*IN VITRO MORPHOGENESIS OF PHAGE P22 FROM HEADS AND BASE-PLATE PARTS**

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The *in vitro* reconstitution of tobacco mosaic virus from its RNA and protein subunits[1, 2] has presaged the *in vitro* assembly of structurally more complex viruses like T4,[3] P22,[4] and λ[5] bacteriophages from various component parts. Here we give a detailed report of experiments[4] demonstrating the efficient *in vitro* assembly of P22 bacteriophage whose DNA is normally contained in a polyhedral head to which a small base plate is attached (Fig. 1*A*). Viewed end on, the base plate appears to consist of a central core around which are arranged six spikes.[6] We have purified both the tail molecules of which these base plates are composed and noninfectious DNA-containing P22 heads that have short necks but no base plates. When the two preparations are mixed, a number of tail molecules combine at the neck to make the base plate required for infectivity. P22 particles thus made *in vitro* are fully active and are morphologically indistinguishable from particles that have been assembled *in vivo*.

Materials and Methods.—Bacterial and phage strains: Salmonella typhimurium strain LT2 was used for all infections and as background for counting plaque-forming units (PFU). As a source of phage heads, a lysogenic bacterium carrying a temperature-sensitive c_2 mutant prophage[7] was prepared. This lysogen is stable at 25°C, but is thermally induced at 40°C. As a source of tail parts, use was made of a newly isolated temperature-sensitive mutant, *G5*.[8] Phage carrying this mutation are defective at 37°C, but are able to produce normal yields of phage at 25°C. The double mutant $G5c_1$ was employed to prevent lysogenization[9] and thus to ensure high yields of material. In addition, wild-type phage was used as a standard in the determinations of serum-blocking power described in a later section.

Media: Buffered saline, nutrient agar, soft agar for top layers, L broth,[9] and supplemented M9[10] have all been previously described.

Preparation of heads: Bacteria lysogenic for the temperature-sensitive c_2 prophage were grown in L broth at 25°C with aeration to a titer of 4×10^8/ml. The cells were sedimented and resuspended at 10^8/ml in L broth prewarmed to 40°C. Aeration was resumed until lysis was complete. Such lysates contained approximately 3–4 $\times$ 10^{10} heads and 10^{10} active phage per ml. Active phage were removed by repeated adsorptions with cells at a final concentration of 10^9/ml for 1 hr at 37°C in 0.01 *M* NaCN to prevent phage multiplication. After the background titer had been lowered by several decades, the heads were sedimented for 90 min at 19,000 rpm, and the pellets were resuspended in buffered saline. Further purification was achieved by banding in CsCl.

Preparation of tail parts: Cells were grown at 37°C in supplemented M9 with aeration to a concentration of 10^8 cells/ml and were infected with a multiplicity of 10 $G5c_1$ phage. After 35 min the infected cells were rapidly chilled by pouring over ice and quickly concentrated by centrifugation at 17,000 rpm for 5 min in the Servall RC-2 centrifuge. The viscous pellet was resuspended in $^1/_{100}$ the original volume of buffered saline and frozen twice in a bath of acetone and dry ice. The lysed cells were then treated for 10 min with DNase (10 γ/ml) at room temperature. Debris and active phage particles were removed by centrifugation for 1 hr at 17,000 rpm.

Crude preparations could be purified further by zone sedimentation of a 1-ml sample placed on a 5–20% sucrose gradient in a SW25 rotor at 25,000 rpm for 25 hr at a rotor temperature of 25°C. The peak fraction (see below) was purified approximately 200-fold relative to the original frozen and thawed pellet.

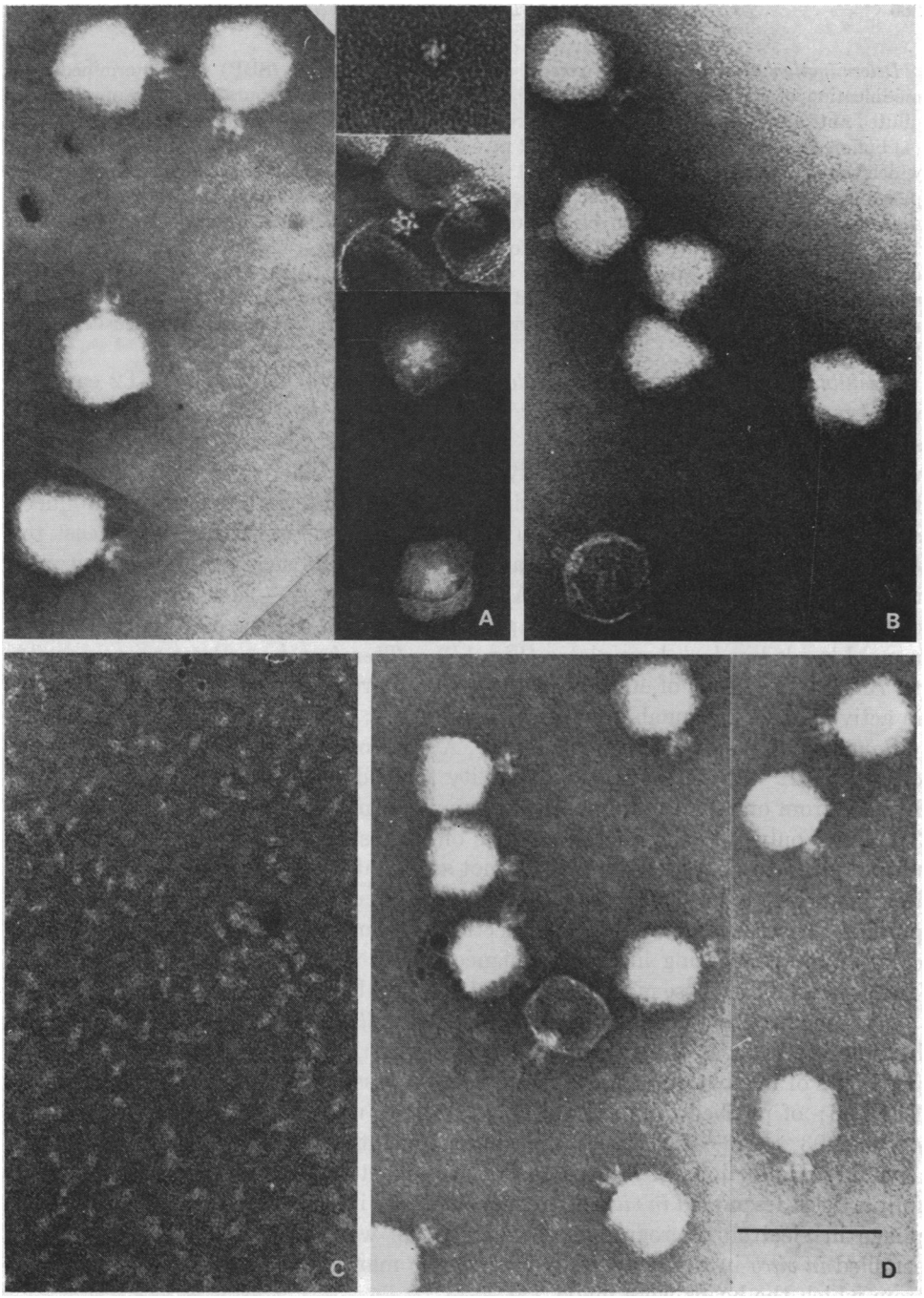

FIG. 1.—The *in vitro* assembly of P22 as seen in the electron microscope. (*A*) On the left are four normal wild-type P22 particles seen from the side; the base plates have the appearance of tridents. On the top right are two isolated P22 base plates and on the bottom right are two P22 base plates seen through the heads. When viewed end on like this, the base plates are seen to have hexagonal symmetry. (*B*) Noninfectious P22 heads prepared by thermal induction of LT2 (tsc_2). Most particles have short necks or tubes attached to the head, while one particle at the top left seems to have a partial base plate. (*C*) P22 tail parts from a 37°C P22*G5* lysate that had been purified by zone centrifugation through a sucrose gradient. (*D*) Infectious P22tsc_2 particles assembled *in vitro* by mixing purified head and tail preparations like those shown in (*B*) and (*C*). The particles are indistinguishable from wild-type particles shown in (*A*). The bar represents 1000 Å.

Determination of serum blocking power: Serum blocking power (SBP) was determined by a modification of the method of DeMars.[11] The reaction mixture contained (1) appropriately diluted antiphage serum, (2) a serum-blocking agent, (3) 3×10^8 $c+$ particles as test phage, and (4) buffered saline in a total volume of 1.2 ml. The mixture was incubated for 2 hr at 37°C, at which time the reaction was stopped by a 1:100 dilution into cold buffered saline. Surviving test phage were assayed at 37°C. When no serum-blocking agent was added, the test phage was inactivated to about 10^5 PFU per ml. More survived when SBP was present. By using 10^9 to 5×10^{10} phage $G5c_1$ (which formed few plaques at 37°C) as the serum-blocking agent, a standard curve was constructed in which the titer of surviving $c+$ test phage was plotted against the amount of $G5c_1$ added. The amount of SBP in an unknown sample could then be estimated by reference to the standard curve. This procedure is faster, but somewhat less sensitive than DeMar's method.

Results.—Source of heads without tail structures: When lysogenic LT2 carrying wild-type P22 is induced to lyse by ultraviolet light, the yield of active phage is strongly dependent on temperature. Incubation at 25°C gives burst sizes of 300, while at 40°C the burst size is usually less than one. Thermally induced temperature-sensitive lysogens behave in a similar manner. Phage preparations produced at 40°C and purified by banding in CsCl have an OD_{260}/PFU ratio at least ten times that of normal phage preparations. This result suggests that many noninfectious particles containing DNA are made at 40°C. Such preparations are devoid of SBP and electron micrographs show that most of the 40°C particles have normal heads but lack base plates (Fig. 1*B*). *Therefore, base plates are responsible for SBP.* The heads of 40°C phage possess a short neck which is not usually seen in active phage, presumably because it is normally obscured by the tail assembly.

Source of tail parts not attached to heads: A series of temperature-sensitive mutants of phage P22 were screened for the ability to produce SBP or tail parts at 37°C. Lysates from one such mutant, *G5*, contained normal levels of SBP when made at 37°C, but only 10^{-6} the infectious titers of lysates made at 25°C. The SBP was not attached to phage heads, as it did not sediment when centrifuged under conditions sufficient to pellet 90 per cent of normal phage. Electron micrographs of a purified SBP preparation show a collection of homogeneous spindle-shaped molecules (Fig. 1*C*) bearing little resemblance to the tail structure of mature phage. The dimensions of these molecules are estimated to be approximately 50 Å by 180 Å.

Morphogenesis of active phage from head and tail preparations: Mixing purified head and tail preparations results in a millionfold rise in the infectious titer after a few hours of incubation at room temperature: assay of purified heads, 2.5×10^5 PFU/ml; of purified tail parts, 3.23×10^3 PFU/ml; of mixture of heads + tail parts, 1.93×10^{11} PFU/ml. This increase in titer clearly represents efficient assembly of infectious P22 particles from the inactive head and tail preparations, for particles assembled *in vitro* are morphologically indistinguishable from wild-type phage in electron micrographs (Fig. 1*D*). As was expected, P22 particles assembled *in vitro* in this way carry the genetic markers characteristic of the stock from which the heads were made.

Stoichiometry: The number of heads in a preparation can be estimated by activating them all with excessive amounts of a tail preparation and counting the PFU's. As long as the number of phage equivalents of tail parts (T) remains greater than the number of heads (H_0), PFU is constant since all the heads then become equipped with base plates. However, when T is further decreased, an equivalence point is reached where $T = H_0$. Below this point, where $T < H_0$,

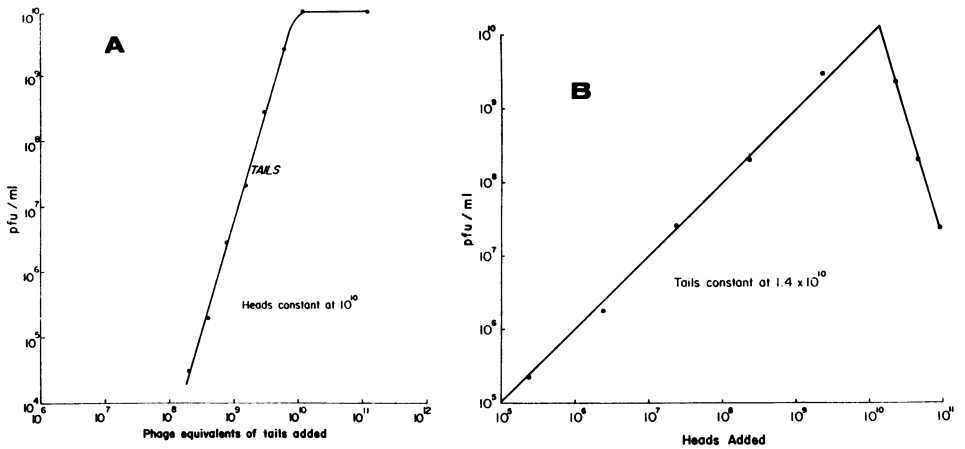

FIG. 2.—Stoichiometry of assembly of P22 from heads and base plate parts. (*A*) Varying amounts of tails were rapidly mixed with approximately 10^{10} heads and the reactions were allowed to go to completion. Final volume, 1 ml. The number of heads was equated to the maximum titer (10^{10} PFU/ml) attained when the tail parts were in excess. The number of phage equivalents of tails added was equated to the number of heads at the equivalence point where the break in the curve occurred, and for other points it was estimated from the volumes and dilutions of the tail parts added. (*B*) Varying amounts of heads were rapidly mixed with approximately 10^{10} tail equivalents and the reactions were allowed to go to completion. Final volume, 1 ml. In both (*A*) and (*B*) each point represents a separate determination from which background titers have been subtracted.

there are not enough tail parts to equip all the heads with base plates, and log (PFU) drops abruptly with a slope of 3.3 when plotted vs. log T (Fig. 2*A*) according to the empirical relation

$$\log (\text{PFU}) = 3.3 \log T - 2.3 \log H_0. \quad (1)$$

T can thus be estimated by titrating a known number of heads with tail parts to locate the equivalence point or, more conveniently, by adding tail parts to a larger number of heads (H_0), determining PFU, and calculating T from (1).

The results of adding varying amounts of heads to constant amounts of tail parts (Fig. 2*B*) are consistent with the results of Figure 2*A*. In the region of low head concentrations, where tail parts are in excess, the titer is proportional to the number of heads added (log PFU = log H) until the equivalence point is reached. Then when the heads are in excess, the titers of the mixtures drop abruptly with a slope of -3.3 on the log–log plot of Figure 2*B*.

These results would be expected for the general reaction $A + nB \rightarrow AB_n$ in which one unit of component A is able to react with up to n units of component B and in which each successive step involving the addition of B to A is essentially irreversible. The slope of 3.3 in our case indicates that three or more tail parts are required to make each PFU.[12]

PFU's formed with an excess of heads over tail parts adsorb on host cells very slowly compared to PFU's formed with an excess of tail parts. This suggests that most PFU's formed with an excess of heads have incomplete base plates which are able to function with low efficiency.

That intermediates exist in our system is also shown by the observation that

when tail parts are equilibrated with an excess of heads, all the SBP can be sedimented at speeds that sediment heads, but not tail parts.

Estimation of the molecular weights of the tail assembly and of the individual tail parts: The molecular weight of the tail assembly (M_T) was taken as the difference between the molecular weights of the total P22 protein (M_p) and of P22 head protein (M_p'). These, in turn, can be estimated from the buoyant densities (ρ_{P22} and ρ_H) of complete phage and of tailless heads and the known molecular weight of P22 DNA (M_d) as follows. By definition

$$\rho_{P22} = \frac{M_d + M_p}{V_d + V_p} = \frac{M_d + M_p}{\frac{M_d}{\rho_d} + \frac{M_p}{\rho_p}} \qquad (2)$$

where V_d and V_p are the volumes occupied by DNA and protein with densities ρ_d and ρ_p. Writing a similar equation in primes for ρ_H, solving both equations for M_p and M_p', and subtracting one from the other, we obtain

$$M_T = M_p - M_p' = M_d \frac{\rho_p}{\rho_d}\left\{\frac{\rho_d - \rho_{P22}}{\rho_{P22} - \rho_p}\right\} - M_d' \frac{\rho_p'}{\rho_d'}\left\{\frac{\rho_d' - \rho_H}{\rho_H - \rho_p'}\right\} \qquad (3)$$

for the molecular weight of the tail structure. Taking $\rho_{P22} = 1.5100$,[13] $\rho_H = 1.5158$ (Fig. 3), $\rho_d = \rho_d' = 1.7000$,[14] $\rho_p = \rho_p' = 1.3000$, and $M_d = M_d' = 27.5 \times 10^6$ daltons,[15] we obtain $M_T = 1.2 \times 10^6$ daltons as the molecular weight of the tail structure.

Tail-part preparations produce a symmetrical peak on zone sedimentation through a sucrose gradient (Fig. 4), suggesting that the tail component is a single

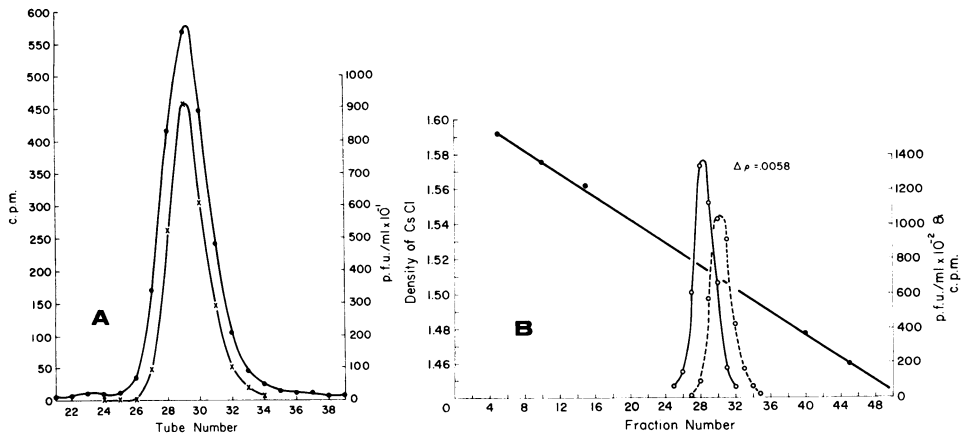

FIG. 3.—CsCl density gradients of heads and whole phage. Approximately 2×10^9 UV-inactivated H^3 thymidine-labeled tsc_2 phage (4×10^4 cpm) and 10^6 heads or 10^4 tsc_2 phage previously assembled *in vitro* were mixed with 3 ml of CsCl, density 1.5. Two ml mineral oil were layered on top of each tube. Centrifugation was carried out at 21°C for 20 hr at 22,000 rpm. Fractions of 50 λ were collected, diluted to 0.25 ml, and assayed for radioactivity. They were then diluted to 1 ml and assayed for phage or heads. (*A*) Control showing identity of density of reconstituted tsc_2 phages (X—X) and UV-inactivated H^3 tsc_2 phage (●—●). (*B*) Difference in density between heads (O----O) and whole phage using UV-inactivated H^3 tsc_2 phage as a marker (O—O). The densities of various fractions are plotted using the left ordinate, while their contents of PFU or cpm are plotted using the ordinate on the right.

homogeneous molecular species. The calculated $S_{20,w}$ value is found to be 9.5 using bovine liver catalase ($s_{20,w}$ = 11.4)[16] as a standard. Estimating the axial ratio from Figure 1*C* as 3.6, we may take the frictional coefficient, f/f_0, to lie between 1.15 and 1.55 corresponding to hydrations of 0.00 and 1.00.[17] If we assume a partial specific volume of 0.73, the molecular weight, M_t, of a tail part would then lie between 1.6×10^5 and 2.6×10^5 daltons.[18] The estimated number of tail parts per tail (M_T/M_t) would be in the range between 7.5 and 4.6 with the value of 6 corresponding to the reasonable hydration of 30 per cent.

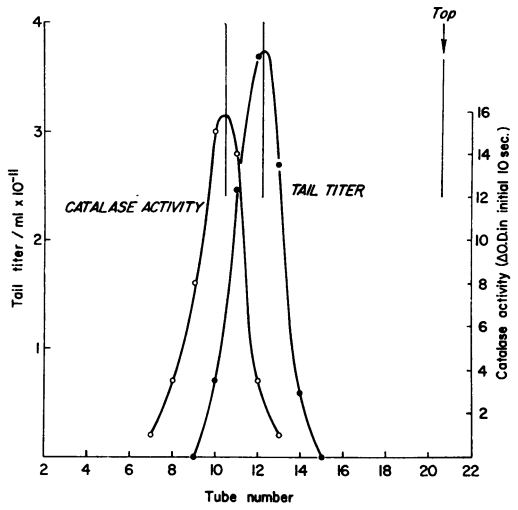

FIG. 4.—Zone sedimentation of tail parts through a sucrose gradient. Bovine liver catalase (100 μg) (Worthington) and approximately 10^{11} tail equivalents were layered on a 5-ml 5–20% sucrose gradient buffer and centrifuged 6 hr at 39,000 rpm at a rotor temperature of 21°C. The gradient was collected in fractions of 0.25 ml. Catalase was assayed according to the method of Martin and Ames.[16]

Characteristics of the reaction: We have scanned the effects of temperature, pH, ionic strength, and various inhibitors on the *in vitro* assembly of P22. The kinetics themselves exhibit the initial lag that is characteristic of multistep reactions (cf. Fig. 8). Assembly is easily detectable at 0°C and the rate increases steadily with temperature (Fig. 5). Assembly proceeds to completion between pH 3.1 and 10 and exhibits a maximum initial rate around pH 5 (Fig. 6). The initial reaction rate is also strongly dependent on NaCl concentration (Fig. 7), being maximal at 0.03 *M* and strongly depressed at 0.5 *M*, even though the reaction eventually proceeds to completion. On the other hand, in 10^{-4} *M* NaCl, assembly is not detectable, and the heads plus tails slowly become inactivated. Urea at concentrations as low as 0.5 *M* also inhibits assembly (Fig. 8). In 4 *M* urea the reaction is very slow, but neither head nor tail protein is irreversibly damaged, so the reaction eventually goes to completion.

The rate of the reaction is not inhibited by mercaptoethanol, which argues against the possibility that disulfide linkages are formed between the head and tail parts.

Discussion and Summary.—The *in vitro* assembly does not appear to require the intervention of an enzyme, for it is extremely efficient when purified head and tail-part preparations are mixed. Similar findings by Edgar and Wood[3] and by Weigle[5] suggest that nonenzymatic reactions may be of general significance in the morphogenesis of viral particles. The firm bonds that hold P22 tail parts to the head apparently do not involve disulfide bonds. They could well be similar to the specific polar, hydrophobic, and cooperative interactions[19] which hold antigens and antibodies together, but the effects of salt concentration seem to be unusual. Further work is required to determine the significance of these interesting salt effects as well as those of temperature, pH, and urea on the initial rate of assembly.

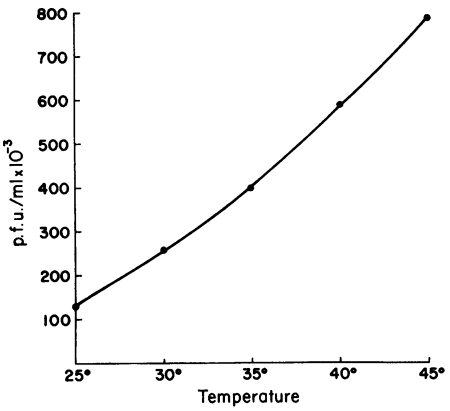

FIG. 5.—Effect of temperature on assembly. Reaction mixture contained 0.03 *M* NaCl, 0.01 *M* Tris pH 8, 5×10^6 heads, and 2×10^{10} tail equivalents in a final volume of 1 ml. The reaction was stopped after 2 min by a 1/100 dilution into buffered saline. The background phage level was approximately 3×10^3.

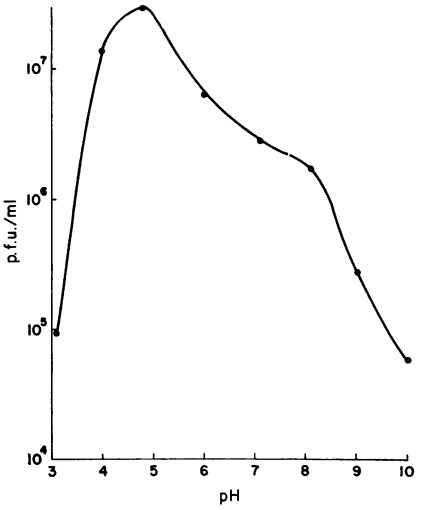

FIG. 6.—Effect of pH on assembly. The reaction mixture contained 0.03 *M* NaCl, 0.05 *M* buffer, 5×10^7 heads, and 1×10^{10} tail equivalents in a final volume of 1 ml. The reaction was run at room temperature and stopped after 2 min by a 1/100 dilution into buffered saline. The background phage level was approximately 1×10^3. Buffers used were as follows: citrate, pH 3.1; acetate, pH 4.0 and 4.8; phosphate, pH 6; Tris, pH 7.1, 8.1, and 9.0; glycine, pH 10.

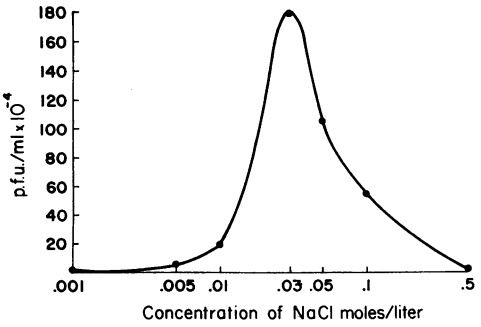

FIG. 7.—Effect of NaCl concentration on assembly. The conditions are the same as in Fig. 5, except that NaCl concentration was varied and Tris was at a concentration of 0.001 *M*.

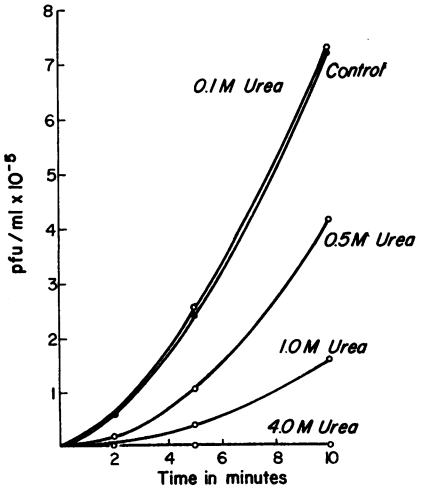

FIG. 8.—Effect of urea on assembly Reaction mixture contained 0.03 *M* NaCl, 0.1 *M* Tris pH 8, 2×10^6 heads, and 2×10^{10} tail equivalents in a final volume of 1 ml. The reaction was run at room temperature. The background phage level was approximately 3×10^3.

The initial lag in the formation of infectious particles after heads and tail parts are mixed (Fig. 8), shows that the assembly of P22 (like the addition of tail fibers to T4[3] but unlike the addition of tails to λ[5]) is a multistep reaction that involves the successive addition of a number of tail parts. The number of tail parts has proven surprisingly difficult to derive without ambiguity. Both the electron microscopic observations and the determinations of molecular weights suggest that six tail parts are required to make a complete base plate, but the stoichiometric experiments suggest that as few as three may suffice for infectivity. We are thus faced with the interesting possibility that incomplete base plates, with as few as three tail parts, may confer infectivity on some of the heads to which they are attached.

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[12] The form of equation (1) can easily be derived from probability considerations. If on each head there are n equivalent sites for the irreversible binding of tail parts and the sites must all be filled to make a PFU, the probability that any one site will be occupied will be:

$$p = \frac{\text{total number of tail parts}}{\text{total number of sites}} = \frac{t}{nH_0} = \frac{T}{H_0}$$

where $nT = t$ is the total number of tail parts combined with heads. Then the probability that a given head acquire n tail parts to form a PFU is given by $p^n = (\text{PFU}/H_0)^n = (T/H_0)^n$, or $\log \text{PFU} = n \log T - (n - 1) \log H_0$.

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