THE EFFECTS OF PROFLAVINE AND ACTINOMYCIN D ON TRANSFORMATION IN *BACILLUS SUBTILIS*

By

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1950

Submitted to the faculty of the Graduate School of
the Oklahoma State University
in partial fulfillment of the requirements
for the degree of
MASTER OF SCIENCE
August, 1963

THE EFFECTS OF PROFLAVINE AND ACTINOMYCIN D ON TRANSFORMATION IN BACILLUS SUBTILIS

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ACKNOWLEDGEMENT

The author wishes to express his sincere appreciation to Dr. Norman N. Durham, under whose direction this study was conducted, for his patience, counseling and supervision. Indebtedness is acknowledged to Dr. Franklin R. Leach for his suggestions and guidance.

He also wishes to express his appreciation to the United States Army for the opportunity to further his education.

The author extends gratitude to his wife, Mary, for her encouraging help in numerous ways.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER I

INTRODUCTION

Transformation is a process by which certain strains of bacteria, exposed to deoxyribonucleic acid (DNA) from a related strain, acquire and manifest in succeeding generations, certain properties of the related strain. This phenomenon was first described by Griffith in 1928. He succeeded in transforming an attenuated and non-encapsulated variant derived from one specific type into a fully encapsulated and virulent strain of a heterologous specific type. Avery, MacLeod, and McCarthy (1944) demonstrated that nucleic acid of the deoxyribose type was responsible for transformation.

Since 1944 a number of studies have been performed to improve the techniques of transformation. These consisted of improving the quantitative determination of transformants by using drug-resistant and nutritionally deficient organisms; understanding and improving the environmental factors which influence the development of competency (ability of cells to be transformed); improving methods for purification of DNA; and freeing transformants from competition with non-transformed populations. To supplement these studies, workers have been investigating how various mutagens and antibiotics affect transformation.

Studies during the past few years have contributed a wide variety of characters to proflavine. It has been known as a mutagenic agent

for many years (Witkin, 1947). Goldie, Walker, Graham, and Williams (1954) reported that addition of 10^{-3} M acriflavine (of which proflavine is a major constituent) to Sarcoma 180 cells *in vitro* prevented their subsequent proliferation. Bellin, Mohos, and Oster (1961) reported that the addition of 10^{-5} M proflavine to tumor cells was sufficient to produce a rapid inactivation. Schaffer (1962) concluded that proflavine was firmly bound within the polio virus particles when present during synthesis of the virus. The quantity bound was dependent on the dye concentration in the medium. The maximum quantity bound was estimated to be 200 molecules of dye per virus particle or about 1 dye molecule per 30 nucleotide molecules.

If proflavine was present during phage infection, few or no infectious progeny were liberated, but the infected cells synthesized DNA at about the same rate as observed in the absence of proflavine. The DNA synthesized was believed to be phage DNA since it contained hydroxymethyl cytosine instead of cytosine (deMars, 1955). deMars (1955) summarized his work with proflavine and T2 phage by stating that the mode of action of proflavine in preventing the production of infectious T2 particles is unknown. It is possible that proflavine may interfere with either the hypothetical "assembly mechanism" of the three organized macromolecular phage components, that is the phage DNA, the head and the tail, or with the synthesis of some phage constituent as yet unknown.

Hurwitz, Furth, Malamy, and Alexander (1962) stated that the *in vitro* effects of proflavine suggest that "messenger" ribonucleic acid (RNA) production occurs to some extent while DNA synthesis is blocked. Proflavine concentrations of 30 micromoles per ml will inhibit DNA synthesis approximately 85 per cent and RNA polymerase about 30 per cent.

In an extension of de Mars work, Kay (1959) stated that proflavine is known to complex with the DNA and it is possible that it displaces the naturally occurring polyamines. Further, the mutagenic action of proflavine may occur by this mechanism. In his experiments Kay (1959) found that some natural polyamines could reverse the action of proflavine and he explained this reversing action as a possible displacement of the "foreign" polyamine proflavine and the reestablishment of the normal polyamine DNA relationship.

Astrachan and Volkin (1957) found a slight difference in the polynucleotide sequence of normal phage DNA and the DNA synthesized from phage which were grown in the presence of proflavine.

Bellin and Oster (1960) studied the photodynamic inactivation of transforming principles in Diplococcus pneumoniae using various dyes and found that proflavine decreased the transforming activity, even in the absence of light.

Bradley (1961) stated that nucleic acids are known to bind strongly with basic dyes, but there appears to be no general agreement on the details of this interaction.

Actinomycin D was first isolated from cultures of Streptomyces parvullus (Manaker, Gregory, Vining, and Waksman, 1955). Gregory, Pugh, Hata, and Thielen (1956) reported that actinomycin exerted a significant therapeutic effect on two types of ascitic tumors in the mouse. Merker, Teller, Palm, and Woolly (1957) found the antibiotics were effective inhibitors of human tumors growing in conditioned rats. Journey and Goldstein (1961) proposed that the effectiveness of actinomycin D might be due to its ability to inhibit ribonucleic acid (RNA) and protein synthesis. Kirk (1960) elaborated on this proposed mechanism and suggested

that actinomycin D, added to cultures of Staphylococcus aureus growing exponentially, stopped RNA synthesis immediately. Reich, Franklin, Shatkin, and Tatum (1962) have reported that actinomycin D, at appropriate concentrations, suppressed RNA synthesis but did not influence DNA and protein synthesis in L cells. Concentrations which suppressed RNA synthesis by 99% allowed substantial protein and DNA synthesis for prolonged periods. At these concentrations all fractions of the cellular RNA, that is nuclear, ribosomal and "soluble" RNA, are completely inhibited. Hurwitz et al., (1962) substantiated these results when they reported that the actinomycin D-DNA complex formed has very little effect on DNA synthesis, while RNA synthesis is markedly inhibited. Haywood and Sinsheimer (1963) reported that actinomycin D at 10 μg/ml strongly inhibited protein synthesis in protoplasts of Escherichia coli strain C-3000 without inhibiting production of the RNA bacteriophage MS2. They conclude that DNA dependent RNA synthesis is not necessary for MS2 replication.

Both proflavine and actinomycin D have attracted attention as antitumor drugs due to their ability to inhibit nucleic acid synthesis. This study was conducted to determine the influence of actinomycin D and proflavine on transformation in Bacillus subtilis.

CHAPTER II

MATERIALS AND METHODS

Organism

Bacillus subtilis strain 168, an indole-negative auxotroph whose nutritional requirement can be satisfied by tryptophan, has been reported by Anagnostopoulos and Spizizen (1961) to be a highly transformable strain of this bacterium. B. subtilis strain 23 and strain 168WT, nutritionally independent organisms, were used as a source of donor DNA. All strains were obtained from Dr. William McDonald, Washington State University, Pullman, Washington.

Isolation of Transforming Deoxyribonucleic Acid.

Stock cultures were maintained on potato extract agar slants prepared as follows: Fresh diced potatoes (200 grams) were heated at 100° C for 5 minutes in 1 liter of water and filtered through 2 sheets of Whatman No. 1 filter paper. To this preparation, 20 grams of N-Z Case peptone and 2 grams of yeast extract were added. The pH was adjusted to 7.2, the solution diluted to 2 liters, agar added to give a final concentration of 2 per cent, and the medium sterilized by autoclaving.

For DNA preparations the organisms were grown in a minimal medium containing 0.2 per cent $(NH_4)_2SO_4$, 1.4 per cent K_2HPO_4, 0,6 per cent KH_2PO_4, 0.1 per cent sodium citrate, 0.02 per cent $MgSO_4 \cdot 7H_2O$, 0.5 per

cent glucose and 0.1 per cent yeast extract. The glucose was autoclaved separately and added aseptically to the medium prior to use. The organisms were grown for 12 to 14 hours at 37° C under forced aeration and harvested by centrifugation in the Sharples centrifuge. Approximately 5-6 grams of wet packed cells were normally obtained from 7 liters of medium. The cells were washed once with 100 ml of a saline-versene (0.15 M versene-0.1 M saline, pH 8.0) solution, then suspended in 50 ml of the saline-versene solution. Lysozyme (10 mg/ml) was added to give a final concentration of 100 μg/ml and the mixture placed on a reciprocating shaker for 60 minutes at 37° C. Duponal (30 per cent) was added to give a final concentration of 3 per cent. After complete lysis had occurred, as observed microscopically, sodium perchlorate (5M) was added to give a final concentration of 1 M and the solution permitted to stand at room temperature for 1 hour. The following operations were carried out at room temperatures unless otherwise indicated. The mixture was shaken with an equal volume of chloroform-isoamyl alcohol (20:1) for 30 minutes and centrifuged at 3500 x G for 10 minutes. The emulsion separated into 2 layers. The top portion contained the nucleic acids while the bottom layer contained denatured protein and chloroform-isoamyl alcohol. The upper layer was carefully removed with a pipette, placed in a beaker, and 2 volumes of cold 95% ethanol were layered over the nucleic acid containing solution. The nucleic acid was collected by spinning on a stirring rod and dissolved in a 0.015 M NaCl-0.0015 M sodium citrate solution. Sufficient 1.5 M NaCl-0.15 M sodium citrate solution was added to give a final diluent concentration of 0.15 M NaCl-0.015 M sodium citrate. An equal volume of chloroform-isoamyl alcohol was added to the mixture and the depro-

teinization repeated. This process was repeated until the bottom layer (the protein layer) had almost completely disappeared.

The nucleic acid strands were dissolved in a minimal amount of 0.015 M NaCl-0.0015 M sodium citrate solution. Ribonuclease (1 mg/ml), previously heated at 100° C for 10 minutes, was added to the solution to give a final concentration of 50 μg/ml and the solution incubated at 37° C for 1 hour. The deproteinization was repeated, followed by alcohol precipitation, until no denatured protein appeared at the interface between the DNA layer and the chloroform-isoamyl alcohol layer. The DNA was stored in ethanol at 4° C until used in transformation experiments at which time the DNA was dissolved in the citrate-saline solution.

DNA was quantitatively estimated by the diphenylamine reaction as modified by Burton (1956) using salmon sperm DNA as a standard. Ribonucleic acid was determined by the orcinol method using d-ribose as a standard. These determinations were then compared with the nucleic acid concentration calculated from the nomograph prepared by E. Adams (distributed by California Corporation for Biochemical Research, Los Angeles, California). The nomograph permits determination of nucleic acid concentrations by measuring the absorbancy of the solution at 260 and 280 mμ. The absorbancies of all solutions were measured in the Beckman DU spectrophotometer. A comparison of the two methods was favorable to the extent that absorbancy readings and the nomograph were used to confirm the DNA concentrations prepared from the stock solution.

Development of Competent Cells for Transformation.

Bacillus subtilis strain 168 was grown overnight (12-14 hours) at 37° C on a reciprocating shaker in 10 ml of penassay broth

(Anagnostopoulos and Spizizen, 1961). The penassay broth was composed of 0.4 per cent nutrient broth, 0.15 per cent yeast extract, 0.25 per cent peptone, 0.35 per cent NaCl, 0.368 per cent K_2HPO_4, 0.132 per cent KH_2PO_4, and 0.1 per cent glucose. An aliquot of the overnight culture (0.1 ml) was transferred to 5 ml of minimal broth containing 0.1 per cent yeast extract. The cells were incubated for 4 hours at 37° C with constant shaking. Then 1 ml of the 4 hour culture was transferred to 9 ml of minimal broth plus 0.01 per cent yeast extract and incubated for 1 hour at 37° C with shaking. Results indicated these cells were competent for transformation by DNA extracted from B. subtilis strain 23 or strain 168WT.

Test Compounds and Chemicals

Proflavine (proflavine sulfate), purchased from the National Aniline Division, Allied Chemical and Dye Corporation, New York, N.Y. was dissolved in sterile water immediately prior to use.

Actinomycin D, supplied by Merck, Sharp and Dohme Research Laboratory, West Point, Penn., was dissolved in sterile water. The stock solution containing 120 μg/ml was stored at 4° C. Lysozyme, (IUB 3.2.1.17) was purchased from the Worthington Biochemical Corp., Freehold, New Jersey. DNase (IUB 3.1.4.5) and RNase (IUB 2.7.7.16) were purchased from the California Corporation for Biochemical Research, Los Angeles, California.

Transformation Procedure and Scoring of Transformants

Cells were grown to competency as previously outlined. The standard transforming mixture contained: 0.6 ml of competent cells

(approximately 30 x 10^6 cells/ml), 0.2 ml of DNA solution (approximately 40 μg/ml), 1.0 ml of minimal broth plus 0.01 per cent yeast extract, and water or test compound dissolved in water to a total volume of 2.0 ml.

This system was incubated for 15 minutes at 37° C, then 0.2 ml of DNase (200 μg/ml) was added to the transforming mixture to destroy residual extracellular DNA. The mixture was incubated at 37° C for 10 minutes, placed in an ice bath for 5 minutes, and then 0.05 ml and 0.1 ml aliquots plated directly on minimal agar. Total counts were made by plating 10^{-5} and 10^{-6} dilutions on penassay agar.

Determination of Thermal Denaturation Temperatures

Tubes containing DNA, DNA plus the test compounds, or the test compounds without DNA were prepared in a total volume of 2.5 ml (2.3 ml citrate-saline + 0.2 ml water or test compound). These mixtures were incubated 30 minutes at 37° C. An equal volume of methanol (2.5 ml) was added to each tube, mixed thoroughly, and the tube plugged with a rubber stopper. Thermal denaturation temperatures were determined by heating the DNA-containing mixtures and the controls to the desired temperature in a laboratory water bath. When the desired temperature was attained, a 5 minute temperature equilibration period was observed. The tubes were removed from the water bath and placed in an ice bath for 5 minutes. Absorbancies were read on the Beckman DU spectrophotometer at 260 mμ and compared to appropriate controls.

CHAPTER III

RESULTS

Influence of Time and Temperature on Transformation

The initial investigations were concerned with defining the conditions which would give an efficient and relatively constant method of transformation.

The time at which the recipient cells were the most competent was determined by the following procedure. Cells were grown as described under Methods and Materials and the 4 hour culture was inoculated into minimal medium plus 0.01 per cent yeast extract. After 45 minutes growth at 37° C with constant shaking in the minimal medium plus 0.01 per cent yeast extract, aliquots of the cell suspension were removed at various times, exposed to DNA (4 μg/ml) for 15 minutes, treated with DNase, chilled, and plated. These results (Figure 1) indicate that maximal cell competence is achieved after approximately 60 minutes following dilution of the 4 hour culture into the minimal medium plus 0.01 per cent yeast extract. Deviations from this time interval produced fewer transformants indicating a less competent culture. Extreme care was taken in later experiments to use cells in the most competent state of development.

The optimum temperature for indole transformation in B.

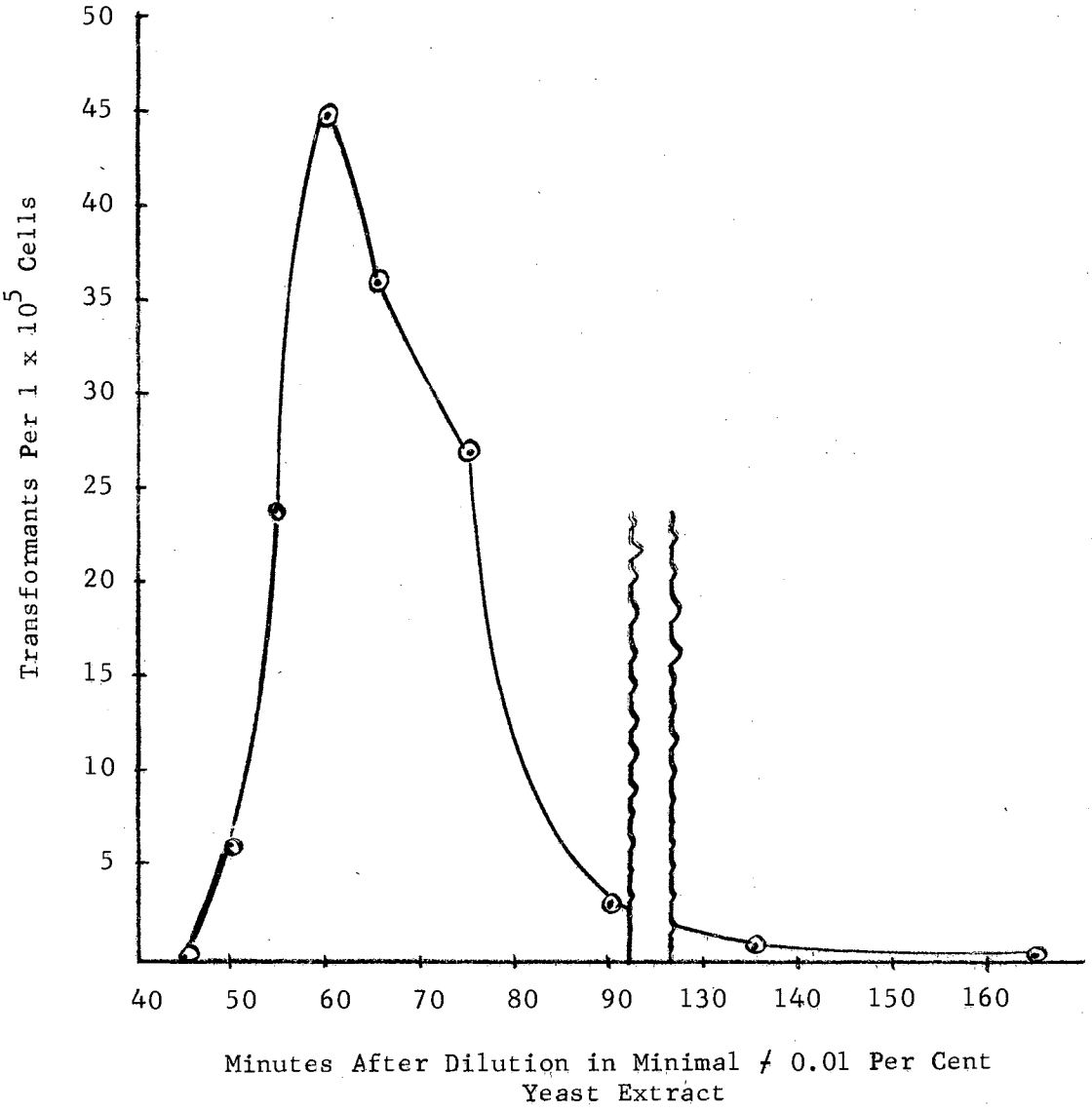

Figure 1. Effect of Cultivation Time on Competency.

subtilis strain 168 was determined by incubating competent cells with DNA (4 μg/ml) at the temperatures indicated in Table I and the per cent transformants calculated on the basis of the total cell count. A temperature of 37° C was optimal for transformation of the indole character.

TABLE I

EFFECT OF TEMPERATURE ON TRANSFORMATION

Temperature °C	Per cent Transformation
25	0.0082
30	0.0085
37	0.0234
40	0.0102
45	0.0050

Influence of DNA Concentration

The optimum concentration of DNA for transformation was determined by adding 0.5 μg to 20 μg/ml of DNA to competent cells and testing for transforming ability as outlined in Methods and Materials. Results presented in Figure 2 illustrate the maximum transformation was obtained with a DNA concentration of approximately 5 μg/ml. Increasing the DNA concentration to 20 μg/ml was without significant effect.

Comparison of DNA Transforming Capacity from Different Donor Stains

Deoxyribonucleic acids isolated from both B. subtilis strain 23 and strain 168WT were compared for their ability to transform B.

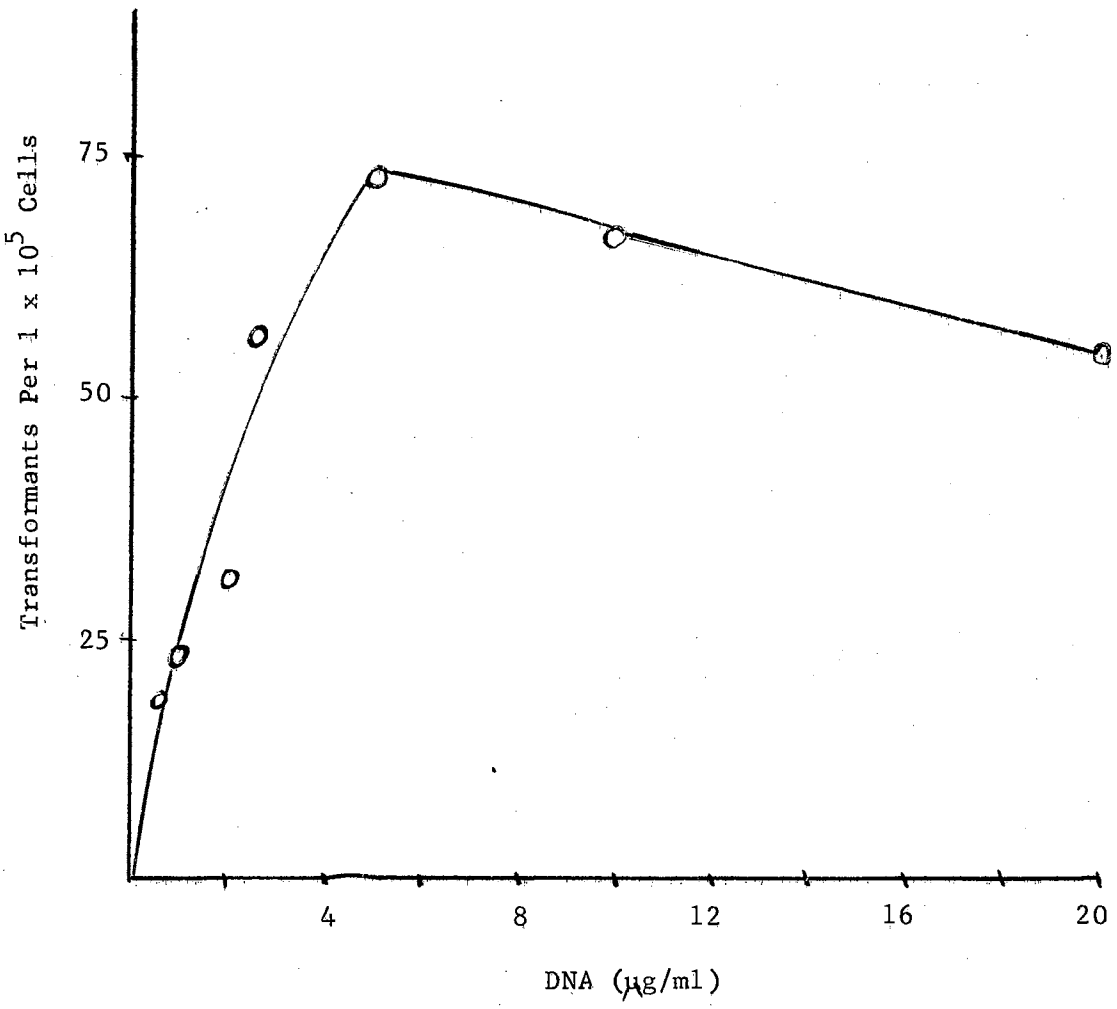

Figure 2. Effect of DNA Concentration on Transformation.

subtilis strain 168 to prototrophy. DNA solutions (40 μg/ml) were prepared from each donor and added to a suspension of competent cells to give a final concentration of 4 μg/ml. Results from a typical experiment are presented in Table II and indicate that the DNA prepared from B. subtilis strain 168WT is more active in transforming the tryptophan auxotroph to prototrophy than is the DNA isolated from B. subtilis strain 23.

TABLE II

TRANSFORMING ABILITY OF DNA ISOLATED FROM BACILLUS SUBTILIS STRAIN 23 AND STRAIN 168WT

DONOR	TRANSFORMANTS PER 1×10^5 CELLS
B. subtilis strain 23	7.3
B. subtilis strain 168WT	11.0

Determination of Optimum DNase Concentration

Since DNase was added to the transforming mixture to destroy extracellular DNA that had not been taken up by the cells, experiments were conducted to ascertain the DNase concentration that would inactivate 4 μg/ml DNA.

Different concentrations of DNase were incubated with 0.2 ml of DNA (40 μg/ml) at 37° C for 10 minutes prior to its addition to competent cells. The results are listed in Table III.

TABLE III

EFFECT OF VARIOUS CONCENTRATIONS OF DNase ON TRANSFORMING ABILITY OF DNA ISOLATED FROM BACILLUS SUBTILIS STRAIN 168WT

Concentration DNase (μg/ml)	Transformants per 1×10^5 cells	Total Counts 1×10^5
0	21.6	160
2	16.3	175
5	7.4	133
8	2.46	203
20	0.0	213
50	0.0	206

Transformation was obtained with DNA treated with 8 μg/ml DNase or less. However, no transformants resulted when 20 μg/ml DNase was used. A DNase concentration of 20 μg/ml was sufficient to destroy the transforming activity of the DNA concentration (4 μg/ml) used in these experiments without affecting the total viable cell count.

Influence of Proflavine and Actinomycin D on Cells

Various concentrations of proflavine and actinomycin D were tested with B. subtilis strain 168 to determine the sensitivity of the organism to the test compounds and to permit selection of an appropriate concentration that could be used in transformation

studies. Tubes containing various concentrations of the test compounds were inoculated from a 4 hour culture to give a cell concentration of approximately 20×10^6 cells/ml. The tubes were incubated at 37° C, aliquots were removed at 15 and 30 minutes, chilled in an ice bath, diluted, and plated for total counts. Figure 3 shows results obtained after exposure of the cells to the inhibitors for 30 minutes. Viable cell counts indicated that proflavine or actinomycin D concentrations up to and including 10 μg/ml did not significantly decrease the number of viable cells. However, exposure to higher concentrations (20 μg/ml) of both inhibitors showed a marked decrease in cell counts. Total counts in the control tube remained constant over the 30 minute period.

Studies were conducted to ascertain if either proflavine or actinomycin D influenced the spontaneous conversion of B. subtilis strain 168 from auxotrophy to prototrophy. Cells were exposed to concentrations up to and including 10 μg/ml of the test compound for time periods equivalent to those used during transformation. Results indicated no reversions under the conditions used in these investigations. However, total counts and frequency of spontaneous reversion were run concurrently with scoring of transformants in all experiments.

Influence of Proflavine on Transformation

Experiments were conducted using DNA from B. subtilis strain 168WT, to determine the effect of proflavine on transformation

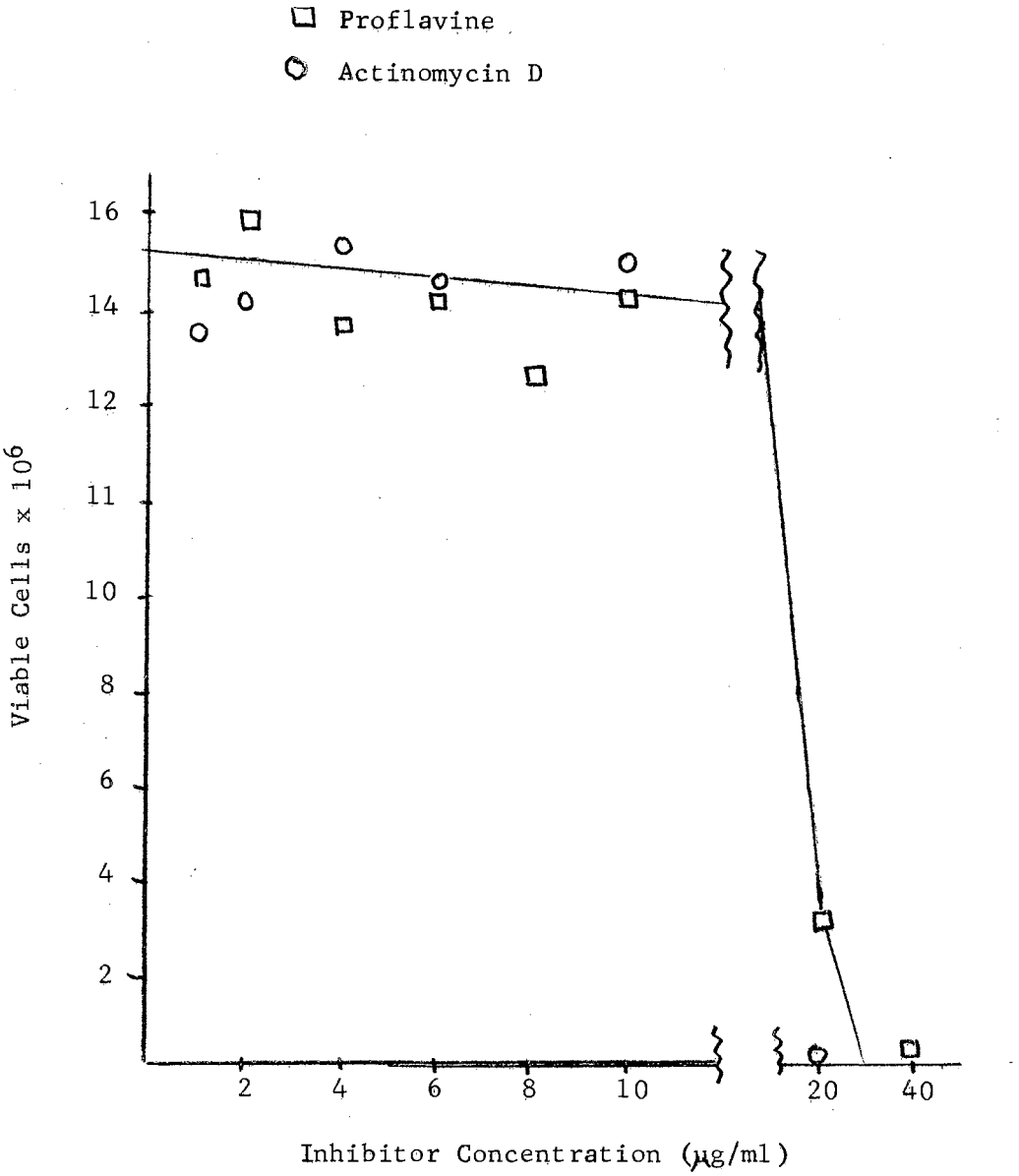

Figure 3. Effects of Proflavine and Actinomycin D on Proliferation of Bacillus subtilis strain 168.

under the following three conditions:

1. DNA was exposed to various concentrations of proflavine for 30 minutes at 37° C prior to addition to competent cells.
2. Recipient cells were exposed to various concentrations of proflavine for 30 minutes at 37° prior to the addition of DNA.
3. Various concentrations of proflavine were added to a competent cell suspension simultaneously with the DNA.

Results from these experiments are presented in Figure 4. When the proflavine-DNA incubation mixture is added to the competent cell suspension, the concentration of proflavine and DNA is reduced by a 2.5 fold dilution factor. The amount of proflavine indicated in Figure 4 is the concentration of proflavine in the transforming mixture while the DNA concentration in the transforming mixture is 4 μg/ml. When proflavine is incubated with DNA (Prof/DNA) for 30 minutes at 37° C prior to addition of the mixture to the competent cells, the biological activity (frequency of transformation) is decreased. The effect of proflavine on the transforming ability of DNA does not appear to be directly related to concentration in the range tested in this experiment. Inhibition when DNA is incubated with proflavine could suggest formation of a proflavine-DNA "complex." Under these conditions it is possible that the "complex" cannot penetrate the permeability barrier or if the "complex" does enter the recipient cell, it is unable to effect transformation from auxotrophy to prototrophy. It is also possible that the proflavine simply exerts its influence on the organism and renders the cell less competent.

When the cells were incubated with proflavine for 30 minutes at 37° C prior to addition of DNA (Prof/cells), or when the proflavine and DNA are added to competent cells simultaneously (Prof/cells/DNA), similar results were obtained in that low concentrations of proflavine showed a pronounced inhibition on transformation. With all three systems a proflavine concentration of 2 μg/ml showed transformation approaching approximately 30 per cent of that observed in the control system. These values remained relatively constant as the proflavine concentration was increased to 5 μg/ml.

The similarity in results obtained with all three systems suggests that treating either the cells or DNA with proflavine was no more effective than adding the inhibitor simultaneously with the other components. Thus, proflavine may be preventing transformation by one of the following mechanisms:

1. The transforming DNA is bound or complexes with the proflavine.
2. The proflavine enters the cell and either directly or indirectly selectively interacts with the mechanism which "incorporates" information from the donor DNA into the genome material of the recipient cell.
3. Alters cell permeability.

Influence of Actinomycin D on Transformation

Similar experiments were conducted with actinomycin D. (Figure 5). When transforming DNA is incubated with actinomycin D for 30 minutes prior to addition to competent cells (Actin D / DNA), there is a

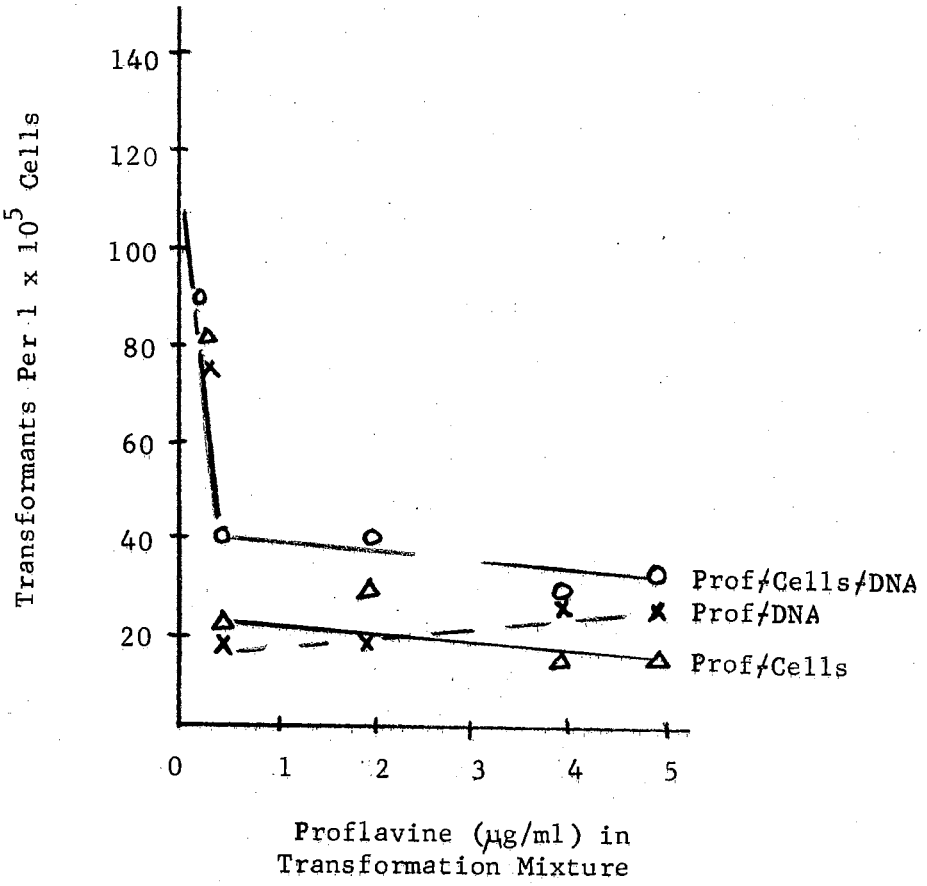

Figure 4. Effect of Proflavine on Transformation.

significant decrease in the biological activity of the DNA. The actinomycin D concentrations in Figure 5 are the final concentrations of the inhibitor in the transforming mixture and there is a 2.5 fold dilution of the actinomycin D-DNA solution as previously explained for the proflavine-DNA solution. Recipient cells incubated with actinomycin D for 30 minutes at 37° C prior to the addition of DNA (Actin D / cells) showed approximately the same number of transformants as the system in which DNA and actinomycin D (Actin D / DNA) were incubated together. A similar observation was evident when actinomycin D and transforming DNA were added to the competent cells simultaneously (Actin D/cells/DNA).

The results obtained with actinomycin D were similar to those observed with proflavine in that exposure of either DNA or cells to actinomycin D for 30 minutes prior to adding to transformation mixture was no more effective for inhibiting transformation than when actinomycin D, DNA, and competent cells are mixed simultaneously.

Tentatively the inhibition of transformation by actinomycin D could be explained in the same manner as proposed for proflavin by complexing with donor DNA, by interfering with the "incorporation" of donor information, or by altering permeability.

Nature of Reaction Between DNA and Test Compounds

Previous workers (Kay, 1959; Bradley, 1961; Hurwitz et al., 1962) have indicated that proflavine or actinomycin D complexes with nucleic acids. Adding DNA and the test compounds simultaneously or incubating DNA with either compound prior to adding to competent

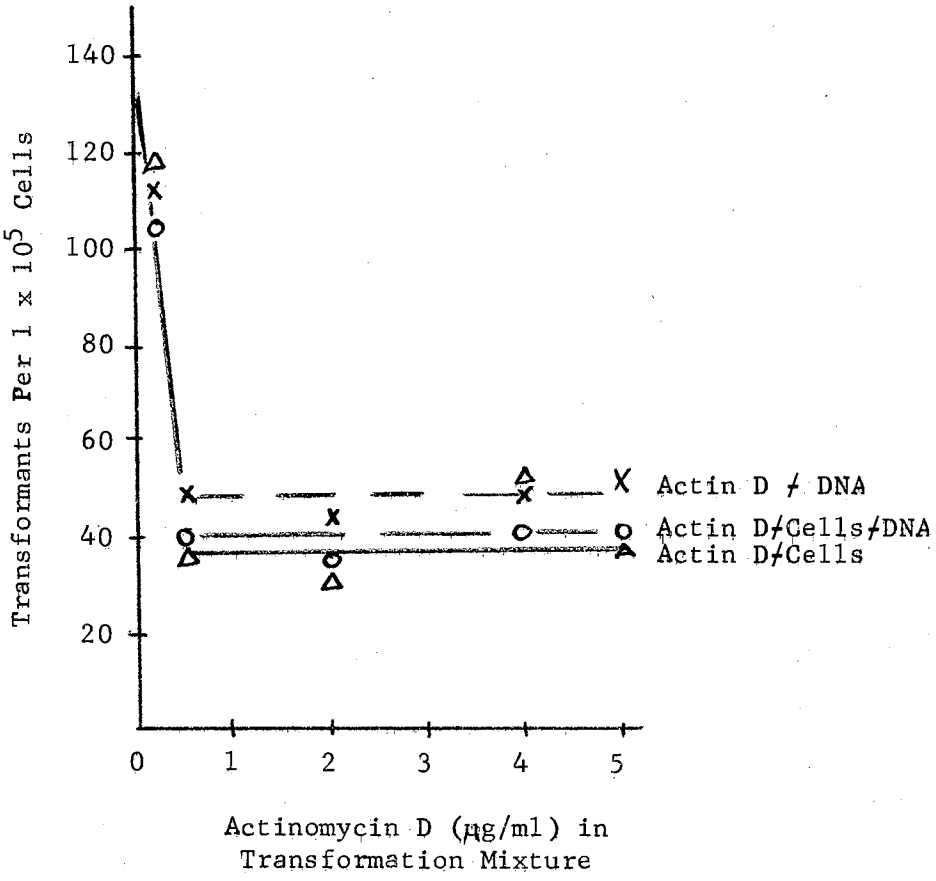

Figure 5. Effect of Actinomycin D on Transformation.

cells resulted in a loss of biological activity. This observation could indicate that the test compounds interfere with transformation by complexing or interacting with the DNA molecule. Studies were conducted to determine the nature of such a "complex" and to determine if its formation is reversible. To determine reversibility of the "complexes," the test compound was incubated with DNA and then the mixture was dialyzed to ascertain if the inhibitor could be removed and the biological activity restored. Another approach was to determine the thermal denaturation temperature of the DNA-test compound "complexes" before and after dialysis as compared with the thermal denaturation temperature of native DNA. A change in the thermal denaturation temperature of the inhibitor treated DNA could indicate a structural alteration in the DNA molecule. To supplement these studies, the biological activity of the DNA-test compound mixture was measured both before and after dialysis.

Absorbancy Spectra of DNA and DNA-Test Compound "Complexes"

Absorbancy spectra were determined on the DNA and DNA-test compound "complexes" before and after dialysis. Studies indicated characteristic absorption peaks for actinomycin D at 240 mμ and 440 mμ and peaks for proflavine were observed at 260 mμ and 445 mμ (Figure 6). Studies indicated as little as 1 μg/ml of actinomycin D and 0.5 μg/ml of proflavine could be detected by absorbancy measurements. A characteristic absorption peak for DNA was located at 260 mμ (Figure 7). These peaks were used for detecting the test compounds.

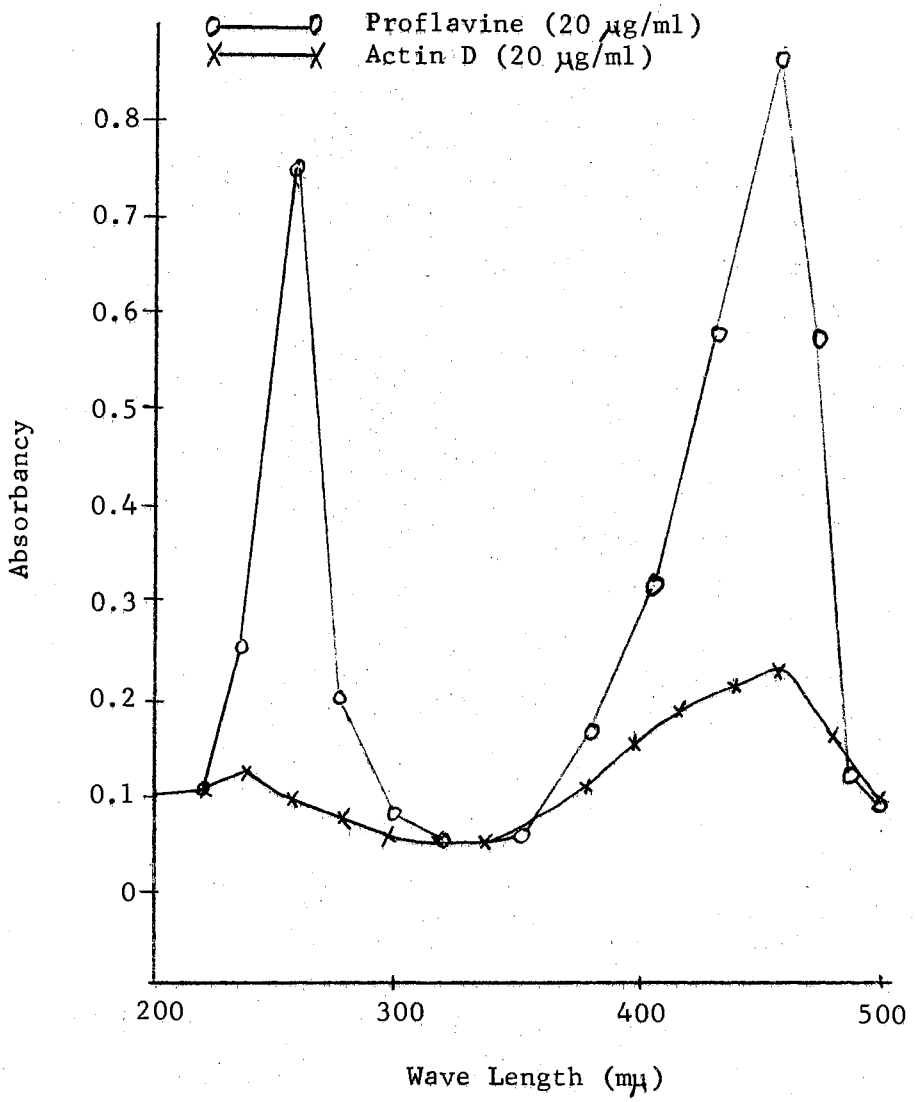

Figure 6. Absorption Spectra of Proflavine and Actinomycin D.

DNA (16.6 μg/ml) was incubated with either water, proflavine (20 μg/ml), or actinomycin D (20 μg/ml) for 30 minutes at 37° C and a spectrum determined for each (Figure 7). All three mixtures showed a peak characteristic of DNA at 260 mμ and the inhibitors showed characteristic peaks at the higher wave lengths. The mixtures were then dialyzed for 48 hours against 0.15 M sodium chloride 0.015 M sodium citrate solution at 4° C. Spectra were again measured and the characteristic peak (440 mμ) for actinomycin D showed an increase over the DNA control indicating that some of the actinomycin D might still be present (Figure 8). The peak at 240 mμ also characteristic of actinomycin D, was either absent or masked by the DNA absorption peak at 260 mμ. Since a significant increase in absorption at 260 mμ over the DNA control was not observed, it could be that the absorption at 240 mμ is no longer evident. The characteristic absorption peak for proflavine at 445 mμ was not observed after dialysis and no increase was observed at 260 mμ over that measured in the DNA control indicating that most of the proflavine had apparently dialyzed through the diffusion membrane.

The presence of a small peak at 440 mμ in the actinomycin D-treated DNA after dialysis could indicate that all of the inhibitor did not dialyze out of the mixture, or a minute, yet detectable, amount of actinomycin D complexed with the DNA. The inability to detect a peak for proflavine could indicate that if proflavine is bound to the DNA molecule the quantity is too small to be detected by absorbancy measurements.

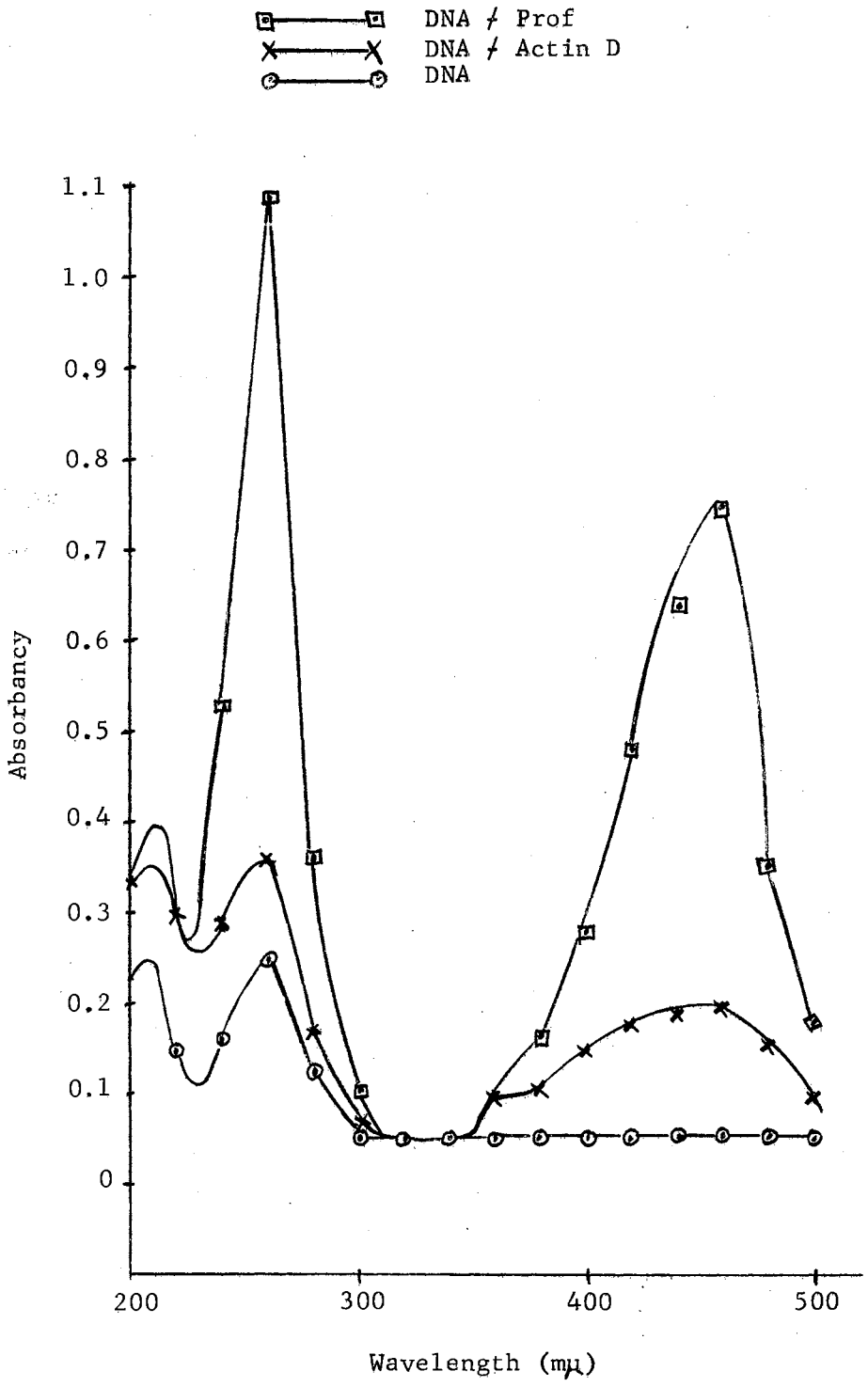

Figure 7. Absorption Spectra of DNA and DNA-Test Compound "Complexes" before Dialysis.

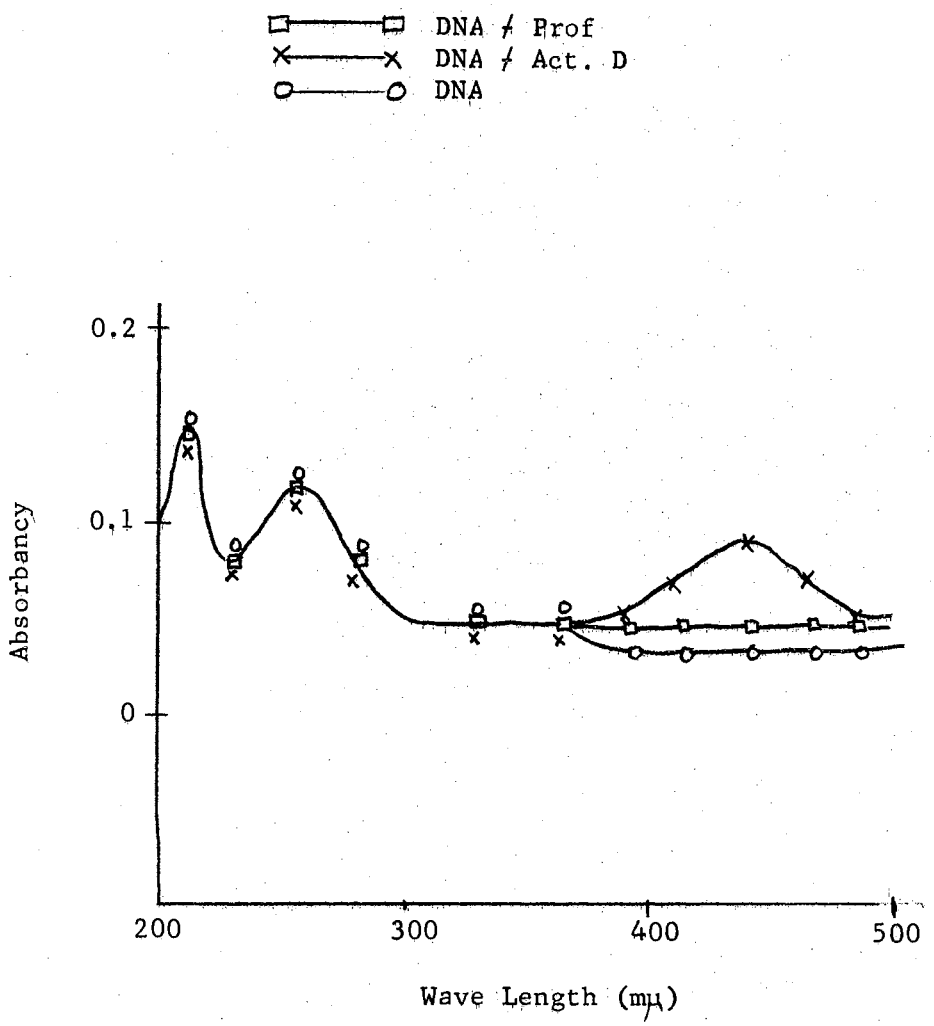

Figure 8. Absorption Spectra of DNA and DNA-Test Compound "Complexes" After Dialysis.

Effect of Dialysis on the Biological Activity of DNA and DNA-Test Compound "Complexes"

Since both proflavine and actinomycin D might decrease the biological activity of DNA by interacting with DNA, studies were conducted to determine if the complexes formed between the test compounds and DNA were reversible with respect to biological activity. Five ml DNA (24 μg/ml) were incubated with 1 ml of proflavine (120 μg/ml) and 5 ml DNA (24 μg/ml) were incubated with 1 ml actinomycin D (120 μg/ml) for 30 minutes at 37° C. Then 0.2 ml was added to 1.8 ml of a competent cell suspension to determine the transforming ability of the mixture. A 3.0 ml sample of the DNA control and the DNA-test compound "complexes" were dialyzed with four changes against a solution of 0.15 M sodium chloride-0.015 M sodium citrate solution for 48 hours at 4° C. The dialyzed solutions were recovered and adjusted to the same volume (3.0 ml). The biological activity of the DNA and DNA-test compound mixtures was determined by the addition of 0.2 ml of these solutions to 1.8 ml of a competent cell suspension. The results of these experiments are outlined in Table IV.

TABLE IV

EFFECT OF DIALYSIS ON BIOLOGICAL ACTIVITY OF DNA AND DNA-TEST COMPOUND "COMPLEXES"

Transforming Material	Number of Transformants per 1 x 10^5 cells	
	Before dialysis	After dialysis
DNA	53	119
DNA/proflavine	36	113
DNA/actinomycin D	26	99

Results indicate that dialysis of the DNA-test compound mixtures restored the biological activity of the transforming DNA molecule. Reversal was complete in the case of proflavine and virtually complete with actinomycin D, although a slight difference was still noted. Thus, the DNA-test compound "complex" appears to be reversible, or if a complex still exists it apparently does not influence the biological activity of the transforming DNA.

Effect of Dialysis on Thermal Denaturation Temperatures of DNA and DNA-Test Compound "Complexes"

Optical density change was determined by dividing the optical density of the mixtures at each temperature by the optical density of the mixtures at 37° C. Thermal denaturation temperatures, as indicated by these changes in optical density, were determined to ascertain possible structural modifications of the DNA molecule by proflavine or actinomycin D. Thermal denaturation studies were conducted before and after dialysis of the DNA-test compound mixtures. The thermal denaturation

of the various mixtures before dialysis (Figure 9) indicate that DNA has a lower denaturation temperature than DNA-actinomycin D "complex". DNA treated with proflavine did not show a characteristic increase in optical density at high temperatures.

After dialysis, DNA treated with proflavine and actinomycin D appear to have a higher denaturation temperature than DNA (Figure 10).

Effect of Time of Addition of Inhibitors to Transforming Mixture

Studies were conducted to determine the time the inhibitors could be added to the transforming system and still prevent transformation. The inhibitors were added to the competent cell suspension at 0 minutes (simultaneously with DNA) and at 5 and 15 minutes after the addition of DNA. Results from this study are presented in Table V.

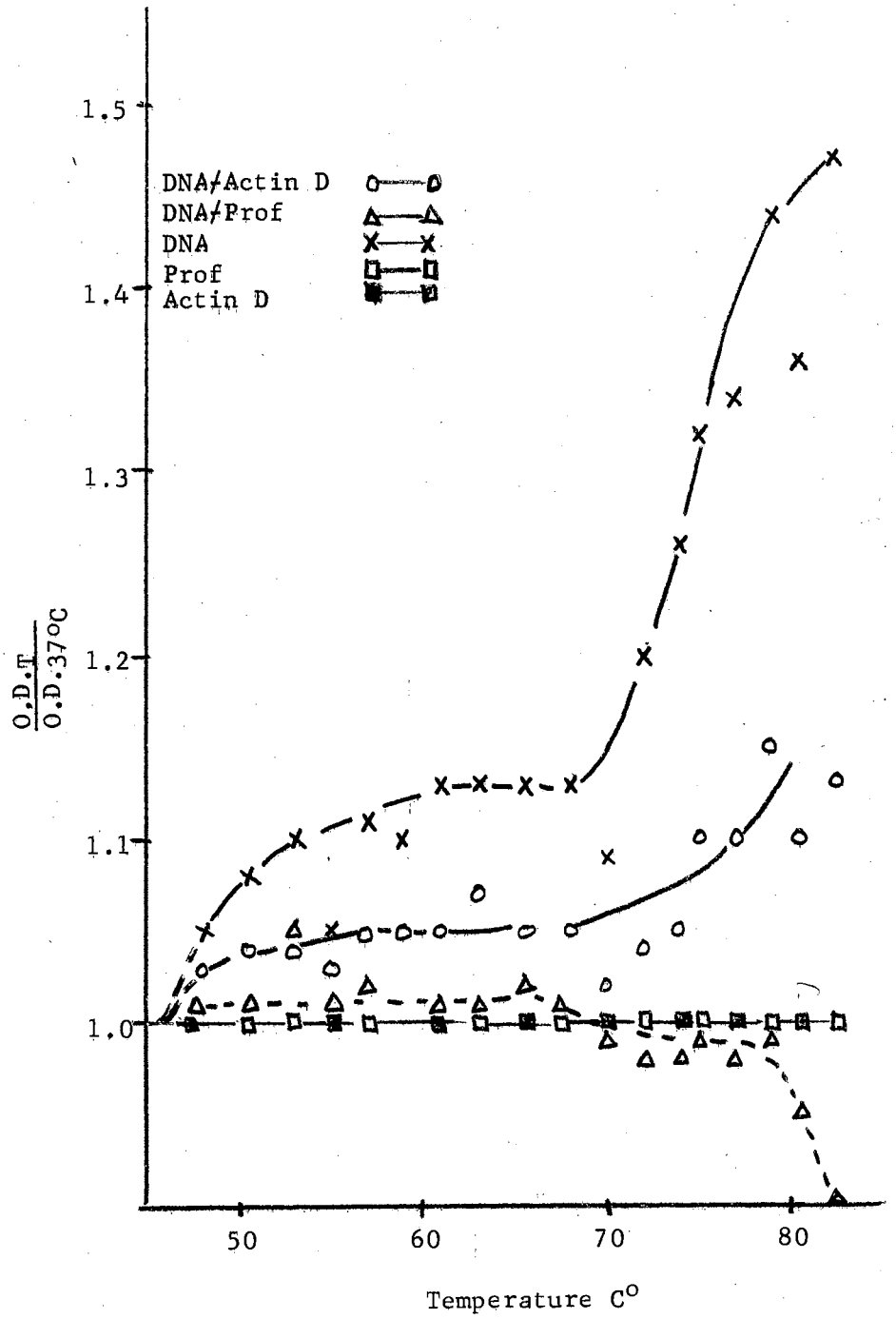

Figure 9. Effect of Temperature on Absorbancy (260 mμ) of DNA, Test Compounds and DNA-Test Compounds "Complexes" Before Dialysis.

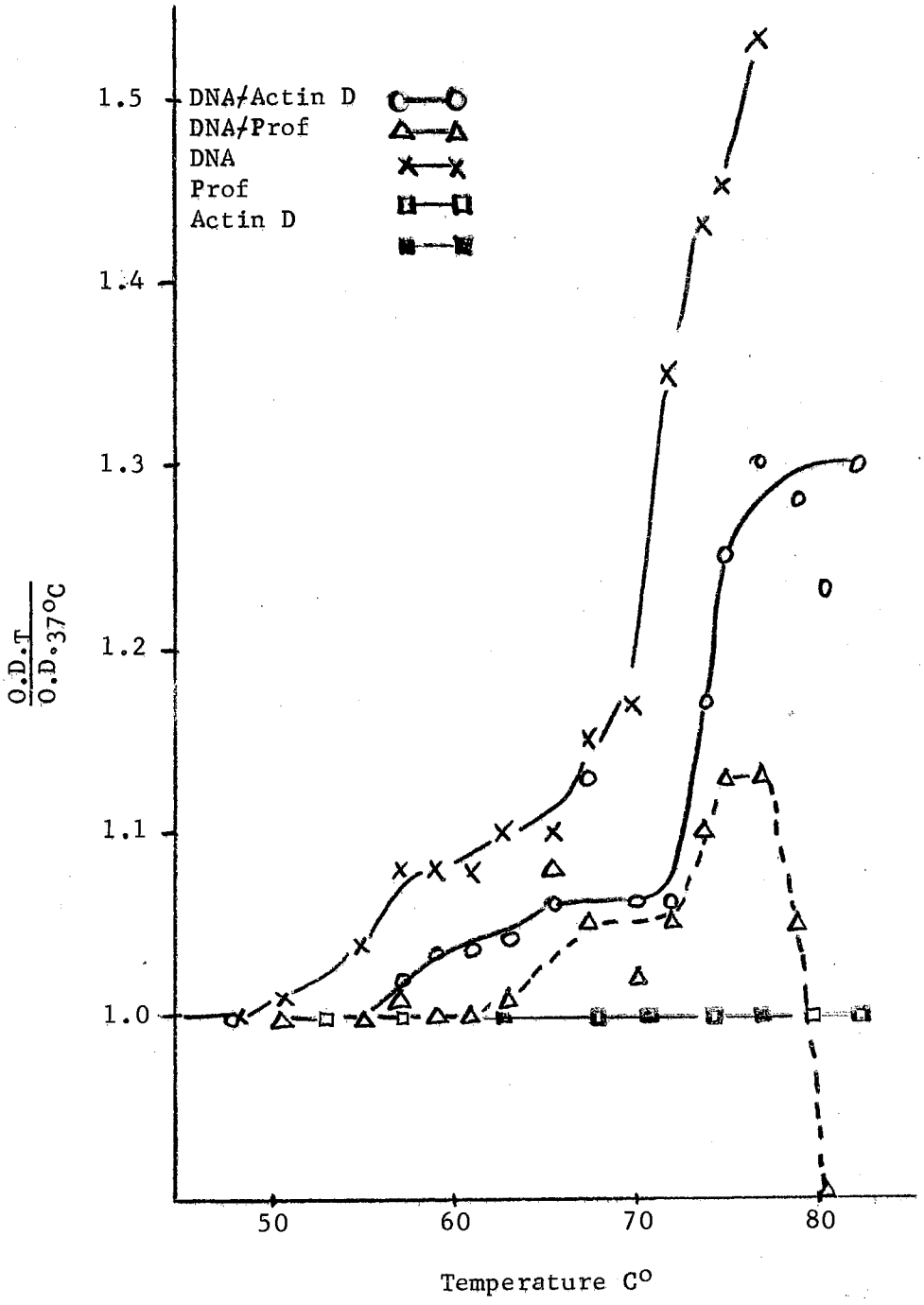

Figure 10. Effect of Temperature on Absorbancy (260 mμ) of DNA, Test Compounds and DNA-Test Compounds "Complexes" After Dialysis.

TABLE V

EFFECT OF TIME OF ADDITION OF INHIBITORS

TO TRANSFORMING MIXTURE

	TRANSFORMANTS PER 1 x 10^5 CELLS Time of Addition of Text Compound		
	0 Min	5 Min after DNA	15 Min after DNA
Control	122	-	-
Actinomycin D	7	0	0
Proflavine	47	76	67

The results of these experiments establish that both actinomycin D and proflavine may be added as late as 15 minutes after addition of DNA and still exert an inhibitory effect. It would appear that neither of these inhibitors interfere with penetration of the transforming DNA through the permeability barrier since similar inhibition patterns were obtained at the different time intervals. These findings may indicate that the incorporation of the DNA into the genome of the recipient cell has not taken place within 15 minutes after the addition of transforming DNA to the recipient cells or that the transformed cells are more sensitive to the inhibitors than are the recipient cells.

CHAPTER IV

SUMMARY AND CONCLUSIONS

The ability of proflavine and actinomycin D to inhibit transformation has been established and the phenomenon investigated in an attempt to elucidate the mechanism(s) by which these compounds exert their effect. In this transforming system, Bacillus subtilis strain 168, an indole-negative auxotroph, was used as the recipient cell and B. subtilis strain 168WT, a nutritionally independent prototroph, was used as the donor for transforming DNA. The optimum temperature for transformation was 37° C.

Exposure of B. subtilis strain 168 to low concentrations (10 μg/ml or less) of proflavine or actinomycin D for a period of 30 minutes did not show a decrease in viable cell counts. However, high concentrations (20 μg/ml) showed a pronounced killing.

Addition of either actinomycin D or proflavine to the transformation mixture inhibited transformation from auxotrophy to prototrophy. Recent reports (Kay, 1959; Bradley, 1961; and Horwitz et al., 1962) have indicated that both of these inhibitors are able to complex with nucleic acids, and we observed a decrease in transformation following incubation of transforming DNA with either inhibitor. This decrease in the biological activity of the DNA,

would suggest the possible formation of DNA-test compound "complexes" which would interfere with transfer or "incorporation" of genetic information into the recipient cell genome. Investigations showed that the biological activity of the DNA-test compound "complexes" could be restored if the mixtures were dialyzed for 48 hours against a sodium chloride-sodium citrate solution at 4^{o} C.

Absorbancy spectra studies conducted on the DNA-test compound "complexes" before and after dialysis revealed that a minute, yet detectable amount of actinomycin D was present after dialysis. These studies suggested that a irreversible complex was formed between DNA and actinomycin D but since nearly all biological activity was restored by dialysis, the complexing of actinomycin D with DNA obviously did not permanently influence the transforming ability of the DNA.

Thermal denaturation studies using the dialyzed mixtures showed that the thermal denaturation temperatures of the DNA-test compound "complexes were higher than the DNA control, establishing that exposure of the transforming DNA to either proflavine or actinomycin D influences the heat stability of the molecule.

Since the inhibitors do not appear to inhibit the biological activity of the DNA molecule by complexing or interacting with that site of the molecule functional in this transformation system, it was proposed that the inhibitors exert their effect primarily on the recipient cell. This conclusion is augmented by the observation that similar inhibition patterns were observed regard-

less of whether the cells or DNA were incubated with the inhibitors prior to initiating transformation or when all components were added simultaneously. Also, results indicated that addition of either proflavine or actinomycin D to the transforming mixture at 0, 5, or 15 minutes after the addition of DNA showed similar patterns of inhibition. These findings eliminate the possibility that the inhibitors interfered with penetration of the DNA through the cell permeability barrier and suggest that the inhibitor action was intracellular.

The results suggest that 15 minutes after addition of DNA the recipient cells have not been "transformed" since both proflavine and actinomycin D, added at this time, prevent expression of transformants. Thus, it would appear that both of these compounds function by inhibiting the "incorporation" of donor information into the genome of the host cell.

Hurwitz et al., (1962) reported that actinomycin D and proflavine inhibited DNA dependent RNA and DNA polymerases. Both polymerase systems were inhibited, but the RNA polymerase was somewhat more sensitive to actinomycin D while the DNA polymerase was more sensitive to proflavine. A plausible explanation for these results would be that nucleic acid polymerases are important in the "incorporation" of donor information into the recipient cell genome and that actinomycin D and proflavine inhibit transformation by influencing these enzyme systems.

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