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edited by

A. H. ROSE

School of Biological Sciences
Bath University
England

J. GARETH MORRIS
Department of Botany and Microbiology
University College Wales
Aberystwyth

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Contributors

ANTHONY F. CACCIAPUOTI *Department of Microbiology and Immunology, University of Oregon Health Sciences Center, Portland, Oregon 97201, U.S.A.*

W. FORD DOOLITTLE *Department of Biochemistry, Dalhousie University, Halifax, Nova Scotia, Canada*

PAUL G. LYSKO *Department of Microbiology and Immunology, University of Oregon Health Sciences Center, Portland, Oregon 97201, U.S.A.*

J. MANDELSTAM *Microbiology Unit, Department of Biochemistry, University of Oxford, OX1 3QU, U.K.*

STEPHEN A. MORSE *Department of Microbiology and Immunology, University of Oregon Health Sciences Center, Portland, Oregon 97201, U.S.A.*

TAIJI NAKAE *Department of Parasitology, Tokai University School of Medicine, Isehara, Kanagawa-ken, Japan*

HIROSHI NIKAIDO *Department of Microbiology and Immunology, University of California, Berkeley, California 94720, U.S.A.*

M. YOUNG *Department of Botany and Microbiology, University College of Wales, Aberystwyth SY23 3DA, Dyfed, U.K.*

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Contents

The Cyanobacterial Genome, its Expression, and the Control of that Expression by W. FORD DOOLITTLE

I. Introduction 2
A. Preamble 2
B. What cyanobacteria are 3
C. Why they are of interest 4
II. Cyanobacterial anatomy 5
A. Sheaths, walls and membranes 5
B. Phycobiliproteins and phycobilisomes 6
C. Cellular inclusions 9
D. Differentiated cells 11
E. Morphology, taxonomy and phylogeny 11
III. Cyanobacterial metabolism 14
A. Photoautotrophic metabolism 14
B. Endogenous metabolism 16
C. Photoheterotrophy and facultative chemoheterotrophy . . . 17
D. Controls on metabolism 18
E. The biochemical basis of obligate photoautorophy 19
IV. The cyanobacterial genome 20
A. "Chromosome" structure 20
B. Chromosomal DNA: base composition and base modification . . 21
C. Chromosomal DNA: sequence complexity 22
D. Plasmid DNAs 24
E. DNA synthesis 26
V. Cyanobacterial ribonucleic acids synthesis, processing and sequence characterization 32
A. RNA polymerase 32
B. Ribosomal RNA 34
C. Transfer RNA 39
D. Conditionally-stable RNAs of unknown function 40
E. Unstable (presumed messenger) RNA 41
VI. Ribosomes and their functions 43
A. Ribosome structure 43
B. Ribosome function 44
VII. Control of gene expression 45
A. General remarks: an expression of faith 45
B. Effects of light on net macromolecular synthesis 48
C. Effects of light intensity and colour ("quantity" and "quality") on components of the photosynthetic apparatus 55
D. Effects of carbon source 60
E. Effects of nitrogen source 66
F. Effects of sources of phosphate, sulphate and iron 78
G. Effects of anaerobiosis and oxygen 79

H. Effects of amino acids 79
I. Concluding remark 81
VIII. Genetics 81
A. Introductory remarks 81
B. "Mating" 82
C. Transformation 83
D. Transduction 84
E. Cyanobacterial genetic maps 85
IX. Prospects 86
X. Acknowledgements 88
References 88

Early Events During Bacterial Endospore Formation by M. YOUNG and J. MANDELSTAM

I. Introduction. 104
II. Morphological changes during sporulation 105
III. Biochemical changes during stages 0 to II 110
A. Categories of events 111
B. Marker events associated with stages 0 and I 113
C. Marker events associated with stage II 117
D. Later events 119
IV. Changes in transcription and translation apparatus 119
A. Transcription 119
B. Translation 124
C. Summary 127
V. Genetics and regulation of spore formation 127
A. Estimates of molecular complexity 127
B. Methods and nomenclature 129
C. Number of sporulation-specific genetic loci 131
D. Pleiotropy, dependent sequence and phenotypic exclusion sporulation mutants 132
E. Genetic loci concerned with stages 0 to II 134
VI. Induction of sporulation 142
A. Population probability factor and the hunt for the repressor . . 142
B. Glutamine synthetase 144
C. Highly phosphorylated nucleotides 145
D. The effect of purine starvation 146
E. Summary of biochemistry of induction 147
F. Requirement for DNA replication 148
VII. Compartmentation and division of labour 151
VIII. Conclusion 153
IX. Acknowledgements 154
References 154

The Outer Membrane of Gram-negative Bacteria by HIROSHI NIKAIDO and TAIJI NAKAE

I. Introduction 164
II. Preparation and composition 164
A. Preparation 164
B. Composition 166
III. Structure 168
A. Phospholipids 168
B. Lipopolysaccharides (LPS) 168
C. Proteins 171
D. Supramolecular organization of the components 181
IV. Functions of the outer membrane 187
A. "Cell surface" functions 187
B. Multiplicity of diffusion pathways 189
C. Barrier properties against hydrophobic molecules 192
D. Non-specific permeability toward small, hydrophilic molecules . . 196
E. Specific diffusion mechanisms 216
F. Penetration of macromolecules 218
G. Other functions 219
H. The structure of the outer membrane revisited 221
V. Assembly of the outer membrane 228
A. Biosynthesis of lipopolysaccharides 228
B. Biosynthesis of proteins 231
C. Assembly and growth of the outer membrane 233
VI. Acknowledgements 239
References 240

Physiology of *Neisseria gonorrhoeae* by STEPHEN A. MORSE, ANTHONY F. CACCIAPUOTI and PAUL G. LYSKO

I. Introduction 252
II. Taxonomy and ultrastructure 253
III. Colonial types of *Neisseria gonorrhoeae* 254
IV. Cell division and growth patterns 258
V. The cell envelope 260
A. Capsule 260
B. Outer membrane 261
C. Peptidoglycan composition 264
D. Pili 265
E. Lipopolysaccharide 266
VI. Peptidoglycan turnover and synthesis 268
VII. Autolysis 268
A. Whole-cell studies 268
B. Mechanism of autolysis 269
C. Characteristics of the autolysin 272
D. *In vivo* significance 274

VIII. Carbon dioxide requirement 274
IX. Carbohydrate metabolism 276
A. General 276
B. Glucose transport 276
C. Glucose metabolism 277
D. Pentose synthesis 282
E. Lactate and pyruvate metabolism 283
F. Acetate metabolism 284
X. Terminal electron transport 286
XI. Inorganic nitrogen and amino-acid metabolism 290
A. Inorganic nitrogen metabolism 290
B. Amino-acid metabolism 290
C. Miscellaneous enzymes 293
XII. Nucleotide precursors 294
A. Pyrimidines 294
B. Purines 296
XIII. Lipid composition and metabolism 299
A. Phospholipids and whole-cell fatty acids 299
B. Lipase and phospholipase activity 305
C. Inhibitory activity of phospholipids and fatty acids on growth . . 307
D. Lipid A 309
E. Steroids 310
XIV. Inorganic ions 311
XV. Acknowledgements 313
References 314

Author index 325

Subject index 347

The Cyanobacterial Genome, its Expression, and the Control of that Expression

W. FORD DOOLITTLE

Department of Biochemistry, Dalhousie University, Halifax, Nova Scotia, Canada

I. Introduction 2
A. Preamble 2
B. What cyanobacteria are 3
C. Why they are of interest 4
II. Cyanobacterial anatomy 5
A. Sheaths, walls and membranes 5
B. Phycobiliproteins and phycobilisomes 6
C. Cellular inclusions 9
D. Differentiated cells 11
E. Morphology, taxonomy and phylogeny 11
III. Carbon and energy metabolism 14
A. Photoautotrophic metabolism 14
B. Endogenous metabolism 16
C. Photoheterotrophy and facultative chemoheterotrophy. 17
D. Controls on metabolism 18
E. The biochemical basis of obligate photoautotrophy 19
IV. The cyanobacterial genome 20
A. "Chromosome" structure 20
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C. Chromosomal DNA: sequence complexity 22
D. Plasmid DNAs 24
E. DNA synthesis 26
V. Cyanobacterial ribonucleic acids synthesis, processing and sequence characterization. 32
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B. Ribosome function 44
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B. Effects of light on net macromolecular synthesis 48
C. Effects of light intensity and colour ("quantity" and "quality") on components of the photosynthetic apparatus 55
D. Effects of carbon source 60
E. Effects of nitrogen source 66
F. Effects of sources of phosphate, sulphate and iron 78
G. Effects of anaerobiosis and oxygen 79
H. Effects of amino acids 79
I. Concluding remark 81
VIII. Genetics 81
A. Introductory remarks 81
B. "Mating" 82
C. Transformation 83
D. Transduction 84
E. Cyanobacterial genetic maps 85
IX. Prospects 86
X. Acknowledgements 88
References 88

I. Introduction

A. PREAMBLE

I have two purposes in writing this review. The first is to convince molecular biologists, many of whom are aware only vaguely of the existence, and not at all of the nature, of cyanobacteria ("blue-green algae"), that this ancient and diverse group of organisms should be of interest to them. The second is to persuade cyanobacteriologists that blue-greens have begun to acquire a truly "molecular" biology of their own, and that fertile application of molecular biological and genetic techniques could now be made in fields of more traditional cyanobacteriological concern; physiology, metabolism and ecology.

The bulk of this review concerns the cyanobacterial genome, the machineries of its expression, and the ways in which that expression appears to be controlled. Fear that the cyanobacterial literature is becoming "over-reviewed" underlies my decision to restrict my topic so narrowly. A number of excellent articles of broad or limited scope have

TABLE 1. Reviews of the cyanobacteriological literature 1973–1978.

Bogorad (1975)	phycobiliproteins, chromatic adaptation
Carr and Bradley (1973)	differentiation
Carr and Whitton (1973)	book; many aspects
Delaney *et al.* (1976a)	genetics
Fogg *et al.* (1973)	book; many aspects
Gantt (1975)	phycobilisomes
Glazer (1976)	phycobiliproteins
Glazer (1977)	phycobiliproteins
Haselkorn (1978)	heterocysts, N_2-fixation
Knoll (1977)	fossil cyanobacteria
ÓCarra and ÓhEocha (1976)	phycobiliproteins
Padan and Shilo (1973)	cyanophages
Sherman and Brown (1978)	cyanophages
Smith and Hoare (1977)	autotrophy, metabolism
Stanier (1977)	photosynthesis, phylogenetic status
Stanier and Cohen-Bazire (1977)	morphology, development, metabolism
Stewart (1976)	*Anabaena cylindrica*
Stewart (1977a)	storage products, N_2-fixation, symbioses
Stewart (1977b)	nitrogen fixation
Stewart *et al.* (1975)	nitrogen fixation, metabolism
Stewart *et al.* (1977)	nitrogen fixation, metabolism
Walsby (1975)	gas vesicles
Walsby (1977a)	gas vesicles
Whittenbury and Kelly (1977)	autotrophy
Wolk (1973)	all aspects
Wolk (1975)	differentiation

appeared in the five years since the publication of two entire volumes devoted to the cyanobacteria (Table 1). None save that of Carr (1973) has dwelt at length on the subjects covered here, and my biases are different from those of Carr.

B. WHAT CYANOBACTERIA ARE

Oxygen-evolving photosynthetic prokaryotes existed unnamed for at least two, and possibly more than three, billion years and played perhaps *the* most important role in preparing the earth for the evolution of "higher" forms (Schopf, 1974, 1975; Margulis *et al.*, 1976; Knoll and Barghoorn, 1977; Knoll, 1977). The controversy about whether such organisms should be called blue-green *algae* or blue-green *bacteria* may seem in this context a matter of some inconsequence (Stanier, 1977; Brock, 1973; Stewart, 1977a). Each author should state his prejudices

and mine are these. Blue-greens are unquestionably prokaryotes. More specifically they are photosynthetic *bacteria* which can be distinguished from other (green and purple) photosynthetic bacteria by their photosynthetic machinery and biochemistry (Stanier, 1977). In this last respect, they more closely resemble the chloroplasts of eukaryotic algae (especially rhodophytes and cryptomonads). The resultant taxonomic ambiguity is neatly resolved by the endosymbiont hypothesis; rhodophytan and cryptomonad chloroplasts (and possibly others) descend from endosymbiotic, once free-living blue-greens. In spite of early suggestions concerning both the prokaryotic nature of blue-greens (Cohn, 1853) and the blue-green ancestry of chloroplasts (Mereschowksy, 1905), none of this was very obvious before the distinguishing characters of prokaryotes and eukaryotes had been properly defined (Stanier and van Niel, 1962) and the tools for examining their fine structure (principally the electron microscope) were widely available (Drews, 1973; Echlin and Morris, 1965). It is thus understandable that blue-greens have until recently been the wards of botanists and phycologists, and have been considered by them a class or division (Cyanophyceae, or Myxophyceae) of the algae (Fritsch, 1944), all the rest of which are eukaryotes. In this review I shall call them cyanobacteria or occasionally blue-greens, regarding them, as does Margulis (1974), as a phylum of the kingdom Monera (Prokaryota). I shall refer to the rest of the prokaryotes as "bacteria" for convenience and not to imply a true dichotomy in the kingdom.

C. WHY THEY ARE OF INTEREST

Cyanobacteria are of molecular biological interest for a number of independent reasons, each sufficient to justify their more intensive investigation:

(1) *Evolutionary position.* Cyanobacteria are phylogenetically remote from the best studied bacteria, and phylogenetically close to chloroplasts of at least some eukaryotic algae (Bonen and Doolittle, 1975, 1976; Zablen *et al.*, 1975). Comparative biochemical and molecular sequence studies thus have relevance in both prokaryotic and eukaryotic domains.

(2) *Autotrophic physiology.* Cyanobacteria require, in addition to inorganic nutrients, only CO_2 for carbon and light for energy, and markedly prefer these to organic sources of carbon and energy. Gene expression is regulated in response to light, CO_2 and inorganic nutrients, and seldom

in response to organic substances. This pattern of response is quite different from that shown by heterotrophic bacteria, from which almost all of our knowledge of genetic regulation in prokaryotes derives. Genetic regulatory responses to light and nitrogen availability are remarkably complex and pleiotropic in cyanobacteria, and have no obvious analogue at all in well-studied heterotrophs.

(3) *Differentiation.* Selected cells in the filaments of nitrogen-fixing blue-greens give rise to heterocysts whose differentiation is terminal, and both temporally and spatially controlled. In these respects, heterocyst formation provides a good general model for cellular differentiation.

(4) *Ecology.* There is probably more information on ecological variables governing distribution and population dynamics for cyanobacteria than for any other group of prokaryotes (see, for instance, Keating, 1977, 1978, and early work reviewed in Fogg *et al.*, 1973, and by Brock, 1973). An integration of such information with molecular biological data concerning, for instance, the role of light and anaerobiosis in controlling cyanobacterial gene expression or the biological function of cyanobacterial plasmids, seems feasible. Blue-greens may represent the most promising group for the development of a comprehensive and multi-faceted "molecular ecology".

(5) *Photosynthesis and nitrogen fixation.* Although methods for genetic analysis in cyanobacteria are yet inadequate, there seems no reason why they cannot be developed so as to permit a sophisticated and comprehensive biochemical genetic dissection of oxygenic photosynthesis, something which eukaryotic systems have so far failed to provide. Increasing facility in genetic analysis should also allow us to manipulate nitrogen fixation in the many naturally-important symbioses in which cyanobacteria are involved, and to construct free-living cyanobacterial strains which, using only solar energy, fix N_2 or evolve H_2 in significant quantity.

II. Cyanobacterial Anatomy

A. SHEATHS, WALLS AND MEMBRANES

Vegetative cyanobacterial cells are in most fundamental respects similar to those of other bacteria, especially Gram-negative bacteria. In this and the next two subsections I will briefly describe features which are largely peculiar to cyanobacteria and/or are of particular "molecular biological" interest.

Although they may occasionally stain Gram-positive (Drews, 1973), cyanobacteria have cell walls similar in structure to those of Gram-negative bacteria (Frank *et al.*, 1962; Allen, 1968b). The cell membrane is surrounded by a peptidoglycan layer which is in turn enclosed in a protein- and lipopolysaccharide-containing membrane (Wolk, 1973; Weckesser *et al.*, 1974). The major membrane protein is comparable in molecular weight to that similarly isolated from a great number of Gram-negative bacteria (Golecki, 1977) although the major lipopolysaccharide component (O-antigen) differs from its Gram-negative homologues in a number of ways (Katz *et al.*, 1977). Cyanobacteria are usually sensitive to lysozyme (especially in the presence of EDTA, e.g. Lindsey *et al.*, 1971; Doolittle, 1972), to penicillin, which can be used in auxotrophic and other mutant selections (e.g. Herdman and Carr, 1972; Herdman *et al.*, 1973; Stevens *et al.*, 1975; Singer and Doolittle, 1975b; Currier *et al.*, 1977), and even to lytic enzymes elaborated by *Bdellovibrio bacteriovorus* (Burnham *et al.*, 1976).

Beyond the cell wall are polysaccharide-containing "mucilages", "investments" or "sheaths" of various degrees of structural definition (Wolk, 1973; Fogg *et al.*, 1973; Dunn and Wolk, 1970; Martin and Wyatt, 1974). Sheath characteristics can determine the positioning of daughter cells after division and, although environmentally variable, have been employed in making taxonomic distinctions. (Sheath material may copurify with nucleic acids and can be mistaken by the unwary for "satellite" DNA in cesium-chloride gradients: Kaye *et al.*, 1967; Edelman *et al.*, 1967.)

Cyanobacterial photosynthetic functions are conducted in and on flattened, membranous sacs (thylakoids) which may or may not be topologically continuous with the cell membrane (Stanier and Cohen-Bazire, 1977; Lang and Whitton, 1973; Allen, 1968a, b; Jost, 1965). Most components of the photosynthetic apparatus (e.g. chlorophyll, carotenoids, photosynthetic electron-carriers) are located within the thylakoids (Krogmann, 1973). However, *phycobiliproteins* appear to be outside, although closely attached to, these lamellar structures. These proteins are of considerable "molecular biological" interest, and I shall discuss their properties at some length.

B. PHYCOBILIPROTEINS AND PHYCOBILISOMES

Phycobiliproteins are photosynthetic accessory pigments; chromophore-bearing proteins which trap light-energy of wavelength between 500 and

650 nm and transfer it (primarily) to chlorophyll *a* (P680) at the reaction centre of photosystem II (Fig. 1; see Bogorad, 1975; Gantt, 1975; ÓCarra and ÓhEocha, 1976; Glazer, 1976, 1977 for reviews). Most of the energy captured by blue-greens is harvested by these "antenna" pigments, which comprise up to 20 per cent of the dry weight (40 per cent of the total soluble proteins) of the cell (Myers and Kratz, 1955; Bennett and Bogorad, 1971).

All cyanobacterial biliproteins are, under reasonable *in vitro* conditions, trimers $(\alpha\beta)_3$ or hexamers $(\alpha\beta)_6$ of α and β apo-protein subunits (molecular weights 14–20,000 daltons), each with covalently attached prosthetic chromophores. These latter are open chain tetrapyrolles (bilins), most commonly (but not always; Bryant *et al.*, 1976) phycocyanobilin and phycoerythrobilin. All cyanobacteria appear to contain small amounts of the biliproteins allophycocyanin (APC) and allophycocyanin-B (APC-B), along with very much larger amounts of phycocyanin (PC). Many cyanobacteria also contain a second major biliprotein, phycoerythrin (PE). PC is intensely blue and PE very red; the relative abundances of these two pigments in the cell determine not only its light-capturing abilities but its sensible colour.

The various spectral classes of biliproteins (PE, PC, APC, APC-B) can be defined by the amino acid sequences of their α and β subunits and by the number and types of attached chromophores. The classes are immunologically distinct: antisera against PC will not precipitate PE or APC subunits, although they will precipitate all other PC subunits, regardless of source (cyanobacterial, rhodophytan or cryptomonad; Glazer *et al.*, 1971; Cohen-Bazire *et al.*, 1977). Nevertheless, all α-type subunits appear to show amino acid sequence homology at their amino termini and bilin-attachment sites; there is similar conservatism among β subunits (Williams and Glazer, 1978; Bryant *et al.*, 1978; Glazer, 1977). The β subunit of *Anacystis nidulans* PC has been fully sequenced (Friedenreich *et al.*, 1978), and efforts at X-ray crystallographic analysis of its higher-order structure have begun (Hackert *et al.*, 1977).

Two types of intracellular localization of phycobiliproteins are found in cyanobacteria. The simplest and rarest is that exhibited by *Gloeobacter violaceus* (Rippka *et al.*, 1974). Here the phycobiliproteins appear as a continuous, electron-dense, cortical layer (80 nm thick) immediately beneath the cell membrane. (This cyanobacterium is unique in lacking thylakoids. Other, thylakoid-containing strains may however show similar diffuse phycobiliprotein organization; Stanier and Cohen-Bazire,

1977). More typically, phycobiliproteins are almost entirely bundled into disc-shaped (40 nm diameter) *phycobilisomes*, which are attached in regular array along the thylakoid membrane surface (Edwards and Gantt, 1971; Gray and Gantt, 1974; Gray *et al.*, 1973; Wildman and Bowen, 1974).

Phycobilisomes are fascinating organelles. A model for their structure and function has been presented by Gantt and her collaborators (Gantt, 1975; Gantt *et al.*, 1976; Glazer, 1977). In this model (Fig. 1), a hemisphere of APC and APC-B, presumed to be the site of thylakoid membrane attachment, is surrounded by PC subunits which are in turn covered by PE subunits (in those organisms containing this biliprotein). Such a structure is consistent with phycobilisome dissociation studies and

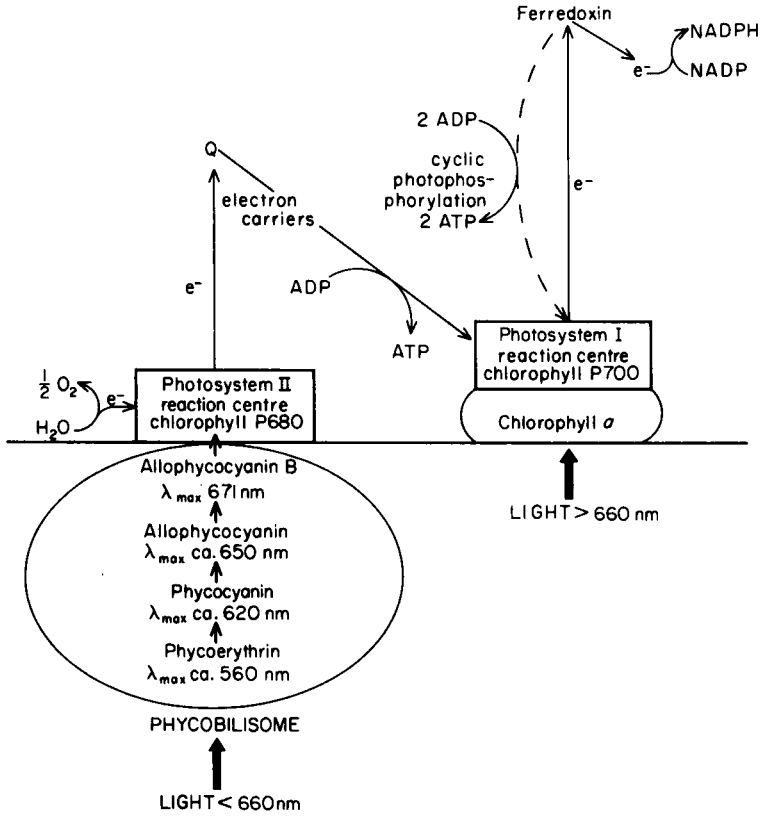

FIG. 1. Light reactions in cyanobacterial photosynthesis.

with spectroscopic data which, in isolated phycobilisomes (Ley *et al.*, 1977), show energy to be transferred sequentially (by "resonance"; Forster, 1960) from PE to PC to APC to APC-B. Such transfer requires close and ordered arrangement of components and can be 80 to 90 per cent efficient (Gantt, 1975). Recently, Tandeau de Marsac and Cohen-Bazire (1977) have shown that cyanobacterial phycobilisomes contain, in addition to phycobiliproteins, four to nine colourless polypeptides which together comprise some 15 per cent of total phycobilisomal protein. It will be very interesting to know the role of these in assembly and function.

Light "quality" (colour) and "quantity" (intensity) regulate cellular phycobilisome number and composition (relative levels of PC and PE). These phenomena ("complementary chromatic adaptation" and "intensity adaptation") offer a wealth of problems for biophysicists, membrane biologists and biochemical geneticists, and are touched on below (Section VIIC).

C. CELLULAR INCLUSIONS

1. *Glycogen Granules, Polyphosphate Granules and Carboxysomes*

Cyanobacterial vegetative cells contain a number of particulate inclusion bodies, and it is now possible to say something about the molecular structure and function of each of them. (1) Glycogen granules ("α granules"; Chao and Bowen, 1971; Weber and Wöber, 1975) store excess photosynthate for use as carbon and energy source during darkness or CO_2-deprivation (Lehmann and Wöber, 1976). (2) Polyphosphate bodies ("metachromatic granules"; Sicko-Goad *et al.*, 1975; Stewart, 1977a), spherical aggregates of high-molecular weight linear polyphosphates, serve as phosphate stores and represent potential, although quite possibly not actual, sources of high-energy phosphate. (3) "Polyhedral bodies" contain much or all of the cyanobacterial ribulose-1,5-diphosphate carboxylase (RUDP carboxylase; the key enzyme in dark CO_2-fixation via the Calvin cycle). They are now, on that basis, considered analogous to the "carboxysomes" of certain other autotrophs (Stewart and Codd, 1975; Codd and Stewart, 1976a). Two further structures, gas vesicles and cyanophycin granules ("structured granules") are of especial molecular biological interest, and merit individual discussion.

2. *Gas Vesicles*

Gas vesicles occur in a variety of planktonic (free-floating aquatic) cyanobacterial and bacterial species, where they appear to confer buoyancy and the ability to assume optimal position in the water column (see reviews by Walsby, 1972, 1975, 1977a, b). Cyanobacterial gas vesicles are cylinders of about 70 nm diameter and variable length, with conical caps. They appear to be self-assembled (Waaland and Branton, 1969) from a single rather small (14,000 dalton) polypeptide (Jones and Jost, 1970, 1971; but see Walsby, 1975; Blaurock and Walsby, 1976) whose amino acid sequence is now partially known (Weathers *et al.*, 1977). It is assumed (Walsby, 1977a) that these subunits have both hydrophilic and hydrophobic surfaces. Subunits aggregate in such a way that the inner surface of the vesicle is strongly hydrophobic, and excludes water during and after assembly. Gases, however, freely diffuse through the walls of vesicles, whose gaseous contents are thus in equilibrium with gases dissolved in the medium. Gas vesicles aggregate into gas "vacuoles" which are visible in the light-microscope, and which show overall densities of about 0.2 gm/cm^3 (about one-fifth that of the cytoplasm). The buoyancy of the cell is thus determined by its gas vacuole (or vesicle) content, which is in turn a function of rates of synthesis and collapse of these structures. This last mentioned process is best understood; vesicles collapse at a given critical pressure which can be externally applied (by the experimenter) or generated by the cell itself as turgor pressure. Rapidly photosynthesizing cells (at the surface of the water column) will have high turgor pressure, due in part to the accumulation of soluble products of photosynthesis (Grant and Walsby, 1977). Some vesicles will then collapse and the cells will sink in the water column to depths where photosynthetic activity (and hence turgor pressure) is less. Walsby and Booker (1976) have recently presented some direct evidence for stable buoyancy regulation through vesicle collapse in *Anabaena flos-aquae*.

3. *Cyanophycin Granules*

"Structured granules", long familiar to electron microscopists, are now known (Lang *et al.*, 1972) to consist of multi-L-arginylpoly(L-aspartic acid), formerly "cyanophycin granule protein" or "cyanophycin". In a series of studies perhaps unique in the cyanobacteriological literature for their thoroughness and depth, Simon has described the structure, mode of synthesis and likely function of this storage substance, which is

probably found only in cyanobacteria (Simon, 1971, 1973a, 1973b, 1976; Simon and Weathers, 1976). Cyanophycin consists of varying lengths of poly-L-aspartate residues to whose β-carboxyl groups arginine is attached in peptide linkage. It is made *in vivo* by a chloramphenicol-insensitive (hence ribosome-independent) mechanism. The enzyme responsible for cyanophycin synthesis has been purified some one hundred-fold, and characterized. Its formation may be constitutive.

D. DIFFERENTIATED CELLS

Cyanobacterial vegetative cells can give rise to two sorts of differentiated structures, heterocysts, which are discussed at length in Section VIID, and akinetes, about which little is yet known. These latter are enlarged cells with thickened envelopes sharing at least some components with heterocysts (Lambein and Wolk, 1973). They form in ageing cultures and are capable of "germination", being in these senses at least analogous to bacterial spores. Simon (1977a, 1977b) has begun to characterize events in the differentiation of these mysterious structures, and Fisher and Wolk (1976) have shown that, in *Cylindrospermum*, sporulation is stimulated by a substance excreted by differentiating filaments.

E. MORPHOLOGY, TAXONOMY AND PHYLOGENY

It is fashionable and also correct to describe the state of cyanobacterial taxonomy as "chaotic" (Stanier and Cohen-Bazire, 1977; Stanier *et al.*, 1971; Fogg *et al.*, 1973; Desikichary, 1973; Lewin, 1974). There is little agreement about what individual organisms should be called and little certainty in constructing higher taxa. Classical cyanobacterial taxonomy is based largely on light-microscopic examination of preserved or freshly collected (and biologically contaminated) specimens. This is an uncertain procedure; microscopic prokaryotes, especially unicells, can offer relatively few distinctive morphological characters. Even within a clonally-derived population, morphology can vary considerably and alter drastically with growth conditions (Lazaroff, 1973; Evans *et al.*, 1976). There are many instances in which the same strain has been identified on separate occasions, or by different authorities, as belonging to different genera or families. It is equally certain that some supposedly closely-related strains are truly very different. This *does* matter to the molecular biologist as well as to the taxonomist; one must know one's experimental

organism to be identical to that of other laboratories before attempting to integrate results, and one would like to know its taxonomic affinity with other cyanobacteria on which conceptually related work has been done. Cyanobacteriologists show a tendency to take all blue-greens as their domain, and to incorporate data derived from many different species into unitary models for biochemical or developmental processes, in a way that modern bacteriologists almost never do.

Stanier *et al.* (1971) have most forcefully stated the need for a blue-green taxonomy based on characters determined with pure (axenic) cyanobacterial cultures grown under defined conditions. Recently this group (Herdman *et al.*, 1979b) have presented GC contents for the DNAs of 175 cyanobacterial species, and have described a provisional grouping based on developmental pattern (Stanier and Cohen-Bazire, 1977). I will use this scheme here and have summarized it in Table 2. I also list there the genera most frequently discussed in this review. It should be noted that the organism usually (and here) called *Anacystis nidulans* is a *Synechococcus*, and that its most commonly studied strains are probably similar to that designated by Stanier *et al.* (1971) as *Synechococcus* 6301. Species previously designated by Stanier *et al.* (1971) as *Aphanocapsa* are now considered by them to be *Synechocystis*. I have no wish to retard taxonomic revisions by adhering to classical and soon-to-be-discarded nomenclature, but I do not feel at liberty to modernize strain designations in the existing literature without more certain knowledge that I am doing so correctly.

It seems generally likely (Desikichary, 1973) that morphologically more complex cyanobacteria evolved from morphologically simpler ones and, where it is relevant, the fossil record bears this out (Knoll, 1977; Schopf, 1974, 1975). However, classical taxonomies based on morphology clearly do not reflect phylogenetic diversity. The simple unicellular species (chroococcaleans) show a range of DNA GC contents (35 to 71 moles per cent) nearly as great as that shown by all the bacteria. The relatively simple filamentous oscillatorians also show considerable diversity on this basis. Heterocystous species, in which diversity of form and proliferation of names is most extreme, appear to comprise a relatively restricted group (limited GC range; see Table 2). We (Bonen and Doolittle, 1975, 1976, 1978; Doolittle *et al.*, 1975) have used T1-oligonucleotide cataloguing (see Section VB) to construct phylogenetic trees for eight cyanobacterial species. There does appear on this basis to be considerable diversity among unicellular species. Two

TABLE 2. Principal cyanobacterial subgroups (after Stanier and Cohen-Bazire, 1977).

Subgroup	Characteristics	DNA GC content[a]	Generic designations
Chroococcacean			
Chroococcales	unicells, binary fission	35–71	*Synechococcus (Anacystis, Agmenellum), Synechocystis (Aphanocapsa), Gloeobacter, Gloeocapsa, Gloeothece, Aphanothece*
Chamaesiphonales	budding unicells	46–47	*Chamaesiphon*
Pleurocapsalean	unicells, reproduce by multiple fission	38–47	*Dermocarpa, Pleurocapsa*
Oscillatorian	simple, undifferentiated filaments	40–67	*Oscillatoria, Pseudanabaena Spirulina, Plectonema, Lyngbya, Phormidium*
Heterocystous subgroups			
Nostocacean	unbranched filaments, uniform width, intercalary and terminal heterocysts	38–47	*Anabaena, Nostoc, Cylindrospermum, Nodularia, Scytonema, Aphanizomenon, Tolypothrix*
Rivularian	tapering filaments, basal heterocysts	39–45	*Calothrix, Fremyella*
Stigonematalean	branched filaments	41–47	*Fischerella, Chlorogloea, Mastigocladus*

[a] In moles per cent, from M. Herdman *et al.* (1979b). Genera listed are those referred to in this review; GC contents have not yet been determined for some of them.

morphologically quite distinct filamentous forms (*Nostoc* MAC and *Fischerella ambigua*) classically considered to be members of different orders are, on the other hand, very close to each other and to unicellular species of the *Synechocystis* (*Aphanocapsa*) type. These data also show that red algal chloroplasts are of definite cyanobacterial origin, and may have arisen from *Synechocystis*-like unicellular forms.

III. Carbon and Energy Metabolism

A. PHOTOAUTOTROPHIC METABOLISM

This review is not "about" cyanobacterial metabolism, but I will later try to interpret regulation of cyanobacterial gene expression in terms of the peculiar biochemical needs and abilities of these organisms, and cannot do so without a brief discussion of those needs and abilities. Much more thorough treatments are presented by Stanier and Cohen-Bazire (1977), Smith and Hoare (1977), Fogg *et al.* (1973) and many contributors to the volume of Carr and Whitton (1973).

Cyanobacteria characteristically live by aerobic or microaerophilic (Stewart and Pearson, 1970; Weller *et al.*, 1975) oxygenic photosynthesis, although they are capable of other modes. Cyanobacterial photosynthetic light reactions are analogous and (in the evolutionary sense) homologous to those in eukaryotic algae and higher plants (Fig. 1). In all three groups, there are two coupled photosystems, photosystem II, for which (in blue-greens, rhodophytes and cryptomonads) phycobiliproteins are the major light harvesters, and photosystem I, for which chlorophyll *a* is the major light harvester (Glazer, 1977). Joint operation of the two photosystems results in the generation of ATP and NADPH. During noncyclic electron-flow, electrons derived ultimately from water reduce ferredoxin, which in turn reduces NADP. Oxygen is evolved as a byproduct and ATP is generated ("noncyclic photophosphorylation") as electrons traverse the electron-carrier chain linking photosystem II and photosystem I. When photosystem II is poisoned, as by DCMU [3′-(3,4-dichlorophenyl)1′, 1′-dimethylurea], or is absent, as in heterocysts (Tel-Or and Stewart, 1977), only photosystem I operates. ATP is generated by "cyclic photophosphorylation" but reductant is not available for CO_2 fixation, and cell carbon must be derived from endogenous stores or exogenous supplies of carbohydrate. In cyanobacteria capable of anoxygenic photosynthesis, sulphide or

hydrogen can provide reductant for CO_2 fixation. Their photosystem I-dependent metabolism in reducing environments may be both analogous and homologous to that of bacterial photosynthesizers (Cohen *et al.*, 1975a, b; Garlick *et al.*,1977; Belkin and Padan, 1978). In any growth mode, cyclic and non-cyclic photophosphorylation *and* oxidative phosphorylation are poisoned by CCCP (carbonyl cyanide *m*-chlorophenyl hydrazone).

ATP and NADPH are used in light-independent reactions for fixation of CO_2. These proceed predominantly via the "Calvin" (reductive pentose phosphate) cycle (Pelroy and Bassham, 1972, 1973a, b; Smith, 1975, see Fig. 2). Some carboxylation of phosphoenolpyruvate (Fig. 5, p. 63) may also occur (Jansz and Maclean, 1973; Döhler 1974b, 1976, Colman *et al.*, 1976). When light is plentiful (and especially when nitrogen is simultaneously limited), excess photosynthate accumulates as glycogen, a storage polymer which can be mobilized readily in adversity to provide both carbon and energy (Lehmann and Wöber, 1976).

Although phosphoenolpyruvate can be derived from intermediates of

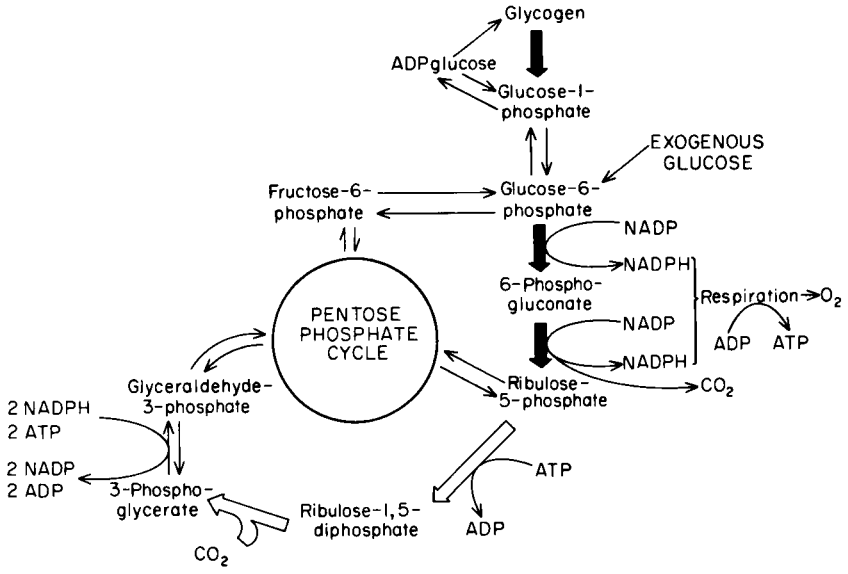

FIG. 2. Light and dark carbon metabolism in cyanobacteria. Reactions specific to photoautotrophic metabolism are indicated by white arrows. Reactions specific to dark endogenous or heterotrophic metabolism are indicated by black arrows.

the reductive pentose phosphate cycle and thus contribute to the formation (via acetyl CoA) of lipid and the amino acids of the glutamate and aspartate families, it does not undergo terminal oxidation through the tricarboxylic acid cycle, since this is interrupted (Fig. 5, p. 63). Blue-greens, and a variety of other autotrophic prokaryotes, lack α-ketoglutarate (oxoglutarate) dehydrogenase, and in them the broken tricarboxylic acid cycle can serve only a biosynthetic role (Smith *et al.*, 1967; Hoare *et al.*, 1967; Pearce *et al.*, 1969; Van Baalen *et al.*, 1971; Ihlenfeldt and Gibson, 1977). (There may be exceptions; patterns of labelling by exogenous acetate strongly indicate a functional cycle in *Chlorogloea fritschii*, Miller and Allen, 1972.)

B. ENDOGENOUS METABOLISM

Microbial survival requires the maintenance of internal ATP pools ("energy charge", Atkinson, 1971) and in the absence of exogenous sources of energy such maintenance depends on the "endogenous metabolism" of internal reserves (Dawes, 1976). Cyanobacteria are accustomed to daily interruptions in energy supply (light) and maintain high energy charge in darkness (Biggins, 1969, Pelroy and Bassham, 1972; Ihlenfeldt and Gibson, 1975a; Imafuku and Katoh, 1976; Bottomley and Stewart, 1976). They appear normally to do so at the expense of glycogen (Lehmann and Wöber, 1976). The major, and possibly only, route of glycogen metabolism is the oxidative pentose phosphate cycle (Fig. 2). The majority of steps in this cycle can be interpreted as converting six moles of pentose (ribulose-5-phosphate) to five moles of hexose. These reactions are common also to the reductive pentose phosphate (Calvin) cycle. Only the glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase reactions are unique to endogenous metabolism and only these two generate reductant (NADPH) and, through its oxidative phosphorylation, ATP. The key role of the oxidative pentose phosphate cycle in endogenous metabolism has been established through measurements of pools of sugar phosphate intermediates in cells transferred from light to darkness, through demonstrations that glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase are present at high levels even in photoautotrophically growing cells (Pelroy and Bassham, 1972; Pelroy *et al.*, 1972; Pearce and Carr, 1969), and through our finding that double mutants of *A. nidulans* lacking both dehydrogenases grow normally in the

light but neither respire nor incorporate significant label into RNA and protein in the dark (Doolittle and Singer, 1974).

The fact that this same double mutant nevertheless survives in darkness may suggest that there is another, low activity, anaerobic (fermentative?) pathway capable of providing minimal maintenance energy. There is some other, indirect, evidence that such an elusive fermentative pathway may exist in cyanobacteria. Hoare *et al.* (1971) reported that *Nostoc* strain MAC was capable of survival, in fact could grow, on glucose under dark anaerobic conditions. Cyanobacteria contain enzymes of the Embden-Meyerhof-Parnas pathway, although phosphofructokinase is characteristically low (Pelroy *et al.*, 1972). At least one cyanobacterium (*Synechococcus* 6716) possesses all the necessary enzymes for fermentation of glucose to lactate (Sanchez *et al.*, 1975). However, lactate production at the expense of endogenous glycogen or exogenous glucose is very low in this strain, ATP levels fall dramatically in a number of cyanobacteria subjected to anaerobiosis (Biggins, 1969; Pelroy *et al.*, 1976a; Bottomley and Stewart, 1976) and dark viability is not long maintained in the absence of oxygen (Rippka, 1972).

C. PHOTOHETEROTROPHY AND FACULTATIVE CHEMOHETEROTROPHY

Some cyanobacteria are photoheterotrophs, capable of satisfying carbon requirements from exogenous sources (usually glucose) but dependent on light-generated ATP for measurable growth. This metabolic capacity can be demonstrated as exogenous-substrate dependent growth (1) in the absence of CO_2 (Ingram *et al.*, 1973a, b), (2) at a light intensity too low to support photoautotrophic growth (Van Baalen *et al.*, 1971), or (3) in the presence of DCMU, which allows cyclic photophosphorylation but prevents CO_2 fixation (Rippka, 1972).

Some photoheterotrophs are also facultative chemoheterotrophs, capable of deriving both carbon and energy from exogenous glucose (or sucrose, fructose, gluconate, ribose or glycerol) and thus of dark growth on these substances. Such substrates are catabolized under these or photoheterotrophic conditions via the oxidative pentose phosphate cycle, as is endogenous glucose (Pelroy and Bassham, 1973a, 1973b; Pearce and Carr, 1969; Pelroy *et al.*, 1972). Heterotrophic growth is, nevertheless, always slower than substrate-dependent photoheterotrophic growth and very much slower than photoautotrophic growth.

D. CONTROLS ON METABOLISM

The enzymes of both reductive and oxidative pentose phosphate cycles are present both in light and darkness and, except for glucose-6-phosphate dehydrogenase, do not vary much in amount with growth conditions (see Section VIID). Yet CO_2 is not fixed in the dark (Ihlenfeldt and Gibson, 1975a; Pelroy *et al.*, 1976b) and glycogen is not mobilized in the light (Lehmann and Wöber, 1976). Rapid switches between reductive and oxidative pentose phosphate cycle activities must thus be controlled at the level of enzyme activity. Six years ago, Pelroy *et al.* (1972; see also Stanier, 1973) proposed a simple and very attractive model to describe the regulatory interplay between light and dark metabolism in cyanobacteria. In the light, carbon flow through the oxidative pentose phosphate cycle is restricted because ribulose-1,5-diphosphate (RUDP), the only sugar phosphate unique to photoautotrophic metabolism, inhibits glucose-6-phosphate dehydrogenase. In the dark, RUDP levels fall (perhaps because phosphoribulokinase is inactivated) and the oxidative pentose phosphate cycle is switched on. This model was supported by the observations that (1) RUDP levels are high in the light and negligible in the dark, (2) illumination inhibits respiration and (in heterotrophs) formation of CO_2 from exogenous glucose; these effects are reversed by addition of DCMU, which also blocks RUDP formation, (3) light-dark transitions result in rapid appearance of 6-phosphogluconate, the product of glucose-6-phosphate dehydrogenase, and (4) cyanobacterial glucose-6-phosphate dehydrogenase is inhibited by RUDP in crude extracts.

It now seems that the regulatory interactions are more complex than this (Schaeffer and Stanier, 1978; Pelroy *et al.*, 1976a; Grossman and McGowan, 1975). It does remain clear that switches between light and dark metabolic modes are controlled at the level of enzyme activity by low-molecular weight effectors, and that the first key enzyme unique to the oxidative pentose phosphate cycle (glucose-6-phosphate dehydrogenase) and at least the first key enzyme unique to the reductive cycle (phosphoribulokinase) are major targets for these effectors. Cyanobacterial glucose-6-phosphate dehydrogenase is a complex enzyme, showing several states of aggregation, the highest being the most active (Schaeffer and Stanier, 1978). Glucose-6-phosphate and glutamine favour aggregation; NADP and RUDP favour disaggregation. NADPH and ATP inhibit catalytic activity regardless of quaternary structure, and pH affects both aggregation and activity. The effect of

RUDP initially observed in crude extracts is not seen in more purified preparations (Grossman and McGowan, 1975). Schaeffer and Stanier (1978) suggest that in photosynthesizing cells, elevated pH and intracellular pools of RUDP, NADPH and ATP all serve to suppress glucose oxidation. However, Pelroy *et al.* (1976a) observed that exogenous glucose actually enters the oxidative pentose phosphate cycle more readily in light-grown than in dark-grown cells of *Aphanocapsa* 6714, and that NADPH levels are lowest in the light while ATP pools remain constant. A more direct light-modulated inactivation of glucose-6-phosphate dehydrogenase by disulphide-bond reduction has been suggested by Duggan and Anderson (1975). There is general agreement that the activity of 6-phosphogluconate dehydrogenase, the second enzyme specific to dark metabolism, is not modulated.

Inhibition of CO_2 fixation in the dark has been attributed by Ihlenfeldt and Gibson (1975a) to NADPH modulation of the activity of glyceraldehyde-3-phosphate dehydrogenase; by Duggan and Anderson (1975) to inactivation (through disulphide-bond oxidation) of phosphoribulokinase and, additionally, by Pelroy *et al.* (1976b) to dark inactivation of fructose-1,6-diphosphate and sedoheptulose-1,7-diphosphate phosphatases. Dark inhibition of CO_2 fixation may also in part be effected by 6-phosphogluconate, which inhibits RUDP carboxylase (Tabita and McFadden, 1972; Codd and Stewart, 1977a).

E. THE BIOCHEMICAL BASIS OF OBLIGATE PHOTOAUTOTROPHY

It becomes increasingly apparent that many cyanobacteria previously thought to be obligately photoautotrophic can grow photoheterotrophically or chemoheterotrophically on glucose or other sugars metabolized via the oxidative pentose phosphate cycle (Smith and Hoare, 1977). Nevertheless, some (like *A. nidulans*) remain obdurate obligate photoautotrophs and none grows as well heterotrophically as photoautotrophically. This behaviour is teleologically offensive and much of the cyanobacteriological literature is devoted to attempts to rationalize it. This literature has undergone an interesting evolution. The assumption that obligate autotrophs are impermeable to all exogenous substrates was disproved in specific cases by Smith *et al.* (1967). The suggestion of these authors that obligate autotrophy reflected metabolic deficiency (specifically an interrupted tricarboxylic acid cycle and the lack of NADH oxidase) lost credence with the discovery that NADP is the

principal electron carrier to oxygen in cyanobacteria and that the oxidative pentose phosphate cycle provides ample NADPH from the oxidation of glucose (Leach and Carr, 1968; Biggins, 1969; Pelroy *et al.*, 1972; Carr, 1973). This leaves us with a variant of the initial notion; obligate autotrophs cannot assimilate *energy-yielding* substrates while heterotrophs can. This, at least for glucose, seems to be true (Pelroy *et al.*, 1972; Stanier, 1973; Beauclerk and Smith, 1978).

It is unlikely that permeability barriers are the only barriers to heterotrophic growth, and they cannot explain the inefficient utilization of glucose by those strains which do take it up well (Pelroy *et al.*, 1976a; Stanier and Cohen-Bazire, 1977). A teleologically satisfying explanation based on the cell's need to restrict utilization of endogenously-generated glucose may be possible. In the light, negative controls on glucose-6-phosphate dehydrogenase activity are sensible; they limit unnecessary oxidation of photosynthetically generated glucose and allow glycogen storage. In the dark, strong controls on glucose oxidation also make sense; glycogen must be used sparingly (perhaps only at a rate sufficient to provide maintenance energy) in order that it not be exhausted before light is once again available. To ask the cell to use exogenous glucose at a higher rate is to ask that it be able to distinguish exogenous glucose from endogenous (glycogen-derived) glucose. Since both present themselves within the cell as glucose-6-phosphate, this may be asking too much.

IV. The Cyanobacterial Genome

A. "CHROMOSOME" STRUCTURE

Cytochemical and ultrastructural studies (well reviewed by Whitton *et al.*, 1971; Fuhs, 1973; Leach and Herdman, 1973; Wolk, 1973; and Fogg *et al.*, 1973) dominated the literature until the late 1960's. These showed convincingly that the frequently centrally located cyanobacterial "nucleoplasm" is not membrane bounded and contains loosely-organized DNA fibrils. Attempts to identify within it structural analogues of eukaryotic chromosomes (Leak and Wilson, 1960; Hofstein and Pearson, 1965; Gavrila, 1977) became less frequent with the growing recognition that cyanobacteria are not, after all, merely simple plants (Stanier and van Niel, 1962). This is not to say that either cyanobacterial or bacterial chromosomes are "naked DNA." A "histone-like" DNA-binding protein is present in cyanobacteria (Haselkorn and Rouvière-

Yaniv, 1976), although cytochemically identifiable histones may be absent (Biswas, 1957; De and Ghosh, 1965; Makino and Tsuzuki, 1971). This protein is immunologically indistinguishable from the *E. coli* histone-like protein HU_E and very similar to it and a *Thermoplasma* "histone" (Searcy *et al.*, 1978) in amino acid composition (Rouvière-Yaniv *et al.*, 1977). There is very preliminary evidence (Makino and Tsuzuki, 1971) that the DNA of *Anabaena cylindrica* can be isolated as an RNA- and protein-containing "nucleoid" analogous to that of *E. coli* (Worcel and Burgi, 1972).

B. CHROMOSOMAL DNA: BASE COMPOSITION AND BASE MODIFICATION

Base compositions have been determined (from buoyant density, thermal denaturation and, at least in one case, direct chemical estimation) for the DNAs of more than 175 cyanobacteria (Biswas, 1960; Edelman *et al.*, 1967; Kaye *et al.*, 1967; Craig *et al.*, 1969; Whitton *et al.*, 1971; Kung *et al.*, 1972; Siu *et al.*, 1974; Roberts *et al.*, 1975, 1977; Charles, 1977; Stanier and Cohen-Bazire, 1977; Herdman *et al.*, 1979b). As mentioned above, such values show a considerable range among chroococcalean (35–71 moles per cent GC) and oscillatorian (40–67 moles per cent) species, but a more restricted diversity for pleurocapsalean (39–47 moles per cent) and heterocystous (38–47 moles per cent) strains.

Pakhomova *et al.* (1968) reported that the DNAs of *Aphanizomenon flos-aquae* and *Coelospherium dubium* contained 1 to 2 moles per cent 5-methylcytosine and about 0.50 moles per cent 6-methylaminopurine. These very likely result from methylation ("modification") occurring after replication, a suggestion consistent with earlier work of Kaye *et al.* (1967) showing *in vivo* incorporation of the [^{3}H] methyl group of exogenous labelled methionine into 5-methylcytosine and 6-methylaminopurine in the DNA of *Plectonema boryanum*. A more highly modified, novel adenine derivative (*N*-6-dimethylaminopurine) has since been reported at relatively high levels in the DNAs of *A. flos-aquae*, *C. dubium*, and *Spirulina platensis* (Pakhomova, 1974).

The modified bases 5-methylcytosine and 6-methylaminopurine occur also in many bacterial DNAs. Their presence there reflects, in part, the operation of sequence-specific restriction-modification (R-M) systems (Arber, 1974; Roberts, 1976). In general, such systems comprise (1) a restriction endonuclease which recognizes (and commonly introduces a double-strand scission within) a specific four-to-six base pair

"palindromic" region and (2) a modification methylase which recognizes and methylates that same sequence, preventing such scission. Unmodified foreign DNA (introduced into the cell by conjugation, transformation or viral infection) is in most cases "restricted" and subsequently fully degraded. Non-replicating (modified on both strands) or newly replicated (modified on one strand) indigenous "host" DNA is protected (and in the latter case, further modified; Vovis *et al.*, 1974). There is good evidence (see Arber, 1974) that bacterial R-M systems do indeed protect against foreign DNA. They may also function in promoting site-specific recombination within and between chromosomes (Chang and Cohen, 1977).

The obvious supposition that modified bases in cyanobacterial DNA reflect the presence of R-M systems like those of bacteria is supported (although not proven) by the finding of specific "restriction nucleases" in several strains. Murray *et al.* (1976) isolated two distinct sequence-specific endonucleases from *Anabaena variabilis* (*Ava* I and *Ava* II) and have also detected nucleases of different but as yet unknown (or undescribed) sequence specificity in *A. subcylindrica* (*Asu* I) and *A. catenula* (*Aca* I) (unpublished, cited in Roberts, 1977). There are at least two other strains in which the existence of R-M systems may be inferred. J. S. Miller and M. Cestari (personal communication) find that the DNA of *Fremyella diplosiphon* is not cleaved by bacterial restriction nucleases *Bam* I, *Hae* II and *Sal* I. The simplest interpretation is that the DNA of this strain is modified in sequences recognized by these enzymes, and preliminary experiments with coliphage λ DNA indicate that *F. diplosiphon* produces one or more site-specific nucleases. The DNA of *Agmenellum quadruplicatum* (strain PR-6) is similarly not cleaved by *Sal* I; no search had yet been made for nucleases in this strain.

C. CHROMOSOMAL DNA: SEQUENCE COMPLEXITY

From the kinetics of renaturation of purified DNA one can derive measurements of sequence complexity (information content). In bacteria, which probably contain few multiple-copy genes (Starlinger, 1977), determinations of kinetic complexity allow simple definition of genome size (Britten *et al.*, 1974; Roberts *et al.*, 1977). Bacterial genome sizes, determined from renaturation experiments, range from minima of 0.37 to 0.44×10^9 daltons for rickettsiae (Kingsbury, 1969) and mycoplasmas (Bak *et al.*, 1969) to maxima of 4 to 8×10^9 daltons (Bak *et*

al., 1971; Zusman *et al.*, 1978). Early renaturation studies with DNA from the filamentous cyanobacterium *Lyngbya* (Kung *et al.*, 1971) indicated that the genome of this organism was of a complexity comparable to that of *E. coli*. Herdman and Carr (1974) estimated genome sizes of 2.27 and 2.47×10^9 daltons for *Anacystis nidulans* and *Anabaena cylindrica*, respectively, values again comparable to that of *E. coli* (2.7×10^9 daltons; Klotz and Zimm, 1972). Clearly the most thorough studies of an individual species were those carried out by Roberts *et al.*, 1975, 1977) on the DNA of the marine unicellular cyanobacterium *Agmenellum quadruplicatum* (strain PR-6). Renaturation was monitored both optically (decreased hyperchromicity) and by separation of single- and double-stranded DNA on hydroxyapatite. These two independent methods yielded values of 2.8 and 2.3×10^9 daltons, respectively. Unfortunately, measurements of viscoelastic retardation time (Roberts *et al.*, 1976) on these same DNA preparations indicated that they contained intact molecules of molecular weights as high as 3.9×10^9 daltons (Roberts *et al.*, 1977). This value is intermediate between that expected if the *Agmenellum* chromosome were a single (presumably circular) DNA of about 2.8×10^9 daltons and that expected if it (or some intermediate in its replication) were a molecule twice as large in which most sequences are present in two or more identical (or nearly identical) copies.

Recent and fascinating results of Herdman (Herdman 1976; Herdman *et al.*, 1979a, cited in Stanier and Cohen-Bazire, 1977) indicate that characterization of cyanobacterial genomes may provide information of great evolutionary interest. Herdman and co-workers determined (by optical monitoring of DNA renaturation) genome sizes for more than 118 representatives of 25 cyanobacterial genera. These fell into four groups with averages of 2.2, 3.6, 5.0 and 7.4×10^9 daltons, which can be interpreted as corresponding to multiples of two, three, four and six times a basic unit of 1.2×10^9 daltons. In general, the "simplest" chroococcaleans were in the first (dimeric) or second (trimeric) group, those chroococcaleans which can fix nitrogen anaerobically fell into the second, while those which do so aerobically were in the third. Pleurocapsalean species (as defined by Stanier and Cohen-Bazire, 1977) generally showed "trimeric" genomes. Oscillatorians were distributed among the first three classes but showed, on average, larger genomes than unicellular species. Heterocystous blue-greens (with the exception of *Nodularia* (3.3×10^9 daltons) and *Anabaena* (3.7×10^9 daltons) showed "tetrameric" and "hexameric" genomes, with those of the structurally

complex genera *Calothrix* and *Scytonema* being often as complex as the largest known bacterial genomes (*Myxococcus xanthus*, 8.4×10^9 daltons, Zusman *et al.*, 1978). Herdman (1976) suggested that these results reflect repeated duplication of a basic 1.2×10^9 dalton genomic unit during the evolution of nutritionally (e.g. nitrogen fixing) and/or structurally complex cyanobacteria from simple nonfixing unicellular ancestors. The suggestion is not without precedent. Wallace and Morowitz (1973) proposed that duplication of an approximately 0.5×10^9 dalton genome occurred during the evolution of modern bacteria from a primitive mycoplasm-like ancestor, while the distribution of functionally related genetic markers on the linkage maps of *Streptomyces coelicolor* and *E. coli* are consistent with the notion that these arose by duplication or quadruplication (Hopwood, 1967; Zipkas and Riley, 1975).

The suggestion that structural or functional complexity requires a large genome bears thought. In bacteria, the number of genes required for nitrogen fixation is not large (Dixon and Postgate, 1972; Dixon *et al.*, 1977). In cyanobacteria, filamentous forms can arise quite easily by mutation from unicellular species (Ingram and Van Baalen, 1970; Kunisawa and Cohen-Bazire, 1970), and the phylogenetic distance between even highly-differentiated, heterocyst-bearing filamentous species and simple unicellular forms may not be very great (Bonen and Doolittle, 1978). Nevertheless there is in eukaryotes (Ohno, 1970; Britten and Davidson, 1971) and now in prokaryotes, a rough but believable correlation between genome size and organizational or developmental "complexity", however we might care to define them. In eukaryotes, much DNA does not code for protein and may play an "evolutionary role" (Britten and Davidson, 1971; Gilbert, 1978). Is it possible that complex ("advanced") prokaryotes also carry large amounts of noninformational DNA?

D. PLASMID DNAS

The existence in bacteria of autonomously replicating and frequently transmissible extrachromosomal genetic elements (plasmids) was first inferred from genetic evidence and later confirmed by their identification as covalently-closed, circular (CCC) DNAs with molecular weights ranging from 1 to more than 100 million daltons (Helinski and Clewell, 1972; Falkow, 1975; Bukhari *et al.*, 1977). Plasmid DNAs are most commonly separated from chromosomal DNA by dye-buoyant-density

gradient centrifugation (Radloff *et al.*, 1967) or by differential precipitation (of chromosomal material) followed by agarose-gel electrophoresis (e.g. Myers *et al.*, 1976). Such methods have also allowed the identification of CCC (presumably plasmid) DNAs of as yet unknown (cryptic) genetic composition in many prokaryotes including, quite recently, numerous cyanobacteria.

Asato and Ginoza (1972, 1973) demonstrated that a fraction of *Anacystis nidulans* DNA was separable from the bulk by ethidium-bromide cesium-chloride gradient centrifugation. This fraction was not separated from the main band in gradients lacking ethidium bromide, reannealed rapidly after alkali denaturation, and was deoxyribonuclease-sensitive, all properties expected of a plasmid of GC content similar to that of the chromosome. Heaton and Frampton (1976; see also, Restaino and Frampton, 1975) confirmed the presence of a CCC DNA in *A. nidulans* and estimated its molecular weight (by neutral sucrose gradient centrifugation) to be $35.5–38 \times 10^6$. Most recently, we (R. H. Lau and W. F. Doolittle, in preparation) have shown that this plasmid occurs in two strains of *A. nidulans* and in the closely related *Synechococcus cedrorum*. The strains differ, however, in their content of a previously undescribed 5×10^6 dalton plasmid, which appears to have been lost from one of the strains of *A. nidulans*.

Roberts and Koths (1976), in the most thorough study published to date, showed that *Agmenellum quadruplicatum* (strain PR-6) harbours at least six CCC DNAs, which together comprise about 5 per cent of the total DNA. Their molecular weights (3, 6.5, 10, 20, 24 and $65–80 \times 10^6$) were determined by electron microscopy and agarose-gel electrophoresis. Neutral cesium-chloride gradient centrifugation showed a more heterogeneous band for pooled plasmids than for chromosomal DNA, suggesting that plasmids differ (slightly) in GC content from each other and from chromosomal DNA. These workers also showed that lower-molecular-weight plasmids accumulate preferentially late in growth of a culture.

Simon (1977c, and personal communication) has undertaken a survey of CCC DNA content of diverse filamentous cyanobacteria using the differential-precipitation and agarose-gel electrophoretic procedures of Myers *et al.*, (1976), and we (R. H. Lau and W. F. Doolittle, in preparation) have begun a similar survey of chroococcalean species. It is clear that many blue-greens, including oscillatorian and heterocystous filamentous species as well as *Synechococcus*- and *Synechocystis*

(*Aphanocapsa*)-like unicells, do contain plasmids. Those which do, tend to contain several. Plasmid content is not obviously correlated with any physiologically or ecologically important trait such as nitrogen fixation or heterotrophic capacity (Simon, 1977c) and there is as yet no way to assign genetic functions to any cyanobacterial plasmid.

E. DNA SYNTHESIS

1. *Labelling*

Studies of cyanobacterial nucleic acid synthesis are hampered by the relative impermeability of cyanobacterial cells to exogeneous nucleic acid precursors other than [^{32}P]orthophosphate and by certain metabolic idiosyncrasies. There is, it must be admitted, confusion as to just *how* impermeable blue-greens really are. Piggott and Carr (1971) found (in agreement with most others) that intact *A. nidulans* incorporates exogenous uracil into acid precipitable material more readily than it incorporates other precursors. Nevertheless, maximum rates were less than one one-thousandth those found for *E. coli* by these authors, who suggested that a uracil permease, although present and inducible, limited uptake. Glaser *et al.* (1973) and Stevens *et al.* (1975) obtained rates of uracil incorporation some three- five-fold greater than those reported by Piggott and Carr (1971). Stevens *et al.* (1975), who used a uracil-requiring auxotroph, pointed out that such values were only slightly "worse" (one-fourth) than values obtained (by a different method) for *E. coli*. The fact that their auxotrophic strain grew, at optimal uracil concentrations, with rates approaching that of the parent wild-type organism indeed suggests that uracil uptake is not severely limiting.

Incorporated uracil appears predominately in uridylate and cytidylate residues in RNA but (not surprisingly) also in thymidylate and deoxycytidylate residues of DNA, and in most cyanobacteria uracil is probably the best vehicle for introducing label into either nucleic acid. It is, however, manifestly non-specific, and several workers have attempted to develop techniques for the specific labelling of DNA, with variable and not fully consistent results. Piggott and Carr (1971) found ring-labelled thymine and thymidine to be only very poorly incorporated into total nucleic acids of *A. nidulans*, as did Glaser *et al.* (1973) and Restaino and Frampton (1975). The former precursor was, however, incorporated at

relatively high rates by *Synechocystis aquatilis*, where (as with *A. nidulans*) it appeared in both RNA (as uridylate and cytidylate) and DNA, a phenomenon attributed by Glaser *et al.* (1973) to endogenous demethylating activity. Thymidine seemed equally susceptible to demethylation but the product(s) appeared ultimately only in DNA (as deoxycytidylate and thymidylate), through the operation of an, as yet, uncharacterized pathway which may also be present in certain fungi and eukaryotic algae.

Glaser *et al.* (1973) suggested that blue-greens incorporated neither thymine nor thymidine directly into DNA because they lack thymidine phosphorylase and thymidine kinase, a suggestion confirmed by Restaino and Frampton (1975) for *A. nidulans*. At odds with these results is the claim of Ssymank *et al.* (1977) that both methyl-labelled thymidine and ring-labelled adenine are incorporated efficiently into DNA by *their* strain of *A. nidulans*. These workers did not show thymidine incorporation to be solely into DNA, and it is difficult to make quantitative comparisons between these and earlier results.

Deoxyadenosine and deoxyguanosine are known to stimulate thymine incorporation in certain bacteria, possibly, although not certainly, through their provision of deoxyribose-1-phosphate as substrate for thymidine phosphorylase or by direct participation in a non-phosphorylytic exchange reaction (Boyce and Setlow, 1962; Friesen, 1968; Yagil and Rosner, 1970; Kornberg, 1974). Predictably, these nucleosides do not stimulate incorporation of either thymine or thymidine in blue-greens. High levels of deoxyadenosine are inhibitory to growth, DNA synthesis, and the incorporation of uracil label into DNA (Ingram and Fisher, 1972; Restaino and Frampton, 1975), as they can be in other systems (Friesen, 1968). However, [8-^{14}C] deoxyadenosine, when present at low levels, is incorporated specifically (greater than 90 per cent) into the DNA of *A. nidulans*, and should find extensive use in future studies of DNA metabolism in cyanobacteria (Restaino and Frampton, 1975).

2. *Enzymology*

I can find but one report describing DNA-dependent DNA polymerase activity in cyanobacteria (Schönherr and Keir, 1972). *Anabaena variabilis* and *A. nidulans* were shown to contain enzymes which required, for optimal activity, low magnesium, high pH and all four

deoxyribonucleoside triphosphates. Denatured DNA proved a better template than did native DNA, and deoxyribonuclease activities in the preparations were low.

3. *DNA Replication and the Cell Cycle*

There is a considerable literature bearing more-or-less directly on the regulation of cyanobacterial DNA replication. Many workers attempt, prematurely I think, to interpret results in terms of existing models for regulation of DNA replication and cell cycle events in *E. coli*. (These models are themselves often in conflict, see Copeland, 1977, and Mendelson, 1977, for what appear to be even-handed treatments of them.) There is general, but by no means complete, agreement that *in bacteria*; (1) chromosome replication is controlled primarily at initiation, either positively or negatively, (2) in *E. coli*, at least, the rate at which replication proceeds is independent of growth rate except possibly when this is very low, and (3) replication initiation and the very last stages of termination require protein synthesis, but the much more time-consuming elongation process does not (Jacob *et al.*, 1969; Pritchard *et al.*, 1969; Cooper and Helmstetter, 1968; Gudas and Pardee, 1974; Lark *et al.*, 1963; Marunochi and Messer, 1973). DNA replication cycles must, perforce, be coordinated with cell cycles (division times), which vary with nutritional status. Cooper and Helmstetter (1968) determined, for *E. coli* growing at medium to high rates, two constants: C, the time required for chromosome replication (approximately 40 minutes) and D, the time between termination of replication and cell division (approximately 20 minutes). Cell division requires prior termination of chromosome replication in *E. coli* but not in *Bacillus subtilis* (Donachie *et al.*, 1971). More rapidly growing cells initiate replication and (probably independently) cell cycle events more frequently. For cells with generation times less than C plus D, this means that new rounds of replication and division-related processes must be initiated while previous rounds are still in progress; cell size may be the trigger for initiation (Donachie, 1974). Elucidation of these, admittedly here too sketchily described, relationships has required experiments with synchronously growing and asynchronous cultures and with cultures in which DNA replication has been arrested either randomly (by inhibitors) or at termination (by amino acid starvation). Similar techniques have been employed with blue-greens, especially *A. nidulans*.

It seems generally true that DNA replication (monitored as accumulation of chemically measurable DNA or by incorporation of radioactive precursors) is ultimately arrested when asynchronously growing *A. nidulans* is placed in darkness. Although Hayashi *et al.* (1969) and Asato and Folsome (1970) observed nearly complete cessation of DNA synthesis after removal of light, more sensitive measurements by Herdman *et al.* (1970) indicated that accumulation of DNA (mass) continued for about 50 min (19 per cent increase) after removal of light. Ssymank *et al.* (1977) observed residual incorporation of label into density-gradient-purified DNA for at least eight hours after darkening; actual rates of synthesis could not be calculated because precursor pool specific activities were unknown (and were likely to have varied).

When chromosomes resume replication after reillumination of darkened *A. nidulans*, they appear to do so in *genetic synchrony*. Asato and Folsome (1970) measured the frequency of nitrosoguanidine-induced forward mutations at several rather loosely defined genetic loci after reillumination; they found sequential and more-or-less discrete stepwise increases in frequency, and ordered their markers in a "temporal genetic" map. A similar and more extensive (17 marker) map has been constructed by Delaney and Carr (1975), using ethyl methanesulphonate mutagenesis. The fact that such maps can be constructed at all indicates that, upon reillumination, chromosomes resume replication at a unique origin or (at worst) a few origins. It further suggests that initiation (but not elongation) is light dependent, and that darkened cells are thus synchronously arrested at termination, or between termination and initiation.

Both Asato and Folsome (1970) and Herdman *et al.* (1970) observed two to three cycles of synchronous cell-division and DNA synthesis in reilluminated cells, although the former found periods of DNA synthesis to coincide with cell division and the latter found these to alternate. It is also noteworthy that the DNA synthetic period lasted only 60 to 100 mins in the rapidly growing (two hour doubling time) cultures of Herdman *et al.* (1970) but approximately 400 to 500 mins in the slowly growing (eight to nine hour doubling time) cultures of Asato and Folsome (1970). Differences in apparent DNA replication time of that order (two *versus* eight hours) also characterize the work of Hayashi *et al.* (1969) and Ssymank *et al.* (1977). Both these groups, moreover, *imposed* synchrony by alternating light and dark treatments and it is unclear to what extent their results reflect natural cycles. It would be of interest to study cell

division and DNA replication in cultures synchronized by methods which do not disrupt these processes, such as centrifugal separation by cell size (Sitz and Schmidt, 1973; Brewer *et al.*, 1976). This is especially important in view of the observation of Herdman *et al.* (1970; see also, Herdman and Carr, 1971b) that doublings in cell number observed during the first and second cycles after reillumination of darkened cells were each preceded only by an approximately fifty per cent increase in DNA content, and that up to one-third of the progeny of the first division were inviable. This they interpreted in terms of a complex model which, although it fits the data, does not address the (to me more interesting) question of why cyanobacteria should be so effectively killed by an environmental alteration (darkness) which they have encountered daily throughout their long evolutionary history.

Detailed studies on asynchronously growing cultures of *A. nidulans* have been recently presented by Mann and Carr (1974, 1977; see also Carr and Mann, 1975). When cultures of *A. nidulans* were "shifted-up" by increasing light intensity, cell divison continued at the original rate for 178 mins, in accordance with the notion (Cooper and Helmstetter, 1968) that such a shift should immediately induce new rounds of DNA replication but that these can only be manifested in cell division after an interval C+D (in this case 178 mins). Rough values of 65 and 115 minutes for C and D respectively could be derived from the earlier synchronous culture studies of Herdman *et al.* (1970). Measurements of cell volume at different growth rates also indicated that achievement of a critical cell volume triggers new rounds of initiation, as suggested by Donachie (1974) for *E. coli*.

When asychronously growing cells were treated with mitomycin C at levels sufficient to inhibit DNA synthesis but not RNA synthesis, cell

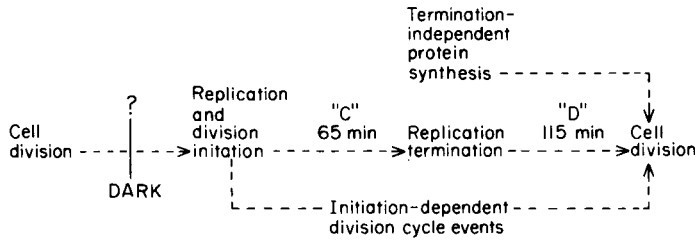

FIG. 3. DNA replication and cell division cycles in *Anacystis minulans* (after Mann and Carr, 1977).

division ceased in about 180 mins, indicating that in *A. nidulans*, as in *Salmonella typhimurium* but not *E. coli*, commitment to cell division occurs simultaneously with initiation of chromosome replication. A further chloramphenicol-sensitive but mitomycin C-insensitive cell division event may occur simultaneously with, but is not dependent upon, termination of chromosome replication. Figure 3 (adapted after Mann and Carr, 1977) summarizes these results. Although the model is attractive in its simplicity, it must be remembered that rather different values for C would be derived from the results of Ssymank *et al.* (1977) and Asato and Folsome (1970).

4. *The Problem of DNA Content*

At generation times much longer than C+D (60 mins), cells of *E. coli* appear to contain between one and two genome-equivalents of DNA, while cells growing with maximum rapidity may contain two to three times that amount (Maaløe and Kjeldgaard, 1966; Kjeldgaard and Gausing, 1974). Vigorous effort is required to convince *A. nidulans* to grow with generation times less than *its* C+D (180 mins) and yet this organism (and perhaps most cyanobacteria) appears multinucleate even when growing very slowly. Mann and Carr (1974) found that DNA content per cell extrapolated to about 5×10^{-15} g (approximately one genome equivalent) at infinite generation time but was already twice this at generation times of about nine hours, while cells growing with maximum rapidity (two hour generation time) might contain more than 20 genome equivalents per cell. Preliminary experiments with rapidly and slowly growing cultures of *Anabaena variabilis* similarly showed that the former contained 7.5 times as much DNA per cell as did the latter (Whitton *et al.*, 1971; Craig *et al.*, 1969). Roberts *et al.* (1977) found that very slowly growing *Agmenellum quadruplicatum* contained close to three genome equivalents of DNA (although they detected no increase in DNA content with increased growth rate). These chemical determinations complement ultrastructural studies showing the presence of multiple "nucleoids" in vegetative cyanobacterial cells (Ueda, 1971; Fuhs, 1973; Gavrila, 1977).

It is not clear why cyanobacteria contain so much DNA. It has often been suggested that excess DNA serves as protection against ultraviolet irradiation, and that its presence is one of the reasons why it is so difficult to isolate auxotrophic and other mutants of cyanobacteria (Vonshak and

Richmond, 1976; Delaney *et al.*, 1976b). Genome multiplicity should, however, only increase the segregation time required for the appearance of mutations (once fixed), and cyanobacteria do possess efficient light and dark mechanisms for repairing radiation damage to DNA (for reviews see Van Baalen, 1973; Shestakov *et al.*, 1975a,b; Asato, 1976; Delaney *et al.*, 1976a). The possession of extra genomes would facilitate recombinational repair (Howard-Flanders 1975). This is the only mechanism for repair of DNA molecules which have suffered lesions affecting complementary regions in both strands (e.g. double strand scission or "post-replicational gaps" opposite unexcised pyrimidine dimers), which might be expected in brutally irradiated cells. Shestakov *et al.* (1976) have presented evidence for recombinational repair in *A. nidulans*.

V. Cyanobacterial Ribonucleic Acids: Synthesis, Processing and Sequence Characterization

A. RNA POLYMERASE

RNA polymerase activity was first demonstrated in *A. nidulans* more than ten years ago (Capesius and Richter, 1967; von der Helm and Zillig, 1967) and this enzyme is now relatively well characterized. Herzfeld and Zillig (1971) achieved purification to near homogeneity of the *A. nidulans* enzyme and reported it to be composed of subunits of 147, 125, 86 and 39×10^3 daltons, which they designated β, β', σ and α respectively. This is of course the *E. coli* terminology; *its* RNA polymerase is described as $[\beta'\beta\alpha_2]\sigma$, and subunit molecular weights are $\beta' = 160$, $\beta = 155$, $\sigma = 89$ and $\alpha = 40 \times 10^3$ (Burgess, 1976). Although the smaller *A. nidulans* polypeptides resembled their *E. coli* homologues in molecular weight, the larger did not, and the β' subunit was so named because it was (like *E. coli* β') the most basic. In any event Herzfeld and Rath (1974) have recently drastically revised their estimates of the molecular weights of *A. nidulans* RNA polymerase subunits, to 190 ("β_{190}"), 145 ("β_{145}"), 72 (σ) and 38 (α) $\times 10^3$ (the last being present in two copies), and have abandoned attempts to define either of the two larger subunits as homologues of *E. coli* β'. A fifth polypeptide (175,000 daltons) appeared to result from *in vitro* proteolysis of β_{190}. The cyanobacterial enzyme further differed from that of *E. coli* in that (1) σ subunit is present in stoichiometric amounts, and the enzyme cannot be separated into σ and "core" ($\beta'\beta\alpha_2$) on

phosphocellulose, (2) rifampicin-resistant initiation complexes can be formed at 0°C, (3) *in vitro* reconstitution of urea-denatured subunits is very slow and dependent upon the presence of σ (Herzfeld and Kiper, 1976).

Early work (Herzfeld and Zillig, 1971) indicated that *A. nidulans* σ could partially substitute for *E. coli* σ in encouraging specific promoter-recognition by *E. coli* "core" polymerase. It now appears (Hertzfeld and Kiper, 1976) that neither σ nor α subunits will function in a hybrid polymerase in which all other subunits are from the heterologous source (*E. coli* or *A. nidulans*). There is, nevertheless, evidence that *A. nidulans* RNA polymerase specifically recognizes the same sites (promoters) on the DNAs of coliphages T4 and λ that are recognized by *E. coli* polymerase. Von der Helm and Zillig (1969) showed that both polymerases bind the same number of sites on T4 DNA (approximately one per 2000 nucleotide-pairs), and these appear to be the *same* sites, although the *A. nidulans* enzyme initiates RNA synthesis efficiently at only about half of the sites at which it binds. More recently, S. S. Miller, F. Ausubel and L. Bogorad (in preparation) have found that *A. nidulans* and *Fremyella diplosiphon* RNA polymerases bind specifically to the P_R promoter borne by a restriction fragment of λ DNA, and initiate λ-repressor repressible transcription from this promoter, as does *E. coli* RNA polymerase. The RNA products appear identical in size and sequence, if not in relative amounts, to those of the homologous enzyme.

S. S. Miller and L. Bogorad (in preparation) have recently purified an RNA polymerase from *Fremyella diplosiphon* (a filamentous rivularian species). It shows five subunits of molecular weights 161, 134, 91, 72 and 41×10^3. Loss of the smallest polypeptide in terminal purification steps results in a four-fold reduction in specific activity. Antisera directed against RNA polymerases from *E. coli* and *B. subtilis* do not precipitate the *F. diplosiphon* enzyme.

A. nidulans and *F. diplosiphon* enzymes were both shown to be rifampicin- (and other rifamycin derivative-) sensitive and α-amanitin insensitive *in vitro*. Neither cyanobacterium appears to produce multiple distinct RNA polymerases, as eukaryotes characteristically do, although there is evidence from Bogorad's laboratory (Bogorad, 1975) showing that some rifampicin-resistant mutants of *Fremyella diplosiphon* produce both resistant and sensitive forms of the enzyme. Cyanobacterial transcription is also generally rifampicin- and streptolydigin-sensitive *in vivo* (Rodriguez-Lopez *et al.*, 1970; Doolittle, 1972). I see no reason not to

regard cyanobacterial RNA polymerases as typically "bacterial", especially given the known variation in molecular weights of subunits of "typically" bacterial enzymes (Burgess, 1976). It would be most interesting to see if these polymerases, like those of bacteria, undergo subunit modification and/or substitution under conditions where transcriptional specificity might be expected to change. Such conditions could include chromatic adaptation (Bogorad, 1975), heterocyst (and spore) differentiation and, most likely, phage infection. AS-1 phage may offer an excellent model for studying such changes (Sherman and Brown, 1978). So may the recently described phage S-2L (Kirnos *et al.*, 1977) whose modified DNA (in which adenine is completely replaced by 2-aminoadenine) might require an RNA polymerase with DNA-binding capabilities quite different from those of its uninfected host.

B. RIBOSOMAL RNA

1. *Synthesis and Maturation*

Cyanobacterial ribosomes, like those of other prokaryotes (including even the otherwise bizarre "methanogens" (Fox *et al.*, 1977a), contain 5S, 16S and 23S ribosomal RNAs (rRNAs) with molecular weights of approximately 0.04, 0.55 and 1.05–1.1 $\times 10^6$ (Pace, 1973; Loening, 1968; Howland and Ramus, 1971; Payne and Dyer, 1972; Doolittle, 1972; Dobson *et al.* 1974). Cyanobacterial large (50S) ribosomal subunits carry only 5S rRNA and 23S rRNA (or its cleavage products) and lack the "5.8S" species characteristic of eukaryotic large ribosomal subunits (Payne and Dyer, 1972).

Again as in bacteria, mature blue-green 5S, 16S and 23 rRNA appear to be the products of post-transcriptional processing (maturation) of slightly larger precursors. In short-term labelling experiments with *A. nidulans*, radioactivity is first found in RNA species which migrate somewhat more slowly on polyacrylamide gels than mature rRNAs present in the same sample (Doolittle, 1972; Szalay *et al.* 1972, 1973a,b; Seitz and Seitz, 1973). The kinetics of the disappearance of these species and the appearance of molecules which co-migrate with mature rRNAs during prolonged labelling or "chaseing" are consistent with the idea that (1) 23S rRNA derives from a rapidly-maturing precursor some ten per cent larger ("p23," approximately 1.2 $\times 10^6$ daltons), (2) 16S rRNA derives from a more stable precursor some 20% larger ("p16," 0.65–

0.70×10^6 daltons), and (3) 5S rRNA derives from a precursor ("p5") carrying a few (one to four) extra nucleotide residues. Both precursor and mature forms of *A. nidulans* 5S have been "fingerprinted" (by two-dimensional separation of the products of T1 ribonuclease digestion). The 5′-terminal T1 oligonucleotide of p5 contains one or two additional nucleotides; our initial suggestion (Dobson *et al.*, 1974) that precursor and mature species also differ at the 3′ end was very likely incorrect (Corry *et al.*, 1974a). The kinetics of p5 labelling *in vivo* suggest that there is no significant pool of precursor molecules larger than p5. Thus 5S maturation in *A. nidulans* appears very similar to the homologous process in the Gram-negative bacteria *E. coli* (Feunteun *et al.*, 1972) and *Salmonella typhimurium* (Raué and Gruber, 1971) and quite unlike the complex maturational pattern exhibited by certain of the bacilli (Pace *et al.*, 1973). Maturation of all *A. nidulans* rRNA precursors is inhibited by chloramphenicol (as is maturation of bacterial rRNA precursors) and retarded by darkness (Doolittle, 1972, Dobson *et al.*, 1974).

A single 23S precursor (1.2×10^6 daltons) and two putative 16S precursors (0.76 and 0.68×10^6 daltons) have been reported for *Tolypothrix distorta*, a filamentous cyanobacterium (Grierson and Smith, 1973). Kinetic experiments are consistent with the notion that the last two are sequential intermediates in the formation of *T. distorta* 16S rRNA.

No rRNA precursors containing sequences of more than one mature rRNA have been found in cyanobacteria, but then none is found in wild-type *E. coli*, whose rRNAs are now well known to be coded by large transcriptional units, transcribed from a single promoter in the order 5′–16S–(tRNA)–23S–5S–3′ and processed even while transcription of the unit is in progress (see Nomura *et al.*, 1977 for review). I would have until recently assumed that such an arrangement (which is probably also found in the bacilli; Colli *et al.*, 1971) was so likely also to characterize cyanobacteria that experiments designed to prove it would be singularly unexciting. However, Lamfrom *et al.* (1978) have presented good evidence that in the marine bacterium *Beneckea harveyi* rRNA genes (and probably certain tRNA genes) are linked, but in an order different from that in *E. coli*. The question of the transcriptional organization of rRNA genes (and associated tRNA genes) in prokaryotes thus becomes an open and evolutionarily interesting one; it should be investigated in cyanobacteria.

2. *Postmaturational Cleavage*

There have been numerous reports that cyanobacterial 23S rRNA is unusually "labile". When *A. nidulans* total RNA is prepared at low temperature in magnesium-containing buffer and resolved on polyacrylamide gels in the presence of this cation, the expected 23S (1.1×10^6 dalton) and 16S (0.55×10^6 dalton) species are detected in more-or-less the expected equimolar amounts. However, heating or the omission of magnesium results in the disappearance of a portion of the 23S material and the appearance of two new species of apparent molecular weights 0.88 and 0.17×10^6 which, like 16S rRNA, are stable to further mistreatment of this kind (Szalay *et al.*, 1972, 1973a, b; Payne and Dyer, 1972; Doolittle, 1973; Seitz and Seitz, 1973; Wollgiehn and Munsche, 1974). The obvious conclusion, that these new species are derived by fragmentation of 23S rRNA, is supported by the observations that (1) both are found associated, together with intact 23S rRNA, on gradient-purified 50S (but not 30S) ribosomal subunits (Singer and Doolittle, 1974) and (2) preliminary T1-ribonuclease generated two-dimensional fingerprint analyses indicate that each fragment contains a subset of the oligonucleotides found in a T1-digest of intact 23S rRNA (L. Bonen and W. F. Doolittle, unpublished).

Fragmentation could *a priori* occur either *in vivo* or *in vitro* (during and after lysis and extraction). Available evidence strongly favours the former interpretation (Doolittle, 1973). In cells sequentially labelled with [^{32}P] orthophosphate and [^{3}H] uracil, 23S fragmentation is always most extensive in the "older" (first-labelled) rRNA. Pulse-chase kinetics show an exponential decay ($t_{\frac{1}{2}}$ approximately 5 h) in that fraction of total 23S-related material which migrates as intact 23S rRNA on polyacrylamide gels, and a concomitant equimolar rise in the fractions represented by each of the fragments. It seems most likely that chain-scission is a random *in vivo* event; each intact 50S subunit in an exponentially-growing culture having an equal, age-independent, probability of suffering endonucleolytic attack.

Chain-scission of 23S rRNA is probably universal in cyanobacteria. We (P. R. Dobson and W. F. Doolittle, unpublished) have found that *Agmenellum quadruplicatum* and the unicellular species designated by Stanier *et al.* (1971) as *Synechococcus* 6312, *Aphanocapsa* 6714 and *Aphanocapsa* 6701 all produce 23S fragments comparable in size to those of *A. nidulans*. Similarly-sized fragments (approximately 0.9 and 0.2×10^6

daltons) are found in the filamentous cyanobacteria *Phormidium persicinium* (Howland and Ramus, 1971), *Anabaena flos-aquae, Oscillatoria tenuis, Nostoc* (unidentified species) and *Anabaena cylindrica* (Payne and Dyer, 1972; Grierson and Smith, 1973). Other, often more complex, fragmentation patterns are observed with *Calothrix membranacea, Mastigocladus laminosus, Plectonema boryanum, Scytonema javanicum, Tolypothrix distorta* and *Nostoc muscorum* (Payne and Dyer, 1972; Grierson and Smith, 1973).

Although 23S rRNA cleavage has been reported in two species of *Rhodopseudomonas* (Lessie, 1965; Marrs and Kaplan, 1970), in *Agrobacterium tumefaciens* (Grienenberger and Simon, 1975; Schuch and Loening, 1975), in *Bdellovibrio bacteriovorus* (Meier and Brownstein, 1976) and in *Paracoccus denitrificans* (R. MacKay, in preparation) it is nevertheless quite likely to be rare among the bacteria (Pace, 1973). Such cleavage is not at all rare in chloroplasts (see Whitfeld, 1977, for review) although differences in fragmentation patterns among chloroplast rRNAs and between these and cyanobacterial rRNAs make me reluctant to draw any phylogenetic conclusions from this coincidence.

I am similarly reluctant to draw conclusions about the functional significance of 23S cleavage. Both intact and fragmented molecules are found on apparently functioning ribosomal subunits (Singer and Doolittle, 1974). Cleavage is more rapid in illuminated, growing cells of *A. nidulans* than in darkened or DCMU-poisoned cells (Doolittle, 1973) and, in the hands of Wollgiehn and Munsche (1974), is more rapid in stationary than in exponentially growing cells. A fairly dramatic inhibition of 23S cleavage in cyanophage-infected *A. nidulans* has recently been reported by Borbely *et al.* (1976). All this could, however, simply reflect growth-related variations in levels of the (quite possibly non-specific) nuclease(s) responsible for cleavage, and in the occupation of the ribosomes. A truly intersting role for 23S scission has yet to be shown in any prokaryote.

3. *Sequences of Ribosomal RNAs*

The nucleotide sequence of *A. nidulans* 5S rRNA has been completely determined (Corry *et al.*, 1974a) and an initial characterization (catalogues of sequences of T1 ribonuclease- and pancreatic ribonuclease A-generated oligonucleotides) of *Oscillatoria tenuis* 5S rRNA has been described (Corry *et al.*, 1974b). The *A. nidulans* sequence is demonstrably

homologous to bacterial 5S sequences (e.g. it shares 63 per cent homology with the 5S rRNA of *E. coli*) and conforms to a general secondary-structural model proposed for all prokaryotic 5S rRNAs (Fox and Woese, 1975a, b). *A. nidulans* and *Oscillatoria tenuis* 5S rRNAs contain the universal prokaryotic sequence pyrimidine-GAAC often considered to be involved in tRNA-binding (Richter *et al.*, 1973) but do lack strong homology (to each other and to bacterial 5S rRNAs) in a region otherwise highly conserved among prokaryotes (*E. coli* positions 67–80).

The *A. nidulans* 5S sequence has been fitted on several occasions into phylogenetic trees relating known prokaryotic and eukaryotic 5S molecules (Hori, 1976; Larue *et al.*, 1977; Schwartz and Dayhoff, 1978). In all such trees it diverges from bacterial 5S rRNAs after the divergence of eukaryotic cytoplasmic 5S rRNAs. However, the analyses of Hori (1976) and of Larue *et al.* (1977) show *A. nidulans* diverging from the common prokaryotic trunk prior to any of the modern bacteria; Schwartz and Dayhoff (1978) would have it diverge after the clostridia and bacilli.

We have now assembled T1-oligonucleotide catalogues (sequences of all G-terminated fragments) for the 16S rRNAs of eight cyanobacterial species: the unicells *A. nidulans* (*Synechococcus* 6301), *Synechococcus* 7502, *Agmenellum quadruplicatum*, *Aphanocapsa* 6701, *Aphanocapsa* 6714, *Aphanocapsa* 6308; and the filamentous strains *Fischerella ambigua* and *Nostoc* (strain MAC) (Doolittle *et al.*, 1975; Bonen and Doolittle, 1975, 1976, 1978, and in preparation). In addition to provoking the speculations about phylogenetic relationships among blue-greens and between blue-greens, bacteria and chloroplasts discussed earlier, these data bear on the relationship between structure and function in cyanobacterial and bacterial ribosomes. In general, oligonucleotides which are common to all ("universal"), or most ("conserved"), cyanobacterial 16S rRNAs are identical or closely related in sequence to oligonucleotides which are similarly universal or conserved in bacteria (Woese *et al.*, 1975). By alignment of such sequences against the positions of homologous oligonucleotides in the known primary sequence of *E. coli* 16S rRNA, it is possible to show that conservation is particularly strong in nine relatively restricted regions of the 16S molecule, six of which occur in the 3′-terminal one-half (Bonen and Doolittle, 1978). Identical regions of conservatism characterize the bacilli (Fox *et al.*, 1977b) and a much more diverse collection of prokaryotes analyzed by Woese *et al.* (1975). Modified oligonucleotides are especially prevalent among sequences common to blue-greens and bacteria. I feel it safe to conclude that no

major, functionally-significant structural differences exist between bacterial and cyanobacterial ribosomes and thus that ribosome function was largely perfected ("modern") at the time of divergence of blue-greens and bacteria (perhaps three billion years ago). Ribosome function appears not to have been perfected at the (thus very likely even more ancient) times of divergence of the three lineages now represented by (1) blue-greens and most bacteria, (2) archaebacteria (methanogens, halobacteria and certain mycoplasmas) and (3) eukaryotic cytoplasmic components (Woese and Fox, 1977).

C. TRANSFER RNA

Studies of cyanobacterial tRNAs are of two kinds; (1) those which compare the extent of charging in *in vitro* systems made up of heterologous combinations of cyanobacterial and other (prokaryotic or eukaryotic) tRNAs and aminoacyl-tRNA synthetases to charging in systems with components from a single source, and (2) those directed at sequence determination. Both have phylogenetic implications. Beauchemin *et al.* (1973) found that *E. coli* aminoacyl-tRNA synthetase preparations charged two $tRNA^{met}$ isoacceptors, five $tRNA^{leu}$ isoacceptors and four $tRNA^{ser}$ isoacceptors from *A. nidulans* as efficiently as did homologous (*A. nidulans*) synthetase preparations. Godzicka-Jozefiak *et al.* (1975) found, somewhat more unexpectedly, that synthetase preparations from barley and yeast could efficiently aminoacylate three *A. nidulans* $tRNA^{val}$ isoacceptors. Similar, although more complex, heterologous charging of at least one *Nostoc* $tRNA^{ile}$ by *Euglena* synthetases has been described by Selsky (1976).

The most extensive studies are those of Cedergren and his collaborators on the $tRNA^{met}$ species of *A. nidulans* (Beauchemin *et al.*, 1973; Ecarot and Cedergren, 1974a, b; Ecarot-Charrier and Cedergren, 1976). These are, of course, of particular interest since, in all prokaryotes, eukaryotic cytoplasms and organelles, one $tRNA^{met}$ isoacceptor is used for the initiation of protein synthesis. In bacteria (not including halobacteria, Heckman *et al.*, 1978), in cyanobacteria (Bachmeyer and Kreil, 1968; Sala *et al.*, 1970), and in mitochondria and chloroplasts, the initiator functions as formylmethionyl-$tRNA^{met}$. In eukaryotic cytoplasmic systems, initiator methionyl—$tRNA^{met}$ is not formylated *in vivo*, although it often can be formylated *in vitro* by enzymes from prokaryotes. *A. nidulans* contains three separable isoaccepting species of $tRNA^{met}$. Only one of

these ($tRNA_f^{met}$, comprising two-thirds of the total) can be formylated *in vitro* by enzyme preparations from *A. nidulans* or *E. coli*. *A. nidulans* transformylase will also formylate *E. coli* $tRNA_f^{met}$. More interestingly, the *A. nidulans* formylating enzyme also recognizes initiator $tRNA^{met}$ from wheat germ; something the *E. coli* enzyme will not do.

The initiator $tRNA_f^{met}$ from *A. nidulans* has now been sequenced (Ecarot-Charrier and Cedergren, 1976), and shares with other prokaryotic (but not eukaryotic) initiator tRNAs the apparently diagnostic noncomplementarity between the 5′-terminal nucleotide and nucleotides forming the 3′ end of the molecular stalk. The molecule is a remarkably conservative one, differing by only eight or nine residues (out of 77) from $tRNA_f^{met}$ species of *E. coli* and *B. subtilis*.

D. CONDITIONALLY-STABLE RNAS OF UNKNOWN FUNCTION

Cyanobacteria produce an intriguing class of "conditionally stable" RNAs which are, to my knowledge, unique to these organisms and, I suspect, universal among them. These were first detected in experiments in which we (Singer and Doolittle, 1974) labelled darkened cells of *A. nidulans* with [^{3}H] uracil and (for reasons which I can no longer recall) took care to exclude light during cell-harvesting, lysis and the initial stages of RNA extraction. Subsequent SDS-polyacrylamide gel electrophoresis of the RNA resolved, in addition to the expected ribosomal species, four "novel" RNAs (molecular weight 0.33, 0.24, 0.16 and 0.12×10^6). These had never been seen in RNA labelled or prepared in the light, but far exceeded in molar amounts rRNAs synthesized during the same period of darkness. Dark-stabilized RNAs, similar in abundance although variable in number and molecular weight, have been seen in each of five other unicellular cyanobacteria examined in this way (Bonen *et al.*, 1976).

In *A. nidulans*, these novel RNAs appear to be synthesized both in light and darkness but in the light they, like mRNA, turn over rapidly and can only be demonstrated during very short labelling periods. In darkness they appear completely stable. Novel RNAs accumulated in darkened cells are rapidly ($t_{\frac{1}{2}}$ 6 min) and apparently completely degraded when cells are re-illuminated. Degradation after re-illumination is retarded ($t_{\frac{1}{2}}$ 14 min) by the addition of chloramphenicol.

It is not, however, darkness *per se* which is required for the accumulation of novel RNAs. They accumulate in illuminated *A.*

nidulans and *Aphanocapsa* 6714 when photosynthetic electron flow is blocked by DCMU. Furthermore, dark accumulation can be prevented by the addition of glucose in *Aphanocapsa* 6714 (which can use glucose for carbon and energy), but not in *A. nidulans* (which cannot). Thus, accumulation of novel RNAs is symptomatic of carbon and energy deprivation, in much the same way that guanosine tetraphosphate (ppGpp) accumulation is symptomatic of nitrogen or energy deprivation in *E. coli*.

I feel that the behaviour of these RNAs is telling us something important about blue-green physiology and that an understanding of their nature and function is essential. The least interesting possibility, that they are aberrant and somehow dark-stabilized degradation products of nascent rRNAs, has been ruled out by (1) comparisons of T1-ribonuclease generated oligonucleotides from all four novel *A. nidulans* RNAs with *A. nidulans* 16S and 23S rRNA oligonucleotides (L. Bonen and W. F. Doolittle, unpublished) and (2) a similar comparison of T1-ribonuclease generated oligonucleotides from a purified (0.09×10^6 dalton) *Aphanocapsa* 6714 novel RNA with oligonucleotides from mature rRNAs of that species (Bonen *et al.*, 1976). In each case, the novel species contained many oligonucleotide sequences absent from rRNA. It is tempting to speculate that novel RNAs are stabilized messengers for proteins needed in large amounts upon subsequent restoration of conditions allowing growth, or that they play a role in controlling (limiting) gene expression in non-growing cells. Both hypotheses could be, but have not yet been, tested.

E. UNSTABLE (PRESUMED MESSENGER) RNA

A fraction of cyanobacterial RNA is unstable and is presumably messenger although, strictly speaking, there is no proof that it is. Estimates of the fraction of RNA which is unstable, and its half-life, have been made on several occasions. I (Doolittle, 1972) showed that addition of streptolydigin (an inhibitor of the RNA polymerase elongation reaction) to *A. nidulans* resulted in loss ($t\frac{1}{2}$ 2 to 5 min) of a large fraction (more than fifty per cent) of acid-insoluble material labelled with [^{3}H] uracil during a preceding ten minute pulse. Although this implied that unstable RNA comprised a much larger fraction of total RNA than expected (by analogy with *E. coli*), Leach and Carr (1974) found a similarly large fraction of pulse-labelled *Anabaena variabilis* RNA to be

unstable after proflavine addition. Even after six to seven generations of labelling, some 17.5 per cent of total labelled RNA in this strain was unstable to proflavine, a proportion more than five times higher than that expected for *E. coli*. In *A. variabilis* growing rapidly, mRNA half-lives measured by (1) loss of acid-precipitable [^{3}H] uracil, (2) loss of the ability to incorporate [^{14}C] valine into protein and (3) loss of templating ability of total RNA in *in vitro* protein-synthesizing systems all fell between ten and fifteen minutes.

More "reasonable" (i.e. expected) values for the fraction of total RNA which is unstable have recently been reported for *A. nidulans* by Smith and Carr (1977a), using RNA:DNA hybridization. In RNA isolated after a four minute pulse, some forty per cent was "unstable" (see also Smith, 1977), while RNA from continuously-labelled cultures showed an unstable fraction comprising only two per cent of the total. From such values a $t_{\frac{1}{2}}$ for the unstable fraction of approximately seven minutes could be calculated. These hybridization experiments also permitted determination of the fraction of total DNA coding for rRNA (about 0.6 per cent, or twice the value usually found for *E. coli*), tRNA (approximately 0.06 per cent) and unstable RNAs. In the case of these last, some ninety per cent of unstable material was complementary to only one per cent of the DNA (two per cent of the coding capacity) while the remaining ten per cent was complementary to only an additional eighteen per cent of the coding capacity. These values are quite similar to those reported by Kennell (1968) for *E. coli*.

The fact that blue-green mRNAs are no more stable than bacterial mRNAs (especially when half-lives are expressed as fractions of generation time) is perhaps a surprise. Cyanobacteria clearly do not respond to environmental alterations by alterations in patterns of gene expression as often or as rapidly as does *E. coli* (Carr, 1973), and it is presumably the ability to respond quickly that necessitates rapid destruction of mRNA in bacteria (Koch, 1971). There would, furthermore, seem to be some advantage in stabilizing messenger RNAs for phycobiliproteins which (if not stable) would have to comprise up to twenty per cent of total instantaneous RNA synthesis and thus would most likely have to be transcribed from multiple structural genes. (There is, in fact, evidence for relatively stable PE mRNA in *Fremyella diplosiphon*, Gendel *et al.*, 1978). Even if phycobiliprotein mRNAs are unstable, they should comprise approximately one per cent of total cellular RNA. It would be refreshing to see some more courageous attempts made to find

them, either as discrete bands on polyacrylamide gels of pulse-labelled RNA, as a particularly prominent species hybridizing to restriction nuclease-digested DNA (Southern, 1975), or as gradient- or gel-purified fractions capable of directing phycobiliprotein synthesis *in vitro*.

I should point out that the experiments of Smith and Carr (1977a), which involved comparing hybridization (as a function of RNA:DNA ratio) by pulse-labelled RNA and by "chased" stable RNA (Kennell, 1968), did not allow detection of possibly stable messenger RNAs. Conversely, those experiments which measure RNA stability after inhibitor addition cannot distinguish between mRNA and other unstable species (e.g. nascent rRNA). It would be desirable to perform, as well, hybridization experiments using purified unlabelled rRNAs and tRNAs as competitors. It would be desirable to repeat messenger decay experiments with inhibitors which block transcription initiation (e.g. rifampicin; Pato and von Meyenburg, 1970) rather than elongation (streptolydigin and proflavine). This last may be difficult; rifampicin is, in our hands and those of Smith and Carr (1977a), quite light sensitive and only transiently effective in inhibiting RNA synthesis.

VI. Ribosomes and their Functions

A. RIBOSOME STRUCTURE

Early ultrastructural (electron microscopical) studies showed cyanobacterial ribosomes and polysomal aggregates to be similar in size and disposition (no associated endoplasmic reticulum) to their bacterial homologues (Ris and Singh, 1961; Pankratz and Bowen, 1963; Jost, 1965). The demonstration that, *in vitro*, cyanobacterial ribosomes show bacteria-like (70S) and not eukaryotic cytoplasmic-like (80S) sedimentation constants and dissociate into 50S and 30S (not 60S and 40S) subunits, lent strength to the developing argument (Stanier and van Niel, 1962) for the fundamentally prokaryotic nature of blue-greens (Taylor and Storck, 1964; Craig and Carr, 1968; Rodriguez-Lopez and Vasquez, 1968; Vasconcelos and Bogorad, 1971; Carlton and Herson, 1972; Gulikova *et al.*, 1976). Cyanobacterial ribosomes are further like those of bacteria in their sensitivity (*in vivo* or *in vitro*) to known 70S inhibitors (streptomycin, chloramphenicol, erythromycin, lincomycin and others, see Leach and Herdman, 1973), and insensitivity to the 80S-ribosome-specific inhibitor cycloheximide. Cyanobacterial ribosomal

proteins have been resolved in one-dimensional electrophoretic systems at least twice (Vasconcelos and Bogorad, 1971; Yurina and Odintsova, 1974). They are different from ribosomal proteins of *E. coli* and chloroplasts and show little reactivity with antisera directed against *E. coli* ribosomes (Wittmann, 1970).

B. RIBOSOME FUNCTION

The ability of crude cyanobacterial preparations and purified ribosomes to mediate polyuridylate-directed synthesis of polyphenylalanine in appropriately constructed *in vitro* systems has been demonstrated (Rodriguez-Lopez *et al.*, 1970; Leach and Carr, 1974; Gray and Herson, 1976). Heterologous hybrids made from *E. coli* 50S and *A. nidulans* 30S subunits (or the reverse) are also functional. Streptomycin inhibition of hybrid ribosomes reflected, as expected, the characteristics of the 30S component (Gray and Herson, 1976).

Translation templated (apparently) by endogenous ribosome-bound mRNA was first described by Bazin (1970) for *A. nidulans* and more recently and carefully by Leach and Carr (1974) for *Anabaena variabilis*. These latter authors demonstrated, in extracts previously incubated to exhaust endogenous message and supplemented with saturating levels of tRNA, strong stimulation of amino acid incorporation upon addition of total RNA extracted from *A. nidulans*. This stimulation was presumably due to unstable (messenger) RNA in the preparation, since proflavine treatment prior to lysis and extraction reduced the stimulatory effect, the reduction being the more severe the longer the interval between proflavine addition and cell lysis.

Studies of *in vitro* translation would benefit greatly from the availability of defined "natural" messengers such as the RNA bacteriophage genomes used to characterize bacterial translation systems. Unfortunately no RNA cyanophages are known. We could find, in preliminary experiments, no coliphage MS-2 RNA stimulation of protein synthesis in crude *A. nidulans in vitro* systems which *did* respond well to polyuridylate and endogenous (ribosome-bound) *A. nidulans* mRNA. This is perhaps not surprising. Bacterial translation systems exhibit considerable specificity; ribosomes from one species are frequently incapable of initiating translation of messenger (or phage) RNAs from a second, distantly related, species (Stallcup *et al.*, 1976; Leffler and Szer, 1973). In fact, Schweiger *et al.* (1969) demonstrated several years ago

that purified *A. nidulans* DNA directed RNA but not protein synthesis when incubated in a coupled transcription-translation system from *E. coli*. Translational specificity is now thought to reside both in the 3′-terminal oligonucleotides of 16S rRNA (Shine and Dalgarno, 1974; Steitz and Jakes, 1975) and in ribosomal proteins, predominantly S1 (Steitz *et al.*, 1977; Isono and Isono, 1976).

If cyanobacterial and bacterial (especially *E. coli*) ribosome: mRNA recognition specificities are indeed different, then one might expect difficulties in achieving expression of cyanobacterial genes cloned in *E. coli* or, indeed, in attempts to translate cyanobacterial mRNAs in the, as yet, much better characterized *E. coli in vitro* translation systems. It is encouraging therefore to note that S. Gendel and L. Bogorad (personal communication) have obtained what appears to be specific translation, in an *E. coli* system, of mRNAs from *Fremyella diplosiphon* (a filamentous cyanobacterium).

VII. Control of Gene Expression

A. GENERAL REMARKS: AN EXPRESSION OF FAITH

It is with most molecular biologists an article of faith that selection favours the evolution of controls on gene expression which maximize an organism's potential to grow and survive in environments to which its nutritional capabilities confine it. Blue-greens are of especial interest because they sorely try this simple faith. They very often do not alter enzyme levels in response to environmental manipulations which, if inflicted upon *E. coli*, would dramatically affect rates of enzyme formation (gene expression). N. G. Carr and his collaborators interpret this behaviour as not only characteristic of blue-greens but as a cause of their obligately photoautotrophic tendencies.

> "With only one or two exceptions the rate of synthesis of enzymes of both intermediary metabolism and of biosynthesis have been shown to be unaltered by the availability of substrates or by the endproduct of the biosynthetic pathway respectively. We may see in this behaviour the reason for the absence of response, in terms of both growth and respiration, to external organic substrates that so characterizes the obligate phototrophic blue-green algae. No matter how favourable the environment or suitable a mix of macromolecular precursors are

> supplied, the organisms elaborate the same range and quantity of enzymes and, within the constraints derived from allosteric inhibition and activation, those enzymes effect the catalytic interchange of metabolites as if carbon dioxide was the only carbon source available. The obligate phototroph presents its characteristic physiology not because of failure of substrate to enter or be metabolized, but because of failure to adjust its metabolic activities to take advantage of such a potentially favourable change in its environment. For such an explanation to be correct it is not necessary for there to be no control over the synthesis of specific enzymes, and, indeed, there are cases where this is likely to happen; the enzymes of nitrogen assimilation being the examples of which we have most information. It may be noteworthy that the enzymes of which there is best evidence of transcriptional control, nitrogenase, nitrate reductase and perhaps phosphatase, are all concerned with inorganic assimilatory processes" (Carr, 1973).

Although Carr's hypothesis has stimulated much research, underlies many discussions of cyanobacterial physiology, has been endorsed by a "correspondent" to Nature (Anonymous, 1973), and has even found its way into the popular press (Anonymous, 1975), I find it difficult to accept. That is, I prefer to see the failure to regulate gene expression in certain pathways as a consequence rather than a cause of autotrophy (thereby begging the question of the "true cause", which I have attempted to come to grips with in Section III). As Carr (1973) in fact pointed out, and has demonstrated experimentally, there are many assimilatory pathways in which cyanobacteria *do* regulate gene expression. I find it hard to believe that they could not have evolved controls on gene expression in other pathways, were it to their selective advantage. Furthermore, there appears to be no peculiarity of the cyanobacterial genome or the machinery for its expression (Sections IV-VI) which could stand in the way of the evolution of such controls. In bacteria, genetic regulatory mechanisms are clearly of multiple and independent origins.

It is useful to distinguish, as Tomkins (1975) did, between two modes of genetic regulation. In "simple" regulatory systems (e.g. the *lac* and *ara* operons, amino-acid biosynthetic operons), there is a direct chemical relationship between regulatory effector molecules and the regulated genetic system. These effectors are either substrates or end products (or

metabolic derivatives or precursors of substrates or end products) of the enzymes whose synthesis is controlled. In "complex" systems, the regulatory effector is not a metabolite of the regulated system; it serves as a "symbol" of general nutritional status and regulates a "domain" of chemically and genetically distinct systems related only in their biological role in maintaining that status. For instance, cyclic AMP is a symbol of glucose deficiency in *E. coli* and its domain includes a number of operons devoted to the catabolism of less efficient sources of carbon and energy (Pastan and Adhya, 1976). "Magic spot" nucleotides (ppGpp and pppGpp) act as symbols of amino acid (nitrogen) and/or energy starvation in *E. coli* and regulate a complex domain including macromolecular synthesis, membrane transport and a variety of enzyme activities (Cashel, 1975; Gallant and Lazzarini, 1976).

As photoautotrophs (Smith and Hoare, 1977; Whittenbury and Kelly, 1977) cyanobacteria require no sources of cell substance other than CO_2, nitrogen, phosphorus and (in lesser amounts) other inorganic nutrients, and no energy source other than light. Alterations in the availability of any of these have consequences in multiple domains extending beyond the pathways directly involved in their utilization. I would expect to find controls of the "complex" kind mediating responses (at the level of gene expression and enzyme activity) to such alterations. Light is of course not a ligand and genetic regulatory responses to it must be via a "symbol" (e.g. products of photosynthesis or photosensitive effector molecules). "Complex" genetic regulatory responses to nitrogen source starvation are so well documented in bacteria (Cashel, 1975; Cashel and Gallant, 1974; Gallant and Lazzarini, 1976) and the consequences of removal of fixed nitrogen are so pleiotropic in cyanobacteria (especially nitrogen fixers) that I expect these too to be mediated by "symbols". One might also anticipate controls of the "simple" sort which regulate the synthesis of proteins involved in assimilation of the various acceptable sources of fixed nitrogen (NO_3^-, NO_2^-, NH_3, urea, certain amino acids (Fogg *et al.*, 1973; Kapp *et al.*, 1975) and phosphate. Whether or not "simple" genetic regulatory controls (and it is really in "simple" controls that cyanobacteria are most obviously deficient) will be found which regulate the synthesis of enzymes for the utilization of carbon sources other than CO_2 or for the biosynthesis of macromolecular precursors should, I think, depend on several factors: (1) the extent to which (in the case of carbon sources) limited available catabolic pathways will allow useful substitution for CO_2, (2) the frequency with which, and concentrations in

which, the carbon source or macromolecular precursor is encountered in habitats in which cyanobacteria have evolved, and (3) the genetic and biosynthetic expense of maintaining controls (operator sites, regulator genes and their products).

B. EFFECTS OF LIGHT ON NET MACROMOLECULAR SYNTHESIS

1. *Metabolic Effects of Shifts between Light and Darkness*

When cyanobacteria are deprived of light, they quickly switch metabolic modes (Stanier, 1973; Stanier and Cohen-Bazire, 1977). Light-driven NADPH formation, CO_2 fixation and ATP generation are replaced by "endogenous metabolism". This latter can be interpreted as serving functions required of all resting cells; maintenance of ATP levels (adenylate "energy charge", Atkinson, 1971), and perhaps resynthesis of unstable macromolecular components (Dawes, 1976). Endogenous metabolism is powered by glycogen (Lehmann and Wöber, 1976; Weber and Wöber, 1975; Frederick, 1970) and dependent upon the oxidative pentose-phosphate cycle, which produces NADPH and from this (through respiration) ATP (Stanier and Cohen-Bazire, 1977; Pelroy and Bassham, 1972; Pelroy *et al.*, 1972; Biggins, 1969; Doolittle and Singer, 1974). ATP pools remain, after a very transient decline, as high in darkened cells as in illuminated ones (Biggins, 1969; Pelroy and Bassham, 1972; Ihlenfeldt and Gibson, 1975a; Sentsova *et al.*, 1975; Imafuku and Katoh, 1976; Bottomley and Stewart, 1976). However, the capacity of the oxidative pentose phosphate cycle to generate ATP appears very limited, and demands on ATP pools must also therefore be severely reduced (Bottomley and Stewart, 1976). Similarly, flow of carbon through the pentose phosphate cycle and into RNA, protein and lipid is severely curtailed in darkened cells; the principal bottleneck appears to be the glucose-6-phosphate dehydrogenase reaction, which is sensitive to a number of negative effectors (Pelroy *et al.*, 1976a; Schaeffer and Stanier, 1978).

The analogy between light-dark (or dark-light) transitions in cyanobacteria and shifts between good and poor organic energy sources ("downshifts" and "upshifts") in *E. coli* is an obvious one, even though there are few if any experiments with this latter organism involving downshifts to media so poor that only maintenance metabolism persists. In downshifted *E. coli*, cell division and DNA synthesis proceed unabated

for a time, while RNA and protein synthesis are immediately curtailed, later gradually to assume the rates characteristic of cells growing continuously in the new (poorer) medium (Maaløe and Kjeldgaard, 1966). Controls on the accumulation of stable RNA and the components of the translation apparatus are probably (although not certainly) mediated in part (if not entirely) through the "symbol" ppGpp, whose concentrations quickly rise upon shiftdown (for reviews see Nomura *et al.*, 1977; Gallant and Lazzarini, 1976; Travers, 1976).

I would anticipate that downshifts (light-dark transitions) in cyanobacteria would result in orderly curtailment of macromolecular synthesis. RNA and protein accumulation cannot persist unabated because ATP generation by the oxidative degradation of endogenous glycogen is too weak to support them; the maintenance of high ATP levels indicates that they *do not* persist. A limited number of proteins (for instance unstable ones or those specific to dark endogenous metabolism) may have to be preferentially synthesized in the dark. Gross changes in protein synthetic patterns are not expected since darkened cyanobacteria are not adapting to a new growth mode (unless they are supplied with glucose and can use it). They need only maintain the ability to grow rapidly when light is restored.

2. *Light and RNA Synthesis*

In experiments monitoring, at rather long intervals, the accumulation of chemically measurable (orcinol reaction) RNA during light-dark and dark-light transitions in *A. nidulans*, Hayashi *et al.* (1969) found net RNA synthesis to be strictly light-dependent. Carr's group (Herdman *et al.*, 1970) observed a residual 22 per cent increase in RNA during the first hour after removal of light, but have more recently reported (Mann *et al.*, 1975) that a shift from high to *low* light intensity results first in a transient drop in total RNA per cell followed, some 50 minutes later, by a resumption in accumulation at the (lower) rate characteristic of cells growing continuously in dim light. Chemical determinations of RNA accumulation are of course (because of the large amount of RNA already present) relatively insensitive to transient light-mediated effects.

Measurements of labelled-precursor incorporation are more sensitive, and with such measurements Hayashi *et al.* (1969) found a very rapid increase in accumulation of labelled RNA during transitions from dark to light and a rather modest reduction in accumulation upon subsequent

removal of light. In their high-light to low-light shift experiments, Mann *et al.* (1975) noted a complete but transient (20 min) cessation in net label incorporation followed by a resumption of accumulation at rates *higher* than those observed at high intensity. This anomaly they attributed to an increase in UTP pool specific radioactivities resulting, perhaps, from diminished endogenous synthesis of UTP in dimly-illuminated cells. Indeed, any measurements of the effect of experimental manipulations on RNA synthesis which monitor label incorporation without simultaneously determining pool specific activities (e.g. Doolittle, 1972; Doolittle and Singer, 1974; Adams *et al.*, 1977) must be considered suspect. (This is true even when the radioactive precursor is [^{32}P] orthophosphate, since possible light effects on the mobilization of internal stores of (unlabelled) polyphosphate cannot be discounted.) Results of a preliminary experiment (M. A. Torres and W. F. Doolittle, unpublished) in which we attempted simultaneously to measure [^{3}H] uracil incorporation and UTP and CTP pool specific radioactivities in a culture continuously labelled with [^{32}P] orthophosphate during light-dark-light transition are shown in Fig. 4. Although resolution of UTP and CTP (by the method of Cashel *et al.*, 1969) was poor, and the consequent determination of pool specific radioactivities untrustworthy, it is safe to

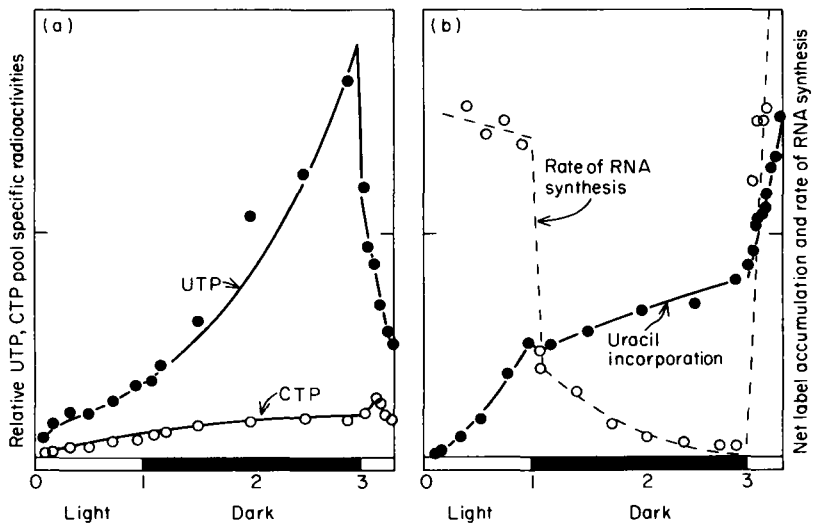

FIG. 4. UTP and CTP pool specific radioactivities, net label accumulation and calculated rates of nucleic acid (RNA) synthesis in *Anacystis nidulans* undergoing light-dark, and dark-light transitions. (M. A. Torres and W. F. Doolittle, unpublished.)

make the following qualitative statements: (1) pool equilibration is slow, (2) pool specific radioactivities rise in the dark and plummet upon reillumination, and (3) the *rate* of the net synthesis of RNA (calculated from pool specific radioactivities and net label accumulation) responds more dramatically to light-dark and dark-light transitions than total label accumulation would indicate. Such rates fell progressively during darkness and increased rapidly upon reillumination. In other (unpublished) experiments, we have shown that dark-light upshifts provoke an apparent ten-fold increase in rates of label accumulation in less than 30 seconds. Although pool specific radioactivities were not determined for such short intervals, the possibility that this rapid response was due to a sudden increase in pool specific radioactivity was eliminated by showing that the same response occurs when excess unlabelled uracil is added just before illumination.

Such rapid responses require explanation. Are they the trivial consequence of increased availability of RNA precursors (UTP pools obviously do expand upon reillumination; Fig. 4), or do they reflect the operation of light-mediated controls on RNA polymerase activity? Inhibitors of light-driven NADP reduction (DCMU) and ATP synthesis (CCCP) should be useful in dissecting such early responses; they have yet to be so employed.

In *E. coli*, energy source downshift preferentially inhibits stable RNA synthesis (Gallant and Lazzarini, 1976; Gallant *et al.*, 1976), and we can ask to what extent light affects the relative synthesis of the major classes of RNA (rRNA, tRNA and mRNA) in cyanobacteria. Mann and Carr 1973) reported that *A. nidulans* tRNA:rRNA ratios remained constant over a five-fold range in growth rate, when this was varied by illumination. Carr and his collaborators (e.g. Smith and Carr, 1977a) have often cited this as evidence for lack of transcriptional control, since tRNA:rRNA ratios were early reported to vary with growth rate in heterotrophic bacteria. It is now however generally thought that both kinds of stable RNA are under "stringent" control and regulated coordinately in *E. coli* (Kjeldgaard and Gausing, 1974; Nomura *et al.*, 1977).

More puzzling is the observation (Mann and Carr, 1974; Leach *et al.*, 1971) that, both in *A. nidulans* and *Anabaena variabilis*, RNA:DNA ratios remain constant over the same light-dependent five-fold range in growth rate. It is hard to know what to say about this. In heterotrophic bacteria, translation rate (ribosome number) is presumed to limit growth (Koch,

1971). DNA per cell is proportional to growth rate, but so is, roughly, volume (and protein) per cell. Thus the number of ribosomes per cell (as reflected in total RNA) must increase more rapidly than DNA in order to accomplish the synthesis of more protein in less time (Maaløe and Kjeldgaard, 1966; Kjeldgaard and Gausing, 1974). In cyanobacteria (Mann and Carr, 1974), DNA per cell increases with growth rate somewhat more rapidly than it does in *E. coli*, but cell volume (and thus presumably protein per cell) increases considerably more slowly. From the data of Mann and Carr (1974), one can see that the number of ribosomes (as reflected in total RNA) per cell is proportional to the rate at which protein (assuming it to be proportional to cell volume) must be synthesized, just as it is in *E. coli*. (My assumption that protein per volume is constant does *not* hold when growth rate falls below 0.06 h^{-1} (Slater, 1975), but most of the data of Mann and Carr 1974, are for cells growing more rapidly than that.) The peculiarity for cyanobacteria is that DNA per cell, and even more, DNA per volume, increases "too rapidly".

Does this mean, as Mann and Carr (1974) suggested, that cyanobacteria can only increase the rate at which they make ribosomes by increasing the number of rRNA genes? Probably not; subsequent reports from Carr's group and from Smith show that the rate of stable RNA synthesis can be differentially controlled, as it is in *E. coli*, by energy and aminoacyl-tRNA availability, and that this control may be mediated by the guanosine nucleotides ppGpp and pppGpp.

Mann *et al.* (1975) showed that downshifts from high to low light in *A. nidulans* provoked (as they do in *Rhodopseudomonas spheroides*, Eccleston and Gray, 1973); (1) a decrease in total RNA accumulation and (2) a seven- to eight-fold increase in cellular levels of the "magic spot" nucleotides ppGpp and pppGpp, as well as in a third unidentified phosphorylated compound. Levels of ppGpp and pppGpp were maximal at about 20 min after downshift and then slowly declined to concentrations four- to five-fold greater than preshift values. Smith (1977) found a rapid and more permanent increase in magic spot nucleotides after the addition of DCMU (which blocks photosystem II) to illuminated cells. Concomitant with this was a decline from about 60 to 40 per cent in the fraction of rapidly-labelled RNA comprising stable species (as measured by "coincident hybridization"). We (M. A. Torres and W. F. Doolittle, unpublished) have also demonstrated ppGpp accumulation after light-dark transitions or DCMU inhibition in *A. nidulans* (an obligate autotroph) and *Aphanocapsa* 6714 (a facultative chemoheterotroph

capable of dark growth on glucose). Glucose addition prevented ppGpp accumulation in the latter but not in the former.

Inhibition of tRNA charging, which provokes a "stringent response" (magic spot accumulation and reduction in stable RNA synthesis) in wild-type *E. coli* (Cashel, 1975; Gallant and Lazzarini, 1976) appears also to do so in *A. nidulans*. Smith and Carr (1977b) reported that both leucine deprivation of a leucine auxotroph of *A. nidulans* and addition to the wild-type of 5-methyltryptophan (which probably inhibits $tRNA^{trp}$ charging) provoke (1) magic spot accumulation, (2) curtailment of net RNA and protein synthesis, and (3) a decrease in the fraction of newly-synthesized RNA which is stable RNA. In *E. coli*, stringent synthesis of magic spot nucleotides is mediated by a protein ("stringent factor") in "idling" ribosomes provided with mRNA but lacking at least one charged tRNA (Block and Haseltine, 1974). *E. coli* stringent factor also stimulates magic spot synthesis with *A. nidulans* ribosomal preparations. It does not appear to do so with ribosomes made from *Aphanocapsa* 6308, which shows no ppGpp synthesis *in vivo* (Adams *et al.*, 1977).

None of this need mean that downshift provoked ppGpp and pppGpp accumulations are stringent factor-mediated. *E. coli relA*$^-$ strains, which lack stringent factor, do not show amino acid deprivation-induced ppGpp accumulation but *do* accumulate this nucleotide when negotiating energy-source downshift. There appear to be two at least partially independent mechanisms governing ppGpp accumulation (Gallant and Lazzarini, 1976; Gallant *et al.*, 1976) and mutants affected in energy-source response have now been identified (Pao and Gallant, 1978). It is not immediately apparent why light-dark transitions should result in reduced charging of tRNA in blue-greens (there is no relevant data), and I would like to suggest that *A. nidulans* also manifests two modes of regulating the levels of ppGpp and pppGpp.

There is as yet no compelling evidence that magic spot nucleotides are more than coincidentally related to changes in rates of rRNA synthesis in blue-greens. Mann *et al.* (1975) felt that kinetic correlation of ppGpp accumulation and reduction in net RNA synthesis did not justify such an assumption. Adams *et al.* (1977) showed that at least one cyanobacterium (*Anabaena cylindrica*) which reduces net RNA synthesis after a light-dark downshift produced no ppGpp. The situation is confused, but then it remains, after nearly a decade, confused for *E. coli* as well (Nomura *et al.*, 1977; Gallant *et al.*, 1976). I would like to consider magic spot nucleotides

guilty of exerting control over cyanobacterial macromolecular metabolism until they are truly proven innocent.

3. *Light and Protein Synthesis*

Many early studies of light-dark transitions in cyanobacteria (e.g. Hayashi *et al.*, 1969) showed that net protein synthesis, like RNA synthesis, requires light. We (Singer and Doolittle, 1975a) monitored protein synthesis using pulse-incorporation of [^{3}H] leucine into a leucine-requiring auxotroph. In such a strain, light-modulated alterations in aminoacid pools (assuming little breakdown of preformed, unlabelled protein) presumably cannot confuse measurements of protein synthetic rates. These fell some seven-fold during the first hour after removal of light, remaining constant for at least 10 hours thereafter. Double-label experiments (continuous exposure to [^{14}C] leucine and short exposure to [^{3}H] leucine) showed that some (at least twelve) polypeptides reproducibly constituted a somewhat larger fraction of total newly synthesized protein in the dark than they did in the light. There is as yet no way to assign function to any of these polypeptides. Assays for glycogen phosphorylase, glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase showed modest (1.5 to 2.0-fold), chloramphenicol-inhibitable increases in specific activities for the first two. Since net protein synthesis was decreased, these increases represent more substantial (ten- to twenty-fold) elevations in differential rates of synthesis. The two dehydrogenases are, of course, uniquely involved in dark endogenous metabolism; changes in their synthesis might be expected. There are however two important *caveats*: (1) Cyanobacterial glucose-6-phosphate dehydrogenase is a complex "hysteretic" enzyme (Schaeffer and Stanier, 1978), which may also be light-inactivated (through disulphide-bond reduction; Duggan and Anderson, 1975). It may thus be difficult to assay this enzyme reliably. (2) A multiplicity of allosteric effectors modulate the activity of glucose 6-phosphate dehydrogenase and the utility of a mere two-fold increase in cellular levels is unclear (Schaeffer and Stanier, 1978; Pelroy *et al.* 1976a; Grossman and McGowan, 1975).

As suggested earlier, gross changes in patterns of protein synthesis are perhaps not expected in light-dark downshifts. Darkened cells are resting cells and, if glycogen stores are ample, should derive no advantage from altering their protein composition. Major changes might be expected

under conditions where (1) alterations in light intensity or wavelength necessitate adjustments in components of the light-harvesting apparatus, or (2) continuous dark or photoheterotrophic growth are permitted by the presence of an exogenous metabolizable energy source. Changes are indeed found and are discussed in the next two sections.

C. EFFECTS OF LIGHT INTENSITY AND COLOUR ("QUANTITY AND QUALITY") ON COMPONENTS OF THE PHOTOSYNTHETIC APPARATUS

1. *Light Intensity Control*

The photosynthetic lamellar structure with its attached biliproteins is the single most expensive piece of molecular equipment cyanobacteria possess. Estimates of the maximum fraction of total soluble protein which can be represented by the latter alone range from 40 per cent (Myers and Kratz, 1955) to more than 60 per cent (Bogorad, 1975). Selection pressure must be intense for controls which modulate the content and composition of the light harvesting machinery to provide maximum light-capture at minimum biosynthetic expense. Controls are indeed multiple and strong, affecting both protein and non-protein components (chlorophyll, carotenoids, membrane lipids; Jones and Myers, 1965; Öquist, 1974b; Goedheer, 1976).

Quite early, Myers and Kratz (1955) showed that phycocyanin (PC) content varies over a four-fold range in response to variation in white light intensity, less being present at higher levels of illumination, where presumably less is needed to capture sufficient energy to saturate the biosynthetic capabilities of the cell. (The effect may only be apparent at quite high light levels; Goedheer, 1976; Döhler, 1976; Stanier and Cohen-Bazire, 1977). Chlorophyll varies roughly in parallel with phycocyanin, and Allen (1968a, b) has shown that lamellar content (area and volume) is controlled coordinately with pigment content.

Two enzymes are unique to CO_2 fixation via the Calvin cycle; phosphoribulokinase and ribulose-1,5-diphosphate carboxylase (RUDP carboxylase). Slater (1975) found that, at least for the latter protein, light does not affect differential rates of synthesis. Specific activities remained constant over a five-fold range in growth rate when this was governed by intensity of illumination, but then so did total protein per cell. Slater (1975) interpreted this as evidence for a lack of transcriptional (or translational) control, but did not clearly state how (in which direction)

he might expect RUDP carboxylase specific activities to vary with growth rate if synthesis *were* controlled. (Döhler, 1976, has more recently reported that RUDP carboxylase levels in fact do vary with light quantity and quality in *A. nidulans*.)

2. *Light Quality Control: Chromatic Adaptation*

All cyanobacteria can produce phycocyanin (PC) which absorbs light in the red (λ max around 620 nm). Some also produce, as a second major accessory pigment phycoerythrin (PE), which absorbs in the blue (λ max around 565 nm). Both transfer energy primarily to the reaction centre of photosystem II, chlorophyll *a* being the major light harvester for photosystem I (Glazer, 1977). In strains with PC alone (such as *A. nidulans*), light quality appears not to affect PC synthesis, although it may (Jones and Myers, 1965; Döhler, 1976) or may not (Goedheer, 1976) alter chlorophyll *a* levels so as to balance light capture by the two photosystems. In strains with both biliproteins, light quality can drastically alter PC:PE ratios to maximize light-harvesting ability. This phenomenon, "complementary chromatic adaptation", has been long known and often reviewed, most recently by Bogorad (1975).

PC and PE producing strains appear capable of three types of differential response to red and green light (Tandeau de Marsac, 1977) and can be divided on that basis into three groups:

I (no response); PC and PE contents are unaffected by light quality.
II (only PE controlled); PE synthesis is induced by green light but it is always measurable, even in red light. PC content is constant.
III (PE and PC controlled); PE synthesis is induced by green light and can be undetectable in red light. PC synthesis is induced by red light but is always measurable, even in green light. There appears to be no obvious correlation between type of response and taxonomic position. Groups I and III include some facultative chemoheterotrophs which can grow in darkness on glucose. When allowed to do so, they resemble red-light grown cells in pigment content.

Light colour affects more than PC and PE in chromatically adapting species. Allophycocyanin (APC) appears to vary coordinately with PC in at least one type III strain. In this same strain (7409, a *Phormidium* like filamentous cyanobacterium), green light also markedly reduces the relative content of two of the colourless proteins found to comprise a

significant component of purified phycobilisomes, while strongly increasing the content of a third (Tandeau de Marsac and Cohen-Bazire, 1977).

During the last ten to twenty years, two chromatically adapting filamentous cyanobacteria, *Tolypothrix tenuis* and *Fremyella diplosiphon*, have received considerable attention. Both are of Tandeau de Marsac's type III, modulating both PC and PE synthesis. Fujita and Hattori (1960a, b, 1962, 1963) have thoroughly characterized controls on phycobiliprotein synthesis in *T. tenuis*. This organism produces more PE and less PC (and APC) when grown in green light (maximum PE production at 541 nm) while it elaborates more PC (and APC) and less PE when grown in red light (maximum effect at 641 nm). It will continue such preferential PC or PE synthesis, at the expense of endogenous glycogen stores, when placed in the dark. A brief (six minute) exposure of green-light grown cells to red light just before darkening results in dark synthesis of PC, just as if prior growth had been continuously in red light. Similarly, brief illumination with green light reverses the effect of prior growth in red light on subsequent dark phycobiliprotein synthesis (favouring PE production). Demonstration of these effects required removal of nitrate during illumination (which results in destruction of existing biliprotein and accumulation of glycogen), and its restoration during dark incubation.

Fremyella diplosiphon, studied extensively by Bogorad and collaborators Bennett and Bogorad, 1971, 1973; Haury and Bogorad, 1977; Gendel *et al.*, 1978; Bogorad, 1975) shows a grossly similar pattern of response. Spectrophotometric pigment determinations (which measure intact, chromophore-containing phycobiliprotein) were combined with immunochemical measurements which permitted monitoring of rates of synthesis and stability of PE and PC apoproteins. Red-light grown cultures contained no PE, *de novo* synthesis of this biliprotein (measured by the above methods or by fluorescense spectroscopy) being rapidly induced upon transfer to green light, which also depresses PC accumulation. Induced PE synthesis can be blocked by rifampicin or by transfer back to red light. Loss of the ability to produce PE shows similar kinetics ($t\frac{1}{2}$ approximately 50 min) under both conditions, suggesting that (1) PE mRNA is relatively stable, and (2) green to red transitions block initiation of PE mRNA synthesis (Gendel *et al.*, 1978). PE synthesis continues in the presence of red *and* green light, suggesting, as did the results of Tandeau de Marsac (1977), that PE production is

positively controlled by green light, and not negatively controlled by red. PE, once produced, is stable under all illumination conditions, and decline in cellular content during growth in red light is attributable to dilution.

Since chromatic adaptation involves *de novo* phycobiliprotein synthesis and is sensitive to both chloramphenicol and rifampicin, it almost certainly reflects controls operating at the level of phycobiliprotein apoprotein structural gene transcription (as well as at the level of cognate chromophore synthesis; Bogorad, 1975). Equally clearly, this transcriptional control must be mediated (although there is no way to say how *directly*) by photoreceptive pigments ("adaptochromes", Bogorad, 1975). There is information concerning the likely nature of these. Diakoff and Scheibe (1973), in extending the work of Fujita and Hattori, found PE synthesis to be stimulated by a photoreceptor absorbing at 550 and 350 nm, while the PC stimulating photoreceptor absorbed at 660 and 360 nm. A photoreversible pigment (perhaps a phytochrome-like biliprotein) in *Tolypothrix tenuis* extracts was previously described by Scheibe (1972). The pigment exists in two spectral forms, P_G and P_R, which can be interconverted *in vitro* by appropriate illumination. Characteristics of this pigment are similar but not identical to those predicted for the *T. tenuis* adaptochrome on the basis of *in vivo* action spectra. Action spectra for PC and PE synthesis in *Fremyella diplosiphon* are different (PC production maximal at 463 and 641 nm, PE production maximal at 375 and 550 nm) and a different photoreceptor(s), which may be a metalloporphyrin(s) or metalloporphyrin-protein complex(es), are likely to be involved (Haury and Bogorad, 1977).

There remain a host of interesting questions here. Chromophore and apoprotein synthesis are probably regulated coordinately since neither has been observed to accumulate in its free state. In principle, (1) adaptochromes could regulate the activity of enzymes involved in chromophore synthesis, chromophore concentrations then governing apoprotein structural gene expression, (2) adaptochrome-regulated apoprotein concentration could control chromophore synthesis, or (3) apoprotein gene expression and chromophore synthesis could be separately regulated by adaptochrome (Bogorad, 1975). It should be possible to isolate mutants deficient in the ability to adapt (because they lack apoprotein, chromophore, adaptochrome or other regulatory elements). It should also be possible to clone and amplify apoprotein structural genes in *E. coli*. Cloned genes could serve as probes in

DNA:RNA hybridization experiments and as templates in *in vitro* transcription-translation systems. Chromatic adaptation should be comprehensible, and soon, at the molecular level.

3. *Other Light Quality Effects: Photomorphogenesis*

Certain cyanobacterial species exhibit extensive pleiomorphism. Cell shape, filament length and structure, mobility and aggregation of cells and filaments can all vary with culture age or history (Lazaroff, 1973; Evans *et al.* 1976). In some strains, such behaviour may be controlled by photoreversible adaptochromes. Lazaroff (1973, see also Ginsburg and Lazaroff, 1973) has carefully described "developmental" changes in *Nostoc muscorum*. When grown in darkness on glucose or sucrose, this filamentous cyanobacterium appears as packets of coccoid cells. When illuminated, it begins (after several days) to grow as long heterocystous filaments which subsequently fragment, yielding motile hormogonia (short, heterocyst-free filaments). Hormogonia swarm for a time and then become non-motile, reforming "aseriate" packets. This complex cycle, once triggered by light, can proceed in its absence. Triggering light need not be sufficiently intense to support photosynthesis, and the action spectrum is different, maximal effect being in the red (650 nm). Furthermore, induction of development can be reversed by green light given after red. Lazaroff (1973) suggested that APC might be the inducing photoreceptor, PC and PE the induction-reversing photoreceptors. Scheibe (1972) proposed that a single photoreversible pigment similar to that which he isolated from *Tolypothrix tenuis* was responsible. There is yet no compelling evidence in favour of either hypothesis.

There are also striking differences in morphology between red light- and green light-grown filaments of *Fremyella diplosiphon*. Certain rifampicin-resistant mutants, when incubated in the presence of this RNA polymerase inhibitor, retain the capacity for chromatic adaptation, but no longer show photomorphogenesis (unpublished work cited in Bogorad, 1975). This tantalizing preliminary observation suggests that different RNA polymerases, or more likely different RNA polymerase subunits, are involved in the two responses. Diakoff and Scheibe (1975) reported that the rate of dark heterotrophic growth of *F. diplosiphon* is also under light quality control. A single daily irradiation with green light for as little as five minutes depressed dark growth. This depressing effect could be reversed by a similar brief illumination with red light.

D. EFFECTS OF CARBON SOURCE

1. *Carbon Dioxide*

CO_2 is (by definition) the only carbon source supporting photoautotrophic growth, and we expect it to be used efficiently. However, when CO_2 is low and O_2 is high, RUDP carboxylase (from any source) can catalyze the apparently inefficient oxidative cleavages of RUDP to produce one mole of phosphoglycerate and one of phosphoglycollate (Tolbert, 1973; Codd and Stewart, 1976b, 1977a). Phosphoglycollate is dephosphorylated to glycollate, which can either be excreted or, less wastefully, retained or reassimilated to contribute to cell carbon *via* one of several routes (Codd and Stewart, 1973; Tolbert, 1973; Stanier and Cohen-Bazire, 1977). Net glycollate excretion can be curtailed by (1) increasing the availability of CO_2 in its photosynthetically active form, and (2) increasing the activity of glycollate-utilizing pathways.

There is preliminary evidence that cyanobacteria can do both, and that controls at the level of gene expression may be involved. Döhler (1974a) reported that specific activities of glycollate dehydrogenase, malate dehydrogenase, serine-pyruvate aminotransferase and aspartate-α-ketoglutarate aminotransferase were all two- to four-fold higher in cells grown in low CO_2 than in cells grown in high CO_2. Carbonic anhydrase was also elevated some three-fold. This enzyme has been described as important in maintaining high levels of CO_2 (rather than bicarbonate) at the site of carboxylation in photosynthetic eukaryotes (Everson, 1970; Raven, 1973). Substantially more dramatic changes in its apparent synthesis have been documented by Ingle and Colman (1976). These workers found that, in cells of the unicellular blue-green *Coccochloris peniocystis* transferred from high to low CO_2, there is a very rapid increase (from immeasurably low base levels) in carbonic anhydrase activity. This is kinetically correlated with a decrease in the rate of glycollate excretion (initially very high in cells transferred from high to low CO_2) and an increase in net CO_2 fixation. This appears to represent a good example of induced enzyme synthesis; it should be further characterized at the molecular level.

Carbon dioxide limitation also affects phycobiliprotein synthesis. A long-standing casual observation in many laboratories, that cells supplied with CO_2 in excess of that available to cultures shaking in air look "healthier" (are bluer), has been formally documented by Eley

(1971). *A nidulans* grown in one per cent CO_2 elaborates twice as much PC as it does when grown in air (0.03 per cent CO_2), while showing similar levels of chlorophyll and carotenoids. The cell's ability to capture light-energy is thus, at least qualitatively, adjusted to its ability to use captured energy productively in CO_2 fixation.

Total CO_2 deprivation has more dramatic effects, resulting ultimately in the complete dismantling of the photosynthetic apparatus, a phenomenon recently described in detail by Miller and Holt (1977). Four days after CO_2 removal, PC and chlorophyll levels in *A. nidulans* began a precipitous decline, becoming negligible within about 24 hours. Shortly thereafter, thylakoids were disassembled and became undetectable in the electron microscope. Restoration of CO_2 resulted in very rapid reassembly of these structures, followed by preferential resynthesis of chlorophyll *a* and PC. Growth did not resume until these attained normal levels.

Destruction of PC is a typical response of cyanobacteria deprived of essential growth factors *other* than light (e.g. nitrogen, phosphorous, iron, sulphur, see below). The amino acids provided by the degradation of PC presumably function as both carbon and nitrogen source for the resynthesis of (unknown) unstable components whose loss would be even more detrimental to cell survival. A phycocyanin-specific protease has been identified in *Anabaena cylindrica*; it may be induced during nitrogen-source starvation (Foulds and Carr, 1977). It is tempting to suggest that all nutrient deficiencies provoke phycobiliprotein degradation by increasing the activity, or inducing the synthesis, of such an enzyme, and that they do so through a metabolic "symbol" which is independently sensitive to levels of each of the required nutrients. Miller and Holt (1977) suggested that cessation of protein synthesis directly or indirectly provides the "symbol" during CO_2, nitrogen and sulphur limitation, but it is less clear how it could do so quickly in the case of iron and, possibly, phosphorus starvation. Isolation of mutant strains carrying conditionally-lethal mutations which block nutrient-deficiency induced phycobiliprotein degradation might reveal whether there is indeed a common "symbol", and provide clues to its identity.

2. *Carbon Sources other than CO_2: Those which do not Support Heterotrophic Growth*

Cyanobacteria can assimilate a number of organic acids which, because of limitations imposed by an interrupted tricarboxylic acid cycle and

exclusive reliance on the oxidative pentose phosphate cycle, cannot replace CO_2 or glucose as sole carbon source (Ihlenfeldt and Gibson, 1977; Butler and Capindale, 1975; Smith, 1973; Smith *et al.*, 1967; Smith and Hoare, 1977; Carr, 1973). In general, such compounds are much more readily assimilated in the light ("photoassimilation") than in the dark. They do not, even when readily taken up and incorporated into cell substance, increase growth rate above values observed under optimal photoautotrophic conditions, and they appear not to effect induction or repression of any of the enzymes responsible for their own assimilation (Carr, 1973).

Acetate provides perhaps the best example. In heterotrophic bacteria possessing a functioning tricarboxylic acid cycle, acetate can serve as sole carbon and energy source, and shifts from growth on glucose to growth on acetate are known to affect levels of tricarboxylic acid cycle enzymes (see Carr, 1973). In cyanobacteria and many other autotrophs, the interrupted tricarboxylic acid cycle is considered to serve a purely biosynthetic role (Fig. 5, Smith *et al.*, 1967; Pearce *et al.*, 1969; Smith and Hoare, 1977). Label from acetate appears only in leucine, glutamate (and amino acids derived therefrom) and in lipids (Smith *et al.*, 1967; Pearce and Carr, 1967; Ihlenfeldt and Gibson, 1977; see Miller and Allen, 1972 and Ingram *et al.* 1973a, b, for exceptions). Its failure to appear in aspartate not only betokens the absence of α-ketoglutarate dehydrogenase but suggests that two other potential routes to aspartate, the glyoxylate cycle (dotted arrows in Fig. 5) and ferredoxin-dependent decarboxylation of α-ketoglutarate to succinyl CoA are dysfunctional, even though the requisite enzymatic activities have been found at low levels (Pearce and Carr, 1967; Bothe *et al.*, 1974).

In photoautotrophically growing cells in low CO_2, acetate can contribute up to 17 per cent of total cell carbon (Pearce and Carr, 1967; Carr, 1973), a value gratifyingly close to the theoretically maximum value of 16 per cent calculated by Ihlenfeldt and Gibson (1977) on the assumption that acetate goes only into lipid, leucine, and amino acids of the glutamate family. Uptake is strictly light-dependent. Incorporation of label from acetate into protein requires CO_2 (for the regeneration of oxaloacetate); lipid-labelling is relatively CO_2-independent (Ihlenfeldt and Gibson, 1977).

Even though acetate can thus effectively "spare" CO_2 (endogenous acetyl CoA production from CO_2 *is* apparently reduced), it does not stimulate growth (Hoare *et al.*, 1967; Smith *et al.*, 1967; Carr, 1973), nor

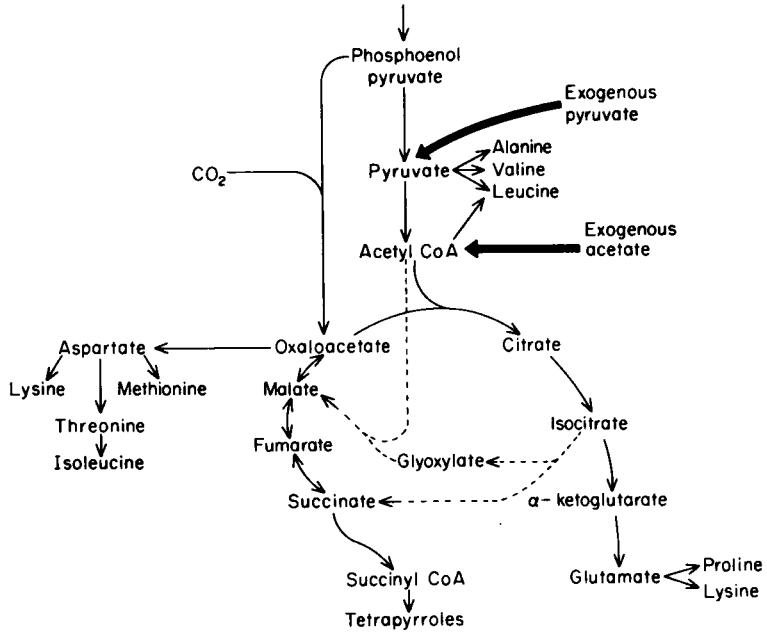

FIG. 5. The interruped tricarboxylic acid cycle and fate of exogenous acetate and pyruvate in most cyanobacteria.

does its inclusion in the medium alter levels of enzymes responsible for its uptake and utilization, including acetyl CoA synthetase, acetate kinase, phosphotransacetylase, isocitrate lyase, malate synthetase, and enzymes of the interrupted tricarboxylic cycle (see Carr, 1973; Ihlenfeldt and Gibson, 1977). If ("uninduced") levels of enzymes for assimilation of acetate and its conversion to amino acids and lipids already allow theoretically maximal (16 per cent) contribution to total carbon, the cell may in fact have no "reason" to increase them. It would however have reason to increase its very low (Miller and Allen, 1972) levels of isocitrate lyase and malate synthetase (glyoxylate cycle enzymes) to promote acetate incorporation into amino acids of the aspartate family. Failure to do so indeed seems unadaptive and supportive of Carr's argument for the regulatory inflexibility of cyanobacteria.

3. *Carbon Sources other than CO_2: Those which do Support Heterotrophic Growth*

Facultatively chemoheterotrophic cyanobacteria will grow in the dark using, as carbon and energy source, glucose or other sugars which can

feed into the oxidative pentose phosphate cycle. Heterotrophic growth is always very much slower than photoautotrophic growth (Stanier and Cohen-Bazire, 1977) and it is not clear except in a few cases (e.g. Hoare *et al.*, 1971; Silvester, 1976) that cyanobacteria are evolutionarily adapted to it. For dark heterotrophically grown cells, metabolic brakes on the activity of glucose-6-phosphate dehydrogenase (Pelroy *et al.*, 1976a) and the necessity of producing massive amounts of unused photosynthetic paraphenalia seem unnecessarily burdensome. However, releasing the brakes is risky unless exogenous carbohydrate supplies are dependable, and abandonment of the light harvesting apparatus exacts its toll when photoautotrophic growth must be resumed. The extent to which cyanobacteria do these things should therefore reflect the extent to which prolonged heterotrophic growth has been important in their evolutionary history; one should expect no single pattern to apply to all species.

Addition of glucose to autotrophically grown *Anabaena variabilis* (Pearce and Carr, 1967, 1969; Pearce *et al.*, 1969) has no effect on levels of the two enzymes unique to the oxidative pentose phosphate cycle (glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase), nor on any of several enzymes of the Embden-Meyerhof-Parnas pathway. In the facultative chemoheterotroph *Aphanocapsa* 6714, however, glucose-6-phosphate dehydrogenase levels are elevated some two-fold by the addition (to illuminated cells) of glucose; some two- to four-fold by the addition of glucose and DCMU, and some five- to six-fold by the simultaneous addition of glucose and removal of light. These treatments have no effect on levels of 6-phosphogluconate dehydrogenase, hexokinase or triosephosphate dehydrogenase (Pelroy *et al.*, 1972) nor does dark heterotrophic growth of this strain depress levels of PC (Rippka, 1972). Dark heterotrophic growth of several other, chromatically adapting, species also did not alter total phycobiliprotein content (Tandeau de Marsac, 1977).

In cultures of *Plectonema boryanum* transferred from photoautotrophic to heterotrophic (dark with glucose) conditions, Raboy *et al.* (1976) observed: (1) a chloramphenicol-inhibitable seven-fold increase in rates of glucose incorporation, maximum rates being achieved at 16 h, (2) a ten- to twenty-fold increase in glucose-6-phosphate dehydrogenase, maximum activity being achieved in about ten days (one dark generation), and (3) a decrease in generation times (from 200 to 70-100 h)

shortly after achievement of maximal glucose-6-phosphate dehydrogenase levels. Again no change in pigmentation or RUDP carboxylase levels was observed during this adaptation process. However, prolonged (several years) maintenance under dark heterotrophic conditions resulted in selection of faster-growing mutants with one-half the wild-type levels of RUDP carboxylase and chlorophyll but, surprisingly, normal amounts of PC. Similar prolonged dark growth (on sucrose) of *Chlorogloea fritschii* has produced cells with only modestly altered RUDP carboxylase content but drastically reduced levels of PC (see Carr, 1973; Evans and Carr, 1975; Evans *et al.*, 1976; Joset-Espardellier *et al.*, 1978).

Long-term laboratory maintenance of dark heterotrophic cultures may not tell us much about naturally-evolved controls on synthesis of the photosynthetic apparatus. However, Nature herself has apparently maintained several cyanobacterial species under dark heterotrophic conditions, the best studied being *Nostoc* strain "MAC" (Hoare *et al.*, 1971). This organism was isolated from roots of the cycad *Macrozamia lucida* (Bowyer and Skerman, 1968) at soil depths were photoautotrophic growth was precluded. *Nostoc* MAC is a very good heterotroph, showing dark heterotrophic generation times of 48 h in the presence of glucose and 24 h in the presence of glucose and casein hydrolysate (Hoare *et al.*, 1971; Pulich and Van Baalen, 1974). It will apparently also grow anaerobically in the dark (!) and on a greater variety of carbon sources (including acetate) in the light when it is deprived of exogenous CO_2 (Hoare *et al.*, 1971; Ingram *et al.*, 1973a). Unlike most blue-greens, it shows repression-like controls for at least one amino acid biosynthetic pathway (N. G. Carr, personal communication). In spite of this strong heterotrophic potential and the likelihood that *Nostoc* MAC has a long history of dark heterotrophic growth at the expense of carbohydrates provided by the cycad with which it associates, no differences in thylakoid structure were noted between photoautotrophic and dark heterotrophic cells, and total phycobiliprotein content was the same in both. However, PE:PC ratios were higher in dark-grown than in light-grown cells (Hoare *et al.*, 1971). The reluctance of even evolutionarily adapted heterotrophs to part with the photosynthetic machinery strikes me as remarkable, especially since we know that production of its most expensive single component, phycobiliprotein, *can* be controlled at the level of gene expression under other conditions (e.g. chromatic adaptation).

E. EFFECTS OF NITROGEN SOURCE

1. *Combined Nitrogen*

Cyanobacteria will utilize, as sole nitrogen source, nitrate, nitrite, ammonia, urea, and a variety of amino acids and purines (Berns *et al.*, 1966; Fogg *et al.*, 1973; Kapp *et al.*, 1975). Nitrate is the nitrogen source most commonly employed in the laboratory and probably in nature. It is presumably reduced by nitrate reductase to nitrite, and this by nitrite reductase to ammonia. Both enzymes in *A. nidulans* are particulate and depend on reduced ferredoxin as immediate electron donor (Manzano *et al.*, 1976). Both are clearly "adaptive" (inducible/repressible) enzymes.

Hattori (1962) demonstrated that when N_2-fixing *Anabaena cylindrica* was transferred to nitrate-containing medium, nitrate and nitrite reductase activities (in whole-cell assays) increased some 60-fold over the course of 10 h. Transfer from N_2 to nitrite also "induced" both activities. No increase was observed if transfer was to ammonia- or glutamate-containing media. Induction of nitrite reductase was inhibited by chloramphenicol, by darkness and by anaerobiosis (nitrate reductase was not measured in such experiments), and the enzyme appeared to be unstable, disappearing rapidly when nitrate was exhausted. Ohmori and Hattori (1970), using cell-free assay systems, found that nitrate reductase activity reached maximal levels before that of nitrite reductase, but that the appearance of the latter was considerably accelerated when N_2-grown cells were transferred directly to nitrite, rather than nitrate. They suggested that nitrate induced nitrite reductase only indirectly, after its conversion to nitrite.

Such experiments have been extended by Stevens and Van Baalen (1974) using an apparent nitrite reductase mutant of *Agmenellum quadruplicatum* (Stevens and Van Baalen, 1970; 1973), in which nitrite excretion can be taken as a measure of nitrate reductase activity. Transfer from growth in ammonia-containing media to nitrate-containing media resulted in a dramatic increase in enzyme activity (so measured), beginning at about one hour after transfer and completed in about 10 h. This was prevented if chloramphenicol, rifampicin or ammonia were also present in the medium, and further synthesis of the enzyme could be blocked by their addition during the induction period. Induction required illumination (maximum at 680 nm) and was markedly diminished in blue (430-480 nm) light, even though the activity of the enzyme was nearly as high in blue light as in light at 680 nm.

This is clearly a very easily manipulated system for the study of cyanobacterial gene expression. It seems likely that ammonia represses nitrate reductase formation (Carr, 1973) and that, as Ohmori and Hattori (1970) suggested, nitrite induces nitrite reductase. Of course more complex regulatory mechanisms may underlie these apparently simple effects. The synthesis of pyrurate:ferredoxin oxidoreductase, which may in *Anabaena cylindrica* provide electrons for reduction of nitrate, is also repressed by ammonia (Bothe, 1975; Bothe *et al.*, 1974). Mutants deficient in either nitrate or nitrite reductase are easy to isolate (Stevens and Van Baalen, 1973, 1974) and among them one might hope to find many altered in the regulation of this essential assimilatory pathway.

2. *Deficiency of Combined Nitrogen: Degradation of Endogenous Stores*

Nitrogen-source deprivation is a threat to the integrity of all organisms and many respond to it by preferential degradation of one or more nitrogen-containing macromolecules, which may or may not serve other functions during balanced growth (Dawes, 1976; Goldberg and Dice, 1974). Cyanobacteria appear to turn to at least two major endogenous nitrogen sources when so deprived; cyanophycin granule protein (multi-L-arginyl-polyaspartic acid) and phycocyanin (PC). For N_2-fixing species, degradation is (if N_2 is available) a temporary measure; for species which do not fix N_2 it may be a terminal act. In either it is likely that specific degradative systems are induced.

Cyanophycin granule protein has been well characterized by Simon and co-workers (Simon, 1971, 1973a, b, 1976; Simon and Weathers, 1976; Lang *et al.*, 1972). Much is known about its structure and synthesis; less about its degradation or, with certainty, its function. This nitrogen-rich polypeptide consists of a polyaspartic acid core, to each of the free carboxyl groups of which arginyl residues are attached. It is synthesized by a chloramphenicol-insensitive, ribosome- and tRNA-independent enzyme system and packaged in the "structured granules" long familiar to electron microscopists. It appears unique to, but may be universal among, cyanobacteria (Lang *et al.*, 1972; Stanier and Cohen-Bazire, 1977). Evidence that cyanophycin granule protein functions as a nitrogen store is circumstantial but compelling: (1) Chemically measurable cyanophycin granule protein accumulates (up to eight per cent of total dry weight) in stationary phase cultures and is rapidly depleted when these resume growth upon dilution. (2) Cyanophycin

granule protein levels fall precipitously upon nitrogen starvation of *Aphanocapsa* 6308 and rise rapidly upon restorations of combined nitrogen in any of several forms (M. Allen, personal communication). There is marked preferential accumulation of cyanophycin granule protein in such restoration experiments (Allen *et al.*, 1977), reminiscent of the "Polyphosphate Überkompensation" observed for phosphate storage (Harold, 1966). (3) Nitrogen-starved cyanobacteria characteristically lack "structured granules".

Phycobiliproteins are also rapidly and specificially degraded in nitrogen starved cyanobacteria. Allen and Smith (1969) showed that combined-nitrogen deprivation provoked, in *A. nidulans*, immediate and ultimately complete loss of spectrophotometrically measurable PC, while levels of chlorophyll and carotenoid were maintained. Nitrate restoration promoted, after a short lag, rapid repigmentation which had to be completed before growth could resume. We (Lau *et al.*, 1977) have used immunochemical methods to confirm that starvation results in the coordinate loss of both PC apoprotein subunits and to show that (1) during starvation, PC apoprotein synthesis is *specifically* depressed, presumably at the level of gene expression, and (2) this depression persists for several hours after nitrogen-source restoration. Proteases which specifically degrade PC and which appear to be activated or preferentially synthesized during nitrogen-starvation have been described for species of *Anabaena* (Foulds and Carr, 1977; Wood and Haselkorn, 1976, 1977). It is usually assumed that PC degradation functions to provide amino acids for (in the case of species incapable of N_2-fixation) maintenance and (in the case of N_2-fixing species) formation of nitrogenase and the accessories necessary for its effective operation (Allen and Smith, 1969; Haselkorn, 1978; Stewart and Lex, 1970; Neilson *et al.*, 1971).

It is probably true that degradation of both cyanophycin granule protein and phycobiliprotein can serve this function. One would like to assume that the former storage product, which does not have any other obvious cellular role, is degraded *before* the latter, which does. I can find no published evidence which shows that this is so. The possibility, suggested by Stanier and Cohen-Bazire (1977), that cyanophycin granule protein is really an energy reserve capable of providing ATP under anaerobic conditions is an intriguing one, especially in the light of evidence that cyanobacteria can generate some ATP anaerobically from endogenous reserves (Bottomley and Stewart, 1976; Doolittle and Singer,

1974), and that at least one species possesses the appropriate (arginine dihydrolase) pathway (Weathers *et al.*, 1978).

3. *Deficiency of Combined Nitrogen: Induction of Nitrogenase in Nonheterocystous Cyanobacteria*

Removal of combined nitrogen not only triggers degradation of endogenous reserves but, in a great many blue-greens, induces the synthesis of nitrogenase, a multi-component enzyme system catalyzing the ATP- and reductant-dependent conversion of N_2 to ammonia. The ability to produce nitrogenase is latent in cyanobacteria of diverse taxonomic position as well as in Gram-negative and Gram-positive bacteria and thus may have arisen early in an ancestor common to all three (Silver and Postgate, 1973; Cole, 1976; but see Postgate, 1974). All prokaryotic nitrogenases (there are no eukaryotic ones) are oxygen-sensitive. The extent to which nitrogenase, once produced, will actually function in providing the cell with combined nitrogen depends upon the availability of combined-nitrogen deficient anaerobic habitats otherwise permissive to growth, or on the ability to elaborate structures which protect the enzyme from environmental or endogenously produced oxygen. The cyanobacteria include species which show no protective mechanisms, species which elaborate complex differentiated cellular factories (heterocysts) devoted exclusively to N_2 fixation, and species which can be seen as intermediate.

In 1970, Stewart and Lex showed that the oscillatorian *Plectonema boryanum*, which cannot grow under air in media lacking combined nitrogen, could grow photoautotrophically on such media when atmospheric and photosynthetically generated O_2 were removed by periodic purging with N_2-CO_2. Nitrogenase activity (which is completely destroyed by readmission of O_2) developed not only under these conditions but also when purging was with argon-CO_2. It did not develop in the presence of ammonia. Nitrogenase activity was detectable after two days but maximal levels were not achieved for four to six days. Appearance of nitrogenase was preceded by the degradation of PC, this pigment being replenished when N_2-fixing competence developed. N_2-fixation and growth under these conditions (which must be described as microaerophilic, since intracellular photosynthetic O_2 evolution persists) has since been demonstrated for a number of additional oscillatorians by Kenyon *et al.* (1972).

Oxygen can be more rigorously excluded by the addition of DCMU (which blocks photosynthetic O_2 evolution) to argon-CO_2-flushed cultures previously allowed to accumulate glycogen during combined-nitrogen source starvation in air-CO_2. With such techniques, Rippka and Waterbury (1977) demonstrated that eight of 55 chrococcaleans, 19 of 32 pleurocapsaleans and 24 of 44 oscillatorians, most of which had not previously been identified as N_2-fixers, in fact do produce nitrogenase under strictly anaerobic conditions. Eight of these were shown to be able to fix nitrogen and grow microaerophilically (when flushed with N_2-CO_2). It is possible that all strains producing nitrogenase under strict anaerobiosis can do this. Rippka and Waterbury (1977) also demonstrated that imposition of strict anaerobiosis upon previously nitrogen-starved but aerobic cultures of *Plectonema boryanum* resulted in very rapid (lag less than one hour) appearance of detectable nitrogenase. This apparent induction was completely blocked by O_2, ammonia or chlorampenicol.

Nitrogen fixation under anaerobic or microaerophilic conditions is almost certainly not a mere laboratory curiosity. Stewart and Pearson (1970) and Weller *et al.*, (1975) have demonstrated that many cyanobacteria can grow well photoautotrophically in highly reducing environments where they may well contribute to nitrogen balance (Stewart *et al.*, 1977). Some cyanobacteria, including several anaerobic fixers, can in fact grow completely anaerobically, using sulphide as an electron donor, and an anoxygenic, photosystem II-independent process similar to that shown by green and purple-sulphur photosynthetic bacteria (Garlick *et al.*, 1977).

Five members of the unicellular cyanobacterial "genus" *Gloeothece* (formerly *Gloeocapsa*) and one of *Aphanothece* can fix N_2 aerobically (Rippka and Waterbury, 1977; Rippka *et al.*, 1971; Wyatt and Silvey, 1969; Singh, 1973; Gallon *et al.*, 1975). They lack the thick outer envelope which, in heterocystous species, is *assumed* to be necessary for the exclusion of O_2, although *Gloeothece*, at least, shows an extensive polysaccharide capsule (M. M. Allen, personal communication). Transfer from combined nitrogen to N_2 provokes (temporary) PC degradation and nitrogenase synthesis (Wyatt and Silvey, 1969; Rippka *et al.*, 1971), but no obvious difference in cell structure. *Gloeothece* nitrogenase is, unlike that of *Anabaena* and *Plectonema* species, particulate, but nevertheless is highly O_2-sensitive *in vitro*. Nitrogenase activity and photosynthetic O_2 evolution are maximal at different stages in the

development of batch cultures and Gallon *et al.* (1975) suggested that aerobic N_2-fixation is favoured by this temporal separation, although resistance to atmospheric O_2 remains to be explained.

It might be illuminating to determine, with techniques like those of Fleming and Haselkorn (1973, 1974), the numbers and kinds of polypeptides whose synthesis is induced by nitrogen starvation in, respectively, nonfixers, anaerobic fixers, non-heterocystous aerobic fixers and heterocystous species (under aerobic and anaerobic conditions), especially since there may now be evidence that the early stages of the development of N_2-fixing ability are comparable in the last three groups (Rippka and Stanier, 1978). That is, it might be instructive to view sequential stages in the development of heterocystous species as analogous to the total response to nitrogen deficiency represented by the "simpler" forms, as if, here, ontogeny recapitulated phylogeny.

4. *Deficiency of Combined Nitrogen in Heterocystous Cyanobacteria*

a. *Heterocyst structure and function.* All aspects of heterocyst structure, biochemistry and development have been reviewed at least once in the last five years (Haselkorn, 1978; Stewart, 1977a, b, 1976, 1977a, b; Stewart *et al.*, 1977; Stewart *et al.*, 1975; Stanier and Cohen-Bazire, 1977; Wolk, 1975, 1973; Fay, 1973; Wilcox *et al.*, 1975a; Carr and Bradley, 1973). My own coverage will therefore be brief, and in many cases lack citations to the earlier literature. Heterocysts are differentiated structures (derived from vegetative cells) which occur at regular, genetically and environmentally determined, intervals along the filaments of all filamentous cyanobacteria which can fix nitrogen in air (Wilcox *et al.*, 1975a; Wolk, 1975). They differ in structure from adjacent vegetative cells in that they: (1) possess a thick three-layered envelope containing several unique structural components, and probably serving as a barrier to oxygen (Cardemil and Wolk, 1976; Wolk, 1973), (2) exhibit a different pattern of thylakoid organization and lack phycobilisomes or, indeed, phycobiliproteins, (3) contain large "polar granules" (probably consisting of cyanophycin granule protein) at the site of attachment to vegetative cells, (4) are connected to the latter only by narrow intercytoplasmic channels ("microplasmadesmata"), and (5) *may* contain little if any (!) DNA (See Haselkorn, 1978).

Heterocysts also differ biochemically from vegetative cells in several important ways: (1) They lack photosystem II activity, both because of a

deficiency in phycobiliproteins and because of depletion of manganese, essential to the photolysis of water (Tel-Or and Stewart, 1977). However, they retain components of the electron transport chain linking the two photosystems and a functional photosystem I, allowing cyclic photophosphorylation (ATP formation) at wavelengths absorbed by chlorophyll *a*, which are optimal for N_2-fixation in short-term experiments (see Stewart, 1977a, b). They also retain the capacity for oxidative phosphorylation. (2) They lack RUDP carboxylase activity, show no carboxysomes and no immunologically detectable RUDP carboxylase (Codd and Stewart, 1977b). (3) They contain higher levels of nitrogenase (which may or may not also be present in vegetative cells; see Haselkorn, 1978, for discussion) and of glutamine synthetase, and lower levels of glutamine-oxoglutarate amido transferase (glutamate synthase). (4) They contain elevated levels of hexokinase, and activities of glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase are six- to eight-fold greater than in vegetative cells (Lex and Carr, 1974; Winkenbach and Wolk, 1973; Apte *et al.*, 1977).

The current consensus on heterocyst function appears to be this: Nitrogenase requires protection from O_2 and this is ensured by the heterocyst envelope and by the absence in heterocysts of photosystem II (O_2-evolving) activity. Nitrogenase requires for function ATP and a reductant. The former is provided in the light by photosystem I activity (cyclic photophosphorylation) within the heterocyst and in the dark perhaps by oxidative phosphorylation at the expense of NADPH, produced by the oxidative pentose phosphate cycle using carbohydrate imported from adjacent vegetative cells. Reductant also derives from the oxidation of imported carbohydrate (perhaps maltose; Jüttner and Carr, 1976) *via* the oxidative pentose phosphate cycle. The ultimate electron donor for nitrogenase is probably a reduced ferredoxin (which may be heterocyst-specific; Haselkorn, 1978). This is itself reduced either by ferredoxin-NADP oxidoreductase (Tel-Or and Stewart, 1976; Apte *et al.*, 1977) or pyruvate-ferredoxin oxidoreductase, or both. The product of nitrogenase is ammonia, and its export form is glutamine; heterocyst levels of glutamine synthetase are thus high. In adjacent vegetative cells, glutamine-oxoglutarate amido transferase (GOGAT, or glutamate synthetase) produces from one mole of glutamine and one of α-ketoglutarate (oxoglutarate), two moles of glutamate. One of these must return to the heterocyst for further transport of amino groups to occur; the second is free to provide fixed nitrogen for synthesis of cell substance (Wolk *et al.*, 1976).

b. *Heterocyst formation.* In heterocyst-forming cyanobacteria, removal of combined nitrogen results first in degradation of phycobiliprotein in all cells of the filament and subsequently in induction of nitrogenase and heterocyst formation. Under anaerobic conditions, nitrogenase is probably formed in all cells of the filament. This occurs even in mutants of normally heterocystous cyanobacteria incapable of forming these structures (Rippka and Stanier, 1978), while heterocyst production can occur even in mutants lacking nitrogenase (Currier *et al.*, 1977; Singh *et al.*, 1977). The two processes are thus at least partially independent and heterocyst formation can be viewed as a relatively recent evolutionary development superimposed upon the general response of nitrogen-fixers to nitrogen limitation.

Progressive ultrastructural changes during heterocyst formation have been documented for a number of species (Fay, 1973; Wilcox *et al.*, 1973; Fleming and Haselkorn, 1974; Haselkorn, 1978). Cells on their way to becoming heterocysts are commonly called "proheterocysts" but it is not clear whether all authors use this term in the same sense. Wilcox *et al.* (1973) distinguish seven stages in heterocyst maturation in *Anabaena catenula*; Fleming and Haselkorn (1974) distinguish four in *Anabaena* 7120. It is clear that the process usually involves, at least: (1) sequential elaboration of the three layers of the thick heterocyst envelope, (2) loss of granular inclusions (glycogen, polyphosphate, cyanophycin granules, carboxysomes, phycobilisomes, but not ribosomes), (3) enlargement of the developing proheterocyst and constriction of its contact with neighbouring cells, (4) changes in the organization and perhaps amount of DNA, and (5) in very late stages, reorganization of photosynthetic lamellae and construction of polar granules, the latter frequently being considered diagnostic of heterocyst maturity. Proheterocysts, by usual operational definitions, do not fix N_2 aerobically and can, at least until late stages, be persuaded to "regress" to vegetative status by restoration of ammonia (Bradley and Carr, 1976, 1977) or detachment from adjacent vegetative cells (Wilcox *et al.*, 1973, 1975a).

Fleming and Haselkorn (1973, 1974) have characterized in some detail the numbers and kinds of polypeptides synthesized by developing heterocysts and vegetative cells in differentiating filaments of *Anabaena* 7120 (at that time called *Nostoc muscorum*). [^{35}S] sulphate was used to label protein at various periods ("pulse" and "pulse-chase" experiments) before and during adaptation to a medium deficient in combined nitrogen. Polypeptides were resolved by one-dimensional sodium

dodecyl sulphate-polyacrylamide gel electrophoresis. Lysozyme treatment (to which heterocysts and late proheterocysts are resistant) was used to identify polypeptides confined to heterocysts or to vegetative cells, or common to both. Several developmental classes were thus identified: (1) proteins present in vegetative cells but degraded in heterocysts by proteases which appear to be induced by nitrogen starvation (Wood and Haselkorn. 1976, 1977), (2) proteins synthesized early, late or continuously in heterocysts but not vegetative cells, (3) proteins synthesized early, late or continuously in vegetative cells but not heterocysts, and (4) proteins synthesized in both vegetative cells and proheterocysts but enjoying continued synthesis only in the latter. Nitrogenase components (tentatively identified by electrophoretic mobility, see Tsai and Mortenson, 1977) appear to be among these. Failure of nitrogenase polypeptides to persist in vegetative cells may reflect either degradation by a protease specific to oxygen-inactivated proteins and confined to vegetative cells or specific repression of nitrogenase structural gene transcription (Haselkorn. 1978). However, the early appearance of nitrogenase components in proheterocysts is surprising in view of the finding of Rippka and Stanier (1978) that, even under strict anaerobiosis, nitrogenase activity does not appear for some 20 h after the removal of combined nitrogen.

For many developmental processes it is possible to identify commitment times as those after which differentiative events, once induced, cannot be reversed or aborted by transfer to non-inducing conditions. Such commitment times have been identified for both proheterocyst and mature heterocyst formation in cyanobacteria. These differ from each other and also vary with the manipulations used to define them. Bradley and Carr (1976) found that proheterocysts first appeared in ammonia-starved filaments of *Anabaena cylindrica* in about 5.5 h and were maximal in number at about 14 h. However, the filaments were committed to produce some proheterocysts by 2.3 h (i.e. would do so even if ammonia were restored at that time) and were maximally committed at about 6 h. Commitment to form at least some mature heterocysts occurred at 5 h and maximal commitment was at about 10 h, even though mature heterocysts themselves were not first seen until 14.5 h and were not maximal in number until 28 h.

Illumination is required for the initial commitment to differentiation in *Anabaena cylindrica* (Bradley and Carr, 1977). A mere 0.5 to 1.0 h of illumination after removal of ammonia allows proheterocyst formation in

subsequent darkness, although this commitment period is prolonged by about one hour if proflavin, rifampicin or ammonia is added at the time of exclusion of light. A minimum of 3 h of illumination is required for the appearance of *any* mature heterocysts, while 7–8 h is necessary for maximum heterocyst formation. Proflavin, fluorouracil and ammonia can block final differentiation if added between 8 and 10 h in the dark. It is not clear whether the light requirement is an energetic or "photomorphogenetic" one, and it would be interesting to determine action spectra for this effect.

Oxygen is also required for the formation of mature heterocysts in certain cyanobacteria. Nitrogen starvation under strict anaerobiosis (DCMU in argon-CO_2) promotes hyperinduction of (O_2-sensitive) nitrogenase in all cells of the filament (Rippka and Stanier, 1978), but proheterocysts are arrested at an early stage. The role of oxygen in heterocyst maturation is unlikely to be secondary (i.e. energetic) since O_2-dependence is observed even in heterotrophic strains provided with glucose as a source of reductant and light as a source of ATP.

c. *Control.* There is good circumstantial evidence that glutamine, the major export product of functioning heterocysts, is a negative effector controlling both nitrogenase synthesis and heterocyst differentiation. Molecular nitrogen clearly does not induce these processes, since both occur in its absence (e.g. under argon-CO_2). Sources of combined nitrogen (especially ammonia) do normally block them, even in the presence of N_2 (Haselkorn, 1978; Bradley and Carr, 1976; Rippka and Waterbury, 1977; Rippka and Stanier, 1978; Fay, 1973). Their effect is not direct, however. Nitrogenase synthesis and heterocyst formation are stimulated, even when combined nitrogen is present, by methionine sulphoximine, an inhibitor of glutamine synthetase. Stewart and Rowell (1975) showed that the addition of this amino acid analogue to N_2-fixing cultures of *Anabaena cylindrica* resulted in: (1) loss of glutamine synthetase activity, (2) depletion of glutamine and glutamate pools, and (3) excretion of N_2 fixed, as ammonia. Accumulated ammonia did not repress (as it normally would) nitrogenase synthesis or heterocyst formation in such methionine sulphoximine-intoxicated cells. When ammonia-grown cultures lacking nitrogenase and heterocysts were exposed to methionine sulphoximine, mature heterocysts and functioning nitrogenase appeared after 40 h, as they would do if ammonia had been removed. When N_2-grown cells were rid of nitrogenase, but not heterocysts, by brief exposure to ammonia (which

represses *de novo* synthesis and allows rapid *in vivo* destruction of nitrogenase) and *then* treated with methionine sulphoximine, nitrogenase activity appeared in the existing heterocysts within 10 h (Stewart and Rowell, 1975; Rowell *et al.*, 1977). Glutamine synthetase was shown to be sensitive to methionine sulphoximine *in vitro*, although only at much higher concentrations. This discrepancy was attributed by Stewart and Rowell (1975) to an ability of the cells to concentrate the analogue, and by Haselkorn (1978) to its possible *in vivo* conversion to a more active derivative.

These results are consistent with the hypothesis that either glutamine synthetase itself, or one of its products, or both, negatively control(s) nitrogenase and heterocyst formation. In some bacteria, glutamine synthetase may be directly involved in activation of nitrogenase structural gene transcription (Ausubel *et al.*, 1977). This appears not be be true in *Anabaena cylindrica*, for several reasons: (1) activity of glutamine synthetase is negatively, not positively, correlated with nitrogenase synthesis, (2) glutamine synthetase does not appear to be adenylylated, as bacterial glutamine synthetases serving regulatory roles characteristically are, and (3) it shows no immunological cross-reactivity with such covalently modifiable enzymes, which usually show cross-reactivity among themselves. On the other hand, levels of *glutamine* do correlate well (and inversely) with nitrogenase synthesis, suggesting that this amino acid may negatively regulate (repress) nitrogenase- and heterocyst-specific gene-expression (Rowell *et al.*, 1977; Ownby, 1977).

d. *Approaches to the study of heterocyst differentiation.* An understanding of molecular events during nitrogenase induction and heterocyst differentiation is, and should be, a major goal of modern cyanobacteriology, for reasons which are both academic and practical. Differentiation is not only temporal but, as in multicellular eukaryotes, spatial. Rules governing the recruitment of proheterocysts from vegetative cells have been defined and a formal understanding of this useful model system is beginning to emerge (Wilcox *et al.*, 1973, 1975a; Wolk, 1975; Wolk and Quine, 1975; well reviewed by these authors and by Haselkorn, 1978, and thus not covered here). The possibility of isolating conditionally lethal mutants of heterocyst-formers which behave (under non-permissive conditions) as do methionine-sulphoximine intoxicated wild-type cells, is a real one. A temperature-sensitive glutamine synthetase mutant should, for

instance, efficiently carry out a light-dependent conversion of N_2 to ammonia and thus provide fixed nitrogen for agricultural applications at little cost.

N_2-fixing cyanobacteria might also be persuaded to convert light energy to chemical energy. Cyanobacterial nitrogenase catalyzes a "wasteful" ATP-dependent, N_2-inhibitable, but CO-insensitive evolution of molecular hydrogen ($2H^+ + 2e \rightarrow H_2$; Peterson and Burris, 1978; Lambert and Smith, 1977). Evolved hydrogen can be recycled ($H_2 \rightarrow 2e24 + 2H^+$) to provide reductant for nitrogenase activity and perhaps oxygen-scavenging. This activity is promoted by an inducible "uptake" hydrogenase which is ATP-independent and CO-inhibitable (Tel-Or *et al.*, 1977; Bothe *et al.*, 1977a, b). Conditions which inhibit this latter activity promote net H_2 evolution and both chemical and genetic manipulations might be employed to develop biological systems for generation of H_2 in useful quantities (Benemann and Weare, 1974).

Further progress in understanding and manipulating N_2-fixation and differentiation in cyanobacteria will depend strongly on the application of molecular genetic techniques, as has the current and still incomplete understanding of sporulation in bacilli (e.g. Young, 1978). It would be useful to isolate, as Currier *et al.* (1977), Wilcox *et al.* (1975b) and Singh *et al.* (1977) have begun to do, mutants blocked in successive steps in heterocyst formation and to characterize, by polyacrylamide gel electrophoresis, heterocyst and vegetative cell-specific polypeptides synthesized before and after phenotypic expression of the block. It would be useful to identify, by Southern (1975) hybridization, mRNAs specific to differentiating cells and to clone in *E. coli*, DNA fragments from which they are transcribed. Cloned DNAs can serve initially as probes for heterocyst-specific transcription *in vivo* and, later, as templates in experiments designed to duplicate heterocyst transcription patterns *in vitro*. It would be *most* useful to develop a system for genetic analysis in at least one heterocystous cyanobacterium and concentrate further efforts on such a species. There is no evidence, except for very preliminary reports (e.g. Stewart and Singh, 1975) that such a system exists for any heterocystous form. In the absence of genetics, it might be best to concentrate biochemical and molecular biological efforts either on *Anabaena cylindrica* (because it is best known), *Anabaena variabilis* (because it is facultative chemoheterotroph; Wolk and Shaffer, 1976) or the newly isolated *Anabaena* species described by Stacey *et al.* (1977a, b) because it grows very rapidly.

F. EFFECTS OF SOURCES OF PHOSPHATE, SULPHATE AND IRON

Deficiency of phosphate (Ihlenfeldt and Gibson, 1975b) sulphate (Prakash and Kumar, 1971) and iron (Öquist, 1971, 1974a) all provoke chlorosis (depigmentation) in cyanobacteria. In the case of phosphate, the response appears the same as that to nitrogen or CO_2 deficiency, since PC is preferentially lost, viability is retained, and PC is replenished when nutrient is restored. (Data for sulphate- and iron-starved cells are insufficient to allow one to decide whether the response is similar or simply reflects progressive cellular disorganization.) Deficiency of each of the three nutrients also appears to provoke "simple" regulatory responses in systems involved in uptake or utilization.

Bone (1971) reported that alkaline phosphatase levels varied (over a 20-fold range) inversely with extracellular phosphate in chemostat-grown *Anabaena flos-aquae*. Healey (1973) and Ihlenfeldt and Gibson (1975b) observed dramatic increases in cellular alkaline phosphatase upon exhaustion of exogenous orthophosphate in batch cultures of *Anabaena* or *A. nidulans*. Readdition of nutrient repressed this apparent *de novo* synthesis, but existing activity was lost only by dilution.

Prior sulphate starvation substantially enhances sulphate uptake in *A. nidulans* (Jeanjean and Broda, 1977). This enhancement is inhibited (although incompletely) by addition of chloramphenicol during starvation, and it seems likely that it requires *de novo* synthesis of a component of the sulphate transport system.

Iron starvation provokes, in many prokaryotes, excretion of iron chelators (siderochromes) which function to release ferric iron from insoluble complexes (e.g. ferric hydroxide) and promote its assimilation. Siderochromes have been identified in several cyanobacteria (Simpson and Neilands, 1976; Armstrong and Van Baalen, 1975, 1976). In at least one, siderochrome production is completely suppressed by addition of 0.01m*M* ferrous sulphate to the medium, although there is no certainty that this reflects repression of the formation, rather than inhibition of the activity, of siderochrome-producing enzymes. A more certain effect of iron on patterns of protein synthesis was shown for ferredoxins and flavodoxin in *Nostoc* (strain MAC) by Hutber *et al.* (1977). At low exogenous iron concentrations, flavodoxin synthesis increases dramatically, while that of the two ferredoxins is depressed, presumably at the level of apoprotein structural gene expression.

G. EFFECTS OF ANAEROBIOSIS AND OXYGEN

A number of cyanobacterial strains exhibit photosystem II-independent, sulphide-dependent, facultative anoxygenic photosynthesis in sulphide-rich environments (Garlick *et al*, 1977; Cohen *et al.*, 1975a, b). Adaptation to such environments after growth under aerobic conditions requires, at least in *Oscillatoria limnetica, de novo* protein synthesis. Addition of sulphide immediately blocks oxygenic photosynthesis (presumably by poisoning photosystem II), but permits limited (photosystem I-dependent) protein synthesis. After two hours, sulphide-dependent CO_2 photo-assimilation develops; this development is completely prevented by chloramphenicol added at zero time, but becomes chloramphenicol-insensitive after 90 min (Oren and Padan, 1978). It seems important to determine the numbers and kind, and ultimately the nature, of polypeptides synthesized during the adaptation period.

Although no cyanobacterium is obligately anaerobic, too much oxygen and light, in the presence of too little CO_2, leads to "photooxidative death". Eloff *et al.* (1976) believe this to be an important cause of "die off" of cyanobacterial blooms and a determinant of cyanobacterial distribution in nature. Superoxide dismutase plays a major role in protecting against photooxidation in many microorganisms. Its levels in cyanobacteria are correlated with resistance to high light and oxygen and, not surprisingly, are controlled by oxygen tension. Transfer of *A. nidulans* from growth under N_2 to growth in air results in a dramatic increase in superoxide dismutase specific activity, beginning at two hours and completed at four. No induction occurs in the dark in the presence of chloramphenicol (Abeliovich *et al.*, 1974).

H. EFFECT OF AMINO ACIDS

In the best studied heterotrophic bacteria, amino acid biosynthesis is characteristically controlled by feedback inhibition of an early (usually the first) pathway-specific enzyme and by repression of the transcription of structural genes coding for most or all pathway-specific enzymes. Although feedback inhibition is common in cyanobacterial amino acid biosynthesis, repression appears to be very rare indeed (Carr, 1973). This "failure" to exert control remains a major argument for the regulatory inflexibility of cyanobacteria, and it is well documented: (1) Exogenous arginine, although incorporated into protein by *Anabaena variabilis*, fails

to repress the formation of enzymes of arginine biosynthesis in this organism (and in *A. nidulans, Chlorogloea fritschii* and *Gloeocapsa alpicola*; Hood and Carr, 1971; Hood *et al.*, 1969). Furthermore, no significant induction by exogenous arginine of the enzymes of arginine degradation is observed (Hood and Carr, 1971; Weathers *et al.*, 1978). (2) Enzymes of the isoleucine, valine and leucine pathway fail to show repression by exogenous amino acids (Hood and Carr, 1968, 1972). (3) DAHP (3-deoxy-D-arabinoheptulosonic acid) synthetase, the first enzyme of the pathway leading to tryptophan, tyrosine and phenylalanine, is not repressed by addition of these aromatic amino acids (Weber and Böck, 1969).

It can always be argued (Carr, 1973) that the wild-type strains used in such experiments were already fully repressed by endogenously generated amino acids, or that exogenous amino acids simply are assimilated too slowly to elevate internal pools. Studies with amino acid auxotrophs circumvent these problems, and I am aware of four such: (1) In a methionine-requiring mutant of *A. nidulans*, levels of the first enzyme of the methionine pathway were not elevated during acute methionine-starvation, nor were they lowered by methionine excess (Delaney *et al.*, 1973). The enzyme was, however, subject to end product inhibition. (2) levels of enzymes involved in phenylalanine biosynthesis were not affected by phenylalanine starvation or excess in a prephenate dehydratase mutant of *Synechococcus cedrorum* (Kaney and Jhabvala, 1975). (3) Synthesis of the second and third enzymes specific to leucine formation was essentially unresponsive to leucine in several *A. nidulans* auxotrophs deficient in the first enzyme (α-isopropylmalate synthetase) although the wild-type enzyme did show feedback inhibition by leucine (Singer and Doolittle, 1975b). (4) A tryptophan synthetase A protein deficient mutant of the unicellular cyanobacterium *Agmenellum quadruplicatum* (strain BG-1) showed, when starved for tryptophan, four-fold derepression of the first four enzymes of the tryptophan pathway and twenty-fold derepression of the tryptophan synthetase B protein (Ingram *et al.*, 1972).

This last is, to my knowledge, the only published report describing control at the level of gene expression of amino acid biosynthesis in any cyanobacterium. It may however be possible to explain the apparent lack of control in other amino acid biosynthetic pathways. One can argue that the rather restricted number of cyanobacterial species examined have not in nature seen the rather limited variety of amino acids found not to exert repression often enough, or in sufficiently high concentration, to justify

the evolution of controls on the enzymes of the relevant structural genes. To say this is to say that in heterotrophs like *E. coli*, the "purpose" of repression is to eliminate biosynthetic enzymes when *exogenous* amino acids render them superflous and that controls on enzyme activity alone should be sufficient to adjust rates of *endogenous* amino production.

I. CONCLUDING REMARK

I have devoted rather a lot of space to the description of specific instances in which cyanobacterial gene expression appears to be, or appears not to be, controlled, and this may seem strange to the reader more familiar with the bacterial than the cyanobacterial literature. I have done so because the notion that blue-greens are fundamentally different from bacteria, that they are more primitive and poorly adapted, is still very prevalent in the cyanobacterial literature. This notion derives in part from the fact that structures resembling modern cyanobacteria appear very early in the fossil record (Knoll, 1977), and in part from the demonstration by Carr (1973) that aspects of cyanobacterial metabolism which one might expect to be regulated at the level of gene expression are not (although Carr himself does not describe this as a "primitive" trait). I feel that modern cyanobacteria are in no meaningful sense more primitive than modern bacteria, that they can and do regulate gene expression in sophisticated ways when this is to their advantage, and that cases in which they fail to do so can be rationalized. This is not to say that I have, in the preceding sections, successfully rationalized them. Nor is it to say that cyanobacteria are simply blue-green *E. coli* and that further study of the ways in which they regulate gene expression will not be intellectually rewarding. The "complex" controls involved in adjusting to light colour and intensity are perhaps unique to cyanobacteria, and those involved in the response to nitrogen deficiency are uniquely well developed in them. Those of us interested in cyanobacterial gene expression would do well, I think, to specialize in those areas in which blue-greens themselves appear to have specialized.

VIII Genetics

A. INTRODUCTORY REMARKS

Cyanobacterial molecular biology is now where *E. coli* molecular biology was in the 1950's and it is not likely to progress rapidly until genetic

analyses and manipulations can be performed in cyanobacteria with the ease with which they have long been performed in *E. coli*. Cyanobacteria do "have genetics". Much of the cyanobacterial genetic literature has recently been reviewed by Delaney *et al.* (1976a) to whom the reader should turn for a more complete discussion.

Cyanobacteria are sensitive to a number of mutagens, nitrosoguanidine-(*N*-methyl-N'-nitro-*N*-nitrosoguanidine) perhaps being the most frequently used in spite of its propensity to produce multiple lesions. A reasonably large collection of mutants (described by Delaney *et al.*, 1976a) have been isolated from a limited number of species. These include mutants resistant to antibiotics and inhibitors, mutants with altered morphology or development (most recently those of Currier *et al.*, 1977), and mutants with altered light-harvesting characteristics (most recently those of Sherman and Cunningham, 1977). Penicillin selection allows the isolation of a variety of auxotrophs (Herdman *et al.*, 1973; Herdman and Carr, 1972; Singer and Doolittle, 1975b, Currier *et al.*, 1977; Stevens *et al.*, 1975). Initial fears that blue-greens are for some reason (e.g. "polyploidy" or impermeability, Kaney and Dolack, 1972) incapable of sporting auxotrophic mutants appear unjustified (Delaney *et al.*, 1976b).

Mutants alone do not give genetics. Cyanobacteria can exchange genetic information, possibly by mating and certainly by transformation. They have not yet been shown to do so by transduction. Development of *useful* systems for genetic analysis will require the concentrated efforts of many laboratories; this has barely begun.

B. "MATING"

In 1962, Kumar reported "genetic recombination" between streptomycin and penicillin resistant mutants of *A. nidulans* to produce double-resistant progeny. These studies were performed before adequate cloning techniques were available , and Pikalek (1967) was unable to reproduce Kumar's observations, which he attributed to the instability of penicillin. Singh and Sinha (1965) performed experiments with the same methods, conclusions and limitations as Kumar's with streptomycin and penicillin resistant strains of *Cylindrospermum majus*. Clonally derived (axenic) polymyxin B and streptomycin resistant mutants of *A. nidulans*, and plating techniques which allowed quantitation, were first used by Bazin (1968). He obtained what he considered evidence of

recombination in this organism, but the frequencies observed were extraordinarily low (5×10^{-9} to 5×10^{-7} per cell.) More recently, Stewart and Singh (1975) obtained apparent transfer of nitrogen fixation (*nif*) genes in *Nostoc muscorum* at frequencies up to 5×10^{-5}, some 200-fold greater than the frequency of spontaneous reversion.

It is not clear that any "mating" experiments, including those of Bazin (1968) and Stewart and Singh (1975), which were probably "successful", truly demonstrate conjugational processes, and Delaney *et al.* (1976a) attribute these results to transformation by "extracellular" nucleic acid (see below). It is distressing that successful mating systems have not been further developed, and the conception underlying them all may be naive. Even if cyanobacteria possess fertility factors analogous to the *E. coli* F factor which promote conjugation, matings between clonally-related mutants are not likely to be particularly fertile. Both "donor" and "recipient" will, except for rare spontaneous Hfr or F^- variants, be of the same "sex" (see Stent, 1971). It might be profitable to attempt crosses between mutants derived from distinct natural isolates of what appear to be the same species, some of which may differ in content or state of putative fertility factors.

C. TRANSFORMATION

Although the existence of genetic exchange between intact cyanobacterial cells remains to be convincingly shown, there *is* now ample evidence that DNA-mediated transformation does occur in at least three species; and reason for confidence that this system can be exploited successfully in genetic analysis. Eight years ago, Shestakov and Khyen (1970) documented transformation of wild-type *A. nidulans* to erythromycin resistance, using DNA isolated from an erythromycin resistant donor. Streptomycin resistance and a morphological marker, *fil*, could also be transferred to wild-type and mutant recipient cells. The process was deoxyribonuclease-sensitive and enhanced by light. Although transformation frequencies were initially low, more efficient procedures have recently been developed by these and other workers (e.g. Grigorieva and Shestakov, 1976). The techniques and results described by Orkwiszewski and Kaney (1974) are particularly straightforward and encouraging. They found that streptomycin resistance and wild-type alleles of lesions affecting phenylalanine and arginine biosynthesis were transferred at high-frequency (one in 10^3) to appropriate recipient cells,

and the transformants obtained were stable. Recipients were maximally "competent" at the end of the logarithmic phase of growth.

Results presented by Herdman and Carr (Herdman and Carr, 1971a; Herdman, 1973a, b) are less straightforward. These workers described two transformation systems in *A. nidulans*, one mediated by chemically extracted DNA and the other by nucleic acids apparently excreted into the medium by growing cultures of *A. nidulans*. Although transformation in the former system was, as expected, completely deoxyribonuclease-sensitive, that in the latter was only partially so and Herdman and Carr (1971a) evoked a DNA:RNA hybrid as transforming principle. RNA and DNA:RNA hybrid mediated transformation *have* been reported sporadically in bacterial systems (see Notani and Setlow, 1974), and Szalay *et al.* (1973a, b) have described a DNA:RNA hybrid of unknown function in total nucleic acid extracts of *A. nidulans*. However, both transformation systems described by Herdman and Carr (Herdman, 1973a, b; Herdman and Carr, 1971a) show still another and more disturbing peculiarity; a very high frequency (up to 100 per cent) of recipients transformed acquired easily detected mutations which were present in neither parent. Mutation during recombination has been reported in bacterial systems (e.g. Yoshikawa, 1966) but never at such high frequency, and Orkiszewski and Kaney found *no* evidence for mutation in their studies of DNA-mediated transformation in *A. nidulans* (A. R. Kaney, personal communication).

Transformation with extracted DNA has also been shown for the facultatively chemoheterotrophic unicellular species *Aphanocapsa* 6714 (Astier and Espardellier, 1976) and most recently, for *Gloeocapsa alpicola*, by Devilly and Houghton (1977). These workers found that wild-type cells of this strain could only be transformed to streptomycin resistance by DNA from streptomycin-resistant donors after cold-shock and calcium chloride treatment, but after such treatment could be transformed at high efficiency (3×10^{-3} transformants per recipient cell). Similarly high efficiency with DNA from streptomycin-resistant *Gloeocapsa alpicola* could be obtained "intergenerically", with *A. nidulans* as recipient. Transformation was, as in the experiments of Herdman (1973a, b), partially sensitive to both deoxyribonuclease and ribonuclease.

D. TRANSDUCTION

Phage-mediated transduction has in fact never been shown in cyanobacteria, but my organizational scheme allows me no other place to

alert the reader to the fact that cyanobacteria are sensitive to a number of specific viruses; some of these are temperate and some may someday prove capable of transduction. The extensive cyanophage literature has been reviewed very recently by Sherman and Brown (1978), and less recently by Padan and Shilo (1973), Safferman (1973) and Brown (1972). In fact, the most reliable genetic analyses and perhaps the most sophisticated "molecular" genetics yet performed in cyanobacteria are those performed with the temperate cyanophage LPP2-SP1. This virus efficiently lysogenizes *Plectonema boryanum* but lysogens cannot be "induced" by usual treatments (Padan *et al.*, 1972). A mutant (SP1ctsl) which is virulent at high temperature and temperate at low has been isolated (Rimon and Oppenheim, 1975). Lysogens formed after low-temperature infection by this mutant can be induced by temperature shift. There is some very interesting molecular biology here: heat-induction of SP1ctsl prophage requires light and is inhibited by DCMU or CCCP. Carbon dioxide is not required. After induction, phage development can proceed in darkness, and productive lytic infection can occur in the total absence of light (Rimon and Oppenheim, 1975; Cocito and Goldstein, 1977). Since DCMU (which allows continued ATP production but no photosynthetic electron flow) blocks induction but CO_2-deprivation does not, the light requirement is either very direct or mediated through NADPH.

Rimon and Oppenheim (1974, 1976) isolated and characterized 23 temperature-sensitive mutants of LPP2-SP1. These fell into 14 complementation groups, and were mapped by two-factor crosses. Characterization of proteins synthesized at permissive and non-permissive temperature allowed preliminary identification of regions of the apparently linear genetic map corresponding to early and late proteins.

E. CYANOBACTERIAL GENETIC MAPS

Very preliminary maps of some markers on the genome of *A. nidulans* have been prepared in two ways. Herdman (1973a, b) ordered four auxotrophic and one antibiotic-resistance marker by cotransformation, although the high frequency of attendant mutation precluded measurement of recominational distance. Asato and Folsome (1970) and Delaney and Carr (1975) have constructed "temporal genetic maps" using nitrosoguanidine or ethyl methanesulphonate mutagenesis during

synchronous genome replication induced by prior dark incubation (see Section IVE3). Delaney *et al.* (1976a) pointed out that the relative position of ten *A. nidulans* markers correlated well with the position of what they took to be homologous genetic loci in *E. coli*. The coincidence is striking but should not, I think, be considered more than coincidence until biochemical lesions in the cyanobacterial mutant strains are more precisely defined.

IX. Prospects

If I have taken a "position" in this review, it is this: Blue-greens are prokaryotes not only in their ultrastructure and physiology but in their molecular biology. They are (at least on the basis of 16S rRNA homology) as remote from the enteric bacteria or the bacilli as these two groups are from each other, but they are no *more* remote than that (Bonen and Doolittle, 1976; Woese and Fox, 1977). Recognizable blue-greens are common in the early fossil record and their apparent structural conservation prompts some to regard cyanobacteria as primitive "living fossils". They are not, and there is no reason to believe that they do important things (like regulate the expression of their genomes) in "primitive" ways. Cyanobacteria have however adapted to ecological niches quite different from those occupied by heterotrophic bacteria, and individual adaptive mechanisms and the ways in which they are integrated are of considerable interest. To study them we will need to make more aggressive use of the techniques of bacterial biochemical genetics and molecular biology. We need more mutants altered in their response to environmental variables such as light and nitrogen availability. We desperately need a usable system for genetic analysis in cyanobacteria. It seems likely that the transformation system known for *A. nidulans* can be made more reliable if sufficient effort is devoted to optimization and standardization of conditions for DNA uptake and intergration. Similar efforts must be made to develop systems for genetic exchange in heterocystous nitrogen-fixing species, and the search for transducing cyanophages must continue and intensify.

As, I believe, this review makes apparent, the cyanobacterial molecular biological literature has considerable breadth but little depth. Few of the fascinating molecular biological problems which blue-greens pose have been investigated with the full battery of techniques now

available, and none has been "run into the ground". There are reasons for this: general techniques for obtaining pure (axenic and unialgal) cultures, for isolation of mutants, and for plating on solid media to produce single clones are all of fairly recent origin (see Delaney *et al.*, 1976a; Stanier *et al.*, 1971). The absence of useful methods for genetic analysis is a constant limitation. Perhaps equally important is the tendency of cyanobacteriologists to be generalists, to study many aspects of the physiology of many species and not (as bacterial molecular biologists do) to exploit a single organism and concentrate on a limited number of biochemical or biological processes within it. Although the holistic and eclectic approach of cyanobacteriologists may be philosophically laudable, I fear that it is less productive than the narrow, reductionist attitude shown by most bacterial molecular biologists.

If I were to select areas in which molecular biological progress is liable to be most rapid in the next several years, I would select these: (1) Nitrogen fixation. Our understanding of the biochemistry of this process is already well advanced and characterization of the changes in morphology and patterns of protein synthesis accompanying heterocyst differentiation proceeds at an accelerating pace. Mutants blocked at various stages in heterocyst formation (e.g. Currier *et al.*, 1977) should tell us much, even in the absence of useful genetics, and I would not be surprised soon to see genes involved in nitrogen fixation and differentiation cloned in *E. coli*. (2) Chromatic adaptation and phycobiliprotein synthesis. Bogorad's group seems well on its way to isolating mRNAs specific for phycoerythrin and phycocyanin in *Fremyella diplosiphon*. With these it should be possible to identify and clone DNA fragments carrying apoprotein structural genes, and investigate controls on their expression *in vivo* and *in vitro*. (3) Plasmid function. Plasmids are common in cyanobacteria and it seems not unreasonable to suppose that they carry genes of significance in determining responses to and effects upon the natural environment, as they do in bacteria (Falkow, 1975). Likely candidates for cyanobacterial plasmid-borne determinants are those for gas vacuole production (Walsby, 1977b), penicillinase production (Kushner and Breuil, 1977) and, I anticipate, toxin production (see work of Gorham and others reviewed by Fogg *et al.*, 1973). Some plasmid-determined genetic functions should be identified within the next few years. Such identification will allow the determination of plasmid transmissibility and an assessment of the role of extrachromosomal elements in cyanobacterial ecology.

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Early Events During Bacterial Endospore Formation

M. YOUNG* and J. MANDELSTAM

Microbiology Unit, Department of Biochemistry, University of Oxford, OX1 3QU, U.K.

I. Introduction 104
II. Morphogenetic changes during sporulation 105
III. Biochemical changes during stages 0 to II 110
A. Categories of events 111
B. Marker events associated with stages 0 and I 113
C. Marker events associated with stage II 117
D. Later events 119
IV. Changes in transcription and translation apparatus 119
A. Transcription 119
B. Translation 124
C. Summary 127
V. Genetics and regulation of spore formation 127
A. Estimates of molecular complexity 127
B. Methods and nomenclature 129
C. Number of sporulation specific genetic loci 131
D. Pleiotropy, dependent sequence and phenotypic exclusion in sporulation mutants 132
E. Genetic loci concerned with stages 0 to II 134
VI. Induction of sporulation 142
A. Population probability factor and the hunt for the repressor . . . 142
B. Glutamine synthetase 144
C. Highly phosphorylated nucleotides 145
D. The effect of purine starvation 146
E. Summary of biochemistry of induction 147
F. Requirement for DNA replication 148
VII. Compartmentation and division of labour 151
VIII. Conclusion 153
IX. Acknowledgements 154
References 154

*Present Address: Department of Botany and Microbiology, University College of Wales, Aberystwyth SY23 3DA, Dyfed, U.K.

I. Introduction

Bacterial sporulation was last reviewed in this series by Murrell (1967). Since then there have been a considerable number of reviews, many of them very comprehensive and extending over the whole intervening period up to 1977 (Kornberg *et al.*, 1968; Mandelstam, 1969, 1971, 1976; Murrell, 1969; Schaeffer, 1969; Hanson *et al.*, 1970; Balassa, 1971; Dawes and Hansen, 1972; Freese, 1972; Szulmajster, 1973a; Aronson and Fitz-James, 1976; Hoch, 1976; Piggot and Coote, 1976; Doi, 1977a; Strauss, 1977). The most recent advances in the subject, particularly those arising out of the new genetic techniques that are being developed by the use of restriction enzymes, are covered by the papers read at the 7th International Spores Conference and published in Spores VII, 1978. Any reader who wishes to bring himself up to date on the basic facts of sporulation can therefore do so quite easily. It is noteworthy that the number of people actively working on sporulation has been increasing very rapidly. This is reflected in the fact that the number of contributions to the Spore Conference has increased considerably over the years. There has been a corresponding increase in the number of papers on the subject appearing in the Journals.

Because of the volume of publications, a detailed coverage even of fairly recent work would result in an unreadable catalogue of references which would, anyway, be incomplete. We decided instead to restrict the scope of the review to a consideration of spore formation as a simple example of cell differentiation. Even so, we have omitted a number of topics that many readers may consider important, such as the problem of "commitment" and the effects of intercalating agents and other inhibitors. These are covered at least partly in other reviews, e.g. Piggot and Coote (1976); Doi (1977a). Furthermore, we have concentrated our discussion on the earlier parts of the process. This is because they pose many of the basic problems that need to be answered in any study of cell differentiation.

In the last ten years the whole study of the subject has changed radically. This has been the result of the development of effective methods of genetic analysis in *Bacillus subtilis*. These were established as a consequence of the cumulative efforts of Spizizen (1958) who established the technique of genetic transformation, Takahashi (1963) who described the use of transducing phages and Dubnau *et al.* (1967) who used a combination of these techniques to establish a fairly complete

linkage map of *B. subtilis*. The chromosome was shown by autoradiographic studies to be circular (Wake, 1973). The recent experiments of Lepesant-Kejzlarovà *et al.* (1975) and of Harford (1975) have resulted in the resolution of several anomalies in the earlier mapping data and the revision of the circular linkage map. As a result of all these studies it has been possible to place upon the chromosome virtually all the known mutations affecting sporulation. From this information one can now draw conclusions about the probable number of sporulation genes and discuss possible modes of regulation of the chain of sporulation events in a way that was not previously possible.

Our purpose in this chapter will be to regard sporulation not only as an inherently worthwhile subject for investigation but as a "feasibility study" in the biochemistry and genetics of differentiation of more complex organisms. We shall attempt to determine how far we are now able to assess the molecular complexity of sporulation. The genetic information we now have makes it possible for us to do this, and a rough estimate of complexity of this sort is worth making because it indicates which types of question are likely to be worth pursuing with present day techniques and which are not. Because of the defined bias of this article much of the discussion will be founded on findings obtained with *B. subtilis*, on which virtually all the genetic analysis has been done. Nevertheless, the course of sporulation is much the same in all spore-forming bacilli and any general conclusions that are valid for one species should be valid for the others.

II. Morphogenetic Changes During Sporulation

Spore formation is triggered by starvation, and two different experimental procedures are in use. In the first, the culture is allowed to grow until some nutrient becomes limiting. The time at which growth ceases to be exponential is taken as the time of induction (t_0) and hourly periods after this are denoted by t_1, t_2, etc. The "exhaustion" method has the advantage of simplicity; the disadvantages are considerable and two will be mentioned. First, t_0 is usually ill-defined and vegetative growth may drag on at a declining rate for some time. Second, when the cells do begin to sporulate they are in a complex chemical environment which is changing continuously. This will be true even when the initial growth phase takes place in a defined medium. The reason is that cells

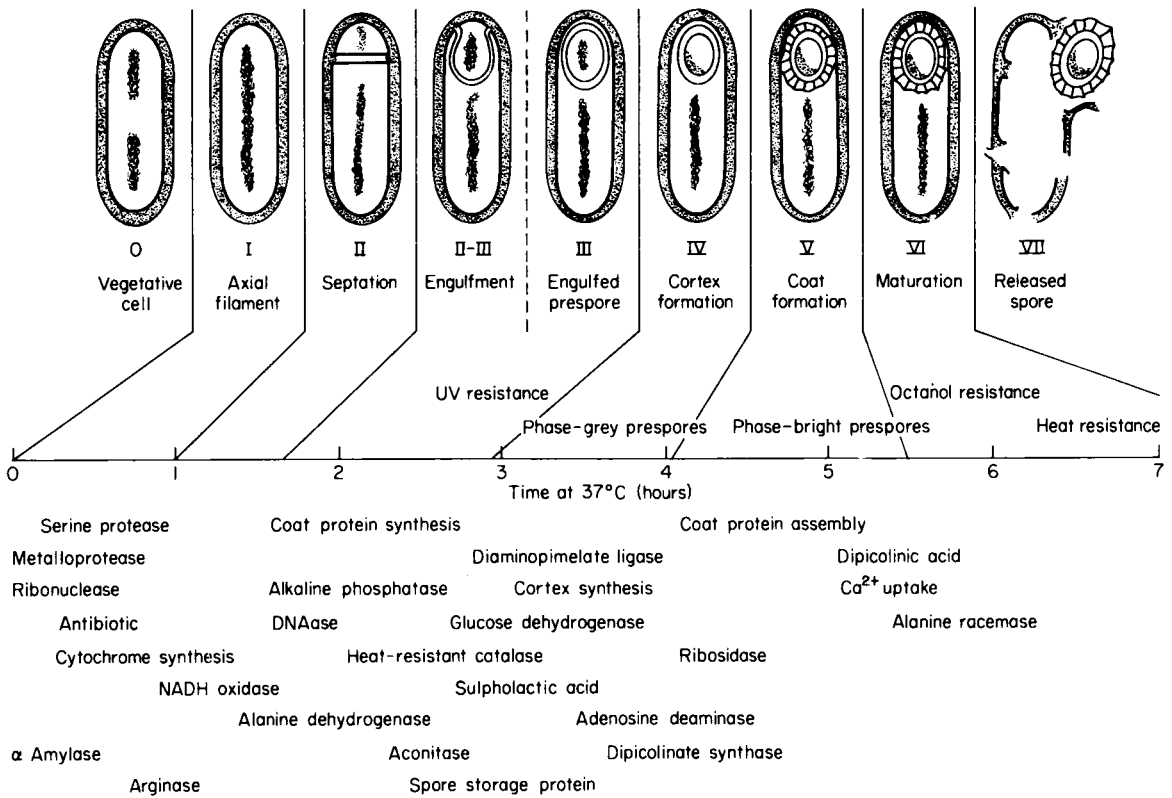


FIG. 1. Schematic diagram of morphological stages of sporulation in *Bacillus* spp. as seen by electron microscopy. The biochemical events are common to most aerobic spore-formers. Some of them are essential for sporulation, some are inessential but appear to be integrally associated, and some are vegetative responses of the cells following the imposition of the nutritional "step-down" that initiates sporulation (see text). The times at which the events occur are somewhat variable and depend on the type of organism and on the experimental conditions.

growing in, say, a solution containing glucose and inorganic ions will accumulate metabolic products of glucose in the medium. The amounts of these are unknown and they are metabolized during the subsequent sporulation. That biochemical changes of this type are going on is shown by the fall in pH that can be observed during growth and its subsequent rise (see Szulmajster, 1964). A highly buffered medium will conceal the underlying changes, but will not prevent them. These metabolites obviously complicate the measurement and interpretation of the biochemical changes that accompany sporulation.

To avoid some of these difficulties, a resuspension technique was introduced by Sterlini and Mandelstam (1969). A culture in exponential growth in a rich medium is centrifuged and the cells are transferred to a resuspension medium containing a suitable carbon substrate such as glutamate. Since carry-over of medium is small, even when the cells are not washed, this method largely removes the complications of having present in the medium a large number of metabolities whose identities and concentrations are unknown. The method also gives a precise time for t_0 and an improved synchrony of sporulation. Even so, difficulty arises because at the time of resuspension the cell population will contain chromosomes at all stages of replication. Since sporulation is tied to chromosome replication (see later) there is an inherent asynchrony in the population that cannot be overcome even when the resuspension method is used.

Although the general order of sporulation events is the same with the exhaustion technique and with resuspension, the precise time at which a particular event occurs may differ somewhat with the two methods. What follows is a summary description of the morphological events that constitute sporulation. These events are based largely on electron microscopy; they have been described for *Bacillus cereus* by Young and Fitz-James (1959 a, b, c), for *B. subtilis* (Ryter, 1965; Ryter *et al.*, 1961, 1966a) and for *Bacillus coagulans* (Ohye and Murrell, 1962). In all these species the general picture is the same and indeed resembles quite closely that found during sporulation in the anaerobic clostridia. For a diagrammatic comparison of sporulation in clostridia and bacilli see Fitz-James and Young (1969).

The events are depicted schematically in a composite diagram based on these studies (Fig. 1). The durations of the stages refer specifically to *B. subtilis* at 37°C (Dawes *et al.*, 1969) and are only approximate.

Stages 0 and I (t_0 to $t_{1.0}$)

Stage 0 refers to the vegetative cell. This contains two or more replicating copies of the chromosome which are seen as discrete, compact and roughly spherical bodies in the cytoplasm. After transfer of the cells to resuspension medium they fuse to form an axial filament that is conventionally described as stage I of sporulation. However, despite a quite intensive search (see Mandelstam *et al.*, 1975), no mutants have ever been discovered which are specifically unable to form axial filaments. Futhermore, similar filaments can often be seen in electron micrographs of *Escherichia coli* and other non-sporulating species. It is therefore probable that the axial filament represents a non-specific response of the DNA when the cells are transferred to a poor medium. For these and other reasons, Yamamoto and Balassa (1969) proposed that the axial filament should no longer be called stage I. However, this usage is now so embedded in the literature that a change in convention would produce confusion without bringing any conceptual advantage. It has therefore been retained by most workers in the field.

Whatever the reasons for filament formation, the chromosomes subsequently separate, again for unknown reasons, and one of them migrates to a position near one of the poles of the cell. In electron micrographs new small mesosomes can often be discerned at sub-polar positions at this stage (Mandelstam *et al.*, 1975). Except for these structures which are, in any case, not visible in all electron micrograph sections, the cells are not noticeably different from those seen at t_0. Nevertheless, the physical changes that have taken place, and the biochemical events that have presumably caused them, are critical in the sense that they have placed the chromosomes in the correct position for the asymmetric septation that is to follow and that is the basis for the whole of the subsequent development. The occurrence of the events up to this point depends on the proper functioning of about ten sporulation-specific genetic loci (see later) and a mutation in any one of them will prevent sporulation altogether.

Stage II ($t_{1.0}$ to $t_{1.5}$): septum formation

A membranous septum forms in a sub-polar position, the site being presumably determined by the fact that the chromosome is now at the end of the cell with its accompanying mesosome. The septum is formed by an invagination of the cell membrane in a manner resembling that which normally takes place at cell division, but it differs from the cell-division

septum in two important respects. First, it is sub-polar rather than central. Second, cell wall material is clearly present in the annular invagination of the incipient spore septum (seen in thin sections as cell wall spikes) but there is very little further deposition of wall material as the septum grows. As seen in electron micrographs the spore septum contains no detectable peptidoglycan. It is, nevertheless, significant that inhibitors of cell wall synthesis, e.g. penicillin, will prevent the formation of the spore septum (Hitchins and Slepecky, 1969a; Lawrence, 1974). Apparently, then, there is a need for peptidoglycan synthesis; possibly it is required to direct membrane growth as the septum forms (Freese, 1972). More direct evidence for this is provided by the fact that in thin sections of some species, e.g. *Bacillus sphaericus*, cell wall material is clearly visible in the spore septum at stage II (Holt *et al.*, 1975).

Stage II–III ($t_{1.5}$ *to about* $t_{2.5}$): *engulfment*
At the completion of stage II there are two cells of unequal size side by side. The larger cell now proceeds to engulf the smaller, and in thin sections the two points of abutment of the spore septum to the cell wall appear to move towards the pole. Synthesis of mother cell membrane must account for a large proportion of the new material that is needed.

Stage III (*about* $t_{2.5}$ *to* t_3): *spore protoplast*
The engulfment culminates in membrane fusion at the pole of the cell and the whole engulfed structure becomes detached from the membrane of the mother cell to give the spore protoplast. The net effect is the emergence of an organism consisting of a cell within a cell. It should be noted in this context that the two membranes of the spore protoplast are oriented in opposition, i.e. outer membrane surfaces are face to face and the space between them is "extracellular" in relation both to mother cell and to spore protoplast (Fitz-James and Young, 1969; Wilkinson *et al.*, 1975; see also review by Freese, 1972). This leads to obvious difficulties in deciding which of the two cells is responsible when structures are formed in the "no-man's land" during the ensuing stages.

Stage IV (t_3 *to* t_4): *cortex formation*
Peptidoglycan is laid down between the membranes of the spore protoplast to form the cortex. Tipper and his colleagues have made a detailed investigation of this process in *Bacillus sphaericus*. In this species the cortex contains *meso*-diaminopimelic acid, whereas the peptidoglycan

of the vegetative cell wall is devoid of this compound and contains lysine instead. The enzyme diaminopimelate ligase appears during sporulation at the time of cortex formation and is found only in the mother cell (Tipper and Linnett, 1976). This implies that cortex biosynthesis takes place on the outer membrane of the prespore and that it is directed by the chromosome in the mother cell (Tipper *et al.*, 1977b). The innermost layer of the cortex (germ cell wall) does not contain spore-specific linkages. Since the prespore apparently contains all the enzymes required for the production of the peptidoglycan in this layer, synthesis could take place on the inner membrane of the prespore and it could be directed by the chromosome in the prespore.

At this stage of development the spore can be seen in the phase-contrast microscope as a grey body at one end of the cell.

Stage V (t_4 *to* $t_{5.5}$): *coat formation*
Successive layers of protein are laid down around the cortex and a laminar structure is often visible in electron micrographs, particularly in some species, e.g. *Bacillus popilliae*. During this stage the spore becomes phase-bright. Aronson and Fitz-James (1976) have provided a recent review of spore coat structure and morphogenesis.

Stage VI ($t_{5.5}$ *to* t_7): *maturation*
The spore shrinks somewhat and becomes resistant to high temperature and other adverse physical conditions. This represents the end of the differentiation.

Stage VII: release of spore
The mother cell lyses and releases the mature spore. This is not really a stage of sporulation at all. Lysis of the cells will occur anyway under conditions of nutrient deprivation, and it does not depend on the completion of sporulation or indeed upon its occurrence at all.

III. Biochemical Changes During Stages 0 to II

Our understanding of the precise biochemical events that underlie spore development is very poor despite the apparent wealth of information on the biochemical characteristics of sporulating bacteria that has accumulated particularly during the last two decades.

The first problem we have to consider is that not all the measurable changes that occur during sporulation are an integral part of the process.

Even with the many mutants that are available, it is not always easy to unravel those events that are specific from those that are not.

The biochemical events can be divided into different categories of relevance. The scheme that we have adopted is based on several previous classifications (Mandelstam, 1969, 1976; Hanson *et al.*, 1970; Freese, 1972; Piggot and Coote, 1976).

A. CATEGORIES OF EVENTS

1. *Events that are Essential and Sporulation-specific*

On the assumption that the morphological changes are the result of underlying molecular processes, we have to assume that there is a category of biochemical events specifically concerned with sporulation that determines the physical development at each stage. Also, at each point it has to be assumed that a biochemical messenger is generated that acts as a signal for turning on the genes needed if the process is to continue (see later). An interesting analysis of sporulation in these terms was given by Halvorson (1965). None of these messengers has been identified, but we can infer their existence from the fact that mutations at any of a large number of genetic loci cause sporulation to stop. Although they are, in principle, distinguishable from the class of "structural" molecules it may well be that there is functional overlap, i.e. a species of molecule may play an essential part in a structural change and also act as an "inducer" for a later stage. The biochemical events that underlie the production and location of the spore septum at stage II and the subsequent engulfment of the spore protoplast (stages II–III) are presumably essential sporulation-specific events. The enzyme diaminopimelate ligase, which is involved in cortex biosynthesis in *Bacillus sphaericus* (Tipper and Pratt, 1970), and the spore coat protein(s) are certainly sporulation-specific, but how essential they are is a matter of semantics. Some mutants that fail to make either the cortex or the coat can produce spores at the end of differentiation. These are sometimes viable but have altered properties (see Pearce and Fitz-James, 1971; Millet and Ryter, 1972, and the review by Piggot and Coote, 1976).

2. *Events that are Sporulation-specific and Dispensible*

Events in this category differ from those in the former category in that they are not required for normal sporulation. This is shown by the fact

that mutants with specific defects in these functions can sporulate normally. Their times of appearance are precisely controlled and they therefore serve as useful marker events in experiments. The production of metalloprotease at the onset of sporulation and of dipicolinic acid and alanine dehydrogenase at later stages, are examples of events in this category (see Hageman and Carlton, 1973; Freese *et al.*, 1964; Warren, 1968; Hanson *et al.*, 1972; Zytkovicz and Halvorson, 1972). Dipicolinic acid and alanine dehydrogenase may be required for spore germination and thus be distinct from other events in this group (see Piggot and Coote, 1976).

3. *Events that are Essential but not Sporulation-specific*

This category includes all those events that are required for sporulation and which may also be expressed during vegetative growth. The cytochromes, and the enzymes of the tricarboxylic acid cycle, are needed to maintain adequate supplies of energy during sporulation (Hanson *et al.*, 1970; Freese, 1972) and they therefore fall into this category.

4. *Irrelevant Events*

The production of arginase and amylase at the onset of sporulation is occasioned by the nutritional step-down conditions used to obtain sporulation (Laishley and Bernlohr, 1966; Schaeffer, 1969). These enzymes are otherwise quite unrelated to the process of differentiation.

Some sporulation-associated events are shown in relation to their times of appearance in Fig. 1. An attempt to assign them to categories was made by Mandelstam (1976), but many of the assignments were provisional. This is because it is difficult to decide into which category to place an event unless specific mutants are available for study. In an attempt to circumvent this problem Dancer and Mandelstam (1975b) have suggested an additional criterion which is particularly useful for distinguishing events in the third and fourth categories from other events. Since the induction of sporulation depends on DNA replication, whereas the induction of vegetative functions does not (see later), they proposed that only those events whose appearance is prevented by inhibition of DNA synthesis during sporulation should be considered part of the sporulation sequence. They found, for example, that extracellular serine

protease and RNAase did not appear in thymine-starved cells, whereas amylase did appear. This last event was accordingly designated an irrelevant event. It is curious that in some later experiments by Leighton *et al.* (1975) and by G. Dunn and B. N. Dancer (unpublished work) it was found that the addition of 6-(*p*-hydroxyphenylazo)-uracil (HPUra) to sporulating cells to stop DNA synthesis did not prevent the synthesis of extracellular serine protease. The reason for the discrepancy between the results of the thymine-starvation experiments and the HPUra experiments is not known. Whatever the reason may be, it remains true that thymine starvation provides a useful criterion for distinguishing events that are sporulation-specific from those that are not.

The distinctions we are making are obviously operational, and some events would fall into different categories depending on the medium used to obtain sporulation. For example, sporulation in a medium containing histidine as the sole nitrogen source would depend on the production of histidase. Under these specialised conditions histidase production would not be an "irrelevant event", but would be listed in the third category.

Nevertheless, by means of the criteria we have outlined it is generally possible to distinguish sporulation-specific events (categories I and II) from non-specific events (categories III and IV). Even then we are left with a problem of major dimensions—the problem of knowing how the biochemical changes specifically associated with spore development produce the complex morphogenetic changes that are observed. An analogous question arises in all studies of differentiation and, except in the case of bacteriophage assembly, no significant progress has been made towards its solution.

We shall now enumerate some of the biochemical events specifically associated with the early stages of sporulation; in view of what has been said, it will be clear that we do not know their role, if any, in the causal chain of events.

B. MARKER EVENTS ASSOCIATED WITH STAGES 0 AND I

1. *Proteases*

One of the first events to occur after the induction of sporulation is the production of proteolytic activity. Many species produce several distinct enzymes, some of which may be secreted. It is generally assumed that one of these enzymes (presumably an intracellular protease) participates in

the extensive protein turnover that occurs throughout sporulation in many species. Reports on protein turnover go back to Foster and Perry (1954); they have been reviewed among others by Kornberg *et al.* (1968) and Schaeffer (1969). Little or no protease activity may be produced by those species in which there is negligible protein turnover during sporulation; e.g. *Bacillus brevis* ATCC9999 (Slapikoff *et al.*, 1971) and *Clostriduim pasteurianum* (Mackey and Morris, 1974). *Bacillus licheniformis* is a protease producer that, nevertheless, shows negligible protein turnover (Bernlohr and Clark, 1971).

Many species produce two distinct classes of protease. One of these, a metalloprotease, is not required for either protein turnover or sporulation. Mutants of *B. subtilis* that lack this enzyme can still sporulate normally (Michel and Millet, 1970; Hageman and Carlton, 1973). Similarly, there is normal sporulation in mutants of *Bacillus cereus* and *Bacillus megaterium* that produce little or no extracellular metalloprotease even though this is the only extracellular protease produced by these organisms (Millet and Aubert, 1969; Aronson *et al.*, 1971).

In *B. subtilis*, two serine proteases are produced at the beginning of sporulation, one extracellularly and the other intracellularly (Szulmajster and Keryer, 1975), and at least one of them seems to be essential. Consequently, serine protease inhibitors such as phenyl methyl sulphonyl fluoride and *m*-amino-benzeneboronic acid block sporulation although they do not affect vegetative growth (Dancer and Mandelstam, 1975a; Geele *et al.*, 1975). Mutations in two of the ten known stage 0 sporulation loci abolish extracellular enzyme synthesis; mutations in four more of these loci result in decreased production (Table 1) (Michel and Millet, 1970; Piggot and Coote, 1976). A mutant of *B. subtilis* that was temperature-sensitive for production of extracellular serine protease was also temperature-sensitive for sporulation; both characters apparently reverted in a single step (Leighton *et al.*, 1972; 1973). On the other hand, Millet *et al.*, (1976) isolated a phenotypically similar mutant also in *B. subtilis*, that was temperature-sensitive for both serine protease production and sporulation. This strain was found to harbour two mutations, one conferring temperature-sensitive serine protease production and the other temperature-sensitive sporulation. When these mutations were separated by transformation the Spo^+ transformants retained the temperature-sensitive protease phenotype. This indicates that the sporulation phenotype of the original mutant was not caused by the mutation that affected the protease. The authors point out that their

results do not rule out the possibility that the extracellular serine protease has a vital role to play in sporulation (see, for example, Mandelstam and Waites, 1968) because there was a slight residual activity at the non-permissive temperature. As noted previously, several species sporulate without production of detectable amounts of extracellular serine protease (Levisohn and Aronson, 1967; Millet and Aubert, 1969; Slapikoff *et al.*, 1971; Mackey and Morris, 1974). On balance, therefore, the evidence suggests that the enzyme is not essential for sporulation.

In those species that show extensive protein turnover during sporulation there is an intracellular serine protease (see for example Cheng and Aronson, 1977). Reysset and Millet (1972) have characterized a Ca^{2+}-requiring enzyme of chymotrypsin-like specificity from *B. subtilis*. A similar enzyme is produced in *B. megaterium* (Millet, 1971). The relationship between the intra- and extracellular enzymes is not clear. It seems likely that they are quite distinct proteins since they are not related immunologically (Szulmajster and Keryer, 1975) and a protein inhibitor of the intracellular enzyme has no effect on the extracellular enzyme (Millet, 1977). On the other hand, a more recent report indicates that there is extensive sequence homology between the intracellular serine protease and the extracellular subtilisins of *B. subtilis* (Strongin *et al.*, 1978).

2. *Antibiotics*

Sporulating bacteria elaborate an extraordinary variety of substances with antibiotic activity; moreover, a number of different compounds may be produced by a single organism (Bérdy, 1974). Many of the antibiotics are linear or cyclic peptides that are synthesized enzymically, i.e. not on ribosomes. Katz and Demain (1977) have recently reviewed the biogenesis and possible functions of antibiotics.

Antibiotic synthesis in a variety of species starts soon after the end of exponential growth in exhaustion media. In resuspension experiments with *B. subtilis* no measurable amounts of antibiotic are found (J. Mandelstam, unpublished findings). Strains of *B. subtilis* that harbour mutations in six of the ten known stage 0 loci are impaired in antibiotic production (Table 1). A variety of different roles have been proposed for antibiotics in sporulation (see, for example, Hodgson, 1970; Sarkar and Paulus, 1972). However, mutants of *B. subtilis*, *B. licheniformis* and *B. brevis* that fail to produce mycobacillin, bacitracin and gramicidin-S,

respectively, sporulate normally (Ray and Bose, 1971; Haavick and Thomassen, 1973, Demain et al., 1976). *Cl. pasteurianum* is an example of a species which sporulates without the production of any detectable antibiotic (Mackey and Morris, 1974). Although it is apparent that synthesis of antibiotic is not obligatory for spore formation, it has recently been claimed that gramicidin affects the quality of the spores produced in *B. brevis* (Mukherjee and Paulus, 1977; Sarkar *et al.*, 1978). The authors isolated a mutant that produced no antibiotic and had reduced amounts of protease and dipicolinic acid. The spores produced by this strain were slightly less heat-resistant than those of the wild type organism. All of these phenotypic defects reverted in a single step, and they were corrected when the antibiotic was supplied at the end of growth.

3. *Other Extracellular Products*

The RNAase that is produced at the onset of sporulation has been little studied since the review of Schaeffer (1969). Nevertheless, it is probably one of the sporulation-associated events. This follows from the fact that if thymine-starvation is imposed at t_0 it prevents the formation of RNAase in the same way as it does with the known biochemical markers of sporulation (Dancer and Mandelstam, 1975b).

Amylase, another enzyme that appears in *B. subtilis* at the beginning of sporulation, seems to be induced by the starvation conditions used (Schaeffer, 1969). It is quite clearly not necessary for the process since mutants that fail to produce the enzyme sporulate normally. Furthermore, it appears in thymine-starved cells and in stage 0 mutants; it is therefore probably a "vegetative" response to starvation.

A variety of toxins are excreted, mainly by clostridia but also by aerobic spore-formers such as *B. cereus*. Some of these are produced at the end of exponential growth, but their relation to sporulation remains obscure (Schaeffer, 1969).

4. *Metabolic Changes*

Sporulation is normally induced in response to nutrient starvation and many of the enzymes that appear at the beginning of spore development are correlated with the transition from a feast to a famine existence. Some of these play no obvious role in spore development, e.g. α-amylase and the enzymes of arginine catabolism (Schaeffer, 1969; Laishley and Bernlohr,

1966). Others, such as the TCA cycle enzymes, are required to maintain adequate supplies of ATP during development (Hanson *et al.*, 1970; Freese, 1972; Ohné and Rutberg, 1976). Mutants defective at various steps in the TCA cycle were blocked at stage 0–I of sporulation (Freese and Marks, 1973) and the block could be overcome in certain cases by the addition of TCA cycle intermediates (Yousten and Hanson, 1972; Ohné and Rutberg, 1976). The failure to cure certain TCA cycle mutants by the addition of malate (Freese *et al.*, 1969) may have been due to the repressive effect that this compound has on sporulation (Ohné and Rutberg, 1976). Other substances that are required for ATP generation during sporulation also increase in activity soon after induction, e.g. particulate NADH oxidase, various cytochromes and menaquinones (see Szulmajster and Schaeffer, 1961; Taber *et al.*, 1972; Felix and Lundgren, 1973; Weber and Broadbent, 1975). Mutants of *B. subtilis* that lack cytochrome *a* do not sporulate (Taber and Freese, 1974).

Several enzymes that are normally present during vegetative growth appear to undergo selective inactivation, or possibly degradation, during the early stages of sporulation (Szulmajster, 1964; Szulmajster and Hanson, 1965; Deutscher and Kornberg, 1968; Bernlohr and Gray, 1969).

C. MARKER EVENTS ASSOCIATED WITH STAGE II

1. *Spore Coat Protein(s)*

The protein material that comprises the spore coat undergoes continuous synthesis from about stage II. It accumulates as some kind of precursor and assembly does not occur until stage V (Cheng and Aronson, 1977; Pandey and Aronson, 1979). Aronson and Fitz-James (1968) used a pulse-chase technique to demonstrate that radioactivity was incorporated into the spore coat of *B. cereus* T at a more or less constant rate from about the time of prespore engulfment onwards. Wood (1972) showed that, in *B. subtilis*, protein which was precipitated by anti-serum raised against an alkali-soluble coat protein fraction appeared during stage II. A *spoIIE* mutant failed to produce this material, whereas *spoIIA* and *spoIID* mutants did produce it (Wood, 1972; Piggot and Coote, 1976). In some recent experiments Munoz *et al.* (1978) identified coat protein messenger RNA (mRNA) and nascent coat protein in polysomes extracted from sporulating cells of *B. subtilis*. Their findings

suggest that translation of spore coat protein mRNA may begin even before stage II. For the time being we shall include coat protein synthesis among the stage II events in accordance with the phenotypic properties of the mutants (see Table 1). A comprehensive review of coat structure and morphogenesis has recently appeared; it should be consulted for a more detailed discussion of this topic (Aronson and Fitz-James, 1976).

2. *Alkaline Phosphatase*

In *B. subtilis*, alkaline phosphatase is synthesized during stage II of sporulation. Mutants with defects in four of the seven known stage II loci fail to produce the enzyme (Waites *et al.*, 1970; Piggot and Coote, 1976). Mutants with an altered structural gene have not been reported, in spite of intensive searches for them (Le Hégarat and Anagnostopoulos, 1969, 1973; Grant, 1974). The enzyme produced by phosphate-starved vegetative cells is indistinguishable from that produced during sporulation (Glenn and Mandelstam, 1971). While this suggests that the "vegetative" and "sporulation" enzymes are transcribed from the same stuctural gene, we can provisionally assign the enzyme to the second category, i.e. sporulation-specific but non-essential. This is because of the quite different controls to which it is subjected during growth and sporulation. Alkaline phosphatase is repressed by inorganic phosphate during vegetative growth but not during sporulation (Anagnostopoulos, 1960; Warren, 1968; Glenn and Mandelstam, 1971; Ichikawa and Freese, 1974). Mutants have been described that are unable to produce the enzyme during vegetative growth but that display the normal pattern of enzyme synthesis during sporulation (Ichikawa and Freese, 1974; Grant, 1974). Conversely, mutants that show premature enzyme synthesis during sporulation can still have a normal pattern of synthesis during growth. This is a property that characterises some of the *sap* mutations reported by Piggot and Taylor (1977). These mutations allow synthesis of alkaline phosphatase in mutants blocked early in sporulation that are otherwise unable to produce the enzyme.

We do not know whether alkaline phosphatase plays an active role in sporulation. The premature synthesis that occurs in constitutive phosphatase mutants of *B. megaterium*, and also in some of the *sap* mutants of *B. subtilis* mentioned above, has no detrimental effect on sporulation (Millet, 1963; Piggot and Taylor, 1977). These facts do not necessarily mean that the enzyme is produced fortuitously during sporulation. It is

probably significant that phosphatases appear at characteristic times during the development of a variety of other organisms (Mandelstam, 1971). Also, the periplasmic alkakine phosphatase of *Pseudomonas aeruginosa* appears to play a role in cell division (Bhatti *et al.*, 1976).

3. *Extracellular DNAase*

Akrigg (1978) has recently isolated and characterised an extracellular nuclease that is produced by sporulating cells and excreted into the culture medium, and which is active against double-stranded DNA. The enzyme shows the same pattern of production in sporulation mutants as does alkaline phosphatase, and its synthesis is therefore classified as a stage II event (see Table 1) even though it does not appear until about one hour after the phosphatase (Akrigg and Mandelstam, 1978).

D. LATER EVENTS

Once the prespore has been engulfed, several sporulation-associated events occur, e.g. production of glucose dehydrogenase, spore storage protein, heat-resistant catalase, diaminopimelate ligase and, in *B. subtilis*, sulpholactic acid (see Lawrence and Halvorson, 1954; Bonsen *et al.*, 1969; Tipper and Pratt, 1970; Waites *et al.*, 1970; Wood, 1971; Coote, 1972a; Setlow, 1974; 1975). These events seem to be characteristic of the transition from stage III to IV, as far as has been ascertained by studies with sporulation mutants (Waites *et al.*, 1970; Coote, 1972a).

IV. Changes in Transcription and Translation Apparatus

A. TRANSCRIPTION

During the early stages of spore development, up to the time of engulfment, some twenty sporulation-specific genetic loci are activated (Piggot and Coote, 1976). Phenotypic exclusion experiments suggest that these loci are expressed in a number of separate transcriptional steps (see later, V). There may also be a selective silencing of certain vegetative genes during this initial period although there is, at present, no evidence for this. In theory, some, or perhaps even all, of these transcriptional changes during early sporulation could be mediated by alterations in the template specificity of RNA polymerase (Szulmajster, 1973a), such that

the enzyme recognises new classes of promoter sites or "reads through" termination sites (antitermination), as occurs, for example, during development of bacteriophage lambda (Roberts, 1970; 1976). Losick and Pero (1976) have recently summarized the case for the regulatory role of RNA polymerase in sporulation. As will become apparent, this is a controversial subject; much of the supporting evidence is circumstantial and open to alternative interpretations. The critical experiment, which is to examine whether RNA polymerase from vegetative and sporulating cells can discriminate *in vitro* between purified vegetative and sporulation genes, has yet to be done. Several lines of evidence that have been adduced in support of the polymerase model will now be summarized.

1. *Bacteriophage Infection of Sporulating Cells*

Several virulent bacteriophages that grow in vegetative cells of *B. subtilis* cannot multiply in sporulating cells (Yehle and Doi, 1967; Sonenshein and Roscoe, 1969; Ito *et al.*, 1973). Some of these phages that do not productively infect sporulating cells can become "trapped", i.e. the phage DNA is sequestered in the developing spore before the lytic sequence is set in train, and phage development is delayed until spore outgrowth occurs (Cohen *et al.*, 1973). Similarly, sporulating cells that harbour a lysogenic phage will not support multiplication of the phage when it is induced during sporulation. The phage may become trapped after induction (Hendry and Fitz-James, 1974) or, alternatively, the spores of the host may be re-lysogenised during outgrowth (Osburne and Sonenshein, 1976).

The behaviour of these phages in sporulating cells has been attributed to changes in the host polymerase which is held to have been modified during sporulation so that it is unable to transcribe phage DNA. However, transcription of phage DNA in sporulating cells of *B. subtilis* has been demonstrated by Kawamura and Ito (1974, 1975) for the small virulent phage ϕ29, which is trapped soon after the induction of sporulation, and for the large virulent phage ϕe, which is not trapped until about stage II (Sonenshein and Roscoe, 1969). In this context it is important to remember that nutrient starvation will largely prevent phage multiplication even in *E. coli* (see for example Stent, 1963), and this is precisely the condition under which sporulation occurs in bacilli.

2. *Changes in Template Specificity of RNA Polymerase*

The RNA polymerase from sporulating cells of *B. subtilis* has less activity than the enzyme from vegetative cells when ϕe DNA is used as a template. The enzyme from both sources is equally active using poly(dA-dT) as a template (Losick and Sonenshein, 1969; Brevet and Sonenshein, 1972). The change in template specificity of the "sporulation" polymerase was correlated with the loss of the ability of sporulating cells to support ϕe multiplication *in vivo* (see above) and also with a decrease in the sigma-factor activity in extracts of sporulating cells (Sonenshein and Losick, 1970; Brevet, 1974). Stage 0 sporulation mutants with defects in the *spoOA*, *spoOB* and *spoOH* loci did not show the change in template specificity (Brevet and Sonenshein, 1972). Interpretation of this result is obscured by the finding that extracts of sporulating bacteria contain an RNAase that can degrade ϕe RNA but not poly(A-U). This led Kerjan and Szulmajster (1974) to suggest that the apparent change in template specificity was an artefact. In answer to this criticism Tjian and Losick, quoted by Losick and Pero (1976), reported that in their system there was no apparent degradation of newly synthesised ϕe RNA when it was incubated with a crude extract of sporulating cells. In any case, the relevance to sporulation of the reported change in template specificity is uncertain because it does not seem to occur at all when sporulation is induced by the resuspension technique (Murray *et al.*, 1974). Consequently, it might simply be a characteristic of the stationary phase of growth rather than of sporulation (Szulmajster, 1973b). Rexer *et al.* (1975) failed to detect any change in template specificity or subunit composition (see below) of RNA polymerase during sporulation of *B. cereus*.

3. *Changes in Subunit Composition of RNA Polymerase*

The sigma subunit of RNA polymerase co-purifies with the core enzyme from vegetative cells of *B. subtilis*. When the same technique of purification was applied to sporulating cells it led to the isolation of core polymerase largely deficient in sigma activity (Tjian and Losick, 1974). This change in subunit composition of the extracted polymerase was correlated with the inability of RNA polymerase from sporulating cells to support transcription of phage ϕe DNA *in vitro* and also with the failure of the phage to multiply in sporulating cells (see above). In apparent contradiction to these results are those of Maia *et al.* (1971) who reported

that RNA polymerase from dormant spores did contain the sigma factor. Also, Duie *et al.* (1974) found that an anti-sigma antibody would precipitate RNA polymerase from sporulating cells. Their obsevation is complicated by the fact that their anti-sigma antibody formed a precipitin line with purified core polymerase in an immunodiffusion assay. In spite of all the experimental difficulties and conflicting interpretations it now seems to be generally agreed that the sigma polypeptide is present in extracts of sporulating cells. It is still in doubt whether it forms a functional association with the RNA polymerase core *in vivo*. In any case, it is clear that the postulated change in sigma activity does not occur until stage II, by which time about ten sporulation-specific loci have been activated, apparently in a specific sequence (see later), and the cells have long since lost the ability to support growth of the virulent phage ϕ29 (Kawamura and Ito, 1974).

The proteolytic cleavage of the β' subunit of RNA polymerase that was originally thought to herald the onset of sporulation (Leighton *et al.*, 1972; Millet *et al.*, 1972) has since been shown to be an artefact resulting from proteolysis during enzyme extraction (Linn *et al.*, 1973; Orrego *et al.*, 1973).

Recently, attention has been focussed on the isolation and characterization of polypeptides from sporulating cells that bind to RNA polymerase. These could, in principle, modulate the transcriptional specificity of the polymerase (Doi, 1977a,b). For example, late exponential cells of *B. subtilis* yield RNA polymerase containing a new factor (Holland and Whiteley, 1973). Greenleaf *et al.* (1973) have also found a new RNA polymerase-binding protein that is produced in the wild type and in mutants blocked at stage II or later, but not in stage 0 mutants (*spoOA*, *spoOB*). Other factors that may be associated with the polymerase at later stages of sporulation have also been described (Klier and Lecadet, 1974; Nishimoto and Takahashi, 1974; Fukuda *et al.*, 1975; Linn *et al.*, 1975a; Fukuda and Doi, 1977).

4. *Drug-resistant Mutants*

There have been numerous reports of mutants, resistant to the drugs rifampicin, streptolydigin or streptovaricin, that are also affected in sporulation (e.g. Leighton, 1973; Sonenshein *et al.*, 1974; Rothstein *et al.*, 1976; Sumida-Yasumoto and Doi, 1977; Leighton, 1977). Many of these strains harbour single point mutations, and those that confer resistance

to rifampicin do so by affecting the β-subunit of RNA polymerase (Linn *et al.*, 1975b). This establishes that bacteria with an altered RNA polymerase may be unable to sporulate; by contrast, many mutants resistant to these drugs and also containing an altered RNA polymerase can sporulate normally. The sporulation-defective mutants are often blocked at stage 0 (Sonenshein, *et al.*, 1974), although mutants blocked at later stages have also been reported (Leighton, 1973, 1977; Santo *et al.*, 1973; Sumida-Yasumoto and Doi, 1977). The existence of such mutants has been used to support the contention that RNA polymerase undergoes sequential changes during sporulation that could, in principle, modulate the specificity of transcription during development (Doi, 1977a). An alternative explanation would be that as the internal cellular environment changes during sporulation it becomes progressively less conducive to polymerase function; hence, a mutant with a slightly defective polymerase may be able to "get by" during vegetative growth, whereas the altered conditions during nutrient starvation could prevent efficient transcription during sporulation. Since there is evidence that certain gene products are required at precise times during spore development (Young, 1976), inefficient transcription could drastically reduce sporulation. It is significant in this context that the Spo^- phenotype of one rifampicin-resistant (Rif^r) mutant was overcome by the addition of a mixture of four amino acids to the sporulation medium (Pun *et al.*, 1975). This suggests that the sporulation defect in this particular mutant might be due to an inability of the polymerase to transcribe efficiently genes concerned with the biosynthesis of the four amino acids rather than sporulation genes.

These uncertainties might yet be resolved by the study of mutants resistant to new drugs that block transcription (Sonenshein *et al.*, 1977) and by attempts to identify polymerase subunits that are specifically concerned with transcription during sporulation (Rothstein *et al.*, 1976). Perhaps an examination of *spo* mutants for defects in some of the putative sporulation-specific protein factors that were mentioned above would be worthwhile. It is curious that a search for drug-resistant mutants has yielded a subclass that are also affected in sporulation whereas, as far as we are aware, none of the *spo* mutants studied so far has simultaneously acquired drug resistance. In particular, it is surprising that among the very large number of stage 0 mutants with which fine structure mapping has been done, no mutation has been found to be in the *rif* locus which corresponds to the structural gene for the β subunit (Linn *et al.*, 1975b).

5. *Phage Conversion of Sporulation Mutants*

Bramucci *et al.*, (1977a) have described a bacteriophage with the property of being able to convert naturally-occurring oligosporogenous strains of *Bacillus pumilis* into strains that sporulate at a high frequency. Another phage (PMB12) has also been found which converted all Rif^r Spo^- strains of *B. subtilis* on which it was tested to Spo^+ (Bramucci *et al.*, 1977b). This phage is related to PBS2 which codes for a new RNA polymerase that can substitute for the altered, and presumably defective, host enzyme (Clark *et al.*, 1974; Clark, 1978). The phage also apparently converted a *spoOJ* mutant to Spo^+ (Bramucci *et al.*, 1977b). This raises the interesting possibility that the *spoOJ* locus codes for a subunit of RNA polymerase.

B. TRANSLATION

There have been numerous reports that the protein-synthetic machinery changes during sporulation, but it still has to be shown that the observed changes are essential for sporulation. A convincing demonstration would first require the preparation of reagent quantities of mRNA corresponding to "vegetative" and "sporulation" genes. The translation systems from vegetative and sporulating cells would then have to be tested for the ability to discriminate between them.

If translational controls exist, they could function in one or more of the following ways: (a) by regulation of translation of particular mRNA molecules (if stable mRNA species exist, this type of control might be necessary), (b) by directed alterations in ribosomes, (c) by directed alterations in tRNA or tRNA synthetases. These will be discussed in turn.

1. *Half-life of Sporulation Messenger RNA*

It is difficult to make generalisations about the half-life of mRNA in procaryotes. The first evidence came from experiments with vegetative cells (Gros *et al.*, 1961; Levinthal *et al.*, 1962). These reports concluded that bacterial mRNA has a short half-life (about 3 minutes). Although doubts were raised about these experiments (see Harris, 1974) this estimate has been generally accepted. More recently, however, it has been shown unequivocally by experiments with mini-cells of *E. coli* that at least some types of mRNA have a longer half-life, estimated at 40 to 80 min (Levy, 1975).

The evidence on sporulating bacteria is conflicting. Aronson and del Valle (1964) first suggested that the mRNA concerned in sporulation might be long-lived in *B. cereus*. This was followed by experiments in *B. subtilis* which showed that biochemical events associated with sporulation were able to continue for periods up to an hour after the addition of actinomycin D (Sterlini and Mandelstam, 1969). These findings were contradicted by results obtained using higher concentrations of actinomycin D and also rifampicin (Szulmajster *et al.*, 1963; Balassa, 1966; Leighton and Doi, 1971; Leighton, 1974a).

It is now fairly clear than an unambiguous answer cannot be expected from experiments involving the use of inhibitors such as actinomycin D or rifampicin. At low concentrations the inhibition of RNA synthesis by these drugs is incomplete; this leaves open the possibility that the residual synthesis of mRNA is sufficient to allow sporulation to continue. Conversely, when the concentrations of the drugs are high enough to stop RNA synthesis completely, the damage to the cells is so gross that it is quickly manifested in a falling respiration rate and in lysis (Coote *et al.*, 1973). Failure to sporulate under these conditions is therefore not surprising.

Some individual mRNA molecules seem to have intermediate or long half-lives, e.g. 15 mins for dipicolinate synthetase mRNA in *B. subtilis* (Chasin and Szulmajster, 1969); 10 min for the messenger for parasporal crystal protein in *Bacillus thuringiensis* (Glatron and Rapoport, 1972); about one hour for the messenger for enterotoxin and for spore coat protein in *Clostridium perfringens* (Labbe and Duncan, 1977). Another long-lived mRNA species was purified by Segall and Losick (1977) as a prelude to a DNA cloning experiment.

In the light of the information we now have, it is reasonable to conclude that most species of mRNA molecules synthesised either during vegetative growth or during sporulation have a short half-life. On the other hand there is an increasing list of independent observations to suggest that in bacilli—and indeed, as we have noted, in *E. coli*—some mRNA molecules have half-lives of up to an hour. The existence of these will entail a serious consideration of the possibility that translational as well as transcriptional controls are important during sporulation.

2. *Ribosomal Changes During Sporulation*

Several investigators have reported that the protein composition of the ribosomes of sporulating cells and spores differs from that of vegetative

cells (Kobayashi, 1972, 1973; Fortnagel and Bergmann, 1973; Fortnagel *et al.*, 1975; Jerez *et al.*, 1976). There have also been reports that ribosomes from sporulating cells have an altered specificity compared with those from vegetative cells when tested with *in vitro* translation systems (Chambliss and LeGault-Demare, 1977; Guha and Szulmajster, 1977).

Changes in ribosomes can also occur as a consequence of the acquisition of resistance to a variety of antibiotics. There have been several reports of mutants of *B. subtilis* resistant to ribosome-directed drugs that are also defective in sporulation. In many, but not all, cases (see, for example, Staal and Hoch, 1972) these phenotypic properties result from a single-site mutation in a ribosomal protein or an associated factor. Leighton (1974b) described a streptomycin-resistant mutant with ribosomes that did not bind dihydrostreptomycin *in vitro*. This mutant was also temperature-sensitive for sporulation and the temperature-sensitivity extended over almost the entire period of sporulation. A large number of independently isolated erythromycin-resistant mutants were studied by Tipper *et al.* (1977a). Two representative mutants had single-site defects in the L17 protein of the 50S subunit and their ribosomes failed to bind erythromycin *in vitro*. All of these mutants apparently had a temperature-sensitive period that spanned the latter half of the sporulation sequence, but they were also defective in the accumulation of extracellular products during the initial stages of sporulation. The erythromycin-resistant mutant of *B. subtilis* studied by Domoto *et al.* (1975) also had an altered protein in the 50S ribosomal subunit. This mutant was sensitive to the drug during the first five hours of sporulation but refractory at later times. Graham and Bott (1975) have obtained similar results with respect to the sporulation of their erythromycin- and spectinomycin-resistant mutants. Mutants of *B. subtilis* that are resistant to fusidic acid (as a result of an alteration of the elongation factor G (Goldthwaite and Smith, 1972) are oligosporogenous and blocked at stage 0 (Fortnagel and Bergmann, 1973; Fortnagel and Freese, 1977; Kobayashi *et al.*, 1977). Wild-type bacteria become refractory to fusidic acid at about the time of spore septum formation, and this is also the time at which the ribosomes no longer bind to the drug *in vitro* (Fortnagel and Freese, 1977, but see also Guha and Szulmajster, 1974). Under different sporulation conditions (Sterlini-Mandelstam resuspension technique) cells become refractory to fusidic acid before septum formation (M. Young and P. Jeffs, unpublished data). In all such studies it is

important to demonstrate that cells do not become refractory to a drug as a result of an inability to take it up. In some cases the observed phenotypes have been correlated with ribosomal changes *in vitro*, which does much to allay such doubts. Even so, these findings cannot be taken as unequivocal evidence for the operation of sporulation-specific translational control. The reasons for this have been mentioned previously with reference to the work on RNA polymerase.

3. *Alterations in Transfer RNA and tRNA Synthetases*

There are several reports of altered tRNA species in sporulating cells (Vold, 1974, 1975; Yeng and Doi, 1975; McMillian and Arceneaux, 1975; Menichi and Heyman, 1976), and of modification to the tRNA synthetase enzymes (Steinberg, 1975). In principle, these changes could also play a role in the control of translation of sporulation mRNA.

C. SUMMARY

Different workers have described alterations during sporulation in any one of the following: RNA polymerase, the polypeptides associated with RNA polymerase, the ribosomes and associated factors, tRNA, the tRNA synthetases. These observations, together with those on mutants affecting one or more of the functions, could mean that directed alterations in either the transcription or the translation machinery, or in both, are determining factors in the ordered regulation of the sporulation sequence. However, the evidence is equivocal. It may simply be that the sporulating cell requires a completely functional apparatus for protein synthesis in the same way that it requires a completely functional ATP-generating system. Cells changed in these essential functions by mutation may be asporogenous for non-specific reasons. The definitive experiments have yet to be done.

V. Genetics and Regulation of Spore Formation

A. ESTIMATES OF MOLECULAR COMPLEXITY

An intellectually satisfying solution to the problem of sporulation would be one in which most of the genes had been identified and mapped and their protein products, when they have any, characterised. When the latter are enzymes, their small molecule substrates and products would

also need to be identified and their functions established. Finally, it would be necessary to know by what means and in what sequence the genes are activated. A molecular approach of this type was first applied successfully in the case of inducible and repressible enzymes and then in the elucidation of bacteriophage development. In the latter, although some details may be lacking, it is in general clear what the chain of events is from the time the phage particle infects the host until the time when a burst of progeny is liberated. The enzymes responsible for producing multiple copies of the phage nucleic acid are known and so is the process of activation of early and late genes. The structure of the completed phage largely results from a process of self-assembly.

Before going on, it will be useful to consider the molecular complexity of some of these systems. In each instance genes are active in some situations but inactive in others. For their expression these genes need to be in a cell in which the machinery for generating ATP and for synthesising macromolecules is fully functional. In studies of phage development this is so obvious that it is seldom, if ever, mentioned, but in the case of sporulation, where it is equally important, the distinction between specific and non-specific requirements for complete expression is sometimes not clear.

The best studied example of an inducible system is the *lac* operon, which contains three structural genes, together with a promoter and an operator. In addition, there is a regulator gene and a catabolite repression mechanism that involves cyclic AMP. The arginine biosynthetic system in coliform bacteria, which does not involve cyclic AMP, consists of about ten structural genes. They lie in several operons scattered about the chromosome and the number of operators is therefore greater. Again there is a separate regulator. In both *lac* and *arg* systems the functions of the enzymes, their substrates and their products are known.

The next stage of complexity is represented by phage assembly. In the case of T4 the number of genes identified is about 140 and, from the size of the genome, this must be near the total number. The number of protein products is similar. Specific small molecule products appear to play little part in the process. The current state of knowledge has been summarised by Wood and Revel (1976).

We now need to enquire whether we can reasonably expect that sporulation can, in the forseeable future, be made intelligible at this level of molecular definition.

B. METHODS AND NOMENCLATURE

We have already emphasised that we are dealing with elements of a system that is only very partially characterised biochemically. Consequently, it is not possible to identify operons in the precise way that has been done for many vegetative functions in bacteria. It is therefore more correct to speak of genes, or of loci which may contain one or more genes. Later, in discussing possible modes of regulation it will be useful for simplicity to assume that the loci are operons.

With the methods of genetic analysis now available, it is possible to establish the position of any *spo* mutation on the chromosome. This is done by a combination of transduction and transformation. The generalised transducing phage PBS1 transfers about 5% of the donor chromosome (see Lepesant-Kejzlarová *et al.*, 1975) and can therefore be used for rough mapping. Fine structure mapping can be done by transformation which spans 20 to 30 genes (Bodmer and Ganesan, 1964).

Genetic loci are indicated by the morphological stages that they affect. Thus, stage 0 mutants present the appearance in electron microscopy of vegetative cells and the loci containing mutations producing this phenotype specifically (i.e. with no apparent effect on any vegetative function) are denoted *spoOA*, *spoOB*, etc. Loci containing mutations that block sporulation at other stages are denoted *spoIIA*, etc. The choice of a morphological criterion was arbitrary and it is important to realise that other criteria would, in some instances, produce a different classification of a genetic locus. The clearest example, mentioned earlier, is provided by the protein of the spore coat. By the criterion of morphology, deposition of this protein is a stage V event. However, synthesis of the protein commences during stage II (Aronson and Fitz-James, 1968; Wood, 1972), or perhaps at an even earlier stage (Munoz *et al.*, 1978). So by this criterion it is a stage II event and the genetic locus corresponding to the structural gene would have a *spoII* designation. Clearly, the latter is the more relevant consideration if one's primary interest is the time of activation of the gene and its translation into protein. Although coat protein is thus a clear example of a protein whose synthesis starts long before it assumes its physiological function (see also Young, 1976), there is no reason to believe that this kind of discrepancy is a common occurrence. Fortunately, in the case of many marker events connected with sporulation, we are concerned with the measurement of enzymes, e.g. alkaline phosphatase, or glucose dehydrogenase, or with a product of enzyme action such as sulpholactic acid or dipicolinate. Furthermore, the

bulk of the messenger RNA in sporulating cells appears to have a short half-life (see earlier). We shall therefore assume that for most sporulation genes the time of activation of the gene, the time of appearance of its protein product, and the time of intervention of the product in the morphological development are not significantly separated.

We can now proceed to the criteria for distinguishing one locus from another. These were proposed initially by Piggot (1973) on the assumption that the loci were functioning as operons, that is that the genes within a particular locus were controlled as a unit. The criteria are as follows. (1) If two sporulation mutations are separated on the chromosome by an auxotrophic or other "vegetative gene" they lie in different loci. (2) Mutations that block sporulation at different stages and which are presumably activated at different times lie in separate loci. As we have indicated, this may on occasion be misleading but in general it is probably correct. (3) If two mutations are unlinked by transformation they are presumed to lie in separate loci even if they affect sporulation at the same stage. This is based on the estimate that mutations lying 10 to 15 genes apart are about 50% linked by transformation (Bodmer and Ganesan, 1964). The assumption is probably correct unless the gene clusters controlling sporulation are much larger than those involved in vegetative functions. In fact, those sporulation loci that have been analysed in detail seem to contain only a few genes each.

Sporulation mutants may be either asporogenous or oligosporogenous. The former produce no spores at all, whereas the latter produce spores at a reduced frequency, most of the cells being blocked at a defined stage of development. The frequency of sporulation in oligosporogenous mutants varies from about 50% to $1:10^8$ (these are effectively the limits of detection), but for a particular mutant the frequency is constant and reproducible under standard cultural conditions. When a spore produced by an oligosporogenous mutant is used to establish a new population of cells, sporulation will occur at the same frequency as it did in the original population. In a study of a number of oligosporogenous mutants Coote (1972b) obtained evidence that mutations causing oligosporogency may lie very close to those causing asporogeny on the genetic map. This suggests that the two phenotypes may be the expression of different mutations in the same gene (Piggot and Coote, 1976). It is most probable, therefore, that oligosporogenous mutants are leaky mutants in which a defective protein is produced in normal amounts. Temperature-sensitive mutants, which have been used to determine

times of gene activation during sporulation (see, for example, Szulmajster *et al.*, 1970; Young, 1976) can be regarded as a class of oligosporogenous mutants in which the degree of oligosporogeny is temperature-dependent.

C. THE NUMBER OF SPORULATION-SPECIFIC GENETIC LOCI

Although it is not our intention to deal in detail with the later stages of sporulation, for the sake of completeness we will summarise the information now available on the genetics of the whole process. The

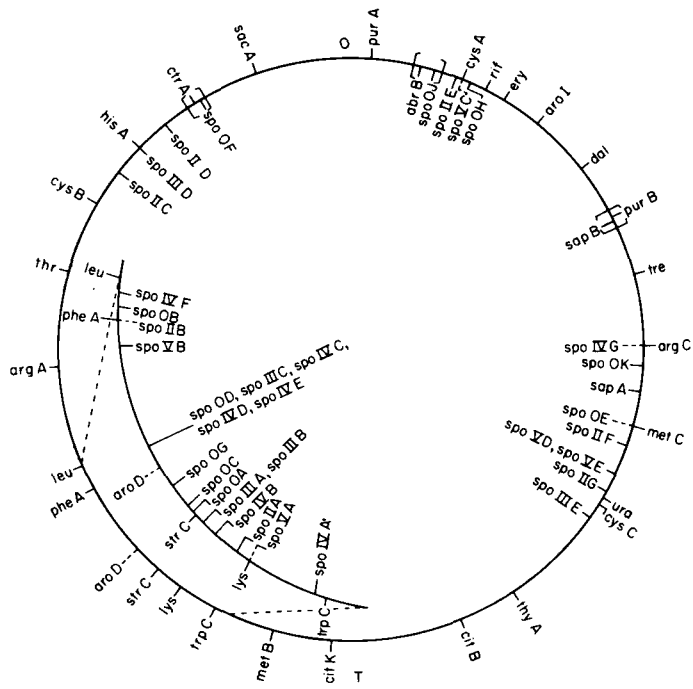


FIG. 2. Genetic map of *Bacillus subtilis*. Loci concerned with sporulation functions are inside the circle and those concerned with vegetative functions are outside. Where the order of markers has not been determined, they are bracketed together. Loci that are indicated by a dashed line have not been ordered relative to neighbouring markers. The replication origin and terminus are denoted O and T, respectively. This map is based on previous compilations by Young and Wilson (1975), Lepesant-Kejzlarovà *et al.* (1975) and Piggot and Coote (1976); these papers may be consulted for further details.

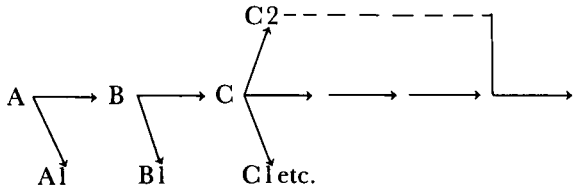
number of known loci associated with sporulation up to the point at which a complete protoplast has been formed in the mother cell is 16, of which nine are classified as stage 0–I and the remaining seven as stage II (see below). A roughly equal number of loci are associated with the remaining stages (Piggot, 1973; Piggot and Coote, 1976). The total number of identified loci is thus about 32; the actual total is certainly higher. A statistical estimate of this number was made by Hranueli *et al.* (1974). Sixteen *spo* mutants were isolated at random and the mutations mapped in relation to 23 identified loci. Ten of the 16 fell into previously identified loci and six into new loci. The crude total was accordingly estimated at 37 ($23 \times 16/10$). Allowance then had to be made for the fact that some loci are more liable to mutate than others. The revised value for the probable total then became 42 with an upper limit of 59 for a confidence coefficient of 68%. The same data were used to calculate that "saturation mapping" to determine the actual number of loci would necessitate the isolation and characterization of about 2000 mutants. The map positions of identified loci are shown in Fig. 2.

D. PLEIOTROPY, DEPENDENT SEQUENCE AND PHENOTYPIC EXCLUSION IN SPORULATION MUTANTS

It has been known since the earliest studies that sporulation mutants are invariably pleiotropic (see for example Balassa, 1969; Waites *et al.*, 1970). Furthermore, if a series of mutants blocked at different stages is scored for the marker events associated with sporulation, each mutant exhibits the characteristics normally associated with all the earlier stages but not those associated with later stages (see Table 1). Sporulation can thus be viewed as a dependent sequence of events, i.e. one in which failure at any one point will cause the whole sequence to fail (Mandelstam, 1969).

On *a priori* grounds, one can therefore infer the existence of a primary sequence of dependent events designated A, B, C, etc. (see below). Each event in this sequence is considered to act directly or indirectly as a trigger for the next (Mandelstam, 1976). Although a primary sequence of biochemical effectors is conceptually necessary to explain the fact of dependence none of these effectors has yet been identified. Indeed, we do not even know whether they are proteins, nucleic acids, or small molecules. (The absence of cross-feeding between mutants probably precludes the last of these possibilities—Mandelstam, 1969.) Whatever their nature, some of the effectors will trigger, in addition, the production

of other substances that are not part of the main sequence. These are, nevertheless, specifically associated with sporulation and hence useful as marker events. They are designated A1, B1, etc., in the diagram below. This group includes, for instance, the metalloprotease and dipicolinic acid. These two groups of substances correspond to the first two categories of sporulation-associated events defined earlier. In general terms, then, we are dealing with a system that can be represented as follows:



As might be expected, a more detailed consideration shows that this is an oversimplification. We have already referred to the fact that coat protein is produced at stage II but is not incorporated into the structure of the spore until stage V (Aronson and Fitz-James, 1968; Wood, 1972). To accomodate this fact, we have introduced a loop into the chain of events. This is indicated as C2 above (Coote and Mandelstam, 1973). Piggot and Coote (1976) have considered the possibility not only that loops might exist, but also that they might constitute subsidiary sequences of dependent events. The consequences of a more complex model of this sort have been set out by Piggot and Coote (1976, Table 4). One consequence is that mutants should be obtainable in which the phenotype does not fit the pleiotropic hierachy that is generally observed (see below). If secondary chains of dependent events are discovered, the simple scheme we have set out will have to be amended. For the time being we can assume that we are dealing with a single chain of events, at least in the early stages of sporulation. In the later stages there may be more than one chain of dependent events (Coote and Mandelstam, 1973; Piggot, 1978).

If we disregard this complication and assume a single chain of dependent events then, obviously, a mutation that affects an early event will have a greater number of pleiotropic consequences than one that affects a later event. In principle, therefore, the order of expression of sporulation loci, presumptive operons, can be deduced from the pleiotropic hierachy. (We comment elsewhere on some factors that might lead to erroneous conclusions when procedures of this sort are adopted. In

particular, we are not measuring the time at which the mRNA molecules are formed; instead we observe only the consequences, biochemical or morphological, after they have been translated and the products have entered into the sporulation sequence. In more rigorous terms, therefore, we should say that we are not measuring the order of expression of loci or genes but, rather, that we are measuring the order in which they intervene in the sporulation sequence.)

An alternative method for determining the order of intervention of loci also follows as an obvious implication of the dependent sequence. If two mutations that block sporulation at different points in the sequence are introduced into the same organism and if sporulation is then induced it will proceed normally until it is blocked by the expression of the earlier of the two mutations. The phenotype of the double mutant will thus resemble that of the parent blocked at the earlier stage and will exclude that of the later blocked mutant. From the phenotypic exclusion patterns (epistatic relationships) some of the stage 0 and stage II mutations could be placed in an order that was internally consistent and that was consistent also with the biochemical marker events in the pleiotropic sequence (Coote and Mandelstam, 1973). The tentative order of expression of stage 0 and stage II loci given below is based both upon the pleiotropic hierachy and the epistatic relationships. When double mutants are constructed from two parents blocked at later stages, anomalous results are sometimes obtained in the sense that the double mutant differs in phenotype from both parents. There are various possible explanations for this (see later, p. 152).

E. GENETIC LOCI CONCERNED WITH STAGES 0 TO II

1. *Stage 0 Loci*

Morphologically, all stage 0 mutants present the appearance of vegetative cells, in the sense that they do not get as far as making a spore septum. Nowadays, no distinction is made between stage 0 and stage I for the reasons given earlier.

Phenotypically, stage 0 mutants can be divided into several categories, the distinctions being made partly on biochemical grounds. Thus exoproteases and antibiotics appear definitely to be sporulation-associated substances. In addition to these biochemical markers there are some other cell functions that are not involved in normal growth or in

normal sporulation but which are, nevertheless, altered in stage 0 mutants. These include the size of the cells in starvation conditions, their competence in genetic transformation and their ability to support the multiplication of certain bacteriophages. Properties under these latter headings appear to straddle the demarcation line between those that are sporulation-linked and those that are "vegetative". On a strict definition they should perhaps be excluded from consideration until it has been definitely established that they are secondary consequences of a block in the sporulation sequence. This is an assumption that has been made, sometimes tacitly and sometimes explicitly, by workers using these criteria. In fact, the position of these events in the pleiotropic sequence, as well as their occurrence or non-occurrence in double mutants (q.v.), supports the assumption that they are, in fact, consequences of interference in the sporulation sequence. For these reasons, they will be included in our discussion of mutations affecting stage 0 and are included in Table 1.

Some of the stage 0 marker events have already been mentioned: viz. protease, antibiotic and cytochrome. The others require a little elaboration. These are (1) *pha*. This characterises the ability of the cells to support growth of phages $\phi 2$ and $\phi 15$. Some stage 0 mutants are susceptible to infection and plaques are formed on agar plates (Ito and Spizizen, 1971, 1972). Mutants blocked at later stages, and also wild type cells, are more resistant and no plaques are formed. (2) *cpt*. This denotes competence in genetic transformation; some stage 0 mutants have largely lost this property (Spizizen, 1965; Michel and Cami, 1969; Schaeffer, 1969). (3) Cell size. Some stage 0 mutants, when exposed to conditions that induce sporulation, divide to form half-sized cells even though, during vegetative growth, their size is normal (Balassa and Yamamoto, 1970; Coote, 1972a; Kretschmer and Fiedler, 1974; Dunn, *et al.*, 1976). Other characteristics include sensitivity to antibiotics (Guespin-Michel, 1971a, b; Ito, *et al.*, 1971), sometimes denoted *abs*, and constitutive synthesis of nitrate reductase (Guespin-Michel *et al.*, 1970; Bohin *et al.*, 1976). This perplexing spectrum of changes has been interpreted as an indication of some kind of membrane alteration in these mutants (Ito and Spizizen, 1971; Ito *et al.*, 1971; Bohin *et al.*, 1976). There is independent evidence to support this now (Rigomier *et al.*, 1974) and to suggest that changes in membrane proteins may be of importance during the early stages of sporulation (Goldman, 1976).

These characteristics, associated with asporogeny, can be suppressed

by a variety of mutations e.g. *cpsX*, Guespin-Michel (1971a, b); *abs* or *abr*, Ito *et al.*, (1971), Trowsdale *et al.*, (1978); *tol*, Ito (1973). Curiously, none of these mutations restores normal sporulation. Suppression is a complex subject, and a detailed discussion is beyond the scope if this article. The reader is referred to Piggot and Coote (1976) for a summary and for references to the original literature. Recent work has suggested that in one class (designated *abrB*) the mutations that confer antibiotic resistance on *spoOA* and *spoOB* strains lead to alterations in ribosomal proteins (Trowsdale *et al.*, 1978). This raises the possibility that the gene products of the *spoOA* and *spoOB* loci interact in some way with the ribosomes at the onset of sporulation (Hoch *et al.*, 1978).

We can now consider the ten loci affecting stage 0. These have been arranged in Table 1 according to their phenotypes. The most pleiotropic

TABLE 1. Properties associated with asporogenous mutants of *Bacillus subtilis*. These are denoted as follows: *cpt*, competence in genetic transformation; *pro*, extracellular protease; *pha*, reaction of cells to infection by phages $\phi 2$ and $\phi 15$ (see text); *cyt*, cytochrome formation under sporulation conditions; *ab*, antibiotic production; *asc*, alkali-soluble coat protein; *pho*, alkaline phosphatase; *DNAase*, Mn^{2+}-dependent endonuclease; *gdh*, glucose dehydrogenase; *ref*, phase brightness. These are scored as: +, wild-type; −, mutant; var, variable. Properties that have not been determined but are presumed positive or negative are given in parentheses.

Locus	*cpt*	*pro*	*pha*	*cyt*	*ab*	*asc*	*pho*	*DNAase*	*gdh*	*ref*
spoOA	−	−	−	−	−	(−)	[a]−	(−)	−	−
spoOB	+	±	−	−	−	(−)	[a]−	(−)	(−)	−
spoOG	+	−	[a]−	(−)	−	(−)	[a]−	(−)	(−)	−
spoOD	+	+	[a]−	(−)	−	(−)	[a]−	(−)	(−)	−
spoOE	(+)	±	+	+	var	(−)	[a]−	(−)	(−)	−
spoOF	+	±	var	+	−	(−)	[a]−	(−)	(−)	−
spoOH	(+)	±	+	+	+	−	−	(−)	−	−
spoOC	+	+	(+)	(+)	+	−	−	(−)	(−)	(−)
spoOJ	(+)	+	(+)	(+)	+	(−)	[a]−	(−)	(−)	−
spoOK	(+)	+	(+)	(+)	+	(−)	[a]−	(−)	(−)	−
spoIIE	[a]+	+	(+)	(+)	+	−	−	−	−	−
spoIIF	[a]+	(+)	(+)	(+)	(+)	?	−	(−)	−	−
spoIIG	[a]+	(+)	(+)	(+)	(+)	?	−	(−)	−	−
spoIIA	[a]+	+	(+)	(+)	+	+	−	−	−	−
spoIIB	(+)	+	(+)	(+)	+	(+)	+	+	−	−
spoIIC	(+)	(+)	(+)	(+)	(+)	(+)	+	+	−	−
spoIID	(+)	(+)	(+)	(+)	(+)	+	+	+	−	−
spoIIIA	(+)	+	(+)	(+)	+	+	+	+	+	−
spoIVG	(+)	+	(+)	(+)	+	+	+	+	+	±

[a] P. J. Piggot (personal communication).

of all is *spoOA*. Mutations in this class have been studied independently in several laboratories (Ionesco *et al.*, 1970; Hoch, 1971; Coote, 1972b; Brehm *et al.*, 1973). They are negative for all marker events associated with stage 0. In an adjacent locus linked by transformation there is another group of mutations affecting the same morphological stage. Strains that harbour these mutations differ considerably in their biochemical properties from strains harbouring *spoOA* mutations (Hoch *et al.*, 1978). They are designated *spoOC* (Table 1). Although this locus is adjacent to the *spoOA* locus, further study might be expected to show that it is under the control of a separate operator. It would be of interest therefore to determine whether the two loci are parts of a single transcriptional unit. It should be noted that some *spoOA* mutations are suppressible by a nonsense suppressor (Hoch and Spizizen, 1969; Hoch, 1971). Unless the mutations are polar, the results suggest that failure to produce a single functional protein can lead to a pleiotropic phenotype.

Fine structure analysis of the *spoOB* locus has also been undertaken by Hoch and his colleagues (Hoch and Matthews, 1973; Hoch *et al.*, 1978). This locus is near to *phe-1* and is thought to be immediately adjacent to the *spoIVF* locus.

Another well-characterised region containing *spoO* mutations is that near *cysA14* (Piggot, 1973; Hoch and Matthews, 1973; Hranueli *et al.*, 1974). Two stage 0 loci designated *spoOH* and *spoOJ* lie in this sector. They are about 50% linked by PBS1-mediated transduction but are unlinked by transformation. Mutations in these loci are among the least pleiotropic of those affecting stage 0 (see Table 1). From recent evidence it seems possible that one of the loci (*spoOJ*) codes for a subunit of RNA polymerase (Bramucci *et al.*, 1977b).

The remaining loci involved in stage 0 have not been studied so extensively. Details of the mapping of the loci are given by Piggot (1973); Piggot and Coote (1976); Hoch (1976) and Hoch *et al.*, (1978).

As a working basis, we can assume that the marker events listed in Table 1 are sporulation-associated and that they are part of a linear sequence of gene expression. The *spoO* loci can accordingly be placed in an order which reflects the sequence of their activation. More precisely, for reasons already given, it may be presumed to indicate the order of intervention of the gene products in the sporulation sequence.

Piggot and Coote (1976), on the basis of the data in their review, suggested the following order:

$$spoOA \rightarrow spoOG \rightarrow spoOB \rightarrow (spoOF, spoOD) \rightarrow spoOE \rightarrow$$
$$spoOH \rightarrow (spoOJ, spoOK)$$

The order depends somewhat on the phenotypic criteria that are considered significant. If for instance the criterion of cell size were adopted, some further discrimination might be possible. This is because it has been shown that if a single strain harbours two *spoO* mutations, one associated with the "small cell" phenotype and the other with the "large", then in this double mutant the "small" phenotype excludes the "large" (Dunn *et al.*, 1976).

2. *Stage II Loci*

Stage II represents the formation of the spore septum and a variety of morphological malformations are observed in mutants blocked at this stage. Recapitulation of the morphological occurrences preceding and accompanying formation of the spore septum will help us to infer some of the topological and biochemical signals that must operate and thus help to clarify the types of failure in development that occur.

As seen in the electron microscope, this stage begins with invagination of the membrane. Spikes of cell wall material are deposited, but they do not develop further. At this point the septum is indistinguishable from an early cell division septum, except that it has begun to form in a sub-polar position instead of the centre of the cell. The growth of membranes continues in an inward direction but is unaccompanied by the further deposition of large amounts of cell wall material. There is now clear divergence from the appearance presented by a cell division septum. (The rest of the process ending in engulfment of the prespore at stage III has already been described).

For the successful completion of stage II a number of essential signals are required. (1) A signal that blocks the central division site of the cell and prevents construction of a septum in the central, i.e. cell division, position. This signal may be given when sporulation is triggered. Strains that harbour a mutation at the *spoOA* locus—the earliest in the pleiotropic sequence—appear to be defective in this signal, and, consequently, they lay down a septum in the central position. This produces the small cell phenotype to which we have referred. It is possible that mutations at other stage 0 loci also produce this phenotype, e.g.

spoOK (Coote, 1972a). (2) A topological signal that places the spore septum in the correct position near one of the poles of the cell. (3) A biochemical signal that prevents the laying down of large amounts of cell wall in the space between the membranes. (4) A signal that we shall call "numerical" for want of a better term. This is a signal specifying that only one septum is to be formed.

Mutations interfering with these signals have been known from the earliest electron microscope studies of sporulation. Many mutants of *B. subtilis* and also of *B. cereus* blocked at stage II have a phenotype that has been called "abortively disporic" (see Ryter *et al.*, 1966a; Fitz-James and Young, 1969; Waites *et al.*, 1970; Piggot and Coote, 1976). The mutants manifest a malfunction of two of the signals (3) and (4); they form septa at both poles instead of one and they insert cell wall into the space between the membranes. The resultant structures differ from minicells in that they contain chromosomes and are apparently capable of growing out into normal vegetative cells (Young, 1964). They have accordingly been called "pygmy" cells (Waites *et al.*, 1970). Mutations at several loci will produce this phenotype (see below). A variety of other morphological phenotypes are encountered in stage II mutants (see Fig. 3 for a diagrammatic representation). Some of these mutants are affected in several of the signals (1–4) referred to. It is not easy to suggest in molecular terms the cause for a misreading of several different signals. It seems likely, however, that some of the more complex forms that are encountered may arise by abnormal development from simpler morphological forms. This can be simply demonstrated by electron microscope examination of samples removed at intervals from cultures of stage II mutants incubated in sporulation medium (M. Young and S. A. Higgs, unpublished work). In mutants blocked at stage II there is thus a tendency for development not simply to stop, but to continue to give rise to abnormal forms that do not form part of the normal sequence of development in wild type strains.

We can now proceed to a summary description of the stage II loci.

spoIIA. Mutations here lead to the disporic phenotype. However, interpretation is made difficult by the fact that in a single preparation one can also see cells exhibiting one, two or multiple septa (Piggot, 1973; J. Mandelstam and S. Clarke, unpublished observations). Strains that harbour a mutation in this locus are blocked in the expression of alkaline phosphatase, extracellular DNAase and all later events (Table 1).

spoIIB. Only one mutation at this locus has been described. The strain

that harbours it is oligosporogenous (Coote, 1972a, b) and forms alkaline phosphatase and DNAase (Akrigg and Mandelstam, 1978). The cells have a single septum in the correct position but it contains cell wall. This mutant seems to be affected in only one of the signals (3).

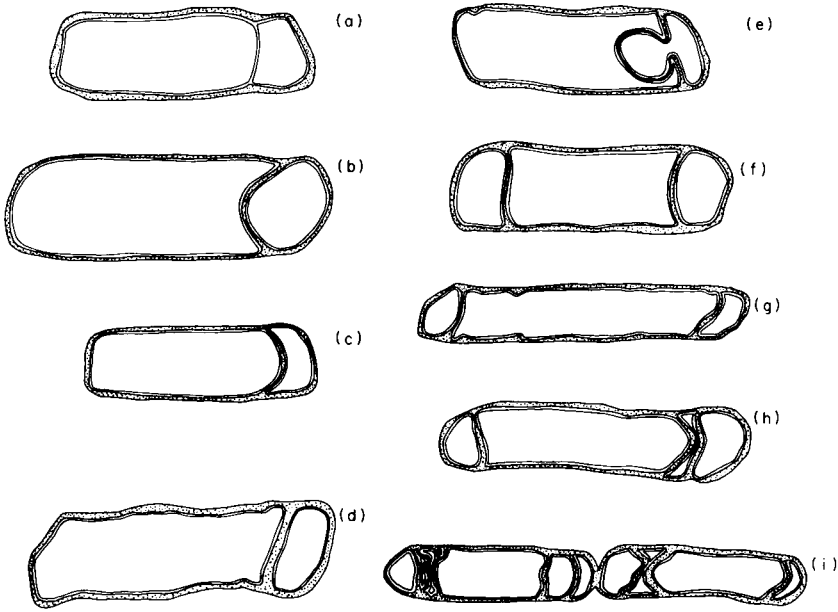


FIG. 3. Schematic diagrams of common abnormalities seen in electron micrographs of mutants blocked at stage II (see text). Mutants showing these and similar phenotypes have been described, among others, by Ryter *et al.* (1966a), Fitz-James and Young (1969), Waites *et al.* (1970) and Coote (1972a).

(a) Development has stopped after the formation of a normal sporulation septum in the correct position.
(b) and (c) A septum has been formed in the correct position but cell wall material has been inserted into the space between the membranes resulting in the formation of a "pygmy" cell.
(d) The septum has become massively rigidified by the insertion of large amounts of cell wall material between the membranes.
(e) The septum has bulged into the mother cell, but the points of abutment to the cell wall have not migrated towards the cell pole. Cell wall material has been inserted into the distorted septum.
(f) and (g) "Abortively disporic" phenotype. Two septa, both containing cell wall material, have been formed at opposite poles of the cell.
(h) A variant of the "abortively disporic" phenotype in which two septa are formed at one pole and one at the other.
(i) Cells with multiple septa are commonly seen in cultures of "abortively disporic" mutants. The relative proportions of (f), (g) and (i) depend on the sampling time and can vary from one experiment to another (see text).

spoIIC. A single mutation mapping at this locus has been reported (Coote, 1972a,b). It is found in an oligosporogenous mutant that forms a single septum in the correct position. However, the intermembranal space is filled with cell wall material and the structure forms a hemispherical bulge in the cytoplasm of the mother cell. The phenotype is very characteristic, but it has again to be noted that in the same preparation some of the cells may show other phenotypes resembling those seen in cells mutated in the *spoIIB* locus. Furthermore, the proportion of cells exhibiting each of the phenotypes may vary considerably from one experiment to another (J. Mandelstam and S. Clarke, unpublished findings).

spoIID. Mutations in this locus seem to give rise to a single septum containing wall. The cells form alkaline phosphatase and also DNAase but are blocked in the expression of later events.

spoIIE. Mutations in the *purA-cysA* region have been reported by Takahashi (1965; 1969) as well as by Piggot (1973). They give rise to a variety of phenotypes and once again the relative proportions of cells exhibiting these may vary in different experiments (J. Mandelstam and S. Clarke, unpublished work). One of the mutants, N25, produces excess membrane (Waites *et al.*, 1970). The cells present the appearance of being blocked just before the growing membrane fuses at the pole to give a spore protoplast. While the morphological phenotype suggests that the mutant is blocked very late in stage II, the biochemical characteristics, failure to produce phosphatase and DNAase, place it earlier in the pleiotropic sequence (see Table 1). The latter interpretation is supported by the fact that in double mutants this phenotype excludes those of several other *spoII* loci (Coote and Mandelstam, 1973).

Several other mutations in the *spoIIE* locus produce a disporic phenotype. In transformation crosses these mutations gave recombination indexes that were very low (less than 0.1) implying that they lie in the same gene as N25 (Piggot, 1973). Mutations giving rise to a single septum massively rigidified with cell wall material are also found at this locus. It is important not to over-interpret these results. First, there is the fact, already noted, of day to day variation in appearance in the electron microscope. Second, is the possibility that two mutations lying at the adjacent ends of two contiguous genes may have a lower recombination index than two mutations lying at opposite ends of the same gene.

spoIIF. Mutations in this region are also characterised by the abortively

disporic phenotype. The locus has been mapped by Hranueli *et al.*, (1974). Mutations lying in this region of the chromosome which were mapped by Ionesco *et al.*, (1970) have been provisionally assigned to this locus by Piggot and Coote (1976).

spoIIG. Several mutations studied by Piggot (1973) and by Young (1975) lie in this locus. One of them (*spo-279*) gives rise to a temperature-sensitive phenotype and this was used to establish the time at which this locus is activated (Young, 1976). Apparently, a product of this locus (the product that is defective in strains harbouring the *spo-279* mutation) is required for only some 15 min at about the time of septum formation. Mutations at this locus also result in the abortively disporic phenotype.

On the basis of the pleiotropic hierarchy and the phenotypic exclusion relations in double mutants (Coote and Mandelstam, 1973) the following sequence of expression of the stage II loci has been suggested (Piggot and Coote, 1976).

$$(spoIIE,\ IIF,\ IIG) \rightarrow spoIIA \rightarrow (spoIIB,\ IIC,\ IID)$$

VI. Induction of Sporulation

In this section, we will summarise evidence to show that the probability that a cell will choose the sporulation pathway in preference to continued vegetative growth is governed by two separate and quite different types of factor. The first factor is the nutritional state of the culture, which is presumably reflected in the concentration of an intracellular biochemical effector and this, in turn, determines the proportion of cells in the population that will be induced to sporulate. This factor we might term the population probability factor. Superimposed upon this is a second factor. This is the probability, at any particular moment, that a cell will be in the right physiological condition to respond to the biochemical effector. The capacity for this response is determined by the state of chromosome replication in the individual cell. The population factor and the physiological factor will be considered separately.

A. POPULATION PROBABILITY FACTOR AND THE HUNT FOR THE REPRESSOR

The suggestion that spore formation is caused by lack of nutrients is often attributed to Knaysi (1948) but it goes back a good deal further, having

been proposed by Buchner (1890). No conceptual progress was made until Aubert *et al.* (1961) discovered that even when a culture of *B. megaterium* was growing exponentially a very small fraction of the cells formed spores. The next advance was made by Schaeffer *et al.* (1965). Batch cultures of *B. subtilis* were grown in a number of different media and the growth rate and spore incidence were measured in each culture while it was in exponential growth. When the medium contained a good carbon source and a good nitrogen source the probability of inducing sporulation was very low, but it increased with the poorer nitrogen sources. The data also indicated the importance of the carbon source, and the conclusion was drawn that sporulation in a growth medium is controlled by some catabolite which has its intracellular concentration determined by both the carbon and nitrogen sources.

This line of investigation was pursued in a study in which chemostat cultures of *B. subtilis* were subjected to nutrient limitations at a variety of growth rates in a defined medium. In these experiments the spore incidence was determined only after the culture had reached a steady state (Dawes and Mandelstam, 1970). Some degree of sporulation was found at all growth rates tested and it was highest at the lowest growth rates. A medium limited in either nitrogen or carbon gave the best yields of spores. When growth was limited by a single amino acid (tryptophan) or magnesium ions or inorganic phosphate, the incidence of spores was very much lower.

These two sets of experiments allow the following conclusions to be drawn. (1) The incidence of sporulation is related to growth rate by a continuous curve; there is no evidence of a threshold effect. (2) At any specified growth rate a definite fraction of the cells will be induced to sporulate. (3) The results are consistent with the assumption that sporulation is a repressible function and that the repressor is at a high concentration when the cells grow in a medium containing good sources of both nitrogen and carbon.

The last conclusion attracted the obvious comparison of the regulation of spore formation with the well-studied catabolite repression mechanisms that control the synthesis of inducible enzymes such as β-galactosidase and histidase. The analogy inspired a search for the catabolite effector which still continues and which we shall attempt to summarise only partially. One of the most favoured compounds, cyclic AMP, is not found in appreciable quantities in bacilli (Ide, 1971; Setlow, 1973). Other possibilities including ATP, cyclic GMP and the adenylate

energy charge (Hutchison and Hanson, 1974,; Bernlohr *et al.*, 1974) seem to have fallen from fashion and have been little studied in recent years. Some other possible effectors will now be considered in turn.

B. GLUTAMINE SYNTHETASE

Aubert and his colleagues have suggested that glutamine synthetase is involved in the induction of sporulation in *B. megaterium*. Initially, interest was directed towards the idea that glutamine synthetase is an early enzyme in the pathway concerned with the production of the catabolite effector substance. Elmerich and Aubert (1972, 1973) proposed that the trigger substance is glutamine itself or, alternatively, an intermediate in purine biosynthesis whose nitrogen atoms are derived from the amide group of glutamine. There is a possible link here with the finding that partial starvation for purine nucleotides will induce sporulation (see below).

More recent experiments indicate that glutamine synthetase itself may be involved in the induction of sporulation. Reysset and Aubert (1975) studied a number of glutamine-requiring mutants of *B. megaterium* and showed by a variety of different criteria that they harboured defects in the structural gene for glutamine synthetase. Most of these mutants did not sporulate, even in the presence of glutamine. Several strains yielded single-step revertants that could synthesise glutamine and that had simultaneously regained the ability to sporulate. Very recently, Reysset *et al.* (1978) have extended these reversion studies. When the primary selection was made for the ability to sporulate as opposed to glutamine prototrophy, there was no simultaneous reversion of the glutamine requirement. The explanation that Aubert and his colleagues propose for these complex patterns of reversion is that glutamine synthetase plays an important regulatory role in sporulation that is quite separate from its catalytic role in glutamine biosynthesis. Reysset *et al.* (1978) have also obtained evidence that the protein may play a similar role in *B. subtilis*, although it appears that in this species most of the glutamine-requiring mutants that are defective in the glutamine synthetase structural gene will sporulate normally in the presence of added glutamine (Dean *et al.*, 1977; Fisher and Sonenshein, 1977).

In these studies there is an obvious analogy with the regulatory role of glutamine synthetase in the control of transcription of the genes coding for enzymes of amino acid catabolism in *Klebsiella aerogenes* (Magasanik *et al.*, 1974).

C. HIGHLY PHOSPHORYLATED NUCLEOTIDES

Rhaese and his collaborators have identified a group of highly phosphorylated nucleotides (HPNs) that are synthesised at the onset of sporulation but not during growth. Two of these compounds (HPN I or ppApp and HPN IV or pppAppp) are synthesised *in vitro* from ATP by purified plasma-membrane vesicles (Rhaese and Groscurth, 1976). The authors have speculated that HPN IV may be the "chemical messenger" that is released from the membrane when the growth medium is exhausted and that it induces sporulation.

The enzymic machinery for HPN IV production is present in the plasma membrane during both growth and sporulation. Rhaese and Groscurth (1976) suggest that the burst of HPN IV synthesis that occurs at the start of sporulation is occasioned by a fall in the cellular concentration of inhibitory phosphorylated glucose metabolites that occurs when glucose is exhausted. These compounds (glucose-1-phosphate, glucose-6-phosphate and fructose-1,6-diphosphate) inhibit HPN IV synthesis *in vitro*. A mutant that is defective in phosphoglucoisomerase and phosphofructokinase, and which therefore accumulates fructose-6-phosphate, is unable to produce HPN IV and does not sporulate (Rhaese *et al.*, 1978).

It has recently been shown that a stage 0 sporulation mutant with a defect in the *spoOF* locus does not synthesise HPN IV (Rhaese *et al.*, 1977). A single step Spo^+ revertant regained the ability to produce the compound. The authors proposed that the *spoOF* locus might contain the gene that codes for "HPN IV synthetase". The recent observation that a temperature-sensitive *spoOF* mutant was somewhat temperature-sensitive *in vitro* for HPN IV production adds substance to their proposal (Rhaese *et al.*, 1978). Confirmation that the *spoOF* locus in fact codes for the enzyme must await the purification and characterization of "HPN IV synthetase" from wild-type bacteria and from *spoOF* mutants.

A mutant with a defect in the *spoOE* locus also produced little or no HPN IV, whereas a defect in the *spoOA* locus caused over-production of HPN IV (Rhaese *et al.*, 1977). The roles of these genetic loci in the control of HPN IV synthesis are not known. Another compound, HPN III (with the tentative structure ppZpUp, where U is uridine and Z is an unknown sugar) with unknown function is also synthesised at the beginning of sporulation.

The two compounds, HPN I and II (ppApp and ppAppp), that also appear at the start of sporulation, are produced by the "idling" reaction

of ribosomes from sporulating cells (Rhaese and Groscurth, 1974). Ribosomes from vegetative cells produce the corresponding guanine nucleotides (MS I and II). When a variety of stage 0 mutants (with defects in the *spoOA*, *OB*, *OE*, *OF* and *OH* loci) were incubated under conditions in which wild-type strains sporulated, they all failed to produce HPN I and II; instead they retained the capacity to synthesise MS I and II which is normally lost at the beginning of sporulation (Rhaese *et al.*, 1978; Rhaese and Groscurth, 1978). It now seems clear that MS nucleotides are not involved in sporulation (Schaeffer *et al.*, 1973; Fortnagel and Bergmann, 1974; Rhaese *et al.*, 1975); the roles of HPN I and II remain a matter of conjecture.

D. THE EFFECT OF PURINE STARVATION

A variety of compounds that interfere with purine biosynthesis can stimulate sporulation. For example, Sacks and Thompson (1977) have demonstrated that several methylxanthines (theophylline, caffeine and isobutylmethylxanthine) increase the spore yield of *Clostridium perfringens*. The effectiveness of these compounds varied from one strain to another and with the carbon source. Similarly, sporulation in *B. subtilis* can be induced in the presence of excess carbon, nitrogen and phosphorus (see earlier part of this section) by the addition of decoyinine or hadacydin to the growth medium (Mitani *et al.*, 1977). Decoyinine and hadacydin are specific inhibitors of GMP synthetase and adenylosuccinate synthetase and the enhancement of sporulation that they promote can be antagonised by the simultaneous addition of guanine and adenine, respectively. More recently, Freese and his collaborators (Freese *et al.*, 1978) have demonstrated that a variety of compounds that lead to purine starvation will induce sporulation, whereas starvation for pyrimidines has no effect. All of the compounds that enhance sporulation in the presence of excess carbon, nitrogen and phosphorus are effective at concentrations which are inhibitory to growth. This is consistent with the long-established fact (already referred to) that the probability that a cell will sporulate increases with decreasing growth rate (Schaeffer *et al.*, 1965; Dawes and Mandelstam, 1970). However, growth rate cannot be the only controlling factor in these experiments because it does not explain why starvation for pyrimidines instead of purines failed to induce sporulation.

Mutations that affect purine biosynthesis can also affect sporulation.

For example, Levisohn and Aronson (1967) and Elmerich and Aubert (1975) have described purine auxotrophs which sporulate under conditions that prevent sporulation of wild-type strains. The glutamine synthetase mutants that show impaired sporulation have been discussed earlier. Freese *et al.* (1978) have shown that purine auxotrophs can be made to sporulate by incubation in media containing excess carbon and nitrogen, but limiting concentrations of purines. It has been proposed that partial starvation for purines creates "the proper biosynthetic balance" conducive to the siting of a septum in a polar rather than a central position (Mitani *et al.*, 1977).

E. SUMMARY OF BIOCHEMISTRY OF INDUCTION

The data we have summarised are difficult to interpret because the intermediary metabolism of the bacterial cell is a complex of interdependent pathways. Consequently, altering the concentration of one intermediate is likely to affect the concentration of a great many others and hence to affect also the biosynthesis of cell polymers. Particularly large fluctuations are likely to be measured when the intermediates are such sensitive indicators of nutrition as phosphorylated compounds or so centrally placed in metabolism as glutamine, adenine, etc. For example, any interference with the supply of glutamine will affect the supply of all the amino acids, purines, pyrimidines, etc. for which glutamine acts as an amino donor. Similarly, an alteration in the intracellular concentration of ATP will have consequential effects on the pool concentrations of all metabolic intermediates as well as on the rates of formation of proteins, nucleic acids and other polymers. The complexity of these interactions makes it impossible to decide which of the manifold variations in molecular concentrations is the "real" trigger event.

This investigation is reminiscent of the hunt for the catabolite repressor of the *lac* operon. A great deal of effort was expended and a great many compounds were erroneously suggested as the real repressor by a great many investigators*. In fact, correlations could be shown between the concentrations of a diversity of compounds and the degree of catabolite repression. In the end, cyclic AMP was identified as the active substance, but proof of its function was only possible because by that time the regulation of the *lac* operon had been elucidated both biochemically and genetically.

*Including one of the authors of the present review (J.M.).

It is apparent that whatever the key substance may be in the catabolite repression of sporulation, there are likely to be large numbers of metabolites whose concentrations will fluctuate with it in parallel and a comparable number of others whose concentrations will fluctuate inversely. Until we know whether the control of sporulation is positive or negative and until we have elucidated the functioning of the first operon concerned in sporulation at a molecular level, it is unlikely that the catabolite effector will be identified.

F. REQUIREMENT FOR DNA REPLICATION

In this section, we consider the evidence showing that the induction of sporulation requires DNA replication. This fact clearly distinguishes sporulation from other catabolite-repressed functions of *B. subtilis* (Coote, 1974).

When *B. subtilis* was grown in a chemostat with carbon or nitrogen as the limiting nutrient, a mixed population was established that comprised growing cells, sporulating cells and spores (Dawes and Mandelstam, 1970). The relative proportions of these cell types depended on the growth (dilution) rate (see earlier). If rapidly growing cells were exposed to repeated short periods of starvation (by changing from a fast to a slow dilution rate for a fraction of a generation time) only a proportion of the population of cells was induced to sporulate during each exposure. It thus appeared that some cells in the growing population were sensitive to the starvation stimulus whereas others were not. Dawes and Mandelstam (1970) drew the inference that cells acquire sensitivity only at a specific stage of the cell cycle. (See Haber and Halvorson, 1972; Holzer *et al.*, 1972; Reinert and Holzer, 1975, for other examples of the cell cycle dependency of differentiation processes.)

Additional information was obtained by examining the sporulation behaviour of sister cells. When growing cells of *B. subtilis* are transferred to a sporulation medium the sister cells tend to remain attached. They either go through a cycle of vegetative growth together or they proceed to sporulate in almost perfect synchrony. These observations also suggested that the induction stimulus was effective only at a critical period during the cell cycle. If sporulation had not been induced the cell would divide and the daughters would be committed to another round of growth: if sporulation had been induced the cell would divide and the daughters would sporulate in unison (Dawes *et al.*, 1971).

The fact that a cell division must occur after induction of sporulation will also explain why outgrowing spores of *B. subtilis* could not resporulate until a second round of replication had been initiated, i.e. until the DNA replication potential of the outgrowing spore was sufficient to generate two daughter cells each containing two chromosomes (Keynan *et al.*, 1976). These experiments therefore suggest that in *B. subtilis* sporulation is expressed not in the cell in which induction occurs but in the next generation.

From what has already been said, it is reasonable to suppose that the starvation stimulus results in either the production of an inducer or the removal of a repressor of sporulation. Although we do not know whether the regulation is positive or negative, we do know that it differs from the regulation systems of catabolite repressible vegetative functions in several important respects. We will now discuss evidence to show that: (1) cells can only be induced to sporulate during a very limited period in the growth cycle and this period seems to be determined by the state of chromosome replication; (2) even if sporulation has been induced, the ensuing sequence of events will not occur if completion of chromosome replication is prevented.

These represent fundamental differences from other inducible or repressible systems. From the kinetics of induction of such enzymes as β-galactosidase in *E. coli*, it is apparent that the whole population is induced and begins to form the enzyme a few minutes after induction. The population is homogeneous in enzyme content even after so short a period as 15 min. This was shown in a classic and elegant experiment carried out by Benzer (1953). A comparably rapid production of enzyme by the whole population was also shown after induction of penicillinase in *B. cereus* (Collins *et al.*, 1964). Finally, it has been demonstrated that inducible "vegetative" enzymes such as sucrase and histidase are formed freely in *B. subtilis* even when DNA synthesis is prevented by thymine starvation (Coote, 1974).

We can now consider in more detail the evidence linking sporulation to the DNA replication cycle.

1. *Requirement for DNA Synthesis*

If thymine-requiring cells of *B. subtilis* are transferred to a sporulation medium lacking thymidine, sporulation does not occur (Mandelstam *et al.*, 1971). Even the earliest known sporulation-associated event, namely

production of extracellular serine protease, is blocked unless thymidine is supplied (Dancer and Mandelstam, 1975b). It is thus clear that even if sporulation is induced by exposing the cells to a suitable environment there is additionally a requirement for DNA synthesis. From the experiments described below it is apparent that this is because existing rounds of chromosome replication must be terminated to allow sporulation to proceed. Presumably, segregation of the presumptive prespore and mother cell chromosomes before formation of the polar spore septum depends upon chromosome termination, as does chromosome segregation during normal cell division (see review by Donachie, 1973). Other parallels have been drawn between sporulation and cell division by Hitchins and Slepecky (1969b).

2. *Induction and the Critical Stage in Chromosome Replication*

Induction of sporulation seems to occur only while a critical region of the genome, fairly near the origin, is undergoing replication. In one set of experiments Mandelstam and Higgs (1974) used a temperature-sensitive DNA-initiation mutant to obtain a population of cells in which a single complete synchronous round of chromosome replication occurred in a rich medium. Samples of the culture were transferred at intervals to a poor medium to test the ability of the cells to sporulate. The maximum sporulation potential developed about 15 min after the initiation of replication (i.e. after about a quarter to a third of the chromosome had been replicated) and it declined thereafter. The experiment has since been repeated with more time points and an essentially similar result was obtained (M. Young and P. Jeffs, unpublished data).

In the experiments with the temperature-sensitive mutant the frequency of sporulation was low and it was possible, if unlikely, that the results were artefacts arising from the temperature shifts used to obtain synchrony. For these reasons a quite different approach was used to obtain independent confirmation of these findings. Sporulation was induced in a growing asynchronous population of cells by the resuspension technique. Samples were removed at intervals and treated with enough HPUra to block further DNA replication (HPUra is thought to be a specific inhibitor of replicative DNA synthesis in *B. subtilis* and other Gram-positive organisms (Brown, 1970, 1971; see also review by Cozzarelli, 1977). After about 35 min in the sporulation-inducing medium, the population of the cells began to escape from the inhibitory

effect of the drug (Dunn *et al.*, 1978; Young and Jeffs, 1978). The authors concluded that the first cells to escape from the inhibitory effect of the drug had replication forks about 35 min from the replication terminus at the time when they were transferred to the poor medium. Since the chromosome replication time in *B. subtilis* is 50 to 55 min (Ephrati-Elizur and Borenstein, 1971; Keynan *et al.*, 1976; Dunn *et al.*, 1978) this would correspond to a region of the chromosome about 15 to 20 min from the replication origin. This agreed with the experiments carried out with the temperature-sensitive mutant.

When experiments are done by the exhaustion method rather than by resuspension, the results are less clear cut since t_0 is ill-defined and the time of induction is difficult to pinpoint in relation to chromosome replication. Nevertheless, HPUra, added at t_0, blocks sporulation in *B. subtilis* (Leighton *et al.*, 1975; Shibano *et al.*, 1978).

VII. Compartmentation and Division of Labour

In this section we consider some of the recent evidence suggesting that different genes are expressed in the prespore and mother cell compartments of sporulating organisms. Evidently, this is not a new idea; it is only recently, however, that persuasive evidence to support it has been obtained.

The two cells that are generated at stage II of sporulation by an unequal division of the parent cell are presumably rather similar in biochemical terms. As sporulation proceeds they diverge from each other. At the end of development their composition and metabolic activities are very different indeed. This could have at least two explanations. First, different genes might be transcribed and translated in the two cells. An alternative explanation is that the prespore genome is "silent" once engulfment has occurred and that transcription is not reactivated until the mature spore has germinated. It now seems likely that the former of these alternatives is correct. The reasons for this are as follows.

1. Piggot (1978) has recently described a new marker rescue method for examining gene expression in the prespore and mother cell compartments during sporulation in *B. subtilis*. He used DNA from a sporogenic strain to obtain genetic transformation of asporogenous mutants and added it when the cells were exposed to sporulation-inducing conditions. After allowing adequate time for sporulation of any organisms that had rescued the relevant *spo*$^+$ gene from the transforming

DNA, the cultures were heat-treated to kill vegetative cells and then plated on a minimal medium. The spores thus selected then germinated and gave rise to colonies. Piggot found that strains bearing mutations at certain loci (*spoIIIA*, *spoIIIE* and *spoVA*) gave rise almost exclusively to Spo^+ colonies whereas others (*spoVB* and perhaps *spoIVC*) gave rise predominantly to Spo^- colonies. Presumably in those instances where Spo^+ colonies arose the rescued spo^+ allele had been incorporated into the prespore genome and it was therefore perpetuated and expressed in the resulting progeny. In those cases where predominantly Spo^- colonies arose, the rescued spo^+ allele had presumably been incorporated into the mother cell genome and for this reason it was not perpetuated.

The assumption underlying the experiments is that if the sporulating organism, which is a two-celled structure, bears one wild type and one mutant allele for a sporulation gene, then a spore can be produced only if the spo^+ allele is segregated into that compartment in which it has to be expressed during sporulation. Thus *spoIIIA*, *spoIIIE* and *spoVA* need to be expressed in the prespore, whereas *spoVB* and perhaps *spoIVC* need to be expressed in the mother cell. As more information becomes available, it will be of interest to correlate these results with those of the epistasis experiments. Some of the epistasis experiments with double sporulation mutants, which have been discussed previously, suggest that there may be more than one dependent sequence of gene expression during the later stages of sporulation (Coote and Mandelstam, 1973; Piggot and Coote, 1976). This is consistent with the idea that different genes are activated in the two compartments of the developing sporangium.

2. When radioactively labelled phenylalanine was supplied to sporulating cells of *B. megaterium* at late stages of development, trichloracetic acid-precipitable activity was found in purified preparations of prespores (Ellar *et al.*, 1975). This indicates the deposition of newly-synthesized protein in the prespore, even at late stages of development. Although it seems probable that this protein is synthesized *in situ* we cannot, at present, rule out the possibility that it is synthesized in and exported from the mother cell. Ryter *et al.* (1966b) have shown that RNA synthesis occurs in stage III prespores of *B. subtilis*. It is now important to know what proportion of this RNA is messenger RNA, and it would be of interest to use hybridization-competition techniques to determine whether this messenger RNA is transcribed from the same genes as that in the mother cell.

In addition to this information on general protein and RNA synthesis,

it is also significant that many of the substances elaborated during sporulation are of restricted distribution; several compounds are found exclusively in one or other of the two compartments of the developing sporangium. Of course, it is not possible to infer the site of synthesis of a compound from its distribution. For instance, dipicolinic acid is found only in the prespore compartment (Ellar and Posgate, 1974), whereas the enzyme concerned with its biosynthesis, dipicolinate synthetase, is found only in the mother cell (Andreoli *et al.*, 1975). Presumably, this small molecular weight compound is made in the mother cell and transported across the prespore membranes. The transport of macromolecules from one compartment to the other, at least on a large scale, is less likely. It therefore seems probable that spore-specific proteins, such as the low molecular weight basic proteins that accumulate in the prespore from about stage III onwards and which finally comprise up to 20 per cent of the total spore protein (Setlow, 1975; M. G. Shepherd and H. A. Foster, personal communication), are synthesized exclusively from messenger RNA that is transcribed and translated in the prespore. The enzyme diaminopimelate ligase in *Bacillus sphaericus*, which is concerned with cortex biosynthesis and is found only in the mother cell (Tipper and Pratt, 1970; see p. 109), has been discussed previously.

VIII. Conclusion

In this review we have considered sporulation mainly as a problem in the regulation of a complex sequence of events. Until now, progress towards a solution of this problem has been slow. Although as a result of genetical analysis we know the dimensions of the problem, progress has been held up on the biochemical side. In particular, we know nothing about the molecules that control the operons that initiate sporulation, and nothing about those that act as messengers in the ordered sequence that follows. The latest techniques in genetic manipulation have opened up the possibility of cloning sporulation genes and identifying the products for which they code (Segall and Losick, 1977). It is now reasonable to hope that within the next few years we shall have identified at least some of the molecules that govern expression of the sequence and we might understand, at least in principle, how they do so. Even a partial elucidation of the mechanism should greatly increase our understanding not only of sporulation but of biochemical differentiation in more complex organisms.

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See p. 321 for Note added in proof.

The Outer Membrane of Gram-negative Bacteria

HIROSHI NIKAIDO

Department of Microbiology and Immunology, University of California, Berkeley, California 94720, U.S.A.

and

TAIJI NAKAE

Department of Parasitology, Tokai University School of Medicine, Isehara, Kanagawa-ken, Japan

I. Introduction 164
II. Preparation and composition 164
A. Preparation 164
B. Composition 166
III. Structure. 168
A. Phospholipids 168
B. Lipopolysaccharide (LPS) 168
C. Proteins 171
D. Supramolecular organization of the components 181
IV. Functions of the outer membrane 187
A. "Cell surface" functions 187
B. Multiplicity of diffusion pathways 189
C. Barrier properties against hydrophobic molecules. 192
D. Non-specific permeability toward small, hydrophilic molecules . . . 196
E. Specific diffusion mechanisms 216
F. Penetration of macromolecules 218
G. Other functions 219
H. The structure of the outer membrane revisited 221
V. Assembly of the outer membrane 228
A. Biosynthesis of lipopolysaccharides 228
B. Biosynthesis of proteins 231
C. Assembly and growth of the outer membrane 233
VI. Acknowledgments 239
References 240

I. Introduction

All bacterial cells except those of mycoplasma and L-forms are surrounded by cell wall. It is a common knowledge that a fundamental difference exists in the structure of cell wall between the Gram-positive and Gram-negative prokaryotes, and that in the latter the cell wall contains an outer membrane layer in addition to the underlying peptidoglycan (or "murein") layer (see, for example, Glauert and Thornley, 1969; Freer and Salton, 1970). The outer membrane, especially that of *Escherichia coli* and *Salmonella typhimurium*, has been studied extensively during the last several years, and at present we can claim to understand its structure and function fairly well at the molecular level. In fact it would not be an exaggeration to say that, together with the erythrocyte membrane, the outer membrane is one of the best studied or best understood biological membranes. This review attempts to synthesize our current knowledge of the outer membrane, emphasizing the structural and molecular basis of various functions. Some of the earlier works have been reviewed (Costerton *et al.*, 1974). Recently, excellent reviews on the genetics (Stocker and Mäkelä, 1978) as well as the biogenesis (DiRienzo *et al.*, 1978) of the outer membrane have been written; consequently only bare outlines of these aspects will be given here. The number of recent publications in the area is quite large even when the scope is limited in this manner. We therefore had to be somewhat selective in citing literature.

II. Preparation and Composition

A. PREPARATION

Although morphological studies showed that the cell wall of Gram-negative bacteria was composed of the outer membrane and the underlying peptidoglycan layer (Murray *et al.*, 1965; De Petris, 1967), cell disruption followed by differential centrifugation usually produced "cell envelopes", containing not only the outer membrane and the peptidoglycan, but also the cytoplasmic membrane. Thus detailed biochemical studies of the outer membrane had been impossible until Miura and Mizushima (1968, 1969) devised a method of separating the outer membrane away from other components of the envelope. Various procedures and modifications have since been published.

1. *Methods based on the Buoyant Density Differences*

In the original procedure of Miura and Mizushima (1968), cells of *E. coli* were disrupted by the osmotic lysis of lysozyme-EDTA sphaeroplasts, and the membranes were separated by an equilibrium sucrose density gradient centrifugation. The outer membrane banded at a higher density presumably because it contains much more carbohydrate in the form of lipopolysaccharide (LPS), and possibly more proteins, than does the inner membrane. Ethylenediaminetetra-acetic acid is known to extract significant portions of LPS (Leive, 1965c; Gray and Wilkinson, 1965a,b), but Osborn *et al.* (1972a) carefully examined the various steps of the Miura-Mizushima procedure, and established a modified procedure in which the loss of LPS is negligible, at least in *S. typhimurium.*

The outer membrane is preferentially shed during production of lysozyme-EDTA sphaeroplasts, and the released material can be recovered by aggregation at pH 5 (Wolf-Watz *et al.*, 1973) or by high-speed centrifugation (Mizushima and Yamada, 1975). Although this method is convenient, the composition of the released material seems to be significantly different from the average composition of the outer membrane (Mizushima and Yamada, 1975).

Instead of effecting lysis of lysozyme-EDTA sphaeroplasts, intact cells of *E. coli* and *S. typhimurium* can be directly disrupted by using a French pressure cell, and the outer-membrane-peptidoglycan complex can be separated from the inner membrane by equilibrium density-gradient centrifugation. This procedure, originally described by Schnaitman (1970) and modified by Koplow and Goldfine (1974) and by Smit *et al.* (1975), has several advantages over the lysozyme-EDTA method: (a) large amounts of cells can be handled easily; (b) significant amounts of intermediate density material are not generated, so that discontinuous gradients involving large "steps" can be used; (c) presumably because of the presence of the peptidoglycan, the density of the outer-membrane-containing complex remains quite high even in "deep rough" mutants (see below) that produce outer membranes of lower density, thus facilitating its separation (Smit *et al.*, 1975); (d) in the procedure described by Smit *et al.* (1975) EDTA is not added at any stage. The one drawback of this method is the frequently poor recovery of the inner membrane, due to its fragmentation into very small vesicles (see van Heerikhuizen *et al.*, 1975; Jones and Osborn, 1977b).

Finally, both the Osborn and Schnaitman procedures have been

successfully used for the isolation of outer membranes from such non-enteric bacteria as *Neisseria gonorrhoeae* (Johnston and Gotschlich, 1974; Wolf-Watz *et al.*, 1975; Walstad *et al.*, 1977), *Pseudomonas aeruginosa* (Stinnett and Eagon, 1973; Hancock and Nikaido, 1978), *Caulobacter crescentus* (Agabian and Unger, 1978), and *Thermomicrobium roseum* (G. J. Merkel, D. R. Durham and J. J. Perry, personal communication). In *Acinetobacter* sp., the outer membrane could be isolated by repeated differential centrifugation because French pressure cell treatment broke the cytoplasmic membrane into many small fragments (Thornley *et al.*, 1973).

2. *Methods based on the Differences in Electric Charges*

Outer membrane has a very large number of negatively charged groups owing to the presence of LPS, and can be separated from the less negatively charged inner membrane by use of a preparative particle electrophoresis apparatus (White *et al.*, 1972).

3. *Methods based on Selective Solubilization with Detergents*

Ionic interactions presumably play an important role in stabilization of the structure of the outer membrane (see Section III,D), which therefore often resists attacks by non-ionic detergents. It was originally found by DePamphilis and Adler (1971) and by Schnaitman (1971) that treatment of the cell envelope with Triton X-100 in the presence of Mg^{2+} results in complete solubilization of the inner membrane, whereas the outer membrane retains its morphological integrity. An ionic detergent, Sarkosyl, was found to produce a similar selective solubilization effect in the absence of added Mg^{2+} (Filip *et al.*, 1973). Triton X-100, however, removes significant fractions of phospholipids and LPS from the outer membrane. Thus, although most outer membrane proteins remain unextracted, there is no guarantee that every protein component behaves in this fashion.

B. COMPOSITION

The major constituents of the outer membrane are proteins, LPS and phospholipids. The weight ratios between these components, however, mean very little, as they are strongly affected by the lengths of the carbohydrate chains of LPS. A more meaningful figure is the number of

these molecules found per unit area of the outer membrane. Such a calculation has been done in *S. typhimurium*, which was found to contain about 1.5×10^6 molecules of phospholipids and 5.8×10^5 molecules of LPS *monomers* (see Section III.B) per μm^2 section of the outer membrane, which represents 2 μm^2 of total surface area (Smit *et al.*, 1975). The surface area of a single molecule of phospholipid is approximately 0.54 nm^2. The surface area of LPS monomer was reported to be 0.77 nm^2 (Romeo *et al.*, 1970); this value, however, is too low (see Section IV.C.3), and here we will assume a molecular area of 1.29 nm^2 based on the 0.433 nm spacing observed in a recent X-ray diffraction study (Emmerling *et al.*, 1977). It thus follows that phospholipids and LPS cover 0.9 and 0.7 μm^2, respectively, of the 2 μm^2 area of both sides of the membrane. The remaining portion of the surface of the outer membrane is presumably covered with proteins.

The protein content of the outer membrane is very high, and Haller and Henning (1974) showed that some bifunctional reagents could crosslink almost all of the outer membrane proteins into a single, cell-sized complex. Quantitative analysis (Smit *et al.*, 1975) gave a figure of 12 $fg/\mu m^2$. For comparison, the human red-cell membrane, which is generally considered to be a protein-rich membrane, contains only 4 fg of protein/μm^2, according to the published values of protein-to-lipid weight ratio (Rosenberg and Guidotti, 1968), lipid content per cell (Ways and Hanahan, 1964) and cellular surface area (Westerman *et al.*, 1961). If the specific volume of the outer membrane proteins is assumed to be 0.74 cm^3/g, then 12 fg of protein will occupy a surface area of 0.9×2 μm^2, on the assumption that the thickness of the protein layer is 10 nm. This seems rather excessive, and it is likely that the true surface area is larger than the estimate of Smit *et al.* (1975), presumably because of small-scale curvature or undulations. As a very rough approximation, we might calculate the total membrane area by adding up the areas calculated above for various components; this yields 3.4 μm^2 for a material that should occupy 2.0 μm^2, suggesting that the surface area was underestimated by about 40%. When combined with the information on the asymmetric distribution of LPS and phospholipids (Section III.D), we come to a very provisional estimate that, of the outer surface, 41% and 59% are covered by LPS and proteins, respectively, and that of the inner surface, 53% and 47% are covered by phospholipids and proteins, respectively.

An often neglected component of the outer membrane of members of

the Enterobacteriaceae is the "enterobacterial common antigen". This substance, which comprises as much as 0.2% of the dry weight of the cell, is present in all members of this family and is a polymer containing *N*-acetyl-D-glucosamine, *N*-acetyl-D-mannosaminuronic acid and palmitic acid residues (Mäkelä and Mayer, 1976). It is present on the outside surface of the outer membrane, as it reacts with antibodies added to whole cells. It is probably anchored to the membrane through hydrophobic interactions mediated by palmitic acid residues. The role of this polymer in the structure and function of the outer membrane remains to be studied.

III. Structure

A. PHOSPHOLIPIDS

In members of the Enterobacteriaceae, the phospholipid composition of the outer membrane is very similar to that of the inner membrane, and thus it contains mostly phosphatidylethanolamine, some phosphatidyglycerol, and very small amounts of cardiolipin (Osborn *et al.*, 1972a). This is not surprising in view of the recent finding of the Osborn group (see Section V.C) that there is a rapid exchange of phospholipids between the outer and inner membrane.

B. LIPOPOLYSACCHARIDE (LPS)

The structure of LPS has been the subject of a number of recent reviews (Lüderitz *et al.*, 1971; Nikaido, 1973, 1975; Jann and Westphal, 1975; Lindberg *et al.*, 1975; Galanos *et al.*, 1977; Ørskov *et al.*, 1977), to which the reader is referred for details. The lipopolysaccharide is an amphipathic molecule, with the hydrophilic, polysaccharide portion and a hydrophobic portion, called lipid A (Fig. 1). The hydrophilic portion can usually be considered to consist of two parts. The peripheral portion often consists of oligosaccharide repeating units, and shows extreme variability even within a single species (or "genus" such as *Salmonella* and *Escherichia*). For this reason, the structure of this portion has been used, as "O-antigens", in the fine serological typing of the strains, especially in the Enterobacteriaceae. In contrast, the proximal portion of the polysaccharide, called "R-core", shows a lesser degree of evolutionary divergence, and almost always contains an eight-carbon sugar acid,

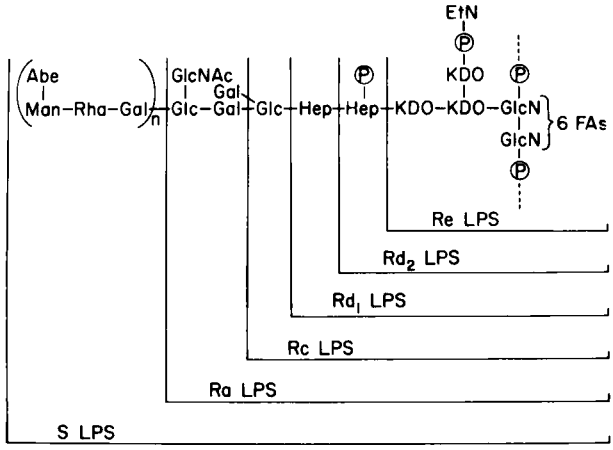

FIG. 1. Structure of LPS from *Salmonella typhimurium*. Biosynthesis proceeds generally toward the left in this figure. Thus wild-type cells complete the synthesis of the entire structure ("S LPS"), whereas R mutants blocked at various steps of biosynthesis produce incomplete structures ("Ra through Re LPS"). Abbreviations: Abe, abequose; Man, D-mannose; Rha, L-rhamnose; Gal, D-galactose; GlcNAc, *N*-acetyl-D-glucosamine; Glc, D-glucose; Hep, L-*glycero*-D-*manno*heptose; P, phosphate; KDO, 3-deoxy-D-*manno*octulosonic acid (2-keto-3-deoxy-octonic acid); EtN, ethanolamine; and FA, fatty acid.

3-deoxyoctulosonic acid (or 2-oxo-3-deoxyoctonic acid) with, in most cases, a seven-carbon sugar, L-glycero-D-mannoheptose (see the compilation in Nikaido, 1975). This portion also contains phosphate, ethanolamine phosphate and ethanolamine pyrophosphate (Fig. 1), and it is important to realize that this portion contains a very high density of charged groups, which include the carboxyl groups of 3-deoxyoctulosonic acid and the charged groups of "lipid A" (see below) in addition to those mentioned above. The binding of divalent cations by LPS was recently examined by the use of LPS derivatized with a fluorescent probe (Schindler *et al.*, 1978).

The structure of lipid A has been studied in detail in the Enterobacteriaceae. It is an unusual glycolipid, with a D-glucosaminyl-$\beta(1\rightarrow6)$-D-glucosamine backbone to which two and three fatty acid residues are attached through amide and ester linkages, respectively (Fig. 2). The amide-linked fatty acids are 3-hydroxytetradecanoic acids, and one of the ester-linked residues has a branched structure, i.e. 3-tetradecanoyl-3-hydroxytetradecanoic acid. In other bacteria, the 3-hydroxytetradecanoic acid is sometimes replaced by other 3-hydroxy acids, and *Brucella* spp. reportedly do not contain any hydroxy fatty

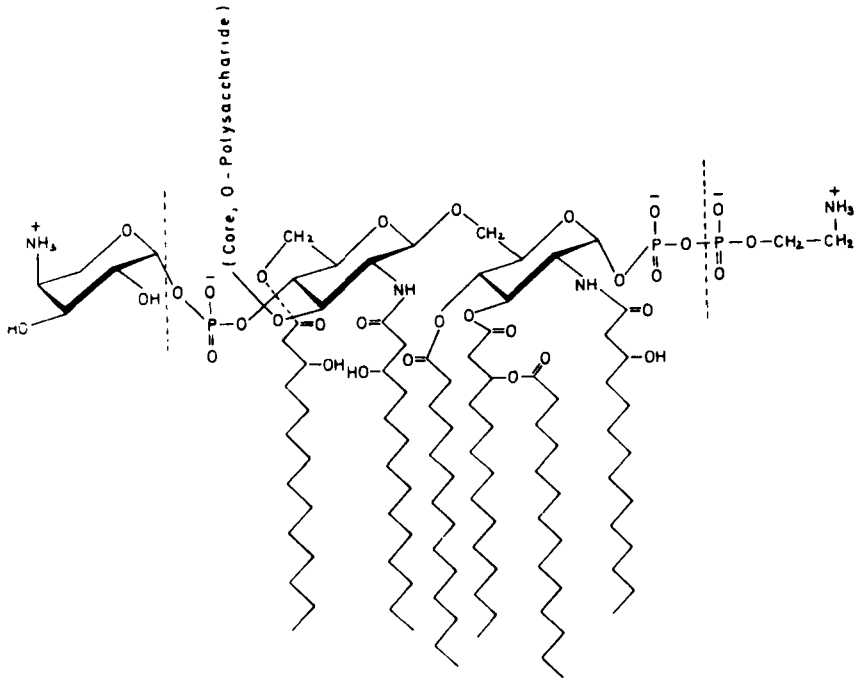

FIG. 2. Structure of lipid A from *Salmonella minnesota*. In this organism, three fatty acyl residues, i.e. dodecanoyl, hexadecanoyl (palmitic), and 3-O-tetradecanoyl-tetradecanoyl residues, are linked to the hydroxyl groups of the glucosamine disaccharide, but their exact locations are unknown and the arrangement shown here is arbitrary. Some units lack the arabinosamine residue, the phosphorylethanolamine residue, or both (dotted lines). From Mühlradt *et al.* (1977).

acids in lipid A. The glucosamine disaccharide apparently constitutes the backbone in most other bacteria, except in *Rhodopseudomonas palustris* where it is replaced by 2, 3-diaminohexose (or its oligomer) (Weckesser *et al.*, 1973). It should here be emphasized that all fatty-acid chains in lipid A are saturated. Together with the fact that as many as six chains are linked to a common backbone, this absence of unsaturated fatty acids probably contributes to the low fluidity exhibited by the hydrophobic portions of LPS (Nikaido *et al.*, 1977c).

A recent development in the definition of the structure of LPS concerns the possibility of "cross-linking" between monomeric units. When LPS is treated under mildly acidic conditions, the polysaccharide portion is split off from lipid A, but ion-exchange chromatography of such polysaccharide preparations produces an extremely complex pattern

(Osborn, 1963). Since ultracentrifugation of acetylated LPS in acetone gave a molecular weight three times higher than expected from the structure of the monomeric unit (Romeo *et al.*, 1970), it was first assumed that, on average, three monomeric units were crosslinked at the level of "R core" polysaccharide, and evidence was presented which apparently supported this hypothesis (Cherniak and Osborn, 1966). However, gel filtration of the acid-released polysaccharide did not support the presumed structure (Mühlradt, 1969), and it was then proposed that crosslinking occurred at the level of lipid A, presumably via pyrophosphate bridges (Lüderitz *et al.*, 1973). But, more recent studies using ^{31}P nuclear magnetic resonance failed to substantiate the presence of any such pyrophosphate bridges (Mühlradt *et al.*, 1977). Still more studies are obviously necessary, but in this review it will be assumed that the "oligomers" observed earlier were actually aggregates possibly held together by divalent cation bridges. The data of Smit *et al.* (1975), for example, are thus presented in a recalculated form, assuming that no crosslinking exists between LPS "monomers".

It has been claimed by one laboratory (Wu and Heath, 1973) that LPS is covalently bound to a protein. The basis of this conclusion is the coelectrophoresis of LPS and the "protein", and the possibility of fortuitous association has not been ruled out. Furthermore, LPS prepared by organic-solvent extraction at 0°C (Galanos *et al.*, 1969) does not contain any protein, and so far no other laboratory has been able to confirm the conclusion of Wu and Heath (1973). We feel that at present there is no compelling evidence for this covalent association.

C. PROTEINS

Very rapid progress has been made in the identification and quantitation of protein components of the outer membrane, mainly owing to the technique of sodium dodecyl sulphate (SDS)-poly-acrylamide gel electrophoresis (Schnaitman, 1970; Bragg and Hou, 1972; Uemura and Mizushima, 1975). The improved resolution of the slab gel technique, first exploited in studies of bacterial membranes by Giovanna Ames (1974), was especially useful in the study of outer membranes which contained not too many, yet often difficult-to-resolve, protein components (for example, see Ames *et al.*, 1974; Lugtenberg *et al.*, 1975).

Electropherograms of the outer membrane proteins are usually

dominated by only a few prominent bands (Schnaitman, 1970) (Fig. 3). We owe a great deal to the pioneering studies of Braun (reviewed in Braun, 1975) and Schnaitman (1973 a,b; 1974 a,b) for identification and elucidation of the properties of these major proteins. Some of these proteins are briefly discussed below.

1. *Murein Lipoprotein (Braun Lipoprotein)*

This small protein, containing 58 amino-acid residues in *E. coli*, is often covalently linked to the underlying peptidoglycan via the ε-amino group of its C-terminal lysine residue. Its *N*-terminal amino acid, cysteine, carries an amide-linked fatty acid as well as a diglyceride moiety connected through a thioether linkage. These and other features of this very unusual protein have been reviewed in detail by Braun (1975), by whom it was discovered and characterized. In an *E. coli* cell, this is probably the protein that exists in the largest number of copies (7×10^5), two-thirds of which exist in a form not covalently linked to the peptidoglycan (Inouye *et al.*, 1972). The isolated lipoprotein is very rich in α-helical regions (Braun, 1975). The structural gene for this protein, *lpp*, is located at 36.5 min on the *E. coli* chromosome (Inouye *et al.*, 1977a) (Fig. 4).

Similar lipoproteins are present in species of *Salmonella* and *Serratia* in bound as well as in "free" forms, but they have not been detected in *Proteus mirabilis* (Braun *et al.*, 1970; Halegoua *et al.*, 1974). However, more recent studies showed that *Proteus mirabilis* does contain a smaller quantity of "bound" lipoprotein, whose detection was made even more difficult because it aggregated in SDS and did not migrate at expected rates in SDS gel electrophoresis experiments (Gmeiner *et al.*, 1978).

2. *"Heat-Modifiable" Proteins*

Both *E. coli* and *S. typhimurium* contain a set of several prominent proteins with apparent molecular weights in the range of 32-37,000. These are often called "major proteins" (Fig. 3). One of these (3a in *E. coli* and "33K" in *S. typhimurium*) attracted attention because on SDS acrylamide gel electrophoresis it moved like an approximately 25,000 dalton protein when samples were solubilized in SDS at room temperature, but paradoxically migrated more slowly when the samples were heated in SDS prior to electrophoresis (Schnaitman, 1973a). The nomenclature

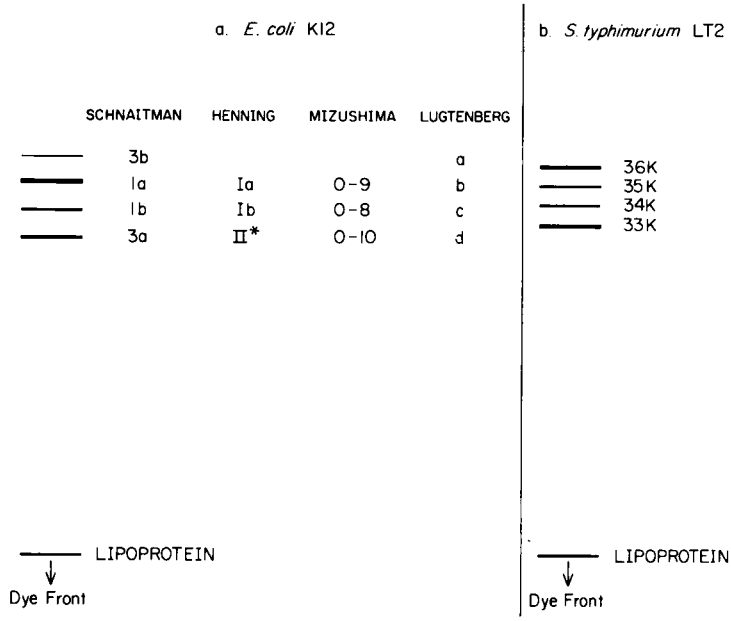

FIG. 3. SDS-Polyacrylamide gel electrophoresis pattern of major outer membrane proteins and their nomenclature. The *Escherichia coli* pattern shown is obtained with the gel system of Lugtenberg *et al.* (1975), the *Salmonella typhimurium* pattern by the method described by Ames (1974). The samples were treated in SDS at 100°C before electrophoresis. It should be emphasized that even slight modifications of the gel system sometimes change the order of migration of these bands. Protein 3b does not appear in Henning's gels, because it is not synthesized at 30°C, the temperature of cultivation used by Henning's group (Lugtenberg *et al.*, 1976).

for these proteins is summarized in Fig. 3; here we follow the nomenclature of Schnaitman. The protein has been purified (Schnaitman, 1973b; Reithmeier and Bragg, 1974; Hindennach and Henning, 1975). The amino-acid composition of the protein is not markedly hydrophobic (Garten *et al.*, 1975), and the protein is unusually rich in β-structure (Nakamura and Mizushima, 1976) when solubilized in SDS at a low temperature. Effects of heating on the conformation of this protein have been studied (Schnaitman, 1973a, Nakamura and Mizushima, 1976; Reithmeier and Bragg, 1977a). A significant portion (6-24%) of the lysine residues in this protein is converted to allysine (α-amino adipic acid semialdehyde) by a post-translational modification reaction (Diedrich and Schnaitman, 1978). The structural gene for this protein in *E. coli* is the *ompA* locus (earlier called *tolG* and *con*) (Henning *et*

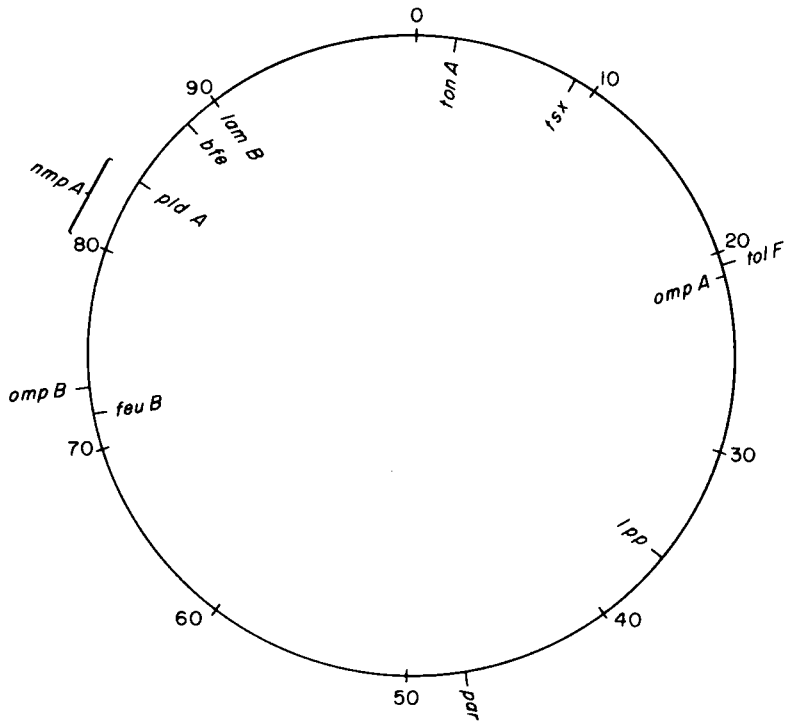

FIG. 4. Genetic loci involved in the synthesis of outer membrane proteins in *Escherichia coli*. The loci involved in the biosynthesis of porins are shown *outside* the circular map: for *tolF, par*, and *ompB* see Section III.C.3. The locus *nmpA* allows the expression of an "alternative porin" in the molecular weight range of 35,000 (J. Foulds, personal communication; see also Section IV. D.5). The loci involved in the biosynthesis of non-porin proteins are shown *inside* the circle: for *ompA* and *lpp*, see Sections III.C.1 and 2; for *tonA, tsx, feuB, bfe*, and *lamB*, see Section III.C.4 and Table 1; the gene *pldA* codes for phospholipase A (Section III.C.6).

al., 1976), located at 21.5 min on the genetic map (Foulds, 1974) (Fig. 4). The structural gene for the 33K protein is located in the corresponding region of the *S. typhimurium* chromosome, viz, at 20 units on the 100 units map (B. A. D. Stocker, M. Nurminen, P. H. Mäkelä and K. Lounatmaa, personal communication).

Another "heat-modifiable" protein, 3b, with an apparent molecular weight of 40,000, is synthesized in *E. coli* K12 only when the culture is grown at 37°C or at a higher temperature (Lugtenberg *et al.*, 1976). Little is known about the properties of this protein.

3. *"Peptidoglycan-Associated" Proteins, or Porins*

Among the "major" outer membrane proteins of 32–37,000 dalton range, the proteins other than the "heat-modifiable" ones show a set of distinctive, common properties. Firstly, these proteins have a tendency to associate with the underlying peptidoglycan. Thus, treatment of the cell envelope with SDS at temperatures below 70°C solubilizes all of the outer membrane components except lipoprotein molecules and "peptidoglycan-associated" ~35,000 dalton proteins that are associated with peptidoglycan through covalent and non-covalent linkages, respectively (Rosenbusch, 1974). The "peptidoglycan-associated" proteins can be released from this complex in 0.5*M* NaCl containing SDS; when the released proteins are added to lipoprotein-free peptidoglycan sheets, they rapidly become reassociated (Hasegawa *et al.*, 1976). Secondly, unless heat-denatured, these proteins do not tightly bind much SDS (Rosenbusch, 1974) and mostly remain oligomeric (Nakae, 1976 a, b; Palva and Randall, 1978). Thirdly, these proteins have the unusual property of producing transmembrane diffusion pores when added to a phospholipid-LPS mixture (Nakae, 1976a, b), and therefore are called "porins". In this article, the name porin will be used for these peptidoglycan-associated, *ca.* 35,000 dalton, pore-producing proteins. It should be understood that some other outer-membrane proteins (e.g. λ-receptor) also tend to remain associated with peptidoglycan, and other proteins may also produce diffusion pores of less than absolute specificity (Section IV. D.5).

The presence of more than one porin species was first noticed in *S. typhimurium* by the use of slab gel electrophoresis (Ames, 1974; Ames *et al.*, 1974; Nakae, 1976a), and the three species of porin were called "36K" "35K" and "34K", on the basis of their *apparent* molecular weights (Fig. 3). These proteins are clearly differentiated from the "33K", heat-modifiable" protein, as the former associate with the peptidoglycan and heating in SDS increases their mobility in the gel (as a consequence of dissociation into monomers), in contrast to its decreasing the mobility of the "heat-modifiable" protein. *Escherichia coli* B produces a single species of porin (Rosenbusch, 1974) that is similar to the protein 1a of *E. coli* K12 (Schmitges and Henning, 1976). Strain K12 of *E. coli* produces two species of porins, 1a and 1b (Uemura and Mizushima, 1975; Lugtenberg *et al.*, 1975: Schmitges and Henning, 1976; Bassford *et al.*, 1977) (Fig. 3). In addition, *E. coli* O111B4, as well as *E. coli* K12 strains lysogenic for

phage PA-2, produce another porin, protein 2 (Schnaitman, 1974 a, b; Schnaitman *et al.*, 1975; Diedrich *et al.*, 1977). The total number of porin molecules found in a cell is quite large, approximately 10^5 (Rosenbusch, 1974). Porins are usually isolated by using SDS (Rosenbusch, 1974; Hindennach and Henning, 1975; Hasegawa *et al.*, 1976), but it is possible to isolate them without the use of ionic detergents (Nurminen, 1978).

In *E. coli* K12, there are three genetic loci involved in porin synthesis (Fig. 4). Mutations at the *tolF* locus, located at 21 min on the genetic map, results in the absence of protein 1a (Foulds, 1976) Bassford *et al.*, 1977). Mutations at the *par* (or *meoA*) locus, located at 48 min, result in the loss of protein 1b (Bassford *et al.*, 1977; Verhoef *et al.*, 1977) Mutations at the *ompB* locus, located at 73.7 min, result in the absence of both proteins 1a and 1b (Sarma and Reeves, 1977; Bassford *et al.*, 1977; Bavoil *et al.*, 1977). In *S. typhimurium*, the mutations causing the loss of the 34K porin mapped near *trp*, and most of the mutations causing the loss of the 36K porin mapped near *nalA*, presumably at a locus corresponding to *par* in *E. coli* (M. Nurminen, and P. H. Mäkelä, personal communication).

Interpretation of these genetic results depends on the mechanism of biosynthesis of these proteins. Since various species of porins were quite similar in size, isoelectric point, and amino-acid composition (Schmitges and Henning, 1976), many workers tended to believe that the different species were generated by post-translational modification, in different manners, of a common precursor polypeptide (Bassford *et al.*, 1977; Henning *et al.*, 1977). According to this hypothesis, *ompB* has to be the structural gene for the precursor, although available evidence did not support such a conclusion (Bavoil *et al.*, 1977). More recently, further pieces of evidence against the common precursor hypothesis have appeared. Thus, Ichihara and Mizushima (1978) have shown that porins 1a and 1b are significantly different in their amino-acid compositions, and one of the authors of this paper has found a similar situation with the 34K and 36K porins of *S. typhimurium* (H. Tokunaga, M. Tokunaga, and T. Nakae, unpublished observations). The presence of many more alanine residues in the smaller 34K porin, for example, is difficult, though not impossible, to explain by the common precursor hypothesis. Even more decisive is the finding, by Ichihara and Mizushima (1978), of the difference in the NH_2-terminal amino-acid sequences of porins 1a and 1b, isolated from the same strain: thus the third and the eleventh residues are Ileu and Phe in porin 1a, but Val and Leu in porin 1b. These results clearly establish that multiple species of porins are coded for by separate

structural genes (which probably originated by duplications of an ancestral gene). Accordingly, the best hypothesis at present is to consider *tolF* and *par* as structural genes and *ompB* as a regulatory gene in *E. coli*. Most of the data are consistent with this hypothesis. Thus transduction of the *ompB*$^+$ allele from *E. coli* B/r to *E. coli* K12 decreases production of *both* 1a and 1b (Pugsley and Schnaitman, 1978), and altered porins with a different electrophoretic mobility or iso-electric point have not been found among revertants of *ompB* mutants (Bavoil *et al.*, 1977). Although some *par* mutants did not allow synthesis of protein 2 when lysogenized with phage PA-2 (Bassford *et al.*, 1977), this phenotype occurred only in a fraction of *par* mutants and the result can be explained by assuming that a part of the *par* "locus" contains gene(s) responsible for modification of both porin 1b and protein 2. Recently T. Sato, K. Ito and T. Yura (personal communication) found that replacement of the *tolF*-containing region of the *E. coli* chromosome with the corresponding region of the *S. typhimurium* chromosome resulted in the replacement of porin 1a with protein(s) similar to *S. typhimurium* porin(s). There was no alteration in porin 1b, and introduction of an *ompB* allele into the hybrid strain caused the disappearance of porin bands, including those of Salmonella-like porins; these results strongly support the hypothesis presented above. For a further comparison of porins of *E. coli* and *Salmonella* spp., see Section IV. D.3 and Table 4).

The porins so far purified are not noticeably hydrophobic in their amino-acid compositions (Rosenbusch, 1974; Garten and Henning, 1974; H. Tokunaga, M. Tokunaga, and T. Nakae, to be published). When isolated in SDS at low temperature, they existed as oligomers but the sizes of these oligomers remained ambiguous. Recent sedimentation equilibrium studies showed conclusively that the *E. coli* B porin as well as *S. typhimurium* 34K and 36K porins existed as stable trimers in 0.25% SDS (T. Nakae, J. Ishii, and M. Tokunaga, to be published). This conclusion is supported by the study of Palva and Randall (1978), who showed, by crosslinking, that in 0.1% SDS the porins were predominantly trimers. The circular dichroism spectrum of these proteins is very similar to that of pure β-structural sheets of protein (Rosenbusch, 1974; Nakamura and Mizushima, 1976), and the presence of β-structure is also indicated by the infrared spectra (Nakamura *et al.*, 1974; Rosenbusch, 1974). The β-structure disappears upon heating in SDS, and at the same time the oligomer dissociates into monomers (Nakamura and Mizushima, 1976). Recently Diedrich and Schnaitman (1978) found that in *E. coli* K12 some

of the lysine residues in this protein were replaced by allysine, i.e. α-aminoadipate semialdehyde.

Since the diffusion channels are always necessary, porins may be expected to comprise a major part of the protein in the outer membrane of any Gram-negative bacterium. Indeed, major proteins of 25–40,000 daltons have been described in the outer membranes of other genera of the Enterobacteriaceae (Lugtenberg *et al.*, 1977), *Spirillum serpens* (Schweizer *et al.*, 1975)., *Neisseria meningitidis* (Frasch and Gotschlich, 1974), *Neisseria gonorrhoeae* (Johnston and Gotschlich, 1974; Wolf-Watz *et al.*, 1975; Walstad *et al.*, 1977), *Pseudomonas aeruginosa* (Stinnett *et al.*, 1973), *Campylobacter fetus* (McCoy *et al.*, 1976), *Caulobacter crescentus* (Agabian and Unger, 1978), *Bordetella pertussis* (Parton and Wardlaw, 1975) and *Acinetobacter* sp. (Thorne *et al.*, 1976).

4. *Proteins Presumably Facilitating Specific Diffusion Processes*

Growing *E. coli* in the presence of maltose induces production of *lamB* or λ-receptor protein. Under these conditions, this protein becomes a major protein, comparable in its abundance to the porins (Braun and Krieger-Brauer, 1977). This protein, with an apparent molecular weight of 47,000 (Braun and Krieger-Brauer, 1977), also resembles porins in its affinity towards peptidoglycan and in its tendency to form stable oligomers in SDS (Palva and Randall, 1978). The role of λ-receptor in maltose transport will be discussed in Section IV.E.

Escherichia coli, when deprived of available sources of iron, will drastically increase production of several outer-membrane proteins in the 70–85,000 dalton range (see Table 1) (Uemura and Mizushima, 1975; Hancock and Braun, 1976a; Pugsley and Reeves, 1976; McIntosh and Earhart, 1976). Among these, *feuB* and *tonA* proteins are known, primarily from mutant studies, to be involved in uptake of Fe^{3+}-enterochelin (Hancock *et al.*, 1976; Wayne *et al.*, 1976; Pugsley and Reeves, 1977; Ichihara and Mizushima, 1977; Wookey and Rosenberg, 1978) and (Fe^{3+})-ferrichrome (Wayne and Neilands, 1975; Hantke and Braun, 1975a; Luckey *et al.*, 1975), respectively, and the *tonA* protein has been purified (Braun and Wolff, 1973). The *cit* protein is assumed to participate in transport of ferric citrate, as production of this protein is induced by citrate (Hancock *et al.*, 1976). The remaining proteins are also presumed to be involved in Fe^{3+} transport, but their precise role is unknown (Braun *et al.*, 1976b; Hancock and Braun, 1976a). One of them,

TABLE 1. Outer Membrane proteins involved in specific diffusion processes

Facilitates the diffusion of:	Molecular weight of solutes transported:	Gene coding for the protein	Apparent molecular weight of protein	Protein used as receptor by:
Maltose and Maltodextrins	342 and higher	*lamB*	47,000	λ
Ferrichrome	740	*tonA*	78,000	T5, T1, ϕ80, colicin M
Fe^{3+}-Enterochelin	746	*feuB*	81,000	colicin B
Fe^{3+}-Citrate	245?	*cit*	80,500	
Fe^{3+}-Complex?			83,000	
Fe^{3+}-Complex?		*cir*	74,000	colicins I and V
Nucleosides	230–290	*tsx*	25,000	T6, colicin K
Vitamin B_{12}	1357	*bfe*	60,000	BF23, E group colicins

"colicin I receptor" or *cir* protein, has been purified (Konisky and Liu, 1974). Another "receptor" protein, T6-receptor or *tsx* protein, was found to facilitate the diffusion of nucleosides across the outer membrane (Hantke, 1976). This protein has an apparent molecular weight of 25,000. The "receptor" for colicin E and phage BF23 was the first outer membrane protein found to participate in uptake of nutrients, in this case vitamin B_{12} (Di Masi *et al.*, 1973). This 60,000 dalton protein ("*bfe* protein") has been purified (Sabet and Schnaitman, 1973).

Addition of *sn*-glycerol 3-phosphate to *E. coli* induces production of a 40,000 dalton protein designated GLPT. Although this protein is released by "osmotic shock", like other periplasmic proteins, it exists as stable oligomers in SDS solutions at room temperature, much like porins or λ-receptor. Possibly this protein also is involved in the formation of rather specific channels through the outer membrane (Argast *et al.*, 1977).

5. *Other "Minor" Proteins*

"Protein G" of 15,000 daltons was reported to become incorporated into the outer membrane, in association with elongation of the cell wall (James, 1975). A protein of approximately 80,000 daltons becomes incorporated into the outer membrane at a specific point in cell cycle (Gudas *et al.*, 1976; Churchward and Holland, 1976). Although this protein was initially implicated in the initiation of DNA synthesis, this conclusion was based on procedural artifact, and the protein is actually identical with the Fe^{3+}-enterochelin transport protein or *feuB* protein described in the preceding section (Boyd and Holland, 1977).

6. *Enzymes*

In members of the Enterobacteriaceae, phospholipase A had been the only outer-membrane protein with known enzymic activity. This 28,000 dalton protein requires Ca^{2+} for activity, and has been purified extensively (Scandella and Kornberg, 1971; Nishijima *et al.*, 1977). Very recently, a protease activity was detected in the outer membrane of *E. coli* K12, by using as an assay the proteolytic release of nitrate reductase complex from the inner membrane of the same organism (C. MacGregor, personal communication). In *Neisseria meningitidis*, tetramethylphenylenediamine oxidase activity was found in the outer membrane (DeVoe

and Gilchrist, 1976), an interesting finding in view of the widespread use of "oxidase reaction" in bacterial taxonomy.

D. SUPRAMOLECULAR ORGANIZATION OF THE COMPONENTS

The lipid bilayer structure, in which the hydrocarbon chains are arranged approximately at right angles to the plane of the membrane to form its hydrophobic interior, seems to correspond to the basic continuum of the outer membrane as in other biological membranes. The data supporting this conclusion include the following:

(i) Electron microscopy of stained thin sections of the outer membrane reveals a typical trilaminar image characteristic of "unit membranes". The thickness of the trilaminar structure is about 8.0nm, similar to, or slightly thicker than, most biological or "unit" membranes (Glauert and Thornley, 1969).

(ii) At least some of the lipid hydrocarbons of the outer membrane are arranged in one orientation, and the hexagonal quasicrystalline packing of these chains disappears at high temperature through the process of cooperative "melting", as judged by X-ray diffraction studies (Forge *et al.*, 1973; Overath *et al.*, 1975).

(iii) With lipid bilayer membranes, freeze-fracturing produces cleavage in the centre of the membrane (Branton, 1966). Similar cleavage of the outer membrane has repeatedly been observed (DeVoe *et al.*, 1971; van Gool and Nanninga, 1971; Smit *et al.*, 1975).

The lipid bilayer in the outer membrane, however, appears to be quite asymmetric in the Enterobacteriaceae. From labelling studies with LPS-specific, ferritin-labelled antibody (Mühlradt and Golecki, 1975), as well as degradation experiments with galactose oxidase (Y. Funahara and H. Nikaido, unpublished results), it is now clear that LPS is located only in the outer half of the membrane. Furthermore, covalent labelling with cyanogen bromide-activated dextran, which cannot diffuse across the outer membrane, showed that, at least in wild-type *S. typhimurium*, most phospholipid molecules are located in the inner half of the outer membrane (Kamio and Nikaido, 1976). The presence of this rather extreme form of asymmetry, LPS outside and phopholipids inside, is also supported by other lines of evidence.

(i) The total amount of phospholipids present in the outer membrane is not even sufficient to cover the one side of the membrane (Smit *et al.*, 1975; see Section II.B).

(ii) Freeze-fracture of the outer membranes of *E. coli* (van Gool and Nanninga, 1971) and of *S. typhimurium* (Smit *et al.*, 1975) reveals a concave fracture face densely filled with particles, a major constituent of which was shown to be proteins (Smit *et al.*, 1975). In *Pseudomonas aeruginosa*, similar particles seen on the concave fracture face appear to be protein-LPS complexes, since EDTA treatment which releases such complexes from the whole cell (Rogers *et al.*, 1969; Roberts *et al.*, 1970) results in the disappearance of a major fraction of these particles (Gilleland *et al.*, 1973). Thus it seems likely that the outer half of the outer membrane (corresponding to the concave fracture face) of *E. coli* and *S. typhimurium* is filled with protein-LPS complexes (or "particles"). It should be emphasized that smooth faces corresponding to the phospholipid monolayer were found abundantly on the convex face, but not on the concave face.

(iii) Electron-spin resonance (e.s.r.) studies with spin-labelled probes show that the hydrocarbon chains of LPS have rather low fluidity. Thus, artificial mixed bilayers, with LPS molecules interdigitated among phospholipid molecules, exhibit significantly lower fluidity than the bilayers made of phospholipids alone (Nikaido *et al.*, 1977c). Yet phospholipid hydrocarbon chains in the outer membrane show a degree of fluidity very similar to that in the cytoplasmic membrane (Overath *et al.*, 1975; Nikaido *et al.*, 1977c), a finding which indicates that phospholipids in the outer membrane occupy a domain completely separated from LPS. This conclusion was also supported by the titration of the size of domains by exchange broadening of e.s.r. line widths (Nikaido *et al.*, 1977c). Another electron-spin resonance study (Rottem and Leive, 1977) concluded that the amount as well as the saccharide chain length of LPS affects the fluidity of hydrocarbons in the outer membrane. However, in this study the membranes were prepared by lysozyme digestion, and it is likely that extensive reorganization of membrane components took place (see below). Indeed comparison of the spectra which they obtained (Rottem and Leive, 1977) with their own earlier results (Rottem *et al.*, 1975) and our own (Nikaido *et al.*, 1977*c*) indicates very strongly that in their new preparation the phospholipid hydrocarbons had a much more restricted mobility, presumably due to the formation of artefactual, LPS-phospholipid mixed bilayer regions.

Recently, a study has been undertaken of LPS thermal transition using X-ray diffraction and fluorescent probe techniques (Emmerling *et al.*, 1977). This interesting work describes a cooperative "melting" of

LPS hydrocarbons at around 22°C. This result appears contradictory to our spin-label experiments in which no cooperative transition was found (Nikaido *et al.*, 1977c). However, Emmerling *et al.* (1977) found that even 0.1 m*M* Mg^{2+} almost abolished transition and most probably it is significant that our experiments were performed in 10 m*M* Mg^{2+}. Interestingly these workers showed that some hydrocarbon chains in the intact *E. coli* cell "melted" at 25°C, not at the much lower temperature expected for LPS-phospholipid mixed bilayers, and this result is consistent with our model predicting complete segregation of LPS domains and phospholipid domains.

The structure of the outer membrane (in wild type *E. coli* and *S. typhimurium* at least) can thus be visualized as in Fig. 5A. It is seen that the outer half of this membrane is composed of polyanionic molecules

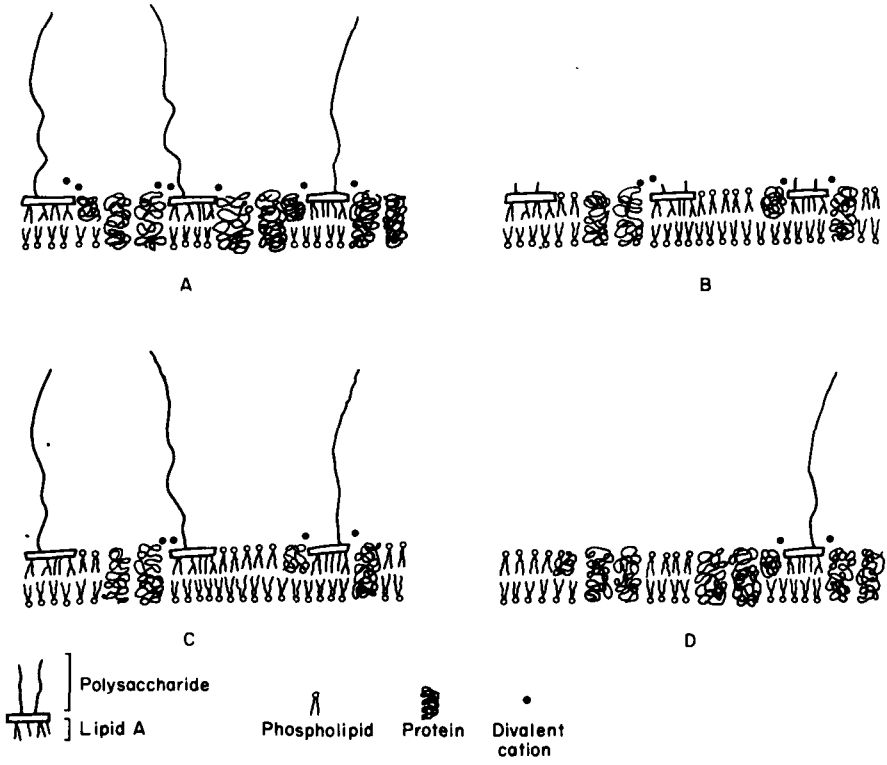

FIG. 5. Schematic structure of the outer membrane of *Escherichia coli* and *Salmonella typhimurium*. A. Wild-type strains. B. "Deep rough" mutants producing LPS of Rd- or Re-type. C. Mutants incorporating reduced amounts of proteins into the outer membrane. D. Wild type strains after EDTA treatment. For details see text.

(LPS) and proteins, the major members of which, porins, are again strongly acidic (Schmitges and Henning, 1976). The strong electrostatic repulsion between these components is probably the major destabilizing force in the outer membrane, which would explain the important role that divalent cations play in the stabilization of the outer membrane structure, most probably through neutralization of, and bridging between, the anionic groups (Gray and Wilkinson, 1965b; Nikaido, 1973). Thus, removal of these cations by EDTA treatment will result in the release of predominantly LPS in *E. coli* (Leive, 1965c) and LPS-protein complexes in *Ps. aeruginosa* (Rogers *et al.*, 1969; Roberts *et al.*, 1970), presumably because the electrostatic repulsion becomes stronger than the cohesive forces of hydrophobic interaction. Furthermore, even in the absence of EDTA, the stronger electrostatic repulsion at the outer surface might produce a tendency for the formation of "blebs", a phenomenon repeatedly observed under various growth conditions (see De Petris, 1967; Smit *et al.*, 1975). It is possible that one of the major functions of the Braun lipoprotein is to pull down the outer membrane layer onto the underlying peptidoglycan layer. Indeed an *E. coli* mutant lacking the Braun lipoprotein, owing to a deletion through the *lpp* gene (Hirota *et al.*, 1977), produces very large "blebs" or "bulges" of the outer membrane, which phenomenon is prevented by adding high concentrations of Mg^{2+} to the medium (Suzuki *et al.*, 1978; Fung *et al.*, 1978). It is also known that in *E. coli* the digestion of peptidoglycan with lysozyme produces extensive relocation of components within the outer membrane, apparently decreasing the degree of asymmetry in its organization (Shands, 1966; Mühlradt and Golecki, 1975). It thus seems clear that *E. coli* and *S. typhimurium* maintain the highly unstable, asymmetric structures of their outer membranes by the help of the lipoprotein-peptidoglycan system. The outer membrane of *Ps. aeruginosa* contains even higher number of anionic groups than does that of the enteric bacteria (Gray and Wilkinson, 1965b), and thus usually requires high concentrations of Mg^{2+} for stabilization. Growth in low concentrations of Mg^{2+} "induces" production of a new outer membrane protein (Gilleland *et al.*, 1974); it would be most interesting to see whether this protein contains many cationic groups.

The extent of asymmetry becomes reduced and a significant number of phospholipid molecules appear in the outer leaflet in certain mutants of *E. coli* and *S. typhimurium*. A well-characterized case involved "deep rough" mutants producing very defective LPS of Rd_1, Rd_2, or Re type

(see Fig. 1). In these mutants much of the protein components fail to get incorporated into the outer membrane (Ames *et al.*, 1974; Koplow and Goldfine, 1974), presumably because the defective LPS cannot interact properly with the proteins. This decrease in protein content is compensated for by increases in phospholipid content, which now is more than that which can be accommodated on one side of the membrane (Smit *et al.*, 1975) (see Fig. 6b). The presence of phospholipids in the outer leaflet was confirmed by covalent labelling of their head groups with a non-penetrating reagent (Kamieo and Nikaido, 1976) as well as by freeze-fracture studies revealing smooth, "phospholipid monolayer" areas between particles in the concave fracture face (Smit *et al.*, 1975). The structure of the outer membrane in deep rough mutants is shown schematically in Fig. 5B.

The reduction of outer membrane protein levels caused by uncharacterized mutations similarly produces a membrane with significant amounts of phospholipids in the outer leaflet (Kamio and Nikaido, 1976). Freeze-fracture electron microscopy suggests the production of a similar type of membrane in *S. typhimurium* and *E. coli* mutants lacking major proteins (Nurminen *et al.*, 1976; L. van Alphen *et al.*, 1978). Outer membranes of this type, containing a phospholipid bilayer, are probably also produced by wild-type strains of some non-enteric bacteria. This is suggested, for example, by the observation that the concave fracture face of *Ps. aeruginosa* outer membrane shows large areas which are smooth and particle-free (Gilleland *et al.*, 1973). The structure of this type of outer membrane is shown in Fig. 5C.

Finally, removal of LPS (Leive, 1965c) or of LPS-protein complexes (Rogers *et al.*, 1969; Roberts *et al.*, 1970) by treatment with EDTA produces an empty space which, presumably, is immediately filled with phospholipids. This is suggested by freeze-fracture electron microscopy (Gilleland *et al.*, 1973; Bayer and Leive, 1977), and also by the permeability properties of such membranes (see Section IV.C). The presumed structure of EDTA-treated outer membrane is shown in Fig. 5D.

At this point we should like to discuss the question of "coverage" of cell surface by the polysaccharide side chains of LPS. There is no doubt that in *wild-type E. coli* or *S. typhimurium* the cell surface is effectively covered by such carbohydrate chains, as in these organisms more proximal structures on the outer membrane are often inaccessible to antibodies (Smit and Nikaido, 1978) and phages (Lindberg, 1973). However, this concept is

sometimes extrapolated to rough mutants that produce LPS carrying only tri- to decasaccharide chains (for example, see L. van Alphen *et al.*, 1978; Magnusson and Johannisson, 1977). [Readers are here reminded that *E. coli* K12 and B strains are rough mutants, and are *not* wild-type in terms of LPS biosynthesis (Prehm *et al.*, 1975, 1976.)] The cross-section of a sugar residue in an extended carbohydrate chain may be calculated from the space-filling model to be about 0.4 nm × 0.6 nm = 0.24 nm^2. Since there are 3.4×10^5 molecules of LPS per μm^2 of cell surface when correction is made for the underestimation of cell surface area (Section II.B.), a maximum of $3.4 \times 10^5 \times 0.24 \times 10^{-6} = 0.08\ \mu m^2$, or only 8% of the surface will be covered by the saccharide chains of LPS. Although it is not impossible to visualize a near-complete coverage through the complex bending of saccharide chains, this calculation shows that caution is needed when the idea of "coverage by carbohydrate" is to be extended to rough stains.

The distribution of the proteins in a direction perpendicular to the plane of the membrane has become reasonably clear. Incubation of whole cells of *S. typhimurium* with cyanogen bromide-activated dextran covalently links the dextran to ε-amino groups in lysine residues of the proteins exposed on the cell surface. This approach could demonstrate that the majority of outer-membrane proteins, including all species of porins and several species of iron-chelator transport proteins, are indeed exposed, and the experiment failed to label only four or five proteins out of a total of about 20 proteins seen in one-dimensional SDS acrylamide gel electrophoresis (Kamio and Nikaido, 1977). This finding is supported by the fact that most of the known outer-membrane proteins serve as receptors for phages or colicins (see Table 1). The sole discrepancy between the covalent labelling study and "receptor" activity concerns the "heat-modifiable", protein 3a, the Salmonella equivalent of which did not appear to be labelled (Kamio and Nikaido, 1977), yet which is known to serve as a receptor for bacteriocin 4–59 (B. A. D. Stocker, personal communication). It seems likely that its binding to dextran went unnoticed, as on gel electrophoresis this protein migrated with one prominent inner membrane protein. Indeed, protein 3a of *E. coli* B appears to have reacted with the dextran (Kamio and Nikaido, 1977). It is not clear whether a part of the Braun lipoprotein is exposed on the outside surface. Braun *et al.* (1976a) found that the lipoprotein was immunogenic in rough mutants with short LPS side chains, and concluded that it extruded through the membrane bilayer but was

normally covered by the polysaccharide chains. Closer examination of their data shows, however, that the most prominent rise in immunogenicity occurred in "deep rough" mutants with very unstable outer membranes (See Section IV.F), and suggests that the data can be interpreted equally well, possibly even better, by assuming that the lipoprotein is not normally exposed and became accessible only through the structural imperfections of the outer membrane in deep rough mutants.

Less information is available on the exposure of proteins on the inside surface of the outer membrane, although the porins appear to be tightly associated with the underlying peptidoglycan (Section III.C.3), and a part of protein 3a also appears to be exposed on the inside surface, as it was recently crosslinked to the peptidoglycan layer with dithio-*bis*-(succinimidyl propionate) (Reithmeier and Bragg, 1977b; Endermann *et al.*, 1978).

How are the proteins distributed, and possibly organized, in the plane of the membrane? We shall defer the discussion of this question until we finish the description of the functions of the outer membrane (See Section IV.H).

IV. Functions of the Outer Membrane

A. "CELL SURFACE" FUNCTIONS

The outer surface of the outer membrane corresponds to the outer surface of the cell in many bacteria, although in others the cells are further surrounded by the loosely bound capsular layer. The outer membrane, or at least the outer half of this membrane, therefore has to confer on the cells the properties characteristic of the cell surface of unicellular micro-organisms. The first of such properties is the negatively charged surface. Thus, LPS contains many anionic groups (see Fig. 1), and it may be significant that the most abundant proteins in the outer membrane, porins, are quite acidic proteins (Section III.D). Secondly, the surface of unicellular micro-organisms is usually covered with a carbohydrate layer. Obviously, the O side chains of LPS form this layer in organisms lacking capsules. One important function of the cell-surface carbohydrate is to increase the hydrophilicity of the cell surface. Actually, the *S. typhimurium* cell surface is already quite hydrophilic without the side chains, as shown by the water droplet contact angle of

about 21° (Cunningham *et al.*, 1975), which could be contrasted with the contact angle of a totally hydrophobic surface, for example paraffin that gives 106° (Adamson, 1967). However, a further increase in hydrophilicity introduced by the addition of O chains and indicated by a decrease of the contact angle to 16.5°, is apparently crucial in enabling cells to escape phagocytosis (Cunningham *et al.*, 1975). Avoiding phagocytosis is obviously necessary for survival of animal pathogens such as *Salmonella* spp.; presumably it is also important for free-living microorganisms to escape phagocytic attack by eukaryotic predators such as amoebae. Thirdly, the surface of unicellular organisms usually undergoes a very rapid change during evolution. Thus the structure of the exposed portion of LPS, i.e. the O chain, often shows a tremendous diversity. Since attachment of specific antibodies to a bacterial surface enhances phagocytosis by promoting attachment to the Fc- and C'3-receptors on the phagocyte surface (Silverstein *et al.*, 1977) and by decreasing the hydrophilicity of the bacterial surface (van Oss and Gillman, 1972), the "invention" of a new surface structure, to which the host animal population has not been exposed, will give a significant selective advantage to pathogenic bacteria, and the diversity is particularly pronounced in animal symbionts and pathogens (Roantree, 1971; Nikaido, 1970). In this context, we note that the exposed proteins of the outer membrane, such as porins, also seem to have undergone this process of evolutionary diversification, as antibodies (Smit and Nikaido, 1978) and phages (Siitonen *et al.*, 1977) specific for *S. typhimurium* porins do not interact with *E. coli* porins, and phages that adsorb to *E. coli* protein 3a do not use *S. typhimurium* 33K proteins as receptor (Datta *et al.*, 1976).

The first step in animal infection by pathogenic bacteria is usually the specific attachment to the mucous membrane of the host. It is logical that specific structures on the outer membrane surface should play a role in this process. However, so far, most of the components found to be involved in the "recognition" process are pili or pili-like structures (Swanson, 1973; Buchanan and Pearce, 1976; Stirm *et al.*, 1967) in Gram-negative bacteria, and the deficiency in porins was not found to have any discernible effect on the virulence of a strain of *S. typhimurium* (Valtonen *et al.*, 1977). On the other hand, in *N. gonorrheae* the presence or absence of a 28,000–29,000 dalton outer-membrane protein was found to correlate with the ability of the bacteria to associate with human polymorphonuclear leucocytes (King and Swanson, 1978). Furthermore, another set of outer membrane proteins in the 24,500–28,000 dalton

range were found to be correlated with the opacity of the colony, (Walstad *et al.*, 1977; Swanson, 1978). There is a frequent change (or "variation") from the opaque to the translucent form, and *vice versa*, among the progeny of a single cell. Interestingly, James and Swanson (1978) found that opaque forms were usually isolated from male urethra, whereas cultures of female cervical exudates showed periodic variations in the transparent/opaque ratio depending on the time within the menstrual cycle. The same tendency was observed when even male and female patients infected with a single, distinctive strain were examined. Apparently, increasing proteolytic activity of cervical secretions towards the end of the menstrual cycle selects for the transparent forms that are without the "opacity protein" and are protease-resistant (James and Swanson, 1978). The opaque form is favoured in urethral infections, possibly because the presence of the "opacity protein" in the outer membrane makes the bacterial surface strongly adhesive to the surfaces of epithelial cells of the host (J. James, personal communication). In *Pseudomonas aeruginosa*, Homma and his co-workers have isolated a protein, called OEP, which resembles major outer-membrane proteins in terms of its release from cells with EDTA and its association with LPS (Homma and Suzuki, 1966), and showed that it is a more potent protective antigen than LPS (Abe *et al.*, 1977). Clearly the role of outer-membrane proteins in pathogenesis appears to be an intriguing topic that requires further investigation.

B. MULTIPLICITY OF DIFFUSION PATHWAYS

The availability of single-step mutants with defined biochemical defects in LPS synthesis made it possible experimentally to alter the structure of a component of the outer membrane, and to study the effect of such alterations on the permeability of the membrane. These studies, initiated by Roantree and Stocker and their co-workers (Roantree *et al.*, 1969; 1977), as well as by Schlecht, Schmidt and Westphal (Schlecht and Schmidt, 1969; Schmidt *et al.*, 1969: Schlecht and Westphal, 1970). revealed the following: (a) Wild-type strains of *S. typhimurium*, *E. coli*, and other related bacteria are naturally sensitive to a number of antibiotics including neomycin, cycloserine, ampicillin and cephalothin; the sensitivity to these antibiotics was not greatly affected by alterations in LPS structure. (b) These strains, in contrast, are much more resistant than most Gram-positive bacteria to some other antibiotics including

actinomycin D, erythromycin, novobiocin and rifamycin SV, to such dyes as crystal violet, and to such detergents as bile salts and sodium dodecyl sulphate. Indeed, this difference in dye and detergent sensitivity is the basis for various selective media for Gram-negative enteric bacteria such as *S. typhimurium* and *E. coli*, for example deoxycholate agar and eosin-methylene blue agar. The sensitivity to these agents, however, increased drastically in "deep rough" mutants of *S. typhimurium* that produced extremely defective LPS of Rd_1, Rd_2, and Re-type (Fig. 1), and approached the level of sensitivity seen in Gram-positive bacteria. A similar observation was subsequently made with mutants of *E. coli* (Tamaki *et al.*, 1971).

In addition to the genetic approach already described, the structure of the outer membrane can be altered by removing up to 50% of LPS molecules by briefly treating cells of *S. typhimurium* or *E. coli* with EDTA (Leive, 1965c). Removal of LPS by this treatment increases the sensitivity of the organisms to actinomycin D (Leive, 1965a, b). Subsequent studies, summarized by Leive (1974), showed that the treated cells also became sensitive to novobiocin, rifampicin, long-chain fatty acids and detergents.

Since the outer membrane is the only subcellular structure that contains LPS in substantial amounts, and since the targets of action of those antibiotics and dyes examined are usually located in the cytoplasmic membrane or in the cytoplasm, the results described above suggest that the outer membrane normally acts as a penetration barrier against certain kinds of molecules and that this barrier can be breached by mutational alteration of LPS structure or by removal of LPS with EDTA. Furthermore, the presence of another group of agents whose efficacy is not influenced by mutations in LPS synthesis suggests a multiplicity of mechanisms of diffusion across the outer membrane. Thus there would be at least one diffusion pathway that is influenced by the structure or the amount of LPS in the outer membrane, and at least another that is not.

How, then, is any compound assigned to one of these pathways? A review of the structure of various tested compounds suggested to us that the compounds whose efficacy is increased in deep rough mutants and in EDTA-treated cells are usually hydrophobic. When the hydrophobicity of these compounds was determined quantitatively by measuring their partition coefficients in 1-octanol/phosphate buffer (pH 7.0), it was indeed found that most of these compounds had partition coefficients

higher than 0.07 (Table 2). In contrast, the majority of the compounds whose efficacy was unaffected by LPS structure were quite hydrophilic, with partition coefficients lower than 0.02 (Nikaido, 1976) (Table 2). The simplest explanation of these results is as follows. (a) There are at least two *general* pathways for diffusion of small molecules across the outer membrane, one for hydrophilic compounds, and one for hydrophobic

TABLE 2. Hydrophobicity, size, and relative efficacy against deep rough mutants, of various inhibitory agents[a]

Agent	Efficacy ratio (deep rough/wild type)	Partition coefficient[c]	Molecular weight
Actinomycin D		>20	1255
Novobiocin		>20	613
Phenol		>20	94
Crystal Violet		14.4	408
Rifamycin SV	≥10[b]	8.8	698
Malachite Green		4.2	365
Nafcillin		0.31	414
Oxacillin		0.07	418
Vancomycin		>0.01[d]	≃3300
Penicillin G		0.02	334
Ampicillin		>0.01	349
Cephalothin		>0.01	395
Carbenicillin	0.5–1.0	>0.01	378
Neomycin		>0.01	615
Cycloserine		>0.01	102
Chloramphenicol		12.4[d]	323
Tetracycline		0.07[d]	444

[a] Modified from Nikaido (1976).

[b] Other hydrophobic agents to which deep rough mutants, EDTA-treated cells, or both become sensitive include fatty acids (Sheu and Freese, 1973), 3,4-benzpyrene (Coratza and Molina, 1978), and a large number of polycyclic carcinogens (Ames *et al.*, 1973).

[c] Determined in a system containing equal volumes of 1-octanol and 0.05 *M* sodium phosphate buffer, pH 7.0, at 24°C.

[d] These compounds (for unknown reasons) do not follow the general tendency.

compounds. (b) The hydrophilic pathway is not much affected by the structure of the LPS present. (c) The hydrophobic pathway is almost inactive in wild-type strains producing complete LPS; it becomes fully active only in deep rough mutants or in EDTA-treated cells. The nature of this hydrophobic pathway is described in the next section.

C. BARRIER PROPERTIES AGAINST HYDROPHOBIC MOLECULES

1. *Measurement of Transmembrane Diffusion Rates*

Most biological membranes are known to allow the transmembrane diffusion of small , hydrophobic compounds (Danielli, 1952); the outer membrane of deep rough mutants apparently behave in a similar manner. In contrast, the results described in the preceding section suggest that the outer membranes of wild-type *S. typhimurium* and *E. coli* constitute an exception in that they are not penetrated by many hydrophobic compounds.

These conclusions, however, were based on inhibitory effects measured using whole cells, and we could only infer that the difference in sensitivity reflected difference in penetration rates. A more direct approach was taken by Gustafson *et al.* (1973), who showed that the rates of uptake of crystal violet by intact cells were dependent on the nature of the LPS present. These results, however, were complicated by massive adsorption of the dye to unidentified cellular constituents. We have overcome this difficulty by using a high concentration of a semi-synthetic penicillin, nafcillin, as the permeant (Nikaido, 1976). When nafcillin was added to thick suspensions of the deep rough mutants, its extracellular concentrations at zero time and at infinite time (actually five to ten minutes at 22°C), determined in supernatants after centrifugation, were equal to those predicted on the assumption that nafcillin existed only in the extracellular space at zero time and was distributed uniformly in extracellular and intracellular spaces at infinite time. Adsorption, degradation or active transport is therefore negligible under these conditions. Furthermore, the kinetics of diffusion followed a theoretical equation, and first-order rate constants for permeation could thus be calculated. These quantitative diffusion assays led us to the following conclusions (Nikaido, 1976). (a) Permeation through the cytoplasmic membrane, measured with sphaeroplasts, was at least ten times more rapid than the penetration rates into intact deep rough mutant cells. Thus in whole cells permeation through the outer membrane is the rate-limiting step. (b) The permeation rate constants were low (0.005–0.009 min^{-1} at 22° under our assay conditions) on the wild-type (S) as well as in Ra and Rc mutants (see Fig. 1), but were much higher (0.1–1.8 min^{-1}) in deep rough (i.e. Rd_1, Rd_2, and Re) mutants. There was a perfect correlation between the increase in permeability and the increase in the phospholipid content of the outer membrane (Fig. 6).

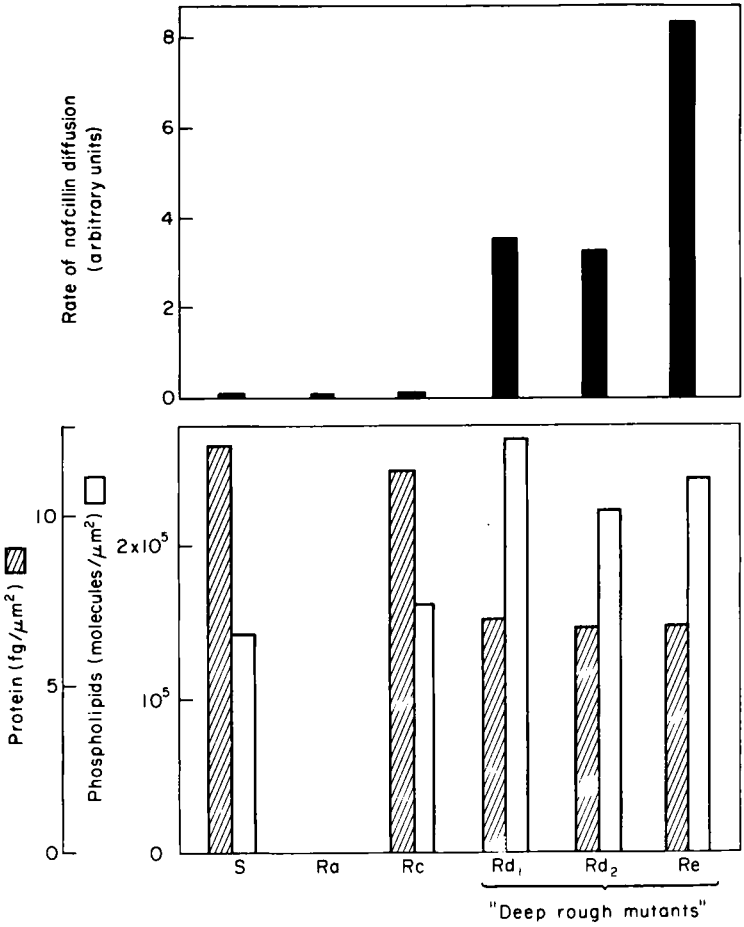

Fig. 6. (a) Rate of diffusion of a hydrophobic solute, nafcillin, across the outer membranes of *Salmonella typhimurium* strains, and (b) the composition of the outer membrane in these strains. Rates of diffusion into intact cells were actually determined, but they reflect the diffusion rates across the outer membrane (for details see Nikaido, 1976). The diffusion rate does not increase significantly even when about 90% of the saccharide residues are lost as in the Rc mutant, but increases suddenly in parallel with the increase in phospholipid content (and decrease in protein content) occurring in the deep rough mutants. The data for (b) were taken from Smit *et al.* (1975).

2. *Mechanism of Diffusion of Hydrophobic Compounds*

The mechanism of transmembrane diffusion of hydrophobic molecules has been studied extensively since the pioneering work of Collander and Bärlund (1933), and it is known that the diffusion rates are fastest with

molecules of small size, with those of high hydrophobicity, and at high temperature (Danielli, 1952; Stein, 1967; Sha'afi *et al.*, 1971). Critical analysis of these results suggested that the permeant molecules first dissolve in the hydrophobic interior of the membrane, diffuse through the thickness of the hydrocarbon layer, and then cross the membrane by partitioning into the aqueous phase on the other side of the membrane (Stein, 1967). To test whether hydrophobic substances penetrate through the outer membrane of deep rough mutants by a similar mechanism, we measured the influence of various conditions on diffusion rates, and obtained the following results. (a) Among compounds with similar size, diffusion rate increased with increasing hydrophobicity. (b) The diffusion rates were extremely dependent on temperature, with a Q_{10} value close to 10. (c) Molecules of fairly large size apparently penetrated the outer membrane by this mechanism, and there was no indication of a clear-cut size limit. These properties, especially the very high temperature coefficient (Galey *et al.*, 1973), are well known properties of the diffusion process effected by dissolution into the membrane interior, and we conclude that hydrophobic compounds penetrate through the deep rough outer membrane by a similar mechanism.

3. *Structural Basis for the Lack of Permeability Toward Hydrophobic Compounds*

The unusual barrier property of the outer membrane of wild-type *E. coli* or *S. typhimurium* against hydrophobic compounds can be understood quite well on the basis of the asymmetric bilayer model presented in Section III.D (Fig. 5). In fact, this model was in part created in an effort to explain this barrier property. Thus, the hydrocarbon chains of LPS are likely to be tightly clustered together because at least six chains are connected to a single backbone structure and because unsaturated fatty-acyl residues are entirely absent. This idea is supported by spin labelling studies which showed that the motion of the segments of LPS hydrocarbon chains near the carboxyl end is severly restricted (Nikaido *et al.*, 1977c). It is also consistent with the monolayer studies of Romeo *et al.* (1970), who found a smaller cross-sectional area for LPS hydrocarbons than for phospholipid hydrocarbon chains. [The absolute magnitude of their value (0.13 nm^2 for a LPS hydrocarbon), however, is obviously an underestimate, since even hexagonal crystalline packing with 0.433 nm spacing (see Emmerling *et al.*, 1977) would require an area of 0.216 nm^2

per chain.] Since a major portion of the resistance in the transmembrane diffusion of hydrophobic compounds arises because of the difficulty in making a hole between the associated hydrocarbon chains (Galey *et al.*, 1973), it is easily seen that the presence of the tightly clustered hydrocarbon chains of LPS will drastically slow down the permeation process.

In deep rough mutants, the extremely abbreviated structure of LPS hinders proper interaction between LPS and proteins at the time of outer membrane assembly (Section V.C). This results in decreased incorporation of outer-membrane proteins, which in turn produces a compensatory increase in phospholipids, some of which are now found in the outer half of the membrane (Section III.D). Hydrophobic molecules can obviously diffuse through these "phospholipid bilayer" regions of the membrane (Fig. 5B).

Treatment of wild-type cells with EDTA releases LPS-protein complexes (Section III.D). This selective removal of components of the outer half of the membrane is presumably quickly followed by flip-over of phospholipids from the inner half, or by the lateral diffusion of phospholipids from the inner membrane in a manner similar to that described by Jones and Osborn (1977b). Thus "phospholipid bilayer" regions are again generated also in this case, and the outer membrane will become permeable to hydrophobic molecules.

The low permeability to hydrophobic compounds of the outer membrane of wild-type *E. coli* or *S. typhimurium* must confer an enormous selective advantage to these organisms by making them resistant to bile salts and long-chain fatty acids that must be abundant in the intestinal tract. There is no *a priori* necessity, however, that all Gram-negative bacteria should produce hydrophobe-impermeable outer membranes. In fact, some non-enteric Gram-negative bacteria, for example *N. gonorrhoeae*, are extremely sensitive to such hydrophobic agents as erythromycin, rifampin, acridine orange, ethidium bromide (Maness and Sparling, 1973) and free fatty acids (Miller *et al.*, 1977). Furthermore, there is usually good correlation between the sensitivities to these various agents (Maness and Sparling, 1973). A hydrophobic dye, crystal violet, was shown to diffuse as rapidly into *N. gonorrhoeae* as into deep rough mutants of *E. coli* (Wolf-Watz *et al.*, 1975). These observations suggest that the outer membrane of *N. gonorrhoeae*, in contrast to that of enteric bacteria, contains phospholipid bilayer regions, and that the sensitivity to these hydrophobic agents is determined by the relative size of the

phospholipid bilayer areas. This hypothesis should be testable by the techniques developed with enteric bacteria (Nikaido, 1976; Kamio and Nikaido, 1976).

According to our hypothesis, selection for mutants with higher permeability toward hydrophobic compounds should produce mutants whose outer membrane contained less protein and more phospholipids. No such experiment has been reported, but the recent work of McIntosh *et al.* (1978) *might* be relevant. By selecting for mutants able to grow in the absence of the outer-membrane transport system for Fe^{3+}-enterochelin (see Section IV.E), these workers obtained mutants with much diminished levels of porins. Although it is not clear how the porin deficiency contributes to iron transport across the outer membrane, it seems possible that some Fe^{3+}-chelator complexes pass through the interior of the membrane. These porin-deficient mutants, however, were reportedly not unusually sensitive to inhibition by hydrophobic agents (McIntosh *et al.*, 1978).

D. NON-SPECIFIC PERMEABILITY TOWARD SMALL, HYDROPHILIC MOLECULES

1. *Equilibrium Studies with Intact Cells*

Gram-negative bacteria are sensitive to a number of hydrophilic antibiotics (Table 2), which must penetrate through the outer membrane in order to reach their targets of action. Similar diffusion is also required for nutrients including sugars, amino acids and inorganic salts, as well as for waste products of metabolism. It seems very unlikely that these compounds penetrate via the "hydrophobic pathway" described above, since this pathway is essentially inoperative in wild-type organisms and since, even if the pathway were open, the diffusion of these compounds would be extremely slow owing to their small partition coefficients. These considerations led us to postulate the existence of a separate penetration mechanism for hydrophilic compounds, a mechanism presumed to operate fully in the wild-type organism as well as in deep rough mutants. We studied the properties of this postulated pathway by using oligosaccharides as permeants (Decad *et al.*, 1974; Decad and Nikaido, 1976).

In order to study their penetration through the outer membrane, it was necessary to use oligosaccharides that neither penetrated through

nor were actively transported across the cytoplasmic membrane. Oligosaccharides of the sucrose-raffinose series fulfilled these conditions in *S. typhimurium*, and were used in most of our experiments. Raffinose (galactosyl-sucrose), stachyose (galactosyl-galactosyl-sucrose), and verbascose (galactosyl-galactosyl-galactosyl-sucrose) have an added advantage that they can be easily labelled with tritium by oxidation of C-6 of the terminal galactose residue with galactose oxidase, followed by its reduction by NaB^3H_4. Since the space between the outer membrane and the cytoplasmic membrane (i.e. the "periplasmic space") is small, the diffusion into this space was difficult to measure. [A recent paper by Stock *et al.* (1977) concludes that the periplasmic space corresponds to 20–40% of the total cell volume in normal, unplasmolysed *S. typhimurium* and *E. coli*. In our hands, however, the space never exceeded 5% of the cell volume, except in starved cells or stationary-phase cells in which up to 13% of the cell volume became the periplasmic space (Decad, 1976). The cause for this discrepancy is not clear at present]. We, therefore, expanded the periplasmic space by plasmolysing cells in 0.3 to 0.5 *M* NaCl or in 0.5 *M* sucrose (Decad and Nikaido, 1976). Under these conditions, 40–50% of the cell volume was occupied by the periplasmic space, and the extent of penetration of oligosaccharides into this space could easily be measured by centrifuging plasmolysed cells after incubation with the radioactive oligosaccharides, and by determining the concentration of the radioactive compound both in the supernatant and the pellet. Corrections for intercellular space in the pellet were made by adding, to the incubation mixture, large, uncharged polymers (e.g. [^{3}H]-dextran) which were assumed to be impermeable through the outer membrane.

These studies showed the existence of a clear size limit in the penetration process through this pathway (Decad and Nikaido, 1976). Sucrose (342 daltons) and raffinose (504 daltons) penetrated fully into the periplasmic space after 5 min incubation at room temperature, but stachyose (666 daltons) and larger oligosaccharides showed only partial penetration. The partial penetration was apparently due to heterogeneity of the cell population, as prolonged incubation did not increase the degree of penetration. Furthermore, much of the heterogeneity seemed to have been caused by damage produced during the preparation of plasmolysed cells. Thus, in experiments in which the cells were prepared rapidly and the degree of plasmolysis was not extreme, stachyose penetrated little (less than 25% penetration) whilst

verbascose (828 daltons), as well as inulins, or dextrans did not penetrate at all. It seems likely that the slight penetration of stachyose represents diffusion into damaged cells, and we conclude that the cell wall of *S. typhimurium* is permeable to a disaccharide (sucrose) and a trisaccharide (raffinose), but is essentially impermeable to tetrasaccharides and higher oligosaccharides. There thus seems to be a sharp exclusion limit around 550–650 daltons for the raffinose series of oligosaccharides.

It was gratifying to note that these results were consistent with the results of a pioneering study by Payne and Gilvarg (1968), who found that peptides larger than about 600–650 daltons could not enter *E. coli* and postulated the presence of a barrier "external to the peptide transport system". However, neither the study of Payne and Gilvarg (1968) nor our results already described told us which of the two layers of the cell wall, the outer membrane or peptidoglycan, acted as the limiting barrier for penetration of hydrophilic molecules. Our later studies, however, clearly showed that the outer membrane, rather than the peptidoglycan layer, acted as a limiting molecular sieve (Nakae and Nikaido, 1975). In one series of experiments, the peptidoglycan layer was degraded by lysozyme treatment or by growth in the presence of penicillin. The plasmolysed cells, or sphaeroplasts produced from these bacteria still showed the same molecular sieving properties seen before, in spite of the absence of the intact peptidoglycan layer. In another series of experiments, outer membrane, containing only traces of peptidoglycan fragments, was isolated in the form of closed vesicles. These vesicles apparently had a number of "cracks", probably produced as a result of incomplete resealing of membrane fragments. Heating in the presence of Mg^{2+}, however, was found to seal most of these cracks, and produced vesicles that had permeability properties resembling those of plasmolysed cells.

How do the hydrophilic molecules pass through the outer membrane? As stated earlier, diffusion via dissolution into the membrane interior is impossible, as this would require breaking a great many hydrogen bonds between the oligosaccharides and water molecules, thermodynamically a very unfavourable operation. The only possible mechanisms seem to be diffusion through water-filled pores and carrier-mediated diffusion. It is not possible to distinguish absolutely between these two mechanisms with the data already described. Even the theoretical line of demarcation is sometimes unclear, as the latter mechanism can be considered as diffusion through specialized pores (Singer, 1974). We found, however, that

diffusion of oligosaccharides through the outer membrane was extremely rapid at 0°C (Decad and Nikaido, 1976), and this observation, as well as the identical size limit for widely different kinds of compounds, seemed to favour the aqueous pore mechanism. We sought a more definitive answer to this question through reconstitution studies described in the next section.

2. *Reconstitution Studies*

When the outer membranes are dissociated by detergents and the detergents are then slowly removed in the presence of Mg^{2+}, one observes reformation of structures morphologically resembling the outer membrane. Experiments of this type were performed on *E. coli* outer membranes first by DePamphilis (1971) by using dissociation by Triton X-100 and EDTA, then by Bragg and Hou (1972) as well as by Sekizawa and Fukui (1973) using SDS as the dissociating agent, and more recently by Nakamura and Mizushima (1975) with partially purified components. These experiments established that the supramolecular structure of the outer membrane was ultimately determined by the structure and properties of the component molecules. However, they did not throw much light on the nature of the interaction between the component molecules, or on the functions of individual components.

In our laboratory, reconstitution experiments were undertaken to identify the components that are responsible for the characteristic hydrophilic permeability of the outer membrane. Since both the hydrophilic permeability and the presence of LPS are unique attributes of the outer membrane, we first examined the possibility that LPS might be involved in formation of pores. We prepared liposomes containing both LPS and phospholipids in the bilayer structure, by resuspending a dried film of phospholipids in an aqueous suspension of LPS, as described by Kinsky's group (Kataoka *et al.*, 1971). These liposomes bounded by the mixed phospholipid-LPS bilayer, were, however, as impermeable toward hydrophilic compounds as the liposomes made only of phospholipid (Nikaido and Nakae, 1973); that is, both of them allowed a fairly rapid penetration of glycerol and a slow diffusion of erythritol, but were essentially impermeable to glucose, sucrose and higher oligosaccharides. These results led us to the alternative hypothesis that outer-membrane proteins play an essential role in formation of pores. This hypothesis was tested by one of us (Nakae, 1975) by making

liposomes in the presence of outer-membrane proteins. For this purpose, the outer membrane proteins were extracted by the use of 0.7 *M* lithium diiodosalicylate. Removal of the chaotropic agent by extensive dialysis resulted in the precipitation of proteins, which were then recovered by centrifugation and resuspended by sonication in an aqueous buffer. Membrane vesicles were reconstituted by adding this protein suspension and an aqueous suspension of LPS to a dried film of phospholipids. When the reconstitution medium contained both [^{3}H]-dextran and [^{14}C]-sucrose, both were presumably present in intravesicular as well as extravesicular space. When the vesicles were then separated from the medium by gel filtration through Sepharose 4B, a large portion of intravesicular [^{14}C]-sucrose diffused out through the protein-containing membrane during the filtration step, so that the ^{3}H/^{14}C ratio of the recovered liposome preparation was usually several times higher than the ^{3}H/^{14}C ratio in the initial reconstitution mixture. A control mixture lacking proteins produced liposomes essentially impermeable to sucrose, that is with hardly any increase in the ^{3}H/^{14}C ratio. In another control experiment, proteins from sheep red-blood cell membranes were completely inactive in the production of sucrose-permeable pores. We conclude from these results that outer-membrane proteins are essential in formation of water-filled pores.

We then used partially purified preparations of outer-membrane proteins for reconstitution, in order to identify the proteins(s) involved in pore formation (Nakae, 1976a). As described already, treatment of *S. typhimurium* cell envelope with SDS at 37°C leaves behind an insoluble complex that contains peptidoglycan with covalently linked lipoprotein as well as the "peptidoglycan-associated" major proteins or porins. We found that the SDS-insoluble complex was much more active than the SDS-solubilized proteins in producing pores. We then degraded the former with lysozyme and fractionated the products by gel filtration in SDS. This produced a protein aggregate containing only the porins from *S. typhimurium* (Nakae, 1976a) as well as from *E. coli* K12 (T. Nakae, unpublished results), and a similar oligomer composed only of the 36,500 dalton porins from *E. coli* B (Nakae, 1976b), all of which were extremely active in producing pores in the reconstitution experiments (Table 3). Inouye (1974) has proposed from theoretical considerations that oligomers of Braun lipoproteins may contain aqueous channels in the centre and thus produce pores by penetrating through the thickness of the outer membrane; our active complex, however, contained very few

TABLE 3. Results of a reconstitution experiment[a]

	[^{3}H]-dextran (cpm)	[^{14}C]-sucrose (cpm)
Added to the Reaction Mixture	240,000	260,000
Recovered in Vesicles:		
Exp. I (without porins)	2,400	2,100
Exp. II (with 10 μg porin)	3,350	>30

[a] Membrane vesicles were reconstituted from 0.8 mg LPS and 0.8 mg phospholipids, with or without 10 μg of porin oligomer, ("Complex I" of Nakae, 1976a) from *S. typhimurium*, in the presence of [^{3}H]-dextran and [^{14}C]-sucrose. Vesicles were recovered after gel filtration, which removed radioactive saccharides in the extravesicular space as well as those able to diffuse out through the vesicle membrane. For details see Nakae (1976a).

lipoproteins. In fact, the porin oligomer prepared from a lipoprotein-deficient mutant (Hirota *et al.*, 1977) of *E. coli* K12 was fully active (T. Nakae, unpublished results). Furthermore, aggregates containing mostly lipoproteins and little porin were only very weakly active in pore formation, and purified lipoprotein was completely inactive.

The pores produced by addition of porins had the expected size, in that they allowed a free diffusion of sucrose and raffinose yet essentially excluded stachyose and larger oligosaccharides. The vesicles formed were also permeable to a wide variety of small, hydrophilic molecules, including galactose, glucosamine, glucose 1-phosphate, lysine, tryptophan, uridine, UMP, GDP and polyethyleneglycol (600 daltons), but not to polyethyleneglycol (1540 daltons) (Nakae, 1976a). Since complexes containing only one to three protein species can produce permeability to such a wide variety of compounds, the mechanism of diffusion cannot be the facilitated diffusion that requires a specific carrier protein for each compound. This lack of specificity is indeed one of the strongest pieces of evidence suggesting the involvement of aqueous pores.

The channel-forming ability of porins has recently been confirmed by a different approach. Benz *et al.* (1978) added an extremely dilute (e.g. 0.5 ng/ml) solution of *E. coli* B porin to the aqueous solution in which a planar lipid bilayer membrane was bathed. Interestingly, the conductance increased in a stepwise manner, and the single incremental step of 1.7 nanosiemens (in 1 *M* KCl), suggesting the opening of a channel of about 1 nm in diameter, was interpreted to result from

insertion of one molecule or oligomer of the porin into the membrane. At slightly higher (e.g. 20 ng/ml) concentrations of porin, the conductance eventually increased about 10^5-fold, clearly indicating that the effect of porin is distinct from the non-specific effect of many hydrophobic proteins that increase conductance only up to 100-fold (Montal, 1976).

Reconstitution of hydrophilic pores has been achieved also by using outer-membrane proteins of species other than *E. coli* or *S. typhimurium*. Decad (1976) showed that proteins in the "SDS-non-extractable fraction" of *Proteus morganii* cell envelope, as well as the Triton X-100-EDTA-extracted proteins of the *Ps. aeruginosa* cell envelope, could be used to reconstitute sucrose-permeable membranes. More recently, Nixdorff *et al.* (1977) achieved similar results with a 39,000 dalton outer-membrane protein from *Proteus mirabilis*, extracted with 50% acetic acid. A partial reconstitution from isolated phospholipids, LPS and a small amount of outer-membrane fragments has recently been performed by using components from *Ps. aeruginosa* (Hancock and Nikaido, 1978). The results suggested, interestingly, that the exclusion limit of *Ps. aeruginosa* pores is several thousand daltons, much higher than in enteric bacteria. The *Ps. aeruginosa* porin with the large exclusion limit has recently been purified by ion-exchange chromatography in Triton X-100, and was identified as 35,000 to 37,000 dalton major protein(s) of the outer membrane (Hancock *et al.*, 1979).

3. *Rate Studies with Intact Cells*

If all of the pores in the outer membrane are surrounded by porin molecules, mutational loss of the porin is expected to be lethal. However, the apparent loss of these proteins (Davies and Reeves, 1975; Foulds 1976; Nurminen *et al.*, 1976), or of all "major" proteins (Henning and Haller, 1975), reportedly did not produce any marked reduction in growth rates. The solution to this puzzle was provided in 1977 by studies utilizing two different methods of approach. Both of these methods enabled us to estimate the *rates of diffusion across the outer membrane, rather than the equilibrium distribution* of solutes described in Section IV.D.1. and gave us new insights into the function of the outer-membrane pores.

As described already, diffusion rates across the outer membrane were too rapid to measure with the equilibrium method described earlier. Zimmermann and Rosselet (1977) recently devised an elegant method to measure these rates. They measured the rates of hydrolysis of β-lactam

antibiotics by intact cells as well as by broken cells of *E. coli* harbouring an R-factor. The β-lactamase coded for by the R-factor is located in the periplasmic space. Thus, with intact cells, the rate of diffusion across the outer membrane should be balanced, at the steady state, by the rate of hydrolysis in the periplasmic space. Zimmermann and Rosselet (1977) made a very important contribution in showing that the rates of hydrolysis by intact cells indeed followed this prediction, and could be calculated by assuming that the diffusion and hydrolysis, respectively, follow Fick's law and Michaelis-Menten kinetics. It is also necessary to point out that this quantitative treatment is absolutely required for the discussion of outer membrane permeability. For example, determination of "crypticity" (ratio of rates of hydrolysis in intact cells and in broken cells) at an arbitrarily chosen substrate concentration, a procedure unfortunately still followed by some workers, means very little because the mathematical equations show us that in any system the numerical values of "crypticity" can be altered freely depending on the substrate concentration used (Zimmermann and Rosselet, 1977, see also Fig. 7).

We have followed the Zimmermann-Rosselet approach and determined permeability coefficients of the outer membrane in various strains of *S. typhimurium* (Nikaido *et al.*, 1977b). The results showed clearly that the permeability of the outer membrane toward a hydrophilic β-lactam of 415 daltons, cephaloridine, decreased significantly when one of the three porins was lost through mutation, and that mutants with reduced levels of all three porins usually showed permeability coefficients less than 10% of that found in the wild type. Similar results were independently obtained with *E. coli* K12 by workers who approached the problem from the opposite direction, i.e. by examining membrane alterations in "cryptic" mutants. The mutants studied by Beacham *et al.*, (1977) showed an increased K_m but unaltered V_{max} values for hydrolysis, by intact cells, of nucleotides by periplasmic nucleotidases. When outer membranes of these mutants were analysed, they were found to have drastically reduced levels of porins (Beacham *et al.*, 1977).

In the approach already described, the inherent difficulty in determining diffusion rates across the outer membrane was solved by the use of enzymes that rapidly hydrolysed substrates as they came into the periplasmic space. The requirement for the assay of outer membrane permeability is thus the efficient removal of diffusing substrates from the periplasmic space; only under these conditions will the overall rates of the process be predominantly determined by the diffusion rates across the

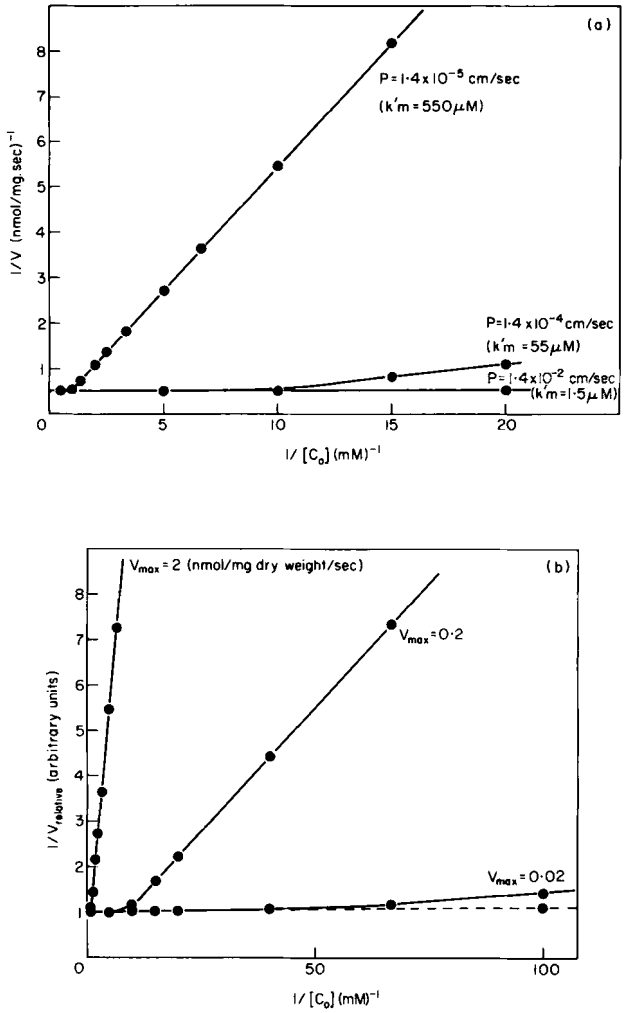

FIG. 7. Theoretically predicted behaviour of overall transport systems. Part a shows the behaviour of a high V_{max} (2 nmol/mg dry weight/sec), low K_m(1 μM), transport system in mutants producing 1% and 0.1% of the normal amount of porin. In the wild type cell, where an outer membrane permeability of 1.4×10^{-2} cm/sec was assumed, the presence of the outer membrane raises the overall K_m' from 1.0 μM to only 1.5 μM. In mutants, apparent K_m' values are increased, but V_{max} does not change. The plots approximately follow straight lines except at very high values of C_o. Because of this deviation from linearity, the apparent K_m' values were obtained as C_o values giving the half-maximal transport (or growth) rates, rather than by extrapolation of the linear portion. The values of V_{max}, P, and K_m used approximate those obtained experimentally or assumed for

outer membrane, thus allowing measurement of the latter rates. Removal of substrates can also be performed by active transport systems located in the cytoplasmic membrane, and this forms the basis of the second approach to be described, i.e. the study of overall transport kinetics and "apparent transport K_m" values. It should be emphasized again that uptake assays at a single, arbitrary concentration does not give much information. The diffusion rate (V) across the outer membrane is determined by Fick's law, and if we denote by C_o and C_p the substrate concentrations in the outside medium and in the periplasmic space, then

$$V = P \cdot A \cdot (C_o - C_p)$$

where P and A are the permeability coefficient and the area of the membrane, respectively. The rate of active transport follows Michaelis-Menten kinetics, so that

$$V = \frac{C_p \cdot V_{max}}{K_m + C_p}$$

At steady state the two rates are equal, and thus we can eliminate C_p. This gives

$$V = \tfrac{1}{2}[V_{max} + P \cdot A \cdot K_m + P \cdot A \cdot C_o - \sqrt{(V_{max} + P \cdot A \cdot K_m + P \cdot A \cdot C_o)^2 - 4 \cdot C_o \cdot P \cdot A \cdot V_{max}}]$$

This equation looks rather unwieldy, but results of calculations using reasonable values of A, K_m and V_{max} show that the decrease in outer-membrane permeability (P) produces changes in the overall transport rate (V), changes suggesting an increase in the overall "K_m" of transport (K'_m) without alteration in V_{max} (Fig. 7a). These considerations suggest that the effect of porin deficiency on transport rates will become apparent only at low substrate concentrations (or high values of $1/C_o$). Furthermore, the effect will be difficult to detect even at low values of C_o, if the V_{max} value of the inner membrane, active-transport system happens to be low (Fig. 7b).

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glucose uptake in *E. coli* B/r (Bavoil *et al.*, 1977). (However a more recent study (H. Nikaido and E. Y. Rosenburg, to be published) has shown that in this earlier report values of P and K_m were overestimated and underestimated, respectively). Part b shows the effect of the V_{max} on the transport in a mutant containing 0.1% of the normal number of porins. The other parameters were the same as in part a. The overall transport rates are shown as relative values, $V_{relative}$, which equal V/V_{max}. It is seen that even a loss of 99.9% of the porins does not produce striking retardation of overall transport, if the V_{max} of the active transport system (located in the inner membrane) is low.

Actually, mutants with the predicted change, i.e. increased "K_m" values for transport of a number of substrates, had been isolated some time ago by von Meyenburg (1971) without the expectation of their possessing any outer membrane defect. These mutants were recently shown to be deficient in porins, in the 36.5 K porin in *E. coli* B/r and in the two porins (proteins 1a and 1b) in *E. coli* K12 (Bavoil *et al.*, 1977). Since porin-deficient mutants have increased "K_m" values for transport of various sugars, a sugar alcohol, a sugar phosphate, amino acids, a pyrimidine and even inorganic anions like phosphate and sulphate (von Meyenburg, 1971), this approach establishes the role of porins in the transmembrane diffusion of a very wide range of hydrophilic compounds. In addition, if we assume that the active transport systems in the cytoplasmic membrane have very high affinities (or low K_m values) for the substrate, we can estimate the minimal values of permeability coefficient towards various substrates (Bavoil *et al.*, 1977).

The transport studies and the β-lactamase approach in our laboratory have so far produced the following pieces of information.

(a). The permeability coefficient (P) for glucose in the wild-type *E. coli* B/r strain was calculated to be larger than 1.4×10^{-2} cm/s (Bavoil *et al.*, 1977), but this estimate should be changed to larger than 0.3×10^{-2} cm/s in view of the more recently obtained values of growth K_m values for glucose (H. Nikaido and E. Y. Rosenberg, unpublished observations). We can interpret the significance of this figure as follows. If the outer membrane did not offer any resistance to the diffusion of glucose, the value of P can be estimated from $P = D/d$, where D and d are the diffusion coefficient of glucose in water and the thickness of the membrane, respectively. Thus at 25°C, $P = (0.673 \times 10^{-5}\ \text{cm}^2/\text{sec}) \div (5 \times 10^{-7}\ \text{cm}) = 13.5$ cm/s. The observed value is lower by a factor of at least 10^3, and we see that the outer membrane does constitute a significant barrier, as predicted.

Since the outer membrane is permeable to raffinose, the hydrated radius of which is 0.60 nm (Schultz and Solomon, 1961), as a first approximation we can assume the presence of pores 0.60 nm in radius. Since there are about 10^5 molecules of porin per cell (Rosenbusch, 1974), the total area of the cross-section of pores per cell will be $(0.6 \times 10^{-3})^2 \times 3.14 \times 10^5\ \mu\text{m}^2$, if one molecule of porin is assumed to produce one pore. Since the surface area of the average *E. coli* cell is of the order of 3 μm^2, the expected permeability coefficient can be calculated by multiplying the "free diffusion" permeability coefficient described

above, by the relative proportion of the pore area. Thus P (expected) = $13.5 \times 0.11 \div 3 = 0.5$ cm/s. The observed value is lower than this. But, even molecules with a smaller radius than the pore radius are significantly hindered in their diffusion because of their collision with the rims of the pore and of the viscous drag along the pore wall. According to the so-called Renkin equation (Renkin, 1954) the area *available for diffusion* is obtained by multiplying the geometrical area by a factor, $(1 - a)^2 (1 - 2.104a + 2.09a^3 - 0.95a^5)$, where a is the ratio of the solute radius to the radius of the pore. If we calculate this correction factor by using the hydrated radius of glucose, namely 0.42 nm (Schultz and Solomon, 1961) and correct, by this factor, the expected P value obtained above, a value of 0.37×10^{-2} cm/s is obtained, which is very close to the "observed" value viz. larger than 0.3×10^{-2} cm/s.

(b). The temperature dependence of the diffusion rate was quite low, in contrast to the diffusion of hydrophobic substances described in Section IV.C. Diffusion of a β-lactam antibiotic, cephaloridine, gave a Q_{10} value of about two (H. Nikaido and E. Y. Rosenburg, unpublished observation); this small temperature dependence is a strong evidence that permeation occurs through the water-filled pores. The simplest explanation for the observation that the Q_{10} value was slightly higher than that expected for free diffusion in water, i.e. $Q_{10} = 1.2$, is that the water inside the pore is more structured or more strongly associated than the bulk water (see below).

(c). The most remarkable observation is the presence among permeable solutes of a very large difference in permeability coefficients. Thus, in wild-type cells, the permeability coefficient of $\geq 0.3 \times 10^{-2}$ cm/s for glucose can be contrasted with several hundred-fold lower coefficient for 6-amino-penicillanic acid (1.4×10^{-5} cm/s), whose size (216 daltons) is not much different from that of glucose (180 daltons). We do not yet fully understand the factors that influence the rate of penetration through these pores, but a few parameters are already known to be important. The first factor is obviously the size of the solute. In the "apparent transport K_m" experiments, calculation of outer membrane P values on the basis of data on wild-type cells gives only minimal values with a large margin of uncertainty. In contrast, the overall transport rates in intact cells of porin-deficient (*ompB*) mutants are limited essentially by diffusion rates through the outer membrane, and thus allow a fairly accurate calculation of P values (Bavoil *et al.*, 1977). Since the *ompB* gene is most probably a regulatory gene (Section III.C), the mutants are

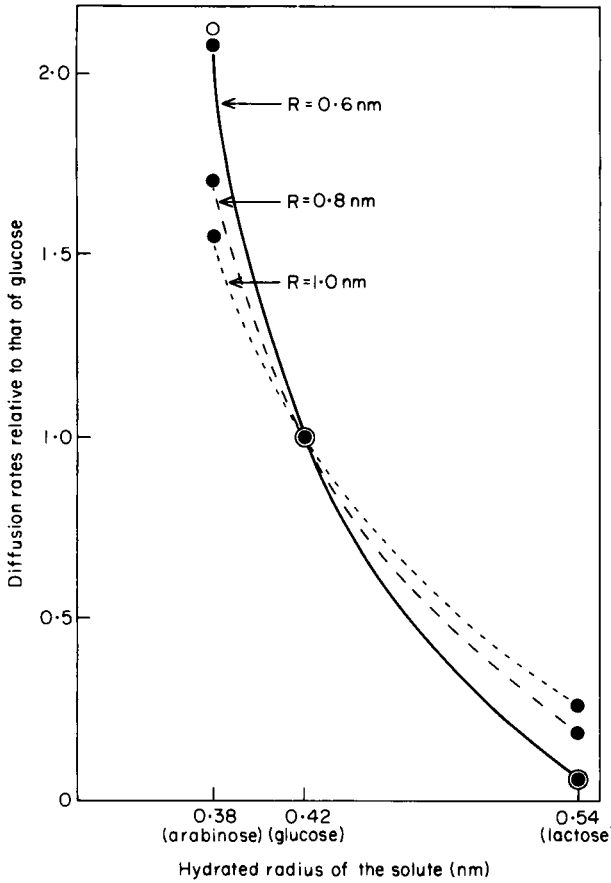

FIG. 8. Theoretical and observed permeability toward solutes of various sizes. The theoretical values (●——●) were calculated by multiplying the free diffusion coefficients for arabinose, glucose, and lactose by appropriate Renkin factors calculated for various pore sizes. The values of hydrated radius used were according to Schultz and Solomon (1961). The observed permeability (○) was calculated from the "growth K_m" of porin-deficient (*ompB*) mutant CM7 on these carbon sources (H. Nikaido and E. Y. Rosenberg, to be published), and from the growth rate and growth yields (for details, see Bavoil *et al.*, 1977). R = pore radius.

expected to produce smaller numbers of unaltered pores. Thus, if we assume that hydrophilic solutes diffuse through the mutant outer membrane via the remaining porin channels, the data shown in Fig. 8 clearly indicate the effect of solute size and, furthermore, there is a remarkably good fit with the theoretical values calculated on the basis of

the Renkin equation, assuming the presence of pores of 1.2 nm diameter.

The second factor is hydrophobicity. Hydrophobic antibiotics could not rapidly penetrate through the outer membrane of wild-type *E. coli* or *S. typhimurium* even when their size was clearly below the exclusion limit of the pores (Nikaido, 1976). This suggested that the pores tended to exclude hydrophobic solutes, an idea put to a direct test by Zimmermann and Rosselet (1977). These workers compared the permeability of the outer membrane of *E. coli* to a number of semisynthetic cephalosporin derivatives, and their findings, shown in a graphic form in Fig. 9, confirmed the inverse correlation between hydrophobicity and transmembrane diffusion rates (through pores), at least for a series of mono-anionic cephalosporins. At present, it is not known why pores exclude hydrophobic molecules. An obvious possibility is that these hydrophobic antibiotics cannot penetrate into the pores because they form micelles. However, although these substances do indeed form micelles at very high concentrations, they seem to exist mostly as monomers at the concentrations used for permeability studies (H. Nikaido and P. Bavoil, unpublished observations). Perhaps the

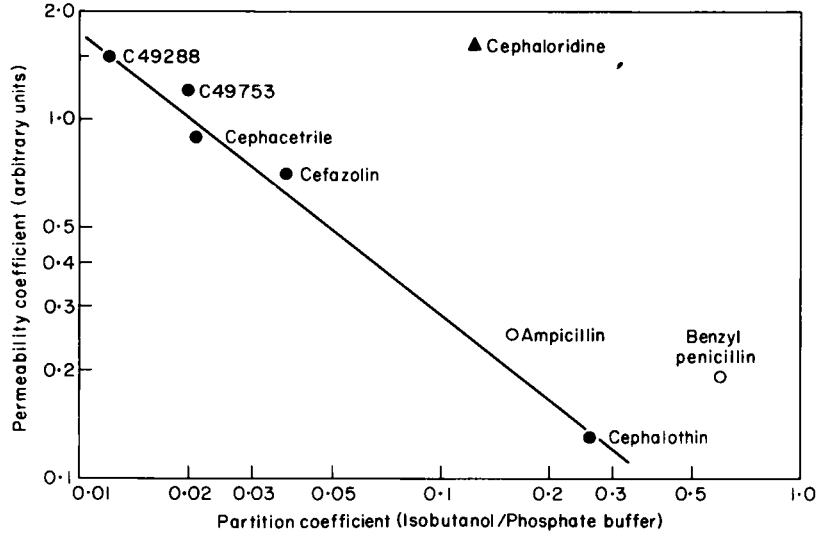

FIG. 9. Hydrophobicity and the rate of diffusion across the outer membrane of β-lactam antibiotics. Based on the results of Zimmermann and Rosselet (1977). The partition coefficients were determined in iso-butanol/0.02 *M* phosphate buffer, pH 7.4, containing 0.9% NaCl, at 37°C.

important thing to realize here is that the diameter of the pore is not much different from the diameter of solute molecules. Thus the walls of the pore are probably lined with hydrophilic or hydrogen-bonding structures, so that transfer of hydrophilic solutes from bulk water to the interior of the pore can be achieved without the net breakage of many hydrogen bonds. In the "empty" pore, it is reasonable to expect that the structures on the wall are rather strongly hydrogen-bonded to water molecules in the pore, thus making the water in the channel more structured or more strongly hydrogen-bonded than the bulk water. Penetration of hydrophobic molecules into the pore will break this structure, and thus may be energetically unfavourable.

In this connection, the apparently larger pores found in *Ps. aeruginosa* may play a significant role in facilitating diffusion of hydrophobic molecules (Hancock and Nikaido, 1978). It is known that *Ps. aeruginosa* can grow on a number of organic acids and alcohols (Stanier *et al.*, 1966), and even on paraffinic hydrocarbons and camphor in the presence of a suitable "degradative" plasmid (Chakrabarty *et al.*, 1973). Possibly a larger pore may permit passage of hydrophobic molecules enveloped in a "cage" of structured water molecules, or even of micelles. "Crypticity" measurements in *Haemophilus influenzae* containing R-plasmids suggest that the outer membrane of this species permits a much more efficient diffusion of β-lactams (Medeiros and O'Brien, 1975); it would be most interesting to determine the exclusion limit of pores in this organism.

The third factor is the electrical charge. In the experiments of Zimmermann and Rosselet (1977), the zwitterionic cephaloridine penetrated through outer membrane much faster than the mono-anionic cephalosporins of comparable hydrophobicity (Fig. 9). It seems possible that the pore walls contain a number of anionic groups; in this connection we recall the observation that porins are the most acidic proteins found in the outer membrane, both in *E. coli* (Rosenbusch, 1974; Schmitges and Henning, 1976) and *S. typhimurium* (G. F-L. Ames, personal communication). However, a significant part of the resistance against anionic solutes may come from the fact that the periplasmic space contains many negatively charged macromolecules, and there thus exists a Donnan equilibrium favouring exit of diffusible anions from the periplasmic space (Stock *et al.*, 1977).

(d). These considerations help us to understand the functions of porins in a physiological context. A very large number (about 10^5) of porins exist

in a single cell. If we consider cells growing in a medium containing a typical concentration (say 0.2%) of glucose, the porins are of course present in a large excess (perhaps 10^3-fold) over that which is needed for inflow of glucose at a rate needed for exponential growth at the doubling time of about 45 min (for calculation, see Bavoil *et al.*, 1977). But production of these large numbers of porins is not wasteful, because they are needed under two conditions. One is for diffusion of less permeable compounds. The Renkin equation predicts that diffusion rates of disaccharide will be about 9% of that of glucose. Hydrophobicity and electric charges, for example in some peptides that are a major source of nutrition for enteric bacteria in their natural habitat, will further lower the diffusion rates by several orders of magnitude. It thus seems that for the diffusion of certain classes of compounds the presence of 10^5 copies of porins is indeed necessary. Another important factor is the effect of the concentration of nutrients. We are so used to active-transport systems that it is often hard for us to understand the simple fact that the passive diffusion rate is *proportional* to the concentration difference across the membrane. Thus, although 10^5 copies of porins might seem an unnecessary excess in a medium containing 0.2% (roughly 10^{-2} *M*) glucose, all of these porins will become necessary if *E. coli* is to keep growing at its maximal rate in the presence of 10^{-5} *M* glucose. We all know that most of the active-transport systems of *E. coli* appear as though they have been designed to scavenge the last traces of nutrients from the environment. But without the larger number of porins, the diffusion through the outer membrane would quickly become limiting at low substrate concentrations and the high affinity of the active transport system would become a useless luxury.

In addition to the study in our laboratory (Bavoil *et al.*, 1977), Lutkenhaus (1977) has independently shown that porin-deficient mutants of *E. coli* B/r and K12 had increased "transport K_m" values for methionine, leucine, histidine, as well as glucose. Furthermore, porin-deficient mutants were resistant to several inhibitors, which therefore are *likely* to diffuse through porin channels in wild type cells. These compounds include Cu^{2+} (Lutkenhaus, 1977) and chloramphenicol, the latter because mutations (*cmlB*) known to confer a moderate resistance to chloramphenicol (Reeve and Doherty, 1968) were found to be probably identical with *tolF* (Foulds, 1976) causing the deficiency in porin 1a.

The discussions so far suggest that the permeability of the outer

membrane might be easily modified, especially for compounds showing marginal normal permeability. There exist two lines of evidence hinting at the possibility that some R-factors may alter the permeability of the outer membrane through alterations of the porins. Firstly, Rajul Iyer and her co-workers have made an exciting discovery that some R-factors belonging to the N compatibility group drastically reduce the level of the porin when introduced into *E. coli* B/r (Iyer, 1977; Iyer *et al.*, 1978). Although these workers favour the interpretation that pre-existing porin-deficient *mutants* acted as better recipients of these conjugative plasmids, we are more attracted to the possibility that "repression" of porin synthesis by the plasmid genome is involved, a process that may be useful in making the strain more antibiotic-resistant. Secondly, Curtis and Richmond (1974) found that introduction into *E. coli* of a mutant Pseudomonas R-factor RP-1, defective in β-lactamase synthesis, made the strain slightly more resistant to penicillins. Furthermore, when this plasmid was introduced into *E. coli* AS19, a hydrophobic antibiotic-supersensitive mutant whose outer membrane presumably possesses a diminished protein content as in the "deep rough" mutants discussed in Sections III.D and IV.C, the resistance toward hydrophobic agents was increased. These results suggest rather extensive changes in the protein make-up of the outer membrane, caused by the presence of the R-factor genome. In a superficially similar situation, a new protein appears in the cell envelope in very large numbers in cells containing tetracycline-resistant R-factors (Levy and McMurry, 1974). This protein, however, is a cytoplasmic membrane protein (S. B. Levy, personal communication).

Both *E. coli* K12 and *S. typhimurium* LT2 synthesize more than one species of "peptidoglycan-associated" major protein. In the case of *S. typhimurium* each of the three species ("36K", "35K", and "34K") has been purified from mutants isolated by Nurminen *et al.* (1976) and Smit and Nikaido (1978), and was shown to produce channels of indistinguishable exclusion limit in the reconstitution assay (Nakae and Ishii, 1978). In *E. coli* K12, the presence of either protein 1a or 1b confers on the mutants significant permeability to amino acids (Lutkenhaus, 1977; Pugsley and Schnaitman, 1978), and both are therefore porins. Lysogenization of K12 with temperate phage PA-2 (Schnaitman *et al.*, 1975) causes the appearance of a new peptidoglycan-associated protein, protein 2, which was recently shown to confer permeability to amino acids, carbohydrates and sulphate (Pugsley and Schnaitman, 1978), and thus should be classified as a porin.

Production of the various species of porin appears to be determined by a different set of physiological conditions. Similar parameters seem to control synthesis of each of the porins of *E. coli* and *S. typhimurium*, and these regulatory properties, as well as the genetics, suggest rather strongly the correspondence between each porin species in these two organisms (Table 4). In any case, these complex control mechanisms suggest different functions and properties for pores produced by each species of porin. The evidence so far obtained, however, is not clear-cut, and this is basically because all transport *rate* assays have to be done with intact cells of mutants, which often show compensating increases in remaining species of porins whenever one or more porins are absent. Nevertheless, the results strongly suggest that the "salt-repressible", 35K, porin produces the channel most efficient for diffusion of cephaloridine (Nikaido *et al.*, 1977b), and the similarly "salt-repressible" 1a porin of *E. coli* K12 again seems to make a better pore for the diffusion of another kind of charged molecule, *viz.* nucleotides (W. van Alphen *et al.*, 1978). The latter authors, however, unfortunately used only one substrate concentration for permeability assay, and further made the unwarranted assertion that protein 1b does not serve as porin for nucleotide diffusion. The effect of the mutational loss of 1b might have been obscured by increases in 1a, and the effect of the presence of 1b in a 1a-deficient background could have escaped detection because of the use of an unfavourable substrate concentration. The results of Lutkenhaus (1977) and of Pugsley and Reeves (1978) suggest that 1b makes rather efficient pores for diffusion of various amino acids.

It has been claimed that the "heat-modifiable protein" 3a produces pores (Manning *et al.*, 1977). This conclusion, however, is based on decreased transport rates of only two amino acids, tested at a single concentration, in a mutant lacking this protein. As we have emphasized earlier, the decrease in outer membrane permeability produces an increase in "apparent K_m" values of the overall transport process; thus, in order to detect any difference between the wild type and mutants, substrate concentrations in the range of the K_m, or lower, must be used (Fig. 7a). Although amino-acid transport in *E. coli* has K_m values usually around 1 μM or lower (Kaback and Hong, 1973; Oxender and Quay, 1975), Manning *et al.* (1977) used a very high substrate concentration of 500 μM and still found a large difference, a result not expected for mutants with diminished outer-membrane permeability. Since a large fraction of the mutant population was not viable, it seems much more

TABLE 4. Comparison of Porins of *Escherichia coli* and *Salmonella typhimurium*

Escherichia coli K12	*Salmonella typhimurium* LT2	Location of gene[a]	Physiological control of synthesis
1a		21	Repressed by salts (Nakamura and Mizushima, 1976; van Alphen and Lugtenberg, 1977)
	35K	21 (?)[b] (T. Sato, K. Ito and T. Yura, pers. comm.)	Repressed by salts (Smit and Nikaido, 1978)
1b		48	Production enhanced in Trypticase-Soy-Broth; decreased in Difco Nutrient Broth (Bassford *et al.*, 1977)
	36K	47 (P. H. Mäkalä, pers. comm.)	Decreased in Difco Nutrient Broth (P. Bavoil and H. Nikaido, unpublished)
2		Unknown (coded for by a prophage)	cAMP-dependent (Schnaitman, 1974; Schnaitman *et al.*, 1975)
	34K	34 (P. H. Mäkalä, pers. comm.)	cAMP-dependent (B. Rotman and G. F.-L. Ames, pers. comm.)

[a] Map location of *E. coli* genes is based on the 100 min map of Bachmann *et al.* (1976). In order to facilitate comparison between the two species, map locations of *S. typhimurium* genes were converted to a 100 "unit" map.

[b] Although a *Salmonella* porin gene is known to be located in this region, the identity of the porin is not known. We suspect that it is the 35K porin gene, because genes for other porins are located elsewhere.

plausible that the low transport rates simply reflect the low proportion of viable cells. We thus conclude that, so far, there is no reliable evidence that protein 3a forms pores. In *S. typhimurium*, the mutational loss of 33K protein, presumably corresponding to 3a, did not produce any change in the transmembrane diffusion rates of cephaloridine (Nikaido *et al.*, 1977b).

It was proposed, on the basis of the amino-acid sequence, that Braun lipoprotein may form oligomers with a central channel, which would then serve as transmembrane pores (Inouye, 1974). So far, no experimental evidence favouring this hypothesis has appeared. On the contrary, all efforts to reconstitute the channel-containing membrane by the use of Braun lipoprotein have been unsuccessful (Nakae, 1976 a, b). The hypothesis seems implausible also on theoretical grounds, as the Braun lipoprotein contains a large number of charged amino acid residues, in contrast to the sequenced, intramembranous regions of known intrinsic membrane proteins (Tomita and Marchesi, 1975; Ozols and Gerard, 1977; Nakashima and Konigsberg, 1974), from which charged amino-acid residues are completely absent. Recently, by using a mutant lacking the lipoprotein (Hirota *et al.*, 1977), we have shown that the absence of this protein did not produce any decrease in the hydrophilic permeability of the outer membrane, measured by the diffusion of 6-aminopenicillanic acid (Nikaido *et al.*, 1977a).

4. *Alternative Porins*

In porin-deficient mutants there exists a very strong selective pressure for the emergence and outgrowth of cells capable of producing alternative kinds of channels. Thus Bavoil *et al.* (1977) described "suppressor" mutants, which are capable of producing at least three kinds of proteins different from the wild-type porin, and are able to grow better at low concentrations of nutrients than the original, porin-deficient mutants. The "new" proteins appear to have molecular weights of 110K, 50K, or 35K, and the "50K" protein is identical with the λ-receptor protein (von Meyenburg and Nikaido, 1977). The appearance of approximately 36K protein, Ic, has been reported by Henning *et al.*, (1977), and similar observations were also made by W. van Alphen *et al.* (1978) as well as by Pugsley and Schnaitman (1978). On the basis of electrophoretic mobility these proteins seem similar to the "35K" protein of ours (Bavoil *et al.*, 1977). Foulds and Chai (1978), in contrast, found that some

pseudorevertants of porin-deficient *E. coli* K12 strains produced "protein E", which moved slightly more slowly than porin 1a on SDS polyacrylamide gel electrophoresis. Henning *et al.* (1977) showed that the gene controlling the appearance of "Ic" was not located near the known genes of porin synthesis. J. Foulds (personal communication) recently found that the mutations for his "protein E" and that for "Ic" are located near the *ilv* region of the chromosome, and are possibly alleles of the same gene.

E. SPECIFIC DIFFUSION MECHANISMS

The discovery that some outer-membrane proteins are involved in specific transport of nutrients was made by Bradbeer's group (White *et al.*, 1973), when they found that a vitamin B_{12}-binding protein, located in the outer membrane, is essential for transport of this substrate into the cytoplasm. This was rapidly followed by many other works describing various systems and defining their components (Table 1, p. 179).

We have observed in the previous section that passive diffusion through porin channels is likely to become limiting if the solute has a large size or if the external concentrations of the solutes normally encountered are quite low. We can thus expect that specific transport systems across the outer membrane will be needed in these cases, and it is gratifying to find that in all cases listed in Table 1 one or more of the above conditions is satisfied. Thus, iron-chelator complexes (ferrichrome, 740 daltons; Fe^{3+}-enterochelin, 746 daltons) as well as vitamin B_{12} (1,357 daltons) are very large and also exist in very low concentrations. Maltodextrin oligosaccharides are also large, and although maltose can diffuse through porin channels (Szmelcman *et al.*, 1976) such diffusion becomes limiting for disaccharides if the external concentration falls below 10^{-4} M or so (see Section IV.D.3.). Thus, for the proper functioning of the maltose transport system which has a very high affinity (K_{diss} of the periplasmic maltose binding protein is 2×10^{-9} M for maltotriose, Szmelcman *et al.*, 1976) at low substrate concentrations, the porin channels are clearly insufficient. Although the presence of a specific transport protein for nucleosides (Hantke, 1976) which are not so large (less than 300 daltons) may at first appear puzzling, the necessity for such a system becomes clear when one considers that the "overall transport K_m" value of the nucleoside transport system is very low (3 to 5×10^{-6} M) and that, more importantly, its V_{max} value is very high (Peterson *et al.*, 1967). Apparently

only a part (either the ribose residue or the amino-group) of the nucleoside molecule is rapidly utilized by the cell as the source of carbon or nitrogen (Koch, 1971), and this fact explains the observation that the V_{max} value is in the range expected for the transport of compounds serving as major carbon sources, such as glucose, and is much higher than expected for compounds serving merely as building blocks in nucleic acid synthesis. Thus in the case of nucleosides and maltose, diffusion through porin channels is sufficient at high external concentrations of these compounds, but specific systems become necessary at low external concentrations because of the low K_m and high V_{max} values of the active transport systems located in the cytoplasmic membrane (see also Fig. 7b).

The maltose transporter, or the "λ-receptor", transports not only maltose but also maltotriose and possibly higher oligosaccharides (Szmelcman *et al.*, 1976). Since this protein shares several properties with porin (Section III.C), it seems possible that it also acts essentially as a large pore with a discriminating mechanism in it. If so, the molecules of this protein have to be present in large numbers, comparable to porins, in order to allow rapid diffusion of the carbon source, maltose, at concentrations of the order of 10^{-6} *M*. Furthermore, the discriminating mechanism may allow structurally similar compounds occasionally to slip through the pore. Both of these predictions seem to be fulfilled, as λ-receptor is produced in large amounts in maltose-induced cells (Palva and Randall, 1978; Braun and Krieger-Brauer, 1977), and its presence allows diffusion, albeit very slow, of glucose and lactose (but not of histidine) across the outer membrane of porin-deficient mutants (von Meyenburg and Nikaido, 1977). Recently, it was also shown that λ-receptor forms ion-permeable channels in planar lipid films (W. Boos, personal communication).

The transport processes carried out by other specific transporter proteins may also involve pores, but little information is available on this point. Since the absolute amounts of substrates transported by some systems are small, we cannot exclude the involvement of the rotating carrier protein, either. In this connection, we note that binding of the substrate to the outer membrane transporter has been demonstrated with the vitamin B_{12}-*bfe* protein system (White *et al.*, 1973; Di Masi *et al.*, 1973), ferrichrome-*tonA* protein system (Wayne and Neilands, 1975; Luckey *et al.*, 1975; Hollifield and Neilands, 1978) either by direct binding assay or by competitive binding assay with phages and colicins

that bind to the same protein, but so far all efforts to demonstrate binding of maltose and maltodextrins to λ-receptor have failed.

An interesting finding is that several of these systems, including the ferrichrome-transporting *tonA* system, the ferric enterochelin-transporting *feuB* system and the vitamin B_{12}-transporting *bfe* system, all require the function(s) of another gene, *tonB* (Hantke and Braun, 1975b; Bassford *et al.*, 1976). The functioning of this gene is also required for irreversible adsorption of phages T1 and φ80 (Garen and Puck, 1951; Hancock and Braun, 1976b), as well as for killing of *E. coli* by colicins B, I, V, and M (Davies and Reeves, 1975). Cells of *E. coli* can take up iron through the ferric enterochelin transport system without *tonB* function, if the iron is introduced to the periplasmic space as a chelate with dihydroxybenzoic acid (Hancock *et al.*, 1977). Thus, *tonB* function seems to be necessary for "activating" the specific diffusion systems in the outer membrane, not for transport through the cytoplasmic membrane. Since the irreversible adsorption of T1 and φ80 also needs "energized cytoplasmic membrane", it has been suggested that the function of the *tonB* gene might be to couple the outer membrane proteins to the energized inner membrane (Hancock and Braun, 1976b). In any case, this system may provide valuable clues in our quest to more fully understand the dynamic aspects of outer membrane structure and function.

F. PENETRATION OF MACROMOLECULES

Under certain conditions, macromolecules can apparently cross the outer membrane barrier. Thus increased leakage of periplasmic enzymes into the surrounding medium has been reported in a large number of mutants, including the "deep rough" mutants with very incomplete LPS (Lindsay *et al.*, 1973), mutants lacking lipoprotein (Suzuki *et al.*, 1978) and mutants with defects of an unknown nature (Lopes *et al.*, 1972; Weigand and Rothfield, 1976). Many of these mutants are also more sensitive to lysozyme (Tamaki and Matsuhashi, 1973); their outer membrane must thus also allow inward penetration of macromolecules from the external medium.

The molecular mechanism of this "leakage" process is not presently understood. However, the most likely possibility is that it involves transient rupture and resealing of the membrane. If so, penetration by macromolecules will be enhanced by conditions favouring the

destabilization of membrane organization. This seems to be the case, as "deep rough" mutants produce membranes wherein normally strong interactions between LPS and "major proteins" (see Section III.D) are lacking, and the outer membrane of lipoprotein-deficient mutants does not get stabilized by anchoring to the peptidoglycan layer. Furthermore, destabilization of the outer membrane by removal of divalent cations enhances its "leakiness" even in wild type cells, as evidenced by the well known "osmotic shock" procedure (Nossal and Heppel, 1966) used to release periplasmic enzymes into the suspending medium. Finally, a major cause of the instability of outer membrane structure, viz. electrostatic repulsion between negatively charged groups, can be minimized by adding excess Mg^{2+} to the medium. This treatment is known to decrease the "leakage" in "deep rough" mutants (Lindsay *et al.*, 1973), lipoprotein-deficient mutants (Nikaido *et al.*, 1977a) and porin-deficient mutants (Nikaido *et al.*, 1977b).

In conclusion, it is important to keep in mind the *multiplicity* of diffusion pathways across the outer membrane. There seem to be at least three non-specific pathways (porin channels, hydrophobic pathway through membrane interior, and probably rupture-and-resealing) and at least several specific pathways. Use of vague words, such as "leaky" and "abnormally permeable", should be discouraged as much as possible, as they tend to give a false impression that there is only one mechanism of passage.

G. OTHER FUNCTIONS

Protein 3a was found to be necessary for conjugation, as *E. coli* mutants deficient in this protein could not act as recipients (Skurray *et al.*, 1974). Furthermore, purified 3a protein complexed with LPS (Schweizer *et al.*, 1978) inhibits conjugation when added to the medium (Schweizer and Henning, 1977; van Alphen *et al.*, 1977). According to Achtman and Skurray (1977), protein 3a is involved in the stabilization of the mating cell aggregate, and it is not the receptor for F-pilus.

Cells of *E. coli* containing F factor fail to form stable mating aggregates with cells of another male strain, owing to the function of the *traS* and *traT* gene (Achtman and Skurray, 1977). Interestingly, the product of the latter gene has recently been characterized as a 25,000 dalton outer-membrane protein (Achtman *et al.*, 1977). It is not yet known how the presence of this protein results in the prevention of aggregate stabilization. Apparently the protein is identical to the one

independently found by Minkley and Ippen-Ihler (1977), and erroneously identified as the product of *traS* gene.

The "major proteins" (1a, 1b and 3a) were once thought to determine the shape of *E. coli* cells (Henning *et al.*, 1973a), but it was later found that mutants lacking all three of the major proteins did not show any abnormality in terms of morphology (Henning and Haller, 1975). There is thus no reason to assume that the outer membrane or its protein plays any major role in the *morphogenesis* of bacterial cell. However, some lines of evidence suggest that the outer membrane helps to *maintain* the cell shape, once it is determined. For example, Feingold *et al.* (1968) showed that treatment of *E. coli* K12 with lysozyme resulted in extensive degradation of the peptidoglycan layer without alteration of cell shape. In this work, as well as in the work of Birdsell and Cota-Robles (1967), disruption of both the peptidoglycan layer and the outer membrane was shown to be necessary for transformation of rods into spherical forms, a result strongly suggesting that the outer membrane contributes to the rigidity of the cell wall. A similar conclusion was reached by Asbell and Eagon (1966), who showed that EDTA, which releases protein-LPS complexes from the outer membrane, converted *Ps. aeruginosa* into osmotically sensitive forms. Although degradation of peptidoglycan by autolytic enzymes cannot be ruled out in this experiment, the mechanical role of the outer membrane was further supported by the observation that incubation of these osmotically fragile forms in the presence of EDTA-released protein-LPS complex, under non-metabolizing conditions, made them once again osmotically resistant (Stinnett and Eagon, 1975).

Some workers proposed that Braun lipoprotein played a crucial role in cell division (Torti and Park, 1976; Weigand *et al.*, 1976; Fung *et al.*, 1978). The argument, however, appears less than convincing for the following reasons. (i) The mutation studied by Torti and Park (1976) affects not only the lipoprotein, but also a number of other membrane proteins (S. V. Torti, personal communication). (ii) The *lkyD* mutants described by Weigand *et al.* (1976) showed diminished covalent binding of lipoprotein to the peptidoglycan, and large bulges over the septum sites where the outer membrane was pulled apart from the peptidoglycan layer. So far, the authors have not considered the possibility that the latter phenomenon is the cause, rather than the result, of the former. (iii) A mutant strain containing a deletion mutation through the structural gene of the lipoprotein was found to divide in a normal manner (Hirota *et al.*, 1977).

H. THE STRUCTURE OF THE OUTER MEMBRANE REVISITED

The striking lack of permeability of the outer membrane of *E. coli* or *S. typhimurium* to hydrophobic compounds can be explained very well by the asymmetric construction of this membrane (Sections III.D and IV.C). Thus, the low fluidity of the LPS hydrocarbon chains, which constitute the outer half of the membrane, hinders the dissolution therein of hydrophobic molecules and their diffusion into the membrane interior. In addition, the presence of a high concentration of charged groups in the proximal region of LPS saccharide chain, and possibly the presence of the polysaccharide chain "layer" (see Section III.D), will further interfere with the diffusion process.

The asymmetric structure of the outer membrane in the enteric bacteria was deduced, as described in Section IV.C, mainly from the properties of "deep rough" mutants. There are other types of mutants that may also be useful in understanding the overall structure of the outer membrane. In *E. coli* K12, *envA* mutations are known to make the cells more sensitive to a number of hydrophobic agents (Boman *et al.*, 1971); the LPS content was found to be lower than normal in these mutants (S. Normark, personal communication), and the appearance of phospholipid bilayer regions in the outer membrane might explain the altered sensitivity. A more drastic decrease in LPS was observed in polymyxin-resistant strains of *Ps. aeruginosa*; the resistance may be related to the possible, accompanying decrease in the level of porin (H. E. Gilleland, Jr., personal communication). Alternatively, the resistance could be related to the strong affinity of polymyxin toward LPS (Bader and Teuber, 1973).

Another remarkable property of the outer membrane is the non-specific permeability toward small, hydrophilic substances. As we have seen in Section IV.D, for most substances this permeability can be explained almost completely by the presence of a special class of proteins, porins, that span the membrane. How are these proteins organized in the membrane? We have seen that the solubilized *E. coli* porins exist mostly as trimers even in SDS (Section III.D.3). They also seem to exist as trimers in the outer membrane. Thus, crosslinking studies with bifunctional reagents when undertaken with cell envelopes or outer membrane produced dimers and trimers of porin (Reithmeier and Bragg, 1977b). What was thought to be a "tetramer" in this study was later found to be another form of the trimer by Palva and Randall (1978), who also found

that, among higher oligomers, hexamers and nonamers are preponderant. Chopra *et al.* (1977) found that larger amounts of stable porin oligomers were produced in the presence of lysozyme. Our interpretation of this observation is that the basic protein (lysozyme) stabilizes the oligomer of acidic proteins, porins, by binding to it. Their results do not prove their conclusion that all porin oligomers are artefacts, a conclusion which is certainly untenable in view of the various studies already described.

Does each monomer of porin contain a channel, or does a channel get formed in the central space after the association of three subunits? A decisive answer is not available at present. We have not been able to reconstitute sucrose-permeable membranes by using monomeric porins (T. Nakae, unpublished results). On the other hand, some published results are at least consistent with the one-porin one-channel hypothesis. For example, Benz *et al.* (1978) calculate that the 1.7 nS increment in conductivity in 1 *M* KCl indicates the presence of a single pore, 0.93 nm in diameter. Superficially this seems to favour the hypothesis of one central channel per porin trimer. However, they did not take into account the fact that hydrated ions have diameters that are not negligible in comparison with the diameter of the pore. If we use the hydrated ionic radius of 0.24 nm, calculated from the hydration number (2) given by Bockris (1949) for both K^+ and Cl^-, and make corrections according to the Renkin equation, assuming a pore radius of 0.6 nm, we find that *three* pores of this radius will give a conductivity of 1.4 nS in 1 *M* KCl, which is in reasonable agreement with the observed value of 1.7 nS. Furthermore, although the most common incremental step corresponded to 1.7 nS, many increments of smaller magnitude were seen (Benz *et al.*, 1978). Possibly the latter were caused by insertion of porin monomers or dimers. More recently, Schindler and Rosenbusch (1978) have incorporated porins into planar lipid bilayers in the absence of detergents and organic solvents, and observed clearly that the smallest increment in conductance consisted of three equal steps. They furthermore made observations suggesting that the porin channels open and close in a hitherto unsuspected manner. Thus the channels were closed when the model bilayers were made, and the application of "initiation voltage" was needed to open them up. After the initiation, application of voltage exceeding 140mV resulted in the reversible closing of some of the channels and therefore the porin channels must be classified as voltage-gated channels.

The existence of the "triplet of pores" is also suggested by recent structural studies. When the cell envelope of *E. coli* B is extracted with SDS at room temperature, peptidoglycan sheets with hexagonally arranged oligomers of porin are left behind (Rosenbusch, 1974). Because of this regular arrangement, Steven *et al.* (1977) were able to use image-enhancement techniques in electron microscopy, and obtained the negatively stained picture schematically shown in Fig. 10. The proteins (porins) clearly exist as trimers, and a central triplet of holes is produced by each monomeric unit contributing a hole or indentation of approximately 2 nm in diameter. Although Steven *et al.* (1977) argued that these holes were probably occupied by lipoproteins, this appears unlikely because the wall of the holes then must be hydrophobic and would repel the hydrophilic particles of the negative stains. Ueki *et al.* (1979) examined the small-angle X-ray scattering pattern produced by oriented layers of intact outer membranes of *S. typhimurium*. Such a method is advantageous in that extraction artefacts can be avoided. The study revealed a series of in-plane reflexions that appeared to correspond to protein aggregates of about 11 nm in diameter. A most interesting feature was the presence of a central electron-transparent area or a "hole" of 5–6 nm in diameter. This could correspond to the single channel in the centre of the porin trimer. But it is also possible that the triplet of holes observed by Steven *et al.* (1977) appeared as one large hole in this low-resolution study. Detailed analyses of scattering patterns are

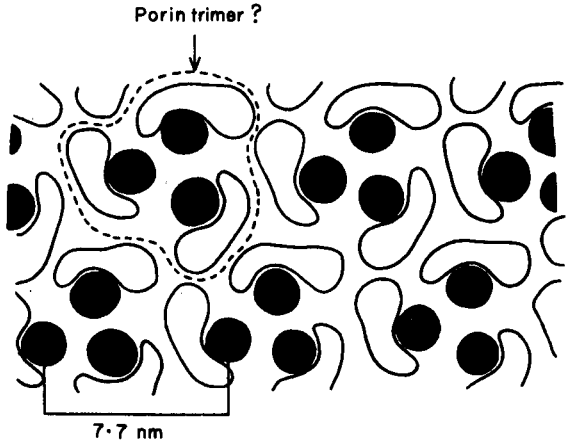

FIG. 10. Negatively stained image of the porin-lipoprotein-peptidoglycan complex, after image enhancement. After Steven *et al.* (1977).

underway, and could reveal basic features of the oligomeric assembly of the porins and other proteins.

The porin trimers in the outer membrane are probably associated with LPS, in view of the known affinity of porin for LPS (Yu and Mizushima, 1977). However, there is so far no decisive evidence that the trimer is strongly associated with any other protein, or that the "stoicheiometric aggregates" containing all major proteins, proposed some time ago (Henning *et al.*, 1973b), actually exist. Cross-linking studies (Palva and Randall, 1976, 1978; Reithmeier and Bragg, 1977b) consistently failed to cross-link porins to any other protein. DeMartini and Inouye (1978) observed that the porin molecules were more easily dissociated from the complex composed of porins, lipoproteins and peptidoglycan sheets, if the lipoprotein-to-peptidoglycan association was broken with trypsin, which did not cleave any bonds in the porin. This observation, which is a confirmation of earlier work (see Fig. 1 of Nakae, 1976b) led the authors to the conclusion that *in situ* the porins are associated with Braun lipoprotein molecules. Although it is still possible that such associations are artefacts caused by the coexistence of two hydrophobic substances in an aqueous environment, these results are at least consistent with the presence of some interaction between lipoprotein and porins.

What do we know about the arrangement of the other "major" proteins of 30–40,000 dalton range, protein 3a and "33K" protein? Cross-linking studies reveal that 3a is readily cross-linked to Braun lipoprotein (Palva and Randall, 1976), and that it forms higher oligomers. Thus it seems likely that 3a exists as an aggregate containing several copies both of itself and the Braun lipoprotein. Protein 3a shows a strong affinity for LPS, and the detailed study of this interaction suggests that it is associated with LPS in the intact outer membrane (Schweizer *et al.*, 1978).

In spite of our greater knowledge of the chemistry and biosynthesis of Braun lipoprotein, its precise location in the cell envelope is still unknown. For the reasons given in Section IV.D.3, it is difficult to imagine that lipoproteins penetrate deeply into the interior of the outer membrane. On the other hand, the regularity seen in the amino acid sequence of this protein (Braun, 1975), as well as its high α-helix content, certainly favours the idea that a few lipoprotein helices must be wound together, perhaps not exactly in the way originally proposed by Inouye (1974) but rather in the manner found, for example, in tropomyosin, i.e. without the central pore (Hodges *et al.*, 1972). With this structure,

however, bending would be difficult and the insertion of only the fatty-acid chains into the membrane as proposed by Braun and Bosch (1972) would place the outer membrane about 7 nm away from the peptidoglycan layer. Inouye (1974) considered that this situation was extremely unlikely, and rejected this model. Examination of electron micrographs of *E. coli*, however, suggests that it is not so implausible. Thus, according to De Petris (1967), the space between the peptidoglycan layer and the outer membrane is at least 5 nm. Murray *et al.* (1965) found that the distance from the inner surface of peptidoglycan to the outer border of the inner "dark line" of the outer membrane is about 10 nm, and thus the space between the peptidoglycan and the outer membrane would be about 6–7 nm. The arrangement that seems most likely at present is as shown in Fig. 11; obviously this is a speculative scheme and should not be taken as an established structure.

One technique for observing the distribution and organization of proteins in the plane of the membrane is freeze-fracture electron

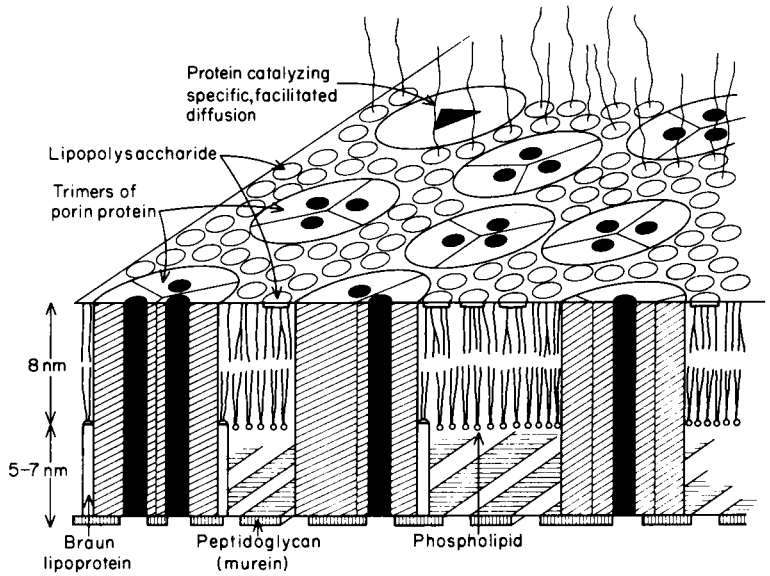

FIG. 11. A tentative structure of the cell wall of *Escherichia coli* and *Salmonella typhimurium*. Some features of this figure (e.g. the length of the polysaccharide chains of LPS) are not drawn to scale. Some features have not been established experimentally: these include the assumptions that each porin molecule produces a channel, that Braun lipoprotein is joined to the membrane only via its hydrocarbon chains, that Braun protein associates with porin, and that there is a considerable distance between the plane containing the phospholipid head groups and the peptidoglycan layer.

microscopy. The structure shown in Fig. 11 is expected to be quite resistant to freeze-fracture cleavage, because so many protein molecules traverse the membrane. Indeed, the outer membranes of wild-type *E. coli* or *S. typhimurium* are quite difficult to cleave, but those of "deep rough" LPS mutants are easily cleaved (Smit *et al.*, 1975; Irvin *et al.*, 1975; Bayer *et al.*, 1975), possibly because many fewer transmembrane proteins are present (see Section III.D). In any case, the freeze-fracture analysis showed a number of densely packed particles on the concave fracture face, and very few particles on the convex side (van Gool and Nanninga, 1971; Smit *et al.*, 1975). Smit *et al.* (1975) showed, by using deep rough mutants, that there was a proportionality between the decrease in total protein content of the outer membrane and the decrease in particle density. This observation suggests very strongly that at least the major component of the "particles" is protein. Which proteins do they contain? One μm^2 area of *S. typhimurium* outer membrane contained about 9000 particles (Smit *et al.*, 1975). The same area is expected to contain about 10,000 porin trimers, on the basis of Rosenbusch's data of 10^5 porin monomers per cell (Rosenbusch, 1974). Our recent determination on *S. typhimurium* gave a value also in this range (P. Bavoil and H. Nikaido, unpublished data). Thus, at least a major fraction of these particles should contain porins. This hypothesis also accords with the results of X-ray scattering experiments (Ueki *et al.*, 1979) which revealed the regular aggregate of protein with hole(s) in the centre, and with an overall diameter of about 10 nm; the "particles" seen by freeze-fracture also had a diameter of 8–10 nm (Smit *et al.*, 1975). But porins are not the only transmembrane protein in the outer membrane, and other proteins could either form a part of the porin-containing aggregate or form independent aggregates. In any case, this explains the failure of Verkleij *et al.* (1977) and of Schweizer *et al.* (1976) to observe a decrease in particle density in *E. coli* mutants mostly deficient in single species of porin. In these mutants there frequently are compensating increases in other proteins, and the reduction obviously cannot be expected. Verkleij *et al.* (1977), however, claimed that their results indicated that the particles were composed, not of proteins, but of LPS "micelles". We cannot imagine that inverse micelles (hydrophobic part outside) could even be made of LPS, with its very large hydrophilic segment. In their most recent work, L. van Alphen *et al.* (1978) showed that treatment with Ca^{2+} of a mutant of *E. coli* lacking all of the major proteins of the outer membrane could induce formation of numerous particles on the concave fracture face. But they

concluded that particles of this type "probably do not occur in substantial amounts in wild type cells", and now concur with our original conclusion (Smit *et al.*, 1975) that the majority of such particles are composed mainly of proteins. Their additional claim that each particle contains protein-LPS complexes seems reasonable in view of the known affinity of some outer-membrane proteins for LPS (see p. 224), and in view of the large amount of work performed with *Ps. aeruginosa*, in which system identification of freeze-fracture particles as LPS-protein complexes was established beyond much doubt (Rogers *et al.*, 1969; Gilleland *et al.*, 1973; Stinnett *et al.*, 1973); none of their own data, however, directly supports this hypothesis.

Finally, it should be emphasized that details of the freeze-fracture morphology often show unpredictable alterations in some mutants (Nurminen *et al.*, 1976; Lounatmaa and Nurminen, 1977). Although we consider the predominantly proteinaceous nature of "particles" as essentially established, these interesting morphological variations obviously require further study.

We thought earlier (Smit *et al.*, 1975) that the presence of most "intramembranous particles" on the concave fracture face meant the localization of major part of the outer membrane proteins in the outer half of the membrane. But an equally plausible explanation is that in the outer half the proteins are associated with each other, either directly or indirectly via LPS, and the lack of such strong sidewise association in the inner half produces the asymmetric partition seen upon freeze-fracture.

The hypothetical structure in Fig. 11 explains most of what we know at present of the structure and function of the outer membrane. It should be emphasized, however, that this model structure is simply a first approximation, and therefore cannot be expected to satisfactorily explain all the details of outer membrane physiology. For example, certain mutations make cells of *S. typhimurium* more sensitive to anionic detergents, such as SDS, cholate, or deoxycholate, but not to other hydrophobic agents such as crystal violet and erythromycin (Mäkelä *et al.*, 1976). Similar, specific enhancement of deoxycholate sensitivity is seen in many colicin-tolerant mutants, as summarized by Holland (1975). Sodium cholate, but not other anionic detergents, was found to be more active on *E. coli* strains carrying R plasmids in an integrated state than on those containing autonomous plasmids (Yoshida *et al.*, 1978). Subtle changes like these cannot be explained by such a wholesale alteration of the outer membrane structure as the one found in "deep

rough" mutants (see Sections III.D and IV.C) It is hoped that studies on these subtle alterations of outer-membrane functions will give us more insight into its detailed structure.

V. Assembly of the Outer Membrane

A. BIOSYNTHESIS OF LIPOPOLYSACCHARIDES

Since this topic has been reviewed in detail (Wright and Kanegasaki, 1971; Osborn and Rothfield, 1971; Nikaido, 1973), only a brief outline will be given here.

Biosynthesis of lipopolysaccharide begins at its hydrophobic end, lipid A. Despite many attempts, the enzymic reactions involved in formation of the glucosamine disaccharide backbone as well as in the addition of 3-hydroxytetradecanoic acid residues to the backbone have not been characterized, although particulate fractions from *Ps. aeruginosa* were shown to incorporate ^{14}C-labelled 3-hydroxydodecanoic acid residues into lipopolysaccharides when supplied with [1-^{14}C] dodecanoic acid and CoA (Humphreys *et al.*, 1972). More recently, some later steps in lipid A synthesis have been elucidated by Osborn's group. They first isolated a temperature-sensitive mutant in the enzyme 2-oxo-3-deoxyoctanoate(KDO)-8-phosphate synthetase; this mutant cannot produce enough KDO at 37°C due to an increased K_m value for one of its substrates (Rick and Osborn, 1972, 1977). The mutant accumulated a lipopolysaccharide precursor, which in addition to the absence of KDO residues and all other components of the "saccharide" portion, also lacked dodecanoic and tetradecanoic acid residues (Rick *et al.*, 1977). Thus, after transfer of two 3-hydroxytetradecanoic acid residues to the amino groups of the backbone and of one such residue to one hydroxyl group, the next reaction is apparently transfer of KDO residues, a reaction that could be demonstrated *in vitro* by using the "precursor" molecule as the acceptor (Munson *et al.*, 1978). Apparently, transfer of dodecanoic and tetradecanoic acid residues takes place after transfer of KDO, but the former reactions have not been demonstrated *in vitro*. A temperature-sensitive mutant accumulating similar precursors, yet apparently unaltered in KDO 8-phosphate synthetase, has also been isolated (Lehmann *et al.*, 1977).

The core portion of LPS is synthesized by the sequential and direct

transfer of sugars from nucleoside diphosphate sugars (Osborn and Rothfield, 1971). A few of the transferases have been purified, and the reaction seems to proceed only when both LPS and the enzyme molecules become embedded in a common bilayer of phospholipids.

Biosynthesis of the O chain portions has been studied in most detail in *S. typhimurium* and *S. anatum*. In these organisms, the repeating unit oligosaccharide is built up on a C_{55} polyisoprenol (undecaprenol) pyrophosphate carrier. The repeating units are then polymerized by transfer of a growing chain onto the non-reducing end of the repeating unit monomer linked to the carrier lipid, and finally the polymeric O chain is transferred to the core LPS. In some cases, glucosyl branches are attached to the O chain; this is accomplished by transfer of glucose to a lipid carrier to form undecaprenol monophosphate glucose, which then acts as a direct donor of glucose.

Biosynthesis of O side chains in the species described are controlled by genes in the *rfb* gene cluster (65 min on the *S. typhimurium* genetic map; Sanderson, 1972) as well as by *rfc* gene(s) determining the polymerase activity, approximately 50 min on the map. In contrast, biosynthesis of O side chains in Group C_1 and group L *Salmonella* does not require the function of gene(s) located at 50 min, and instead requires the function of *rfe* gene(s) located 124 min on the genetic map (Mäkelä *et al.*, 1970). The *rfe* gene product does not seem to be involved in the polymerization of repeating units, as the same wild-type allele of this gene functions in the biosynthesis of side chains of widely different composition, for example the O chain of *S. montevideo* containing mannose, *N*-acetylglucosamine, and glucose; the O chain of *E. coli* O9 containing mannose and isopropylidene ribose (Schmidt *et al.*, 1976); the T_1-chain found in various *Salmonella* strains containing galactofuranose and ribofuranose residues (P. H. Mäkelä, personal communication); and "enterobacterial common antigen" (Mäkelä and Mayer, 1974), present in all species of the Enterobacteriaceae, and containing *N*-acetylglucosamine and *N*-acetylmannosaminuronic acid (Mäkelä and Mayer, 1976). It is possible that the *rfe* gene specifies a modified undecaprenol, or a carrier molecule of a totally different type. The *in vitro* synthesis of *S. montevideo* O chain (J. Gmeiner and H. Nikaido, unpublished results) and of *E. coli* O9 O chain (Kopmann and Jann, 1975) is not inhibited by bacitracin, which blocks cyclic utilization of undecaprenol pyrophosphate carrier (Stone and Strominger, 1971). Genes of the *rff* cluster (located close to the *ilv* genes) are necessary for common antigen synthesis (Mäkelä *et al.*, 1976); analysis

of *rff* mutants showed that the cluster contains genes for the two enzymes needed for conversion of UDP-*N*-acetylglucosamine into UDP-*N*-acetylmannosaminuronic acid (Lew *et al.*, 1978), through the pathway described earlier by Kawamura *et al.* (1975).

All of the known glycosyl transferases involved in LPS biosynthesis are located in the cytoplasmic membrane (Osborn *et al.*, 1972b). However, most of the important topological questions in LPS biosynthesis remain to be solved. For example, some workers have proposed that the role of the lipid carrier might be to transport the oligosaccharide-lipid complex from the inner to the outer surface of the cytoplasmic membrane (Wright and Kanegasaki, 1971; Nikaido, 1973). If so, the repeating unit polymerase should be located on the outer surface of the cytoplasmic membrane, whereas the enzymes involved in building up the repeating units should be found on the inner surface. Furthermore, the hypothesis predicts that the O chain linked to the carrier should be located on the outer surface of the cytoplasmic membrane. Although the finding that an O chain-specific phage could adsorb to sphaeroplasts of core-defective mutants of *S. typhimurium*, which accumulates the O-chain-carrier complex (Kent and Osborn, 1968), is consistent with the latter prediction, more studies are needed as it is difficult to rule out the possibility of artifactual rearrangement of membrane components under some experimental conditions.

Physiological regulation of LPS synthesis, as well as the way in which biosynthesis of the various parts of the LPS are coordinated, is another area where practically no information is available. Although it has been claimed that the absence of enzymes of core synthesis interferes with the synthesis, or integration into the membrane of the first enzyme of O chain synthesis (Rundell and Shuster, 1975), this conclusion is unwarranted, because at least one of the strains used was a double mutant with a known mutation in the latter enzyme (Wilkinson and Stocker, 1968).

Recently, an interesting effect of growth temperature on LPS synthesis was described by McConnell and Wright (1979). When *S. anatum* strains were grown at a suboptimal temperature, for example 20°C, the LPS that was synthesized contained many fewer, though longer than normal O side chains. This alteration exposed significant portions of R core structure on the cell surface, and made the cells susceptible to R-specific phages. If this temperature effect is a general phenomenon, it may explain why sewage material very often contains R-specific phages, in spite of the rarity of R mutants in Nature.

B. BIOSYNTHESIS OF PROTEINS

Rapid progress has been made during the last few years in this area, mainly owing to the efforts of M. Inouye and his co-workers. They first established a toluenized *E. coli* system which synthesizes only membrane proteins (Halegoua *et al.*, 1976). When the Braun lipoprotein was synthesized in this system, the product behaved as though it was larger than the lipoprotein, and amino-acid analysis, as well as the sequencing of carboxyl-terminal residues, suggested the existence of about 20 extra residues at the amino-terminal end of the protein (Halegoua *et al.*, 1977). Inouye's group has also been able to isolate the specific messenger RNA for lipoprotein (Hirashima *et al.*, 1974), and cell-free protein synthesis with this RNA as the template also produced the apparently identical prolipoprotein. These results are in perfect agreement with the "signal" hypothesis of Blobel and Dobberstein (1975), who proposed that secreted proteins are synthesized with an extra amino-terminal sequence that guides the protein to its destination across the membrane and is split off during the transport process. The 20-residue, extra segment at the amino-terminal end has been analysed and found to consist of a long sequence of mostly hydrophobic amino acids, preceded by a short amino-terminal region containing two lysine residues at a close interval (Inouye *et al.*, 1977b). The messenger RNA is about 120 nucleotides longer than expected from the size of the prolipoprotein (Takeishi *et al.*, 1976). The first 89 nucleotides of this RNA have been sequenced (Pirtle *et al.*, 1978) in a study that revealed the presence of a non-coding sequence of 38 nucleotides including the expected stretch thought to interact with 16S ribosomal RNA (Steitz and Jakes, 1975) as well as a nine nucleotide segment immediately preceding the initiation codon which was identical with the initiation sequence found in brome mosaic virus RNA 4 (Dasgupta *et al.*, 1975).

Lipoprotein is not the only outer membrane protein that is synthesized in a precursor form, with the "leader" sequence. Thus by slightly modifying the toluenized cell technique, Sekizawa *et al.* (1977) could show that both the porin(s) and protein 3a are synthesized in precursor forms, which appear to contain about 20 extra amino-acid residues. *In vitro* completion of protein synthesis on polysomes bound to the cytoplasmic membrane resulted in production of larger precursors of an outer-membrane protein, λ-receptor, and two periplasmic proteins, maltose-binding protein and arabinose-binding protein (Randall and

Hardy, 1977; Randall *et al.*, 1978). The extra sequence of the arabinose-binding protein was found to be located at the amino-terminal end (Hardy and Randall, 1978). Evidence for larger precursors has also been obtained for a periplasmic enzyme, viz. alkaline phosphatase (Inouye and Beckwith, 1977).

Genes for two outer-membrane proteins have been cloned. Gayda and Markovitz (1978) cloned a fragment that specifies synthesis of protein 3b in minicells. Achtman *et al.* (1977) cloned a gene derived from the F-factor. This gene, *traT*, produced in an *in vitro* system a protein normally found in the outer membrane of F-factor-containing *E. coli*. Most interestingly, the *in vitro* and the *in vivo* products behaved identically on SDS-polyacrylamide gel electrophoresis, in apparent contradiction to the Blobel-Dobberstein hypothesis.

Silhavy *et al.* (1977) developed an ingenious test for the "signal" hypothesis. They showed, by fusing the genes, that some of the hybrid proteins containing the amino-terminal portion of λ-receptor (an outer-membrane protein) and the carboxyl-terminal portion of β-galactosidase (a cytoplasmic protein) became located in the outer membrane. Further analysis is expected to pinpoint the sequence necessary for the transport information.

The precursor proteins with "leader" sequences must obviously become modified by proteolytic cleavage, but reactions causing "maturation" of outer-membrane proteins have not been demonstrated in cell-free systems. However, in biosynthesis of a periplasmic protein, alkaline phosphatase, the larger precursor, synthesized in a cell-free system, was processed to form the protein of the mature size by incubation with the outer, but not inner, membranes (H. Inouye and Beckwith, 1977). In an interesting study, C. H. MacGregor (personal communication) purified a protease from the outer membrane of *E. coli*, by using the proteolytic release of the nitrate reductase complex from the cytoplasmic membrane of the same organism; the role of this enzyme in maturation of outer membrane or periplasmic proteins remains to be established. The lipoprotein, in addition to cleavage, must be modified by addition of a fatty-acyl residue to the terminal amino-group and of a diglyceride group to the thiol group of a cysteine residue. The presence of cyclopropane fatty-acyl residues in the diglyceride moiety (Lin and Wu, 1976) and the results of pulse-chase studies (Schulman and Kennedy, 1977) suggested that the diglyceride group is derived from phospholipids. A more recent study by Chattopadhyay and Wu (1977), however,

indicates that the glycerol moiety is derived from the non-acylated glycerol residue of phosphatidylglycerol. In this connection, a mutant that was originally thought to produce lipid-deficient lipoprotein (Suzuki *et al.*, 1976) turned out to contain an amino-acid substitution (Inouye *et al.*, 1977a), together with another mutation that allowed attachment of the glycerol residue to the amino-terminal cysteine but not further transfer of the two ester-linked fatty acids (Rotering and Braun, 1977). The nature of the latter mutation is obviously consistent with the biosynthetic pathway proposed by Chattopadhyay and Wu (1977). Another mutant producing a lipoprotein lacking the entire diglyceride group is known; the altered lipoprotein apparently cannot become bound to the peptidoglycan layer (Wu *et al.*, 1977).

One-third of the lipoprotein that is synthesized finally becomes covalently linked to the peptidoglycan. After pulse-chase treatment, about 40% of the labelled lipoprotein is found to have been chased into the bound form after one generation, but, interestingly, the percentage does not increase even after a longer chase (Inouye *et al.*, 1972). This observation suggests either that there is an equilibrium between the bound and free forms because of the reversibility of the binding reaction, or that the lipoproteins become compartmentalized.

Since many outer-membrane proteins are subject to post-translational clipping (see p. 231), protease inhibitors are expected to interfere with this process of proteolytic cleavage. Ito (1977, 1978) treated growing *E. coli* with such inhibitors, and observed inhibition of incorporation of various outer-membrane proteins. For example, antipain, an inhibitor produced by an actinomycete, inhibits specifically synthesis (or incorporation) of porins in *E. coli* K12 without affecting the rate of overall protein synthesis. However, accumulation of higher molecular weight "precursors" was never observed, and the nature of the inhibited biosynthetic step remains to be determined.

C. ASSEMBLY AND GROWTH OF THE OUTER MEMBRANE

1. *Translocation from the Cytoplasmic Membrane*

Since all known outer-membrane components are synthesized in or on the cytoplasmic membrane, the major problem in assembly of the outer membrane is that of translocation or export from the sites of synthesis. The mechanism is perhaps most approachable in the case of

phospholipids, which appear to move rapidly and reversibly between the outer and inner membranes (Jones and Osborn, 1977a, b). Since there is no evidence of discrimination on the basis of lipid structure, it is supposed that transport occurs via zones of adhesion between the outer and inner membranes. A possible alternative pathway, however, was described recently (D. R. Leuking, L. K. Cohen and S. Kaplan, personal communication). These workers found protein fractions from *Rhodopseudomonas sphaeroides* and from *E. coli* capable of catalysing phospholipid exchange. The physiological function of such proteins in inner-to-outer membrane translocation remains to be studied.

Translocation of LPS has been studied by Leive and her associates. Such a study was made possible by the existence of *galE* mutants of *E. coli* and *S. typhimurium*. These mutants produced very defective LPS when grown in the absence of galactose, but immediately began to produce complete LPS upon addition of galactose (Fukasawa and Nikaido, 1961). Leive took advantage of the fact that regions of the outer membrane containing the complete LPS are of higher density than regions containing incomplete LPS. Thus, when a *galE* mutant of *E. coli* O 111 was grown in the absence of galactose, and outer membrane fragments were isolated after a short pulse of galactose, fragments of higher density could be isolated; a result suggesting that newly made LPS enters the outer membrane at a small number of discrete sites (Kulpa and Leive, 1972, 1976).

Mühlradt and his coworkers used a similar galactose-induced switching of LPS structure but detected the newly made "complete" LPS with ferritin-labelled antibody. Thus when a *galE* mutant of *S. typhimurium* was pulsed with galactose and then labelled with ferritin-conjugated antibody that binds to the complete LPS but not to the "old", incomplete LPS, newly made LPS was found to appear at discrete sites on the surface of outer membrane (Mühlradt *et al.*, 1973). Several hundred such sites were found in a single cell, and electron microscopy of thin sections revealed that these sites corresponded to the sites of fusion between outer and inner membrane, previously discovered by Bayer (1968) as the preferential sites of attachment of bacteriophages.

In contrast to the translocation of phospholipids, export of LPS is an irreversible process. This was established by Osborn *et al.* (1972b) with another conditional LPS synthesis mutant. They first grew this mutant, lacking phosphomannoisomerase, in the presence of [^{3}H]-glucose, which labelled the incomplete LPS produced under these conditions. Most of

the incomplete LPS was exported to the outer membrane. The cells were now shifted to a [^{14}C]-mannose-containing medium, in which complete LPS labelled with [^{14}C]-mannose was synthesized. Analysis of these complete LPS molecules showed that they did not contain significant amounts of ^{3}H, thus ruling out the possibility that the LPS "comes back" from the outer membrane to be re-used as the substrate for further elongation at the surface of the inner membrane. Such irreversible transport may of course be due to the presence of a discriminating mechanism at the site of export. However, an easier explanation (M. J. Osborn, personal communication) might be that the LPS, once exported, is retained in the outer membrane because LPS becomes associated strongly with the proteins of the outer membrane (Yu and Mizushima, 1977; Schweizer *et al.*, 1978). That the LPS does not move from the outer to the inner membrane has been confirmed by introducing labelled LPS and the KDO-deficient "LPS precursor" (Section V. A, p. 228) into the outer membrane of intact cells and then examining the inner membrane for their presence (Jones and Osborn, 1977b).

The kinetics of insertion of newly made proteins was examined by the pulse-labelling technique (Ito *et al.*, 1977). At 20°C, a near-complete "chase" of label into the inner-membrane proteins took less than 1 min, whereas that into the outer-membrane proteins needed 3 min. Integration of porins required an exceptionally long time. It is also interesting that the "chase" into periplasmic proteins required 10 min, a result which does not support the idea that outer-membrane proteins go through the periplasmic space on their way to their final destination.

The mode of translocation of a major outer-membrane protein, 35K porin, was studied in *S. typhimurium* (Smit and Nikaido, 1978). The culture was grown in the presence of sodium chloride so as completely to repress synthesis of this porin, and soon after the shift into a medium lacking sodium chloride the cells were labelled with ferritin-labelled antibody specific for the porin. The newly made porins were found to become exported at numerous but discrete sites, corresponding to the fusion sites between the outer and inner membrane. Thus, translocation of porin seems to take place by a mechanism very similar to that of LPS.

Since proteins of the outer membrane are completely distinct from those of the inner membrane, export through fusion sites requires a precise mechanism of discrimination, but the nature of such a mechanism is unknown at present. In this connection, L-forms of *Proteus mirabilis* that are covered by a single cytoplasmic membrane were found to contain, in

the membrane, some LPS but *no* outer-membrane proteins (Gmeiner *et al.*, 1976). Obviously, incorporation of pore-forming proteins would have been lethal, but it is not known how L-forms avoid this.

If *E. coli* is infected with phage λ after a brief induction of the λ-receptor with maltose, the phages are seen mainly over the region of the division septum (Ryter *et al.*, 1975). It appears that λ-receptor is synthesized only during a short period in the cell cycle just before cell division. Similar cell-cycle-dependent synthesis (or insertion) of outer-membrane components has been reported: (a) for a 76–80,000 dalton protein (Churchward and Holland, 1976) which was originally thought to be a DNA-initiation protein (Gudas *et al.*, 1976) but later turned out to be an iron-chelator transporter (Boyd and Holland, 1977); and (b) for the "free form" lipoprotein (James and Gudas, 1976). In the latter case, however, the rate of incorporation merely doubled or tripled during the cell cycle and the observed effect may simply be the result of the increased gene dosage due to synchronous replication of the lipoprotein structural gene. It should be added that synthesis and insertion of porin continue to occur all through the cycle (Churchward and Holland, 1976).

2. *Growth of the Outer Membrane*

Many workers have tried to elucidate the mode of growth, and to locate the zone of growth, of the outer membrane. Some studies used phospholipids as markers (Green and Schaechter, 1972; Tsukagoshi *et al.*, 1971) and the conclusions derived from them do not seem very convincing today in view of the "fluid" nature of membrane phospholipids and reversible exchange between the outer- and inner-membrane phospholipids (Jones and Osborn, 1977b). In contrast, Begg and Donachie (1977) examined the behaviour of a presumably much less mobile marker, namely the T6 receptor protein, and concluded that the "framework" or the matrix of the outer membrane is added exclusively at the pole (or end) of the cell. However, our results (Smit and Nikaido, 1978) show clearly that the major component of such a supposedly immobile framework, the porin, is inserted at discrete sites that are distributed more or less uniformly over the entire cell surface. Close

examination of Begg and Donachie's work reveals that the behaviour of their marker protein showed unexpected anomalies, which make their conclusion less than convincing (Smit and Nikaido, 1978). We therefore believe that the predominant mode of outer-membrane growth is by insertion at fusion sites that are found all over the cell surface.

Earlier studies with fluorescent antibody specific for LPS showed that there were no distinct growth zones in the outer membrane of *E. coli* and Salmonella species (Cole, 1964; Beachey and Cole, 1966). These studies have often been criticized on the grounds that LPS molecules floating in the fluid membrane would rapidly diffuse away from the sites of insertion and thus produce the appearance of diffuse intercalation. The lateral diffusion rate of LPS, however, is quite slow, and from the value given by Mühlradt *et al.* (1974) we can calculate that the LPS molecules will need about 10 h in order to travel a distance of 1 μm. This slow rate is caused probably by the low fluidity of LPS hydrocarbon chains (Nikaido *et al.*, 1977) and possibly by the presence of a very high concentration of proteins in the membrane (see Section III.D). In any case, this finding shows that one can use LPS as a more or less "immobile" marker, and validates the conclusion of earlier workers on the absence of discrete growth zones. In fact, Mühlradt *et al.* (1973), who with the ferritin antibody technique were able to get a much finer resolution than the previous workers both in terms of time and space, showed clearly that LPS appeared at discrete sites distributed evenly all over the cell surface.

It thus seems certain that the outer membrane grows predominantly by addition of new material through the "fusion sites", and that there are no distinct "growth zones" (polar or septal) for this membrane. However, the situation may be different for some non-porin proteins. We have seen that λ-receptor, which seems to be synthesized only during a short period preceding cell division, is added predominantly to the septal region of the membrane (Ryter *et al.*, 1975). It seems possible that the iron-chelator transporters, that are also synthesized during the same period (Churchward and Holland, 1976; Boyd and Holland, 1977), share the same mode of insertion; this point, however, remains to be established. It is not known why there are two different modes of insertion of outer-membrane components. It may be pertinent, however, to realize that newly synthesized peptidoglycan also seems to be added in *E. coli* in two different ways, i.e. by diffuse intercalation as well as by accumulation in septal zones (Schwarz *et al.*, 1975). Furthermore, newly made peptidoglycan at the septum seems to

be slightly different from that in the cylindrical region of the cell (Mirelman *et al.*, 1976). It is tempting to speculate that the different modes of insertion of the outer-membrane proteins may be related to their different ways of interaction with the peptidoglycan layer, but no information is at present available on this point.

3. *Re-arrangement and Stablization of Component and the Maintenance of the Outer Membrane Structure*

As we have seen in Section III.D, the outer membrane, or at least the outer membrane of members of the Enterobacteriaceae, has features that could make its structure unusually unstable. Perhaps because of this inherent thermodynamic instability, the outer-membrane components seem to undergo various modification reactions which tend to stabilize membrane organization. This point is most clearly seen in the covalent linking of Braun lipoprotein to the underlying peptidoglycan. Since the Braun lipoprotein is firmly integrated into the outer membrane through its three fatty-acyl chains, the linkage to peptidoglycan "pulls down" the outer membrane onto a mechanically strong supportive layer. We can see the significance of this stabilization process in lipoprotein-deficient mutants, as already described in Section III.D. As we have already seen (Section III.C), porins associate tightly with the peptidoglycan. The ferritin-antibody labelling studies of newly made porins suggest that the porin molecules become "fixed" soon after their insertion into the outer membrane, and do not show significant lateral diffusion (Smit and Nikaido, 1978); this could be a consequence of interaction with neighbouring molecules and/or association with the peptidoglycan layer. In this connection, Diedrich and Schnaitman (1978) have made the most interesting discovery that some of the lysine residues in the porin molecules are oxidized into α-aminoadipic acid semi-aldehyde (allysine). This reaction was demonstrated in a cell-free system by Mirelman and Siegel (1979). This is a well known post-translational modification in mammalian structural proteins such as collagen and elastin (Gallop *et al.*, 1972). Possibly these aldehyde groups are involved in intramolecular as well as intermolecular crosslinking for the stabilization of porin oligomer; evidence indicating the presence of Schiff base structures between lysine and allysine has been obtained (Mirelman and Siegel, 1979).

Lipopolysaccharide can also go through a spatial rearrangement. Leive (1977) has indeed shown that wild-type LPS molecules with long O

side chains tend to cluster together after insertion into the outer membrane. If this is due to the interaction between the polysaccharide chains, it could also have some stabilizing effect on the structure of the outer membrane. Possibly LPS could also become crosslinked covalently. Although pyrophosphate cross-bridges appear to be absent (Mühlradt *et al.*, 1977), the published molecular weights of LPS (Romeo *et al.*, 1970) are difficult to understand unless some covalent crosslinks are present between the monomeric units. Again this could be viewed as a part of the "stabilization" reaction.

Finally, the inherent thermodynamic instability of the structure means that the organization of the outer membrane can be altered drastically by very small amounts of perturbation. This idea might be able to explain a number of observations that have been puzzling up to the moment. Thus, the binding of colicins and phages to "receptors" present in the outer membrane suddenly triggers a series of complex reactions, culminating sometimes in the apparent penetration of colicin molecules into cytoplasm (Holland, 1975) or in the "movement" of phages to the fusion region (Tomita *et al.*, 1977) followed by injection of phage nucleic acid (Bayer, 1975). The triggering is difficult to understand if we consider the outer membrane as a stable, static structure, but not so incomprehensible if we adopt the ideas presented above. An interesting observation perhaps relevant to the idea described above has been reported (Nieva-Gomez *et al.*, 1976; Nieva-Gomez and Gennis, 1977). These workers found that the binding of a number of hydrophobic fluorescent probes to intact *E. coli* cells is strongly affected by the energy state of the cell. Perhaps one might speculate that energy is continuously used in intact cells in order to maintain the thermodynamically unfavourable structure of the outer membrane, a structure nevertheless necessary for the survival of the organisms in their natural habitat; this aspect of outer membrane structure and functions remains a topic for future investigation.

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Physiology of *Neisseria gonorrhoeae*

STEPHEN A. MORSE, ANTHONY F. CACCIAPUOTI and PAUL G. LYSKO

Department of Microbiology and Immunology, University of Oregon Health Sciences Center, Portland, Oregon 97201, U.S.A.

I. Introduction 252
II. Taxonomy and ultrastructure 253
III. Colonial types of *Neisseria gonorrhoeae*. 254
IV. Cell division and growth patterns 258
V. The cell envelope 260
A. Capsule 260
B. Outer membrane 261
C. Peptidoglycan composition 264
D. Pili 265
E. Lipopolysaccharide 266
VI. Peptidoglycan turnover and synthesis 268
VII. Autolysis 268
A. Whole-cell studies 268
B. Mechanism of autolysis 269
C. Characteristics of the autolysin 272
D. *In vivo* significance 274
VIII. Carbon dioxide requirement 274
IX. Carbohydrate metabolism 276
A. General 276
B. Glucose transport 276
C. Glucose metabolism 277
D. Pentose synthesis 282
E. Lactate and pyruvate metabolism 283
F. Acetate metabolism 284
X. Terminal electron transport 286
XI. Inorganic nitrogen and amino-acid metabolism 290
A. Inorganic nitrogen metabolism 290
B. Amino-acid metabolism 290
C. Miscellaneous enzymes 293

XII. Nucleotide precursors 294
A. Pyrimidines 294
B. Purines 296
XIII. Lipid composition and metabolism 299
A. Phospholipids and whole-cell fatty acids 299
B. Lipase and phospholipase activity 305
C. Inhibitory activity of phospholipids and fatty acids on growth . . . 307
D. Lipid A 309
E. Steroids 310
XIV. Inorganic ions 311
XV. Acknowledgements 313
References 314

I. Introduction

Gonorrhoea is one of the most commonly reported communicable diseases. The incidence of gonorrhoea has reached epidemic proportions in several areas of the World. In the United States alone, one million new cases were reported in 1977 (Center for Disease Control, 1977). With the recent appearance of antibiotic-resistant strains of *Neisseria gonorrhoeae*, which are thought to be the result of an intergeneric conjugal transfer (Elwell *et al.*, 1977), we have been forewarned of the possible loss of efficacy of drug treatment in control of this disease. The development of a vaccine must follow a basic understanding of human immunity to gonococcal infection, for, unlike some diseases, gonorrhoea has no one virulence factor against which one could be easily immunized. The multifactorial nature of virulence and the mechanisms of pathogenesis can be examined through an understanding of the basic biology of the gonococcus. The more we unravel the physiology of this organism, the better equipped we shall become to deal with problems of a clinical nature.

This review is a summary of the important aspects of gonococcal physiology that relate directly or indirectly to the disease process. Certain aspects of the physiology and metabolism may be as yet unrelated to pathogenesis, but they provide a base from which to plan future research. We have also attempted to show differences and similarities between the physiology of *N. gonorrhoeae* and other pathogenic and non-pathogenic *Neisseria* spp., where this information is available and pertinent. Knowledge of the physiology of related species may help to delineate subject areas of gonococcal physiology which require additional effort. Those readers who are interested in other aspects of gonococcal biology

are referred to the following references: Morton, 1977; Roberts, 1977; Morse, 1978; Brooks *et al.*, 1978.

II. Taxonomy and Ultrastructure

Members of the family Neisseriaceae are spherical organisms which occur either in pairs or masses with the adjacent sides flattened or are rod-shaped organisms occurring in pairs or short chains (Reyn, 1974). These organisms are non-flagellated although some demonstrate twitching motility resulting from the presence of pili (Swanson, 1977a,b; Bøvre *et al.*, 1970). All species are Gram-negative by staining, and possess a typical Gram-negative cell envelope. The family Neisseriaceae was extensively reorganized with publication of the 8th edition of Bergey's Manual in 1974 (Reyn, 1974). At present, the four genera, *Neisseria*, *Branhamella*, *Moraxella* and *Acinetobacter* comprise the family Neisseriaceae. Species of *Neisseria*, *Branhamella* and *Moraxella* are parasitic, and are commonly found on the moist mucous membranes of man and of other mammals (Griffiss and Artenstein, 1976; Berger and Husmann, 1972; Bøvre, 1970; Henriksen, 1973; Vedros *et al.*, 1973). Species of *Acinetobacter* have been temporarily associated with this family and are saprophytic or opportunistic pathogenic organisms. All members of this family, with the exception of *Acinetobacter* sp., are oxidase positive.

Only two species, *N. gonorrhoeae* and *N. meningitidis*, are normally pathogenic for humans. These species can be readily differentiated from the non-pathogenic species (Reyn, 1974). The presumptive identification of *N. gonorrhoeae* is based upon the appearance of the organism in Gram-stained smears, typical colonial morphology and a positive oxidase test. Confirmatory identification is based upon production of acid from glucose and not other carbohydrates, or by staining with fluorescein-labelled antibodies.

Thin sections of *N. gonorrhoeae* grown *in vitro* exhibit cell structures in electron micrographs similar to those of other Gram-negative bacteria. An undulating outer membrane, approximately 7.5 to 8.5 nm in thickness, appears as a bilayered structure (Morse *et al.*, 1977; Fitz-James, 1964; Swanson *et al.*, 1971). The periplasmic space, the space between the cytoplasmic membrane and the outer membrane, contains a thin, electron-dense layer, approximately 6.0 nm in diameter, which corresponds to the peptidoglycan layer of the bacterial cell envelope (Swanson *et al.*, 1971). The peptidoglycan layer and outer membrane

appear to adhere to each other at regular intervals around the periphery of the cell (Fitz-James, 1964; Wolf-Watz *et al.*, 1975). Membrane-bound vesicles resembling mesosomes have been seen in the cytoplasm of some cells proximal to the site of septum formation (Morse, 1976; Fitz-James, 1964; Murray *et al.*, 1963).

Cell-wall blebs produced by budding of the outer membrane have been seen both in log phase broth- or agar-grown cultures of *N. meningitidis* (Devoe and Gilchrist, 1973). The blebs were found to consist of outer membrane components including lipopolysaccharide, and were formed only by rapidly growing cells since no blebs were seen on stationary-phase cells. Membrane blebs were also present on the cell surface of *N. gonorrhoeae* (Morse, 1976). The blebs were approximately 70 nm in diameter and were similar in size to those reported for *N. meningitidis* (Devoe and Gilchrist, 1973). Scanning electron microscopy of gonococcal colonies has revealed the presence of spherical bodies which range in diameter from 20 to 60 nm and which exist either free or in association with the cell surface of organisms from all colony types (Kraus and Glassman, 1974). The spherical bodies are least numerous on type 4 gonococci.

III. Colonial Types of *Neisseria gonorrhoeae*

As early as 1904, differences were noted in the colonial appearance of *N. gonorrhoeae* (Lipschutz, 1904). This initial report led to further observations regarding the size, colour, elevation, opacity and consistency of gonococcal colonies which were summarized by Hill (1948). More recently, Kellogg *et al.* (1963, 1968) reported an apparent association between virulence and colonial morphology. Four morphologically distinct colonial types were initially described and designated types 1, 2, 3 and 4 (T1, T2, T3, T4). Jephcott and Reyn (1971) identified a fifth colonial type (T5). Another colonial type, designated T1′, has recently been described by Chan and Wiseman (1975). The differences in the gross morphology of colonial types 1, 2, 3 and 4 have been reported by Kellogg *et al.* (1963). Scanning electron microscopy (Elmros *et al.*, 1975) has distinguished five different gonococcal colony types analogous to light-microscopic observations. Excellent critical-point drying of the colonies allowed superior observations of colonial morphology. Intercellular filamentous strands were observed among the virulent colonial types, and were rare among the avirulent derivatives. Such strands were present in both normal and

microcolonies, and were thought to be the reason why virulent gonococci formed colonies with characteristic rounded, raised margins and highly convex upper surfaces. Since treatment with trypsin, Pronase, deoxyribonuclease, β-glucuronidase, α-amylase or hyaluronidase did not affect the morphology of the strands, the authors suggested that they were not aggregated pili, but may have been elongated protrusions of biochemically complex cell-wall material.

Colonial types 1 and 2 predominated in isolates from the urethra of males (Kellogg *et al.*, 1963, 1968), the endocervix of females (Sparling and Yobs, 1967) and from the rectum (Kovalchik and Kraus, 1972). Types 1 and 2 colonial morphology could be maintained by selective subculture at daily intervals whereas, during non-selective transfer, the predominant colonial type was rapidly replaced by another type. Most often, these shifts were from the virulent colonial types (T1 and T2) to the avirulent colonial types (T3 and T4). Chan and Wiseman (1975) reported that the typical patterns for colonial variation which they observed were: T1→T4; T2→T3; T3→T4; and T′→T5. The presence of antimicrobial agents in the medium caused profound changes in colonial morphology so as to make colony typing difficult (James *et al.*, 1973).

Dark- and light-coloured colonial variants of *N. gonorrhoeae* have been observed. Diena *et al.* (1974) reported that the dark variants of colonial types 1 and 2 reverted only to colonial type 3, whereas the light variants of these colonial types reverted only to colonial type 4. Swanson (1977a,b, 1978) investigated the properties of these colouration variants. Colour differences were determined using the diffusing substage reflector of a dissecting microscope; differences in opacity were determined by using the smooth, planar, polished side of the substage mirror. In general, most dark colonies were opaque (*Op*), while light-coloured colonies were transparent (*Tr*). These optical properties were independent of piliation, and were apparently related to the degree of cellular aggregation within a colony. Electron microscopy demonstrated zones of adhesion between neighbouring gonococci from *Op* colonies, but not from *Tr* colonies. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis of gonococcal outer membrane proteins indicated that *Op* colony forms possessed two proteins, with molecular weights of approximately 26,000 and 28,000, which were not found in *Tr* colony forms. Treatment of opaque forms with trypsin resulted in a loss of these cell-envelope protein bands. Opaque colony types grown on solid or in liquid medium were more susceptible to growth inhibition by trypsin than were *Tr* colony

types. When outer membrane proteins were radiolabelled with ^{125}I and then exposed to trypsin, the loss of label from *Op* colony types was roughly twice that of *Tr* colony types. Treatment with trypsin decreased the opacity of *Op* colony forms, and twitching motility of small clumps of organisms was noted along the periphery of the colonies. Without trypsin treatment, twitching motility in young *Op* colony forms was difficult to detect. Twitching motility of *Tr* colony forms was readily observed without prior treatment with trypsin, and occurred only among piliated gonococci, regardless of opacity type. Swanson concluded that these data suggested that differences in colony colour, opacity and trypsin sensitivity were due to cell-wall surface proteins or glycoproteins not associated with pili.

James and Swanson (1978) found that gonococci cultured from male urethrae and those from female cervices differed with regard to colony opacity and sensitivity to trypsin. Isolates from males consisted of predominantly *Op* colony forms and were trypsin sensitive, while colony forms isolated from females were predominantly *Tr* and were trypsin resistant. The occurrence of trypsin-resistant organisms from females was markedly increased around the time of menstruation, suggesting that selective forces in the cervix, particularly the presence of proteolytic enzymes, may be responsible for the differences observed. The implication of these cyclical changes are of great importance to the study of gonococcal pathogenesis and the immune response to gonorrhoea.

It has yet to be firmly established whether the shift in colonial morphology and its attendant properties are due to a genetic change or to phenotypic variation. Kellogg *et al.* (1968) implied that the variation in colonial type was a result of genetic change. Mayer *et al.* (1974) demonstrated that loss of virulence and transition of colony type did not result from irreversible loss of a plasmid. However, it was not ascertained whether these properties were influenced by the state of plasmid integration into the chromosomal DNA. Tyeryar *et al.* (1974) studied the reversion of avirulent gonococci propagated in mammalian cell cultures to phenotypically virulent cells. In addition to colonial morphology, the criteria used for classification as type 1 included the presence of pili and the ability to undergo DNA-mediated transformation. Their results demonstrated that the type 4 to type 1 reversion was an infrequent event, and was a strain-related phenomenon. However, reversion was favoured by an acid pH value and was apparently a true phase transition and not just a population shift.

Hafiz *et al.* (1977) reported that colonial type 4 gonococci reverted to type 1 organisms when incubated under static conditions and an atmosphere of 10% carbon dioxide in a complex liquid medium supplemented with 0.1% (w/v) ferric citrate. Under these conditions, cultures remained viable for several weeks. The lack of information concerning piliation and transformability of the type 1 revertants indicated that further confirmation was required.

The development of chemically defined (Catlin, 1973; LaScolea and Young, 1974) and complex (Hart and Goldberg, 1975; Hafiz *et al.*, 1977) media which promote colonial-type stability and rapid, simple methods for distinguishing gonococcal colonial types (Juni and Heym, 1977) will greatly aid studies concerned with the physiology, metabolism, antigenic analyses and pathogenicity of *N. gonorrhoeae*. Both the physical and chemical environment have an important role in maintaining the colonial type of the inoculum. Jephcott (1972) noted that carbon dioxide concentration markedly affected the proportion of type 1 organisms in the population. Maximum stability was obtained with an initial carbon dioxide concentration in air of 10%. More recently, Chan *et al.* (1975) showed the importance of maintaining a constant atmospheric concentration of carbon dioxide. Growth in an atmosphere of 16% carbon dioxide maintained an inoculum of colonial type 1 organisms as type 1 throughout 24 hours of growth. These results are in agreement with the earlier observations of Kellogg *et al.* (1963) that higher carbon dioxide tensions favoured growth of colonial type 1 and 2 organisms on solid medium. The molecular basis for the stabilizing effect of high concentrations of carbon dioxide is not known at the present time.

Growth temperature was also important in maintaining the colonial type. Jephcott (1972) observed that incubation at suboptimal temperatures (30° C) afforded the least variation of colonial types from an initial inoculum of type 2 organisms.

Lim *et al.* (1977) studied macromolecular synthesis and incorporation of radioactive compounds into specific cellular components of colonial types 1 and 4 gonococci during growth in a chemically defined medium. In this medium, cultures of type 4 organisms had a shorter lag period than those of type 1 organisms. The authors suggested that type 4 cells could utilize nutrients more efficiently than type 1 cells, and that the two colonial types may be unique in growth and metabolism. The pattern of incorporation of labelled compounds was similar for both colonial types. By selecting the appropriate labelled compound, it was possible to obtain

cells with a high proportion of the label in specific macromolecules, e.g. DNA, RNA, protein. Undoubtedly, further studies need to be conducted with a greater number of strains.

IV. Cell Division and Growth Patterns

Cell division in *N. gonorrhoeae* occurred by septation rather than by constriction (Westling-Haggstrom *et al.*, 1977) and was initiated by ingrowth of the cytoplasmic membrane enclosing a fold of peptidoglycan. Jyssum (1972, 1973a,b) showed that initiation of cell division in *N. meningitidis* was dependent upon replication of a particular region or locus on the chromosome. Protein synthesis was also required for replication. Evidence for the presence of both partial and complete septa in *N. gonorrhoeae* was presented by Westling-Haggstrom *et al.*, (1977). Addition of mecillinam to dividing gonococci either induced formation of one sphaeroplast or two individual sphaeroplasts, one lysing before the other. In the former case, a communicating cytoplasm was probably present whereas, in the latter, a complete septum must exist. Complete septa were seen rather infrequently. Annular structures perpendicular to the plane of new septum formation have been observed on the surface of dividing cells of *N. meningitidis* (Devoe and Gilchrist, 1974). If the peptidoglycan septum of *N. gonorrhoeae* was synthesized as a series of concentric rings, then thin sections through the equatorial plane of the cell would rarely show a completed septum, while thin sections above or below the equatorial plane would show complete septa more frequently. These latter sections can often be recognized by the unequal size of the cells in a single diplococcus.

Division planes in gonococci are formed consecutively at right angles to each other resulting in a transient tetrad form (Murray *et al.*, 1963; Westling-Haggstrom, 1977; Catlin, 1975), a pattern also observed in other *Neisseria* species such as *N. meningitidis* and *N. pharyngitis* (Westling-Haggstrom *et al.*, 1977). By following growth of individual gonococci, Westling-Haggstrom *et al.* (1977) determined that the cell expanded in only one dimension throughout virtually the entire cell cycle. However, expansion in two dimensions was observed in some cells towards the end of the division period. Initiation of growth in the second dimension, i.e. the former width, began before completion of the first septum. The rate of growth in each dimension appeared to be linear. Using penicillin

treatment, additional evidence was obtained showing that the active areas of peptidoglycan synthesis were restricted to the septal regions. In some cells, areas of weakened cell wall occurred perpendicular to each other suggesting that these organisms had begun a second septum before completion of the first. It has further been suggested (Westling-Haggstrom *et al.*, 1977) that the change in growth direction observed in *N. gonorrhoeae* enabled the organism to avoid a rod-sphere-rod transition, which would occur if growth were unidirectional. It was postulated that such a transition would cause large oscillations in the volume-to-mass ratio, resulting in large changes in internal pressure.

Penicillin G is known to induce filament formation in Gram-negative rods and has been used to differentiate coccobacilli from cocci (Catlin, 1975). Addition of penicillin G (Westling-Haggstrom *et al.*, 1977; Catlin, 1975) or nalidixic acid (Westling-Haggstrom *et al.*, 1977) did not induce filament formation in *N. gonorrhoeae*. Nevertheless, Westling-Haggstrom *et al.* (1977) proposed that *N. gonorrhoeae* should be regarded as a short rod which always extends parallel to the longitudinal axis and which never undergoes a rod-sphere-rod transition.

Strains of gonococci have different growth patterns in liquid medium. In some strains, growth is characterized by a homogenous (i.e. smooth) suspension, whereas in others it is characterized by various degrees of clumping. The growth pattern is related to the colony type of the organism (Swanson *et al.*, 1971). Type 1 organisms exhibit slight or moderate clumping, while type 4 organisms do not exhibit clumping. There is no correlation between the relative extent of piliation and the degree of clumping. Swanson *et al.* (1971) observed that clumping was mediated through adhesion zones between the outer membranes of adjacent cells. A colloidal suspension of alkaline lanthanum nitrate was used to estimate the intercellular space of the adhesion zones as 20 nm. They further postulated that zones of adhesion represented specialized regions of stickiness on the gonococcal wall. Zones of adhesion were seen less frequently in broth-grown cells than in agar-grown cells, suggesting that this was a growth rate-related phenomenon.

Temperature is an environmental parameter which can markedly influence growth of *N. gonorrhoeae*. Previous results (Morse, 1976) indicated that the gonococcus has a maximum growth temperature of 41° C, a minimum of approximately 25° C, and an optimum of 38.5° C. The optimum temperature varied from strain to strain, and ranges from about 36° to 39° C. Temperature-sensitive mutants of *N. gonorrhoeae*, capable of

growth at 30° C but not 37° C, have been isolated following mutagenesis with nitrosoguanidine (Wharton and Zubrzycki, 1976).

Morse and Hebeler (1978) studied the effect of pH value on growth and composition of *N. gonorrhoeae* in glucose-containing medium over a pH range of 6.0 to 8.0 The optimum pH value for *N. gonorrhoeae* was between pH 7.0 and 7.5. However, the total hexose content of gonococci was 56% higher in cells grown on agar plates at pH 6.3 than in those grown at pH 7.2 or 8.0. This difference was related to alterations in glucose metabolism and is discussed in Section IX. C. (p. 277).

Studies on the biochemistry and antigenic composition of *N. gonorrhoeae* will require large quantities of bacteria grown under reproducible conditions, such as in continuous culture. Brookes and Sikyta (1967) studied growth of gonococci in continuous culture at pH values ranging from 6.3 to 7.2 in a complex medium containing excess glucose. These investigators did not determine conclusively which medium constituent was the growth-limiting factor, but amino-acid analyses indicated that lysine was exhausted at all dilution rates tested; threonine and methionine were exhausted at a dilution rate of 0.08 h^{-1}, but their concentrations increased with increasing dilution rate. At constant pH value, the highest cell yields at all dilution rates tested (0.08–0.46 h^{-1}) were obtained at pH 6.75. Maximum yield (1.12 mg dry wt/ml) was obtained with a dilution rate of 0.26 h^{-1}. Significant decreases in cell concentration were observed with other pH values at dilution rates outside the range 0.20–0.25 h^{-1}. At all pH values studied, there was an increase in acetic acid production with decreasing dilution rate which corresponded to an increase in glucose consumption.

V. The Cell Envelope

A. CAPSULE

Capsules are produced by each serogroup of *N. meningitidis* which are chemically and immunologically distinct from those of other serogroups. The meningococcal capsules have been characterized as polysaccharide polymers made up of one or two sugars (Liu *et al.*, 1971a,b; Bundle *et al.*, 1973, 1974; Bhattacharjee *et al.*, 1974, 1976). The presence of a capsule on *N. meningitidis* suggested that a capsule might also be found on *N. gonorrhoeae*. Several investigators have reported the presence of gonococcal capsules (Hendley *et al.*, 1977; James and Swanson, 1977;

Richardson and Sadoff, 1977; Demarco de Hormaeche *et al.*, 1978). Capsules have been observed on gonococci of both virulent and avirulent colonial types. James and Swanson (1977) observed capsules most easily on recent clinical isolates, but they were also evident on laboratory strains. *In vivo* passage in guinea-pig chambers enhanced capsule production (Demarco de Hormaeche *et al.*, 1978). Hendley *et al.* (1977) reported capsule production during the exponential growth phase of gonococci grown in medium containing acid-hydrolysed casein. The presence of "viridans streptococci" was shown by Richardson and Sadoff (1977) to stimulate capsule production by *N. gonorrhoeae*, *N. sicca* and *N. subflava*. Viridans streptococci excrete lactic and acetic acids when growing on a glucose-containing agar medium (Donoghue and Tyler, 1975). Accumulation of these compounds produces a marked decrease in the pH value of the medium. Growth at an acid pH value (pH 6.0) was shown to increase the total carbohydrate content of *N. gonorrhoeae* (Morse and Hebeler, 1978). The chemical structure of the gonococcal capsule has not yet been determined. However, James and Swanson (1977) showed that the capsule was resistant to trypsin, chymotrypsin, lysozyme, hyaluronidase, neuraminidase and glucuronidase.

B. OUTER MEMBRANE

Negatively stained preparations of the gonococcal outer membrane revealed the presence of hexagonal and ring-like structures (Swanson *et al.*, 1971; Novotny *et al.*, 1975; Swanson, 1972). These ring-like structures may be related to the pits or holes seen in freeze-fractured, freeze-etched preparations (Swanson, 1972). Novotny *et al.* (1975) observed that the rings had an average internal diameter of 6.7 nm and an outer diameter of 11.7 nm. The mean distance between the ring structures, when they were distributed in a regular pattern, was 26 nm. These workers have also isolated the hexagonal structures free from their matrix. The inner radius of the hexagons was calculated to be 3.3 to 4.0 nm. Hexagonal structures with a diameter of 0.8 nm were observed by Swanson (1972).

Johnston and Gotschlich (1974) isolated the gonococcal outer membrane, which was composed of lipopolysaccharide, phospholipids and protein, by means of isopycnic centrifugation of osmotically ruptured sphaeroplasts formed after treatment of cells with EDTA and lysozyme. The majority of the outer membrane protein consisted of three proteins having apparent molecular weights, as determined by SDS-polyacryla-

mide gel electrophoresis, of 34,500, 22,000 and 11,500. The protein with a molecular weight of 34,500 accounted for over 60% of the total outer membrane protein, and was termed the principal outer-membrane protein. These investigators were unable to resolve the 34,500 protein into subunits, but felt that it had immunological significance due to its quantity, location on the outer membrane and apparent electrophoretic homogeneity. Later studies (Johnson *et al.*, 1976) ascertained that the apparent molecular weight of the principal outer-membrane protein was strain dependent and ranged from 32,000 to 39,000.

Swanson (1977b) reported that the major determinant in gonococcal-neutrophil interactions was a protein on the gonococcal surface that had an apparent molecular weight of 28,000. This "leucocyte-association" (LA) factor was found in both dark and light colony types (see Section III, p. 255).

Recently, Hildebrandt *et al.* (1978) transformed resistance to the complement-dependent bactericidal activity of normal human serum into a serum-sensitive strain. Transformation to serum resistance was accompanied by simultaneous structural and antigenic changes in the principal outer-membrane protein. The apparent molecular weight of the principal outer-membrane protein in the recipient strain was decreased from 39,500 to 36,500 after transformation. The antigenicity, as measured by an enzyme-linked immuno-absorbent assay, also converted to that of the donor strain. These results suggested that serum resistance in gonococci was mediated, at least in part, through the principal outer-membrane protein.

Wolf-Watz *et al.* (1975) compared the outer-membrane composition of penicillin-sensitive and -resistant strains of *N. gonorrhoeae* by means of an isolation procedure originally developed for use with *E. coli* (Wolf-Watz *et al.*, 1973). The only apparent differences in the outer membranes were the distribution of the fatty-acyl residues in lipid A and phospholipids. The penicillin-resistant strain contained a higher relative content of lauryl and myristyl residues and a lower content of *cis*-vaccenyl residues. These differences may be related to increased non-specific antibiotic resistance. Differences in outer-membrane components were also noted between cells grown *in vivo* and *in vitro*. Electron microscopy indicated that *in vivo* grown organisms possessed a more electron-dense cell envelope (Arko *et al.*, 1976; Penn *et al.*, 1976) and a specific envelope-associated protein which was not detected in the same strain grown *in vitro* (Penn *et al.*, 1976).

Heckels and Everson (1978) reported the presence of a major outer-membrane protein (mol. wt 60,000) which was lost during repeated subculture. The iso-electric point of this protein was 5.0, consistent with its position in the outer membrane since the surface pI value of whole gonococci (5.3) was similar (Heckels *et al.*, 1976).

Wolf-Watz *et al.* (1975) suggested that the outer membrane of *N. gonorrhoeae* was more permeable than that of *E. coli* and other members of the family Enterobacteriaceae. This hypothesis was in agreement with their findings and those of others (Guymon and Sparling, 1975; Miller and Morse, 1977) that *N. gonorrhoeae* demonstrated an exceedingly high uptake and cytoplasmic concentration of crystal violet. This dye did not penetrate the cell envelope of wild-type *E. coli* K12 (Wolf-Watz *et al.*, 1976) or *Salmonella typhimurium* DB21 (Miller and Morse, 1977); the bound dye was associated primarily with the cell-envelope fraction. Wolf-Watz *et al.* (1976) compared the cell envelope of *N. gonorrhoeae* with that of *E. coli*. The main difference between their outer membranes appeared to reside in the proteins. Whereas outer-membrane proteins of *N. gonorrhoeae* were easily extracted by chaotropic agents, those of *E. coli* were not. The outer membrane of *E. coli* was extracted only by detergents, suggesting that the outer-membrane proteins of this organism were considerably more hydrophobic than the corresponding proteins of *N. gonorrhoeae*. The authors postulated that the passage of charged molecules, such as crystal violet, may be facilitated by the presence of hydrophilic proteins in the outer membrane of *N. gonorrhoeae*.

There is other evidence indicating that gonococcal and meningococcal outer membranes are normally more permeable than those of most other wild-type Gram-negative bacteria. The gonococcus and meningococcus bind, and are sensitive to growth inhibition by, certain gonadal steroids (Morse and Fitzgerald, 1974; Miller and Morse, 1977) and long-chain free fatty acids (Miller *et al.*, 1977). Concentrations of progesterone which markedly inhibited growth of *N. gonorrhoeae*, *N. meningitidis* and *N. flavescens* only moderately inhibited growth of *N. subflava*, *N. perflava* and *N. flava*, and had no effect upon growth of *N. mucosa*. Other Gram-negative bacteria tested were apparently insensitive to growth inhibition by progesterone.

Inhibition of reduced nicotinamide adenine dinucleotide (NADH) oxidase activity in gonococcal membranes exposed to progesterone suggested that electron transport may be inhibited (Morse and Fitzgerald, 1974). Further studies indicated that the cytoplasmic

membrane bound four times more progesterone than did the outer membrane (Miller and Morse, 1977), suggesting that the outer membrane allowed free transport of progesterone to the inner membrane.

C. PEPTIDOGLYCAN COMPOSITION

Hebeler and Young (1976a) showed that the peptidoglycan of *N. gonorrhoeae* from cells grown at neutral pH values comprised from 1 to 2% of the cellular dry weight. No significant variations were observed between colony types. The peptidoglycan of *N. perflava* represented 0.4% of the dry weight of the cell (Martin *et al.*, 1973). Hebler and Young (1975) and Wolf-Watz *et al.* (1975) found that the peptidoglycan of *N. gonorrhoeae* consisted of residues of muramic acid, glutamic acid, alanine *meso*-diaminopimelic acid and glucosamine in approximate molar ratios of 1:1:2:1:1, respectively. The average chain length of the glycan backbone was between 80 and 110 disaccharide units (Hebeler and Young, 1976a). Hebeler and Young (1976a) observed that lysozyme, but not lysostaphin, completely solubilized the gonococcal peptidoglycan. Residues of amino acids and aminosugars comprised more than 96% of the total weight of the peptidoglycan of cells grown at neutral pH values; trace amounts of residues of aspartic acid, threonine and glycine were also present (Hebeler and Young, 1976a). Wolf-Watz *et al.* (1975) also reported trace amounts of glycine and aspartic acid residues in the peptidoglycan. Under the growth conditions studied, neither group of investigators was able to detect covalently-attached lipoprotein in the peptidoglycan. The loose association of the outer membrane to the peptidoglycan, as observed in electron micrographs of gonococci (Wolf-Watz *et al.*, 1975), was thought to be related to the absence of linkage molecules like lipoprotein between these cell envelope structures. Recently, Hebeler *et al.* (1978) observed that the dry weight of the peptidoglycan fraction increased markedly in several strains of *N. gonorrhoeae* grown at pH 6.0 and comprised between 4 and 18% of the total dry weight. The increased dry weight of the peptidoglycan fraction was due to an increase in the amount of peptidoglycan, coupled with the presence of peptidoglycan-associated protein(s) (Table 1.). The protein(s) produced at acid pH values may be involved in anchoring the outer membrane to the peptidoglycan.

TABLE 1. Effect of growth pH values on the apparent peptidoglycan content of *Neisseria gonorrhoeae*. From Hebeler *et al.* (1978)

Strain	Peptidoglycan (percent of dry weight) pH 8.0	pH 7.2	pH 6.0
RUG 40	0.9 (9.5%)	1.0 (9.1%)	8.0 (42.2%)
F62	ND	1.4	13.4
JW-31	1.9	2.5	11.4
RUG 50	ND	1.1	4.5
CS-7	ND	9.2	18.5

Values in parentheses represent percentage of protein associated with peptidoglycan. ND indicates that the value was not determined.

D. PILI

Pili are present on both pathogenic and non-pathogenic *Neisseria* spp. (Jephcott *et al.*, 1971; Swanson *et al.*, 1971; Weistreich and Baker, 1971; Devoe and Gilchrist, 1974; McGee *et al.*, 1977). Short pili (175–210 nm in length) were seen only on non-pathogenic species, whereas long pili (up to 4,300 nm) were seen on both non-pathogenic and pathogenic species (McGee *et al.*, 1977). These researchers also suggested that it was unlikely that pili were responsible for the characteristic colonial morphology of *N. gonorrhoeae*.

Several functions for pili have been proposed to explain the greater virulence of piliated gonococci. There is a considerable amount of evidence that gonococcal pili play an important role in attachment of the gonococcus to host cells (Buchanan, 1977). Gonococci attach to many different cell types, but the most extensive attachment was to cells which were histologically most similar to the sites of gonococcal infection (Pearce and Buchanan, 1978).

Payne and Finkelstein (1975) observed that the ability to acquire iron *in vivo* was a significant factor in gonococcal virulence. They found that piliated gonococci were able to acquire sufficient iron *in vivo*, and suggested that pili might be one of the factors responsible for the binding of this element. Subsequently, Buchanan (1977) reported that purified gonococcal pili were able to bind ^{59}Fe, and that 0.1 *M* solutions of Fe^{2+} and Fe^{3+} salts (pH 7.2) produced a marked aggregation of pili.

The presence of pili was also associated with genetic transformation of *N. gonorrhoeae* to streptomycin resistance (Sparling, 1966). Acceptable transformation frequencies were obtained only with the piliated types 1 and 2 gonococci. Non-piliated types 3 and 4 gonococci had a very low

frequency of transformation. Biswas *et al.* (1977) reported that only competent, piliated gonococci exhibited uptake and incorporation of exogenous deoxyribonucleic acid into a deoxyribonuclease-insensitive state. However, genetic transformation of colonial type 4 gonococci has recently been accomplished by Baron and Saz (1978). The frequency of transformation approached 2%, which was similar to the frequency observed with piliated, virulent colonial types 1 and 2. Piliation and virulence were characters transformed to recipient organisms as determined by electron microscopy and lethality for chicken embryos, respectively. That different characters may be transformed at different frequencies was a reasonable explanation for the previous inability to transform non-piliated colonial types. This report suggested that a re-examination of the central role of pili in transformation and the expression of virulence was in order.

Gonococcal pili are composed of a single repeating subunit protein with a molecular weight of approximately 19,000 (Robertson *et al.*, 1977; Buchanan, 1977). Hermodson *et al.* (1978) determined the amino-acid composition of purified pili from four antigenically dissimilar strains of *N. gonorrhoeae* and *N. meningitidis*. The gonococcal and meningococcal pili had an unusual N-terminal amino acid, namely N-methyl-phenylalanine. A similar finding has been reported (Frøholm and Sletten, 1977) for pili from *M. nonliquifaciens*. The amino-terminal amino-acid sequence of gonococcal and meningococcal pili was identical for the first 29 residues and was highly homologous to the sequence of *M. nonliquifaciens* pili protein (Frøholm and Sletten, 1977). However, it was totally unrelated to the amino-acid sequence of pili from *E. coli*. The character of the sequenced region was highly hydrophobic, suggesting that the region was buried deep inside the subunit, or involved in subunit interactions. The amino terminus may be important in facilitating attachment or necessary for physiological functions.

E. LIPOPOLYSACCHARIDE

Investigations of the lipopolysaccharides of *Neisseria* species have revealed the existence of compositional variations between all species. Wiseman and Caird (1977) analysed the glycose and fatty-acyl contents of lipopolysaccharide (LPS) from isogenic colonial variants of several strains of *N. gonorrhoeae*. They found intrastrain variations in the contents of residues of glucose, galactose and mannose, and noted that the LPS

from the virulent colonial types 1 and 2 contained greater glycose contents than the LPS from the avirulent colonial types 3, 4 and 5. The ratios of residues of mannose: 2-oxo-3-deoxyoctulosonic acid (KDO), galactose:KDO and glucose:KDO in the polysaccharides from virulent gonococci were greater than the ratios in those from avirulent organisms. The LPS of gonococci from colony types 1 and 2 did not contain rhamnose residues, in contrast to the LPS of gonococci from avirulent colony types and the non-pathogenic species *N. sicca* and *N. lactamica.* Gonococcal LPS did not contain fucose, a residue which was characteristically present in the LPS of the non-pathogenic species. Total glycose contents and mannose:KDO ratios of the LPS from non-pathogenic species of *Neisseria* were much higher than those in the LPS from *N. gonorrhoeae.*

Perry *et al.* (1975) reported that the core oligosaccharide was common to all strains of *N. gonorrhoeae* tested, in contrast to the findings of Wiseman and Caird (1977). Differences in culture medium and growth conditions may have been responsible for the discrepancies. McDonald and Adams (1971) observed that the LPS of *N. sicca,* grown at a rapid rate, had a greater content of residues of hexosamine and KDO, and a higher ratio of galactosamine to glucosamine residues than LPS from cells grown more slowly. In addition, bacteria from highly aerated cultures contained a greater percentage of unsaturated fatty-acyl residues than bacteria from cultures grown under low aeration suggesting that an oxygen-dependent desaturation of fatty acids had occurred (Kates, 1966). These results indicated that environmental conditions can affect the content and composition of LPS.

Recently, Apicella *et al.* (1978) analysed a polysaccharide antigen purified from gonococcal LPS. Chemical analysis showed that the polysaccharide contained residues of glucose, galactose, glucosamine, galactosamine, glucosamine 6-phosphate, heptose, KDO and ethanolamine, and was the polysaccharide component of LPS. Apicella *et al.* (1978) have also reported that gonococci and group B and C meningococci contain enzymes which degrade the polysaccharide component of gonococcal LPS. Similar extracts from *E. coli, Staphylococcus aureus, Klebsiella pneumoniae* and *Streptococcus pneumoniae* type II were not active. The authors postulated that the ability to degrade a major antigenic component of the cell wall may permit the gonococcus to evade or impair the host's immune response. Further studies are being conducted to substantiate these speculations.

VI. Peptidoglycan Turnover and Synthesis

Peptidoglycan turnover was observed in cells of *N. gonorrhoeae* strain RD_5 labelled during growth in medium containing [^{3}H]diaminopimelic acid (Hebeler and Young, 1976a). Turnover followed first-order kinetics with a rate of almost 50% per generation. Since [^{3}H]diaminopimelic acid specifically labelled the tetrapeptide chain of the peptidoglycan, the data indicate turnover of this portion of the peptidoglycan. Wegener *et al.* (1977b) labelled the peptidoglycan of *N. gonorrhoeae* strain JW-31 during growth in a medium containing [6-^{3}H]glucose. Turnover of peptidoglycan was observed during growth, but the rate of turnover (10 to 20% per generation) was lower than that reported by Hebeler and Young (1976a). These differences may be due to strain variation, differences in the composition of the media, or location of the label in the peptidoglycan.

Wegener *et al.* (1977b) grew cells under conditions whereby old peptidoglycan was labelled with [6-^{3}H]glucose and newly synthesized peptidoglycan with [1-^{14}C]glucose. They demonstrated that the ratio of ^{3}H (old): ^{14}C (new) in purified peptidoglycan remained essentially constant during 60 min incubation in growth medium, which suggested that previously synthesized peptidoglycan turned over at about the same rate as newly synthesized peptidoglycan.

D-Alanine carboxypeptidase may be involved in gonococcal peptidoglycan biosynthesis. A cell envelope D-alanine carboxypeptidase has been characterized by Davis and Salton (1975). The enzyme exhibited optimal activity between pH 8 and 9 in the presence of Mg^{2+}, and was inhibited by penicillin, ampicillin, cloxacillin and methicillin. Although the physiological role of this enzyme has yet to be determined, it may function by decreasing the relative degree of peptidoglycan cross-linking by removing the terminal D-alanine residue.

VII. Autolysis

A. WHOLE-CELL STUDIES

Unlike many Gram-positive and Gram-negative organisms, *N. gonorrhoeae* does not survive for long periods after cessation of growth (Catlin, 1973; Morse and Bartenstein, 1974). The decrease in viability was often correlated with cellular lysis which can occur following the depletion of glucose in the medium (Morse and Bartenstein, 1974). Non-

autolytic strains of *N. gonorrhoeae* have been isolated (S. A. Morse, unpublished data). Autolysis of *N. gonorrhoeae* has been studied by measuring the decrease in optical density of washed cells suspended in an appropriate buffer as a function of time (Hebeler and Young, 1975; Elmros *et al.*, 1976a; Wegener *et al.*, 1977a). Elmros *et al.* (1976a) observed that gonococci harvested in the logarithmic or late-logarithmic growth phases showed a similar lytic behaviour. However, when cells were harvested one hour after reaching the stationary phase of growth, the initial rate of lysis was twice as rapid as with logarithmic-phase cells. Rates of autolysis followed first-order kinetics with half-times varying from 23 to 35 min (Hebeler and Young, 1975) and were dependent upon several variables. Whole-cell autolysis was enhanced by K^+ (100 to 500 mM) (Hebeler and Young, 1975); lower concentrations of K^+ (<10 mM) and other monovalent cations (Li^+, Na^+, NH_4^+) had either no effect or were slightly inhibitory (Hebeler and Young, 1975; Wegener *et al.*, 1977a). Divalent cations (Mg^{2+}, Ca^{2+}, Mn^{2+}, Ba^{2+} and Cu^{2+}) markedly inhibited autolysis (Elmros *et al.*, 1976a; Wegener *et al.*, 1977a). The inhibitory effect of divalent cations on autolysis could be reversed by addition of a chelating agent such as EDTA. Trivalent cations (Fe^{3+} and Al^{3+}) showed only slight inhibition of autolysis (Wegener *et al.*, 1977a).

The nature of the buffer was another important variable. Buffers, such as Tris, which chelate divalent cations, enhanced the rate of autolysis over buffers, such as Hepes, which do not chelate divalent cations as readily (Wegener *et al.*, 1977a). Whole-cell autolysis was optimum at pH 9.5 and at 40° C. Autolysis was also inhibited by sulphydryl poisons such as Hg^{2+}. The observation that the rate of autolysis was temperature dependent and could be irreversibly inactivated by heating (80° C, 10 min) suggested that autolysis was enzyme mediated (Hebeler and Young, 1975).

B. MECHANISM OF AUTOLYSIS

Wegener *et al.* (1977a) examined the relationship between autolysis in buffer and peptidoglycan hydrolysis. The rates of autolysis in Hepes buffer were lowest at an acid pH value (6.0) and in the presence of divalent cations (Mg^{2+}), and were highest at an alkaline pH value (8.5). The rates of peptidoglycan hydrolysis in intact cells under stabilized (in the presence of Mg^{2+}) and non-stabilized conditions (no Mg^{2+}) were similar under both conditions, and clearly demonstrated that Mg^{2+} did

not prevent autolysis by inhibiting peptidoglycan hydrolysis. Peptidoglycan hydrolysis also occurred at acid pH values (pH 6.0) but at half of the maximum rate. As with autolysis, the rate of peptidoglycan hydrolysis was maximal at pH 8.5. Wegener *et al.* (1977b) reported that addition of penicillin G to logarithmic-phase gonococci at pH 7.2 stimulated immediate hydrolysis of peptidoglycan and loss of viability, and was followed, after a 30 min lag, by cell lysis. Addition of penicillin inhibited peptidoglycan synthesis, promoting peptidoglycan hydrolysis and weakening the cell wall to a point where lysis occurred. Addition of penicillin to a suspension of cells grown at pH 6.0 resulted in less lysis even though the viability decreased at the same rate as at pH 7.2, and peptidoglycan hydrolysis still occurred (at 50% of its rate at pH 7.2). These studies on peptidoglycan hydrolysis and autolysis in gonococci indicated that stabilization of these cells against autolysis at acid pH values occurred by a mechanism other than by inhibition of peptidoglycan hydrolysis. Goodell *et al.* (1978) reported similar effects of benzylpenicillin on gonococci grown at pH 8.0. Addition of penicillin immediately inhibited peptidoglycan synthesis, increased the release of glucosamine, and resulted in a loss of viability. Penicillin also enhanced release of lipids and lipopolysaccharide into the medium. These were followed by cell lysis. In cells grown at pH 6.4 in medium containing Mg^{2+}, lysis did not occur but glucosamine was released and viability decreased.

The characteristics of peptidoglycan hydrolysis have also been examined (Wegener *et al.*, 1977a). Dual labelling of the peptidoglycan enabled the rates of hydrolysis of old and newly synthesized peptidoglycan to be compared. Old peptidoglycan was labelled during growth in the presence of [6-^{3}H]glucose, newly synthesized peptidoglycan with [1-^{14}C]glucose. The ratio of ^{3}H:^{14}C in purified peptidoglycan remained relatively constant during a 60 min incubation period which indicated that both the old and new peptidoglycan of cells suspended in buffer underwent hydrolysis at similar rates. Of significance was the comparison of the rates of hydrolysis of the peptide and glycan moieties of the peptidoglycan. The peptide moiety was specifically labelled during growth in the presence of [^{3}H]diaminopimelic acid, and the glycan moiety with [1-^{14}C]glucosamine. The total activity of the labelled peptidoglycan decreased by 50% over a 100-minute incubation period, while the ^{3}H:^{14}C ratio remained relatively constant. These data indicated that hydrolysis of peptidoglycan resulted in solubilization of

both the peptide chain and the glycan backbone. However, the initial rate of ^{3}H loss was greater than that of ^{14}C, which suggested that cleavage of the peptide chain preceeded solubilization of the glycan backbone. Thus, the findings of Wegener *et al.* (1977a) are in accord with a previous report (Hebeler and Young, 1976b) that a principal peptidoglycan hydrolase in gonococci is an N-acetylmuramyl-L-alanine amidase.

Hebeler and Young (1975) previously reported that glucosamine was incorporated primarily into the glycan backbone of the peptidoglycan. Solubilization of ^{14}C from [1-^{14}C]glucosamine-labelled peptidoglycan suggested that gonococci possessed a hexaminidase in addition to an amidase. The radioactive fragments released into the suspending medium were not precipitated by cold 10% trichloroacetic acid followed by centrifugation (Wegener *et al.*, 1977a) suggesting the presence of short glycan chains. In addition, Hebeler and Young (1976b) calculated, from the increase in the number of reducing groups, that incubation of purified

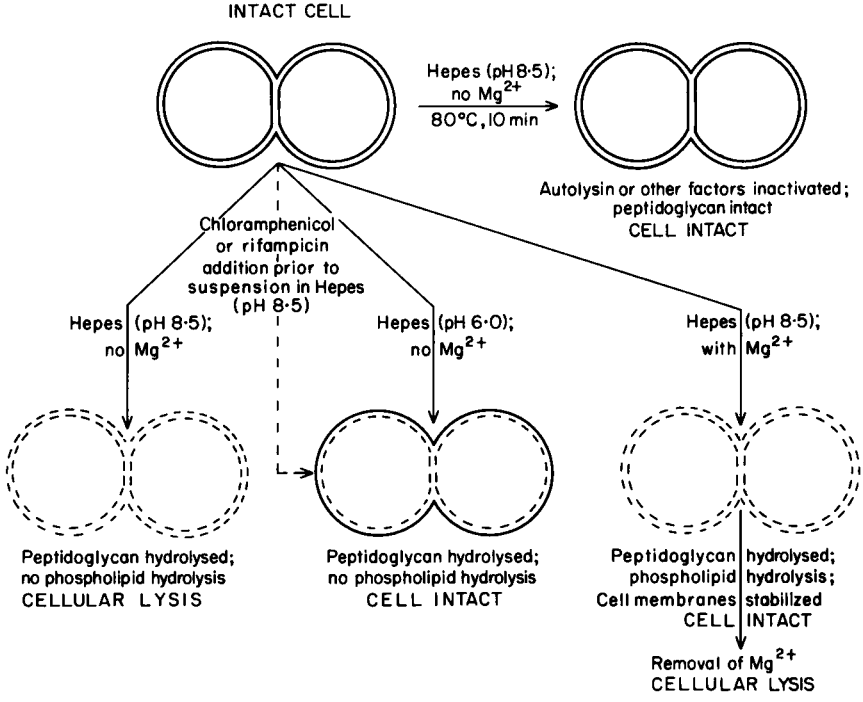

FIG. 1. Tentative model showing the relationship between autolysis and peptidoglycan hydrolysis in *Neisseria gonorrhoeae*.

peptidoglycan with a crude amidase preparation extracted from cell walls shortened the average glycan chain length from 105 to 32 disaccharide units.

Incubation of gonococci with chloramphenicol or rifampicin suppressed autolysis in buffer but did not eliminate peptidoglycan hydrolysis (Wegener *et al.*, 1977a). Inhibition of protein synthesis permitted continued activity of the autolysin(s) indicating that the enzyme(s) did not have a rapid rate of turnover and that it was not synthesized in a latent form and subsequently activated by a regulatory protein. The relationship between peptidoglycan hydrolysis and cellular autolysis is summarized in Fig. 1. The combined data indicate that peptidoglycan hydrolysis *per se* does not account for autolysis, and that other factors, such as stabilization of the cytoplasmic membrane in the presence of divalent cations or at low pH values, may regulate a second event required for cellular autolysis. The fact that both peptidoglycan hydrolysis and autolysis did not occur in heat-inactivated cells (Wegener *et al.*, 1977a) suggested that autolysis was the result of enzyme-catalysed reactions. Senff *et al.* (1976) recently demonstrated phospholipase A activity in envelope preparations from *N. gonorrhoeae*. The phospholipase A was heat sensitive, required Ca^{2+} for activity and exhibited optimal activity between pH 8.0 and 9.0. This pH range was also optimal for autolysis. The enzyme was inactive at an acid pH value (6.5). Lysophospholipase activity has also been reported in the outer membrane of *N. gonorrhoeae* (Wolf-Watz *et al.*, 1975). Phospholipid hydrolysis, mediated by endogenous phospholipase activities, did not appear to be involved in autolysis of gonococci in buffer (Cacciapuoti *et al.*, 1978). Significant *in vivo* phospholipid hydrolysis, characterized by decreased cellular contents of phosphatidylethanolamine and phosphatidylglycerol and increased lysophosphatidylethanolamine and free fatty acids, occurred under non-autolytic conditions (Table 2). Phospholipid hydrolysis was minimal under autolytic conditions. At this time, the mechanism(s) by which destabilization of the cytoplasmic membrane and cellular autolysis occur is unknown.

C. CHARACTERISTICS OF THE AUTOLYSIN

Autolysin activity was recovered from crude cell envelopes following cell disruption (Hebeler and Young, 1976b). No detectable activity was present in the soluble cell fraction or in the culture medium. The

TABLE 2. Relationship between autolysis and phospholipid hydrolysis in *Neisseria gonorrhoeae* strain JW-31. From Cacciapuoti *et al.* (1978)

Conditions	Autolysis	Rate of autolysis ($k \times 10^{-3}$)	Phospholipid composition (%)			
			Phosphatidyl-ethanolamine	Phosphatidyl-glycerol	Lysophosphatidyl-ethanolamine	Free fatty acids
Control	–	–	72.6	22.4	2.5	1.1
Hepes, pH 8.5	+	17.0	71.0	21.7	1.3	4.5
Hepes, pH 8.5; with $MgCl_2$ (20 m*M*)	–	<0.2	62.1	27.7	2.8	12.0
Hepes, pH 8.5; with $CaCl_2$ (20 m*M*)	–	<0.2	36.2	17.4	7.3	36.9
Hepes, pH 6.0	–	<0.2	72.6	21.5	1.3	2.0
Hepes, pH 6.0; with EDTA (10 m*M*)	+	11.4	70.5	25.1	1.2	2.2

Control conditions used exponential-phase cells grown in glucose-containing medium without resuspension in Hepes buffer.

autolysin has been partially purified by treatment of crude cell envelopes with 10 m*M* Tris-maleate buffer (pH 8.5) containing 2% Triton X-100 and 0.5 *M* NaCl for 30 min at room temperature. Solubilization of the enzyme(s) was dependent upon a combination of detergent and salt, suggesting that the enzyme was retained in the cell envelope by a combination of hydrophobic and ionic bonds. The autolysin probably contained an essential sulphydryl group since its activity was completely inhibited by Hg^{2+} (Wegener *et al.*, 1977a). Hydrolysis of peptidoglycan in a cell-free assay system did not exhibit a sharp optimal pH value (Hebeler and Young, 1976b). This may be due to the presence of more than one enzyme having different pH optima. Magnesium ions did not inhibit peptidoglycan hydrolysis by the partially purified enzyme. Similar results (see Section III B, p. 269) were obtained using whole cells (Wegener *et al.*, 1977a).

D. *IN VIVO* SIGNIFICANCE

The relationship between bacterial persistence and autolytic activity has been documented in a number of species. Activation of autolysis may produce cells with damaged envelopes which can remain viable due to stabilization with divalent cations (Elmros *et al.*, 1976b). Cells with damaged envelopes could undergo repair and resume growth under appropriate conditions. Autolytic activity may enhance conversion of gonococci to L-forms. Thus, L-forms may be produced without the selective pressure of penicillin. Scanning electron-microscope studies (Elmros *et al.*, 1976c) have shown the presence of cells resembling L-forms in colonies grown on agar.

VIII. Carbon Dioxide Requirement

Neisseria gonorrhoeae has long been known to have a carbon dioxide requirement for growth. Either carbon dioxide (Platt, 1976) or bicarbonate (Morse, 1976; Talley and Baugh, 1975; Earl *et al.*, 1976) can exert a stimulatory effect on initiation of gonococcal growth. A critical concentration of carbon dioxide seemed to be necessary for growth to begin (Tuttle and Scherp, 1952); increasing the concentration of

bicarbonate resulted in a shorter lag phase (Morse, 1976; Talley and Baugh, 1975).

Jones and Talley (1977) studied the carbon dioxide/bicarbonate requirements of 34 clinical isolates and laboratory strains of *N. gonorrhoeae*, and found that different strains of gonococci had widely differing requirements for these compounds. Some strains grew well with carbon dioxide, but not with bicarbonate; others grew as well with carbon dioxide as with bicarbonate; still others did not appear to require either carbon dioxide or bicarbonate. The basis for these differences is not yet known, but they suggest genetic and/or metabolic variations in the molecular basis of the carbon dioxide/bicarbonate requirement of *N. gonorrhoeae*. Platt (1976) observed a relationship between growth phase and carbon dioxide requirement; carbon dioxide was required during both the lag and stationary phases, but not during the exponential phase. Cellular metabolism during exponential growth may eliminate the need for an exogenous source through production of metabolic carbon dioxide (Morse *et al.*, 1974). Morse (1976) showed that the amount of [^{14}C] HCO_3^- incorporated by *N. gonorrhoeae* into cellular components was lower during the exponential phase than in either the lag or early exponential phases due to dilution of the label in the carbon dioxide pool with metabolically produced carbon dioxide formed during exponential growth.

Two enzymes known to be involved in carbon dioxide assimilation have been described in *Neisseria* species. Carbonic anhydrase (EC 4.2.1.1), a periplasmic enzyme, was detected in *N. sicca* by Adler *et al.* (1972), and phosphoenolpyruvate carboxylase (EC 4.1.1.31), a cytoplasmic enzyme, has been found in pathogenic species of *Neisseria* (Jyssum and Jyssum, 1961; Hebeler and Morse, 1976; Cox and Baugh, 1977). Assimilation of carbon dioxide by phosphoenolpyruvate carboxylase in *N. gonorrhoeae* was an irreversible reaction which required phosphoenolpyruvate, bicarbonate and a metal cation (Ca^{2+}, Mg^{2+}, Mn^{2+}) (Cox and Baugh, 1977). The enzyme has a high affinity for bicarbonate (K_m, 0.27 m*M*), higher than that of phosphoenolpyruvate carboxylases from *S. typhimurium* (Maeba and Sanwal, 1969), *E. coli* (Smith, 1968) and *Thiobacillus thiooxidans* (Suzuki and Werkman, 1958). The affinity of the gonococcal enzyme for magnesium ions (K_m, 1.16 m*M*) and phosphoenolpyruvate (K_m, 4.36 m*M*) was similar to that of the enzyme from *E. coli* (Canovas and Kornberg, 1966).

IX. Carbohydrate Metabolism

A. GENERAL

A major characteristic of the genus *Neisseria* is the limited variety of carbohydrates which its members can utilize. The pathogenic and non-pathogenic species differ in the types of carbohydrates used as sources of carbon and energy. *Neisseria gonorrhoeae* can metabolize only glucose; *N. meningitidis* can use both glucose and maltose. Corbett and Catlin (1968) reported that a number of strains which resembled *N. meningitidis* were able to produce acid from lactose. These strains were found to have a constitutive β-galactosidase activity. In addition to glucose, non-pathogenic strains metabolize fructose and sucrose (Morse, 1978). The non-saccharolytic *Neisseria* species, *N. elongata*, *N. flavescens* and *N. cinerea*, do not metabolize glucose (Holten, 1975).

B. GLUCOSE TRANSPORT

Transport of substrates by *N. gonorrhoeae* has not been studied in great detail. The non-metabolizable analogue of glucose, 2-deoxy-D-glucose (2-DOG), has been used to study glucose transport (Morse, 1976). It was transported into gonococcal cells in an unaltered state. Transport of 2-DOG was competitively inhibited by glucose which suggested that 2-DOG was transported by a glucose-transport system. There was a close similarity between Arrhenius plots of growth rates and 2-DOG uptake rates, suggesting that the rate of glucose uptake dictated the rate of growth (S. A. Morse *et al.*, 1977; Morse, 1976).

Glucose was apparently transported into gonococcal cells in an unaltered state and then phosphorylated by hexokinase and ATP (Morse *et al.*, 1974; Holten, 1974). *Neisseria gonorrhoeae* lacks glucose oxidase (Morse *et al.*, 1974) which would be required for conversion of glucose to gluconate upon transport into the cell. In addition, gonococci do not metabolize gluconate (Morse, 1976). The absence of a phosphoenol-pyruvate-dependent phosphotransferase system in *N. gonorrhoeae* was suggested by the inability to detect 2-deoxyglucose 6-phosphate in cells incubated in the presence of phosphoenolpyruvate (Morse, 1976). This was not unexpected since strictly aerobic organisms, like the gonococcus, are normally devoid of such a transport system (Romano *et al.*, 1970).

C. GLUCOSE METABOLISM

Glucose metabolism in *N. gonorrhoeae* has been investigated using radiorespirometric techniques. A discussion of the advantages of radiorespirometry over conventional manometric techniques has been presented by Wang (1971). Application of radiorespirometry to gonococci has provided data concerning production of carbon dioxide from specifically-labelled carbon atoms of the substrate. The pathways involved in catabolism of the substrate may be charted by the relative yields of labelled carbon dioxide. Morse *et al.* (1974) used radiorespirometry to study glucose catabolism during growth of *N. gonorrhoeae*. Holten (1975) used radiorespirometry to examine glucose catabolism in both growing and non-growing cells. Rates of carbon dioxide production from specifically labelled [^{14}C]glucose by gonococci growing in medium at pH 7.2 were found to be: C-1 > C-4 ≫ C-6 > C-2 > C-3. The Entner-Doudoroff pathway was apparently the major route of glucose catabolism in *N. gonorrhoeae* since production of carbon dioxide was greatest from carbons 1 and 4. A minor contribution by the pentose-phosphate pathway was suggested by the greater production of carbon dioxide from carbon 1 than from carbon 4.

The relative participation of both pathways was determined for various species of *Neisseria* under growth conditions by means of the equations developed by Wang (1971). The data are summarized in Table 3. The Entner-Doudoroff pathway was the principal route of glucose catabolism at pH 7.2. The remaining glucose was catabolized via the pentose-phosphate pathway. Holten (1975) reported that the relative pathway participation in *N. meningitidis* under similar conditions resembled that in *N. gonorrhoeae*. The non-pathogenic species, *N. sicca* and *N. perflava*, utilized the pentose-phosphate pathway to a greater extent at pH 7.2 than did the pathogenic species (46 and 42%, respectively). When *N. gonorrhoeae* was grown on pyruvate, the contribution of the pentose-phosphate pathway was increased to 32% (S. A. Morse *et al.*, 1977). The basis for this change was not known.

The pH value of the environment markedly affected catabolism of glucose (Morse and Hebeler, 1978). Cells grown at pH 6.0 utilized both pathways to the same extent (Table 3). This effect was not due to the decreased growth rate at pH 6.0. Cells grown at 28° C in medium buffered at pH 7.2 had decreased growth rates yet dissimilated glucose similarly to cells grown at 37° C. Growth at pH 6.0 also resulted in a marked increase in the total hexose content of cells.

TABLE 3. Relative participation of glucose catabolic pathways in *Neisseria gonorrhoeae* under varied growth conditions

Organism	pH Value	Temperature (°C)	Participation (%) Entner-Doudoroff pathway	Pentose-phosphate pathway	Reference
Neisseria gonorrhoeae	7.2	37	84	16	Morse *et al.* (1974)
	7.2	28	76	24	Morse and Hebeler (1978)
	8.0	37	76	24	Morse and Hebeler (1978)
	6.0	37	50	50	Morse and Hebeler (1978)
Neisseria meningitidis	7.4	37	85	15	Holten (1975)
Neisseria sicca	7.4	37	54	46	Holten (1975)
Neisseria perflava	7.4	37	58	42	Holten (1975)

Pathway participation was calculated from the original carbon dioxide yield data at 1 relative time unit (Kitos *et al.*, 1958) and the equations developed by Wang (1971). Pentose-phosphate pathway participation equals % $^{14}CO_2$ from [1-^{14}C]glucose minus % $^{14}CO_2$ from [4-^{14}C]glucose. Entner-Doudoroff pathway participation equals 100 minus pentose-phosphate pathway participation.

Active glucose dissimilation by gonococci during growth at neutral or alkaline pH values (S. A. Morse *et al.*, 1977; Morse, 1976; Morse *et al.*, 1974; Holten, 1975) appeared to repress activity of the TCA cycle. There were several lines of evidence which suggested that this inhibition occurred at the level of acetyl-CoA. Concomitant peaks of carbon dioxide production from carbons 2, 3 and 6 of glucose were not observed during active glucose dissimilation (Morse, 1976; Morse *et al.*, 1974; Holten, 1975) indicating that endogenously formed acetyl-CoA was not oxidized via the TCA cycle. Instead, acetate was formed and excreted (Morse *et al.*, 1974). Under these experimental conditions, exogenous acetate was not oxidized to any significant degree until either growth ceased or glucose was depleted from the medium (Hebeler and Morse, 1976). However, cells grown at pH 6.0 dissimilated glucose with concomitant TCA-cycle activity (Morse and Hebeler, 1978). Preliminary data suggested that regulation of the TCA cycle involved both enzyme induction and effects on enzyme activity.

Under non-growing conditions, *Neisseria* spp. use the same pathways for glucose catabolism as they use during growth (Table 4). Pentose-phosphate pathway activity was increased under non-growth conditions except in the presence of 0.15 *M* NaCl. An important difference was that all carbon atoms appeared as carbon dioxide during active glucose dissimilation under non-growth conditions, which suggested that there was no regulation of the TCA cycle at the level of entry of acetyl-CoA. In most cases, there was a significant increase in the amount of glucose carbon incorporated by non-growing cells, suggesting further that carbon skeletons from glucose were being utilized as the major source of carbon in this deficient medium.

Glucokinase, glucose 6-phosphate dehydrogenase (G6PD), 6-phosphogluconate dehydrogenase (6PGD), 6-phosphogluconate dehydratase and 2-oxo-3-deoxy-6-phosphogluconate aldolase (KDPG-aldolase have been found in cell extracts of both pathogenic and non-pathogenic species of *Neisseria* (Morse *et al.*, 1974; Holten, 1974a,b). Morse *et al.* (1974) detected all of the Embden-Meyerhof pathway enzymes in *N. gonorrhoeae*, although no activity from this pathway was detected in radiorespirometric studies. Cell extracts of *N. gonorrhoeae* consistently contained higher specific activities of G6PD than of 6PGD (Morse *et al.*, 1974; Holten, 1974a,b). Increasing the concentration of glucose in the medium increased the specific activities of both G6PD and 6PGD (Holten, 1974a,b). Glucose 6-phosphate dehydrogenase activity

TABLE 4. Relative pathway participation of catabolic pathways of glucose catabolism in non-growing cells of *Neisseria* spp.[a]

Organism[b]	Medium A[c]		Medium B[c]		Medium C[c]	
	Entner-Doudoroff	Pentose-phosphate	Entner-Doudoroff	Pentose-phosphate	Entner-Doudoroff	Pentose-phosphate
Neisseria gonorrhoeae						
Strain 1a	60	40	75	25	70	30
Strain 21308/70	67	33	83	17	63	37
Neisseria meningitidis	81	19	92	8	90	10
Neisseria sicca	54	44	80	20	61	39

[a]Percentage pathway participation was calculated at one relative time unit from the data of Holten (1975).

[b]Organisms were initially grown on blood-agar plates prior to resuspension in minimal media.

[c]Medium A contained, per litre: K_2HPO_4, 7g; KH_2PO_4, 3g; $MgSO_4.7H_2O$, 0.1g; NH_4Cl, 1.5g; $FeSO_4$, 0.001g; $MnSO_4$, 0.001g; $CaCl_2.H_2O$, 0.1g; and $Na_2S_2O_3$, 0.025g. In Medium B, phosphates were replaced by 0.01 *M* Tris·HCl containing 0.15 *M* NaCl. In Medium C, phosphates were replaced by 0.01 *M* Tris-HCl containing 0.15 *M* KCl. The final pH value of these media was 7.4.

from *N. gonorroheae* was similar to that from *N. meningitidis* (Jyssum *et al.*, 1961), *Leuconostoc* spp. (Olive and Levy, 1967; Ragland *et al.*, 1966) and *Pseudomonas* spp. (Ragland *et al.*, 1966) in its capacity to utilize both NAD^+ and $NADP^+$ as cofactors (Morse *et al.*, 1974). The activity of the gonococcal G6PD was higher with $NADP^+$ than with NAD^+. Temperature-inactivation studies suggested that the gonococcal G6PD was a single enzyme with both NAD^+- and $NADP^+$-specific activity, and that a multiplicity of enzymes may not be present in *N. gonorrhoeae*, as has been reported for *Ps. cepacia* (Lessie and VanderWyk, 1972).

The specific activity of 6-phosphogluconate dehydrase was enhanced during growth of *N. gonorrhoeae* in glucose-containing medium, but that of KDPG-aldolase remained constant (Holten, 1974a). 2-Oxo-3-deoxy-6-phosphogluconate aldolase appeared to be a constitutive enzyme in gonococci.

Morse *et al.* (1974) reported that, during exponential growth of *N. gonorrhoeae*, acetate was the only non-gaseous end product formed in significant quantities from [^{14}C]glucose. Acetate production may be decreased or inhibited by the presence of certain ratios of cystine and sodium sulphite in the medium (Baron and Saz, 1976). Substitution of L-cysteine for cystine did not result in a decrease in acid production. The mechanism by which acetate formation was inhibited is not known. C. D.

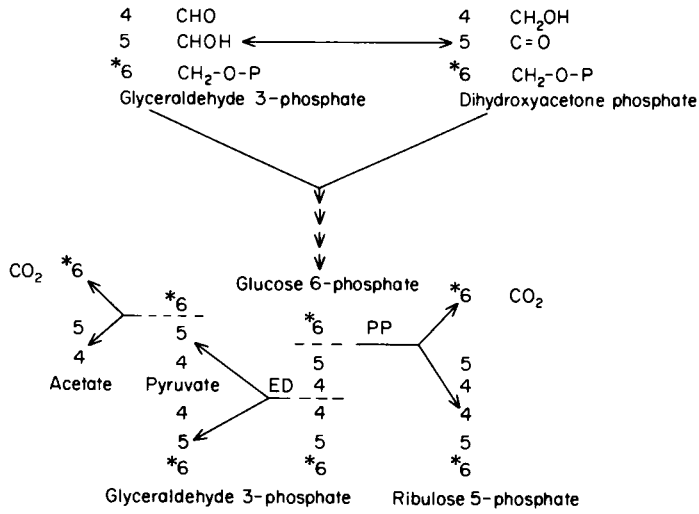

FIG. 2. Pathways for production of [^{14}C]carbon dioxide from [6-^{14}C]glucose by *Neisseria gonorrhoeae*.

Morse *et al.* (1976, 1977) reported the presence of acetylmethylcarbinol and 2,3-butanediol in supernatants of 16-h cultures of bacteria grown in defined medium (Catlin, 1973). They postulated that acetylmethylcarbinol and 2,3-butanediol were derived from glucose; substitution of maltose for glucose decreased the amount of acetylmethylcarbinol produced. Direct evidence indicating that glucose was the only source of these compounds was lacking. Acetylmethylcarbinol was not a major end product, and was produced in low concentrations only during late logarithmic and early stationary phases of growth in a glucose-containing medium (Morse, 1976). Approximately 1.4 mmoles of acetylmethylcarbinol were produced per mole of glucose metabolized. Thus, acetylmethylcarbinol accounted for about 0.14% of the end products from glucose.

C. D. Morse *et al.* (1977) reported that the gonococcus could utilize maltose for growth in a chemically-defined medium without concomitant production of acidic end products. These results must be confirmed by alternate methodology in view of the fact that the defined medium used contained alternate energy sources (Catlin, 1973) and that commercially obtained maltose usually contains trace amounts of glucose.

D. PENTOSE SYNTHESIS

Pentoses are not utilized as carbon and energy sources by pathogenic *Neisseria* species (Kingsbury and Duncan, 1967). In *N. gonorrhoeae*, glucose catabolized via the Entner-Doudoroff pathway results in formation of pyruvate from carbons 1, 2 and 3, and 3-phosphoglyceraldehyde from carbons 4, 5 and 6. Formation of carbon dioxide from carbon 6 of glucose coincided with formation of carbon dioxide from carbons 1 and 4 suggesting that a portion of 3-phosphoglyceraldehyde was recycled by conversion to glucose 6-phosphate (Morse, 1976; Morse *et al.*, 1974; Holten, 1975). The avirulent colony types 3 and 4 may be more active in recycling 3-phosphoglyceraldehyde than the virulent colony types 1 and 2 (Morse, 1976). The extent of recycling may be approximated by assuming that label from [6-^{14}C]glucose will be equally distributed between carbons 1 and 6 after formation of glucose 6-phosphate from 3-phosphoglyceraldehyde (Fig. 2). Acetate was not oxidized during glucose catabolism (Hebeler and Morse, 1976). Therefore, [^{14}C]-labelled carbon dioxide produced from [6-^{14}C]glucose must arise from subsequent catabolism of recycled glucose 6-phosphate.

Morse *et al.* (1974) have estimated that as much as 40% of the 3-phosphoglyceraldehyde was recycled. Some recycled 3-phosphoglyceraldehyde may be utilized for pentose synthesis as well as for energy production, but the proportion of this intermediate used for each purpose has not been determined.

Morse *et al.* (1974) and Holten (1975) suggested that pentose synthesis in *N. gonorrhoeae* can occur by both non-oxidative pentose-phosphate pathway, and a pathway that would utilize 3-phosphoglyceraldehyde. Both pathways may be active simultaneously (Morse, 1976; Morse *et al.*, 1974). Carbons 4, 5, and 6 of glucose were incorporated into the ribose and deoxyribose moieties of nucleic acids to a greater extent than carbons 2 and 3 (Morse *et al.*, 1974; Holten and Jyssum, 1974). If pentoses were being produced only via the pentose-phosphate pathway, incorporation from carbons 2–6 would be equal.

E. LACTATE AND PYRUVATE METABOLISM

Smyth *et al.* (1976) provided immuno-electrophoretic evidence for the presence of two forms of lactate dehydrogenase in gonococcal cell envelopes. Morse and Fitzgerald (1974) and Winter and Morse (1975) reported the presence of membrane-associated lactate dehydrogenases (EC 1.1.2.3) which were specific for the L- and D-isomers of lactate. The L-lactate dehydrogenase, and possibly the D-lactate enzymes, were associated with the electron-transport chain. A cytoplasmic nicotinamide nucleotide-dependent L-lactate dehydrogenase (EC 1.1.1.27) has also been found in *N. gonorrhoeae* (Holten and Jyssum, 1974; Morse *et al.*, 1974). The activity of this enzyme was greater with NADH than with NAD^+ (Holten and Jyssum, 1974), thus favouring the formation of lactate from pyruvate. Therefore, the initial reaction in gonococcal lactate metabolism may be oxidation of lactate to pyruvate by the membrane-associated forms of lactate dehydrogenase. Hoshino *et al.* (1976) reported the presence of a different pathway of lactate metabolism in a species of *Neisseria* isolated from dental plaque. In this pathway, lactic oxidase, an enzyme not reported in gonococci, converted lactate directly to acetate.

Little work has been done on pyruvate metabolism in *Neisseria* spp. Radiorespirometric analysis of pyruvate metabolism in *N. gonorrhoeae* (Holten, 1976) indicated that the carboxyl carbon (C-1) of pyruvate was readily converted to carbon dioxide while the carbonyl (C-2) and methyl

(C-3) carbons, which accumulated as acetate in the medium, were not converted to carbon dioxide as quickly. The rate and extent of carbon dioxide production from pyruvate was: C-1 > C-2 > C-3. The slow conversion of C-2 and C-3 of pyruvate to carbon dioxide, relative to the rapid release of carbon dioxide from C-1, suggested that C-2 and C-3 were being metabolized via the TCA cycle.

F. ACETATE METABOLISM

Acetate accumulated in the medium during glucose metabolism by *N. gonorrhoeae* (Morse *et al.*, 1974). The excreted acetate was not oxidized until growth ceased or glucose was depleted (Morse, 1976; Morse *et al.*, 1974; Holten, 1975). Acetate oxidation was also inhibited during utilization of pyruvate (Holten, 1976b) or lactate (Morse, 1976). Carbon 1 of acetate was converted to carbon dioxide at a faster rate than C-2 after glucose depletion, which suggested that the TCA cycle was involved in its oxidation (Morse, 1976; Morse *et al.*, 1974; Hebeler and Morse, 1976; Holten, 1975). All TCA-cycle enzymes, except for a soluble, nicotinamide nucleotide-dependent malate dehydrogenase (EC 1.1.1.37), have been detected in extracts of gonococci. This enzyme was also absent from *N. gonorrhoeae* and *N. cinerea* (Holten and Jyssum, 1974) and from *N. meningitidis* (Jyssum, 1960). However, most non-pathogenic *Neisseria* species possess nicotinamide nucleotide-dependent malate dehydrogenase activity (Holten and Jyssum, 1974). Even though such activity was lacking in gonococci and meningococci, these species contain antigens which cross-reacted with antiserum prepared against partially purified *N. perflava* malate dehydrogenase (Holten, 1974c). The purity of the antigen from which the antiserum was prepared makes these data suspect.

Holten (1976c) reported that gonococci possessed a membrane-associated nicotinamide nucleotide-independent malate oxidase (EC 1.1.3.3). This enzyme was also detected in *N. meningitidis* and a number of non-pathogenic species including *N. sicca*, *N. cinerea* and *N. subflava*. The malate oxidase in *N. gonorrhoeae* was found by Hebeler and Morse (1976) to require FAD, and was associated with the electron-transport chain.

As stated earlier, acetate was not oxidized by *N. gonorrhoeae* in the presence of glucose, pyruvate or lactate. Growth of gonococci on these energy sources repressed the synthesis of some TCA-cycle enzymes (Morse *et al.*, 1977; Morse, 1976). Glucose-grown gonococci were

capable of oxidizing TCA-cycle intermediates from α-oxoglutarate to oxaloacetate, but incapable of oxidizing acetate, cis-aconitate, citrate and isocitrate (Tonhazy and Pelczar, 1953). Hebeler and Morse (1976) found that only a portion of the TCA cycle functioned during growth in a glucose-containing medium. Under these conditions, glutamate was converted to α-oxoglutarate by either glutamate dehydrogenase (EC 1.4.1.2 and EC 1.4.1.4) (Hebeler and Morse, 1976; Holten, 1973) or aspartate aminotransferase (EC 2.6.1.1) (Hebeler and Morse, 1976; Holten and Jyssum, 1974). The rate and extent of conversion of C-1 of glutamate to carbon dioxide was greater than that of C-5 as determined by radiorespirometry (Hebeler and Morse, 1976). This difference would be expected if glutamate were oxidized via a partial TCA cycle. S. A. Morse *et al.* (1977) and Hebeler and Morse (1976) were unable to detect aconitase, isocitrate dehydrogenase, or malate oxidase activities in extracts of glucose-grown gonococci; other TCA-cycle enzyme activities were present. However, extracts of gonococci harvested after glucose-depletion (or during the stationary growth phase) contained activities of all TCA-cycle enzymes. After glucose depletion, the rate of carbon dioxide production from C-1 of acetate was approximately twice that from C-2, indicating that acetate was oxidized via the TCA cycle. Holten (1977) observed a similar radiorespirometric pattern for carbon dioxide release from acetate in *N. elongata* subsp. *glycolytica*.

The TCA cycle in *N. gonorrhoeae* appeared to be regulated at a branch point in the cycle where acetyl-CoA and oxaloacetate are condensed to citrate by the action of citrate synthase (EC 4.1.3.7). This was suggested by inhibition of acetate oxidation, but not glutamate oxidation, upon addition of glucose to acetate-oxidizing gonococci (S. A. Morse *et al.*, 1977). Partially purified citrate synthase (molecular weight 200,000) was non-competitively inhibited, with respect to acetyl-CoA, by NADPH, NADH and ATP (K_i values of 3.3 m*M*, 5.4 m*M* and 3.5 m*M*, respectively). With respect to oxaloacetate, the enzyme was competitively inhibited by α-oxoglutarate (apparent K_i value of 9.1 m*M*). These inhibitory compounds, which are readily produced during metabolism of glucose, pyruvate and lactate, may become present in intracellular concentrations sufficient to inhibit citrate synthase activity in *N. gonorrhoeae*.

Regulation of the TCA cycle was not apparent in non-growing gonococci. Holten (1975) showed that carbons 2, 3 and 6 of glucose were simultaneously oxidized during glucose catabolism by gonococci

suspended in a buffered salts medium. In addition, glutamate oxidation by non-growing cells revealed a pattern of carbon dioxide release of: C-1 > C-5 ≥ C-2 > C-3,4 (Holten, 1976a). Fumarate oxidation to carbon dioxide was in the order C-1,4 > C-2,3. These data indicated that the TCA cycle was functioning in non-growing cells.

The activity of the TCA cycle under non-growing conditions depended upon the ability of the gonococcus to form oxaloacetate. Reactions which depleted C_4 intermediates for biosynthesis had an adverse effect on TCA-cycle activity. Holten (1976a,b) reported that oxidation of TCA-cycle intermediates was enhanced by the presence of other cycle intermediates.

Neisseria gonorrhoeae lacks a glyoxylate bypass as a means of synthesizing C_4 intermediates from acetate to supply the TCA cycle. This was shown by production of only small amounts of carbon dioxide from acetate when cells were suspended in buffered salts solution (Holten, 1976b). Most other species of *Neisseria* have also been shown to lack a glyoxylate bypass (Holten, 1976b), except for *N. elongata* subsp. *glycolytica* (Holten, 1977). The glyoxylic acid-cycle enzymes, isocitrate lyase (EC 4.1.3.1) and malate synthetase (EC 4.1.3.2), have not been detected in extracts of either glucose-grown or glucose-depleted *N. gonorrhoeae* (Hebeler and Morse, 1976).

Although *N. gonorrhoeae* does have a functional TCA cycle, it cannot utilize intermediates of the cycle as energy sources for growth. Gonococci were apparently able to transport these intermediates into the cell (Hebeler and Morse, 1976; Tonhazy and Pelczar, 1953; Holten, 1976a), which suggested that their inability to grow on these compounds was related to something other than a lack of permeability. *Neisseria meningitidis* lacks phosphoenolpyruvate carboxykinase (Jyssum and Jyssum, 1961), an enzyme required for synthesis of phosphoenolpyruvate from cycle intermediates (Hansen and Juni, 1974). The absence of this enzyme from *N. gonorrhoeae* would prevent growth on TCA-cycle intermediates by limiting the synthesis of phosphoenolpyruvate.

X. Terminal Electron Transport

The cell membrane-associated respiratory electron-transport chain of *N. gonorrhoeae* has been examined by means of electron paramagnetic spectroscopy (e.p.r.) at liquid helium temperatures and by difference spectroscopy at both liquid nitrogen and room temperatures (Winter and

Morse, 1975). The general line shape of the e.p.r. spectra of dithionite-reduced particles was very similar to that of mammalian mitochondria. Prominent signals corresponding to centres N-1 and N-3 in the site I region of the respiratory chain were observed, but signals corresponding to centre N-2 were not present. Electron paramagnetic spectroscopy spectra of succinate-reduced particles revealed the existence of centre S-1 from the succinate cytochrome *c* reductase segment. The low potential centre S-2 was not observed in gonococcal membrane fragments due to an overlap with site I e.p.r. signals. Free radicals resembling those due to flavin semiquinone were observed with both reductants.

Low temperature (77°K) difference spectroscopy of *N. gonorrhoeae* cell-membrane fragments resolved maxima at 549 nm (*c*-type cytochrome) and 557 nm with a shoulder at 562 nm (Winter and Morse, 1975). The latter two, together with the *beta* maximum at 528 nm, are indicative of *b*-type cytochromes. A broad absorption band centred around 600 nm and a shoulder of 440 nm may indicate the presence of an *a*-type cytochrome. Room temperature dithionite-reduced plus carbon monoxide minus dithionite-reduced difference spectra showed absorption maxima and minima which were attributed to cytochrome *o*. The carbon monoxide difference spectra did not indicate the presence of cytochromes *a* or a_3. The respiratory chain contains coenzyme Q (Beebe and Wlodkowski, 1976), several non-haem iron centres, a cytochrome *c*, two *b*-type cytochromes, and cytochrome *o*, which probably serves as the terminal oxidase.

Recently, Kenimer and Lapp (1978) reported the effects of inhibitors on electron transport in *N. gonorrhoeae*. Oxidation of NADH by sonic extracts of both glucose- and acetate-grown cells was inhibited by rotenone, amytal, 2-heptyl-4-hydroxyquinoline (HQNO), antimycin A and potassium cyanide. Cytochromes were not reduced in the presence of class II inhibitor HQNO unless succinate was present. A slight reduction indicative of *b*-type cytochrome was noted when succinate was added to HQNO-treated samples. Furthermore, succinate-stimulated oxygen uptake was not inhibited by HQNO. These data indicated that HQNO probably blocked the gonococcal electron-transport chain on the NADH dehydrogenase side of cytochrome *b*. The inability of potassium cyanide totally to inhibit either NADH oxidation or NADH-stimulated oxygen uptake indicated the existence of cyanide-insensitive respiration, which may be due to a flavoprotein oxidase. Kenimer and Lapp (1978) suggested that cyanide-insensitive respiration was due to a cyanide-

insensitive *d*-type cytochrome, which they observed in ascorbate-tetramethyl-*p*-phenylenediamine (TMPD)-reduced minus oxidized cytochrome spectra. However, the authors did not state whether their results were obtained with acetate- or glucose-grown cells. Different growth conditions may affect the cytochrome composition of gonococci. Further studies are required to determine the role of cytochrome *d* in gonococcal respiration.

Approximate concentrations of the various cytochromes were computed from low-temperature dithionite-reduced minus ferricyanide-oxidized difference spectra (Winter and Morse, 1975). The high amount of cytochrome *c* which was found reflects the high cytochrome *c* oxidase activity present in members of the genus *Neisseria*, which Jurtshuk and Milligan (1974b) observed by using a quantitative manometric TMPD oxidase reaction, an assay which was functionally analogous to measuring cytochrome *c* oxidase. The results of the quantitative determination of TMPD oxidase activities for several species of *Neisseria*

TABLE 5. Quantitation of the ascorbate tetramethyl-*p*-phenylenediamine (TMPD) oxidase activity of various *Neisseria* species. From Jurtshuk and Milligan (1974b) and Jurtshuk and McQuitty (1976)

Organism	Respiratory quotient (Q_{O_2} value) divided by the value for TMPD oxidase (endogenous value subtracted)
Branhamella (Neisseria) catarrhalis (25238)	1736
Branhamella catarrhalis Gp4	1598
Branhamella catarrhalis NC31	1613
Neisseria lactamica (23970)	2595
Neisseria flava (14211)	1322
Neisseria gonorrhoeae (F62)	
colony type 1	1077
colony type 2	1260
colony type 3	934
colony type 4	872
Neisseria sicca (H.H.D.)	857
Neisseria mucosa (H.H.D.)	755
Neisseria elongata (25295)	635

Numbers in parentheses are the A.T.C.C. strain designations. H.H.D. represents cultures obtained from the Houston Health Department. Strains Gp4 and NC31 were from Rita Colwell at the University of Maryland, U.S.A. Respiratory quotient (Q_{O_2} values) are expressed as microlitres of oxygen per hour per milligram (dry weight) at 30°C. Endogenous value represents cellular respiration obtained in the absence of ascorbate-tetramethyl-*p*-phenylenediamine.

are listed in Table 5 (from Jurtshuk and Milligan, 1974b, and Jurtshuk and McQuitty, 1976).

Recently, Devoe and Gilchrist (1976) reported that a portion of the TMPD oxidase was found in association with evaginations of the outer membrane of *N. meningitidis*. Similar evaginations or wall blebs have been observed in *N. gonorrhoeae* (Morse, 1976). The ratio of succinate dehydrogenase to TMPD oxidase activities in cell-envelope preparations of *N. meningitidis* was approximately 100 times that in isolated wall blebs, suggesting that the outer membrane preparations were relatively devoid of cytoplasmic membrane contamination. Whether the TMPD oxidase was an integral part of the outer membrane, or was present in the periplasmic space and incorporated as the outer membrane evaginates, was not evident. There are precedents for the location of *c*-type cytochromes in the periplasm of Gram-negative bacteria (Knowles *et al.*, 1974). However, it is difficult to conceive of an oxidase located in the periplasm (or outer membrane) which could be a terminal electron acceptor for a respiratory chain which is located in the cytoplasmic membrane. It is conceivable that the TMPD oxidase represents one terminal oxidase of a branched respiratory pathway or that it has some other, as yet unknown, function.

The terminal electron-transport chain of *Neisseria* (*Branhamella*) *catarrhalis* and *N. sicca* have also been characterized by Jurtshuk and Milligan (1974a). Dithionite-reduced-minus-oxidized difference spectra revealed an α-maximum at 550 nm and a β-maximum at 524 nm, which indicated the presence of a *c*-type cytochrome. Shoulders at 555 nm and 530 nm indicated a possible combination of *b*- and *c*-type cytochromes, while a small broad peak in the 600 nm region suggested the presence of an *a*-type cytochrome. Carbon monoxide-dithionite-reduced-minus-reduced difference spectra were typical of cytochrome *o*, and did not indicate the presence of an *a*-type cytochrome. All spectra were similar for the two species studied.

Branhamella catarrhalis and *N. sicca* were found by Jurtshuk and Milligan (1974a) to have a moderately active succinate, as well as a non-nicotinamide nucleotide-dependent DL-lactate oxidoreductase. Oxidation of L-malate and L-glutamate was dependent on nicotinamide nucleotides, and was more active with $NADP^+$ than with NAD^+. The activity was due to soluble L-malate and L-glutamate dehydrogenases. A malate oxidase was not found.

Membrane-associated D- and L-lactate dehydrogenase (Johnston and

Gotschlich, 1974; Morse and Fitzgerald, 1974; Miller and Morse, 1977), succinate dehydrogenase (Johnston and Gotschlich, 1974; Hebeler and Morse, 1976) and malate oxidase (Hebeler and Morse, 1976; Holten, 1976c) activities are associated with the electron-transport chain of *N. gonorrhoeae*. The characteristics of these enzymes are discussed more fully in other sections of this review.

XI. Inorganic Nitrogen and Amino-Acid Metabolism

A. INORGANIC NITROGEN METABOLISM

Metabolism of inorganic nitrogen by *N. gonorrhoeae* has not been extensively studied. Glutamate dehydrogenase, which enables most bacteria to incorporate ammonium ions into organic material, has been detected in both *N. gonorrhoeae* (Holten, 1973; Hebeler and Morse, 1976) and *N. meningitidis* (Holten and Jyssum, 1973; Jyssum and Borchgrevink, 1960). Jyssum (1959) adapted meningococci to grow on a synthetic medium containing glucose as the carbon and energy source and ammonium ions as the sole nitrogen source. However, this may be strain dependent since some strains of *N. meningitidis* require specific amino acids for growth (Catlin, 1973). Some species of *Neisseria* reduce nitrites or nitrates (Reyn, 1974). Berger (1970) reported that *N. gonorrhoeae* and *N. meningitidis* were unable to reduce nitrates. However, nitrite, in low concentration, was reduced without formation of gaseous nitrogen by *N. gonorrhoeae* and by *N. meningitidis* serogroups A, D and Y.

B. AMINO-ACID METABOLISM

The majority of the information concerning amino-acid metabolism in *N. gonorrhoeae* was derived from isolation and study of naturally occurring amino-acid auxotrophs. All strains of *N. gonorrhoeae* exhibit an absolute requirement for cysteine (or cystine) (Catlin, 1973; Carifo and Catlin, 1973); cysteine is not required by *N. meningitidis*, *N. lactamica* or non-pathogenic species (Catlin, 1973; McDonald and Johnson, 1975). The nature of the block in the cysteine biosynthetic pathway is not known. Since some strains of *N. gonorrhoeae* are capable of synthesizing serine (LaScolea and Young, 1974), it is possible that the gonococcus lacks the enzyme cysteine synthetase which converts serine to cysteine. Other enzymes involved in cysteine metabolism were reported by Tauber and Russell (1962) who detected activities corresponding to cysteine oxidase

and cysteine desulphydrase in all strains of *N. gonorrhoeae* examined. The role of these enzymes in the amino-acid metabolism of *N. gonorrhoeae* remains to be determined.

Biosynthesis of amino acids by *N. gonorrhoeae* most likely occurs by pathways similar to those in most micro-organisms. Only 19% to 59% of the clinical isolates surveyed (Catlin, 1973, Carifo and Catlin, 1973; Knapp and Holmes, 1975) synthesized all amino acids with the exception of cysteine (or cystine). Requirements for amino acids (Catlin, 1974, 1976; Young *et al.*, 1977; Catlin and Pace, 1977; Short *et al.*, 1977) have been frequently observed, and the proportion of amino acid-requiring strains has varied with geographical location. Therefore, the growth requirements of isolates, i.e. auxotype, can be used as an epidemiological marker for strain identification. Strains exhibiting a requirement for a particular amino acid would be phenotypically identical, but might be genotypically different. Young *et al.* (1977) used DNA-mediated transformation to show that there were at least eight classes of naturally-occurring arginine-requiring mutants, and two classes of proline-requiring mutants. Many arginine-requiring strains did not result from a single mutation but rather had multiple blocks in the arginine biosynthetic pathway.

An examination of specific enzymes involved in amino-acid metabolism has been limited to studies on aspartate and glutamate metabolism. Formation of aspartate from fumarate via the enzyme aspartase (EC 4.3.1.1) has been demonstrated in *N. gonorrhoeae* and most other species of *Neisseria* (Holten and Jyssum, 1974). Aspartate can undergo a transamination reaction with α-oxoglutarate via the enzyme aspartate aminotransferase (EC 2.6.1.1), which is present in *N. gonorrhoeae* and other species of *Neisseria* (Holten and Jyssum, 1974; Hebeler and Morse, 1976).

The presence of an NAD^+-dependent (EC 1.4.1.2) and an $NADP^+$-dependent (EC 1.4.1.4) glutamate dehydrogenase in *N. gonorrhoeae* was initially reported by Holten (1973) and confirmed by Hebeler and Morse (1976). A study of these enzymes in *N. meningitidis* (Holten and Jyssum, 1973; Jyssum and Borchgrevink, 1960) suggested that the NAD^+-dependent enzyme served mainly a degradative function, while the $NADP^+$-dependent enzyme was used for formation of glutamate.

In a survey of the glutamate dehydrogenase system in different *Neisseria* species, Holten (1973) found that all species except *N. catarrhalis*, *N. ovis* and *N. caviae* contained both NAD^+- and $NADP^+$-dependent

enzymes. The latter three species contained a single enzyme which reacted with both coenzymes, and provided additional evidence that these species should not be included in the genus *Neisseria*.

Holten (1973) observed that the specific activity of the NAD^+-dependent enzyme of *N. gonorrhoeae*, *N. lactamica* and *N. flavescens* decreased with increasing concentrations of glucose in the medium, whereas the specific activity of the $NADP^+$-dependent enzyme increased. *In vitro* studies suggested that these enzymes had different functions. The activity of glutamate dehydrogenase(s) was dependent upon the nicotinamide nucleotide coenzyme used in the assay procedure. Enzyme activity was greater with the coenzyme NADP(H) than with NAD(H) (Holten, 1973; Hebeler and Morse, 1976). The reduced form of the coenzyme in the *in vitro* assay was generally more active than the oxidized form, thereby favouring formation of glutamate from α-oxoglutarate. *In vivo* studies utilizing radiorespirometry (Hebeler and Morse, 1976) demonstrated that, in a medium containing excess glutamate, this amino acid entered the TCA cycle and was partially oxidized. Both the rate and extent of glutamate oxidation increased following glucose depletion. It appeared that an important factor which determined whether the enzyme served a catabolic or biosynthetic function was the concentration of exogenous glutamate and not the coenzyme specificity of the enzyme.

Despite the central role which glutamate and aspartate play in amino-acid metabolism and the distribution of nitrogen within the cell, exogenous sources of these two amino acids were not required for growth by most strains of *N. gonorrhoeae* (LaScolea and Young, 1974). The gonococcus has sufficient biosynthetic capabilities to obtain the necessary carbon skeletons and nitrogen from metabolism of glucose and other amino acids.

Transaminases may be important for biosynthesis of amino acids in *Neisseria* spp. Transaminations between the amino-nitrogen donors aspartate, alanine, tyrosine, phenylalanine, tryptophan, isoleucine, valine, leucine, cysteine and arginine, and the amino-nitrogen acceptor, α-oxoglutarate, have been observed in cell extracts of *N. meningitidis* (Jyssum, 1959). Alanine aminotransferase (Hebeler and Morse, 1976) and phenylalanine aminotransferase (Brooks *et al.*, 1971) have not been detected in *N. gonorrhoeae*. However, production of α-oxo-isocaproic acid from L-leucine (Brooks *et al.*, 1971) suggested the presence of leucine aminotransferase activity in gonococci.

Putrescine and cadaverine have been tentatively identified in supernatants from cultures of gonococci grown in defined medium (C. D. Morse *et al.*, 1977). Putrescine did not accumulate in most cultures (Brooks *et al.*, 1978) which suggested that it might be an intermediate. The presence of these amines indicates the presence of specific amino-acid decarboxylases.

Weaver and Herbst (1958) reported an amine oxidase produced by strains of *N. perflava* which oxidized the polyamines spermine and spermidine to 1,3-diaminopropane. They further speculated that α-aminobutyraldehyde was formed by oxidation of putrescine, and that this compound could undergo spontaneous oxidation to form pyrroline. Pyrroline has also been detected in cultures of *N. gonorrhoeae* and *N. meningitidis* grown in defined medium (Brooks *et al.*, 1978).

C. MISCELLANEOUS ENZYMES

Degradation of proteins or peptides requires specific enzymes. Very few studies have investigated this aspect of gonococcal metabolism. Swanson (1977b) reported preliminary data on the presence of a gonococcal protease in spent-culture media; the enzyme had activity against gelatin and artificial trypsin substrates. Gelatinase activity was also found by Hafiz *et al.* (1977) in 169 of 170 gonococcal isolates, but not in six strains of *Branhamella catarrhalis*. Six strains of *N. meningitidis* were very weakly positive. Plaut *et al.* (1975, 1977) reported that gonococci produced an extracellular enzyme which cleaved a proline-threonine bond on the heavy chain of the immunoglobulin A_1 subclass of immunoglobulin A. Cleavage of this susceptible bond resulted in loss of antibody activity. The highly specific nature of this enzyme suggested that it may be important in resisting host defences. However, the primary function of this enzyme may be related to gonococcal metabolism. Evidence presented as to the extracellular nature of these enzymes is weak. In light of the highly autolytic nature of this organism, additional studies are required to ascertain the cellular location of these proteolytic enzymes.

Watson and Perrine (1978) examined aminopeptidase activity in *Neisseria* spp. utilizing L-amino-acid-β-naphthylamide derivatives as substrates. *Neisseria gonorrhoeae* was found to contain an aminopeptidase(s) active against L-alanine-, L-arginine-, L-aspartate-, L-glutamate-, L-leucine-, L-lysine- and L-methionine-β-naphthylamide derivatives. D'Amato *et al.* (1978) reported the presence of L-

hydroxyproline aminopeptidase, L-arginine aminopeptidase, L-serine aminopeptidase, glycyl-glycine aminopeptidase, 4-methoxyleucine aminopeptidase and glycine aminopeptidase in *N. gonorrhoeae*. *Neisseria gonorrhoeae* and *N. meningitidis* could be distinguished from each other, and from the non-pathogenic *N. flava*, *N. sicca* and *Branhamella catarrhalis*, by both qualitative and quantitative differences in their abilities to hydrolyse L-amino acid-β-naphthylamides. Data as to the cellular location (e.g. periplasmic, intracellular) or number of aminopeptidases were not given. Davis and Salton (1975) reported the presence of a D-alanine carboxypeptidase in envelope fractions of *N. gonorrhoeae*. This enzyme may be involved in cell-wall biosynthesis.

XII. Nucleotide Precursors

A. PYRIMIDINES

Studies on pyrimidine metabolism in members of the genus *Neisseria* have been primarily concerned with *N. meningitidis*, which does not require exogenous pyrimidine (or purine) bases for growth (Frantz, 1942; Catlin, 1973). The absence of a requirement for exogenous pyrimidines may be responsible, in part, for the low uptake and incorporation of these compounds by meningococci. Kingsbury and Duncan (1967), using labelled pyrimidines, observed that uracil was the only pyrimidine incorporated to a significant degree into nucleic acids by a wild-type strain of *N. meningitidis*. Cytidine, deoxycytidine and deoxyuridine were incorporated to a much lesser degree. Pyrimidine nucleotides in *N. meningitidis* may be synthesized *de novo* or from exogenous uracil (see Fig. 3).

Jyssum and Jyssum (1970) reported similar low levels of incorporation of exogenous thymine, thymidine and thymidine monophosphate (TMP). Their results suggested that meningococci lacked certain enzymes which are required for uptake and incorporation of pyrimidine bases. Jyssum (1971) failed to detect thymidine phosphorylase (EC 2.4.2.4), nucleoside deoxyribosyl transferase (EC 2.4.2.6) and thymidine kinase (EC 2.7.1.21) in *N. meningitidis*.

Neisseria meningitidis appeared to lack enzymes for uridine incorporation. Jyssum and Jyssum (1970) observed that inhibition of meningococcal growth by 5-fluorouracil was reversed by addition of uracil, but not by addition of uridine or thymidine. It was suggested that

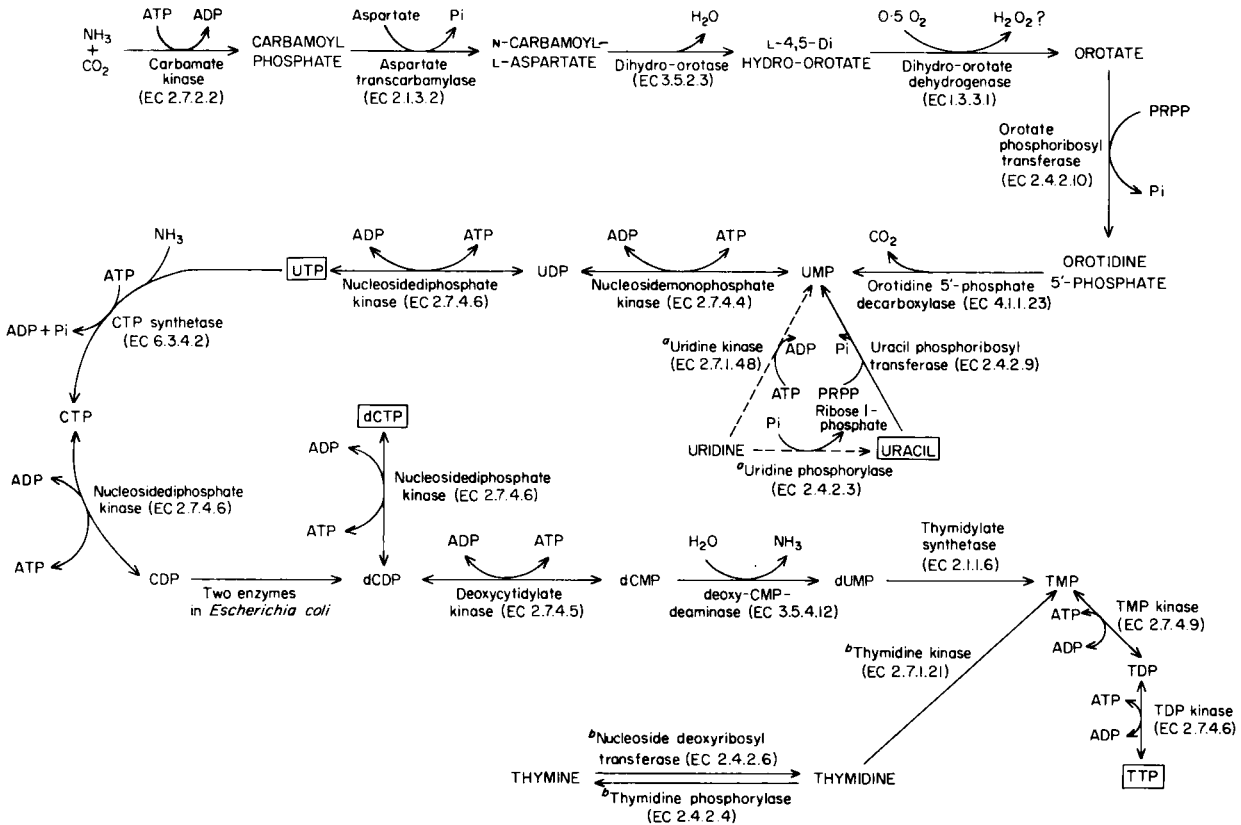

FIG. 3. Pathways for pyrimidine nucleotide biosynthesis in *Neisseria gonorrhoeae*. *a* indicates that the activity was probably absent, *b* that it was absent.

uridine phosphorylase and uridine kinase were absent from *N. meningitidis*, and that uracil phosphoribosyl transferase may be involved in uracil incorporation. Usually, both uracil and thymine are required to overcome 5-fluorouracil inhibition (Wachsman *et al.*, 1964). In *N. meningitidis*, uracil is probably a precursor of TMP (see Fig. 3).

Low levels of radioactive thymidine and thymine were incorporated into TMP, thymidine diphosphate (TDP) and thymidine triphosphate (TTP) by extracts and whole cells of *N. meningitidis* (Jyssum, 1971). The radioactivity from exogenous thymidine and thymine was incorporated into both RNA and DNA (Jyssum, 1972) indicating that breakdown products of these bases were being utilized by cells.

Uracil appeared to be an important precursor for nucleic-acid synthesis in strains of *N. gonorrhoeae* which require uracil, arginine and hypoxanthine for growth. These $arg^-hyx^-ura^-$ strains were reported by Knapp and Holmes (1975) to account for 87% of disseminated gonococcal infections, but for only 38% of uncomplicated infections, in Seattle, Washington, U.S.A. The uracil requirement of a number of these strains was not replaced by carbamoyl phosphate, carbamoyl aspartate, dihydro-orotate, orotate, orotidine 5′-monophosphate, uridine or cytosine (S. A. Morse *et al.*, 1977). Griffin and Racker (1956), using other uracil-requiring strains, were not able to replace the uracil requirement with either thymine, thymidine, uridine, uridylic acid or orotic acid. Growth of these gonococcal strains in medium supplemented with arginine and hypoxanthine was directly proportional to the concentration of uracil (S. A. Morse *et al.*, 1977). High concentrations of uracil (40 to 50 μg per ml) were necessary to obtain maximum growth. The high concentrations of uracil required by $arg^-hyx^-ura^-$ strains of *N. gonorrhoeae* suggested that uracil may be required for biosynthesis of all pyrimidine nucleotides.

B. PURINES

Purine metabolism has also been extensively studied in *N. meningitidis*. Adenosine monophosphate (AMP) and guanosine monophosphate (GMP) are synthesized by *N. meningitidis* both *de novo* (Catlin, 1973) and from exogenous adenine (Jyssum, 1974a). In contrast to the low level of incorporation of exogenous pyrimidines, a wild-type strain of *N. meningitidis* readily incorporated significant amounts of exogenously added adenine (99.5%) and guanine (69.9%) into the nucleic acid-containing cell fraction (Kingsbury and Duncan, 1977).

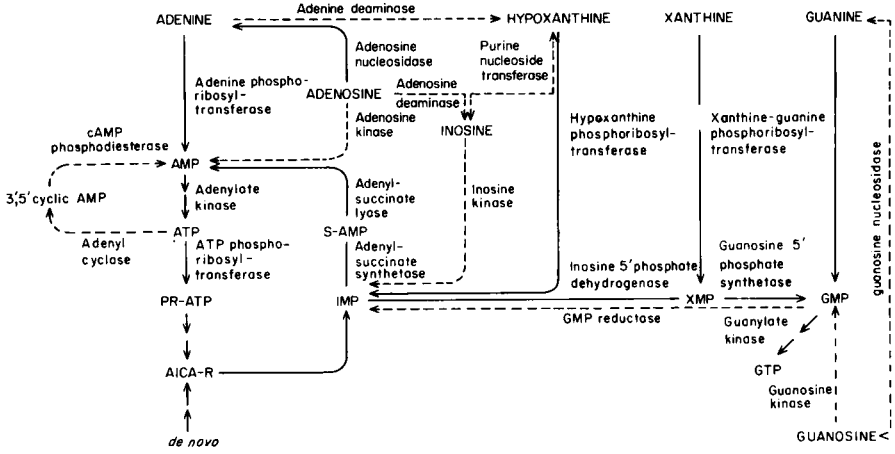

FIG. 4. Pathways for purine nucleoside biosynthesis in *Neisseria* spp. Dashed lines indicate the reactions reported to be missing in *Neisseria gonorrhoeae* or *Neisseria meningitidis*.

Figure 4 depicts the pathways of purine synthesis and interconversion proposed by Jyssum and Jyssum (1975). Adenine or hypoxanthine can serve as precursors for synthesis of purine nucleotides in meningococci. Adenine is converted to AMP by the action of adenine phosphoribosyl transferase (EC 2.4.2.7) (Jyssum, 1974a). Adenosine monophosphate is then phosphorylated to adenosine diphosphate (ADP) and adenosine triphosphate (ATP) by adenylate kinase (EC 2.7.4.3) (Jyssum and Jyssum, 1963). Adenosine triphosphate is phosphorylated by phosphoribosyl transferase (EC 2.4.2.2.c), converted to aminoimidazole-carboxamide ribonucleotide and then to inosine monophosphate (IMP). The last compound is a key intermediate which is also produced from hypoxanthine by hypoxanthine phosphoribosyl transferase (EC 2.4.2.8). It can be converted to AMP with adenylosuccinate as an intermediate or to GMP with xanthine 5'-phosphate as an intermediate. Guanosine monophosphate cannot be converted to AMP in *N. meningitidis* (Jyssum, 1974b) due to the absence of GMP reductase (Jyssum and Jyssum, 1975). Recent data indicate that GMP and AMP are not interconverted in *N. gonorrhoeae* (Table 6). Hypoxanthine replaces the requirements for adenine and guanine in a purine-requiring auxotroph of *N. meningitidis* (Jyssum, 1975) and can serve as a source of GMP and AMP in *N. gonorrhoeae* (Table 6).

Some strains of *N. gonorrhoeae* have a requirement for hypoxanthine

TABLE 6. Incorporation of purines by *Neisseria gonorrhoeae* CS-7. From unpublished data of S. A. Morse.

Fraction	Additions to defined medium				
	Adenine and guanine [8-^{14}C]adenine	Adenine and guanine [8-^{14}C]guanine	Guanine [8-^{14}C]guanine	Adenine and hypoxanthine [8-^{14}C]adenine	Hypoxanthine [8-^{14}C]hypoxanthine
Cold trichloroacetic acid soluble	13.1	15.6	16.5	13.7	7.8
Ethanol soluble	0.9	0.9	0.9	0.9	0.7
Ethanol-ether soluble	0.3	0.3	0.4	0.2	0.2
Hot trichloroacetic acid soluble	83.8	81.5	79.8	83.5	90.0
Papain soluble	1.9	1.6	2.3	1.6	1.2
Residue	0.1	0.1	0.1	0.1	0.1
Recovery (%)	113.0	109.0	102.0	107.0	105.0
Adenine (%)	97.0	2.0	5.0	98.0	46.0
Guanine (%)	3.0	98.0	95.0	2.0	54.0

Cells were grown in a purine-free defined medium similar to that described by Catlin (1973). Purines were incorporated at a final concentration of 40 μg/ml. The nucleic acid fraction (hot trichloroacetic acid-soluble cell fraction) was subjected to acid hydrolysis and the labelled purines identified by thin-layer chromatography.

(Griffin and Racker, 1956; Carifo and Catlin, 1973; Knapp and Holmes, 1975) which cannot be overcome by addition of guanine, guanosine, adenosine, AMP, ADP, ATP, xanthine, inosine, IMP, IDP or ITP (Griffin and Racker, 1956). However, addition of both adenine and guanine can replace the requirement for hypoxanthine (S. A. Morse, unpublished data).

Although adenosine 3′,5′-cyclic monophosphate (cAMP) plays an important metabolic role in many bacteria, it appears to lack any role in metabolism of *N. gonorrhoeae*. Neither cAMP nor its associated enzyme activities, adenyl cyclase (EC 4.6.1.1) and cyclic AMP phosphodiesterase (EC 3.1.4.c) was detected in gonococcal cells or culture supernatants (S. A. Morse *et al.*, 1977). These enzymes have also not been detected in *N. meningitidis* and non-pathogenic *Neisseria* species. Cyclic-AMP was not required for growth of *N. gonorrhoeae* (Catlin, 1973; LaScolea and Young, 1974), and it had no effect on metabolism of glucose or on metabolism of substrates not normally utilized by gonococci (S. A. Morse *et al.*, 1977).

XIII. Lipid Composition and Metabolism

A. PHOSPHOLIPIDS AND WHOLE-CELL FATTY ACIDS

Neisseria gonorrhoeae can synthesize all of its lipids, as evidenced by growth in chemically-defined media lacking fatty acids (Catlin, 1973; LaScolea and Young, 1974; Hunter and McVeigh, 1970). Lipid comprised between 8.2 and 10.2% of the dry weight of gonococci (Sud and Feingold, 1975; Beebe and Wlodkowski, 1976). More than 80% of the lipids were phospholipids (Sud and Feingold, 1975). The proportion of phospholipid to total lipid in gonococci was greater during the stationary phase than during the exponential phase of growth (Sud and Feingold, 1975). The phospholipid composition of *N. gonorrhoeae* has been evaluated by several groups of investigators (Wolf-Watz *et al.*, 1975; Senff *et al.*, 1976; Beebe and Wlodkowski, 1976; Sud and Feingold, 1975; Walstad *et al.*, 1974) and, with some exceptions, was similar to that of other Gram-negative bacteria. The results of quantitative phospholipid analysis are summarized in Table 7. Phosphatidylethanolamine and phosphatidylglycerol were the major phospholipids in all strains of *N. gonorrhoeae* examined. Phosphatidylethanolamine comprised approximately 74% of the cellular phospholipids; low concentrations (approx. 2.6%) of diphosphatidylglycerol (cardiolipin) were also present.

TABLE 7. Phospholipid composition of *Neisseria gonorrhoeae*

Strain	Growth phase	Percent of total phospholipids PA	CL	PE	PG	PC	LPE	LCL	LPG	Reference
NYH002	stationary	ND	8.6	74.2	15.4	ND	1.8	NR	NR	Beebe and Wlodkowski (1976)
NYH006	stationary	ND	5.9	82.2	9.1	ND	2.2	NR	NR	Beebe and Wlodkowski, (1976)
2686 (T1)	exponential	NR	2.0	77.0	19.0	NR	2.0	ND	ND	Senff *et al.* (1976)
2686 (T4)	exponential	NR	1.0	76.0	22.0	NR	1.0	ND	ND	Senff *et al.* (1976)
BI-21	exponential	NR	0.8	74.6	22.2	2.3	NR	NR	NR	Sud and Feingold (1975)
BI-21	stationary	NR	0.3	69.7	18.5	11.4	NR	NR	NR	Sud and Feingold (1975)
Copenhagen V	exponential	NR	1.3	75.9	22.6	NR	NR	NR	NR	Wolf-Watz *et al.* (1975)

Phospholipid abbreviations are: PA, phosphatidic acid; CL, cardiolipin; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PC, phosphatidylcholine; LPE, lysophosphatidylethanolamine; LCL, lysocardiolipin; LPG, lysophosphatidylglycerol. ND indicates that the phospholipid was not detected, and NR that its content was not reported.

TABLE 8. Effect of medium pH value on the phospholipid composition of *Neisseria gonorrhoeae*. Unpublished data of A. F. Cacciapuoti, W. S. Wegener and S. A. Morse

pH Value	Percent of total phospholipids			
	Lysophosphatidyl-ethanolamine	Phosphatidyl-ethanolamine	Phosphatidyl-glycerol	Ratio of previous two analyses
6.0	1.2	78.8	17.4	4.5:1
7.2	2.0	73.1	21.5	3.4:1
8.0	2.1	68.3	25.8	2.6:1

Data were obtained from freshly extracted, exponential phase cells of *Neisseria gonorrhoeae* strain JW-31.

The phospholipid composition of *N. gonorrhoeae* varied with pH value of the growth medium (Table 8). Significant differences were observed in the relative amounts of phosphatidylethanolamine and phosphatidylglycerol in exponential-phase cells grown at pH values of 6.0, 7.2 and 8.0. These differences were markedly apparent by the change in the ratios of the two phospholipids.

Sud and Feingold (1975) detected small quantities of phosphatidylcholine in exponential-phase cells. The reported presence of this phospholipid in *N. gonorrhoeae* was unusual since it is rarely found in bacteria (Rizza *et al.*, 1970). Its identification was based upon a comparison of the R_f value with that of a known standard after thin-layer chromatography on silica-gel plates. The presence of phosphatidylcholine was further verified by a positive Dragendorff reaction and the presence of choline on paper chromatograms of acid-hydrolysed phospholipid. However, this finding has not been confirmed (Senff *et al.*, 1976; Beebe and Wlodkowski, 1976). Phosphatidylcholine was not detected in either exponential- or stationary-phase cells of recent isolates or laboratory strains of *N. gonorrhoeae* even when the procedure described by Sud and Feingold (1975) was used. The phospholipid was present in cells of *Neisseria* (*Branhamella*) *catarrhalis* (Beebe and Wlodkowski, 1976). About 30% of the cellular lipid in *N. catarrhalis* was neutral lipid whereas, in *N. gonorrhoeae*, it comprised about 8%. In addition, gonococci had roughly twice the amount of phosphatidylethanolamine as *N. catarrhalis*, the latter having variable levels of both phosphatidylethanolamine and phosphatidyglycerol depending on the strain. Lysophosphatidylethanolamine was not detected in *N. catarrhalis*.

Lysophosphatidylethanolamine is present in *N. gonorrhoeae* (Senff *et al.*,

1976; Beebe and Wlodkowski, 1976) but only in low concentrations unless the cells were subjected to physical manipulations. For example, Senff *et al.* (1976) detected only 1–2% of the compound in actively growing cells which were extracted directly for analysis of cellular lipids; higher concentrations (6 to 11%) were observed in exponential-phase cells lyophilized prior to extraction. These results suggested that physical manipulations of the cells, such as centrifugation, cell suspension or lyophilization, resulted in increased concentrations of the lysophospholipid, which also increased in content as a function of growth. When growth was limited by depletion of the energy source (glucose), an increase in the content of lysophosphatidylethanolamine occurred and was coincidental with the onset of autolysis (Beebe and Wlodkowski, 1976). Walstad *et al.* (1974) reported the presence of lysophosphatidylethanolamine in 24-h cultures grown on agar plates. Once colonies became visible, agar-grown cells were no longer in the exponential phase of growth. Turnover of phospholipids was balanced by their synthesis during exponential growth. Factors, e.g. glucose-depletion and growth phase which decreased their rate of synthesis, did not appear to have a similar effect on their rate of hydrolysis.

During growth in liquid medium, changes occurred in the phospholipid composition of *N. gonorrhoeae*. As cells entered the stationary phase, the content of phosphatidylethanolamine decreased slightly and was accompanied by a concomitant increase in the lyso derivative of this compound (Beebe and Wlodkowski, 1976). The proportion of phosphatidylglycerol declined approximately 40% throughout growth and that of cardiolipin increased three-fold. A similar increase in cardiolipin has been observed in other organisms (Randle *et al.*, 1976).

The phospholipid composition of the inner (cytoplasmic) membrane of *N. gonorrhoeae* differs from that of the outer membrane. Wolf-Watz *et al.* (1975) reported that cardiolipin is located primarily in the outer membrane, and that the inner membrane has a slightly higher proportion of phosphatidylglycerol relative to the other phospholipids. Since cardiolipin is synthesized directly from phosphatidylglycerol, it was not surprising that there was a decreased content of the latter phospholipid in the outer membrane. The inner and outer membrane did not differ significantly with respect to content of phosphatidylethanolamine.

Sud and Feingold (1975) determined the fatty-acid composition of isolated gonococcal phospholipids. The major fatty acids were palmitic

(16:0) and palmitoleic (16:1) acids which comprised 75 to 80% of the total fatty acids detected. Octadecanoic (18:1) and myristic (14:0) acids were found to be minor components. Cyclopropane fatty acids, and those with greater than 18 carbon atoms commonly found in other Gram-negative bacteria (Kates, 1964; Asselineau, 1966), were not detected in *N. gonorrhoeae*. β-Hydroxylauric acid (12:0-hydroxyl) was found to be a minor constituent in phospholipids, but to be present in large amounts after whole-cell saponification. Wolf-Watz *et al.* (1975) compared the outer membrane fatty-acid composition of three strains of *N. gonorrhoeae*, and reported that lauric (12:0), β-hydroxymyristic (14:0-hydroxyl) and stearic (18:0) acid residues were not major components of outer-membrane phospholipids but were probably associated with the lipid A.

Concerning the fatty-acid composition of whole cells, Yamakawa and Ueta (1964) observed close similarity between *N. gonorrhoeae*, *N. meningitidis* and several non-pathogenic *Neisseria* species. The common fatty acids were lauric (12:0), myristic (14:0), palmitic (16:0), palmitoleic (16:1), octadecanoic (18:1), β-hydroxylauric (12:0-hydroxyl) and β-hydroxymyristic (14:0-hydroxyl), with palmitic, palmitoleic and occasionally octadecanoic being the predominant fatty acids in these bacteria. A number of later studies (Moss *et al.*, 1970; Lambert *et al.*, 1971; Jantzen *et al.*, 1974; Walstad *et al.*, 1974; Sud and Feingold, 1975) were in general agreement. *Neisseria gonorrhoeae*, *N. meningitidis* and most of the non-pathogenic species studied formed a homogenous group with respect to fatty-acid composition. Only *N.* (*Branhamella*) *catarrhalis*, *N. caviae*, *N. ovis*, *N.* (*Gemella*) *haemolysans* and *N. dentrificans* differed markedly from the fatty-acid composition of *N. gonorrhoeae* (Lewis *et al.*, 1968) and, in fact, showed a closer similarity to fatty-acid fingerprints of organisms in the genus *Moraxella* (Jantzen *et al.*, 1974). Bryn *et al.* (1977) reported that waxes, i.e. simple esters of fatty alcohols and fatty acids, were not present in *N. gonorrhoeae* and other species of *Neisseria*. However, they were present in *B. catarrhalis*, *N. caviae*, *N. ovis* and *Moraxella* spp.

A summary of the fatty-acid composition of *N. gonorrhoeae* is presented in Table 9. The high proportion of even-numbered fatty-acid residues in the gonococcus was in agreement with studies on the fatty-acyl composition of other Gram-negative bacteria (Kates, 1964; Cho and Salton, 1966). However, the contents of the six major fatty-acyl residues present in the gonococcus were rather distinct from that of other bacteria. Moss *et al.* (1970) examined 20 strains of *N. gonorrhoeae* and reported that approximately 78% of the total cellular fatty-acyl residues were made up

TABLE 9. Fatty-acid composition of *Neisseria gonorrhoeae*[a]

Fatty acid	Percent of total fatty acid				
	Yamakawa and Ueta (1964)[b] average of two strains	Moss *et al.* (1970)[c] average of 20 strains	Jantzen *et al.* (1974)[d] average of two strains	Walstad *et al.* (1974)[e] average of two strains	Sud and Fenigold (1975)[f] average of two strains
Lauric (12:0)	6	15	3	ND	10
Myristic (14:0)	8	8	3	7	8
Palmitic (16:0)	43	19	34	42	28
Stearic (18:0)	3	1	3	1	1
Palmitoleic (16:1)	32	22	26	37	33
Octadecanoic (18:1)	8	7	12	8	5
Octadecadienoic (18:2)	ND	ND	2	ND	ND
β-Hydroxylauric (12:0-hydroxyl)	+	22	8	ND	14
β-Hydroxymyristic (14:0-hydroxyl)	+	2	7	ND	1
Others	ND	4	4	7	Tr

[a]ND indicates that the acid was not detected. Tr indicates that a trace amount of the acid was detected; + indicates the presence of these acids, but no percentage values were available.

[b]Data recalculated as percent of total fatty acids. Hydroxy acids are not included in the total. Cells were grown on GC base medium (Eiken) containing 10% horse serum at 37°C for 20 h.

[c]Cells were grown on GC agar (Difco) supplemented with cocarboxylase, glutamine and ferric nitrate (defined supplement, 1% v/v) for 18 h at 36°C in a carbon dioxide-containing atmosphere.

[d]Cells were grown on blood agar at 37°C for 20 h in a carbon dioxide-containing atmosphere.

[e]Data were rounded off to the nearest percent. Experimental techniques to detect hydroxy acids were not used in this study. Cells were grown on GC agar (Difco) including 1% defined supplement at 37°C for 24 h in an atmosphere containing 5% carbon dioxide.

[f]Cells were grown aerobically in liquid medium (similar to GC base medium, Difco) supplemented with glucose, sodium bicarbonate and Difco supplement B at 37°C to a cell density of $2 - 10^8$ to $4 - 10^8$ colony-forming units/ml.

of palmitic (19%), palmitoleic (22%), β-hydroxylauric (22%) and lauric (15%) acid residues. Myristic (8%) and octadecanoic (7%) acid residues were present in moderate amounts, while only small or trace amounts of β-hydroxymyristic, pentadecanoic, heptadecanoic, stearic and other acid residues were detected.

The fatty-acyl residue patterns of *N. gonorrhoeae* were reproducible under constant growth conditions. However, it is a well documented phenomenon (Kates, 1964) that alteration in environmental parameters, or change in the growth phase, can alter the fatty-acyl composition. Such changes have been observed in members of the genus *Neisseria*. Lambert *et al.* (1971) observed that the composition of the growth medium affected the fatty-acyl composition of nonpathogenic *Neisseria* spp. Sud and Feingold (1975) reported the appearance of $C_{18\,2}$ residue as a major constituent of *N. gonorrhoeae* as cells entered the stationary phase of growth.

B. LIPASE AND PHOSPHOLIPASE ACTIVITY

Piotrowski and Berger (1973) suggested that lipolytic activity was present in *N. gonorrhoeae* and most other species of *Neisseria* by observing the hydrolysis of Tween 20 (polyoxyethylene sorbitan monolaurate) and glyceryltributyrate. No mention was made of the degree of hydrolysis, location of the enzymes or utilization of the products of hydrolysis. However, Miller *et al.* (1977) reported that saturated fatty-acids (C_1-C_{20}) and the C_{18} unsaturated fatty-acids, elaidic and oleic acids, failed to stimulate oxygen consumption by cell suspensions of *N. gonorrhoeae*.

Medium and long chain-(C_{10}-C_{15}) length fatty acids accumulated in the medium during growth of *N. gonorrhoeae* (Brooks *et al.*, 1971). In addition, substances which inhibited growth of most strains of *N. gonorrhoeae* and *N. meningitidis* were released during growth of *N. gonorrhoeae* (Walstad *et al.*, 1974). These inhibitors were identified as free fatty acids and a minor phospholipid component, lysophosphatidylethanolamine. The mechanism by which these inhibitory free fatty acids were produced was not determined, but the authors suggested that there were phospholipases present which degraded phosphatidylethanolamine to lysophosphatidylethanolamine and glycerophosphatidylethanolamine with the release of one or both fatty acids. Wolf-Watz *et al.* (1975) subsequently reported the presence of phospholipase A and lysophospholipase activities in outer-membrane preparations from *N.*

gonorrhoeae. The properties of gonococcal phospholipase A (EC 3.1.1.4) were studied by Senff *et al.* (1976). Similar phospholipase activities were present in partially purified outer-membrane preparations. Enzyme activity required the presence of Ca^{2+} (5 m*M* for optimal activity) and exhibited an optimum pH value range between 8.0 and 9.0. Enzyme activity decreased markedly at pH values less than 8.0. Optimum activity was obtained with 10% methanol; higher concentrations were increasingly inhibitory. Ethanol and isopropanol could substitute for methanol but were only 75% and 23% as effective, respectively. *N*-Propanol, n-butanol and isobutanol were ineffective when substituted for methanol. Phospholipase A activity was sensitive to Triton X-100 (0.1%) and other detergents which further substantiated the association of this enzyme with the membrane.

Senff *et al.* (1976) compared the activity of gonococcal phospholipase A for the major outer-membrane phospholipids, phosphatidylethanolamine, cardiolipin and phosphatidylglycerol. The activity of the enzyme was two to three times greater with phosphatidylethanolamine than with phosphatidylglycerol. It could not be determined whether this was caused by a single enzyme with multiple substrate specificities or multiple enzymes with single substrate specificity. The cellular function of this enzyme remains to be elucidated. Manipulations such as centrifugation and resuspension of cells or lyophilization appear to stimulate the activity of phospholipase A. However, this may be nothing more than an imbalance in the relationship between phospholipid synthesis and degradation. Beebe and Wlodkowski (1976) observed a similar increase in the content of lysophosphatidylethanolamine as cultures entered the stationary phase of growth. In addition, end products of phospholipase A activity were apparently released during growth of *N. gonorrhoeae* (Brooks *et al.*, 1971; Walstad *et al.*, 1974).

Studies in our laboratory (A. F. Cacciapuoti, W. S. Wegener and S. A. Morse, unpublished data) indicated that the phospholipids of intact cells of *N. gonorrhoeae*, grown to late exponential phase and resuspended in Hepes buffer at pH 8.5 in the presence of Ca^{2+} or Mn^{2+} (20 m*M*), underwent hydrolysis. Hydrolysis of phosphatidylethanolamine and phosphatidylglycerol appeared to be mediated by the combined action of phospholipase A and lysophospholipase, which was apparent by accumulation of lysophospholipids, and free fatty acids in intact cells, with a corresponding decrease in the concentrations of phosphatidylethanolamine and phosphatidylglycerol. In the presence of

Ca^{2+}, phospholipase A activity was greater with phosphatidylethanolamine than with phosphatidylglycerol (see Table 10). These results were similar to those obtained by Senff *et al.* (1976) with cell-envelope preparations. The activity in the presence of Mn^{2+} was greater with phosphatidylglycerol than phosphatidylethanolamine. Manganese ions also stimulated lysophospholipase activity. Methanol, ethanol, propanol and isopropanol, added at concentrations which inhibited growth by 50%, markedly stimulated phospholipase A but not lysophospholipase activity. Differential heat inactivation and pH value optima suggested that phospholipase A activities with either phosphatidylethanolamine or phosphatidylglycerol and lysophospholipase were separate enzymes.

C. INHIBITORY ACTIVITY OF PHOSPHOLIPIDS AND FATTY ACIDS ON GROWTH

The presence of free fatty acids in the growth medium (Catlin, 1973; Bacigalupi and Lawson, 1973; Hunter and McVeigh, 1970), often as contaminants in agar, were inhibitory to growth of *N. gonorrhoeae.* Because of this inhibition, agents such as starch, serum albumin or

TABLE 10. *In vivo* Phospholipid hydrolysis in *Neisseria gonorrhoeae*

Additions	Percent of ^3H recovered as: Phosphatidyl-ethanolamine	Phosphatidyl-glycerol	Lysophosphatidyl-ethanolamine	Free fatty acids
None	71.3	21.7	1.3	4.5
Ca^{2+}	36.2	17.4	7.3	36.9
Mn^{2+}	45.1	10.7	3.4	38.1
Ca^{2+} + methanol (1.25 *M*)	25.6	14.9	9.9	47.8
Ca^{2+} + ethanol (0.7 *M*)	21.5	17.1	14.3	42.5
Ca^{2+} + propanol (0.2 *M*)	21.1	16.8	13.4	44.0
Ca^{2+} + isopropanol (0.3 *M*)	18.0	14.8	14.5	48.1

Tritium acetate-labelled exponential-phase cells of *Neisseria gonorrhoeae* strain JW-31 were suspended in Hepes buffer (pH 8.5). Divalent cations (20 m*M*) were added as indicated. Alcohols were added at the concentrations indicated. Cells were extracted with chloroform-methanol (1:1) after incubation for two hours. Distribution of tritium in phospholipids was determined by thin-layer chromatography and scintillation counting of scraped spots as previously described (Cacciapuoti *et al.*, 1978).

charcoal were added to the medium in order to bind these compounds. An inhibitory substance excreted from gonococcal cell pellets was completly inactivated by 2% (w/v) bovine serum albumin and partially inactivated by addition of 1% soluble starch (Walstad *et al.*, 1974). Additional studies, which showed the insensitivity of this inhibitor to inactivation by pronase, trypsin or deoxyribonuclease and its stability after autoclaving, suggested that it was lipid in nature. Chromatographic separation of chloroform-methanol extracts of washed whole cells revealed that the material was composed primarily of phospholipids and free fatty acids. Of the phospholipids, only lysophosphatidylethanolamine was inhibitory. The inhibition produced by free fatty acids increased with their chain length to a maximum with C_{14}-C_{16}. Unsaturated fatty acids were also inhibitory. It was suggested that the inhibition was due to the detergent-like activity of lysophosphatidylethanolamine and free fatty acids on the surface of the gonococcus. These observations were confirmed by Miller *et al.* (1977), who studied the inhibition produced by saturated fatty acids. The inhibitory effectiveness increased with fatty-acid chain length to a maximum obtained with palmitic acid (C_{16}). Stearic (C_{18}) acid and saturated fatty acids with longer chains were relatively ineffective in inhibiting growth of *N. gonorrhoeae*. However, unsaturated fatty acids with chain lengths of 16 to 20 carbon atoms were inhibitory.

Palmitic (C_{16}) acid and the unsaturated 16:1 and 18:1 fatty acids were among the major fatty acids of *N. gonorrhoeae* (see Table 9, p. 304). Both Walstad *et al.* (1974) and Miller *et al.* (1977) observed that these fatty acids were very inhibitory for growth of *N. gonorrhoeae*. Production of inhibitory concentrations of these fatty acids may, in part, explain the relatively short survival time of this organism.

The permeability of the cell envelope was related to inhibition of growth by free fatty acids. Wild-type Gram-negative organisms, such as *S. typhimurium* and *Ps. aeruginosa*, were relatively resistant to medium-chain (C_7-C_{10}) fatty acids and completely resistant to long-chain (C_{12}-C_{18}) fatty acids. However, a deep-rough mutant of *S. typhimurium* and the Gram-positive *Bacillus subtilis* were inhibited in a fashion similar to *N. gonorrhoeae* (Miller *et al.*, 1977).

Inhibition of gonococcal growth by short-chain (C_1-C_6) fatty acids was pH-dependent and increased with decreasing pH value (Miller *et al.*, 1977) which suggested that this inhibition was attributable to the undissociated form of the acid. Saturated fatty acids also inhibited

oxygen consumption by logarithmic-phase cells of *N. gonorrhoeae* (Miller *et al.*, 1977). Inhibition of oxygen consumption increased with fatty-acid chain length to a maximum with myristic (C_{14}) acid. Both saturated (C_1 to C_{12}) and unsaturated (C_{16} to C_{20}) fatty acids inhibited reduced nicotinamide adenine dinucleotide oxidase activity *in vitro* (Miller *et al.*, 1977). However, since the concentrations of fatty acids required to inhibit this enzyme were generally higher than those required to inhibit growth or oxygen consumption, fatty acids may inhibit growth at sites other than the electron-transport chain.

D. LIPID A

Approximately 37% of the dry weight of gonococcal lipopolysaccharide was comprised of lipid A (Stead *et al.*, 1975; Wiseman and Caird, 1977). There were no significant differences in the amount of lipid A isolated from the various colonial variants of *N. gonorrhoeae* (Stead *et al.*, 1975). The lipopolysaccharide of *N. meningitidis* contained an amount of lipid A similar to that of *N. gonorrhoeae* (Jennings *et al.*, 1973). Wiseman and Caird (1977) reported that the lipid A of *N. sicca* and *N. lactamica* comprised 35% and 33%, respectively, of the dry weight of the lipopolysaccharide. Adams *et al.* (1968) reported a value of 59% for *N. perflava*.

A major portion (44–74%) of the lipid A in *N. gonorrhoeae* and *N. meningitidis* strains was composed of fatty acids (Stead *et al.*, 1975; Jennings *et al.*, 1973). The major fatty acids identified in the lipid A of *N. gonorrhoeae* were 10:0, 12:0, 14:0, 14:0-hydroxyl, 16:0 and 18:0 (Wolf-Watz *et al.*, 1975; Wiseman and Caird, 1977; Stead *et al.*, 1975). There was no significant difference in fatty-acyl composition of lipid A from different colonial variants of *N. gonorrhoeae* (Wiseman and Caird, 1977). Both qualitative and quantitative differences in fatty-acyl composition of lipid A have been observed between strains of various *Neisseria* species. McDonald and Adams (1971) found that environmental conditions, such as aeration rate, markedly affected the lipid composition of the lipopolysaccharide of *N. sicca*. Cells grown at a high rate of aeration contained a higher percentage of unsaturated fatty-acyl residues than cells grown at a low rate of aeration. Environmental conditions also affected the fatty-acyl composition of lipid A in *N. gonorrhoeae*. Stead *et al.* (1975) found that *N. gonorrhoeae* grown with 15% oxygen saturation contained 16:1 and 18:1 fatty-acyl residues in its lipid A. Wolf-Watz *et al.*

(1975), using gonococci grown under normal atmospheric conditions, and Wiseman and Caird (1977), using cells grown with increased tensions of carbon dioxide, were not able to detect these fatty-acyl residues in lipid A. These results suggested that *N. gonorrhoeae* (and other *Neisseria* species) synthesized mono-unsaturated fatty acids by a direct oxygen-dependent desaturation of the corresponding saturated fatty acids.

A number of investigators have reported conflicting data concerning the presence of β-hydroxy acids in *N. gonorrhoeae*. Moss *et al.* (1970) observed that β-hydroxylauric acid remained associated with cells after continuous chloroform-methanol treatment, whereas other fatty acids were completely extracted. β-Hydroxy-acyl residues were major components of lipid A and required saponification or acid extraction to release them from their stable amide linkages. Also, β-hydroxycapryl (10:0-hydroxyl), β-hydroxylauryl (12:0-hydroxyl) and β-hydroxymyristyl (14:0-hydroxyl) residues have been reported in lipid A of gonococci by Stead *et al.* (1975), but only β-hydroxymyristyl residues were detected by Wolf-Watz *et al.* (1975) and Wiseman and Caird (1977). The reason for this difference may be related to variations in the gas chromatographic analytical techniques used for identification of the fatty-acyl methyl esters. For example, Wiseman and Caird (1977) used a stainless-steel column which was reported to destroy the β-hydroxylauric methyl ester (Moss *et al.*, 1970). Stead *et al.* (1975) detected the 10:0-hydroxyl and 12:0-hydroxyl fatty acids using a glass column.

E. STEROIDS

Cholesterol and other steroids are not incorporated into cell membranes by most bacteria (Kates, 1964). The presence of *N. gonorrhoeae* within the genito-urinary tract exposes these organisms to relatively large concentrations of gonadal steroids. In addition, disseminated infections expose blood-borne gonococci to relatively high concentrations of cholesterol. Interaction of gonadal steroids with *N. gonorrhoeae* was suggested by the early studies of Koch (1947) which showed a correlation between isolation of gonococci from cervical cultures and the phase of the menstrual cycle from which the cultures were isolated. The lowest isolation rate for *N. gonorrhoeae* corresponded with the peak of progesterone activity. Gonadal steroids and related azasteroids inhibited growth of Gram-positive but not Gram-negative bacteria (Smith *et al.*,

1964; Varrichio *et al.*, 1967; Yotis and Stanke, 1966; Yotis and Waner, 1968). Members of the genus *Neisseria*, particularly *N. gonorrhoeae* and *N. meningitidis*, were exceptions to this rule (Morse and Fitzgerald, 1974; Fitzgerald and Morse, 1976). Growth of *N. gonorrhoeae* was inhibited by progesterone and several other gonadal steroids. The extent of inhibition and its reversibility were dependent upon the ratio of progesterone-to-cell mass (Morse and Fitzgerald, 1974). Bound progesterone was associated with the lipid-containing cell fractions, i.e. the cell membrane. Membrane fractions from progesterone-sensitive organisms bound this steroid more readily than membranes from insensitive cells. Miller and Morse (1977) found that the cytoplasmic membrane bound four times more progesterone than the outer membrane. In addition, isolated cytoplasmic-membrane proteins bound three times more progesterone per milligram of protein than the intact membrane. The interaction of progesterone with the cytoplasmic membrane resulted in an inhibition of NADH oxidase activity (Morse and Fitzgerald, 1974). Inhibition of this enzyme system would severely restrict growth of an obligately aerobic organism such as *N. gonorrhoeae*. The major site of inhibition appeared to be close to the NADH dehydrogenase region of the electron-transport chain, since addition of lactate partially reversed inhibition of growth by progesterone.

The ability to bind progesterone was related, in part, to the permeability of the outer membrane. Whole cells of *Ps. aeruginosa* and *S. typhimurium* were unable to bind progesterone, and their growth was not inhibited. However, isolated membrane fractions from these organisms bound progesterone, as did membranes from *N. gonorrhoeae*. Thus, the outer membrane of many Gram-negative bacteria may prevent progesterone from binding to sites in the cytoplasmic membrane.

XIV. Inorganic Ions

Inorganic ions are required for growth and maintenance of all cells. There have been relatively few studies on the effects of inorganic ions on growth of *N. gonorrhoeae*. Divalent cations (Mg^{2+}, Ca^{2+}) markedly inhibited autolysis (Elmros *et al.*, 1976a; Wegener *et al.*, 1977a) presumably by stabilization of the cell membrane. Phospholipase A from *N. gonorrhoeae* required the presence of a divalent cation (Senff *et al.*, 1976; Cacciapuoti *et al.*, 1978) for optimal activity. The activity of phospholipase A in the presence of Ca^{2+} was greater with

phosphatidylethanolamine than with phosphatidylglycerol as substrate. In the presence of Mn^{2+}, phospholipase A activity was greater with phosphatidylglycerol as substrate. Neither Mg^{2+} nor Zn^{2+} enhanced phospholipase A activity. Ferrous ions were not as effective as Ca^{2+} or Mn^{2+} in stimulating phospholipase A activity.

Kellogg *et al.* (1968) reported that ferric ions stimulated growth of *N. gonorrhoeae* as measured by an increase in colony diameter. The types of anions accompanying the ferric ions had no effect. Haemin and ferrous gluconate were not stimulatory. The only observable difference in colonial morphology was an increase in the darkness of the colonial colouration. Payne and Finkelstein (1977) observed that Imferon, an iron-dextran complex, could replace ferric nitrate and significantly increased the average colony size. The stimulatory effect of Imferon appeared to be attributable to both the iron and dextran components. The function of the dextran was not known; however, these authors suggested that it might serve to bind fatty acids and other toxic compounds in the medium.

Iron has been implicated in the expression of virulence by both *N. gonorrhoeae* and *N. meningitidis*. Payne and Finkelstein (1975) showed that intravenous inoculation of iron compounds with gonococci from colonial types 3 and 4 increased the lethality of these avirulent organisms for chicken embryos. Furthermore, the lethality of virulent organisms from colony type 1 was lowered or delayed when cells were inoculated together with conalbumin, an iron-binding protein. More recently, Payne *et al.* (1978) observed that another laboratory strain and three of six urogenital isolates behaved in a similar fashion when inoculated with conalbumin. In contrast, none of six isolates from disseminated gonococcal infections was inhibited by conalbumin. The urogenital isolates which were unaffected by conalbumin may have represented strains which had the potential to disseminate. These results suggested that certain strains of gonococci probably contained siderophores which competed for available iron with iron-binding proteins *in vivo*. Another recent report by Payne and Finkelstein (1978a) mentioned that siderophores from *Vibrio cholerae* stimulated growth of *N. gonorrhoeae* and *N. meningitidis*, whereas enterochelin from *E. coli*, *Salmonella* sp. and *Shigella* sp. did not. Gonococci also responded to homologous and meningococcal culture filtrates. However, gonococcal filtrates did not stimulate growth of other Gram-negative bacteria tested (Payne and Finkelstein, 1978b). Calver *et al.* (1976) demonstrated that prior injection of ferrous sulphate or

concomitant injection of iron sorbitol citrate or iron dextran with meningococci increased the lethality of several different meningococcal sero-groups for mice by up to a million-fold. The effect of added iron was found to be partially abrogated by prior incubation of meningococci with desferal, an iron-chelating compound.

Hafiz *et al.* (1977) found that high concentrations of ferric citrate stimulated reversion of type 4 gonococci to type 1 during growth in a liquid medium. Odugbemi and Hafiz (1978) further demonstrated that both iron and an iron chelator were necessary for this reversion. These authors suggested that iron chelators would affect the availability of iron to the gonococcus, but whether the iron content of cells would be increased or decreased was not known. Odugbemi and Dean (1978) found that the iron content of virulent gonococci from colonial types 1 and 2 averaged 0.13 μg Fe/mg dry weight, while gonococci from the avirulent types 3 and 4 averaged 0.17 μg Fe/mg dry weight. These authors suggested that an increase in the concentration of iron in the host might provide the gonococcus with the necessary concentration of iron to express virulence.

Copper ions are inhibitory to *in vitro* growth of *N. gonorrhoeae* (Fiscina *et al.*, 1973; Johannisson *et al.*, 1976). The presence of serum was found to protect gonococci from the toxic effects of copper ions.

Mitzel *et al.* (1972) tested nasopharyngeal isolates of *N. meningitidis* for growth on nutrient agar with or without sodium chloride, and found a requirement in some strains for either sodium or chloride, or both. An early study by Miller *et al.* (1932) on the influence of inorganic salts on growth of the gonococcus found that either sodium or potassium supported growth. Calcium and magnesium were not essential and even inhibitory at higher concentrations; chloride could be replaced by either nitrate or sulphate. Since this study used a single isolate, it is possible that some strains of gonococci possess requirements for inorganic ions as do some strains of meningococci.

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Early Events During Bacterial Endospore Formation

M. YOUNG and J. MANDELSTAM

Note added in proof

In the time that has elapsed since this article went to press, the only report of a successful DNA cloning experiment in *Bacillus subtilis* that has come to our attention has been that of Keggins, Lovett and Duvall (*Proceedings of the National Academy of Sciences of the United States of America* **75**, 1423, 1978). The authors cloned fragments of DNA isolated from *B. subtilis, B. licheniformis* and *B. pumilis* that were able to complement the *trpC2* mutation in *B. subtilis*. The vector used in these experiments was one of a group of plasmids originally found in *Staphylococcus aureus* and subsequently transferred to *B. subtilis* (Ehrlich, *Proceedings of the National Academy of Sciences of the United States of America* **74**, 1680, 1977; Gryczan and Dubnau, Ibid **75**, 1428, 1978). No indigenous plasmids with selectable genetic markers are known in *B. subtilis*. In order to clone fragments of *B. subtilis* DNA that do not contain a readily selectable genetic marker (e.g. DNA containing *spo* genes, which are expressed *only* during sporulation, and cannot therefore be selected for during growth) the investigator would normally rely on the expression of heterospecific genetic information viz. an antibiotic resistance marker on the plasmid. Unfortunately, in contrast to *Escherichia coli, B. subtilis* has proved to be far from catholic in its ability to express non-homologous genetic information (Ehrlich, *Proceedings of the National Academy of Sciences of the United States of America* **75**, 1433, 1978; Ehrlich and Sgaramella, *Trends in Biochemical Sciences* **3**, 259, 1978), and this may account for the fact that so few successful cloning experiments have yet been reported.

One possible way in which this difficulty could be circumvented is by the use of a suitable bacteriophage as an alternative cloning vehicle. A promising start has been made by Kawamura, Saito and Ikeda (*Gene* **5**, 87, 1979), who mixed and ligated EcoRI-restricted DNA obtained from the bacteriophage ρ11 and from *B. subtilis* (this latter DNA was obtained from particles of the defective phage PBSX). The DNA was then used to transform a ρ11 lysogen of *B. subtilis*. Some, at least, of the transformants to *lys*$^+$ and *hisA*$^+$ were found to harbour a specialised transducing phage that had presumably been generated by recombination *in vitro* (i.e. when the restricted phage DNA and the restricted *B. subtilis* DNA were ligated). It is not yet known whether the transforming molecules are linear or circular, nor is it clear precisely where the incoming copy of the *lys* or *hisA* gene is integrated—the authors suggest that the most likely place is the site of the ρ11 prophage in the lysogen. If this example of specialised transduction proves to be of general application in *B. subtilis*, it will provide a long-awaited and convenient method for investigations of the dominance relationships of the *spo* genes and for complementation testing.

A number of interesting papers have been published by Balassa and his colleagues. The majority of these (*Molecular and General Genetics* **163** 35, 45, 57, 65 and 285) deal with what appear to be sporulation control (*sco*) mutations in *B. subtilis*. These are "mutations that affect the quantitative regulation of sporulation-associated products and/or the time course of the morphogenesis". The mutants that harbour these defects were selected on the basis of their overproduction of extracellular protease activity. The mutants overproduce alkaline phosphatase and, apparently, spore coat protein as well as extracellular serine protease and metalloprotease (Sousa, Silva and Balassa, *Molecular and General Genetics* **128**, 261, 1974). One group (*scoA*) lies between *metC* and *argC* on the *B. subtilis* chromosome. The overproduction of alkaline phosphatase in *scoA* strains is reminiscent of the phenotype associated with the *sapA* mutations that also lie in this region

(Piggot and Taylor, 1977). Another group (*scoC*) closely resembles the *catA* mutants described by Ito and Spizizen (*Colloques Internationaux du Centre National de la Recherche Scientifique* **227**, 81, 1973) both in their map position and their associated phenotype. A third group (*scoB,D*) lies between *ura* and *metC* in a region where a number of *spo* loci have been located (Fig. 2, p. 131).

Balassa and his colleagues have also described a class of "decadent" sporulation mutants that seem to differ from *spo* mutants in that populations of sporulating cells contain individuals apparently blocked at a number of different stages (Balassa, Milhaud, Sousa and Silva, *Journal of General Microbiology* **110**, 381, 1979). Such a phenotype could arise if, for example, these mutants harbour defects in a general mechanism of transcriptional control, such that the efficiency of activation of successive *spo* loci is reduced.

In another paper, Balassa, Milhaud, Raulet, Silva and Sousa (*Journal of General Microbiology* **110**, 365, 1979) describe a late-blocked mutant of *B. subtilis* that will produce heat-resistant spores in the presence of added dipicolinic acid. This mutant has a similar phenotype to mutants previously described in *B. megaterium* and *B. cereus* (Fukuda and Gilvarg, *Journal of Biological Chemistry* **243**, 3871, 1968; Wise *et al.*, *Journal of Bacteriology* **94**, 2075, 1967; Zytkovic and Halvorson, 1972). These studies strongly suggest that dipicolinic acid does indeed play an important role in the development of heat resistance in the bacterial spore. We have previously mentioned a mutant of *B. subtilis* that apparently lacks dipicolinic acid but can nevertheless produce heat-resistant spores (Zytkovic and Halvorson, 1972 – see p. 112). The contradiction might possibly arise because of small quantitative differences in the amounts of dipicolinic acid present in the two mutants, since very little of the compound may suffice to confer heat resistance (Balassa *et al.*, 1979). The mutant described by Balassa harbours a genetic defect that appears to lie on the side of *ura* distal to *metC*. A *spoVF* mutant which can also be "cured" by the addition of dipicolinic acid was recently found to lie in this region (P. J. Piggot, personal communication).

There have been two reports of new classes of rifampicin-resistant mutants. Ryu (*Journal of Bacteriology* **135**, 408, 1978) has isolated a group of mutants that also require amino acids of the glutamate or aspartate families for growth. Apparently, the altered RNA polymerase in these mutants fails to transcribe efficiently the glutamate synthetase gene. Kane *et al.* (*Journal of Bacteriology* **137**, 1028, 1979) have described a class of rifampicin-resistant mutants that are also resistant to trimethoprim. These mutants have an elevated dihydrofolate reductase activity; six other enzymes that were measured had normal activities. In this case the altered RNA polymerase seems to transcribe the dihydrofolate reductase gene with increased efficiency. These studies, together with those of Pun *et al.* (1975) clearly show that alterations to the β subunit of the RNA polymerase can selectively affect the ability of the enzyme to transcribe certain genes. The differences observed presumably reflect subtle differences between the promoters of different genes. The existence of rifampicin-resistant mutants that are unable to sporulate may well be a non-specific consequence of the fact that different classes of sporulation promoters exist. These new findings, thus weaken further the notion that alterations to the transcriptional specificity of RNA polymerase are of primary importance in determining the precise sequence of gene expression during spore development.

Nakayama *et al.* (*Journal of Bacteriology* **135**, 952, 1978) have confirmed the finding of Cheng and Aronson (1977) and Pandey and Aronson (1979) that the spore coat protein is synthesised as a precursor, although there are differences in the molecular weights of the precursors obtained by the two groups.

Trowsdale, Chen and Hoch (*Journal of Bacteriology* **135**, 99, 1978) have used the rather

complicated and difficult procedure developed by Anagnostopoulos and his colleagues for the construction of merodiploid strains of *Bacillus subtilis*, in order to establish that mutations at the *spoOA* locus are in fact recessive to their wild type alleles, and not dominant as was previously reported.

The capacity of prespores for germination and outgrowth has been examined by Dion, Kay and Mandelstam (*Journal of General Microbiology* **107**, 203–210, 1978). If sporulating cells at stage IV are first cold-shocked and then exposed to an enriched medium the immature spores germinate rapidly. Outgrowth occurs while the spores are still within the sporangia. Other types of pre-treatment (e.g. with toluene) are equally effective in inducing intra-sporangial outgrowth. The experiments show that prespores, at least from stage IV onwards, can revert to the vegetative state while they are in the sporangium and this raises the question why they do not do so during sporulation. One possibility is that the mother cells normally contain an anti-germinant and that this is lost as a result of the cold shock.

Finally, Hitchins (*Canadian Journal of Microbiology* **24**, 1104, 1978) has provided a review of recent evidence to support the idea that the early stages of sporulation are a modified cell division (Hitchins and Slepecky, 1969b).

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Author Index

Numbers in italics refer to the pages on which references are listed at the end of each article.

A

Abad-Zapatero, C., 7, *93*
Abe, C., 189, *240*
Abeliovich, A., 79, *88*
Abelson, J., 35, *95*
Abramov, Z. T., 115, *161*
Achtman, M., 219, 232, *240*
Adams, D. G., 50, 53, *88*
Adams, G. A., 267, 309, *314*, *316*, *318*
Adamson, A. W., 188, *240*
Adhya, S., 47, *97*
Adhya, S. L., 24, *89*
Adler, J., 166, *241*
Adler, L., 275, *314*
Agabian, N., 166, 178, *240*
Akrigg, A., 119, 140, *154*
Albro, P. W., 302, *319*
Alexander, H. B., 123, *161*
Alexandrushkina, N. I., 34, *95*
Allen, G. V., 40, 41, *89*
Allen, M., 68, *88*
Allen, M. W., 6, 16, 55, 62, 63, 68, 69, 80, *88*, *96*, *101*
Alley, C. C., 253, 293, *314*
Al-Nuri, M. A., 26, 27, *92*
Alphen, L., van, 171, 173, 175, 185, 186, 214, 219, 226, *245*, *249*, *250*
Alphen, W., van., 213, 215, *249*
Amaro, A. M., 126, *157*
Ames, B. N., 171, 191, *240*
Ames, G. F. L., 171, 173, 175, 185, *240*
Anagnostopoulos, C., 118, *154*, *158*
Anderson, L. E., 19, 54, *91*
Andreoli, A. J., 153, *154*
Angelo, N., 114, *154*
Apell, G. S., 7, *92*
Apicella, M. A., 267, *314*
Apte, S. K., 3, 70, 71, 72, *88*, *100*
Araya, S., 283, *316*
Arber, W., 21, 22, *88*
Arceneaux, J. L., 127, *158*
Argast, M., 180, *240*
Arko, R. J., 262, *314*
Armstrong, J. E., 78, *88*
Aronson, A. I., 104, 110, 114, 115, 117, 118, 125, 129, 133, 144, 147, *154*, *155*, *158*, *159*
Artenstein, M. S., 253, 293, *315*, *319*
Asato, Y., 25, 29, 31, 32, 85, *88*
Asbell, M. A., 220, *240*
Ashton, F. E., 255, 267, *315*, *319*
Asselineau, J., 303, *314*
Astier, C., 65, 84, *88*, *94*
Atkinson, D. E., 16, 84, *88*
Aubert, J-P., 114, 115, 122, 143, 144, 146, 147, 152, *154*, *155*, *159*, *160*
Austin, S. M., 72, *102*
Ausubel, F., 76, *88*

B

Bachmann, B. J., 214, *240*
Bachmeyer, H., 39, *88*
Bacigalupi, B. A., 307, *314*
Bader, J., 221, *240*
Baecker, P. A., 153, *154*
Bagi, G., 39, *93*
Bak, A. L., 22, *88*, *89*
Baker, R. F., 265, *320*
Balassa, G., 104, 108, 125, 132, 135, *154* *162*
Balch, W. E., 34, *92*
Ball, P. R., 222, *241*
Baratova, L. A., 115, *161*
Barghoorn, E. S., 3, *95*
Bärlund, H., 193, *241*
Barnett, W. E., 39, *93*
Baron, E. S., 266, 281, *314*
Barrett, A. J., 178, *249*
Bartenstein, L., 268, *318*
Barth, P. T., 28, *97*

Bassford, P. J., Jr., 175, 176, 177, 214, 218, *240*
Bassham, J. A., 15, 16, 17, 18, 19, 20, 48, 54, 64, *97*
Bauer, W., 25, *97*
Baugh, C. L., 274, 275, *315*, *319*
Baumstark, B., 39, *93*
Bavoil, P., 176, 177, 205, 206, 207, 208, 211, 215, 219, 237, *240*, *246*
Bayer, M. E., 185, 226, 234, 239, *240*
Bazin, M., 44, 82, 83, *89*
Beacham, I. R., 203, *240*
Beachey, E. H., 237, *240*
Beam, W. E., 313, *318*
Beauchemin, N., 39, *89*
Beauclerk, A. D., 20, *89*
Beckwith, J., 232, *243*, *248*
Beebe, J. L., 287, 299, 300, 301, 302, 306, *314*
Begg, K. J., 28, *91*, 236, *240*
Béguin, S., 7, *90*
Belkin, S., 15, *89*
Belozerskii, A. N., 21, *97*
Belvanova, L. P., 115, *161*
Bender, R. A., 144, *158*
Benemann, J. R., 77, *89*
Bennett, A., 7, 57, *89*
Benz, R., 201, 222, *240*
Ben-Zéev, H., 120, *155*
Benzer, S., 149, *154*
Bérdy, J., 115, *154*
Berendsen, W., 173, 174, *245*
Bergan, T., 253, 303, 304, *314*, *316*
Berger, U., 253, 290, 305, *314*, *319*
Bergmann, R., 126, 146, *156*
Bernheimer, H., 173, 174, *245*
Bernlohr, R. W., 112, 114, 116, 117, 144, *154*, *158*
Berns, A. A., 149, 151, *157*
Berns, D. S., 66, *89*
Bhattacharjee, A. K., 260, *314*
Bhatti, A. R., 119, *154*
Biggins, J., 16, 17, 20, 48, *89*
Bijvelt, J., 226, *250*
Billault, A., 105, 129, 131, *158*
Birch-Andersen, A., 254, 258, 265, *316*, *318*
Birdsell, D. C., 220, *240*
Biswas, B. B., 21, *89*
Biswas, G. D., 266, *314*
Bittner, R., 51, 53, *92*
Black, F. T., 22, *88*
Blackett, B., 263, *316*
Blackman, E., 266, *314*
Blaurock, A. E., 10, *89*
Blicharska, J., 125, *161*
Blobel, G., 231, *240*
Block, R., 53, *89*
Bloom, B., 152, *160*
Bloom, D., 263, *320*
Bloom, G. D., 165, 166, 178, 195, 221, *240*, *250*, 254, 262, 263, 264, 269, 272, 299, 300, 302, 303, 305, 309, 310, 311, *315*, *320*
Blumenfeld, O. O., 238, *242*
Böck, A., 80, *101*
Bockris, J. O., 222, *240*
Bodmer, W. F., 129, 130, *154*
Bogorad, L., 3, 7, 33, 34, 42, 43, 44, 55, 56, 57, 58, 59, *89*, *92*, *93*, *101*
Bohin, A., 135, *154*
Bohin, J-P., 135, *154*
Boman, H. G., 186, 221, *240*, *247*
Bonamy, C., 131, *161*
Bone, D. H., 78, *89*
Bonen, L., 4, 12, 24, 38, 40, 41, 86, *89*, *91*, *102*
Boniface, J., 216, *247*
Bonsen, P. P. M., 119, *154*
Booker, M. J., 10, *101*
Boos, W., 180, 201, 216, 217, 222, *241*
Borbely, G., 37, 39, *89*, *93*
Borchgrevink, B., 281, 290, 291, *317*
Borenstein, S., 151, *156*
Bosch, V., 186, 225, *241*
Bose, S. K., 116, *159*
Bothe, H., 62, 67, 77, *89*
Bott, K. F., 126, 144, *156*, *160*
Bottomley, P. J., 16, 17, 48, 68, *89*
Bøvre, K., 253, 303, 304, *314*, *316*
Bowen, C. C., 8, 9, 43, *90*, *97*, *101*
Bowyer, J. W., 65, *89*
Boxtel, R., van, 214, 219, 226, *249*
Boyce, R. P., 27, *89*
Boyd, A., 180, 236, 237, *240*
Bradbeer, C., 180, 216, 217, 218, *240*, *241*, *250*
Bradley, S., 3, 71, 73, 74, 75, *89*, *90*
Bragg, P. D., 171, 173, 187, 199, 221, 224, *240*, *247*
Bramucci, M. G., 124, 137, *154*
Brandt, E., 16, 17, *101*

Branton, D., 10, *101*, 181, *240*
Braun, V., 172, 178, 186, 217, 218, 224, 225, 233, *240*, *241*, *243*, *247*
Breen, J. F., 267, *314*
Brehm, S. P., 137, *154*
Brenchley, J. E., 144, *158*
Brenner, M., 181, 182, *246*
Brenner, S., 28, *94*
Breuil, C., 87, *95*
Brevet, J., 121, 122, 123, 146, *154*, *160*, *161*
Brewer, M. E., 30, *89*
Britten, R. J., 22, 24, *89*
Broadbent, D. A., 117, *162*
Brock, T. D., 3, 5, 14, 70, *89*, *101*
Broda, E., 78, *94*
Bronstein, H., 178, *245*
Brookes, R., 260, *314*
Brooks, G. F., 272, 299, 300, 301, 302, 306, 307, 311, *319*
Brooks, J. B., 253, 282, 292, 293, 305, 306, *314*, *318*
Brorson, J. E., 313, *316*
Brown, D. M., 7, *90*
Brown, J. S., 22, *96*
Brown, K. E., 195, *245*, 263, 308, 309, *318*
Brown, L., 254, 255, 257, *317*
Brown, N., 113, 151, *158*
Brown, N. C., 150, *154*
Brown, R. W., Jr., 3, 34, 85, *89*, *98*
Brownstein, B. H., 37, *96*
Bruce, S. A., 22, *96*
Bruggen, E. F. J., van, 165, *249*
Brundell, J., 275, *314*
Bryant, D. A., 7, 9, *89*, *96*
Bryn, K., 303, 304, *314*, *316*
Buchanan, T. M., 188, *241*, 262, 265, 266, *314*, *316*, *318*
Buchner, H., 143, *154*
Buetow, D. E., 4, *102*
Bukhari, A. I., 24, *89*
Bullard, J. C., 262, *314*
Bundle, D. R., 260, *314*
Burgess, R. R., 32, 34, *90*
Burgi, F., 21, *102*
Burman, L., 274, *315*
Burman, L. G., 269, 311, *315*
Burnham, J. C., 6, *90*
Burris, R. H., 77, *97*
Butler, M., 62, *90*
Butler, W. L., 9, *96*

C

Cacciapuoti, A. F., 272, 273, 307, 311, *314*
Caird, J. D., 257, 266, 267, 309, 310, *315*, *320*
Calcott, P. H., 289, *317*
Calder, J. A., 17, 62, 65, *94*
Calver, G. A., 312, *314*
Cami, B., 122, 123, 135, 137, 142, 146, *157*, *159*, *160*, *161*
Campisi, J., 233, *249*
Candau, P., 66, *96*
Canfield, R. E., 125, *161*
Cannon, F., 76, *88*
Canovas, J. L., 275, *314*
Capesius, I., 32, *90*
Capindale, J. B., 62, *90*
Capra, J. D., 293, *319*
Cardemil, L., 71, *90*
Carifo, K., 290, 291, 299, *315*
Carlton, B. C., 112, 114, *157*
Carlton, J. R., 43, *90*
Carr, N. G., 3, 6, 11, 14, 16, 17, 20, 21, 22, 23, 26, 29, 30, 31, 32, 41, 42, 43, 44, 46, 49, 50, 51, 52, 53, 59, 61, 62, 63, 64, 65, 67, 68, 71, 72, 73, 74, 75, 79, 80, 81, 82, 83, 84, 85, 86, 87, *88*, *89*, *90*, *91*, *92*, *93*, *94*, *95*, *96*, *97*, *99*, *101*
Carson, J., 165, 168, *246*
Cashel, M., 47, 50, 53, *90*, 112, *156*
Castles, R., 305, 313, *318*
Castoriadis-May, C., 143, *154*
Catlin, B. W., 257, 258, 259, 268, 276, 282, 290, 291, 294, 296, 299, 307, *315*
Cedergren, R. J., 38, 39, 40, *89*, *91*, *95*
Centre for Disease Control, 252, *315*
Chai, T-J., 215, *242*
Chakrabarty, A. M., 210, *241*
Chambliss, G. H., 126, *154*
Chan, K., 254, 255, 257, *315*
Chang, S., 22, *90*
Chao, L., 9, *90*
Chappelle, E., 117, *156*
Charles, D., 21, *90*
Chasin, L. A., 125, *155*
Chatterjee, A. K., 226, *243*
Chattopadhyay, P. K., 232, 233, *241*
Chee, J. L., 69, 80, *101*
Cheldelin, V. H., 278, *317*
Chen, K. C. S., 266, *316*
Chen, S. M. H., 136, 137, *157*

Chenault, S. S., 196, *245*
Cheng, K. H., 15, *90*
Cheng, K. J., 164, 218, 219, *241*, *244*
Cheng, Y-S. E., 115, 117, *155*
Cherniak, R., 171, *241*
Chiang, K-S., 21, *99*
Cho, K. Y., 303, *315*
Chopra, I., 222, *241*
Chou, G., 210, *241*
Choudhary, G., 253, 293, *314*
Christiansen, C., 22, *88*, *89*
Churchward, G. G., 180, 236, 237, *241*
Clark, J. B., 265, *318*
Clark, S., 124, *155*
Clark, V., 114, *154*
Clewell, D. B., 24, *93*
Cocito, C., 85, *90*
Codd, G. A., 9, 19, 60, 72, *90*, *100*
Cohen, A., 120, *155*
Cohen, I. R., 254, 255, 256, 312, *317*
Cohen, S. N., 22, *90*
Cohen, Y., 15, 79, *90*
Cohen-Baizire, G., 3, 6, 8, 9, 11, 12, 13, 14, 20, 21, 23, 24, 36, 48, 55, 57, 60, 64, 67, 68, 70, 71, 87, *90*, *93*, *95*, *97*, *98*, *99*, *100*
Cohn, F., 4, *90*
Cohn, Z. A., 188, *248*
Cole, J. A., 69, *90*
Cole, R. M., 237, *240*, *241*
Collander, R., 193, *241*
Colli, W., 35, *90*
Collins, J., 28, *97*
Collins, J. F., 149, *155*
Colman, B., 15, 60, *90*, *94*
Cooper, S., 28, 30, *90*
Coote, J. G., 104, 111, 112, 114, 117, 118, 119, 125, 130, 131, 132, 133, 134, 135, 136, 137, 139, 140, 141, 142, 148, 149, 152, *155*, *159*
Copeland, J. C., 28, *90*
Coratza, G., 191, *241*
Corbett, W. P., 276, *315*
Corry, M. J., 35, 37, *90*
Costerton, J. W., 164, 181, 218, 219, 226, *241*, *242*, *243*, *244*
Cota-Robles, E. H., 220, *240*
Cote, R., 122, 123, *161*
Cox, D. L., 275, *315*
Cozzarelli, N. R., 150, *155*
Craig, I. W., 20, 21, 31, 43, *90*, *101*
Crang, R. E., 9, *99*
Crowlesmith, I., 169, *248*
Cumsky, M., 23, 24, *102*
Cunningham, J., 82, *98*
Cunningham, R. K., 188, *241*
Currier, T. C., 6, 73, 77, 82, 87, *90*
Curry, M. V., 112, *157*
Curtis, N. A. C., 212, *241*
Cuzin, F., 28, *94*

D

Dalgarno, L. 45, *99*
D'Amato, R. F., 293, *315*
Dancer, B. N., 112, 114, 116, 150, *155*
Danielli, J. F., 192, 194, *241*
Daoust, V., 267, *319*
Darby, V., 212, *244*
Dasgupta, R., 231, *241*
Datta, D. B., 188, *241*
Davidson, E. H., 24, *89*
Davies, J. K., 202, 218, *241*
Davis, R. H., 268, 294, *315*
Dawes, E. A., 16, 48, 67, *90*
Dawes, I. W., 104, 107, 118, 119, 132, 139, 140, 141, 143, 146, 148, *155*, *162*
Dayhoff, M. O., 38, *98*
De, D. N., 21, *90*
Deacon, W. E., 254, 255, 257, *317*
Dean, B., 313, *318*
Dean, D. R., 144, *155*
Deans, J. A., 109, 152, *155*, *162*
Decad, G., 196, 197, 199, 202, *241*, *243*
Dedonder, R., 105, 129, 131, *158*
Delaney, S. F., 3, 6, 29, 32, 80, 82, 83, 85, 86, 87, *91*, *94*
De Leo, A. B., 144, *158*
Del Valle, M. R., 125, *154*
Demain, A. L., 115, 116, *155*, *157*
Demarco de Hormaeche, R., 261, *315*
De Martini, M., 224, *241*
Dennison, D., 274, *315*
De Pamphilis, M. L., 166, 199, *241*
De Petris, S., 154, 184, 225, *241*
Desikichary, T. V., 11, 12, *91*
Deutscher, M. P., 104, 114, 117, *155*, *158*
Devilly, C. I., 84, *91*
De Voe, I. W., 119, *154*, 180, 181, *241*, 254, 258, 265, 289, *315*
Dharmawardene, M. W. N., 3, 71, *100*

Diakoff, S., 58, 59, *91*
Dice, J. F., 67, *93*
Dichtelmüller, H., 146, *160*
Dickson, A., 80, *91*
Diedrich, D. L., 173, 175, 176, 177, 214, 238, *240*, *241*
Diena, B. B., 255, 267, *315*, *319*
Di Girolamo, P. M., 216, 217, *250*
Di Masi, D. R., 180, 217, *241*
Dingle, S. L., 276, *319*
Di Rienzo, J., 164, *241*
Dittmer, J. C., 302, *319*
Dixon, R. A., 24, *91*
Dobberstein, B., 231, *240*
Dobson, P. R., 34, 35, 40, 41, *89*, *91*
Doemel, W., 14, 70, *101*
Doherty, P., 211, *247*
Döhler, G., 15, 55, 56, 60, *91*
Doi, R. H., 104, 114, 117, 120, 122, 123, 125, 127, 129, *155*, *156*, *158*, *159*, *160*, *161*, *162*
Dolack, M. P., 82, 95
Domoto, T., 126, *155*
Donachie, W. D., 28, 30, *91*, 150, *155*, 236, *240*
Donoghue, H. D., 261, *315*
Doolittle, W. F., 4, 6, 12, 17, 24, 33, 34, 35, 36, 37, 38, 40, 41, 48, 50, 54, 68, 80, 82, 86, *89*, *91*, *95*, *99*
Doorenbos, N. J., 310, 311, *319*, *320*
Doudoroff, M., 17, *98*, 210, *248*
Dourmashkin, R. R., 265, *318*
Drews, G., 4, 6, *91*, *95*, *101*, 170, *250*
Dubnau, D., 104, *155*
Duggan, J. X., 19, 54, *91*
Duie, P. 122, *155*
Duncan, C. L., 125, *158*
Duncan, J. F., 282, 294, 296, *317*
Duncan, W. P., 262, *314*
Dunn, G., 135, 138, 149, 151, *155*, *157*
Dunn, J. H., 6, *91*
Dunne, F. T., 260, *318*
Durham, D. R., 166, *245*
Durston, W. E., 171, 191, *240*
Dyer, T. A., 34, 35, 36, 37, *90*, *97*
Dynga, L. O., 43, *93*

E

Eagon, R. A., 220, *240*
Eagon, R. G., 166, 178, 182, 184, 185, 220, 227, *242*, *247*, *249*
Earhart, C. F., 166, 178, 196, *242*, *245*
Earl, R. G., 274, *315*
Eaton, M. W., 152, *155*
Ecarot, B., 39, *91*
Ecarot-Charrier, B., 39, 40, *91*
Eccleston, E. D., 52, *91*
Echlin, P., 4, *91*
Edelman, M., 6, 21, *91*
Edwards, M. R., 8, *91*
Eisbrenner, G., 77, *89*
Eiserling, F. A., 7, *89*
Elanskaya, I. A., 32, *98*
Eley, J. H., 60, *91*
Ellar, D. J., 109, 152, 153, *155*, *162*
Elmerich, C., 144, 147, *155*
Eberhard, S. J., 276, *319*
Elmros, T., 166, 178, 195, *250*, 254, 258, 259, 262, 263, 264, 269, 272, 274, 299, 300, 302, 303, 305, 309, 310, 311, *315*, *320*
Eloff, T. N., 79, *91*
Elson, H. E., 164, 255, *246*
Elwell, L. P., 25, *96*, 252, *315*
Emmerling, G., 167, 182, 183, 194, *241*
Endermann, R., 187, *242*
Enriquez, L. A., 293, *315*
Enticott, S., 76, *98*
Ephrati-Elizur, E., 151, *156*
Epstein, H. T., 6, 21, *91*
Erdmann, V. A., 38, *97*
Erick, K., 178, *250*
Ermakova, L. M., 115, *161*
Escamilla, E., 153, *154*
Espardellier, F., 84, *88*
Evans, E. H., 11, 59, 65, *91*, *94*
Everson, J. S., 263, *316*
Everson, R. G., 60, *91*

F

Falkbring, S. O., 275, *314*
Falkenburg, B., 62, 67, *89*
Falkow, S., 24, 25, 87, *91*, *96*, 252, 256, *315*, *318*
Farrand, S. K., 117, *161*
Farshy, D. C., 303, 304, 310, *318*
Faulkner, B. M., 29, 30, 49, *93*

Fay, P., 3, 5, 6, 11, 14, 20, 47, 66, 71, 73, 75, 87, *92*
Fazio, M., 270, *315*
Feingold, D. S., 220, *242*, 299 300, 301, 302, 303, 304, 305, *319*
Felix, J. A., 117, *156*
Feunteun, J., 35, *92*
Fielder, G., 135, *158*
Fielding, P., 236, *249*
Filip, C., 166, *242*
Finkelstein, R. A., 265, 312, *318*
Finnerty, W. R., 272, 299, 300, 301, 302, 306, 307, 311, *319*
Fiscina, B., 313, *315*
Fischer, R. W., 11, *92*
Fisher, S. H., 123, 144, *156*, *161*
Fisher, W. D., 27, *94*
Fitzer, H., 202, *246*
Fitzgerald, T. J., 263, 283, 290, 311, *315*, *318*
Fitz-James, P. C., 104, 107, 109, 110, 111, 117, 118, 120, 129, 133, 139, 140, *154*, *156*, *157*, *159*, *162*, 253, 254, *315*
Fleck, J., 264, *318*
Fleming, H., 71, 73, *92*
Fletcher, G., 166, *242*
Fogg, G. E., 3, 5, 6, 11, 14, 20, 47, 66, 87, *92*
Folsome, C., 29, 31, 85, *88*
Forge, A., 181, *242*
Fortnagel, P., 117, 126, 146, *156*
Forster, T., 9, *92*
Forster, J. W., 114, *156*
Foulds, I., 11, 59, 65, *91*
Foulds, J., 174, 176, 202, 211, 215, *242*
Foulds, I. J., 61, 68, *92*
Fox, C. F., 236, *249*
Fox, G. E., 34, 38, 39, 86, *92*, *102*
Fox, J. L., 7, 47, 66, *93*, *95*
Frakas, G. L., 37, 39, *89*, *93*
Frampton, E. W., 25, 26, 27, *93*, *97*
Frank, H., 6, *92*
Frantz, I. D. Jr., 294, *315*
Frasch, C. E., 178, *242*
Frederick, J. F., 48, *92*
Freer, J. H., 164, *242*
Freese, E., 104, 109, 111, 112, 117, 118, 146, 147, *156*, *157*, *159*, *161*, 191, *248*
Freese, E. B., 117, 126, 146, 147, *156*
Freese, P. K., 114, 122, *158*
Freundt, E. A., 22, *88*
Fridlander, B., 6, 21, *95*
Friebel, T. E., 116, *155*
Friedenreich, P., 7, *92*
Friedman-Kien, A. E., 283, *315*
Friesen, J. D., 27, *92*
Fritsch, F. E., 4, *92*
Frøholm, L. D., 266, *315*
Frøholm, L. O., 253, *314*
Fromme, I., 6, *101*, 170, *250*
Fu, M. L., 216, 217, *250*
Fuhs, G. W., 20, 31, *92*
Fujita, Y., 57, *92*
Fukasawa, T., 234, *242*
Fukuda, R., 122, *156*
Fukui, S., 199, *248*
Fung, J., 184, 220, *242*
Fung, L. W-M., 228, *247*

G

Gagliardi, N. C., 267, *314*
Garlanos, C., 168, 171, *242*, *245*
Galey, W. R., 194, 195, *242*
Gallant, J., 47, 49, 51, 53, *90*, *92*, *97*
Gallon, J. R., 70, 71, *92*
Gallop, P. M., 238, *242*
Galonsky, A., 72, *102*
Gander, J. E., 165, 168, 230, 234, *246*
Ganesan, A. T., 129, 130, *154*
Gantt, E., 3, 7, 8, 9, *91*, *92*, *93*
Garen, A., 218, *242*
Garlick, S., 15, 70, 79, *92*
Garner, J. V., 112, *157*
Garrett, E., 114, *156*
Garten, W., 173, 177, 219, 224, 235, *242*, *248*
Gary-Bobo, G. M., 194, *248*
Gausing, K., 31, 51, 52, *95*
Gauthier, J. J., 30, *89*, 109, *157*
Gavrila, G., 20, 31, *92*
Gayda, R., 232, *242*
Geary, I., 257, 293, *315*
Geele, G., 114, *156*
Gendel, S., 42, 57, *92*.
Gennis, R. B., 239, *246*
Gerard, C., 215, *247*
Ghosh, S. N., 21, *90*
Ghuysen, J-M., 264, *318*
Gibson, J., 16, 18, 19, 48, 62, 63, 78, *94*
Gilbert, J. V., 293, *319*

Gilbert, W., 24, *92*, 124, *156*
Gilchrist, J. E., 180, *241*, 254, 258, 265, 289, *315*
Gilleland, H. E., Jr., 178, 182, 184, 185, 227, *242*, *247*, *249*
Gillman, C. F., 188, *241*, *249*
Gilvarg, C., 198, *247*
Ginoza, H. S., 25, *88*
Ginsburg, R., 59, *92*
Ginther, C. L., 126, *161*
Girard, A., 167, 171, 194, 239, *247*
Glaser, V. M., 26, 27, *92*
Glassman, L. H., 254, *317*
Glatron, M. F., 125, *156*
Glauert, A. M., 164, 166, 181, *242*, *249*, 261, *315*
Glazer, A. N., 3, 7, 8, 9, 14, 56, *89*, *90*, *92*, *93*, *96*, *101*, *102*
Glenn, A. R., 118, *156*
Gmeiner, J., 172, 202, 236, *242*, *246*
Godzicka-Jozefiak, A., 39, *93*
Goedheer, J. C., 55, 56, *93*
Goldberg, A. L., 67, *93*
Goldberg, I. D., 257, *316*
Goldberg, N. D., 144, *154*
Goldfine, H., 165, 185, 226, *240*, *244*
Goldman, J. N., 220, *242*
Goldman, R. C., 135, *156*
Goldstein, D., 85, *90*
Goldthwaite, C., 104, 126, *155*, *156*
Golecki, J. R., 6, *93*, 181, 184, 234, 237, *245*
Gomez-Moreno, C., 66, *96*
Goodell, E. W., 270, *315*
Gool, A. P., van, 181, 182, 226, *249*
Gorodetsky, D. I., 115, *161*
Gotschlich, E. C., 166, 178, *242*, *244*, 253, 259, 260, 261, 262, 265, 290, *314*, *317*, *318*, *319*
Gottfried, S., 218, *244*
Grade, R., 146, *160*
Graff, P. J., de, 176, *250*
Graham, D. E., 22, *89*
Graham, R. S., 126, *156*
Grant, N. G., 10, *93*
Grant, W. D., 118, *156*
Gray, B. H., 8, *93*, 117, *154*
Gray, E. D., 52, *91*
Gray, G. W., 165, 182, 184, 185, *242*, *247*
Gray, J. E., 44, *93*
Green, E. W., 236, *242*
Green, G. R., 21, *98*
Greenleaf, A. L., 122, *156*, *158*
Grienenberger, J. M., 37, *93*
Grierson, D., 35, 37, *93*
Griffin, P. J., 296, 299, *315*
Griffiss, J. M., 253, *315*
Grigorieva, G. A., 83, *93*
Gros, F., 21, *98*, 124, *156*
Groscurth, R., 145, 146, *160*
Groshev, V. V., 26, 27, 32, *92*, *98*
Gross, J. G., 265, *318*
Grossman, A., 18, 19, 54, *93*
Gruber, M., 35, *97*
Gudas, L., 180, 236, *242*
Gudas, L. J., 28, *93*, 236, *244*
Guespin-Michel, J. F., 135, 136, *156*
Guha, S. 126, *156*
Guidotti, S., 167, *247*
Guinand, M., 110, *161*
Gulik-Krzywicki, T., 167, 181, 182, 183, 194, *241*, *246*
Gulikova, D. M., 43, *93*
Gunsalus, I. C., 210, *241*
Gusev, M. V., 48, *98*
Gustafson, P., 192, *242*
Guymon, L. F., 166, 178, 189, *250*, 263, *315*

H

Haas, D., 203, *240*
Haavik, H. I., 116, *156*
Haber, J. E., 148, *156*
Hackert, M. L., 7, *93*
Haddox, M. K., 144, *154*
Hafemann, B., 126, *156*
Hafiz, S., 257, 293, 313, *315*, *316*, *318*
Hageman, J. H., 112, 114, *156*, *157*
Halegoua, S., 172, 231, *242*, *243*, *248*
Halfenger, G. M., 117, *161*
Haller, I., 167, 173, 202, 220, *242*, *243*
Halvorson, H. O., 111, 112, 119, 148, *156*, *157*, *158*, *162*
Hanahan, D. J., 167, *250*
Hancock, I. C., 228, *243*
Hancock, R. E. W., 166, 178, 202, 210, 218, 219, *241*, *243*, *248*
Hansen, E. J., 286, *316*
Hansen, J. N., 104, *155*

Hanson, R. S., 104, 111, 112, 117, 144, *157*, *161*, *162*
Hantke, K., 178, 180, 216, 218, *241*, *243*
Hardy, S. J. S., 232, *243*, *247*
Hareford, N., 105, *157*
Harold, F. M., 68, *93*
Harris, H., 124, *157*
Hart, E. J., 257, *316*
Hartmann, A., 178, *241*
Hasegawa, Y., 175, 176, *243*
Heselkorn, R., 3, 20, 21, 68, 71, 72, 73, 74, 75, *92*, *93*, *98*, *102*
Haseltine, W. A., 53, *89*
Hasin, M., 182, *247*
Hastings, A. B., 305, 313, *318*
Hathaway, G., 120, *157*
Hattori, A., 57, 66, 67, *92*, *93*, *96*
Haury, J. F., 6, 57, 58, 73, 77, 82, 87, *90*, *93*
Hawes, G. B., 309, *316*
Hayashi, F., 29, 49, 54, *93*
Hayes, J., 274, *315*
Haystead, A., 3, 71, *100*
Healey, F. P., 78, *93*
Heath, E. C., 171, *250*
Heaton, D., 25, *93*
Hebeler, B. H., 253, 260, 261, 264, 265, 268, 269, 270, 271, 272, 274, 275, 276, 277, 278, 279, 282, 285, 286, 290, 291, 292, 296, 299, 311, *316*, *318*, *320*
Heckels, J. E., 263, *316*
Hecker, L. I., 39, *93*
Heckman, J. E., 39, *93*
Heerikhuizen, H., van, 165, *249*
ten Heggeler, B., 223, *249*
Heinze, J. E., 146, 147, *156*, *159*
Helsinki, D. R., 24, *93*
Hellio, R., 237, *248*
Helmsetter, C. E., 28, 30, *90*
Hendley, J. O., 260, 261, *316*
Hendry, G. S., 120, *157*
Henning, U., 167, 173, 175, 176, 177, 178, 182, 183, 184, 187, 188, 194, 202, 210, 215, 216, 219, 220, 224, 226, 235, *241*, *242*, *243*, *248*
Henriksen, S. D., 253, *316*
Heppel, L. A., 219, *246*
Herbst, E. J., 293, *320*
Herdman, M., 3, 6, 12, 13, 20, 21, 23, 24, 29, 30, 32, 43, 49, 82, 83, 84, 85, 86, 87, *91*, *93*, *94*, *95*
Hermodson, M. A., 266, *316*
Herrlich, P., 44, *98*
Herson, D. S., 43, 44, *90*, *93*
Herzfeld, F., 32, 33, *94*
Heym, G. A., 257, *317*
Heyman, T., 127, *159*
Hiatt, H., 124, *156*
Higa, A., 124, *158*
Higgs, S. A., 150, *159*
Hildebrandt, J. F., 262, *316*
Hill, J. H., 254, *316*
Hindennach, I., 173, 176, 215, 216, 219, 224, 235, *242*, *243*, *248*
Hines, J., 275, 276, 277, 278, 279, 281, 282, 283, 284, *318*
Hirashima, A., 231, 233, *242*, *243*, *249*
Hirota, Y., 172, 184, 201, 215, 218, 219, 220, 233, 237, *243*, *246*, *248*, *249*
Hitchins, A. D., 109, 150, *157*
Hixon, C. S., 7, *89*
Ho, C., 228, *247*
Hoare, D. L., 17, 64, 65, *94*
Hoare, D. S., 3, 14, 16, 17, 19, 47, 62, *94*, *99*, *101*
Hoare, S. L., 16, 62, *94*
Hoch, J. A., 104, 126, 136, 137, 144, 145, *154*, *155*, *157*, *160*, *161*, *162*
Hock, P., van der, 171, 173, 175, *245*
Hodges, R. S., 224, *243*
Hodgson, B., 115, *157*
Hoffman, E. J., 28, *95*
Hofstein, A. V., 20, *94*
Hogarth, C., 152, *155*
Hogg, L., 296, *320*
Höhn, B., 220, 224, *243*
Holland, I. B., 180, 212, 227, 236, 237, 239, *240*, *241*, *243*, *244*
Holland, M. J., 122, *157*
Hollifield, W. C., Jr., 217, *243*
Hollis, D. G., 303, 305, *317*
Holmes, K. K., 256, 262, 291, 296, 299, 312, *314*, *317*, *318*
Holohan, P., 66, *89*
Holt, S. C., 61, *96*, 109, 110, 114, *154*, *157*, *161*
Holten, E., 276, 277, 278, 279, 280, 281, 282, 283, 284, 285, 286, 290, 291, 292, *316*
Holzer, H., 148, *157*, *159*
Holzgrefe, H. H., 260, 261, *316*
Homma, J. Y., 189, *240*, *243*
Hong, J., 213, *244*

Honjo, M., 151, *160*
Hood, W., 80, *94*
Hopwood, D. A., 24, *94*
Hori, H., 38, *94*
Horiuchi, K., 22, *101*
Horstedt, P., 254, *315*
Hoshino, E., 283, *316*
Hou, C., 171, 199, 233, *240*, *250*
Houghton, J. A., 84, *91*
Howard-Flanders, P., 32, *94*
Howe, T. G. B., 222, *241*,
Howland, G. P., 34, 37, *94*
Hranulei, D., 108, 132, 137, 142, *157*
Hughes, S. G., 22, *96*
Humphreys, G. O., 228, *243*
Hunter, J. A., 313, *318*
Hunter, K. M., 299, 307, *316*
Husmann, D., 253, *314*
Hutber, G. N., 78, *94*
Hutchinson, F., 68, *88*
Hutchinson, K. W., 144, *157*
Hutson, K. G., 78, *94*

I

Ichihara, N., 230, *244*
Ichihara, S., 176, 178, *243*
Ichikawa, T., 118, *157*
Ide, M., 143, *157*
Ihlenfeldt, M. J. A., 16, 18, 19, 48, 62, 63, 78, *94*
Iketani, H., 172, 233, *243*, *249*
Imafuku, H., 16, 48, *94*
Ingle, R. K., 15, 60, *90*, *94*
Ingram, J. M., 119, *154*, 164, *241*
Ingram, L. O., 17, 24, 27, 62, 64, 65, 80, *94*
Inoue, K., 199, *244*
Inouye, H., 232, *243*
Inouye, M., 164, 172, 200, 215, 224, 225, 231, 233, *241*, *242*, *243*, *247*, *248*, *249*
Inouye, S., 172, 231, 233, *243*, *248*
Ionesco, H., 107, 137, 139, 140, 142, *157*, *160*
Ippen-Ihler, K., 220, *245*
Irvin, R. T., 226, *243*
Ishida, M. R., 29, 49, 54, *93*
Ishii, J., 212, *246*
Ishimoto, N., 230, *244*
Isono, K., 45, *94*
Isono, S., 45, *94*
Ito, E., 230, *244*
Ito, J., 120, 122, 135, 136, *157*
Ito, K., 233, 235, *243*, *244*
Iwashita, S., 239, *249*
Iyer, R., 212, *244*
Izotova, L. S., 115, *161*

J

Jacob, F., 28, *94*
Jahkola, M., 229, *245*
Jakes, K., 45, *100*, 231, *249*
James, A. N., 255, 257, *316*, *318*
James, J. F., 189, *244*, 256, 260, 261, *316*
James, R., 180, 236, *242*, *244*
Janko, K., 201, 222, *240*
Jann, B., 168, 186, *246*, *247*
Jahn, K., 168, 186, 229, *244*, *246*, *247*
Jansz, E. R., 15, *94*
Jantzen, E., 303, 304, *314*, *316*
Janvier, M., 12, 13, 21, 23, *94*
Jaskunas, S. R., 35, 49, 51, 53, *96*
Jeanjean, R., 78, *94*
Jeffs, P., 150, 151, *155*, *162*
Jennings, H. J., 260, 309, *314*, *316*
Jensen, R. A., 80, *94*
Jensen, T. E., 9, *99*
Jenssen, W. N., 167, *250*
Jephcott, A. E., 254, 257, 265, 313, *316*
Jerez, C. A., 126, *157*
Jhabvala, P., 80, *95*
Johannisson, G., 186, *245*, 313, *316*
Johansson, V., 188, *248*, *249*
Johnson, C. W., 126, *161*
Johnson, K. G., 290, *318*
Johnston, D. G., 253, *320*
Johnston, K. H., 166, 178, *244*, 261, 262, 290, *317*
Jones, D. D., 10, 30, *89*, *94*
Jones, L. W., 55, 56, *94*
Jones, N. C., 165, 195, 234, 235, 236, *244*
Jones, R. T., 275, *317*
Jonssen, E. K., 260, *318*
Jonsson, S., 221, *240*
Jordan, B. R., 35, *92*
Jørgensen, B. B., 15, 79, *90*
Josefsson, L-G., 232, *247*
Joset-Espardellier, F., 65, *94*

Jost, M., 6, 10, 43, *94*, *101*
Juni, E., 257, 286, *316*, *317*
Jurasek, L., 242, *243*
Jurtshuk, P., 288, 289, *317*
Jurtshuk, P., Jr., 288, 289, *317*
Jüttner, F., 72, *95*
Jyssum, K., 258, 275, 281, 283, 284, 285, 290, 291, 292, 294, 297, *316*, *317*
Jyssum, S., 275, 281, 286, 294, 296, 297, *317*

K

Kaback, H. R., 213, *244*
Kadner, R. J., 218, *240*
Kaesberg, P., 231, *240*
Kalbacher, B., 50, *90*
Kaminski, M., 122, *155*
Kamio, Y., 165, 167, 171, 181, 182, 184, 185, 186, 193, 196, 226, 227, *244*, *248*
Kane, J. F., 80, *94*
Kanegasaki, S., 228, 230, 239, *249*
Kaney, A. R., 80, 82, 83, *95*, *97*
Kaplan, S., 37, *96*
Kapp, R., 47, 66, *95*
Karamustafa, A., 313, *316*
Karbisheva, E. A., 32, *98*
Kataoka, T., 199, *244*
Kates, M., 267, 303, 305, 309, 310, *314*, *317*
Katoh, T., 16, 48, *94*
Katz, A., 6, *95*, *101*
Katz, E., 115, *157*
Kaushik, B. D., 27, 29, 31, *99*
Kawamura, F., 120, 122, *157*
Kawamura, T., 230, *244*
Kawasaki, T., 281, *319*
Kay, D., 107, 108, 118, 119, 132, 139, 140, 141, 148, 149, *155*, *159*, *162*
Kaye, A. M., 6, 21, *95*
Keating, K. I., 5, *95*
Keeler, C. L., 122, 123, *160*
Keeler, J. S., 6, *96*
Keggins, K. M., 124, 137, *154*
Keilman, G., 122, *156*
Kier, H. M., 27, *98*
Kellenberg, D., 79, *88*
Kelln, R. A., 114, 122, *158*
Kellogg, D. S., 292, 305, 306, *314*
Kellogg, D. S., Jr., 253, 254, 255, 256, 257, 282, 293, 303, 304, 310, 312, *314*, *317*, *318*
Kelly, D. P., 3, 47, *101*
Kemp, S., 296, *320*
Kenimer, E. A., 287, *317*
Kennedy, C., 24, *91*
Kennedy, E. P., 232, *248*
Kennedy, N., 219, 232, *240*
Kennell, D., 42, 43, *95*
Kenny, C. P., 260, 309, 312, *314*, *316*
Kent, J. L., 230, *244*
Kenyon, C. N., 69, *95*
Kerjan, P., 121, 122, *157*, *158*, *159*
Kerr, K. A., 181, *242*
Keryer, E., 114, 115, *161*
Keynan, A., 124, 149, 151, *157*, *158*
Khachatourians, G., 113, 151, *158*
Khudyakov, I. Y., 34, *95*
Khyen, N. T., 83, *98*
Kikuchi, T., 29, 49, 54, *93*
King, G. J., 188, *244*
King, T. E., 278, *317*
Kingsbury, D. T., 22, *95*, 282, 294, 296, *317*
Kinsky, S. C., 199, *244*
Kiper, M., 33, *94*
Kirk, M. R., 17, 18, 19, 20, 48, 54, 64, *97*
Kirnos, M. D., 34, *95*
Kissel, M. S., 4, *102*
Kistler, J., 223, *249*
Kitos, P. A., 278, *317*
Kjeldgaard, N. O., 31, 49, 51, 52, *95*, *96*
Klier, A., 122, *157*
Klofat, W., 117, *156*
Klotz, L. C., 21, 22, 23, 31, *95*, *98*
Klumpp, E. R., 186, *241*
Knapp, J. S., 291, 296, 299, *317*
Knaysi, G., 142, *157*
Knoll, A. H., 3, 12, 81, *95*
Knowles, C. J., 289, *317*
Kobayashi, H., 126, *157*
Kobayashi, K., 126, *155*, *157*
Kobayashi, Y., 126, *155*, *157*, *158*
Koch, A. L., 42, 51, *95*, 216, 217, *244*, *247*
Koch, M. L., 310, *317*
Kolcsei, M., 37, *89*
Komano, T., 151, *160*
Kondrosi, A., 24, *91*
Konigsberg, W., 215, *246*
Konsky, J., 180, 239, *244*, *246*

Koplow, J., 165, 185, 226, *240*, *244*
Kopmann, H. J., 229, *244*
Kornberg, A., 27, *95*, 104, 114, 117, 119, *154*, *155*, *158*, 180, *248*
Kornberg, H. L., 276, *314*
Koths, K. E., 25, *98*
Kovalchik, M. T., 255, *317*
Kramer, C., 187, 188, *241*, *242*
Kratz, W. A., 7, 55, *96*
Kraus, S. J., 253, 254, 255, 259, 261, 265, *317*, *319*
Kreil, G., 39, *88*
Kretschmer, S., 135, *158*
Krieger-Brauer, H. J., 178, 217, *241*
Krishnapillai, V., 24, *91*
Krogmann, D. W., 6, *95*
Kroll, H-P., 172, 236, *242*
Krotoski, D. M., 23, 24, *102*
Kulpa, C. F., Jr., 234, *244*
Kumar, H. D., 73, 77, 78, 82, *95*, *97*, *99*
Kung, S. D., 21, 23, *95*
Kunisawa, R., 11, 12, 24, 36, 70, 87, *95*, *97*, *100*
Kuo, T-T., 189, *247*
Kuritz, H. M., 220, *242*
Kurland, C. G., 124, *156*
Kurz, W. G. W., 70, 71, *92*
Kushner, D. J., 87, *95*
Kwak, E., 165, *249*

L

Labbe, R. G., 125, *158*
Labuda, D., 39, *93*
Ladha, J. K., 73, 77, *99*
Laishley, E. J., 112, 116, *158*
Lambein, F., 11, *95*
Lambert, G. R., 77, *95*
Lambert, M. A., 303, 304, 305, 310, *317*, *318*
Lamfrom, H., 35, *95*
Lamport, D. T. A., 10, *101*
La Nauze, J., 152, *155*
Lang, N. J., 6, 10, 67, *95*
Langley, D., 116, *160*
Laporte, J., 131, *161*
Lapp, D. F., 287, *317*
Lark, K. G., 28, *95*
Larribe, M., 114, *159*
Larue, B., 38, 39, *89*, *95*
Larue, T. A., 70, 71, *92*
La Scolea, L. J., 257, 290, 292, 299, *317*
Lau, R. H., 68, *95*
Lauer, G., 23, *98*
Läuger, P., 201, 222, *240*
Laughrea, M., 45, *100*
Lavergne, G., 312, *314*
Lawrence, N. L., 119, *158*
Lawrence, P. J., 109, *158*
Lawson, J. W., 307, *314*
Lazaroff, N., 11, 59, *92*, *95*
Lazzarini, R. A., 47, 49, 50, 51, 53, *90*, *92*
Leach, C. K., 16, 20, 21, 31, 41, 43, 44, 51, 62, 64, *90*, *95*, *97*
Leak, L. V., 20, *95*
Leaver, A. G., 80, *94*
Lecadet, M-M., 122, *157*
Lee, F. D., 171, 191, *240*
Lee, N., 172, 233, *243*
Leefler, S., 44, *96*
Lefort, M., 6, *92*
Le Gault-Demare, L., 126, *154*
Le Hegarat, J-C., 118, *158*
Lehmann, M., 9, 15, 16, 18, 48, *96*
Lehmann, V., 170, 171, 228, 239, *244*, *245*
Leighton, T. J., 113, 114, 122, 123, 125, 126, 151, *158*, *160*
Leive, L., 165, 182, 184, 185, 190, 234, 238, *240*, *244*, *247*
Lengelsen, C., 126, *156*
Lennarz, W. J., 166, *250*
Lepesant, J. A., 105, 129, 131, *158*
Lepesant-Kejzlarovà, J., 105, 129, 131, *158*
Lessie, T. G., 37, *96*, 281, *317*
Letellier, L., 181, 182, *246*
Leunissen-Bijvelt, J., 226, *249*
Levine, G. A., 18, 19, *97*
Levinthal, C., 124, *158*
Levisohn, S., 115, 147, *158*
Levy, H. R., 281, *318*
Levy, S. B., 124, *158*, 212, *244*
Lew, H., 230, *244*
Lewin, R. A., 11, *96*
Lewis, B. J., 38, *102*
Lewis, V. J., 303, *317*
Lex, M., 68, 69, 72, *96*, *100*
Ley, A. C., 9, *96*
Liddle, J. A., 253, 293, *314*
Lim, D. V., 257, *318*
Lin, J. J-C., 232, 233, *244*, *250*

Lindberg, A. A., 185, *244*
Lindberg, B., 168, *244*
Lindsay, S., 218, 219, *244*
Lindsey, J. K., 6, *96*
Linn, T. G., 122, 123, *156*, *158*
Linnett, P. E., 110, *161*
Lipschultz, C. A., 8, *92*, *93*
Lipschultz, B., 254, *318*
Liu, C. T., 180, *244*
Liu, T-Y., 260, *318*
Locher, G., 6, *90*
Loeblich, A. R., III., 21, 22, 23, 31, *98*
Loening, U. E., 34, 37, *96*, *98*
London, J., 16, 19, 62, *99*
Lönngren, J., 168, *244*
Lopes, J., 218, *244*
Lorenz, H., 27, 29, 31, *99*
Losada, M., 66, *96*
Losick, R., 120, 121, 122, 123, 124, 125, 153, *155*, *156*, *158*, *160*, *161*
Lounatmaa, K., 185, 202, 212, 227, *244*, *246*
Lovett, P. S., 124, 137, *154*
Low, K. B., 214, *240*
Lowenstein, J. M., 281, *319*
Lubochinski, B., 135, *160*
Luckey, M., 178, 217, *245*
Luderitz, O., 168, 171, 199, 229, *242*, *244*, *245*
Lugtenberg, B., 171, 173, 174, 175, 176, 178, 185, 186, 213, 214, 215, 219, 226, *245*, *249*, *250*
Luijk, L. W., 77, *101*
Lundgren, D. G., 117, *156*
Lutkenhaus, J. F., 211, 212, 213, *245*
Lyles, R., 260, 261, *316*

M

MacAlister, J. T., 184, 220, *242*
McConnell, M., 230, *245*
McCoy, E. C., 178, *245*
McDonald, I. J., 267, 290, 309, *318*
McDowell, T. D., 276, *319*
McEntegart, M. G., 257, 293, 313, *315*, *316*
McFadden, B. A., 19, *100*
McGee, Z. A., 265, *318*
McGowan, R. E., 18, 19, 54, *93*
McIntosh, M. A., 178, 196, *245*
Mackenzie, M., 68, *95*
Mackey, B. M., 114, 115, 116, *158*
McKibbin, J., 35, *97*
Maclean, F. I., 15, *94*
MacLeod, R. A., 181, *241*, 289, *317*
McMillian, R. A., 127, *158*
McMurry, L., 212, *244*
MacPhee, D. G., 189, *247*
McQuitty, D. N., 288, 289, *317*
McVeigh, I., 299, 307, *316*
McVey, E., 122, *156*
Maaløe, O., 31, 49, 52, *96*
Maeba, P., 275, *318*
Magasanik, B., 144, *158*
Magnusson, K-E., 186, *245*
Magrum, L. J., 34, *92*
Maia, J. C. C., 121, *158*
Main, J. S., 309, 310, *319*
Mäkelä, P. H., 164, 168, 185, 188, 202, 212, 227, 229, 230, *244*, *245*, *246*, *248*, *249*
Makino, F., 21, *96*
Makula, R. A., 272, 299, 300, 301, 302, 306, 307, 311, *319*
Manca de Nadra, M. C., 122, *159*
Mandel, M., 11, 12, 36, 87, *94*, *100*
Mandelstam, J., 104, 107, 108, 111, 112, 114, 115, 116, 118, 119, 125, 132, 133, 134, 135, 137, 138, 139, 140, 141, 142, 143, 146, 148, 149, 150, 151, 152, *154*, *155*, *156*, *157*, *158*, *159*, *161*, *162*
Maness, M. J., 195, *245*
Mann, N., 30, 31, 49, 50, 51, 52, 53, *90*, *96*
Mann, N. H., 151, *155*
Manning, P. A., 213, *245*
Mansa, B., 188, *249*
Manzano, C., 66, *96*
Marchesi, V. T., 215, *249*
Mardones, E., 126, *157*
Margolskee, R., 76, *88*
Margulis, L., 3, 4, *96*
Markovitz, A., 232, *242*
Marks, C. L., 117, *156*
Marmur, J., 104, *155*
Marrs, B., 37, *96*
Martin, A., 260, *314*
Martin, D. T. M., 28, *91*
Martin, H. H., 6, *92*, 172, 202, *242*, *246*
Martin, H. M., 236, *242*
Martin, J-P., 264, *318*

Martin, T. C., 6, *96*
Marunochi, T., 28, *96*
Mason, D. B., 149, *155*
Matsuhashi, M., 190, 218, *249*
Matteo, C. C., 116, *155*
Matthews, J. L., 137, *157*
Mayer, H., 6, *95*, *101*, 168, 170, 186, 227, 229, *241*, *245*, *248*, *250*
Mayer, L. W., 252, 256, 262, *315*, *316*, *318*
Mayne, R., 148, *157*
Meadow, P. M., 228, *243*
Medeiros, A. A., 210, *245*
Meier, J. R., 37, *96*
Meijers, J., 171, 173, 175, *245*
Meinke, W., 120, *157*
Mendelson, N. H., 28, *96*
Menichi, B., 127, *159*
Menzel, J., 234, 237, *245*
Mereschowsky, C., 4, *96*
Merkel, G. J., 166, *245*
Merrick, M., 24, *91*
Messer, W., 28, *96*
Meyenburg, K., von, 43, *97*, 176, 177, 205, 206, 207, 208, 211, 215, 217, *240*, *250*
Michel, J., 137, 142, *157*
Michel, J. F., 114, 135, *159*
Midgeley, J. E. M., 49, 50, 52, 53, *96*
Mildner, G., 135, 136, *157*
Miller, C. P., Jr., 305, 313, *318*
Miller, J. S., 16, 63, *96*
Miller, L. S., 61, 62, *96*
Miller, R. D., 195, *245*, 253, 263, 264, 275, 276, 277, 279, 284, 285, 289, 290, 296, 299, 308, 309, 311, *318*
Millet, J., 111, 114, 115, 118, 122, 143, 146, *154*, *159*, *160*
Milligan, T. W., 288, *317*
Minkley, E. G., Jr., 220, *245*
Mirelman, D., 238, *245*
Mitani, T., 146, 147, *156*, *159*
Mitchison, G. J., 71, 73, 76, 77, *101*
Mitrconova, T. N., 32, *98*
Mitsui, T., 223, 226, *249*
Mitzel, J. R., 313, *318*
Miura, T., 164, 165, *245*
Miyazawa, T., 177, *246*
Mizushima, S., 164, 165, 171, 173, 175, 176, 177, 178, 199, 214, 224, 235, *243*, *245*, *246*, *249*, *250*
Mochan, B., 148, *157*
Mock, M., 264, *318*
Mohler, B. A., 278, *317*
Molina, A. M., 191, *241*
Monier, R., 35, *92*
Monner, D., 221, *240*
Montal, M., 202, *245*
Moore, P. B., 45, *100*
Moore, R. B., 16, 62, *94*
Morgan, E. A., 35, 49, 51, 53, *96*
Morowitz, H. J., 24, *101*
Morris, I., 4, *91*
Morris, J. G., 114, 115, 116, *158*
Morse, C. D., 282, 293, *318*
Morse, S. A., 195, *245*, 253, 254, 259, 260, 261, 263, 264, 265, 268, 269, 270, 271, 272, 273, 274, 275, 276, 277, 278, 279, 281, 282, 283, 284, 285, 286, 287, 288, 289, 290, 291, 292, 296, 299, 307, 308, 309, 311, *314*, *315*, *316*, *318*, *320*
Mortenson, L. E., 74, *101*
Morton, R. S., 253, *318*
Moscarello, M. A., 21, 23, *95*
Moss, C. W., 303, 304, 305, 310, *317*, *318*
Mühlradt, P. F., 170, 171, 181, 184, 234, 237, 239, *245*
Mukhersee, P. K., 116, *159*, *160*
Muller, R., 223, *249*
Munoz, L. E., 117, 129, *159*
Munoz, M. L., 33, 44, *98*
Munsche, D., 34, 36, 37, 84, *100*, *102*
Munson, R. S., Jr., 228, *246*
Murray, C. D., 121, 123, *159*
Murray, K., 22, *96*
Murray, R. G. E., 164, 225, *246*, 254, 258, *318*
Murrell, W. G., 104, 107, *159*
Myers, J. A., 6, 7, 26, 55, 56, 82, *94*, *96*, *100*

N

Nadler, H., 23, *95*
Nakae, T., 170, 175, 182, 183, 185, 194, 196, 198, 199, 200, 201, 202, 212, 215, 224, 227, 237, *241*, *246*
Nakaike, S., 180, *246*
Nakamura, K., 164, 173, 177, 199, 214, *241*, *246*
Nakashima, Y., 215, *246*
Nakayama, T., 117, 129, *159*
Nanninga, N., 181, 182, 226, *249*
Neff, I., 186, *241*

Neilands, J. B., 78, *99*, 178, 217, *243*, *245*, *250*
Neilson, A., 68, 70, *96*, *97*
Nelson, D. L., 104, 114, 119, *154*, *158*
Neter, E., 227, 229, *245*
Neufeld, B. R., 22, *89*
Nichols, J. M., 50, 53, *88*
Niel, C. B., van, 4, 20, 43, *99*
Nieva-Gomez, D., 239, *246*
Nikaido, H., 165, 166, 167, 168, 169, 170, 171, 175, 176, 177, 181, 182, 183, 184, 185, 186, 188, 191, 192, 193, 194, 196, 197, 198, 199, 202, 203, 205, 206, 207, 208, 209, 210, 211, 212, 213, 214, 215, 217, 219, 223, 226, 227, 228, 229, 230, 234, 235, 238, *240*, *241*, *242*, *243*, *244*, *245*, *248*, *249*, *250*
Nikitina, K. A., 48, *98*
Nishijima, M., 180, *246*
Nishimoto, H., 122, *159*
Nishimura, A., 184, 218, *249*
Nishimura, Y., 172, 184, 201, 215, 218, 220, 233, *243*, *249*
Nixdorff, K., 202, *246*
Nojima, S., 180, *246*
Nolteernsting, U., 62, 67, *89*
Nomura, M., 35, 49, 51, 53, *96*
Nordstrom, K., 192, *242*
Norins, L. C., 254, 255, 256, 312, *317*
Normark, S., 165, 166, 178, 192, 195, 221, *240*, *242*, *250*, 254, 258, 259, 262, 263, 264, 272, 274, 299, 300, 302, 303, 305, 309, 310, *315*, *320*
Nossal, N. G., 219, *246*
Notani, N. K., 84, *96*
Novotny, P., 261, *318*
Nurminen, M., 171, 176, 185, 188, 202, 203, 212, 213, 215, 219, 227, 237, *244*, *245*, *246*, *248*, *249*
Nyman, P. O., 275, *314*

O

O'Brien, T. F., 210, *245*
ÓCara, P., 3, 7, *96*
Odintsova, M. S., 44, *102*
Odugbemi, T. O., 313, *318*
Ohad, I., 42, 57, *92*
ÓhEocha, C., 3, 7, *96*
Ohmori, K., 66, 67, *96*
Ohné, M., 117, *159*
Ohnishi, S-I., 170, 182, 183, 194, 237, *246*
Ohno, S., 24, *96*
Ohye, D. F., 107, *159*
Oishi, M., 35, *90*
Old, J. M., 51, *95*
Olive, C., 281, *318*
Oliver, R. C., 178, *249*
Oppenheim, A. B., 85, *97*
Öquist, G., 55, 78, *96*
Oren, A., 15, 70, 79, *92*, *96*
Orkwiszewski, K. G., 83, *97*
Orrego, C., 122, *159*
Ørskov, F., 168, 188, *246*, *249*
Ørskov, I., 168, 188, *246*, *249*
Osborn, M. J., 165, 168, 169, 171, 195, 228, 230, 234, 235, 236, *241*, *244*, *246*, *247*, *248*
Osburne, M. S., 120, *159*
Oss, C. J., van, 188, *241*, *249*
Oster, G., 313, *315*
Oster, G. K., 313, *315*
Ostrovsky, D. N., 177, *246*
Overath, P., 181, 182, *246*
Owen, J. D., 194, 195, *242*
Ownby, J. D., 76, *97*
Oxender, D. L., 213, *247*
Ozols, J., 215, *247*

P

Pace, N. R., 34, 35, 37, *97*
Pace, P. J., 291, *315*
Packer, L., 77, *101*
Padan, E., 3, 15, 64, 70, 79, 85, *89*, *90*, *92*, *96*, *97*
Pakhomova, M. V., 21, 43, *93*, *97*
Palleroni, N. J., 17, *98*, 210, *248*
Palva, E. T., 175, 177, 178, 217, 221, 224, *247*
Pandey, N. K., 117, *159*
Pankratz, H. S., 43, *97*
Pao, C. C., 53, *97*
Pardee, A. B., 28, *93*, 180, 236, *242*
Paris, C. G., 144, *158*
Parisi, B., 39, *98*
Parisi, E., 165, 168, 230, 234, *246*
Park, J. T., 220, *249*
Park, S. W., 112, *156*
Parker, P. L., 17, 62, 65, *94*
Parsons, N. J., 262, *319*
Parthier, B., 34, 36, 84, *100*

Parton, R., 178, *247*
Pastan, I., 47, *97*
Pato, M. L., 35, 43, *97*
Paulus, H., 115, 116, *159*, *160*
Payne, J. W., 198, *247*
Payne, P. I., 34, 35 36, 37, *90*, *97*
Payne, S. M., 265, 312, *318*
Peacock, W. L., Jr., 254, 255, 257, *317*
Pearce, J., 16, 17, 62, 64, *97*
Pearce, S. M., 111, *159*
Pearce, W. A., 188, *241*, 265, *318*
Pearson, H. W., 14, 70, *100*
Pearson, L. C., 20, *94*
Pechman, K. R., 38, *92*, *102*
Pelczar, M. J., Jr., 285, 286, *319*
Pelroy, R. A., 15, 16, 17, 18, 19, 20, 48, 54, 64, *97*
Penn, C. W., 262, *319*
Perkins, W. J., 149, *155*
Pero, J., 120, 121, 124, *155*, *158*
Perrine, S., 293, *320*
Perry, J. J., 114, *156*, 166, *245*
Perry, M. B., 267, *319*
Peskin, A., 76, *88*
Peters, R., 171, 173, 174, 175, 178, *245*
Peterson, J. A., 104, 111, 112, 117, *157*
Peterson, R. B., 77, *97*
Peterson, R. N., 216, *247*
Philips, D. O., 50, 53, *88*
Picciolo, G., 117, *156*
Pickett, C. L., 196, *245*
Piehaud, M., 135, *156*
Pierce, L. E., 167, *250*
Pierson, D., 80, *94*
Piggot, P. J., 104, 111, 112, 114, 117, 118, 119, 130, 131, 132, 133, 136, 137, 139, 141, 142, 151, 152, *157*, *159*
Piggott, G. H., 26, *97*
Pikalek, P., 82, *97*
Piotrowski, H. D., 305, *319*
Piret, J. M., 116, *155*
Pirkle, C. I., 254, 255, 257, *317*
Pirtle, I. L., 231, *247*
Pirtle, R., 231, *249*
Pirtle, R. M., 231, *247*
Platt, D. J., 274, 275, *319*
Plaut, A. G., 293, *319*
Ploscowe, V., 291, *320*
Ploscowe, V. B., 291, *319*
Plucker, F. E., 17, 62, 65, *94*
Polukhina, L. E., 32, *98*
Posgate, J. A., 153, *155*
Postgate, J. R., 24, 69, *91*, *97*, *99*
Postnova, T. I., 32, *98*
Powell, K. R., 260, 261, *316*
Prakash, G., 78, *97*
Pratt, I., 110, 111, 119, 153, *161*
Prehm, P., 186, *247*
Preston, Y. A., 216, 217, *250*
Pritchard, R. H., 28, *97*
Prival, M. J., 144, *158*
Puck, T. T., 218, *242*
Pugsley, A. P., 177, 178, 212, 213, 215, *245*, *247*
Pulich, W. M., 65, *97*
Pun, P., 121, *159*
Pun, P. P. T., 123, *159*

Q

Quan, A. L., 256, *320*
Quay, S., 213, *247*
Quine, M. P., 76, *102*

R

Rabinowitz, J. C., 44, *99*
Raboy, B., 64, *97*
Racker, E., 296, 299, *315*
Radcliffe, C. W., 35, *97*
Radloff, R., 25, *97*
Ragland, T. E., 281, *319*
Raj Bhandary, U. L., 39, *93*
Rambach, A., 237, *248*
Rambler, M., 3, *96*
Ramus, J., 34, 37, *94*
Randall, L. L., 175, 177, 178, 217, 221, 224, 232, *243*, *247*
Randle, C. L., 302, *319*
Rapoport, G., 125, *156*
Rasmussen, N. S., 228, *246*
Rasul, S. A., 32, *99*
Rath, N., 32, *94*
Rauch, B., 197, 210, *249*
Raué, H. A., 35, *97*
Raven, J. A., 60, *97*
Ray, B., 116, *159*
Razin, S., 182, *247*
Reeves, E. C. R., 211, *247*
Reeves, P., 175, 176, 177, 178, 202, 213, 214, 218, 219, *240*, *241*, *245*, *247*, *248*
Renn, K., 172, 220, *241*, *243*

Reinert, J., 148, *159*
Reising, G., 254, 255, 256, 312, *317*
Reiss, C., 21, *98*
Reithmeier, R. A. F., 173, 187, 221, 224, *247*
Reitz, R. C., 299, 302, 303, 304, 305, 306, 308, *320*
Renkin, E. M., 207, *247*
Relimpio, A. M., 66, *96*
Rene, A. A., 256, *320*
Repko, T., 28, *95*
Restaino, L., 25, 26, 27, *97*
Revel, H. R., 127, *162*
Rexer, B., 121, *159*
Reyn, A., 253, 254, 258, 265, 290, *316*, *318*, *319*
Reysset, G., 115, 144, *160*
Rhaese, H. J., 145, 146, *160*
Richardson, W. P., 261, *319*
Richmond, A. E., 31, *101*
Richmond, M. H., 212, *241*
Richter, D., 38, *97*
Richter, G., 32, *90*
Rick, P. D., 228, *247*
Riedel, G., 76, *88*
Rietschel, E. T., 168, 171, *242*, *245*
Rigomier, D., 135, *160*
Riley, M., 24, *103*
Rimon, A., 85, *97*
Rimon, S., 7, *90*
Rippka, K., 23, *94*, *95*
Rippka, R., 7, 12, 13, 16, 17, 18, 20, 21, 48, 64, 68, 69, 70, 71, 73, 74, 75, *94*, *96*, *97*, *98*
Ris, H., 43, *98*
Risebrough, R. W., 124, *156*
Rizza, V., 301, *319*
Roantree, R. J., 188, 189, *247*
Roberts, J. W., 120, *160*
Roberts, M., 252, *315*
Roberts, N. A., 182, 184, 185, *247*
Roberts, R. B., 253, *319*
Roberts, R. J., 21, 22, *98*
Roberts, T. M., 21, 22, 23, 25, 31, *98*
Robertson, J. N., 266, *319*
Rodewald, R., 260, 261, *316*
Rodriguez-Lopez, M., 33, 43 44, *98*
Rogers, L. S., 78, *94*
Rogers, S. W., 182, 184, 185, 227, *247*
Romano, A. H., 276, *319*
Romeo, D., 167, 171, 194, 239, *247*
Roscoe, D. H., 120, *161*
Roseman, S., 197, 210, *249*
Rosenberg, H., 178, *250*
Rosenberg, S. A., 167, *247*
Rosenbusch, J. P., 175, 176, 177, 206, 210, 222, 223, 226, *247*, *248*, *249*
Rosenfelder, G., 171, *245*
Rosner, A., 27, *102*
Rosselet, A., 202, 203, 209, 210, *250*
Rotering, H., 233, *247*
Roth, I. L., 182, 185, 227, *242*
Rothfield, L., 167, 171, 194, 218, 228, 229, 239, *244*, *246*, *247*
Rothfield, L. I., 184, 218, 220, *242*, *250*
Rothstein, D. M., 122, 123, *160*, *161*
Rottem, S., 182, *247*
Rouviere-Yaniv., J., 20, 21, *93*, *98*
Rowell, P., 3, 70, 71, 72, 75, 76, *88*, *98*, *100*
Rumpf, G., 145, 146, *160*
Rundell, K., 230, *247*
Rupprecht, E., 228, *244*
Russell, H., 290, *319*
Rutberg, B., 117, *159*
Ryan, A., 255, *315*
Ryan, R., 21, *99*
Ryter, A., 107, 111, 139, 140, 152, *159*, *160*, 236, 237, *247*, *248*

S

Sabet, S. F., 180, *247*
Sacks, L. E., 146, *160*
Sadoff, J. C., 261, *319*
Safferman, R. S., 85, *98*
Sala, F., 39, *98*
Salsas, M. F., de, 176, 212, 214, *248*
Salton, 303
Salton, M. R. J., 164, *242*, 268, 283, 294, 303, *315*, *319*
Sanchez, D., 25, *96*
Sanchez, J. J., 17, *98*
Sanderson, K. E., 218, 219, 226, 229, *243* *244*, *247*
Sandstrom, G., 274, *315*
Sankoff, D., 38, *95*
Santo, L., 123, *160*
Sanwal, B. D., 276, *318*
Sarabhai, A., 35, *95*
Saranto, J., 153, *154*
Saris, C., 231, *241*

Sarkar, N., 115, 116, *160*
Sarma, V., 177, *248*
Sarvas, M., 185, 202, 212, 227, *246*
Sato, T., 190, 235, *244*, *249*
Sawyer, W. D., *314*
Saz, A. K., 266, 281, *314*
Scandella, C. J., 180, *248*
Schaechter, M., 236, *242*
Schaeffer, F., 18, 19, 48, 54, *98*
Schaeffer, P., 104, 107, 112, 114, 116, 117, 135, 139, 140, 142, 143, 146, *154*, *156*, *157*, *160*, *161*
Scheibe, J., 58, 59, *91*, *98*
Scherp, H. W., 274, *319*
Schiff, J. A., 6, 21, *91*
Schindler, H., 222, *248*
Schindler, M., 169, *248*
Schlecht, S., 186, 189, *241*, *248*
Schmidmayr, W., 176, 215, 216, *243*
Schmidt, G., 189, 227, 229, *245*, *248*
Schmidt, R. R., 30, *99*
Schmitges, C. J., 175, 176, 184, 210, *248*
Schnaitman, C. A., 165, 166, 171, 173, 175, 177, 180, 212, 214, 215, 217, 218, 238, *240*, *241*, *247*, *248*, *250*
Schmitt, R., 117, *156*
Scholes, V. E., 6, *96*
Schönherr, O. T., 27, *98*
Schopf, J. W., 3, 12, *98*
Schroeter, A. L., 254, 255, 256, 312, *317*
Schunch, W., 37, *98*
Schulman, H., 232, *248*
Schultz, S. G., 206, 207, 208, *248*
Schumacher, G., 180, *240*
Schwartz, M., 216, 217, 232, 236, 237, *247*, *248*, *249*
Schwartz, R. M., 38, *98*
Schwartzbach, S. D., 39, *93*
Schwarz, H., 226, *248*
Schwarz, U., 237, 238, *245*, *248*
Schweiger, M., 44, *98*
Schweizer, M., 178, 219, 224, 226, 235, *248*
Scott, E., 66, *89*
Searcy, D. G., 21, *98*
Segall, J., 125, 153, *160*
Seifter, S., 238, *242*
Seitz, U., 34, 36, *98*
Sekizawa, J., 172, 199, 231, *242*, *243*, *248*
Selm, N., van, 178, 185, 186, 213, 215, *245*, *249*
Selsky, M. I., 39, *98*
Sen, D., 262, *319*
Senff, L. M., 272, 299, 300, 301, 302, 306, 307, 311, *319*
Sensi, S., 39, *98*
Sentsova, O. Y., 48, *98*
Setlow, J. K., 84, *96*
Setlow, P., 119, 143, 153, *160*
Setlow, R. B., 27, *89*
Sha'afi, R. I., 194, *248*
Shaffer, P. W., 72, 77, *102*
Shaknabatian, L. G., 32, *98*
Shaltiel, L., 203, 213, 215, 219, 237, *246*
Shands, J. W., 184, *248*
Shapiro, J. A., 24, *89*
Sharrock, W. J., 44, *99*
Shaw, D. H., 309, *314*
Shaw, J., 172, 233, *243*
Shay, D. E., 310, *319*
Shechter, E., 181, 182, *246*
Shell, J., 51, 53, *92*
Shen, C., 172, 233, *243*
Sherman, L. A., 3, 34, 82, 85, *98*
Shestakov, S. V., 26, 27, 32, 83, *92*, *93*, *98*
Sheu, C. W., 191, *248*
Shibano, Y., 151, *160*
Shiflett, M., 136, *162*
Shiflett, M. A., 136, 137, *157*
Shih, D., 231, *241*
Shilo, M., 3, 15, 64, 79, 85, *88*, *90*, *91*, *97*
Shine, J., 45, *99*
Shorenstein, R. G., 122, *158*
Short, H. B., 291, *319*, *320*
Short, J. A., 261, *318*
Shuman, H., 236, 237, *247*
Shuman, H. A., 232, *248*
Shuster, C. W., 230, *247*
Sicko-Goad, L. M., 9, *99*
Siegel, R. C., 238, *245*
Siitonen, A., 188, *248*
Sikyta, B., 260, *314*
Silhavy, T. J., 216, 217, 232, *248*, *249*
Silver, W. S., 69, *99*
Silverstein, S. C., 188, *248*
Silvester, W. B., 64, *99*
Silvey, J. H. G., 70, *102*
Simon, D., 37, *93*
Simon, M., 171, *245*
Simon, R. D., 10, 11, 25, 26, 67, *95*, *99*
Simpson, F. B., 78, *99*

Singer, R. A., 6, 17, 36, 37, 40, 48, 50, 54, 68, 80, 82, *91*, *99*
Singer, S. J., 198, *248*
Singerman, E., 293, *315*
Singh, H. N., 73, 77, 83, *99*, *100*
Singh, P. K., 70, *99*
Singh, R. N., 43, 82, *98*, *99*
Sinha, R., 82, *99*
Sitz, T. O., 30, *99*
Siu, C. H., 21, *99*
Skerman, V. R. D., 65, *89*
Skurray, R., 219, 232, *240*, *248*
Slapikoff, S., 114, 115, *160*
Slater, J. H., 52, 55, *99*
Slepecky, R. A., 109, 150, *157*
Sletten, K., 266, *315*
Sleytr, U. B., 166, *249*
Smillie, L. B., 224, *243*
Smit, J., 165, 167, 171, 181, 182, 184, 185, 188, 193, 212, 214, 226, 227, 235, 236, 237, 238, *248*
Smith, A. J., 3, 14, 15, 16, 19, 20, 47, 62, 68, *88*, *89*, *99*
Smith, D., 176, 212, 214, *248*
Smith, G. D., 77, *95*
Smith, H., 35, 37, *93*, 262, *319*
Smith, I., 35, *90*, 104, 126, *155*, *156*
Smith, I. C. P., 260, *314*
Smith, R. F., 310, *319*
Smith, R. J., 42, 43, 51, 52, 53, 71, 73, 76, 77, *99*, *101*
Smith, T. E., 275, *319*
Smyth, C. J., 283, *319*
Söderström, T. O., 188, *241*
Sogin, M. L., 12, 34, 35, 38, *91*
Solek, J., 224, *243*
Solomon, A. K., 194, 195, 206, 207, 208, *242*, *248*
Solomon, R., 6, 21, *95*
Sonenshein, A. L., 120, 121, 122, 123, 144, *154*, *156*, *158*, *159*, *160*, *161*
Song, S. A., 203, 213, 215, 219, 237, *246*
Sonntag, I., 178, 224, 226, *243*, *248*
Southern, E. M., 43, 77, *99*
Sox, T., 266, *314*
Sparling, P. F., 166, 178, 189, 195, *245*, *250*, 255, 263, 265, 266, 299, 302, 303, 304, 305, 306, 308, *314*, *315*, *319*, *320*
Speth, V., 234, 237, *245*
Spinzl, M., 38, *97*
Spitzer, J. L., 114, 115, *160*
Spizizen, J., 104, 120, 135, 136, 137, *157*, *161*
Spudich, E.N., 175, 185, *240*
Spudich, J. A., 104, 114, 119, *154*, *158*
Sprinivasan, V. R., 121, *159*
Ssymank, V., 27, 29, 31, *99*
Staal, S. P., 126, 137, *154*, *161*
Stacey, G., 77, *99*
Stahl, D., 12, 38, *91*, *102*
Stallcup, M. R., 44, *99*
Stanier, R. Y., 3, 4, 6, 7, 8, 11, 12, 13, 14, 16, 17, 18, 19, 20, 21, 23, 36, 43, 48, 54, 55, 60, 62, 64, 67, 68, 69, 71, 73, 74,75, 87, *93*, *94*, *95*, *96*, *97*, *98*, *99*, *100*, 210, *248*
Stanke, R., 311, *320*
Starlinger, P., 22, *100*
Staub, A-M., 168, *245*
Stead, A., 309, 310, *319*
Steed, P., 164, 225, *246*
Stein, D. B., 21, *98*
Stein, S., 276, 277, 278, 279, 281, 282, 283, 284, *318*
Stein, W. D., 194, *248*
Steinberg, W., 127, *161*
Steiner, A., 153, *154*
Steinitz, Y., 79, *91*
Steinman, R. M., 188, *248*
Steitz, J. A., 45, *100*, 231, *249*
Stenderup, A., 22, *89*
Stent, G. S., 83, *100*, 120, *161*
Stepanov, V. M., 115, *161*
Sterlini, J. M., 107, 125, 149, *159*, *161*
Stetak, T., 6, *90*
Steven, A. C., 223, *249*
Stevens, A., 311, *320*
Stevens, C. L. R., 6, 26, 82, *100*
Stevens, S. E., Jr., 6, 7, 26, 47, 66, 67, 82, *93*, *95*, *100*
Stewart, W. D. P., 3, 5, 6, 9, 11, 14, 16, 17, 19, 20, 47, 48, 60, 66, 68, 69, 70, 71, 72, 75, 76, 83, 87, *88*, *89*, *90*, *98*, *100*
Stinnett, J. D., 166, 178, 182, 184, 185, 220, 227, *242*, *249*
Stirm, S., 186, 188, *247*, *249*
Stock, J. B., 197, 210, *249*
Stocker, B. A. D., 164, 189, 230, *247*, *249*, *250*
Stone, T. J., 229, *249*
Storck, R., 43, *100*
Strauss, N., 104, 121, 123, *159*, *161*

Streicher, S. L., 144, *158*
Strominger, J. L., 229, *249*
Strongin, A. Y., 115, *161*
Sud, I. J., 299, 300, 301, 302, 303, 304, 305, *319*
Suehiro, S., 153, *154*
Sumida-Yasumoto, C., 122, 123, *161*
Summers, A. O., 176, *241*
Suzuki, H., 172, 184, 201, 215, 218, 220, 233, *243*, *249*
Suzuki, I., 275, *319*
Suzuki, N., 189, *243*
Svensson, S., 168, *244*
Swanson, J., 188, 189, *244*, *249*, 253, 255, 256, 259, 260, 261, 262, 265, 293, 313, *315*, *316*, *319*
Swift, H., 21, *99*
Swinton, D., 6, 21, *91*
Szalay, A., 34, 36, 84, *100*
Szer, W., 44, *96*
Szmelcman, S., 216, 217, *249*
Szulmajster, J., 104, 107, 114, 115, 117, 119, 121, 122, 125, 126, 131, *155*, *156*, *158*, *159*, *161*

T

Taber, H., 117, *161*
Taber, H. W., 117, *161*
Tabita, F. R., 19, 77, *99*, *100*
Takahashi, I., 104, 122, 141, *159*, *161*
Takamatsu, N., 227, *250*
Takeishi, K., 231, *249*
Takeuchi, Y., 170, 182, 183, 194, 237, *246*
Talley, R. S., 274, 275, *317*, *319*
Tamaki, S., 190, 218, *249*
Tamori, Y., 180, *246*
Tamura, K., 151, *160*
Tanamoto, K., 189, *240*
Tandeau de Marsac, N., 9, 56, 57, 64, *100*
Tauber, H., 290, *319*
Taylor, A. L., 214, *240*
Taylor, M. M., 43, *100*
Taylor, S. Y., 118, *159*
Taylor-Robinson, D., 265, *318*
Tel-Or, E., 14, 72, 77, *100*, *101*
Tennigkeit, J., 77, *89*
Teuber, M., 221, *240*
Thacker, L., 292, 305, 306, *314*
Thayer, J. D., 303, 304, 310, *318*
Thomas, J., 72, *102*
Thomas, M. L., 303, 305, *317*
Thomassen, S., 116, *156*
Thompson, P. A., 146, *160*
Thorne, K. J. I., 178, *249*
Thornley, M. J., 164, 166, 181, *242*, *249*, 261, *315*
Thurston, E. L., 17, 64, 65, *94*
Tjian, R., 121, *161*
Tipper, D. J., 109, 110, 111, 119, 126, 153, *157*, *161*
Tolbert, N. E., 60, *101*
Tomasz, A., 270, *315*
Tomfohrde, K. M., 293, *315*
Tomita, M., 215, *249*
Tomita, T., 239, *249*
Tomkins, G. M., 46, *101*
Tonhazy, N. E., 285, 286, *319*
Torgerson, D. M., 135, 138, 151, *155*
Torti, S. V., 220, *249*
Travers, A., 49, *101*
Trowsdale, J., 136, 137, *157*, *162*
Tsai, L-B., 74, *101*
Tsukogoshi, N., 236, *249*
Tsuziki, J., 21, *96*
Tucker, A. N., 301, *319*
Turner, E. M., 292, 305, 306, *314*
Tuttle, D. M., 274, *319*
Tyeryar, F. J., Jr., 256, *320*
Tyler, B. M., 144, *158*
Tyler, J. E., 261, *315*

U

Uchida, T., 38, *102*
Ueda, K., 31, *101*
Ueki, T., 223, 226, *249*
Uemura, J., 171, 175, 178, *249*
Ueta, N., 303, 304, *320*
Unger, B., 166, 178, *240*

V

Vaccaro, D., 114, 115, *160*
Valtonen, M. V., 188, *249*
Van Baalan, C., 16, 17, 24, 32, 62, 65, 66, 67, 77, 78, 80, *88*, *94*, *97*, *99*, *100*, *101*
Vance, B. D., 6, *96*
Vandamme, E. J., 116, *155*

Van der Wyk, J. C., 281, *317*
Vanyushin, B. F., 34, *95*
Varricchio, F., 311, *320*
Vasconcelos, A. C. L., 43, 44, *101*
Vasquez, D., 33, 43, 44, *98*
Veale, D. R., 262, *319*
Vedros, N. A., 253, *320*
Verhoef, C., 176, *250*
Verhoef, K., 214, 219, *249*
Verkleij, A., 226, *249*, *250*
Vincent, P., 266, *319*
Vinci, K. D., 220, *250*
Vinograd, J., 25, *97*
Vold, B., 127, *162*
Vold, B. S., 127, *162*
von der Helm, K., 32, 33, *101*
Vonshak, A., 31, *101*
Volvis, G. F., 22, *101*

W

Waaland, J. R., 10, *101*
Wachsman, J. T., 296, *320*
Wahba, A. J., 45, *100*
Waites, W. M., 115, 118, 119, 132, 139, 140, 141, *159*, *162*
Wake, R. G., 105, *162*
Walker, J. C. G., 3, *96*
Walker, P. D., 261, *318*
Walkup, R., 17, 64, 65, *94*
Wallace, D. C., 24, *101*
Wallace, R., 255, 267, *315*, *319*
Walle, J., 105, 129, 131, *158*
Walsby, A. E., 3, 10, 87, *89*, *93*, *101*
Walsby, A. W., 3, 5, 6, 11, 14, 20, 47, 66, 87, *92*
Walstad, D. L., 166, 178, 189, *250*, 299, 302, 303, 304, 305, 306, 308, *320*
Waner, J., 311, *320*
Wang, C. H., 277, 278, *317*, *320*
Wang, S., 231, *243*
Wang, S. P., 262, *316*
Ward, M. E., 263, 266, 309, 310, *316*, *319*
Wardlaw, A. C., 178, *247*
Warren, P. I., 253, *320*
Warren, R. A. J., 114, 122, *158*
Warren, S. C., 112, 118, 119, 132, 139, 140, 141, *162*
Waterbury, J., 7, *98*
Waterbury, J. B., 12, 13, 21, 70, 75, *94*, *97*
Watson, J. D., 124, *156*
Watson, R. R., 293, *320*
Watt, P. J., 309, 310, *319*
Wayne, R., 178, 217, *245*, *250*
Ways, P., 167, *250*
Weare, N. M., 77, *89*
Weathers, P., 11, 67, 68, *88*, *99*
Weathers, P. J., 10, 69, 80, *101*
Weaver, R. E., 303, 305, *317*
Weaver, R. H., 293, *320*
Weber, H-L., 80, *101*
Weber, M., 9, 48, *101*
Weber, M. M., 117, *162*
Weckesser, J., 6, *95*, *101*, 170, *250*
Wegener, W. S., 268, 269, 270, 271, 272, 273, 274, 299, 300, 301, 302, 306, 307, 311, *314*, *319*, *320*
Weigand, R. A., 218, 220, *250*
Weintraub, H., 148, *157*
Weiss, E., 256, *320*
Weiss, J. A., 291, *319*
Weistreich, G. A., 265, *320*
Weller, D., 14, 70, *101*
Wende, R. D., 255, *316*
Werkman, C. H., 275, *319*
Westerman, M. P., 167, *250*
Westling-Haggstrom, B., 258, 259, *320*
Westphal, O., 168, 171, 189, *242*, *244*, *245*, *248*
Whadford, V., 274, *315*
Whang, H. Y., 227, 229, *245*
Wharton, R. D., 260, *320*
Wheeler, B., 218, 219, *244*
White, D. A., 166, *250*
White, D. C., 301, *319*
White, J. C., 180, 216, 217, *241*, *250*
Whiteley, H. R., 122, *157*
Whitfeld, P. R., 37, *101*
Whittenbury, R., 3, 47, *101*
Whitton, B. A., 3, 6, 14, 20, 21, 31, *90*, *95*, *101*
Wilcox, M., 71, 73, 76, 77, *101*
Wildman, R. B., 8, *101*
Wilkinson, B. J., 152, *155*
Wilkinson, B. T., 109, *162*
Wilkinson, R. G., 230, *250*
Wilkinson, S. G., 165, 182, 184, 185, *242*, *247*
Williams, J. P., 21, 23, *95*
Williams, R. P., 255, 257, *316*, *318*
Williams, V. P., 7, *101*, *102*
Wilson, G. A., 131, 162

Wilson, G. B., 20, *95*
Wilberger, H. A., 178, *245*
Winblad, B., 254, 258, 259, 274, *315*, *320*
Winkenbach, F., 72, *102*
Winter, A. J., 178, *245*
Winter, D. B., 283, 287, 288, *320*
Wiseman, G. M., 254, 255, 257, 266, 267, 309, 310, *315*, *320*
Wistar, R., Jr., 293, *319*
Witholt, B., 165, *249*
Whittman, H. G., 44, *102*, 126, *161*
Wlodkowski, T. J., 287, 299, 300, 301, 302, 306, *314*
Wöber, G., 9, 15, 16, 18, 48, *96*, *101*
Woese, C. R., 4, 12, 34, 38, 39, 86, *91*, *92*, *102*
Wolfe, R. S., 34, *92*
Wolff, H., 172, 178, *241*
Wolf-Watz. H., 165, 166, 178, 195, *250*, 254, 262, 263, 264, 272, 299, 300, 302, 303, 305, 309, 310, *320*
Wolk, C. P., 3, 6, 10, 11, 20, 67, 71, 72, 73, 76, 77, 82, 87, *90*, *91*, *92*, *95*, *102*
Wollgiehn, R., 34, 36, 37, 84, *100*, *102*
Wong, W., 264, 265, *316*
Wood, D. A., 117, 118, 119, 125, 129, 132, 133, 139, 140, 141, *155*, *162*
Wood, N. B., 68, 74, *102*
Wood, W. B., 127, *162*
Wookey, P., 178, *250*
Worcel, A., 21, *102*
Wray, V., 170, 171, 239, *245*
Wright, A., 228, 230, *245*, *250*
Wu, H-C., 172, 232, 233, *241*, *243*, *244*, *250*
Wu, M-C., 171, *250*
Wulff, J. L., 166, *242*
Wyatt, J. T., 6, 70, *96*, *102*
Wysocki, J. R., 260, *318*

Y

Yagil, E., 27, *102*, 203, *240*
Yaguchi, M., 309, *314*
Yamada, H., 165, 175, 176, *243*, *245*
Yamada, M., 184, 218, *249*
Yamada, T., 283, *316*
Yamakawa, T., 303, 304, *320*
Yamamoto, T., 108, 135, *154*, *162*
Yashouv, J., 120, *155*
Yashouv-Gan, Y., 238, *245*
Yasuda, S., 184, 201, 215, 218, 220, *243*, *249*
Yasumura, M., 231, *249*
Yates, M. G., 77, *89*
Yehle, C. O., 120, *162*
Yem, D. W., 233, *250*
Yeng, Y-H., 127, *162*
Yobs, A. R., 255, *319*
Yoshida, Y., 227, *250*
Yoshikawa, H., 84, *102*
Yoshikawa, M., 227, *250*
Yotis, W. W., 311, *320*
Young, E., 107, 109, 139, 140, *156*
Young, F. E., 131, *162*, 257, 264, 265, 268, 269, 271, 272, 274, 275, 290, 291, 292, 299, *314*, *316*, *317*, *319*, *320*
Young, I. E., 107, 139, *162*
Young, M., 77, *102*, 123, 129, 131, 142, 149, 150, 151, *155*, *157*, *162*
Yousten, A. A., 104, 111, 112, 117, *157*, *162*
Yu, F., 224, 235, *250*
Yura, T., 235, *244*
Yurina, N. P., 44, *102*

Z

Zablen, L. B., 4, 38, *102*
Zaitseva, G. N., 21, 43, *93*, *97*
Zeldin, B., 6, 21, *91*
Zhevner, V. D., 32, *98*
Zilinskas, B., 8, *92*
Zillig, W., 32, 33, 44, *98*, *101*, 121, *159*
Zimm, B. H., 23, *95*
Zimmermann, W., 202, 203, 209, 210, *250*
Zinder, N. D., 22, *101*
Zipkas, D., 24, *102*
Zubrzycki, L., 260, *320*
Zullig, W., 32, 33, *94*
Zusman, D. R., 23, 24, *102*
Zytkovicz, T. H., 112, *162*

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Subject Index

A

Abnormalities, morphological, in bacterial sporulation mutants, 140
Abortively disporic phenotype in bacterial sporulation, 138
Accessory photosynthetic pigments of cyanobacteria, 6
Acetate, excretion of by gonococci, 279
Acetate metabolism by *Neisseria gonorrhoeae*, 284
Acetate, sparing effect of on carbon dioxide in cyanobacteria, 62
Acetate utilization by cyanobacteria, 62
N-Acetylmuramyl-L-alanine amidase, activity of in gonoccocci, 271
Acridine orange, resistance of bacteria to, 195
Acidic nature of outer membrane porins in Gram-negative bacteria, 184
Acidity
  changes in during bacterial sporulation, 107
  effect of on glucose catabolism by gonococci, 277
  effect of on growth of *Neisseria gonorrhoeae*, 260
  effect on on peptidoglycan content of *Neisseria gonorrhoeae*, 265
Acidity of medium, effect of on phospholipid composition of *Neisseria gonorrhoeae*, 301
Acinetobacter, as a genus, 253
Actinomycin D, effect of on bacterial sporulation, 125
Actinomycin D sensitivity of Gram-negative bacteria to, 190
Activation of autolysis in gonococci, 274
Adaptochromes, and pleiomorphism in cyanobacteria, 59
Adenine incorporation by meningococci, 297
Adenylate charge, and induction of bacterial sporulation, 143
Adherence of layers in envelope of *Neisseria gonorrhoeae*, 254
Adhesion zones between inner and outer membranes in Gram-negative bacteria, 234
Aerobic metabolism of cyanobacteria, 14
Aerobic nitrogen fixation by cyanobacteria, 71
Affinity of Gram-negative bacteria for transported solutes, 211
Agmenellum quadruplicatum, nitrite reductase mutant of, 66
Agmenellum quadruplicatum, plasmid DNA in, 25
Aggregates, mating, formation of by *Escherichia coli*, 219
Agmenellum quadruplicatum, size of chromosome of, 23
Akinetes, formation of by cyanobacteria, 11
Alanine aminotransferase activity of *Neisseria gonorrhoeae*, 291
Alanine dehydrogenase, production of during bacterial sporulation, 112
Alkaline phosphatase activities of cyanobacteria, 78
Alkaline phosphatase, production of by sporulating bacteria, 118
Allophycocyanin in cyanobacterial photosynthesis, 8
Allophycocyanin, production of by cyanobacteria, 56
Allysine residues in porins, biosynthetic origins of, 238
Allysine residues in proteins of outer membrane of Gram-negative bacteria, 173
α-Amanitin, lack of effect of on cyanobacteria, 33
Amino-acid incorporation into, cyanobacterial proteins, 44

Amino-acid metabolism of *Neisseria gonorrhoeae*, 290
Amino-acid-requiring strains of *Neisseria* spp., 291
Amino-acid utilization by cyanobacteria, 66
Amino acids, effect of on cyanobacteria, 79
Aminoacyl-tRNA synthetases from cyanobacteria, 39
6-Aminopenicillanic acid, permeation of into Gram-negative bacteria, 207
Aminopeptidase activity of *Neisseria* spp., 293
Ammonia utilization by cyanobacteria, 66
Ampicillin, diffusion of into Gram-negative bacteria, 209
Ampicillin sensitivity of Gram-negative bacteria, variations in, 189
Amylase, production of during bacterial sporulation, 112
Amytal, effect of on electron transport in *Neisseria gonorrhoeae*, 287
Anabaena cylindrica, 3
  DNA of 21
  effect of light on differentiation of, 74
  nitrogen fixation by, 66
Anabaena flos-aquae, behaviour of gas vesicle in, 10
Anabaena nidulans
  cessation of DNA synthesis by in the dark, 29
  light-dependent RNA synthesis in, 49
  properties of RNA polymerase from, 32
  RNA polymerase from, 32
Anabaena variabilis
  DNA synthesis in, 27
  half life of mRNA in, 42
Anacystis minulans, DNA replication and cell cycle in, 30
Anacystis nidulans
  changes in nucleotide pools in during light-dark transitions, 50
  effect of light on synthesis of magic-spot nucleotides by, 52
  effect of light on synthesis of ribulose disphosphate carboxylase by, 56
  genetic map of, 85
  plasmid DNA in, 25
  taxonomic position of, 12
  transformation in, 84
Anaerobic conditions, nitrogenase activity of cyanobacteria under, 70
Anaerobiosis, effect of on cyanobacteria, 79
Anatomy of cyanobacteria, 5
Antibiotic resistance in bacteria, role of plasmids, 212
Antibiotic-resistant mutants of cyanobacteria, 82
Antibiotic sensitivity of Gram-negative bacteria, variation in, 189
Antibiotics
  hydrophilic, effect of on Gram-negative bacteria, 196
  production of by sporulating bacilli, 115
  production of in Stage 0 of bacterial sporulation, 134
Antigen, enterobacterial common, in outer membrane of Gram-negative bacteria, 168
Antigens, O, of Gram-negative bacteria, 168
Antimycin A, effect of on electron transport in *Neisseria gonorrhoeae*, 287
Aphanizomenon flos-aquae, base composition of DNA of, 21
Aphanocapsa sp.
  effect of light on synthesis of magic-spot nucleotides by, 52
  nitrogen starvation of, 68
  pentose phosphate cycle in, 19
Apoprotein synthesis in the cyanobacterial photosynthetic apparatus, 58
Arabinose, permeation of into bacteria, 208
Arabinose-binding protein of Gram-negative bacteria, biosynthesis of, 232
Archaebacteria, ribosome structure in, 39
Area of outer membrane of Gram-negative bacteria occupied by protein, 167
Arginine biosynthesis in cyanobacteria, 80
Arginine catabolism, enzymes for and bacterial sporulation, 116
Arginine, incorporation of into *Anabaena variabilis*, 79
Arginase, production of during bacterial sporulation, 112
Aspartase activity of *Neisseria* spp., 291
Asporogenus mutants of bacteria, 130
Asporogenous mutants of *Bacillus subtilis*, properties associated with, 136
Assembly of the outer membrane of Gram-negative bacteria, 228
Asymmetric distribution of molecules in

the outer membrane of Gram-negative bacteria, 181
Asymmetry in the plasma membrane of *Neisseria gonorrhoeae*, 302
Autolysin from gonoccocci, properties of, 272
Autolysis of gonococci, 268
Autolysis of gonococci, effect of antibiotics on, 272
Autotrophic physiology of cyanobacteria, 4
Autotrophy of cyanobacteria, 3
Auxotrophy for purines, and bacterial sporulation, 147
Axenic cyanobacterial cultures, use of, 12
Axial filament, formation of in bacterial sporulation, 106

B

Bacillus cereus, morphology of sporulation by, 107
Bacillus coagulans, morphology of sporulation by, 107
Bacillus popilliae, coat formation during spore formation by, 110
Bacillus sphaericus, spore formation by 109
Bacillus subtilis
genetic analysis of, 104
genetic map of, 131
morphology of sporulation in, 107
properties of asporogenous mutants of, 136
Bacillus thuringiensis, half life of mRNA in, 125
Bacitracin, production of by sporulating bacilli, 115
Bacteria, Gram-negative, outer membrane of, 163
Bacterial endospore formation, early events during, 103
Bacterial genomes, size of, 22
Bacteriophage infection of sporulating bacteria, 120
Base composition of cyanobacterial DNA, 21
Base modification in cyanobacterial DNA, 21
Beneckea harveyi, ribosomal RNA genes in, 35
Benzylpenicillin, diffusion of into Gram-negative bacteria, 209
Bicarbonate, effect of on growth of *Neisseria gonorrhoeae*, 275
Bilayers, lipid, incorporation of porins into, 222
Bile salts, resistance of Gram-negative bacteria to, 195
Bilins, in cyanobacterial photosynthesis, 7
Biliproteins, cyanobacterial, location of, 7
Biochemical basis of obligate photoautotrophy in cyanobacteria, 19
Biochemical changes during early events in bacterial sporulation, 110
Blebs in the outer membrane of Gram-negative bacteria, significance of, 184
Blue-green algae as experimental tools, 2
Bordetella pertussis, proteins of outer membrane of, 178
Branhamella, as a genus, 253
Braun lipoprotein of outer membrane of Gram-negative bacteria, 172
Braun lipoprotein, possible formation of channels by, 215
Brucella spp., structure of lipid A in, 169
Buffers, effect of on lysis of *Neisseria gonorrhoeae*, 269
Buoyant density methods for preparing outer membranes of Gram-negative bacteria, 165
Buoyancy, gas vesicles and in cyanobacteria, 10

C

Cadaverine production by gonococci, 293
Caffeine, effect of on bacterial sporulation, 146
Calcium ions, need for in activity of phospholipase in gonococci, 272
Calothrix sp., genome size of, 24
Campylobacter fetus, proteins of outer membrane of, 178
Capsule of *Neisseria gonorrhoeae*, 260
Carbohydrate metabolism of *Neisseria gonorrhoeae*, 276
Carbohydrates in the lipopolysaccharide of Gram-negative bacteria, 166
Carbon dioxide, and cyanobacterial metabolism, 60
Carbon dioxide, effect of deprivation of on the cyanobacterial photosynthetic apparatus, 61

Carbon dioxide, effect of on *Neisseria gonorrhoeae*, 257
Carbon dioxide fixation by cyanobacteria, 4, 15
Carbon dioxide requirement of *Neisseria gonorrhoeae*, 274
Carbon metabolism of cyanobacteria, 14, 15
Carbon source, deprivation of and induction of bacterial sporulation, 143
Carbon source, effect of on cyanobacterial metabolism, 60
Carbonic anhydrase activity of cyanobacteria, 60
Carbonic anhydrase activity of gonococci, 275
Carboxysomes in cyanobacteria, 9
Cardiolipin content of *Neisseria gonorrhoeae*, 300
Catabolite repression, and induction of bacterial sporulation, 143
Catalase, production of by sporulating bacteria, 119
Cations, divalent, binding of to lipopolysaccharide in Gram-negative bacteria, 169
Cations, effect of on lysis of *Neisseria gonorrhoeae*, 269
Caulobacter crescentus, proteins of outer membrane of, 178
Cefazolin, diffusion of into Gram-negative bacteria, 209
Cell cycle in cyanobacteria, and DNA synthesis, 28
Cell division of *Neisseria gonorrhoeae*, 258
Cell division, role for Braun lipoprotein in, 220
Cell division, synchronous, in cyanobacteria, 29
Cell envelope of *Neisseria gonorrhoeae*, 260
Cell shape, role of outer membrane in maintaining in Gram-negative bacteria, 220
Cell volume, role of in initiation of cell division in cyanobacteria, 30
Cells, size of during bacterial sporulation, 135
Cell-wall blebs in *Neisseria gonorrhoeae*, 254
Cellular inclusions in cyanobacteria, 9
Cephaloridine, diffusion of into Gram-negative bacteria, 209
Cephalothin, diffusion of into Gram-negative bacteria, 209
Cephalothin sensitivity of Gram-negative bacteria, variations in, 189
Cervical proteolytic enzymes, effect of on *Neisseria gonorrhoeae*, 256
Chain scission of ribosomal RNA in cyanobacteria, 36
Chamaesiphonales, as a cyanobacterial subgroup, 13
Channel in outer membrane of Gram-negative bacteria, number of porin molecules in, 222
Channels in walls of Gram-negative bacteria, 215
Channel-forming ability of porins in Gram-negative bacteria, 201
Characterization of cyanobacterial RNAs, 32
Chemoheterotrophy, facultative, in cyanobacteria, 17
Chloramphenicol, effect of on cyanobacterial ribosomes, 43
Chloramphenicol, effect of on gonococci, 272
Chlorogloea fritschii, dark growth of on sucrose, 65
Chlorogloea fritschii, operation of the tricarboxylic acid cycle in, 16
Chlorosis, induction of in cyanobacteria, 78
Chromatic adaptation in the cyanobacterial photosynthetic apparatus, 56
Chromophore synthesis in cyanobacteria, 58
Chromosomal DNA of cyanobacteria, 21
Chromosome structure in cyanobacteria, 20
Chroococcales, as a subgroup of the cyanobacteria, 13
Citrate synthase activity of *Neisseria gonorrhoeae*, 285
Cleavage, postmaturational, of ribosomal RNA in cyanobacteria, 36
Clinical isolates of *Neisseria gonorrhoeae*, bicarbonate requirements of, 275
Clostridium pasteurianum, turnover of protein during sporulation by, 114
Clostridium perfringens, half life of mRNA in, 125
Clumping of *Neisseria gonorrhoeae* in liquid medium, 259

Coat formation in bacterial sporulation, 106, 110
Coccochloris peniocystis, variations in the carbonic anhydrase activity of, 60
Coelospherium dubium, base composition of DNA of, 21
Coenzyme Q in *Neisseria gonorrhoeae*, 287
Colicin receptors on outer surface of outer membrane of Gram-negative bacteria, 186
Colicins death of *Escherichia coli* caused by, 217
Colonial types of *Neisseria gonorrhoeae*, 254
Colour of light, effect of on cyanobacterial photosynthetic apparatus, 55
Combined nitrogen sources for cyanobacteria, 66
Commitment to cell division in cyanobacteria, 31
Compartmentation in bacterial sporulation, 151
Competence, changes on during bacterial sporulation, 135
Complexity, molecular, and bacterial sporulation, 127
Composition of the outer membrane of Gram-negative bacteria, 164
Conditionally stable RNAs in cyanobacteria, 40
Conjugation of *Escherichia coli*, role for outer membrane protein in, 219
Continuous culture of gonococci, 260
Content of DNA in cyanobacteria, 31
Control of gene expression in cyanobacteria, 45
Control of light intensity, and the cyanobacterial photosynthetic apparatus, 55
Cortex formation in bacterial sporulation, 106, 109
Cross linking in lipopolysaccaride of Gram-negative bacteria, 170
Cryptomonad chloroplasts, 4
Crystal violet, effect of lipopolysaccharide on uptake by bacteria of, 192
Crystal violet uptake by *Neisseria gonorrhoeae*, 263
Cyanide, effect of on electron transport in *Neisseria gonorrhoeae*, 287
Cyanobacteria, Gram-negative nature of, 5
Cyanobacterial anatomy, 5
Cyanobacterial genome, expression of, 1
Cyanobacterial genome, properties of, 20
Cyanobacterial ribosome recognition, differences from the bacterial system, 45
Cyanobacterial taxonomic subgroups, 13
Cyanophages, 3
Cyanophages, lack of RNA-containing, 44
Cyanophages, use of in genetics, 85
Cyanophycin as a reserve nitrogen source in cyanobacteria, 67
Cyanophycin, chemical nature of, 10
Cyanophycin granules in cyanobacteria, 9
Cyclic AMP, and induction of bacterial sporulation, 143
Cyclic AMP, effect of on porin synthesis, 214
Cyclic GMP and induction of bacterial sporulation, 143
Cyclic photophosphorylation in cyanobacteria, 14
Cycloserine sensitivity, of Gram-negative bacteria variations in, 189
Cylindrospermum majus, antibiotic-resistant mutants of, 82
Cylindrospermum sp., sporulation by, 11
Cysteine requirement by gonococci, 290
Cysteine synthase, lack of in *Neisseria gonorrhoeae*, 290
Cysteine composition of medium, effect of on acetate excretion by *Neisseria gonorrhoeae*, 281
Cytochrome *c* oxidase activity in *Neisseria gonnorrhoeae*, 288
Cytochromes, need for during bacterial sporulation, 112
Cytochromes of *Neisseria gonorrhoeae*, 287
Cytoplasmic membrane, site for synthesis of outer-membrane components in Gram-negative bacteria, 233

D

Dark endogenous metabolism in cyanobacteria, 54
Dark-light shifts, metabolic effects of in cyanobacteria, 48
Death, photo-oxidative in cyanobacteria, 79
Deep rough mutants, instability of outer membrane of, 219

Deep rough mutants of Gram-negative bacteria, properties of, 184
2-Deoxy-D-glucose transport into *Neisseria gonorrhoeae*, 276
Deoxyoctulosonic acid residues in lipopolysaccharides of Gram-negative bacteria, 169
Deoxyribonuclease, production of by sporulating bacteria, 119
Deoxyribonucleic acid biosynthesis, requirement for in bacterial sporulation, 149
Deoxyribonucleic acid content, cellular, and growth rate in cyanobacteria, 52
Deoxyribonucleic acid content of cyanobacteria, 31
Deoxyribonucleic acid, cyanobacterial chromosomal, sequence complexity in, 22
Deoxyribonucleic acid, plasmid, cyanobacterial, 24
Deoxyribonucleic acid polymerase from cyanobacteria, 27
Deoxyribonucleic acid repair in cyanobacteria, 32
Deoxyribonucleic acid replication in cyanobacteria, 28
Deoxyribonucleic acid replication, requirement for in bacterial sporulation, 148
Deoxyribonucleic acid synthesis in cyanobacteria, 26
Deoxyribonucleic acid synthetic period in the cyanobacterial cell cycle, 29
Deoxyribonucleic acid, uptake of by *Neisseria gonorrhoeae*, 266
Deprivation of energy, and synthesis of conditionally stable RNAs in cyanobacteria, 41
Detergents, sensitivity of Gram-negative bacteria to, 190
Detergents, use of to dissociate components of the outer membrane in bacteria, 199
Detergents, use of to separate envelopes of Gram-negative bacteria, 166
Diaminopimelate ligase, formation of by bacterial spores, 110
Meso-Diaminopimelic acid, formation of by bacterial spores, 109
Differentiation of cyanobacteria, 3, 5
Diffusion mechanisms specific, in walls of Gram-negative bacteria, 216
Diffusion of hydrophobic molecules into Gram-negative bacteria, 192
Diffusion pathways in Gram-negative bacteria, multiplicity of, 189
Diffusion, rate of, of nafcillon into *Salmonella typhimurium*, 193
Diffusion through the outer membrane of Gram-negative bacteria, 178
Dipicolinate synthetase, half life of in bacteria, 125
Dipicolinic acid, production of during bacterial sporulation, 112
Disporic phenotype in bacterial sporulation, 139
Division, cell, of *Neisseria gonorrhoeae*, 258
Division planes in gonococci, 258
Drugs, ribosome-directed, and bacterial sporulation, 126
Drug-resistant mutants of bacteria, and sporulation, 122
Duplication of cyanobacterial genomes, 24
Dyes, sensitivity of Gram-negative bacteria to 190.

E

Early events during bacterial endospore formation, 103
Ecology of cyanobacteria, 5
Electric charges, methods based on for separating the envelope of bacteria, 166
Electron-spin resonance studies on the outer membrane of Gram-negative bacteria, 182
Electron transport, terminal, in *Neisseria gonorrhoeae*, 286
Electrophoresis of proteins of outer membrane of Gram-negative bacteria, 173
Endogenous metabolism of cyanobacteria, 16
Endonuclease action on cyanobacterial DNA, 21
Endospore formation, bacterial, early events during, 103
Energy charge, and induction of bacterial sporulation, 143

Energy charge in cyanobacteria, 16
Energy metabolism of cyanobacteria, 14
Engulfment in bacterial sporulation, 106, 109
Enterobacterial common antigen, in the outer membrane of Gram-negative bacteria, 168
Entner-Doudoroff pathway, operation of in *Neisseria gonorrhoeae*, 277
Envelope, cell, of *Neisseria gonorrhoeae*, 260
Envelope structure of *Neisseria gonorrhoeae*, 253
Envelopes of Gram-negative bacteria, preparation of, 164
Environmental factors, lack of effect of on enzyme synthesis by cyanobacteria, 46
Enzyme synthesis in cyanobacteria, induction of, 45
Enzyme turnover during bacterial sporulation, 117
Enzymes in the outer envelope of Gram-negative bacteria, 180
Enzymology of DNA synthesis by cyanobacteria, 27
Erythromycin, effect of on cyanobacterial ribosomes, 43
Erythromycin-resistant mutants of bacteria, and sporulation, 126
Escherichia coli
  formation of axial filaments in, 108
  genetic loci involved in synthesis of outer-membrane proteins of, 174
  glucose transport into, 206
  lipoprotein of outer membrane of 172
  model for structure of wall of, 225
  outer membrane of, 164
  particulate nature of outer monolayer of outer membrane of 182
  penetration of peptides into, 198
  phospholipid exchange by a fraction from, 234
  properties of porins of, 214
  structure of outer membrane of, 183
Ethylenediamine tetraacetic acid, effect of on outer membrane of Gram-negative bacteria, 184
Eukaryotic algae, chloroplasts of and cyanobacteria, 4
Events, sequence of during bacterial sporulation, 107
Evolution, and cyanobacterial genomes, 23
Evolutionary position of cyanobacteria, 4
Excess DNA in cyanobacteria, significance of, 31
Exoproteases, production of in stage O of bacterial sporulation, 134
Expression of bacterial sporulation genes, 130
Expression of cyanobacterial genome, 1

F

Ferrichrome-*ton*A protein in outer membrane of Gram-negative bacteria, 217
Ferrichrome transport into Gram-negative bacteria, 218
Filament formation by cyanobacteria, 59
Filamentous cyanobacteria, diversity among, 12
Filaments, axial, formation of during bacterial sporulation, 108
Fixation of carbon dioxide by cyanobacteria, 15
Fixation of nitrogen by cyanobacteria, 5
Faculative chemoheterotrophy in cyanobacteria, 17
Fatty acids, inhibitory effect of on growth of *Neisseria gonorrhoeae*, 307
Fatty-acyl composition of phospholipids in *Neisseria gonorrhoeae*, 302
Fatty-acyl residues in *Neisseria gonorrhoeae*, 299
Fermentation pathways in cyanobacteria, 17
Ferric enterochelin transport system in *Escherichia coli*, 218
Ferrichrome diffusion, and outer-membrane proteins in Gram-negative bacteria, 179
Fluidity of the outer membrane of Gram-negative bacteria, 182
Formylation of methionine tRNA in cyanobacteria, 40
Freeze fracture of the outer membrane of Gram-negative bacteria, 181
Freeze fracture, use of to study organization of proteins in the wall of Gram-negative bacteria, 225

Fremyella diplosiphon, action of restriction nucleases on DNA of, 22
Fremyella diplosiphon, chromatic adaptation in, 57
Fremyella diplosiphon, light-induced pleimorphism in, 59
Fremyella diplosiphon, rifampicin-resistant mutants of, 33
Fremyella diplosiphon, RNA polymerase from, properties of, 33
function of heterosysts in cyanobacteria, 71
Function of ribosomes in cyanobacteria, 43
Functions of the outer membrane in Gram-negative bacteria, 187
Fusidic acid-resistant mutants of bacteria and sporulation, 126

G

Beta-Galactosidase activity of *Neisseria* spp., 276
Beta-Galactosidase synthesis in bacteria, induction of, 149
Gas vesicles of cyanobacteria, 3, 10
Gelatinase activity of gonococci, 293
Generation times in cyanobacteria, 31
Gene expression, cyanobacterial, control of, 45
Gene expression in cyanobacteria, 80
Genes for bacterial sporulation, 128
Genes for lipopolysaccharide biosynthesis in Gram-negative bacteria, 229
Genes for outer-membrane proteins of Gram-negative bacteria, 232
Genetic competence, changes in during bacterial sporulation, 135
Genetic loci involved in sythesis of outer-membrane proteins of *Escherichia coli*, 174
Genetic map of *Bacillus subtilis*, 131
Genetic map of cyanobacteria, 29
Genetic maps of cyanobacteria, 85
Genetic recombination among cyanobacteria, 82
Genetic synchrony in *Anabaena cylindrica*, 29
Genetics and regulation of bacterial sporulation, 127
Genetics of cyanobacteria, 3, 81
Genome, cyanobacterial, expression of, 1
Genome, cyanobacterial, properties of, 20
Genomes, cyanobacterial, duplication of, 24
Gloeothece spp., nitrogen fixation by, 70
Gloeobacter violaceus, location of phycobiliproteins in, 7
Glucokinase activity in *Neisseria* spp., 279
Glucosamine disaccharide residues in lipopolysaccharide of Gram-negative bacteria, 170
Glucosamine, incorporation of into the peptidoglycan of gonococci, 271
Glucose assimilation by cyanobacteria, 20
Glucose, as the only energy source for *Neisseria gonorrhoeae*, 276
Glucose catabolism by *Neisseria gonorrhoeae*, 277
Glucose dehydrogenase, production of by sporulating bacteria, 119
Glucose metabolism by *Neisseria gonorrhoeae*, 277
Glucose oxidase, lack of from *Neisseria gonorrhoeae*, 276
Glucose, permeation of into bacteria, 208
Glucose 6-phosphate dehydrogenase activity and endogenous metabolism in cyanobacteria, 16
Glucose 6-phosphate dehydrogenase activity, and heterotrophic growth of cyanobacteria, 64
Glucose 6-phosphate dehydrogenase activity of *Neisseria* spp., 279
Glucose transport into *Escherichia coli*, 206
Glucose utilization by *Anabaena variabilis*, 64
Glutamate dehydrogenase activity of *Neisseria gonorrhoeae*, 290
Glutamate oxidation by *Neisseria gonorrhoeae*, 286
Glutamine as a possible trigger of bacterial sporulation, 144
Glutamine, effect of on nitrogenase synthesis in cyanobacterial heterocysts, 75
Glutamine, excretion of by cyanobacteria heterocysts, 75
Glutamine-requiring mutants of bacilli, induction of sporulation in, 144
Glutamine synthetase and induction of bacterial sporulation, 144

Glutamine synthetase, role of in cyanobacterial heterocyst formation, 76
L-Glycero-D-mannoheptose residues in lipopolysaccharides of Gram-negative bacteria, 169
Glycerol, penetration of into liposomes, 199
Glycogen as an endogenous reserve in cyanobacteria, 48
Glycogen as an energy reserve in cyanobacteria, 16
Glycogen granules in cyanobacteria, 9
Glycollate dehydrogenase from cyanobacteria, 60
Glycosyl transferases involved in lipopolysaccharide biosynthesis in Gram-negative bacteria, location of, 230
Gonococci, turnover of peptidoglycan in, 268
Gonorrhoea, incidence of World-wide, 252
Gramicidin, production of by sporulating bacilli, 115
Gram-negative bacteria, model for structure of wall of, 225
Gram-negative bacteria, outer membrane of, 163
Granules, cyanophycin, in cyanobacteria, 10
Green light, response of cyanobacterial photosynthetic apparatus to, 56
Growth patterns of *Neisseria gonorrhoeae*, 258
Growth phase, effect of on phospholipid composition of *Neisseria gonorrhoeae*, 302
Growth phase, role of in bicarbonate requirement of *Neisseria gonorrhoeae*, 275
Growth rate and induction of bacterial sporulation, 143
Growth rate, effect of on lipopolysaccharide composition of *Neisseria gonorrhoeae*, 267
Growth rate and pore size in Gram-negative bacteria, 202
Growth rate of cyanobacteria and cellular DNA content, 52
Guanosine-plus-cytosine contents of cyanobacterial DNA, and taxonomy, 12

H

Haemophilus influenzae, size of pores in outer membrane of, 210
Half life of sporulation mRNA in bacteria, 124
Heat-modifiable proteins of outer membrane of Gram-negative bacteria, 172
Heterocystous, non, cyanobacteria, induction of nitrogenase in, 69
Heterocysts, cyanobacterial, effect of light on formation of, 75
Heterocysts, formation of cyanobacterial, 11, 71
Heterotrophic growth of cyanobacteria, 64
Hexaminidase activity of gonococci, 271
Hexosamine residue content of the lipopolysaccharide from gonococci, 267
Hexose phosphates, inhibition of highly phosphorylated nucleotides, by, 145
Highly phosphorylated nucleotides, role of in induction of bacterial sporulation, 145
Histidase synthesis by bacilli, 149
Histidase, synthesis of during bacterial sporulation, 113
Histone-like proteins in cyanobacteria, 20
Hormogonia formation by *Nostoc muscorum*, 59
Hybridization of cyanobacterial ribonucleic acids, 42
Hydrocarbon chains in outer membrane of Gram-negative bacteria, as a barrier, 195
Hydrogen evolution by cyanobacterial nitrogenase, 77
Hydrogenase activity of cyanobacterial heterocysts, 77
Hydrophilic antibiotics, effect of on Gram-negative bacteria, 196
Hydrophilicity, role of outer membrane in conferring on Gram-negative bacteria, 187
Hydrophobic molecules, entry of into Gram-negative bacteria, 192
Hydrophobic nature of porins, from Gram-negative bacteria, 177
Hypoxanthine requirement of some strains of *Neisseria gonorrhoeae*, 297

I

Inclusions, cellular, in cyanobacteria, 9
Induction of bacterial sporulation, 142
Induction of morphogenetic changes in bacterial sporulation, time of, 105
Induction of nitrogenase in non-heterocystous cyanobacteria, 69
Infection of sporulating bacteria by phage, 120
Inhibition of cleavage of ribosomal RNA in cyanobacteria, 37
Initiation of carbon-dioxide fixation in cyanobacteria, 19
Initiation of cell division in *Neisseria gonorrhoeae*, 258
Initiator of transcription, role of formylmethionine-tRNA, 39
Inorganic ions, and *Neisseria gonorrhoeae*, 311
Inorganic nitrogen metabolism of *Neisseria gonorrhoeae*, 290
Ions, inorganic, and *Neisseria gonorrhoeae*, 311
Iron, and metabolism of *Neisseria gonorrhoeae*, 312
Iron, and virulence of gonococci, 265
Iron, effect of on cyanobacteria, 78
Irrelevant events during bacterial sporulation, 112
Isobutylmethylxanthine, effect of on bacterial sporulation, 146
Intensity of light, effect of on cyanobacterial photosynthetic apparatus, 55
Interrupted tricarboxylic-acid cycle in cyanobacteria, 63
Intracellular proteases, and bacterial sporulation, 115
Inulin, penetration of into Gram-negative bacteria, 198
Invagination of membrane in bacterial sporulation, 108
Investments surrounding cyanobacteria, nature of, 6

K

Kinetics of transport across the outer membrane of Gram-negative bacteria, 205

L

Labelling of DNA in cyanobacteria, 26
Labour, division of in bacterial sporulation, 151
β-Lactam antibiotics, rates of hydrolysis of by bacteria, 203
Lactate dehydrogenase activity of *Neisseria gonorrhoeae*, 283
Lactate metabolism by *Neisseria gonorrhoeae*, 283
Lactose, permeation of into bacteria, 208
Leakage of periplasmic proteins from Gram-negative bacteria, 218
Leucine incorporation into cyanobacteria, and protein synthesis, 54
Leucocyte-association factor of gonococci, 262
Light-dark shifts, metabolic effects of in cyanobacteria, 48
Light, effect of on differentiation of *Anabaena cylindrica*, 74
Light, effect of on macromolecule synthesis in cyanobacteria, 47
Light, effect of on protein synthesis in cyanobacteria, 54
Light, effect of on RNA synthesis in cyanobacteria, 49
Light-induced pleiomorphism in cyanobacteria, 59
Light intensity control, and the cyanobacterial photosynthetic apparatus, 55
Light intensity, effect of on cyanobacterial photosynthetic apparatus, 55
Light-mediated controls on RNA synthesis in cyanobacteria, 51
Light reactions in cyanobacterial photosynthesis, 8
Light, use of to obtain synchronous cultures of cyanobacteria, 29
Lincomycin, effect of on cyanobacterial ribosomes, 43
Linear sequence of gene expression in bacterial sporulation, 137
Linkage map of *Bacillus subtilis*, 105
Lipase activity of *Neisseria gonorrhoeae*, 305
Lipid A, biosynthesis of in Gram-negative bacteria, 228
Lipid A, of *Neisseria gonorrhoeae*, structure of, 262, 309

Lipid A, structure of in lipopolysaccharide of Gram-negative bacteria, 169
Lipid carrier in biosynthesis of lipopolysaccharide in Gram-negative bacteria, 229
Lipid composition of *Neisseria gonorrhoeae*, 299
Lipopolysaccharide
biosynthesis of from outer membrane of Gram-negative bacteria, 228
composition of from gonococci, 266
composition of outer membrane of Gram-negative bacteria, of, 168
of the cyanobacterial wall, 6
extent of coverage of surface of Gram-negative bacteria by, 185
extraction of from Gram-negative bacteria, 166
of gonococci, degration of, 267
of Gram-negative bacteria, possible linking of to protein, 171
of Gram-negative bacteria, translocation of, 234
of the outer membrane of *Neisseria gonorrhoeae*, 261
use of as a marker in studies on outer-membrane growth in Gram-negative bacteria, 237
Lipoprotein, Braun, of outer membrane of Gram-negative bacteria, 172
Liposomes, preparation of, 199
Lithium diiodosalicylate, use of to extract bacterial proteins, 200
Loci, genetic, and sporulation by bacteria, 119
Long-lived mRNA in bacteria, 125
Lyngbya sp., size of genome in, 23
Lysine, requirement by gonococci, 260
Lysis, release of bacterial spores by, 110
Lysis of *Neisseria gonorrhoeae*, 269
Lysogenic phage, and sporulating bacteria, 120
Lysostaphin, inability of to lyse gonococci, 264
Lysozyme resistance of cyanobacterial heterocysts, 74
Lysozyme-sensitivity of cyanobacteria, 6
Lysozyme sensitivity of deep rough mutants of Gram-negative bacteria, 218
Lytic enzymes, sensitivity of cyanobacteria to, 6

M

Macromolecule synthesis in cyanobacteria, effect of light on, 47
Macromolecules, penetration of the outer membrane of Gram-negative bacteria by, 218
Magic-spot nucleotides and regulation of microbial metabolism, 46
Malachite green, inhibitory effect of on Gram-negative bacteria, 191
Malate dehydrogenase activity of *Neisseria gonorrhoeae*, 284
Malate oxidase activity of *Neisseria gonorrhoeae*, 284
Maltodextrin diffusion and outer-membrane proteins in Gram-negative bacteria, 179
Maltose diffusion, and outer membrane proteins in Gram-negative bacteria, 179
Maltose transport into Gram-negative bacteria, 216
Maltose utilization by *Neisseria meningitidis*, 276
Maltotriose transport into Gram-negative bacteria, 217
Mapping of bacterial spore genes, 130
Maps, genetic, of cyanobacteria, 85
Markers, biochemical, and bacterial sporulation, 116
Mating aggregates, formation of by *Escherichia coli*, 219
Mating among cyanobacteria, 82
Maturation of bacterial spores, 110
Maturation of cyanobacterial ribosomal RNA, 34
Maturation of outer-membrane proteins of Gram-negative bacteria, 232
Maturation of ribosomal RNA in cyanobacteria, 34
Mecillinam, effect of on *Neisseria gonorrhoeae*, 258
Medium composition, effect of on glucose catabolism by *Neisseria* spp., 280
Membrane, excess formation of by bacterial sporulation mutants, 141
Membrane, outer, of Gram-negative bacteria, 163
Membranes of cyanobacteria, 5

Membrane proteins, changes in during bacterial sporulation, 135
Mesosomes, formation of in bacteria, 108
Messenger RNA for bacterial spore coat proteins, 117
Messenger RNA for the Braun lipoprotein in Gram-negative bacteria, 231
Messenger RNA, half life of in sporulating bacteria, 124
Messenger RNA in cyanobacteria, 41
Metabolic changes during bacterial sporulation, 116
Metabolic regulation in cyanobacteria, 18
Metabolism, endogenous, of cyanobacteria, 16
Metalloprotease, production of during bacterial sporulation, 112
Methionine sulphoxime, effect of on nitrogenase formation by cyanobacterial heterocysts, 75
6-Methylaminopurine in cyanobacterial DNA, 21
Methylase action on cyanobacterial DNA, 22
Methylation of cyanobacterial DNA, 21
Methylcytosine in cyanobacterial DNA, 21
N-Methylphenylalanine, presence of residues of in proteins from gonococcal pili, 266
5-Methyltryptophan, effect of on RNA synthesis in cyanobacteria, 53
Methylxanthines, effect of on bacterial sporulation, 146
Micro-aerophilic growth of cyanobacteria, 70
Mitomycin C, effect of on cyanobacteria, 30
Modification of bases in cyanobacterial DNA, 21
Molecular biology, and the cyanobacteria, 4
Molecular complexity of bacterial sporulation, 127
Moraxella, as a genus, 253
Morphogenetic changes during bacterial sporulation, 105
Morphological abnormalities in bacterial sporulation mutants, 140
Morphological stages in sporulation of bacilli, 106
Morphology mutants of cyanobacteria, 82
Morphology of cyanobacteria, 11
Murein lipoprotein of outer membrane of Gram-negative bacteria, 172
Mutagens, effect of on cyanobacteria, 81
Mutants, bacterial sporulation, phage conversion of, 124
Mutants, defective in steps in cyanobacterial heterocyst formation, 77
Mutants of cyanobacteria, isolation of, 31
Mutations in bacterial spore genes, 129
Mycobacillin, production of by sporulating bacilli, 115
Mycoplasmas, size of genome in, 22

N

Nafcillin, penetration of into Gram-negative bacteria, 192
Negative charge, role of outer membrane of Gram-negative bacteria in conferring, 187
Neisseria, as a genus, 253
Neisseria catarrhalis, phosphatidylethanolamine content of, 301
Neisseria gonorrhoeae
antibiotic-resistant strains of, 252
carbon dioxide requirement of, 274
fatty-acyl composition of 304
hydrolysis of phospholipids in, 273
model for autolysis of, 271
as a pathogen, 253
pathway for pyrimidine nucleotide synthesis in, 295
physiology of, 251
proteins of outer membrane of, 178
outer membrane of, 261
sensitivity of to hydrophobic compounds, 195
viability of, 268
Neisseria meningitidis
as a pathogen, 253
proteins of outer membrane of, 178
pyrimidine metabolism of, 294
Neisseria sicca, pathways of glucose catabolism by, 277
Neisseria spp., non-pathogenic species of, 252
Neomycin sensitivity of Gram-negative bacteria, variations in, 189

Nitrate, effect of on cyanobacterial photosynthetic pigments, 57
Nitrate reductase, changes in during bacterial sporulation, 135
Nitrate reductase formation by cyanobacteria, repression of by ammonia, 67
Nitrate reductase of cyanobacteria, 66
Nitrate reduction by gonococci, 290
Nitrate utilization by cyanobacteria, 66
Nitrite reductase activity of cyanobacteria, 66
Nitrite reduction by gonococci, 290
Nitrite utilization by cyanobacteria, 66
Nitrogen deprivation of cyanobacteria, effects of, 67
Nitrogen fixation, aerobic, by cyanobacteria, 71
Nitrogen fixation by cyanobacteria, 3, 5
Nitrogen metabolism of *Neisseria gonorrhoeae*, 290
Nitrogen source, deprivation of and induction of bacterial sporulation, 143
Nitrogen sources for cyanobacteria, 66
Nitrogenase in cyanobacterial heterocysts, 74
Nitrogenase, induction of in non-heterocystous cyanobacteria, 69
Nitrogenase structural gene expression, and glutamine synthetase, 76
Nodularia sp., size of genome of, 23
Non-cyclic photophosphorylation in cyanobacteria, 14
Non-heterocystous cyanobacteria, induction of nitrogenase in, 69
Nomenclature of oxygen-evolving prokaryotes, 3
Nostoc muscorum, light-induced pleiomorphism in, 59
Nostoc sp. NAC, heterotrophic growth of, 65
Nucleases, restriction, in cyanobacteria, 22
Nucleic acid ratios, effect of light on in cyanobacteria, 51
Nucleotide precursors in *Neisseria* spp., 294
Nucleotide sequences in cyanobacterial ribosomal RNAs, 37
Nucleoside transport into Gram-negative bacteria, 216
Nucleosides, diffusion of and outer-membrane proteins in Gram-negative bacteria, 179
Nucleotides, phosphorylated, role of in induction of bacterial sporulation, 145
Number of genetic loci concerned with bacterial sporulation, 132
Nutrient limitation as a trigger in bacterial endospore formation, 105
Nutrient utilization by *Neisseria gonorrhoeae*, 257
Nutrition, and induction of bacterial sporulation, 142

O

O antigens, bacterial, nature of, 168
Obligate photoautotrophy, biochemical basis of in cyanobacteria, 19
Oligosporogenous bacteria, 126
Opacity of colonies of *Neisseria gonorrhoeae*, 255
Opacity of colonies of Gram-negative bacteria, and surface molecules, 189
Operator control in expression of sporulation genes in bacilli, 137
Order of expression of bacterial sporulation genes, 133
Oscillatoria limnetica, protein synthesis in, 79
Oscillatoria tenuis, sequencing of ribosomal RNA from, 37
Outer membrane, functions of in Gram-negative bacteria, 187
Outer membrane in Gram-negative bacteria, passage of hydrophilic molecules across, 198
Outer membrane of Gram-negative bacteria, 163
Outer membrane of Gram-negative bacteria as a barrier to hydrophobic molecules, 192
Outer membrane of Gram-negative bacteria, assembly of, 228
Outer membrane of Gram-negative bacteria, growth of, 233
Outer membrane of Gram-negative bacteria, permeability of to raffinose, 206
Outer membrane of Gram-negative bacteria, protein content of, 167
Outer membrane of Gram-negative bacteria, structure of, 168

Outer membrane of *Neisseria gonorrhoeae*, 261
Outer-membrane proteins, role of in pore formation in Gram-negative bacteria, 200
2-Oxo-3-deoxyoctonoate (KDO)-8-phosphate synthetase from Gram-negative bacteria, 228
2-Oxo-3-deoxyoctulosonic acid residues in the lipopolysaccharide from gonococci, 267
α-Oxoglutarate dehydrogenase, absence of from cyanobacteria, 16
Oxygen, effect of on cyanobacteria, 79
Oxygen, requirement for in heterocyst formation in cyanobacteria, 75

P

Palmitic acid residues in phospholipids of *Neisseria gonorrhoeae*, 302
Particles in membrane of Gram-negative bacteria, proteinaceous nature of, 226
Penetration barrier, outer membrane of Gram-negative bacteria as a, 190
Penetration of macromolecules in the outer membrane of Gram-negative bacteria, 218
Penicillin, prevention of septum formation by in sporulating bacilli, 109
Penicillin-resistant strains of *Neisseria gonorrhoeae*, 262
Pentose-phosphatepathway, operationofin *Neisseria gonorrhoeae*, 277
Pentose synthesis by *Neisseria gonorrhoeae*, 282
Peptides, antibiotic, production of by sporulating bacilli, 115
Peptides, penetration of into *Escherichia coli*, 198
Peptidoglycan
-associated proteins in outer membrane of Gram-negative bacteria, 175
composition of *Neisseria gonorrhoeae*, 264
content of *Neisseria gonorrhoeae*, 265
of the cyanobacterial wall, 6
of gonococci, labelling of, 270
role of in maintaining asymmetry in the outer membrane of Gram-negative bacteria, 184
synthesis in gonococci, 259
turnover in gonococci, 259, 268, 270
Periplasmic proteins, leakage from Gram-negative bacteria, 218
Periplasmic space in bacteria, estimated volume of, 197
Permeability coefficients of outer membrane in *Salmonella typhimurium*, 203
Permeability of membrane in Gram-negative bacteria, differences in, 189
Permeability of the outer membrane of *Neisseria gonorrhoeae*, 263
Phage conversion of bacterial sporulation mutants, 124
Phage infection of sporulating bacteria, 120
Phage-mediated transduction in cyanobacteria, 84
Phage receptors on outer surface of the outer membrane in Gram-negative bacteria, 186
Phages, growth of in sporulating bacterial cells, 135
Phagocytosis, basis for ability of bacteria to avoid, 188
Phase brightness, development of during bacterial sporulation, 110
Phase brightness, in bacterial sporulation, 136
Phenol, inhibitory effect of on Gram-negative bacteria, 191
Phenotypic exclusion in bacterial sporulation mutants, 132
Phormidium persicinium, fragments of ribosomal RNA in, 37
Phosphatase, alkaline, production of by sporulating bacilli, 118
Phosphate, effect of on cyanobacteria, 78
Phosphate, inorganic, repression of alkaline phosphatase synthesis by, 118
Phosphate transport into Gram-negative bacteria, 206
Phosphatidic acid content of *Neisseria gonorrhoeae*, 300
Phosphatidylcholine content of *Neisseria gonorrhoeae*, 300
Phosphatidylethanolamine content of *Neisseria gonorrhoeae*, 300
Phosphatidylethanolamine in outer membrane of Gram-negative bacteria, 168

Phosphoenolpyruvate carboxylase activity of gonococci, 275
6-Phosphogluconate dehydratase activity of *Neisseria* spp., 279
6-Phosphogluconate dehydrogenase and endogenous metabolism in cyanobacteria, 16
Phospholipase A, activity of in gonococci, 272
Phospholipase activity of *Neisseria gonorrhoeae*, 305
Phospholipase A in the outer membrane of Gram-negative bacteria, 180
Phospholipid composition of outer membrane of Gram-negative bacteria, 168
Phospholipid hydrolysis in gonococci, 272
Phospholipid molecules, concentration in inner half of the outer membrane of Gram-negative bacteria, 181
Phospholipid molecules in outer membrane of *Salmonella typhimurium*, number of, 167
Phospholipids, breakdown of in *Neisseria gonorrhoeae*, 273
Phospholipids, inhibitory effect of on growth of *Neisseria gonorrhoeae*, 307
Phospholipids, movement from inner to outer membrane in Gram-negative bacteria, 234
Phospholipids of *Neisseria gonorrhoeae*, 299
Phosphomannoisomerase-less mutants of Gram-negative bacteria, 234
Phosphorylated nucleotides, role of in induction of bacterial sporulation, 145
Photoassimilation of carbon sources by cyanobacteria, 62
Photoautotrophic metabolism of cyanobacteria, 14
Photoautotrophy, obligate, biochemical basis for, 19
Photoheterotrophy in cyanobacteria, 17
Photomorphogenesis in cyanobacteria, 59
Photo-oxidative death in cyanobacteria, 79
Photosynthesis by cyanobacteria, 5
Photosynthetic bacteria, nature of, 4
Photosystems in cyanobacterial photosynthesis, 8, 14
Photosystems in heterocysts of cyanobacteria, lack of, 72
Phycobiliprotein degradation in cyanobacteria, 61
Phycobiliprotein synthesis, and chromatic adaptation in cyanobacteria, 58
Phycobiliprotein synthesis, effect of carbon dioxide on in cyanobacteria, 60
Phycobiliproteins, cyanobacterial, 3
Phycobiliproteins, degradation of in nitrogen starvation in cyanobacteria, 67
Phycobiliproteins of cyanobacteria, location of 6
Phycobilisomes, cyanobacterial, structure and function of, 8
Phycobilisomes of cyanobacteria, 3
Phycocyanin content of cyanobacterial photosynthetic apparatus, and light intensity, 55
Phycocyanin-specific protease from cyanobacteria, 61
Phycoerythrin, production of by cyanobacteria, 56
Phylogeny, and the cyanobacterial genome, 24
Phylogeny of cyanobacteria, 11
Phylogenetic status of cyanobacteria, 3
Physiology, autotrophic, of cyanobacteria, 4
Physiology of *Neisseria gonorrhoeae*, 251
Pili, and virulence of Gram-negative bacteria, 188
Pili of *Neisseria gonorrhoeae*, 265
Pilus, F protein receptor for in *Escherichia coli*, 219
Plasmid DNA, and cyanobacteria, 24
Plasmids, of *Neisseria gonorrhoeae*, 256
Plasmids, possible effect on permeability of bacteria, 212
Plectonema boryanum, nitrogen requirements of, 69
Plectonema boryanum, transition of from photoautotrophic to heterotrophic growth, 64
Pleiomorphism in cyanobacteria, 59
Pleiotropy in bacterial sporulation mutants, 132
Polyamines, oxidation of by *Neisseria perflava*, 293

Poly-L-aspartate nature of cyanophycin, 11
Polymerase, RNA, and regulation of bacterial sporulation, 120
Polymyxin-resistant strains of *Pseudomonas aeruginosa*, 220
Polypeptides in cyanobacterial heterocysts, 73
Polypeptides that bind to RNA polymerase, isolation of from sporulating bacteria, 122
Polyphosphate granules in cyanobacteria, 9
Polyribosomes in cyanobacteria, 43
Population probability factor, and induction of bacterial sporulation, 142
Pores, diameter of in Gram-negative bacteria, 202
Pores, formation of in outer membrane of Gram-negative bacteria, 175
Porin-deficient mutants of Gram-negative bacteria, 206
Porin molecules, insertion of in the outer membrane of Gram-negative bacteria, 238
Porins
  alternatives to in walls of Gram-negative bacteria, 215
  association of with peptidoglycan in the outer membrane of Gram-negative bacteria, 187
  as pore-forming molecules in Gram-negative bacteria, 201
  incorporation of into lipid bilayers, 222
  in outer membrane of Gram-negative bacteria, 175
  number of molecules in each Gram-negative bacterium, 206
  structural genes for in Gram-negative bacteria, 177
  synthesis of in Gram-negative bacteria, 213
Postmaturational cleavage of ribosomal RNA in cyanobacteria, 36
Post-translational clipping of proteins of outer membrane of Gram-positive bacteria, 233
Precursors of cyanobacterial ribosomal RNA, 35
Preparation of the outer membrane of Gram-negative bacteria, 164
Prespore formation by bacilli, 106
Proflavin, effect of on synthesis of messenger RNA in cyanobacteria, 42
Progesterone, inhibitory effect on *Neisseria gonorrhoeae*, 263
Prokaryotes, oxygen-evolving, 3
Protase, metallo, production of during bacterial sporulation, 112
Proteases, production of during bacterial sporulation, 113
Protein composition of gonococcal pili, 266
Protein content of outer membrane of Gram-negative bacteria, 167
Protein G of the outer membrane of Gram-negative bacteria, 180
Protein synthesis in cyanobacteria, effect of light on, 54
Proteins
  asymmetric distribution of in the outer membrane of Gram-negative bacteria, 186
  from cyanobacterial ribosomes, isolation of, 44
  membrane, changes in during bacterial sporulation, 135
  of cyanobacterial gas vesicles, 10
  of lipopolysaccharide of Gram-negative bacteria, 171
  of the outer envelope of *Neisseria gonorrhoeae*, 255
  of the outer membrane in Gram-negative bacteria, biosynthesis of, 231
  of the outer membrane in Gram-negative bacteria, insertion of, 235
  spore coat, production of by sporulating bacilli, 117
Proteolysis, role of in biosynthesis of proteins of the outer membrane in Gram-negative bacteria, 231
Proteolytic cleavage of RNA polymerase, and bacterial sporulation, 122
Protoplast, spore, formation of by bacilli, 109
Proteus mirabilis, lipoprotein of outer membrane of, 172
Proteus morganii, outer-membrane proteins of, 202
Pseudomonas aeruginosa
  biosynthesis of lipopolysaccharide in, 228
  particle-free areas in the outer membrane of, 185

presence of large pores in outer membrane of, 210
proteins of outer membrane of, 178
polymyxin-resistant strains of, 220
Purine
incorporation into *Neisseria gonorrhoeae*, 298
metabolism by *Neisseria* spp. 296
nucleoside biosynthesis, pathway for in *Neisseria gonorrhoeae*, 297
starvation, effect of on bacterial sporulation, 146
utilization by cyanobacteria, 66
Putrescone production by gonococci, 292
Pygmy cells, formation of in sporulation mutants of bacterial, 139
Pyrimidine metabolism in *Neisseria* spp. 294
Pyruvate, ferrodoxin-oxidoreductase activity of cyanobacteria, 67
Pyruvate metabolism by *Neisseria gonorrhoeae*, 283

Q

Quality of light, effect of on cyanobacterial photosynthetic apparatus, 56

R

Radiorespirometry of *Neisseria gonorrhoeae*, 277
Raffinose, diffusion of into *Salmonella typhimurium*, 197
Re-arrangements of components of the outer membrane of Gram-negative bacteria during growth, 238
γ-Receptor in Gram-negative bacteria, nature of, 217
Recombination, genetic, among cyanobacteria, 82
Recombination repair of cyanobacterial DNA, 32
Reconstitution of components of the Gram-negative outer membrane, 199
Red light, response of cyanobacterial photosynthetic apparatus to, 56
Regulation and genetics of bacterial sporulation, 127
Regulation of cyanobacterial enzyme synthesis, problems in, 46
Regulation of expression of cyanobacterial genome, 1
Repair of damaged DNA in cyanobacteria, 32
Replication of DNA in cyanobacteria, 28
Replication of DNA, requirement for in bacterial sporulation, 148
Repression, catabolite, and induction of bacterial sporulation, 143
Repression of amino-acid biosynthesis in cyanobacteria, 80
Repressor, possible existence of for bacterial sporulation, 142
Requirement for DNA replication in bacterial sporulation, 148
Reserve nitrogen sources of cyanobacteria, 67
Resistance to antibiotics, and ribosomes in sporulating bacteria, 126
Restriction and modification of cyanobacterial DNA, 22
Restriction enzymes, and research on bacterial endospore formation, 104
Rhodopseudomonas palustris, structure of lipid A in, 170
Rhodopseudomonas sphaeroides, phospholipid exchange by a fraction from, 234
Rhodophytan chloroplasts, 4
Ribonuclease, production of by sporulating bacilli, 116
Ribonucleic acid polymerase, and regulation of bacterial sporualtion, 120
Ribonucleic acid polymerase, changes in subunit composition of and bacterial sporulation, 121
Ribonucleic acid polymerase from cyanobacteria, 32
Ribonucleic acid polymerase, specificity of template of and bacterial sporulation, 121
Ribonucleic acid polymerases, and light-induced pleiomorphism in cyanobacteria, 59
Ribonucleic acid synthesis, effect of light on in cyanobacteria, 49
Ribonucleic acid synthesis in bacterial sporulation, 125
Ribonucleic acid, unstable, in cyanobacteria, 41
Ribonucleic acids, conditionally stable, in cyanobacteria, 40

Ribonucleic acids in cyanobacteria, 32
Ribosomal changes in bacterial sporulation, 125
Ribosomal function in cyanobacteria, 44
Ribosomal RNA in cyanobacteria, 34
Ribosomal RNA synthesis in cyanobacteria, 53
Ribosomal RNAs, cyanobacterial, sequences in 37
Ribosome content and growth rate in cyanobacteria, 52
Ribosome evolution in prokaryotes, 39
Ribosome structure in cyanobacteria, 43
Ribosomes, cyanobacterial, properties of, 34
Ribosomes of cyanobacteria, properties of, 43
Ribulose diphosphate carboxylase, effect of light on synthesis by cyanobacteria, 56
Rickettsiae, size of genome in, 22
Rifampicin
  effect of on bacterial sporulation, 125
  effect of on gonococci, 272
  effect of on RNA polymerase from cyanobacteria, 33
Rifampicin-resistant mutants of bacteria, and sporulation, 122
Rigidity, cell-wall, role of outer membrane in maintaining, 220
Rod nature of the cell of *Neisseria gonorrhoeae*, 259
Rotating proteins in the outer membrane of Gram-negative bacteria, 217
Rotenone, effect of electron transport in *Neisseria gonorrhoeae*, 287

S

Salmonella anatum, biosynthesis of O side chains in lipopolysaccharide of, 229
Salmonella minnesota, structure of lipid A in, 170
Salmonella spp., lipoprotein of outer membrane of, 172
Salmonella typhimurium
  bicarbonate affinity for, 275
  biosynthesis of O side chains in lipopolysaccharide of, 229
  model for structure of wall of, 225
  number of phospholipid molecules in outer membrane of, 195
  outer membrane of, 164
  particulate nature of outer monolayer of outer membrane of, 182
  properties of porins of, 214
  rate of diffusion of nafcillin into, 193
  structure of lipopolysaccharide of, 169
  structure of outer membrane of, 183
  translocation of major outer-membrane protein in, 235
Salmonella spp., avoidance of phagocytosis by, 188
Salts, effect of on porin synthesis, 214
Sarkaosyl, use of to extract outer membranes of Gram-negative bacteria, 166
Scytonema sp., size of genome of, 24
Septation by *Neisseria gonorrhoeae*, 258
Septation in bacterial sporulation, 106
Septum formation in bacterial sporulation, 108
Sequence complexity in cyanobacterial chromosomal DNA, 22
Sequences of ribosomal RNAs in cyanobacteria, 37
Serine protease, production of during bacteria sporulation, 113
Serological typing of Gram-negative bacteria, 168
Serratia spp., lipoprotein of outer membrane of, 172
Serum resistance in gonococci, 262
Shape, cell, role of outer membrane in maintaining in Gram-negative bacteria, 220
Sheaths of cyanobacteria, 5
Siderochromes, excretion of by cyanobacteria, 78
Sigma factor, and transcription during bacterial sporulation, 122
Silence in the bacterial genome, 151
Size of cells during bacterial sporulation, 135
Sources of nitrogen for cyanobacteria, 66
Spacing between layers in the wall of Gram-negative bacteria, 225
Specific diffusion mechanisms in walls of Gram-negative bacteria, 216
Specificity, template, of RNA polymerase, and bacterial sporulation, 121

Spherical bodies associated with colonies of *Neisseria gonorrhoeae*, 254
Spirillum serpens, proteins of outer membrane of, 178
Spo mutants of bacteria, 123
Spore coat proteins, production of by sporulating bacilli, 117
Spore septum formation in bacterial sporulation, mutants of, 137
Spores, release of by bacilli, 106
Sporulation, bacterial, induction of, 142
Sporulation, bacterial, modification of by phage, 120
Sporulation, bacterial, ribosomal changes in, 125
Sporulation mutants of bacteria, 130
Sporulation-specific events in bacteria, 111
Sporulation-specific genetic loci in bacteria, 131
Stability of mRNA in cyanobacteria, 42
Stachyose, diffusion of into *Salmonella typhimurium*, 197
Stage O genetic loci in bacteria, 134
Stage II loci in bacterial sporulation, 137
Starvation as a trigger for bacterial endospore formation, 105
Starvation of nutrients, induction of chlorosis in cyanobacteria by, 78
Starvation of purines, effect of on bacterial sporulation, 146
Steroids, effect of on incidence of isolation of *Neisseria gonorrhoeae*, 310
Storage products of cyanobacteria, 3
Streptolydigin, effect of on cyanobacteria, 33
Streptolydigin, effect of on synthesis of messenger RNA in cyanobacteria, 41
Streptolydigin-resistant mutants of bacteria, and sporulation, 122
Streptomycin, effect of on cyanobacterial ribosomes, 43
Streptomycin resistance of gonococci, 265
Streptovaricin-resistant mutants of bacteria, and sporulation, 122
Stringent control of RNA synthesis in cyanobacteria, 51
Structure and function in the cyanobacterial genome, 38
Structure of heterocysts in cyanobacteria, 71
Structure of outer membrane of Gram-negative bacteria, 168, 183
Subunit composition of RNA polymerase, changes of during bacterial sporulation, 121
Subunits of cyanobacterial RNA polymerase, 34
Succinate dehydrogenase activity of gonococci, 289
Sucrase synthesis by bacilli, 149
Sucrose, dark growth of by cyanobacteria, 65
Sugar residues, transfer of in lipopolysaccharide biosynthesis, 229
Sugars in lipopolysaccharide of Gram-negative bacteria, nature of, 229
Sulphate, effect of on cyanobacteria, 78
Sulphate transport into Gram-negative bacteria, 206
Sulphite, effect of on acetate excretion by *Neisseria gonorrhoeae*, 280
Sulpholactic acid, production of by sporulating bacteria, 119
Suppression of bacterial sporulation, 136
Supramolecular organization of components of the outer membrane in Gram-negative bacteria, 181
Survival of *Neisseria gonorrhoeae*, 268
Switch from light to dark metabolism in cyanobacteria, 18
Synchrony, genetic, in *Anabaena cylindrica*, 29
Synchrony of bacterial sporulation, 107
Synechococcus cedrorum, plasmid DNA in, 25
Synechococcus sp., lactic fermentation by, 17
Synechococcus sp., taxonomic position of, 12
Synthesis of DNA in cyanobacteria, 26
Synthesis of DNA, requirement for in bacterial sporulation, 149
Synthesis of peptidoglycan in gonococci, 268
Synthesis of RNA, effect of light on in cyanobacteria, 49

T

Taxonomy of cyanobacteria, 11
Taxonomy of *Neisseria gonorrhoeae*, 253
Temperature-dependence of diffusion into Gram-negative bacteria, 207

Temperature, effect of on diffusion of hydrophobic compounds into bacteria, 194
Temperature, effect of on glucose catabolism by *Neisseria* spp., 278
Temperature, effect of on growth of *Neisseria gonorrhoeae*, 257
Temperature, effect of on lysis of *Neisseria gonorrhoeae*, 269
Temperature-sensitive sporulation mutants of bacilii, 114
Template specificity of RNA polymerase, and bacterial sporulation, 121
Terminal electron transport in *Neisseria gonorrhoeae*, 286
Tetramethylphenylenediamine reductase, presence in the outer membrane of Gram-negative bacteria, 180
Theophylline, effect of on bacterial sporulation, 146
Thermal transitions in lipopolysaccharide of Gram-negative bacteria, 182
Thylakoids of cyanobacteria, properties of, 6
Thymidine, incorporation of into cyanobacteria, 27
Thymidine kinase, absence of from cyanobacteria, 27
Thymidine phosphorylase, absence of from cyanobacteria, 27
Thymine incorporation into *Neisseria* spp., 294
Thymine starvation, and bacterial sporulation, 116
Tolypothrix distorta, precursors of ribosomal RNA in, 35
Tolypothrix tenuis, chromatic adaptation in, 57
Topological signal in septum formation in bacterial sporulation, 138
Toxins, production of by sporulating bacilli, 116
Transaminases of *Neisseria* spp., 291
Transcriptional control in cyanobacteria, 46
Transcription during bacterial sporulation, 119
Transcription in cyanobacteria, 33
Transduction among cyanobacteria, 84
Transduction, use of to map bacterial spore genes, 129
Transfer RNA, alterations in during bacterial sporulation, 127
Transfer RNA in cyanobacteria, 39
Transformation in bacilli, 104
Transformation in cyanobacteria, 83
Transformation, use of to map bacterial spore genes, 129
Transition from dark to light, effect of on RNA synthesis by cyanobacteria, 49
Translation during bacterial sporulation, 124
Translation in cyanobacteria, 44
Translocation of lipopolysaccharide of Gram-negative bacteria, 234
Translocation of outer-membrane components from the cytoplasm in Gram-negative bacteria, 233
Transport of glucose into *Neisseria gonorrhoeae*, 276
Tricarboxylic acid-cycle enzymes, and bacterial sporulation, 117
Tricarboxylic acid cycle, interrupted, in cyanobacteria, 63
Tricarboxylic acid cycle, regulation of in gonococci, 279
Trilaminar structure of outer membrane in Gram-negative bacteria, 181
Trimer nature of porins from Gram-negative bacteria, 177
Trimers of porins, association of with lipopolysaccharide in the outer membrane of Gram-negative bacteria, 224
Triplets of pores, existence of in the outer membrane of Gram-negative bacteria, 223
Triton X-100, use of to separate envelopes of Gram-negative bacteria, 166
Trypsin, effect on outer-membrane proteins of *Neisseria gonorrhoeae*, 255
Turnover of mRNA in cyanobacteria, 42
Turnover of peptidoglycan in gonococci, 268
Turnover of protein during bacterial sporulation, 114
Twitching motility of *Neisseria* spp., 253

U

Ultrastructure of *Neisseria gonorrhoeae*, 253

Undecaprenol monophosphate, role of in lipopolysaccharide biosynthesis in Gram-negative bacteria, 229
Unstable RNA in cyanobacteria, 41
Uracil, incorporation of into cyanobacteria, 26
Uracil utilization by *Neisseria* spp., 296
Urea utilization by cyanobacteria, 66

V

Vaccine against gonorrhoea, 252
Variants, colonial of *Neisseria gonorrhoeae*, 255
Verbascose, diffusion of into *Salmonella typhimurium*, 197
Vesicles, gas, of cyanobacteria, 3, 10
Viability of *Neisseria gonorrhoeae*, 268
Viridans streptococci, 261
Virulence of gonococci, and presence of pili, 265
Vitamin B_{12}-binding protein, location of in Gram-negative bacteria, 216
Vitamin B_{12}, diffusion of and outer-membrane proteins in Gram-negative bacteria, 179

W

Wall, model for structure of in Gram-negative bacteria, 225
Walls of cyanobacteria, 5
Water-filled pores, permeation into Gram-negative bacteria through, 207
Wavelength, effect of in response of cyanobacteria to light, 55

Z

Zones of growth in outer membrane in Gram-negative bacteria, 236

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