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Volume 8

A. H. Rose &
D. W . Tempest

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MICROBIAL PHYSIOLOGY

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Advances in
MICROBIAL PHYSIOLOGY

Edited by
A. H. ROSE
School of Biological Sciences
Bath University
England

and

D. W. TEMPEST
Microbiological Research Establishment
Porton, Salisbury
England

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Contributors to Volume 8

J. R. BENEMANN, *Department of Chemistry, University of California, San Diego, La Jolla, California, 92037, U.S.A.*

H. GOLDFINE, *Department of Microbiology, School of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania 19104, U.S.A.*

J. C.-C. HUANG, *Department of Bacteriology, University of California, Davis, California 95616, U.S.A.*

M. P. STARR, *Department of Bacteriology, University of California, Davis, California 95616, U.S.A.*

R. E. STRANGE, *Microbiological Research Establishment, Porton, Nr. Salisbury, Wiltshire, England.*

I. W. SUTHERLAND, *Department of General Microbiology, University of Edinburgh, Edinburgh, Scotland.*

R. C. VALENTINE, *Department of Biochemistry, University of California, Berkeley, California 94720, U.S.A.*

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Contents

Contributors to Volume 8 v

Comparative Aspects of Bacterial Lipids. HOWARD GOLDFINE

I. Introduction 1
II. Lipids of Bacteria Compared With Those of Higher Organisms . 2
A. Apolar Components: Fatty Acids, Alk-1-Enyl Ethers and Alkyl Ethers 2
B. Extractable Polar Lipids of Bacteria 5
C. Non-Extractable Lipids in Bacteria 16
D. Neutral Lipids of Bacteria 17
E. Poly-β-Hydroxybutyric Acid 18
III. Biosynthesis of Bacterial Lipids 19
A. Biosynthesis of the Apolar Components 19
B. Biosynthesis of the Complex Lipids 28
IV. Bacterial Lipid Compositions 35
A. Lipids of Gram-Negative Bacteria 35
B. Complex Lipids of Gram-Positive Bacteria 42
C. Conclusions 50
V. Acknowledgements 50
References 51

The Pathways of Nitrogen Fixation. JOHN R. BENEMANN and R. C. VALENTINE

I. Introduction 59
A. Nitrogen-Fixing Micro-Organisms 60
B. Biochemistry of Nitrogen Fixation 64
II. Electron Donors 70
A. Pyruvate 70
B. Formate 72
C. Nicotinamide Nucleotides 73
D. Hydrogen 76
E. Photosynthetic Electron Donors 76
III. Electron Carriers 78
A. Ferredoxins 80
B. Flavodoxins 83
C. Coupling Factors of *Azotobacter* 85
IV. Nitrogenase 88
A. Isolation and Properties 88
B. Mechanism of Action 91

V. Conclusions and Future Outlook 94
VI. Acknowledgements 97
References 98

Rapid Detection and Assessment of Sparse Microbial Populations. R. E. STRANGE

I. Introduction 105
II. Principles of Rapid Microbial Assessment Methods . . . 107
A. Physical Properties of Microbes 108
B. Chemical Composition of Microbes 109
C. Microbial Growth and Metabolism 111
D. Bacterial Enzymes 113
E. Immunological Properties of Microbes 114
III. Rapid Broad Spectrum Methods 118
A. Luminol Chemiluminescence 118
B. Determination of ATP 120
C. Staining Methods 121
D. Physical Methods 124
E. Methods Depending on Growth and Metabolism . . . 125
IV. Rapid Specific Identification Methods 126
A. Immunofluorescence-Membrane Filtration Techniques . 126
B. Radioactive Antibody Technique 127
C. Analysis of Bacterial Growth Products 130
D. Gas Chromatography-Pyrolysis Methods 131
E. Analysis of Phosphorescent Decay 131
V. Rapid Determination of Microbial Viability 131
A. Indirect Methods 131
B. Direct Methods 132
VI. Rapid Detection and Determination of Viruses . . . 132
A. Immunofluorescent-Monolayer Techniques . . . 132
B. Immuno-Adherence 133
C. Radioactive Antibody Method Applied to Bacteriophage T7 133
D. Detection of Virus Activity with Gas Chromatography . 135
VII. Conclusions and Prospects 136
VIII. Acknowledgements 137
References 137

Bacterial Exopolysaccharides. I. W. SUTHERLAND

I. Introduction 143
II. Production 146
A. In Growing Cultures 146
B. In Washed Cell Suspensions 148

III. Properties 149
A. Isolation 149
B. Purification 149
C. Composition 150
D. Structural Features 156
E. Enzymic Hydrolysis 158
F. Structures of Some Exopolysaccharides 165
IV. Biosynthesis 185
A. Enzymes and Precursors 185
B. Cell-Free Synthesis. 188
C. Control 201
V. Function of Exopolysaccharides 206
VI. Unanswered Questions 206
VII. Conclusions 207
VIII. Acknowledgements 208
References 208

Physiology of the Bdellovibrios. M. P. STARR and J. C.-C. HUANG

I. Introduction 215
II. Isolation, Cultivation, Distribution, and Taxonomy of *Bdellovibrio* 218
A. Isolation 218
B. Culture Media 218
C. Maintenance of *Bdellovibrio* Cultures. 219
D. Distribution and Importance in Nature 220
E. A Taxonomic and Terminological Note 221
III. Structure and Chemical Composition of *Bdellovibrio* . . 222
A. Morphology and Ultrastructure 222
B. Chemical Composition of *Bdellovibrio* Cells. . . . 226
IV. Symbiosis Between Host-Dependent Bdellovibrios and Host Cells 228
A. Life Cycle of Host-Dependent Bdellovibrios . . . 229
B. Effects of *Bdellovibrio* on the Host Cell 239
C. Mechanisms of Lysis of the Host Cell by *Bdellovibrio* . . 242
D. Kinetics of Host-Dependent Growth 246
E. Nutrition of *Bdellovibrio* 246
F. Host Specificity 249
V. Metabolism of *Bdellovibrio* 252
VI. Host-Independent Derivatives of *Bdellovibrio* 254
VII. *Bdellovibrio* Bacteriophages 256
VIII. Acknowledgements 257
References 257
Author Index 263
Subject Index 277

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Comparative Aspects of Bacterial Lipids

HOWARD GOLDFINE

Department of Microbiology, School of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania 19104, U.S.A.

I. Introduction 1
II. Lipids of Bacteria Compared With Those of Higher Organisms . . 2
A. Apolar Components: Fatty Acids, Alk-1-Enyl Ethers and Alkyl Ethers 2
B. Extractable Polar Lipids of Bacteria 5
C. Non-Extractable Lipids in Bacteria 16
D. Neutral Lipids of Bacteria 17
E. Poly-β-Hydroxybutyric Acid 18
III. Biosynthesis of Bacterial Lipids 19
A. Biosynthesis of the Apolar Components 19
B. Biosynthesis of the Complex Lipids 28
IV. Bacterial Lipid Compositions 35
A. Lipids of Gram-Negative Bacteria 35
B. Complex Lipids of Gram-Positive Bacteria 42
C. Conclusions 50
V. Acknowledgements 50
References 51

I. Introduction

A decade of intensive work on the lipid components of the bacterial cell has revealed major differences in the lipids of prokaryotic and eukaryotic organisms and in the lipid compositions of different groups of prokaryotic organisms. In this review, I shall attempt to outline these differences, to describe the biosynthetic systems the presence or absence of which lead to the observed compositions, and to assess the significance of these differences. Bacterial lipids have been discussed extensively in monographs by Asselineau (1966) and O'Leary (1967). Several review articles concerned with various aspects of bacterial lipids have appeared in the recent past (Kates, 1964, 1966; Macfarlane, 1964b; Lennarz, 1966;

In the shorthand designations of unsaturated fatty acids and aldehydes, the number preceding the colon is the chain length. Following the colon is the number of double bonds. In the designation (n-x) for mono-unsaturated fatty acids, the position of the double bond is x carbon atoms from the methyl end of the chain. Abbreviations used for bacterial phospholipids are given in Fig. 4 (p. 6).

Law, 1967; Ikawa, 1967; Lennarz, 1970). A review on the membranes of bacteria with particular reference to the phospholipids has also appeared (Op den Kamp *et al.*, 1969c).

II. Lipids of Bacteria Compared With Those of Higher Organisms

A. Apolar Components: Fatty Acids, Alk-1-Enyl Ethers and Alkyl Ethers

1. Fatty Acids

The fatty acids of bacteria are generally 10–20 carbons in length, with 15–19 carbon chains predominating. These are mainly of four types: straight-chain saturated, straight-chain mono-unsaturated, branched

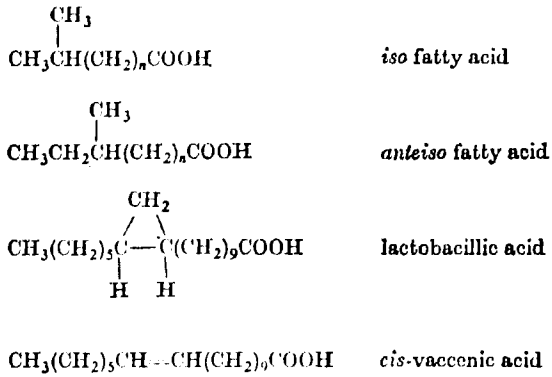


Fig. 1. Formulae of branched, cyclopropane and mono-unsaturated fatty acids of bacteria.

chains, predominantly *iso* and *anteiso*, and cyclopropane fatty acids (Fig. 1). Several differences from the fatty acyl chains of higher organisms are immediately apparent. With few exceptions, which will be discussed later, the longer chain polyunsaturated fatty acids typical of the lipids of animal tissues are absent, as are the C_{18} di- and tri-unsaturated acids. The cyclopropane and branched-chain fatty acids, on the other hand, are rarely encountered in higher organisms, with the exception of the cyclopropene fatty acids of plants (Law, 1971). Among the mono-unsaturated fatty acids, the *n*-7 series (counting from the methyl group) is the predominant type. Thus the C_{18} mono-unsaturated fatty acid most commonly found is *cis*-vaccenic acid, 18:1 Δ^{11} (Fig. 1) rather than oleic acid, 18:1 Δ^{9} (Scheuerbrandt and Bloch, 1962). The C_{16} mono-unsaturated acid most frequently found is palmitoleic acid, 16:1 Δ^{9}, which is also the most common 16:1 isomer in plants and animals.

The cyclopropane fatty acids are widely distributed among both Gram-positive and Gram-negative species, for example in the lactobacilli, streptococci, clostridia, enterobacteria, and Brucellaceae (see Section III. A. 1. d. p. 26). The predominant cyclopropane fatty acids are *cis*-9,10-methylenehexadecanoic acid and *cis*-11,12-methylene octadecanoic acid (lactobacillic acid), but *cis*-9,10-methyleneoctadecanoic acid (dihydrosterculic acid) has also been found (Law, 1971). The *iso*- and *anteiso*-branched acids predominate in the Bacillaceae and Micrococcaceae.

The distribution of saturated and mono-unsaturated fatty acids on phosphatidylethanolamine in several Gram-negative species was studied by Hildebrand and Law (1964). In *Azotobacter agilis*, *Escherichia coli*, and *Serratia marcescens* the fatty acids linked to C-1 of glycerol were mainly saturated and those linked to C-2 were mainly unsaturated and cyclopropane. *Agrobacterium tumefaciens* had over 90% unsaturated plus cyclopropane fatty acids, and there was only a slight degree of difference between the residues on C-1 and C-2. The only exception to the general rule, which is also obeyed in higher organisms, that the fatty acids linked to C-1 tend to be more saturated than those linked to C-2, was *Clostridium butyricum*, an anaerobic, Gram-variable species that has large amounts of plasmalogens (see p. 4).

Another group of fatty acids common to the Gram-negative bacteria, and not usually found in the lipids of higher organisms, are the β-hydroxy fatty acids. The most common of these are β-hydroxydecanoic, β-hydroxylauric, and β-hydroxymyristic acids which are found in the lipid A (see Section II. C. p. 16) of *Escherichia coli* (Burton and Carter, 1964), *Proteus* (Nesbitt and Lennarz, 1965), *Pseudomonas* (Hancock *et al.*, 1970) and other Gram-negative species.

2. *Alk-1-Enyl Ethers*

It has recently been shown that a wide variety of anaerobic bacteria contain plasmalogens, which are glycerolipids containing an α,β un-

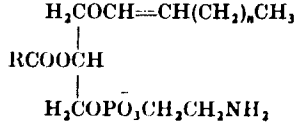


FIG. 2. Formula of an ethanolamine plasmalogen.

saturated or alk-1-enyl ether on the C-1 of glycerol (Fig. 2) (Allison *et al.*, 1962; Wegner and Foster, 1963; Goldfine, 1964; Kamio *et al.*, 1969). The other carbons of the glycerol backbone of plasmalogens are linked

in the usual fashion to a fatty acid at C-2 and a phosphate ester at C-3. This structure has been verified for the plasmalogens of a number of higher organisms (Rapport and Norton, 1962) but in only one species of bacteria, *Clostridium butyricum* (Hagen and Goldfine, 1967). Presumably the same structure will be found for the plasmalogens of other anaerobes, but this remains to be confirmed. On acid hydrolysis, plasmalogens yield long-chain fatty aldehydes. I shall refer to the alk-1-enyl ether-linked chains as aldehydes for convenience despite the fact that the evidence that these chains arise from long-chain aldehydes during the formation of plasmalogens is not completely convincing (see Section III. A. 2. p. 28).

At present it is difficult to generalize about the variety and distribution of aldehyde chains in bacterial plasmalogens. Allison *et al.* (1962), Wegner and Foster (1963), and Katz and Keeney (1964) demonstrated the formation of branched-chain fatty aldehydes with 13–17 carbon atoms in pure cultures of rumen bacteria and in the mixed flora isolated from cow's rumen. These branched-chain aldehydes were shown to be derived from branched volatile fatty acids, which are known to be nutritional requirements for this group of organisms (Allison *et al.*, 1962; Wegner and Foster, 1963).

The long-chain aldehydes derived from the plasmalogens of *C. butyricum* are similar in composition to the long-chain fatty acids of this organism. The major components are 16:0, 16:1, 17:cyc, 18:0, 18:1, and 19:cyc (Goldfine, 1964; Goldfine and Panos, 1971).

The fatty aldehydes released from the plasmalogens of *Selenomonas ruminantium* have been analysed by Kanegasaki and Takahashi (1968) and Kamio *et al.* (1970a). They consist of normal saturated and monounsaturated fatty aldehydes, with 12–18 carbon chain lengths predominating. A response to volatile fatty acids added to the growth medium was seen in that cells grown on odd-chain volatile fatty acids or lactic acid contained predominantly odd-numbered chains, and cells grown on even-numbered volatile fatty acids contained mainly even numbered long-chain aldehydes. The long-chain fatty acid composition reflected the added volatile fatty acids in a similar manner.

3. Alkyl Ethers

Long-chain alcohols bound to phospholipids in alkyl ether linkage have been found in the extremely halophilic bacteria. Kates *et al.* (1966) have made a thorough study of extreme halophiles, which require 4 *M* sodium chloride for growth, and moderate halophiles requiring 1 *M* sodium chloride. The latter group had normal fatty acid ester-containing lipids but the extreme halophiles had very little if any fatty acids in their lipids.

Instead, the lipids contained predominantly di-*O*-alkyl analogues of phosphatidylglycerophosphate. The major alcohol was found to be dihydrophytyl alcohol (Fig. 3). This lipid appears to be unique to extreme halophiles. *O*-Alkyl ether lipids are found in varying amounts in animal tissues, but these are usually of the mono-alkyl, mono-acyl type, have no dihydrophytyl alcohol, and are of the normal *sn*-glycerol 3-phosphate configuration while the di-*O*-alkyl phosphatides of *Halobacterium cutirubrum* are of the opposite *sn*-glycerol 1-phosphate configuration (Joo and Kates, 1969).

Small amounts of *O*-alkyl, acyl, glycerophosphatides have recently been found in anaerobic bacteria (Hagen and Blank, 1970; Kamio *et al.*, 1970b; Kim *et al.*, 1970).

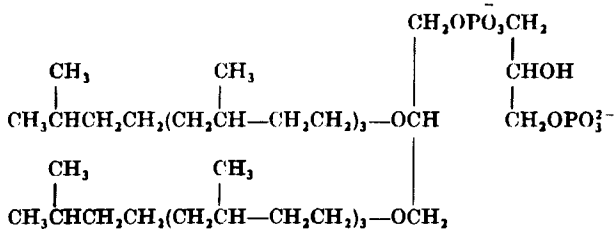


FIG. 3. Formula of the diether analogue of phosphatidylglycerol phosphate.

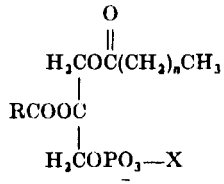
B. EXTRACTABLE POLAR LIPIDS OF BACTERIA

1. Diacyl Phosphoglycerides

As in the lipids of higher organisms, lipids built around a *sn*-glycerol 3-phosphate backbone are commonly found and are indeed the most abundant form of polar lipids in most groups of bacteria. Figure 4 illustrates the basic structure of these lipids and the major types of substituents found to be attached to the phosphate of the glycerophosphate backbone. Although most of the common glycerophospholipids of higher organisms are also found in bacteria there are major differences in their abundances, which will be described below.

a. *Phosphatidylethanolamine* is distributed widely among bacteria as it is in the tissues of higher organisms. In Gram-negative bacteria it is frequently the major glycerophospholipid. The exceptions are some of the Gram-negative bacteria that are capable of carrying out a stepwise methylation of phosphatidylethanolamine to yield phosphatidyl-N-methylethanolamine, phosphatidyldimethylethanolamine, phosphatidylcholine or mixtures of these (Fig. 4). As shown recently by Randle *et al.* (1969), the relative amounts of phosphatidylethanolamine

and phosphatidylcholine in *Agrobacterium tumefaciens* varies during the growth of batch cultures. In the log phase, phosphatidylethanolamine was predominant, but in the stationary phase the ratio became reversed and phosphatidylcholine predominated. A similar change was reported by Shively and Benson (1967), who found that the percentage of phosphatidylethanolamine decreased from 20 to 3%, while the percentage of phosphatidyl-N-methylethanolamine increased from 36 to 54% between the first and third day in a culture of *Thiobacillus thio-oxidans*.



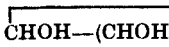
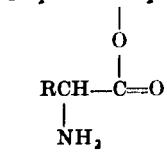
X =	Name of phospholipid
H	Phosphatidic acid
$CH_2CH_2NH_3$	Phosphatidylethanolamine
$CH_2CH_2NHCH_3$	Phosphatidyl-N-methylethanolamine
$CH_2CH_2N(CH_3)_2$	Phosphatidyl-N,N′-dimethylethanolamine
$CH_2CH_2\overset{+}{N}(CH_3)_3$	Phosphatidylcholine (lecithin)
$CH_2CHOHCH_2OH$	Phosphatidylglycerol
Phosphatidyl glycerol	Cardiolipin (diphosphatidyl glycerol)
	Phosphatidylinositol
	O-Amino acyl phosphatidylglycerol

FIG. 4. Structures of bacterial phosphoglycerides.

From the data amassed in many laboratories, it appears that all of the Gram-negative organisms of the order Eubacteriales (Breed *et al.*, 1957) are capable of synthesizing phosphatidylethanolamine. Among these are the large and important groups of enterobacteria, which have been studied in great detail, and the medically important family Brucellaceae which includes species of *Brucella* and *Haemophilus*. Unfortunately, the lipids of only a few members of this family have been studied with modern analytical techniques. Most members of the other orders of Gram-negative bacteria also appear to be capable of synthesizing phosphatidylethanolamine, but the data on these other groups are less

complete. The phospholipid compositions of individual species will be described in greater detail in the last section of this article.

The distribution of phosphatidylethanolamine in the Gram-positive organisms is more complex. It is a major phospholipid constituent of the spore-forming genus *Bacillus*, but is not found in most of the non-spore-forming Gram-positive groups including the large groups of Micrococcaceae, Lactobacillaceae, and some of the Corynebacteriaceae (Ikawa, 1967).

b. *Phosphatidylcholine*, a major component of the membranes of fungi, plants, and animals is a relatively rare component of bacteria (Goldfine and Ellis, 1964; Hagen *et al.*, 1966; Ikawa, 1967; Randle *et al.*, 1969). Among the exceptions to this generalization are a number of Gram-negative bacteria including the agrobacteria (Kaneshiro and Marr, 1962; Goldfine and Ellis, 1964), *Nitrosocystis oceanus*, Hyphomicrobiales (Hagen *et al.*, 1966; Goldfine and Hagen, 1968; Park and Berger, 1967), *Brucella abortus* Bang (Thiele *et al.*, 1968), *Thiobacillus novellus* (Barridge and Shively, 1968), and some of the photosynthetic bacteria (Wood *et al.*, 1965; Gorchein, 1964; Lascelles and Szilagyi, 1965). The other N-methylated derivatives of phosphatidylethanolamine, phosphatidyl-N-methylethanolamine and phosphatidyl-N,N′-dimethylethanolamine, are also being found in an increasing number of Gram-negative bacteria, either with or without phosphatidylcholine. For example, phosphatidyldimethylethanolamine occurs in *Hyphomicrobium vulgare* in substantial amounts along with phosphatidylcholine (Hagen *et al.*, 1966). Phosphatidylmethylethanolamine has been found in *Clostridium butyricum* (Baumann *et al.*, 1965), several species of *Thiobacillus* (Barridge and Shively, 1968) and in *Proteus vulgaris* (Goldfine and Ellis, 1964; Randle *et al.*, 1969) in the absence of phosphatidylcholine, and in *Agrobacterium tumefaciens*, *Azotobacter agilis* (Law *et al.*, 1963; Randle *et al.*, 1969), *Thiobacillus novellus* (Barridge and Shively, 1968) and *Ferrobacillus ferro-oxidans* (Short *et al.*, 1969), along with phosphatidylcholine.

In animal tissues phosphatidylmethylethanolamine and phosphatidyldimethylethanolamine are rarely found as major lipid components. Rather, they appear to be present in small amounts as metabolic intermediates between phosphatidylethanolamine and phosphatidylcholine in those tissues that contain the phosphatidylethanolamine methylation pathway (Bremer and Greenberg, 1960; Artom and Lofland, 1960; Gibson *et al.*, 1961). This pathway was first described in rat liver and is now known to occur in *Neurospora* (Hall and Nyc, 1959), yeast (Letters, 1966), and protozoa (Lust and Daniel, 1966; Smith and Law, 1970).

c. *Phosphatidylglycerol* is widely distributed among both Gram-positive and Gram-negative bacteria but the proportions of this phospholipid

found in bacteria vary widely. In general, a higher proportion of the phosphoglycerides of Gram-positive cells is accounted for by phosphatidylglycerol (or the phosphoglycerides derived from it, cardiolipin and amino-acyl phosphatidylglycerol) than with the Gram-negative organisms where phosphatidylethanolamine and its methylated forms tend to be the predominant class of phospholipids by a ratio of roughly 2:1 to 4:1. Phosphatidylglycerol is a major phosphoglyceride in the spore-forming bacilli and in the staphylococci that have been studied. The lipid composition of a number of other Gram-positive groups is not as clear, but phosphatidylglycerol has been found in streptococci, micrococci, *Sarcina*, and pneumococci. It is also present in *Clostridium butyricum* and in *Cl. welchii*. Its distribution among the other clostridia has not been reported.

d. *Cardiolipin* or diphosphatidylglycerol occurs in many of the organisms in which phosphatidylglycerol occurs. Since the two lipids are difficult to separate, they have often been reported as their sum. It is known that cardiolipin is derived from phosphatidylglycerol in *E. coli* (Stanacev *et al.*, 1967). As batch cultures of Gram-negative cells go from the logarithmic phase of growth to the stationary phase, the proportion of phosphatidylglycerol tends to fall while that of cardiolipin tends to rise (Shively and Benson, 1967; Cronan, 1968; Randle *et al.*, 1969), but not necessarily stoicheiometrically (Randle *et al.*, 1969).

Both cardiolipin and phosphatidylglycerol are well known components of the cells of higher organisms (Macfarlane, 1964b). Animal mitochondria have more cardiolipin (16% of total phospholipids; Fleischer *et al.*, 1961) than phosphatidylglycerol, whereas phosphatidylglycerol predominates in plant subcellular organelles (Benson, 1964).

e. *Amino-acyl phosphatidylglycerol.* These derivatives of phosphatidylglycerol appear to be unique to bacteria. The structure of this class of compounds was elucidated by Macfarlane (1962) in a study of the previously ill-defined lipo-amino acids of bacteria. She showed that the amino acids were esterified to one of the free hydroxyl groups of glycerol in phosphatidylglycerol in the lipids of *Clostridium welchii* and *Staphylococcus aureus*. Since then these lipids have been detected in a number of other Gram-positive bacteria including *Bacillus subtilis* (Bishop *et al.*, 1967; Op den Kamp *et al.*, 1969a), *B. megaterium* MK 10D (Op den Kamp *et al.*, 1965), *B. cereus* (Houtsmuller and van Deenen, 1963; Lang and Lundgren, 1970), *B. natto* (Urakami and Umetani, 1968), *Streptococcus faecalis* (Vorbeck and Marinetti, 1965; Kocun, 1970) and in *Mycoplasma laidlawii* (Koostra and Smith, 1969). There are very few well-documented reports of the occurrence of this class of compounds in Gram-negative bacteria. The amounts of this compound in Gram-positive bacteria differ

from species to species and varies within a given species (see Section IV. B. p. 42).

Only a limited range of amino acids has been found in this class of compounds. The most common is lysine which has been found in *Staph. aureus*, *B. megaterium* MK 10D, *B. subtilis* and *Strep. faecalis* (see references above). Alanylphosphatidylglycerol has been reported in *Strep. faecalis*, *Cl. welchii*. *Bifidobacterium bifidum* (Exterkate and Veerkamp, 1969) and *B. cereus*. The ornithine derivative has been found in *B. cereus* and tentatively identified in *B. natto* (Urakami and Umetani, 1968).

The amino acids in this group of compounds are probably linked to the 3′-hydroxyl group of the unacylated glycerol moiety. In confirmation of enzymic studies carried out by Lennarz *et al.* (1967) on the biosynthesis of these compounds with deoxy analogues of phosphatidylglycerol, Molotkovsky and Bergelson (1968) produced evidence with chemically synthesized lysylphosphatidylglycerols that the natural compound has the amino acid on the 3′-hydroxyl group.

A glucosaminyl derivative of phosphatidylglycerol occurs in *B. megaterium* (Op den Kamp *et al.*, 1965, 1969b; Gurr *et al.*, 1968) and in *Pseudomonas ovalis* Chester (Phizackerley *et al.*, 1966). A compound from *B. megaterium* has the glucosamine linked glycosidically at the 2′-hydroxyl of the glycerol moiety. Other isomers have been reported (Phizackerley and MacDougall, 1969).

f. *Phosphatidylinositol*. This important lipid of higher organisms is not a common phosphoglyceride of bacteria and appears to be absent from Gram-negative bacteria. Among the Gram-positive bacteria, there are two reports of its occurrence in the Micrococcaceae, namely *Sarcina lutea* (Huston *et al.*, 1965) and *Micrococcus lysodeikticus* (Macfarlane, 1961a, b), but it appears to be absent from staphylococci or at least from *Staph. aureus*, the best studied member of that genus. Ikawa (1963) did not find inositol in hydrolysates of *Lactobacillus casei*, *L. plantarum*, *Leuconostoc mesenteroides*, and *Strep. faecalis*.

There are groups of bacteria that generally do contain phosphatidylinositol or lipids containing this structure. The acid-fast mycobacteria, the related propionibacteria and some corynebacteria are among these groups which will be discussed below (see Section IV. B. 3. p. 48).

g. *Minor phospholipid components and biosynthetic intermediates*. Phosphatidic acid is rarely present as more than a few per cent of the total phosphoglycerides of bacteria, but if the pathway for the biosynthesis of phosphatides in *E. coli* is universally present in bacteria (see Section III. B. 1. p. 28), then all bacteria that contain phosphatidylethanolamine, phosphatidylglycerol, and the other phosphatides derived from

them should have trace amounts of phosphatidic acid as an intermediate in these pathways. Randle *et al.* (1969) recently surveyed stationary-phase cells of eight species of Gram-negative bacteria and found trace amounts up to 4·1% of their total phospholipids as phosphatidic acid. Early log-phase cells of *E. coli* growing in a mineral salts-glycerol medium were reported to have 10% of their total phosphoglycerides as phosphatidic acid, and log-phase cells of *Proteus vulgaris* were reported by the same authors to have 7% phosphatidic acid. These findings are in accord with the concept of phosphatidic acid as a biosynthetic intermediate.

Phosphatidylserine is an intermediate between phosphatidic acid and phosphatidylethanolamine (Fig. 11, p. 29) and is usually detected in very small amounts or missed entirely unless isotopic tracers are used. There are two other phosphorus-containing lipids of the phosphoglyceride type. These are phosphatidylglycerophosphate and cytidine diphosphate diglyceride, both of which are intermediates in the biosynthesis of the major phospholipids of *E. coli* (see Section III. B. 1. p. 28).

Mono-acylphosphoglycerides or lysophosphatides are also generally found in trace amounts. Lysophosphatidic acid or mono-acylglycerophosphate is an intermediate in the biosynthesis of phosphatidic acid in bacteria (see Section III. B. 1. p. 28). Other lysophosphatides, such as lysophosphatidyl ethanolamine, are probably formed as a result of the partial hydrolysis of the major phosphoglycerides of the cell.

2. Alk-1-Enyl, Acyl Phosphoglycerides: Plasmalogens

Until recently there were only a few reports of the presence of aldehydogenic lipids in bacteria, but it had been noted that bacteria that contained plasmalogens were all anaerobes (see Section II. A. 2. p. 3). This weak generalization was made much stronger in a recent survey by Kamio *et al.* (1969) who found molar ratios of aldehyde:phosphorus of 0·14 to 0·43 in mixed populations of cells obtained from the sheep rumen by differential centrifugation of the contents. This confirmed an earlier observation of Katz and Keeney (1964). They also studied anaerobic bacteria obtained by enrichment of mixed rumen bacteria and mixed soil bacteria grown on various energy sources. The range for the rumen bacteria enrichments was 0·19–0·89 (aldehyde:phosphorus); the range for the soil bacterial enrichments was 0·25–1·38 (Kamio *et al.*, 1969). These authors also studied the occurrence of plasmalogens in a wide range of pure cultures of anaerobic bacteria and compared these lipids with those obtained from a smaller number of aerobic or facultative organisms. Among the latter were *Pseudomonas fluorescens*,

E. coli (grown either aerobically or anaerobically), *Corynebacterium sepedonicum*, *Bacillus subtilis*, *Rhodospirillum rubrum*, and *Streptomyces aureofaciens*. None of the aerobic or facultative organisms has plasmalogens, in confirmation of the results obtained in many laboratories (see for example, Gray and Wilkinson, 1965; White, 1968; Randle *et al.*, 1969). Plasmalogens were also absent from the microaerophilic lactic acid bacteria, which included *Strep. faecalis*, *Leuconostoc mesenteroides* and two species of *Lactobacillus* (Kamio *et al.*, 1969). The phospholipids of all the anaerobes tested, however, did contain plasmalogens with ratios of aldehyde : phosphorus varying from 0·004 to 1·04 (Table 1).

TABLE 1. Occurrence of plasmalogens in bacteria

Bacterial strain	Aldehyde : phosphorus ratio in phospholipid (molar)	References
Bacteroides ruminicola	0·004	Kamio *et al.* (1969)
Bacteroides succinogenes	0·71	Wegner and Foster (1963)
Clostridium acetobutylicum 179–121	0·72	Kamio *et al.* (1969)
Clostridium acetobutylicum 314–48	0·92	Kamio *et al.* (1969)
Clostridium butyricum	0·40	Baumann *et al.* (1965)
Clostridium kainantoi	0·86	Kamio *et al.* (1969)
Clostridium kaneboi	0·82	Kamio *et al.* (1969)
Clostridium perfringens	0·04	Kamio *et al.* (1969)
Clostridium saccharoperbutyl-acetonicum	0·80	Kamio *et al.* (1969)
Desulfovibrio sp.	0·09	Kamio *et al.* (1969)
Peptostreptococcus elsdenii	1·04	Kamio *et al.* (1969)
Propionibacterium freudenreichii		
Anaerobic culture	0·68	Kamio *et al.* (1969)
Standing culture	0·60	Kamio *et al.* (1969)
Propionibacterium shermanii		
Anaerobic culture	0·37	Kamio *et al.* (1969)
Standing culture	0·45	Kamio *et al.* (1969)
Ruminococcus flavefaciens	0·56–0·80	Allison *et al.* (1962)
Selenomonas ruminantium		
Lactate-grown	0·50	Kamio *et al.* (1969)
Glucose-grown	0·25	Kamio *et al.* (1969)
Sphaerophorus ridiculosus	0·25	P.-O. Hagen (unpublished data)
Treponema pallidum (Reiter)	0·14	Meyer and Meyer (1971)
Veillonella gazogenes	0·69	Kamio *et al.* (1969)

The problem that remains is to determine the structures of the polar groups of these bacterial plasmalogens. In animal tissues these are generally ethanolamine or choline. Only a small amount of information is available on the bacteria. Wegner and Foster (1963) reported that almost all of the phospholipid in *Bacteroides succinogenes* was ethanolamine phosphatide, and that approximately 70% of the total phosphatide was ethanolamine plasmalogen. Baumann *et al.* (1965) analysed the three major lipids of *Clostridium butyricum* for plasmalogen content, and found that 78% of the N methylethanolamine phosphatides, 55% of the ethanolamine phosphatides and 9% of the phosphatidylglycerol were of the plasmalogen type.

In their extensive survey of the plasmalogens of anaerobic bacteria, Kamio *et al.* (1969) subjected the phospholipids from enrichment cultures and from five pure cultures to thin-layer chromatography on silica gel H in chloroform–methanol-water (62:25:4) and found, in each extract, a major component (R_f 0·45–0·50) which gave positive reactions for fatty aldehyde, phosphate, and amino nitrogen. These data are consistent with the presence of an ethanolamine plasmalogen, but are only indicative, since N-methylethanolamine plasmalogen behaves similarly upon thin-layer chromatography (Baumann *et al.*, 1965).

3. Alkyl Ether Lipids

As mentioned above (see Section II. A. 3. p. 4) a unique type of dialkyl glycerylphosphoryl lipid occurs in the extremely halophilic bacteria. The predominant member of this phospholipid class in *Halobacterium cutirubrum* has been found by Kates and his associates to be an analogue of phosphatidylglycerophosphate (Fig. 3, p. 5). The alcohol attached to C-3 and C-2 of glycerol (the configuration being opposite to that of the usual phosphoglycerides) is dihydrophytol (Kates *et al.*, 1965). A similar, probably identical, lipid has been found in six other species of extremely halophilic bacteria (Kates *et al.*, 1966). Minor lipids in these halophiles are the diether analogue of phosphatidylglycerol, a diether glycolipid, and its sulphate ester (Kates *et al.*, 1967).

Other phosphorus-containing lipids that are not of the glycerophospholipid type, such as lipid A of Gram-negative bacterial cell walls and polyisoprenoid phosphates involved in cell-wall synthesis, will be discussed elsewhere.

4. Other Types of Extractable Polar Lipids

a. *Glycolipids*

i. *Glycosyl diglycerides.* These compounds seem to be the most common form of extractable glycolipid in bacteria. Figure 5 illustrates the struc-

tures of two of these bacterial glycolipids. A compilation and review of the distribution and structures of the glycolipids in bacteria has recently appeared (Shaw, 1970). The most common type, which is also commonly found in plants, is a diglycosyl diglyceride, composed of a disaccharide glycosidically linked to C-3 of a diglyceride. According to Shaw and Baddiley (1968) five types of disaccharide residue are widely distributed in the glycolipids of Gram-positive bacteria. These are: α-glucosyl-glucosyl, β-glucosylglucosyl, α-galactosylglucosyl, β-galactosylgalactosyl

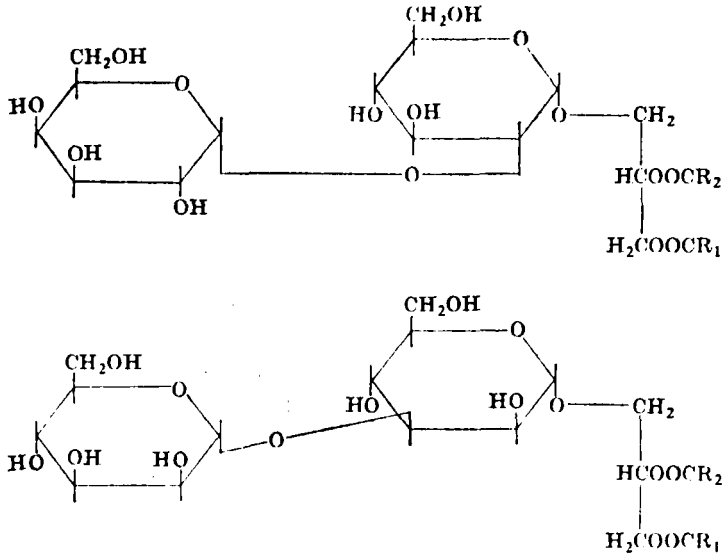


FIG. 5. Structural formulae of some bacterial glycosyl diglycerides. Top: α-D-galactosyl-(1 → 2)-α-D-glucosyl-(1 → 3)-diglyceride, which has been detected in pneumococci and lactobacilli. Bottom: α-D-mannosyl-(1 → 3)-α-D-mannosyl-(1 → 3)-diglyceride, which has been detected in *Micrococcus lysodeikticus*, *Microbacterium lacticum* and three species of *Arthrobacter*.

and α-mannosylmannosyl diglycerides. On further structural investigation, these authors and others have found that the two anomeric centres in the glycosides have the same configuration, and the α-linked disaccharides containing glucose or galactose have a 1 → 2 linkage between the two sugars but the β-linked disaccharides have a 1 → 6 linkage.

A large group of Gram-negative bacteria has been examined for these glycosyl diglycerides (K. Heatherington, unpublished observations cited by Shaw and Baddiley, 1968). None of those examined contained glycosyl diglycerides. Glycolipids have, however, been reported in two species of *Pseudomonas* (Wilkinson, 1968a, b; 1969). In addition to an α-glucopyranosyl-D-2,3-diglyceride there is a hexuronyl diglyceride in these

organisms. This has been identified as 1-*O*-α-D-glucopyranuronosyl-D-2,3-diglyceride in *Ps. diminuta* (Wilkinson, 1969) and a β-linked species in *Ps. rubescens* (Wilkinson, 1968b). The taxonomy of these pseudomonads is, however, in doubt (Wilkinson, 1968b). Glycolipids have also been reported in photosynthetic bacteria (Wood *et al.*, 1965; Gorchein, 1968c; Radunz, 1969). On the other hand, all of the Gram-positive organisms examined at the University of Newcastle upon Tyne (Shaw and Baddiley, 1968; Shaw, 1970) contain glycosyldiglycerides.

The amounts of glycolipid in a given species may range from just one to three per cent of the total lipid in the lactobacilli and staphylococci (Brundish *et al.*, 1966; Shaw and Baddiley, 1968) to 34% in a pneumococcus (Brundish *et al.*, 1965) and 40% in *Microbacterium lacticum* (Shaw, 1968) and *Mycoplasma laidlawii* B (Shaw and Smith, 1967).

Kates and his coworkers (1967) demonstrated the presence of a diether glycolipid and its sulphate ester in *Halobacterium cutirubrum* and in other extreme halophiles (Kates *et al.*, 1966) and have characterized the lipid from *H. cutirubrum* as a 2,3-di-*O*-dihydrophytyl-L-glycerol-1-*O*-(glucosyl-mannosylgalactosyl) sulphate. Like the diether analogue of phosphatidylglycerophosphate of these organisms, the glycolipids have the polar substituents on the C-1 of L-glycerol.

ii. *Mycosides*. Among the complex mixture of lipids characteristic of the mycobacteria are mycosides. The Mycoside B from bovine strains of mycobacteria contains a benzene ring linked to a sugar residue (2-*O*-methylrhamnose) and to a long-chain hydrocarbon containing hydroxyl groups to which are esterified palmitic acid and mycocerosic acids (branched fatty acids of 29–32 carbon atoms) (Asselineau, 1966). It is beyond the scope of this article to discuss the lipids of mycobacteria in detail. The interested reader is referred to the monograph of Asselineau (1966).

b. *Sphingolipids*. Until recently it was thought that this class of lipid was not synthesized by bacteria. This rule, like most, has now found its exceptions. White and his coworkers (LaBach and White, 1969; White *et al.*, 1969; Rizza *et al.*, 1970) have shown that the lipids of *Bacteroides melaninogenicus* contain a family of sphingolipids which represent over half of the total extractable lipids. These compounds have been carefully characterized as ceramide phosphorylethanolamine and ceramide phosphorylglycerol, the major sphingolipid components, and ceramide phosphorylglycerol phosphate, a minor component. It is of interest that the water-soluble moieties resemble the phosphoglyceride water-soluble moieties of bacteria, rather than phosphorylcholine which is more commonly found in animals. The long-chain bases of *B. melaninogenicus* sphingolipids have been characterized as 17-methyloctadeca-

sphinganine (63%), *n*-octadecasphinganine (21%) and 15-methylhexadecasphinganine (12%) (White *et al.*, 1969). A similar group of sphingolipids have been found in *B. ruminicola* (Iannoti *et al.*, 1970).

The more complex sphingolipids common to the tissues of higher organisms, the cerebrosides and gangliosides, have not been found in bacteria.

c. *Ornithine amides.* Lanéelle *et al.* (1963) found an ornithine-containing lipid in a strain of atypical mycobacteria which yielded an ether-soluble ornithine derivative upon saponification. Infrared spectra indicated the presence of an amide bond. Gorschein (1964, 1968a, b, c) isolated a similar non-saponifiable ornithine-containing lipid from the photosynthetic bacterium, *Rhodopseudomonas spheroides*. This lipid is thought to have a long-chain alcohol esterified to the carboxyl group of ornithine and a long-chain fatty acid in amide linkage to the α-amino group of ornithine. Alkali-stable ornithine-containing lipids were also reported in *Rhodomicrobium vannielii* (Park and Berger, 1967), *Rhodospirillum rubrum*

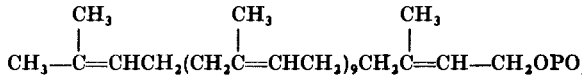


FIG. 6. Formula of undecapronyl phosphate.

(Depinto, 1967), two other photosynthetic bacteria, and *Streptomyces sioyaensis* (Kimura and Otsuka, 1969) where a similar lysine-containing lipid was also found. Prome *et al.* (1969) have proposed that a fatty acid is linked to the carboxyl group of ornithine through 1,2-propanediol in *Brucella melitensis*. They reported a similar lipid with an ethylene glycol bridge in *Mycobacterium bovis*.

d. *Glycosyl derivatives of polyisoprenols.* In two important series of investigations on the biosynthesis of the peptidoglycans of Gram-positive bacteria and of the *O*-antigens of the lipopolysaccharides of the enteric Gram-negative bacteria, Strominger and Robbins and their colleagues characterized a novel class of sugar derivatives of polyisoprenols. In the biosynthesis of the peptidoglycans, a phospho-N-acetyl-muramylpentapeptide is transferred from UDP-N-acetyl-muramylpentapeptide to a polyisoprenoid phosphate to form a pyrophosphate linkage. The polyisoprenoid was established as an undecaprenol by mass spectroscopy (Higashi *et al.*, 1967). In the case of the biosynthesis of the lipopolysaccharides, the repeating polysaccharides of the *O*-antigen of *Salmonella typhimurium* were shown to be built up on a similar, if not identical, undecaprenyl phosphate (Fig. 6) (Wright *et al.*, 1967). Again a pyrophosphate bridge links the polyisoprenol to the polysaccharide. This type

of lipid represents only a fraction of a per cent of the total extractable lipids of bacteria and was not recognized until its functions were explored. Thorne and Kodicek (1966) had, however, isolated a non-phosphorylated form of a C_{55} polyisoprenoid alcohol in lactobacilli, where it was shown to become labelled when the cells were fed radioactive mevalonic acid. Since this type of lipid is involved in the biosynthesis of the universal peptidoglycan or rigid layer of bacterial cells, it is implicit that it or a similar lipid is present in all bacterial cells that have cell walls. In the species that contain *O*-antigenic polysaccharides, this lipid appears to play a dual role. Scher *et al.* (1968) have reported the involvement of a similar lipid intermediate in the biosynthesis of a mannan of the membrane of *Micrococcus lysodeikticus*. The lipid and hexose of this intermediate are linked through a phosphodiester bond rather than a pyrophosphate link.

These findings have led to a search for similar lipid intermediates involved in the biosynthesis of other complex polysaccharides. The results thus far seem to be encouraging. Baddiley and his coworkers (Watkinson *et al.*, 1971) have obtained indirect evidence for the involvement of a similar lipid in teichoic acid biosynthesis in Gram-positive bacteria, and Troy *et al.* (1971) have reported the involvement of these lipids in capsular polysaccharide biosynthesis in *Aerobacter aerogenes*.

C. Non-Extractable Lipids in Bacteria

A broad survey of the literature reveals that a portion of the fatty acids of many bacteria can be released from cells by acid or alkaline hydrolysis after the cells have been exhaustively extracted with lipid solvents. In many bacteria, the materials to which the fatty acids are bound are not known nor is the location of these materials in the cell understood. There is one type of bound fatty acid that is better understood than most, but the structures of these materials are far from clear. This lipid is obtained from the lipopolysaccharide of the cell envelope of Gram-negative bacteria by treatment with dilute acid. It was given the name lipid A by Westphal and Lüderitz (1954) in order to distinguish it from the lipid extracted from lipopolysaccharide prior to acid hydrolysis, which was called lipid B and is predominantly phosphatidylethanolamine (Kurokawa *et al.*, 1959). The lipid A component contains phosphate, glucosamine and fatty acids, and the fatty acids are qualitatively different from those found in the glycerophosphatides of Gram-negative bacteria. There is a high proportion of 12:0 and 14:0 fatty acids and β-hydroxymyristic acid (Ikawa *et al.*, 1953; Burton and Carter, 1964) in the lipid A of *E. coli*. The longer chain saturated, unsaturated and cyclopropane fatty acids, which are common to the phosphoglycerides

of *E. coli*, are present in small amounts. The fatty acids appear to be linked to the amino and hydroxyl groups of glucosamine. The linkages between glucosamines are not clear. The possibilities of glycoside and phosphodiester bonds have been considered (Burton and Carter, 1964) and recent work favours the glycosidic linkage (Lüderitz, 1970).

Lipid A is distributed widely in Gram-negative bacteria. Material of similar composition was isolated by Nowotny (1961a, b) from a number of strains of *Salmonella* and from other species of Enterobacteriaceae, Neisseriaceae and Pseudomonadaceae. A review of the structure and function of the lipopolysaccharides has recently appeared (Lüderitz, 1970).

D. Neutral Lipids of Bacteria

Several important generalizations have emerged from examination of the neutral lipids of bacteria and have been noted by previous authors including Kates (1964), Bloch (1965), Asselineau (1966), and O'Leary (1967). Bacteria generally contain either no sterols or amounts very much smaller than are found in the tissues of higher organisms. It is quite clear that bacteria are capable of synthesizing polyisoprenoids. These are found to be widely distributed in prokaryotes in the form of quinone coenzymes, carotenoids, and the recently discovered undecaprenol phosphates needed for peptidoglycan and lipopolysaccharide synthesis (see Section II. B. 4. p. 12). The missing reactions are those required for forming the ring systems of sterols. These reactions are oxygen-dependent and were evolved at a later period than the time of evolution of the forerunners of present-day bacteria (Goldfine and Bloch, 1963). The presence of trace amounts of sterols in bacteria has recently been re-investigated by Schubert *et al.* (1968). They found sterols present as 0·01% of the dry weight of *Azotobacter chroococcum* and smaller amounts (0·0035%) in *Streptomyces olivaceus* and (0·0004%) in *E. coli*. A number of other bacteria were investigated and were found to have less than 0·0001% sterol. When one considers that lipids represent anywhere from 3 to 20% of the dry weight of many bacteria, these amounts appear to be of slight significance in terms of membrane structure. That the sterols may play some other role cannot be denied. There is still no conclusive proof of the ability of any bacterium to synthesize a sterol *de novo*.

The mycoplasmas, a group of bacteria that lack a rigid cell wall, almost universally have a requirement for sterols. Of 31 species recently examined by Razin and Tully (1970), all but three required cholesterol for growth, but none appeared to be capable of synthesizing sterols. These organisms are parasitic and have ready access to sterols, which

may function to strengthen the membranes of these wall-less cells (Smith, 1964; Bloch, 1965).

Bloch (1965) has outlined the sterol-synthesizing capacity of higher protists. The blue-green algae do not synthesize sterols (Levin and Bloch, 1964), but all the other algae do (Carter *et al.*, 1961). The fungi are also generally capable of synthesizing sterols. As one goes up the phylogenetic scale, sterols are universally found, but many insects have lost the ability to synthesize them (Bloch, 1965).

With the exception of the strictly anaerobic bacteria, the quinone coenzymes, coenzymes Q and vitamins K, and the carotenoids are found in a wide variety of bacteria (Goldfine, 1965; Liaaen-Jensen, 1965).

Another significant difference between the lipids of bacteria and those of higher organisms is a quantitative one. Although bacteria have been widely reported to contain glycerides (Kates, 1964), the amounts of the neutral fats per cell are usually low, and mono- and diglycerides are reported more often than triglycerides. Bacteria do not appear to store glycerides as energy reserves. A form of lipid, unique to bacteria, poly-β-hydroxybutyric acid, does however appear to serve this function (see p. 19).

The proportion of neutral lipids, including glycerides, hydrocarbons, quinones, carotenoids, free fatty acids and poly-β-hydroxybutyric acid varies widely from species to species. One problem in assessing these data is the variation in reporting poly-β-hydroxybutyric acid, which has somewhat different solubility properties from the other neutral lipids and may or may not be included with neutral lipids depending on the isolation procedures used. With the exception of *Corynebacterium diphtheriae*, Kates' (1964) summary of the data on Eubacteriales and Pseudomonadales gives the neutral-lipid content of bacteria as anywhere from 3 to more than 50% of the total lipid, if poly-β-hydroxybutyric acid is excluded. Among the species containing large amounts of neutral lipids are *Sarcina lutea* (>50% of total lipids; Akashi and Saito, 1960; Huston and Albro, 1964), *Clostridium welchii* (30% of total lipid; Macfarlane, 1962), and a number of the lactic acid bacteria (Ikawa, 1963; Thorne and Kodicek, 1962). The Gram-negative bacteria generally have smaller amounts of neutral lipids, usually 5–15% of total lipid, if poly-β-hydroxybutyric acid is excluded. The distribution and structures of hydrocarbons in bacteria have recently been reviewed (Albro and Dittmer, 1970).

E. Poly-β-Hydroxybutyric Acid

As noted above, although glycerides are generally a minor component of bacterial lipids, a storage form of lipid does occur. Poly-β-hydroxybutyric acid is widely distributed in both Gram-positive and Gram-

negative bacteria (Dawes and Ribbons, 1964). Among the genera in which is has been reported are *Bacillus*, *Azotobacter*, *Pseudomonas*, *Rhodopseudomonas*, *Rhodospirillum*, *Spirillum*, and *Vibrio*. The polymers can reach a molecular weight of 250,000 and are found in cells as granules (Merrick *et al.*, 1965; Lundgren *et al.*, 1965). In some species of *Spirillum* (*S. repens*), in *Micrococcus halodenitrificans*, and in *B. megaterium* inclusions of poly-β-hydroxybutyrate attain 40–60% of the cell dry weight when the cells are grown with good aeration on an appropriate carbon source (Hayward *et al.*, 1959; Sierra and Gibbons, 1962; Lemoigne *et al.*, 1949).

III. Biosynthesis of Bacterial Lipids

A number of important differences in the lipid composition of bacteria and higher organisms were described in the preceding sections of this review. Since many bacteria synthesize their own complex lipids as well as the precursors of these lipids, the differences in lipids must be the result of differences in biosynthetic pathways. For obvious reasons, studies on the biosynthesis of bacterial lipids have not kept pace with knowledge of their composition and, although many of the biosynthetic pathways are known, there is little comparative data between groups of bacteria. In most cases, we have nothing more than the hope that what is known about *Escherichia coli* or *Staphylococcus aureus* will also apply to closely related species and the even more fragile assumption that it will apply to more distantly related genera.

A. Biosynthesis of the Apolar Components

1. Fatty Acids

a. *Saturated, straight-chain fatty acids.* The formation of fatty acids in bacteria has been studied intensively in the past decade in a number of laboratories. An outline of the biosynthesis of saturated fatty acids is given in Fig. 7. This pathway begins with the formation of malonyl-CoA by carboxylation of acetyl-CoA in a manner analogous to the reaction found in animal systems (Wakil, 1958; Brady, 1958). Transfer of malonyl and acetyl residues to the free sulphydryl of an acyl carrier protein of molecular weight 9600 is carried out in *E. coli* by separate transacylases (Alberts *et al.*, 1964). The reactions involve transfer of the acyl moieties from the 4′-phosphopantetheine residue of coenzyme-A to an identical residue on an acyl carrier protein (Majerus *et al.*, 1964, 1965; Sauer *et al.*, 1964). In essence, this process does not differ from fatty-acid synthesis in higher organisms in which acetyl and malonyl residues are transferred to similar 4′-phosphopantetheine prosthetic groups on a multi-enzyme complex (Majerus and Vagelos, 1967). The subsequent

condensation between acetyl-S-acyl carrier protein and malonyl-S-acyl carrier protein to yield aceto-acetyl-S-acyl carrier protein and carbon dioxide, the reduction of the β-keto acid to a D(−)-β-hydroxy acid, followed by dehydration to give the α,β unsaturated acid, and reduction to the saturated fatty acid, all occur with the acyl groups esterified to the acyl carrier protein (Majerus and Vagelos, 1967). All of these reactions are similar to those known to occur in the synthesis of saturated fatty acids in higher organisms. In animal tissues and in yeast, a multi-enzyme complex carries out the reactions of the cycle, and the acyl carrier protein is firmly bound to the complex (Majerus and Vagelos, 1967; Brindley *et al.*, 1969). In bacteria and plants, the acyl carrier proteins are small proteins and are easily separated from the other enzymes of fatty-acid synthesis. Most of the enzymes involved in fatty acid synthesis in *E. coli* have been extensively purified (Majerus and Vagelos, 1967).

The formation of the 16- and 18-carbon saturated, straight-chain fatty acids commonly found in bacteria involves repetition of this cycle (Fig.

Malonyl-S-CoA + ACP-SH $\rightleftharpoons$ Malonyl-S-ACP + CoA-SH
Acetyl-S-CoA + ACP-SH $\rightleftharpoons$ Acetyl-S-ACP + CoA-SH
Acetyl-S-ACP + Malonyl-S-ACP $\rightleftharpoons$ Aceto-acetyl-S-ACP + CO_2 + ACP-SH
Aceto-acetyl-S-ACP + $TPNH_2$ $\rightleftharpoons$ D(−)-β-Hydroxybutyryl-S-ACP + TPN
D(−)-β-Hydroxybutyryl-S-ACP $\rightleftharpoons$ Crotonyl-S-ACP + H_2O
Crotonyl-S-ACP + $TPNH_2$ $\rightleftharpoons$ Butyryl-S-ACP + TPN

FIG. 7. Initial steps in the pathway for biosynthesis of straight-chain fatty acids in bacteria. ACP indicates acyl carrier protein.

7) in which the addition of two-carbon units occurs by condensation of molecules of malonyl-S-acyl carrier protein with the fully reduced acyl-S-acyl carrier protein formed in the preceding round of condensation and reduction. The saturated products of this cycle are similar in chain length to those produced in higher organisms (Brindley *et al.*, 1969). The factors that control the chain length are not well understood. Free fatty acids are formed by the action of thiolesterases in the fatty-acid synthetase from *E. coli* during *in vitro* experiments. The chain-length specificity of such thiolesterases may play a role in determining the lengths of the fatty acids formed. As will be described below (see Section III. B. 1. p. 28) the fatty acids can be transferred directly from acyl carrier protein to glycerophosphate and mono-acylglycerophosphate to yield phosphatidic acid. The chain-length specificity of these acyl transferases may also play a key role in determining the chain length of the fatty acids synthesized. In higher organisms, the controlling factor may be the transfer from the fatty acid synthetase complex to coenzyme-A (Lynen, 1961). Coenzyme-A appears to be required for the transfer of

fatty acids from the synthetase to glycerol 3-phosphate in yeast (Kuhn and Lynen, 1965).

b. *Terminally branched fatty acids.* Synthesis of saturated, terminally branched fatty acids occurs in the Gram-positive aerobic bacilli and in the Micrococcaceae in which they are the predominant fatty-acid type. Rumen bacteria are also known to contain substantial amounts of branched fatty acids (Allison *et al.*, 1962; Wegner and Foster, 1963). In theory, the problem of their biosynthesis was solved by Horning *et al.* (1961), who showed that a fatty acid synthetase from rat adipose tissue could utilize branched-chain volatile fatty acyl-CoA derivatives in place of acetyl-CoA as initiators of fatty acid synthesis. For example, initiation with isobutyryl-CoA yielded *iso*-16:0, and incubation with isovaleryl-CoA yielded *iso*-15:0 plus *iso*-17:0 as the major products. The branched precursors provided the branched end of the fatty acid molecule, and malonyl-CoA provided the straight chain portion. Lennarz (1961)

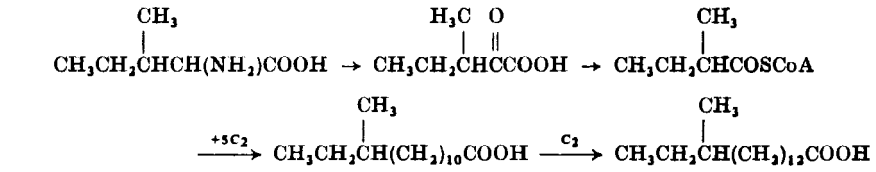


FIG. 8. Outline of the proposed pathway for synthesis of *anteiso*-C_{15} and *anteiso*-C_{17} fatty acids from isoleucine by bacteria.

demonstrated the formation of *anteiso*-15:0 and *anteiso*-17:0 (Fig. 8) from isoleucine by cells of *Micrococcus lysodeikticus*. Presumably the coenzyme-A ester of 2-methylbutyrate, which is postulated to be the chain initiator, was formed by transamination and decarboxylation. Similar findings have been reported on the formation of *anteiso*-branched fatty acids in rumen bacteria (Allison *et al.*, 1962). The *iso*-branched, odd-numbered fatty acids are formed in bacilli from isovaleric acid or leucine and the *iso*-branched, even-numbered fatty acids from isobutyrate or valine (Fig. 1, p. 2; Kaneda, 1963). Initiation of fatty-acid synthesis by these branched-chain acyl-CoA derivatives rather than acetyl-CoA poses a problem of specificity. Are the branched precursors favoured over acetyl-CoA as a result of greater availability, or as a result of enzyme specificity? The answer is still not completely clear. The work of Kaneda (1963, 1966, 1967) with bacilli provided evidence that the availability of precursors determined the types and amounts of saturated fatty acids produced by these organisms. He showed that feeding butyrate to *B. subtilis* increased the amount of myristic and palmitic acids synthesized relative to the branched-chain fatty acids, whereas

feeding isobutyrate increased the relative amounts of isomyristic and isopalmitic acids, and feeding 2-methylbutyrate increased the relative amounts of *anteiso*-15:0 and *anteiso*-17:0. These findings are in accord with the chain-elongation mechanism shown above (Fig. 8). The fatty acid and fatty aldehyde compositions of the lipids of *Selenomonas ruminantium* are also under the influence of volatile fatty acids added to the medium. When grown on glucose, this organism requires fatty acids of 3–10 carbon chain length (Kamio *et al.*, 1970a). Kanegasaki and Numa (1970) investigated the fatty-acid synthetase from *S. ruminantium*, *in vitro*, and have shown that the synthetase is much more active with C_4 to C_8 acyl-CoA derivatives as primers than it is with acetyl-CoA or propionyl-CoA, and that the relative effectiveness is related to the Michaelis constants of the synthetase for these substrates. They suggest that the fatty-acid synthetase may lack the acetyl-CoA: acyl carrier protein transacylase found in *E. coli* (Alberts *et al.*, 1964), which was shown to transacylate poorly with propionyl-CoA, butyryl-CoA, hexanoyl-CoA and octanoyl-CoA (Williamson and Wakil, 1966), and that instead it has a transacylase specific for longer chain acyl-CoA derivatives. No experiments with acetyl-acyl carrier protein were done, however.

A similar study on the fatty-acid synthetase system of *B. subtilis* was carried out by Butterworth and Bloch (1970), who showed that the enzymes utilized branched-chain acyl-CoAs in preference to acetyl-CoA as primers for long-chain fatty acid synthesis. However, when acetyl-acyl carrier protein was provided, normal straight-chain fatty acids were produced. The specificity of the acyl-CoA : acyl carrier protein transacylase, therefore, seems to be a determining factor in directing the cells toward the almost exclusive production of branched-chain fatty acids. The products obtained from the enzymic incubations were exactly as predicted by the scheme shown in Fig. 8 when isobutyryl-CoA, isovaleryl-CoA and 2-methylbutyryl-CoA were used as primers.

c. *Mono-unsaturated fatty acids.* Fundamental differences in the biosynthesis of mono-unsaturated fatty acids in most, but not all, bacteria on the one hand and in higher organisms on the other, were revealed by work done in the past two decades in a number of laboratories. These studies have been described in considerable detail in several reviews and monographs (Bloch *et al.*, 1961; Hofmann, 1963; O'Leary, 1967; Bloch, 1969).

Derivation of mono-unsaturated fatty acids from saturated fatty acids in animal tissues had long been suspected (Schoenheimer and Rittenberg, 1936). This pathway was first confirmed by Bloomfield and Bloch (1960) who demonstrated a TPNH- and oxygen-dependent desaturation of the

coenzyme-A esters of palmitate and stearate to palmitoleate and oleate, respectively, using extracts from yeast.

A similar desaturation involving $TPNH_2$ and molecular oxygen has since been demonstrated in a variety of organisms including *Mycobacterium phlei* (Lennarz *et al.*, 1962; Scheuerbrandt and Bloch, 1962; Fulco and Bloch, 1964), *Corynebacterium diphtheriae*, *Micrococcus lysodeikticus*, and *Bacillus megaterium* (Fulco *et al.*, 1964). Bloch (1964) has also reported that desaturation of preformed fatty acids occurs in *Streptomyces venezuelae*, the blue-green alga *Anabena variabilis* and the protozoan *Tetrahymena pyriformis*. It also occurs in rat liver (Bernhard *et al.*, 1959; Imai, 1961), and in *Euglena* (Nagai and Bloch, 1966). Using extracts of *Euglena*, Nagai and Bloch (1968) showed that preparations from dark-grown cells desaturated stearyl-CoA to yield oleyl-CoA but did not desaturate stearyl-acyl carrier protein. On the other hand, preparations from light-grown, photo-auxotrophic cells desaturated stearyl-acyl carrier protein but not stearyl-CoA. The oxygen-dependent desaturation usually leads to the formation of the Δ^9 mono-unsaturated fatty acids (Bloch *et al.*, 1961). An interesting exception occurs in *B. megaterium* KM which desaturates 18:0 and 16:0 fatty acids to the corresponding Δ^5 compounds. This reaction is more extensive in cells growing at 23° than at 30° (Fulco *et al.*, 1964). Fulco (1967, 1969, 1970) has subsequently demonstrated that this temperature-dependent desaturation is characteristic of a number of species of bacilli. It should be recalled that these organisms have mainly terminally branched fatty acids at their optimal growth temperatures. *Bacillus licheniformis* is capable of desaturating 16:0 to yield 16:1 Δ^8, 16:1 Δ^9, and 16:1 Δ^{10}. At 20° it also is capable of desaturating at the 5,6 position and thus can produce di-enoic acids, e.g. 16:2 $\Delta^{5,10}$, at lower temperatures (Fulco, 1969, 1970). The capacity to desaturate at the 9,10 position is present in several other bacilli (Fulco, 1967). These findings represent an exception to the rule that bacteria do not synthesize polyunsaturated fatty acids. This rule should perhaps be amended to state that bacteria do not produce the types of polyunsaturated fatty acids characteristic of plants and animals, such as linoleic, linolenic and arachidonic acids.

The major pathway for formation of mono-unsaturated fatty acids in bacteria does not depend on molecular oxygen (Goldfine and Bloch, 1961), does not involve desaturation of preformed saturated fatty acids (Hofmann *et al.*, 1959; Bloch *et al.*, 1961), and usually results in the formation of the *n*-7 series of mono-unsaturated fatty acids, i.e. 16:1 Δ^9 and 18:1 Δ^{11} (*cis*-vaccenic acid) or a mixture of the *n*-7 series and the *n*-9 series (Kates, 1964). This pathway has often been called the "anaerobic pathway" because it does not require molecular oxygen. Despite this name it should be emphasized that it also occurs in aerobic

and facultative bacteria (Bloch *et al.*, 1961). A key feature of this pathway is the introduction of the double bond in the fatty acid during the process of chain elongation. Nutritional experiments by Hofmann and his coworkers on the replacement of biotin by medium chain-length and long-

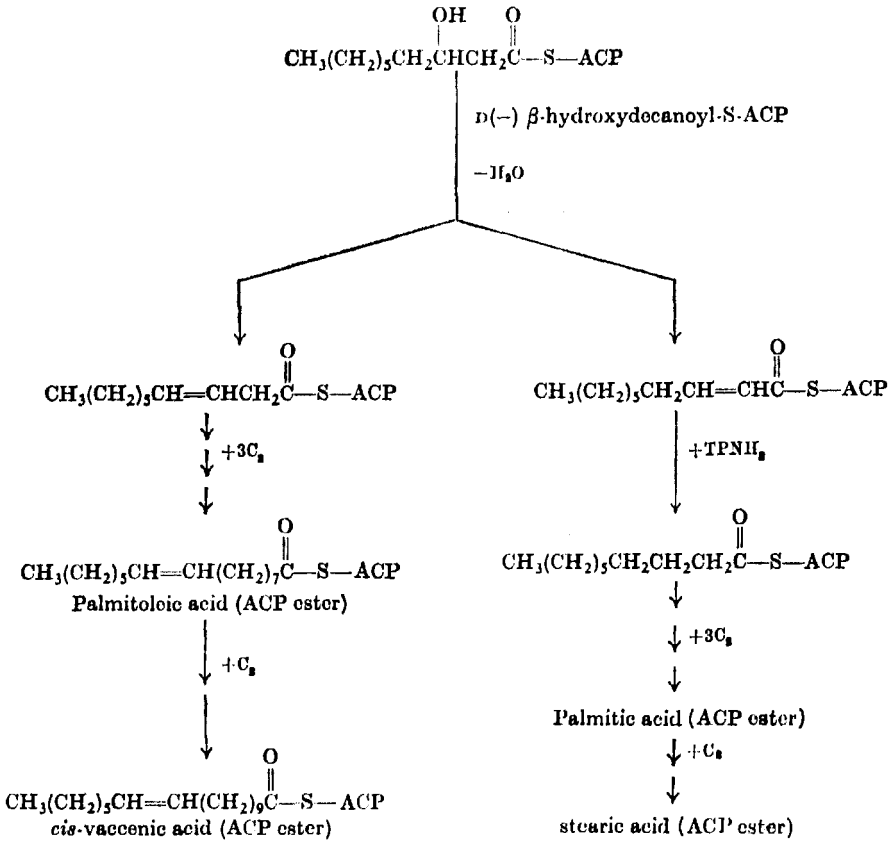


FIG. 9. Terminal steps in the synthesis of mono-unsaturated and saturated fatty acids in *Escherichia coli*. ACP indicates acyl carrier protein.

chain mono-unsaturated fatty acids in lactobacilli had strongly suggested the existence of such a pathway (Hofmann *et al.*, 1959). Experiments by Bloch and his coworkers (1961), first with isotopically labelled precursors and whole cells and then with enzyme preparations, demonstrated that the formation of mono-unsaturated fatty acids was linked to the synthesis of saturated fatty acids, and that the former branched off from the latter at the C_8 or C_{10} stage of fatty acid synthesis (Fig. 9).

The branch-point reaction of this pathway in *E. coli* is the dehydration of the D(−)-β-hydroxydecanoate intermediate to yield *cis*-β,γ-unsaturated decenoate, which is not subsequently reduced, as are the *trans*-α,β-unsaturated intermediates formed before and after this step. Subsequent work with crude and purified enzymes has substantiated this pathway (Lennarz *et al.*, 1962a; Norris *et al.*, 1964). The intermediates are now known to be acylthio-esters of an acyl carrier protein rather than coenzyme-A. Further evidence for this pathway has recently come from work with an unsaturated fatty acid auxotrophic mutant of *E. coli* that lacks the key β-hydroxydecanoyl thio-ester dehydrase and is therefore unable to form unsaturated fatty acids but is capable of synthesizing the saturated fatty acids (Silbert and Vagelos, 1967).

Depending on the chain length of the β-hydroxyacyl-acyl carrier protein at which β,γ dehydration occurs, a variety of unsaturated fatty acids can be produced. For example, β,γ dehydration of both β-hydroxydecanoyl-acyl carrier protein and β-hydroxydodecanoyl-acyl carrier protein and subsequent chain elongation provides the mixture of 16:1 Δ^7, 16:1 Δ^9, 18:1 Δ^9 and 18:1 Δ^{11} found in *Clostridium butyricum* (Scheuerbrandt *et al.*, 1961) and presumably provides mixtures of 18:1 Δ^9 and 18:1 Δ^{11} found in some strains of *E. coli* (Bloch, 1970). Many of the mono-unsaturated fatty acids predicted by this scheme have been found in bacteria (O'Leary, 1967). Bishop and Still (1963) have also found 16:1 Δ^8, 18:1 Δ^8, and 18:1 Δ^{10} acids in *Serratia marcescens*. They postulate the formation of these unusual mono-enes by the retention of α,β double bonds at the C_{10} and C_{12} level of chain elongation. There is as yet no enzymic evidence for this proposal.

Evidence for the existence of the "anaerobic pathway" for mono-unsaturated fatty-acid biosynthesis has been largely obtained by analysis of the positional isomers of the mono-unsaturated fatty acids of bacteria or by isotopic labelling experiments with whole cells. Most of the bacteria that are believed to have this pathway are of the orders Pseudomonadales and Eubacteriales. There are, however, bacteria of these orders that make very little or no mono-unsaturated fatty acids. For example in the aerobic bacilli and a number of Micrococcaceae, the terminally branched acids are the predominant types. When these organisms form mono-unsaturated fatty acids, it is by an oxygen-dependent mechanism (see p. 23). The few species of corynebacteria and mycobacteria that have been studied also have an oxygen-dependent desaturation mechanism that forms Δ^9 mono-enoic fatty acids (Fulco and Bloch, 1964; Fulco *et al.*, 1964). As one goes up the evolutionary scale, the aerobic desaturation mechanism predominates as has been outlined above.

The β-hydroxy C_{10}, C_{12} and C_{14} fatty acids found in the cell-wall "lipid A" of Gram-negative bacteria are also provided by the normal

anaerobic pathway for saturated fatty-acid synthesis. β-Hydroxystearic acid and other β-hydroxy long-chain acids have been found in amide linkage in the ornithine-containing lipids of *Brucella melitensis*, *Mycobacterium bovis* (Prome *et al.*, 1969) and *Thiobacillus thio-oxidans* (Knoche and Shively, 1969).

d. *Cyclopropane fatty acids.* Biosynthesis of cyclopropane fatty acids has been recently reviewed by Law (1971). These compounds are derived from the corresponding mono-unsaturated fatty acids by addition of a C_1 unit across the double bond. Liu and Hofmann (1962) demonstrated

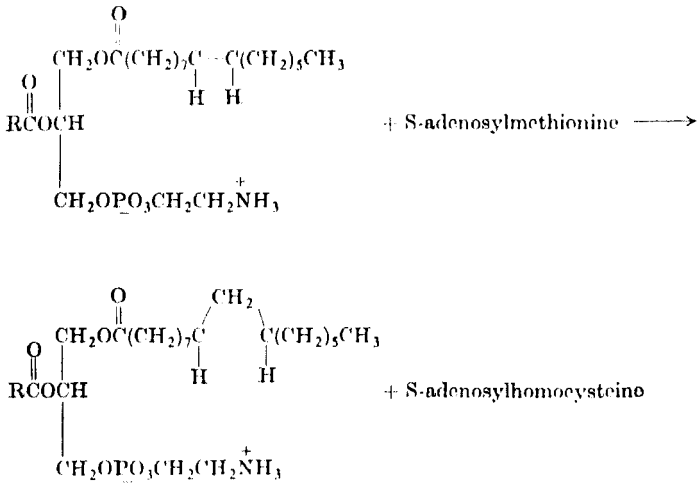


FIG. 10. Synthesis of a cyclopropane fatty-acid residue on phosphatidylethanolamine.

the derivation of the C_1 unit from methionine in the formation of lactobacillic acid from its precursor, *cis*-vaccenic acid. O'Leary (1962) provided evidence that S-adenosylmethionine was the active donor of the C_1 unit and this was confirmed by Zalkin *et al.* (1963) who showed that a C_1 unit derived from S-adenosylmethionine was utilized in the formation of cyclopropane fatty acids catalysed by extracts from *Serratia marcescens* and *Clostridium butyricum*, and that the C_1 unit was incorporated into mono-unsaturated fatty acid moieties of phosphatidylethanolamine. Working with a purified enzyme from *Cl. butyricum*, Chung and Law (1964) provided strong evidence that the actual acceptor was a fatty-acid residue bound to a phospholipid (Fig. 10). Subsequent work has shown that aldehyde residues in plasmalogens (Chung and Goldfine, 1965) and alcohol residues in alkyl ether lipids (Thomas and Law, 1966)

could also serve as acceptors of the C_1 unit to form the corresponding cyclopropane aldehydes and cyclopropane alcohols. Cyclopropane aldehydes (Goldfine, 1964) and cyclopropane alcohols (Day *et al.*, 1970) in addition to cyclopropane fatty acids have been found in the lipids of *Cl. butyricum*. Various phospholipids have been shown to be potential precursors of cyclopropane fatty acids provided they contain unsaturated fatty acids (Thomas and Law, 1966). Phosphatidylglycerol, phosphatidic acid, cardiolipin and phosphatidylserine as well as phosphatidylethanolamine were active precursors *in vitro* in the presence of the cyclopropane synthetase from *Cl. butyricum*. Cronan (1968) has since shown that growing cells of *E. coli* form cyclopropane fatty acids on phosphatidylglycerol and cardiolipin in addition to phosphatidylethanolamine. Cyclopropane fatty-acid synthetase has also been demonstrated in extracts of *Aerobacter aerogenes* and *Lactobacillus arabinosus* (O'Leary, 1965).

Cyclopropane fatty acids have been found in a variety of bacteria. The Gram-negative species include many members of the Enterobacteriaceae, including *E. coli* (Dauchy and Asselineau, 1960; Kaneshiro and Marr, 1961), *A. aerogenes* (O'Leary, 1962), *Salmonella typhimurium* (Gray, 1962), *Serratia marcescens* (Bishop and Still, 1963; Kates *et al.*, 1964) and the Brucellaceae, including *Pasteurella pestis* (Asselineau, 1961), *Brucella abortus* and *B. melitensis* (Thiele *et al.*, 1969). Among the Gram-positive bacteria, cyclopropane fatty acids have been found in a number of the Lactobacillaceae, including *Lactobacillus acidophilus*, *L. arabinosus*, *L. casei*, *L. delbrueckii* (Hofmann, 1963), *Streptococcus lactis* and *S. cremoris* (MacLeod and Brown, 1963). The most commonly found cyclopropane fatty acids are *cis*-11,12-methylene-octadecanoic acid (lactobacillic acid), which is derived from *cis*-vaccenic acid (Hofmann, 1963), and *cis*-9,10-methylenehexadecanoic acid, which is derived from palmitoleic acid (Kaneshiro and Marr, 1961). *Cis*-9,10-Methyleneoctadecanoic acid (dihydrosterculic acid) has also been reported (Gray, 1962; Goldfine and Panos, 1971). The latter authors, in a study of the positional isomers of the mono-enoic and cyclopropane fatty acids and aldehydes of *Cl. butyricum*, have provided evidence for a strong preference of the cyclopropane synthetase for the *n*-7 series of mono-enes. *Cis*-9,10-Methylenehexadecanoic acid was the major 17-carbon cyclopropane fatty acid formed despite a preponderance of the 16:1 Δ^7 over 16:1 Δ^9 precursors. A similar preference was seen in the cyclopropane aldehydes. A similar sequence of reactions may be involved in the formation of cyclopropene fatty acids in higher plants (Hooper and Law, 1965). Cyclopropane fatty-acid synthetases do not appear to be present in animal tissues.

The mid-chain, branched fatty acid, tuberculostearic acid (10-methyl stearic acid) is formed from oleic acid in an analogous manner. Lennarz

et al. (1962b) demonstrated the formation of this compound in *Mycobacterium phlei* from oleic acid and the methyl group of methionine. A similar reaction was shown to occur in *M. tuberculosis* (Lederer, 1964). In two recent papers, Akamatsu and Law (1968, 1970) presented evidence for the formation of tuberculostearic acid on an oleyl residue of a phospholipid. The methyl donor was S-adenosylmethionine, and phosphatidylglycerol, phosphatidylinositol, and phosphatidylethanolamine all served as acceptors. In crude extracts, the tuberculostearate formed enzymically on endogenous lipids was found in both phosphatidylethanolamine and an oligomannoside of phosphatidylinositol. 10-Methylenestearyl (phospholipid) is an intermediate in this reaction (Jaurégui-berry *et al.*, 1966).

2. *Alk-1-Enyl Ethers*

The aldehydogenic chains of plasmalogens are thought to be derived from long-chain fatty acids. Since the biosynthesis of the alk-1-enyl ether bond of bacteria has not been achieved *in vitro*, the proposed route is based almost entirely on experiments with whole cells. Labelled long-chain fatty acids were incorporated into the alk-1-enyl ether chains of plasmalogens in *Clostridium butyricum* (Baumann *et al.*, 1965). Kinetic experiments with [^{14}C]acetate were also consistent with the concept that the alk-1-enyl ethers are derived from the corresponding fatty acids (Hagen and Goldfine, 1967). Compositional data on the chain lengths and positional isomers of the unsaturated and cyclopropane fatty acids and aldehydes also lend support to this hypothesis (Goldfine and Panos, 1971). The major unresolved problem is the stage at which the fatty acid is converted to the corresponding aldehyde. Some evidence points to a conversion of the ester bond of a diacylphospholipid to an alk-1-enyl ether (Baumann *et al.*, 1965), but recent work suggests that the fatty acid or their activated thioester derivatives undergo reduction prior to the formation of the vinyl ether linkage (Hagen and Goldfine, 1967). Day *et al.* (1970) have demonstrated the enzymic reduction of palmityl-CoA to palmitaldehyde and cetyl alcohol by extracts prepared from *Cl. butyricum*. The biosynthesis of plasmalogens in bacteria has been reviewed recently (Goldfine and Hagen, 1971).

B. Biosynthesis of the Complex Lipids

1. *Phosphoglycerides*

a. *Phosphatidylethanolamine, phosphatidylglycerol and cardiolipin.* The assembly of the diacylphosphoglycerides begins in bacteria, as it does in higher organisms, with a two-step acylation of glycerol 3-phosphate

to yield sequentially lysophosphatidic acid (mono-acylglycerol 3-phosphate) and phosphatidic acid (Fig. 11) (Van den Bosch and Vagelos, 1970; Ray *et al.*, 1970; Hechemy and Goldfine, 1971). In experiments with particulate enzymes derived from *Escherichia coli*, both acyl-CoA and acyl-acyl carrier protein derivatives have been shown to serve as acyl donors (Pieringer *et al.*, 1967; Ailhaud and Vagelos, 1966; Kito and Pizer, 1969; Van den Bosch and Vagelos, 1970). The glycerol 3-phosphate

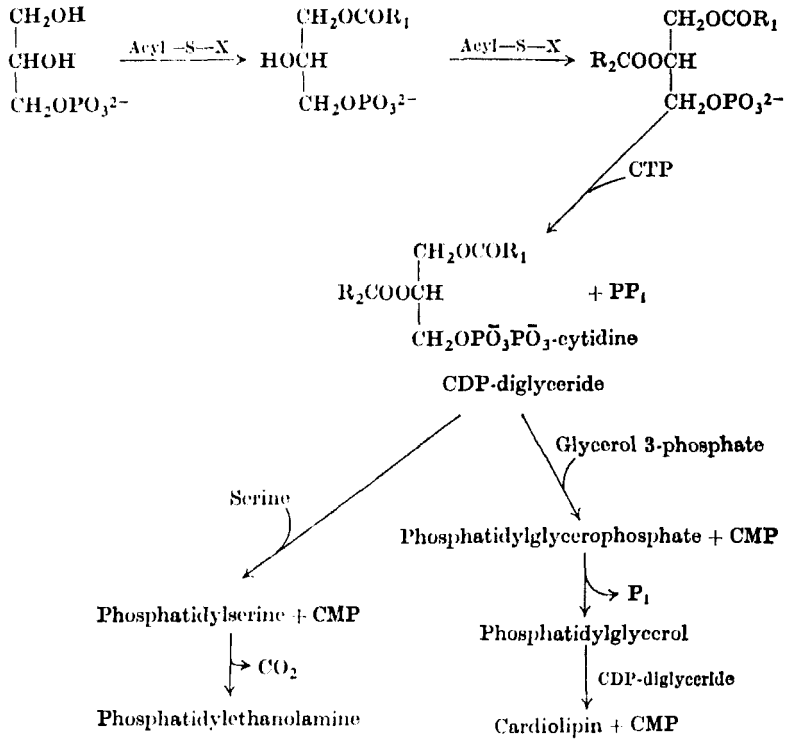


FIG. 11. Pathways for phospholipid synthesis in *Escherichia coli*.

acyltransferase of *Cl. butyricum* was shown to be highly specific for the acyl-S-acyl carrier protein derivatives (Goldfine, 1966; Goldfine *et al.*, 1967). Since *E. coli* synthesizes fatty acids *de novo* as the acyl-S-acyl carrier protein derivatives, and no transacylation between acyl carrier protein and Co-ASH has been demonstrated, it is reasonable to assume that the fatty acids synthesized *de novo* are transferred directly from acyl carrier protein to glycerol 3-phosphate. The recent work of Van den Bosch and Vagelos (1970) has shown that transacylation from acyl-acyl carrier protein by *E. coli* enzymes is more specific for the placement of unsaturated fatty acids on the C-2 of 1-mono-acyl glycerol 3-phosphate

than transacylation from acyl-CoA. In earlier reports on the specific acylation of glycerol 3-phosphate by palmityl-S-acyl carrier protein, catalysed by membrane particles from *Cl. butyricum*, the reaction proceeded only as far as lysophosphatidic acid (Goldfine *et al.*, 1967). Recent work (H. Goldfine and G. P. Ailhaud, unpublished observations) has demonstrated the formation of phosphatidic acid with acyl-S-acyl carrier protein derivatives as the acyl donors.

Exogenous fatty acids can be used by *E. coli* for both phospholipid synthesis and energy production, via β-oxidation. It is now clear that these exogenous fatty acids become activated by a fatty acyl-CoA synthetase and not as the acyl carrier protein derivatives (Overath *et al.*, 1969; Samuel and Ailhaud, 1969; Samuel *et al.*, 1970). Thus transacylation from coenzyme-A esters to glycerophosphate provides a mechanism for utilizing exogenous fatty acids directly for phospholipid synthesis. Samuel and Ailhaud (1969) were unable to demonstrate activation of exogenous fatty acids by *Cl. butyricum* extracts with either coenzyme-A or acyl carrier protein, despite the ability of these cells to utilize exogenous long-chain fatty acids for lipid synthesis (Goldfine and Bloch, 1961).

In higher organisms, the acyl-CoA derivatives have long been regarded as the acyl donors in the formation of phosphatidic acid (Kornberg and Pricer, 1953) and indirect evidence for two enzymes has been obtained (Lands and Hart, 1965). Kuhn and Lynen (1965) demonstrated an obligatory requirement for coenzyme-A in the acylation of glycerol 3-phosphate by fatty acyl thio-esters of the yeast fatty-acid synthetase, in a reaction catalysed by a particulate preparation from yeast.

From phosphatidic acid, phospholipid synthesis in *E. coli* proceeds by way of CDP-diglyceride (Fig. 11) which is formed by the reaction of phosphatidic acid with CTP (Carter, 1968; McCamen and Finnerty, 1968). An analogous reaction was previously demonstrated in guinea-pig liver (Carter and Kennedy, 1966). Kennedy and his coworkers showed that two pathways of phospholipid biosynthesis branch off from CDP-diglyceride in *E. coli*. One pathway leads first to phosphatidylserine by reaction of CDP-diglyceride with free L-serine (Kanfer and Kennedy, 1964). Phosphatidylserine is then rapidly decarboxylated to yield phosphatidylethanolamine; thus, the steady-state pool of phosphatidylserine in *E. coli* is always very small (Fig. 11). A similar sequence of reactions occurs in two species of bacilli (Lennarz, 1970; Patterson and Lennarz, 1971), and in *Micrococcus cerificans* (Makula and Finnerty, 1970).

The other branch of phospholipid synthesis in *E. coli* leads first to phosphatidylglycerophosphate by reaction of CDP-diglyceride with glycerol 3-phosphate (Kanfer and Kennedy, 1964; Chang and Kennedy,

1967a, b). Phosphatidylglycerophosphate is then rapidly converted to phosphatidylglycerol by a specific phosphatase (Fig. 11). Evidence for these reactions in two species of bacilli has also been obtained (Lennarz, 1970; Patterson and Lennarz, 1971). Stanacev *et al.* (1967) showed that a reaction between phosphatidylglycerol and another molecule of CDP-diglyceride catalysed by particles from *E. coli* leads to the formation of cardiolipin (diphosphatidylglycerol). These two sequences of reactions account for all of the known phosphoglycerides in *Escherichia coli*. The formation of phosphatidylglycerol and cardiolipin by a similar route has been postulated for *Micrococcus cerificans* (Makula and Finnerty, 1970).

In bacteria, ethanolamine is derived from serine by the sequence of reactions described above. No evidence for a reaction involving CDP-ethanolamine has been found in bacteria, although many groups of bacteria contain phosphatidylethanolamine. It appears that the reaction described by Kennedy and Weiss (1956) in which a 1,2-diglyceride reacts

$$\text{Phosphatidylethanolamine} \xrightarrow{\text{AMe}} \text{Phosphatidyl-N-methylethanolamine} \quad (1)$$

$$\xrightarrow{\text{AMe}} \text{Phosphatidyl-N,N'-dimethylethanolamine} \quad (2)$$

$$\xrightarrow{\text{AMe}} \text{Phosphatidylcholine} \quad (3)$$

FIG. 12. Reactions leading to phosphatidylcholine synthesis in Gram-negative bacteria. AMe indicates S-adenosylmethionine.

with CDP-ethanolamine to form phosphatidylethanolamine, did not arise at the prokaryotic stage of evolution.

Formation of phosphatidylglycerophosphate from CDP-diglyceride and glycerol 3-phosphate and the subsequent removal of inorganic phosphate that occur in *E. coli* were also found in animal tissues (Kiyasu *et al.*, 1963). Guinea-pig liver mitochondria are apparently capable of synthesizing cardiolipin by the pathway that operates in *E. coli*, according to a recent report by Stanacev and Davidson (1971).

b. *Phosphatidyl-N-methylethanolamine, phosphatidyl-N,N'-dimethylethanolamine and phosphatidylcholine.* As discussed in Section II. B. 1. (p. 5), phosphatidylcholine and the N-methylated phosphatidylethanolamines are found in a number of Gram-negative organisms. Their biosynthesis in bacteria, in every organism so far studied, is through a stepwise methylation of phosphatidylethanolamine (Fig. 12), a pathway demonstrated earlier by Bremer and Greenberg (1960), Arton and Lofland (1960) and Gibson *et al.* (1961) in the mammalian liver, and by Hall and Nyc (1959) in *Neurospora*. The same pathway also occurs in yeast (Letters, 1966; Waechter *et al.*, 1969) and in protozoa (Lust and Daniel, 1966; Smith and Law, 1970). Studies with growing cells of *Cl.*

butyricum (Goldfine, 1962), *Agrobacterium tumefaciens* (Law *et al.*, 1963), two other species of agrobacteria, *Proteus vulgaris* (Goldfine and Ellis, 1964), *Hyphomicrobium*, *Nitrosocystis oceanus* (Hagen *et al.*, 1966; Goldfine and Hagen, 1968), *Micrococcus cerificans* (Makula and Finnerty, 1970), and *Rhodopseudomonas spheroides* (Gorchein *et al.*, 1968) have demonstrated the utilization of the methyl group of methionine in the formation of N-methylated ethanolamine phosphatides. The enzymic formation of these compounds has been studied in two species of bacteria. Kaneshiro and Law (1964) obtained a soluble enzyme that catalysed the first methylation of phosphatidylethanolamine and a particulate fraction that catalysed the conversion of phosphatidylmethylethanolamine to phosphatidyldimethylethanolamine and phosphatidylcholine from extracts of *A. tumefaciens*. The methyl donor in each reaction was S-adenosylmethionine. Work with choline-deficient mutants of *Neurospora* suggested that a single enzyme catalyses steps (2) and (3) (Fig. 12) (Scarborough and Nyc, 1967). Extracts of *Hyphomicrobium* sp. also catalyse steps (1), (2) and (3) (Fig. 12). This organism is somewhat unusual in that phosphatidyldimethylethanolamine is present in the cell in quantities equalling those of phosphatidylcholine (Hagen *et al.*, 1966; Goldfine and Hagen, 1968). The regulation of these reactions has not been studied in bacteria, but the recent studies of Lester and his coworkers (Waechter *et al.*, 1969; Steiner and Lester, 1970) suggest that the methylation pathway is repressed in yeast when the organism is grown with adequate choline. It should be noted, however, that yeast has both the methylation pathway and the enzymes for forming phosphatidylcholine *via* CDP-choline, while no such scavenging pathway has been demonstrated in bacteria, despite at least two attempts (Sherr and Law, 1965; Shieh and Spears, 1967). It seems clear that the methylation pathway evolved in the prokaryotic organisms, but the evolutionary development of animals has led to a partial loss of this pathway, and thus to a nutritional dependence on choline.

c. *O-Amino Acyl Phosphatidylglycerols.* The biosynthesis of these compounds was studied by Lennarz and his colleagues with several Gram-positive bacteria. A particulate fraction obtained from *Staphylococcus aureus* catalysed the transfer of lysine from lysyl-t-RNA to phosphatidylglycerol (Lennarz *et al.*, 1966; 1967). Lysyl-t-RNA from several organisms served as the lysine donor. Among the natural lipids tested, only phosphatidylglycerol served as an acceptor of the lysyl group. Synthetic 2′-deoxy-, but not 3′-deoxy-, phosphatidylglycerol served as an acceptor, suggesting that the amino acid normally becomes attached to the 3′-hydroxyl group of phosphatidylglycerol. Specificity for the t-RNA carrier was also shown. Studies on the alanylphosphatidylglycerol

synthetase of *Clostridium welchii* showed that ala-t-RNA^{ala}, but not ala-t-RNA^{cys} was active. Lactyl-t-RNA^{ala} and N-acetyl-ala-t-RNA^{ala} were also inactive. Enzymic degradation of t-RNA inactivated the carrier (Gould *et al.*, 1968). Extracts of *Bacillus cereus*, *B. megaterium* and *Streptococcus faecalis* also catalysed formation of lysylphosphatidylglycerol. With the last preparation, arginylphosphatidylglycerol formation was also observed (Gould and Lennarz, 1967). Koostra and Smith (1969) demonstrated the formation of L-alanylphosphatidylglycerol by extracts of *Mycoplasma laidlawii* from L-ala-t-RNA. Synthesis of D-alanylphosphatidylglycerol was also observed, but the mechanism of activation did not appear to require t-RNA.

d. *Phosphatidylinositol.* Information is lacking on the route of formation of phosphatidylinositol in the limited range of bacteria that synthesize this phospholipid or compounds containing it. In animal tissues it is formed by a reaction analogous to the bacterial pathways leading to phosphatidylethanolamine and phosphatidylglycerol, i.e. reaction of free inositol with CDP-diglyceride (Paulus and Kennedy, 1960).

e. *Plasmalogens.* The detailed mechanism of formation of these 1-alk-1′-enyl-2-acyl-glycerophosphatides in anaerobic bacteria and in animal tissues has not been elucidated. As mentioned in Section III. A. 2. (p. 28), the alk-1-enyl ether chains in bacteria are derived from long-chain fatty acids. Experiments in which the kinetics of incorporation of ^{32}Pi into the diacylphosphatides and plasmalogens of *Clostridium butyricum* were measured provided evidence that the diacylphosphatides were precursors of the corresponding plasmalogens (Baumann *et al.*, 1965; Goldfine and Hagen, 1971). When [1-^{3}H-1-^{14}C]palmitaldehyde was fed to this organism, much of it was oxidized to palmitic acid which was then incorporated into both acyl and alk-1-enyl chains of the phospholipids; however, some was incorporated into the alk-1-enyl chains without prior oxidation (Hagen and Goldfine, 1967). The enzymic reduction of palmityl-CoA to palmitaldehyde and cetyl alcohol in extracts of *Cl. butyricum* has recently been demonstrated (Day *et al.*, 1970). Based on these and other experiments, Goldfine and Hagen (1971) have proposed that the 1-linked fatty-acid chains of the diacylphosphatides are replaced by chains at the oxidation level of aldehydes or possibly alcohols, but no direct demonstration of this pathway has been accomplished. This is somewhat different from the pathway proposed for animal tissues based on the experiments of a number of workers (Thompson, 1968; Wood and Healy, 1970; Hajra, 1970; Wykle *et al.*, 1970) in which 1-alkyl glycerol 3-phosphate is formed by reaction of 1-acyl dihydroxyacetone phosphate and a long-chain fatty alcohol. 1-Alkyl-2-acyl-glycerol

3-phosphate is then formed, and the plasmalogen is formed subsequently either before or after the addition to the lipid of a water-soluble base, e.g. ethanolamine. In view of the large evolutionary gap between the anaerobic bacteria and the animals, it is conceivable that different mechanisms for the formation of plasmalogens may have evolved (Goldfine and Hagen, 1971).

f. *Glycolipids.* It is beyond the scope of this review to discuss the extensive work on the biosynthesis of the lipopolysaccharides of Gram-negative bacteria. An excellent review has recently appeared (Lüderitz, 1970). The formation of glycosyl diglycerides was shown by Lennarz (1964), Lennarz and Talamo (1966), and Kaufmann *et al.* (1965) to involve the transfer of a sugar from its appropriate nucleotide derivative to a diglyceride. The system from *Micrococcus lysodeikticus* studied by Lennarz and Talamo (1966) resulted in the sequential formation of an α-D-mannosyl (1 → 3) diglyceride and a dimannosyl diglyceride from GDP-mannose and a 1,2-diglyceride. In the studies of Kaufman *et al.* (1965) on a *Pneumococcus* sp., the stepwise formation of a galactosyl-glucosyl diglyceride was demonstrated in the presence of a cell-free preparation and the appropriate UDP-sugars. Pieringer (1968) reported the formation of α-D-glucopyranosyl diglyceride and 2-*O*-α-D-glucopyranosyl-α-D-glucopyranosyl diglyceride from UDP-glucose and 1,2-diglycerides catalysed by a particulate preparation from *Streptococcus faecalis*. A similar mechanism presumably exists in plants. Synthesis of galactolipids by spinach chloroplasts was studied by Neufeld and Hall (1964) who showed that galactose from UDP-galactose was transferred to endogenous acceptors.

g. *Polyisoprenoids.* The building blocks for polyisoprenoid synthesis are similar to those needed for sterol biosynthesis, which does not occur in bacteria. Bloch and his colleagues (Kandutsch *et al.*, 1964; Allen *et al.*, 1967) studied the formation of geranylgeraniol, a C_{20} terpenoid, from isopentenyl pyrophosphate, which was catalysed by enzymes from *Micrococcus lysodeikticus*. Polyisoprenoids up to C_{40} in length were also formed by enzymes from this organism. Similar reactions are presumably involved in the formation of carotenoids, quinone coenzymes and the undecaprenylphosphate sugar carriers (Fig. 6, p. 15) needed for *O*-antigen (Wright *et al.*, 1967), peptidoglycan (Higashi *et al.*, 1967), mannan (Scher *et al.*, 1968), and capsular polysaccharide (Troy *et al.*, 1971) biosynthesis. The related bactoprenol, isolated by Thorne and Kodicek (1966) from lactobacilli, is also derived from mevalonic acid. Gough *et al.* (1970) have recently characterized a similar compound from *Lactobacillus plantarum*.

IV. Bacterial Lipid Compositions

During the past five years, quantitative data on the lipids of a number of important groups of bacteria have become available. Earlier observations have been confirmed, extended and corrected. These efforts have been aided by a greater awareness of the types of lipids that may be found in bacteria, along with the application of a variety of recent techniques for the identification and quantitative analysis of complex lipids. Especially important has been the realization that chromatographic comparisons of intact lipids, though valuable, are not sufficient for the identification of complex lipids. Nor is the examination of total hydrolysates of purified lipids sufficient for identification, since a number of these compounds yield identical hydrolysis products, albeit in different proportions. The increasing use of the deacylation procedures pioneered by Dawson (1960), followed by paper- or thin-layer chromatography of the water-soluble products, or more recently by anion-exchange chromatography as developed by R. L. Lester and by Wells and Dittmer (1966), has aided considerably in the analysis of complex bacterial lipids. Several reviews and monographs cover the information available up to 1966 (Kates, 1964; Asselineau, 1966; Ikawa, 1967; O'Leary, 1967), and the reader is referred to these for data from the earlier literature. In the tables and discussion that follow, I have attempted to select from the recent literature the most complete data available for a given species. In some cases similar data may be found either in the older or more recent literature, but repetition has been avoided in order that the outlines may stand out more clearly. The classification of bacteria given by Breed *et al.* (1957) is used throughout.

A. Lipids of Gram-Negative Bacteria

1. Complex Lipids

Tables 2 and 3 present the compositions of the complex lipids of Gram-negative bacteria of the Orders Pseudomonadales, Hyphomicrobiales, and Eubacteriales. It is apparent from these data that no outstanding quantitative or qualitative generalizations can be made that would enable us to distinguish the Pseudomonadales and Gram-negative Eubacteriales on the basis of their phosphatide compositions. Organisms from both orders usually contain phosphatidylethanolamine, phosphatidylglycerol, and cardiolipin (diphosphatidylglycerol). Bacteria in the Pseudomonadales have somewhat more phosphatidylglycerol plus cardiolipin, average 34% (range 17–44%), than members of the Eubacteriales, average 25% (range 20–33%). If the family of lipids derived from phosphatidylethanolamine, including phosphatidylethanolamine,

TABLE 2. Compositions of Lipids from Gram-Negative Bacteria of the Orders Pseudomonadales and Hyphomicrobiales

	Phosphatidylethanolamine	Phosphatidyl-N-methylethanolamine	Phosphatidylcholine	Phosphatidylglycerol	Cardiolipin	Others	References
PSEUDOMONADALES							
Thiorhodaceae							
Chromatium Strain D	55[a]			39	5	[b]	Steiner *et al.* (1970b)
Athiorhodaceae							
Rhodopseudomonas spheroides	41		19	36		[c]	Gorchein (1968a, b)
Rhodopseudomonas capsulata	46		13	41			Steiner *et al.* (1970a)
Rhodospirillum rubrum[d]	19			10	5	17[c]	Hirayama (1968)
Rhodospirillum capsulatus[d]	31			9	1		Hirayama (1968)
Nitrobacteraceae							
Nitrosocystis oceanus	67		3	←28→			Hagen *et al.* (1966)
Nitrosomonas europaea	78			←17→			Hagen *et al.* (1966)
Thiobacteriaceae							
Thiobacillus neapolitanus[e]	42–45	20–27		11–15	17–23		Barridge and Shively (1968)
Thiobacillus thioparus	65			24	11		Barridge and Shively (1968)

Thiobacillus intermedius[e]	55–60	12–15		2–20	10–26		Barridge and Shively (1968)
Thiobacillus thiooxidans (log-phase)	20	36		37	7	[c]	Shively and Benson (1967)
Thiobacillus thiooxidans (stationary-phase)	4	53		27	16	[c]	Shively and Benson (1967)
Thiobacillus novellus[e]	23–27	3–11	33–37	24–30	5–7		Barridge and Shively (1968)
Pseudomonadaceae							
Pseudomonas aeruginosa	69		1·4	15	9·4		Randle *et al.* (1969)
Siderocapsaceae							
Ferrobacillus ferrooxidans	20	42	1·5	23	13		Short *et al.* (1969)
HYPHOMICROBIALES							
Hyphomicrobium vulgare NQ521	23		29	10		[f]	Goldfine and Hagen (1968)
Rhodomicrobium vanniellii	4·5		27	10		[c]	Park and Berger (1967)

[a] Values are expressed as per cent of lipid phosphorus.
[b] Glucosyldiglyceride, 8–10% of total lipid; smaller amounts of other glycolipids.
[c] Ornithine-containing lipids.
[d] As per cent of total lipids.
[e] Ranges for different media.
[f] Phosphatidyldimethylethanolamine, 36%.

TABLE 3. Compositions of Lipids from Gram-Negative Bacteria of the Order Eubacteriales

	Phosphatidylethanolamine	Phosphatidyl-N-methylethanolamine	Phosphatidylcholine	Phosphatidylglycerol	Cardiolipin	Others	References
Azotobacteraceae							
Azotobacter agilis (log-phase)	64[a,b]	5	1	27	2·4		Randle *et al.* (1969)
Azotobacter agilis (stationary-phase)		5	2·4	13	23		Randle *et al.* (1969)
Azotobacter vinelandii	53			←7→		[c]	Jurtshuk and Schlech (1969)
Rhizobiaceae							
Agrobacterium tumefaciens (log-phase)	45	14	7	29			Randle *et al.* (1969)
Agrobacterium tumefaciens (stationary-phase)	18	16	28	13	19		Randle *et al.* (1969)
Chromobacterium violaceum	77			18	4·6		Randle *et al.* (1969)
Enterobacteriaceae							
Escherichieae							
Escherichia coli (log-phase[d])	76			20	1·1		Randle *et al.* (1969)
Escherichia coli (stationary-phase[d])	77			11	6·6		Randle *et al.* (1969)
Enterobacter aerogenes ATCC 13048	74			21	3·2		Randle *et al.* (1969)
Serratieae							
Serratia marcescens	66			14	17		Randle *et al.* (1969)
Proteeae							
Proteus vulgaris (log-phase)	63	4·0		17	2·9		Randle *et al.* (1969)
Proteus vulgaris (stationary-phase)	63	12·1		5·8	14·8		Randle *et al.* (1969)
Salmonelleae							
Salmonella typhimurium	78			18	3·2		Ames (1968)
Brucellaceae							
Brucella abortus Bang 1119	←27→		37	16	5·8	13·8	Thiele *et al.* (1968)
Haemophilus parainfluenzae	78	0·4	0·4	18	3		White (1968)

[a] Values are expressed as per cent of lipid phosphorus.
[b] Includes phosphatidyldimethylethanolamine.
[c] Unidentified.
[d] Cells grown on glucose-minimal media.

phosphatidylmonomethylethanolamine and phosphatidylcholine are added, the organisms in Pseudomonadales have an average of 66% (range 55–78%) compared with an average of 72% for the Eubacteriales (range 64–79%). When these major biosynthetic families of phospholipids in the two orders are compared, the similarities are probably more significant than the small differences in phosphatide compositions noted above.

Within each order there are organisms that can carry out the three-step methylation of phosphatidylethanolamine that yields phosphatidylcholine, organisms that can carry out only the first methylation and organisms that appear to be incapable of N-methylating phosphatidylethanolamine. Even within some of the families of the Order Pseudomonadales there are species differences in biosynthetic capacities. Among the Athiorhodaceae, for example, two species of *Rhodopseudomonas* contain phosphatidylcholine but two species of *Rhodospirillum* do not. Among the Thiobacteriaceae one species of *Thiobacillus* stops at phosphatidylethanolamine, three at phosphatidylmethylethanolamine, and one synthesizes phosphatidylcholine. In this regard, there is conflicting evidence on *Pseudomonas aeruginosa*. Randle *et al.* (1969) and Sinha and Gaby (1964) found small amounts of phosphatidylcholine, but Hancock and Meadow (1969) and Goldfine and Ellis (1964) did not. Among the Eubacteriales, some organisms in the families Azotobacteraceae and Rhizobiaceae are capable of forming phosphatidylcholine (Table 3). In addition to *Agrobacterium tumefaciens*, phosphatidylcholine has also been found in *A. radiobacter*, *A. rhizogenes* (Goldfine and Ellis, 1964), and *Rhizobium japonicum* (Bunn *et al.*, 1970). Most of the organisms in the Enterobacteriaceae have a relatively simple phospholipid composition consisting of phosphatidylethanolamine, phosphatidylglycerol and cardiolipin. The only exception to date is *Proteus vulgaris*, which synthesizes phosphatidylmethylethanolamine (Goldfine and Ellis, 1964; Randle *et al.*, 1969). Hagen *et al.* (1966) pointed out that a number of the organisms that contain phosphatidylcholine also have complex intracytoplasmic membrane systems; for example, *Hyphomicrobium vulgare*, *Nitrosocystis oceanus*, *Azotobacter agilis*, and the photosynthetic bacteria *Rhodomicrobium vannielii*, *Rhodopseudomonas spheroides*, *R. capsulata* and *R. palustris* (Wood *et al.*, 1965). They postulated that the unique size and/or charge of the polar head group of phosphatidylcholine may facilitate folding of the cell membrane. However, this correlation is not universal. *Agrobacterium tumefaciens* (Kurkdjian *et al.*, 1966) and *Thiobacillus novellus* (Caeseele and Lees, 1969) synthesize phosphatidylcholine but do not possess complex intracytoplasmic membrane structures. There are other organisms which contain complex intracytoplasmic membrane systems but do not have

phosphatidylcholine. Included in this group are some of the photosynthetic bacteria, including *Rhodopseudomonas gelatinosa*, *Rhodospirillum rubrum*, and *Chromatium* strain D, and *Nitrosomonas europaea*. Another view of the distribution of phosphatidylcholine in bacteria was suggested by Ikawa (1967), who pointed out that most of the organisms that have phosphatidylcholine also possess efficient electron-transport systems. This would bring together *T. novellus* and the photosynthetic bacteria as well as *N. oceanus*, the hyphomicrobia and *Azotobacter agilis*. The exact function of phosphatidylcholine is not clear, but these relationships, as well as its known presence in the intracytoplasmic membranes of higher organisms, suggest an important role for this lipid in membrane structure and function. The evidence from the comparative lipid compositions of bacteria favours the idea that phosphatidylcholine biosynthesis evolved more recently than the biosynthesis of phosphatidylglycerol and phosphatidylethanolamine.

The polar lipids of several species of Gram-negative bacteria have been found to contain ornithine. Among these are *Rhodopseudomonas spheroides*, *Rhodospirillum rubrum*, *Thiobacillus thio-oxidans*, *Rhodomicrobium vanniellii* (Table 2), *Pseudomonas rubescens* (Wilkinson, 1968a) and *Brucella melitensis* (Prome *et al.*, 1969). In all organisms except *Rhodomicrobium vannielii*, the purified ornithine-containing lipid does not contain phosphorus, and a fatty acid in amide linkage is usually found. The detailed structure is still unclear (see Section II. B. 4c. p. 15).

For several Gram-negative organisms, data on the lipid composition of cells in the logarithmic and stationary phases of growth in batch cultures are given in Tables 2 and 3 (p. 36) and illustrated in Fig. 13. Data for *Azotobacter agilis*, *Agrobacterium tumefaciens*, *E. coli* and *Proteus vulgaris* are taken from Randle *et al.* (1969) and data on *Thiobacillus thio-oxidans* from Shively and Benson (1967). Changes in the phospholipid composition of these organisms during different stages of growth are evident, and it is clear that no single set of compositional data for an organism is likely to hold up under all conditions. As batch cultures of Gram-negative cells become older, the proportion of cardiolipin in phosphatidylglycerol plus cardiolipin increases, while that of phosphatidylglycerol decreases. The sum of the two is not always constant, for example in *E. coli* and *A. agilis*. It is known that both phosphatidylglycerol (Kanfer and Kennedy, 1963) and cardiolipin (Kanemasa *et al.*, 1967) undergo turnover in *E. coli*. Another observation from Tables 2 and 3 is the increased methylation of phosphatidylethanolamine in ageing cultures. In *A. tumefaciens*, the proportion of phosphatidylcholine increases markedly in stationary-phase cells, while that of phosphatidylethanolamine decreases. The content of phos-

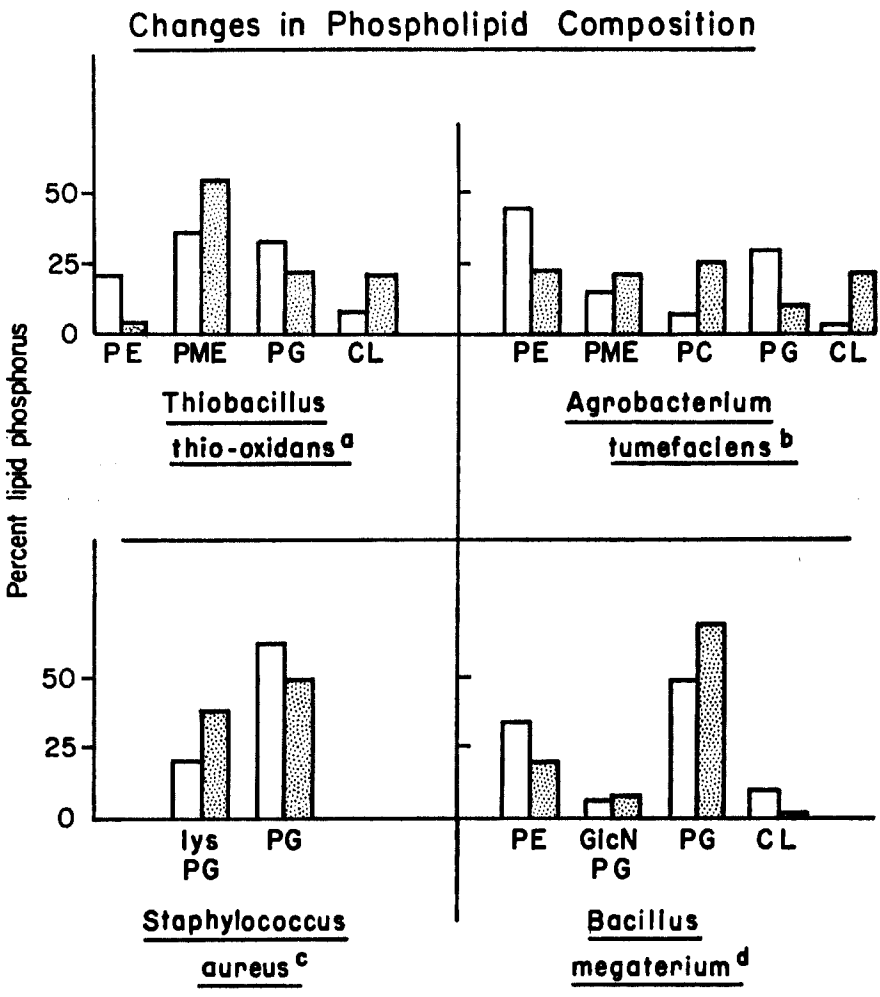


FIG. 13. Examples of the alteration of the phospholipid compositions of bacteria during growth of batch cultures. Open bars indicate the composition of bacteria from logarithmic-phase cultures, and stippled bars of bacteria from stationary-phase, or presporulation-phase cultures with *Bacillus megaterium*. CL, indicates cardiolipin; GlcNPG, glucosaminephosphatidylglycerol; lysPG, lysylphosphatidylglycerol; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PME, phosphatidylmonomethylethanolamine. References: a, Shively and Benson (1967); b, Randle *et al.* (1969); c, Houtsmuller and Van Deenen (1965); d, Bertsch *et al.* (1969).

phatidylmethylethanolamine increases in both *Proteus vulgaris* and *T. thio-oxidans* and the proportion of phosphatidylethanolamine either remains constant (*P. vulgaris*) or decreases (*T. thio-oxidans*; Fig. 13).

Glycolipids are not usually found in Gram-negative bacteria. One exception noted in Table 2 is *Chromatium* Strain D. Glycolipids have also been found in several other photosynthetic bacteria (Constantopoulos and Bloch, 1967; Radunz, 1969), and Wilkinson (1968a, b, 1969) has found glycolipids containing glucuronic acid in two species of *Pseudomonas* (see Section II. B. 4. p. 12).

2. *Fatty Acids of the Gram-Negative Bacteria*

There are abundant data on the fatty acids of this group of organisms (Kates, 1964; Asselineau, 1966; O'Leary, 1967). The major saturated fatty acid is usually 16:0, with lesser amounts of 14:0 and 18:0. The major unsaturated fatty acids are 16:1 Δ^9 and 18:1 Δ^{11}. Cyclopropane fatty acids are frequently encountered in the lipids of the Gram-negative bacteria of the order Eubacteriales and these are usually mixtures of 17:cyc-9,10 and 19:cyc-11,12. Among the Pseudomonadales, the cyclopropane acids have not been found in photosynthetic bacteria (Wood *et al.*, 1965), in *Pseudomonas aeruginosa* or in *Ps. diminuta* (Wilkinson, 1969); however, Thiele *et al.* (1969) reported the presence of a 17:cyc fatty acid in *Hydrogenomonas eutropha*.

B. Complex Lipids of Gram-Positive Bacteria

On the basis of their lipid compositions, the Gram-positive bacteria divide into two well-recognized groups. The first is the group that does not form endospores, including the lactic acid bacteria and the Micrococcaceae (Table 4). These organisms generally contain phospholipids of the phosphatidylglycerol family, which here includes *O*-amino acyl phosphatidylglycerol as well as cardiolipin, but they do not contain the phospholipids of the phosphatidylethanolamine biosynthetic family. The second major group is the endospore-forming Bacillaceae. The complex lipids of the aerobic members of this family have been intensively studied and are characterized by the presence of phosphatidylethanolamine in addition to phosphatidylglycerol and the lipids derived from it. Other groups of Gram-positive organisms will be discussed separately.

1. *Lactic Acid Bacteria and the Micrococcaceae*

These organisms do not appear to contain the phosphatidylethanolamine family of lipids. The major components are phosphatidylglycerol, cardiolipin and the *O*-amino acyl derivatives of phosphatidylglycerol. In addition, glycosyl diglycerides may represent from a few per cent up to 35% by weight of the total lipid (Table 4). As can be seen, the

TABLE 4. Bacterial Lipid Compositions. Gram-Positive Bacteria and Spirochaetales

	Phosphatidylethanolamine	Phosphatidylcholine	Phosphatidylglycerol	*O*-Aminoacyl phosphatidylglycerol	Cardiolipin	Phosphatidylinositol	Other	References
EUBACTERIALES								
Micrococcaceae								
Micrococcus lysodeikticus			72[a]		4	13	[b]	Macfarlane (1961a, b)
Micrococcus cerificans HOI-N[c]	47–87	12–31			1–15		[d]	Makula and Finnerty (1970)
Staphylococcus aureus[c]			10–60	18–80[e]	0–20		[f]	Gould and Lennarz (1970) Houtsmuller and Van Deenen (1965)
Staphylococcus aureus			63	16–18[e]	11–14		[f]	Joyce *et al.* (1970)
Sarcina lutea		1	14		17	5·5	[g]	Huston *et al.* (1965)
Lactobacillaceae								
Streptococceae								
Diplococcus (*Pneumococcus*)			About 25		About 70		[h]	Brundish *et al.* (1965, 1967)
Streptococcus faecalis			Major	Major[i]	Minor		[f]	Vorbeck and Marinetti (1965) Santos Mota *et al.* (1970)
Bifidobacterium								
Bifidobacterium bifidum			8·6–19	About 3 (ala)	42–52		[k]	Exterkate and Veerkamp (1969)
Propionibacteriaceae								
Propionibacterium shermanii			+			+	[j]	Brennan and Ballou (1968) Shaw and Dinglinger (1969) Prottey and Ballou (1968)

TABLE 4—*continued*

	Phosphatidylethanolamine	Phosphatidylcholine	Phosphatidylglycerol	*O*-Aminoacyl phosphatidylglycerol	Cardiolipin	Phosphatidylinositol	Other	References
Corynebacteriaceae								
Listeria monocytogenes			Major		Major		[k]	Carroll *et al.* (1968)
Microbacterium thermosphactum	+		+		+		[b, l]	Shaw and Stead (1970)
Arthrobacter simplex (stationary phase)	–		About 85					Yano *et al.* (1970)
Bacillaceae								
Bacillus cereus	46		35	8 (orn)	+		[m]	Houtsmuller and van Deenen (1963)
Bacillus cereus ATCC 4342	45–64		25–32	1–5 (ala)	5–25			Lang and Lundgren (1970)
Bacillus licheniformis	23		About 60	2 (lys)	2–12			Morman and White (1970)
Bacillus megaterium QM B1551	34–20		49–69		10–2		7–8[n]	Bertsch *et al.* (1969)
Bacillus megaterium MK10D, pH 5	+		5–10	+			30–35[n]	Op den Kamp *et al.* (1965)
Bacillus megaterium MK10D, pH 7	36–45		35–45	8–14 (lys)				Op den Kamp *et al.* (1965)
Bacillus natto (log-phase)	22		27		34			Urakami and Umetani (1968)
Bacillus subtilis	40		13	10 (lys)	38		[o]	Bishop *et al.* (1967)
Bacillus stearothermophilus	14–32		22–30		46–57			Card *et al.* (1969)
Clostridium butyricum	14[p]		26[p]		+		38[p]	Baumann *et al.* (1965)
Clostridium welchii	←Major lys, ala→							Macfarlane (1962)

SPIROCHAETALES						
Treponemataceae						
Treponema pallidum Kazan 5[q]	5–10	30–40			44–55[r]	Johnson *et al.* (1970a)
Treponema pallidum Reiter[q]		41[s]	7	4	25[r]	Meyer and Meyer (1971)
Treponema zuelzerae[q]			31	16	37[t]	Meyer and Meyer (1971)
Leptospira canicola	60–65					Stern *et al.* (1969)
Leptospira patoc	80–90		5–10	1–5	[u]	Johnson *et al.* (1970b)

[a] Values are expressed as per cent of lipid phosphorus except where otherwise noted.
[b] Dimannosyldiglyceride (Lennarz and Talamo, 1966).
[c] Ranges for different media or growth conditions.
[d] Traces to 9% of phosphatidylglycerophosphate. More than 50% of total lipid by weight is neutral lipid.
[e] Lysylphosphatidylglycerol.
[f] Diglucosyldiglyceride and "phosphatidylglucose". See Fisher (1970) and Shaw *et al.* (1970) for recent work on the structure of this glycolipid.
[g] Lipo-amino acids, including perhaps *O*-aminoacylphosphatidylglycerol and other uncharacterized polar lipids. Over 70% of total lipids by weight are neutral lipids.
[h] Up to 30% of lipids by weight is galactosylglucosyldiglyceride.
[i] Lysylphosphatidylglycerol and alanylphosphatidylglycerol.
[j] Mainly phosphatidylinositol mannoside and acylated inositol mannoside.
[k] Glycolipid.
[l] Acylated glucose.
[m] 30–50% Neutral lipid and small amount of diglucosyldiglyceride.
[n] Glucosaminyl phosphatidylglycerol.
[o] Diglycosyldiglyceride accounts for 15% of lipid weight.
[p] 38% Phosphatidylmethylethanolamine of which 78% is plasmalogen; 55% of phosphatidylethanolamine and 9% of phosphatidylglycerol are also of the plasmalogen form.
[q] Per cent of total lipid by weight.
[r] Monogalactosyldiglyceride.
[s] 20% of phosphatidylcholine is in plasmalogen form.
[t] Glucosyldiglyceride.
[u] Cells contain 30% by weight of neutral lipids.

proportions of the phospholipids tend to vary greatly from one species to another and it is now becoming increasingly apparent that the proportions may vary within a given species. In this regard, *Staphylococcus aureus* has been the most carefully studied. Houtsmuller and van Deenen (1965) confirmed the wide variations in the proportions of phosphatidylglycerol and its lysyl derivative at different stages of growth that were earlier observed by Macfarlane (1964a). These changes (Fig. 13, p. 41) were ascribed to changes in the pH value of the growth medium, which could be induced either by fermentation of glucose or by artificial means (Houtsmuller and van Deenen, 1965). Lysylphosphatidylglycerol was found to predominate at lower pH values and phosphatidylglycerol was predominant when the medium was near neutrality. Cardiolipin appeared mainly at or around pH 4·9. Gould and Lennarz (1970) showed that these changes in the proportions of phosphatidylglycerol and lysylphosphatidylglycerol were mainly the result of a large decrease in the absolute amount of phosphatidylglycerol per cell, concomitant with a small increase in lysylphosphatidylglycerol. Comparing growing and resting cells at pH 7 and pH 5, they observed the largest changes at the lower pH value in resting cells. Joyce *et al.* (1970) saw little change in the proportions of the three major phospholipids of *Staph. aureus* during exponential growth in temperature shift-down experiments.

The phospholipid composition of *Micrococcus cerificans* is obviously anomalous (Table 4). The classification of this organism has been re-evaluated (Baumann *et al.*, 1968), and its phospholipid composition also suggests that it may be more closely related to Gram-negative bacteria. Its fatty acid composition, consisting largely of normal saturated and unsaturated fatty acids rather than the branched *iso* and *anteiso* fatty acids usually found in the Micrococcaceae, also suggests membership in a different group (Makula and Finnerty, 1968). These cells stain Gram-negative (Finnerty *et al.*, 1962).

Sarcina lutea was found to contain relatively large proportions of neutral lipids, consisting mainly of hydrocarbons and glycerides (Huston and Albro, 1964). The hydrocarbon composition of this species and that of other bacteria was recently reviewed by Albro and Dittmer (1970).

The coccal members of the Family Lactobacillaceae that have been studied resemble the Micrococcaceae in having large amounts of phosphatidylglycerol, amino acyl-phosphatidylglycerol, and cardiolipin. The lipids of *Pneumococcus* I-R144 were studied by Ishizuka and Yamakawa (1968) who confirmed the findings of Brundish *et al.* (1967) with another strain of *Pneumococcus* (Table 4). Diglycosyldiglycerides are found in both groups of organisms (Shaw, 1970). Unfortunately, the phospholipid composition of organisms in the genus *Lactobacillus* is still not clear. A

large proportion of the total lipid in several species of lactobacilli was found to be "bound", i.e. not extractable without prior hydrolysis (Asselineau, 1966). The nature of these bound lipids is unknown. Ikawa (1963) found no ethanolamine, serine, choline, or inositol in hydrolysates of the phospholipids of *L. plantarum* and *L. casei*. Instead, the nitrogen-containing products of hydrolysis were found to be amino acids. Thorne (1964), however, reported the presence of phosphatidylethanolamine, phosphatidylcholine, and cardiolipin in *L. casei*. These conflicting results have not been resolved. No evidence for the biosynthesis of phosphatidylcholine in these organisms was obtained by growing *L. casei* and *L. plantarum* in medium containing [^{14}C]-CH_3-methionine (Goldfine and Ellis, 1964).

Exterkate *et al.* (1971) have recently analysed the extractable phospholipids of nine *Lactobacillus* strains and compared them with a number of *Bifidobacterium* strains of human, insect and bovine origins. All of the *Lactobacillus* strains contained a large proportion (54–83%) of ^{32}P bound to lipid in phosphatidylglycerol, and from 3 to 15% in cardiolipin. Seven species contained 3–32% of lipid-bound ^{32}P in lysylphosphatidylglycerol, but *Lactobacillus helveticus* and *L. delbrueckii* did not synthesize this compound. The phospholipid compositions of some *Bifidobacterium bifidum* strains of human origin are given in Table 4 (p. 43). Cardiolipin and phosphatidylglycerol were also found in the bovine and bee strains, but alanylphosphatidylglycerol was not present in the three bee strains and one of the two bovine strains.

2. *Bacillaceae*

This group of spore-forming bacteria, especially the aerobic bacilli, has been analysed extensively during the past five years. Unlike the lactic acid bacteria and the Micrococcaceae, members of the genus *Bacillus* all synthesize phosphatidylethanolamine, which usually comprises 20–40% of the total phospholipids. The average is considerably lower than that for components of the phosphatidylethanolamine biosynthetic family in Gram-negative organisms. Another important difference is the absence from the aerobic bacilli of the methylated members of the phosphatidylethanolamine family that are found in a wide variety of Gram-negative bacteria. Conversely, the phosphatidylglycerol family occupies a more prominant place in the bacilli, and the *O*-amino acyl esters of phosphatidylglycerol are found in many species. A glucosamine-containing derivative of phosphatidylglycerol has been found in *B. megaterium* (Table 4). Major variations in the phospholipid composition of *B. megaterium* MK 10D were reported by Op den Kamp *et al.* (1965). A large decrease in the proportion of phosphatidylglycerol was counter-

balanced by the appearance of the glucosaminyl derivative of phosphatidylglycerol when cells were grown on a peptone-yeast extract medium that was supplemented with 2% glucose and 0·2% ammonium sulphate. These supplements resulted in a lower pH value (5 versus 7) at the time of harvesting the cells. A smaller change in the phospholipid composition, in the same direction, was achieved by artificially lowering the pH value of the medium with hydrochloric acid. Changes in the phospholipid composition of *B. megaterium* QM B1551 as cultures go from log to early stationary phase (Bertsch *et al.*, 1969) are shown in Fig. 13 (p. 41).

The phospholipids of a large number of clostridial species were examined by Kamio *et al.* (1969) and many were found to contain plasmalogens (Table 1, p. 11). The phospholipid compositions of only two species have been studied in depth. Baumann *et al.* (1965) found that the diacyl and plasmalogen forms of phosphatidylethanolamine and phosphatidylmethylethanolamine were major constituents of phospholipids from *Cl. butyricum* along with substantial amounts of phosphatidylglycerol mainly in the diacyl form. Cardiolipin was found in stationary-phase cells. The complex lipids of *Cl. welchii* (*perfringens*) were studied by Macfarlane (1962) who demonstrated the presence of phosphatidylglycerol, lysyl- and alanylphosphatidylglycerol, and cardiolipin as the major components. This species has little plasmalogen (Kamio *et al.*, 1969). Some species of clostridia appear to have 70–90% plasmalogen according to these authors (Table 1, p. 11). Radioactive N-methylated derivatives of phosphatidylethanolamine were not found in *Cl. histolyticum*, *Cl. propionicum*, *Cl. acetobutylicum* and *Cl. tetanomorphum* when these species were grown on [^{14}C]CH_3-methionine (Goldfine and Ellis, 1964). *Clostridium butyricum* seems to be unusual among clostridia in this regard, but more species will have to be examined before general conclusions can be drawn.

3. Corynebacteria and Propionibacteria

The lipids of *Corynebacterium diphtheriae* have been studied intensively in a number of laboratories and the results have been summarized by Asselineau (1966). Free fatty acids (16:0, 16:1), corynomycolic acid and corynomycolenic acid represent much of the free lipids. The latter two are branched C_{32} fatty acids related to the mycolic acids of mycobacteria. Neutral glycerides and esters of ethylene glycol have also been found. The phosphatides represent less than half of the total free lipids. From strain P.W. 8 of *Corynebact. diphtheriae*, Gomes *et al.* (1966) isolated three phosphoglycolipids which contained 40–50% fatty acids and 10–20% hexoses. On hydrolysis, mannose and inositol were found in addition to

glycerol. *Corynebacterium xerosis* and *C. diphtheriae* were shown to contain acylated dimannophosphoinositides and phosphoinositides (Brennan, 1968; Brennan and Lehane, 1969). Similar acylated dimannophosphoinositides also occur in the mycobacteria (Pangborn and McKinney, 1966; Brennan and Ballou, 1967) and in the propionibacteria Brennan *et al.* (1970) have recently identified mono-acylglucose as a major lipid in *Corynebact. diphtheriae* and *Mycobacterium smegmatis*. The major fatty acid esterified to glucose is corynomycolic acid. *Corynebacterium diphtheriae* does not contain phosphatidylethanolamine or phosphatidylcholine. The lipids of several other organisms classified as Corynebacteriaceae have been studied and appear to more closely resemble the lipids of other Gram-positive organisms. In both *Listeria monocytogenes* and *Arthrobacter simplex*, phosphatidylglycerol is reported to be a major lipid. It is also found in *Microbacterium thermosphactum*. Cardiolipin is found in *L. monocytogenes* and *M. thermosphactum* and the latter is also reported to contain phosphatidylethanolamine (Table 4). Stanier *et al.* (1970) have discussed the indefinite nature of the relationship of other genera of Corynebacteriaceae to *Corynebact. diphtheriae*.

In a number of ways the propionibacteria are also related to the mycobacteria (Brennan and Ballou, 1968) and this is reflected in their phospholipid composition. *Propionibacterium shermanii* has phosphatidylinositol, phosphatidylmyoinositolmonomannoside, phosphatidylglycerol, and phosphatidylglycerophosphate (Brennan and Ballou, 1968; Prottey and Ballou, 1968). Similar lipids were found in *P. freudenreichii* (Lanéelle and Asselineau, 1968). In addition to these phospholipids, Prottey and Ballou (1968) and Shaw and Dinglinger (1968) found an acylated inositol mannoside in *P. shermanii*, in which it represents 40% of the total lipid. Shaw and Dinglinger (1969) demonstrated smaller amounts of the same glycolipid in five other propionibacteria.

The broader subject of the lipids of the mycobacteria is beyond the scope of this discussion. Lipids are present in large amounts in these organisms and have a complexity which is unique to the acid-fast bacteria. The interested reader will find much material on this subject in the monograph by Asselineau (1966) and in a review by Lederer (1967).

4. Spirochaetales

Recent work on the medically important Treponemataceae has begun to clarify their complex lipid composition. Meyer and Meyer (1971) examined the lipids of *Treponema pallidum* Reiter, and have reported the presence of 25% monogalactosyldiglyceride, 41% phosphatidylcholine and smaller amounts of phosphatidylglycerol and cardiolipin (Table 4). One-fifth of the phosphatidylcholine is in the plasmalogen

form. Large amounts of the galactosyldiglyceride, a similar phosphatidylcholine content and 5–10% phosphatidylethanolamine were found in the Kazan 5 strain (Johnson *et al.*, 1970a). Similar amounts of the galactosyldiglyceride were found by these authors in other strains of *T. pallidum*. *Treponema zuelzerae*, a free-living spirochete, has monoglucosyldiglyceride, phosphatidylglycerol and cardiolipin as its major lipid components (Table 4). The two species of *Leptospira* that have been studied resemble the Gram-negative bacteria in having phosphatidylethanolamine as their major phospholipid. In addition, small amounts of phosphatidylglycerol and cardiolipin were found in the patoc strain (Johnson *et al.*, 1970b).

C. Conclusions

The study of bacterial membrane lipids has advanced beyond the descriptive and analytical stage, and research on the biosynthesis of the component parts and on the assembly of the complex lipids is moving rapidly forward. Research on bacterial lipids has begun to focus on such questions as their location in functional membrane units, the relationship of individual lipid species to various enzymes, multi-enzyme complexes and transport systems. The regulation of membrane-lipid biosynthesis at the enzyme level is receiving more attention in bacteria, and the isolation of a variety of mutants with lesions in the synthesis of lipids promises to lead to a better understanding of the genetics and control of lipid biosynthesis. Studies of such cell functions as wall synthesis, *O*-antigen synthesis and the transport of sugars have revealed several roles for individual species of lipids. These exciting areas of research have barely been touched in this review in order to limit its size and scope.

Despite the new wealth of data on complex bacterial lipids, it is obvious that many important groups of organisms have received little or no attention. From the data presented in Tables 2, 3 and 4 and discussed in this section, it seems certain that knowledge of lipid composition will add much to studies on the relationships between cell chemistry and taxonomy. It has long been recognized that cell diversity among the bacteria tends to increase towards the periphery of the cell, and the membrane lipids clearly offer a fertile ground for the study of comparative biochemistry.

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The Pathways of Nitrogen Fixation

JOHN R. BENEMANN

Department of Chemistry, University of California, San Diego, La Jolla, California, 92037, U.S.A.

and

R. C. VALENTINE

Department of Biochemistry, University of California, Berkeley, California, 94720, U.S.A.

I. Introduction 59
A. Nitrogen-Fixing Micro-organisms 60
B. Biochemistry of Nitrogen Fixation 64
II. Electron Donors 70
A. Pyruvate 70
B. Formate 72
C. Nicotinamide Nucleotides 73
D. Hydrogen 76
E. Photosynthetic Electron Donors 76
III. Electron Carriers 78
A. Ferredoxins 80
B. Flavodoxins 83
C. Coupling Factors of *Azotobacter* 85
IV. Nitrogenase 88
A. Isolation and Properties 88
B. Mechanism of Action 91
V. Conclusions and Future Outlook 94
VI. Acknowledgements 97
References 98

I. Introduction

Biological nitrogen fixation is the enzymic reduction of atmospheric nitrogen to ammonia. Besides the obvious scientific interest of nitrogen fixation as a fundamental biochemical reaction, it is of great ecological and agricultural importance, since it is the most important source of the metabolizable nitrogen needed by all living organisms. Nitrogen fixation is catalysed by nitrogenase, which requires energy in the form of ATP

This article is dedicated to H. A. Barker, and the Van Niel school of microbiology.

and a biologically strong reductant for the formation of ammonia. The flow of electrons (the electron-transport chain) from an electron donating-substrate to molecular nitrogen is referred to here as the pathway of nitrogen fixation. Nitrogen fixation differs from the more familiar pathways of carbon and nitrogen metabolism in that it is primarily an electron-transport chain coupled to ammonia synthesis. How the various physiological groups of nitrogen-fixing micro-organisms generate the strongly reducing electrons required for reduction of molecular nitrogen and the nature of the electron carriers and enzymes which handle these electrons is the main subject of this review. We will also give a general review of the field of nitrogen fixation with emphasis on recent developments. For previous reviews on the biochemistry of nitrogen fixation see Hardy and Knight (1968), Hardy and Burns (1968), Burris (1969) and Benemann and Valentine (1970).

A. *Nitrogen-Fixing Micro-organisms*

Nitrogen fixation is a property found only among the prokaryotic micro-organisms, namely bacteria, and blue-green algae. No higher or eukaryotic organisms have been shown to fix nitrogen (Millbank, 1969). There are many, taxonomically quite different, types of free-living and symbiotic bacteria and blue-green algae able to fix nitrogen. Almost every physiological group of prokaryotic micro-organisms has at least some representatives in the still expanding list of nitrogen fixers.

The first scientific proof of nitrogen fixation came in the 19th century when it was shown that peas, soybeans, and other leguminous plants were capable of growing in the absence of any nitrogen source besides air when their roots bore characteristic nodules. The root nodules appeared to contain bacteria, and were produced by inoculating the plants with soil. A bacterium able to nodulate leguminous plants was isolated in pure culture by Beijerinck (1888) and later named *Rhizobium japonicum*. Many species of *Rhizobia* have since been isolated and, in all cases, nitrogen fixation is a strictly symbiotic process; the bacterium is unable to fix nitrogen without the plant. The process of nodulation involves profound physiological changes in the bacteria (which become "bacteroids") and root tissue (such as the leghaemoglobins produced which colour the nodules pink), but little is understood about the biochemical basis of this symbiosis. For reviews, see Stewart (1966, 1967), Bergersen (1969, 1971) and Dilworth and Parker (1969).

Many different types of plants besides the legumes are capable of forming nitrogen-fixing root nodules (for reviews, see Stewart, 1966, 1967; Becking, 1970a; Silver, 1969; Bond, 1967). Often these plants are found to grow in adverse environments and to pioneer the establishment of a more varied vegetation. The endophytes responsible for nitrogen

fixation in non-leguminous plants have not yet been isolated, apparently because they are difficult to grow in the absence of the plant. The nodulating bacteria appear to be actinomycetes and/or streptomycetes, and have recently been classified in one genus named *Frankia* (Becking, 1970b).

Free-living nitrogen-fixing bacteria have been known since Winogradski (1893) isolated the strict anaerobe *Clostridium pasteurianum* and Beijerinck (1901) showed that several *Azotobacter* species fix nitrogen aerobically. *Clostridium pasteurianum* and *A. vinelandii* have been the subjects of much of the research, both physiological and biochemical, in nitrogen fixation because they are easily grown under laboratory conditions, and also because for almost 50 years they were the only known genera of free-living bacteria able to fix nitrogen. Nitrogen fixation, under anaerobic conditions, by species of the facultative anaerobes *Klebsiella* and *Bacillus* was not confirmed until the 1950s, although earlier reports existed (see Stewart, 1966, for references).

These free-living bacteria are heterotrophs which must obtain carbohydrates (or other carbon and energy sources) directly or indirectly from plants. Since carbohydrates are usually in short supply in the environments (soil, water) where these bacteria are normally found, their contribution to the total nitrogen fixation in the biosphere has been considered minor. Indeed, the use for several decades of *Azotobacter* in Russian agriculture has had at best a marginal effect on crop yields (Mishustin, 1970). However, many of these bacteria are often found in close association with plants (see Stewart, 1969, for a review). *Azotobacter* (and related *Beijerinckia*) *spp.* are found on root and leaf surfaces of tropical plants. Clostridia can fix large amounts of nitrogen in water-logged soils supplemented with straw. *Klebsiella* species are the endophytes of nitrogen-fixing leaf nodules of many tropical plants (see Silver, 1969) and Douglas Fir (Jones, 1970) and have even been found in the guts of animals and man where they fix nitrogen (Bergersen and Hipsely, 1970). Thus the heterotrophic nitrogen fixers should not be discounted when estimating nitrogen fixation in the biosphere.

Some nitrogen-fixing bacteria do not use carbohydrates for growth. For example, some methane-oxidizing bacteria (Davis *et al.*, 1964; Whittenbury *et al.*, 1970) are capable of fixing nitrogen aerobically, and *Desulfovibrio vulgaris* can grow, and fix nitrogen, by sulphate reduction with organic acids (Sisler and Zobell, 1951; Riederer-Henderson and Wilson, 1970). More widespread and numerous are the photosynthetic bacteria able to grow on light and carbon dioxide in the presence of a reductant such as molecular hydrogen, hydrogen sulphide or some organic compounds. Kamen and Gest (1949) first showed that *Rhodospirillum rubrum* fixed nitrogen, and most photosynthetic bacteria

tested have this ability (Lindstrom *et al.*, 1950). The ecological importance of nitrogen fixation by these types of bacteria remains to be established.

The blue-green algae are the most widespread and versatile group of nitrogen fixers. They are strict photo-autotrophs able to grow and fix nitrogen under many different, often extreme, environmental conditions, including the Arctic, oceans, lakes, hot springs, and especially in rice paddies, where they are of great agricultural importance (see Stewart, 1970, for a review). Filamentous nitrogen-fixing blue-green algae contain heterocysts, peculiarly large thick-walled, empty looking cells occurring at intervals in the filaments of the algae. The correlation between heterocyst occurrence and development and nitrogen fixation led to the theory that heterocysts are the site of nitrogen fixation in these blue-green algae (see p. 67 for a discussion). Like nitrogen-fixing bacteria, the blue-green algae are often found in symbiotic associations such as with fungi to form lichens and with many plants (Holm-Hansen, 1968; Silvester and Smith, 1969).

The list of nitrogen-fixing micro-organisms is not yet complete, since new names are being added (Wyatt and Silvery, 1969; Federov and Kaliniskaya, 1961) and old ones removed (Hill and Postgate, 1969; Parejko and Wilson, 1968; Millbank, 1969). Thus, besides *Azotobacter*, the genera of free-living aerobic nitrogen-fixing heterotrophs now includes *Derxia*, *Beijerinckia*, *Mycobacterium* and *Azomonas*. However, there are still only two species of the large genus *Bacillus* known to fix nitrogen (*B. polymyxa* and *B. macerans*). Better assays for nitrogen fixation are encouraging the isolation and study in both laboratory and field of an increasing number of nitrogen-fixing micro-organisms. Some representative nitrogen fixers, selected because they have been used in

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FIG. 1. Some nitrogen-fixing micro-organisms and symbiotic associations.

A. Cells of *Clostridium pasteurianum* (Winogradsky, 1893).

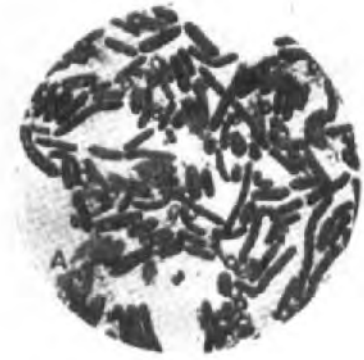
B. A thin section through *Azotobacter vinelandii* showing internal membrane of typical molecular nitrogen-grown cells. Visible are the cell wall (CW), projections of the cell wall (CWP), the cytoplasmic membrane (CM) and the invaginating internal membrane (IM). From Oppenheim and Marcus (1970).

C. A micrograph showing *Fischerella*, a heterocystous blue-green algae. Magnification approximately ×800. From Stewart (1966).

D. Nodulated leaf of *Psychotria bacteriophila* containing *Klebsiella*. From Silver (1969).

E. Root nodules of soybean containing *Rhizobium*. The photograph was provided by courtesy of J. Burton, Nitrigin Company, Evanston, Illinois, U.S.A.

F. New Guinea aborigines reported to have nitrogen-fixing *Klebsiella* in their guts (Bergerson and Hipsley, 1970). The photograph was provided by courtesy of V. Sarich.



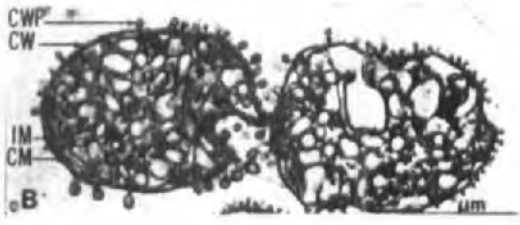


FIG. 1.

biochemical investigations, are listed in Table I. Figure 1 illustrates some free-living nitrogen fixers and symbiotic associations.

TABLE 1. Examples of Nitrogen-Fixing Organisms

Physiological group	Representative genus	Most studied species	Habitat	References reporting cell-free nitrogen fixation
Strict anaerobes	*Clostridium*	*pasteurianum*	Soil	Carnahan *et al.* (1960a, b)
Strict aerobes	*Azotobacter*	*vinelandii*	Soil	Bulen *et al.* (1964)
Symbiotic fixation	*Rhizobium*	*japonicum*	Root nodules of leguminous plants	Koch *et al.* (1967)
Blue-green algae	*Anabaena*	*cylindrica*	Water	Smith and Evans (1970)
Facultative anaerobes	*Klebsiella*	*pneumoniae*	Soil, water, and leaf nodules	Mahl and Wilson (1968)
Photosynthetic bacteria	*Rhodospirillum*	*rubrum*	Water	Bulen *et al.* (1965)

B. *Biochemistry of Nitrogen Fixation*

Before reproducible cell-free bacterial extracts capable of fixing nitrogen were prepared in 1960 (Carnahan *et al.*, 1960a, b), studies using whole cells and nodulated plants had established the following facts (Wilson, 1969): (1) Molybdenum and iron are specifically required for nitrogen fixation; vanadium is able to replace molybdenum in some cases (Becking, 1962); (2) ammonia appears to be the first product of nitrogen fixation; (3) a relationship exists between nitrogen fixation and hydrogen metabolism; and (4) a large number of compounds (such as oxygen, ammonia, hydrogen, cyanide, nitrous oxide) specifically inhibit nitrogen fixation (Bradbeer and Wilson, 1963).

In 1960, Carnahan and his coworkers achieved reproducible nitrogen fixation in cell-free extracts of *Clostridium pasteurianum* by supplementing the extracts with high concentrations of pyruvate. Pyruvate was shown to have a dual function. It was an electron donor providing a source of reductant (electrons) and also a source of ATP (Hardy and D'Eustachio, 1964; Mortenson, 1964). The electrons derived from pyru-

vate were not used directly in the nitrogen reduction reaction but were passed through an intermediate "electron carrier" which later was isolated and named ferredoxin (Mortenson *et al.*, 1962). Other electron carriers such as flavodoxin (Knight *et al.*, 1966) and the artificial dye methyl viologen (D'Eustachio and Hardy, 1964) were also shown to function in this reaction. The ATP generated from pyruvate through acetyl phosphate proved to be an essential (and previously unsuspected) substrate in the nitrogen fixation reaction. The requirements for ATP and a suitable strong reductant for nitrogen fixation in cell-free extracts has since been found in all extracts tested (see Hardy and Knight, 1968, for a review). Since ATP in high concentrations (above 10 m*M*) inhibits nitrogen fixation (Carnahan, 1960b), it is usually generated in the assay vessel from acetyl phosphate or creatine phosphate (Hardy and D'Eustachio, 1964; Mortenson, 1964).

Nitrogen fixation in cell-free extracts does not depend on the presence of pyruvate; indeed pyruvate did not support nitrogen fixation in extracts of aerobic, symbiotic or some photosynthetic organisms, due to their lack of enzymes that catalyse a pyruvate phosphoroclastic reaction (see Section II.A. p. 70). In the presence of ATP any substrate, natural or artificial, which can supply the reducing electrons required is able to drive nitrogen fixation. Thus, substrates able to reduce ferredoxin (or flavodoxin or methyl viologen) such as hydrogen (D'Eustachio and Hardy, 1964), nicotinamide nucleotides (D'Eustachio and Hardy, 1964; Benemann *et al.*, 1971), and even illuminated chloroplasts (Yoch and Arnon, 1970), can serve as electron donors in nitrogen fixation. Bulen *et al.* (1965) found that even the electron carriers could be dispensed with if sodium dithionite (an inorganic reducing agent) was used as a direct reductant of nitrogenase. The use of dithionite has now become the method of choice in the study of cell-free nitrogen fixation.

The enzyme system present in cell-free extracts which can catalyse the reduction of nitrogen to ammonia in the presence of the reductant (which is oxidized) and ATP (which is hydrolyzed to ADP) is called nitrogenase. Nitrogenase binds molecular nitrogen and converts it into ammonia in a series of redox reactions requiring six electrons and about 15 moles of ATP. Nitrogenase, which will be described in detail later, has very similar properties in all organisms where it has been studied. It is the only protein which is known to be unique to the nitrogen fixation pathway; the others (such as the electron carriers) have other functions outside the pathway and, unlike nitrogenase, their biosynthesis is usually not repressed by the presence of ammonia or other nitrogenous compounds. A large number of reactions, besides nitrogen reduction, are catalysed by nitrogenase. The enzyme hydrolyses ATP to ADP and inorganic phosphate (Hardy and Knight, 1966; Kennedy *et al.*, 1968) in

a reaction which is dependent on the presence and oxidation of reductant. However, some ATP hydrolysis can be independent of reductant, especially at low pH values (Jeng *et al.*, 1970). Reductant is oxidized by nitrogenase only during ATP hydrolysis, and ATP-independent reductant oxidation is not known. Nitrogenase reduces, to varying extents, acetylene, cyanide, nitrous oxide, azide, and many other small (stearically unhindered) triple bonded molecules (see Hardy and Knight, 1968, for a review). In the absence of a reducible substrate, nitrogenase continues to hydrolyse ATP and oxidize reductant (at undiminished rates), and releases electrons in the form of hydrogen (Burns and Bulen, 1965). This ATP-dependent hydrogen evolution is found also when nitrogen fixation is inhibited by carbon monoxide, as a side reaction during substrate reduction, and in whole cells or nodules incubated under an inert atmosphere. Finally, nitrogenase catalyzes a $D_2 - H_2$ exchange reaction which occurs only during nitrogen reduction (not during hydrogen evolution) (Hoch *et al.*, 1960; Jackson *et al.*, 1968; Kelly, 1968; Turner and Bergerson, 1969).

Assays for nitrogenase take advantage of the reactions which it catalyses, and the most important ones are: (1) ammonia formation (Mortenson, 1961); (2) $^{15}N_2$ incorporation (Burris and Wilson, 1957); (3) acetylene reduction (Dilworth, 1966; Hardy *et al.*, 1968; Schöllhorn and Burris, 1967); and (4) ATP-dependent hydrogen evolution (Burns, 1965). The $^{15}N_2$ incorporation assay introduced by Burris and Wilson almost 30 years ago is the most rigorous method, but also the most difficult and laborious. The acetylene assay, which takes advantage of the fact that nitrogenase reduces acetylene to ethylene, involves a simple and very sensitive gas chromatographic determination and has become the method of choice for *in vitro*, *in vivo* and even *in situ* assays for nitrogenase activity.

Nitrogen fixation by broken-cell preparations (cell-free extracts) is a strictly anaerobic process. This is not surprising considering that: (1) the strong reductants (reduced ferredoxin, dithionite) used in this process are easily oxidized in air; (2) nitrogenase itself is irreversibly inhibited by oxygen; (3) oxygen immediately and irreversibly inhibits whole-cell anaerobic nitrogen fixation; (4) competitive inhibition of nitrogen fixation in whole cells of *Azotobacter vinelandii* has been observed (Parker and Scott, 1960), and other aerobic nitrogen fixers also fix nitrogen better at oxygen tensions below atmospheric (Dalton and Postgate, 1969a; Bergersen, 1969; Stewart and Pearson, 1970; Biggins and Postgate, 1969; Drozd and Postgate, 1970a) indicating that nitrogen fixation is also inhibited by oxygen in aerobes. How then do strict aerobic and oxygen evolving (blue-green algae) organisms protect the nitrogen-fixation reaction from oxygen? The available evidence

indicates that, in these organisms, oxygen is excluded from the actual site of nitrogen fixation. Thus Dalton and Postgate (1969a) concluded from studies of continuous cultures of *Azotobacter chroococcum* that nitrogen fixation is protected in *Azotobacter* by a respiratory system which "scavenges" oxygen from the neighbourhood of the nitrogen-fixing site. Their conclusions were supported by Yates (1970a), who found that inhibition of oxygen uptake also inhibited nitrogen fixation, and by Drozd and Postgate (1970b), who showed that the organism is able to vary its respiratory activity (depending on pO_2) so as to prevent oxygen from reaching the nitrogen fixation site. Nitrogenase activities in cell-free extracts of *Azotobacter* and *Mycobacterium flavum* are present in particulate (sedimentable) form and are the only instances of nitrogenases being relatively stable in air (for a matter of hours instead of minutes) (Bulen *et al.*, 1964; Biggins and Postgate, 1969). Purification of *Azotobacter* nitrogenase results in its becoming as sensitive to oxygen as the nitrogenases from other organisms (Bulen and LeComte, 1966; Kelly, 1969a). The nitrogenase of *Azotobacter* is thus present in cell-free extracts as part of a large complex (possibly containing other proteins besides the nitrogenase proteins) which is irreversibly dissociated during purification and which apparently gives the nitrogenase its unusual properties of oxygen (and heat) resistance. However, if the nitrogenase was obtained by osmotic lysis of the cells (instead of using the French pressure cell), activity was lost about twice as fast on exposure to oxygen and it was not sedimentable or associated with membrane fragments (Oppenheim *et al.*, 1970). Therefore, the mechanism of the reversibility of oxygen inhibition of nitrogenase in whole cells of *Azotobacter* is yet to be established.

Other nitrogen fixers living under aerobic conditions (rhizobia, blue-green algae) do not manifest oxygen-resistant nitrogenase activity in cell-free extracts; therefore oxygen scavenging, dependent on a high rate of oxygen consumption, cannot be their method of aerobic nitrogen fixation. They must have evolved other methods of oxygen protection. Leghaemoglobin could have a function in protecting nitrogen fixation in bacteriods, which have been shown to require oxygen for nitrogen fixation (Bergersen, 1969). The cell membranes found in root nodules and the cell wall of heterocysts of blue-green algae (Dum and Wolk, 1970; Kale *et al.*, 1970) might play a role in protecting the nitrogenase in these organisms which also fix nitrogen under aerobic conditions.

The heterocysts of blue-green algae were proposed as the sites of nitrogen fixation (Fay *et al.*, 1968; Stewart *et al.*, 1969; Pringsheim, 1968) because all nitrogen-fixing blue-green algae were thought to be heterocystous and because there was a definite correlation between heterocyst formation and nitrogen fixation (Fogg, 1949), both being

inhibited by ammonia and appearing in nitrogen-limited cultures (Stewart *et al.*, 1968; Neilson *et al.*, 1971). Heterocysts are thought not to evolve oxygen, but to contain a reducing environment (Stewart *et al.*, 1969), an excellent place for the nitrogenase to be located. However, non-heterocytous blue-green algae were discovered to fix nitrogen (Wyatt and Silvery, 1969; Stewart and Lex, 1970; Haystead *et al.*, 1970; Rippka *et al.*, 1971). Also Smith and Evans (1970, 1971) found that, in cell-free extracts of anaerobically grown *Anabaena cylindrica*, the nitrogenase was not associated with heterocysts, which led them to conclude that the heterocysts were not the site of nitrogen fixation. Therefore, a role for heterocysts in nitrogen fixation is not yet established.

The regulation and metabolic control of nitrogen fixation is an interesting and important, but relatively little studied, aspect of this reaction. It has been known for a long time that addition of nitrogen-containing compounds (especially ammonia) to cultures of nitrogen-fixing organisms inhibits nitrogen fixation (Wilson *et al.*, 1943; Kamen and Gest, 1949) indicating that nitrogenase was an inducible enzyme whose synthesis is repressed by ammonia (Strandberg and Wilson, 1968; Munson and Burris, 1969; Daesch and Mortenson, 1968). Indeed, nitrogen-fixing organisms show typical diauxic growth curves (indicating induction of an enzyme) when grown on limiting ammonia (Yoch and Pengra, 1968). This explains the absence of nitrogenase from ammonia-grown cells (Bulen *et al.*, 1964). Nitrogenase does not appear in the cells until ammonia is exhausted (Strandberg and Wilson, 1968; Daesch and Mortenson, 1968). Even the ammonia produced by the nitrogen-fixation process is enough to repress synthesis of nitrogenase, as seen from the increased nitrogenase levels of cells grown in continuous cultures on limiting ammonia in the absence of molecular nitrogen (Munson and Burris, 1969; Daesch and Mortenson, 1968). In addition to biosynthetic control, nitrogenase is also feedback inhibited, as in *Rhodospirillum rubrum* which immediately stops nitrogen fixation or the related evolution of molecular hydrogen upon addition of ammonia (Gest *et al.*, 1950, 1962; Schick, 1971a). Nitrogen fixation by whole cells of *Azotobacter* is only slowly inhibited (over several hours) by addition of ammonia (Hardy *et al.*, 1968; Benemann, 1970) indicating some indirect (and unknown) regulation mechanism. Nitrogen fixation by *Clostridium pasteurianum*, however, is not inhibited by ammonia, but further nitrogenase biosynthesis is stopped (Daesch and Mortenson, 1968). In photosynthetic bacteria (Schick, 1971b) and blue-green algae (Cox and Fay, 1969) nitrogen fixation is dependent also on the availability of carbon skeletons (and thus on photosynthesis); in their absence, ammonia accummulates and inhibits nitrogen fixation. The biochemical

basis for these regulatory mechanisms is not understood, since neither ammonia nor metabolites derived from it are specific inhibitors of nitrogenase in cell-free extracts (Carnahan *et al.*, 1960b, Benemann, 1970). It is therefore possible that the feedback inhibition is in some other protein of the nitrogen-fixation pathway. Regulation of symbiotic nitrogen fixation is even less understood, although it is well known that in the presence of sufficient metabolizable nitrogen, root nodules will either not form or be "ineffective" (non-fixing). Bergersen (1969) recently showed that ammonia does not inhibit nitrogen fixation by bacteroids.

The cell provides nitrogenase with a steady supply of ATP whose source is the cellular ATP pool. Using purified nitrogenase, about 12 to 15 moles of ATP are hydrolysed per mole of molecular nitrogen reduced (Bulen and LeComte, 1966; Winter and Burris, 1968; Kennedy *et al.*, 1966). This is a remarkably high value, and would make nitrogen fixation an extremely inefficient process. The large amount of ATP hydrolysed could be an artifact produced by the alteration of nitrogenase during cell breakage and isolation. Unfortunately carbohydrate balance studies comparing cells grown on molecular nitrogen and ammonia have not resolved this issue, since contradictory results have been obtained. Twenty moles of ATP are reportedly required per mole of molecular nitrogen reduced in *Clostridium pasteurianum* cells (Daesch and Mortenson, 1968) while only four moles per mole of molecular nitrogen in *Azotobacter chroococcum* cells (Dalton and Postgate, 1969b). Therefore the question of the amount of ATP required for nitrogen fixation by the native nitrogenase functioning *in vivo* is still unresolved. The role of ATP in nitrogen fixation is discussed in Section IV (p. 92). An interesting observation is that ADP is an inhibitor of nitrogenase action. This inhibition might well represent a feedback control of nitrogen fixation under conditions of ATP starvation (Moustafa and Mortenson, 1967).

The supply of reductant required for nitrogen fixation comes from relatively few cellular metabolites ("electron donors"), such as pyruvate. They do not donate electrons directly to nitrogenase; rather they are dehydrogenated by specific dehydrogenases or reductases and their electrons are trapped by specialized electron-carrying proteins, the ferredoxins and flavodoxins. These electron carriers are redox proteins which are able to reduce nitrogenase and are the actual reductants of nitrogenase. Thus an electron-transport chain linking the reducing power of the electron donors to nitrogenase is established. Nitrogen-fixing organisms differ in the electron donors and electron carriers used in this chain, but the pathways of nitrogen fixation in all nitrogen-fixing organisms can be summarized as an electron transport chain as shown in Fig. 2. This picture of nitrogen fixation comes from a study of a variety

of nitrogen-fixing organisms, and appears to be representative of the pathways of nitrogen fixation in Nature. The various steps of the pathway will be discussed in the following sections.

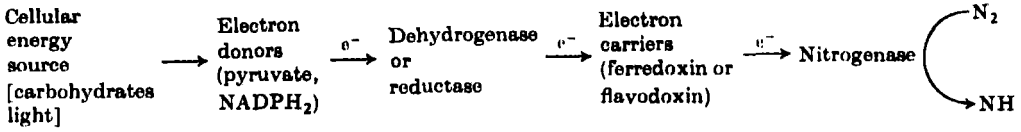


FIG. 2. General scheme of the pathways of nitrogen fixation.

II. Electron Donors

The origin and nature of the electron donors used in nitrogen fixation vary among the different physiological groups of nitrogen-fixing organisms. The strictly anaerobic *Clostridium pasteurianum*, for example, produces the electron donor (pyruvate) from available carbohydrates such as sucrose or glucose by fermentation. Strictly aerobic bacteria must produce electron donors during oxidative catabolism. Photosynthetic nitrogen fixers must be capable of photochemically generating electron donors for nitrogen fixation. In symbiotic organisms, the metabolites needed to generate the electron donors are supplied by the host plant. Whatever the growth physiology of the organism, it has to channel electrons from some electron donor to the nitrogen fixation pathway. In spite of the diverse nature and patterns of synthesis of the electron donors by the different metabolic groups of microbes, the donors themselves show one common property. They are all potentially strong reducing agents able to reduce the low-potential electron carriers (ferredoxins and flavodoxins). We now believe that any substrate capable of reducing these electron carriers is a possible source of electrons for nitrogen fixation.

A. *Pyruvate*

Carnahan and his coworkers (1960a, b) prepared the first active and reproducible cell-free nitrogen-fixing extract (using *Cl. pasteurianum*) by adding pyruvate in high concentrations as a substrate. A large head of carbon dioxide and molecular hydrogen accumulated in the reaction vessel, indicating that an extremely active fermentation of pyruvate was occurring simultaneously with nitrogen fixation. Earlier workers studying pyruvate fermentation in clostridia had described a complex enzyme system which converted pyruvate to acetyl phosphate with the liberation of carbon dioxide and molecular hydrogen (Wolfe and O'Kane, 1953). Because inorganic phosphate was the final acetyl acceptor, this reaction was named the "phosphoroclastic" reaction.

Strong reductants were produced during pyruvate metabolism by extracts of *Cl. pasteurianum*, as was evident from the evolution of molecular hydrogen and the additional observation that some low-potential redox dyes (such as methyl viologen) were reduced by this process (Mortlock *et al.*, 1959). That the phosphoroclastic reaction was the driving force in nitrogen fixation by the pyruvate-supplemented clostridial extracts became obvious when the same cofactor requirements (TPP, coenzyme A, ADP, Mg^{2+}, Pi and ferredoxin) were found for both reactions (Munson *et al.*, 1965). The reaction sequence is complex involving a number of steps catalysed by several enzymes (Fig. 3). First

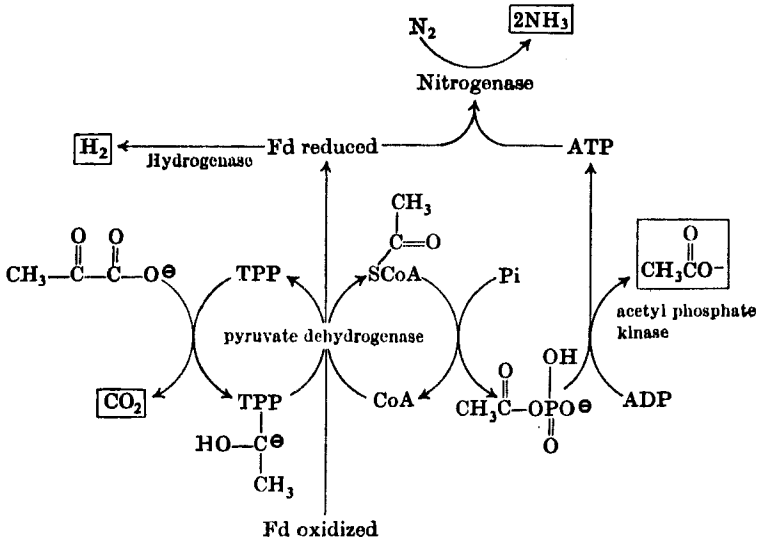


FIG. 3. The phosphoroclastic reaction in nitrogen fixation in clostridia. Fd indicates ferredoxin.

pyruvate is decarboxylated by the TPP-containing pyruvate dehydrogenase. The acetylated dehydrogenase then passes the acetyl moiety to coenzyme-A while the electrons are used to reduce ferredoxin. The acetyl group is next transferred to inorganic phosphate forming acetyl phosphate and regenerating coenzyme-A. Finally acetyl kinase generates ATP from ADP and acetyl phosphate. The decarboxylation step is the key step in the phosphoroclastic reaction, since the release of the COO^- group as carbon dioxide gas provides the driving force for ATP formation and the reduction of ferredoxin. The phosphoroclastic reaction is not specific for pyruvate; α-ketobutyrate is also a substrate and was found to drive nitrogen fixation, although at half the rate of pyruvate (Carnahan *et al.*, 1960b).

Both requirements of nitrogenase (reduced ferredoxin and ATP) are met by a single substrate (pyruvate or α-ketobutyrate) through the phosphoroclastic reaction. It should be noted that the ratio of ATP to reduced ferredoxin formed in this reaction is unity, while that needed for nitrogen fixation is about five. The excess reduced ferredoxin is used to make molecular hydrogen, thus accounting for the formation of this gas observed during nitrogen fixation, and for the high levels of pyruvate needed. Indeed, addition of acetyl phosphate (or other suitable ATP generators) decreased the pyruvate requirement in nitrogen fixation. Pyruvate will also support nitrogen fixation by whole cells and extracts of many organisms besides *Cl. pasteurianum* (see Hardy and Knight, 1968; Fischer and Wilson, 1970).

The historical position of pyruvate as the first known electron donor of nitrogen fixation and its high activity may have impeded the search for alternative electron donors in nitrogen fixation. Thus although molecular hydrogen and nicotinamide nucleotides were shown to be effective (but less so than pyruvate) electron donors in nitrogen fixation by extracts of *Cl. pasteurianum* (D'Eustachio and Hardy, 1964), they were thought to have no real physiological significance. But, under certain conditions of cell growth, these substrates might well take a leading role in the pathway of nitrogen fixation, as is discussed below.

B. *Formate*

Formate is a common fermentation product, derived from pyruvate, among the facultative and strictly anaerobic bacteria. It can be a strong reducing agent, and was first shown to drive nitrogen fixation (because of the presence of a formate: ferredoxin oxidoreductase) in cell-free extracts of *Cl. pasteurianum* (Mortenson, 1966).

In our laboratory we showed that formate could support nitrogen fixation in two organisms, namely *Klebsiella pneumoniae* and *Bacillus polymyxa* (D. C. Yoch and R. C. Valentine, unpublished data), both of which are known to metabolize formate. Activity was found to be highest (approaching that of pyruvate) in freshly prepared extracts, and rapidly deteriorated with ageing of the extracts. This might be an explanation for the low activities observed by Fischer and Wilson (1970) for this reaction. Formate dehydrogenase and pyruvate dehydrogenase activities were clearly separate enzymes in these organisms. For example, an extract of *Klebsiella* frozen under argon for 48 hours lost its formate reaction while the pyruvate-driven nitrogen fixation was affected but little. The activities were approximately equal in fresh extracts. In extracts of *Bacillus*, formate dehydrogenase was sedimentable (145,000 *g* for 2 hours) and was easily separated from the pyruvate

system which was soluble. The mechanisms of formate-driven nitrogen fixation in these organisms are not known.

C. *Nicotinamide Nucleotides*

Until recently, no electron donors were known which were involved in nitrogen fixation by aerobic or symbiotic organisms. The lack of a clastic-type reaction in such species suggested that donors other than pyruvate were supplied by these cells. With the recent isolation in our laboratory and in other laboratories, of electron carriers from *Azotobacter vinelandii* and soybean root nodule-extracts (*Rhizobia*) able to reduce nitrogenase (see Section III.A. p. 78), the way opened for the investigation of the pathways of nitrogen fixation in these organisms. Our experiments indicated that $NADPH_2$ was the electron donor in nitrogen fixation by *A. vinelandii* (Benemann *et al.*, 1971c) and that it plays a major role in other nitrogen-fixing organisms (D. C. Yoch and R. C. Valentine, unpublished observations). Strictly defined, $NADPH_2$ is an electron carrier, since it transfers electrons from a donor to an acceptor, but for the purpose of this review we will consider it an electron donor in the category of formate or pyruvate.

There is a major objection in considering the nicotinamide nucleotides as potential electron donors in nitrogen fixation. The redox potential of the nicotinamide nucleotides is −0·32 V, while that of ferredoxin (at least the clostridial type) is −0·42 V (Tagawa and Arnon, 1962; Sobel and Lovenberg, 1966). Therefore, transfer of electrons between nicotinamide nucleotides and ferredoxin should be in favour of nicotinamide nucleotide reduction, not ferredoxin reduction. Since reduced ferredoxin is the actual reductant of nitrogenase ($NADH_2$ or $NADPH_2$ being unable to reduce nitrogenase by themselves), this seemed to present a thermodynamic barrier to the involvement of nicotinamide nucleotides in nitrogen fixation, unless one postulated some kind of energy (ATP)-driven reaction similar to the reverse electron flow in photosynthetic bacteria (see Gest, 1972). However, the redox potentials in question are only halfway potentials; therefore if the ratio of oxidized:reduced species is increased, the effective potential is lowered. Thus a ratio of 100:1 $NADPH_2$:NADP would give an effective potential of −0·35 V. Indeed, ratios of over 100:1 (NADPH:NADP) are not uncommon in living cells (Veech *et al.*, 1969). Secondly, the actual potential of the reductant of nitrogenase is not yet known; it could well be higher than the potential of clostridial ferredoxin. Indeed, flavodoxin with a redox potential above −0·40 V (Mayhew *et al.*, 1969a) is an effective reductant of nitrogenase albeit less so than ferredoxin (Knight and Hardy, 1966). There are several lines of experimental evidence to indicate that

nicotinamide nucleotides are able to reduce ferredoxin both *in vitro* and *in vivo*. In the ethanol-fermenting bacterium, *Clostridium kluyveri*, ATP can only be produced if excess electrons from nicotinamide nucleotides are removed by ferredoxin and disposed of as molecular hydrogen by the ferredoxin-linked hydrogenase enzyme. *In vitro*, this reaction was readily reversible (although subject to regulatory control) depending on the partial pressure of molecular hydrogen and required no additional ATP source (Thauer *et al.*, 1969, 1971; Jungermann *et al.*, 1969, 1971). Even in sucrose-fermenting clostridia, some $NADH_2$ appears to reduce ferredoxin and be liberated as molecular hydrogen (Mortenson, 1968). Therefore, as long as there is an active reduced nicotinamide nucleotide-generating system which keeps the reduced:oxidized ratio high, $NADPH_2$ (or $NADH_2$) could be an effective electron donor in nitrogen fixation. This was clearly demonstrated by a simple experiment in which $NADPH_2$, generated by glucose 6-phosphate dehydrogenase and linked to clostridial ferredoxin by the spinach chloroplast NADP-ferredoxin reductase, reduced nitrogenase from *A. vinelandii* with an efficiency of about 40% of the maximal dithionite reduction rate (Benemann *et al.*, 1971c). Although the system was non-physiological in that purified enzymes from many different sources were used, the results clearly showed that there was no thermodynamic barrier to $NADPH_2$ being an electron donor in nitrogen fixation.

The role of $NADPH_2$ in nitrogen fixation by *A. vinelandii* was discovered only after its electron carriers, azotoflavin and azotobacter ferredoxin, were isolated (see Section III.A. p. 78). A cell-free extract, containing both electron carriers in sufficient concentrations, exhibited nitrogenase activity when supplied with various substrates. Dialysis showed that NADP (but not NAD) was a required cofactor and all active substrates were known to be NADP linked. Addition of $NADPH_2$, either in substrate quantities or as part of a generating system (from glucose 6-phosphate) resulted in comparatively high activities. Reduced nicotinamide adenine dinucleotide (or $NADH_2$-linked metabolites) was inactive. The substances most active in generating $NADPH_2$ in cell-free extracts of *A. vinelandii* were isocitrate (isocitrate dehydrogenase makes up about 1% of the soluble cell protein; Chung and Franzen, 1969), malate, and glucose 6-phosphate (Benemann *et al.*, 1971c). Probably no single metabolite has an exclusive role in generating the $NADPH_2$ used for nitrogen fixation by *Azotobacter*.

We have recently extended the investigation of $NADPH_2$ as electron donor in nitrogen fixation to other organisms (Table 2), and found that it is an effective electron donor in extracts of all organisms studied, except for the soybean root nodules where the lability of the system made such experiments difficult. The finding of $NADPH_2$-driven

nitrogen fixation in extracts of so many different organisms makes it a most important electron donor of nitrogen fixation.

Prior to this work, $NADH_2$ had been implicated in nitrogen fixation by several laboratories. D'Eustachio and Hardy (1964) found that $NADH_2$ would drive molecular nitrogen fixation in extracts of *Cl. pasteurianum* with about one-third the activity of pyruvate. Klucas and Evans (1968) reported low rates of acetylene reduction in soybean root nodule and extracts of *A. vinelandii* supplemented with $NADH_2$ as electron donor and the dyes methyl- or benzyl viologen as electron carriers. The use of

TABLE 2. Nicotinamide Nucleotides as Electron Donors for Nitrogen Fixation

Organism	Electron Donor	Nitrogenase activity (mμmoles ammonia or ethylene evolved/min./mg. protein)	Reference
Clostridium pasteurianum	$NADPH_2$	1.0	D'Eustachio and Hardy (1964)
	$NADH_2$	2·0	
Klebsiella pneumoniae	$NADPH_2$	0·91	H. Nagatani and R. C. Valentine, (unpublished observations)
Bacillus polymyxa	$NADPH_2$	1·4	D. C. Yoch and R. C. Valentine (unpublished observations)
Azotobacter vinelandii	$NADPH_2$	0·36	
	$NADH_2$	0	Benemann *et al.* (1971)
Rhizobium japonicum	$NADH_2$	0·18	Klucas and Evans (1968)
Anabaena cylindrica	$NADPH_2$	0·21	H. Bothe and R. C. Valentine (unpublished observations)

artificial electron carriers (dyes), which are known to interact with many enzymes non-specifically, raises the possibility that these reactions were not physiological. Indeed, the recently isolated bacteroid ferredoxin could not substitute for the dyes in these reactions (D. C. Yoch and R. C. Valentine, unpublished results; P. Wong, H. J. Evans, R. Klucas and S. Russel, unpublished observations), and recent reports from Evans' laboratory suggest that the bacteroid nitrogen-fixation pathway is very similar to the one found in *A. vinelandii*. Yates and Daniel (1970) reported that $NADH_2$ was the electron donor in nitrogen fixation by *A. vinelandii*; however, the activities they observed could be accounted for by the presence of NADP in the crude extracts used (M. G. Yates, personal communication).

D. *Hydrogen*

Molecular hydrogen is a common fermentation product of many anaerobic bacteria and could be an electron donor in nitrogen fixation as a result of activity of the reversible ferredoxin-linked hydrogenase reaction. Indeed, molecular hydrogen was an effective electron donor in cell-free extracts of clostridia (Mortenson, 1964) and was even used (together with a crude nitrogenase-free clostridial extract) in the early studies with *Azotobacter* extracts (Bulen *et al.*, 1964).

Several aspects of molecular hydrogen as an electron donor must be considered: (1) molecular hydrogen is an inhibitor of nitrogen fixation; and (2) the gas is a by-product of nitrogen fixation itself; even in the presence of molecular nitrogen a significant amount of ATP-dependent evolution of molecular hydrogen often occurs as a side reaction. But these findings pose no serious obstacle to molecular hydrogen being considered as an electron donor in nitrogen fixation since nitrogenase inhibition is significant only at high partial pressures of molecular hydrogen, while uptake and re-use of this gas would be an efficient method of recycling a waste product of the nitrogenase reaction. This could also account for the failure to observe molecular hydrogen evolution during *in vivo* nitrogen fixation by many organisms.

The *Azotobacter* hydrogenase has the unusual property of only taking up hydrogen, being unable to evolve it (Hyndman *et al.*, 1953; Peck *et al.*, 1956). It is associated with the cell membrane (Cota-Robles *et al.*, 1958) and is preferentially synthesized during growth on molecular nitrogen, induction of nitrogen fixation causing a stimulation in hydrogenase synthesis (Green and Wilson, 1953; Lee and Wilson, 1943). This clearly indicates a relationship between hydrogenase and nitrogenase in this aerobic nitrogen fixer, but the nature of this relationship remains to be elucidated. Photosynthetic bacteria can grow and fix nitrogen with molecular hydrogen as the electron donor; the hydrogenase appears to be similar to the Azotobacter enzyme.

E. *Photosynthetic Electron Donors*

The nature and origin of the electron donors in nitrogen fixation by the blue-green algae and the photosynthetic bacteria are not yet established. The dependence of nitrogen fixation on photosynthesis observed in long-term studies of whole cells led to suggestions that photosynthesis was directly linked to nitrogen fixation in blue-green algae (Fay and Fogg, 1962; Fogg and Than-Tun, 1958) and photosynthetic bacteria (Arnon *et al.*, 1961; Pratt and Frenkel, 1959). A direct way of generating reductant for nitrogenase in these organisms might be by a photochemical reduction of ferredoxin as occurs in chloroplasts (Arnon,

1967). However, such a reaction has not been shown in photosynthetic bacteria, and more recent studies suggest that photosynthesis is not directly coupled to nitrogen fixation since nitrogenase activity of whole cells continues in the dark (Fogg, 1961; Schick, 1971a; Cox, 1966). The available evidence indicates that, in the photosynthetic nitrogen-fixers, the same electron donors drive nitrogen fixation as in other organisms.

In photosynthetic bacteria, attempts to link *in vitro* the photosynthetic apparatus, located in the chromatophores, and nitrogen fixation have had only limited success. Yoch and Arnon (1970) using *Chromatium* extracts were able to provide the ATP requirement for nitrogen fixation through photophosphorylation by illuminated chromatophores; however, the reductant had to be provided through others means. One such method was the use of illuminated chloroplast fragments (heated to avoid evolution of molecular oxygen) which, in the presence of ferredoxin or other suitable electron carriers, are able to provide the reductant requirement of nitrogenase (Yoch and Arnon, 1970; Benemann *et al.*, 1969), indicating that a ferredoxin-linked photoreduction of molecular nitrogen is at least theoretically possible. The only known case of photosynthetic ferredoxin reduction in photosynthetic bacteria was reported (at low rates) in carbon dioxide fixation by cell-free extracts of *Chlorobium thiosulfatophilum* (Evans and Buchanan, 1965; Buchanan and Evans, 1965). Recently Evans and Smith (1971) have been able to couple the photosynthetic apparatus of another green sulphur bacterium, *Chloropseudomonas ethylicum*, to nitrogenase in a ferredoxin-mediated reaction. However, in the purple bacteria, the mechanism of ferredoxin reduction is not known, but pyruvate can drive nitrogen fixation in cell-free extracts of *Chromatium* (Bennett *et al.*, 1964). Thus the question of the relation between photosynthesis and the reductant requirement for nitrogen fixation in photosynthetic bacteria is not yet settled.

Evidence against a role for direct photoreduction of ferredoxin is strongest for the blue-green algae. Nitrogen fixation in *Anabaena cylindrica* takes place in the dark (after light has been turned off) (Cox, 1966), and some blue-green algae can slowly grow and fix nitrogen heterotrophically on sucrose in the dark (Fay, 1965; Watanabe and Yamamoto, 1967). Also, the absence of photosystem II in heterocysts (deduced from the absence of oxygen evolution, carbon dioxide fixation and phycocyanin) (Fay, 1969; Wolk and Simon, 1969; Fay and Walsby, 1966; Wolk, 1968) would rule out generation in these cells of a photosynthetic reductant. The action spectra of nitrogen fixation by *Anabaena cylindrica* (Fay, 1970) indicates that the primary involvement of light in this pathway is due to cyclic photophosphorylation (photosystem I) and that the direct contribution of photosystem II is, at best, minor. Recently

it was found that $NADPH_2$, generated from glucose 6-phosphate by an endogenous dehydrogenase, functions as an effective electron donor in nitrogen fixation by *A. cylindrica* (Bothe, 1970). Earlier work had indicated that decarboxylation of pyruvate would support nitrogen fixation in whole cells or extracts of this organism (Cox and Fay, 1969; Cox, 1966).

In conclusion, then, it appears that, in the photosynthetic nitrogen fixers, the photosynthetic system is not directly involved in the generation of reductant for nitrogenase with the possible exception of green sulphur bacteria. However, photosynthesis, through photophosphorylation, does provide the ATP requirement of nitrogenase and, through carbon dioxide fixation, the carbon skeletons needed for ammonia incorporation. In the absence of suitable ammonia acceptors, nitrogen fixation is quickly inhibited due to buildup of ammonia (Cox and Fay, 1969; Schick, 1971b), thus accounting for some of the dependence of nitrogen fixation on photosynthesis. We believe that the electron donors which drive nitrogen fixation in these organisms appear to be the same as those found in other nitrogen-fixing bacteria. However, further studies are required to prove this contention, and definitely establish the relationship between photosynthesis and nitrogen fixation, two of the most important biological processes.

III. Electron Carriers

The natural reductants of nitrogenase are electron carriers of the ferredoxin and flavodoxin type. These electron carriers have been found in bacteria, blue-green algae, and plants, and have many functions in cellular metabolism in addition to nitrogen fixation. The ferredoxins and flavodoxins of nitrogen-fixing organisms can be easily purified as coloured acidic proteins which bind to DEAE-cellulose. Their common characteristics are a low-redox potential (below nicotinamide nucleotides) and the reversibility of their reduction and oxidation. They have no catalytic function of their own; they act only in transferring electrons from one enzyme to another. Among bacteria these electron carriers had, until recently, been isolated only from strictly anaerobic and photosynthetic bacteria (Valentine, 1964). During the last year, work in our laboratory (and in others) led to the discovery of ferredoxin- and flavodoxin-type electron carriers in facultative anaerobic, aerobic, and symbiotic nitrogen-fixing bacteria. Although the study and characterization of these new electron carriers is not yet finished, they extend the concept of low-potential electron carriers to all nitrogen-fixing organisms and bacterial types. A summary of the properties of the ferredoxins and flavodoxins from nitrogen-fixing organisms is presented in Table 3.

TABLE. 3. Properties of Electron Carriers used for Nitrogen Fixation

	Ferredoxin					Flavodoxin				
Organism	Molecular weight	Iron and (sulphide)	Visible Spectra Maximum (nm.)	NADP Reduction by chloroplasts	References	Name	Molecular weight	Coenzyme	Activity (chloroplast-NADP)	References
Clostridium pasteurianum	6,000	8(8)	390	+	Lovenberg *et al.* (1963)	Flavodoxin	14,600	FMN	+	Knight and Hardy (1966)
Azotobacter vinelandii	14,500	8(8)	375–415	+	D. C. Yoch (personal communication)	Azotoflavin	31,000	FMN	–	Benemann *et al.* (1969); Hinkson and Bulen (1967)
Rhizobium japonicum	about 10,000	?	shoulder 300–400	–	Burton *et al.* (1970); D. C. Yoch and R. C. Valentine (unpublished observations)	Rhizoflavin	?	?	–	D. C. Yoch and R. C. Valentine (unpublished observations)
Anabaena cylindrica	10,000	2(1)	330, 420 463	+	Yamanaka *et al.* (1969)	Phytoflavin	–	FMN	+	Smillie (1965)
Bacillus polymyxa	11,000	4–5(4–5)	385	+	Shethna (1970a); D. C. Yoch and R. C. Valentine (unpublished observation)	–	–	–	–	–
Chromatium	5,600	8(8)	385	+	B. B. Buchanan (personal communication)	–	–	–	–	–
Desulfovibrio gigas	6,500	4(4)	400	not cited	Laishley *et al.* (1969)	Flavodoxin	16,000	FMN	not cited	Dubourdieu and LeGall (1970)

A. *Ferredoxins*

Ferredoxins have been isolated from plants, blue-green algae, and many bacteria. They can be best defined on the basis of their prosthetic group and redox properties. They are iron-sulphur (Fe-S) proteins with a redox potential of about −0·40 V. and are able to undergo reversible oxidation and reduction. The non-haem iron and "labile" sulphide are released upon acidification and are present in approximately equivalent amounts. Definitions based on their electron-carrier properties in a simple specific enzymic system such as the phosphoroclastic reaction, the NADP reduction by illuminated chloroplasts, or even their ability to reduce nitrogenase, are limited by the enzymic nature of these reactions since ferredoxin-linked enzymes often do not interact with ferredoxins of different species. However, activity in at least one of the characteristic reactions of ferredoxin, such as reduction of nitrogenase, hydrogenase, or NADP-ferredoxin reductase, is essential to establish a newly isolated iron-sulphur protein as a ferredoxin. Not all iron-sulphur proteins are ferredoxins, and even the iron-containing protein of nitrogenase does not fall into this classification. The isolation, properties and functions of ferredoxins have been extensively reviewed (Valentine, 1964; Buchanan and Arnon, 1970; Hall and Evans, 1969; Malkin and Rabinowitz, 1967). This discussion will emphasize recent developments of interest to nitrogen fixation.

Bacterial ferredoxin was discovered by its ability to link pyruvate oxidation to hydrogen evolution in extracts of *Cl. pasteurianum* (Mortenson *et al.*, 1962). Clostridial ferredoxin was first seen as a dark brown band which adsorbed to a DEAE-cellulose column and could be eluted at high concentrations of salt. The protein thus obtained was a required cofactor in the pyruvate phosphoroclastic reaction. That ferredoxin transported electrons from pyruvate or hydrogen to nitrogenase was shown independently by D'Eustachio and Hardy (1964) and by Mortenson (1964). Ferredoxins participate in a large number of biochemical reactions, nitrogen fixation being only one of them (see Benemann and Valentine, 1971; Buchanan and Arnon, 1970). Clostridial ferredoxin is a small (mol. wt. 6,000) acidic protein containing eight iron and eight sulphide atoms per molecule. It has a characteristic adsorption spectrum with maxima at 280, 300, and 380 nm. All ferredoxins can be reduced by dithionite (with a decrease of absorbance at 380 nm.) and completely re-oxidized in air. Clostridial ferredoxin is typical of the ferredoxins isolated from anaerobic bacteria. If iron and sulphide are removed (Lovenberg *et al.*, 1963), the remaining ferredoxin apoprotein is biologically inactive. Malkin and Rabinowitz (1966) have been able to reconstitute native ferredoxin from the apoprotein by addition of

mercaptoethanol, iron, and sulphide. This is also the way ferredoxin biosynthesis occurs; the iron is incorporated after the apoprotein is completely synthesized and released from the polysomes (Trakatellis and Schwartz, 1968). Reconstitution experiments are now routine (Hong and Rabinowitz, 1967; Devanathan *et al.*, 1969).

The ferredoxins isolated from the various nitrogen-fixing organisms (Table 3) differ in many properties including molecular weight, iron and sulphide contents, redox properties, and biological activity. Some of the recently isolated ferredoxins have no activity in the clostridial phosphoroclastic reaction, a reaction which until recently was thought to be characteristic of the bacterial ferredoxins. However, all ferredoxins isolated from the nitrogen-fixing organisms reacted with their homologous nitrogenases and in most experiments showed reactivity with other nitrogenases. Several of the new ferredoxins were isolated by their activities in the coupled chloroplast-nitrogenase assay developed by Yoch and Arnon (1970) and first used in the isolation of an electron carrier by Benemann *et al.* (1969). In this assay, illuminated washed chloroplast fragments reduce the electron carrier which then serves as a reductant for nitrogenase. Azotobacter ferredoxin (Yoch *et al.*, 1969) and Rhizobium ferredoxin (Yoch *et al.*, 1970) (from soybean root nodules) were first discovered by this method.

Azotobacter ferredoxin was isolated from *A. vinelandii* by the normal procedures of DEAE-cellulose chromatography (Yoch *et al.*, 1969). It reduced both nitrogenase or $NADPH_2$ in the presence of illuminated chloroplasts, but had only low activity in the clostridial phosphoroclastic reaction. It has been crystallized, and contains eight atoms of iron and eight sulphide residues and has a molecular weight of 14,500 (D. C. Yoch, personal communication), higher than any other ferredoxin previously known. It undergoes reversible reduction and re-oxidation; however, re-oxidation in air seemed to occur in two steps. About 50% re-oxidation took less than a minute while complete re-oxidation proceeded only slowly and took about an hour. This sluggish re-oxidation of Azotobacter ferredoxin appears to be an adaptation which enables this aerobic organism to conserve, in the presence of oxygen, the strong reducing power needed for nitrogen fixation. Azotobacter ferredoxin is distinct from other iron-sulphur proteins which have been previously purified from *A. vinelandii* (Shethna *et al.*, 1966, 1968). Shethna (1970a) confirmed the presence of Azotobacter ferredoxin, and Yates (1970b) reported finding a similar protein in *Azotobacter chroococcum*. Whether or not Azotobacter ferredoxin is a new type of ferredoxin (Yoch *et al.*, 1969; Buchanan and Arnon, 1970) remains to be confirmed by further work; however, it represents the first example of such an electron carrier from an aerobic nitrogen-fixing organism.

Rhizobium ferredoxin, isolated from soybean root nodule bacteroids, was more difficult to purify since it was sensitive to oxygen (Yoch *et al.*, 1970). This redox protein is an example of a ferredoxin which was not active in NADP reduction by illuminated chloroplasts, although it worked in the coupled chloroplast-nitrogenase assay (with both Rhizobium and Azotobacter nitrogenases; Yoch *et al.*, 1970). Apparently the spinach chloroplast NADP-ferredoxin reductase cannot react with this electron carrier, accounting for the lack of $NADPH_2$ reduction (Burton *et al.*, 1970). It was also inactive in the phosphoroclastic reaction. It contains non-haem iron and sulphide (Burton *et al.*, 1970), but no data on its physical properties are yet available.

Ferredoxins have also been isolated from several types of photosynthetic nitrogen fixers. Those from blue-green algae are very similar to the plant-type ferredoxin, containing two atoms of iron, one of sulphide and a molecular weight of about 10,000 (Yamanaka *et al.*, 1969; Smillie, 1965). This similarity probably reflects a similarity between the photosynthetic systems of blue-green algae and plants. The ferredoxins of the green photosynthetic bacteria (*Chlorobium* species) resemble those isolated from clostridia in physical properties and amino-acid sequences (Buchanan *et al.*, 1969). The ferredoxin of the purple photosynthetic bacterium *Chromatium* is also similar, containing about eight atoms of iron and eight sulphide groups with a molecular weight of 9,000 (Bachofen and Arnon, 1966). Two distinct membrane-bound ferredoxins, one similar to plant-type ferredoxin, the other (absent during aerobic growth) similar to clostridial ferredoxin, have been found in *Rhodospirillum rubrum* (K. T. Shanmugam, personal communication).

Ferredoxin has also been recently isolated from the facultative anaerobe, *Bacillus polymyxa*, by methods similar to those used for Azotobacter and Rhizobia ferredoxins (Shethna, 1970b; D. C. Yoch and R. C. Valentine, unpublished data). This ferredoxin was distinct from the clostridial type but showed similar biological activity. The electron carrier(s) from *Klebsiella pneumoniae*, which links to nitrogenase in this organism, has two unique properties which have made isolation difficult. Unlike all other ferredoxins and flavodoxins it does not bind tightly to DEAE-cellulose and it is not reduced in crude extracts by illuminated chloroplasts. Further work is needed to elucidate the nature of the electron carriers in this nitrogen fixer.

Clostridial-type ferredoxins can transfer up to two electrons with a reducing potential of about −400 mV (Evans *et al.*, 1968; Mayhew *et al.*, 1969b; Eisenstein and Wang, 1969) while plant-type ferredoxins (containing only two iron-sulphur groups) transfer only one electron (Whatley *et al.*, 1963). The nature of the prosthetic iron-sulphur groups

of ferredoxins, which give these proteins their characteristic properties, has been the subject of many investigations (see review by Malkin and Rabinowitz, 1967). The evidence points to each iron atom being bonded to two cysteine sulphur groups and two labile sulphides in a tetrahedral arrangement. Physical measurements indicate the presence of two distinct types of irons in clostridial ferredoxins, which led Bloomstrom *et al.* (1964) to propose a structure in which the iron atoms were linked in a single linear array by alternating bridges of cysteine sulphur and labile sulphide (the central and terminal irons having different environments). However, alternative arrangements are possible (such as two clusters of iron groups) and X-ray diffraction analysis will have to be undertaken to provide the final answers. The mechanism of electron transfer by ferredoxins (and other iron-sulphur proteins) also remains to be elucidated. A number of artificial non-haem iron proteins with ferredoxin-like absorption spectra have been synthesized by treating proteins with S^{2-} and Fe^{2+} (Suzuki and Kimura, 1967; Lovenberg and McCarthy, 1968), but they lacked biological activity. The protein itself is not even required in models, since mercaptoethanol, iron and sulphide, at pH 9, form a soluble complex whose spectrum was similar to those of the non-haem iron proteins (Yang and Huennekens, 1970). Whether biologically active models can be made remains to be seen.

With the discovery of ferredoxins in aerobic, facultative, and symbiotic organisms, the already extensive work on ferredoxins will undoubtedly expand in the near future. The evolutionary history of some of the new ferredoxins in relation to that established (see Matsubara *et al.*, 1968; Hall *et al.*, 1971; Buchanan and Arnon, 1970) for the anaerobic and photosynthetic ferredoxins will be of special interest. The presence of ferredoxins in all types of nitrogen-fixing organisms makes it clear that they are indeed an important part of the pathway of nitrogen fixation.

B. *Flavodoxins*

Several years after the discovery of ferredoxins, another electron carrier able to function in the phosphoroclastic reaction was isolated from *Cl. pasteurianum* (Knight *et al.*, 1966). This electron carrier was a flavoprotein which was found only in cells grown in media containing a low concentration of iron. It was named flavodoxin since it could replace ferredoxin *in vitro* in a large number of reactions. It apparently also replaces ferredoxin *in vivo*, during conditions of iron starvation, as suggested by the almost complete absence of ferredoxin from cells grown under these conditions. Clostridial flavodoxin (molecular weight, 14,000) contains one flavin mononucleotide (FMN) group but no iron or sulphide (Knight and Hardy, 1966, 1967). The FMN group is bound to

the single cysteine residue in the molecule and complexed with one or two of the four tryptophan residues (McCormick, 1970). Flavodoxin was similar in physical and catalytic properties to a flavoprotein named phytoflavin, which had been previously isolated from blue-green algae (Smillie, 1965) grown on media containing a low concentration of iron (Trebst and Bothe, 1966). Flavodoxins have recently been isolated from other anaerobic bacteria (*Peptostreptococcus elsdenii*; Mayhew and Massey, 1969; and two *Desulfovibrio* species; Dubourdieu and LeGall, 1970) and a closely related protein, named azotoflavin, from *Azotobacter vinelandii* (Benemann *et al.*, 1969). Flavodoxins can be defined (by criteria very similar to those used for ferredoxins) as flavoproteins which function as low redox potential (below that of nicotinamide nucleotides) electron carriers in at least one of the biochemical reactions of ferredoxins. Thus azotoflavin and the known flavodoxins form a new group of electron carriers with similar biological activity to the ferredoxins. We propose the general name "flavodoxin" for this class of electron carrier.

On a molar basis, clostridial flavodoxin is only about 30% as effective as ferredoxin in the pyruvate phosphoroclastic reaction, NADP reduction by hydrogen, or nitrogen fixation reactions (see Hardy and Knight, 1968). This, coupled with a higher molecular weight, makes flavodoxins much less efficient electron carriers than the ferredoxins. The reasons for this could be due to a difference in the specificity of the carrier in these reactions, but more likely to its redox properties. The results of Mayhew *et al.* (1969a) showed that flavodoxin from *Peptostreptococcus elsdenii* accepted two electrons at different oxidation levels. Addition of one reducing equivalent of sodium dithionite (or $NADPH_2$ in the presence of NADP-ferredoxin reductase) generates a new species of flavoprotein, the flavin semiquinone. This reduction is almost instantaneous and can be easily observed since the colour of the flavodoxin changes from yellow to blue. This step in the reduction of flavodoxin has a potential of $-0{\cdot}115$ V at pH 7. Further reduction with two reducing equivalents of dithionite gave the colourless fully reduced semiquinone. This second step in the reduction of flavodoxin is probably the more important in reactions in which this protein substitutes for bacterial ferredoxin because the oxidation-reduction potential for this second step was $-0{\cdot}373$ V, at pH 7, somewhat above that of ferredoxin ($-0{\cdot}42$ V) but below that of the $NADP/NADPH_2$ couple ($-0{\cdot}32$ V). This could account for the lower reactivity of flavodoxin when substituted for ferredoxin as electron carrier. But differences in specificity can, of course, not be excluded.

Azotobacter vinelandii contains a flavoprotein of unusual redox properties (Hinkson and Bulen, 1967; Shethna *et al.*, 1964) which

Benemann *et al.* (1969) found to function as reductant of nitrogenase in the coupled chloroplast-nitrogenase assay, and named "azotoflavin". The most unusual property of azotoflavin was its oxidation-reduction behaviour. It was reduced only slowly by excess sodium dithionite to give a strikingly blue-coloured protein, the semiquinone flavoprotein. A very large excess (100-fold) of dithionite for several hours was required for reduction to the colourless fully reduced form. Re-oxidation in air was also very slow; the semiquinone form was extremely resistant, taking over 12 hours for complete oxidation to the yellow oxidized form. These properties are distinctly different from the behaviour of the flavodoxins which are easily reduced and oxidized. The flavin prosthetic group, although easily released from azotoflavin (Hinkson, 1968), appears to be complexed in this protein in such a way that the reduced species are not accessible to oxygen. The resistance of reduced azotoflavin to oxidation by air may be a distinctive adaptive advantage for transporting high-energy electrons to nitrogenase in the highly aerobic cellular environment of *Azotobacter*. At first (Benemann *et al.*, 1969) it was thought that azotoflavin was unrelated to flavodoxin because of: (1) its unusual redox properties (stable semiquinone); (2) virtual inability to replace ferredoxin in the clostridal phosphoroclastic reaction or in the reduction of NADP by chloroplasts; (3) the high molecular weight (31,000) of azotoflavin; and (4) the presence of azotoflavin, together with azotobacter ferredoxin in cells grown in media containing either high or low concentrations of iron. However, these differences are not crucial to the electron-carrier nature of the protein, and azotoflavin, although quite distinct from flavodoxin in both chemical and biological properties, can be grouped along with phytoflavin in the flavodoxin class of electron carriers.

Flavodoxins have now been found in most groups of nitrogen-fixing organisms (anaerobes, aerobes, and photosynthetic bacteria; Table 3, p. 79), and we may expect in the future to find more examples of these electron carriers. Thus, a flavoprotein with electron carrier properties appears to be present in extracts from soybean root nodules (D. C. Yoch and R. C. Valentine, unpublished data) and a flavoprotein from *E. coli* (Knappe *et al.*, 1969) has flavodoxin-like properties (D. C. Yoch, personal communication).

C. *Coupling Factors of* Azotobacter

Although both Azotoflavin and Azotobacter ferredoxin could function independently and separately as reductants of nitrogenase when reduced artificially with illuminated chloroplasts, this was not the case when endogenous substrates, such as $NADPH_2$, were the electron donor. Both Azotobacter ferredoxin and azotoflavin were needed in the

transfer of electrons from $NADPH_2$ to nitrogenase using a crude extract of *A. vinelandii*. This was most clearly shown when the electron carriers were removed from the crude extract by absorption on DEAE-cellulose; both were required to restore $NADPH_2$-driven nitrogenase activity. This indicated that the two *Azotobacter* electron carriers acted together in an electron chain extending from $NADPH_2$ to nitrogenase (Benemann *et al.*, 1971c).

Two other factors (called "coupling factors") present in the crude extracts were also needed in the complex *Azotobacter* pathway of nitrogen fixation, and will be discussed briefly. When $NADPH_2$ is the electron donor, the first coupling factor is NADP-ferredoxin reductase which catalyses reversible electron transfer between ferredoxin and NADP (Shin and Arnon, 1965). It has only been isolated and crystallized from spinach chloroplasts (Shin *et al.*, 1963) where it participates in the photosynthetic NADP reduction by illuminated chloroplasts (Keister *et al.*, 1960). The spinach enzyme is a flavoprotein, and was shown to form a complex with spinach ferredoxin and also NADP (Nelson and Neuman, 1969; Shin and San Pietro, 1968; Foust *et al.*, 1969). Spinach NADP-ferredoxin reductase is able to replace an enzyme present in *A. vinelandii* (removed by DEAE-cellulose treatment) which was required for the transfer of electrons from $NADPH_2$ to nitrogenase (Benemann *et al.*, 1971c). Although it remains to be isolated and characterized, it is logical to assume that the *Azotobacter* and spinach enzymes are at least functionally similar. Azotoflavin, Azotobacter ferredoxin and NADP-ferredoxin reductase were not sufficient for the transfer of electrons from $NADPH_2$ to nitrogenase. A soluble heat-labile factor present in a centrifuged (nitrogenase-free) extract was also required. Although no further information about this coupling factor is presently known, its function is most likely the transfer of electrons between the two *Azotobacter* electron carriers, since they do not interact spontaneously. Thus, a closer look at the *Azotobacter* system has shown that the pathway is much more complex than anticipated; a proposed scheme, indicating the functional groups involved, is given in Fig. 4. Even the order of the chain is not yet definite; however, the fact that spinach NADP-ferredoxin reductase is unable to transfer electrons to azotoflavin would put Azotobacter ferredoxin next to $NADPH_2$ with azotoflavin being the actual reductant of nitrogenase. Although this pathway shows all components working independently of each other, they might well be complexed and arranged in some functional structure in the cell. Indeed, preliminary evidence indicates that Azotobacter ferredoxin and azotoflavin are present in the *Azotobacter* cell complexed to each other and possibly to other factors of the pathway (J. R. Benemann, unpublished data). In an aerobic environment the extremely

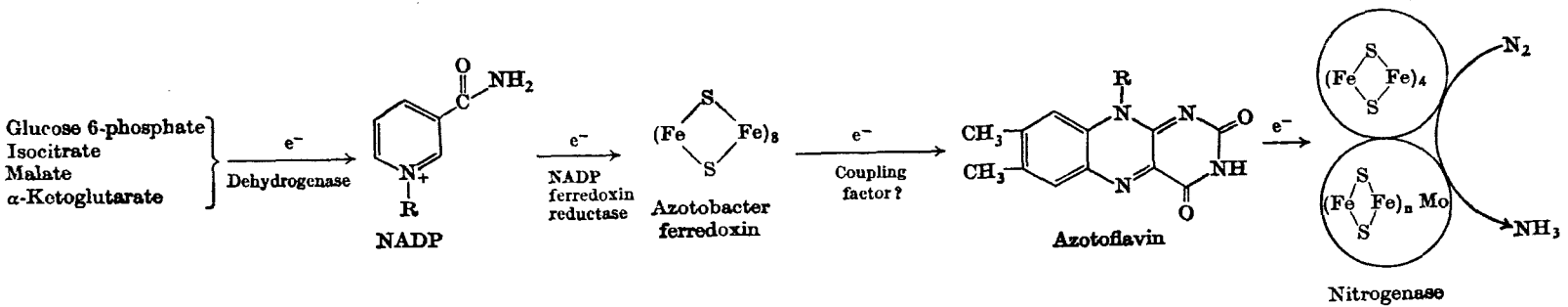


FIG. 4. The pathway of nitrogen fixation *Azotobacter vinelandii*.

reductive process of nitrogen fixation may best be accomplished by an enzyme-carrier complex.

IV. Nitrogenase

For many years, the term nitrogenase had been used to describe the hypothetical enzyme involved in the reduction of molecular nitrogen to ammonia. This central catalyst of the nitrogen-fixation pathway turned out to be a complex of two metal-containing proteins which are surprisingly similar in all the organisms from which it has been isolated. However, the actual mechanisms of the reactions catalysed by nitrogenase have not yet been elucidated.

A. *Isolation and Properties*

The first two nitrogenases investigated in detail were those of *Clostridium pasteurianum* and *Azotobacter vinelandii*. From early work with cell-free extracts, it appeared that these two nitrogenases, although they catalysed the same reactions, were quite different. Heat, cold, and oxygen (Dua and Burris, 1965; Carnahan *et al.*, 1960a) quickly inactivated nitrogenase activity in extracts of *Cl. pasteurianum* but not *A. vinelandii* (Bulen *et al.*, 1964). The Azotobacter enzyme was particulate (it could be sedimented at 144,000 *g* for 6 hours) while the clostridial enzyme was soluble. However, during purification Azotobacter nitrogenase lost its particulate character, and the solubilized Azotobacter nitrogenase was just as air-, heat-, and cold-sensitive as the clostridial enzyme (Bulen and LeComte, 1966). The nature of the Azotobacter nitrogenase complex which appears to give it its unique properties is not understood.

Purification of nitrogenase progressed slowly until the introduction of sodium dithionite as an artificial reductant (Bulen *et al.*, 1965) simplified the assay system for nitrogenase by eliminating the need for an electron carrier and phosphoroclastic system. Not surprisingly, similar purification schemes were developed almost simultaneously by Mortenson (1966) for nitrogenase from *Cl. pasteurianum* and by Bulen and LeComte (1966) for the enzyme from *A. vinelandii*. The most significant finding was that nitrogenase was made up of two different proteins which interacted to form the active enzyme. The basic technique in the purification involved precipitation of nucleic acids in the crude extracts with protamine sulphate (a basic protein) followed by precipitation of the nitrogenase by further additions of protamine sulphate. The nitrogenase was resuspended with cellulose phosphate (which removed the protamine sulphate) and chromatographed on DEAE-cellulose. This chromatography resulted in separation of the nitrogenase activity into two

protein fractions, easily visible as two brown bands on the DEAE-cellulose column (Fig. 5). Each fraction was further purified by ammonium-sulphate fractionation and Sephadex chromatography. Anaerobic procedures had to be used throughout due to the oxygen sensitivity of the components. These two proteins were found to contain non-haem iron and labile sulphide (in approximately equivalent amounts); they therefore both belong in the family of ion-sulphur proteins. The larger nitrogenase protein also contained molybdenum (Fig. 5), a finding

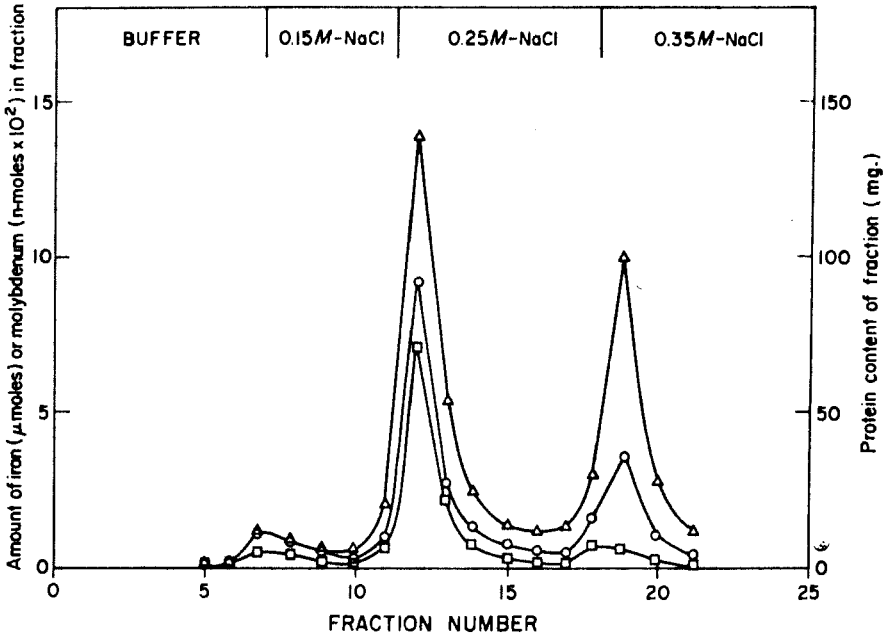


FIG. 5. Separation of the two nitrogenase proteins from *Azotobacter vinelandii* on DEAE cellulose (Bulen *et al.*, 1966). △ indicates content of protein; ○, of iron; □ of molybdenum.

which explains the long known molybdenum requirement of nitrogen-fixing organisms. Purification of nitrogenase has been extended to other organisms (Detroy *et al.*, 1968; Klucas *et al.*, 1968; Kelly, 1969b) and by modified purification procedures. Thus, Vandecasteele and Burris (1970) have eliminated the protamine sulphate step completely and chromatographed a crude extract of *Cl. pasteurianum* directly on DEAE-cellulose, followed by further purification of each fraction. With *Azotobacter* (Kelly *et al.*, 1967), heat treatment (60°; 10 min.) can be used to remove a large amount of contaminating protein. Nitrogen-fixing extracts of soybean root nodules were obtained, and nitrogenase purified,

by adding polyvinyl pyrrolidone to remove interfering phenolic compounds (Koch *et al.*, 1967).

The two protein fractions obtained by these purification procedures are enzymically inert in any of the reactions catalysed by nitrogenase; they have to be recombined for activity in the many different assays for this enzyme. The names "Fe-protein" (for the smaller protein) and "MoFe-protein" (for the larger one) seem to be most popular now, although other names are still being used. The proteins have not been purified to complete homogeneity in most cases, but their general properties have been established. Recently Nakos and Mortenson (1971) found that the Fe-protein of the nitrogenase of *Cl. pasteurianum* contains about four labile iron-sulphide groups, and is made up of two, apparently identical, subunits. The Fe-protein from *A. vinelandii* has not yet been studied as closely, but the data available (Bulen *et al.*, 1966) agree with earlier reports of the clostridial Fe-protein (Moustafa and Mortenson, 1969; Vandecasteele and Burris, 1970) suggesting that they are similar. The spectrum is of the Fe-S-protein type, and although the protein can be reduced by dithionite (however reportedly not by ferredoxin; Moustafa and Mortenson, 1969), it is not reversibly re-oxidized by oxygen. The Fe-protein is cold-labile and extremely oxygen-sensitive, but can be stored in liquid nitrogen. The MoFe-protein from *A. vinelandii* has been crystallized as white crystals from a low ionic-strength solution, by Burns *et al.* (1970). It appears to be a protein of about 270,000 molecular weight containing two molecules of molybdenum, 34–38 molecules of iron, and 26–28 molecules of S^{2-}. The protein can be dissociated into more than one type of subunit. Similar values for metal content (and minimum molecular weight) have been published for impure preparations of the clostridial protein (Mortenson *et al.*, 1967; Vandecasteele and Burris, 1970).

The Fe- and MoFe-proteins are present only in cells grown under nitrogen-fixing conditions; growth in the presence of ammonia prevents their synthesis. In all cases, both the Fe-protein and the MoFe-protein were required for reduction of molecular nitrogen, substrate analogues (acetylene, cyanide, azide), ATP-dependent evolution of molecular hydrogen, or reductant-dependent ATPase activity (Kennedy *et al.*, 1968). The molar ratios of the two proteins needed in the various reactions are not yet definite, but a ratio of 2 : 1 Fe : MoFe proteins (calculated on a molecular weight of 150,000 for the MoFe-protein) has been found to give highest activity (Vandecasteele and Burris, 1970; Kelly, 1969a).

Several workers (Mortenson, 1966; Taylor, 1969) reported a third component (found also in cells grown on ammonia) which was part of the nitrogenase *Cl. pasteurianum*; however, Jeng *et al.* (1969) refuted these claims. Kajiyama *et al.* (1969) claimed that the nitrogenase of *A.*

vinelandii was made up of three proteins (one a ZnFe-protein), but their experiments could not be reproduced independently (M. Kelly, private communication).

Isolation of the two proteins which make up nitrogenase from *Azotobacter* and *Clostridium* and the finding of similar proteins in other bacteria raised the possibility of cross-reacting the Fe- and MoFe-proteins from the different organisms. The first results with *Azotobacter* and *Clostridium* proteins proved negative (see Burris, 1969). The Fe-protein of one showed no activity in the presence of the MoFe-protein of the other. However, the proteins from *Klebsiella pneumoniae* and *Bacillus polymyxa* did cross-react with each other, and even with *Azotobacter* and *Clostridium* proteins to a limited extent (Detroy *et al.*, 1968; Dahlen *et al.*, 1969). These results were confirmed by Kelly (1969b) who also found that the cross reactions were independent of the assay used for nitrogenase activity. Cross reactions between the nitrogenase proteins of blue-green algae and photosynthetic bacteria have also been carried out (Biggins *et al.*, 1971; Smith *et al.*, 1971). These experiments strikingly demonstrated the homology both in structure and function which exists between the nitrogenases of the various organisms. They indicate a good deal of functional and chemical similarity between the nitrogenase proteins of even distantly related organisms, a situation perhaps dictated by stringent structural and catalytic requirements for nitrogen reduction. This idea is supported by the fact that the catalytic activity of all nitrogenases is very similar.

B. *Mechanism of Action*

The question of how nitrogenase reduces molecular nitrogen to ammonia (the mechanism of action of nitrogenase) is central to the study of nitrogen fixation. There have been two main approaches in the quest to discover the mechanisms of nitrogen fixation by nitrogenases, namely the study of nitrogenase itself and the reactions it catalyses, and the study of model systems involving inorganic complexes of molecular nitrogen.

Nitrogenase catalyses the transfer of electrons from a suitable reductant (which is oxidized) to a variety of electron acceptors (N_2, H^+, CN^-) in a reaction which is absolutely dependent on the hydrolysis of ATP to ADP and inorganic phosphate. It also catalyses other reactions, including ATP hydrolysis independent of reductant oxidation and molecular nitrogen-dependent H_2-D_2 exchange. It is quite obvious that the function of nitrogenase is the reduction of molecular nitrogen; all other reactions are secondary and are the result of the structural and catalytic properties of the enzyme. However, the study of these secondary reactions can yield valuable clues, and any proposed mechanisms must

account for them. Before a detailed mechanism of nitrogen fixation by nitrogenase can be worked out, the functions of ATP, molybdenum, and iron will have to be understood; the order of the reaction between substrate, reductant, ATP, and the two nitrogenase proteins known; and the active sites for the various reactions determined. Much work remains to be done, but recent experiments have added to our knowledge in this area.

The role of ATP has been one of the most puzzling questions. From thermodynamic considerations, ATP should not be required since the overall reaction: $3H_2 + N_2 \rightarrow NH_3$ is slightly exothermic at physiological conditions and thus, assuming perfect catalysis by nitrogenase, only a reductant of redox potential about that of molecular hydrogen (such as ferredoxin) would be required. However, this argument does not take into account the energy of activation of the reaction, which in the absence of catalysis, is very high due to the great strength of the triple bond in molecular nitrogen. Although this energy of activation could theoretically be overcome completely by a perfect catalyst, practically it is not too surprising that nitrogenase uses an energy source such as ATP to help drive the reaction. The following facts have been recently established about the role of ATP: (1) the rate of ATP hydrolysis by nitrogenase is independent of the substrate reduced but dependent on the presence of reductant; in the absence of a reductant, nitrogenase still hydrolyses some ATP, especially at low pH values (about 5) where the requirement for a reductant disappears (Bui and Mortenson, 1969); (2) the stoichiometry of ATP utilization by nitrogenase is complex; the ratio of the amount of ATP hydrolysed for each electron pair transferred to an electron acceptor (ATP/$2e^-$ ratio) is dependent on pH value (Winter and Burris, 1968) and temperature (Hadfield and Bulen, 1969), and values ranging from 4 to 5 have been obtained. If allowances are made for the ATP hydrolysed, which does not result in product formation (that is, no electron transfer), the ATP/$2e^-$ ratio is decreased to only two (Jeng *et al.*, 1970; Hadfield and Bulen, 1969; Kelly, 1969a); (3) both proteins can bind ATP (Biggins and Kelly, 1970; Bui and Mortenson, 1968). Four different roles for ATP in the nitrogenase reaction have been proposed: (i) as a proton source for nitrogenase (Brintzinger, 1966); (ii) as an "electron activator" (Mortenson, 1964; Hardy and Knight, 1968); (iii) as a source of hydrated electrons (Bui and Mortenson, 1969); and (iv) as an inducer of conformational change (Bulen *et al.*, 1965). Which of these four proposals comes closest to representing the actual mechanism of action of ATP is not yet clear.

Nitrogenase appears to function in a stepwise fashion adding two electrons at each step to the substrate. The evidence for this is that all substrates are reduced to products which contain an added even

number of electrons. Thus acetylene is reduced to ethylene in a two-electron step, nitrogen to ammonia in three two-electron steps, and isocyanide to a complex mixture of products in as many as six two-electron steps (see Hardy and Knight, 1968). Intermediates of nitrogen reduction (diimide and hydrazine) have never been observed (Burris *et al.*, 1965), presumably because they are strongly enzyme-bound. However, substrate analogues are often reduced to varying degrees, the intermediates being able to dissociate from the enzyme. It is clear that there must be several distinct sites on the nitrogenase enzyme which involve ATP hydrolysis, reductant oxidation, and substrate reduction, since these processes are affected by different inhibitors. Thus carbon monoxide stops all substrate reduction, but ATP hydrolysis continues undiminished and molecular hydrogen is evolved (Hardy *et al.*, 1965; Burns and Bulen, 1965).

The nature of the substrate-binding site, where molecular nitrogen is reduced to ammonia, is at the centre of the study of the nitrogenase reaction, with the key questions being the nature of the metal(s) and ligands at this site and the mechanism of action. A large amount of work has been, and continues to be, done on the inorganic chemistry of complexes of molecular nitrogen (mostly of transition metals) with the hope that these would result in model systems of nitrogenase which would answer these questions (see reviews by Calderazzo, 1969; Kuchynka, 1969; Hardy and Knight, 1968; Allen and Bottomley, 1968; Van Tamelen, 1971). These inorganic models, which in some cases exhibited nitrogen-fixing capabilities, were not very fruitful in the solution of the nitrogenase problem. On the basis of theoretical considerations, many workers considered iron to be the key metal in the nitrogen binding and reduction site, with molybdenum playing only an incidental role (Chatt, 1969; Hardy and Knight, 1968). Some recent studies have suggested molybdenum as the primary metal involved in binding and reduction of molecular nitrogen. Chatt *et al.* (1969) have made complexes of molybdenum and molecular nitrogen. Shrauzer and Schlesinger (1970) developed a simple model system of nitrogenase involving molybdenum-thiol complexes which catalysed (at pH 9 to 10, room temperature, and several atmospheres pressure) reduction of molecular nitrogen with dithionite or sodium borohydride as reductant. Activities were low (Schrauzer *et al.*, 1971); however, the model had the unique property of reducing all of the substrate analogues of nitrogenase tested (acetylene, azide, nitrous oxide) with similar stereospecificities. Also, ATP stimulated the acetylene-reduction activity of the model system (Schrauzer and Doemeny, 1971). Newton *et al.* (1971) reported, without giving experimental details, that simple organic-iron complexes can reduce some molecular nitrogen at physiological conditions in the

presence of potassium borohydride and the absence of molybdenum. Shilov *et al.* (1971) reduced $^{15}N_2$ to ^{15}N-hydrazine in strongly alkaline solutions with either molybdenum or vanadium as catalyst. Hill and Richards (1971) have confirmed all three model systems and were able to improve on the yield of the Schrauzer model over a thousand-fold by use of 2-aminoethanethiol as ligand of molybdenum. With these latest results, it would appear that nitrogenase model chemistry is going to contribute significantly to the study of the mechanisms of this enzyme.

A more direct approach to the question of the metal in the active site of nitrogenase was taken by McKenna *et al.* (1970) who prepared a vanadium-containing nitrogenase (obtained by growing *A. vinelandii* on media lacking molybdenum but containing vanadium). The "vanadium nitrogenase" differed in substrate-binding affinity and reduction kinetics from the "molybdenum-nitrogenase", findings which were confirmed by Burns *et al.* (1971). Further investigations revealed that the enzymic activity of "vanadium-nitrogenase" could be attributed to the presence of contaminating molybdenum; the vanadium present in the enzyme appeared to stabilize the *Azotobacter* nitrogenase complex allowing the small amounts of molybdenum to function (Benemann *et al.*, 1971b). This accounted for the ability of vanadium to replace the molybdenum requirement of some nitrogen-fixing strains of *Azotobacter*. These experiments with "vanadium-nitrogenase" gave the first direct evidence of a role for molybdenum in the active site of nitrogenase (McKenna *et al.*, 1970; Burns *et al.*, 1971). That iron is also involved in the active site of nitrogenase is suggested from the work of Ward *et al.* (1971) who found that the reductive activity of the MoFe-protein of *Cl. pasteurianum* nitrogenase was lost upon removal of six iron atoms while the ATPase activity remained intact. No detailed mechanism of nitrogenase action which can account for all of the known data and observations has been proposed. It is obvious that, despite the large amount of work done in the past decade, we do not yet understand how this enzyme reduces molecular nitrogen.

V. Conclusions and Future Outlook

Table 4 gives a summary of the biochemical pathways of nitrogen fixation in micro-organisms. The pathway is divided into three parts: (i) the electron donors (derived from the metabolic pathways of the cells); (ii) the electron carriers (ferredoxin or/and flavodoxins); and (iii) nitrogenase (made up of the Fe- and MoFe-proteins). The most striking aspect is the unity of the pathways which underlie the more apparent physiological diversity of the various nitrogen-fixing micro-organisms.

TABLE 4. Summary of the Pathways of Nitrogen Fixation in Micro-organisms

Organism	Electron donors	Carriers	References to the nitrogenase proteins
Clostridium pasteurianum	Pyruvate, H_2, $NADH_2$, $NADPH_2$	Ferredoxin or flavodoxin	Mortenson *et al.* (1967)
Klebsiella pneumoniae	Pyruvate, formate, $NADPH_2$, H_2	?	Detroy *et al.* (1968); Kelly (1969b)
Bacillus polymyxa	Pyruvate, formate, $NADPH_2$	Ferredoxin	Detroy *et al.* (1968); Kelly (1969b)
Azotobacter vinelandii	$NADPH_2$	Ferredoxin and azotoflavin	Bulen and LeComte (1966); Burns *et al.* (1970)
Rhizobium japonicum	$NADH_2$(?)	Ferredoxin	Klucas *et al.* (1968)
Anabaena cylindrica	Pyruvate, $NADPH_2$	Ferredoxin (phytoflavin ?)	Smith *et al.* (1971)
Rhodospirillum rubrum	Pyruvate, H_2 (?)	Ferredoxin	Smith *et al.* (1971)

The basic components of the pathway are very similar in all organisms. In the case of the aerobic nitrogen-fixing *Azotobacter* (and probably others) the pathway involves two electron carriers in a complex sequence of reactions (Benemann *et al.*, 1971). The question marks in Table 4 will have to be answered by further research. It should be noted that the pathways of nitrogen fixation were elucidated by studies with cell-free extracts; physiological studies with whole cells are needed to confirm the results, especially with regard to the electron donors used.

It is hazardous to predict the future of scientific research, but recent advances point the way to developments in our understanding of nitrogen fixation which can be expected in the coming years. The mechanism of nitrogen fixation will be one of the most exciting areas. Now that pure nitrogenase proteins have been obtained (Burns *et al.*, 1970; Nakos and Mortenson, 1971), the physical and chemical characterization of this interesting enzyme can proceed. The sequence of interaction between ATP, reductant, substrate, and nitrogenase proteins still needs to be elucidated, as does the location and structure of the various sites (ATP hydrolysis, reductant oxidation, substrate binding) on nitrogenase. This, together with careful kinetic studies and development of model systems, should result in the solution of the question of the mechanism of biological nitrogen fixation. It is likely that a detailed mechanism will be elucidated before all of these data are accumulated.

The development of the acetylene assay has led to a resurgence of physiological and ecological research in nitrogen fixation. Studies on the distribution and activities of nitrogen-fixing micro-organisms are becoming more numerous and quantitative (for examples see Stewart *et al.*, 1967, Brezonik and Harper, 1969; Howard *et al.*, 1970; Silver and Mague, 1970; Horne and Fogg, 1970; Bunt *et al.*, 1970) and should soon give us a more detailed picture of the true extent and importance of this microbial reaction in nature. The biochemical relationships of symbiotic nitrogen fixation present a fertile area of research for biochemists. A recent report by Holsten *et al.* (1970) that soybean root-cell tissue cultures are able to form nitrogen-fixing symbiotic associations with *Rhizobium* could provide the wedge needed for entry into this problem.

The use of modern genetic techniques in nitrogen fixation is just beginning. The study of the biochemical genetics of nitrogen fixation could yield important basic knowledge regarding the pathway, mechanism and regulation of this process. Biochemical mutants blocked in the nitrogen-fixation pathway have been obtained from *A. vinelandii* (Fischer and Brill, 1969; Sorger and Trofimenkoff, 1970; Benemann *et al.*, 1971a), *Cl. pasteurianum* (Simon and Brill, 1971), and *Klebsiella pneumoniae* (Streicher *et al.*, 1971). Streicher *et al.* (1971) were able to develop a genetic system in *K. pneumoniae*, using the transducing phage P_1^-,

and achieved the transfer of nitrogenase genes from one strain to another. Mapping of the nitrogenase genes is now possible. In symbiotic nitrogen fixation, both plant and host bacterium carry genetic information relevant to this process, making the genetics of the system complex (see review by Nutman, 1969). The acetylene assay has been used (Schwinghamer *et al.*, 1970) to study nitrogen fixation by *Rhizobium* mutants.

The potential contribution of research in nitrogen fixation to the welfare of mankind is immense. The major nutritional problem associated with the exponentially growing world population is a deficiency in protein supplies, especially in technologically underdeveloped countries where the use of synthetic fertilizers is limited because of their expense. Although production of chemical fertilizers is rapidly expanding, it appears unlikely that major improvements will be made in their production, since the basic processes have remained unchanged for over 50 years. Also, chemical fertilizers are threatening to pollute the environment by upsetting the biological nitrogen cycle (see Delwiche, 1970, for a review). Considering that one acre of nitrogen-fixing soybeans produces over thirty times as much protein as one acre used for cattle raising (Dawson, 1970), it is obvious that a solution to the problem of world protein supply could come from practical applications of research on biological nitrogen fixation. The past record in this area is already impressive. Seeds of leguminous plants are now routinely inoculated with specific strains of *Rhizobium* which are available commercially. Molybdenum is added to deficient soils to facilitate nitrogen fixation. Future applications could be the development, through genetic techniques, of hardier, more active strains of nitrogen-fixing organisms (symbiotic and free living) which could be used as cheap non-polluting sources of nitrogenous fertilizers. Understanding of the biochemical relationships in symbiotic nitrogen fixation might allow the extension of this process to non-leguminous agricultural crops. The recent report of nitrogen-fixing *Klebsiella* in human and animal intestines (Bergersen and Hipsely, 1970) and the availability of a genetic system in these organisms (Streicher *et al.*, 1971) raises the fascinating prospect of solving the protein supply problem directly. However, scientific and technological advances alone cannot provide the ultimate solution to the problems they created. That will have to come from political and moral adjustments of our societies.

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Rapid Detection and Assessment of Sparse Microbial Populations

R. E. STRANGE

Microbiological Research Establishment, Porton, Nr. Salisbury, Wiltshire, England

I. Introduction 105
II. Principles of Rapid Microbial Assessment Methods . . . 107
A. Physical Properties of Microbes 108
B. Chemical Composition of Microbes 109
C. Microbial Growth and Metabolism 111
D. Bacterial Enzymes 113
E. Immunological Properties of Microbes 114
III. Rapid Broad-Spectrum Methods 118
A. Luminol Chemiluminescence 118
B. Determination of ATP 120
C. Staining Methods 121
D. Physical Methods 124
E. Methods Depending on Growth and Metabolism . . . 125
IV. Rapid Specific Identification Methods 126
A. Immunofluorescence-Membrane Filtration Techniques . . 126
B. Radioactive Antibody Technique 127
C. Analysis of Bacterial Growth Products. 130
D. Gas Chromatography-Pyrolysis Methods 131
E. Analysis of Phosphorescent Decay 131
V. Rapid Determination of Microbial Viability 131
A. Indirect Methods 131
B. Direct Methods 132
VI. Rapid Detection and Determination of Viruses 132
A. Immunofluorescent-Monolayer Techniques 132
B. Immuno-Adherence 133
C. Radioactive Antibody Method Applied to Bacteriophage T7 . 133
D. Detection of Virus Activity with Gas Chromatography . . 135
VII. Conclusions and Prospects 136
VIII. Acknowledgements 137
References 137

I. Introduction

The significant words in the title of this review are "rapid" and "sparse". If the concentration of microbes in the sample is high enough, and/or sufficient time is available for a detailed examination to be made,

their detection, specific identification and quantitative assessment (i.e. determination of total number or mass of microbes/unit volume of sample and/or viability) present no problems to the microbiologist. On the other hand, immediate or rapid detection or specific identification of a sparse microbial population is much more difficult and requires the use of specially developed methods; it is with these methods, and their physiological basis, that this review is mainly concerned.

If a sparse population of microbes is to be detected and determined within a few minutes it might be possible initially to increase the microbial concentration in a sample by centrifugation or membrane filtration; but augmentation of the population by growth (on which most conventional techniques depend) must be excluded. Since, during unimpeded growth in a suitable culture medium, bacteria with a mean generation time of 30 min. could increase 10^{14}-fold during 24 hours it is not surprising that, in general, the sensitivity of rapid methods does not approach that of the more conventional time-consuming techniques.

Many of the known chemical, physical and physiological characteristics of microbes have been exploited for rapid microbial detection or identification purposes. Methods based on widely differing principles, capable of detecting within a few minutes 10^3–10^4 bacteria (and, in some cases, yeasts) have been reported (Oleniacz *et al.*, 1966; Mitz, 1969; Strange *et al.*, 1971). In this connexion, a much sought after capability is the rapid detection of microbes in the atmosphere, and automatic monitoring systems have been developed for this purpose (Nelson *et al.*, 1962; Oleniacz *et al.*, 1966; Mitz, 1969). As Oleniacz *et al.* (1966) state, there are certainly a number of potential applications for a reliable microbial detection device (monitoring of the atmosphere, the air in dairy and other industries, "clean" rooms and operating theatres). There may be grounds for believing that monitoring of the air for the presence of bacteria, and other micro-organisms of similar or larger size, is at least feasible but this is not the case with viruses. The rapid detection and identification of viruses is usually achieved with immunofluorescence techniques, but with extremely small viral populations a propagation step that takes several hours is necessary before detection is possible.

A major problem with rapid, highly sensitive, microbial detection methods (and with sub-micro analysis in general) is interference by extraneous material in the sample. An assay may be accurate and reproducible when it is applied to a small number of purified microbes suspended in a bland medium, but may give meaningless results in the presence of extraneous particulate material and/or certain soluble substances. The use of a highly specific assay does not necessarily resolve the problem of interference. For example, if 10^3–10^4 bacteria/ml. of sample are to be detected, the equivalent bacterial dry weight ranges

around 10^{-9} g./ml.; even if the concentration of extraneous matter in the sample is only 10^{-6} g/ml., significant interference will occur unless the response of the bacteria is above 1000-fold that of the extraneous matter (on a dry weight basis). If readout depends on microscopic examination, microbes might be distinguished from interfering matter but, if response is determined by other means, interpretation of the results depends on the "blank value" of the assay. Provision of suitable blank samples presents no problems when samples of washed bacterial suspension are assayed, but can be difficult or impossible with, for example, samples from natural environments.

It is convenient to divide detection methods into two main classes. "Broad spectrum" methods detect and/or estimate microbes in general, or a taxonomic group of microbes (bacteria, yeasts, fungi, viruses), without specifically identifying them; "specific identification" methods detect and identify microbes in one step. Broad-spectrum methods have potential application in situations where the identity of microbes likely to be present is known or not of immediate importance. Specific identification methods at present available vary very much with respect to the time taken to complete them, and their overall capability. Methods based on immunological principles are rapid and sensitive but their application to the detection of microbes depends on having a specific reagent for each of the species or even strains of microbes that may be present. Methods depending on other principles are generally less sensitive and, although more rapid than conventional techniques, they are too slow to be adapted for monitoring purposes.

In general, the rapid methods for bacterial and viral assessment discussed in this review are far less precise than the longer conventional methods, and several give results only in terms of the order of magnitude of the number of microbes present. A few are established methods based on proven principles (e.g. immunofluorescence) for which adequate published methodology, sensitivity and performance data are available; others are either "in the experimental stage" or based on principles of questionable validity.

II. Principles of Rapid Microbial Assessment Methods

Properties of microbes that have been utilized in rapid assessment methods include their characteristic size and mass ranges, chemical composition, growth characteristics, metabolic activities, specific enzymic activities and immunological activities. For practical purposes, the only rapid viral detection methods available are based exclusively on immunological properties although other approaches have been proposed.

Consideration of the actual amount of biomass, mass of a particular component, or physiological activity in a sparse microbial population, makes it clear why rapid assessment poses difficult analytical problems.

A. Physical Properties of Microbes

The size ranges of the different taxonomic groups of microbes overlap (for example, the larger viruses are similar in size to the smallest bacteria) but dimensions of most individual bacterial cells are 1–3 μm. and of virus particles, 20–200 nm. Similarly, values in terms of equivalent dry weight are extremely variable but the ranges 10^{-13}–10^{-12} and 10^{-17}–10^{-16} g. are representative of many individual bacterial cells and viral particles, respectively. The shape, dimensions and dry weight equivalent of bacteria vary not only in different species but also in the same species or strain depending on growth conditions and past history. In spite of the large variations, ranges of values for bacterial dimensions and mass are sufficiently characteristic to be used as the basis of assessment. The concentration, size distribution pattern and volumes of small particles in liquid suspension can be rapidly determined with the Coulter counter (Coulter Electronics Inc., Hialeah, Florida, U.S.A.) (see Kubitschek, 1969). Similar information for air samples can be obtained with the Royco particle counter (Royco Instruments, Inc., Menlo Park, California, U.S.A.).

Extremely sparse microbial populations must be concentrated before assay and, in the case of bacteria and larger organisms, this can usually be done either by centrifugation or membrane filtration. Membrane filtration techniques are particularly useful for concentrating small numbers of microbes (contained in large volumes of liquids) on to small areas where the microbes can be stained, or allowed to multiply, and then examined microscopically (see Mulvany, 1969). Sparse viral populations can be concentrated by ultracentrifugation, electrophoresis (Bier *et al.*, 1965), treatment with polyethylene glycol to remove water (Cliver, 1965a), ultrafiltration through an aluminium alginate gel soluble in sodium citrate (Gärtner, 1965), fractionation in aqueous two phase polymer systems (Albertsson, 1958; Philipson *et al.*, 1960; Schmidt, 1968; Shuval *et al.*, 1969), gauze samplers (Liu *et al.*, 1971) or membrane chromatography (Cliver, 1965b).

Interference by extraneous particulate matter is a major problem in many rapid assessment methods and it may be necessary to fractionate the sample before analysis. Particles collected from air samples are fractionated on a size basis with a liquid pre-impinger (May and Druett, 1953) that removes particles of >4 μm. diameter, a multistage liquid impinger (May, 1966) that separates particles into size ranges of >6, 3–6

and <3 μm. diameter or through filters of graded pore size. Liquid samples are fractionated by membrane filtration, differential or gradient centrifugation, liquid two phase polymer systems (Albertsson, 1958; Philipson *et al.*, 1960; Tiselius *et al.*, 1963; Schmidt, 1968; Schuval *et al.*, 1969) or magnetically stabilized electrophoresis (Kolin, 1960). Two phase aqueous polymer systems are particularly useful for purifying microbes and virus can be concentrated up to 100-fold in one step with the method (Philipson *et al.*, 1960). If membrane filtration is adopted for fractionation purposes it is important to realize that even though bacteria and viruses have smaller diameters than the mean pore size of the filter they may still be retained. Rao and Labzoffsky (1969) found that more than 50% of poliovirus type 1 (Mahoney strain) were absorbed by Millipore membrane filters (HA; 0·45 μm.) when suspensions also containing a certain concentration of electrolytes were filtered through the filters and pre-filter pads (AP25). The technique was used to detect low concentrations of viruses in large volumes of water. Cliver (1965b) reported that 99% or more of enterovirus in de-ionized water, tapwater or saline phosphate buffer failed to pass through Millipore membranes with pore sizes of 0·45 μm. Absorption of virus by membrane filters was strongly inhibited by serum or whey protein in the input suspension. Similarly, a significant number of *Bacillus subtilis* spores are retained when saline phosphate buffer suspensions containing 10^3–10^4 spores/ml. are filtered (and washed) by suction through Millipore membrane filters of 3 μm. pore size (i.e. much larger than the spore diameter).

B. Chemical Composition of Microbes

Broad-spectrum methods depending on analysis for a particular constituent common to bacteria in general have been reported. With few exceptions, the various constituents of bacteria are present in other life forms and this type of method may give a response with other biological material present in the sample. Unless samples are free from, or contain, a known amount of interfering material, bacteria must be separated before analysis.

The chemical composition of bacteria varies markedly in different species, or with growth conditions and past history in the same species, so that quantitative compositional data are of little value without reference to these factors. In all cases, the biomass is largely accounted for by protein, RNA, DNA, lipid and carbohydrate but, with the exception of DNA, the relative concentrations of these macromolecules vary widely. Compositional data for *Aerobacter aerogenes* grown to the stationary phase in a defined mannitol-salts medium and tryptone-glucose medium are given in Table 1.

If a microbial detection method based on analysis for the whole biomass or a major constituent(s) is required to detect 10^4 *A. aerogenes* cells, the respective amounts of substances that must give a measurable response are shown in Table 1. If 10^4 bacteria are concentrated into a sufficiently small volume and are not obscured by extraneous matter, these small amounts of material can be detected microscopically after staining or treatment with histochemical reagents but not by the microscale methods used in biochemical analysis. Only methods with the sensitivity and precision to measure nanogram to picogram quantities can be applied, for example gas chromatography, mass spectroscopy and radiochemical techniques.

TABLE 1. Biomass or Weight of Constituent in Equivalent Dried Weight of 10^4 Stationary-Phase *Aerobacter aerogenes* NCTC 418 Cells Grown in a Mannitol-Limited Defined Medium and Tryptone-Glucose Medium. Data from Strange *et al.* (1961) and Strange *et al.* (1963).

	Grams component/10^4 cells	
	Defined medium	Tryptone-glucose medium
Biomass	$3{\cdot}5 \times 10^{-9}$	$2{\cdot}4 \times 10^{-9}$
Protein	$2{\cdot}3 \times 10^{-9}$	$1{\cdot}4 \times 10^{-9}$
RNA	$7{\cdot}0 \times 10^{-10}$	$2{\cdot}9 \times 10^{-10}$
DNA	$1{\cdot}0 \times 10^{-10}$	$9{\cdot}0 \times 10^{-11}$
Carbohydrate	$2{\cdot}0 \times 10^{-10}$	$3{\cdot}6$–$4{\cdot}8 \times 10^{-10}$
Lipid[a]	$2{\cdot}8 \times 10^{-10}$	$1{\cdot}5 \times 10^{-10}$
ATP[b]	$5{\cdot}6$–$7{\cdot}7 \times 10^{-12}$	[c]

[a] Extracted with chloroform-methanol (3 : 1, v/v).
[b] In fully aerated bacteria.
[c] Not determined.

Gas chromatography has been applied to the analysis of the pyrolysis products of bacteria with results that suggest this is an acceptable method for rapidly and specifically identifying bacterial species (Oyama, 1963; Garner and Gennaro, 1965; Reiner, 1965, 1967). Specific identification of small numbers of bacteria involving pyrolysis and product analysis with gas chromatography and mass spectroscopy was proposed for rapid biological detection purposes by Mitz (1969).

Other constituents of bacteria comprise a wide spectrum of substances most of which individually account for <1% of the equivalent bacterial dry weight. Certain of these constituents can be accurately measured in extremely low concentrations and analyses for them have been proposed

as rapid microbial detection methods. Examples are ATP and haematin, compounds which are apparently universally present in bacteria and that can be detected at a minimum concentration of about 10^{-11} g./ml. in each case. Even if these compounds each account for only 0·1% of the equivalent bacterial dry weight, analysis should allow detection of a minimum of 10^4–10^5 bacteria/ml.

The major constituents of viruses are proteins, nucleic acids (RNA or DNA), lipids and carbohydrates; estimates of the relative amounts of these components in Semliki Forest virus and bacteriophage T7 are

TABLE 2. Biomass or Weight of Constituent in Single Dried Particles of Phage T7 and Semliki Forest Virus (SFV). Data for Semliki Forest Virus are from D. Titmuss, K. H. Grinstead and J. D. Oram (unpublished work)

	Grams component/particle ($\times 10^{16}$)	
	Phage T7	SFV
Biomass	1·9	3·7
Protein	0·91	2·4
RNA	0·08[a]	0·2
DNA	0·64	—
Lipid	[b]	0·84
Carbohydrate	—	0·29

[a] Probably due to host cell contamination and/or nterference by other phage constituents in assay.
[b] Not determined.

given in Table 2. An extremely large number of virus particles is required to provide a sufficient mass of material for analysis and this, together with the likelihood that virus preparations will contain interfering host cell material, eliminates the chemical analysis approach to viral detection except with relatively concentrated virus suspensions.

C. Microbial Growth and Metabolism

Expression of the ability to reproduce is one of the most reliable indicators of the presence of microbes and attempts have been made to modify classical microbiological methods, based on growth, to give results in a shorter time than usual. Provided that the microbes in the sample are viable and results are not required in less than a few hours, growth methods are to be preferred.

The various methods available for determining microbial growth were analysed by Merck and Oyama (1968) who were concerned with the detection of growth in the search for extraterrestrial life. The methods were determination of dry weight, "silting index" (developed by Millipore Corp., Bedford, Mass., and depending on the principle that particles plug membrane filter pores and decrease the rate of flow of liquid), electronic particle counting (Coulter counter; see Kubitschek, 1969), monitoring for metabolic activity (rate of substrate consumption, rate of product formation, changes in pH value, redox potential changes), chemical analysis of the particles (e.g. nitrogen determination; see Ferrari *et al.* 1965), optical techniques applied to a surface (Glaser and Wattenburg, 1966) and optical techniques applied to a liquid (proposed by Dr. Wolf Vishniac; see Quimby, 1964). Merck and Oyama (1968) consider optical monitoring in a system free of interfering particles is the best of these methods for their purpose, and they discuss the problems of formulating suitable culture media.

Measurement of the light scattered when small particles are exposed to a beam of light is a sensitive method for their detection and determination, and the principle has been used in automated microbial detection instruments. The sensor includes an optical assembly that permits only light scattered by particles to produce a signal in a phototube connected to a pulse recorder and/or a digital readout system. In the "Wolf Trap" (invented by Dr. Wolf Vishniac, University of Rochester, New York; see Mitz, 1969) captured microbes are cultured in liquid medium and in the "capillary tube scanner instrument" (Bowman *et al.*, 1967) microcolonies are formed in gelled nutrient agar held in capillary tubes; in both instruments, growth is measured with a scattered light sensor.

Bacterial growth in the presence of carbon-energy sources like glucose results in the production of organic acids that affect the pH value of the medium, and measurement of changes in pH value in appropriately formulated growth medium is a potential method for detecting bacteria. In addition to scattered light sensors, the Wolf Trap is provided with pH value probes and growth can be monitored with either one or both types of sensor (Mitz, 1969).

Products of bacterial metabolism include gases (carbon dioxide, hydrogen sulphide, methane, molecular hydrogen and ammonia) and detection of gas evolution is another potential growth monitoring and/or microbial detection method. With a sparse microbial population, however, the amount of gas evolved is extremely small (10^7 fast growing *E. coli* cells produce about 1 μl. CO_2/hr) and can only be detected with, for example, radiochemical techniques. Scientists of the National Aeronautics and Space Administration in the U.S. have developed an automated detection device based on this principle called "The Gulliver" (see

Mitz, 1969) that monitors the $^{14}CO_2$ evolved from a small number of bacteria growing in a medium containing a ^{14}C-labelled substrate.

During bacterial growth, metabolic products that vary qualitatively and quantitatively with the particular species or strain of microbe and the growth conditions are released into the culture medium. An intriguing discovery made concerning products released under a given set of growth conditions was that they were qualatitively and quantitatively distinctive of a particular bacterial species (Henis *et al.*, 1966). A highly sensitive gas chromatographic technique was used to analyse the growth products extracted from the medium, and several workers have confirmed that the profiles ("signatures", "fingerprints") are sufficiently distinctive to be used for specifically identifying bacteria (Moore *et al.*, 1966; Moore, 1967; Mitruka and Alexander, 1967; 1968; 1969). The minimum readout time depends on the species, initial concentration of bacteria and the sensitivity of the gas chromatographic detector; in certain circumstances results can be obtained in 2–4 hours.

Detection of metabolites elaborated by virus-infected cells during the course of infection in tissue culture or host animal was reported as a means of rapidly detecting viral infections (Mitruka *et al.*, 1968; 1969).

D. Bacterial Enzymes

Growth and metabolism of bacteria depend upon numerous endogenous enzymes, the activities of which can be specifically and quantitatively determined in whole bacteria, homogenates and/or extracts with simple, highly sensitive methods. Many of these enzymes are apparently present in all bacteria although there may be some differences in the physical and chemical structure of the enzyme molecules in different bacterial species. In principle it is feasible to detect bacteria with an assay for a particular common enzyme but in most cases the amount of enzyme present, rate of substrate breakdown per bacterium and sensitivity of the assay are too low to allow detection of a few bacteria. Scientists of the National Aeronautics and Space Administration (see Mitz, 1969) have considered enzyme activities that might be exploited for rapid microbial detection purposes and selected two that can be assayed rapidly and have high "turnover numbers" (molecules substrate decomposed/bacterium/min.) with their respective substrates. One is phosphatase (the activity of which is used as an index of bacterial action in milk) and the assay depends on hydrolysis of *p*-nitrophenyl phosphate with release of *p*-nitrophenol that is measured spectrophotometrically; alternatively a fluorescent organic phosphate can be used which releases a highly fluorescent product that can be measured fluorimetrically. Use

of the latter substrate allowed detection of 10^7 bacteria within a few minutes. The second useful enzyme is bacterial esterase(s) which Mitz (1969) and his colleague (Dr. Gordon Blanchard) found in 12 representative bacterial species; the assay depends on hydrolysis of phenyl acetate with release of acetic acid that can be measured by automatic titration with standard potassium hydroxide to maintain the pH value of the reaction mixture at 7·4. The minimum number of bacteria detectable with the esterase assay varied from 10^3 to 10^6.

E. Immunological Properties of Microbes

1. Bacteria

Immunological methods are indispensable for the *specific* identification of microbes. Simple agglutination tests with one or more antisera and appropriate controls often suffice to identify specifically a homogeneous bacterial population. A relatively dense bacterial suspension (10^8–10^9 bacteria/ml.) is required, but bacteria in liquid samples can be concentrated by centrifugation or membrane filtration and this, combined with microscopic observation of agglutination, allows identification of bacteria in an initial concentration of about 10^5/ml. However, with sparser populations, some other indicator of an immune reaction must be employed.

Bacteria usually have several distinct immunospecific surface components (e.g. O, K and H antigens) and on exposure to homologous antiserum each combines with its specific antibody to an extent depending on the amounts of respective antigens present, their accessibility and the dissociation constants of the immune complexes. Surface adsorption of specific antibodies precedes bacterial agglutination that in dilute bacterial suspensions occurs at a slow rate because of the low cell collision rate. A solution to the problem of detecting antibody specifically attached to bacteria in liquid suspension, clinical specimens and tissue sections was found by Dr. Albert Coons and his colleagues (Coons *et al.*, 1941; Coons *et al.*, 1942; Coons, 1961) who invented the fluorescent antibody technique. The numerous successful applications and modifications of the method are adequately reviewed elsewhere (e.g. see Nairn, 1964; Goldman, 1968); only broad principles and a few pertinent applications are mentioned in this review.

Ultraviolet microscopy of samples treated with fluorescent antibody, followed by washing with alkaline buffer, shows homologous bacteria as strongly fluorescent particles whereas heterologous bacteria are unstained. The sensitivity attained is extremely high depending on ones ability to find the stained particles and the extent of interference due to autofluorescing material (and/or non-specific uptake of the tagged

antibody by supporting media and extraneous particles or substances in the samples). Immune globulins and other proteins in crude antibody preparations are macromolecules of relatively high molecular weight ($1{\cdot}6 \times 10^5$–10^6 daltons) that tend to adhere to many types of material and complete elimination of background fluorescence may not be attained. Background fluorescence may be decreased by counter-staining preparations with Evan's blue dye (White and Kellogg, 1965).

In attempts to improve the reproducibility of standard immuno-fluorescent techniques, emphasis is now being placed on standardization of reagents, methodology and instruments (Nairn, 1968; Holborow, 1970; Beutner, 1971). Important factors include the quality of antisera, globulin fraction or purified antibody, optimum intensity of labelling with fluorochrome (Spendlove, 1966; Brighton, 1966; Beutner *et al.*, 1968) illumination (Johnson and Dollhopf, 1968; Tomlinson, 1970; Johnson, 1970), and design of optical equipment (Lidwell *et al.*, 1967).

Immunofluorescence can be quantitatively determined with automatic microscopic scanning techniques (Mansberg and Kusnetz, 1966) and microfluorimetric techniques were reported by Goldman (1967), Pearse and Rost (1969) and Ploem (1970). The fluorescent antibody test for syphilis has been automated (Coffey *et al.*, 1971).

Immunofluorescent methods are applied to heat- or chemically-fixed samples on microscope slides or to microbes filtered on to non-fluorescent membrane filters (Danielsson, 1965; Danielsson and Laurell, 1965; Guthrie and Reeder, 1969). Increasing the size of the bacterial population by a short growth step before fluorescent antibody staining greatly increases sensitivity (Danielsson & Laurell, 1965).

"Direct" or "indirect" immunofluorescence techniques can be used for identifying bacteria. In the direct method, the sample is treated with fluorochrome-labelled antibody specific for the bacteria being looked for; in the indirect method, the sample is treated with unlabelled antibacterial serum, and after washing, with fluorochrome-labelled antibody against the whole serum or serum globulin fraction of the animal in which the antibacterial serum was produced. An advantage of the latter method is that only one labelled antibody reagent is required to detect each of any number of different bacterial species provided that antiserum against each of them is prepared in the same species of animal.

Radioisotopes of iodine have also been used for labelling antibody proteins (Miles and Hales, 1968) and a ^{125}I-labelled antibody-membrane filtration method was reported for the rapid specific detection and determination of small numbers of vegetative bacteria and bacterial spores (Strange *et al.*, 1971). As with immunofluorescence techniques, the major sensitivity-limiting factor in the radioassay is variable non-specific uptake of labelled antibody by materials used in the assay and

extraneous particulate matter in samples; nevertheless, sensitivity compared favourably with that attained with other rapid methods.

2. *Viruses*

Viral particles have surface antigens that are detectable immunologically. Concentrated poliovirus preparations (Smith *et al.*, 1956) and phage particles (Jerne and Avegno, 1956) both flocculate in the presence of homologous antiserum. However the large number of viral particles required to produce a discernable precipitate usually excludes the use of flocculation tests for detection and identification purposes. A concentration of about 10^{11} phage T7 particles/ml. is required to produce a visible precipitate with antiphage serum. The sensitivity of flocculation tests for viruses is increased by adsorbing either viruses or antiviral globulins on to various kinds of larger particles (for details, see Kwapinski, 1965). Segre (1957) used ion-exchange resin particles coated with specific virus antibodies for detecting relatively small numbers of hog cholera and vesicular stomatitis viruses; the method was modified from that of Evans and Haines (1954) who employed ion-exchange resin particles coated with polysaccharide antigens.

Under certain conditions, viruses cause agglutination of erythrocytes, a phenomenon first shown with influenza virus (Hirst, 1941; McClelland and Hare, 1941). Scott *et al.* (1957) used the passive haemagglutination test to detect viral antibody by coating tannic acid-treated erythrocytes with virus antigen. Smith and Courtney (1965) detected viruses with tanned erythrocytes coated with anti-viral globulin and markedly increased the sensitivity of the passive haemagglutination assay.

In studies designed to demonstrate phagocytosis of virulent *Treponema pallidum, in vitro*, observations on control preparations led to the discovery of an immunologically specific adherence reaction between normal human erythrocytes and treponemes sensitized with antibody from syphilis serum (Nelson, 1953). The reaction required a heat-labile substance in normal serum, presumably complement; erythrocytes played a specific role since, when they were replaced by human platelets, charcoal, magnesium silicate particles or cells of *Candida albicans*, no adherence reaction was observed. It was found that the reaction occurred with several other bacterial species and the phenomenon was called "the immune adherence reaction". Nelson (1953) suggested the reaction of erythrocytes and micro-organisms could be used to detect circulating antibody.

Since Nelson's (1953) discovery, the immune adherence reaction has been shown to occur with yeast cells, starch granules, rickettsia, several viruses and proteins (Nelson and Woodworth, 1957). It has been used as a

sensitive method for titrating animal virus antigens and antibodies (Ito and Tagaya, 1966; Nishicka *et al.*, 1967) and a small-scale adaptation of the principle was used for the detection of Moloney virus on monolayer cells (Tachibana and Klein, 1970).

Immunofluorescence methods (see Nairn, 1964; Goldman, 1968; Carter, 1971) are extensively used for the rapid detection and identification of viruses—indeed no other similarly rapid and reliable methods are available. There are three main ways in which immunofluorescence can be used in virus identification. First, serum virus antibody can be detected with indirect immunofluorescence or by neutralization tests based on fluorescent cell-counting assays. Second, viral antigen may be detected in smears and impressions from exudates and tissues and less commonly in cryostat tissue sections. Third, viral antigens may be detected after inoculation and incubation of tissue culture monolayers on coverslips or slides.

The aim in immunofluorescent detection methods is to achieve the highest sensitivity, and methods employing cell monolayers facilitate this by providing conditions that augment the initial viral population by replication. Sensitivity for a particular virus depends on many factors of which susceptibility of the tissue cells to virus infection is of particular importance. Depending on the type of virus and nature of the sample, pretreatment such as ultrasonics or repeated freeze–thaw cycles may be necessary to release viruses from cells, or cell debris, before applying the sample to the monolayer. The rate of adsorbtion by and penetration of virus into a cell monolayer is increased by a centrifugation technique first described by Hahon and Nakamura (1964). Adsorption of psittacosis virus was carried out with a centrifugal force of 500 **g** using centrifuge adaptors for coverslips. More recently Hahon and his colleagues described the use of centrifugal forces up to 30,000 **g** for other viruses (Hahon, 1965, 1966, 1969; Hahon and Cooke, 1965; Hahon and Hankins, 1970). As the rate of virus-cell contact is virtually independent of inoculum volume, a relatively large volume of sample can be used, allowing detection of very low concentrations of virus.

The total period of monolayer incubation from inoculation to fixation is called the detection time and consists of two phases: that of adsorption and penetration of virus, and that during which the virus replicates to detectable levels. Infected cells are demonstrated by staining with fluorescent antibody and counted with the microscope at relatively low magnification, using ultraviolet radiation or blue light. Under appropriate conditions, a linear proportionality exists between the concentration of virus in the inoculum and the number of fluorescent cells. The mean count is multiplied by the field factor (i.e. area of the coverslip monolayer divided by the area of field for a given microscope objective

and ocular system) to give the number of fluorescent cells on the entire monolayer. The value multiplied by dilution and volume factors provides the titre of the virus in some convenient units.

The fluorescent antibody-cell monolayer method is sensitive, rapid and reliable. In many cases, readout is possible within less than 24 hours and, in the case of foot and mouth disease virus, the assay is completed within 4 hours (Mohanty and Cottral, 1970).

The feasibility of rapidly detecting virus particles in liquid suspension with ^{125}I-labelled homologous antibody was investigated with phage T7 as the test particle (R. E. Strange, unpublished observation). A major technical problem was the rapid and quantitative separation of virus particles after attachment of labelled antibody and, although tentative solutions were found for phage T7, the methods may not be applicable to viruses in general. Since the uptake of antibody by a microbial particle depends on its size, the theoretical sensitivity of the radioassay in terms of the minimum number of particles detectable is much lower than with bacteria.

III. Rapid Broad-Spectrum Methods

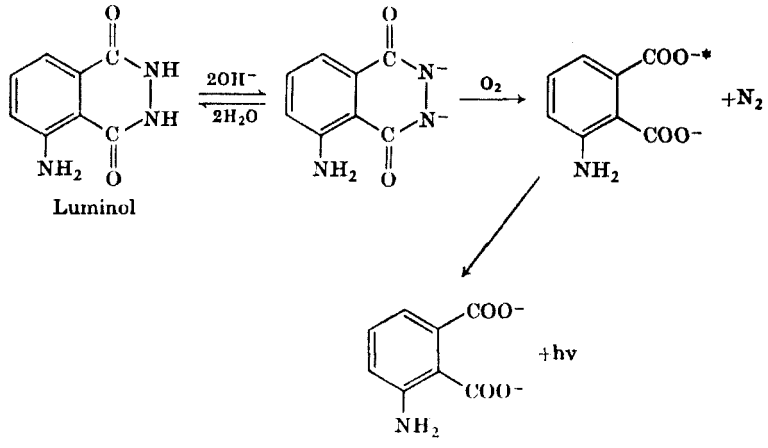
A. Luminol Chemiluminescence

The catalytic effect of haematin on the chemiluminescence of luminol has been discussed by White (1961) and this principle can be used to determine extremely small amounts (minimum 10^{-11} g.) of various haematin compounds (Neufeld *et al.*, 1965). Bacteria and yeasts contain haem compounds (e.g. catalase) and Pisano *et al.* (1965) reported that cell-free extracts of these microbes activate luminol.

Luminol (5-amino-2,3-dihydro-1,4 phthalazinedione) in the presence of alkali hydrogen peroxide (White, 1961) or sodium perborate (Goldenson, 1957) and an activating agent (sodium hypochlorite, potassium ferricyanide or a transition metal) produces photons (White *et al.*, 1964). If the reaction is allowed to take place in a dark chamber, the emitted light can be measured photometrically.

Oleniacz *et al.* (1966) described an automated luminol system for the detection of small numbers of bacteria and yeasts based on a Technicon Autoanalyser (Technicon Corporation, Ardsley, New York). Samples of 11 representative species of bacteria and two species of yeasts all activated luminol to give a light emission peak that decayed in about 100 seconds. In all cases, the light signal minus the assay blank value was directly proportional to microbial concentration. Of the Gram-positive bacteria tested, *Bacillus stearothermophilus* gave the most light emission per cell and about 500 cells could be detected; *Micrococcus lysodeikticus*

and *Sarcina lutea* gave the smallest response, the minimum number of cells detectable being greater than 10^4. The response of Gram-negative bacteria also varied with species but 5×10^4 or fewer of the species tested (Enterobacteriaciae, *Serratia marcescens*, *Pseudomonas fluorescens*, *Neisseria catarrhalis*) were detected. *Saccharomyces cerevisiae* gave more light per cell than *Candida albicans* but, again, less than 10^4 cells of each could be detected. Thus, in general, the automated luminol system was sensitive to 10^3–10^4 cells of each microbial species tested. Oleniacz *et al.* (1966) suggest that, if the system is attached to an efficient air collector, the detection of biological aerosols is feasible.



Luminol

Aminophthalate

The simplicity of the procedure and the high sensitivity and short readout time are features of the luminol chemiluminescence method that make it worth considering for rapid microbial detection purposes. A less attractive feature is that the reaction is activated by substances other than haem compounds and interference can be a serious problem. Our own laboratory tests with a manual method confirm that haematin compounds and washed suspensions of various bacterial species in distilled water can be determined with the sensitivity claimed by Neufeld *et al.* (1965) and by Oleniacz *et al.* (1966). However, it was our experience that: (a) assay blank values determined by mixing the luminol reagent with distilled water varied markedly with different batches of water from the same still; (b) the reproducibility of the signal with the same amount of haemin or bacteria was poor from day to day and often from one series of test and blank determinations to the next; and (c) if bacteria were suspended in solutions of sodium chloride or phosphate, blank values were extremely high and small numbers of bacteria could not be detected.

Oleniacz *et al.* (1966) state that in their automated luminol system, the effects of chemical contaminants in samples that activate luminol could be eliminated by splitting the sample stream with a microbial filter and monitoring the sample before and after filtration. However, this procedure could not eliminate interference by extraneous particulate matter in samples that is retained by the microbial filter.

Oleniacz *et al.* (1967) investigated the effect of non-bioluminescent micro-organisms on other chemiluminescent compounds besides luminol. Intact cells and cell-free extracts of *Serratia marcescens* activated lucigenin (10,10′-dimethyl-9,9′-biacridylium nitrate) luminescence in alcoholic solvents in the absence of either added hydrogen peroxide or alkali. Peak light transmission was attained three seconds after initiation of the reaction followed by rapid decay to a low constant light level. Sensitivity of the reaction in the context of bacterial detection was not reported.

B. Determination of ATP

Adenosine triphosphate is apparently present in all living organisms and it can be determined with the firefly luminescence technique (McElroy, 1947; Strehler and Totter, 1952). Light emission when the enzyme luciferase reacts with luciferin (both in firefly tails), Mg^{2+} and ATP is directly proportional to the amount of ATP present. The chemistry of the reaction is as follows (McElroy *et al.*, 1969; E indicates luciferin; LH_2, luciferase):

$$E + LH_2 + ATP \xrightleftharpoons{Mg^{2+}} E.LH_2.AMP + PP \qquad (1)$$

$$E.LH_2.AMP + O_2 \longrightarrow E + \text{product} + CO_2 + AMP + \text{light} \qquad (2)$$

In its simplest form, the method involves adding a crude buffered extract of firefly tails, containing magnesium ions, to a sample contained in a light-proof chamber adjacent to a phototube, and measuring the peak light emission with a photometer. Certain extraneous reactions (e.g. involving ATP production in the reaction mixture) are largely eliminated if purified luciferase and luciferin are used instead of crude extracts of firefly tails.

The "760 Luminescence Biometer" (E. I. DuPont de Nemours & Co. Inc., Wilmington, Del. 19898) is based on firefly luminescence and is claimed to detect down to 10^{-13} g. ATP per 10 μl. sample. Extraction of bacteria with butan-1-ol is recommended and an aqueous phase is introduced to partition the hydrophilic ATP. A sample (10 μl.) of the aqueous phase is injected into the luciferin-luciferase reaction mixture

and the resulting light flash is automatically converted to concentration of ATP or micro-organisms. Assuming (as DuPont state) that bacterial ATP falls within a range of 2·2 to $10·3 \times 10^{-16}$ g. per cell, detection of ATP from 100–500 bacteria is feasible. However, because an extraction process has to be used, a larger number of bacteria is required and about 2×10^4 is probably a realistic estimate of the minimum number of bacteria detectable.

Another automated firefly luminescence assay is being developed at the Goddard Space Flight Centre of the National Aeronautics and Space Administration (see Mitz, 1969) and it is stated that the instrument will detect 10^5 bacteria within a few minutes.

The fact that the bacterial ATP content varies not only in different species, but also in the same species with the physiological state and environment of the bacteria (Strange *et al.*, 1963), means that with bacteria from, for example, natural environments, the minimum number required to give a detectable response in the firefly luminescence assay may be considerably higher than is the case with freshly grown bacteria in the laboratory.

C. Staining Methods

1. Partichrome Instrument

Detection of bacteria by staining with dyes, followed by microscopic examination, is routine microbiological practice and this principle was used by Nelson *et al.* (1962) in their rapid detection device, the "Partichrome" ("particle " and "colour") instrument designed to detect airborne microbes. With this apparatus, samples of air are drawn in (17·5 l./min.) and the particles deposited by impaction on to a moving tape (Cronar from E. I. DuPont de Nemours & Co., Inc., Wilmington, Del. U.S.A.) coated with a thin film of immersion oil. Impaction of particles larger than 5 μm. is prevented by means of a cyclone-type separator placed ahead of the impactor. The deposited microbes are treated successively with HCl (1·9% aqueous) at 60° for 1 min., water, ethyl violet (1% in 0·5% aqueous Triton X-100, 85°, 45 sec.), water and, after drying, with nitrobenzene for 5 seconds. The treated tape is then scanned with white light through an oil-immersion lens and the transmitted light beam is split. The two beams are directed towards phototubes made sensitive, by means of filters, to blue and green light, respectively. A scanning pattern is obtained by rotation of a mirror system placed after a zirconium arc lamp used as the light source. When white light passes through an unstained particle, both the blue and green

components are absorbed and the output of both phototubes is decreased; if light passes through a blue-stained particle, the output of the green phototube is decreased more than that of the blue phototube and, therefore, blue particles may be counted in the presence of other particles. Pulses from the photomultipliers are analysed with a signal analyser (base-line straightener, amplifier, discriminator and anti-coincidence circuit). Nelson *et al.* (1962) state that low "blue counts" were obtained with carbon and dust particles but the maximum sensitivity of the instrument is not clear from their report.

2. *BioSensor*

The BioSensor, like the Partichrome instrument, is an automated detector based on staining micro-organisms and observing them electronically with microscopical optics (Whittick *et al.*, 1967). It was developed by scientists of the National Aeronautics and Space Administration for space biology, and Mitz (1969) suggests that the use of a fluorescent dye for staining could improve detection sensitivity; sensitivity attained with the present staining method was not reported.

3. *Fluorescin Isothiocyanate Staining*

The use of fluorescein isothiocyanate in a direct staining method for microbial detection was reported by Pital *et al.* (1966). In principle, the method provides broad-spectrum detection capability in contrast to the high specificity of immunofluorescent staining. Heat-fixed smears of bacteria and proteins either alone or in the presence of atmospheric debris and soil were stained with the dye, washed with alkaline buffer (pH 9·6) and examined microscopically with ultraviolet radiation. A stable and apparently specific linkage formed with protein, and non-protein substances were readily destained. A number of bacterial species, hamster kidney cells, wheat germ, and egg albumin were detected but sensitivity data are not given. Pital *et al.* (1966) mention two potential sources of error, namely, reaction of fluorescein isothiocyanate with non-protein substances of unusual chemical configuration, and emission of green autofluorescence by non-protein substances. The method is presented as a "possible life-detection technique".

4. *Membrane Filtration-Staining Methods*

The detection and counting of bacteria in very low concentrations by microscopical observation is difficult, tedious and often inaccurate. Methods for concentrating sparse bacterial populations include evaporation under reduced pressure (Kuzentsov and Karsinkin, 1931; Collins and Kipling, 1957), centrifugation and membrane filtration. Using the latter

method, Cholodny (1928, 1929) quantitatively analysed bacteria in natural waters by transferring the residue retained by a membrane filter to a slide and examining it microscopically. Rasumov (1932) improved Cholodny's technique by directly observing bacteria filtered on to a membrane filter after the filter was rendered transparent with immersion oil. Erlich (1955) investigated several methods for concentrating bacterial suspensions, and considered membrane filtration had advantages over sedimentation or vacuum distillation. He developed a quantitative membrane filtration-staining method that involved staining bacteria on the membrane with Victoria pure blue dye, removing excess stain under suction, and microscopical examination. Better contrast was obtained by prestaining membrane filters with basic fuchsin. The bacteria present in 50 microscopic fields (×800) were counted and the total converted to number in unit volume of original sample by means of a simple formula. Earlier workers who reported membrane filter-staining techniques for counting sparse bacterial populations include Tietz (1949), Jannasch (1953) and Richards and Krabek (1954). Jannasch (1958) describes the use of the technique to study planctonic bacteria.

Ecker and Lockhart (1959) described a rapid membrane filter method for direct counts of micro-organisms in small samples. Millipore membranes (0·45 μm. pore size; 1 in. diameter) are divided into 12 individual filtering areas (2·5 mm. diameter) by pressing with a die having a series of circular indentations smeared with 10% paraffin in petroleum jelly. The membrane filter is placed on the sintered glass of a Millipore microanalysis filter holder and accurately measured volumes (50–100 μl.) of sample (or dilutions of it) are measured with a microlitre pipette on to the marked areas. The samples are filtered under reduced pressure (5–10 mm. Hg). Samples are diluted in 0·85% (w/v) sodium chloride containing 1% (w/v) picric acid to fix the bacteria but, if undiluted sample is used, bacteria are fixed on the membrane filter by adding a drop of saline-picric acid solution. The bacteria are stained with acid fuchsin (0·1% w/v, in H_2O, pH 3·0; 1 min.) and the excess stain sucked through under reduced pressure (50–60 mm. Hg). The stained membranes are taped to glass slides and dried (37°; 15–20 min.). A drop of immersion oil is placed on the dried filter and the bacteria counted under the microscope. To calculate bacteria/ml. original sample (n), the following formula is used:

$$n = \frac{\text{conversion factor} \times \text{dilution factor} \times \text{cells counted}}{\text{number of fields counted}}$$

The conversion factor depends on the size of the field at the magnification used. If a calibrated Whipple ocular is used and the grid area is taken as the standard field, then C is equal to the filtering area divided by the area of the Whipple field at the magnification used.

A rapid membrane filtration staining method for determining the number of viable microbes in food and food processing equipment was reported by Winter *et al.* (1971). Bacteria are rinsed from food or swab samples with sterile diluent and concentrated by filtration on to membrane filters. The filters are incubated on suitable media for 4 hours at 30°, heated at 105° for 5 minutes and stained. Comparison of counts on the dried membranes (rendered transparent) with those of the standard plate count method showed a correlation coefficient of 0·906.

D. Physical Methods

1. Royco Particle Counter

This instrument (Royco Instruments, Inc., Menlo Park, California, U.S.A.) measures quantity and diameter of micrometre-sized particles present in the air or other gases and can be programmed to count all particles within one or more size ranges giving a separate total for each range or all particles larger than any selected range. Full operating instructions are given in the manufacturer's "Operating and Service Manual". One of fifteen individual channels that cover a band from 0·3 μm. to 10 μm. and above can be selected to measure particles within a particular size and 30,000 particles/min. can be counted with a coincidence loss of <10% using a sample flow rate of 0·01 cu. ft./min. The air sample is passed through a light beam where the measured particles scatter light on to a phototube, the pulses from which are analysed, sorted and counted electronically according to the particle size selected for the count. The instrument is calibrated with uniformly sized polystyrene latex particles (graded from 0·5 to 5 μm.; Dow Chemical Company, Physical Research Laboratory, Midland, Michigan, U.S.A.) disseminated with an aerosol generator consisting of an atomiser that forms a fine mist of distilled water in which the particles are carried. The mist is passed down a dryer tube to give a fine dispersion of the calibration particles that then go to the counter. Examples of potential uses of the instrument are monitoring of "clean rooms" and detection of biological aerosols resulting from leaks in culture apparatus.

2. Coulter Counter

Rapid and accurate determinations of cell volumes and concentration of micro-organisms in liquid suspension can be made with the Coulter counter (Coulter Electronics Inc., Hialeah, Florida, U.S.A.). The principle, theory of operation and applications of the instrument are discussed by Kubitschek (1969). Sensitivity of detection depends on the cell volumes of the particles; with bacteria having cell volumes greater

than 1–2 μm^3., bacterial concentrations down to several hundred cells/ml. can be determined but, with smaller cell volumes, the background noise levels are relatively larger and the necessity to increase sensitivity of the instrument increases the detection of foreign particles that are undetected at lower sensitivity settings.

E. Methods Depending on Growth and Metabolism

1. Wolf Trap

This instrument was invented by Dr. Wolf Vishniac for operation on the Martian surface. It consists of a culture tube containing nutrient medium that is automatically monitored for changes in acidity and/or turbidity with pH-value probes and scattered light sensors respectively. Samples are collected and delivered with water and nutrients into five culture tubes. In actual tests, an inoculum of 10–20 bacteria grew to 10^3–10^4 in a few hours to give a signal detectably greater than background. Some details of the instrument and sensor systems are described by Mitz (1969).

2. Gulliver

The device (Mitz, 1969) automatically measures radioactive gases evolved during microbial metabolism of radioactive substrates. A medium was developed in which detectable levels of $^{14}CO_2$ were evolved by the metabolic activity of representative bacteria, streptomycetes, fungi and algae within minutes to several hours. Mixed microbial populations give rise to several population curves that can be distinguished with time.

The early detection of bacterial growth with ^{14}C-labelled glucose was described by Deland and Wagner (1969) who developed an automated radiometric assay (Deland and Wagner, 1970). However, Washington and Yu (1971) evaluated the production of $^{14}CO_2$ as an index of bacterial growth and were unable to detect $^{14}CO_2$ from blood cultures within 6 hours with inoculum sizes ranging from 4 to 4250 colony-forming units.

3. Uptake of ^{32}P-Labelled Phosphate

Macleod *et al.* (1966) reported a membrane-filtration method for the rapid detection of small numbers of viable bacteria based on their ability to take up ^{32}P as orthophosphate. A medium containing potassium chloride, magnesium sulphate, glucose and $^{32}PO_4^{3-}$ was inoculated with a small number of bacteria and incubated with shaking at 37° in parallel with controls without bacteria. After incubation (usually 1 hour), samples (1 ml.) were filtered and washed on sterile Millipore membrane

filters (HA; 0·45 μm.). The membrane filters were placed on planchets, air dried and retained radioactivity was determined with a thin end window Geiger counter attached to a Picker scaler.

Macleod *et al.* (1966) claimed that, under optimum conditions, as few as 23 viable cells/ml. were detected, but this claim was later withdrawn (Macleod *et al.*, 1970). Nevertheless, several thousand bacteria of different species can be detected with the method. An important question is the extent of interference that would be encountered if, for example, samples of natural water were analysed with the technique.

IV. Rapid Specific Identification Methods

A. Immunofluorescence-Membrane Filtration Techniques

Danielsson (1965) reported a methodological study of a membrane filter-immunofluorescence method for the specific identification of bacteria in tapwater or broth cultures within 1 hour. Samples are filtered through non-fluorescent black Millipore membrane filter (HAB(P)G047) and circular pieces (12·5 mm. diameter) cut out with a metal die. The bacteria are stained with fluorescent antibody for 45–60 minutes, washed, mounted and examined with a Zeiss fluorescence microscope. By means of the formula $\chi = NR^2/20r^2$ (N, number of bacteria/20 fields; R = 20 or 40 mm. depending on the diameter of the membrane surfaces used; r, diameter of field of vision, 0·32 mm.; with 40 mm. diameter filtering surface, $\chi = 781{\cdot}25$ N; with 20 mm. diameter filtering surface, $\chi = 195{\cdot}31$ N) the concentration of bacteria specifically reacting with antibody can be determined. The technique easily allowed quantitative determination of bacteria present in a minimum concentration of 10^5/l. but, with 10^3 bacteria/l., the method was time consuming. Danielsson and Laurell (1965) described the application of this method to the detection of small numbers of bacteria in water. When bacteria, separated from a sample by filtration through a membrane filter, were eluted by the method of Miller (1963) that involves a simple washing procedure with glass beads or a magnetic stirrer, the lower limit of sensitivity for direct fluorescence microscopy was about 5000 bacteria/l. water. By direct staining of bacteria on non-fluorescent membranes with fluorescent antibody, the lower limit was about 1000 bacteria/l. water; if this technique was combined with an enrichment procedure, it was possible to demonstrate 2–50 bacteria/l. water within 4–6 hours.

Closs (1968) reported a membrane filter-immunofluorescence technique that is claimed to overcome certain disadvantages of Danielsson and Laurell's (1965) two-step technique. Membrane filters (black Sartorius, MF50) on to which the bacteria were collected were cultured on a filter soaked with growth medium containing fluorescent antibody

conjugate. Specifically stained microcolonies developed within 2–4 hours and were detected with the fluorescence microscope. *Haemophilus influenzae*, *Proteus rettgeri* and *Pasteurella haemolytica* were detected in this way. Sensitivity limitations are discussed and possible uses of the technique mentioned (Closs, 1968).

Guthrie and Reeder (1969) reported a further modification of the membrane filter-fluorescence technique. Water from a small pond was filtered through a Millipore membrane filter (HABG 047) that was then cultured on Trypticase Soy agar at 35°. Colonies appeared after 5 hours but incubation was continued for 12 hours. The membrane was overlayed with 1–2 ml. pooled normal rabbit serum for 5 minutes at 20°. The serum was removed by suction and the membrane overlayed with fluorescent rabbit antiserum for 5–20 minutes. The antiserum was sucked through, and the membrane washed with 10–15 ml. phosphate-buffered saline. The stained membrane was overlayed with mounting fluid and examined with a dissecting microscope (magnification ×10) using visible light to determine the total count and ultraviolet radiation to determine fluorescent colonies. According to the authors, adaptation of the technique for use with lower magnification takes somewhat longer than the method of Danielsson and Laurell (1965) but has the advantage that all colonies on the entire filter can be counted. Critical factors are: (i) washing filters during staining by filtration under reduced pressure; (ii) using glycerol-containing mounting fluid to prevent drying, while enhancing fluorescence; and (iii) rapid counting of colonies once the preparations are exposed to ultraviolet radiation (because bleaching occurs).

The rapid detection of a small number of airborne bacteria with a membrane filter-fluorescent antibody method was described by Jost and Fey (1971). Calibrated air samples from aerosols of *Serratia marcescens* were drawn through (10 l./min.) a Millipore aerosol filter holder fitted with a non-fluorescent membrane filter, and the organisms were rapidly identified with Danielsson's (1965) method using a high power incident light ultraviolet microscope.

B. Radioactive Antibody Technique

Analysis of data that have been obtained for antibody uptake/bacterial cell, maximum ^{125}I or ^{131}I-labelling intensity of antibody protein commensurate with retention of immunological activity, and the efficiency of radioactivity counting instruments with ^{125}I, indicate that extremely high sensitivity is theoretically attainable with microbial detection methods based on the use of ^{125}I-labelled homologous antibodies. In practice, however, determination of a few bacteria is possible

only if, after separation of the labelled immune complex, the variation in the level of non-specifically attached radioactivity is less than the radioactivity of the immune complex. If residual non-specifically attached radioactivity (determined by testing "blank" samples without bacteria) is high compared with the radioactivity of the immune complex, even relatively small variations will swamp the signal from the complex. Strange *et al.* (1971) largely resolved the problem of high erratic assay blank values with a radioactively-labelled antibody-membrane filtration technique and were able to detect and determine specific bacteria and bacterial spores (down to a minimum of 500–1000 organisms) within 8–10 minutes. This order of sensitivity was only obtained with radiolabelled immuno-purified antibodies having extremely high specific immunological activity.

1. Immuno-Purified Antibodies

When bacteria were incubated at 25°–37° with ^{125}I- or ^{131}I-labelled homologous antisera or crude salt-precipitated antiserum globulin fractions for 6–15 minutes, and the resulting immune complexes separated by filtering and washing on a membrane filter, a radioactive signal higher than the assay blank (no bacteria) value was not obtained with less than about 10^5 bacteria in the sample. Iodination of such crude antibody preparations results in products containing a large proportion of labelled non-immune proteins, more of which is taken up non-specifically by a membrane filter than the amount of labelled antibody that combines specifically with a few bacteria. The amount of anti-bacterial globulin in a high-titre antiserum may account for only 1–2% of the total protein which means that up to 99% of the isotope used for labelling is wasted and relatively large amounts of isotope must be used to obtain antibody of sufficiently high specific radioactivity for rapid detection purposes.

Methods investigated for purifying antibody proteins included electrophoresis, isoelectric focusing and immunopurification. Immunopurification provided the best products for the radio-assay and preparative methods, based on the previous work of Dr. B. T. Tozer and Dr. A. P. MacLennan (unpublished), were described by Strange *et al.* (1971).

2. Radioactive Iodination of Antibacterial Globulins

Purified antibodies were iodinated with carrier-free ^{125}I or ^{131}I (obtained from the Radiochemical Centre, Amersham, Bucks, England) using the chloramine-T method (Hunter and Greenwood, 1962; Bocci, 1964; Glover *et al.*, 1967) as described by Strange *et al.* (1971). Optimum

specific radioactivity of antibody protein for radio-assay purposes was 15–30 μCi./μg. protein. Although a higher labelling intensity increased the signal from a given number of bacteria, the assay blank value also increased and no improvement in sensitivity was obtained.

3. Radio-assay

The simple assay procedure consists of incubating samples of bacterial suspension (0·1–1·0 ml.) and saline buffer suspending medium alone, with labelled antibody reagent (50–100 μl. containing 10–25 μg. ^{125}I-labelled antibody protein and 0·16 ml. clarified normal rabbit serum/ml.) for the selected reaction time at 25°–30°. Reaction mixtures are diluted with saline phosphate buffer (0·11 *M*-sodium chloride and 0·02 *M*-sodium phosphate buffer, pH 7·7) containing 1% Brij 35 (polyoxyethylene lauryl ether; British Drug Houses Ltd., Poole, Dorset, England) and rapidly filtered by suction through black or white Millipore membrane filters (0.75 in. diameter) held in special stainless steel filter jigs (Strange *et al.*, 1971). After a standard washing procedure with saline-phosphate containing Brij 35, the washed membrane filter is placed between discs of linen tracing paper and a central area 8% less than the filtration area is accurately cut out, with a compound blanking punch, and collected into a glass vial (3·5 × 1 cm.). Test and blank samples should be tested at least in duplicate. The radioactivity of the membrane discs is measured with a conventional well-type sodium iodide scintillation counter and scaler, or liquid scintillation counting can be used (Ashcroft, 1970).

The method allows the specific detection and determination of a minimum of 500–1000 washed bacteria, or bacterial spores, contained in 0·1 ml. samples, to be obtained within 8–10 minutes. Of course, very small bacteria (e.g. species of *Brucella*) tend to give a lower signal per cell than larger bacteria, but, provided that the assay blank value is relatively low, and reproducible, less than 10^4 cells of all species are detectable with a significant signal to blank ratio.

The relationship between number of bacteria (500–10^5) and radioactive signal is usually linear, but sometimes the signal per bacterium decreases progressively as the number of bacteria in the sample is increased. Non-linear relationships are presumed to be due to the immunological heterogeneity of antibody molecules in certain purified antibody preparations.

The sensitivity and accuracy of the assay decrease if samples contain particulate matter that non-specifically attaches antibody and is retained by a membrane filter. This type of interference is decreased by pretreating samples with clarified normal rabbit serum (0·1 to 0·2 ml./ml. sample) for a few minutes before assay.

C. Analysis of Bacterial Growth Products

Detection and identification of bacteria by examination of their growth products with gas chromatography has been reported by several workers (Henis *et al.*, 1966; Moore *et al.*, 1966; Brien, 1967; Moss and Lewis, 1967; Mitruka and Alexander, 1967; 1968; 1969). Mitruka and Alexander (1968) were able to detect a small number of bacteria of several different species within 2–12 hours depending on the species. A modified Proom and Knight (1955) liquid medium (10 ml.) was inoculated with a washed suspension of bacteria (0·1 ml.; 4–810 × 10^2 cells) and incubated at 35°. The culture was acidified (5 *M*-HCl and 0·2 *M*–HCl–KCl buffer, pH 2·0) and centrifuged (2000**g**; 15 min.). The supernatant liquid (5 ml.) was saturated with anhydrous sodium sulphate and extracted with ethyl ether (3 × 10 ml.). The combined extracts were acidified (pH 2·0) and concentrated to 5 ml. on a rotary evaporator at room temperature. The concentrate was saturated with anhydrous sodium sulphate and samples (3 μl.) injected into a dual channel gas chromatograph fitted with two detectors (electron capture, "ECD"; flame ionization, "FID") each of which received the equivalent of 1·5 μl. of the input. Eluted compounds were identified by comparing their retention times and "Q values" (area of ECD peak)/(area of FID peak) with those of authentic standard compounds. The sensitivity limit was the quantity of metabolite or number of viable bacteria giving a peak of 10 mm^2. area with appropriate settings of the instrument. With inocula of less than 10^4 cells/ml., the presence of *Proteus vulgaris*, *Streptococcus faecalis*, *Strep. liquefaciens*, *Escherichia coli* B, *Bacillus cereus* and *B. popilliae* was detected within 2–4 hours but a 7–12 hours growth period was necessary to detect products formed by *Serratia marcescens*, *Aerobacter aerogenes*, *E. coli* K12, *Staphylococcus aureus* and *Salmonella typhimurium*. Metabolites formed by the equivalent of less than a single cell of *B. cereus*, *Strep. faecalis*, *P. vulgaris* or *E. coli* B were sensed with the ECD; the FID was generally less sensitive. Letters were assigned to the gas–liquid chromatography peaks ("Bacterial signatures"; Henis *et al.*, 1966) in the order of their retention times to provide a "formula" for each bacterial species.

Gas chromatographic analysis of bacterial growth products provides a relatively rapid means of specifically identifying bacteria and, indeed, there is evidence that different strains of a single species can be differentiated. In certain genera of anaerobic bacteria, the fermentation patterns do not differ between species in each genus but the patterns may be useful to establish the identity of the genus with certainty (Moore, 1967). Mixed bacterial species in a sample may be difficult to deal with but Fuller (1967) suggests that " . . . perhaps new media that bring about

the generation of unique dead-end metabolites, or application of sophisticated cross-correlational techniques to a maze of peaks may provide a path to the answer".

D. Gas Chromatography-Pyrolysis Methods

Identification of bacteria by means of "pyrograms", that is profiles of the products separated from pyrolysed bacteria with gas–liquid chromatography, has been reported (Garner and Gennaro, 1965; Reiner, 1965, 1967). Pellets of freeze-dried killed bacteria are heated in an inert atmosphere on a nickel filament at 850° for 10 seconds in a pyrolysis module attached to a gas chromatograph, and peaks representing the eluted products are recorded. Differences in the pyrograms of different bacterial species are mainly quantitative, not qualitative, and Reiner (1967) states that several papers dealing with gas-liquid chromatographic analyses of microbial extracts or metabolic products either ignore quantitative aspects or they simply lack rigorous data. According to Reiner (1967), pyrolysis-gas-liquid chromatography is not a rapid detection-identification method but it allows unequivocal identification of bacteria within a considerably shorter time than is possible with conventional techniques.

In contrast, Mitz (1969) proposes pyrolysis coupled with gas–liquid chromatography and mass spectroscopy for the rapid detection of minute amounts of bacterial components (protein, carbohydrates, lipids, nucleic acids). The "projected characteristics" of potential instrumented systems are compactness, and analyses every few minutes with sensitivity and specificity to detect a few hundred micro-organisms.

E. Analysis of Phosphorescent Decay

When proteins or aromatic amino acids are irradiated with ultraviolet radiation, they exhibit fluorescence and phosphorescence (Hoerrman and Balekjian, 1966). Adelman *et al.* (1967) irradiated intact living or killed cells of five bacterial strains, and found that the general forms of the total and phosphorescent emission curves were different, if not unique, from one species or strain to another. They proposed analysis of phosphorescent decay curves as a means of identifying bacteria.

V. Rapid Determination of Microbial Viability

A. Indirect Methods

Methods for determining microbial viability not depending on cell multiplication were reviewed by Postgate *et al.* (1961) and Postgate (1967). They include vital staining (Gilliland, 1959), differential staining

(Strugger, 1948; Wade and Morgan, 1954), reduction of redox dyes (Greenburg *et al.*, 1958), leakage of purines (Koch, 1959), changes in refractive index (Barer *et al.*, 1953; Fikhman, 1959a, b), changes in extinction values of bacteria in solutions of salts compared with extinction values in distilled water (Mager *et al.*, 1956) and growth without division (Valentine and Bradfield, 1954). None of these methods has been accepted by microbiologists as a valid alternative to the more lengthy but reliable procedures involving microbial growth, although all of them may give useful information concerning microbial structure and function.

B. Direct Methods

A comprehensive survey of microbial microculture methods is given by Quesnel (1969). Microcultures on cellophan irrigated with nutrients (Powell, 1956) or thin layers of nutrient agar (Postgate *et al.*, 1961) will give results (in terms of the percentage of viable bacteria in a population) within 2–2·5 hours with fast-growing bacteria. The simple elegant slide culture method of Postgate *et al.* (1961) has found wide acceptance as a rapid reliable alternative to conventional plate count methods for determining viability. A small loopful of a bacterial suspension containing about 5×10^7 cells/ml. is required as the inoculum, but less dense suspensions are easily concentrated by centrifugation or membrane filtration. The method may give inaccurate results with populations of bacteria having a wide range of generation times (e.g. certain stressed populations) and cannot be used with filamentous bacteria (e.g. certain *Bacillus* species). The method cannot be used to determine the total number of bacteria or the number of viable bacteria in a population.

Membrane filtration techniques provide a means of determining the viability of sparse microbial populations in many cases within a shorter time than is possible with conventional viable cell plate counts (Mulvany, 1969).

VI. Rapid Detection and Determination of Viruses

A. Immunofluorescent-Monolayer Techniques

Early quantitative methods based on counting fluorescent cells after viral infection and fluorescent antibody staining were reported by Deibel and Hotchkin (1959) and Rapp *et al.* (1959) but the first fully characterized assay was described by Wheelock and Tamm (1961) for Newcastle disease virus. Application of the principle to the detection and determination of a large variety of viruses and rickettsiae has been reported by many virologists (see Carter, 1971). The sensitivity of the fluorescent antibody-monolayer technique with vaccinia and variola

virus was investigated by Carter (1965). Coverslip monolayers of HeLa cells were infected with varying concentrations of virus and the minimum incubation time required for the appearance of foci detectable with fluorescent antibody was determined. Infection with from 2–10^6 plaque-forming units of vaccinia or variola viruses/ml. was detectable within 16 hours decreasing to 4 hours, and 24 hours decreasing to 10 hours, respectively.

As mentioned above, centrifugal force may be used to promote adsorption of virus on to coverslip monolayers (Hahon and Nakamura, 1964). Under these conditions, adsorption is highly efficient and rapid. Plastic chambers for the cultivation of tissue cells on microscope slides were reported by Sattar and Westwood (1967a). Fluorescent cells are counted in a random 5–10% sample of the monolayer and the use of a simple counting grid and pointer system to select a uniform pattern and number of randomized fields (Sattar and Westwood, 1967b) improves the accuracy of results.

B. Immuno-Adherence

The "immuno-adherence phenomenon" and its applications in virology were discussed in Section II, E, (p. 114). An "immuno-adherence assay" depending on the agglutination of antibody-coated erythrocytes by viruses (Smith and Courtney, 1965) was reported by Mitz (1969). Erythrocytes sensitized with antiserum, in the presence of a small number of homologous viruses, formed dimers in numbers depending on the viral concentration. The present laboratory method is claimed to detect as few as 10^3 viruses/ml. in a few minutes, ultimate sensitivity depending on the device to count the number of erythrocyte dimers formed. Mitz (1969) suggests the possibility of adapting antibody-coated materials as a staining reagent in an instrument similar to the BioSensor or Partichrome instrument. He also suggests the development of broad-spectra antisera to overcome the limitation of the method, i.e. the requirement for a large number of specific antisera.

C. Radioactive Antibody Method Applied to Bacteriophage T7

Bacteria can be rapidly detected and determined with homologous ^{125}I- or ^{131}I-labelled homologous antibodies (Strange *et al.*, 1971), and in principle there is no reason why the method cannot be applied to viruses. However, the relatively small size of virus particles presents additional problems that are not easily resolved. The amount of antibody taken up by a virus particle relative to that taken up by a bacterium is one to several orders of magnitude lower and this affects the theoretical attainable sensitivity in terms of the minimum number of virus particles detectable. Then there is the problem of rapidly separating labelled

virus-immune complex from excess labelled antibody. The feasibility of detecting viruses with radiolabelled antibodies was studied using bacteriophage T7 (60 × 80 nm. diameter) as a model particle in the virus size range (R. E. Strange, unpublished work).

Purified antiphage T7 globulin was obtained by adsorbing high-titre antiphage serum with purified phage T7, separating and washing the agglutinate by centrifugation and dissociating the complex with 0·1 *M* hydrochloric acid—1% (w/v) sodium chloride solution (pH 1·2) at 2°. Released soluble antibody was separated by centrifugation, neutralized, concentrated by pressure dialysis and fractionated with ammonium sulphate. The purified protein fraction (precipitated at 0–50% saturation of ammonium sulphate) was dialysed and iodinated with ^{125}I to give a product with a specific radioactivity of 30–40 μCi./μg. protein.

The problem of separating labelled phage immune complex after reaction of phage suspension with ^{125}I-labelled antibody was resolved in three ways. (a) Phage was adsorbed on to host bacterial cells (*Escherichia coli* MRE 160) for 15 minutes at 37° and the phage-cell complex reacted with ^{125}I-labelled antiphage globulin (exhaustively pre-adsorbed with *E. coli* MRE 160). The labelled immune complex could then be easily separated by filtration and washing on a Millipore membrane filter (0·45 μm.). (b) A suspension of phage particles was reacted with ^{125}I-labelled antiphage globulin and the reaction mixture filtered and washed on a Millipore membrane filter (0·45 μm.) impregnated with heat-fixed (80°, 10–15 min.) purified antibody. This "immunofiltration technique" retained ^{125}I-labelled phage immune complex on the membrane filter but allowed excess labelled antibody to pass through. (c) Phage suspensions in distilled water were freeze-thawed several times and the broken phage reacted with ^{125}I-labelled antiphage globulin. On filtration, and washing reaction mixtures through Millipore membranes (0·22 μm.), ^{125}I-labelled immune complex was retained but excess labelled antibody filtered through. The method was apparently specific since ^{125}I-labelled heterologous antibodies were not taken up by broken phage.

Results obtained with the three assay procedures are shown in Table 3. In each case the radioactive signal given by the immune complex minus the mean assay blank value was proportional to the phage concentration. The highest sensitivity was obtained with the broken phage method mainly due to the fact that the assay blank value was relatively low; a minimum of 5×10^4 to 10^5 plaque-forming units (about 2·5 to 5×10^5 total phage particles according to electron microscope counts kindly done by our colleague Mr. J. Harris) were detected.

Thus, particles in the virus size range can be rapidly and quantitatively detected with the radiolabelled antibody method, and sensi-

TABLE 3. Detection of Bacteriophage T7 with ^{125}I-Labelled Antiphage Globulin by Three Methods.

Number of plaque-forming units	Radioactivity (counts/sec.; blank subtracted)		
	1 (Adsorption on to host cells)	2 (Immuno-filtration)	3 (Broken phage)
5×10^4	—	—	3·5
10^5	—	—	3·9
5×10^5	45·0	13·5	16·8
10^6	82·0	27·1	41·0
5×10^6	—	124·0	256·0
10^7	206·0	217·0	474·0
Blank	11·5	22·0	4·0

Method 1: Sample (0·1 ml) treated with *Escherichia coli* MRE 160 cells (60 μg. dry wt.; 25 μl.) for 15 min. at 37°; ^{125}I-labelled antibody (0·5 μg.; 80 μCi./μg. protein) was added and after 15 min. at 25° the mixture was filtered and washed through a Millipore (HA) membrane.

Method 2: Sample (0·1 ml.) treated with ^{125}I-labelled antibody (0·5 μg.; 40 μCi./μg. protein) for 15 min. at 25°; the reaction mixture was filtered and washed through a Millipore (HA) membrane, the centre impregnated with 20 μg. of heat-fixed purified antibody.

Method 3: Sample (0·1 ml.) of phage T7 broken by six freeze-thaw cycles in distilled water was treated with saline phosphate buffer (0·1 ml.) and ^{125}I-labelled antibody (0·5 μg.; 40 μCi./μg. protein) for 15 min. at 25°. The reaction mixture was filtered and washed through a Millipore (GS) membrane. In each case, the radioactivity of the punched out membrane filtration area was measured.

tivity, on a particle mass basis at least, is extremely high. In principle, one or other of the methods would appear to be applicable to the detection of animal viruses but this has not yet been tested.

D. Detection of Virus Activity with Gas Chromatography

Application of gas chromatography to the detection of viral infections was reported by Mitruka *et al.* (1968, 1969). Samples of serum from infected animals or tissue cultures were acidified, dried under vacuum ,dissolved in pyridine and treated with hexamethyldisilazane and trimethylchlorosilane. Microlitre samples were then examined by gas chromatography with electron capture or flame-ionization detectors. Gas chromatographs of serum extracts of dogs inoculated with canine infectious hepatitis showed two metabolites not present in the serum from uninoculated animals. Chromatographs of extracts of tissue cultures of dog kidney cells inoculated with canine hepatitis, herpes, distemper or parainfluenza virus each showed two or more different metabolites not present in uninoculated cultures. Certain unique metabolites

detected *in vivo* were chromatographically similar or identical to those noted *in vitro*.

VII. Conclusions and Prospects

Detection of a few microbes in a few minutes is a difficult problem for which a general solution has not yet been found. Methods designed to have this capability may give entirely satisfactory results with washed microbes in the laboratory but fail with samples from natural or other environments. Extremely fast readout requires the use of near instantaneous reactions such as luminol chemiluminescence or firefly luminescence, but neither reaction is specifically activated by microbes or even a component that is unique to microbes. A microbiologist would be unwise to report the presence of microbes in a sample of unknown composition solely on the basis of a positive response with either method. It is inevitable that the maximum sensitivity claimed for detection methods is the threshold response of the reaction involved determined under optimum conditions, but in practice sensitivity depends on the relative concentration of interfering material in the sample. Microgram concentrations of extraneous matter with a specific reactivity below that of the microbial compound being detected may still interfere in the presence of only nanogram to picogram amounts of that compound. Certain broad-spectrum methods can therefore only be applied if it is known that samples are free from or contain a constant amount of interfering matter, or following fractionation of samples to separate the microbes. The inclusion of automatic sample-fractionation devices in fast readout detectors based on broad-spectrum principles would at least partially resolve the interference problem.

Problems associated with rapid specific identification methods depending on immunological principles differ from those with broad-spectrum methods. The microbial components detected are usually unique to a particular species and extraneous biological material in samples will not specifically react with the reagent. However, interference is caused by adherence and occlusion of the reagent by materials used in the assay and extraneous particles in samples, and this may prevent qualitative or quantitative assessment of a small number of microbes. Application of such methods to microbial detection is restricted because the micro-organisms likely to be present in samples must be predicted so that the necessary reagents are available. It has been suggested that the development of antibodies with decreased specificity that react with a component universally present in bacteria or in viruses would remove this restriction, but suitable antibodies are not at present available.

If the requirement is not for immediate detection, and results within several hours are acceptable, more precise and reliable methods, depending on microbial growth and replication, are available. The large amplification of a population resulting from a short period of growth shifts down several orders of magnitude the sensitivity required of an analytical method; for example, a population of 10^3 viable *Escherichia coli* cells will increase to about 10^5 cells during unrestricted growth in culture medium for about 3 hours. The larger the population, the smaller the problem of interference by extraneous material initially present in the sample. The interesting discovery that the growth products of bacteria grown under controlled conditions are characteristic of the bacterial species (Henis *et al.*, 1966) is an important advance in the context of rapidly detecting and specifically identifying microbes.

Considerable effort is being expended on automating microbial detection methods, for example, for use in aerosol detection (Nelson *et al.*, 1962; Oleniacz *et al.*, 1966) or space research (see Mitz, 1969) and several working detectors are now in existence. It is clear that the relevant engineering problems have been largely resolved but it remains to be seen whether the biological principles involved will prove satisfactory in practice.

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Bacterial Exopolysaccharides

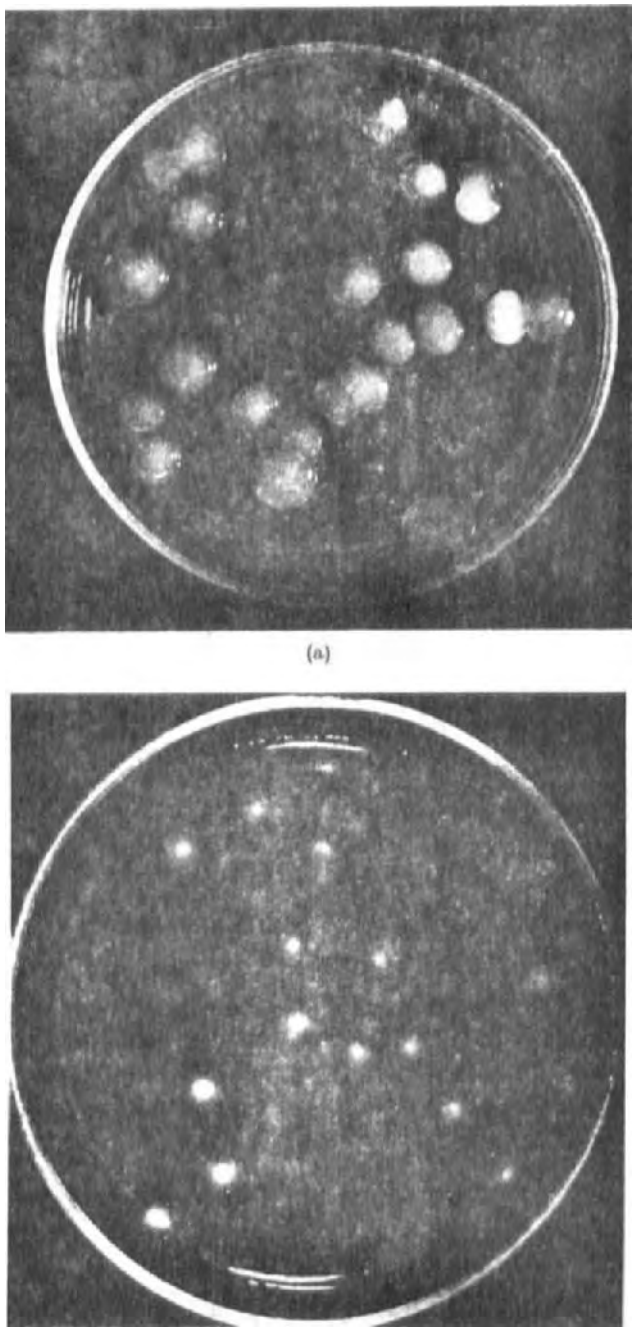
I. W. SUTHERLAND

Department of General Microbiology, University of Edinburgh, Edinburgh, Scotland

I. Introduction 143
II. Production 146
A. In Growing Cultures 146
B. In Washed Cell Suspensions 148
III. Properties 149
A. Isolation 149
B. Purification 149
C. Composition 150
D. Structural Features 156
E. Enzymic Hydrolysis 158
F. Structures of Some Exopolysaccharides 165
IV. Biosynthesis 185
A. Enzymes and Precursors 185
B. Cell-Free Synthesis 188
C. Control 201
V. Function of Exopolysaccharides 206
VI. Unanswered Questions 206
VII. Conclusions 207
VIII. Acknowledgements 208
References 208

I. Introduction

The bacterial cell resembles cells of higher organisms and of other micro-organisms in forming a number of polysaccharides. These are either components of cell structures, such as the teichoic acids and lipopolysaccharides which form integral components of prokaryotic cell walls, or they may provide mechanisms for storing carbon or energy in polymeric form. In addition, numerous cells synthesize polysaccharides which lie outside the cell wall or are found secreted into the environment. These polymers form the subject of the present article. Depending on their structural relationship to the bacterial cell, they have been variously termed slime, capsular or microcapsular polysaccharides. The name *exopolysaccharide* provides a general term for all these forms of bacterial polysaccharide found outwith the cell wall and will be used in this context.



(a)

(b)

FIG. 1. (a) Mucoid colonies and (b) non-mucoid colonies of wild-type *Klebsiella aerogenes* grown on nutrient agar (72 hr. at 35°).

Bacterial exopolysaccharides have been the subject of numerous studies over a period of many years (see reviews by Wilkinson, 1958; Stacey and Barker, 1960; Lüderitz *et al.*, 1968). Some studies, such as those on *Diplococcus pneumoniae*, have been primarily concerned with the role of the exopolysaccharide in bacterial virulence for the host animal and with the use of immunochemistry as a tool in the identification of strains. Other studies on soil bacteria have been more concerned with the possible role of the exopolysaccharide in soil fertility and in microbial survival. Despite much expenditure of effort, little is known about the *in vivo* role of these polymers and few attempts have been made to correlate the large amount of information obtained on their chemistry and biology.

In the laboratory, it is clear that exopolysaccharides are not essential for bacterial growth and survival. Non-mucoid mutants, unable to form exopolysaccharides, are readily isolated. They occur spontaneously or after mutagenesis and can be readily recognized by their altered colonial appearance (Figs. 1a and b). In mucoid cultures, capsules or slime can be removed physically or enzymically without adverse effect on bacterial growth. Further incubation leads to synthesis of new exopolysaccharide.

Because of their highly hydrophilic nature and their chemistry, most methods of staining exopolysaccharides are of only limited application. Capsules can be observed using light microscopy either in unstained preparations examined directly or by use of phase-contrast illumination. Exopolysaccharides are best detected by negative staining techniques. The India Ink method (Duguid, 1951) has the advantage of distinguishing between cells or cultures which form a discrete capsule (Fig. 2) and those which excrete an amorphous polysaccharide into the environment where it is observed in the form of slime, unattached to the bacteria. Use has also been made of the "Quellung" reaction, often wrongly termed the "capsule-swelling" technique. In this, deposition of a precipitate of

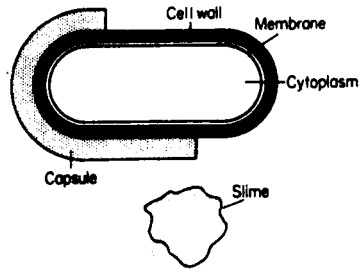


FIG. 2. Diagrammatic representation of a bacterial cell with capsule and slime.

homologous antibody at the periphery of the capsule permits its visualization on microscopic examination (Cruickshank, 1966). It is also unfortunate that electron microscopy and freeze etching techniques are of limited application with regard to bacterial exopolysaccharides. As a result, little information on their possible internal structures is available.

In cultures of capsulate cells, some slime is obtained due to its gradual release from the capsules. This material, like the slime obtained from non-capsulate mutants, is chemically indistinguishable from capsular material. Production of either capsules or slime is found in many species of Gram-positive and Gram-negative bacteria. It is assumed, in the absence of evidence to the contrary, that these polymers are physically identical and that polysaccharides produced under different growth techniques are constant in size and other properties. Whether this assumption is justified must await improved techniques and further experimentation.

The purpose of the present review is to discuss some of the results obtained from studies on the structure and synthesis of exopolysaccharides in order to present a model for their biosynthesis within the bacterial cell.

II. Production

A. In Growing Cultures

Although most, but not all, mucoid bacteria produced some exopolysaccharide under all cultural conditions, the growth environment is very important for maximal exopolysaccharide production. The influence of environmental conditions has been studied in a number of bacterial species using both growing cultures and alternatively, conditions under which the cells can produce polysaccharides but do not grow. Most exopolysaccharide-synthesizing bacteria are either aerobes or facultative anaerobes, and polysaccharide production is normally highest when limitation of oxygen is not imposed. Perhaps, as a result of this, more polymer is frequently excreted in solid media than is obtained from comparable amounts of cells grown in liquid media. Studies on *Klebsiella aerogenes* (Wilkinson *et al.*, 1955) established that the composition of the exopolysaccharide was independent of the carbon and energy source provided for growth and polymer synthesis. This is probably true for most if not all heteropolysaccharides found as bacterial slimes and capsules. Where, however, more than one exopolysaccharide is formed, variations in the proportions of the different polymers synthesized may occur. In contrast, synthesis of a number of

homopolysaccharides, such as levans and dextrans, requires the provision of a specific substrate, thus reflecting the involvement of highly specific enzymes acting on oligosaccharide carbon sources.

In defined media, exopolysaccharide production was stimulated by nutrient limitation in the presence of excess carbohydrate (Duguid and Wilkinson, 1953; Wilkinson *et al.*, 1954). Limitation of the carbon and energy source resulted in minimal polysaccharide production. Deficiency of nitrogen, phosphorus, or sulphur sources in the presence of carbohydrate, all led to increased exopolysaccharide production by strains of *K. aerogenes* or *Escherichia coli* until a maximum was reached. This value was not exceeded even when the cultures still apparently possessed sufficient carbohydrate and oxygen to permit further synthesis. The maximum value, as measured by the polysaccharide : cell nitrogen ratio, depended on the limiting nutrient. It was highest for phosphate limitation (48) and lower for nitrogen (29) and sulphate (17) limitations. Deprivation of potassium limited growth in a manner similar to deprivation of other nutrients, but was much less effective in stimulating polysaccharide synthesis (Duguid and Wilkinson, 1954). This may have been due to restricted uptake of the carbohydrate substrate, as potassium limitation also results in cells with a very low content of intracellular glycogen even though the other growth conditions were expected to favour polysaccharide synthesis (Dicks and Tempest, 1967). The cause of the lowered synthesis of polysaccharide was attributed to an antagonism between potassium and ammonium ions.

For *K. aerogenes* in liquid media, the *rate* of polysaccharide production was greatest during the exponential phase of growth and gradually decreased thereafter (Duguid and Wilkinson, 1953). The maximal *amount* of expolysaccharide was, however, accumulated after cell multiplication had effectively ceased (after 14–48 hr. growth). This was also reflected in the increased diameter of the capsules during the later stages of growth.

Although a high carbon : nitrogen ratio in the medium favoured exopolysaccharide production in mucoid strains of *Chromobacterium violaceum* (Corpe, 1964) in a manner similar to that observed for *K. aerogenes*, this was not true for cellulose production by *Acetobacter acetigenum* (Du Iman, 1959). Both growth and cellulose synthesis were only slightly affected by changing the glucose concentrations in a defined medium. Cellulose synthesis was stimulated under these conditions by addition of acetate, citrate or succinate. Although supplementation with ethanol increased the cell yield, it did not affect cellulose synthesis. Results obtained with one bacterial species should only be extrapolated with care when considering other strains, but the response of exopolysaccharide production by *Rhizobium meliloti* to the nitrogen concentration

of the medium resembled that of *K. aerogenes* and *E. coli* (Dudman, 1964). Where the *Rhizobium* sp. showed a markedly different response from the enteric bacteria was in the effect of aeration. Both growth and polysaccharide production were favoured, under the conditions employed, by low aeration. It is probable that similar conditions, i.e. carbohydrate excess and low aeration together with low concentrations of nitrogen, are found in many of the natural environments in which production of large amounts of exopolysaccharide present an effluent problem. The micro-organisms responsible for this are mainly strains of *Sphaerotilus natans* and related species.

In his studies using *C. violaceum*, Corpe (1964) observed that the presence of Ca^{2+} strongly stimulated polysaccharide production. When Fe^{2+} was omitted from the culture media, increased polysaccharide synthesis was detected despite a decrease in bacterial growth. Both growth and polymer production required the presence of Mg^{2+} ions which could not be replaced by other divalent cations such as Mn^{2+}

B. In Washed Cell Suspension

Studies on exopolysaccharide synthesis in nutrient media showed that the polymers continued to be excreted some time after growth and cell division had ceased. It was thus logical to use non-nutrient suspending liquids (washed suspensions) to determine some of the parameters necessary for optimal polysaccharide production. Using *K. aerogenes* type 54 (strain A3 (S1)), Wilkinson and Stark (1956) obtained uniform production of exopolysaccharide over a period of 4 hours in washed cell suspensions. Under optimal conditions, about 0·75% of the available carbohydrate was converted to exopolysaccharide each hour. A further 0·25% of the of the glucose was utilized to form intracellular polysaccharide (glycogen). Such high conversion rates were only obtained in aerated cell suspensions with excess utilizable carbohydrate in the presence of K^+, Mg^{2+} and Ca^{2+}. The greatest decrease in such levels followed the exclusion of oxygen or the omission of K^+. Using a similar slime-forming mutant of *K. aerogenes*, Norval (1969) obtained similar results and also showed that the rate of polysaccharide synthesis in washed cell suspension by log-phase and stationary-phase cells grown in several different media was very similar (Fig. 3). The ability to synthesize extracellular polysaccharide was lost from older cultures (48 hr.) in synthetic media and was very low in cells grown in media lacking a utilizable carbohydrate. Production of exopolysaccharide was independent of glycogen-synthesizing capability, as log-phase cells contained little, if any, glycogen (Koeltzow *et al.*, 1968; Norval, 1969).

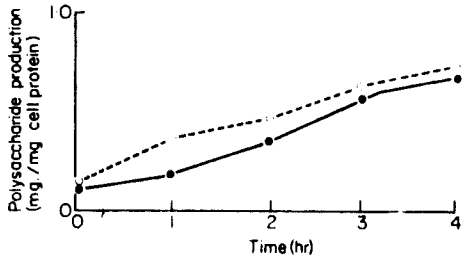


FIG. 3. Time-course of polysaccharide synthesis in washed cell suspensions by Log-Phase (•–•) and Stationary-Phase cells of *Klebsiella aerogenes* A1(S1). From Norval (1969).

III. Properties

A. Isolation

The isolation of exopolysaccharide presents few problems when it is secreted as an extracellular slime. The lack of physical attachment between polysaccharide and cell enables differential centrifugation to be employed. The main problem in such preparations tends to be the high viscosity of the slime solutions which hinders deposition of the cells. Different slime preparations vary greatly in their intrinsic viscosities and no firm rules for centrifugal speeds required to achieve adequate separation can be given. The polymers are recovered from the supernatant fluids by addition of acetone or ethanol. If the exopolysaccharide is in the form of a capsule it must be detached from the cells. Again it is difficult to generalize, as capsules are much more readily removed from some strains than from others. Gentle stirring or mixing in a homogenizer may suffice or more drastic procedures may have to be employed. Boiling has frequently been used, as has treatment with dilute alkali. Such methods inevitably lead to production of a polymer containing various contaminants and some degradation may also occur.

B. Purification

The removal of extraneous matter from exopolysaccharide preparations, whether of slime or capsular origin, presents several problems some of which are again due to the high viscosity of the polymers in aqueous solutions. The deproteinization technique of Sevag (1934) has been widely applied and it can be reasonably successful provided that dilute (<0·5%, w/v) solutions of the polysaccharide are used. Fractional precipitation with organic solvents has been of only limited value and gives poor separation from other linear polymers which may be present. These may include nucleic acids if the cells have undergone lysis.

Removal of either nucleic acids or protein is probably best accomplished by enzymic digestion and a succession of treatments with deoxyribonuclease, ribonuclease, trypsin and pronase may be required. After mild heating to destroy the enzymes, the polysaccharides are recovered from the supernatant fluids after centrifugation. Use can also be made of the ability of acidic polysaccharides to combine with quaternary ammonium salts (Scott, 1965). The complexes thus formed precipitate and can be separated from soluble neutral material. Recovery of the polysaccharides is obtained in strong salt solutions. It is also possible to apply the phenol-water extraction procedure developed for the extraction of lipopolysaccharides from the cell walls of Gram-negative bacteria (Westphal *et al.*, 1952). On addition of the crude material in aqueous solution to 90% (w/v) phenol at 60° and subsequent centrifugation at 0°, the exopolysaccharides are found in the upper aqueous phase of the biphasic system. Nucleic acids will also be found in this layer if they were present in the original mixture. An alternative approach is found in the use of dimethyl sulphoxide, a dipolar aprotic solvent showing selective solubility for polysaccharides. Selective absorption of either the polysaccharides or of contaminating material on ion-exchange absorbents has found relatively little application in the purification of bacterial exopolysaccharides although it proved successful during the purification of polysaccharides from other sources (Jermyn, 1962). It was also employed by Guy *et al.* (1967) to separate the capsular polysaccharide of *D. pneumoniae* type I from contaminating C substance (another polysaccharide) and from polyglutamic acid. Use of a sodium chloride gradient in 0·1 *M*-sodium acetate (pH 7·0) with DEAE-Sephadex as absorbent, produced pure polysaccharide. On a micro scale, as in the study of polymer produced by cell-free synthesis, precipitation with specific antisera provided an elegant method for recovering the polysaccharide formed (Smith *et al.*, 1960).

C. Composition

1. Sugars

Several different types of sugar residue have been detected in exopolysaccharides, but the commonest monosaccharides are undoubtedly the hexoses, D-glucose, D-galactose, and D-mannose. In addition, the methylpentoses fucose and rhamnose have frequently been reported. They provide an interesting example of L-isomers of sugars found in Nature, as opposed to the more usual D-forms. Less commonly found have been the pentoses ribose, arabinose and xylose, while the 3,6-dideoxyhexoses appear to be confined to the lipopolysaccharides of Gram-negative bacterial cell walls (Lüderitz *et al.*, 1968b). Also normally

TABLE 1. Specific Enzymic Methods for the Assay of Monosaccharides Found in Bacterial Exopolysaccharides

Sugar	Enzyme	Method	Reference
D-Glucose	Glucose oxidase	Colorimetric	Hugget and Nixon (1957)
D-Galactose	Galactose oxidase	Colorimetric	Amaral *et al.* (1966)
	Galactose dehydrogenase	NAD reduction	Wallenfels and Kurz (1966)
D-Mannose	Mannose isomerase	Colorimetric for fructose	Palleroni and Doudoroff (1956)
L-Fucose	Fucose epimerase	Colorimetric for keto sugars	
L-Rhamnose	Rhamnose isomerase	Colorimetric for keto sugars	Domagk and Zech (1966)
D-Glucosamine	Glucosamine phosphate acetylase	Colorimetric	Lüderitz *et al.* (1964)
D-Galactosamine	Galactose oxidase	Colorimetric	Sempere *et al.* (1965)

absent appear to be the heptoses and octa-oses again found associated with lipopolysaccharides. However, exopolysaccharides do contain both amino sugars and sugar acids, either separately or together in the same polymer. The amino sugars, in the *N*-acetylated form, are usually D-glucosamine, D-galactosamine or D-mannosamine. Although D-glucuronic acid has been most frequently noted, D-galacturonic acid, D-mannuronic acid and L-guluronic acid have also been found. These sugar acids contribute to the net negative charge commonly found in exopolysaccharides. The role of uronic acids in this respect may also be filled by methyluronic acids (Humphrey, 1959). It is certain that other sugars and sugar derivatives are components of these polymers, and that careful re-examination of many such preparations will reveal further monomers some of which may resemble or be identical with the unusual amino sugars found during re-appraisal of some bacterial lipopolysaccharides (Volk *et al.*, 1970; Lüderitz *et al.*, 1968b).

Most sugars in hydrolysates of exopolysaccharides have been identified initially by paper- or thin-layer chromatography. In many cases, provided suitable solvent systems were used, these gave a satisfactory and perhaps the simplest means of identification. It should be remembered that these techniques do not distinguish between D- and L-isomers. It is desirable to characterize the sugars either by preparation of suitable deritatives or by specific quantitative enzymic methods. Many of these enzymic assays are both extremely rapid and sensitive thus being very valuable in studies of oligosaccharides where only small amounts of the product may be available. In some of the assays (Table 1) the enzymic reaction can be followed directly, but in others the specific enzyme product is determined by non-specific colorimetric procedures. It is unfortunate that, in many studies of polysaccharide composition, only group-specific colorimetric tests have been used. An alternative method of sugar characterization and assay involves the preparation of trimethylsilyl ethers and the identification of these volatile compounds by gas–liquid chromatography.

2. *Non-Sugar Components*

Two types of non-sugar components are found in bacterial exopolysaccharides, namely organic and inorganic. The organic substituents are organic acids or, in a few polysaccharides, methyl groups. The simplest of these, and at the same time the most recently discovered, are formyl residues. These had been detected earlier in the *N*-formylated antigens isolated from *Brucella melitensis* (Miles and Pirie, 1939) and had also been found as part of a glycoprotein from ovomucoid (Marshall and Neuberger, 1960). In bacterial exopolysaccharides, formate was charac-

terized in the slime from a *K. aerogenes* type 54 strain but is now also known to occur in *Klebsiella* type 2 exopolysaccharides (Sutherland, 1971b). The formyl residues in these polymers are attached to either a neutral sugar or to glucuronic acid, and the absence of amino sugars precludes *N*-formylation. Formate is therefore most probably linked as an ester, attached to one of the several available free hydroxyl groups on a sugar residue. The presence of formyl residues confers characteristic chromatographic properties on oligosaccharides in which they are present, while having little effect on the electrophoretic mobility (Sutherland and Wilkinson, 1968, Sutherland, 1971b).

Acetate has long been known as a component of exopolysaccharides, and O-acetyl residues are probably among the most widespread non-carbohydrate modifications found on neutral or acidic sugars. They are

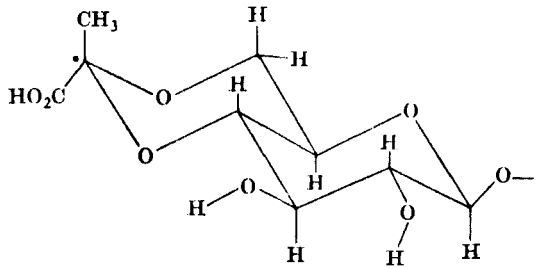


FIG. 4. Linkage of pyruvate to glucose as a ketal in a polysaccharide from *Xanthomonas campestris*. From Sloneker and Orentas (1962a).

found in polysaccharides from diverse organisms such as *D. pneumoniae*, *Rhizobium radicicolum*, *Azotobacter vinelandii* and *K. aerogenes*. As with *O*-formyl residues, acetylation affects the chromatographic mobility of oligosaccharides derived from the exopolysaccharides by enzymic hydrolysis. They also cause sufficient alteration to their charge : mass ratio to retard them slightly when subjected to paper electrophoresis. In those polymers containing amino sugars, *N*-acetyl groups are consistently found.

Pyruvate was first found in a polysaccharide in agar (Hirase, 1967) but was subsequently detected in capsular polysaccharides from *Xanthomonas campestris* (Sloneker and Orentas, 1962a). The pyruvate in these polymers is linked as a ketal to glucose (Fig. 4). The carboxylic acid group is thus free and contributes to the overall charge on the polymer as well as being available for the possible binding of salts. Pyruvate is widely found in exopolysaccharides and has been found in many of the organisms in the polysaccharides of which acetate is present. Pyruvylgalactose appears to be the most frequently encountered residue, but pyruvylglucose has also been identified (Sloneker and

Orentas, 1962b). Unlike *O*-acetyl groups which are extremely labile to alkali and to acid treatment and are consequently never found in oligosaccharides derived from partial acid hydrolysis of the polysaccharides, pyruvylated sugar residues are relatively stable to acid or alkaline hydrolysis. It is thus simpler to obtain pyruvylated sugars or pyruvylated oligosaccharides by partial acid hydrolysis (0·25 *M* H_2SO_4 at 100° for 20–30 min.) of the polymers. High yields of such derivatives may also be obtained by the autohydrolysis of the polysaccharide

TABLE 2. Properties of Pyruvylated Sugars Isolated from *Klebsiella* Polysaccharides

Type	Strain	Pyruvylated Sugar	Rglc in Solvent		Mobility relative to glucuronic acid in Pyridinium acetate: pH 5·3
			B	C	
1	1S	Fucose [?]	0·81	2·20	0·91
	19				
	A1				
	1803				
2	22	Glucose	1·00	1·80	0·97
8	8S	Galactose	0·68	1·65	0·98
30	889	Galactose	0·68	1·64	0·85
69	7824	Galactose	0·93	1·88	0·87
		Galactose	0·68	1·64	0·87
?	W	Galactose	0·68	1·64	0·87
Escherichia coli	K12	Galactose	1·02	1·95	0·92
Salmonella typhimurium	395M2	Galactose	0·68	1·64	0·87

Solvent B: butan-1-ol: acetic acid: water (4:1:5, v/v/v). Solvent C: ethyl acetate: acetic acid: formic acid: water (18:3:1:4, v/v/v/v).

solutions in the free acid form for 10–16 hours at 100°. As well as pyruvylated galactose and glucose, pyruvylfucose is thought to occur (I. W. Sutherland, unpublished results) in the polysaccharide of one *Klebsiella* serotype. Pyruvyl sugars have high chromatographic mobilities and, on paper electrophoresis in pyridine-acetic acid buffers, have a mobility close to that of glucuronic or galacturonic acid (Table 2). These properties may have led to confusion in the examination of partial acid hydrolysates of polymers in which pyruvate was not originally known to be present. The properties of pyruvylated oligosaccharides also differ considerably from the corresponding compounds from which pyruvate is absent (Table 3).

The fourth type of non-carbohydrate organic substituent to be detected was succinate. To date, this has been found in a single exopolysaccharide produced by a strain of *Alcaligenes faecalis* var. *myxogenes* (Harada, 1965). The polymer contained glucose as the principal sugar along with smaller amounts of galactose (Misaki *et al.*, 1969). Uronic acids and amino sugars were absent, so the polymer differed from most others in which acyl sugars have been found and which

TABLE 3. Pyruvylated Oligosaccharides and their Properties
From Sutherland (1969 and unpublished data)

Structure	Substituent	Mobility relative to glucuronic acid (in pyridinium acetate; pH 5·3)	R_{Glc} in Solvent: A	B	C
GAL→GlcA→GAL	—	0·54	0·13	0·19	0·08
Gal→GlcA→Gal	Pyruvate	0·87	0·73	0·28	0·34
Gal→GlcA→Gal→Fuc	Pyruvate	0·76	—	0·32	0·53
Gal→GlcA→Gal→Fuc ↑ Glc	Pyruvate	0·64	—	0·09	0·42
Glc→Gal→Gal	—	0	0·20	0·11	0·09
Glc→Gal→Gal	Pyruvate	0·41	0·28	0·23	0·15

Solvents: A. ethyl acetate : pyridine : acetic acid : water (5 : 5 : 1 : 3);
B. butanol : acetic acid : water (4 : 1 : 5);
C. acetic acid : formic acid : ethyl acetate : water (3 : 1 : 1 8 : 4).

contain various charged sugars. The succinate was probably linked as an ester to free hydroxyl groups of glucose moieties. The succinyl groups resembled *O*-acetyl groups in being labile to alkali (0·1 *N* NaOH at 70° for 2·5 hr.) and to mild acid treatment (Saito *et al.*, 1970). Succinylated fragments were not isolated.

It is probable that other non-carbohydrate components will be found in exopolysaccharides, since Lindberg and his colleagues have recently shown that in colanic acid, a heteropolysaccharide secreted by a number of species of Enterobacteriaceae, various non-sugar substituents were present. These included methylene and ethylidene groups identified by mass spectrometry (Garegg *et al.*, 1971a, b).

3. Inorganic Substituents

Crude preparations of exopolysaccharides almost all contain a considerable amount of salts. It is sometimes difficult to determine whether

these form an essential part of the polymer or whether they are non-specifically absorbed to it. Phosphate is certainly a component of one group of capsular polysaccharides from *D. pneumoniae*. These are exemplified by the specific polysaccharide of type XVIIIA Pneumococcus which contains D-galactose, D-glucose, rhamnose, *N*-acetyl-D-glucosamine, glycerol and phosphate in the approximate molar ratio 2 : 3 : 5 : 1 : 1 : 1 (Heidelberger *et al.*, 1964). This type of polymer bears a resemblance to the teichoic acids found in the cell walls of many Gram-positive bacterial species. The presence of other inorganic compounds remains unproven. It does seem surprising that sulphate, which is found in a number of polysaccharides derived from the higher algae and from mammalian tissues (Percival and McDowell, 1967), has not so far been found in bacterial exopolysaccharides.

D. Structural Features

A number of features define the structure of a polysaccharide. These are: (i) the monosaccharides present; (ii) the type of linkage by which each monosaccharide is joined to the adjacent sugar residues; (iii) the sequence of the oligosaccharides formed from the specifically linked monosaccharides; (iv) the presence of non-carbohydrate substituents as esters or ketals or ester-linked phosphate; and (v) the molecular weight. This combination of properties may provide a number of problems when attempts are made to elucidate the structure of bacterial exopolysaccharides. Structural studies are also rendered difficult by the lack of well-characterized enzymes breaking down the exopolysaccharides. It is thus more difficult to determine whether repeating units occur in such polymers and whether variations occur within such units.

1. Methylation

One method for structural determination requires the methylation of all free hydroxyl groups in the polymer. The methylated polysaccharide is then hydrolysed and the methylated sugars identified, the presence of free hydroxyl groups indicating the nature of the glycosidic bonds in the native polysaccharide. From the identification of the methyl sugars and determination of the relative amounts, it should be possible to deduce the amount and type of the glycosidic linkages in the polysaccharide. Unfortunately, earlier work using this technique was affected by several problems which resulted in incomplete methylation and consequent wrong deductions as to polysaccharide structure. In particular, the frequent references to exopolysaccharides as "highly branched structures" derive from these artifacts and should be regarded with some caution. The problems in the methylation of polymers

containing uronic acid residues or amino sugars are much greater than in neutral polysaccharides which lack the highly acid-resistant aldobiouronic acid linkages. These can be overcome by conversion of the uronic acid residues to the corresponding neutral sugars (carboxyl reduction) prior to methylation. One of the major problems in the methylation procedure, that of incomplete methylation, has been overcome through the development of an improved methylating agent by Hakamori (1964). He employed the strongly basic methylsulphymyl carbanion to give efficient alkoxide formation in complex polysaccharides. The procedure was applied to studies on *Klebsiella* exopolysaccharides with much more satisfactory results than those obtained by earlier workers (Sandford and Conrad, 1966). The methylated polymer should then be hydrolysed under conditions where demethylation and destruction of methylated sugars is minimal. A further development is seen in the formation of acetates from methylated sugars and their characterization through combined gas–liquid chromatography and mass spectrometry. This method was initially applied to lipopolysaccharides from bacterial cell walls (Björndal *et al.*, 1967) but is also applicable to studies on exopolysaccharides.

2. *Periodate Oxidation and Carboxyl Reduction*

Periodate oxidation can provide a considerable amount of information about exopolysaccharide structure and, when used under conditions where "over-oxidation" is prevented, has been a valuable technique. It has also been followed by reduction and partial acid hydrolysis. Not all bacterial exopolysaccharides, however, possess structures which are oxidized by periodate (Barker *et al.*, 1963a). Further information in uronic acid-containing polymers can be obtained by esterification of the carboxylic acid and subsequent reduction. The neutral polymer so obtained can then be examined without the problems associated with the acid-stable aldobiouronic acid. When the native polymer contained glucuronic acid, and glucose was already present, it can be distinguished from that formed on carboxyl reduction by the use of sodium borotritide as reductant (Sutherland, 1970b). This is equally true for other uronic acids and the corresponding neutral sugars. Determination of the specific activity of fragments obtained on partial acid hydrolysis then indicated their origin.

Partial acid hydrolysis has been widely applied to the study of exopolysaccharide structure. It has provided much useful information but suffers also from certain drawbacks. Where uronic acids or amino sugars are present, the resultant glycosidic bonds to the adjacent neutral-sugar residues resist hydrolytic conditions which cleave all other glycosidic bonds in the polymer. This is especially true when furanosidic

bonds are present but is also seen in highly acid-labile fucosidic, mannosidic or ribosidic linkages. It is thus difficult to devise hydrolysis conditions yielding significant amounts of oligosaccharides larger than the aldobiouronic acids or the corresponding amino sugar-containing disaccharides. A similar problem is seen when the D-galactopyranosyl 1→3 D-glucose configuration is present (Osborn *et al.*, 1964). The yield of fragments from partial acid hydrolysates can however be increased considerably through use of polystyrene sulphonic acid (Painter and Morgan, 1961) or through controlled continuous removal of the oligosaccharides as they are formed (Galanos *et al.*, 1969).

Biological methods of structural determination have been surprisingly slow to gain acceptance. This may in part be due to the small number of enzymes known to hydrolyse exopolysaccharides (see p. 159) and to the enormous volume of work necessary to obtain the basic information required prior to the use of the immunochemical approach. However the painstaking work of Heidelberger and his colleagues is seen in the increasingly frequent use of immunochemical methods as an adjunct to other techniques in the determination of exopolysaccharide structure (e.g. Heidelberger *et al.*, 1970).

E. Enzymic Hydrolysis

Relatively few enzymes that hydrolyse exopolysaccharides have been isolated and fewer still have been characterized. One of the first examples cited was an enzyme obtained from a soil bacillus capable of growth on the capsular polysaccharide of *D. pneumoniae* type III (Dubos and Avery, 1931). The enzyme destroyed the capsular structure, and resulted in complete loss of the immunochemical activity of the polysaccharide. It was however inactive against serologically related polysaccharides such as that from type VIII Pneumococcus. Subsequently a soil organism, to which the name *Bacillus palustris* was given, was found, depending on the polysaccharide used as inducer, to excrete specific enzymes that hydrolyse either type III or type VIII pneumococcal polysaccharides (Shaw and Sickles, 1950; Sickles and Shaw, 1950; Campbell and Pappenheimer, 1966). The bacterial strain producing the enzymes was subsequently identified as a strain of *B. circulans* (T. Gibson, personal communication). A thorough investigation of these two enzymes revealed that each was highly specific both with regard to its induction and to its substrate (Torriani and Pappenheimer, 1962). In both cases, induction only occurred when the substrate polymer or its hydrolysis products were added to the medium. Substrate activity did extend to chemically related substances such as oxidized cellulose in the case of the enzyme active against type VIII polysaccharide. The mode of action

of the two enzymes was shown to differ, although each acted as an exo-enzyme removing the repeating units from the polymer chain (Becker and Pappenheimer, 1966). The enzyme active on type III pneumococcal polysaccharide was a hydrolase that splits the *O*-β-D-glucosyl 1→4 bonds in the substrate. The second enzyme was a lyase or eliminase that catalyses formation of products containing terminal 4,5 unsaturated glucuronic acid residues. These were recognized by their absorption maxima at 230 nm.

Another inducible polysaccharide-hydrolysing enzyme was obtained from an organism identified as *K. aerogenes* (Barker *et al.*, 1964). The substrate was the capsular polysaccharide from type XIV *D. pneumoniae*. Initially the enzyme released galactose and a trisaccharide which was characterized as *O*-β-D-glucopyranosyl-(1→4)-*O*-(2-acetamido-2-deoxy-)-β-D-glucopyranosyl-(1→3)-D-galactopyranose. Prolonged exposure of the trisaccharide to the *Klebsiella* cells led to induction of a β-glucosidase which removed the terminal non-reducing glucosyl residue, releasing a disaccharide.

Another successful attempt to hydrolyse a bacterial exopolysaccharide with enzyme(s) from heterologous bacteria was reported by Lesley (1961). The polysaccharide from *Xanthomonas phaseoli* was degraded by an inducible enzyme formed by a *Bacillus* species isolated from soil. This enzyme resembled the enzyme active against *D. pneumoniae* type VIII polysaccharide (Becker and Pappenheimer, 1966) in its mode of action. The products contained terminal 4,5 unsaturated glucuronic acid residues. The oligosaccharide obtained in highest yield was a trisaccharide composed of 4,5 unsaturated glucuronic acid, mannose and glucose which probably derived from the repeating unit of corresponding structure in the original polysaccharide. The eliminase type of action observed for this enzyme also resembled the mode of action of bacterial and other hyaluronidases examined by Linker and his colleagues (1956) and by Ludoweig *et al.* (1961).

The relative lack of success in finding polysaccharide hydrolases from conventional sources led to examination of another possible system, namely phage-infected bacteria. A crude enzyme preparation capable of removing capsules from *K. pneumoniae* type 1 was obtained by Humphries (1948). A similar system for *K. pneumoniae* type 2 was examined by Park (1956) and Adams and Park (1956). Although a considerable amount of knowledge relating to enzyme synthesis and phage specificity was obtained, hydrolysis products were not characterized from either polysaccharide. This was also true for some other systems such as those for *Azotobacter* sp. (Eklund and Wyss, 1962) in which phage-infected bacteria were a source of enzymes affecting the capsular or slime polysaccharides of the host bacteria. Some of these are listed in Table 4.

TABLE 4. Phage-induced Enzymes that Hydrolyse Exopolysaccharides

Bacteria	Phage	Products characterized	Reference
Klebsiella pneumoniae type 1		—	Humphries (1948)
Klebsiella pneumoniae type 2	Kp	—	Park (1956)
Klebsiella pneumoniae type 2		Tetrasaccharide	Watson (1966), Sutherland (1972a)
Klebsiella aerogenes type 54	F31, F39	Tetrasaccharides, Octasaccharide	Sutherland (1967)
Escherichia coli | *Aerobacter cloacae*	F1, F12	Hexasaccharide	Sutherland and Wilkinson (1965), Sutherland (1972b)
Azotobacter sp.	A22	—	Eklund and Wyss (1962)
Pseudomonas aeruginosa	2	—	Bartell *et al.* (1966, 1968)
Pseudomonas putida			Chakrabarty *etal.* (1967)
Alcaligenes faecalis	A6	Trisaccharides	Mare and Smit (1969)

The *Azotobacter* system (Eklund and Wyss, 1962) produced phage-induced enzymes which caused a rapid drop in the viscosity of polysaccharide solutions. Although hydrolysis products were not isolated, certain characteristics of the enzyme system, such as pH optimum, were determined using viscosity measurements. Experiments to determine whether the polysaccharide depolymerase was also associated with the phage particles indicated that only 0·1% of the enzyme produced by phage-infected bacteria was actually incorporated into the viral particles. Later work (Barker *et al.*, 1968) showed that the enzymes induced by different phages infecting the same host bacteria had certain physical differences, indicating that their synthesis was under the

TABLE 5. Host Range of Bacteriophages that Induce Synthesis of Polysaccharases Acting on Colanic Acid

		Growth on						
		Escherichia coli strains					*Aerobacter cloacae* strains	
Phage	Original host	S23	S53	S53C	Other K12	Others	5920	Others
	Escherichia coli							
F1	S53	+	+	+	+	−	±	−
F5, 26, 27	S53	+	+	+	+	−	−	−
	Aerobacter cloacae							
F12, 13, 14	5920	−	−	−	−	−	+	−

+ indicates confluent lysis; −, no lysis; ±, variable response.

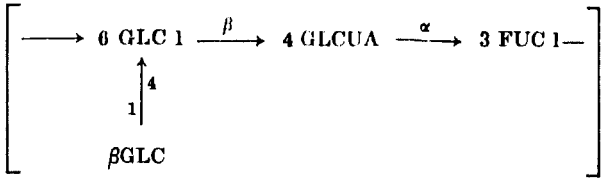
control of the phage and not the bacterial genome. Liberation of reducing material by the depolymerases from the *A. vinelandii* polysaccharide was observed, and it was recently reported (Pike and Wyss, 1971) that despite the marked drop in the viscosity of the polysaccharide solutions no noticeable decrease in the molecular size of the substrate occurred. Enzyme digests contained fragments of different size, resembling the original polysaccharide in their carbohydrate composition but lacking acetate. This suggests that the enzymes are exo-enzymes that remove material from the ends of the polymer chains. The known irregularity in the substrate molecules (see p. 179) may account for the failure to produce higher yields of oligosaccharides, as tends to occur in the hydrolysis of exopolysaccharides comprised of repeating units.

From earlier work in our own laboratory (Sutherland and Wilkinson, 1965), it was clear that, where a polysaccharide of similar composition was excreted by a number of different bacterial strains, there was no

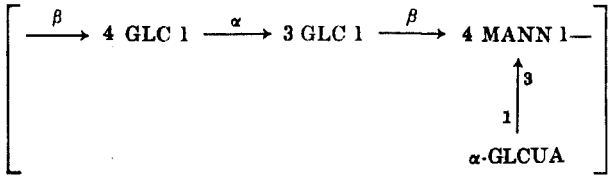
correlation between the ability of a phage to infect a bacterial strain and the ability of the enzyme produced after phage infection to "depolymerize" the polysaccharide. Enzyme produced in one strain of bacterium could act on polysaccharide from another bacterial strain which was phage resistant. Typical results for *E. coli* and other strains that produce the exopolysaccharide "colanic acid" are shown in Table 5. Although hydrolysis products were not obtained, improved techniques have recently led to the isolation of the repeating unit from preparations of *E. coli* and other "colanic acid" (Sutherland, 1972b). The repeating unit of this exopolysaccharide is a complex hexasaccharide of approximate molecular weight 1100 (Sutherland, 1969; Lawson *et al.*, 1969). The failure to isolate oligosaccharides initially may have been due to the size and configuration of the hydrolysis products. Unlike many other polysaccharide hydrolases including those for slime polysaccharides from *Klebsiella* type 54 (Sutherland, 1967) the colanic acid hydrolases only released 30–35% of the polymer as oligosaccharides. This may be due to irregularities in the substrate structure. At least two hydrolysis products have been identified from *E. coli* K12 colanic acid, one of which has the same structure as the repeating unit of the polymer. The enzymes are active against colanic acid from a number of different strains of *E. coli*, *Salmonella typhimurium* and *Aerobacter cloacae* although a number of variations in the non-carbohydrate components of colanic acid were later shown (Garegg *et al.*, 1971a, b). De-acetylated polysaccharide prepared by mild alkali treatment was also a substrate, but no activity was obtained using carboxyl-reduced polysaccharide in which all the glucuronic acid residues were converted to glucose.

A phage which induced a capsule depolymerase for *K. pneumoniae* type 2 strains was studied by Watson (1966). The enzyme destroyed the immunochemical activity of the polysaccharides. Further examination of this enzyme (Sutherland, 1972a) revealed that the polysaccharides from a large number of type 2 *Klebsiella* strains were all hydrolysed to their component tetrasaccharides. As the linkage cleaved is: mannosyl-glucose, the enzyme is a mannosidase. Enzyme activity was unaffected by the presence or absence of a number of non-carbohydrate residues on the polysaccharides. Activity was not detectable against carboxyl-reduced type 2 *Klebsiella* polysaccharide or against a number of other polysaccharides tested, including some such as *Klebsiella* serotypes 30 and 69 which show serological cross-reaction with type 2 material. (I. Orskov, personal communication). As with the *E. coli* system described above, there was no correlation between phage-sensitivity of the bacteria and susceptibility of the capsular polysaccharide to the phage-induced enzyme.

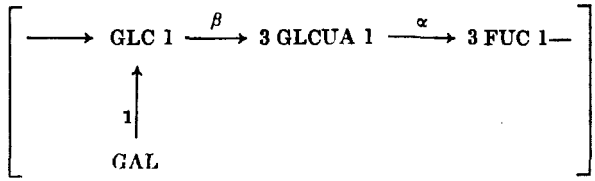
Probably the phage-induced enzyme systems which proved most useful in providing information about their substrates were those active against *K. aerogenes* type 54 exopolysaccharide (Sutherland, 1967; Sutherland and Wilkinson, 1968). Two distinct enzyme types were found, exemplified by those induced by phages F31 and F39 respectively. The F39-induced enzymes hydrolysed the substrate into octasaccharide repeating units, each of which contained one acetyl group and two other groups subsequently identified as formyl (Sutherland, 1970a). In



(i) *Klebsiella* Type 54 polysaccharide



(ii) *Klebsiella* Type 2 polysaccharide



(iii) *Escherichia coli* K27 polysaccharide

FIG. 5. Structures of substrates for *Klebsiella* phage F34-induced enzyme.

contrast, the F31-induced enzyme hydrolysed the polysaccharide to equal quantities of two tetrasaccharides, one of which was acetylated. The enzyme was also active against the octasaccharide repeating unit. Both enzymes hydrolysed de-acetylated polysaccharide and polymer from which both acetyl and formyl groups had been removed by acid treatment. As with the other phage-induced polysaccharide depolymerases, activity was not found using carboxyl-reduced polysaccharide. The discovery of an *E. coli* exopolysaccharide with a structure showing some similarity to *K. aerogenes* type 54 slime provided a possible substrate for the F31- and F39-induced enzymes (Jann *et al.*, 1968). Although one of the glucose residues in the natural substrate had been replaced in

E. coli K27 material by galactose, both enzymes were active in hydrolysing it (Sutherland *et al.*, 1970). The products were an acetylated tetrasaccharide and an octasaccharide, respectively. An extension of these studies (Sutherland, 1972a) showed that type 2 *Klebsiella* polysaccharides also acted as substrates. The two type 54 hydrolases thus

TABLE 6. Some Enzymes of Value in Determining Oligosaccharide Structure

EC No.	Name	Specificity
3.2.1.57	Lactase	Hydrolyses β-galactosyl 1→4 glucose.
3.2.1.4.	Cellulase[a]	Hydrolyses some β glucosyl 1→4 linked residues resistant to other β-glucosidases.
3.2.1.20	α-Glucosidase[a]	Acts on many α-D-glycopyranosides.
3.2.1.21	β-Glucosidase[a]	Acts on many β-D-glucopyranosides, but activity is lower when the terminal reducing sugar is not a hexose. Commercial preparations are normally impure, containing α-glucosidase and β-galactosidase activity as well as other glycosidases.
3.2.1.22	α-Galactosidase	Hydrolyses several α-D-galactopyranosides.
3.2.1.23	β-Galactosidase[a]	Hydrolyses numerous β-D-galactopyranosides.
3.2.1.24	α-Mannosidase	Hydrolyses α-D-mannopyranosides.
3.2.1.25	β-Mannosidase	Hydrolyses β-D-mannopyranosides.
3.2.1.27	α-1,3-Glucosidase	Acts on several α-1,3-D-glucosides.
3.2.1.31	β-Glucuronidase[a]	Although this enzyme hydrolyses many β-D-glucuronides, commercial preparations frequently contain α-glucuronidase and other glycosidases.
3.2.1.38	β-L-Fucosidase	Acts on β-L-fucosides.
—	α-L-Fucosidase	Hydrolyses α-L-fucosides but, depending on the source, may contain β-L-fucosidase and hexosaminadases.
—	α-*N*-acetylglucosaminidase	The preparations from limpets contain other glycosidases.
—	β-*N*-acetylglucosaminidases	This enzyme, prepared from various sources, hydrolyses many β-*N*-acetyl-D-glucosaminides.

[a] Preparations of these enzymes can be obtained commercially.

appear to have the lowest specificity of any of the enzymes of this type studied so far. Their activities are unaffected by the presence of acetyl, formyl or pyruvyl substituents, and they hydrolyse three distinct polysaccharide types (Fig. 5), all apparently to completion. Although these enzymes can be classified as fucosidases, they can also hydrolyse mannoside bonds provided certain criteria are fulfilled. The L-fucose

or D-mannose residue must form part of an aldobiouronic acid with D-glucuronic acid and the terminal reducing residue of the fucoside or mannoside should be D-glucose.

The hydrolase enzymes, whether from bacterial sources or induced in bacteriophage-infected bacteria, provide a very useful tool in studies on bacterial exopolysaccharides. They enable isolation of fragments from the native polysaccharides by mild methods. Acetyl and other labile groups are not detached as they are by virtually all of the classical chemical techniques. The enzymes are thus extremely valuable in structural studies, and may yield fragments which can in turn be degraded by other hydrolytic enzymes. The polysaccharide hydrolases also provide a highly sensitive and specific assay method for use in biosynthetic studies on the polysaccharides. From the results outlined above, it seems that different types of enzyme exist, including hydrolases and eliminases; endoenzymes and exoenzymes. It is possible that all types can be found among either the bacterial or the bacteriophage-induced enzymes.

The products of the polysaccharide hydrolases and of partial acid hydrolysis can yield further information on treatment with other glycosidases. A number of terminal non-reducing sugars can be removed in this way. It should be noted that such glycosidases show variations in their activity due to aglycone specificity and they may also be inhibited by the presence of acyl or ketal substituents. Several commercial glycosidases are available and others can be isolated and purified in the laboratory (Table 6). Many of these enzymes remain impure and the inclusion of adequate control substrates is necessary if accurate results are to be obtained.

F. Structures of Some Exopolysaccharides

Very many bacterial exopolysaccharides have been examined to determine their qualitative and quantitative sugar composition. In many preparations the presence of monosaccharides in a simple molar ratio has enabled authors to predict that the polymers contain a repeating unit whose size and composition can be postulated. Despite this, relatively few polysaccharides have been rigorously investigated with a view to complete elucidation of their structure. It is seldom possible to predict in advance what the composition, still less the structure, of the polysaccharide isolated from a particular bacterial species or strain will be. But, it is clear from structural determinations on a number of exopolysaccharides that they are formed from repeating units of varying complexity. Recent results elucidating the mode of biosynthesis of these polymers would seem to indicate that all heteropolysaccharides and

possibly even some homopolysaccharide which are found in bacterial slime and capsules may be constructed in this way. In the following pages, some examples are taken from those bacterial genera in which the complete or partial structures of a number of exopolysaccharide types have been elucidated.

All polysaccharides, including bacterial exopolysaccharides, may be divided into homopolysaccharides and heteropolysaccharides, i.e. into polymers composed of a single type of monomer unit, and those formed from several types of monomer, respectively. Homopolysaccharides most widely studied have been cellulose excreted by *Acetobacter* species and the levans and dextrans synthesized from sucrose and similar substrates by *Leuconostoc mesenteroides* and other bacterial species. Glucans from *Agrobacterium* species and other soil bacteria have also been studied (Putman *et al.*, 1950; Gorin *et al.*, 1961). Other homopolysaccharide slimes and capsules undoubtedly exist and further knowledge of their structure and synthesis must await improved methods of analysis. By their very simplicity, being composed of a single uniform type of monomer, studies on homopolysaccharides present problems which are either absent when studying heteropolysaccharides or are more readily attacked with these polymers.

Most bacterial exopolysaccharides studied, whether in detail or merely by detection of the component sugars, are heteropolysaccharides. These polymers can vary greatly in their complexity. Some, such as the capsular polysaccharide of *D. pneumoniae* type III, contain only two types of monosaccharide, D-glucose and D-glucuronic acid (Reeves and Goebel, 1941). The presence of five or six different sugars has been reported in some bacterial exopolysaccharides but it is possible that all of these may not have been truly derived from capsule or slime. Polysaccharides containing three or four different monosaccharides are commonly found. It is certain that exopolysaccharides in general are much less complex than are the polysaccharide entities of the lipopolysaccharide or somatic antigen found in the cell walls of most Gram-negative bacteria. In these, even the so-called "basal structure" in the genus *Salmonella* contains five different sugars, while a further three or four different monosaccharides may form the *O*-antigenic determining "side-chains" (Lüderitz *et al.*, 1968).

1. Structure of Homopolysaccharides

Determination of the structure of homopolysaccharides may often be more difficult that that of heteropolysaccharides due to the presence of a single monomer unit. Bacterial cellulose, produced by *Acetobacter* species, has received much study and was identified as a linear polymer

of β-glucosyl 1→4 glucose residues (Barclay *et al.*, 1952). The polysaccharide chains have an average length of 600 glucose residues and closely resemble the product from green plants. The difference between the plant and bacterial polymers lies in their location in the cells in which they are synthesized. In bacteria, cellulose is an extracellular polymer and does not form part of the cell structure as in plant cells. Loss of the ability to synthesize cellulose does not adversely affect the bacteria.

Other extracellular homopolysaccharides which have been the subject of much study are the levans and dextrans. Levans are produced by many plant-pathogenic bacteria of the genera *Pseudomonas* and *Xanthomonas* as well as the Gram-positive genus *Bacillus* and *Streptococcus salivarius*. Levans are polyfructoses, often with very high molecular weights of one million or greater, in which the predominant linkage is β-D-fructosyl 2→6 D-fructose (Cooper and Preston, 1935). The products from *Bacillus* species have chain lengths of 10–12 fructose residues and are extremely labile to acid hydrolysis being completely hydrolysed by 0·02 *N* HCl at 60° in only 30 min. (Lyne *et al.*, 1940). Dextrans are essentially linear chains of α-D-glucosyl 1→6 D-glucose with branch points at positions 2, 3 or 4. Their formation as extracellular polymers is confined to a small number of Gram-positive bacteria including, *Leuconostoc mesenterioides*, *L. dextranicum* and *Streptococcus viridans*. The chain length shows considerable variation, some preparations having average chain length of 5–40 monosaccharides while others contain up to 550 glucose residues (Peat *et al.*, 1939).

Examination of a polysaccharide excreted by *Agrobacterium tumefaciens* indicated that it consists mainly of β-D-glucopyranosyl 1→2 D-glucopyranose linkages (Putman *et al.*, 1950). Further work on polysaccharides from a number of *Agrobacterium* species confirmed that they too are similar linear glucans (Gorin *et al.*, 1961), but this type of 1→2 linked structure has not been widely found outside the genus *Agrobacterium*.

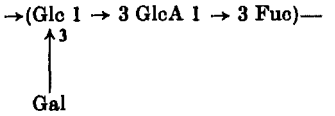
2. *Structure of Heteropolysaccharides*

a. Gram-negative bacteria

i. *Escherichia coli*. The large number of strains which are classified as *E. coli* are differentiated on the basis of the lipopolysaccharide (somatic) antigens, found in the cell walls. Within these many serotypes, two forms of exopolysaccharide production were recognized. A large number of strains, together with strains of most *Salmonella* species and *Aerobacter cloacae*, are capable, under suitable conditions, of excreting an extracellular slime to which the name colanic acid was given (Goebel, 1963; Anderson and Rogers, 1963; Grant *et al.*, 1969). This polymer will be

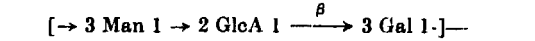
considered separately. In addition, a minority of *E. coli* strains are capsulate and may be recognized as such by negative staining techniques. For diagnostic purposes, the capsulate strains of *E. coli* are subdivided into three groups termed A, B and L (Kauffmann and Vahlne, 1945). Of these the B and L types form very small capsules which could not be discerned using the light microscope except after growth under special cultural conditions (Ørskov, 1956) but could be identified by serological methods. The A type formed larger capsules visible by light microscopy.

Escherichia coli type 27. The acidic polysaccharide from this strain contains four sugars, namely D-glucuronic acid, D-glucose, D-galactose and L-fucose in equimolar proportions (Jann *et al.*, 1968). *O*-Acetyl groups were also detected. Acid hydrolysis yielded a number of charged oligosaccharides but no neutral oligosaccharides. On the basis of methylation, periodate oxidation and other data, a tetrasaccharide repeating unit was suggested but the site of the *O*-acetyl groups was not determined. The native polysaccharide has the repeating unit:



It has a high molecular weight which is decreased to about 10,000 by alkali treatment. This led to the suggestion that chains of about 140 repeating units are linked through ester bonds between the hydroxyl groups of monosaccharides and the carboxyl groups of glucuronic acid residues.

Escherichia coli type 30. Another *E. coli* capsular polysaccharide was also an acid macromolecule which contained D-glucuronic acid, D-galactose and D-mannose in the molar ratio 1 : 1 : 1 (Hungerer *et al.*, 1967). Although acetate was present, the molar ratio was less than one, indicating either that not every repeating unit is acetylated or that some of the labile *O*-acetyl groups were lost during isolation and purification of the polysaccharide. The repeating unit proved to be a trisaccharide:



It was suggested that the polysaccharide is formed from linear subunits and has a molecular weight of about 150,000. Because of the lability of the mannosidic bonds in the polymer and the relative stability of the aldobiouronic acid 3-*O*-β-D-glucuronosyl-D-galactose, partial acid hydrolysates of type 30 material were unusual in yielding a single product, the aldobiouronic acid. No other oligosaccharides were isolated.

E. coli type 42. Another simple trisaccharide repeating unit was found in type 42 *E. coli* exopolysaccharide (Ørskov *et al.*, 1963; Jann *et al.*, 1965).

This polymer contains galacturonic acid, galactose and fucose. On the basis of chemical studies on the polymer, a repeating unit structure was deduced:

$$\text{-[3 Gal 1} \rightarrow \text{3 GlcA 1} \rightarrow \text{2 Fuc 1-]—}$$

As well as sugars, *O*-acetyl groups (6·4% of the dry weight) are present in this polysaccharide. Probably because of the structure of the aldobiouronic acid, serological cross-reaction with *D. pneumoniae* type I capsular polysaccharide occurred (Heidelberger *et al.*, 1968).

These three *E. coli* capsular polysaccharides, all of the A-type, thus have fairly simple repeating units composed of 3–4 monosaccharides and all are acetylated. Much more complicated polymers were found when two of the B-type polysaccharides were examined. *E. coli* type 85 was found to be an acid macromolecule containing glucuronic acid, mannose, *N*-acetylglucosamine and rhamnose in a molar ratio of 1 : 2 : 1 : 1 (Jann *et al.*, 1966). A number of oligosaccharides were obtained by partial acid hydrolysis and, on the basis of their structure and other data, a pentasaccharide repeating unit was proposed:

$$\rightarrow \text{2 or 4 [GlcA 1} \rightarrow \text{2 or 6 Man 1} \rightarrow \text{3 Man 1} \rightarrow \text{3 GlcNAc]—}$$
$$\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\quad |$$
$$\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\quad \text{Rha}$$

Later work (Heidelberger *et al.*, 1968) indicated that the structure might be even more complex and thus account for a very strong cross-reaction with antisera to type II and type V *D. pneumoniae* polysaccharides.

Another *E. coli* exopolysaccharide of unknown type contains rhamnose, galactose, glucosamine, glycerol and phosphate, but these components are also thought to be present in the cell-wall lipopolysaccharide of the same strain (Jann *et al.*, 1970). It was suggested that a similar structure exists in both the lipopolysaccharide and the exopolysaccharide. Whether this is indeed so, or whether the "exopolysaccharide" represents an extracellular secretion of incomplete lipopolysaccharide, remains to be established.

"Colanic acid". It was long thought that a polysaccharide is produced which is common to many species in the bacterial family Enterobacteriaceae. The initial serological observations of Kauffmann (1936) were confirmed and extended by Henriksen (1949, 1950). Further work (Anderson, 1961; Anderson and Rogers, 1963) indicated that many strains of *Salmonella* or *Escherichia* could form a slime polysaccharide when cultured under suitable conditions. It was also known that mucoid mutants could be obtained from *E. coli* strain K12 (Beiser and Davis, 1957). A similar polysaccharide was isolated from another *E. coli* strain along with a type-specific capsular antigen (Ørskov *et al.*, 1963) which was serologically and chemically distinct. Goebel (1963) applied the name "colanic acid" to the slime and observed that it contains 30–32% fucose,

33–34% galactose, 16–17% glucose and 17–20% glucuronic acid. It was also confirmed that antigenically similar material could be isolated from mucoid mutants of *E. coli* K12 (Sapelli and Goebel, 1964). Material of the same chemotype was obtained from *Aerobacter cloacae* strains and, along with other colanic acid preparations, served as substrates for a group of phage-induced enzymes (Sutherland and Wilkinson, 1965). The control of colanic acid synthesis has been the subject of a number of studies (e.g. Kang and Markovitz, 1967; Grant *et al.*, 1969) which showed that, with the exception of strains which have a defect in a gene coding for an enzyme responsible for synthesis of a portion of the

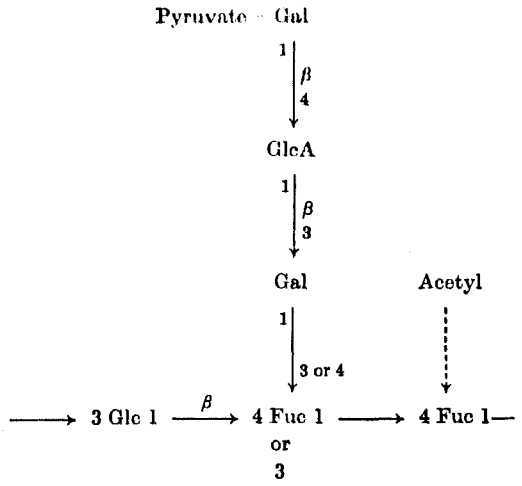


FIG. 6. Structure of colanic acid.

molecule, all *E. coli* K12 strains possess the genetic ability to synthesize colanic acid. Although strains were normally non-mucoid, under suitable conditions polysaccharide synthesis was induced in numerous *Salmonella* "species" and *E. coli* strains. In all cases the polymer proved to contain the same monosaccharides and acted as a substrate for phage-induced enzymes. Despite the large number of studies on colanic acid, its structure was for long neglected. Partial acid hydrolysis of the *E. coli* capsular polymer yielded two charged oligosaccharides, one of which was identified as the aldobiouronic acid, β-D-glucuronosyl 1→3 D-galactose (Roden and Markovitz, 1966). Re-examination showed that three preparations from *E. coli*, *A. cloacae* and *S. typhimurium* each contain the four sugars already mentioned, together with acetate and pyruvate (Sutherland, 1969). The presence of the polysaccharide constituents in an approximate molar ratio of fucose : galactose : glucuronic acid : acetate : pyruvate, 2 : 2 : 1 : 1 : 1 : 1, indicated the possibility of hexasaccharide repeating

unit which was both acetylated and pyruvylated. Methylation and base-catalysed fragmentation indicated that pyruvate was present as a ketal forming 4,6-*O*(1′-carboxyethylidene)-D-galactose (Lawson *et al.*, 1969). The overall results confirmed that the basic structure was a hexasaccharide (Fig. 6) which was constructed as a trisaccharide branch on a trisaccharide portion of a linear chain composed of residues of glucose and fucose. The position of the acetyl group remained ambiguous despite the recent isolation of the repeating unit by enzymic hydrolysis (Sutherland, 1972b). Further studies showed that, although the carbohydrate composition of colanic acid from a number of *E. coli* and *S. typhimurium* strains is constant, variations occurred among the non-carbohydrate constituents (Garegg *et al.*, 1971a, b). Thus, one strain of *Salmonella* produced a polysaccharide lacking acetate, while other preparations were acetylated on the non-branching fucose residue. The greatest variations were in the substituents on the terminal galactose residue. In two mucoid mutants of *S. typhimurium* of common parentage, the polysaccharides contain a 3,4-*O*-ethylidene group and a 3,4-*O*-carboxyethylidene group respectively. In all, six out of nine preparations tested contain a 4,6-*O*-carboxyethylidene group, while one polysaccharide is unusual in containing a methylene group linked to positions 4 and 6. The various structures detected are shown in Fig. 7.

From these results, it is clear that, at least in the strains examined, it is possible for a number of dissimilar bacterial strains to synthesize an exopolysaccharide of similar if not identical carbohydrate structure. The polymer does, however, show considerable variation in the non-carbohydrate residues present, both in regard to the nature of these residues and to their position on the carbohydrate entities. It is unfortunate that, so far, extensive studies have not been made on the modifications to polysaccharides from strains of common ancestry.

ii. *Klebsiella aerogenes and K. pneumoniae.* All strains of *Klebsiella* are mucoid, and in many cases large amounts of capsular polysaccharides are formed. Like the polymers from the closely related *E. coli* several of the structures have been determined, and it has been shown that, unlike most other bacterial general, the type of extracellular polymer is remarkably uniform. Few contain more than three sugars (Table 7) and those in which the structure has been completely determined possess tetrasaccharide repeating units.

Type 1. The monosaccharides in this expopolysaccharide were identified as fucose, glucose and glucuronic acid (Eriksen and Henriksen, 1959) but more recent work has shown that pyruvate is also present (W. F. Dudman, unpublished work). The polymer is unusually susceptible to alkali and is unaffected by periodate (Barker *et al.*, 1963a, b).

This was interpreted as being due to the predominance of (1→3) linkages between the sugars. One observation which appears unusual was that some glucuronic acid was released on mild acid hydrolysis (0·01 *N* H_2SO_4 at 80° for 30 min.). This would seem to indicate that at least some of the glucuronic acid in the polymer occurs linked in a manner different from the normally acid-resistant aldobiouronic acids. Alternatively, if

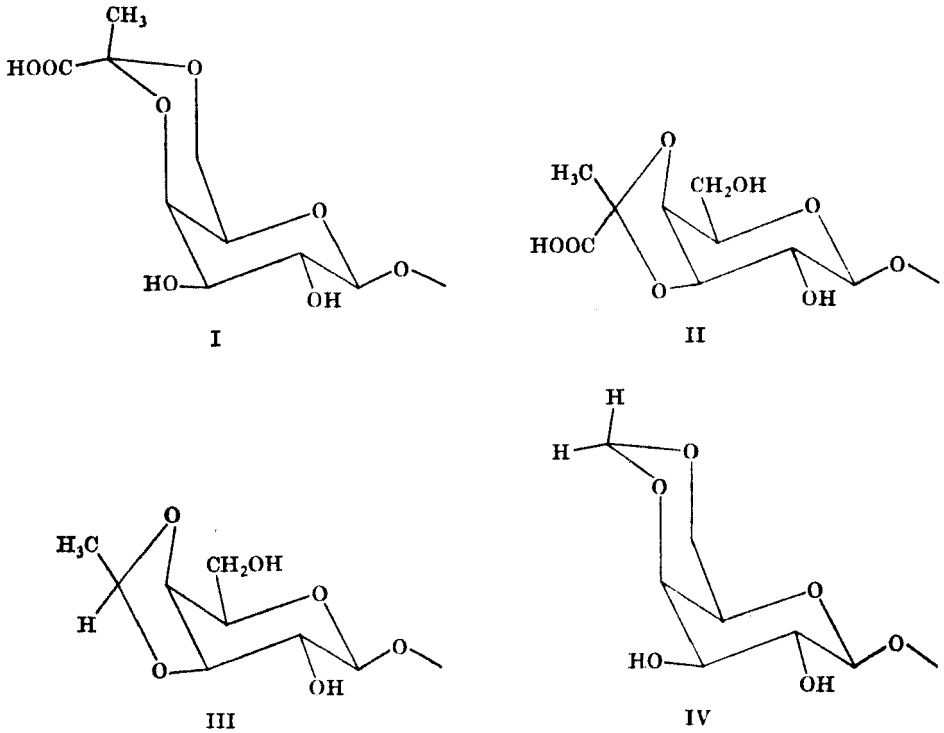


FIG. 7. Ketals attached to the terminal reducing galactose residues of colanic acids from different strains of *Escherichia coli* and *Salmonella typhimurium*. From Garegg *et al.* (1971a, b).

electrophoretic separation was used, a pyruvylated fragment might have been mistaken for glucuronic acid. A number of oligosaccharides have been isolated from the polymer produced by strain A1, by partial acid hydrolysis and by autohydrolysis (I. W. Sutherland, unpublished results). Several of these were found to contain pyruvate but their complete structures have not yet been determined.

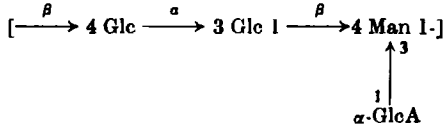
Type 2. The monomer constituents of this serotype were shown to be mannose, glucose and glucuronic acid (Barker *et al.*, 1958). On the basis of methylation, periodate oxidation and oligosaccharide sequences, it

was suggested that the exopolysaccharide contains a large "repeating unit" containing about 40 monosaccharides. By contrast, examination

TABLE 7. Monosaccharides Residues Found in *Klebsiella* Exopolysaccharides

Type	Monosaccharides	References
1	Fucose, glucose, glucuronic acid	Barker *et al.* (1963a)
2	Mannose, glucose, glucuronic acid	Gahan *et al.* (1967)
3	Mannose, galactose, galacturonic acid	Henriksen and Eriksen (1962)
4	Mannose, galactose, glucose, uronic acid	Eriksen (1962)
5	Mannose, galactose, glucose, uronic acid	Henriksen *et al.* (1961)
8	Galactose, glucose, glucuronic acid	Dudman and Wilkinson (1956)
9	Rhamnose, galactose, glucuronic acid	Heidelberger *et al.* (1970)
11	Mannose, galactose, glucose, glucuronic acid	Nimmich (1969); Eriksen and Henriksen (1963)
21	Mannose, galactose, uronic acid	Eriksen and Henriksen (1963)
23	Rhamnose, glucose, glucuronic acid	Nimmich (1969)
26	Mannose, galactose, glucose, uronic acid	Dudman and Wilkinson (1956)
29	Mannose, galactose, uronic acid	Dudman and Wilkinson (1956)
30	Mannose, galactose, glucose, uronic acid	I. W. Sutherland (unpublished data)
32	Rhamnose, galactose	Heidelberger *et al.* (1970)
45	Rhamnose, glucose, glucuronic acid	Nimmich (1969)
47	Rhamnose, galactose, glucuronic acid	Heidelberger *et al.* (1970)
52	Rhamnose, galactose, glucuronic acid	Heidelberger *et al.* (1970)
54	Fucose, glucose, glucuronic acid	Dudman and Wilkinson (1956)
57	Mannose, galactose, uronic acid	Dudman and Wilkinson (1956)
61	Galactose, glucose, glucuronic acid	Nimmich (1959)
64	Rhamnose, mannose, glucose, glucuronic acid	Barker *et al.* (1958)
69	Mannose, glucose, galactose, glucuronic acid	I. W. Sutherland (unpublished data)
71	Rhamnose, glucose, glucuronic acid	Nimmich (1969)
72	Rhamnose, glucose, glucuronic acid	Nimmich (1969)

of material from four different strains showed that each contained a relatively simple tetrasacharide repeating unit (Gahan *et al.*, 1967):

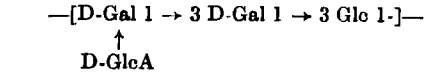


The availability of a phage-induced enzyme (Watson, 1966) depolymerizing type 2 *Klebsiella* polysaccharides permitted a further study of their structures. A preliminary examination (Sutherland, 1971b) showed that different polysaccharide preparations contained acetate, formate and, in one case, pyruvate. All acted as substrates for the phage-induced

enzymes as well as for similar enzymes from type 54 *Klebsiella* strains. The hydrolysis products differed, depending on the polysaccharide composition but, in each case, a tetrasaccharide with the same carbohydrate structure as the repeating unit of the polysaccharide was isolated These oligosaccharides were either formylated, acetylated or pyruvylated and their properties varied with the different substituents. All the type 2 polysaccharides contained formate, but three distinct types were distinguished, namely (i) containing formate only; (ii) containing formate and acetate, and (iii) containing formate and pyruvate. The preparations in the first group appeared to possess formate uniformly distributed on the polymer, as a single formylated tetrasaccharide was found in enzymic digests. The second group of type 2 exopolysaccharides were also uniformly acetylated and formylated, as enzymic hydrolysis yielded only one tetrasaccharide composed of the same monomers in the same molar ratio as the polysaccharide. The single polymer which contained formate and pyruvate was unusual in that enzymic hydrolysis produced equal amounts of two tetrasaccharides, one of which was formylated and the other pyruvylated. The formylated oligosaccharide was indistinguishable from that isolated from the other type 2 polysaccharides. It is therefore probable that the polymer is composed of tetrasaccharide repeating units which are alternately formylated and pyruvylated. There is some similarity to a *K. aerogenes* type 54 strain in which alternate tetrasaccharides are acetylated (Sutherland and Wilkinson, 1968).

Type 3. The polysaccharides from six type 3 *Klebsiella* strains all contain mannose, galactose and galacturonic acid (Eriksen, 1965a). Although the complete structures were not elucidated, the results indicated that the polymers probably contain a relatively simple repeating unit in which there is an aldobiouronic acid containing galacturonic acid and mannose. The polymers from type 3 strains classified as *K. ozaenae*, *K. pneumoniae*, *K. rhinoscleromatis* and *K. aerogenes* proved to be serologically indistinguishable (Eriksen, 1965b).

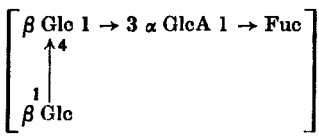
Type 8. The component sugars of a type 8 exopolysaccharide were identified as glucose, galactose and glucuronic acid in the molar ratio 1 : 2 : 1 (Dudman and Wilkinson, 1956). On the basis of periodate oxidation, carboxyl reduction and partial acid hydrolysis, a tetrasaccharide repeating unit was proposed (Sutherland, 1970b):



Acetyl, formyl or pyruvyl residues were not found in the preparation examined. Recent studies (I. W. Sutherland, unpublished work) on a number of other *Klebsiella* type 8 strains showed that the exopoly-

saccharide from one contained acetate and pyruvate; the others were devoid of acyl groups. The pyruvylated sugar was galactose and, as it could be obtained by mild acid hydrolysis, it was concluded that it did not form part of the aldobiouronic acid. From the chromatographic mobility of the carboxyethylidene galactose in a number of solvents (Sutherland, 1971a) it appeared to be identical with 3,4(1'-carboxyethylidenegalactose). If this observation is correct, it implies that the carbohydrate structure of the polysaccharide must differ slightly from that previously reported for *K. aerogenes* type 8 (strain A4) (Sutherland, 1970b) in at least some of the glycosidic linkages.

Klebsiella aerogenes type 54. Studies using the A3 strain of this serotype showed that the slime and capsular polysaccharides have the same chemical composition, which is unaffected by the carbon source in the growth medium (Wilkinson *et al.*, 1955; Aspinall *et al.*, 1956). It was thought to be a highly branched polymer containing 10% fucose, 27% glucuronic acid and 46% glucose. A re-examination of the polymer by Sandford and Conrad (1966) using improved methylation and other procedures indicated that fucose, glucose and glucuronic acid were present in the simple molar ratio 1 : 2 : 1. The presence of a tetrasaccharide



repeating unit was later confirmed (Conrad *et al.*, 1966). This structure was complicated by the presence of acetate first indicated from serological properties of the polysaccharides (Davies *et al.*, 1958) and later confirmed by analysis (Sutherland, 1967; Sutherland and Wilkinson, 1968), and of formate (Sutherland, 1970b). Enzymic hydrolysis produced equal quantities of two tetrasaccharides, both of which contained formate and one of which was acetylated. The isolation and characterization of an octasaccharide which could be further hydrolysed to the two tetrasaccharides indicated that every alternate tetrasaccharide repeating unit is acetylated and that formate is present uniformly on each tetrasaccharide. Although the positions of the acyl groups were not identified, the isolation of trisaccharides (Fig. 8) showed that they are not attached to the terminal non-reducing glucose residues. Examination of the products of enzymic hydrolysis of several other type 54 exopolysaccharides (I. W. Sutherland, unpublished results) showed that all of the polysaccharides are formylated. Acetate is absent from some of the polysaccharides and is present on all the tetrasaccharides of other preparations. Pyruvate does not appear to be present in this serotype.

Another *K. aerogenes* exopolysaccharides of unknown serotype was recently shown to contain a tetrasaccharide repeating unit of the same general form as those found in other capsular preparations from this genus (Yurewicz *et al.*, 1971; Troy *et al.*, 1971). The polymer contains D-galactose, D-mannose and D-glucuronic acid in the molar ratio 2 : 1 : 1. As with other preparations studied, the polysaccharide consists essen-

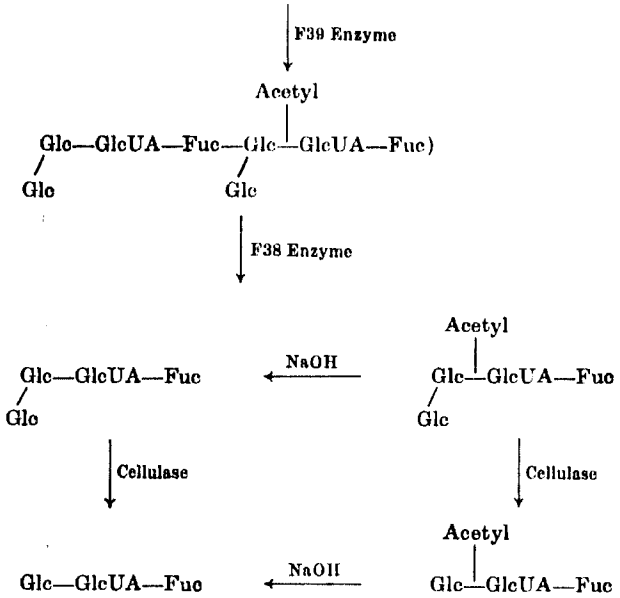
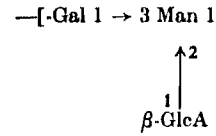


FIG. 8. Course of hydrolysis of acetylated fractions from *Klebsiella* A3 polysaccharide (type 54). From Sutherland and Wilkinson (1968). All oligosaccharides later proved to be formylated.

tially of a linear chain to which single monomer residues are attached. The structure was apparently free from acyl substituents:



A number of *Klebsiella* polysaccharides were found to contain galactose, mannose, glucose and a uronic acid (Table 7). In some preparations, the uronic acid was identified as D-glucuronic acid. Types 30 and 69 are known to cross-react with serotype 2 (I. Ørskov, personal communication) but the chemical structures on which the serological cross-reactions are dependent are not known. Neither of those two exopolysaccharides is hydrolysed by the type 2 polysaccharide depolymerase (I. W. Sutherland, unpublished results).

It is clear that from the results obtained with serotypes 2, 8 and 54, as with colanic acid, numerous strains can synthesize a polysaccharide with the same carbohydrate structure. The non-carbohydrate substituents, such as acetate, formate and pyruvate, are however dependent on the strain examined, and show a considerable degree of variation. The exopolysaccharides of the genus *Klebsiella* which have received detailed study to date are remarkable since they all conform to a uniform general pattern, usually a tetrasaccharide repeating unit from which a trisaccharide forms a linear molecule with a side chain consisting of a single carbohydrate residue. The number and nature of the monomer constituents are also much more limited than in other bacterial genera which have received comparable study.

iii. *Miscellaneous bacterial exopolysaccharides from Gram-negative species.* Despite the increasing knowledge of the structure of bacterial exopolysaccharides, it is still not possible to predict their physical properties. Very few exopolysaccharides, other than the dextran group, have been examined with a view to determining their actual or potential commercial use. A few polymers from diverse bacterial genera are now being investigated to determine their suitability as replacements or substitutes for the traditional industrial polysaccharides such as starch and alginates. As yet, insufficient is known about their structure and physical chemistry to enable the production of tailor-made polymers for a particular purpose as can be done with dextrans and starches, but some knowledge of their structures is now available.

Arthrobacter viscosus. One of the few bacterial exopolysaccharides known to possess gelling, as opposed to pseudo-gelling, properties, is the polymer from *A. viscosus* strain NRRL B-1973. Solutions were shown to form a gel after autoclaving and cooling (Cadmus *et al.*, 1963). At certain concentrations, various salts promoted gel formation and the viscosity of the solutions was unaltered by heating in the presence of these salts. Despite these unusual properties, the chemical composition of the polysaccharide shows no unusual features. The components found were D-glucose, D-galactose and D-mannuronic acid in equimolar amounts (Jeanes *et al.*, 1965). As well as sugars, the polymer contains 25% acetate. Thus, almost two-thirds of the available free hydroxyl groups possess *O*-acetyl groups, a higher proportion than in any similar polymer so far examined. The only monomer in common with other gel-forming polysaccharides is D-mannuronic acid which is a major constituent of alginates, but the possibility of other common properties such as specific glycosidic linkages has not yet been reported.

Xanthomonas campestris. A second polysaccharide with gel characteristics resembling those of sodium alginate or other commercial plant gums was isolated from cultures of *X. campestris* strain NRRL B-1459

by Jeanes *et al.* (1961). It resembles that of *A. viscosus* in the atypical resistance of its viscosity in solutions to heat and to salt effects. The viscosity of solutions is enhanced by monovalent cations at neutral pH values. Although *O*-acetyl groups are present, their removal does not affect these properties. The polysaccharide contains D-glucose, D-mannose, D-glucuronic acid, acetate and pyruvate in the approximate molar ratio 2·8 : 3·0 : 2·0 : 1·7 : 0·6 (Sloneker and Jeanes, 1962). Graded acid hydrolysis released one mole of mannose and eventually several charged oligosaccharides, the major one being an aldobiouronic acid 2-*O*-β-D-glucuronosyl-D-mannose. Smaller amounts of a trisaccharide and a tetrasaccharide were also isolated. These both contain the aldobiouronic acid together with one and two moles of glucose, respectively. Further studies (Sloneker *et al.*, 1964) using periodate oxidation and other techniques led to the suggestion that the polysaccharide consists of a structure with a repeating unit of 16 monomer residues. Most of the polymer was thought to be a linear molecule of glucuronic acid, mannose and glucose residues to which were attached other mannose residues, *O*-acetyl groups and the pyruvate ketal. If this structure is correct, it indicates the possible involvement of a highly complex biosynthetic system.

Azotobacter species. Azotobacter vinelandii normally produces exopolysaccharides in laboratory culture, but shows some variability as to whether discrete capsules or slime are produced. The polymers were thought to contain various sugars depending on the strain studied. Cohen and Johnstone (1964) reported the presence of glucose, rhamnose and galacturonic acid as well as a lactone resembling mannuronolactone in some of its properties. The galacturonic acid was the major component of the polymer, as identified by colorimetric assay. Acetate was also found in several preparations. An unconfirmed report (Claus, 1965) suggests that polysaccharides from several *A. vinelandii* strains contain rhamnose and a keto acid, which was reactive in the thiobarbiturate assay. It was identified as 2-keto-3-deoxy-galactonic acid on the basis of its chromatographic behaviour, oxidation and reduction products. It was preferentially released from the polysaccharide on mild acid hydrolysis.

Several exopolysaccharides from strains of *A. agile*, *A. vinelandii* and *A. chroococcum* have also been examined. It was suggested that, unlike most other bacterial exopolysaccharides, their composition varies with the culture medium, in particular with regard to the presence of fructose in the polymers (Zaitseva *et al.*, 1959). Glucose is present in all preparations as a major component, while rhamnose has also been detected. Although traces of other sugars were identified on the basis of their chromatographic mobilities, the possible presence of uronic acids was

apparently not considered. It is, however, probable that these polymers are similar to those described by Cohen and Johnstone (1964). An extracellular polysaccharide from *A. indica* (*Beijerinckia indica*) was examined by Quinnell *et al.* (1957) and was initially thought to contain D-glucose, D-glucuronic acid and a heptose in the molar ratio 3 : 2 : 1. Later the polymer was subjected to methylation and periodate oxidation, and it apparently contains a trisaccharide repeating unit composed of glucuronic acid, glucose and D-glycero-D-mannoheptose (Parikh and Jones, 1963):

—[-*O*-D-GlcA (1 → 3) *O*-D-Glc (1 → 2) *O*-D-glycero-D-mannoheptose-]—

A further preparation studied by Haug and Larsen (1970) resembled this *A. indica* polysaccharide in containing the same neutral sugars, but lacked D-glucuronic acid, which was replaced by L-guluronic acid, identified by its optical rotation and other properties. Acetate was also present.

The exopolysaccharide from another *A. vinelandii* strain differs from those described earlier from this bacterial species by its close similarity to alginic acid (Gorin and Spencer, 1966). It is a polyuronide in which D-mannuronic acid is the major component with lesser amounts of L-guluronic acid and acetate. The kinetics of periodate uptake were very similar to those for alginic acid from *Laminaria* species. The mannuronosyl residues are linked together through the 4-position and the only appreciable difference from the algal polymer is the higher mannuronic acid: guluronic acid ratio and the presence of acetate. These particular results were extended by the report from Larsen and Haug (1971) on alginate-like polysaccharides from three further strains of *A. vinelandii*. A fractionation procedure was adopted to separate the uronic acid-containing polysaccharide from polymeric material which contains glucose and protein. The ratio of mannuronic acid to guluronic acid in all three preparations was very similar (about 0·56), while the acetyl content was calculated as one per five uronic acid residues. When partial acid hydrolysis was applied in a manner analagous to that used for seaweed alginate, it appeared that each polymer contains sequences of each uronic acid, i.e. sequences of contiguous D-mannuronic acid residues separated from similar L-guluronic acid sequences by portions of the molecule in which the two types of monomer alternated. These structures are very similar to those described for other alginate preparations, although minor differences between the three *Azotobacter* exopolysaccharides were observed. In view of these results, some of the earlier reports of other monomer constituents in the exopolysaccharides from *Azotobacter*, species may have been due to degradation products or to other artefacts and should be re-evaluated. Another important feature of the *Azotobacter* alginate is the characterization of an exopolysaccharide which

apparently is not formed from identical blocks of repeating units but which is a linear molecule with differing structure in different portions of the polymer. Associated with this feature was the isolation of an extracellular enzyme capable of modifying the polysaccharide structure (Haug and Larsen, 1971). The implications of this are considered with other work on exopolysaccharide biosynthesis (p. 198).

Pseudomonas species. The production of polymers resembling alginic acid is not confined to a single bacterial genus. Linker and Jones (1964, 1966) isolated a mucoid Gram-negative bacterium from a patient with cystic fibrosis and identified it as a *Pseudomonas* species. The polysaccharide hydrolysate resembled hydrolysates of alginic acid, containing mannuronic acid as the major component, together with guluronic acid and the two corresponding lactones. Infrared spectra of the polysaccharide closely resembled those obtained from sodium alginate and both polymers are susceptible to an inducible "alginase" prepared from a yellow-pigmented bacterial strain. Comparison with similar preparations from other mucoid *Pseudomonas* isolates revealed one polymer containing mannuronic acid only, and others in which the molar ratio of mannuronic acid to guluronic acid ranged from 4 : 1 to 20 : 1, respectively. In a commercial sodium alginate, the ratio was 5 : 2. The initial preparation method used alkaline extraction with consequent loss of *O*-acetyl groups. Omission of this preparative stage gave a product containing 9–11% acetate, which was much more resistant to digestion with the bacterial alginase.

A further examination of mucoid *Ps. aeruginosa* strains (Carlson and Matthews, 1966) confirmed that the exopolysaccharides secreted by these bacteria are polyuronic acids in which the principal monomer component is D-mannuronic acid. Varying amounts of L-guluronic acid were detected. The identities of the uronic acids were further confirmed by esterification, reduction and characterization of the methyl esters. Unlike the production of "colanic acid" by various species of *Enterobacteriaceae* most of which are sufficiently closely related to permit genetic compatibility, "alginic acid" production appears to be a feature of bacterial genera which are not at present considered to be closely related. Thus, the dissimilarity of the genera *Azotobacter* and *Psuedomonas* is indicated by their respective DNA G–C proportions of 56 and 64%. By comparison, the bacterial strains which excrete colanic acid all belong to a group with 50–55% G + C in their DNA.

b. Gram-Positive Bacteria

Diplococcus pneumoniae (Pneumococcus). Although a number of genera of Gram-positive bacteria produce exopolysaccharides, few have been

extensively studied. Due mainly to their medical importance, and to the pioneering studies of Griffith (1928) on bacterial transformation, a considerable amount of information about pneumococcal polysaccharides has been obtained. The polysaccharides contain a much wider range of monosaccharides and other components, and are more varied in structure than the *Klebsiella* group, although so far the structures of only a few serotypes have been completely established.

Type I. The capsular polysaccharide of this strain was one of the first such polymers to receive a considerable degree of chemical and immunochemical investigation. Both *O*-acetyl and D-galacturonic acid residues are present and immunologically important (Avery and Goebel, 1933; Heidelberger, 1962). Further studies (Guy *et al.*, 1967) identified both glucosamine and galactosamine as polysaccharide components. The presence of D-glucose, earlier tentatively identified, was confirmed, but galactose was not found. Although the complete structure of *D. pneumoniae* type I exopolysaccharide has not yet been elucidated, part of the structure is considered to be a trisaccharide sequence:

—[-D-galacturonosyl-(1 → 3)-glucosaminyl-(1 → 3) galacturonosyl-]—

Type II. This exopolysaccharide contains L-rhamnose (48%), D-glucose (35%) and D-glucuronic acid (16%) (Barker *et al.*, 1965). From methylation studies, it was concluded that the L-rhamnose is linked through positions 1 and 3, while the D-glucuronic acid is linked at positions 1 and 4 as well as occurring as the non-reducing terminal residue. The D-glucose is involved in 1, 4, 6 branch points. A β-linked trisaccharide, isolated by enzymic hydrolysis, contained only L-rhamnose. The application of sequential enzyme induction revealed further facets of the structure not detected by earlier studies (Barker *et al.*, 1967). The trisaccharide sequence: β-L-Rha (1→3) β-L-Rha (1→3) α-L-Rha, was attached to the remainder of the exopolysaccharide molecule by an α (1→4) linkage. On the basis of these and other results, a complex repeating unit structure was postulated for the polymer (Fig. 9).

Type III. This polymer possesses the simplest known repeating unit found in a bacterial extracellular heteropolysaccharide. In a series of studies, Goebel and his colleagues (Hotchkiss and Goebel, 1937; Adams *et al.*, 1941; Reeves and Goebel, 1941) found that the polymer is composed of D-glucose and D-glucuronic acid, forming a disaccharide repeating unit which is a polymer of cellobiuronic acid and is, as already mentioned, the substrate for a hydrolase produced by a *Bacillus* species:

⊢—[-3-β-D- GlcA (1 → 4) β-D-Glc-]1—

Type V. The capsular material of this serotype proved to contain a more complex pattern of sugars. Acid hydrolysis of the purified polymer released D-glucose, D-glucuronic acid and two amino sugars, namely

L-fucosamine (2-amino-2,6 dideoxy-L-galactose) and L-pneumosamine (2-amino 2,6 dideoxy-L-talose) (Barker *et al.*, 1960). Although acetate was present, this formed part of *N*-acetylamino sugars and not *O*-acetyl groups. By the use of methylation studies and periodate oxidation, some structural details were determined by Barker *et al.* (1966b). A disaccharide repeating unit of 3-*O*-D-glucuronosyl-*N*-acetyl-L-fucosamine was found, to which were attached residues of D-glucose and *N*-acetyl-L-pneumosamine.

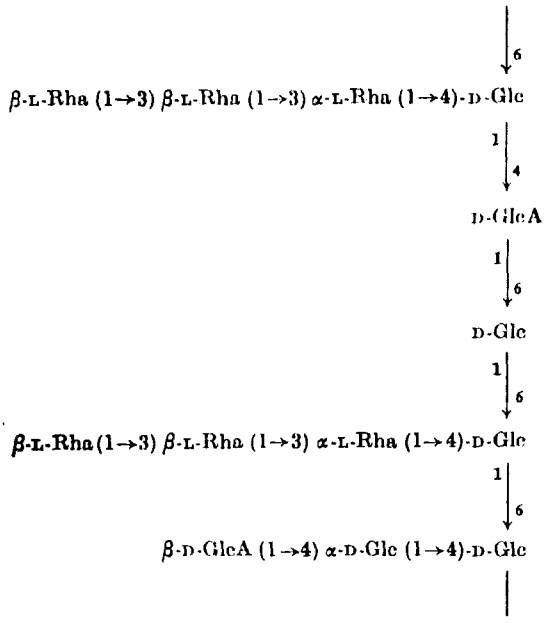
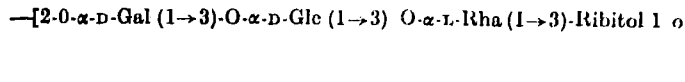


FIG. 9. Structure of the repeating unit of the polysaccharide from *Diplococcus pneumoniae* type 2. From Barker *et al.* (1967).

Diplococcus pneumoniae type VI polysaccharide is one of a group which contains ribitol phosphate. Unlike many other such polymers, the only other sugars present were the neutral hexoses D-glucose and D-galactose and the methylpentose L-rhamnose (Rebers and Heidelberger, 1959, 1961). On the basis of chemical analyses and other techniques, a simple tetrasaccharide repeating unit was proposed:



Types XIA and XVIII. Exopolysaccharides of these serotypes are among several which contain glycerol phosphate residues (Shabarova

et al., 1962) and thus bear a superficial resemblance to the teichoic acids which are of widespread occurrence in the cell walls of Gram-positive bacteria. Type XIA exopolysaccharide contains in addition, D-glucose, D-galactose and acetate (Kennedy *et al.*, 1969). The molar proportions of D-glucose, D-galactose, glycerol, phosphate and acetate are 2 : 2 : 1 : 1 : 2. A partial structure for the polymer, based on the results of periodate oxidation and alkaline hydrolysis, is

—[-3-*O*-D-Gal (1 → 4) D-Glc (1 → 6) D-Glc (1 → 4) D-Gal-]—
4
P
3
glycerol

In a series of studies on capsular material from *D. pneumoniae* type XVIII (Markowitz and Heidelberger, 1954; Estrada-Parra *et al.*, 1962; Estrada-Parra and Heidelberger, 1963), a sugar composition showing some similarities to that of type XIA was noted. D-Glucose, D-galactose, L-rhamnose and glycerol phosphate were detected along with *O*-acetyl groups. Treatment with alkali removed the glycerol phosphate but left the sugars intact. As a result, it was concluded that the polymer contains a linear chain of neutral sugars to one of which is attached the glycerol phosphate. On the basis of the chemical and immunochemical studies, the main chain of the polysaccharide from *D. pneumoniae* type XVIII may consist of one of two possible structures:

—[-3-D-Gal (1 → 4) α-D-Glc (1 → 6)-D-Glc (1 → 3)-L-Rha (1 → 4)-D-Glc 1-]—

or

—[-3-D-Gal (1 → 4)-D-Glc (1 →3)-L-Rha (1 → 4) α-D-Glc (1 → 6)-D-Glc 1-]—

Although the sugars and other components present in a number of other *D. pneumoniae* exopolysaccharides have been determined (Table 8), in most other serotypes no attempt has been made to determine what repeating units, if any, are present in the polymers. It is clear that these capsular polysaccharides are exceedingly heterogenous with regard to their monomer components, containing a number of sugars and other substances not previously found in exopolysaccharides. Care should probably be taken in assessing some of the results, as contamination of capsular polysaccharide preparations with other polymers has been observed and a cell-wall polysaccharide from *D. pneumoniae* (Pneumococcal C polysaccharide) is known to contain among other substances *N*-acetylgalactosamine phosphate (Gottschlich and Liu, 1967). A large volume of data on the immunochemical relationships of pneumococcal polysaccharides has been amassed. This was reviewed by Heidelberger (1960) who showed that there were serological cross-reactions between different *D. pneumoniae* polymers and with a number of other

TABLE 8. Components of Some Expolysaccharides from *Diplococcus pneumoniae*

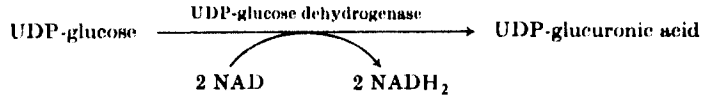
Serotype	Monomer components	Reference
I	*N*-acetyl-D-glucosamine, D-galacturonic acid, acetate	Heidelberger (1960)
II	L-rhamnose, D-glucose, D-glucuronic acid	Barker *et al.* (1966b)
III	D-glucuronic acid, D-glucose	Adams *et al.* (1941)
V	*N*-acetyl-L-fucosamine, pneumosamine, D-glucose, D-glucuronic acid	Barker *et al.* (1966a, b)
VI	D-galactose, D-glucose, L-rhamnose, ribitol, phosphate	Rebers and Heidelberger (1961)
VII	D-galactose, D-glucose, L-rhamnose, *N*-acetyl-D-glucosamine, *N*-acetyl-D-galactosamine	Tyler and Heidelberger (1968) Heidelberger and Tyler (1964)
VIII	D-galactose, D-glucose, D-glucuronic acid	Heidelberger (1960)
IX	D-glucose, D-glucuronic acid, *N*-acetyl-D-glucosamine	Rao and Heidelberger (1966)
XIV	D-galactose, D-glucose, *N*-acetyl-D-glucosamine	Heidelberger (1960)
XVIII	D-galactose, D-glucose, L-rhamnose, glycerol, phosphate	Estrada-Parra *et al.* (1962)
XIX	D-glucose, L-rhamnose, *N*-acetyl-D-mannosamine, phosphate	Miyazaki and Yadomae (1971)
XXXI	L-rhamnose, D-galactose, D-glucuronic acid.	Roy *et al.* (1970)
XXXIII	D-galactose, D-glucose, D-galacturonic acid, amino sugar	Mills and Smith (1962)
XXXIV	D-galactose, D-glucose, ribitol, phosphate	Roberts *et al.* (1966)

polysaccharides of bacterial and plant origin. The chemical similarities known in some of these polymers accounted for some of these serological relationships, while others could only be postulated.

IV. Biosynthesis

A. Enzymes and Precursors

The importance of sugar nucleotides as glycoside donors for the synthesis of most polysaccharides and glycan-containing molecules has been known for many years. The first of these compounds to be isolated and characterized by Caputto *et al.* (1950) was UDP-glucose. Along with many other such compounds it has since been shown to function in, among other systems, exopolysaccharide synthesis. As well as their direct role in providing sugars for transfer to form polymers, the sugar nucleotides provide a mechanism for synthesis of particular types of monosaccharides. This is seen in the conversion of UDP-glucose to UDP-galactose by UDP-galactose-4-epimerase and in the formation of GDP-fucose from GDP-mannose by the two enzymes of the GDP-fucose synthetase complex. Many of these reactions are involved in the provision of sugars for other polymers in the cell, but the action of UDP-glucose dehydrogenase in the formation of glucuronic acid is normally



specifically associated with its provision for exopolysaccharides. A comprehensive list of sugar nucleotides and their isolation from bacterial (and other) cells can be found in the review by Ginsburg (1964).

It is obvious that, in provision of the precursors for polysaccharide formation, a considerable number of enzymes are involved. Some of these are concerned primarily with the catabolism of monosaccharide substrates but are equally important in the early reactions leading to sugar nucleotide synthesis. A scheme for the enzymes leading from a carbohydrate substrate to the synthesis of the exopolysaccharide of *K. aerogenes* type 8 (Fig. 10) shows that, out of a probable total of at least ten enzymes, six are specific to the processes of capsule formation.

The enzymes responsible for synthesis of sugar nucleotides involved in capsule production were studied in several strains of *D. pneumoniae* (Smith *et al.*, 1967, 1959). Even in non-capsulate strains, high levels of sugar nucleotides were detected, indicating that control was probably exerted at an early stage in precursor formation and not at the sugar-transferase level. Defects in enzymes responsible for sugar-nucleotide

formation led to failure to produce polymer. If an alternative monosaccharide is incorporated into the culture medium, synthesis of exopolysaccharide can sometimes be restored. Thus, if galactose is a component of the slime or capsule, lack of the enzyme UDP-galactose 4-epimerase results in non-capsulate cells. Growth in galactose-containing media can, in *E. coli* and *K. aerogenes* at least, circumvent the

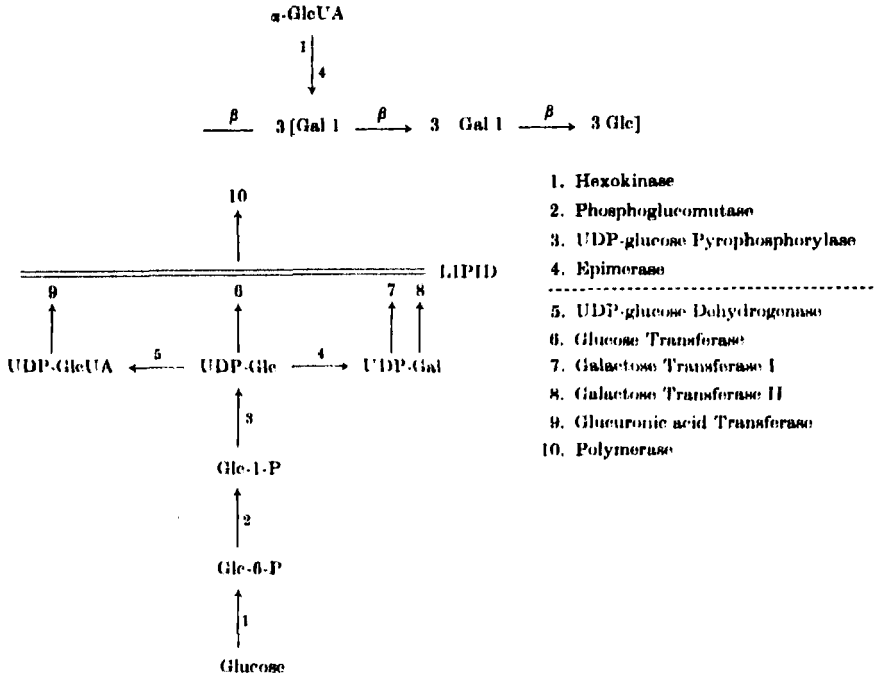


FIG. 10. Pathway for synthesis of the capsular polysaccharide of *Klebsiella aerogenes* A4. The enzymes listed include several (1–4) which are involved in precursor formation for several different polymers. The remainder are involved solely in exopolysaccharide synthesis.

defect and restore capsulation. The same effect is seen with mutants defective in UDP-glucose pyrophosphorylase or phosphoglucomutase (Norval, 1969). In these species, a galactokinase and other enzymes permit the formation of UDP-galactose and its epimerization to glucose. In an analogous manner, growth in mannose-containing medium renders phosphomannose isomerase mutants mucoid.

Synthesis of sugar nucleotide precursors requires systems of varying complexity. From glucose as the starting substrate, only three enzymes,

namely hexokinase, phosphoglucomutase and UDP-glucose pyrophosphorylase, are required to produce UDP-glucose. On the other hand, fucose, a frequent component of bacterial exopolysaccharides, is transferred from GDP-fucose. This necessitates the formation of GDP-mannose, involving two enzymes of the GDP-fucose synthetase complex, as well as prior stages in the conversion of glucose to mannose. Most of the potential glycosyl donors and the systems by which they are synthesized are well recognized and further details can be found in the review by Ginsburg (1964). Some gaps in our knowledge still remain, as nucleotide sugars containing several of the amino sugars recently discovered in *D. pneumoniae* polysaccharides have not yet been identified.

The sugar nucleotides play a dual role in exopolysaccharide synthesis, as in the formation of other glycan-containing polymers. They provide a mechanism for sugar interconversion. This is seen in the formation of galactose and glucuronic acid as their UDP derivatives from glucose as UDP-glucose. Similarly, L-fucose is formed from D-mannose through the mediation of the GDP-linked sugars (Ginsburg, 1960). The second role of the sugar nucleotides is that of activating the monosaccharides. The simple sugars cannot be transferred directly to acceptors during polymer synthesis. The nucleotide diphospho sugars with relatively high (negative) free energy of hydrolysis provide suitably activated monosaccharides. The free energy of hydrolysis of UDP-glucose is −7600 calories, and other sugar nucleotides probably give similar values. The only exopolysaccharides which are apparently not formed from activated glycosyl donors are levans and dextrans. Their synthesis requires the utilization of sucrose or related oligosaccharides from which part of the molecule (fructose for levans and glucose for dextrans) is polymerized. Other extracellular homopolysaccharides (see p. 189) conform to the standard pattern, utilizing sugar nucleotides as the glycosyl donors.

In the same way that several of the enzymes of carbohydrate metabolism are essential for exopolysaccharide formation, the involvement of isoprenoid lipids or glycosyl carrier lipid demonstrated by Troy *et al.* (1971) extends the range and number of enzymes which must be considered. The C_{55} isoprenoid alcohol bactoprenol, which contains one saturated isoprene residue, was identified in *Lactobacillus casei* (Thorne and Kodicek, 1966) where it was the major biosynthetic product from mevalonic acid. Recently complete synthesis of metabolically active glycosyl carrier lipid from Δ^3-isopentenyl pyrophosphate and farnesyl pyrophosphate was achieved (Christenson *et al.*, 1969). Particulate enzyme preparations from *Salmonella newington* catalysed synthesis of a C_{55} isoprenoid alcohol phosphate containing 11 unsaturated isoprene residues. A soluble enzyme system yielded lipids of shorter chain length.

Whether the bactoprenol isolated from *L. casei* serves the same function *in vivo* as glycosyl carrier lipid is not clear. Cytological studies (Barker and Thorne, 1970) indicated that it was almost all associated with the sphaeroplast membrane. This would be the expected site for synthesis of polymers found outwith the cell membrane. Insufficient is yet known about the synthesis of glycosyl carrier lipid and bactoprenol to determine whether enzymic defects in their synthesis are lethal, and what secondary effects occur on polymer formation. Conditional mutants should provide information of the effect of altered synthesis of glycosyl carrier lipid on polysaccharide formation. We believe that a group of mutants (see p. 205) showing temperature-sensitive (low temperature restrictive) polysaccharide synthesis fall within this category (Norval and Sutherland, 1969).

As well as sugar nucleotides and glycosyl carrier lipid, a third type of precursor must be considered for exopolysaccharide synthesis. These are required for the acyl substituents commonly present. It is not yet clear what these precursors are. Formate might be added from a tetrahydrofolate compounds or from formyl-CoA. Similarly, two possible donors for acetate are acetyl phosphate and acetyl CoA. The latter is more probably involved as it is known to be the precursor of *O*-acetyl groups found in the lipopolysaccharide of *S. newington* (Keller, 1965). Transfer of acetate from acetyl-CoA to oligosaccharides from *K. aerogenes* type 54 exopolysaccharide was also noted (Sutherland and Wilkinson, 1968). The most probable donor for pyruvate is phosphoenolpyruvate. Although polymers with the same carbohydrate structure but different acyl groups have been studied, only one example has been reported where the bacterial strains were of common ancestry. In these strains, changes in the acyl groups present on colanic acid from *S. typhimurium* were observed, one polymer being acetylated and the other not (Garegg *et al.*, 1971a). Thus a defect in the acetylase may still permit synthesis of a non-acetylated polymer. Whether this is also true for other polysaccharides and for other acyl groups has still be be examined.

B. Cell-Free Synthesis

Several attempts have been made to study the synthesis of exopolysaccharides by cell fractions. The earlier work on these aspects provided some information, but a real stimulus was obtained from the systematic studies on heteropolysaccharide synthesis by Mills and Smith (1962) and their colleagues using preparations from *D. pneumoniae*. The value of specific methods of assay, particularly immunochemical and related methods, was recognized during these studies and also in work on lipopolysaccharide synthesis, but has not proved widely applicable in

exopolysaccharide studies. Only recently, following the recognition of the role of lipid intermediates as polysaccharide precursors in numerous systems, have further developments been made with exopolysaccharide systems. The methods used have the disadvantages of all work which necessitates the use of particulate enzyme preparations which cannot be separated into their active components. It should also be recognized that *in vitro* results may not proceed in exactly the same manner as is normal for whole cells. Few of the cell-free systems studied so far convert appreciable quantities of the substrates provided into polymeric material, when compared with the rates of polysaccharide synthesis by whole cells in culture or in washed suspension. It is therefore open to question whether the glycosyl donors and other substrates used in such experiments are necessarily the sole compounds involved in the intact cells. At the same time, it should be remembered that preparation of the particulate enzyme systems necessitates disruption of what, in the intact cell, is a well ordered functional system for the formation and supply of the polymer precursors and their transfer from aqueous to lipophilic environments.

1. Homopolysaccharides

Despite the large number of bacteria which secrete exopolysaccharides few cell-free systems have been studied and most of the work on homopolysaccharides has been concerned with levan and dextran systems which will not be considered here. Some of the results from studies on bacterial synthesis of cellulose by *Acetobacter xylinum* are particularly relevant in view of the recent discovery of the involvement of lipids in polysaccharide formation. Glaser (1958) prepared an insoluble enzyme system which catalysed the transfer of glucose from UDP-glucose to polymeric material. The product was soluble in water but insoluble in alkali. It was sensitive to cellulase and, on hydrolysis, formed the characteristic oligosaccharides obtained from cellulose. When the enzyme system was pretreated to remove endogenous polysaccharide, cellodextrins acted as primers. It was of particular interest that the glucosyl donor in the system was UDP-glucose in contrast to cellulose synthesis in green plants in which GDP-glucose functions (Barber *et al.*, 1964). Another intermediate was also detected in the *A. xylinum* system (Khan and Colvin, 1961a, b). Ethanol extracts of the cells yielded a product which, on incubation with soluble cell material, formed fibrils. These fibrils were insoluble in alkali and hydrolysates showed the presence of glucose. No further evidence for the involvement of lipid intermediates in bacterial cellulose synthesis has been reported.

Synthesis of a glucan by *Rhizobium japonicum* was studied by Dedonder and Hassid (1964). A particulate system was involved, and incubation with UDP-glucose led to the formation of a product identical with the extracellular glucan. Optimal synthesis required the presence of Mg^{2+} or Mn^{2+} and a pH value of 7·5. The polymer closely resembled the material isolated from *Agrobacterium* species (Putman *et al.*, 1950) in its high content (80%) of β 1→2 linked glucose residues. Some of the glucose was also β 1→3 or β 1→6 linked.

2. *Heteropolysaccharides*

From the few exopolysaccharides which have received study using biosynthetic systems, most information has been gleaned from studies on *D. pneumoniae* and *K. aerogenes*. Using cell-free preparations from *D. pneumoniae* type III, Smith *et al.* (1960) obtained net synthesis of polysaccharide from UDP-glucose and UDP-glucuronic acid. Incorporation of the two sugars in a 1 : 1 molar ratio was comparable to that found in the native polymer, while the product, identifiable through the use of ^{14}C-sugar nucleotides, was precipitable with antisera to type III Pneumococcus polysaccharide. Attempts to replace the UDP-sugars with glucose or glucose 1-phosphate were unsuccessful. Further examination (Smith *et al.*, 1961) showed that the enzyme complex had a pH optimum at 8·35 and an optimal temperature for polymer formation at 32°. For full activity, Mg^{2+} were essential; in their absence, polysaccharide synthesis was negligible. Differential centrifugation of the particulate enzyme preparation showed that it sedimented between 30,000 and 115,000 **g**, although slight activity (less than 10%) was found in the supernatant solution. Perhaps because of the simple structure of the polymer and its requirements for only two glycosyl donors, in exactly equimolar proportions, a very high proportion of the sugars (90%) was incorporated into polymer under optimal conditions, Another feature of the system was a requirement for preformed polymer, which was presumably essential as an acceptor for the sugars. The dependence was highly specific, as addition of pure exopolysaccharide had no effect. Stimulation of polymer synthesis was noted following the addition of polysaccharide which had been depolymerized by enzymic treatment (Smith and Mills, 1962a). After chromatographic separation of the products of enzymic hydrolysis and testing fragments of different size, it was concluded that oligosaccharides composed of 8–12 disaccharide units were active as acceptors. The nature and role of such acceptors is still not clear, as we observed (Sutherland and Norval, 1970) that a *K. aerogenes* strain, known to be defective in phosphoglucomutase, was unable to form polymer when cell-free preparations from glucose-grown cells were used and

the appropriate sugar nucleotides supplied. Cells grown in galactose-containing media resembled wild-type bacteria in their ability to form polymer. This may indicate that failure to form glucose 1-phosphate results in the inability to form some acceptor or primer molecule. On the other hand, UDP-glucose pyrophosphorylase-less mutants showed normal polymer synthesis. In similar studies (Troy *et al.*, 1971), UDP-galactose epimerase-less mutants also had normal polymer-synthesizing capacity, which apparently indicates the presence of suitable acceptors.

In other polysaccharide-synthesizing systems it has not always been clear whether the polymer produced by cell-free systems was of comparable size to that formed by intact cells. The polysaccharide from *D. pneumoniae* type III preparations behaved on immunoelectrophoresis in exactly the same way as the native polysaccharide and it was therefore thought to be of identical molecular weight.

Attempts to obtain polymer synthesis in other *D. pneumoniae* strains did not give as good incorporation of sugars into polymeric material (Smith and Mills, 1962b; Mills and Smith, 1962). In *D. pneumoniae* type I, addition of UDP-galacturonic acid and UDP-*N*-acetylglucosamine to a particulate fraction obtained from a non-capsulate mutant yielded polymeric material precipitable by homologous antisera. Some synthesis occurred if UDP-galacturonic acid alone was present in the incubation mixture, but it was not clear whether this was due to its addition to a pre-existing molecule or to the presence of some membrane-bound UDP-*N*-acetyl-glucosamine. *Diplococcus pneumoniae* type VIII preparations also showed polymer synthesis when the uridine diphosphate derivatives of glucose, galactose and glucuronic acid were added to particulate enzyme preparations, but the incorporation of isotopically labelled glucose only reached 5% of the total available as UDP-glucose. Some of the glucose was epimerized to galactose, as investigation of the fate of the labelled sugar showed the presence of ^{14}C-glucose and ^{14}C-galactose in hydrolysates of enzymically formed polymer. One unexplained result from these studies was the report that, in type VIII preparations, some of the glucose from UDP-glucose was converted to a non-reducing compound thought to be a disaccharide, which produced only glucose on hydrolysis. This reaction was non-specific, being observed in several *D. pneumoniae* strains of different serotype. It was not clear whether this compound was in any way associated with exopolysaccharide synthesis, and it may have been rather associated with some intracellular or cell-wall component. No further observations on its nature and function have been reported.

The identification of lipid-linked intermediates in the biosynthesis of a number of polysaccharides or polysaccharide-like molecules containing repeating oligosaccharides has been well documented. These compounds,

which have recently been termed glycosyl carrier lipids in the review of the subject by Rothfield and Romeo (1971), were first identified as intermediates in the synthesis of lipopolysaccharides (Wright *et al.*, 1965; Weiner *et al.*, 1965) and of mucopeptide (Anderson *et al.*, 1965). Ethanol-soluble compounds had already been recognized in bacterial cellulose synthesis (Khan and Colvin, 1961a, b). It was to be expected that a role might also be found for glycosyl carrier lipids in exopolysaccharide synthesis, as these polymers resemble lipopolysaccharides and mucopeptides in three respects, namely: (i) they are composed of oligosaccharides polymerized into a linear macromolecule; (ii) they are found outwith the bacterial cell membrane; and (iii) the glycosyl donors are mainly found in the intracellular aqueous environment. The first

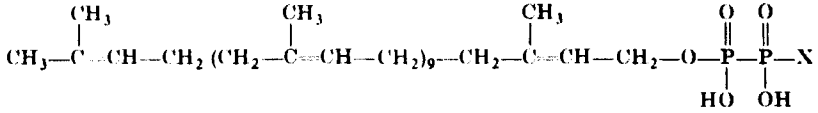
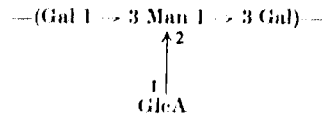


FIG. 11. Structure of glycosyl carrier lipids involved in polysaccharide synthesis in *Klebsiella aerogenes*. X indicates a monosaccharide residue.

report of the involvement of glycosyl carrier lipid in exopolysaccharide synthesis (Troy and Heath, 1968) was followed by its subsequent identification on the basis of its mass spectrum as an isoprenoid alcohol (Fig. 11; Troy *et al.*, 1971). In its active form, the terminal isoprenoid alcohol group is attached through a pyrophosphate bridge to a monosaccharide (Fig. 11). The compound is thus similar, if not identical, to that participating in the formation of the oligosaccharide repeating units of both the mucopeptide and lipopolysaccharide constituents of bacterial cell walls (Rothfield and Romeo, 1971). It differs from the glycosyl carrier lipids responsible for mannolipid synthesis and for certain modifications to lipopolysaccharide (in some lysogenic bacterial strains) as these are linked to the reducing end of monosaccharides or oligosaccharides through a phosphate and not a pyrophosphate group.

Using a strain of *K. aerogenes* which synthesizes a polysaccharide with a tetrasaccharide repeating unit:



Troy *et al.* (1971) found that sequential transfer of sugars from nucleotide donors to glycosyl carrier lipid (GCL) occurred. The sequence of reactions is:

UDP-Gal + GCL-P → GCL-P-P-Gal + UMP

GCL-P-P-Gal + GDP-Man → GCL-P-P-Gal-Man + (GDP)

GCL-P-P-Gal-Man + UDP-GlcA → GCL-P-P-Gal-Man-GlcA + (UDP)

GCL-P-P-Gal-Man-GlcA + UDP-Gal → GCL-P-P-Gal-Man-Gal + (UDP)

↑

GlcA

The isolation and study of a series of mutants from another *K. aerogenes* strain that synthesizes a tetrasaccharide repeating unit (Sutherland and Norval, 1970) confirmed these results. The unit has the structure:

—(Gal I → 3 Gal I → 3 Glc)—

↑

1

GlcA

The first reaction in the sequence for this strain is:

UDP-Glc + GCL-P → GCL-P-P-Glc + UMP

This was shown by isolation of UMP from the reaction mixture and by its inhibitory effect on glucose 1-phosphate transfer. Subsequently two moles of galactose were transferred to the glycosyl carrier lipid and their transfer was partially inhibited by UDP. Mutants blocked at various stages of transfer of sugar to lipid were isolated and characterized (Table 9). As in other forms of sequential transfer, as found in lipopolysaccharide formation, failure to transfer one sugar led to non-transfer of subsequent monosaccharides. Thus, mutants unable to transfer glucose 1-phosphate to the lipid phosphate failed to transfer galactose, and all such transferase-less mutants were unable to form polymer. One noticeable difference in this respect from the lipopolysaccharide-synthesizing systems was in the process for forming the monosaccharide side-chains. In the preparations used by Troy *et al.* (1971) the addition of the side chain (glucuronic acid) was a prerequisite for the addition of

TABLE 9. Transfer of Sugars to Lipid in Mutants of *Klebsiella* Type 8 (Strain A4)

Strain	Sugar transfer (nmol./hr./mg. protein)			Enzyme defect in mutant
	Glucose	Galactose	Ratio	
A4	0·124	0·284	2·3	—
034	0·053	0·090	1·8	?
036	0·053	0·124	2·3	?
027	0·022	0·054	2·5	?
037	0	0	—	Glucose transferase
029	0·013	0	—	Galactose transferase I
038	0·020	0	—	
032	0·061	0·070	1·1	Galactose transferase II
031	0·051	0·065	1·3	

the second galactose residue. By contrast, in the formation of the antigenic portion of *S. typhimurium* lipopolysaccharide, the trisaccharide mannosyl-rhamnosyl-galactose is formed on the glycosyl carrier lipid and could be polymerized even in the absence of the side-chain sugar, which in this polymer was the 3,6 dideoxyhexose abequose attached to the mannosyl residues (Osborn and Weiner, 1968).

The lipid requirement for the exopolysaccharide enzyme system was demonstrated by the use of solvent extraction and careful reconstruction of an active complex (Troy *et al.*, 1971). The largest oligosaccharide identified as being attached to lipid was an octasaccharide (i.e. two units of the polymer) and it is still not clear how large a fragment is eventually accumulated on the lipid prior to transfer to an acceptor. In the only comparable system which has been studied, the antigen portion of lipopolysaccharides (Kent and Osborn, 1968), the entire side-chain of about eight tetrasaccharide units attached to glycosyl carrier lipid was isolated from mutant strains unable to transfer it to acceptor portions of the macromolecule. Unfortunately the acceptor molecule in capsule synthesis has not been identified, and selection of mutants similar to these is not possible. It is however certain from the isolation of slime-forming mutants unable to attach the polysaccharide formed to the cell surface (Wilkinson *et al.*, 1954; Norval, 1969) that some specific site is involved. The possible disorganization of this site during particulate enzyme preparation may account for the relatively low levels of polymer formed by cell-free systems.

Of particular interest in the exopolysaccharide biosynthetic system is the role of the glycosyl carrier lipid *vis à vis* its function in lipopolysaccharide and mucopeptide synthesis. It is possible that there are slight anomeric differences between the glycosyl carrier lipids which render them specific to the different systems which utilize them. The amount of glycosyl carrier lipid in bacterial cells is extremely low; a value of 0·02% of the cell mass was suggested for that involved in lipopolysaccharide formation in *S. newington* (Dankert *et al.*, 1966) and of 40 nmol./g. wet weight for the analogous compound in *S. typhimurium* (Kent and Osborn, 1968), while Wright (1971) indicated 10^5 molecules/cell as the value for glucosyl lipid in lysogenic *Salmonella*. The glycosyl carrier lipid from *K. aerogenes* membranes (Troy *et al.*, 1971) functioned *in vitro* in the biosynthesis of a mannan in *Micrococcus lysodeikticus*, while interchange between the mucopeptide- and lipopolysaccharide-forming systems was earlier reported. In the growing cell, mucopeptide and lipopolysaccharide syntheses are assumed to occur simultaneously, and would therefore require glycosyl carrier lipid at the same time. Exopolysaccharide formation could occur when there is no direct requirement for glycosyl carrier lipid in the other systems and certainly occurs in non-growing

cells. If the lipid were used first for synthesis of the cell-wall components, then for extracellular polymers it might be expected that bacitracin, which prevents dephosphorylation of the glycosyl carrier lipid pyrophosphate, would have an inhibitory effect on exopolysaccharide synthesis. In fact, 25% inhibition of transfer of sugars involved in exopolysaccharide synthesis to lipid was observed (Sutherland and Norval, 1970). This may indicate that a portion of the glycosyl carrier lipid normally utilized for mucopeptide synthesis can function in exopolysaccharide formation. Other evidence comes from *K. aerogenes* serotype 2 (strain 243) in which galactose is absent from the exopolysaccharide

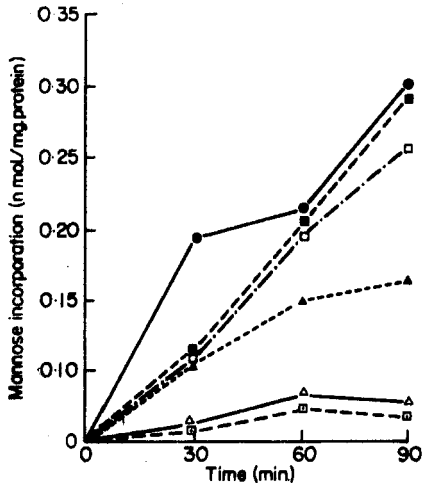


FIG. 12. Time-course of incorporation of mannose from GDP-mannose into lipid. Cell membrane from Type 2 *Klebsiella* was incubated at 18° and pH 8·0 with the following compounds:

(i) □, UDP-glucose, GDP-mannose;
(ii) ⊟, GDP-mannose;
(iii) △, UDP-glucose, GDP-mannose, UMP;
(iv) ▲, UDP-glucose, GDP-mannose, UDP-galactose;
(v) ■, UDP-glucose, GDP-mannose, GMP;
(vi) ●, UDP-glucose, GDP-mannose, GDP.

but is the major component of the lipopolysaccharide (Koeltzow *et al.*, 1968). Addition of UDP-galactose to cell-free systems depressed the incorporation of glucose and mannose into glycosyl carrier lipid and exopolysaccharide (Sutherland *et al.*, 1971). Thus, formation of galactosylated glycosyl carrier lipid appears to decrease the amount available for exopolysaccharide synthesis. In this system, as in *K. aerogenes* type 8, glucose is the first sugar transferred to lipid in exopolysaccharide formation. Incorporation is again diminished in the presence of UMP.

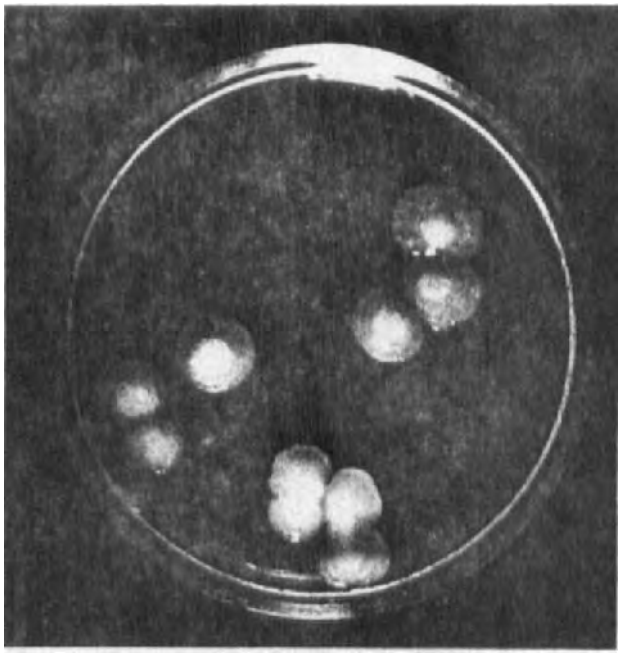


Fig. 13(a)

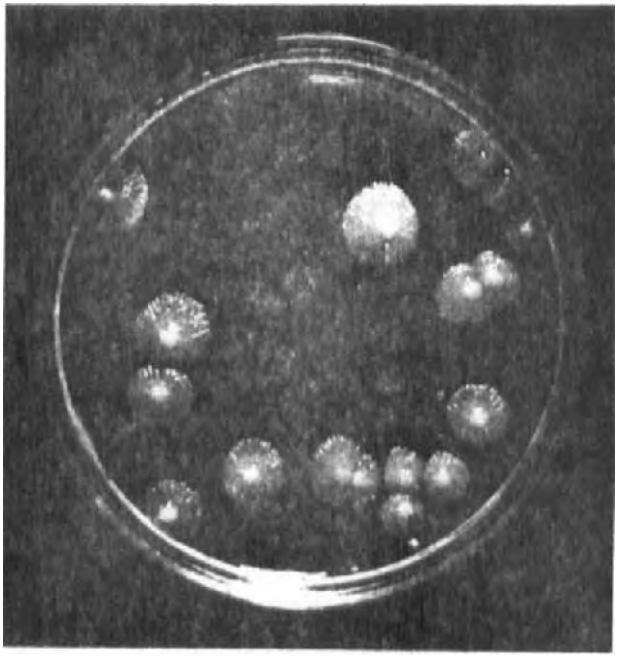
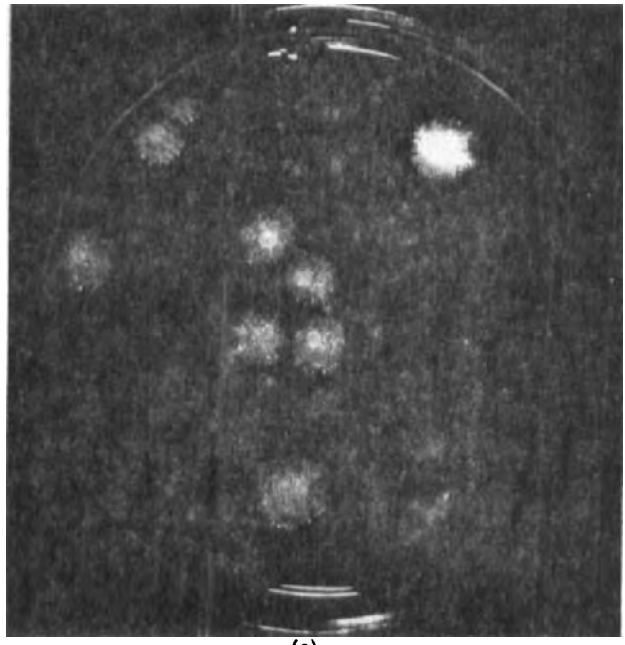


Fig. 13(b)

Mannose, from GDP-mannose, is transferred after glucose, and little is transferred in the absence of prior glucosylation (Fig. 12). Further support for the sharing of the glycosyl carrier lipid between the systems is seen in some recent results from our laboratory (Norval and Sutherland, 1969). A new group of mutants were isolated from three different *K. aerogenes* strains of different serotype. These mutants, mainly obtained after aminopurine mutagenesis, had curious culture morphology, resembling the wild type at 35° but possessing characteristic colony type

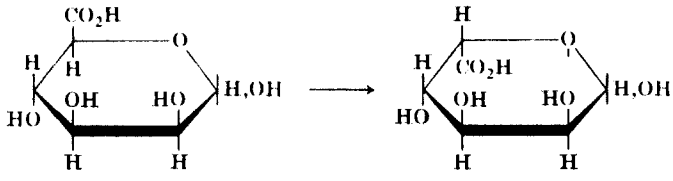


(c)

FIG. 13. Colonies of *Klebsiella aerogenes* CR mutant grown on a nitrogen-deficient agar-containing medium for (a) 72 hr. at 35°, (b) for 72 hr. at 30°, (c) for 96 hr. at 20°.

at 30° and lower temperatures (Fig. 13). In liquid media autoagglutination was seen at temperatures below 30°. At 35° synthesis of both lipopolysaccharide and exopolysaccharide was normal but, at 20° on solid media, the amount of exopolysaccharide synthesized was decreased by approximately 75% and in liquid media by about 50%. At the same time, the lipopolysaccharide content was decreased by 50–60%. From these results, it is probable that this type of mutant (CR mutant) involves a conditional fault in glycosyl carrier lipid synthesis. At the lower temperature insufficient glycosyl carrier lipid may be available to enable

all three polysaccharide-synthesizing systems to function, and there may be priority for mucopeptide formation which is presumably essential for cell viability. In non-proliferating cells (washed suspension) grown originally at 20°, normal exopolysaccharide synthesis occurred and cell-free synthesis was also possible (M. Norval and I. W. Sutherland, unpublished results). This result, too, would fit with the concept of limitation of glycosyl carrier lipid, priority going to cell-wall components in growing cells but lipid becoming available for exopolysaccharide synthesis when the cells were not growing. These results do not however indicate whether the limitation of glycosyl carrier lipid is due to a decrease in the total amount or in the loss of one particular type functioning primarily in exopolysaccharide formation.



D-Mannuronic acid L-Guluronic acid

FIG. 14. Conversion of D-mannuronic acid to L-guluronic acid.

A recent paper (Haug and Larsen, 1971) presented several features which were not previously thought to occur in exopolysaccharide synthesis. In particular, the discovery of an extracellular enzyme, a polymannuronic acid C_5-epimerase capable of modifying the polysaccharide after synthesis and extrusion from the cell, may be the first of a new type so involved. Such activity would only be expected if the enzyme either required no coenzyme or the coenzyme was firmly bound to the enzyme molecule. Further investigation of the extracellular epimerase should thus prove interesting in this respect. The result of the enzyme action was to covert D-mannuronic acid residues to L-guluronic acid in the substrate polysaccharide (Fig. 14). The partially purified enzyme was dependent on Ca^{2+} for full activity. Their replacement by Na^{+} or Mg^{2+} had little effect in restoring enzyme activity, but partial re-activation was obtained in the presence of Sr^{2+} ions. Various alginate preparations, including those of algal origin, were used as substrates despite differing initial mannuronic acid contents. There may well be differences between the *in vivo* and *in vitro* systems, especially with regard to the sequence of modifications on the final product. An analogous system in which the products of cell-free and whole-cell synthesis showed some differences was that involved in lipopolysaccharide formation.

Examination of acylation of exopolysaccharides should prove interesting and fruitful in this respect, especially since the recent discovery of polysaccharides with various acyl modifications (Garegg *et al.*, 1971b). It is possible that such polysaccharides are formed with a basic acyl group, probably pyruvate, which could then be modified by enzymes found either extracellularly or in the periplasmic region. A comparible system was described by Keller (1965) involving the acetylation of some *Salmonella* lipopolysaccharides. In this system, the donor was acetyl-CoA and various compounds acted as acceptors. These included deacetylated lipopolysaccharide, oligosaccharides, oligosaccharide-1-phenylflavozoles and a number of compounds in which L-rhamnosyl (1→3)-D-galactosyl sequences were present. Surprisingly, the intermediate involved in lipopolysaccharide synthesis, namely L-rhamnosyl-D-galactosyl-phospholipid, was not an effective acceptor. This result, combined with the use of various mutants defective in complete lipopolysaccharide synthesis, suggested that, normally, acetylation might occur either on the complete lipopolysaccharide or at the stage of polymerized trisaccharide attached to the carrier lipid. If the exopolysaccharide biosynthetic system is similar, acetyl-CoA from the cell membrane or the periplasmic space might again function as the acyl donor, while the enzymes involved might be part of the membrane or alternatively, some of the small molecular-weight proteins discovered in the periplasm. The variety of acyl substituents so far discovered indicates the possibility of either a range of suitable donors or, more probably, a number of different enzymes capable of modifying a smaller number of added groupings such as acetate and pyruvate, i.e.:

$$PS + (X) \rightarrow PS - X$$

or

$$PS + (A) \rightarrow PS - A \xrightarrow[\text{enzyme}]{\text{modifying}} PS - X$$

Preliminary experiments with a serotype 8 *K. aerogenes* strain, in which the exopolysaccharide contains acetate and pyruvate in addition to the normal carbohydrate structure (p. 193), showed that addition of the potential acyl donors, acetyl CoA or acetyl phosphate and phosphoenolpyruvate did not stimulate transfer of sugars to lipid or polymer (I. W. Sutherland, unpublished results).

From all these results, one can propose a generalized model for exopolysaccharide synthesis in bacteria (Fig. 15).

In this model, almost all of the functions are primarily associated with the bacterial cell membrane. Only the early stages in non-specific carbohydrate metabolism (in the example cited, those leading to UDP-glucose formation) are intracellular in location. All others are located at

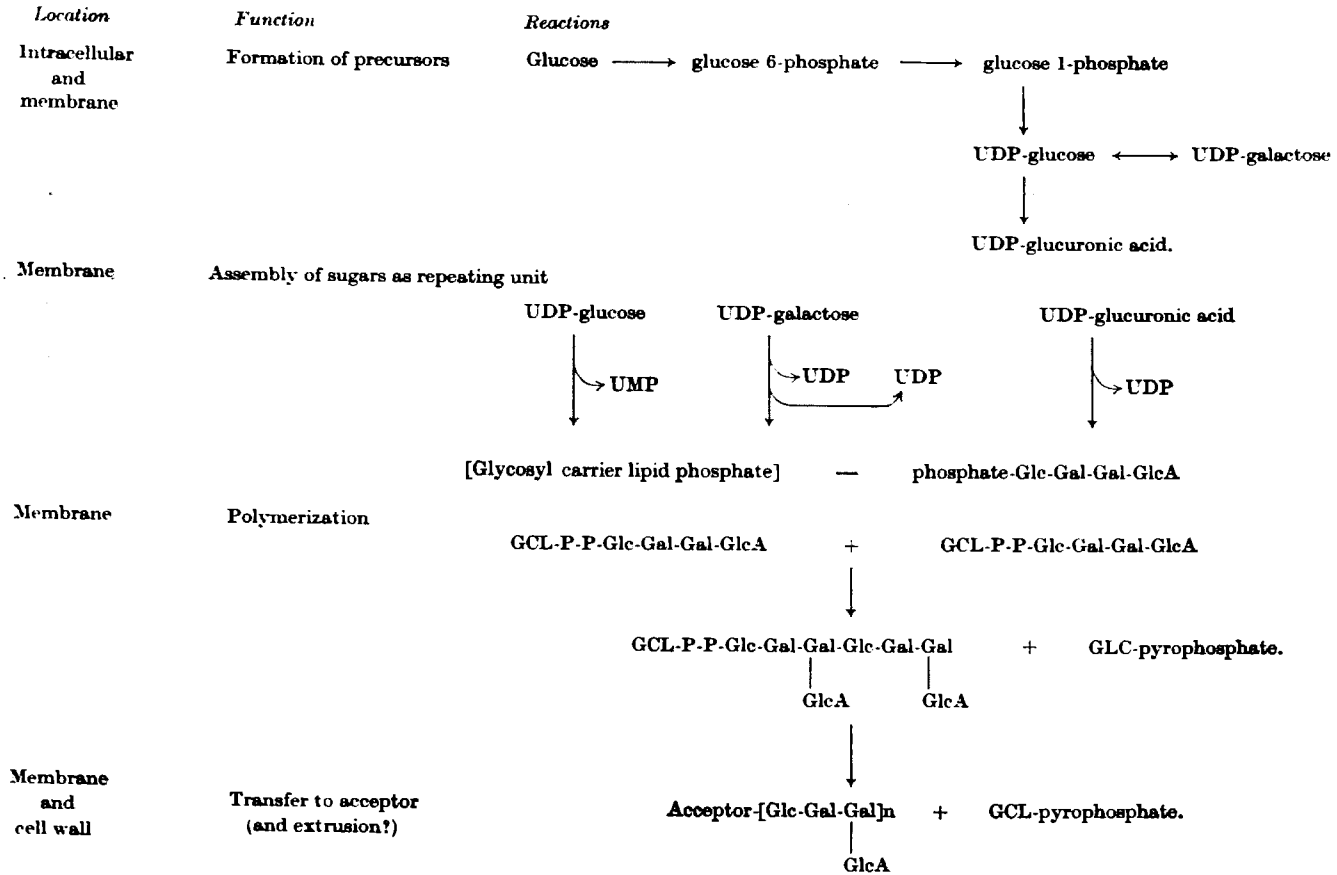


FIG. 15. A model for exopolysaccharide synthesis. Based on known reactions for *Klebsiella aerogenes* type 8 and related species.

the cell membrane, and suggest a high degree of localization and regulation within the membrane. It is also clear that, for the synthesis of any heteropolysaccharide capsule or slime, a considerable number of highly specific enzymes are involved. This in turn implies that a significant portion of the bacterial genome is devoted to provision of the necessary genetic information.

C. Control

One can make an arbitrary division of the enzymes leading to exopolysaccharide formation into several groups: (1) enzymes such as hexokinase responsible for the initial metabolism of a carbohydrate substrate; (2) enzymes involved in sugar nucleotide synthesis and interconversion, e.g. UDP-glucose pyrophosphorylase and UDP-glucose dehydrogenase; (3) transferases forming the repeating unit attached to glycosyl carrier lipid; and (4) the translocases or polymerases that form the polymer.

Possibilities exist for exerting control over polysaccharide synthesis at any of these four levels, and mutants lacking enzymes of any group fail to synthesize exopolysaccharide. The first group, being involved in so many other cell processes, cannot be involved in exerting any specific control and are generally found intracellularly. Insufficient is known about the final group to consider them here. They are almost certainly particulate, as are the glycosyl transferases and some enzymes in the second group. Those enzymes which have been shown to exert control are the nucleotide phosphorylases. They were shown (Ginsburg, 1964; Bernstein and Robbins, 1965) to control the transfer of monosaccharides into polymer either through the base specificity of the sugar nucleotide or through negative feedback mechanisms operating at the level of enzyme action. The actual levels of these enzymes remain constant even in non-mucoid mutants of several of the exopolysaccharide-synthesizing strains examined. Exceptions are found in *E. coli* and will be considered separately. In a study of synthesis of GDP-mannose and GDP-fucose (Kornfeld and Ginsburg, 1966) a feedback inhibition mechanism permitted independent control of the rate of formation of the two sugar nucleotides from the same precursors. Such fine-control mechanisms would obviously be extremely valuable to the cell in regulating exopolysaccharide precursor formation, especially in those bacterial strains, such as some *Salmonella* species, in which mannose is present in the lipopolysaccharide of the cell wall and fucose is found in colanic acid.

An interesting example of the control of polysaccharide synthesis was seen in the observation of binary capsulation in *D. pneumoniae* (Austrian and Bernheimer, 1959). Following transformation of a non-capsulated strain (originally type III) with DNA from a heterologous capsulate

(type I) strain, a small number of binary capsulated colonies were detected. These produced a normal amount of type III capsular polysaccharide together with a small amount of type I polymer. Use of the DNA from the binary capsulated recombinants for the transformation of heterologous (type II) non-mucoid strains produced three types of progeny, namely capsulate type I, non-capsulate type III, and binary type (I + III) cells. Capsulate type III cells were not detected. From these results, it was concluded that repair of the type III genome had not taken place, and consequently no recombination of the type I DNA with the imperfect type III genome. It was assumed that the type I DNA coexisted with the non-capsulate genome, permitting the formation of some type I material from several of the precursors normally used for the type III polysaccharide. There are thus two parallel and interacting biosynthetic pathways in the binary cells (Fig. 16). Some of the binary capsulate recombinants were unstable and reverted to the non-mucoid

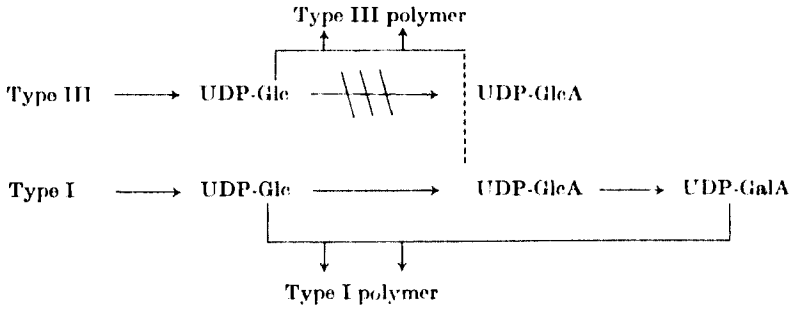


FIG. 16. Pathways of polysaccharide synthesis in binary capsulated *Diplococcus pneumoniae* cells.

type at high frequency (Bernheimer and Wermundsen, 1969). The instability was thought to be caused by the type of mutation in the non-mucoid (type III) strains. The heterologous genetic material was probably integrated at at least two loci, one of which was adjacent to the part of the recipient genome responsible for capsule synthesis and the other some way from it.

Production of colanic acid in the Enterobacteriaceae seems to possess a unique regulation mechanism. A regulator gene, designated *cap R* (Markovitz, 1964; Markovitz and Rosenbaum, 1965), was identified on the chromosome of *E. coli* adjacent to the *pro* locus (responsible for proline biosynthesis). It controlled several enzymes thought to be involved in colanic acid synthesis. These included UDP-galactose-4-epimerase and GDP-fucose synthetase. Transduction was used to form heterozygotes. When the gene was integrated on the chromosome, the non-mucoid condition was dominant. Transfer of the *capR* locus as part

of an episome produced the reverse effect, mucoidness, i.e. production of colanic acid, being dominant. By culture in medium containing *p*-fluorophenylalanine, the regulator gene produced an inactive product, resulting in colanic acid production in strains which were normally non-mucoid (Kang and Markovitz, 1967; Grant *et al.*, 1969). Simultaneously an increase was noted in the level of several of the enzymes involved in colanic acid synthesis, including phosphomannose isomerase, UDP-glucose dehydrogenase, UDP-galactose-4-epimerase, GDP-mannose pyrophosphorylase and GDP-fucose synthetase. The increase in enzyme levels was 2·5–3·3-fold for some of the enzymes, but as high as 13-fold for UDP-glucose pyrophosphorylase (Lieberman *et al.*, 1970). The capacity to form colanic acid seems to extend to most *E. coli* and *Salmonella* species as Grant *et al.* (1969, 1970) showed that, under appropriate conditions, colanic acid was produced unless a recognized defect was present in an enzyme essential for its synthesis. This was also reflected by high levels of GDP-fucose and UDP-glucuronic acid in the nucleotide pools of cells synthesizing the exopolysaccharide. The increase in the two sugar nucleotide levels occurred simultaneously when non-mucoid repressed cells were cultured in the presence of *p*-fluorophenylalanine. It thus seemed that control was normally through the reaction of a single repressor rendered inactive by the amino-acid analogue. These results were therefore in good agreement with those of Markovitz and his colleagues. The genera *Escherichia* and *Salmonella* together with *Aerobacter cloacae* thus fall into three groups: (1) strains normally producing colanic acid, including *A. cloacae* and many *E. coli* K12 substrains; (2) strains producing the polymer when grown under nitrogen-deficient, energy-rich conditions, including most *E. coli* and some *Salmonella* types; and (3) strains which give rise to colonies which were only mucoid in media containing fluorophenylalanine. It still remains to be determined whether the repressor is part of an operon controlling all of the genes involved in colanic acid synthesis. If this is so, an appreciable part of the bacterial genome may be involved, as a large number of enzymes are thought to be involved in the formation of the complex oligosaccharide unit which is polymerized to colanic acid (Fig. 17).

Numerous mutations can affect the production of bacterial exopolysaccharides. The use of several such mutants in work on polymer production has already been mentioned. Non-mucoid colonies were frequently noticed during subculture of mucoid bacterial strains. Such cultures provided much information during studies on capsule synthesis by *Diplococcus pneumoniae*, a system which had the advantage that transformation provided a means of genetic interchange between non-mucoid strains.

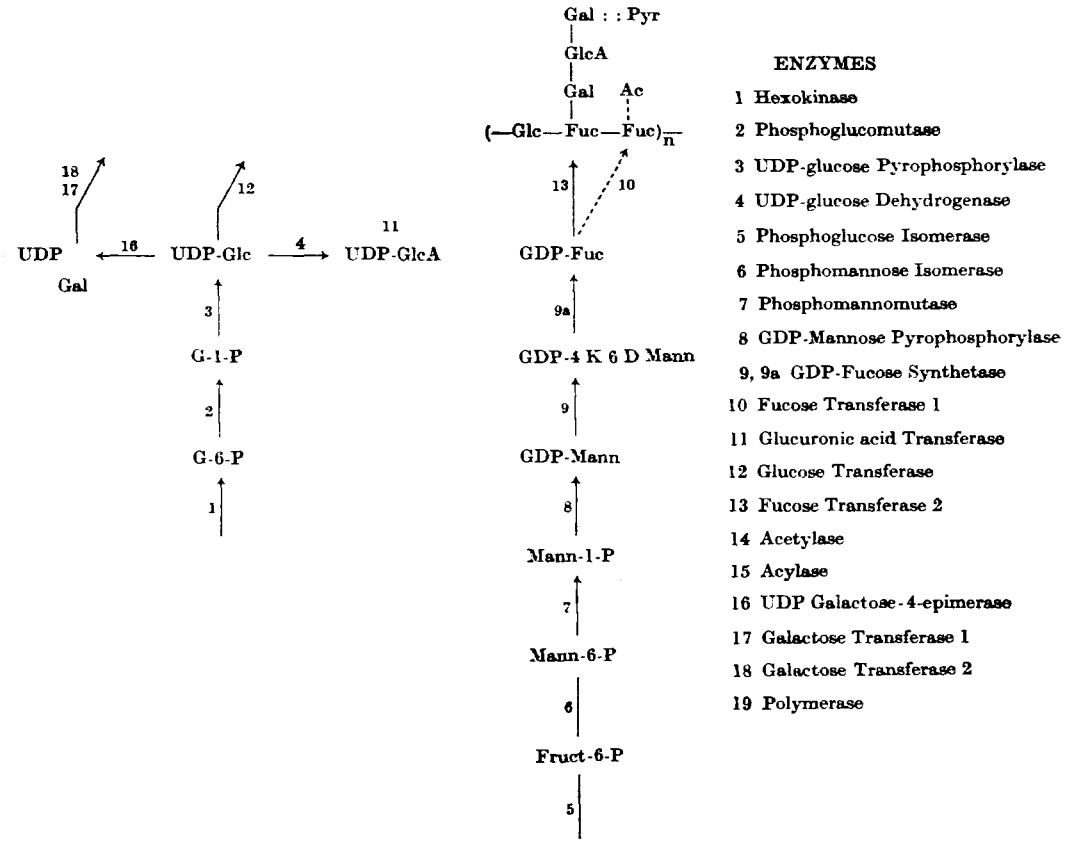


FIG. 17. Pathway for the biosynthesis of colanic acid from its precursors. Several enzymes (1–3) and (5–7) are involved in general carbohydrate metabolism. Others are involved in the synthesis of precursors for exopolysaccharide and lipopolysaccharide (16) or exopolysaccharide alone (4,8,15. 17–19). GDP-4K6DMan indicates guanosine diphosphate-4-keto-6-deoxymannose.

Most mutations affecting enzymes known to participate in exopolysaccharide synthesis lead to non-mucoid (O) derivatives. Such mutants resemble the wild-type bacteria with regard to most of the enzymes leading to exopolysaccharide formation. Thus, in *D. pneumoniae*, wild-type and non-capsulate cells both contain the enzymes involved in metabolism of UDP-sugar nucleotides, and the actual sugar nucleotides were isolated and identified (Smith *et al.*, 1957). The loss of exopolysaccharide synthesis may be due to deletions in precursor synthesis, in transferases, or in polymerase. Because of interrelated systems coming under specific control mechanisms, it might be expected that loss of one enzyme in a sequence would lead to altered levels of others. Few attempts to measure the levels of enzymes involved in different stages of polysaccharide synthesis have been reported. Changes in levels of several enzymes leading to colanic acid formation in *E. coli* were observed (Markovitz *et al.*, 1967) in mutants. Non-capsulate mutants of *E. coli* K27 varied greatly in the levels of UDP-glucose pyrophosphorylase,

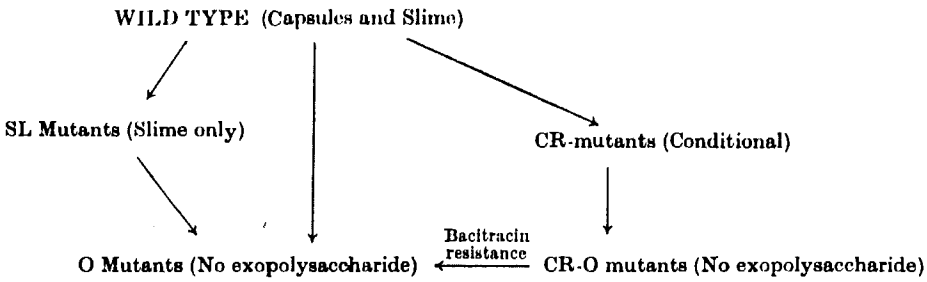


FIG. 18. Mutations affecting exopolysaccharide synthesis in *Klebsiella aerogenes*.

phosphoglucomutase, UDP-galactose-4-epimerase and GDP-mannose pyrophosphorylase (Olson *et al.*, 1969) indicating a lack of correlated control in this strain at least. Similar results were obtained using non-mucoid derivatives from two different serotypes of *K. aerogenes* (Norval, 1970).

The mutants already mentioned, which have lost the ability to form capsule but retain the ability to secrete slime, can undergo further mutation involving loss of polymer-synthesizing capacity. There are thus two pathways of mutation leading to production of O (non-mucoid) mutants (Fig. 18). The conditional mutants (CR-mutants) also mentioned can also undergo a second mutation to produce cells completely unable to form polymer. These CR-O mutants resemble the CR type in auto-agglutinability at low incubation temperature and in their altered phage-sensitivity (Norval and Sutherland, 1969). They also resemble O mutants in their stability. Few revertants to mucoidness have been obtained (e.g. Norval, 1970; I. W. Sutherland, unpublished results).

The CR mutants differ in that they are less stable, and revertants to capsulation at low temperature occur. Despite the low levels of reversion of CR-O mutants to mucoidness, it is possible to isolate revertants to the O-characteristic by selecting for bacitracin resistance (I. W. Sutherland, unpublished results; Fig. 18). This tends to confirm the postulated involvement of a glycosyl carrier lipid defect in the CR mutants. Bacitracin-resistant mutants probably have elevated levels of glycosyl carrier lipid to circumvent the failure to dephosphorylate glycosyl carrier lipid-pyrophosphate which has been identified as the mode of action of this antibiotic (Siewert and Strominger, 1967).

V. Function of Exopolysaccharides

Various hypothetical functions have been suggested for bacterial exopolysaccharides. Most of these have implied a protective function, such as against desiccation, against phagocytosis or against bacteriophages. While there may certainly be some possibility of the first two roles being correct, the occurrence of a number of phages capable of inducing capsule-destroying enzymes suggests that capsules frequently present no real barrier to phage infection. They may even, in fact, act as a receptor for certain phages (M. L. Wilson, unpublished results) as a *Klebsiella* phage absorbed with much greater efficiency to capsulate than to non-capsulate strains. Thus the capsule might function as a primary viral receptor while the cell wall is a secondary receptor.

Protection against phagocytosis may also be associated with a function for the exopolysaccharides as agressins in pathogenic bacterial strains. They might inhibit lysozyme, the acid polysaccharide combining with the basic protein, and other antibacterial substances. The capsules may also inhibit bacterial engulfment by amoebae. Protection against desiccation is most likely to be of importance to soil species, and it is certainly true that many of these produce exopolysaccharides. A further role could be in the adsorption and provision of nutrients and ions. The numerous free carboxylic acid groups of the uronic acids and ketals available in many of these polymers could permit this. Also, the amount of water bound to the capsule (which is 99% water) might allow some of it to be utilized by the cell. Further elucidation of these possible roles must await adequate data on the binding and exchange of molecules to exopolysaccharide material.

VI. Unanswered Questions

Despite the volume of work on bacterial exopolysaccharides which has been amassed, there are still a large number of unanswered questions. These include:

(1) The real function of exopolysaccharides is still obscure and it remains perplexing that microbial cells should expend so much energy and exert such complex regulatory and genetic mechanisms on their synthesis.

(2) The difference between slime and capsule is thought to exist in the loss of a specific receptor or binding site from mutant cells which, although originally capsulate, become slime-forming. This remains unproven and other possibilities such as loss of a binding enzyme, may be considered.

(3) When a capsulate cell divides, does the capsule undergo some parallel form of division? It would be interesting to know whether, and to what extent, the capsule on either daughter cell is derived from existing and new polysaccharide, and to what extent the process resembles deposition of mucopeptide in the bacterial cell wall.

(4) The mechanism of polysaccharide acylation and modification is still obscure. In particular, it remains to be seen whether acylation normally occurs through a limited range of donors and subsequent modification of the acylated polymer. The stage at which polymerization occurs is also unknown.

(5) It is clear that, during exopolysaccharide synthesis, carrier lipids are involved and that these polyisoprenoid phosphates are interchangeable in the synthesis of other repeating unit polymers, including lipopolysaccharide and mucopeptide, *in vitro*. What is not clear is whether there is any specificity in the use of these lipids, either through slight modifications to the lipid or through spatial separation of polymer-synthesizing sites on the cell membrane. Alternatively, is there discontinuous synthesis of lipid-requiring polymers?

(6) The size of the repeating unit formed on the lipid is not clear, nor is the nature of the acceptor to which it is transferred. This aspect may be related to (2). The loss, through mutation, of an acceptor molecule might lead to release of soluble slime from the glycosyl carrier lipid instead of normal capsule formation.

VII. Conclusions

Bacterial exopolysaccharides are a complex group of polymers containing a variety of monosaccharides and acyl and other substituents. They are essentially linear strands, many of which possess side-chains of one or more monosaccharides attached at regular intervals to the chain. Almost all such exopolysaccharides are probably formed from

repeating units of 2–6 monosaccharide residues which are assembled from glycosyl donors on to a polyisoprenoid phosphate carrier by membrane-bound enzymes. After polymerization, the molecules are extruded from the cell surface to form either a slime unattached in any way to the cell or a discrete capsule which has an attachment mechanism as yet undetermined, binding it to the outer layer of the cell wall. As a result of their regular structure, several exopolysaccharides are the substrates of enzymes from heterologous micro-organisms or from phage-infected bacteria, which degrade them to their component oligosaccharides. One group of bacterial exopolysaccharides, the bacterial alginates, are exceptional in that their components, and consequently their properties, can be modified by extracellular enzymes after their excretion into the medium.

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Physiology of the Bdellovibrios

M. P. Starr and J. C.-C. Huang

Department of Bacteriology, University of California, Davis, California 95616, U.S.A.

I. Introduction 215
II. Isolation, Cultivation, Distribution, and Taxonomy of *Bdellovibrio* . 218
A. Isolation 218
B. Culture Media 218
C. Maintenance of *Bdellovibrio* Cultures 219
D. Distribution and Importance in Nature 220
E. A Taxonomic and Terminological Note 221
III. Structure and Chemical Composition of *Bdellovibrio* . . . 222
A. Morphology and Ultrastructure 222
B. Chemical Composition of *Bdellovibrio* Cells 226
IV. Symbiosis Between Host-Dependent *Bdellovibrios* and Host Cells . 228
A. Life Cycle of Host-Dependent *Bdellovibrios* 229
B. Effects of *Bdellovibrio* on the Host Cell 239
C. Mechanisms of Lysis of the Host Cell by *Bdellovibrio* . . 242
D. Kinetics of Host-Dependent Growth 246
E. Nutrition of *Bdellovibrio* 246
F. Host Specificity 249
V. Metabolism of *Bdellovibrio* 252
VI. Host-Independent Derivatives of *Bdellovibrio* 254
VII. *Bdellovibrio* Bacteriophages 256
VIII. Acknowledgements 257
References 257

I. Introduction

Heinz Stolp was honored in 1968 with the prestigious Robert Koch Prize for his discovery (Stolp and Petzold, 1962) in German soils of a small vibrioid bacterium which is parasitic upon other bacteria. Additional cultures of this unusual organism were isolated in California, it was further characterized, and it was named *Bdellovibrio bacteriovorus* by Stolp and Starr (1963b). "*Bdello-*" is a combining form which means "leech"; "*-vibrio*" represents the comma shape of the parasite; "*bacteriovorus*" means bacteria-eater.

Host-dependent ("parasitic") bdellovibrio populations (characterized by the impossibility of cultivating them in the absence of host bacteria) outwardly resemble virulent bacteriophages in their ability to lyse living bacterial cells. The lysis is made evident by the decrease in optical density of suspensions of living host bacteria in broth cultures, and by the development of plaques (Figs. 1 and 2) or confluent lysis in lawns of

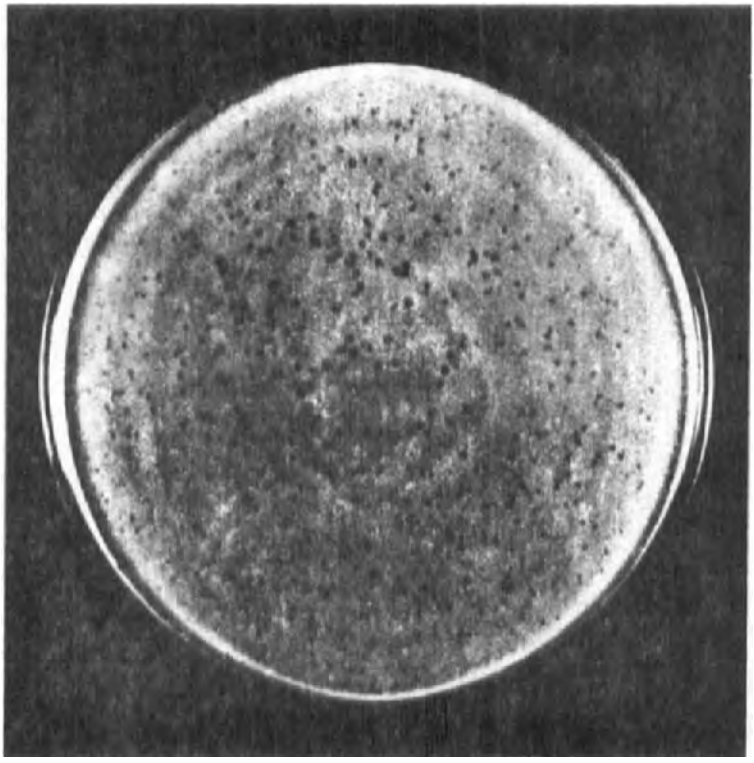


FIG. 1. Plaque formation by *Bdellovibrio starrii* (strain *Bd.* A3.12) on a lawn of *Pseudomonas putida* (strain A3.12). Fig. 4 of Stolp and Starr (1963b).

living host bacteria in solid media (more rarely and only under special conditions, as related below, with heat-killed host bacteria). *Bdellovibrio* could be distinguished from bacteriophages by the kinetics of development of the plaques on host lawns (Stolp and Petzold, 1962; Stolp and Starr, 1963b). Phage plaques are usually fully developed within 12–24 hr., while *Bdellovibrio* plaques become visible on lawns only after 2–4 days and then enlarge progressively with continued incubation up to six days. *Bdellovibrio* was also characterized by its being an actively motile, rather small, vibroid bacterium which attaches to the bacterial

host cell (Stolp and Petzold, 1962; Stolp and Starr, 1963b). As will be made clear presently, the entrance of *Bdellovibrio* into the bacterial host cell and its development therein are necessary elements in the life of this micro-organism. It has been possible to obtain host-independent ("saprophytic") bdellovibrio populations (defined by the ability to grow in the absence of host bacteria) from host-dependent bdellovibrio

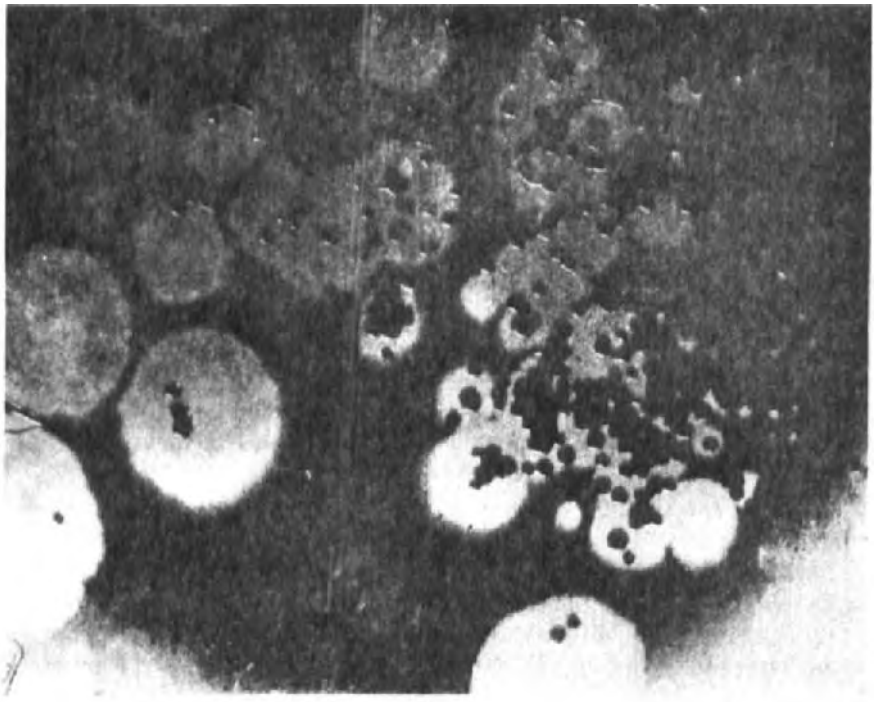


FIG. 2. Lytic action of *Bdellovibrio bacteriovorus* (strain *Bd.* 109) on colonies of *Escherichia coli* B. A mixture of bdellovibrios and host bacteria was streaked on nutrient agar and the plate was photographed after four days. Fig. 7 of Stolp and Starr (1963b).

populations; host-independent populations can revert to host-dependent populations.

Additional insights about microbial interrelationships in general would be gained if host-parasite interactions were better understood. Pertinent to this better understanding are the studies of host-parasite relationships in host-dependent protista, including malaria protozoa (Trager, 1960; Moulder, 1962); rust (Shaw, 1963) and other fungi (Barnett, 1963); *Mycobacterium* (Hanks, 1966); rickettsiae and psittacine bacteria (Moulder, 1962, 1964, 1966); *Mycoplasma* (Hayflick,

1969); *Dictyobacter*, *Cyclobacter*, and other predatory bacteria (Perfil'ev and Gabe, 1969); parasites and commensals of green algae (Gromov and Mamkaeva, 1966, 1971); and bacteriophage (Adams, 1959). Also pertinent is the on-going conceptual analysis of symbiosis, including parasitism (H. R. Heise and M. P. Starr, unpublished data).

Bdellovibrio thus merits extensive study because it parasitizes bacteria. *Bdellovibrio* has been the subject of other reviews and summaries (Stolp and Starr, 1963b; Starr and Skerman, 1965; Shilo, 1966, 1969; Stolp, 1968, 1969a, b; Uematsu and Wakimoto, 1969a; Wakimoto, 1970; Starr and Seidler, 1971). The present treatment, which emphasizes the physiology and biochemistry of the bdellovibrios, is complementary to another essay from our group (Starr and Seidler, 1971) which stresses the morphological, ecological, and taxonomic aspects of the burgeoning literature on the bdellovibrios.

II. Isolation, Cultivation, Distribution, and Taxonomy of *Bdellovibrio*

A. Isolation

The procedure first used for isolating *Bdellovibrio* (Stolp and Petzold, 1962; Stolp and Starr, 1963a, b; Stolp, 1965) involved centrifugation and differential filtrations of suspensions of soil, sewage, or other materials through a set of Millipore membrane filters with pore sizes ranging through 3, 1·2, 0·8, 0·65, and 0·45 μm. The filtrate from the 0·45 μm. filter was plated with prospective host cells in semi-solid agar using the double-layer technique as for bacteriophage (Adams, 1959). Enrichment of the *Bdellovibrio* population with susceptible bacteria was considered undesirable by Stolp and Starr (1963b) because it increased interference from bacteriophages, while Huang (1969) found such enrichment beneficial when host cultures were used which were resistant to bacteriophages. The cultures thus obtained were subsequently purified by three successive single-plaque isolations and were further checked microscopically for the presence of *Bdellovibrio*, based on the small size, active motility, and parasitic/predatory behaviour (Stolp and Starr, 1963b). Other isolation and enumeration procedures have been used (see Section II.D, p. 220).

B. Culture Media

Stolp and Petzold (1962) isolated *Bdellovibrio* in nutrient broth-agar using the double-layer technique. The top and bottom layers contained, in addition to nutrient broth, 0·9% and 1·8% (w/v) Bacto agar, respectively. A somewhat richer peptone-yeast extract agar was used by Stolp and Starr (1963b) to grow host-independent bdellovibrios;

it consists of 1·0% (w/v) peptone, 0·3% (w/v) yeast extract (pH 6·8). Sullivan and Casida (1968) used the Campbell-Hoffer medium or a modified Brown's medium. Burger *et al.* (1968) grew *Bdellovibrio* W in prospective host bacteria which had been cultivated in five different culture media.

Yeast peptone broth (0·3%, w/v, yeast extract, 0·06%, w/v, peptone, pH 7·2; Stolp and Starr, 1963b) is widely used (Dias and Bhat, 1965; Klein and Casida, 1967; Parker and Grove, 1970) because its relatively low nutrient content gives host lawns of proper density and physiological conditions to favour *Bdellovibrio* development. A 10-fold dilution of nutrient broth (Stolp and Petzold, 1962) or a four-fold dilution of yeast peptone (Shilo and Bruff, 1965) are also used for the same reason.

Starr and Baigent (1966) incorporated tris-HCl buffer (0·05 *M*; pH 7·5) into the yeast peptone broth to obtain a constant pH value. Scherff *et al.* (1966) cultivated host bacteria separately in a complex medium and then grew *Bdellovibrio* in cell suspensions of host bacteria in distilled water, hoping thereby to avoid unknown effects of the complex culture medium. Guélin *et al.* (1967) employed the same concept, but suspended the host bacteria in distilled water containing salts (KCl, $CaCl_2$, $MgSO_4$, and NaCl). Water agar has been used successfully by Gillis and Nakamura (1970).

The survey of culture media leads to the conclusion that the complex media have some limitations in connection with the undefined composition of the medium, the metabolism of the host bacterium, and the accumulation of metabolic products possibly inhibitory for the bdellovibrio. Some of these problems might be avoided by suspending washed cells of the host and *Bdellovibrio* in tris-HCl (Stolp and Starr, 1963b) or HEPES (N-2-hydroxyethyl-piperazine-N′-2-ethane sulphonic acid)-NaOH buffer (0·025 *M*; pH 7·5) in the presence of required cations, Ca^{2+} and/or Mg^{2+} (Simpson and Robinson, 1968; Huang, 1968; Huang and Starr, 1971a). In any case, rational design of culture media must await exploration of the exact nutritional requirements of *Bdellovibrio* (see Section IV.E, p. 246).

C. Maintenance of *Bdellovibrio* Cultures

In the present state of knowledge, maintaining cultures of *Bdellovibrio* is a rather exasperating art. Host-dependent *Bdellovibrio* isolates were maintained (Stolp and Starr, 1963b) by depositing a drop of fresh *Bdellovibrio* lysate on the top layer of yeast peptone agar containing host bacteria in a flask; subculture was made monthly. Host-independent cultures were maintained at room temperature on peptone-yeast extract agar plates and transferred monthly (Stolp and Starr, 1963b).

Burger *et al.* (1968) found that the viable count of host-dependent *Bdellovibrio* W cells decreased from 10^8/ml. to 10/ml. when kept as a lysate for 36 days. Abram and Davis (1970) report that more than 90% of the *Bdellovibrio* cells lost their infectivity in the lysates after three days at room temperature. On the other hand, Much (1965), Seidler (1968), and Huang (1969) indicate that they were able to maintain *Bdellovibrio* lysates at 4° for four to eight months.

Lyophilization of skim-milk suspensions of *Bdellovibrio* obtained from confluently lysed plates has generally been satisfactory. However, the results are sometimes erratic. The milk suspension of *Bdellovibrio* in this case contains also the semi-solid agar from the lawn, and the viability seems to be influenced by the composition of the medium employed for propagation of the host and bdellovibrio. Media which support limited growth of the host bacteria usually give maximal recovery of the *Bdellovibrio* after lyophilization. A horse serum-nutrient broth plus 7·5% glucose (final concentration) has been employed successfully as a suspending medium in lyophilization (Huang, 1969). Sullivan and Casida (1968) reported that the slime produced by *Azotobacter* helped to preserve *Bdellovibrio* in the frozen state. Freezing *Bdellovibrio* lysates with 10% dimethylsulphoxide or glycerol added, either alone or plus glucose as is the practice with bacteriophage (Yehle and Doi, 1965), has been suggested by H. Stolp (personal communication). *Bdellovibrio* may be preserved frozen at −20° and −78° for at least 40 days with excellent recovery (K. Nakamura, personal communication). However, Abram and Davis (1970) found that five strains of *Bdellovibrio* were highly susceptible to a freeze-thaw cycle.

D. Distribution and Importance in Nature

Bdellovibrios are widely distributed in nature. They have been found in sewage, river and lake water, seawater, soil, and in rice paddy soil and irrigation water. The population has been estimated at about 40–200 bdellovibrios/g. of Californian soils (Stolp and Starr, 1963b); about 1,000–70,000 bdellovibrios/g. in 23 soils from 13 states in eastern and central United States (Klein *et al.*, 1966; Klein and Casida, 1967); Western Australian soils scored 2–1,200 bdellovibrios/g. (Parker and Grove, 1970); sewage in India contained up to 864 bdellovibrios/ml. (Dias and Bhat, 1965) and sewage from France as much as 100,000 bdellovibrios/ml. (Guélin *et al.*, 1967); about 40–50 bdellovibrios/ml. were found in sea or pond water in Israel (Shilo, 1966).

The several methods which have been used result in much variation in recovery of bdellovibrios. The organism has been enumerated by the double-layer plaque technique directly without filtration (Klein and

Casida, 1967), by the most-probable-number method after centrifugation of a sewage suspension (Dias and Bhat, 1965), or on double-layer lawns after centrifugation followed by differential filtration (Stolp and Starr, 1963a, b; Parker and Grove, 1970). Varon and Shilo (1966) and Varon (1968) have applied differential centrifugation in linear Ficoll gradients, and have suggested that two successive differential filtrations (first 1·2 μm. and then 0·22 μm. pore size) of the upper layer of the Ficoll gradient could be used for quantitative assay of *Bdellovibrio* in nature (Shilo, 1969).

The possible importance of *Bdellovibrio* in the biological control of pathogenic and saprophytic microflora in nature was suggested by Stolp and Petzold (1962) and Stolp and Starr (1963b). This has been established to some extent in river water (Guélin *et al.*, 1967, 1968a, b, c, 1969a, c; Guélin and Lamblin, 1966, 1967, 1968), in seawater (Mitchell *et al.*, 1967; Mitchell and Morris, 1969; Guélin and Cabioch, 1970), in soil (Klein and Casida, 1967), and probably also in sewage (Dias and Bhat, 1965). Further remarks on this subject will be found in Section IV.F (p. 249).

E. A Taxonomic and Terminological Note

The possible relationship of the genus *Bdellovibrio* to the genera *Spirillum* and *Vibrio* has been analysed by Starr and Seidler (1971), who also have attempted to develop a concept and a definition of *Bdellovibrio*.

A plurality of species has now been discerned within the genus *Bdellovibrio* (Seidler and Starr, 1968b; Seidler *et al.*, 1969, 1972; Starr and Seidler, 1971). The use of the epithet "*Bdellovibrio bacteriovorus*" should be limited to the nomenclatural type specimen of that species—strain *Bd.* 100, as designated by Stolp and Starr (1963b)—and to closely related strains (e.g. *Bd.* 109). On the basis of molecular and other evidence, Seidler *et al.* (1972) have recently delineated two additional species; namely *Bdellovibrio stolpii* (with strain *Bd.* UKi2 designated by them as the nomenclatural type specimen) and *Bdellovibrio starrii* (with strain *Bd.* A3.12 designated by them as the nomenclatural type specimen). To avoid nomenclatural infelicities in the present essay, we will simply use strain designations: e.g. *Bdellovibrio* strain A3.12 (or *Bd.* A3.12). In addition, where necessary for clarity, we will use the prefixes H-I (for host-independent), H-D (for host-dependent), and F-P (for facultatively-parasitic). Sections IV.A.5 (p. 238) and IV.E (p. 249) contain remarks about the differences which exist between the Davis and Jerusalem strains of *Bd.* 109. Further confusion would be avoided if they were separately designated in the future as *Bd.* 109 (Davis) and *Bd.* 109 (Jerusalem), respectively.

III. Structure and Chemical Composition of *Bdellovibrio*

A. Morphology and Ultrastructure

Bdellovibrios are usually small, curved, Gram-negative rods, 0·25–0·4 μm. wide and 0·8–1·2 μm. long (Stolp and Petzold, 1962). Vibrios of slightly different proportions have also been described (Stolp and

Fig. 3. *Bdellovibrio bacteriovorus* (strain *Bd.* 109), illustrating its flagellum. The preparation was fixed with 1% formaldehyde and stained with 0·5% uranyl acetate. Magnification ×41,280. Fig. 1 of Seidler and Starr (1968a).

Starr, 1963b; Murray, 1964; Burnham *et al.*, 1970; Abram and Davis, 1970). The variation depends on strain and species differences, on the stage in the life cycle at which the cells are measured (Starr and Baigent, 1966), on the medium used (rich media, e.g. trypticase-yeast extract or nutrient broth, give 5–20% long forms which are straight or coiled, according to Abram and Davis, 1970), and on incubation conditions (Mishustin and Nikitina, 1970).

Bdellovibrio has an extremely thick flagellum (Fig. 3; 28 nm. according to Seidler and Starr, 1967, 1968a; 21–25 nm. according to Abram and

Davis, 1970), which consists of a sheath (7·5 nm. thick) surrounding a core (13 nm. diameter) (Seidler and Starr, 1967, 1968a). Slightly different proportions have also been reported (Shilo, 1966; Burger *et al.*, 1968; Burnham *et al.*, 1970; Abram and Davis, 1970; Uematsu and Wakimoto, 1970). The sheath, which is continuous with the cell wall (Murray, 1964; Seidler and Starr, 1968a; Burnham *et al.*, 1968b; Abram and Davis, 1970), may swell, break, and separate from the core in the presence of 6 *M*-urea (Seidler and Starr, 1968a). Even though the wall appears to be morphologically continuous with the sheath, it is not known why urea affects only the sheath but not the cell wall. However, Abram and Davis (1970) believe that the flagellar sheath material might be more susceptible to disruption than the cell wall.

Appendages or extrusions have frequently been observed in preparations stained with phosphotungstic acid in the electron microscope (Huang *et al.*, 1966; Shilo, 1966). Shilo (1966) and Abram and Shilo (1967) found spike-like filaments, 4·5–5·5 nm. in diameter and 0·8 μm. in length, located at the pole distal to the flagellum, and suggested that the filaments may play a role in the attachment and penetration of the host cell since this pole acts in attachment to the host cell. These structures are believed by some writers to be artifacts formed during fixation or staining. Murray (1968) noted that zinc ions were required in the fixative for preserving the cell wall and cell membrane of *Bdellovibrio*; in the absence of zinc, the cell wall may become loosened. *Bdellovibrio* has a cell-wall profile typical of Gram-negative bacteria, but it has been said to lack the usual peptidoglycan layer commonly illustrated in other Gram-negative bacteria (Murray and Maier, 1965), although chemical analysis (Tinelli *et al.*, 1970) of the cell wall of *Bd.* 109 has proven the presence of peptidoglycan components.

Abram and Davis (1970) observed intricate surface projections from the cell of negatively stained bdellovibrios. These projections were varyingly induced (depending upon the type of stains applied), and they were easily detached from the cells and fused to form vesicles and tubules which surrounded the cell. Uranyl acetate, which stains *Bdellovibrio* cells positively and negatively, was found not to have these effects and preparations stained with it show smooth surfaces with many folds. The stains which cause the formation of projections from *Bdellovibrio* cells also caused dispersion of the isolated cell wall. An undulate outermost surface was seen in freeze-etched preparations. The isolated cell wall has two smooth surface layers. The inner-wall layer is separate from the outer layer. Scattered particles (6–10 nm. in diameter) and patches of inner layer from the wall cover the outside of the cytoplasmic membranes (Abram and Davis, 1970).

A "holdfast" has been reported at the anterior (aflagellated) end of

some *Bdellovibrio* strains (Shilo, 1966; Abram and Shilo, 1967; Huang, 1969; "infection cushion", Scherff *et al.*, 1966, Scherff, 1966; "large convolution", Burnham *et al.*, 1968b). Scherff *et al.* (1966) and others have suggested that this "holdfast" structure might mediate attachment and penetration of *Bdellovibrio*. However, Starr and Baigent (1966) and Abram and Davis (1970) were unable to find such a device in their preparations, and the current writings from two groups (Burnham *et al.*, 1970; Abram and Davis, 1970) suggest that this "holdfast" may be an artifact, caused by disorganization of the cell surface.

At the anterior end of *Bdellovibrio* cells, two distinct features have been observed (Abram and Davis, 1970). Fibres which vary in length (up to 1·5 μm.) and in diameter (8–10 nm.) emerge from the anterior end. They may be straight or curved with angular bends. A cell generally bears two to three fibres and occasionally as many as six. These structures are not seen in the round form of *Bdellovibrio* or in aged cells. Six to 12 electron-dense circles (ring structures) with an outer diameter of 9 to 12 nm., which are built into the cell wall and associated with the protoplast, are also visible at the anterior ends; two to three fibres emerge from it. The ring structure may be scattered or in clusters. Abram and Davis (1970) believe that these ring structures and the fibres may be related to the parasitic activity of *Bdellovibrio*.

Compact bodies (150–300 nm. long, 70–120 nm. wide) are frequently observed in a certain negatively stained preparation; these structures were not seen in freeze-etched cells (Abram and Davis, 1970). Vibrioid cells have an average of two, and long forms have several, such structures. These bodies show a regular laminated structure which appears as "finger-print" patterns when stained with lithium tungstate and potassium phosphotungstate at pH 7·5, whereas both laminated and vesiculated structures were seen in preparations stained with potassium phosphotungstate at pH 8·1. These bodies and intracellular laminated structures are believed to extrude from the protoplast as a result of an osmotic effect exerted on the cells by the stain (Abram and Davis, 1970).

Densely stained regions are frequently seen embedded in the nuclear areas which are surrounded by ribosomes. Electron-dense inclusion bodies are often seen (Huang, 1969; Abram and Davis, 1970). Reiner and Shilo (1969) found that formation of these inclusion bodies was favoured by growing *Bdellovibrio* cells in one-tenth strength nutrient both containing heat-killed cells (65°, 30 min.) of *Pseudomonas aeruginosa*. Mesosomes have been observed at the anterior end of the cell (Starr and Baigent, 1966; Burnham *et al.*, 1968b; Huang, 1969). The mesosomes are thought to be associated with attachment (Burnham *et al.*, 1968b) and cell division (Burnham *et al.*, 1970).

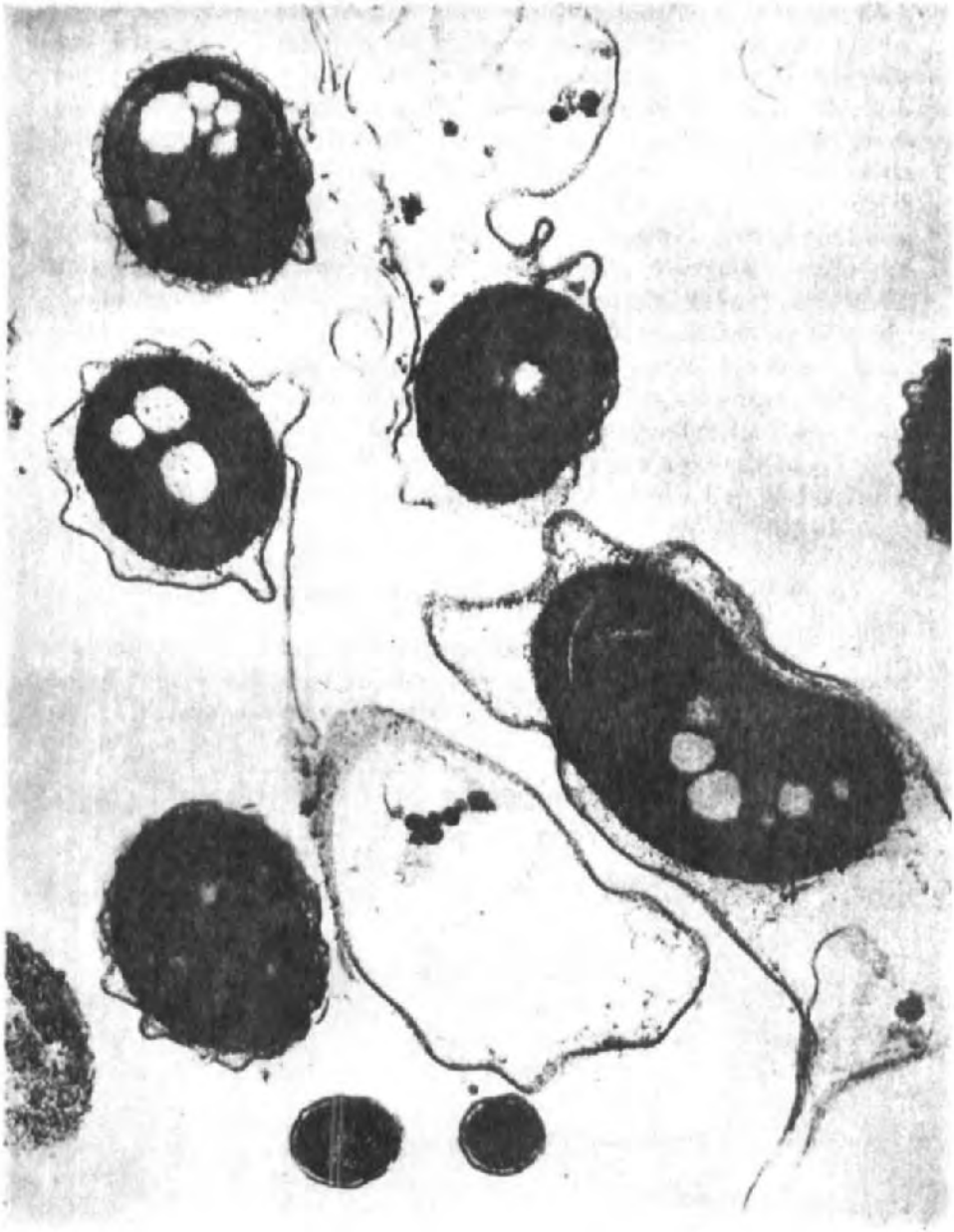


FIG. 4. A group of cysts ("resting bodies") of *Bdellovibrio* sp. (strain *Bd.* W) in a 21-hour culture with *Rhodospirillum rubrum*. All cysts have a thick, dark, outer layer and contain storage granules. At the bottom of the micrograph, two normal bdellovibrio cells are seen in cross section. Magnification ×48,000. From Hoeniger *et al.* (1972).

Burger *et al.* (1968) observed that some cells of *Bd.* W were "encysted" within the disrupted host cells of *Rhodospirillum rubrum;* the fine structure of these encysted cells (also called "resting bodies") is rather different from that of normal intracellular bdellovibrio cells. Hoeniger *et al.* (1972), who studied in detail the development and structure of these resting bodies or cysts in *Bd.* W grown in *R. rubrum* (Fig. 4), report that the resting body or cyst is 1·2 μm. long by 0·6 μm. wide, and that it starts forming some three hours after the establishment of host-*Bdellovibrio* culture by deposition of amorphorus materials around the periphery of the *Bdellovibrio* cell. Then two layers, an outermost cell wall and an innermost plasma membrane, are formed and they enclose the granular cytoplasm and fibrillar nucleoplasm regions. Upon maturation, the resting body (cyst) consists of a thick outer layer (30–40 nm.) and a cell wall differentiated into a tripartite inner layer. The resting body (cyst) has been observed only in *Bd.* W grown in *R. rubrum*, but not in *Bd.* W grown in *E. coli* nor in *Bd.* 109 grown in *E. coli* B (Hoeniger *et al.*, 1972).

B. Chemical Composition of *Bdellovibrio* Cells

The DNA content of host-independent *Bd.* A3.12 was found by Seidler *et al.* (1969) to be 5·01 ± 0·04% DNA on a dry weight basis. Chromatography of the acid hydrolysate of the nucleic acids (Fig. 5).

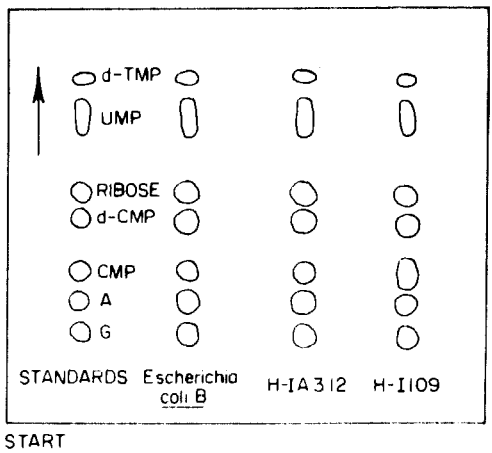


Fig. 5. Chromatograph of the acid-hydrolysis products of the nucleic acids of *Escherichia coli* B, *Bdellovibrio bacteriovorus* (strain *Bd.* 109), and *Bdellovibrio starrii* (strain *Bd.* A3.12). Figure 3 of Seidler *et al.* (1969). The solvent system was *tert*-butanol-HCl-water (700:132:168, v/v/v). The chromatogram was developed at the rate of 2 cm. per hr.

isolated from host-independent *Bd.* A3.12 and host-independent *Bd.* 109 by Marmur's (1961) method, revealed the presence of d-TMP, UMP, ribose, d-CMP, adenine and guanine (Seidler *et al.*, 1969). Based on the GC content of DNA as determined by the buoyant density in caesium chloride gradients (Mandel *et al.*, 1968) and by an optical melting method

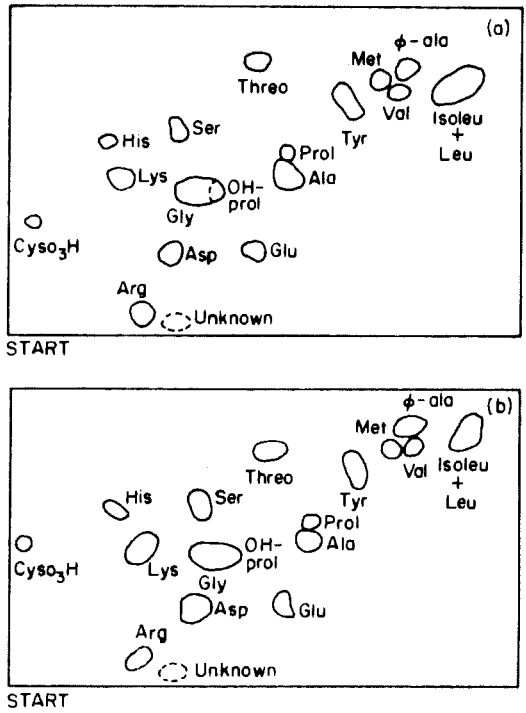


FIG. 6. Two-dimensional chromatograph of the acid-hydrolysis products of the proteins of *Escherichia coli* B (a) and *Bdellovibrio starrii* (strain *Bd.* A3.12) (b). Unpublished observations of R. J. Seidler and M. P. Starr. The chromatograms were developed in the vertical direction with n-butanol-acetone-diethylamine-water (10:10:2:5, v/v/v/v; 0·2 cm. per min.) and in the second direction with isopropanol-formic acid-water (4:2:10, v/v/v; 0·07 cm. per min.).

(Marmur and Doty, 1962), Seidler *et al.* (1969, 1972) were able to divide the strains of host-dependent and host-independent bdellovibrios into two distinctive groups, namely a high-GC group (most strains) with 50–51% GC and a low-GC group (*Bd.* A3.12, *Bd.* UKi2, and *Bd.* 321) with 42–43% in their DNAs. These studies did not, however, show any unusual structure or composition in the DNAs of the bdellovibrios.

Rittenberg and Shilo (1970) found that *Bd.* 109 contains 0·42 ± 0·05

mg. protein per 10^{10} cells [10 min. digestion in NaOH (by the method of Lowry *et al.*, 1951)]. Host-independent strains of *Bdellovibrio* have protein contents of 60–65% dry weight (Seidler and Starr, 1968b). All of the usual amino acids are present (Fig. 6).

The major phospholipids of *Bd.* UKi2 are phosphatidylethanolamine and phosphatidylglycerol; there is a lesser amount of phosphatidic acid (Steiner and Conti, 1970). Acetate- or serine-labelled lipid was prepared by incorporating [1,2-^{14}C]acetate, [3-^{14}C]serine, or [^{32}P]orthophosphate into yeast extract-peptone medium. Alkaline hydrolysis of the lipid revealed the presence of fatty acids on the chromatogram. The alkali-stable residue of the lipid contains sphingolipid, which is rare in bacteria (Steiner and Conti, 1970). The polar portion of the two major sphingolipids of *Bd.* UKi2, upon degradation, give rise to the same phosphorus-containing products (Steiner *et al.*, 1971). After hydrolysis with 2N-HCl (100°, 4 hr.), the water-soluble portion of the product consists of a single major compound which contains phosphorus and is ninhydrin-positive. Alkaline phosphatase (from *E. coli*) or hydrolysis in 2N-HCl (125°, 100 hr.) does not release inorganic phosphorus from this compound. This characteristic of tolerance to hydrochloric acid and alkaline phosphatase suggests that the uncharacterized compound contains a carbon-phosphorus bond, and that phosphonolipids are present in *Bd.* UKi2. A third phosphorus-containing sphingolipid was found, which is alkali-labile like other phospholipids and capable of releasing inorganic phosphorus when hydrolysed with hydrochloric acid.

Tinelli *et al.* (1970) determined the chemical composition of the cell wall of *Bd.* 109 harvested from a 20 hr.-culture with *E. coli* B. The isolated cell walls, after hydrolysis in 6N-HCl (120°, 20 hr.), contain two amino sugars (muramic acid and glucosamine) in addition to 13 other amino acids, all of which are similar to the usual cell-wall components of prokaryotic micro-organisms.

IV. Symbiosis Between Host-Dependent Bdellovibrios and Host Cells

In the earliest studies on the symbiotic association between *Bdellovibrio* and its bacterial host, Stolp and Petzold (1962) followed the interaction by means of phase-contrast microscopy and shadowed preparations in the electron microscope. They found that the bdellovibrio collided violently with, and attached to, the host cell surface, like a leech attaches to an animal's skin, within a few minutes after establishment of the two-membered culture. The host cells subsequently lysed. Further observations of this sort (Fig. 7) led Stolp and Starr (1963b) to describe *Bdellovibrio* as a predatory, ectoparasitic, and bacteriolytic micro-organism. The intermediate intracellular steps

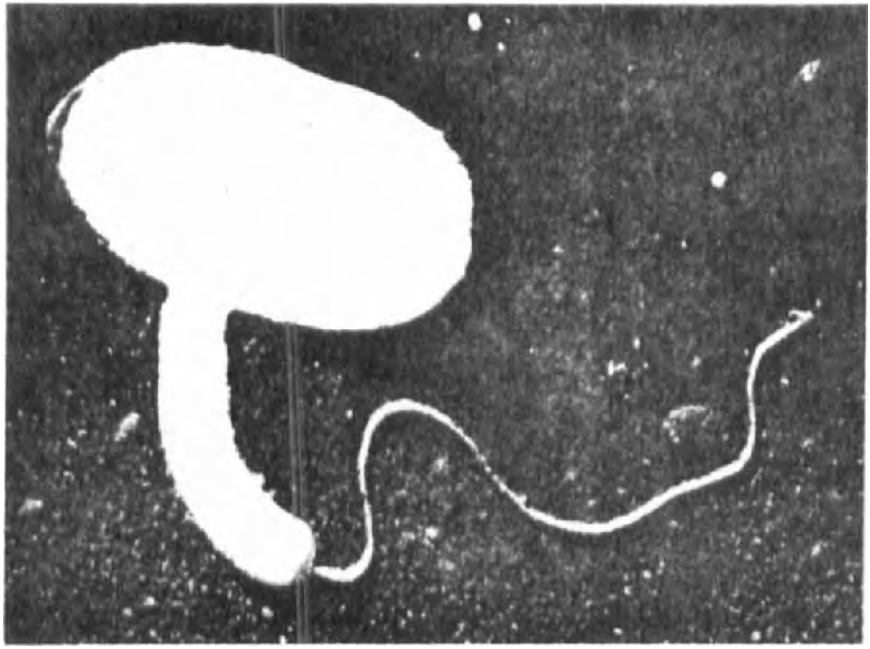
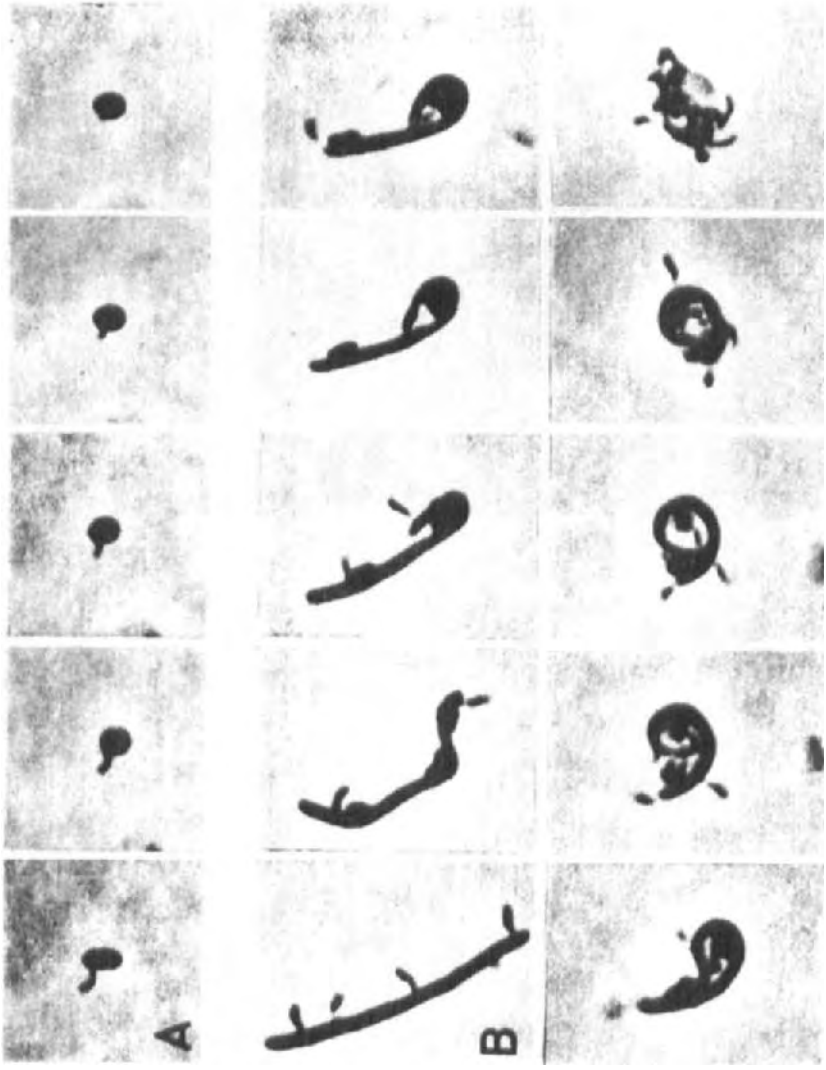


FIG. 7. Shadowed electron micrograph of *Bdellovibrio bacteriovorus* (strain *Bd.* 100) attached to a cell of *Erwinia amylovora* (ICPB EA137). Fig. 3 of Stolp and Starr (1963b).

between attachment and disintegration of the infected host cell, however, were not then (1962–1963) known.

A. Life Cycle of Host-Dependent Bdellovibrios

Starr and Baigent (1966), Huang *et al.* (1966), and Scherff *et al.* (1966) reported simultaneously and independently that several distinct steps are involved in the host-*Bdellovibrio* interaction (Fig. 8). In the light of current knowledge and our present purpose, these steps might be designated as follows: (1) "recognition of prey" and movement toward it; (2) attachment of *Bdellovibrio* to its host cell; (3) entrance into the host cell by the bdellovibrio; (4) rounding up ("sphaeroplasting") or other morphological changes in the host cell; (5) development and multiplication of the bdellovibrio within the host cell; and (6) disruption of the host cell and release of the *Bdellovibrio* progeny. These reports show that *Bdellovibrio* definitely is an "endoparasite", more correctly [Starr and Seidler, 1971, citing the opinion of H. R. Heise and M. P. Starr (unpublished observations)], an intramural or intra-integumental


A
B

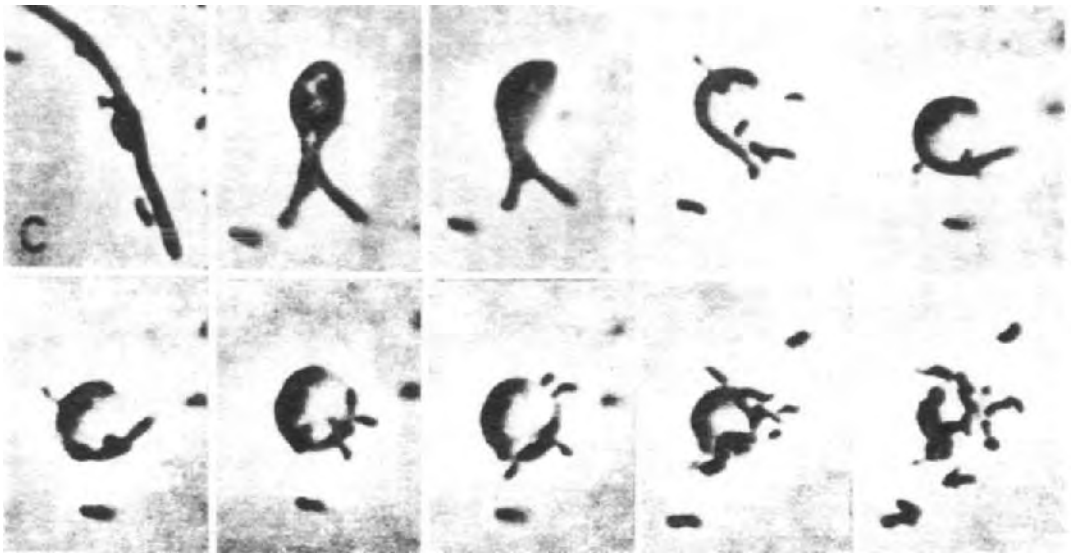


FIG. 8. Time sequence, recorded by phase-contrast photomicrography, of the interactions (A) between *Bdellovibrio bacteriovorus* (strain *Bd.* 100) and *Erwinia amylovora* (ICPB EA137) and (B and C) between *Bdellovibrio* sp. (strain *Bd.* 233) and *Pseudomonas tabaci* (ICPB PT1). Magnification ×2,750. A (top row): after 5, 20, 25, 25·5, and 30 minutes. B (second and third rows): after 15, 25, 26, 33, 48, 69, 75, 80, 150, and 275 minutes. C (fourth and bottom rows): after 25, 35, 39, 42, 48, 48·5, 69, 80, 150, and 275 minutes. Fig. 1 of Starr and Baigent (1966).

parasite, not an "ectoparasite" as described in the first papers (Stolp and Petzold, 1962; Stolp and Starr, 1963a, b; Shilo and Bruff, 1965; Klein and Casida, 1967) and, rather puzzlingly, in a recent article (Uematsu and Wakimoto, 1970).

1. Attachment

Chemotaxis (presently under study by M. P. Starr and his colleagues) has been suggested as playing a role in attachment, because *Bdellovibrio* behaves as though it can "recognize" the prospective host cell in a mixed culture of non-susceptible and susceptible bacteria (Starr and Baigent, 1966; Stolp, 1968; see, also, the remarks about host specificity in Section IV.F, p. 249).

Generally the non-flagellated end of the bdellovibrio attaches to the host cell (Scherff *et al.*, 1966; Starr and Baigent, 1966; Stolp and Petzold, 1962; Stolp and Starr, 1963b; Burnham *et al.*, 1968b). Murray (1964), however, seems to say that the flagellated end attaches, but this is probably a *lapsus calami*. Attachment of *Bdellovibrio* to the host cell occurs in seconds. An actively motile bdellovibrio, moving at an unusually high velocity (Stolp, 1967a, b; Simpson and Robinson, 1968), hits a host cell with such force that it may push it for several cell lengths (Stolp and Starr, 1963b). Stolp and Starr (1963b) suggested that the actual collision between bdellovibrio and host, and the drilling action (or an arm-in-socket type of motion, according to Starr and Baigent, 1966) of the motile bdellovibrio against the host cell, are necessary factors in establishing the attachment. The excellent time-lapse films of Stolp (1967a, b) seem to support this hypothesis.

In the earliest stages, attachment is reversible (Stolp and Starr, 1963b; Starr and Baigent, 1966; Burnham *et al.*, 1968b). Infection of a single host cell by several *Bdellovibrio* cells has been noted (Stolp and Starr, 1963b; Stolp, 1967c; Scherff *et al.*, 1966; Shilo and Bruff, 1965); this depends in part upon the relative number of host and parasite cells in the two-membered cultures (Stolp and Starr, 1963b; Stolp, 1964). Sometimes, such multiple attachments to a single host cell lead to rapid disintegration of the host cell—"lysis-from-without"—with no apparent intracellular multiplication (Abram and Shilo, 1967). *Bdellovibrio* attaches, penetrates, and multiplies in all growth phases of the host culture (Shilo, 1969).

More *Bdellovibrio* cells attach to host cells when the relative concentrations are one bdellovibrio cell to 10 host cells (multiplicity of infection = 0·1) than under any other conditions tested (Varon and Shilo, 1968). Unattached and attached *Bdellovibrio* cells, in this case, were enumerated after differential filtration of the two-membered culture.

The multiplicity of infection, however, is not critical for overall growth since an initially few *Bdellovibrio* cells are able to cause the complete lysis of a concentrated bacterial cell suspension (Stolp and Starr, 1963b). The multiplicity of infection affects the growth rate, but not the yield, of *Bdellovibrio*.

Many other factors are known to affect the attachment of *Bdellovibrio* to the host cell in addition to multiplicity of infection; these include composition and pH value of the medium, oxygen tension, and incubation temperature (Varon and Shilo, 1968). Motility of *Bdellovibrio* is essential for attachment (Stolp and Starr, 1963b; Stolp, 1964; Varon and Shilo, 1968; Diedrich *et al.*, 1969, 1970; Abram and Davis, 1970). Heat-killed host cells are less suitable for attachment by *Bdellovibrio* than are living cells. *Bdellovibrio* is reported generally not to attach to Gram-positive bacteria (see Section IV.F, p. 251); however, there have been observations in our laboratory which show that *Bdellovibrio* can attach to the cells of *Bacillus subtilis* which are uncongenial for *Bdellovibrio* growth, and even to cover glasses and slides.

Starr and Baigent (1966) postulated that host and parasite were held together by "a strong surface bonding", since a cytological device which might mediate the attachment seemed to be lacking. However, as detailed in Section III.A (p. 223), other workers have variously reported (or denied the existence of) such "holdfast" devices.

Varon and Shilo (1969a) measured the ability of *Bd.* 109 and *Bd.* GB to attach to mutant cells of *E. coli* B and *Salmonella typhimurium* genetically different in cell-wall chemical composition (chemotypes). They found that chemotype Ra (containing a complete "rough" core but lacking O-specific side chains) was a better receptor for *Bdellovibrio* than the wild-type (smooth) host strains. Chemotype Rb (absence of glucosamine) showed a less firm attachment. Additional deficiencies in the R antigen (chemotype Re) further decreased receptor activity. Varon and Shilo (1969a) interpreted their data as suggesting that the location of receptors for *Bdellovibrio* is in the R antigen layer. Addition of rough host cells to a system containing smooth strains of *Salmonella typhimurium* and *Bd.* 109 increased the attachment. The high level of attachment, however, seems not to be essential for growth of *Bdellovibrio* in the host cell, because *Bdellovibrio* develops equally well in cells deficient and adequate in attachment sites. J. C.-C. Huang (unpublished data), based on a similar working hypothesis, found that *Bd.* 6-5-S parasitized equally well the wild-type and 11-chemotype mutants of *Salmonella typhimurium* (Kessel *et al.*, 1966), using cell suspensions in tris-HCl buffer containing Ca^{2+} and Mg^{2+}. Klein and Casida (1967) found that *Bd.* OX9-1 and *Bd.* 167-1 parasitized members of all 25 *E. coli* subgroups which they tested as well as the S and R forms of *E. coli*

MGH6. Encapsulation, which occurred in a mutant strain of *Aerobacter cloacae* in the presence of 1% lactose, did not afford protection against attack by *Bd.* Ac as determined by plaque formation (Much, 1965).

The outermost structured layer on the cell wall of *Spirillum serpens* MW1 and MW11, similar to the outmost layer on the wall of *S. serpens* VHA (Murray, 1963; Murray *et al.*, 1967; Buckmire and Murray, 1970), appears to protect the host cells from parasitization by *Bdellovibrio* (Huang, 1969; Buckmire, 1971). Removal of the structured layer (protein in nature; Buckmire and Murray, 1970) by treatment of host cells with EDTA and sodium lauryl sulphate, by heating, or by spontaneous mutation, rendered them susceptible to parasitization. This factor might account for our being able to show the susceptibility of *S. serpens* VHL (defective structured layer) to *Bd.* W, whereas Burger *et al.* (1968) reported it would not parasitize their strains of *S. serpens* (which may have had a complete structured layer).

Surviving host clones resistant to *Bdellovibrio* have not yet been found in two-membered cultures (Stolp and Starr, 1963b; Shilo, 1969). Such a *Bdellovibrio*-resistant clone would require the capacity to block the approach, attachment, penetration, and assimilation of host materials for growth and reproduction by *Bdellovibrio*, and/or the ability to release itself from the host envelopes. J. E. Snellen and M. P. Starr (unpublished data) examined many preparations of the survivors of *Bdellovibrio* attack; in every case, the propagated clones of the survivors were still seemingly as susceptible to attack by *Bdellovibrio* as the original host strains.

2. *Penetration into Host Cell*

Most observers agree that, following attachment to a congenial host cell, the *Bdellovibrio* breaches the host cell wall within a few minutes and completes the intramural (intra-integumental) penetration of the host cell several minutes thereafter (Fig. 8). Stolp (1967a, b), using cinematography, observed that penetration was completed within seconds in the system he studied but, more typically, the penetration is reported to begin within 1–20 min. after attachment (3–20 min.; Burger *et al.*, 1968) and to be complete within 5–60 min. after attachment (Starr and Baigent, 1966; 10–60 min.; Burger *et al.*, 1968). The parasite loses its flagellum during attachment or invasion.

Varon and Shilo (1968) measured the penetration of *Bdellovibrio* into

FIG. 9. Interaction of *Bdellovibrio bacteriovorus* (strain *Bd.* 100) and *Erwinia amylovora* (ICPB EA137) as shown in thin-section electron micrographs. Magnification ×54,000. (A) Entrance of the bdellovibrio through a pore in the host cell wall. (B) A more advanced state of the interaction in a multiple parasitization. Fig. 3 of Starr and Baigent (1966).

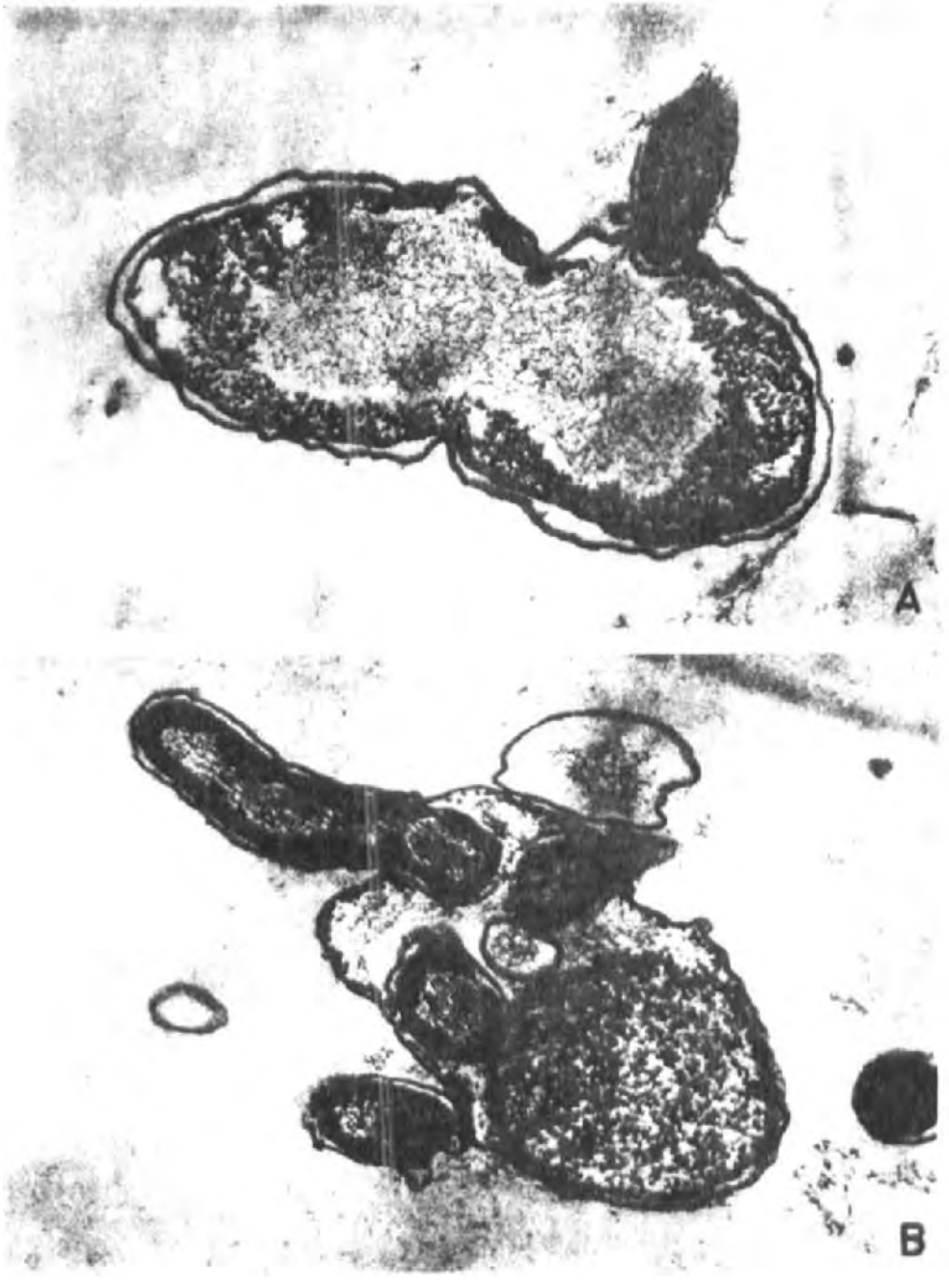


FIG. 9.

the host cell by preparing ^{14}C-labelled *Bdellovibrio* and determining radioactivity in the host cell after severe mechanical agitation. Since penetration is completely blocked by inhibitors of protein synthesis, such as streptomycin, puromycin and chloramphenicol, they suggest that inducible enzymes have to be formed by the parasite before it is able to penetrate the host cell. This induction is believed by them to require a direct contact between *Bdellovibrio* and its host cell or between *Bdellovibrio* and the inducer; the postulated inducer is masked by the host cell wall and is exposed to the *Bdellovibrio* only after attachment. It would be of interest to see whether such postulated inducible enzymes are formed in a system containing disrupted host cells and intact *Bdellovibrio* cells.

The mechanism for the formation of a pore (Fig. 9) in the host cell wall is not immediately clear. Stolp and Starr (1963b) postulated that the violent ballistic collision of the parasite with the host cell and/or the subsequent rotation (up to 100 revolutions per sec. according to Stolp, 1967a, b) of the parasite are responsible for pore formation. The work of Lépine *et al.* (1967) and Burnham *et al.* (1968b) seems to support this concept, since the peptidoglycan, which is responsible for rigidity of the host cell wall, was observed unchanged in electron micrographs during the earliest stages of the host-parasite interaction; not all of the host cells attacked by *Bdellovibrio* turned into spherical bodies at this stage. An abstract by Abram and Chou (1971) indicates that the *Bdellovibrio* must be able to approach and attach to the host protoplast in order for penetration to be completed.

Burnham *et al.* (1968a,b) postulated that the *Bdellovibrio* penetrated through a bulge preformed on the host cell wall. At the point of attachment, the *Bdellovibrio* flattens against its host cell at the outermost membrane in the holdfast region. The *Bdellovibrio* then pushes into the centre of the bulge and breaks through the host cell wall. Lépine *et al.* (1967) reported that bulge formation on the host cell during the early stages of attachment was induced by the internal pressure of the host cell. Huang (1969) also observed a bulge formed in some host cells infected by *Bdellovibrio*. A combination of factors, including damage of the host cell and turgor pressure, likely contribute to the development of the bulge on the cell wall of the host.

Constriction of the *Bdellovibrio* cell is usually seen as the bdellovibrio advances through its restricted point of entry into the host cell. An intimate "arm-in-socket" arrangement or "strong bonding" between the *Bdellovibrio* and its host cell has been postulated for this stage of the interaction. Thus, after primary penetration, the *Bdellovibrio* is not readily separated from its host cell by violent shaking or mixing (Burnham *et al.*, 1968b). Scherff *et al.* (1966) reported that the "infection cushion", which they believe is an integral part of the parasite, becomes enlarged;

it is this part of the parasite which penetrates the host cell wall first. Thereafter, the *Bdellovibrio* cell pushes into the host cell; the host cell wall becomes distorted and is separated from the plasma membrane, forming vacuolated areas ("bubbles"; "balloons") between the cytoplasmic membrane and the cell wall where the bdellovibrios develop. Scherff *et al.* (1966) observed that as many as six *Bdellovibrio* cells entered and reproduced within a single pseudomonad host cell.

3. Growth of Bdellovibrio *Within Host Cells*

In the days before the intramural or intra-integumental locus of *Bdellovibrio* was known, Stolp and Starr (1963b) suggested that *Bdellovibrio* multiplied extracellularly by binary fission. Starr and Baigent (1966) subsequently showed the intermediate formation within the host cell of a large helical *Bdellovibrio* cell, which divided by multiple constriction to form several vibrioid progeny. Although there are variations in the details, Scherff *et al.* (1966), Lépine *et al.* (1967), Burnham *et al.* (1969, 1970), and Huang (1969) also report that the bdellovibrios penetrate the host cell wall (but not the membrane) and develop intracellularly by thickening and elongation to form a helical filament. The filament, upon maturation, segments into individual vibrioid units, presumably by constriction (Abram and Shilo, 1967) since cross-wall formation has not been observed. The division starts with an asymmetric constriction of the cytoplasmic membrane of the filaments, according to Burnham *et al.* (1969, 1970). Then, the outer layer of the mother cell wall breaks at the dividing regions. The flagellum and the flagellar sheath form at one end of the daughter cell before it separates completely from the filamentous mother cell (Burnham *et al.*, 1969, 1970). The bdellovibrio progeny within the ghosts of the host cells move actively (Starr and Baigent, 1966; Burger *et al.*, 1968; and others). Whether or not the daughter cells are infective before their flagella are formed is still obscure. Scherff *et al.* (1966) claim that growth was observed to occur from both ends of the intracellular *Bdellovibrio*; the end which entered the host cell was allegedly the first to grow. The number of *Bdellovibrio* progeny produced within a cell of *Pseudomonas fluorescens* varies between 6 and 30 although a more usual range is 8 to 12 (Scherff *et al.*, 1966), 14 (Abram and Shilo, 1967) or 5·7 (Seidler and Starr, 1969a) in *E. coli*.

4. Long Forms of Bdellovibrio

Abram and Davis (1970) noted that trypticase-yeast extract or nutrient broth supports both intracellular and extracellular development of *Bdellovibrio*. According to these authors, the long forms (6–20

μm.) which appear in the two-membered culture are too long to have emerged from a host cell. This type of facultatively parasitic growth has been reported at various times in several strains of *Bdellovibrio* (*Bd.* 100, Murray, 1964; *Bd.* W, Burger *et al.*, 1968; *Bd.* UKi2, Burnham *et al.*, 1969, 1970; *Bd.* 6-5-S, Crothers *et al.*, 1970; *Bd.* 109, Reiner and Shilo, 1969, Varon and Shilo, 1969b, Amemiya and Kim, 1970; *Bd.* N-6801, Uematsu and Wakimoto, 1969b, 1971; see also Section VI, p. 256).

Reiner and Shilo (1969) found that 5% of *Bd.* 109, *Bd.* GB, and *Bd.* T28 cells grew into long forms when the bdellovibrios were incubated in cell-free extracts of *Pseudomonas aeruginosa*, *E. coli*, *M. lysodeikticus*, *Saccharomyces cerevisiae*, or *Bd.* 109. Tritiated thymidine is incorporated into these long forms only in the presence of the cell-free extract with a lag period of several hours; a rapid incorporation then follows. An active factor which can induce the formation of long forms has been isolated from the aforementioned cell-free extracts. This factor is non-dialysable and stable to heat (autoclaving), deoxyribonuclease, ribonuclease and Pronase. Under certain osmotic conditions, the long forms may segment into chains of *Bdellovibrio* cells. Some chains divide into individual cells. A tightly coiled non-segmented long form was induced by omitting amino acids from the incubation mixture.

5. *Disruption of Host Cells and Release of* Bdellovibrio *Progeny*

When host cells are challenged with a high multiplicity of *Bdellovibrio* cells, multiple attacks occur and the multiply-attacked host cells lyse within a few minutes ("lysis-from-without", according to Abram and Shilo, 1967). In such cases, *Bdellovibrio* progeny would not be produced (if the parasitic cycle is the only way of growth) because the possible host is destroyed before the parasitic phase is established. The empty envelopes of the host cells either remain in the lysate or disappear with time. The mechanism of lysis under these conditions remains unknown. The manner in which the host cell is disrupted in the normal parasitic growth cycle with the consequent release of mature *Bdellovibrio* is also not well understood. Possible mechanisms for the bacteriolysis caused by *Bdellovibrio* are described in Section IV.C (p. 242).

The size and shape of *Bdellovibrio* cells have been said to vary according to the recency of their release from the host cell. The newly released cells of *Bd.* 109 have been reported by Varon and Shilo (1968) to be usually motile and small; older cells were immotile and relatively large. However, Starr and Baigent (1966) found just the opposite size relationship. It should be noted here that S. C. Rittenberg (personal communication) and others (including personnel in our laboratory)

have observed that *Bd.* 109 as kept in our laboratory in Davis and "*Bd.* 109" as kept in M. Shilo's laboratory in Jerusalem are markedly different in cell morphology and other physiological characters (see also Section IV.E, p. 249). The Davis strain of *Bd.* 109, which had been kept lyophilized and presumably unchanged since it was described by Stolp and Starr (1963b), shows its typical curved appearance for a considerable period of time after completion of lysis in a two-membered culture, whereas the Jerusalem strain of *Bd.* 109 changes very shortly after complete lysis of the hosts to a straight elongated rod.

B. Effects of *Bdellovibrio* on the Host Cell

Stolp and Starr (1963b) observed that *Rhodospirillum rubrum* lost its motility five seconds after attachment by *Bd.* 100. The motility of *Spirillum serpens* strain VHL, similarly, was reported to cease 12 min. after attachment by *Bdellovibrio* 6-5-S (Huang, 1969). How *Bdellovibrio* infection in its initial stages could affect the motility of host cells is still unknown.

Most students of *Bdellovibrio* have observed that the host cells form into spherical bodies ("sphaeroplasts") within a few minutes after attachment by *Bdellovibrio*. In the case of long host cells, a localized, sometimes multiple "ballooning" or "bubble formation" has been observed, rather than a rounding up of the entire host cell. Most writers on the subject have suggested that this morphological change in the host cell is caused mainly by damage to the rigid peptidoglycan component of the host cell wall by *Bdellovibrio* (although, as noted in Section IV.A.2, p. 236, the electron microscope evidence is still equivocal). These spherical bodies may be related morphologically to sphaeroplasts produced from bacterial cells by either lysozyme or penicillin in media containing sucrose and cations as supporting agents. The term "sphaeroplast" is conventionally used to designate the spherical, osmotically sensitive form of the bacterial cell in which the cell wall has been modified (McQuillen, 1960; Martin, 1963). There is still some question (see Section IV.C.1, p. 242, and Starr and Seidler, 1971) as to whether the spherical host form resulting from attack by *Bdellovibrio* is actually a conventional sphaeroplast, since it does not seem to be osmotically sensitive.

Shilo and his coworkers investigated the early effects of *Bdellovibrio* on cells of *E. coli*. Shilo (1966) and Abram and Shilo (1967), using phosphotungstic acid-stained and shadow-cast preparations in an electron microscope, showed that holes or pits appear on the host cell surface shortly after attack by *Bdellovibrio* is initiated. Varon *et al.* (1969) found that *Bdellovibrio* incorporated amino acids and uracil poorly, and produced insignificant amounts of β-galactosidase, whereas

the host cell (either *E. coli* B or *E. coli* K-12) was competent with respect to these characters. They enhanced the early effects of *Bdellovibrio* by infecting the host culture with a high multiplicity of infection (8–17), so that 50% of the host cells became non-viable within 2–4 min. after infection, at which time no biosynthesis of bdellovibrios had yet begun. In the presence of an inducer, ethyl-β-D-thiogalactoside, *E. coli* synthesized β-galactosidase which is detectable by the increase of red pigment as the destruction of *o*-nitrophenyl-β-D-galactopyranoside proceeds. Synthesis of β-galactosidase halted when the culture of *E. coli* was infected with *Bd.* 109 (Jerusalem), even in the presence of inducer. These authors interpreted their data as indicating that *Bdellovibrio* infection interferes with the synthesis of β-galactosidase-specific m-RNA of the host. To prove the point that synthesis of RNA and protein in the host were affected, they further examined incorporation of ^{3}H-uracil into an acid-precipitable portion of *E. coli* B, and incorporation of ^{14}C-leucine as specific markers for RNA and protein syntheses, respectively. They found that RNA synthesis in the host was inhibited 3 min. after *Bdellovibrio* infection; protein synthesis was inhibited at 8–9 min. Varon and Shilo (1968) found that streptomycin specifically prevents the penetration of *Bdellovibrio* into, but not attachment to, the host cell. Since the rate of ^{3}H-uracil incorporation was decreased in the presence of streptomycin, Varon *et al.* (1969) concluded that the host RNA synthesis halted before the parasite penetrates the host cell wall, i.e. the attachment of *Bdellovibrio* on the surface of the host cell was sufficient to cause the cessation of the host's RNA synthesis.

Rittenberg and Shilo (1970) examined the effects of *Bd.* 109 (Jerusalem) infection on the rate of respiration of lactose, succinate, or acetate by *E. coli* ML35 or *E. coli* B and on the permeability to lactose, acetate, succinate, or *O*-nitrophenylgalactoside. The host strain, *E. coli* ML35, is lactose-cryptic (*lac* $i^{-}z^{-}y^{-}$), i.e. capable of synthesizing β-galactosidase (z^{+}) but lacking galactoside permease (y^{-}) and a regulatory gene (i^{-}) which would otherwise produce repressors to control the β-galactosidase operator. In their systems, a high multiplicity of infection (two or more) was used. A low concentration (5 μmoles/ml.) of the substrate (lactose, succinate, or acetate) was used to avoid its possible effect on the growth or respiration of the host cell, but it did serve as an indicator to detect the changes which occurred in cultures of the host *Bdellovibrio*.

The respiration rate rose steadily in the control culture (no added substrate) of *E. coli-Bdellovibrio*, and reached a maximum at 3 hr. at which time the lysis of host cells began. Lysis was completed in 4 hr, and the respiration rate dropped when the *Bdellovibrio* progeny were released from the ghosted host cell. Addition of lactose to the host-

Bdellovibrio culture rapidly stimulated respiration over a 24 min.-period after the two-membered culture was established; this increase was then followed by a rapid decrease. Addition of succinate, lactate, glucose, or malate to the host-*Bdellovibrio* system gave no increase in respiration; instead, starting at 10 min. after infection, the rate of respiration dropped rapidly (Rittenberg and Shilo, 1970).

Close scrutiny of the changes in respiratory patterns showed that the increase in the lactose respiration rate was detected between 5 and 10 min. and reached a maximum 20 min. after the establishment of the two-membered culture. The decline in succinate respiration started at the time when an increase in the rate of lactose respiration was noticed. The changes in respiration were influenced by several factors including age of the host cells, number of cells at a fixed multiplicity of infection, or the multiplicity of infection at a fixed host-cell population (Rittenberg and Shilo, 1970).

The rate of *O*-nitrophenylgalactoside hydrolysis, which is indicative of unmasking of β-galactosidase activity in the cryptic host, continued to rise for 45 min., although the rate of respiration of lactose increased rapidly and started dropping at 25 min. *Beta*-galactosidase activity was detected only in trace amounts in the supernatant fluids of the host-*Bdellovibrio* system. Addition of sodium chloride or phosphate, which prevent attachment of the *Bdellovibrio* to host cells, did not damage permeability control and the respiration of the host cell. The earlier the addition of streptomycin (250 μg./ml.) to the system, the slower the unmasking of β-galactosidase activity. Puromycin and chloramphenicol had similar effects. Rittenberg and Shilo (1970) state that the increase in permeability and disruption of respiratory activity of the host cell might suggest that one early effect of *Bdellovibrio* attack is damage to the host cell membrane. Manifestation of these effects did not require the complete penetration of the *Bdellovibrio* into the host cell. Leakage of ultraviolet-absorbing materials, ^{14}C-labelled host cell components, amino acids, and host β-galactosidase was low, but these substances could be detected 20 min. after infection and before completion of the first growth cycle of *Bdellovibrio* (Drucker, 1969). Alkaline phosphatase, an enzyme localized in the periplasm of *E. coli*, did not leak out immediately after penetration of the host cell and its conversion into a spherical body. These data suggest to Shilo (1969) that the enzyme is effectively bound within the periplasm and/or that immediate repairing of the damaged wall takes place.

During growth of *Bd.* 6-5-S in a living culture of *E. coli* ML35, compounds absorbing at 260 nm. and reacting with Folin-Ciocalteau reagent were released, and total cell protein (presumably host-plus-*Bdellovibrio*) decreased (Crothers and Robinson, 1970, 1971). The rate

of *O*-nitrophenylgalactoside hydrolysis increased, but soluble β-galactosidase was not detected in the supernatant. The *Bdellovibrio*-infected cell of *E. coli* became brightly fluorescent when a fluorescent dye (8-anilino-1-naphthalene sulphonic acid) was added. These data lead to the suggestion that, upon damage of the cell membrane by the attack of *Bdellovibrio* (which would allow passage of *O*-nitrophenylgalactoside and the fluorescent dye), a large amount of host cellular components is still held intracellularly for nourishing the host-dependent *Bdellovibrio* cells.

C. Mechanisms of Lysis of the Host Cell by *Bdellovibrio*

1. Mode of Bacteriolytic Action

Lysis of bacteria by biological or non-biological agents, and the possible mechanisms involved, have been reviewed extensively by McQuillen (1960), Work (1961), Martin (1963, 1966), Perkins (1963), Brown (1964), Salton (1964), Weidel and Pelzer (1964), MacLeod (1965), Rogers (1965), Ghuysen *et al.* (1966, 1968), Stolp and Starr (1965), Shockman (1965), Strominger and Ghuysen (1967), Ghuysen (1968), and Guze (1968). Lysis of filamentous fungi by *Streptomyces* sp. (Aguirre *et al.*, 1963; Hsu and Lockwood, 1969; Jones *et al.*, 1968), by other soil micro-organisms (Carter and Lockwood, 1957; Ko and Lockwood, 1970), by *Bacillus circulans* (Horikoshi and Iida, 1959), and by other bacteria (Mitchell and Alexander, 1963) has been well documented.

Actually, little is yet known about the mechanism of bacteriolysis by *Bdellovibrio*. We might first review the facts about the conversion of the host cell into a spherical body (see Section IV.B, p. 239, and Starr and Seidler, 1971). Existence of the spherical body suggests that the rigid peptidoglycan layer of the wall has been softened and that the pore formed in the host cell wall during penetration by *Bdellovibrio* may have been sealed by some agglomerating material (Guélin *et al.*, 1967) or by a wall-repairing mechanism (Shilo, 1969). The mechanism of formation of the spherical body, as well as the role of this structure in the host-parasite relationship, are still unknown. The nature of these spherical bodies and particularly their existence in non-osmotically supported media, if they are indeed sphaeroplasts, requires further investigation. The identity of these spherical bodies to the sphaeroplasts formed by lysozyme-EDTA is questionable, although Shilo (1969) has suggested that the spherical bodies formed during *Bdellovibrio* infection resemble the penicillin- or cycloserine-induced sphaeroplasts which retain the alkaline phosphatase.

The continued interaction with *Bdellovibrio* eventually results in considerable disorganization of the host cells (Starr and Baigent, 1966;

Scherff *et al.*, 1966). Nuclear materials disperse and disappear. Ribosomes and cytoplasmic materials become granular and unevenly distributed, and the peptidoglycan layer of the host cell wall is partially digested. Although extensive disintegration of the host cell wall is evident in later stages, this disintegration might be caused by enzymes produced by *Bdellovibrio* (see Section IV.C.2, p. 243), by autolytic enzymes produced by the damaged host cell (Stolp and Starr, 1965), or by both.

The lytic enzymes produced by *Bdellovibrio* may also affect its own viability, and might cause the rapid decline in numbers of viable cells observed in liquid cultures. This hypothesis is based on the fact that dilution of *Bdellovibrio* cultures prolongs this viability. Alternative or additional explanations occur to us; these include accumulation of metabolic wastes and other toxic substances, and exhaustion of nutrients. Further work on this subject is clearly required.

2. Muramidase

Early attempts to demonstrate muramidase and other lytic enzymes in *Bdellovibrio* and to relate these enzymes to the disintegration of the host cell were hampered by the use of two-membered systems containing living host cells, with the consequent uncertainty as to whether the enzymes originated in the bdellovibrio or host cells. Several *Bdellovibrio* strains were shown (Huang and Starr, 1971a, b) to multiply readily in heat-killed (70° or 100°; 10 min.; 121°, 5 or 15 min.) cell suspensions of the host in tris-HCl buffer (0·025 *M*; pH 7·5, containing 2 m*M* each of $MgSO_4$ and $CaCl_2$) and to lyse the host cells. Growth of *Bdellovibrio* and simultaneous dissolution of the heat-killed host cells were accompanied by the appearance in the culture of soluble muramic acid and of submicroscopic particles which contained amino sugars. An enzyme or a mixture of enzymes which is released by the growing *Bdellovibrio* and which degrades the host cells is believed to liberate the particulate matter containing amino sugars (Huang and Robinson, 1969; Huang and Starr, 1971b). The crude enzyme preparation precipitated from filtrates of a culture of *Bd.* 6-5-S grown on heat-killed host cells by ammonium sulphate or cold acetone, solubilizes the peptidoglycan isolated from the cell of *Spirillum serpens* VHL and releases reducing sugars into the supernatant solution. The crude enzyme preparation also lyses ^{14}C-labelled peptidoglycan and releases soluble ^{14}C-labelled materials into the filtrate (Huang and Starr, 1971b). Using this radiochemical analysis as indicator, lysozyme-like enzymes have been isolated and characterized (Huang and Starr, 1971b) from the crude enzyme preparation by chromatography on a DEAE-cellulose column

or Sephadex (G-100) gel filtration. The molecular weight of this lysozyme-like enzyme is 12,500, as determined by Sephadex gel filtration. There is no question, as there may have been with preliminary studies on this subject, that these are *Bdellovibrio* enzymes; any such enzymes from the host cells would have been inactivated by the heat treatment, and the heat-killed host cells could, of course, not produce any such enzymes.

An abstract by Fackrell *et al.* (1970) also reports the purification (60–70 fold), from *Bdellovibrio*-host culture lysates, of a bacteriolytic enzyme which degrades isolated cell walls of *Spirillum serpens* VHL. The enzyme, which is said to have a molecular weight of approximately 40,000, also degrades purified peptidoglycan isolated from *S. serpens*.

3. Protease

Many, if not all, strains of *Bdellovibrio* produce large amounts of proteases (Stolp and Starr, 1963b; Shilo and Bruff, 1965; Seidler and Starr, 1969b; Diedrich *et al.*, 1969, 1970) which are capable of lysing heat-killed or acid-, butanol-, or EDTA-treated bacteria. These proteases have no apparent effect on living host cells, but are believed to be responsible for the lysis of dead host cells (Shilo and Bruff, 1965). Such proteases become effective only when the *Bdellovibrio* cell is in contact with the host cell.

Twelve strains of host-dependent *Bdellovibrio* were shown to have a strong proteolytic action on gelatin and casein, as well as a lytic action on heat-killed bacteria (Stolp and Starr, 1963b; Stolp, 1964). Some host-independent derivatives from these host-dependent bdellovibrios are proteolytic (Seidler and Starr, 1969b); others are reported to be not proteolytic (Stolp, 1964). *Bdellovibrio* A3.12 and other strains form protease in full strength and one-tenth strength nutrient broth in the presence or absence of the host cell; this protease lyses heat-killed bacteria and hydrolyses Azocoll (an insoluble cowhide substrate) and is said to be responsible for dissolving most components of the host bacteria except the empty hulls of cell walls (Shilo and Bruff, 1965).

To allow detection of protease produced by host-dependent bdellovibrios, without interference from host cell protease or autolytic enzymes, a suspension was used of non-proteolytic (or heat-inactivated) *Spirillum serpens* in tris-HCl buffer (25 m*M*, ph 7·5), containing 2 m*M* each of $CaCl_2$ and $MgSO_4$ (Huang and Starr, 1971b). Proteolytic enzymes, as assayed by the hydrolysis of Azocoll and of casein or N,N-dimethyl-casein, are produced by *Bd.* 6-5-S growing in living or heat-killed cell suspensions, and also in cell-free extracts from *S. serpens*. *Bdellovibrio* 100, *Bd.* 109, and *Bd.* A3.12 also were shown to produce protease in suspensions of *S. serpens* VHL.

Lysis of the host culture usually precedes the detectable formation of protease in the two-membered culture (Huang and Starr, 1971b). At a low multiplicity of infection (0·2), a mild lysis was observed over a period of 0·5 to 8 hr.; however, significant amounts of the protease were not produced until 9 hr. after mixing *Bdellovibrio* with the host cells. When a high multiplicity of infection (50) was used, immediate lysis of the host culture occurred (within 0·5 hr.) and yet the protease could not be detected until 3·5 hr. later. These results indicate that the host cells lyse extensively before protease production reaches a maximum. When the production of a casein-hydrolysing zone by a normally protease-positive *Bdellovibrio* is inhibited by addition of ammonium sulphate, plaques are still formed on the host lawn (Huang and Starr, 1971b). "Protease-negative" mutants, in this case *Bdellovibrio* strains which do not hydrolyse Azocoll or casein (collagenase- and caseinase-negative), were isolated from protease-positive *Bd.* 6-5-S using nitrosoguanidine treatment or by the selection of spontaneous mutants. "Protease-negative" mutants grew slowly and formed small plaques (less than 1 mm. in diameter in five days), while the protease-positive strains formed plaques 6 mm. in diameter in two days in a moist environment. Although "protease-negative" bdellovibrios do not hydrolyse collagen or casein, they do, like the protease-positive strains, produce carboxypeptidase which hydrolyses N,N-dimethylpolypeptides obtained from trypsin-treated casein (Huang and Starr, 1971b).

The protease produced by host-dependent *Bd.* 6-5-S has been purified 65-fold using acetone fractionation and DEAE cellulose separation (Huang and Starr, 1971b). One major peak, which retains a strong activity in hydrolysing Azocoll, was observed. The partially purified enzyme releases groups from haemoglobin, gelatin, and albumin which react with dinitrofluorobenzene. The protease obtained from the fractionation on DEAE cellulose loses its Azocoll-digesting ability (about 50%) after dialysis for 24 hr. in tris-HCl or distilled water. Calcium, but not magnesium, ions are required to stabilize the protease activity during dialysis. Addition of Ca^{2+} and Mg^{2+} to the reaction mixture of tris-HCl buffer containing protease and Azocoll activates enzyme activity. The optimal pH value is 8·5 and the optimal concentration of tris-HCl buffer is 0·25 *M*. The Michaelis constant of the protease for N,N-dimethylcasein is 5.1×10^{-5} *M*. The molecular weight, as measured by Sephadex gel filtration, is about 11,000 (Huang and Starr, 1971b).

4. Lipase

A lipase, which is capable of hydrolysing tributyrin incorporated into agar, was detected in the cold-acetone precipitate of the

Bdellovibrio-host culture fluid (Huang and Starr, 1971b). Enzymes of this type are likely to participate in degradation of the host-cell membrane.

In summary, *Bdellovibrio* produces lysozyme-like enzyme(s) which can lyse the peptidoglycan of the cell envelope of the host, lipase(s) which might hydrolyse lipids of the cell membrane, and proteolytic enzyme(s) which can consume proteins of the host cells. Such enzymic capabilities are probably all pertinent to the bacteriolytic action of *Bdellovibrio*.

D. Kinetics of Host-Dependent Growth

The kinetics of growth of *Bdellovibrio* and of death of the host cell were first illustrated by Stolp and Starr (1963b) before the intracellular nature of the *Bdellovibrio* parasitism was known. Bruff (1964) found that plaque formation by *Bdellovibrio* followed the formula $NP = CN_V^x$ (where NP = number of plaque; sN_V = concentration of the bdellovibrio; x = the number of bdellovibrios necessary to form a plaque; and C = a constant) with x equal to 1·2. This means that one bdellovibrio was sufficient to initiate formation of a single plaque in the system she used.

Seidler and Starr (1969a) followed the non-synchronous growth of *Bd.* 109 in *E. coli* B in terms of plaque-forming units. There was a lag period of about 4 hr., and a mean generation time of 1·3 hr. (the calculation assumes that bdellovibrio divides by binary fission, which it does not). On average, each cell of *E. coli* B gives rise to 5·7 *Bdellovibrio* cells. The lag period depends on the physiological state of the parasite, the pH value, and the temperature of incubation. In the temperature range of 25–38°, the average burst size and lag period are inversely proportional to the temperature; at 42°, there is no burst. The burst coefficient (a quotient of the average burst size and the latent period in hours) is highest in the range 30–35° (an optimum range of growth temperature).

An optimum temperature ranging from 30° to 32° and optimum pH value range of 7·0–8·5 were noted for *Bd.* N-6801 (Uematsu *et al.*, 1971). The thermal death point of one strain of *Bdellovibrio* is reported to be approximately 10 min. at 51° (K. Nakamura, personal communication).

E. Nutrition of *Bdellovibrio*

Early attempts to grow host-dependent *Bdellovibrio* on non-living host cells or on extracts of cells did not succeed (Stolp and Starr, 1963b). In these trials, *Bdellovibrio* was unable to lyse and grow in host cells

killed by heat (100°), chloroform, toluene, or Roccal disinfectant. This inability to grow was not related to differences of pH value or redox potential in the cultures of host bacteria. In only one case did Stolp and Starr (1963b) find that plaques developed on heat-killed cells, and this case may have depended on the carry-over of nutrients from the *Bd.* A3.12 lysate (host-independent growth has been reported with this strain; Shilo and Bruff, 1965). The nature of the material supposedly carried over has not yet been determined.

The results of the single extant study of the nutritional requirements of a host-independent *Bdellovibrio* might profitably be related here. Yeast extract was shown to be an excellent substrate for growth of host-independent *Bd.* A3.12 (Bruff, 1964; Shilo and Bruff, 1965), but casein hydrolysate could not be utilized as a sole source of carbon and energy. Small quantities of yeast extract were needed to obtain good growth yields when peptone was used as the major carbon and energy source; this suggests that yeast extract contains one or more growth factors for *Bdellovibrio.* A mixture of B-group vitamins (riboflavin, calcium pantothenate, B_{12}, biotin, *p*-aminobenzoic acid, pyridoxal phosphate, and pyridoxamine hydrochloride) could replace yeast extract in this system.

A number of workers (Huang, 1969; Varon and Shilo, 1969b; Crothers *et al.*, 1970; Huang and Starr, 1971a) have observed that washed cells of host-dependent bdellovibrios develop in the presence of, and lyse, washed host cells which have been heat-killed (70° or 100°, 10 min., or autoclaved, Huang and Starr, 1971a; 70°, 15 min., Varon and Shilo, 1969b). However, extensive autoclaving (e.g. 40 min.) renders such cells unsuitable as *Bdellovibrio* nutrient (Huang and Starr, 1971a). Lysis of such preparations by *Bdellovibrio* in broth or tris-HCl buffer suspension is extensive, but generally with very low plaquing efficiencies on lawns. Growth of *Bd.* W on heat-killed host cells (80°, 20 min., *Rhodospirillum rubrum*) was observed by Burger *et al.* (1968); again the plaquing efficiency was very low. Although *Bdellovibrio* attaches very poorly to heat-killed cells (70°, 15 min., Varon and Shilo, 1968), some development of *Bdellovibrio* was observed in such preparations (Varon and Shilo, 1969b). Gillis and Nakamura (1970) reported that *Bdellovibrio* grew on lawns of heat-killed (65°, 20 min.; 121°, 15 min.) cells of *Shigella boydii* in non-nutrient agar. These observations eliminate the likelihood that bdellovibrios require nutrients from the culture medium in addition to those they get from the bacterial host cells, and they support the view that development of *Bdellovibrio* depends primarily on some host-cell material or metabolic products, as suggested originally by Stolp and Starr (1963b). This concept is further supported by the growth of *Bdellovibrio* in host bacteria at all phases of the host growth

cycle (mid-log phase growth of the host, as with bacteriophages, is unnecessary), by *Bdellovibrio* development in host bacteria that had been inactivated by streptomycin or by ultraviolet irradiation (Huang, 1969; Varon and Shilo, 1969b), and by multiplication of *Bdellovibrio* in cell-free extracts of susceptible or non-susceptible host organisms (see Section IV.A.4, p. 238, and the next paragraph).

Bdellovibrio N-6801 multiplies in suspensions of autoclaved cells or in cell-free extracts of bacteria which were either susceptible or non-susceptible as hosts for the parasitic phase; these bacteria include *Corynebacterium michiganense*, *Bacillus subtilis*, *Bacillus megaterium*, *Bacillus cereus*, *Agrobacterium tumefaciens*, and *Pseudomonas fluorescens*. *Bdellovibrio* N-6801 also grows and forms colonies on agar containing autoclaved host cells, a kind of host-dependent but not parasitic growth (Uematsu and Wakimoto, 1969a, b, 1971). The colonies produced in these trials were light yellow in colour and produced lytic zones, probably due to proteolytic activity on the dead cells. The lytic zone was not seen when cells of autoclaved bacteria, which were non-susceptible to parasitic attack by *Bd.* N-6801, were incorporated into the agar. Since the number of plaque-forming units on the lawn of living host cells corresponded with the number of colony-forming units on the lawn of autoclaved host cells (the ratios being unity), *Bd.* N-6801 is considered to be a facultatively parasitic strain of *Bdellovibrio*. It is, however, still dependent on nutrients from the host cell or other non-susceptible bacteria. Thus *Bd.* N-6801 behaves differently from the host-independent bdellovibrio strains or facultatively parasitic *Bd.* UKi2 (see Section VI, p. 256).

Growth and lytic activity in liquid culture are influenced considerably by the composition of the medium used for cultivating the host bacteria (Stolp and Starr, 1963b). Somewhat retarded growth of the host bacteria generally favours development of the parasite; a more rapid host growth rate might somehow suppress the more slowly growing *Bdellovibrio*, and host metabolites might inhibit the *Bdellovibrio*. One-tenth strength nutrient broth or quarter strength yeast peptone medium generally are best for this purpose.

There have been several studies regarding the role of divalent cations in *Bdellovibrio* development. Robinson and Huang (1967) reported that suspensions of washed *E. coli* B/r harvested from complex and from synthetic media and suspended in 0·025 *M* tris-HCl buffer (pH 7·5) supported growth of *Bdellovibrio* only when Ca^{2+} and/or Mg^{2+} were added (similarly with *E. coli* ML35; Crothers and Robinson, 1970). Huang and Starr (1971a) noted that *Bd.* 6-5-S, *Bd.* 100, *Bd.* 109 (Davis), and *Bd.* A3.12 all multiplied in the presence of living or heat-killed (70° or 100°, 10 min.; 120°, 5 or 15 min.) *Spirillum serpens* VHL in HEPES-

NaOH (for *Bd.* 6-5-S, see p. 243) or tris-HCl buffers or distilled water, when supplemented with Ca^{2+} and/or Mg^{2+}.

Multiplication of *Bdellovibrio* in the presence of Ca^{2+} and Mg^{2+} is associated with the release of ultraviolet-absorbing materials, presumably from the host cell, and the release into the culture supernatant solution of amino sugars; soluble amino sugars were not released in the absence of added cations in this system. Even in the presence of added cations, the growth rate of *Bdellovibrio* in heat-killed host cells is lower than in viable but non-proliferating host cells. Late addition of the cations shortens the growth lag time of *Bdellovibrio*. Calcium or magnesium ions (chloride or sulphate) each support growth independently. *Bdellovibrio* 100, *Bd.* 109 (Davis and Jerusalem strains), *Bd.* A3.12, and *Bd.* 6-5-S require Ca^{2+} for growth in either their homologous or heterologous host bacteria. *Bdellovibrio* 109 (Jerusalem), but not always *Bd.* 109 (Davis), *Bd.* 100, *Bd.* A3.12, or *Bd.* 6-5-S, is capable of growing in the presence of the low concentration of Ca^{2+} bound *in situ* on its host *E. coli* B (when grown in complex medium). After repeated washings of such host cells or upon growing the host cells in a Ca^{2+}-depleted minimal medium, *Bd.* 109 (Jerusalem) requires added cations for development (Huang and Starr, 1971a). This distinction in cation requirements between *Bd.* 109 (Davis) and *Bd.* 109 (Jerusalem) supports the view expressed in Sections I.E and IV.A.5 (pp. 221 and 239) to the effect that these two strains are markedly different. Addition of EDTA (0·01 *M*; pH 7·5) prevents growth of *Bd.* 6-5-S; growth is restored by addition of Ca^{2+} and Mg^{2+}. A requirement for Mg^{2+} and/or Ca^{2+} in the system *Bd.* 109 (Davis)—*E. coli* B was also observed by Seidler and Starr (1969a), who suggest that the possible effects of cations in this system might be associated with attachment to the host cells, diminution of the latent period, support of the host "sphaeroplasts", and increase in the average burst size. The fact that *Bd.* 6-5-S multiplies in dead host cells only in the presence of added cations suggests to Huang and Starr (1971a) that these cations are needed, also, for other aspects of *Bdellovibrio* development and in addition, since release of soluble amino sugars into the culture supernatant occurs only in the presence of added cations, also for activating lytic enzymes.

One other fact on *Bdellovibrio* mineral nutrition might be recorded. Marine bdellovibrios isolated from the Mediterranean Sea (Shilo, 1966) or the Atlantic Ocean (Mitchell *et al.*, 1967; Mitchell and Morris, 1969) form plaques only on lawns containing 3% (w/v) NaCl.

F. Host Specificity

Several procedures are used for determining the host range of bdellovibrios, namely (1) attachment of *Bdellovibrio* to the host cell as observed

in a phase-contrast microscope (Stolp and Starr, 1963b) or by the differential filtration method (Varon and Shilo, 1968); (2) clearing of a host culture in broth (Stolp and Starr, 1963b) or a host cell suspension in buffer (Huang, 1969); (3) plaque formation (Klein and Casida, 1967; using Thornton's medium) or confluent lysis (Stolp and Starr, 1963b; using yeast-peptone medium); and (4) total count by Coulter counter (Bruff, 1964; Shilo and Bruff, 1965). Shilo and Bruff (1965) found that the host range was broader in liquid media (turbidity change) than on agar media (plaques); the broader host range might be attributed to the higher multiplicity of the parasite. The use of washed host cells suspended in buffer together with washed cells of *Bdellovibrio* generally gives a consistently wider host spectrum than the other procedures (Huang, 1969). G. Drews (personal communication), however, observed that the growth rate of *Bd.* W is very low when the host cells (*Rhodospirillum rubrum*) were washed, suspended in buffer, and infected with the *Bdellovibrio*; the rates of growth and infection are increased somewhat by addition to the buffered cell suspension of a carbon source, but much more so by ammonium chloride.

Many factors affect the development of *Bdellovibrio* and, consequently, lysis of the host cell. These would have a bearing, too, on host specificity. These factors include: number (Stolp and Starr, 1963b), age (Wood and Hirsch, 1966), and metabolic activity of the host cell (Stolp and Starr, 1965); accumulation of metabolic products of the host; composition of the medium including the presence of Ca^{2+} and/or Mg^{2+} (Robinson and Huang, 1967; Seidler and Starr, 1969a; Crothers *et al.*, 1970; Crothers and Robinson, 1971; Huang and Starr, 1971a; see also Section IV.E, p. 249). The efficiency of plaque formation is affected also by the host density on the lawn, water content and thickness of the semi-solid agar top layer, incubation temperature, and humidity of the incubator (Uematsu *et al.*, 1971). Burger *et al.* (1968) expressed host specificity in terms of plaquing efficiency. The plaquing efficiency of their *Bd.* W varied from 0·00001 with *Serratia marcescens* to 10 with *Proteus vulgaris*; the plaquing efficiency with *Rhodospirillum rubrum* was arbitrarily designated as unity.

To facilitate plaque counting, 0·1% (w/v), 2,3,5-triphenyltetrazolium chloride has been incorporated into media (Seidler and Starr, 1969a; Jackson, 1967). J. C.-C. Huang and M. P. Starr (unpublished data) found that addition of skim milk (2%) to the semi-solid agar also increased the contrast for plaque counting.

Despite the undeniable relevance of host specificity to an understanding of the *Bdellovibrio*-host symbiosis, very little systematic work has been done on this subject. Twelve strains of bdellovibrios could be divided into five groups on the basis of host specificity (Stolp and Starr,

1963b). Of 120 strains of pseudomonads tested, 23% were unaffected by *Bd.* 321 (Stolp and Petzold, 1962). *Bdellovibrio* N-6801, *Bd.* N-6804, and *Bd.* N-6805 could attack about 50%, while *Bd.* N-6802 and *Bd.* N-6806 could attack only about 5%, of the 42 strains of *Pseudomonas* tested (Uematsu and Wakimoto, 1970). *Xanthomonas campestris* and *X. cucurbitae* are resistant to all strains of *Bdellovibrio* isolated by these workers, and *Bd.* N-6802 and *Bd.* N-6806 could not attack *Erwinia* sp., *E. coli*, or *Aerobacter* sp.

Bdellovibrios which parasitize *Azotobacter* and *Rhizobium* were sought but at first not found (Stolp and Starr, 1963b; Postgate, 1967). However, a *Bdellovibrio* isolated by using *E. coli* as the host can parasitize *Azotobacter chroococcum*, but not *Azotobacter vinelandii* or *Rhizobium* species (Sullivan and Casida, 1968). Recently, Parker and Grove (1970) isolated, from Western Australian soils, strains of *Bdellovibrio*, which are capable of parasitizing *Rhizobium meliloti*, *R. trifolii*, *Agrobacterium tumefaciens*, and *A. radiobacter*.

Using an agar-block transfer method, Gillis and Nakamura (1970) studied the susceptibility of *Shigella boydii*, *S. flexneri*, and *S. sonnei* to parasitization by bdellovibrios isolated from sewage. An agar block (one cm. square) is cut from the centre of the *Bdellovibrio* plaque on one host lawn and transferred to another. The agar block, according to these authors, provided little or no carry-over of the first host, and formed a clear zone on lawns of the latter. All *Shigella* species tested were susceptible to these bdellovibrios. J. R. Gillis and K. Nakamura (personal communication) injected *Bd.* 109 into a rabbit eye infected with *Shigella flexneri* and found that the *Bdellovibrio* partially protected the eye from *Shigella* keratoconjunctivitis.

The presently known bdellovibrios usually attack only Gram-negative bacteria. The existence of bdellovibrios capable of attacking Gram-positive bacteria was expected by Stolp and Starr (1963b), even though these workers did not find any such strain. Guélin *et al.* (1968c, 1969b, c) found that bacterial parasite Xpfr (which they at first claimed was a *Bdellovibrio* species) could parasitize the Gram-positive bacteria, *Clostridium perfringens* and *C. histolyticum*. There is no evidence in any of their papers that this bacterial parasite (Xpfr) actually enters the clostridial cells and there is some question as to its being a *Bdellovibrio* species (A. Guélin, personal communication; Starr and Seidler, 1971). Burger *et al.* (1968) isolated *Bd.* W which they claimed could parasitize, in addition to some Gram-negative bacteria (including *Agrobacterium tumefaciens*), the Gram-positive *Streptococcus faecalis* and *Lactobacillus plantarum*. *Bdellovibrio* W was reported not able to parasitize *Pseudomonas aeruginosa* or *Spirillum serpens*. We have found that *Bd.* W multiplies in *Rhodospirillum rubrum* and *Spirillum serpens*

VHL, but that it does not parasitize or lyse available strains of one of the alleged Gram-positive hosts, *Streptococcus faecalis* (we have not tried *L. plantarum*). The reasons for the discrepant findings are presently unknown (see Section IV.A.1, p. 234 for comments about *Bd.* W and *Spirillum serpens* wall structure).

It is interesting to note that *Bdellovibrio* (or organisms which have some possibly superficial resemblance to *Bdellovibrio*) have been reported to parasitize, beside the bacteria referred to above, *Hyphomicrobium* (Wood and Hirsch, 1966), *Cytophaga* (Much, 1965), and *Chlorella vulgaris* (Mamkaeva, 1966; Gromov and Mamkaeva, 1966, 1971). Comments on these findings will be found in Starr and Seidler (1971).

The ability of *Bd.* 6-5-S to infect mice, fertilized eggs, and a variety of tissue cultures has been tested (F. J. Simpson, personal communication). The results show that the *Bdellovibrio* does not attack any of the mammalian cells tested with the exception of one egg which was believed to be infected with a Gram-negative bacterium.

V. Metabolism of *Bdellovibrio*

Since host-dependent bdellovibrios do not grow on any as yet defined medium, studies of their metabolism and nutrition have been possible only in the presence of the living or dead host cells. Somewhat more has been achieved in studying the metabolism of host-independent strains, although their precise nutritional requirements are also not yet known.

That *Bdellovibrio* grows only aerobically has been noted by Stolp and Starr (1963b), Simpson and Robinson (1968), and others. Burger *et al.* (1968) observed that *Bd.* W grew and infected the host cell only under oxygen partial pressures of 4–5 mm. Hg and higher.

Freshly harvested *Bd.* 6-5-S has an endogenous respiration rate of 20×10^{-12} μl. O_2/cell/hr. at 35° (Simpson and Robinson, 1968). Thirty-five per cent of this respiration rate was lost when *Bd.* 6-5-S was stored in tris-HCl buffer (2°, 4 days). Potassium cyanide and sodium azide did, but carbon monoxide did not, inhibit the endogenous respiration of *Bd.* 6-5-S.

Under their experimental conditions, Simpson and Robinson (1968) found that *Spirillum serpens* started lysing at 120 min. after *Bd.* 6-5-S and host cells were mixed. The lysis, which was completed at 200 min., was accompanied by a large increase in total respiration, and this increase continued after the host was completely disintegrated. They believe that the material released from the host likely supported respiration, since the consumption of oxygen was too great to be accounted for by endogenous respiration of the *Bdellovibrio* alone. Asparagine and

glutamate, and especially glutamine, stimulate respiration. Rittenberg and Shilo (1970) reported that peptone, yeast extract, or casein hydrolysate increased the respiration rate of another strain.

According to Simpson and Robinson (1968), the particulate fraction of extracts of *Bd.* 6-5-S contains cytochromes *a* (absorption peak at 605 mm.), a_3 (445 nm.), *b* (559, 528, and 426 nm.), and *c* (556–552, 526–522, and 424–422 nm.). Seidler and Starr (1969b) showed that 16 strains of host-independent *Bdellovibrio* contain cytochrome *c* (522–524 nm.). Two patterns were found among the host-independent bdellovibrios at the absorption peaks corresponding to cytochrome *c* and the Soret region; host-independent *Bd.* A3.12 has peaks at 607 nm. and 417 nm., and the rest of these bdellovibrios have peaks at 596–600 nm. and 421–423 nm. (Seidler and Starr, 1969b). *Bdellovibrio* 6-5-S possesses cytochrome oxidase, as determined with N,N′-dimethyl-*p*-phenylene diamine. A cell-free extract of *Bd.* 6-5-S contains $NADH_2$ and $NADPH_2$ oxidases; the rate of oxidation was twice as high with $NADH_2$ than with $NADPH_2$; the oxidation of $NADH_2$ by *Bd.* 6-5-S extracts was markedly inhibited by potassium cyanide and sodium azide, but rotenone or a 4:1 carbon monoxide-oxygen gas mixture did not inhibit it; the oxidation of $NADPH_2$ was not inhibited by any of these reagents; all of the $NADPH_2$ oxidase and 10% of the $NADH_2$ oxidase were found in the soluble fraction (Simpson and Robinson, 1968).

Polarographic studies showed that glutamate, α-ketoglutarate, succinate, fumarate, malate, pyruvate, acetate, α-glycerophosphate, β-hydroxybutyrate, NAD, or NADP could not support consumption of oxygen by the crude *Bd.* 6-5-S cell extracts; $NADH_2$ and $NADPH_2$, however, did support it (Simpson and Robinson, 1968). Cell extracts of *Bd.* 6-5-S contained ATPase which mostly resided in the particulate fraction. The extracts were also capable of catalysing an ATP $\leftrightarrows$ $^{32}P_i$ exchange. Since arsenate, azide, and 2,4-dinitrophenol did not inhibit this exchange, the process is probably catalysed by reactions other than those associated with oxidative phosphorylation. The cell extracts possibly contain two systems for oxidizing $NADH_2$; one is stimulated by flavins (FAD or FMN), is insensitive to potassium cyanide and leads to the production of hydrogen peroxide; the other is sensitive to potassium cyanide, and likely leads to the production of hydrogen peroxide *via* the cytochromes (Simpson and Robinson, 1968). Some strains of *Bdellovibrio* are rich in catalase (Simpson and Robinson, 1968; Seidler and Starr, 1969b; Diedrich *et al.*, 1969, 1970), while others lack it (Stolp and Starr, 1963b; Seidler and Starr, 1969b).

Bdellovibrio 6-5-S apparently contains the enzymes of the tricarboxylic acid cycle in soluble from and succinate dehydrogenase in bound

form. Six enzymes of the cycle (isocitrate dehydrogenase, α-ketoglutarate dehydrogenase, succinyl-CoA synthetase, succinatedc hydrogenase, fumarate hydratase, and malate dehydrogenase) were found to be present. The absence of a glyoxylate cycle and the inability to utilize carbohydrates, alcohols, and similar sources of carbon and energy suggests that *Bdellovibrio* might be dependent on proteins, peptides, amino acids, and nucleic acids (Simpson and Robinson, 1968). Mitchell *et al.* (1967), on the other hand, report that a marine *Bdellovibrio* was capable of utilizing cell walls of *E. coli* as a sole source of organic carbon.

Seidler *et al.* (1972) identified, from seven strains of host-independent bdellovibrios, four dehydrogenases (alanine, glutamate, malate, isocitrate), fumarate hydratase, adenylate kinase, quinone reductase, and tetrazolium oxidase. By comparison of thin-layer electrophoretic migration rates through starch gels, these authors were able to classify these seven strains of *Bdellovibrio* into five distinctive zymogen groups with important taxonomic utility (Seidler *et al.*, 1972; Starr and Seidler, 1971).

Wehr and Klein (1971) used the disc-assay technique to test the activity of commercial herbicides against a *Bdellovibrio* strain (not specified) and its pseudomonad host. The sensitivity of the *Bdellovibrio* to the herbicides was measured: (1) by growth inhibition of the *Bdellovibrio* plaque obtained by placing tangentially to a plaque edge (six days old) a herbicide test disc and a control disc and incubating further for 4–14 days at 27°; and (2) by enumerating the decrease of plaque-forming units in the host-*Bdellovibrio* cultures containing a graded concentration of herbicide. Of the 17 herbicides tested, 11 including urea, carbamate bromacil, and three phenoxyacetic acid herbicides inhibited plaque formation of the *Bdellovibrio* on lawns of *Pseudomonas* sp., while only 4,6-dinitro-O-*sec*-butylphenol inhibited the pseudomonad host. Linuron [3-(3,4-dichlorophenyl)-1-methoxy-1-methylurea] decreased the net multiplication of *Bdellovibrio*. Wehr and Klein (1971) showed that free *Bdellovibrio* cells, in the absence of host cells, were sensitive to linuron thus indicating to them "that linuron primarily increased the death rate rather than influencing only the secondary characteristics of motility, attachment and penetration".

VI. Host-Independent Derivatives of *Bdellovibrio*

Stolp and Petzold (1962) and Stolp and Starr (1963b) selected host-independent derivatives from cultures of host-dependent bdellovibrios. These host-independent derivatives were unable to lyse living bacteria (Stolp and Starr, 1963b). They were generally less homogeneous in

appearance than the parental type. Although they usually have a single sheathed flagellum, one strain has been reported to have as many as three flagella at either one or both ends (Stolp and Petzold, 1962; Stolp, 1968).

The frequency of occurrence of host-independent bdellovibrios in host-dependent populations was at first reported to vary from strain to strain. Values of $1:10^6$ to $1:10^9$ have been quoted (Stolp and Starr, 1963b; Stolp, 1967c, 1968) but most workers now agree that it is about $1:10^6$ in many strains (Seidler and Starr, 1969b). The reversion of host-independent populations to host-dependent populations occurs at about the same frequency when host-independent bdellovibrios are massively inoculated into congenial host cultures. Whether the shift is genetic or environmental or both is not yet conclusively known, nor is it clear whether individual cells (as opposed to populations) are both independent and dependent on a host. These and related questions are considered in more detail by Starr and Seidler (1971).

Three methods have been used for isolating host-independent derivatives. The first method (Stolp and Starr, 1963b) involves the inoculation of a concentrated suspension of host-dependent cells (over 10^9 cells/ml.) on to a yeast-peptone agar plate or into nutrient broth. The second method (Shilo and Bruff, 1965) uses an enrichment technique. Host-independent derivatives are concentrated by the successive addition of host cells and host-dependent *Bdellovibrio* cells, and the host cells plus the *Bdellovibrio* cells (those attached to the host cell) are separated from the independent derivatives by differential filtration. The host-independent cells in the filtrate are transferred to full strength or one-tenth strength nutrient both containing heat-killed (120°, 15 min.) host cells (10^9/ml.) and shaken at 28°. The third method (Seidler and Starr, 1969b) consists first in selecting streptomycin-resistant (Sm^r) host-dependent *Bdellovibrio* clones on Sm^r host cells. Streptomycin-resistant host-independent bdellovibrios develop spontaneously in the two-membered culture. Then, the Sm^r host-dependent *Bdellovibrio* culture is grown on streptomycin-sensitive (Sm^s) host cells. A lysate containing large numbers of Sm^r host-dependent *Bdellovibrio* cells and some remaining Sm^s host cells, as well as Sm^r host-independent *Bdellovibrio* cells, is transferred to a nutrient selection medium which contains streptomycin. The Sm^s host cells in the lysate are killed, the Sm^r host-dependent *Bdellovibrio* cells cannot grow under these conditions in the absence of viable host cells, and the Sm^r host-independent *Bdellovibrio* clones develop.

Whether the host-independent strains of *Bdellovibrio* isolated by these several methods are genetically and physiologically identical remains to be investigated. A still somewhat sketchy assemblage of the bacteriological traits of the host-independent bdellovibrios will be found in the

review by Starr and Seidler (1971); much remains to be done in depicting these organisms completely.

A facultatively parasitic strain of *Bdellovibrio* (strain UKi2) has been selected from obligately parasitic *Bd.* UK, using *E. coli* B/r as a host bacterium (Burnham *et al.*, 1969, 1970; Diedrich *et al.*, 1969, 1970). The cells are 0·8–1·2 μm. in length and 0·3–0·4 μm. in width; they are motile, curved rods each with a single polar flagellum. Long spirals (up to 50 μm.) are regularly observed. The growth curve in yeast peptone broth has a lag period of 6 hr. During this period of time, the comma-shaped cell develops into spirals, then 7–10 vibrioid cells are produced from each spiral cell. The host-independent phase in this exceptional strain involves thickening, coiling, and fragmenting into new progeny, quite similar to the parasitic intracellular phase which has been observed in most *Bdellovibrio* strains. Facultatively parasitic *Bd.* UKi2 produces a whitish-grey colony surrounded by a circular plaque-like clearing zone, a trait which enabled Diedrich *et al.* (1969, 1970) to differentiate it from the obligate parasite which forms a clear plaque. This facultatively parasitic strain has physiological characteristics very similar to those of host-independent strains. Proteases are produced in the supernatant of yeast-peptone broth culture which lyse autoclaved *E. coli* B/r cells and degrade Azocoll. Further remarks about this very interesting *Bd.* UKi2 strain and about facultative parasitism in *Bdellovibrio* will be found in Starr and Seidler (1971).

VII. *Bdellovibrio* Bacteriophages

Hashimoto *et al.* (1970) isolated from sewage a hexagonal, tail-less phage (HDC-1) which is infective for facultatively parasitic *Bd.* UKi2. The phage has a size of between 60 and 70 nm. and appears in preparations stained with 0·5% uranyl acetate to be composed of two distinct coats (capsomer layers). Acridine orange staining followed by molybdic acid treatment revealed that the phage contains single-stranded DNA. *Bdellovibrio* bacteriophage HDC-1 infects and lyses *Bd.* UKi2 specifically and yields 5×10^{10} phage particles per ml. Strain HDC-1 is relatively stable to heat (99% inactivation at 60°, 2 hr.), osmotic shock, storage, and over a wide range of pH 2 to 10·5 (Diedrich *et al.*, 1971).

Twelve additional phages for host-independent bdellovibrios were isolated from raw sewage in Kentucky (Althauser and Conti, 1971). Negative stains of these phages show three distinctive morphological types, designated B, C, and E, according to Bradley's nomenclature. A positive correlation between the three morphological types and the host range was observed when the 13 phages were tested against 19 strains of host-independent bdellovibrios.

The 13 strains of bdellovibrio phages were further classified into five groups according to their host specificity: (1) MAC-9 and MAC-6 lyse only host-independent *Bd.* A3.12 and *Bd.* OX9-2; (2) HDC-1 and HDC-2 attack only *Bd.* UKi2; (3) MAC-7 attacks host-independent *Bd.* B and H-I *Bd.* E; (4) the remaining seven strains have a broad host range, encompassing most available *Bdellovibrio* strains; and (5) MAC-3, the only phage that attacks host-dependent *Bd.* 120, has the same wide host range as (4) except that it does not lyse host-independent *Bd.* Xty. Some of the bdellovibrio phages attack parasitic strains of bdellovibrios in three-membered (bdellovibrio, host, and phage) systems, a very interesting example of hyperparasitism.

The analysed correlations among phage typing (as studied by Conti and his collaborators), base composition of *Bdellovibrio* DNA (Seidler *et al.*, 1969), zymogen analysis (Seidler *et al.*, 1972), molecular heterogeneity in terms of DNA-DNA or RNA-DNA hybridization (Seidler and Mandel, 1971; Seidler *et al.*, 1972), host specificity, and other traits of *Bdellovibrio* are eagerly awaited, particularly for the clarification of important issues in the taxonomy and parasitism of *Bdellovibrio* which these should provide.

VIII. Acknowledgements

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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

Abram, D., 220, 222, 223, 224, 232, 233, 236, 237, 238, 239, *252*
Adams, G. A., 12, 14, 27, *54*
Adams, M. H., 159, 181, 184, *208*, 218, *257*
Adelberg, E. A., 49, *57*
Afanas'eva, T. P., 178, *213*
Aguirre, M. J. R., 242, *257*
Ailhaud, G., 29, 30, *57*
Ailhaud, G. P., 30, *51*, *52*
Akagi, J. M., 81, *99*
Akamatsu, Y., 28, 40, *54*
Akashi, S., 18, *51*
Alberts, A. W., 19, 22, *51*, *55*, *56*
Albertsson, P. A., 108, 109, *137*, *140*, *141*
Albro, P. W., 5, 7, 8, 10, 11, 18, 37, 38, 39, 40, 41, 43, 46, *51*, *53*, *56*
Aldelman, S. L., 131, *137*
Aleem, M. I. H., 7, 37, *57*
Alexander, M., 113, 130, 135, *138*, *139*, 242, *259*
Alkamatsu, Y., *51*
Allen, A. D., 93, *98*
Allen, C. M., 34, *51*
Allison, M. J., 3, 4, 21, *51*
Alper, R., *55*
Althauser, M., 256, *257*
Alworth, W., 34, *51*
Amaral, D., 151, *208*
Amemiya, K., 238, *257*
Ames, G. F., 38, *51*
Anderson, E. S., 167, 169, 192, *208*
Anderson, J. S., *208*
Anderson, R. F., 176, *209*
Arnon, D. I., 65, 73, 74, 76, 77, 79, 80, 81, 82, 83, 84, 85, 86, *98*, *99*, *103*, *104*
Arsenio, C., 151, *212*
Artom, C., 7, 31, *51*
Ashcroft, J., 129, *137*
Aspinall, G. O., 146, 175, *208*, *213*
Asselineau, J., 1, 14, 15, 17, 27, 35, 42, 47, 48, 49, *51*, *52*, *55*, *57*
Austrian, R., 150, 185, 190, 201, *208*, *212*
Avegno, P., 116, *139*
Avery, O. T., 158, 181, *208*, *209*
Avi-Dor, Y., 132, *139*
Azorad, R., 28, *53*

B

Bachofen, R., 82, *98*
Bacon, J. S. D., 242, *259*
Baddiley, J., 13, 14, 16, 43, 46, *51*, *57*, *58*, 182, 183, 184, *211*, *212*
Baigent, N. L., 222, 224, 229, 231, 232, 233, 234, 237, 238, 242, *261*
Bakal, M., 67, *101*
Balekjian, A. Y., 131, *138*
Ballou, C. E., 43, 49, *51*, *56*
Bamburg, J. R., 175, *209*
Baptist, J. N., 257, *260*
Barber, C. A., 189, *208*
Barber, G. A., *208*
Barclay, K. S., 167, *208*
Barer, R., 132, *137*
Barker, D. C., 188, *208*
Barker, S. A., 145, 157, 159, 172, 173, 181, 182, 184, *208*, *209*, *212*
Barker, T., 161, *209*
Barnett, E. V., 115, *137*
Barnett, H. L., 217, *257*
Barney, J. A., 116, *140*
Barnick, J. A., 108, *139*
Baronowsky, P., 22, *51*
Baronowsky, P. E., 23, 24, 25, *57*
Barridge, J. K., 7, 36, 37, *51*
Bartell, P. F., 160, *209*
Baumann, N. A., 7, 11, 12, 28, 33, 44, 46, 48, *51*
Becking, J. H., 60, 61, 64, *98*
Beijerinck, M. W., 60, 61, *98*
Beinert, H., 81, 84, *103*
Becker, G. E., 159, *209*
Beiser, S. M., 169, *209*
Belozerskii, A. N., 178, *213*

Benemann, J. R., 60, 65, 68, 73, 74, 75, 77, 79, 80, 81, 82, 84, 85, 86, 94, 96, *98*, *102*, *104*
Bennett, R., 77, *98*
Benson, A. A., 6, 8, 37, 40, 41, *51*, *57*
Berger, L. R., 7, 15, 37, *56*
Bergerson, F. J., 60, 61, 66, 67, 68, 69, 97, *104*
Bergerson, F. J., 62, 66, *98*
Bergelson, L. D., 9, *56*
Bernhard, K., 23, *51*
Berliner, E., 114, *138*
Bertsch, L. L., 41, 44, 48, *51*
Bernheimer, H. P., 150, 185, 190, 201, 202, *208*, *209*, *212*
Bernstein, R. L., 201, *209*
Beutner, E. H., 115, *137*
Bhat, J. V., 220, 221, *258*
Bick, S. M., 182, 184, *208*, *209*
Bier, M., 108, *137*
Biggins, D. R., 66, 67, 91, 92, *98*
Bishop, D. G., 25, 27, 44, *51*
Bister, F., 150, *213*
Björndal, H., 157, *209*
Blank, M. L., 5, 33, *53*, *58*
Bloch, K., 2, 17, 18, 20, 22, 23, 24, 25, 27, 28, 30, 34, 42, *51*, *52*, *54*, *55*, *56*, *57*
Bloch, W., 25, *52*
Blomstrom, D. C., 83, *98*
Blume, P., 112, *137*
Bocci, V., 128, *137*
Bolduan, O. E. A., 106, 121, 122, *140*
Bond, C., 60, *98*
Bonner, H., Jr., 29, *56*
Bonsen, P. P. M., 9, 32, 41, 44, 48, *51*, *53*, *55*, *56*
Bourne, E. J., 167, *208*
Bowman, R. L., 112, *137*
Bloomfield, D. K., 22, *51*
Bothe, H., 78, 82, 84, *98*, *100*, *103*
Bottomley, F., 93, *98*
Bradbeer, C., 64, *98*
Bradfield, J. R. G., 132, *141*
Bradford, L. L., 115, *138*
Brady, R. O., 19, *51*
Brashear, D. A., 108, *139*
Braun, W., 233, *259*
Breed, R. S., 6, 35, *51*
Bregoff, H. M., 68, *100*
Bremer, J., 7, 31, *51*
Brennan, P. J., 43, 49, *51*
Brewer, A. K., *137*
Brezonik, P. L., 96, *98*
Brien, R. T., 130, *137*
Brierley, G., 8, *52*
Brighton, W. D., 115, *137*
Brill, W. J., 96, *100*, *103*
Brimacombe, J. S., 182, 184, *208*, *209*
Brindley, D. N., 20, *51*
Brintzinger, H., 92, *98*
Brown, A. D., 242, *257*
Brown, J. P., 27, *55*
Brown, R., 156, *210*
Bruckner, G. C., 108, *137*
Bruff, B., 244, 247, 250, 255, *260*
Bruff, B. S., 232, 246, 247, 250, *257*
Brundish, D. E., 14, 43, 46, *51*
Bryant, M. P., 3, 4, 21, *51*
Buchanan, B., 80, *101*
Buchanan, B. B., 77, 80, 81, 82, 83, *98*, *99*, *100*
Buchanan, J. G., 182, 183, 184, *211*, *212*
Buckmire, F. L. A., 234, *258*
Bui, P. T., 92, *99*
Bullen, W. A., 64, 65, 66, 67, 68, 69, 76, 84, 88, 89, 90, 92, 93, 95, *99*, *100*, *102*
Bunn, C. R., 39, *51*
Bunt, J. R., 96, *99*
Burger, A., 219, 223, 226, 234, 237, 238, 247, 250, 251, 252, *258*
Burnham, J. C., 36, *57*, 222, 223, 224, 232, 236, 237, 256, *258*
Burns, R. C., 60, 64, 65, 66, 67, 68, 76, 88, 90, 92, 93, 94, 95, 96, *99*, *100*, *101*
Burris, R. H., 60, 66, 67, 68, 69, 71, 76, 88, 89, 90, 91, 92, 93, *99*, *100*, *101*, *102*, *103*, *104*
Burton, A. J., 3, 16, 17, *51*
Burton, P., 82, *99*
Busse, D., 7, 38, 42, *57*
Butterworth, P. H. W., 22, *52*

C

Cabioch, L., 221, *258*
Cadmus, M. C., 177, *209*
Caeseele, L., 39, *52*
Calderazzo, F., 93, *99*
Cammack, R., 83, *100*
Campbell, G. K., 244, *258*
Campbell, J. H., 158, *209*
Cantor, C. R., 83, *102*
Caputto, R., 185, *209*
Card, G. L., 44, *52*
Cardini, C. E., 185, *209*
Carlson, D. M., 180, *209*
Carmichael, L. E., 113, 135, *139*
Carnahan, J. E., 64, 65, 69, 70, 71, 80, 88, *99*, *102*
Carroll, K. K., 44, *52*
Carroll, T. W., 224, 229, 232, 236, 237, 243, *260*
Carroll, W. R., 184, *212*

Carter, G. B., 132, 133, *137*
Carter, H. E., 16, 18, *51*, *52*
Carter, H. P., 242, *258*
Carter, J. R., 3, 17, 30, *52*
Carter, J. R., Jr., 30, *52*
Casida, L. E., Jr., 219, 220, 221, 232, 233, 250, 251, *259*, *261*
Castle, J. E., 64, 68, 70, 71, 88, *99*
Cato, E. P., 113, 130, *139*
Cavanaugh, L. A., 122, *141*
Chakrabarty, A. M., 160, *209*
Chang, Y.-Y., 8, 30, 31, *52*, *57*
Chatt, J., 93, *99*
Cholodny, N., 123, *138*
Chou, D., 236, *257*
Christenson, J. G., 187, *209*
Chung, A. E., 26, *52*, 74, *99*
Churcher, G., 116, *140*
Clark, S. P., 115, *139*
Claus, D., 178, *209*
Cliver, D. O., 108, 109, *138*
Closs, O., 126, 127, *138*
Coffey, E. M., 115, *138*
Cohen, G. H., 178, 179, *209*
Cohen-Bazire, G., 68, *102*
Collins, V. G., 122, *138*
Colvin, J. R., 189, 192, *211*
Conklin, C. J., 118, 119, *140*
Conrad, H. E., 148, 157, 173, 175, 195, *209*, *210*, *211*, *212*
Constantopoulos, G., 42, *52*
Conti, S. F., 36, *57*, 222, 224, 228, 232, 236, 237, 238, 244, 253, 256, *257*, *258*, *259*, *261*
Cook, T., 15, *53*
Cooke, K. O., 117, *138*
Cooksey, K. E., 96, *99*
Coons, A. H., 114, *138*
Cooper, E. A., 167, *209*
Cooper, F. C., 108, *137*
Corbin, J. L., 93, *102*
Corpe, W. A., 147, 148, *209*
Cota-Robles, E. H., 76, *99*
Cottral, G. E., 118, *139*
Coty, V. F., 61, *99*
Courtney, R. J., 116, 133, *140*
Cox, R. M., 68, 77, 78, *99*
Creech, H. J., 114, *138*
Cronan, J. E., Jr., 8, 27, 29, *52*, *56*
Croson, M., 19, *55*
Crothers, S. F., 241, 247, 248, 250, *258*
Cruickshank, R., 146, *209*
Crumpton, J. E., 131, 132, *140*
Crumpton, M. J., 175, *209*
Currie, J. F., 125, 126, *139*
Cutts, J. H., 44, *52*
Cymbalista, S., 108, *140*

D

Daesch, G., 68, 69, *99*
Dahlen, J. V., 91, *99*
Dalton, H., 66, 67, 69, 94, *99*, *104*
Daniel, J. J., 7, 31, *55*
Daniel, R. M., 75, *104*
Danielsson, D., 115, 126, 127, *138*
Dankert, M., 15, 34, *58*, 192, 194, *209*, *213*
Dark, F. A., 110, 121, *140*
Dauchy, S., 27, *52*
Davidson, J. B., 31, *52*
Davies, D. A. L., 175, *209*
Davis, B. D., 169, *209*
Davis, B. K., 220, 223, 224, 233, 237, *257*
Davis, J. B., 61, *99*
Dawes, E. A., 19, *52*
Dawson, M. D., 97, *103*
Dawson, R. C., 97, *99*
Dawson, R. M. C., 35, *52*
Day, J. I. E., 27, 28, 33, *52*
Decker, K., 74, *101*, *103*
Dedonder, R. A., 190, *209*
Deibel, R., 132, *138*
Deland, F. H., 125
Delaney, R., 19, *57*
Delwiche, C. C., 97, *99*
Denisov, N., 94, *103*
Dennis, S. C., 220, *259*
Denny, C. F., 233, 244, 253, 256, *258*
Depinto, J. A., 15, *52*
Der Vartanian, D. V., 81, *103*
Detroy, R. W., 89, 91, 95, *99*
D'Eustachio, A. J., 64, 65, 72, 75, 80, 83, 93, *99*, *100*, *101*
Devanathan, T., 66, 81, 90, *99*, *101*
De Vay, J. E., 224, 229, 232, 236, 237, 243, *260*
Dias, F. F., 220, 221, *258*
Dicks, J. W., 147, *209*
Diedrich, D. L., 233, 244, 253, 256, *258*, *259*
Diena, B. B., 132, *138*
Dilworth, J. R., 93, *99*
Dilworth, M. J., 60, 66, 71, *99*, *100*, *102*
Dinglinger, F., 43, 49, *57*
Distler, J., 34, *54*
Dittmer, J. C., 5, 7, 8, 10, 11, 18, 35, 37, 38, 39, 40, 41, 46, *51*, *56*, *58*
Doomony, P., 93, *103*
Doi, R. H., 220, *261*
Dollhope, F. L., 115, *139*
Domagk, G. F., 151, *209*
Dorfman, A., 159, *211*
Doty, P., 227, *259*
Doudoroff, M., 46, 49, *51*, *57*, 151, *211*

Drews, G., 223, 226, 234, 237, 238, 247, 250, 251, 252, *258*
Drozd, J., 66, 67, *100*
Drucker, E., 239, *258*
Drucker, I., 240, 241, *261*
Druett, H. A., 108, *139*
Dua, R. D., 88, *100*
Dubos, R., 158, *209*
Dubourdieu, O., 79, 84, *100*
Duckworth, D. H., 176, *213*
Dudman, W. F., 146, 147, 148, 158, 173, 174, 175, *209*, *210*, *213*
Dugan, P. R., 96, *101*
Duguid, J. P., 145, 147, 194, *209*, *213*
Dum, J. H., 67, *100*

E

Ecker, R. E., 123, *138*
Edenharder, Q., 85, *101*
Edmunds, P. N., 147, 194, *213*
Eggebraten, L., 45, *53*
Efimov, O., 94, *103*
Eggleston, L. V., 73, *104*
Eidus, L., 132, *138*
Eisenstein, K. K., 82, *100*
Eklund, C., 159, 160, 161, *209*
Elbein, A. D., 189, *208*
Elgart, R. L., 118, *140*
Elkan, G. H., 39, *51*
Ellis, M. E., 7, 32, 39, 47, 48, *52*
El Nakhal, H., 124, *141*
Epley, J. D., 148, 175, 195, *209*, *211*
Eriksom, J., *209*
Eriksen, J., 171, 173, *209*, *210*
Eriksen, J. L., *208*
Erlich, R., 123, *138*
Estrada-Parra, S., 156, 183, 184, *209*, *210*
Estroumza, J., 30, *57*
Evans, E. E., 116, *138*
Evans, H. C., 82, *99*
Evans, H. J., 64, 75, 89, 90, 95, 97, *101*, *103*
Evans, M. C. W., 64, 68, 77, 80, 82, 91, 95, *98*, *100*, *103*
Exterkate, F. A., 9, 43, 47, *52*

F

Fattal, B., 108, *140*
Fackrell, H. B., 244, 247, 250, *258*
Farmer, V. C., 242, *259*
Farr, A. L., 228, *259*
Fay, P., 67, 68, 76, 77, 78, *99*, *100*
Federov, M. V., 62, *100*
Felton, F. G., 116, *140*
Fennessey, P., 15, 34, *58*
Ferrari, A., 112, 132, *138*
Fey, H., 127, *139*
Fikhman, B. A., 132, *138*
Finnerty, W. R., 30, 31, 32, 43, 46, *52*, *55*, *56*
Fisher, R. J., 67, *102*
Fischer, R. J., 72, 96, *100*
Fischer, W., 45, *52*
Fitzgerald, G. P., 67, 68, *103*
Fleischer, S., 8, *52*
Fogg, G. E., 67, 76, 77, 96, *100*, *101*
Forsyth, W. G. C., 19, *53*
Foster, A. B., 172, *208*
Foster, E. M., 3, 4, 12, 21, *58*
Foust, G. P., 82, 84, *100*, *102*
Fraenkel, A. W., 76, *102*
Francis, M. J. O., 9, *56*
Franzen, J. S., 74, *99*
Frea, J. I., 96, *101*
Freedman, H. H., 233, *259*
Frerman, F. E., 16, 34, *57*, 176, 187, 191, 192, 193, 194, *213*
Frick, G., 108, 109, *140*
Fuchsman, W. H., 94, *99*
Fulco, A. J., 23, 25, *52*
Fuller, C. E., 130, *138*
Fuller, R. C., 77, *98*
Furukawa, Y., 44, *58*

G

Gabe, D. R., 218, *260*
Gaby, W. L., 39, *57*
Gahan, L. C., 173, *210*
Galanos, C., 152, 158, *210*, *213*
Gancedo, C., 151, *212*
Garcia-Acha, I., 242, *257*
Garcia-Rivera, J., *99*
Garegg, P. J., 155, 162, 171, 172, 188, 199, *210*
Garner, W., 110, 131, *138*
Gärtner, H., 108, *138*
Gasdorf, H., 176, *209*
Gennaro, R. M., 110, 131, *138*
Georgi, C. E., 44, *52*
Gerke, J. R., 112, *138*
Gest, H., 36, *57*, 61, 68, 73, 76, *100*, *101*, *102*
Ghalambor, M. A., 176, *213*
Ghuysen, J. M., 223, 228, 242, *258*, *261*
Gibbons, N. E., 4, 12, 14, 19, *54*, *57*
Gibson, K. D., 7, 31, *52*
Gilliland, R. B., 131, *138*
Gillis, J. R., 247, 251, *258*
Ginsburg, V., 185, 187, 201, *210*, *211*
Glaser, D. A., 112, *138*

Glaser, L., 189, *210*
Glaudemans, C. P. J., 184, *212*
Goebel, W. F., 166, 167, 169, 170, 181, 184, *208*, *210*, *212*
Goldblum, N., 108, *140*
Goldenson, J., 118, *138*
Goldfine, H., 3, 4, 7, 11, 12, 17, 18, 22, 23, 24, 25, 26, 27, 28, 29, 30, 32, 33, 34, 36, 37, 39, 44, 47, 48, *51*, *52*, *53*, *57*, *58*
Goldman, M., 114, 117, *138*
Gomes, N. F., 48, *52*
Gorchein, A., 7, 14, 32, 36, *52*
Gordon, I., 132, *140*
Gorin, P. A. J., 167, 179, *210*
Gottschlich, E. C., 183, *210*
Gough, D. P., 34, *53*
Gould, J. R., 113, 130, *138*
Gould, R. M., 33, 43, 46, *53*
Grant, W. D., 167, 170, 203, *210*
Gray, G. M., 27, *53*
Gray, G. W., 11, *53*
Green, M., 76, *100*
Greenberg, D. M., 7, 31, *51*
Greenburg, L., 132, *138*
Greenwood, F. C., 128, *139*
Griffith, F., 181, *210*
Grindey, G. B., 43, *53*
Gromov, B. V., 218, 252, *258*
Gross, S. K., 187, *209*
Grove, P. L., 220, 221, 251, *260*
Guélin, A., 220, 221, 236, 237, 242, 251, *258*, *259*
Gunsalus, I. C., 160, *209*
Gurney, E., 96, 97, *103*
Gurr, M. I., 9, *53*
Guthrie, R. K., 115, 127, *138*
Guze, L. B., 242, *259*
Guy, R. G. E., 150, 181, *210*

H

Hadfield, K. L., 92, *100*
Hagen, P. O., 4, 5, 7, 11, 12, 27, 28, 32, 33, 34, 36, 37, 39, 44, 48, *52*, *53*
Hahon, N., 117, 133, *138*
Haines, R. F., 116, *138*
Hajra, A. K., 33, *53*
Hakamori, S., 157, *210*
Hales, C. N., 115, *139*
Hall, C. W., 34, *56*
Hall, D. O., 80, 82, 83, *100*
Hall, M. O., 7, 31, *53*
Hammond, R. K., 43, 46, *54*
Hancock, I. C., 3, 39, *53*
Hankins, W. A., 117, *138*
Hanks, J. J., 217, *259*
Hansen, R. E., 84, *103*
Hara, I., 16, *55*
Harada, T., 155, *210*, *211*, *212*
Hardy, R. W. F., 60, 64, 65, 66, 68, 72, 73, 75, 79, 80, 83, 84, 90, 92, 93, 94, 95, 96, *99*, *100*, *101*
Hare, A., 116, *139*
Harper, C. L., 96, *98*
Harper, E. M., 205, *212*
Hart, P., 30, *55*
Hashimoto, T., 222, 224, 232, 233, 236, 237, 238, 244, 253, 256, *258*, *259*
Haskin, M., 192, *208*
Hassid, W. Z., 167, 189, 190, *208*, *209*, *212*
Haug, A., 179, 180, 198, *210*
Hawtrey, E., 46, *52*
Hayflick, L., 217, 218, *259*
Haystead, N., 68, *100*, *103*
Hayward, A. C., 19, *53*
Healy, K., 33, *58*
Heath, E. C., 16, 34, *57*, 177, 187, 191, 192, 193, 194, *213*
Hebert, R. R., *101*
Hechemy, K., 29, *53*
Hees, M. A., 96, *99*
Heidolberger, M., 150, 156, 158, 169, 173, 181, 182, 183, 184, *209*, *210*, *211*, *212*, *213*
Heinor, G. V., 115, *139*
Henis, Y., 113, 130, *138*
Henriksen, S. D., 169, 171, 173, *209*, *210*
Hersh, R. T., 81, *99*
Hemming, F. W., 34, *53*
Higashi, Y., 15, 34, *53*
Higuchi, T., 192, *213*
Hildebrand, J. G., 3, *53*
Hill, J. H. M., 118, *141*
Hill, R. E. E., 94, *100*
Hill, R. L., 19, *57*
Hill, S., 62, 94, *100*
Himes, R. H., 81, *99*
Himmelspach, K., 158, *210*
Hinkson, J. W., 84, 85, *100*
Hipsely, E. H., 61, 62, 97, *98*
Hirase, S., 153, *210*
Hirayama, O., 36, *53*
Hirsch, P., 250, 252, *261*
Hirst, G. K., 116, *138*
Hoch, G. W., 66, *101*
Hodgson, R., 167, 190, *212*
Hoeniger, J. F. M., 225, 226, *259*
Hoerman, K. C., 131, *137*
Hoerrman, K. C., 131, *138*
Hofmann, K., 22, 23, 24, 26, 27, *53*, *55*
Hoffmann, K., 7, 38, 42, *57*
Hoffman, P., 159, *211*

Holborow, E. J., 115, *138*
Holdeman, L. V., 113, 130, *139*
Holm-Hansen, O., 62, *101*
Holme, T., 155, 162, 171, 172, 188, 199, *210*
Holsten, R. D., 66, 68, 90, 95, 96, *99*, *100*, *101*
Hong, J., 81, *101*
Hooper, N. K., 27, *53*
Hopner, Th., 85, *101*
Hopton, J. W., 159, 181, *208*
Horecker, B. L., 151, 158, 192, *208*, *211*, *213*
Horikoshi, K., 242, *259*
Hörhold, C., 17, *57*
Horne, A. J., 96, *101*
Horning, M. G., 21, *53*
Hotchin, J. E., 132, *138*
Hotchkiss, R. D., 181, *210*
Hotta, K., 16, *55*
Houtsmuller, U. M. T., 8, 9, 41, 43, 44, 46, *53*, *56*
How, M. J., 150, 181, 184, *208*, *210*
Howard, D. L., 96, *101*
Howard, E., 82, 96, *99*
Hsu, S. C., 242, *259*
Huang, J. C.-C., 218, 220, 223, 224, 229, 234, 236, 237, 239, 243, 244, 245, 246, 247, 248, 249, 250, *259*, *260*
Hudak, C. E., 122, *140*
Huennekens, F. M., 83, *104*
Hugget, A. S. G., 151, *210*
Hull, J. F., 68, *104*
Humphrey, B. A., 152, *210*
Humphreys, G. O., 3, *53*
Humphries, J. C., 159, 160, *210*
Hungerer, D., 168, *210*
Hunter, J. R., 131, 132, *140*
Hunter, W. M., 128, *139*
Hussey, H., 16, *58*
Huston, C. K., 9, 18, 43, 46, *53*
Hutchison, A. M., 175, *209*
Hyndman, L. A., 76, *101*

I

Ianotti, E., 15, *53*
Iida, S., 242, *259*
Ikawa, M., 2, 7, 9, 16, 18, 35, 40, 47, *53*
Ikekawa, N., 17, *57*
Imai, Y., 23, *53*
Insolera, R. V., 120, *140*
Ioneda, T., 48, *52*
Ishizuka, I., 46, *53*
Ito, M., 117, *139*
Ito, T., 155, *211*

J

Jackson, E. K., 66, 68, *100*, *101*
Jackson, H. R., 250, *259*
Jackson, R. W., 177, *209*
James, A. T., 7, 14, 39, 42, *58*
Jamieson, R. S. P., 175, *208*
Jann, B., 163, 164, 168, 169, 205, *210*, *211*, *213*
Jann, K., 145, 163, 164, 166, 168, 169, *210*, *211*, *213*
Jannasch, H. W., 123, *139*, 221, 249, 254, *260*
Janowitz, S. L., 122, *140*
Jaross, L. B., 132, *140*
Jauréguiberry, G., 28, *53*
Jeanes, A., 177, 178, *210*, *211*, *212*
Jeng, D. Y., 66, 90, 92, *101*
Jenkin, H. M., 45, 50, *53*, *54*
Jermyn, M. A., 150, *210*
Jerne, N-K., 116, *139*
Johnson, G. D., 115, *139*
Johnson, R. C., 45, 50, *53*, *54*
Johnstone, D. B., 178, 179, *209*
Jones, D., 242, *259*
Jones, J. K. N., 179, *211*
Jones, K., 61, *101*
Jones, R. N., 114, *138*
Jones, R. S., 180, *211*
Joo, C. N., 4, 5, 12, 14, *54*
Jost, R., 127, *139*
Jukes, T. H., 83, *102*
Jungermann, K., 74, *101*, *103*
Jurtshuk, P., 38, *54*
Joyce, G. H., 43, 46, *54*

K

Kägi, H. H., 118, *141*
Kajiyama, S., 90, *101*
Kalo, S., 67, *101*
Kaliniskaya, T. A., 62, *100*
Kallio, R. E., 46, *52*
Kamen, M. D., 61, 68, 94, *98*, *100*, *101*
Kamio, Y., 3, 4, 5, 11, 12, 22, 48, *54*
Kandutsch, A. A., 34, *54*
Kaneda, T., 21, *54*
Kanegasaki, S., 3, 4, 11, 22, 48, *54*
Kanemasa, Y., 40, *54*
Kaneshiro, T., 7, 27, 32, *54*
Kanfer, J., 30, 40, *54*
Kang, S., 170, 203, *211*
Karmen, A., 21, *53*
Karsinkin, G. S., 122, *139*
Kates, M., 1, 4, 5, 12, 14, 17, 18, 23, 27, 35, 42, *54*
Katz, I., 3, 4, 10, 21, *51*, *54*

Kauffmann, F., 168, 169, *211*
Kaufmann, 34, *54*
Keeney, M., 3, 4, 10, 15, 21, *51*, *53*, *54*
Keister, D. L., 86, *101*
Keller, J. M., 188, 199, *211*
Kelley, W. S., 194, *209*
Kellogg, D. S., 115, *141*
Kelly, M., 66, 67, 89, 90, 91, 92, 95, *98*, *101*
Kelly-Falcoz, F., 151, *208*
Kennedy, D. A., 183, *211*
Kennedy, E. P., 8, 30, 31, 33, 40, *52*, *54*, *56*, *57*
Kennedy, I. R., 65, 68, 69, 90, *101*
Kent, J. L., 194, *211*
Khan, A. W., 189, 192, *211*
Kessel, R. W. I., 233, *259*
Kim, J., 238, *257*
Kim, K. C., 5, 10, 12, *54*
Kimura, A., 15, *54*
Kimura, T., 83, *103*
Kindt, T. J., 175, *209*
Kipling, C., 122, *138*
Kirby, A. L., 34, *53*
Kito, M., 29, *54*
Kiyasu, J. Y., 31, *54*
Klein, D. A., 220, 221, 232, 233, 250, 254, *259*, *261*
Klein, E., 117, *141*
Klouwen, H., 8, *52*
Klucas, R. V., 75, 89, 95, *101*
Knappe, J., 85, *101*
Knight, B. C. J. G., 130, *140*
Knight, E., 83, *98*
Knight, E., Jr., 60, 65, 66, 72, 73, 79, 83, 84, 93, *100*, *101*
Knight, S. G., 179, *212*
Knoche, H. W., 26, *54*
Knutson, C. A., 177, *211*
Ko, W.-H., 242, *259*
Koch, A. L., 132, *139*
Koch, B., 64, 90, *101*
Koch, R., 89, 95, *101*
Kocun, F. J., 8, *54*
Kodicek, E., 16, 18, 34, *57*, 187, *213*
Koeltzow, D. E., 148, 195, *211*
Koepfli, J. B., 16, *53*
Kok, H., 82, *99*
Kolin, A., 109, *139*
Koostra, W. L., 8, 33, *54*
Kornberg, A., 30, 41, 44, 48, *51*, *54*
Kornfeld, R. H., 201, *211*
Krabek, W. B., 123, *140*
Krebs, H. A., 73, *104*
Kubitschek, H. E., 108, 112, 124, *139*
Kuchynka, K., 93, *101*
Kuczynski, M., 132, *139*
Kuhn, N. J., 21, 30, *54*
Kundig, F. D., 34, *54*
Kunisawa, R., 68, *102*
Kunnes, R. S., 29, *56*
Kurkdjian, A., 39, *54*
Kurokawa, M., 16, *55*
Kurz, G., 151, *213*
Kushner, D. J., 4, 12, 14, *54*
Kusnetz, J., 115, *139*
Kusunose, M., 44, *58*
Kuzentsov, S., 122, *139*
Kwapinski, J. B., 116, *139*

L

Labach, J. P., 14, *55*
Labzoffsky, N. A., 109, *140*
Lacave, C., 15, 26, 27, 40, *56*, *57*
Ladwig, R., 223, 225, 226, 234, 237, 238, 247, 250, 251, 252, *258*, *259*
Lagoda, A. A., 176, *209*
Lam, G. K. H., 160, *209*
Lamblin, D., 220, 221, 236, 237, 242, 251, *258*, *259*
Lands, W. E. M., 30, *55*
Laneele, G., 15, 49, *55*
Laneéle, M. A., 15, 26, 40, *55*, *56*
Lang, D. R., 8, 44, *55*
Larsen, B., 179, 180, 198, *210*, *211*
Lascelles, J., 7, *55*
Laurell, G., 115, 126, 127, *138*
Laurent, M., 223, 228, *261*
Law, J. H., 2, 3, 7, 26, 27, 28, 31, 32, *51*, *52*, *53*, *54*, *55*, *57*, *58*
Lawson, C. J., 162, 171, *211*
Le Comte, J. R., 64, 65, 67, 68, 69, 76, 88, 89, 90, 92, 95, *99*
Lederer, E., 28, 49, *53*, *55*
Lee, C. C., 96, *99*
Lee, L. H., 116, *140*
Lee, S. B., 76, *101*
Lees, H., 39, *52*
Le Gall, J., 79, 84, *100*
Lehane, D. P., 49, *51*
Leiberman, M. M., 202, 205, *211*
Leloir, L. F., 185, *209*
Lemoigne, M., 19, *55*
Lenfant, M., 28, *53*
Lennarz, W. J., 1, 2, 3, 9, 16, 21, 22, 23, 24, 25, 27, 28, 30, 31, 32, 33, 34, 43, 45, 46, *51*, *53*, *55*, *56*, *57*
Lépine, P., 220, 221, 236, 237, 242, 251, *258*, *259*
Lesley, S. M., 159, *211*
Lester, R. L., 31, 32, 35, 36, *57*, *58*, 228, *261*
Letters, R., 7, 31, *55*
Levin, E., 34, *54*

Levin, E. Y., 18, *55*
Levy, R., 23, 25, *52*
Lewis, E. E., 122, *140*
Lewis, V. J., 130, *139*
Lex, M., 68, *103*
Liaaen-Jensen, S., 18, *55*
Lidwell, O. M., 115, *139*
Lie, R., 94, *98*
Liebermann, M. M., 203, *211*
Liepkalns, V., 33, *53*
Light, M., 125, 126, *139*
Light, R., 22, 23, 24, *51*
Light, R. J., 23, 25, *55*
Lindberg, B., 155, 157, 162, 171, 172, 188, 199, *209*, *210*
Linker, A., 159, 180, *211*
Lindstrom, E. S., 62, *101*
Liu, O. C., 108, *139*
Liu, T. Y., 23, 24, 26, *53*, *55*, 183, *210*
Livermore, B. P., 45, 50, *53*, *54*
Lockhart, W. R., 123, *138*
Lockwood, J. L., 242, *258*, *259*
Lofland, H. B., Jr., 7, 31, *51*
Losada, M., 76, *98*
Lovenberg, W., 73, 80, 83, *101*, *103*
Lowry, O. H., 228, *259*
Lüderitz, O., 16, 17, 34, *55*, *58*, 145, 150, 151, 152, 158, 166, *210*, *211*, *213*
Ludoweig, J., 159, *211*
Lundgren, D. G., 8, 19, *55*, *56*
Lust, G., 7, 31, *55*
Lyne, R. R., 167, *211*
Lynen, F., 20, 21, 30, *54*, *55*

M

McCamen, R. E., 30, *55*
McCarthy, K., 83, *101*
McClelland, 116, *139*
McCormick, D. B., 84, *102*
MacDougall, J. C., 9, *55*, *56*
McDowell, R. H., 156, *212*
McElroy, W. D., 120, *139*
MacFarlane, M. G., 1, 8, 18, 43, 44, 46, 48, *55*
McKenna, C. E., 94, *98*
McKenna, E. C., 86, 94, *102*
McKinney, J. A., 49, *56*
McLeary, C. W., 162, 171, *211*
MacLeod, P., 27, *55*
MacLeod, R. A., 125, 126, *139*, 242, *259*
McNeill, J. J., 39, *51*
McQuillen, K., 239, 242, *259*
MacPherson, I. A., 175, *209*
Macrae, A., 34, *51*
Mager, J., 132, *139*
Magee, T., 96, *103*
Mahl, M. C., 64, *102*
Maier, S. M., 223, 234, *260*
Majerus, P. W., 19, 20, 22, *51*, *55*, *56*
Malkin, R., 80, 83, *102*
Makita, M., 16, *55*
Makula, R., 30, 31, 32, 43, 46, *56*
Mamkaeva, K. A., 218, 252, *258*, *259*
Mandel, M., 226, 227, 254, 257, *259*, *260*
Manigault, P., 39, *54*
Mansberg, H. P., 115, *139*
Marchessault, R. H., 19, *55*
Marcus, L., 62, 67, *102*
Marc, I. J., 160, *211*
Marinetti, G. V., 8, 43, *58*
Markovitz, A., 202, 203, 205, *211*, *212*
Markowitz, H., 170, 183, *211*
Marmur, J., 227, *259*
Marr, A. G., 7, 27, *54*, 76, *99*
Marshall, R. D., 152, *211*
Martin, D. B., 21, *53*
Martin, H. H., 239, 242, *259*
Martin, S. M., 27, *54*
Massey, V., 73, 82, 84, *102*
Massey, W., 84, *100*
Matsubara, H., 82, 83, *98*, *102*
Matsuhashi, M., 192, *208*
Matsumara, S., 20, 25, *51*, *56*
Matsuki, T., 90, *101*
Matthews, L. W., 180, *209*
Mavis, R. D., 29, *56*
May, K. R., 108, *139*
Mayhew, S. G., 73, 82, 84, *100*, *102*
Meadow, P. M., 3, 39, *53*
Merck, E. L., 112, *139*
Merrick, J. M., 19, *56*
Metcalf, T. G., 108, *139*
Meyer, F., 11, 45, 49, *56*
Meyer, H., 11, 45, 49, *56*
Meyer, K., 159, *211*
Miles, A. A., 152, *211*
Miles, L. E. M., 115, *139*
Milhaud, G., 19, *55*
Militzer, W. A., 44, *52*
Millbank, J. W., 60, 62, *102*
Miller, E. J., 126, *139*
Mills, G. T., 150, 184, 185, 188, 190, 191, 205, *211*, *212*
Misaki, A., 155, *211*, *212*
Mishustin, E. N., 61, *102*, 222, *259*
Mitchell, R., 221, 242, 249, 254, *259*, *260*
Mitruka, B. M., 113, 130, 135, *139*
Mitz, M. A., 106, 110, 112, 113, 114, 121, 122, 125, 131, 133, *139*
Miyazaki, T., 184, *211*
Möckel, W., 101, *101*

Mohanty, G. C., 118, *139*
Molotkovsky, J. G., 9, *56*
Moor, H., 225, 226, *259*
Moore, W. E. C., 113, 130, *139*
Morgan, D. M., 132, *141*
Morgan, W. T. J., 158, *211*
Morman, M. R., 44, *56*
Morris, J. A., 65, 69, 90, 92, 95, *101*, *102*
Morris, J. C., 221, 249, *260*
Mortenson, L. E., 64, 65, 66, 68, 69, 70, 71, 72, 74, 76, 80, 88, 90, 92, 94, 95, 96, *99*, *101*, *102*, *104*
Mortlock, R. P., 71, *102*
Moss, C. W., 130, *139*
Moulder, J. W., 217, *260*
Moustafa, E., 69, 90, *102*
Movaca, R. F., 122, *141*
Mower, H. F., 64, 68, 70, 71, 88, *99*
Much, A. M., 220, 234, 252, *260*
Mudd, S. G., 16, *53*
Mulvany, J. G., 108, 132, *139*
Munson, T. O., 68, 71, 93, *99*, *102*
Murray, E. G. D., 6, 35, 44, *51*, *52*
Murray, R. G. E., 222, 223, 229, 232, 234, 238, *259*, *260*

N

Nagai, J., 23, *56*
Nairn, R. C., 114, 115, 117, *139*
Nakada, H. I., 162, 171, *211*
Nakamura, K., 247, 251
Nakamura, R. M., 117, 133, *138*
Nakos, K., 90, 96, *102*
Naritomi, L. S., 115, *138*
Neilsen, A., 68, *102*
Nelson, N., 86, *102*
Nelson, R. A., 116, 121, 122, *139*, *140*
Nelson, S. S., 106, 121, *140*
Nesbitt, J. A. III, 3, 32, *55*, *56*
Neuberger, A., 32, *52*, 152, *211*
Neufeld, E. F., 34, *56*
Neufeld, H. A., 118, 119, *140*
Neuman, J., 86, *102*
Newton, W. E., 93, *102*
Niblack, J. F., 160, *209*
Nichols, B. W., 7, 14, 39, 42, *58*
Nielson, A., 68, *102*
Niemann, C., 16, *53*
Nikitina, E. S., 222, *259*
Nimich, W., 158, 173, *210*
Nimmich, W., 173, *211*
Nishioka, K., 117, *140*
Nison, E. H., 76, *99*
Nixon, D. A., 151, *210*
Nojima, S., 40, *52*, *54*
Norris, A. T., 22, 23, 24, 25, *51*, *56*
Norton, W. T., 4, *56*
Norval, M., 148, 149, 186, 188, 190, 193, 194, 195, 197, 205, *211*, *212*
Nosoh, Y., 90, *101*
Nowotny, A. H., 17, *56*
Nozaki, M., 76, *98*
Numa, S., 22, *54*
Nutman, P. S., 96, *102*
Nyc, J. F., 7, 31, 32, *53*, *57*

O

Ohno, K., 18, *52*
Ohrloff, C., 74, *101*, *103*
O'Leary, W. M., 1, 17, 22, 23, 24, 25, 26, 27, 35, 42, *53*, *56*
O'Kane, D. J., 70, *104*
Okunuki, K., 82, *104*
Oleniacz, W. S., 106, 118, 119, 120, *140*
Olson, A. C., 205, *211*
Onn, T., 155, 162, 171, 172, 188, 199, *210*
Op den Kamp, J. A. F., 2, 8, 9, 43, 44, *53*, *56*, *57*
Oppenheim, J., 62, 67, *102*
Orentas, D. G., 153, 154, 178, *212*
Ormerod, J. G., 68, *100*
Ormerod, K. S., 68, *100*
Orr, T. E., 160, *209*
Ørskov, F., 163, 168, 169, *210*, *211*
Ørskov, I., 163, 168, 169, *210*, *211*
Osborn, M. J., 158, 192, 194, *211*, *213*
Otsuka, H., 15, *54*
Otten, B. J., 47, *52*
Overath, P., 30, *56*
Oyama, V. I., 110, *140*
Oyama, Y. I., 112, *139*

P

Painter, T. J., 158, *211*
Paladini, A. C., 185, *209*
Palamota, B., 4, 12, 14, *54*
Palleroni, N. J., 151, *211*
Palmer, G., 84, *102*
Pangborn, M. C., 49, *56*
Panos, C., 4, 27, 28, *52*
Pappenheimer, A. M., 158, 159, *209*, *213*
Pardoe, G. I., 159, *208*
Parejko, R. A., 62, 89, 91, 95, *99*, *102*
Parikh, V. M., 179, *211*
Park, B. H., 159, 160, *208*, *211*
Park, C. E., 7, 15, 37, *56*
Parker, C. A., 60, 66, *100*, *102*, 220, 221, 251, *260*
Parshall, C. W., 66, *101*
Patterson, P. H., 30, 31, *56*
Pauli, G., 30, *56*

Paulus, H., 31, 33, 34, *54*, *56*
Pearce, T. W., 106, 115, 128, 129, 133, *140*
Pearse, A. G. E., 115, *140*
Pearson, H. W., 66, 68, *103*
Peat, S., 167, *211*, *212*
Peck, H. D., Jr., 76, *102*
Pelzer, H., 242, *261*
Pengra, R. M., 68, *104*
Percival, E., 156, *212*
Perfil'ev, B. V., 218, *260*
Perkins, H. R., 242, *260*
Perry, M. P., 12, 14, *54*
Petering, D., 84, *102*
Petitprez, A., 251, *258*
Petzold, H., 215, 216, 217, 218, 221, 222, 228, 232, 251, 254, 255, *261*
Pfister, R. M., 19, *56*, 96, *101*
Philipson, L., 108, 109, *140*
Phillips, K. C., 61, *104*
Phillips, W. D., 83, *98*
Phizackerley, P. J. R., 9, *55*, *56*
Pieringer, R. A., 29, 31, 34, *54*, *56*
Pike, L., 161, *212*
Pirie, N. W., 152, *211*
Pisano, M. A., 106, 118, 119, 120, *140*
Pital, A., 122, *140*
Pittsley, J. E., 177, 178, *210*, *211*
Pizer, L., 29, *54*
Ploem, J. S., 115, *140*
Porath, J., 109, *141*
Postgate, J. R., 62, 66, 67, 69, 91, *98*, *99*, *100*, 131, 132, *140*, 251, *260*
Potter, A. L., 167, 190, *212*
Powell, E. O., 106, 115, 128, 129, 133, *140*
Poxton, I. R., 195, *213*
Pratt, D. C., 76, *102*
Preston, J. F., 167, *209*
Pricer, W. E., Jr., 30, *54*
Pringsheim, E. G., 67, *102*
Prome, J. C., 15, 26, 40, *56*
Proom, H., 130, *140*
Prottey, C., 43, 49, *56*
Pudles, J., 48, *52*
Pugh, E. L., 19, *57*
Putman, E. W., 166, 167, 190, *212*

Q

Quesnel, L. B., 132, *140*
Quimby, F. A., 112, *140*
Quinnell, C. M., 179, *212*

R

Rabinowitz, J. C., 80, 81, 83, *101*, *102*
Radunz, A., 14, 42, *56*
Randall, R. J., 228, *259*
Randle, C. L., 5, 7, 8, 10, 11, 37, 38, 39, 40, 41, *56*
Rao, C. V. N., 184, *212*
Rao, K. K., 83, *100*
Rao, N. V., 109, *140*
Rapp, F., 132, *140*
Rapport, M. M., 4, *56*
Rasumov, A. S., 123, *140*
Ray, T. K., 29, *56*
Razin, S., 17, *57*
Robers, P. A., 182, 183, 184, *209*, *212*
Redai, I., 8, *56*
Reeder, D. J., 115, 127, *138*
Rees, D. A., 162, 171, *211*
Reeves, R. E., 166, 181, 184, *208*, *212*
Reiner, A. M., 224, 238, *260*
Reiner, E., 110, 131, *140*
Reis, J., 32, *55*
Ribbons, D. W., 19, *52*
Richards, J. B., 34, *53*
Richards, O. W., 123, *140*
Richards, R. L., 93, 94, *99*, *100*
Riederer-Henderson, M. A., 61, *102*
Rigopoulos, N., 77, *98*
Ripke, R., 68, *102*
Rippka, R., 68, *102*
Rittenberg, D., 22, *57*
Rittenberg, S. C., 227, 240, 241, 253, *260*
Rizza, V., 14, *57*
Robbins, P. W., 187, 192, 194, 201, *209*, *213*
Roberts, J. B., 19, *53*
Roberts, W. K., 184, *212*
Robinson, J., 223, 229, 232, 241, 243, 244, 247, 248, 250, 252, 253, 254, *258*, *259*, *260*
Robinson, R., 68, *100*
Robbins, P. W., 15, 34, *58*
Roden, L., 170, *212*
Rogers, A. H., 167, 169, *208*
Rogers, H. J., 242, *260*
Romeo, D., 192, *212*
Rose, G., 17, *57*
Rosebrough, N. J., 228, *259*
Roseman, S., 34, *54*
Rosen, S. M., 158, *211*
Rosenfeld, M. H., 106, 118, 119, 120, *140*
Rosenbaum, N., 202, 205, *211*
Ross, K. F. A., 132, *137*
Rost, F. W. D., 115, *140*
Rothfield, L., 158, 192, *211*, *212*, *213*
Roy, H. E., 108, *137*
Roy, N., 184, *212*
Rupprecht, E., 74, *101*, *103*
Russel, H., 82, *99*
Russel, S. A., 82, *101*

Russell, S., 64, 90, 95, *101*
Russell, S. A., 89, *104*
Rutberg, L., 44, *51*
Ryter, A., 39, *54*

S

Saito, H., 155, *211*, *212*
Saito, K., 18, *51*
Salter, D. N., 128, *138*
Saltmarsh-Andrew, M., 192, *213*
Salton, M. R. J., 242, *260*
Samuel, D.. 30, *57*
Samuelsson, B., 44, *51*
Sandborn, W., 131, *137*
Sanders, J. R., 93, *99*
Sandford, P. A., 157, 173, 175, *210*, *212*
San Pietro, A., 76, 86, *101*, *102*, *103*
Santos Mota, J. M. dos, 43, *57*
Sapelli, R. V., 170, *212*
Sastry, P. S., 12, *54*
Sattar, S. A., 133, *140*
Sauer, F., 19, *57*
Scarborough, G. A., 32, *57*
Schairer, H. U., 30, *56*
Schacht, J., 101, *101*
Schatzberg, G., 132, *139*
Scher, M., 16, 34, *57*
Scherff, R. H., 224, 229, 232, 236, 237, 243, *260*
Scheuerbrandt, G., 2, 23, 24, 25, 27, 28, *51*, *55*, *57*
Schick, H. J., 68, 77, 78, *102*
Schildkraut, C. L., 227, *259*
Schlech, B. A., 38, *54*
Schlesinger, G., 93, *103*
Schluchterer, E., 167, *212*
Schmidt, G., 169, 205, *210*, *211*
Schmidt, W. A. K., 108, 109, *140*
Schnaitman, C., 19, *55*
Schneider, K. C., 66, *101*
Schneider, K. F., 163, 168, *210*
Schneider, P. W., 93, *102*
Schoenheimer, R., 22, *57*
Schöllhorn, R., 66, *102*
Schrauzer, G. N., 93, *103*
Schubert, K., 17, *57*
Shieh, H. S., 32, *57*
Schwartz, G., 81, *103*
Schwinghamer, E. A., 97, *103*
Scott, J. E., 150, *212*
Scott, L. V., 116, *140*
Scott, P. B., 66, *102*
Segre, D., 116, *140*
Seidler, R. J., 218, 220, 221, 222, 223, 226, 227, 228, 229, 237, 239, 242, 244, 246, 249, 250, 251, 252, 253, 254, 255, 256, 257, *260*, *261*
Seligman, S. J., 132, *140*
Selinger, H. H., 120, *139*
Sempere, J. M., 151, *212*
Senti, F. R., 178, *210*
Sepulveda, M. R., 115, *137*
Seraichekas, H. R., 108, *139*
Sevag, M. G., 149, *212*
Shabarova, Z. A., 182, 183, *212*
Shaparis, A., 203, *211*
Shaw, M., 158, *212*, *217*, *260*
Shaw, N., 13, 14, 43, 44, 45, 46, 49, *51*, *57*
Sheffield, F. W., 116, *140*
Shenberg, E., 45, *57*
Shepherd, B. P., 128, *138*
Sherr, S. I., 32, *57*
Shethna, Y. I., 81, 82, 84, *103*
Sheu, C. W., 96, *98*
Shilo, M., 218, 220, 221, 223, 224, 227, 228, 232, 233, 234, 237, 238, 239, 240, 241, 242, 244, 247, 248, 249, 250, 253, *257*, *260*, *261*
Shilov, A., 94, *103*
Shilova, A., 94, *103*
Shin, M., 86, *103*
Shiomi, T., 246, 250, *261*
Shively, J. M., 6, 7, 8, 26, 36, 37, 40, 41, *51*, *54*, *57*
Shockman, G. D., 242, *260*
Short, S. A., 7, 37, *57*
Shrauzer, G. N., 93, *103*
Shurcliff, W. A., 106, 121, 122, *140*
Shuval, H. I., 108, 109, *140*
Shuvalov, N., 94, *103*
Shuvalova, N., 94, *103*
Sickles, G. M., 158, *212*
Siddiqui, I. R., 172, *208*
Sierra, G., 19, *57*
Siewert, G., 206, *212*
Silbert, D. F., 25, *57*
Silver, W. S., 60, 61, 62, 96, *103*
Silvery, J. K. G., 62, 68, *104*
Silvester, W. B., 62, *103*
Simmons, D. A. R., 151, *211*
Simon, M. A., 96, *103*
Simon, R. D., 77, *104*
Simpson, F. J., 232, 252, 253, 254, *260*
Sinha, D. B., 39, *57*
Sisler, F. D., 61, *103*
Sisman, J. 236, 237, 251, *258*, *259*
Skorman, V. B. D., 218, *261*
Sloneker, J. H., 153, 154, 178, *212*
Smillie, R. M., 79, 82, 84, *103*
Smit, J. A., 160, *211*
Smith, D. R., 62, *103*
Smith, E. E. B., 150, 184, 185, 188, 190, 191, 205, *211*, *212*

Smith, J. D., 7, 31, *57*
Smith, J. E., 116, *140*
Smith, N. R., 6, *51*
Smith, P. F., 8, 14, 18, 33, 45, *54*, *57*
Smith, R. V., 64, 68, 77, 91, 95, *100*, *103*
Smith, W., 116, *140*
Snyder, F., 33, *58*
Sobel, B. E., 73, *103*
Sojka, G. A., 36, *57*
Somers, P. J., 181, 182, 184, *208*, *209*
Sorger, G. J., 96, *103*
Spears, D., 32, *57*
Spencer, J. F. T., 166, 179, *210*
Spendlove, R. S., 115, *140*
Stacey, M., 145, 150, 159, 167, 172, 181, 182, 184, *208*, *209*, *210*, *211*, *212*
Stanacev, N. Z., 8, 31, *52*, *57*
Stanier, R. Y., 46, 49, *51*, *57*
Stanley, J. P., 61, *99*
Stark, G. H., 148, *213*
Starr, M. P., 215, 216, 217, 218, 220, 221, 222, 223, 224, 226, 227, 228, 229, 231, 232, 233, 234, 236, 237, 238, 239, 242, 243, 244, 245, 246, 247, 248, 249, 250, 251, 252, 253, 254, 255, 256, 257, *259*, *260*, *261*
Stead, D., 44, *57*
Steed, P. D., 234, *260*
Steiner, M. R., 31, 32, *57*, *58*
Steiner, S., 36, *57*, 228, *261*
Sterling, R., 82, *99*
Stern, N., 45, *57*
Stewart, W. D. P., 60, 61, 62, 66, 67, 68, 96, *100*, *103*
Still, J. L., 25, 27, *51*
Stock, C. C., 117, *140*
Stolp, H., 215, 216, 217, 218, 220, 221, 222, 228, 229, 232, 233, 234, 236, 237, 239, 242, 243, 244, 246, 247, 248, 250, 251, 252, 253, 254, 255, *261*
Stolzenbach, F. E., 86, *101*
Strandberg, C. W., 68, *103*
Strange, R. E., 106, 110, 115, 121, 128, 129, 133, *140*
Strehler, B. L., 120, *140*
Streicher, S., 96, 97, *103*
Strominger, J. L., 15, 34, *53*, 151, 192, 206, *208*, *211*, *212*, 242, *258*, *261*
Strugger, S., 132, *141*
Sullivan, C. W., 219, 220, 251, *261*
Sutherland, I. W., 153, 155, 157, 160, 161, 162, 163, 164, 167, 170, 171, 172, 173, 174, 175, 176, 188, 190, 193, 195, 197, 203, 205, *210*, *211*, *212*, *213*
Suzuki, K., 83, *103*
Svesson, S., 157, *209*
Sweeley, C. C., 14, 15, 16, 34, *53*, *57*, *58*
Szilagy, J. F., 7, *55*

T

Tachibana, T., 117, *140*, *141*
Tagawa, K., 73, 76, 82, 86, *98*, *103*, *104*
Tagaya, I., 117, *139*
Tait, G. H., 32, *52*
Takahashi, H., 3, 4, 5, 10, 11, 12, 22, 48, *54*
Takenami, S., 82, *104*
Talamo, B., 19, 22, 34, 45, *51*, *55*
Talpasayi, E. R. S., 67, *101*
Tamm, I., 132, *141*
Taylor, B. F., 96, *99*
Taylor, C. E., 115, *139*
Taylor, K. B., 90, *103*
Telfer, A., 95, *103*
Tempest, D. W., 147, *209*
Than-Tun, 76, *100*
Thaver, R. K., 74, *101*, *103*
Thiele, O. W., 7, 27, 38, 42, *57*
Thomas, D. W., 49, *51*
Thomas, P. J., 26, 27, 49, *57*
Thompson, G. A., Jr., 33, *57*
Thorne, K. J. I., 16, 18, 34, 47, *57*, 187, 188, *208*, *213*
Thornton, M. P., 33, *53*
Tietz, A., 45, *57*
Tietz, C. J., 123, *141*
Tinelli, R., 223, 228, *261*
Tipper, D. J., 242, *258*
Tipton, C. L., 18, *52*
Tiselius, A., 109, *141*
Tkaczyk, S., 132, *137*
Tomasi, V., 2, *56*
Tomlinson, A. H., 115, *141*
Torriani, A., 158, *213*
Totter, J. R., 120, *140*
Toubiana, R., 28, *53*
Tove, S. R., 62, *101*
Towner, R. D., 118, 119, *140*
Trager, W., 217, *261*
Trakatellis, A. C., 81, *103*
Traylor, T. G., 94, *98*, *102*
Trobst, A., 84, *103*
Trofimenkoff, D., 96, *103*
Troy, F. A., 16, 34, *57*, 176, 187, 191, 192, 193, 194, *213*
Tucker, A. N., 14, 15, *52*, *58*
Tully, J. G., 17, *57*
Turner, G. L., 66, *104*
Tyler, J. M., 184, *210*, *213*

U

Udenfriend, S., 7, 31, *52*
Uematsu, T., 218, 223, 232, 238, 246, 248, 250, 251, *261*

Ulfeldt, M. V., 115, *138*
Umbreit, W. W., 112, *138*
Umetani, K., 8, 9, 44, *57*
Urakami, C., 8, 9, 44, *57*

V

Vagelos, P. R., 19, 20, 21, 22, 25, 29, 30, *51*, *52*, *53*, *55*, *56*, *57*, *58*
Vahlne, G., 168, *211*
Valentine, R. C., 60, 65, 71, 73, 74, 77, 78, 79, 80, 81, 82, 84, 85, 86, 96, 97, *98*, *102*, *103*, *104*, 132, *141*
Van Deenen, L. L. M., 2, 8, 9, 32, 41, 43, 44, 46, *53*, *55*, *56*, *57*
Vandecasteele, J. P., 89, 90, *104*
Van den Bosch, H., 29, *58*
Van Tamelen, E. E., 93, *104*
Varon, M., 221, 232, 233, 234, 238, 239, 240, 247, 248, 250, *261*
Veech, R. L., 73, *104*
Veerkamp, J. H., 9, 43, 47, *52*
Vennesland, B., 159, *211*
Verheij, H. M., 43, 45, *57*
Vetter, H., Jr., 85, *101*
Villanueva, J. R., 242, *257*
Volk, W. A., 152, *213*
Von Bulow-Koster, J., 23, *51*
Vorbeck, M. L., 8, 43, *58*
Vurek, G. G., 112, *137*

W

Wachtel, H., 17, *57*
Wada, K., 82, *104*
Wade, H. E., 110, 121, 132, *140*, *141*
Waechter, C. J., 31, 32, *58*
Wagner, H., 11, 23, *51*
Wagner, H. N., 125, *138*
Wakil, S. J., 19, 22, *57*, *58*
Wakimoto, S., 218, 223, 232, 238, 246, 248, 250, 251, *261*
Walby, J. K., 45, 50, *54*
Wallenfels, K., 151, *213*
Walsby, A .E., 67, 77, *100*
Wang, J., 82, *100*
Ward, M. A., 94, *104*
Washington, T. A., 125, *141*
Watanabe, A., 77, *104*
Watkinson, R. J., 16, *58*
Watson, P. R., 160, 162, 173, 177, *211*
Watson, R. W., 112, *138*
Wattenburg, W. H., 112, *138*
Webb, M. A., 167, *208*
Webley, D. M., 242, *259*
Wegner, G. H., 3, 4, 12, 21, *58*
Wehr, N. B., 254, *261*
Weidel, W., 242, *261*
Weiner, I., 192, 194, *213*
Weiner, I. M., 194, *211*
Weiss, S. B., 31, *54*
Wells, M. A., 35, *58*
Wermundsen, I. E., 202, *209*
Westlake, D. W. S., 166, *210*
Westphal, O., 16, *58*, 150, 151, 168, 169, *210*, *211*, *213*
Westwood, J. C. N., 133, *140*
Whatley, F. R., 80, *100*
Whatley, R. F., 82, *104*
Wheat, R., 145, 166, *211*
Wheelock, E. F., 132, *141*
Wheiner, J. R., 83, *98*
White, D. C., 7, 11, 14, 15, 37, 38, 43, 44, 46, *54*, *55*, *56*, *57*, *58*
White, E. H., 118, 120, 126, *141*
White, L. A., 115, 125, *139*, *141*
Whittenbury, R., 61, *104*
Whittick, J. J., 122, *141*
Wilkinson, J. F., 61, *104*, 145, 146, 147, 148, 153, 160, 161, 162, 163, 167, 170, 171, 173, 174, 176, 188, 194, 203, *209*, *210*, *211*, *212*, *213*
Wilkinson, S. G., 11, 13, 14, 40, 42, *53*, *58*
Williams, J. M., 182, *208*
Williams, P. J. le B., 7, 32, 36, 39, *53*
Williamson, I. P., 22, *58*
Willinson, J. F., 175, *208*
Wilson, J. D., 7, 31, *52*
Wilson, P. W., 61, 62, 64, 66, 67, 68, 72, 76, 81, 84, 89, 91, 95, *98*, *99*, *100*, *101*, *102*, *103*, *104*, 179, *212*
Winogradski, S., 61, *104*
Winter, F. H., 124, *141*
Winter, H. C., 69, 92, 93, *99*, *104*
Witz, D. F., 89, 95, *99*
Wolfe, R. S., 70, 71, *102*, *104*
Wolk, C. P., 67, 77, *100*, *104*
Wong, P., 75, 82, *99*
Wood, B. J. B., 7, 14, 39, 42, *58*
Wood, O. L., 250, 252, *261*
Wood, R., 33, *58*
Wood, R. M., 115, *138*
Woodworth, H. C., 116, *140*
Work, E., 242, *261*
Wright, A., 15, 34, *58*, 192, 194, *209*, *213*
Wyatt, J. T., 62, 68, *104*
Wykle, R. L., 33, *58*
Wyss, O., 159, 160, 161, *209*, *212*

Y

Yadomae, T., 184, *211*
Yamakawa, T., 46, *53*
Yamanaka, T., 82, *104*

Yamomoto, Y., 77, *104*
Yang, C. S., 83, *104*
Yankofsky, S., 221, 249, 254, *260*
Yano, I., 44, *58*
Yates, M. G., 67, 75, 81, *104*
Yehle, C. O., 220, *261*
Yeng, D. Y., 65, 90, 95, *102*
Yengoyan, L. S., 12, *54*
Yoch, D. C., 65, 68, 73, 74, 77, 79, 81, 82, 84, 85, *98*, *104*
Yoho, C. W., 23, 24, *53*
York, G. K., 124, *141*
Yoshimura, T., 16, *55*
Yu, P. K. W., 125, *141*
Yurewicz, E. C., 176, *213*

Z

Zaferiou, O., 118, *141*
Zaitseva, G. N., 178, *213*
Zalkin, H., 7, 26, 32, *58*
Zech, R., 151, *209*
Zeleznick, L. D., 158, *211*
Zobell, C. E., 61, *103*

SUBJECT INDEX

A

Acetate in bacterial exopolysaccharides, 153
Acetobacter acetigenum, extracellular polysaccharide production by, 147
A. xylinum, cellulose synthesis by cell-free extracts of, 189
Acetobacters, cellulose excretion by, 166
Acetyl residues in bacterial exopolysaccharides, 153
Acetylated dehydrogenase in nitrogen fixation, 71
Acetylation of bacterial exopolysaccharides, 188
Acetylene reduction, nitrogenase assay by, 66
Acetylene, reduction of, by nitrogenase, 66
Acidic proteins, ferredoxins and flavodoxins as, 78
Acidity of medium and bdellovibrio attachment, 233
Actinomycetes, nitrogen fixation by, 61
Action spectra of nitrogen fixation in *Anabaena cylindrica*, 77
Activation of luminol by microbes in relation to detection, 118
Active sites on the nitrogenase enzyme, 93
Acyl carrier protein in fatty acid synthesis, 19
Acyl thio-esters in bacterial fatty-acid synthesis, 25
Acylation of bacterial exopolysaccharides, 188
Adenosine diphosphate as an inhibitor of nitrogen fixation, 69
Adenosine triphosphate, and nitrogen fixation, 65
  content of *Aerobacter aerogenes*, 110
  contents of microbes, 121
  determination of, 120
  hydrolysis by nitrogenase, 91
  requirement of, in nitrogen fixation, 92
  role of, in nitrogen fixation, 69
Adenosylmethionine as a methyl donor in phospholipid biosynthesis, 32
Aeration, effect of, on bacterial exopolysaccharide production, 148
Aerobacter aerogenes, weight per cell relationship with, 110
A. cloacae, attachment of bdellovibrios to, 234
  colanic acid produced by, 170
Aerobic nitrogen fixers, 67
Age, effect of, on lipid composition of bacteria, 40
  of cells, effect of, on bdellovibrio growth, 250
Agglutination of erythrocytes, use of, to detect viruses, 116
Agricultural importance of nitrogen fixation, 59
Agrobacteria, phospholipid composition of, 7
Agrobacterium spp., extracellular glucan production by, 166
Agrobacterium tumefaciens, fatty acids in, 3
  growth of bdellovibrios on, 248
  phosphatidylethanolamine in, 6
  phospholipid composition of, 41
Alanylphosphatidylglycerol in bacteria, 9
Alanylphosphatidylglycerol synthetase in bacteria, 32
Alcaligenes faecalis, exopolysaccharide of, 155
Aldehydes, long chain, in bacterial lipids, 4
Aldobiuronic acid in exopolysaccharides of bacteria, 175
Algal sterols, 18
Alginases of bacteria, 180
Alginic acid, 179
Alkaline phosphatase activity of host cell-bdellovibrio cultures, 241
Alk-1-enyl ethers, in bacteria, 3, 28
  in bacterial lipids, 2
Alk-1-enyl glycerides in bacteria, 10
Alkyl ether lipids in bacteria, 12
Alkyl ethers in bacterial lipids, 4
Amino-acyl phosphatidylglycerol in bacteria, 8
Aminophthalate, production of, from luminol, 119
Amino sugars, in bacterial exopolysaccharides, 152
  in bdellovibrios, 228
Ammonia, as a suppressor of nitrogenase synthesis, 90
  as the first product of nitrogen fixation, 64

Ammonia—*continued*
detection of, in estimating microbial populations, 112
formation, assay of nitrogenase by, 66
inhibition of nitrogen fixation by, 64
inhibitory effect of, on nitrogen fixation, 68
Anabaena cylindrica, nicotinamide nucleotides as electron donors in nitrogen fixation by, 75
nitrogen fixation by, 64, 77
nitrogenase in, 68
pathway of nitrogen fixation in, 95
properties of electron carriers in, 79
Anaerobes, plasmalogens in, 10
Anaerobic bacteria, plasmalogens in, 3
Anaerobic nitrogen fixation, 61
Anaerobic pathway of fatty-acid synthesis in bacteria, 23
Analysis of bacterial growth products, 130
Anteiso fatty acids in bacterial lipids, 2
Antibacterial globulins, iodinated, use of, in estimation of microbial populations, 128
Antibodies, immune-purified, use of, in estimating microbes, 128
Antibody method for detecting bacterial capsules, 146
Antibody technique, radioactive, for estimating microbes, 127
Antigens, formylated, of bacteria, 152
use of, in detecting microbes, 114
Apolar components of bacterial lipids, 2
Apolar lipids, bacterial, biosynthesis of, 19
Appendages on bdellovibrios, 223
Arthrobacter spp., glycolipids in, 13
Arthrobacter viscosus, exopolysaccharide from, 177
Asparagine, respiration of, by bdellovibrios, 252
Assessment of sparse microbial populations, 105
Athiorhodaceae, lipids of, 36
Atmosphere, monitoring of, for small concentration of microbes, 106
Atmospheric nitrogen, fixation of, 59
Attachment of bdellovibrios, 229, 232
Automated detectors for stained microbes, 121
Azide, reduction of, by nitrogenase, 66
Azocoll, hydrolysis of, by bdellovibrio protease, 244
Azomonas spp., nitrogen fixation by, 62
Azotobacter agile, exopolysaccharide from, 178
A. agilis, fatty acids in, 3
A. chroococcum, exopolysaccharide from, 178
nitrogen fixation in, 67
sterols in, 17
Azotobacter, coupling factors from, 85
ferredoxin, properties of, 81
Azotobacter indica, exopolysaccharide from, 179
Azotobacter spp., particulate nitrogen fixation system in, 67
poly-β-hydroxybutyrate in, 19
production of enzymes by, that hydrolyse bacterial exopolysaccharides, 161
use of, in preserving bdellovibrios, 220
Azotobacter vinelandii, azotoflavin from, 84
electron carrier from, 73
exopolysaccharide from, 178
nitrogen fixation by, 61, 64
pathway of nitrogen fixation in, 95
properties of electron carriers in, 79
properties of nitrogenases from, 88
role of nicotinamide nucleotides in nitrogen fixation by, 74
separation of nitrogenase proteins from, 89
Azotobacteriaceae, lipids of, 38
Azotobacters, parasitization of, by bdellovibrios, 251
Azotoflavin, nature of, 84
structure of, 87

B

Bacillaceae, lipid composition of, 44, 47
Bacilli, endospore formation in, and lipid composition, 42
production of extracellular polysaccharides by, 167
Bacillus cereus, chromatograpny of products of, 130
B. circulans, production of enzymes by, that hydrolyse bacterial exopolysaccharides, 158
B. macerans, nitrogen fixation by, 62
B. megaterium, phospholipid composition of, 41
B. palustris, production of enzymes by, that hydrolyse bacterial exopolysaccharides, 158
B. polymyxa, nicotinamide nucleotides as electron donors in nitrogen fixation by, 75
nitrogen fixation by, 62
pathway of nitrogen fixation in, 95
properties of electron carriers in, 79
Bacillus spp., nitrogen fixation by, 61
poly-β-hydroxybutyrate in, 19

Bacillus stearothermophilus, detection of, by chemiluminescence, 118
B. subtilis, growth of bdellovibrios on, 248
lipids in, 11
Bacteria, immunological properties of, in relation to detection, 114
methylation of phosphatidylethanolamine in, 32
nitrogen fixing, 60
terminal steps in synthesis of unsaturated fatty acids in, 24
virulence and extracellular polysaccharides, 144
Bacterial acyl carrier proteins, 20
Bacterial cellulose, structure of, 166
Bacterial enzymes, use of, in estimating microbial populations, 113
Bacterial exopolysaccharides, 143
biosynthesis of, 185
cell-free synthesis of, 188
production of, 146
structures of, 165
Bacterial ferredoxin, properties of, 80
Bacterial glycolipids, formulae of, 13
Bacterial growth products, analysis of, 130
Bacterial heteropolysaccharides, structures of, 167
Bacterial hosts of bdellovibrios, 228
Bacterial lipid compositions, 35
Bacterial lipids, biosynthesis of, 19
comparative aspects of, 1
Bacterial neutral lipids, 17
Bacterial phosphoglycerides, formulae of, 6
Bacterial spores, methods for rapid assessment of sparse populations of, 109
Bacteriolytic attack, mechanism of, by bdellovibrios, 242
Bacteriolytic nature of bdellovibrios, 228
Bacteriophage, assay of, 133
Bacteriophages, for bdellovibrios, 256
that induce synthesis of enzymes that hydrolyse bacterial exopolysaccharides, 161
Bacteroid ferredoxin, 75
Bacteroides melaninogenicus, sphingolipids in, 14
B. ruminicola, plasmalogens in, 11
B. succinogenes, plasmalogens in, 11
Bacteroids, leghaemoglobin in, 67
nitrogen fixation by, 60
Bactoprenol, in exopolysaccharide biosynthesis by bacteria, 187
nature of, 34
Ballooning of host cells in bdellovibrio invasion, 239
Base composition of bdellovibrio deoxyribonucleic acid, 226
Bdellovibrio bacteriophages, 256
Bdellovibrio bacteriovorus, lytic action of, 217
Bd. bacteriovorus, 215, 221
Bdellovibrio cultures, maintenance of, 219
Bdellovibrio cysts, 225
Bdellovibrio starrii, 221
plaque formation by, 216
Bd. stolpii, 221
Bdellovibrios, chemical composition of, 226
composition of, 222
cultivation of, 218
distribution of, 218
encystment of, 226
facultatively parasitic growth of, 238
growth of, in host cells, 237
host specificity of, 249
importance of, in nature, 220
isolation of, 218
lipase of, 245
long forms of, 237
marine, 249
mechanisms of lysis of host cells by, 242
metabolism of, 252
muramidase of, 243
nutrition of, 246
physiology of, 215
protease-negative mutants of, 245
protease of, 244
rate of respiration of, 240
structure of, 222
taxonomy of, 221
terminology of, 221
vitamin requirements of, 247
Beijerinckia indica, exopolysaccharide from, 179
Beijerinckia spp., nitrogen fixation by, 61
Bifidobacterium bifidum, lipid composition of, 43
Binary capsulation in diplococci, 201
Biochemistry of nitrogen fixation, 64
Biological control of microflora in nature, 221
Biomass in methods for rapid assessment of sparse microbial populations, 110
Biosensor, use of, in detecting microbes, 122
Biosynthesis, of bacterial exopolysaccharides, 185
of bacterial lipids, 19
of capsular polysaccharide of *Klebsiella aerogenes*, pathway for, 186
of colanic acid, 170
of cyclopropane fatty acids in bacteria, 26
of ferredoxins, 81
of plasmalogens in bacteria, 28
Biotin, involvement in fatty acid synthesis in bacteria, 24

Blue-green algae, electron donors from, during nitrogen fixation, 76
nitrogen fixing, 60
Branched-chain acids as primers in bacterial fatty-acid biosynthesis, 21
Branched fatty acids in bacterial lipids, 2
Broad-spectrum methods for detecting microbes, 107
Broken cell preparations, nitrogen fixation by, 66
Bromacil, effect of, on bdellovibrios, 254
Brucella melitensis, lipids of, 26
Brucella spp., phosphatidylethanolamine in, 6
Brucellaceae, fatty acids in, 3
lipids of, 38
Bubble formation in bdellovibrio invasion, 239

C

Calcium, effect of, on bacterial polysaccharide production, 148
effect of, on bdellovibrios, 249
Candida albicans, detection of, by chemiluminescence, 119
Capillary tube scanner, nature of, 112
Capsular polysaccharide of *Klebsiella aerogenes*, pathway for biosynthesis of, 186
Capsular polysaccharides, of bacteria, 143
of *Escherichia coli*, 169
of pneumococci, 150
Capsule swelling technique, 146
Carbamate, effect of, on bdellovibrios, 254
Carbohydrate content of *Aerobacter aerogenes*, 110
Carbon dioxide, detection of, in estimating microbial populations, 112
Carbon monoxide and nitrogen fixation, 66
Carbon : nitrogen ratio in medium, effect of, on bacterial exopolysaccharide production, 147
Carboxyl reduction of bacterial exopolysaccharides, 157
Cardiolipin, biosynthesis of, 28
composition of bacteria, 46
formula of, 6
in bacteria, 8
Carotenoids in bacteria, 17
Catalase in bdellovibrios, 253
Cations, effect of, on bdellovibrios, 248
Casein, hydrolysis of, by bdellovibrio protease, 244
Cell-free extracts, nitrogen fixation by, 64
Cell-free synthesis of bacterial exopolysaccharides, 188
Cell membranes, effects of bdellovibrios on, 241
Cellulase, use of, in determining oligosaccharide structure, 164
Cellulose, excretion by acetobacters, 166
synthesis by bacteria, 147
synthesis of bacterial, in cell-free systems, 189
Cell volumes, estimation of, 124
Cell walls, and bdellovibrio penetration, 236
host, lysis of, by bdellovibrios, 243
of bdellovibrios, chemical composition of, 228
Centrifugation in methods for rapid assessment of microbes, 108
Ceramide phosphorylethanolamine in bacteria, 14
Cetyl alcohol, biosynthesis of, in bacteria, 33
Chain elongation in bacterial fatty acid biosynthesis, 22
Chain length of fatty acids in bacterial lipids, 2
Chain lengths of bacterial fatty acids, 20
Chemical composition of bdellovibrios, 222, 226
Chemical composition of microbes in relation of rapid assessment methods, 107
Chemiluminescence of luminol, use of, in microbial detection, 118
Chemotaxis of bdellovibrios, 232
Chemotypes of bacteria and bdellovibrio attachment, 233
Chloramphenicol, effect of, on bdellovibrio penetration, 236
Chlorella vulgaris, effect of bdellovibrios on, 252
Chromatium sp., lipids of, 36
Chromatium spp., properties of electron carriers in, 79
Chromatographic properties of pyruvylated sugars, 154
Chromatophores, nitrogen fixation by, 77
Chromobacterium violaceum, extracellular polysaccharide production by, 147
Clostridia, fatty acids in, 3
phospholipid composition of, 8, 48
phosphoroclastic reaction in, 71
plasmalogens in, 11
Clostridial ferredoxin, properties of, 80
Clostridial flavodoxin, 83
Clostridium butyricum, biosynthesis of plasmalogens in, 33
fatty acids in, 3
plasmalogen in, 4
unsaturated fatty acids in, 25

Cl. kluyveri, adenosine triphosphate production in, 74
Cl. pasteurianum, electron donors in nitrogen fixation by, 70
flavodoxin from, 83
nitrogen fixation by, 61, 64, 68
nitrogenase, properties of, 88
pathway of nitrogen fixation in, 95
properties of electron carriers in, 79
use of adenosine triphosphate in nitrogen fixation by, 69
Coenzyme A esters of fatty acids as acyl donors, 30
Colanic acid, biosynthesis, 202
hydrolysis of, 162
in bacterial exopolysaccharides, 155
nature of, 167
structure of, 162, 169
Collagen, hydrolysis of, by bdellovibrio protease, 245
Collision of bdellovibrios and host cells, 228
Commensals of green algae, 218
Compact bodies and bdellovibrios, 224
Comparative aspects of bacterial lipids, 1
Complex lipids, bacterial, biosynthesis of, 28
of Gram-negative bacteria, 35
of Gram-positive bacteria, 42
Components of exopolysaccharides of pneumococci, 184
Composition of bacterial exopolysaccharides, 150
and medium composition, 146
Composition of bdellovibrios, 222
Composition of colanic acid, 171
Composition of medium, effect of, on lysis of host cells by bdellovibrios, 248
Composition of microbes in relation to rapid assessment of sparse populations, 109
Compositions, lipid, of bacteria, 35
Concentration, effect of, in bdellovibrio attachment, 233
Conditional mutants in bacterial exopolysaccharide biosynthesis, 205
Continuous culture of nitrogen-fixing bacteria, 68
Control of exopolysaccharide biosynthesis in bacteria, 201
Corynebacteria, lipid composition of, 48
Corynebacteriaceae, lipid composition of, 44
Corynebacterium diphtheriae, lipid composition of, 48
neutral lipids in, 18
Corynebact. michagenense, growth of bdellovibrios in, 248
Corynebact. sepedonicum, lipids in, 11
Corynomycolic acid in bacteria 48
Coulter counter, use of, in estimation of sparse microbial populations, 124
Coupling factors of *Azotobacter*, 85
Coverslip monolayers, use of, in estimating viruses, 133
Cross reacting of nitrogenases, 91
C substance from pneumococci, 150
Cultivation of bdellovibrios, 218
Culture media for bdellovibrios, 218
Cyanide, as an electron acceptor with nitrogenase, 91
inhibition of nitrogen fixation by, 64
reduction of, by nitrogenase, 66
Cyclopropane alcohols in bacteria, 27
Cyclopropane aldehydes in bacteria, 27
Cyclopropane fatty acids, in bacteria, 26, 42
in bacterial lipids, 2
in plants, 2
Cyclopropane synthetase in bacteria, 27
Cysteine residues of ferredoxins, 83
Cysts on bdellovibrios, 225
Cytidine diphosphate diglyceride in bacteria, 10
Cytochromes of bdellovibrios, 253
Cytophaga, effect of bdellovibrios on, 252

D

Davis strain of bdellovibrio, 239
Decarboxylation of phosphatidylserine in bacteria, 30
Decay, phosphorescent, use of, in detection of bacteria, 131
Dehydrogenases, in nitrogen fixation, 70
of bdellovibrios, 254
Deoxyribonucleic acid content, of *Aerobacter aerogenes*, 110
of bdellovibrios, 226
of microbes in rapid assessment methods, 109
Depolymerases of bacterial exopolysaccharides, 161
Deproteinization of bacterial exopolysaccharides, 149
Derivatives, host-independent, of bdellovibrios, 254
Desaturation of fatty acids in bacteria, 23
Desiccation, exopolysaccharides and protection against, 206
Desulfovibrio gigas, properties of electron carriers in, 79
Desulfovibrio spp., flavodoxins from, 84
plasmalogens in, 11

Desulfovibrio vulgaris, nitrogen fixation by, 61
Determination of adenosine triphosphate, 120
Dextrans, bacterial, 166
  biosynthesis of, in cell-free systems, 189
Diacyl phosphoglycerides of bacteria, 5
Diauxic growth in nitrogen-fixing organisms, 68
Dictyobacter spp., host–parasite relationships with, 218
Dideoxy sugars in bacterial exopolysaccharides, 150, 182
Diether analogue of phosphatidylglycerol phosphate, formula of, 5
Diether glycolipid in *Halobacterium cutirubrum*, 14
Diglycosyl diglycerides in bacteria, 13
Dihydrophytyl alcohol in halophilic bacteria, 5
Dihydrosterculic acid in bacteria, 3, 27
Diimide, inability to demonstrate as an intermediate in nitrogen fixation, 93
Dimannophosphoinositides in bacteria, 49
Dimer nature of nitrogenase, 88
Diphosphatidylglycerol, formula of, 6
  in bacteria, 8
Diplococcus pneumoniae, capsular polysaccharides of, 166
  cell-free synthesis of polysaccharides of, 190
  exopolysaccharides of, 180
  extracellular polysaccharides of, 150
  polysaccharides of, 144
Disruption of host cells by bdellovibrios, 238
Distribution of bdellovibrios, 218
  in nature, 220
Distribution of phosphatidylcholine in bacteria, 40
Dithionite, and nitrogen fixation, 65
  in cell-free nitrogen fixation, 66
  reduction of, by ferredoxins, 80
Divalent cations, effect of, on bdellovibrios, 248
Division of bdellovibrios in host cells, 237
Douglas fir, nitrogen fixation by, 61
Dyes, use of, to stain and detect microbes, 121

E

Ecological importance of nitrogen fixation, 59
Ectoparasitic nature of bdellovibrios, 228
Effect of pH value on lipid composition of bacteria, 46
Effects of bdellovibrios on host cells, 239
Efficiency of plaque formation by bdellovibrios, 250
Electron activation, adenosine triphosphatase as in nitrogen fixation, 92
Electron carriers, in nitrogen fixation, 78
Electron donors, in nitrogen fixation, 70
  need for, in nitrogen fixation, 69
  nicotinamide nucleotides as in nitrogen fixation, 75
Electron microscopy of bdellovibrios, 223
Electronic particle counters, use of, in estimation of microbial populations, 112
Electrophoresis in rapid assessment of virus populations, 108
Encapsulation and bdellovibrio attachment, 234
Encystment of bdellovibrios, 226
End products of metabolism, detection of, in estimating microbial populations, 113
Endophytes, nitrogen fixation by, 60
Endospore formation in bacilli, and lipid composition, 42
Energy generation by bdellovibrios, 253
Enterobacteria, fatty acids in, 3
Enterobactericeae, cyclopropane fatty acids in, 27
  lipids of, 38
  production of colanic acid by, 169
Entrance of bdellovibrio into host cells, 217, 229
Environment and nitrogen fixation, 97
Enzymes, and bdellovibrio penetration, 236
  involved in biosynthesis of bacterial exopolysaccharides, 185
  lytic, of bdellovibrios, 243
  that hydrolyse bacterial exopolysaccharides, 158
Enzymic activities of microbes in relation to rapid assessment methods, 107
Enzymic digestion of protein from bacterial exopolysaccharides, 150
Enzymic hydrolysis of bacterial exopolysaccharides, 158
Epimerases in biosynthesis of bacterial exopolysaccharides, 186
Erwinia amylovora, and bdellovibrios, 229
  attachment of bdellovibrios to, 234
Escherichia coli, attachment of bdellovibrios to, 233
  biosynthesis of phospholipids in, 29
  chromatography of products of, 130
  extracellular heteropolysaccharides of, 167

Escherichia coli—continued
extracellular polysaccharide production by, 147
fatty acid synthesis in, 24
fatty acids in, 3
lipids in, 11
Escherichieae, lipids of, 38
Esterases, use of, in estimating microbial populations, 114
Ester-containing lipids of halophilic bacteria, 4
Ethanolamine phospholipids in *Bacteroides succinigenes*, 12
Ethanolamine plasmalogen in bacteria, 3,12
Eubacteriales, lipid composition of, 43
lipids of, 38
phosphatidylethanolamine in, 6
Eukaryotic micro-organisms, inability of, to fix nitrogen, 60
Eukaryotic organisms, lipids of, 1
Excretion of bacterial polysaccharides, 146
Exogenous fatty acids, use of, by bacteria, 30
Exopolysaccharides, bacterial, 143
biosynthesis of, 185
cell-free synthesis of, 188
structures of, 165
functions of, 206
Exothermic nature of the nitrogen fixation reaction, 92
Extractable polar lipids, in bacteria, 12
of bacteria, 5
Extraterrestrial life, detection of, 112
Extreme halophiles, lipids of, 4
Extrusions on bdellovibrios, 223

F

Facultatively parasitic growth of bdellovibrios, 238
Fatty acid desaturases in bacteria, 23
Fatty acids, bacterial, biosynthesis of, 19
in bacterial lipids, 2
of Gram-negative bacteria, 42
Fatty aldehydes in bacterial lipids, 4
Feedback inhibition of nitrogen fixation in bacteria, 69
Ferredoxin, in cell-free nitrogen fixation, 66
in nitrogen fixation, 65, 78
photochemical reduction of, 76
reduction of, by nicotinamide nucleotides, 74
Ferredoxins, properties of, 80
reaction of, with nitrogenases, 87
Ferrous ions, effect of, on bacterial polysaccharide production, 148
Fertility, soil, and bacterial exopolysaccharides, 144
Fibres on bdellovibrios, 224
Filter-immunofluorescence methods for estimating microbes, 126
Firefly luminescent method for determining adenosine triphosphate, 120
Fischerella spp., micrograph of, 63
Fixation of nitrogen, pathways of, 59
Flagella of bdellovibrios, 222
Flagellation in bdellovibrios, 232
Flavodoxin, and nitrogen fixation, 65
in nitrogen fixation, 78
Flavodoxins, properties of, 83
Flavoproteins as electron carriers in nitrogen fixation, 83
Fluorescin isothiocynate method for detecting microbes, 122
Formate, as a reducing agent with nitrogenase, 72
dehydrogenase activities in bacteria, 72
Formyl residues in bacterial exopolysaccharides, 152
Formylated antigens of bacteria, 152
Formylation of bacterial exopolysaccharides, 188
Freezing of bdellovibrios, 220
Fucosamine in bacterial exopolysaccharides, 182
Fucose epimerase, use of, in sugar analysis, 151
Fucose in *Klebsiella* exopolysaccharides, 173
Fucosidases, use of, in determining oligosaccharide structure, 164
Fucosidic linkages in bacterial exopolysaccharides, 158
Functions of exopolysaccharides, 206
Fungal sterols, 18
Fungi, phosphatidylcholine in, 7

G

Galactosamine in bacterial exopolysaccharides, 152
Galactose dehydrogenase, use of, in sugar analysis, 151
Galactose, in bacterial exopolysaccharides, 150
in *Klebsiella* exopolysaccharides, 173
oxidase, use of, in sugar analysis, 151
β-Galactosidase activity of host cell–bdellovibrio cultures, 241
β-Galactosidase synthesis by bdellovibrios, 240

Galactosyldiglycerides in treponemes, 50
Galacturonic acid in *Klebsiella* exopolysaccharides, 173
Gas chromatography of bacterial products, 130
Gas chromatography, use of, to detect viruses, 135
Gelatin, hydrolysis of, by bdellovibrio protease, 244
Genetics of nitrogen fixation, 96
Glucan of *Rhizobium japonicum*, cell-free synthesis of, 190
Glucans produced extracellularly by bacteria, 166
Glucosamine, in bacterial exopolysaccharides, 152
in bdellovibrios, 228
Glucosaminidases, use of, in determining oligosaccharide structures, 164
Glucosaminyl derivatives of phosphatidylglycerol in bacteria, 9, 48
Glucose, in bacterial exopolysaccharides, 150
in *Klebsiella* exopolysaccharides, 173
oxidase, use of, in sugar analysis, 151
Glucosidases, use of, in determining oligosaccharide structure, 164
Glucuronic acid in *Klebsiella* exopolysaccharides, 173
Glutamine, respiration of, by bdellovibrios, 253
Glycerol in bacterial exopolysaccharides, 156
Glycerol 3-phosphate in bacterial phosphoglyceride synthesis, 28
Glycolipids, biosynthesis of, in bacteria, 34
in bacteria, 12
Glycosyl carrier lipids, nature of, 192
Glycosyl carriers in bacterial exopolysaccharide biosynthesis, 188
Glycosyl derivatives of polyisoprenols in bacteria, 15
Glycosyl diglycerides in bacteria, 12, 42
Glycosyl donors in bacterial exopolysaccharide biosynthesis, 189
Glyoxylate cycle in bdellovibrios, 254
Gradient centrifugation in rapid assessment of microbial populations, 109
Gram-negative bacteria, cyclopropane fatty acids in, 27
fatty acids of, 42
lipid A in, 17
lipids of, 35
miscellaneous exopolysaccharides from, 177
phosphatidylethanolamine in, 5
structures of exopolysaccharides produced by, 167
Gram-positive bacteria, bdellovibrios and, 251
complex lipids of, 42
cyclopropane fatty acids in, 27
exopolysaccharides of, 180
Green algae, commensals of, 218
Growing cultures, production of bacterial exopolysaccharides in, 146
Growth characteristics of microbes in relation to rapid assessment methods, 107
Growth, kinetics of, in host-dependent bdellovibrios, 246
use of, to estimate microbial populations, 125
Growth of bdellovibrios in host cells, 237
Growth phase, effect of, on bacterial exopolysaccharide production, 147
effect of, on bacterial lipid composition, 40
Growth products, bacterial, analysis of, 130
Gulliver, nature of, 112, 125
Guluronic acid, biosynthesis of, 198

H

Haematin content of micro-organisms, 111
Haemophilus influenzae, estimation of, 127
Haemophilus spp., phosphatidylethanolamine in, 6
Halobacterium cutirubrum, lipids in, 12
lipids of, 5
Halophilic bacteria, lipids of, 4
Heat-killed cells, for growth of bdellovibrios, 247
of host, growth of bdellovibrios on, 247
Herbicides, activity against bdellovibrios, 254
Heterocysts, and nitrogen fixation in blue-green algae, 62
role of, in nitrogen fixation in blue-green algae, 67
Heteropolysaccharide synthesis by cell-free systems, 188
Heteropolysaccharides, bacterial, 165
bacterial extracellular, 146
microbial, cell-free synthesis of, 190
Hexokinase in biosynthesis of bacterial exopolysaccharides, 186
Hexoses in bacterial exopolysaccharides, 150
Higher organisms, lipids of, 2
Holdfast on bdellovibrios, 223
Homology of nitrogenases, 91
Homopolysaccharides, bacterial, 165
extracellular, 147
structures of, 166
biosynthesis of, in cell-free systems, 189

Horse serum, use of, in preserving Bdellovibrio cultures, 220
Host cell membrane, effect of bdellovibrios on, 241
Host cell wall, disintegration of, by bdellovibrios, 243
Host cells, disruption of, by bdellovibrios, 238
  effects of bdellovibrios on, 239
  growth of bdellovibrios in, 237
  mechanisms of lysis by bdellovibrios, 242
  penetration of bdellovibrios into, 234
Host-dependent bdellovibrios, and symbiosis, 228
  life cycle of, 229
Host-dependent growth of bdellovibrios, kinetics of, 246
Host-independent bdellovibrio populations, 217
Host-independent derivatives of bdellovibrios, 254
Host range of bacteriophages that induce synthesis of enzymes that hydrolyse bacterial exopolysaccharides, 161
Host specificity groups of bdellovibrios, 250
Host specificity of bdellovibrios, 249
Hydrated electrons, adenosine triphosphate as in nitrogen fixation, 92
Hydrazine, inability to demonstrate, as an intermediate in nitrogen fixation, 93
Hydrocarbon composition of bacteria, 46
Hydrocarbons in micro-organisms, 18
Hydrogen, and nitrogen fixation, 76
  concentration of medium, effect of, on lipid composition of bacteria, 46
  evolution, nitrogenase assay by, 66
  inhibition of nitrogen fixation by, 64
  metabolism and nitrogen fixation, 64
  sulphide, detection of, in estimating microbial populations, 112
Hydrogenase, relation of, to nitrogenase, 76
Hydrolases, use of, in studying oligosaccharide structure, 165
Hydrophilic nature of bacterial capsules, 144
β-Hydroxydecanoic acid in bacterial fatty-acid synthesis, 25
β-Hydroxydecanoic acids in bacteria, 3
β-Hydroxy fatty acids in bacteria, 3
β-Hydroxylauric acid in bacteria, 3
β-Hydroxymyristic acid in bacteria, 3
Hyphomicrobiales, lipids of, 36, 37
Hyphomicrobium, effect of bdellovibrios on, 252
Hyphomicrobium sp., phospholipids of, 32
Hyphomicrobium vulgare, phospholipid composition of, 7

Immune adherence reaction, nature of, 116
Immuno-adherence, assay, 133
  use of, in estimating viruses, 133
Immunofluorescence methods for estimating microbial growth, 126
Immunofluorescent method for determining viruses, 132
Immunological activities of microbes in relation to rapid assessment, 107
Immunological detection of viruses, 116
Immunological properties, of bacteria in relation to detection, 114
  of microbes in relation to detection, 114
Immuno-purified antibodies, use of, in estimating microbes, 128
Importance of bdellovibrios in nature, 220
India ink method for visualizing bacterial capsules, 145
Indirect methods for rapid determination of microbial viability, 131
Induction of synthesis of enzymes that hydrolyse bacterial exopolysaccharides, 158
Infection cushion on bdellovibrios, 224
Inhibition of nitrogen fixation by hydrogen, 76
Inorganic phosphate, hydrolysis of, by nitrogenase, 65
Inorganic substituents in bacterial exopolysaccharides, 155
Intracytoplasmic membranes of bacteria, and phospholipid composition, 39
Intra-integumental parasite, Bdellovibrio as, 229
Intramural parasite, Bdellovibrio as, 229
Iodination of antibacterial globulins, 128
Iodine, radioactive, use of, in estimating microbes, 127
  radio-isotopes of, use of, in estimating microbial populations, 115
Ions and bdellovibrio attachments, 233
Iron content of ferredoxins, 80
Iron-protein nature of nitrogenase, 90
Iron, requirement for, in nitrogen fixation, 64
Iso fatty acids in bacterial lipids, 2
Isolation, of bacterial exopolysaccharides, 149
  of bdellovibrios, 218
  of host-independent derivatives of bdellovibrios, 255
  of nitrogenases, 88
Isoleucine in bacterial fatty-acid biosynthesis, 21
Isoprenoid alcohols, as glycosyl carrier lipids, 192

Isoprenoid alcohols—*continued*
in biosynthesis of bacterial exopolysaccharides, 187
Isovaleric acid in bacterial fatty-acid biosynthesis, 21

J

Jerusalem strain of bdellovibrio, 239

K

Ketals attached to terminal reducing galactose residues of colanic acids, 172
α-Ketobutyrate as a substrate with nitrogenase, 72
Killed host cells, growth of bdellovibrios in, 247
Kinetics of extracellular polysaccharide production by bacteria, 148
Kinetics of host-dependent growth of bdellovibrios, 246
Klebsiella aerogenes, capsular anatomy of, 145
cell-free synthesis of heteropolysaccharide of, 190
mutants deficient in exopolysaccharide biosynthesis, 196
pathway of biosynthesis of capsular polysaccharide of, 186
production of enzymes by, that hydrolyse bacterial exopolysaccharides, 159
time-course of polysaccharide production by, 149
Klebsiella ozaenae, exopolysaccharides of, 174
Klebsiella phages, substrates for, 163
Klebsiella pneumoniae, and formate as an electron donor in nitrogen fixation by, 72
ferredoxin from, 82
nitrogen fixation by, 64
pathway of nitrogen fixation in, 95
Klebsiella polysaccharides, monosaccharides in, 173
Klebsiella rhinoscleromatis, exopolysaccharides of, 174
Klebsiella spp., extracellular polysaccharides of, 171
nitrogen fixation by, 61

L

Labile sulphide in nitrogenase, 89
Lactase, use of, in determining oligosaccharide structure, 164
Lactic acid bacteria, complex lipids of, 42
Lactobacillaceae, lipid composition of, 43
Lactobacilli, fatty acids in, 3
glycolipids in, 14
polyisoprenoid alcohol in, 16
Lactobacillic acid, biosynthesis of, 26
formula of, 2
Lactobacillus casei, bactoprenol production by, 187
Lactose respiration by host cell-bdellovibrio cultures, 240
Leakage of purines by bacteria, 132
Lecithin, formula of, 6
Leghaemoglobin, function of, in bacteroids, 67
Legumes, nitrogen fixation by, 60
Leguminous plants, nitrogen fixing, 60
Leptospira canicola, lipid composition of, 45
Leuconostoc mesenteroides, polysaccharide production by, 166
Levans, bacterial, 166
biosynthesis of, in cell-free systems, 189
Life cycle, of bdellovibrios, 222
of host-dependent bdellovibrios, 229
Light scattering methods in estimations of microbial populations, 112
Limitations, nutrient, effect of, on bacterial exopolysaccharide production, 147
Linkages in bacterial exopolysaccharides, 156
Linuran, effect of, on bdellovibrios, 254
Lipase of bdellovibrios, 245
Lipid A in bacteria, 16
Lipid compositions of bacteria, 35
Lipid content of *Aerobacter aerogenes*, 110
Lipid intermediates in exopolysaccharide biosynthesis, 189
Lipid-linked intermediates in exopolysaccharides synthesis, 191
Lipids, bacterial, comparative aspects of, 1
complex, of Gram-positive bacteria, 42
of bdellovibrios, 227
of Gram-negative bacteria, 35
Lipo-amino acids in bacteria, 8
Lipopolysaccharides, biosynthesis of, in bacteria, 15
extracellular, of Gram-negative bacteria, 167
lipid intermediates in biosynthesis of, 192
of bacteria, 143
Listeria monocytogenes, lipid composition of, 44
Long-chain bases in bacterial lipids, 14
Long forms of bdellovibrios, 237
Low redox potential of ferredoxins and flavodoxins, 78
Luciferase, use of, in determining adenosine triphosphate, 120

Luciferin, use of, in determining adenosine triphosphate, 120
Lucigenin, activation of, by micro-organisms, 120
Luminol chemiluminescence in microbial detection, 118
Lyophilization of Bdellovibrio cultures, 220
Lysine, in bacterial lipids, 9
Lysine-containing lipid in bacteria, 15
Lysis, from without by bdellovibrios, 238
mechanisms of, by bdellovibrios, 242
of bacteria, 242
of host cells by bdellovibrios, 240
Lysophosphatides in bacteria, 10
Lysophosphatidic acid in biosynthesis of bacterial phosphoglycerides, 29
Lysylphosphatidylglycerol, biosynthesis of, in bacteria, 33
Lysyl-t-RNA ribonucleic acid in bacterial lipid biosynthesis, 32
Lytic action of *Bdellovibrio bacteriovorans*, 217
Lytic enzymes of bdellovibrios, 243

M

Magnesium, effect of, on bdellovibrios, 249
Maintenance of Bdellovibrio cultures, 219
Malaria protozoa, 217
Malonyl-coenzyme A in fatty-acid biosynthesis, 19
Mannan, biosynthesis of, in *Micrococcus lysodeikticus*, 16
Mannolipid biosynthesis, 192
Mannosamine in bacterial exopolysaccharides, 152
Mannose, in bacterial exopolysaccharides, 150
in Klebsiella exopolysaccharides, 173
isomerase, use of, in sugar analysis, 151
Mannosidases, use of, in determining oligosaccharide structures, 164
Mannosidic linkages in bacterial exopolysaccharides, 158
Mannosyl diglycerides in bacteria, 13
Mannuronic acid, biosynthesis of, 198
in bacterial exopolysaccharides, 180
Marine bdellovibrios, 249
Mass of microbes in relation to rapid assessment methods, 107
Mechanism of action of nitrogenases, 91
Mechanisms of lysis of host cells by bdellovibrios, 242
Media for culturing bdellovibrios, 218
Membrane filters, use of, in staining methods for detecting microbes, 122
Membrane filtration in methods for rapid assessment of microbes, 108
Membranes, cell, effect of bdellovibrios on, 241
Mesosomes of bdellovibrios, 224
Metabolic activity, effect of, on bdellovibrio growth, 250
Metabolism, and microbial growth, 111
microbial, use of, to estimate microbial populations, 125
of bdellovibrios, 252
Methane-oxidizing bacteria, nitrogen fixation by, 61
Methane production in estimation of microbial populations, 112
Methylated sugars in bacterial exopolysaccharides, 150
Methylation, of bacterial exopolysaccharides, 156
of phosphatidylethanolamine, by bacteria, 5
in bacteria, 31
2-Methylbutyrate in bacterial fatty-acid biosynthesis, 21
Methyloctadecasphinganine in bacteria, 15
Methylsulphymyl carbanion as a methylating agent, 157
Methyluronic acids in bacterial exopolysaccharides, 152
Methyl viologen, and nitrogen fixation, 65
reduction of, by nitrogen-fixing bacteria, 71
Microbacterium lacticum, glycolipids in, 13
M. thermosphactum, lipid composition of, 49
Microbes, immunological properties of, in relation to detection, 114
Microbial concentration, increase in assessments, 106
Microbial growth and metabolism, 111
Microbial microculture methods, 132
Microbial populations, sparse, rapid detection of, 105
Microbial viability, rapid detection of, 131
Microcapsular polysaccharides of bacteria, 143
Micrococcaceae, complex lipids of, 42
fatty acids in, 3
lipid composition of, 43
phosphatidylinositol in, 9
Micrococcus cerificans, anomalous lipid composition of, 46
M. lysodeikticus, biosynthesis of glycolipids in, 34
biosynthesis of mannan in, 16
detection of, by chemiluminescence, 118
glycolipids in, 13

Microculture methods, use of, in estimating microbes, 132
Micro-organisms, nitrogen fixing, 60
Minimum level of detection with microbes, 106
Minor phospholipids in bacteria, 9
Miscellaneous bacterial exopolysaccharides, 177
Mode of bacteriolytic attack by bdellovibrios, 242
Model for exopolysaccharide biosynthesis, 200
Model systems of nitrogenase, 93
Molecular hydrogen, as an electron donor in nitrogen fixation, 76
  detection of, in estimating microbial populations, 112
Molecular nitrogen, fixation of, 60
Molecular oxygen, involvement of, in biosynthesis of bacterial unsaturated fatty acids, 23
Molecular weight of nitrogenase, 90
Molecular weights, of bacterial exopolysaccharides, 156
  of ferredoxins and flavodoxins, 79
Molybdenum in nitrogenase, 89
Molybdenum-iron nature of nitrogenase, 90
Molybdenum, requirement for, in nitrogen fixation, 64
  role of, in nitrogen fixation, 93
Mono-unsaturated fatty acids, biosynthesis of, in bacteria, 22
  in bacterial lipids, 2
Morphology of bdellovibrios, 222
Motility of bdellovibrios, 232
Multiple attachments of bdellovibrios, 232
Multiplication of bdellovibrios, 229
Multiplicity of infection with bdellovibrios, 233
Muramic acid in bdellovibrios, 228
Muramidase of bdellovibrios, 243
Mutants, and nitrogen fixation, 96
  of *Klebsiella aerogenes* deficient in exopolysaccharide biosynthesis, 196
  protease-negative, of bdellovibrios, 245
Mycobacteria, host–parasite relationships with, 217
  lipids of, 14, 49
  ornithine-containing lipid in, 15
Mycobacterium bovis, lipids of, 26
Mycobacterium spp., nitrogen fixation by, 62
Mycolic acids in bacteria, 48
Mycoplasmas, host–parasite relationships with, 217
  sterols in, 17
Mycosides in bacteria, 14

N

Nature, distribution of bdellovibrios in, 220
Neisseria catarrhalis, detection of, by chemiluminescence, 119
Neurospora sp., methylation of phosphatidylethanolamine in, 31
Neutral lipid composition of *Sarcina lutea*, 46
Neutral lipids of bacteria, 17
Nicotinamide nucleotides, and nitrogen fixation, 65
  in nitrogen fixation, 73
  of bdellovibrios, 253
Nitrobacteriaceae, lipids of, 36
Nitrocystis oceanus, phospholipid composition of, 7
Nitrogen fixation, biochemistry of, 64
  genetics of, 96
  in *Azotobacter*, pathway of, 87
  mechanism of, 91
  mutants and, 96
  pathways of, 59
Nitrogen-fixing micro-organisms, 60
Nitrogen incorporation, nitrogenase assay by, 66
Nitrogenase, assay of, 66
  inducible nature of, 68
  nature of, 59
  properties of, 65, 88
  purification of, 88
Nitrogenases, cross reacting, 91
  mechanism of action of, 91
Nitrogenous compounds, effect of deficiency of, on bacterial exopolysaccharide production, 147
Nitrous oxide, inhibition of nitrogen fixation by, 64
  reduction of, by nitrogenase, 66
Non-capsulate mutants of bacteria, 146
Non-extractable lipids in bacteria, 16
Non-haem iron in nitrogenase, 89
Non-mucoid mutants, of bacteria, 144
  of *Klebsiella aerogenes*, 205
Non-sugar components in bacterial exopolysaccharides, 152
Non-synchronous growth of bdellovibrios, 246
Nucleic acid content of micro-organisms, 111
Nucleic acids, of bdellovibrios, 226
  removal from bacterial exopolysaccharides, 149
Nucleotides, in bdellovibrios, 226
  sugar, in biosynthesis of bacterial exopolysaccharides, 185
Nutrients, effect of, on bacterial exopolysaccharide production, 147

Nutrition of bdellovibrios, 246
Nutritional requirements of host-independent *Bdellovibrio*, 247

O

O-Amino acyl phosphatidylglycerols, biosynthesis of, in bacteria, 32
Octadecasphinganine in bacteria, 15
Octasaccharide attached to glycosyl carrier lipid, 194
Oligosaccharide structure, determination of, 164
Organic acids in bacterial exopolysaccharides, 152
Ornithine amides in bacteria, 15
Ornithine in bacterial lipids, 9
Oxygen, effect of, on bacterial exopolysaccharide production, 148
  inhibition of nitrogen fixation by, 64
  inhibitory effect of, on nitrogen fixation, 66
  partial pressure, effect of, on bdellovibrios, 252
  sensitivity of nitrogenases, 67
  tension of medium and bdellovibrio attachment, 233

P

Palmitaldehyde, biosynthesis of, in bacteria, 33
Palmitoleic acid, occurrence of, in bacteria, 2
Parasitic bdellovibrios, 216
Partial acid hydrolysis of bacterial exopolysaccharides, 157
Partichrome instrument, use of, 121
Particle counter, Royce, 124
Pasteurella haemolytica, estimation of, 127
Pathways of nitrogen fixation, 59
  in micro-organisms, 95
Peas, nitrogen fixation by, 60
Penetration of bdellovibrios into host cells, 234
Pentoses in bacterial exopolysaccharides, 150
Peptidoglycan, and bdellovibrio penetration, 236
  biosynthesis of, in bacteria, 15
  lysis of, by bdellovibrios, 243
  softening of, in bacteriolysis, 242
Peptone as a growth substrate for bdellovibrios, 247
Peptostreptococcus elsdenii, flavodoxin from 84
  plasmalogens in, 11
Periodate oxidation of bacterial exopolysaccharides, 157
Permeability of host cells to solutes, effect of bdellovibrios on, 240
Phage T7, composition of, 111
Phages, estimation of, 134
  production of enzymes by, that hydrolyse bacterial exopolysaccharides, 159
Phagocytosis, protection against, exopolysaccharides in, 206
Phase-contrast microscopy of bdellovibrio invasion, 231
Phenoxyacetic acid herbicides, effect of, on bdellovibrios, 254
Phosphatase, use of, in estimating microbial populations, 113
Phosphatases of bdellovibrios, 228
Phosphate, in bacterial exopolysaccharides, 156
  labelled, estimation of, in assay of microbial growth, 125
Phosphatidic acid, formula of, 6
  in bdellovibrios, 227
  in biosynthesis of bacterial phosphoglycerides, 29
Phosphatidylcholine, biosynthesis of, in bacteria, 31
  formula of, 6
  in bacteria, 7
Phosphatidylethanolamine, biosynthesis of, 28
  formula of, 6
  in bacilli, 47
  in bacteria, 5
  in bdellovibrios, 227
Phosphatidylglycerol, biosynthesis of, 28
  formula of, 6
  in bacteria, 7
  in bdellovibrios, 227
Phosphatidylglycerophosphate in bacteria, 10, 30
Phosphatidylinositol, biosynthesis of, 33
  formula of, 6
  in bacteria, 9
Phosphatidylserine, biosynthesis of, in bacteria, 29
  in bacteria, 10
Phosphoenolpyruvate as a pyruvate group donor in biosynthesis of bacterial exopolysaccharides, 188
Phosphoglucomutase in biosynthesis of bacterial exopolysaccharides, 186
Phosphoglycerides, biosynthesis of, 28
  polar, of bacteria, 5
Phospholipid compositions of bacterial changes in, 41
Phospholipids of bdellovibrios, 227

4-Phosphopantetheine as a prosthetic group, 19
Phosphorescent decay, use of, in detection of bacteria, 131
Phosphoroclastic reaction, as the driving force in nitrogen fixation, 71
in bacteria, 70
in nitrogen fixation in clostridia, 71
Phosphorus compounds, effect of deficiency of, on production of bacterial exopolysaccharides, 147
Photochemical reduction of ferredoxin, 76
Photosynthetic bacteria, electron donors during nitrogen fixation by, 76
glycolipids in, 14
nitrogen fixing, 61
phospholipid composition of, 7
Photosynthetic electron donors in nitrogen fixation, 76
Physical attachment of exopolysaccharides to bacteria, 149
Physical methods for determination of sparse microbial populations, 124
Physical properties of microbes in relation to rapid assessment methods, 108
Physiology of the bdellovibrios, 215
Phytoflavin, nature of, 84
Plant ferredoxins, properties of, 82
Plaque formation, by *Bdellovibrio starrii*, 216
by bdellovibrios, 250
Plasmalogens, biosynthesis of, in bacteria, 33
in bacteria, 3, 10
Pneumococcal capsule depolymerases, 162
Pneumococcal exopolysaccharides, hydrolysis of, 159
Pneumococci, components of exopolysaccharides of, 184
phospholipid composition of, 8
Pneumococcus spp., biosynthesis of glycolipids in, 34
Pneumosamine in exopolysaccharides of pneumococci, 182
Polyfructoses produced extracellularly by bacteria, 167
Poly-β-hydroxybutyric acid in bacteria, 18
Polyisoprenoids, biosynthesis of, in bacteria, 34
Polyisoprenols, glycosyl derivatives of, in bacteria, 15
Polymerases in biosynthesis of bacterial exopolysaccharides, 186
Polysaccharide biosynthesis in diplococci, 202
Polysaccharides, extracellular, bacterial, 143
Polyunsaturated fatty acids in bacterial lipids, 2
Ponds, bdellovibrios in, 220
Pores and bdellovibrio penetration, 236
Potassium, effect of, on bacterial exopolysaccharide production, 148
limitation, effect of, on production of bacterial exopolysaccharides, 147
Precursors involved in biosynthesis of bacterial exopolysaccharides, 185
Predatory bacteria, 218
Principles of rapid microbial assessment methods, 107
Production of bacterial oxopolysaccharides, 146
Progeny, release of bdellovibrio, 238
Prokaryotic micro-organisms, nitrogen fixing, 60
Prokaryotic organisms, lipids of, 1
Properties of bacterial exopolysaccharides, 149
Properties of electron carriers in nitrogen fixation, 79
Properties of nitrogenases, 88
Propionibacteria, lipid composition of, 48
plasmalogens in, 11
Propionibacteriaceae, lipid composition of, 43
Prosthetic groups of ferredoxins, 82
Protease, lipids of, 38
Protease-negative mutants of bdellovibrios, 245
Protease of bdellovibrios, 244
Protection of respiratory systems in nitrogen fixers, 67
Protein content of *Aerobacter aerogenes*, 110
Protein content of bdellovibrios, 227
Protein, release of, following host cell combination with bdellovibrios, 241
Proteins, immune adherence reaction with, 116
Proteus rettgeri, estimation of, 127
P. vulgaris, chromatography of products from, 130
phospholipid composition of, 10, 41
Proton source, adenosine triphosphate as, in nitrogen fixation, 92
Protozoa, methylation of phosphatidylethanolamine in, 31
Pseudomonadaceae, lipids of, 37
Pseudomonadales, lipids of, 36
Pseudomonas aeruginosa, exopolysaccharide from, 180
growth of bdellovibrios in, 238
Ps. diminuta, glycolipids in, 14
Ps. fluorescens, detection of, by chemiluminescence, 119
growth of bdellovibrios in, 248

Ps. fluorescens—continued
lipids in, 10
Ps. putida, attack of, by *Bdellovibrio starrii*, 216
Ps. rubescens, glycolipids in, 14
Pseudomonas spp., exopolysaccharides from, 180
glycolipids in, 13
Psittacosis virus, detection of, 117
Psychotria bacteriophila, and nitrogen fixation, 63
Purification, of bacterial exopolysaccharides, 149
of nitrogenase, 88
Purines, leakage of, by bacteria, 132
Puromycin, effect of, on bdellovibrio penetration, 236
Purple photosynthetic bacteria, ferredoxins from, 82
Pyrograms, use of, in microbial detection, 131
Pyrolysis methods for detecting bacteria, 131
Pyrolysis products of bacteria, and estimation 110
Pyruvate, as a substrate with nitrogenase, 72
as an electron donor in nitrogen fixation, 64, 69, 70
in bacterial exopolysaccharides, 153
Pyruvyl residues in bacterial exopolysaccharides, 153
Pyruvylated sugars, properties of, 154, 155
Pyruvylfucose in bacterial exopolysaccharides, 154
Pyruvylgalactose residues in bacterial exopolysaccharides, 153

Q

Quaternary ammonium salts, use of, to purify bacterial exopolysaccharides, 150
Quellung reaction, 145
Quinone coenzymes in bacteria, 18

R

Radioactive antibody technique for estimating microbes, 127
Radioactive gases, estimation of, to assay microbial populations, 125
Radioassay of microbial populations, 129
Radioisotopes of iodine, use of, in estimating microbial populations, 115
Rapid broad-spectrum methods in microbial detection, 118
Rapid detection, of sparse microbial populations, 105
of viruses, 132
Rapid determination of microbial viability, 131
Rapid specific identification methods for microbes, 126
Rate of respiration of bdellovibrios, 240
Recognition of host by bdellovibrios, 232
Recognition of prey by bdellovibrios, 229
Recovery of bdellovibrios, 220
Redox dyes, reduction of, by bacteria, 132
Redox properties of flavodoxins, 84
Redox proteins in nitrogen fixation, 69
Reducing electrons in nitrogen fixation, 60
Reducing site in blue-green algal heterocysts, 68
Reducing potential of ferredoxins, 82
Reductant, need for, in nitrogen fixation, 69
Reduction of atmospheric nitrogen, 59
Reduction of nitrogenase by nicotinamide nucleotides, 73
Reduction of redox dyes by bacteria, 132
Refractive index, changes of, due to microbial activity, 132
Regulation of nitrogen fixation in bacteria, 69
Release of bdellovibrio progeny, 238
Release of compounds following bdellovibrio invasion, 241
Repeating units in pneumococcal exopolysaccharides, 182
Respiration of bdellovibrios, 252
Respiration rate of host-bdellovibrio associations, 240
Resting bodies in bdellovibrios, 226
Rhamnose, in capsular polysaccharides of *Escherichia coli*, 169
in Klebsiella exopolysaccharides, 173
isomerase, use of, in sugar analysis, 151
Rhizobia, parasitization of, by bdellovibrios, 251
Rhizobiaceae, lipids of, 38
Rhizobium ferredoxin, properties of, 82
Rhizobium japonicum, cell-free synthesis of glucan by, 190
nicotinamide nucleotides in nitrogen fixation by, 75
nitrogen fixation by, 60, 64
pathway of nitrogen fixation in, 95
properties of electron carriers in, 79
R. meliloti, extracellular polysaccharide production by, 147
Rhodopseudomonads, lipids of, 36

Rhodopseudomonas spheroides, ornithine-containing lipid in, 15
Rhodospirillum rubrum, effect of bdellovibrio invasion on motility of, 239
infection of, by bdellovibrios, 225
lipids in, 11
nitrogen fixation by, 61, 64
nitrogen fixation in, 68
pathway of nitrogen fixation in, 95
Rhodospirillum spp., lipids of, 36
Ribitol phosphate in pneumococcal exopolysaccharides, 182
Ribonucleic acid content of *Aerobacter aerogenes*, 110
Ribosidic linkages in bacterial exopolysaccharides, 158
Ribosomes of bdellovibrios, 224
Rickettsia, immune adherence reaction with, 116
Rivers, bdellovibrios in, 220
Root nodules, nitrogen fixing, 60
Royce particle counter, 124
Rumen bacteria, branched fatty acids in 21
Ruminococcus flavefaciens, plasmalogens in, 11

S

Saccharomyces cerevisiae, detection of, by chemiluminescence, 119
growth of bdellovibrios in cell-free extracts of, 238
Salmonella newington, polyisoprenoids of, 187
S. typhimurium, attachment of bdellovibrios to, 233
Salmonelleae, lipids of, 38
Saprophytic bdellovibrio populations, 217
Sarcina lutea, detection of, by chemiluminescence, 119
neutral lipid composition of, 46
Sarcina spp., phospholipid composition of, 8
Saturated fatty acids in bacteria, 3, 42
Seawater, bdellovibrios in, 220
Selemononas ruminantium, plasmalogens in, 11
Semliki forest virus, composition of, 111
Separation of nitrogenase proteins from *Azotobacter vinelandii*, 89
Sequences in bacterial exopolysaccharides, 156
Serotypes, of *Escherichia coli*, 167
of pneumococci, 181
Serratia marcescens, biosynthesis of cyclopropane fatty acids in, 26
Serratia marcescens—continued
detection of, by chemiluminescence, 119
fatty acids in, 3
unsaturated fatty acids in, 25
Serratieae, lipids of, 38
Serum virus antibody, detection of, 117
Sewage, as a source of bdellovibrio bacteriophages, 256
bdellovibrios in, 220
Sheath on flagella of bdellovibrios, 223
Shigella boydii, growth of bdellovibrios on, 247
Sideroeapsaceae, lipids of, 37
Silting index, nature of, 112
Sites, active, on the nitrogenase enzyme, 93
Size of microbes in relation to rapid assessment methods, 107
Skim milk, use of, in preserving *Bdellovibrio* cultures, 220
Slime polysaccharides of bacteria, 143
Sodium chloride requirements of marine bdellovibrios, 249
Soil bacteria, extracellular glucan production by, 166
extracellular polysaccharides of, 144
Soil, bdellovibrios in, 220
fertility, importance of bacterial exopolysaccharides and, 144
Solute penetration by bdellovibrios, 240
Soybean root nodule, ferredoxins isolated from, 82
Soybean root nodules, nicotinamide nucleotides and nitrogen fixation in, 74
Soybeans, nitrogen fixation by, 60
Sparse microbial populations, rapid detection of, 105
Specific enzymic methods for assay of bacterial exopolysaccharide sugars, 151
Specific identification methods for detecting microbes, 107
Specific methods, rapid, for microbes, 126
Specificity in rapid methods for detecting microbes, 106
Specificity of bdellovibrio attachment, 233
Sphaerophorus ridiculosus, plasmalogens in, 11
Sphaeroplasting of bdellovibrios, 229
Sphaerotilus natans, extracellular polysaccharide production by, 148
Sphingolipids, in bacteria, 14
of bdellovibrios, 228
Spinach chloroplast ferredoxin reductase, 82
Spirillum spp., relations of bdellovibrios to, 221
serpens, attachment of bdellovibrios to, 234

Spirochaetales, lipid composition of, 43, 45
lipids in, 49
Spore-forming bacilli, phosphatidylglycerol in, 8
Staining methods for detecting microbes, 121
Staining of bacterial capsules, 144
Staphylococci, glycolipids in, 14
Staphylococcus aureus, phospholipid composition of, 41
Starch granules, immune adherence reaction with, 116
Sterol requirement of mycoplasmas, 17
Sterols, possible presence in bacteria, 17
Straight-chain fatty acids, biosynthesis of, in bacteria, 19
Streptococci, fatty acids in, 3
production of extracellular polysaccharides by, 167
Streptococcus faecalis, biosynthesis of glycolipids in, 34
chromatography of products of, 130
Streptomyces aureofaciens, lipids in, 11
Strep. olivaceus, sterols in, 17
Streptomycetes, nitrogen fixation by, 61
Streptomycin, effect of, on bdellovibrio penetration, 236
Structural features of bacterial exopolysaccharides, 156
Structure of bdellovibrios, 222
Structures, of bacterial exopolysaccharides, 165
of bacterial heteropolysaccharides, 167
of bacterial homopolysaccharides, 166
Substrate-binding site of nitrogenase, 93
Subunit nature of nitrogenase, 90
Succinate dehydrogenase in bdellovibrios, 253
Succinate in bacterial exopolysaccharides, 155
Sugar nucleotides in biosynthesis of bacterial exopolysaccharides, 185
Sugar residues in bacterial exopolysaccharides, 150
Sulphate ester lipids in halophilic bacteria, 12
Sulphur compounds, effect of deficiency of, on production of bacterial exopolysaccharides, 147
Sulphur content of ferredoxins, 80
Surface bonding and bdellovibrio attachment, 233
Survival, microbial, and extracellular polysaccharides, 144
Symbiosis and bdellovibrios, 228
Symbiotic nitrogen fixation, 60
Syphilis, fluorescent antibody test for, 115

T

Talose of bacterial exopolysaccharides, 182
Taxonomic significance of bacterial lipid compositions, 35
Taxonomy of bdellovibrios, 221
Teichoic acids of bacteria, 143
Temperature, and bdellovibrio attachment, 233
effect of, on bdellovibrio-host cell kinetics, 246
Terminal steps in synthesis of unsaturated fatty acids in bacteria, 24
Terminally branched fatty acids, biosynthesis of, in bacteria, 21
Terminology of bdellovibrios, 221
Thiobacillus novellus, phospholipid composition of, 7
T. thio-oxidans, lipids in, 26
phosphatidylethanolamine in, 6
phospholipid composition of, 41
Thiobacteriaceae, lipids of, 36
Time-course of polysaccharide synthesis by *Klebsiella aerogenes*, 149
Transacylation in bacterial phospholipid biosynthesis, 30
Transfer of sugars to lipids in polysaccharide biosynthesis, 193
Transferases in biosynthesis of bacterial exopolysaccharides, 193
Treponema pallidum, lipids in, 49
plasmalogens in, 11
Treponemataceae, lipid composition of, 45
Tricarboxylic acid cycle in bdellovibrios, 253
Triglycerides in micro-organisms, 18
Tuberculostearic acid in bacteria, 27
Two-membered cultures of bdellovibrios, 241
Typing of bdellovibrio bacteriophages, 257
Types of pneumococcal exopolysaccharides, 181

U

Ultracentrifugation in methods for rapid assessment of viral populations, 108
Ultrastructure of bdellovibrios, 222
Ultraviolet microscopy in detecting microbes, 114
Undecaprenol phosphates in bacteria, 17
Uranyl acetate and staining of bdellovibrios, 223
Urea, effect of, on bdellovibrios, 254
Uridinediphospho sugars in biosynthesis of bacterial exopolysaccharides, 185

Uronic acid in Klebsiella exopolysaccharides, 173
Uronic acids in bacterial exopolysaccharides, 152
Use of adenosine triphosphate in nitrogen fixation, 69
Utilization of extracellular polysaccharides by bacteria, 148

V

cis-Vaccenic acid, formula of, 2
Vaccinia virus, determination of, 133
Vanadium and nitrogen fixation, 64
Vanadium-containing nitrogenases, 94
Variation of lipid composition in a species of bacterium, 46
Variola virus, determination of, 133
Veillonella gazogenes, plasmalogens in, 11
Viability, microbial, rapid determination of, 131
Vibrio spp., relation of bdellovibrios to, 221
Viral antigen, detection of, 117
Viruses, detection of, 116
  immune adherence reaction with, 116
  rapid detection of, 106, 132
Viscous slimes produced by bacteria, 149
Vishniac, Wolf, contribution of, to estimation of microbial populations, 125
Vital staining of bacteria, 131
Vitamin requirements of bdellovibrios, 247

W

Walls of bdellovibrios, composition of, 228
Washed cell suspensions, production of polysaccharides by bacteria in, 148
Weights of microbes in relation to rapid assessment methods, 108
Wolf trap, nature of, 112, 125

X

Xanthomonas campestris, composition of exopolysaccharide from, 153
  oxopolysaccharide from, 177
X. phaseoli, production of enzymes by, that hydrolyse bacterial oxopolysaccharides, 159
X-Ray diffraction analysis of ferredoxins, 83

Y

Yeast extract as a substrate for bdellovibrios, 24
Yeast, fatty acid synthesis in, 20
  methylation of phosphatidylethanolamine in, 31
Yeasts, immune adherence reaction with, 116

Z

Zinc, and nitrogenase, 91
  and preservation of bdellovibrios, 223