THE UNIVERSITY OF MANITOBA

Nutritional Requirements for Spore Outgrowth and

Effect of Netropsin on Sporulating Cells of a Clostridium sp.

by

Charlie A. Naccarato

A Thesis Submitted to the Faculty of Graduate Studies in

Partial Fulfillment of the Requirements for the Degree of

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ABSTRACT

A modification of a chemically-defined medium (Lee1975) was devised to study the nutrition requirements for germination, growth and sporulation of a non-toxigenic, sporogenic strain of C. botulinum type E Msp$^+$. The CDM 11 consisted of a mixture of amino acids, vitamin factors, purines, pyrimidines, trace minerals, 0.4% glucose, 1.0% sodium acetate, 0.1% sodium bicarbonate and 0.2% sodium thioglycollate in a phosphate buffer pH 7.0. The effect of single trace mineral deletions showed that $MnSO_4$, $CaCl_2$, $CuSO_4$, or $ZnSO_4$ prevented germination of the Msp$^+$ strain while omission of NaCl, $MgSO_4$ or $FeSO_4$ only delayed outgrowth. The omission of para-amino benzoic acid or inositol had no effect on outgrowth and subsequent vegetative development and the single deletions of uracil, xanthine or adenine suggests that an exogenous supply of purines and pyrimidines was not essential for germination or outgrowth of the Msp$^+$ strain. Changing the concentration of isoleucine from 5mM to 0.4mM had no effect on the O.D.. The very rapid incorporation of C^{14}-isoleucine into cells in trypticase-peptone-glucose-yeast extract (TPGY) and in CDM during outgrowth indicates that isoleucine may be likely used for a process other than protein synthesis. The delay in outgrowth with the substitution of isoleucine and threonine by 2-Keto butyrate implies that the threonine deaminase system was being induced during the lag-phase preceeding the rise in optical density.

Derepression and turnover of alkaline phosphatase in the presence of netropsin was only slightly delayed. The assays for acid phosphatase were inconclusive. Netropsin did prevent the synthesis of dipicolinic acid (DPA) a specific metabolite required for sporulation.

Netropsin had an effect on stage lll and stage lV of sporulating cells of Msp$^+$ but did not significantly affect the growth rate.

Compared with untreated cells two types of damage were observed in electron-micrographs of netropsin-treated cells. In cells inhibited at stage lll a number of lesions were observed at the site of the fore-spore membrane. In those cells that had already entered stage lV, the cellular change observed was an abnormal aggregation of the nuclear material into long parellel cords aligned along the forespore membrane together with the arrest of sporulation.

TO MY WONDERFUL FAMILY

ACKNOWLEDGEMENTS

APPENDIX

Reagents required for electron microscopy

1. Vernal -Acetate Buffer (Michaelis)

    sodium barbitone   2.94g

    sodium acetate   1.94g   Make up to 100ml H_2O D

    sodiume chloride   2.94g

2. Kellenberger Buffer   Must be used within 24 h

    Vernal-acetate buffer   5ml

    Distilled water   13ml

    HCL 0.1 N   7ml

    $CaCl_2$ 0.1 M   0.25ml

    Adjust pH of this buffer to 6.1 using 0.1 N HCL

3. Kellenberger Fixative

    Prepare 8-10 h before use: keep at RT^O until all had dissolved

    Store at 4^OC: Must be used within 48 h.

    Wash the osmic acid vial (OsO_4) with soap or acetone

    Add the complete vial to a glass stopped bottle.

    Place in 10ml of the Kellenberger buffer, shake well on rotor

    until all the crystals have dissolved. Store at 4^OC.

4. Wahsing fluid

    Uranyl acetate   0.5g

    Kellenberger buffer   100ml

    Stable at 4^OC for several weeks.

5. Tryptone Medium

Bacto-tryptone	1.0g
NaCl	0.5g
H_2O D	100ml

6. Infilteration

Propylene Oxide	75ml
Propylene Oxide/Araldite	50/50
Propylene Oxide/Araldite	25/75
Araldite	

   Water bath at 45°C. Oven at 60°C.

7. Embedding

   Prepare 50ml of Araldite for capsules

   31g Araldite Resin

   22g DDSA

   Place in dark dessicator to remove bubbles

   Then add DMP-30

8. Polymerization

   24 h at RT$^{\circ}$

   24 h at 48°

   24 h at 60°

Protocol for Electron Microscopy

Day One

Add equal volumes of culture and 6% glutaraldeyde in Vernal-acetate buffer. Fix for 2-4 h at RT$^{\circ}$.

Centrifuge at 1800 g for 15 min. Wash 2x in Vernal-acetate buffer.

Resuspend in 1% osmic acid dissolved in Vernal-acetate.

Use 1ml of this Kellenberger Fixative, add in 0.1ml tryptone medium.

Stopper tightly and leave at 4° for 8-16 h.

Day Two

Dilute suspension with 8ml Kellenberger buffer

Centrifuge immediately at 1800 g for 5 min.

Add one to two drops of a 0.005% toluidine blue dye for 5 min.

Warm samples in a 45° water bath

Add 4-6 drops of a 3% Noble Agar solution (Difco)

Suck up sample with prewarmed pipet, place on ice and expel.

Cut into blocks and check for visible purple grandules

Place in 2.0ml Uranyl-acetate washing fluid for 2 h at RT$^{\circ}$.

Dehydration Steps:

30% ethyl alcohol	15 min 2x
50% ethyl alcohol	15 min 2x
70% ethyl alcohol	10 min 3x
100% ethyl alcohol	10 min 3x

During this procedure rotate Araldite with Propylene Oxide in a vacuum desiccator to remove all the bubbles, store in the dark.

Infilteration:

Propylene Oxide   1 x 15 min   (three separate times)

Rotation:

Rotation:

Propylene Oxide/Araldite	50/50	One hour
Propylene Oxide/Araldite	25/75	8 h – 16 h
Araldite		10 h

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INTRODUCTION

The growth cycle occurring in <u>Bacillus</u> and <u>Clostridium</u> <u>spp</u>.

consists of vegetative growth leading to the formation of an endo-

spore, lysis of the mother cell to release the mature spore and germ-

ination of the spore followed by outgrowth into a new vegetative cell,

(18,23,27,28). Germination is the conversion of a resistant and

dormant spore into a sensitive and metabolically active form (27,28).

Research on spore germination has been largely concerned with elucid-

ating the biochemical and biophysical steps comprising the trigger

reaction and with describing the resultant changes recognizable as

germination. The part of the growth cycle following germination and

terminating in the formation of the first mature vegetative cell is

referred to as outgrowth. Many aerobic <u>Bacillus</u> <u>spp.</u> have simple

growth requirements and lend themselves more easily to study the various

aspects of germination and outgrowth. Anaerobic <u>Clostridium</u> <u>spp.</u>are

more difficult to deal with and until recently had never been cultured

in a simple medium. The nutritional components of a double strength

cooked meat medium (75) provided optimal growth and spore formation

but Roberts had difficulty in harvesting "clean spores" from this com-

lex medium. A synthetic or chemically-defined medium offers the most

direct route to the elucidation of nutritional requirements. The early

literature concerning this was mainly restricted to strains of type A

and B of <u>C</u>. <u>parabotulinum</u> (7,8). Germination and growth of strain D8

in asynthetic medium was reported by Ward and Carroll (94) in 1966.

Burrows (8) introduced an inorganic salts medium supplemented with

glucose, vitamins, nucleic acids, and various amino acids which supp-
orted the growth of C. botulinum. Eventhough chemically-defined med-
ium to support the growth of Clostridium spp. have been developed;
little is known about the roles of trace mineral, purines, pyrimidines,
vitamin factors and amino acids in thegermination and outgrowth process.
Trace minerals are known to be required for a variety of metabolic func-
tions and although spores of some bacterial species are able to germin-
ate in medium lacking cations, the addition of trace minerals increased
the rate of germination of many strains of Bacillus spp.(28,38). Sodium
ions accelerated the growth of the Msp$^+$ strain and was shown to be ess-
ential for outgrowth of C. bifermentans.(28). Inakai and Haga (40) used
a semi-sythetic medium containing vitamin-free casamino acids to study
the requirements of the Iwanai strain of type E. In accordance with the
studies on C. botulinum type E D8 strain there is no absolute require-
ment for any single vitamins. Biotin, thiamine and para-amino benzoic
acid on the other hand, were shown to be essential for the outgrowth of
five strains of C. parabotulinum (68). Purines and pyrimidines were not
required for the type E D8 strain (94) and Lee (51) reported that out-
growth of the Msp$^+$ strain was stimulated when purines and pyrimidines
were included into the chemically-defined medium.

The amino acid requirements for outgrowth of B. stereothermophilus,
B. coagulans, and B. cereus included isoleucine, valine, methionine,
andglutamate (28), whereas B. notto only required isoleucine(94).
B. subtilis and B. mycoides had no absolute amino acid requirements. In

single amino acid deletion studies (51), isoleucine was shown to be

an absolute requirement for outgorwth of the Msp$^{,+}$ strain. The purpose

of this research was to test the ability of the Msp^{+} strain to grow

under various nutritional conditions, in hope to further elucidate the

roles of trace minerals, purines,pyrimidines, vitamin factors and amino

acids in germination, outgrowth, and vegetative development of the

bacterial spore.

There is a remarkable similiarity in the sporulation of species of

Bacillus and Clostridium both from a morphological and the physiological

points of view. Sporulation is a complex process which is initiated at

the end of the exponential phase of growth (To) when many enzymes are

released from catabolite-repression (14,18,23). The genetic localization

of spore-specific genes and their arrangement and regulation in the

bacterial chromosome is not yet known. Thus far no single-gene or gene-

product has definitely been identified as an initiator of the sporulation

process,(64). No methods are available to properly separate the enzymes

of the developing spore from the mother cell due to the difficulty in

isolating the various compartments and their contents intact, (18,28).

Due to the overlap in the levels of many spore-specific enzymes during

the change from exponential to the stationary growth phases (57), the

specific role of the derepression of enzymes is unknown. It has prev-

iously been shown that enzymes were found to occur prior to sporulation

although it was not essential for the initiation of sporulation (45,44,

94). The antibiotic,netropsin known to bind to only A-T base pairs

has further strengthened the view that sporulation is inhibited by the

drug possibly by binding to sporulation-specific genes that are rich in A-T for the RNA polymerase binding sites. Netropsin had little effect on growth but significantly affected sporulation in B.sublitis (44). Inhibition of the rate of expression of sporulation genes may be only slightly affected but the cummulative effects are that of unbalancing the delicate sporulation process, thus resulting in abortive sporulation. In this study we used netropsin in order to further understand the phenomenon of catabolite-derepression, since netrospin did not prevent expression of catabolic functions but was shown to selectively inhibit the sporulation process as a whole.

HISTORICAL

HISTORICAL

The intracellular differentiation of dormant bacterial spores
into metabolically active vegetative cells has been extensively
studied (3,14,31). It is now clear that germination of the bact-
erial spore is essentially a degradative process which does not involve
extensive synthesis of new macromolecules (32,33). The distinquishing
feature of germination is the development from a phase-bright to a
phase-dark state as a result of a change in the structure of the cortex
(27,28), which is accompanied by a loss of resistance to heat, pressure,
desiccation, extreme pH, ultraviolet and ionizing radiation. Whereas
outgrowth of the emerging cell is characterized by a rise in respira-
tory activity and an ordered synthesis of macromolecules such as nucleic
acids, proteins and cell-wall peptidoglycans (20,38,62). The initial
events are dependant on enzymes pre-existing in the spore and the later
events require enzymes that are absent or present in limiting amounts
and are contingent on the development of the protein synthesizing
capacity, (63). It is evident that outgrowth is accompanied by
changes in the patterns of RNA synthesis, classes of ribosomes, the
relative amounts of enzymes and overall metabolic activity and some,
though probably not all are unique to spore outgrowth, (28,78,80).

Most of the work on sporogenesis of bacteria has been carried out
on species of Bacillus and Clostridium (3,5,13,14,23). It consists
of an ordered sequence of structural changes coupled with biochemical
events which occur in seven stages, (Fig. 1). Sporulation begins

Figure 1

STAGE 0	STAGE I	STAGE II	STAGE III	STAGE IV	STAGE V	STAGE VI
Vegetative cell	Chromatin filament	Spore septum	Spore protoplast	Cortex formation (refractility)	Coat formation	Maturation
	Antibiotic Exo-protease Protein turnover Ribonuclease Amylase	Alanine dehydrogenase	Alkaline phosphatase Glucose dehydrogenase Aconitase Heat-resistant catalase	Ribosidase Adenosine deaminase Dipicolinic acid Uptake of Ca^{2+}	Cysteine incorporation Octanol resistance	Alanine racemase Heat resistance

by the formation of an axially disposed filament of condensed chromatin (stage1) followed by a unique type of membrane-dependent, internal cell-division with an infolding of the cell-membrane leading to the formation of a forespore (stage 111). The engulfment of the forespore protoplast occurs by unidirectional growth, so that the two membrane surfaces that previously faced the exterior of the cell now face each other at the surface of the forespore cytoplasm. Peptido-glvcan precursors are transported to the space between the membranes while cortical peptidoglycans and germ cell-walls are being synthesized during stage 111 and stage 1V. In _Bacillus_ _spp._ the cortex develops in stage 1V followed by the coat in stage V (14), whereas in the _Clostridium_ _spp._ (15,23) segments of spore coat appear at the same time as the cortex. Maturation is associated with stage V1 when the endospore undergoes a change in the structure of the cortex, development of heat-resistance, refractility and an exosporium.

The nutritional components of complex double strength cooked-meat medium (Roberts) provide for optimal growth and spore formation of _C._ _botulinum_ (75). However a chemically-defined medium is essential for defining the requirements for growth and spore formation. Burrows introdused an inorganic salts medium supplemented with glucoe, vitamins, nucleic acids and various amino acids which supported the growth of _C._ _botulinum_ (7,8). Biotin, thiamine and para-amino benzoic acid were required by five strains of type A _C._ _parabotulinum_ and in addition pyridoxin and nicotinic acid by a sixth strain (68,69). Magnesium, mangenese, calcium and iron were essential for the germination

and optimal growth (94), but were neither required nor stimulatory
for outgrowth. Only amino acids have been shown to be a prerequisite
for outgrowth of spores of Clostridium spp. and many Bacillus spp.
(4,5,7,16). The minimum amino acids for outgrowth of B. stereo-
thermophilus, B. coagulans, and B.cereus (28,29) included isoleucine,
valine, methionine, and glutamate. Isoleucine was essential for the
outgrowth of B. notto while B. subtilis and B. mycoides had no absol-
ute amino acid requirements, (71,72).

The amino acid requirements for Clostridium spp., C. botulinum
type A and C. botulinum type E was shown to be isoleucine, valine, and
leucine, (7,40). In a recent study outgrowth of Msp[+] strain and
other type E strains was acheived in a chemically-defined medium cont-
aining 19 amino acids, including isoleucine, valine and leucine. Single
deletion studies showed that isoleucine was essential for outgrowth of
the Msp[+] strain (51), while omission of arginine, methionine,tyrosine
and threonine seemed only to delay the outgrowth process.

The vast majority of recent studies in the area of amino acid met-
abolism has been concerned with either the regulation of biosynthetic
enzymes (10,33), or with amino acid transport (4.5,16). Most of
the early work on amino acid pools was qualitative and designed only to
determine which amino acids occurred naturally (31,43). From studies
designed specifically to estimate the concentrations of the individual
amino acids in the pools (10,16), it was found that most were present
at all times and that a few amino acids made up the majority of the pool.
Glutamte and alanine comprised 60-90% of the total free amino acid pool

of growing cells, whereas other amino acids were present in low conc-

entrations (16,80   ). In spores, L-glutamate but not alanine was

the predominant component of the free amino acid pool of several <u>Bacillus</u>

<u>spp.</u> (16,20   ). The level of glutamate in spore or vegetative cells

was not affected by the concentration of glutamate present in the medium,

(16). Exogenous amino acids were shown to first <u>equilibrate</u> with

native amino acids prior to incorporation into protein (4,16,43,79).

The individual rates of transport varied as much as 40 fold and the

intracellular/extracellular ratios of 100-200 fold were maintined during

growth of a population of cells in a glucose minimal salt medium (16).

The total intracellular amino acid pool during growth of <u>B</u>. <u>licheniformis</u>

in a minimal media rapidly decreased near the end of exponential growth,

(10   ). The intracellular amino acid pools have been shown to be

dependant upon the composition of the salts as well as the age of the

culture, (10). A ten-fold increase in exracellular amino acids

occurs by the extrusion from the cell during the change from vegetative

to sporulation metabolism, (4). The uptake of isoleucine, leucine

and valine by <u>Escherichia</u> <u>coli</u> is required for protein synthesis and

when synthesis is blocked these branched amino acids are recovered

unmodified from the intracellular fluid (50). The kinetic analysis

of active transport shows that these branched amino acids have more

than on transport system, (4). Transfer of vegetative cells to a

medium lacking isoleucine completely inhibited the sporulaation process

in <u>B</u>. <u>subtilis</u> (16). Isoleucine did not affect early processes like

the protease formation of septum but prevented the increase in enzyme

levels needed for spore-specific protein biosynthesis. The exact role

of this amino acid still remains unclear. (10,16).

The intracellular turnover of proteins in B. subtilis (16,47),

showed that an exoprotease was produced in stage 1 of the sporulation

process degrading existing proteins at the rate of 8-10% per hour. The

protein in the mother cell was degraded so that by 6 h all the cyto-

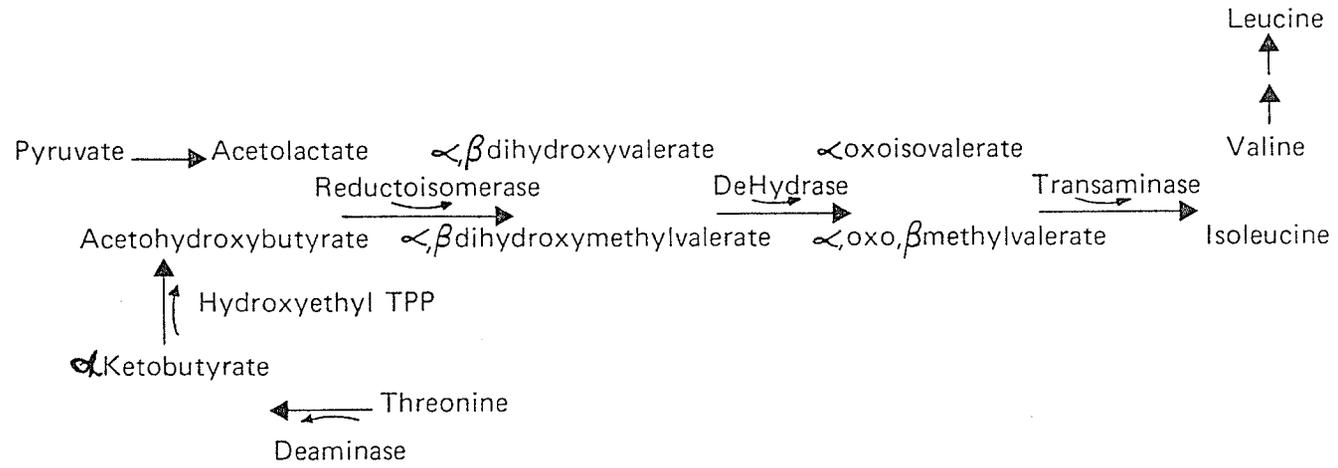plasm was made up of "de novo" synthesized proteins.

The routes of synthesis of valine, leucine and isoleucine are

closely related. The biosynthesis of valine and leucine proceeds through

the common intermediate $\propto$ oxoisovaleric acid, (Fig.2). The thiamine

pyrophosphate-dependent condensation of pyruvate with a two-carbon

fragment yields acetoaectic acid which is converted directly to the

$\propto,\beta$ -dihydroxyisovaleric acid. The transformation, which closely is

related to the classical pinacol rearrangement, is catalyzed by aceto-

hydroxy-acid isomeroreductase and requires Mg^{+2} and a reduced pyridine

nucleotide. By analogy with the known mechanism for the pinacol rearr-

angment, Mg^{+2} may function as a superacid catalyst, coordinating with

the carbonyl oxygen of acetolactate and consequently aiding the methyl

migration to an electron-deficient carbon atom. Dihydroxy acid dehydr-

ase (2,3-dihydroxy acid hydro-lyase) catalyzes the dehydration of

$\propto,\beta$ -dihdroxyisovaleric acid, yeilding$\propto$ -oxoisovaleric acid, which, by

transamination, vields valine directly. $\propto$ -oxoisovaleric acid is cond-

ensed with acetvl-SCoA, yielding$\propto$ -hydroxy-β-carboxyisocaproic acid.

The substance is converted to$\propto$ -hydroxy- β-carboxyisocaproicacid by a

dehydration-rehydration sequence closely related to that for the citrate-

isocitrate conversion catalyzed by aconitase. However, the$\propto$ -hydroxyβ-

carboxyisocaproic acid isomerase has been purified and shown to be

distinct from aconitase (67). Oxidative decarboxylation and trans-

amination complete the synthesis of leucine. Threonine dehydratase

(L-threonine hydro-lyase (deaminating)) catalyzes the conversion

of threonine to 2-Keto-butyrate. The latter substance is condensed

with hydroxyethyl TPP, yielding α -aceto β -hydroxybutyric acid, a key

intermediate for the synthesis of isoleucine. The synthesis of iso-

leucine from this intermediate closely parellels the synthesis of

valine from acetolactate, and, indeed, the same enzymes appear to be

involved in both reaction sequences except for the terminal transamin-

ases since two transaminases for valine but only one for isoleucine

has been found. This may account for mutants that require isoleucine

but not valine.(67).

Basic questions in the areaof spore morphogensis are geared towards

understanding how new spore components are synthesized and how many

new sporulation specific genes are required for the process (14,18   ).

The rates of synthesis and types of spore layers has been shown to

vary from species to species (2,3,33). The membranous components of

spores contain several polymers not found in vegetative cells, and the

synthesis of specific spore peptidoglycans have been shown to involve

new enzyme activities (31,33). Specific inhibitors such as rifampcin,

and the actinomycin series have been widely used to elucidate the role

of various enzymes in the sporulation sequence (36). Netropsin was

first found to inhibit sporulation of B. subtilis (Keilman 1975). He

found that 0.5ug/ml would permit growth and catabolite-derepression

while specifically inhibiting sporulation at stage 111.,(44).

Figure 2

Biosynthesis of the Branched Amino Acids

Leucine

Pyruvate ⟶ Acetolactate   ∝,β dihydroxyvalerate   ∝oxoisovalerate   Valine

    Reductoisomerase   DeHydrase   Transaminase

Acetohydroxybutyrate   ∝,β dihydroxymethylvalerate   ∝,oxo,β methylvalerate   Isoleucine

    Hydroxyethyl TPP

∝Ketobutyrate

    ⟵ Threonine

Deaminase

Netropsin, an antibiotic obtained from culture filterates of a hitherto undescribed actinomycin, Streptomyces netropsis (21), is a tetraacidic base with a formula approximating $C_{32}H_{48}N_{18}O_4$, (Fig.3). Alkaline titration of netropsin indicates that all basic groups are of equal strength, hydrolysis with $Ba(OH)_2$ provide evidence of an amide linkage and a negative Van Slyke reaction suggests the absence of any amino acids. Netropsin exhibits a wide range of antibacterial and anti-fugal activity and prevents DNA and RNA tumor viruses from replicating in mamalian cell systems,(21). Whereas 3μg/ml of the crystalline hydrochloride was sufficient to inhibit growth of Staphylococcus albus, 1000μg/ml was required for Pseudomonas aeroginosa (21).

The mode of action of netropsin differs from the actinomycins series of antibiotics. It binds to DNA by a noninterchelating mechanism since it has been shown not to cause unwinding of supercoiled DNA,(96). A marked specificity for DNA's which contained adenine-thymine or inosine-cytosine base pairs was shown by the use of spectral titration, analytical density gradient centrifugation, thermal denaturation and equilibrium dialysis, (96). The netropsin molecule is predominantly planar as a result of hypercongugation. The propionamidino and the guanidino groups interact with the phosphate oxygens on opposite strands of the same A-T pair, with the subsequent formation of hydrogen bonding between the 2 basic groups and the negetively charged backbone of the Duplex DNA. This would explain the necessity for Duplex DNA,(Fig4). The pyrolle rings, both in the same plane are tilted 30° from the plane of the base pairs. A hydrogen bond connects the amide to the 2-Keto group of thymidine. This model explains the marked preference of the

Figure 3
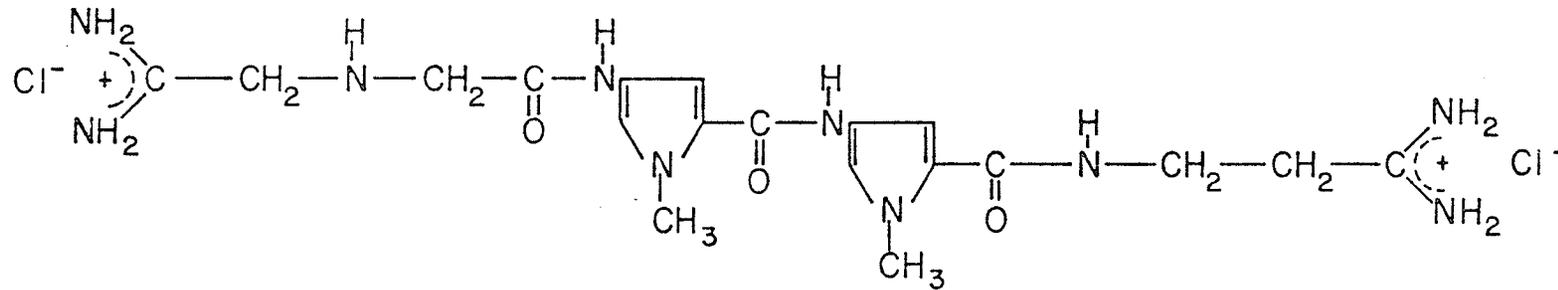
NETROPSIN

antibiotic for A-T or I-C base pairs since the presence of the 2 amino group on Guanine is too large to permit close association of the drug with the minor groove of Duplex DNA,(Fig. 4). Analogues of netropsin showed that by extending the length of the methyl-pyrolle group. the binding affinity for DNA was increased whereas eliminating the end basic groups resulted in a marked decrease in the binding affinity, (97).

Bacterial sporulation is a complex process where initiation occurs at the end of exponential phase of growth (T_o) when some enzymes are released from catabolite-repression (31). The identification of events which occur during the early stages of sporulation have provided a clearer picture of the initial regulatory processes in the sporulation cycle, (32). Although DNA-RNA hybrid competition experiments have demonstrated that differential transcription occurs during sporuation it has been difficult to distinquish between fortuitous expression of genes during post logrithmic growth and specific expression of the sporulation genes,(33). The subsequent development may be regulated at the level of transcription, translation or both (33). Arnoson (3) suggested later development was contigent on a long-lived m-RNA species. Mandelstam et al (57) showed that successive events included commitment to the formation of alkaline phosphatase, synthesis of dipicolinic acid, heat-resistance and refractility and each point of committment was followed by a 30 minute period in which the event concerned ceased to be inhibited by Actinomycin D, (53).

The biochemical events associated with sporulation were described as three main types, (14). The primary sequence depended on events specifically concerned with spore formation such as serine proteases,