ADVANCES IN PROTEIN CHEMISTRY

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Protein Gels[1]

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I. Introduction

Gels are differentiated from other systems in which small proportions of solid are dispersed in relatively large proportions of liquid by the property of mechanical rigidity, or the ability to support shearing stress at rest. The rigidity may be accompanied by viscous retardation, which delays the response to stress; it may be associated with slow deformation under constant stress, resulting in flow and permanent set; it may be reduced or destroyed under high stresses, resulting in thixotropic effects. Among these complicated mechanical phenomena, however, rigidity is the characteristic property common to all gels.

This review describes gels in which the solid component is protein; the

liquid component is usually water or an aqueous solution. Such systems include normal physiological structures, such as fibrin, vitreous humor, Wharton's jelly, and protoplasm itself, gels prepared *in vitro* from what are usually considered native proteins, such as collagen, myosin, and tobacco mosaic virus, and gels whose preparation involves some degree of chemical modification, such as those of gelatin and denatured proteins.

1. *General Theories of Gel Structure*

Attempts to explain the remarkable fact that a gel, which consists mostly of fluid, behaves as a rigid solid and yet retains many properties characteristic of the fluid component (such as compressibility, vapor pressure, and electrical conductivity, which are but little altered), have resulted in many contributions to the colloid chemical literature and numerous hypotheses concerning the nature of gel structure. Most prominent among these (*cf.* Goodeve, 1939) have been those based on (*a*) immobilization of solvent through adsorption by the solute, (*b*) the presence of a three-dimensional network of solute, and (*c*) the operation of long-range forces between solute particles. Each of these theories may be applicable to some gel systems but not to others.

a. Immobilization of Solvent by Adsorption. According to the adsorption theory, the units of the disperse phase (particles, molecules, or micelles) are surrounded by shells of immobilized solvent. Under conditions in which gels are formed, the extent of solvation is considered to be so great that the shells touch or overlap, thus immobilizing the entire system. Although some writers (Du Nouy, 1945; *cf.* von Buzagh, 1937) have continued to interpret gelation in terms of this theory, there is now good evidence that it could not be applicable to protein solutions except in very concentrated systems.

The amount of water which, in aqueous solution, is bound to a protein molecule tightly enough to accompany it in translatory and rotatory movement can be estimated from measurements of sedimentation and diffusion, viscosity, and dispersion of the dielectric constant (Oncley, 1941). The interpretation of any one of these measurements is ambiguous, involving both hydration and asymmetry, but permits setting an upper limit for hydration; for proteins whose shape is not very asymmetric, this is about 1.5 g. water per g. protein. By combining the different measurements the most probable values are found to be considerably less than 0.5 for serum albumin, lactoglobulin, hemoglobin, insulin, and egg albumin (*cf.* Bull and Cooper, 1943). Immobilization of this much water by solvation might conceivably account for gelation of a system containing 20% or 30% of protein, but hardly of a 1% gelatin

gel and certainly not of a fibrin clot prepared from a solution containing 0.004% of fibrinogen.

Furthermore, application of the solvation theory necessitates assumption of large changes in solvation when gelation occurs. In the case of gelatin, this means an enormous increase in hydration upon lowering the temperature a few degrees in a particular temperature range, which would be difficult to understand. In the case of denatured protein gels, it means an increase in hydration upon denaturation, which is inadmissible; denatured proteins are *less* hydrophilic than their native precursors (Neurath, Greenstein, Putnam, and Erikson, 1944; Bull, 1944).

Additional evidence against immobilization of solvent by adsorption in dilute protein gels is found in rates of diffusion and electrical transport of salts through gelatin and fibrin (Kraemer, 1931; Gelfan, 1930), which differ but little from values obtained for the same systems before gelation; evidently there are interconnecting regions, large compared with ionic diameters, where the solvent has its normal viscosity and cannot, therefore, be subject to restraining forces.

Most of the gels discussed in this review are so dilute that their properties cannot be attributed to immobilization of solvent by adsorption.

b. Three-Dimensional Networks. The postulate of a three-dimensional network of solute, or disperse phase, pervading the system can account for the properties of dilute and concentrated gels alike. The earlier concepts of a "brush-heap" or "ramifying aggregate" (Zsigmondy and Bachmann, 1912; Laing and McBain, 1920; Darke, McBain, and Salmon, 1921) have been clarified by the precise theoretical formulations by Flory (1941, 1942) of the conditions for formation of three-dimensional networks from molecules or particles carrying reactive groups which combine with each other. The kinetic theory of rubberlike elasticity (James and Guth, 1943; Flory and Rehner, 1943a) has provided further clarification in showing that the strands of such a network need not be stiff to endow the system with rigidity; a network of flexible strands resists deformation because the latter involves a decrease in entropy (see Section III, 4, *b*, page 14).

In many protein gels, including those of gelatin, fibrin, and denatured proteins, the essential structural feature appears to be a three-dimensional network. The network may be held together by primary bonds, by secondary forces localized at certain points on the molecules, or by nonlocalized secondary attractive forces. These alternatives are discussed in Section III below, together with the results of some investigations of synthetic polymers, rubbers, and cellulose derivatives which facilitate the understanding of protein systems.

c. Long-Range Forces. In certain gels, optical and X-ray evidence

shows that long-range forces between the solute particles act to hold them in a stable, regular array, spaced at surprisingly large distances. Among protein systems of this type, tobacco mosaic virus has been the most thoroughly investigated. Ordinary protein crystals have some properties in common with these highly ordered gels, which are treated in the following section.

II. Gels with Long-Range Forces

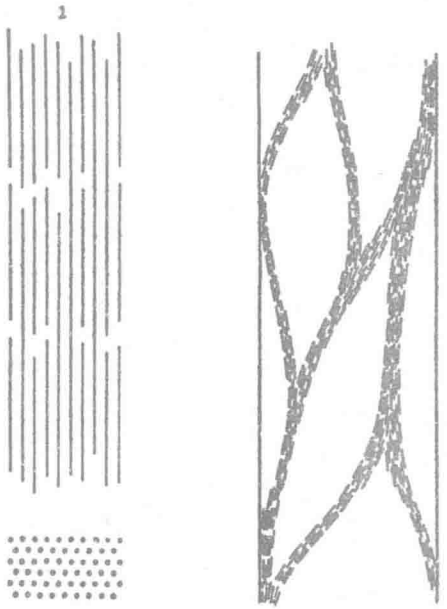
1. *Tobacco Mosaic Virus*

The molecules of tobacco mosaic virus are rods of constant diameter (150 A.) and variable length (ranging from 2700 A. to considerably higher values), as deduced from measurements of viscosity, sedimentation, diffusion, X-ray diffraction, and electron micrography (Lauffer, 1944). In aqueous solution, they associate in several different stages of aggregation, depending on the concentration, to form the following systems (Bernal and Fankuchen, 1941): (*a*) an isotropic liquid, in which no association occurs, at concentrations below 1.6 to 4% (depending on the purity of the virus), and in which the molecules can be oriented by flow; (*b*) a spontaneously doubly refracting liquid, containing, in addition to unassociated molecules, spindle-shaped tactoids (of the order of 0.1 mm. in size) in which the rodlike molecules are associated parallel to each other in hexagonal arrays, at concentrations higher than in *a* but below 34%; (*c*) a gel, formed under certain conditions in the same concentration range as in *b*, in which the tactoids are thought to have fused together here and there, converging and diverging to form a three-dimensional network; (*d*) gels ranging in concentration from about 13% up to 100% (dry virus protein) in which the hexagonal array of the molecules pervades the entire structure. Of these different states described by Bernal and Fankuchen, we are concerned here only with the gels.

X-ray diffraction photographs of fully oriented gels (*d*) demonstrate the existence of a two-dimensional lattice. There is no regularity in the direction of the axis of the molecule, but in the plane perpendicular to this axis there is a hexagonal array in which the spacing depends on the concentration of virus (Fig. 1*a*). The intermolecular distance is, in fact, proportional to the square root of the volume of solution per molecule, showing that the addition of solvent swells the lattice laterally without any other change. The quality of the diffraction photographs shows that remarkably precise regularity is maintained even at dilutions where the intermolecular distance is 460 A. Since the molecular diameter is 150 A. (in agreement with the intermolecular distance of 150 A. in a fully dried, oriented gel), when the molecule axes are 460 A. apart,

their surfaces are separated by over 300 A. The forces which hold the molecules in strict alignment at this distance certainly merit the designation "long-range."

In these fully oriented gels, easily recognizable rigidity is manifested at a concentration of about 30%, and increases with increasing concentration; its appearance is not abrupt, and it is possible that sensitive



(a) (b)

FIG. 1. Schematic diagrams of the structure of tobacco mosaic gels. (a) Fully oriented gel; (b) network gel. From Bernal and Fankuchen (1941).

rheological measurements would detect it at concentrations below 30%. At 30%, the intermolecular distance is about 300 A. and the molecular surfaces are accordingly about 150 A. apart. The rigidity may be attributed to the same long-range forces which maintain the lattice.

While addition of water to a fully oriented gel results in increased swelling of the lattice without limit and eventual dissolution, in some other solvents there is an equilibrium separation which is not exceeded. Decreasing the pH to the isoelectric point (3.4), for example, or addition of ammonium sulfate to a concentration of 3 *M*, reduces the equilibrium separation to about 180 A. with the formation of a rather densely packed hexagonal array.

When the molecules in a 4% solution of virus are oriented by flow, and the pH is lowered by addition of acid, the molecules assume their

equilibrium spatial relationships in a hexagonal lattice by associating into tactoids, which constitute a separate phase. With suitable agitation, the solution develops considerable rigidity, attributed to fusion of the tactoids into a three-dimensional network (state *c*, above; Fig. 1*b*). This type of gel is allied to the other network gels which will be described in Section III. It evidently differs from them, however, in that the coherence of the strands of the network is based on the same long-range forces which, in the more concentrated, homogeneous gels, act throughout the structure.

The only intermolecular forces which can act over the distances which the X-ray evidence reveals are coulombic. Since the virus molecules are all negatively charged in the systems described, the electrostatic forces between them should be repulsive. It is reasonable that such forces between rod-shaped molecules should lead to a hexagonal array in order to fill the available space as efficiently as possible, and that the spacing should increase progressively upon dilution, as observed for gels in the absence of salt. The existence of an equilibrium spacing in the presence of salt is more difficult to understand; it has been discussed by Levine (1939, 1941) and Hamaker (1946).

2. *Other Protein Crystals*

The swelling of the hexagonal lattice of tobacco mosaic virus upon addition of solvent, with increase in the spacing while the regularity is maintained, finds a parallel to some extent in the behavior of other protein crystals. As pointed out by Katz (1925), crystals of proteins usually contain considerable amounts of water of hydration, and when it is removed their macroscopic dimensions shrink while the crystal shapes are unchanged. X-ray measurements comparing wet and dry crystals demonstrate this shrinkage; both the dimensions and the angles of the unit cell may change as the water is removed (Fankuchen, 1945). However, the dimensional changes here are much smaller than in the case of tobacco mosaic virus. For example, lactoglobulin crystals contain 45% water by weight (McMeekin and Warner, 1942); X-ray measurements on horse methemoglobin show a water content of 42% by volume (Perutz, 1942). In the latter crystal, the molecules appear to be arranged in sheets separated by layers of water 15 A. thick (Boyes-Watson and Perutz, 1943). The forces responsible for the coherence and rigidity of the crystal thus act over distances which are small compared with those of virus gels, but rather large compared with those of crystals of substances of low molecular weight. This is probably true of most hydrated protein crystals. No rigidity measurements on protein crystals appear to have been reported.

III. Formation and Properties of Network Gels

Before describing those protein gels whose structures appear to involve three-dimensional networks, it will be useful to review the properties of some simpler network systems formed by the junction of long threadlike or rodlike molecules.

1. *Networks Bound Together by Primary Chemical Bonds*

a. Branching Polymerization of Small Molecules. When polycondensation occurs between small molecules which are bifunctional (*i.e.*, carry two reactive groups, such as, in the case of esterification, a mixture of a glycol and a dibasic acid), long chains are formed and the product is soluble in suitable solvents. If the mixture contains a substance with three or more reactive groups (such as glycerol or a tribasic acid), branched chains are formed; if the reaction proceeds far enough, the branched chains produce a network and the system gels—develops rigidity—and thereafter contains material which is insoluble in all solvents (Carothers, 1931, 1936). Flory (1941) showed by statistical theory that, when polyfunctional molecules are involved in the reaction, a branched network infinite in extent will appear suddenly after the reaction has proceeded to a certain point. This point, measured by the proportion of total groups which has by then reacted, can be calculated in terms of the proportion of polyfunctional molecules present and their functionality. When it is identified with the experimentally observed gel point, the agreement with experimental data in a number of cases, together with the qualitative evidence of polymerization practice, leaves little doubt that the necessary and sufficient condition for a gel in such a system is a three-dimensional network which is infinite (*i.e.*, pervades the entire sample).

At the gel point, when in the course of a three-dimensional polymerization reaction a network first appears, its mass is negligible; practically all of the polymerizing material still exists as monomers, dimers, trimers, and larger groups which are not bound to the network. As the reaction proceeds *after* the gel point, more and more of these loose groups become attached to the network, so that its relative mass (the "gel fraction") gradually increases and approaches unity. Approximate equations for the increase in gel fraction with extent of reaction have been given by Flory (1941) and Stockmayer (1943).

Even after the gel fraction has become 1, all of the polymerizing material having been bound to the network, the reaction may still proceed, as the network strands react with each other to form additional

cross-links with an attendant increase in the rigidity of the gel. This process is illustrated in the clotting of fibrinogen (Section VI).

b. Cross-linking of Long Linear Molecules. Shortly after the gel point in a branching polymerization reaction, the system is a gel in the sense that it contains a small proportion of "solid" (the network responsible for the rigidity) in a large proportion of "liquid" (unreacted molecules, together with polymer chains of various lengths which have not yet attached to the network). A similar geometrical arrangement can be obtained by starting with long linear molecules, cross-linking them at a few points by primary chemical bonds, and then adding liquid in the form of inert solvent.

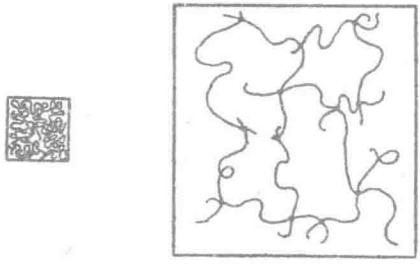


FIG. 2. Schematic diagram of the structure of a swollen rubber gel.

The latter sequence is illustrated by the vulcanization of rubber and subsequent swelling of the product in a solvent such as cyclohexane. The long linear molecules of raw rubber are joined by primary chemical bonds (cross-linked) at a few widely separated points during vulcanization, forming a flexible network in which the strands are irregularly coiled and crumpled. When solvent enters the network, the strands are straightened out to a slight extent, permitting the structure to increase in size, without disturbing the network junctions or cross-links; the result is a swollen gel (Fig. 2).

The above picture of the network structure of vulcanized rubber is supported by the success of the kinetic theory of rubberlike elasticity (see part 4, page 14); calculations based on this model agree well with experimental measurements of stress–strain curves and other properties (James and Guth, 1943; Flory, 1944). Excellent evidence that the swollen gel contains the same network as the unswollen rubber has been presented by Flory (1944, 1946), based on studies of butyl rubber. Using the network model, the number of cross-links in the structure can be calculated in three ways: (*a*) from measurements of the proportions of insoluble (network) and soluble (unattached) material in samples of different initial molecular lengths; (*b*) from the elastic modulus of the unswollen rubber; (*c*) from the maximum amount of liquid imbibed by the gel when swollen in equilibrium with pure solvent. The results of these three calculations for butyl rubber samples were in good agreement.

A third method for obtaining the same geometrical arrangement of a three-dimensional network interspersed by liquid is to start, again, with

long linear molecules and (reversing the order of operations in preparing a rubber gel) dissolve them first and cross-link them afterward. This has been carried out with cellulose derivatives, using reagents which combine chemically with two hydroxyl groups on different molecules and thus effect junction. Signer and von Tavel (1943) cross-linked acetylcellulose and methylcellulose in solution with oxalyl chloride and silicon tetrachloride; Jullander (1945) cross-linked nitrocellulose in solution with silicon tetrachloride and titanium chloride. The course of the reaction was qualitatively similar to that of branching polymerization, described above; first the viscosity rose gradually, corresponding to formation of cellulose dimers, trimers, etc.; gelation (appearance of rigidity) occurred suddenly at a definite point; as the reaction continued further, the rigidity of the gel increased. The products were insoluble in all solvents, confirming the presence of networks held together by primary chemical bonds.

The existence of a stable, dilute gel with a network of long molecules cross-linked by primary bonds demands that the cross-links be spaced *far apart*. In the butyl rubber samples described above, the average length of strands between cross-links was of the order of a thousand carbon atoms. If too many cross-links are introduced in the vulcanization of rubber, the strands will be tied together too closely and the network cannot imbibe enough solvent to form a dilute gel. If too many cross-links are introduced in the treatment of dilute nitrocellulose with silicon tetrachloride, the progressively closer binding of the network strands results in expulsion of solvent and contraction of the gel to a more concentrated state—*i.e.*, syneresis.

2. *Networks Bound Together by Secondary Attractive Forces at Widely Separated Points*

The presence of loci of strong secondary forces of attraction at widely spaced points along threadlike molecules might be expected to result in gelation; association of the loci would produce a network very similar to those described above, except that the cross-links, instead of permanent, would be dissociable.

Gloor and Spurlin (1936) described the gelation of nitrocellulose by copper ions in the presence of a reducing agent. Complex formation between a copper ion and (reduced) groups on two nitrocellulose chains is considered to cause association and eventual network formation. The requirement that the cross-links be widely spaced is met by introducing only a small amount of copper; the minimum required for gelation corresponded to 1 atom per 60 glucose residues, for the sample of highest molecular weight employed. Of course, it is probable that not every