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IN BACILLUS SUBTILIS: AN ENDONUCLEASE
INHIBITED DURING INFECTION BY BACTERIOPHAGE SP82G

by

JOHN J. KING

B.S. University of Rochester, 1968

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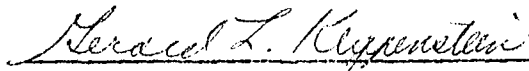
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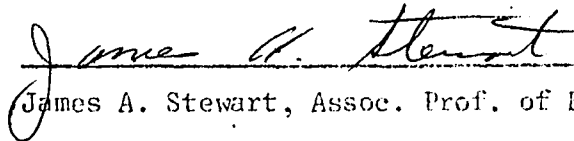
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ABSTRACT

THE MOLECULAR BASIS OF INTRACELLULAR INACTIVATION
IN BACILLUS SUBTILIS: AN ENDONUCLEASE
INHIBITED DURING INFECTION BY BACTERIOPHAGE SP82G

by

JOHN J. KING

Successful transfection of Bacillus subtilis with SP82G DNA requires 2-4 phage genomes per competent cell, while successful infection requires only one bacteriophage per cell. The transfecting DNA has been shown to be degraded within the cell by a process (intracellular inactivation) that is inhibited at early times after infection with bacteriophage SP82G. This inhibition is chloramphenicol sensitive and thus presumed to involve a phage-induced protein. If phage infection is allowed to proceed in the presence of chloramphenicol, the phage genome is degraded in a manner similar to the degradation of transfecting DNA.

Analysis of the molecular weight changes of SP82G DNA at various times after infection in the presence of chloramphenicol shows that the number of double strand breaks per DNA molecule increase as a function of time after infection. This suggests that intracellular inactivation was the result of an endonuclease capable of causing double strand breaks in phage DNA.

The total nuclease activity of uninfected cell extracts of B. subtilis was characterized and shown to decrease following infection with bacteriophage SP82G. This inhibition was maximal at 6 minutes after infection at 37° and 9 minutes after infection at 33°. The bulk

(90%) of the inhibition was chloramphenicol sensitive but a small part (10%) was shown to be chloramphenicol insensitive. Infected cell extracts were shown to have decreased nuclease activity on a variety of substrates.

An endonuclease was partially purified from uninfected cell extracts of B. subtilis on the basis of its inhibition in extracts infected with SP82G for 6 minutes at 37°. The endonuclease has a molecular weight in the range of 10-12,000, an absolute requirement for divalent cation, and is stimulated by Mg^{2+} and Ca^{2+} but not Mn^{2+} . The endonuclease attacks native SP82G and Bacillus subtilis DNA. DNAs attacked by the endonuclease show an equal number of double strand and single strand breaks. Denatured SP82G and Bacillus subtilis DNA, single stranded polio virus RNA and ØX 174 RFI DNA are all substrates for this endonuclease.

INTRODUCTION

The role of intracellular nucleases as protection against invasion by foreign nucleic acid would seem primary to the survival of a bacterial organism.

Luria (1953) was the first to describe 'host controlled modification' in E. coli. It was observed that phage grown in one strain of E. coli grew very slowly or not at all on a closely related strain. The few phage that did grow on a restrictive host were somehow modified and were subsequently able to grow on either their original host or the previously restrictive host.

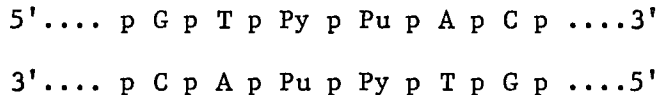
Arber and Dussoix (1962) defined the process, now called restriction and modification, in molecular terms. The host was shown to have the ability to both degrade and modify unmodified DNA. The modification process made the DNA immune from degradation in subsequent infections.

Meselson and Yuan (1968) isolated the restriction endonuclease from E. coli K. It required Mg^{+2} , ATP and S-adenosylmethionine and produced double stranded breaks in unmodified DNA. Restriction endonucleases have subsequently been purified from E. coli B (Linn and Arber, 1968), Haemophilus influenzae (Smith and Wilcox, 1970), and Haemophilus aegyptiae (Middleton et al., 1972). Boyer (1971) classifies the restriction endonucleases in two classes. Type I (E. coli B and K) are complex enzymes with stringent requirements for ATP, Mg^{2+} and S-adenosylmethionine. They have molecular weights of approximately 250,000 and are part of a multisubstrate complex consisting of endo-

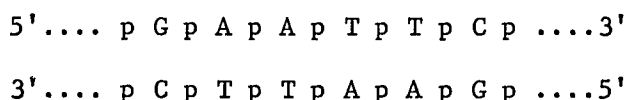
nuclease, modification methylase and a recognition protein. Type II (H. influenzae and E. coli $\text{fi}^{-\text{R}}$) have a molecular weight in the range of 72,000 and require only Mg^{+2} . These are quite clearly uni-substrate enzymes. Both classes produce double stranded breaks in unmodified DNA.

The modification methylase from E. coli B was isolated and found to add a methyl group, donated by S-adenosylmethionine, to the 6 position of adenine at a very few, specific sites on the genome (Kuehnlein et al., 1969). Modification methylases have also been purified from E. coli K and E. coli lysogenized with bacteriophage P1 (Haberman et al., 1972).

Kelly and Smith (1970), using the H. influenzae endonuclease and T7 DNA obtained the following nucleotide sequence for the substrate site of restriction and modification:



Hedgpeth et al. (1972), using the E. coli $\text{fi}^{-\text{R}}$ endonuclease and λ DNA, presented the following as the substrate site in that system:



Both of the sequences show the "two-fold rotational symmetry" which is assumed to be common to all restriction modification substrate sites.

The effectiveness of the restriction process can be reduced either by ultraviolet irradiation (Bertani and Weigle, 1953) or heating the host cells in low salts medium (Schell and Glover, 1966) prior to infection. Schell (1969) showed that pre-infection of E. coli B with bacteriophage T3 protects unmodified λ phage in a restrictive host. This inactivation of restriction was due to a phage induced enzyme which

degrades S-adenosylmethionine (SAM) and thus inhibited the restriction endonuclease which requires SAM for activity.

Boyer (1971) lists fifteen gram-negative bacterial strains, but only one gram-positive strain (Staphylococcus aureus) that have a proven restriction-modification system. A restriction-modification mechanism has been suggested in Bacillus stearothermophilus on the basis of efficiencies of plating of various TP bacteriophage, although as yet no biochemical evidence has been found (Lees and Welker, 1973).

Cells of Bacillus subtilis 168 lysogenic for bacteriophage SP02 have been shown to restrict growth of bacteriophage ϕ_e (Rettenmier, 1973) and $\phi 1$ and $\phi 105$ (Yasbin and Young, 1974). This has been termed 'interference' rather than restriction since there is no evidence of nuclease action or a modification system. Rettenmier and Hemphill (1974) has shown that when $\phi 1$ attacks B. subtilis (SP02), the phage adsorbs, penetrates and kills but does not productively infect the bacteria. A general failure of host and phage nucleic acid synthesis, occurring 15 min after infection is responsible for the non-productive infection.

The transformation process, in which cells can be made "competent" to take up and incorporate extracellular DNA, is also affected by the restriction system (Arber, 1965). DNA purified from an unmodified strain of E. coli is restricted, and thus cannot transform, in a restrictive strain.

In the Challis strain of Streptococcus, Chen and Ravin (1968) found that DNA of this strain could be aided in transformation by a variety of other DNA molecules added to the competent cells prior to the donor DNA. These "helper" DNA molecules act by tying up an inactivation activity within the cell and preventing degradation of the donor

DNA. Since Challis strain DNA is the most effective "helper" DNA, this inactivation process is quite distinct from a restriction modification process.

A positive correlation between intracellular nuclease activity and transformation levels has been found in E. coli and Diplococcus pneumoniae, where mutants lacking an ATP dependent DNase (rec^- mutants) have very low levels of transformation (Friedman and Smith, 1973). No differences, up to the step of association of donor DNA with recipient chromosome, were observed in comparing these rec^- strains with wild type (Vovis, 1973). In Haemophilus influenzae an MMS (methyl methane sulfonate) sensitive mutant lacks an ATP dependent nuclease and is virtually transformation deficient, even though DNA uptake is completely unimpaired. When this mutant is transformed back to MMS resistance, both the ATP dependent nuclease and transforming ability return (Greth and Chevallier, 1973). Chestukhin et al. (1973) have partially purified an ATP dependent nuclease from Bacillus subtilis and have isolated one rec^- mutant with a 5-10 fold decrease in the activity of this enzyme. Conversely, Dubnau et al. (1973) looked at 16 rec^- mutants of this strain, all blocked in late steps of transformation, and didn't find any with a decrease in ATP dependent nuclease activity.

In Diplococcus pneumoniae, double strand breakage of the transforming DNA precedes entry into the cell and produces fragments of approximately 2.8×10^6 daltons/strand in size (Morrison, 1973a). These fragments then enter the cell without further degradation. The extent of degradation of donor DNA on entry varied with the size of the donor DNA molecule (Morrison and Guild, 1972) and breakage was not genetically specific (Morrison and Guild, 1973b). Some point mutations

in Diplococcus pneumoniae fall into two very distinct classes: those with high efficiencies of transformation (HE), and those with low efficiencies (LE) (Green, 1959). An excision-repair system which acts on some donor recipients (LE), but not others (HE) was postulated in explanation (Ephrussi-Taylor and Gray, 1966). Lacks (1970) showed that the LE-HE differences were only found in recipients strains with the hex⁺ genotype. In hex⁻ strains, all markers are transformed with a high efficiency. As with the Challis strain of Streptococcus mentioned previously, the hex system can be easily saturated by addition of excess homologous DNA with a resultant 6-7 fold increase in the efficiency of LE markers.

In Bacillus subtilis, EDTA is required for transformation with single-stranded DNA in order to inactivate a Ca⁺² dependent extracellular nuclease which degrades the single-stranded DNA (Tevethia and Mandel, 1970). Morrison (1970) suggested a surface bound endonuclease which was capable of producing double strand breaks in transforming DNA. This endonuclease was thought to be originally bound to the cells, and released when the cells came in contact with transforming DNA. An endonuclease associated specifically with competent cells is also indicated by data which shows a decrease in the efficiency of transduction when competent rather than non-competent cells are used (Adams, 1972).

Haseltine and Fox (1971) found that while both competent and non-competent B. subtilis cells could bind DNA, the non-competent cells degraded the DNA while the competent cells did not. The endonucleolytic breaks were double stranded in nature and the resulting product had a molecular weight of approximately 10×10^6 . Ayad and Shimmin (1974) describe a factor capable of inducing competence in non competent

cultures ("competence factor"), which shows both a lytic activity against isolated cell walls and a nuclease activity against transforming DNA.

Two endonucleases are postulated from the work of Davidoff-Abelson and Dubnau (1973b). The first endonuclease attacks donor DNA within 30 seconds of attachment and results in a double stranded fragment (DSF) with a molecular weight of approximately 9×10^6 . A second nuclease, acting between one and two minutes after uptake, results in the formation of single stranded fragments (SSF) which occur simultaneously with the release of acid soluble nucleotides to the medium. An endonuclease has been isolated from B. subtilis which may be one of the nucleases described (Scher and Dubnau, 1973). It is located in the periplasm and results in double strand breaks and a limit product in excess of 3×10^6 . It is stimulated by Mn^{2+} and Ca^{2+} , but not by Mg^{2+} .

Strains of Bacillus subtilis lysogenic for bacteriophage $\phi 105$ and SPO2 have greatly reduced rates of transformation (Yasbin and Young, 1972; Yasbin et al., 1973). The effect is additive, i.e., double lysogens have lower rates of transformation than either of the single lysogens. A differential inactivation of bacterial genes after lysogenization is offered in possible explanation. The process of transfection, in which competent cells take up and incorporate viral DNA in such a way that a productive infection results, is not affected by host lysogenization.

Transfection is, however, affected by restriction-modification mechanisms. Benzinger and Kleber (1971), using spheroplasts of E. coli and Salmonella typhimurium observed host controlled restriction of bacteriophage P22 during transfection. This restriction results in a

strong inhibition of transfection even at high DNA concentrations.

In Bacillus subtilis, two modes of transfection were observed, dependent upon the source of the DNA. A single molecule of DNA from bacteriophage $\phi 29$ and SP02 is sufficient to produce an infectious center (Okubo and Romig, 1965; Reilly and Spizizen, 1965), while two or more molecules are required with DNA from bacteriophage SP82G (Green, 1964), SP01 (Okubo, 1964), SP50 (Földes and Trautner, 1964) SP3 (Romig, 1962) and $\phi 1$ (Reilly and Spizizen, 1965).

Analysis of progeny from infectious centers using DNA from SP01 (Okubo et al., 1964) and SP82G (Green, 1964) has shown that genetic recombination between two or more DNA molecules is necessary for successful transfection. DNA from phage with a "multiple molecule" requirement is unable to transfect recombination deficient strains (rec^-) while DNA from $\phi 29$ or SP02 can successfully transfect either rec^+ or rec^- strains (Spatz and Trautner, 1971). A 12-fold increase in recombinants in progeny produced by transfection as opposed to infection was observed with SP82G (Green and Urban, 1972).

Marker rescue studies done with SP82G, in which genetic markers introduced into the cell by transfecting DNA are "rescued" by superinfection with appropriate mutant phage, show that the probability of two linked markers being rescued together decreases as the map distance between the markers increases. A process within the cell which inactivates infective DNA ("intracellular inactivation") was postulated (Green, 1966). Gene dislinkage was shown to occur during this inactivation, with the phage genome being broken into at least 40 fragments (Green, 1968). Intracellular inactivation has been shown to effect the DNA of $\phi 105$ (Rutberg et al., 1969) and SP50 and SPP1 (Spatz and Trautner, unpublished).

Infection with SP82G bacteriophage before addition of DNA was shown to inhibit intracellular inactivation and lead to unimolecular kinetics with SP82G DNA ("preinfection protection") (Green, 1966). This inhibition was maximized if transfecting DNA was added 6 minutes after phage infection (Green, 1966; McAllister and Green, 1972). Inhibition of intracellular inactivation is sensitive to chloramphenicol thus implicating the need for protein synthesis. Further, infection with intact bacteriophage in the presence of chloramphenicol showed that the infecting genome was sensitive to the inactivation process under these conditions (McAllister and Green, 1972). Studies comparing infection and transfection show that although the rate of marker entry is the same, the order of entry during infection is reversed during transfection (Williams and Green, 1972). Presumably, the region on the genome coding for the inhibitory protein is the first to enter the cell during infection and the last to enter during transfection, possibly explaining the lack of inhibition of intracellular inactivation during transfection.

Enhancement of transfection rates of SP82G following ultraviolet irradiation of competent cells prior to addition of DNA has been reported, although such treatments resulted in a 99-99.5% loss of cell viability (Epstein, 1967). Incubation of competent cells with UV irradiated heterologous or homologous DNA was reported to result in enhancement of transfection to first order, or uni-molecular, dose response. This protective effect was thought to be due to a sequestering of host intracellular inactivation activities by the UV irradiated DNA (Epstein and Mahler, 1968). The recent work of Arwert and Venema (1974) while showing a 100-fold enhancement of transfection using preincubation

of competent cells with UV irradiated DNA, shows no change to first order dose response. Further, the enhancement is not seen at all in ultraviolet sensitive strains (uvr^-). The enhancement in uvr^+ is postulated to be caused by the tying up of exonuclease activities by the UV damaged DNA after incision by a UV repair enzyme. In uvr^- strains, this incision is not made and the exonucleases remain free to attack transfecting DNA. These experiments make it clear that transfection enhancement afforded by pre-incubation with UV irradiated DNA is quite different from phage induced inhibition of intracellular inactivation.

Several nucleases from B. subtilis have been described (Table 1). The fact that only exonuclease Bs II has been purified to any extent is rather surprising, since some of the nucleases were first investigated over 10 years ago. Although the existence of a number of endonucleases has been suggested, none have been purified greater than 10-fold. The reason for this lack of purified nucleases is not clear, but may involve nuclease instability following fractionation of cell extracts.

The present work describes an endonuclease from B. subtilis that is inhibited at early times after infection with bacteriophage SP82G. The endonuclease has been partially purified from extracts of uninfected cells on the basis of its inhibition in extracts of infected cells. Characterization of the nuclease makes it quite clear that this particular nuclease has not been previously described.

Table 1. NUCLEASES OF BACILLUS SUBTILIS

Location	Reference	Substrate	Mode of Attack; Requirements	Purification (if any)	Remarks
Intracellular	Birnboim (1966)	denatured DNA	exonuclease; Ca ²⁺ dependent	100X	Activity released during protoplast formation.
	McCarthy & Nester (1966)	native DNA	endonuclease-d.s. breaks only; Mg ²⁺ dependent	3-9X	Activated by high temperatures (80°)
	Chestukhin <u>et al.</u> , (1972)	native DNA	exonuclease; ATP, Mg ²⁺ dependent	30X	Strict requirement for dATP
	Scher & Dubnau (1973)	native DNA	endonuclease-d.s. breaks only; Mn ²⁺ or Ca ²⁺ dependent	7X	Activity released during protoplast formation; limit size of product; 3 x 10 ⁶ daltons
Extracellular	Kerr <u>et al.</u> , (1967)	native and denatured DNA; RNA	exonuclease; Ca ²⁺ dependent on native DNA; Ca ²⁺ stimulated on denatured DNA & RNA	71X	See also: Kerr <u>et al.</u> , (1965) Okazaki <u>et al.</u> , (1966)

Table 1. (cont'd)

Location	Reference	Substrate	Mode of Attack; Requirements	Purification (if any)	Remarks
Extracellular	Morrison, D. (1966)	native DNA	endonuclease-d.s. and s.s. breaks	----	Nuclease bound to competent cells-released upon addition of transforming DNA
	Haseltine & Fox (1971)	native DNA	endonuclease-d.s. breaks only	----	Attacks transforming DNA bound to non-competent cells; limit product size: 10^7 daltons
	Ayad & Shimmin (1973)	native DNA	exonuclease	----	Activity associated with competence inducing factor
	Kanamori <u>et al.</u> , (1973)	native and denatured DNA; RNA	exonuclease; Ca^{2+} dependent on native DNA; Ca^{2+} stimulated on denatured DNA;	BsII-3400X BsIA-27X BsIB-326X	Probably slight modifications of Kerr <u>et al.</u> , (1967) enzyme

RNA

Table 1. (cont'd)

Location	Reference	Substrate	Mode of Attack; Requirements	Purification (if any)	Remarks
Extracellular	Kanamori <u>et al.</u> , (1973) Bs10	denatured DNA	exonuclease; Ca ²⁺ dependent	----	

Abbreviations:

d.s. - double strand

s.s. - single strand

MATERIALS AND METHODS

Bacteria and Bacteriophage

Bacillus subtilis strain SB-1 (his_1^- , ind_1^-) was used to prepare all crude extracts and was the host for bacteriophage SP82G preparations. The media for bacterial growth was Nomura salts (NM) (Nomura, 1962) supplemented with 0.5% glucose, 0.2% casein hydrolysate, 2×10^{-3} M $MgCl_2$, 0.1% yeast extract, 0.05 mg/ml of DL-tryptophan, 2 mg/ml of arginine and 0.2 mg/ml of L-histidine.

For preparation of phage lysates, bacteria were grown at 37° to 10^8 cells/ml and infected with SP82G at an m.o.i. = 1. Incubation at 37° was continued until lysis was complete. Phage lysates were concentrated by the method of Yamamoto et al. (1970). Lysates were made 0.5 M in NaCl and the debris removed by centrifugation at $5000 \times g$ for 10 minutes. Polyethylene Glycol 6,000 (Matheson, Coleman and Bell) was added to the supernatant to a final concentration of 10% (w/v), and the solution was allowed to sit overnight at 4° . The solution was centrifuged at $5,000 \times g$ for 20 minutes and the pellet was redissolved in a small amount of NM salts plus $MgCl_2$ ($2 \times 10^{-3}M$) and EDTA ($10^{-3}M$). The phage were pelleted by centrifugation at $27,000 \times g$ for 60 minutes, redissolved in the same buffer and centrifuged at $3,000 \times g$ for 10 minutes to remove debris. The phage, in approximately 4-5 ml of buffer, were layered onto a preformed $CsCl_2$ gradient, consisting of 1.0 ml layers of $CsCl_2$ with approximate densities of 1.65, 1.6, 1.55, 1.50, 1.46, 1.42, and 1.38 g/ml and centrifuged at $37,000 \times g$ for 30 minutes. Phage bands were withdrawn with a pasteur pipet and dialyzed against 1 x NM salts plus

MgCl₂ (2×10^{-3} M) and EDTA (10^{-3} M). Phage preparations, usually between $1 - 4 \times 10^{12}$ phage/ml, are stored at 4°.

DNA Preparations

Bacillus subtilis strain G-1 (adenine⁻), isolated from gamma irradiated spores of strain 168, was the host used for production of radioactively labeled SP82G phage. The cells were grown to approximately 10^8 cells/ml in NM salts supplemented with 0.2% casein hydrolysate, 2×10^{-3} M MgCl₂ and 5 µg/ml adenine. At this time ³H-labeled adenine (New England Nuclear; 5 mCi/.025 mg), 1 mCi/liter, was added along with SP82G phage at an m.o.i. = .1. After completion of lysis, phage were concentrated using a low speed centrifugation (5,000 x g for 15 minutes) to remove debris, and a high speed centrifugation (15,000 x g for 3 hours) to pellet phage. Pellets were redissolved in 1 x NM with 2×10^{-3} M MgCl₂ and 10^{-3} M EDTA and banded on a step-wise CsCl₂ gradient. After dialysis against 1 x NM plus 10^{-2} M EDTA, solid sodium perchlorate was added to a final concentration of 3M and the solution was left at room temperature for 30 minutes. Following dialysis against 0.05 M Tris-HCl (pH 8.0) plus 10^{-1} M EDTA, debris was removed by centrifugation at 10,000 x g for 15 minutes. The DNA solutions were stored at 4°. DNA concentrations were determined from A₂₅₅ readings, assuming a solution of 20 µg/ml of DNA has an A₂₅₅ of 0.4. An A₂₆₀/A₂₈₀ ratio of 1.7-1.9 was considered a satisfactory indication of purity.

DNA, diluted to 25 µg/ml with .05 M Tris-HCl (pH 8.0) was denatured by heating in a boiling water bath for 10 minutes followed by rapid cooling in an ice bath.

DNA, diluted to 50 µg/ml with 0.05 M Tris-HCl (pH 8.0) was irradiated at a distance of 24 inches from a UV germicidal lamp (G15T8, Sylvania)

at a dose of 1200 erg/mm²/min.

³H-DNA from Bacillus subtilis G-1 was extracted using the method of Marmur (1963). ³²P-labeled T₄ DNA was a gift from Dr. Donald Nuss. ³H-labeled ØX174 RFI circular DNA was a gift from Dr. David Dressler.

³²P-labeled silk worm larvae ribosomal RNA, and ³H-labeled single stranded polio virus RNA were gifts from Dr. Donald Nuss.

Sedimentation Velocity of DNA

Sedimentation velocities of double stranded and single stranded DNAs were determined by centrifugation through 5-20% neutral sucrose (1.0 M NaCl) and 5-20% alkaline sucrose (0.9 M NaCl, 0.1 M NaOH) gradients respectively, using the methods of Reznikoff and Thomas (1969). Unless otherwise noted, centrifugation was done in a Beckman SW65 rotor at 35,000 rev/min for 105 minutes at 23°. Tubes were punctured and 8 drop fractions collected directly into scintillation vials. Ten mls of an Omnifluor (New England Nuclear) solution (5.6 g/liter of a toluene-Triton X-100 (2:1) base) were added and the samples counted in a Nuclear Chicago liquid scintillation counter.

Calculation of double strand breaks was done using the following equation from Charlesby (1954):

$$\frac{(MW)_t}{(MW)_n} = \frac{2(e^{-P} + P - 1)}{P^2}$$

where (MW)_n is the untreated molecular weight, (MW)_t is the molecular weight after treatment (with endonuclease) and P is the number of double strand breaks. The molecular weights were determined using Studier's equation (1965):

$$S_{20}^0, w = 0.0882 M^{0.346}$$

where $S_{20,w}^0$ is the sedimentation coefficient of the DNA and M is its molecular weight.

Single strand breaks were measured by using the nomogram of Litwin et al. (1969).

Amino Acid Incorporation

B. subtilis SB-1 cells were grown in NM salts supplemented with 0.5% glucose, $2 \times 10^{-3}M$ $MgCl_2$, 0.05 mg/ml, DL tryptophan and 0.2 mg/ml of L-histidine to about 5×10^7 cells/ml. Chloramphenicol (200 μ g/ml) was added to a concentration of 200 μ g/ml 4 minutes previous to the first time point and a mixture of 3H -labeled amino acids (New England Nuclear) (5 μ Ci/100 mls) was added 1 minute later. SP82G bacteriophage were added at an m.o.i. = 10 thirty seconds prior to the first time point. Incorporation of the amino acids into protein was measured using the method of Levinthal et al. (1962). At each time point, 2 ml of cells were diluted into 2 mls of cold 10% trichloroacetic acid (TCA) - 1% casamino acid solution and stored at 4° for at least 30 minutes. The samples were centrifuged at $12,000 \times g$ for 10 minutes and the pellets resuspended in 1.5 ml of 1.0 N NaOH. Samples were maintained at room temperature for 20 minutes, then 6 ml of 10% TCA-1% casamino acids were added and the samples were heated to 95° for 30 minutes in a water bath. Precipitates were collected on membrane filters (B-6, Schleicher and Schuell) and washed twice with cold 10% TCA-1% casamino acids. The filters were placed in liquid scintillation vials and dried at room temperature overnight. Ten milliliters of an Omnifluor-toluene solution (4 g/liter) were added and the radioactivity determined in a Nuclear Chicago liquid scintillation counter.

Preparation of Cell Extracts

Uninfected. Ten milliliters of an overnight growth of SB-1 were added per liter of media and incubated with aeration at 37° until late log phase (5×10^8 cells/ml). Suspensions were centrifuged at 5,000 x g for 15 minutes and pellets washed once with .05 M Tris-HCl (pH 8.0). Cells were resuspended (3 ml/g wet weight of cells) in a lysozyme (Miles Serovac) (1 mg/ml) 0.05 M Tris (pH 8.0) solution. Cells were frozen at -20°, thawed slowly at room temperature and refrozen at -20°. After again slowly thawing, the solution was sonicated (MSE 100 watt Ultrasonic Disintegrator) to reduce viscosity and centrifuged at 10,000 x g for 20 minutes to remove debris. Extracts were stored at -20°.

Infected. Cells were grown, as above, to 10^8 cells/ml, centrifuged at 5,000 x g for 15 minutes at 37° and resuspended in warm media to a final concentration of 2.5×10^9 cells/ml. SP82G bacteriophage were added at an m.o.i. = 7.5-10 and allowed to adsorb for 1 minute at 37° after which the cells were diluted to 10^8 cells/ml and infection was allowed to proceed for 6 minutes at 37°. At the end of this period, chloramphenicol was added to a final concentration of 200 µg/ml and the solutions were quickly cooled by pouring over ice. Cells were collected, lysed and stored as above. Assays for bacterial cell titre, done before and after the 1 minute phage adsorption period, showed that 95-98% of the cells were successfully infected in the first minute.

Protein concentrations were determined by the Lowry method modified by Oyama and Eagle (1956).

Nuclease Assays

Total Nuclease Assay. The total nuclease assay was used to

measure the total nucleolytic activity of a particular extract. Incubation mixtures (1.0 ml) contained 2.5 μg ^3H -SP82G denatured DNA or 10.0 μg ^3H -SP82G native DNA, 50 mM Tris-HCl (pH 8.0) and 10 mM MgCl_2 . Reactions were started by addition of .01-2.0 mg of crude extracts and allowed to proceed for 15 minutes at 37°. Reactions were terminated by addition of 0.5 ml cold 1% bovine serum albumin and 0.5 ml cold 20% trichloroacetic acid. Samples were centrifuged at 2,000 x g for 15 minutes at 4°, and 1 ml of supernatant was added to liquid scintillation counting vials. After neutralization with 1 drop of concentrated NH_4OH , 10 mls of Aquasol (New England Nuclear) were added and the radioactivity determined in a Nuclear Chicago liquid scintillation counter. Efficiencies of counting were determined using the external standard method.

Standard Assay. The standard assay was developed to measure nuclease activity present in uninfected but missing in infected cell extracts. Incubation mixtures (1.0 ml) contained 2.5 μg ^3H -SP82G denatured DNA, 50 mM Tris-HCl (pH 8.0), 10 mM MgCl_2 , 1.0-2.0 mg extract of infected cells and .01-2.0 mg extract of uninfected cells. The reaction is started by the addition of the uninfected and infected cell extracts in quick succession and allowed to proceed for 15 minutes at 37°. Reactions were terminated and radioactivity determined as above.

Enzyme Isolation

Crude extracts were prepared as described above. The frozen extract was thawed and 15.5 ml were layered onto a 1.5 x 55 cm. Sephadex G-200 column, previously equilibrated with .05 M Tris-HCl (pH 8.0). Elution was carried out with .05 M Tris-HCl (pH 8.0) at a flow rate of 12 ml/hr maintained by a peristaltic pump (LKB Instruments, Inc.). 3 ml fractions were collected and assayed for both total nuclease and standard

assay activity. Fractions containing activity in the standard assay were pooled and concentrated at 4° under nitrogen using a Pellicon PSAC filter (Millipore Filter, Inc.) in an Amicon pressure filtration device. Isolated endonuclease was stored at 4°.

Molecular Weight Determination

The molecular weight of the isolated nuclease was determined by gel filtration on Sephadex G-200 using the method of Whitaker (1963). A Sephadex G-200 column (1.5 x 55 cm) was eluted with .05 M Tris-HCl (pH 8.0) at a flow rate of 12 ml/hr with 3 ml fractions collected. The following proteins were used to construct a standard curve: bovine serum albumin (Sigma Chemicals) 68,000 MW, ovalbumin (Sigma Chemicals) 43,000 MW, β -lactoglobulin (Mann Research Lab.) 36,800 MW, and myoglobin (Calbiochem) 17,200 MW. Sodium azide was run as an internal volume indicator. The elution volume of the isolated endonuclease was determined, and the molecular weight calculated from the standard curve. Protein peaks were detected by A_{280} readings, sodium azide was read at A_{254} .

Protoplast Formation

Protoplasts were made using the procedure of Scher and Dubnau (1973). Cells of B. subtilis 168 were grown to 5×10^9 cells/ml in 50 ml of NM media. The cells were centrifuged (5,000 x g for 15 min.) and resuspended in 25 ml of 0.6 M sucrose containing .05 M Tris-HCl (pH 8.0), .005 M $MgCl_2$, .001 M KCl and 12.5 mg lysozyme. The mixture was incubated at 37° for 30 minutes and then centrifuged at 10,000 x g for 15 minutes. The pellet was washed once with the sucrose-Tris buffer and then resuspended in 2.5 ml of cold .05 M Tris-HCl (pH 8.0). Resuspended cells were placed in an ice bath for 10 minutes, and the remaining pellet was dispersed by a short burst of sonication (MSE 100 watt Ultrasonic Dis-

integrator), which was insufficient to break whole cells. Centrifugation at 10,000 x g for 15 min. removed any cells not ruptured by exposure to the hypotonic Tris buffer. Thus, the supernatant is a crude extract derived only from those cells which were, in fact, protoplasts.

RESULTS

Molecular Weight Changes of SP82G During
Infection in the Presence of Chloramphenicol

McAllister and Green (1972) have shown that phage inhibition of intracellular inactivation was sensitive to chloramphenicol (CM). Furthermore, the DNA of phage allowed to infect cells in the presence of chloramphenicol was susceptible to an inactivation process similar to the one affecting transfecting DNA. To establish the nature of this inactivation process the fate of phage DNA with respect to molecular weight changes during infection in the presence of CM was examined.

Cells were grown to 5×10^7 cells/ml and concentrated to 10^8 cells/ml using NM media containing 200 μ g/ml chloramphenicol (CM). After incubation for 10 minutes at 37°, the cells were infected with radioactively labeled (3 H-adenine) SP82G at an m.o.i. = 10. The phage were allowed to adsorb for 1 minute and the cells were diluted 1:4 with warm NM plus CM (200 μ g/ml). At various times after infection, 5 ml aliquots were withdrawn and chilled rapidly by a 1:10 dilution into ice cold NM plus CM. Cells were collected by centrifugation (5,000 x g for 15 min.), resuspended in 1 ml of lysis buffer (1 mg/ml lysozyme, 50 mM Tris-HCl pH 8.0, 10 mM EDTA) and frozen overnight at -20°. Cells were slowly thawed at room temperature and then incubated for 30 minutes at 37° in the presence of Actinomycin D (1 mg/ml). After addition of .05 ml of a 20 mg/ml protease solution (Type VI, Strep. griseus, Sigma) in 1% sodium dodecyl sulfate (Matheson, Coleman and Bell), incubation was continued for 120 minutes at 47°. Phage DNA molecular weight changes

were analyzed by sedimentation through sucrose gradients.

To determine the effect of the extraction procedure on phage DNA, a "reconstruction" type of experiment was done. In this case, a small amount of ^3H -adenine labeled SP82G bacteriophage was phenol extracted and the DNA dialyzed against 0.05 M Tris-HCl (pH 8.0). A portion of the DNA was left untreated, while the rest was mixed with an appropriate amount of uninfected *B. subtilis* cells and treated with lysozyme and SDS-pronase. The two DNA preparations were then analyzed on neutral sucrose gradients. The results (Figs. 1A, 1B) show a small but significant drop in the molecular weight of the DNA caused by the SDS-pronase procedure. Approximately 1.0-1.5 double strand breaks per DNA molecule are introduced by the extraction procedure.

The profiles of phage DNA extracted at 2.5, 5, 25 and 40 minutes following infection (Figs. 1C - 1F) show a progressive shift towards smaller molecular weights, indicating that double strand breakage of the infecting DNA increases with time.

Table 2 summarizes the information contained in the sedimentation profiles of Fig. 1. The number of double strand breaks were calculated using the phage DNA put through the SDS-pronase extraction procedure as a control. Percent acid soluble nucleotides were calculated by determining the percentage of the total counts in the gradient that stayed at the top of the gradient.



It is clear that the infecting DNA is subject to a large number of endonucleolytic breaks during infection in the presence of CM. In addition, as the DNA is fragmented, cellular exonucleases would have an increased number of sites to attack and thus the percent acid soluble nucleotides should increase with time. It appears that intracellular

Fig. 1. Molecular weight changes of SP82G DNA during infection in the presence of chloramphenicol as determined on neutral sucrose gradients.

Cell lysates were prepared as described in text. Aliquots (0.2 ml) were carefully layered onto 5-20% neutral sucrose gradients and centrifuged at 35,000 rev/min for 105 min at 23° in an SW65 rotor.

Fig. 1A: sedimentation profile of phenol extracted SP82G DNA;

Fig. 1B: phenol extracted SP82G DNA mixed with approximately 10^9 cells and treated with lysozyme and SDS-protease;

Figs. 1C-1F: infection allowed to proceed for 2.5 min (1C), 5 min (1D), 25 min (1E) and 40 min (1F). Arrow () in 1A denotes sedimentation value for phenol extracted DNA; arrows () in 1B-1F denote sedimentation value for lysozyme-protease treated control DNA.

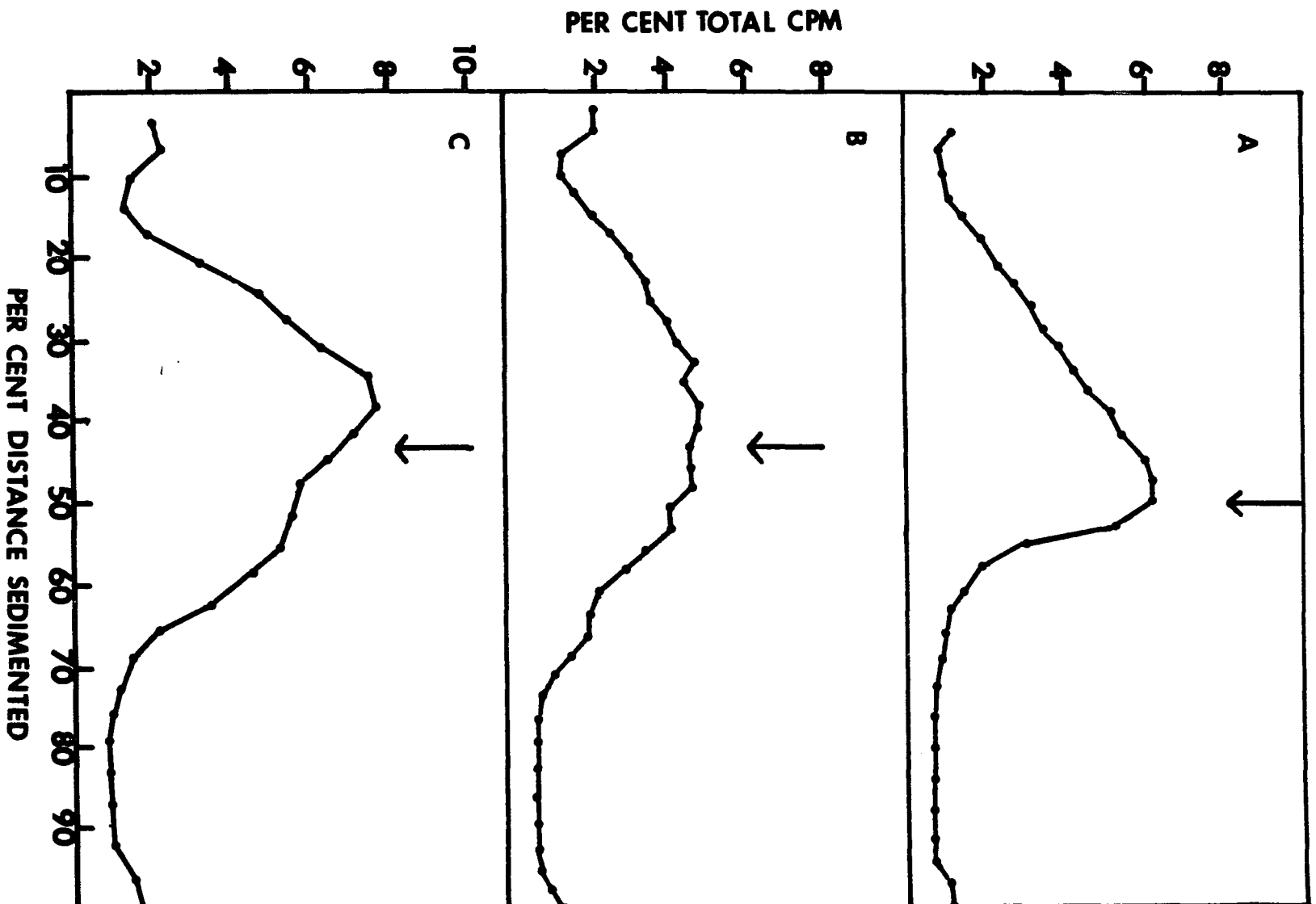


Fig. 1

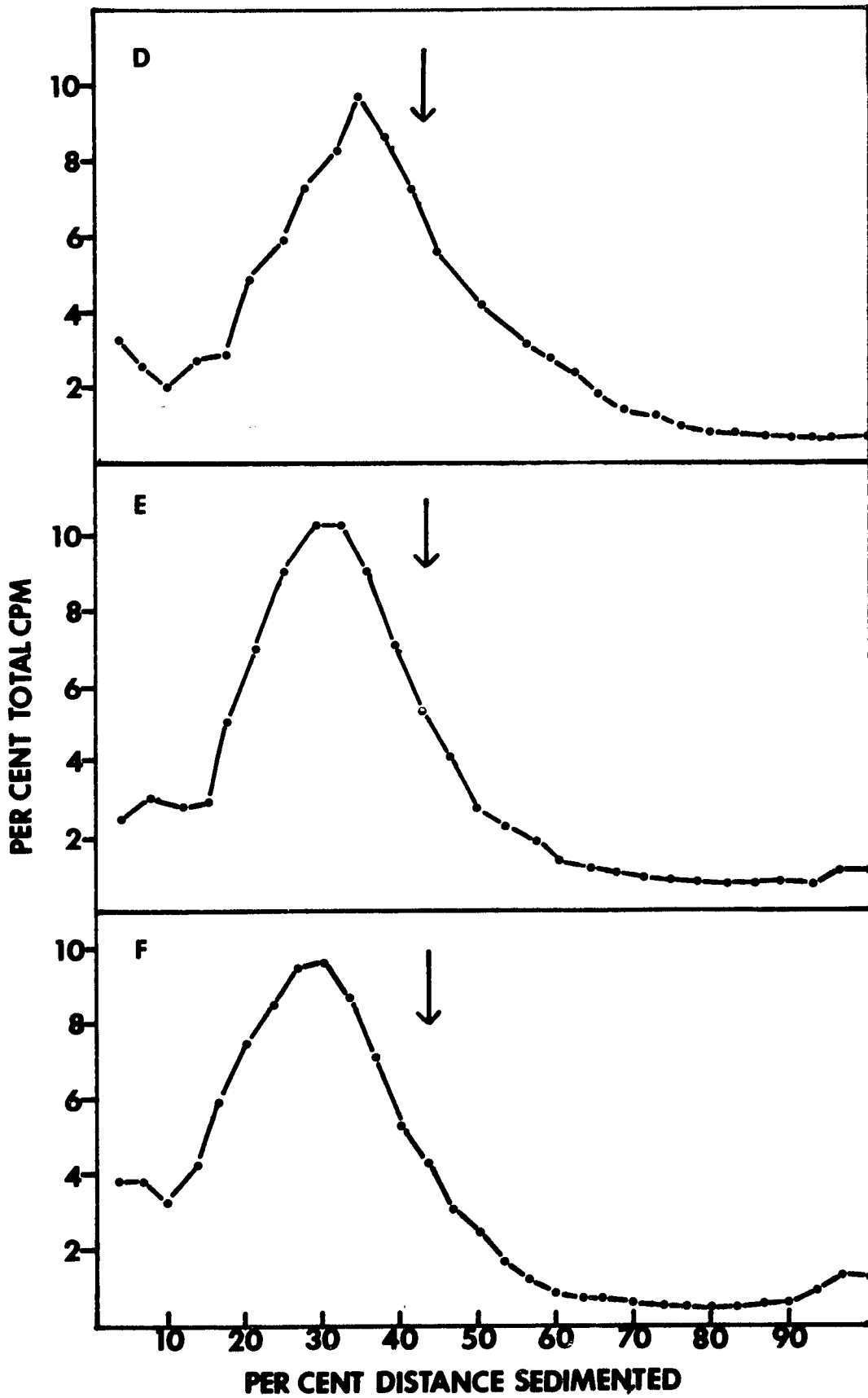


Fig. 1 (cont'd)

Table 2. DAMAGE TO PHAGE GENOME DURING
INFECTION IN THE PRESENCE OF CHLORAMPHENICOL

Time after Infection (min)	No. of Double Strand Breaks/DNA Molecule*	% Acid Soluble Nucleotides
Control	----	4.37
2.5	1	4.37
17	2-2.5	5.85
25	4.0-4.5	6.32
40	7.0	7.64

* calculated by method of Charlesby (1953)

inactivation is the result of an endonuclease capable of causing double strand breaks in SP82G DNA.

An experiment to quantitate single strand breaks by sedimentation through alkaline sucrose gradients is shown in Fig. 2. The control showed a large number of breaks, probably induced by the extraction procedure. Thus, quantitation of single strand breaks with respect to time is impossible.

Effect of Divalent Cations on the Total Nuclease

Activity of Uninfected Cell Extracts

Before attempting to look at the effect of infection on the nuclease activity of crude extracts, conditions must be found to optimize the total nuclease activity of uninfected extracts. The concentration of divalent cation was found to be critical in this respect and an experiment was designed to measure this effect using various concentrations of $MgCl_2$ and $CaCl_2$. The results (Fig. 3) show that:

- 1) essentially no acid soluble product is formed in the absence of divalent cations and,
- 2) uninfected extracts are stimulated to a greater extent by 10 mM $MgCl_2$ than by 10 mM $CaCl_2$. For this reason, 10 mM $MgCl_2$ is used in all total nuclease assays.

Effect of Infection on Total Nuclease

Activity of Cell Extracts

Having shown that intracellular inactivation involves an endonucleolytic attack on phage DNA, the question of how phage infection inhibits this activity could be investigated. The most straightforward explanation, that of a phage induced protein directly inhibiting the

Fig. 2. Molecular weight changes of SP82G DNA during infection in the presence of chloramphenicol as determined by alkaline sucrose gradients.

Cell lysates were prepared as described in text. Aliquots (0.2 ml) were layered onto 5-20% alkaline sucrose gradients and centrifuged at 35,000 rev/min. for 105 min at 23° in an SW65 rotor. Sedimentation profiles are shown for lysozyme protease treated control (Δ), 5 min (\blacktriangle) and 40 min (\square) after infection.

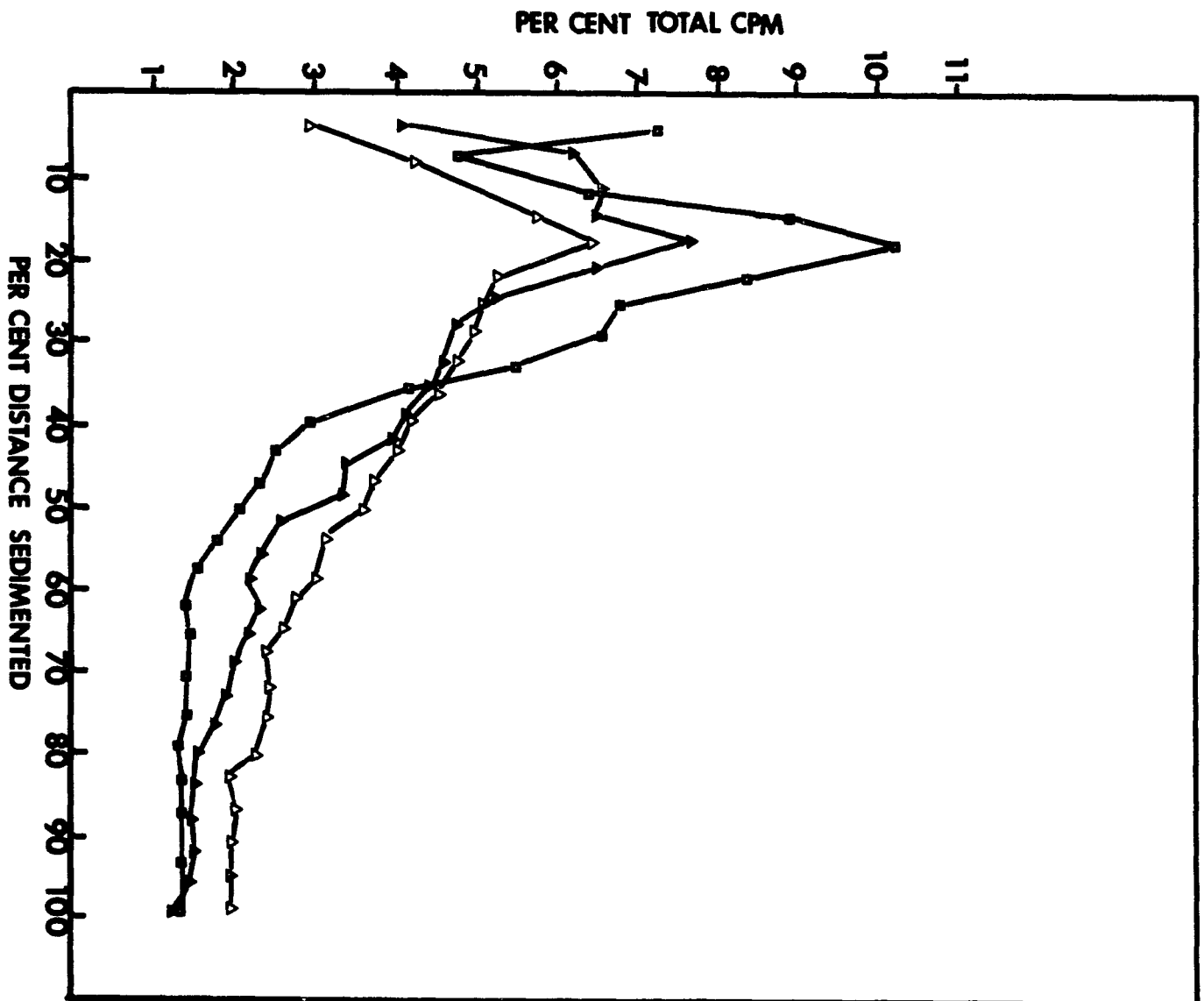


Fig. 2

Fig. 3. Effect of divalent cations on the total nuclease activity of uninfected cell extracts.

Total nuclease assays were done as described in Materials and Methods using native SP82G DNA and various amounts of $MgCl_2$ and $CaCl_2$. Acid soluble product formed is shown as a function of Mg^{2+} (●) and Ca^{2+} (□) concentrations.

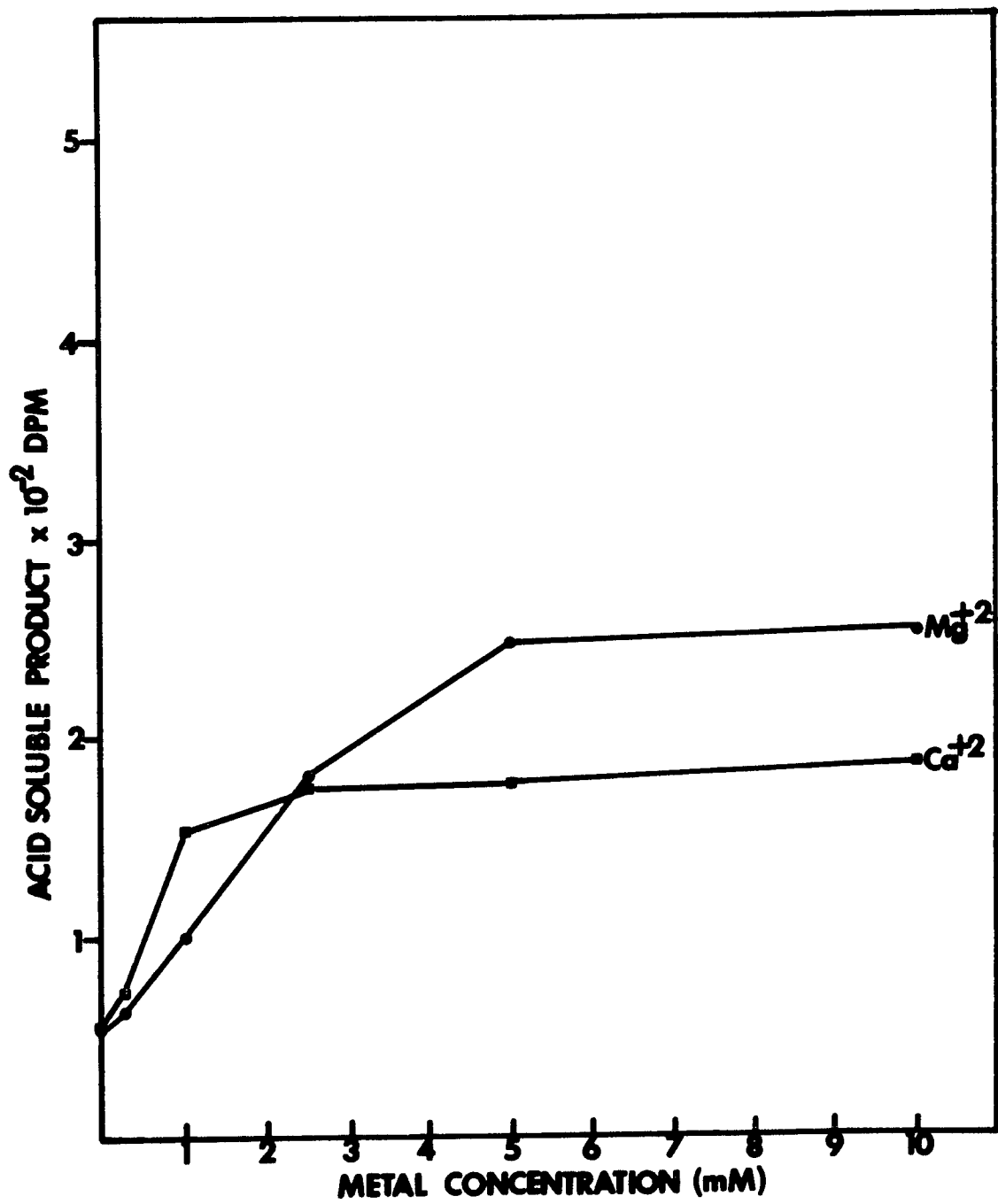


Fig. 3

intracellular inactivation nuclease, was the first to be investigated.

The ability of a crude extract to degrade substrate DNA into acid soluble nucleotides is a function of both cellular exonucleases and endonucleases. The exonucleases, which attack only free ends of DNA molecules, are responsible for all of the degradation to acid soluble nucleotides. The endonucleases, although producing no acid soluble nucleotides, supply more substrate sites for exonucleases by generating more free DNA ends. Thus, the inhibition or loss of an endonuclease in a crude extract should be detectable using an assay of the amount of acid soluble product.

A comparison of total nuclease activity between uninfected cell extracts and extracts of cells infected with SP82G for various lengths of time was done. Cells were infected at a multiplicity of infection of 10 and infection stopped by rapid cooling in the presence of chloramphenicol (200 $\mu\text{g}/\text{ml}$). Cells were washed with 0.05 M Tris-HCl (pH 8.0) to remove extracellular nucleases and lysed with lysozyme.

Fig. 4 shows total nuclease activity as a function of time after infection at 33° and 37°. Uninfected extracts show a linear increase with time. With extracts of cells infected at 37°, no change in nuclease activity is observed up to 1 1/2 minutes after infection. By 3 minutes, a distinct decrease in total nuclease activity is observed which reaches a maximum at about 6 minutes. After 6 minutes, nuclease levels slowly rise again and are approximately equal to uninfected levels by 20 minutes after infection (not shown). When infection occurs at 33°, the process appears much more complex. At early times after exposure to phage a marked increase in nuclease activity is observed. This increase peaks at 2 minutes after infection, and rapidly decreases to uninfected

Fig. 4. Effect of infection on the total nuclease activity of cell extracts.

Cells were grown to 10^8 cells/ml and infected with SP82G phage at an m.o.i. = 10. At various times after infection, chloramphenicol (200 μ g/ml) was added and cells cooled rapidly in ice. Cells were lysed and extracts assayed using the total nuclease assay with native SP82G DNA. Acid soluble product is shown as a function of time after infection for extracts of infected (●) and uninfected (■) cells. Fig. 4A: infection at 33°; 4B: infection at 37°.

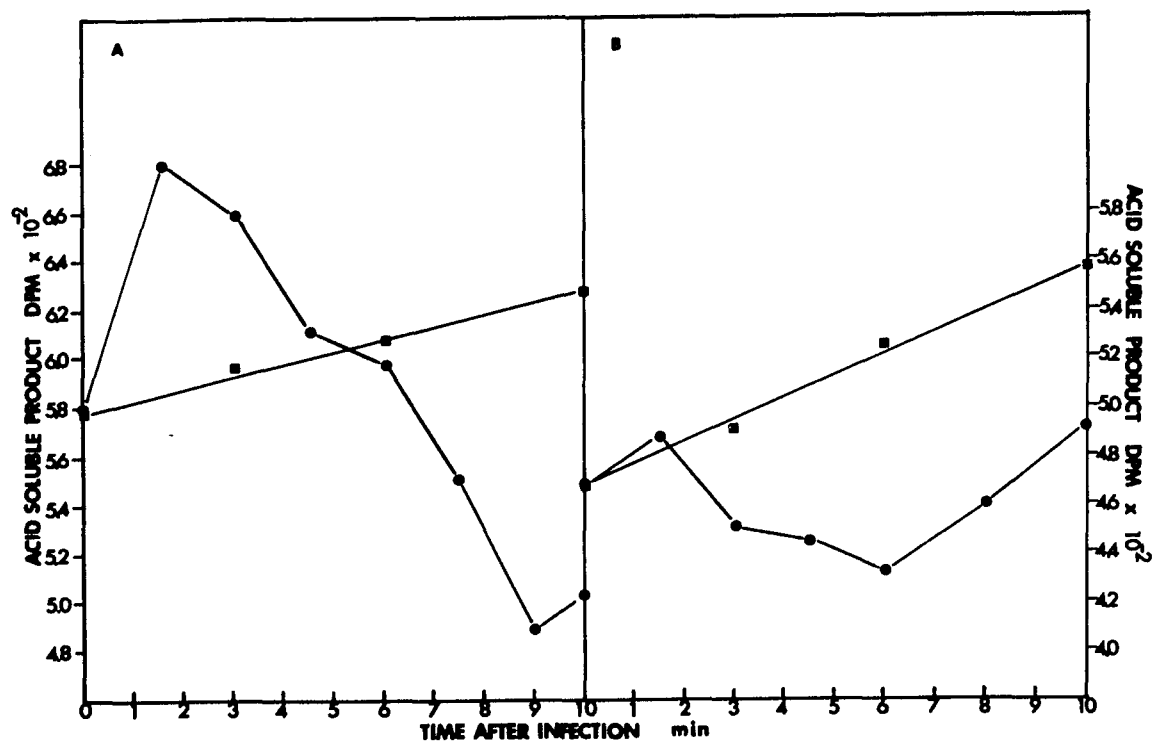


Fig. 4

levels by 5 minutes. Nuclease levels continue to fall until about 9 minutes after infection, after which a slow increase in activity is seen. As at 37°, by 20 minutes after infection, levels of nuclease activity in the two extracts are approximately identical.

Thus, at both 33° and 37° during phage infection, there occurs a marked decrease in the total nuclease activity of the infected cell extract when compared to identical uninfected cell extracts.

Quantitation of Total Nuclease Activity in Cell Extracts

One possible explanation of the decrease in nuclease activity observed above is that a certain population of cells is prematurely lysed or structurally weakened by infection, so that the decreases observed simply reflect a drop in total protein concentration. In addition, since the inhibition of intracellular inactivation is chloramphenicol sensitive (McAllister, 1972), phage induced inhibition of a host nuclease thought to be responsible for intracellular inactivation should also be chloramphenicol sensitive.

To investigate these two questions, three large extracts of cells grown at 37° were prepared. These were: uninfected, infected for 6 minutes, and infected for 6 minutes in the presence of CM (200 µg/ml). Fig. 5 shows total nuclease activity as a function of mg of protein per ml of reaction mixture. Two conclusions can be drawn from the data.

First, there is at least twice as much nuclease activity per mg of protein of uninfected cell extract as there is per mg of protein of infected cell extract. This shows that nuclease activity in extracts of cells infected for 6 minutes at 37° is significantly inhibited in comparison to uninfected cell extracts. That the inhibited activity is probably endonucleolytic in nature can be inferred from the fact that at

Fig. 5. Quantitation of total nuclease activity in cell extracts.

Total nuclease assays were done as described in Materials and Methods using heat denatured SP82G DNA as substrate. Acid soluble product formed is shown as a function of mg of protein in extracts from cells infected (■), uninfected (▼) and infected in the presence of CM (●).

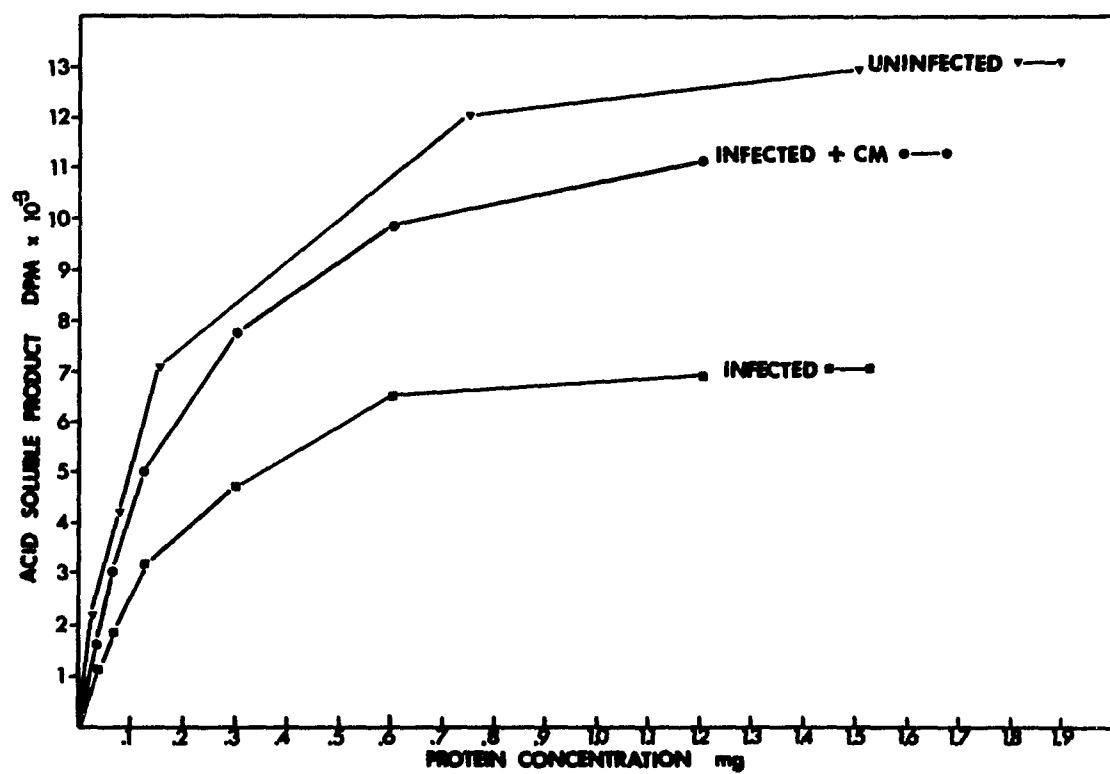


Fig. 5

high concentrations of infected cell extract, degradation is clearly substrate limited at a point where 50% of the total DNA has been hydrolyzed. The uninfected cell extract, on the other hand, undergoes no substrate limitation and degradation ceases to increase only after >90% of the DNA has been hydrolyzed. The substrate limitation seen with extracts of infected cells is clearly attributable to an inhibited endonuclease.

Secondly, as expected, extracts made from cells infected in the presence of CM are almost identical with uninfected cell extracts. Nevertheless, a small (10%) but reproducible inhibition of total nuclease activity is seen in these extracts. This raises the possibility that the phage-induced inhibition has two components: a CM-sensitive inhibition accounting for about 90% of the observed decrease, and a CM-insensitive inhibition accounting for 10% of the decrease in host nuclease activity. That the nuclease inhibited by the CM-insensitive process is also an endonuclease can be implied from the same argument outlined above.

Effect of Infection in Presence of CM on Total

Nuclease Activity of Cell Extracts

To determine at what time after infection the CM-insensitive inhibition is maximally manifest within the cell, nuclease activity as a function of time after infection in the presence of CM was measured. Experimental procedures were the same as used previously, with the exception that CM (200 µg/ml) was added to the cell suspension 5 minutes prior to infection. McAllister (1972) has shown that CM does not affect phage adsorption or DNA injection and does no irreversible harm to the bacteria.

The results (Fig. 6) indicate that within two minutes after infection at 37°, CM-insensitive inhibition is fully realized. No further change in nuclease levels occurs after this initial decrease. The rapidity with which this decrease is brought about argues against the formation of some phage induced inhibitor, and the fact that its onset coincides with the time needed for phage DNA injection suggests the possibility that the phage itself transfers something into the cell which causes the inhibition directly.

Studies were attempted using Actinomycin D to determine if RNA synthesis was required for this CM-insensitive inhibition. The experiments could not be evaluated, however, because residual levels of Actinomycin D in cell extracts inhibited all nuclease levels to such an extent that comparisons between extracts were impossible. Attempts to remove these residual levels of Actinomycin D by extensive dialysis were unsuccessful. Again, it should be pointed out that the speed with which the inhibition occurs argues against any cell mediated macromolecular synthesis of inhibitor.

Chloramphenicol and Amino Acid Incorporation

Levinthal et al. (1962) has shown that CM concentrations similar to those used in the above experiments (200 µg/ml) essentially stop all incorporation of radioactive amino acids into acid precipitable protein. Because of the unexpected discovery of a CM-insensitive inhibition, Levinthal's experiment was repeated to confirm that the CM treatment was, in fact, blocking protein synthesis in this system, and to see whether phage infection in any way modified the CM effect on protein synthesis.

The results (Fig. 7) show that chloramphenicol at a dose of 200 µg/ml effectively blocks at least 95% of protein synthesis in unin-

Fig. 6. Effect of infection in presence of CM on total nuclease activity of cell extract.

Cells were grown to 10^8 cells/ml and treated with CM (200 μ g/ml) 5 minutes previous to infection with SP82G. Infection was carried out at an m.o.i. = 10 at 37°. At various times after infection, aliquots were withdrawn and chilled rapidly. Cells were lysed and extracts assayed using the total nuclease assay with native SP82G DNA. Acid soluble product formed is shown as a function of time after infection for extracts of infected (●) and uninfected (■) cells.

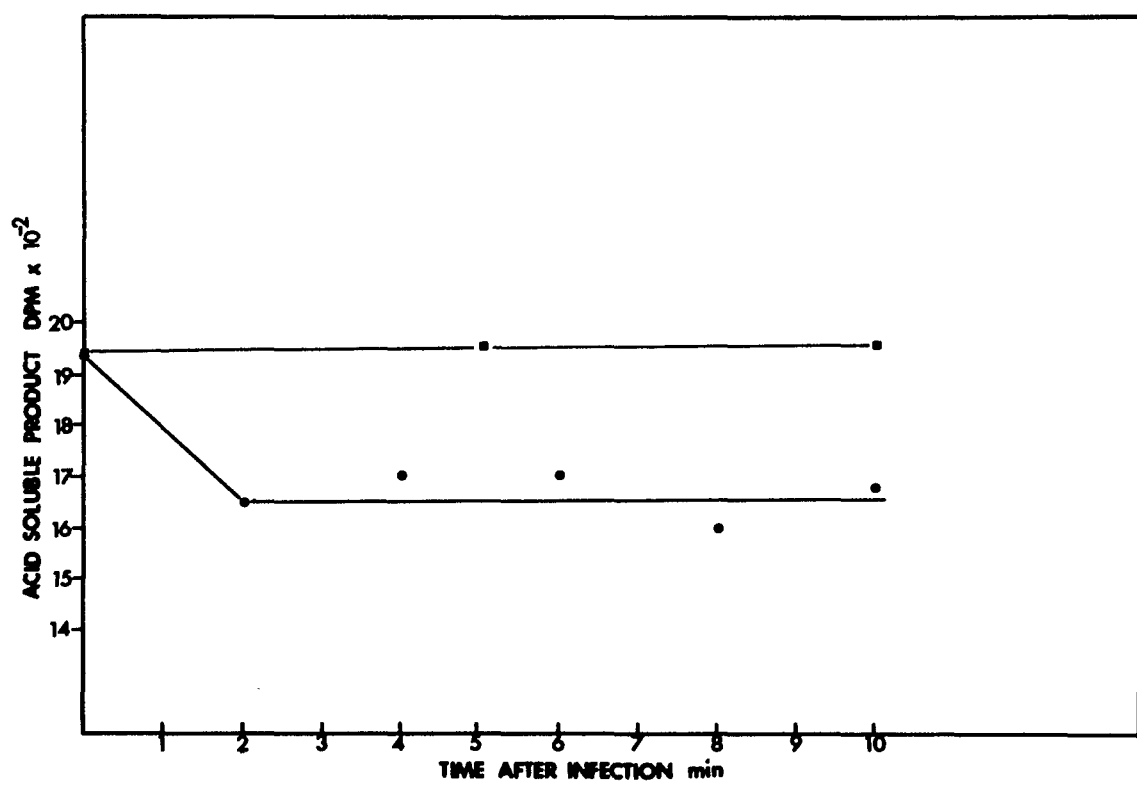


Fig. 6

Fig. 7. Effect of chloramphenicol on amino acid incorporation

Experimental procedure described in Materials and Methods. The amount of ^3H -amino acid incorporated into acid precipitable protein is shown as a function of time for control cells (no CM) (O), cells treated with CM (■) and cells treated with CM and infected with SP82G at time zero (●).

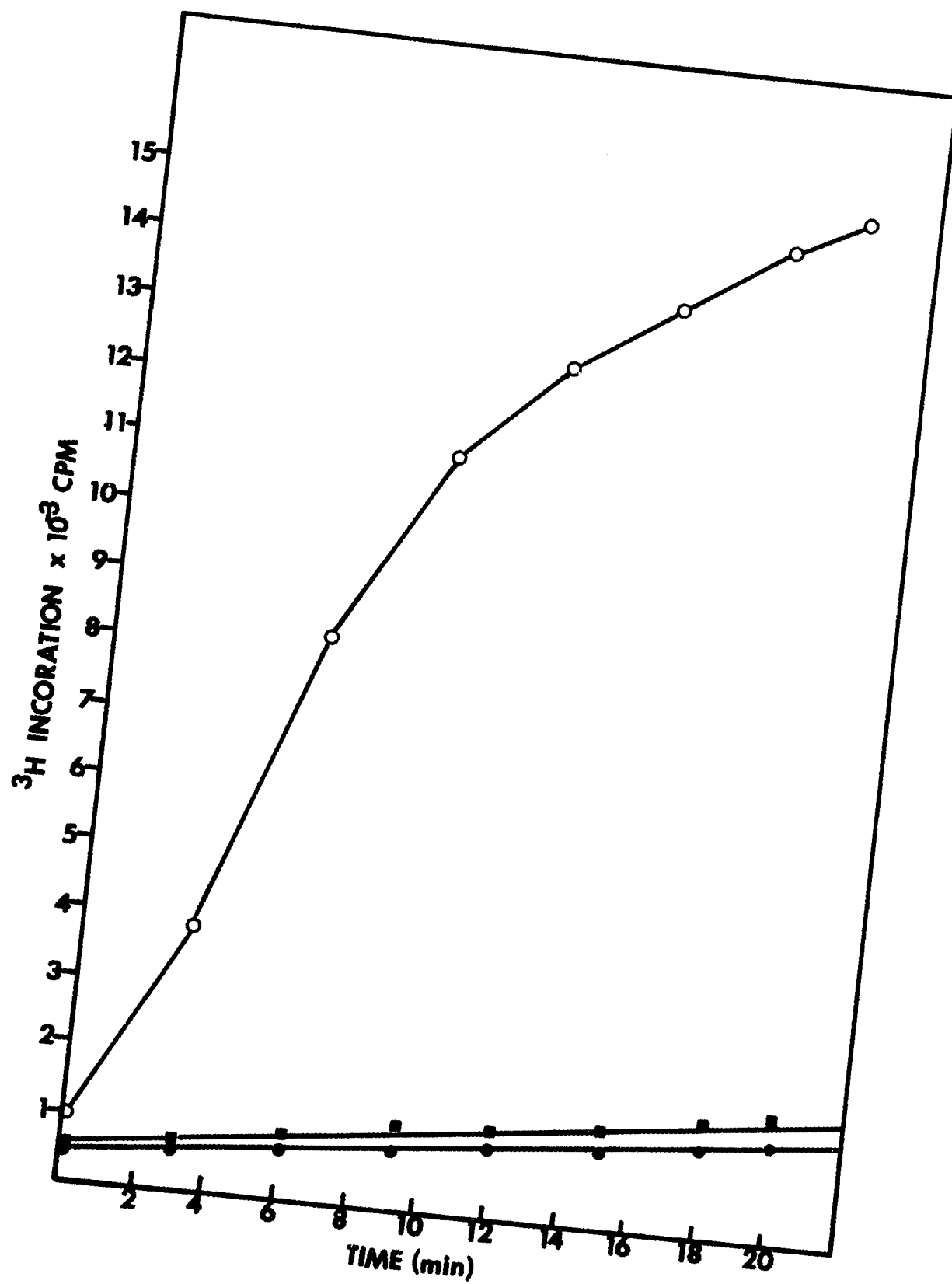


Fig. 7

fecting cells. In addition, infection with SP82G does not modify chloramphenicol's effect on protein synthesis. These results reinforce the idea that the CM-insensitive inhibition is independent of host mediation and is probably carried into the cell with the infecting genome.

Comparison of Infected and Uninfected

Cell Extracts on Various Substrates

Since a striking quantitative difference in total nuclease activity between infected and uninfected cell extracts is evident with SP82G DNA as a substrate, an attempt was made to investigate the substrate specificity of the inhibited nuclease. The rationale of the experiment was that if the nuclease which is inhibited by infection is unable to attack a specific substrate, e.g. B. subtilis DNA, the differences between extracts from uninfected and infected cells, when measured on that substrate, should disappear. To investigate this, the following substrates were tested:

(1) SP82G native and denatured DNA. As seen in Fig. 8, a distinct, quantitative difference between extracts of infected and uninfected cells does exist using both denatured and native SP82G DNA. A distinct difference is noted between the total amount of degradation with each substrate. With equivalent amounts of crude extracts, about 10 times more acid soluble product is formed using denatured DNA as substrate as when native DNA is used. That this increase is almost certainly due to a greater activity on denatured DNA of the cellular exonucleases is shown by the fact that the percent inhibition in infected extracts is the same using either substrate. Thus, the nuclease inhibited by phage infection is able to degrade both native and denatured SP82G DNA.

Fig. 8. Comparison of extracts from infected and uninfected cells on native and denatured SP82G DNA.

Total nuclease assays were done as described in Materials and Methods using denatured or native ^3H -SP82G DNA. Acid soluble product formed is shown as a function of mg of protein of uninfected (O) and infected (●) cell extracts. Fig. 8A: denatured SP82G DNA used as substrate; 8B: native SP82G DNA used as substrate.

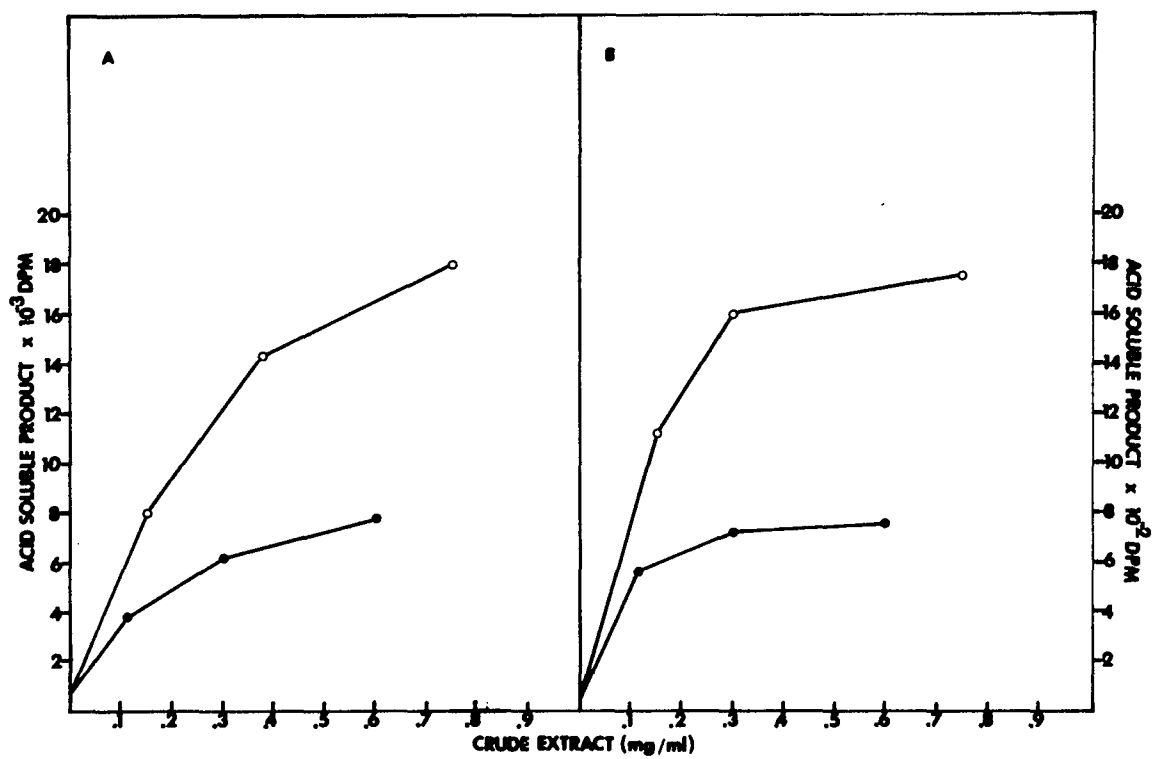


Fig. 8

(ii) UV irradiated and un-irradiated native SP82G DNA. The work of Epstein (1967) suggested that a nuclease with a preference for UV irradiated DNA played an important role in intracellular inactivation. To test this possibility, the nuclease activities of extracts from infected and uninfected cells were tested on equal amounts of UV irradiated and un-irradiated native SP82G DNA. If the enzyme shows a preference for UV irradiated substrate, extracts from uninfected cells should show greater activity with UV DNA than with unirradiated DNA while extracts from uninfected cells should show no change. The net result expected would be an increase in the difference between extracts from uninfected and infected cells when using UV DNA as substrate when compared with the differences seen using un-irradiated DNA as substrate.

The results (Fig. 9) show that this is not the case. The degree of inhibition does not increase when UV irradiated DNA is used as substrate. Thus, the nuclease inhibited by phage infection does not exhibit a preference for UV irradiated DNA.

(iii) Other nucleic acids. The question whether this inhibition of nuclease activity would be seen when using nucleic acids other than SP82G DNA as substrate was the next to be investigated.

Fig. 10 shows results comparing the nuclease activities of infected and uninfected extracts on three different substrates. Fig. 10a and 10b show the results using Bacillus subtilis and bacteriophage T4 DNA respectively. In both cases, there is a significant difference between extracts, thus implying that the nuclease inhibited by phage infection is able to degrade either of the substrates. Fig. 10c shows results using silk worm larvae ribosomal RNA as the substrate. Again, there is clearly a distinct difference between the two extracts. The only interpretation possible is that the nuclease activity inhibited during phage

Fig. 9. Comparison of extracts from infected and uninfected cells on UV irradiated and un-irradiated native SP82G DNA.

Total nuclease assays were done as described in Materials and Methods using unirradiated and UV irradiated ($36,000 \text{ ergs/mm}^2$) native SP82G DNA. Acid soluble product formed is shown as a function of mg. of protein of extracts from infected (●) and uninfected (○) cells. Fig. 9A: UV irradiated native SP92G DNA used as substrate; 9B: un-irradiated native SP82G DNA used as substrate.

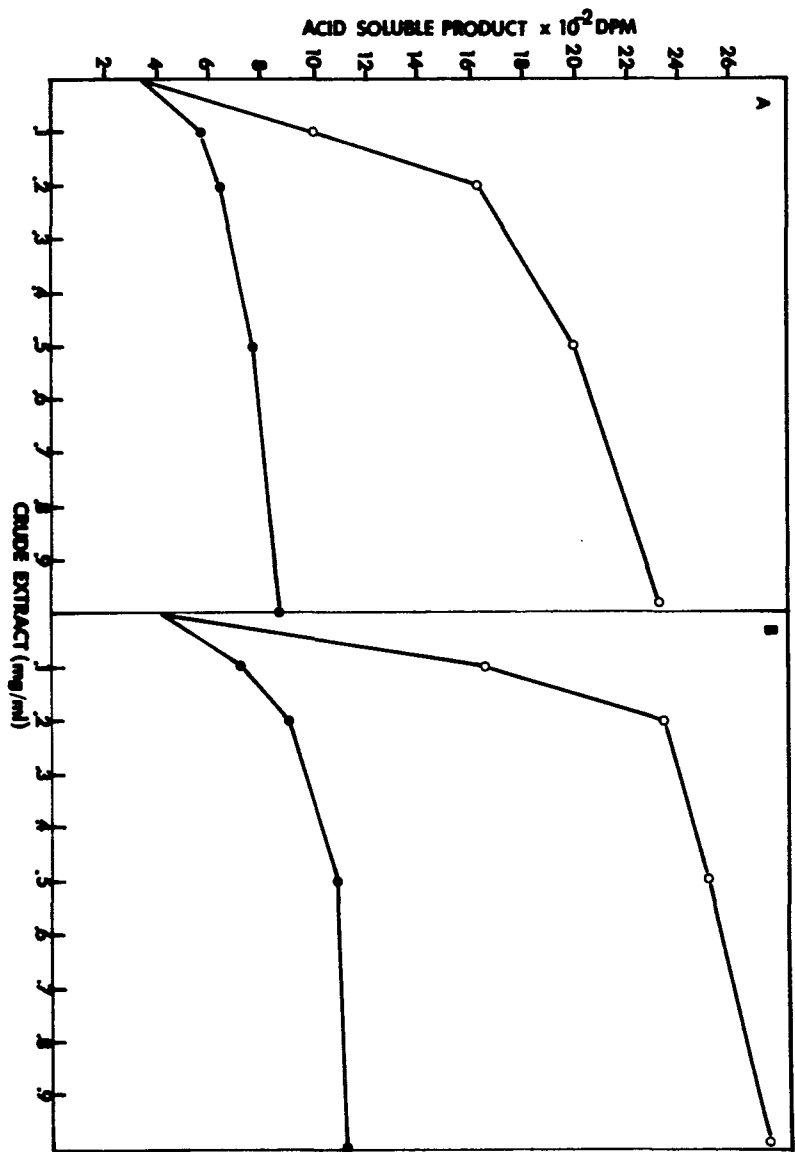


Fig. 9

Fig. 10. Comparison of extracts from infected and uninfected cells on B. subtilis and T4 DNA and silk worm larvae ribosomal RNA.

Total nuclease assays were done as described in Materials and Methods using native Bacillus subtilis DNA, native T4 DNA and silk worm larvae ribosomal RNA. Acid soluble product formed is shown as a function of mg of protein of extracts from infected (●) and uninfected (○) cells. Fig. 10A: native B. subtilis DNA used as substrate; 10B: native T4 DNA; 10C: silk worm larvae ribosomal RNA.

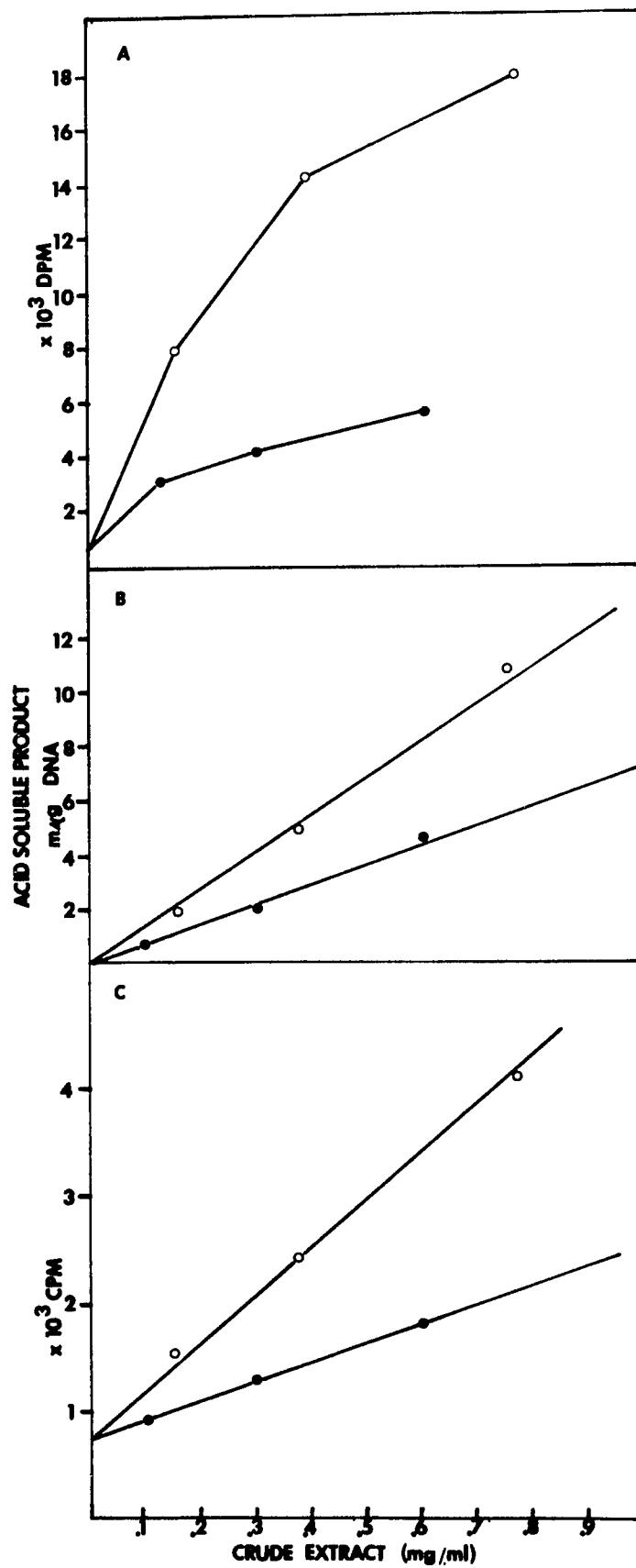


Fig. 10

infection will attack RNA as well as DNA.

Taken in total, this series of experiments implies that the nuclease activity inhibited by phage infection shows no preference as to the kind of nucleic acid it will attack (within the limitation of the substrates tested).

Standard Assay

Once the existence of an inhibited nuclease had been established, experiments were undertaken to develop an assay that would specifically detect that nuclease.

As described earlier and seen in Fig. 11A, extracts from infected cells show a "leveling off" (saturation) of acid soluble product formation at protein concentrations greater than 5-6 mg/ml. The substrate, in this case free ends of DNA molecules which exonucleases attack, is clearly limiting the extent of degradation. Since the potentially inhibited nuclease can clearly "open up" new substrate sites, it should be possible to add small amounts of extract from uninfected cells to "saturating" amounts of extracts from infected cells and observe an increase in acid soluble product. This increase would be due to the formation of more ends of DNA molecules by the endonuclease in the extract from uninfected cells, thus releasing the substrate limitation.

Fig. 11B shows that such an increase is seen when extract from uninfected cells is added to a saturating amount of extract from infected cells. This increase in acid soluble product is directly proportional to the quantity of uninfected cell extract added, thus making the assay suitable for the quantitation of nuclease activity in cell extracts.

The standard assay developed is simple and convenient. To each tube in an experiment, a saturating amount of extract from infected cells

Fig. 11. Standard Assay

Fig. 11A: total nuclease assay done as described in Materials and Methods using denatured SP82G DNA and extract from infected cells (■); 11B: standard assay done as described in Materials and Methods using denatured SP82G DNA with extracts from infected (■) and uninfected (□) cells.

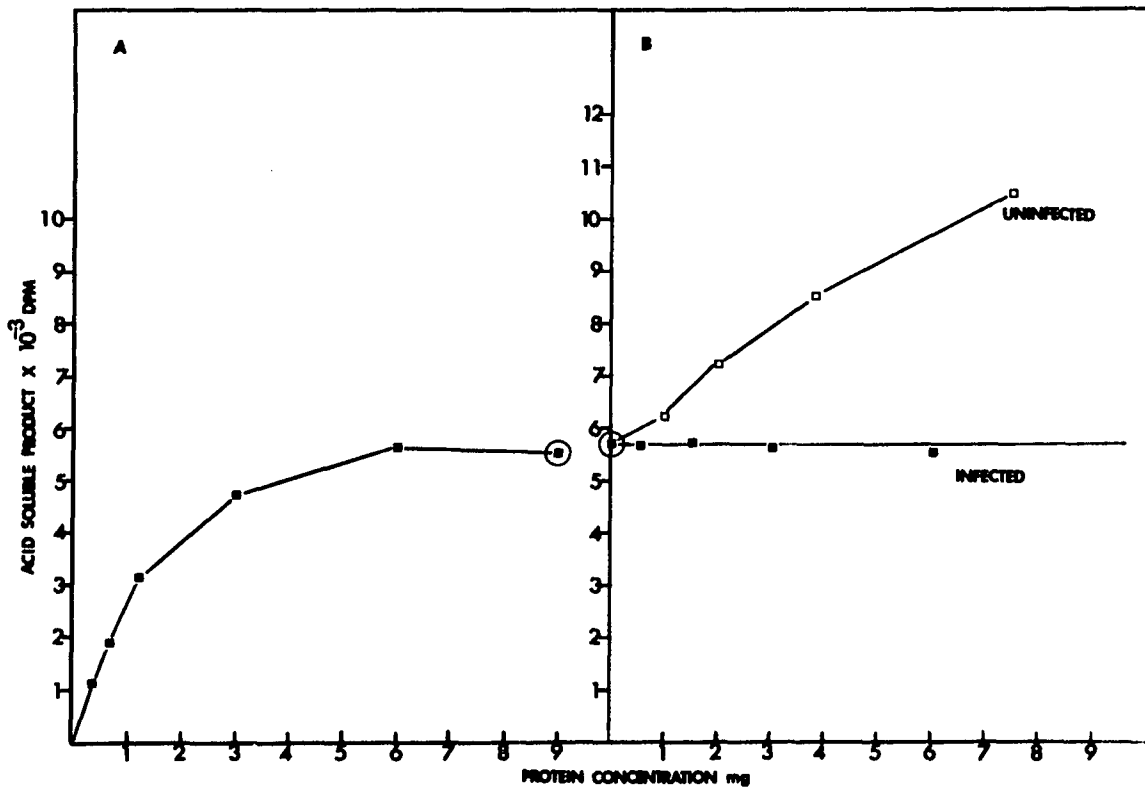


Fig. 11

is added. The first tube, to which no additional extract is added, serves to establish a base line of activity. Only those tubes to which additional endonucleolytic activity has been added will show amounts of acid soluble product in excess of the baseline amount.

Cellular Location of Inhibited Nuclease

Studies by Birnboim (1966) and Scher and Dubnau (1973) described cellular nuclease activities in Bacillus subtilis which were released to the media upon protoplast formation, thus suggesting a periplasmic location. Since Scher and Dubnau's nuclease was an endonuclease thought to play an important role in the "trimming" of transforming DNA molecules, it was felt that the nuclease presently being studied should be classified as to cellular location.

Experiments to test whether the standard assay activity was membrane bound or "soluble" are shown in part I of Table 3. Uninfected cells were lysed, centrifuged at 10,000 x g for 10 minutes to remove debris and then centrifuged at 140,000 x g for 60 minutes to remove all non-soluble components. Characteristically, 95-100% of the nuclease activity found in the cell extract was recovered in the 144,000 x g supernatant fluid. Thus, a stable membrane associated nuclease is ruled out.

Dubnau (1973) has pointed out that such a test is not adequate proof of a cytoplasmic nuclease, since a nuclease located between the cell wall and the cell membrane would be released upon lysis with lysozyme and appear in the soluble fraction. Periplasmic enzymes can, however, be identified by their release during protoplast formation, resulting in a decreased activity in crude extracts made from washed protoplasts.

To test this, protoplasts were made, washed with 0.6 M sucrose-

Table 3. INTRACELLULAR LOCATION OF INHIBITED NUCLEASE

Part I

Fraction	Standard Assay Activity	
	(DPM/mg protein/hr at 37°)	
Crude	18,176	
Soluble (144,000 x g)	17,855	

Part II

Extract	Standard Assay	Total Nuclease
	(DPM/mg protein/hr at 37°)	
Whole cells	10,376	55,703
Protoplasts	10,504	54,829

0.05 M Tris-HCl (pH 8.0) and then lysed by addition of ice cold 0.05 M Tris-HCl (pH 8.0). The total nuclease and standard assay activities were compared on a per mg protein basis to cell extracts prepared as usual. The results (Table 3, part II) show no loss of activity in the extract made from protoplasts in either assay. Thus, the nuclease inhibited by phage infection appears to have a cytoplasmic location.

Effect of Salt on Standard Assay

Several unexpected results led to an investigation of the effects that various salts (KCl, NH₄Cl and NaCl) had on the total nuclease activity of extracts from uninfected and infected cells. As seen in Fig. 12, increasing the concentration of KCl in reaction mixtures increases the formation of acid soluble products in both extracts. Further, this increase is seen using either native or denatured DNA as substrate. Both NH₄Cl and NaCl also show this stimulation with KCl stimulating the most and NaCl stimulating the least.

Since the effects on the two extracts are parallel, it is assumed that the salt stimulates a nuclease common to both extracts. Although not particularly interesting in so far as the inhibited nuclease is concerned, this effect constitutes a serious drawback to the standard assay since it causes a fluctuation in the amount of degradation caused by saturating amounts of extract from infected cells. Since standard assay activity is defined as any increase in acid soluble product above this saturating "baseline," any increase in salt concentration would be seen as a burst of standard assay activity. Thus, any protein separation (elution) procedure resulting in unequal salt concentrations among samples to be assayed would have to be avoided. Specifically, any kind of salt gradient, as used, for example, in ion-exchange chromatography,

Fig. 12. Effect of salt on standard assay.

Total nuclease assays were done as described in Materials and Methods using various amounts of KCl and extracts from infected (●) and uninfected (○) cells. Fig. 12A: denatured SP82G DNA used as substrate; 12B: native SP82G DNA used as substrate.

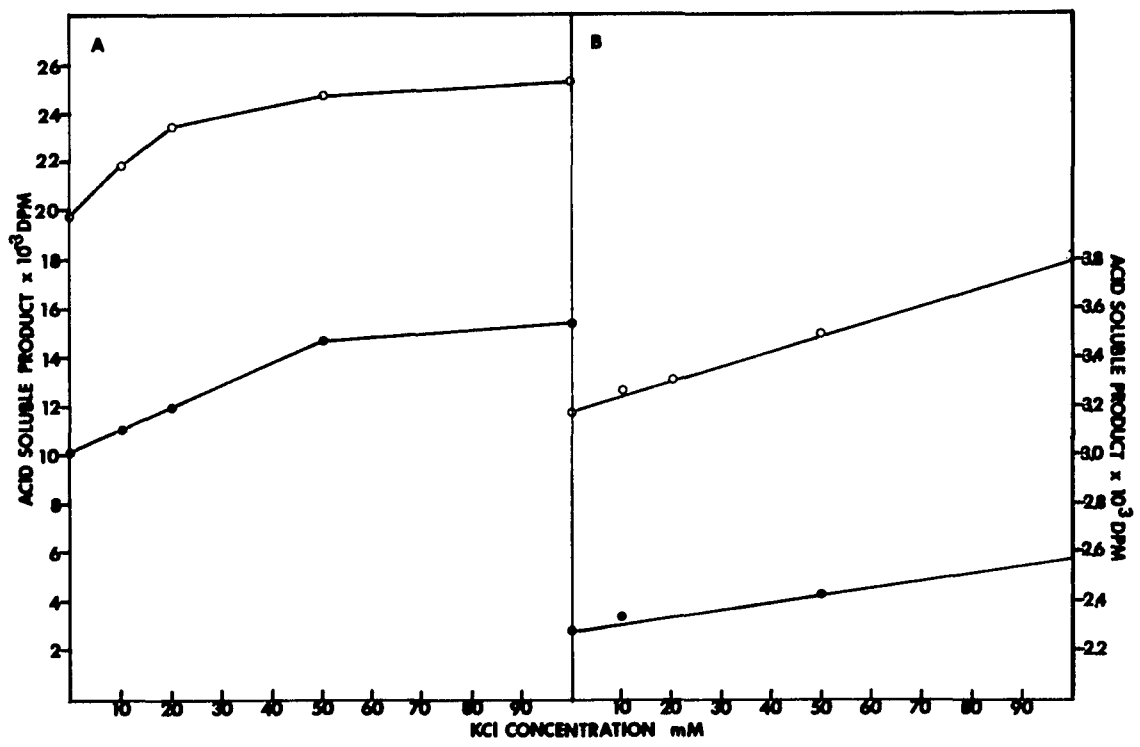


Fig. 12

could not be used. The sensitivity of the standard assay to salt severely limits the number of methods of protein separations that can be employed.

Nuclease Isolation

In a typical preparation, 15-16 ml of an extract from uninfected cells were put on a Sephadex G-200 column (1.5 x 55 cm). The column was eluted with 0.05 M Tris-HCl (pH 8.0) at a flow rate of 12 mls/hr and 3 ml fractions were collected. All work was done at 4°. Alternate fractions were assayed for A_{280} , total nuclease and standard assay activity.

Fig. 13A shows the A_{280} and the standard assay activity of the eluant from the Sephadex G-200 column. The bulk of the A_{280} material elutes within the first 15 fractions, while all of the standard assay activity elutes in a single peak around the fortieth fraction.

A comparison of total nuclease activity and standard assay activity in the eluant (Fig. 13B) shows that almost all exonuclease activity elutes in a broad peak encompassing the first twenty fractions. This peak is quite well separated from the slower eluting standard assay peak. It should be noted that the samples exhibiting the most exonuclease activity show no activity in the standard assay.

Fractions showing activity in the standard assay were pooled and concentrated using a Pellicon PSAC membrane filter, which retains compounds of 1,000 molecular weight and larger. Table 4 shows that the gel filtration-concentration step results in an approximate 27-fold purification of the nuclease and approximately 50% recovery of the starting activity.

Further attempts at purification, involving $(\text{NH}_4)_2\text{SO}_4$ fractionation, batch-wise preparations using DEAE-cellulose, phosphocellulose and DNA-cellulose, and even further gel filtration on Sephadex G-75 or

Fig. 13. Nuclease isolation

15.4 ml of extract from uninfected cells were put onto a Sephadex G-200 column (1.5 x 55 cm). The column was eluted with 0.05 M Tris-HCl (pH 8.0) at a flow rate of 12 mls/hr and 3 ml fractions were collected. Fig. 13A: shows A_{280} (●) and standard assay activity (□) of eluted fractions; 13B: shows total nuclease activity (■) and standard assay activity (□) of eluted fractions.

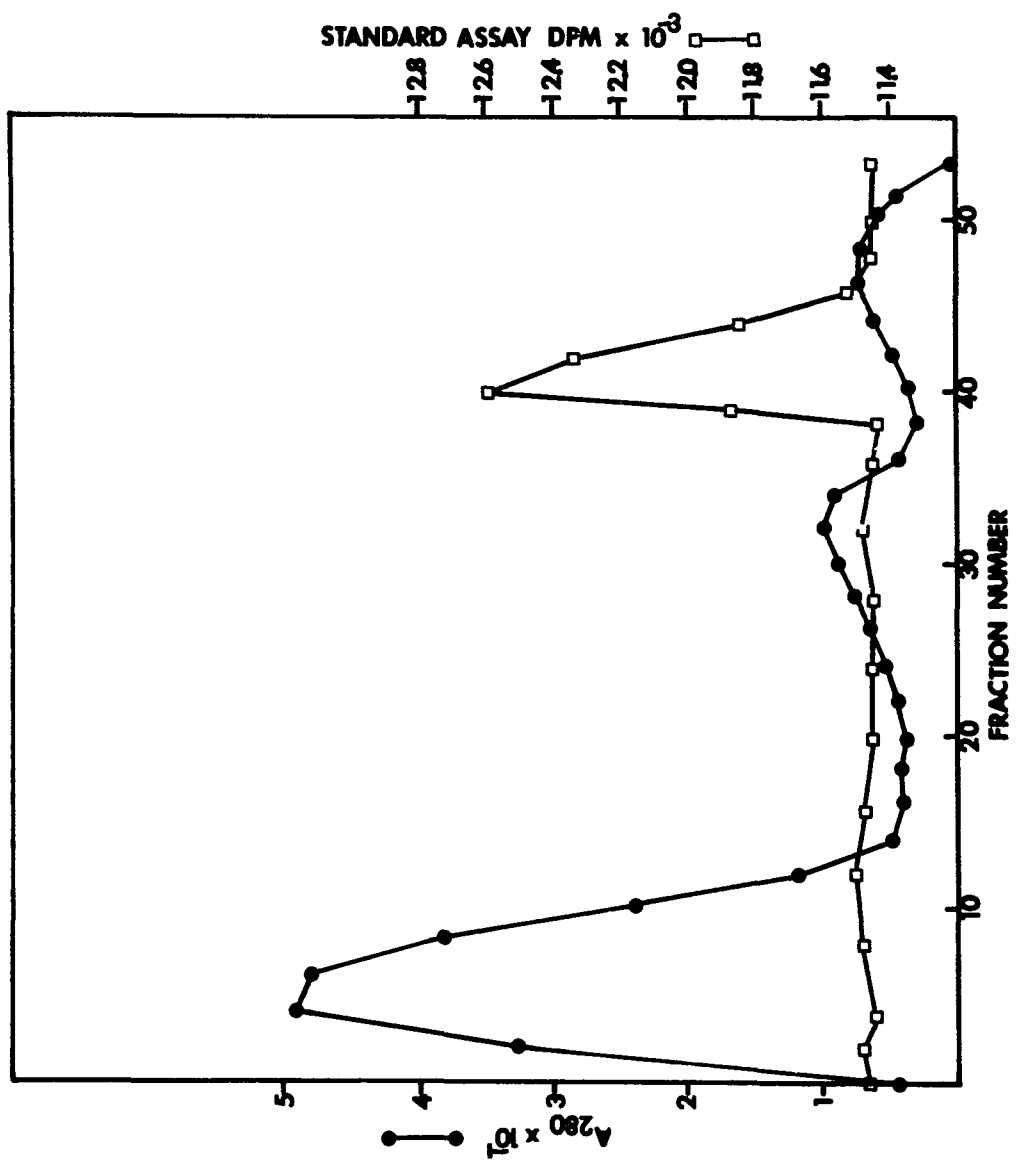


Fig. 13A

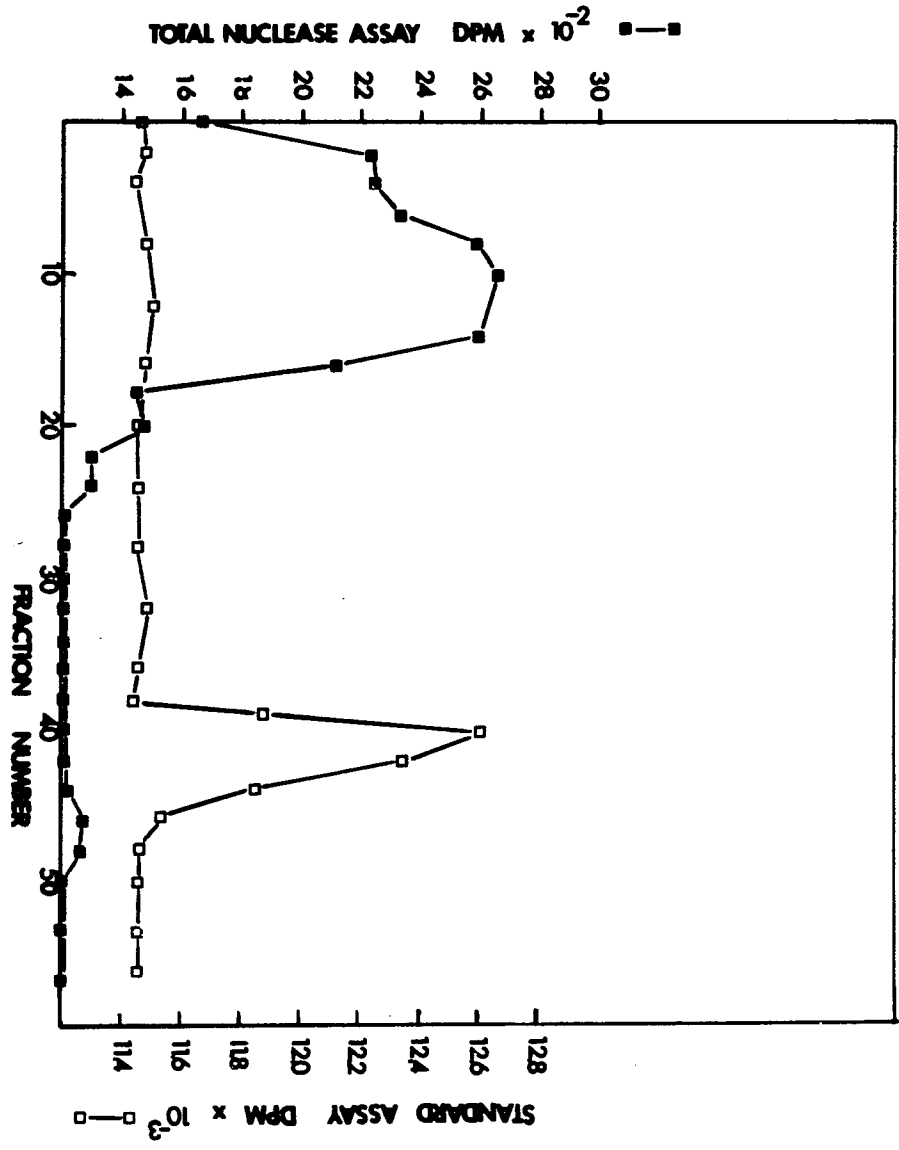


Fig. 13B

Table 4. NUCLEASE ISOLATION

<u>Fraction</u>	Volume (ml)	Protein Conc. (mg/ml)	Total Protein (mg)	Units* /ml	Total Units	Recovery %	Specific Activity (units/ mg)
Crude	15.5	25.7	399.0	495.2	7676.4	100	19.2
G-200 (conc.)	4.6	1.6	7.5	848.8	3904.6	50.8	520.7

* 1 unit is that amount of isolated nuclease causing the formation of 10.0 mug/hr of acid soluble product in the standard assay at 37°.

G-200, uniformly resulted in large, if not total, losses in activity, and no further increase in specific activity. The reasons for this apparent instability are not clear, but may result from the loss of some stabilizing factor during gel filtration.

Effect of Freezing on Isolated Nuclease

The isolated nuclease's instability is evident with storage. Within one week at 4°, the nuclease activity is completely lost. Freezing the enzyme, however, produces an even more dramatic loss of activity. The data shown in Table 5 shows that overnight storage at -20° results in a drop in activity of more than 60% when compared to an unfrozen extract. The addition of 20% glycerol (v/v) had little effect on this drop in activity. Subsequent freeze-thaw cycles resulted in no further drop in activity.

Molecular Weight of Isolated Nuclease

In an effort to determine the approximate molecular weight of the isolated nuclease, the gel filtration technique of Whitaker (1963) was employed, using Sephadex G-200. A standard curve (Fig. 14) plotting elution volumes as a function of the log of molecular weight, was derived, using a series of pure proteins of known molecular weight. The elution volume of the isolated nuclease was determined and its molecular weight was calculated. As shown in Fig. 14, the purified nuclease ran quite slowly and its molecular weight was calculated to be in the range of 10,000 - 12,000. However, no proteins of molecular weight less than that of the purified nuclease were used to derive the standard curve, and thus the linearity of the curve in regions of low molecular weight cannot be guaranteed. Therefore, it should be emphasized that

Table 5. EFFECT OF FREEZING ON PURIFIED NUCLEASE

Extract	Standard Assay Activity (DPM/mg protein/hr at 37°)	Activity loss (%)
G-200	24,294	0
G-200 stored overnight at:		
4°	23,070	<5
-20°	9,423	62
-20° plus 20% (v/v) glycerol	13,619	44

Fig. 14. Molecular weight of purified nuclease.

Experimental procedure described in Materials and Methods.

The standard curve was made using proteins of known molecular weight (●). The V/V_i of the isolated nuclease is shown by the arrow (↓).

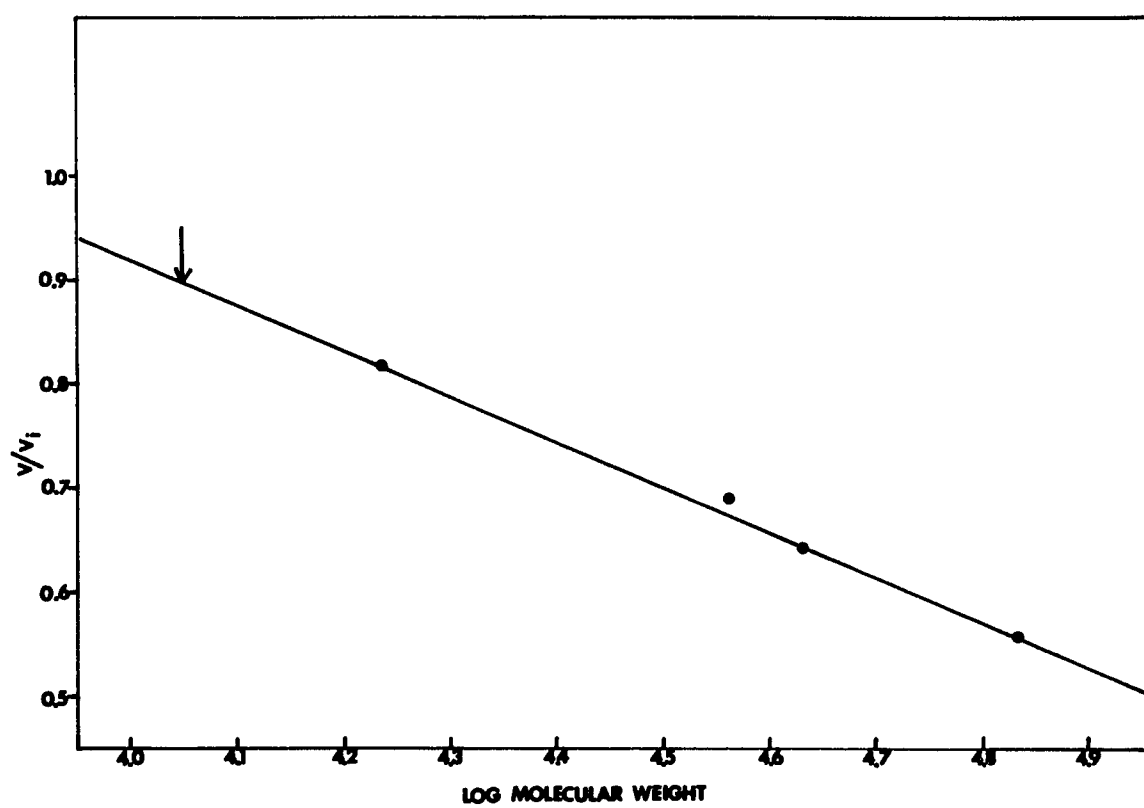


Fig. 14

the molecular weight range calculated for the purified nuclease is only an estimate.

Divalent Metal Requirement of Isolated Nuclease

Since extracts from uninfected cells required divalent cation for acid soluble product formation, it was of interest to determine whether the partially purified endonuclease had a similar requirement. The standard assay, however, was not suitable for this determination because of the mixture of nuclease activities involved. Therefore, DNA treated with purified nuclease under varying cation conditions was analyzed by sedimentation through sucrose gradients.

To determine if the purified nuclease had a strict requirement for divalent cations, DNA was incubated with the nuclease in the presence of 10 mM EDTA. The results (Fig. 15A) show that the nuclease is unable to cause even a slight change in the molecular weight of the DNA when EDTA is present, thus indicating an absolute requirement for divalent cation.

The question of the specificity of this cation requirement was investigated by adding equal amounts (2.5 mM) of $MgCl_2$, $CaCl_2$ and $MnCl_2$ to reaction mixtures and subsequent product analysis using sucrose gradients. From the results (Fig. 15B) it can be seen that while Mg^{2+} and Ca^{2+} can equally stimulate the action of the nuclease, Mn^{2+} affords very little, if any, stimulation.

Exonuclease Activity of Purified Nuclease

To determine whether the purified nuclease fractions exhibited any exonucleolytic activity .1-.2 mg (80-160 units) of the nuclease were incubated with native or denatured SP82G DNA, 10 mM Mg^{+2} and 50 mM Tris-

Fig. 15. Divalent metal requirements of purified nuclease.

Nuclease was isolated as described above. ^3H native SP82G DNA (10 μg) was incubated with 50 mM Tris-HCl (pH 8.0) various divalent cations and 160 units of isolated nuclease for 60 mins at 37° . Reactions were terminated by the addition of EDTA to a final concentration of 10 mM. Product size of DNA was determined by layering 0.2 ml of the incubation mixture on a 5-20% neutral sucrose gradient and centrifuging at 35,000 rev/min for 105 minutes at 23° in an SW65. Fig. 15A: control DNA (no nuclease) (●) and DNA incubated with nuclease in the presence of 10 mM EDTA (○); 15B: DNA from incubation mixtures containing 2.5 mM Mg^{2+} (●), Ca^{2+} (■) and Mn^{2+} (□). The arrow (↓) shows the sedimentation position of control DNA.

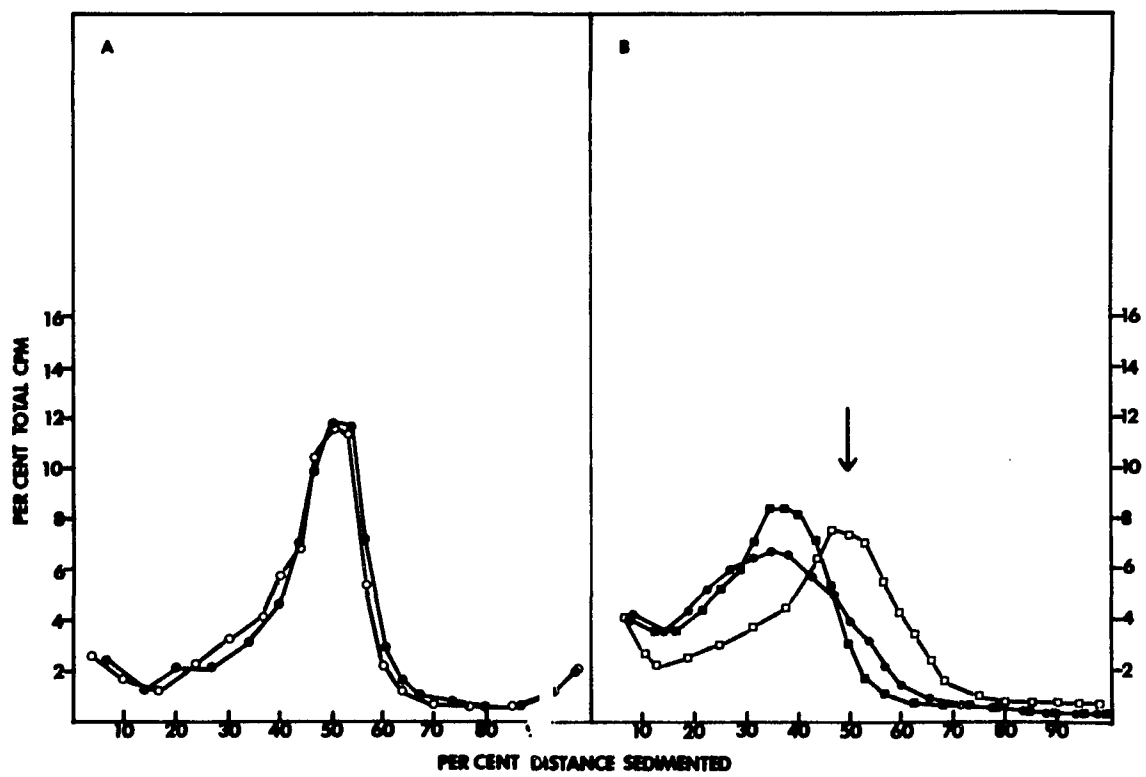


Fig. 15

HCl (pH 8.0). The ability to produce acid soluble nucleotides was determined as in the total nuclease assay. With incubation times up to 60 minutes, no acid soluble counts above background were detected using either substrate, thus showing the purified nuclease to be completely devoid of exonuclease activity.

Nature of Nuclease Damage to DNA

The degradation of native DNA by the isolated nuclease could conceivably be of 3 types: (i) simultaneous scission of 2 closely associated phosphodiester bonds, located on opposite strands of the helix, resulting in a double strand break, (ii) scission of a single phosphodiester bond on either strand of the helix, resulting in a single strand break, or (iii) a combination of the two events.

To determine the nature of the phosphodiester breaks SP82G native DNA was incubated with purified nuclease and 10 mM Mg^{2+} for various lengths of time, and the change in molecular weight determined by sedimentation through neutral and alkaline sucrose gradients. The neutral gradient, by maintaining the structure of the helix, allows determination of double strand breaks, while the alkaline gradients, which denature the helix into single strands, allow calculation of single strand breaks.

Fig. 16 shows the sedimentation profiles (in neutral sucrose gradients) of nuclease treated DNA. An upfield shift, readily apparent after 60 minutes incubation, demonstrates that a significant number of double strand breaks have been induced into the DNA. A further decrease in molecular weight is noted at 120 minutes of incubation, beyond which no further decreases were noted.

Fig. 17 shows sedimentation profiles of the same incubation mix-

Fig. 16. Nature of nuclease damage to native SP82G DNA as determined by neutral sucrose gradients.

Nuclease was isolated as described above. ^3H -native SP82G DNA (10 μg) was incubated with 10 mM Mg^{2+} , 50 mM Tris-HCl (pH 8.0) and 160 units of purified nuclease for various lengths of time at 37°. Reactions were terminated by addition of EDTA to a final concentration of 10 mM. Aliquots (0.2 ml) of incubation mixtures were layered onto 5-20% neutral sucrose gradients and centrifuged at 35,000 rev/min for 105 min at 23° in an SW65 rotor. Fig. 16A: control DNA (no nuclease); 16B: 30 min incubation; 16C: 60 min incubation; 16D: 120 min incubation.

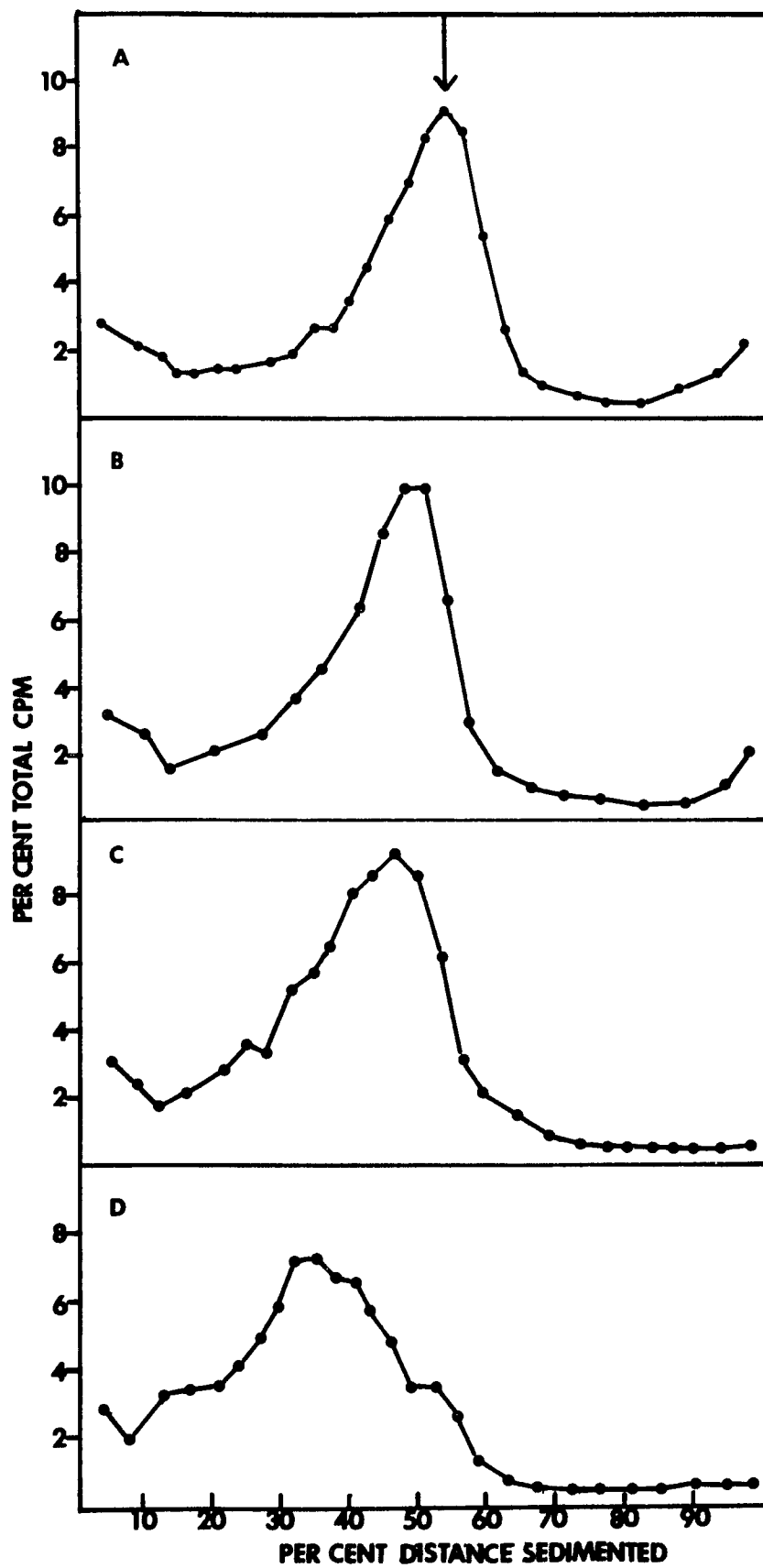


Fig. 16

Fig. 17. Nature of nuclease damage to native SP82G DNA as determined by alkaline sucrose gradients.

Nuclease was isolated as described above. ^3H -native SP82G DNA (10 μg) was incubated with 10 mM Mg^{2+} , 50 mM Tris-HCl (pH 8.0) and 160 units of isolated nuclease for various lengths of time at 37°. Reactions were terminated by addition of EDTA to a final concentration of 10 mM. Aliquots (0.2 ml) of incubation mixtures were layered onto 5-20% alkaline sucrose gradients and centrifuged at 35,000 rev/min for 105 min at 23° in an SW65 rotor. Fig. 17A: control DNA (no nuclease); 17B: 30 min incubation; 17C: 60 min incubation.

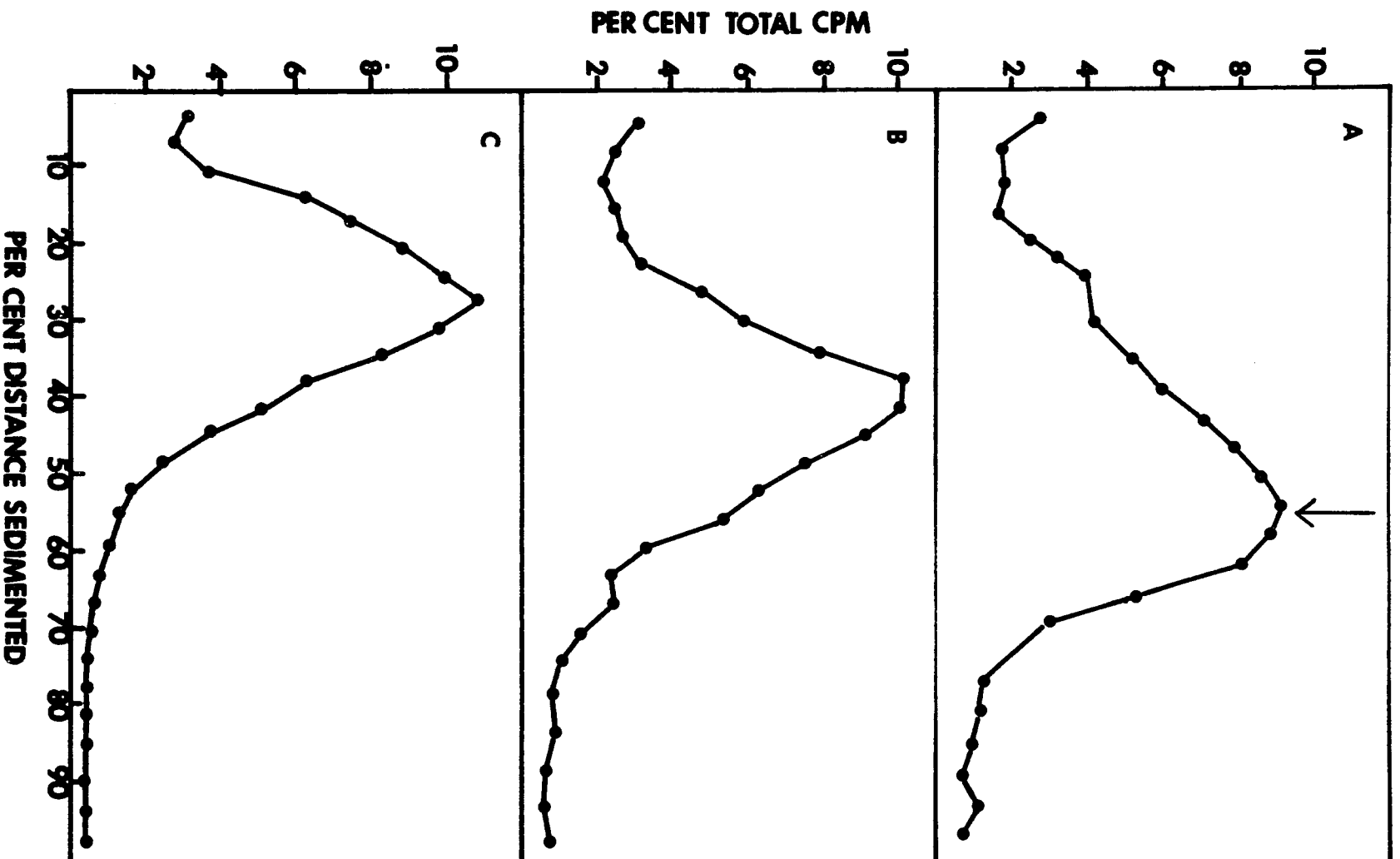


Fig. 17

tures in alkaline sucrose gradients. These results indicate that there is a more rapid production of single strand breaks in the DNA than double strand breaks.

Table 6 shows the actual numbers of double and single strand breaks per DNA molecule as calculated from Figs. 16 and 17. The results clearly show that the DNA has been subject to both single and double strand breaks. It is also clear that these breaks occur in approximately equal numbers: for every event leading to double strand scission, there is a separate event resulting in a single strand scission.

Finally, the ability of the nuclease to degrade heat denatured DNA was investigated. Fig. 18 shows the sedimentation profiles of treated and untreated heat denatured DNA on alkaline sucrose gradients. From the data two observations can be made: (i) when compared to single strand molecules derived from native DNA (Fig. 17B), single strand molecules from heat denatured DNA are at least 50% smaller. This decrease in size is attributed to the fact that the DNA was heated at 100° for 10 minutes to induce denaturation. This treatment is thought to produce regions of depurination which account for the numerous phosphodiester breaks; (ii) even with the small size of the control DNA, the purified nuclease is able to cause a significant decrease in its molecular weight.

Substrate Specificity of Purified Nuclease

Since previous data (Figs. 8, 9, 10) has shown that phage infection decreases the ability of unfractionated cell extracts to degrade several species of DNA, as well as RNA, experiments were done to determine the substrate specificity of the purified nuclease.

Already shown to be able to degrade native and denatured SP82G

Table 6. NATURE OF DAMAGE TO NATIVE SP82G
DNA CAUSED BY PURIFIED NUCLEASE

<u>Time of Incubation</u> <u>(min)</u>	<u>Single Strand Breaks</u> <u>Per DNA Molecule</u>	<u>Double Strand Breaks</u> <u>Per DNA Molecule</u>
30	3-4	1.5-2
60	7-8	4
120	---	6

Fig. 18. Nature of nuclease damage to denatured SP82G DNA.

Nuclease was isolated as described above. Heat denatured ^3H -SP82G DNA (10 μg) was incubated with 10 mM Mg^{2+} , 50 mM Tris-HCl (pH 8.0) and 160 units of purified nuclease for 30 minutes at 37°. Reactions were terminated by addition of EDTA to a final concentration of 10 mM. Aliquots (0.2 ml) were layered on 5-20% alkaline sucrose gradients and centrifuged at 35,000 rev/min for 105 min at 23° in an SW65 rotor. The diagram compares the sedimentation profiles of control DNA (no nuclease) (■) and DNA treated with nuclease (□).

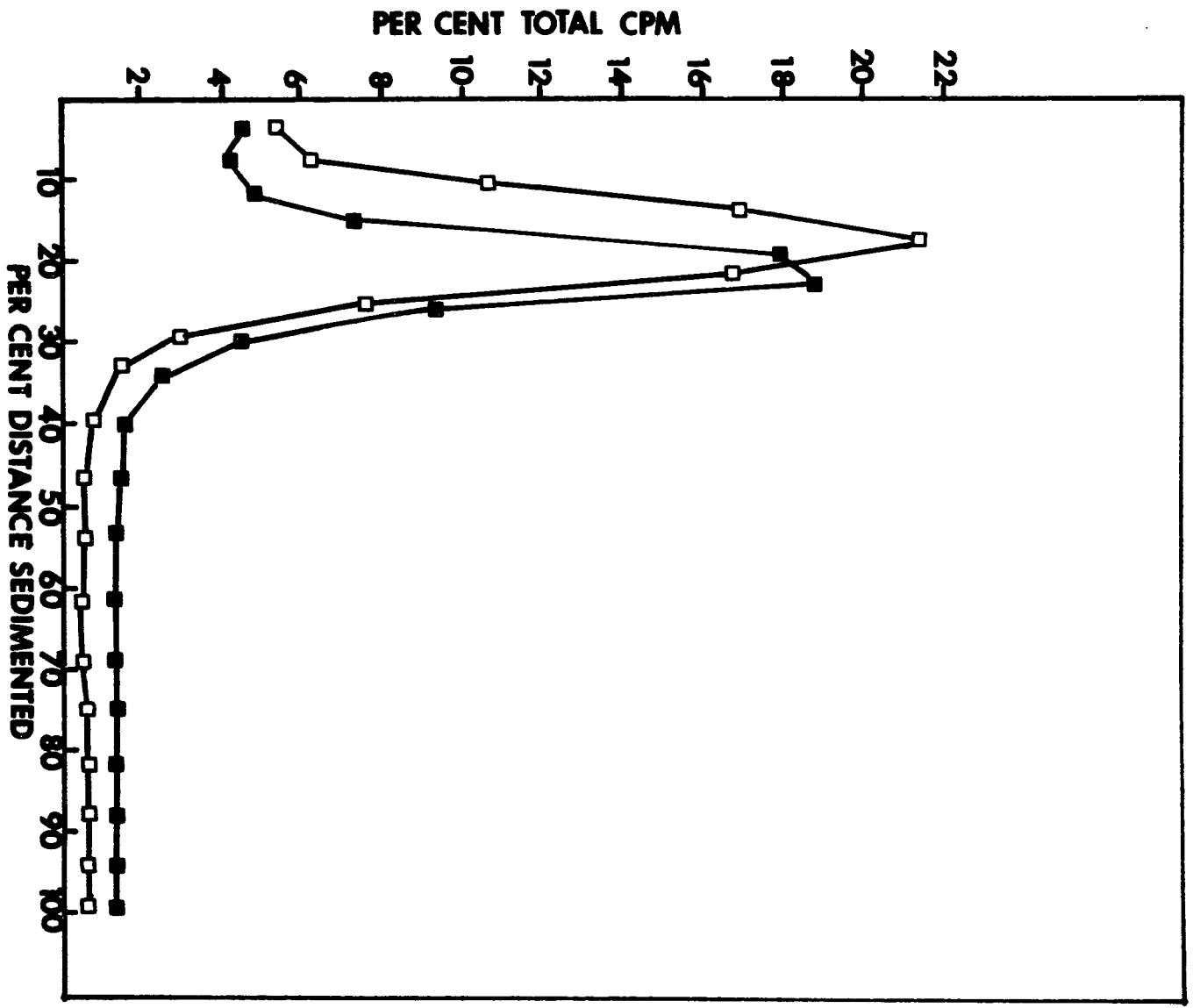


Fig. 18

DNA, the nuclease was tested on DNA from B. subtilis strain G-1, an adenine requiring mutant of strain 168. Using neutral and alkaline sucrose gradients, the results (Fig. 19) show that both native and denatured B. subtilis DNA is subjected to a considerable decrease in molecular weight during incubation with the isolated nuclease.

The question of whether the nuclease could attack closed circular molecules, a classic test of an endonuclease, was examined by incubating the purified nuclease with the RFI form of bacteriophage ϕ X 174, a double stranded closed circle. From the sedimentation profiles in neutral sucrose (Fig. 20A) it is obvious that the nuclease has the ability to put double strand breaks into circular DNA.

The ability of the nuclease to attack RNA is shown in Fig. 20B. In this case, single stranded polio virus RNA was incubated with the nuclease and the product analyzed on alkaline sucrose gradients. The results clearly show a large decrease in the molecular weight of the RNA treated with the isolated nuclease.

In summary, an endonuclease which is inhibited in cells infected with SP82G has been isolated and partially purified. This endonuclease shows the same remarkable lack of substrate preference that was observed by comparing extracts from uninfected and infected cells (Figs. 8, 9, 10). In addition, the nuclease has been shown to attack circular DNA.

Fig. 19. Nature of nuclease damage to B. subtilis DNA

Nuclease was isolated as described above. Native and denatured ^3H -B. subtilis DNA (10 μg) was incubated with 10 mM Mg^{2+} , 50 mM Tris-HCl (pH 8.0) and approximately 160 units of purified nuclease for 30 minutes at 37°. Reactions were terminated by addition of EDTA to a final concentration of 10 mM. Aliquots (0.2 ml) of incubation mixtures were layered onto 5-20% neutral and alkaline sucrose gradients and centrifuged at 35,000 for 120 minutes at 23° in an SW65 rotor. The diagram shows untreated DNA (□) and DNA treated with nuclease (●). Fig. 19A: neutral sucrose gradients of native DNA; 19B: alkaline sucrose gradients of denatured DNA.

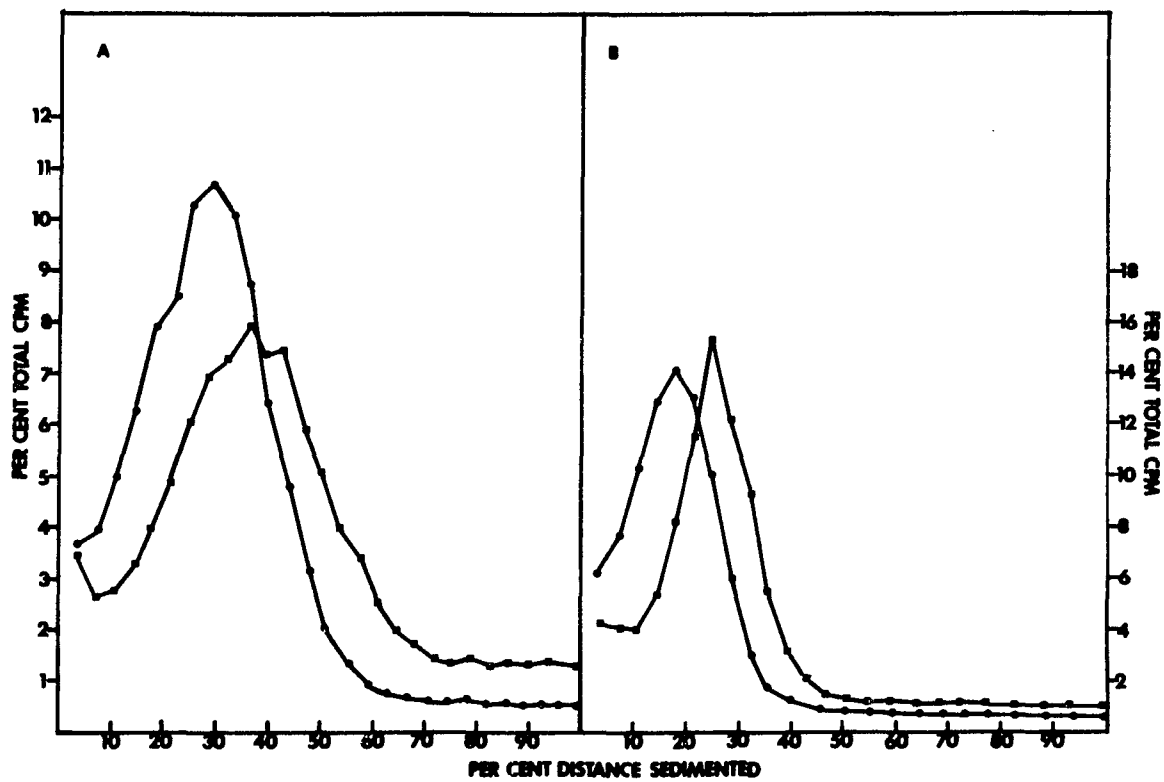


Fig. 19

Fig. 20. Nature of damage to ϕ X 174 RFI DNA and polio virus RNA

Nuclease was isolated as described above. ^3H - ϕ X 174 RFI DNA or ^3H -polio virus RNA was incubated with 10 mM Mg^{2+} , 50 mM Tris-HCl (pH 8.0) and 160 units of purified nuclease for 60 minutes at 37°. Reactions were terminated by addition of EDTA to a final concentration of 10 mM. Aliquots were layered onto 5-20% sucrose gradients. ϕ X 174 RFI DNA was centrifuged at 38,000 rev/min for 6 hours at 8° in an SW65 rotor. Single strand polio virus was centrifuged at 50,000 rev/min for 110 minutes at 23° in an SW65 rotor. The diagram shows sedimentation profiles of control DNA or RNA (●) and DNA or RNA treated with nuclease (▲). Fig. 20A: neutral sucrose gradients of ϕ X 174 RFI DNA; 20B: alkaline sucrose gradients of polio virus RNA.

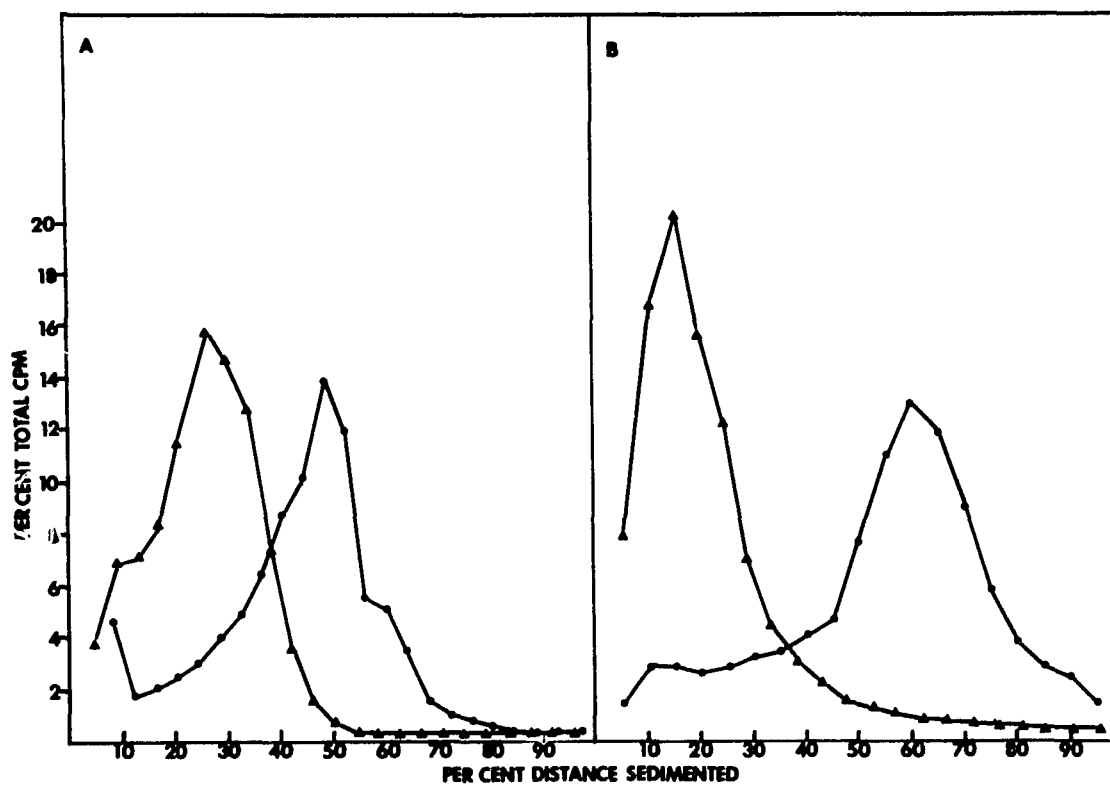


Fig. 20

DISCUSSION

These experiments indicate that the molecular basis for intracellular inactivation in Bacillus subtilis (Green, 1966) is a host endonuclease capable of attacking a wide range of nucleic acids in vitro. This endonuclease is inhibited at very early times following infection with bacteriophage SP82G. Furthermore, this phage mediated inhibition consists of two distinct processes, one requiring host protein synthesis and the other not requiring it.

Early investigations into the process of intracellular inactivation in B. subtilis made it clear that the processes involved were quite distinct from the restriction-modification systems of E. coli and Haemophilus influenzae. Using B. subtilis strain 168, it was shown (Green, 1966) that transfecting DNA was being restricted, i.e., degraded. However, it was also quite clear that no modification processes were taking place, since the transfecting DNA was purified from phage grown on strain 168. Also, the gene dislinkage events occurring during transfection, at least 40 per genome, were randomly distributed (Green, 1966), while the restriction-modification system recognizes only a few very specific substrate sites per genome.

Observations (McAllister and Green, 1972) that phage DNA will undergo intracellular inactivation if infection is done in the presence of chloramphenicol, allowed investigations into the nature of the inactivation process. Intracellular inactivation is shown to be an endonucleolytic attack upon phage DNA resulting in double strand, and possibly single strand, breaks in the infecting genome. Recent results

(Arwert and Venema, 1974), using H1 bacteriophage, which is closely related to SP82G, have indicated the appearance of both single and double strand breaks in the transfecting genome within five minutes after uptake. These experiments, using the same extraction procedure as the one described above, did not take into account single strand breakage induced during extraction, a major problem in the present work. Davidoff-Abelson and Dubnau (1973) have reported that single strand breakage was a serious problem when attempts were made to recover exogenously added DNA from Bacillus subtilis cells. Thus, at this time, only double strand breaks can be unequivocally reported as the damage caused by intracellular inactivation.

Infection with SP82G has been shown to have a marked inhibitory effect on intracellular inactivation (Green, 1966; McAllister and Green, 1972) that is manifest quite early in the infection process. Some general effects of phage infection on Bacillus subtilis metabolism can be summarized as follows (Pène, 1968; Levinthal, 1967):

- 1) no significant degradation of host genome occurs until 30 minutes after infection;
- 2) there is no immediate shutdown in host DNA synthesis, although ³H-thymine incorporation reaches a peak at 6 minutes after infection and then steadily decreases;
- 3) there is a rapid shut off of host protein synthesis shortly after infection which is correlated with depression of host mRNA production.

In addition, McAllister (1970) has shown that during infection with SP82G there is a linear order of marker entry, with the left end of the genetic map always entering the cell first. The SP82G genome can be divided

into three functional areas: the left end is associated with DNA synthesis, the middle with tail synthesis and the right end with head synthesis (Green and Laman, 1972).

Several mechanisms can be entertained to explain the protective effect of pre-infection protection. The phage may induce a protein which binds to sites on the phage genome, thus preventing the binding of the host cell nuclease. Alternatively, a phage-induced protein may interact directly with the host nuclease, or one of its co-factors, thus inhibiting the nuclease. Results shown above favor the latter idea since the total in vitro nuclease activity in extracts of infected cells is significantly less than in extracts of uninfected cells. A similar process occurs during λ infection (Sakaki et al., 1973), in which a λ induced protein, the γ protein, specifically inhibits the host rec BC DNase. Also, Israel et al., (1972) have shown a decrease in total host nuclease activity 5-10 minutes after Salmonella typhimurium is infected with bacteriophage P22.

Time course studies following the changes in nuclease activity after infection resulted in some interesting observations. At 33°, an initial increase in nuclease activity after infection is followed by a rather rapid decrease which maximizes at 9 minutes after infection. At 37° the initial increase is not apparent, but could have been masked by the rapid production of inhibitor at this temperature which results in a minimizing of host nuclease activity by 6 minutes after infection. Similar nuclease increases following bacteriophage infection also occur with P22 infection of S. typhimurium where an increase has been noted up to 2 minutes after infection, and then followed by a rapid decrease (Israel et al., 1972).

Previous work (Green, 1966; McAllister and Green, 1972) done at 33°, demonstrated that the maximal effect of pre-infection protection on marker rescue occurred at 6 minutes after infection. The present work, looking at nuclease levels after infection, shows a maximal inhibition at 9 minutes after infection at 33°. Since it seems clear that pre-infection protection must involve a direct inhibition of host nuclease activities, the differences in times of maximal inhibition at 33° is somewhat puzzling. Although no direct experimental evidence is available to explain this discrepancy, it is useful to review the techniques used to arrive at these slightly varying conclusions. Green (1966) added phage DNA at intervals after infecting cells with marker rescue phage and looked for the maximum number of wild type recombinants. McAllister (1972) preinfected cells with a triply mutant phage and then stopped protein synthesis at various times by addition of CM (100 µg/ml). At a later time (9 minutes after infection), cells were exposed to appropriate transfecting DNA and superinfecting phage, and wild type recombinants were scored.

It is possible that both of these techniques have a lag period between the time that a particular event is initiated and the time at which the effects of that event are manifest within the cell. For example, DNA added to cells at 6 minutes after infection would not be intracellular until some few minutes later, due to adsorption and uptake times. Thus, the DNA would not be "in position" to be affected by intracellular inactivation (and thus monitor the effects of phage infection on intracellular inactivation) until some time after 6 minutes. Therefore the time at which pre-infection protection is maximized is some time after 6 minutes.

A lag period is also quite possible with McAllister's work, since the addition of CM at 6 minutes does not mean an instantaneous stoppage of protein synthesis. The argument is less clear-cut in this case, however, due to the fact that in the present experiments, infection is also stopped by addition of CM and thus a similar lag period could be envisioned. However, there are two differences in experimental procedure which may be significant:

- 1) CM levels are doubled in the present work - 200 $\mu\text{g}/\text{ml}$ as opposed to 100 $\mu\text{g}/\text{ml}$;
- 2) in the present work, in addition to adding CM, cells were rapidly cooled by pouring over ice and then quickly centrifuged at 4°. In McAllister's experiment, cells remained at 33° after addition of CM.

Thus, there seems to be the possibility of a lag period between addition of CM and stoppage of metabolic processes in McAllister's work that is not present in the work described above. Therefore, I feel that the discrepancies in the time of onset of maximal pre-infection protection at 33° can be explained and that 9 minutes after infection may be a more accurate estimation of time of maximal inhibition of intracellular inactivation.

In addition, it should be recognized that while previous work looked at events taking place only in competent cells, the present work involves only regular log phase cells, none of which are competent. There is evidence (Nester and Stocker, 1963) that competent cells have altered rates of macromolecular synthesis and the discrepancies detailed above may be a function of these altered rates.

The fact that intracellular nuclease levels rise again after

sharply decreasing at early times after infection is in accord with the findings of Green (1966) and McAllister and Green (1970) that the levels of wild-type recombinants fall off sharply after a peak at 6 minutes after infection. Although no firm evidence is available, it is assumed that the phage induces a nuclease which itself inactivates transfecting DNA. This situation has been reported with P22 infection of S. typhimurium (Woodworth-Gutai et al., 1972). In these studies, a host nuclease is inhibited at 5 minutes after infection and a new, phage induced, nuclease appears at about 10 minutes after infection. The overall time course of nuclease activity in P22 infected S. typhimurium very strongly resembles the one described above, with a rapid initial increase (0-2 mins.) followed by a rapid decrease (2-10 mins.), and then a gradual increase to uninfected levels by 20 minutes after infection.

The finding that a small amount of inhibition of intracellular inactivation takes place during infection even in the presence of CM was unexpected, but does not contradict McAllister's finding of total CM sensitivity of pre-infection protection, since the amount of inhibition detected would probably be too small to cause a significant change in the number of wild type recombinants in marker rescue experiments.

The fact that the CM-insensitive inhibition is complete within 2 minutes after infection argues against an inhibitor synthesized intracellularly. There would, therefore, seem to be two possible explanations for the mechanism of inhibition. Phage-induced inhibitor could be packaged with the phage DNA during maturation and then enter the cell with the DNA during infection. (McAllister (1970) has shown that chloramphenicol does not affect the injection of SP82G DNA.) An analogous mechanism is known to occur with the T4 v₁ gene product, a UV

specific endonuclease which is packaged within the head of the T4 bacteriophage (Shames et al., 1973). Alternatively, it is possible that a region of the infecting genome is able to selectively bind without reacting with, or releasing, host nuclease molecules, thus inhibiting host nuclease activity.

There is no firm data to differentiate between these two mechanisms but the speed with which the inhibition occurs seems to favor the latter explanation. Extrapolation of McAllister's time of entry work to 37° suggests that only 25% of the genome enters the cells by 2 minutes after infection (Green and Laman, 1973). Since all of the CM insensitive degradation occurs within the first 2 minutes, pre-packaged inhibitor would have to be uniformly associated with the entering end of the DNA and be primarily packaged in the tail of the bacteriophage. On the other hand, a region at the left end of the genome that could selectively bind host nuclease would always enter the cell immediately after infection and thus be totally manifest within the first 2 minutes.

This CM-insensitive inhibition would seem to be quite important to bacteriophage survival in that it would protect the phage genome during the critical period between the time of entry and the time at which the CM-sensitive inhibition is manifest within the cell. Without this early protection, it is quite conceivable that the phage genome would be lethally damaged before the phage induced (CM-sensitive) inhibitor was fully expressed. It should be noted that transfecting DNA would most probably not enjoy this protection since an inhibitor packaged within the bacteriophage would be lost during DNA purification, and an inhibitory region on the left end of the genome would enter the cell last, rather than first, due to the reversed order of entry.

One of the questions posed by McAllister's work (1972) concerned

the fact that during infection in the presence of CM, the infecting genome is subject to considerably less damage than occurs during transfection. It has been shown (Williams and Green, 1972) that transfecting DNA enters the cell at approximately the same speed, albeit with opposite polarity, as infecting DNA, thus suggesting that degradation during entry is not an important aspect.

It should be noted that infection in the presence of CM is an artificial event, and the degradation of the phage genome is probably dependent on the half-life of the host nucleases, since no new proteins can be synthesized. This is obviously quite different from the situation in competent cells where protein synthesis continues unabated. Also, there have been reports (Morrison, 1970; Arwert and Venema, 1974) of new endonucleases appearing with the onset of competence. These new nucleases could account for some of the additional damage to transfecting DNA.

To these two possibilities another can now be added: infection in the presence of CM results in approximately a 10% inhibition in host nuclease activity. This inhibition, which is not enjoyed by transfecting DNA, could account for a large portion of the protection afforded phage DNA during infection in the presence of chloramphenicol.

The failure to achieve a high degree of purity in the isolated nuclease, while very disappointing, was not totally unexpected. As can be seen in Table 1, only one nuclease of Bacillus subtilis, Bs II, has been purified to any great extent. The problem of enzyme stability, a major one in this work, was mentioned by Chestukhin et al. (1973) and Dubnau and Scher (1973) as being major obstacles to further purification.

The nuclease's marked sensitivity to freezing (Table 5), rather unexpected in light of its surprisingly low molecular weight, together

with the fact that crude extracts could be frozen for weeks without any significant loss of activity, suggests the possibility that some stabilizing molecule may be purified away from the nuclease during gel filtration.

The question of whether all of the activities reported for the nuclease are really associated with the nuclease or are contaminants, cannot be answered without further purification. However, the fact that extracts from infected cells have a quantitatively lower ability than extracts from uninfected cells to degrade native DNA, denatured DNA and ribosomal RNA lends credence to the contention that the nuclease should, in fact, be able to degrade all of these substrates.

The ability of the purified nuclease to degrade Bacillus subtilis DNA in vitro raises the question of how the cell protects its own DNA in vivo. There is the possibility that the ability to distinguish between host and viral DNA resides in the repair rather than the restriction system. That is, all intracellular DNA is attacked to an equal extent, but the host repair system preferentially repairs host DNA. There would seem to be at least two major difficulties with this system. First, it is not readily apparent how the repair system could differentiate between random breaks in host DNA and similar breaks in phage DNA. In addition, the extent of double strand breakage occurring in phage DNA during infection in the presence of CM suggests that the nuclease involved is quite active, and would necessitate the constant repair of a significant number of double strand breaks. This amount of repair would seem to constitute an intolerable burden on the metabolism of the cell.

Alternatively, it should be recognized that the in vitro degrada-

tion of host DNA does not prove that this degradation occurs in vivo. In fact, with the exception of the restriction endonucleases, all the nucleases purified from E. coli have the ability to degrade E. coli DNA in vitro. Bacterial DNA may be protected from nucleolytic damage in vivo by some mechanism which is lost during DNA extraction. It seems reasonable to suggest that the cell membrane system may supply just such a protective effect. Host DNA bound in vivo to the cell membrane would be protected from nucleolytic degradation. Purification of the DNA away from this membrane would render it susceptible to nucleases in vitro, even though the DNA was not attacked in vivo.

Thus, it is suggested that intracellular inactivation will attack any DNA not bound to the cellular membrane system. The phage induced inhibition of host nuclease activity may have developed as a way of protecting the phage genome until it becomes bound to the cell membrane, after which, it is afforded the same protection as host DNA.

In summary, the investigations undertaken allow the following conclusions to be drawn:

- 1) during infection in the presence of chloramphenicol, the phage genome is subject to a large number of double strand breaks.
- 2) by 6 minutes after infection at 37°, cell extracts have significantly less nuclease activity than extracts from uninfected cells. This inhibition is maximal at 9 minutes after infection at 33°.
- 3) the phage mediated inhibition of host nuclease activity proceeds by two distinct pathways. A large proportion (90%) of the inhibition is sensitive to chloramphenicol and thus depends upon protein synthesis. The remaining inhibition is insensitive

to chloramphenicol and is thought to involve an inhibitor brought in with the infecting phage genome.

- 4) an endonuclease, isolated on the basis of its inhibition in extracts infected with SP82G, has been purified 27 fold. The nuclease has a molecular weight of approximately 10-12,000. It attacks native and denatured DNA, and single strand RNA. This endonuclease is believed to be the basis for the process of intracellular inactivation in Bacillus subtilis.

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