STUDIES ON GLUTAMINE SYNTHETASE AND GLUTAMATE SYNTHASE DURING GROWTH AND SPORULATION IN *BACILLUS SUBTILIS*

PAN FUI-LIN

Presented for the degree of Master of Science in the Faculty of Science, University of Glasgow.

Department of Microbiology February, 1978

ACKNOWLEDGEMENTS

Neither the project nor the thesis would have reached completion but for the conscientious supervision and enthusiastic support of Dr. J.G. Coote. I thank Professor Wardlaw for his helpful review of the manuscript and for the use of the facilities in the department.

I much appreciate the help of Mr. F.W. Park in demonstrating the use of certain laboratory equipment and techniques.

The British Council generously contributed a grant, without which this study could not have been undertaken, and for this I am most grateful.

My deepest gratitude is due to my family, especially my late father, my beloved mother and brother for their love, constant encouragement and for financial support.

Mrs. Anne Strachan is to be thanked for the final typing of the manuscript.

Finally, my sincere thanks are due to all those who have contributed in various ways to my happy and successful stay in the United Kingdom.

SUMMARY

The purpose of this work was to investigate the activity of two enzymes, glutamine synthetase (GNS) and glutamate synthase (GTS) during growth and sporulation in Bacillus subtilis 168. The coupled activity of these two enzymes is known to be one route by which ammonia is assimilated in Bacillus spp. Ammonia is known to be a repressor of sporulation and the overall aim of the work was to assess the possibility that one or both of these enzymes may play a role in the repression of sporulation. The investigation adopted two broad approaches. 1. Determination of the specific activities of the enzymes; firstly, during growth of the organism in minimal medium with various nitrogen sources and secondly, during sporulation induced by either carbon or nitrogen limitation. 2. Characterisation of a GTS-defective mutant and observations on its ability to sporulate under conditions of carbon and nitrogen deprivation.

Assay procedures for GNS and GTS were developed based on previously published methods. Both enzymes were found almost entirely in the soluble fraction of broken cell suspensions. GTS activity was found to be slightly unstable on storage and for this reason all enzyme assays were performed on freshly prepared cell-free extracts.

B. subtilis 168 grew well in a minimal medium with glucose as carbon source and either ammonia, L-arginine or L-glutamine as nitrogen source. It grew more slowly with L-glutamate or nitrate and only very slowly with L-histidine as source of nitrogen. The specific activity of either GNS or GTS could be altered by varying the source of nitrogen supplied for growth. GNS was repressed when either ammonia, L-glutamine or L-arginine were used as nitrogen source, but was derepressed when

L-glutamate or nitrate were used. GTS was repressed by L-glutamate or L-arginine and to a lesser extent by L-glutamine, but was relatively derepressed in the presence of ammonia or nitrate. These observations supported previous suggestions that GNS was repressed by L-glutamine and GTS repressed by L-glutamate in Bacillus spp.

Sporulation was obtained using the replacement technique whereby exponentially growing cells in a rich medium were resuspended in a poor medium to induce sporulation. This procedure gives a clearly defined point of initiation to the process and allows good synchrony throughout the cell population during sporulation. The composition of the resuspension medium was adjusted so that sporulation could be induced either by starvation for an adequate carbon source or by starvation for a source of nitrogen. No significant alterations in GNS or GTS specific activities were detected at 20 min or 40 min after initiation to sporulation by either carbon or nitrogen starvation. This result indicated that repression or derepression of GNS or GTS played no role in the onset of sporulation, although transient changes in enzyme specific activity could not be excluded by these experiments.

A mutant strain (glt-100), isolated originally as an auxotroph requiring L-glutamate for growth, was more fully characterised and shown to lack GTS activity. This strain was unable to grow in minimal medium with ammonia as source of nitrogen. This fact, coupled with a failure to detect glutamate dehydrogenase activity in cell-free extracts, indicated that B. subtilis 168 lacks glutamate dehydrogenase capable of assimilating ammonia. Ammonia assimilation was assumed to occur via the coupled reactions catalysed by GNS and GTS as strain glt-100, which lacked a functional GTS, was unable to grow with ammonia. Strain glt-100 was

unable to sporulate under conditions where the process was assumed to be initiated by starvation for an adequate nitrogen supply, although it sporulated normally under conditions of carbon deprivation. The deficiency in GTS activity in strain glt-100, which prevented successful sporulation under conditions of nitrogen deprivation, was active during the initial 2.5 hours of sporulation. L-glutamine has previously been implicated as a repressor of sporulation, but the activities of GNS found during the onset of sporulation in the wild-type and strain glt-100 and the sporulation behaviour of the mutant strain in various resuspension media tended to conflict with this hypothesis.

CONTENTS

INDEX OF TABLES

INDEX OF FIGURES

ABBREVIATIONS

ADP	=	Adenosine 5'-diphosphate
AMP	=	Adenosine 5'-monophosphate
AP	=	Alkaline phosphatase
ATP	=	Adenosine 5'-triphosphate
BSA	=	Bovine serum albumin
cAMP	=	Adenosine 3'5'-cyclic monophosphate
CAP	=	Catabolite gene activator protein
cGMP	=	Guanosine 3'5'-cyclic monophosphate
CTP	=	Cytidine 5'-triphosphate
DNA	=	Deoxyribonucleic acid
DPA	=	Dipicolinic acid
E	=	Extinction coefficient
EDTA	=	Ethylenediaminetetraacetate
GDH	=	Glucose dehydrogenase
GHA	=	γ-glutamyl hydroxamic acid
GNS	=	Glutamine synthetase
GTS	=	Glutamate synthase
Km	=	Michaelis constant
NADH	=	Reduced Nicotinamide adenine dinucleotide
NADPH	=	Reduced Nicotinamide adenine dinucleotide phosphate
nm	=	Nanometre
nmole	=	Nanomole
p.s.i.	=	Pound per square inch
RNA	=	Ribonucleic acid
rpm	=	Revolutions per minute
TME buffer	=	Tris-HCl + β-mercaptoethanol + EDTA buffer
t_s	=	Temperature-sensitive
Tris	=	2-Amino-2-(hydroxymethyl)-1,3-propanediol

w/v	=	Weight/volume
µg	=	Microgram
µmole	=	Micromole
γGT	=	γ-glutamyl transferase

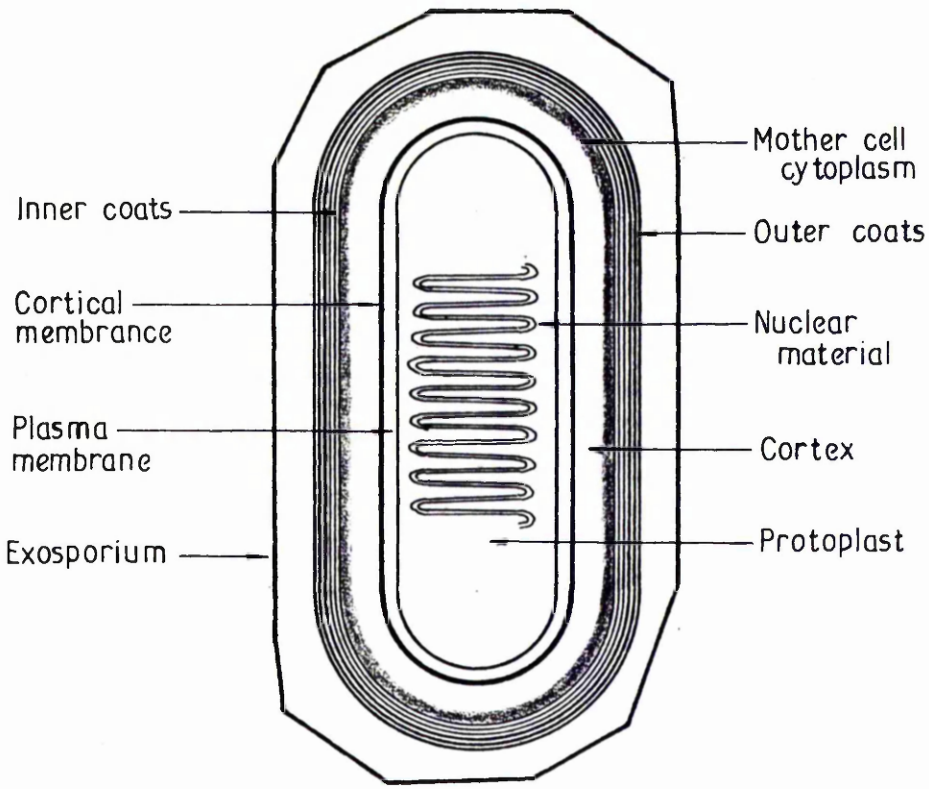
INTRODUCTION

Morphology and biochemistry of sporulation

At the end of growth, the vegetative cells of certain Gram positive bacteria, notably aerobic rods (*Bacillus* spp.), anaerobic rods (*Clostridium* Spp.) and a few cocci (*Sporosarcina* spp.) are converted to endospores through a time-ordered sequence of morphological and biochemical steps. The final product, the dormant endospore, differs cytologically and physiologically from the vegetative cell (Murrell, 1967). In micro-organisms spores are generally formed as a response to starvation and are used by the organism to survive a period of nutrient deprivation in its life-cycle. Bacterial endospores can remain dormant for many years and are very resistant to extremes of the environment including heat, pressure, desiccation and radiation. These properties, together with the pathogenic activity of some sporulating species, have made them of interest medically and in the food industry.

When an environment more suited to growth is encountered again, the endospore may germinate very rapidly, lose its resistant properties and during the process of outgrowth return to the vegetative state once again (Gould & Hurst, 1969). Figure 1 is an illustration of the structure of an endospore. Originally it was thought that the outermost layer, the exosporium, was absent from spores of *B. subtilis*, but recent evidence suggests that *B. subtilis* 168 spores, at least, are surrounded by a tightly wrapped exosporium-like outerlayer (Balassa, 1976; Aronson & Fitz-James, 1976). Beneath this are the coat layers which are protein in nature. The polypeptides making up the coat layers are unique to the spore. They are rich in the amino acid cysteine which is able to form a disulphide bridge with a cysteine residue in an adjacent polypeptide and this cross-linking between polypeptides gives the coat layers rigidity.

Figure 1. Diagram illustrating the structural components of a typical bacterial spore (a modified drawing taken from Mandelstam & McQuillen, 1973)


Mother cell cytoplasm
Inner coats
Outer coats
Cortical membrance
Nuclear material
Plasma membrane
Cortex
Exosporium
Protoplast

Aronson & Fitz-James (1976) have recently reviewed the structure of the spore coat layer. Beneath the coat layer is the cortex which is a peptidoglycan structure similar to that of the vegetative cell wall. However, there is less cross-linking of tetrapeptide residues in the spore cortex and this, together with the absence of teichoic acids, makes it a less rigid structure than the cell wall (Tipper & Gauthier, 1972). This flexibility is important because during maturation of the spore the cortex structure is thought to expand and dehydrate the inner protoplast as a result of high osmotic pressure (Gould & Dring, 1975). The consequent low water content of the protoplast is thought to help to give the spore many of its resistant properties and is the reason the spores appear refractile when viewed in the phase-contrast microscope. The innermost layer of the cortex, the germ cell wall, resembles the vegetative cell in structure (Tipper & Gauthier, 1972). It becomes the new emerging cell on germination of the spore when the dormant state is broken and the cortex and coat layers are lost (Gould & Hurst, 1969). All these structures surround the protoplast which is bounded by a membrane and contains all the necessary materials for the outgrowth of a new cell such as ribosomes, protein synthesis machinery, enzymes and a complete chromosome for replication. The protoplast also contains many of the enzymes found in the growing cell (Kornberg et al., 1968) and these are thought to be stabilised during dormancy by chelation with a compound unique to the endospore, calcium dipicolinate. This compound can make up 5-15% of the dry weight of the endospores depending on the species (Murrell, 1969). It is thought to inactivate reversibly the enzymes within the spore and so allows them to withstand the extremes of environment which would denature them in the vegetative cell (Keynan, 1972).

As its name indicates, the endospore is formed within the vegetative cell and when mature is released by lysis of the mother cell. The structural changes which accompany the transition from growth to spore formation have been examined by electron microscopy. They can conveniently be divided into seven stages (Ryter, 1965) which are represented in Figure 2. The work of Young & Fitz-James (1959) with B. cereus, Ryter (1965) with B. subtilis and Ohye & Murrell (1962) with B. coagulans showed that the morphological changes during the process are similar for each species. The process can be timed from the end of exponential growth (t-zero) and usually takes 7-8 hours at 37°C.

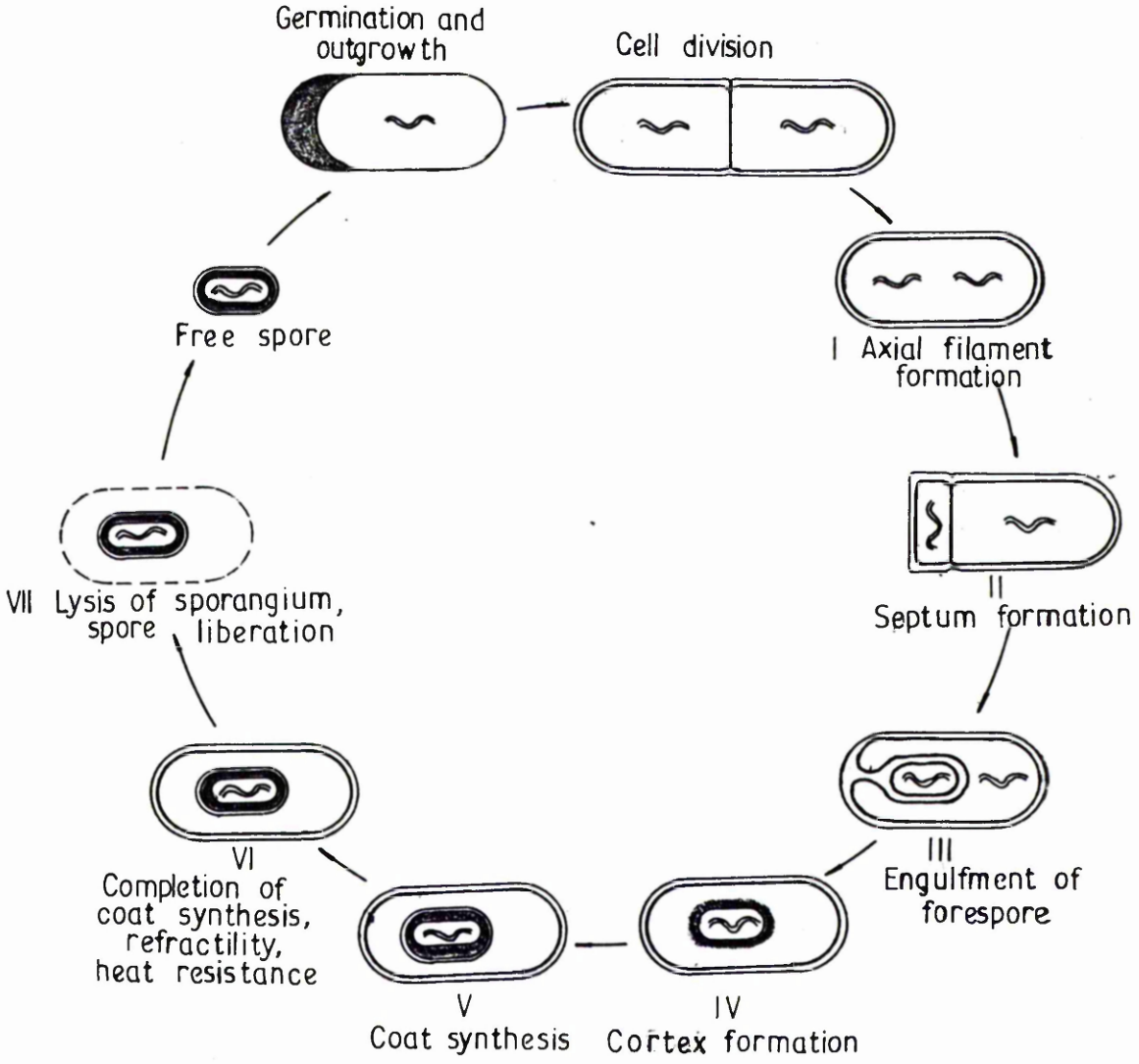
Stage 0. This is the vegetative stage at the end of growth when each cell will contain at least two chromosomes as compact bodies.

Stage I. Formation of axial chromatin. The chromosomes form a single axial filament of chromatin in the centre of the cell and this is probably connected to the cell membrane by mesosome attachments (Mandelstam, Kay & Hranueli, 1975).

Stage II. Septation. A septum is formed by membrane invagination at one pole of the cell. This septum is clearly distinguishable from a normal cell division septum by its polar position and the fact that little peptidoglycan is laid down between the septum membranes. The septum separates one chromosome from the other in what can be considered now a double-celled organism or sporangium.

Stage III. This is the formation of the protoplast free within the mother cell brought about by growth of the membrane and movement of the septum, at the point of attachment to the mother cell membrane, towards the pole of the cell. One chromosome is now enclosed within the protoplast and the other left in the mother cell.

Figure 2. Schematic representation of the morphological changes associated with the stages of sporulation (shown by the Roman numerals). Taken from Mandelstam & McQuillen (1973).


Germination and outgrowth
Cell division
Free spore
I Axial filament formation
VII Lysis of sporangium, spore liberation
II Septum formation
VI Completion of coat synthesis, refractility, heat resistance
III Engulfment of forespore
V Coat synthesis
IV Cortex formation

Stage IV. The cortex and germ cell wall are laid down between the double membrane of the protoplast. At this stage the spore begins to appear refractile under phase-contrast microscopy.

Stage V. The coat layers are deposited around the cortex and at this stage the spore develops resistance to organic chemicals such as n-octanol (Ryter, 1965).

Stage VI. This is the stage of maturation when the spore develops resistance to heat and becomes fully phase-bright in the microscope.

Stage VII. The mother cell lyses and releases the fully mature spore.

To obtain sporulation in liquid medium one of two techniques are commonly used, both involving starvation of the cells. The first is the "exhaustion" procedure in which bacteria are grown in a medium until they use up some essential nutrient and then sporulate (Schaeffer, 1969). The second is the "replacement" technique in which the cells are first grown in a rich medium and then during the exponential phase are transferred to a poor medium (Sterlini & Mandelstam, 1969). This "shift-down" procedure triggers sporulation and may involve starvation for a good carbon or nitrogen source. The experiments on sporulation in this work were performed using the replacement technique (see Materials and Methods section). This was preferred as it gives a clearly defined starting point, better synchrony throughout the process and allows the composition of the media to be conveniently altered.

Use of sporulation defective mutants

The process of sporulation in Bacillus spp. has received so much

attention because it can be considered as a primitive form of cellular differentiation (Mandelstam, 1976). As discussed above the spore has many characteristics which differ markedly from those of the growing cell. It might be hoped that information about the regulation of this relatively simple process might give some insight into the control mechanisms governing growth and differentiation in higher eukaryotes and help towards an understanding of such things as cancer where cells fail to differentiate in the normal manner. The ultimate aim of the study of sporulation will be to describe how the structural changes are controlled by the biochemical reactions within the cell. For this to happen it will be essential to distinguish between the biochemical activities which are necessary for sporulation and those which occur incidentally. Schaeffer (1969) suggested that the following were necessary pre-requisites for sporulation to occur: a) Nucleotides and amino acids for nucleic acid and protein synthesis. As sporulation occurs under starvation conditions these will not be supplied by the medium and must be derived by degradation of existing polymers. b) A ready supply of energy in the form of ATP to drive the biosynthetic reactions. Only a functional tricarboxylic acid cycle and an intact respiratory chain will provide enough energy for sporulation. c) Expression of sporulation specific genes (which remain repressed during growth) to allow synthesis of new proteins which direct the manufacture of the spore.

Apart from the general requirements given above it is difficult to know which biochemical changes are essential for sporulation. Enzymes synthesising the components unique to the spore, e.g. DPA or spore coat protein will be the only obvious requirement. The study of mutants which are unable to form normal spores has helped to categorise some of the biochemical changes which accompany sporulation. Sporulation mutants

can easily be obtained as they fail to produce a brown pigment characteristic of wild-type spores and many have been studied (see Piggot & Coote, 1976 for review of sporulation mutations). Mutations in events not required for sporulation should have no effect on the ability of the strain to form spores. Conversely, mutations in events required for sporulation should prevent spore formation and if the mutated event is specific to sporulation it should have no effect on growth. In addition, biochemical events involved in sporulation should not be expressed in sporulation defective mutants. Some examples will serve to illustrate these points. A mutant of B. subtilis which is temperature-sensitive for sporulation has an accompanying temperature-sensitive serine protease (Leighton & Doi, 1973). Another B. subtilis mutant with reduced extracellular protease activity showed reduced intracellular protein turnover (Mandelstam & Waites, 1968). The effect of these mutations on sporulation may reflect the requirement for protein turnover during the process. In the mutants lacking the necessary protease this turnover presumably does not occur, with a consequent effect on sporulation. Mutations in many enzymes of the tricarboxylic acid cycle cause a block at stage 0 or I of sporulation (Fortnagel & Freese, 1968). They cannot maintain sufficient quantities of ATP for sporulation (Freese, 1972). Mutations which give rise to a defective α-amylase do not affect sporulation (Schaeffer, 1969). Thus unlike the protease activities mentioned above this enzyme is not associated with sporulation, but it appears during the process probably as a response to the starvation conditions used to induce the process.

Many biochemical activities associated with sporulation increase in activity at particular times during the process (Warren, 1968; Waites

et al., 1970). Thus an increase in exo-protease activity is associated with the very earliest stage (0 - I), an increase in alkaline phosphatase (AP) activity with stage II, an increase in glucose dehydrogenase (GDH) activity with stage III and the synthesis of dipicolinic acid with stage IV - V (Waites et al., 1970; Coote, 1972). Many sporulation defective mutants have been isolated. The morphological stage at which the cells became blocked was determined by electron microscopy and the activity of various biochemical markers such as those above was also assayed in the mutants (Waites et al., 1970; Coote, 1972). It was clear from this work that for any one mutant the structural changes and biochemical activities would proceed normally up to the block, but when development stopped as a result of the mutation so the increase in biochemical activities normally associated with later events did not occur.

Thus the study of sporulation defective mutants established that a number of biochemical activities such as exoprotease, AP and GDH were involved in some way with the process because they failed to occur in mutants blocked before the stage in the process at which they increased in activity. In addition, the fact that a mutation prevented, in most cases, all subsequent development indicated that the process could be regarded as a dependent sequence of events where later steps depended on the successful completion of earlier ones.

More recently the study of mutants has played an increasingly important role in providing information on the regulation of the process. The construction of strains carrying two sporulation mutations (Coote & Mandelstam, 1973) substantiated for the most part the idea of a dependent sequence of events for the control of the process. A mutation which affected an early event in sporulation, when introduced into a strain

containing a mutation which affected a later stage, determined the phenotype of the double mutant. This at least was true when mutations up to and including stage III were used to construct the double mutants. When mutations affecting later stages were used the phenotypes of the double mutants in some cases were different from either of the parent strains. This type of result was explained by suggesting that the two mutations were not part of a dependent sequence, but were involved in separate pathways each independent of the other. This indicated that sporulation was not composed of a single pathway of dependent events during its later stages. It was suggested that two or more pathways might run in parallel especially during the later stages (Coote & Mandelstam, 1973).

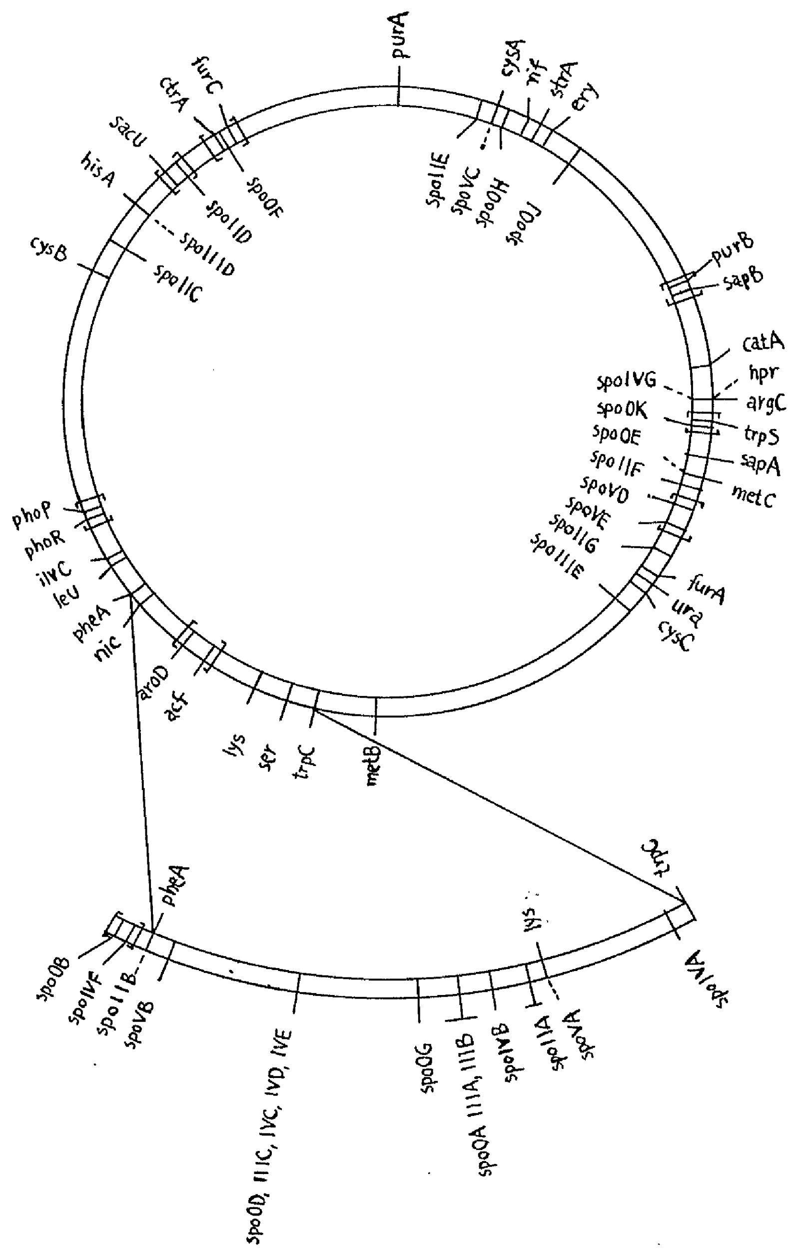
The work discussed above indicated that spo mutations were pleiotropic, i.e. a sporulation mutation prevented all subsequent events on the pathway after its expression. The use of constructed double mutants was able to show, in many cases, the order of expression of different spo loci identified by the pleiotropic spo mutations. This work did not indicate the exact time when a particular locus, characterised by a spo mutation, was expressed during the process. Sporulation could reasonably be expected to continue for some time after a mutated gene had been expressed. The study of conditional temperature-sensitive (ts) mutants provided some information on the exact time that a spo mutation that affected a particular stage was expressed and also for how long during the process it was expressed. The work with ts mutants has been reviewed elsewhere (Piggot & Coote, 1976), but the most significant observation seems to be that the spo loci vary greatly in the length of time they are expressed during the process. Thus a ts spo mutation which affected serine protease activity was in a locus whose expression was

required for at least the first 3 hours of the process (Leighton et al., 1972). On the other hand another ts spo mutation, which affected some event during stage II of the process, was in a locus whose expression was only required for 15 min (Young, 1976).

The studies of sporulation defective mutants have clearly thrown some light on the manner in which spore formation may be regulated. Scores of mutants have been characterised morphologically and biochemically. In addition, the positions of the spo mutations on the chromosome of B. subtilis 168 have been determined in many cases by a combination of transduction and transformation techniques. The mapping data relating to spo mutations is summarised elsewhere (Piggot & Coote, 1976). To date over 30 spo loci have been located on the genetic map and the position of these in relation to non-sporulation loci are shown in Figure 3.

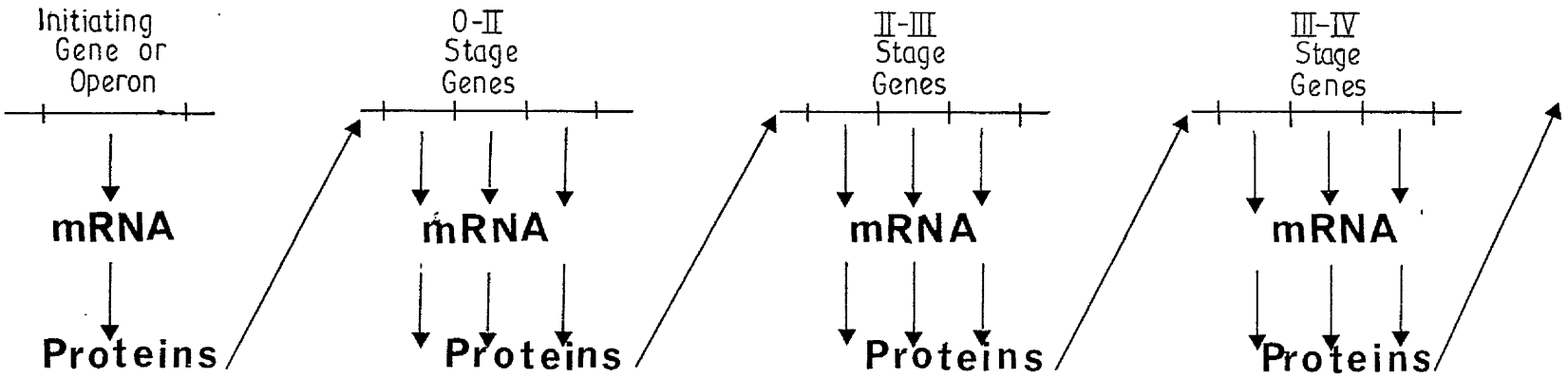
The loci have been identified by the spo mutations which are recognised by their effect on the sporulation behaviour of the mutant strains. For this reason the products of the genes specified by the spo mutations are for the most part unknown and this means that it is difficult to determine if a particular locus contains more than one gene. Mutations were placed in separate loci if they were: a) separated by a non-sporulation genetic marker, b) unlinked by transformation, c) blocked at different stages in the sporulation process (Piggot, 1973). A statistical approach (Hranueli, Piggot & Mandelstam, 1974) suggested that the most probable number of sporulation loci was 42 which means that the great majority of sporulation loci have been identified. If it is assumed that all the loci contain at the most three genes, then a little over 100 genes will be involved in the synthesis of the endospore.

Figure 3. Genetic map of the known sporulation loci in *B. subtilis* 168 identified by mutations interfering with sporulation (*spo*). The designation includes the stage of blockage as defined by Ryter *et al.* (1966) and shown in Figure 2 (Roman numerals). In the expanded map of the *pheA* to *trpC* region the groups of *spo* loci which have not been orientated relative to one another are shown as mapping at a single site. Where no orientation is known relative to a non-sporulation marker, then the linkage is indicated by a dotted line. Markers placed in parentheses have not been ordered relative to outside markers. Reproduced from Piggot & Coote (1976).


purA
cysA
rif
strA
ery
spoIIE
spoVC
spo0H
spo0J
purB
sapB
catA
hpr
argC
trpS
sapA
metC
spoIVG
spo0K
spo0E
spoIIF
spoVD
spoVE
spoIIG
spoIIIE
furA
ura
cysC
metB
trpC
ser
lys
acf
aroD
nic
pheA
leu
ilvC
phoR
phoP
cysB
spoIIC
hisA
spoIIID
sacU
spoIID
ctrA
furC
spo0F
trpC
spoIVA
lys
spoIIA
spoVA
spoIVB
spo0A IIIA, IIIB
spo0G
spo0D, IIIC, IVC, IVD, IVE
pheA
spo0B
spoIVF
spoIIB
spoVB

The genetic map of the spo loci (Figure 3) shows that the spo loci are not clustered together into one area of the chromosome and, in addition, loci affecting totally different stages in the process can often be found grouped together. This observation is important when a model for the overall control at the process is considered. Information derived from the genetic work using spo mutants discussed above suggests that the temporal sequence of sporulation events is controlled by a process of sequential induction (Halvorsan, 1965; Mandelstam, 1976). This process is illustrated in Figure 4. It is presumed that there is an initiating locus whose transcription is blocked during growth by repressing compounds derived from the constituents of the growth medium. Starvation lowers the concentration of repressing metabolites and the process is initiated by expression of the first locus. It can be assumed that the product will be a protein or set of proteins and that one of these will be responsible for activating the next locus. Expression of this locus will in turn be responsible for activating the next locus required in the process and so on. The activation at each step could occur in two ways. First, the protein product itself could interact with the DNA at a promoter site adjacent to the locus. This might allow binding of RNA polymerase to the promoter site and so encourage transcription. This positive activity by a regulatory protein in aiding transcription occurs with the catabolite gene activator protein (CAP) during expression in E. coli of the lac operon (Pastan & Adhya, 1976) and during the positive regulation of the ara operon (Englesberg & Wilcox, 1974). Second, it could be the product of the action of the protein, if it was an enzyme for example, that might allow activation of the new locus. In this respect the effector might combine with a protein repressor and

Figure 4. Diagram of the sequential induction model for the regulation of sporulation. It is assumed that the initiating gene (or operon) is under some form of catabolite repression. Starvation allows transcription of this region of the chromosome. The protein product is responsible for activating the next set of genes required in the process at a regulatory region which controls the transcription of all the genes involved in that step. One of the products of the transcription of the activated set of genes will then be responsible for activation of a further set required later in the process.


Initiating Gene or Operon
mRNA
Proteins
0-II Stage Genes
mRNA
Proteins
II-III Stage Genes
mRNA
Proteins
III-IV Stage Genes
mRNA
Proteins

so lift the negative control of a locus by a repressor molecule, as occurs when the inducer combines with the repressor protein to allow transcription of the lac operon in E. coli.

A consequence of this type of model is that each separately located spo locus must have its own control sections of DNA. For this reason the spo loci have been termed operons (Piggot, 1973) as each would represent a number of genes regulated by common control elements. This model is also consistent with the fact that the spo loci are widely scattered on the chromosome (Figure 3). It is assumed that the activators are freely diffusible in the cytoplasm and can therefore reach any area of the genome at any one time. This would also allow more than one locus, situated on different areas of the chromosome, to be switched on together at the same time.

Initiation of sporulation

The process of sporulation is strongly influenced by the nutrients in the medium (Mandelstam & McQuillen, 1973). Sporogenesis generally is repressed by easily metabolisable carbon or nitrogen sources and starvation for either will allow sporulation to occur (Grelet, 1957; Schaeffer, Millet & Aubert, 1965). In 1965 Schaeffer et al. proposed that at some point in the growth cycle each cell had a choice between whether to continue growth or to sporulate and the decision was influenced by the rate of metabolism of the available carbon and nitrogen sources. Later work (Dawes & Mandelstam, 1970; Dawes & Thornley, 1970) using chemostat cultures showed that the incidence of sporulation in a particular medium was a function of the growth rate. These results are compatible with the idea that the initiation of sporulation is repressed by catabolites during growth and that the cell makes a repressor(s) or

inhibitor(s) from components in the medium. Schaeffer et al. (1965) suggested the notion of a key gene or operon subject to catabolite repression which had to be expressed to set the whole process in chain. In this way the intracellular concentration of at least one nitrogen-containing metabolite would repress directly or indirectly the expression of the sporulation associated genes. Depletion of a supply of carbon or nitrogen is able to relieve the repression of sporulation. At the present time it is not clear whether there is a common repressing metabolite(s) derived from both the carbon and nitrogen sources or whether each supplies a repressor and both have to be present to prevent sporulation. This point is taken up again later.

At present the nature of the repressing metabolites is unknown, but many experiments have been done to attempt to define them clearly. Grelet (1957) pointed out that sporulation can be initiated by phosphate starvation which suggests that the repressing metabolites might be phosphorylated compounds. Freese and his co-workers have attempted to define the repressors by the use of mutant strains which restrict the metabolism of carbon and nitrogen sources and allow particular intermediates to accumulate. Thus the accumulation of α-glycerol phosphate by a strain lacking glycerol phosphate dehydrogenase blocked sporulation at stage II (Oh, Freese & Freese, 1973) and the accumulation of glucose-6-phosphate by a triple mutant which could metabolise this compound no further blocked sporulation before stage II (Freese et al., 1972). During growth, intermediates of this sort would be present in only low concentrations and it is not known if their depletion during starvation plays a role in the initiation of sporulation.

A more interesting observation has been made by Rhaese and his

colleagues. They have found that a number of highly phosphorylated nucleotides (HPNI to HPNIV) appear at the start of sporulation (Rhaese et al., 1975). They are mainly phosphorylated adenosine derivatives and seem analogous to the phosphorylated guanosine derivatives (ppGpp and pppGpp) that accumulate on amino acid starvation (Gallant & Margason, 1972). They seem directly associated with the onset of sporulation under carbon or phosphate depletion and are not formed by a spo mutant blocked at stage 0 (Rhaese, Hoch & Groscurth, 1977). Their exact relationship to initiation remains to be established. They may simply accumulate as a response to a functional change in the protein synthesising machinery of the cell which has been demonstrated in B. subtilis (Chambliss & Legault-Delmare, 1977).

The biochemical studies described above have failed to reveal the nature of the initiating events of sporulation. The isolation of mutant strains, which proved effective for examination of the regulation of sporulation, has also been attempted for events associated with initiation. Michel, Cami & Schaeffer (1968) were able to enrich selectively for mutants blocked at stage 0 by shifting cells from a good nitrogen source to a poor one (ammonia to nitrate) or from a good carbon source to a poor one (glucose to citrate or histidine). This shift-down encouraged the continued growth of the mutant strains whereas the wild-type cells were induced to sporulate. The mutations in these strains are located in the spo0A locus (see Figure 3), but as yet the gene products at this locus have not been identified. These strains show no characteristics of sporulating cells and must undoubtedly be defective in a component required for one of the earliest events of initiation.

Mutations in the spo0A locus may be considered as hyper-repressed or uninducible for the initiation of sporulation. The converse type of

mutation, the derepressed mutant which sporulated under all conditions, would be lethal unless the mutation was conditional. No ts mutants have been described which grow at the permissive temperature, but which sporulate when shifted to a restrictive temperature. Several mutants of B. cereus have been described which sporulate in media which only allow growth with the wild-type (Levisohn & Aronson, 1967). Several of these were purine auxotrophs, an observation similar to that made by Elmerich & Aubert (1975) using B. megaterium where purine auxotrophs were found to sporulate under conditions where the wild-type would not. They have suggested that a component of the purine biosynthetic pathway may be involved in the repression of sporulation (see below). Two mutations (cat A & cat B) have been described in B. subtilis which allow sporulation in the presence of glucose or amino acids that normally repress sporulation. These mutations mapped in areas of the genome distinct from any known spo loci, but again the nature of the products of the mutated genes is unknown (Ito & Spizizen, 1973).

The original hypothesis of Schaeffer et al. (1965) was that sporulation was under some form of catabolite repression, somewhat similar to the catabolite repression of inducible enzyme synthesis (Magasanik, 1961) which is also brought about by easily degraded carbon or nitrogen sources. Evidence has accumulated, however, to suggest that the mechanisms operating to overcome repression of sporulation and repression of inducible enzyme synthesis are not identical. This evidence can be usefully summarised as follows. In the hyper-repressed spoOA mutants described above the ability to synthesise many inducible enzymes was unimpaired (Brehm, Staal & Hoch, 1973). Conversely, the cat A and cat B mutants insensitive to glucose as amino acid repression of sporulation were normal with respect to catabolite repression of inducible enzyme

synthesis (Ito & Spizizen, 1973). Sporulation can only be initiated during chromosome replication (Mandelstam, Sterlini & Kay, 1971; Mandelstam & Higgs, 1974), whereas the induction of enzyme synthesis is independent of DNA replication (Coote, 1974). This evidence does not rule out the possibility that there may be some common factor involved in both repression of sporulation and the catabolite repression of inducible enzymes, but it is also likely that many of the initial events of sporulation are a modification of those which normally occur during vegetative cell division (Hitchins & Slepecky, 1969; Freese, 1972). The starvation conditions promote new gene expression and the gene products are responsible for the synthesis of the asymmetrical spore septum (see Figure 2).

From the biochemical investigations and the mutant studies outlined above no clear picture has emerged of the nature of the initial reactions which operate during the onset of sporulation nor of the nature of the repressor molecules which are presumed to be made from easily metabolised carbon and nitrogen sources in the medium. As stated earlier the possibility exists that a common repressor metabolite is made by the cells from both the carbon and nitrogen source in the medium. In this case depletion of carbon or nitrogen would lower the level of repressor and initiate sporulation. Alternatively, there may be two distinct repressors, one made from the carbon source and the other from the nitrogen source. Depletion of either will allow initiation to occur, but this could occur in 2 possible ways. First, the two repressors together may be necessary to prevent transcription by a single mechanism and depletion of either will allow transcription by this one mechanism. Second, each may act at a common site on the chromosome by influencing the activity of two entirely different mechanisms for transcription of the

common locus. The precedent for this latter type of control is found in Gram negative organisms where it has been reported that the induction of certain enzymes is promoted by different mechanisms depending on whether the stimulus is carbon or nitrogen limitation (Magasanik et al., 1974; Magasanik, 1977). A third possibility, that the two repressors may act at separate sites on the chromosome and depletion of either can induce sporulation at separate sites by different mechanisms is essentially a refinement of the second possibility.

Regulation of enzyme synthesis by glutamine synthetase

It was originally observed that Klebsiella aerogenes was unable to synthesise the inducible enzyme histidase when grown in a medium containing glucose and ammonia with L-histidine added as the inducer (Niedhardt & Magasanik, 1957). In this medium histidase synthesis was subject to catabolite repression by glucose. However, if the ammonia was omitted from the medium and histidine used as the nitrogen source then histidase was synthesised and escaped the repression by glucose. Histidine is degraded to L-glutamate in this organism by four inducible enzymes:

$$\text{Histidine} \xrightarrow{A} \text{Urocanate} \xrightarrow{B} \text{Imidazolone-propionate} \xrightarrow{C} \text{Formimino-glutamate} \xrightarrow{D} \text{Glutamate} + \text{Formamide}.$$

The enzymes are: A, Histidase; B, Urocanase; C, Imidazolone-propionate hydrolase; D, Formimino-glutamate hydrolase. The genes responsible for the synthesis of these enzymes are grouped together into two operons controlled by a common repressor protein which is synthesised by a gene situated between the two operons of structural genes (Smith & Magasanik, 1971; Smith, Halpern & Magasanik, 1971).

Evidence had accumulated to show that in Gram negative organisms

the catabolite repression of inducible enzymes was mediated via adenosine 3,5' cyclic monophosphate (cAMP) and a protein factor (the catabolite gene activator protein or CAP) which was able to bind cAMP (Reznikoff, 1972). These two factors in combination were necessary to promote binding of RNA polymerase at the promoter site on the DNA and so allow transcription of the structural genes. Easily metabolisable carbon sources, such as glucose, were shown to lower the intracellular concentration of cAMP and so transcription was prevented (De Crombrugghe et al., 1969). Mutants of E. coli and K. aerogenes have been described which have either a defective CAP protein or are unable to synthesise cAMP as a result of a defective adenylate cyclase. These mutants are unable to grow on carbon sources such as lactose or L-arabinose which require inducible enzymes for their metabolism (Perlman & Pastan, 1969).

Prival & Magasanik (1971) found that a mutant of K. aerogenes with a defective adenylate cyclase was unable to synthesise the histidine degrading enzymes when L-histidine was used as carbon source and ammonia as nitrogen source. This was in agreement with the observations described above. However, the histidine degrading enzymes were synthesised in this strain when histidine was supplied as a nitrogen source with glucose as a carbon source. Therefore an alternative to the cAMP mediated mechanism was being used by the cells to overcome the catabolite repression by glucose in this medium. The factor promoting synthesis of the histidine degrading enzymes under conditions of nitrogen limitation was shown to be glutamine synthetase (GNS) (Tyler et al., 1974). Mutants deficient in GNS activity were unable to produce histidase under nitrogen limiting conditions and mutants constitutive for the enzyme had high levels of histidase even in the presence of glucose and ammonia (Prival, Brenchley & Magasanik, 1973). Mutants which produced an

enzymically inactive GNS protein were nevertheless able to produce the histidine-degrading enzymes which indicated that the defective GNS protein still retained the ability to stimulate transcription (Streicher, Bender & Magasanik, 1975).

The transcription of the structural genes for histidine degradation is thus controlled in three ways. The repressor exerts negative control and prevents transcription unless converted to an inactive state by combination with the inducer urocanate (formed from the histidine in the medium by a basal level of histidase present in the cells). Transcription can then be activated by either the CAP protein in combination with cAMP or by GNS. In the former case this will only occur in a medium with a poor carbon source which allows cAMP to accumulate. In the latter case transcription will only occur in a medium with a poor nitrogen source. In this way the production of the histidine degrading enzymes is regulated according to the needs of the cell. The enzymes are not formed unless histidine is available and are formed by different mechanisms depending on whether histidine is required as a carbon or as a nitrogen source.

This regulatory effect of GNS on inducible enzyme synthesis has been found to occur with other enzymes which can be used to metabolise compounds as a source of nitrogen. Thus the synthesis of urease (Magasanik, 1977), L-asparaginase (Resnick & Magasanik, 1976) and nitrogenase (Tubb, 1974) in K. aerogenes are all subject to regulation by this enzyme.

The manner in which GNS is able to regulate transcription has been recently elucidated by Magasanik and his co-workers. It had previously been shown that Gram negative bacteria produce two forms of GNS

(Kingdon, Shapiro & Stadtman, 1967) depending on the growth medium. In ammonia-rich media an enzyme adenylated on each subunit with AMP is produced and lack of ammonia causes deadenylation of the enzyme. The enzyme is able to regulate its own synthesis (an example of autogenous regulation) by this mechanism because the adenylated enzyme can repress GNS synthesis, whereas deadenylation converts it to a form no longer able to exert this repression (Janssen & Magasanik, 1977; Foor, Janssen & Magasanik, 1975). Thus ammonia starvation promotes increased synthesis of nonadenylated GNS enzyme which in turn can promote synthesis of enzymes whose regulatory genes are susceptible to activation by GNS.

The regulatory activity of GNS in Gram negative bacteria outside its normal catalytic role in the metabolism of the cell is of particular relevance to a discussion of the initiation of sporulation because this enzyme plays a major role in the assimilation of ammonia in Gram positive bacteria. It must therefore be involved in the synthesis of any metabolites derived from ammonia which repress sporulation. This is discussed more fully below.

Metabolism of nitrogenous compounds

In order to grow in a minimal medium in which ammonia serves as the only source of nitrogen, bacteria must possess a mechanism for combining the ammonia with intermediates of the central metabolic pathways in order to be able to synthesise amino acids. Two main pathways for the incorporation of ammonia have been shown to operate in bacteria.

1. Direct reductive amination of 2-oxoglutarate catalysed by glutamate dehydrogenase (E.C.1.4.1.4.).

$$NH_3 + \text{2-oxoglutarate} + NADPH + H^+ \rightleftharpoons \text{L-glutamate} + NADP^+ + H_2O.$$

2. A coupled reaction involving first the amination of L-glutamate catalysed by glutamine synthetase (E.C.6.3.1.2) followed by the reductive transfer of the amide nitrogen to 2-oxoglutarate catalysed by glutamine (amide); 2-oxoglutarate aminotransferase oxidoreductase (NADP) or glutamate synthase (E.C.2.6.1.53)

$$\text{L-glutamate} + \text{ATP} + NH_3 \rightleftharpoons \text{L-glutamine} + \text{ADP} + \text{Pi} + H_2O$$

$$\text{L-glutamine} + \text{2-oxoglutarate} + \text{NADPH} + H^+ \rightleftharpoons 2\ \text{L-glutamate} + NADP^+.$$

Glutamate dehydrogenase has a poor affinity for ammonia (Tempest, Meers & Brown, 1970; Brenchley & Magasanik, 1974) which means that when cells are growing in low concentrations of ammonia this enzyme becomes inoperative. Tempest et al. (1970) estimated that in ammonia-limited chemostat cultures of K. aerogenes the free intracellular ammonia concentration was below 0.5 mM, well below the Km value of 4 mM for K. aerogenes glutamate dehydrogenase. This observation prompted the authors to search for an alternative pathway for ammonia incorporation which they showed involved the combined action of glutamine synthetase (GNS) and glutamate synthase (GTS). An important observation was that, compared to glucose-limited cultures, ammonia-limited cultures of K. aerogenes showed a ten-fold decrease in glutamate dehydrogenase specific activity accompanied by an eight-fold increase in GNS activity. In addition, the measured Km for ammonia incorporation in the reaction catalysed by GNS was shown to be less than 1 mM which substantiated its role in ammonia incorporation when the intracellular concentration of ammonia was low. The net synthesis of glutamate in ammonia limited cultures of K. aerogenes was shown by Tempest et al. (1970) to be the result of the action of a second enzyme, glutamate synthase. This enzyme catalysed the transfer of the amide group of glutamine to 2-oxoglutarate, thereby forming two molecules of glutamate. Although both routes, the

glutamate dehydrogenase reaction or the coupled GNS and GTS reaction, assimilate ammonia to form glutamate the second pathway involves the participation of ATP. It was suggested by the authors that this expenditure of energy was the penalty necessary for assimilation of low intracellular concentrations of ammonia.

A subsequent investigation (Meers, Tempest & Brown, 1970) revealed that certain species of bacteria, notably Erwinia carotovora, Bacillus megaterium KM and Bacillus subtilis W23 lacked noticeable glutamate dehydrogenase activity. These species, together with Pseudomonas fluorescens, synthesised GTS under both conditions of ammonia or glucose limitation. The authors concluded that the ability to synthesise glutamate via GNS and GTS was not restricted to ammonia limited cultures and could be the predominant pathway in certain species, particularly those lacking glutamate dehydrogenase.

Previous work had noted the absence of glutamate dehydrogenase activity in B. subtilis (Freese, Park & Cashel, 1964) and the work of Elmerich (1972) confirmed that in B. megaterium ammonia was incorporated solely via GNS and GTS. The evidence for this can be usefully summarised.

a) B. megaterium when grown in minimal medium with glucose as carbon source and ammonia as nitrogen source had no detectable glutamate dehydrogenase or aspartase activity. Aspartase (E.C.4.3.1.1) catalyses the reaction:

$$\text{Fumarate} + NH_3 \rightleftharpoons \text{L-aspartate}.$$

b) A mutant lacking alanine dehydrogenase activity grew normally in the glucose-ammonia medium. Alanine dehydrogenase (E.C.1.4.1.1) catalyses the reaction:

$$\text{Pyruvate} + NH_3 + NADH + H^+ \rightleftharpoons \text{L-alanine} + H_2O + NAD^+.$$

This observation was supported by other work which indicated that in Bacillus spp. this enzyme appears to function catabolically to produce pyruvate and is not used for ammonia assimilation (Berberich, Kaback & Freese, 1968; Meers & Pedersen, 1972).

c) Mutants of B. megaterium were isolated which lacked either GNS or GTS activity. Neither of these strains was able to grow on glucose-ammonia minimal medium.

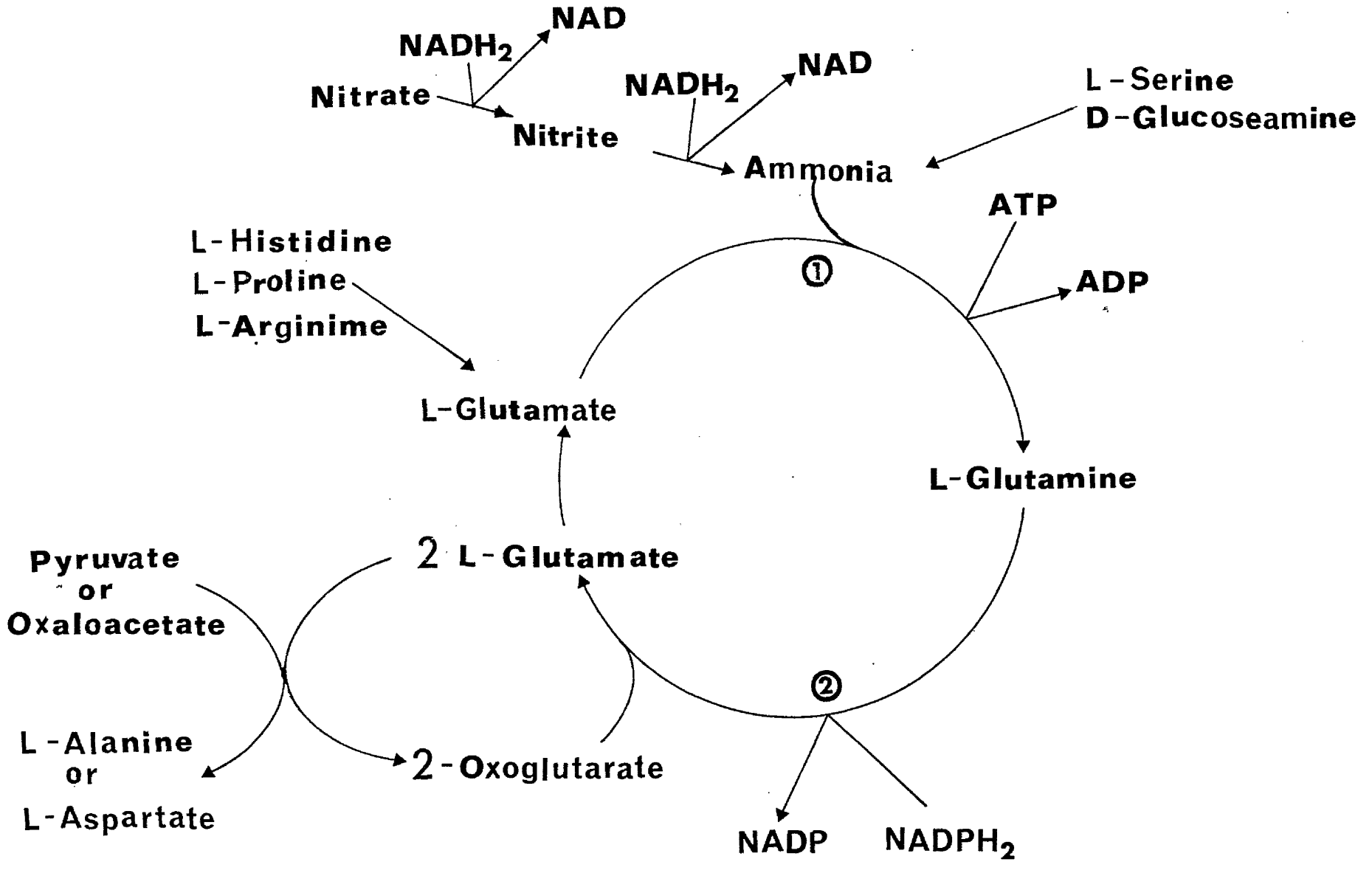
Following the assimilation of ammonia via GNS and GTS to give L-glutamate, Elmerich showed that the glutamate was transaminated using pyruvate or oxaloacetate to yield L-alanine or L-aspartate respectively. This reaction can be illustrated as :

$$\text{L-glutamate} + \text{R. CO. COOH} \rightleftharpoons \text{R.CHNH}_2\text{.COOH} + \text{2-oxoglutarate.}$$

The transamination reactions yield 2-oxoglutarate which is made available again for the GTS reaction, so forming a cycle called the glutamate cycle by Elmerich (1972). This cycle is illustrated in Figure 5. The diagram also illustrates that bacteria do not necessarily depend on the provision of ammonia in the medium for the synthesis of L-glutamate. They can obtain this amino acid by the degradation of other amino acids provided in the growth medium if they are able to induce the necessary enzymes. Thus L-histidine, L-proline and L-arginine, for example, yield L-glutamate as the end-product of their degradative pathways. The degradative pathway for L-histidine in K. aerogenes was mentioned earlier. Alternatively, compounds can be metabolised to yield ammonia. Glucosamine and L-serine can be degraded to yield ammonia. Nitrate can be assimilated to ammonia by many micro-organisms via the combined action of nitrate and nitrite reductases.

With Gram negative bacteria such as E. coli or K. aerogenes the

Figure 5. Diagram of the pathways of ammonia assimilation and glutamate metabolism in Bacillus spp. which lack glutamate dehydrogenase activity. Numbers 1 and 2 represent the enzymes glutamine synthetase and glutamate synthase respectively.


NAD
NADH2
Nitrate
Nitrite
NADH2
NAD
L-Serine
D-Glucoseamine
Ammonia
ATP
ADP
L-Histidine
L-Proline
L-Arginime
①
L-Glutamate
L-Glutamine
2 L-Glutamate
Pyruvate
or
Oxaloacetate
②
L-Alanine
or
L-Aspartate
2-Oxoglutarate
NADP
NADPH2

combined action of GNS, and GTS is not required when the level of ammonia in the medium is high (above 1 mM). At high concentrations of ammonia glutamate dehydrogenase will operate satisfactorily. The cell has evolved appropriate control mechanisms to allow continued assimilation of ammonia at low concentrations. As mentioned previously a fall in ammonia concentration promotes deadenylation of GNS in the cell which in turn allows an increased synthesis of the non-adenylated GNS which now takes on the role of glutamate provider together with GTS. In fact, the increase in the level of non-adenylated GNS has been shown to be accompanied by repression of glutamate dehydrogenase synthesis (Brenchley, Prival & Magasanik, 1973). Thus the transfer of the Gram negative cell from high to a low ammonia condition brings about an increase in the activity of the enzymes capable of assimilating the ammonia at low concentrations (GNS and GTS) and a fall in the activity of the enzyme (glutamate dehydrogenase) only useful at high ammonia concentrations. In addition, the increase in GNS is able to relieve certain enzymes from catabolite repression which can be used to degrade compounds to glutamate (see above) for use as an alternative nitrogen source.

It is apparent that the enzyme GNS in addition to its enzymic function, plays a critical role in the adaptation by the Gram negative cell to a condition of nitrogen limitation. In the Gram positive Bacillus spp. which lack glutamate dehydrogenase activity it is clear that the enzymes GNS and GTS will operate for ammonia assimilation under all conditions and therefore supplementary control mechanisms would only be expected to occur to relieve inducible enzymes from catabolite repression under conditions of nitrogen limitation. This possibility has not, as yet, been investigated.

Possible role of glutamine synthetase and glutamate synthase in initiation of sporulation

In the metabolism of the cell GNS plays an important role because the amide group of glutamine, the compound it synthesises, is a source of nitrogen in the biosynthesis of a variety of major cell components such as the amino acids tryptophan and histidine, nucleotides such as CTP and AMP and peptidoglycan via glucosamine-6-phosphate. As a result of its pivotal role or branch-point in metabolism it is subject to feed-back inhibition by a variety of metabolites. The enzyme has been purified from E. coli (Woolfolk, Shapiro & Stadtman, 1966) and from B. subtilis (Deuel & Prusiner, 1974). Both enzymes are cumulatively inhibited by final products of glutamine metabolism such as AMP, histidine and CTP. Both are subject to regulation by L-glutamine, but by different mechanisms. An increase in L-glutamine concentration stimulates adenylation of the enzyme from E. coli (Schutt & Holzer, 1972) which, as has been noted already, results in an autogenous repression of GNS synthesis. The enzyme from B. subtilis, however, is subject to direct inhibition by its product, L-glutamine (Deuel & Prusiner, 1974) which provides a greatly simplified mechanism for the overall regulation of L-glutamine synthesis by the cell. Thus the Gram positive cell appears to regulate the amount of glutamine in the cell mainly via a change in enzyme activity, whereas a change in the rate of enzyme synthesis plays a greater role in the Gram negative cell. Indeed, there is no evidence that the GNS from Bacillus spp. exists in an adenylated form (Gancedo & Holzer, 1968; Deuel et al., 1970). In addition, synthesis of the enzyme has been found to be only marginally sensitive to regulation (Rebello & Strauss, 1969); its synthesis being partially repressed by glutamine, asparagine,

arginine and, to a lesser extent, glutamate. These obvious differences between the regulation of GNS activity in the Gram positive and Gram negative cell make it difficult to draw a possible analogy between the behaviour of the enzyme in the two cell types with regard to inducible enzyme synthesis and, from the point of view of this discussion, the induction of sporulation under conditions of nitrogen limitation. The situation is complicated further by the observation that cAMP is absent from Bacillus spp. (Ide, 1971; Bernlohr, Haddox & Goldberg, 1974). It presumably plays no role in the catabolite repression of inducible enzymes as it does in Gram negative bacteria growing with a good carbon source. It is thus possible that a different mechanism of catabolite repression operates in Gram positive bacteria, although an analogous compound to cAMP may play essentially the same role. In this context it is interesting to note that the intracellular concentration of 3'5' cyclic guanosine 5'-monophosphate (cGMP) changes sharply at the start of spore formation in B. licheniformis (Bernlohr et al., 1974), although it was not clear from this work whether sporulation was induced by carbon or nitrogen deprivation.

The enzymes GNS and GTS appear to be solely responsible for ammonia assimilation in B. subtilis and B. megaterium. It must be assumed, therefore, that these enzymes are responsible in some way for the repression of sporulation which is exerted by ammonia. They could conceivably operate in this respect in two ways. a) They could operate enzymically in the formation at metabolite repressors derived from the ammonia in the medium. b) One or the other could operate in a similar manner to the role of GNS in Gram negative bacteria by promoting the expression of genes required for the initiation of sporulation. By analogy this would require a change in enzyme levels as a result of the depletion of ammonia (nitrogen limitation) from the medium. A convenient

procedure for establishing whether GNS and GTS play a role in the manufacture of metabolite repressors would be to isolate mutant strains which possessed either an inactive GNS or GTS enzyme. A defective GNS would not allow ammonia to be metabolised at all and a defective GTS would allow ammonia to be metabolised only as far as glutamine. The effects of these restrictions on the sporulation capacity of the mutant cells could then be assessed. This approach has been adopted in both B. megaterium and B. subtilis and will be discussed below. The examination of mutants defective in GNS or GTS would also help to establish a possible role for either enzyme as an effector outside its catalytic role. Production of a GNS enzyme protein inactive catalytically still allowed it to function normally in enzyme induction (see above) whereas a regulatory mutant producing no enzyme protein was inactive in both ways. In addition, any change in enzyme level caused by changes in enzyme synthesis at the onset of sporulation should be detectable as changes in enzyme specific activity by direct assay in cell-free extracts of sporulating cells.

As far as is known no attempts have been made to detect changes in GNS or GTS specific activities during the onset of sporulation. However, Rebello & Strauss (1969) investigated the control of GNS synthesis under conditions of carbon and nitrogen deprivation in a chemostat; conditions which would be expected to encourage the onset of sporulation. Compared with the specific activity of the enzyme from cells growing in glucose-ammonia medium in batch culture, nitrogen limitation (using ammonia or L-glutamine as the limiting component) caused a 2.5-fold increase in specific activity, whereas carbon limitation (using glucose as the limiting component) caused an 8-fold decrease in specific activity. In addition, they investigated the change in specific activity of GNS during transition

from a fast to a slow growth rate. This was accomplished by growing B. subtilis in a glucose-ammonia minimal medium containing a limiting amount of casein hydrolysate. Exhaustion of the casein hydrolysate reduced the growth rate and was accompanied by a transient increase in the specific activity of GNS followed by a lag in enzyme synthesis for a short period as growth slowed. Finally, enzyme synthesis resumed at a reduced rate. The authors also showed that these observed changes in GNS specific activity were due to specific changes in enzyme synthesis and not to general changes in the rate of protein degradation. This work indicated that the level of GNS in B. subtilis changed on transition from a rich to a poor growth environment and almost a 10-fold difference in specific activity of the enzyme was found between conditions of carbon and nitrogen starvation. Shift-down conditions involving carbon or nitrogen limitation are known to trigger sporulation, but the authors did not seek any correlation between sporulation and the activity of GNS.

A possible role for GNS and GTS during the onset of sporulation has also been sought by the isolation of GNS and GTS defective strains in B. megaterium by Aubert and his colleagues (Elmerich & Aubert, 1971, 1972, 1973, 1975; Reysset & Aubert, 1975). These authors first reported that a GNS-negative mutant was able to sporulate efficiently in a medium containing glucose and ammonia which prevented sporulation in the wild-type (Elmerich & Aubert, 1972). Addition of L-glutamine to this medium lowered the level of sporulation in the GNS-negative strain, but the addition of L-glutamate did not. The authors concluded that the repression of sporulation depended on L-glutamine and not L-glutamate. In keeping with this a GTS-negative strain was unable to sporulate even in the presence of glutamate because, the authors suggested, the absence of GTS caused an accumulation of L-glutamine which repressed sporulation under

all conditions. A subsequent report (Elmerich & Aubert, 1973) suggested that it was not L-glutamine itself, but rather a component in the early part of the purine biosynthetic pathway that was involved in the repression of sporulation. L-glutamine was implicated only as an amido-group donor. Mutants were selected from the GNS-negative strain which sporulated in the glucose ammonia medium plus added L-glutamine where the GNS-negative strain was unable to sporulate. These were often blocked in the purine biosynthetic pathway. Similarly, mutants were selected from the GTS - strain which sporulated in the glucose-ammonia medium where the GTS - strain was unable to sporulate because of the presumed accumulation of L-glutamine. Again these mutants were blocked in an early step of purine biosynthesis. It was concluded that there is a component of the purine pathway, formed using L-glutamine, which acts as a repressor or permits the synthesis of a repressor for sporulation. This hypothesis was tested directly by the isolation of purine deficient strains from the wild-type B. megaterium. Classes of mutants blocked in the steps between 5-phosphoribosyl-1-pyrophosphate and 5-aminoimidazole ribonucleotide were able to sporulate in the glucose-ammonia medium which prevented sporulation in the wild-type.

In a later report Reysset & Aubert (1975) examined the properties of a number of GNS defective strains. All degrees of sporulation were found in these mutants from 100% down to 0.001%. There was no correlation between the degree of sporulation and the activity of GNS nor was there any correlation between the degree of sporulation and the amount of GNS protein as estimated by immunological means. It was noted earlier that in K. aerogenes the regulatory function of GNS in histidase synthesis was independent of its enzyme activity so that a mutant which synthesised enzymically inactive GNS was still able to control histidase synthesis normally. If GNS was playing an analogous role in B. megaterium in the

control of sporulation it might have been expected that the presence or absence of GNS antigen would have correlated with the level of sporulation, but there was no correlation in this respect. Thus one GNS-negative strain had an antigen level equivalent to the wild-type, but sporulated only at a level of 5×10^{-5}, another strain lacked any detectable antigen and sporulated at a level of 5×10^{-4} and yet another possessed little antigen, but sporulated normally. The conclusion of Aubert and his colleagues (Reyssel & Aubert, 1975) from the study of GNS and GTS defective strains in B. megaterium is that GNS is the first enzyme involved in the synthesis of a low molecular weight effector derived from an early intermediate of the purine biosynthetic pathway. They also suggest that the GNS protein has a regulatory role independent of its catalytic activity and may itself be the receptor of the low molecular weight effector molecule which, it is postulated, may alter its regulatory function. Thus combination with the effector may prevent transcription of essential genes required for sporulation. This is analogous to K. aerogenes where similar regulatory effects are caused by adenylation. It is difficult, however, to reconcile this hypothesis with Aubert's own observations on the lack of correlation between the degree of sporulation and the level of GNS protein.

The situation is complicated further by a recent report (Dean, Hoch & Aronson, 1977) which stated that a large number of GNS defective mutants of B. subtilis were able to sporulate normally which is the opposite to what was found in B. megaterium (Reysset & Aubert, 1975). The authors noted that all the B. subtilis mutants contained some GNS activity, but suggested that active GNS may not be necessary for sporulation in B. subtilis.

It should be noted that L-glutamate is a major constituent of the dormant spore. Spores of B. subtilis contain about 1% of their dry weight as free glutamic acid (Nelson & Kornberg, 1970). A requirement for the synthesis of L-glutamate during sporulation would be expected to be an essential part of the sporulation process. Thus mutants deficient in the first three enzymes of the tricarboxylic acid cycle, citrate synthase, aconitase and isocitrate dehydrogenase, are unable to sporulate even in nutrient broth. They are unable to synthesise 2-oxoglutarate which can then be transaminated to form L-glutamate (Fortnagel & Freese, 1968; Carls & Hanson, 1971). In minimal medium they have to be supplied with a source of L-glutamate. This means that they are glutamate auxotrophs and will not grow on glucose-ammonia minimal medium. They can be distinguished from glutamate synthase negative strains which have a similar phenotype by the fact that L-aspartate will serve as an alternative to L-glutamate in the latter strains (Young & Wilson, 1975), but not in the TCA cycle mutants. L-aspartate can be transaminated to form L-glutamate only if a supply of 2-oxoglutarate is available (see Figure 5).

No clear picture has evolved of the possible role of GNS or GTS in the onset of sporulation. The work with GNS defective mutants has presented a confusing picture. In B. megaterium a single mutation can render the cell defective in GNS activity and sometimes in sporulation whereas in B. subtilis lack of GNS activity has apparently no effect on sporulation. However, in both these studies sporulation was estimated by exhaustion in broth medium which meant that a distinction between carbon and nitrogen limitation as a means of promoting sporulation was not made. For the reasons discussed above GNS and GTS would be expected to play a role in repression of sporulation via metabolism of nitrogenous compounds. The work of Rebello & Strauss (1969) suggested a difference in

behaviour of GNS under carbon and nitrogen limitation. This is the only work available which has investigated the activity of GNS in B. subtilis under these different conditions. It seemed important to make a distinction between carbon and nitrogen limitation as a means of inducing sporulation and to examine the role of both GNS and GTS under these two separate situations.

The work described in this thesis was done with the possibility in mind that sporulation may be induced under carbon or nitrogen limitation by different mechanisms. The work centred on the activity of GNS and GTS during the onset of sporulation. Lack of a readily metabolisable carbon or nitrogen source will initiate sporulation. Conversely, a good source of nitrogen, such as ammonia, will repress sporulation and as ammonia is apparently assimilated via the action at GNS and GTS in B. subtilis it might be expected that these enzymes would play a role in the repression of sporulation. This possibility was investigated in two ways. First, by studies on the specific activity of the enzymes during the onset of sporulation induced by either carbon or nitrogen starvation. In Gram negative bacteria there is an increase in specific activity of GNS associated with the relief from catabolite repression of certain inducible enzymes under nitrogen limitation. On the assumption that the initiation events of sporulation are under some form of catabolite repression, it might be anticipated that a role for one or other of these enzymes in the relief from this repression might be reflected in an increase in specific activity during the onset of sporulation induced by nitrogen limitation. Second, by studies on a mutant strain which was presumed defective in GTS activity. Previously reported work has concentrated on strains defective in GNS activity and there has been little emphasis placed on mutants defective in GTS. It might be anticipated that the study of a GTS defective strain under sporulation conditions might also indicate whether this enzyme has a role in sporulation.

OBJECT OF THE RESEARCH

The main object of this work was to determine the role of the enzymes glutamine synthetase (GNS) and glutamate synthase (GTS) during the initiation of sporulation in B. subtilis under nitrogen limitation. In order to do this it was necessary first, to obtain experimental conditions which would allow sporulation to occur equally well under carbon or nitrogen limitation; second, to establish satisfactory assay procedures for GNS and GTS and to assay the enzymes during growth of B. subtilis under conditions where a change in specific activity would be expected to occur; third, to assay the enzymes at intervals after the onset of sporulation induced by either carbon or nitrogen limitation; fourth, to characterise fully a mutant strain of B. subtilis which was presumed deficient in GTS activity and to note its sporulation behaviour under conditions of carbon and nitrogen limitation. Finally, to compare the enzyme activities during growth and sporulation of the mutant strain with those of the wild-type.

MATERIALS AND METHODS

Organisms

Bacillus subtilis 168 (*trpC2*) was used as the wild-type strain. This strain requires indole or L-tryptophan for growth. It forms spores normally in media containing L-tryptophan. Strain *glt-100* was made available by Dr. J.G. Coote. It was isolated originally from *B. subtilis* 168 (*trpC2*) as unable to grow on a number of carbon sources, such as D-glucose or L-arabinose, with ammonia as nitrogen source. It was later identified as a glutamate auxotroph, i.e. it required L-glutamate as a nitrogen source for growth. The mutation was transferred to *B. subtilis* 168 (*phe-12*, *leu-8*) by transformation (Coote, 1972). In this cross, high concentrations of DNA were used (congression) and selection was made for *leu*$^+$ transformants. These were then tested for a requirement for L-glutamate.

Work described here identifies the absence of glutamate synthase (glutamine : 2-oxoglutarate aminotransferase) in this strain. The genotypic designation *glt* was that used by Hoch & Mathews (1972) to describe a similar glutamate synthase negative strain and this designation has been incorporated into the genetic map of *B. subtilis* 168 (Lepesant *et al.*, 1975; Young & Wilson, 1975). The strain used in the experiments described here was *glt-100*, *phe-12* and for growth and sporulation all media were supplemented with L-phenylalanine and L-glutamate (or amino acids which could replace L-glutamate).

Maintenance of organisms

Stock cultures of the organisms were maintained at 4°C on nutrient agar (Oxoid Ltd., London, England). Both *B. subtilis* wild-type

and strain glt-100 formed spores on this medium so they remained viable for long periods and were only subcultured onto fresh nutrient agar at approximately four month intervals. The cultures were periodically checked for contamination by streaking the wild-type strain onto minimal agar (see below) and the glt-100 strain onto minimal agar with added phenylalanine. Both strains were unable to grow on these media and any contaminating colonies or revertants would become visible after incubation at 37°C for 48 hours.

Growth media

1) Casein hydrolysate medium (CH medium)

The basal medium contained 10 g of casein hydrolysate, 3.68 g L-glutamic acid, 1.25 g L-alanine, 1.39 g L-asparagine, 1.36 g KH_2PO_4, 0.107 g Na_2SO_4, 0.001 g $FeCl_3 6H_2O$, 0.1 g NH_4NO_3 and 0.54 g NH_4Cl in 1 litre of distilled water. The pH was adjusted to 7.0 with 40% NaOH and the medium sterilised by autoclaving at 15 psi for 15 min.

Additions to the CH medium

CH_3. This contained 2.0 g $MgSO_4.7H_2O$ plus 0.4 g $CaCl_2.2H_2O$ made up to one litre and sterilised by autoclaving at 15 psi for 15 min.

CH_4. This contained 1.1 g $MnSO_4.4H_2O$ made up to 100 ml and sterilised by autoclaving at 15 psi for 15 min.

Complete CH medium

To each 100 ml of basal medium was added 5.0 ml of CH_3, 0.2 ml CH_4, 1.0 ml 0.2% (w/v) L-tryptophan or L-phenylalanine (sterilised by filtration through cellulose acetate membrane filters of pore size 0.45 µm

Oxoid Ltd., Basingstoke, Hants). The medium is almost identical to that described by Sterlini & Mandelstam (1969).

2) Minimal salts media

This medium has been described previously (Schaeffer et al., 1965). The basal medium contained 10.5 g K_2HPO_4, 3.5 g KH_2PO_4 and 0.5 g NH_4Cl made up to 1 litre with distilled water and adjusted to pH 7.0 with 40% NaOH. It was sterilised by autoclaving at 15 psi for 15 min.

Additions. The following salt solutions were prepared; $FeSO_4.7H_2O$ (0.05% w/v), $MnCl_2.4H_2O$ (0.05% w/v), $MgSO_4.7H_2O$ (0.5% w/v), $CaCl_2.2H_2O$ (2.2% w/v). These were all sterilised by autoclaving at 15 psi for 15 min.

Complete minimal medium. To each 100 ml of basal medium was added 1.0 ml of $FeSO_4.7H_2O$ solution, 1.0 ml $MnCl_2.4H_2O$ solution, 1.0 ml $MgSO_4.7H_2O$ solution, 0.25 ml $CaCl_2.2H_2O$ solution and 1.0 ml L-tryptophan or L-phenylalanine (0.2% w/v). It was supplemented by aseptic addition of a carbon source at 0.5% final concentration (5.0 ml 10% solution added per 100 ml of medium). All carbon sources were sterilised by filtration.

When an alternative to ammonia was required as a source of nitrogen a basal medium was made up in the same way except that NH_4Cl was omitted. Alternative nitrogen sources were used at 0.1% (w/v) final concentration (1.0 ml 10% solution added asceptically per 100 ml of medium). All amino acid solutions were sterilised by filtration, except L-glutamate which was autoclaved at 15 psi for 15 min.

3) Resuspension media

The following stock solutions were prepared:

Solution A. This contained: $FeCl_3.6H_2O$, 0.1 g; $MgCl_2.6H_2O$, 0.83 g; $MnCl_2.4H_2O$, 1.98 g made up to 100 ml with distilled water.

Solution B. This contained: NH_4Cl, 53.5 g; Na_2SO_4, 10.6 g; KH_2PO_4, 6.8 g; NH_4NO_3, 9.7 g made up to 1 litre with distilled water.

Solution C. This contained: Na_2SO_4, 10.6 g; KH_2PO_4, 6.8 g; $NaNO_3$, 10.0 g made up to 1 litre with distilled water.

These stock solutions were stored at $4^{\circ}C$.

Basal salts solutions

(1) S(C) Medium. This salts medium was used as a basal medium for the preparation of the resuspension medium containing an adequate source of nitrogen, but lacking a good carbon source (see below). To 900 ml of distilled water was added 1.0 ml of stock solution A followed by 10.0 ml of stock solution B. The pH was adjusted to 7.0 with 40% NaOH and the medium sterilised by autoclaving at 15 psi for 15 min.

(2) S(N) Medium. This salts medium was used as a basal medium for the preparation of the resuspension medium containing an adequate source of carbon but lacking a good nitrogen source (see below). The medium was prepared in the same way as the S(C) medium, except that stock solution B was replaced by solution C.

Additions to the basal salts solution

The following solutions were prepared separately:

(1) L-glutamate solution. A 1.5% (w/v) solution of L-glutamate was brought to pH 7.0 with 40% NaOH.

(2) Calcium chloride solution. A 2.2% (w/v) solution of $CaCl_2.2H_2O$.

(3) Magnesium sulphate solution. M $MgSO_4.7H_2O$ solution.

These solutions were sterilised by autoclaving at 15 psi for 15 min.

Resuspension medium for induction of sporulation by carbon deprivation (S(C) medium). This is the original replacement medium described by Sterlini & Mandelstam (1969). To each 100 ml of S(C) basal salts medium was added 10 ml L-glutamate solution, 1.0 ml $CaCl_2$ solution, 4.0 ml $MgSO_4.7H_2O$ solution and 1.0 ml 0.2% (w/v) L-tryptophan or L-phenylalanine. This medium contained an adequate nitrogen source in the form of ammonia (NH_4Cl and NH_4NO_3 from stock solution B), but only a poor carbon source in the form of L-glutamate.

Resuspension medium for induction of sporulation by nitrogen deprivation (S(N) medium). To each 100 ml of S(N) basal salt medium was added 1.0 ml $CaCl_2$ solution, 4.0 ml $MgSO_4.7H_2O$ solution, 1.0 ml 0.2% (w/v) L-tryptophan or L-phenylalanine and 2.0 ml 10% (w/v) D-glucose solution. This medium contained a good carbon source, D-glucose, but only a poor nitrogen source in the form of $NaNO_3$ from stock solution C.

Buffers

TME buffer. 0.05 M tris-HCl (pH 7.4) containing 0.01 M β-mercapto-ethanol and 0.1 M EDTA. This buffer has been used previously (Rebello & Strauss, 1969).

Imidazole buffer. 0.5 M imidazole adjusted to pH 7.5 with concentrated HCl.

Growth of organisms

Inoculation procedure for growth in casein hydrolysate medium

From the stock plate a fresh nutrient agar plate was inoculated

and incubated at 37°C for approximately eight hours. Two loops of bacteria were then transferred to 1.0 ml of sterile Penassay broth (antibiotic medium 3, Difco Laboratories, P.O. Box 14B, West Molesey, Surrey KT8 OSE), mixed, and one drop of the suspension transferred to 10.0 ml of Penassay broth with a sterile pasteur pipette. After mixing, one drop of this suspension was added to a further 10 ml of Penassay broth. Four drops of this final diluted suspension were used to inoculate 20 ml CH medium in a 100 ml conical flask. The flask was incubated overnight at 37°C on an orbital shaker (Gallenkamp Ltd., Braeview Place, Nerston, East Kilbride, Glasgow) with the platform set to shake at 125 rpm. The following day 1 ml of the overnight culture was used to inoculate 50 ml of fresh CH medium contained in a 250 ml dimpled conical flask which was shaken at 37°C.

Inoculation procedure for growth in minimal medium

Essentially the same procedure was adopted except that the initial 1.0 ml bacterial suspension was not further diluted and 4 drops were used to inoculate 20 ml minimal medium in a 100 ml conical flask. After overnight growth at 37°C on an orbital shaker, 2.5 ml of the culture was used to inoculate fresh minimal medium (50 ml).

Estimation of growth

The growth of an organism was followed by measuring the absorbance of aseptically removed portions of a culture at 600 nm in a Pye Unicam SP 500 spectrophotometer using 1.0 cm cuvettes and reading against a water blank.

Conditions for obtaining sporulation

The organisms were grown with shaking at 37°C in 50 ml CH medium

contained in 250 ml dimpled conical flasks. When the exponentially growing cells had reached an E_{600} of 0.6 to 0.8 the culture was centrifuged at 4000 rpm for 15 min and the pelleted cells taken up in a resuspension medium (S or S(N)) at the same density. The time at which the cells were resuspended was taken as the start of the sporulation process (t-zero). The cells were shaken at 37°C and the extent of sporulation determined after overnight incubation.

Determination of sporulation

Resistance to heat and organic chemicals is a characteristic property of the mature spore and reflects the structural and physiological changes which occur during the final stages of sporulation. Resistance to heat above 80°C, which kills vegetative cells, can therefore be used as a convenient assay for detection of mature spores in a culture.

Assay for heat-resistance. A 0.1 ml sample of the bacterial suspension was transferred to 0.9 ml of sterile minimal salts (see above). The sample was heated at 85°C for 15 min in a water bath (Grant Instruments Ltd., Barrington, Cambridge CB2 5Q2, England) and the heated sample was then diluted in minimal salts and plated out on nutrient agar (Oxoid). For a 10^{-2} dilution 0.1 ml of the heated suspension was spread directly onto nutrient agar. After incubation overnight at 37°C the number of colonies appearing on the agar represented the number of heat resistant cells per ml in the original culture x 10^{-2}. Further dilutions were made in minimal salts when required.

Total viable count. This was assessed in a similar manner to the above assay for heat-resistance except that the heating step was omitted.

The degree of sporulation in a culture was expressed as a percentage of the viable cell count.

Microscopic examination of cells. Endospores are characterised by extreme refractility and this fact can be used as a simple and rapid means to assess the extent of sporulation in a culture. A drop of culture was placed on a microscope slide and viewed by oil immersion in a phase contrast microscope (Vickers Instruments, Haxby Road, York, England).

Preparation of cell-free extracts

The procedure routinely employed was that of ultrasonic disintegration. Bacteria from 20 ml or 50 ml cultures at an E_{600} of 0.6 - 0.8 were centrifuged at 5000 rpm for 15 min, the tubes drained well and the pellet taken up in 2.0 or 3.0 ml of TME buffer. The cell suspension was then disrupted by sonic oscillation using a MSE 100 watt ultrasonic disintegrator. It was used at 30% of its maximum amperage for 2 min and the cell suspension was cooled throughout the procedure by standing the tube in ice. The suspension of broken cells was then centrifuged at 18,000 rpm for 30 min at $4^{\circ}C$ in a MSE High Speed 18 centrifuge. The supernatant was used directly as a source of enzyme activity or stored frozen at $-20^{\circ}C$.

Occasionally, two other procedures were used to break the bacterial cells: a) X-press. Cells from a 50 ml culture were prepared as above, taken up in 5.0 ml TME buffer and sheared by passage through an X-press (Biotic Ltd., Croydon, England) at $-25^{\circ}C$ at a pressure of 28,000 psi. Cell debris was removed by centrifugation at 18,000 rpm for 30 min at $4^{\circ}C$. b) Toluene treatment. Cells from a 50 ml culture were prepared as above and taken up in 2.0 ml TME buffer. Toluene (0.2 ml) was added to the cell suspension which was then mixed vigorously for one min.

Enzyme assays

Glutamine synthetase (L-glutamate-ammonia ligase, E.C.6.3.1.2)

Glutamine synthetase activity was determined by the formation of γ-glutamylhydroxamic acid (GHA) (Elliot, 1955). The assay routinely employed was the forward or biosynthetic reaction measuring the ability of the enzyme to form glutamine. The reaction mixture (3.0 ml) contained 0.5 M L-glutamate in 0.5 M imidazole-HCl buffer, pH 7.5 (2.05 ml), 0.05 M ATP (0.5 ml), M $MgSO_4 7H_2O$ (0.1 ml), M hydroxylamine, pH 7.0 (0.1 ml), and a suitable aliquot of cell extract (0.25 ml). The reaction was started by the addition of the enzyme preparation after 5 min preincubation of the reaction mixture at 30°C and the incubation continued for 30 min. The reaction was stopped by the addition of 0.75 ml of ferric chloride reagent. The reagent was made up of equal parts of 10% (w/v) $FeCl_3.6H_2O$ in 0.2 M HCl, 24% (w/v) trichloroacetic acid and 50% HCl. The mixture was centrifuged at 5000 rpm for 15 min and the extinction at 510 nm of the supernatant was determined in 1 cm cuvettes on a Pye Unicam SP 500 spectrophotometer against a blank reaction mixture lacking L-glutamate.

On occasion, an assay which measures the γ-glutamyl transferase activity of the enzyme was used (Prusiner & Millet, 1972). The reaction mixture (3.0 ml) contained 0.4 M L-glutamine in 0.02 M imidazole-HCl buffer, pH 7.4 (2.35 ml), 0.004 M ADP (0.1 ml), 0.003 M $MnCl_2.4H_2O$ (0.1 ml), 0.06 M hydroxylamine, pH 7.0 (0.1 ml), 0.02 M sodium arsenate (0.1 ml) and a suitable aliquot of cell extract (0.25 ml). The assay procedure was identical to that given above.

Glutamate synthase (L-glutamine(amide) : 2-oxoglutarate aminotransferase oxidoreductase (NADP), E.C.2.6.1.53)

Activity of this enzyme was determined by following the rate of oxidation of reduced NADP at 340 nm (Meers et al., 1970; Elmerich & Aubert, 1971). The reaction mixture (3.0 ml) contained 0.5 M imidazole-HCl buffer, pH 7.5 (usually 2.6 ml), 0.00012 M NADPH (0.1 ml), 0.00125 M L-glutamine (0.1 ml), 0.005 M 2-oxoglutarate (0.1 ml) and a suitable aliquot of cell extract (0.1 ml). The reaction was started by the addition of substrate (L-glutamine) after 5 min preincubation of the reaction mixture at 30°C. The decrease in extinction at 340 nm was followed on a Pye Unicam SP 800 recording spectrophotometer at 30°C in 1 cm cuvettes using a reaction mixture lacking L-glutamine as a control to monitor endogenous NADPH oxidation.

Glutamate dehydrogenase (E.C.1.4.1.4)

Activity of this enzyme was assayed in a reaction mixture identical to that used for glutamate synthase, except that L-glutamine was replaced by 0.04 M NH_4Cl (0.1 ml). The decrease in extinction at 340 nm was again followed on a SP 800 recording spectrophotometer at 30°C in 1 cm cuvettes using a reaction mixture lacking NH_4Cl as a control to monitor endogenous NADPH oxidation.

Protein estimation

The protein concentration of cell extracts was estimated by the method of Lowry et al. (1951) which is a modification of that used by Folin & Ciocalteau (1927). The following solutions were required:

A. 2% (w/v) Na_2CO_3 in 0.1 M NaOH.

B. 1% (w/v) $CuSO_4.5H_2O$.

C. 2% (w/v) NaK tartrate.$4H_2O$.

D. B.D.H. Folin-Ciocalteau's reagent diluted with an equal volume of distilled water.

E. 25 ml of A, 0.25 ml of B and 0.25 ml of C made up immediately before use.

The protein solution (normally 0.1 ml) was diluted to 1.0 ml with distilled water. Four ml of Solution E was added and the mixture allowed to stand at room temperature for 10 min. Solution D (0.5 ml) was then added with rapid mixing. After standing the mixture at room temperature for a further 30 min the extinction of the solution at 750 nm was measured against a reagent blank on a Pye Unicam SP 500 spectrophotometer using 1.0 cm cuvettes. The protein concentration was estimated by reference to a calibration curve obtained with bovine serum albumin over a concentration range of 0-200 µg.

Enzyme units

An enzyme unit was defined as the amount of enzyme which catalyses the formation of product or the disappearance of substrate at the rate of 1.0 nmole per min at 30°C. Specific activity for the glutamine synthetase assay was expressed as nmoles of glutamylhydroxamate formed/min/mg protein, and specific activities for the glutamate synthase and glutamate dehydrogenase assays were expressed as nmoles NADPH oxidised/min/mg protein.

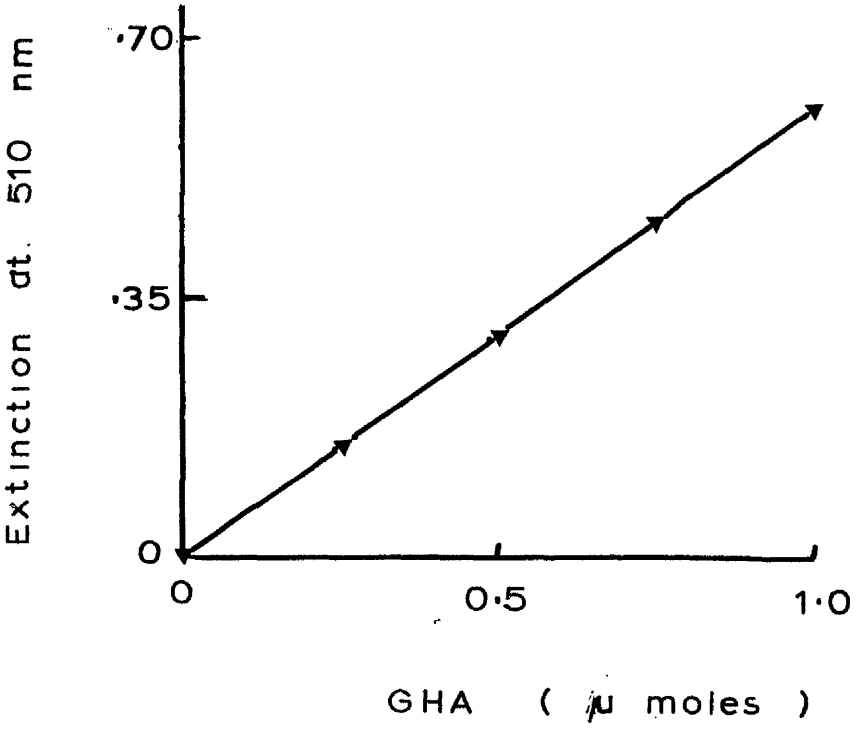
For the calculation of enzyme activities the molar extinction coefficient of NADPH was taken as 6,200 at 340 nm (Weber, 1961). The concentration of γ-glutamylhydroxamate was estimated by reference to a

standard curve prepared using γ-glutamylhydroxamate (Sigma, Norbiton Station Yard, Kingston upon Thames, London) (Figure 6). To dilutions of γ-glutamylhydroxamate in distilled water (total volume 1.0 ml) was added 0.75 ml of $FeCl_3$ reagent (see glutamine synthetase assay), and the extinction at 510 nm measured in a Pye Unicam SP500 spectrophotometer using 1.0 cm semi-micro cuvettes. One μmole of γ-glutamylhydroxamate gave an E_{510} of 0.6 under these conditions.

Chemicals

Casein hydrolysate, nutrient agar, sterile disposable Petri-dishes were obtained from Oxoid Ltd. L-glutamate, L-glutamine, L-alanine, L-asparagine, L-tryptophan, ATP, imidazole, hydroxylamine, NADPH, 2 oxo-glutarate, ADP, BSA, GHA, L-phenylalanine and β-mercapto-ethanol were obtained from Sigma. All other chemicals were obtained from British Drug Houses Ltd., England.

Figure 6. Calibration curve relating extinction at 510 nm to concentration of γ-glutamylhydroxamate (GHA). Increasing concentrations of GHA in distilled water were treated with 0.75 ml ferric chloride reagent (see glutamine synthetase assay) and the extinction measured in a Pye Unicam SP 500 spectrophotometer.


Extinction at 510 nm
·70
·35
0
0
0·5
1·0
GHA (µ moles)

RESULTS

Examination of glutamine synthetase and glutamate synthase during growth of *Bacillus subtilis* 168 wild-type

As a preliminary to an investigation of possible changes in specific activity of glutamine synthetase (GNS) or glutamate synthase (GTS) during the onset of sporulation, it was decided to investigate the behaviour of the enzymes under various conditions of nitrogen availability during vegetative growth. Previous evidence (Rebello & Strauss, 1969) had indicated that GNS synthesis was sensitive to induction or repression, but apparently no evidence was available on the regulation of GTS in *B. subtilis*. It was anticipated that an investigation of enzyme behaviour under various growth conditions would establish a pattern of regulation for the enzymes with regard to nitrogen availability and provide useful information on the extent of repression and de-repression of these enzymes.

Growth rate of *B. subtilis* wild-type with different nitrogen sources

In preliminary experiments the growth response of *B. subtilis* to various sources of nitrogen in liquid media was determined. Cells were grown overnight in 20 ml cultures of minimal medium each containing glucose as sole carbon source with various combinations of nitrogen source. Ammonia was used alone or in combination with either glutamine, glutamate, arginine or histidine. Alternatively, each of the four amino acids was used as sole source of nitrogen . Finally, nitrate was used instead of ammonia as source of nitrogen. After overnight growth 2.5 ml of each culture was used to inoculate fresh 50 ml of the same medium. The cells were shaken at 37^{o}C and growth was followed by

measuring the E_{600} of 1 ml samples withdrawn aseptically from each medium at 30 min intervals.

The results obtained from the growth experiments are summarised in Table 1. B. subtilis generally grew rapidly in the glucose-ammonia minimal medium, although occasionally a much slower growth rate was obtained. Addition of an extra source of nitrogen to this medium, in the form of arginine, glutamine, glutamate or histidine did apparently promote a somewhat slower growth rate. Substitution of ammonia with either arginine or glutamine as sole nitrogen sources had no marked effect on the cell doubling time. Glutamate, however, would not serve as well and there was a marked tendency for the cells to clump together in this medium. A pronounced increase in cell doubling time using nitrate as the only available nitrogen source was observed. Histidine would not serve as a satisfactory source of nitrogen with glucose as carbon source. This result is in agreement with previous results (Chasin & Magasanik, 1968) which showed that histidine was readily utilised as source of nitrogen only with those carbon sources, such as citrate or arabinose, which did not exert a strong catabolite repression on the synthesis of the histidine degrading enzymes. The results here indicate that the enzymes degrading arginine to glutamate are not as susceptible to glucose catabolite repression as those used for histidine degradation as the cells grew well in the glucose-arginine medium. This result is also in agreement with Chasin & Magasanik (1968).

Preliminary studies on GNS and GTS enzyme activities

a) Comparison of methods for cell breakage

Three procedures were used to disrupt the bacteria in order

TABLE 1

Growth rates of B. subtilis 168 wild-type on media containing various nitrogen sources

The cells were grown in minimal medium (see Materials and Methods section). The carbon source in each case was glucose (0.5% w/v) and each nitrogen source was used at a concentration of 0.1% (w/v) except ammonium sulphate (0.05%). The values given are the mean generation or cell doubling times determined by following the increase in turbidity of a culture at 600 nm. Each value is the average from the number of separate determinations shown. The extreme ranges of the values found are also included.

Nitrogen source	Number of determinations	Cell doubling time (min)	Range
Ammonia	9	56	50-100
Ammonia & L-arginine	3	66	54-72
Ammonia & L-glutamine	4	67	60-84
Ammonia & L-glutamate	5	71	60-90
Ammonia & L-histidine	3	66	60-72
Nitrate	3	147	144-150
L-arginine	5	69	51-90
L-glutamine	5	58	48-66
L-glutamate	4	114	95-150
L-histidine	1	360	360

to obtain a broken cell suspension or cell-free extract which would give maximum activity in the GNS assay. Exponentially growing cultures (50 ml) of B. subtilis wild-type in glucose-ammonia minimal medium were harvested, the cells broken in an ultrasonic disintegrator or an X-press and a cell-free extract prepared as described in the Materials and Methods section. It was found that extracts prepared by sonication had a high protein content (average 200 µg/ml), whereas the extract from the X-press had a much lower protein content (50 µg/ml). This was presumably a reflection of the greater volume required for passage of a cell suspension through the X-press rather than a difference in efficiency of cell breakage. In addition, although extracts prepared by sonication were active in both the GNS and GTS assay, no activity was detectable in extracts prepared by the X-press method.

Treatment of cell suspensions with toluene gave rise to a suspension which when added to the assay mixture for GNS activity gave rise to a turbid solution. It was therefore decided that ultrasonic disintegration was the method of choice and all the results presented in the thesis were obtained using cell-free extracts prepared by this method.

b) Solubility of enzymes

An exponentionally growing 50 ml culture of B. subtilis wild-type in glucose-ammonia minimal medium was harvested and the cells disrupted by sonication (see Materials and Methods section).

GNS activity by the biosynthetic assay and GTS activity were assayed in the crude broken cell suspension before centrifugation and in the cell-free extract after centrifugation. For the GNS assay a change in E_{510} of 0.0035/min was obtained for both the broken cell suspension and

cell-free extract indicating that the enzyme was confined to the soluble fraction. For the GTS assay a change in E_{340} of 0.087/min was obtained using the broken cell suspension and a value of 0.064/min for the cell free extract. These values indicated that the GTS activity was again mostly to be found in the soluble fraction.

c) Comparison of γ-transfer and biosynthetic assay for glutamine synthetase

GNS activity may be assayed either by noting the γ-glutamyl transferase activity of the enzyme or by measuring the biosynthetic ability of the enzyme to form glutamine. This distinction is important in enteric bacteria because both the adenylated and deadenylated form of the enzyme are active in the γ-glutamyl transferase assay, but adenylation markedly lowers the biosynthetic activity of the enzyme (Stadtman et al., 1970; Kingdon et al., 1967; Schutt & Holzer, 1972). As adenylation of the enzyme from Bacillus spp. does not appear to occur as a means of regulation (see Introduction) these different assay procedures are not as significant. Nevertheless a comparison of the γ-GT and biosynthetic activities was done using a cell-free extract prepared from B. subtilis wild-type grown on glucose-ammonia medium. The assay procedures are given in the Materials and Methods section.

A specific activity of 90 nmoles/min/mg protein was obtained from the γ-GT assay and a value of 250 nmoles/min/mg from the biosynthetic assay. As the biosynthetic assay procedure yielded a higher activity this assay was used routinely in all subsequent experiments.

d) Stability of enzymes

Cell-free extracts were prepared in TME buffer (see Materials and Methods section). This buffer had previously been shown by Rebello &

Strauss (1969) to stabilise the GNS of B. subtilis. To test the stability of the enzyme preparation a cell-free extract was prepared from cells grown in glucose-ammonia medium. In the GNS assay, fresh extract gave a change in E_{510} of 0.016/min and after 24 hours storage at -20°C the same extract gave a value of 0.018/min. This indicated that there was no loss in GNS activity after 24 hours in this buffer which agreed with the observation of Rebello & Strauss (1969). In the GTS assay some evidence of loss of activity on storage was found for this enzyme. The fresh cell extract gave a change in E_{340} of 0.08/min which dropped to 0.028/min on storage at -20°C for 24 hours. Because of the apparent loss of GTS activity on storage, all subsequent assays for both GNS and GTS activities were performed on a freshly prepared extract.

e) Control experiments for GNS and GTS assays

A cell-free extract was prepared from glucose-ammonia grown cells. In the GNS assay a change in E_{510} of 0.016/min was observed for the complete system. If ATP was replaced by buffer, no change in E_{510} was observed over a 30 min period. ATP was therefore essential for the biosynthetic assay for GNS. It was found that the ATP solution used in the assay could be safely stored at -20°C. Comparison of freshly prepared ATP solution and a ATP solution stored frozen for 72 hours gave identical rates of reaction using the same cell extract. It was also noted that heating a portion of the extract to 100°C for 5 min gave a negligible change in E_{510} of 0.0003/min. This low rate of reaction was comparable to the change in E_{510} when enzyme preparation was replaced by buffer in the assay mixture. It was concluded that heating the extract caused a total loss of GNS activity.

In the GTS assay a change in E_{340} of 0.084/min was observed for the complete system. Replacement of the substrate L-glutamine, by buffer gave a value of 0.005/min. This represented the endogenous oxidation of NADPH and all subsequent GTS estimations were corrected for this endogenous oxidation which was determined at the same time as the GTS assay by the inclusion of a control lacking L-glutamine (see Materials and Methods section). The co-factor specificity of the GTS reaction for NADPH or NADH was also tested. Replacement of the NADPH in the assay mixture by an equal quantity of NADH gave a low change in E_{340} of 0.006/min. The enzyme therefore had a high specificity for NADPH as co-factor.

f) Assay for glutamate dehydrogenase

GDH can conveniently be assayed using the system for the GTS assay, but replacing L-glutamine with NH_4Cl (see Materials and Methods section). Using the cell-free extract prepared for the experiment described in (e) above it was found that there was no change in E_{340} over and above the endogenous rate when NH_4Cl replaced L-glutamine as substrate or when NADH replaced NADPH as co-factor. It was concluded that GDH was undetectable in extracts of B. subtilis 168 grown in glucose-ammonia medium. This result is in agreement with that of other workers (see Introduction).

A summary of some of the results from this section is presented in Table 2.

Enzyme specific activity during growth of B. subtilis

The object of this section of the work was to look for changes

TABLE 2

Summary of the properties and specificity of GNS and GTS activities from B. subtilis 168

Cell-free extracts were prepared from cells growing exponentially in minimal medium with glucose (0.5% w/v) as carbon source and $(NH_4)_2SO_4$ (0.05% w/v) as nitrogen source. The details of the assay for GNS and GTS are given in the Materials and Methods section. Activities are expressed as ΔE_{510}/min for GNS and ΔE_{340}/min for GTS. In the GTS assay all values are corrected for endogenous oxidation of NADPH or NADH which was a change in E_{340} of 0.006/min using NADPH as co-factor.

Glutamine synthetase assay	
Complete system	0.016
ATP omitted	0.0
Enzyme preparation heated to 100°C	0.0003
Enzyme preparation stored at -20°C for 24 hours	0.018

Glutamate synthase assay	
Complete system	0.08
Complete system with NADPH replaced by NADH	0.006
Complete system with L-glutamine replaced by NH_4Cl (glutamate dehydrogenase assay) (corrected for endogenous NADPH oxidation)	0.0
Enzyme preparation stored at -20°C for 24 hours	0.028

in the specific activities of GNS and GTS under growth conditions where repression and derepression of the enzymes might be expected to occur. If changes associated with nitrogen availability during growth were detectable this would be a suitable prelude to an investigation of possible changes during the onset of sporulation.

Enzyme levels were determined in cell-free extracts prepared from exponentially growing B. subtilis wild-type using glucose as carbon source and various nitrogen sources used for the growth determinations (Table 1). The specific activity of GNS and GTS under the various growth conditions are presented in Table 3. As some fluctuation in specific activity of both enzymes from separate cell extracts was observed, average values are presented together with the extreme values for each enzyme. The figures show that cells grown with ammonia as nitrogen source possessed quite high levels of both enzymes. Addition of arginine, glutamate, glutamine or histidine to the medium containing ammonia produced a decreased activity for both enzymes. In the case of GNS this repression was greatest with glutamine and arginine which were in turn greater than glutamate or histidine. This result is exactly in agreement with that of Rebello & Strauss (1969) who obtained a similar pattern of GNS repression in B. subtilis 168 by arginine, glutamate and glutamine in the presence of ammonia, although the degree of repression obtained here was slightly greater. For example, they reported a 55% repression of GNS activity by L-glutamine in the presence of ammonia compared with the activity obtained from cells grown with ammonia alone. The results in Table 3 show an average 66% repression. The repression by the added amino acids of GTS was not identical to that of GNS. With GTS a six-fold reduction in activity was obtained by the addition of arginine while glutamate and glutamine caused only a two-fold reduction.

TABLE 3

Levels of glutamine synthetase and glutamate synthase in B. subtilis wild-type under different growth conditions

The cells were grown in minimal medium containing glucose (0.5% w/v) as carbon source and the appropriate nitrogen source given below at 0.1% (w/v) except for ammonium sulphate which was used at 0.05% (w/v). Enzyme assays were performed using freshly prepared cell-free extracts as described in the Materials and Methods section. The number of determinations represents the number of separate enzyme assays, each using an extract prepared from a different culture. Values are specific activities expressed as nmoles/min/mg protein and represent the average of the total number of determinations. Generally both GNS and GTS assays were performed on each extract.

Nitrogen source	Number of determinations	Glutamine synthetase (range)	Number of determinations	Glutamate synthase (range)
Ammonia	12	170 (100-250)	10	740(650-800)
Ammonia & L-arginine	2	63 (63,63)	3	130(110-140)
Ammonia & L-glutamine	3	70 (60-88)	3	300(180-400)
Ammonia & L-glutamate	3	116 (88-150)	3	340(300-430)
Ammonia & L-histidine	1	138	1	700
Nitrate	3	516 (413-575)	3	400(260-500)
L-arginine	4	146 (88-150)	3	113(100-130)
L-glutamine	4	105 (63-100)	3	343(200-500)
L-glutamate	1	325	1	80
L-histidine	N.D.	-	-	-

*N.D. means not done. As B. subtilis grew very slowly with L-histidine as nitrogen source no attempt was made to prepare a cell-free extract.

The obvious repression of GTS synthesis by glutamate is in agreement with Elmerich (1972) who reported repression of GTS synthesis by glutamate in B. megaterium. The greater repression caused by the inclusion of arginine in the glucose-ammonia medium was somewhat surprising as it could be assumed that arginine would be degraded to glutamate under these circumstances. Histidine had only a marginal effect on the synthesis of both enzymes. This would be in keeping with its lack of metabolism in the presence of glucose due to catabolite repression of the histidine degrading enzymes.

When nitrate replaced ammonia in the growth medium, a 3-fold increase in GNS specific activity, accompanied by almost a 2-fold reduction in GTS activity was found, compared to ammonia grown cells. In fact, the highest specific activity for GNS was consistently found for cells grown with nitrate as source of nitrogen. As cells grow only slowly with nitrate as nitrogen source (Table 1), they were presumably in a condition of nitrogen deprivation. Starvation for a ready source of nitrogen is known to cause sporulation in B. subtilis (see Introduction) and in fact was used in later work as a mean for obtaining sporulation. It was thus of interest to note at this stage the derepression of GNS when cells were grown with nitrate as source of nitrogen.

In other experiments extracts were prepared from cultures grown with glutamate or glutamine or arginine as sole source of nitrogen. As noted previously B. subtilis grew very slowly with histidine as sole source of nitrogen and a culture suitable for preparation of a cell-free extract was not obtained. Also, as noted before, the cells did not grow in a satisfactory manner with glutamate as sole nitrogen source, they tended to clump together and for this reason the GNS and GTS activities

obtained from these cells may be unreliable. The use of either L-glutamine or L-arginine as sole nitrogen source produced enzyme specific activities similar to those obtained with cultures grown with ammonia together with the amino acid. Thus with L-arginine as sole nitrogen source both GNS and GTS were extensively repressed over the values obtained with ammonia as nitrogen source and with L-glutamine GNS was even more repressed and GTS somewhat less so.

This work clearly established that changes in specific activity of both enzymes could be detected during growth with different nitrogen sources. Growth limitation by the use of nitrate as nitrogen source prompted derepression of GNS. Previous results of Rebello & Strauss (1969) had shown a transient increase in GNS activity during shift-down from a rich to a poor growth medium. Shift-down conditions involving transfer of cells from a rich to a poor growth medium with limiting carbon or nitrogen sources are known to induce sporulation (see Introduction). It was now necessary to examine the behaviour of GNS and GTS during the transition from growth to sporulation.

Sporulation behaviour of B. subtilis wild-type in various resuspension media

The technique used for obtaining sporulation is the replacement or resuspension procedure (see Materials and Methods section) whereby bacteria that are growing exponentially in a rich medium are transferred to a poor medium. The resuspension medium described by Sterlini & Mandelstam (1969) contains an adequate supply of ammonia in the form of NH_4NO_3 and NH_4Cl, but only a poor carbon source in the form of L-glutamate (see Materials and Methods section). This resuspension medium is referred to as S(C) and in this medium sporulation is assumed to be triggered by starvation for a carbon source. This is supported by the

fact that inclusion of D-glucose (0.2% w/v) in this medium at first allowed the cells to grow rather than sporulate (see below).

In order to obtain sporulation by nitrogen deprivation the S(C) resuspension medium was modified. The NH_4Cl and NH_4NO_3 constituents were replaced by $NaNO_3$ and the L-glutamate was replaced by D-glucose (0.2% w/v). This medium is referred to as S(N) medium and a fuller description is given in the Materials and Methods section. Supplementation of this S(N) medium with L-glutamate (0.15% w/v) as an extra nitrogen source again promoted growth of the cells rather than sporulation. The behaviour of B. subtilis wild-type in the different resuspension media is illustrated in Table 4. Cultures were grown in CH medium and resuspended in S(C) medium, S(C) medium supplemented with glucose, S(N) medium or S(N) medium supplemented with L-glutamate. Cell densities were measured after resuspension at hourly intervals for 5 hours. Heat-resistance was assayed at 5 hours after resuspension and after overnight incubation. The results show that in the S(C) and S(N) media, the E_{600} roughly doubled in the first 4 - 5 hours. This is in agreement with previous results obtained with the S(C) medium where the O.D. doubled and then levelled off (Sterlini & Mandelstam, 1969). Heat-resistant colonies were detectable at 10^{-6} dilution 5 hours after resuspension in both these media and examination of the cultures in the phase-contrast microscope revealed that 1 - 5% of the cells contained refractile spores. Heat-resistance determinations at 24 hours revealed that in this experiment slightly better sporulation was achieved in the S(C) resuspension medium than in the S(N) medium, although this was not consistently found (see Table 5).

The S(C) medium was assumed to trigger sporulation by carbon deprivation and Table 4 shows that supplementation of this medium with

TABLE 4

Sporulation behaviour of B. subtilis wild-type in various resuspension media

Four cultures (50 ml) growing in rich CH medium were harvested at E_{600} of approximately 0.6 and each resuspended in an equal volume of one of four resuspension media. The S(C) medium contained ammonia as the main nitrogen source and glutamate as carbon source and to this was added glucose (0.2% w/v) to give the S(C) + glucose medium. The S(N) medium contained nitrate as nitrogen source and glucose as carbon source and to this was added glutamate (0.15% w/v) to give the S(N) + glutamate medium. Optical density readings were taken every hour by aseptic removal of 3 ml volumes. Heat-resistance was used as a measure of spore formation. This procedure and a fuller description of the resuspension media are given in the Materials and Methods section.

Time after resuspension (hr)	E_{600}				Heat-resistance x 10^{-6}			
	S(C)	S(N)	S(C) + glucose	S(N) + glutamate	S(C)	S(N)	S(C) + glucose	S(N) + glutamate
0	.62	.57	.52	.53	-	-	-	-
1	.76	.64	1.02	.82	-	-	-	-
2	.93	.72	1.9	1.04	-	-	-	-
3	1.16	.8	> 2.0	2.0	-	-	-	-
4	1.32	.92	> 2.0	>2.0	-	-	-	-
5	1.3	1.08	> 2.0	>2.0	5	2	0	0
24	-	-	-	-	197	69	24	181

glucose allowed a rapid increase in E_{600} to a value well above that of the S(C) medium alone (E_{600} >2.0). The cell doubling time over the first 2 hours was calculated as 63 minutes, a value similar to that obtained for B. subtilis growing in glucose-ammonia minimal medium (see Table 1). The S(N) medium was assumed to trigger sporulation by nitrogen deprivation and again Table 4 shows that supplementation of this medium with a good source of nitrogen (L-glutamate) caused a rapid increase in E_{600} with an initial cell doubling time calculated as 90 min, similar to the value obtained for B. subtilis growing in glucose-glutamate minimal medium (see Table 1). The presumption that cells resuspended in S(C) + glucose or S(N) + glutamate continued to grow rather than initiate sporulation was supported by the heat-resistance determinations made 5 hours after resuspension. Table 4 shows that no colonies were detected at 10^{-6} dilution at 5 hours in either medium and examination of the cells in the phase-contrast microscope revealed only an occasional spore. However, by 24 hours the heat-resistance values showed that the cells had successfully sporulated by this time. The final level of sporulation in the glucose supplemented S(C) medium was lower than in the other three media (Table 4).

Enzyme levels during the onset of sporulation

The results above had satisfactorily demonstrated that sporulation could be induced by carbon starvation in the S(C) medium and by nitrogen starvation in the S(N) medium. Changes in specific activity of GNS and GTS associated with the initiation of sporulation might not be expected to occur during the onset of sporulation under conditions of carbon starvation. Assay of enzyme levels in the S(C) medium would therefore act as a control as any changes in enzyme levels detected in this

medium would be more likely to reflect derepression of enzyme synthesis following transfer of cells from the rich CH medium with its numerous sources of nitrogen to the minimal S(C) resuspension medium.

For the assay of the enzymes during the onset of sporulation 200 ml cultures of B. subtilis wild-type in 1 litre dimpled flasks were used. These cells were resuspended in the same volume of either S(C) or S(N) resuspension medium. Portions (50 ml) were removed at time zero and at 20 and 40 minutes after time zero and a cell free extract prepared from each. The results of the enzyme assays from many separate experiments together with the percentage sporulation finally obtained in each medium are shown in Table 5. As some variation in specific activity of both enzymes from cell extracts from separate experiments was observed, the extreme values for each enzyme are included in the table.

The results for the enzyme assays in Table 5 show that no marked changes in specific activity of either enzyme had occurred at either 20 min or 40 min after the initiation of sporulation in either the S(C) or S(N) media. In the S(C) medium it did appear that the GNS activity had increased slightly by 20 min and then fallen back to roughly the original activity by 40 min. This fluctuation was not so apparent in the S(N) medium, although the specific activity of GNS was on average slightly higher at each time interval in the S(N) medium than in the S(C) medium. No obviously significant difference in GTS activity was detected in the two media.

Therefore no striking differences were found in GNS or GTS activities when sporulation was induced by either carbon or nitrogen starvation. Therefore it must be concluded that changes in the amount of GNS or GTS enzymes play no role in the onset of sporulation, at least at the times studied.

TABLE 5

Levels of glutamine synthetase and glutamate synthase in B. subtilis wild-type at the onset of sporulation induced by either carbon or nitrogen starvation

Cells growing exponentially in 200 ml cultures of CH medium were harvested and resuspended in the same volume of either S(C) or S(N) resuspension medium. A full description of these media is given in the Materials and Methods section. Immediately (time zero) and at 20 and 40 minutes past time zero, 50 ml portions of the culture were removed and cell-free extracts prepared from each as described in the Materials and Methods section. Enzyme assays and protein determinations were made with the cell extracts. The remaining 50 ml of each culture was incubated overnight and viable count and heat-resistance determinations performed as described in the Materials and Methods section. Values for the enzyme activities are expressed as nmoles/min/mg protein and represent the average of 10 separate determinations at each time for S(C) medium and 3 determinations for the S(N) medium. The values in brackets represent the range of activities found.

Resuspension medium	Glutamine synthetase activity. Time (min) after resuspension			Glutamate synthase activity. Time (min) after resuspension			Percentage sporulation $\frac{\text{Heat-resistant colonies}}{\text{Total viable count}} \times 100$
	0	20	40	0	20	40	
S(C) medium. Sporulation induced by carbon deprivation.	140 (88-188)	180 (100-225)	123 (88-225)	49 (35-70)	47 (32-60)	68 (50-100)	35%
S(N) medium. Sporulation induced by nitrogen deprivation.	188 (113-225)	191 (175-213)	169 (113-225)	48 (32-60)	57 (40-80)	55 (40-60)	41%

An alternative approach to seek a role for these enzymes in sporulation is to examine the behaviour of a mutant strain lacking the particular enzyme activity in question. This approach has already been applied in B. megaterium in the case of GNS (see Introduction). A possible role for GTS activity during sporulation was investigated using the glutamate auxotroph, glt-100, tentatively identified as lacking GTS activity.

Examination of glutamine synthetase and glutamate synthase during growth and sporulation of strain glt-100

Growth characteristics of the presumed glutamate synthase negative strain (glt-100)

This strain was originally isolated by Dr. J. Coote. It was unable to grow in glucose-ammonia minimal medium but grew normally in glucose-glutamate minimal medium. For these reasons it was presumed to have a defective glutamate synthase by analogy with a previously described GTS negative strain of B. megaterium (Elmerich & Aubert, 1971).

The growth pattern of this strain was investigated in more detail. The organism was inoculated into 20 ml portions of various media and incubated overnight at 37°C. A turbid culture was scored as positive growth and no obvious increase in turbidity was scored as a negative growth response to the medium in question. The wild-type B. subtilis was inoculated and grown in each medium in an identical manner as a control. The results of the growth experiments are given in Table 6. The results with the wild-type organism will be considered first. Some growth properties of B. subtilis wild-type using glucose as carbon source and a variety of nitrogen sources were more fully examined earlier (Table 1) whe

TABLE 6

Growth pattern of B. subtilis wild-type and glutamate synthase negative strain (glt-100) in liquid media

The cells were grown overnight in minimal medium containing a carbon source at 0.5% (w/v) and nitrogen source at 0.1% (w/v) except for ammonia which was used as $(NH_4)_2SO_4$ at 0.05% (w/v). After overnight incubation each culture was scored as +++ for a heavy turbidity, ++ for a distinctly turbid culture, + for slightly turbid and - for no obvious increase in turbidity over that of the original inoculum.

Carbon source	Nitrogen source	B. subtilis wild-type	B. subtilis glt-100
Glucose	Ammonia	+++	-
Citrate	Ammonia	++	-
Histidine	Ammonia	++	++
Glucose	Histidine	+	-
Glucose	Arginine	+++	+++
Glucose	Glutamate	+++	+++
Glucose	Glutamine	+++	+++
Glucose	Proline	+++	+++
Glucose	Aspartate	+++	+++
Glucose	Glucosamine	+++	-
Citrate	Histidine	+++	++
Citrate	Arginine	+++	+++
Citrate	Glucosamine	+++	-
Glycerol	Histidine	++	-
Glycerol	Arginine	+++	+++
Glycerol	Glucosamine	+++	-

the cell doubling times were calculated. In Table 1 it was seen that the wild-type grew quite rapidly using glucose as carbon source and either arginine, glutamate or glutamine as nitrogen source. It grew only slowly with histidine as nitrogen source. Similar results were obtained in the experiments presented in Table 6. A low turbidity was produced in the glucose-histidine medium, but more vigorous growth was obtained using all other nitrogen sources including proline, aspartate and glucosamine. The use of L-histidine as nitrogen source, but with the substitution of glucose by either citrate or glycerol allowed more vigorous growth. This is in keeping with earlier published results (Chasin & Magasanik, 1968) which showed that the histidine degrading enzymes were strongly catabolite repressed by glucose, less so by glycerol and not at all by citrate. It should also be noted that histidine used as a carbon source, with ammonia as nitrogen source, supported better growth than when histidine was used as a nitrogen source. In this case ammonia would be exerting minimal catabolite repression on the induction of the histidine degrading enzymes.

The growth response of the proposed GTS negative strain glt-100 is also given in Table 6. This strain was unable to grow in a glucose or citrate medium with ammonia as nitrogen source. It was able to grow in the histidine-ammonia medium. This is because the breakdown of histidine to glutamate will allow the cell to use glutamate as a nitrogen source and also as a carbon and energy source, and so the presence of an extra nitrogen source, ammonia, is not necessary. Conversion of L-glutamate to 2-oxoglutarate will allow entry to the tricarboxylic acid cycle, where energy can be generated in the form of $NADH_2$ and subsequently ATP via the respiratory chain. Thus, amino acids such as L-histidine, L-arginine or

L-proline, which are degraded to L-glutamate, can serve as sources of both nitrogen and energy (Magasanik et al., 1974). The strain glt-100 was able to grow normally using L-arginine, L-glutamate, L-glutamine, L-proline and L-aspartate as nitrogen sources. L-arginine and L-proline will give rise to L-glutamate by degradation and L-aspartate is transaminated with 2-oxoglutarate to give oxaloacetate and L-glutamate (see Figure 2). L-glutamine would be expected to yield glutamate by the action of a glutaminase (Prusiner, 1975). Unlike the wild-type strain glt-100 was unable to use glucosamine as a source of nitrogen for growth. This is because glucosamine is degraded to yield ammonia which this strain is unable to utilise. L-serine is also degraded to ammonia (see Figure 2) and so it would be expected that glt-100 would also be unable to use this amino acid as a nitrogen source. However, it was found that the wild-type strain would not grow with L-serine as nitrogen source and so this proposition could not be tested. The other point to notice when comparing the results obtained with the wild-type and with strain glt-100 is that L-histidine allowed poorer growth of the mutant when used as a nitrogen source. Thus in the glucose-histidine medium the wild-type grew poorly, but the mutant did not grow at all. Similarly, although in the citrate-histidine and glycerol-histidine media the wild-type grew well, the mutant showed no growth in the latter medium and poorer growth in the former.

In summary, the growth response of the mutant strain glt-100 was what would be expected of a strain lacking the ability to assimilate ammonia. The strain was unable to grow in media containing ammonia as sole nitrogen source or in a medium containing glucosamine which acts as a nitrogen source by degradation to yield ammonia. It grew well with

nitrogen sources which yielded glutamate either by degradation or transamination.

Enzyme activity of strain glt-100 during growth

As a preliminary to an examination of glt-100 under sporulation conditions it was decided to assay GNS and GTS activities in the mutant during growth. The enzymic activities were examined in cell-free extracts prepared from cells grown in the rich CH medium and also in the minimal medium containing D-glucose with ammonia and L-glutamate as nitrogen sources. The wild type was included as a control. The results are presented in Table 7.

In the extracts from cells grown in the CH medium the specific activity of GNS was higher in the wild-type than in the mutant. There was no detectable GTS activity in the mutant extract, although a clear activity was obtained using the wild-type extract. With the extracts from cells grown in the minimal medium a slightly higher GNS activity was obtained in the mutant rather than the wild-type. Again no GTS activity was detectable in the mutant strain, although a high activity was given by the wild-type.

These results firmly established that strain glt-100 lacked GTS activity. Lack of this activity had the apparent effect of repressing GNS activity in the CH medium while allowing a slight derepression in the minimal medium when activities of these enzymes were compared in the wild-type and mutant.

Enzyme activity in strain glt-100 during the onset of sporulation

The experimental procedure for the assay of GNS and GTS

TABLE 7

Levels of glutamine synthetase and glutamate synthase in *B. subtilis* wild-type and mutant *glt*-100 under different growth conditions

The cells were grown in 50 ml cultures of either rich CH medium or minimal medium containing D-glucose (0.5% w/v) as carbon source and $(NH_4)_2SO_4$ (0.05% w/v) with L-glutamate (0.1% w/v) as nitrogen sources. Enzyme assays were performed using freshly prepared cell-free extracts as described in the Materials and Methods section. Values are specific activities expressed as nmoles/min/mg protein.

Growth medium	*B. subtilis* wild-type		Strain *glt*-100	
	Glutamine synthetase	Glutamate synthase	Glutamine synthetase	Glutamate synthase
CH medium	63	88	32	0
Minimal medium	88	100	143	0

activities in glt-100 during the onset of sporulation was identical to that described earlier for the assays in the wild-type. Resuspension cultures (200 ml) in S(C) and S(N) media were set up and 50 ml portions removed at time zero and at 20 and 40 min after time zero. Cell-free extracts were prepared and the enzymes assayed on the fresh extracts. L-glutamate is an important constituent of the mature spore in Bacillus spp. (see Introduction) and it seemed reasonable to assume that strain glt-100 would require a supply of L-glutamate in the S(N) medium as it would be unable to synthesise the amino acid from nitrate, the only nitrogen source supplied. For this reason additional resuspension cultures were set up using the S(N) medium, but with L-glutamate added. The results of the enzyme assays together with the percentage sporulation finally obtained in the three resuspension media are shown in Table 8.

For the GNS activity the results in Table 8 can be compared with those in Table 5 obtained from the wild-type under similar conditions. In the S(N) resuspension medium, the level of GNS activity in the mutant was the same at all time intervals as was found in the wild-type and was only slightly lower than the wild-type activity. Addition of L-glutamate to this medium had no obvious effect on GNS activity. With regard to the S(C) medium, a difference between GNS behaviour in the wild-type and strain glt-100 was noticeable. With the wild-type GNS activity had increased by 20 min after time zero, whereas with strain glt-100 the activity had fallen by this time.

In accordance with the results in Table 7, no detectable GTS activity was obtained at any time during sporulation of the glt-100 strain. An interesting result was obtained when the level of sporulation was determined in the resuspension media. Good sporulation was obtained in

TABLE 8

Levels of glutamine synthetase and glutamate synthase in strain glt-100 at the onset of sporulation

Cells growing exponentially in 200 ml cultures of CH medium were harvested and resuspended in the same volume of either S(C) or S(N) or S(N) + L-glutamate (0.15% w/v) resuspension media. Immediately (time zero) and at 20 and 40 min past time zero, 50 ml portions of the cultures were removed and cell-free extracts prepared. Enzyme assays and protein determinations were done on the fresh extracts. The remaining 50 ml of each culture was incubated overnight and viable count and heat-resistance determinations done the next day. Values for enzyme activities are expressed as nmoles/min/mg protein and represent the average of at least 2 separate determinations at each time. The values in brackets represent the range of activities found.

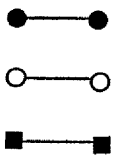
Resuspension medium	Glutamine synthetase activity. Time (min) after resuspension			Glutamate synthase activity. Time (min) after resuspension			Percentage sporulation $\frac{\text{Heat-resistant colonies}}{\text{Total viable count}} \times 100$
	0	20	40	0	20	40	
S(C) medium	134 (88-175)	94 (30-138)	82 (50-113)	0.0	0.0	0.0	32%
S(N) medium	144 (100-187)	138 (100-175)	138 (100-175)	0.0	0.0	0.0	< 0.1%
S(N) medium + L-glutamate	138 (138)	163 (100-213)	150 (113-186)	0.0	0.0	0.0	0.2%

the S(C) medium and as expected almost no spores were detected in the S(N) medium. However, in the S(N) + L-glutamate medium, where L-glutamate was added as a nitrogen source to overcome the mutation in strain glt-100, again a very low level of sporulation was obtained (Table 8).

Results presented earlier using the wild-type organism had shown that the cell densities in the S(C) and S(N) resuspension media roughly doubled and then levelled off (Table 4). The cell densities of strain glt-100 during sporulation in the three resuspension media described in Table 8 were followed for comparison. The results are shown in Figure 7. As with the wild-type the E_{600} values for strain glt-100 resuspended in the S(C) medium roughly doubled over the first 3 hours and then began to level off. Similarly, the E_{600} values in the S(N) medium, supplemented with L-glutamate, increased rapidly, but began to level off at a somewhat lower extinction than the wild-type. An E_{600} value of 2.0 was obtained in the S(N) + glutamate medium 3 hr after resuspension with the wild-type (Table 4), but strain glt-100 gave only a value of 1.7 at a similar time (Figure 7) and then began to level off. Comparison of the behaviour of the wild-type and the mutant strain in the S(N) medium shows roughly a doubling in E_{600} for the wild-type (Table 4), whereas the E_{600} for the mutant increased slightly over the first hour and then abruptly levelled off (Figure 7).

In summary, it was found that strain glt-100 sporulated normally in the S(C) resuspension medium, but only poorly in the S(N) medium even if this medium was supplemented with L-glutamate. Poor sporulation was expected in the S(N) medium alone as the cells had no utilisable source of nitrogen except that accumulated by the cells during

Figure 7. Increase in extinction of cells of strain glt-100 during resuspension in various media. Cells were grown in CH medium to an E_{600} value of about 0.6. They were then resuspended in an equal volume of one of the following media and the E_{600} noted at hourly intervals.

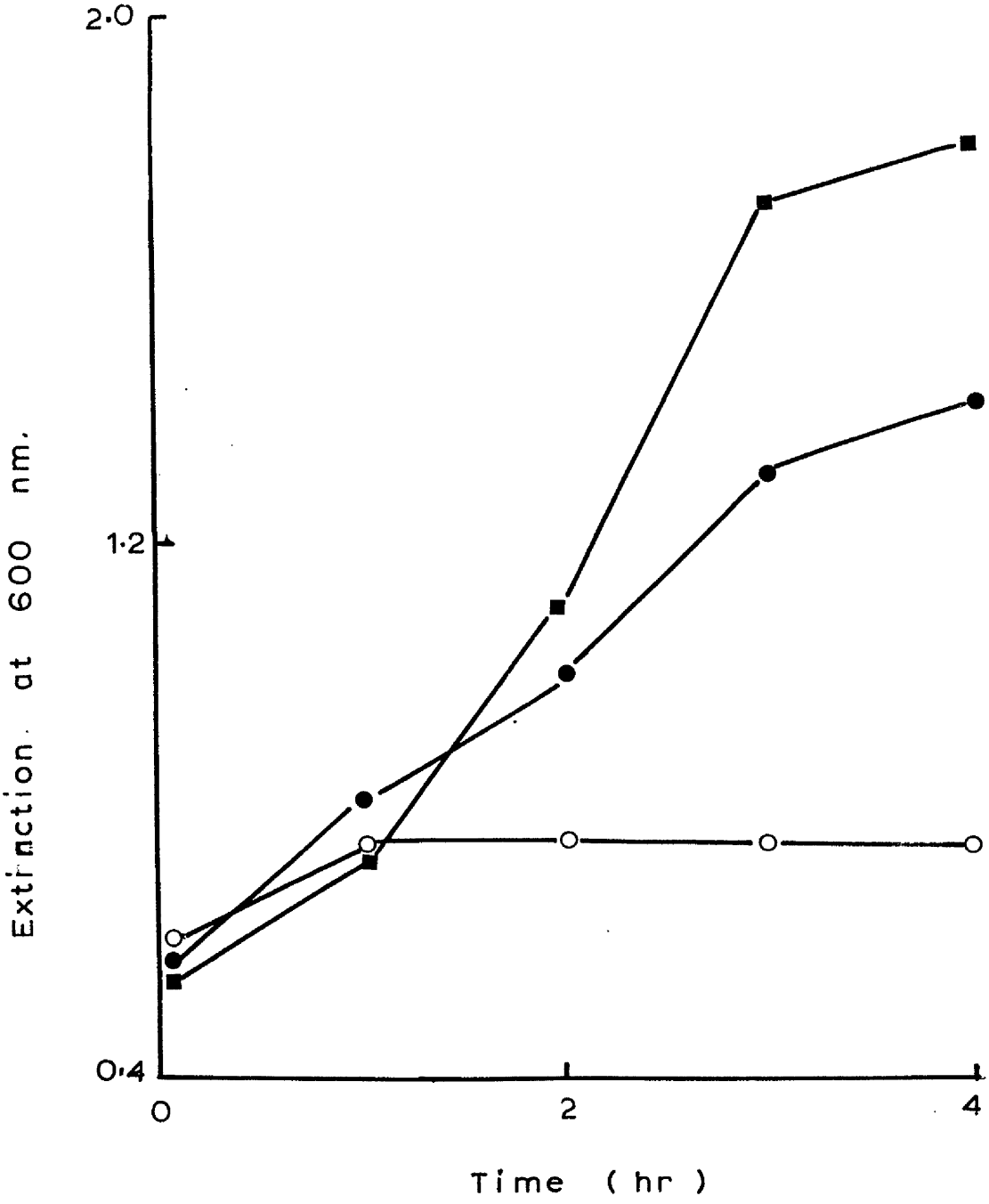


S(C) medium

S(N) medium

S(N) medium + L-glutamate (0.15% w/v)

A full description of the S(C) and S(N) media is given in the Materials and Methods section.


2·0
1·2
0·4
Extinction at 600 nm.
0
2
4
Time (hr)

growth. In keeping with this there was only a slight increase in cell density in the S(N) resuspension medium (Figure 7). However, it was anticipated that addition of L-glutamate to the S(N) medium would allow the cells to behave normally as the block in nitrogen assimilation in the mutant would be by-passed by the addition of exogenous L-glutamate. In keeping with this the cell density readings were similar to those obtained with wild-type (Figure 7). Surprisingly, however, a very low level of sporulation was obtained (Table 8). It could be concluded that strain glt-100 sporulated satisfactorily under conditions of carbon deprivation (S(C) medium), but not under conditions of nitrogen deprivation (S(N) medium + glutamate).

Sporulation behaviour of B. subtilis wild-type and strain glt-100

Percentage sporulation in various media

The lack of sporulation of the glt-100 strain in the S(N) + glutamate medium suggested that it would be useful to compare the sporulation capacity of the wild-type and mutant strains in other media. For this purpose 50 ml cultures in CH medium were resuspended in S(C) or S(N) media in which the carbon or nitrogen sources were varied. The cultures were incubated overnight and viability and heat-resistance determinations done the next day. The results are presented in Table 9. They show that strain glt-100 sporulated normally in the S(C) medium (ammonia as nitrogen source and L-glutamate as carbon source) but not in the S(N) medium (nitrate as nitrogen source and D-glucose as carbon source). This result together with the observation that addition of L-glutamate still did not allow sporulation to occur, had been noted earlier (Table 8). If glucose was omitted from the S(N) medium and L-glutamate used as carbon source in its place, then sporulation occurred normally. A similar result

TABLE 9

Sporulation behaviour of strain glt-100 in various resuspension media

Cultures (50 ml) growing in CH medium were each harvested and resuspended in an equal volume of one of the resuspension media given below. A full description of the S(C) and S(N) media is given in the Materials and Methods section. When L-glutamate and L-glutamine were added it was to a final concentration of 0.15% (w/v). In one case the S(N) medium was used with D-glucose (0.2% w/v) omitted and in another S(N) medium the glucose was replaced by citrate (0.2% w/v). The resuspension media were incubated overnight and total viable count and heat-resistance determinations were done the next day. Values are percentage sporulation expressed as number of heat-resistant colonies/total viable count x 100. Each value is the average of at least 2 determinations.

Resuspension Medium	Percentage sporulation	
	wild-type strain	strain glt-100
S(C) medium	35.0	72.0
S(C) medium + glucose	93.0	8.0
S(N) medium	40.0	< 0.1%
S(N) medium + glutamate	50.0	0.2
S(N) medium + glutamine	85.0	5.0
S(N) medium (glucose omitted) + glutamate	66.0	88.0
S(N) medium (glucose replaced by citrate) + glutamate	93.0	74.0

was obtained if glucose was replaced by citrate. It seemed that the presence of the easily metabolised carbon source glucose in the resuspension medium was the determining factor which suppressed sporulation in strain glt-100. Thus inclusion of glucose in the S(C) medium also suppressed sporulation in strain glt-100, but not in the wild type. Interestingly, the presence of L-glutamine instead of L-glutamate in the S(N) medium allowed a much greater degree of sporulation to occur in strain glt-100. It should also be noted that the S(N) medium + glutamate is essentially identical to the S(C) medium + glucose, except that nitrate serves as the main nitrogen source in the former and ammonia in the latter (see Materials and Methods section). This difference between nitrate and ammonia in the two media caused a marked difference in the sporulation capacity of glt-100 (8.0% for the S(C) + glucose and 0.2% for the S(N) medium + glutamate). It is concluded that strain glt-100, lacking GTS activity, is unable to sporulate in media containing a good carbon source such as glucose where sporulation would be expected to occur as a result of nitrogen starvation.

Influence on sporulation of strain glt-100 of addition of glucose at intervals during resuspension

The results presented in Table 9 showed that the inclusion of glucose in the resuspension medium prevented successful sporulation with strain glt-100. It was thought to be of interest to determine if addition of glucose at any time during the resuspension period would prevent the cells forming spores or whether the cells would escape the repression of sporulation by added glucose after a certain time. To investigate this the following experiment was performed. Two 50 ml

cultures of strain glt-100 were grown in CH medium. When the E_{600} reached 0.6 - 0.8 the cells from each culture were resuspended in equal volumes of S(N) + L-glutamate from which glucose was omitted. At time intervals 10 ml portions were removed from the S(N) + L-glutamate (no glucose) media and placed in a pre-warmed sterile 100 ml flask to which glucose had previously been added to give a final concentration of 0.2% (w/v). Each flask was shaken at 37°C. A final 10 ml in the original medium was transferred to a 100 ml flask and incubated as a control. The 10 ml cultures were all incubated overnight and the degree of sporulation determined the next day. The results (Table 10) show that addition of glucose at any time up to 2 hr after resuspension prevented the cells from forming spores. After this time the cells began to escape the effects of the added glucose. By 3 hr after resuspension it had essentially no effect and cells sporulated as well as the control cells incubated in the S(N) medium + glutamate with glucose omitted. It is concluded that the inhibitory effects of glucose are not restricted to the initiation events, but are effective during the first 2-3 hr of sporulation. This is on the assumption that sporulation is initiated immediately on resuspension in the S(N) + glutamate medium with glucose omitted. This is a reasonable assumption as this medium is deprived of both a good carbon and nitrogen source; L-glutamate serves as both carbon and nitrogen source in this medium if it is assumed that strain glt-100 lacking GTS activity cannot utilise the ammonia derived from the nitrate in the medium.

TABLE 10

Effect of addition of glucose at time intervals during resuspension on the subsequent sporulation of strain *glt*-100

The experimental procedure is described in the text. The control culture was resuspended in S(N) medium + L-glutamate (0.15% w/v) from which glucose was omitted. This medium was known to give successful sporulation (see Table 9). Percentage sporulation was determined after 24 hr incubation and is expressed as number of heat-resistant colonies/ml of culture.

Time (min) of addition of glucose after resuspension	Sporulation Heat-resistant colonies/ml of culture x 10^{-4}
15	4
30	1
60	6
90	1
120	6
150	16
180	1600
210	4200
240	1300
270	2800
Control (no glucose added)	14,800

DISCUSSION

In order to investigate any possible changes in specific activity of GNS and GTS during the onset of sporulation it was necessary first of all to develop satisfactory assay procedures for these two enzymes. Once this was done the assay procedures could then be tested under conditions where changes in specific activity might be expected to occur. Previous results using B. subtilis (Deuel et al., 1970; Rebello & Strauss, 1969) and other micro-organisms (Elmerich, 1972; Wu & Yuan, 1968; Pateman, 1969; Meers et al., 1970) had indicated that the activity of GNS and GTS varied depending on the availability and type of nitrogen source used during growth. Therefore, the assay procedures were tested during growth of B. subtilis under various conditions of nitrogen availability to ensure that the expected differences in specific activity were detectable. Only then would enzyme activity found during the onset of sporulation be acceptable as a realistic reflection of events.

The first experiments were concerned with determining the rate of growth of B. subtilis in a minimal salts medium supplemented with glucose as carbon source and various sources of nitrogen. Generally, many separate estimations of the growth rate were made for each combination of carbon and nitrogen source (Table 1). Considerable variation was often found for any one medium from one experiment to another and for this reason an average cell doubling time is presented in Table 1 for each medium together with extreme values in each case. The variations may have arisen because the cells grown overnight for each experiment may have been at different stages of growth when inoculated into fresh medium. Cells well into the stationary phase had a longer lag time when inoculated into fresh medium than cells still in

the exponential phase and this caused difficulties in deciding where to draw the line on the growth curve which in turn affected the calculation of the mean generation time.

B. subtilis grew well with ammonia as nitrogen source and the addition of an extra source of nitrogen in the form of either L-arginine, L-glutamate, L-glutamine or L-histidine promoted a slightly slower growth rate. This may have been due to the fact that both GNS and GTS activity were repressed by these additional nitrogen sources (Table 3) and so the ammonia in the medium could not perhaps be assimilated quite so rapidly. However, because of the variation in cell doubling time from one experiment to another it is difficult to assess these slight differences and they may only be a reflection of experimental variation. This is borne out by the fact that when L-glutamine replaced ammonia as nitrogen source the cell doubling times were very similar in each case (Table 1), yet L-glutamine as sole nitrogen source caused repression of both GNS and GTS activities compared to the activities in ammonia-grown cells (Table 3). Neither L-histidine nor nitrate would serve as good nitrogen sources for growth of B. subtilis. In the case of L-histidine this result is in agreement with that of Chasin & Magasanik (1968) who showed that it was caused by glucose repression of the enzymes required for L-histidine utilisation. It is known that nitrate reductase is inducible by nitrate in B. subtilis (Guespin-Michel, Piechaud & Schaeffer, 1970) and the slow growth of the organism on nitrate in the presence of glucose may be due to glucose repression of the nitrate reductase. Alternatively one of the enzymic steps, nitrate or nitrite reductase, which convert nitrate to ammonia may be rate-limiting. In Aspergillus nidulans these enzymes are repressed by ammonia which is more powerful than the induction by nitrate (Pateman & Cove, 1967). Thus the growth

rate of B. subtilis on nitrate may be limited by the rate of production of ammonia.

The method of ultrasonic disintegration of whole cells was chosen for preparation of cell-free extracts. This method gave an extract with a high protein content which was active in both GNS and GTS assays. X-press treatment for cell-free extract preparation gave lower protein values and little detectable activity in either assay. Although toluene treatment of whole cells was used by Rebello & Strauss (1969) to give preparations active in the GNS assay, in this work toluene was found to give a turbid cell preparation unsuitable for use, particularly in the GTS assay which monitored a fall in extinction at 340 nm.

All enzyme assays were taken from previously published procedures. The GNS assay was that described by Rebello & Strauss (1969) measuring the biosynthetic ability of the enzyme to form L-glutamine. These authors had previously shown that a tris buffer containing EDTA and β-mercaptoethanol (TME), both at 0.01 M, favoured stability of the enzyme. This buffer was used here during the preparation of cell-free extracts and experiments showed that GNS activity was stable in this buffer after storage at -20°C for 24 hours (Table 2). In certain Gram negative species a distinction has been drawn between the capacity of the enzyme to catalyse the γ-glutamyl transfer reaction (equation 1) and the biosynthetic capacity catalysed in vivo (equation 2) which can be conveniently assayed by hydroxamate formation when ammonia is replaced by hydroxylamine (equation 3).

1. L-glutamine + hydroxylamine $\longrightarrow$ γ-glutamylhydroxamate + ammonia

2. L-glutamate + ATP + ammonia ⟶ L-glutamine + ADP + Pi.

3. L-glutamate + ATP + hydroxylamine ⟶ γ-glutamylhydroxamate + ADP + Pi.

The adenylated form of GNS enzyme, formed during growth of E. coli or K. aerogenes in the presence of ammonia (see Introduction) is relatively inactive in the biosynthetic reaction (equations 2 and 3), while remaining fully active in the γ-GT assay (equation 1) (Kingdon et al., 1967; Bender et al., 1977). In an extract prepared from B. subtilis grown in glucose-ammonia medium a higher activity was detected in the biosynthetic assay than in the γ-GT assay. This agrees with results obtained from Aspergillus nidulans and Neurospora crassa where biosynthetic activities were higher than γ-GT activities in extracts prepared from cells grown under similar conditions (Pateman, 1969). In these organisms, as in B. subtilis, there is no evidence for two forms of the GNS enzyme. In Gram negative bacteria the adenylated form of the enzyme produced under conditions of growth with excess ammonia is relatively inactive in the biosynthetic assay. The greater activity of GNS from B. subtilis in the biosynthetic assay rather than the γ-GT assay supports previous work which has found no evidence for adenylation of the B. subtilis enzyme (see Introduction).

In the GNS biosynthetic assay ATP was found to be an essential co-factor for the reaction and in the GTS assay NADPH was a far superior co-factor than NADH (Table 2). Both these results are in agreement with previous work (Woolfolk et al., 1966; Tempest et al., 1970; Rebello & Strauss, 1969; Elmerich, 1972). In addition, no detectable glutamate dehydrogenase activity was found in an extract prepared from glucose-ammonia grown cells. The absence of glutamate dehydrogenase

activity in B. subtilis 168 was supported by the fact that the mutant strain, glt-100, which lacked GTS activity was unable to grow with ammonia as nitrogen source (Table 6). These observations were similar to those previously obtained by Elmerich (1972) using B. megaterium where glutamate dehydrogenase activity was also undetectable. In addition, Meers et al. (1970) had found little glutamate dehydrogenase activity in B. megaterium KM or in B. subtilis W23. The same authors did, however, find appreciable activity in B. subtilis var. niger and activity has also been reported in B. licheniformis (Meers & Pedersen, 1972) and B. thuringiensis (Borris & Aronson, 1969). It would seem that not all Bacillus spp. rely on the combined action of GTS and GNS to assimilate ammonia. Some possess glutamate dehydrogenase activity and so may have similar control mechanisms operating as Gram negative bacteria to regulate the operation of the three enzymes (see Introduction). Alternatively, the enzyme may only be used in a degradative capacity and not for the assimilation of ammonia (Meers & Pedersen, 1972).

With satisfactory assay procedures established for both GNS and GTS enzymes, their activity during the growth of B. subtilis with various sources of nitrogen was examined (Table 3). It was noted at an early stage that extracts prepared from separate cultures of cells grown in glucose-ammonia medium showed obvious variations in both GNS and GTS specific activity. For this reason enzyme activities were determined from as many separate cultures as possible for each growth condition and an average specific activity calculated. For example, twelve separate extracts were prepared in all from cells grown in the glucose-ammonia medium. Table 3 shows that the GNS assay gave extreme specific activities of 100 and 250 and the GTS assay extreme values of 650 and 800. In fact, these values show that GTS specific activity

was reasonably reproducible from glucose-ammonia grown cells, whereas GNS activity was less so. Cells from other media gave the opposite result. Thus glucose-L-glutamine grown cells gave extreme GTS specific activities of 200 and 500 whereas the GNS extreme values were 63 and 100. The reason for the variation in enzyme activity from identically grown cells is not obvious as the cells were all harvested during the mid-exponential phase of growth. It may reflect differences in stability of each enzyme to sonication during preparation of cell-free extracts. Perhaps differences in the degree of cooling during sonication may have caused differences in enzyme stability from one extract to another.

As a result of the obvious variation in enzyme specific activity from one cell extract to another it would seem necessary not to take small differences in average activities from extracts from different media as meaningful changes due to repression or derepression of enzyme synthesis. Nevertheless, some differences in average specific activity of GNS and GTS from one medium to another are large enough to justify certain conclusions. In general, the results presented in Table 3 are in agreement with previous work on GNS and GTS activities in Bacillus spp. However, as far as is known, there are no published reports combining assays for both enzymes and so a comparison has to be made from reports where only one enzyme activity was examined. It had been reported previously that GNS was derepressed by ammonia-limitation in B. subtilis i.e. a low activity was obtained from cells grown in glucose-ammonia minimal medium but a high activity given by cells grown in glucose-glutamate medium where glutamate was the sole nitrogen source (Deuel et al., 1970). This result was confirmed here. Elmerich (1972) reported that GTS in B. megaterium was repressed when L-glutamate served as sole nitrogen source. Again the values in Table 3 agree with this

observation, although it should be noted that difficulty was found in obtaining a satisfactory cell preparation from glucose-glutamate grown cells and for this reason only assays from one cell extract are included in Table 3. These results are also in agreement with those obtained from Gram negative species. Nitrogen limitation (using glutamate as sole nitrogen source) caused derepression of GNS in K. aerogenes and E. coli (Tempest et al., 1970; Woolfolk et al., 1966; Pateman, 1969; Miller & Stadtman, 1972; Wu & Yuan, 1968), but repressed GTS (Meers et al., 1970; Miller & Stadtman, 1972).

Use of L-arginine or L-glutamine as sole nitrogen source gave lower specific activities for GNS and GTS than those obtained with ammonia. This repression was most marked in the GTS activity from cells grown with L-arginine. It can be concluded that GTS is most susceptible to repression by L-glutamate and to a lesser extent by L-arginine and L-glutamine. The repression by arginine may be a consequence of its metabolism via L-glutamate. The repression of GNS by L-glutamine and L-arginine is in contrast to the derepression by L-glutamate, but is in agreement with results obtained in E. coli (Wu & Yuan, 1968; Pateman, 1969) and is discussed again below.

When L-arginine or L-glutamine was used as nitrogen source in combination with ammonia repression of both enzymes was obtained. In both cases the values were lower than those obtained when the amino acid was used alone. Enzyme activities from cells grown with glucose and ammonia plus L-glutamate were also repressed compared to ammonia grown cells and markedly different to the values obtained when L-glutamate was used alone. These results are similar to previous results using B. subtilis (Deuel et al., 1970; Rebello & Strauss, 1969) where

repression of GNS synthesis was found by L-glutamine, L-glutamate and L-arginine in combination with ammonia, but are here extended to include GTS as well. It should be noted that no obvious repression of either enzyme was obtained over and above the values with ammonia alone by the use of L-histidine in combination with ammonia. This result is in keeping with the lack of metabolism of L-histidine due to the catabolite repression of the histidine degrading enzymes by glucose in the medium (Chasin & Magasanik, 1968).

The use of nitrate as sole nitrogen source allowed marked depression of GNS activity together with a pronounced repression of GTS activity. This contrasts with the report by Pateman (1969) that E. coli, A. nidulans and N. crassa grown with nitrate as sole nitrogen source had GNS activities similar to those obtained in ammonia-grown cells. It was suggested in this report that glutamine repressed GNS synthesis in these organisms and that the high activity of GNS from cells grown with glutamate alone was due to the low concentration of ammonia in these cells which would limit the production of L-glutamine and so in turn derepress GNS synthesis. This hypothesis is substantiated by the observation that in E. coli and K. aerogenes the ratio of intracellular 2-oxoglutarate to L-glutamine is critical in determining the adenylation state and thus the biosynthetic activity of GNS (Senior, 1975). High glutamine levels enhance adenylation of GNS and so reduce its biosynthetic capacity (see Introduction). B. subtilis has a high level of GNS when grown on L-glutamate or nitrate as sole nitrogen source. In each case this can be explained by supposing that the cell has a low intracellular concentration of ammonia which limits glutamine production and so allows derepression of GNS. B. subtilis grows more slowly on

nitrate than ammonia which would suggest that the intracellular concentration of ammonia would be low as its production from nitrate would limit growth of the organism. L-arginine is ultimately degraded to L-glutamate, but the degradative pathway releases ammonia (Dagley & Nicholson, 1970). B. subtilis can grow rapidly using L-arginine as sole nitrogen source (Table 1), so ammonia production would not be limiting. This would explain why L-arginine has a strong repressive effect on GNS synthesis whereas L-glutamate alone does not. The pattern of GNS specific activities in Table 3 can also be explained on the supposition that ammonia, and not L-glutamine, represses GNS synthesis. Other work (Rebello & Strauss, 1969) favours L-glutamine as the repressing compound.

In summary, the results presented in Table 3 do not conflict with the hypothesis made previously (Rebello & Strauss, 1969; Pateman, 1969; Adler, Purich & Stadtman, 1975) that L-glutamine is able to repress GNS synthesis. The results also show that GTS synthesis is repressed by L-glutamate or compounds like L-arginine and L-glutamine which would be expected to give rise to L-glutamate in the cell. It is difficult to explain, however, why cells grown with nitrate as sole nitrogen source should have a markedly lower GTS activity than cells grown with ammonia. Under the hypothesis presented above cells growing with nitrate should have limiting production of ammonia which would in turn limit the production of L-glutamine and L-glutamate by GNS and GTS respectively. It would be expected then that GTS synthesis would be fully derepressed. The possibility is suggested by some of the values in Table 3 that there is an inverse relationship between the activities of the two enzymes, such that when one is high the other is low. This

does not hold in all instances though, as the values for cells grown with L-arginine show.

The determination of the enzyme levels in B. subtilis during growth established clearly that significant changes in specific activity were detectable. The results indicated that synthesis of each enzyme was subject to regulation. GNS synthesis was repressed by ammonia or L-glutamine (or both) and GTS synthesis was repressed by L-glutamate. These observations provided a firm base from which to examine the behaviour of the enzymes during the onset of sporulation. In addition the observed derepression of GNS synthesis when nitrate was used as nitrogen source during growth suggested that there might well be a correlation between increased GNS synthesis and the capacity of cells to sporulate. Shift-down of cells from a medium where ammonia was sole nitrogen source to one where nitrate was used induced sporulation in B. subtilis (Michel et al., 1968).

The method used for obtaining sporulation was the replacement procedure originally described by Sterlini & Mandelstam (1969). This involved the transfer of cells growing exponentially in a rich casein hydrolysate (CH) medium to a minimal salts medium containing an adequate supply of ammonia as nitrogen source, but only a poor carbon source in the form of L-glutamate (S(C) medium). It was assumed that sporulation would be initiated by deprivation of an adequate carbon source. This assumption was tested by including a good carbon source, glucose, in the resuspension medium. This caused the optical density of the culture to increase rapidly to a value well above that obtained in the normal medium (Table 4). In addition, heat-resistance determinations showed that sporulation was delayed by the inclusion of glucose in the

medium. It was concluded therefore that the S(C) resuspension medium initiated sporulation by carbon deprivation.

The original resuspension medium of Sterlini & Mandelstam (1969) was modified in an attempt to promote sporulation by nitrogen deprivation. To this end the ammonia in the medium was replaced by nitrate (a poor nitrogen source) and the L-glutamate was replaced by D-glucose (a good carbon source). Good sporulation was obtained in this medium (Table 4) and supplementation of the S(N) medium with an additional nitrogen source in the form of L-glutamate had a similar effect to glucose supplementation of the S(C) medium. Optical density of the culture increased rapidly to a level well above that attained by the normal S(N) medium and sporulation was again delayed (Table 4). These results led to the conclusion that sporulation could be initiated by resuspension of growing cells in either the S(C) or S(N) medium, but that initiation was achieved by carbon starvation in the former case and by nitrogen starvation in the latter case.

Cell-free extracts prepared from 50 ml portions of resuspension cultures in both S(C) and S(N) media were assayed for GNS and GTS activities at zero time and at 20 min and 40 min following initiation. For practical reasons it was only convenient to prepare and assay three extracts during one experiment. The times at 20 min and 40 min after time zero were arbitrarily chosen as times during which changes in enzyme specific activity might be expected to occur if they were involved in the earliest steps of sporulation. Again, considerable variation in specific activity of both enzymes was detected from one experiment to another and for this reason average values are tabulated in Table 5 together with the extreme values obtained. The results in

Table 5 show that there is no marked alteration in activity for either enzyme when the average values at each time interval are compared. It should be noted that when extreme values were obtained all 3 time intervals gave similar enzyme levels. Thus taking an average of values has not evened out differences between time intervals in individual experiments. Each separate experiment indicated little change in enzyme activities in either S(C) or S(N) media, at the time intervals studied.

These results were somewhat surprising in view of the fact that previous results using growing cells had suggested that growth limitation by the use of nitrate as nitrogen source caused derepression of GNS synthesis (Table 3). In addition, Rebello & Strauss (1969) using chemostat cultures of B. subtilis had shown that nitrogen limitation caused derepression and carbon limitation repression of GNS synthesis (see Introduction). Possible explanations for the results represented in Table 5 are that the times chosen to investigate the enzymes were inadequate to demonstrate a transient change in specific activity or that changes occurred after the 40 min period. If the enzymes were involved in the initiation events of sporulation the latter possibility can reasonably be excluded. It could be assumed that by 40 min after time zero the majority of cells were engaged in forming spores. The former possibility, that the times chosen missed a transient change in enzyme specific activity, is suggested by the observation of Rebello & Strauss (1969) that transition from a fast to a slow growth rate was accompanied by a brief increase in GNS specific activity (see Introduction). The results presented in Table 5 allow the tentative conclusion that prolonged changes in GNS or GTS specific activity do not accompany the initiation of sporulation by either carbon or nitrogen deprivation. The possibility of transient changes taking place are not excluded by these

experiments. Unfortunately, time did not allow a more thorough investigation of enzyme levels at closer intervals during sporulation.

Determination of the specific activities of GNS and GTS had not established any role for these enzymes during the onset of sporulation. For this reason it was decided to investigate the sporulation behaviour of a mutant strain, tentatively identified as lacking GTS activity. A similar approach, using both GNS and GTS defective strains of B. megaterium, had suggested a role for L-glutamine as the precursor of a sporulation repressor (see Introduction). Estimation of the in vitro activity of enzymes gives no indication of their activity in vivo which will be governed by substrate availability and the effects of allosteric activators and inhibitors. Therefore, although the results presented above suggested that an increase in enzyme protein was not a controlling influence during sporulation, they did not rule out the possibility that each enzyme played a role in the control of sporulation by their enzymic activity. If it was established that the strain glt-100 lacked GTS activity then the effect of this defect (which would not allow the further metabolism of L-glutamine derived from GNS activity) on sporulation could be assessed.

The defect in strain glt-100 was established by direct assay for GTS activity (Table 7) and by noting the growth response of the strain to various sources of carbon and nitrogen (Table 6). Previous results had shown that a GTS-negative strain of K. aerogenes was unable to grow with ammonia or glucosamine which is degraded to yield ammonia (Magasanik et al., 1974). A GTS-negative strain of B. subtilis was reported to require L-glutamate or L-aspartate for growth (Young & Wilson, 1975). The growth pattern of strain glt-100 was in keeping with these observations. Strain

glt-100 grew normally when L-glutamate or a compound which could be degraded to yield L-glutamate, such as L-glutamine, L-arginine, or L-proline, was supplied as nitrogen source. It would not grow with ammonia or glucosamine. The ability to grow with L-aspartate as nitrogen source meant that it could be distinguished from the glutamate requiring auxotrophs defective in one of the first three enzymes of the tricarboxylic acid cycle (see Introduction). An interesting observation was made when L-histidine was used as source of nitrogen. The strain glt-100 showed a consistently worse growth response than the wild-type with L-histidine as nitrogen source, yet it grew as well as the wild-type with the other sources of nitrogen and also when L-histidine was used as a carbon source with ammonia as nitrogen source (Table 6). L-histidine serves as a poor source of nitrogen in the presence of carbon compounds that are easily metabolised and so repress synthesis of the histidine degrading enzymes (Chasin & Magasanik, 1968). This is clearly shown in Table 6 where the wild-type grew only poorly with L-histidine and glucose or glycerol, but normally with citrate as carbon source. The mutant strain would not grow at all with L-histidine and glucose or glycerol and its response to citrate was poorer than the wild-type. This suggested that the defective GTS activity in the mutant was causing a greater repression of the L-histidine degrading enzymes than occurred in the wild-type. Lack of time prevented this possibility from being investigated, but a similar observation was made by Brenchley et al., (1973) who found that a GTS-negative strain of K. aerogenes was unable to grow in a glucose-histidine minimal medium. They explained their observation by saying that the lack of GTS activity would not allow depletion of glutamine and therefore no non-adenylated GNS would be available to allow transcription of the histidine degrading enzymes

(see Introduction). By analogy lack of GTS activity would allow glutamine accumulation in B. subtilis which apparently affected the rate of histidine metabolism by the cells.

No GTS activity was found in the cell-free extracts of strain glt-100 (Table 7). As only one extract was assayed for the results presented in Table 7 little significance can be attached to the differences in GNS activity between the wild-type and strain glt-100. It can be noted, however, that with both strains enzyme specific activities are very low in extracts prepared from cells grown in the rich casein hydrolysate (CH) medium. This is in agreement with Rebello & Strauss (1969) who found maximum repression of GNS synthesis in a casein hydrolysate medium supplemented with ammonia, L-glutamate and L-asparagine.

The results in Tables 6 and 7 established that strain glt-100 lacked GTS activity. In addition, the fact that this strain was unable to grow with ammonia as nitrogen source showed that B. subtilis 168 lacked a glutamate dehydrogenase that could be used to assimilate ammonia. This was in keeping with the negative result obtained earlier when cell extracts were assayed for this enzyme activity.

The effect on sporulation of the lack of GTS activity was investigated next. First the levels of GNS were investigated in the glt-100 strain in S(C) and S(N) resuspension media (Table 8). Just as in the wild-type (Table 5) variation in enzyme specific activity was found from one experiment to another, but individual cell extracts gave GNS values which showed little variation at time zero, 20 min and 40 min intervals after resuspension, although there was a suggestion of a slight fall in GNS activity in the S(C) medium with strain glt-100 which did not

occur in the wild-type. It is difficult to assess the significance of this observation because of the large variations found in specific activity from one experiment to another.

Strain glt-100 was unable to sporulate in the S(N) medium (Table 8) and this was expected because the only nitrogen source available, nitrate, could not be assimilated in this strain. In keeping with this the E_{600} of the culture in S(N) medium increased only slightly (Figure 7). It was anticipated that addition of L-glutamate, which by-passed the defect in GTS activity, would restore normal sporulation to the strain. Figure 7 shows that strain glt-100 behaved normally with regard to the increase in E_{600} in the S(N) + L-glutamate resuspension medium, but normal sporulation was not restored (Table 8). This observation was entirely unexpected and suggested the possibility that strain glt-100 was able to sporulate normally when initiated under conditions of carbon deprivation but was not able to do so under nitrogen deprivation. This possibility was investigated further by comparing the extent of sporulation of the wild-type and strain glt-100 in various resuspension media (Table 9). It was found that omission of glucose or its replacement by citrate in the S(N) medium + glutamate allowed normal sporulation in strain glt-100. This was firm evidence that the presence of an easily metabolisable carbon source was suppressing sporulation in the mutant strain. Similarly, addition of glucose to the S(C) medium suppressed sporulation. This would be expected because in this medium only L-glutamate could serve as a nitrogen source as the ammonia present would not be assimilated. Addition of glucose would ensure sporulation was induced by nitrogen deprivation. In the case of the S(C) medium alone sporulation would normally be expected to occur by carbon deprivation. With strain glt-100, though, glutamate would have to serve as both carbon

and nitrogen source as ammonia would not be utilisable. Presumably therefore sporulation in strain glt-100 could be triggered by either means in the S(C) medium. This situation also applies to the S(N) medium + glutamate where glucose is omitted. In this case the nitrate in the medium would not be utilised. The S(N) + glutamate with glucose omitted and the S(C) medium are identical except the former has nitrate present and the latter ammonia, but as the mutant can utilize neither of these it must rely on the glutamate present to serve as sole source of carbon or nitrogen. In both media strain glt-100 sporulates normally, presumably because sporulation can be initiated by carbon starvation.

The extent of sporulation achieved by strain glt-100 in the various resuspension shown in Table 9 suggested strongly that the strain would only sporulate under conditions where the cells were starved of an adequate carbon source. Conditions of nitrogen deprivation alone in the presence of a good carbon source did not allow sporulation to occur. Besides this primary observation, two other points are worth discussion. First, Table 9 shows that strain glt-100 sporulated to a greater extent in the S(C) + glucose medium (8%) than in the S(N) + glutamate medium (0.2%). Second, use of L-glutamine as nitrogen source in the S(N) medium instead of L-glutamate allowed a greater degree of sporulation to occur (5% instead of 0.2%).

The above observations are best discussed with regard to the hypothesis that L-glutamine plays a major role in the repression of sporulation (Elmerich & Aubert, 1972). Both Elmerich & Aubert (1972) and Rebello & Strauss (1969) have suggested that depletion of L-glutamine acts as the primary trigger for the initiation of sporulation. The experimental basis for this hypothesis is given in detail in the Intro-

duction. Elmerich & Aubert (1972, 1973) noted that a GTS-negative strain of B. megaterium was unable to sporulate even in the presence of L-glutamate. They suggested the reason for this was that the strain would accumulate L-glutamine which was a repressor of sporulation. The results in Table 9 can only partly be reconciled with this hypothesis. It could be argued that strain glt-100 was unable to sporulate properly under conditions of adequate carbon supply (S(N) medium) because L-glutamine accumulated. Where an adequate carbon source was not supplied (S(C) medium) it would not have accumulated because it had to be metabolised to provide carbon and energy. However, better sporulation was obtained in the S(N) medium + L-glutamine (5.0%) than in the S(N) medium + L-glutamate (0.2%). This does not fit with the hypothesis linking L-glutamine to the sporulation repressor(s). Similarly, better sporulation was obtained in the S(C) medium + glucose (8%) than in the S(N) medium + glutamate (0.2%). These media are identical except that ammonia is supplied as the main nitrogen source in the former and nitrate in the latter medium. However, as the mutant is unable to assimilate either of these two sources of nitrogen, L-glutamate supplies the only source of utilisable nitrogen to the cells. Therefore it would be assumed that as strain glt-100 can utilise only glucose and L-glutamate from each medium, the level of sporulation attained should be similar. Apparently though, the presence of ammonia allows a greater degree of sporulation to occur. It can be assumed that the ammonia would be readily converted to L-glutamine by the GNS in the cells. It therefore follows that L-glutamine would be more likely to accumulate in the S(C) medium + glucose, yet greater sporulation was achieved in this medium.

Some of the results presented in Table 9 therefore did not fit readily into the hypothesis that L-glutamine is responsible for the repression of sporulation. It was clear, though, that the defective GTS in strain glt-100 was preventing the cells from sporulating in certain circumstances. From the data available it seemed that these circumstances involved conditions where an adequate source of carbon ensured that sporulation would have to be initiated by nitrogen deprivation. It would follow from this that a defective GTS activity prevents the initiation of sporulation under conditions of nitrogen limitation.

The specific activities of GNS and GTS obtained under various conditions of growth (Table 3) did not conflict with the hypothesis that L-glutamine repressed GNS synthesis. Under growth conditions where L-glutamine would not be expected to accumulate (glutamate or nitrate as nitrogen source) the GNS specific activity was found to be high. The specific activities of GNS and GTS during the onset of sporulation (Table 5 and 8) do not support the idea that initiation to sporulation is accompanied by a depletion of L-glutamine. No derepression of GNS synthesis was observed in the wild-type at least at 20 and 40 min into sporulation. If L-glutamine was depleted during sporulation it should have been indicated by a depression of GNS synthesis. In addition, L-glutamine would be expected to accumulate in strain glt-100 because of the block in GTS activity. In keeping with this a slight fall in GNS activity was noticeable at 20 min and 40 min after initiation to sporulation in the S(C) medium which was not apparent in the wild-type (compare GNS activities in the S(C) medium in Table 5 and 8). This fall in GNS activity may have been due to repression of GNS synthesis by the

accumulated L-glutamine, yet strain glt-100 sporulated normally in this medium. These observations, taken together with the levels of sporulation obtained in strain glt-100 in the various resuspension media (Table 9), lend little support to the idea that depletion of L-glutamine acts as a trigger for sporulation.

It was noted earlier that strain glt-100 was not able to utilise L-histidine as a nitrogen source as effectively as the wild-type strain in the presence of glucose or glycerol (Table 6). By analogy with a similar observation with a GTS-negative strain of K. aerogenes it was suggested that this may be because the L-histidine degrading enzymes were more severely repressed in this strain by the presence of easily metabolised carbon sources such as glucose. It may not be coincidental that strain glt-100 is also unable to sporulate in the presence of a good carbon source. It was noted that strain glt-100 grew normally when L-histidine was supplied as carbon source with ammonia as nitrogen source (Table 6). This is a condition essentially of carbon deprivation as L-histidine is metabolised slowly. Likewise the mutant sporulates normally under conditions of carbon deprivation (S(C) medium). This obvious similarity in behaviour of the mutant under these two conditions (normal growth and sporulation under conditions of carbon starvation and abnormal behaviour during nitrogen starvation) suggests there might be a common link between enzyme induction and initiation of sporulation under conditions of nitrogen limitation in B. subtilis.

An experiment was done to determine for how long during sporulation strain glt-100 was inhibited by the presence of glucose. The results in Table 10 show that glucose addition to the S(N) medium + L-glutamate (glucose omitted) during the initial two hours of sporulation prevented

successful sporulation. With glucose omitted from the S(N) + glutamate medium strain glt-100 could only use L-glutamate as source of both carbon and nitrogen. Sporulation therefore could presumably be triggered by either carbon or nitrogen deprivation. Addition of glucose to this medium would only allow a condition of nitrogen starvation to occur. The fact that glucose was able to suppress sporulation for 2-3 hours was somewhat surprising. Later addition of glucose would mean that it was effectively suppressing sporulation in cells which must have embarked on the sporulation process. This would suggest that stages later than the initiating events of sporulation are also required to be triggered by either carbon or nitrogen starvation. Addition of glucose would prevent them being triggered by carbon deprivation and the mutation affecting GTS activity would prevent them being triggered by nitrogen deprivation. It is known that the sporulation process can be halted and growth restored by the addition of nutrients up to about stage III (Frehel & Ryter, 1969) which must mean that sporulation loci remain susceptible to repression by catabolites up to this stage. It can be concluded that whatever it is that prevents sporulation in the GTS deficient strain operates not just at early initiating events, but at later events along the sequential pathway of sporulation. It is possible therefore that the mechanism that initiates sporulation during nitrogen starvation may not be a unique event, but may operate in the same way at regular intervals up to stage III to trigger the various stages in the process.

In summary, the results obtained with strain glt-100 suggest that GTS activity may be essential for sporulation to occur under conditions of nitrogen starvation. The lack of this activity in the mutant at first sight supports the hypothesis that L-glutamine is a repressor of

sporulation as this compound would be expected to accumulate in the mutant. However, some observations obtained with wild-type and strain glt-100 do not support this hypothesis. These can be summarised as follows. Strain glt-100 sporulates normally in conditions of carbon starvation, although there is an apparent repression of GNS synthesis at the onset of sporulation presumably caused by L-glutamine accumulation. Strain glt-100 is able to sporulate more satisfactorily under nitrogen deprivation if L-glutamine is supplied instead of L-glutamate or ammonia is supplied instead of nitrate. These conditions do not favour depletion of L-glutamine as a trigger for sporulation. In addition, depletion of L-glutamine would be expected to allow depression of GNS synthesis at the onset of sporulation, yet this did not occur even in the wild-type. These observations therefore conflict with the idea that L-glutamine depletion triggers sporulation, although the obvious reason why strain glt-100 is unable to sporulate under certain circumstances in that its lack of GTS activity will cause L-glutamine to accumulate. It is difficult to assess what other reason could cause the lack of sporulation. If L-glutamine accumulation caused the lack of sporulation there is no obvious reason why L-glutamine should not accumulate under all circumstances and prevent sporulation. The results obtained with strain glt-100 suggest that the GTS defect has no effect on sporulation induced by carbon deprivation. This may mean that sporulation is triggered by alternative routes during carbon or nitrogen deprivation. In conclusion, the determination of enzyme specific activities of GNS and GTS during sporulation have suggested in either case that an alteration in enzyme synthesis plays no role during the onset of sporulation. The sporulation behaviour of the GTS-negative strain glt-100 does suggest that GTS has an enzymic role during the onset of

sporulation induced by nitrogen starvation. Lack of this enzyme presumably altered the metabolism of the cells under these circumstances so as to prevent sporulation. This may have been caused by an accumulation of L-glutamine, although some of the results were not consistent with this idea.

Conclusions

The main conclusions arising from this work are as follows. Both GNS and GTS are susceptible to repression and derepression during growth of B. subtilis. Variation in specific activity of both enzymes can be brought about by alteration of the nitrogen source in the medium. The pattern of enzyme activities under various growth conditions suggest that GNS is repressed by ammonia or L-glutamine and GTS is repressed by L-glutamate. Glutamate dehydrogenase activity, capable of assimilating ammonia, is absent from B. subtilis 168. There is no significant variation in GNS or GTS specific activity during the onset of sporulation induced by either carbon or nitrogen starvation. A strain lacking GTS activity is unable to sporulate under conditions where sporulation is presumed to be initiated by starvation of an adequate nitrogen supply. The sporulation behaviour of this mutant strain and the activity of GNS during the onset of sporulation do not altogether support the idea that depletion of L-glutamine is involved in the onset of sporulation. The factor which prevents the GTS-negative mutant from sporulating under conditions of nitrogen deprivation is active during the initial 2-3 hour of the process.

Future work

If this project were to be continued, attention would be given to

the following points. GNS and GTS enzyme activities would be determined at closer intervals and for a longer period during resuspension to try and ensure that transient alterations in enzyme activity could be detected. The stage during sporulation at which cells of strain glt-100 become blocked under conditions of nitrogen deprivation could be determined by examination of cells in the electron microscope and by noting the appearance of specific sporulation markers such as exo-protease and alkaline phosphatase activity. It would be of interest to note the rate of histidase synthesis during conditions of nitrogen deprivation which prevent sporulation in strain glt-100. Histidase is the first enzyme involved in the degradation of L-histidine to L-glutamate and there was the possibility that the poor growth of strain glt-100 on minimal media when L-histidine was used as a nitrogen source was due to lack of synthesis of the histidine degrading enzymes. The isolation of a mutant strain lacking GNS synthesis would allow an assessment of the role of this enzyme during sporulation in B. subtilis 168. In addition, a double mutant could be constructed (using genetic exchange by transformation) which contained mutations affecting both GNS and GTS activity. This would provide additional information on the role of these enzymes during sporulation.

REFERENCES

Adler, S.P., Purich, D. and Stadtman, E.R. (1975) Cascade control of Escherichia coli glutamine synthetase: Properties of the P_{II} regulatory protein and the uridylyltransferase-uridylyl-removing enzyme. Journal of Biological Chemistry, 250, 6264-6272.

Aronson, A.I. and Fitz-James, P. (1976) Structure and morphogenesis of the bacterial spore coat. Bacteriological Reviews, 40, 360-402.

Balassa, G. (1976) An exosporium-like outer layer in Bacillus subtilis spores. Nature, 263, 53-54.

Bender, R.A., Janssen, K.A., Resnick, A.D., Blumenberg, M., Foor, F. and Magasanik, B. (1977) Biochemical parameters of glutamine synthetase from Klebsiella aerogenes. Journal of Bacteriology, 129, 1001-1009.

Berberich, R., Kaback, M. and Freese, E. (1968) D-amino acids as inducers of L-alanine dehydrogenase in Bacillus subtilis. Journal of Biological Chemistry, 243, 1006-1011.

Bernlohr, R.W., Haddox, M.K. and Goldberg, N.D. (1974) Cyclic guanosine -3'5'-monophosphate in Escherichia coli and Bacillus licheniformis. Journal of Biological Chemistry, 249, 4329-4331.

Borris, D.P. and Aronson, J.N. (1969) Short Communication : Relationship of L-alanine and L-glutamate dehydrogenase of Bacillus thuringiensis. Biochimica et Biophysica Acta, 191, 716-718.

Brehm, S.P., Staal, S.P. and Hoch, J.A. (1973) Phenotypes of pleiotropic-negative sporulation mutants of Bacillus subtilis. Journal of Bacteriology, 115, 1063-1070.

Brenchley, J.E. and Magasanik, B. (1974) Mutants of Klebsiella aerogenes lacking glutamate dehydrogenase. Journal of Bacteriology, 117, 544-550.

Brenchley, J.E., Prival, M.J. and Magasanik, B. (1973) Regulation of the synthesis of enzymes responsible for glutamate formation in Klebsiella aerogenes. Journal of Biological Chemistry, 248, 6122-6128.

Carls, R.A. and Hanson, R.S. (1971) Isolation and characterization of tricarboxylic acid cycle mutants of Bacillus subtilis. Journal of Bacteriology, 106, 848-855.

Chambliss, G.H. and Legault-Demare, L. (1977) Functional modifications of the translational system in Bacillus subtilis during sporulation. Journal of Bacteriology, 132, 13-22.

Chasin, L.A. and Magasanik, B. (1968) Induction and repression of the histidine-degrading enzymes of Bacillus subtilis. Journal of Biological Chemistry, 243, 5165-5178.

Coote, J.G. (1972) Sporulation in Bacillus subtilis. Characterization of oligosporogenous mutants and comparison of their phenotypes with those of asporogenous mutants. Journal of General Microbiology, 71, 1-15.

Coote, J.G. (1972) Sporulation in Bacillus subtilis. Genetic analysis of oligosporogenous mutants. Journal of General Microbiology, 71, 17-27.

Coote, J.G. (1974) Comparative studies on induction of sporulation and synthesis of inducible enzymes in Bacillus subtilis. Journal of Bacteriology, 120, 1102-1108.

Coote, J.G. and Mandelstam, J. (1973) Use of constructed double mutants for determining the temporal order of expression of sporulation genes in Bacillus subtilis. Journal of Bacteriology, 114, 1254-1263.

Dagley, S.E. and Nicholson, D.E. (1970) In An Introduction to Metabolic Pathways. pp. 77. Oxford: Blackwell Scientific Publication.

Dawes, I.W. and Mandelstam, J. (1970) Sporulation of Bacillus subtilis in continuous culture. Journal of Bacteriology, 103, 529-535.

Dawes, I.W. and Thornley, J.H.M. (1970) Sporulation in Bacillus subtilis. Theoretical and experimental studies in continuous culture systems. Journal of General Microbiology, 62, 49-66.

de Crombrugghe, B., Perlman, R.L., Varmus, H.E. and Pastan, I. (1969) Regulation of inducible enzyme synthesis in Escherichia coli by cyclic adenosine 3'-5'-monophosphate. Journal of Biological Chemistry, 244, 5828-5835.

Dean, D.R., Hoch, J.A. and Aronson, A.I. (1977) Alteration of the Bacillus subtilis glutamine synthetase results in overproduction of the enzyme. Journal of Bacteriology, 131, 981-987.

Deuel, T.F., Ginsburg, A., Yeh, J., Shelton, E. and Stadtman, E.R. (1970) Bacillus subtilis glutamine synthetase purification and physical characterisation. Journal of Biological Chemistry, 245, 5195-5205.

Deuel, T.F. and Prusiner, S. (1974) Regulation of glutamine synthetase from Bacillus subtilis by divalent cations, feed-back inhibition and L-glutamine. Journal of Biological Chemistry, 249, 257-264.

Elliott, W.H. (1955) Glutamine synthesis. In Methods in Enzymology, Vol. 2. pp. 337-342. Edited by Colowick, S.P. and Kaplan, N.O. New York. Academic Press Inc.

Elmerich, C. (1972) Le cycle du glutamate, point de depart du metabolisme de l'azote, chez Bacillus megaterium. European Journal of Biochemistry, 26, 216-224.

Elmerich, C. and Aubert, J.P. (1971) Synthesis of glutamate by a glutamine: 2 oxo-glutarate aminotransferase (NADP oxidoreductase) in Bacillus megaterium. Biochemical and Biophysical Research Communications, 42, 371-376.

Elmerich, C. and Aubert, J.P. (1972) Role of glutamine synthetase in the repression of bacterial sporulation. Biochemical and Biophysical Research Communications, 46, 892-897.

Elmerich, C. and Aubert, J.P. (1973) Involvement of the biosynthetic pathway of purine nucleotides in the repression of bacterial sporulation. Biochemical and Biophysical Research Communications, 55, 837-842.

Elmerich, C. and Aubert, J.P. (1975) Involvement of glutamine synthetase and the purine nucleotide pathway in repression of bacterial sporulation. In Spores, Vol. VI pp. 385-390. Ed. by Gerhardt, P., Costilow, R.N. and Sadoff, H.L. Washington, D.C.: American Society for Microbiology.

Englesberg, E. and Wilcox, G. (1974) Regulation Positive Control. Annual Review of Genetics, 8, 219-242.

Folin, O. and Ciocalteau, V. (1927) On tyrosine and tryptophane determinations in protein. Journal of Biological Chemistry, 73, 627-650.

Foor, F., Janssen, K.A. and Magasanik, B. (1972) Regulation of synthesis of glutamine synthetase by adenylylated glutamine synthetase. Proceedings of the National Academy of Sciences of the U.S.A., 72, 4844-4848.

Fortnagel, P. and Freese, E. (1968) Analysis of sporulation mutants II. Mutants blocks in the citric acid cycle. Journal of Bacteriology, 95, 1431-1438.

Freese, E. (1972) Sporulation of Bacilli, a model of cellular differentiation. Current Topics in Developmental Biology, 7, 85-124.

Freese, E., Ichikawa, T., Oh, Y.K., Freese, E.B. and Prasad, C. (1974) Deficiencies or excesses of metabolites interfering with differentiation. Proceedings of the National Academy of Sciences of the U.S.A. 71, 4188-4193.

Freese, E., Oh, Y.K., Freese, E.B., Diesterhaft, M.D. and Prasad, C. (1972) Suppression of sporulation of Bacillus subtilis. In: Spores Vol. V. pp. 212-221. Ed. by Halvorson, H.O., Hanson, R. and Campbell, L.L. Washington, D.C.: American Society for Microbiology.

Freese, E., Park, S.W. and Cashel, M. (1964) The developmental significance of alanine dehydrogenase in Bacillus subtilis. Proceedings of the National Academy of Sciences of the U.S.A., 51, 1164-1172.

Frehel, C. and Ryter, A. (1969) Reversibilite de la Sporulation chez B. subtilis. Annales de l'Institut Pasteur, 117, 297-311.

Friedrich, B. and Magasanik, B. (1977) Urease of Klebsiella aerogenes. Control of its synthesis by glutamine synthetase. Journal of Bacteriology, 131, 446-452.

Gallant, J. and Margason, G. (1972) Amino acid control of messenger ribonucleic acid synthesis in Bacillus subtilis. Journal of Biological Chemistry, 247, 2289-2294.

Gancedo, C. and Holzer, H. (1968) Enzymatic inactivation of glutamine synthetase in Enterobacteriaceae. European Journal of Biochemistry, 4, 190-192.

Gould, G.W. and Dring, G.J. (1975) Heat resistance of bacterial endospores and concept of an expanded osmoregulatory cortex. Nature, 258, 402-405.

Gould, G.W. and Hurst, A. (1969) In the Bacteria Spore, Chapter 11. London : Academic Press.

Grelet, N. (1957) Growth limitation and sporulation. Journal of Applied Bacteriology, 20, 315-324.

Guespin-Michel, J.F., Piechaud, M. and Schaeffer, P. (1970) Constitutivite' vis-avis du nitrate de la nitrate-reductase chez les mutants asporogenes precoces de Bacillus subtilis. Annales de l'Institut Pasteur, 119, 711-718.

Halvorson, H.O. (1965) Sequential expression of biochemical events during intracellular differentiation. Symposia of Society of General Microbiology, 15, 343-368.

Hitchins, A.D. and Slepecky, R.A. (1969) Bacterial sporulation as a modified procaryotic cell division. Nature, 223, 804-807.

Hoch, J.A. and Mathews, J. (1972) Genetic studies in Bacillus subtilis. In Spores Vol. V. pp. 113-116. Ed. by Halvorson, H.O., Hanson, R. and Campbell, L.L. Washington, D.C.: American Society for Microbiology.

Eranueli, D., Piggot, P.J. and Mandelstam, J. Statistical estimate of the total number of operons specific for Bacillus subtilis sporulation. Journal of Bacteriology, 119, 684-690.

Ide, M. (1971) Adenyl cyclase of bacteria. Archives of Biochemistry and Biophysics, 144, 262-268.

Ito, J. and Spizizen, J. (1973) Genetic studies of catabolite repression insensitive sporulation mutants of Bacillus subtilis. Colloques Internationaux Centre Nat. Rech. Sci., 227, 81-82.

Janssen, K.A. and Magasanik, B. (1977) Glutamine synthetase of Klebsiella aerogenes. Genetic and physiological properties of mutants in the adenylylation system. Journal of Bacteriology, 129, 993-1000.

Keynan, A. (1972) Cryptobiosis. A review of the mechanisms of the ametabolic state in bacterial spores. In Spores Vol. V. pp. 355-362. Ed. by Halvorson, H.O., Hanson, R. and Campbell, L.L. Washington, D.C.: American Society for Microbiology.

Kingdon, H.S., Shapiro, B.M. and Stadtman, E.R. (1967) Regulation of glutamine synthetase & ATP : glutamine synthetase adenylyltransferase, an enzyme that catalyses alterations in the regulatory properties of glutamine synthetase. Proceedings of the National Academy of Sciences of the U.S.A., 58, 1703-1710.

Kornberg, A.J., Spudich, A., Nelson, D.L. and Deutscher, M.P. (1968) Origin of proteins in sporulation. Annual Review of Biochemistry, 37, 51-78.

Leighton, T.J. and Doi, R.H. (1973) The relationship of serine protease activity to RNA polymerase modification and sporulation in Bacillus subtilis. Journal of Molecular Biology, 76, 103-122.

Leighton, T.J., Freese, P.K., Doi, R.H., Warren, R.A.J. and Kelln, R.A. (1972) Initiation of sporulation in Bacillus subtilis. Requirement for

serine protease activity and ribonucleic acid polymerase modification. In Spores, Vol. V. pp. 238-246. Ed. by Halvorson, H.O., Hanson, R. and Campbell, L.L. Washington, D.C. : American Society for Microbiology.

Lepesant-Kejzlarova, J., Lepesant, J.A., Walle, J., Billault, A. and Dedonder, R. (1975) Revision of the linkage map of Bacillus subtilis 168 indications for circularity of the chromosome. Journal of Bacteriology, 121, 823-834.

Levisohn, S. and Aronson, A.J. (1967) Regulation of extracellular protease production in Bacillus cereus. Journal of Bacteriology, 93, 1023-1030.

Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) Protein measurement with the Folin phenol reagent. Journal of Biological Chemistry, 193, 265-275.

Magasanik, B. (1961) Catabolite repression. Cold Spring Harbor Symposia Quantitative Biology, 26, 249-256.

Magasanik, B. (1977) Regulation of bacterial nitrogen assimilation by glutamine synthetase. Trends in Biochemical Sciences, 2, 9-12.

Magasanik, B., Prival, M.J., Brenchley, J.E., Tyler, B.M., Deleo, A.B., Streicher, S.L., Bender, R.A. and Paris, C.G. (1974) Glutamine synthetase as a regulator of enzyme synthesis. Current Topics in Cellular Regulation, 8, 119-138.

Mandelstam, J. (1976) The Leeuwenhoek lecture, 1975 : bacterial sporulation : a problem in the biochemistry and genetics of a primitive developmental system. Proceedings of the Royal Society of London (Biology), 193, 89-106.

Mandelstam, J. and Higgs, S.A. (1974) Induction of sporulation during synchronized chromosome replication in Bacillus subtilis. Journal of Bacteriology, 120, 38-42.

Mandelstam, J., Kay, D. and Hranueli, D.H. (1975) Biochemistry and morphology of stage I in sporulation of Bacillus subtilis cells. In Spores VI. pp. 181-186. Ed. by Gerhardt, P., Costilow, R.N. and Sadoff, H.L. Washington, D.C. : American Society for Microbiology.

Mandelstam, J. and McQuillen, K. (1973) In Biochemistry of Bacterial Growth. Chapter 9. Oxford, London, Edinburgh and Melbourne : Blackwell Scientific Publications.

Mandelstam, J., Sterlini, J.M. and Kay, D. (1971) Sporulation in Bacillus subtilis. Effect of medium on the form of chromosome replication and on initiation to sporulation in Bacillus subtilis. Biochemical Journal, 125, 635-641.

Mandelstam, J. and Waites, W.M. (1968) Sporulation in Bacillus subtilis. The role of exoprotease. Biochemical Journal, 109, 793-801.

Meers, J.L. and Pedersen, L.K. (1972) Nitrogen assimilation by Bacillus licheniformis organisms growing in chemostat cultures. Journal of General Microbiology, 70, 277-286.

Meers, J.L., Tempest, D.W. and Brown, C.M. (1970) Glutamine (amide): 2-oxoglutarate amino transferase oxido-reductase (NADP), an enzyme involved in the synthesis of glutamate by some bacteria. Journal of General Microbiology, 64, 187-194.

Michel, P.J.F., Cami, B. and Schaeffer, P. (1968) Selection de mutants Bacillus subtilis. Bloques au debut de la sporulation. Annales de -'Institut Pasteur, 114, 11-20.

Miller, R.E. and Stadtman, E.R. (1972) Glutamate synthase from Escherichia coli. An iron-sulfide flavoprotein. Journal of Biological Chemistry, 247, 7407-7419.

Murrell, W.G. (1967) The biochemistry of the bacterial endospore. Advances in Microbial Physiology, 1, 133-251.

Murrell, W.G. (1969) Chemical composition of spores and spore structures. In The Bacterial Spore. pp.215-273. Ed. by Gould, G.W. and Hurst, A. London and New York _ Academic Press Inc.

Neidhardt, F.C. and Magasanik, B. (1957) Reversal of the glucose inhibition of histidase biosynthesis in Aerobacter aerogenes. Journal of Bacteriology, 73, 253-259.

Nelson, D.L. and Kornberg, A. (1970) Biochemical studies of bacterial sporulation and germination. Journal of Biological Chemistry, 245, 1128-1136.

Oh, Y.K., Freese, E.B. and Freese, E. (1973) Abnormal septation and inhibition of sporulation by accumulation of L-α-glycerophosphate in Bacillus subtilis mutants. Journal of Bacteriology, 113, 1034-1045.

Ohye, D.F. and Murrell, W.G. (1962) Formation and structure of the spore of Bacillus coagulans. Journal of Cell Biology, 14, 111-

Pastan, I. and Adhya, S. (1976) Cyclic adenosine 5'-monophosphate in Escherichia coli. Bacteriological Reviews, 40, 527-551.

Pateman, J.A. (1969) Regulation of synthesis of glutamate dehydrogenase and glutamine synthetase in micro-organisms. Biochemical Journal, 115, 769-775.

Pateman, J.A. and Cove, D.J. (1967) Regulation of nitrate reduction in Aspergillus nidulans. Nature, 215, 1234-1237.

Perlman, R.L. and Pastan, I. (1969) Pleiotropic deficiency of carbohydrate utilization in an adenyl cyclase deficient mutant of Escherichia coli. Biochemical and Biophysical Research Communications, 37, 151-157.

Piggot, P.J. (1973) Mapping of asporogenous mutations of Bacillus subtilis. A minimum estimate of the number of sporulation operons. Journal of Bacteriology, 114, 1241-1253.

Piggot, P.J. and Coote, J.G. (1976) Genetic aspects of bacterial endospore formation. Bacteriological Reviews, 40, 908-962.

Prival, M.J. and Magasanik, B. (1971) Resistance to catabolite repression of histidase and proline oxidase during nitrogen-limited growth of Klebsiella aerogenes. Journal of Biological Chemistry, 246, 6288-6296.

Prival, M.J., Brenchley, J.E. and Magasanik, B. (1973) Glutamine synthetase and the regulation of histidase formation in Klebsiella aerogenes. Journal of Biological Chemistry, 248, 4334-4344.

Prusiner, S. (1975) Regulation of glutaminase levels in Escherichia coli. Journal of Bacteriology, 123, 992-999.

Prusiner, S., Miller, R.E. and Valentine, R.C. (1972) Adenosine 3'5'-cyclic monophosphate control of the enzymes of glutamine metabolism in Escherichia coli. Proceedings of the National Academy of Sciences of the U.S.A., 69, 2922-2926.

Rebello, J.L. and Strauss, N. (1969) Regulation of synthesis of glutamine synthetase in Bacillus subtilis. Journal of Bacteriology, 98, 683-688.

Resnick, A.D. and Magasanik, B. (1976) L-asparaginase of Klebsiella aerogenes: Activation of its synthesis by glutamine synthetase. Journal of Biological Chemistry, 251, 2722-2728.

Reysset, G. and Aubert, J.P. (1975) Relationship between sporulation and mutation impairing glutamine synthetase in Bacillus megaterium. Biochemical and Biophysical Research Communications, 65, 1237-1241.

Reznikoff, W.S. (1972) The operon revisited. Annual Review of Genetics, 6, 133-152.

Rhaese, H.J., Dichtelmullur, H., Grade, R. and Groscurth, R. (1975) High phosphorylated nucleotides involved in regulation of sporulation in Bacillus subtilis. In Spores VI. pp. 335-340. Ed. by Gerhardt, P., Costilow, R.N. and Stadoff, H.L. Washington, D.C.: American Society for Microbiology.

Rhaese, H-J, Hoch, J.A. and Groscurth, R. (1977) Studies on the control of development. Isolation of Bacillus subtilis mutants blocked early in sporulation and defective in synthesis of highly phosphorylated nucleotides. (Differentiation/initiation of sporulation/regulatory nucleotides/vegetative and sporulation type ribosomes/adenosine 3' (2')-triphosphate 5'-triphosphate synthetase. Proceedings of the National Academy of Sciences of the U.S.A., 74, 1125-1129.

Ryter, A. (1965) Etude morphologique de la sporulation de Bacillus subtilis. Annales de l'Institute Pasteur, 108, 40-60.

Schaeffer, P. (1969) Sporulation and the production of antibiotics, exoenzymes, and exotoxins. Bacteriological Reviews, 33, 48-71.

Schaeffer, P., Millet, J. and Aubert, J-P. (1965) Catabolic repression of bacterial sporulation. Proceedings of the National Academy of Sciences of the U.S.A., 54, 704-711.

Schutt, H. and Holzer, H. (1972) Biological function of the ammonia-induced inactivation of glutamine synthetase in Escherichia coli. European Journal of Biochemistry, 26, 68-72.

Senior, P.J. (1975) Regulation of nitrogen metabolism in Escherichia coli and Klebsiella aerogenes : Studies with the continuous-culture technique. Journal of Bacteriology, 123, 407-418.

Smith, G.R. and Magasanik, B. (1971) Nature and self-regulated synthesis of the repression of the hut operons in Salmonella typhimurium. Proceedings of the National Academy of Sciences of the U.S.A. 68, 1493-1497.

Smith, G.R., Halpern, Y.S. and Magasanik, B. (1971) Genetic and metabolic control of enzymes responsible for histidine degradation in Salmonella typhimurium. Journal of Biological Chemistry, 246, 3320-3329.

Stadtman, E.R., Ginsburg, A., Ciardi, J.E., Yen, J., Henning, S.B. and Shapiro, B.M. (1970) Multiple molecular forms of glutamine synthetase produced by enzyme catalyzed adenylylation and deadenylylation reactions. Advances in Enzyme Regulation, 8, 99-118.

Sterlini, J.M. and Mandelstam, J. (1969) Commitment to sporulation in Bacillus subtilis and its relationship to development of Actinomycin resistance. Biochemical Journal, 113, 29-37.

Streicher, S.L., Bender, R.A. and Magasanik, B. (1975) Genetic control of glutamine synthetase in Klebsiella aerogenes. Journal of Bacteriology, 121, 320-331.

Tempest, D.W., Meers, J.L. and Brown, C.M. (1970) Short communications: Synthesis of glutamate in Aerobacter aerogenes by a hitherto unknown route. Biochemical Journal, 117, 405-407.

Tipper, J. and Gauthier, J.J. (1972) Structure of the bacterial endospore. In Spores Vol. V. pp. 3-12. Ed. by Halvorson, H.O., Hanson, R. and Campbell, L.L. Washington, D.C. : American Society for Microbiology.

Tubb, R.S. (1974) Glutamine synthetase and ammonium regulation of nitrogenase synthesis in Klebsiella. Nature, 251, 481-485.

Tyler, B., Deleo, A.B. and Magasanik, B. (1974) Activation of transcription of hut DNA by glutamine synthetase. Proceedings of the National Academy of Sciences of the U.S.A., 71, 225-229.

Warren, S.C. (1968) Sporulation in Bacillus subtilis. Biochemical changes. Biochemical Journal, 109, 811-818.

Waites, W.M., Kay, D., Dawes, I.W., Wood, D.A., Warren, S.C. and Mandelstam, J. (1970). Sporulation in Bacillus subtilis. Correlation of biochemical events with morphological changes in asporogenous mutants. Biochemical Journal, 118, 667-675.

Weber, G. (1961) In Biochemists Handbook. pp. 81. Edited by Long, C. London: E. and F.N. Spon. Ltd.

Woolfolk, C.A., Shapiro, B. and Stadtman, E.R. (1966) Regulation of glutamine synthetase. I. Purification and properties of glutamine synthetase from Escherichia coli. Archives of Biochemistry and Biophysics, 116, 177-192.

Wu, C. and Yuan, L.H. (1968) Regulation of synthesis of glutamine synthetase in Escherichia coli. Journal of General Microbiology, 51, 57-65.

Young, M. (1976) Use of temperature-sensitive mutants to study gene expression during sporulation in Bacillus subtilis. Journal of Bacteriology, 126, 928-936.

Young, E.I. and Fitz-James, P.C. (1959) Chemical and morphological studies of bacterial spore formation. I. Formation of spores in Bacillus cereus. Journal of Biophysical and Biochemical Cytology, 6, 467-

Young, F.E. and Wilson, G.A. (1975) Chromosomal maps of Bacillus subtilis. In Spores Vol. VI. pp. 596-614. Ed. by Gerhardt, P., Costilow, R.N. and Sadoff, H.L. Washington, D.C. : American Society for Microbiology.