BIOCHEMICAL STUDIES

ON BACTERIOPHAGE PBS 1

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By

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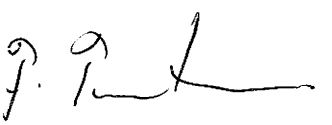
SCOPE AND CONTENTS: This investigation was carried out to obtain information on biochemical changes in Bacillus subtilis infected with phage PBS 1. The survival of PBS 1 DNA in cells in the carrier state may be explained by the fact that an inhibitor for deoxyribonuclease which specifically hydrolyzes PBS 1 DNA is produced after phage infection.

A novel enzyme, dCTP deaminase, is found in PBS 1-infected cells. Deoxyuridine triphosphate which is derived from dCTP by the action of this enzyme appears to be incorporated directly into PBS 1 DNA.

PREFACE

This thesis describes studies carried out in the Research Unit in Biochemistry, Biophysics and Molecular Biology, McMaster University, from September 1964 to June 1968. Except where others are specifically mentioned, it consisted entirely my own original work. No similar thesis has been submitted at any other university.

My grateful thanks are due to Dr. I. Takahashi for his unstinting support, guidance and critical insight during this investigation. I am deeply indebted to the Research Unit in Biochemistry, Biophysics and Molecular Biology of McMaster University and the National Research Council of Canada for the award of Scholarships covering the period of the research herein. My thanks are also due to Miss D. Danoff for her help in proof reading and to Mrs. P. Scott for her skillful typing.



Research Unit in Biochemistry,
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CHAPTER I

INTRODUCTION

Deoxyribonuclease

Bacteriophages may be divided, on the basis of their interaction with host bacteria, into two major classes; virulent phages and temperate phages (1,2,3). When virulent phages infect sensitive bacteria, they multiply in the host and eventually host cells lyse and release progeny phages. On the other hand, temperate phages may establish a symbiotic relation (lysogeny) with host cells (4). In this case, bacteria which survive phage infection normally carry the phage genome (prophage) in their chromosomes. Since the prophage becomes a part of the bacterial chromosome, the replication of the prophage is in harmony with that of host genome.

In addition to these two types of interaction, one can observe another type of interaction which is called carrier state or pseudolysogeny where some phage-infected cells survive and they are able to produce free phages for many generations. However the characteristics of lysogenic cells, such as the stable incorporation of phage genome into the bacterial chromosome and the synchronous replication of phage and host chromosomes are absent in cells in the carrier state. Lwoff (4) defines the carrier state simply as a mixture of bacteria and phage which are in a more or less stable equilibrium. Phage T3 (5)anda virulent mutant of P22 for Salmonella (6) have been reported to show this type of interaction.

Bacteriophages for Bacillus subtilis have been known for decades. In 1961 Romig and Brodetsky (7) initiated a systematic investigation on bacteriophages active on B. subtilis. Among six phage isolates, two of them (SP6 and SP13) formed turbid plaques, suggesting that they might be temperate phages. Their results showed, however, that cells isolated from the turbid plaques were not truly lysogenic, although spores derived from such cells were able to produce free phages even after heat treatment which would inactivate all free phages.

Transducing phages SP 10 and PBS 1 for B. subtilis were isolated in 1961 by Thorne (8) and by Takahashi (9) respectively. It was thought earlier that PBS 1 isolated by Takahashi was a temperate phage and lysogenic cultures could retain prophages through repeated subcultures (9). However, it was found later that the individual lysogenic bacterium was not as stable as previously thought. When apparently lysogenic cells were grown in broth containing phage antiserum, they reverted to the sensitive state at an extremely high frequency (10). The proportion of lysogenic (phage-carrying) cells remained constant for about 60 minutes and thereafter it decreased by 50% at each generation time (10).

Further studies on the PBS 1 system were carried out by Takahashi (11) with phage-carrying spores obtained from B. subtilis cultures infected with PBS 1. Centrifugation in a CsCl gradient of DNA extracted from such spore preparations, in which free phages were eliminated by heat treatment and thorough washing, yielded two distinct DNA bands. From their buoyant densities they were identified as the bacterial DNA (ρ=1.703) and the PBS1 DNA (ρ=1.722). No DNA of intermediate densities was found. From

the relative amount of the PBS 1 DNA banded in the CsCl gradient, it has been estimated that each spore contained PBS 1 DNA equivalent to several copies of the phage genome.

From these and other observations, Takahashi (11) has suggested that the PBS 1 system can be a case of pseudolysogeny (carrier state) in which the phage genome present in B. subtilis cells or spores is not attached to the host chromosome and the replication of PBS 1 DNA is independent.

Studies carried out with another transducing phage SP10 also showed that cells carrying this phage were unstable and they were in the carrier state (12,13,14).

When non-replicating phage DNA is present without stable integration into the host chromosome as in the case of the restricted phage genome in a non-permissible host or in the case of superinfecting phage genome, the phage DNA is usually degraded into acid-soluble fragments by the action of DNase (15-19). In order to prevent breakdown of a superinfecting genome, addition of streptomycin or reduction of Mg^{2+} in the medium is necessary to inhibit the action of DNase (20).

In contrast to the above situations, PBS 1 DNA can remain in the cells for several generations without being degraded, although it is not integrated into the host chromosome. Therefore in the present study DNases in uninfected B. subtilis cells and cells infected with PBS 1, were investigated to obtain information on the mechanism by which PBS 1 DNA can remain intact in B. subtilis cells.

In addition to the ability to create the carrier state, phage PBS 1

can mediate generalized transduction in B. subtilis (10). It has been shown by Takahashi (21) that freqencies of joint transfer, as expressed by the cotransfer index or by percentage of joint transfer, are higher in transduction than in transformation mediated by free DNA molecules. With a pair of closely linked markers, the frequency of joint transduction was only slightly higher than that of joint transformation. On the other hand, a considerably higher degree of linkage was obtained by transduction when loosely linked markers were examined. Thus it appeared that host DNA, during the phage multiplication was not degraded extensively so that transducing particle could incorporate relatively undamaged host DNA.

In the present thesis results of investigations on DNases active on B. subtilis DNA and on the fate of the bacterial DNA after infection with PBS 1 will be presented to substantiate the above genetic observation.

Although DNases in Escherichia coli have been investigated extensively (22,23), comparatively little is known about DNases in B. subtilis. During studies on DNA polymerase of B. subtilis, Okazaki and Kornberg (24) found a DNase which had several fold greater activity on native DNA than on heat-denatured DNA. This DNase has a DNA-phosphatase activity which facilitates the priming action of template DNA in DNA synthesis by eliminating phosphoryl groups at the 3'-termini. This enzyme may be similar to exonuclease III found in E. coli (25,26). Birnboim (27) found a DNase which was associated with the fraction containing cell walls and membranes of mechanically disrupted cells of B. subtilis. This DNase has an exonucleolytic activity on heat-denatured DNA. Strauss et al. (28,29) has reported that extracts of B. subtilis degrade methylated DNA

obtained by methyl methanesulphonate treatment, and these authors have suggested that this DNase activity may be responsible for the repair of alkylated DNA. No attempts have been made to purify this enzyme.

In addition to these intracellular DNases, B. subtilis produces an extracellular DNase (30). This enzyme has been purified and its properties have been investigated by Kerr et al. (31) and by Okazaki et al. (32). The extracellular DNase preferentially degrades heat-denatured DNA in the absence of Ca^{2+} producing 3'-deoxyribonucleotides by attacking from the 5'-terminus in a stepwise manner. This enzyme also can degrade exonucleolytically native DNA from the 3'-terminus in the presence of Ca^{2+}.

Upon infection of B. subtilis with SP3, DNase activity on heat-denatured DNA increases 50-fold (33). The increase in the DNase activity can not be observed when cells are infected in the presence of chloramphenicol. Therefore the enzyme appears to be synthesized after phage infection. Unlike DNase normally present in extracts of B. subtilis, which has an absolute requirement for Ca^{2+}, the phage-induced enzyme requires Mg^{2+}.

Infection with SP10 also resulted in a marked increase of DNase activity in B. subtilis (14). No nuclease activity was detected in SP10-infected cells of strain 168 in which the phage was unable to grow. The SP10-induced DNase could degrade both native host DNA and SP10 DNA in vitro. However DNA extracted from the cells 25 minutes after infection did not show any decrease in the frequency of joint transformation, indicating that bacterial DNA was not degraded extensively at least during the early stages of infection in spite of the induction of DNase which

showed an elevated activity *in vitro*. The SP10-induced DNase therefore may be involved in some processes related to the development of phage SP10 rather than the destruction of host DNA. The isolation and characterization of this DNase have not been attempted as yet.

Changes in DNase activity in *E. coli* induced by phage infection have been reported by many authors. In *E. coli* infected with T-even phages, nuclei are fragmented to yield small blocks of chromatin materials at very early stages of infection (34). Protein synthesis is necessary for the disruption of bacterial nuclei, for cells which are infected with T-even phages in the presence of chloramphenicol retained their morphological integrity (35). The above cytological observation is in agreement with the finding that during the multiplication of T-even phages the major part of host DNA is degraded to small molecules and that these are subsequently reutilized in the synthesis of phage DNA (36-44).

Pardee and Williams (38) have reported that the DNase activity of extracts of *E. coli* infected with T2 is significantly higher than that of extracts prepared from uninfected cells. Later Stone and Burton (45) have reexamined this problem and concluded that the increased DNase activity observed in extracts of *E. coli* infected with T2 was attributable to the induced synthesis of DNase after infection. One of the DNases in the infected cells has been found to be an exonuclease (oligonucleotide diesterase) which degrades partially-degraded DNA by the action of pancreatic DNase, into mononucleotides. This enzyme does not degrade either native or heat-denatured DNA (46,47). In addition to the exonuclease, Bose and Nossel (48) described a new endonuclease induced by T2

infection. The partially purified enzyme attacks preferentially heat-denatured DNA and the principal products of hydrolysis are oligonucleotides with 5'-phosphoryl ends. Thus the phage-induced endonuclease and exonuclease seem to act together on E. coli DNA to produce deoxyriboside 5'-monophosphates which can be converted to triphosphates, the immediate precursors of phage DNA (49). Therefore in T-even phages the phage-induced destruction of host DNA seems to be due to two potent induced enzymes.

Recently Wiberg (50) examined several conditional lethal mutants (amber) of T4 which could not cause degradation of bacterial DNA. These mutants produced progeny phages at less than 10% of the yield of the corresponding wild type phage. The production of new enzymes which degrade host DNA therefore appears to be under the control of phage genes.

Upon infection of E. coli with T5, more than 90% of host DNA was converted to acid-soluble fragments within 5 minutes (51,52). The degradation of host DNA appeared to be dependent on the synthesis of a new protein, since infection of E. coli in the presence of chloramphenicol did not cause the destruction of host DNA. In contrast to cells infected with T-even phages, no increase in DNase activity was detectable in the early stages of infection where a rapid destruction of host DNA was observed. Nevertheless, DNase activities increased at a later stage of phage multiplication. In this case too, the increase in DNase activity was attributable to the synthesis of a new enzyme. The observation that appearance of the new DNase closely paralleled the synthesis of T5 DNA, suggested that the new DNase might play a role in DNA synthesis rather than in degradation. Recently, T5-induced DNase has been purified

and its properties have been examined in detail (53). The mode of attack of the enzyme appears to be both endonucleolytic and exonucleolytic, yielding a mixture of small oligonucleotides and mononucleotides terminated with a 5'-phosphoryl group (53). It was found that DNA isolated from T5 by a very mild technique, contains four single-strand breaks (54). It is suggested that the T5-induced DNase is responsible for the introduction of single-strand breaks in T5 DNA (53).

Changes in DNase activities during the development of phage lambda in lysogenic bacteria have been studied by Weissbach and Korn (55,56). They found that induction of lambda prophage in E. coli by mitomycin C or thymine deprivation caused a 10-fold increase in the activity of a DNase which was distinct from all other DNases present in uninduced E. coli K12 (λ). The induced DNase has been isolated and found to be a new exonuclease. The DNase appeared in the early vegetative growth cycle of the phage, and its increase paralleled the DNA synthesis. Enzymes similar to the lambda-induced exonuclease were also found in induced cells which were lysogenic to lambdoid phages such as 434, 21 or ø80 (57,58). The exonuclease induced by lambda phage was purified extensively and its properties were studied in detail (59,60). The enzyme specifically degrades native DNA by attacking at the 5'-termini in a stepwise manner and releasing 5'-mononucleotides. The sequential degradation of DNA molecules by this DNase generated molecules which bore protruding 3'-terminated single-stranded regions at both ends of a double-stranded segment. Since protruding 5'-terminated single-stranded regions are required for the cohesion of DNA molecule, this mode of action appears to rule out a possible role of the enzyme in the

formation of cohesive ends of mature lambda DNA. Shuster et al. (61) have reported that non-inducible temperate phages (186 and 299) fail to produce detectable amounts of the exonuclease. It has also been known that bacteria carrying the defective lambda prophage T_{11}, which directs the synthesis of an excessive amount of exonuclease upon induction, have a very high rate of curing (64,65). Thus it has been proposed that the exonuclease induced by lambdoid phages might keep the prophage detached from the bacterial chromosome after induction either by destroying the cohesive ends of the phage DNA which might be necessary for prophage attachment (63), or by destroying the prophage attachment site in the host DNA (61).

In the present thesis the isolation and characterization of a DNase active on native PBS 1 DNA will be described. The possible biological role of this enzyme in the pseudolysogeny of B. subtilis will also be discussed.

Biosynthesis of PBS 1 DNA

Biochemical investigations on the multiplication of T-even phages were initiated about 20 years ago by Cohen (66). In his earlier work it was found that DNA synthesis ceased immediately after infection, but commenced again at a rate five times greater than the preinfection rate. This rapidly synthesized DNA was later indentified as viral DNA (36). No net synthesis of RNA was observed, although a rapid turnover of RNA having a base composition similar to that of viral DNA was found later by Volkin and Astrachan (67). Protein synthesis continued from the inception of infection. However, very little of the protein which was

formed before viral DNA synthesis began, appeared in the phage particle. The necessity of protein synthesis in early stages of infection for phage multiplication was shown by using various inhibitors of protein synthesis (68,69,70,71). One of the proteins synthesized during early stages of infection was identified as deoxycytidylate hydroxymethylase which provided hydroxymethylcytosine (HMC), a unique constituent of the phage DNA (72,73). After Cohen's pioneer work, many enzymes which are essential for the synthesis of T-even phage DNA were discovered in the T-even phage system. They are dHMCMP kinase (74,75), dCTPase (74,75,76), dTMP kinase (76), dGMP kinase (76) thymidylate synthetase (77,78), dCMP deaminase (79,80), DNA polymerase (81) and glucosyltransferase (76). These enzymes which are responsible for the rapid synthesis of phage DNA are detectable as early as 5 minutes after infection with a T-even phage.

The DNA of T-even phages contains HMC (82) and can be readily distinguished from the host DNA. For this reason most biochemical investigations on phage multiplication have been carried out with T-even phages and very little is known in other phage systems where the chemical compositions of viral DNA is similar to that of host DNA. Recently, however, unusual bases have been found in several B. subtilis phages. Hydroxymethyluracil (HMU) replaces thymine in the DNA of SP8 (83) and uracil replaces thymine in the DNA of phage PBS 1 (84).

It has been found, in B. subtilis after infection with phages, SP8, ϕe or SP5C which contain HMU in their DNA, that the level of dCMP deaminase which provides dUMP increased markedly (85,86,87). It has been suggested that the incorporation of thymine into DNA is prevented

by the action of dTTPase or dTMPase or the production of a specific inhibitor for thymidylate synthetase in cells infected with those phages (85,86,87). Nishihara et al. (88) have reported that dCMP deaminase isolated from cells infected with SP8 is not inhibited by dTTP or dHMUTP nor stimulated by dCTP, although dCMP deaminase induced by T-even phages is inhibited by dTTP and stimulated by dHMCTP or dCTP (89,90).

Since PBS 1 DNA contains uracil in place of thymine, biochemical changes similar to those observed in cells infected with SP8 may be expected. However, according to Kahan (85) in cells infected with PBS 2, a clear-plaque mutant of PBS 1, dCMP deaminase was not detectable. He has observed a high level of enzymes, dUMP kinase and dTMPase which are virtually absent in uninfected cells (91). Other pathways for dUTP synthesis in PBS 1-infected cells have not been investigated. A ribonucleotide reductase may give rise to dUTP by the following reactions: UDP$\longrightarrow$dUDP$\longrightarrow$dUTP (92). The existence of this pathway in various systems has been shown (93,94,95). The dC deaminase (96) and dU kinase (97) may also yield dUMP. Therefore the latter part of the present study was devoted to the biochemical pathways for the synthesis of dUTP in B. subtilis cells infected with PBS 1.

CHAPTER II

MATERIALS AND METHODS

Symbols

Symbols to designate the genotype of mutant bacteria are according to Demerec *et al.* (98).

The abbreviations used for amino acids, bases, nucleosides, nucleotides, and nucleic acids are those recommended by the NAS-NRC Office of Biochemical Nomenclature (99).

Bacteria

	Strains	Characteristics
Bacillus subtilis	SB19E	wild type, str^r, ery^r
	SB202	aro_2, try_2, his_2, tyr_1
	MIL	*met*, *ile*, *leu*
Escherichia coli	B	wild type

Bacteriophages

	Host	Characteristics
PBS 1	*B. subtilis*	Uracil replaces thymine in DNA. Single-strand breaks in DNA.
PBS 15	*B. subtilis*	Hydroxymethyluracil replaces thymine.

T4	E. coli	Hydroxymethylcytosine replaces cytosine
T5	E. coli	Single-strand breaks in DNA.

Culture media

1 Penassay Broth

2 Tryptose Blood Agar Base (TBB agar)

3 TBB soft agar

This medium contained the same ingredients as TBB agar except that the content of agar was 10 g per litre.

4 Nutrient Broth

5 Spizizen's Minimal Medium (SMM) (100)

$(NH_4)_2SO_4$	2.0 g
K_2HPO_4	14.0 g
KH_2PO_4	6.0 g
Sodium citrate $(Na_3C_6H_5O_7 \cdot 2H_2O)$	1.0 g
$MgSO_4 \cdot 7H_2O$	0.2 g
Glucose (10%)	50 ml
Distilled water	1.0 l

The pH was adjusted to 7.0. To prepare minimal agar, 15 g of Bacto-Agar (Difco) were added. Where biochemical supplements were required in the medium, they were autoclaved separately and added to a final concentration of 50 µg/ml unless otherwise stated.

Glucose was autoclaved separately and added prior to use.

6 A-medium

Nutrient Broth	8.0 g
Yeast Extract	1.0 g
NaCl	4.0 g
$MgSO_4 \cdot 7H_2O$	0.2 g
KH_2PO_4	1.5 g
$Na_2HPO_4 \cdot 7H_2O$	5.7 g
Distilled water	1.0 l

The pH was 7.5.

7 Tris-medium

Yeast Extract	0.5 g
Casamino Acids	0.5 g
$(NH_4)_2SO_4$	2.0 g
Sodium citrate ($Na_3C_6H_5O_7 \cdot 2H_2O$)	1.0 g
$MgSO_4 \cdot 7H_2O$	0.2 g
Tris-Cl Buffer (1 M, pH 7.5)	50 ml
Potassium phosphate buffer (0.05 M, pH 7.5)	2 ml
Glucose (10%)	50 ml
Distilled water	1.0 l

Glucose was autoclaved separately and added prior to use.

8 Sporulation medium (101)

Nutrient Broth	8.0 g

$MgSO_4 \cdot 7H_2O$	250 mg
KCl	1.0 g
$MnCl_2$ (1.9%)	0.1 ml
Distilled water	1.0 l

The pH was adjusted to 7.0. Before use, the following sterile solutions were added per one litre of the medium.

$FeSO_4 \cdot 7H_2O$ (0.27%)	0.1 ml
$Ca(NO_3)_2 \cdot 4H_2O$ (2.3%)	10 ml

To prepare sporulation agar 15.0 g of Bacto-Agar (Difco) were added to one litre of the above medium.

9 Adsorption medium (10)

Yeast Extract	1.0 g
NaCl	4.0 g
K_2SO_4	5.0 g
KH_2PO_4	1.5 g
Na_2HPO_4	3.0 g
$MgSO_4 \cdot 7H_2O$	120 mg
$CaCl_2$(1.0%)	1.0 ml
$FeCl_3$ (0.5%)	2.0 ml
Distilled water	1.0 l

10 M-9 medium (modified) (75)

NH_4Cl	1.0 g
KH_2PO_4	3.0 g
Na_2HPO_4	11.3 g

$FeSO_4 \cdot 7H_2O$	0.5 mg
Glucose (10%)	50 ml*
$MgSO_4 \cdot 7H_2O$	490 mg*
$CaCl_2 \cdot 6H_2O$	109 mg*
Distilled water	1.0 l

* added after sterilization.

All dehydrated culture media, yeast extract and casamino acids were obtained from Difco Laboratories.

Chemical Reagents

Amino acids were purchased from Calbiochem Co. Bases, nucleosides, nucleotides were products of Sigma General Biochemical Co. or Nutritional Biochemical Co. Ltd. All the chemical compounds used were analytical grade. 3, 5-Diaminobenzoic acid and methyl methoxysulphonate were products of Aldrich Chemical Co. β-Mercaptoethanol was a product of Eastman Organic Chemicals. The CsCl (optical grade) was a product of Harshaw Chemical. Albumin (Bovine Fraction V) was obtained from Calbiochem Co. Chloramphenicol was a product of Parke, Davis and Co. Streptomycin and erythromycin were obtained from Merck and Sharp, and Abott Laboratory Ltd. respectively. Calf thymus DNA and yeast RNA were products of Worthington Biochemical Co.

Labelled compounds (H^3-dU, H^3-dC, H^3-dCMP and H^3-UDP) were obtained from Schwarz BioResearch Co. Methyl-H^3-dT was obtained from New England Nuclear Co. Orthophosphate ($H_3P^{32}O_4$) was obtained from Atomic Energy of

Canada Ltd.

Enzymes

Lysozyme (egg white) was obtained from Calbiochem. Co. DNase (pancreatic), RNase (pancreatic), snake venom phosphodiesterase, pronase and trypsin were products of Worthington Biochemical Co.

Other materials

DEAE-cellulose, CM-cellulose and ECTEOLA-cellulose were products of Mann Laboratories. Sephadex G-75, G-200 and DEAE-Sephadex A-25 were products of Pharmacia. Hydroxylapatite-C was obtained from Clarkson Chemical Co. Inc.

Methods

1 Culture methods

Broth cultures were obtained by inoculating a medium with cells grown on TBB agar overnight and incubating with shaking for 4 to 5 hours at 37°C. Bacterial growth was measured by either viable counts made on TBB agar after an appropriate dilution or optically with a Klett Summerson colorimeter.

2 Phage technique

Phage PBS 1 was assayed by the method of Takahashi (10). Phages T4 and T5 were assayed by the method of Adams (102).

Phage lysates of PBS 1 were prepared in the following way:

bacterial cultures grown in A-medium for 4 hours at 37°C were diluted 10 times in fresh A-medium and infected with PBS 1 at a multiplicity of infection (m.o.i.) of about 0.5. The infected cultures were incubated with shaking for 60 minutes and incubation continued overnight without shaking at 37°C. A phage titre of 3 to 7 x 10^9/ml could be obtained consistently. Phage lysates of T4 and T5 were prepared according to Adams (102).

The presence of free phages was detected by streaking samples on TBB agar and overlaying soft TBB agar containing sensitive bacteria.

3 Preparation of cell free extracts

Uninfected cells were prepared by growing SB19E in A-medium. Cells were collected by centrifugation at 7,400 x g for 5 minutes and were washed once with 0.15 M NaCl-0.015 M sodium citrate (1 x SSC). Cells were stored at -5°C.

Infected cells were prepared by infecting 4 hour cultures of SB19E at m.o.i. of 3 to 5. At various time intervals, samples were withdrawn, quickly cooled and centrifuged. The infected cells were washed and frozen as described above.

The frozen cells were resuspended in a suitable buffer solution. The thick suspensions of bacteria were disrupted by a French Press (Carver Laboratory Press) at 15,000 lb/in^2 three times. Unbroken cells and cell debris were removed by centrifugation at 15,000 x g for 30 minutes. The supernatant fluids were used as crude extracts.

4 Preparation of bacterial DNA

The crude DNA obtained by the method of Takahashi (103) was

purified further by the following method. The crude DNA preparation was treated with RNase (pancreatic) at concentration of 50 µg/ml at 37°C for 1 hour and deproteinized by phenol saturated with 1 x SSC. The deproteinized DNA was treated with isopropanol as described by Marmur (106). The treatment with RNase, phenol extraction and isopropanol precipitation was repeated until the amount of RNA was reduced to less than 1.0% as measured by the orcinol reaction (104).

5 Preparation of DNA from phages

Preparation of DNA from phages was described by Takahashi (105). For the DNA of PBS 1, the isopropanol precipitation technique (106) was employed to eliminate teichoic acids (107).

6 Preparation of labelled DNA

Bacteria and phages for the preparation of P^{32} labelled DNA were grown in Tris-medium containing P^{32} (10 µC/ml). In order to obtain DNA of high specific activities, the amount of phosphate was limited to 18.2 µg P per ml in the labelling medium. Extraction and purification procedures were identical to those of unlabelled DNA.

7 Transformation technique

Transformation was carried out by the methods of Spizizen (100) and of Takahashi (108).

8 Analytical procedures

The amount of protein was estimated by the method of Lowry et al. (109), or by measuring the optical density at 280 mµ assuming $E^{1\%}_{1\ cm} = 11.4$.

The amount of DNA was estimated by the method of Dische (110), or by measuring the optical density at 260 mµ assuming $E^{0.1\%}_{1\ cm} = 20.0$.

In some instances, the following fluorometric method was employed (111). 3, 5-Diaminobenzoic acid (0.6 g) was dissolved in 2 ml of 4 N HCL. The darkbrown solution was decolourized by acid-washed active charcoal (Norit-A). The decolourization was repeated 5 times with 0.02 g of charcoal for each treatment. Samples of DNA (0.02 ml) in 0.1 M NaCl was mixed with 0.03 ml of 1.8 N perchloric acid and 0.05 ml of decolourized 3, 5-diaminobenzoic acid. The reaction mixture was incubated at 60°C for 30 minutes, then 1 ml of 0.6 N perchloric acid was added. The fluorescence was measured by Farrand Fluorometer Mode A-2 using primary filter (Corning filters 5970,4308,3060) and secondary filter (Corning filters 5031,3384). Under these conditions, with calf thymus DNA as standard, a linear relation was obtained between the amount of fluorescence produced and the concentration of DNA ranging from 1.0 µg to 50 µg per tube.

The concentration of RNA was measured by the orcinol reaction (104) or optical density at 260 mµ, assuming $E^{0.1\%}_{1\ cm} = 21.0$.

Phosphorous was measured by King's method (112).

9 Assay of enzymes

DNase

i) Radioisotope method

The method was based on the release of acid-soluble radioactivity from a labelled substrate after enzymic reaction. When P^{32}-labelled DNA was used acid soluble P^{32} was measured by Nuclear Chicago gas flow counter Model D 47. Unless otherwise stated, the incubation mixtures consisted of 0.05 ml of substrate (DNA,330 µg/ml), 0.015 ml of

β-mercaptoethanol (0.1M), 0.025 ml of $CaCl_2$ (5×10^{-3}M), 0.1 ml of appropriate buffer, aliquots of enzyme preparation and distilled water to make up 0.27 ml. The buffers for assaying activity on native PBS 1 DNA, heat-denatured DNA (PBS 1 or host), and native host DNA were 1.0 M Tris-Cl (pH 8.50), 1.0 M Tris-Cl (pH 7.0) and 1.0 M glycine-KOH (pH 8.50) respectively. The reaction was terminated by the addition of 0.2 ml of 1.8 N perchloric acid, o.2 ml of calf thymus DNA (0.5 mg/ml) and 0.04 ml of bovine albumin (5 mg/ml). The mixtures were kept at 0°C for 15 minutes. After centrifugation at 14,500 x g for 5 minutes, radioactivity in the supernatant fluid was measured.

ii) Optical method

The method was based on the release of acid-soluble nucleotides from the substrate after enzymic reaction. According to Lehman (113), oligonucleotides of less than 21 nucleotide-length were acid-soluble. The reaction mixture was the same as in the radioiosotope method, except that non-radioactive DNA was added as substrate. Increase in absorption at 260 mμ in the supernatant fluid after centrifugation was measured by a Beckman spectrophotometer Model DU.

iii) Fluorometric method

The amount of acid-soluble deoxyribose after enzymic reaction was measured by the method described in section 8 of this chapter.

RNase

RNase was measured by the same technique employed in the assay of DNase except that Mg^{2+} was added at a concentration of 0.01 M.

dU kinase

The modified procedure of Sköld (97) was used. The reaction mixtures consisted of 1.4 μM H^3-dU (2.5 μC), 1.25 mM ATP, 1.25 mM $MgCl_2$, 50 mM Tris-Cl buffer (pH 7.50) and aliquots of enzyme, in a total of 0.5 ml. The reaction was stopped after 15 minutes incubation at 37°C by heating at 100°C for 3 minutes and the resulting precipitates were removed by centrifugation. The supernatant fluids were analyzed by paper chromatography (Whatman No. 1) with a mixture of ethanol (95%)-saturated sodium tetraborate-5 M ammonium acetate-0.5 M EDTA (220:80:20:0.5 by vol.) as solvent. The spots corresponding to dU and dUMP were cut out and their radioactivities were measured.

UDP reductase

The modified method of Bertani et al. (92) was used. The reaction mixtures contained 1.3 mM ATP, 1.2 mM $CaCl_2$, 25 mM Tris-Cl buffer (pH 7.50), 0.14 μM NADPH (or 0.17 μM NADH or 0.47 μM reduced lipoic acid), 1.1 μM H^3-UDP (3 μC) and aliquots of enzyme, in a total of 0.5 ml. The reaction was terminated after 30 minutes incubation at 37°C by heating at 100°C for 3 minutes and the resulting precipitates were removed by centrifugation. The supernatant fluids were chromatographed as in the case of dU kinase. Spots of dUMP, dUDP, dUTP and UDP were cut out and their radioactivities were measured.

dCMP deaminase

The assay method described by Wang (96) was used with modification. The reaction mixtures contained 125 μ M $MgCl_2$, 805 μM dCMP, 25 mM Tris-Cl buffer (pH 7.50) and aliquots of enzyme, in a total of 1.0 ml.

At various time intervals optical density measurements were made on 0.1 ml-samples after mixing with 1.5 ml of 1 M HCl. The following molecular extinction coefficients were used: for dUMP A_{280}= 4.0 x 10^3 (pH 2.0), 3.33 x 10^3 (pH 6.70), 3.84 x 10^3 (pH 7.50) and for dCMP A_{280}= 13.1 x 10^3 (pH 2.00), 7.78 x 10^3 (pH 6.70) and 7.25 x 10^3 (pH 7.50).

In some instances, the assay of dCMP deaminase was made with H^3-dCMP as substrate. The reaction mixtures contained 1.5 µM of H^3-dCMP (5 µC), 125 µM $MgCl_2$, 25 mM Tris-Cl buffer (pH 7.50) and aliquots of enzyme, in a total of 0.5 ml. The enzymic reaction was terminated after 15 minutes incubation at 37°C by heating at 100°C for 3 minutes and the resulting precipitates were removed by centrifugation. The supernatant fluids were chromatographed with a mixture of conc. HCl-isopropanol-water (41:170:28 by vol.) as solvent. The enzyme activity was estimated by measuring the radioactivity of the spot corresponding to dUMP.

dC deaminase

The reaction mixtures consisted of the same components as those of dCMP deaminase except that 1.5 µM of H^3-dC (5 µC) replaced dCMP. The enzymic reaction was terminated after 15 minutes incubation at 37°C by heating at 100°C for 3 minutes and the resulting precipitates were removed by centrifugation. The supernatant fluids were chromatographed as in the case of dCMP deaminase. The enzymic activity was estimated by measuring the radioactivity of the spot corresponding to dU.

dCTP deaminase

When crude extracts were assayed, the reaction mixtures contained 250 μM $MgCl_2$, 380 μM dCTP, 25 mM Tris-Cl buffer (pH 7.50) and aliquots of enzyme, in a total of 1.2 ml. At various time intervals, optical density measurement were made on 0.1 ml-samples after mixing with 1.5 ml of 1 M HCl. The molecular extinction coefficients for dUMP and dCMP described above were also used for dUTP and dCTP respectively. When partially purified preparations were used for the enzymic assay, decrease in the optical density at 280 mμ was measured by means of a Gilford multi-sample absorbance recorder. The reaction mixtures consisted of 250 μM $MgCl_2$, 280 μM dCTP, 67 mM potassium phosphate buffer (pH 6.70) and aliquots of enzyme, in a total of 1.2 ml.

CHAPTER III

STUDIES ON DNASES IN *B. SUBTILIS*

Preliminary Experiments

As the properties of phage PBS 1 reported by Takahashi (10) were determined in Penassay Broth, it was necessary to establish the growth characteristics of the phage in the A-medium used in the present study.

A single-step growth experiment was carried out by the following procedure. Cells of fully grown SB19E (5.3×10^8 cells/ml) were suspended in the adsorption medium (5.3×10^8 cells/ml) and infected at a m.o.i. of 0.1. After 5 minutes at 37°C, unabsorbed phages were removed by centrifugation at 5,900 x g for 5 minutes. The infected cells were resuspended and diluted at 10^{-3} in the A-medium. The infected cultures were incubated in a water bath with aeration at 37°C and samples were withdrawn at appropriate time intervals for plaque assay. As shown in Fig. 1, in the A-medium the latent period was found to be approximately 38 minutes and the burst size was 8, which was much smaller than that found in Penassay Broth by Takahashi (10).

Changes in the number of viable cells, the cell density as expressed by Klett units and the proportion of phage-carrying cells in a culture of *B. subtilis* infected with PBS 1 are shown in Fig. 2. The cell density decreased only slightly during the first 20 minutes, although at the 20 th. minute 90% of cells were killed. During the first 20

minutes, more than 50 % of the surviving cells were in the carrier state. Thereafter the cell density decreased as a result of lysis of infected bacteria.

The chemical and physical properties of PBS 1 DNA differ considerably from those of the host DNA (11,84). It was therefore desirable to obtain information on the sensitivity of PBS 1 DNA and B. subtilis DNA to well characterized DNases such as pancreatic DNase (an endonuclease) and snake venom phosphodiesterase (an exonuclease). Degradation of DNA was measured by the fluorometric method. As shown in Table 1, the initial velocity of pancreatic DNase on both PBS 1 and host DNA (native or heat-denatured DNA) was very similar. On the other hand, snake venom phosphodiesterase degraded heat-denatured PBS 1 DNA faster than heat-denatured B. subtilis DNA. Since snake venom phosphodiesterase degrades from 3'-hydroxyl ends, PBS 1 DNA seems to increase the number of 3'-hydroxyl ends upon heating.

DNases active on PBS 1 DNA

DNases in crude extracts prepared from uninfected cells were fractionated on a column of Hydroxylapatite-C. After the column chromatography, DNase activities were found in three fractions (Fig. 3). Effluent from the column (Fraction I) had no DNase activity. Fraction II which was eluted with 0.05 M potassium phosphate buffer (pH 6.80) degraded only native B. subtilis DNA. Fraction III which was eluted with 0.16 M potassium phosphate buffer (pH 6.80) showed a DNase activity on native PBS 1 DNA and on heat-denatured DNA (PBS 1 and B. subtilis DNA). Fraction IV

which was eluted by 0.32 M potassium phosphate buffer (pH 6.80) showed a DNase activity on heat-denatured PBS 1 and B. subtilis DNA (Table 2).

Fraction III had an optimal pH of 8.0 with native PBS 1 DNA as substrate. Fraction IV showed an optimal pH of 7.0 for the degradation of heat-denatured PBS 1 DNA. Both DNase activities were dependent on the presence of Ca^{2+}. Other bivalent cations, Mn^{2+}, Mg^{2+}, Fe^{2+} and Cu^{2+} could not replace Ca^{2+}. The optimal concentration of Ca^{2+} was found to be 5×10^{-3} M for both DNase activities. Results were summarized in Fig. 4 and 5, and Tables 3 and 4.

With the above optimal conditions, changes in the level of DNases active on PBS 1 DNA in infected cells were investigated with crude extracts. A DNase active on native PBS 1 DNA which was detectable in uninfected cells disappeared 20 minutes after infection, whereas a DNase active on heat-denatured PBS 1 DNA remained at the same level during the multiplication of PBS 1 (Fig. 6).

An inhibitor for DNase active on native PBS 1 DNA

The disappearance of the DNase activity on native phage DNA in PBS 1-infected cells may be due to the production of an inhibitor. Alternately the disappearance of the DNase activity on native phage DNA may also be explained by the cessation of enzyme synthesis followed by the degradation of the enzyme. As it was found that a boiled crude extract from infected cells strongly inhibited the DNase activity on native phage DNA in uninfected cells (Fig. 7), it appeared that the production of an inhibitor was responsible for the disappearance of the DNase activity.

The time of appearance of the inhibitor coincided with the time of disappearance of the DNase (Fig. 7). The second possibility could not be tested, since the inhibitor bound tightly to the enzyme and the DNase could not be separated from the inhibitor-enzyme complex.

When chloramphenicol was added (100 μg/ml) to a culture at 5 or 15 minutes after infection, the production of the inhibitor was completely blocked. However chloramphenicol had no effect on the production of the inhibitor if added 20 minutes after infection, suggesting that the inhibitor was synthesized at early stages of phage multiplication.

Partial purification of the inhibitor was achieved by chromatography on Sephadex G-75 (Fig. 8). The partially purified inhibitor was destroyed by pronase and trypsin, while it was resistant to RNase, DNase, lysozyme and to heat treatment at 100°C for 15 minutes (Table 5). The inhibitor seems to be a protein and its molecular weight was estimated to be about 1.5×10^4 as judged by its behavior on the column of Sephadex G-75.

The inhibitor was further purified as follows: the partially purified inhibitor from Sephadex G-75 was pooled and concentrated <u>in vacuo</u> at 60°C. Nucleic acids were eliminated from the concentrated fraction by the method of Razzell (114). Precipitates resulting from the treatment were removed by centrifugation, then the supernatant fraction was concentrated <u>in vacuo</u> at 60°C. The concentrated fraction was dialyzed against distilled water several times and finally against 0.05 M Tris-Cl buffer (pH 7.50). The precipitates resulting from the method of Razzell consisted mostly of nucleic acids and did not show any inhibitory activity.

Since the concentrated fraction still contained nucleic acids as

judged by the ratio of $O.D._{260}$ to $O.D._{280}$, the inhibitor was treated with pancreatic RNase (50 µg/ml) and pancreatic DNase (50 µg/ml) at 37°C for 2 hours. After the treatment with the nucleases, the inhibitor was separated from DNase, RNase and degradation products by gel filtration on Sephadex G-75(Fig. 9). The ultraviolet absorption spectrum of the most purified fraction still showed an absorption maximum at 260 mµ, suggesting the presence of nucleic acids (Fig. 10). Further purification has not been attempted.

The purified inhibitor showed a non-competitive type of inhibition, suggesting that the DNase active on native PBS 1 DNA might be an allosteric enzyme (Fig.11). The inhibitor affected the DNase active on native PBS 1 DNA, but not other DNase activities (Table 6).

The binding of the inhibitor to the DNase active on native PBS 1 DNA was examined with a purified preparation. As shown in Fig. 12, the inhibitor emerged from the column of Sephadex G-75 at fractions 9 through 11, and the DNase emerged at fractions 6 through 8, when they were applied on the column separately. When a mixture of the inhibitor and the DNase was applied on Sephadex G-75, the peak of protein corresponding to the inhibitor disappeared and only the peak corresponding to the DNase was observed. This observation showed that the binding of the inhibitor and the DNase was very tight. The tight association of the inhibitor and the DNase seems to explain the failure to recover the DNase active on native phage DNA from infected cells.

DNase of B. subtilis in the carrier state

As reported by Takahashi (10,11), PBS 1 can establish pseudolysogeny. The pseudolysogenic cultures are unstable and phage-carrying cells segregate sensitive cells at a fairly high frequency. Thus a population of cells infected with PBS 1 consists of cells in the carrier state and cells in the lytic cycle, and their proportion changes with incubation time. For this reason spores derived from infected cultures were chosen to investigate DNase in B. subtilis in the carrier state.

The spores carrying PBS 1 were prepared by the following technique: fully grown SB19E in Penassay Broth was infected at m.o.i. of 20 and was incubated at 37°C for one hour. The infected culture (0.5 ml) was spread on the sporulation agar and incubated at 37°C for 30 hours (11). The spores were collected in 0.01 M potassium phosphate buffer (pH 7.50) containing 0.85 % NaCl and treated with lysozyme (1 mg/ml) and pancreatic DNase (100 µg/ml) for 30 minutes at 37°C to lyse vegetative cells. After the enzymic treatment, the spores were washed three times with the same buffer. Upon heat treatment at 85°C for 10 minutes and plating on TBB agar, it was found that 72 % of germinated spores carried free PBS 1 particles.

The purified spores were resuspended in 8 M urea-0.1 M β-mercapto-ethanol at pH 3.0 (115,116), and were incubated at 37°C for 30 minutes. The spores were washed five times with 0.85 % NaCl and were finally resuspended in 0.01 M potassium phosphate buffer (pH 7.50) containing 0.85 % NaCl. The lysozyme was added to the spore suspension at 2 mg/ml and incubated at 37°C for 30 minutes. After treatment most spores lost their refractility. The lysozyme-treated spores were washed once with 0.05 M

Tris-Cl (pH 7.50) containing 0.01 M β-mercaptoethanol and resuspended in the same buffer. The treated spores were crushed with a French Press (Carver Laboratory Press) four times at 15,000 lb/in^2. The supernatant fluids obtained after centrifugation at 12,000 x g for 30 minutes were dialyzed against 0.05 M Tris-Cl buffer (pH 7.5). The dialysates were used as enzyme or inhibitor preparations.

Crude extracts from spores carrying PBS 1 had no DNase activity on native PBS 1 DNA and had a considerable amount of the inhibitor (Table 7). On the other hand, crude extracts of spores prepared from uninfected cultures showed the DNase activity on native PBS 1 DNA and the inhibitor was absent.

Purification of the DNase active on native PBS 1 DNA

Foregoing results indicated that B. subtilis cells contained a DNase which hydrolyzed native phage DNA but not native host DNA. Attempts were made to purify and characterize this DNase.

The frozen uninfected cells were resuspended in 0.001 M potassium phosphate buffer (pH 6.80) and cell free extracts were prepared as described in Methods. The cell free extract was dialyzed against 0.001 M potassium phosphate buffer (pH 6.80) overnight and was applied on a column of Hydroxylapatite-C which was previously equilibrated with 0.001 M potassium phosphate buffer. The charged column was first eluted with 0.001 M potassium phosphate buffer, then 0.05 M and finally 0.16 M buffer. The DNase active on native PBS 1 DNA was found only in the fraction eluted with 0.16 M potassium phosphate buffer. The fraction eluted with 0.16 M buffer was precipitated with 40 to 60 % saturation of ammonium sulphate. Ammonium

sulphate was removed by dialysis in 0.001 M potassium phosphate buffer (pH 6.80) overnight.

The dialysate was applied on a column of DEAE-cellulose (100 ml in a column of 20 mm in diameter) previously equilibrated with 0.001 M potassium phosphate buffer. The column was washed first with 120 ml of 0.001 M potassium phosphate buffer, then eluted with a linear gradient of NaCl (0 M to 0.3 M NaCl in 0.001 M potassium phosphate buffer). The total volume of the gradient was 400 ml. Five ml fractions were collected. The DNase activity on native PBS 1 DNA appeared as a broad peak, which included two separate protein peaks (Fig. 13). The active fractions were pooled and concentrated with ammonium sulphate at 60 % saturation. Ammonium sulphate was removed by dialysis in 0.001 M potassium phosphate buffer (pH 6.80) overnight.

The specific activity and recovery of the DNase during the purification steps are shown in Table 8. The final product had a specific activity 50 times higher than that of crude extracts and contained almost no nucleic acid as judged from its ultraviolet absorption spectrum.

Further purification of the DNase with CM-cellulose, ECTEOLA-cellulose Sephadex G-200 or DEAE-Sephadex A-25 was unsuccessful.

Properties of the purified DNase active on native PBS 1 DNA

All preparations except the final product from DEAE-cellulose chromatography could be stored at -5°C without any loss of activity. The purified DNase after chromatography on DEAE-cellulose was stable at 4°C, but was inactivated in the frozen state. The inactivation took place even in an enzyme preparation kept in liquid nitrogen.

The rate of hydrolysis of native PBS 1 DNA in Tris-Cl buffer is shown in Fig. 14. The highest DNase activity was obtained at pH between 8.00 and 8.50. The rate of hydrolysis was affected by the concentration of buffer used. The optimal concentration of Tris-Cl buffer for the enzyme activity was 0.5 M (Fig. 15). As in the case of partially purified preparations, the DNase required the presence of Ca^{2+} for its activity (Table 9). Among other cations tested, Cu^{2+} was slightly effective, but Mg^{2+} and Mn^{2+} could not replace Ca^{2+}. Monovalent cations, K^{+} and Na^{+} did not stimulate the enzyme activity.

The enzymic activity was strongly inhibited in the presence of potassium phosphate (Fig. 16). The inhibition of the DNase by phosphate probably is due to the removal of Ca^{2+} from the mixture by forming insoluble complexes.

The addition of yeast RNA to the reaction mixture did not inhibit the DNase activity at concentrations up to 30 µg/ml.

As it was shown earlier , the DNase active on native PBS 1 DNA did not degrade native host DNA. The specificity of this DNase was studied further with the purified preparation. This DNase degraded native PBS 1 DNA rapidly and reached complete degradation after 90 minutes of incubation at 37°C. PBS 15 DNA in which hydroxymethyluracil replaces thymine was degraded to a small extent. Calf thymus, B. subtilis and T4 DNA were not degraded by this enzyme. Results are summarized in Table 10 and Fig. 17.

The DNase active on native PBS 1 DNA was shown to have a strict specificity toward substrate. In order to investigate whether DNA from other sources could compete with PBS 1 DNA in the enzymic reaction, calf

thymus DNA was added to the reaction mixture. As shown in Table 11, thymus DNA had no effect on the degradation of PBS 1 DNA even at a concentration three times higher than that of the substrate. Therefore it seems that thymus DNA does not interact with active site(s) of this enzyme.

No RNase or nucleotidase activity was detectable in the purified preparation. The purified preparation, however, contained a DNase activity on heat-denatured DNA. As shown in Fig. 18, heat-denatured DNA's from T4, PBS 15 and B. subtilis were degraded by this preparation. The initial rate of degradation of PBS 1 DNA was greatest among DNA's tested. It was not clear whether the DNase activity on heat-denatured DNA was due to the presence of another enzyme. The sensitivity of the two enzyme activities (on native and heat-denatured PBS 1 DNA) to heat was found to be different. As shown in Fig. 19, the DNase active on native PBS 1 DNA was completely inactivated after 30 minutes of incubation at 55°C. On the other hand, 12% of the original activity on heat denatured PBS 1 DNA was found after the same heat treatment. In addition to the difference in heat sensitivity, the DNase active on native PBS 1 DNA was inhibited specifically by the inhibitor, whereas the DNase activity on heat-denatured DNA was not affected by the same inhibitor (Table 6). These observations suggest that the most purified preparation might still contain two enzymes; one active on native PBS 1 DNA and the other active on heat-denatured DNA.

The mode of action of the DNase active on native PBS 1 DNA

It has been shown that the PBS 1 DNA molecule has four single-strand breaks (117) in its linear structure, while T4 and B. subtilis DNA

which are resistant to the enzyme have no single-strand breaks. In order to examine whether the specificity of this enzyme toward substrate was due to the presence of single-strand breaks in PBS 1 DNA, single-strand breaks were artificially introduced into B. subtilis DNA.

Single-strand breaks were introduced by the treatment with a low concentration of pancreatic DNase (118). The incubation mixture (1.52 ml) contained DNA from SB19E (1.5 ml of A_{260}=8.6 in 0.01 M Tris-Cl, pH 8.0-0.02 M NaCl), 0.01 ml of 1 M $MgCl_2$ and 0.01 ml of pancreatic DNase (0.13 or 0.66 units). The mixture was incubated for 30 minutes at room temperature. The reaction was terminated by adding 0.01 ml of 0.1 M EDTA and the mixture was dialyzed against 0.1 M NaCl.

Zone sedimentation analysis of DNA treated with pancreatic DNase was carried out in a alkaline sucrose gradient (5-20 %) in 0.02 M K_3PO_4 at pH 12.1 (pH was adjusted with 4 M KOH). Samples of DNA were layered on the top of the gradient and were centrifuged for 24 hours at 24,000 r.p.m. in a Spinco SW 25.1 rotor at 15°C. The sedimentation profile of DNA treated with various amounts of pancreatic DNase is shown in Fig. 20. The DNA treated with pancreatic DNase sedimented in alkaline sucrose gradients at a much slower rate than the control. Since the control DNA and the DNA treated with 0.13 units/ml of pancreatic DNase sedimented at the same rate in neutral sucrose gradient, the slower rate of sedimentation of treated DNA in alkaline sucrose gradients would indicate the presence of single-strand breaks.

Single-strand breaks were also introduced by ascorbate as described by Bode (119). The reaction mixture (1.0 ml) contained 0.5 ml of 0.01 M Tris-Cl buffer (pH 7.50)-10^{-3} M EDTA, 0.5 ml of DNA (A_{260}=6.2) and various

amounts of ascorbic acid. The mixture was incubated for 7 hours at 37°C and the reaction was terminated by dialyzing it against 0.1 M NaCl. The zone sedimentation in sucrose gradients was made in the same way as in the case of DNA treated with pancreatic DNase. As shown in Fig. 21, DNA treated with ascorbate sedimented at a greatly reduced rate in alkaline sucrose gradients, indicating again the presence of single-strand breaks in the treated DNA.

B. subtilis DNA treated with 0.13 units/ml of pancreatic DNase or with 10^{-3} M of ascorbate, as well as T5 DNA which contained 4 single-strand breaks were resistant to the action of the DNase (Table 12). B. subtilis DNA treated with higher concentrations of pancreatic DNase or ascorbate was degraded by the DNase. Presumably higher concentrations of these agents produce not only single-strand breaks but also single-stranded regions which are sensitive to the DNase active on denatured DNA.

Another unique property of PBS 1 DNA which is absent in DNA of other sources is the substitution of thymine by uracil. In order to examine whether the strict specificity of the DNase toward native PBS 1 DNA was due to the presence of uracil in PBS 1 DNA, cytosine was converted to uracil in thymus DNA by nitrous acid according to the method of Shapiro and Chargaff (120). Since low pH causes depurination and back bone breakage of DNA (121-123), DNA treated with low pH without nitrous acid was examined as a control. The treatment of DNA with nitrous acid was as followed ; 135 mg of $NaNO_2$ were added to a solution of 5 mg of calf thymus DNA in 2.5 ml of distilled water. The pH of the mixture was adjusted to an appropriate value by glacial acetic acid. The mixture was incubated at 37°C for 30

or 72 hours in a securely closed test tube. The treated DNA was dialyzed against 1 x SSC and finally against 0.1 M NaCl.

Extensive hydrolysis was obtained with DNA's treated at pH 3.35 with or without addition of nitrous acid (Table 13). Under this condition the DNA was fragmented and could not be recovered as fibre by the addition of ethanol. On the other hand, under a milder acidic condition (pH 4.0 for 30 hours), control DNA was not degraded, while the deaminated DNA was degraded extensively.

As nitrous acid deaminates also guanine and adenine (124), it is not clear whether the sensitivity of deaminated calf thymus DNA to the DNase is due to only the presence of uracil in the molecule. Therefore it was necessary to examine the digestion products to obtain further information on the mode of action of the DNase.

PBS 1 DNA labelled with P^{32} was hydrolyzed with the purified enzyme for 7 hours at 37°C. The products of hydrolysis were examined by paper chromatography with a mixture of ethanol (95 %)-saturated sodium tetraborate-5 M ammonium acetate-0.5 M EDTA (220:80:20:0.5 by vol.) as solvent. Results are shown in Fig. 22. The products were mostly oligonucleotides and smaller amounts of mononucleotides. The mononucleotides were dGMP and dAMP.

The digestion products were also examined by chromatography on DEAE-Sephadex A-25 column. A large amount of digestion products was obtained by hydrolyzing unlabelled PBS 1 DNA. Reaction mixtures were the same as described in Methods except that 0.45 M Tris-Cl buffer was replaced by 0.12 M glycylglycine-KOH buffer (pH 8.30) in order to lower

the amount of total solute in the mixture. The extent of hydrolysis in glycylglycine-KOH buffer was the same as in 0.45 M Tris-Cl buffer. After an incubation at 37°C for 10 hours, urea was added to the reaction mixture to a concentration of 7 M. After the addition of urea, the total volume became three times the original volume and the concentration of all the solute other than urea was approximately 0.04 M. The hydrolysate was applied on DEAE-Sephadex A-25 (1 x 15 cm) previously equilibrated with 0.02 M Tris-Cl (pH 7.60) containing 7 M urea.(125). After washing the column with 0.02 M Tris-Cl (pH 7.60)-7 M urea, elution was accomplished by a linear gradient of 0.14 M to 0.28 M NaCl in 0.02 M Tris-Cl-7 M urea, and finally with 1 M NaCl in the same buffer. As shown in Fig. 23 (A), digestion products consisted of nucleoside, mononucleotides and oligonucleotides. Each fraction was concentrated by absorbing on a column of DEAE-cellulose previously equilibrated with 0.01 M NH_4HCO_3 and by eluting with 1 M NH_4HCO_3. The ammonium bicarbonate was removed by evaporating in vacuo. The concentrated fractions were treated with snake venom phosphodiesterase (100 μg/ml) at 37°C for 12 hours in order to examine mononucleotides present in the fractions. The hydrolysates with phosphodiesterase were examined by paper chromatography using a mixture of isopropanol-conc. HCl-H_2O (170:41:39 by vol.) as solvent. The results of the analysis are summarized in Table 14. The nucleoside fraction contained only dU. In the mononucleotide fraction and oligonucleotide fractions up to tetranucleotide, only dGMP and dAMP were present. In the fraction of oligonucleotides larger than hexanucleotides, dAMP, dGMP and dCMP were present but dUMP was absent.

As described previously the purified DNase still contained a DNase active on heat-denatured DNA. In order to investigate the mode of action of the DNase active on heat-denatured DNA, digestion products of heat-denatured PBS 1 and host DNA were examined by chromatography on DEAE-Sephadex A-25 as in the case of native PBS 1 DNA. The digestion products of heat-denatured PBS 1 DNA were very similar to those of native PBS 1 DNA (Fig. 23 (B)). In the hydrolysate of B. subtilis DNA, mononucleotides and oligonucleotides smaller than hexanucleotides were found to contain only dAMP and dGMP. Nucleosides were absent. Oligonucleotides larger than hexanucleotides contained dAMP, dGMP, dCMP, and dTMP. Results are summarized in Fig. 23 (C) and Table 14.

DNases active on B. subtilis DNA

Crude extracts from uninfected cells were fractionated by chromatography on a column of Hydroxylapatite-C (Fig. 3 and Table 2). Fraction II had a DNase activity on native B. subtilis DNA and Fractions III and IV had a DNase activity on heat-denatured B. subtilis and PBS 1 DNA. The optimal pH for the DNase active on native B. subtilis DNA (Fraction II) was 8.5. The optimal pH for the DNase active on heat-denatured host DNA (Fraction IV) was 7.0 (Fig. 24 and 25). Both DNase activities were dependent on the presence of Ca^{2+}. Other cations, Mn^{2+}, Mg^{2+}, Fe^{2+} and Cu^{2+} could not replace Ca^{2+}. The optimal concentration of Ca^{2+} was found to be 5×10^{-3}M for both cases (Tables 15 and 16). The optimal pH and cation requirement of Fraction III were described earlier.

Using these optimal conditions, changes in the level of DNase

activity on B. subtilis DNA in cells infected with PBS 1 were investigated. The DNase activity on heat-denatured host DNA remained constant. The DNase activity on native host DNA was barely detectable throughout PBS 1 multiplication (Fig. 26). It has been shown that RNA and a heat sensitive protein are inhibitors for some DNases (113,126). The low level of DNase activity on native host DNA could be due to the presence of these inhibitors. However, the treatment with RNase or mild heat treatment of crude extracts did not change the DNase activity (Table 17).

DNase activity on B. subtilis DNA in cells infected with PBS 1 was also examined by measuring the release of radioactive nucleotides in the acid-soluble fraction from H^3-labelled cells. Strain SB19E was grown in Tris-medium containing H^3-dT and shortly before the end of log phase a large amount of non-radioactive dT was added. The labelled culture was incubated further for 100 minutes in order to reduce the radioactive nucleotide pool. Cells were infected with PBS 1 and samples were taken at various time intervals. After the addition of an equal volume of 10 % trichloroacetic acid to the culture, the radioactivity in the acid-soluble fraction was determined (127). As shown in Fig. 27, the radioactivity in the acid-soluble fraction remained constant throughout PBS 1 multiplication.

The assay methods employed in the above experiments were based on the amount of acid-soluble nucleotides released from the substrate. Thus a small amount of DNase activity which causes fragmentation of DNA without producing detectable amounts of nucleotides cannot be measured. It is known that transforming DNA damaged by a relatively low level of DNase or by shearing forces shows a considerably reduced specific transforming

activity. The DNase activity was therefore determined by measuring transforming activity of B. subtilis DNA treated with crude extracts. Both the frequencies of joint transformation (aro$_2$, try$_2$, his$_2$, tyr$_1$) and single transformation decreased when DNA was treated with cell extracts (Table 18). The number of single transformants (str^r) obtained with DNA preparation treated either with infected cell extracts or with uninfected cell extracts was the same. The number of joint transformants, on the other hand, was lower with DNA treated with infected cell extracts than that obtained with DNA treated with uninfected cell extracts. This was reflected in the ratio of the number of single transformants to that of joint transformants.

The above result indicates that there is a slight increase in the DNase activity on the host DNA, when tests are made in vitro. Nevertheless, the specific transforming activity and the frequency of joint transformation of DNA extracted from infected B. subtilis were the same as those of the control (Table 19). It appears that the host DNA in PBS 1-infected cells is protected from the action of DNase.

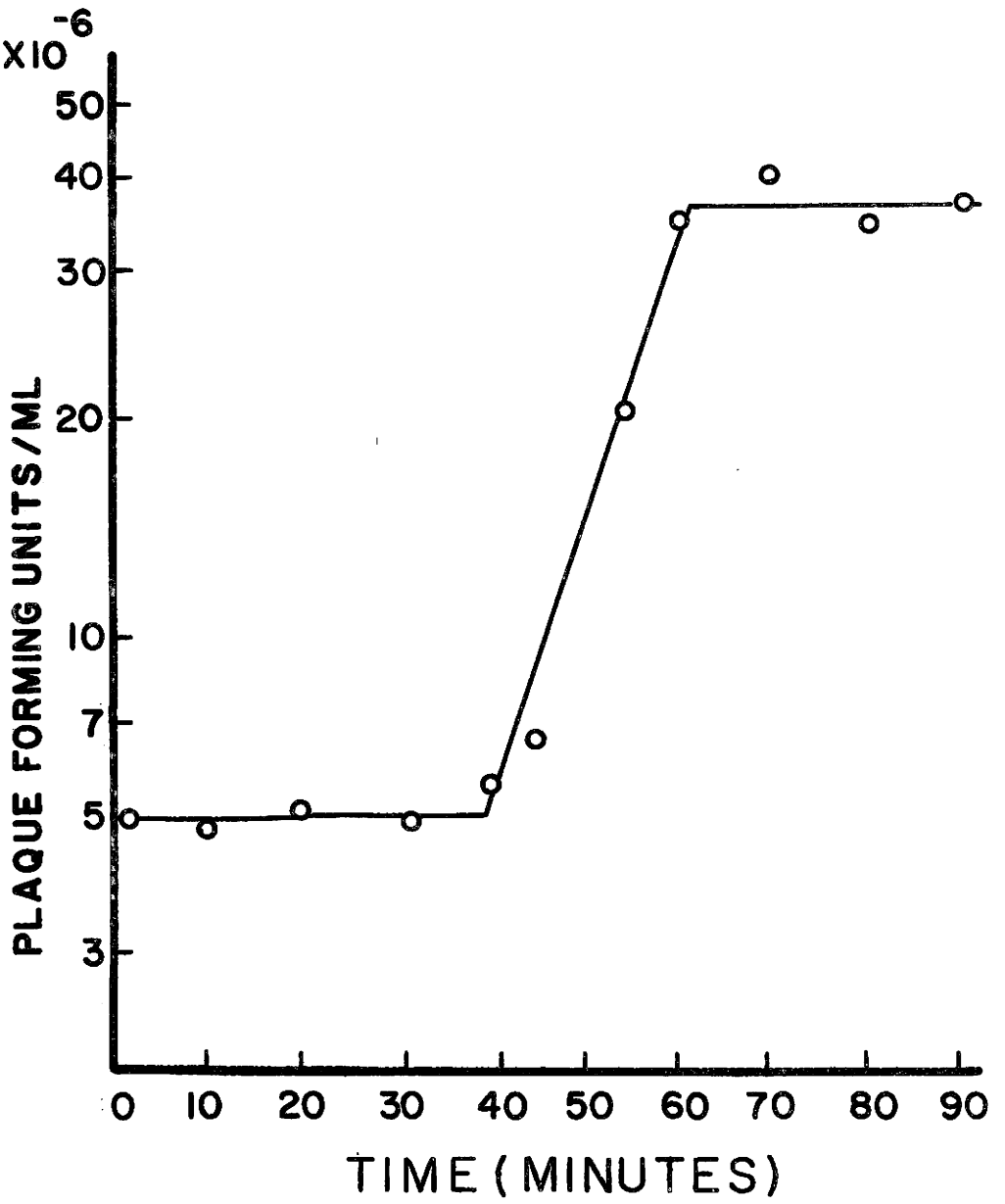

Fig. 1. Single-step growth curve of PBS 1 in the A-medium.

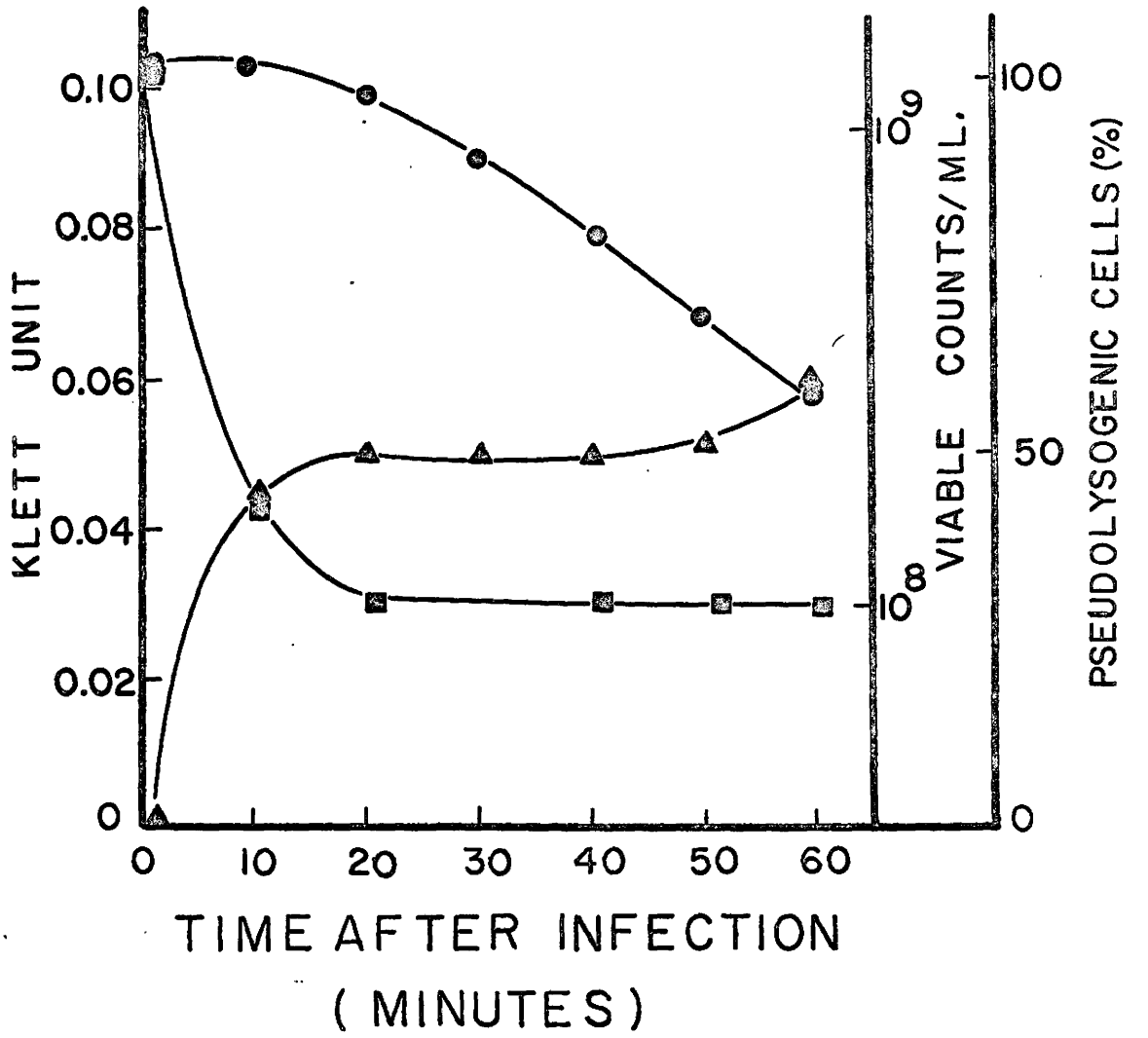

Fig. 2. Changes in the viable counts, cell density and percentage of phage-carrying cells after PBS 1 infection.

Klett units (●—●); viable counts (■—■); pseudolysogenic cells (%) (▲—▲).

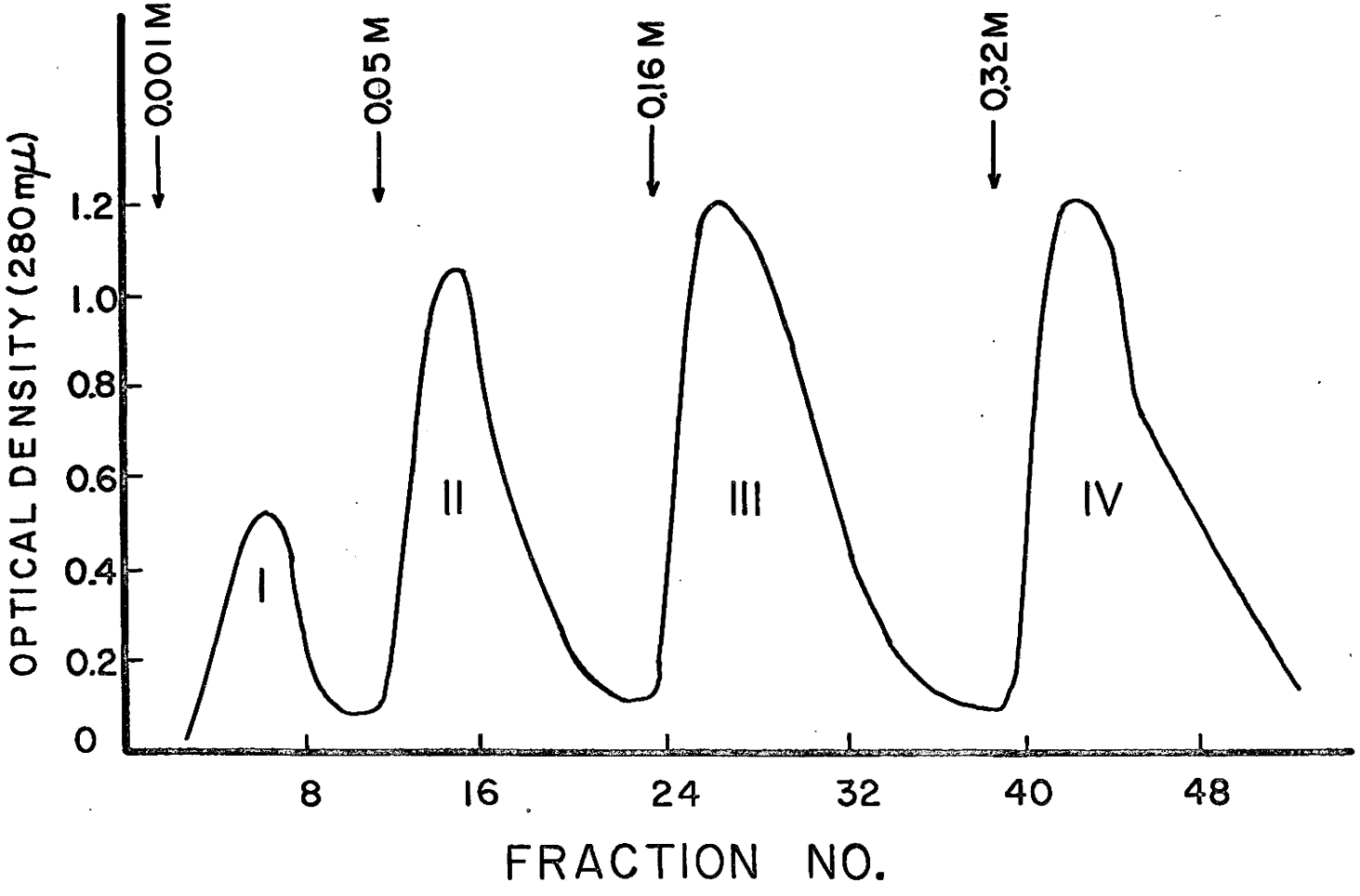

Fig.3. Chromatography of crude extract from uninfected cells on Hydroxylapatite-C. The amount of protein applied on the column (2 x 34 cm) was 4.2 g.

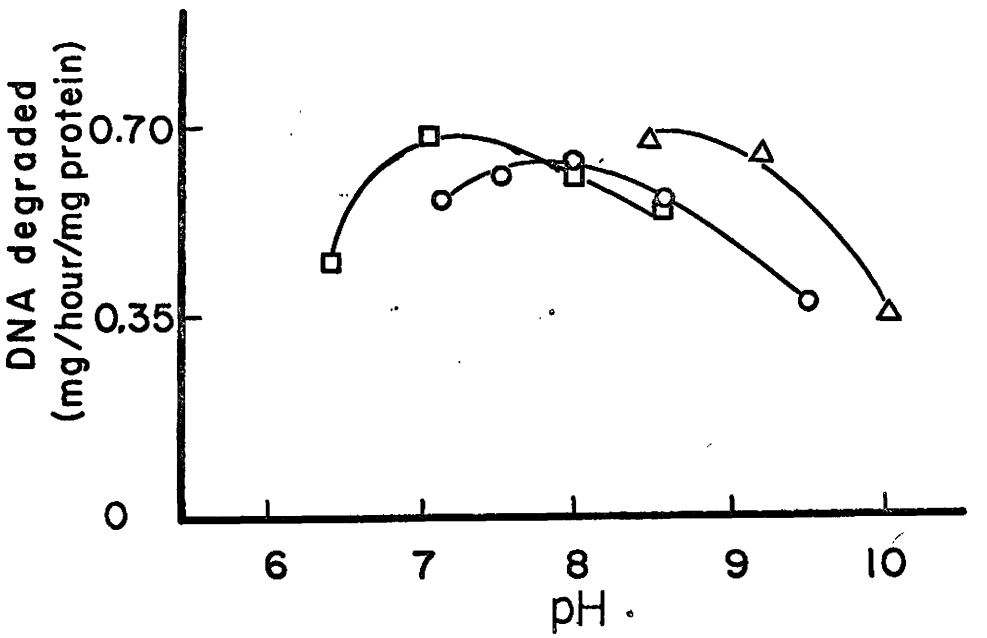

Fig. 4. Effect of pH on DNase active on native PBS 1 DNA.

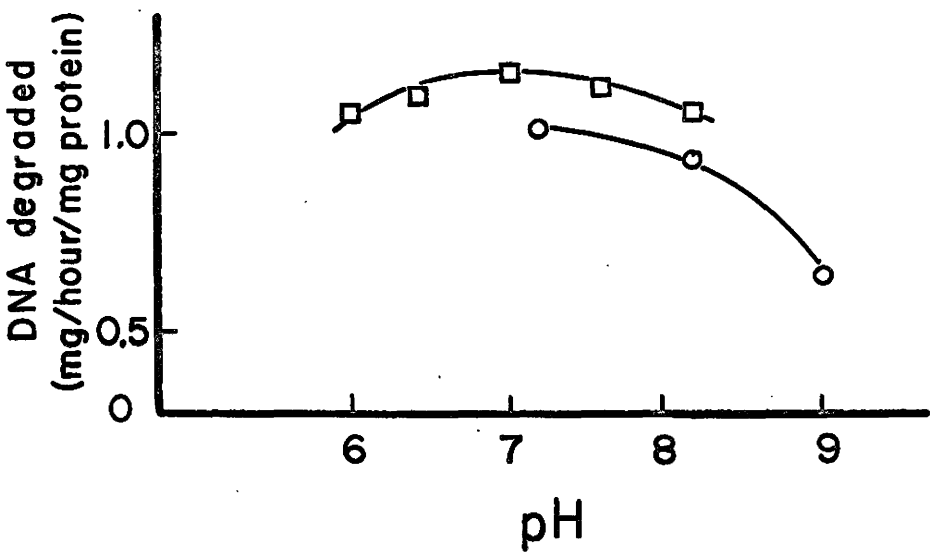

Fig. 5. Effect of pH on DNase active on heat-denatured PBS 1 DNA.

Fig. 4. Effect of pH on DNase active on native PBS 1 DNA. Fraction III in Table 2 was used. The assays of DNase were made by the radioisotope method. Buffers used were Tris-maleate-KOH (□—□), Tris-Cl (○—○) and glycine-KOH (△—△).

Fig. 5. Effect of pH on DNase active on heat-denatured PBS 1 DNA.

Fraction IV in Table 2 was used. The assays of DNase were made by the radioisotope method. Buffers used were Tris-maleate-KOH (□—□) and Tris-Cl (○—○). The initial velocity of enzymic reaction was proportional to the amount of enzyme added in the ranges of 0.005 to 1 μg/min for native PBS 1 DNA and 0.005 to 1.3 μg/min for heat-denatured PBS 1 DNA.

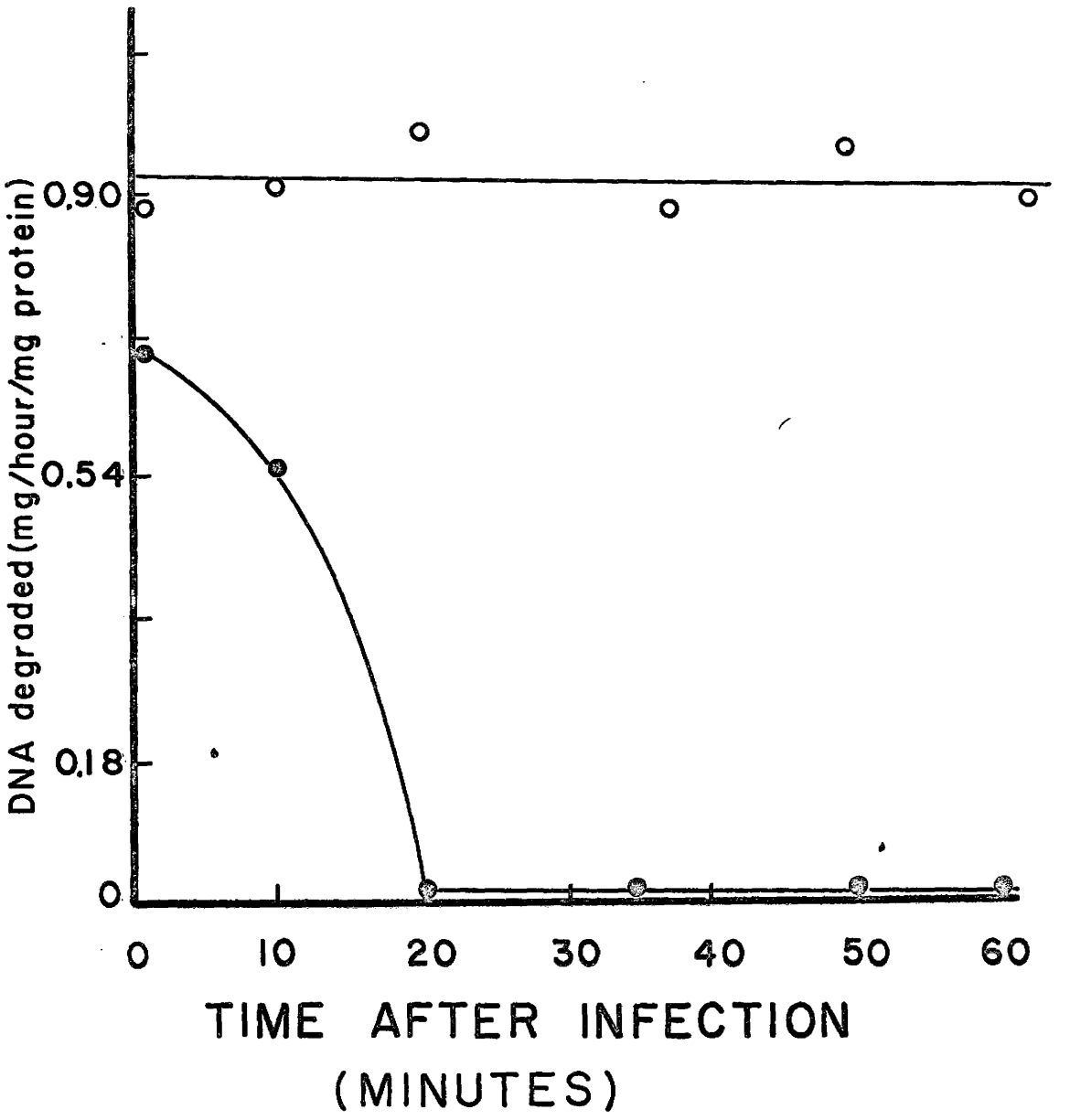

Fig. 6. DNase activities on PBS 1 DNA in crude extracts of B. subtilis infected with PBS 1.

The assays of DNase were made by the radioisotope method. Native PBS 1 DNA (⊙—⊙); heat-denatured PBS 1 DNA (○—○).

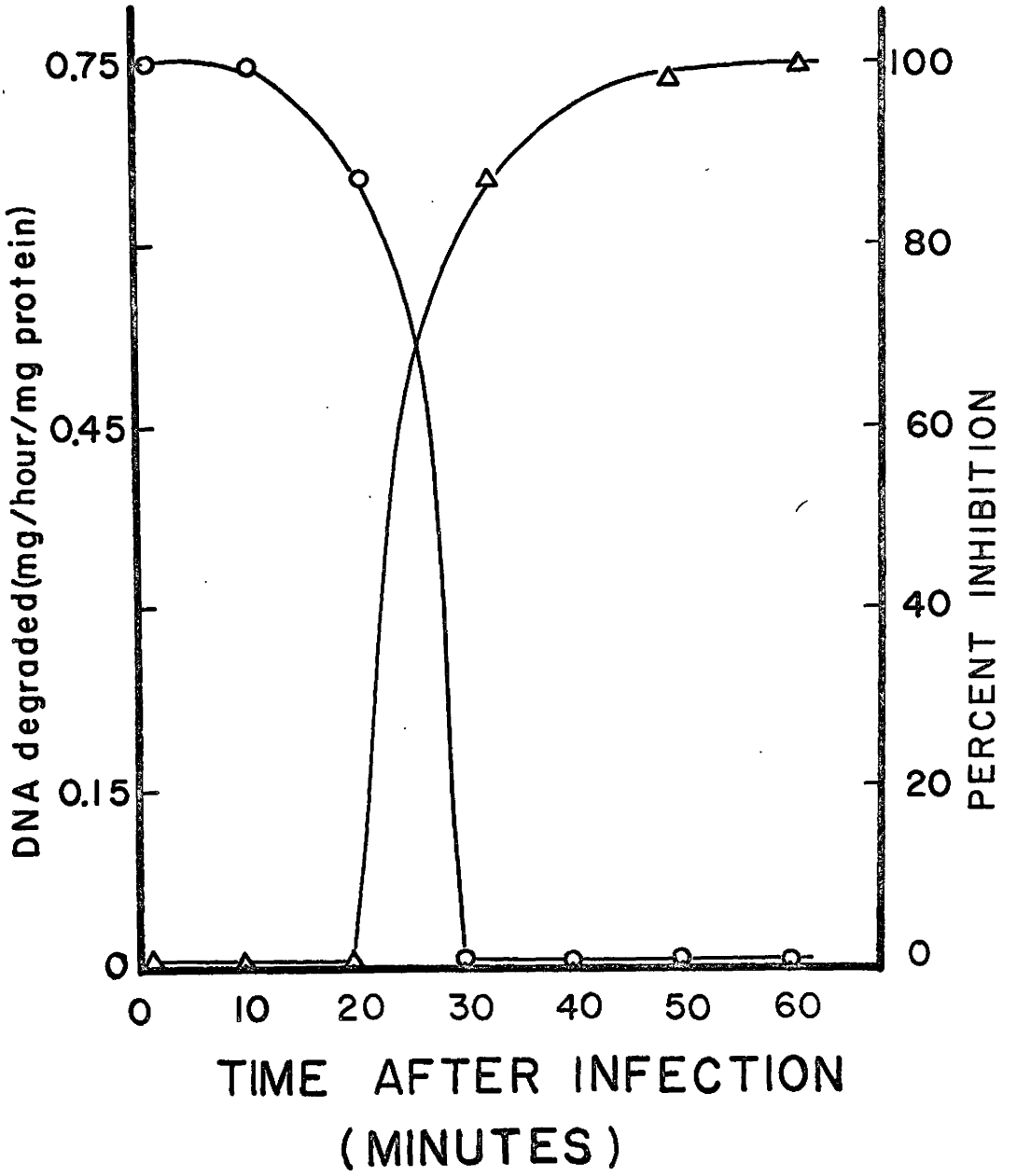

Fig. 7. Appearance of inhibitory activity after phage infection.

Crude extracts from infected cells were mixed with an equal volume of crude extracts from uninfected cells and decrease in the DNase activity was measured by the radioisotope method. Crude extracts from infected cells were heated at 100°C for 15 minutes in order to destroy DNase. DNase (O—O); Inhibitor (△—△). The DNase activity without the inhibitor was taken as 100.

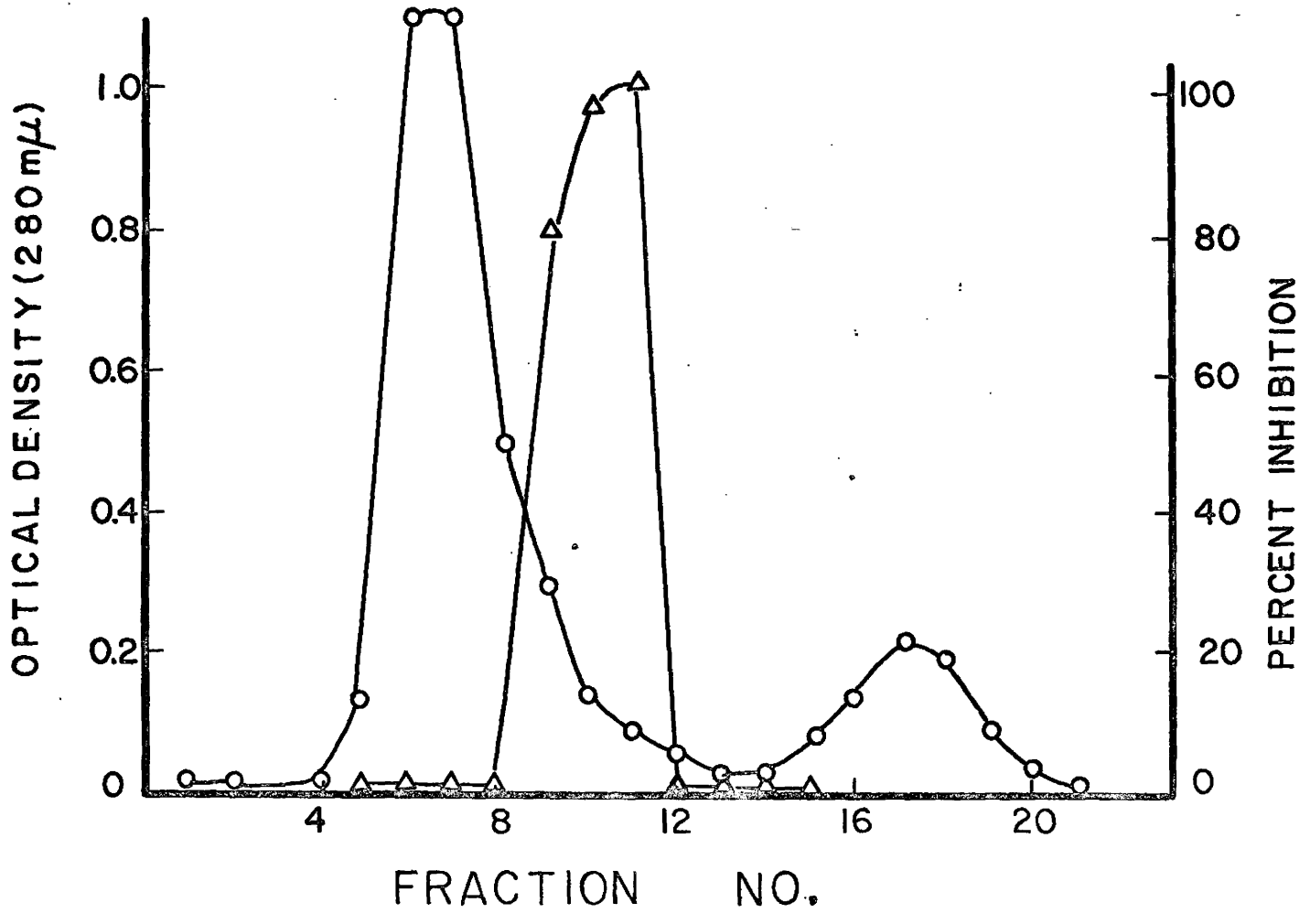

Fig. 8. Separation of the inhibitor on Sephadex G-75.

Fig. 8. Separation of the inhibitor on Sephadex G-75.
A crude extract (40 minutes after infection) was applied on a column of Sephadex G-75 previously equilibrated with 0.05 M Tris-Cl buffer (pH 7.50). The assay of the inhibitor was made by adding 0.1 ml-samples of each fraction to the DNase assay mixture containing aliquots of crude extract from uninfected cells. Protein (O—O); Inhibitory activity (Δ—Δ).
The size of the column was 1 x 27 cm and 0.5 ml-fractions were collected.

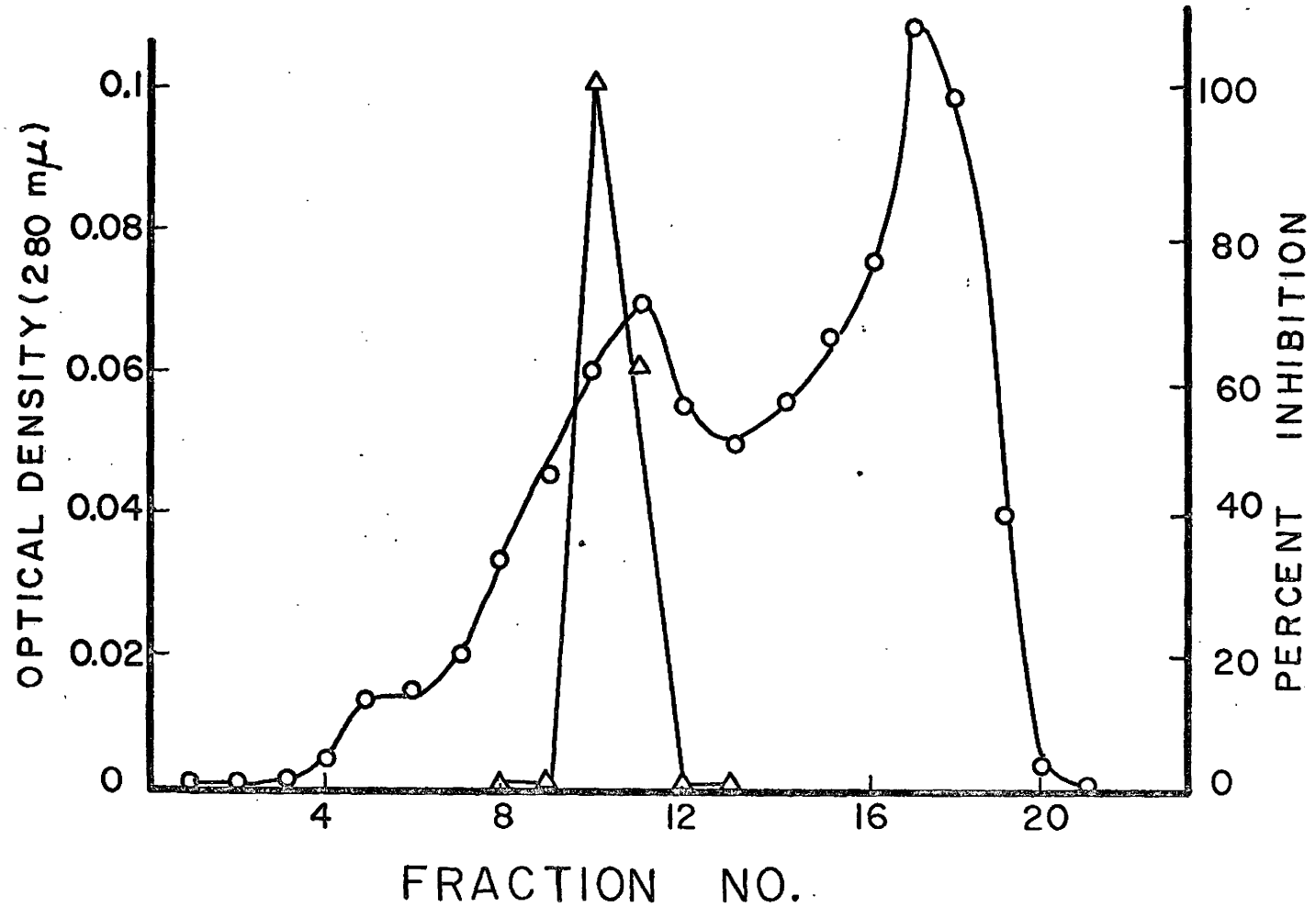

Fig. 9. Separation of the inhibitor from DNase, RNase and smaller molecules on Sephadex G-75.

Fig. 9. Separation of the inhibitor from DNase, RNase and smaller molecules on Sephadex G-75.

Sephadex G-75 was equilibrated with 0.05 M Tris-Cl buffer (pH 7.50). DNase and RNase were eluted out between fraction No. 4 and No. 8. The assay of the inhibitor was made as described in Fig. 8.

Protein (O—O); Inhibitor (Δ—Δ).

The size of the column was 1 x 27 cm and 0.5 ml-fractions were collected.

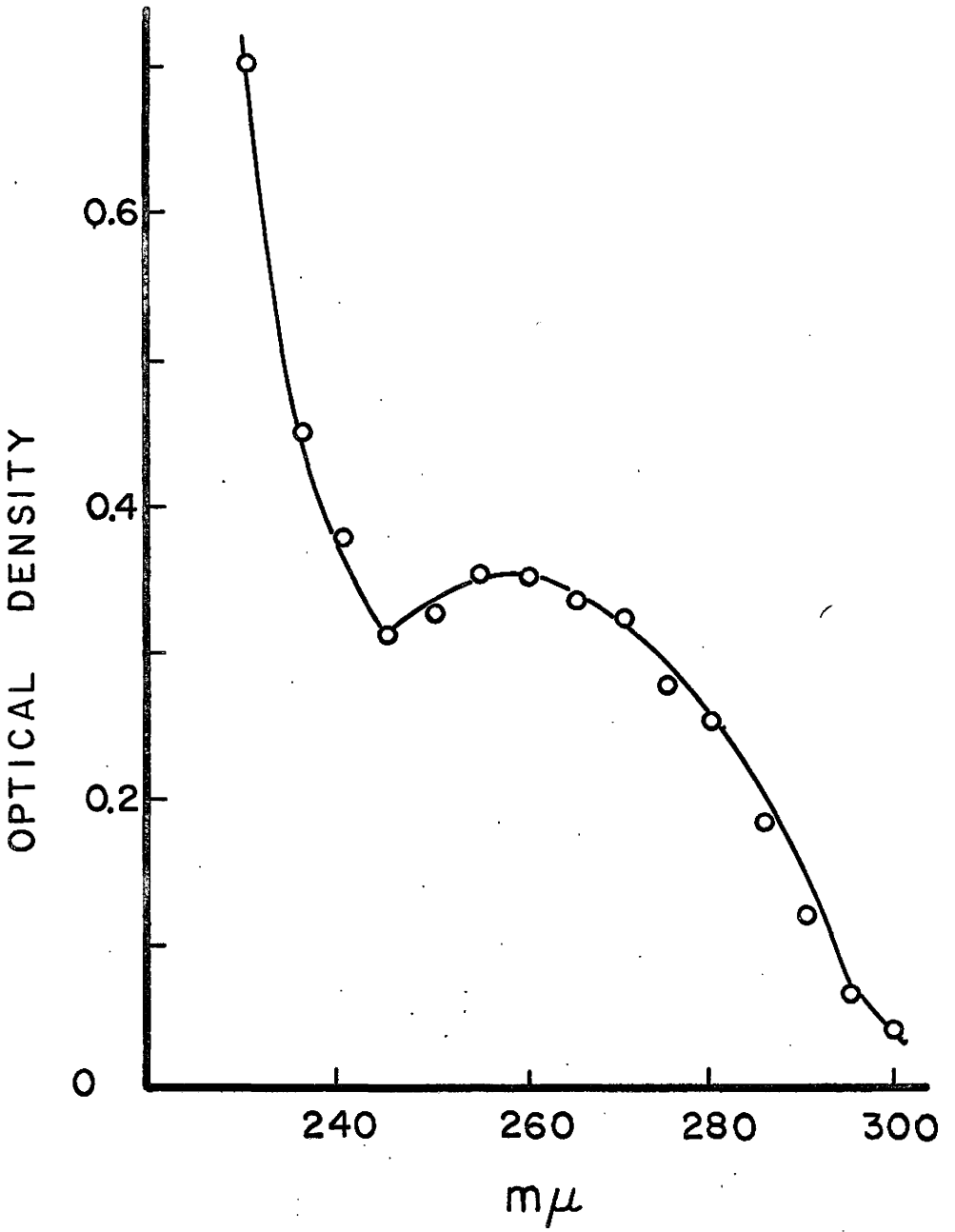

Fig. 10. Absorption spectrum of the purified inhibitor.

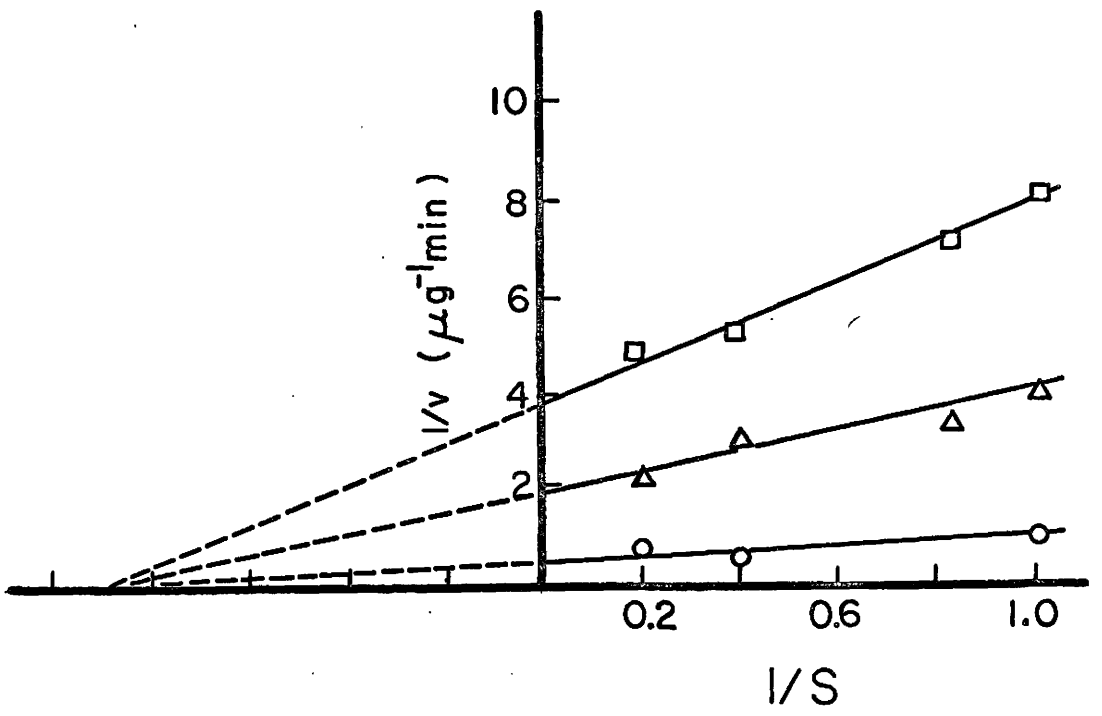

Fig. 11. Effect of the inhibitor on the DNase active on native PBS 1 DNA.

The assays of the DNase were made by the radioisotope method. Fraction III in Table 2 was used as the enzyme preparation. The concentrations of DNA varied from 60 μg/ml to 12 μg/ml. The amounts of the inhibitor were 0.08 ml/0.27ml (□—□) and 0.06 ml/0.27 ml (△—△); no inhibitor (○—○). The inhibitor was the purified fraction which had been treated with DNase and RNase and gel filtration as shown in Fig. 9.

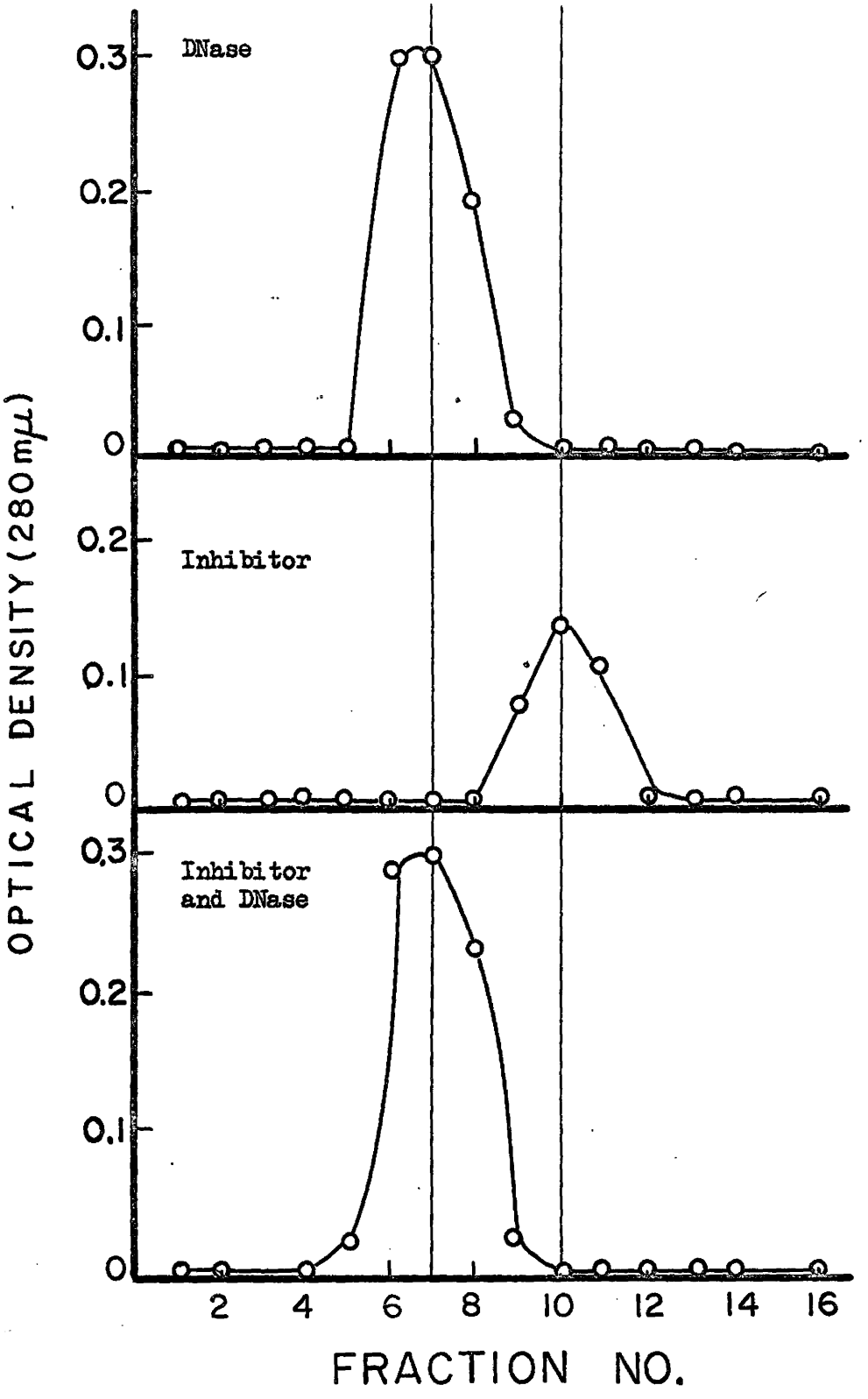

Fig. 12. Chromatography of the DNase and the inhibitor on Sephadex G-75.

The purified DNase (Table 8) and the purified inhibitor (Fig. 9) were subjected to Sephadex G-75 which was previously equilibrated with 0.05 M Tris-Cl, pH 7.50.

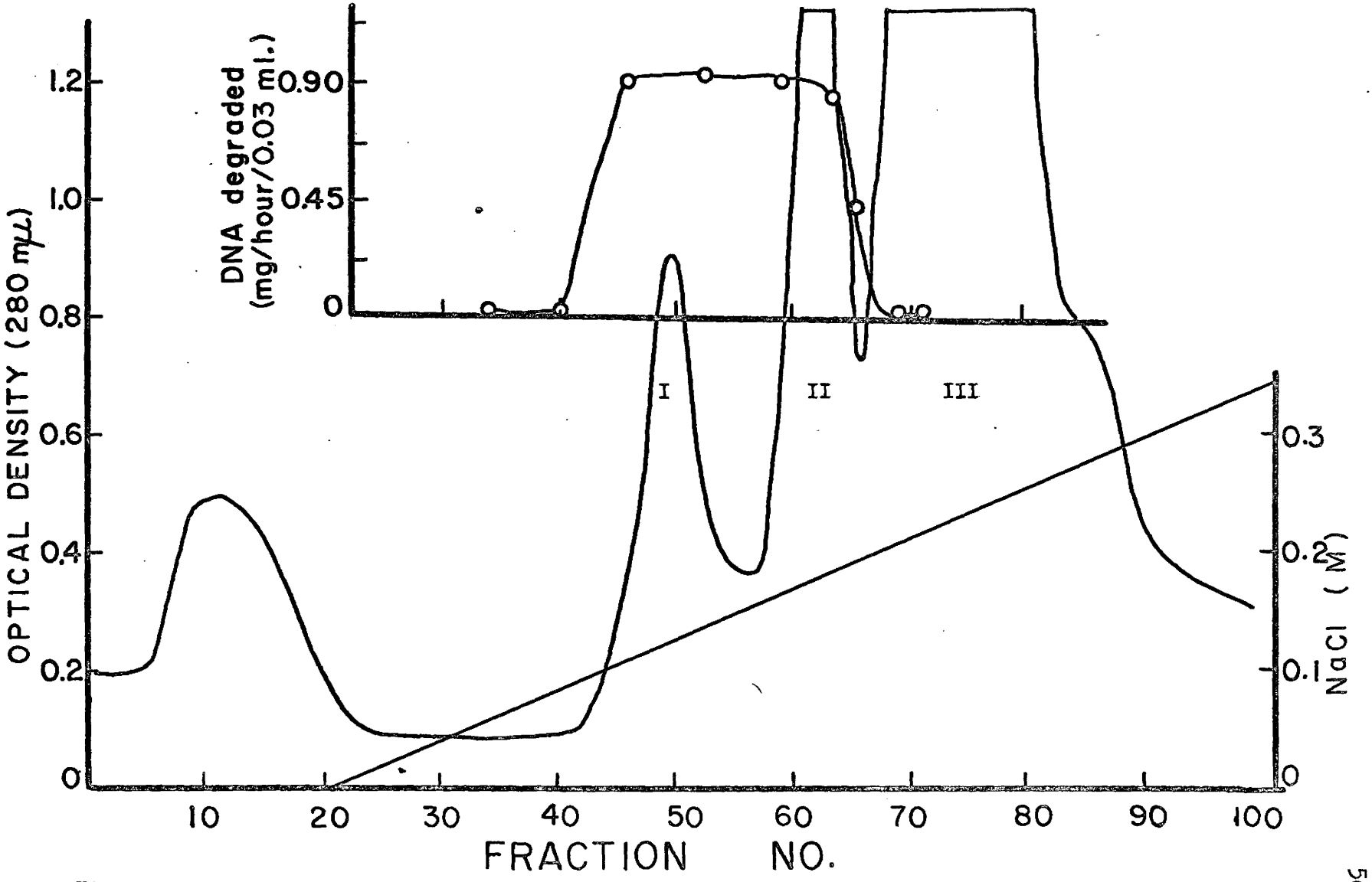

Fig. 13. Elution profile of the DNase on DEAE-cellulose.

The assays of the DNase were made by the radioisotope method.
The amount of protein applied on the column (2 x 34 cm) was 600 mg.

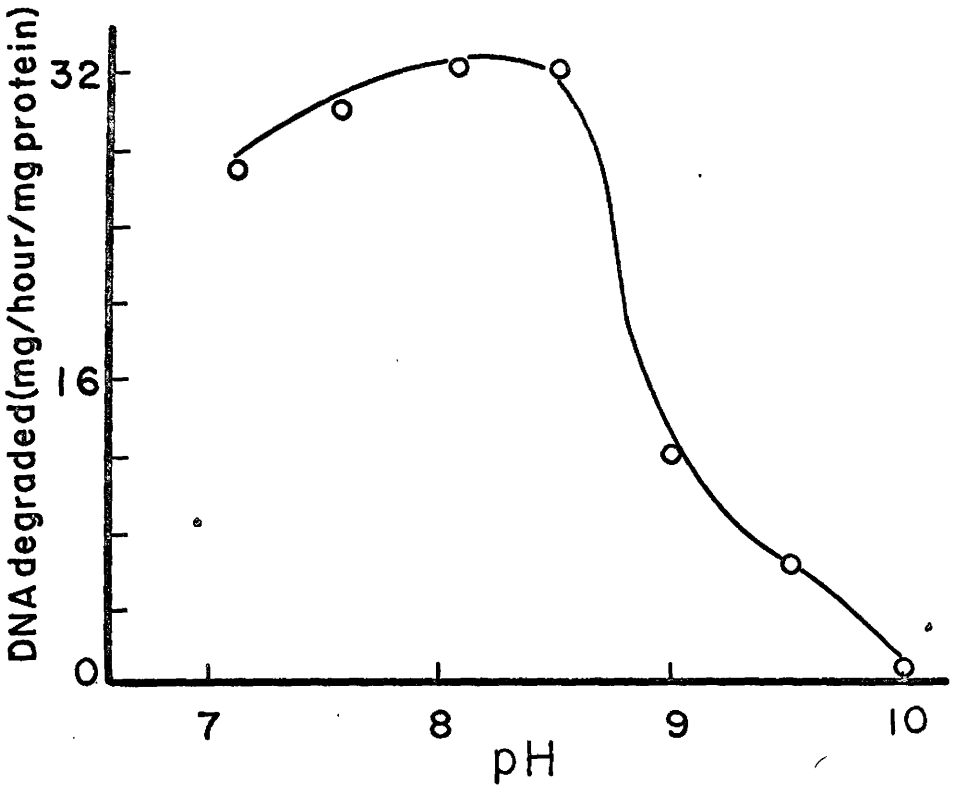

Fig. 14. Effect of pH on the purified DNase active on native PBS 1 DNA.

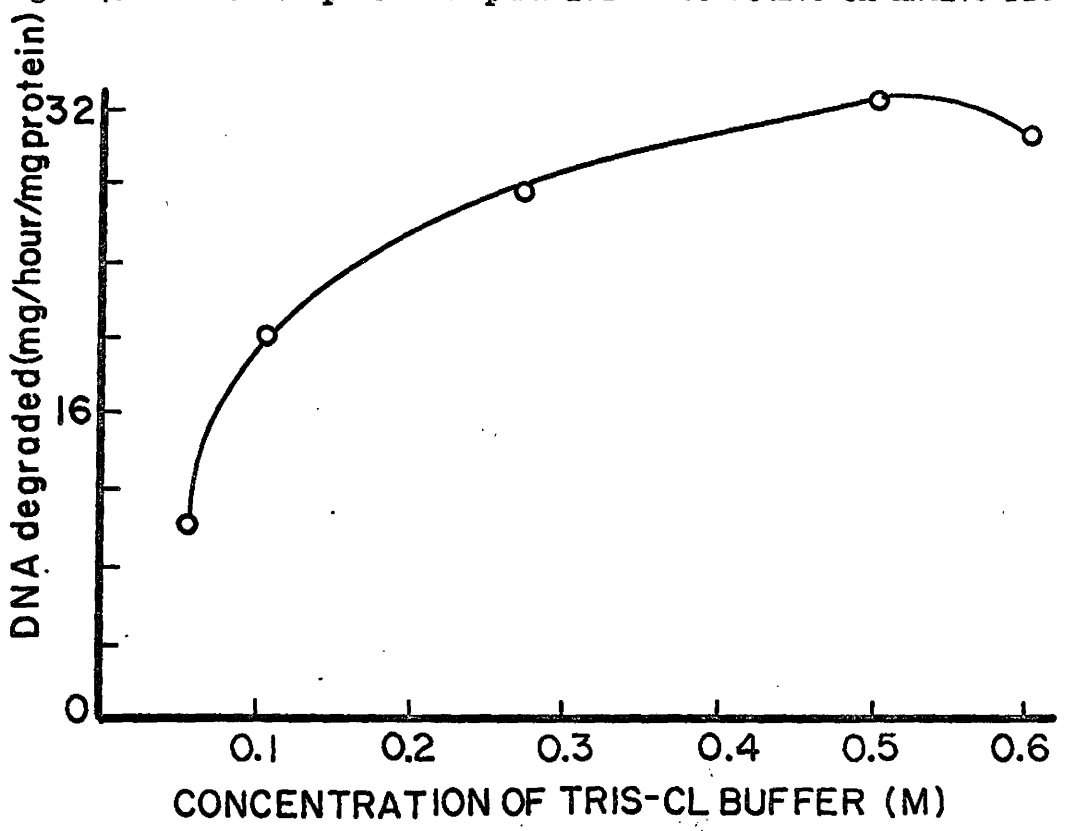

Fig. 15. Effect of concentration of buffer on the DNase.

Fig. 14. Effect of pH on the purified DNase active on native PBS 1 DNA.

The assays of DNase were made by the optical method.

Fig. 15. Effect of concentration of buffer on the DNase.

The assays of DNase were made by the optical method.

The pH of Tris-Cl buffer was 8.0.

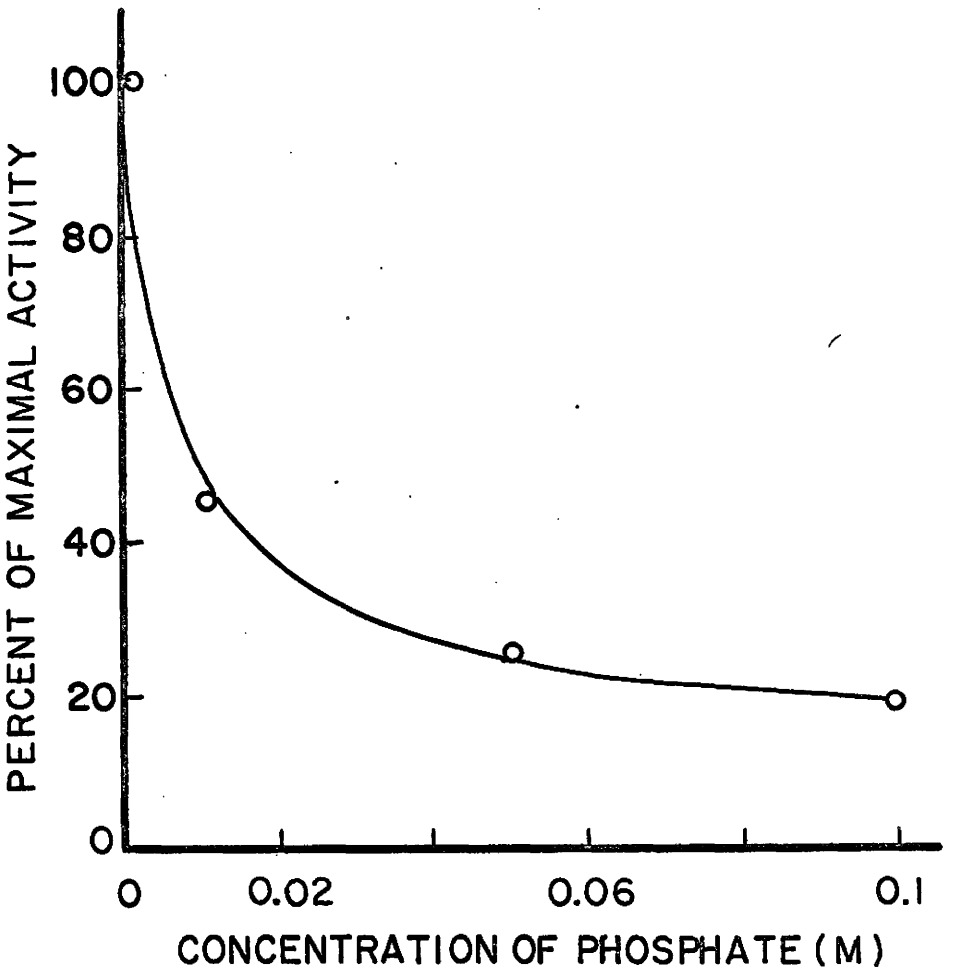

Fig. 16. Effect of phosphate on the DNase active on native PBS 1 DNA.

The assays of the DNase were made by the optical method. The reaction mixtures consisted of 0.5 M Tris-Cl buffer (pH 8.00). Potassium phosphate buffer (pH8.00) was added. The activity obtained in the absence of potassium phosphate was taken as 100.

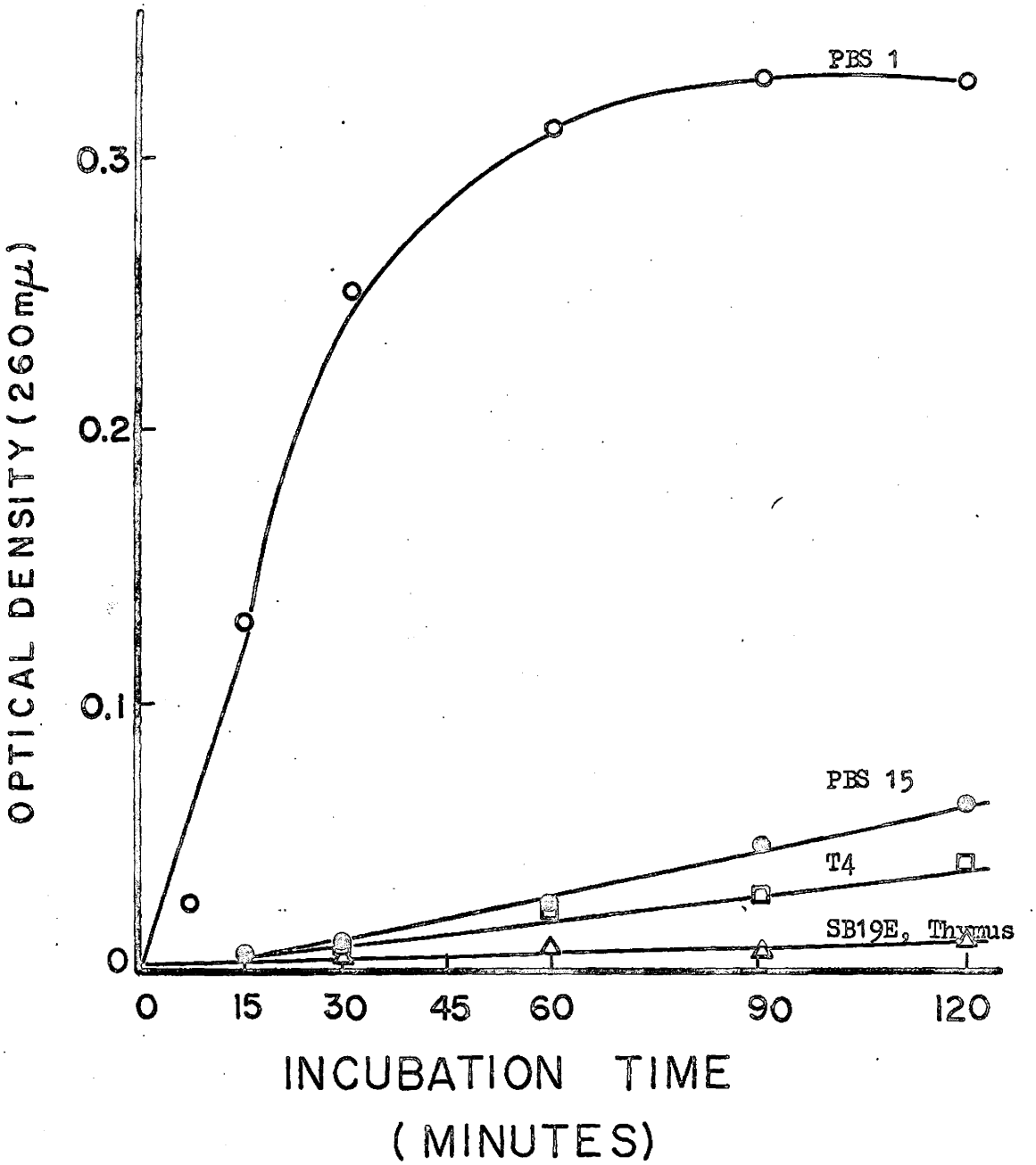

Fig. 17. Degradation of various DNA's by the purified DNase active on native PBS 1 DNA.

The assays of DNase were made by the optical method.

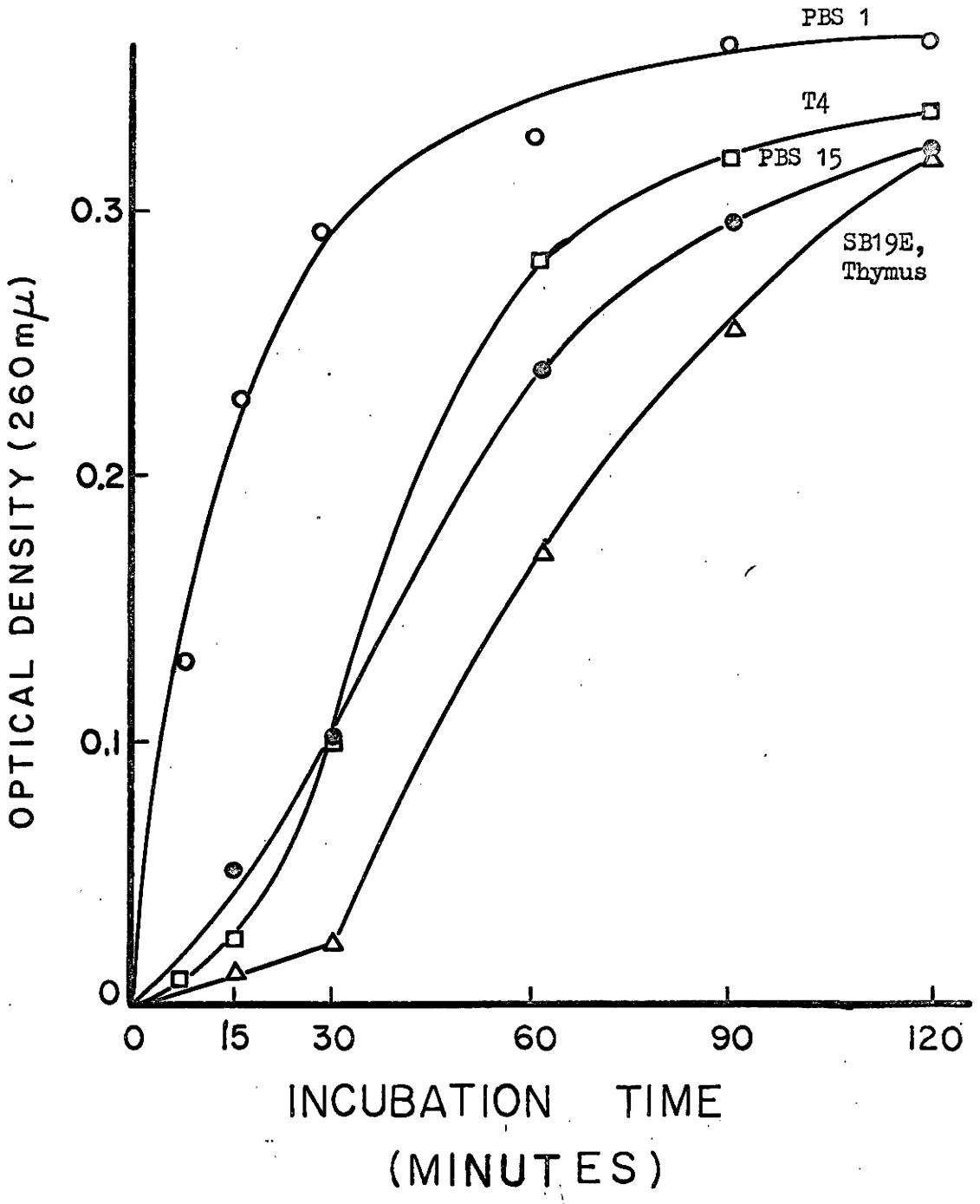

Fig. 18. Degradation of various heat-denatured DNA's by the purified DNase.

The assays of DNase were made by the optical method.

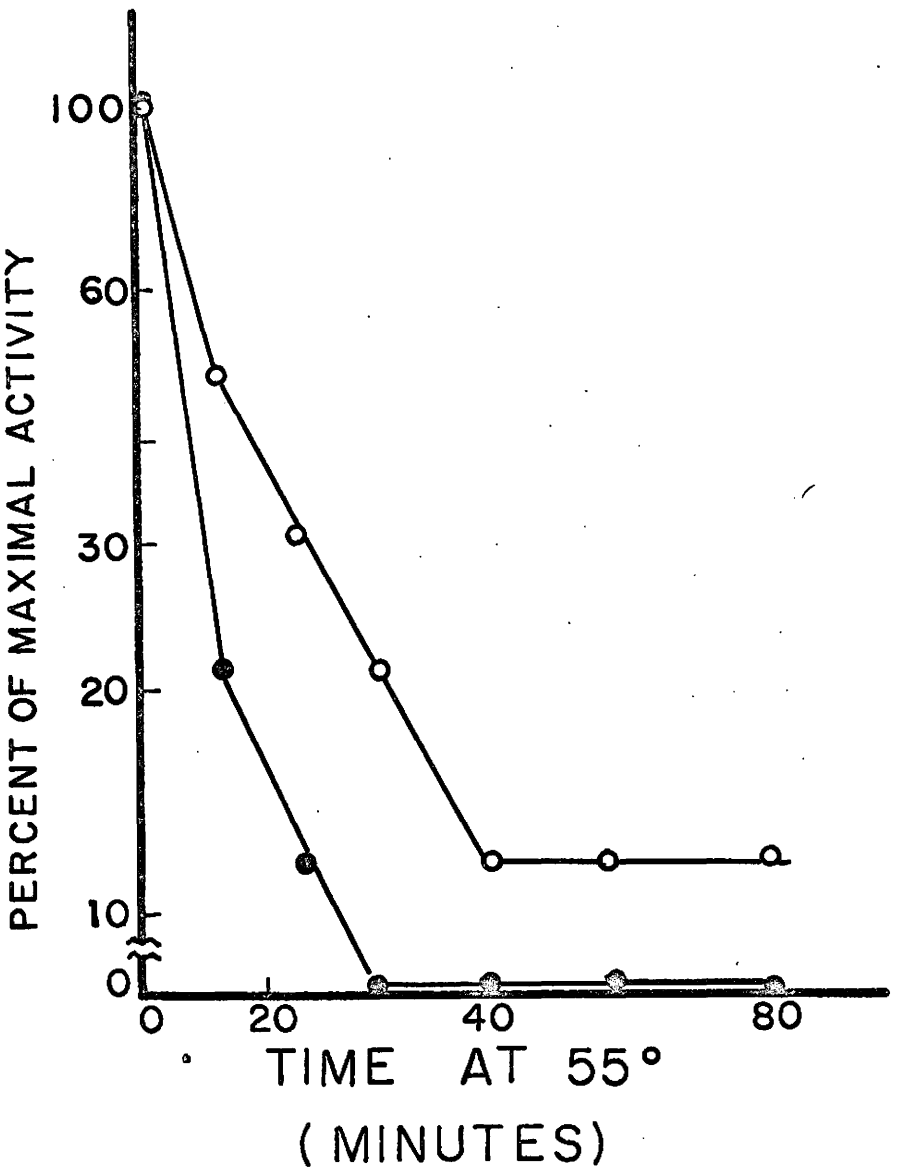

Fig. 19. Heat inactivation of DNases active on PBS 1 DNA.

Heating was performed on the complete reaction mixture without substrate as described in Materials and Methods. The reaction was initiated by transferring the mixture to 37°C and adding the substrate to the mixture. Substrates were native PBS 1 DNA (●—●) and heat-denatured host DNA (○—○).

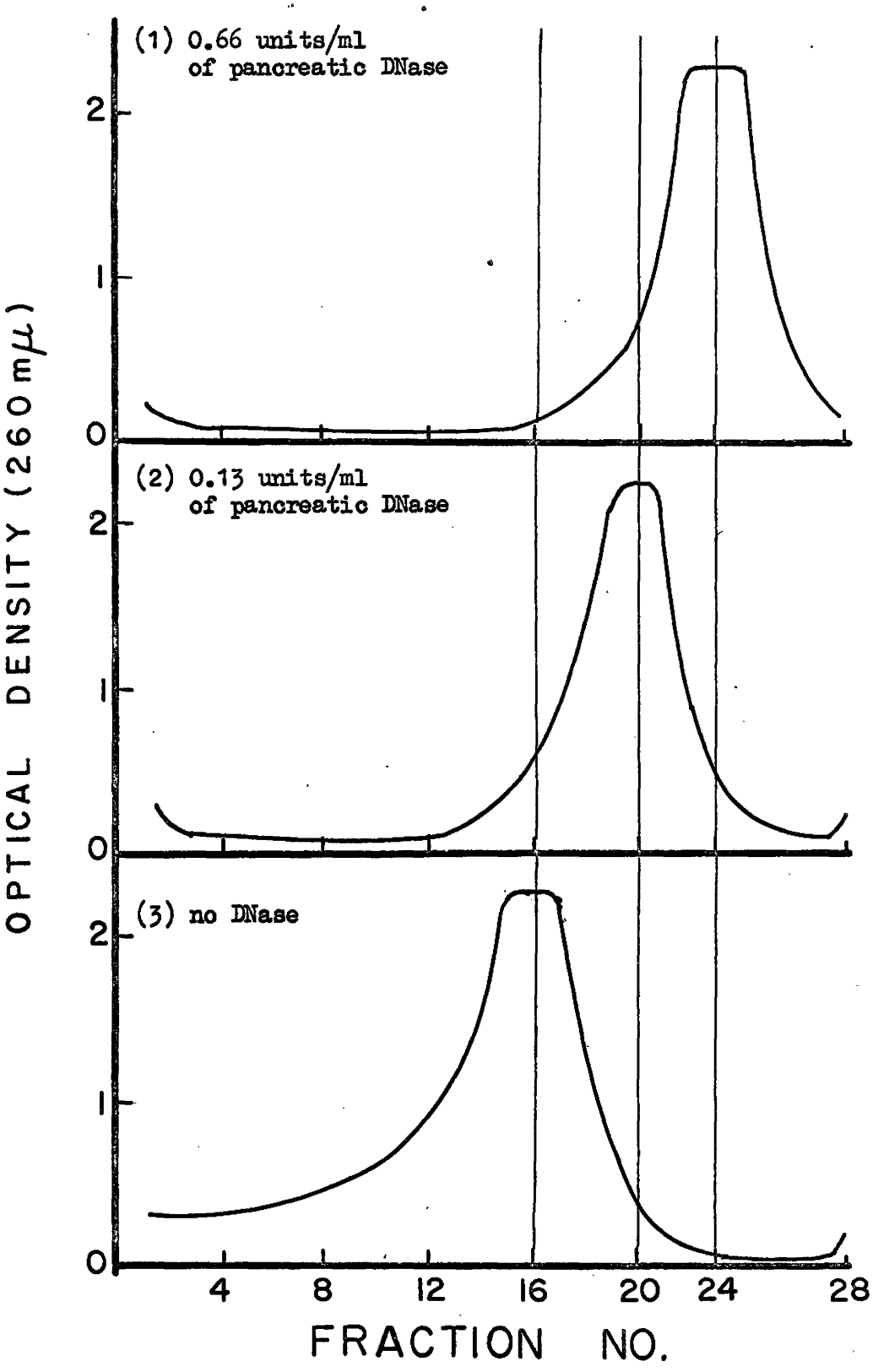

Fig. 20. Sedimentation analysis of B. subtilis DNA treated with pancreatic DNase.

Fig. 20. Sedimentation analysis of B. subtilis DNA treated with pancreatic DNase.

Bacterial DNA was sedimented after treatment with pancreatic DNase (0.66 units/ml) (1), or (0.13 units/ml) (2), or prior to enzymic treatment (3). Sedimentations were performed in 0.02 M K_3PO_4 at pH 12.1 in a gradient of sucrose (5-20 %).

DNA (0.5 ml, $O.D._{260}$=8.7) was layered on the top of the gradient.

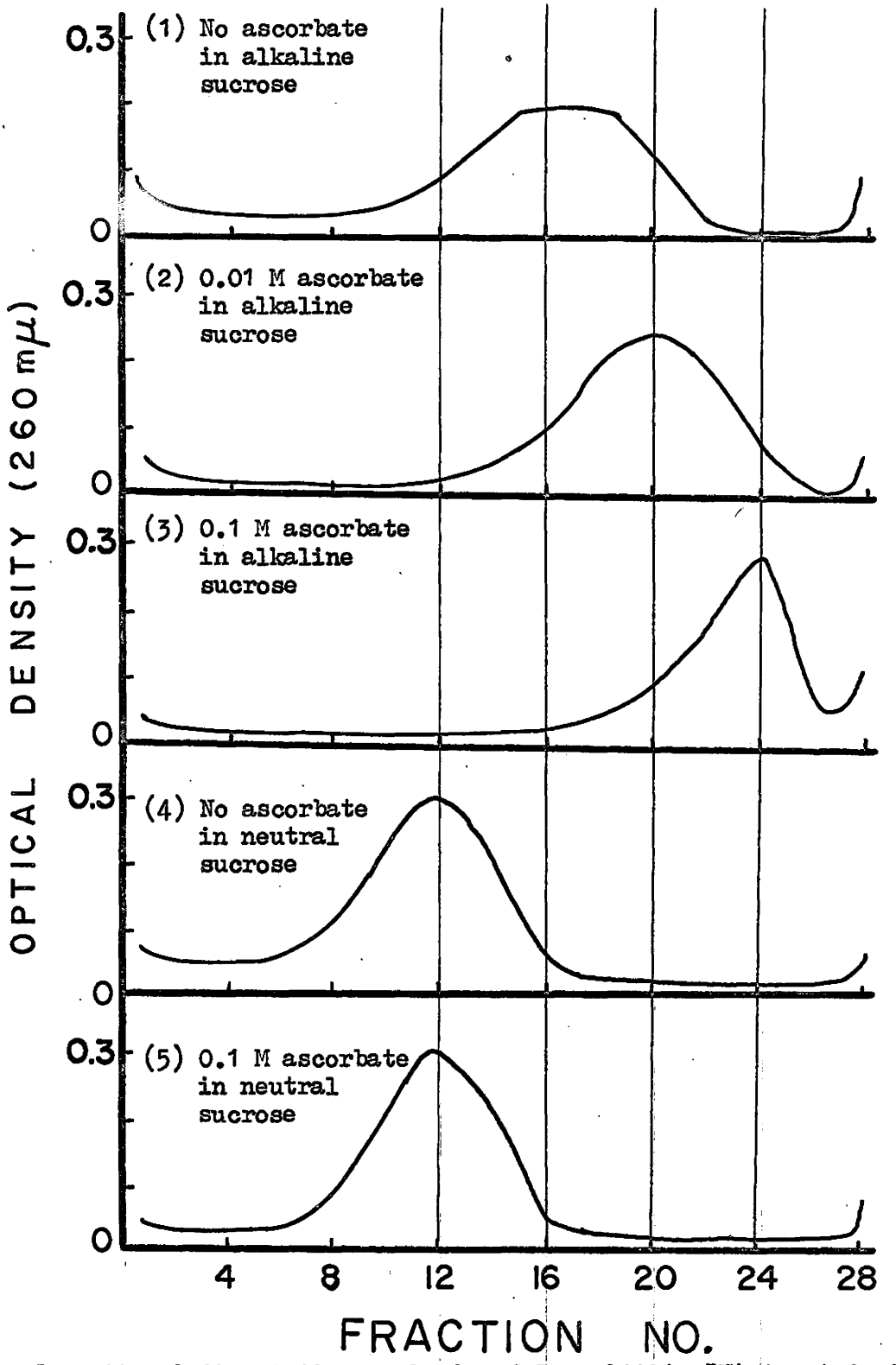

Fig. 21. Sedimentation analysis of B. subtilis DNA treated with ascorbate.

Fig. 21. Sedimentation analysis of B. subtilis DNA treated with ascorbate.

Bacterial DNA was sedimented in sucrose gradients at neutral or alkaline pH, prior to the treatment (1), (4), after the treatment with 0.01 M ascorbate (2), or 0.1 M ascorbate (3),(5). Conditions of the treatment and sedimentation were described in the text.

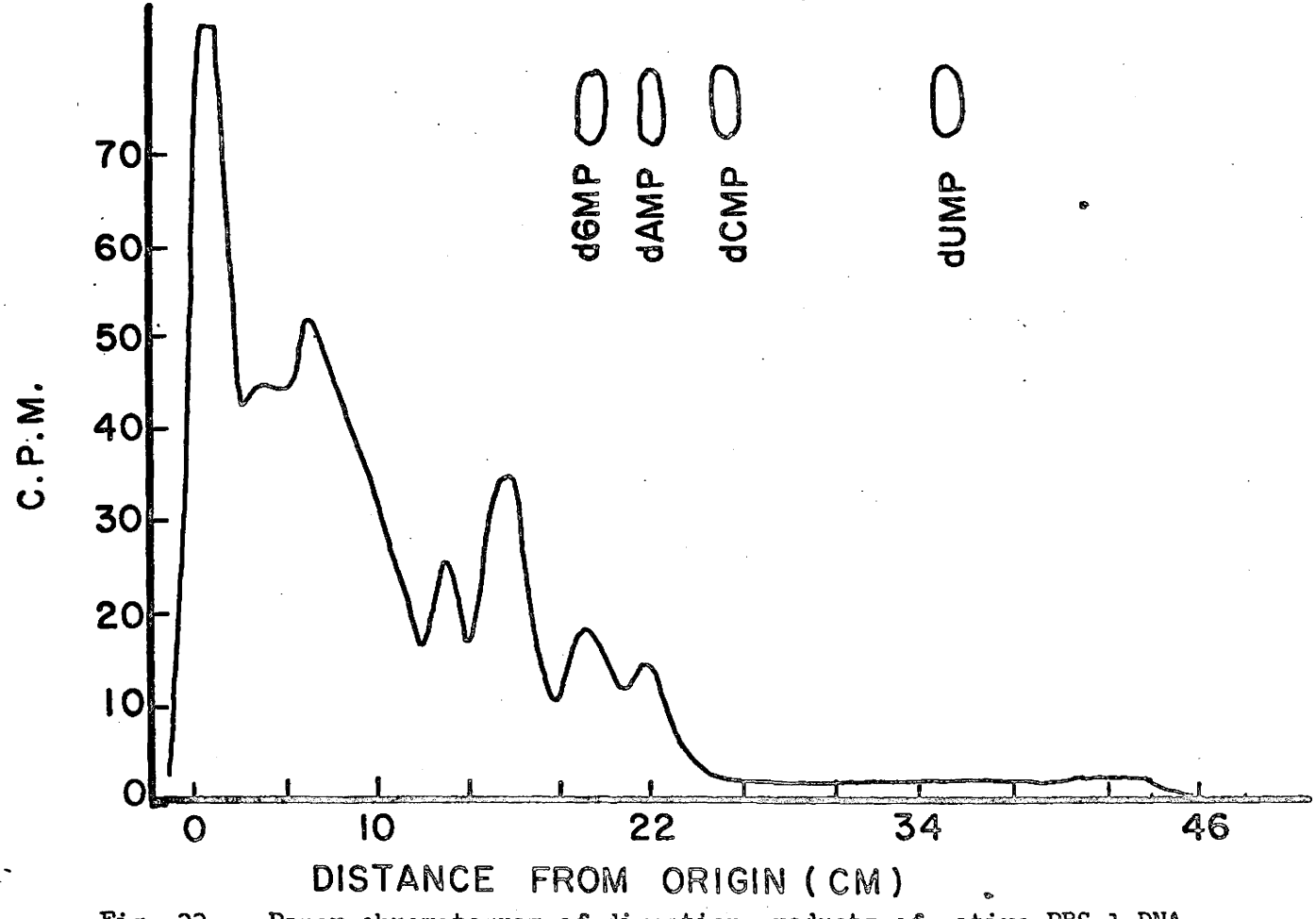

Fig. 22. Paper chromatogram of digestion products of native PBS 1 DNA.

The hydrolysate of DNA labelled with P^{32} was chromatographed in a mixture of ethanol (95%)-saturated sodium tetraborate-5 M ammonium acetate-0.5 M EDTA (220:80:20:0.5 by vol.) as solvent. The positions of reference compounds, dGMP, dAMP, dCMP and dUMP were located by scaning under a ultraviolet lamp and shown on the Figure.

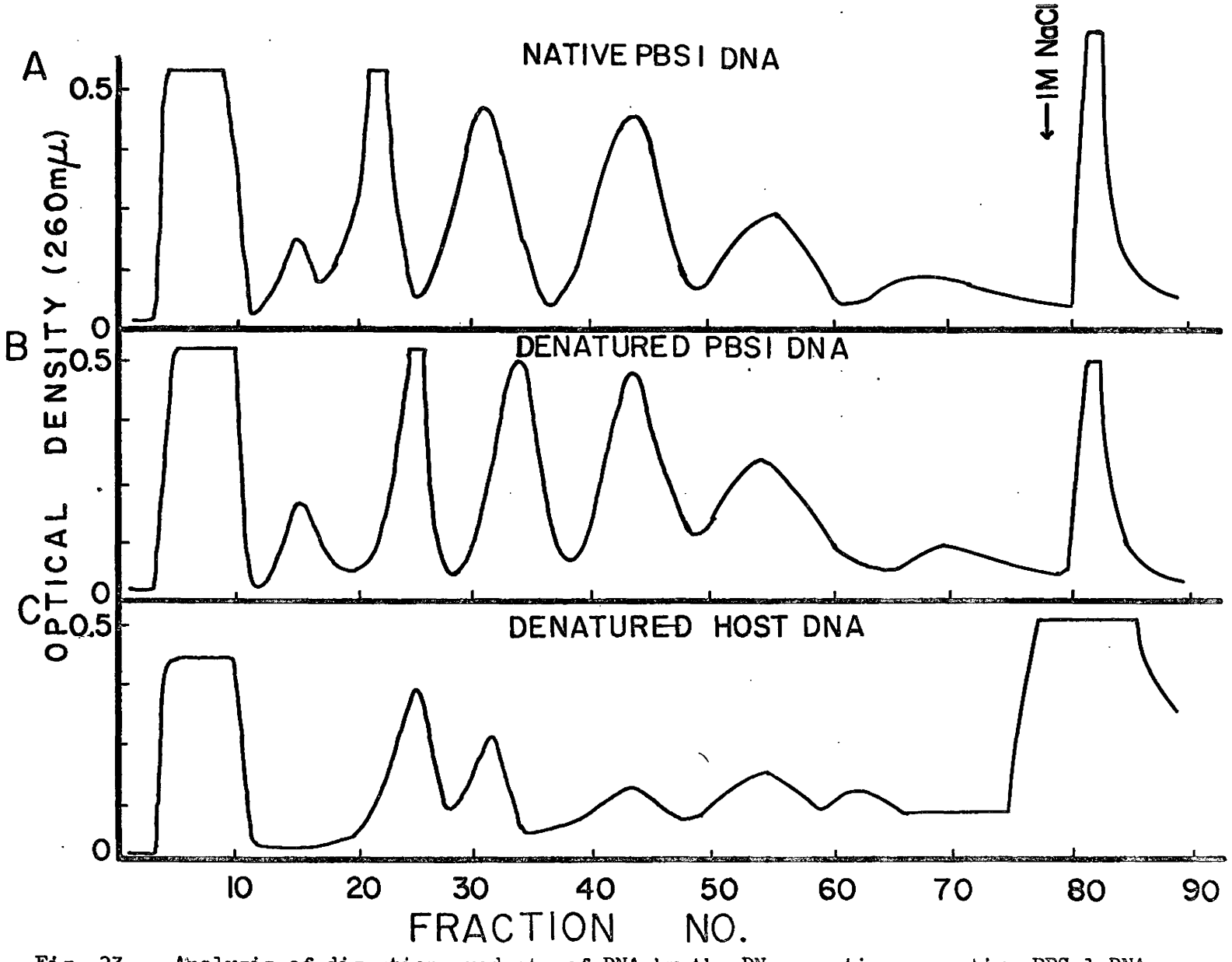

Fig. 23. Analysis of digestion products of DNA by the DNase active on native PBS 1 DNA on the DEAE-Sephadex A-25 chromatography.

Fig. 23. Analysis of digestion products of DNA by the DNase active on native PBS 1 DNA on the DEAE-Sephadex A-25 chromatography.

A column (1 x 15 cm) of DEAE-Sephadex A-25, pre-equilibrated with 7 M urea and 0.02 M Tris-Cl, pH 7.6, was used with a linear gradient of 140 ml (total volume) of 0.14 M to 0.28 M NaCl, in 7 M urea-0.02 M Tris-Cl (pH 7.6). Two ml fractions were collected.

The amount of sample applied on the column was 45 O.D. units at 260 mμ.

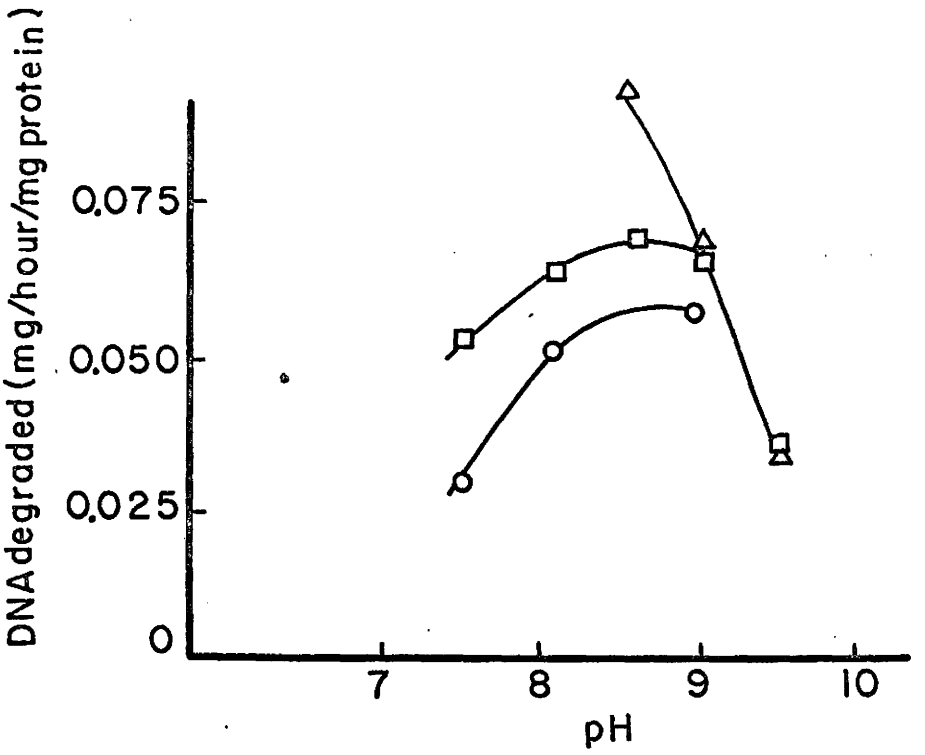

Fig. 24. Effect of pH on the DNase active on native host DNA.

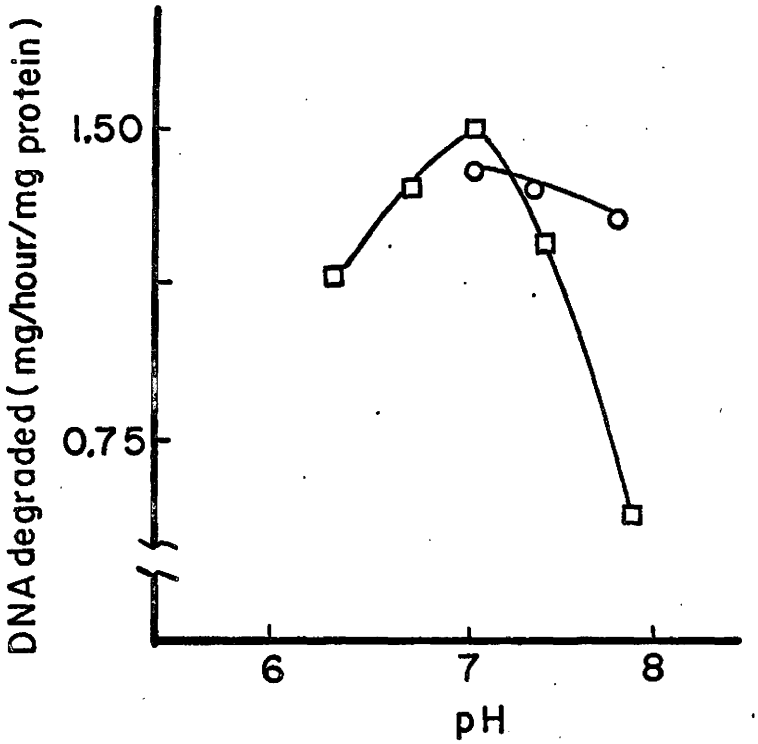

Fig. 25. Effect of pH on the DNase active on heat-denatured host DNA.

Fig. 24. Effect of pH on the DNase active on native host DNA.

The assays of DNase were made by the radioisotope method. Buffers used were Tris-maleate-KOH (□—□), Tris-Cl (○—○) and glycine-KOH (△—△).

Fig. 25. Effect of pH on the DNase active on heat-denatured host DNA.

The assays of the DNase were made by the radioisotope method. Buffers used were Tris-maleate-KOH (□—□) and Tris-Cl (○—○).

The initial velocity of enzymic reaction was proportional to the amount of enzyme added in the ranges of 0 to 0.015 μg/min for the DNase active on native host DNA and 0.005 to 1.3 μg/min for heat-denatured host DNA.

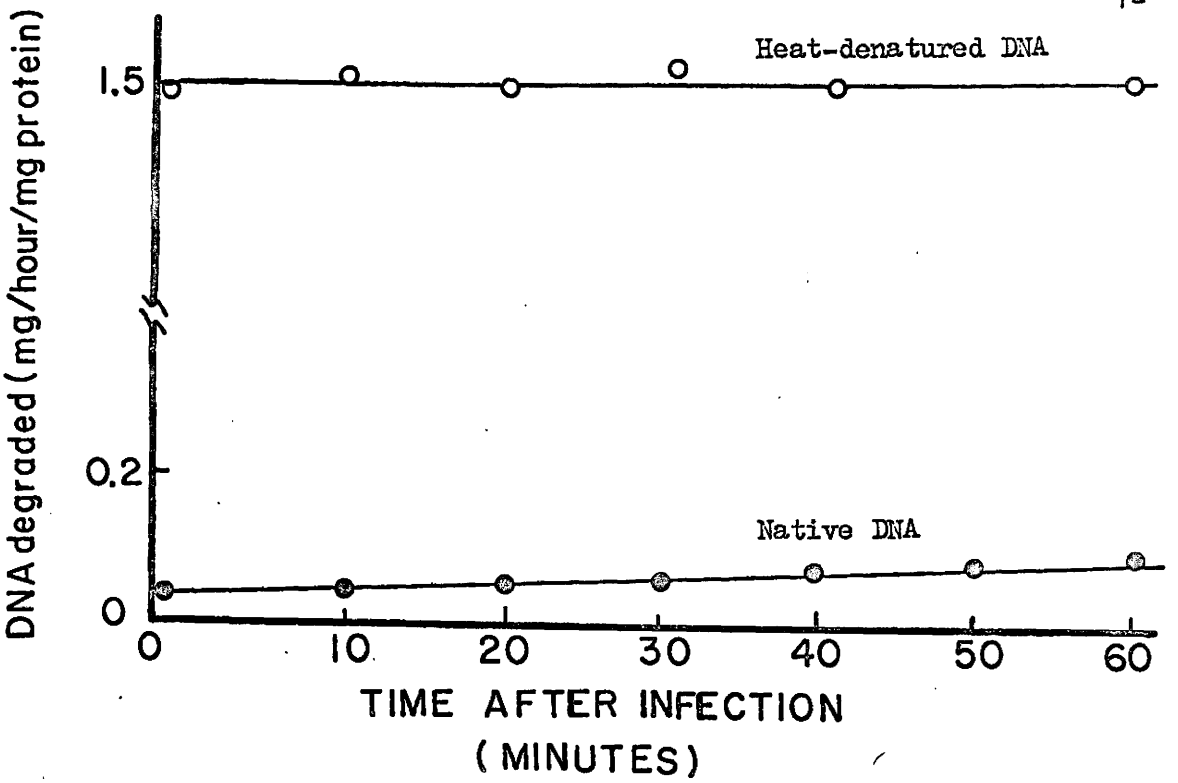

Fig. 26. DNase activities on host DNA in crude extracts of B. subtilis infected with PBS 1.

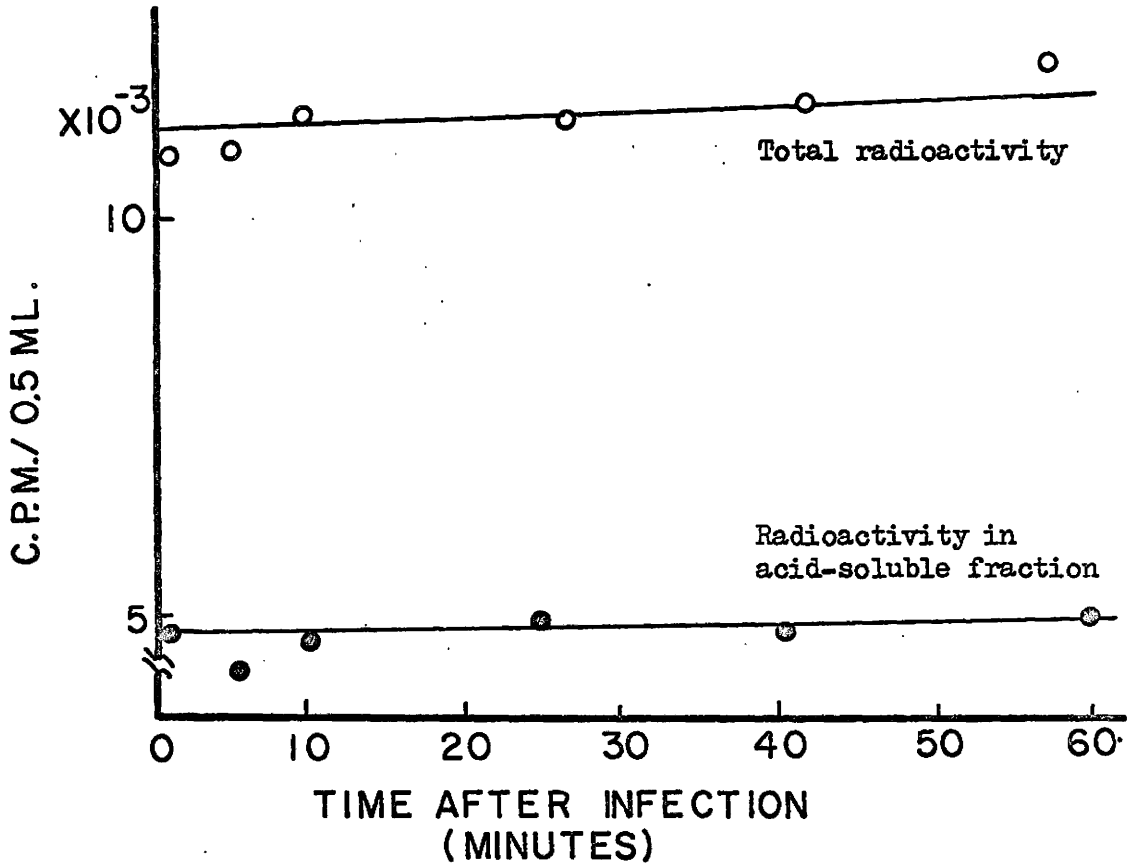

Fig. 27. The release of radioactive nucleotides in acid-soluble fraction from H^3-labelled cells.

Fig. 26. DNase activities on host DNA in crude extracts of B. subtilis infected with PBS 1.
The assays of DNase were made by the radioisotope method. Native host DNA (●—●); heat-denatured host DNA.(○—○).

Fig. 27. The release of radioactive nucleotides in the acid-soluble fraction from H^3-labelled cells.
For determination of total amounts of radioactivity in the cells, 0.5 ml-samples, at various time intervals after infection, were pipetted onto Millipore filters of 0.45 μ pore diameter, and washed with 10 ml of cold Tris-medium, 10 ml of cold distilled water. For determination of the amounts of radio-activity in nucleic acids, 0.5 ml-samples of cells were mixed with an equal volume of cold trichloroacetic acid (10 %) and kept at 0°C for 10 minutes. Then the filters were washed with 10 ml of cold trichloro-acetic acid (5 %) and 10 ml of cold distilled water. The filters were dried and the radioactivity was measured.

Table 1

Degradation of PBS 1 and *B. subtilis* DNA by pancreatic DNase and snake venom phosphodiesterase

Enzyme	(μg/ml)	DNA	K (minutes^{-1})
Pancreatic DNase	0.91	Native PBS 1	1.14
	0.18	Native PBS 1	0.17
	0.18	Native host	0.14
	0.91	Denatured PBS 1	0.12
	0.91	Denatured host	0.16
Snake venom phosphodiesterase	22.7	Denatured PBS 1	0.62
	9.1	Denatured PBS 1	0.23
	22.7	Denatured host	0.30
	9.1	Denatured host	0.09

Reaction mixtures for pancreatic DNase contained 2.0 ml of DNA ($O.D._{260}$= 2.0), 2.0 ml of 0.15 M Tris-Cl (pH 7.2) containing 0.05 M $MgCl_2$ and 0.01 M β-mercaptoethanol, and 0.4 ml of enzyme preparation. Reaction mixtures for snake venom phosphodiesterase contained 2.0 ml of DNA ($O.D._{260}$ =2.0), 2.0 ml of 0.05 M Tris-Cl (pH 8.7) containing 0.01 M NaCl and 0.001 M $CaCl_2$, and 0.4 ml of enzyme preparation.
K is the velocity constant expressed as minutes^{-1}.

Table 2

DNase activity of fractions obtained by

Hydroxylapatite-C chromatography

Fraction	Substrate			
	Host DNA		PBS 1 DNA	
	Native	Denatured	Native	Denatured
I	0	0	0	0
II	0.09	0	0	0
III	0	1.04	0.71	0.87
IV	0	1.48	0.01	0.73

Activity was expressed as mg of DNA degraded/hour/mg protein.
DNase activity was assayed by the radioisotope method.

Table 3

Effect of bivalent cations on DNase active on native PBS 1 DNA

Cations	(mM)	DNA degraded (mg/hour/mg protein)	Relative activity
$CaCl_2$	0.5	0.63	90
	1.0	0.69	99
	5.0	0.70	100
	10.0	0.66	95
$MgCl_2$	0.5	0.01	2
	1.0	0.09	13
	5.0	0.13	19
	10.0	0.10	14
$FeCl_2$	0.1	0	0
	1.0	0	0
$CuCl_2$	0.1	0	0
	1.0	0	0
$MnCl_2$	0.1	0.10	15
	1.0	0	0
None	-	0.01	2

Enzyme preparation used was Fraction III in Table 2.
DNase activity was assayed by the radioisotope method.

Table 4

Effect of bivalent cations on DNase active on heat-denatured PBS 1 DNA

Cations	(mM)	DNA degraded (mg/hour/mg protein)	Relative activity
$CaCl_2$	0.5	0.89	90
	1.0	0.98	99
	5.0	0.99	100
	10	0.97	98
$MgCl_2$	0.5	0	0
	1.0	0	0
	5.0	0	0
	10	0	0
$FeCl_2$	0.1	0	0
	1.0	0	0
$CuCl_2$	0.1	0	0
	1.0	0	0
$MnCl_2$	0.1	0	0
	1.0	0	0
None	-	0.02	2

Enzyme preparation used was Fraction IV in Table 2.
DNase activity was assayed by the radioisotope method.

Table 5

Inactivation of the inhibitor by various treatments

Treatment	PBS 1 DNA degraded (mg/hour/mg protein)	Inhibition (%)
Control	0.69	0
Pancreatic RNase	0	100
Pancreatic DNase	0	100
Lysozyme	0.01	98
Pronase	0.66	3
Trypsin	0.68	2
Heat (100°C for 15 minutes)	0	100

The inhibitor was treated with various enzymes at a final concentration of 10 µg/ml for RNase and DNase, and 100 µg/ml for lysozyme, pronase and trypsin for 30 minutes at 37°C. After the treatment, the reaction mixtures were heated at 100°C for 15 minutes. The control tube contained only the DNase and PBS 1 DNA. Enzymic activity was measured by the radioisotope method.

Table 6

Specificity of the inhibitor

Substrate	Inhibitor	DNA degraded (mg/hour/mg protein)	Inhibition (%)
Denatured PBS 1	-	0.98	0
DNA	+	0.98	0
Denatured host	-	1.46	0
DNA	+	1.43	2
Native PBS 1	-	0.72	0
DNA	+	0	100

Enzymic activity was measured by the radioisotope method. Fraction III in Table 2 was used as an enzyme preparation. A purified inhibitor preparation (Fig. 9) was used.

Table 7

The inhibitory activity of crude extracts from spores carrying PBS 1 genomes

Preparations	DNase activity on native PBS 1 DNA
Extracts from uninfected spores (I)	0.34
Extracts from spores carrying PBS 1 genomes (II)	0
Extracts from uninfected cells (III)	0.67
(I) + (III)	0.67
(II) + (III)	0

The activity of DNase was expressed as DNA degraded (mg/hour/mg protein). DNase activity was assayed by the radioisotope method.

Table 8

Purification of the DNase active on native PBS 1 DNA

Purification steps	Specific activity (mg/hour/mg protein)	Protein (mg)	Recovery (%)
Crude extract	0.67	40000	100
Hydroxylapatite-C	0.67	16000	60
$(NH_4)_2SO_4$ 0.4-0.6 saturation	6.7	1650	41
DEAE-cellulose (Fractions II and III)	19.5	30	2.2
$(NH_4)_2SO_4$ 0.6 saturation	33.3	10	1.3

The DNase activity was assayed by the radioisotope method.

Table 9

Effect of cations on the purified DNase active on native PBS 1 DNA

Cation	(mM)	DNA degraded (mg/hour/mg protein)	Relative activity
$CaCl_2$	0.5	25.8	76
	1.0	29.5	88
	5.0	34.0	100
	10.0	19.8	58
	100.0	3.5	10
$MgCl_2$	0.5	0	0
	1.0	0	0
	10.0	0	0
	100.0	0	0
$MnCl_2$	0.1	0	0
	1.0	0	0
	10.0	0	0
	100.0	0	0
$CuCl_2$	0.1	0	0
	1.0	5.8	17
	10.0	10.6	31
KCl	100.0	0	0
	330.0	0	0
NaCl	100.0	3.4	10
	330.0	2.4	7
None	-	3.4	10

Table 10

Degradation of various DNA's by the purified DNase active on native PBS 1 DNA

Source of DNA	DNA degraded (mg/hour/mg protein)	Relative activity
B. subtilis SB19E	0	0
Calf thymus	0	0
Phage T4	0.34	1
Phage PBS 15	1.70	5
Phage PBS 1	34.0	100

The DNase activity was assayed by the optical method. In these experiments 0.05 ml of DNA (470µg/ml) was used in a total volume (0.27 ml) of reaction mixtures.

Table 11

Effect of thymus DNA on the degradation of PBS 1 DNA by the purified DNase

Substrate	Calf thymus DNA added (µg/ml)	DNA degraded (mg/hour/mg protein)	Relative activity
Native PBS 1 DNA (60 µg/ml)	None	34.0	100
Native PBS 1 DNA (60 µg/ml)	30	33.6	99
	60	34.6	102
	170	34.4	101
Native thymus DNA (60 µg/ml)	-	0	0

The DNase activity was assayed by the optical method.

Table 12

Degradation of DNA carrying single-strand breaks by the purified DNase active on native PBS 1 DNA

Souce of DNA	Treatment	DNA degraded (mg/hour/mg protein)	Relative activity
PBS 1	None	34.2	100
SB19E	Pancreatic DNase		
	0.13 units/ml	0	0
	0.66 units/ml	11.0	32
	Ascorbate		
	10^{-2} M	0	0
	10^{-1} M	8.9	26
T5	None	1.7	5

The DNase activity was assayed by the optical method.

Table 13

Degradation of nitrous acid-treated calf thymus DNA by DNase active on native PBS 1 DNA

Treatment	% cytosine converted	Total content of uracil (mole %)	DNA degraded (mg/hour/mg protein)	Relative activity
72 hours at pH 3.3 with nitrous acid	75	15.4	26.2	77
without nitrous acid	0	0	26.8	79
30 hours at pH 4.0 with nitrous acid	30	6.2	27.2	80
without nitrous acid	0	0	0	0
None (PBS 1 DNA)	-	36.0	34.0	100
None (thymus DNA)	-	0	0	0

The DNase activity on native PBS 1 DNA (34.0 mg/hour/mg protein) was taken as 100. The DNase activity was assayed by the optical method. The amount of cytosine converted was determined by paper chromatography of the treated DNA after complete hydrolysis by the mixture of pancreatic DNase (100 µg/ml) and snake venom phosphodiesterase (100 µg/ml).

Table 14

Analysis of digestion products

Substrate	Fraction	Mole %				
		dAMP	dGMP	dCMP	dTMP	dU
Native PBS 1 DNA	Nucleosides	0	0	0	-	100
	Mononucleotides	89	11	0	-	0
	Dinucleotides	70	30	0	-	0
	Trinucleotides	72	28	0	-	0
	Tetranucleotides	81	19	0	-	0
	Larger than hexa-nucleotides	53	19	28	-	0
Denatured PBS 1 DNA	Nucleosides	0	0	0	-	100
	Mononucleotides	89	11	0	-	0
	Dinucleotides	74	26	0	-	0
	Trinucleotides	73	27	0	-	0
	Tetranucleotides	80	20	0	-	0
	Larger than hexa-nucleotides	44	25	31	-	0
Denatured host DNA	Nucleosides	None				
	Mononucleotides	91	9	0	0	-
	Dinucleotides	53	47	0	0	-
	Trinucleotides	47	53	0	0	-
	Tetranucleotides	61	49	0	0	-
	Larger than hexa-nucleotides	26	18	26	30	-

Table 15

Effect of bivalent cations on the DNase active on native host DNA

Cation	(mM)	DNA degraded (µg/hour/mg protein)	Relative activity
$CaCl_2$	0.5	46	53
	1.0	57	65
	5.0	87	100
	10.0	9.6	11
$MgCl_2$	0.5	7.2	8
	1.0	11	13
	5.0	10	12
	10.0	11	13
$FeCl_2$	0.1	7.2	8
	1.0	0.0	0
$CuCl_2$	0.1	0.0	0
	1.0	0.9	1
$MnCl_2$	0.1	6.5	7
	1.0	7.9	9
None	-	7.2	8

Enzyme preparation used was Fraction II in Table 2. Glycine-KOH buffer (0.1 M, pH 8.53) was used. The DNase activity was assayed by the radioisotope method.

Table 16

Effect of bivalent cations on the DNase active on denatured host DNA

Cations	(mM)	DNA degraded (mg/hour/mg protein)	Relative activity
$CaCl_2$	0.5	1.21	80
	1.0	1.35	95
	5.0	1.42	100
	10.0	1.41	99
$MgCl_2$	0.5	0.16	11
	1.0	0.10	7
	5.0	0.05	4
	10.0	0.02	2
$FeCl_2$	0.1	0.02	2
	1.0	0	0
$CuCl_2$	0.1	0.10	7
	1.0	0.10	7
$MnCl_2$	0.1	0.11	8
	1.0	0.05	4
None	-	0.08	6

Enzyme preparation used was Fraction IV in Table 2. Tris-maleate-KOH (0.2 M, pH 7.00) was used. The DNase activity was assayed by the radioisotope method.

Table 17

Effect of RNase and mild heat treatment on DNase active on host DNA

Treatment	Substrate	DNase activity DNA degraded (mg/hour/mg protein)	
		Uninfected	Infected
None	Native	0.085	0.125
	Denatured	1.42	1.41
RNase 10 µg/ml	Native	0.085	0.085
	Denatured	1.41	1.40
Heat 55°C for 10 minutes	Native	0.043	0.065
	Denatured	0.81	0.83

The assays were performed by the radioisotope method. Crude extracts were prepared from cells 40 minutes after infection.

Table 18

Transforming activity of B. subtilis DNA treated with crude extracts or pancreatic DNase

Treatment	Number of transformants/ml (1) Str^r	(2) Prototroph ($aro_2^+, try_2^+, his_2^+, tyr_1^+$)	Ratio (2)/(1)
None	2050	3850	1.9
Extracts from 0 minutes	417	228	0.55
15 minutes	512	179	0.35
30 minutes	450	170	0.38
Pancreatic DNase			
10^{-3} µg	150	200	1.3
10^{-2} µg	44	19	0.43

Recipient bacterium was SB202 (aro_2, try_2, his_2, tyr_1) The mutations borne by this strain were linked to each other. Incubation mixtures for the treatment consisted of 0.03 ml of DNA (100 µg/ml), 0.08 ml of enzyme preparation (crude extracts, 5 mg/ml in protein contents) and 0.05 ml of buffer (0.3 ml of 1 M Tris-Cl, pH 8.50, 0.15 ml of 5×10^{-2} M $CaCl_2$ and 0.09 ml of 0.1 M β-mercaptoethanol). Incubation mixtures for pancreatic DNase contained 0.05 M Tris-Cl (pH 7.50), 0.03 ml of DNA (100 µg/ml) and pancreatic DNase in 100 mM $MgCl_2$ to make a final voume of 0.16 ml. After incubation at 37°C for 20 minutes, mixtures were diluted to obtain a concentration of 1 µg DNA/ml and 0.1 ml of the treated DNA was added to 0.9 ml of competent cultures. The number of transformants was linearly related to the amount of DNA within a range of 0 to 0.1 µg/ml.

Table 19

Transforming activity of DNA extracted from infected cells at various stages of phage multiplication

Experiment I

Time of DNA extraction after phage infection (minutes)	Number of transformants/ml (1) *Str*^{r}	(2) Prototroph (*aro*$_2^+$, *try*$_2^+$, *his*$_2^+$, *tyr*$_1^+$)	Ratio (2)/(1)
0	2000	4400	2.2
10	1590	3800	2.4
20	1590	3800	2.4
30	2090	4600	2.2

Recipient bacterium was SB202 (*aro*$_2$, *try*$_2$, *his*$_2$, *tyr*$_1$). The mutations borne by this strain were linked to each other. Amounts of DNA used for transformation were 0.1 µg/ml.

Experiment II

Time of DNA extraction after phage infection (minutes)	Number of transformants/ml Total *ile*$^+$	*met*$^+$, *ile*$^+$	Percent double*
0	2270	910	39.9
10	2140	825	38.5
20	2250	920	40.8
30	2090	924	44.2

Recipient bacterium was MIL (*met*, *ile*, *leu*). The mutations (*met*, *ile*) borne by this strain were linked to each other. Total *ile*$^+$ transformants were scored on minimal agar plus leucine and methionine, and *met*$^+$, *ile*$^+$ transformants were scored on minimal agar plus leucine.

* Percent double : the frequency of transformants which received the two markers simultaneously. Amounts of DNA used for transformation were 0.1 µg/ml. The number of transformants was linearly related to the amount of DNA within a range of 0 to 0.1 µg/ml.

CHAPTER IV

ENZYMIC STUDIES ON dUTP SYNTHESIS IN CELLS INFECTED WITH PBS 1

Formation of deoxyuridine compounds by various enzymes in cells infected with PBS 1

Bacteriophage PBS 1 contains DNA in which thymine is completely replaced by uracil (84). This is the only DNA known to contain deoxyuridylic acid. Two enzyme activities which are virtually absent in uninfected cells, have been observed in B. subtilis infected with PBS 2, a clear-plaque type mutant of PBS 1 (91). One of them is dUMP kinase, which phosphorylates dUMP. This enzyme has been considered to be responsible for the formation of dUTP. The other enzyme is dTMPase, which dephosphorylates dTMP. This enzyme seems to prevent the incorporation of thymine into the phage DNA.

Other pathways which would yield dUTP in PBS 1-infected cells have not been investigated. A ribonucleotide reductase system can give rise to dUTP by the following reactions: UDP $\rightarrow$ dUDP $\rightarrow$ dUTP (92). The existence of ribonucleotide reductases has been shown in various organisms (93-95,128). The dCMP deaminase would also give rise to dUMP as shown in E. coli infected with T-even phages (79,89,129) and B. subtilis infected with phages SP8 (85), SP5C (86) or ϕe (87). The dC deaminase (96) and dU kinase (97) may also yield dUMP.

The formation of deoxyuridylates in B. subtilis cells infected

with PBS 1 was investigated by determining the activities of dU kinase, dC deaminase, dCMP deaminase and UDP reductase in crude extracts. These enzymes were examined at various pH values (6.5-8.5) and in the presence of different bivalent cations (Mg^{2+}, Mn^{2+} and Ca^{2+}), but there were no appreciable differences in the enzyme activities between infected and uninfected cells. The highest enzymic activities were obtained under the conditions described in Materials and Methods. Results are shown in Table 20. Elimination of small molecules from crude extracts by passage through Sephadex G-75 made no difference in the level of enzymic activities. In no case dCMP deaminase was detectable, even when the sensitivity of the assay method was increased by the use of radioactive substrate.

Deamination of dCTP in PBS 1-infected cells

As shown in the previous section, dCMP deaminase was not detectable in either infected or uninfected cells. It has been reported that dCMP deaminase from a variety of sources such as sea urchin eggs (130), chick embryo (131) and *E. coli* infected with T-even phages (89) is stimulated by the presence of dCTP. Therefore the effect of dCTP on dCMP deaminase in *B. subtilis* infected with PBS 1 was examined. As shown in Table 21 (Expt. I), the addition of dCTP into the reaction mixtures apparently stimulated the deamination of deoxycytidylate. However, when increasing amounts of dCMP were used with a relatively large amount of dCTP, the amounts of deoxycytidylate deaminated remained almost constant (Table 21, Expt. II). These results clearly indicated

that dCTP rather than dCMP was the substrate of the enzyme.

The addition of chloramphenicol at 100 µg/ml to PBS 1-infected cultures during infection prevented further increase in the deaminase activity (Fig. 28).

Purification of dCTP deaminase

Partial purification of dCTP deaminase was achieved with crude extracts from cells harvested at 30 minutes after infection by passing through a column of DEAE-cellulose which had been equilibrated with 0.05 M Tris-Cl buffer (pH 7.50). The enzyme was not absorbed on the column. The fractions which emerged immediately from the column were pooled and concentrated with ammonium sulphate (60% of saturation). The concentrated fraction had a 10-fold specific activity (1.33 µ moles/hour/mg protein) over the crude extract. Attempts to purify the enzyme by Hydroxylapatite-C, CM-cellulose and Sephadex G-200 were unsuccessful.

Properties of partially purified dCTP deaminase

Effect of pH: The optimum pH for dCTP deaminase was found to be 6.65 to 6.85 and the type of buffer had little effect on the enzymic activity (Fig. 29). It was observed during the study on the effect of pH that high ionic strength of buffer inhibited the activity of dCTP deaminase (Fig. 30).

Effect of cations: The effect of bivalent cations was tested in 0.067 M phosphate buffer (pH 6.70). The enzyme activity was stimulated by Mn^{2+}, Mg^{2+}, and Ca^{2+} (Table 22). Among them, Mn^{2+} showed the strongest

stimulatory effect at the concentrations tested.

Substrate specificity: The susceptibility of dCDP, dCMP, dC and CTP to the enzyme was tested. None of these four compounds was deaminated.

Reaction product and stoichiometry of deamination of dCTP

The product of deamination of dCTP was separated from the other compounds by paper chromatography with a mixture of isobutyric acid-2.3 N NH_4OH (66:34 by vol.) as solvent. Only two spots which showed the R_f values of dCTP and dUTP were found on the chromatogram. Furthermore the material extracted from the spot corresponding to dUTP had ultraviolet absorption spectra identical to those of authentic dUTP. When quantitative determination of dCTP and dUTP by paper chromatography was made, it was found that for each mole of dUTP formed an equivalent amount of dCTP was lost from the reaction mixture (Fig. 31). These observations indicated that the partially purified enzyme was free of phosphatases and other enzymes which degrade dUTP.

Inhibition of dCTP deaminase by dTTP

It has been shown that dCMP deaminase from various organisms (130,131) can be inhibited by dTTP in a non-competitive manner. Therefore the effect of various compounds on the activity of dCTP deaminase was examined. As shown in Table 23, dTTP inhibited markedly the dCTP deaminase activity. A slight inhibition was observed with dTDP and dTMP at rather high concentrations. Other compounds (dT, dUDP, dUMP and dU) had no effect on the enzyme activity.

The mode of inhibition by dTTP appeared to be non-competitive. The K_m and V_{max} were found to be 360 μM and 2.32 mμ moles/minute respectively (Fig. 32). The K_i was found to be 22 μM.

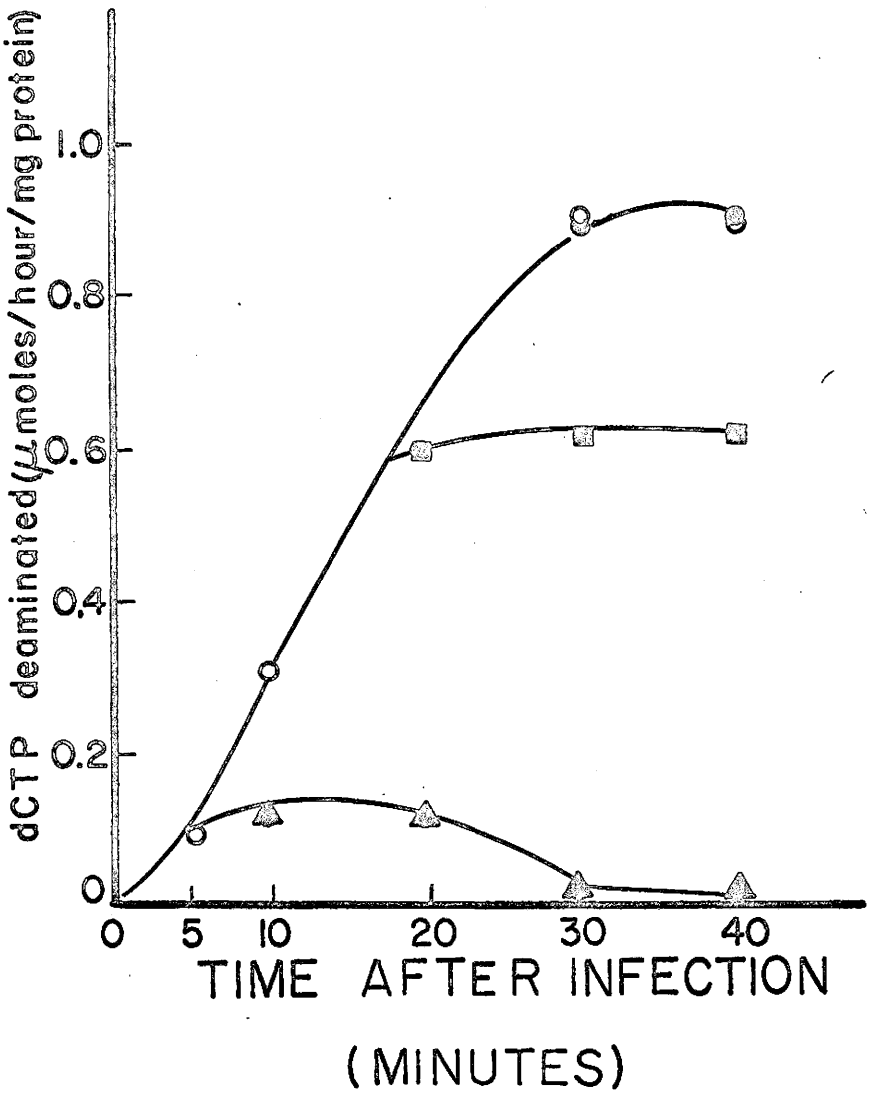

Fig. 28. Effect of chloramphenicol on the appearance of dCTP deaminase.

Fig. 28. Effect of chloramphenicol on the appearance of dCTP deaminase.

The assays of dCTP deaminase were made with preparations partially purified by passing through a column of Sephadex G-75. Extracts were prepared from infected cultures of B. subtilis at various time intervals after the following additions: chloramphenicol (100 µg/ml) added at 5 minutes (△—△), 15 minutes (□—□) and 25 minutes (◎—◎); no chloramphenicol added (○—○).

The initial velocity of enzymic reaction was proportional to the amount of enzyme added in the range of 0 to 3 mµ moles/min.

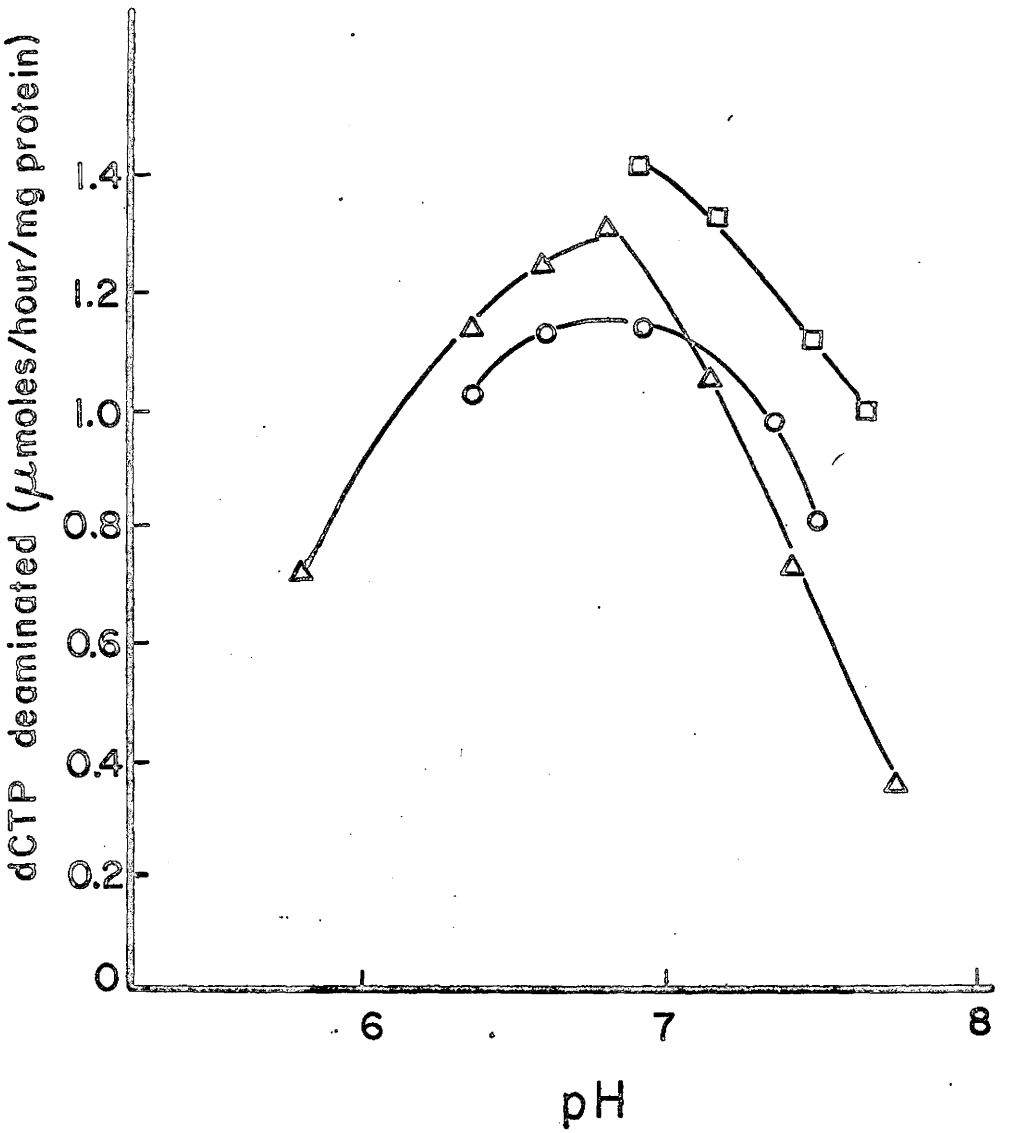

Fig. 29. Effect of pH on dCTP deaminase.

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Fig. 29. Effect of pH on dCTP deaminase.
Buffers examined were M/20 Tris-Cl (□—□), M/20 borate-phosphate (○—○) and M/15 potassium phosphate (△—△). The assays were made with an enzyme preparation partially purified by the DEAE-cellulose chromatography and concentrated by ammonium sulphate precipitation. Cells for the preparation of crude extracts were harvested 30 minutes after infection.

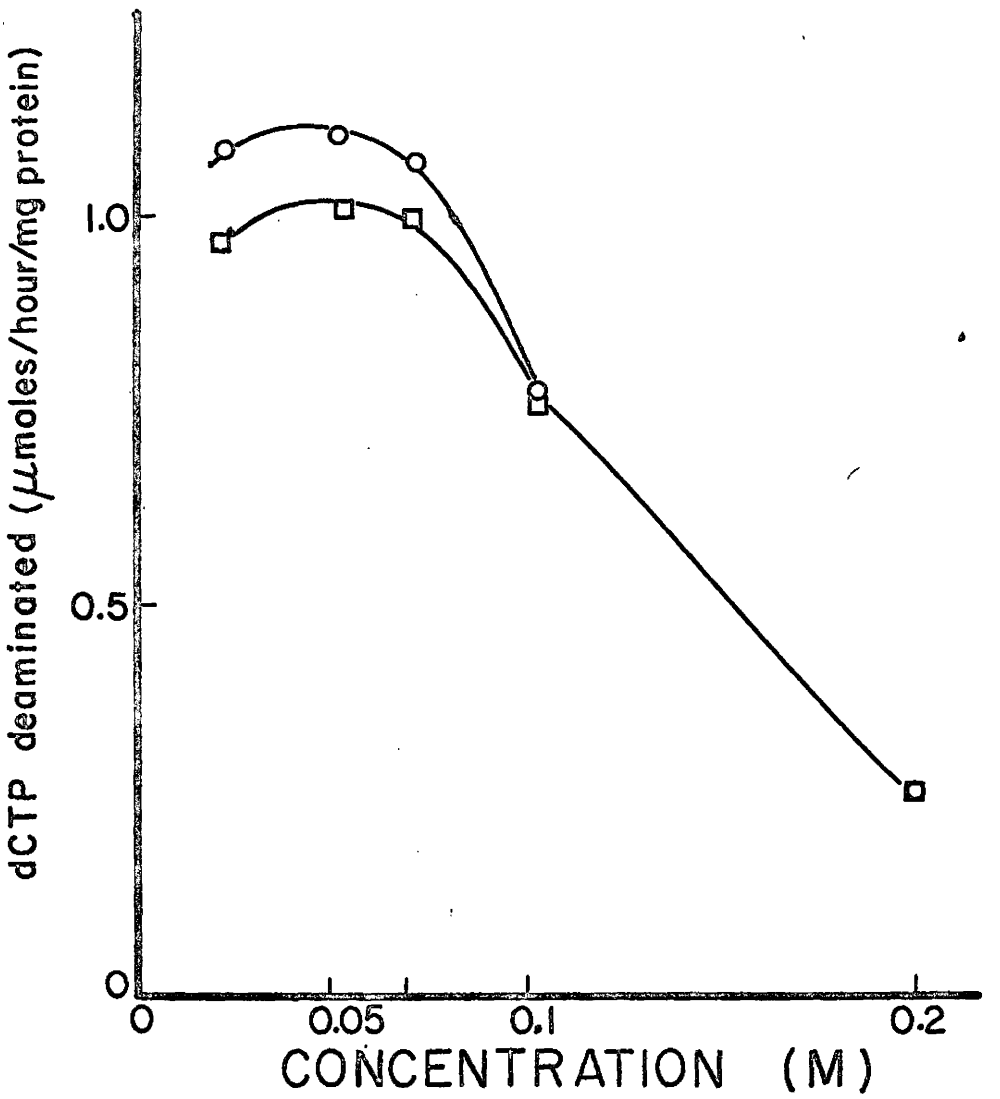

Fig. 30. Effect of ionic strength of buffer on the dCTP deaminase.

Buffers used were Tris-Cl (pH 7.20) (O—O) and potassium phosphate buffer (pH 6.80) (□—□). An enzyme preparation partially purified as in Fig. 29 was used.

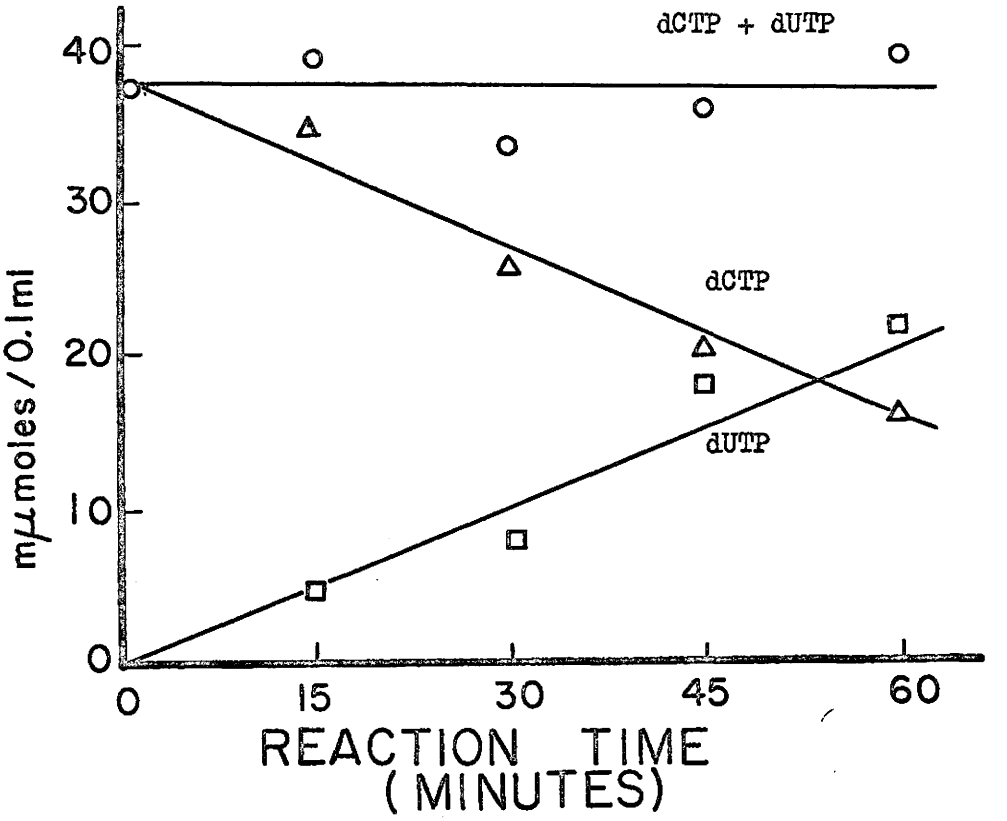

Fig. 31. Stoichiometry of dCTP deamination.

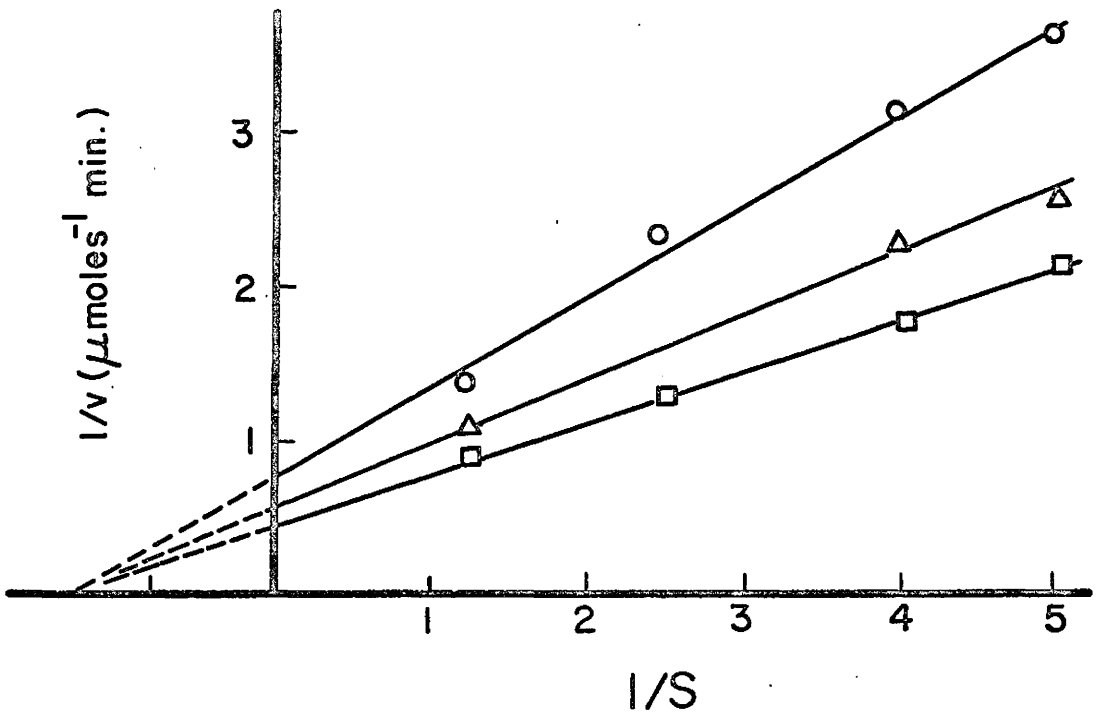

Fig. 32. Effect of dTTP on dCTP deaminase.

Fig. 31. Stoichiometry of dCTP deamination.

The initial concentration of dCTP was 380 μM. An enzyme preparation partially purified as in Fig.29 was used.

Fig. 32 Effect of dTTP on dCTP deaminase.

The assays of dCTP deaminase were made with a Gilford multi-sample absorbance recorder and an enzyme preparation partially purified as in Fig. 29. The concentration of dCTP varied from 360 μM to 90 μM. The concentrations of dTTP were 20 μM (O—O) and 10 μM (Δ—Δ); no dTTP added (□—□).

Table 20

Formation of deoxyuridine compounds by various enzymes in infected and uninfected cells

Enzyme	Specific activity (mμ moles/hour/mg protein)	
	Infected	Uninfected
dU kinase	0.31	0.31
dC deaminase	1.1	1.1
dCMP deaminase	0	0
UDP reductase	0.36	0.35

Infected cells for the preparation of crude extracts were harvested 30 minutes after infection.
The initial velocity of enzymic reaction was proportional to the amount of enzyme added: for UDP reductase 0 to 0.04 mμ moles/min, for dC deaminase 0 to 0.06 mμ moles/min, for dU kinase 0 to 0.06 mμ moles/min, and for dCMP deaminase 0.01 to 2 mμ moles/min (using PBS15-infected cell extracts for enzyme preparation).

Table 21

Deamination of dCTP by crude extracts

Expt. No.	Substrate (μM)		Amounts deaminated
	dCMP	dCTP	(mμ moles/hour/mg protein)
I	805	0	0
	805	122	20
	805	244	127
	805	488	140
II	0	488	127
	8	488	127
	80	330	118
	805	330	118

Cells for the preparation of crude extracts were harvested 30 minutes after infection.

Table 22

Effect of bivalent cations on dCTP deaminase

Cation	Concentration (mM)	dCTP deaminated (mμ moles/hour/mg protein)	Relative activity
None	-	690	48
$MgCl_2$	0.125	1130	78
	0.250	1300	90
	1.25	1130	78
	2.50	1130	78
	25.0	710	49
$MnCl_2$	0.025	1450	100
	0.25	1230	85
$CaCl_2$	0.125	1070	74
	1.25	810	56

An enzyme preparation partially purified as in Fig. 29 was used.

Table 23

Effect of nucleotides and nucleosides on dCTP deaminase

Compound tested	Concentration (μM)	dCTP deaminated (mμ moles/hour/mg protein)	Inhibition (%)
dTTP	44	0	100
	22	625	52
	13	860	26
	6	1140	12
dTDP	81	900	31
dTMP	80	860	26
	27	1250	4
dTT	55	1310	0
dUDP	55	1300	0
dUMP	55	1300	0
dU	55	1300	0

An enzyme preparation partially purified as in Fig. 29 was used.

CHAPTER V

DISCUSSION AND CONCLUSIONS

DNases in B. subtilis before and after infection with PBS 1

Results of the present study show that B. subtilis cells contain a DNase which hydrolyzes native PBS 1 DNA and that this enzyme activity disappears completely 20 minutes after infection by PBS 1. The disappearance of the enzyme activity seems to be due to the production of an inhibitor which interacts specifically with the DNase. Other DNases found in B. subtilis are not affected by the inhibitor. The inhibitor may be at least partly responsible for the survival of the PBS 1 genome in cells or spores in the carrier state.

DNases active on B. subtilis DNA in PBS 1-infected cells have also been investigated. A considerable amount of DNase activity on heat-denatured B. subtilis DNA is detectable in crude extracts of both uninfected and infected cells, but there is no difference in the activity between the two types of cells. Only a small amount of activity on native B. subtilis DNA is found in both infected and uninfected cells. It has been shown that RNA and a heat sensitive protein are inhibitors for DNases in some instances (113,126). These possibilities were tested by adding RNase to the reaction mixture (100 µg/ml) or by heating the enzyme preparation at 55°C for 10 minutes. These treatments do not change the level of DNase activity on native host DNA in both infected and uninfected

cells.

Degradation of host DNA during the multiplication of PBS 1 was also investigated by infecting cells which were previously labelled with H^3-dT. Almost no radioactivity is released into the acid-soluble fraction throughout PBS 1 multiplication, indicating that the host DNA is not degraded into small polynucleotides. In another experiment, it is found that DNA extracted from infected B. subtilis has a specific transforming activity which is comparable with that of DNA from uninfected cells, although crude extracts of infected cells can reduce considerably the specific transforming activity of the DNA in vitro. These observations suggest that the host DNA is protected in the cells and is not degraded extensively during the multiplication of PBS 1.

The above results will account for the presence of relatively large fragments of host DNA in transducing particles of PBS 1. Yamagishi and Takahashi (117) estimated that the total molecular weight of DNA in the transducing particles would be 2×10^8 daltons.

It has been reported by Bott and Strauss (14) that the general level of DNase increases markedly in cells infected with phage SP10 which is also able to create the carrier state in B. subtilis. Although the increase in DNase activity is detectable as early as 10 minutes after infection, there is no decrease in the frequencies of joint transformation of linked markers in the host DNA extracted at later stages. Therefore it appears that this enzyme is not involved in the destruction of host DNA. Other DNases in B. subtilis infected with SP10 have not been investigated.

Properties of DNases in B. subtilis

According to Laskowski (132,133), DNases may be divided into two classes: exonucleases which catalyze successive removal of mononucleotides from the end of a DNA molecule, and endonucleases which catalyze hydrolysis of phosphodiester bonds randomly within a DNA molecule. Depending on the site of hydrolysis, the digestion products would have 3'-hydroxyl ends or 5'-hydroxyl ends. It has been shown that some DNases attack heat-denatured DNA faster than native DNA or vice versa. It has been found that an exonuclease can attack oligonucleotides but not DNA of higher molecular weight (46). Some endonucleases can hydrolyze both strands of native DNA simultaneously at the same locus (134). Some DNases are specific for UV-irradiated DNA which contains pyrimidine dimers (29,135) or methylated DNA obtained by the methyl methanesulphonate treatment (28).

Recently an endonuclease specific for DNA lacking a host-controlled modification which confers immunity to the host specific restriction mechanism has been found by Meselson and Yuan (136). Since the modification of a DNA molecule seems to be determined by its pattern of methylation (137, 138), Meselson and Yuan suggest that this endonuclease may recognize a specific pattern of methylation in a DNA molecule . However, no DNases are known to have specificity toward purine or pyrimidine bases in DNA.

As mentioned earlier, a DNase present in uninfected B. subtilis has a specific affinity toward PBS 1 DNA. The DNA from PBS 15 which contains hydroxymethyluracil in place of thymine is degraded only to a small extent and DNA's from calf thymus, B. subtilis, phages T4 and T5 are completely resistant to this DNase.

It has been shown that PBS 1 DNA contains four single-strand breaks in the molecule (117). It is possible that the enzyme may initiate hydrolysis at the single-strand breaks. However, an introduction of artificial single-strand breaks by the treatment with ascorbate or with a small amount of pancreatic DNase does not make B. subtilis DNA susceptible to the DNase. Thus the possibility of single-strand breaks as a primary site for hydrolysis may be ruled out.

After an exhaustive digestion of native PBS 1 DNA with the purified enzyme, the digestion products are found to be dU, mononucleotides (dAMP and dGMP), oligonucleotides (di- to penta-) consisting only of dAMP and dGMP, and oligonucleotides larger than hexanucleotides containing dCMP, dAMP and dGMP. No uridine nucleotides are found.

It appears that native PBS 1 DNA is hydrolyzed first at dUMP in double stranded DNA molecules. The observation that thymus DNA in which cytosine is converted to uracil by nitrous acid becomes susceptible to this enzyme suggests that the sensitivity toward the enzyme is determined by the presence of uracil in the molecule. The release of dU by the initial reaction may be followed by strand separation of small DNA fragments, for both dAMP and dGMP are present in the digestion products (Table 14). Presumably these mononucleotides are released by the DNase active on heat-denatured DNA still present in the purified preparation.

When heat-denatured PBS 1 DNA is used as substrate, digestion products are identical to those of native PBS 1 DNA. Digestion products of heat-denatured B. subtilis DNA are quite different from those of native or heat-denatured PBS 1 DNA. Nucleosides are absent in digestion products.

Mononucleotides and oligonucleotides smaller than hexanucleotides contain only purine nucleotides, showing that purines are removed preferentially from the DNA. It is probable that heat-denatured PBS 1 DNA is hydrolyzed at purine nucleotides and dU is removed as in the case of native PBS 1 DNA. Since cytosine is absent in the oligonucleotide fractions of less than 5 nucleotide-chain length, it seems that cytosine runs of less than 5 nucleotides are infrequent in PBS 1 DNA.

The most purified DNase still contains the activity toward heat-denatured DNA. At present it is not known whether this activity is due to a contaminating enzyme. Differential inactivation by heat and the presence of a specific inhibitor for the DNase active on native PBS 1 DNA suggest that it may be a contaminating enzyme.

The properties of DNases active on host DNA were not investigated extensively in the present study. Our DNase active on host DNA appears to be different from the enzyme found by Okazaki and Kornberg (24). The enzyme found by these authors is an exonuclease degrading DNA from 3'-hydroxyl ends, and it exhibits also DNA-phosphatase activity as in the case of exonuclease III of E coli (26). Since the DNase found in this study is inhibited by RNA, this enzyme may be similar to endonuclease I of E. coli (113). A DNase active only on heat-denatured DNA found in the present study is similar to the enzyme reported by Birnboim (27) in regard to the substrate specificity and cation requirement. Our enzyme, however, has an optimal pH of 7.0, while that of Birnboim shows the maximal enzyme activity at 9.5.

dUTP synthesis in B. subtilis infected with PBS 1

Enzymes which convert uridine nucleotides to corresponding deoxyribonucleotides have been found in bacteria (92-94). Studies on DNA polymerase of E. coli show that dUTP can be incorporated into DNA in vitro (139). However, no DNA of bacterial origin which contains uracil in place of thymine has been found so far. It has been suggested that dUTPase in E. coli may prevent the incorporation of dUTP into DNA by dephosphorylating the triphosphate (92,140). It is also probable that the inability of dUMP to serve as a precursor of DNA results from the apparent absence of a phosphotransferase which converts dUMP to dUTP (139-141).

Reductases for ribonucleotides are also present in animal cells. It has been found that a heat stable extract from rat liver converts dUMP to dUDP (142) and unheated extracts from normal and regenerating rat liver efficiently phosphorylate dUDP to form dUTP (143). In these cases the presence of an active dUTPase and inhibition by UMP and CMP in the phosphorylating system appear to be responsible for preventing dUTP from being incorporated into DNA (143). However, in some systems such as sea urchin eggs, dUTP may be actually incorporated into DNA and then methylated by transmethylation with methionine (144), although the amount of thymine thus formed in the DNA may be very small.

Since PBS 1 DNA contains uracil in place of thymine, infected cells should contain enzyme(s) which synthesize dUTP and this triphosphate must be protected from the action of phosphatases. The present study with PBS 1-infected B. subtilis reveals the presence of a dCTP deaminase which specifically reacts with dCTP to form dUTP. The addition of chloramphenicol

into PBS 1-infected cultures prevents further increase in the deaminase activity. Furthermore the enzyme activity was completely absent in uninfected cells. Thus it appears that the formation of dCTP deaminase is induced by PBS 1 infection.

According to Kahan (91), dUTP may be formed by the phosphorylation of dUMP in PBS 2-infected cells. In crude extracts, the specific activity of dUMP kinase has been reported to be 15 mμ moles/hour/mg protein (91). On the other hand, dCTP deaminase found in the present study shows a specific activity of 127 mμ moles/hour/mg protein in crude extracts. These results apparently suggest that the deamination of dCTP is a major pathway for the formation of dUTP in PBS 1-infected cells. The real evaluation of dCTP deaminase in the dUTP synthesis, however, should be made by comparing the following two pathways: UDP $\longrightarrow$ dUDP $\longrightarrow$ dUTP and CDP $\longrightarrow$ dCDP $\longrightarrow$ dCTP $\longrightarrow$ dUTP in PBS 1-infected B. subtilis cells.

Since in PBS 1 DNA thymine is completely substituted by uracil and since dCTP deaminase is strongly inhibited by dTTP, dTTP must be eliminated from the system in order to maintain the production of PBS 1 DNA. Various mechanisms for the elimination of dTTP have been suggested for other cases in which the phage DNA lacks thymine. Cells infected with SP8 contain dTTPase (85), SP5C-infected cells contain dTMPase (86) and dTMP synthetase is inhibited in ϕe-infected cells (87). It has been reported that thymine deprivation derepressed CDP reductase (145), resulting in the accumulation of dCTP in the intracellular pool of deoxyribonucleotides (146). Since dTTP must be eliminated from PBS 1-infected cells, these cells will probably have a lower concentration of thymidylates than that in uninfected

cells. This situation may cause an accumulation of dCTP which subsequently is converted to dUTP by the deaminase.

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