation of whole yolk at room temperature, has a similar effect on yolk plasma. Yolk plasma has been separated into aggregating (gelling) and soluble fractions by delipidation, using formic acid. Each of these fractions consists of three or four protein components, as observed by gel filtration, ultracentrifugation, and agar electrophoresis. The proteins are glycoproteins and contain bound hexoses, hexosamine, and sialic acid. The gelation of yolk has been attributed to the interactions between protein molecules following disruption of lipid-protein bonds.

One of the striking structural changes that take place in biological fluids upon freezing and thawing is the gelation of hen's egg yolk fluid when stored frozen below $-6°$ C. and thawed (Lopez et al., 1954; Moran, 1925). The gelation has been attributed to structural changes in the lipoproteins of egg yolk, primarily in the low-density lipoproteins or lipovitellenins (Fevold and Lausten, 1946; Powrie et al., 1963; Saari et al., 1964). The inhibition of gelation by proteolytic enzymes such as papain, chymotrypsin, trypsin (Lopez et al., 1955), and phospholipase-A (Feeney et al., 1954), but not by ribonuclease (Marion, 1958), lipase or erepsin (Lopez et al., 1955), and the induction of gelation by treatment with phospholipase-C at room temperature (MacFarlane and Knight, 1941; Mahadevan and Kumar, 1967), suggest that lipoproteins are involved in gelation. Addition of certain compounds such as sodium chloride, glycerol, sugars, and other polyhydroxy compounds to whole yolk inhibits gelation to varying degrees (Lopez et al., 1954).

During investigations on the phenomenon of gelation of hen's egg yolk, a group of proteins obtained from yolk plasma was shown to have the property of aggregation resulting in the formation of gels. These studies are reported in this paper.

MATERIALS AND METHODS

Material. One- to 2-day-old unfertilized White Leghorn eggs were procured from a local market.

Egg yolk fluids (lipid yolk) were obtained by the procedure of Powrie et al. (1963).

Separation of yolk granules from plasma (soluble phase) was achieved by high speed centrifugation, essentially according to the method of Schmidt et al. (1956). Liquid yolk was centrifuged either at 20,000 × G for 12 hours or at 300,000 × G for 1 hour in a preparative ultracentrifuge. The temperature during centrifugation was held at 25° to 30° C. The clear

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supernatant yolk plasma was carefully decanted. The sedimented granules were resuspended in five volumes of water, and resedimented by centrifugation. The resedimented granules were washed further by two repetitions of the foregoing procedure.

Analytical Methods. Protein was determined either by the biuret method (Gornall et al., 1949) or by using Folin's reagent (Lowry et al., 1951) with crystalline bovine serum albumin as the standard. Nitrogen was determined by the micro-Kjeldahl method, and lipid and protein phosphorus (Marinetti, 1962), hexose and hexosamine (Winzler, 1961), and sialic acid (Saifer and Gerstenfeld, 1962) were measured by published methods. Thin-layer chromatography of phospholipids was carried out by the standard procedure on silicic acid plates using chloroform–methanol–water (30:6:0.2). The spots were visualized by exposure to iodine vapors and identified with reference to authentic samples of phospholipids. Total lipid content was estimated by extraction of the sample with ether-ethanol (3 to 1) and determining the dry weight of the extracted lipids. Total cholesterol was determined by the method of Liebermann and Burchard (Stadtman, 1957). Amino acid analysis was carried out as follows. Protein samples were hydrolyzed with constant-boiling HCl in sealed, evacuated tubes at 105° C. for 22 hours. Hydrochloric acid was removed from the hydrolyzate by evaporation in vacuo and the residues were made up to 10 ml. in $0.2M$ citrate buffer, pH 3.26. Amino acid analysis was carried out by ion exchange chromatography using a Beckman amino acid analyzer, according to the method of Spackman et al. (1958). Results are expressed as micromoles of amino acid per 100 grams of protein. Corrections for the destruction of amino acids such as cystine, serine, and threonine have not been made.

Analyses of protein samples by ultracentrifugation were performed in a Spinco Model E analytical ultracentrifuge. The sedimentation constants were calculated according to Schachman (1957). Gel filtration through Biogel P-200 was carried out according to standard procedures. Biogel P-200 (50- to 100-mesh) was soaked in 0.5% sodium dodecyl sulfate for at least 24 hours and deaerated before packing into a column of 2-cm.

diameter to a height of 66 cm. A layer of Sephadex G-25, coarse grade and 0.5 cm. in depth, was placed on top of the Biogel bed to afford stability to the surface. Biogel chromatography was carried out at room temperature. Void volume was determined by using blue dextran 2000 (Pharmacia). Other pertinent details are given in the appropriate context. Agar gel electrophoresis of proteins was carried out according to the method of Giri (1956) in veronal buffer, pH 8.6, ionic strength 0.025, and containing 0.25% sodium dodecyl sulphate (SDS).

RESULTS

Gelation of Yolk Plasma. Initial experiments showed that plasma stored frozen below $-8°$ C. was gelled upon thawing. However, it was not gelled upon thawing when the plasma was rapidly frozen to $-150°$ C. in liquid oxygen, even after keeping it for several hours at that temperature. To determine whether granules had the property of gelation, they were dispersed in an equal weight of water, stored frozen at $-20°$ C. for 24 hours, and thawed. There was no gelation, although plasma had formed a gel under exactly similar conditions. Addition of monosaccharides such as glucose, galactose, or ribose at 6% w./w., disaccharides (sucrose, maltose, etc.) at 12% w./w., and sodium chloride at 3% w./w. inhibited gelation of the whole yolk, and also of the plasma. Methanol at 3% w./w., but not ethanol at 5% w/w., inhibited gelation of yolk plasma. Proteolytic enzymes such as crystalline trypsin, chymotrypsin, and papain, when added at 0.15% w./w. level to yolk plasma and incubated at 25° C. for 2 hours, arrested gelation upon subsequent freezing and thawing. Addition of crude phospholipase-A, ex Russel's viper venom, at 0.15% w. w. level to yolk plasma, followed by incubation at 25° C. for 2 hours, resulted in an opaque but free-flowing liquid after freezing and thawing, in contrast to the translucent gel obtained with untreated yolk plasma control. Phospholipase-C, ex *Clos. weichii*, at the same level induced gelation of yolk plasma during 30 minutes of incubation at room temperature, even without freezing. These results indicate that the fraction of yolk that is responsible for gelation upon freezing and thawing is the yolk plasma.

Delipidation of Yolk Plasma Using Formic Acid. In view of the observations that proteolytic enzymes inhibit gelation and that phospholipase-C induces gelation of yolk plasma, it was thought that gelation is probably an intrinsic property of the proteins of yolk plasma and that gelation is induced by the dissociation of lipids. Several organic solvents and organic acids, such as acetic and formic acid, have been used for the delipidation of many membrane lipoproteins (Green, 1961; Weber, 1963). Formic acid has also been used for solubiliz: g lipid-free low-density lipoproteins of egg yolk (Martin *et al.*, 1959). While glacial acetic acid is not suitable for delipidating yolk plasma, formic acid (98 to 100% w./w.) is a good delipidating agent. Available evidence (Josefsson and Edman, 1957; Martin *et al.*, 1959; Narita, 1959; Smillie and Neurath, 1959) indicates that peptide bonds are not cleaved and extensive denaturation does not take place in formic

acid solutions. Hence, formic acid was used for obtaining delipidated yolk plasma proteins.

Yolk plasma, 20 grams, was added drop by drop with continuous stirring to 60 ml. of formic acid (Merck, 98 to 100%), precooled to 4° C. After addition (the final concentration of formic acid being 85 to 86%), the mixture was left at 0° C. for 2, 24, or 36 hours. At the end of this period, the mixture was centrifuged at 20,000 $\times$ G for 30 minutes in a refrigerated centrifuge. A top yellow lipid layer and a clear formic acid subnatant were separated. The clear formic acid layer was carefully withdrawn, recentrifuged to remove traces of floating lipids, and dialyzed in the cold against 80 liters of running distilled water, or until the pH inside the dialysis bag had reached 5.5. At this stage the dialysis bag contained two phases, a liquid on top (the soluble fraction) and a firm gel (the gel fraction) at the bottom. The contents of the dialysis bag were mixed thoroughly in a beaker and centrifuged at 20,000 $\times$ G for 30 minutes. The clear supernatant (soluble fraction) was poured off and the gel fraction was washed three times with three volumes of cold water. The washings were separately pooled and the protein contents of the gel, the soluble fractions, and the washings determined. Table I gives the protein contents of the gel and soluble fractions of the delipidated yolk plasma. The gel and soluble fractions thus obtained were lyophilized and stored in the cold for further studies.

Chemical Composition of Gel and Soluble Proteins. The gel and soluble proteins contained lipids which could be extracted with ether-ethanol solvent mixture. In Table II the lipid content and composition of lipids extracted from gel and soluble fractions are given.

Examination of the lipids by thin-layer chromatography indicated that the phospholipids consisted mainly of phosphatidyl choline with traces of phosphatidyl ethanolamine, and some neutral lipids.

The totally delipidated gel and soluble protein fractions were analyzed for their nitrogen, phosphorus, hexoses, hexosamine, and sialic acid contents (Table II).

Abraham *et a¹.* (1960), who have analyzed the contents of hexoses, hexosamine, and sialic acid in low density lipoproteins of hen's egg yolk having an S_f value of 25 to 30, have given a ratio of 1:0.5:0.3 for these constituents. Although the absolute concentrations shown in Table II are somewhat higher, their ratio works out as 1:0.46:0.21, which is comparable to that given by Abraham *et al.* (1960).

The amino acid compositions of the totally delipidated gel and soluble protein fractions are given in Table III. Sulfur-containing amino acids—viz., half cystine and methionine—were low in both fractions, particularly in the gel fraction. The contents of amino acids containing hydrophobic side chains such as valine, leucine, isoleucine, and phenylalanine were high in both fractions. The observed amino acid compositions were similar to those reported for lipoproteins.

In comparing the amino acid compositions of the gel and soluble protein fractions, gross similarities were found between the two fractions. Both fractions were rich in aspartic and glutamic acids, as well as lysine and arginine.

Molecular Sieve Filtration on Biogel P-200. One of

Table I. Protein Contents of Gel and Soluble Fractions Isolated by Formic Acid Treatment

Treatment with Formic Acid, Hours	G./100G. Yolk Gel Fraction, Plasma	G./100G. Yolk Soluble Fraction,[a] Plasma
2	4.86 (53.4)[b]	4.23 (46.6)
24	4.89 (56.3)	3.80 (43.7)

[a] Includes the washings of gel fraction.
[b] Figures in parentheses indicate per cent of total protein.

Table II. Content and Composition of Gel and Soluble Fractions

Constituents Analyzed	Per Cent of Dry Weight			
	Gel Fraction		Soluble Fraction	
	2 hr.[a]	24 hr.[b]	2 hr.[a]	24 hr.[b]
Lipids				
Total lipids	18.90	7.20	25.40	14.50
Lipid phosphorus	0.18	0.11	0.44	0.35
Phospholipid[c]	4.50	2.70	11.00	8.70
Total cholesterol	0.98	0.38	1.42	0.56
Nitrogen, Phosphorus, and Carbohydrate Contents				
Protein nitrogen	14.27	14.30	14.20	14.50
Protein phosphorus	0.11	0.08	0.05	0.06
Hexoses	2.25	2.42	2.38	2.37
Hexosamine	1.02	1.12	1.07	1.26
Sialic acid	0.46	0.43	0.69	0.65

[a] Formic acid treatment for 2 hours.
[b] Formic acid treatment for 24 hours.
[c] Phospholipid = lipid P × 25.

Table III. Amino Acid Composition of Gel and Soluble Protein Fractions

Amino Acid	Mmoles/100 G. Protein	
	Gel Protein[a]	Soluble Protein[a]
Lysine	59.6	50.3
Histidine	13.2	12.3
Arginine	38.6	38.1
Aspartic acid	96.4	94.0
Threonine	53.8	46.9
Serine	74.7	69.7
Glutamic acid	101.2	99.6
Proline	27.9	31.6
Glycine	43.8	45.6
Alanine	54.9	67.7
Half cystine	7.6	20.5
Valine	48.0	52.5
Methionine	17.1	17.7
Isoleucine	42.3	45.5
Leucine	83.3	76.6
Tyrosine	24.5	28.2
Phenylalanine	29.8	32.3

[a] For this experiment, gel and soluble fractions were prepared by treatment with formic acid for 24 hours.

the major experimental difficulties in the characterization of apolipoproteins is their insolubility (Granda and Scanu, 1966; Martin et al., 1959). A suitable solvent was therefore necessary for the physical characterization of the gel protein fraction. The following compounds were tested for solubilizing the gel proteins—sodium dodecyl sulfate, dibutyl sodium sulfosuccinate, dioctyl sodium sulfosuccinate, n-hexylpyridinium bromide, and cetyl trimethyl ammonium bromide. Of these, only two compounds, an anionic detergent, sodium dodecyl sulfate, and a cationic detergent, cetyl trimethyl ammonium bromide, were able to solubilize the gel fraction. Sodium dodecyl sulfate is commonly used in

studies on the physical characterization of proteins. Such detergents are commonly used for solubilizing delipidated membrane lipoproteins (Bakerman and Wasemiller, 1967; Thornber et al., 1967). Sodium dodecyl sulfate has been used in particular for the solubilization of delipidated low-density lipoprotein from human serum (Granda and Scanu, 1966). Accordingly, sodium dodecyl sulfate was used for solubilizing the gel protein fraction. Soluble protein fraction was also dissolved in SDS for comparative purposes.

One hundred to 200 mg. of gel or soluble fraction were dissolved in 2 ml. of 10% SDS and dialyzed against 500 ml. of 0.5% SDS for 18 hours or more. These were placed on Biogel columns described earlier, and eluted with 0.5% SDS at a flow rate of about 1 ml. per 20 minutes. One-milliliter fractions were collected and the absorbance at 280 mμ was recorded. Figures 1 and 2 illustrate typical elution patterns of gel and soluble fractions. The patterns for the gel fraction and for the soluble fraction were qualitatively similar, but quantitatively different. A major peak came with the void volume, which was followed by a shoulder, and finally a well-separated peak. The relative amounts, as measured by the areas under each peak (Figures 1 and 2), were 58:31:11 for the gel fraction and 40:27:33 for the soluble fraction.

Ultracentrifugal Analysis of Gel and Soluble Protein Fractions. For these studies, delipidated gel and soluble protein fractions were dissolved separately in 0.2 ml. of formic acid (98 to 100%). Urea (1.8 ml., 10M)
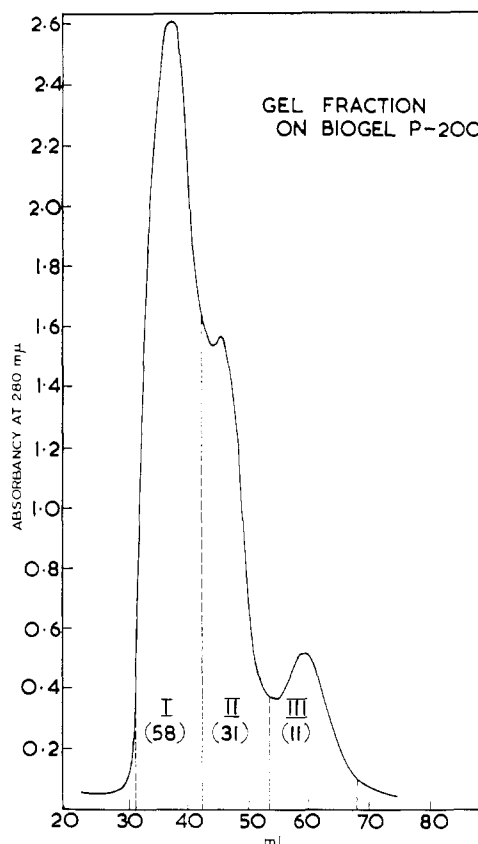

Figure 1. Molecular sieve filtration of gel fraction on Biogel P-200

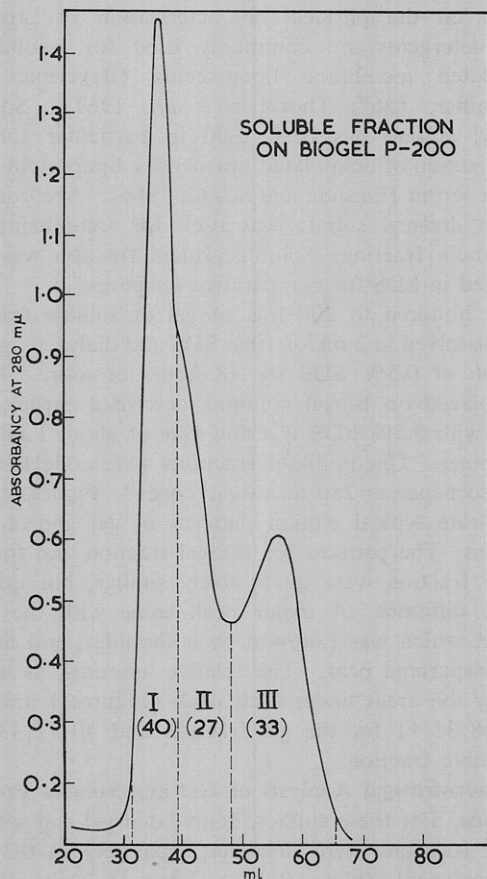Values in parentheses indicate relative per cent of each fraction

Figure 2. Molecular sieve filtration of soluble fraction on Biogel P-200

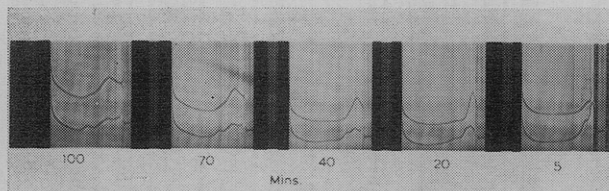Values in parentheses indicate relative per cent of each fraction

Figure 3. Sedimentation patterns of lipid free gel and soluble proteins

Gel and soluble fractions were prepared by treatment with formic acid for 36 hours. Upper curves represent soluble proteins; lower curves represent gel proteins

was added to each solution to give a final concentration of 1.5%. These solutions were dialyzed for equilibration against $6M$ urea. Ultracentrifugal runs were carried out at 59.780 r.p.m., using ordinary and wedge cells. Photographs were taken at varying intervals and are reproduced in Figure 3.

There were three components in the soluble protein fraction, and possibly four in the gel protein fraction. There was a small fast-sedimenting component (Figure 3; 5- and 20-minute frames) in both gel and soluble fractions, with a S_w^{20} value of 7 to 11S. Another component (40-, 70- and 100-minute frames) with a S_w^{20} value of 3.7 to 4.5S seemed to constitute the major difference between the gel and soluble protein fractions, for whereas it sedimented out as a separate peak in the gel fraction, it constituted only a shoulder in the soluble fraction. A third component (40-, 70- and 100-minute
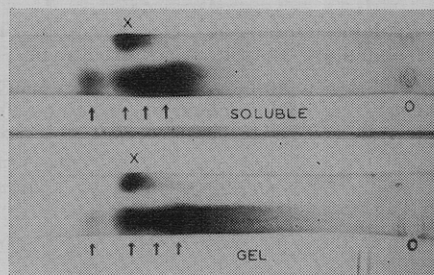

Figure 4. Agar electrophoresis of soluble and gel proteins

O refers to origin, X refers to BSA reference spot; arrows indicate positions of components separated

frames) with a sedimentation value of 1.4 to 2.0S formed the most prominent component in the soluble fraction, while it was smaller in the gel sample. A fourth component (100-minute frame) was noticed as a kink in the gel sample only.

Agar Gel Electrophoresis of Gel and Soluble Fractions. Ten-milligram lots of solvent-delipidated samples of gel and soluble protein fractions were separately dissolved overnight in 0.2 ml. of 10% SDS solution. Five to 10 μl. of this sample was placed in slots made in agar gel. A reference sample of crystalline bovine serum albumin, also dissolved in SDS, was placed in another slot. Electrophoresis was carried out for 6 to 7 hours at 250 v. and about 6 ma. per plate. At the end of this period the plates were air-dried, stained with amido black stain for 2 hours, and destained with methanol–acetic acid, 90 to 10. Photographs of these patterns are given in Figure 4.

There were four components in the soluble sample, one of them moving ahead of the BSA reference spot. This component was present only in traces in the gel sample, and could be observed only when large quantities of the sample were used. There were three distinct spots in the gel sample which were of the same mobilities as the corresponding bands in the soluble sample, one of which corresponded to the mobility of BSA reference, while the other two had lower mobilities. The component with the least mobility appeared to be most intense in both cases.

DISCUSSION

Low-density lipoproteins of egg yolk plasma have been implicated as the components responsible for gelation of yolk upon freezing and thawing. The investigations reported here confirmed this, and have demonstrated that all agents which inhibit gelation on freezing and thawing or induce gelation of whole yolk also bring about similar effects with yolk plasma. Yolk granules do not appear to have the property of gelation upon freezing and thawing under conditions where plasma gels. It is, therefore, established that yolk plasma is the component of yolk which has the property of gelation.

The separation of aggregating and nonaggregating species of proteins by delipidation using formic acid indicates that there are soluble protein components in the yolk plasma which do not aggregate. It is probable that the soluble proteins contain the nonlipoprotein components of the yolk plasma—viz., the livetins

(McCully et al., 1962). However, the presence of protein-bound lipids in this fraction suggests that there are also partially delipidated lipoproteins which do not aggregate under these conditions. These may be the partially delipidated forms of $FLPL_1$ and $FLPL_2$ lipoproteins described by Saari et al. (1964), which were obtained by them in a nonaggregated form from a mass of low-density lipoproteins gelled by freezing and thawing.

The gelling proteins may constitute the major lipoproteins of yolk plasma, and it appears that when the lipids are removed, the areas on the protein molecules normally bound to lipids interact with each other and form insoluble aggregates. These interactions are both nonpolar and polar in nature, since treatment with phospholipase-C, which splits off the polar moiety of lecithin, induces aggregation (Mahadevan and Kumar, 1967). It is possible, therefore, that phospholipids are mainly responsible for keeping these apolipoproteins in solution in the native state. This concept is supported by observations reported here that the gel protein is solubilized by sodium dodecyl sulfate (SDS), which has a nonpolar chain with a polar end, similar to the phospholipids. Granda and Scanu (1966) have suggested that addition of SDS would prevent the aggregation of delipidated human serum low-density lipoprotein by favoring interactions between the protein and the detergent.

The possibility that polymerization may occur due to the oxidation of cysteine residues, as in the case of gelation of proteins of concentrated milk preparations, is eliminated because of the low content of half cystine residues seen in the amino acid analysis of the gel proteins.

A review of literature of the chemical composition of lipoproteins indicates that they are in general lipoglycoproteins (Ito and Fujii, 1962; Marshall and Kummerow, 1962; Scanu, 1965). This observation is further supported in the present findings by the demonstration of protein-bound hexoses, hexosamine, and sialic acid, particularly in the gel proteins. These findings are in agreement with the analyses of low-density lipoproteins of egg yolk (Abraham et al., 1960). Although it has not been possible to assign any particular role for these carbohydrate moieties in the gelation process per se, their presence in most lipoproteins suggests that they may play an important role in the binding of lipids.

It is of interest to determine the structural features of the gelling proteins which are responsible for the binding of such quantities of lipids, as well as of their ability to aggregate so extensively upon removal of lipids.

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