STUDIES ON THE INTERACTION OF SUBVIRAL PARTICLES OF THE BACTERIOPHAGE ϕX174 WITH PROTOPLASTS OF ESCHERICHIA COLI

Thesis by

George Drake Guthrie

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ABSTRACT

Interaction of Subviral Particles of Bacteriophage ϕX174 with Protoplasts of Escherichia coli. Part I: Infection of Protoplasts of Escherichia coli by Subviral Particles of Bacteriophage ϕX174. G. D. Guthrie and R. L. Sinsheimer, J. Mol. Biol., (1960), 2, 297-305. Infection of protoplasts of E. coli leading to the production of mature phage can occur with subviral particles of phage ϕX174, including the purified DNA of the phage. Methods for obtaining the proper conditions for infection are described. Results of experiments describing the characteristics of the infection and the nature of the subviral particles causing the infection are presented. Evidence is presented that net synthesis of mature ϕX174 can be obtained from infection of protoplasts by purified ϕX174 DNA.

Part II: Initial Studies on Protoplasts and ϕX-DNA as a system for investigating virus replication. G. D. Guthrie (unpublished thesis research). Further investigations on the interaction of ϕX-DNA with protoplasts are described. General techniques to be employed in using the system for the study of the replication of virus are outlined.

Data from investigations on the initial "infection" process of ϕX-DNA are presented. From these data, factors influencing the level of infection are discussed and a simple mathematical relationship relating these parameters to the infection is proposed.

ACKNOWLEDGMENTS

The encouragement and guidance of Dr. Robert Louis Sinsheimer have been a source of continued strength throughout my graduate study and research.

The interest and assistance of Mr. Thomas E. Creighton was of considerable value in the initial investigations of the techniques described in Part II.

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The unfailing support of my wife, Shirley, manifests itself in many ways. The completion of this thesis is in no small part due to her continued encouragement and valuable suggestions.

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INTRODUCTION

Initial Investigations of Biological Activity of Free Nucleic Acids: In a report concerned with the biological activity of deoxyribonucleic acid it would seem appropriate to begin with a quotation from the paper by O. T. Avery, C. M. MacLeod, and M. McCarthy (1) on the transformation of pneumococcal types:

> "The data obtained by chemical, enzymatic, and serological analyses together with the results of preliminary studies by electrophoresis, ultracentrifugation, and ultraviolet spectroscopy, indicate that, within the limits of the methods, the active fraction contains no demonstrable protein, unbound lipid, or serologically reactive polysaccharide and consists principally, if not solely, of a highly polymerized, viscous form of desoxyribonucleic acid."

This statement laid the foundation for what has become a continued and increasingly diverse series of investigations on the biological activity of both ribonucleic and deoxyribonucleic acids (RNA and DNA). It also outlines the limitations governing conclusions which are drawn, on the one hand from macroscopic physical chemical analyses on a preparation, and on the other from a biological response in which apparently only a small fraction of the population of molecules exhibits activity. It is evident that any conclusions based on data from both of these different approaches rest heavily on the hoped for homogeneity of a particular preparation which will permit the identification of a biologically active fraction in terms of its physical and chemical properties.

The phenomenon of transformation has been utilized by many investigators for the study of the ability of DNA to produce in an

organism, through genetic change, particular phenotypic modifications. It is a method for investigating one or a few genes of the total genetic complement of an organism, using free nucleic acid. Such a system also permits the study of the effect of nucleic acid which has been altered physically or chemically in an isolated state.

Fraenkel-Conrat (2) and Gierer and Schramm (3, 4) demonstrated that the ribonucleic acid from Tobacco Mosaic Virus (TMV-RNA) was infectious. Innoculation of sensitive leaves with the RNA caused the production of complete TMV in the plant cells. This achievement proved that not only could a particular genetic function be transferred by nucleic acid, as in the transforming system, but in fact all the necessary information for the production of a complete virus could be carried to the cellular metabolic mechanism by the viral nucleic acid alone.

Methods for purifying large amounts of both TMV and TMV-RNA were developed, and such a system offered many advantages for the characterization of free nucleic acid. In addition a great deal of knowledge has been gained about the biological effects and the mode of action of chemical and physical mutagenic agents on the genetic material with and without the presence of its associated protein.

In attempting to investigate the replication of nucleic acid, in vivo, however, the efficiency of TMV-RNA infection and the relative instability of TMV-RNA, even in a purified state, present significant technical difficulties. Partially for these reasons the

possibility of developing another system is of interest. In addition DNA, which appears to be the genetic material in most instances, might yield more general information about the replication of the genetic code.

The Bacteria-Bacteriophage System and the Protoplast-Subviral System: The extent of development of the bacteria-bacteriophage system is generally known. Beginning with the discovery of bacteriophage by d'Herelle, Twort, and others (5), many data on bacteriophage have been reported by a large number of investigators. With the evolution of this background of information bacterial viruses have become increasingly valuable as tools in the study of the replication of macromolecules.

In 1953 Weibull (6) reported that the bacteria, Bacillus megaterium, when exposed to the enzyme, lysozyme, under hypertonic conditions, formed spherically shaped bodies which he called protoplasts. He and other investigators have shown that these protoplasts offered certain advantages over intact bacteria for metabolic studies. Repaske (7) extended the technique of obtaining protoplasts from whole cells to include some gram negative organisms. Furthermore, Salton and McQuillen (8) and Brenner and Stent (9) demonstrated that protoplasts formed from cells of B. megaterium previously infected with bacteriophage C could carry out all the metabolic steps necessary for making mature phage. Zinder and Arndt (10) found similar results using E. coli (a gram negative organism) and T4 phages.

These data suggested the possibility of using a protoplast-subviral particle* system for the study of phage production. Spizizen (11) and Fraser, Mahler, Shug, and Thomas (12) published descriptions of such a system using protoplasts of Escherichia coli B and subviral particles from T2. While these reports represented the initial success in the development of a method for studying the activity of subviral particles, the particular virus used has proved to be somewhat limited. The only subviral particles of T2 exhibiting biological activity also have been shown to contain a significant fraction of the viral protein still associated with the DNA. Lack of evidence for infection by free T2 DNA may result from the ease of breakage of the T2 DNA during purification with concomitant loss of biological activity or may actually be inherent in the nature of the mechanism of infection of the T2 phage. Whatever the reason, as yet the only subviral particles of T2 demonstrated to be biologically active have only been slightly modified from their normally active virus state.**

The Bacteriophage ϕX174: V. Sertic and N. Boulgokov (13) published a series of papers on the isolation and initial characterization of a group of minute bacteriophages. In the last of these

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* Subviral particle, as used herein, is any particle incapable of infecting bacteria but capable of infecting protoplasts and causing the production of mature virus as a result of this infection.

** Since the work on this dissertation was started, other systems for the assay of free nucleic acid or "markers" from nucleic acid extracts have been reported. Because these systems were not a part of the original point of departure, they are not summarized.

papers and in two later publications (14, 15), the bacteriophage ϕX174 is mentioned and described briefly as to its salt requirements, plaque size, probable particle size, and other basic characteristics. In 1955, R. L. Sinsheimer obtained the phage and began a detailed characterization of the physical and chemical properties of both the phage and its nucleic acid (16, 17, and 18). Briefly, ϕX174 is a minute, polyhedral particle approximately 25 mμ in diameter; Hall, Maclean, and Tessman (19) report 248 $\mathring{A} \pm 10$. The virus contains only one molecule of single-stranded deoxyribose nucleic acid with a molecular weight of 1.7×10^6 g/mole.

Since these publications, other data have been reported concerning ϕX174. Several papers compare ϕX174 with S13. Notable are publications by Zahler (20) comparing growth, serology, and other properties of the two phages and Tessman (21) comparing the inactivation of the phages labeled with radioactive phosphorous. Woodley (22) first investigated the ultraviolet action spectrum of ϕX174; recently Setlow and Boyce (23) published ultraviolet inactivation data correlating their results with pH during irradiation.

Because of the size of ϕX174 and the fact that each virus particle contains only one molecule of DNA, it was felt that this phage might be more useful in a protoplast-subviral system than T2. As a consequence, research was begun in 1958 on the development of such a system. The results and characterization of

the system are contained herein. In addition to these results, there are three other publications which report similar findings with subviral particles of ϕX174. Hofschneider (24) describes a system quite similar to the one described in Part I of this dissertation. R. Wahl, J. Huppert, and L. Emerique-Blum (25) report the finding of an infectious unit in the lysate of cells infected with ϕX174 and prematurely lysed, while a preliminary note by M. Sekiguchi, A. Taketo, and Y. Takagi (26) describes infection by a subviral particle under conditions similar to Hofschneider's. At least two of the investigators mentioned above are continuing to work with protoplasts and ϕX-DNA.

The work described herein is divided into two parts. Part I is a reprint of developments up to June, 1960. Part II describes some of the results found since that time and outlines the proposed uses of the system in future investigations.

Mol. Biol. (1960) **2**, 297-305

FECTION OF PROTOPLASTS OF *ESCHERICHIA COLI* BY SUBVIRAL PARTICLES OF BACTERIOPHAGE ØX174

By

George D. Guthrie and Robert L. Sinsheimer

J. Mol. Biol. (1960) **2**, 297-305

Infection of Protoplasts of *Escherichia coli* by Subviral Particles of Bacteriophage ϕX174†

GEORGE D. GUTHRIE AND ROBERT L. SINSHEIMER

Division of Biology, California Institute of Technology, Pasadena, California, U.S.A

(*Received 11 July 1960*)

Infection of protoplasts of various strains of *Escherichia coli* may be obtained with purified DNA preparations from bacteriophage ϕX174 made by two distinctly different methods. Infection of such protoplasts can also be obtained with nucleoprotein particles made from ϕX174 by heat treatment. None of the particles, infective to protoplasts, are infective to whole cells and all are infective to protoplasts of strains of bacteria usually resistant to whole ϕX174 virus.

The protoplast-infective agents are in all cases destroyed by enzymatic treatment with DNase. The infective DNA is not inactivated by treatment with enzymatic amounts of trypsin but is by treatment with stoichiometric amounts, suggesting the formation of an inactive DNA-trypsin complex.

Studies of the properties of protoplast infection indicate certain general characteristics which distinguish it from the more familiar virus infection of whole bacterial cells.

1. Introduction

The use of protoplasts for the study of microbiological phenomena is becoming increasingly widespread. Investigations conducted more or less simultaneously by Spizizen (1957) and Fraser, Mahler, Shug & Thomas (1957) demonstrated that protoplasts could be infected by subviral particles of the bacteriophage T2. Fraser has discussed the general characteristics of such infections.

Bacteriophage ϕX174 has been characterized by Sinsheimer (1959*a*) as a minute particle containing a single molecule of DNA. This DNA molecule has been characterized physically and chemically (Sinsheimer, 1959*b*) as a small single-stranded polynucleotide of some 5,500 nucleotides. For these reasons, attempts have been made to obtain, from ϕX174, subviral particles with the ability to infect protoplasts. Such infective particles have been obtained by three different methods. These particles are of two types. One type is known from its behavior during sedimentation and from its density to have associated with it a large part of the original viral protein. The other type is the free nucleic acid of the virus. Evidence for the fact that this latter particle is the free DNA rests to a large extent on the physical-chemical characterization of the molecule as reported by Sinsheimer.

2. Materials and Methods

Stock solutions

All stocks including nutrient media are made up using either deionized water or double distilled water.

Tris buffers: Sigma 121 grade (Sigma Chemical Co., St. Louis, Mo.) is used in stock solutions of tris (2-amino-2-hydroxymethylpropane-1:3-diol) buffer.

† This research has been supported by U.S. Public Health Service grants C-3441 and 2G86. One of us (GDG) has been supported by a U.S. Public Health Predoctoral Fellowship (No. 10, 078) during most of this work.

I. Heated particle† (*HTϕX*): purified ϕX (at a concentration of 10^{15}/ml.) is diluted 1/100 into 0·1 M-tris buffer pH 7·5. It is then heated at 70°C for 5 min and cooled. Samples treated in this manner show a loss of infectivity (as assayed by normal plating procedure) of between 10^6 and 10^8/ml. From electron micrographs of this particle (Hall, Maclean & Tessman, 1959) it appears that, on heating, the virus breaks open and that the DNA, possibly with some protein, is extruded from the protein coat. HTϕX has a density in rubidium chloride of 1·42 g/cm^3 (0·02 g/cm^3 greater than the whole virus). Its sedimentation coefficient in 0·1 M-tris pH 8·0 is 47 S, less than that of either the whole virus (114 S) or the protein "coat" (70 S), a result which would be expected for a particle of the type seen in the micrographs. Upon treatment with DNase the sedimentation coefficient of the residual protein particle rises to 70 S. Also present in the HTϕX preparation is a small amount of material which is probably free DNA. This material may be separated from the HTϕX particle by banding in a cesium chloride density gradient (*vide infra*); both the DNA and HTϕX particle are biologically active when tested with protoplasts.

II. Phenol-prepared DNA: the preparation of this particle (the free DNA) is described and the molecule is characterized as the free DNA by Sinsheimer (1959*b*).

III. Calcium particle (*CaϕX-DNA*): a third method for obtaining a biologically active subviral particle is the following: purified ϕX is diluted tenfold (from a stock of 10^{14} to 10^{15} virus/ml.) into cold 4 M-$CaCl_2$ (analytical reagent grade recrystallized once from 95% ethanol and dissolved in deionized water), allowed to stand at room temperature for 30 min and stored in the cold for another 90 min. A heavy precipitate forms which contains almost all of the virus. This is centrifuged in the cold for 30 min (12,000 *g* in the Servall SS 1 centrifuge). The supernatant‡ is discarded. The pellet is resuspended in 0·1 M-tris buffer pH 7·5 to 8·0, centrifuged and the supernatant retained. This supernatant contains from 30 to 50% of the original viral DNA. Repeated extractions of the pellet, which presumably contains the rest of the DNA, are largely unsuccessful.

The ultraviolet absorption spectrum of this calcium-prepared DNA is the same as the spectrum of the DNA from the phenol treatment with a minimum at 230 mμ and a maximum at 257 mμ. Its sedimentation coefficient is that of phenol-prepared DNA (23 S). In the cesium chloride density gradient it bands at a density of 1·72 g/cm^3 as does phenol-prepared DNA. On the basis of these criteria the CaϕX-DNA is believed to be the same molecule as that prepared by the phenol method. Data obtained using either of these particles are reported as data resulting from the infection of protoplasts by free ϕX-DNA.

General Method for Infecting Protoplasts with Subviral Particles of ϕX174

Cells are grown and converted to protoplasts as described above. The infecting particle, which is stored in 0·1 M-KCl, 0·001 M-tris buffer, pH 7·5 (under these conditions infectivity is retained for weeks if the sample is kept in the cold), is diluted at least tenfold into 0·01 M-tris pH 8·0 to 8·3. To a volume of infecting particles is added an equal volume of "protoplast stock." The mixture is incubated at 37°C for 10 to 15 min, after which it is diluted fivefold into nutrient broth. After another 90 min incubation the sample is titered for mature ϕX phage. If infective centers are to be assayed the sample is diluted and plated after the 10 min adsorption period. Dilutions for infective centers are made through nutrient broth plus 2% BSA and plating is performed as described above.

Experiments have shown that, while 2% BSA is necessary for the initial stabilization of the protoplasts, the BSA concentration during incubation of the infected protoplasts in liquid medium must be below 0·2% for maximum final phage yield. During incubation of the protoplasts on agar plates, however, BSA at a concentration of from 1 to 2% is used.

3. Experiments and Results

Banding of the infective particles in the cesium chloride density gradient: each of the three particles has been banded by cesium chloride density gradient centrifugation (Meselson, Stahl & Vinograd, 1957). After fractionation by drop collection from the

† This method for obtaining a subviral particle was suggested to us by Dr. Irwin Tessman.

‡ This first supernatant has ultraviolet absorption with a maximum at 270 mμ and apparently contains a small amount of viral protein.

the manner ordinarily observed with whole infected cells (due possibly to the presence of sucrose in the nutrient medium), and that a portion of the virus produced by the protoplasts is not released from them unless they are shocked into water.

Since the adsorption of DNA by protoplasts occurs in the presence of nutrient, events are measured from the time of mixing. The "zero time" infective centers simply represent those infections occurring between the time of mixing the DNA and protoplasts and the time the first sample is plated, i.e. within about the first minute. The mature phage curve (curve B of Figs. 3 and 4) is extrapolated back from the time of the first appearance of mature phage.

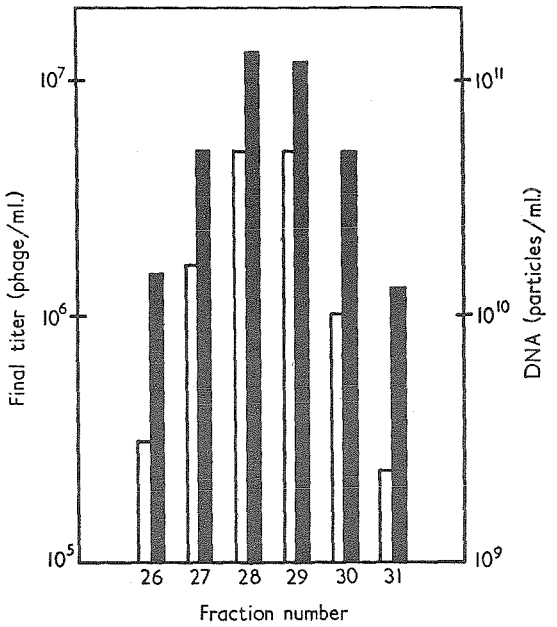

FIG. 2. Infectivity and particle concentration of samples from a cesium chloride density gradient band of CaϕX-DNA. Mean density of the band is 1·72 g/cm^3. Biological activity is shown by open bars; particle concentration, calculated from the u.v. absorption, by solid bars.

There is an obvious difference between the two growth curves at later times of incubation. The curve from the K12 experiment resembles more closely the shape of the growth curve of virus in whole cells, while the *E. coli* C curve continues to rise. *E. coli* C is a normal host for ϕX while K12 is not; also, protoplasts made by our method seem to show some capability of resynthesizing cell wall. These facts, coupled with the observation that the infected tube of C protoplasts remains clear while the control (uninfected protoplasts) is found to contain a high concentration of whole cells after incubation, lead to the conclusion that residual C cells or protoplasts which have regrown a portion of their cell wall are infected by the phage initially produced by the infected protoplasts, resulting in the continued production of phage. For this reason it is preferable in these experiments to use protoplasts of an organism which is resistant to mature ϕX174.

Dilution experiments: if successive dilutions of DNA are used to infect a constant number of protoplasts, activity curves of the general shape of those shown in Fig. 5 result. At high concentrations of DNA there is an apparent saturation of protoplasts susceptible to infection. At lower concentrations of infecting particle a linear

At the lowest concentration represented in Fig. 5 the number of infective centers obtained is equivalent to 3% of the DNA particles (the number of DNA particles is calculated from the u.v. absorption). The final phage yield at this concentration contains 5·5 times as much ϕX-DNA in mature phage particles as was initially

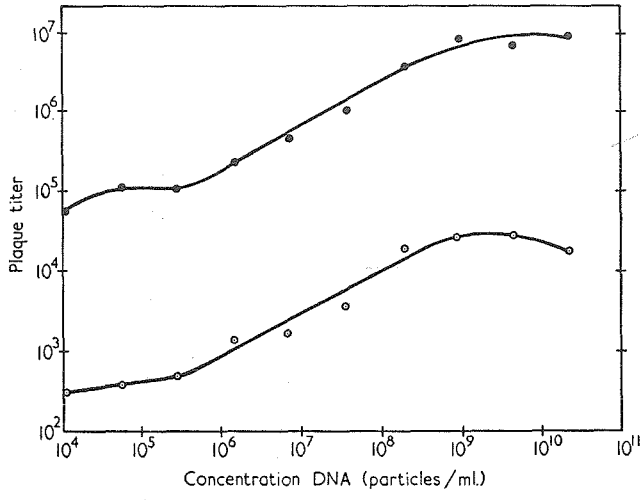

FIG. 5. Dilution curve (nonlinearity of assay). Curve A (⊙——⊙) indicates infective centers (plated after 10 min adsorption), curve B (●——●) represents mature phage produced after 2 hr incubation.

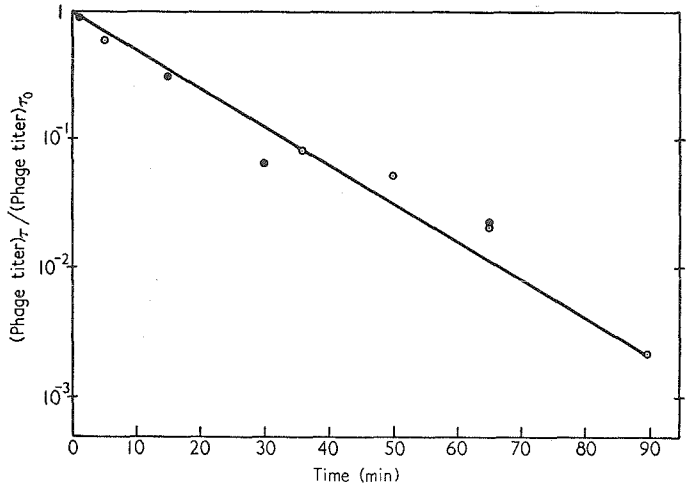

FIG. 6. DNase inactivation of CaϕX-DNA. The curve represents the normalized decline in final phage titer as a result of DNase inactivation of the infective particle. The actual inactivation is somewhat greater since the nonlinearity of assay tends to make lower concentrations of infective particles more efficient. Temperature 25°C, reaction mixture contained 0·005 M-$MgSO_4$, 0·1 M-tris pH 7·5, approx. 10^{13} CaϕX-DNA particles/ml. and 10^{11} DNase molecules/ml.

present in the infection mixture as free DNA, clearly indicating that new DNA is being made (rather than input DNA simply being "coated" with viral protein).

Reactions of DNase and trypsin with subviral particles: studies have been made of the actions of DNase and trypsin on CaϕX-DNA and HTϕX particles. Fig. 6 demonstrates that the action of DNase on CaϕX-DNA follows the kinetics of an enzymatic

The evident ability of these protoplasts to take up a single-stranded DNA suggests that bacterial transformation or infection with the DNA of other viruses might be achieved if the DNA employed were first converted to the single-stranded form (Doty, Marmur, Eigner & Schildkraut, 1960).

It is a pleasure to acknowledge the capable technical assistance of Miss Faith Poole. Several talks with Dr. Dean Fraser, Indiana University, about the preparation and use of protoplasts were of considerable value in developing the techniques for this research. Dr. Wolfgang Mundry's contributions in numerous discussions are gratefully acknowledged.

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-7 to 15-

In the original copy of this thesis the pages indicated above contain the text of a published article: "Infection of Protoplasts of Escherichia coli by Subviral Particles of Bacteriophage ϕX174" by George D. Guthrie and Robert L. Sinsheimer, J. Mol. Biol., (1960), 2, 297-305.

PART II

The original thesis "that ϕX174 DNA can exhibit biological activity under the proper conditions" has been demonstrated in Part I. Therefore, it would seem advisable at this point to include an apologia for Part II.

As a result of the publication of Part I, the use of the protoplast system has become important as an assay method in several other phases of ϕX174 research. Because of the variable conditions in different investigations, further characterizations and improvements were needed in order to extend application of the assay system. A method for making more reproducible and active protoplasts was developed and will be described. In addition, the effect of salts and other agents was studied.

To go beyond this generalization of the assay system, a second thesis must be put forth--namely "that the protoplast system itself can be used to study the replication of a virus".

Technically, therefore, the system must be able to turn back upon itself making possible the study of replication of infectious subviral units within infected protoplasts. This could then lead to the study of the replication of altered DNA and possibly to the effect of isolated specific RNA or protein during replication. While this second thesis is not proved in Part II, the preliminary data presented offer support to the idea.

Such a proposal is made at a time when significant advances are being reported in <u>in vitro</u> systems. Not only are the early

DNA and RNA polymerase systems being further developed and refined, but recently combined studies on DNA, RNA, and protein in protein synthesizing systems have provided new thoughts and theories concerning their interrelationships during replication. In the face of these advances, the reasons for continuing with a relatively involved and unknown system, such as protoplasts, might seem less obvious than before.

The ultimate hope of this general area of research is to understand the biological processes involved in specific replication. Already the DNA polymerase systems have been shown to be specific from chemical tests. For example, the base ratios and nearest neighbor frequencies of synthesized molecules agree with those of the "primer" and physical studies have demonstrated the new molecules to be highly polymerized. With protein in vitro systems antigenic tests give evidence for specific antigen-antibody reactions with material which contains the radioactive label of the precursor amino acids. Finally, control of the base composition of the RNA in in vitro protein synthesizing systems has resulted in preferential incorporation of one or another amino acid from the precursor pool.

These data offer evidence of controlled specificity in the replication taking place in the in vitro systems. However, the ultimate specificity is production of biologically active molecules. As yet, no convincing evidence has been found for the presence of such biological specificity in in vitro synthesized molecules.

The opposite approach to this problem presents contrary advantages and difficulties. The protoplast-subviral system described here is one example of such a contrary approach. The first step has been taken in the reduction of the bacterial-viral system of replication to an in vitro system by removal of part of the bacterial cell wall, thereby permitting infection of the resulting protoplast by subviral particles.

While this "first step" is a minor one toward the final goal, already the protoplast system is coupled to the in vitro system by providing a test for the biological activities of products from in vitro synthesis. For example, the product of the Kornberg DNA polymerase system, using ϕX-DNA as primer, has been tested for biological activity, (unpublished results). The results indicated that by the time the ϕX-DNA had served as primer once, all biological activity was lost. No activity returned even after incorporation had reached 10 times the initial level.

It is hoped that further steps can be taken in reducing the protoplast system toward an in vitro system. Certain of the results reported in Part II may be of value in furthering this reduction. However, the primary purpose of Part II is to outline the basic techniques used in studying the initial process of replication in the intact protoplasts.

MATERIALS AND METHODS

Several new techniques and changes in stock solutions have

been introduced subsequent to Part I which need to be described.

Lysozyme: Worthington lysozyme is dissolved at a concentration of 2 mg/ml. in 0.25M Sigma 121 "tris" buffer pH 8.1.

Nutrient Broth: The "nutrient broth" contains 0.2 % $MgSO_4$ instead of 0.1%. For making protoplasts, "nutrient broth" without $MgSO_4$ is used. Otherwise it is the same as described in Part I.

Protoplasts: Cells of E. coli K12, W6, are grown at 35-37°C with aeration in 3XD medium as in Part I. Twenty ml. of cells at 5×10^8/ml. are centrifuged. The pellet is resuspended in 0.35 ml. of 1.5M sucrose. The following solutions are added in order with gentle mixing after each: 0.17 ml. of 30% BSA, 0.02 ml of 2 mg/ml. lysozyme in 0.25 M tris pH 8.1, 0.04 ml. 4% versene, and 10 ml. of "nutrient broth" without $MgSO_4$. After the mixture is incubated at room temperature for 10-15 minutes, 0.2 ml. of 10% $MgSO_4$ is added to remove the versene and stop the reaction. This is the "protoplast stock" and may be kept at room temperature for about an hour or stored on ice for several hours. Note that 20 ml. of cells at 5×10^8/ml yield 10 ml. of protoplasts at 1×10^9/ml.

Protoplasts made in this way produce approximately 10 times as many virus per DNA molecule added as the method described in Part I or any of the several intervening methods. Infection versus DNA concentration is always or nearly always linear as opposed to the drastically non-linear assay shown in Part I. The results of a representative infection experiment are presented in fig. 7.

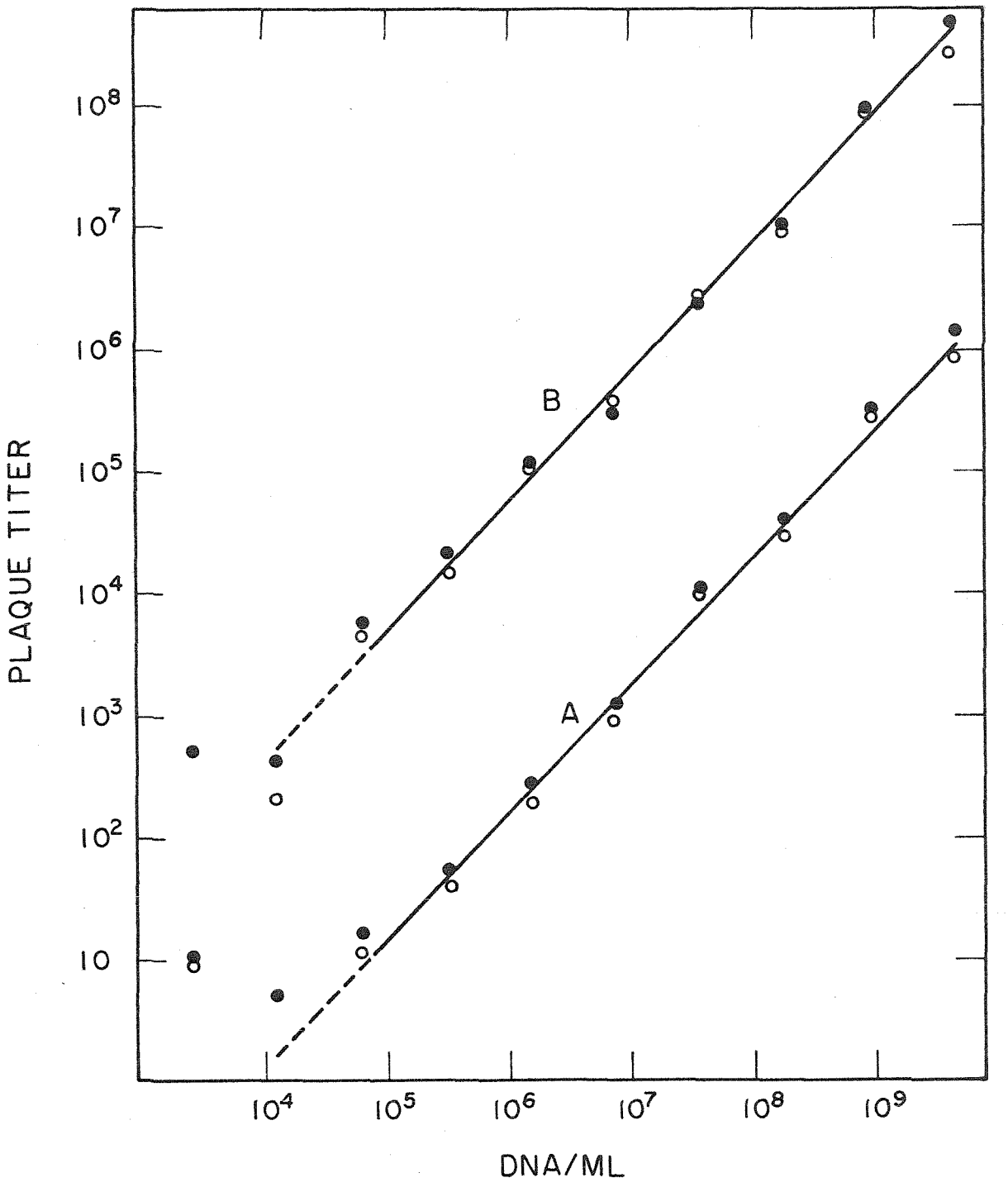

Figure 7. Results of an infectivity versus DNA concentration experiment. Curve A represents the infective centers at the end of the adsorption period, i.e., the infected protoplasts. Curve B presents the final titer of mature phage. The two sets of data (● and o) are independently diluted, duplicated runs.

Large Scale Assay: One of the primary uses of the protoplast system is the assay of "drop fractions" from the preparative density gradient. For this reason it is often desirable to assay many samples with one protoplast stock. Protoplasts are made by scaling up the described protoplast procedure. Drop samples are diluted into 0.4 ml. of 0.05M tris buffer, pH 8.1. By means of an automatic syringe (aupipette)*, 0.4 ml. of protoplast stock are added per sample tube. After 10 minutes at 35° C, 3.2 ml. of "nutrient broth" containing 0.2% $MgSO_4$ and prewarmed to 35° C are added with another aupipette. In this way 150-200 samples can be infected and diluted within the 10 minute time limit. By adding solutions with an automatic syringe, no further mixing is required.

Hot Phenol Extraction of DNA: Previously described methods (cold phenol and 4M $CaCl_2$) of extraction of φX-DNA are limited in use to concentrations of virus of 0.1 mg/ml. or greater and yield 40-60% DNA. By use of a hot phenol extraction, first suggested by Huppert (27), lower concentrations of virus may be used and yields above 60% DNA are obtained.

One volume of borate (sodium tetra borate saturated at 4° C) at 70° C is mixed with 5 volumes of phenol (redistilled and liquefied phenol) also at 70° C. The mixture is allowed to equilibrate, and the phases are allowed to separate at room temperature.

* Aupipettes are available in various sizes from Becton, Dickinson, and Co., Rutherford, N. J.

One volume of the equilibrated phenol is brought to 70° C. An equal volume of virus sample is separately brought to 70° C*. The two volumes are combined and alternately shaken and re-heated to 70° C for 3 minutes. The mixture is then brought to room temperature and centrifuged at 1000 g for 5 minutes to separate the layers. Two additional room temperature phenol extractions are performed on the aqueous layer, and the three phenol layers are serially re-extracted with 1/2 volume saturated borate. The two aqueous layers are combined and the residual phenol extracted at 0° C with an equal volume of ether, three to five times. The ether is then removed by dialysis or bubbling nitrogen through the sample. Such extractions can be successfully carried out on as little as 10^{-4} mg/ml. of virus.

Centrifugation and Recovery of Infected Protoplasts: Infected protoplasts can be centrifuged and washed by use of a sucrose layer technique and a swinging bucket centrifuge. For this centrifugation the Spinco Model L SW 25 and SW 39 rotors have been used. The rotor used depends on the volume needed: the SW 25 has three tubes of about 35 ml. each; the SW 39, three tubes of about 5 ml. A diagram of a sucrose layered tube is shown in Figure 8.

Depending on the particular experiment, layering can be

* For low concentrations of virus, about 1 mg of powdered BSA is previously added to each ml. virus stock.

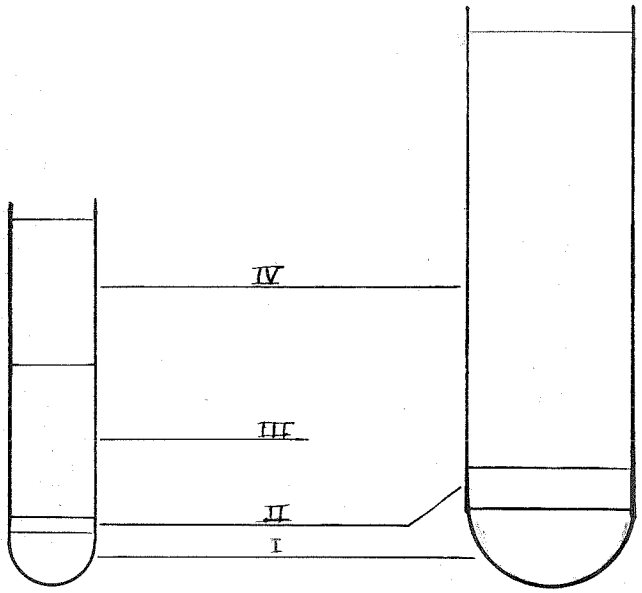

Figure 8. Sucrose layered tubes for centrifugation of protoplasts. Layer I is 4M sucrose and must be high enough to at least fill the rounded portion of the tube. Layer II, a mixture of nine parts 4M sucrose and one part 30% BSA, is 2-5 mm thick. The protoplasts sediment into this layer. Layer III, a mixture of equal volumes of 1.5M sucrose and "nutrient broth", serves as a wash for the protoplasts provided the sample volume is small enough to allow for inclusion of this layer. Layer IV is the sample.

done from bottom to top or by under layering with a capillary pipette. For larger sample volumes the latter is often easier; the order of addition therefore being IV, I, II (omit III).

For removal of layers a Pasteur pipette, whose tip has been bent at a right angle to the axis, is used to avoid undue convection. The layers may also be fractionated by drop collection from the bottom of the tube.

EXPERIMENTS AND RESULTS

Optimum Conditions for Adsorption and Incubation: Four

essential, externally controlled parameters are influential in the adsorption and incubation mixture in protoplast infection: the concentration of sucrose, $MgSO_4$, bovine serum albumin, and tris buffer. The optimum concentration of tris will be discussed in the next section.

Bovine serum albumin, at a concentration of 0.5% in the "protoplast stock", gives highest yields of phage in the final incubation mixture. This assumes the standard dilution sequence of equal volumes of protoplasts and DNA for the adsorption mixture followed by a five fold dilution into "nutrient broth" for further incubation. No significant difference in the production of phage exists among the sterile 30% BSA solution, which contains 0.1% sodium azide; the purified powdered "fraction V" Bovine plasma (obtained from Armour and Co.); and a sample of bovine albumin obtained from J. Huppert which gives optimum results in his system.

The optimum concentration of $MgSO_4$ in weight/volume percent varies slightly with different protoplast stocks but is approximately 0.1% for adsorption and 0.2% for incubation.

In order to achieve good infection of protoplasts with ϕX-DNA, the concentration of sucrose must be reduced during adsorption and raised again for the incubation period. Average values for these optima are 0.15 to 0.05M for adsorption and 0.3 to 0.5M for incubation.

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*The sodium azide is added to the BSA solution by Armour and Co. as a bateriostatic agent.

Effect of the Presence of Various Substances on Infectivity:

Several substances have been tested for their effect on the infection of protoplasts by ϕX-DNA during the adsorption period. Some of these substances are unavoidably present in samples of ϕX-DNA. For example, ϕX-DNA recovered from a column fractionation is in a solution containing 1M NaCl, while fractions from a density gradient contain CsCl.

Initially the optimum concentration of tris buffer in which the DNA is added to the adsorption mixture was determined. Fig. 9 demonstrates that an optimum concentration for tris exists. All other experiments investigating the effect of salt concentration were done in the presence of 0.05M tris pH 8.1. In the absence of tris the shapes of the curves for other salts are the same but the phage production is lower. The reason for this tris optimum has not been investigated.

Fig. 10 shows the effect of three salts present in the DNA samples at the time of infection. The molar values are those in the DNA sample tube, i.e., the sample half of the adsorption tube. As with the tris buffer, there is a marked inhibition of infection at about 0.1M or greater. Other experiments indicate that the inhibition is most pronounced during the adsorption period; however, if the adsorption is carried out in low salt and the subsequent incubation in high salt, a partial inhibition is still observed. Unlike tris, these salts do not cause the infection to exhibit an optimum value.

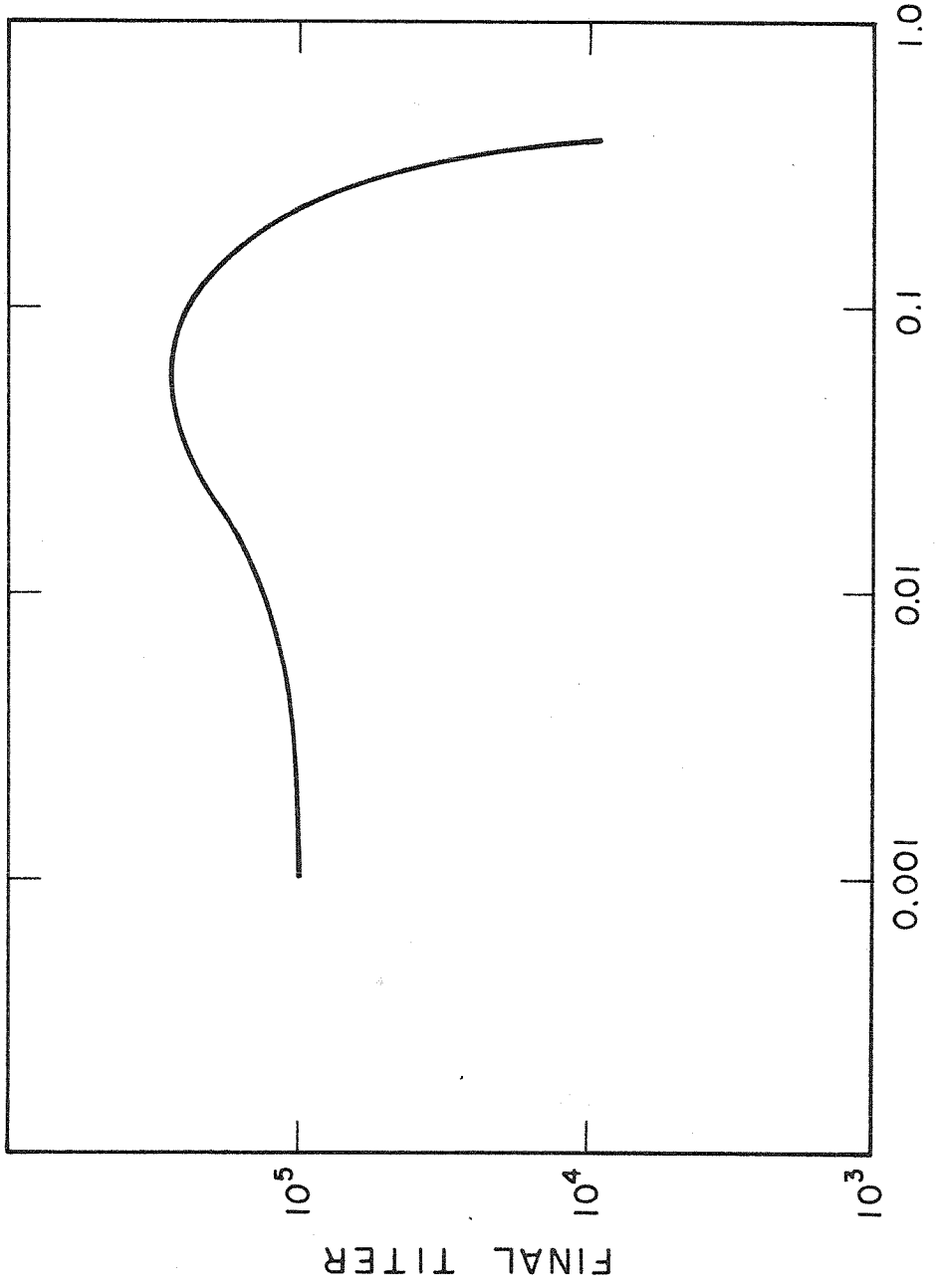

Figure 9. The effect of the tris concentration in the DNA stock on phage production. Sigma 121 grade tris at pH 8.1 was used.

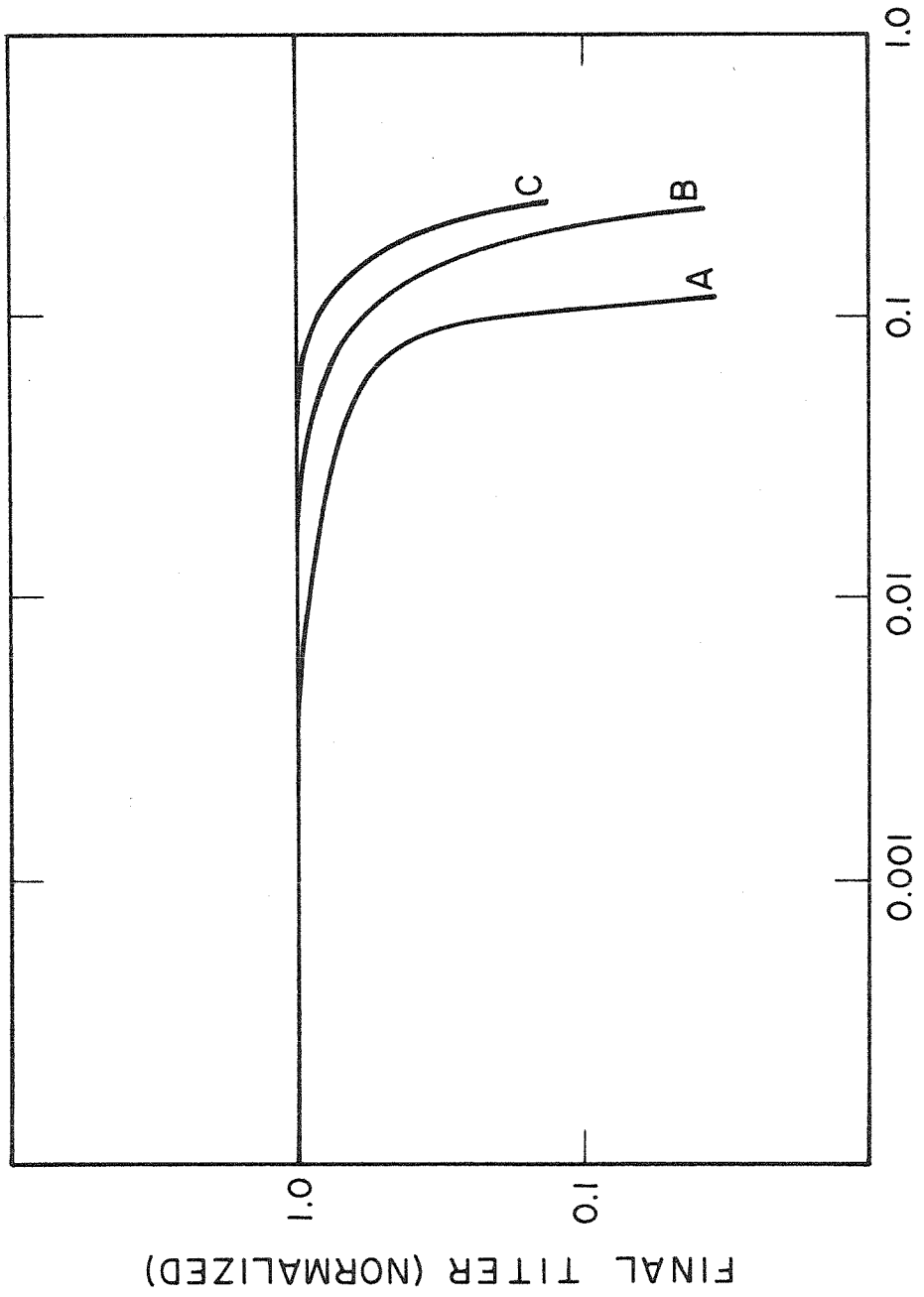

Figure 10. The effect of the presence of various salts on infectivity. Curve A - CsCl; Curve B - NaCl; Curve C - KCl. Each DNA sample also contained 0.05M tris pH 8.1. The titers represent the fraction of phage produced as compared to the standard DNA assay in 0.05M tris pH 8.1.

The pH optimum is rather broad; its limits seem to be determined primarily by the precipitation of BSA at low values and the lysis of protoplasts at high values. Infection is constant between pH 7 and 8.5. Since the normal buffering of the adsorption mixture is due to the BSA and is in the region of pH 7, any adjustment of the pH must be accomplished by the addition of a significant amount of salt. For this reason critical studies on the effect of pH on adsorption have not been done.

Table 1 summarizes the effects on phage titer of various other substances present during the adsorption period. The two E. coli extracts were selected because of the possibility of their presence during infection. The results with irradiated ϕX-DNA do not necessarily reflect the effect of ϕX-DNA inactivated in other ways. The "70S extract" was made by repurifying the 70S sample (17) using an additional isolation from a RbCl density gradient and then extracting by the hot phenol method. Cohen and co-workers (28) reported that addition of the nucleic acid from the 70S fraction increased the infectivity of the ϕX-DNA even though this fraction was not active alone. The results in Table 1 do not confirm this finding.

The optimum temperatures for adsorption and incubation after adsorption were determined. Table 2 summarizes the results of infection and incubation at several temperatures. The first column of titers in the table gives the concentration of infected protoplasts at five temperatures. For the remaining data each adsorption mixture was diluted and incubated at all five temperatures.

TABLE 1

Substance Added	Amount	Inhibition in % of Control
Extract of E. coli K 12 nucleic acid[1]	~ 40 ug	94
	4 ug	16
	0.4 ug	0
Lysed, uninfected protoplasts	Equivalent to 4X "protoplast stock" added	97
	2 X stock	75
	1 X stock	30
	0.5 X stock	0
Ultraviolet inactivated ϕX-DNA[2]	100 X infecting ϕX-DNA	0 to 18
	10 X	0
	1 X	0
"70 S DNA"	Equivalent in optical density to:	
	2×10^7 X infecting ϕX-DNA	96
	2×10^6	32
	2×10^5	16
	2×10^4	8

Table 1. Summary of the effect of various extracts present in the adsorption mixture on the infection by ϕX-DNA.

1a). The nucleic acid extract was isolated from protoplasts. After the protoplasts were lysed by dry ice-acetone freezing three times the lysate was extracted with phenol at room temperature, precipitated with isopropanol at 0°C and redissolved to the original volume in 0.1M tris pH 8.1. The extract was then heated for 15 minutes at 70°C to remove any residual DNAase activity.

b). Infection of protoplasts by RF (29) is inhibited to essentially the same degree when these concentrations of extract are used.

c). The amount of nucleic acid added is estimated from the absorbency; 40 μg is assumed to be the nucleic acid in 1 ml. having an optical density of 260 mμ of 0.8.

2). Since the infection was performed with 10^7 unirradiated ϕX-DNA molecules/ml. and 5×10^8 protoplasts/ml., interference due to a random selection of inactivated molecules would have caused a drastic inhibition in the 100X and 10X samples. However, since the multiplicity of total DNA (irradiated plus unirradiated) to protoplasts is never more than two, interference resulting from multiple infection would not be observed.

The final titer for each initial sample is listed in the last five columns under the appropriate incubation temperature. These data suggested that the incubation temperature exhibited the greatest influence on the production of mature phage. This interpretation was checked with data from a growth curve experiment on infection mixtures at 25° and 30° C. The results, shown in fig. 11 demonstrate that the titers at 120 minutes, the time at which the assays listed in Table 2 were performed, are very dependent on temperature; however, if the production of virus is allowed to continue until phage titer remains constant, the difference in concentration of mature virus approaches the difference of the initial infective center titer. Further investigations have shown that the titer of mature virus decreases above 37° C. From the above data the optimum temperature for adsorption is considered to be 30° C and for subsequent incubation 35° C.

TABLE 2

Adsorption Temperature	Infective Center* Titers X 10^{-4}	Final Titer x 10^{-6}*				
		25°	27.5°	30°	32.5°	35°
25°	1.1	1.6	6.5	13.4	14.8	17.5
	0.91	0.8	6.7	11.5	9.4	12.3
Average	1.0	1.2	6.6	12.5	12.1	15.4
27.5°	1.8	1.2	7.4	13.8	13.5	16.8
	1.47	0.9	4.9	8.4	12.7	11.7
Average	1.64	1.1	6.2	11.1	13.1	14.3
30°	2.5	1.5	7.0	13.9	17.4	17.6
	2.28	1.3	7.2	13.6	13.8	13.5
Average	2.39	1.4	7.1	13.8	15.6	15.5
32.5°	2.84	1.4	8.7	8.0	10.7	15.7
	2.18	1.4	7.3	10.3	10.7	14.9
Average	2.51	1.4	8.0	9.2	10.7	15.4
35°	2.1	1.5	6.3	8.5	11.4	10.8
	2.1	2.1	5.6	7.7	11.2	9.5
Average	2.1	1.8	6.0	8.1	11.3	10.2

Table 2. Results from infection of protoplasts at various temperatures. Each infection mixture was diluted after the adsorption time and incubated at the five temperatures.

*Infective center assay was made at 15 min. after mixing DNA and protoplasts; final titers were made at 120 min.

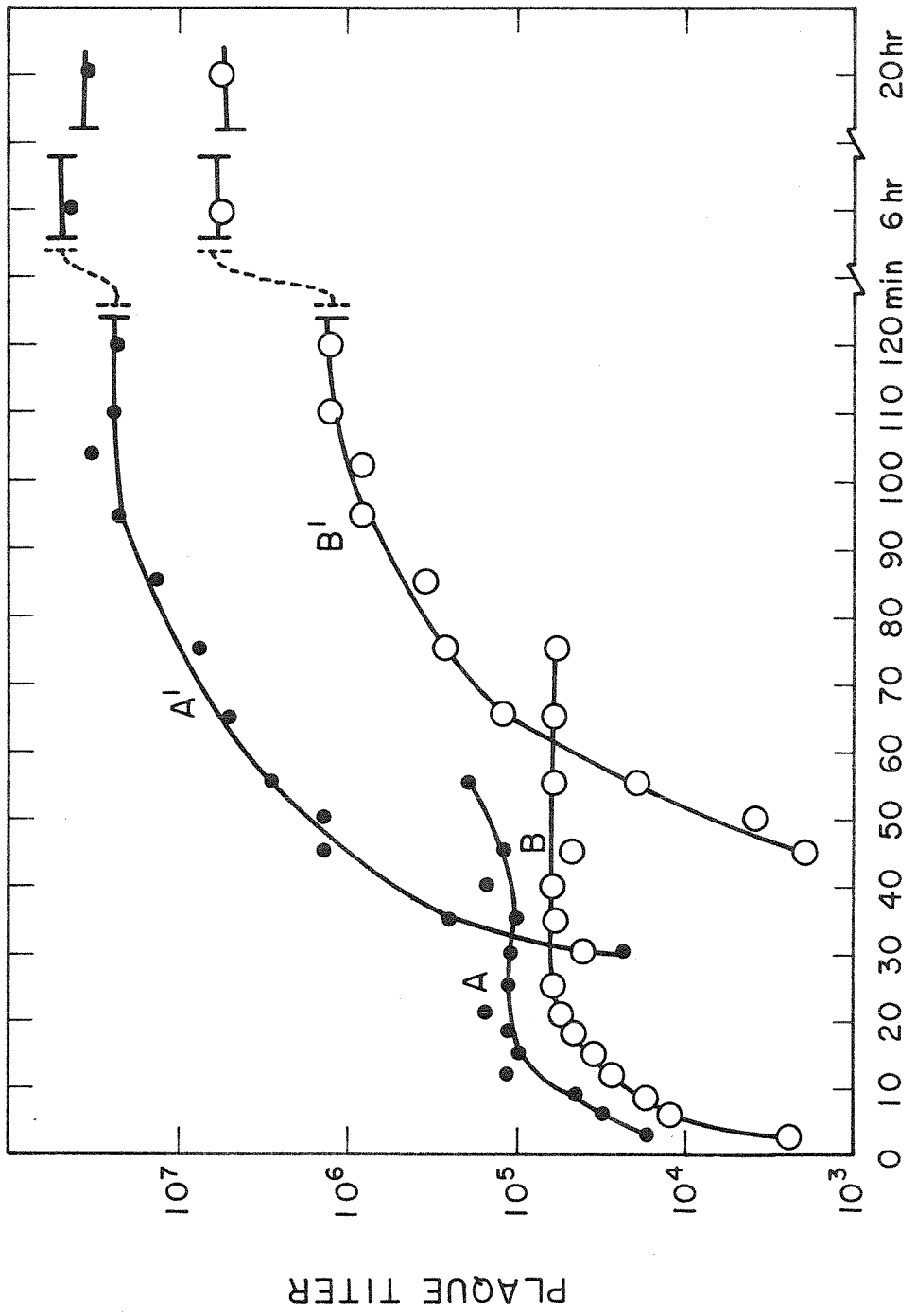

Figure 11. Growth curves of φX174 from adsorption mixtures at 25° and 30° C. Curve A represents infected protoplasts at 30°; Curve A' mature phage at 30°. Curves B and B' represent the same titers at 25° C.

FIRST STEPS TOWARD THE STUDY OF INFECTED PROTOPLASTS AS A REPLICATING SYSTEM

Fate of Non-infecting φX-DNA: The experimental results presented thus far have been for the purpose of defining an assay system which can be used in conjunction with a standard φ X-DNA dilution curve to determine the concentration of infectious sub-viral particles in an unknown sample.

Before proceeding further and attempting even the initial steps in the study of the protoplast replicating system, the current status of the understanding of φ X-DNA will be reviewed. It was shown in Part I that the infectivity of a preparation of φ X-DNA banded in a cesium chloride density gradient coincides with the ultraviolet adsorption of the φX-DNA. Furthermore, for several, independently isolated samples the specific infectivity (infective units per optical density unit) has been constant across the band within the accuracy of the assay system. Preparations of φ X-DNA, therefore, appear to be homogeneous in infectivity with respect to density isolation.

Recently, however, Fiers (30) has shown that phenol extracted φ X-DNA contains two species of DNA, called S_1 and S_2 because of their differing sedimentation coefficients. The amount of infectious φ X-DNA in any sample correlates with the amount of S_1 component present. The S_2 fraction has the same molecular weight and the same density in CsCl as the S_1 fraction but contains no infectious DNA.

Specific activities (infected protoplasts per DNA molecule)

in protoplast infection have always been calculated on the basis of the optical density of a φX-DNA sample* or (vide infra) from the specific radioactivity according to ^{32}P label. Such methods were based on the previous assumption that the material isolated from a CsCl density gradient was homogeneous with respect to physical properties and therefore could not be further separated into infectious and non-infectious fractions. As a result of Fiers' investigations, it is clear that only the S_1 fraction contains infectious molecules. However, the S_1 fraction always represents a significant percent of the total. Thus the fraction containing the biological activity (the S_1 fraction) still represents from 10-80% of the total absorbency. Biological specific activities (calculated from infective center data at early times, vide infra) range from 10^{-3} to 10^{-4} infective centers per DNA. Therefore even correcting for the probable S_1 content of a particular DNA sample, only a small fraction of these S_1 molecules infect protoplasts to form infective centers.

Early experiments (cf. fig. 5, Part I and fig. 12, Part II) had indicated that such low efficiency at higher concentrations of φX-DNA might reflect a heterogeneity in the protoplast population.

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* The number of particles/optical density unit can be calculated from the E(p) for φX-DNA and the influence of salt concentration and temperature on the E(p). These data are given by Sinsheimer (18). For 0.2M NaCl at 25° C the conversion factor is: 1.33×10^{13} particles per optical density unit (Beckman DK 2 Spectrophotometer, one centimeter cell). The conversion for optical densities taken in 0.1M tris buffer at 25° C is 1.0×10^{13} particles per optical density unit.

** The S_1 content of φX-DNA samples used throughout this work was not determined. In all cases where number of DNA molecules is given the calculations are made from the total absorbency using the conversion method described in the preceding footnote.

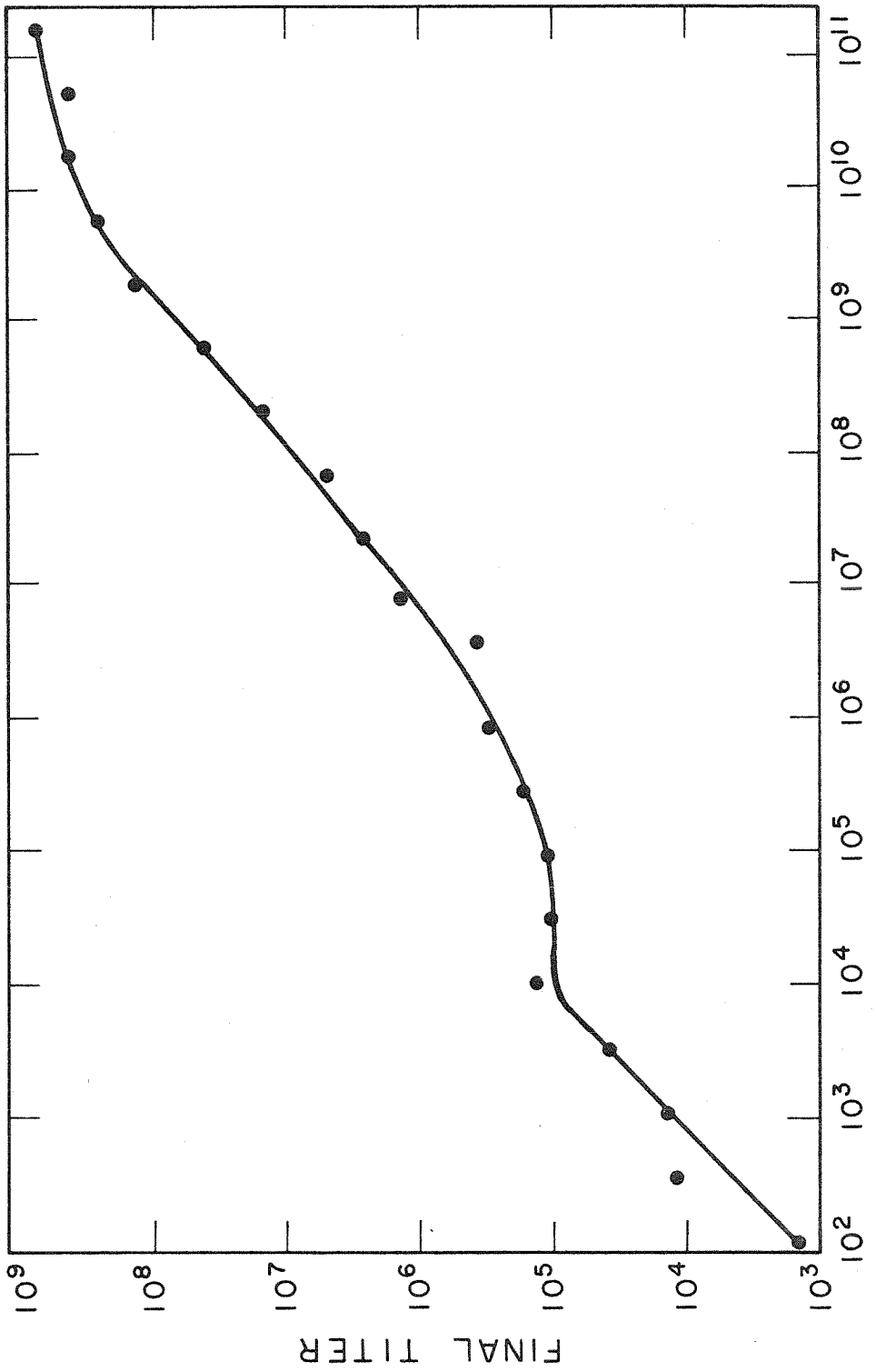

Figure 12. Results of a dilution curve of φX-DNA using protoplasts made by the method in Part I. The values are titers of mature phage/ml. of the adsorption tube.

This conclusion was supported by the increase in efficiency at low DNA concentrations, if some selective infection process were postulated.

In addition, these data demonstrate that under proper conditions a net synthesis of DNA, normalizing to the amount of DNA added to the adsorption tube, does occur, suggesting that a significant fraction of the initial DNA was infectious. Furthermore, a change in the method of forming protoplasts changes the response of the protoplasts to infection at both high and low concentrations of DNA (causing an increase in efficiency at high concentrations and removing the "ultra-efficient" segment of the dilution curves formerly observed--comparing figs. 12 and 7). The results of salt and buffer studies (figs. 8 and 9) demonstrate a strong dependence of infection on the ionic conditions in the adsorption tube. All of these data permit the hypothesis that possibly all DNA molecules of the S_1 fraction are potentially infectious and that their infection is limited by other parameters. Therefore, experiments have been done to investigate both the fate and nature of the DNA which does not initiate infection in the adsorption tube.

Infection of Protoplasts by Phosphorus 32 Labeled ϕX-DNA: Tessman (19) has shown that α, the efficiency of inactivation from ^{32}P decay, is 1 for ϕX174. Therefore, since a single radioactive decay will inactivate a DNA molecule, the specific activity of ϕX174 is limited to an average of 0.3 ^{32}P atoms per DNA molecule or a maximum of about 10^{-6} cpm per plaque forming phage*.

* Sinsheimer, et al. (29) discusses the method of preparing ϕX174 labeled with ^{32}P for experiments involving the use of the labeled nucleic acid.

With this limit for the specific activity it is not possible to study the infecting DNA molecules, but it might be possible to gain information about the non-infecting molecules which make up more than 99% of the total.

ϕX-DNA was extracted from labeled ϕX174* by the hot phenol method. The number of DNA molecules present was calculated from the radioactivity and the known specific activity of the virus sample. Such a calculation implies essentially the same assumptions as those used in calculating the number of particles from ultraviolet absorbency.

Protoplasts at 5×10^8/ml were infected with labeled DNA at 1×10^9/ml. After adsorption for 10 minutes at 30° C, the mixture was centrifuged in the SW 25 rotor at 10,000 rpm for 5 minutes. The supernatant was removed from each tube; the protoplasts were resuspended to the original volume in "nutrient broth" containing 0.5% BSA and centrifuged again. The second supernatant was removed, and the protoplasts resuspended as before.

Biological assay indicated quantitative recovery of the infected protoplasts as compared with an uncentrifuged control. The combined radioactivity of the first and second supernatants accounted for approximately 50% of the input radioactivity; that of the resuspended protoplasts for 10%. The infected protoplasts indicated that 10^{-4} of the total input DNA or 10^{-3} of the DNA remaining with the protoplast fraction after centrifugation had

* Labeled ϕX174 was provided by Mr. D. Denhardt and Dr. A. Burton.

formed infective centers.

From such results it can be concluded that (a) a significant fraction of the initial DNA particles remains in the supernatant after infection is complete, and (b) even though only 10% of the radioactivity remains with the protoplast fraction, this 10% still represents 10^3 DNA molecules per infective center.

Investigations of the Supernatant from Infected Protoplasts: In order to observe directly the state of the ϕX-DNA which does not remain associated with protoplasts after infection, the supernatants of adsorption mixtures were investigated after centrifugation.

Sucrose layer tubes were made up as shown in fig. 8 excluding the sample layer. Protoplasts and DNA (multiplicity = 1) were mixed and incubated for 15 minutes at 35° C. Sufficient concentrated sucrose was added at 15 minutes to increase the sucrose concentration from the 0.15M of the adsorption mixture to 0.3M. Part of the sample was then diluted to incubate as the control. One and one half ml. of the sample were added to the centrifuge tube and centrifuged for 12 minutes at 5,000 rpm in the SW 39 rotor.

After centrifugation, the top layer was removed with a pipette, then isolation of the protoplast band (layer II) was accomplished by drop collection through a hole in the bottom of the tube. The protoplast fraction was adjusted to the original volume and diluted for incubation. The top layer which should contain the

unadsorbed ϕX-DNA was diluted 125 times into 0.05M tris and used to reinfect protoplasts. At the same time a control sample of ϕX-DNA which had been diluted 1:1 into broth-BSA was also diluted 125 times and used to infect protoplasts.

The summary of the results of this experiment is given in Table 3. Other experiments of this kind have also indicated a recovery of supernatant ϕX-DNA equal to about 50%.

Protoplast control (infected, uncentrifuged protoplasts)	2.5×10^7
Centrifuged protoplasts	2.14×10^7
Percent recovery of infected protoplasts	85.5%
Control ϕX-DNA (ϕX-DNA mixed with an equal volume of broth containing 0.5% BSA)	2.32×10^7
Fraction IV (the adsorption mixture after removel of protoplasts by centrifugation)	1.67×10^7
Percent infectious ϕX-DNA remaining in the adsorption mixture:	
compared to the ϕX-DNA control	72%
compared to the original infectivity	66.8%

Table 3. The results given have been corrected to the original stock ϕX-DNA tube in order that comparisons may be made directly.

These results clearly indicate that a significant fraction of the initial ϕX-DNA remains in the adsorption tube as infectious particles. The exact fraction undoubtedly varies, and occasionally no infectious material has been recovered. These occasional failures are presumed to be caused by unstable protoplasts or

by poor technique in the centrifugation steps.

Investigations on the Protoplast Fraction of Centrifuged Infection Mixtures: The results of infection with ^{32}P labeled ϕX-DNA have shown that a significant fraction (at least 10%) of the DNA added to the adsorption mixture remains associated with the protoplasts after centrifugation. In order to investigate the fate of this DNA directly, density gradient centrifugation in CsCl was performed on the protoplasts recovered from a centrifuged adsorption mixture. Table 4 outlines the procedure used for infecting and centrifuging the protoplasts.

The initial adsorption mixture contained protoplasts and ϕX-DNA at 6×10^8/ml each. Infective center assay indicated 10^5 infected protoplasts/ml. In the next step of the experiment each density gradient tube contained 3 ml. of the final frozen samples. Since these frozen samples are 3.5 times as concentrated as the initial mixtures, each density gradient tube contained: $3.5 \times 6 \times 10^8$ protoplasts/ml x 3 ml = 6.3×10^9 protoplasts. From the infective center data the two tubes containing infected samples had 3.5×10^5 infective centers/ml x 3 ml = 1.05×10^6 infective centers. From these data, at least 1×10^6 infective DNA molecules were known to be present in each gradient tube. However, from the results of the experiment with ^{32}P labeled ϕX-DNA, at least 10% of the initial ϕX-DNA or 6.3×10^8 ϕX-DNA molecules should be present in each gradient tube. Since the only evidence for the presence of this fraction of the

1. Chloramphenicol, to a final concentration of 10 μg/ml. was added to the protoplast stock 10 minutes before infection.
2. Protoplast stock and φX-DNA in 0.05M tris pH 8.1 were mixed in a volume ratio of 3 to 2. For a control protoplasts were mixed in the same ratio with 0.05M tris.
3. The mixtures were incubated at 30° C for 15 minutes.
4. 50 ml. of infected protoplasts (2 tubes) and 25 ml. of control (1 tube) were centrifuged in the SW 25 rotor at 10,000 rpm for 10 minutes.*
5. The supernatants were removed, and the protoplast layers were resuspended from the sucrose pad which covered the bottom of the tube. Resuspension was made in "nutrient broth" containing 0.2% $MgSO_4$, 0.5% BSA, and 10 μg/ml. chloramphenicol. Samples were resuspended to their original volume.**
6. The samples were centrifuged as before.
7. The supernatant was removed and the protoplasts resuspended in the same medium except that chloramphenicol was omitted.
8. The samples were centrifuged a third time.
9. The protoplasts in the three tubes (1 control and 2 infected samples) were then resuspended in about 7 ml. "nutrient broth" per tube.
10. A sample of the infected protoplasts was incubated to determine the recovery from the centrifugation. The three samples were frozen individually in a dry ice-acetone mixture and stored in the freezer (-40° C).

Table 4. An outline of the procedure used for centrifuging, washing, and recovering infected protoplast samples.

* Samples were centrifuged as outlined in fig. 8 using the "large sample" method. The refrigerator of the Model L centrifuge was regulating at 2.5° C during the entire experiment; however, the rotor was at room temperature. All other operations except adsorption were performed at room temperature (about 25° C).

** 0.5% BSA contains sufficient sodium azide to inhibit the metabolism of the protoplasts. It should be noted that for the production of mature viruses by protoplasts it is necessary to dilute the adsorption mixture five fold thus reducing the concentration of sodium azide to a value which is not inhibitory.

DNA is radioactive label, nothing is known about its biological condition. The two values, 6.3×10^8 and 1×10^6 DNA molecules set the upper and lower limits for the added infectious ϕX-DNA per gradient tube. Because the infection and washing was done in the presence of 0.0017% sodium azide (from the BSA) and, for all but one centrifugation, in the presence of chloramphenicol, very little multiplication of ϕX-DNA in infected protoplasts would be expected.

The density gradient banding experiment consisted of three samples: For number one, 3 ml of the control, uninfected protoplasts, was dialyzed against saturated borate in the cold to remove the sucrose. The sample was then brought to a density of 1.637 g/cm^3 with CsCl. To the final volume of 4.9 ml. was added a total of 10^9 ϕX-DNA molecules. For number two, 3 ml. of the infected sample was dialyzed against saturated borate in the cold and brought to a density of 1.652 g/cm^3 with CsCl giving a final volume of 4.9 ml. For number three, 3 ml. of the infected sample was extracted at room temperature with phenol three times. The phenol was re-extracted serially with about 1 ml. borate. The re-extraction borate was added to the sample and the mixture dialyzed against saturated borate in the cold. Sample three was then brought to a density of 1.65 g/cm^3 with CsCl in a final volume of 4.9 ml. During the dialysis procedure, each sample received three changes of approximately 30 volumes of saturated borate.

The samples were centrifuged at 2.5° C in the SW 39 rotor--Model L centrifuge for 48 hours at 37,000 rpm. The rotor was brought to rest without braking. Each tube was fractionated dropwise from a small hole made in the tube bottom with the drops individually collected in 1 ml. of borate collection buffer*.

For the biological assay, 0.04 ml. of each 1 ml. fraction was diluted into 0.4 ml. of 0.05M tris pH 8.1. To this was added 0.4 ml. of protoplast stock, and after 10 minutes 3.2 ml. of "nutrient broth". All the fractions from one gradient tube and a standard dilution series of ϕX-DNA were assayed with the same stock of protoplasts. The final titers were then converted to infective particles per original drop.

The patterns of infective particle distribution for the three tubes are shown in figs. 13, 14, and 15. Since the values are corrected to infective particles per drop, the number of particles in the original gradient tube is just the sum of the values shown in these patterns. The control tube (fig. 13), according to the bio-assay of the individual fractions, contains a total of 1.7×10^9 ϕX-DNA particles. Only 1×10^9 DNA molecules had been added to this uninfected control. The ϕX-DNA standard curves for these assays apparently were indicating 70% more DNA than was actually present.

Figure 14, showing the distribution for the infected sample,

* Borate collection buffer is 0.05M sodium tetra borate containing 0.5 µg yeast RNA/ml., 5 µg BSA/ml., and 0.01% disodium versonate.

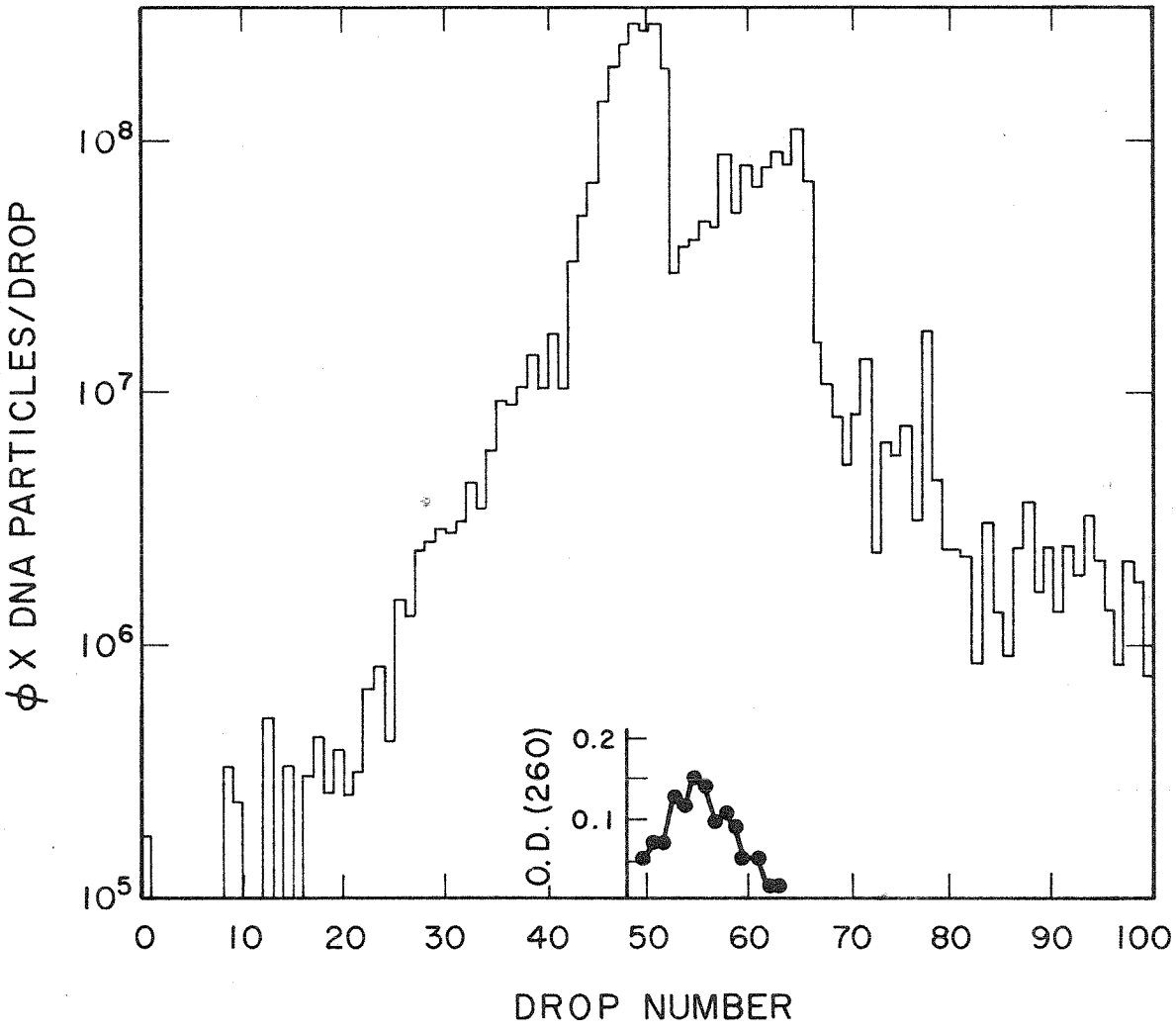

Figure 13. Infectivity pattern from a density gradient fractionation of 10^9 single stranded φX-DNA molecules added to a lysate of uninfected protoplasts. Total number of drops collected was 139. Optical density indicates the location of the E. coli DNA.

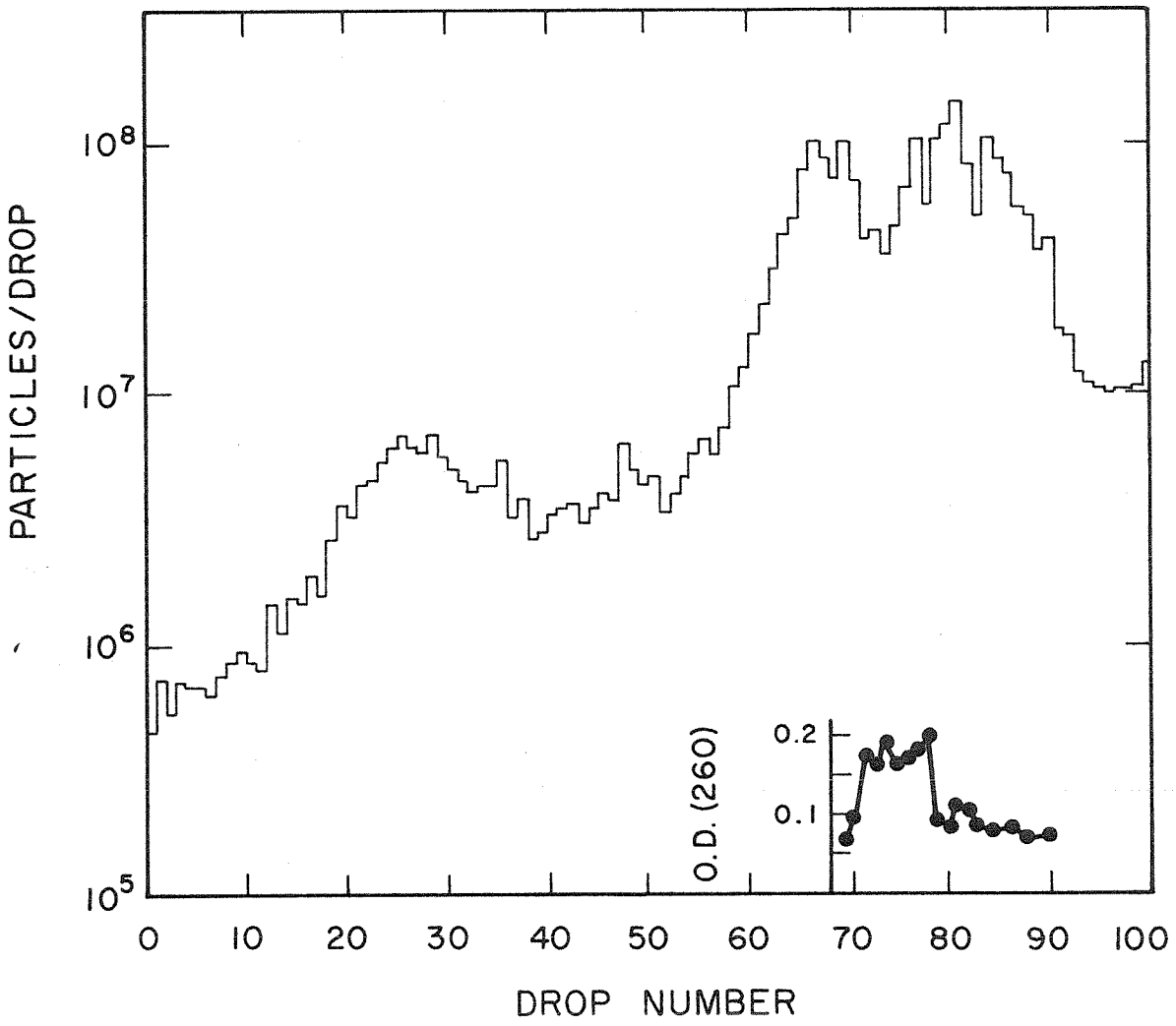

Figure 14. Infectivity pattern from a density gradient fraction of infected protoplasts. Total number of drops collected was 184. Optical density indicates the location of the E. coli DNA.

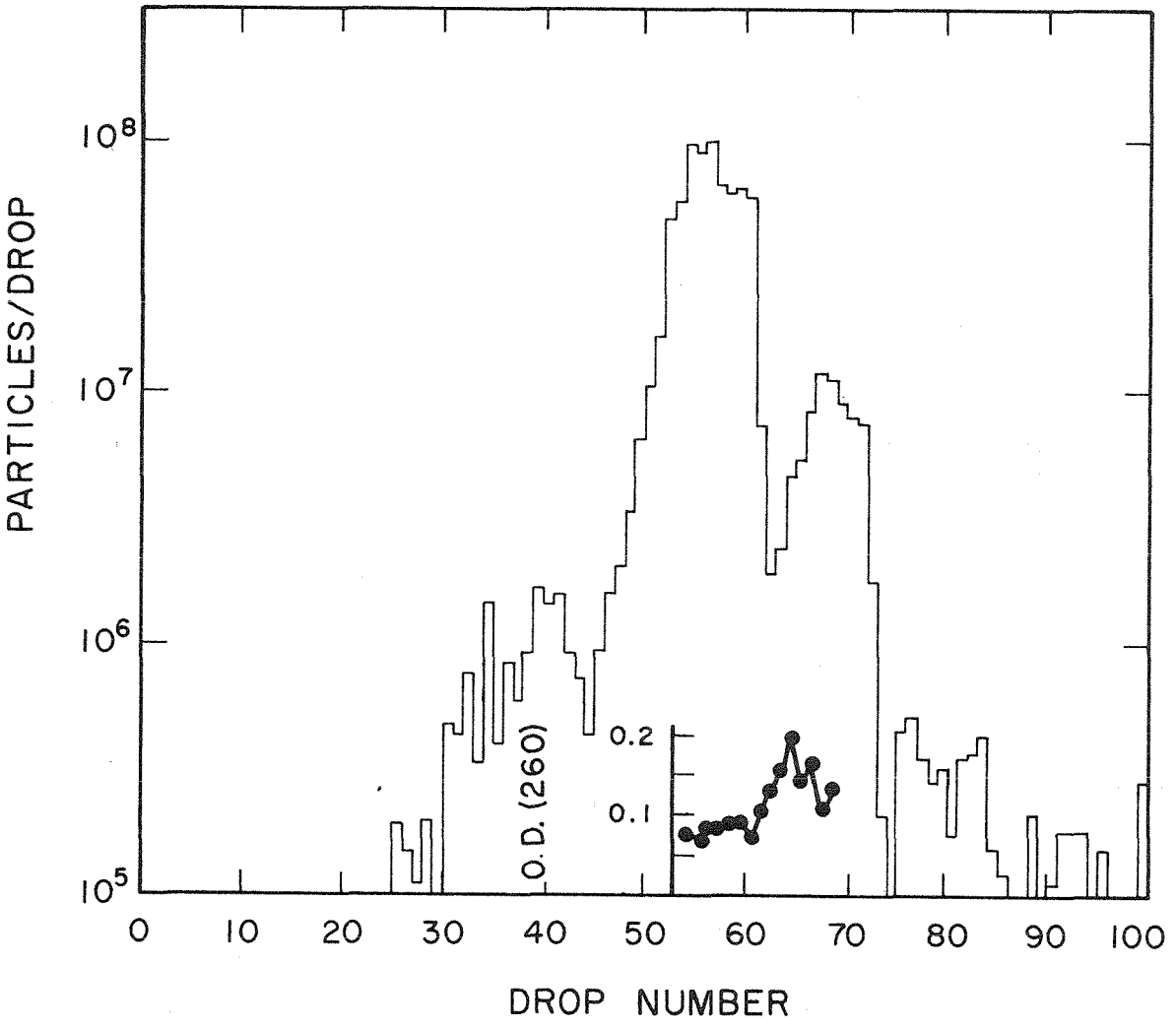

Figure 15. Infectivity pattern from a density gradient fractionation of phenol extracted, infected protoplasts. Total number of drops collected was 136. Optical density indicates the location of the E. coli DNA.

indicates a total of 6.9×10^8 ϕX-DNA particles in drops 59 through 71. Figure 15, giving the data for the infected protoplast treated with phenol, indicates 6.7×10^8 ϕX-DNA particles in drops 54 through 61. If the correction is made for the difference between the known ϕX-DNA added to the control tube and the amount shown by assay, the untreated, infected sample contains $(6.9/1.7) \times 10^8 = 4.05 \times 10^8$ ϕX-DNA and the phenol treated sample $(6.7/1.7) \times 10^8 = 3.95 \times 10^8$ ϕX-DNA.

The gradient tubes were titered before centrifugation and contained no whole virus. It is known (31) that in whole cells the rate of multiplication of phage nucleic acid (as RF) is retarded in the presence of chloramphenicaol. However, the centrifugation of the infected protoplasts prior to freezing required more than one hour. The infectivity pattern of the non-phenol treated sample (fig. 14) shows a higher infectivity in tubes immediately above (less dense) the E. coli band compared to those immediately below than does the control. In addition there appears to be a "shoulder" to the infectivity band on the dense side which is not present in the control. These data suggest that at least limited synthesis of infectious particles may have taken place. The nature of the infectious particles in these bands is not known nor is their presence seen in the phenol treated sample.

The major peaks of the two samples coincide with the single strand ϕX-DNA peak of the control sample. The amount of infectivity located in these peaks indicates that the original ϕX-DNA

added to the protoplasts and subsequently carried along with the protoplast fraction during centrifugation is infectious even though it did not form infective centers initially. The close agreement between the actual amount of this single stranded material found and the amount postulated is considered fortuitous since the ^{32}P label experiment from which the predictions were made was a separate experiment.

Investigations on the Initial Interaction of ϕX-DNA and Protoplasts: Another series of experiments can be cited which offers support to the results of the direct examination of infected protoplasts and the adsorption tube supernatant. These experiments concern the "reinfection" of the adsorption mixture with new protoplasts or new ϕX-DNA.

Earlier investigations (unpublished) had shown that ϕX-DNA could infect protoplasts from about 10 minutes before to 10 minutes after the protoplasts had been diluted 1:1 with 0.05M tris buffer. Therefore, if new ϕX-DNA is added to the adsorption mixture prior to 10 minutes after the initial infection, this DNA should be able to infect protoplasts which are capable of being infected, but which were not infected by the initial ϕX-DNA present at time zero. Conversely, the addition of new protoplasts should reflect the presence of the infectious ϕX-DNA still present in the adsorption mixture.

The experiment is outlined in Table 5.

TUBE	Content of adsorption tube at t = 0. Concentration in particles/ml. of adsorption mixture.	Addition at t = 7 minutes. Concentration in particles/ml of adsorption mixture.	Final titer in phage/ml. of adsorption mixture x 10^{-5}.
1	2.5×10^7 DNA 2.5×10^7 Protoplasts	none	4.5
2	2.5×10^7 DNA 2.5×10^7 Protoplasts	5×10^7 DNA	12
3	2.5×10^7 DNA 2.5×10^7 Protoplasts	5×10^7 Protoplasts	12
4	2.5×10^7 Protoplasts	5×10^7 DNA	3.4
5	2.5×10^7 DNA	5×10^7 Protoplasts	6.6

Table 5. Outline of a "reinfection" experiment. The adsorption tube consists of 1 volume of "nutrient broth" containing 0.5% BSA and 1 volume of 0.05M tris pH 8.1. All samples were diluted five fold for incubation at 14 minutes. Dilutions were made with nutrient broth containing magnesium sulfate. The experiment was performed at 35° C.

The increase in infection from the addition of new DNA or protoplasts is not as great as would be expected if the infection were simply additive. However, the results do demonstrate that in the original mixture there is present both infectious DNA and protoplasts capable of being infected even after the initial infection has been established.

The results of these experiments suggest that other factors may be limiting the level of infection in the adsorption mixture.

For this reason more detailed investigations on the initial interaction of ϕX-DNA and protoplasts have been initiated. These investigations have been concerned with the manner in which the final level of infection is influenced by the concentrations of the interacting particles (i.e. ϕX-DNA and protoplasts) as well as the temperature during adsorption.

The results of a dilution array experiment are shown in figs. 16 and 17. For this experiment a series of three fold dilutions of ϕX-DNA was made through a total of ten tubes. The highest concentration of the DNA stocks was 1.1×10^{10} particles/ml. In a similar manner serial dilutions of protoplasts were made in nutrient broth containing 0.5% BSA through five samples. The highest protoplast stock was at a concentration of 10^9 protoplasts/ml. A 5 x 10 infection array was thus obtained after mixing all combinations of DNA and protoplasts 1:1.

To present the results in graphical form, the titer (infective centers in fig. 16 and final phage yield in fig. 17) was plotted against the multiplicity (i.e. DNA molecules/protoplast). Data plotted in this manner illustrate directly the effects of diluting the DNA while keeping the protoplasts constant, diluting the protoplasts while keeping the DNA constant, and keeping the product [DNA] x [protoplast] constant. These three families of curves are indicated on the two graphs.

It is of particular interest to note that for multiplicities of

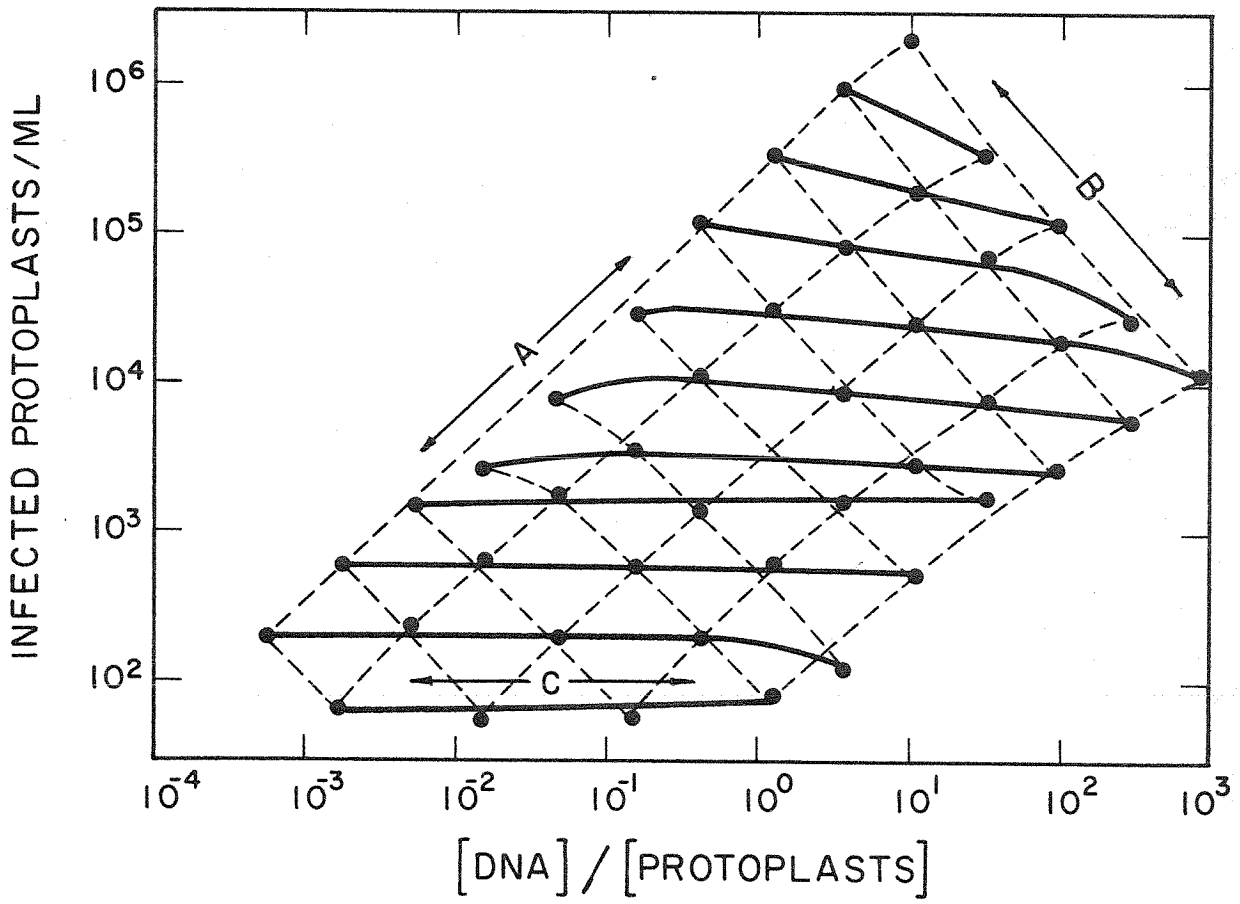

Figure 16. Results of a dilution array. The figure presents initial infective center assay, as infected protoplasts/ml. in the adsorption tube. The families of curves represent data for: dilution of DNA, [protoplasts] = constant (A curves); dilution of protoplasts, [DNA] = constant (B curves); varying the multiplicity, [DNA] X [protoplasts] = constant (C curves).

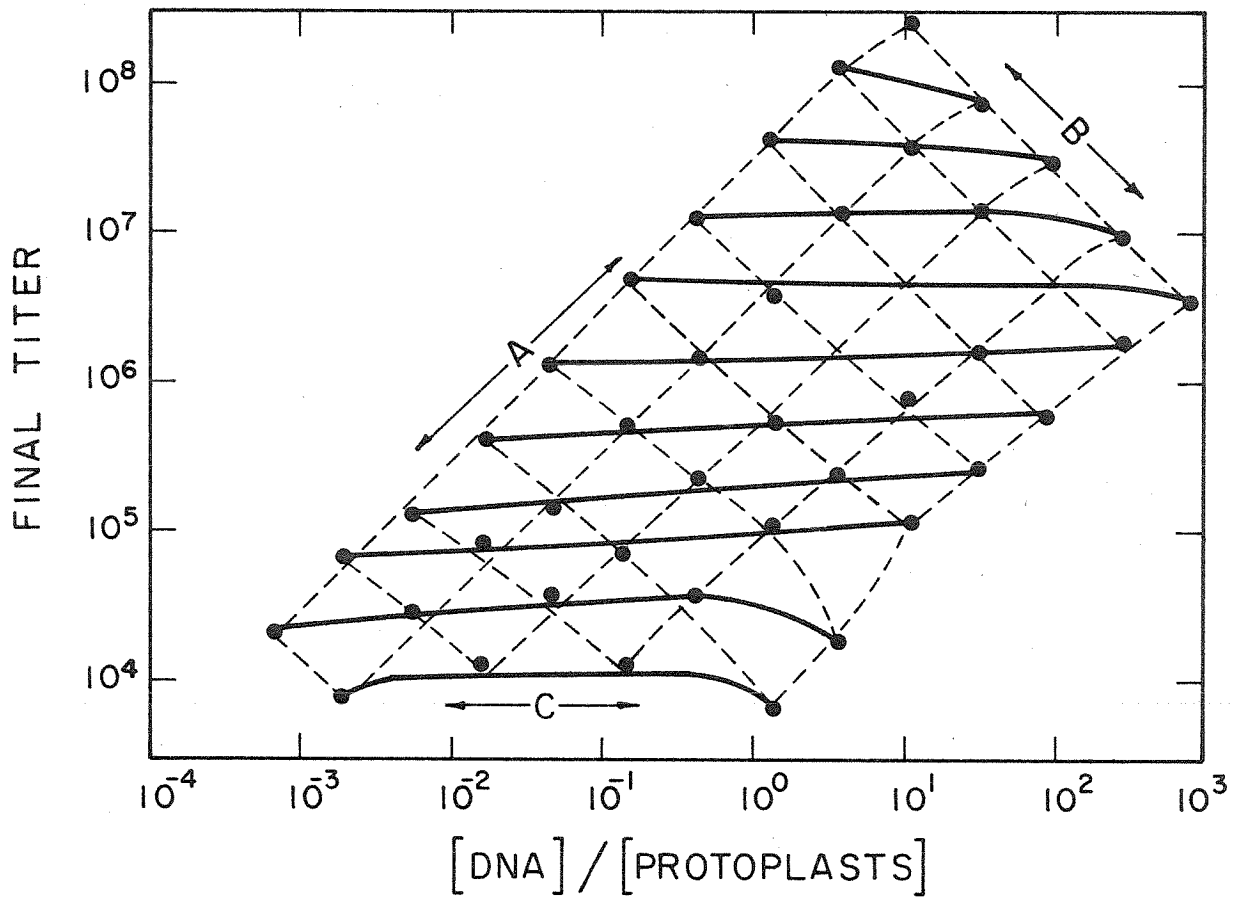

Figure 17. Results of a dilution array. The figure presents data from the experiment of Fig. 16 as mature phage/ml. in the adsorption tube (final titer) after 120 min. incubation. A, B, and C indicate the same families of curves as in Fig. 16.

3.7 or less, the relationship: [DNA] x [protoplast] $\propto$ [infective centers] holds remarkably well with the difference in infected protoplasts and final titer indicating an average burst size of about 200 phage/infected protoplast.

Kinetic Studies of Infection: The results of previous experiments which have been described have shown that the level of infection is determined by the concentrations of DNA and protoplasts in the adsorption mixture (fig. 16) and by the temperature at which adsorption takes place (table 2). In order to investigate these effects in greater detail, the kinetics of adsorption at various DNA concentrations and several temperatures were studied.

Figure 18 presents the results of a study of the rate of infection of protoplasts with DNA at three different concentrations. The final infection level is proportional to the DNA concentration which agrees with the results of the dilution array experiment. After approximately 5 minutes, the rate of infection is linear and, within the limits of the accuracy of the data, is directly proportional to the DNA concentration. The actual values are:

10^6 DNA/ml. -------- 4.8×10^2 infections/10 minutes
10^7 DNA/ml. -------- 6.1×10^3 infections/10 minutes
10^8 DNA/ml. -------- 4.85×10^4 infections/10 minutes

The evidence from these results suggests that the rate of infection is dependent on the concentration of DNA present, and that the dependency of the level of infection on DNA concentration

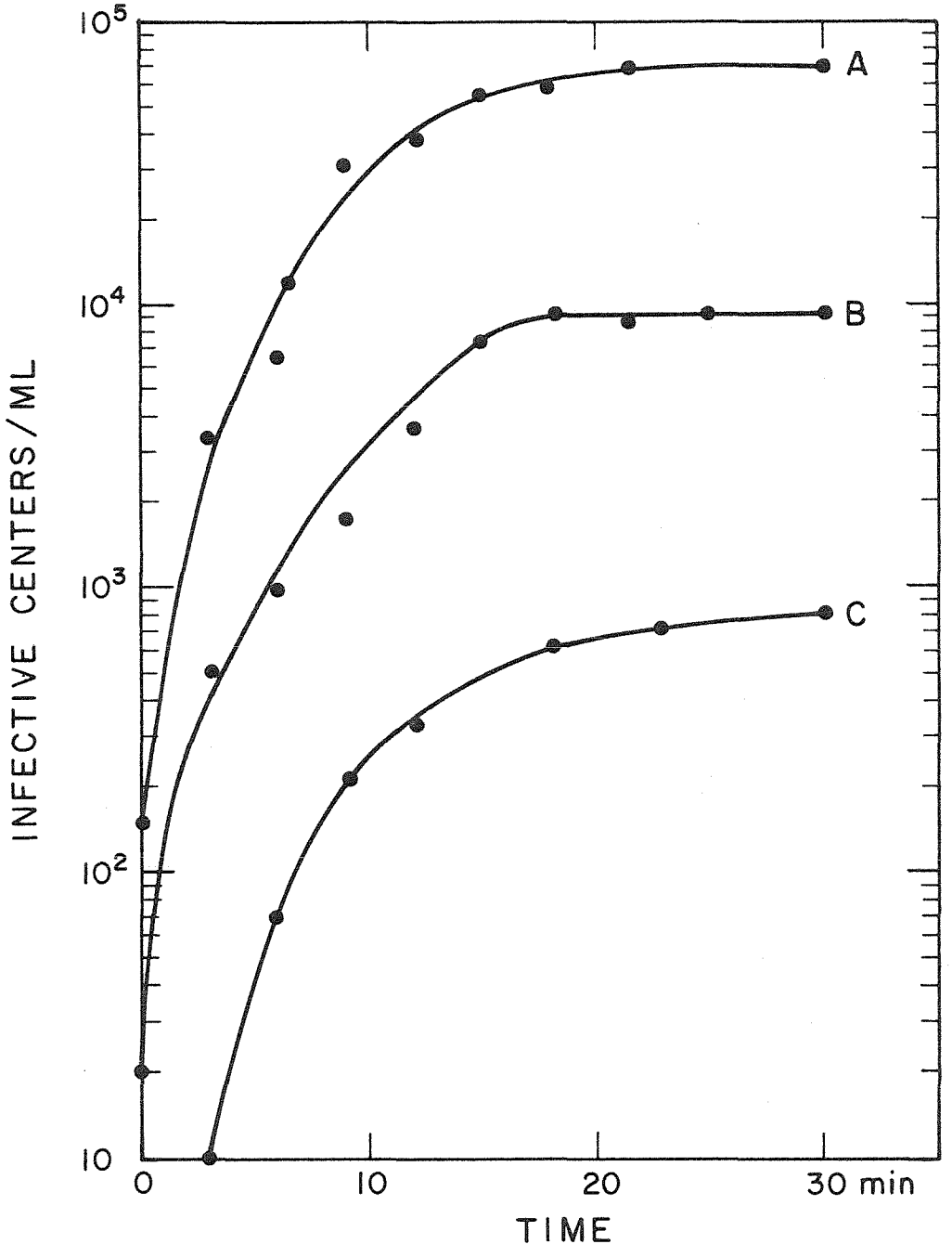

Figure 18. Assay of infected protoplasts/ml. versus time for three concentrations of DNA. Curve A resulted from 10^8 DNA particles/ml.; Curve B from 10^7 DNA particles/ml.; and Curve C from 10^6 particles/ml. The protoplast concentration was 5×10^8/ml in each case.

is due to the cessation of infection at 20 minutes.

Similar experiments were done to investigate the infection of protoplasts by φX-DNA as a function of time at different temperatures. Figures 19, 20, 21, and 22 give the results of these experiments at 0°, 10°, 20°, and 30° C. Each set of data is plotted on semi-logarithmic and linear coordinates.

The results demonstrate that the rate of infection is constant at all four temperatures during most of the adsorption period. Again, as shown in the studies on infection kinetics at different DNA concentrations, the infection process ceases at about 20 minutes except for the 20° sample. For an unknown reason the infection at 20°C reached a higher value than was expected.

The data shown in figures 19, 20, 21, and 22, and other data (cf. Table 2, "Infective Center Titers") demonstrate a greater temperature dependency for infection than would be expected for a simple diffusion mechanism of infection. The results of the dilution array experiment and these kinetics of infection studies would seem to suggest the following mathematical relationship:

$$[\text{Infective Centers}] = K\, e^{-A/T}$$

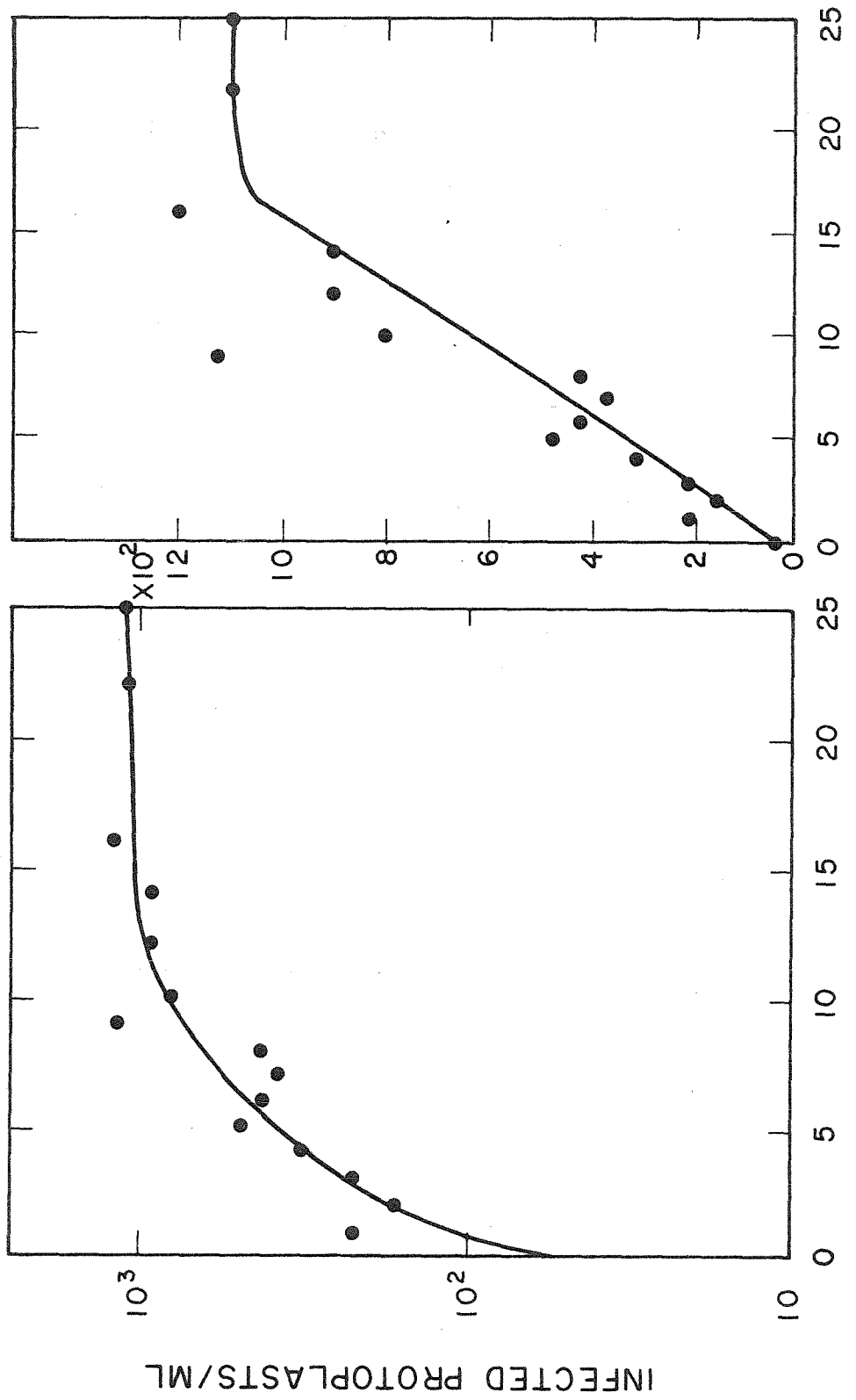

Figure 19. These curves demonstrate the kinetics of infection at 0°C. Curve A is a semi-logarithmic plot; Curve B is a linear plot. The multiplicity equals 0.2.

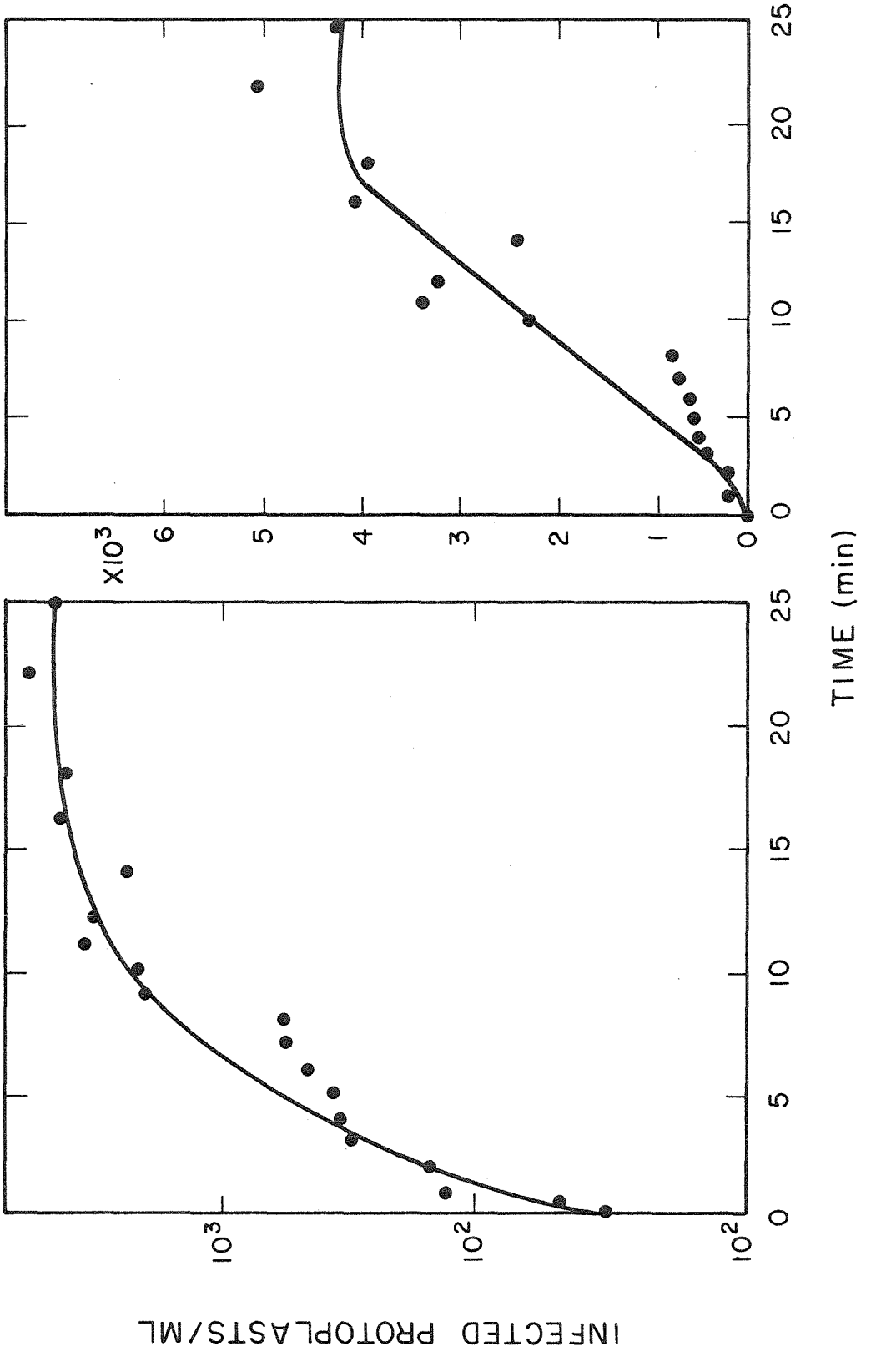

Figure 20. The kinetics of infection at 10°C. All other conditions were the same as those for the experiment represented in Fig. 19.

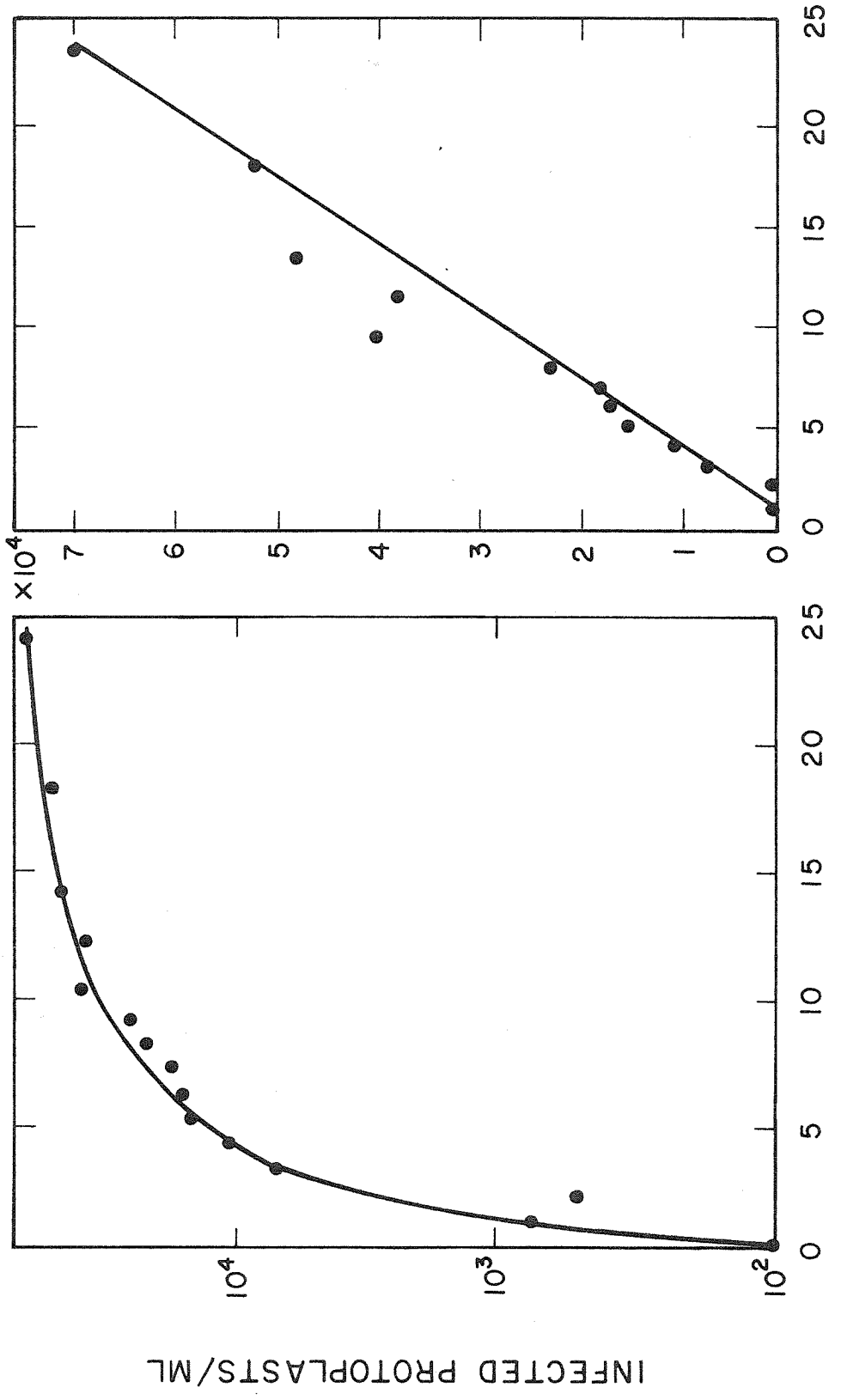

Figure 21. The kinetics of infection at 20°C. All other conditions were the same as those for the experiment represented in Fig. 19.

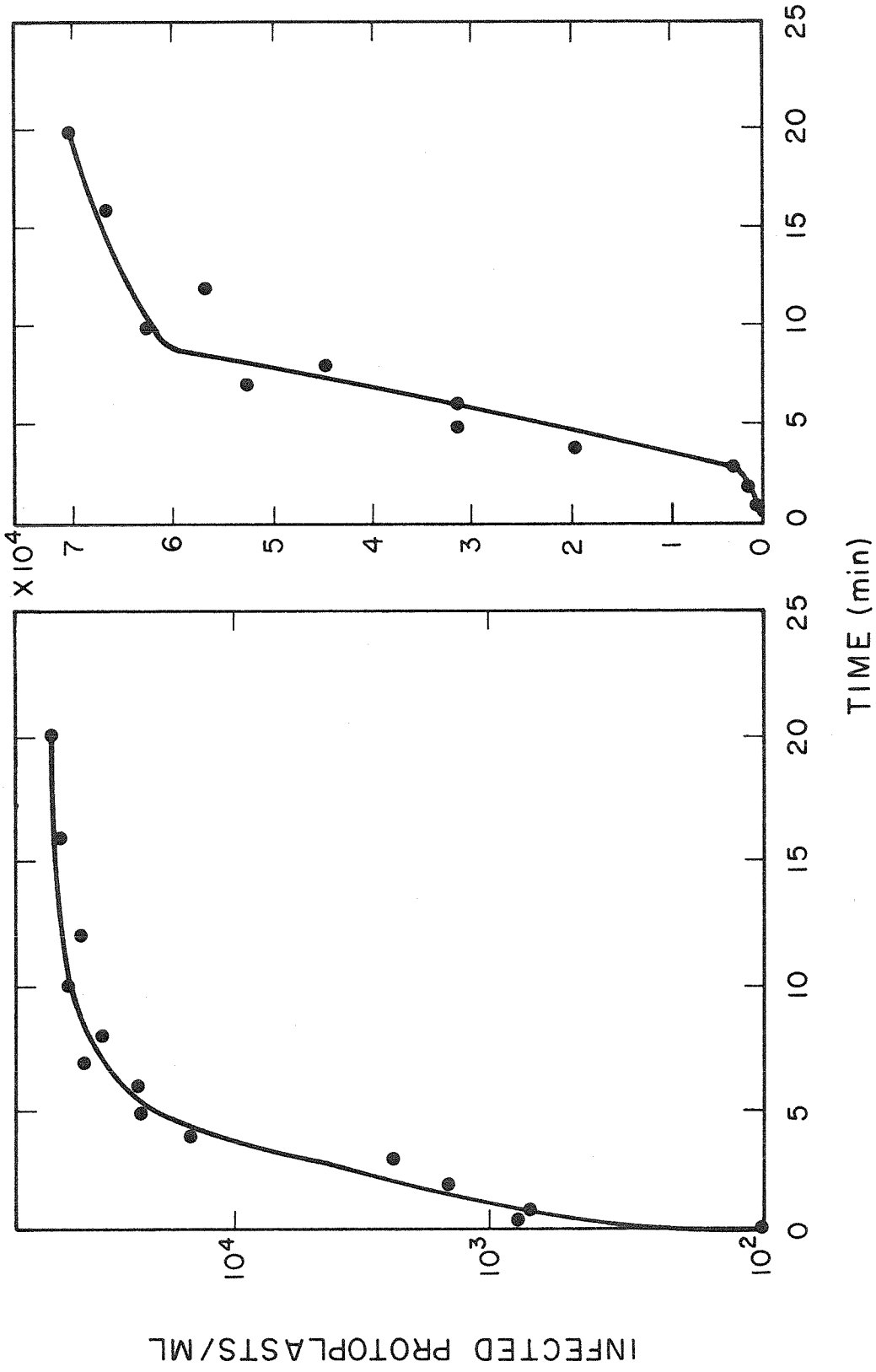

Figure 22. The kinetics of infection at 30°C. All other conditions were the same as those for the experiment represented in Fig. 19.

where the constant K includes both the concentration of the DNA and the concentration of protoplasts and possibly other parameters. At present the nature of the parameters involved in A is not known but could conceivably involve the actual mechanism of uptake for infection. However, further understanding of these two classes of parameters must await new investigations.

SUMMARY AND DISCUSSION

The general thesis adopted for Part II was "that the protoplast system, itself, can be used to study the replication of virus". However, the general goal of the investigations contained in Part II was more modest. The purpose of these initial experiments was (a) to investigate the fate of the ϕX-DNA added to the system and in the process of this investigation (b) to develop techniques which would be of general value in using protoplasts as a system for studying replication in subsequent investigations. In the course of these investigations certain suggestions have arisen concerning the initial interaction of ϕX-DNA and protoplasts.

Early results showed that under certain conditions extremely efficient infection resulting in a net synthesis of DNA in the final yield of virus could occur. This phenomenon seemed to occur for very low concentrations of infecting DNA and only certain samples of "protoplast stock". Even such intermittent results suggested that a significant fraction of the total ϕX-DNA could be infectious. Under routine conditions, however, only a much

smaller fraction of the DNA caused an infection.

With improvement in the method of forming protoplasts, this routine efficiency of infection increased from the former 3×10^{-5} infections/DNA/ml. to 5×10^{-4} or 1×10^{-3} infections/DNA/ml. for protoplast stocks at 10^9/ml.* An increase of more than 10 times in infection resulting only from a change in the method of forming protoplasts suggested that factors other than the amount of infections DNA present were limiting infection.

The distribution of radioactivity after infection with ^{32}P labeled ϕX-DNA suggested that non-infecting DNA was divided between the protoplast fraction and the supernatant fraction. Investigation of both of these fractions has shown that infectious ϕX-DNA, other than that causing infective centers in the original mixture, is present. The amount of this remaining infectious material agrees well with the radioactivity data. However, it should be noted that occasionally no infectious DNA remaining in the supernatant is detectable. This is probably a matter of technique since the frequency of such failures has decreased as the techniques of centrifugation and recovery of the fractions have improved.

* The results presented in fig. 7 indicate 1.5×10^{-4} infections/DNA/ml. However, the protoplast system is still evolving and efficiencies have increased since the data of fig. 7 to give the values mentioned in the text. Such discrepancies are also evident between other data. One such example is with figs. 5 and 6. From fig. 6 treatment with DNAase reduces the titer of DNA molecules to 2×10^{-3} the control concentration; yet according to the dilution curve in fig. 5 such a loss of titer is not possible. The ambiguity arises from the fact that the DNAase inactivation studies and the concentration effect studies were performed at different times on the protoplast developmental scale.

The data from the dilution array experiments have shown that the level of infection is determined by the product of the concentrations of both the DNA and protoplasts. In this respect infection appears to be analogous to a chemical reaction wherein the reacting substances are in high excess due to an equilibrium which is very unfavorable for the formation of the product. However, such an interpretation appears over simplified in the presence of data on infection kinetics.

These kinetic data illustrate the primary limitation to infection. In every case, within the limits of the experimental conditions, infection stops 20 minutes after the mixing of DNA and protoplasts. The velocity of infection during most of this 20 minutes is constant, and this velocity is influenced by parameters such as concentrations of DNA and protoplasts and temperature.

These conclusions suggest several approaches to the problem of improving infection. All parameters, such as the concentration of sucrose, magnesium sulfate, and bovine serum albumin should be investigated for their effects on the kinetics of infection. While either an increase of infection velocity or an extension of the twenty minute "cut off" time would produce the same increase in infection, an increase in velocity is of greater value for practical reasons.

The "cut off" reaction after twenty minutes is of interest. It should be noted that the reinfection experiment (table 3) was

executed by reinfecting the adsorption mixtures at seven minutes, while two-thirds of the adsorption time still remained. Further investigations are also necessary to determine the effect on the infectivity of diluting the adsorption mixture for further incubations. Certain data suggest that this dilution does not influence the adsorption kinetics after the first 10 minutes of the 20 minute infection period. Such experiments are examples of the type which may yield further information about the mechanism of infection of protoplasts by ϕX-DNA.

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