X-RAY AND CRYSTALLOGRAPHIC STUDIES OF PLANT VIRUS PREPARATIONS

I. Introduction and Preparation of Specimens

II. Modes of Aggregation of the Virus Particles

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Plates 1 to 4

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INTRODUCTION

Since their original isolation by Stanley in 1935, the protein preparations from plants suffering from virus diseases have been much studied, but chiefly chemically and biologically. This paper is an account of a physical and crystallographic study of virus preparations which was carried out in conjunction with the work of Bawden and Pirie (1937 *a*, *b*; 1938 *a*, *b*, *c*).

The interest of this study is twofold, physical and biological. The properties of the virus preparations, particularly those that exhibit liquid crystal character, show many novel phenomena that throw much light on physicochemical problems, especially on those of colloid systems. They have revealed unsuspected regularities in the arrangement of long particles in anisotropic solutions, and thrown light on the structure of the various forms such liquids exhibit. The study of these substances has necessitated the development of new techniques of x-ray analysis and has resulted in the extension of their use to the measurement of repeat distances of the order of hundreds of Ångström units, thus going far to bridge the gap between x-ray and microscopic observations.

The properties revealed by these methods are exhibited in perfection by the virus preparations, but there is no reason to believe that they are confined to them, since they might occur in any aggregate of similar and markedly anisotropic particles. But x-ray analysis has revealed internal structures which are characteristic of the virus particles themselves.[1] This is shown by their

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[1] We have designated throughout the particles found in virus preparations as virus particles without prejudice to the question of whether they are or are not the infective agent. This can only be answered by biological means but already the weight of evidence seems to indicate that they are: (see Bawden and Pirie, 1937 *a*, *b*; 1938 *a*, *b*).

relative independence of the state of aggregation and by significant differences between different strains and species of virus. The virus particles have been shown to possess a regular internal structure which can be partially interpreted. These structures show interesting similarities and differences from those of both crystalline and fibrous proteins and help in turn to throw light on their constitution. The x-ray and optical studies are complementary to the biological investigations of the virus and give a more concrete picture of the different states in which it exists both in nature and in artificial preparations. Finally, the comparative study of these structures shows an interesting parallelism with their biological relationships.

The Progress of the Investigation

In giving a rational account of all these somewhat complex phenomena, it is necessary to depart widely from the historical order of discovery. It may therefore not be out of place to preface it with a short account of the actual steps of the work which will also serve as an introduction to all the main phenomena observed.

In 1935 Stanley isolated from tobacco and tomato plants suffering from mosaic disease substances which he described as crystalline and which appeared to possess the properties of the virus. Shortly afterwards, Bawden and Pirie, using different methods of preparation, obtained solutions which separated into two layers, the lower of which exhibited a marked sheen. It was at this stage that we first examined the preparations and observed the spontaneous birefringence of the bottom layer solution and the flow birefringence of the top layer solution. The next stage was the production from these, by drying, of oriented preparations, the so called "wet" and "dry" gels.

An x-ray examination of the dry gel showed a fibre diagram similar to that of many proteins. It also showed what appeared to be strong equatorial reflections at very low angles. X-ray apparatus was developed to study these in detail and this revealed a pattern of five reflections corresponding to a hexagonal close packing of long particles with a diameter of approximately 150 Å.

At this point the x-ray investigation diverged into two lines. One dealt with the high-angle scattering and consequently with the internal structure of the particles, and the other with the low-angle scattering and consequently largely, though not entirely, with their mutual arrangement. These investigations are referred to subsequently as "intramolecular" and "intermolecular" respectively.

It was early shown that the intramolecular x-ray pattern was independent of the state of aggregation; dry and wet gels, bottom and even top solutions giving approximately the same pattern. This showed the persistence of a definite unit which may be properly called the virus particle.

The intermolecular pattern, however, showed a complete and quantitative dependence on the state of aggregation. All preparations, except the top solution, showed a regular hexagonal arrangement of particles, in which the particle distance varied

with the concentration. This showed that even in weak solutions, forces were acting between the particles capable of preserving a parallel and equidistant arrangement. An important negative result was that no indication of the length of the particles could be found by x-rays (Bawden, Pirie, Bernal, and Fankuchen, 1936).

It was next shown that the "crystals" originally described by Stanley (1935, 1937), were identical with the wet gel preparations and possessed therefore regularity only in two dimensions and not in three as in true crystals. It could then be said that all but one of the phases of the virus had substantially the same structure. The one exception was that of the crystals found in some infected plants which were too small for x-ray examination but appeared to be true crystals confirming the idea that the virus existed in a different form in the plant from that of any of its preparations studied by us. (However, see the paper by Loring, Lauffer, and Stanley, 1938.)

Examinations of the intra- and intermolecular patterns of different strains of tobacco mosaic virus, cucumber viruses 3 and 4, and potato virus X (Bawden and Pirie, 1937 *b*, 1938 *a*) were also made and showed significant and illuminating similarities and differences (Bernal and Fankuchen, 1937). Subsequent observations have clarified and to a certain extent corrected these findings. An unexpected discovery was that of the single crystal character and the secondary orientation relative to the water surface of the gels formed by drying. This orientation is not shown by the particles themselves, demonstrating even more clearly the independence of the internal structure and the external arrangement of the virus particles.

More detailed investigation of the inner structure of the particles brought to light a high degree of regularity and complexity. The most perfect results were given by solutions, showing that the internal regularity is a characteristic of the virus particle itself (Bernal, 1938). The complexity of the x-ray pattern for long baffled interpretation until it was discovered that it was due to the presence of intensity maxima not conformable with Bragg's law owing to the small number of cooperating planes. It was then possible to establish a hexagonal unit cell (a = 87 Å, c = 68 Å) and to say something of the internal structure and its resemblances to and differences from those of the crystalline proteins that have been studied by x-rays.

A most fundamental simplification resulted from further study of gels of different water contents and also of those swelled in acid and salt solutions. This showed conclusively that all degrees of interparticle distance were possible and that there was no fundamental distinction between dry gels, wet gels, and bottom solution. These are all one phase with "top layer" solution as the only other phase. It further showed that the particles did not swell on wetting as had been thought at an intermediate stage of the investigation, and that their distance apart was entirely due to the properties of the solution in which they were immersed. A knowledge of this at once made apparent the explanation of the special physical properties of the virus—separation into layers, tactoid formation, etc. Indeed the theory can be further generalised to explain many colloid phenomena, such as thixotropy, and the structure of gels. A very promising explanation has been furnished by the theory of Levine (1939, *a*, *b*, *c*), which accounts for the interparticle forces as due to interpenetration of ionic atmospheres. Finally, one serious gap in the investigation of the virus, namely the question of the length of the particle, has probably been settled by the remarkable achievement of Kausche and coworkers (Kausche *et al.*, 1939), who have succeeded in photograph-

ing isolated virus particles in the electron microscope. Their observations are compatible with the picture of a thin straight particle of 15 mμ thickness, but show that the length is always a multiple of some unit of minimum length of the order of ten times the thickness, lengths beyond the range of observation of the x-ray methods used here.

All these results can be fitted into a general picture in which, however, only the outlines can be claimed to be well established. The views presented here must be taken as superseding in the light of fuller evidence those put forward in our earlier publications.

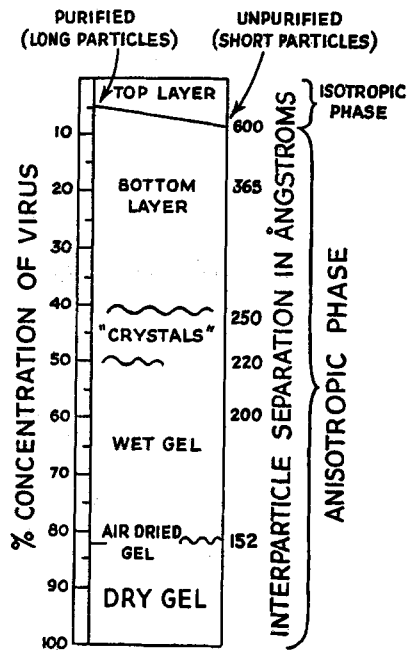
In what follows an attempt is made to achieve a logical order of presentation. In Part I a general description is given of the different phases of the virus preparations and of the methods used for examining them. In Part II the mutual arrangements of the particles in the different phases is discussed as well as the conditions controlling the relations between them. In Part III, Section 1, the evidence for their internal structure is presented; the relation of this to the structure of proteins is considered and on the basis of this, certain suggestions for possible structures are put forward. In the second section of Part III, comparisons are made between the structures of different strains and kinds of virus, and the possible biological implications of the physical investigations are discussed.

Preparation and Description

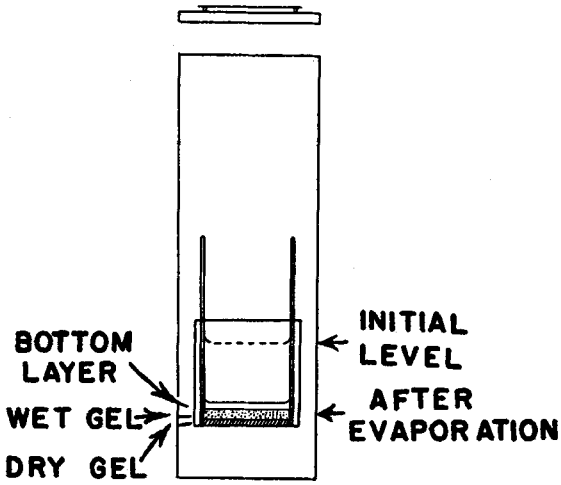
The purified virus preparations examined were (*a*) Tobacco mosaic virus, (*b*) enation mosaic virus, (*c*) aucuba mosaic virus, (*d*) cucumber virus 3, (*e*) cucumber virus 4, (*f*) potato virus X strain P, (*g*) potato virus X strain G, and (*h*) tomato bushy stunt virus. All these were prepared for us by Bawden and Pirie. For their methods the reader is referred to the original papers (Bawden and Pirie 1937 *a*, *b*; 1938 *a*, *b*.).

We received the material except for the bushy stunt virus in the form of liquid crystalline solutions, containing from 5 to 34 per cent of dry weight virus. These several substances were examined in very different degrees of detail. The tobacco mosaic virus, which was not only the easiest to obtain but the stablest preparation, was most studied. In what follows all descriptions will be supposed to refer to it or to its very similar strains unless otherwise specified.

The properties of the virus preparations depend primarily on the concentration of virus. There are only two distinct phases, the disordered, and the ordered. The first is referred to as top layer solution and is an opalescent isotropic sol. The second varies from an anisotropic sol, referred to as bottom layer solution, through various stages of viscosity and rigidity to wet and finally to dry gel. Though all these form one phase and are not as was previously thought separate phases, it is convenient to treat them separately as they require different methods of preparation and examination. Top layer solutions are found for dilutions containing less than 4 per cent dry matter in the less well purified (and also probably less well aggregated) preparations,



TEXT-FIG. 1. General scheme of virus preparations.



TEXT-FIG. 2. Cell for making oriented dry gel.

or less than 1.8 per cent in the purest preparations. In concentrations greater than 1.8 per cent or 8 per cent according to the degree of purity and less than 34 per cent, the solutions referred to as "bottom layer," are spontaneously birefringent (Text-fig. 1). The more concentrated of these solutions were prepared by centrifugation (Bawden and Pirie, 1937 *a*). Bawden and Pirie describe them as jellies. They show, however, complete continuity with the more dilute solutions, differing only in their greater viscosity and we accordingly consider them to be part of the same phase as the bottom layer solution.

Preparation of Wet and Dry Gel

The solutions on drying in ordinary vessels form a skin on the surface, which was early found to be birefringent and therefore showed that the particles were oriented relative to the air-water interface. This property was made use of to prepare oriented specimens of wet and dry gels by controlled evaporation in a simple cell. A drop of virus solution is introduced between a microscope slide and a cover-glass, kept apart by two glass threads, which can be of various thicknesses, according to the amount of material and thickness of gel required. The slide is then gently tilted so that the liquid fills the space between the glass rods down to the lower edge of the cover-slip (Text-fig. 2, and Plate 1 Fig. 1). The upper surface of the liquid being relatively protected from evaporation by the narrow space between the cover-slip and the slide does not form a skin. Most of the evaporation accordingly takes place through the skin at the bottom on which the solid material is deposited forming ultimately a layer of wet gel consisting of a single "crystal." The process is gradual and can be followed in a microscope. At an intermediate stage it could be seen from the birefringence that the concentration of the solution increased up to the sharp boundary of the wet gel where it attains the limiting birefringence of about 0.001. The wet gel forms a layer sharply distinguishable from this with a birefringence of 0.006. This distinction seems, however, simply to be due to the speed of drying and the inability of the more viscous concentrated solution to diffuse into equilibrium. The consistency of the wet gel varies accordingly with the speed of drying. On the outside the wet gel appears to pass also with a sharp boundary into dry gel which has a lower birefringence, 0.003. A shrinkage amounting to about 50 per cent—limited necessarily to one dimension—occurs in the drying out of wet gel. On further drying longitudinal, and less frequently, transverse cracks occur, leaving single pieces of approximate dimensions $1.0 \times 0.5 \times 0.5$ mm., which can then be examined as are ordinary crystals by x-rays. This method of preparation of oriented surface layers of considerable thickness is one we have subsequently found to be of very general utility. By means of it we have prepared, for example, oriented specimens of nucleic acid and of the lipoid containing antigen from *Brucella*.

Wet gel specimens for x-ray examination cannot be obtained in this way, as the material is softer than butter, and could not be removed from the cell without destroying its orientation. This difficulty can be overcome in two ways. A mass of wet gel can be reoriented by rolling between glass plates in a low temperature room to avoid loss of water. The oriented cylinders can then be transferred to the thin glass cells described below (p. 119). Alternatively, rewet gel can be used. A piece

of dry gel can be introduced into a narrow thin-walled tube and a suitable liquid—water, acid, or salt solution—can be introduced through a fine capillary. Except in extreme cases the gel swells without losing its orientation. Swelling can be limited by adding measured quantities of solvent, or equilibrium swelling reached by filling a section of the tube with the appropriate solution.

The "crystals" precipitated from solutions by acid (Bawden and Pirie, 1937*a*)[2] can be separated by centrifuging in the end of an *L2* Chamberland filter candle (Bawden and Pirie, 1937*a*),[3] the buttery mass rolled and transferred into the special cells (described below) in the same way as for the completely wet gel, from which it is indistinguishable as will be subsequently shown (p. 129).

Optical Examination

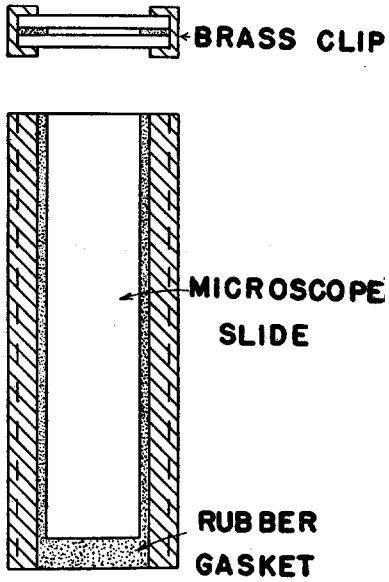
The optical studies on the virus were made with a Swift Dick polarising microscope. For examination of the solutions the simple cell described above was used, but where thicker preparations were required, free from the danger of evaporation, a cell consisting of two microscope slides separated from each other by a rubber sheet gasket and held together by brass clips was used (Text-fig. 3). Capillary tubes are also useful for certain purposes and their refractive effects were neutralised by immersing them between slide and cover-glass in a medium of suitable refractive index (Plate 2, Fig. 11).

X-Ray Examination

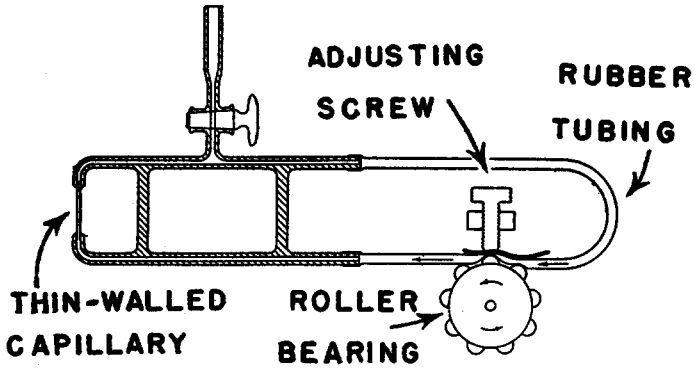
The standard x-ray apparatus used for the study of small crystals requires to be modified in two directions, both in order to measure low angle reflections and to enhance their intensity. To study adequately the large spacings which were observed, it was necessary either to use longer wave-length radiation or to examine the diffraction at smaller angles. The reason many of the phenomena here described were not discovered earlier was probably due to the choice of the first method. In deciding what radiation to use three factors must be borne in mind—the actual dispersion given by the radiation which at small angles is directly proportional to its wave-length, the absorption in all substances which is approximately proportional to the cube of the wave-length used, and the difficulty of producing the radiation which in turn depends on a number of factors but is certainly far greater for long wave-length radiation than for the standard copper K_α radiation. The great advantage of using this radiation is that it is possible to work in air without vacuum cameras, and to use commercial x-ray tubes with large out-put. The necessary dispersion can be obtained most easily merely by increasing the specimen to film distance. It may, in the future, be necessary to work with long wave-length radiation, but for the moment all the preliminary work can most conveniently be done by changing the camera rather than the radiation. In the second place it was found that the reflection from many of the specimens was extremely feeble and means had to be used to increase the intensity of the scattered radiation, and the contrast achieved on the films. This was done by

[2] Page 294.
[3] Page 300.

the adoption, where it was geometrically possible, of slits instead of the usual pinholes, which enabled exposure times to be cut down considerably. Finally, particu-



TEXT-FIG. 3. Small cell for optical studies of tobacco mosaic virus proteins.



TEXT-FIG. 4. Peristaltic pump and glass circulatory system for x-ray examination of oriented top layer solution.

larly in the case of examination of liquids and wet gels, monochromatic radiation was used. The device introduced by one of us (Fankuchen, 1937) provided a source of monochromatic radiation, little if anything weaker than that provided by the direct radiation from the tube. Two types of cameras were accordingly used. A long camera for the intermolecular reflections was adapted from the normal Pye

camera (Bernal, 1928) by the addition of an arm 40 cm. in length, and the corresponding elongation of the slit system. The slits were made by adjusting lead jaws under the microscope to about 0.10 mm. in width. They were placed 15 cm. apart, and the scattering from the second slit was checked by a slit of 0.20 mm. near the crystal specimen. In this way it was possible to obtain a beam which, at a distance of 40 cm. was only 0.7 mm. wide, and therefore subtended an angle of 5′ at the crystal. It was possible to measure diffraction angles of 4.5′ corresponding to a spacing of 1200 Å. In some experiments the whole of the apparatus between the specimen and the plate was replaced by a vacuum camera, but without appreciable improvement. Besides this normal type of long distance camera, monochromatic slitless cameras were also constructed and used. The short cameras used for the intramolecular investigations were of the normal pin-hole type, but with specially small pin-holes giving a beam of divergence 20′, thereby enabling plate distances up to 15 cm. to be used. The longest spacing measurable in this way is about 200 Å. Great care was taken to cut down the lead stop to a size allowing the maximum of pattern to be seen without any overlap of the central beam. It was found useful to use extremely thin lead back stops, leaving a faint trace of the central beam on the plate which could be used as a point of reference. With monochromatic radiation the lead stop could be replaced by one of a definite thickness of aluminium which allowed only a known proportion (1 per cent and 0.01 per cent) of the radiation to be transmitted and could therefore be used to estimate the absolute intensity of reflection.

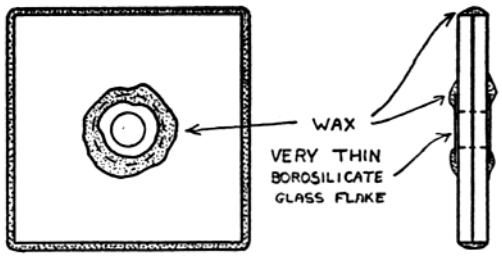
Of the specimens for x-ray examination only the dry gels could be mounted in air. For other preparations thin-walled tubes or special cells were used. It was found possible to make out of a special low absorption borosilicate glass, tubes of wall thickness of the order of 0.02 mm. These tubes were tested for x-ray absorption when empty and only those which absorbed less than a quarter of the incident characteristic copper K_α radiation were used. There is no advantage in pushing the thinness of the capillaries very much further, because the water with which the specimens are enclosed produces more absorption than the glass.

The specimen was usually secured against change in the capillary by sealing the ends in a small flame. It was of great importance to have absolute sealing to avoid any change in the specimen during the long exposures which often proved necessary.

These capillaries were used mainly for specimens of wet gel precipitated crystals, and for bottom layer solutions of various strengths. For top layer solution, in order to produce orientation, it was necessary that the liquid should be flowing. A small apparatus was therefore prepared, consisting of a stout capillary tube system, on to which was sealed a piece of thin capillary, the liquid being maintained in motion by means of a peristaltic rubber tube motor (Text-fig. 4). In this way both top solution and precipitated crystals were examined in the oriented state.

The disadvantage of capillary tube methods is that specimens which are easily deformable cannot conveniently be introduced into them. For x-ray examinations of such specimens a small brass cell was used. This was prepared as follows. Two brass plates of dimensions $7 \times 7 \times 1$ mm. were accurately ground together and a hole of 2 mm. diameter drilled through them. To the ends of this hole were sealed sheets of borosilicate glass blown to bubble thinness. The brass plates were then sealed together with sealing wax. Just before using, the seal was broken with a sharp

pointed knife (Text-fig. 5). Into this cell the specimen was introduced and the plates were then sealed by closing the fine crack in the sealing wax with a heated knife blade. All these operations were carried out in a low temperature room to avoid evaporation. Such cells showed no appreciable loss of water over periods of many days. It might be thought that the scattering of the glass, which though small in volume is of the same order as that of the scattering material to be studied would affect the photographs. This, however, was not found to be so. A certain amount of scattering did occur, but it was in a range equivalent to 4–2 Å, and therefore at angles which were much too high to interfere with any of the significant scattering from the specimens. The same was largely true of the water scattering. It is an interesting but not unexpected fact that prolonged exposures show no appreciable scattering for the water or glass at very low angles. The slight scattering that did occur at low angles was subsequently shown to be largely due to the air in the apparatus, as most of it could be removed by using a vacuum camera.



TEXT-FIG. 5. Small cell for x-ray study of wet gels.

II. The Modes of Aggregation of Virus Particles

This section is conveniently divisible into two main parts, first a description of the x-ray and physicochemical observations on modes of aggregation of the virus, and the second a discussion of these observations in the light of a general theory of equilibrium between colloid particles.

A. The Observations

All the viruses examined, except bushy stunt, have one common characteristic: their capacity for forming doubly refracting aggregates. In the case of the dilute top layer solutions this is only produced by flow orientation. In bottom layer solutions and gels, the orientation is spontaneous, any small region of the material behaving as a uniaxial positive crystal. Normally, these regions have different orientations, but by flowing, rolling, and the action of neighbouring surfaces, for example in narrow tubes or on air-water interfaces, large portions of the material can acquire the same orientation. The orientation of the wet gel is retained on drying. The needle-shaped "crystals"

precipitated by acid or ammonium sulfate, the tactoids of bottom layer solution occurring in top layer solutions (see p. 128), and the hexagonal crystals occurring in infected plants also show positive birefringence.

The simplest explanation of this property is that it is due to the existence in all the phases of markedly anisotropic particles, which have a tendency to set themselves parallel. X-ray examination confirms and gives precision to this hypothesis.

When oriented preparations of dry and wet gels and solutions of strengths down to 8 per cent are examined in the low angle x-ray camera described above, a pattern of reflections is observed characteristic only of the concentration of the specimen (Plate 3, Figs. 15 and 16). This is called, for reasons that will be discussed below, the intermolecular pattern. All these reflections occur on the equatorial line in the photograph, that is they correspond to planes parallel to the axis of orientation. The most careful search was made for other intermolecular reflections, particularly from planes perpendicular to the orientation axis, but it failed to reveal any such reflections. The most complete intermolecular pattern is given by dry gels. There are four strong reflections, but three other weaker ones have been observed, particularly well developed in the cucumber virus (Plate 3, Fig. 18). (The potato virus X gives no observable intermolecular pattern, see below p. 159.)

These reflections correspond to the first four planes of a two dimensional hexagonal packing of side 152 Å (146 Å for the cucumber virus). The agreement of the spacings with those calculated for a hexagonal lattice is perfect (see Table I); any variation from it would lead to blurring of the outer lines, which does not occur. The lines are in fact remarkably sharp, indicating that the areas of regular packing are large compared to the size of the particles. Actually, as will be shown later, the effective area may extend to that of the whole specimen. The patterns for the wet gels, for the precipitated "crystals," and for the solutions, also correspond to a hexagonal packing, with spacings increasing with the dilution; but here it is rarely possible to distinguish more than three lines in the first two cases and two in the last, the ratios of their spacings still indicate, however, the hexagonal nature of the packing. The full data are given in Table I.

The existence of hexagonal packing and the absence of any intermolecular reflections from planes not parallel to the axis of birefringence point to the existence of particles of identical cross-section, not less than 150 Å in diameter which have a tendency to pack in parallel bundles but give little information as to the lengths beyond setting a minimum value of about 1500 Å. We will here attempt to relate the observed properties of the virus at different concentrations to this hypothesis and in particular to discuss the transition between the ordered and disordered phases. It will be convenient to discuss first of all the bottom layer and top layer solutions and from there to pass to a discussion of

the gels, bearing in mind, however, that the bottom layer solutions, the wet and dry gels, all form part of the same ordered phase.

Bottom Layer Solutions

As has already been mentioned, solutions of the virus of concentrations between 1.8 per cent and 34 per cent if purified and of between 8 per cent and 34 per cent if less pure form spontaneously birefringent liquids whose properties change continuously with the concentration. The solution at extreme dilution appears clear except for a characteristic sheen, and mobile, possessing a low but anomalous viscosity. While the more concentrated solutions are extremely viscous, they can be made to flow through capillaries, but they will not flow under their own weight, possessing a certain degree of elasticity which might lead to their being considered as gels (see p. 131). Such centrifuged preparations from the solutions are not distinguishable by x-ray methods from dry gels swelled at high pH or in dilute salt solution.

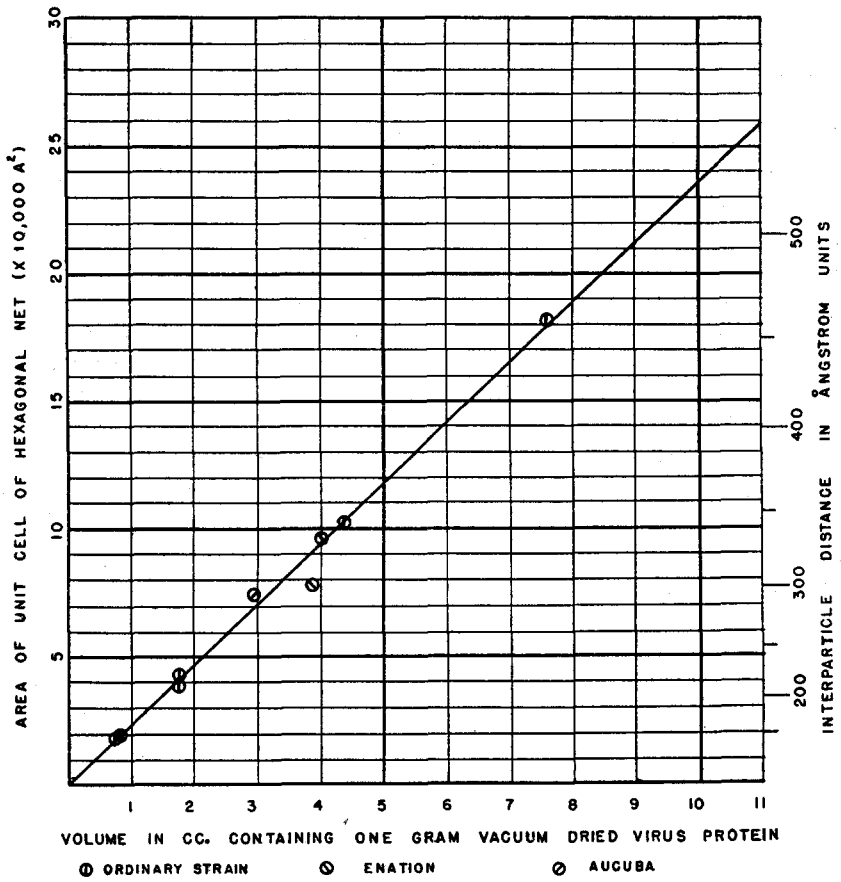
Examination of the liquid in bulk shows that it is divided into birefringent regions, which, in the still liquid, have no discernible shape or relation to each other. The size of these regions depends on the concentration of the virus. It is largest in the most dilute and smallest in the most concentrated solutions. Examined in thin layers, the liquid has the appearance shown in Plate 1, Fig. 4; the areas are seen not to be sharply defined regions, but represent a continuous flux of direction of birefringence in layers of 0.5 mm. or so, showing orders up to the 3rd, but there are no really black regions, indicating that parallelism to the glass sides does not extend through the distance between them but instead that there are twisted formations showing optical activity as can easily be seen by rotating one of the nicols.

In extremely thin layers, regions of approximately constant orientation appeared, the direction of maximum birefringence varying over the field in a sinuous manner. There is a marked tendency for orientation to take place parallel to the glass and optically active layers can be artificially produced by rotating the cover-slide over the glass head as in the case of nematic liquid crystals (Friedel and Friedel, 1931).

In flow the maximum refractive index sets itself along the flow lines, but when the flow ceases this arrangement is maintained indefinitely. Plate 1, Fig. 2 shows the effect of flow very clearly. The liquid is flowing through a narrow aperture between the glass tube spacing rod and a layer of wet gel. On the side from which the flow comes the orientation lies parallel to a set of stream lines converging on the orifice, while on the other side flow consists of loops alternatively to the right and left of the orifice.

In thin tubes there is in general after a little time complete orientation with the direction of maximum refractive index along the axis of the tube. The extinction direction is as sharply defined as in a true crystal, showing a definite

mean orientation of the particles. Pin-hole x-ray photographs taken at film distance of 12 cm. and 15 cm. (Plate 4, Fig. 24, and p. 136) show that most of the particles have this mean orientation; the angular scatter of the particle axis is not more than 1.5° and is probably much less as this measurement makes no allowance for the lack of resolving power of the particle reflections.



TEXT-FIG. 6. Relation between virus concentration and interparticle distance.

Regularity of Structure

Photographs taken with the special camera at 20 cm. or 40 cm. plate distance show two large spacing lines, which are in well oriented specimens, very sharp (Plate 3, Fig. 17) as well as a diffuse line at 93 Å which does not vary with the concentration and is consequently produced by the particle and not by the arrangement of particles (see p. 153). As has already been mentioned, the sharp lines correspond to the two first reflections $(10\bar{1}0)$ and $(11\bar{2}0)$ of hexagonal

packing of variable interparticle distance. Measurements of this distance brought to light an extremely simple regularity. The distance between the particles is inversely proportional to the square root of the concentration by volume and is expressed by the equation $R = 1650/\sqrt{N}$ where R is the interparticle distance in Å units and N the number of grams of vacuum dried virus in 100 cc. of specimen. The immediate consequence of the existence of this relation is that the *particles distribute themselves in a hexagonal array so as to fill the available space as uniformly as possible.*

This is shown in Table II and in Text-fig. 6 by plotting the area of a unit cell of the hexagonal arrangement against the volume of solution associated with unit weight of dried virus. For percentage concentrations ranging from 100 per cent for the dry gel down to 13 per cent the points lie, within the experimental error, on a straight line through the origin.

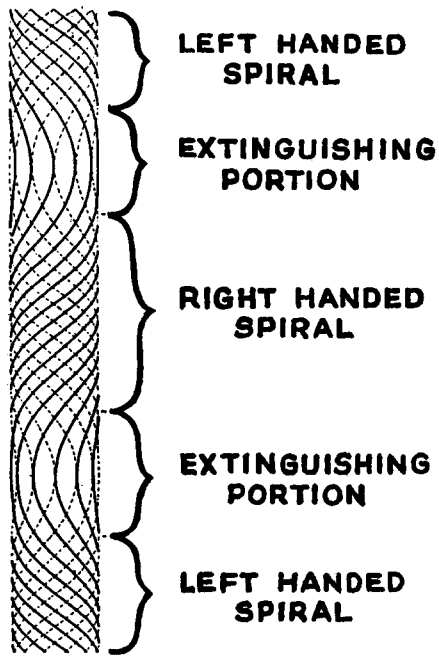
A further implication of the relation between spacing and concentration is that on dilution, the particles separate measurably only in the direction at right angles to their length. If they also separated to any considerable extent in the direction of their length the lateral separation would not increase at the rate observed. This suggests that the particles may form long chains in the solution and even that there may be some form of attachment between their ends. Such attachments have been observed by Kausche *et al.* (1939). The observation of a regular lattice arrangement of colloidal particles in solution and at distances up to 500 Å—more than three times the diameter of the particles—may prove to be of cardinal importance for the theory of such solutions. The discussion of its physical basis will be left till later.

Reversed Spirals

Before leaving the description of the bottom layer solutions, it is worth mentioning another typical structure which they exhibit, that of reversed spirals. When the liquid is made to flow rapidly through a fine tube, or alternatively the tube is set vibrating by bending one end and releasing it, it appears between crossed nicols to show striations which divide it, when the nicols are parallel to the tube, into light and dark regions (Plate 2, Figs. 7 and 8). This is a phenomena first observed by Van Iterson (1934) in cadmium glycerophosphate. On turning both nicols it can be seen that the light regions are never extinguished, but that they can be made to do so if one nicol is turned, showing that the light regions are alternatively dextro- and laevorotary, while the dark regions separating them behave like optically inactive crystals oriented parallel to the tube. The explanation of these optical effects is clearly that here the long axes of the particles set themselves spirally in the tube, these spirals being alternately right and left handed, separated only by regions where the particles lie parallel to the tube axis (Text-fig. 7). The reverse spiral arrangement for liquid flow is a further confirmation of the

existence of very long particles. Eirich and Simha (1937) have shown, experimentally as well as theoretically, that if parallel oriented particles experience resistance on flowing through a tube a periodical change of orientation is the most stable arrangement.

Similar phenomena are shown in the drying gel, in capillary tubes or on plates, (Plate 2, Fig. 10), but in this case a zigzag instead of a spiral arrangement is produced. These phenomena are of interest because of their close resem-



TEXT-FIG. 7. Directions of molecular axes in reversed spiral arrangement on surface of circular cylinder.

blance to many natural structures, for examples, to the striations of muscle (Bernal 1937), and this suggests very strongly that all are due to the same cause—the presence of long particles with a tendency to parallel orientation.

Top Layer Solutions

The top layer solutions are slightly opalescent, markedly more so than the bottom layer though they contain less solid material. This indicates at once their greater optical inhomogeneity due to their lack of regular arrangement. Lauffer (1938a) has studied polarisation of the Tyndall effect on these solutions and confirms the presence of long particles. The birefringence of the particles is, in his opinion, rod birefringence and not intrinsic. The proofs adduced are,

however, not altogether convincing as the influence of the medium on the particles cannot be excluded. Mere loss of birefringence might have been produced by disaggregation and no increase of birefringence after passing a minimum was demonstrated. Nevertheless some rod birefringence must be present to account for the observed diminution of birefringence between wet and dry gels (see p. 129). The chief properties top layer solutions exhibit are flow anisotropy and anomalous viscosity. Both of these and the relation between them have been studied by Robinson (1938; 1939*a* and *b*) and his conclusions will only be mentioned here in so far as they throw light on the shape of the particles.

Flow Anistropy and Viscosity

In any flow having a velocity gradient, the particles are oriented in relation to the stream lines, but do not, except for high speeds or very long particles, lie in them. For the most part they turn end for end in an irregular manner about axes in the planes of flow perpendicular to the stream lines, spending most of the time in a position near to but not precisely at that of the stream lines, as brownian motion tends to produce an equilibrium position making a definite angle with them. This is the complement of the so-called angle of isocline, observed in flow birefringent solutions. This process gives rise to an anomalous viscosity, that is, to a frictional loss of energy increasing less rapidly than the rate of shear. Robinson has shown that these phenomena are affected by temperature in the way this theory would indicate. Rise of temperature brought about diminished flow birefringence, increased deviation of isocline, and increased anomalous viscosity. From his measurements it was possible to obtain an estimate of the ratio of length to thickness of the tobacco mosaic virus particles in the preparation he used as 88 to 1, a measurement which will be discussed later.

The solutions studied by Robinson were extremely dilute—0.02 per cent—and the particles moved completely independently. In more concentrated solutions approaching the limiting concentration of the phase 1.8 per cent to 4 per cent, there is evidence of interaction between the particles in solution, elastic viscosity begins to appear, and the flow birefringence does not disappear immediately on stopping the flow but persists for a longer or shorter time according to the degree of purification of the virus.

Effects of Electric Currents

That there is an appreciable time of relaxation in strong top layer solutions is also shown by their behaviour in electric fields. A small cell with platinum electrodes about 1 mm. apart was used to study this effect and was observed between crossed nicols. The effect of passing a direct current across the cell was an immediate luminosity around the anode. This luminosity was quite

clearly due to the cataphoretic concentration of the virus, the particles of which carry a negative charge, and the consequent formation of spontaneously birefringent bottom layer. With low voltage—less than 1 volt—the effect was reversible. Reversing the current caused the disappearance of luminosity at one electrode, and its appearance at the other, the two being bridged by fine streamers. With higher voltages, irreversible changes took place involving the permanent formation of birefringent gel.

With alternating current, as long as the voltage was low—less than 5 volts—no such appearance of the bottom layer was observable. Instead, the switching on of the current immediately gave rise to a faint luminosity between the electrodes, this luminosity being clearly due to the orientation of the top layer itself (Plate 1, Fig. 6). The luminosity appeared the moment the current was turned on, but did not at once disappear when the current was broken, remaining for a period, estimated to be between 1/10th and 1/5th of a second.

These experiments indicate that the anisotropic virus particles are easily oriented, but that this orientation is brought about, as in many other liquid crystals forms, more by the cataphoretic currents produced by the electric field than by the direct orientation effect of the field in part of it, as is also the case with many liquid crystals (Freedericksz and Zolina, 1933). This is confirmed by our failure to observe any orientation in a magnetic field of 5000 gauss. Lauffer (1939) has made extended observations on electric orientation of tobacco mosaic virus. He ascribes it to dipole effects but his observations are not inconsistent with the explanation given above.

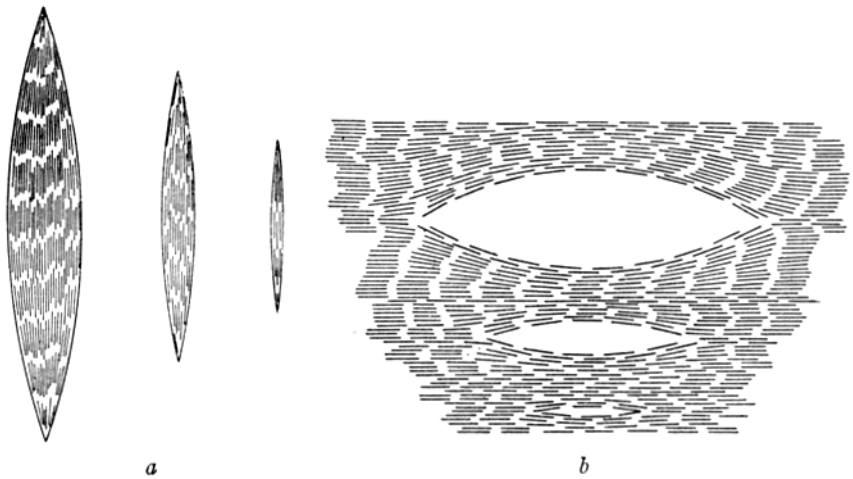
X-Ray Examination

The x-ray examination of the top layer solution carried out in the continuous flow apparatus, already described, confirmed the picture of the almost complete orientation. No intermolecular pattern was found, but this was not surprising as the calculated mean interparticle distance should have been of the order of 1500 Å, nor would regular interparticle distances be expected in any case. On the other hand, the most intense reflection of the intramolecular pattern (0006) (see p. 148) appeared in the form of arcs which were sharp and of small angular width, indicating at the same time the parallel orientation of the particles and their identity with those of the ordered phase.

Equilibria between Top and Bottom Layer Solutions: The Tactoids

In the initial separation of top and bottom layers from a virus solution and in any subsequent shaking together of the layers, characteristic spindle-shaped bodies are observed (Plate 1, Figs. 3 and 4). Similar bodies have been observed in other colloidal solutions and named tactoids by Zocher (1925, 1929) (see also p. 131). The tactoids are of two kinds, which we have called positive and negative. Positive tactoids contain bottom layer in top layer

solution, negative top in bottom. In Plate 1, Figs. 3 and 4, both are shown. Both kinds of tactoids have when undistorted a roughly toroidal surface; in outline they appear as two intersecting circles with slightly cusped ends. Rough measurements show that the minimum radius of curvature of all tactoids in a given preparation diminishes more slowly than their absolute size so that large tactoids approach a spherical shape and small ones are needle-shaped. The radius varies, however, enormously with the age and ionic constitution of the specimen. For fresh bottom layer of 4.5 per cent, salt free and pH 7, it gives the smallest values which are of the order of 0.06



TEXT-FIG. 8. (*a*) Orientation of particles in positive tactoids of different sizes (diagrammatic). (*b*) Orientation of particles around negative tactoids of different sizes (diagrammatic).

mm. The internal structure of the positive tactoids is revealed by their appearance between crossed nicols, the slow extinction position, and consequently the axes of the particles following the meridian lines of the spindles (Text-fig. 8*a*). In the negative tactoids the outline depends on the arrangement in the surrounding bottom layer solution (Text-fig. 8*b*). The tactoids, particularly the larger ones, are easily distorted, but recover their shape; they can also fuse to give larger tactoids. This suggests that they represent an equilibrium form between the conditions of surface energy tending to produce a spherical shape and those of orientation potentials tending to keep all the particles approximately parallel. A roughly quantitative theory of tactoid shape can be built up on this basis, but it is best considered after the discussion of interparticle equilibria (see p. 140).

The Gels

The forms taken by the more concentrated virus preparations prepared by drying and precipitation were originally thought to consist of two different phases, dry and wet gel, showing constant characteristics. This opinion was held both for experimental and theoretical reasons (Bawden *et al.*, 1936). On drying sharp boundaries seemed to separate the drier outer portion from the inner portion and this in turn from the solution. Further, the wet material and the "crystals" prepared by precipitation were alike in their physical properties, having a dry virus content of between 45 per cent and 55 per cent, and giving the same intermolecular x-ray pattern, showing an interparticle distance of 200–210 Å. There seemed therefore to be a definite phase which it was possible to account for only on the assumption of the swelling of the particles, as at that time no other forces of known character could be postulated holding dry particles in equilibrium at such a distance. Subsequent work, however, showed this view to be incorrect, and it has been replaced by a much simpler one, namely that all concentrations of the virus from 100 per cent–2 per cent form one ordered phase, consisting of a regular hexagonal packed assembly of parallel particles their distance apart varying from 150–1000 Å. The particles retain substantially the same internal structure in all states. The nature of the forces between them will be discussed later, but it is first necessary to summarise the experimental evidence.

Dry Gel

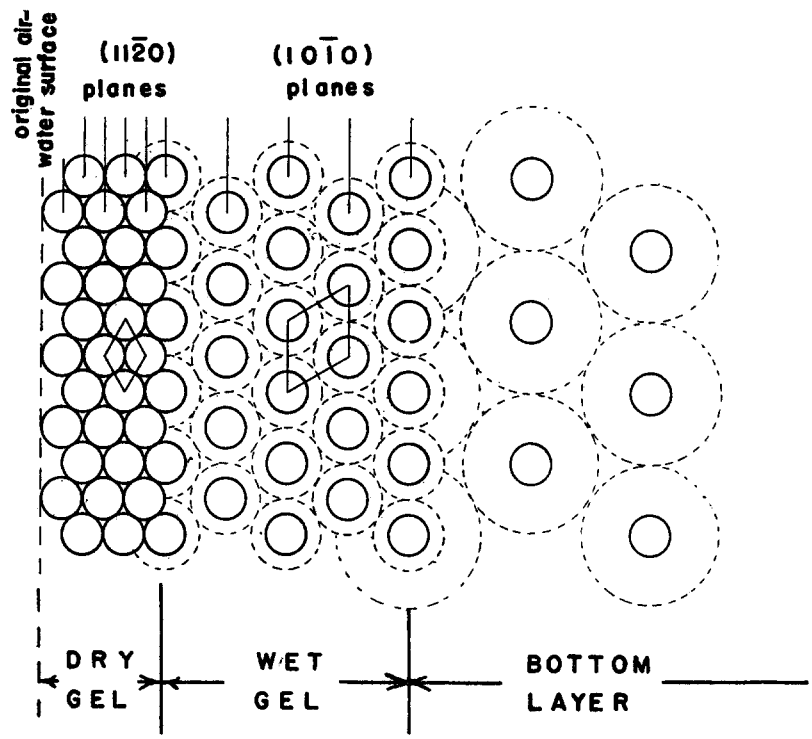
The study of dry gel has been more thorough than that of any of the other phases on account of its ease of manipulation. The intermolecular pattern is particularly sharp and shows the greatest number of lines, indicating extreme regularity of hexagonal packing of the particles with an interparticle distance of 152 Å. This is a surprisingly constant number for air-dried gels of all tobacco mosaic strains, formed in many different ways. Drying over P_2O_5 in a vacuum reduces this distance to 150 Å. There appears to be a sharp distinction in physical properties between dry and wet gel. The material is hard and horny, its birefringence is only half as great, 0.003. This is due to an increase in the transverse refractive index from 1.484 to 1.532 from wet to dry gel due to close apposition of the rod-shaped particles while the longitudinal index rises only from 1.490 to 1.536.

Single Crystal Character

Apart from its intramolecular structure, its chief interest is in its relation to wet gel. For this the cardinal observation (see p. 147) was that pieces of dry gel behaved as single crystals in two dimensions. An intermolecular photograph with the x-rays parallel to the axis of orientation showed a distinct hexagonal pattern (Plate 4, Fig. 22). It is significant that the corresponding

intramolecular pattern (Plate 4, Fig. 23) showed only continuous rings, indicating that at least in the dry gel the orientation did not extend to the inner structure of the particles.

Now, in the mode of preparation of the gel by drying, two factors may affect the transverse orientation of the particles, that of the air-water and glass-water interfaces which are at right angles (Text-fig. 2). Of these, the



TEXT-FIG. 9. Section across particles in successive layers of drying film to illustrate orientation of "single" crystal of dry gel.

effect of the first is likely to be predominating. Now it is at first sight strange that the most densely packed plane of the hexagonal packing (1$\bar{1}$00) is found parallel not to the air-water but to the glass-water interface, while the former is occupied by the less densely packed plane (11$\bar{2}$0). The rigid dry gel, however, cannot have oriented itself in the dry state; its orientation must have been acquired in the wet state and simply fixed by drying. Now the shrinkage of about 50 per cent of the wet gel on drying takes place only in the direction perpendicular to the air-water interface. It is natural, therefore, to assume that this brought about the interchange of the hexagonal packing directions

(Text-fig. 9), and that in wet gel the $(1\bar{1}00)$ plane is parallel to the air-water interface. Thus in the wet gel the particles would be closely packed in this surface, a very reasonable arrangement. The rearrangement could not be perfect owing to the lack of fit of the interparticle dimensions and this may give rise to the cracks that are observed in the dry gel.

Non-Equilibrium Gels

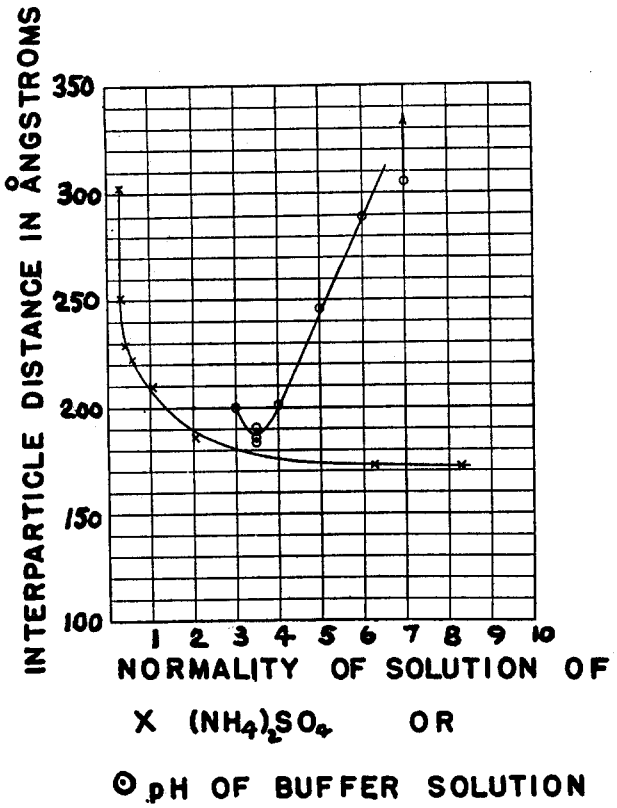
Dry gels formed from solutions of the virus prepared at pH 7 will if placed in salt-free water at the same pH swell indefinitely and dissolve to reform reversibly the oriented phase from which they were prepared. X-ray studies show that here the distance between the particles is always derivable from the concentration (see p. 124, and Text-fig. 6), and this holds as well for the gels as for the more dilute solutions. Additions of small amounts of water to dry gels result in interparticle distances intermediate between the values of 150 Å and 210 Å previously supposed to be characteristic for wet and dry gel and thus makes it very unlikely that they are distinct phases (but see p. 140). Similarly, values between 210 Å and 290 Å, the value for the most concentrated solution, have also been found. This establishes the complete continuity of the non-equilibrium gels and of the bottom layer solutions previously described as one continuous oriented phase of the virus. The state of the particles in this phase is called one of non-equilibrium as they tend to move further apart if given more room as do molecules in solution or particles in a gas, but they differ from them in that they are arranged in a regular lattice structure. They behave in fact as crystals with a long range elasticity.

Equilibrium Gels

Virus solutions near the isoelectric point at pH 3.4 in salt solution are precipitated in the form of fibres or acicular "crystals" containing a variable amount of water. The same phenomenon occurs with a variety of other agents, alcohol, acetone, or merely by ageing. X-ray examination shows that these precipitates are identical with those formed by allowing the dry gel to swell in the same liquids. This indicates that in these cases an equilibrium is attained determining the water content of the gels which may consequently be called equilibrium gels. The study of these gels by x-rays shows that here the distance between the particles is independent of the amount of medium present and depends only on its character. This dependence has been studied in a preliminary manner in two series, that of pH and of ammonium sulfate solution. The results are shown in Table III and Text-fig. 10.

Pieces of dry gel were swelled in a number of solutions of citrate-phosphate buffer of pH varying from 2.5–7. There was a definite minimum at pH 3.4 with an interparticle distance of 185 Å, while at the higher pH the distance increased to 300 Å, greater than that of some of the bottom layer

solutions previously examined. The salt solutions gave similar results except that here there seemed a definite saturation effect, solutions stronger than 6 N giving almost the same minimum distance, 173 Å. The minimum would seem therefore to have some special importance being the least distance of approach of the particles in an aqueous medium. The existence of inter-

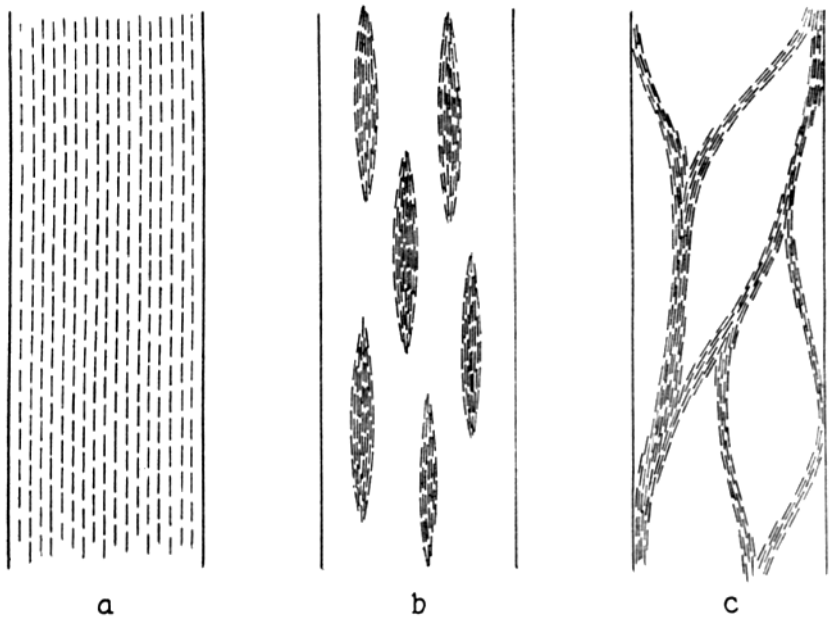


TEXT-FIG. 10. Relation of interparticle spacing and concentration of salt solution and pH.

particle distances of between 210 Å and 230 Å for many concentrations of salt and acid explains how it was possible for so long to consider the gel of this composition, *ca.* 50 per cent dry weight, as a definite phase—wet gel. The state of the virus here is probably physically analogous to that of protein crystals which usually contain about the same proportion of water; it will be discussed in a subsequent section.

It may be pointed out here that the x-ray method gives an exact way of estimating the intrinsic water content of a gel, namely that associated with the

gel particles. The apparent water content may be much greater. Thus Bawden and Pirie (1937*a*) observed that the first effect of decreasing the pH of a virus solution was the formation of a gel which might contain 95 per cent or more water. On shaking these gels separated out as "crystals" which contained 50 per cent water. Our observations, particularly those in which acid solutions were allowed to diffuse into already oriented virus solutions,



TEXT-FIG. 11. Rheopectic effects in acid-precipitated virus solution (diagrammatic). (*a*) Oriented 4 per cent virus solution in capillary tube: free flowing. (*b*) Effect of slow precipitation with acid. Formation of independent tactoids: free flowing. (*c*) Effect of agitation: fusion of tactoids: gel formation.

showed that in all cases submicroscopic fibres of this composition were produced and these being already parallel did not form a gel and flowed readily (Text-fig. 11) though more dilute disoriented solutions did so and showed marked rigidity. It seems clear therefore that in gels of these substances and by inference in those of many others containing long particles of colloidal dimensions we should distinguish two mechanisms of water retention. Such gels contain both intrinsic water lying between the most closely apposed particles and depending only on the nature of the solvent, and trapped water depending on the concentration of gel substance and the conditions of gel formations and ageing.

The observations on the conditions of equilibrium of virus gels are still in

their earliest stage. The effect of other conditions, temperature, non-electrolytes, etc., have still to be studied. Their interest is primarily that of colloid physics; as far as the nature of the virus is concerned they have sufficed to show that all its properties in an aqueous medium are explicable in terms of long particles reacting with each other through the medium in such a way as to keep them parallel and equidistant at all concentrations above a critical one depending on the nature of the medium.

The Crystals in Infected Plants

All the forms of virus preparations hitherto described seem composed of distinct particles of a length far greater than their diameter. This length, however, seems to be a variable quantity and is very probably an artefact (see p. 135). On the state of the virus in the plant we have only two observations to go on; the weakness of flow double refraction (see p. 135) and the existence of crystals in some infected plants. These crystals, which have been studied by Bawden and Sheffield (1939) and Beale (1937) probably do not represent the virus in a pure form. Their solubility is too small, but they may well be compounds of the virus with cell proteins (Bawden and Pirie, 1937 *a*).[4] That they are related to the virus is shown by their association with infection, by their hexagonal habit and positive birefringence, which correspond to the particle arrangement and optical character of the gels, and by their breaking up into bundles of needle-shaped acid precipitate "crystals" on acidifying the sap. More recently, Kausche (1939) has demonstrated the reverse process by producing minute hexagonal crystals from a mixture of infected sap and precipitate "crystal." If we grant this relationship, the hexagonal crystals throw much light on the state of the virus in the plant. Unfortunately, they have proved too small and unstable to be submitted to x-ray examination, but from optical observation certain conclusions can be drawn. The crystals are bounded by hexagonal prisms and basal pinacoids. The existence of the last shows that here we must have a full three dimensional regularity and therefore particles of identical length.

In a few of the many specimens examined it was thought that acute angle pyramids could be observed, but the angle could not be measured so that the axial ratio of the crystals remains undetermined. The habit of the crystals with a width-thickness ratio of approximately four to one indicates according to Fedorof's rule that the length-thickness ratio of the molecules which make it up must be of somewhat the same order, giving a particle length of the order of 1000 Å.

The ready transition to the needle form shows that the particles are already aligned in the crystal and the fact that little swelling is observed shows that their distance apart is not markedly different from that of the isoelectric gel,

[4] Page 304.

namely 185 Å. The observations are necessarily very qualitative, but they point in the same direction as those arrived at in other ways. If sufficiently powerful x-ray apparatus were available the crystals would undoubtedly furnish far more precise information on the nature of the virus than is furnished by the less regular gels and liquid crystals. It is convenient at this point to present evidence as to the length of the particles, since it plays a rôle of cardinal importance in the discussion of interparticle equilibrium.

The Length of the Particles

X-ray evidence as to the length of the particles is all negative, that is to say no reflections attributable to regularities greater than the intramolecular regularities have been observed. The sharpness of these, however, indicates that the particle length must be considerably larger than its width, certainly over 1000 Å. Various physical methods (ultracentrifuge, viscosity, birefringence of flow, diffusion) (Eriksson-Quensel and Svedberg, 1936, Bechhold and Schlesinger, 1933, Wyckoff, 1937*a*, *b*, Wyckoff *et al.*, 1937, Stanley, 1938, Lauffer, 1938*b*, *c*, Takahashi and Rawlins, 1932, 1933*a*, *b*, 1937, Lauffer and Stanley, 1938, 1939, Bawden and Pirie, 1937*a*, *b*, Bawden *et al.*, 1936, Hills and Vinson, 1938, Staudinger, 1932, Neurath, 1938, Neurath and Saum, 1938, Mehl, 1938) provide other bases for estimating the length. All agree in indicating a considerable length for the particles, but although none of the methods was particularly exact, it was clear that the discrepancies between them exceeded the probable experimental error. The conclusion which follows from the remarks made above is that the particles might be of indefinite length depending on the mode of preparation, that they are probably shortest in the living plant, and are increased in length by the methods of purification employed. Fortunately, it is now not necessary to consider this evidence in detail as the study of the virus by means of the electron microscope by Kausche, Pfankuch, and Ruska (1939), and Marton (1941) enables the length to be measured directly. The particles as seen in the electron microscope are rods of different lengths, but they are all multiples of a length approximately 1500 Å, showing that the particles are homogeneous in respect of length, as indicated by the existence of true crystals in the plant. The preparations studied by various workers were all probably aggregated to different degrees. However, recent work of Loring, Lauffer, and Stanley (1938) suggests that preparations may be obtained with little if any aggregation. For instance that studied by Robinson must have consisted of particles containing from 8–10 Kausche units. The length of 1500 Å for the particles is sufficient to contain 22 repeat units as determined from the intramolecular observations (see p. 153), but 24 is not excluded. It is also very likely that the virus as observed by Kausche *et al.* (1939) was already somewhat aggregated and that groups of 12 or less repeat units may occur in the plant, though there is no positive evidence of this.

B. Interpretation

The Nature of the Interparticle Equilibria

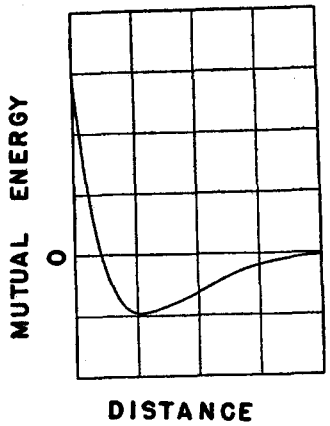
From the point of view of colloid science the most interesting observation to which the x-ray study of virus preparations has led is the regularity of packing of the long virus particles, not only in the concentrated gels but also in solution. That such good order should be attainable in the dry state was unforeseen although not unprecedented, but in the case of the solutions where the particles are separated by water molecules, a distance much greater than their diameter, 150 Å, crystalline regularity was certainly not to be expected on prevailing views. At most it would have been assumed that a roughly even distribution of the particles would be maintained but that anything more regular would have been prevented by thermal motion. Observation shows, however, that the solutions down to a concentration of 20 per cent show as perfect reflections as a crystal. More dilute solutions, down to 13 per cent, have not been so carefully examined and indeed show slightly less sharp maxima, but this is probably due to inhomogeneity rather than irregularity, for similar broadenings were previously observed on stronger solutions but disappeared when well orientated and homogenized specimens were used. As the continuity of physical properties is apparently unbroken down to the critical concentration of 1.8 per cent, it is probable that these are also regular, though, owing to difficulties of observation of such small angles and the weakness of the reflections, this has not yet been proved.

Not only are the particles equidistant, they are arranged in almost exact parallelism. The intramolecular photographs of 36 per cent (Plate 4, Figs. 24 and 25) solution show that the mean deviation from parallelism does not exceed 45′, and that it is probably far less. The same is shown in more dilute solutions by their sharp extinction when observed in polarised light. On the surface of the tactoids the particles are constrained by strong surface forces, but even here the angle of deviation, as measured by the minimum radius of curvature in the longitudinal direction, is still small. The observed radius of curvature of 0.06 mm. gives for a particle of length 1500 Å a maximum particle to particle deviation in this direction of only 8′.

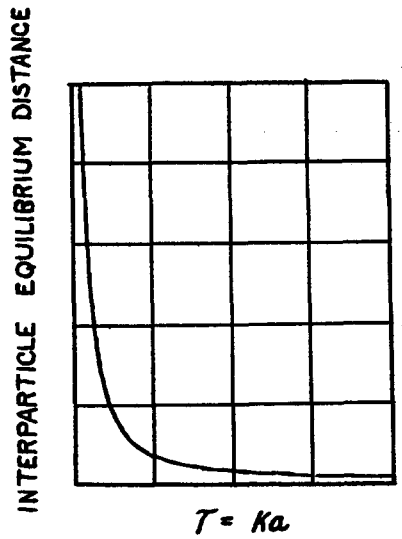
Such equidistance and parallelism can only be maintained by definite forces acting between the particles. For concentrations greater than the critical concentration of 1.8 per cent in the absence of acid or salts, these forces must have a net repulsive character, as is known by the formation of non-equilibrium gels, but at this concentration attractive forces must balance them or no division into layers would be possible. The seat of the forces cannot be in the particles alone. London-Van der Waals forces are ineffective at such distances and in any case give attractive forces. The most probable explanation lies in the Debye ionic atmospheres around the particles which at pH > 3.4 carry a net negative charge.

The Theory of Particle Interaction

The theory of the interaction of particles in an ionic medium has recently been studied by several authors, notably by Langmuir and Levine. Langmuir's theory (Langmuir, 1938) has so far been worked out only in the case of infinite plane particles which would correspond in practice to those of hydroxide sols or clay suspensions. Levine's treatment, which deals with spherical (Levine, 1939*a* and *b*) and later with cylindrical (Levine, 1939*c*)



TEXT-FIG. 12



TEXT-FIG. 13

TEXT-FIG. 12. Mutual energy of two spherical particles in an ionic medium as a function of distance (from Levine, S., *Proc. Roy. Soc. London, Series A*, 1939, **170,** 152).

TEXT-FIG. 13. Interparticle equilibrium distance as a function of ion concentration (from Levine, S., *Proc. Roy. Soc. London, Series A*, 1939, **170,** 152).

particles, is more directly applicable for our case. Physically the problem is very similar to that of the interaction of metal atoms with their positive nuclei and cloud of negative electrons, but on a scale comparable to that of the particles themselves. The simplest case treated by Levine is that of spherical particles. He is able to show that for two charged spherical particles there is always a potential energy distance curve of the type shown in Text-fig. 12, namely one which exhibits attraction at large distances and repulsion at smaller ones, and provides an equilibrium position with a minimum of potential energy. The position and depth of the equilibrium depends only on the product

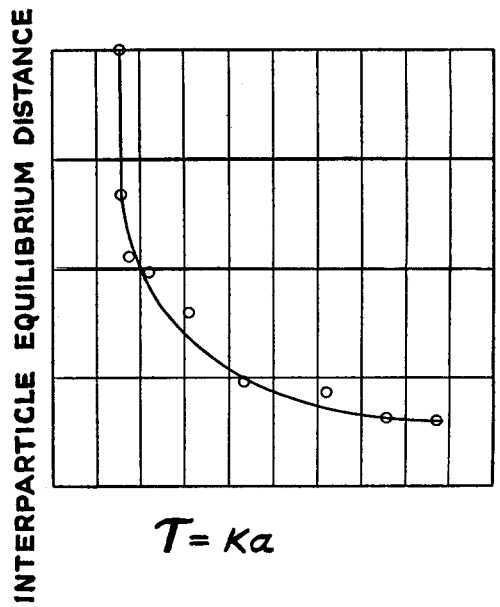
$$\tau = Ka = \frac{a\sqrt{\gamma}}{3.04 \times 10^{-8}}$$

where a is the radius of the particle, and γ the concentration of univalent electrolyte. Increased concentration or charges on ions tend to make the minimum deeper and reduce its distance as shown in Text-fig. 13. At some critical concentration the minimum will move to where the particles are in contact, but at this point Van der Waals forces will play a predominating rôle. Now in most cases with spherical particles the depth of this energy minimum is smaller than the thermal energy kT for the particles, consequently they will behave like gas molecules interacting for short periods, and only in the presence of strong salt solutions are they likely to come together and coalesce in crystals or amorphous precipitates.

The situation for long particles, however, is very different. Levine has so far treated only the case of two long cylindrical particles arranged parallel to each other. It is easy to see that the type of mutual energy curve remains unchanged. But the depth of the energy minimum is for particles of length more than about ten times their thickness, far greater than kT at ordinary temperatures. The result is that here stable equilibrium will occur with a mutual distance depending on the ionic concentration. Methodical treatment so far has only been carried out for parallel particles, but it is easy to see that any displacements from parallelism would, in the case of long particles meet with considerable restoring forces. The energy minimum will occur always in the parallel position and the relative depth of the minimum will be greater the longer the particles. As long as kT for libration is less than a minimum value determined by the length distance ratio, stable equilibrium will result. When it is of the same order, as will occur at high dilutions or with short particles, the libration disturbance will cause the particles to break apart at a distance less than that where they would still be stable for simple lateral displacement. What applies to two particles holds *a fortiori* for a large number of particles. If the energy minima are deep enough, they must arrange themselves in a regular hexagonal lattice, just as they are observed to do in the virus. The mathematical treatment of the equilibrium in this case has yet to be carried out, but it is easy to see that the result will only lead to a slight modification of the equilibrium position in the case of two long particles, the effects of the other particles resulting in a somewhat greater equilibrium distance and lower mutual energy. Levine's theory can therefore be tested directly by the measurements made on the relation of interparticle distance to ionic concentrations for virus particles, and Text-fig. 14 shows how good the agreement is in the shape of curves. Absolute comparisons are impossible at this stage of the theory as computations have not been made for sufficiently high concentrations nor has the character of the tobacco mosaic virus been taken into consideration.

We may, however, say that we have now an adequate semiquantitive explanation of the unexpected phenomena of the regularity observed in the

virus, and it is also clear that this has a far wider application, namely to all fibrous colloid molecules (see p. 145). The arrangement of long particles in equilibrium in a medium furnishes us with a new type of arrangement of matter, namely that of an elastic two dimensional crystalline solution. If a small amount of solvent is present the particles are near together, when the solvent is added they move apart to fill the space, but not through irregular movements as in a gas, but by regular expansion of the particle lattice. It is



TEXT-FIG. 14. Experimental relation between interparticle equilibrium distance and Levine's function.

probable that most phenomena of swelling have the same character. Where platy particles are involved the equilibrium can be maintained at far greater distances than with cylindrical ones, owing to the fact that ion concentration falls off in a linear manner instead of roughly as inverse square of the distance.

In fact in this case the distances reach 5000 Å or more and the phenomenon becomes visible as in iridescent sol.

Hydration of the Particles

It can be seen from Text-fig. 10 that in strong salt solutions the distance between the particles shrinks to a steady value of 173 Å, but that this value is distinctly greater than that between the particles in the dry state. This is not as yet fully accounted for by Levine's theory. It would appear that the

model taken for the theory is too simple in that it does not allow for the finite molecular structure of the water medium. The differences between the two values indicates that there is a layer of some 20 Å in thickness between the particles in the most concentrated solutions. This would correspond to one of 10 Å surrounding the particle or roughly layers of four water molecules. We may consider this water to be bound not by the total charge of the particle but by the positive and negative charges due to amino and carboxy groups which cover it even at the isoelectric point. If this is so, it should be possible to demonstrate the attachment of the water by physical methods, but so far the amounts of material available have not been sufficient to do this. It is the presence of this closely bound water that gave rise, at an earlier stage of the investigation, to the belief in two separate phases, wet gel and dry gel; and indeed the layer may represent one of particular stability as it has up till now proved impossible to prepare specimens with an intermolecular distance of between 159 Å and 169 Å.

Equilibrium between Top and Bottom Layers

The relation between interparticle distance and ionic concentrations can be followed by direct measurement only down to concentrations of 13 per cent, that is to an interparticle distance of 450 Å. At greater dilutions it is clear that equilibrium still exists, the presence of the order disorder phases is enough to show that. Further, the dilution shows that the equilibrium occurs at much greater distances. The attempt, however, to follow the equilibrium by observing the concentrations of top and bottom layers in equilibrium in dilute salt solution led to paradoxical results. Only very small amounts of material were available at this stage and therefore the measurements are too rough to be worth quoting but their trend is unmistakable. On adding dilute salt solution to an equilibrium mixture of top and bottom solutions, the level of separation moves upward, that is the addition of salt solution increases the critical dilution and hence the distance between the particles at equilibrium. When the concentration of the salt solution exceeds a definite value roughly $\tau = 16$, separation of top and bottom solution can no longer be observed. Instead first elongated tactoids and then fibres are formed (Plate 2, Figs. 11–14).

An explanation of this paradoxical behaviour is to be sought in studying more closely the actual conditions of equilibrium of the particles in more dilute solutions. It is here that the tactoid formation, already described above (p. 128), occurs, and consideration of this shows that the phenomenon in question is one which involves the length of the particles.

Theory of Tactoid Form

The tactoid form is characteristic of a liquid crystal of the type formed by the virus particles, that is, of one of long thin particles arranged parallel and

equidistant to each other. This type has not hitherto been described but it corresponds to the class *RRD* in Hermann's (1931) classification of possible liquid crystal systems. Tactoids bear a superficial resemblance to the baton-net of smectic liquid crystals (Friedel and Friedel, 1931), but these have radically different structures, being composed of coaxial cylinders of molecular sheets in which the molecules are perpendicular and not parallel to the axis of elongation.

On the surface of a tactoid the particles of the ordered phase are arranged parallel to each other and parallel to the meridian lines of the tactoids. This follows from direct observation of their birefringence. Now when long particles are involved, the surface of separation can no longer possess the isotropic character of an ordinary liquid interface. The bending of such a surface must require different amounts of work according to whether the bending takes place along the axis parallel to or perpendicular to the orientation of the particles in the surface. *Such a surface will in fact behave as if it possessed an anisotropic interfacial tension.* We may consider such a surface as one possessing two principal surface tensions, σ_1 and σ_2, the first being in general greater than the second, their ratio depending not necessarily in any simple way on the relative length to breadth of the particles. The simplest expression for an equilibrium surface would in this case be

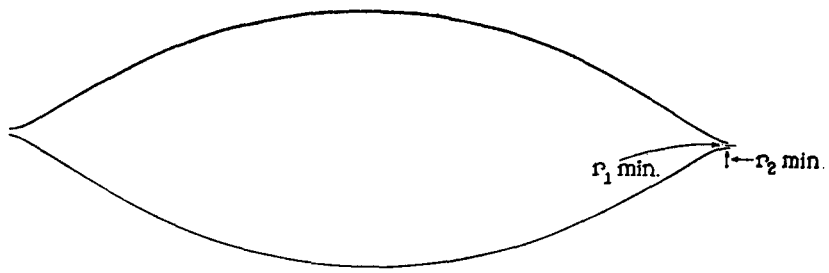
$$\frac{\sigma_1}{r_1} + \frac{\sigma_2}{r_2} = \text{const.}$$

where r_1 and r_2 are the two principal radii of curvature of the surface. Corresponding to a simpler expression for an ordinary interface

$$\sigma\left(\frac{1}{r_1} + \frac{1}{r_2}\right) = \text{constant}$$

This condition is not, however, sufficient to define the surface of the tactoid. It is clearly also necessary that the surface energy for a given volume must be a minimum and the mathematical formulation for this has not yet been found. It is, however, easy to determine the general character of the surface and to describe it approximately graphically (Text-fig. 15). It is a surface of revolution generated by the curve shown in Text-fig. 15. Its curvature is determined by r_1 while r_2 is given by the part of the normal intercepted by the axis of revolution. The minimum positive value of r_1 occurs at the equatorial plane. As r_2 diminishes r_1 increases and passing through an infinite value at a point of inflection, becomes negative, and reaches a minimum negative value where the curve is again parallel to the axis, after which the curve repeats indefinitely. The general shape of the surface is therefore that of a series of barrel-shaped portions separated by hour glass-shaped constrictions. These constrictions, however, in cases where $\sigma_1 > \sigma_2$ almost reduce to cusps and the

anomalous arrangement at these points leads probably to their being occupied by particles of impurities.[5] Where σ_1 is nearly equal to σ_2 the shape approaches the spherical, where σ_1 is much greater it is long and tapering, approaching a fibre-like structure which is observed in old gels and in the fibres studied by Best (1937 *a* and *b*). The shape is dependent not only on the ratio of σ_1 to σ_2 for, unlike the case of a normal liquid, the shape of a tactoid drop also depends on its size. The relation must be complicated but broadly it may be stated that the larger the tactoid the more it resembles a sphere, the smaller the more it resembles a cylindrical or needle-shaped body. How well this geometrical theory fits the observations is shown in Plate 1, Fig. 3, where a number of tactoids in equilibrium can be seen and compared to the theoretical curves. The extreme case is that of the acicular micro-tactoids formed by acid or salt precipitations first described as crystals by Stanley (1935), (1937).



TEXT-FIG. 15. Theoretical axial cross-section for tactoid surface. (The subscripts of r_1 and r_2 should be interchanged.)

The gradations between normal tactoids and fibres can well be seen in the series of preparations with different ionic concentrations as shown in Plate 2, Figs. 11–14. Those containing more salt show more elongated tactoids and a greater tendency to fibre formation. The radius of curvature changes from 0.07 mm. in salt-free solutions to 0.12 mm. in 0.1 N solutions. It is clear that the condition of stability of a tactoid depends on two factors, the length of the particles, and the degree to which mutual influence between the particles maintains parallelism between them. Although it has not yet been calculated the orienting force can be seen to depend according to Levine's theory on the ratio of length to distance apart of the particles. If two particles of finite length be imagined to move away from each other, it is clear that the steepness of the potential trough for angular displacements will diminish, and diminish faster the shorter the particles. For concentrated solutions where the ratio of length of particles to mean distance apart is very great, orientation will

[5] That this is probable is shown by the extrusion of an intermediate layer of impurities on the settling of bottom layer solution, that is, on increasing the size and diminishing the number of independent tactoids.

be perfect, but as the salt concentration falls off the point will be reached when the system will become unstable, not owing to the transverse motion of the particles, but due to their angular oscillation. For particles of finite length, therefore, equilibrium will be reached at greater dilution the longer the particles and the higher the concentration of ions in the solution. This relation will hold until the concentration of ions is so great that particles cannot separate due to oscillatory movements alone. This is the explanation of the apparently anomalous behaviour of weak solutions described below. For different lengths of particles the behaviour at low concentrations will be different, but all will have the same behavior at high concentrations and for every particle length there will be a critical concentration at which the maximum interparticle distance is obtained.

Equilibrium Concentrations of Top and Bottom Layer

The variation of equilibrium concentrations with length of particle is very well shown in the study by Bawden and Pirie (1937*a*) on the effect of purification of the virus. One effect of this purification would be to separate the longer from the shorter particles. The first method used was to allow the virus solution to separate into two layers, to take the bottom layer and to dilute it with water up to the original volume and to allow further separation. If the virus originally contained particles of different lengths in equilibrium there would be a greater concentration of shorter particles in the top layer and the process described would at each stage lead to more uniform and longer particles in the bottom layer. The second method of purification, by means of trypsin digestion also probably led to longer particles, possibly by removing material at the end of the particles which prevented them joining with others. The general observation of Bawden and Pirie was that further purification of the virus altered very markedly the equilibrium concentration between top and bottom layer solutions.

Unpurified virus, *i.e.* salt-precipitated but not subjected to the above methods, remained in the top layer solutions up to concentrations of 4 per cent, when it was in equilibrium with bottom solutions of concentrations of 8 per cent, while highly purified solutions of as little as 1.6 per cent divided into two layers, the difference of concentration between them being as low as 0.02 per cent. Moreover, at this concentration, the top solutions showed a flow birefringence which remained in evidence for some seconds after actual flow had ceased. This suggests that we are dealing with an equilibrium system in which the alternation of one variable causes a continuous transition between a distinct two phase region and a critical condition in which only one phase is possible. One plausible suggestion is that the length of the particles is the variable factor though the effect of unidentified impurities may be of as great or greater importance.

Consider the conditions that determine the limiting concentration in top and bottom layer solutions. In the top layer, this is fixed by the limit of the space available to a particle for free movement. The minimum space may be taken as that of a flat cylinder of height equal to the diameter of the particle (d) and diameter equal to its length (l) (see p. 143). Its volume is accordingly

$$\frac{\pi l^2 d}{4}$$

It may actually be smaller than this if particles on moving around manage to clear others out of their way without jamming, but it is difficult to work this out theoretically. In the bottom layer on the other hand, it is reasonable to suppose that the equilibrium distance between parallel particles is a simple function of their length, that is, that the possibility of maintaining a parallel alignment is a function of the ratio of the distance (r) of the particles apart to their length, and that beyond a certain critical ratio (K) brownian movement would make the arrangement unstable. On the simplest hypothesis, therefore, the volume occupied by a single particle will be:

$$\frac{\sqrt{3}}{2} r^2 l = \frac{\sqrt{3}}{2} K^2 l^3 \quad \text{where} \quad K = \frac{r}{l}$$

The limiting concentrations of the top and bottom layers are therefore $\frac{4m}{\pi l d}$ and $\frac{2m}{\sqrt{3}\, K^2 l^2}$ respectively where m is the mass of dry virus per unit length.

It can be seen that as the length of the particle increases both equilibrium concentrations decrease, and further that at a certain critical concentration the difference between them vanishes, that is where

$$l = \frac{\pi d}{2\sqrt{3}\, K^2}$$

This concentration appears to be 1.8 per cent for tobacco mosaic virus (Bawden and Pirie, 1937 *a*).[6] For a solution of twice this concentration in the top layer, 3.6 per cent, the corresponding bottom layer concentration would be four times, or 7.2 per cent which approximates more closely than should be expected with the values 4 per cent and 8 per cent respectively given by Bawden and Pirie. Moving to higher concentrations we find that a limiting concentration of 9 per cent in the top layer would correspond to one of 45 per cent for the bottom, which is a maximum concentration, being approximately that of wet gel or precipitated crystals. This suggests that the phenomena of separation

[6] Page 287.

into layers, and indeed the existence of bottom layer, can only be observed for particles whose lengths are not less than one-fifth of the critical length.

From the recent measurements of Kausche *et al.* (1939) on tobacco mosaic virus we find this ratio is at least ten to one, so that we may expect orientation always to occur for tobacco mosaic virus at sufficient concentrations. On the other hand the conditions for observing it for other viruses whose particles are not so long though still anisotropic may be more difficult to achieve. It should be possible to check this theory quantitatively when samples of virus of uniform and known length can be obtained, and further to check it by measuring the effect of ionic concentrations and pH on its equilibrium. The influence of temperature on equilibrium would give further information, particularly of the interaction energy. Preliminary measurements show that while changes of temperature affect the equilibrium in the expected way, that is rise of temperature diminishes the amount of the ordered phase, the effect is a small one and therefore corresponds to a very small interaction energy. The whole problem has now acquired such theoretical importance that it deserves an extended study of its own, using uniform and purified materials in large quantities.

The Mechanism of Gel Formation

The conditions of equilibrium between elongated particles in ionic solutions has a very close bearing on the formation of gels. A simple experiment illustrates this in the case of tobacco mosaic virus. If a dilute solution in the oriented phase, of concentration about 4 per cent, is flowed through a capillary tube it behaves like a uniform uniaxial crystal (Text-fig. 11 *a*). If now some acid is introduced in one end of the tube, then as long as the flow continues the same appearance is maintained and there is no notable change in the viscosity. We know, however, from the x-ray study that now the particles are very unevenly distributed and that the liquid is really a two phase one consisting of long tactoids with about 50 per cent dry weight of virus, and of almost pure solvent (Text-fig. 11 *b*). Once the flow is stopped particularly if the tube is jerked, the orientation largely vanishes and the liquid changes to a fairly rigid gel. It is clear that here disturbance of flow lines causes different tactoids to touch and form a combined tactoid framework throughout the liquid. We know that two tactoids in contact fuse into a branched tactoid (see p. 128). We may therefore consider that the whole of the more concentrated phase is a single tactoid (Text-fig. 11 *c*). Now the behaviour described is typical of a rheopectic gel, and it would seem very probable that a similar explanation holds for all cases of gel formation, in which long thin particles are involved. In such cases a gel must be considered to be a two phase system in which one of the phases is a solvent containing a small proportion of particles and the other is a liquid crystalline branched aggregate also containing a

variable amount of the solvent. On standing the aggregation may go further and the gel breaks down into microscopic fibres. Other gels, particularly those showing thixotropic characters, are probably formed under analogous but somewhat different conditions on account of the different nature of the forces between flat and long particles. This type of explanation would seem to offer a solution to the controversy between the rival theories of contact of particles and long distance forces in the explanation of gel formation. Both theories would seem to be right, though in a different sense than that originally propounded. The long distance forces are not exercised through the main bulk of the gel, but only between particles constituting individual tactoids; whereas the interaction between tactoids corresponds to the supposed contacts of colloidal particles. At the present stage, it is only possible to say safely that this applies to tobacco mosaic virus, but the methods of investigation described above could well be used to check the validity of this theory for other colloidal systems.

The Summary and Bibliography appear at the end of Part III.

EXPLANATION OF PLATES

Plate 1

Photographs of orientation phenomena of virus solutions and gels (crossed nicols).

Fig. 1. Tobacco mosaic virus solution in drying cell showing (*a*) bottom layer solution; (*b*) wet gel; and (*c*) dry gel.

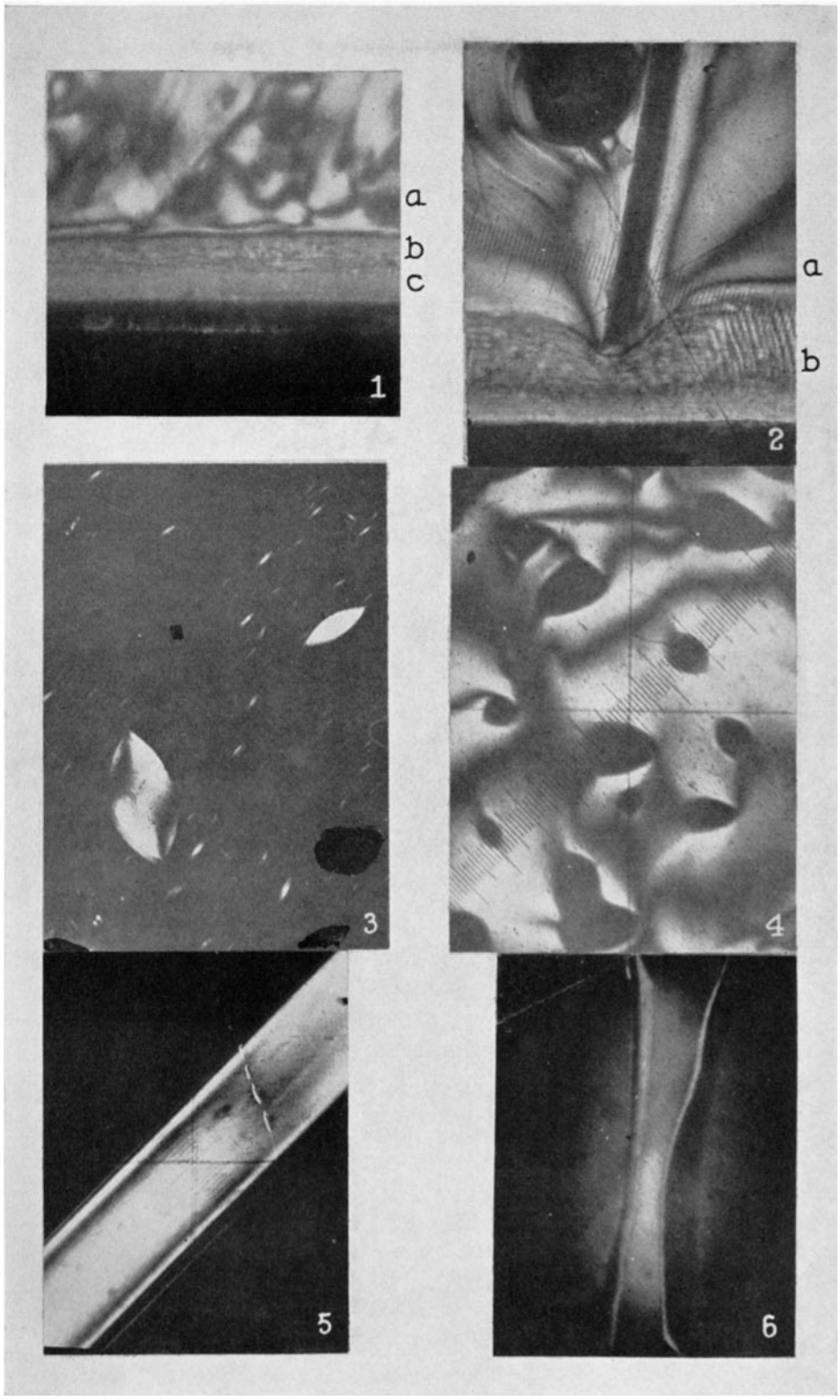
Fig. 2. As Fig. 1, but showing (*a*) flow of bottom layer solution around end of spacer rod from left to right and (*b*) zigzag drying in wet gel.

Fig. 3. "Positive" tactoids of bottom layer solution in top layer.

Fig. 4. "Negative" tactoids of top layer in bottom layer solution showing sinuous orientation directions.

Fig. 5. Oriented bottom layer solution in capillary tube with quartz wedge showing compensation.

Fig. 6. Orientation of top layer solution by alternating current showing electrodes and luminous patch of oriented top layer.



(Bernal and Fankuchen: X-ray and crystallographic studies of plant virus. I, II)

Plate 2

Further orientation phenomena of virus solutions and gels.

Reversed spiral structures—Figs. 7–9.

Fig. 7. Reversed spirals in wide tube. Taken by Professor Van Iterson. (Crossed nicols.)

Fig. 8. Reversed spirals in narrow tube. Taken by Professor Van Iterson. (Crossed nicols.) Showing dark—parallel oriented and light—spirally arranged portions.

Fig. 9. With one nicol rotated showing alternate sections with same direction of rotation of polarisation.

Fig. 10. Zigzag structure of virus dried on plate (unpolarised light) taken by Professor Van Iterson.

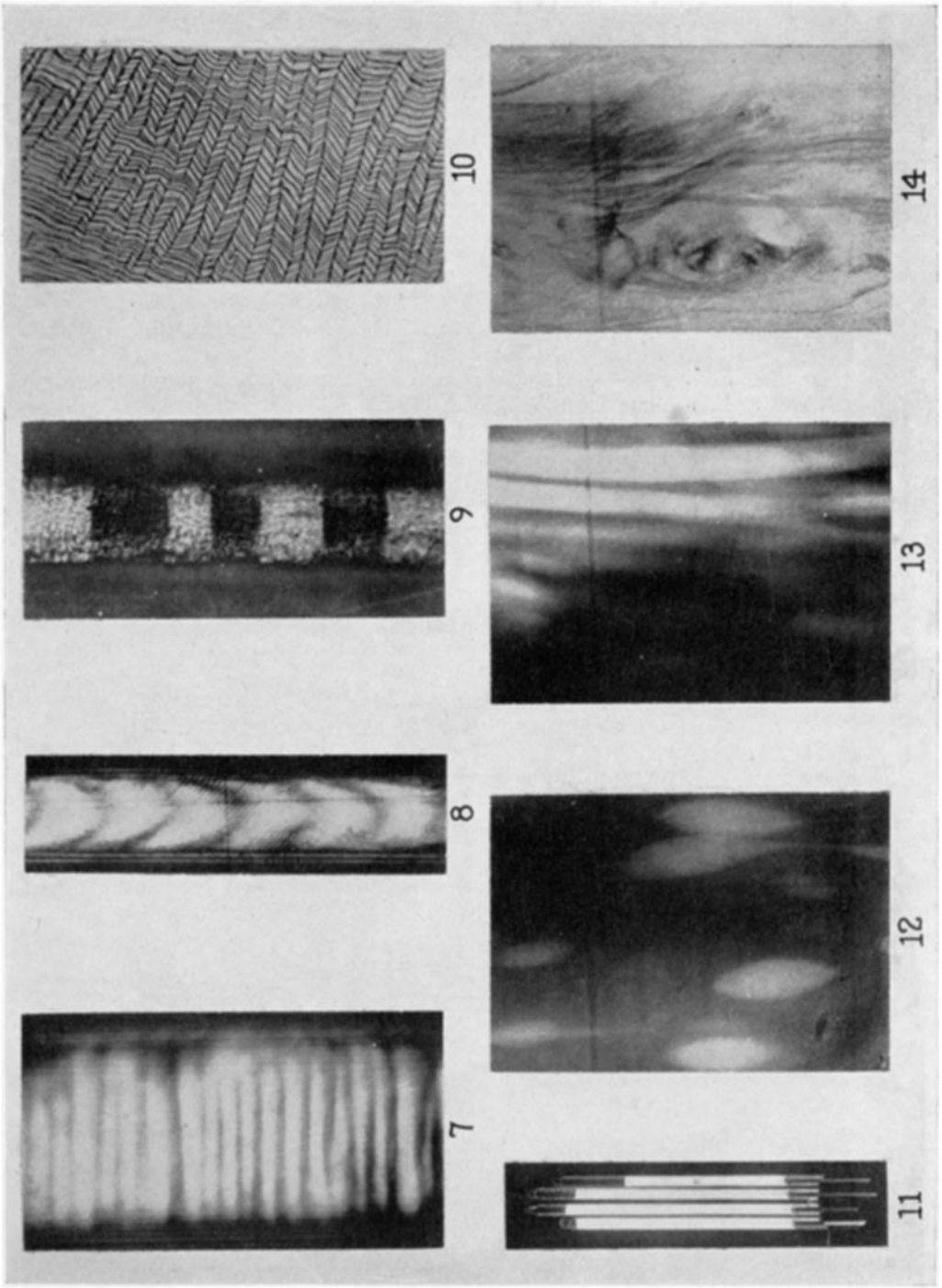
Effects of dilute salt solutions on virus preparations—Figs. 11–14.

Fig. 11. Four capillaries with the same virus concentration but with salt content increasing from right to left. Note the greater amount of bottom solution as salt content increases (crossed nicols).

Fig. 12. 0.1 N $(NH_4)_2SO_4$ solution showing negative tactoids (crossed nicols).

Fig. 13. 0.2 N $(NH_4)_2SO_4$ solution showing elongated negative tactoids (crossed nicols).

Fig. 14. 0.3 N $(NH_4)_2SO_4$ solution showing gel fibres (unpolarised light).



(Bernal and Fankuchen: X-ray and crystallographic studies of plant virus. I, II)

PLATE 3

Inter- and intramolecular x-ray photographs of various virus preparations.

Intermolecular photographs—Figs. 15–18.

FIG. 15. Showing shift of intermolecular reflections on passing from (lowest film) dry gel to (middle film) wet gel and (uppermost film) 13 per cent solution.

FIG. 16. Effect of concentration of virus on spacing.

(*a*) 13 per cent solution. Unfiltered Cu radiation

(*b*) 23 per cent solution. Unfiltered Cu radiation

(*c*) Top half, 34 per cent solution. Unfiltered Cu radiation
Bottom half, 34 per cent solution. Filtered Cu radiation

(*d*) 34 per cent solution. Monochromatic Cu K_α radiation

(*e*) 50 per cent gel. Unfiltered Cu radiation

FIG. 17. Enation mosaic virus solution photographed with monochromatic Cu K_α radiation showing extremely sharp lines due to perfect orientation and arrangement.

FIG. 18. Intermolecular pattern of dry gels of different virus proteins. (*a*) tobacco mosaic virus. (*b*) enation. (*c*) aucuba. (*d*) cucumber.

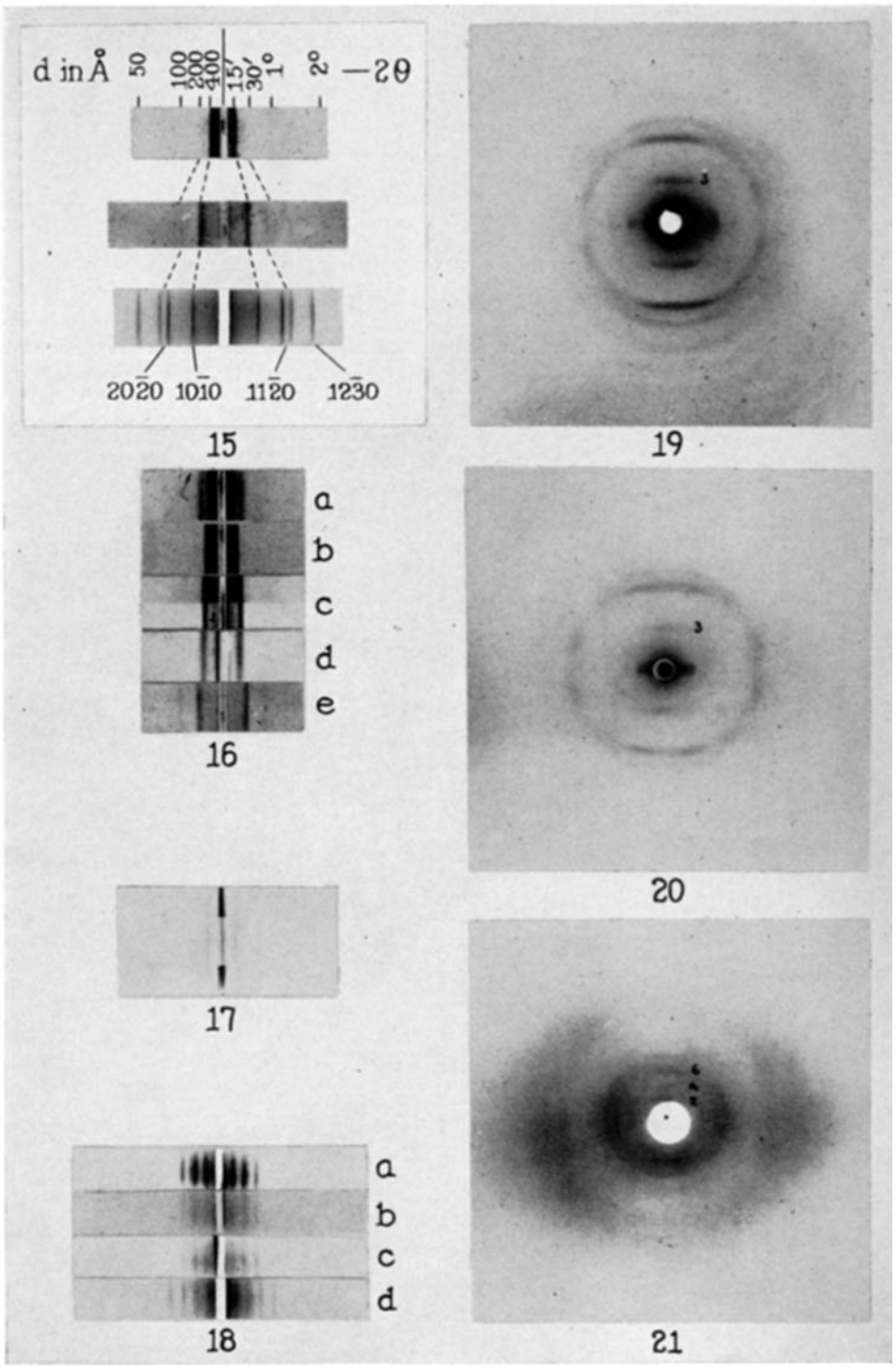
Plate distances: Figs. 15, 16, 17—40 cm.; Fig. 18—16 cm.

Intramolecular photographs—Figs. 19–21.

FIG. 19. Typical early dry gel photograph of tobacco mosaic virus.

FIG. 20. Cucumber mosaic virus.

FIG. 21. Potato virus X (shorter plate distance). Note the two lines 2 and 4 inside the strong meridian line 6 instead of the single line 3 on tobacco and cucumber virus photographs.



(Bernal and Fankuchen: X-ray and crystallographic studies of plant virus. I, II)

PLATE 4

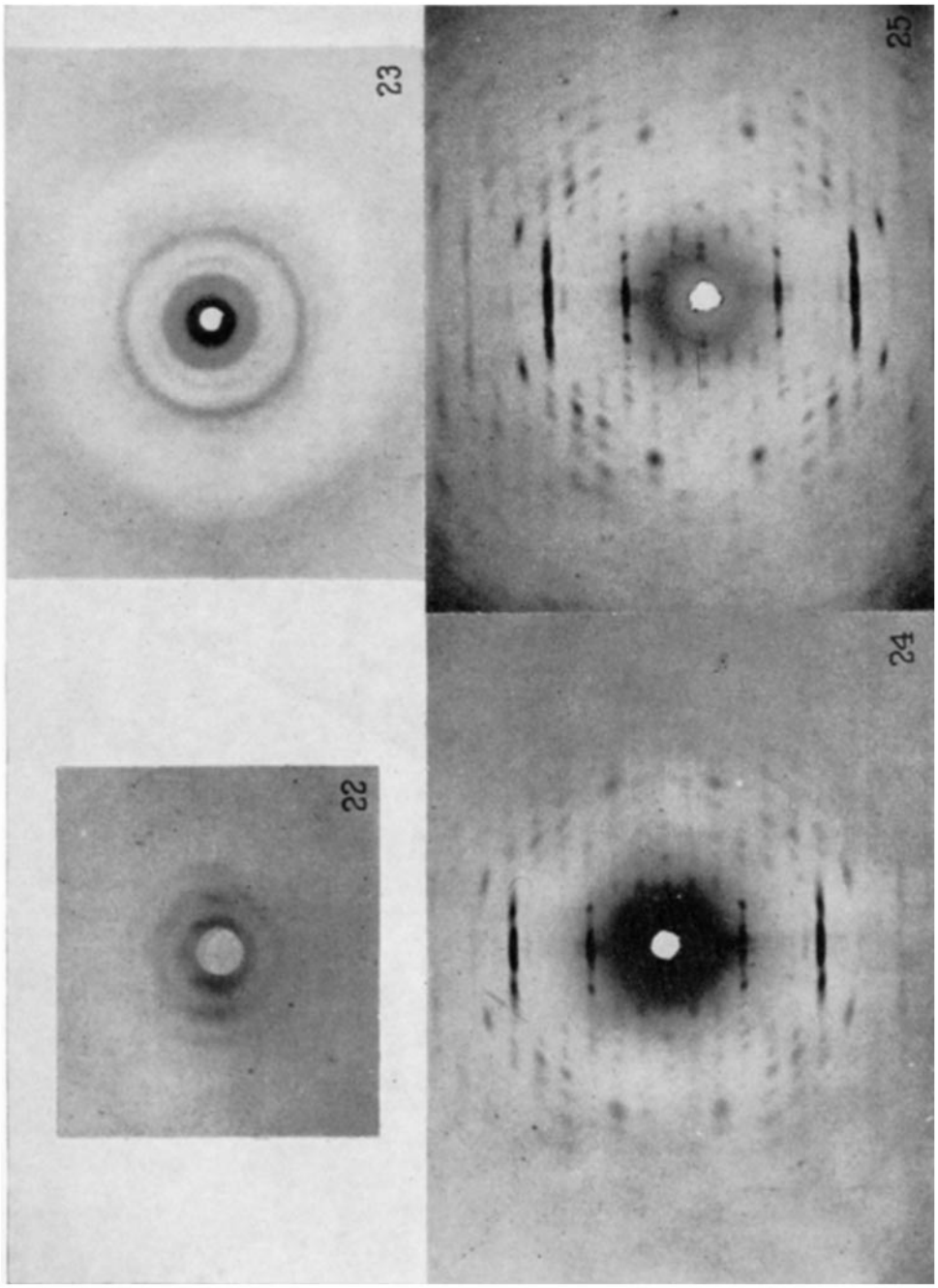
X-ray photographs of tobacco mosaic virus.

FIG. 22. Intermolecular pin-hole photograph (plate distance 30 cm.) of small piece of dry gel mounted with x-rays along particle direction showing orientation relative to surface (vertical in photograph) and hexagonal single crystal character. It can be seen that the $(11\bar{2}0)$ and not the $(1\bar{1}00)$ planes lie parallel to the surface (air-water interface in drying cell (Text-fig 2).

FIG. 23. Intramolecular pin-hole photograph (plate distance 6 cm.) of the same piece showing completely random orientation of individual particles.

FIG. 24. Intramolecular photograph of 36 per cent enation solution in capillary tube (direct print). (Plate distance 14.8 cm.)

FIG. 25. Same as Fig. 24, but printed through a rotating sector disc to show all important reflections of comparable intensity.



(Bernal and Fankuchen: X-ray and crystallographic studies of plant virus. I, II)