# STUDIES ON HEAT-INDUCED DNA INJURY AND

# ITS REPAIR IN *BACILLUS SUBTILIS*

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CONTENTS

Chapter I


INTRODUCTION


   Bacterial spores are extremely resistant to various steriliza-

tion treatments, such as heating (1-3), ultraviolet irradiation (4),

γ-ray irradiation (5), and some chemical agents (6-8), as compared

with vegetative cells of the same strain and other microorganisms.

Therefore, the bacterial spores must be sterilized very carefully in

the processing and storage of foods.

   Of various sterilization agents γ- and UV-irradiation are effec-

tive in some limited cases, but they cannot be used generally for

many reasons.   For example, the transmission of UV is very small,

and it has not yet been established completely that γ-irradiated food

is wholesome.   Some chemical agents were widely used in preservation

of foods before, but they are used in limited cases, because it has

been pointed out that some chemical agents which had been used before

were toxic or carcinogenic.

   Then, the heat treatment which has been widely used in process-

ing and storage of food from old times is now being watched with in-

terest again.   Thus the heating is thought to be a very important

sterilization method in the food preservation.   However, the molec-

ular mechanisms of heat sterilization of bacteria, especially that of

bacterial spores have scarcely been studied.

Many investigators have studied the survival rates and D values of spores of bacteria belonging to genera *Bacillus* and *Clostridium* heated at different temperatures, under different heating conditions since old time (3, 9-14).  Recently, studies concerning the effects of mild heating on cellular components, such as membrane, enzyme proteins, rRNA and DNA, of vegetative cells of bacteria have been performed (15-30).  In spores, however, the damages caused by heat treatment have scarcely been studied except in the case of enzyme proteins (31-33).  As to DNA molecules in bacterial spores, there are few studies concerning the DNA injury and its repair mechanisms, because of the difficulty of mild extraction of DNA from spores.  It has been known that DNA molecule is the most important target in $\gamma$- and UV-irradiation (34-36).  But the action site of heat in bacterial cells has not yet been made clear.

In purified DNA, Tamm et al. (37) found for the first time that acid treatment of DNA protonated the N-glycosidic bonds of purines resulting in hydrolysis of purines from the pentose sugar without breaking the phosphodiester backbone directly (Fig. 1).  Furthermore, Greer and Zamenhof (38) found that purine bases are also slowly re-leased from DNA during incubation at neutral pH and high temperature. Several investigators (39, 40) reported that mutations were induced in spores of *B. subtilis* by heating at high temperatures.  Alteration of requirements for germination in heat-injured spores of *B. stearo-thermophilus* was also reported (41).  Therefore, most of the early
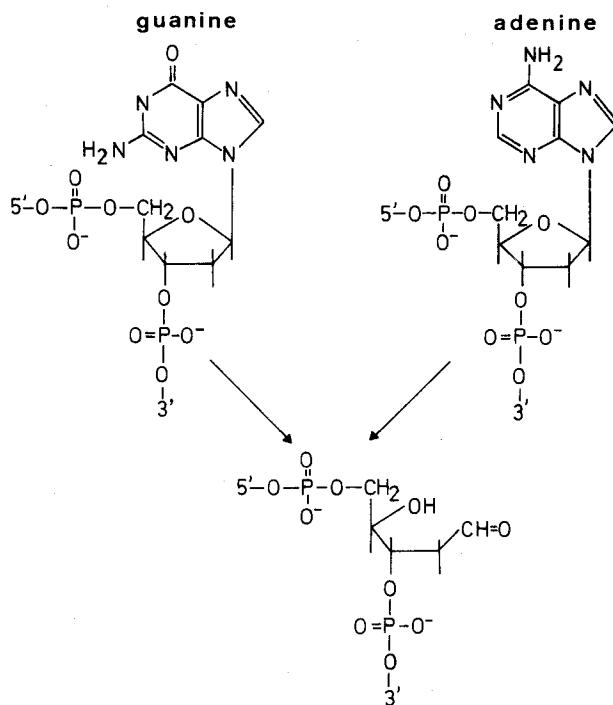
Fig. 1.  Scheme for depurination by heating.

studies attributed heat mutagenesis to depurination (38,39,42,43).

On the other hand, Drake and co-workers (44-46) showed that GC - AT

transition and GC - CG transversion were induced by heat and the

depurination was probably little or not mutagenic in bacteriophage

T4.   In this case, however, the phage was heated at relatively low

temperature and for long time.   Recently, it has been shown that DNA

polymerase can copy past apurinic sites on natural, biologically

active DNA and further, that this leads to increased mutagenesis (47,

48).   At high temperatures as used for sterilization of foods or

near the Tm of DNA, the heating was reported to produce apurinic

sites in *in vitro* DNA (38, 49).   Therefore, it is expected that heat

treatment at high temperatures induces damages of DNA molecules in spores, such as depurination or depyrimidination, and the demaged DNA molecules in spores are repaired during germination of spores under appropriate conditions.

Recently, it has been found that many alkylating and carcinogenic agents modify the bases of DNA and the modified bases are depurinated or depyrimidinated by specific DNA glycosylases (50-52). Accordingly, as a repairing enzyme, endonuclease specific to apurinic sites in DNA (APendodeoxyribonuclease) was paid attention to and extensively investigated. APendodeoxyribonucleases have been isolated from *Escherichia coli* (53-55), *Hemophilus influenzae* (56), *Micrococcus luteus* (57, 58), *Bacillus subtilis* (59), *Bacillus stearothermophilus* (60), higher plant (61), and animals (62-64). These endonucleases have been studied in relation to the DNA injuries induced by alkylating agents, X-ray irradiation, ultraviolet irradiation, or carcinogenic substances, and their repair systems.

In order to make clear the repair system in heat-injured DNA in spores, which perhaps involves depurination reaction, it is necessary to purify and characterize the deoxyribonuclease specific to heat-injured DNA.

The present study was attempted to make clear the following questions ; [1] whether or not mutations are caused by heat treatment in *B. subtilis* spores, [2] whether or not DNA injury of *B. subtilis* can be detected by sedimentation analysis and DNA damage is due to depuri-

nation, [3] whether or not heat induced DNA injury of spores is dif-

ferent from that of vegetative cells, [4] whether or not injured DNA

is repaired during incubation after heating, [5] whether or not the

repair enzyme specific to the heat-damaged DNA is present in *B. sub-*

*tilis*.

Cnapter II


APPEARANCE OF MUTANTS BY HEAT TREATMENT OF *BACILLUS SUBTILIS* SPORES


1.    INTRODUCTION


The bacterial spores are completely inactivated by heat treatment at very high temperature, but they may only be partially inactivated when they were treated with heat under not so drastic conditions. Recent reports have indicated that the heat treatments of *Clostridium* and *Bacillus* spores may result in (i) dead spores, (ii) injured survivors, (iii) uninjured survivors (63-72).   Heat treatment may damage either enzyme systems in the spores or specific sites on the chromosome which are essential for germination and outgrowth.   Under suitable conditions, the injured spores may be recovered and behave as uninjured spores.

In this chapter, the effect of addition of amino acids to the germination media on the recovery of the heat-injured spores of *Bacillus subtilis* was investigated.


2.    MATERIALS AND METHODS


Organism

*Bacillus subtilis* ATCC 6051 was used throughout this work.

## Preparation of spores

B. *subtilis* was grown at 37°C in the nutrient broth containing

0.5 % peptone (Polypeptone, Daigo) and 0.5 % beef extracts (Kyokuto),

supplemented with potato extract.   Spores were harvested after 48 h

incubation and then purified by use of the two-phase Y system which

was reported by Sacks and Alderton (73).   The purified spores were

thoroughly washed with distilled water and lyophilized, and then

stored in a desicator at -20°C.

## Heat treatment and counting of the survivors

Spores suspended in distilled water (0.15 mg of dry spores/ml)

were heated at 80, 90, or 100°C in water baths.   After being heated

for desired periods the spore suspensions were immediately cooled in

ice-water.

Schaeffer's agar medium (74) and Demain's minimal agar medium

(75) were used for counting the spores which survived the heat treat-

ment.   Schaeffer's agar medium was prepared to contain 8 g nutrient

broth, 1 g KCl, 0.25 g $MgSO_4 \cdot 7H_2O$, 1.25 mg $MnCl_2$, $10^{-3}$ M $Ca(NO_3)_2 \cdot$

$4H_2O$, $10^{-6}$ M $FeSO_4 \cdot 7H_2O$, 15 g agar in 1000 ml. Demain's minimal agar

medium was prepared to contain 1 g glucose, 890 mg L-alanine, 1470 mg

L-glutamic acid, 1320 mg L-asparagine, 3 g $K_2HPO_4$, 1 g $KH_2PO_4$, 500 mg

$NH_4Cl$, 100 mg $NH_4NO_3$, 100 mg $Na_2SO_4$, 10 mg $MgSO_4 \cdot 7H_2O$, 1 mg $MnSO_4 \cdot 4$

$H_2O$, 1 mg $FeSO_4 \cdot 7H_2O$, 0.5 mg $CaCl_2$, 15 g agar in 1000 ml.

The spores not being heated germinated and grew well in the

Demain's minimal agar medium.   Amino acid to be tested was supple-

mented to the Demain's minimal agar medium in the concentration of 10 mM. The number of colonies grown on the agar plates was counted after the incubation at 37°C for 48 h.


3. RESULTS


Injury of spores by heating and their recovery

The heat survival curves of *B. subtilis* spores were made by use of Schaeffer's agar medium and Demain's minimal agar medium after being heated at 80, 90, and 100°C for different periods (Fig. 2). Logarithms of the number of survivors were plotted against the heating time. Viale counts of unheated controls were the same on Schaeffer's or Demain's medium. The lower the heating temperature, the more the spores were viable in both agar media. When the spores were heated at 80°C, the survival fraction after 20 min was almost 100 % on Schaeffer's medium but was only approximately 8 % on Demain's medium. In the case of heating at 90°C for 20 min, about $10^{-2}$ of the total spores grew on Schaeffer's medium whereas $10^{-6}$ grew on Demain's medium. At 100°C numbers of survivors on Schaeffer's medium and Demain's medium were similar. These results indicated that some of the spores injured at temperature below 90°C were able to recover when suitable cultural conditions prevailed ; however, spores heated at 100°C showed no recovery at all.

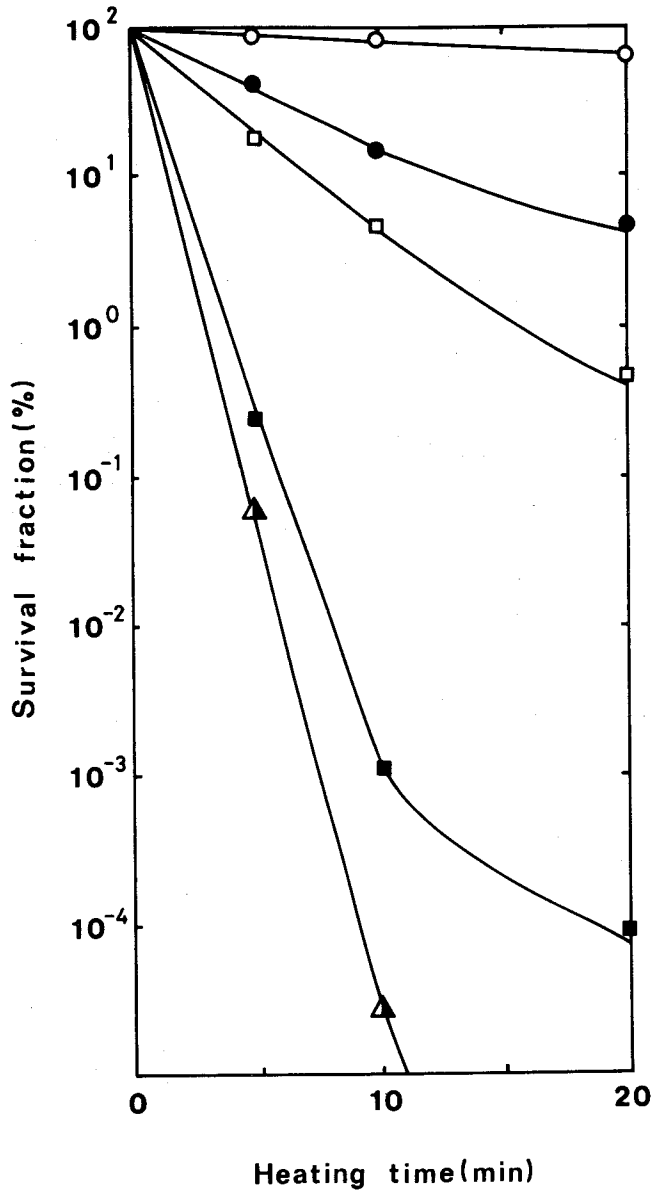Fig. 2. Heat-survival curves of *B. subtilis* spores on Schaeffer's agar and Demain's minimal agar medium.
Symbols : O—O, heated at 80°C and plated on Schaeffer's agar ; ●—●, heated at 80°C and plated on Demain's agar ; □—□, heated at 90°C and plated on Schaeffer's agar : ■—■, heated at 90°C and plated on Demain's agar : △—△, heated at 100°C and plated on Schaeffer's agar ; ▲—▲, heated at 100°C and plated on Demain's agar.
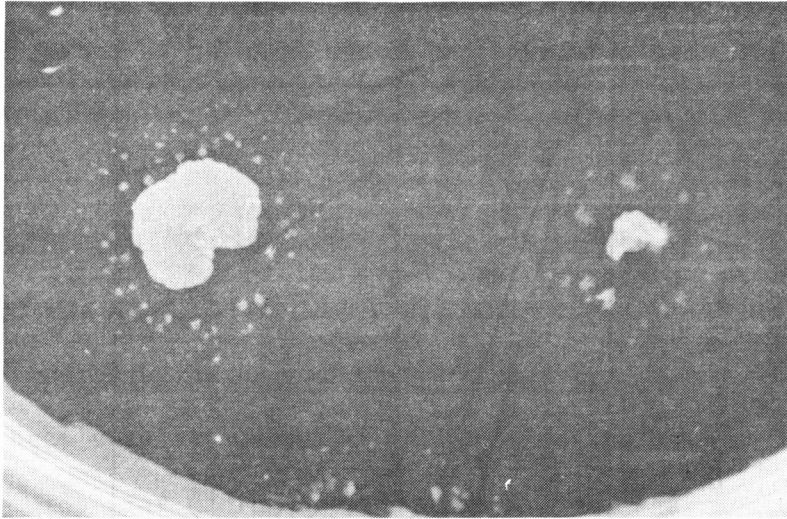
Fig. 3.   Colonies formed from heated (90°C, 10 min) spores of *B.*
*subtilis* on Demain's minimal agar plate (incubated at 37°C for 4 days).

In the case of spores heated at 90°C for 10 min, small satellite
colonies often appeared on Demain's agar medium around a big colony
which probably originated from an uninjured spore (Fig. 3).   This
fact suggests that the big colony excreted some substances which
repaired the heat-injured spores.

Effect of amino acid supplementation on recovery

The principal difference in composition between Schaeffer's me-
dium and Demain's medium is that the former contains most amino acids
but the latter contains only alanine, glutamic acid, and asparagine.
To analyze the effect of Schaeffer's medium on recovery of heat-in-
jured spores, the survivors of the heat-injured spores were counted
by use of the Demain's minimal agar medium supplemented by various

groups of amino acids (Table 1).  As shown in Table 1, the supplement of casamino acids or some groups of amino acids to Demain's minimal agar medium resulted in the significant restoration of the heat-injured spores which were unable to germinate and to grow without the supplement.  Especially, some amino acids contained in groups I and VI were effective.  The effect of individual amino acids on recovery was further examined to determine which amino acid was responsible for recovery of the heat-injured spores.  The results obtained (Table 2) indicated that only glycine and threonine were effective in the recovery of the heat-injured spores.  None of the

Table 1.   Effect of addition of amino acid mixtures to counting media on the number of *B. subtilis* spores surviving after being heated at 90°C for 10 min.

| Medium | Non. of survivors |
|---|---|
| Schaeffer's medium | $1.4 \times 10^6$ |
| Demain's minimal medium (DMM) | $1.3 \times 10^3$ |
| DMM + Casamino acids | $1.4 \times 10^6$ |
| DMM + group I | $8.6 \times 10^5$ |
| DMM + group II | $1.5 \times 10^3$ |
| DMM + group III | $1.0 \times 10^2$ |
| DMM + group IV | $1.1 \times 10^3$ |
| DMM + group V | $2.3 \times 10^3$ |
| DMM + group VI | $1.3 \times 10^6$ |

Group I    : glycine, L-phenylalanine, L-methionine.
Group II   : L-valine, L-leucine, L-tryptophan.
Group III : L-lysine, L-serine, L-cysteine.
Group IV  : L-isoleucine, L-proline, L-tyrosine.
Group V    : L-histidine, L-arginine, L-glutamic acid.
Group VI  : L-aspartic acid, L-threonine.
Each amino acid was added to the Demain's minimal agar medium so as to be 10 mM at the final concentration.

other amino acids tested was effective in this respect.    However, homoserine, which is an intermediate substance in the threonine bio-synthesis pathway, had the same effect as glycine or threonine (data were not shown).    These results suggest that some sites in amino acid metabolism, probably those involved in the metabolism between glycine and serine and/or that between aspartic acid and homoserine, were injured by heating at 80 or 90°C.

Appearance of glycine auxotrophs after heat treatment

In Fig. 4, logarithms of the numbers of survivors and spores which required glycine for germination and outgrowth were plotted against the heating temperature after heating for 10 min.    The sur-

Table 2.    Effect of addition of individual amion acids to counting media on the number of B. *subtilis* spores surviving after being heated at 90°C for 10 min.

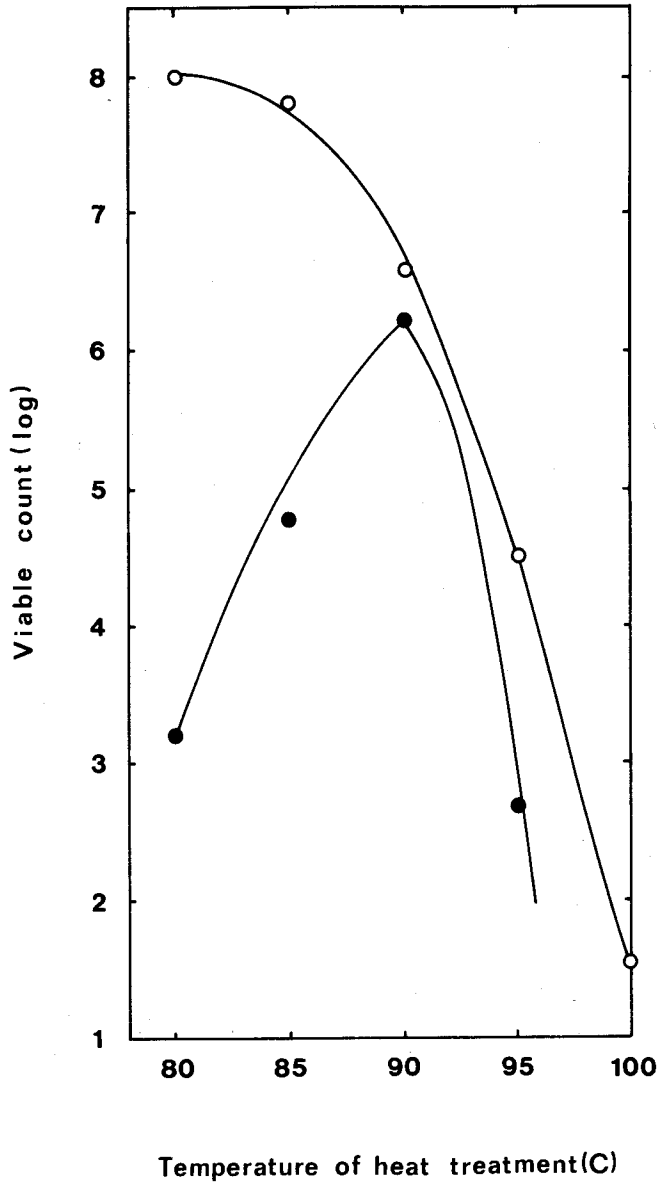| Medium | No. of survivors |
|---|---|
| Schaeffer's medium | $1.4 \times 10^6$ |
| Demain's minimal medium (DMM) | $1.3 \times 10^3$ |
| DMM + glycine | $1.3 \times 10^6$ |
| DMM + L-phenylalanine | $1.4 \times 10^3$ |
| DMM + L-methionine | $1.2 \times 10^3$ |
| DMM + L-aspartic acid | $1.2 \times 10^3$ |
| DMM + L-threonine | $1.5 \times 10^6$ |

Fig. 4.   Development of glycine-requiring mutants of *B. subtilis* by heat treatment at various temperatures.
Symbols : O——O, survivors ; ●——●, glycine-requiring mutants.
The heat treatment was for 10 min.

vival fractions decreased exponentially with the increase in temperature. At 90°C a large percentage of the spores became glycine requiring, and the morphologies of colonies of the glycine requiring cells were different from those of the other cells. Colonies of the heated spores, most of which were glycine requiring, grown on Demain's minimal agar medium supplemented with glycine were very small and amorphous.

The glycine auxotrophy of the cells was maintained after more than five transfers to new agar plates, which indicates that the amino acids requirements of spores induced by the heat treatment were genetically inherited.


4. DISCUSSION


The spores of *Bacillus subtilis* which were injured by heat treatment at 90°C for 10 min were not able to germinate in Demain's minimal agar medium. However, the injured spores underwent recovery and germinated when the medium was supplemented with glycine, threonine or homoserine. On the other hand, when the spores were heated at 100°C, the survival curve obtained with Schaeffer's agar medium was almost the same as that obtained with Demain's minimal agar medium, namely the spores heated at 100°C could not restore the viability at all.

Edwards et al. (76) reported that dipicolinate and $CaCl_2$ were effective in the recovery of heat-injured spores of *B. subtilis*.

Campbell et al. (41) found that glutamic acid and lysine were effective in enhancing the recovery of heated spores of B. *stearothermophilus*. Yokoya and York (77), and Cook and Brown (78) also reported that a high D value was obtained with B. *coagulans* and B. *stearothermophilus*, respectively, when 1 % starch was added to the counting medium.

Zamenhof (39) also found that spores which survived heat treatment formed small colonies on plates. Chiasson and Zamenhof (40), and Northrop and Slepecky(79) reported that, when spores of B. *subtilis* were heated (80°C to 100°C) and then plated, numerous mutants were obtained and these mutants exhibited abnormal sporulation. They proposed that these mutations were due to depurination. Our results were consistent with their hypothesis, since we observed that the spores were inactivated exponentially with an increase in dosage of heat and that most of the spores which survived the heat treatment at 90°C became auxotrophic.

Since the amino acid requirements of spores induced by the heat treatment are genetically inherited, it is suggested that the heat treatment is accompanied by modification of a specific site on the spore chromosome.

## 5. SUMMARY

The spores of *B. subtilis* which had been injured by heat treatment at 90°C for 10 min could not form colonies on Demain's minimal agar medium.  The injured spores, however, recovered and grew when the medium was supplemented with glycine, threonine, or homoserine.  The amino acids requirements of spores induced by the heat treatment were genetically inherited.  It is suggested that the heat treatment is accompanied by the modification of a specified site on chromosome in the spores.

Chapter III


DNA INJURY BY HEAT TREATMENT IN *BACILLUS SUBTILIS*


Section 1.    DEATH AND DNA INJURY OF SPORES BY HEAT TREATMENT


1.    INTRODUCTION


The heat resistance of bacterial spores depends on the inherent

genetic factors (80-82), growth temperature (83), buffer solution in

which spores are suspended (84-86), concentration of spores (87),

heating condition (88-89), and plating medium (69,90) etc.   When the

spores are harvested and plated after heating under the same condi-

tions, the heat resistance of spores is mainly determined by the pH

values of spore suspension exposed to heat.

Xezones and Hutchings (91) studied the heat resistance of

*Clostridium botulinum* spores and found that the decimal-reduction time

(D value) of the spores increased with an increase in pH and the in-

fluence of pH was pronounced at lower heating temperature.   Löwik

and Anema (92) found that when the pH was decreased from 6.0 to 4.8

the D value was decreased by 40 %, irrespective of the heating temper-

ature, and z value was independent of pH.   On the other hand, Cameron

et al. (93) recently reported that increasing acidity was, in general,

accompanied by decreasing heat resistance of *Clostridium sporogenes*

spores, although pH effect was more pronounced at the higher process-
ing temperature.   The effects of low pH on the thermal resistance
of spores were studied by several investigators in relation to the
sterilization of foods (94-96).

In recent years, the biochemical nature of the lesions caused by
heating of bacteria under sublethal and lethal temperature conditions
has attracted attention of investigators.   Although the heat-induced
injuries in bacterial spores have been reported (36, 97-100), most of
those studies have dealt with membrane, protein, and RNA.   And con-
cerning the DNA injury in heated spores few studies have been made.

Then, in this chapter, the author studied the effect of acid-
heating on the survival of *B. subtilis* spores and the heat-injury of
DNA in the spores.


2.   MATERIALS AND METHODS


Organism

*B. subtilis* 168Tt (thy⁻, trp⁻) was used throughout this work.

Preparation of nonlabeled and $^3$H-labeled spores

*B. subtilis* was grown at 37°C in Schaeffer's medium (74) and
spores were harvested after 48 h incubation.   The spores were puri-
fied by treatment with 500 µg of lysozyme per ml at 37°C for 40 min
and 1 % sodium dodecyl sulfate at 37°C for 20 min.   The spores were
washed several times with distilled water and stored at 0°C.   Spores

labeled with $^3$H were harvested after 48 h incubation in Schaeffer's

medium supplemented with 250 μCi of [methyl-$^3$H] thymidine (58 Ci/mmol

: New England Nuclear) per 100 ml and were purified by the same method

used in nonlabeled spores.

Heat treatment and counting of the survivals

　　　Spores suspended in distilled water were centrifuged and the

pellet was suspended again in M/20 sodium citrate-HCl buffer (pH3, 5,

7).　Spore suspension in buffer was heated at 90, 80, 70, or 60°C in

water bath and was immediately cooled in ice bath.　The nutrient

agar medium containing 0.5 % peptone, 0.5 % beef extracts and 1.5 %

agar was used for the enumeration of the spores which survived the

heat treatment.　Colonies were counted after incubation at 37°C for

24 h.

Extraction of DNA from unheated and heated spores

　　　Unheated or heated $^3$H labeled spores were treated with 2 % sodium

dodecyl sulfate in 0.5 N NaOH at 37°C for 1 h and washed two times

with distilled water.　Then spores were treated with 1 mg/ml lysozyme

in 0.01 M Tris-HCl (pH 8.0), 0.01 M EDTA-4Na, 0.2 % Brij-58 and 10 %

sucrose at 37°C for 30 min and centrifuged.　The pellet was resus-

pended and solubilized slowly in 2 % sodium dodecyl sulfate in 0.1 N

NaOH at 37°C for 30 min and then DNA was extracted.

Sedimentation analysis of $^3$H labeled DNA in alkaline sucrose density

gradient

　　　Extracted DNA was layered on linear 5 - 20 % alkaline sucrose

density gradient (pH 12.0) and centrifuged at 40.000 rpm for 2 h at 20°C in a Beckman SW. 50.1 rotor.   The contents were collected from the top in fractions of 0.15 ml on the disks of filter paper with ISCO density gradient fractionator.   The disks were dried and radio-activity was counted in toluene system.


3.   RESULTS


Heat resistance of spores to acid-heat treatment

The thermal death curves resulting from acid-heat treatment of *B. subtilis* spores at 90, 80, 70, 60°C were given in Fig. 5. Logarithmus of the number of survivors after heat treatment in the citrate buffer adjusted to pH 7.0, 5.0, and 3.0 were plotted against heating time.

When the spores were heated at 90°C, the survival fractions after 5 min heating at pH 7.0, 5.0, and 3.0 were about 60, 2, and 0.004 % respectively.   In the case of heating at 80°C, the survivals after 20 min heating at pH 7.0, 5.0, and 3.0 were about 60, 8.3, and 0.008 % respectively.   At 70°C for 20 min, the survivals after heating at pH 7.0, 5.0, and 3.0 were about 90, 60, and 4.6 % respectively.   At 60°C for 20 min, the survivals after heating at pH 7.0, 5.0, and 3.0 were about 95, 72, and 50 % respectively.

These results indicated that at the same temperature spores were more resistant at neutral pH than at lower pH.   Consequently, at the
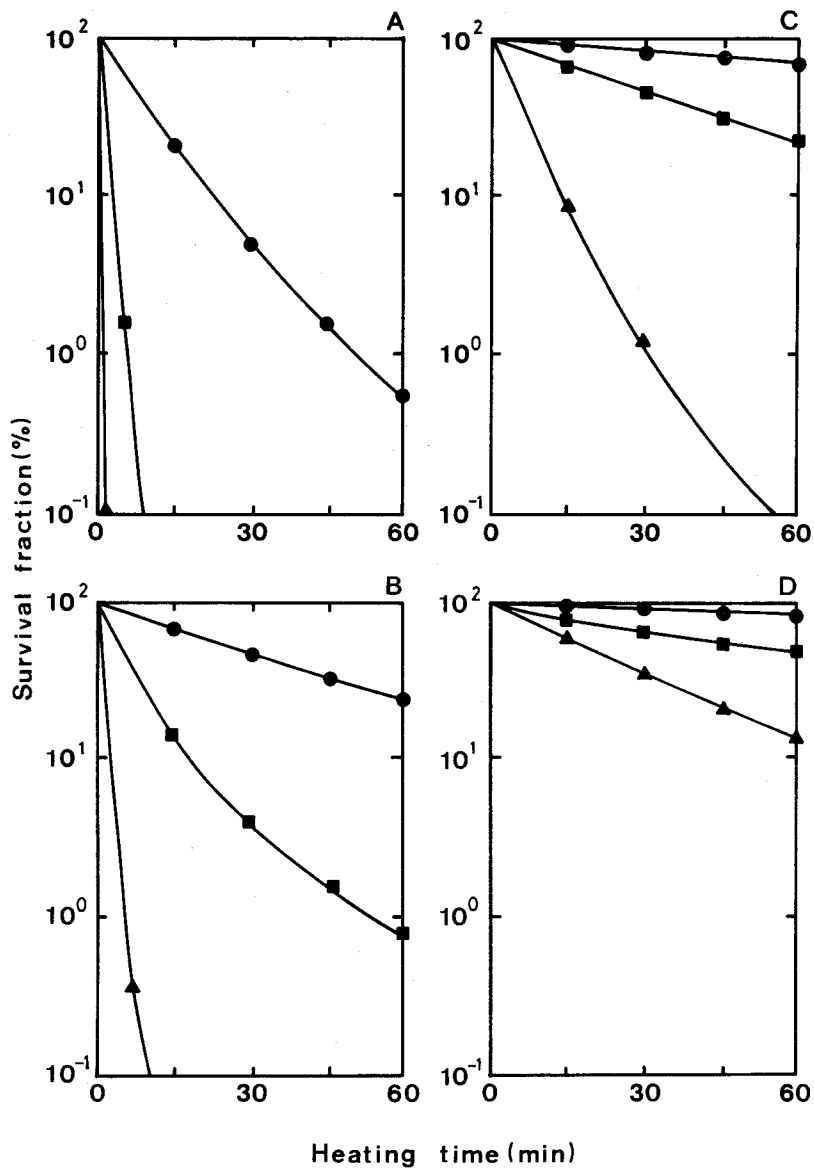
Fig. 5.   Thermal death curves of *B. subtilis* spores.

A, 90°C ; B, 80°C ; C, 70°C ; D, 60°C.

Symbols : ●——●, heated at pH 7.0 ; ■——■, heated at pH 5.0,

▲——▲, heated at pH 3.0.

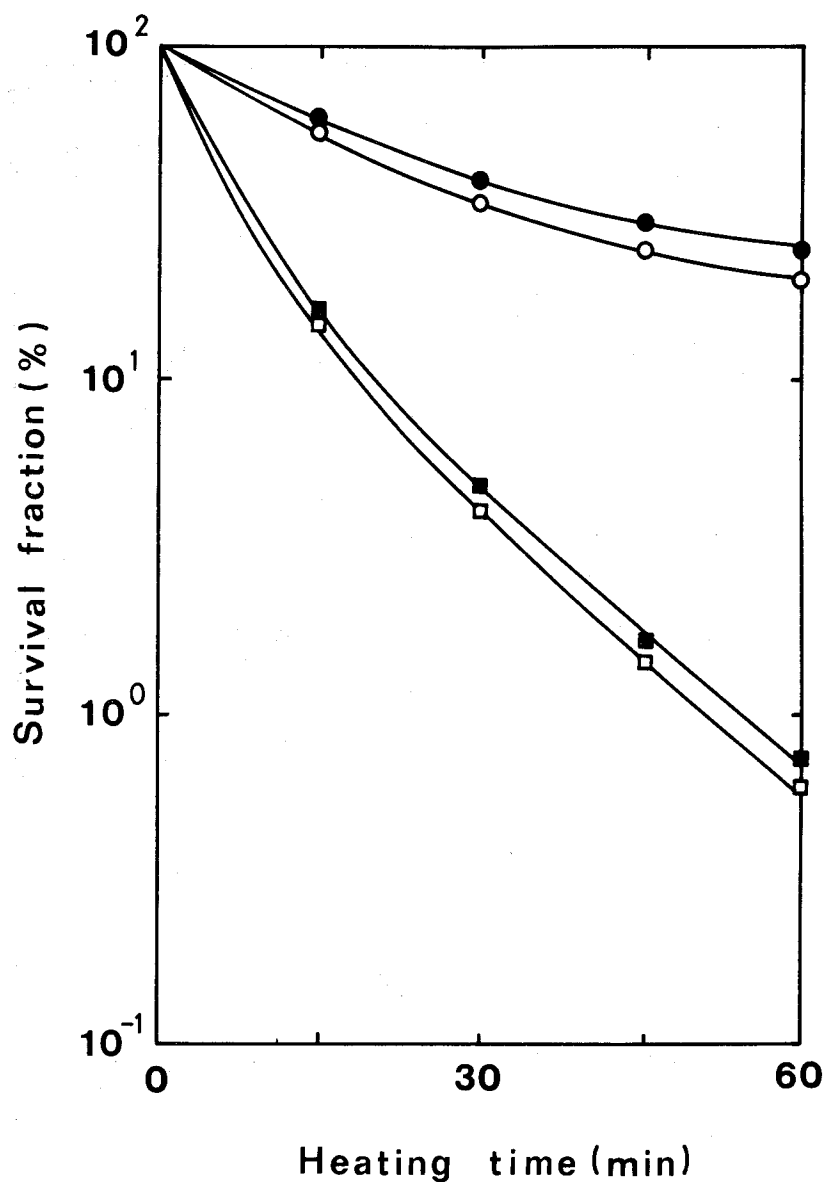Fig. 6.   Effect of incubation time at pH 5.0 on thermal destruction of *B. subtilis* spores heated at 80°C.

Symbols : ●——●, heated at pH 7.0 ; ■——■, heated at pH 5.0 ; ○——○, dialyzed against sodium citrate-HCl buffer (pH 5.0) for 24 h at 5°C and washed, and then resuspended in sodium citrate-HCl buffer (pH 7.0) and heated ; □——□, dialyzed against sodium citrate-HCl buffer (pH 5.0) for 24 h at 5°C and heated.

same pH the higher temperature, the more the spores were inactivated.

Therefore, the lower pH and the higher temperature, the more the

spores were inactivated.

To examine the effect of incubation time at pH 5.0 on survival

of spores, the spores suspension ($1 \times 10^8$ spores/ml) was dialyzed

against sodium citrate-HCl buffer (pH 5.0) for 24 h at 5°C and washed,

and then resuspended in sodium citrate-HCl buffer (pH 7.0) just

before the heating at 80°C.  Fig. 6 shows that the thermal death

curve of spores incubated at pH 5.0 for 24 h was almost the same as

those of spores suspended in the buffer just before the heating.

Similarly in the case of pH 3.0, the same result was obtained.

These results indicates that the water surrounding the spores may

penetrate into the spore inside at just time when the spores are

heated.

Injury of DNA molecules induced by acid-heating in spores

To examine the DNA injury in spores by acid-heating, DNA extract-

ed from heated spores was analyzed in respect to the molecular weight,

by using alkaline sucrose gradient sedimentation.   Sedimentation

patters of DNA in spores heated at 90, 80, 70, and 60°C were given

in Figs. 7, 8, 9, and 10, respectively.

Fig. 7 A shows that single strand scissions of DNA molecules in

spores heated at 90°C at pH 7.0 did not occur even after heating for

30 min, but at low pH single strand breaks of DNA increased according

to the increasing acidity.  Fig. 7 B shows that after 5, 10, and 15

min of incubation at 90°C at pH 5.0, the sedimentation distance of

DNA decreased with the increase of heating time.

Fig. 8 A shows that single strand scissions in DNA molecules in

spores heated at 80°C at pH 7.0 did not occur even after heating for

30 min, but at low pH DNA breaks increased according to the increasing

acidity.   Fig. 8 B shows that after 5, 10, and 15 min of incubation

at 80°C at pH 5.0, the sedimentation distance of DNA decreased with

the increase of heating time.   The number of DNA breaks induced by

heating at 90°C is more than at 80°C.   Fig. 9 A similarly shows

that single strand scissions of DNA in spores heated at 70°C at pH

7.0 did not occur even after heating for 30 min, but at low pH DNA

breaks increased according to the increasing acidity.   Fig. 9 B

shows that after 15, 30, and 60 min of incubation at 70°C at pH 5.0,

the sedimentation distance of DNA decreased with the increase of

heating time.   Fig. 10 A shows that single strand scissions of DNA

in spores heated at 60°C at pH 7.0 and pH 5.0 did not and few occur,

respectively, but at pH 3.0 DNA scissions occurred.   Fig. 10 B

shows that after 15, 30, and 60 min of incubation at 60°C at pH 5.0,

the sedimentation distance of DNA similarly decreased with the in-

crease of heating time, however, the number of DNA breaks induced at

60°C was fewer than at higher temperature.

These results show that after heating at 90, 80, 70, or 60°C at

neutral pH, single strand scissions in DNA did not occur even after

heating for 30 min.   When at the lower pH and the higher temperature

Fig. 7.    Sedimentation patterns in alkaline sucrose density gradients
of DNA from *B. subtilis* spores heated at 90°C for 30 min (A) and at
pH 5.0 (B).    The direction of sedimentation is from right to left.
(A) : ———— , non-heated ; ········, heated at pH 7.0 ; —·—— , heated at
pH 5.0 ; —— —— , heated at pH 3.0.    (B) : ———— , non-heated ; ········,
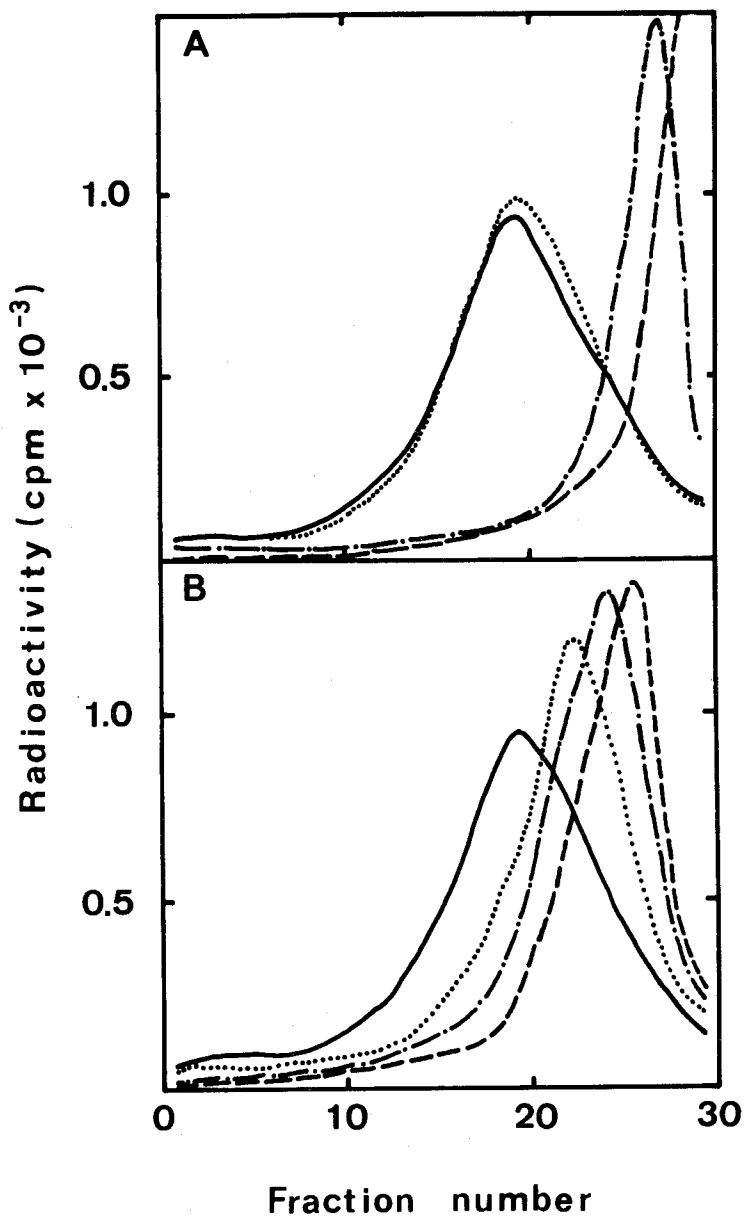heated for 5 min ; —·—, heated for 10 min ; —— ——, heated for 15 min.

Fig. 8. Sedimentation patterns in alkaline sucrose density gradients of DNA from *B. subtilis* spores heated at 80°C for 30 min (A) and at pH 5.0 (B). The direction of sedimentation is from right to left. (A) : ——— , non-heated ; ········, heated at pH 7.0 ; —·—, heated at pH 5.0 ; ―――, heated at pH 3.0. (B) : ——— , non-heated ; ········, heated for 5 min ; —·—, heated for 10 min ; ―――, heated for 15 min.

Fig. 9.　Sedimentation patterns in alkaline sucrose density gradi-
ents of DNA from *B. subtilis* spores heated at 70°C for 30 min (A) and
at pH 5.0 (B).　The direction of sedimentation is from right to left.
(A) : ——, non-heated ; ……, heated at pH 7.0 ; —·—, heated at pH
5.0 ; ———, heated at pH 3.0.　(B) : ——, non-heated ; ……, heated
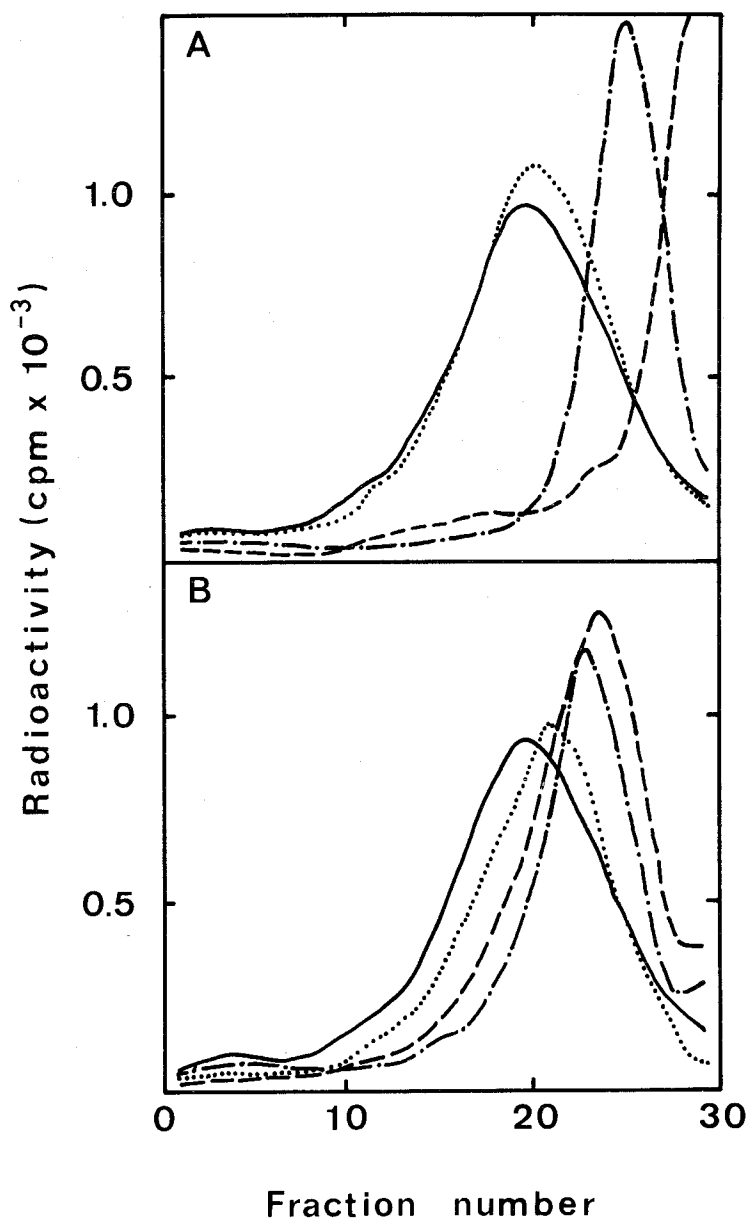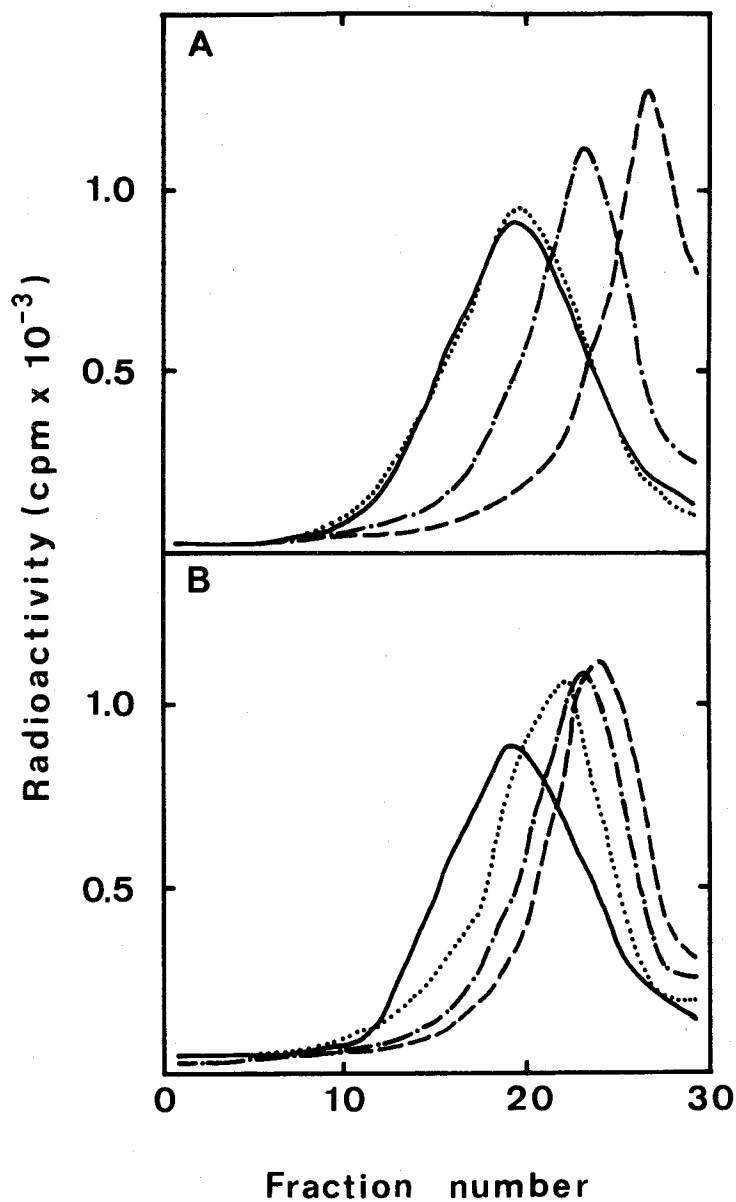for 15 min ; —·—, heated for 30 min ; ———, heated for 60 min.

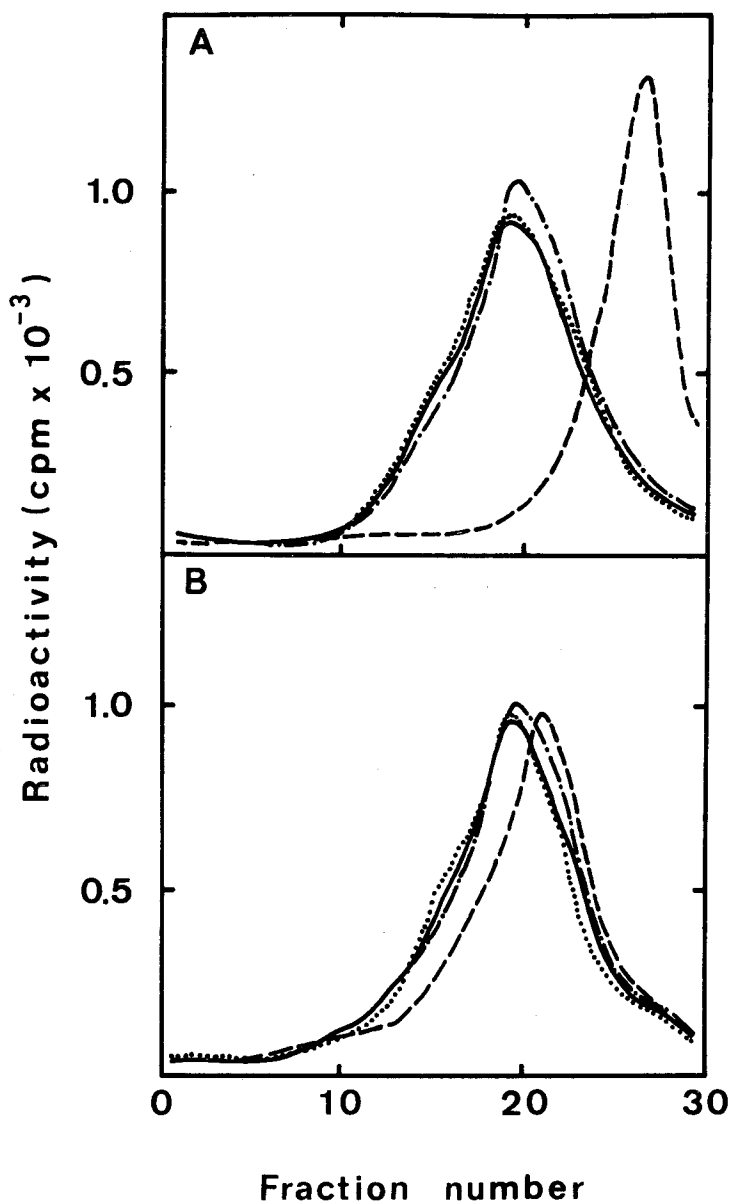Fig. 10.   Sedimentation patterns in alkaline sucrose density gradients of DNA from *B. subtilis* spores heated at 60°C for 30 min (A) and at pH 5.0 (B).   The direction of sedimentation is from right to left. (A) : ———, non-heated ; ⋯⋯, heated at pH 7.0 ; —·—, heated at pH 5.0 ; ———, heated at pH 3.0.   (B) : ———, non-heated ; ⋯⋯, heated for 15 min ; —·—, heated for 30 min ; ———, heated for 60 min.

the spores were heated, the more the single strand scissions of DNA in spores occurred.

It is reported that the main injury of *in vitro* DNA caused by heat treatment is depurination (38) and depurination occurred more frequently at acidic pH than at neutral pH (47). It is generally said that the apurinic sites in DNA are labile to the alkaline treatment resulting chain breaks. Accordingly, it is thought that the apurinic sites in DNA molecules induced by acid-heat treatment of spores, but not at neutral pH, were attacked by the alkaline treatment for the sedimentation analysis and the chain breaks were introduced.

4. DISCUSSION

The effect of low pH on the thermal resistance of *Clostridium botulinum* spores was well reported in relation to the putrefaction of food products and the toxin production. It is a generally accepted fact that a pH of 4.6 or less inhibit the growth and toxin production of *C. botulinum* spores in food (101). Xezones and Hutchings (91) found greater pH effect at the lower rather than at the higher temperature.

On the other hand, Cameron et al. (93) showed that the heat resistance of spores decreased with decreasing pH, but the pH effect was pronounced at the higher processing temperature than the lower

temperature.

The heating causes the damages either enzymes or DNA in spores which are essential for germination and outgrowth. It has been also reported that heat treatment at high temperatures often induced mutation in spores of B. subtilis (39, 40). In the previous chapter, it was found that heat treatment of B. subtilis spores induced the mutants which required amino acids for germination and/or growth.

Therefore, in this chapter, the effects of heat treatment, especially acid-heating, on survivals and DNA injury in spores were studied. As the results, the heat resistance of spores decreased with decreasing pH. Although after heating of spores at 60 - 90°C at neutral pH single strand scissions of DNA molecules did not occur even after heating for 30 min when at the lower pH and the higher temperature the spores were heated, the more the single strand scissions of DNA in spores occurred.

Greer and Zamenhof (38) reported that depurination occurred by heat treatment of DNA at high temperature. Furthermore, Lindahl and Nyberg (47) found that apurinic sites were induced more frequently at acidic than neutral pH. Accordingly, it is strongly suggested that apurinic sites in DNA molecules induced by acid-heat treatment of spores were attacked by the alkaline treatment and phosphodiester bonds at the apurinic sites were broken.

These results indicated that DNA molecules in spores was damaged, perhaps depurination, by heat treatment at acidic pH.

# 5. SUMMARY

The spores of B. *subtilis* suspended in buffer solution of low pH were more sensitive to heat than those in neutral pH.  The correlation of the heating temperature (60, 70, 80, 90°C) and pH value in the range from 3 to 7 on the thermal destruction of spores of B. *subtilis* was examined.  When treated at the lower pH and the higher temperature, the more the spores were inactivated.

To examine the effect of acid-heating on DNA in spores, after heating DNA was extracted from spores and analyzed by alkaline sucrose gradient sedimentation.  The results indicated that when the spores were heated at the lower pH and the higher temperature, the more the single strand scissions of DNA in spores occurred.  It is thought that depurination occurred first by the acid-heat treatment and then the DNA was broken at apurinic sites by the subsequent alkaline treatment.

Chapter III


Section 2.　DEATH AND DNA INJURY OF VEGETATIVE CELLS BY HEAT

　　　　　　　TREATMENT


1.　INTRODUCTION


　　As shown in the previous chapter, it was found that the heat

treatment caused DNA injury in *B. subtilis* spores.　Especially, the

acid-heat treatment caused the single strand scissions of DNA mole-

cules, perhaps through the depurination, in spores.

　　It is expected that such scissions of single strand DNA mole-

cules caused by heating can also be found in vegetative cells of *B.*

*subtilis* under certain conditions.

　　Recently, several papers and a review (23) have been published

concerning the relationship between heat sensitivity and the occur-

rence of single strand scissions in DNA molecules as a consequence

of heating, in some strains of *Escherichia coli* (24-30), *Salmonella*

*typhimurium* (31,32), and *Streptococcus faecalis* (33).　But it is

still uncertain whether the single strand scissions of DNA molecules

in vegetative cells are dependent on either the direct action of

elevated temperature, or the action by nucleases released or activated

by mild heating.

　　In this chapter, experiments were made on the degradation of

single stranded DNA by mild heating and its mechanism in the vegetative cells of B. subtilis.


## 2.  MATERIALS AND METHODS


### Organism

B. subtilis 168Tt (thy⁻, trp⁻) was used throughout this work.

### Preparation of nonlabeled and labeled vegetative cells

Logarithmic-phase culture of B. subtilis was inoculated to 10 ml of the Demain's medium (75) supplemented with 20 μg/ml of tryptophan, 5 μg/ml of thymine and 5 μCi of [methyl-$^{14}$C] thymidine (43.5 mCi/mmol : New England Nuclear).  The cultures were grown at 37°C to an optimal density (660 nm)of 0.6.  The cells were then harvested and washed 3 times with M/15 phosphate buffer (pH 7.2).  Nonlabeled vegetative cells were grown in the same medium without radioisotope and were prepared by the same method used for labeled vegetative cells.

### Heat treatment and counting of the survivals

Washed cells were resuspended in M/15 phosphate buffer (pH 7.2). The suspensions were incubated at 50°C for various periods and then immediately cooled in an ice bath.  The survivors of vegetative cells were counted as described in Chapter III Section 1.

### Preparation of protoplasts from vegetative cells and extraction of DNA

Heated and non-heated cells were converted to protoplasts by the

modification of the method of Weibull (102).   The cells were incu-

bated at 37°C for 15 min in lysozyme solution (500 μg/ml) containing

7 % sucrose and 10 mM EDTA.   After centrifugation, the protoplasts

were suspended in distilled water.

Sedimentation analysis of $^{14}$C labeled DNA in alkaline sucrose density
gradients

Zone sedimentation of DNA was performed by the method of McGrath

and Williams(104).   For sedimentation, a 4.6 ml linear concentration

gradient of 5 - 20 % (w/v) alkaline sucrose (pH 12.0) was used.

A 0.1 ml portion of 0.5 N NaOH containing 2 % SDS and a 0.2 ml por-

tion of ruptured vegetative protoplasts were layered on the top of

the sucrose gradient by using a screwpipette.   The gradients were

centrifuged at 30.000 rpm for 90 min at 20°C in a Hitachi RPS-40T2

rotor.   After centrifugation, the contents were collected from

bottom in fractions of 0.15 ml on the disks of filter paper.   The

disks were dried and counted in toluene system in a liquid scintilla-

tion counter.

3.   RESULTS

Heat sensitivity of vegetative cells of B. *subtilis*

The thermal death curves of B. *subtilis* vegetative cells at dif-

ferent temperatures are given in Fig. 11.   Vegetative cells of B.

*subtilis* are more sensitive to heat than spores.   By heating at 90 °C

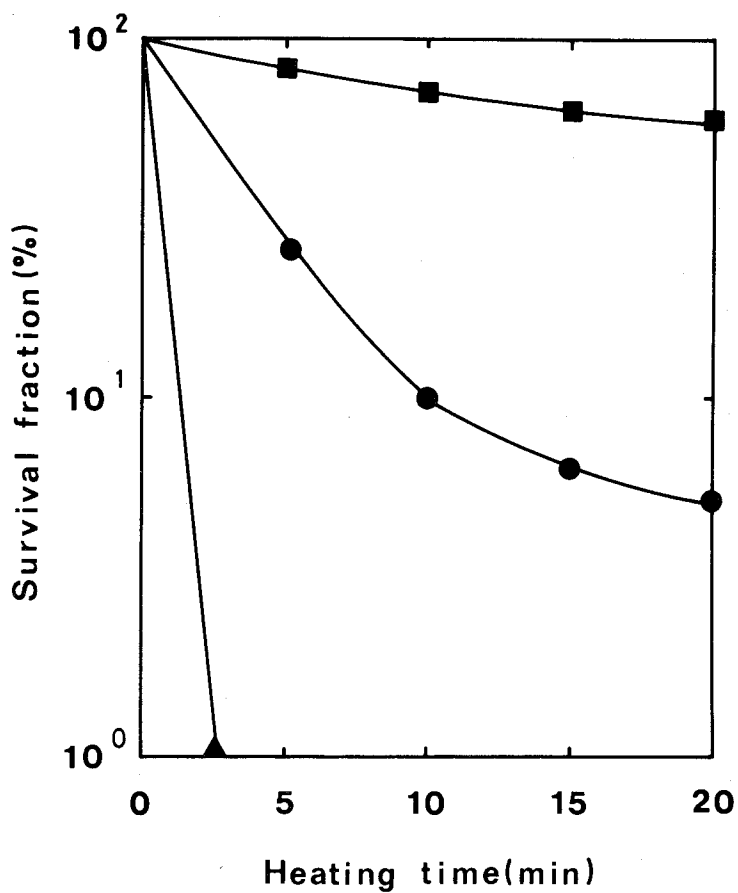Fig. 11. Heat-survival curves of *B. subtilis* vegetative cells. The cells at logarithmic growth phase were used. Symbols : ■—■, heated at 46°C ; ●—●, heated at 50°C ; ▲—▲, heated at 90°C.

for 10 min all the vegetative cells died. After incubation at 50°C for 10 and 20 min, about 10 and 5 %, respectively, of the cells remained viable ; longer incubation times further reduced survival fractions.
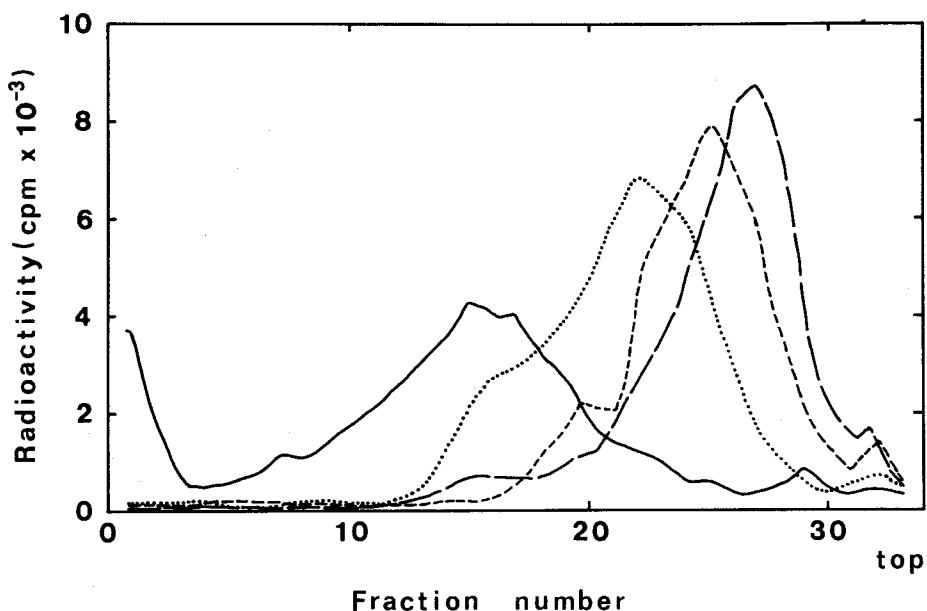
Fig. 12.   Effect of heat treatment at 50°C on sedimentation patterns in alkaline sucrose density gradients of DNA from vegetative cells of *B. subtilis* grown on Demain's medium.
Symbols : ———, not heated ; ⋯⋯⋯, heated at 50°C for 10 min ; ———, heated at 50°C for 20 min ; ——, heated at 50°C for 30 min.


## Sedimentation analysis of DNA of heated vegetative cells

Figure 12 shows the sedimentation profiles for single strand DNA isolated from *B. subtilis* vegetative cells after heated at 50°C for 10, 20, and 30 min.   The sedimentation distance of DNA was decreased in accodance with the decreasing of viability caused by mild heating.   The mild heating either directly or indirectly gave rise to DNA single strand breakages.

Comparing with mild heating (50°C), vegetative cells heated at
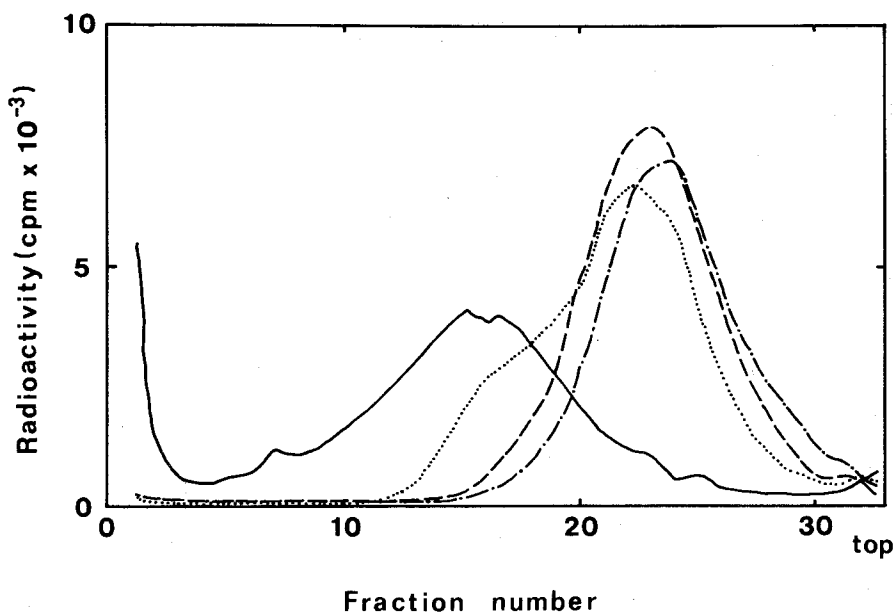
Fig. 13. Effect of heat treatment at different temperatures on sedimentation patterns in alkaline sucrose density gradients of DNA from vegetative cells of *B. subtilis* grown on Demain's medium.
Symbols : ——, not heated ; ........, heated at 50°C for 10 min ; ----, heated at 90°C for 10 min ; —·—, heated at 90°C for 20 min.

90°C for 10 or 20 min were subjected to sedimentation analysis. Figures 13 shows that the sedimentation rate of DNA was decreased by heating at 90°C for 10 min or 20 min, however, the positions of the peaks in DNA from cells heated at 50°C for 10 min, 90°C for 10 min, and 90°C for 20 min were nearly same.

Figures 14 also shows that the sedimentation rate of DNA was decreased by heating at 46°C for 20 or 30 min as well as 50°C for 10 min.

Fig. 14.   Effect of mild heating at 46°C on sedimentation patterns in alkaline sucrose gradients of DNA from vegetative cells of *B. subtilis* grown on Demain's medium.
Symbols : ————, not heated ; ·········, heated at 50°C for 10 min ; ———— , heated at 46°C for 10 min ; ————, heated at 46°C for 20 min ; —·—, heated at 46°C for 30 min.

When vegetative cells which had been heated at 70°C for 10 or 20 min were subjected to sedimentation analysis, DNA in the cells was sedimented at almost the same position with that heated at 50°C for 10 min (data not shown).   However, in the case of vegetative cells grown on Schaeffer's medium to the middle log phase and heated at 50°C for 10 or 30 min, single strand scissions of DNA were not apparent.

These results indicate that the occurrence of single strand scissions of DNA in vegetative cells by heat treatment is dependent on both the growth stage and the physiological state of B. *subtilis* and has no relation to the heating temperature or the heating time.  These data also suggest that the mild heating probably causes indirectly DNA strand breakage in vegetative cells.

Effect of p-CMB on DNA breakage of heated vegetative cells

It is considered that single strand breaks of DNA after heating at 50°C could be the result of nuclease action and not of' the direct action of heat.  In order to ascertain this hypothesis, the effect of p-chloromercuribenzoate (p-CMB) on sedimentation patterns in alkaline sucrose density gradients of DNA from vegetative cells of B. *subtilis* was studied.

Figure 15 A shows that the single strand scissions of DNA took place in vegetative cells when the cells were preincubated for 10 min at 37°C in phosphate buffer (0.067 M, pH 7.2) and then heated at 50°C for 10 or 30 min.  However, when cells were preincubated for 10 min at 37°C in phosphate buffer (0.067 M, pH 7.2) containing p-CMB at a concentration of 1 mM and then heated at 50°C for 10 or 30 min, single strand scissions of DNA scarcely occurred (Fig. 15 B). These data strongly suggest that p-CMB inhibited endonuclease activity, which is probably released or activated by mild heating.
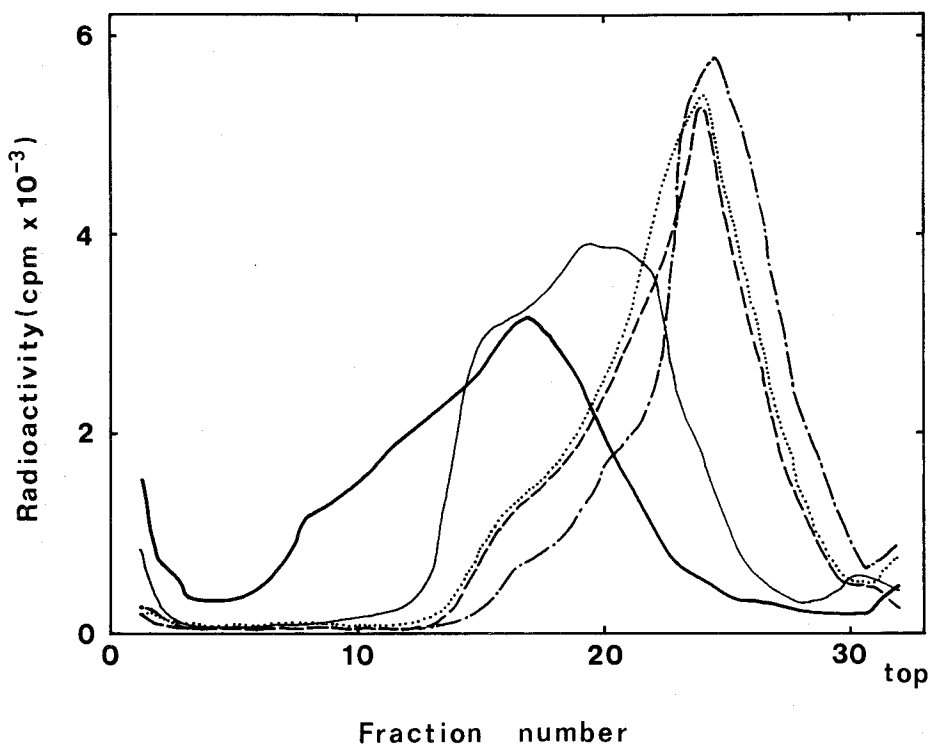
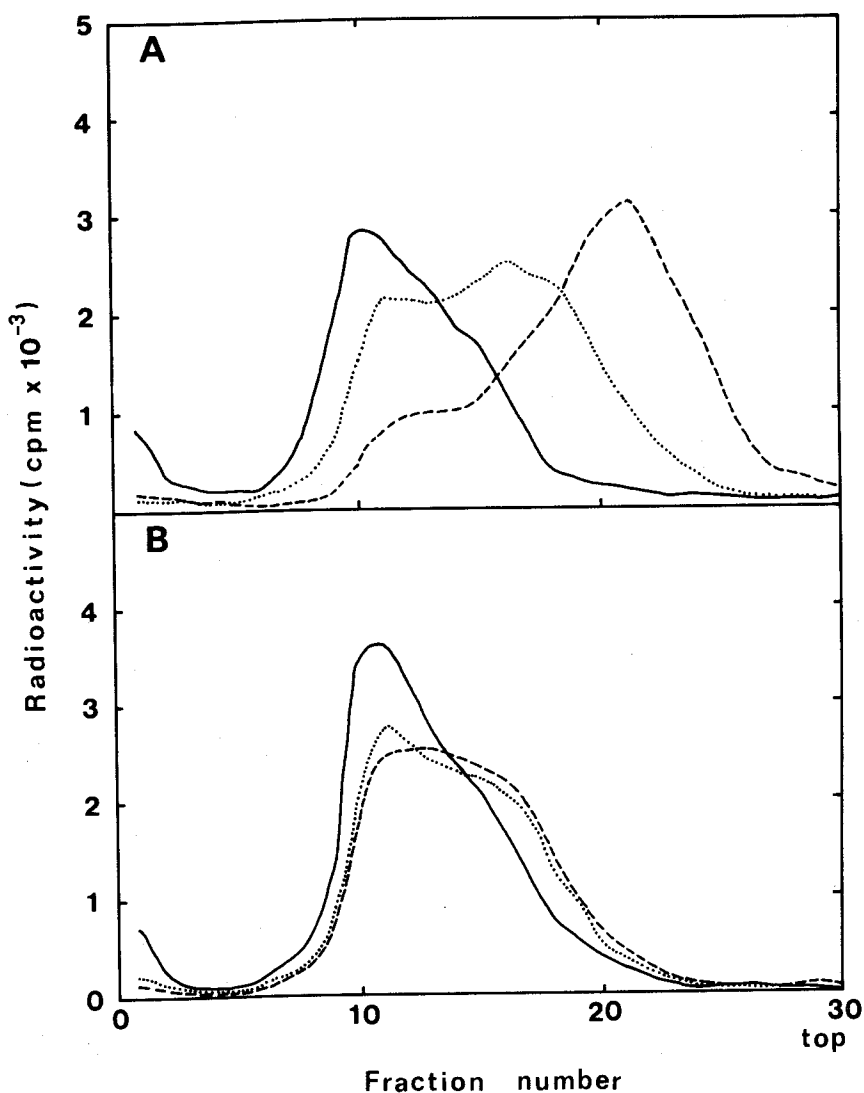Fig. 15. Effect of p-CMB on sedimentation patterns in alkaline su-
crose density gradients of DNA from vegetative cells of *B. subtilis*
grown on Demain's medium.

A : ——— . not heated (-p-CMB) ; ⋯⋯ , heated at 50°C for 10 min (-
p-CMB) ; — — —, heated at 50°C for 30 min (-p-CMB)    B : ———, not
heated (+p-CMB) ; ⋯⋯⋯, heated at 50°C for 10 min (+p-CMB) ; — — —,
heated at 50°C for 30 min (+p-CMB).

4.  DISCUSSION


It has been reported that the heat treatment causes damages of
many cellular components, such as cell wall, membrane, protein, RNA,
and DNA, of bacterial cells (18-22) and that the injured cells were
sensitive to some plating media and sometimes they were repaired in
suitable media (104-114).   Bridges et al. (24,25) demonstrated for
the first time that after incubation of *E. coli* for similarly to
several minutes at 52°C the single strand scissions of DNA occurred,
similarly to the treatment by ionizing radiations.   They proposed
that the production of single strand breaks in DNA by mild heating
may be the primary lethal event in the thermal inactivation of meso-
philic bacteria.

In Chapter III Section 1, it was made clear that DNA breakages in
heated-spores increased with an decrease in pH of heating buffer and
scarcely occurred at neutral pH.   However, in vegetative cells the
single strand scissions of DNA were produced by mild heating at 50 °C
for 10 min.   Furthermore, in vegetative cells, DNA breakages were
not dependent on the heating temperature or the heating time, but on
both the growth stage and the physiological state of the cells.

Since heating of DNA at 52°C does not result in denaturation or
strand breakage *in vitro,* and since the breakage depends on the cells
being in the logarithmic phase of growth, Woodcock and Grigg (27)
postulated that the DNA breakage was enzymatic.

DNA breakage in vegetative cells by mild heating is substantially reduced and scarcely occurred by adding enzyme inhibitor, p-CMB added before heating.　Grecz and Bhatarakamol (30) reported that p-CMB inhibited the DNA breaks of *E. coli* by mild heating and a mutant of *E coli* deficient in apurinic acid endonuclease yielded substantially fewer DNA breaks than wild type cells or cells of ligase deficient strain.

Therefore, it can be said that, in vegetative cells grown on nutrient-poor media, single strand DNA molecules are probably degraded by endonuclease which is activated or released by mild heating.

5.　SUMMARY

Alkaline sucrose gradient sedimentation studies reveal that mild heating at 50°C caused the single strand scissions of DNA molecules in vegetative cells of *B subtilis*.　The DNA breakage was produced by heating at 46 or 90°C as well as at 50°C.　The DNA breakages in vegetative cells were not dependent on the heating temperature and the heating time.　Since the DNA breakages were inhibited by p-CMB, it is thought that the single strand scissions of DNA in vegetative cells are due to the activity of endonuclease, which is probably released or activated by mild heating.

Chapter IV


REPAIR OF DAMAGED DNA IN HEATED B. *SUBTILIS* SPORES DURING GERMINATION


1.    INTRODUCTION


In Chapter III Section 1, the author clarified that when treated
at the lower pH and the higher temperature, the more the spores were
inactivated and the single strand scissions of DNA in spores increased
with decreasing pH in heating.    However, DNA breakage in spores
scarcely occurred by heating at 90°C for 30 min at pH 7.0.    Therefore,
it was thought that the single strand scissions of DNA in spores were
dependent on depurination and the subsequent alkaline treatment.

AS DNA in spores is the most important target of γ-ray or UV,
it is thought that DNA in irradiated spores is subjected to various
injuries and most of the damages are repaired during germination.
In fact, it has been shown that DNA damages caused by radiations and
by chemical agents were repaired *in vivo* (9,11).    However, as to
DNA in spores, such studies were very few, because of the difficulty
to extract DNA from spores in the natural state.    It was shown that
radiation-induced single strand scissions of DNA in spores of B.
*subtilis* were rejoined during postirradiation incubation, and the
rejoining during germination of the spores in nutrient medium occurred
without detectable DNA synthesis (115).

In the present chapter, it was investigated whether or not DNA injuries of spores induced by acid-heating are repaired during germination, and whether or not the repair of DNA injury during germination requires of DNA synthesis.


## 2.  MATERIALS AND METHODS


### Organism

B. *subtilis* 168Tt (thy⁻,trp⁻) was used throughout this work.

### Preparation of nonlabeled and $^3$H-labeled spores

Nonlabeled spores were prepared by growing B. *subtilis* at 37°C in Schaeffer's medium (74) according to the method described in Chapter III.

To obtain the spores labeled with $^3$H-thymidine the same medium supplemented with 2.5 µCi/ml [methyl-$^3$H] thymidine  (58 Ci/mmol : New England Nuclear) was used as described in Chapter III.

### Acid heat treatment

Nonlabeled and labeled spores were heated as described in Chapter III.

### Extraction of DNA from spores and germination spores

The DNA of heated and unheated $^3$H-labeled spores, which were not germinated, was extracted with the method described in Chapter III.

In order to extract DNA from spores incubated after heating, heated spores were centrifuged and resuspended in Schaeffer's medium

to germinate.   After incubation at 37°C for desired periods, the
germinated spores were harvested and treated with 1 mg/ml lysozyme
in 0.01 M Tris-HCl (pH 8.0), 0.01 M EDTA-4Na, 0.2 % Brij-58 and 10 %
sucrose at 37°C for 30 min and centrifuged.   The pellet was suspended
and solubilized slowly in 2 % sodium dodecyl sulfate in 0.1 N NaOH
at 37°C for 30 min and then DNA was extracted.

Sedimentation analysis of $^3$H-labeled DNA in alkaline sucrose density
gradient

Extracted DNA was layered on linear 5 - 20 % alkaline sucrose
density gradient (pH 12.0) and centrifuged at 40,000 rpm for 2 h in a
Beckman SW.50.1 rotor.   The contents were fractionated and radio-
activity was counted as described in Chapter III.

Measurement of DNA synthesis during germination

Heated and unheated spores were incubated at 37°C in 10 ml of
Schaeffer's medium supplemented with 10 µCi of [methyl-$^3$H] thymidine
(58 Ci/mmol : New England Nuclear).   Incorporation of $^3$H-labeled
thymidine into the trichloroacetic acid insoluble fraction was taken
as a measure of the synthesis of DNA.   The bacterial concentration
in the culture was about $10^8$ spores per ml.   At intervals, 0.2 ml
samples were taken in duplicate from the incubation culture.   After
the samples were suspended in 2 ml of 10 % cold trichloroacetic acid
for 10 min, the samples were collected on a Millipore filter (0.22
µm pore size) and washed two times with ethanol ; they were then
dried and counted in a scintillation counter.
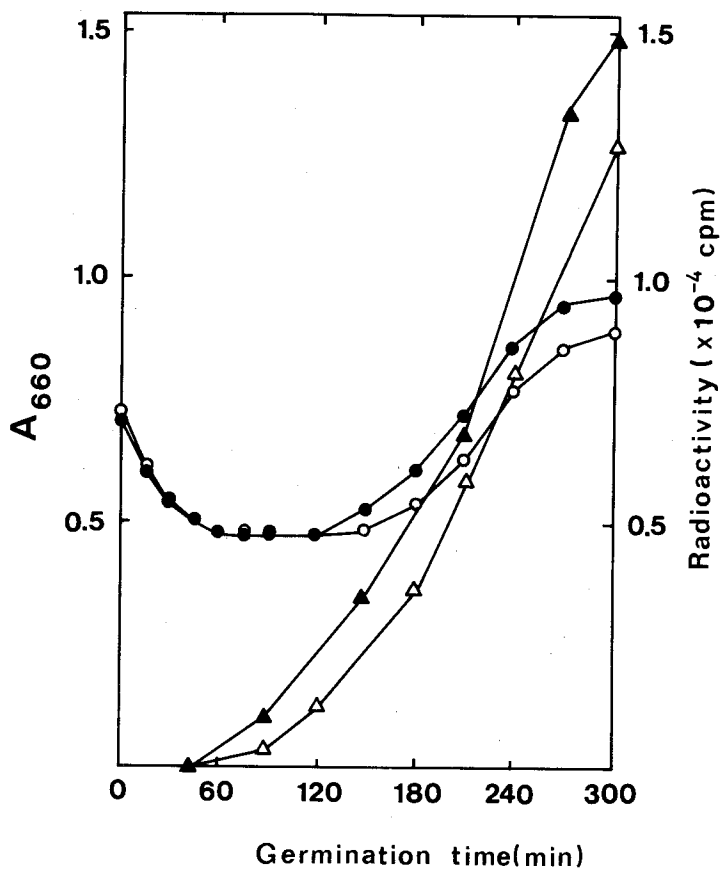
Fig. 16. DNA synthesis of *B. subtilis* spores during postheating incubation in germination medium.

▲——▲ , $^3$H-thymidine incorporated into unheated spores ; △——△ , $^3$H-thymidine incorporated into spores heated at 70°C for 20 min at pH 5.0 ; ●——● , absorbance at 660 nm of unheated spores ; ○——○ , absorbance at 660 nm of spores heated at 70°C for 20 min at pH 5.0.

3.   RESULTS

DNA synthesis during germination of heated spores

In Chapter III Section 1, it was found that the heat resistance of

spores decreased with decreasing pH. When the spores were heated at

70°C, the survival fraction after 20 min heating at pH 7.0 was about

90 %, however at pH 5.0, the survival fraction after 20 min heating

at 70°C was about 60 %. Furthermore, the single strand scissions of

DNA in spores occurred by heating at 70°C for 20 min at pH 5.0, but

not at pH 7.0. Therefore, in order to study the mechanism of DNA

repair during germination, DNA synthesis during postheating incubation

of acid-heated spores was measured by estimative $^3$H-thymidine incorpo-

ration into acid insoluble materials. At the same time the change

in turbidity of the germination culture was measured. The result

is shown in Fig. 16. The decrease in optical density at the initial

stage of germination of acid-heated spores was similar to that of the

spores without heating. However, the subsequent increase in optical

density of heated spores was delayed for 30 min than that of unheated

spores. The DNA synthesis of unheated spores was initiated after

60 min of incubation in germination medium. In spores which was

heated at 70°C for 20 min at pH 5.0, DNA synthesis was initiated after

90 min incubation. In the case of the spores which were heated at

70°C for 30 min at pH 3.0, DNA synthesis in spores was not observed

even after 5 h incubation in germination medium (data is not shown).

Rejoining of DNA single strand breaks during germination

As DNA synthesis of spores heated at 70°C for 20 min at pH 5.0

was initiated after 90 min of incubation in germination meidum, the

change in the molecular weight of single strand DNA from the heated spores was observed with the lapse of time in the post heating incubation.    As shown in Fig. 17, the single strand scissions of DNA in spores incubated by acid-heating were scarcely restored after 1 h postheating incubation at 37°C.    After 2 h postheating incubation, consistently, most of the DNA breakages were restored to the levels of DNA from nonheated spores.    After 3 h postheating incubation, the single strand scissions of DNA were completely repaired.

These results show that in case that most of the heated spores are survival the single strand scissions of DNA in spores which occurred through depurination, are restored during germination.

However, the single strand scissions of DNA in spores heated at 70°C for 30 min at pH 3.0 (about 1 % survival) were hardly repaired even after 6 h incubation in germination medium (data is not shown).


4.    DISCUSSION


The primary objective of this chapter was to investigate the condition under which DNA injury in heated spores was repaired during germination after acid-heat treatment.    For this purpose, $^{3}$H-labeled DNA was extracted from B. *subtilis* spores which were germinated after acid-heating (70°C 20 min, pH 5.0) and the extent of single strand scissions of DNA molecules was measured by alkaline sucrose gradient centrifugation.
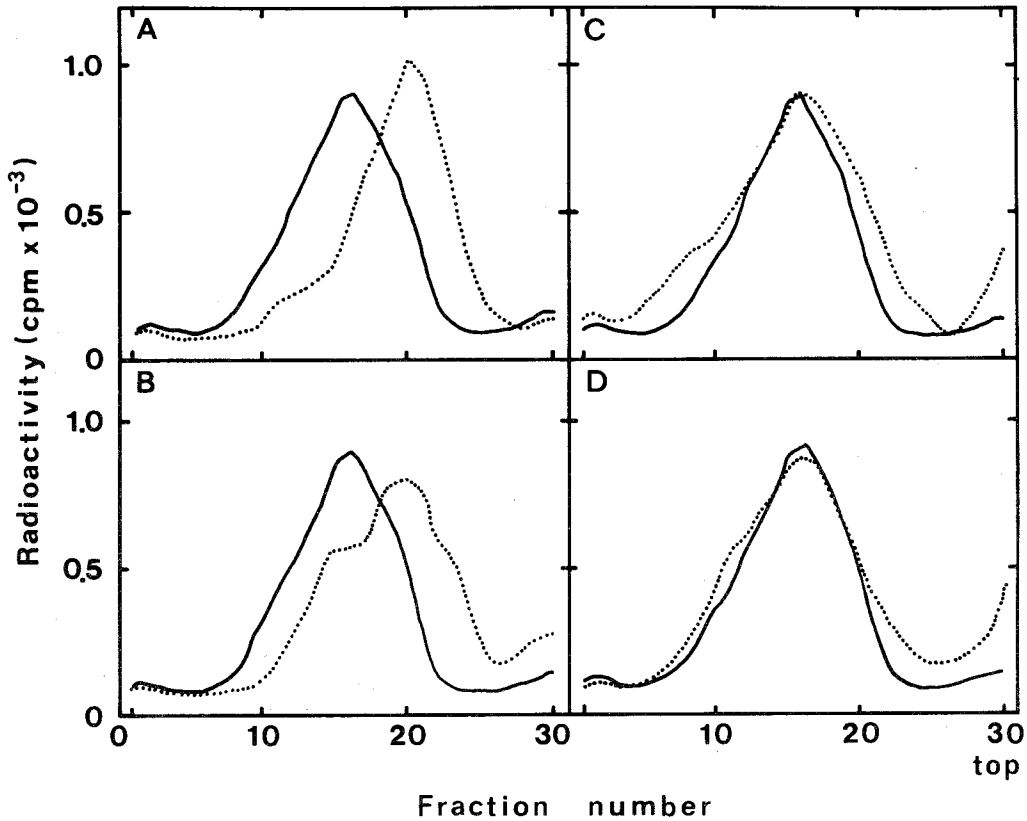
Fig. 17. Alkaline sucrose density gradient profiles for repair of acid-heated DNA in spores of *B. subtilis*.

[3]H-labeled spores were heated at 70°C for 20 min (pH 5.0) and immediately incubated in germination medium at 37°C. At the indicated time, ^{3}H-labeled DNA was extracted and studied by sedimentation analysis. The direction of sedimentation is from right to left.

Symbols : A, ───── , non-heating; ······, just after heating. B, ───── , 1 h incubation of non-heated sproes ; ······ , 1 h incubation of heated spores. C, ───── , 2 h incubation of non-heated spores ; ······, 2 h incubation of heated spores. D, ───── , 3 h incubation of non-heated spores ; ······, 3 h incubation of heated spores.

In Chapter III Section 1, it was found that single strand scissions of DNA in spores heated at 70°C at pH 7.0 did not occur even after heating for 30 min, but at pH 5.0 DNA breakage increased in parallel to the increase in heating time.   When the heated spores were germinated in Schaeffer's medium, the single strand scissions of DNA were repaired in 2 h after germination.   Furthermore, DNA synthesis of acid-heated spores was initiated after 90 min of incubation. When heated at 70°C for 30 min at pH 3.0 (about 1 % survival), however, DNA synthesis in spores was not initiated after 5 h of incubation, and the single strand scissions of DNA in spores were hardly recovered during 6 h incubation.   It was found that the postheating recovery time increased with increasing the DNA breaks in spores.

Terano et al. (115) reported that the single strand breaks of DNA in spores which were irradiated with 190 K rad (8 % survival dose) were rejoined during postirradiation incubation and the rejoining in spores which were germinated in nutrient medium occurred in the absence of detectable DNA synthesis.

Therefore, it is thought that the DNA damages and its recovery system in spores which were treated with γ-ray and heat were different in quality each other.


5.   SUMMARY


When DNA in spores of *B. subtilis* was injured by heat treatment

at 70°C for 20 min at pH 5.0, at which the survivals did not decrease greatly, the molecular weight of single stranded DNA was restored to the level of non-heated spores during germination after heating. The rejoining of DNA in spores during germination in nutrient medium was terminated after about 2 h postheating incubation.

Chapter V


DEPURINATION OF PURIFIED DNA BY HEATING


1.   INTRODUCTION


As described in Chapter III Section 1, it was found that the single strand scissions of DNA in heated spores increased with decreasing pH.   And the scissions hardly occurred at neutral pH.   Therefore, it was thought that acid-heat treatment produced the apurinic sites in DNA in sproes and then DNA was broken at the apurinic sites by the subsequent alkaline treatment.

In the present chapter, the conditions which are responsible for the depurination in purified DNA have been studied.


2.   MATERIALS AND METHODS


Organism

*B. subtilis* 168Tt (thy⁻, trp⁻) and *B. subtilis* (ade⁻, thr⁻, hisA, lys⁻, trp⁻, met⁻) which was kindly provided by H. Tanooka were used.
Sedimentation analysis of purified DNA from vegetative cells in alkaline sucrose density gradient

Non labeled DNA of *B. subtilis* 168Tt vegetative cells was purified according to the method of Marmur (116).   A 20 µl aliquot of 1 M NaOH

was added to 200 µl of depurinated DNA which was heated at various temperatures and various pH values. Then the solution was incubated at 37°C for 15 min. This treatment hydrolyzes a phosphoester bond near the apurinic site (117). The DNA was layered on linear 5 - 20 % alkaline sucrose density gradient (pH 12.0) and centrifuged at 40,000 rpm for 4.5 h at 5°C in a Beckman SW.50.1 rotor. The gradients were analyzed in an ISCO density gradient fractionator (Model 185) and UV-absorbance monitor (Model UA-5).

Preparation of purine-labeled DNA

A purine-requiring mutant of *B. subtilis* (ade⁻,thr⁻,hisA,lys⁻, trp⁻,met⁻) was grown in Schaeffer liquid nutrient broth (74) supplemented with 250 µCi/l of [8-$^3$H] guanine sulfate (15 Ci/mmol ; The Radiochemical center, Amersham) at 37°C, and was harvested at the late logarithmic growth phase. The DNA was extracted according to the method of Marmur (116), dissolved in SSC solution (0.15 M NaCl/ 0.015 M sodium citrate, pH 7.0) and stored at -20°C.

Analysis of depurination

One ml of purine labeled DNA was heated at various pH (pH 3, 5, 7) and chilled. Calf thymus DNA in SSC (400 µg/200 µl) and 200 µl of 12.5 % perchloric acid were added to depurinated DNA. After being kept at 0°C for 10 min, the mixtures were centrifuged at 13,000 x g for 10 min, and the radioactivity of the supernatants was determined in toluene-Triton scintillation fluid in a liquid scintillation counter.

3. RESULTS

## Temperature and pH dependence of depurination

It is generally said that the apurinic/apyrimidinic sites in DNA are labile to the alkaline treatment resulting chain breaks at the 3' -side of apurinic sugar moieties (118) and that these apurinic/apyrimidinic sites are introduced by acid-heat treatment.

In order to ascertain the condition of depurination of purified DNA, the DNA solutions of which pH values were adjusted to 3.0, 5.0, and 7.0 were heated at 50, 60, 70, 80, and 90°C. After the alkaline treatment to break the apurinic sites molecular weights of the DNA were analyzed by alkaline sucrose density gradient centrifugation. The DNA heated at pH 7.0 for 30 min below 90°C, were scarcely depurinated (Fig. 18), although the single strand scissions of DNA in vegetative cells occurred by mild heating at 50°C. On the other hand, at pH 5.0 and 3.0 the sedimentation distance of heated DNA decreased with the increase of temperature and of heating time (Fig. 19).

Consequently, depurination sites increases with decrease in pH value and temperature.

## Release of $^3$H-labeled purines from DNA

In order to measure directly the release of purines from double-stranded DNA in solution, it is necessary to heat the DNA solutions for relatively long periods of time. The [$^3$H] purine-labeled *B. subtilis* DNA was heated in SSC at pH 3, 5, or 7 and 70 or 80°C for 0.5
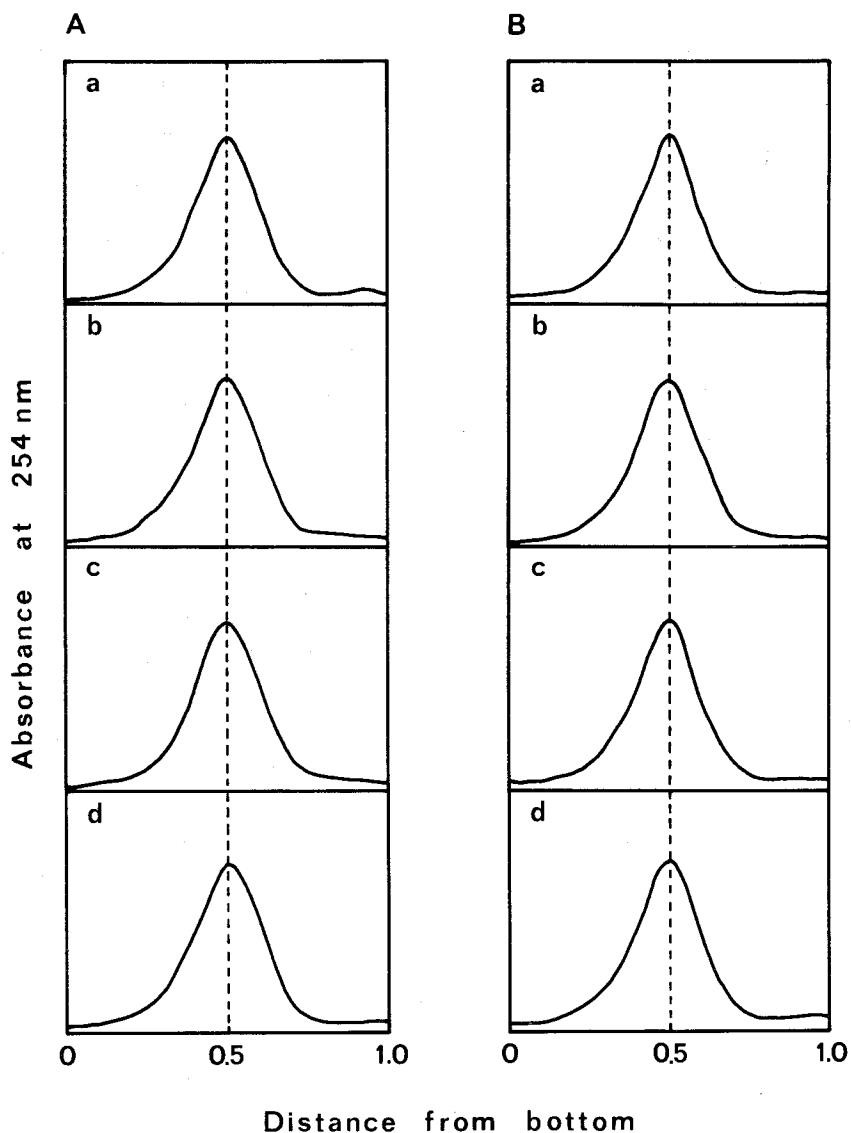
Fig. 18. Effect of temperature on depurination of purified DNA at neutral pH. Following heating at pH 7.0 purified DNA was treated with alkali and studied by sedimentation analysis. The direction of sedimentation is from right to left. A : a, non-heated ; b, heated at 50°C for 10 min ; c, heated at 50°C for 20 min ; d, heated at 50°C for 30 min. B : a, non-heated ; b, heated at 70°C for 30 min ; c, heated at 80°C for 30 min ; d, heated at 90°C for 30 min.

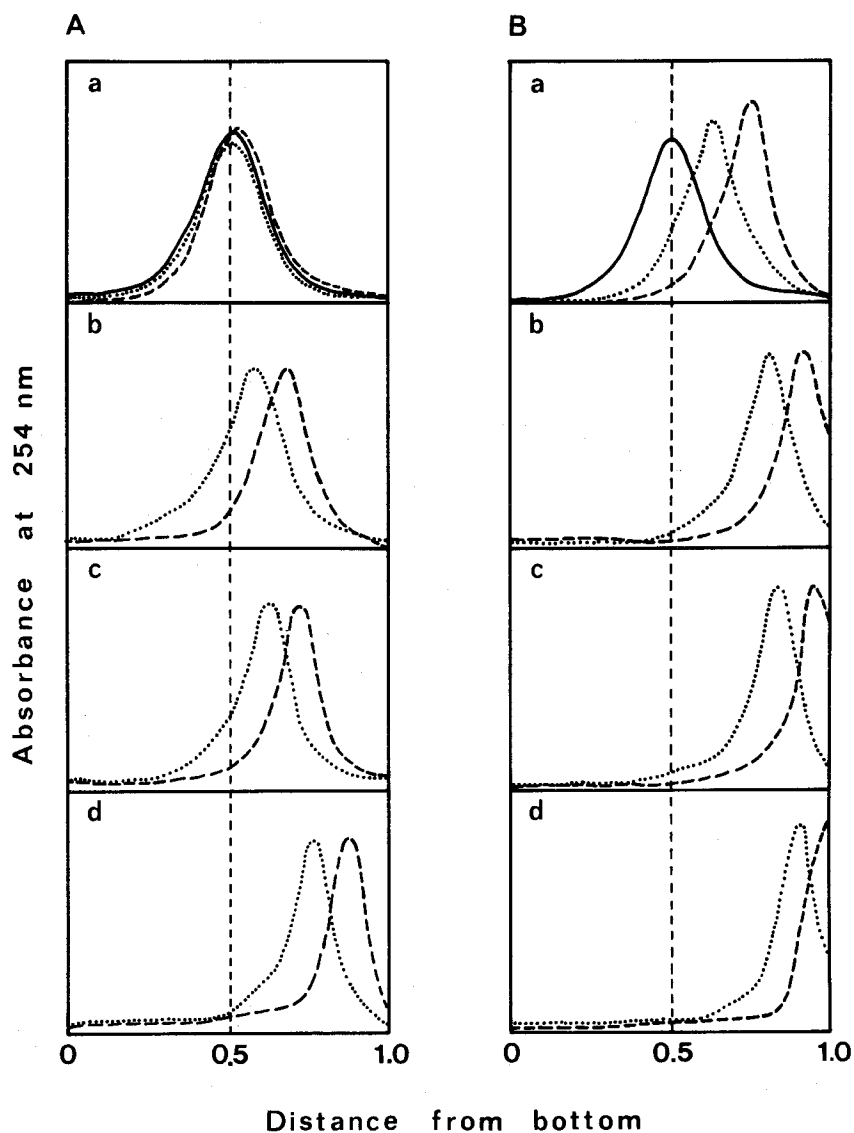Fig. 19.   Effect of temperature on depurination of purified DNA at
pH 5.0 (A) and at pH 3.0 (B).

Following heating at pH 5.0 and at pH 3.0 purified DNA was treated
with alkali and studied by sedimentation analysis.   The direction
of sedimentation is from right to left.   Symbols : a, 60°C ; b, 70°C
; c, 80°C ; d, 90°C.   ———— , non-heated ; ··········· , heated for 10 min ;
————, heated for 30 min.
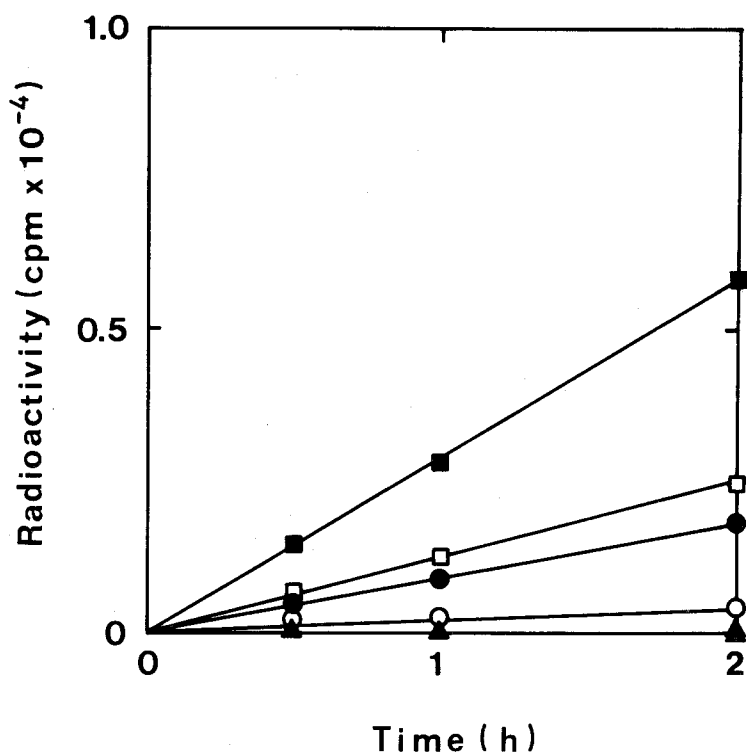
Fig. 20.    Temperature and pH dependences of depurination.
[$^3$H]purine labeled *B. subtilis* DNA was heated at 70°C and 80°C at pH
3 to 7 and radioactive material released from DNA in acid-soluble form
was measured.
Symbols : △—△ , heated at 70°C at pH 7.0 ; ○—○, heated at 70°C at
pH 5.0 ; □—□, heated at 70°C at pH 3.0 ; ▲—▲, heated at 80°C at
pH 7.0 ; ●—●, heated at 80°C at pH 5.0 ; ■—■, heated at 80°C at
pH 3.0.

to 2 h.    Under these conditions, radioactive material was found to

be released from DNA in acid-soluble fraction (Fig. 20).    At pH 5,

the degree of depurination was much slower than at pH 3.    At neutral

pH, however, depurination hardly occurred at 70°C or 80°C for 2 h.

At pH 7, depuration occurred more rapidly at higher temperature.

These results also show that depuration site increases with decrease in pH value and increase in temperature.

The results of acid-heat induced DNA injury of spores, described in Chapter III Section 1, correspond to that of depuration of DNA heated at low pH *in vitro*.

It was concluded, therefore, that DNA in spores of *B. subtilis* was depurinated by acid-heat treatment, but at neutral pH depuration of DNA in spores hardly occurred in the temperature range between 60 and 90°C.


4.  DISCUSSION


In the previous Chapter, it was shown that the single strand scissions of DNA in spores increased with decrease in pH and increase in temperature.  However, at neutral pH DNA breaks hardly occurred.

Greer and Zamenhof (38) demonstrated that purine bases were released in detectable quantities from calf thymus DNA at temperatures near the Tm.  Lindahl and Nyberg (47) reported that in a $Mg^{2+}$-containing buffer of physioligical ionic strength, the rate constant for depuration of DNA was $4 \times 10^{-9}$/sec at 70°C and pH 7.4 and the rate of depuration at pH 5.0 was higher than at pH 7.4.  Therefore, it was thought that depuration likely occurred first by the acid-heat treatment of spores and DNA was broken at apurinic sites by the sub-

sequent alkaline treatment.

In order to study the pH and temperature dependence of depurination, the sedimentation analysis and direct measurement of released [$^3$H]-labeled purines from DNA were made. The results indicate that the depurination site increased with decrease in pH and increase in temperature.

The results shown in Chapter III that the lower pH and the higher temperature, the more the DNA in spores was broken correspond to the results obtained in the present Chapter.

It was concluded, therefore, that acid-heat treatment induced the apurinic sites in DNA in spores.


5.   SUMMARY


In order to study the temperature and pH dependence of *in vitro* depurination of DNA, purified DNA after being heated at various pH and temperature was analyzed by the sedimentation analysis. Furthermore, released [$^3$H] purines from DNA by acid-heating were directly measured. It was found that the number of depuration site increased with decrease in pH value and increase in temperature.

Chapter VI


PURIFICATION AND CHARACTERIZATION OF APURINIC/APYRIMIDINIC ENDODEOXY-

RIBONUCLEASE SPECIFIC TO HEAT INDUCED APURINIC SITES IN DNA OF *B.*

*SUBTILIS*


1.   INTRODUCTION


Under various conditions, nucleoside bases are released from

cellular DNA through the hydrolysis of N-glycosidic bonds, thereby

leaving apurinic or apyrimidinic sites (119,120).   Nucleoside bases

are released by spontaneous hydrolysis, acid hydrolysis, heat treat-

ment (38,47), and γ-irradiation (121,122).   Uracil which appeared

in DNA either by misincorporation of dUMP instead of dTMP residues

(123) or by deamination of cytosine by heat (124), bisulfite (125),

or nitrous acid (126), is removed by a uracil-DNA glycosylase (48).

In addition, some modified bases in DNA which had been treated with

alkylating agents like methylmethane sulfonate were subjected to spo-

taneous or enzymatic release (127).

As shown in Chapter III and V, DNA bases were released by *in vivo*

and *in vitro* acid-heat treatments.   Therefore, the repair of apurinic

or apyrimidinic sites is probably essential for the maintenance of

cellular DNA.

The endonucleases specific to apurinic sites in DNA have been

isolated from *E. coli* (51-53), and *H. influenzae* (54), *M. luteus* ( 55,56), *B. subtilis* (57), and *B. stearothermophilus* (58).   These endonucleases have been studied in relation to the DNA injuries in- duced by alkylating agents, X-ray irradiation, ultraviolet irradiation, or carcinogenic substances and their repair system.

It was found in the previous chapter that acid-heat treatment induced apurinic sites in DNA of spores and the damaged DNA was re- paired during germination.

In this chapter a new APendodeoxyribonuclease (Apurinic/apyri- midinic endodeoxyribonuclease) which acts specifically on the heat injured DNA was isolated from *B. subtilis* and characterized.


## 2.   MATERIALS AND METHODS


### Preparation of substrates

*B. subtilis* 168Tt (thy⁻, trp⁻) was grown at 37°C in Schaeffer liquid nutrient broth (74) containing 0.5 μCi/ml [methyl-$^3$H] thymidine (50 Ci/mmol ; The Radiochemical Center, Amersham), and was harvested at the late logarithmic growth phase.   The DNA was extracted accord- ing to the method of Marmur (116), dissolved in SSC solution (0.15 M NaCl / 0.015 M sodium citrate, pH 7.0) and stored at -20°C.

Alkylated [$^3$H] DNA and alkylated-heated[$^3$H] DNA were prepared according to the method of Verly and Rassart(52).   To the [$^3$H]DNA dissolved in SSC were added 2 volumes of 1 M sodium phosphate buffer,

pH 7.0 / methylmethane sulfonate to a final concentration of 0.3 M
and the solution was incubated at 37°C for 1 h.  The mixture was
cooled on ice and dialyzed three times at 4°C against SSC (alkylated
[³H]DNA).  Under these conditions, the DNA contained about 550 methyl
groups/$10^6$ daltons (128).  The alkylated [³H]DNA in SSC was heated
at 50°C for 6 h and dialyzed three times at 4°C against SSC contining
0.1 M $MgCl_2$.  By this treatment about 160 alkylated purines / $10^6$
daltons of DNA were lost from alkylated DNA.  Approximately 40 % of
the alkylated-heated DNA was recovered as acid-soluble fraction after
treatment with NaOH which caused a strand break near each apurinic
site.  The depurinated (=alkylated heated ) [³H]DNA thus obtained
was stored at -20°C.

In order to obtain single strand DNA or depurinated single strand
DNA, native DNA or a alkylated-heated DNA was dialyzed against 90 %
formamide, 5 mM potassium phosphate (pH 6.8) and was incubated at 37°C
for 2 h.  After chilling, DNA solutions were again dialyzed against 1 %
formaldehyde, 5 mM sodium phosphate (pH 6.8), and were applied to
hydroxyapatite column to isolate  the single strand DNA by use of
method of Miyazawa and Thomas (129).

The DNA which contains small number of apurinic sites was pre-
pared by heating at 60°C for 30 min in SSC (pH 3.5).

Heated DNA was reduced by the modified method described by Hadi
and Goldthwait (130) in order to protect the apurinic sites from al-
kaline treatment.

For the end group analysis, the radioactive reduction of depuri-

nated DNA was prepared according to the modified method of Gossard

and Verly (131).   Sodium boro-[$^3$H]-hydride (260 mCi/mmol : New En-

gland Nuclear) was dissolved in distilled water to a final concentra-

tion of 10 μg/μl.   Solution of depurinated DNA (200 μg/ml) was dia-

lyzed against 0.01 M sodium borate buffer, pH 9.8.   Portions of 50,

25, and 25 μl of sodium boro-[$^3$H]-hydride solution were successively

added and the mixture was left at room temperature for 1 h ; non-

labeled NaBH$_4$ (15 mg/200 μl) in 0.01 M NaOH was finally added.

After 30 min the excess hydride was destroyed with 0.2 ml of 1 M

sodium acetate buffer, pH 5.0.   When the hydrogen release finished,

the solution was dialyzed six times against 2 ℓ SSC containing 0.01

M MgCl$_2$ (pH 7.0) at 4°C for a week.

Enzyme assay

Ten μl of alkylated-heated DNA containing 0.1 M MgCl$_2$ labeled

with [methyl-$^3$H] thymidine (0.67 μg, 11,000 cpm) in SSC (pH 7.0) and

80 μl of buffer A (50 mM Tris-HCl, 0.1 mM EDTA, 0.1 mM 2-mercapto-

ethanol, 10 % glycerol, pH8.0) were mixed with 10 μl of enzyme solu-

tion in buffer A.   After incubation at 37°C for 15 min, the tubes

were chilled and 200 μl of SSC containing 400 μg of calf thymus DNA

and 200 μl of 12.5 % perchloric acid were successively added to each

tube to stop the enzyme reaction.   After being kept at 0°C for 15 min,

the mixtures were centrifuged at 13,000 x g for 10 min, and the radio-

activity of the supernatants was determined in toluene-TritonX-100

scintillation fluid in a liquid scintillation counter. One unit of enzyme activity was defined as the amount of enzyme activity necessary to release 10 % acid-soluble radioactivity of the substrate.

Sedimentation coefficient determination

The sedimentation coefficient and molecular weight of APendo-deoxyribonuclease were determined by glycerol gradient centrifugation by the method of Martin and Ames (132). Linear 10 to 30 % (v/v) glycerol gradient in buffer A was used. Centrifugation was performed at 4°C for 24 h at 38,000 rpm in a Beckman SW.50.1. rotor. The gradients were fractionated with ISCO density gradient fractionator and UV-absorbance monitor (Model UA-5). APendodeoxyribonuclease activity in each fraction was assayed.

Polyacrylamide gel electrophoresis

Electrophoresis was carried out with 7.5 % polyacrylamide gels as described by Gabriel (133). After electrophoresis at 4°C, one of the gels was stained with coomassie brilliant blue for detection of protein and another gel was cut in 3 mm slices. The slices were eluted over night with 0.2 ml of buffer A and APendodeoxyribonuclease activity was measured.

Sodium dodecyl sulfate gel electrophoresis

The method described by Weber and Osborn (134) was followed. Cytochrome c, chymotrypsinogen A, ovalbumin, and bovine serum albumin were used as marker proteins.

Sedimentation analysis of DNA in alkaline sucrose density gradient

DNA was layered on linear 5 - 20 % alkaline sucrose density gradient (pH 12.0) and centrifuged at 40.000 rpm for 4.5 h at 5°C in a Beckman SW.50.1 rotor.   The gradients were analyzed in an ISCO density gradient fractionator and UV-absorbance monitor (Model UA-5).

## Protein determination

Protein was determined by the method of Lowry et al. (135) by using bovine serum albumin as a standard.

## Enzymes

*E. coli* alkaline phosphatase and snake venom phosphodiesterase were bought from Sigma Chemical Co.   Spleen phosphodiesterase and polynucleotide 5'-hydroxyl-kinase were purchased from Boehringer Mannheim Co.

## 3.   EXPERIMENTS AND RESULTS

## Activities of the crude extract

*B. subtilis* 168Tt (thy$^-$, trp$^-$) was grown in nutrient broth (0.5 % peptone and 0.5 % beef extract) at 37°C and was harvested at the late logarithmic growth phase.   The vegetative cells were suspended in buffer A and disrupted with French press.   The suspension was centrifuged at 16,000 x g for 30 min.   The supernatant is referred to as the crude extract.

Fig. 21. gives the acid-soluble radioactivity formed as a function of the amount of enzyme ; the acid-soluble radioactivity increased
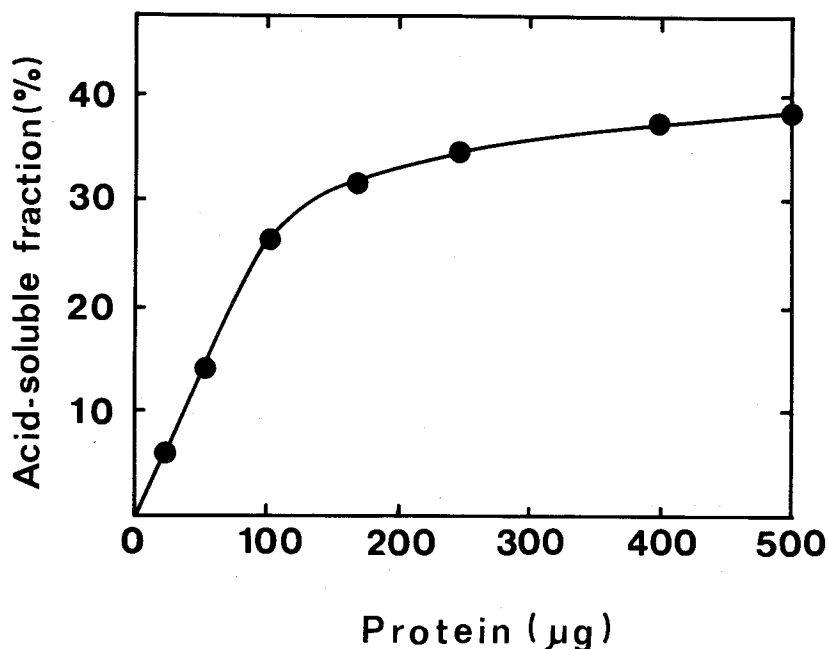
Fig. 21. Acid-soluble radioactivity produced from depurinated [³H] DNA as a function of the enzyme concentration.

To 50 µl of alkylated-depurinated DNA, was added 50 µl of buffer A containing increasing amounts of the crude extract. After 15 min at 37°C, the acid-soluble radioactivity was measured. The acid-soluble radioactivity (in per cent of the substrate radioactivity) is given as a function of the amount of enzyme (expressed in micro-grams of protein).

rapidly first and approached a maximum value of 40 %. This is the acid-soluble radioactivity obtained after an incubation with NaOH in-stead of enzyme. Fig. 21 shows that, as long as the acid-soluble radioactivity remains under 25 %, it is approximately proportional to the amount of enzyme. In all assays used to measure the amount of APendodeoxyribonuclease activity, the enzyme was diluted in
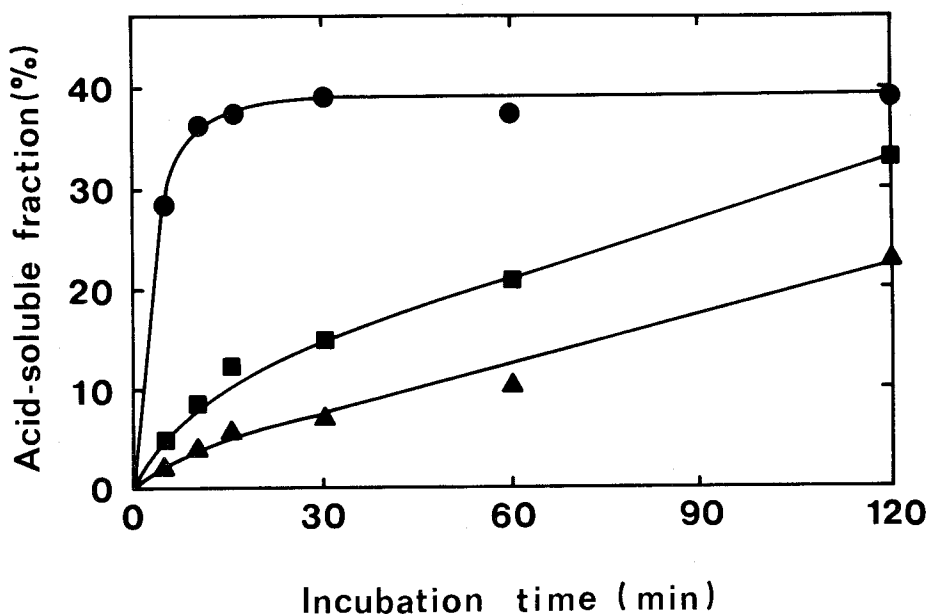
Fig. 22. Action of the crude extract of B. *subtilis* cells on native, alkylated, and alkylated-depurinated DNA.

Samples (100 μl) of the crude extract were mixed with 100 μl of [$^3$H] DNA, either native (▲), alkylated (■), or alkylated-depurinated (●) : the mixtures were incubated at 37°C and aliquots taken from 0 to 120 min to measure the acid-soluble radioactivity.  The results are expressed as fractions of the substrate radioactivity (acid-soluble fraction).

buffer A so that the acid-soluble radioactivity liberated from depuri-nated [$^3$H]DNA fell below 25 %.

Crude extract was mixed with $^3$H-labeled native DNA, alkylated DNA, and depurinated DNA, respectively, and incubated at 37°C ; aliquots were taken after 0 to 120 min to measure the acid-soluble radioactivity.  Fig. 22 shows that the acid-soluble fraction in

depurinated DNA increased rapidly during the first 15 min at 37°C

reaching 38 % of the input radioactivity, but in native DNA only 5 %

of the total radioactivity was made acid-soluble for 15 min.

Alkylated DNA was hydrolyzed more slowly than depurinated DNA but

faster than native DNA.

Purification of enzyme

B. *subtilis* 168Tt (thy$^-$, trp$^-$) was grown in nutrient broth at

37°C by use of jar fermenter.   The cells were harvested at late ex-

ponential phase by centrifugation, washed with cold TM buffer (0.01

M Tris-HCl, 0.06 M NH$_4$Cl, 0.01 M Mg-acetate, and 0.07 M 2-mercapto-

ethanol, pH 7.5), and stored at -20°C.   The frozen cell paste (216

g) was thawed and suspended in 800 ml of buffer A.   Cells were dis-

rupted by four 10 min periods with intermittent cooling with a Tomy

ultrasonic disruptor UR200P at 0°C.   The cell debris was removed by

centrifugation at 17,000 x g for 30 min (Preparation I).   To the

supernatant, 3,2 % streptomycin sulfate in buffer A was added slowly

to a final concentration of 0.8 %.   The suspension was centrifuged

at 17,000 x g for 20 min.   The supernatant fraction was treated with

solid ammonium sulfate at 4°C to reach 50 % saturation ; after centri-

fugation, the supernatant was brought to 80 % saturation.   After

centrifugation, the sediment was resolved in buffer A and dialyzed

against the same buffer (Preparation II).

A column (3.6 x 20 cm) of DEAE-cellulose (Whatman) was equili-

brated with buffer A at 4°C.   Preparation II was applied and the
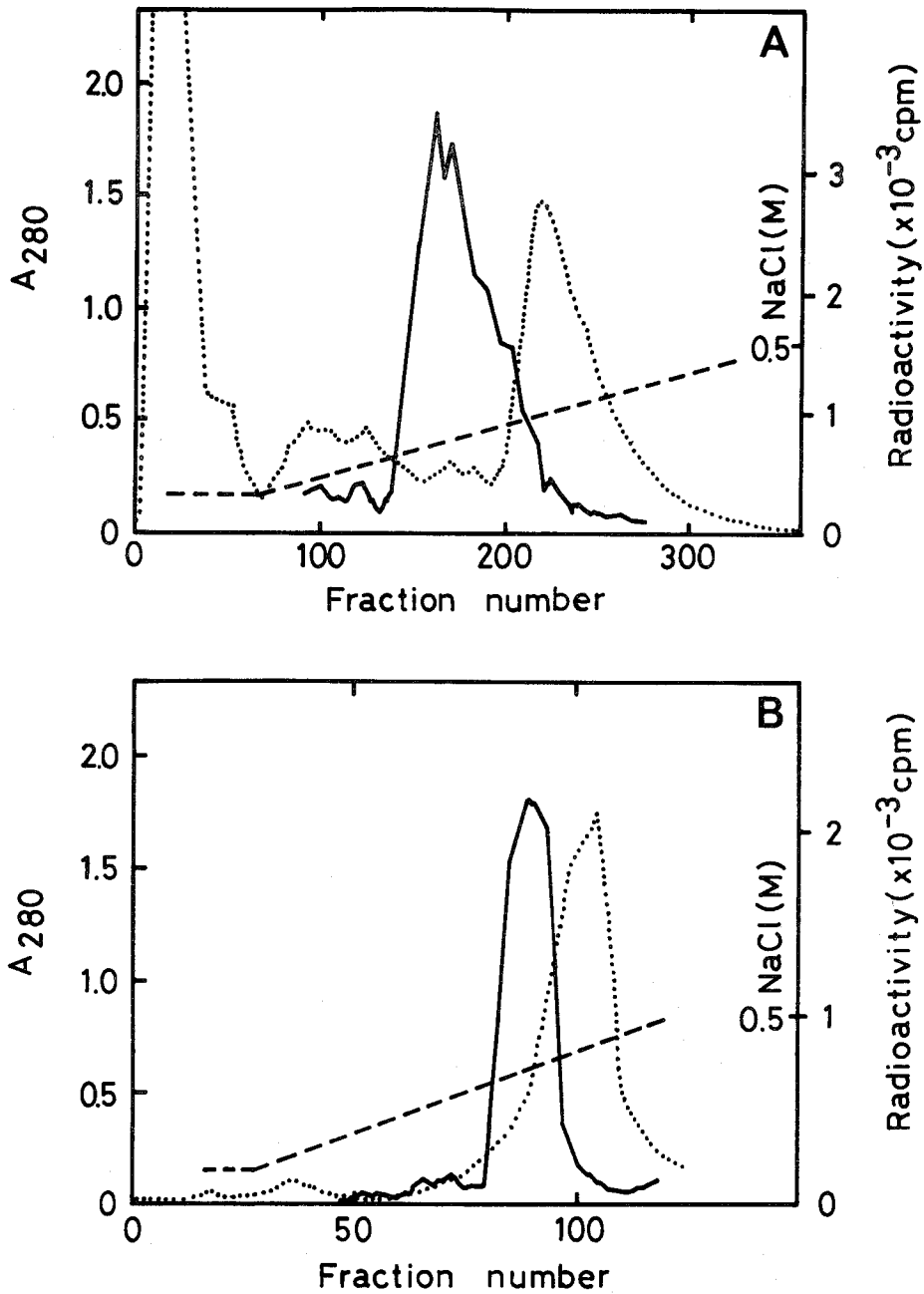
Fig. 23.   Chromatographic purification of APendodeoxyribonuclease.
Symbols : ———— , enzyme activity on depurinated DNA ; ········· , ab-
sorbance at 280 nm ; ——— , concentration of NaCl (M).   A, DEAE-
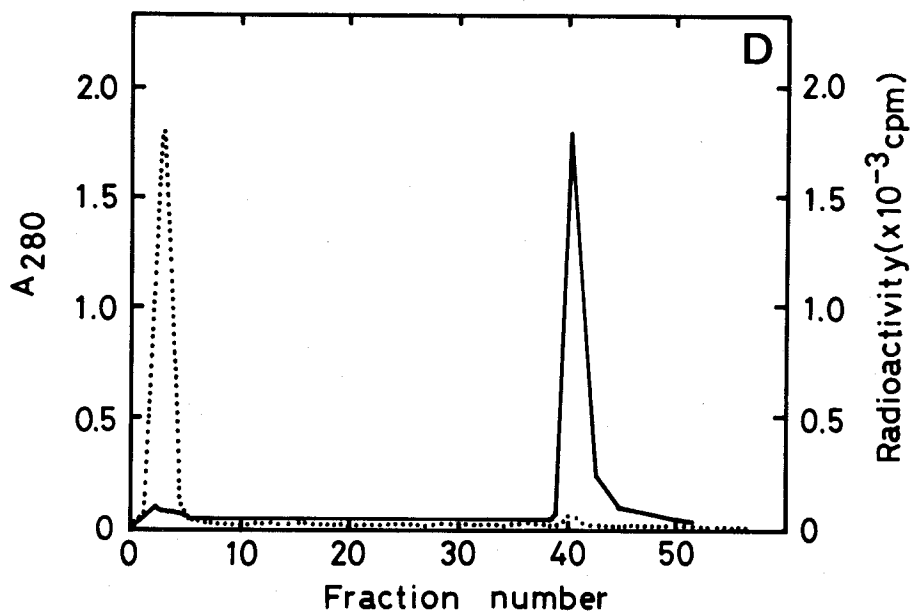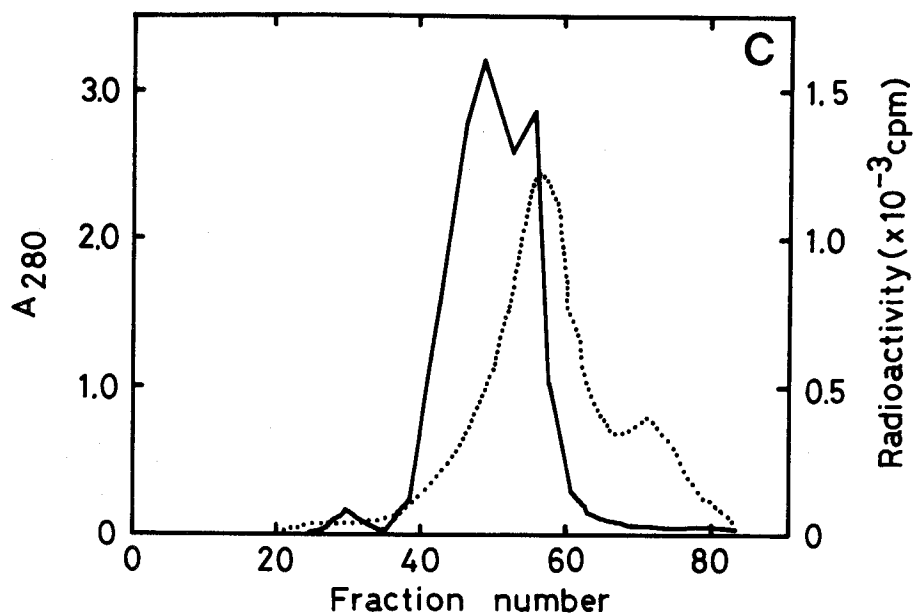cellulose  ;  B, DEAE-Sephadex A-50.

Fig. 23.   Chromatographic purification of APendodeoxyribonuclease.
Symbols : ———— , enzyme activity on depurinated DNA ; ·········, ab-
sorbance at 280 nm.   C, Sephadex G-200  ;  D, DNA-cellulose.

column was washed with 200 ml of buffer A and 500 ml of the same

buffer containing 0.1 M NaCl.   Six liters of a 0.1 M to 0.5 M NaCl

linear gradient in buffer A was eluted at rate of 120 ml/h ; frac-

tions of 20 ml were collected.   Figure 23 A shows that the enzyme

was eluted between 0.25 and 0.32 M NaCl.   Fractions 145 to 200 were

pooled and concentrated to 160 ml with a Millipore ultrafiltration

apparatus with a PSAC filter and then dialyzed against buffer A (Pre-

paration III).

A column (2 x 25 cm) of DEAE-Sephadex A-50 (Pharmacia) was equil-

ibrated with buffer A at 4°C.   Preparation III was applied to the

column at a flow rate of 40 ml/h.   The column was washed with 100 ml

of buffer A containing 0.1 M NaCl and eluted with 1 liter of a 0.1 M

to 0.5 M NaCl linear gradient in buffer A ; fractions of 10 ml were

collected.   Figure 23 B shows that the enzyme was eluted between

0.32 and 0.41 M NaCl.   Fractions 83 to 96 were pooled (Preparation

IV).

Three ml of the concentrated Preparation IV were placed on a

Sephadex G-200 (Pharmacia) column (2.8 x 40 cm) equilibrated with

buffer A ; the elution was carried out with the same buffer at a rate

of 12 ml/h, and 2.5 ml fractions were collected (Fig. 23 C).

Fractions 41 to 50 were pooled (Preparation V).

DNA-cellulose was prepared according to the method of Litman

(136).   Preparation V was concentrated and applied to a DNA-cellulose

(0.8 x 7 cm) equilibrated with buffer A.   The column was washed with

Table 3. Purification of *B. subtilis* endonuclease for apurinic sites.

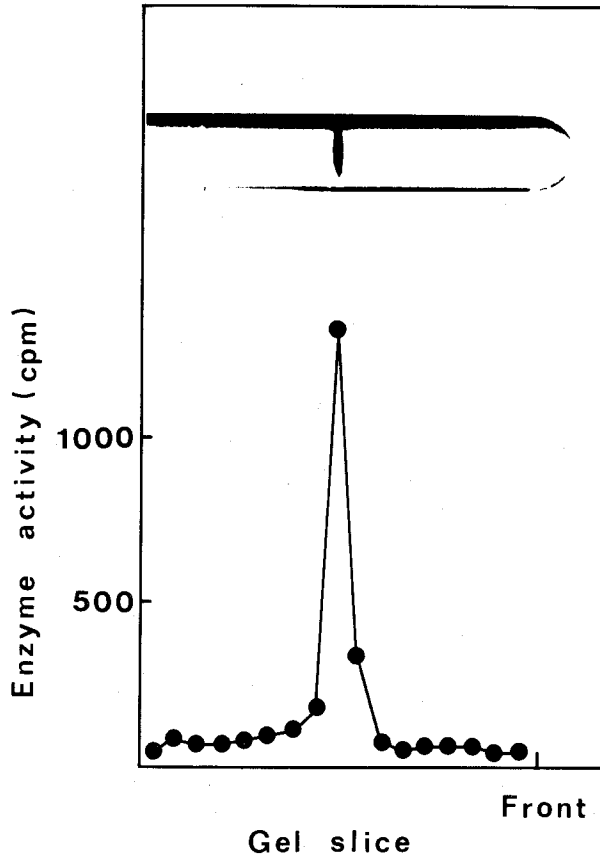| | Preparation | Volume | Total protein | Endonuclease units | Specific activity |
|---|---|---|---|---|---|
| I | Crude extract | 838 ml | 12402 mg | $2022 \times 10^3$ units | 163 units/mg |
| II | Ammonium sulfate | 370 | 7918 | 1679 | 212 |
| III | DEAE-cellulose | 157 | 184 | 1260 | 6850 |
| IV | DEAE-Sephadex | 140 | 57.4 | 1095 | 19100 |
| V | Sephadex G-200 | 35 | 28.4 | 895 | 31500 |
| VI | DNA-cellulose | 8 | 0.304 | 436 | 1430000 |



Fig. 24. Polyacrylamide gel electrophoresis of Preparation VI of APendodeoxyribonuclease.

Upper : a photograph of the gel stained with Commassie brilliant blue ; lower : enzyme activity on alkylated-depurinated [$^3$H]DNA.
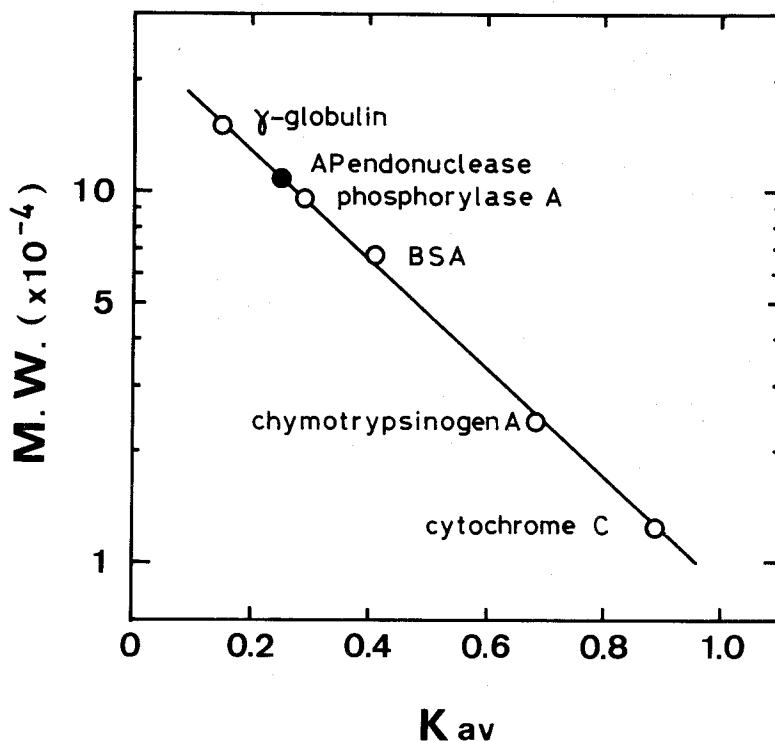
Fig. 25. Molecular weight determination of APendodeoxyribonuclease
on Sephadex G-200.
The abbreviation and molecular weight (in parentheses) of standard
proteins are : γ-globulin (156,000) ; phosphorylase A (96,000) ;
bovine serum albumin(BSA) (68,000) ; chymotrypsinogen A (25,000) ;
cytochrome c (12,000).


80 ml of the same buffer, and then eluted with 0.4 M NaCl in buffer A

at a rate of 4 ml/h ; fractions of 2 ml were collected (Fig. 23 D).

The fractions showing activity were pooled and dialyzed against the

buffer A (Preparation VI), and the enzyme solution was kept at -20°C.

These purification procedures are summarized in Table 3.
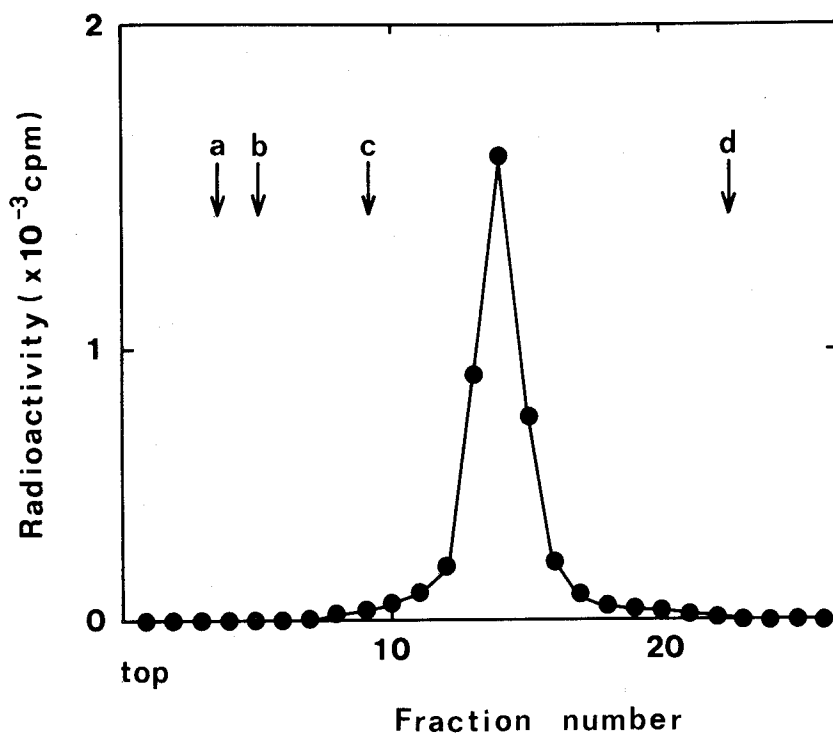
Fig. 26. Glycerol gradient centrifugation of APendodeoxyribonuclease.
Parallel gradients were run with the four marker proteins as stan-
dards. The positions of the standards are given by arrows : (a)
cytochrome c, (b) chymotrypsinogen A, (c) bovine serum albumin, (d)
catalase.


About 8,800-fold purification was achieved with an overall yield of
about 22 %.

The purity of the enzyme was determined by polyacrylamide gel
electrophoresis. Gel electrophoresis revealed a single protein band
which correspond to the APendodeoxyribonuclease activity (Fig. 24).

Properties of the enzyme

Molecular weight      The molecular weight of APendodeoxyribonuclease
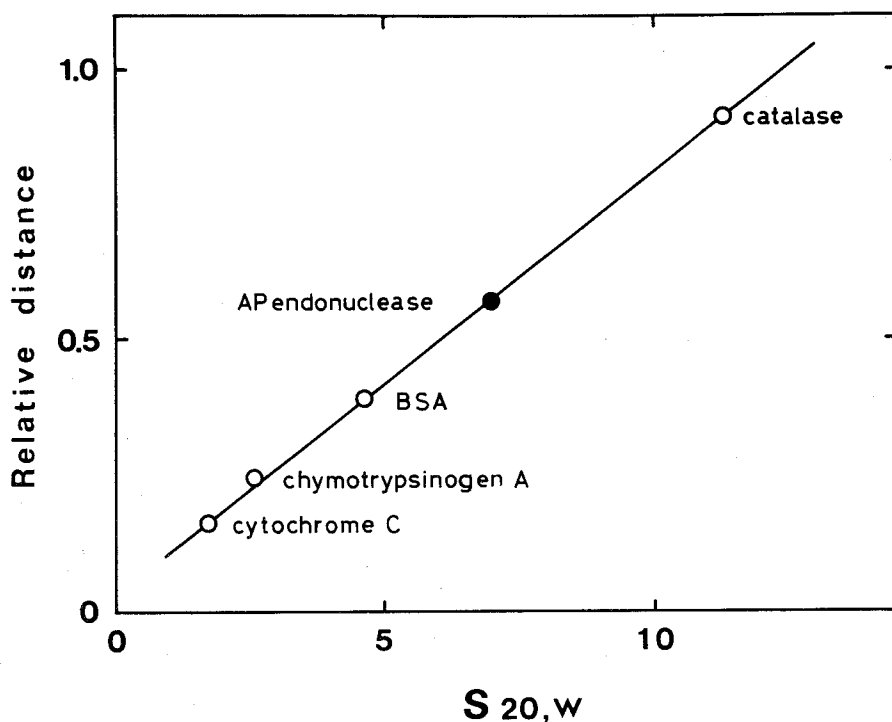
Fig. 27.    Determination of molecular weight of APendodeoxyribonucle-
ase by glycerol gradient centrifugation.

The sedimentation coefficient $(S_{20,W})$ and molecular weight (in paren-
theses) of the standard proteins are : catalase from beef liver, 11.3
S (230,000) ; bovine serum albumin (BSA), 4.6 S (68,000) ; chymotrypsi-
-nogen A, 2.54 S (25,000) : chytochrome c, 1.84 S (12,000)


was determined by Sephadex G-200 column chromatography.    The elution

volumes of APendodeoxyribonuclease and reference proteins are plotted

against the logarithms of their molecular weight (Fig. 25).    From

this plot, the molecular weight of APendodeoxyribonuclease was esti-

mated to be 108,000.

Furthermore, in order to ascertain this result, the molecular
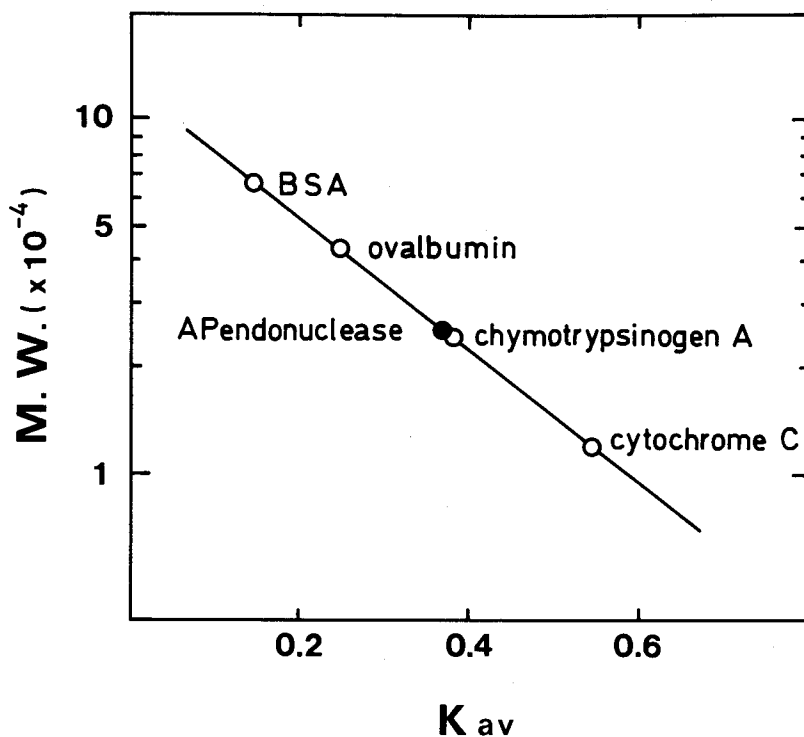
Fig. 28. Determination of molecular weight of denatured APendodeoxy-
ribonuclease by SDS-polyacrylamide gel electrophoresis.
The abbreviation and molecular weight (in parentheses) of standard
proteins are bovine serum albumin(BSA) (68,000) ; ovalbumin (43,000)
; chymotrypsinogen A (25,000) ; cytochrome c (12,000).


weight of APendodeoxyribonuclease was estimated by glycerol gradient

centrifugation.  The enzyme activity of Preparation VI migrated as

a single peak in glycerol gradient centrifugation (Fig. 26).  In

comparison with the marker proteins, the sedimentation coefficient

of APendodeoxyribonuclease was calculated to be 7 S (Fig. 27).  The

molecular weight of APendodeoxyribonuclease was estimated to be

105,000 daltons according to the method devised by Martin amd Ames

(132), assuming that the enzyme had a spherical shape.

Sodium dodecyl sulfate gel electrophoresis revealed that the

purified enzyme (Preparation VI) was composed of a single protein.

Table 4.  Effects of EDTA and $Mg^{2+}$ on APendonuclease
activity.
   Ten µl of alkylated-depurinated DNA in SSC (pH 7.0)
and 10 µl of Preparation VI dialyzed against buffer B
(0.05 M Tris-HCl, pH 8.0/0.1 mM 2-mercaptoethanol/10
% glycerol) were mixed with 80 µl of buffer B ; EDTA
or $MgCl_2$ was added to reach the indicated concentra-
tion.   After incubation at 37°C for 15 min, radio-
activity in the acid-soluble fraction was measured.

| Experiment | Addition | Concentration (mM) | Relative activity (%) |
|---|---|---|---|
| 1 [a] | EDTA | 0 | 100 |
| | | 0.5 | 96 |
| | | 1.0 | 102 |
| | | 5.0 | 103 |
| | | 10.0 | 65 |
| | | 20.0 | 4. |
| 2 [b] | $Mg^{2+}$ | 0 | 10 |
| | | 1.0 | 8 |
| | | 2.0 | 13 |
| | | 5.0 | 28 |
| | | 10.0 | 55 |
| | | 20.0 | 100 |
| | | 40.0 | 91 |
| | | 60.0 | 44 |
| | | 80.0 | 30 |
| | | 100.0 | 19 |

a, In the first experiment, all reaction mixtures
   contained 10 mM $MgCl_2$.
b, In the second experiment, EDTA was absent in all
   reaction mixtures.

The molecular weight of this protein was estimated to be about 26,000
by comparison with the electrophoretic mobilities of standard proteins
(Fig. 28).   Therefore, it is thought that this enzyme consists of
four identical 26,000 daltons subunits.

Requirements for divalent cations

APendodeoxyribonuclease activity on depurinated [$^3$H]DNA was meas-
ured as functions of the concentration of $Mg^{2+}$ (Table 4).   The purified
enzyme absolutely required $Mg^{2+}$ for its activity, and no activity was
detected in the absence of $Mg^{2+}$.   The optimum concentration of $MgCl_2$
was 20 mM and the enzyme activity was inhibited at higher concentrations.

Table 5.  Effects of $Mn^{2+}$ and $Ca^{2+}$ on APendonuclease activity.
    Ten µl of alkylated-depurinated DNA in SSC (pH 7.0) and 10
µl of Preparation VI dialyzed against buffer B were mixed with
80 µl of buffer B ; $MnCl_2$ or $CaCl_2$ was added to reach the in-
dicated concentration.   After incubation at 37°C for 15 min,
radioactivity in the acid-soluble fraction  was measured.

| Addition | Concentration (mM) | Relative activity (%) |
|---|---|---|
| None | 0 | 3 |
| $MnCl_2$ | 1 | 6 |
| | 2 | 10 |
| | 5 | 22 |
| | 10 | 19 |
| | 20 | 8 |
| $CaCl_2$ | 1 | 4 |
| | 2 | 4 |
| | 5 | 3 |
| | 10 | 3 |
| | 20 | 4 |
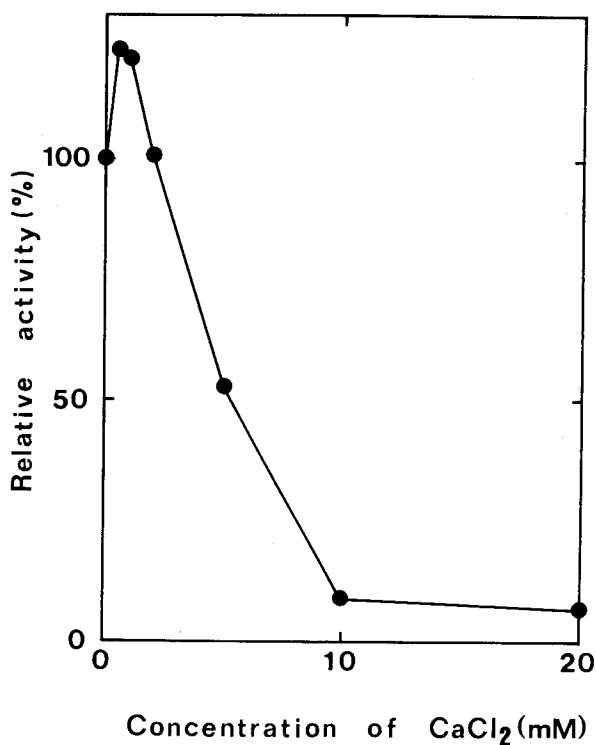
Fig. 29.   Effect of CaCl$_2$ on APendodeoxyribonuclease activity.
The reaction mixtures contained 10 mM MgCl$_2$.   The activity without
CaCl$_2$ was scored as 100 %.


When reaction mixture contained 10 mM MgCl$_2$, 5 mM EDTA did not inhib-

ited the enzyme activity, whereas 20 mM EDTA inhibited completely the

activity.

Among the other divalent cations tested, Ca$^{2+}$ was not effective and

therefore was unable to replace Mg$^{2+}$, although Mn$^{2+}$ had a only slight

activity (Table 5).   When reaction mixture contained 10 mM MgCl$_2$, 10

mM CaCl$_2$ completely inhibited the enzyme activity (Fig. 29).

Effect of ionic strength    The enzyme activity is unusually resistant

to the presence of NaCl in reaction mixtures.   About 60 % of maximum
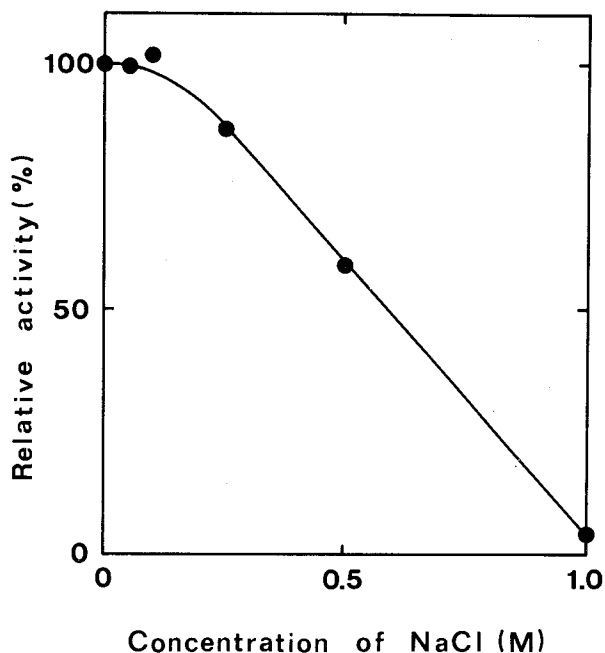
Fig. 30.   Relative activity of APendodeoxyribonuclease (1.5 units) at different NaCl concentrations.

activity was retained in the presence of 0.5 M NaCl and the activity was completely inhibited by 1 M NaCl (Fig. 30).

   Sulfhydryl requirements     The effect of sulfhydryl reagents were summerized in Table 6.   2-mercaptoethanol was not effective to the enzyme activity.   However, about 30 - 40 % of stimulation of the activity was observed in the presence of glutathione or dithiothreitol. In the absence of 2-mercaptoethanol in the reaction mixture, the enzyme activity was completely inhibited by addition of 1 mM p-chloromercuribenzoate but was not influenced by addition of 5 mM N-ethylmaleimide.

   Effect of nucleotides     Table 7 shows that APendodeoxyribonuclease

Table 6. Effect of sulfhydryl reagents on the activity of APendonuclease.

| Additions [a] | Concentration (mM) | Relative activity (%) |
|---|---|---|
| None | 0 | 100 |
| 2-Mercaptoethanol | 1 | 98 |
| | 2 | 102 |
| | 5 | 93 |
| | 10 | 86 |
| Glutathione | 0.1 | 103 |
| | 0.2 | 106 |
| | 0.5 | 129 |
| | 1 | 143 |
| | 2 | 102 |
| Dithiothreitol | 0.1 | 101 |
| | 0.2 | 119 |
| | 0.5 | 137 |
| | 1 | 122 |
| | 2 | 122 |
| N-Ethylmaleimide | 0.2 | 90 |
| | 0.5 | 88 |
| | 1 | 96 |
| | 2 | 87 |
| | 5 | 98 |
| p-Chloromercuri-benzoate-2Na | 0.1 | 23 |
| | 0.2 | 19 |
| | 0.5 | 13 |
| | 1 | 7.5 |
| | 2 | 4.6 |

a, Activity was measured in the standard assay conditions, except that 2-mercaptoethanol was omitted from the assay mixture.

Table 7. Effect of different nucleotides on the activity of APendonuclease.

| Additions [a] | Concentration (mM) | Relative activity (%) |
|---|---|---|
| None | 0 | 100 |
| ATP | 0.01 | 107 |
| | 0.02 | 108 |
| | 0.05 | 113 |
| | 0.1 | 122 |
| | 0.2 | 120 |
| | 0.5 | 105 |
| dATP | 0.01 | 122 |
| | 0.02 | 134 |
| | 0.05 | 120 |
| | 0.1 | 127 |
| | 0.2 | 130 |
| | 0.5 | 130 |

a, Nucleotides were added to the standard assay mixture.

activity was increased slightly (10 to 30 %) in the presence of 0.01 to 0.5 mM of ATP or dATP.
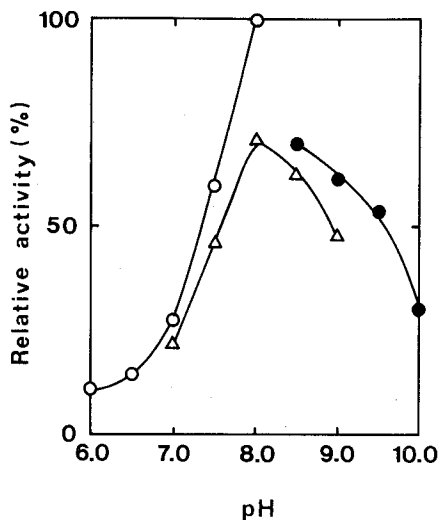
Fig. 31. Effect of pH on APendo-
deoxyribonuclease activity.
Activity was measured in the
standard assay conditions, except
that buffer A was replaced by 50
mM Tris-malate-KOH (O), 50 mM
Tris-HCl (△), or 50 mM glycine-
KOH (●). The activity in Tris-
malate-KOH at pH 8.0 was set at
100 %.
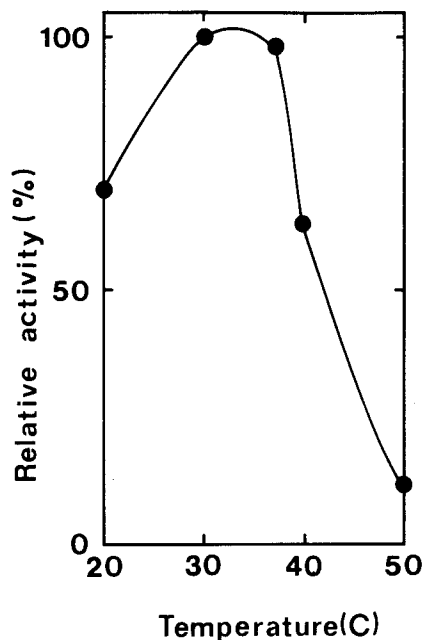
Fig. 32. Effect of reaction tem-
perature on APendodeoxyribonucle-
ase activity. Activity was meas-
ured in the standard assay condi-
tions, except that reaction tem-
perature was varied.

Effect of pH    The optimum pH for the APendodeoxyribonuclease
activity was examined using different buffer solutions ranging from
pH 6.0 to 10.0 (fig. 31).    The optimum pH for the activity of puri-
fied enzyme was 8.0.

Effect of temperature    The effect of incubation temperature on
the enzyme activity is shown in Fig. 32.    The optimum temperature
for the enzyme activity appears to be about 34°C.

- 82 -
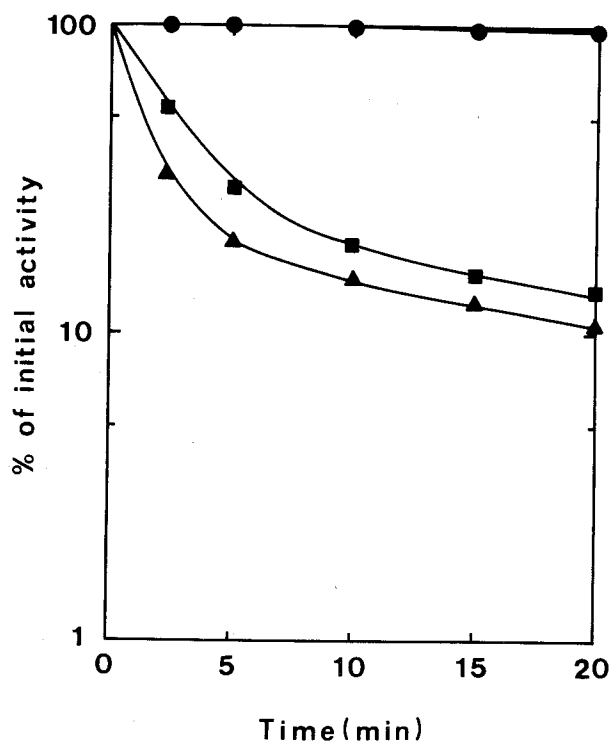
Fig. 33. Heat inactivation of APendodeoxyribonuclease.
APendodeoxyribonuclease was incubated at various temperatures for in-
dicated time and immediately after the incubation, the enzyme activity
was assayed. Symbols : ● , incubated at 0°C ; ■ , incubated at 37
°C ; ▲ , incubated at 40°C.


Thermosensitivity of the enzyme    Fig. 33 shows the thermosensi-

tivity of the enzyme activity at 0, 37, and 40°C.    Aliquots (200 µl)

of Preparation VI (3 units) were heated at 37, or 40°C for 0 - 20 min

in test tubes.    Immediately after the heating, the remaining APendo-

deoxyribonuclease activity was assayed.    The results, expressed as

percentage of the initial activity, were plotted on a semilogarithmic

scale.    At a low enzyme concentration the purified enzyme (Prepara-
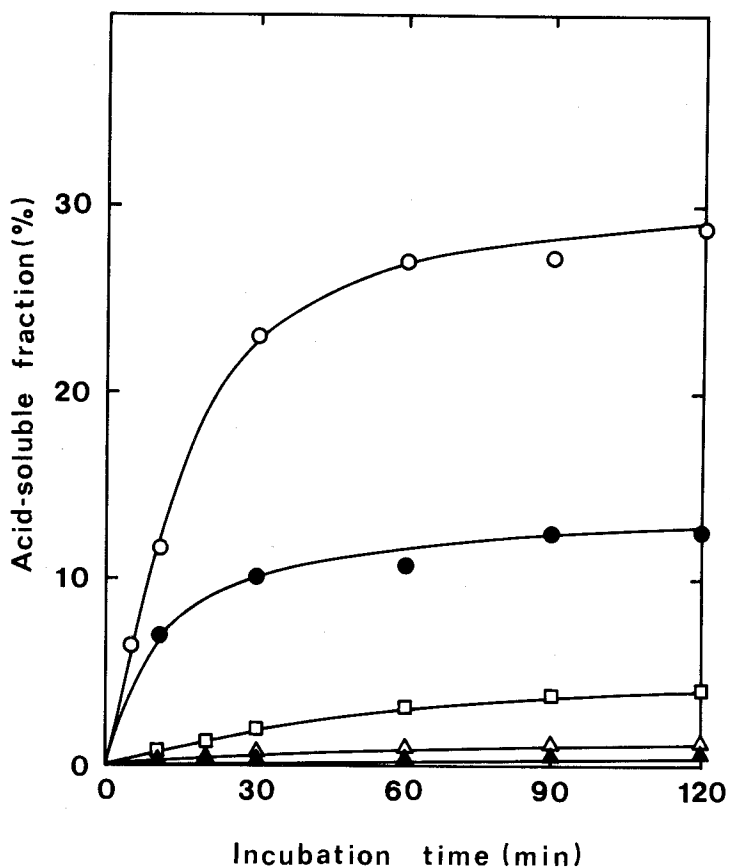
Fig. 34. Activities of the purified enzyme (Preparation VI) on native, denatured, alkylated, depurinated and single strand depurinated DNA. (△), native DNA ; (▲) denatured DNA ; (□), alkylated DNA ; (●), single strand depurinated DNA ; (○), depurinated DNA. (Concentration of substrate DNA was 6.7 µg/ml). Aliquots were taken after 0 to 120 min incubation to measure the acid-soluble radioactivity which was plotted as fraction of the total radioactivity.

tion VI) was sensitive to heat ; the half lives of the enzyme at 37°C and 40°C were about 3 min and 1 min, respectively. However, the enzyme was stable at 0°C. The concentrated Preparation VI (DNA-

cellulose fraction) was stable for three months at -20°C in buffer A. However, the enzyme was easily inactivated by the repeated freezing and melting.

Substrate specificity    Preparation VI was mixed with ^{3}H-labeled native DNA, single strand untreated DNA, alkylated DNA, single strand alkylated-depurinated DNA, and double strand alkylated-depurinated DNA, respectively, and incubated at 37°C ; aliquots were taken after 0 to 120 min incubation to measure the acid-soluble radioactivity. Figure 34 shows that Preparation VI has a remarkable activity to al-kylated-depurinated DNA, but its activity to native, single strand, and alkylated DNA was very little.   It is reported that endonuclease IV, VI of *E. coli* (52,53) and APendonuclease of *B. subtilis* (57) are specific to apurinic sites in double strand DNA and not active to single strand depurinated DNA.   However, Preparation VI is also active to single strand alkylated-depurinated DNA.   Its activity is about a half of that to double strand alkylated-depurinated DNA.

 *E. coli* APendonuclease purified by Verly and his colleagues ( endonuclease VI) (52,131) and *H. influenzae* APendonuclease purified by Clements et al. (54) have an exonuclease activity.   Figure 34 shows that preparation VI's activity to native and single strand DNA is very low.   In order to investigate details of the exonuclease activity, the exonuclease activity to ^{3}H-labeled sonicated DNA of *B. subtilis* which was prepared with the method of Gossard and Verly( 131) was measured in the presence or absence of $MgCl_2$.   Consequently,
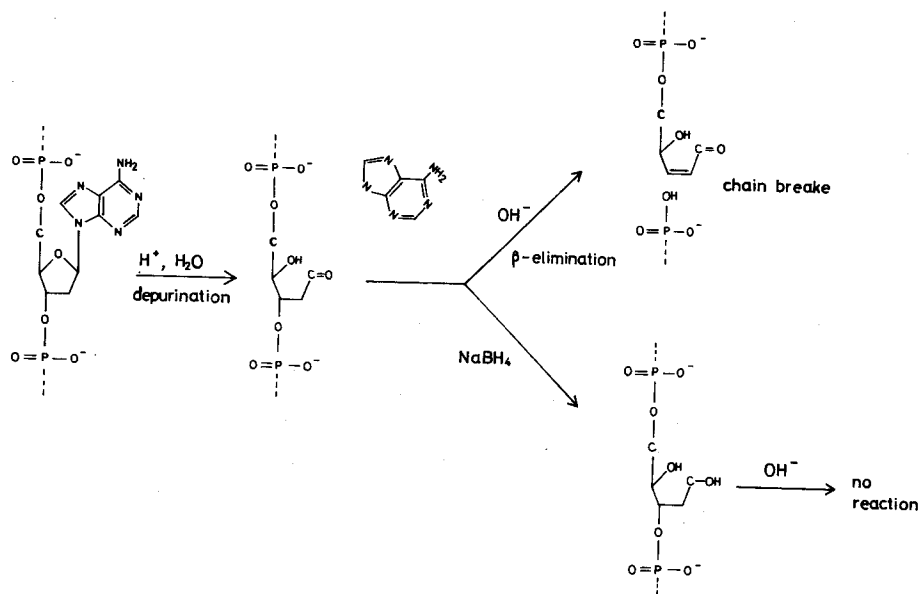
Fig. 35. Molecular mechanism of depurination and subsequent chain breakage.


Preparation VI has no exonuclease activity.

Enzymatic degradation of heated-reduced DNA    It is reported that the deoxyribose residue at an apurinic or apyrimidinic sites in DNA exists in equilibrium between the furanose form and the aldehyde form. In alkaline solution, a β-elimination reaction consequently occurs. In this reaction phosphate is eliminated from the 3' position of an adjacent deoxyribose residue in its aldehyde form, so a chain break taken place at the 3' side of the lesion (118,137,138).   Hadi and Goldthwait (130) reported that it was possible to stabilize the alkali-labile apurinic sites by adding reducing agent $NaBH_4$.   Figure 35 shows molecular mechanism of apurinic sites, β-elimination, and reduction with $NaBH_4$.

Fig. 36. Effect of NaOH and APendodeoxyribonuclease on heated-reduced DNA. *B. subtilis* DNA in SSC(pH 3.5) was heated at 60°C for 30 min and was cooled rapidly.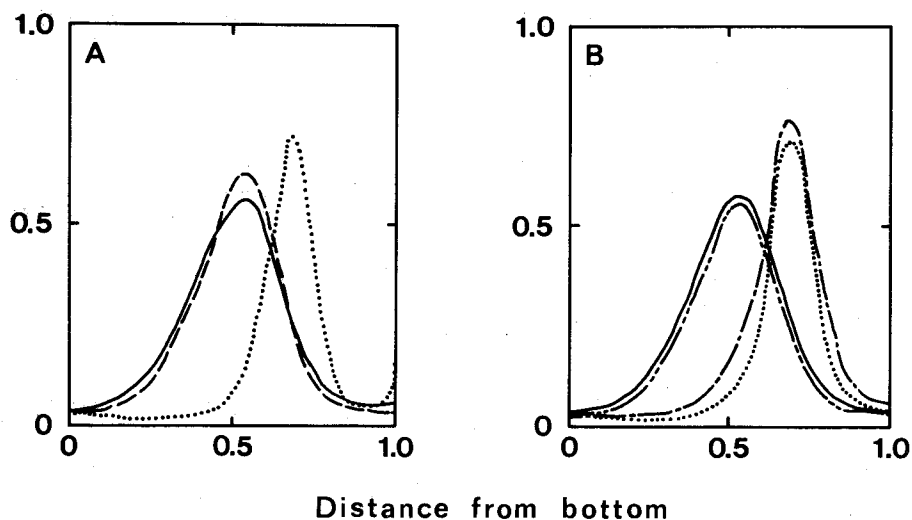 After addition of potassium phosphate buffer (pH 6.5), NaBH$_4$ was added to reduce heated DNA. The solutions were kept at room temperature for 1 h and were dialyzed against SSC containing 10 mM MgCl$_2$(pH 7.0) at 4°C for 12 h. Native or heated-reduced DNA was incubated for 30 min at 37°C with Preparation VI. Prior to analysis in alkaline sucrose gradients, all DNA solutions were mixed with 1/20 volume of 2 N NaOH and incubated at 37°C for 15 min. Each reaction mixture was layered on 5-20 % alkaline sucrose density gradients and centrifuged in an SW50.1 rotor at 40,000 rpm for 4.5 h at 5°C. (A) Alkaline sucrose gradient centrifugation of the native DNA (— — —), heated-reduced DNA (———), and heated (·······). (B) Alkaline sucrose gradient centrifugation of the native DNA + enzyme (—···—), heated-reduced DNA + enzyme (—·—), heated-reduced DNA (———), and heated DNA (······).

In order to know whether this enzyme is able to act on very few apurinic sites in large DNA molecules, depurinated (heated at 60°C for 30 min at pH 3.5) and reduced DNA was mixed with the purified

enzyme and incubated at 37°C for 30 min.   After incubation, the re-action mixture was treated with alkali and subjected to alkaline sucrose density gradient centrifugation.   Native DNA and heated-reduced DNA showed the same sedimentation patterns (Fig. 36 A). About the same number of apurinic sites in the heated-reduced DNA were cleaved by the enzyme as were cleaved by alkaline treatment ( Fig. 36 B).   Single strand DNA breaks were not introduced in native DNA with alkali nor with the purified enzyme.   These data show that this endonuclease specifically cleaves the heat-induced alkali-labile sites (i.e. apurinic sites) in DNA.

Table. 8.   Effect of NaOH and APendonuclease on alkylated-depurinated reduced DNA.
Alkylated-depurinated [$^3$H]DNA or alkylated-depurinated [$^3$H]DNA reduced with NaBH$_4$ was incubated for 15 min at 37° C with APendonuclease or NaOH.   The acid-soluble radio-activity was measured and the results are expressed as fractions of the substrate radioactivity.

| Substrate | Proportion of radio-activity in acid-soluble fraction | |
|---|---|---|
| | NaOH | APendo-nuclease |
| | (%) | (%) |
| Alkylated-depurinated DNA | 39 | 24 |
| Alkylated-depurinated reduced DNA | 0.3 | 20 |

Determination of cleavage sites of APendodeoxyribonuclease    The

following two methods were used for determination of the terminus at

cleaved site of purified APendodeoxyribonuclease.

The first was according to the method of Gossard and Verly (131).

One of two tubes containing 200 µl alkylated-depurinated [$^3$H]DNA in

SSC containing 0.1 M $MgCl_2$ was reduced with sodium borohydride by the

same method that was used for the reduction with sodium boro-[$^3$H]-

hydride of non-labeled depurinated DNA. The other was treated identi-

cally without sodium borohydride.   Both solutions were subsequently

dialyzed against SSC containing 0.01 M $MgCl_2$ at 4°C.   Aliquots (70

µl) of the alkylated-depurinated [$^3$H]DNA reduced or not were mixed

with 30 µl of either Preparation VI, or 0.4 M NaOH, or SSC containing

0.01 M $MgCl_2$ (control).   After 15 min at 37°C the mixtures were

cooled in ice water and the acid-soluble radioactivies were measured

: the results obtained for the enzyme and NaOH were corrected for the

control.   Table 8 shows that reduced-depurinated DNA and depurinated

DNA were similarly cleaved by APendodeoxyribonuclease ; on the other

hand, reduction of depurinated DNA suppressed the action of NaOH.

In order to determine the cleavage site, apurinic sites in DNA

were labeled with sodium boro-[$^3$H]-hydride.   After incubation with

APendodeoxyribonuclease the apurinic DNA was denatured by heating at

100°C for 10 min.   The cleaved apurinic DNA was incubated with snake

venom or spleen phosphodiesterase after pretreatment with *E. coli*

alkaline phosphatase (ALP).   After 30 min incubation, cold perchloric

acid was added to stop the reaction.   The radioactivity in acid-

soluble fraction was determined.

Table 9 shows that snake venom phosphodiesterase, which hydrolyzes DNA strand from 3'-site to 5'-site, releases ^{3}H-labeled apurinic sugar moiety of DNA regardless of the reaction mixtures are pretreated with ALP or not.  On the other hand, spleen phosphodiesterase, which hydrolyzes DNA strand from 5'-site to 3'-site, releases little or no ^{3}H-labeled apurinic sugar moiety whether DNA is pretreated with ALP or not.  These results show that this APendodeoxyribonuclease of *B. subtilis* cleaves the 3'-side of apurinic sites in DNA strand

Table 9.  Action of snake venom and spleen phosphodiesterase on apurinic DNA reduced with sodium boro-[^{3}H]-hydride and treated with APendonuclease. Alkylated-depurinated DNA reduced with sodium boro-[^{3}H]-hydride was incubated with APendonuclease at 37°C for 30 min and subsequently denatured by heating at 100°C for 10 min.  Denatured DNA was incubated with snake venom phosphodiesterase (0.4 unit) or with spleen phosphodiesterase (2 units), after a treatment with alkaline phosphatase (2 units).  The acid-soluble ^{3}H was determined and the results were expressed as percentage of total radioactivity in the substrate DNA ; correction is made for a control.

| ALP | Phosphodiesterase | Acid-soluble fraction ( % ) | Corrected ( % ) |
|-----|-------------------|-----------------------------|-----------------|
| -   | None              | 30                          | 0               |
| -   | Snake venom       | 49                          | 19              |
| +   | Snake venom       | 52                          | 22              |
| -   | Spleen            | 29                          | 0               |
| +   | Spleen            | 36                          | 6               |

giving a 3'-OH and 5'-phosphate.

To confirm this result, another method was used. The DNA containing apurinic sites was incubated with APendodeoxyribonucease at 37°C for 30 min or heated in the Hepes-KOH buffer (pH 7.9) at 70°C for 10 h to break the apurinic sites in DNA. After breakage of the apurinic sites, the DNA was denatured by formamide. After being treated with ALP. DNA was incubated with $[\gamma-^{32}P]$ATP (0.3 mCi/μmol) and polynucleotide 5'-hydroxyl-kinase. The reaction was stopped with addition of cold trichloroacetic acid. $^{32}P$ incorporated into acid-insoluble fraction was determined. Table 10 shows that only when the apurinic DNA which is cleaved with APendodeoxyribonuclease or heat is pretreated with ALP $^{32}P$ was remarkably incorporated into acid-insoluble fraction. Lindahl and Anderson (118) reported that when the DNA containing apurinic sites was heated in neutral pH solution, chain breaks were predominantly due to β-elimination and occurred at the 3'-side of apurinic sugar moiety giving a 3'-OH and 5'-phosphate. Therefore, if the 5'-side of apurinic sugar residue was phosphorylated with $[\gamma-^{32}P]$ATP and polynucleotide 5'-hydroxyl-kinase the apurinic sugar residue would be split off by β-elimination at 100 °C for 60 min in neutral pH solution and $^{32}P$ would be released in acid-soluble fraction. In this experiment, $^{32}P$ incorporated into DNA was not released in acid-soluble fraction even after incubation at 100°C for 60 min under neutral pH. Therefore, these data show that phosphorylated terminal residues were nucleotides but not

apurinic sugar moieties.

The results shown in Tables 9 and 10 indicate clearly that the APendodeoxyribonuclease of *B. subtilis* cleaves the DNA strands on the

Table 10. End group analysis after enzymatic cleavage at apurinic sites in DNA.
Calf thymus DNA (1 mg/ml) in SSC (0.15 M NaCl/0.015 M sodium citrate, pH 5.0) was incubated for 8 h at 70°C to obtain 1 % depurination of the DNA. To obtain chain breaks at the apurinic sites, depurinated DNA was treated with APendonuclease or heated at 70°C for 10 h in 0.09 M Hepes-KOH/0.02 M $MgCl_2$/ 0.1 M glycine (pH 7.9). Control DNA was also incubated in the enzyme reaction mixture, except that no enzyme was added. All DNA solutions were dialyzed at 4°C against 95 % formamide and denatured at 37°C for 2 h and dialyzed at 4°C successively against 0.02 M NaCl/0.01 M Tris-HCl (pH 8.0)/6 % formaldehyde and then dialyzed against the same buffer without formaldehyde and each DNA concentration was determined. One-half of each solution was treated with *E. coli* alkaline phosphatase (ALP, 10 µg/ml) for 30 min at 37°C. To inactivate ALP, EDTA was added (5 mM) and the mixture was heated at 100°C for 2 min. All DNA solutions were then incubated with polynucleotide 5'-hydroxyl-kinase at 37°C for 45 min, in the following reaction mixture (100 µl) : 0.07 M Tris-HCl (pH 7.5), 0.01 M $MgCl_2$, 0.01 M 2-mercaptoethanol, 0.02 M $K_2HPO_4$, 2 x $10^{-5}$ M $[\gamma-^{32}P]$ ATP (0.3 mCi/µmole), calf thymus DNA (35 µg/ml), and polynucleotide 5'-hydroxyl -kinase (5 units). After the reaction was stopped by chilling, one set of sample was added 100 µl of calf thymus DNA (2 mg/ml) and precipitated with 0.5 ml of 7.2 % trichloroacetic acid. The precipitates were collected on Millipore filter (type GS 0.22 µm), washed with cold 7.2 % trichloroacetic acid, dried, and analyzed for radioactivity. While duplicate samples were heated at 100°C for 60 min before cold trichloroacetic acid precipitation.

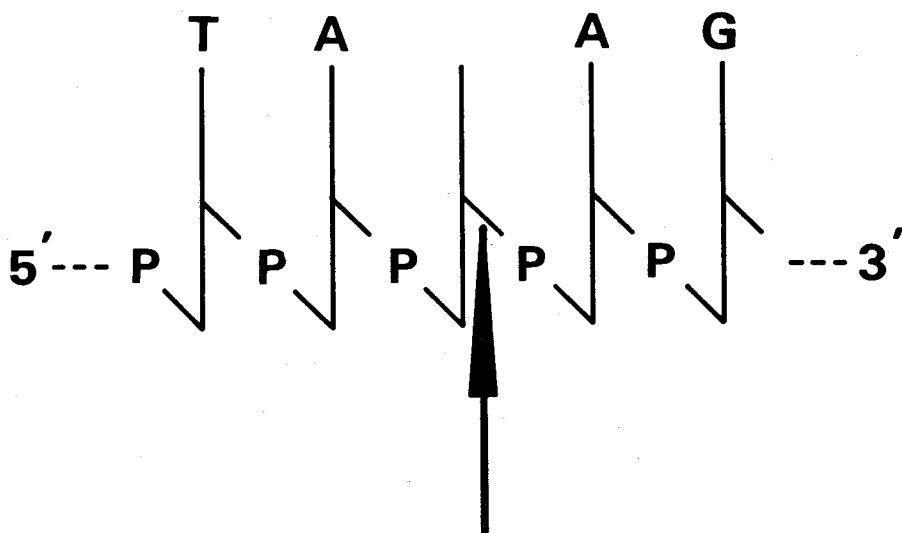| Apurinic DNA | $^{32}P$ incorporated at cleaved apurinic sites | | |
|---|---|---|---|
| | without ALP | with ALP | with ALP and heated at 100°C for 60 min |
| | | p moles | |
| Unhydrolyzed | 4.4 | 10.3 | 8.6 |
| Hydrolyzed with heat (70°C, 10 h) | 7.8 | 34.0 | 23.0 |
| Hydrolyzed with APendonuclease | 8.0 | 45.9 | 42.4 |

Fig. 37.    Cleavage site of APendodeoxyribonuclease.


3'-side of the apurinic sugar residue giving a 3'-OH and a 5'-phos-

phate (Fig. 37).

Gossard and Verly (113) reported that APendodeoxyribonuclease of

*E. coli* (endonuclease VI) cleaves the DNA strand on the 5'-side of the

apurinic sites giving a 3'-OH and 5'-phosphate.    On the other hand,

Ljungquist and Lindahl (61) demonstrated that APendonuclease from

calf thymus incises the DNA strand on the 3'-side of apurinic sites.

This result suggests that the incision mechanism of APendodeoxyribo-

nuclease from *B. subtilis* is similar to that of calf thymus APendo-

deoxyribonuclease.

APendodeoxyribonuclease level during the life cycle of *B. subtilis*

The levels of APendodeoxyribonuclease activity in the intact spores,

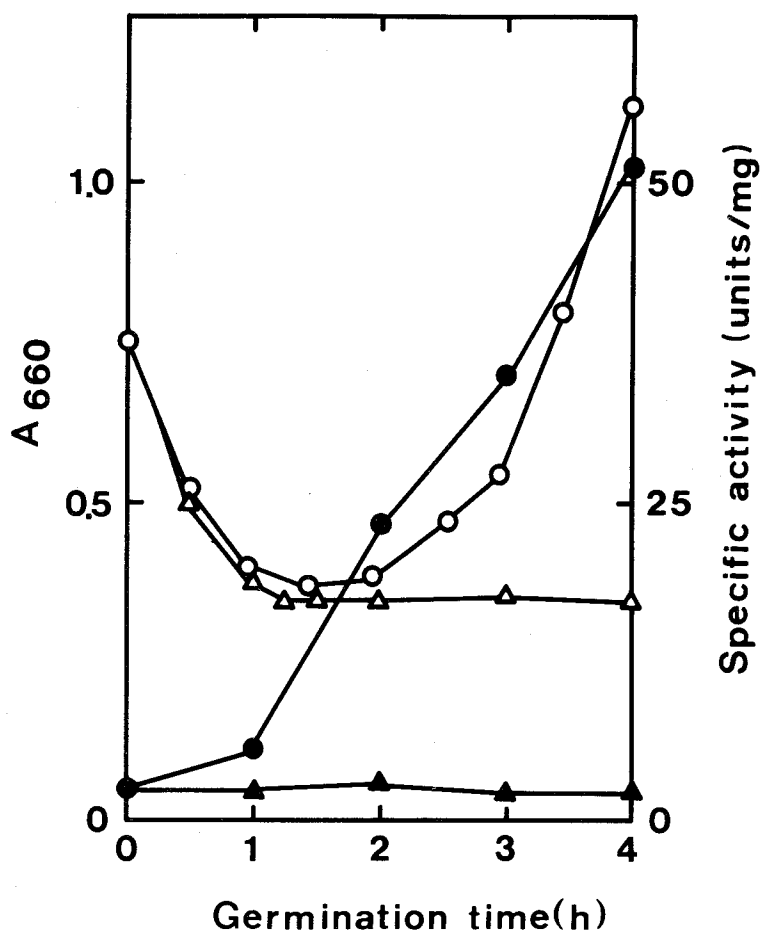germinating spores, vegetative cells, and sporulating cells of *B.*

Fig. 38.　Changes in APendodeoxyribonuclease activity during germi-
nation of B. *subtilis* spores.　　Symbols : O , absorbance at 660 nm
(control) ;　△ , absorbance at 660 nm of germinating spores contain-
ing chloramphenicol (50 μg/ml) ;　● , APendodeoxyribonuclease activity
(control) ;　▲ , APendodeoxyribonuclease activity of germinating
spores containing chloramphenicol (50 μg/ml).


*subtilis* 168Tt were measured.　Cultures were grown at 37°C in the

Schaeffer liquid nutrient broth sporulation medium.　Cells were har-

vested at approximately one doubling time before and at 0, 2, 4, and

Table 11. Variation of APendodeoxyribonuclease level during germination, vegetative growth and sporulation of B. *subtilis*.

| Growth stage | | Total activity (units) | Total protein (mg) | Specific activity (units/mg) |
|---|---|---|---|---|
| Spore | | | | |
| Germination | 0 h | 35 | 10.5 | 3.3 |
| | 1 | 76 | 11.6 | 6.6 |
| | 2 | 315 | 13.6 | 23.2 |
| | 3 | 993 | 28.4 | 35.0 |
| | 4 | 3101 | 60.0 | 51.7 |
| Vegetative cell | | | | |
| Log phase | OD = 0.5 | 9603 | 78.4 | 116 |
| stationary phase | $t_0$ | 4188 | 42.0 | 100 |
| | $t_2$ | 5438 | 52.4 | 104 |
| | $t_4$ | 5625 | 48.8 | 115 |
| | $t_8$ | 3125 | 44.0 | 71 |

8 h after the end of exponential growth. Spores were prepared as described in Chapter III. Spore germination was carried out in the Schaeffer's medium. Germinating cells were harvested at the intervals of every 1 h after the start of germination. The cells were disintegrated by sonication with glass beads for 15 min using the method described by Cobianchi et al. (139). The supernatant fluid obtained by the second centrifugation was used for the enzyme assay.

As shown in Fig. 38, spores have little APendodeoxyribonuclease activity. The activity appears in germinating spores being incubated for 1 h in the Schaeffer's medium. After that, the activity increases rapidly. This process is corresponding to the DNA synthesis during germination (Chapter IV). However, as the APendodeoxyribo-

nuclease activity does not appear during germination in the presence of chloramphenicol which is an inhibitor of protein synthesis (Fig. 38), APendodeoxyribonuclease must be synthesized *de novo* during germination.

The maximum APendodeoxyribonuclease level is deserved in the middle log phase cells. The enzyme activity does not change during sporulation until $t_4$ stage. After $t_4$ stage the activity in the cells extracts decreases. These results are summarized in Table 11.


4.  DISCUSSION


In the previous chapter, it was found that acid-heat treatment caused the single strand scissions of DNA in spores. Considering from the experimental results on the depurination of purified DNA the depurination is responsible for *in vivo* DNA scissions. Then, in case that most of the heated spores are survival the apurinic sites of DNA in spores caused by acid-heating are restored during germination.

Therefore, in order to understand the mechanisms of DNA damage (depurination) by heating and its repair system, the enzyme activity specific to apurinic DNA induced by acid-heating was investigated.

Consequently, an endodeoxyribonuclease which hydrolyzes DNA containing apurinic sites was purified from *B. subtilis* vegetative cells at approximately 8,800-fold with a yield of 22 %. The final prepa-

ration (Preparation VI ; DNA-cellulose fraction) gave a single pro-
tein band corresponding to the position of the enzyme activity on the
gel electrophresis.   By use of Sephadex G-200 and glycerol gradient
centrifugation, it was found that APendodeoxyribonuclease has molec-
ular weight of 105,000 and that this enzyme was dissociated to multi-
ple identical subunits having molecular weight of 26,000 on SDS
electrophoresis.   Those results suggest that APendodeoxyribonuclease
is composed of four identical subunits.

Comparing with APendodeoxyribonuclease from *E. coli* (IV, M.W.
33,000 ; VI, M.W. 32,000), *B. stearothermophilus* (M.W. 28,000) and
*B. subtilis* (M.W. 56,000) molecular weight of this enzyme is much
higher than those of others (52,53,57,58).

The activity of the purified enzyme is strictly restricted to
apurinic sites in double or single strand DNA and not active on
native, denatured, or alkylated DNA.   Previously reported APendo-
deoxyribonucleases were not active to apurinic sites in single
strand DNA.

The purified enzyme strictly requires $Mg^{2+}$ for its activity.
$Mn^{2+}$ and $Ca^{2+}$ were not effective.   However, the enzyme reported by
Inoue and Kada requires no divalent cations and is insensitive to
EDTA (57).   Resistance of the purified enzyme to NaCl is higher than
that of APendodeoxyribonuclease reported by Inoue and Kada (57).

Considering its strict requirement of $Mg^{2+}$, inhibition by EDTA,
resistance to NaCl, substrate specificity, and the molecular weight,

it is thought that the endonuclease specific to apurinic acid DNA isolated from *B. subtilis* is entirely different from that of *B. subtilis* reported by Inoue and Kada (57) and also endonuclease IV and VI of *E. coli* (52,53).

By the end group analysis it was found that the purified APendodeoxyribonuclease of *B. subtilis* cleaves of DNA strands on the 3'-side of the apurinic sugar residue giving a 3'-OH and a 5'-phosphate. However, the endonucleolytic cleavage of endonuclease VI (=exonuclease III) of *E. coli* generates a 3'-OH nucleotide end and a base-free deoxyribose 5'-phosphate at the 5'-terminus (131). On the other hand, APendodeoxyribonuclease from calf thymus (61) incised the DNA strand on the 3'-side of apurinic sites. It is suggested that the incision mechanism of APendodeoxyribonuclease of *B. subtilis* is similar to that of calf thymus APendodeoxyribonuclease.

Initially the apurinic sites are cleaved by APendodeoxyribonuclease, and then the deoxyribose-5'-phosphate residue, and possibly a small number of additional residue, are released by exonuclease action, and the DNA is finally subjected to repair replication and ligation as in the original excision repair madel. The individual steps of repair process in a *B. subtilis* system are schematically shown in Fig. 39. Since the side of cleavage of APendodeoxyribonuclease from *B. subtilis* is different from that of *E. coli* endonuclease VI, it is thought that the catalytic mechanism of exonucleases are different.
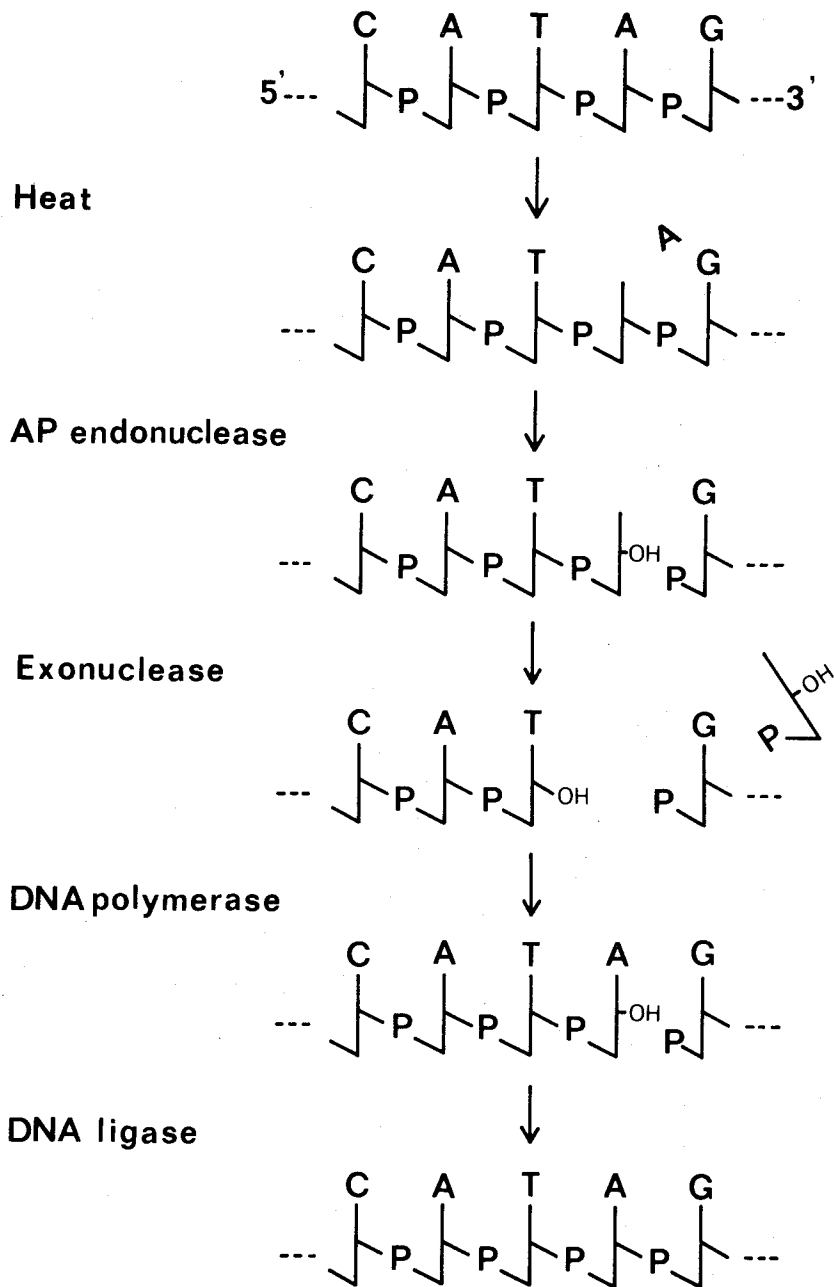
Fig. 39. Scheme for repair process of heat-induced depurinated DNA.

As shown in Chapter III and IV, acid-heat treatment of *B. subtilis* spores induced the depuration of DNA. The depurinated DNA of spores which was heated at 70°C for 20 min (pH 5.0) was repaired completely within 2 h after germination. As shown in Fig. 38, spores have very little APendodeoxyribonuclease activity and this activity increases rapidly after 1 h from germination. As DNA synthesis of the acid-heated spores begins after 90 min of incubation in germination medium, it is strongly suggested that APendodeoxyribonuclease synthesized during germination contributes to the repair mechanism of apurinic/apyrimidinic sites of DNA in spores.

5.    SUMMARY

A new endodeoxyribonuclease specific to apurinic sites in DNA was purified from vegetative cells of *B. subtilis*. The enzyme hydroluzes a phosphodiester bond at the heat-induced apurinic sites in double or single strand DNA ; it does not hydrolyze native nor alkylated DNA. The endonuclease has a molecular weight of around 105,000 and consists of four identical subunits. The enzyme absolutely requires $Mg^{2+}$ for its activity and is inhibited by EDTA. It is completely inhibited by 1 M NaCl or 1 mM p-chloromercurybenzoate. The APendodeoxyribonuclease of *B. subtilis* cleaves the DNA strands on the 3'-side of the apurinic sugar residue giving a 3'-OH and 5'-phosphate. These properties are very different from those of the

endonuclease specific to apurinic sites in DNA isolated from *B. subtilis* by Inoue and Kada and those of endonuclease IV and VI of *E. coli*.

The maximum APendodeoxyribonuclease level is observed in the middle log phase cells. The enzyme activity does not change during sporulation until $t_4$ stage and after $t_4$ stage it decreases. Spores have little APendodeoxyribonuclease activity. But the activity increases rapidly after 1 h from germination. Since the repair of depurinated DNA in spores, DNA symthesis, and appearance of APendodeoxyribonuclease activity are found almost at the same time, it is strongly suggested that APendodeoxyribonuclease synthesized during germination contributes to the repair mechanism of apurinic sites of DNA in spores.

Chapter VII


SUMMARY AND CONCLUSIONS


In order to understand the mechanisms involved in the DNA injury

by heat treatment and its repair, *in vivo* and *in vitro* DNA damages

of B. *subtilis* heated under various conditions were studied.    And

the enzyme acting on heat-injured DNA was purified and characterized.

The results obtained are summarized as follows :

(1)   Spores of B. *subtilis* injured by the heat treatment as 90°C for

10 min could not form colonies on Demain's agar medium unless sup-

plemented with glycine, threonine, or homoserine ; the heated B.

*subtilis* became auxotrophs requiring these amino acids.    These

amino acids requirements were genetically inherited.

Therefore, it is suggested that the appearance of auxotrophic

mutants of spores heated at 90°C for 10 min is due to the minor

modification of DNA molecules by heat, such as depurination in

spores.

(2)   The effect of pH on the thermal damages of spores of B. *subtilis*

was observed.    The results indicate that at the lower pH and the

higher temperature, the more the spores were inactivated.

To know the effect of acid-heating on DNA in spores, DNA in the

heated spores was extracted and analyzed by alkaline sucrose gra-

dient sedimentation.    At neutral pH, single strand scissions in

DNA did not occur after heating for 30 min at 60, 70, 80, and 90°C,
but at low pH DNA breaks increased according to the increasing
acidity.  These results show that at the lower pH and the higher
temperature the spores were heated, the more the single strand
scissions of DNA in spores occurred.

The results of studies on the temperature and pH dependence of
depurination of *in vitro* DNA indicated that depurination increased
with decreasing pH values and increasing temperature.  The acid-
heat induced DNA injury of spores corresponded to that of depurina-
tion of DNA heated at low pH *in vitro*.  Therefore, it is concluded
that DNA in spores of B. *subtilis* was depurinated by acid-heat treat-
ment, but at neutral pH depurination of DNA in spores was scarcely
occurred by heating at 60 - 90°C.  Sedimentation analysis indicated
that in the vegetative cells heating at 50°C induced the single
strand breakages of DNA molecules.  However, DNA breakages in
vegetative cells did not depend on the heating temperature or the
heating time.  Since the heat-induced DNA breakages were inhibited
by p-CMB, it is concluded that DNA in vegetative cells was de-
structed by endonuclease, which might be released or activated by
mild heating.

(3)  When the spores of B. *subtilis* which were heated at 70°C for 20
min at pH 5.0, by the condition of which the survivals were not
greatly reduced, were incubated in germination medium at 37°C for
120 min, the apurinic sites in DNA were restored.  This DNA re-

pairing process corresponds to the DNA synthesis during germination.

(4) As the result of investigation of the enzyme acting on heat-
injured DNA, a new endodeoxyribonuclease specific to apurinic
sites in DNA was purified from vegetative cells of *B. subtilis*.
The enzyme hydrolyzes a phosphodiester bond at the heat-induced
apurinic sites in double or single stranded DNA, but has no action
on normal double stranded DNA, single stranded DNA, nor alkylated
DNA.   It is composed of four identical subunits and has a molecu-
lar weight of about 105,000 daltons.   It strictly requires $Mg^{2+}$
for its activity and is sensitive to EDTA.   It is completely
inhibited by 1 M NaCl or 1 mM p-chloromercuribenzoate.   By the
end group analysis, it was found that APendodeoxyribonuclease of
*B. subtilis* cleaves the DNA strands on the 3'-side of the apurinic
sugar residue giving a 3'-OH and 5'-phosphate.   These properties
are very different from those of the APendodeoxyribonuclease iso-
lated from *B.subtilis* by Inoue and Kada and those of endonuclease
IV and VI of *E. coli*.

APendodeoxyribonuclease level is at its maximum in the middle
log phase cells.   The enzyme activity does not change during
sporulation until the $t_4$ stage.   After the $t_4$ stage the activity
in the cell extracts decreases.   The APendodeoxyribonuclease
activity is very little in spores and synthesized during germination.
The enzyme activity appears after germination for 1 h and after that,
the activity increases rapidly.   This process corresponds to the

DNA synthesis during germination.

These results strongly suggest that apurinic sites in DNA of spores caused by acid heating are nicked by APendodeoxyribonuclease synthesized during germination and repaired by the subsequent excision repair system.

## ACKNOWLEDGMENT

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