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Assembly of tobacco mosaic virus

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The assembly of tobacco mosaic virus requires the presence of a particular protein aggregate, the disk. During the nucleation, a specific region of the RNA interacts with a single disk, to bring about a necessarily cooperative transition from the paired two-layer structure to a short segment of nucleo-protein helix. There is a high selectivity for this region of the TMV RNA, because of the many nucleotides bound at once, and other nucleotide sequences appear only to bind by a different mechanism.

Elongation of the nucleated rods can continue with either further disks or the less aggregated 'A-protein' as the protein source, but the continued cooperativity inherent with disks would have some advantages. The rates of the two processes have been separately determined and growth is faster when disks are still present. New experiments show that the breakdown of disks to yield A-protein is relatively slow and it is concluded that virus growth from disks could not proceed through a prior breakdown in solution, but must involve the direct interaction of the disk with the growing nucleoprotein rod. The detailed mechanism of disk addition is not understood but it may involve a directed breakdown, since there is also evidence for the existence of a non-equilibrium form of A-protein which has aggregation kinetics distinct from those of equilibrium A-protein.

Some implications for the general assembly pathways of viruses both of the specificity and of the assembly/disassembly cycle during the viral infection are considered.

Introduction

Tobacco mosaic virus (TMV) has long been a standard material for the biochemical and, more recently, molecular biological study of viruses, because of both its ready availability and the relative ease with which it can be manipulated. It seems particularly appropriate to discuss it in the context of this meeting, as the whole field of the study of the *in vitro* reassembly of viruses started with the demonstration of the reassembly of TMV by Fraenkel-Conrat & Williams (1955). This established that a virus could self-assemble, without requiring any essential catalytic or morphopoetic function of a cellular component, and with high efficiency – leading to the recovery of more than 50 % of the original infectivity under optimum conditions (Fraenkel-Conrat & Singer 1959). The assembly reaction also showed fair specificity for the RNA, proceeding most readily with the homologous TMV RNA, or with a closely related virus strain, and somewhat less efficiently with certain of the synthetic homopolymers (Fraenkel-Conrat & Singer 1964).

The main objection to extrapolation from this reassembly to the *in vivo* assembly lay in the time required for complete coating and protection of the viral RNA. Even under the optimum conditions the complete reaction took about 6 h. One possible explanation for this slow rate is the difficulty in nucleating the assembly of a helical structure. The TMV particle consists of a single start helix of protein, having $16\frac{1}{3}$ subunits per turn, and with the single stranded viral RNA intercalated between successive turns of the protein. While it is relatively simple to picture how such a structure could elongate, by the addition of subunits either singly or a few at a time onto the 'step' at the growing end of the helix, the formation of a proto-helix

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of two or more turns presents greater problems because of the probable instability of a structure consisting of a large ring of subunits each bonding only with a single subunit on either side. If the formation of such proto-helices were sufficiently slow, this could explain the time course for the formation of infective virus during the reassembly experiments. We therefore set out to examine the aggregation of both the protein and the nucleoprotein in order to try to understand the virus assembly.

Observations on the disk aggregate and its involvement in assembly

Protein aggregation

The isolated protein from TMV shows a considerable polymorphism, with the aggregates varying in size from about trimer up to a protein helix structurally very similar to the virus and of even greater length (Caspar 1963). While the aggregation is largely driven by a strong hydrophobic interaction (see Lauffer & Stevens 1968, for a review), the dominant variable controlling the state and nature of the aggregation at any given temperature is the pH (Durham, Finch & Klug 1971; Durham 1972), with ionic strength only producing a slight modulation of the overall pattern. Thus at low pH the protein helix is always found, while this always dissociates into a mixture of small aggregates ('A-protein') by pH 7.5 (at ionic strengths around 0.1 or 0.2). However, at temperatures above about 15 °C a very interesting protein aggregate is found in the boundary region between the areas where these other aggregates dominate the pattern. This aggregate is known as the 'disk' and up to 80 % of the protein will be in the form of disks at pH 7.0, ionic strength 0.1 and 20 °C i.e. physiologically plausible conditions for the assembly reaction, with the remaining 20 % of the protein being A-protein.

These disks consist of two rings of protein subunits, each of 17 subunits, which form a polar structure with a bonding morphology similar to that found in the virus helix, but with a distortion in the axial direction. In this direction the subunits in the two rings interact more tightly with each other than they do in the helix, giving rise to a 'cross-strutting' between the two layers of the disk. This additional interaction results in the stabilization of the larger two-layer aggregates in the A-protein and allows the formation of disks by a simple linear condensation-polymerization, avoiding the difficulty discussed earlier for the formation of a proto-helix.

The initial stages of the conversion of disks into other protein aggregates, whether smaller or larger, are rapid but the attainment of the final equilibrium aggregate distribution is slower in some cases. Thus the breakdown of disks into material with a sedimentation coefficient of 4S is very rapid when the conditions are changed to ones which disfavour the disk state, either by raising the pH (Durham 1970) or by cooling at pH 7.0 (Durham & Klug 1971; Durham 1972). However, at least under the latter conditions, the freshly produced 4S protein does not behave like the normal equilibrium A-protein but appears to display some 'memory' for its previous state and will reform disks more rapidly than A-protein, if the conditions are changed back. For this reason Butler & Klug (1972) have described this freshly generated mixture of small aggregates as A*-protein. This process of disk breakdown may be relevant to the detailed mechanism of growth of the nucleoprotein rods when a disk preparation is supplied as the protein source and more recent experiments are discussed below.

The other obvious conversion is into the protein helix and this can be brought about by lowering the pH. If this is done slowly, with the pH being lowered from 7.0 to about 5.0 over

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2 min, then normal helix is found, however if the pH is dropped abruptly the aggregates formed are 'nicked helices' consisting of rods made up predominantly of two-turn structures separated by regions of misfit which anneal out over a period of hours (Klug & Durham 1971; Durham & Finch 1972). This observation was interpreted as showing that the disks retained their integrity during the pH drop, but underwent a dislocation into two turns of helix. These 'lockwasher'-like structures could then stack in random azimuth to give the nicked helices which were observed. This sequence is illustrated diagrammatically in figure 1. It was not possible to decide whether or not the dislocation occurred before or upon interaction of the disks with the growing rods, and therefore whether the lockwashers existed free in solution.

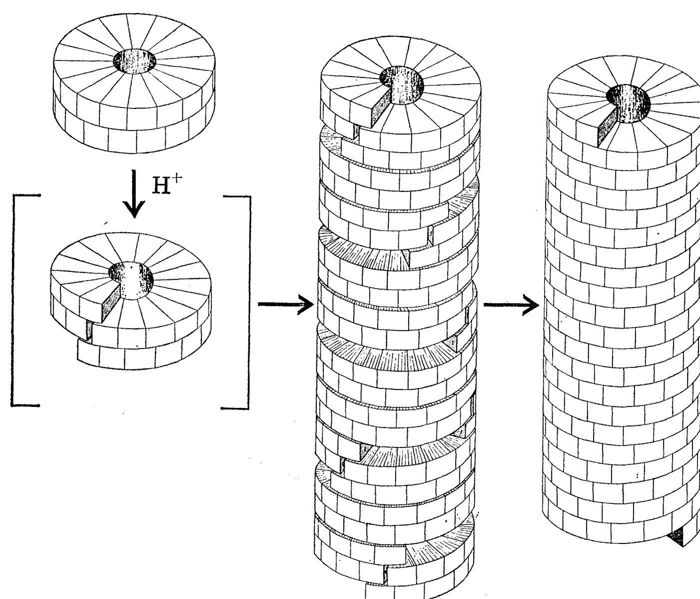

FIGURE 1. A schematic representation of the process in which disks, upon rapid acidification, are converted into single helix (Klug & Durham 1971; Durham & Finch 1972). There is no evidence for the existence of the 'lockwasher' (shown in brackets) free in solution.

Rapid reassembly

The occurrence of disks with a surface morphology similar to that of a turn of the virus helix, in the range of conditions where reassembly had been found to occur, suggested that they might play some role in the nucleation of the nucleoprotein helix, perhaps by acting as a 'jig', and providing a surface upon which the first turns of the nucleoprotein helix could be assembled. This expectation was reinforced and extended by the observation of the formation of lockwashers; if such a reaction occurred during the nucleation of assembly the disk could act as a sub-assembly which was transformed and incorporated into the growing virus structure.

This hypothesis was tested by adding a disk preparation to a mixture of TMV RNA and A-protein under the reassembly conditions described by Fraenkel-Conrat & Singer (1964). The addition of the disks produced a marked increase in the rate, but only when they were added at a concentration similar to that of the A-protein. From this Butler & Klug (1971) concluded that disks were necessary for nucleoprotein helix nucleation. More surprisingly, the omission of the A-protein did not produce any effect upon the rate. The necessity for a significant concentration of disks suggested that their effect was not simply catalytic upon the

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nucleation, while the continued growth in the absence of additional A-protein showed that they could act as a protein source for the elongation, whether directly or indirectly.

The rate of the overall reaction is linearly dependent upon the RNA concentration, as would be expected from the single RNA molecule contained within each nucleoprotein rod. The dependence upon the protein concentration is more complex (figure 2), showing a saturation with increasing protein concentrations. This must mean that the rate limiting step is not controlled solely by the collision frequency, but that some other process is relatively slow. The apparent K_m for the protein was 1.04 mg/ml. From the observation, with the electron microscope, of many incomplete rods even a short time after the mixing of TMV RNA with a disk preparation, Butler & Klug (1971) suggested that the rate limiting step appeared to be the rod elongation and that the slowest step might be the rearrangement of the protein subunits from the disk into the growing nucleoprotein helix. On the basis of the maximum rate of turbidity change, Butler (1972) estimated a time of about 185 ms for the addition of each subunit, corresponding to about $6\frac{1}{2}$ min for the formation of a full length TMV particle at saturating protein concentrations. This is compatible with the observations of full length TMV particles after about 5 min growth at high protein concentrations and of no further elongation continuing beyond 8 min (Butler & Finch 1973), supporting the hypothesis used by Butler to interpret the turbidity data, that elongation is the slower step with nucleation being relatively rapid.

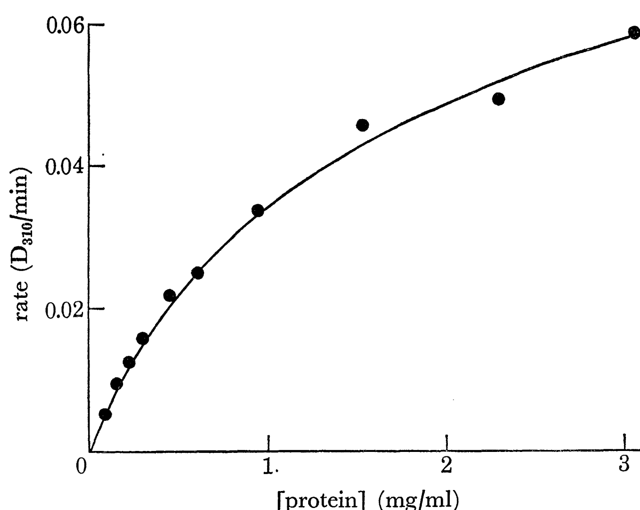

FIGURE 2. Rate of reassembly against concentration of disk preparation at constant TMV RNA concentration (205 μg/ml) (Butler 1972).

The selectivity for its homologous RNA reported by Fraenkel-Conrat & Singer (1964) was also reinvestigated using a disk preparation as the protein source. This gave qualitatively the same results, but a quantitative difference was now found, with the most favourable homopolymers (poly-A and poly-I) only being encapsidated about two orders of magnitude more slowly, on a molar basis, than TMV RNA (Butler & Klug 1971). Since it is improbable that the nucleotide sequence along the whole TMV RNA molecule will be constrained to be the optimum for the reassembly, the hypothesis was proposed that this marked sequence specificity might well lie in the rate of the nucleation reaction and not of the elongation. This hypothesis

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leads to the expectation of a special region of the TMV RNA which will have been evolved to react particularly favourably with the protein disks, to nucleate the whole assembly process. This will be discussed further by Zimmern (this volume).

Kinetics of the assembly

Nucleation

The overall assembly process can be factorized into two conceptually different reactions – the nucleation during which the RNA interacts with protein to form the smallest stable nucleoprotein helix, and the subsequent elongation by the addition of further protein subunits to the nucleoprotein helix until all of the RNA has been incorporated within the helix and the particle is complete. Since a complete TMV particle contains over 2000 protein subunits, while a nucleation reaction to give even a four turn helix will only involve about 70 subunits, it might be expected that elongation will dominate the overall assembly process, in agreement with the results just discussed. However, the observations with poly-A suggested that in this case the nucleation might be the rate limiting step and that it would therefore be susceptible to observation in the overall kinetics of the assembly, using poly-A rather than TMV RNA.

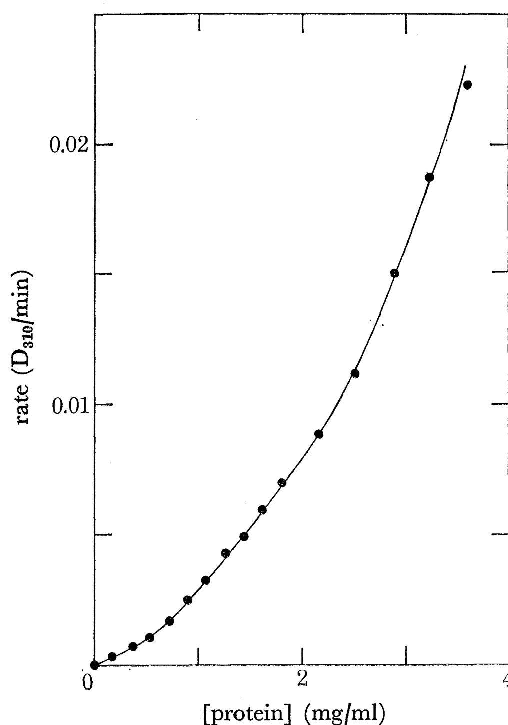

FIGURE 3. Rate of assembly against concentration of disk preparation at constant poly-A concentration (200 μg/ml) (Butler 1972).

The rate of the reaction was again found to be dependent upon the concentration of the poly-A, as with TMV RNA, but in this case the dependence upon the disk protein concentration was greater (figure 3) and approached second order (Butler 1972). This result suggested that the nucleation process involved the interaction of an RNA molecule with two protein disks, and one obvious hypothesis is the 'sandwiching' of the RNA between the disks which

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could then lockwasher to entrap the RNA and start the formation of the nucleoprotein helix. This mechanism for nucleation with an 'artificial substrate', involving two disks, may well occur even with regions of the TMV RNA other than the main nucleation sequence (Richards, Zimmern, this volume). This is suggested by the observations of the Moscow and Strasbourg groups on the encapsidation of fragments of TMV RNA prepared by partial nuclease digestion. These digestions were performed either with pancreatic ribonuclease A to give 'RIS' (Tyulkina *et al.* 1975) or with ribonuclease T1 to give 'SERF' (Guilley, Jonard & Hirth 1974) and the reassembly carried out for some hours at pH 7.25, ionic strength 0.25 and 28 °C or ionic strength 0.5 and 24 °C. Under these relatively drastic conditions, where the disks alone would be tending to stack reversibly to form short stacks of disks (Durham 1972), some of the polynucleotides in the partial digests are encapsidated. However, the resulting nucleoprotein complexes have RNA/protein ratios of half that found in the normal helix (Tyulkina *et al.* 1975; Jonard, Guilley & Hirth 1975), suggesting that their sequences are less favourable than that of the normal nucleation region and that their encapsidation mechanism may be like that found for poly-A.

A rather different picture is found, however, with intact TMV RNA. A count of the number of rods nucleated after short times showed that the rate of nucleation was linearly proportional to the protein concentration (K. E. Richards & R. C. Williams, personal communication) and, in agreement with the conclusion of Butler & Klug, disks were again the necessary protein aggregate for the nucleation reaction (Richards & Williams 1972). This absolute requirement for the disk aggregate for nucleation has been confirmed yet again using both TMV (Rodionova *et al.* 1973) and also a related strain of virus, cucumber green mottle mosaic virus (Ohno, Inoue & Okada 1972*a*).

Another approach to investigating the nucleation reaction with TMV RNA is to study the particles produced after very short times of reassembly, by use of a pulse-chase technique. TMV RNA and radioactively labelled protein, as a disk preparation, were mixed together and after various times the partially assembled rods were 'chased' into complete rods by the addition of a substantial excess of unlabelled disk preparation (Butler 1974*a*). Analysis of the assembly curves showed that the nucleation is a bimolecular reaction, which is first order in disk concentration, with a rate constant of $1.3 \times 10^3\ \mathrm{mol^{-1}\ s^{-1}}$. The significance of this rate constant can be illustrated by the calculation that, at a *constant* protein concentration of 1 mg/ml, the nucleation reaction would be *pseudo*-first order with a half-time of 9.3 s for the RNA, compared to a time of over 10 min for the total elongation.

Elongation

Because of the relatively high rate of the nucleation reaction, the overall assembly process will be kinetically dominated by the repetitive elongation reaction and this can therefore be crudely observed from the overall kinetics, as we had assumed during the initial investigations. More refined and specific investigations of the elongation reaction have been carried out since, using either the pulse-chase technique already described or else pre-nucleated rodlets, to overcome any effect of nucleation on the kinetics observed (Richards & Williams 1973).

Unlike the wide agreement among the various research groups on the necessity of disks for the nucleation reaction, there has been disagreement about which protein aggregates can contribute to the elongation. The cause of this dispute lies in the fact that a disk protein preparation always contains about 20 % of the protein as A-protein and, since there is clearly

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no absolute requirement for disks in the elongation, it is difficult to eliminate the possibility that elongation takes place from the protein in this A-protein pool in the disk preparation supplied. Butler & Klug (1971, 1972) found no increase in the overall rate of reassembly when they added A-protein to the reassembly mixture of disk protein and TMV RNA, even when it was in a 5- or 10-fold excess, and therefore concluded that disks could act directly as the protein source for elongation under these conditions. In their experiments, the rate limiting step appeared to depend only on the disk concentration, with the free A-protein concentration not affecting the overall reassembly rate. This view has, however, been questioned by other groups of workers (Richards & Williams 1972, 1973; Okada & Ohno 1972; Ohno *et al.* 1972*a*, *b*) and these criticisms are discussed later.

The rate of incorporation of protein subunits, as measured in the pulse-chase experiments, becomes dependent upon the elongation at later times if the initial protein concentration is sufficient. In the analysis already mentioned, Butler (1974*a*) determined a value of 7.6 subunits per second for the elongation, with a K_m of 0.66 mg/ml. This elongation rate would again give an overall completion time of about 5 min for a TMV particle. By using these values, together with the nucleation rate constant already described, it is possible to predict the concentration dependence of the overall assembly quite accurately, suggesting that the model of nucleation and then elongation by a saturating process is plausible.

The initial experiments using partially assembled rods were performed by Richards & Williams (1973) with both a disk preparation and A-protein as the protein source. These authors did not observe any difference in rate with these different proteins, nor did they observe any effect of protein concentration on the initial rate of turbidity change, over the range of concentrations used in their experiments (1.5–7 mg/ml with disks and 0.3–1.6 mg/ml with A-protein). One difficulty in interpreting these rates of turbidity change as absolute rates of rod elongation is the failure of the normal Rayleigh light scattering equations with large molecules, due to the molecular dissymmetry. Although an attempt was made to allow for this, the resulting figure must be regarded as only provisional and we believe it to be an overestimate of the actual rate. In order to check on this figure, Richards & Williams measured particle lengths with the electron microscope, after dilution and spray deposition onto grids but again the technique is open to question (see below). A further problem with these experiments was that the rodlet preparation was pelletted before use, in order to concentrate the rodlets. It has, however, been shown that pelletting reduces the overall rate of elongation of rodlets (Butler 1974*b*), probably by causing some step other than the protein addition to become rate limiting.

In a further series of experiments using partially assembled rodlets (Butler 1974*b*), the kinetics of turbidity change were observed with both a disk preparation and A-protein as the protein source and with unpelletted rodlets. This showed that a concentration dependence of the rate of elongation, up to a saturation level, occurred with both protein sources, with K_m values of 0.66 and 0.093 mg/ml for disks and A-protein respectively, and with the maximum rate with the disk preparation about 2.5 times that with the A-protein. This we again interpret as showing that the rate of incorporation of subunits with disks as the 'delivery package' is greater than that with A-protein.

Measurement of absolute rates of elongation

Part of the problem in determining the nature of the aggregate involved in elongation lies in the difficulty in determining the actual rate of rod elongation. Ideally this should be

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monitored continuously, in the reassembly solution. The technique which comes closest to this ideal is the use of turbidity to measure the average particle weight (Butler & Klug 1971; Butler 1972), but this is only suitable as an absolute measurement when the rod lengths are small compared to the wavelength of measurement, i.e. for rods less than about 20 nm in length. While it is possible to obtain some estimate of relative rates of elongation for longer rods (Richards & Williams 1973; Butler 1974*b*), the actual rates cannot be reliably estimated.

An alternative method of measuring the average number of subunits incorporated into rods at any time is by the pulse-chase technique (Butler 1974*a*). Since the chase is carried out with an excess of a protein preparation similar to that used for the initial reassembly, no problem of distortion of the size distribution of the population being sampled will occur and the average rod lengths can be estimated. Both this technique and the measurement of turbidity only determine average values and, while this has the obvious advantage of accuracy, the interpretation of average growth rates in terms of elongation rates depends upon the rate of nucleation. If this latter is fast, as measurements indicate (Butler 1974*a*), then the increase in size will be due solely to elongation and not to a continuing nucleation of particles which could then also participate in the elongation.

One apparently direct approach is to measure the rod lengths in the electron microscope. Unfortunately, however, the preparation of specimens presents certain problems. To measure the absolute concentration of rods, it is necessary to spray small droplets of the sample solution onto grids and then to shadow the particles after the droplets have dried. This spraying can only be performed with a suitable concentration of particles, in order to yield specimens where individual particles can be distinguished and yet sufficient can be seen to get a reasonable measurement of the average length. This concentration is substantially lower than that employed for most reassembly experiments and it has been necessary to dilute samples of the reassembly solution before specimen preparation. Initially this was done into buffer that was both cold and at a high pH (Richards & Williams 1972) and the results apparently showed a relatively slow nucleation. Butler & Klug (1972) pointed out that this could well be due to loss of short rods during the dilution and it has since been confirmed that cooling partially assembled rods, even without dilution or a pH change, will cause their partial disassembly (Butler & Finch 1973).

In a subsequent series of experiments, Richards & Williams (1973) worked with partially assembled rods and prepared their specimens by dilution into buffer at room temperature, although still at higher pH than that for the reassembly. The results that they obtained show a distinct change in rate during quite short times (e.g. their Figure 4 yields growth rates of about 50 nm/min during the first minute, but only 15 nm/min during the second minute), while the comparable plots of turbidity do not show this effect. One possible explanation of this difference would be that the rods were still being partially stripped by the dilution, since this effect would be particularly severe at the zero time point if this sample was withdrawn before the addition of the protein for the elongation.

This hypothesis was tested by observation of the effects of dilution upon a preparation of partially assembled rods, which had been pelletted and then resuspended in order to remove much of the free protein which might be present at equilibrium after the partial reassembly. Such a preparation was preferred to intact virus because of the greater ease in determining any small change in the average rod length. A histogram of the rod lengths, as observed with the electron microscope, both before and after dilution under constant conditions of pH, ionic

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strength and temperature, is shown in figure 4 and demonstrates a distinct reduction in the average rod length. This change in the distribution was found to be significant beyond the 0.1 % level, in both a χ^2 test and an analysis of variance, and corresponded to a reduction in the number average length of about 20 % upon a two-hundredfold dilution. The complementary experiment, of measuring the loss of protein from sedimentable rods into the supernatant, has proved technically difficult because of the very small amounts of protein to be determined, the difficulty in ensuring complete pelleting of all rods and the complication of the measurement of the protein released upon dilution by the protein carried over in the pellet or released upon resuspension of the rods. In consequence, the results obtained are too variable to be used quantitatively, but they do show qualitatively that the rods are always shortened by dilutions between 1:40 and 1:200, in agreement with the length measurements.

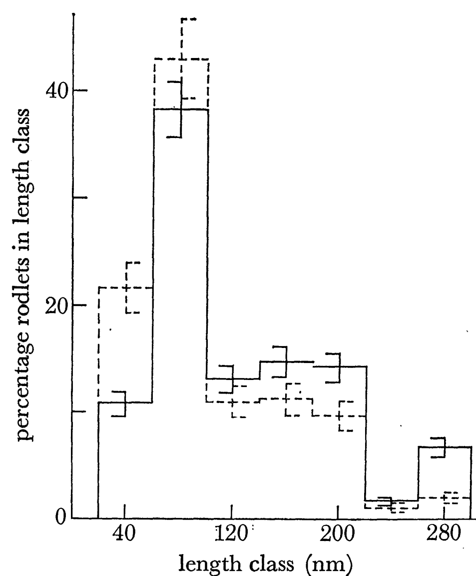

FIGURE 4. Effect of dilution on length distribution of preparation of partially assembled rods. Rods suspended in sodium phosphate buffer, pH 7.0, ionic strength 0.1 and 20 °C, were diluted 200× and specimens prepared for electron microscopy by adsorption onto carbon coated grids, rinsing with water and shadowing with Pt-carbon. Rod lengths were measured on photographs of many different fields and histograms plotted, with bars indicating the standard deviation of numbers in each length class. —┤—, undiluted (N = 1480); --┤--, diluted 200× (N = 859).

These results confirm our suspicions of rod length measurements in the electron microscope using techniques of specimen preparation which involve dilution of the reaction mixture. An alternative procedure is to allow the rods to be adsorbed directly from a drop of the reaction mixture onto the carbon substrate of the specimen grid and then to either negatively stain or shadow the particles. In this way the particles are fixed, by interaction with the carbon film, before they are removed from the reaction mixture. Using this technique, Butler & Finch (1973) measured rates of elongation with a disk preparation as the protein source which were comparable with those estimated from turbidity. They also found that the rate of elongation of rods with A-protein, even after nucleation with disks, was about one quarter of that with the disk preparation. These observations of the rates of rod elongation were further supported by the time at which full length particles first became visible, which is clearly a measure of the fastest elongation in the system.

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Yet a further technique used for studying the reassembly has been to assay the production of infective virus (Okada & Ohno 1972; Ohno *et al.* 1972*a*, *b*). These assays have, however, been carried out after periods of more than 30 min and so are not directly relevant to the rapid elongation occurring within 10 min (Butler & Klug 1973; Richards & Williams 1973), but may well be measuring the rate of some 'finishing' process which is essential for formation of intact infective particles and is not seen by studying the bulk elongation.

New observations on the disk breakdown

One difficulty in designing experiments to resolve the differing interpretations of the protein species required for elongation lies in the nature of the disk, which appears to exist in a rapid micro-equilibrium with the A-protein. Thus, experiments with mixtures of labelled and unlabelled disk preparations and A-protein (Richards & Williams 1972) or with dual-labelled proteins (Butler, unpublished results) always resulted in a random distribution of the labels into the reassembled particles and the remaining free protein. Richards & Williams also performed the control of separating disks and A-protein from a mixture of a labelled disk preparation and unlabelled A-protein on sucrose gradients, and found that the label had again been rapidly redistributed. However, the very observation that the disks and A-protein in a mixture could be separated by a rate zonal centrifugation suggests that, despite this rapid micro-equilibrium, the breakdown of disks on a macro-scale must be relatively slow.

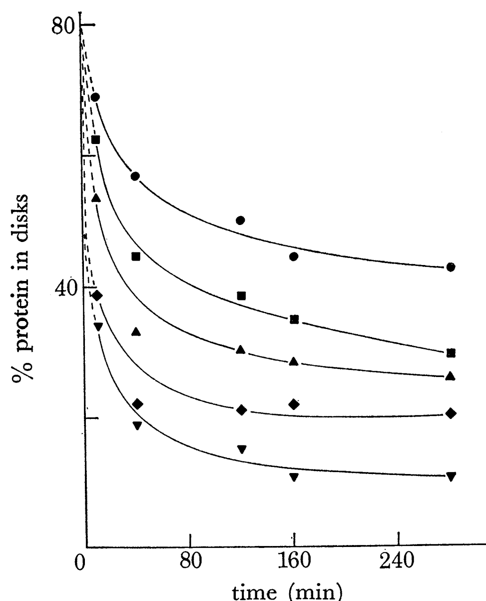

FIGURE 5. Time course for breakdown of protein disks upon dilution. Disk preparation (5 mg/ml) at pH 7.0, ionic strength 0.1 and 20 °C was diluted under same conditions and proportions of various aggregates determined in the analytical ultracentrifuge, by using the photo-electric scanner at 280 nm. Centrifugation was started at the given times after dilution. Final concentrations: ●, 0.40 mg/ml; ■, 0.30 mg/ml; ▲, 0.25 mg/ml; ◆, 0.20 mg/ml; ▼, 0.15 mg/ml.

The nature of the disk/A-protein macro-equilibrium had been studied by Durham (1972), who had found that the aggregation of protein subunits into disks was a quasi-crystallization in which a fixed critical concentration of the smaller material (the A-protein) existed in equilibrium with any concentration of the 'crystals' (the disks) under specific conditions,

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despite the relatively small number of subunits in the disk. Since the micro-equilibrium implies a ready interchange of individual subunits, while this quasi-crystallization requires a stability of the overall integrity of the disk, presumably a critical number of subunits have to be lost simultaneously before total breakdown occurs. This must lead to a high activation energy for the breakdown, which will therefore be a rare event compared to the recapture of replacements for any lost subunits fewer than this critical number. The coexistence of a fixed A-protein concentration in equilibrium with a varying disk concentration requires that the rate of disk breakdown be relatively independent of the number of disks present in the solution, at least at pH 7.0, ionic strength 0.1 and 20 °C – the conditions where this phenomenon is observed.

The breakdown of disks upon changing to conditions which disfavour this form of protein aggregation has already been discussed, together with the memory which the protein retains for its former aggregation state. However, unlike the rapid disaggregation observed on changing the conditions, the disaggregation produced by perturbing the equilibrium by dilution under constant conditions is relatively slow. The time course for disk breakdown at a number of final protein concentrations is shown in figure 5 and is compatible with the fact that, although disk formation under these conditions is slow, the equilibrium mixture contains about 80% disks (Durham *et al.* 1971; Durham 1972). This rate of disk breakdown is also slow compared to the reassembly of TMV, suggesting that any postulated mechanism for elongation must allow for the disks to act as a direct protein source and not require a compulsory breakdown to A-protein first, as this would not allow the observed rate of elongation.

Discussion

Assembly

The *in vitro* reassembly of TMV has been studied in greater detail than that of any other simple virus and therefore the mechanism of assembly can be described more closely. There is a high degree of selectivity by the viral coat protein for its homologous RNA, which can only be mediated by the nucleotide sequence of a special region on the RNA (discussed in later papers). This region interacts with a preformed sub-assembly of the protein, the disk, to nucleate the virus assembly and it may be expected that certain geometric features of the protein and the disk will be reflected in this sequence. The most obvious one is the regular repetition of identical protein subunits, each of which binds to three nucleotide residues thus leading to the expectation of some repeating pattern with a period of three bases. A second such feature is the occurrence of 17 protein subunits in each ring of the disk, and $16\frac{1}{3}$ per turn of the helix, which might lead to similarities after either 50 or 100 nucleotides, if some particular characteristic of the sequence were to be used at a given point either in each turn or in each disk. The possible occurrence of such sequence features is discussed by Zimmern (later paper).

Besides affording the possibility of a much higher specificity for the RNA than could be obtained by single protein subunits each interacting with three nucleotides, the inherent cooperativity of the addition of 34 subunits from the disk allows rapid nucleation of the helix, as discussed before. Such cooperativity could also be valuable during the subsequent elongation, in enabling the interaction energy from neighbouring regions to help to overcome any short, unfavourable regions along the RNA. For this cooperativity to be expressed, the disk would have to be the direct delivery package for the protein to the growing end of the rods and not to break down into A-protein throughout the solution. It does not, of course, require the disk to

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retain its unique structural continuity during the elongation and it might undergo some specific breakdown under the direct influence of the nucleoprotein rod, to yield smaller aggregates with some memory of the previous aggregation state similar to the A*-protein discussed earlier. A mechanism of elongation involving such a 'directed breakdown' of the disks might be very sensitive to the exact experimental conditions and this may supply part of the answer to the conflicting interpretations of the experiments upon the elongation.

Disassembly

In contrast to the situation in the bacteriophages discussed in earlier papers, where the ejection of the nucleic acid from the phage head plays a major and specific part in the normal infection process with the empty head remaining outside the infected cell, the plant viruses and nucleoprotein cores of most animal viruses enter the cell intact and then have to disassemble in order to continue the infective cycle. This will present a particular difficulty in the case of a helical virus such as TMV, because the intimate contact of the RNA with the protein will require that disassembly of the nucleoprotein helix is complete, involving removal of all of the protein subunits, rather than allowing partial disruption of the capsid and expulsion of the nucleic acid as could occur with a spherical virus.

Since this disassembly must take place under similar conditions inside the host cell to those under which assembly will subsequently occur, one obvious hypothesis for the control of this apparently reversible process is that it is a mass action effect due to the dilution of the virus protein upon entry of the virus into a previously uninfected cell. Such a hypothesis will allow for the relative stability of the virus at the high concentration in which it is carried in the natural vector or handled in experimental situations *in vitro*, for under such circumstances the partial breakdown of only a small fraction of the particles would generate sufficient free protein to stabilize the remaining intact virus. The experiments described on the stripping of partially assembled rods support this hypothesis for the virus disassembly.

General considerations

Despite the consideration by Caspar (1963) both of the 'biological misfortune' for a virus if sufficient free protein is not available to coat and protect its nucleic acid and of the requirement in the cycle of a virus infection that the nucleic acid should be both coated and uncoated at the correct times, these points have frequently been overlooked. Biological success requires a fine balance between the tendencies for the aggregation and disaggregation reactions to occur and yet, at particular times, the balance must be tipped decisively one way or the other. One mechanism for achieving such switching is to have a cyclic pathway, so that the two processes are not simple reversals of each other. This is realized in the case of TMV by the requirement for disks for the assembly nucleation coupled with disassembly into A-protein, which forms disks relatively slowly. An alternative, which has been discussed in earlier papers, is the mechanism of the assembly of bacteriophage heads and then the subsequent expulsion of the DNA from the intact structure.

Comparison of the reassembly of TMV with that of cowpea chlorotic mottle virus, also under physiologically plausible conditions (Adolph & Butler 1975, this discussion), suggests that in order to achieve a reasonable rate of assembly, it may well be necessary to supply the protein in the correct aggregation state. This is not, however, necessarily a highly aggregated sub-assembly; while it is the disk for TMV protein, it is the low molecular mass 'hysteresis

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protein' for cowpea chlorotic mottle virus. Only under such relatively mild conditions, with the protein supplied in its optimum form, may the full selectivity of the viral protein for its homologous RNA be observed; under any other conditions the reaction may have to be forced to such an extent that this selectivity is masked. The assembly processes of a number of other viruses, which currently do not appear to show any specificity, might therefore repay a closer investigation. This could establish whether specificity is indeed a general feature of virus assembly.

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