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A study of the states of aggregation of alfalfa mosaic virus protein

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[Plates 22 and 23]

The states of aggregation of alfalfa mosaic virus (AMV) protein have been characterized by sedimentation velocity experiments and electron microscopy. The main association product is a spherical particle with an *s* value of about 30S. It is highly likely that the assembly of this particle starts with dimers of the 25000 molecular mass unit resulting in an icosahedral particle made of 30 dimers. No intermediate aggregation products have been detected.

The clustering pattern of the protein in the cylindrical part of the AMV capsid favours the concept of dimers as the active assembling units.

Introduction

Among the plant viruses alfalfa mosaic virus (AMV) occupies a special position. In purified preparations of this virus a physical heterogeneity exists such that the viral genome is distributed over different particles. (For a recent review see Jaspars & van Kammen 1972.) The rough shape of the nucleoprotein particle can be described by a cylindrical rod with rounded caps at the ends. The length of the cylindrical part of the virus particle is variable and on this basis a characterization into bottom, middle and a number of top components can be carried out (van Vloten-Doting, Dingjan-Versteegh & Jaspars 1970). The bottom component has a length of about 64 nm, whereas the smallest particle of this series measures about 29 nm. It appears that these main components each contain a specific RNA molecule resulting in four types of RNA species with molecular masses of 1.3, 1.1, 0.9 and 0.3×10^6 (Jaspars 1974). However, the total genome consists of the first three types of RNA molecules. It has been shown that the coat of AMV is constructed from one type of polypeptide chain, with a molecular mass of about 25×10^3 (Kruseman *et al.* 1971). In some strains abnormal long particles exist, which contain a complex distribution of different RNA molecules (Hull 1970; Heijtink 1974). The structure of the protein of these virus particles is probably very similar to the 'wild' type protein (B. Kraal, personal communication). Apart from this polymorphism AMV exhibits a number of other features which are unlike most other plant viruses. The coat protein does not fulfil a protective function in the strict sense, as the virus RNA can be hydrolysed *in situ* by RNAse (Bol & Veldstra 1969). The nucleoprotein particle also behaves in an unexpected way towards its RNA, so that when these 2 components are incubated together, the nucleoprotein structure will dissociate (van Vloten-Doting & Jaspars 1972).

We have started to investigate the assembly of this virus over a wide range of conditions, in order to get more information about the pathway of assembly. Previous work on the association of AMV protein with and without RNA has been reported by Bol & Kruseman (1969), Lebeurier, Fraenkel-Conrat, Wurtz & Hirth (1971) and Heijtink (1974). As the main association product of the coat protein a spherical particle was found, with a sedimentation coefficient

of about 30S. Hull, Hills & Markham (1969) have performed electron microscopic studies in order to derive structural parameters for AMV. They postulated the existence of a hexagonal surface lattice with a hexamer clustering pattern of the protein.

In the course of our studies we have been able to determine a model for the structure of the coat of AMV at low resolution by using quantitative methods to analyse the electron microscopic data (Mellema & van den Berg 1974; Mellema 1975).

The scope of this contribution will be to describe the association behaviour of the coat protein, which has been studied in a systematic way by hydrodynamic methods.

Moreover the structural data will be discussed in relation to the different aggregation products found. Future research will be directed towards the study of assembly of protein and RNA which will eventually lead to a better insight into the morphogenesis of this interesting virus at the molecular level.

Materials and methods

Alfalfa mosaic virus was isolated from fresh tobacco leaves (*Nicotiana tabacum*), inoculated 5 days earlier. The procedure for isolating and purifying the virus was according to van Vloten-Doting (1968). The purified virus was dissociated in a solution, containing 0.5 M $MgCl_2$ in phosphate buffer pH 7.0, in which the RNA precipitated, as described by Kruseman (1969).

After centrifugation the supernatant contained residual RNA and $MgCl_2$. Three dialysis steps were required to remove the $MgCl_2$. The protein solution was dialysed against 10^{-3} M sodium acetate/acetic acid buffer pH 5.0 for 2 days, followed by 2 days dialysis against 0.25 M sodium acetate/acetic acid pH 5.0, 5 mM EDTA and finally the protein solution was dialysed against 10^{-3} M sodium acetate/acetic acid pH 5.0, in which the protein solution was stored. All dialyses were carried out at 4 °C. The stock solution contained 5–10 mg/ml protein.

Protein concentrations were determined spectrophotometrically, using an extinction coefficient E_{280} (1 mg/ml) = 0.7.

The residual RNA content was estimated by the E_{260}/E_{280} ratio and determined by the method of Knight & Woody (1958). The RNA content was found to be 0.3–0.6 % and the E_{260}/E_{280} ratio was about 0.7 to 0.8. Efforts to remove the RNA completely from the protein solutions by column chromatography have not been successful yet. According to the procedure recommended by Pfeiffer & Hirth (1974), variations in pH and ionic strength of the AMV protein solutions, were never carried out at the same time. The pH dependence of the aggregation was studied by dialysing the protein solution at constant ionic strength against buffers with different pH values and dialysis of the protein solution against buffers with different ionic strength was always carried out at constant pH.

In the AMV protein system variations of the pH were performed only at low ionic strength. Once the required pH was obtained in this way, the required ionic strength was reached by dialysing the protein solution at constant pH. The period of time necessary for dialysis was found to be 2 days. Several kinds of buffer solutions have been tested to attain the required pH. Sodium acetate/acetic acid in the pH range 4.0–5.5 and sodium pyrophosphate/pyrophosphoric acid in the pH range 5.0–9.0 were the most suitable buffers to study the protein association behaviour (Longchamp, Lebeurier & Hirth 1972). The ionic strength was adjusted by the 'own' buffer ions, i.e. not by adding any other salt. It was found that in the overlapping pH-ranges of the different buffers used, the aggregation states were similar.

Sedimentation velocity and sedimentation equilibrium experiments were carried out with a

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Spinco Model E analytical ultracentrifuge, equipped with both Schlieren optics and an ultraviolet scanning system. S-values were corrected to $s_{20,w}$.

The association behaviour of the protein at low ionic strength has been studied turbidimetrically. The turbidity was measured at a wavelength of 320 nm in a spectrophotometer.

Electron microscopy was performed with a Philips EM 300. Negatively stained specimens were prepared with uranyl-oxalate. The image processing by optical diffractometry and digital methods have been described by Mellema & van den Berg (1974) and Mellema (1975).

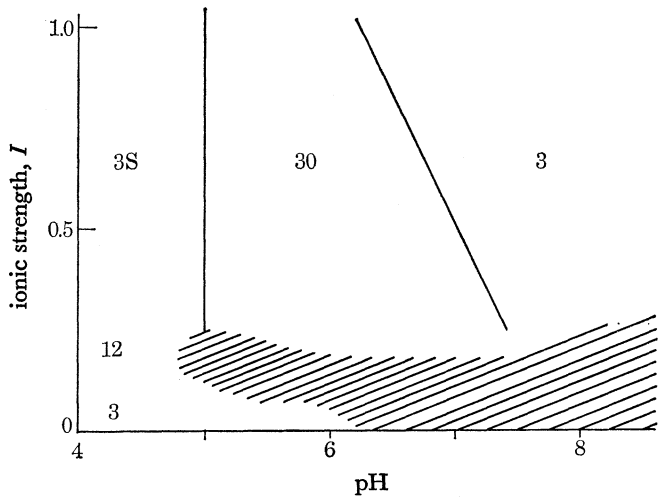

FIGURE 1. Diagram showing the various types of aggregates, existing at different pH and ionic strength values at 4 °C. The lines represent 50% transitions. In the hatched area considerable aggregation occurs and the protein precipitates at rather low concentrations. Regular structures are formed in this zone. Protein concentration was 1 mg/ml in Na pyrophosphate above pH 5.0 and 5–10 mg/ml in Na acetate below pH 5.5.

RESULTS

(*a*) *The aggregation states of AMV protein*

(i) *Phase diagram*

Among the many factors which are important in the association behaviour of AMV protein, the pH and ionic strength play a dominant role. A convenient way to plot the occurrence of the different assembly products is to map the states of aggregation in a diagram. A number of other aggregating protein systems have been described in a similar way, e.g. tobacco mosaic virus protein (Durham, Finch & Klug 1971), brome mosaic virus protein (Pfeiffer & Hirth 1974) and cowpea chlorotic mottle virus protein (Adolph & Butler 1974).

In this diagram, presented in figure 1, all the parameters except pH and ionic strength have been fixed. These parameters are: the temperature, concentration, the time course of dialysis, the pathway for dialysis from the initial conditions of pH and ionic strength towards the final state in the diagram.

To reach equilibrium, two days of dialysis were sufficient as was checked by following the time course of a number of reactions. However, this period depends on the way dialysis was carried out, as will be discussed in §*a*(vi).

The aggregates in the different regions of the state diagram are characterized by their S-values. The phase boundaries represent 50% transitions, i.e. the region indicated as '30-S'

represents the zone in which more than 50 % of the protein is associated into 30S. The hatched zone in the diagram represents an area in which higher aggregation products are formed. Under these conditions of pH and ionic strength the products formed will precipitate even at low protein concentrations. The association behaviour in this zone can not be studied with the ultracentrifuge. Therefore turbidity experiments have been performed, to determine the rough boundaries of this area in the diagram.

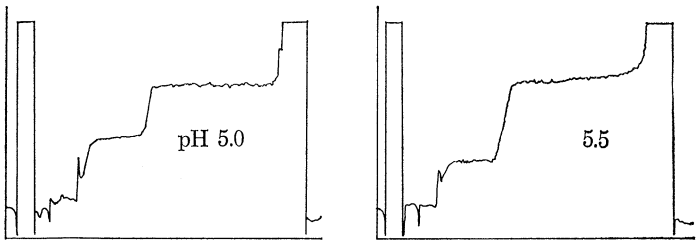

FIGURE 2. An ultraviolet scanning pattern during a sedimentation velocity experiment. Rotor speed was 48000 rev/min. Sedimentation was from left to right and the temperature was 4 °C. The two boundaries in each pattern represent the 3S and 30S components. The experiments were carried out at ionic strength 0.5 and pH 5.0 (left) and 5.5 (right).

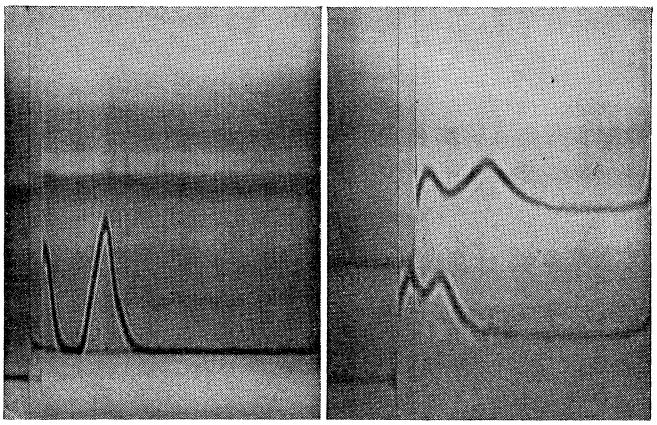

FIGURE 3. Schlieren patterns of AMV protein. The photographs are taken about 20 min after the rotor reached a speed of 48000 rev/min. Left: AMV protein in pyrophosphate buffer, pH 6.8, $I = 0.25$. The peak has a $s_{20,w}$ value of 30S. Protein concentration about 6 mg/ml. Right: AMV protein in acetate buffer, pH 5.5 (upper pattern) and pH 5.0 (lower pattern), $I = 0.1$. The $s_{20,w}$ values in the upper pattern are 3S and 30S (respectively left and right) and in the lower pattern 3S and 12S. Protein concentration about 5 mg/ml. Sedimentation from left to right. Schlieren angle 60°.

(ii) *pH dependence of the aggregation states*

The relative amounts of the aggregation products were calculated from the absorbances at 280 nm measured with the ultraviolet scanning system, or from the peak areas when Schlieren-optics were used. Figure 2 represents an example of a typical pattern of the scanning system and an example of a Schlieren photograph is given in figure 3.

In this way the proportions of 3S and 30S aggregates under various pH conditions have been investigated at constant levels of ionic strength. The results are shown in figure 4.

At low pH the AMV protein aggregates into a product which is designated as 12S. From the Schlieren pattern in figure 3, it is evident that the association behaviour of the protein in the

pH range 5.0–5.5 changes dramatically. At pH 5.0 the protein associates into the 12S component, whereas at pH 5.5 only 30S aggregate together with 3S component exist.

The determination of the precise stability region of the 12S component remains to be done.

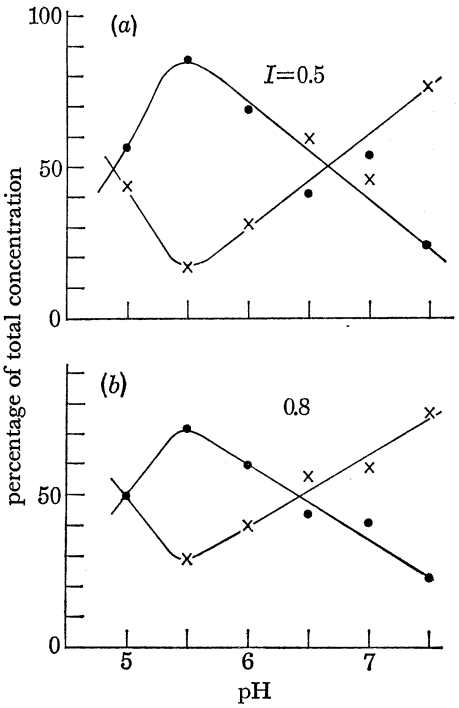

FIGURE 4. Relative amounts of 3S (×) and 30S (●) component as a function of pH in pyrophosphate buffer and at a total protein concentration of 1 mg/ml at ionic strength 0.5 (*a*) and 0.8 (*b*). The points where the two curves intersect indicate the phase boundaries. From these two plots it can easily be seen that the ratio 30S/3S decreases with higher ionic strength values.

(iii) *Effect of ionic strength*

The ionic strength proved to be a very important factor in the behaviour of AMV protein. In all experiments it has been shown that the 30S aggregate breaks down into the 3S form with increasing ionic strength values. Due to the strong tendency of the 30S component to aggregate into very fast sedimenting species at low ionic strength values, the determination of the yield of assembly by sedimentation velocity is not reliable in this case. Therefore the breakdown of the 30S aggregate has been studied turbidimetrically. Some of these results are shown in figure 5. The extinction at 320 nm is given as a function of ionic strength with variable pH.

Apparently at very low ionic strength the extent of aggregation is minimal. The association of protein will take place, after the ionic strength reaches a certain value. The value of the ionic strength at which maximum aggregation occurs is dependent upon pH. This value of the ionic strength increases with lower pH values. In acetate buffers this effect is very clear as no association is detected at ionic strength as low as 0.03.

(iv) *Influence of the temperature*

Preliminary experiments have indicated that an increase of the temperature to 22 °C influences the relative amounts of 30 and 3S components. These experiments were carried

out at a number of different pH and ionic strength values so that the same protein solutions were analysed in order to determine the relative amounts of the 3 and 30S components at different temperatures. In all cases an increase in the amount of 30S component was observed. In due time studies on this temperature dependence will be performed to derive some thermodynamical data.

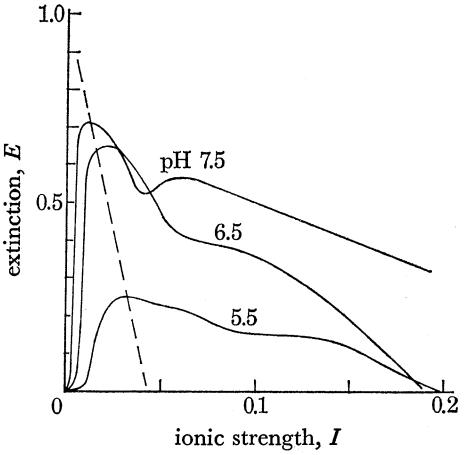

FIGURE 5. The association of AMV protein, measured turbidimetrically as a function of ionic strength. The extinction at 320 nm was measured in a spectrophotometer. Experiments were carried out at various pH values (pH 5.5, 6.5 and 7.5). The dotted line indicates the dependence of the ionic strength values upon pH. Moreover, these plots show that at higher pH values association is more extensive. Protein concentration in all experiments was 0.1 mg/ml.

(v) *Influence of the protein concentration*

The protein concentration has been shown to play a very important role in the association process, as little or no association was observed in the acetate system at protein concentrations lower than 1 mg/ml, whereas considerable association occurred at protein concentrations of 5 mg/ml and higher.

Therefore the effect of the protein concentration has been studied in the pyrophosphate system, as association takes place in pyrophosphate at much lower protein concentrations.

The concentration dependence was studied at three positions in the phase diagram; in the 3S region, on a phase boundary and in the 30S region. The corresponding pH values were 4.5, 5.0 and 5.5, all at ionic strength 0.5. In figure 6 the concentrations of 3S and 30S components are shown as a function of total protein concentration. The graph at pH 5.5 clearly indicates that the 3S component reaches a constant level. This observation agrees with the CCMV-protein system, where Adolph & Butler (1974) have found a similar behaviour of the virus protein. They described this feature as a 'quasi-crystallization'. The scope of this theory in this context is that there is a critical concentration c of the 3S form, beyond which the protein 'crystallizes' into the 30S form. Using the same assumption as Adolph & Butler (1974) and subsequently using the same equation for the free energy change (ΔG) on binding of the polymerizing unit ($\Delta G = RT \ln c$), for the free energy change at pH 5.5 $\Delta G = 36$ kJ/mol (8.6 kcal/mol) has been found. At other pH values we have not yet been able to detect constant 3S level. Although the concept of quasi-crystallization has not been substantially

proven to be valid for the aggregation of AMV protein, it might provide an explanation for the odd behaviour of the protein.

The type of buffer ions probably plays an important role in this concept as well.

(vi) *Confirmation of the equilibrium states*

The foregoing results are only valid when the system is in equilibrium. That equilibrium is reached has been proven by the fact that the relative amounts of 3S and 30S aggregate in protein solutions after two days dialysis remained the same after 4 days dialysis. Moreover, the system has been tested for reversibility. At high ionic strength ($I > 0.2$) it has been observed

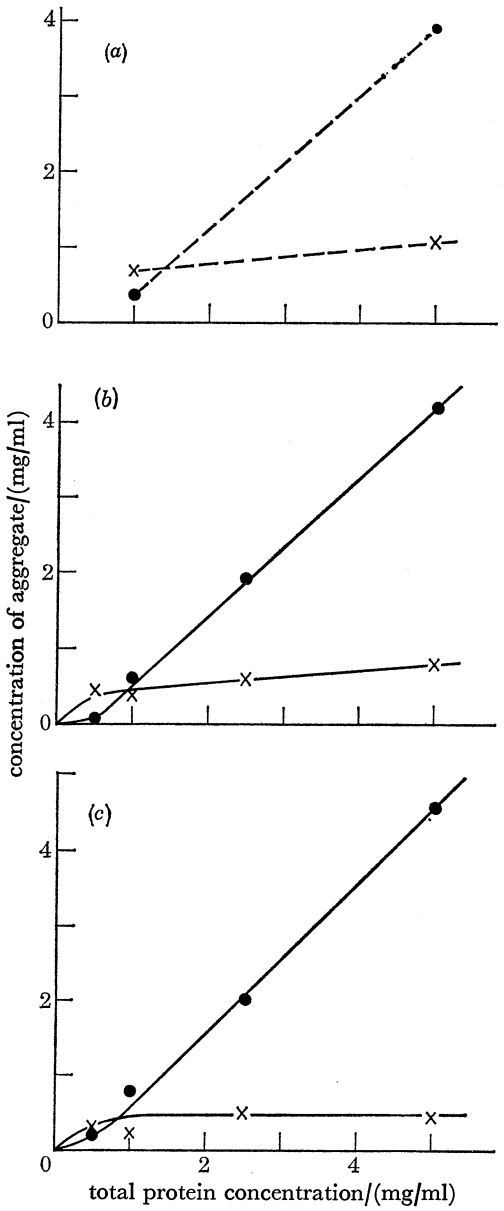

FIGURE 6. The concentrations of the 3S (×) and 30S (●) component as a function of total protein concentration at 4 °C and ionic strength 0.5. Three different pH values were used: (*a*) pH 4.5, (*b*) pH 5.0, (*c*) pH 5.5. Although the 3S concentrations at pH 4.5 and pH 5.0 do not reach a precise critical concentration level, the graph of pH 5.5 favours the theory of quasi-crystallization, described by Adolph & Butler (1974).

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that when protein solutions were dialysed against a buffer with different pH or ionic strength, a new equilibrium state was reached after three or more days.

As has been observed in many cases, the rate of aggregation in the hatched zone of the phase diagram is much higher than at any other point. For this reason, variations with pH were carried out at low ionic strength in all experiments.

(*b*) *Characterization of the aggregation products*

(i) 3S *component*

The identity of this low molecular mass product has been extensively examined by Kruseman (1969) and Kruseman *et al.* (1971). They found for the sedimentation velocity coefficient $s_{20,w} = 2.95$. Our observations are in accordance with these results. Through the text this component will be designated by 3S. The monomer of the protein has a molecular mass of about 25000. Kruseman *et al.* (1971) measured the molecular mass of the 3S component as about 50000 and they concluded that the protein exists in the dimeric form. We have tested whether this value of the molecular mass is correct over a wider range of protein concentrations than determined by the previous mentioned workers. The results are shown in figure 7.

The conclusion that under the experimental conditions the protein exists as dimers seems very likely, although more experiments at other values of pH and ionic strength are necessary.

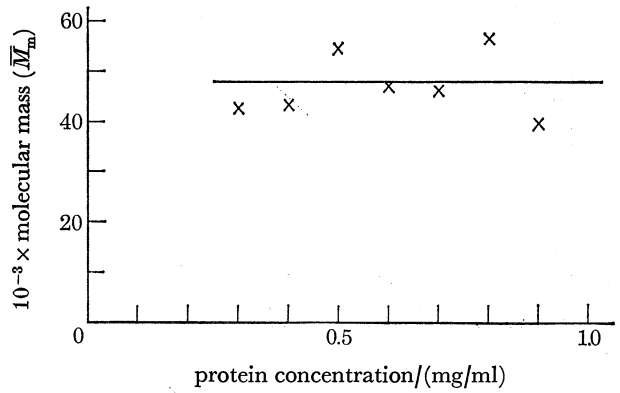

FIGURE 7. Mass average molecular mass of the 3S component as a function of protein concentration. Measurements were performed at pH 5.0 and at an ionic strength of about 0.02. The temperature was 4 °C. Determination of the molecular mass at very low protein concentrations is in progress. The figure shows that the average molecular mass of the 3S component is nearly twice the molecular mass of the monomer, taken as 25000 (Kruseman *et al.* 1971).

DESCRIPTION OF PLATE 22

FIGURE 8. Left: The main product of the AMV-protein association, the 30S particle in pyrophosphate buffer pH 5.5, $I = 0.5$ (magn. ×250000). Right: An example of the bigger spherical particles, sometimes seen under the electron microscope (magn. ×80000).

FIGURE 9. The aggregation products of AMV protein, found at low ionic strength ($I = 0.1$), at different pH values. Top left: 'ribbon-like' structures found between pH 4.0 and 5.0. Top right: an example of one-dimensional arrays of 30S particles, found between pH 5.0 and 5.5. Bottom left: 'sheet-like' structure, mounted over a hole in the carbon film, found over a wide pH-range. Bottom right: crystal of 30S particles, found between pH 6.0 and 7.0.

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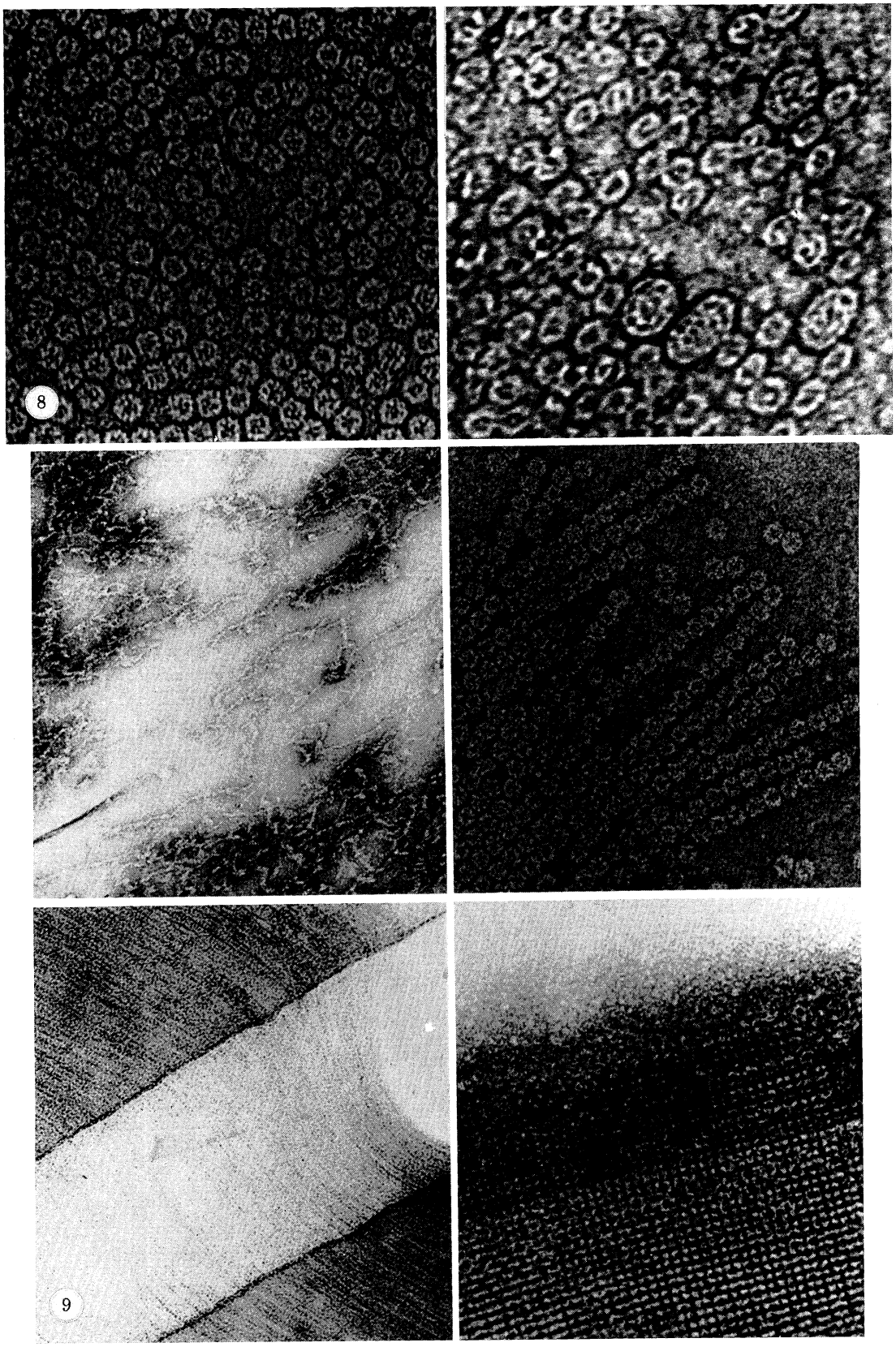

FIGURES 8 AND 9. For description see opposite.

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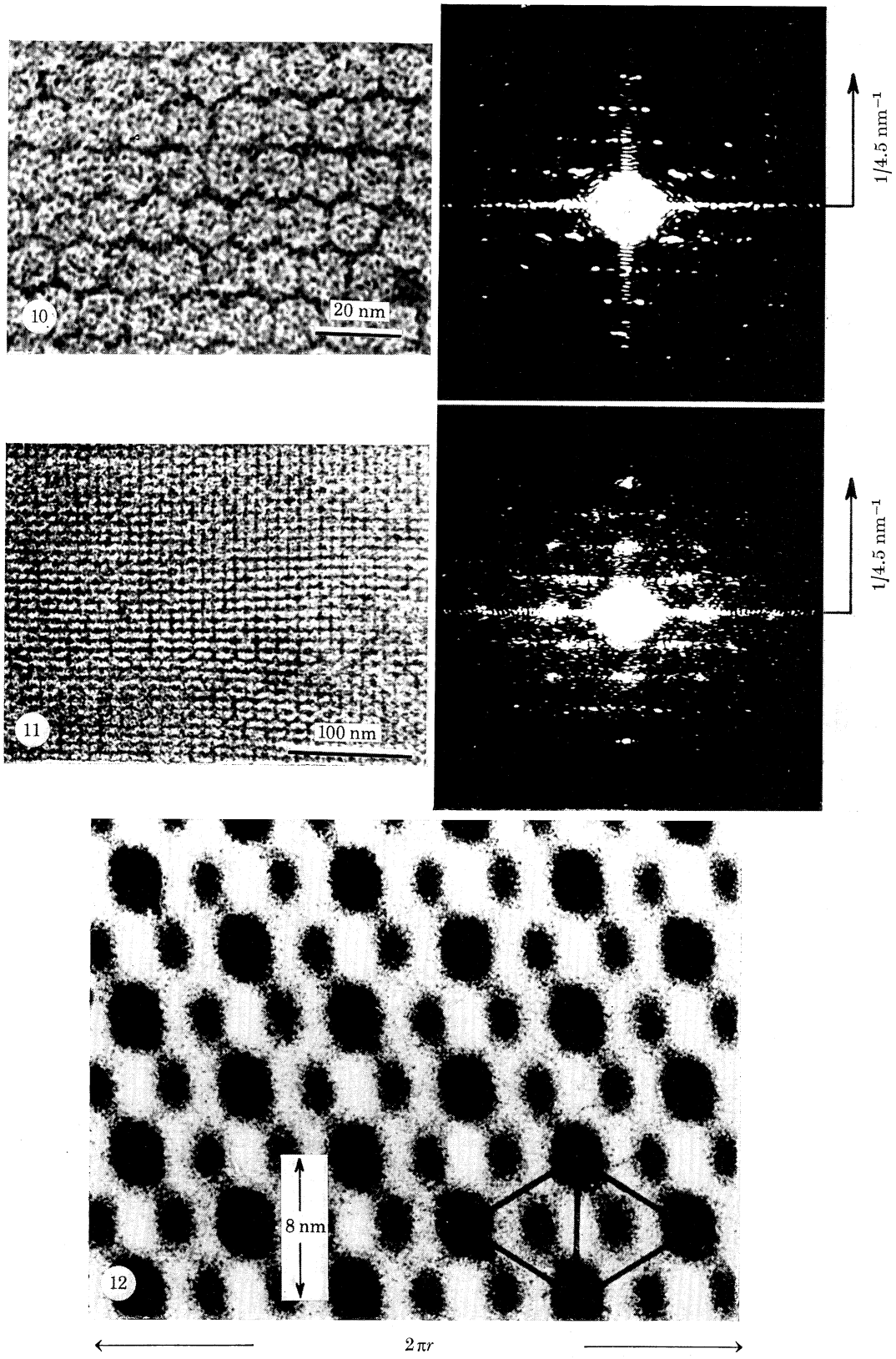

FIGURES 10–12. For description see opposite.

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(ii) 30S *aggregate*

The first observations of this product were described by Lebeurier *et al.* (1971) and Heijtink (1974). Our determination of the $s_{20,w}$ agrees with the value determined by Heijtink (1974). A sedimentation coefficient of about 30S was derived in many experiments. Heijtink (1974) also determined a molecular mass for this 30S component which was close to 1.5×10^6. Therefore, it might be concluded that the 30S component consists of 60 copies of the 25000 unit, which leaves little doubt that the particle itself has a point group symmetry of 532. The electron micrographs (figure 8, plate 22) suggest that the icosahedral particle is hollow. An average diameter of about 18 nm for particles mounted on carbon can be determined. Mounted over holes, in stain, the average particle diameter is about 30% lower.

(iii) *Aggregation states at low ionic strength*

The aggregation products in the hatched zone in the phase diagram have been identified by negative staining in the electron microscope (figures 8 and 9, plate 22). Between pH 4.0 and 5.0 'ribbon'-like components exist, which can be related to the 12S component, detected by ultracentrifugation. Between pH 5.0 and 5.5 well-defined association products are found. The aggregates are one- or two-dimensional arrays of 30S spherical particles. From optical diffraction patterns of 2-dimensional arrays of this kind an average diameter of about 18 nm for the particles has been derived. An example of such a 2-dimensional array and its optical diffraction pattern is given in figure 10, plate 23. At higher pH (between pH 6.0 and 7.0) small crystals have been found. An example is presented in figure 9. Optical transforms of this kind of images show basically the same periodicities as in the arrays of spherical particles (figure 11, plate 23). At the edges of the crystals sometimes arrays are visible, which leaves little doubt, that the crystals are made of the same spherical particles. The projection of the crystals visible in the micrograph therefore show superposition patterns of 2 or more layers of 30S components.

Other types of aggregation products have occasionally been found, for example sheet-like structures of which an example is presented in figure 9. Contrary to the arrays and crystals, it is not clear yet how these products have been formed and what they represent.

Another product which is also spherical can be detected sometimes. Its appearance resembles the multi-shell particles found by Lebeurier *et al.* (1971) and Adolph & Butler (1974). It is likely that the particles are constructed from two or more protein shells. An example of a field of particles in which this kind of aggregate is present, is given in figure 8.

Description of plate 23

Figure 10. A micrograph of a two-dimensional array of 30S particles (left) and an optical transform of this image (right). Negative staining with UO_2 oxalate. Magnification of the micrograph is indicated by a bar which represents 20 nm. The average diameter of the 30S particles in the lattice, derived from the transform, is about 18 nm.

Figure 11. A micrograph of a negatively stained (UO_2 oxalate) crystal. Magnification as indicated at the bottom of the plate. At the right side an optical transform of this image. The intensity maxima are present at the same positions as in the transform shown in figure 10. Apart from these intensities other maxima, which can be indexed on a square lattice, are present as well. This lattice measures about 8.7 nm.

Figure 12. A cylindrical section of the capsid structure of alfalfa mosaic virus as determined by the three-dimensional reconstruction method. The radius of the section is 6 nm. Areas of low density, presumably occupied by negative stain are black. In the lower right hand corner the unit cell is indicated.

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(*c*) *Clustering pattern of the protein coat*

Chemical studies have shown that there is only one kind of polypeptide chain engaged in the architecture of the virus (Kruseman *et al.* 1971). Therefore the coat must be built in a regular way (Crick & Watson 1956).

Modern image analysis methods are quite suitable to extract the periodic information present in the electron images (see, for example, Klug 1971). These methods are based on the decomposition of the electron image into harmonic components. Depending upon the partis cular problem studied, these Fourier components can be combined to yield two- or three-dimensional images of the original object. In this way only the periodic information is used to deduce the image characteristics. Hull, Hills & Markham used optical diffraction to analyse their micrographs of negatively stained AMV. A hexagonal surface lattice was postulated in which the protein was clustered as hexamers. To use these image analysis methods with full advantage, negatively stained images of very long AMV particles were investigated by coherent light and by digital methods (Mellema & van den Berg 1974; Mellema 1975). Also, AMV particles of shorter length and from other strains were analysed in this way.

It was found that a common type of lattice in the AMV images existed, which was approximately hexagonal. The cylindrical symmetry of the particles which possessed this type of lattice was shown to be threefold. The unit cells are arranged in staggered rings, so that a repeat distance of 8 nm along the length of the particle comprises 2 rings of unit cells (stacked type of lattice).

In some images of the longer particles another type of lattice was detected. To a first approximation the unit cell of this type of lattice is isomorphous with the stacked type. Its possible significance remains to be solved.

In order to derive a low resolution map of the density distribution within the cell of the common type of AMV lattice, use was made of the three-dimensional reconstruction technique (De Rosier & Klug 1968).

An average map calculated from 3 different AMV particles is presented in figure 12, plate 23. It clearly shows the clustering of the protein within the lattice, so that large holes are created at the three- and sixfold lattice positions. Protein is present at and close to the twofold lattice positions. Due to the approximation used in the three-dimensional reconstruction method, no reliable information can be derived about the density distribution at different radii.

Discussion

From the previous results the tentative conclusion can be drawn that the assembly of AMV protein into regular aggregates starts from dimers of the 25000 molecular mass unit. This process probably takes place without the existence of well defined intermediate products, as no such aggregates have been detected. A similar behaviour of BMV protein has been reported by Pfeiffer & Hirth (1974).

In some way the RNA plays a role in determining the morphology of the virus as nucleoprotein particles of cylindrical shape are assembled (Lebeurier *et al.* 1971). A low resolution map calculated from the cylindrical part of the virus indicates that the protein is clustered in such a way as to avoid the three- and sixfold lattice positions. This structural result agrees with the assembly process, because the active units are dimers. The dimers are positioned

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within the lattice so that the lattice diads coincide with the dimeric axes. This results in a protein content of 150000 per hexagonal cell, a number which is in good agreement with the experimental molecular mass of the cylindrical lattice (Heijtink & Jaspars, to be published).

It will be interesting to compare the clustering pattern in the icosahedral particle with the cylindrical structure, because this may yield structural information about the role of the RNA in the virus particle. A slight difference in the bonding arrangements of the protein in these two structures seems plausible on the basis of the theory put forward by Caspar & Klug (1962). This theory allows a small deformation of the packing of protein units as a spherical net is formed from a planar or cylindrical one.

The role of the so-called ribbons (12S) remains to be solved. It could be that this product is formed by a process in which the dimers participate after having undergone structural changes. There are no experimental data to assume that the ribbons are precursors of the 30S particles.

Higher values of ionic strength slightly decrease the stability of the 30S particle towards dissociating pH conditions. It is not known at this stage whether the assembly of the AMV protein goes together with a structural change in the reacting species. Therefore, any assignment of the main type of bonding force, out of the delicate balance of physical interactions between the subunits in the 30S particle, which is based upon the form of its stability region in the state diagram must be considered as highly speculative.

Finally we wish to remark that the AMV system offers the unique possibility to study the assembly of a biological system with one of its constituents as a form determining variable. Future research will be directed towards this aspect of molecular morphogenesis.

We gratefully acknowledge the gifts of AMV protein by Dr E. M. J. Jaspars.

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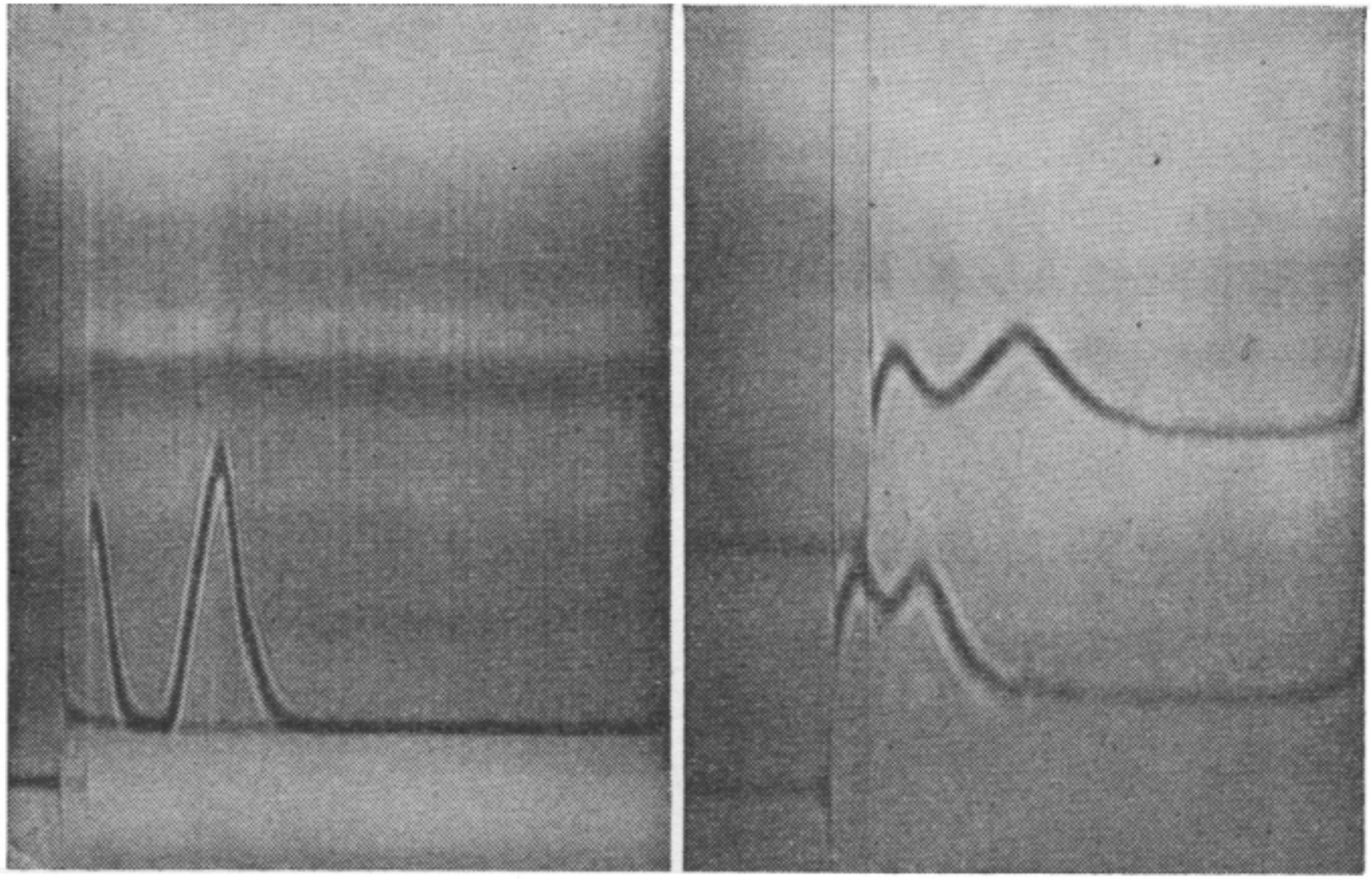
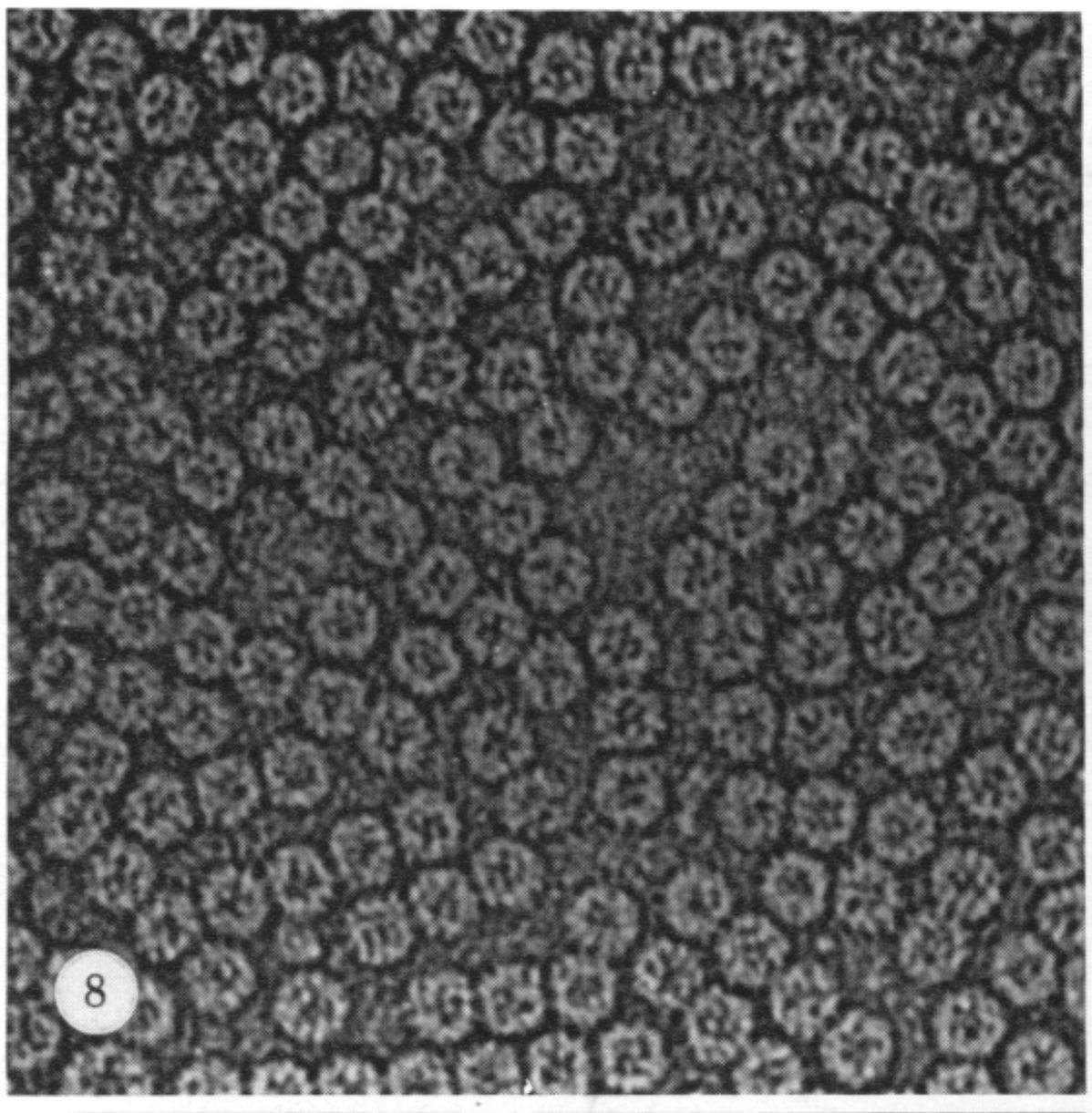
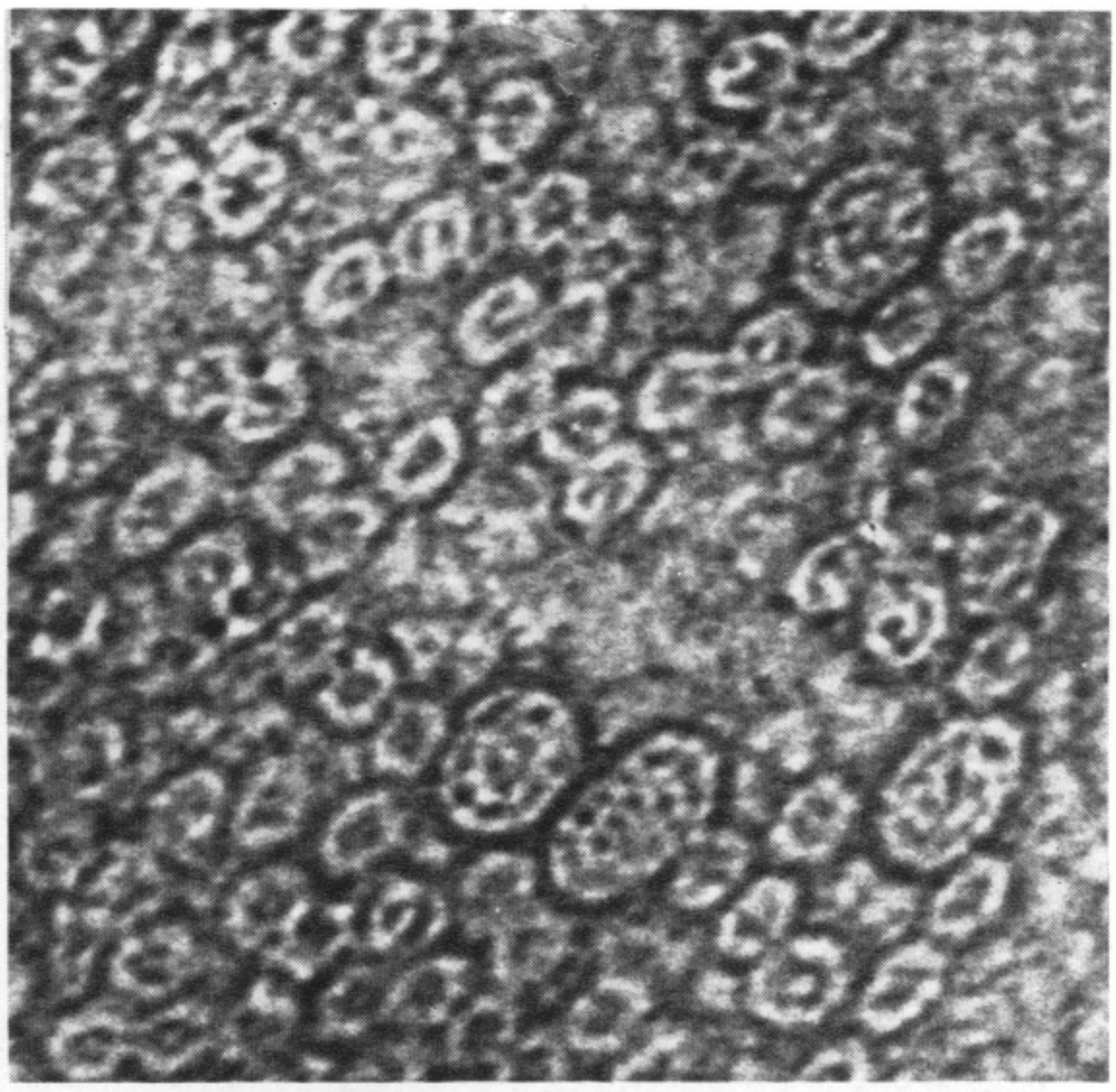
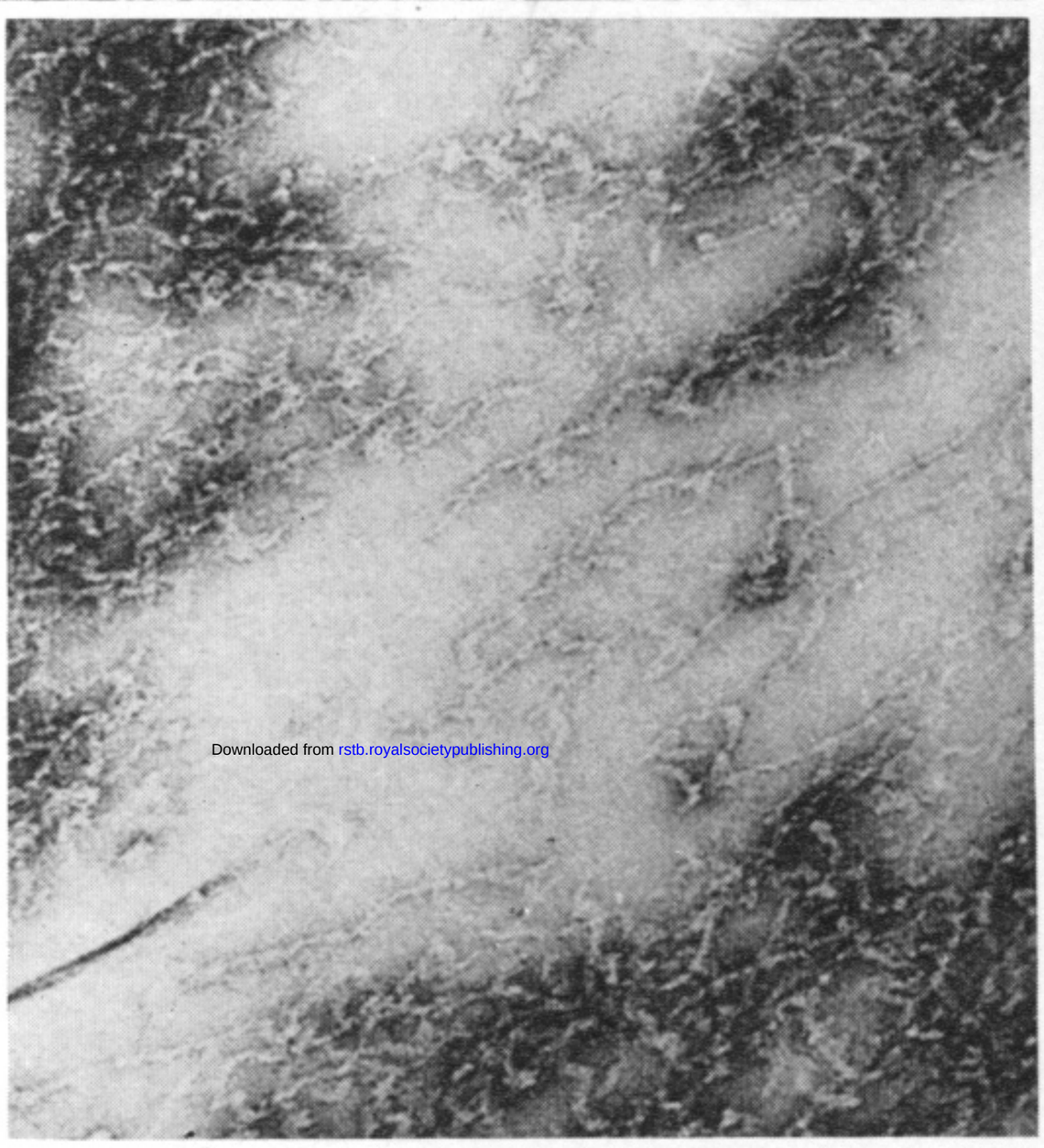
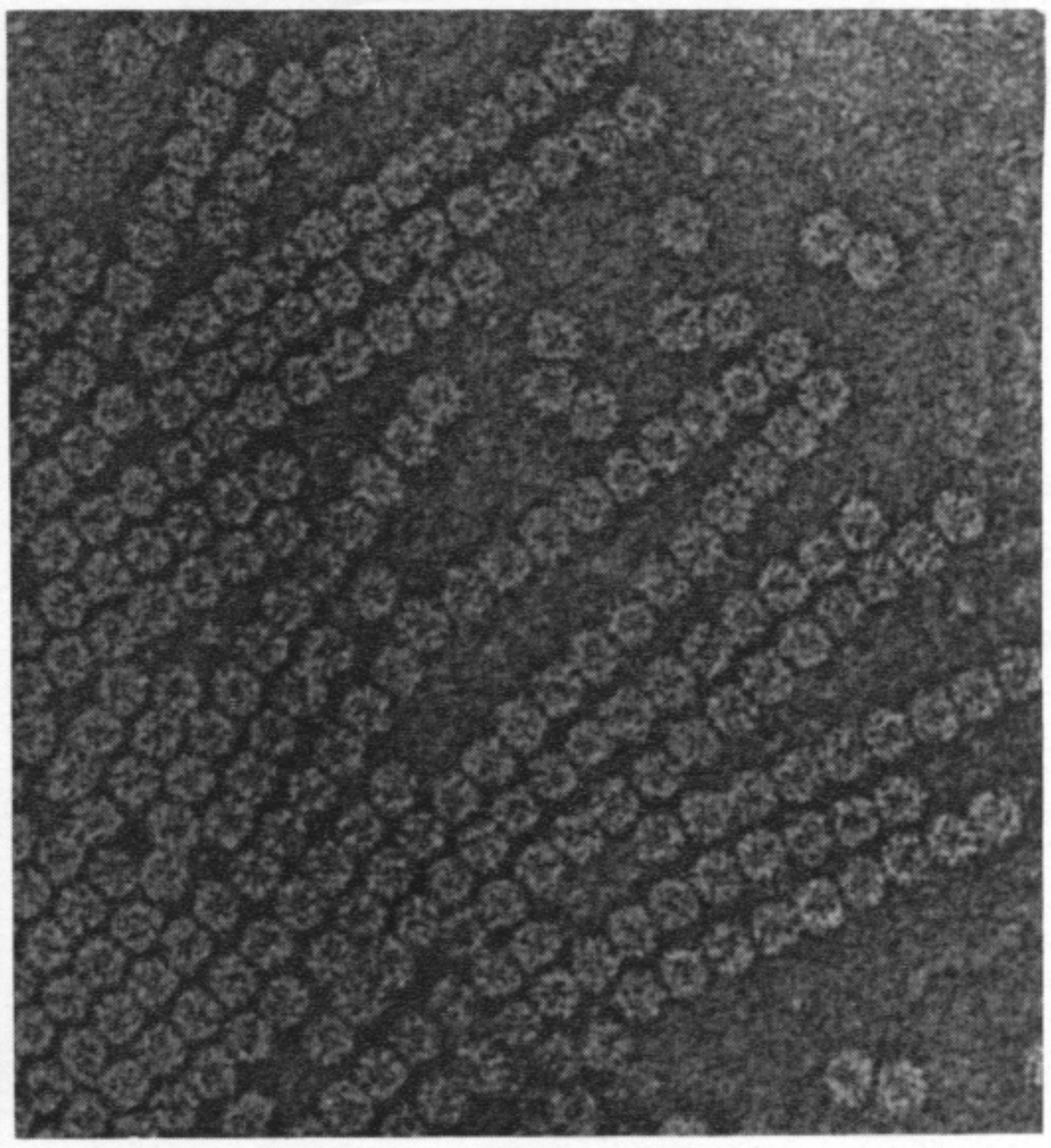


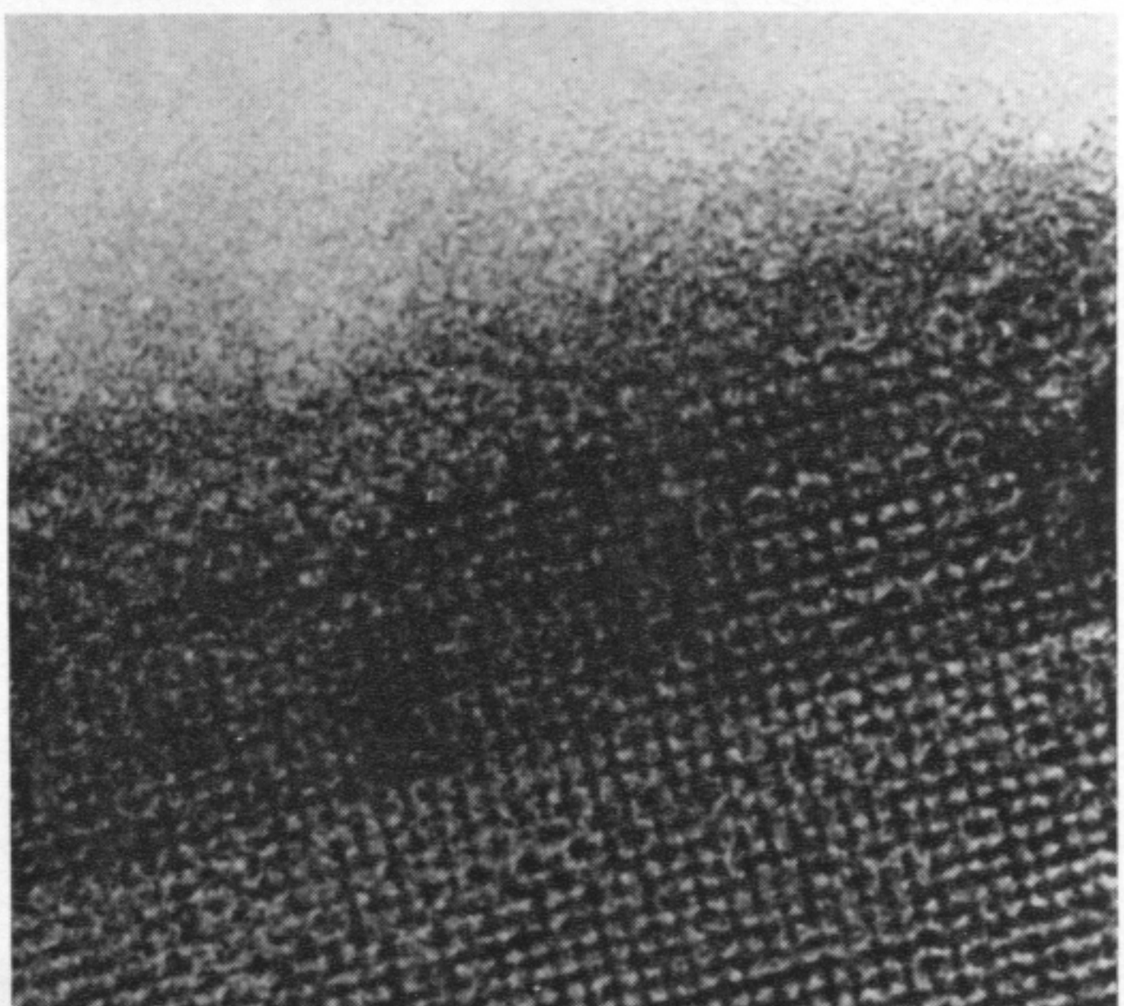
FIGURE 3. Schlieren patterns of AMV protein. The photographs are taken about 20 min after the rotor reached a speed of 48 000 rev/min. Left: AMV protein in pyrophosphate buffer, pH 6.8, $I = 0.25$. The peak has a $s_{20,w}$ value of 30S. Protein concentration about 6 mg/ml. Right: AMV protein in acetate buffer, pH 5.5 (upper pattern) and pH 5.0 (lower pattern), $I = 0.1$. The $s_{20,w}$ values in the upper pattern are 3S and 30S (respectively left and right) and in the lower pattern 3S and 12S. Protein concentration about 5 mg/ml. Sedimentation from left to right. Schlieren angle 60°.











Figures 8 and 9. For description see opposite.

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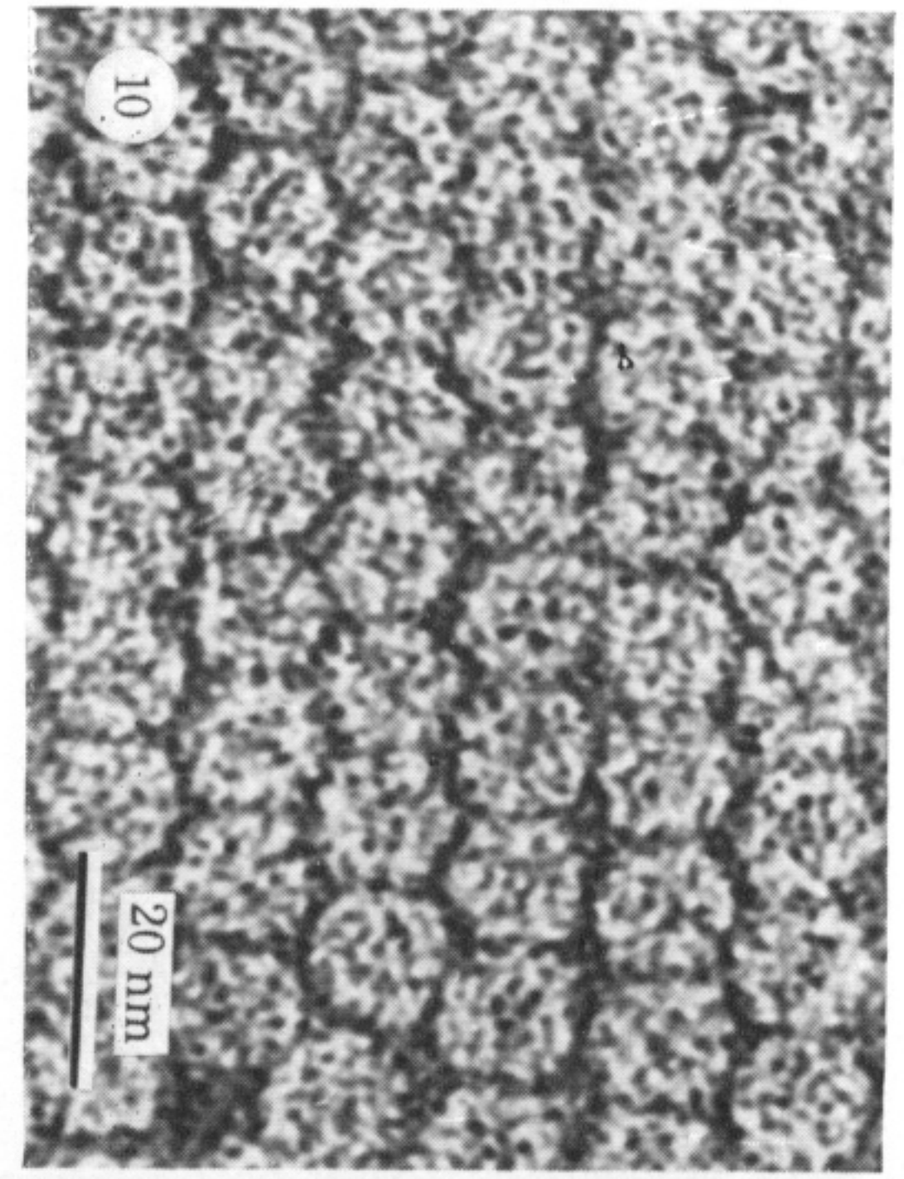

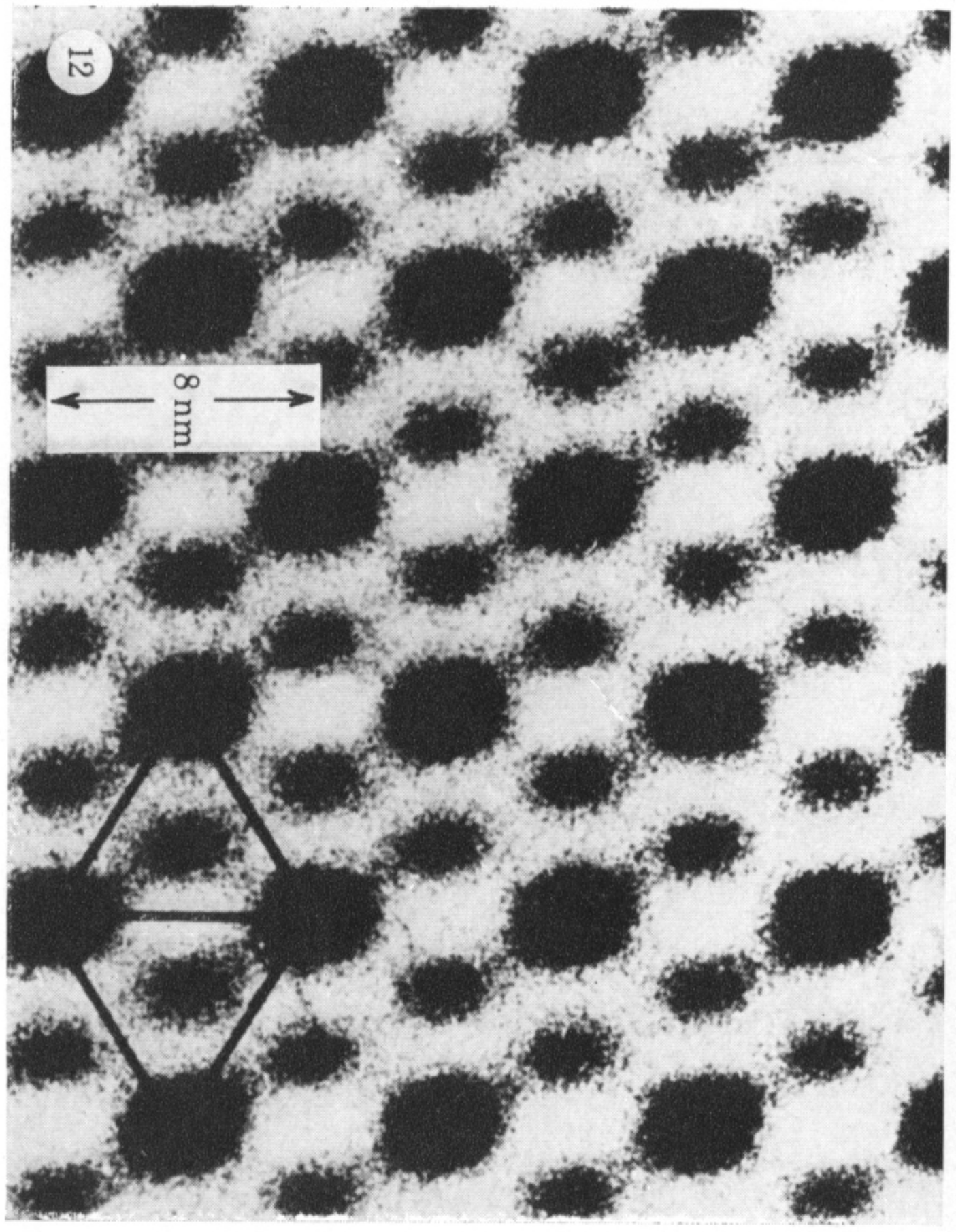

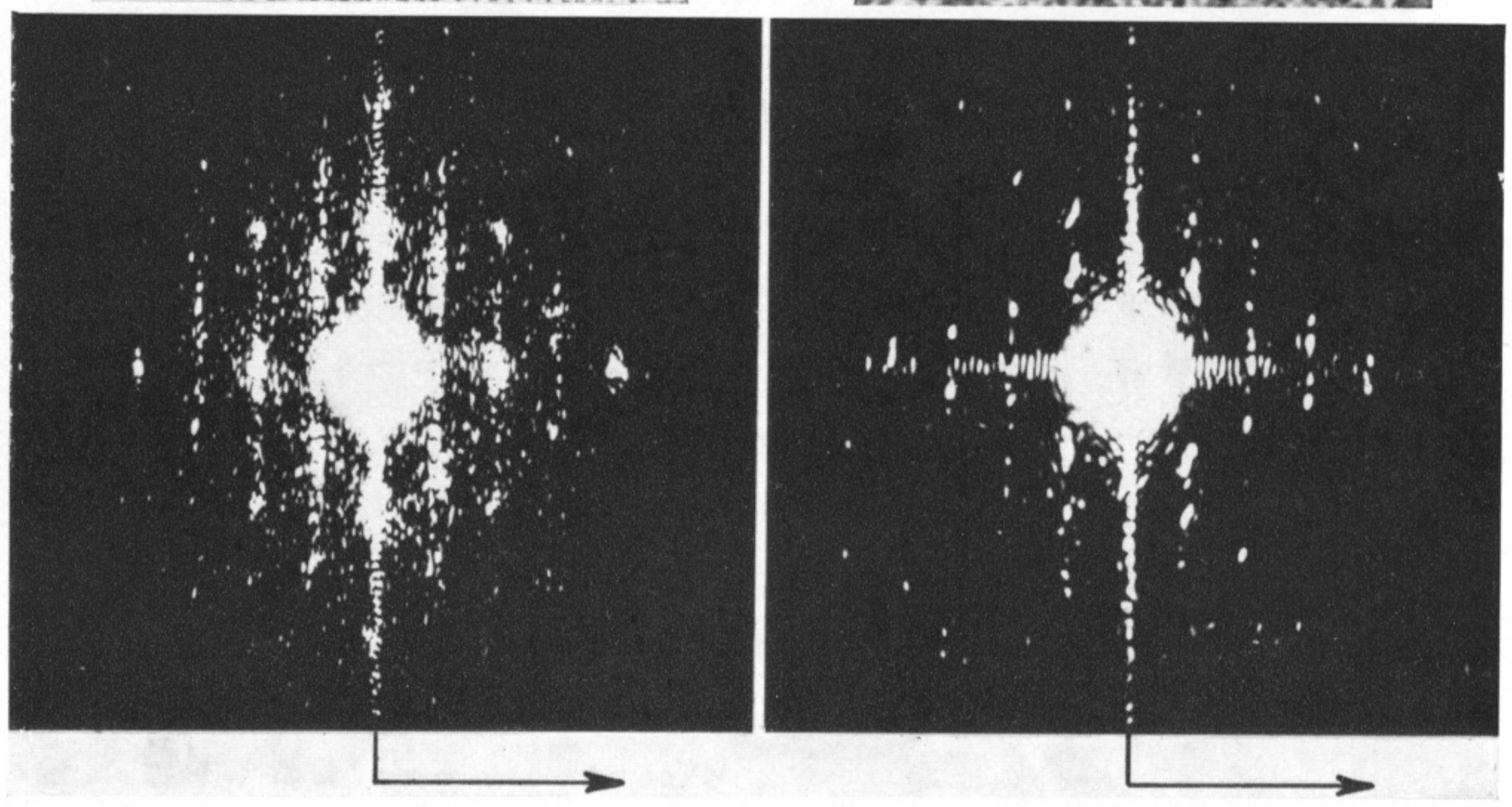

FIGURES 10–12. For description see opposite.