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Purification of Single- and Double-Length M13 Virions by Polyacrylamide Gel Electrophoresis

JACQUES BEAUDOIN[1], TIMOTHY J. HENRY[2], AND DAVID PRATT[3]

Department of Bacteriology, University of Wisconsin, Madison, Wisconsin 53706, and Department of Biophysics and Microbiology, University of Pittsburgh, Pittsburgh, Pennsylvania 15213

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Complete separation of single- and double-length M13 virions from each other and from still-longer phage particles was achieved by electrophoresis of phage stocks through 2.5% polyacrylamide gels.

Multiploid virus particles, i.e., virions containing more than one complete genome, have been reported for many animal and some insect viruses (18). Multiploids are much less common among bacterial viruses, having been found so far only among the filamentous, male-specific coliphages (Ff phages). In Ff phage stocks, multiploid virions were detected as extra-length particles in electron micrographs (4, 5, 10) and also as a few percent of heterozygous phages (hets) among the progeny of genetically mixed infections (G. W. Notani and N. D. Zinder, Bacteriol. Proc., p. 140, 1964; 17).

Salivar, Henry, and Pratt (15) partially purified double-length M13 virions by ion-exchange chromatography and studied some of their biological and physical properties. The double-length virions were called diploids as they contain two individual, circular, single-stranded DNA molecules. It was also shown that among the progeny phages from an infection with two M13 amber mutants, half of the diploid population was heterozygous, whereas the other half was homozygous.

Heterozygotes involving conditional lethal mutant phages constitute an obstacle to genetic mapping of Ff phages, as the hets give rise to plaques when plated under nonpermissive conditions. Such het plaques are in most instances indistinguishable from and much more numerous than the wild-type recombinant plaques. Moreover, Salivar et al. (15) noted that the proportion of hets from mixed infections was greatly increased when certain amber mutants, especially in genes 3 and 6, were involved. One application of a separation of haploid from multiploid virions would be to facilitate estimation of recombination frequencies between Ff phage mutants. Thorough structural and biochemical studies could also be performed on purified diploids. In a companion paper (2), we describe antiserum inactivation of M13 diploid virions.

The presence of slow-migrating phages after electrophoresis of M13 virions through dilute polyacrylamide gels suggested that this technique could be used to separate virions according to their lengths (13). We will in this paper demonstrate that polyacrylamide gel electrophoresis in 2.5% gels is a reliable technique for achieving complete purification of haploid and diploid M13 virions.

MATERIALS AND METHODS

Strains, media, and procedures. The bacteriophages, bacterial host strains, and standard media and procedures for M13 have been described previously (13-16). High-speed centrifugation was performed in Spinco preparative ultra-centrifuge models L and L2 (Beckman Instruments, Inc.).

Chemicals. The following were purchased from Eastman Kodak Co.: acrylamide; *N,N'*-methylenebisacrylamide (bisacrylamide); *N,N,N',N'*-tetramethylethylenediamine (TEMED); glycine; 2,3,5-triphenyl-2H-tetrazolium chloride (TTC); Photo-Flo 200. Ammonium persulfate and sucrose (reagent grade) were purchased from J. T. Baker Chemical Company; Tris(hydroxymethyl)aminomethane (Tris) was purchased from Sigma Chemical Co. 2,5-Diphenyloxazole (PPO) and 1,4-bis-2-(4-methyl-5-phenylazolyl)-benzene (dimethyl POPOP) were purchased from Packard Instrument Co., Inc. Dioxane was purchased from North Central Chemicals, Inc., naphthalene from Mallinckrodt Chemical Works and from Allied Chemical Corporation, and cesium chloride (CsCl) from American Potash & Chemical Company.

Preparation of heterozygote-containing bacteriophage stocks. Mixed infections (crosses) known to yield high proportions of heterozygous diploids were

[1] Present address: Département de Microbiologie et d'Immunologie, Université de Montréal, Case postale 6128, Montréal Qué, Canada H3C 3J7.

[2] Present address: Department of Biochemical Sciences, Princeton University, Princeton, N. J. 08540.

[3] Present address: Department of Bacteriology, University of California, Davis, Calif. 95616.

carried out on *Escherichia coli* K38 (su^-), as described by Salivar et al. (15). Cells mixedly infected with M13 amber mutants *am*2-H2 and *am*3-H5 were incubated at 30 C for 60 min, whereas those infected with *am*5-H3 and *am*6-H1 were incubated for 17 h. Bacteria and large debris were pelleted by low-speed centrifugation. The progeny stocks were kept in the cold and assayed at intervals on both K37 ($su\ 1^+$) and K38 bacteria. These stocks were used for polyacrylamide gel electrophoresis (see below) without further purification.

Analytical electrophoresis of M13 virions. Gels were 12 cm long, 6 mm in diameter, and contained: 2.5% acrylamide, 0.125% bisacrylamide, 0.07% TEMED, and 0.05% ammonium persulfate. The buffer for both gels and reservoirs was 0.37 M Tris adjusted to pH 9.45 to 9.50 with glycine (6). Electrophoresis was in a Polyanalyst apparatus (Buchler Instruments Div., Nuclear-Chicago Corp.), with the lower reservoir cooled with tap water at approximately 12 C. The gels were always pre-subjected to electrophoresis in order to eliminate residual harmful substances to the virions, such as the ammonium persulfate. It was determined experimentally that up to 4×10^{10} PFU per gel could be subjected to electrophoresis with reproducible results. The sample volume was 0.01 or 0.02 ml per gel and was made 10% in sucrose by dissolving sucrose crystals in the phage suspension. Usually, a control gel was layered only with bromophenol blue in 10% sucrose. Because M13 virions migrate slowly, the dye had completely traversed the control gel and had gone into the bottom reservoir buffer before the run was stopped. After electrophoresis, the gel was extruded from the tube by gently squirting water between the gel and the inside wall of the tube as described by Maizel (9), and carefully laid on a piece of Parafilm (American Can Company), on which the gel does not stick and from which it can be picked up easily for loading into the gel fractionator.

Gel fractionation and biological assays. The gels were fractionated in a model AGD-30 Autogeldivider (Savant Instruments, Inc.), after careful insertion into the metal sleeve of the instrument. As suggested by Maizel (9), for fractionating low-percent gels in this apparatus, no metal screen was used in the acrylic extrusion block and the needle-valve was set to give the smallest opening possible. The gels were made deliberately longer than needed for electrophoresis (i.e., 12 cm) and were crushed top end first; this insured that all parts of the gels through which virions had migrated were crushed.

The crushed gel particles were automatically collected with a model UFC-100 Unifrac fraction collector (Savant Instruments, Inc.) into sterile test tubes containing 10 ml of elution buffer (0.05 M Tris chloride, pH 8.9). The phages were allowed to elute from the gel particles overnight, without shaking and at room temperature. After thorough mixing on a Vortex mixer, the larger gel particles were allowed to settle during 10 to 15 min. Samples from the supernatants were either plated directly for spot tests, or further diluted in elution buffer before plating.

Assays for plaque formers were most conveniently performed by plating on K37 and incubating overnight at 33 C. Eosin methylene blue (EMB)-glycerin nutrient agar plates with top nutrient agar were normally used, as M13 plaques then are large and easily visualized (1, 3). When regular nutrient agar plates were used, the plates were incubated overnight and then flooded with 10 ml of a fresh 0.1% aqueous solution of TTC for about 30 min at room temperature (12). Recovery of plaque-forming phages after electrophoresis was on the order of 50 to 70% of the input PFU.

Preparative electrophoresis. Eleven identical gels (12 cm by 6 mm) were simultaneously subjected to electrophoresis after the procedure described above. Each gel was layered with 4×10^{10} PFU. The gels were then fractionated one after the other, and the fractions were collected sequentially into the same test tubes in order to pool corresponding fractions.

For locating rapidly the various phage peaks, a control gel with [^{3}H]thymidine-labeled *am*2-H2 virions was subjected to electrophoresis. The 0.01-ml sample contained a total of 3.2×10^9 PFU and 3.8×10^3 counts/min. This gel was fractionated separately from the others and counted for radioactivity. Fractions from the pooled gels corresponding to peaks in the control gel were assayed for plaque formers on K37. After a total of 5 days of elution at room temperature, four fractions within each peak were pooled for subsequent concentration.

To eliminate gel particles before concentrating the virions, 50 ml of each phage peak was layered over 10 ml of 32% (wt/wt) CsCl solution in cellulose nitrate tubes. Centrifugation was done in a SW 25.2 rotor at 22,500 RPM for 3 h at room temperature. The gel particles neatly concentrated in the dense CsCl solution. The supernatants were transferred into polyallomer tubes and spun for 24 h at 22,500 rpm in the SW 25.2 rotor to pellet the virions. The pellets were finally resuspended in a minimal volume of distilled water and assayed on K37 for plaque formers.

Electron microscopy and particle-length measurement. Samples for electron microscopy were prepared by the agar filtration technique (7) by using 0.005- to 0.01-ml drops of phage suspension containing approximately 5×10^9 PFU/ml (15). The grids were shadowed with platinum at a 25° angle, then turned 180° and shadowed again.

Observations were made on a Phillips-300 electron microscope at low magnifications, usually ×6,840 or 8,094, and pictures were taken on Kodak electron image glass plates. The negative plates were then directly projected onto a flat surface with a lantern projector. The phage particles were traced out on paper, and their lengths were determined with a map measure and recorded.

RESULTS

Separation of hets and haploids. To demonstrate that diploids could be separated from haploids by polyacrylamide gel electrophoresis, as previously suggested by Pratt et al. (13), hets were used as a convenient biological marker for diploids. To obtain hets that could be distinguished by their plaque type when plated on su^- indicator bacteria (15), a mixed infection (or

cross) was performed as described in Materials and Methods. One of the parental phages was an amber mutant in gene 2, which forms turbid plaques, whereas the other phage was a gene 3 amber mutant, which makes clear plaques. This same cross had been shown to yield 3% hets after 17 h of incubation (15). Here, after 60 min of incubation, the progeny stock contained only about 0.6% hets and also 0.03% wild-type plaque formers. Most phages in this stock, therefore, were haploids.

Electrophoresis of the progeny of this cross was performed as described in Materials and Methods. Figure 1 shows the electrophoretic separation of haploids and hets. The titers of each fraction, in PFU/ml, are plotted on a logarithmic scale so that minor peaks can easily be seen. In Fig. 1A, the phages were assayed on the permissive host K37. Of the three peaks that can be seen, the fastest-migrating peak is well separated from the other two and contains most of the phage, tentatively the haploids. An intermediate peak migrated at only a third of the rate for haploids. Finally, the peak at the top of the gel consists of viable particles which have probably not penetrated the gel.

In Fig. 1B, the phages were assayed on the nonpermissive host K38 on which only revertants, wild-type recombinants, and hets can make plaques, the latter by the process of continuous complementation (15, 17). Here again, three peaks can be seen that have the same electrophoretic mobilities as those in part A, although the titers here are much lower. Most hets appear in the two slowest peaks, whereas most of the wild-type plaque formers are found in the fastest peak.

Because hets are necessarily polymeric virions, it can be concluded that single-length virions are indeed in the fastest-migrating peak. Moreover, it is obvious that they are well separated from the longer virions. Het-type plaques in the haploid peak (Fig. 1B) could be explained by mixed infections occurring directly on the su^- (K38) assay plates.

The exact nature of the intermediate peak could not be determined solely by biological experiments. However, preliminary results of serum-inactivation experiments of virions showed that hets in the intermediate peak, assayed on K38, were twice as sensitive as haploid virions in the fastest peak, assayed on K37, as Scott and Zinder (17) had already reported. This strongly suggested that phages in the intermediate peak were double length. Virions in the slowest peak were tentatively classified as multiple length.

Total phage recovery was estimated by plating on K37 and was on the order of 50% of the input PFU. The proportion of wild-type plaque formers after electrophoresis was 0.03%, which is identical to the input ratio. Recovery of hets, as estimated by platings on K38, was only 27%, which would explain the low proportion (0.3%) of the hets after electrophoresis, as compared with the input proportion of 0.6%. This lower recovery of hets could be explained by poorer elution of polymeric virions from the gel particles, due to their sizes, or by the preferential inactivation of hets by the process of gel electrophoresis, or possibly both. Recovery of phages from gels will be discussed later.

Preparative gel electrophoresis. Electron microscopy followed by particle-length measurements was thought to be the most direct approach to establish the physical nature of the virions in each of the three peaks obtained by gel electrophoresis. Because a titer of approximately 5×10^9 PFU/ml is required for preparation of samples by the agar filtration technique (15), a larger scale polyacrylamide gel electrophoresis had to be considered.

In order to obtain high enough titers in each peak, a 17-h mixed infection of K38 cells was carried out with the two amber mutants *am5*-H3 and *am*6-H1, as described in Materials and Methods. This cross yielded 11% hets, in good agreement with the value of 12.5% already reported (15).

Here, all the plaque formers on K38 indicator bacteria were considered as hets, because they give rise to clear plaques which cannot be distinguished from revertant and wild-type recombinant plaque formers.

Electrophoresis was carried out in 12 gels simultaneously, as described in Materials and Methods. Eleven were each loaded with 4×10^{10} PFU of the above mentioned phage stock. The 12th gel was included as a control gel in which [^{3}H]thymidine-labeled *am*2-H2 virions were subjected to electrophoresis. The gel fractionation procedure is described in Materials and Methods.

As illustrated in Fig. 2, three well-separated radioactive peaks were detected in the control gel, with relative mobilities identical to those observed previously with viable phages (Fig. 1). From the position of these peaks in the control gel, the appropriate fractions from the eleven pooled gels were assayed, and unwanted gel particles were eliminated, as described in Materials and Methods.

After concentration, the titer in the diploid peak was 5×10^9 PFU/ml, with 16% hets, whereas the slowest peak contained only 1×10^9 PFU/ml, with 26% hets. This higher proportion

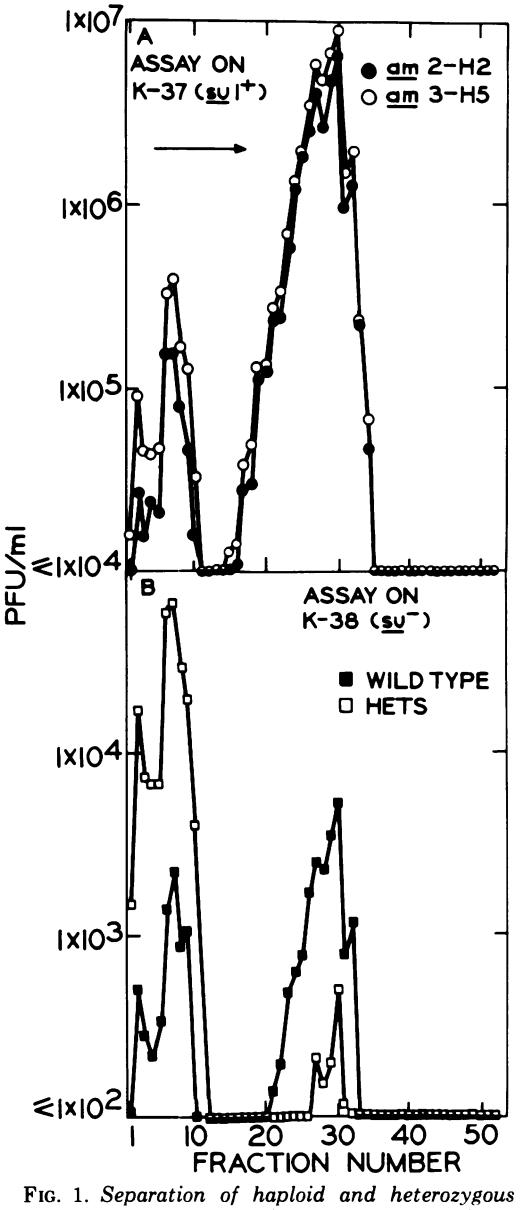

FIG. 1. *Separation of haploid and heterozygous M13 virions by polyacrylamide gel electrophoresis. E. coli K38 was mixedly infected with M13 amber mutants am2-H2 and am3-H5, and incubated for 1 h at 30 C, following the procedure described in Materials and Methods. Electrophoresis of the progeny stock of this cross was performed as described in Materials and Methods, and involved a 0.02-ml sample containing a total of 1.4 × 10⁹ PFU. Recovery was on the order of 50%, as estimated by the K37 platings. Pre-electrophoresis and electrophoresis were as follows: 5.3 V/cm of gel for 15 min, then 21.7 V/cm of gel for 155 min, with a final current of 3.75 mA/gel. Migration was toward the anode (bottom of gel) and is indicated by the arrow. The gel was fractionated top end first, as described in Materials and Methods. Assays for plaque formers were as indicated in parts A and B of this figure.*

of hets in the slowest peak strongly suggested that it contained viable heterozygous particles longer than diploids.

Re-electrophoresis of virions. As a control before proceeding to electron microscopy, the identity and homogeneity of the M13 virions subjected to electrophoresis were biologically ascertained after re-electrophoresis of virions from each of the three peaks from the preparative run. All fractions from the three gels were assayed on K37 for plaque formers. Figure 3 shows the results of this experiment. From the electropherograms, it is obvious that the three peaks have migrated at their expected rates. Moreover, the haploid peak (Fig. 3A) and the diploid peak (Fig. 3B) are essentially electrophoretically pure. On the other hand, the slowest peak (Fig. 3C) is heterogeneous and contains a few haploids, a larger proportion of diploids, and mostly phage particles which remained at the top of the gel. As will be explained below, the slowest peak was called "triploid-rich fraction."

Interestingly, as shown in Table 1, recovery of PFUs after re-electrophoresis is quite markedly different for each of the three gels and seems to be related to electrophoretic mobility. However, the proportion of plaque formers on K38 after re-electrophoresis is much lower than after the initial preparative gel electrophoresis. These results will be discussed later.

Electron microscopy and particle-length measurements. Samples for electron microscopy were prepared by the agar-filtration technique (7) which requires the relatively low concentration of about 5 × 10⁹ PFU/ml for M13 (15). This technique has the advantage of not being selective, because all phage particles are retained on the collodion membrane. Samples were prepared and examined as described in Materials and Methods.

Aggregates involving very many virions were usually numerous in all preparations, and measurement of individual particles was, therefore, often difficult to make. Only virions that could be easily and entirely followed were measured, and their lengths were recorded. Others were left out. Unfortunately, this unavoidable selection of viral particles could bias the results somewhat, because longer particles would probably be left out preferentially. It is now known (J. Beaudoin and A. Niveleau, unpublished data) that formaldehyde prevents clumping of M13 virions.

A control electron micrograph of M13 virions

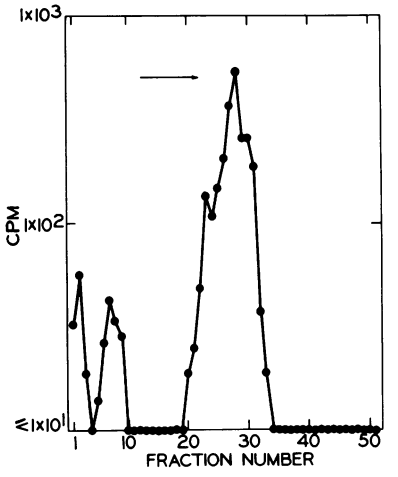

FIG. 2. *Polyacrylamide gel electrophoresis of [³H]thymidine-labeled M13 am2-H2 virions. The 0.01-ml sample of [³H]thymidine-labeled am2-H2 virions contained a total of 3.2 × 10⁹ PFU and 3.8 × 10³ counts/min. Electrophoresis was performed as described in Materials and Methods. Migration was toward the anode (bottom of gel) and is indicated by the arrow. This gel was fractionated as described in Materials and Methods, and counted for radioactivity in a scintillation counter. Recovery was in the order of 70% of the input counts per minute.*

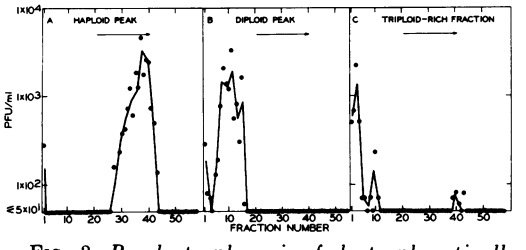

FIG. 3. *Re-electrophoresis of electrophoretically purified M13 virions. The procedure is described in Materials and Methods. The 0.01-ml samples contained a total of 8.2 × 10⁵ PFU in A; 1.2 × 10⁶ PFU in B; and 7.3 × 10⁵ PFU in C. Migration was toward the anode (bottom of gels) as indicated by the arrows. Gel fractionation and assays for plaque formers on K37 were as described in Materials and Methods. Due to malfunctioning of the Autogeldivider, the fractions were very irregular, as can be seen from the scatter of points (the actual experimental values). Therefore, in each of the three parts of this figure, the titers in two consecutive fractions were averaged and the curves were drawn through the averaged values.*

before electrophoresis is shown in Fig. 4, top. Virions of different lengths can be seen, with more or less clumping and overlapping. Diploid virions purified by gel electrophoresis are shown in Fig. 4, bottom. Surprisingly, there were no aggregates in this particular preparation, and the virions were mostly parallel on the grid so that all the particles could be measured easily. Other samples examined after electrophoresis usually showed as much clumping as seen in the control (Fig. 4, top).

Figure 5 illustrates the frequency distribution of the length of the phage particles in the two slowest peaks, as well as in the control unelectrophoresed progeny stock. The procedure followed for particle-length measurements is described in Materials and Methods. As expected, the unelectrophoresed control (Fig. 5A) was heterogeneous, and of the 220 measured particles, 21% were longer than haploids. Because this same progeny stock contained 11% hets, mainly diploids, this agrees quite well with the idea (15) that hets make up 50% of the diploid population. This also suggests that the presence of aggregates, at least in this case, did not dramatically bias the results. In this unelectrophoresed control, the average haploid particle measured about 750 nm. The diploid peak (Fig. 5B) was essentially pure, more than 90% of the 108 measured particles had a length averaging 1,620 nm. This homogeneity corresponds with that observed upon re-electrophoresis of the diploids (see Fig. 3B). Finally, 163 particles were measured in the slowest peak (Fig. 5C). Of these, 45% were triple length, measuring 2,430 nm on the average. There were also 36% shorter particles and 19% longer particles. This slowest

TABLE 1. *Recovery of viable virions and proportion of heterozygotes in the phage peaks after gel electrophoresis*[a]

Phage	Preparative electrophoresis[b]: proportion of hets[c] (%)	Re-electrophoresis	
		Total recovery[d] (%)	Proportion of hets[c] (%)
Haploid peak	0.086[e]	52	
Diploid peak	15.9	24	3.2
Triploid-rich fraction	26.5	16	6.7

[a] The original progeny stock of the cross between mutants *am*5-H3 and *am*6-H1 contained 11% hets. Techniques for preparative gel electrophoresis and for re-electrophoresis of virions are described in Materials and Methods.

[b] Overall recovery of virions after preparative gel electrophoresis was on the order of 50% of the input PFU.

[c] Ratio of plaque formers on K38 over plaque formers on K37.

[d] Ratio of total plaque formers recovered after electrophoresis over the total input PFU.

[e] These are presumably not actually hets but mostly revertants and a few wild-type recombinants.

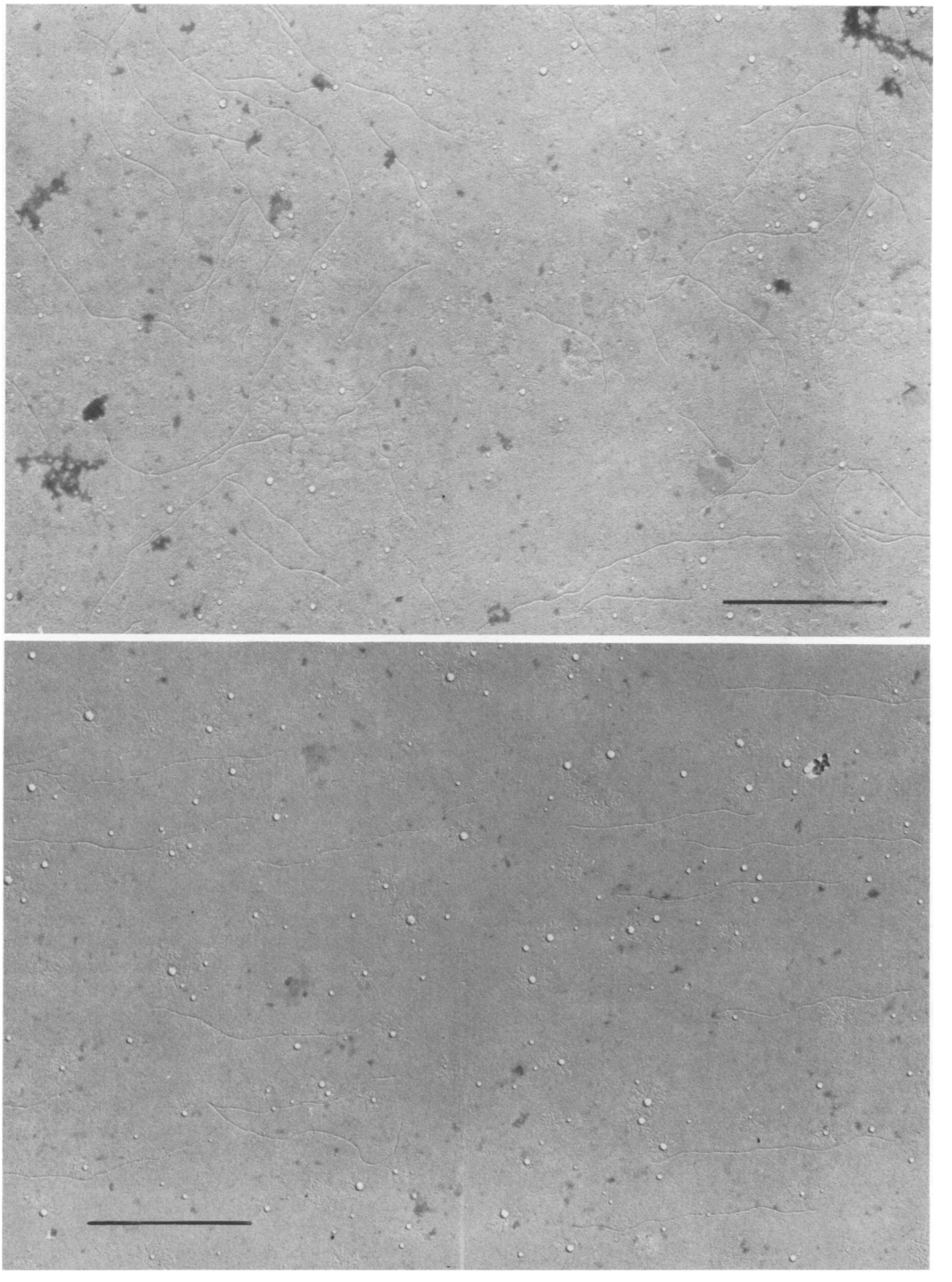

FIG. 4. *M13 virions from the progeny stock of the cross between amber mutants am5-H3 and am6-H1: top, unelectrophoresed virions; bottom, electrophoretically purified diploid virions. The mixed infection, preparative polyacrylamide gel electrophoresis, and electron microscopy were performed as described in Materials and Methods. The reference bars denote 1,000 nm.*

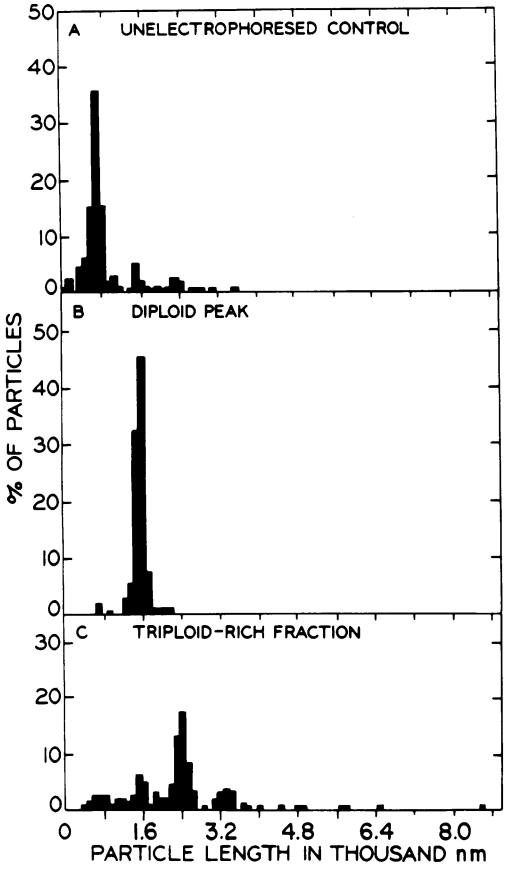

FIG. 5. *Frequency distribution of electrophoretically purified M13 virions according to their lengths. The progeny stock of the cross between amber mutants am5-H3 and am6-H1 was subjected to electrophoresis in 2.5% polyacrylamide gels. Virions were then examined by electron microscopy, and their lengths were measured. The procedures were as described in Materials and Methods.*

peak is therefore called "triploid-rich fraction." Because quantitative electron microscopy was not performed, it is not known at this time if all the phage particles examined were viable or not. It should be pointed out that haploid virions were not measured after electrophoresis as they have been shown to be pure upon re-electrophoresis (see Fig. 3A) and do not contain significant numbers of hets (see Table 1). Moreover, electrophoresis of radioactive virions also yielded a homogeneous peak (see Fig. 2).

The average lengths observed here are somewhat shorter than the reported haploid average length of 870 nm (11), probably because the electron microscope had been calibrated for use at magnifications higher than actually employed here.

DISCUSSION

Complete purification of haploid and of diploid virions has been achieved by analytical polyacrylamide gel electrophoresis. This has been demonstrated both biologically and by electron microscopy. It should be noted that although the numbers of extra-length virions obtained here were barely sufficient for electron microscopy, as described, it has recently become possible to observe M13 virions by using a modified micro-diffusion technique for sample preparation (8), and starting with suspensions containing as little as 2×10^8 PFU/ml (J. Beaudoin and A. Niveleau, unpublished data). Salivar et al. (15) partially purified large numbers of double-length M13 virions. However, their preparations were heterogeneous and contained many still longer viral particles. Complete purification of diploids on a large scale has yet to be achieved. This would allow protein composition analysis and the estimation of the molar proportions of the structural proteins. If, as has been postulated (Beaudoin, Ph.D. thesis, University of Wis., 1970), multiploid virions arise because of an insufficient intracellular pool of A-protein molecules, it can be expected that diploids, just like haploid particles, will contain the same number of A-protein molecules per virion, i.e., three or four (Beaudoin, Ph.D. thesis; 19). Such molecules are presumably present because diploids (albeit perhaps not all) are infectious. On the other hand, diploid virions should contain twice as many B-protein molecules as haploids, which would correlate with their length.

Even though diploids have been completely purified in our system, it is interesting to note that the proportion of heterozygotes never approached the value of 50% reported by Salivar et al. (15). Because their preparations were quite heterogeneous, it is conceivable that many of the still longer viral particles in their samples were indeed viable and thus increased the proportion of heterozygotes. It is difficult, however, to understand why this proportion was never any higher under those conditions. Another possibility is that the proportion of hets is in fact artificially low after gel electrophoresis as M13 genomes could conceivably be chemically inactivated. Hets, which require two complementing genomes when assayed on K38, would be more susceptible to this than the total diploid population assayed on K37, where only one genome is needed for plaque formation. Results shown in Table 1 tend to confirm this preferential inactivation of hets. On the other hand, total recovery seems to be correlated with

the electrophoretic mobility which, in turn, depends on the average particle length. Initial attempts to recover viable phage from gel slices 1 mm thick yielded only 1 to 3% of the input PFU (Beaudoin, Ph.D. thesis), whereas recoveries of about 50% were routinely obtained when the gels were crushed. Thus, it appears that both physical entrapment of virions in the gel particles and chemical inactivation of viral genomes, are important factors in gel electrophoresis of M13 virions. Breakage of the virions, on the other hand, seems to be rare, as can be estimated by electrophoresis of tritium-labeled virions (Fig. 2), re-electrophoresis of virions (Fig. 3), and electron microscopy (Fig. 4).

This is the first report of complete purification of diploid M13 virions. The techniques described, although very cumbersome, could be useful in genetic mapping experiments with Ff phages to separate heterozygotes from recombinants. Also, it will allow the study of the biological properties and physical properties of diploid virions. Serum inactivation of purified diploid virions is described in the companion paper (2).

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