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Biophysical Chemistry 59 (1996) 41–59

Biophysical Chemistry

Mechanism of the long tail-fiber deployment of bacteriophages T-even and its role in adsorption, infection and sedimentation [1]

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Received 17 November 1994; accepted 21 August 1995

Abstract

Models for the tail-fiber deployment of T-even bacteriophages have been experimentally tested by correlating sedimentation constants, adsorption rates, protease inactivation kinetics, and fiber configurations of individual phages observed by electron microscopy. Neither the collective nor the individualistic model, i.e. coordinated fiber retraction and expansion or oscillation of fibers independently of each other, respectively, could satisfactorily account for the results presented. We propose a new intermediary model, in which the base-plate determines a collective behaviour by fixing the hinge angle, around which individual fibers oscillate freely.

The bidisperse, so-called dual sedimentation was shown to occur mainly with nascent high-concentration phage stocks in potassium glutamate containing media. Indeed, when mature intracellular phages are released in 0.5 M potassium glutamate – a condition simulating the intracellular environment – only the fast form appears. Upon storage in the cold or release into 0.5 M chloride, both forms appear. Results confirming that the sedimentation constants of the fast and slow form roughly correspond to those of the monodisperse sedimentation, characteristic of the extreme pH values, i.e. 5 and 8, do not allow to conclude that fiber configuration is the only cause of the bidisperse sedimentation.

Keywords: Bacteriophages T-even; Fiber extension; Sedimentation; Adsorption; Infection; Protease inactivation; Fiber configuration; Electron microscopy; Immobilization; Formaldehyde

1. Introduction

The so-called T-even bacteriophages are endowed with an elaborate tail, a device responsible for the adsorption of the phage to the cell surface and the transfer of its DNA across the envelope. Presently, the morphology, structure, protein composition and functions of the tail and of its components – core, sheath, base plate and fibers – are well known (for review see [1,2]). Although the role of tail fibers in adsorption is now established beyond doubt (for

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[1] This contribution is dedicated to the late Niels K. Jerne, the well known immunologist and 1984 Nobel laureate in medicine, who passed away in the fall of 1994. Niels, who was a co-author on our 1965 publication on the phage tail fiber subject, was a most distinguished thinker. He never lost interest in the problems he was concerned with in his early years and only weeks before his death he was following alertly the evolution of this apparently esoteric problem.

SSDI 0301-4622(95)00117-4

reviews see [3,4]), the kinetics of their movement between the retracted configuration, forming a sort of a jacket around the tail, and the extended configuration remains controversial. It has been well documented that physiological conditions, notably the pH, can shift the equilibrium in favour of one or the other of these states and thus influence the sedimentation velocity of the particles. However, the distinction between the individualistic and the collective behaviour, i.e. fibers moving independently of each other and all fibers simultaneously either retracted or extended, respectively, has remained an open question. In addition, the hypothetical role of the fiber configuration in the so-called dual, bidisperse sedimentation (see below) has not been supported by experimental evidence. In our opinion, the numerous experiments with various biophysical methods [5–10] performed over several decades failed to clarify the situation mainly because the methods, all applicable exclusively to populations of relatively concentrated phage suspensions, did not allow to seek for direct answers to the relevant questions. The reasons for not addressing the latter can be better understood in a historical perspective, of which we will provide a brief summary before giving our reasons for reopening this subject.

Half a century ago, the phenomenon of dual sedimentation was discovered by analytical ultracentrifugation [11–13]. Highly concentrated suspensions of T-even bacteriophages exhibited a bimodal or bidisperse distribution, i.e. two distinct sedimentation boundaries were simultaneously observed. Subsequently, however, improved centrifugation techniques, allowing the investigation of more dilute phage stocks, led to a surprising observation, i.e. phages sedimented with an unimodal and not a bimodal distribution. As it happened, depending on the physiological conditions, the sedimentation constants could take any value between those corresponding to the fast and the slow form observed in dual sedimentation [14]. With the then available electron microscopic techniques, very few experimentalists succeeded in visualising structures that were later shown to be phage tail fibers [15–17]. However, certain observations suggested that fibers may be due to a preparation artefact. For instance, the freeze-drying method revealed a "blob" instead of fibers [15,16]. This was disturbing since the latter technique, widely used in biochemistry, was considered to be rather artefact-free. That oxidative treatments led to tail sheath contraction and the appearance of large numbers of fibers [17], left open the possibility that slight oxidative degradation could occur even during air-drying of phages and be responsible for an artefactual fiber display. Despite additional evidence in favour of tail fibers [16,18], electron microscopic data were considered as ambiguous and not too compelling. This easily explains why fast and slow sedimenting phages were attributed to different tail fiber configurations by one group [19,20], and to different phage particle head sizes by another group [21].

In the mid sixties, when we demonstrated that tail fibers can influence the sedimentation constant of T-even phages [14], an increasing number of high-resolution micrographs obtained by negative staining had established that tail fibers were real and that the oxidation artefact was most unlikely [22–26]. A reasonable physical explanation for the artefactual appearance of blobs was published much later [27]. By using a combination of biophysical and physiological experiments exploiting the adequate phage mutants, obtained at that time [28,29], we had unambiguously shown that tail fibers are essential for phage adsorption and that their configuration plays a dominant role in sedimentation [14]. In addition, we have ruled out the possibility that variable sedimentation rates could be due to head-size variation. This conclusion was confirmed in 1972 [30].

In view of the then predominant controversies, summarized above, one can easily understand why the questions addressed 30 years ago in the contribution of Kellenberger et al. [14] did provide adequate answers neither to the problem of bidisperse dual sedimentation nor to that of the kinetics governing the changes of fiber configuration. In many forthcoming publications, the monodisperse sedimentation, characteristic of the lower phage concentrations, which are usual in physiological experiments, was unfortunately designated, implicitly or even explicitly, as "dual sedimentation". The decision between the two basically satisfactory working hypotheses, i.e. the collective and the individualistic model, was also left open, although the authors [14] were aware of the problem formulated for the first time by Brenner (quoted in [14]).

Since 1965, the quantitative aspects of the fiber involvement in adsorption has been greatly advanced thanks to in vitro complementation experiments, which clearly revealed that phages with only two to three fibers could adsorb to a recipient cell. This led to the conclusion that phages endowed with less than six fibers could be naturally produced. In turn, the electron microscopic observations of phages with less than six extended fibers, considered as supporting the individualistic model [14], were misinterpreted as meaning that these phages were endowed with less than six fibers, all of which were extended [10]. However, the main questions remained unanswered. First, implicitly or even explicitly, the collective model for fiber extension and retraction was accepted without any further experimental evidence, in particular without electron microscopy [5–10,31]. Claims for a first-order kinetics of phage inactivation by protease were in disagreement with the demonstration that proteolysis affected fibers individually and not collectively [32], and rather increased the confusion. Second, bidisperse, dual sedimentation was also implicitly considered as supporting evidence for the collective model of fiber extension. Indeed, monodisperse sedimentations at rates intermediary between those of the fast and the slow form were attributed to rapid changes between the two configurations generating a kinetic equilibrium. A most tempting interpretation would assume that, at high phage concentrations, fibers could have been locked in either the extended or the retracted configuration and thus sediment in two distinct peaks, the slow and the fast, respectively.

The increase in the number of extended fibers of a tryptophan-requiring phage with the tryptophan concentration in the medium to a maximum of 2.7 [14], and the intermediate values of monodisperse sedimentation constants, values which both could only be further increased by higher pH [14,33], was also explored with other biophysical methods applicable to populations [34]. The theory of Jerne in [14] was extended further [9].

In conclusion, over the last three decades, the electron microscopic evidence against the collectivistic behaviour of fibers was ignored, misinterpreted or the experiments again questioned, because of observations using aldehydes in a study of tail-sheath contraction [35].

Under these circumstances, we felt almost obliged to reinvestigate these questions and, more specifically, to provide the methodology for a reliable microscopic inspection of the fiber configuration of individual phages under various experimental conditions. In this contribution, we first demonstrate that formaldehyde treatment apparently irreversibly immobilizes the fiber configuration and allows its analysis by the negative contrast technique. Thus, the fiber configurations and the sedimentation constants for pH values ranging from 5 (most fibers retracted) to 8 (most fibers extended) could be compared. Our results strongly favour an intermediate model in which individual fibers would oscillate around a mean position determined by the pH. We believe that this model can reconcile all available data.

Our results are still insufficient to explain the bidisperse, dual sedimentation. We have established that the phenomenon occurs preferentially with nascent phage stocks. The fast form appears first when the infected cells are lysed into 0.5 M potassium glutamate, which is claimed to best mimic the intracellular conditions (reviewed in [36]).

2. Experimental

2.1. Materials

2.1.1. Bacteriophages

T4D (Doerman), T4B (Benzer), T2L (Luria), and the tail fiberless suppressor sensitive (amber) multiple mutant X4E of T4D [37] were used. T4B requires tryptophan for fiber extension and adsorption. T4D was the reference wild type. The double mutant T4.10amB255.23amH11 was constructed by recombination between the 10^-, tailless, and the 23^-, headless, mutants of the Basel–Geneva collection [28].

2.1.2. Bacteria

Escherichia coli CR63 was the permissive strain for growth of amber mutants, while *E. coli* B was the non-permissive one. Adsorption experiments were performed on *E. coli* B.

2.1.3. Medium

Cells were grown in M9a, i.e. the liquid M9 medium [38] supplemented with 1% Casamino acids

("Oxoid"). Phages were plated on Hershey plates [39] containing Difco nutrient broth, glucose and sodium citrate. They were solidified with 1.5% Gibco Agar. Buffer was phosphate-buffered saline at the adequate pH, with 10^{-3} M $MgSO_4$.

2.2. Methods

2.2.1. General phage techniques

The general phage techniques are described by Eisenstark [39]. Labelled phage stocks were obtained by infecting a bacterial culture in M9a medium at a multiplicity of 0.1. Thirty seconds after infection, 0.5 mCi/ml of a ^{14}C-labelled amino acid cocktail (CFB 104 from Amersham) was added. Lysis inhibition occurring in the second phage growth cycle generated high titre stocks. The latter were purified by centrifugation on a 13 ml linear 5–40% sucrose gradient in the SB 283 rotor of an IEC B-60 centrifuge at 30000 *g* for 20 min at 4°C. Phage-containing fractions were pooled and dialysed against phosphate buffer.

For plating with preadsorption, *E. coli* B was grown in M9a to 5.10^8/ml. Cells were pelleted, washed by two consecutive resuspensions in buffer and sedimentation, and resuspended in 1/10 of the original volume. A volume of 0.1 ml of the thus obtained indicator bacteria was added to 0.9 ml of a buffer phage dilution, incubated for 10 min at 37°C, and plated with top agar containing additional indicator bacteria.

2.2.2. Phage adsorption

A culture in M9a was centrifuged and the pellet resuspended in 2 ml phosphate buffer at a concentration of 10^9/ml. Unfixed or formaldehyde-fixed ^{14}C-labelled phages were added at a multiplicity of five, incubated for 30 min at 37°C, and centrifuged at low speed. The radioactivity of the supernatant and of the resuspended pellet was measured in a scintillation counter.

2.2.3. Attachment of tail fibers

Complementation in vitro of T4 fiberless phage particles was carried out according to Wood and Henninger [40]. Reaction mixtures of 0.5 ml, consisting of Tris buffer (0.1 M *N*-tris(hydroxymethyl)methyl-2-aminoethane sulphonic acid, 0.02 M $MgSO_4$, 0.002 M dithiothreitol, and 0.001 M EDTA), fiberless X4E phage particles, and tail fibers were incubated overnight at 30°C. At this temperature the heat labile factor (gp63) required for efficient fiber attachment remains active. Following incubation, the number of complemented phages was determined by titration of plaque forming units (pfu).

Subsequently, we found that the efficiency of in vitro fiber complementation was considerably increased when potassium chloride was replaced by potassium glutamate.

Tail fibers were produced as follows: a 50-l fermenter culture of *E. coli* B in M9a was infected at 4×10^8/ml with T4.10amB255.23amH11 at a multiplicity of four, followed by two superinfections after 7 and 14 min incubation. After 1 h incubation at 30°C, the culture was sedimented at low speed and the pellet resuspended in 1 l phosphate buffer containing chloroform and DNAse. After homogenisation and lysis, the culture was centrifuged at low speed and the supernatant kept on ice overnight, followed by centrifugation at 140000 *g* for 90 min. The supernatant, containing the fibers, was divided into small portions, rapidly frozen in dry ice–alcohol, and kept at −60°C, a temperature at which the activity is maintained.

2.2.4. Isokinetic sucrose gradients

Isokinetic gradients, in which the sedimentation rate of particles remains constant along the tube, were prepared according to Noll [41]. A 13 ml sucrose gradient (5–26.5%), generated with the mixing chamber, was loaded with 10^{10} particles in 100 μl phosphate buffer, and centrifuged with the IEC SB283 rotor (15 min, 20000 *g*, 20°C). Gradient tubes were punctured, the 280 nm absorbance of the effluent scanned, and the signal plotted directly by a recorder.

2.2.5. Analytical ultracentrifugation

A Beckman Model E analytical ultracentrifuge, equipped with a Schlieren optical system, ultraviolet absorption optics and a photoelectrical scanner was used for moving-boundary sedimentation studies. While the Schlieren optics required at least 10^{12} phages per ml, experiments with absorption could be performed with 10–100 times less concentrated phage suspensions. The 12-mm double sector Epon

cell was chosen for solutions with an OD_{280} of about 0.5. Special care was taken to prevent convection during the run. To avoid precession at low speed runs of 2800 and 4000 rpm, we resorted to the heavy An-J rotor. For the 5000–10000 rpm range, the An-H (titanium) rotor was used. Finally, for centrifugation of up to 20000 rpm, the An-D rotor (aluminium) was preferred since it allows the reduction of the acceleration period to a minimum, allowing enough time to record the sedimentation. To minimize disturbances due to temperature, the rotor was kept at 20°C, a temperature close to that of the chamber (17°C). During runs at about 20°C and speeds of 6000–20000 rpm, the refrigeration system, as well as the "regulate" control of the RTIC system, were switched off. For such short sedimentation runs, the pointer on "indicate" remained at a constant position of ± 0.1°C. To maintain constant temperatures during longer runs at lower speeds, we have resorted to the method described by Chervenka [42].

Measured S values were always expressed as $S_{20,w}$ according to the tables of the CRC Handbook of Chemistry and Physics.

Scans were recorded in two modes, the absorption and its derivative. Because of the delay of the derivative trace [42], S values were calculated from the absorption curves.

2.2.6. Electron microscopy

Phages were maintained at the desired pH for 1 h. They were fixed for an additional hour with either 1, 2 or 5% formaldehyde (Fo), or 2% glutaraldehyde (Glu). When indicated, fixed phages were dialysed against phosphate buffer at a given pH.

For fiber counts, phages were prepared by the agar filtration method [43]. The surface of a collodion-coated agar plate was spread with 10 μl of 5% tryptone. After filtration (5–10 min), 20 μl of the fixed phage suspension were spread and filtered (10–20 min). The agar was exposed for 10 min at room temperature to concentrated formaldehyde vapours. The collodion film was floated on 10% sodium phosphotungstate and picked up onto specimen grids.

Fibers were counted directly in the electron microscope at a magnification of 54000 ×. A plane, perpendicular to the phage axis and encompassing the base-plate, was defined. Fibers above or below this plane were designated as "up" or "out", respectively.

3. Results

3.1. Formaldehyde fixation

To correlate parameters like sedimentation constant, adsorption, infection (plaque forming units, pfu) and protease inactivation to the distribution of phage fibers between the retracted and the extended state, it is essential to resort to direct, electron microscopic observation. However, negative staining, the most suitable method, is not necessarily free of artefacts. First, during drying, the pH of sodium phosphotungstate rises from 7.0 to 7.6, possibly leading to limited additional fiber extension. Second, extended fibers readily adsorb to the supporting film and, once adsorbed, are excluded from the dynamic equilibrium, which is therefore displaced in favour of fiber extension. These phenomena could artefactually increase the actual number of extended fibers. Therefore, it was essential do devise a method for fiber immobilisation, which would ensure that the distribution of fibers is not disturbed by the electron microscopic preparation method.

We have investigated aldehyde fixation on phages under so-called physiological conditions, i.e. buffers or buffered media and concentration not exceeding 10^{10} particles/ml. Formaldehyde was already used for blocking retracted fibers [14,20]. However, no evidence was provided that fibers "out" cannot retract when fixed phages are exposed to conditions which favour retraction. To determine to what extent both extension ("out") and retraction ("up") are blocked by the fixative, phages were fixed at one and dialysed against another pH. The results (Table 1) reveal the following: (i) at pH 7, fiber distributions of fixed and unfixed phages are not significantly different; (ii) when phages were fixed at extreme pH values used in our experiments, i.e. 5.1 and 8.1, their fiber distributions were hardly affected by adjusting the pH to 7 or even bringing it to the opposite, extreme, pH. Interestingly, glutaraldehyde, while irreversibly fixing retracted fibers to the phage particle, did not prevent fiber retraction. At pH 8, glu-

Table 1
Fiber configuration of formaldehyde fixed T4D as a function of pH [a]

Fixation		Observation pH [b]	Number of phages with n fibers "out"							
pH	Formaldehyde concentration (%)		0 [c]	1	2	3	4	5	6	$\bar{x}$ [d]
7.0	0	7.0	2	0	14	26	42	14	2	3.56
7.0	5	7.0	3	4	12	19	32	21	9	3.68
7.8	1	7.8	0	2	9	19	32	31	7	4.02
7.8	1	7.0	1	2	15	17	35	26	4	3.77
7.8	2	7.8	0	0	3	13	32	33	19	4.52
7.8	2	7.0	0	3	11	17	35	27	7	3.93
7.8	5	7.8	0	0	1	10	36	34	19	4.66
7.8	5	7.0	0	0	4	11	34	35	16	4.48
5.1	5	5.1	72	18	6	4	0	0	0	0.42
5.1	5	8.1	56	30	12	2	0	0	0	0.60
8.1	5	8.1	0	0	6	20	30	32	12	4.24
8.1	5	5.1	0	0	2	10	32	36	20	4.92

[a] Phages brought to any given pH were fixed with formaldehyde at the indicated concentration. They were examined by negative staining. By visual inspection, fibers were divided into two classes according to their position relative to the plane encompassing the base-plate. Those above or below this plane were considered as "up" or "out", respectively.
[b] When required, prior to inspection by the electron microscope, phages fixed at a given pH were brought to a different pH by dialysis.
[c] Number $n = 0$–6 of fibers "out".
[d] Mean number of fibers per phage particle. Average of five preparations.

taraldehyde-fixed fibers were mostly in the out position (Table 2), while dialysis against buffers of lower pH increased significantly the retraction. This observation is accounted for by assuming either (i) that extended fibers retain normal mobility and become cross-linked to the phage body as soon as establishing a contact with it, or (ii) a modified hinge angle of the base-plate (see discussion).

After fixation at pH values above 8 we observed various alterations of the phage, including sheath

Table 2
Fiber extension of glutaraldeyde (Glu) fixed T4D as a function of pH [a]

Fixation pH	Concentration of fixation agent	Observation pH [b]	Number of bacteriophages with n fibers "out" 0 [c]							
			0 [c]	1	2	3	4	5	6	$\bar{x}$ [d]
7.0	5% Fo [e]	7.0	0	0	4	20	38	28	10	4.20
7.0	2% Glu	7.0	46	42	12	0	0	0	0	0.66
5.2	2% Glu	5.2	79	17	4	0	0	0	0	0.25
5.2	2% Glu	8.0	79	20	1	0	0	0	0	0.22
5.7	2% Glu	5.7	74	20	6	0	0	0	0	0.32
5.7	2% Glu	8.0	65	33	1	1	0	0	0	0.38
7.0	2% Glu	7.0	32	35	23	9	1	0	0	1.12
7.0	2% Glu	5.2	59	33	8	0	0	0	0	0.49
8.0	2% Glu	8.0	20	37	24	13	5	1	0	1.49
8.0	2% Glu	5.2	47	40	13	0	0	0	0	0.66

[a] Phages brought to the wanted pH were treated with the specified fixation agent.
[b] When required, prior to inspection by the electron microscope, phages were brought to the observation pH by dialysis.
[c] Number $n = 0$–6 of fibers "out".
[d] Mean number of fibers per phage particle. Average of five preparations.
[e] Fo and Glu refer to formaldehyde and glutaraldehyde, respectively.

contraction, confirming observations of Moody [35], who used aldehyde fixation to demonstrate a progressive tail-sheath contraction.

3.2. The distribution of fibers as a function of pH

Based on control experiments reported above, we have reinvestigated the fiber distribution as a function of pH by exploiting formaldehyde fixation. A representative sample of histograms of observed distributions, together with theoretical binomial distributions, is presented in Fig. 1. The latter distributions were calculated with the assumption that each

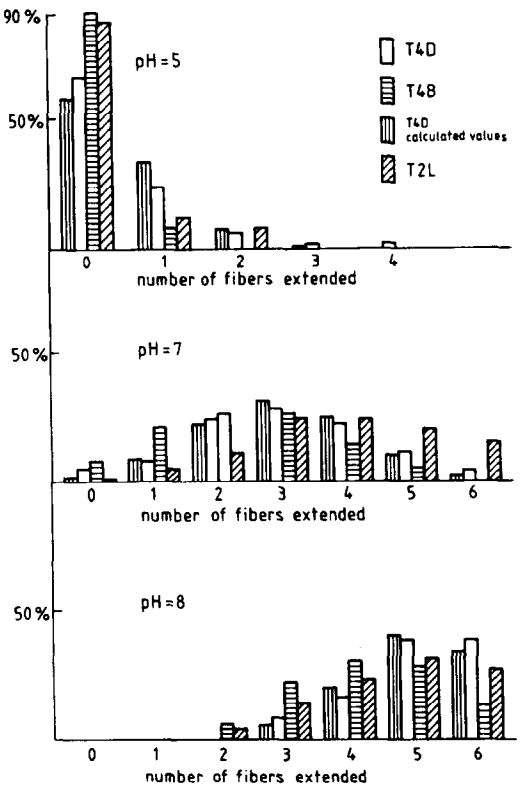

Fig. 1. Configuration of tail fibers of bacteriophages T4D, T4B and T2L as a function of the pH. Bacteriophages were brought at a given pH value, formaldehyde fixed to immobilize their fibers, and examined with the electron microscope by negative staining. According to their position, with respect to the plane comprising the base-plate, fibers were divided into two categories. Those below or above this plane were considered as extended ("out") or retracted ("up"), respectively. One of the bars (indicated) represents the calculated distribution, based on $\bar{x}$ of the experimental counts with T4D.

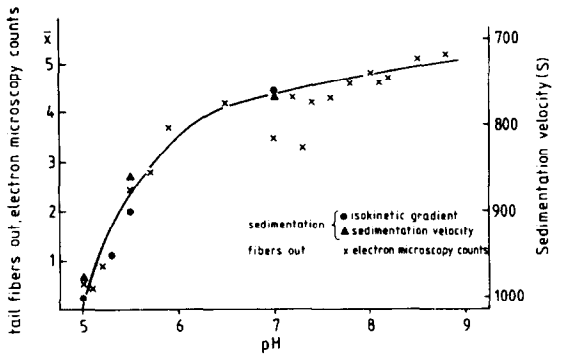

Fig. 2. Correlation between fiber extension and phage sedimentation velocity. The average number $\bar{x}$ of extended fibers per phage (fibers "out") was determined by electron microscopic inspection of negatively stained phages fixed at a given pH (×). Sedimentation constants were determined either in the analytical ultracentrifuge (▲) or in an isokinetic sucrose gradient (●).

phage is endowed with six fibers. Thus, for a given pH, the probability of a phage to exhibit x fibers "up" is:

$$f(x) = \binom{n}{x} p^x q^{n-x}$$

with n taken as 6 and where

$$\binom{n}{x} = \frac{n!}{x!(n-x)!}$$

and

$$\binom{n}{0} = \binom{n}{n} = 1$$

The probability for an individual fiber to be "up" or "out is $p = x/6$ and $q = (6 - \bar{x})/6$, respectively.

The experimental average number $\bar{x}$ of fibers "out" as a function of pH is given in Fig. 2. Inspection of Fig. 1 reveals that the experimental and calculated distributions for T4D are in fair agreement.

The maximal average numbers of fibers "out", i.e. 5.2 and 5.3, were obtained at pH 8.8 and 8.5, respectively. However, at these relatively high pH values, fiber distributions are unreliable, because most of the phages are damaged and have a tendency to aggregate. Therefore, we have not raised the pH above 8, a value associated with an average number of fibers "out" between 4.6 and 5. Never have we observed a major proportion of phages with six

extended fibers, leaving open the possibility that not all phages have a complement of six fibers (see below).

Fiber distributions of formaldehyde-fixed phages are nearly identical to those previously obtained by electron microscopy of shadowed, non-fixed, phages [14].

Our experiments (Fig. 1) reveal a very similar behaviour of the phages T4D, T4B and T2L. Small differences between them are comparable to those between experimental and calculated values, suggesting that they could be accounted for by statistical variations of the experimental data.

3.3. Phage sedimentation constant as a function of pH

The results presented above are apparently compatible with the individualistic model, i.e. under so-called physiological conditions any given fiber can oscillate between the extended and the retracted position, independently of the other fibers.

To assess if the average proportion of the retracted fibers is directly related to the sedimentation behaviour of phage populations, the latter, at a concentration of about 10^{10} particles/ml, were fixed at different pH values and, first, analysed on isokinetic sucrose gradients [41]. Fixed and unfixed phages were examined for pH ranging from 5 to 8 (Fig. 3). As previously described [14], the sedimentation of a fiberless phage mutant (see below) is identical to that of the wild type phage fixed at pH 5 (i.e. with fibers "up"; Fig. 3d and f). As expected, increasing the pH, and thus the proportion of extended fibers, was accompanied by slower sedimentation of the wild type phage, while leaving unaffected that of the fiberless mutant (Fig. 3c, e and f). In contrast to moving-boundary experiments on nascent phage, at a concentration two orders of magnitude higher than that proper to physiological conditions (see below and Fig. 7), we have never observed a bidisperse sedimentation, nor could we generate it by varying the sedimentation speed.

To accurately measure the sedimentation constant as a function of pH and thus of the average number $\bar{x}$ of fibers "out", we resorted to analytical ultracentrifugation. Phages, unfixed or fixed at pH's ranging from 5 to 8, were sedimented at both pH 5 and 7 (Table 3). Control experiments with X4E, the fiberless mutant, provided a remarkably constant S value, independently of the fixation or sedimentation pH, strongly inferring that fibers play a key role in sedimentation. The S values of unfixed, wild type, phages were between 780 and 860 for pH values of 7 and 5.5, respectively. It also appeared that fixation with formaldehyde does not significantly alter the sedimentation constant. Plotting all available S data obtained by moving boundary as well as isokinetic sucrose gradient centrifugations, as a function of pH, together with the average number $\bar{x}$ of fibers "out", obtained by electron microscopy (Fig. 2), reveals

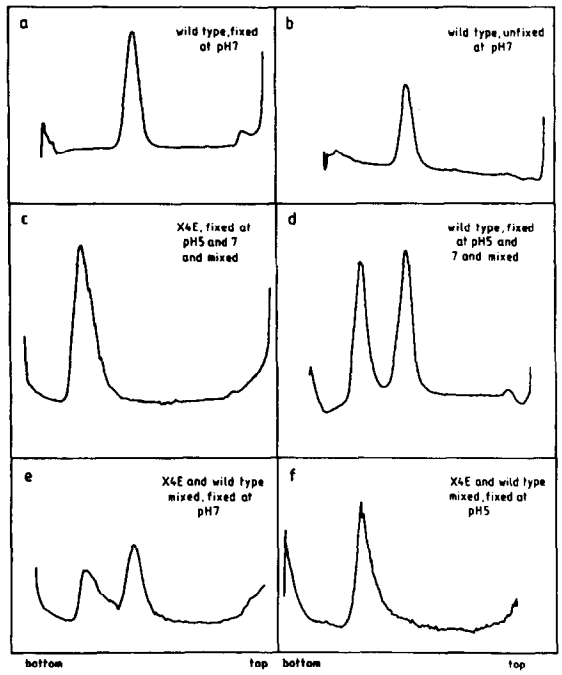

Fig. 3. Sedimentation in isokinetic sucrose gradients. Particles of T4D and of the fiberless mutant X4E were brought to given pH values. Either unfixed or formaldehyde fixed, they were sedimented on isokinetic sucrose gradients (see Material and Methods). The distribution of phages was monitored by absorption at 280 nm. Sedimentation of the wild type phage at pH 7 (a) with and (b) without formaldehyde treatment. Aliquots of either fiberless (X4E) or normal phages were fixed at pH 5 and 7 and mixed prior to centrifugation. Only one peak was visible for X4E (c), while two were present with the wild type (d). Finally, both phage species were mixed and fixed at pH 7 (c) as well as at pH 5 (f). One peak only appeared at pH 5, while two were present at pH 7. Unfixed phages did not yield two peaks.

that, with two exceptions, the correlation between S and the average number of retracted fibers is very good.

In contrast to other claims, we found for different T-even phages no significant differences in the distribution of fiber deployment as a function of pH. For pH values between 5 and 8, the proportion of extended fibers is linearly correlated with pH. Electron microscopic observations seem to be corroborated with sedimentation constant measurements since fiber retraction should reduce the particle's form factor and thus increase its sedimentation speed. However, it is paradoxical that formaldehyde fixed and unfixed phages exhibit the same sedimentation profile, in particular on isokinetic sucrose gradients (Fig. 3). Indeed, if for unfixed phages it can be assumed that the oscillation between the "out" and "up" state occurs with a relatively high frequency, resulting in a relatively constant S for each individual phage, the situation is fundamentally different for fixed phages. For instance, those fixed with 1 or 5 fibers "out" would contribute to the generation of very wide peaks. Therefore, we are forced to conclude that, at a given pH, the position of individual fibers of any phage particle is not fully random. Namely, the exact position of each individual fiber affects the form factor. Therefore, it would appear that, for each particle, the average form factor is nearly constant, i.e. that a higher proportion of fibers "up" is compensated by a more extended position of the other fibers (see Discussion).

3.4. Adsorption with formaldehyde-fixed phages

To investigate the correlation between phage fiber configuration and adsorption, we have resorted to phage populations brought to pH values ranging from 5 to 8 and fixed by formaldehyde (see Table 1). By this method, phages with any given fiber distribution, ranging from all "out" to all "up", can be

Table 3
Sedimentation constants of the wild type T4D and the fiberless mutant X4E, as a function of the fixation agent and the pH

Phage	Fixation		Sedimentation [b]		
	Concentration and agent [a]	pH	pH [c]	rpm	$S_{20,w}$
T4D	5% Fo	5.0	7.0	8000	969
	5% Fo	5.0	7.0	8000	984
	5% Fo	5.3	7.0	8000	1007
	5% Fo	5.3	7.0	14000	942
	–	5.5	–	8000	859
	–	7.0	–	8000	780
	5% Fo	7.0	7.0	8000	747
	5% Fo	7.0	7.0	8000	774
	2% Glu	7.0	7.0	8000	900
	2% Glu	7.0	5.2	8000	920
	5% Fo	7.3	5.2	8000	793
	5% Fo [d]	7.0 + 5.0	7.0	8000	790 + 986
	5% Fo [d]	7.0 + 5.0	7.0	8000	777 + 1035
	5% Fo [d]	8.0 + 5.2	7.0	8000	750 + 900
X4E	5% Fo	5.0	7.0	8000	913
	–	5.8	5.8	8000	918
	–	5.8	5.8	14000	912
	5% Fo	7.0	7.0	8000	918

[a] Fo and Glu refer to formaldehyde and glutaraldehyde, respectively.
[b] The sedimentation constant was determined by moving boundary analytical ultracentrifugation.
[c] When required, phages were brought by dialysis to the specified pH of the supporting sedimentation buffer.
[d] Aliquots of phages fixed at specified, different, pH's were mixed, prior to centrifugation.

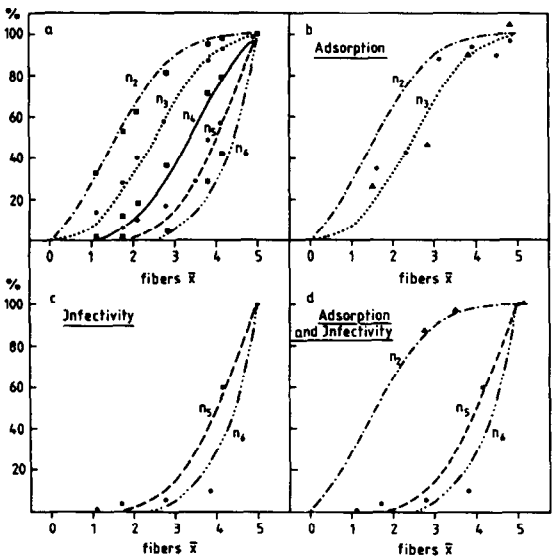

Fig. 4. Correlation between the number of extended fibers and phage adsorption as well as infectivity. Each figure contains relevant, referential theoretical curves obtained as follows: for any given average number $\bar{x}$ of extended i.e. available fibers per virion, the number of particles with n or more than n such fibers was computed according to the theoretical binomial distributions (see Table 6). Curves representing n versus $\bar{x}$, are indexed $n_2 - n_6$. In (a) the number of extended fibers was governed by pH. Fibers were subsequently counted with the electron microscope; the points represent the computed, experimental values relative to corresponding indexed theoretical curves. For (b, c and d) the average number $\bar{x}$ of extended fibers was governed by in vitro complementation and corresponds to the ratio of fibers to virions. The experiments were performed at high pH, so as to obtain the maximum number of extended fibers. In (b) percentages of successful adsorptions are given. In (c) percentages of successful infections, measured as pfu's, are plotted. In (a) the experimental values (●, ▲) were computed from those of Table 6. In (b) the adsorption was measured with (●) normal and (▲) formaldehyde-fixed phages. In (c) measurements of infectivity as a function of $\bar{x}$, for normal phages, were taken from Fig. 5. (d) reports (▲) adsorption and (●) infectivity combined from (b) and (c).

obtained. Adsorption experiments were performed as follows: ^{14}C-labelled phage samples were fixed at different pH values, brought back to pH 7, mixed with aliquots of *E. coli* B at $2–5 \times 10^8$/ml, and incubated for 30 min at 37°C. Cells were harvested by low-speed centrifugation, and the radioactivity was determined in both the pellet and the supernatant. The relative amount of radioactivity in the pellet, corresponding to adsorbed phage, was plotted as a function of the average number of fibers "out" (Fig. 4b). The indicated proportions of phages with three or more fibers "out" were obtained from Fig. 4a. It appeared that the two sets of data are readily superimposable.

The reproducibility of such adsorption experiments was poor (not presented). The hypothesis that the observed variations were due to the highly reversible nature of this adsorption step, and not to reduced adsorption associated to a possible modification of fiber tips by formaldehyde, was indeed confirmed by several experiments aimed to enhance desorption by different shaking procedures (Table 4). Phages with a maximum of fibers "out", fixed at pH 8.2, were compared with those with fewer fibers "out", fixed at pH 5.6. To reduce readsorption during pelleting, samples were diluted 10 and 100 times. Obviously, for each data point, the same amount of phage was considered. Table 4 clearly reveals that phages with a maximum number of fibers "out" adsorbed irreversibly; shaking and dilution did not significantly affect the outcome of the experiment. However, with phages fixed at pH 5.6, 44% were adsorbed without agitation, while only 7% remained adsorbed following agitation and dilution.

Table 4
The reversibility of adsorption of phages with immobilized fibers

Fixation pH	Dilution factor at 30 min	% of phages remaining adsorbed after shaking[b]
8.2	0	99
8.2	100	99
8.2	100	98
5.6	0	44
5.6	10	25
5.6	100	7

[b] ^{14}C-labelled T4 phages were formaldehyde (5%) fixed at the indicated pH, and dialysed against phosphate buffer at pH 7. Less than 10^9/ml phages were added to a 1-ml suspension of 5×10^8/ml bacteria and incubated with agitation by air-bubbling. 30 min after the infection, cells were diluted so as to prevent readsorption. Suspensions were incubated for a further 30 min under strong shaking in a gyratory water bath. Finally, bacteria were pelleted by centrifugation and the radioactivity measured in both the pellet and the supernatant.

3.5. Adsorption and plaque forming ability of phages complemented in vitro with different numbers of fibers

Experiments reported above strongly suggest that the nature of adsorption, more or less reversible, depends on the number of extended fibers. However, with formaldehyde-fixed phages, it was impossible to decide whether aldehyde had any influence on the fiber tips and thus on phage adsorption. Neither was it possible to determine with such phages the infectivity, expressed by the plaque-forming ability. To overcome these limitations, phage particles with a given, average, number of fibers were obtained by in vitro complementation of X4E, the fiberless T4 mutant (Fig. 5). Samples of complemented phages were purified by centrifugation on sucrose gradients, and, careful, stepwise, dialysis to avoid osmotic shock. To insure that most fibers are extended, phages were brought to pH 8. Thus, counting in the electron microscope provided a good estimate of the *total* numbers of fibers attached to a phage. Obviously, at any given time and at pH 7, not all of these attached fibers were in the "out" position. However, once the phage is attached to its cell surface receptor, we may assume that, eventually, by virtue of the dynamic

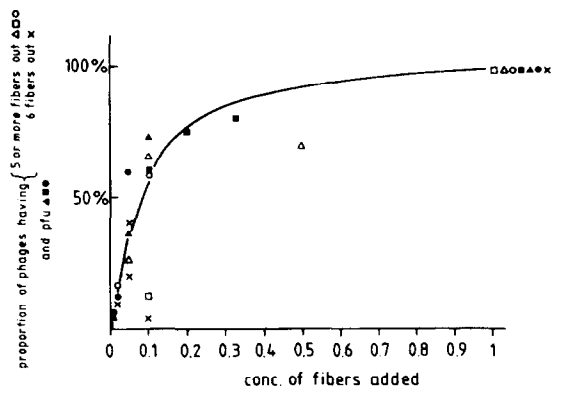

Fig. 5. Correlation between the infectivity (plaque-forming ability) and the proportion of phages with five or more extended fibers. Particles of the fiberless mutant X4E were in vitro complemented with fibers. Phages were purified on sucrose gradients, dialysed against buffer at pH 7.8 and fixed by formaldehyde. The average number of fibers per virion and their distribution were determined by electron microscopy. Thus, the total number of fibers eventually available on complemented phages could be estimated. From distributions given in Table 5, we calculated the proportion of phages with five or more fibers available as a function of the ratio added fibers/fiberless virions. These figures were obtained in three independent experiments represented by open symbols. The proportion of phages with 6 fibers available is also plotted ($\bar{x}$). The infectivity (pfu) of these complemented phages was determined before fixation, and plotted with dark symbols. Comparison with Fig. 4d reveals a good fit between pfu's and phages endowed with at least 5 or even 6 fibers.

Table 5
Adsorption and plaque-forming ability as a function of the number of fibers attached to the fiberless phage X4E by in vitro complementation

Relative amount of fibers added [a]	Unfixed phages			Fixed phages [d]								Ads [i] %	Adsorption normalised to 100% [h]
	pfu after purification [b]	pfu [c] %	Ads [i] %	Distribution of fibers									
				0 [e]	1	2	3	4	5	6	$\bar{x}$ [f]		
1	7×10^9												
0.5	10×10^9		95	0	2	0	6	22	30	40	4.98	74	
0.2	7×10^9	100											100
0.1	8×10^9		77	0	2	2	12	18	26	40	4.84	82	
0.05	4×10^9	50	66	0	8	18	26	22	18	8	3.48	76	97
0.02	5×10^8	6	53	8	12	28	20	18	10	4	2.74	68	87

[a] The figures refer to the original fiber stock which corresponds to 1.
[b] Following the reaction of the fibers with the X4E phage mutant, virions were purified by sucrose gradient centrifugation.
[c] pfu's were normalized with respect to those obtained with relative amounts of fibers equal to or above 0.2.
[d] To ensure that nearly all of the attached fibers are extended, in vitro complemented phages were brought to pH 8 and formaldehyde (5%) fixed. They were brought back to pH 7 by dialysis and the number of fibers per phage counted with the electron microscope. The adsorption was determined with ^{14}C-labelled phages.
[e] The number $n = 0$–6 fibers.
[f] The mean number of fibers per virion.
[g] The figures refer to formaldehyde-fixed phages.
[h] The adsorption measured by radioactivitiy was normalised to 100% as described in [c].
[i] Ads means adsorption.

equilibrium, all fibers will become adsorbed. Under this assumption, the total number of fibers per phage may be relevant.

Comparison of fiber distribution, adsorption (measured by ^{14}C), and plaque-forming ability (pfu) (Table 5) revealed that, with a decreasing average number of $\bar{x}$, pfu's decrease much faster than does the adsorption. This suggests that some phages are able to adsorb, but not necessarily to infect the cell. It should be noted that the experiment was not designed for the detection of very small plaques, which could have occurred as a consequence of a large delay between adsorption and infection, neither was it taken into consideration that the infectivity was strongly dependent on plating conditions. This important aspect will be dealt with below.

To accurately quantify the relationship of adsorption and pfu's to the minimal number of fibers per phage, we have used phages complemented in vitro with different average numbers of fibers. Observed fiber distributions are given in parallel to those calculated according to the binomial distribution given in Section 2 (Tables 6 and 7). The correspondence between the calculated and the experimental values is good. In classes with 5 and 6, as well as in those with 0 fibers experimental values are constantly higher than the theoretical ones. Whether this difference is due to experimental imperfections or to a possible, slight, cooperativity in fiber binding remains an open question (see Discussion).

From the experimental and the theoretical values (Table 6), we have calculated the distribution of the total number of phages, n_i, with i or more fibers "out". The calculated and experimental data are plotted in Fig. 4a, with each n_i normalised to 100%. When comparing these figures to the values of the pfu's, representing successful infections (Fig. 4c), we find that the latter follow the curves of 6 (n_6), or possibly 5 and more fibers (n_5). The adsorption, in agreement with Section 4, follows rather the curves

Table 6
Distributions of the total number of fibers per phage obtained by in vitro complementation; comparison of electron microscopic counts and calculated values (in parentheses)

$\bar{x}$ [a]	Proportion of phages with n fibers [b]							% pfu
	0	1	2	3	4	5	6	
4.96	0	0	4	6	18	34	38	100
	(0.0)	(0.1)	(0.9)	(5.9)	(21.1)	(40.1)	(31.9)	
4.92	0	1	3	7	20	30	39	
	(0.0)	(0.1)	(1.1)	(6.4)	(22.0)	(40.0)	(30.4)	
4.15	0	2	9	18	30	25	16	60
	(0.1)	(1.2)	(6.5)	(19.4)	(32.6)	(29.3)	(11.0)	
3.82	1	4	12	24	24	24	11	10
	(0.2)	(2.4)	(10.6)	(24.8)	(32.5)	(22.8)	(6.7)	
2.80	4	15	24	25	20	10	2	
	(2.3)	(12.1)	(26.4)	(30.8)	(20.2)	(7.1)	(1.0)	
2.75	6	16	23	23	19	10	3	
	(2.5)	(12.8)	(27.1)	(30.6)	(19.4)	(6.6)	(0.9)	6
2.07	18	20	24	22	9	5	2	
	(7.9)	(25.0)	(32.9)	(23.1)	(9.1)	(1.9)	(0.2)	
1.83	19	27	23	17	11	3	0	
	(11.3)	(29.7)	(32.6)	(19.0)	(6.3)	(1.1)	(0.1)	
1.75	21	26	26	16	7	3	1	
	(12.6)	(31.2)	(32.1)	(17.6)	(5.4)	(0.9)	(0.1)	
1.66	12	32	38	15	2	1	0	4
	(14.3)	(32.9)	(31.4)	(16.0)	(4.6)	(0.7)	(0.1)	
1.09	38	30	19	11	2	0	0	1
	(30.0)	(40.0)	(22.2)	(8.6)	(1.1)	(0.1)	(0.0)	

[a] The average number of fibers per phage.
[b] Number n (0–6) fibers "out".

of n_3 or possibly of n_2 (Fig. 4b). In Fig. 4d, both adsorption (Fig. 4b) and infectivity (Fig. 4c) are plotted.

When comparing the data obtained with formaldehyde-fixed phages to those of fiber-complemented X4E, we have to bear in mind that, in the latter case, we are dealing with fibers free to oscillate throughout the experiment, while in the former case, the number of available fibers of a given phage has been definitely fixed.

From these observations, we conclude that more fibers are needed for infectivity than for adsorption, and thus partly confirm the elegant experiments of Crawford and Goldberg [44] performed with phenotypic mixtures of different species of tail fibers. In contrast to our results, their results did not reveal the need of as many as 5 fibers for productive infection. In agreement with results on proteolytic phage inactivation (see below), we believe that this discrepancy might be explained by variations in the physiological conditions of the experiments.

3.6. Phage inactivation by protease

The kinetics of protease inactivation of bacteriophage T4 [32] provides pertinent information relative to the minimal number of fibers required for infection, as well as to any model of fiber retraction. Granboulan [32] first observed that, at 60°C, the proteolysis affected tail fibers individually and not cooperatively, by removing the outer tip (70–80 amino acids) of the gp37 molecule situated at the distal tail fiber end. The curve of protease inactivation of T4 as a function of time, obtained in these experiments, was assimilated to a first-order kinetics, strongly arguing in favour of either the necessity of all (5 to 6) fibers for successful phage infection, in agreement with our results summarized in Fig. 4d, or the existence of a collective mechanism of the protease inactivation.

As reported above, the results of Crawford and Goldberg [44], obtained with phages complemented in vitro with phenotypically mixed fibers, showed

Table 7
Dual, bidisperse ultracentrifugation under different conditions

Phage	Supplements to the cell lysis medium [a]			Age of the stock in days	Slow peak	Fast peak
		pH	Phage titer [b]			
T4D	500 mM NaCl	7	10^{12}	0	+	+ +
				2	+	+
				4	+	–
T2L	500 mM NaCl	7	10^{13}	0	+	+
		6	10^{13}	0	+	+ +
				4	+ +	+
				7	+	–
		5	10^{13}	0	+	+ +
T6	500 mM NaCl	6	10^{13}	1	–	+
				3	+	+
				6	+ +	–
T4D	80 mM NaCl	7	10^{11}	1	+	+
		7	10^{10}	1	+	+
T4D	500 mM potassium glutamate	6.5	10^{12}	0	–	+
				3	–	+
				7	–	+
T4D	200 mM potassium glutamate	7		1	–	+
				7	+ +	–
T4D	80 mM potassium glutamate	6.5	10^{12}	0	–	+
				6	+	–

[a] Cells were lysed in phosphate buffer supplemented with either NaCl or potassium glutamate at the indicated concentrations.
[b] Only the order of magnitude of the phage titer was given. The efficiency of plating of fresh, concentrated lysates are generally low. Upon storage several stocks exhibited a titer increase by a factor of 2–3.

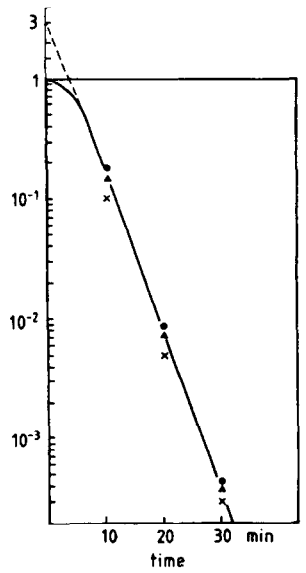

Fig. 6. Effect of the trypsin treatment on T4 adsorption and plaque-forming ability. A T4 stock was incubated with trypsin (0.01%) at 60°C. The adsorption was determined with ^{14}C-labelled phage as described in the text. Plaque-forming units were determined either by direct plating (pouring of 2 ml of soft agar with indicator bacteria) or by preadsorption (see Material and Methods). Both methods give identical results when expressed as relative survival. Symbols (▲, ●, ×) correspond each to a different experiment.

that with less than 5 a successful infection was possible. To resolve this inconsistency we repeated protease inactivation experiments shortly after Granboulan had left our laboratory in 1982 and apparently confirmed the one-hit response. Several years later, with the same operators, we obtained a three-hit inactivation (Fig. 6), this time in agreement with the results of Crawford and Goldberg [44]. When carefully inspecting the data of Granboulan we found that Fig. 1 of reference [32] is actually a multihit curve indistinguishable from that presented here (Fig. 6). This inconsistency over time suggests a variability in the experimental conditions. Despite intensive efforts we could not determine the relevant parameter(s).

3.7. The bidisperse dual sedimentation

To better define the conditions under which a given bacteriophage T-even stock splits into two distinct sedimentation peaks, we have resorted to moving boundary analytical ultracentrifugation. Inspection of the available literature reveals that dual sedimentation occurred with high titre phage stocks. Therefore, the starting material for our experiments were native phage stocks at a concentration of 10^{12} to 10^{13}/ml [45]. To simulate as closely as possible the intracellular conditions, we used high ionic concentrations, first of NaCl and later, when it became known that intracellularly the anionic partner of potassium is glutamate rather than chlorine [46], we used potassium glutamate.

When "pregnant" cells were lysed in 0.5 M NaCl and centrifuged (moving boundary, Schlieren) immediately, fast phages appeared either alone or, most frequently, together with a slow peak. Neither

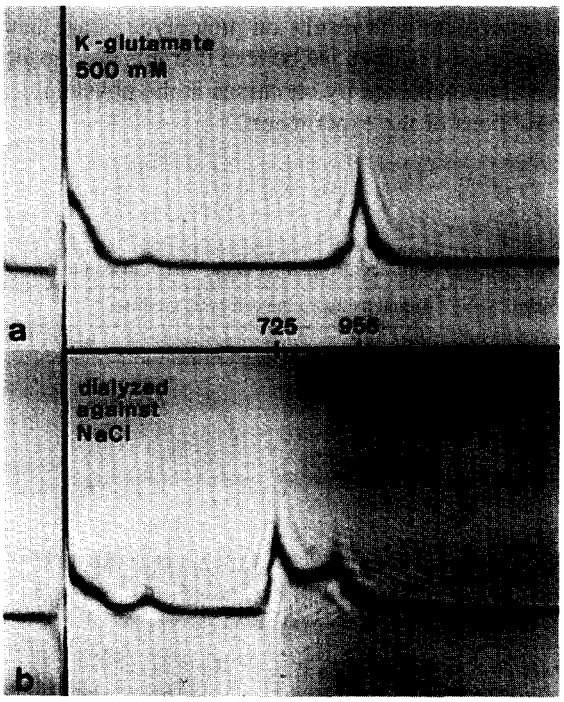

Fig. 7. Bidisperse, dual sedimentation of bacteriophage T4D. Bacteriophage T4D infected bacteria were pelleted and lysed into 500 mM potassium glutamate. The stock was divided into two parts. One was immediately analysed by analytical moving boundary centrifugation (a), while the other was dialysed against 500 mM sodium chloride, for 24 h, before analytical ultracentrifugation (b). The experimental conditions correspond to those of Table 7 and are described in the text. Inspection by Schlieren optics reveals that nascent phages sediment with a single fast peak (a), and that one day dialysis against 500 mM NaCl generates an additional, slow, peak (b).

the choice of the pH – 5, 6 or 7 – nor the replacement of sodium by potassium (data not shown) had a significant, reproducible, influence (Table 7). Within one or a few days at 4°C, the fast peak disappeared progressively in favour of the slow one. Cell lysis in 80 mM NaCl frequently yielded two peaks. However, after one day only at 4°C, all phages appeared in the slow peak. The distribution of phages between the two peaks was apparently dependent on phage concentration; the higher the concentration, the more stable was the fast peak. Supplementing the sedimentation medium with 10% of sucrose also lead to some stabilisation of the fast peak.

The highest stability of the native, fast form was achieved when lysis occurred in potassium glutamate (0.5 M). Dialysing this phage suspension against the same concentration of sodium chloride rapidly generated the slow peak (Fig. 7). In the presence of glutamate, the pH had no significant influence. At the most, low pH delayed the appearance of the slow form. This is in variance with the predominant role

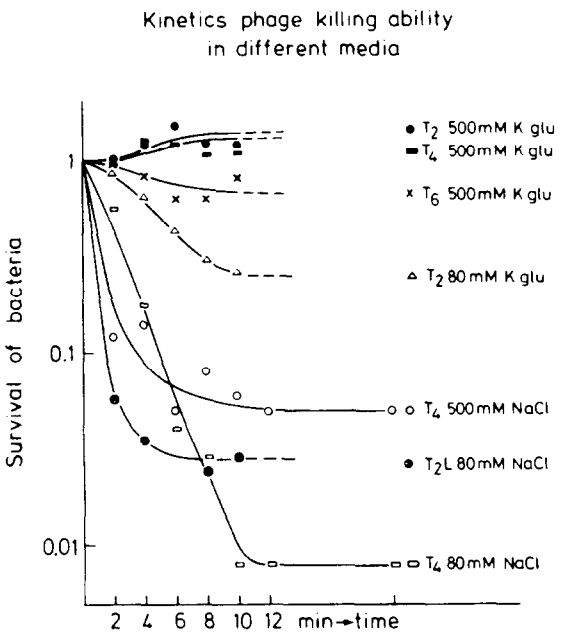

Fig. 8. Kinetics of phage killing ability in different media. The proportion of phages with extended fibers in lysates containing both fast and slow sedimenting phages was monitored by their ability to kill *E. coli* B cells. Bacterial survival was plotted as a function of time. Suspension media, indicated for each curve, are those used in Table 7.

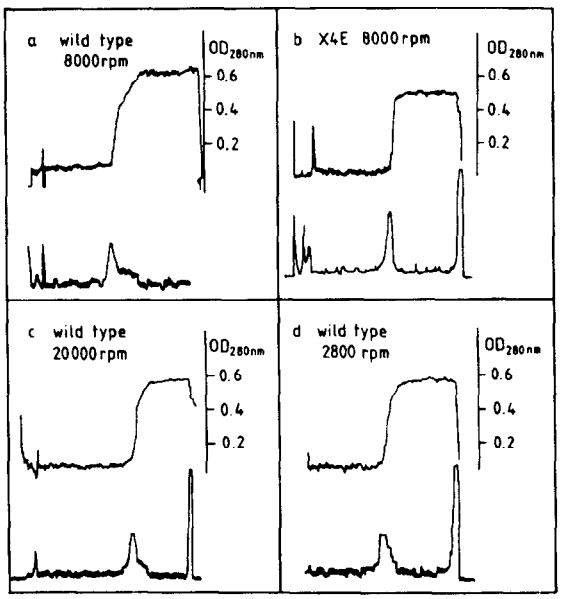

Fig. 9. Comparison of the sedimentation patterns of T4D, the wild type phage, and X4E, the fiberless mutant. Unfixed phage lysates were examined by moving-boundary analytical centrifugation. Absorption at 280 nm was recorded (upper curves). With a relevant accessory (see Material and Methods), rough first-order derivatives of these curves were plotted (lower curves). Curves presented in (a) and, to a lesser extent in (c), reveal a bidisperse sedimentation, expressed as two distinct slopes and a clear shoulder in the upper and the lower curves, respectively. Under the same conditions, the fiberless mutant X4E (b) sediments as one sharp peak. For discussion of this phenomenon, see also Fig. 6 and text.

of pH under so-called physiological conditions, where lowering the pH reduces the sedimentation velocity (see above). At lower concentrations of glutamate (80 mM), the fast peak was appreciably less stable.

Our study did not reveal any significant difference between phages T2, T6 and T4. T4B, which requires tryptophan for adsorption (fibers "out"), behaved like the other phages independently of the presence or the absence of tryptophan as well as glutamate.

In an attempt to correlate the sedimentation properties with the tail fiber configuration, we have tested the biological activity, i.e. adsorption and plaque-forming ability of native phage stocks under different conditions. Direct electron microscopic observation of slow and fast sedimenting peaks was precluded by the high glutamate concentration which is known to strongly interfere with formaldehyde fixation. There-

fore, while maintaining salt concentrations and pH, phage suspensions were mixed with growing *E. coli* cells at a multiplicity of infection of about five and the kinetics of bacterial survival was determined (Fig. 8). In each case, a plateau of surviving bacteria was reached after some 10 min and it appeared clearly that the more abundant the fast peak (see above), the higher was the proportion of non-killing bacteriophages, suggesting that passage from the native, fast form to the slow one corresponded, from a biologically point of view, to a transition from inactive to active phage.

Finally, in agreement with previous reports [14,19], sedimentation of more dilute T4 phage suspensions, followed by absorption optics, has never led to two distinct peaks (Fig. 9). Isokinetic sucrose gradient band centrifugation [41] proved neither to reveal two peaks; at the best small shoulders were observed (Fig. 3a and c). However, when fast or slow sedimenting phages, generated by incubation at high and low pH, respectively, were fixed by formaldehyde and mixed, they sedimented as separate peaks on such gradients (see Fig. 3).

4. Discussion

Experiments on T-even bacteriophages reported above have revealed that dual, bidisperse sedimentation occurs at phage concentration of 10^{12} or more particles per ml, confirming a report of Welch III and Bloomfield [45]. When phages of an infected cell are released directly into 500 mM glutamate only the fast peak is observed. Upon storage in the cold the slow form gradually appeared. When lysis occurred in 500 mM of potassium or sodium chloride both peaks were present; storage led to a steady shift from the fast into the slow form. In many experiments with the analytical centrifuge at somewhat lower phage concentrations, at pH 5.8 a peak with a shoulder was observed (Fig. 9) which, however, did not appear with the fiberless mutant. (To our knowledge this is the only physical evidence for fibers to be involved at least in this sort of dispersity.)

Sedimentation experiments with lower phage concentrations, monitored by UV absorption, show exclusively unimodal distributions. Depending on the suspension media, particularly the pH, a variety of (monodisperse) sedimentation constants is observed. Interestingly, their values were between those characteristic of the dual, bidisperse sedimentation, a reason for which all these phenomena were indistinctly associated with dual sedimentations. At the time of its discovery, the real bidispersity had given rise to fruitful working hypotheses which, subsequently, were thoroughly investigated but at lower phage concentrations only. Our data leave little doubt that we are likely to have two unrelated phenomena, at least two different mechanisms, should the hypothesis of the involvement of fibers be demonstrated.

It is well established, both in vivo and in vitro, that fibers assemble only onto head–tail complexes but not onto free tails [3]. This phenomenon is corroborated by the finding of fiber binding sites on tail sheath, whiskers and the head [47,48]. It is therefore most likely that native phages have the fibers "up", firmly bound to the tail and the head [24,25]. Our finding that lysis into glutamate leads to the fast form only is relevant too. Indeed, it is known that intracellularly K^+ is not balanced by Cl^- but rather by organic compounds [46], suggesting that the fast form is representative of intracellular nascent phage. Presently, there is not enough experimental data to associate the slow, apparently biologically active form with the extended form of the fibers. Other phenomena able to influence the sedimentation velocity, such as phage capable of non-random particle aggregation, as observed with phage P1, a phage endowed with a contractile tail sheath and fibers [49], cannot be ruled out.

Our experiments, performed with phages at low concentrations (about 10^{10}), have revealed that the variations of the (monodisperse) sedimentation constants were well correlated with the average number of extended fibers. The above figures agree well with our confirmative estimates that 2–3 fibers are required for adsorption. However, the actual number of fibers required for productive infection appears to depend strongly on the experimental conditions, although we did not succeed in determining the relevant parameter(s). Under some conditions already 3 fibers suffice for a low probability of infection [44], as we have confirmed for phages with proteolytically inactivated fibers. In other cases 5–6 are needed as shown by our experiments.

Utilisation of formaldehyde-fixed phages with frozen-tail fiber configuration has allowed to study the consequences of a reduced average number of fibers "out" by two different approaches.

The electron microscopy of the formaldehyde-fixed phages revealed that fibers "out" obey a binomial distribution, which is incompatible with the sensu stricto collective model. The pH dependence of fiber extension confirms previous results obtained in combination with the tryptophan dependence of the fiber deployment [14,33]. Indeed, at saturating tryptophan concentration, T4B was found to behave towards the pH like the wild type. However, at that time, electron microscopic counts were interpreted cautiously, because an artefactual alteration of the number of observed extended fibers could not be ruled out. Now, from our experiments, we have learned that with unfixed phages such artefacts go in the direction of an increased number of fibers "out". With formaldehyde fixation the native situation is maintained, while other agents, such as glutaraldehyde, increase the number of fibers "up".

Although the sensu stricto collective model appears incompatible with our results, we cannot explain all available data with the straightforward individualistic model, in which the fibers oscillate independently of each other between the extreme "up" and "out" positions. In particular, isokinetic gradient centrifugation leads to a paradox, i.e. formaldehyde-fixed phages at pH 7, with their broad binomial distribution, sediment with the same sharp peak as do unfixed phages. The straightforward individualistic model would predict a much broader peak for fixed phages. This observation could be accounted for by a new, third, model of fiber deployment based on the hypothesis of different conformational states of the hinge part of the base-plate. The media-dependent conformation would define the given angle around which the fiber can oscillate. Instead of a rotatory articulation (of the mechanical type), which would allow movements of some 130° postulated by the old models, we will now envisage a hinge resting on a flexible polypeptide chain such as observed with other proteins like immunoglobulins. Thus the fiber attachment site on the base-plate, although relatively rigid, is supposed to change the angle that it forms with the axes of the tail. Individual fibers could oscillate freely around this medium position, but only within restricted angular amplitudes, of about 50°–80° rather than the 130°–150° as required for the two classical models. The new model is in agreement with our electron microscopic data obtained under the following pre-set conditions. The positions "up" and "out" were defined as being situated above or below the base-plate, respectively. Indeed, very rarely have we observed fibers that were completely "up", as they would appear, or rather disappear, when wound around the tail to form the jacket configuration. The "up" fibers were rather irregularly arranged in the space above the plane. With the new model the contribution of each of the individually oscillating fibers of a particle to the form factor would thus no longer be between zero and a maximum, but reduced to much smaller variations due to a reduced angular amplitude around a medium position. This model would also explain why at pH's around 7, with the broadest binomial distribution, the values of the sedimentation constants are the least reproducible (see Fig. 2). It also fits with the fact that with extreme pH's all "up" or all "out" configurations were never achieved.

In 1983, a possible role of the base-plate conformation in sedimentation was considered by Kellenberger and Blok (unpublished work). The transition between two extreme states, the hexagonal and the star or hexagram [50], has been described [14,51] and subsequently thoroughly investigated regarding the fine structure [52], and the functions related to the triggering of tail-sheath contraction and its relation to tail fibers [50,53,54]. So far, conformations intermediary between the two extreme base-plate states were not observed (for a recent review see [55]), which is not that surprising, since only free base-plates, detached from the tail, could be used in high-resolution structure analysis.

We conclude that the bidisperse, dual sedimentation that occurs with high phage concentrations cannot be explained by straightforward extrapolating results obtained with lower phage concentrations. The latter are widely used, for instance, in experiments on phage adsorption, infection, multiplication and morphogenesis. They are performed under so-called physiological conditions, i.e. in about 0.1 M saline phosphate buffer suitable for pH adjustments. Because of the limited solubility of phosphates of divalent cations, bidispersity experiments at high

calcium concentrations were not performed, leaving unconfirmed the early claims about their strong promoting influence [12].

Acknowledgements

One of us (E.K.) had advised Philip Granboulan, for genetically identifying his temperature-resistant T4 mutant that finally became located in gene 37. The mutation conveys resistance to individual fiber proteolysis [32]. Without these experiments, for which we are grateful to P.G., E.K., who at that time was involved in quite different problems of form determination of the phage head, would never have reconsidered experimentally the tail-fiber problem.

Many thanks go also to Hans Noll, who has instructed us in the use of his isokinetic sucrose gradient centrifugation [41]; we thank also Theo Staehelin, who had made the same effort already 16 years earlier, at a time when our laboratory had planned experiments with this technique but could not find interested collaborators! Genetic experiments are so much easier!

We are most indebted also to Edward Goldberg who has kindly corrected the English in a previous version of this paper and who made very helpful comments.

Without subsidies of the Swiss National Science Foundation and the support of the Basel University by the Canton Basel Stadt the experiments reported in this paper would never have been possible.

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