Ch 80 Thesis:

Research on the Preparation, Purification, and Properties of the Bacteriophage M13 and its DNA

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1. INTRODUCTION

The bacteriophage M13 is a small rod-shaped virus, originally isolated by Dr. P.H. Hofschneider in Munich. It grows on F^+ and Hfr strains of E. coli and is reported to contain DNA, probably single-stranded. In order for a more extensive investigation to be undertaken of the properties of this virus and its DNA, a suitable quantity of the M13 virus had to be obtained and purified, and its DNA extracted. My research was directed primarily at developing these preparation and purification techniques. This paper describes the methods finally worked out and also presents some preliminary information on the properties of the M13 virus and its DNA.

2. MATERIALS AND METHODS

(a) Media and assay of the virus:

Assay of the virus is performed by the usual layer method, using E.coli strain C-3000, grown on KC broth, as the lawn bacteria. The "bottom" agar is the same as that used for bacteriophage ϕX174 and the "top" agar likewise, with 10^{-3}M $CaCl_2$ added after autoclaving. 2.0 ml of the top agar, 0.4ml lawn bacteria, and 0.1ml of sample to be titered are mixed and poured onto a plate, and incubated at 37°C for six hours or more. MS "top" agar may also be used with approximately the same plating efficiency. All dilutions required in titering a phage sample are done in standard dilution buffer (0.1M NaCl, 0.02M Tris, pH 7.6).

Lysates of titer $2\text{-}3\times10^{12}$/ml can be grown up using E. coli C-3000 at 2×10^{8}/ml, infecting at multiplicity of 5 on a KC broth medium (5g KCl, 10g Tryptone per liter, autoclave, then add 0.5ml of 1M$CaCl_2$ and 10mg Thiamine perliter). Mass cultures are grown on "TPG amino acids" medium: (Mix together in 1liter of H_2O, 0.3g of tyrosine, cystine, and leucine, and 4.5g of an amino acid mixture. Mix in 500ml of H_2O 1.0g NaCl, 16.0g KCl, 2.2g NH_4Cl, 24.2g Tris, 2.0g KH_2PO_4, 1.6g NaPyruvate. Combine volumes, add 2.0ml of 20% solution of $MgCl_2 \cdot 6H_2O$, 0.2ml 1M $CaCl_2 \cdot 2H_2O$, and 36.0ml of adenine (50mg/100ml). Adjust pH to 7.4 with 6N HCl, put in 7 liter culture bottle and bring to 2 liters. Autoclave, add 2.0ml $FeCl_3.6H_2O$ (1μg/ml),

20.0ml of Glucose (20% solution), and 2.0ml thiamine (10mg/ml)).

(b) Growth of the virus:

Bacteriophage M13 was obtained from Dr. P.H. Hofschneider. The virus is grown on E. coli strain C-3000. For mass cultures, 2 liters of the TPG amino acids medium described above are inoculated at 37° from an agar slant of C-3000. The cultures are grown at 37° with vigorous aeration. In 6½ to 7 hours the culture has reached the desired titer of 2×10^8 cells/ml. It is then infected with M13 to provide a final phage concentration of 10^9/ml. Foaming which occurs several hours after infection, is controlled by addition of Dow-Corning anti-foam B. The culture is allowed to incubate, with aeration, for 5 hours, and is then removed from the water bath. The titer of such lysates varies from $4\text{-}9 \times 10^{11}$/ml.

(c) Purification of the virus:

To the 2 liters of the mass culture lysate is added 400ml of Polyethylene Glycol (44g/100ml) and 124ml Dextran Sulfate (20g/100ml). This mixture is put in a 3 liter separatory funnel and after a thorough mixing is placed in the cold room for 48 hours.for the two-phase system to separate. About 75ml of interface can be collected which contains essentially all the cell debris and phage (titer about 3×10^{13}/ml). This interface material is spun at 14,000 rpm for 10 minutes at 5° on the Servall. The pellet is found to contain 99% of the M13 titer. The pellet is resuspended in 80 ml of 1/2 saturated borate buffer, pH 9.1 (containing 2×10^{-3}M Versene), by stirring on a magnetic stirrer in the cold room for 24hrs. The mixture is then spun down and the supernatant is collected and found to contain about 80% of the total phage. MS broth is then added (1/10 volume) to help stabilize phage during later banding. CsCl is added to density of about 1.30. The residual polymer which precipitates out at this ionic strength must be spun out at 14,000 rpm for 10mins. This solution is then placed in the L-1 ultracentrfuge and spun for 20-24hrs at 37,000 rpm (rotor head #40). A white diffuse band is observed about 2/3 the way down the tube. If the contents of the tube are carefully

removed in four portions (the portion below the band, the band, and two equal portions above the band), it is found that 85% of the phage in the tube are located in the portion immediately above the band. (The band appears to contain protein impurities, probably lipoproteins from the bacterial cell walls). This phage-rich portion is then pooled from several tubes and the combined solutions again banded for 20-24hrs at 37,000 rpm at 3°C. Again a difuse band is observed. Drops are collected. The bulk of the titer (80%) is found very slightly above this band. There is only a 50-60% recovery during this second banding. The richest 1ml fraction contains about 20% of the original mass culture titer. The richest fractions are dialyzed against three changes of 500mls each of 0.1M Tris pH 7.6 (a white ppt formed in the dialysis tube was later found to contain only a small number of phage). This dialysate is quite stable for at least 3 months in refrigeration.

(d) Preparation of the M13 DNA:

Two closely related methods of extracting DNA from M13 were used. The first method involved extraction by phenol at room temperature. To 1.0 ml of purified M13 suspension, an equal volume of distilled phenol, saturated with 0.05M Tris pH 7.2, is added. This is vortexed for 3 mins and spun down for 10 mins at 10,000 rpm to separate the two phases. The aqueous phase is removed and to it an equal volume of phenol is added, the mixture vortexed, centrifuged, etc. This procedure is repeated five times until the interface between the phenol and the aqueous phase is clean after centrifugation. To improve the DNA yield a 0.5ml portion of the buffer (0.05M Tris pH 7.2)was added to the phenol phase from the first extraction, vortexed, and spun as above. This aqueous phase is added to the successive phenol phases and the final aqueous layer is pooled with the DNA solution obtained in the first series of extractions. The combined solutions are shaken with an equal volume of cold ether, and the ether layer removed. This ether extraction is repeated 6-8 times to completely remove residual phenol, and the N_2 is bubbled through the solution to evaporate the ether. This solution has about

35% retention of the optical density (260mμ) of the purified phage preparation.

A second method involving hot phenol was used, which was very similar to the procedure above except that after addition of the phenol, the mixture was heated in hot water to 70°, at which temperature the water and phenol are miscible, and vortexed for 1min. It was heated to 70° and vortexed twice more and then spun in the normal fashion. This prodedure also resulted in about 35% recovery of DNA O.D.

(e) Spectra:

All spectra were taken with the Beckman DK2 Spectrophotometer.

(f) Protoplast Technique:

Cells of E. coli strain W6 are grown at 37° with aeration in 3XD medium. 30mls of cells at 2-4x10^8/ml are centrifuged. The pellet is resuspended in 0.35ml of 1.5M sucrose. The following solutions are added, in order, with gentle mixing after each: 0.17ml of 30% BSA, 0.02ml of 2mg/ml lysozyme in 0.25M Tris(pH 8.1), and 0.04ml of 4% Versene. Swirl and after 30 seconds add 9.5mls of PAM medium (10g Casamino acids (Difco), 10g Nutrient Broth (Difco), 10g glucose, and 100g sucrose per liter. Autoclave, then add 10mls of 10% solution of $MgSO_4$ per liter). This is the "protoplast stock", and is kept at room temperature until used (within an hour). DNA dilutions are made in 0.05M Tris pH8.1 and put into a 37° water bath. Then to 0.4ml of DNA solution, add 0.4ml of protoplast stock, incubate at 37° for 10 minutes, add 3.2ml of PAM medium, and incubate at 37° for 1½ hrs. Freeze, thaw, and titer. Phage titers and DNA concentrations are computed for the 0.8ml infection vol.

(g) Millipore Filter Technique:

A culture of C-3000 was grown in KC broth at 37° to 2x10^8cells/ml. It was then infected (t=0) with M13 at an infection multiplicty of M=5. At t=10mins the culture was chilled quickly, centrifuged, and the pellet resuspended in 40mls of cold KC broth. This suspension was diluted down with KC broth resulting in a solution containing 1-4 infected cells/ml. 1ml of the solution was placed on a 25mm type AA Millipore filter (Millipore Filter Corp., Bedford, Mass.). This

filter has a pore size of 0.85μ and passes no bacteria. The infected cell sample was washed with cold KC broth to remove unabsorbed phage and then the stopwatch was started at t=10mins and 1ml of warm 37° KC broth was added to the filter. (The whole filter operation was done in a 37° room). At the end of 1 minute the 1ml portion was pumped through the filter, followed in quick succession by two more 1ml wash portions. These three portions were collected in a tube labeled t=10, and 1ml of fresh broth was added to the cells. This procedure was repeated at one minute intervals, until about t=65 minutes. 1ml from each tube was plated.

A filter efficiency test run under identical conditions showed that only 20% of the M13 placed on the filter were passed through in three 1ml washings. The remaining 80% didn't appear on additional washings and seemed to have been inactivated.

3. PROPERTIES OF M13

(a) Growth Curve:

The growth curve obtained by growing M13 on C-3000 in KC broth is shown in figure 1. The titer increases to $2\text{-}3\text{x}10^{12}$/ml in 3-4hrs. The phage are being produced at the greatest rate (about $5\text{x}10^{11}$/20mins) at t=130mins.

(b) Release of M13 from the host cell:

Most bacteriophage are released by a disruption (lysis) of the host cell, resulting in the simultanious release of all the phage in the cell. It has been observed that lysis doesn't occur in M13 cultures. Hofschneider has provided electron microscope evidence that M13 doesn't lyse cells but is instead released gradually without rupturing the cell. In an attempt to substantiate this observation the course of M13 release was followed using the Millipore filter technique of Hutchison. 1ml of a solution containing a mean of 2.3 infected C-3000 cells/ml was placed on the filter and the phage-release followed as described earlier. The results are shown graphically in figure 2. The M13 were released semicontinuously over a period of at least 45mins, beginning at about t=20. Although the periodic spurts which appear may be nothing more than statistical variation due to the

the small number of phage worked with, it is clear that no definite lysis occurred, as described for similar experiments with ØX174 in which the complete phage release process occurred within 30 seconds. Assuming that only 20% of the M13 released by the cells passed through the filter unharmed, then the total phage released up to 64mins was about 2000. This gives a burst size of 1000-2000, that is, if burst size has any meaning in this case, for the cells might very well have divided during the 64min period covered in the test. It is also noteworthy that in the test shown in fig. 2 and also in earlier tests, the phage release had not stopped when the test was terminated.

(c) Spectra:

An ultraviolet absorption spectrum of purified M13 virus, taken in 0.05M Tris pH 7.2, is shown in figure 3. For purified M13 (dialyzed to remove CsCl) the ratio of absorption at 280mμ to that at 260mμ is 1.10. This roughly corresponds to a DNA content of about 2-4%. (see Colovich and Kaplan, Methods in Enzymology, Vol III, p 451). The absorption minimum is at 247mμ. Various virus preparations vary in their titer/O.D.$_{260}$ ratio, from 1.5×10^{11} to 2.5×10^{12}, showing that some virus preparations contain a large percentage of inactivated phage.

Undialyzed virus preparations, straight from banding, tend to have slightly diff. spectra (see figure 3 also). The O.D.$_{280}$/O.D.$_{260}$ varies from 0.65-0.83 and the absorption minimum is about 244mμ. It has been observed that while the titers of the undialyzed and the dialyzed virus preparations are the same, there is a loss of 60-70% of the O.D.$_{260}$ in the dialyzed portion, and the nucleic acid/protein ratio drops considerably. This could possibly indicate a loss of free nucleotide impurities during dialysis.

4. PROPERTIES OF M13 DNA

(a) Spectra:

The ultraviolet absorption spectra of DNA prepared from purified virus preparations by extraction with phenol (both hot and room temperature extractions) were essentially

identical. In both cases there was a DNA recovery of about 35% as measured by $O.D._{260}$ of the virus and the extracted DNA. The absorption maximum is 258mμ.

(b) Infection of protoplasts:

M13 DNA was found to be infective to protoplasts prepared as described earlier. There was no significant difference between the infectivity of the DNA prepared by hot, or room temperature phenol extraction. (See figure 4). A control was run using ϕX174 DNA to determine the quality of the protoplasts. $3x10^{-2}$ ϕX174 phage were produced per ϕX174 DNA in the reaction volume (0.8ml). Normally the phage/DNA ratio for ϕX174 is about 1, indicating that this particular protoplast preparation was below par. The phage/DNA ratio for M13 DNA was about 10^{-3}, or 1/30 as great as that for ϕX174. But since the "burst size" of M13 is much larger than that of ϕX174, the infected protoplasts/DNA for M13 is much less than 1/30 that of ϕX174. The DNA concentrations used here for M13 were computed on the assumption that M13 DNA has a M.W. of about 10^6 daltons. Then using the fact that 33gamma of DNA/ml yields one O.D./ml, a value of $2x10^{13}$ M13 DNA/O.D. was obtained. From this, the O.D. of the M13 DNA preparation, and the dilution factors, the concentration of DNA was computed for each tube. In figure 4 the M13 DNA curve begins to level off at a DNA concentration of about 10^9/0.8ml, indicating that saturation has begun to occur.

5. Discussion

It seems clear that M13 virus do not lyse cells in the normal way, but rather "leak out" gradually. Evidence obtained both by this research and by Hofschneider point to this conclusion. That this is the case seems reasonable when one considers that M13 contains only a small amount of DNA. It thus would not be expected to code for all the enzymes which T-phage use to accomplish the complex absorption and injection processes, the drastic alteration in cell metabolism, the construction of the complex protein coat, and the lysis of the host cell. M13 is probably able to code only 2 or 3 polypeptides in addition to the DNA polymerase and the protein

coat subunits, and it seems likely that the absorption and infection processes are much simpler than that found in T-phage. I would predict that unlike T-phages, the M13 DNA isn't injected into the cell and the protein coat left behind. Rather I think that the entire M13 phage particle passes into the host cell, by much the same mechanism as that by which the mature phage, produced within the cell, leak out. This leaking out occurs when the mature virus concentration inside the cell reaches a certain level, and continues as long as the cell continues to produce phage. Hofschneider indicates that the cell can even continue to multiply while phage production is taking place, thus indicating that the cell metabolism has not been completely altered.

Electron micrographs taken by Hofschneider show M13 to be a long rod about 1 micron long and 100Å wide. A crude calculation shows that M13 has a volume of about $8 \times 10^{-17} cm^3$. Its density when banded in CsCl is about 1.29. An approximate M.W. can be calculated by $M.W. = V\rho N_o = (8 \times 10^{-17})(1.29)(6 \times 10^{23}) = 60 \times 10^6$ daltons. This is comparable to another rod-shaped virus, TMV, which has a M.W. of about 40×10^6 daltons. The $O.D._{280}/O.D._{260}$ ratio for purified, dialyzed M13 is 1.10, which corresponds to 2-4% DNA. This yields a DNA content of M.W. about 10^6, which is comparable to that of the minute phage ϕX174 (1.6×10^6).

6. SUMMARY

Techniques for the preparation and purification of the small rod-shaped bacteriophage, M13, have been developed. M13 DNA has been successfully extracted with phenol and shown to be infective to protoplasts. Release of M13 from infected E. coli C-3000 cells has been followed and found to take place by a "leaking" process and not by a definite lysis.

7. ACKNOWLEDGEMENTS

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8. REFERENCES

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Fig. 1 M13 Growth Curve

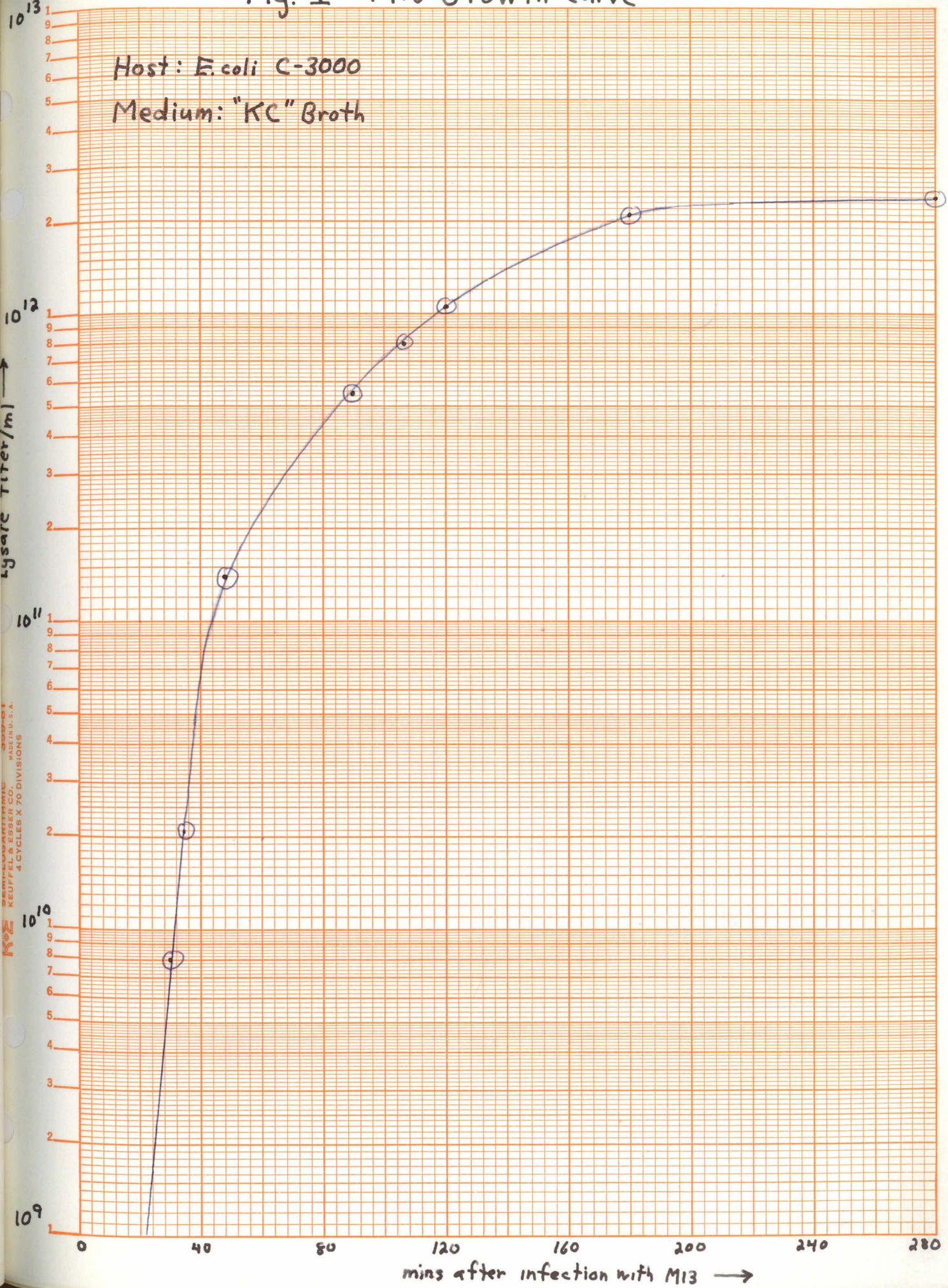

Fig. 2 M13 Release at 37°C

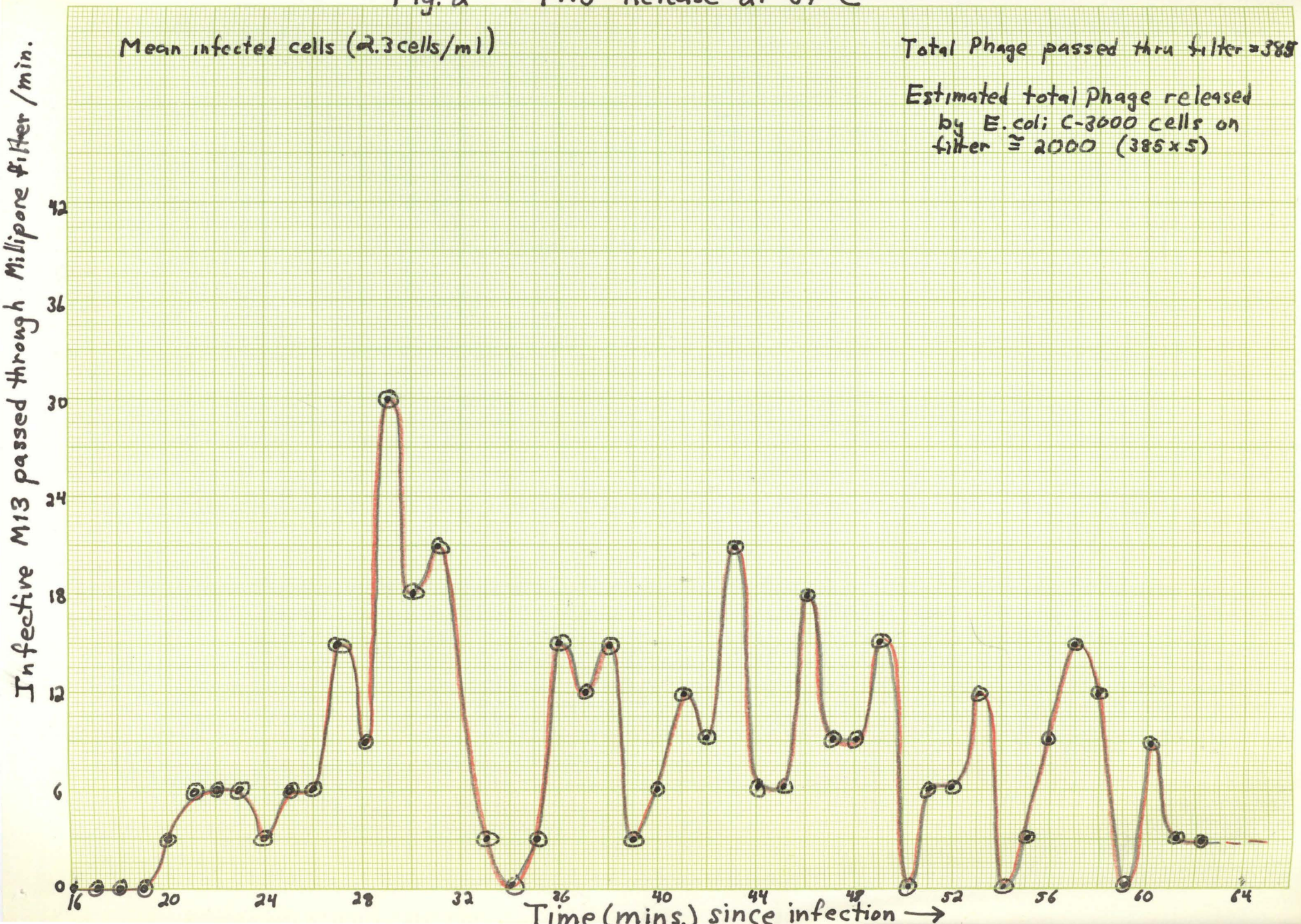

Fig. 3 U.V. Absorption Spectra of M13 Virus and M13 DNA

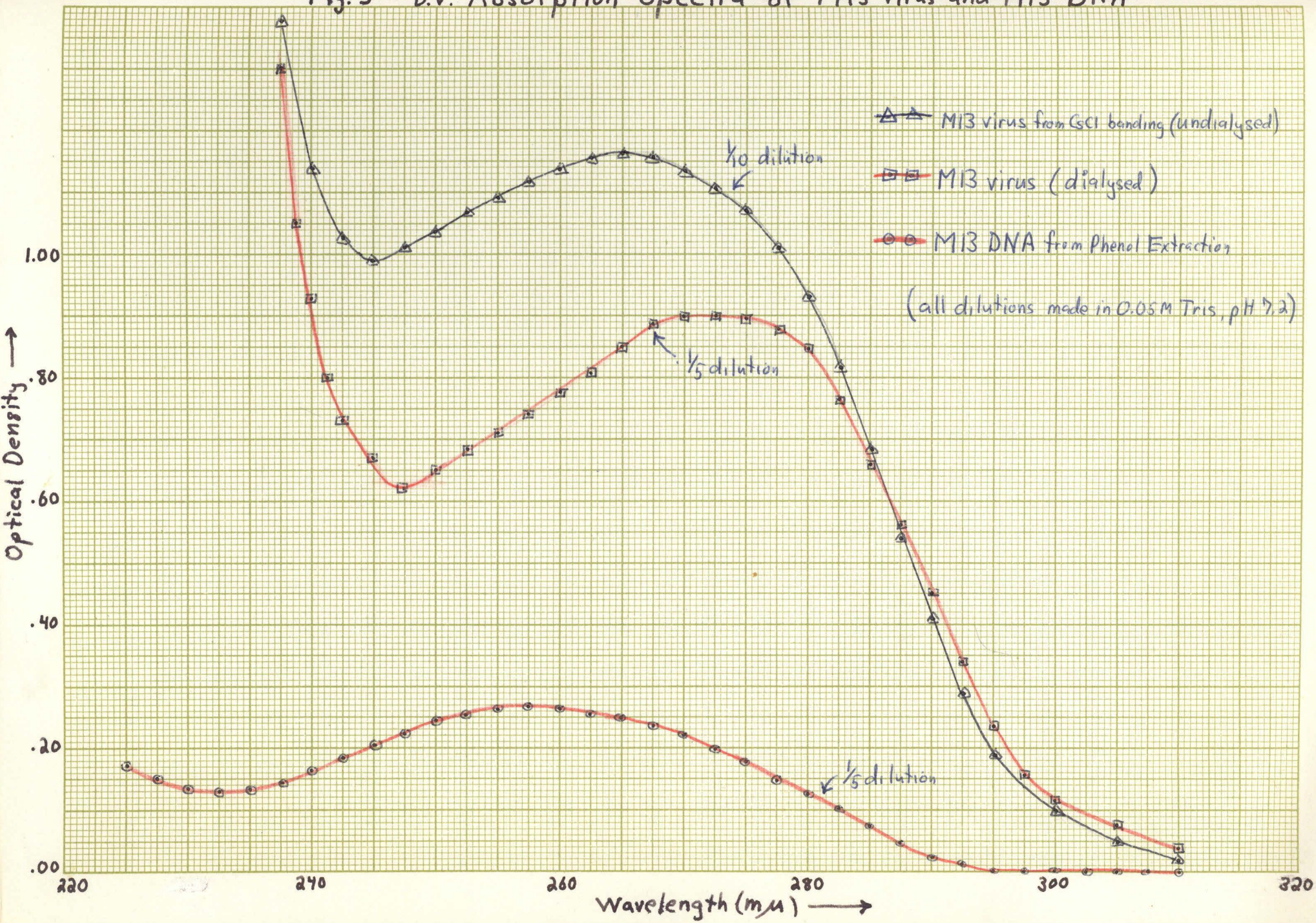

Fig. 4 Infection of Protoplasts by M13 DNA