STUDIES ON THE CELL MEMBRANE OF BACILLUS AMYLOLIQUEFACIENS IN RELATION TO EXTRACELLULAR ENZYME SECRETION

A Thesis

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TABLE OF CONTENTS

SUMMARY

1. Purified cell membrane preparations were isolated from Bacillus amyloliquefaciens by a variety of procedures and various properties of the membrane were studied.

2. Morphological examination of membranes was performed by both conventional and freeze-fracture electron microscopy. Studies were undertaken on intramembranous particle size and distribution, possible membrane-ribosome associations, membrane-associated particles, and on the fine structure of the membrane.

3. Physical properties of the cell membrane were examined by spin-labelling, calorimetric and fluorescence techniques in combination with the activities of membrane-associated enzymes. Arrhenius plots of enzyme activities and spin-label motion yielded 4 discontinuities at 4 critical temperatures. Calorimetry confirmed the occurrence of lipid phase transitions in the membrane. Although the events which occur in the membrane as a function of temperature are not understood at the molecular level, the results are tentatively interpreted in terms of the occurrence of two lipid phase transitions which may be the result of asymmetry in the phospholipid distribution between the two halves of the lipid bilayer of the membrane.

4. The lipid content of the cell membrane was 20% by weight and of this, 70% was phospholipid; cardiolipin was the predominant phospholipid. Branched-chain fatty acids made up over 80% of the total fatty acids and of these iso and anteiso acids of 15 and 17 carbon atoms and iso acids of 14 and 16 carbon atoms were predominant. Less than 1% of the total fatty acids were unsaturated. Straight-chain fatty acids and unsaturated fatty acids were secreted by this organism, however their direct

involvement in extracellular enzyme secretion was excluded.

5. The membrane fatty acid composition and thus presumably membrane fluidity, could be altered. Growth at lower temperatures increased the proportion of branched-chain and unsaturated fatty acids, at the expense of straight-chain acids. Branched-chain monounsaturated-fatty acids were detected in the membrane under these conditions. Inclusion of alcohols in the growth media also affected membrane fatty acid composition.

6. The secretion of extracellular enzymes in cells exhibiting altered fatty acid composition as a result of the above methods was examined. Considerable effects were observed which may be a reflection of changed membrane composition.

7. The phenomenon of cold shock in which cells, subjected to rapid passage across critical temperature zones lose their normal permeability characteristics, was examined. Two critical temperature zones were found which may relate to physical events in the membrane lipid. It was found possible to modulate cold shock in parallel to changes in membrane lipid composition.

STATEMENT

This thesis contains no material which has been accepted for the award of any other degree or diploma in any University.

The work presented in Section 6.3 was carried out in collaboration with Mr. J. Paton. Apart from this, the remainder of the work was carried out by myself.

To the best of my knowledge, this thesis contains no material previously published or written, except where due reference is made in the text.

Signed:

EDWARD JOHN McMURCHIE.

ABBREVIATIONS

The abbreviations used in this thesis are acceptable to the Journal of Molecular Biology, or are defined in the text.

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CHAPTER 1

INTRODUCTION

GENERAL INTRODUCTION

The work described in this thesis is an attempt to gain greater understanding of the process of extracellular enzyme secretion by bacteria. The model system chosen is the secretion of protease, α-amylase and ribonuclease by Bacillus amyloliquefaciens.*.

The bacterial cell membrane is a permeability barrier to the secretion of extracellular enzymes and it may well be indirectly involved in the synthesis of these enzymes. Thus a study on the properties of this membrane may provide greater insight into the mechanism of secretion. In the course of this work, the results obtained were relevant to basic membrane biology, and although attempts were made to relate these to the process of secretion, this study remained principally one on the cell membrane per se. For this reason it was decided that a lengthy review on extracellular enzyme secretion was not warranted. A recent review on extracellular enzyme secretion by bacteria by Glenn (1976), adequately covers this aspect.

As this thesis is concerned with the physical and chemical properties of the bacterial cell membrane and their relation to numerous physiological processes, discussion on these aspects is left to the appropriate chapter.

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* This strain of organism, previously thought a subspecies of Bacillus subtilis was designated Bacillus amyloliquefaciens by Welker and Campbell (1967) on the basis of DNA base composition and hybridization studies.

INTRODUCTION

Bacterial extracellular enzymes are mainly degradative and their most obvious role appears to be the breakdown of large molecular weight compounds which exist in the environment, for subsequent utilization by the cell (Mandelstam, 1969; Engelking and Seidler, 1974), although some claims have also been made for their involvement in sporulation (Mandelstam and Waites, 1968; Hanson et al., 1970). The manner by which these enzymes are synthesized and secreted by the cell is not clear, and the fact that these enzymes are potentially lethal to the cell makes the problem all the more interesting.

This introduction firstly reviews the secretion process in the mammalian system as it is relevant to the bacterial system. This is followed by a review of extracellular enzyme secretion by bacteria.

A. SECRETION IN ANIMAL CELLS

In certain mammalian tissues involved in exoprotein secretion, such as the pancreas, secretory granules may be utilized in which the exoprotein is packaged in membrane vesicles and subsequently released from the cell by a process resembling reverse pinocytosis (Palade et al., 1962; Jamieson and Palade, 1967; Castle et al., 1972). However, in a manner similar to the bacterial cell, the primary secretion process remains one in which the exoproteins are initially secreted through the endoplasmic reticular membrane.

There is a considerable body of evidence to suggest that for mammalian cells engaged in secretion, exportable proteins are synthesized on membrane-bound polysomes whereas proteins not destined for secretion are synthesized on free polysomes (Redman et al., 1966; Ganoza and Williams, 1969;

Takagi et al., 1970; Borgese et al., 1973). Observations together with various models have been made for m-RNA discrimination which allows for this selective synthesis (Cioli and Lennox, 1973; Zauderer et al., 1973).

The experimental basis for the direct translational-extrusion model to account for the transport of exoproteins across the membrane originates from the work on microsomal preparations. Such preparations are vesicular and have ribosomes bound to the external surface by ionic attraction and/or nascent peptide chain (Adelman et al., 1973). They are capable of synthesizing exoprotein in vitro, the exoprotein remaining associated with the microsome as do puromycin-released nascent polypeptides. Both exoprotein and nascent peptides can be subsequently released from the microsome after solubilization of the membrane with deoxycholate (Redman and Sabatini, 1966; Redman et al., 1966; Redman, 1967; Andrews and Tata, 1971; Bevan, 1971). Although such a result provides evidence confirming the point that exoproteins are capable of being synthesized on membrane-bound ribosomes, it does not provide confirmation on a direct transmembrane extrusion of such exoproteins as the exact location of the synthesized material is not known and cannot be established by the solubilization procedures employed (Rothman, 1975).

The signal hypothesis proposed by Blobel and Dobberstein (1975a,b), is a major new concept in the secretion of proteins by eukaryote cells. It is postulated that the information for segregation of a translation product across a membrane is encoded in the m-RNA by means of a unique sequence of codons or signal sequence, rather than in the protein-synthesizing apparatus. The resulting nascent chain is postulated to contain a unique sequence of amino acid residues on the

amino terminal end which may be involved in the attachment of ribosome to membrane and the subsequent transfer of the nascent chain across the membrane. It is further postulated that this amino terminal sequence is then processed and removed by membrane-associated activity (Blobel and Dobberstein, 1975a,b).

B. EXTRACELLULAR ENZYME SECRETION BY BACTERIA

The synthesis and secretion of bacterial extracellular enzymes can be considered analogous to the primary secretion process for the animal system as proposed by Redman (1967). With this in mind, a translational-extrusion model for extracellular enzyme secretion was proposed which was compatible with the known facts on bacterial secretion. The translational-extrusion model proposed by May and Elliott (1968), for the secretion of protease, α-amylase and ribonuclease by Bacillus amyloliquefaciens provides a working model for the study of bacterial exoenzyme secretion. Synthesis of extracellular enzymes is postulated to be carried out by membrane-associated ribosomes with the nascent polypeptide chain being vectorially extruded through the cell membrane to take up its active configuration outside the permeability barrier of the cell membrane. It is proposed that the extracellular enzymes are at no time present within the cytoplasm. Support for this model rests on a number of observations in both this and other bacteria. Firstly, within the cytoplasm, little or no extracellular enzyme activity is detectable (Kushner and Pollock, 1961; McDonald and Chamber, 1966; May and Elliott, 1968; Casas and Zimmerman, 1969; Glew and Heath, 1971). Secondly, there exists a tight coupling between extracellular enzyme synthesis and secretion (Both et al., 1972). Thirdly, the presence of a ribonuclease inhibitor within the cytoplasm which forms a virtually irreversible complex with the

exo-ribonuclease, argues against the free existence of ribonuclease in the cytoplasm (Smeaton et al., 1965; Smeaton and Elliott, 1967b). Fourthly, there is direct evidence that for some extracellular enzymes, the emerging form of the enzyme differs from the native form in regard to susceptibility to proteolytic attack (Bettinger and Lampen, 1975; Sanders and May, 1975).

Although these observations are compatible with the model proposed, the possibility still remains that an inactive precursor or zymogen form of the enzyme exists in the cytoplasm, as is the case for the protease of Streptococcus faecalis (Lin et al., 1963). Alternatively, inactive sub-units of the exoenzyme may exist in the cytoplasm and come together to form the active enzyme only outside the permeability barrier of the cell membrane (Torriani, 1968). At present there is a lack of direct evidence demonstrating that exoenzymes are synthesized on membrane-bound ribosomes. Indeed the existence of membrane-bound ribosomes is still a contentious issue unlike the situation in eukaryotes. Membrane-bound ribosomes have been reported for a variety of bacteria (Schlessinger, 1963; Fitz-James, 1964; Pfister and Lundgren, 1964; Schlessinger, et al., 1965; Moore and Umbreit, 1965; Coleman, 1969a,b; Varricchio, 1972), however, much of this evidence does not take into account the physical entrapment of ribosomes within membrane vesicles, or the possibility that binding to the membrane may be mediated by the presence of lysozyme (Patterson et al., 1970). The apparent level of membrane-bound ribosomes is influenced by the ionic environment (Coleman, 1969a,b), and although it cannot be excluded that the ionic conditions may be important for maintaining possible membrane-ribosome interactions as is the case for mammalian systems (Sabatini et al., 1966), this may only reflect differences in the extent of ribosomal entrapment.

The assumption that membrane-bound ribosomes are responsible for the synthesis of extracellular enzymes in bacteria raises other problems such as the manner by which m-RNA specific for the extracellular enzymes are transported to and recognized by membrane translational sites. A possible "signal" sequence on the 5'-end of the m-RNA which allows initiation to occur only at such sites,may overcome the problem of recognition. The problem of transportation from gene to peripheral translation site is a formidable one in view of the unlikely possibility that exoenzyme m-RNA's are stable (Both et al., 1972; Glenn et al., 1973). A possible means of overcoming this problem is interpreted from the results obtained for B. amyloliquefaciens in which protease secretion is maintained for periods of up to 60 minutes in the abscence of RNA synthesis (Both et al., 1972). The suggestion is made that excess m-RNA is produced and that during migration from gene to membrane translation site, sufficient m-RNA reaches and saturates such sites despite its rapid breakdown in the cytoplasm en route.

Irrespective of the site of extracellular enzyme synthesis, the molecular mechanism of secretion still remains unclear. Although the major permeability barrier to secretion is the cell membrane, the cell wall may be responsible for partially restricting the passage of the exoenzyme out of the cell (Gould et al., 1975) as well as providing the emerging exoenzyme some protection from proteolytic attack (Bettinger and Lampen, 1975; Sanders and May, 1975).

The hydrophobic lipid bilayer of the bacterial cell membrane would be expected to offer the greatest permeability barrier to the secretion of extracellular enzymes. Although the extracellular enzymes are hydrophilic in their native form and in general of molecular weight between 20,000 to 40,000

(Glenn, 1976), this does not exclude the possibility that they may be modified in some manner or have some special properties which facilitate their transport across the membrane. Indeed it has been proposed that due to the lack of cysteine, bacterial extracellular enzymes may acquire a more flexible structure to allow passage through the membrane (Pollock and Richmond, 1962). It has been shown that for E. coli alkaline phosphatase, assembly of the active (dimeric) exoenzyme takes place in the periplasm after secretion of monomers (Schlessinger, 1968; Schlessinger and Olson, 1968; Torriani, 1968), and this would allow for a much smaller part of the exoenzyme to be secreted at any one time.

The attachment of lipid, or the presence of a terminal sequence of hydrophobic amino acids on the exoenzyme could presumably facilitate its transfer through the cell membrane. In this regard Bacillus licheniformis 749/C has been shown to produce two forms of penicillinase (Sawai et al., 1973; Sawai and Lampen, 1974; Yamamoto and Lampen, 1975), although only one structural gene for the enzyme is present (Sherratt and Collins, 1973). One form of penicillinase is a soluble exopenicillinase and the other is a hydrophobic membrane-bound form which differs from the soluble form only by the presence of a phospholipopeptide chain. This segment at the amino-terminal end consists of 25 amino acids and a phosphatidyl serine group (Sawai et al., 1973). The in vitro synthesis of the hydrophobic penicillinase containing the covalently attached phospholipid has also been achieved (Dancer and Lampen, 1975). In addition several forms of α-amylase have been detected associated with the cell membrane of Bacillus subtilis (Marburg) (Nagata et al., 1974) and B. amyloliquefaciens (Fernández-Rivera Río and Arroyo-Begovich, 1975). For this latter organism the need to use detergents to

solubilize one of these forms from the cell membrane, suggests that it may be secreted in a manner similar to that of B. licheniformis penicillinase. Phospholipids have also been implicated in association with extracellular lipase of Acinetobacter O_{16} (Breuil and Kushner, 1975). It has also been suggested that phospholipid may be co-secreted with extracellular enzymes in B. amyloliquefaciens (Glenn and Gould, 1973).

These results suggest that if some form of hydrophobic domain is required for insertion and transport of the exoenzyme across the cell membrane, it could be an intrinsic property of the exoenzyme or it may be acquired by means of co-secretion of a hydrophobic "carrier" molecule. The fluidity of the cell membrane may also influence the secretion of extracellular enzymes as has been suggested for the secretion of alkaline phosphatase in fatty acid auxotrophic strains of E. coli (Kimura and Izui; 1976) and in Bacillus caldolyticus in which changes in protease secretion were correlated to changes in the membrane fatty acid composition (Lauwers and Heinen, 1973).

AIMS OF THE WORK IN THIS THESIS

The aim of this work was to provide greater understanding of the process of extracellular enzyme secretion across the bacterial cell membrane. It was initially intended to study some of the properties of the cell membrane using isolated membranes and then determine whether changes in the cell membrane could influence the secretion of extracellular enzymes. As the cell membrane exhibited some interesting physical properties in regard to the thermal behaviour of the membrane lipids, it was decided to pursue this line of investigation in greater detail. Thus a considerable portion of this thesis is concerned with a study of the physical properties of the membrane and their influence on such physiological processes as extracellular enzyme secretion and cold shock.

Chapter 3 deals firstly with the isolation of cell membranes of Bacillus amyloliquefaciens using both osmotic and mechanical procedures. The morphology of the membrane both in situ and in membrane preparations was examined particularly in regard to the fine structure of the membrane and possible membrane-ribosome associations. As the isolated membranes were found to be vesicular, attempts were made to use these membrane vesicles directly in the study of extracellular enzyme secretion.

The physical properties of the cell membrane were examined in Chapter 4. Of principle interest was the behaviour of the membrane as a function of temperature and this was studied using physical and physiological techniques. As the results of this study indicated that the membrane lipid was important in determining the physical behaviour of the membrane, a study was then made on the lipid components of the cell membrane.

Chapter 5 deals with the lipid components of the membrane and particularly the fatty acids which are considered

important in determining the physical state and the fluidity of the membrane. Attempts were made to alter the fatty acid composition of the membrane (and thus presumably the membrane fluidity), by altering the growth temperature or by including alcohols in the growth medium. These attempts proved successful. Cells which exhibited an altered fatty acid composition as a result of the procedures employed in Chapter 5, were subsequently used to determine the influence of membrane lipid composition on extracellular enzyme secretion. This work is described in Chapter 6. The work in this chapter also includes a study on the phenomenon of cold shock which was thought to be a consequence of temperature-induced changes in the physical state of the membrane. The influence of altered membrane fatty acid composition on cold shock was also investigated with the view of reducing or even eliminating the incidence of cold shock.

CHAPTER 2

MATERIALS AND METHODS

MATERIALS AND METHODS

A. MATERIALS

1. Bacterial Strains

An unclassified strain of Bacillus amyloliquefaciens was obtained from the Takamine Laboratories Inc., Clifton, N.J., U.S.A. This organism was originally classified as a strain of B. subtilis, but has since been identified as B. amyloliquefaciens (Welker and Campbell, 1967) on the basis of DNA base composition, DNA hybridization studies and transduction experiments. A mutant strain of B. amyloliquefaciens designated LF^- which did not produce a surfactin-like lytic factor (May and Elliott, 1970), was routinely used in this work. In addition the wild type (WT) strain of B. amyloliquefaciens was also used for some experiments where indicated in the text.

B. subtilis 168 ($Tryp^-$) was a tryptophan-requiring mutant kindly provided by Dr. A. R. Glenn of this Department.

2. Liquid-growth medium

B. amyloliquefaciens was grown in a salts-maltose-Casamino acids growth medium described by Both et al., (1972).

B. subtilis was grown in a salts-glucose-Casamino acids growth medium described by Bishop et al., (1967), however in addition it contained 0.5 mg/ml Tryptophan.

3. Washed-Cell Suspension Medium

Washed-cell suspension medium (WCSM) was the same as liquid growth medium except that $FeCl_3$ and yeast extract were

omitted thereby limiting cell growth, and the amount of Bacto Casamino acids was reduced to give a final concentration of 0.05% (w/v).

4. Protoplast Medium

Protoplast medium (PM) contained 25mM tris, 0.125mM $CaCl_2$ 0.0125mM $ZnSO_4$, 5.0mM KCl, 3.7mM $(NH_4)_2 HPO_4$, 0.025% (w/v) Bacto Casamino acids, 1%(w/v) maltose, 22%(w/v) sucrose, 5mM $MgSO_4$ and 0.25 ml/litre of the trace metal solution described previously for the liquid growth medium (Both et al., 1972). The medium was adjusted to pH7.3 with HCl.

5. Antibiotics

Chloramphenicol was obtained from Parke Davis and Co., Sydney, Australia, as "chloromycetin".

6. Radioisotopes

All radioactive materials were obtained from Schwartz/ Mann, Orangeburg, New York.

7. Enzymes

Lysozyme was three-times crystallised from egg white and was supplied by Sigma Chemical Co. Ribonuclease-A from Bovine pancreas, Type III-A and Deoxyribonuclease I from Beef pancreas, (noncrystalline) were supplied by Sigma Chemical Co.

8. Buffers

a) Phosphate buffers were prepared from K_2HPO_4 and KH_2PO_4.

b) Crystalline tris was obtained from Sigma Chemical Co. as "Sigma 7-9". Solutions of tris were adjusted to the

required pH by the addition of HCl.

All buffers and solutions were prepared in double distilled water, except for the liquid-growth medium in which monodistilled water was used.

9. Enzyme Substrates

a) Protease substrate: Remazol Brilliant Blue (RBB) was a generous gift from Farbwerke Hoechst AG, Frankfurt and Hide Powder was purchased from Calbiochem, San Diego, California. The Remazol Brilliant Blue-Hide Powder (RBB-Hide) complex was prepared according to the method of Rinderknecht et al., (1968).

b) Ribonuclease substrate: Yeast RNA of high molecular weight was prepared according to the method of Crestfield et al., (1955).

c) α-Amylase Substrate: "Phadebas" tablets were supplied by Pharmacia (South Seas) Pty. Ltd., Lane Cove, N.S.W.

10. Spin Labels

Spin labels were either the methyl ester (m.e.) or acid form (a) of stearic acid with the nitroxide group attached to carbon atom 12, 12NSm.e. or 12NSa [2- (10-carboxydecyl) -2-hexyl-4, 4-dimethyl-3-oxazolidinyloxyl], or to carbon atom 16, or 16NSm.e. or 16NSa [2- (14-carboxytetradecyl)-2-ethyl-4, 4-dimethyl-3-oxazolidinyloxyl]. Spin labels were purchased from Synvar, Palo Alto, California.

11. Gas Liquid Chromatography Columns

All columns were prepacked by Applied Science Laboratories Inc., Pa. U.S.A. The following stationary phases were routinely used for the analysis of fatty acid methyl esters: (Non polar), 3% Apiezon L; 5% Apiezon L; (Polar), 10%

EGSS-X (ethylene succinate-methyl silicone polymer, (low silicone)); 25% DEGS (diethylene glycol succinate). All stationary phases were coated onto 100-120 mesh, acid-washed, silanized Chromosorb P. Columns were 1/8" O.D. by 6 ft. stainless steel.

12. Fatty Acid Standards

The following standards were purchased as the methyl esters from Applied Science Laboratories Inc. Pa. U.S.A., either individually or as a prepared mix. Standards had a stated purity of greater than 96%.

Methyl	12-methyltridecanoate	iso	$C_{14:0}$
"	12-methyltetradecanoate	anteiso	$C_{15:0}$
"	14-methylpentadecanoate	iso	$C_{16:0}$
"	14-methylhexadecanoate	anteiso	$C_{17:0}$
"	16-methylheptadecanoate	iso	$C_{18:0}$
"	16-methyloctadecanoate	anteiso	$C_{19:0}$
"	18-methylnonadecanoate	iso	$C_{20:0}$
"	18-methyleicosanoate	anteiso	$C_{21:0}$
"	caprate	(normal) n	$C_{10:0}$
"	undecanoate	n	$C_{11:0}$
"	laurate	n	$C_{12:0}$
"	tridecanoate	n	$C_{13:0}$
"	myristate	n	$C_{14:0}$
"	pentadecanoate	n	$C_{15:0}$
"	palmitate	n	$C_{16:0}$
"	heptadecanoate	n	$C_{17:0}$
"	stearate	n	$C_{18:0}$
"	nonadecanoate	n	$C_{19:0}$
"	arachidate	n	$C_{20:0}$
"	heneicosanoate	n	$C_{21:0}$
"	behenate	n	$C_{22:0}$
"	palmitoleate, (cis-9-hexadecenoic)	n	$C_{16:1}$
"	oleate, (cis-9-octadecenoic)	n	$C_{18:1}$

Methyl 10-methyldodecanoate (anteiso $C_{13:0}$) was purchased from Analabs Inc., New Haven, Conn. U.S.A.

13. Solvents

Chloroform was A.R. grade supplied by British Drug Houses (B.D.H.) Ltd. Methanol (B.D.H.) was A.R. grade and redistilled over anhydrous $CaCl_2$ before use. Ether was redistilled before use. Petroleum ether (B.D.H.) 40-60° fraction,

was first stirred overnight with concentrated H_2SO_4, dried using NaOH pellets and redistilled over NaOH. Only the fraction distilling between 40^o and 60^o was used. Acetone was redistilled over $KMnO_4$. Hexane (Ajax Chemical Co. Australia) was redistilled and stored in the presence of sodium wire. 14% (w/v) boron trifluoride in methanol was used as supplied by Applied Science Laboratories Inc. Pa. U.S.A. Carbon disulphide was supplied by May and Baker Ltd. and dried using anhydrous $CaCl_2$.

All solvents except the boron trifluoride in methanol were degassed with N_2 before use.

14. Glassware

All glassware used for lipid analysis was chromic acid washed and rinsed with solvent before use.

15. Non-ionic detergent

Tween 80 (sorbitan mono-oleate polyoxyethylene) was supplied by Sigma Chemical Co.

B. METHODS

1. Growth of cells

A. B. amyloliquefaciens

Cultures were grown from spore suspensions in liquid growth medium as described by Both et al., 1972. Cells were grown by shaking in an incubator (Model 461, Paton Industries, S.A.) at 250 cycles per minute. Growth temperature was 30^o unless otherwise stated. Cultures were harvested at an O.D. $_{600nm}^{1cm} = 3.6$, which corresponds to late-log phase. and to a dry weight value of 1.9 mg/ml of cell culture.

B. B. subtilis

Cultures were grown in liquid-growth medium described in the Materials section, from spore suspensions using the procedure of Bishop et al., 1967. Cells were grown by shaking in an incubator as described above, at 37°. Cultures were harvested at an O.D. $\overset{1cm}{}_{600nm}$ = 3.0, which corresponds to late-log phase.

2. Harvest of cells

A. B. amyloliquefaciens

Cells from late-log phase culture were harvested, washed in washed-cell suspension medium (WCSM), recentrifuged and finally resuspended to the required dilution in WCSM.

B. B. subtilis

Cells from late-log phase culture were harvested as described above for B. amyloliquefaciens, however, cells required for lipid extraction were washed in 50mM Tris buffer, pH7.3.

3. Preparation of protoplasts

Protoplasts were prepared from the mutant strain (LF^-) of B. amyloliquefaciens which does not produce the protoplast-bursting factor described by May and Elliott (1970). The culture was harvested, washed once in WCSM and finally resuspended in protoplast medium to the original cell density. The suspension was shaken slowly (50 cycles per minute) in the presence of 133 µg/ml lysozyme. Complete protoplast formation as monitored by phase contrast microscopy occurred after about 60 minutes and at this time the protoplasts were harvested by centrifugation at 15,000 g for 15 minutes and carefully resuspended in PM to the dilution required. All operations were performed at 30°.

4. Preparation of membranes by osmotic lysis of cells

Cells harvested as described were washed by centrifugation and resuspension in 50mM potassium phosphate buffer, pH 7.3 at 30° and finally resuspended to a concentration of 8g (wet weight) per litre. Lysozyme, DNAase and RNAase were added at final concentrations of 300, 10 and 10 g/ml respectively. The suspension was incubated at 30° with vigorous stirring. After 15 minutes $MgSO_4$ was added at a final concentration of 10mM and the suspension left for a further 30 minutes. Sodium ethylenediaminetetraacetic acid (EDTA) was then added to a final concentration of 15mM (to facilitate the release of RNA from ribosomes). The incubation was continued for 15 minutes after which the $MgSO_4$ concentration was increased to a final concentration of 20mM. After a further 15 minutes incubation the membranes were harvested by centrifugation at 35,000g for 30 minutes, the temperature of the suspension being lowered to 1° during this step. The pellet was resuspended in ice-cold 0.1M potassium phosphate buffer pH7.3 by homogenization in a glass-teflon homogenizer. Unlysed cells and cell wall debris were removed by centrifugation of the membrane suspension at 4,000g for 10 minutes. The supernatant remaining after this centrifugation was centrifuged at 35,000g for 30 minutes and the resulting membrane pellet was twice washed by centrifugation and resuspension in ice-cold 0.1M potassium phosphate buffer, pH7.3. Membranes were finally resuspended in this same buffer to a concentration of 6 to 8mg protein/ml by means of a glass-teflon homogenizer. Membranes were either used immediately or otherwise stored at -80° until required. Membranes used after freezing and subsequent thawing were not reused.

5. Preparation of membranes by osmotic lysis of protoplasts

Protoplasts prepared from late-log phase B. amyloliquefaciens LF^- cells were pelletted by centrifugation and resuspended in a small volume of PM at 30°. Protoplasts were lysed in 100 volumes of 50mM potassium phosphate buffer containing 10µg/ml each of DNAase and RNAase. The mixture was vigorously stirred at 30° and membranes were prepared exactly as described above from the stage at which $MgSO_4$ was first added.

6. Preparation of membranes by the French pressure cell

Cells were twice washed by centrifugation (4,000g for 5 minutes) and resuspended in 0.05M potassium phosphate buffer, pH7.3 at 30° to a final concentration of 0.2g wet weight/ml. Cells were slowly cooled to 1° over a period of 5 minutes to avoid any effects due to cold shock (Smeaton and Elliott, 1967a), and then lysed by passage through a French pressure cell at 1° operated at 12,000 p.s.i. The lysate was centrifuged at 35,000g for 30 minutes and the pellet discarded. Membranes were isolated by centrifuging the supernatant at 150,000g for 60 minutes. The membrane pellet was resuspended using a teflon-glass homogenizer in ice-cold 0.1M potassium phosphate buffer, pH7.3. The membranes were twice washed in this buffer by centrifugation (150,000g, 60 minutes) and resuspension. The final membrane pellet was resuspended by homogenization in 0.1M potassium phosphate buffer and stored as described above for the osmotically-prepared membranes.

7. Electron microscopy

This was performed using a Siemens Elmiskop I operated at 80Kv with a 50µ objective aperture.

A. Preparation of negatively stained specimens

A drop of suspension was placed on a carbon-coated grid for about 20 seconds. Excess fluid was then removed with filter paper and several drops of stain (2% aqueous uranyl acetate) were immediately placed on the grid. The fluid from the grid was removed after about 10 seconds with filter paper and the dried grid examined immediately.

B. Preparation of sectioned specimens

Membrane samples to be sectioned were pelletted by centrifugation at 35,000g for 30 minutes and treated for 1 hour in 2% (v/v) glutaraldehyde (0.1M sodium cacodylate buffer, pH7.2). After washing for 30 minutes in this buffer, the pellet was fixed in 1% osmium tetroxide for 1 hour and then made up to 1% uranyl acetate for 30 minutes. The fixed material was progressively dehydrated in acetone and finally embedded in "Araldite". Thin sections were cut on a LKB microtome and post stained for 3 minutes in lead citrate.

8. Solubilization of membrane-associated particles

Membranes suspended in 100mM potassium phosphate buffer, pH7.3, were centrifuged at 35,000g for 30 minutes and resuspended by homogenization in 1mM potassium phosphate buffer, pH7.3. The suspension was recentrifuged as above and the pellet (P_1) resuspended in 1mM potassium phosphate buffer, pH7.3. The resuspended pellet (P_1) was recentrifuged as above yielding a second membrane pellet, (P_2) and supernatant (S_2). The membrane pellet (P_2), was resuspended in 100mM potassium phosphate buffer, pH7.3, and both it and the supernatant (S_2), were examined by electron microscopy.

9. Sucrose density gradient analysis of membrane vesicles

A. Preparation of gradients

12ml linear 25 to 65% sucrose density gradients were prepared using a two chambered density gradient making apparatus. Sucrose density gradients were prepared in either 100mM potassium phosphate buffer pH6.6 or ATPase buffer which contained 75mM tris HCl, 1.25mM $MgCl_2$, pH7.8. Gradients were cooled to 2^{o} before use.

B. Sample loading and centrifugation

0.5ml of the material to be analysed was suspended in either 100mM potassium phosphate buffer, pH6.6 or ATPase buffer, both containing 10% (w/v) sucrose, and loaded onto the top of the gradient. Gradient tubes were loaded into an SW-41 head and centrifuged in a Beckman L2 ultra-centrifuge at 40,500 rpm for 15 hours at 2^{o}.

C. Fractionation of density gradients

Density gradients were fractionated using a density-gradient fractionator (ISCO, Model 40) and the $O.D._{280nm}^{2mm}$ profile recorded using an ISCO, Model UA-4 absorbance monitor connected to a millivolt recorder. 1ml fractions from top to bottom of the gradient were collected.

10. Mg^{2+}-activated ATPase assay

The reaction measured by the liberation of orthophosphate from ATP was carried out using the procedure of Rosenthal and Matheson (1973), however, the final volume of the assay was 1.1ml and the incubation was done at 30^{o} for 30 minutes.

A. ATPase activity of isolated membranes

Membranes were transferred into ATPase buffer by

first centrifuging the membranes at 35,000g for 30 minutes and resuspending the pellet in ATPase buffer, and then repeating this procedure. Between 0.1 and 0.3 mg of membrane protein were used per assay.

B. ATPase activity of gradient fractionated samples

1ml samples from the sucrose density gradient prepared in ATPase buffer were assayed directly by adding 0.1ml of ATP to each sample to give a final concentration of 2.5mM and incubating as described above. For all samples the ATPase activity was corrected for the effect of sucrose on both ATPase activity and non-enzymic hydrolysis. ATPase activity was inhibited by sucrose; the inhibition was directly proportional to sucrose concentration, maximum inhibition in 65% sucrose was 20% of the rate in 10% sucrose. ATP hydrolysis was not influenced by sucrose.

11. Freeze-fracture electron microscopy

Cells (in WCSM) and protoplasts (in PM) were pelletted and cooled to 4^{o} over a period of 5 minutes to avoid cold shock (Smeaton and Elliott 1967a) and transferred into gold sample holders. The samples were then frozen by immersion in liquid Freon followed by immersion in liquid N_2. Samples were freeze fractured in a Blazers freeze-fracture apparatus operated at a vacuum of $2x10^{-6}$ Torr. The sample was maintained at -100^{o} and the knife at -150^{o}. After fracturing, samples were etched for 2 minutes by maintaining the knife directly above the sample. Metal replicas were obtained by shadowing with platinum and carbon at an angle of 45^{o} and thereafter with carbon at an angle of 90^{o}. Metal replicas were removed and cleansed of biological material using 80% (v/v) H_2SO_4 at 60^{o} for 2 hours. Replicas were washed in distilled water then washed in sodium hypochlorite (5% available chlorine) for 2 hours. Replicas were then washed

in distilled water and placed on electron microscope grids coated with 0.5% collodium in amylacetate and thereafter, examined directly under the electron microscope.

12. Spin-labelling experiments

Spin labels were prepared as 10^{-2}M solutions in ethanol. An amount appropriate to give a bulk concentration of between $1x10^{-4}$M and $5x10^{-5}$M spin label was added to a small vial and the ethanol removed by evaporation in a stream of N_2. All samples to be spin labelled were added as aqueous suspensions at 20°. Samples of osmotically isolated membranes were routinely suspended in 0.1M potassium phosphate buffer, pH7.3 and the spin label was present at between 0.2 to 0.5% (w/w) of the membrane protein and between 1 to 2% (w/w) of the total membrane lipid. This ratio yielded spectra free of proximity effects which give rise to line broadening (for discussion see Scandella et al., 1972).

Although spin-label reduction in well-washed membrane vesicles was negligible over a period of 3 hours, potassium ferricyanide $\left[K_3Fe(CN)_6\right]$, at a concentration of 10^{-5}M, was included as a precaution against signal decay. However, with cells or protoplasts suspended in WCSM or PM respectively, almost complete reduction in signal amplitude occurred within 30 minutes at 30° due presumably to cellular reduction of the oxazolidine nitroxide group. This signal decay was abolished by heating the cells at 65° for 10 minutes; $K_3Fe(CN)_6$ was not effective. The amount of spin label added to cells or protoplasts was determined experimentally so that the resulting spectra were similar to those obtained for membrane vesicles.

Phospholipids were spin labelled as follows: A sample of the phospholipid extract in chloroform was taken, the

chloroform removed under a stream of N_2 and the phospholipid was dispersed in 0.1M potassium phosphate buffer, pH6.6, to give a final concentration of 25mg/ml. Dispersion involved a preliminary mixing using a glass bead followed by sonication for 5 minutes. The dispersion appeared faintly white after this treatment. The spin label was then added as described for the isolated membranes.

For the recording of spectra, the sample was inserted into a pasteur pipette sealed at the tip, and during the experiment the sample was not moved whilst in the microwave cavity. Temperature control was as follows: A calibration run was performed by measuring the temperature at different controller settings (Varian temperature controller, V-4557) of a membrane sample using an iron-constantan thermocouple. As the temperature of the N_2 purging the cavity during both the calibration and experimental runs was continually monitored, the sample temperature during the experimental run could be determined. Errors in sample temperature did not exceed $\pm 1^{o}$ during an experiment. For experiments involving spin label motion at various temperatures, an equilibration time of 4 minutes between measurements was used for temperature shifts not exceeding 2^{o}. Results shown for all spin-labelling experiments were recorded using an ascending temperature mode.

Electron paramagnetic resonance spectra were recorded as first-derivative absorption spectra using a Varian E-9 spectrometer operated at 100kHz field modulation, 5mW microwave power (or as indicated), 250 μA detector current and a microwave frequency of 9.18GHz. Modulation amplitude was 2.0 gauss or as indicated. Scans of 100 gauss were made over a period of 2 minutes.

In this study the observed spectra approximate those expected if the spin label were undergoing essentially isotropic motion. A mathematical expression has been derived for analysing such spectra in terms of an empirical motion parameter (τ_o) defined by:

$$\tau_o = 6.5 \times 10^{-10} W_o \left(\sqrt{\frac{h_o}{h_{-1}}} - 1 \right) s$$

where W_o and h_o are respectively, the width (in gauss) and the height of the mid-field hyperfine line and h_{-1} is the height of the high-field hyperfine line. A more complete treatment relating to the use of this expression is given in the following references: Raison et al., (1971a); Eletr et al., (1974); Keith et al., (1975). The values of the hyperfine coupling constant (A_n) for the spin label were determined as described by Raison et al., (1971a).

13. Respiratory studies

Rates of oxygen uptake were measured using an oxygen electrode (Rank Bros., Cambridge, U.K.) connected to a millivolt recorder. The electrode was calibrated with air-saturated water at various temperatures. For membranes prepared either osmotically or by the French pressure cell, reactions were carried out in a total volume of 2ml of 0.1M potassium phosphate buffer, pH7.3. After measurement of the endogenous rates of oxygen uptake, the reaction was started by the addition of 0.005M succinate or 0.001M NADH. Reaction temperatures were measured both before and after the reaction using an iron-constantan thermocouple and temperature variation during the assay did not exceed $\pm 0.2^{o}$. For respiratory studies involving cells or protoplasts, the reaction was carried out in WCSM or PM respectively. Arrhenius plots of succinate and NADH oxidation were carried out using an ascending temperature mode.

14. Fluorescent probe experiments

The fluorescent probe ANS (8-anilinonapthalene-1-sulphonate, sodium salt), was added to osmotically-prepared membrane vesicles as follows: from a 10^{-3}M ethanolic stock solution of ANS, an amount sufficient to give a final concentration of 1.5×10^{-5}M was taken and the ethanol removed by evaporation. Membrane vesicles (3mg membrane protein/ml) suspended in 0.1M potassium phosphate buffer, pH7.3, were added and mixed with ANS for 5 minutes at 20°. Measurements were made using an Aminco-Chance spectrophotometer equipped with a 90° light source using excitation and emission wavelengths of 350 and 450nm respectively. The temperature-controlled cell housing was equipped with a vibrating reed stirrer and a thermocouple directly inserted into the sample. An ascending temperature mode was used, with a heating rate of approximately 1° per minute. Fluorescence intensity was recorded continuously and corrected for intrinsic fluorescence and light scattering effects at all temperatures. Under the conditions described above a sonicated aqueous dispersion of 1,2-dimyristoyl-sn-glycero-3 phosphoryl choline exhibited a pronounced change in fluorescence intensity at 23°, a temperature corresponding to the main endothermic transition of this phospholipid (Hinz and Sturtevant, 1972).

15. Differential scanning calorimetry

Thermal analysis of B. amyloliquefaciens membranes was performed using a differential scanning calorimeter (Perkin - Elmer, DSC-2). Membranes prepared by the osmotic lysis of protoplasts were suspended in 0.1M potassium phosphate buffer, pH7.3 and centrifuged at 35,000g for 30 minutes. The pellet was transferred into a calorimeter sample pan of 100 µl capacity which was then sealed and loaded together with an empty

reference pan into the calorimeter. Both heating and cooling scans were made at a rate of 20° per minute.

16. Adiabatic calorimetry

The thermal analysis of B. amyloliquefaciens membranes was kindly performed by Mr. A. R. Haly, C.S.I.R.O. Division of Textile Physics, in an adiabatic calorimeter, the design of which has been described by Haly and Snaith (1968). Membranes prepared by the osmotic lysis of protoplasts and suspended in 0.1M potassium phosphate buffer, pH7.3, were pelletted by centrifugation at 35,000g for 30 minutes and loaded into the calorimeter sample pan. The sample was heated in steps of approximately 3°, a steady state being reached after each step. The endotherm was determined by measuring the specific heat at each temperature.

17. Lipid extraction

A. Cells

The procedure was a modified version of that used by Houtsmeuller and van Deenen (1965). Harvested cells were resuspended in 50mM tris buffer, pH7.3 and 1 volume of cell suspension was extracted with 1 volume of chloroform and 2.17 volumes of methanol by shaking at 2° for 2 hours. Undissolved material was pelletted by centrifugation and re-extracted two more times. Extracts were pooled and 0.2 volumes of 10% (w/v) NaCl was added to form two phases. The chloroform phase was removed and the aqueous phase was re-extracted with an equal volume of chloroform. The chloroform extracts were pooled, dried over anhydrous sodium sulphate and concentrated by rotary evaporation. Total lipid dry weight was determined after completely drying the extract on a vacuum pump. Lipid extracts

were redissolved in chloroform and stored in sealed tubes under N_2, at a temperature of -20^{o}. Poly-β-hydroxybutyrate was removed from this and other lipid extracts by the method described by Bishop et al., (1967).

B. Culture and washed-cell supernatants

After removal of cells by centrifugation, supernatants were extracted in the ratio of 1 volume to 1 volume of chloroform and 2.17 volumes of methanol. The lipid extract was then processed as described for cells.

C. Membrane suspensions

Suspensions of osmotically prepared membranes were extracted with chloroform : methanol (2:1, v/v) according to the procedure of Folch et al., (1957), and then processed in the manner described for cells. In addition, Sephadex G-25 was also used to remove non-lipid contaminants from the lipid extract using the procedure of Wells and Dittmer (1963).

18. Isolation of phospholipids by acetone precipitation

Phospholipids were isolated from total lipid extracts by virtue of their insolubility in cold acetone. Six volumes of acetone at a temperature of -20^{o} were added to one volume of the particular lipid extract in chloroform. After 30 minutes at that temperature the precipitated phospholipids were washed once with acetone (at -20^{o}), centrifuged and the supernatant pooled with the acetone-soluble lipids. The dry weight of the phospholipids was determined after drying in a vacuum pump. Phospholipids were stored in chloroform under N_2 at -20^{o}.

19. Column chromatography of lipids

Membrane lipids were also fractionated by silicic acid column chromatography. 1g of silicic acid (Mallinckrodt, 100

mesh, acid-washed) was used per 10mg of lipid. The column was equilibrated with chloroform and the sample added in a small volume of the same solvent. Neutral lipids were eluted with chloroform (6ml/g of silicic acid), glycosyl glycerides (glycolipids) with acetone (4ml/g) and phospholipids with methanol (5ml/g) followed by chloroform-methanol-water (10:10:1, v/v/v) (4ml/g). The eluted fractions were dried, weighed, and stored in chloroform until use.

20. Fatty acid analysis

Fatty acid analysis was performed on total lipid extracts and on the fractionated lipids. Lipids were saponified, the fatty acids converted to methyl esters and quantitative analysis was performed by gas-liquid chromatography.

A. Saponification

After removal of the solvent into which they were dissolved, lipids were refluxed for 3 hours in 1N NaOH in 50% (v/v) methanol in sealed tubes under N_2. Non-saponifiable material was removed by extracting three times with hexane. The aqueous layer was acidified with 20N H_2SO_4 and the fatty acids extracted three times with ether. The extract was dried over anhydrous sodium sulphate prior to preparation of methyl esters.

B. Preparation of methyl esters

After removal of solvent by evaporation, the sample was heated in a sealed tube at 75^{o} for 1 hour in the presence of 2 ml of 14% (w/v) BF_3 in methanol. The sample was then cooled in ice, 3 ml of water added and the methyl esters were extracted three times with 5 ml of petroleum ether (40^{o} to 60^{o} fraction). Extracts were combined, dried over anhydrous sodium sulphate and taken up in a small volume of CS_2. Additional

purification by chromatography on Florisil/H_2O, was found not to be necessary.

C. Gas liquid chromatography (GLC)

Fatty acid methyl esters were analysed on a Perkin-Elmer 880 gas chromatograph equipped with a flame-ionization detector, connected to a Rikadenki millivolt recorder. The flame-ionization detector was operated with flow rates of 30 ml/min (H_2) and 450 ml/min (Air). Samples in CS_2 were introduced into the column using a microlitre syringe (S and G, Engineering, Melb. Aust.).

Four separate 6 foot columns were used for the identification of fatty acid methyl esters, (see materials section). The 3% and 5% Apiezon L columns were operated at 190° with a carrier gas (N_2) flow rate of 50 ml/min, the temperature of the injector block and the detector being 240° and 200° respectively. DEGS and EGSS-X columns were operated at 165° (unless otherwise stated) with a carrier-gas flow rate of 40 ml/min, the injection block and detector temperature being 215° and 175°, respectively.

Fatty acid methyl esters were identified by running unknowns and methyl ester standards on both polar and non-polar columns, determining their R_f values relative to methyl palmitate and plotting the logarithm of the R_f value against the carbon number. Unsaturated fatty acids were identified by their different behaviour on the two types of column together with their disappearance after hydrogenation, using palladium on charcoal as catalyst.

Quantitative analysis was performed only on samples run on the non-polar Apiezon L columns as these columns gave relatively greater resolution between the branched-chain fatty acid methyl esters and the straight-chain homologue. Peak areas

were determined by triangulation. Insignificant amounts of lipid material were extracted from the growth medium and the washing buffers in comparison to the amount of lipid extracted from cells, membranes and culture or washed-cell supernatants.

21. Incubation conditions for washed-cell suspension experiments

Sterile conditions were not necessary for washed-cell experiments. Cells harvested from late-log phase culture were resuspended in WCSM, to a dilution equivalent to that in the culture. 20 to 40 ml samples of the cell suspension were shaken at 30° unless otherwise stated, and 0.7 to 2.0 ml samples were withdrawn at appropriate times, centrifuged and the supernatants assayed for extracellular enzyme activity.

22. Measurement of total protein synthesis

To measure total cellular protein synthesis, 2.0 ml of a washed-cell suspension was shaken with 0.5 μCi of uniformly labelled L-(^{14}C) - leucine (spec. act. 316 mCi/m mole), at a temperature of 30° unless otherwise stated. At appropriate times, 0.1 ml samples were withdrawn and processed as described previously (May and Elliott, 1968a), except that the oxoid filters were counted by liquid scintillation in a Packard Tri-Carb spectrometer (90% efficiency) using scintillation fluid containing 3g of 2,5-diphenyloxazole (PPO) and 0.3g of 1,4 -bis-[2- (4 methylphenyloxazolyl)] benzene (POPOP) per litre of toluene.

23. Extracellular enzyme assays

A. α- amylase (α-1-4-D-glucan glucanhydrolase E.C.3.2.1.1.) was assayed as follows: The stock buffer contained 2.5 litres

of 0.1M potassium phosphate, 0.025M NaCl, pH 6.2; 2.0 litres of water; 10 ml of 0.1 M $CaCl_2$; 11 ml of 0.005 M $ZnSO_4$.

An even suspension was prepared by mixing one "Phadebas" tablet with 5 ml of stock buffer, and 1 ml samples of this suspension were pipetted into assay tubes. Suitably diluted enzyme (0.5 ml) was added and the tubes incubated at 37° for 30 minutes. Development of soluble blue product was terminated by the addition of 0.2 ml of 0.5N NaOH. The tubes were centrifuged for 10 minutes at 3,500g and the absorbance of the supernatant determined at 620nm. The relation between enzyme concentration and absorbance at 620nm was linear to 6.0 O.D. units. A unit of α- amylase activity was defined as that amount of enzyme which produces an increase in absorbance at 620nm of 4.0 in 30 minutes at 37°.

B. Protease activity was determined by the Remazol brilliant blue-hide powder assay described by Rinderknecht et al., (1968). A unit of protease activity is defined as that amount of enzyme which produces an increase in the absorbance at 595nm of 5.7 in 40 minutes at 37°.

C. Ribonuclease activity was determined using the method described by Coleman and Elliott (1965). A unit of ribonuclease activity is defined as that amount of enzyme which produces an increase in absorbance at 260nm of 0.8 in 30 minutes at 25°.

24. Cold shock experiments

A. Procedure for rapid cooling

Cells were twice washed in 0.05M tris buffer, pH 8.0 at 30°, or at the temperature stated, with or without supplements depending on the particular experiment. Cells were finally resuspended in one fortieth of their initial volume in the same buffer. The cells were equilibrated at 30° (or other initial

temperatures), and 0.5 ml samples were squirted into 19.5 ml of vigorously stirred buffer at different known temperatures. This buffer was either 0.05M tris, pH 8.0 or WSCM, as is stated in the text. In no case did this procedure cause an increase in the temperature of the buffer of more than 0.5^{o}.

B. Procedure for slow cooling

Washed cells were finally resuspended to their original volume in either 50mM tris buffer pH 8.0 or WCSM. The cells were shaken in an orbital shaking bath at 30^{o} and then slowly cooled by the gradual addition of ice to the bath. The cooling rate was no more than 2^{o} per minute. Samples were withdrawn when the cells reached the desired temperature.

C. Assay for cold shock

Cells cold shocked into tris buffer were assayed by the exposure of intracellular ribonuclease inhibitor (i), while cells cold shocked into WCSM were assayed by their ability to secrete protease (ii).

(i) Exposure of intracellular ribonuclease inhibitor to added ribonuclease

This assay works on the principle that after cold shocking, B. amyloliquefaciens becomes permeable to exogenously added ribonuclease (RNAase) which inactivates the intracellular ribonuclease inhibitor described by Smeaton and Elliot, (1967a). Such exposure becomes maximal after 30 minutes.

After cold shock treatment, 2 ml samples of cells were returned to 30^{o} and a standard amount of dialysed B. amyloliquefaciens culture supernatant (containing RNAase) was added, along with 40µg/ml chloramphenicol to prevent the normal secretion of RNAase. After shaking for 60 minutes, cells were removed by centrifugation and the supernatant

assayed for RNAase activity, the amount of activity being reduced by cold shock treatment. The total level of intracellular inhibitor was determined by assaying for inhibitor exposure in French-press prepared cell lysates. Inhibitor exposure was expressed as a percentage of this level.

(ii) Protease secretion

Cells were cold shocked in WCSM as described, and then returned to 30°. Cells grown at temperatures other than 30°, were returned to a temperature equivalent to the growth temperature. The rate of protease secretion was determined for each sample of cold-shocked cells in the manner described for washed-cell suspension experiments. Protease secretion was expressed as a percentage of the rate of protease secretion in cells continuously maintained at their particular growth temperature.

25. Protein determination

Membrane protein was determined by the method of Lowry et al., (1951).

CHAPTER 3

ISOLATION AND GENERAL FEATURES OF *B. AMYLOLIQUEFACIENS* CYTOPLASMIC MEMBRANES

A. INTRODUCTION

Central to the problem of extracellular enzyme secretion is the role played by the cytoplasmic or cell membrane. As it has been proposed that ribosomes associated with this membrane are responsible for the synthesis of extracellular enzymes (May and Elliott, 1968), the cytoplasmic membrane could be considered as the site of synthesis as well as the site of secretion. Therefore a study of some of the properties of this membrane is warranted for these reasons alone.

To date no detailed study has been made on the cell membrane of B. amyloliquefaciens either as a membrane system per se or with regard to its role in secretion. As the overall aim of the work described in this thesis is the study of both of these aspects, it was first necessary to isolate and characterize the cytoplasmic membrane before detailed studies on the above could be undertaken.

B. RESULTS

1. Isolation of membranes

a. Preparation of membranes by osmotic lysis.

Cytoplasmic membranes from Gram-positive and Gram-negative bacteria have been isolated by a variety of techniques including osmotic lysis and French pressure cell lysis. Membrane vesicles prepared by osmotic lysis have proved useful in the study of active transport (Kaback, 1971,72), and it was decided initially to use this approach for the preparation of cytoplasmic membranes from B. amyloliquefaciens.

Two procedures were used for the preparation of membranes by osmotic lysis, one involving the lysis of cells (Konings et al., 1973), the other the lysis of protoplasts. These procedures which are described in Chapter 2, resulted in membrane vesicles which were free of ribosomes when examined by electron microscopy. These vesicles were used for the studies described in Chapter 4. The substitution of tris buffer for phosphate buffer and the omission of RNAase and EDTA from the lysing medium resulted in a great many ribosomes being visible within the vesicles (see section on membrane morphology in this chapter). Contamination in these preparations mainly consisted of unlysed cells and debris, both of which could be removed by low-speed centrifugation, as described in Chapter 2.

The temperature of the lysing medium, the ratio of protoplasts or cells to lysing medium, the time and sequence of additions of $MgSO_4$ and EDTA, did not affect the final appearance of the membrane vesicles. Membrane vesicles also appeared identical if cells other than those at late-log phase were used in the preparation of membranes. The lysis of protoplasts was instantaneous upon their addition to the lysing medium and it was found that a minimum of 11% sucrose differential was required for complete protoplast lysis. Membrane vesicles formed under these conditions were similar in size to those prepared using the normal osmotic differential of 22% sucrose.

b. Preparation of membranes by the French pressure cell.

The alternative procedure for the preparation of membranes involved lysis of whole cells in the French pressure cell as described in Chapter 2. Membranes so prepared were devoid of ribosomes, although RNAase and EDTA were not included in the lysing buffer. Membranes were vesicular and their diameter was about 1/20th that of intact protoplasts, (1/5th that of osmotically-prepared vesicles). Different pressures for lysis altered the proportion of cells that underwent lysis but did not influence the size of the membrane vesicles obtained. A pressure of 12,000 p.s.i. resulted in virtually complete lysis.

2. Morphology of membranes and membrane-associated particles

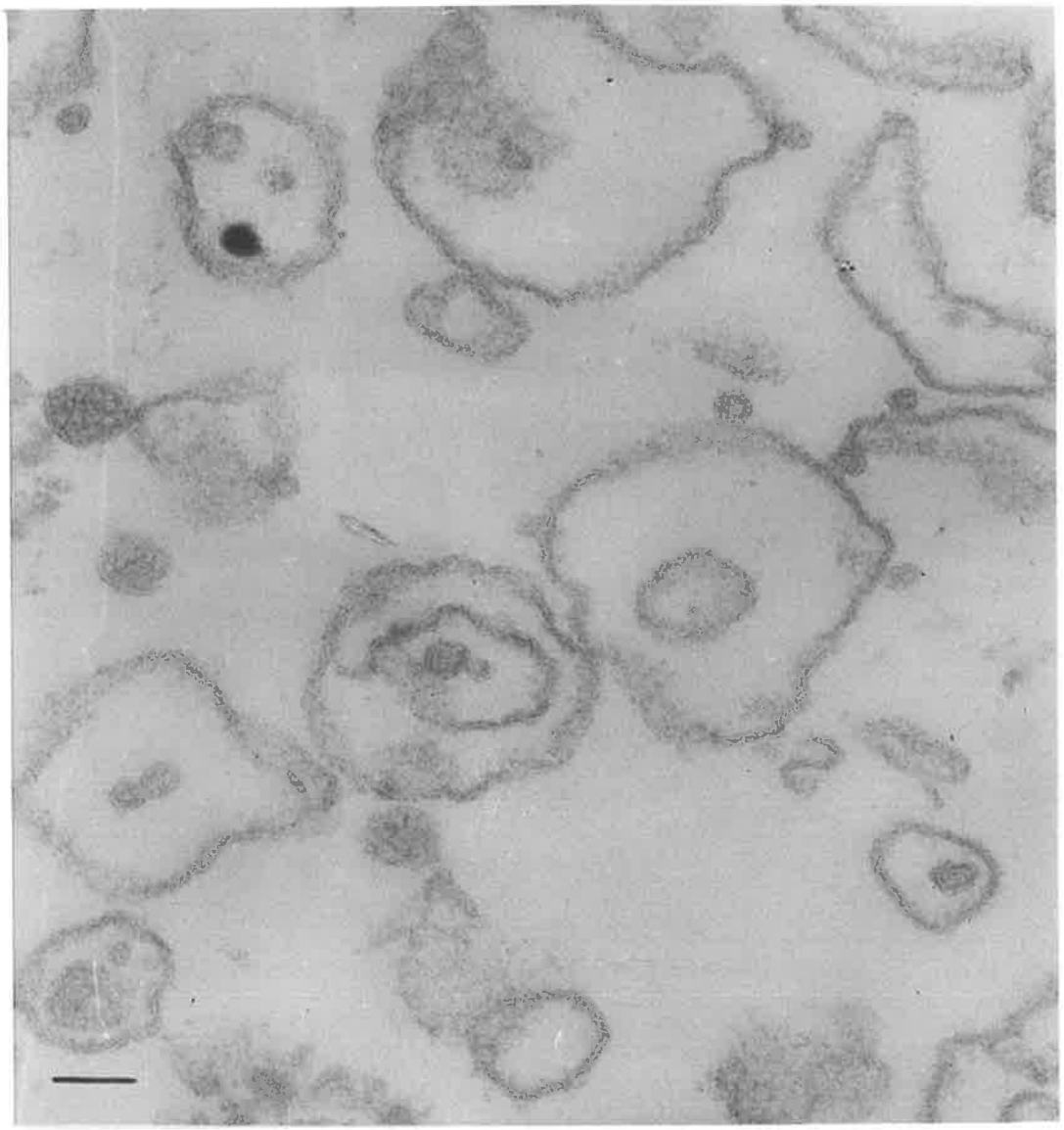
a. Examination of osmotically-prepared membranes after fixation and sectioning.

Membrane vesicles prepared by either of the two osmotic lysis procedures were fixed in 2% glutaraldehyde and sectioned as described in Chapter 2. With both procedures, membranes appeared identical when examined by electron microscopy. As shown in Plate 3.1, membranes were vesicular in form and free of visible cell wall material, unlysed cells and protoplasts. Some entrapment of smaller vesicles within the larger ones was evident. The background and the inside of the vesicles were free of ribosomes when phosphate buffer was used in combination with RNAase and EDTA, as described. The predominant class of

PLATE 3.1. ELECTRON MICROGRAPH OF SECTIONED *B. AMYLOLIQUEFACIENS* MEMBRANES PREPARED BY OSMOTIC LYSIS.

Membranes prepared by the osmotic lysis of protoplasts, suspended in 0.1M potassium phosphate buffer, pH7.3, were fixed, sectioned and examined by the procedures described in Chapter 2.

Bar marker = 0.1μ



vesicles has a diameter of between 0.27 to 0.43μ, in comparison to the diameter of protoplasts (1.0 to 1.5μ).

The average thickness of the membrane was 80A and a "railroad-track" pattern was evident when membranes were observed edge on. The vesicular structure and the staining pattern were observable after extended storage of vesicles at -80^{o}.

b. Examination of osmotically-prepared membranes by negative staining.

When examined by negative-staining electron microscopy using uranyl acetate (as described in Chapter 2), membrane vesicles prepared by either osmotic method, appear as fragments or sheets as is shown in Plate 3.2. Vesicular structure is lost either when the vesicles are applied to the E.M. specimen grid, or when the negative stain is added. The addition of 2% glutaraldehyde to vesicles prior to (but not after) their addition to the grid, affords considerable protection against fragmentation. As shown is Plate 3.2, membranes of differing size, shape, degree of convolution and orientation are observed. The apparent thickness of the membrane, 130 to 140A, is greater than that observed in fixed sectioned material.

Evidence of a periodic staining pattern in the membranes is shown in Plates 3.2 and 3.3. This banded pattern consisted of a light region extending about 180A along the length of the membrane interspaced by a dark region about 34A in width and it was evident when membrane fragments were observed edge on. Within the light region there may also exist some periodicity in the staining pattern.

The particles seen in association with the membrane (bp)

PLATE 3.2. ELECTRON MICROGRAPH OF A NEGATIVELY-STAINED PREPARATION OF B. AMYLOLIQUEFACIENS CYTOPLASMIC MEMBRANES.

Membranes were prepared by the osmotic lysis of protoplasts and negatively stained with uranyl acetate as described in Chapter 2.

Membranes (m) appear as fragments or sheets and are covered with small particles (bp) which appear attached to the membrane via a stalked region. Other particles (fp), morphologically identical to those bound to the membrane appear in the background.

Bar marker = 0.05μ

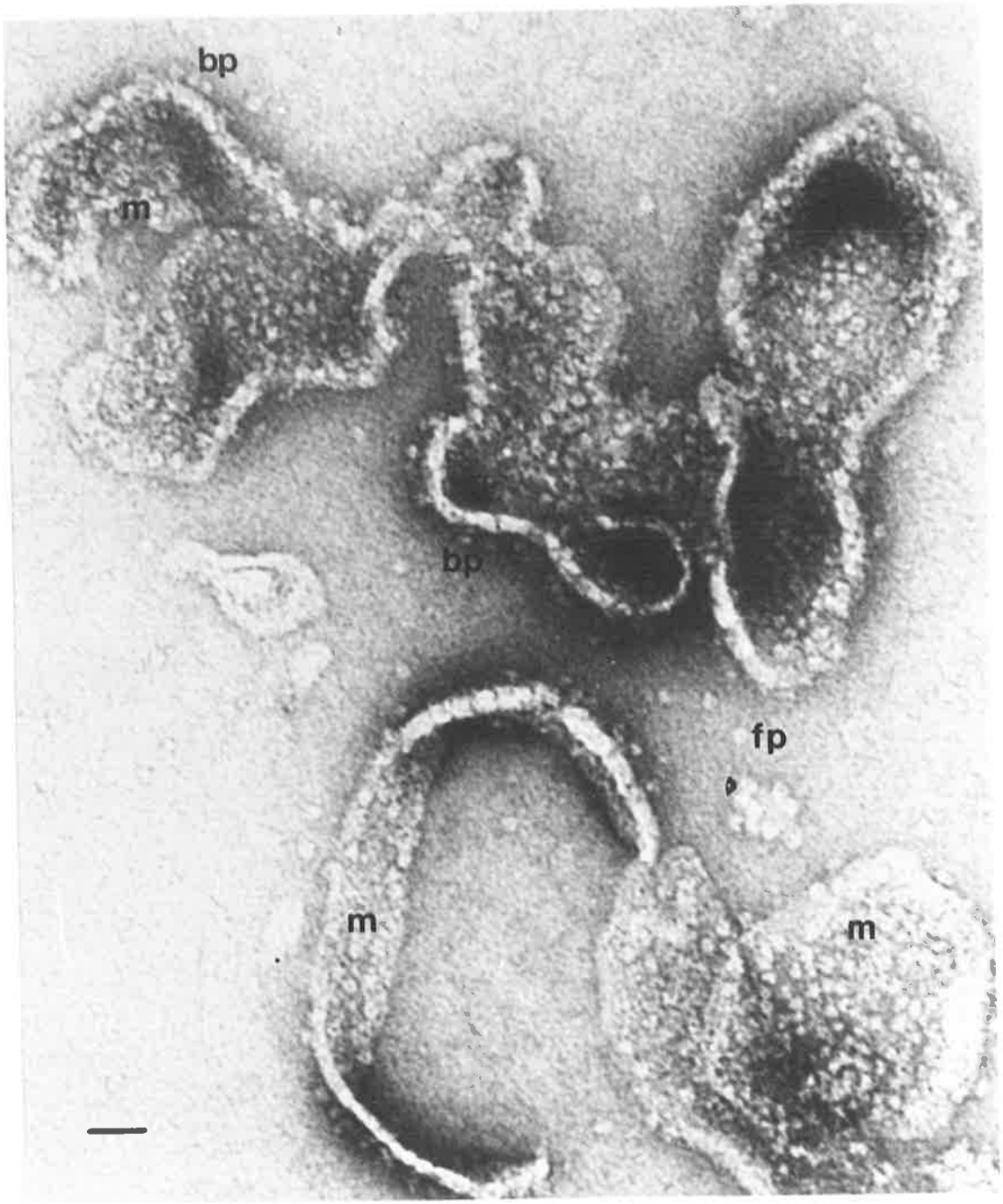
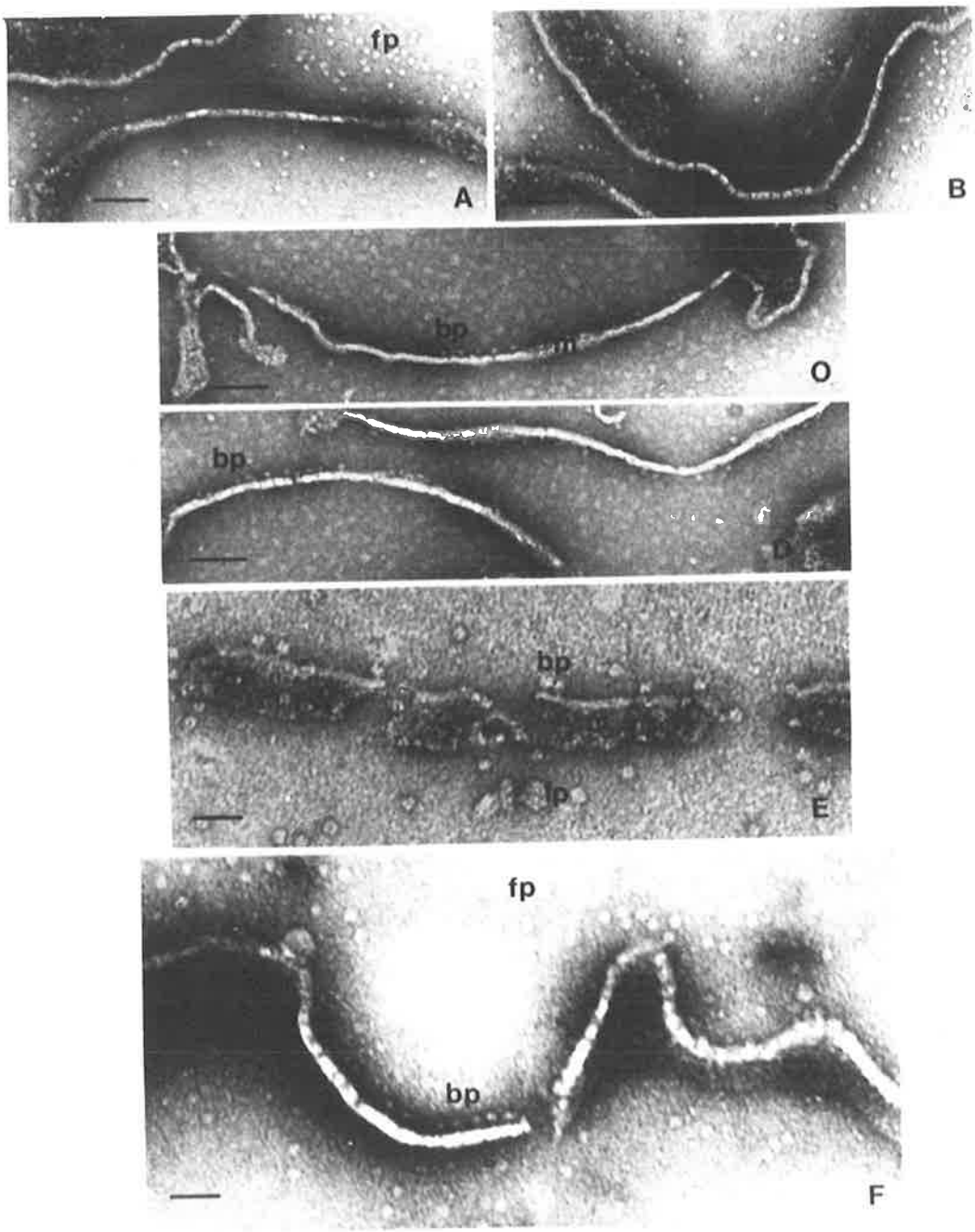

bp
m
bp
fp
m
m

PLATE 3.3. ELECTRON MICROGRAPHS OF NEGATIVELY-STAINED *B. AMYLOLIQUEFACIENS* CYTOPLASMIC MEMBRANES.

Membrane vesicles prepared by the osmotic lysis of protoplasts were negatively stained with uranyl acetate as described in Chapter 2.

The membranes (m) exhibit a banded pattern when observed edge on. Small particles are seen attached to the membrane (bp) and free in the background (fp).

Bar marker = 0.1 μ (Plates, A,B,O,D)
" " = 0.05 μ (Plates E and F)


fp
A
B
bp
O
bp
D
bp
lp
E
fp
bp
F

and in the background (fp), appear identical except that the former were attached to the membrane by a stalk, 55A in length. These particles were 100 to 110A in diameter and spaced 140 to 180A apart. They were evident on only one side of the membrane and in specimens where the fragmentation had followed the circular pattern of the vesicle, the stalked particles were directed inwards. The proportion of bound to free particles was variable; washing the sample with 100mM potassium phosphate buffer, pH7.3, whilst on the E.M. grid, decreased the number of bound particles. It is probable that the particles in the background were released by vesicle fragmentation and subsequent manipulations of the membranes on the E.M. grid. The addition of 1M NaCl to membranes suspended on the grid, resulted in complete release of the particles from the membrane.

These particles resemble those identified as the ATPase complex in other bacteria (Munoz et al., 1968; Schnebli et al., 1970). Removal of these particles from the cell membrane of various bacteria has been achieved by low-ionic strength washes (Munoz et al., 1968,69), and this occurred with B. amyloliquefaciens membranes, (Plate 3.4). The released particles (fp) appearing in the supernatant (Plate 3.4C) appeared identical to those shown in the previous two plates and to the solubilized ATPase complex from other bacteria.

Another distinct particle associated with B. amyloliquefaciens membranes is shown in Plate 3.5. These "daisy-like" particles appeared after membranes were treated with 1M $MgCl_2$ or 1M NaCl, (the latter also released the smaller presumptive ATPase particles from the membrane). Although "daisies" were occasionally observed in untreated membrane preparations, their numbers greatly increased after the high salt treatment. In vesicles which had fragmented on the E.M. specimen

PLATE 3.4. ELECTRON MICROGRAPHS OF NEGATIVELY-STAINED MEMBRANE PREPARATIONS SHOWING RELEASEMENT OF MEMBRANE-ASSOCIATED PARTICLES.

B. amyloliquefaciens cytoplasmic membranes isolated by osmotic lysis were washed with low-ionic strength buffer (1mM potassium phosphate, pH7.3) as described in Chapter 2.

A. Membranes before treatment. The small stalked particles (p) are bound to membrane fragments.

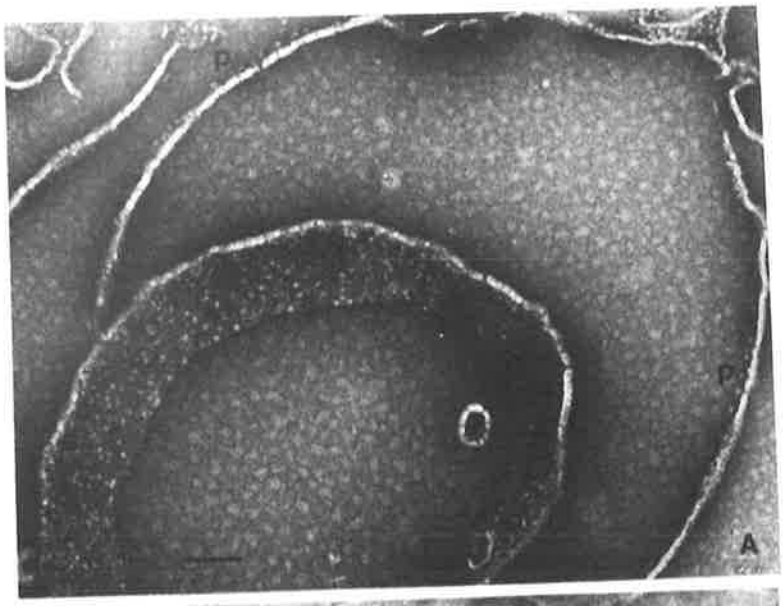
Bar marker = 0.1μ

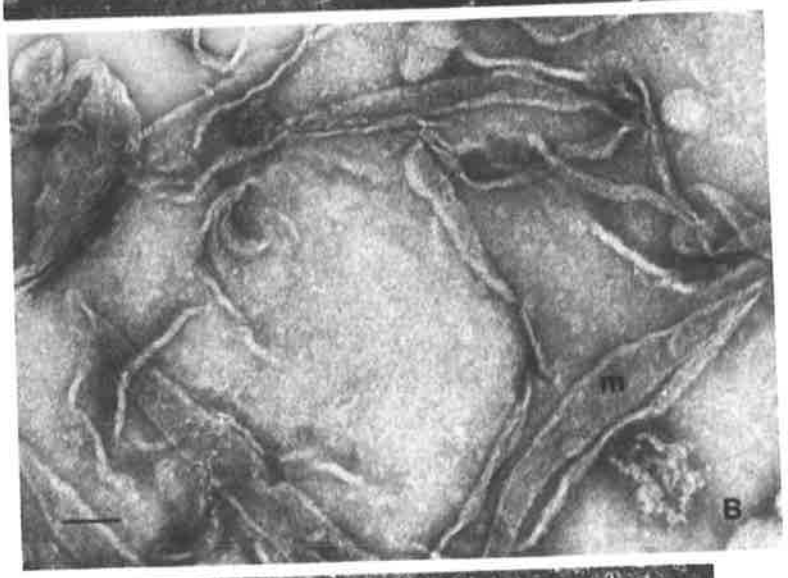
B. Membranes after treatment with low-ionic strength buffer do not possess the small particles either bound to the membrane (m) or in the background.

Bar marker = 0.1μ

C. Supernatant remaining after treating membranes with low-ionic strength buffer. The released particles (fp) are clearly seen.

Bar marker = 0.05μ


A


m
B

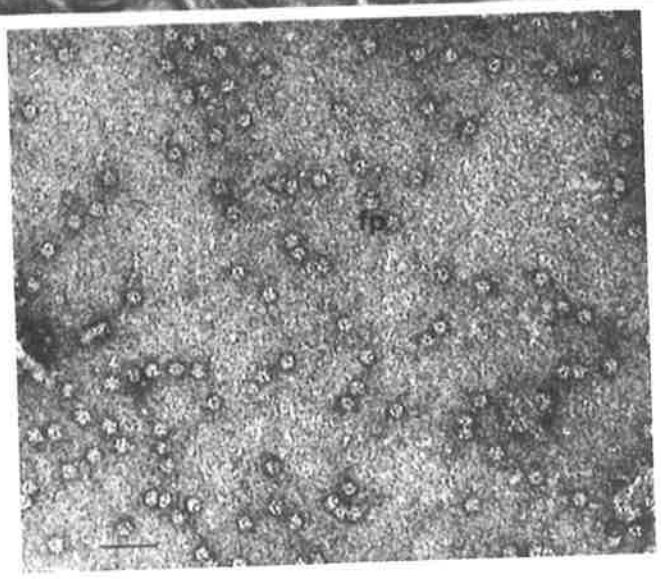

fp

PLATE 3.5. ELECTRON MICROGRAPHS SHOWING THE FINE STRUCTURE OF THE "DAISY-LIKE" PARTICLES AND THEIR ASSOCIATION WITH THE CYTOPLASMIC MEMBRANE OF B. AMYLOLIQUEFACIENS.

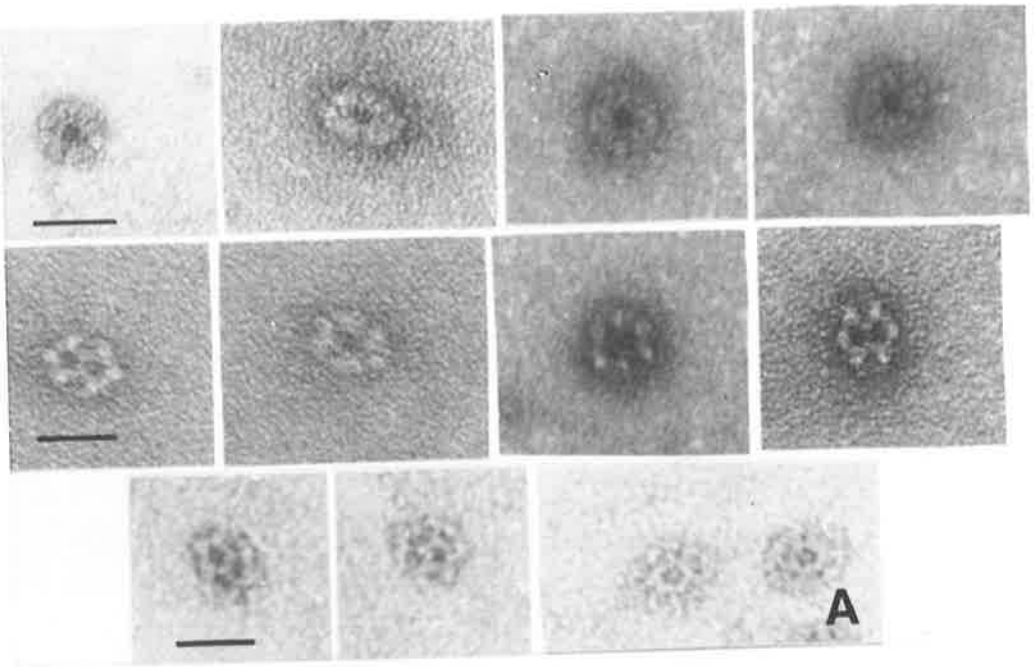
Membranes prepared by osmotic lysis were suspended on an electron microscope specimen grid and treated with 1M $MgCl_2$ prior to the addition of the negative stain.

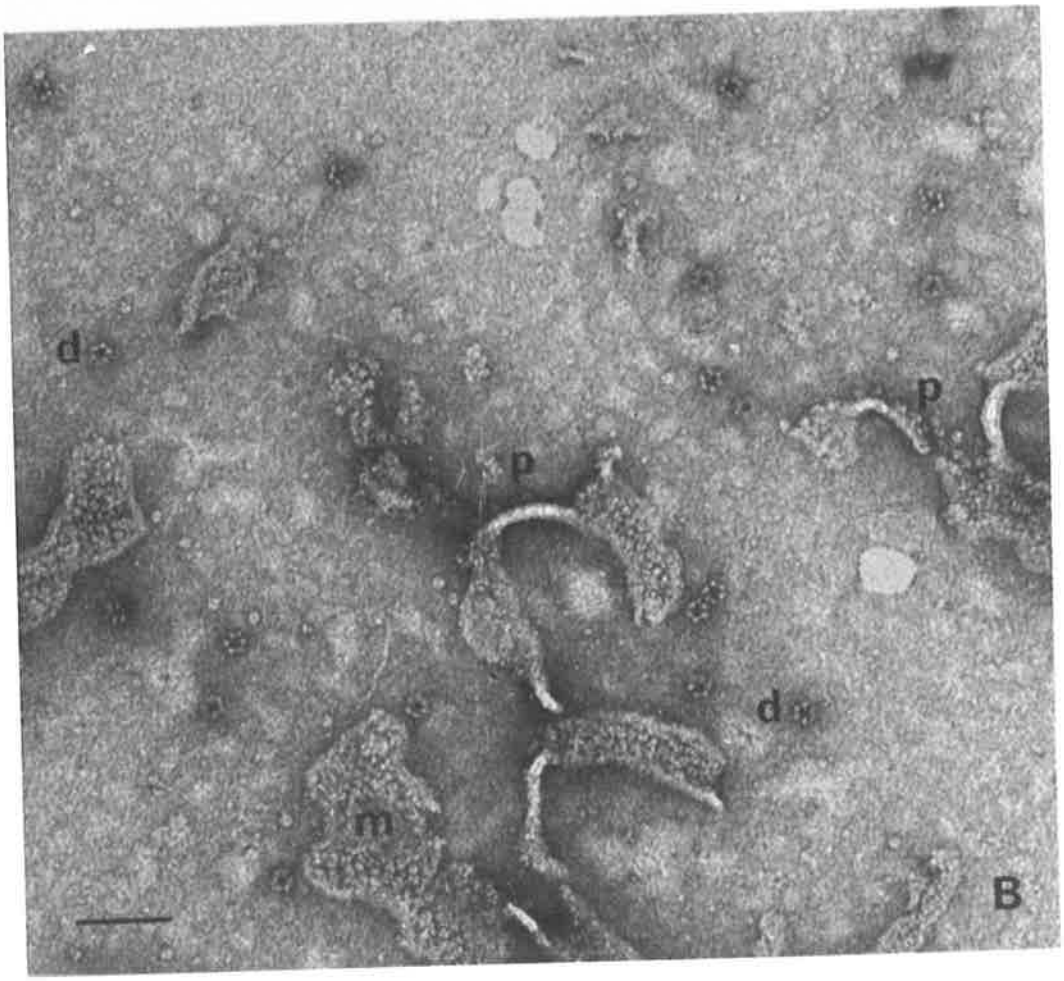
A. High power electron micrographs of "daisies" suspended on a carbon-coated specimen grid. The predominent structure of the particle appears to be one in which four dark central units are each surrounded by possibly five lighter subunits. Considerable shrinkage of the carbon film occurred under the electron beam and this may have resulted in some reduction in the size of the particle.

Bar Marker = 20nM

B. Electron micrograph showing that the "daisy" particles (d) are not observed bound to the cytoplasmic membrane (m) as are the majority of the smaller stalked particles (p). "Daisies" and some of the smaller particles (p) are observed together in the background.

Bar marker = 0.1μ


A


d
p
p
d
m
B

grid, they were not observed until after the addition of high salt buffers and thus they probably do not originate from within the vesicle due to entrapment. The "daisies" were never observed bound to the membrane as were the smaller particles, nor was any discernable hole in or change to the membrane evident after "daisies" were released. "Daisies" released by salt treatment disappeared after about 60 minutes and immediately if the salt concentration was lowered to 0.1M. This is in conflict with the fact that "daisies" were occasionally observed in association with membranes prior to addition of high salt.

The size of the particle was 239± 11A on the short axis and 255± 10A on the long axis. Different views of the particle are shown in Plate 3.5A. Their size in this plate is smaller than the dimensions given above due possibly to shrinkage of the carbon film which was applied to this E.M. grid in an effort to see greater structural detail. Although their morphology is complex, symmetry exists in their structure. They appear to consist of 4 non-dense units 80A in diameter, each surrounded by 5 dense subunits 40A in diameter, 35 to 40A apart. Only two of the large units appear in the same plane. It was impossible to determine whether the views in Plate 3.5A were different views of the same particle or different particles of similar size. As "daisies" always appeared orientated in the same plane, their thickness could not be determined.

The nature of this particle is unknown. Their observation under a wide range of conditions and their constant structural appearance, argues against them being artefacts. Indeed all efforts to produce these particles by combinations of high salt buffers and negative stains alone, were unsuccessful. In comparison to B. amyloliquefaciens ribosomes, "daisies" were morphologically distinct although both were of similar size.

"Daisies" were often seen together with the smaller presumptive ATPase particles as in Plate 3.5B, and in association with the fimbri which extend from the surface of the cell in situ, but which often were observed in the membrane preparations.

A third-type of particle associated with B. amyloliquefaciens membranes, which was observed under a variety of conditions, consisted of a ladder-like complex of subunits (L) seen either attached to the membrane (Plate 3.6A) or free in the background (Plate 3.6B). No treatment was required to bring about their appearance apart from the fragmentation of the membrane vesicles. The number of particles observed bound to the membrane varied as did the number of constituent subunits, (from 3 to 7). Each 140A by 55A subunit was equispaced along a fine strand at distances of about 50A. One of the terminal subunits appeared to be in the shape of an arrowhead.

c. Electron microscopy of membranes in association with ribosomes.

Membrane vesicles which were prepared by the osmotic lysis of cells or protoplasts in which the phosphate buffer was replaced with tris buffer lacking RNAase and EDTA, were used in this study. Such preparations observed in thin section (Plate 3.7A) consisted of sealed membrane vesicles containing many ribosomes. It was impossible to decide whether any of the ribosomes were meaningfully associated with the membrane. (The few clear zones within the larger vesicles were probably due to "pinched-off" invaginations of the membrane). Examination by negative staining procedures resulted in fragmentation and release of the entrapped ribosomes (Plate 3.7B). At only a few places can ribosomes be seen in some form of association with the membrane (Plate 3.7C). At these places (mr), the association

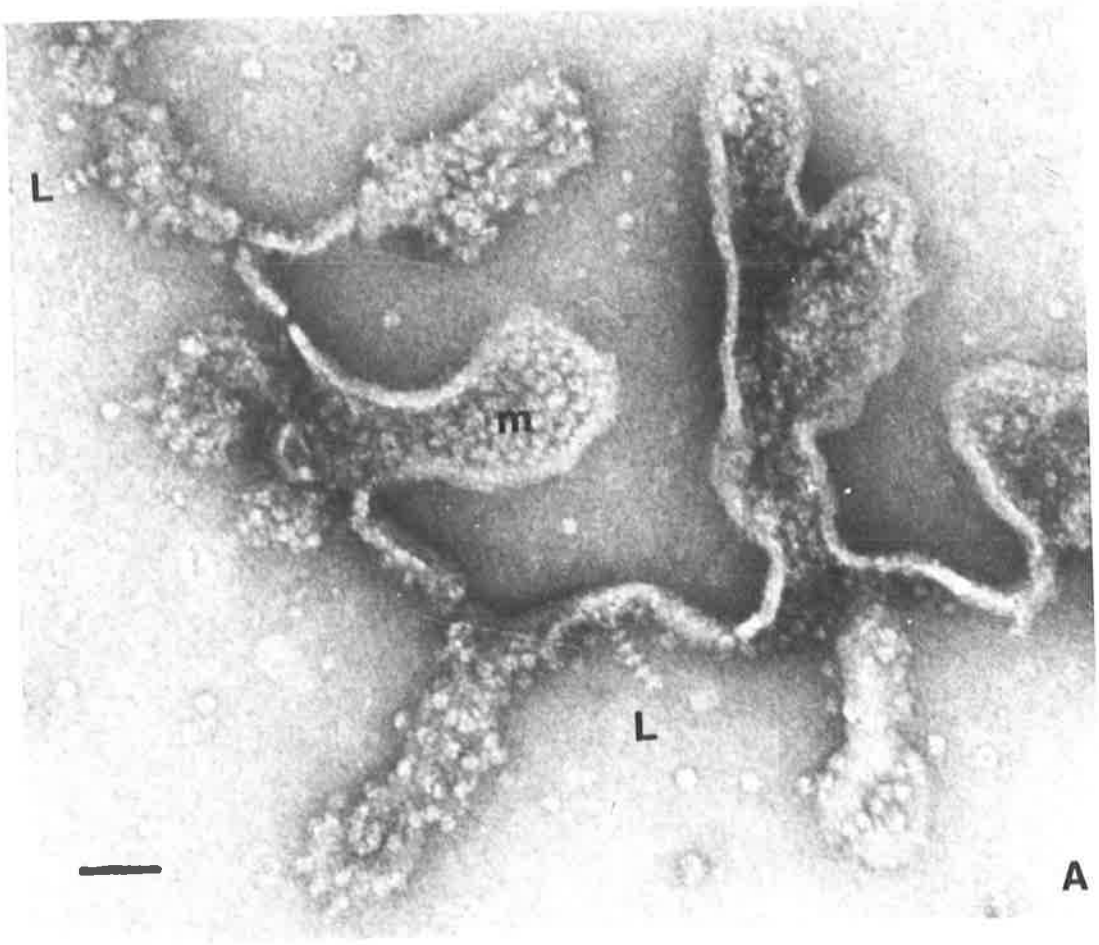
PLATE 3.6. ELECTRON MICROGRAPHS OF NEGATIVELY-STAINED CYTOPLASMIC MEMBRANES OF B. AMYLOLIQUEFACIENS SHOWING LADDER-LIKE COMPLEXES.

A. Two ladder-like complexes (L) are seen protruding from cytoplasmic membranes isolated by osmotic lysis.

Bar marker = 0.073μ

B. Fine structure of ladder complexes. The complex comprises from 3 to 7 subunits attached to a fine thread. One of the terminal subunits is often observed in the shape of an arrowhead.

Bar marker = 0.05μ


L
m
L
A

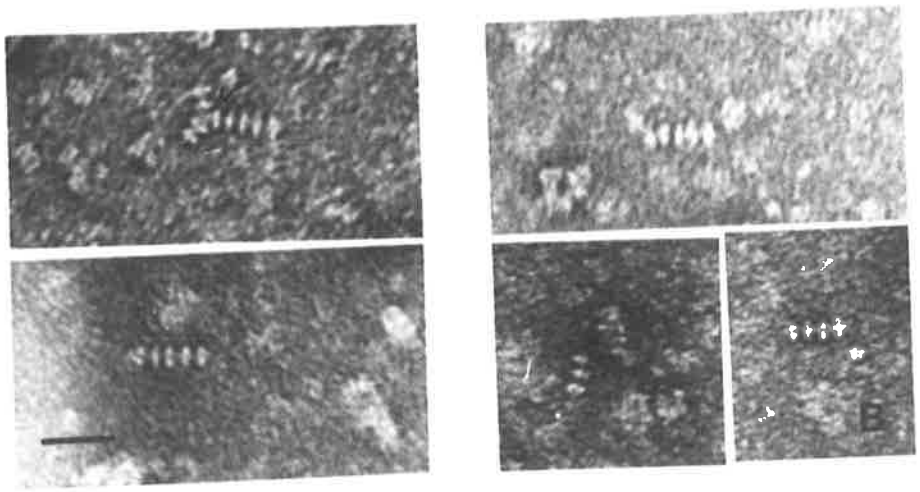

B

PLATE 3.7. ASSOCIATION OF RIBOSOMES WITH OSMOTICALLY-ISOLATED MEMBRANE VESICLES OF *B. AMYLOLIQUEFACIENS*.

Membrane vesicles were prepared by the osmotic lysis of protoplasts as described in Chapter 2, except that 0.05M tris buffer replaced the phosphate buffer throughout the procedure and RNAase and EDTA were omitted.

A. Electron micrograph of sectioned membrane vesicles. Ribosomes are clearly evident trapped within the membrane vesicles. The surrounding background is free of ribosomes.

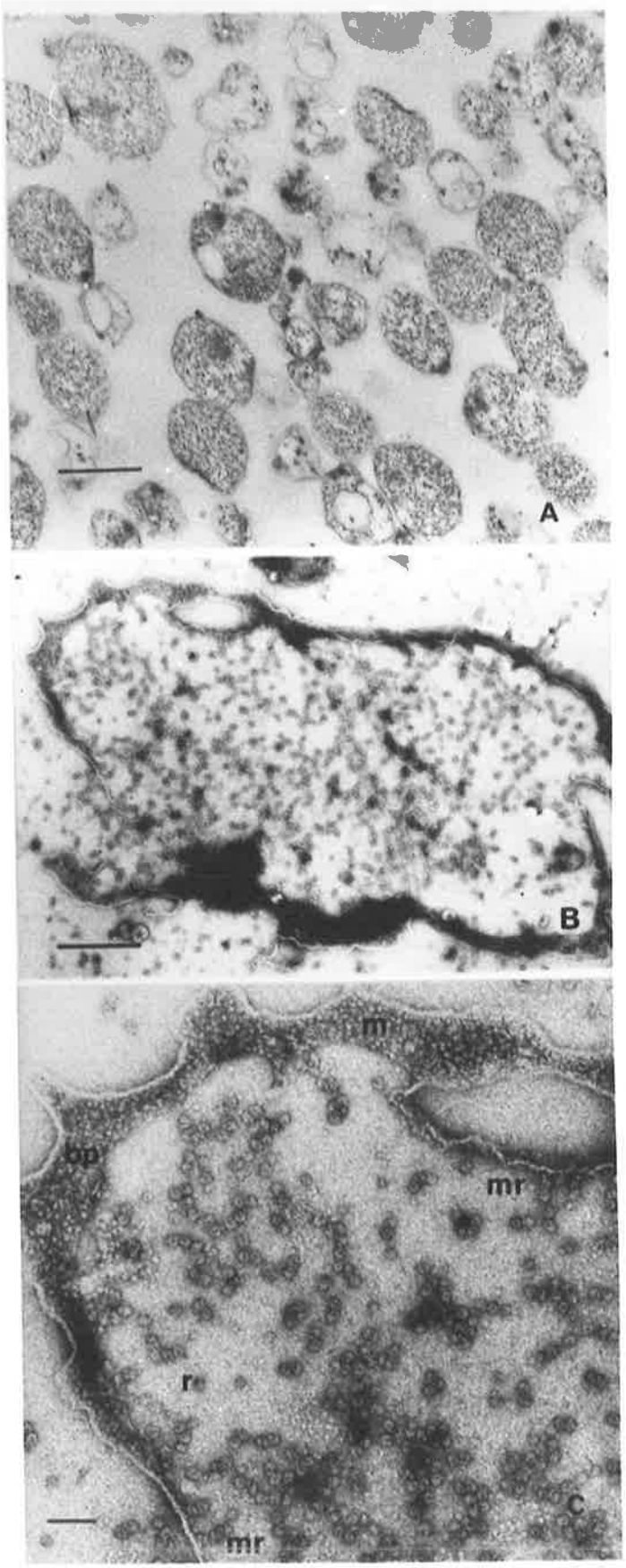
Bar marker = 1.0μ

B. Electron micrograph of negatively-stained membrane vesicles. The membrane vesicles have lysed and fragmented on the E.M. sample grid resulting in the release of the entrapped ribosomes.

Bar marker = 0.5μ

C. High-powered electron micrograph of portion of Plate 3.7B. The fragmented membrane (m) is covered with the small stalked particles (bp), however the majority of the ribosomes (r) are clearly not observed in association with the membrane. There does appear to be some form of association between the cytoplasmic membrane and ribosomes in some places (mr).

Bar marker = 0.1μ


A
B
m
op
mr
r
C
mr

was not as definite as was the case for the small particles.

d. Morphology of membranes prepared by French pressure cell treatment, (F.P. vesicles).

F.P. vesicles were prepared from cells suspended in phosphate buffer not containing RNAase or EDTA, as described in Chapter 2. In contrast to osmotically-prepared membrane vesicles, the majority of F.P. vesicles when examined using negative stains, did not lyse on the E.M. specimen grid, due possibly to their small size (Plate 3.10). Their diameter, 0.035 to 0.08μ was about 1/20th that of intact protoplasts. Little structural detail was evident in intact vesicles or in the small population that underwent fragmentation. There was no evidence of the stalked particles either on or off the membrane or of a periodic staining pattern. No ribosomes were evident in the background or trapped within the vesicle. The cell wall remaining after cell disruption was observed to be split completely open in only one or two places (not shown).

3. Freeze-fracture electron microscopy of the cytoplasmic membrane of cells and protoplasts

Freeze fracturing is believed to split membranes along the central hydrophobic region of the lipid bilayer revealing the inner and outer fracture faces (Pinto da Silva and Branton, 1970). In some instances the fracture plane may pass between the membrane surface and the surrounding ice, revealing after etching, an inside or outside view of the membrane surface (Branton and Southworth, 1967). For most membranes examined to date, the inner and outer fracture faces are studded with particles of various sizes (intramembranous particles) often asymmetrically distributed between the two halves of the bilayer

(Wrigglesworth et al., 1970; Packer et al., 1974). Freeze-fracture electron microscopy of B. amyloliquefaciens cells and protoplasts was undertaken to observe further structural details of the cytoplasmic membrane.

Freeze-fracture electron micrographs of whole cells obtained as described in Chapter 2, are shown in Plate 3.8. The only fracture views obtained were convex and thus represent the inner fracture face (i.f.f.) of the cell membrane. A large number of intramembranous particles were observed on this face. A view of the outer etched face of the cell membrane (c.m.) was quite distinct from the fracture face due to its mosaic appearance and lack of particles. What appears to be cell wall material (c.w.) was also observed associated with the etched cell membrane surface.

Both convex and concave fracture views of the cytoplasmic membrane were obtained when protoplasts were examined, however no etched views of either membrane surface were obtained (Plate 3.9). The convex inner fracture face (i.f.f.), had an appearance similar to that observed for cells with regard to particle size and density; the particles taking up most of the fracture surface. The particle density in this face was 2740 ± 224 particles/μ^2, and the particle distribution was primarily random although some patching was observed. The appearance of the concave outer fracture face (o.f.f.), was distinctly different. The particle density, 338 ± 74 particles/μ^2, was considerably less, the ratio of particle density between inner and outer fracture faces being about 8 to 1. The particle distribution in the outer fracture face was random, with most of the surface area being devoid of particles.

There was also a marked difference in the size of the particles associated with each fracture face. The size class

PLATE 3.8. FREEZE-FRACTURE ELECTRON MICROGRAPHS OF *B. AMYLOLIQUEFACIENS* CELLS.

Whole cells suspended in washed-cell suspension medium were slowly cooled to 4^{o} and then frozen in Freon followed by liquid N_2. Samples were then freeze fractured and etched for 2 minutes as described in Chapter 2. The resulting metal replicas were then observed under the electron microscope.

Only convex views of the cell surface were obtained when using whole cells. These represent either the inner fracture face of the cytoplasmic membrane (i.f.f.) on the external surface view of the cell membrane (cm) and the associated cell wall (cw).

The shadow direction is indicated by the arrows.

Bar markers = 0.2μ

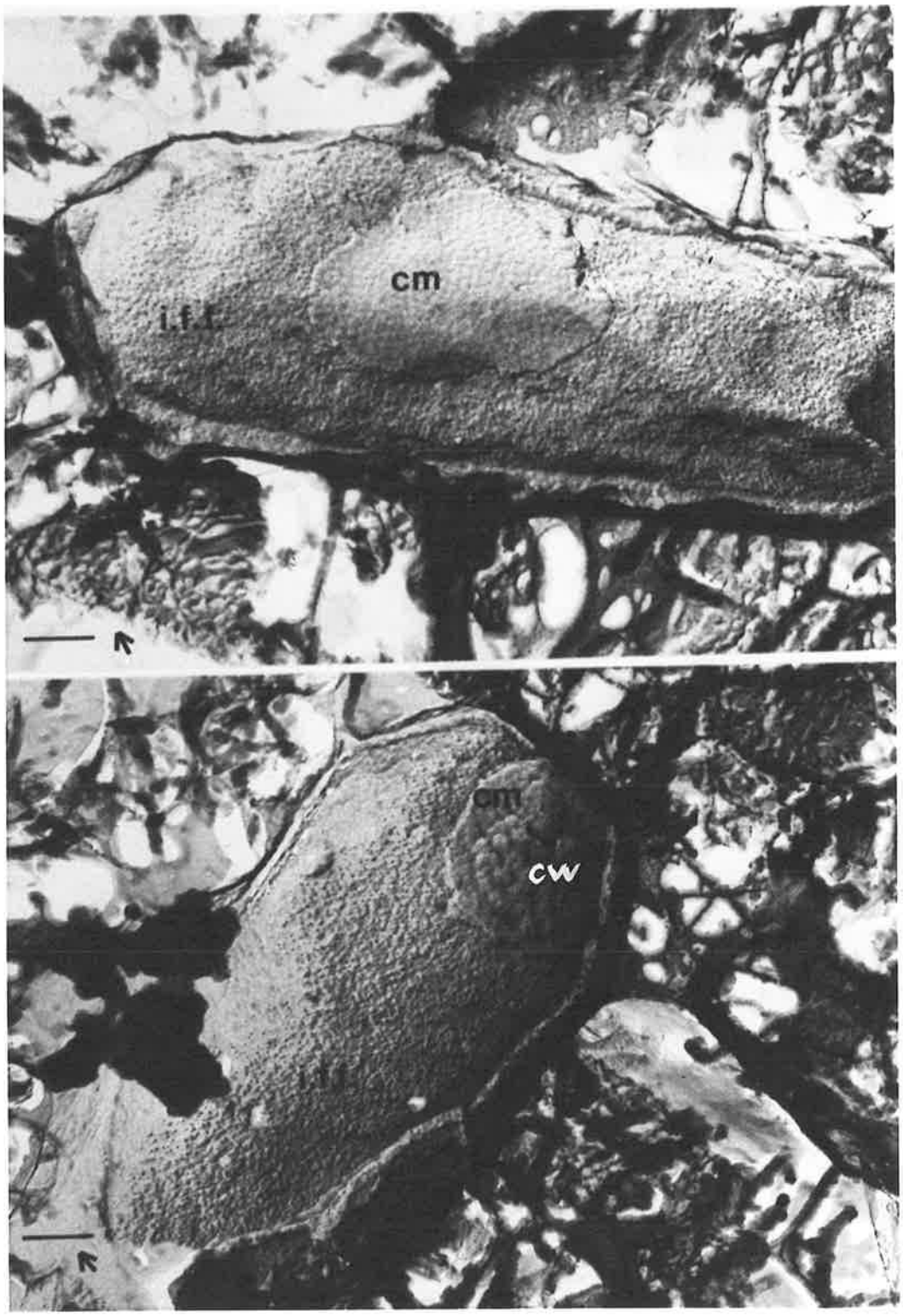

cm
i.f.l.
cm
cw
i.f.l.

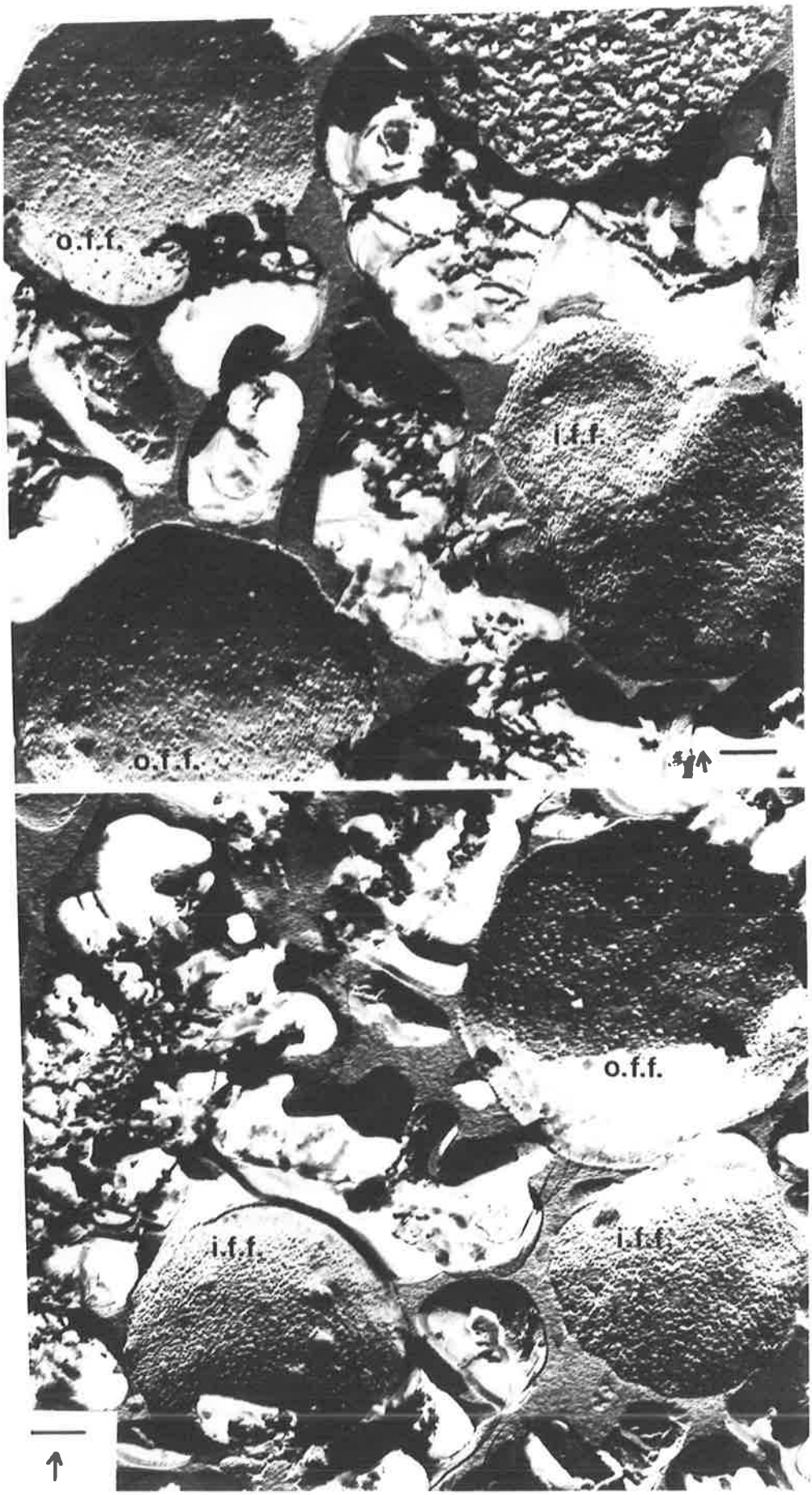
PLATE 3.9. FREEZE-FRACTURE ELECTRON MICROGRAPHS OF *B. AMYLOLIQUEFACIENS* PROTOPLASTS.

Protoplasts in protoplast medium were slowly cooled to 4° and then rapidly frozen in Freon followed by liquid N_2. Freeze fracturing and etching were as described in Chapter 2.

Two different views of protoplasts were obtained. These were either convex representing the inner fracture face of the cytoplasmic membrane (i.f.f.), or concave representing the outer fracture face of the membrane (o.f.f.). Intramembranous particles associated with each of these two fracture faces differed in their distribution and size.

Shadow direction is indicated by the arrows.

Bar markers = 0.2μ


o.f.f.
i.f.f.
o.f.f.
o.f.f.
i.f.f.
i.f.f.

distribution of the intramembranous particles associated with both fracture faces of protoplasts, is shown in Figure 3.1. Particles associated with the outer fracture face (A) were on average, greater in size and exhibited greater size variation than those of the inner fracture face (B). The predominant size of particles associated with the outer fracture face was 151 to 175A (22%) and 226 to 250A (20%). For the inner fracture face, particles were more uniform in size, greatest number being in the 76 to 100A size class (33%).

4. Sucrose density gradient analysis of osmotically-prepared membranes

The osmotic lysis procedures described in Chapter 2, results in clean membrane vesicles, uncontaminated by cells, protoplasts or cell wall debris. However the final membrane pellet was not homogeneous but comprised a tightly packed reddish-brown pellet overlaid by a faintly reddish-brown "fluffy layer". Separation of these was difficult without considerable cross contamination occurring. The pellet consisted of membrane vesicles which exhibited Brownian motion and the fluffy layer which was viscous, comprised membrane vesicles which did not exhibit Brownian motion. The proportion of pellet to fluffy layer varied between preparations and was observed with membranes isolated from the wild type and the LF^- mutant strains of B. amyloliquefaciens.

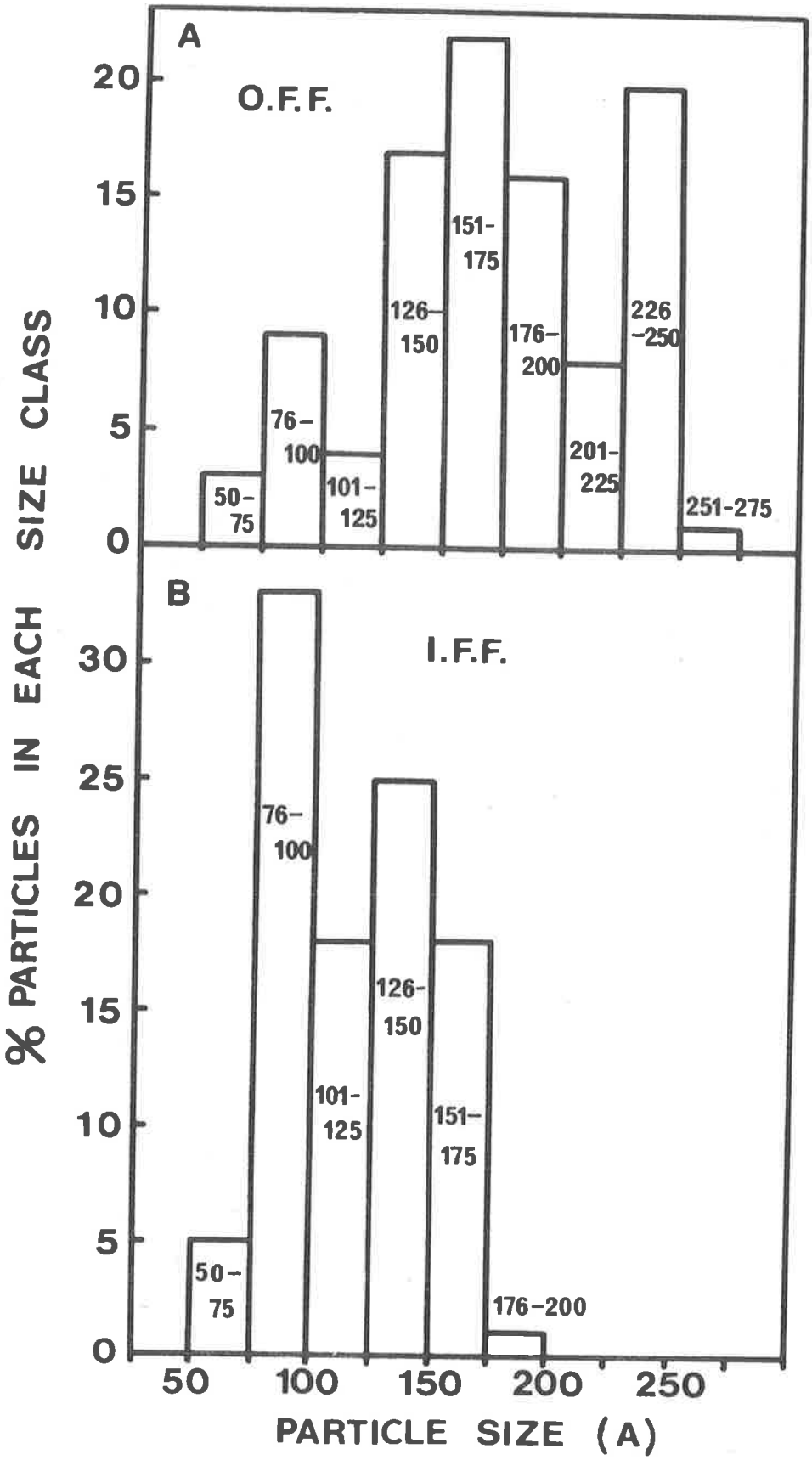
In an effort to separate these fractions. osmotically-prepared membranes were analysed on a linear 25 to 65% (w/v) sucrose density gradient in phosphate buffer, as described in Chapter 2. Two distinct peaks were evident in the O.D. 280_{nm} profile (Figure 3.2A). The peak corresponding to fractions 5 and 6 was symmetrical and smooth, whilst that contained in

FIGURE 3.1. HISTOGRAMS OF THE SIZE CLASS DISTRIBUTION OF INTRAMEMBRANOUS PARTICLES ON BOTH INNER AND OUTER FRACTURE FACES OF THE CYTOPLASMIC MEMBRANE OF B. AMYLOLIQUEFACIENS.

Data for each histogram is based on the diameter of a total of 150 particles counted from a total of 5 separate fracture faces. The % of particles within each 25A size class is shown.

A. Concave or outer fracture face, (o.f.f.).

B. Convex or inner fracture face, (i.f.f.).


A
O.F.F.
20
15
10
5
0
50–75
76–100
101–125
126–150
151–175
176–200
201–225
226–250
251-275
B
I.F.F.
30
25
20
15
10
5
0
50–75
76–100
101–125
126–150
151–175
176–200
50
100
150
200
250
% PARTICLES IN EACH SIZE CLASS
PARTICLE SIZE (A)

fractions 10 and 11 consisted of two main peaks which gave a "noisy" O.D. trace. Fractions from this gradient were dialysed and examined by negative-staining electron microscopy. Fragmented membrane vesicles were clearly evident in fractions 10 and 11. Fractions 5 and 6 consisted of membrane vesicles only some of which had fragmented. However these vesicles were thoroughly trapped within a network of fimbri, and this may have accounted for their position on the gradient and their lack of fragmentation. This network was most likely derived from the fimbri and mucous surrounding the cell. Its presence during lysis cannot be avoided and thus it subsequently acts as a trap for many of the membrane vesicles which are formed. Relatively less fluffy layer was observed when vesicles were prepared from protoplasts rather than cells which may have been due to the partial removal of fimbri and mucous material during protoplast preparation. The separate rerunning of the gradient-purified fractions resulted in a single peak for each sample at the expected position.

The density gradient profile of the supernatant remaining after centrifugation of membrane vesicles at 35,000g for 30 minutes is shown in Figure 3.2B. Very little material remained in the supernatant, however a small peak at fraction 5, which most likely represents contaminating fluffy layer, was evident.

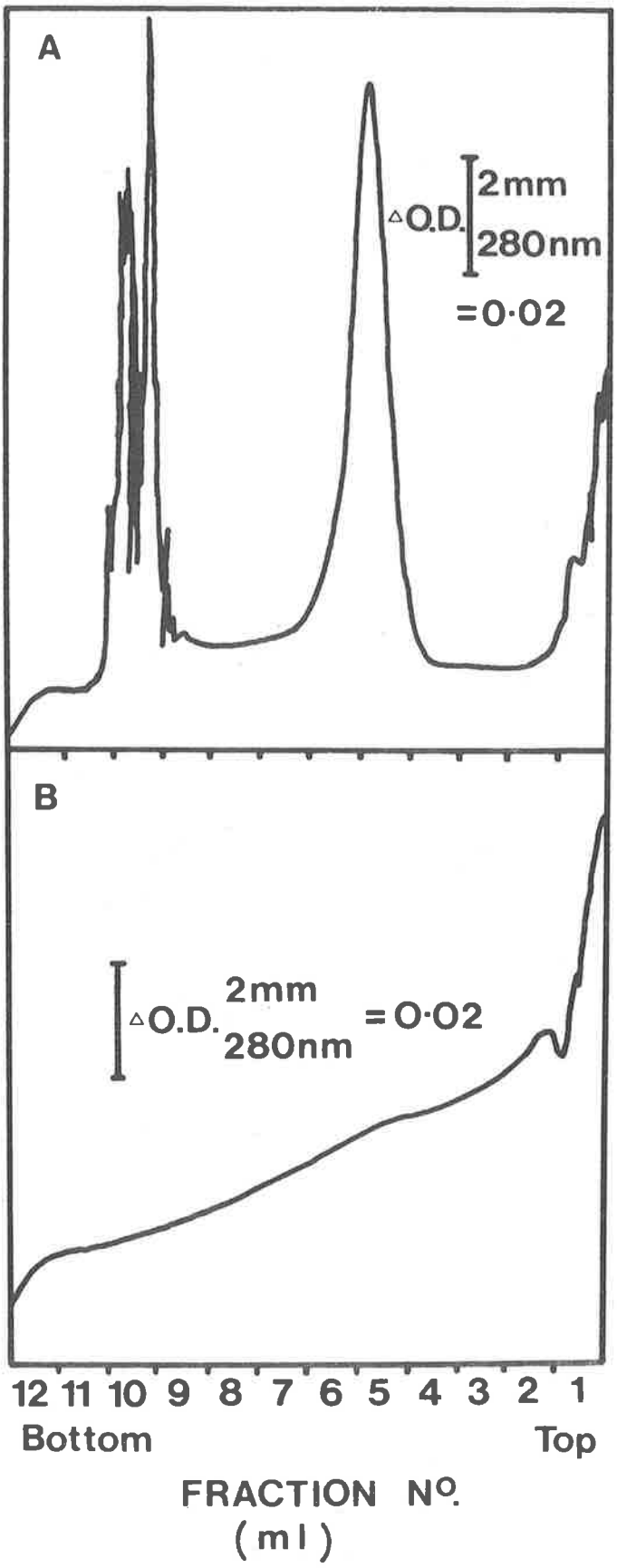
In addition to electron microscopy, Mg^{2+}-activated ATPase activity was used as a marker for determining the position of membrane material on the gradient. The ATPase activity of isolated membranes was first determined by the procedures described in Chapter 2. A value of 0.05μ mole P_i/minute/mg protein at 30^{o} for B. amyloliquefaciens compares favourably with the value of 0.167μ mole P_i/minute/mg protein at 37^{o} obtained for isolated membranes of B. subtilis (Rosenthal and

FIGURE 3.2A. SUCROSE DENSITY GRADIENT PROFILE OF MEMBRANES OF B. AMYLOLIQUEFACIENS.

Membranes were prepared by the osmotic lysis of cells and resuspended in 0.1M potassium phosphate buffer, pH6.6 containing 10% (w/v) sucrose. Membranes (0.5ml) were centrifuged in a linear 25 to 65% (w/v) sucrose density gradient as described in Chapter 2. The OD 2mm/280nm profile was recorded using an ISCO density gradient fractionator/ spectrophotometer unit, 1ml fractions from the top to the bottom of the gradient were collected.

FIGURE 3.2B. SUCROSE DENSITY GRADIENT PROFILE OF THE SUPERNATANT REMAINING AFTER CENTRIFUGATION OF B. AMYLOLIQUEFACIENS MEMBRANES.

Membranes were prepared by the osmotic lysis of cells and resuspended in 0.1M potassium phosphate buffer, pH6.6. 0.5ml of the supernatant remaining after centrifugation of these membranes at 35,000g for 30 minutes was made to 10% (w/v) sucrose and loaded onto a 12ml linear 25 to 65% (w/v) sucrose density gradient which was centrifuged and analysed as described in Chapter 2.


A
△O.D.
2mm
280nm
=0·02
B
△O.D.
2mm
280nm
=0·02
12 11 10 9 8 7 6 5 4 3 2 1
Bottom
Top
FRACTION Nº.
(ml)

Matheson, 1973).

For the analysis of the ATPase activity associated with the density gradient fractions, membrane vesicles were run in a linear 25 to 65% (w/v) sucrose density gradient prepared in ATPase buffer, as described in Chapter 2. The resulting density gradient profile (Figure 3.3A) was similar to that shown in Figure 3.2A however the peak represented by fractions 5 and 6 was comparatively greater, and two peaks were evident in the region of fractions 9 to 11. The profile of the Mg^{2+}- activated ATPase activity is shown in Figure 3.3B after correction was made for the effect of sucrose on enzyme activity and non-enzymic ATP hydrolysis, as described in Chapter 2. ATPase activity was associated with both regions of the gradient and was proportional to the amount of membrane material present within the two regions. Control experiments using density gradients lacking Mg^{2+} confirmed that the ATPase activity was Mg^{2+} dependent.

5. The use of isolated B. amyloliquefaciens cytoplasmic membranes in the study of extracellular enzyme secretion

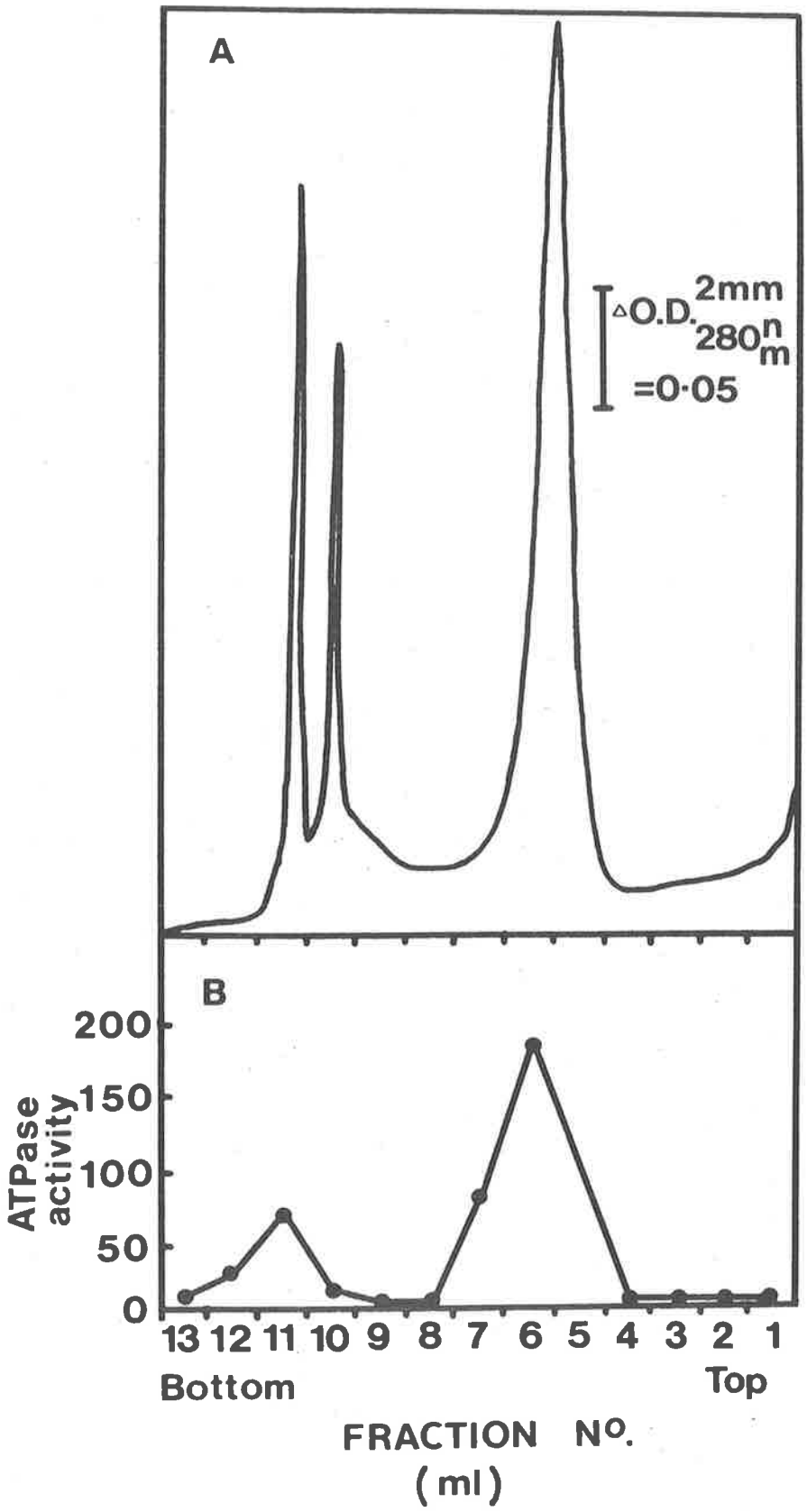
This section deals with a number of experiments involving isolated membrane vesicles designed to provide an experimental system for the study of secretion. Although these experiments were unsuccessful a brief account of these is included in this section as they still may have potential as experimental systems for future work, providing some of the difficulties encountered in this work can be overcome.

FIGURE 3.3A. SUCROSE DENSITY GRADIENT PROFILE OF B. AMYLOLIQUEFACIENS MEMBRANES RUN IN AN "ATPase" DENSITY GRADIENT.

B. amyloliquefaciens membranes were prepared by the osmotic lysis of cells as described in Chapter 2. After the final centrifugation, membranes were resuspended in ATPase buffer (75mM tris, 1.25mM $MgCl_2$, pH7.8) containing 10% (w/v) sucrose. 0.5ml of membranes were centrifuged in a linear 25 to 65% sucrose density gradient (12ml) as described in Chapter 2. The OD2mm/280nm profile was recorded and 1 ml fractions from the top to the bottom of the gradient were collected.

FIGURE 3.3B. PROFILE OF Mg^{2+}-ACTIVATED ATPase ACTIVITY ASSOCIATED WITH FRACTIONS OBTAINED FROM B. AMYLOLIQUEFACIENS MEMBRANES RUN ON A SUCROSE DENSITY GRADIENT.

1 ml fractions were collected from the gradient shown in Figure 3.3A and incubated at 30^{o} for 30 minutes after ATP was added to give a final concentration of 2.5mM. Mg^{2+}-activated ATPase activities of each fraction (expressed as n mole P_i released/30 minutes/ml fraction at 30^{o} were determined as described in Chapter 2.


A
ΔO.D. 280 nm 2mm
=0·05
B
ATPase activity
200
150
100
50
0
13 12 11 10 9 8 7 6 5 4 3 2 1
Bottom
Top
FRACTION No.
(ml)

a. Binding of ribosomes to French pressure cell-prepared vesicles (F.P. vesicles).

There is evidence to suggest that E. coli membrane vesicles prepared by the French pressure cell are inverted (Hertzberg and Hinkle, 1974; Rosen and McClees, 1974; Futai and Tanaka, 1975). If F.P. vesicles of B. amyloliquefaciens are also inverted, the possibility arose that ribosomes may bind to the outside of such vesicles and provide a system analogous to microsomal preparations from animal cells.

The experimental approach involved the separate isolation of F.P. vesicles and ribosomes and incubating these together in conditions considered conducive for the binding of ribosomes to membranes. Ribosome-membrane associations occurring as a result of the incubation were examined by electron microscopy. The results are shown in Plate 3.10. Plate 3.10A shows F.P. vesicles prepared in phosphate buffer as described in Chapter 2. These vesicles were transferred into a tris, Mg^{2+}, K^{+} buffer and incubated with ribosomes prepared by standard procedures. The electron micrographs shown in Plate 3.10B and C which were obtained using ribosomes prepared from E. coli rather than B. amyloliquefaciens, show that although the majority of the ribosomes are not associated with the vesicles, ribosomes do appear to be loosely bound to some of the vesicles. Similar results were obtained using B. amyloliquefaciens ribosomes. As the binding of these few ribosomes to the membrane was not convincing and possibly only non-specific, this line of work was not continued.

b. Ribonuclease transport using membrane vesicles.

It has been shown that B. amyloliquefaciens contains an

PLATE 3.10. BINDING OF RIBOSOMES TO B. AMYLOLIQUEFACIENS MEMBRANE VESICLES PREPARED BY THE FRENCH PRESSURE CELL.

A. Electron micrograph of membrane vesicles (mv) prepared by the French pressure cell procedure as described in Chapter 2 and examined by negative staining.

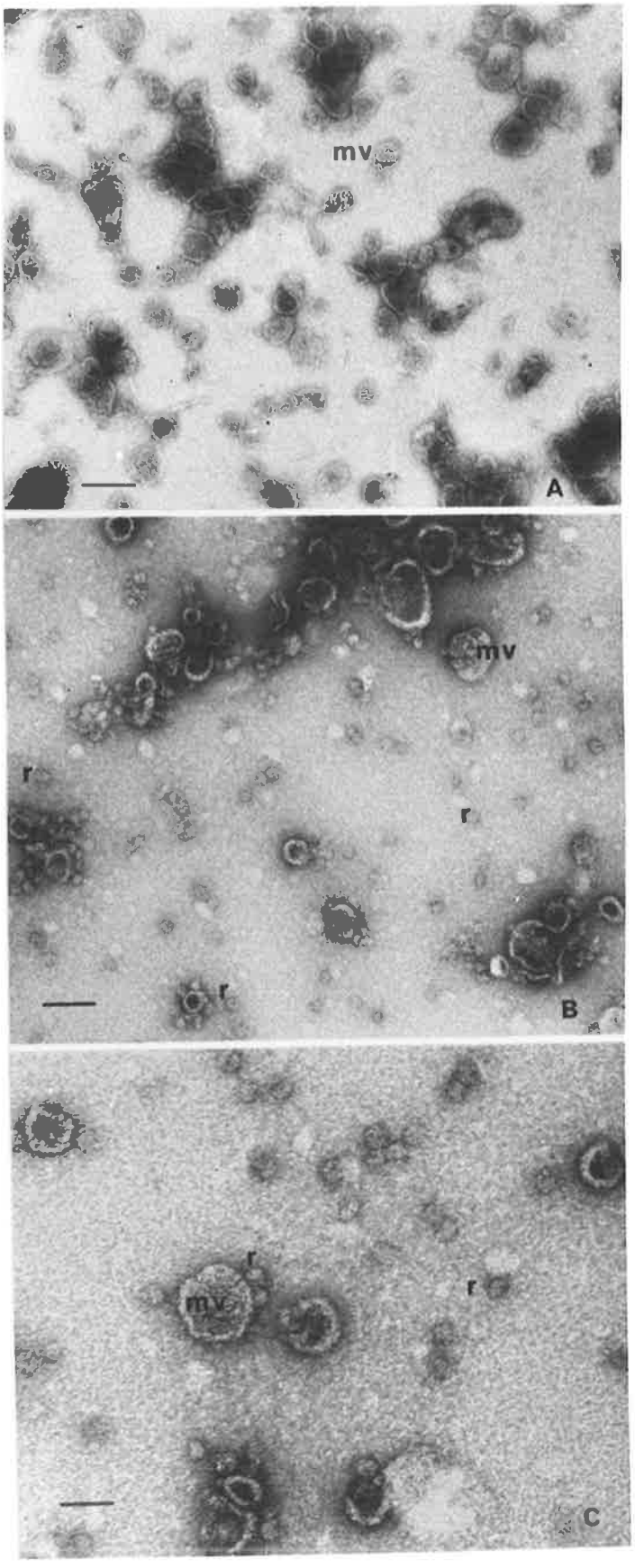
Bar marker = 0.1μ

B. The above membrane vesicles (mv) were transferred from phosphate buffer into a tris, Mg^{2+}, K^{+} buffer and E. coli ribosomes (r) were added and incubated together with the vesicles.

Bar marker = 0.1μ

C. High-powered electron micrograph of E. coli ribosomes (r) in association with B. amyloliquefaciens membrane vesicles (mv) prepared by the French pressure cell.

Bar marker = 0.05μ


mv
A
mv
r
r
r
B
r
mv
r
C

intracellular RNAase inhibitor which can combine virtually irreversibly with the extracellular RNAase of this organism (Smeaton and Elliott, 1967b). For a number of reasons it was thought that an intracellular RNAase inhibitor - RNAase enzyme complex may possibly be a precursor of the extracellular RNAase.

To test this possibility, a complex of RNAase inhibitor and RNAase was prepared in which all RNAase activity was titrated out by the inhibitor. This complex was then tested as a possible precursor for RNAase by determining whether RNAase accumulated inside F.P. vesicles after addition of the complex to the outside of such vesicles. The procedure was as follows: F.P. vesicles were first prepared by lysing cells in the presence of RNA in the hope that some RNA would become trapped within the intravesicular space at cell lysis and subsequently act as substrate for any transported RNAase. However these experiments were not continued when it was found that an insufficient amount of RNA (or ^{32}P-RNA) to act as substrate could be trapped within these vesicles at cell lysis.

Because of the above problem it was decided to use osmotically-prepared vesicles rather than F.P. vesicles to test whether after entrapment of the RNAase inhibitor-RNAase complex within the vesicles, RNAase transport out of the vesicles occurred. Although entrapment of the complex within the vesicles was confirmed, no subsequent transport of RNAase out of the vesicles was detected.

c. Extracellular enzyme secretion using osmotically-prepared vesicles preloaded with cell lysates.

Although cell lysates of B. amyloliquefaciens contain little or no extracellular enzyme activity (May and Elliott, 1968), this does not discount the possibility that an inactive form of

these enzymes exists intracellularly. With this in mind, protoplasts were lysed in medium containing a high concentration of B. amyloliquefaciens cell lysate material such that this material became entrapped within the vesicles at lysis. After thorough washing, the level of extracellular enzyme activity in the supernatant was determined. However these levels were negligible after prolonged incubation.

d. Transport of purified extracellular enzymes across the cell membrane.

The final series of experiment involved the following: 1. Entrapping purified extracellular enzymes within osmotically-prepared vesicles and measuring their possible release from the vesicles. 2. The addition of purified extracellular enzymes to F.P. vesicles and determining whether a time-dependent accumulation of the enzyme within the intravesicular space occurred. However both types of experiment failed principally because of the high level of non-specific adsorption of the enzymes to the surface of the membrane vesicles, which could not be removed by repeated washing.

C. DISCUSSION

The work in this chapter shows that relatively clean cytoplasmic membranes of B. amyloliquefaciens can be isolated using osmotic lysis or French pressure cell treatment. Membranes isolated by the osmotic lysis of cells or protoplasts were vesicular and from their size, it would appear that the cell membrane ruptures into three or four fragments which reseal to form the vesicles. Membranes prepared using the French pressure cell technique were also vesicular but about 20% the average diameter of the osmotically-prepared membrane vesicles.

Membranes examined by negative-stain electron microscopy appear as fragments or sheets rather than in vesicular form as is observed in fixed-sectioned material. The periodic staining pattern observed using negative stains may represent some natural cleavage plane of the membrane. The stalked particles which most likely represent the ATPase complex were only observed when using negative stains and were most likely associated with the inner half of the membrane. The predominant intramembranous particle observed on the inner fracture face of the membrane was of similar size to the presumptive ATPase particle and as such may represent the true in situ location of the ATPase complex. Freeze-fracture studies also indicated that the size of the intramembranous particles differ in each half of the membrane, as did the number of particles associated with each half of the membrane, there being an 8 to 1 asymmetry in favour of the inner fracture face. Asymmetry in intramembranous particle distribution has been observed in a variety of membranes however only sarcoplasmic reticulum membranes display intramembranous particle asymmetry to this extent, (in this instance in favour of the concave face) (Packer et al., 1974).

The identity of the two other particles associated with the membrane, i.e. the "daisies" and the "ladder-like" complexes, is not known, and although they may be of potential interest, no useful comment can be made on these at present.

One of the questions of greatest interest to the secretion of extracellular enzymes is whether membrane-bound ribosomes exist in bacteria. Indeed evidence for membrane-bound ribosomes in isolated membranes often does not take into account the physical entrapment or adsorption of ribosomes to the membrane which may occur during membrane isolation. With the present knowledge that membrane preparations are largely in vesicle

form, the existence of ribosomes (or ribosomal RNA) in exhaustively washed membrane preparations is no longer valid evidence for membrane-associated ribosomes since free ribosomes may be trapped inside as indeed is clearly shown in sectioned vesicles in this chapter. Moreover the situation may be misleading in view of the demonstration that when vesicles are applied to an E.M. specimen grid and negatively stained, they fragment and open out. Thus what is in fact a vesicle containing free ribosomes may appear as a ribosome-studded fragment of membrane. In this present study no meaningful association of ribosomes with the membrane, was observed after fragmentation of the vesicles. This in itself does not disprove the existence of membrane-bound ribosomes in B. amyloliquefaciens, as the association may be destroyed on lysis or alternatively, the association in situ, may only be transitory.

CHAPTER 4

STUDIES ON THE PHYSICAL PROPERTIES OF B. AMYLOLIQUEFACIENS CYTOPLASMIC MEMBRANES

A. INTRODUCTION

One of the aims of this project was to establish the role of the cytoplasmic membrane in the process of extracellular enzyme secretion. A logical first approach was to determine whether changes occurring in the cell membrane could influence the synthesis and/or secretion of extracellular enzymes. Initially this plan involved using cell membrane preparations to characterize some of the physical properties of the membrane. It was hoped that upon characterization, it would be possible to alter the fluidity or physical state of the membrane *in situ*, and determine whether such changes influence extracellular enzyme secretion.

It has been concluded that the activities of a number of membrane-associated enzymes are influenced by changes occurring in the fluidity or physical state of the membrane lipids (Raison, 1973 a,b; Chapman, 1975; Cronan and Gelmann, 1975; Fox, 1975). Thus enzymes such as those of respiratory or transport systems exhibit changes in Arrhenius activation energy at two critical temperatures which correspond to changes occurring in the physical state of the membrane lipid, as determined by physical techniques (Linden *et al.*, 1973 a,b; Raison and McMurchie, 1974). The actual changes which occur at the molecular level may be complex and their nature uncertain. However, by analogy with results obtained using artificial phospholipid vesicles, these changes were postulated to define the initiation and termination of a lipid phase transition in which there is a gradual melting and increasing fatty acyl chain disorder in the hydrocarbon regions of the membrane with increasing temperature (Shimshick and McConnell, 1973; Grant *et al.*, 1974). These boundary temperatures of the phase transition are determined by the physical characteristics of

the lipid (Chapman et al., 1974; Wu and McConnell, 1975). For a number of organisms, the occurrence in their membranes of a lipid phase transition, has been shown to correlate with changes of physiological significance (Raison, 1973 a,b; McElhaney, 1974; Fox, 1975).

The work described in this chapter is aimed at determining whether lipid phase transitions occur in the cell membrane of B. amyloliquefaciens. It was hoped that a combination of, and correlation between physical and physiological methods may provide information on the influence of such changes in the membrane lipids on physiological activities associated with the cell membrane.

B. RESULTS

1. Spin-labelling studies on cells and osmotically-prepared membranes

The technique of spin labelling was used to monitor the lipid components of the membrane. In membrane studies the spin label is usually a lipid molecule containing a reporter group, normally a nitroxide free-radical compound which can be monitored by electron spin resonance (E.S.R.) spectroscopy. The spin label infused into the membrane provides information on the viscosity and polarity of its local environment, as well as the occurrence of phase transitions in the membrane lipid (Keith et al., 1973). Spin labels used in this study were either the methyl ester (m.e.) or acid form (a) of stearic acid with the nitroxide group attached to carbon atom 12, (12NS m.e. or 12NS a), or carbon atom 16, (16NS m.e. or 16NS a), from the carboxyl end.

All attempts to spin label the membrane of intact

cells and protoplasts resulted in rapid loss of the spin-label signal. However by heating cells or protoplasts at 65^{o} for 10 minutes prior to the addition of spin label, the signal remained stable over a period of 3 hours. Spectra recorded at 30^{o} for the spin label 16NS (m.e.) infused into heat-treated cells and protoplasts are shown in Figure 4.1A,B. The hyperfine coupling constant (A_n), provides a measure of the polarity of the spin-label environment, and its value for both cells and protoplasts indicates that the spin label was localized in a highly hydrophobic environment. (A_n values for nitroxide spin labels range from 14.2 gauss for spin label in octadecane, 15.7 gauss in 20% aqueous ethanol to 16.1 gauss in water at 30^{o}, (Raison et al., 1971)). In this environment the spin label underwent restricted isotropic motion; the motion in cells being relatively less restricted as indicated by the lower τ_o value, (τ_o = correlation coefficient, as defined in Chapter 2).

Osmotically-prepared membrane vesicles were spin labelled as described in Chapter 2. For the spin label 16NS (m.e.) (Figure 4.2B), the spectrum is similar to those shown in Figure 4.1 in respect to the hydrophobic localization of the spin label and its restricted isotropic motion within this location. Comparison of the motion of 12NS (m.e.) (Figure 4.2A) and 16NS (m.e.) (Figure 4.2B) in isolated membranes showed that greater motion (lower τ_o value), was obtained using 16NS (m.e.) which is in accord with the more terminal position of the nitroxide group in 16NS (m.e.). These spectra were very similar to those obtained for spin label infused into isolated B. amyloliquefaciens phospholipids (Figure 4.17) and thus indicate that these spin labels must be localized primarily in the phospholipid region of the membrane. Infusion of

FIGURE 4.1. E.S.R. SPECTRA OF THE SPIN LABEL 16NS (m.e.) INFUSED INTO THE CELL MEMBRANE OF B. AMYLOLIQUEFACIENS CELLS AND PROTOPLASTS.

Cells and protoplasts were heated at 65^{o} for 10 minutes prior to the addition of spin label to prevent spin-label reduction. ESR spectra were recorded at 30^{o} using a modulation amplitude of 2.5 gauss. Values of the hyperfine coupling constant (A_n) and of the spin-label motion (correlation coefficient, τ_o) were determined as described in Chapter 2.

A. Cells : A_n=14.6 gauss; $\tau_o = 14.6 \times 10^{-10}$s

B. Protoplasts: A_n=14.5 gauss; $\tau_o = 19.2 \times 10^{-10}$s

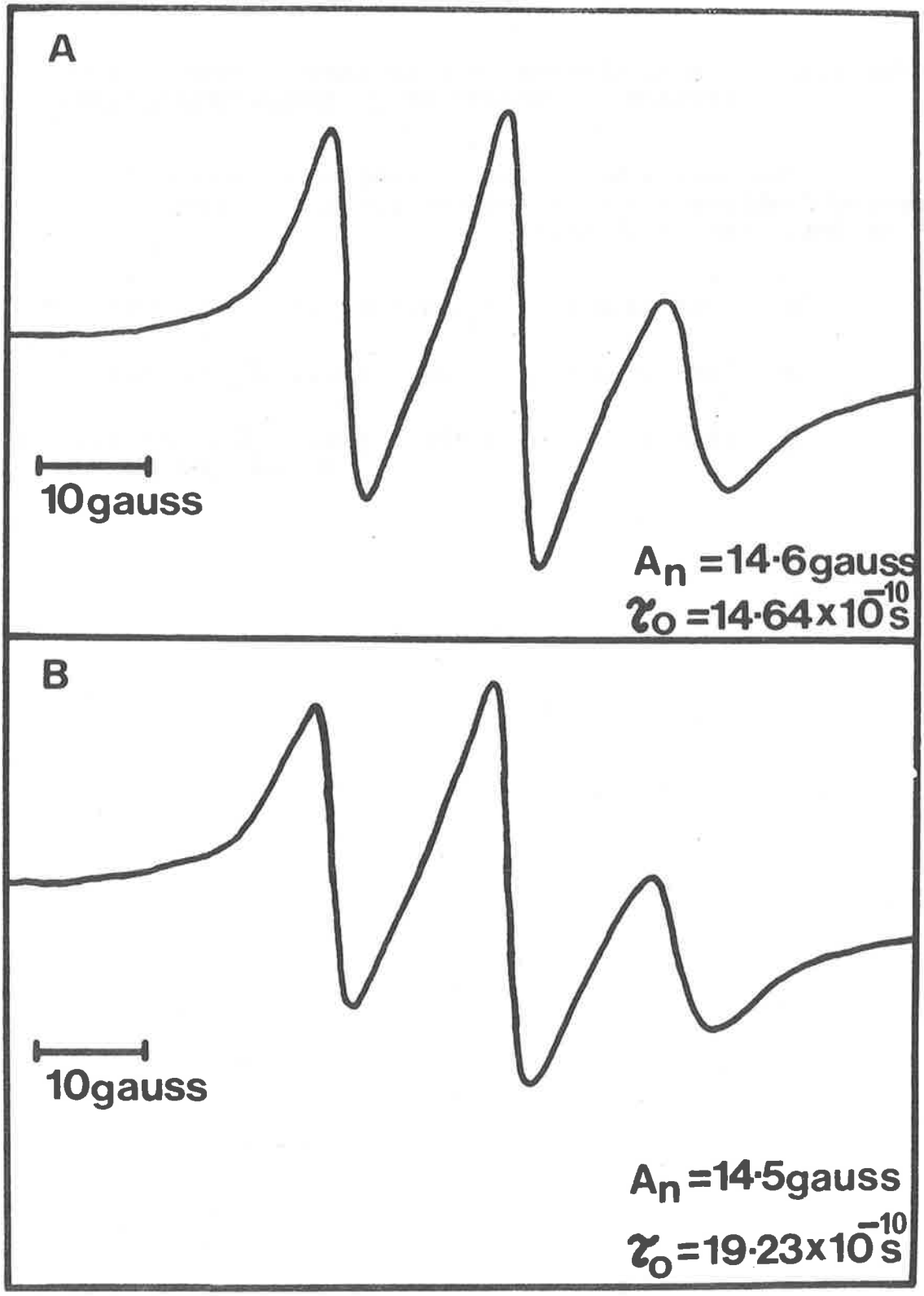

A
10gauss
A_n =14·6gauss
τ_o =14·64x10^{-10}s
B
10gauss
A_n =14·5gauss
τ_o =19·23x10^{-10}s

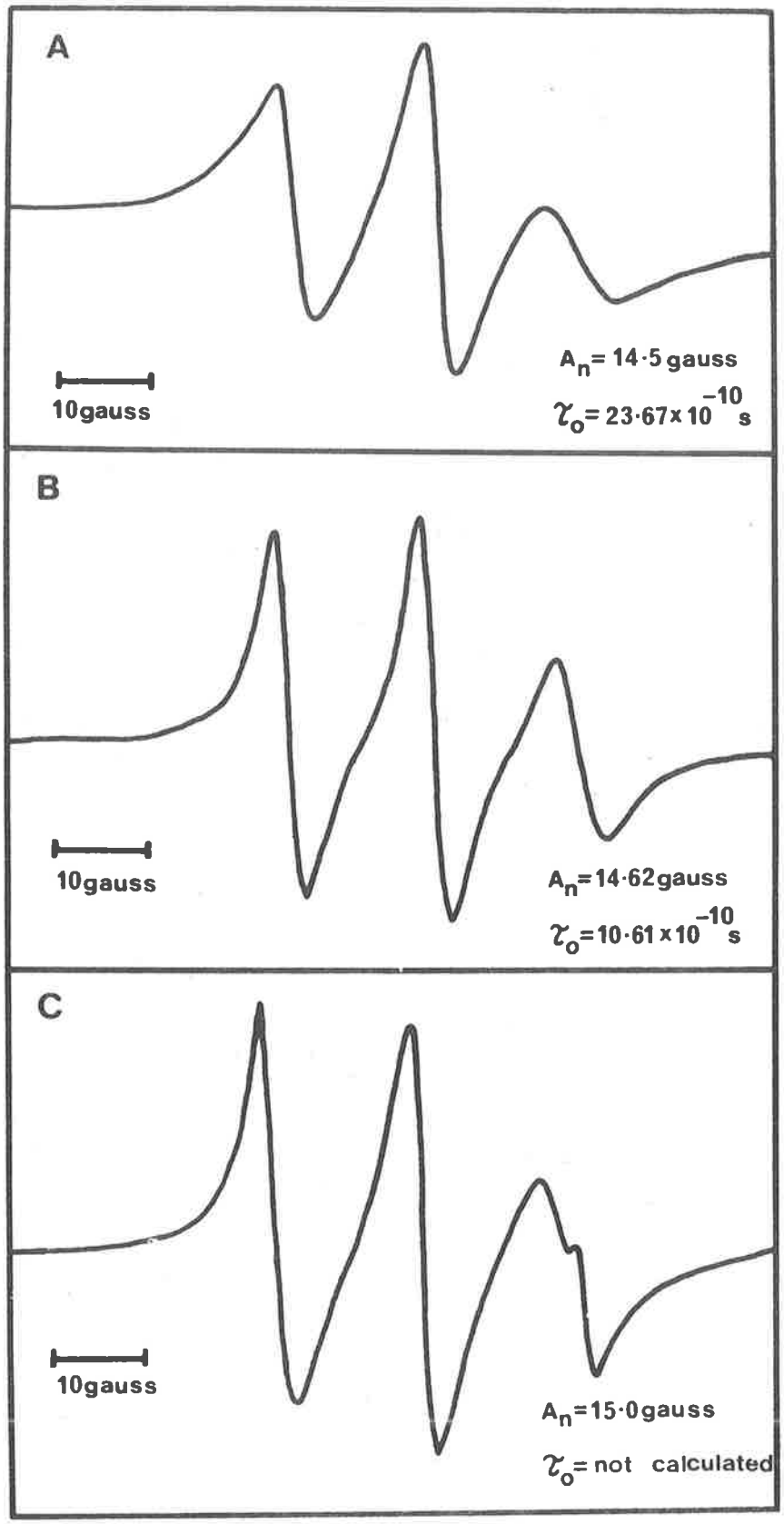
FIGURE 4.2. E.S.R. SPECTRA OF SPIN LABELS INFUSED INTO ISOLATED MEMBRANES OF B. AMYLOLIQUEFACIENS.

The spin labels were infused into osmotically-prepared membrane vesicles and the spectra recorded at 30° as described in Chapter 2.

A. 12NS (m.e.) : A_n=14.5 gauss; τ_o=23.7x10^{-10}s

B. 16NS (m.e.) : A_n=14.6 gauss; τ_o=10.6x10^{-10}s

C. 16NS (a) : A_n=15.0 gauss; τ_o (not able to be calculated)


A
10gauss
A_n= 14·5 gauss
τ_o= 23·67×10^{-10} s
B
10gauss
A_n= 14·62 gauss
τ_o= 10·61×10^{-10} s
C
10gauss
A_n= 15·0 gauss
τ_o= not calculated

16NS (a) into isolated membranes resulted in some aqueous partitioning of the spin label (evident as an extra peak in the high-field hyperfine line), which is in keeping with the slightly less hydrophobic location of this spin label, (A_n = 15.0 gauss, Figure 4.2C). Because of this aqueous partitioning, the acid form of the spin label was not used for studies on membrane phase transitions.

The effect of temperature on spin-label motion is shown in Figure 4.3 in which a series of spectra for the spin label 16NS (m.e.) infused into osmotically-prepared membranes have been recorded at different temperatures. Motion of the spin label increases with increasing temperature, (τ_o decreases). Although spin-label motion at 4.4° is relatively slow, it is still within the range where the correlation coefficient treatment can be employed. 12NS (m.e.) could not be used for measurement at low temperature as its motion was too restricted and meaningful motional analysis could only be performed at temperatures above 12°. Values for the hyperfine coupling constant (A_n) for 16NS (m.e.) infused into these membranes (approximately 14.5 gauss), did not change significantly with temperature. This together with the fact that there was no aqueous partitioning, indicates that 16NS (m.e.) was reporting changes within the hydrophobic region of the membrane at all temperatures tested.

A correspondence has been shown between the changes observed in the kinetics of certain membrane-associated enzymes and temperature-dependent events in the membrane lipid detectable by the spin-labelling technique (Raison et al., 1971a; Mehlhorn and Keith, 1972; Linden et al., 1973a; Raison and McMurchie, 1974; Fox 1975). Arrhenius plots of the motion of spin labels infused into a number of biological membranes

FIGURE 4.3. EFFECT OF TEMPERATURE ON THE E.S.R. SPECTRA OF THE SPIN LABEL 16NS (m.e.) INFUSED INTO B. AMYLOLIQUEFACIENS MEMBRANE VESICLES.

Membrane vesicles were prepared by the osmotic lysis of protoplasts and spin labelled with 16NS (m.e.). The value for the motion of the spin label ($\tau_o \times 10^{-10}$ s) is shown for each spectra together with the temperature at which the spectra were recorded.

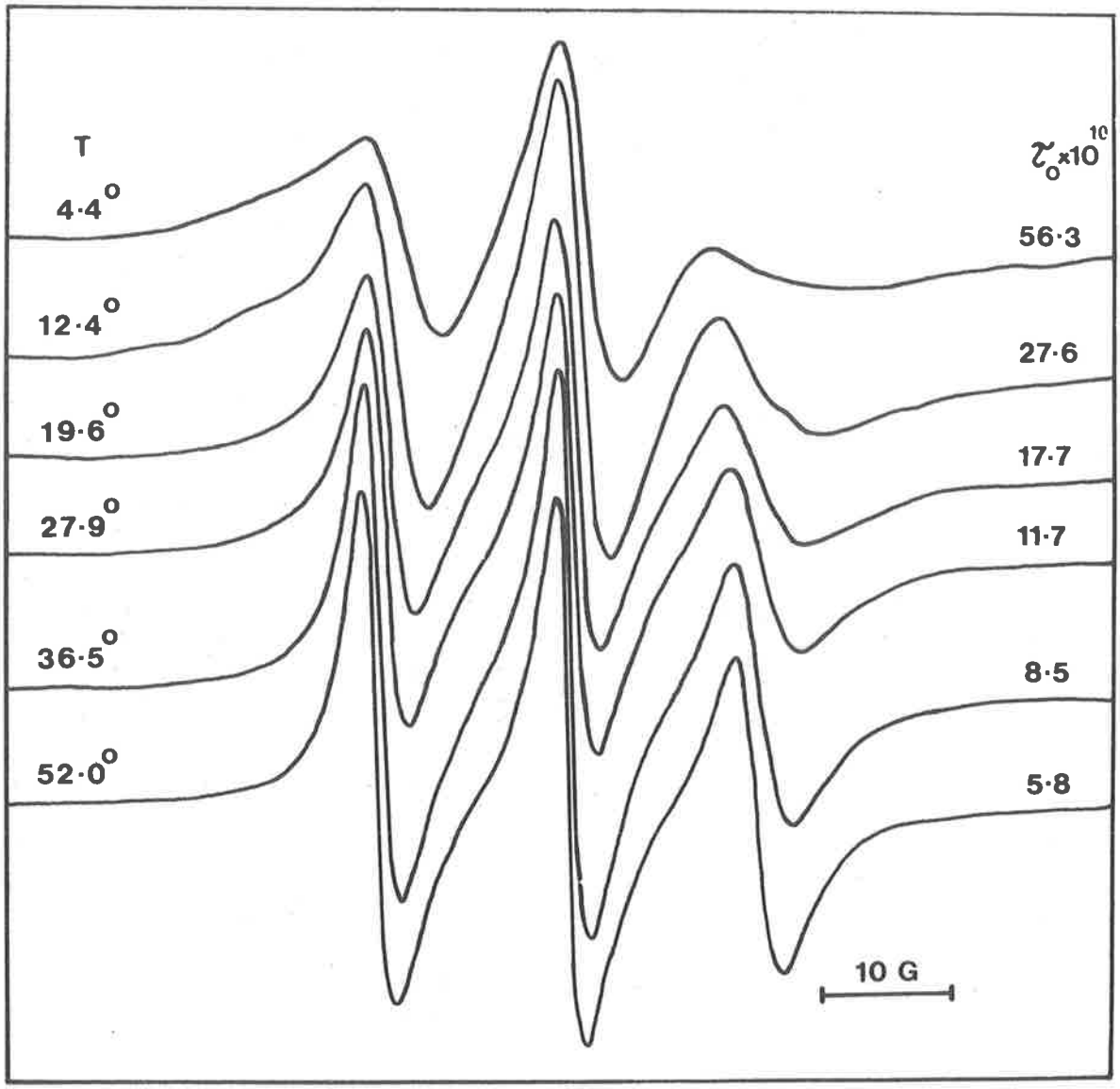

T
4·4°
12·4°
19·6°
27·9°
36·5°
52·0°
$\tau_o \times 10^{10}$
56·3
27·6
17·7
11·7
8·5
5·8
10 G

exhibit discontinuities in slope which have been interpreted as arising from temperature-induced changes (phase changes) in the molecular ordering of the membrane lipid (Raison et al., 1971a; Raison, 1973a,b; Eletr et al., 1974; Keith et al., 1975).

An Arrhenius plot of the change in spin-label motion (τ_o) as a function of temperature for 16NS (m.e.) in osmotically-prepared membrane vesicles of B. amyloliquefaciens, is shown in Figure 4.4. Four discontinuities (changes in slope) are evident at temperatures of about 39^o, 21^o, 15^o and 6^o. An Arrhenius plot of the motion of 16NS (m.e.) infused into the membrane of heat-treated cells is shown in Figure 4.5. Again four discontinuities are observed at approximately the same temperatures as was evident in Figure 4.4. These same discontinuities were observed in cells and isolated membranes irrespective of whether heating or cooling runs were employed.

2. Respiratory studies on isolated cytoplasmic membranes

Many biochemical activities are associated with the cytoplasmic membrane of bacteria. Prominent among these are the succinate and NADH oxidase systems (Machtiger and Fox, 1973) and Mg^{2+} and Ca^{2+} -dependent ATPases (Rosenthal and Matheson, 1973). In addition the electron transport chain is associated with the active transport of many substances across the cell membrane (Kaback, 1972; Konings and Freese, 1972; Bisschop et al., 1975). Monitoring the kinetics of such membrane-associated enzymes has previously been employed for a number of membranes to detect changes occurring in the physical properties of the associated lipid (Raison et al., 1971a; Linden et al., 1973a,b; Raison and McMurchie, 1974; Fox 1975).

This study was aimed at determining whether the electron transport chain of B. amyloliquefaciens, as measured by succinate or NADH oxidation could be used to monitor changes

FIGURE 4.4. ARRHENIUS PLOT OF THE MOTION (τ_o) OF THE SPIN LABEL 16NS (m.e.) INFUSED INTO B. AMYLOLIQUEFACIENS MEMBRANE VESICLES.

Membrane vesicles were prepared by the osmotic lysis of protoplasts and spin labelled as described in Chapter 2. Arrows indicate the approximate temperature at which discontinuities were evident.

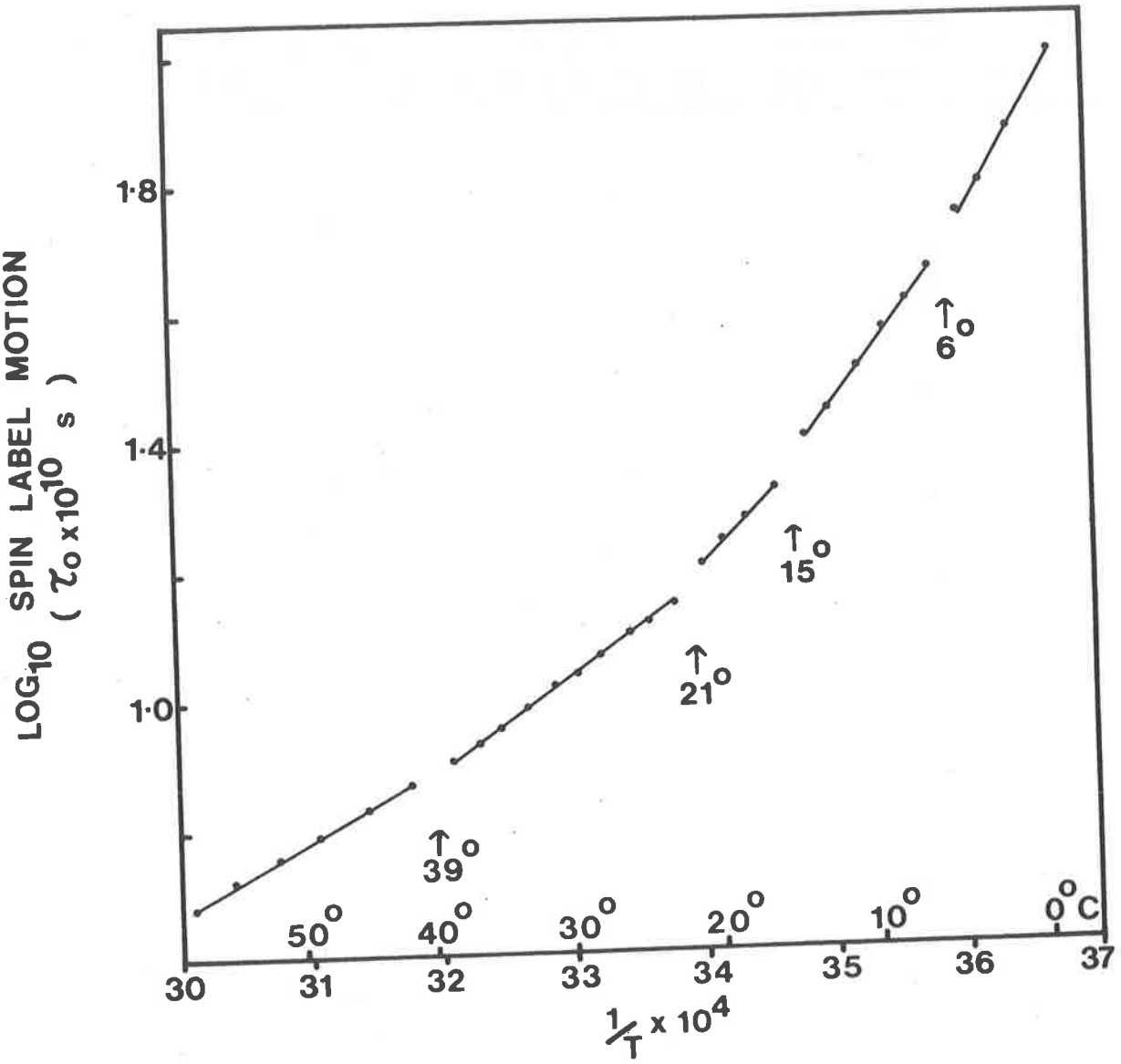
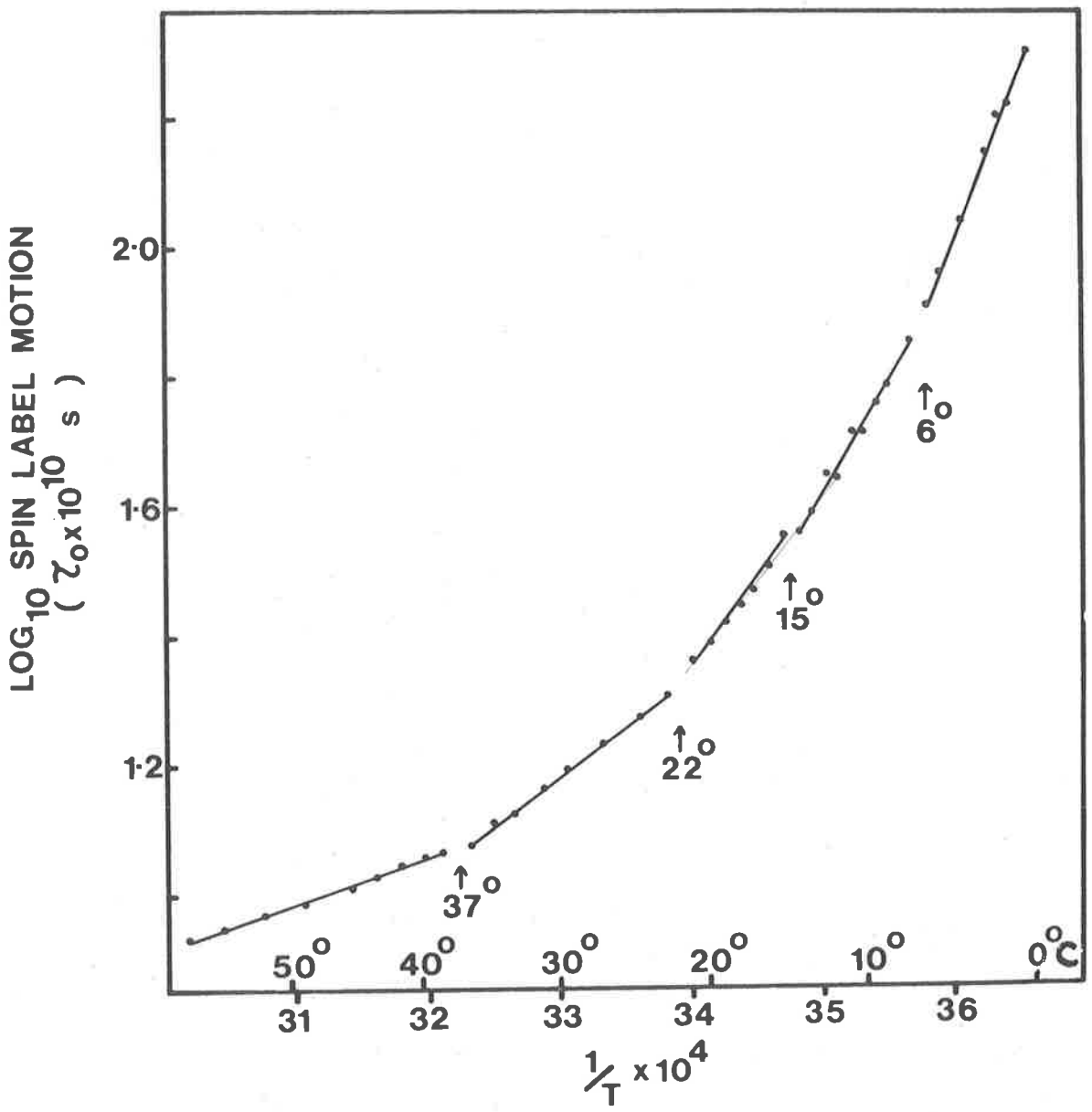

LOG10 SPIN LABEL MOTION
(τo x10^10 s)
1·8
1·4
1·0
39°
21°
15°
6°
50°
40°
30°
20°
10°
0°C
30
31
32
33
34
35
36
37
1/T x 10^4

FIGURE 4.5. ARRHENIUS PLOT OF THE MOTION (τ_o) OF THE SPIN LABEL 16NS (m.e.) INFUSED INTO THE MEMBRANE OF B. AMYLOLIQUEFACIENS CELLS.

Cells were heated at 65° for 10 minutes to prevent spin-label reduction. Arrows indicate the approximate temperatures at which discontinuities were evident.


LOG10 SPIN LABEL MOTION
(τc x 10^10 s)
2·0
1·6
1·2
37°
22°
15°
6°
50°
40°
30°
20°
10°
0°C
31
32
33
34
35
36
1/T x 10^4

occurring in the cell membrane due to alterations in the fluidity or physical state of the membrane lipids. Membranes were prepared by osmotic lysis of protoplasts (O.L. vesicles) or by the French pressure cell lysis of cells (F.P. vesicles) as described in Chapter 2, and rates of oxygen uptake were determined polarographically. A summary of the results is shown in Table 4.1.

The rates of endogenous oxygen uptake in both types of vesicle were extremely low and marked stimulation occurred after the addition of succinate or NADH and the respiration rate was proportional to the amount of membrane vesicles used. (The final concentrations of succinate and NADH which were routinely used were 100 and 200-times higher than the apparent K_m for each substrate respectively). ADP, Mg^{2+}, or 2,4 dinitrophenol did not affect the substrate-stimulated respiratory rate. The oxidation of succinate and NADH was cytochrome linked and was inhibited by cyanide (as was also observed for the endogenous rates of respiration of cells and protoplasts ; results not shown). For O.L. vesicles, NADH resulted in a 10-times greater rate of oxygen uptake than did succinate, however for F.P. vesicles, the rate obtained using NADH was not significantly greater than for succinate. The rate of succinate and NADH oxidation in O.L. vesicles was far greater than that observed for F.P. vesicles and this observation will be discussed in regard to the possible orientation of these two types of vesicle in the discussion section.

The addition of non-ionic detergents to rat liver mitochondrial membranes is believed to result in the abolition of the phase transition associated with these membranes (Raison *et al.*, 1971b). It was anticipated that if phase transitions were detected in *B. amyloliquefaciens* membranes, a similar effect may be seen. Therefore as a preliminary study, the

TABLE 4.1. SUCCINATE AND NADH OXIDATION BY *B. AMYLOLIQUEFACIENS* MEMBRANE VESICLES.

Membrane vesicles were prepared by the osmotic lysis of protoplasts (O.L. vesicles) or by French pressure cell lysis of cells (F.P. vesicles). Rates of respiration were determined polarographically as described in Chapter 2. Final concentrations of succinate and NADH were 5mM and 1mM, respectively.

A. The non-ionic detergent Tween 80 at a final concentration of 0.1% (v/v), was added to membrane vesicles after their preparation.

B. Tween 80 at a final concentration of 0.1% (v/v), was included in the lysing buffer during vesicle preparation and in all subsequent buffers.

Rates of respiration are expressed as n mole O_2/minute/mg membrane protein at 30°, after correction for endogenous oxygen uptake.

TABLE 4.1

O. L. VESICLES

	SUCCINATE OXIDATION	NADH OXIDATION
Apparent Km	0.05mM	0.005mM
Maximum Rate	43.0	434.0
A. Tween 80	63% Inhibition	68% Inhibition
B. Tween 80	86% Inhibition	99.3% Inhibition

F. P. VESICLES

Maximum Rate	11.2	14.0
A. 0.1% (v/v) Tween 80	51% Inhibition	80% Inhibition

affect of the non-ionic detergent Tween 80 on succinate and NADH oxidation by membrane vesicles was undertaken. The addition of 0.1% (v/v) Tween 80 to O.L. vesicles resulted in a 63% and 68% inhibition in the rates of succinate and NADH oxidation respectively (Table 4.1A). However when vesicles were prepared in buffer containing the same concentration of Tween 80 (B), and maintained in the presence of the detergent, succinate and NADH oxidation was inhibited by 86% and 99.3% respectively. In this latter instance the detergent would presumably be available to both sides of the vesicle membrane, whilst in (A) the detergent may only be available to the outside of the membrane. Respiration rates could not be restored to any great extent after repeated washes of the membrane vesicles prepared in the presence of the detergent. Tween 80 added to F.P. vesicles also resulted in an inhibition of the rates of respiration, greatest inhibition being observed for NADH oxidation. Morphological examination of O.L. and F.P. vesicles after treatment with 0.1% (v/v) Tween 80 revealed no obvious morphological changes to either type of vesicle and both still appeared vesicular and intact.

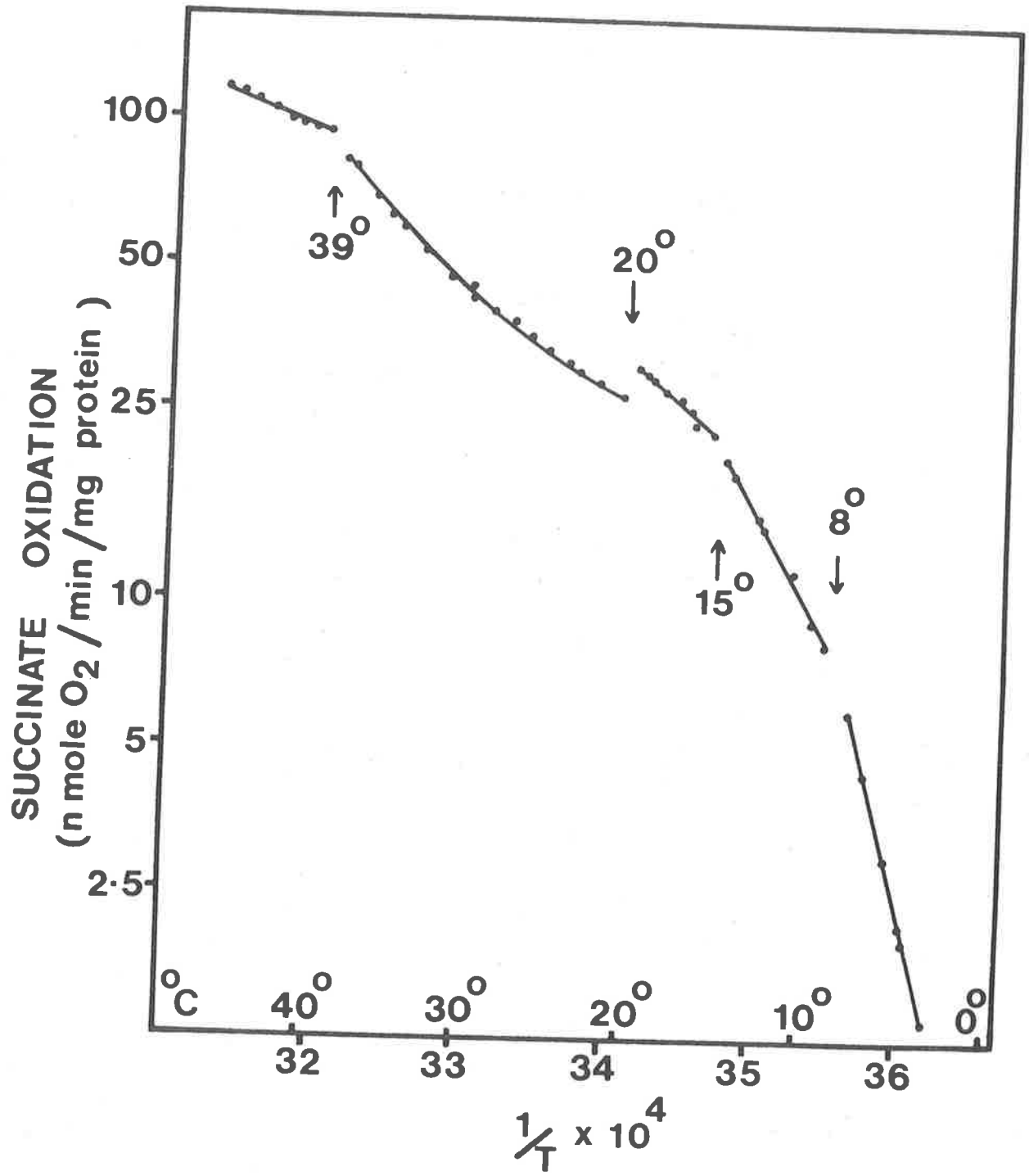
Numerous studies have indicated that changes in the molecular ordering of the membrane lipid may affect the functioning of membrane-associated enzymes and lead to changes in their Arrhenius activation energy at corresponding temperatures (Linden et al., 1973a,b; Raison, 1973a,b).

An Arrhenius plot of the rates of succinate oxidation of O.L. vesicles is shown in Figure 4.6. Discontinuities (changes in activation energy), are evident at temperatures of about 39^{o}, 20^{o}, 15^{o} and 8^{o}. With the exception of the region between 39^{o} and 20^{o}, which displays a concave-upwards curve non-intersecting with the remainder of the graph at 20^{o}, all other temperature regions are linear and exhibit increasing

FIGURE 4.6. ARRHENIUS PLOT OF SUCCINATE OXIDATION BY OSMOTICALLY-PREPARED MEMBRANE VESICLES OF B. AMYLOLIQUEFACIENS.

Rates of succinate oxidation by O.L. vesicles were determined polarographically as described in Chapter 2, with a constant amount of membrane protein being used for all assays. The temperatures indicated by arrows are the average (to the nearest degree) of those on each side of the discontinuities.

Temperature range	Activation energy (kcal/mole)
$> 39^{o}$	5.7
20^{o} to 15^{o}	11.7
15^{o} to 8^{o}	24.3
$< 8^{o}$	50.5


SUCCINATE OXIDATION
(n mole O2 /min /mg protein)
100
50
25
10
5
2·5
39°
20°
15°
8°
°C
40°
30°
20°
10°
0°
32
33
34
35
36
1/T x 10^4

activation energy with decreasing temperature. The Arrhenius plot of NADH oxidation (Figure 4.7) also reveals the presence of four discontinuities at temperatures of about 40°, 22°, 17° and 8° although these are not as pronounced as for succinate oxidation. The characteristic temperatures for both succinate and NADH oxidation correspond with those obtained by spin-labelling techniques (Figures 4.4 and 4.5). For neither respiratory substrate were the discontinuities at about 40° due to irreversible enzyme denaturation since assays on samples used above 40° did not exhibit decreased rates of respiration when re-measured at lower temperatures. The occurrence of non-intersecting discontinuities such as for succinate oxidation at 20° (Figure 4.6) were uncommon and in the majority, the discontinuities were intersecting.

As previously stated low concentrations of non-ionic detergents are believed to abolish lipid phase transitions in membranes by disordering the membrane lipids and thereby decreasing the lipid co-operativity required for the transition (Raison et al., 1971b). Arrhenius plots of membrane-associated enzymes under these conditions do not exhibit discontinuities but rather display a constant activation energy over the assayed temperature range (Raison et al., 1971b). The addition of 0.1% (v/v) Tween 80 results in the inhibition of both succinate and NADH oxidation in B. amyloliquefaciens O.L. and F.P. vesicles (Table 4.1), however it does not appear to alter the morphology of these vesicles, and both appear intact after Tween 80 treatment.

Arrhenius plots for O.L. vesicles assayed in the presence of Tween 80 (but not prepared in its presence), exhibit two discontinuities for succinate (Figure 4.8) and NADH (Figure 4.9) oxidation. The characteristic temperatures, (36° and 15° for succinate; 38° and 15° for NADH), approximate to two of the

FIGURE 4.7. ARRHENIUS PLOT OF NADH OXIDATION BY OSMOTICALLY-PREPARED MEMBRANE VESICLES OF B. AMYLOLIQUEFACIENS.

Membrane vesicles were prepared by the osmotic lysis of protoplasts and rates of NADH oxidation determined as for Figure 4.6.

Temperature range	Activation energy (kcal/mole)
$>40^{o}$	2.8
22^{o} to 17^{o}	20.8
17^{o} to 8^{o}	27.6
$<8^{o}$	37.8

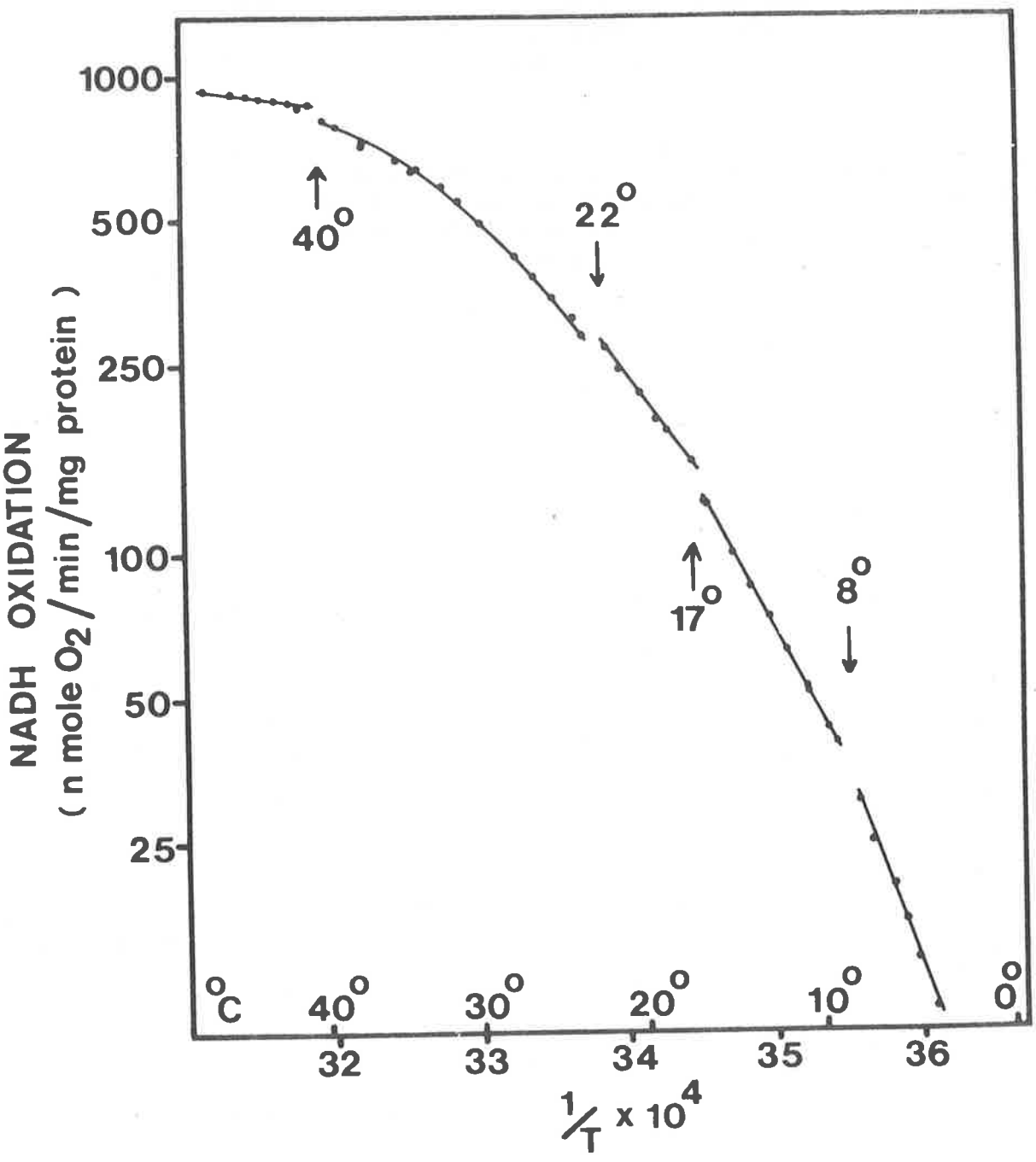

NADH OXIDATION
(n mole O_2 / min / mg protein)
1000
500
250
100
50
25
40°
22°
17°
8°
°C
40°
30°
20°
10°
0°
32
33
34
35
36
$\frac{1}{T} \times 10^4$

FIGURE 4.8. ARRHENIUS PLOT OF SUCCINATE OXIDATION BY OSMOTICALLY-PREPARED MEMBRANE VESICLES OF B. AMYLOLIQUEFACIENS ASSAYED IN THE PRESENCE OF TWEEN 80.

Membrane vesicles were prepared by the osmotic lysis of protoplasts and were assayed as described in Chapter 2, in the presence of 0.1%(v/v) Tween 80.

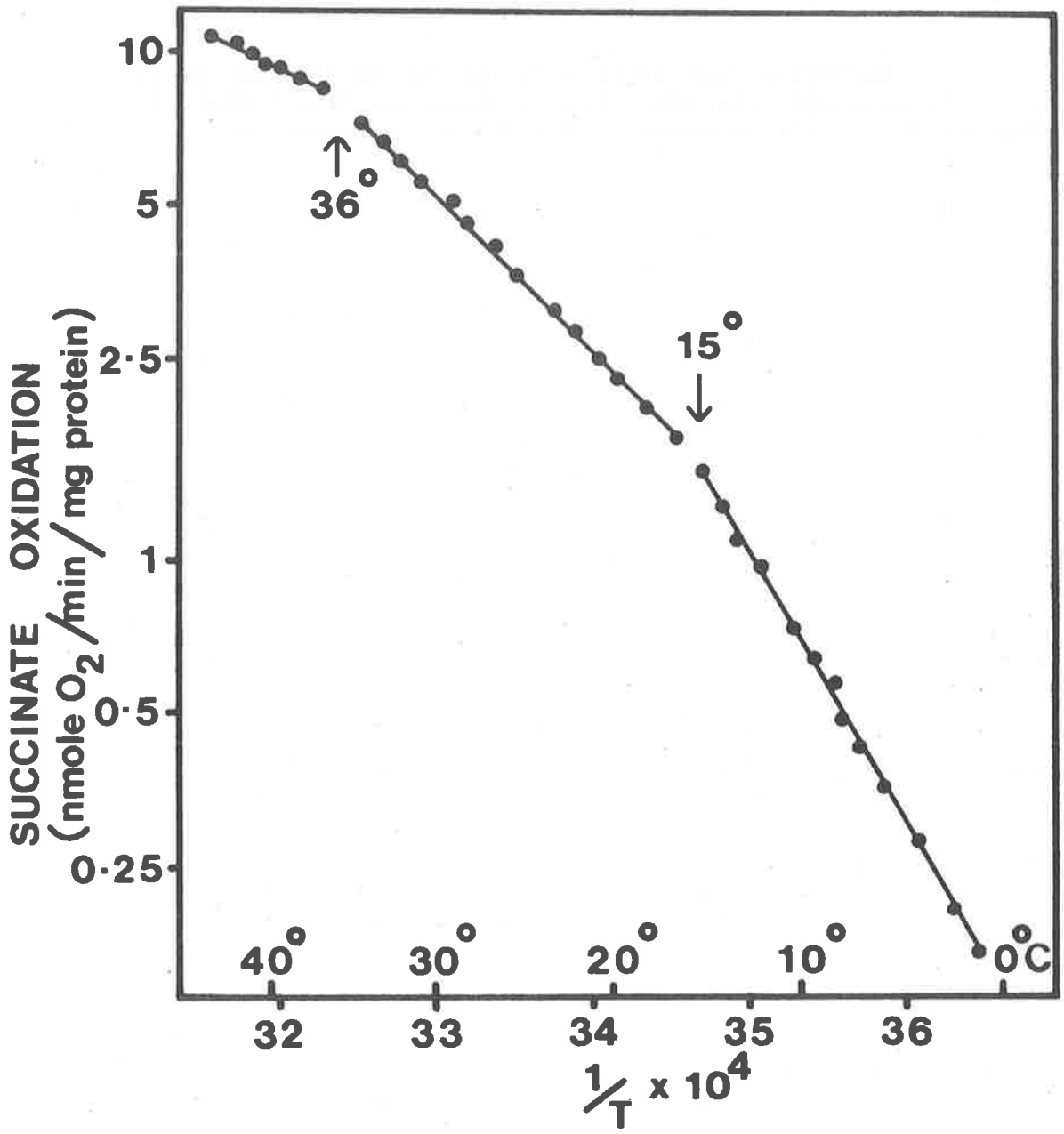
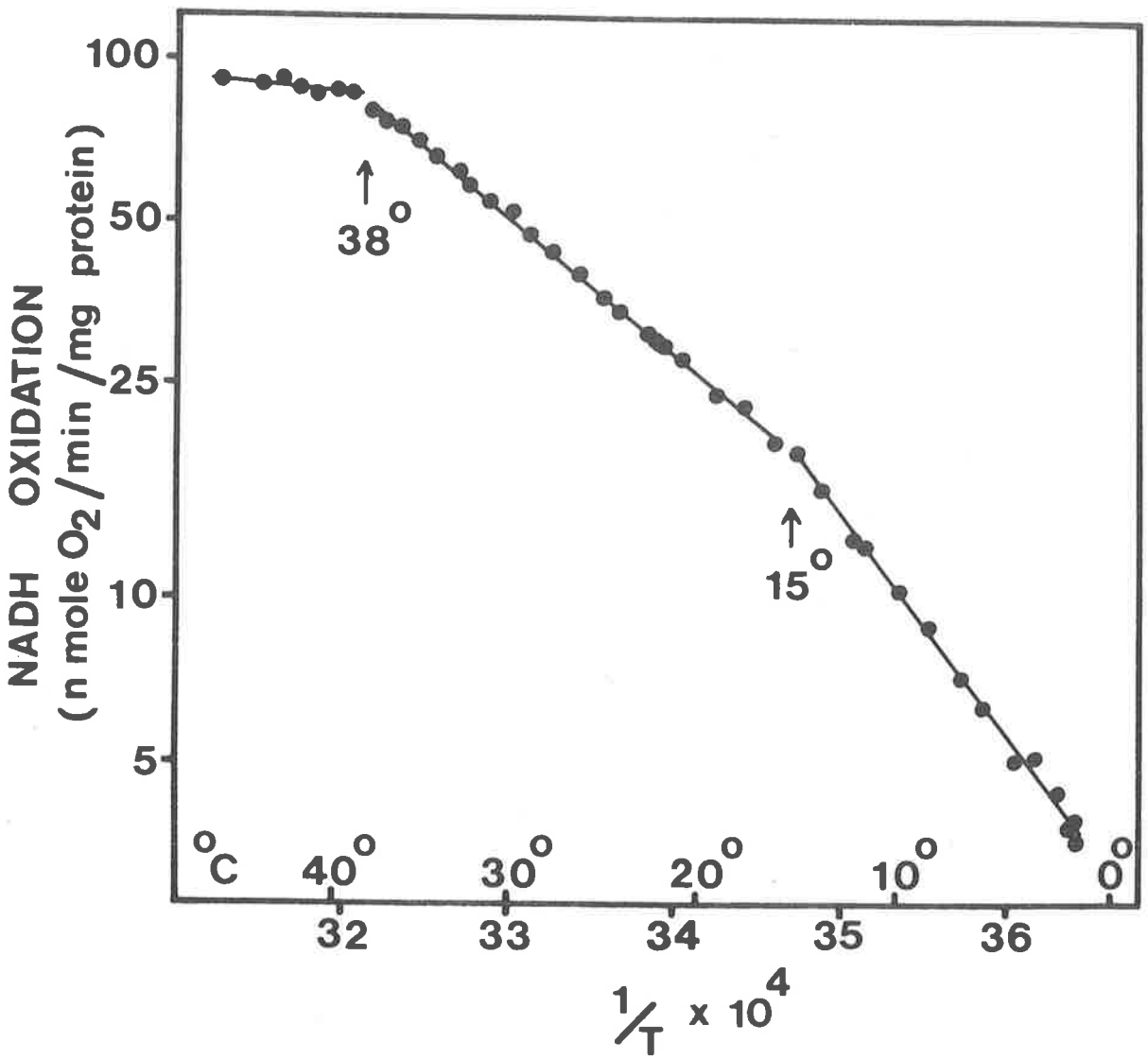

SUCCINATE OXIDATION
(nmole O_2/min/mg protein)
10
5
2·5
1
0·5
0·25
36°
15°
40°
30°
20°
10°
0°C
32
33
34
35
36
$\frac{1}{T} \times 10^4$

FIGURE 4.9. ARRHENIUS PLOT OF NADH OXIDATION BY OSMOTICALLY-PREPARED MEMBRANE VESICLES OF B. AMYLOLIQUEFACIENS ASSAYED IN THE PRESENCE OF TWEEN 80.

Membrane vesicles were prepared by the osmotic lysis of protoplasts and were assayed as described in Chapter 2, in the presence of 0.1%(v/v) Tween 80.


NADH OXIDATION
(n mole O2/min/mg protein)
100
50
25
10
5
38°
15°
°C
40°
30°
20°
10°
0°
32
33
34
35
36
1/T x 10^4

four characteristic temperatures observed by enzyme kinetics (in the absence of Tween 80) and spin-labelling studies. Due to the vesicular nature of the membrane and in the apparent absence of detergent-induced lysis, it is unlikely that Tween 80 would cross the membrane due to its polar sorbitan group. An attempt was therefore made to present the detergent to both sides of the vesicle membrane by lysing protoplasts in buffer containing Tween 80. In these vesicles, comparatively greater inhibition was observed for both succinate and NADH oxidation than if the same concentration of detergent was added to membrane vesicles prepared in the absence of Tween 80 (Table 4.1). In addition, the vesicles still appeared morphologically intact after such treatment. The Arrhenius plot for succinate oxidation in O.L. vesicles prepared in the presence of Tween 80 and assayed in the presence of Tween 80 (Figure 4.10), exhibits no discontinuities of the type seen in the previous figures. Rather a smooth, very shallow concave-downwards curve is observed which displays a slight but constant increase in activation energy with decreasing temperature.

The Arrhenius plot of succinate oxidation by F.P. vesicles (Figure 4.11) reveals four discontinuities at characteristic temperatures of about 39^{o}, 21^{o}, 17^{o} and 6^{o}, whilst four discontinuities are also evident for NADH oxidation at about 35^{o}, 21^{o}, 17^{o} and 7^{o} (Figure 4.12). These characteristic temperatures approximate those for O.L. vesicles obtained by physical and enzymic methods. However, as seen in Figure 4.13, Arrhenius plots of succinate and NADH oxidation in such vesicles, assayed in the presence of 0.1% (v/v) Tween 80, display only two discontinuities. These occur at characteristic temperatures of 19^{o} to 20^{o} and 5^{o} to 6^{o} and approximate to two of the four characteristic temperatures observed in either type of membrane vesicle without Tween 80, but do not approximate to the two

FIGURE 4.10. ARRHENIUS PLOT OF SUCCINATE OXIDATION BY OSMOTICALLY-PREPARED MEMBRANE VESICLES OF B. AMYLOLIQUEFACIENS PREPARED IN THE PRESENCE OF TWEEN 80.

Protoplasts were lysed in buffer containing 0.1%(v/v) Tween 80. Rates of succinate oxidation at different temperatures were then determined in buffer containing the same concentration of Tween 80 and graphed as an Arrhenius plot.

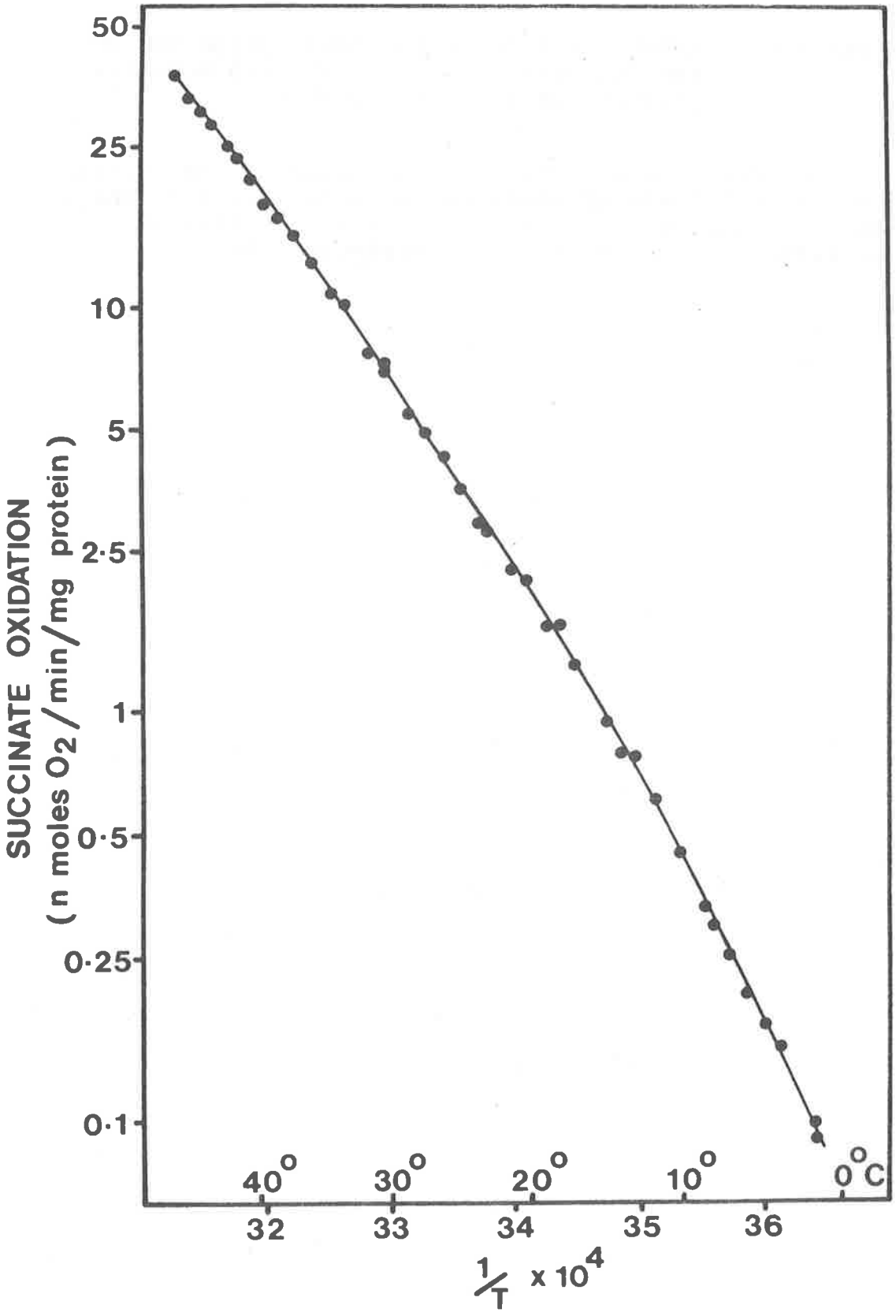

SUCCINATE OXIDATION
(n moles O_2 / min / mg protein)
50
25
10
5
2·5
1
0·5
0·25
0·1
40°
30°
20°
10°
0°C
32
33
34
35
36
$\frac{1}{T} \times 10^4$

FIGURE 4.11. ARRHENIUS PLOT OF SUCCINATE OXIDATION BY FRENCH PRESSURE CELL - PREPARED MEMBRANE VESICLES OF B. AMYLOLIQUEFACIENS.

Membrane vesicles were prepared by the French pressure cell lysis of whole cells as described in Chapter 2 and the rates of succinate oxidation at different temperatures were graphed as an Arrhenius plot.

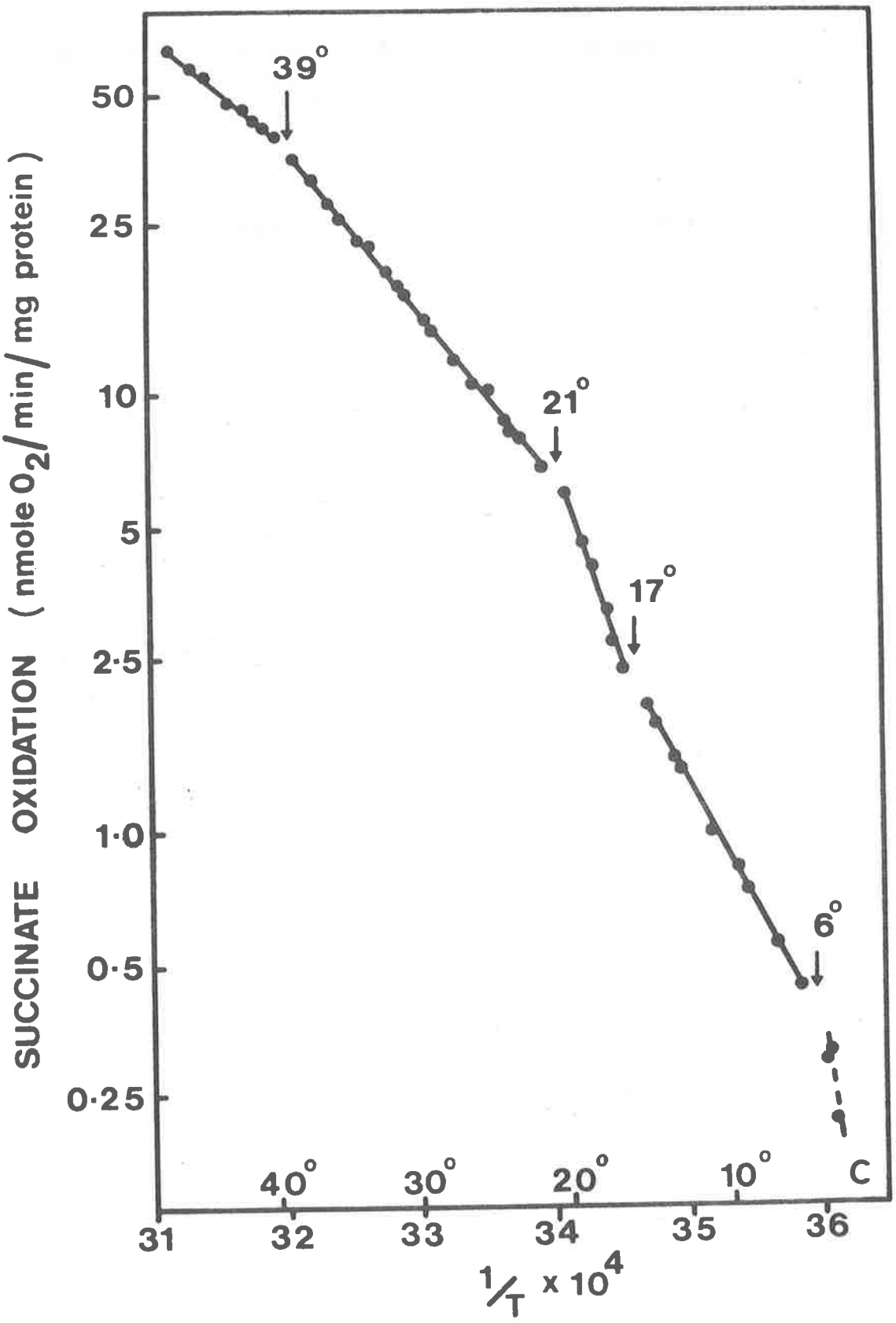

SUCCINATE OXIDATION (nmole O_2/min/ mg protein)
50
25
10
5
2·5
1·0
0·5
0·25
39°
21°
17°
6°
40°
30°
20°
10°
C
31
32
33
34
35
36
1/T x 10^4

FIGURE 4.12. ARRHENIUS PLOT OF NADH OXIDATION BY FRENCH PRESSURE CELL-PREPARED MEMBRANE VESICLES OF B. AMYLOLIQUEFACIENS.

Membrane vesicles were prepared by the French pressure cell lysis of whole cells as described in Chapter 2 and the rates of NADH oxidation at different temperatures were graphed as an Arrhenius plot.

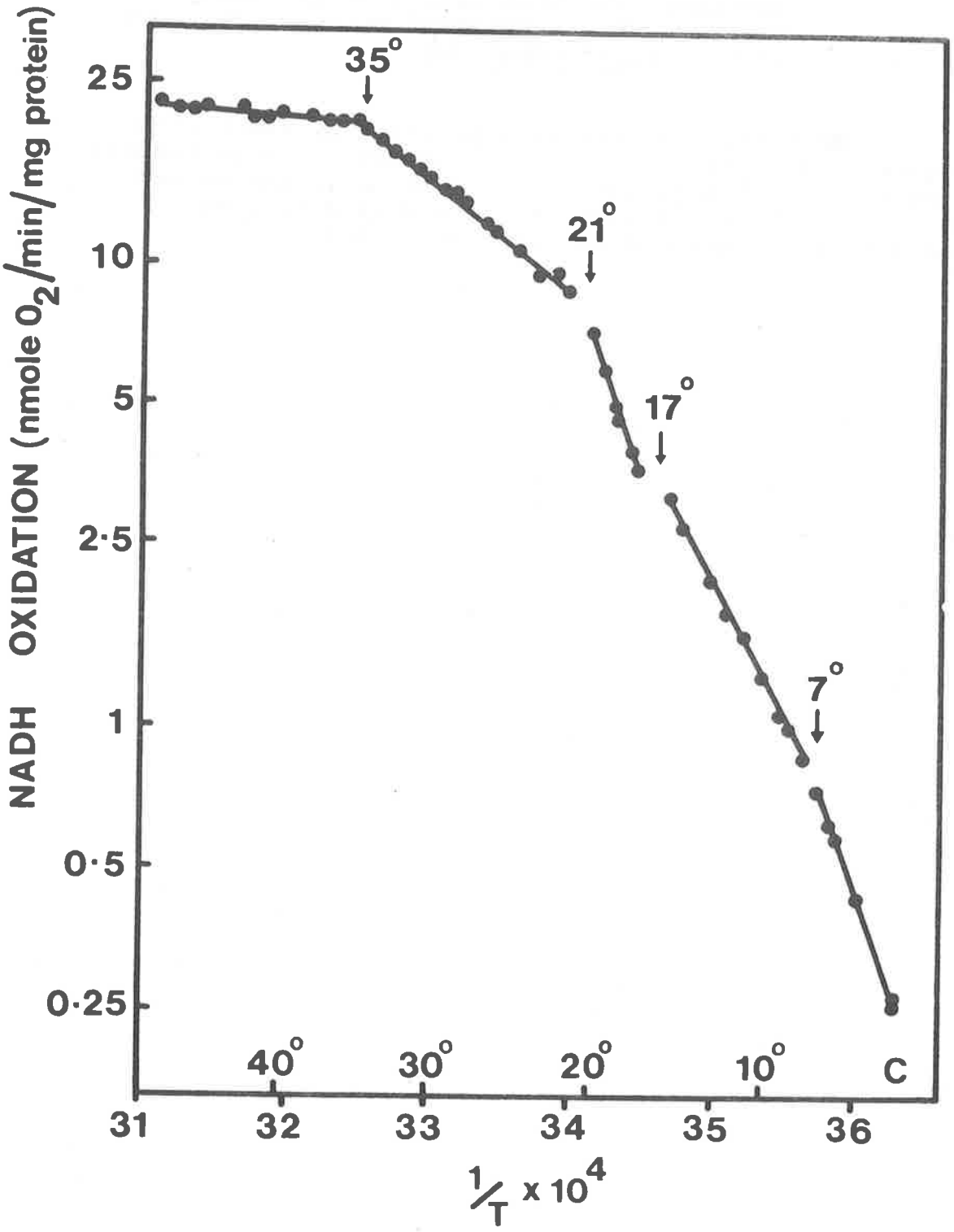
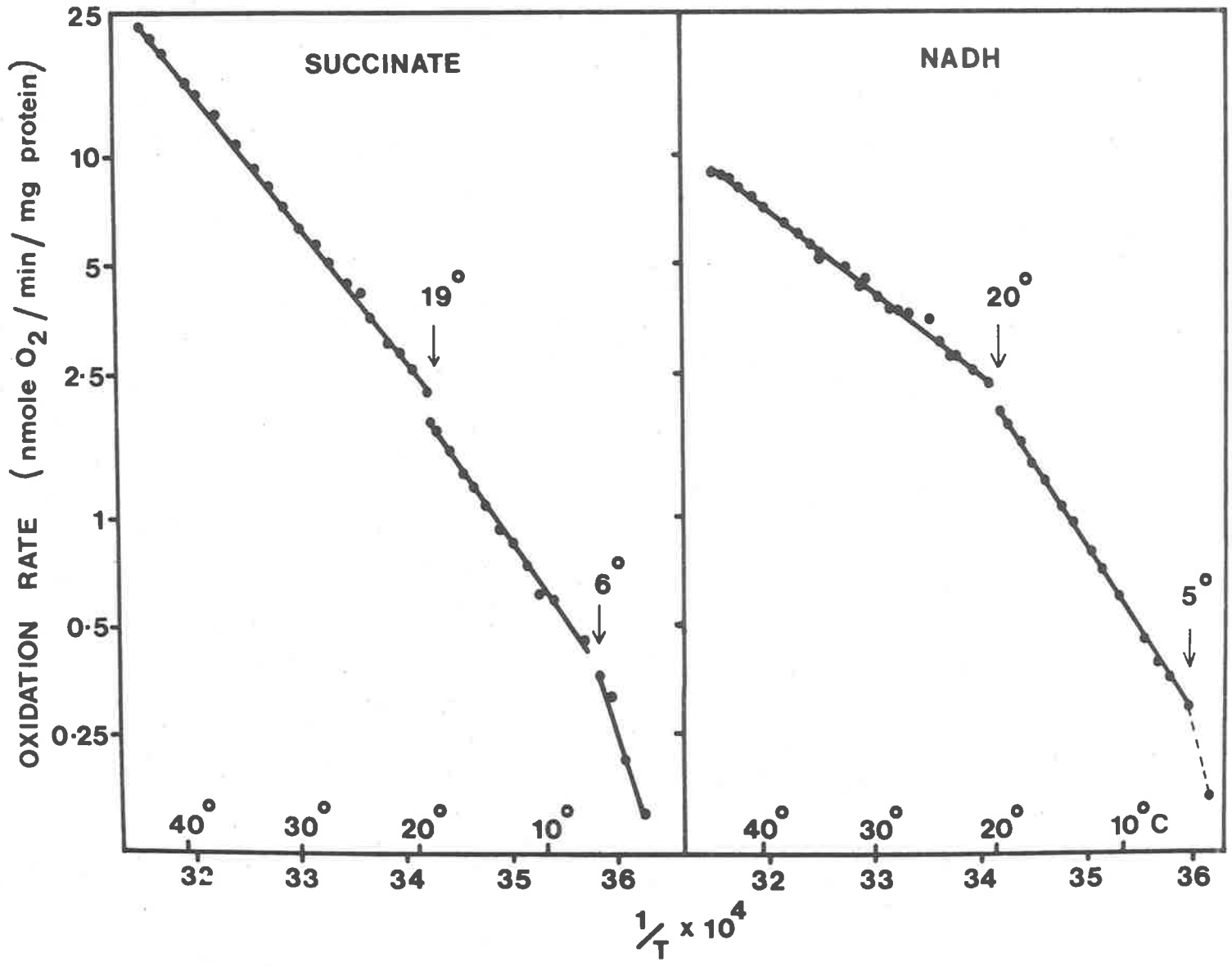

NADH OXIDATION (nmole O_2/min/mg protein)
25
10
5
2·5
1
0·5
0·25
35°
21°
17°
7°
40°
30°
20°
10°
C
31
32
33
34
35
36
$\frac{1}{T} \times 10^4$

FIGURE 4.13. EFFECT OF TWEEN 80 ON ARRHENIUS PLOTS OF SUCCINATE AND NADH OXIDATION BY FRENCH PRESSURE CELL - PREPARED MEMBRANE VESICLES OF B. AMYLOLIQUEFACIENS.

Membrane vesicles were prepared by the French pressure cell lysis of whole cells as described in Chapter 2. Rates of succinate and NADH oxidation in the presence of 0.1% (v/v) Tween 80 were determined at different temperatures and graphed as Arrhenius plots.


SUCCINATE
NADH
OXIDATION RATE (nmole O_2 / min / mg protein)
25
10
5
2·5
1
0·5
0·25
19°
6°
20°
5°
40°
30°
20°
10°
10°C
32
33
34
35
36
$\frac{1}{T} \times 10^4$

characteristic temperatures for O.L. vesicles assayed in the presence of Tween 80. It can again be argued that the detergent, added after vesicle formation, would be unlikely to be present within the vesicle. Thus the characteristic temperatures for succinate and NADH oxidation, assayed in the presence of Tween 80, are dependent on the type of membrane vesicle and indicate a difference between these two types of membrane vesicle in regard to their behaviour in the presence of Tween 80.

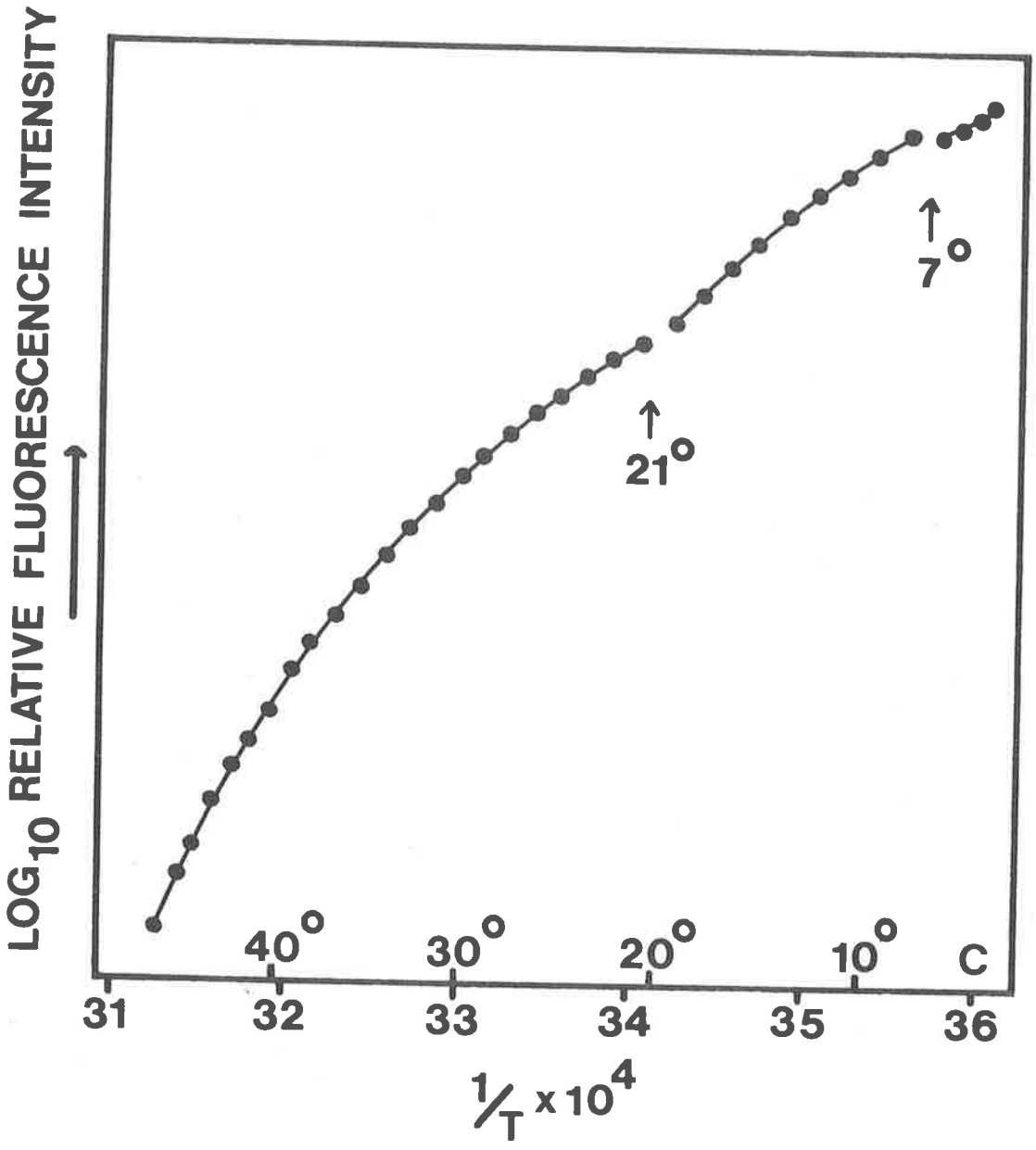
3. Fluorescent probe studies on osmotically-prepared membrane vesicles

The fluorescent probe ANS has been used to monitor phase transitions in membranes and isolated phospholipids, (see review by Azzi, 1975). ANS fluoresces only when portion of the molecule is exposed to a hydrophobic environment such as would be present in the membrane due to the lipid components, and it has been shown to be localized at the polar/apolar interface of the membrane (Lesslauer et al., 1972), with its charged sulphonate group possibly preventing complete penetration of the molecule across the membrane.

An Arrhenius plot of the relative fluorescence intensity of ANS added to O.L. vesicles is shown in Figure 4.14. A series of curves discontinuous at 21^{o} and 7^{o} are evident. These temperatures are coincident with two of the four characteristic temperatures determined by spin-labelling and enzymic methods and approximate those obtained for succinate and NADH oxidation in F.P. vesicles, assayed in the presence of Tween 80. From comparison of the temperature dependence of ANS fluorescence in soybean lecithin in which a lipid phase transition was detected by calorimetric means below 0^{o} (results not shown), ANS fluorescence exhibits a smooth concave-downwards curve when graphed as an Arrhenius plot. As was mentioned in Chapter 2,

FIGURE 4.14. ARRHENIUS PLOT OF THE FLUORESCENCE OF ANS IN OSMOTICALLY-PREPARED MEMBRANE VESICLES OF B. AMYLOLIQUEFACIENS.

Membrane vesicles were prepared by the osmotic lysis of protoplasts. The fluorescent probe ANS was added to membrane vesicles and the relative fluorescence intensity was measured as a function of temperature as described in Chapter 2.


LOG10 RELATIVE FLUORESCENCE INTENSITY
7°
21°
40°
30°
20°
10°
C
31
32
33
34
35
36
1/T x 10^4

the use of ANS clearly detects the phase transition of the phospholipid, dimyristoyl phosphatidyl choline at 23°, and in this instance two smooth curves which were discontinuous at 23°, were evident.

4. Thermal analysis on osmotically-prepared membrane vesicles

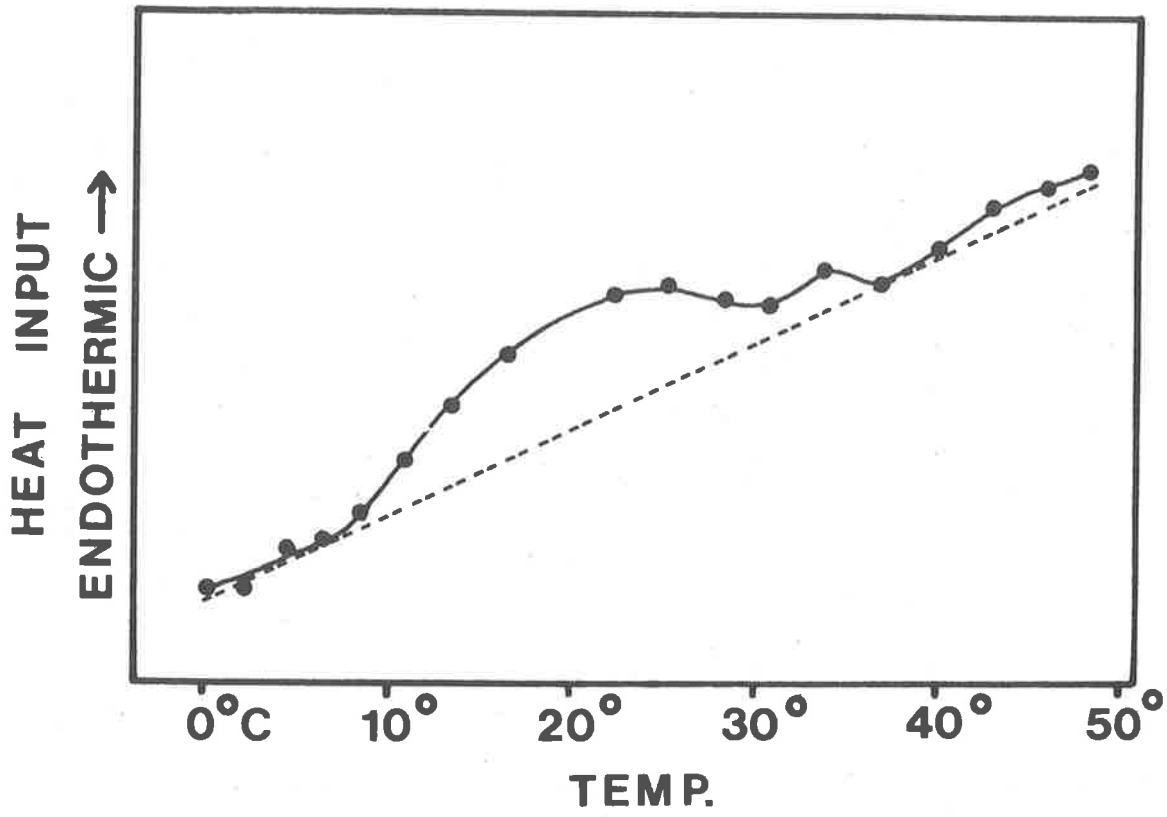
The unique property of phospholipid bilayer structures to undergo a reversible, thermotropic gel ⟶ liquid-crystalline phase transition arising from the co-operative melting of the hydrocarbon chains of the interior of the bilayer, has been investigated by techniques which rely on some form of thermal analysis (Chapman, 1975). In view of the complexity of thermal events evident in the cell membrane of B. amyloliquefaciens by spin-labelling and enzymic studies, it was therefore decided to investigate the behaviour of these membranes by thermal analysis, the aim being to directly observe the lipid phase transition. The techniques of adiabatic calorimetry and differential scanning calorimetry are capable of directly measuring the melting process by recording, as an endothermic peak, the heat input required to bring about a melt.

A. Adiabatic calorimetry.

B. amyloliquefaciens O.L. vesicles were analysed by adiabatic calorimetry as described in Chapter 2, and the result is shown in Figure 4.15. In the heating mode an endothermic peak extending from about 6° to 36°, was clearly evident. This endotherm consisted of a main symmetrical peak extending from 6° to 30°, with a tailing region from 30° to 36°. This endotherm was re-observed if the same membrane sample was cooled and then re-run in the calorimeter. Under the steady-state conditions resulting from this form of calorimetry, it is clear that a phase transition is occurring in B. amyloliquefaciens cell membranes over the temperature range 6° to 36°.

FIGURE 4.15. ADIABATIC CALORIMETRY OF B. AMYLOLIQUEFACIENS MEMBRANE VESICLES.

Membrane vesicles were prepared by the osmotic lysis of protoplasts and adiabatic calorimetry was performed as described in Chapter 2. The endothermic peak was recorded by increasing the temperature of the membrane sample in small steps and measuring the specific heat after allowing the sample to equilibrate at each of the temperatures indicated. The dashed line represents the baseline obtained with aqueous buffer.


HEAT INPUT
ENDOTHERMIC →
0°C
10°
20°
30°
40°
50°
TEMP.

B. Differential scanning calorimetry (D.S.C.).

The results obtained by D.S.C. for O.L. vesicles of B. amyloliquefaciens are shown in Figure 4.16, and they fully support the result obtained by adiabatic calorimetry. Trace A shows the endothermic peak obtained when membranes were heated at a rate of 20° per minute. The peak begins at 9°, rises sharply at 16°, returns to near the baseline at 35° and completely reunites with the baseline at 42°. Due to fast temperature scanning, errors in the actual temperature limits of the endotherm can occur, and this is seen from the fact that the ice ⟶ water transition occurs a few degrees below zero. The exotherm obtained when membranes were cooled from about 45°, is shown in Trace B. (Differences in the magnitude of the peaks obtained in Traces A and B are due to a 2-fold difference in the operating sensitivity of the calorimeter). The exotherm appears similar in shape to the endothermic peak, although the temperature limits, 29° to -1°, are approximately 10 centigrade degrees lower than that obtained when a heating mode was employed. This difference can again be reconciled by the error which is unavoidably introduced by the scanning technique. In actual fact, the temperature limits of the exotherm are virtually identical to those of the endotherm if a correction of plus 10 centigrade degrees is allowed by virtue of the lowered water ⟶ ice transition temperature (-10^0).

5. Spin-labelling studies on isolated phospholipids

Spectra obtained for the spin labels 12NS (m.e.) and 16NS (m.e.) infused into an aqueous sonicated dispersion of B. amyloliquefaciens phospolipids, are shown in Figure 4.17. These spectra are similar to those obtained when spin labelling isolated membranes or heat-treated cells or protoplasts (Figure 4.1 and 4.2). In comparison to the above, A_n values

FIGURE 4.16. DIFFERENTIAL SCANNING CALORIMETRY OF *B. AMYLOLIQUEFACIENS* MEMBRANE VESICLES.

Membranes were prepared by the osmotic lysis of protoplasts as described in Chapter 2. The resulting membrane vesicles suspended in buffer were centrifuged and the membrane pellet loaded into a calorimeter sample pan which was then sealed and loaded with an empty reference pan into a Perkin Elmer (D.S.C.-2) differential scanning calorimeter and calorimetry was performed as described in Chapter 2. Scanning rate was 20^{o} per minute and the dashed lines represent the traces obtained using aqueous buffer alone.

A. Heating mode (endotherm) : Sensitivity 1 mcal/s

B. Cooling mode (exotherm) : Sensitivity 2 mcal/s

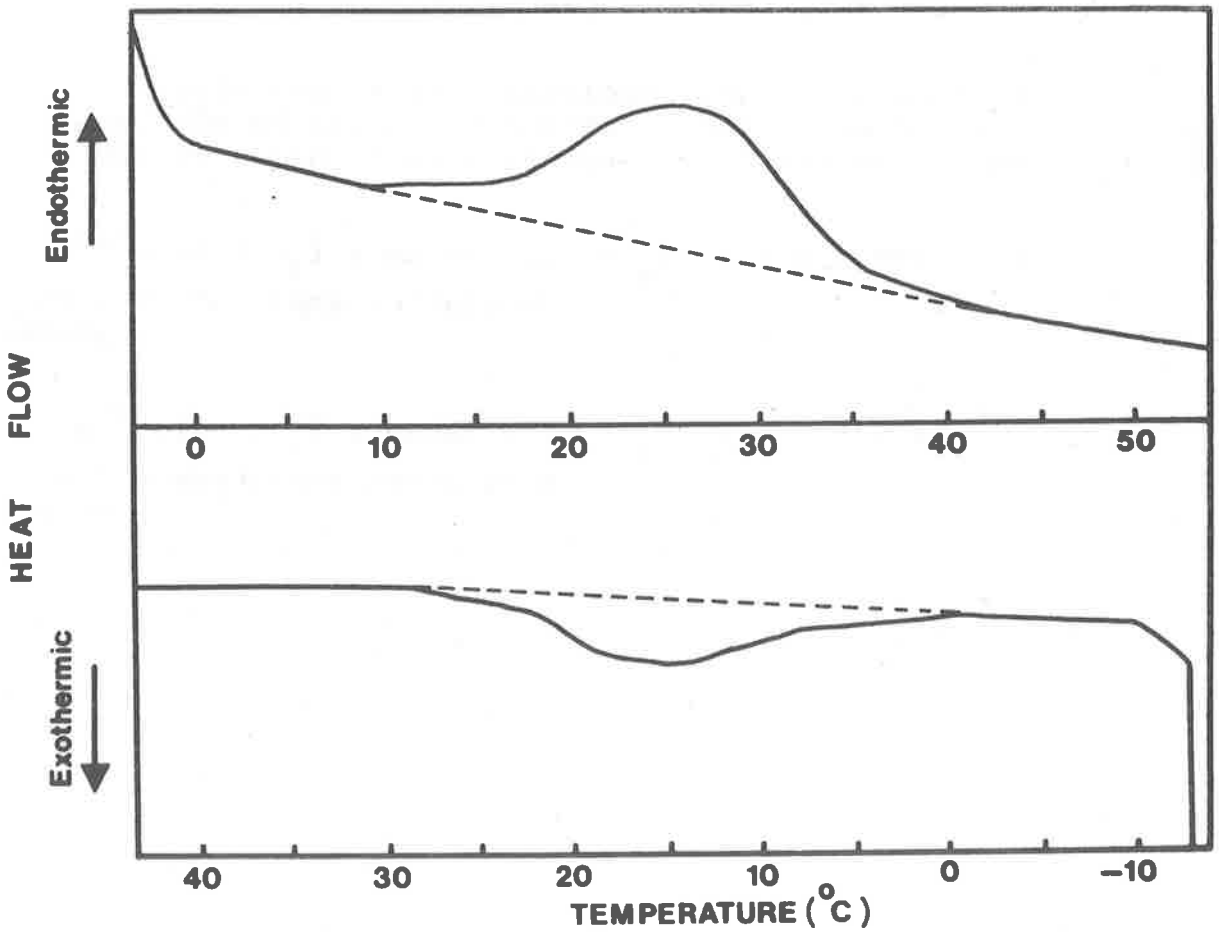
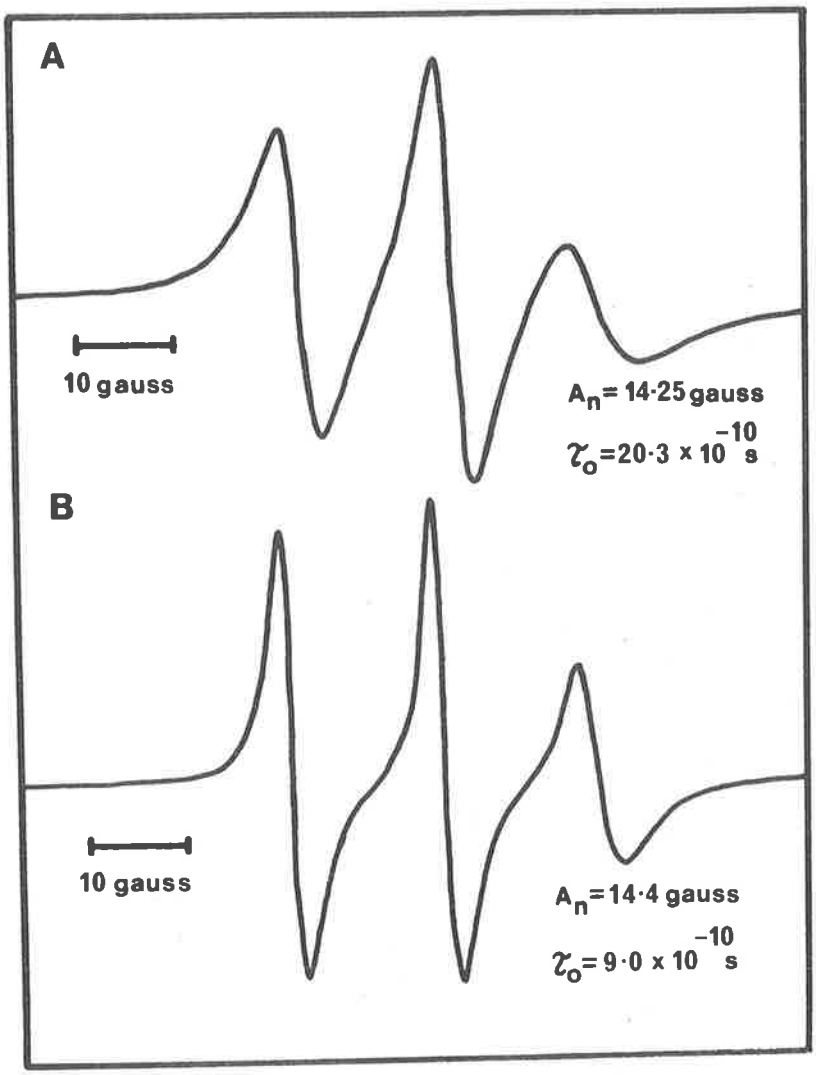

Endothermic
HEAT FLOW
0
10
20
30
40
50
Exothermic
40
30
20
10
0
−10
TEMPERATURE (°C)

FIGURE 4.17. E.S.R. SPECTRA OF SPIN LABELS INFUSED INTO AQUEOUS DISPERSIONS OF PHOSPHOLIPIDS ISOLATED FROM B. AMYLOLIQUEFACIENS.

Phospholipids were isolated from B. amyloliquefaciens cells and suspended in phosphate buffer by sonication as described in Chapter 2 and spectra were recorded at 30°.

A. 12NS(m.e.) : A_n = 14.2 gauss ; $\tau_o = 20.3 \times 10^{-10}$ s
(Modulation amplitude = 1.25 gauss)

B. 16NS(m.e.) : A_n = 14.4 gauss ; $\tau_o = 9.0 \times 10^{-10}$ s
(Modulation amplitude = 1.0 gauss)

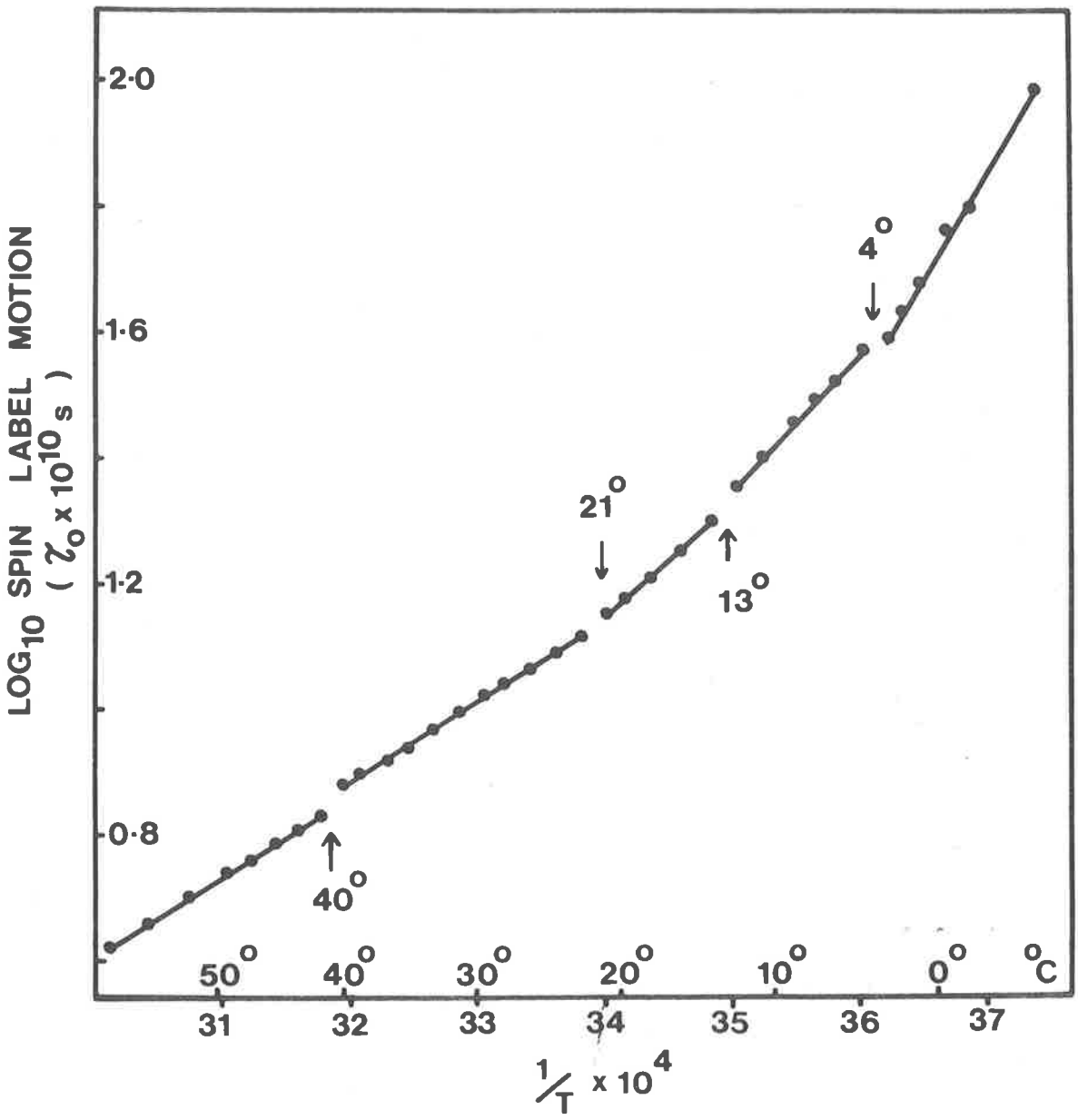

A
10 gauss
A_n = 14·25 gauss
τ_o = 20·3 x 10^{-10} s
B
10 gauss
A_n = 14·4 gauss
τ_o = 9·0 x 10^{-10} s

obtained for the phospholipids indicate a slightly more hydrophobic localization of the spin labels and the relatively faster motion for both spin labels may be due to a greater fluidity in the isolated phospholipids brought about by removal of membrane protein.

An Arrhenius plot of the motion of 16NS (m.e.) in the isolated phospholipids is shown in Figure 4.18. Although the Arrhenius plot is complex, it is possible to visualize four discontinuities at about 40^{o}, 21^{o}, 13^{o} and 4^{o}. Particular difficulty is encountered in ascertaining the actual temperature of the discontinuity at about 4^{o}, although at some temperature within this region, there is a change in slope. The discontinuity at 40^{o} is marked by a non-intersect rather than by a significant change in slope.

FIGURE 4.18. ARRHENIUS PLOT OF SPIN LABEL MOTION IN ISOLATED PHOSPHOLIPIDS OF B. AMYLOLIQUEFACIENS.

Phospholipids were extracted from B. amyloliquefaciens cells and the spin label 16NS(m.e.) was added to an aqueous phospholipid dispersion prepared by sonication. The motion of the spin label (τ_o) was determined at the temperatures indicated and plotted as an Arrhenius plot.


LOG10 SPIN LABEL MOTION (τo x 10^10 s)
2·0
1·6
1·2
0·8
4°
21°
13°
40°
50°
40°
30°
20°
10°
0°
°C
31
32
33
34
35
36
37
1/T x 10^4

C. DISCUSSION

Thermotropic phase transitions in the lipid component of biological membranes have been demonstrated by a variety of physical techniques such as spin labelling (Sackmann et al., 1973; Linden et al., 1973a,b), X-ray diffraction (Engelman, 1970; Esfahani et al., 1971; Shechter et al., 1974), calorimetry (Steim et al., 1969; Ashe and Steim, 1971; Blazyk and Steim, 1972; Haest et al., 1974; Martonosi, 1974) and fluorescent probes (Overath and Träuble 1973; Träuble and Overath, 1973), as well as by studies on membrane-associated enzyme systems such as transport (Linden et al., 1973a) and respiration (Raison, 1973a,b; Watson et al., 1975a,b). In model systems comprising a binary mixture of two phospholipids of differing fatty acyl composition, the order to disorder transition which occurs on raising the temperature has been considered in terms of a lateral phase separation (Shimshick and McConnell, 1973; Grant et al., 1974; Wu and McConnell, 1975). Ideally the lipid is considered to exist in the form of a crystalline or solidus phase below a characteristic temperature, T_s. At T_s, a change in molecular ordering (phase change) is envisaged resulting in the formation of lipid in the liquid-crystalline or fluidus phase which coexists with the solidus phase due to the lateral separation of both phases. On increasing the temperature, the proportion of fluidus phase lipid increases, until a fully "fluid" phase is reached at a second characteristic temperature, T_f.

The temperatures T_s and T_f are determined primarily by the physical characteristics of the acyl chains. Spin-labelled fatty acids of the type used in this study have been employed to probe the physical state of the hydrocarbon regions within biological membranes, particularly in regard to the temperature-dependent fluidity changes associated with the phase

transition (Raison et al., 1971a; Mehlhorn and Keith, 1972; Eletr et al., 1974; Raison and McMurchie, 1974).

The actual molecular events associated with phase transitions in biological membranes are not clearly understood particularly in regard to the following: 1. Whether the "melting" process of the membrane lipids represents a phase transition and/or a lateral phase separation. 2. Whether all or only some of the lipids are involved in the "melt", there being the possibility that specific lipid domains, such as the boundary lipid layer surrounding some membrane proteins (Dehlinger et al., 1974), may behave independently of the bulk lipid. 3. How changes in the membrane lipid influence physiological activities associated with the membrane, i.e. are changes in the lateral compressibility of lipids (as suggested by Linden et al., 1973a), solely responsible for the observed changes in physiological activity. 4. Whether "breaks" in Arrhenius plots for membrane-associated activities correspond to liquid-crystalline to crystalline phase transitions or rather to the formation of lipid "clusters", as suggested by Lee et al., (1974).

At the time the work described in this chapter was commenced, all studies on phase transitions in biological membranes had defined only two critical temperatures which were interpreted in terms of the events occurring in artificial lipid systems, as discussed at the start of this section. The observation of four critical temperatures for B. amyloliquefaciens cell membranes was unprecedented and it was therefore necessary to propose a working hypothesis to account for these unexpected results.

The reality of these four critical temperatures is the first question to be considered. These critical temperatures

are based on the discontinuities in the Arrhenius plots and due to the intersecting nature of these discontinuities, there is difficulty in assessing whether such discontinuities are real, and if so, at what temperature they occur. In most of the Arrhenius plots presented a change in slope was evident at the characteristic temperature, although in some instances a non-intersecting discontinuity was apparent. Although it was not always possible to clearly observe all discontinuities in any one Arrhenius plot, it was possible to clearly detect any one of the discontinuities when repeating the experiments. The fact that essentially the same four critical temperatures (or set of two critical temperatures, depending on the experiment) were found by spin-labelling studies, by enzyme activity studies, by fluorescent probe studies and their existence in agreement with the calorimetry data, all strongly support the reality of the four critical temperatures, although the changes which occur in the membrane lipids at these temperatures remain undefined in molecular terms. The enzyme studies could per se be given alternative explanations such as changes in the physical state of specific enzyme-associated lipid, but it would be difficult to reconcile this with the physical data. Similarly, direct effects of temperature on the enzyme protein would not account for the physical data (unless the assumption was made that the correspondence of critical temperatures determined by the different methods was purely fortuitous).

It is not possible to offer a conclusive interpretation to account for these four critical temperatures at the present time. However, a working hypothesis can be advanced if it is assumed that the critical temperatures represent changes in the physical state of the bulk membrane lipids. By analogy with the

results obtained by other workers for artificial phospholipids systems (as previously discussed), the existence of four distinct temperature-induced changes in the membrane lipid is consistent with the occurrence of two lipid phase transitions which differ in their characteristic temperatures for initiation (T_s) and termination (T_f). This may occur if two different lipid compartments are physically segregated within the membrane such that each is capable of independently undergoing a co-operative phase transition. The presence of two compartments could account for three or four of the characteristic temperatures depending on whether the two phase transitions had one or no phase boundary temperature in common. To achieve this, two obvious possibilities exist. One possibility is that these lipid compartments represent the halves of the membrane in which the lipids are asymmetrically distributed across the bilayer (vertical asymmetry), and the other possibility is that two distinct compartments arise from patching within the lateral plane of the membrane (horizontal asymmetry).

Although as will be discussed, a precedent does exist for vertical phospholipid headgroup asymmetry in some other membranes, no such precedent presently exists for B. amyloliquefaciens. Nevertheless, the evidence is most easily reconciled by the vertical lipid asymmetry hypothesis. The arguments to support this are as follows:

1. Spectral parameters indicate that the spin label is uniquely probing the hydrocarbon region of the membrane and in the absence of any polar group, the spin label probably monitors both the inner and outer lipid monolayers of the membrane.

2. The electron transport systems as measured by succinate and NADH oxidation are believed to span the membrane bilayer (for review see Harold, 1972), and therefore the

possibility exists that they may be influenced by events occurring in both halves of the membrane.

3. The results obtained using the non-ionic detergent Tween 80 are compatible with the vertical lipid asymmetry hypothesis for the following reason. It is anticipated that Tween 80 when added to intact membrane vesicles would position itself with its lipophilic moiety (oleic esters) in the outer half of the membrane, the polar sorbitan moiety possibly preventing diffusion of the molecule to the inner half. If in this position the detergent destroys the co-operativity required for the phase transition, it may be inferred that the characteristic temperatures of 38^{o} and 15^{o} for O.L. vesicles define the boundaries of one of the two phase transitions and this transition is localized in the lipid compartment not influenced by the detergent. Were horizontal asymmetry to exist, it could be argued that both compartments would be exposed to the detergent and thus all discontinuities would be abolished. This is clearly not the case. That Tween 80 does not cross the membrane when added to only the outside of O.L. vesicles is supported by the fact that Tween 80 introduced to both halves of the membrane, by its presence during protoplast lysis, abolishes all discontinuities in the Arrhenius plot of succinate oxidation. (Complementary studies using a spin-labelling approach in combination with the detergent were not performed, as the E.S.R. spectra for membranes in the presence of detergent, were the composite of spin label located both in the membrane and most likely in detergent micelles existing free in the aqueous phase).

4. Results obtained using the fluorescent probe ANS are also compatible with the hypothesis of vertical lipid asymmetry for the following reason. If as suggested above, the characteristic temperatures of 38^{o} and 15^{o} define the boundaries

of the phase transition in the inner half of the membrane of O.L. vesicles, the other two characteristic temperatures of 21° and 7°, may well define the boundaries of the second transition occurring in the outer half of the membrane, if a condition of vertical lipid asymmetry exists. If ANS is unable to completely penetrate O.L. vesicles due to its charged sulphonate group, the characteristic temperatures of 21° and 7° detected using ANS, may be attributed to the phase transition in the outer half of the membrane. If a condition of horizontal lipid asymmetry were to exist, or was ANS to be translocated across the membrane and be present on both sides, four temperature-dependent changes may have been evident.

5. Further evidence supporting the hypothesis of vertical lipid asymmetry comes from the differing effects of Tween 80 using the two types of membrane vesicles. When using F.P. vesicles, Tween 80 abolishes those characteristic temperatures left intact in O.L. vesicles (i.e. 38° and 15°), and the characteristic temperatures of 20° and 6° are similar to those obtained using the fluorescent probe in O.L. vesicles. This difference can be reconciled if it were assumed that the orientation of the two types of vesicle were opposite; (respective orientations of the two types of vesicle will be discussed later in this section). Thus the lipid compartment represented by the inner monolayer of the membrane of F.P. vesicles (i.e. that defined by the characteristic temperatures 22° and 6°) corresponds to the outer monolayer of O.L. vesicles.

6. Adiabatic and differential scanning calorimetry confirm the presence of a lipid phase transition in the membrane, however the boundaries of the two postulated transitions cannot be defined by these techniques. If as suggested, the boundaries of the individual phase transitions are defined by the set of

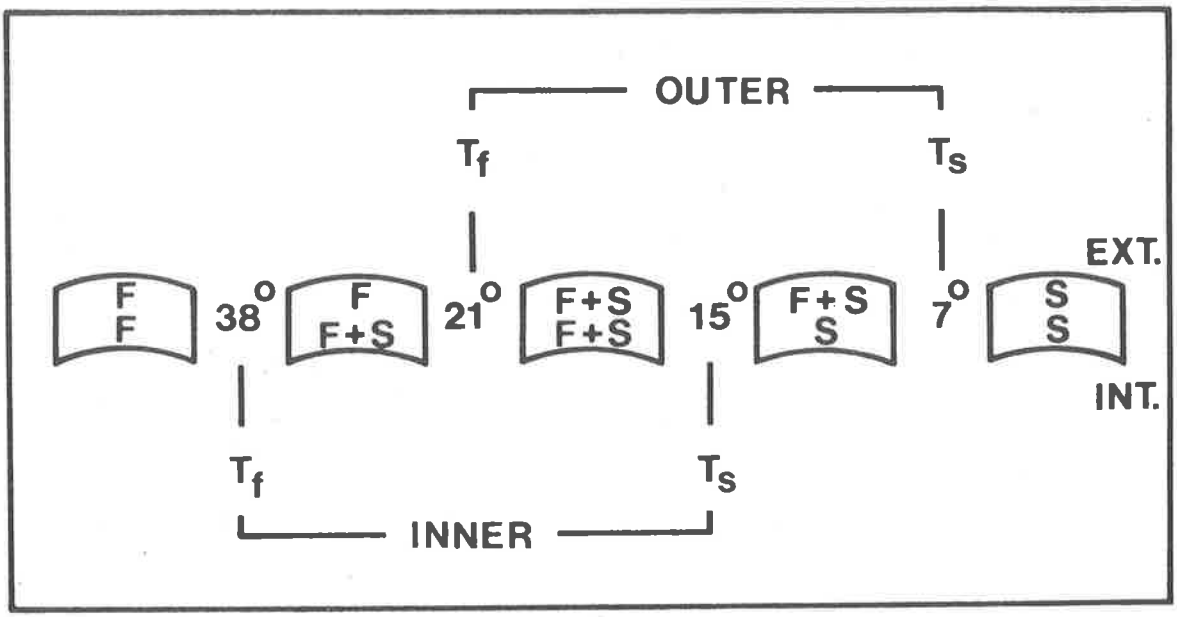
temperatures, 38° and 15°, and 21° and 7°, respectively, the resulting endotherm/exotherm should completely span the temperature range 7° to 38°, as was the case.

The hypothesis formulated from these studies is that the cell membrane of B. amyloliquefaciens exhibits two separate lipid phase transitions and these arise due to a condition of vertical lipid asymmetry. The designation of the characteristic boundary temperatures for the postulated transitions is shown in Figure 4.19. If vertical lipid asymmetry exists, this may arise due to different phospholipid classes (and presumably different acyl fatty acids) in the two halves of the membranes. At the time when this hypothesis was formulated, phospholipid (headgroup) asymmetry had only been reported for the cell membrane of the human red blood cell and in this instance phosphatidyl choline was shown to be predominantly located in the outer half and phosphatidyl serine togehter with phosphatidyl ethanolamine in the inner half (Bretscher, 1972; Gordesky and Marinetti, 1973; Verkleij et al., 1973). More recently these findings have been supported by the work of Marinetti et al.,(1974); Kahlenberg et al.,(1974) and Zwaal et al.,(1975), and the findings extended to rat erythrocyte membranes (Renooij et al., 1976). In addition the membrane envelope of Influenza virus has also been shown to exhibit phospholipid asymmetry (Tsai and Lenard, 1975; Rothman et al., 1976). Although this asymmetry has been demonstrated for particular phospholipid classes, there does not appear to be any direct evidence for asymmetry in the acyl fatty acids.

Independently of this work, Wisnieski et al.,(1974a,b) have reported that animal plasma membranes exhibit two lipid phase transitions, a result considered to reflect differences in the composition of the hydrocarbon compartments in each half

FIGURE 4.19. PHYSICAL STATE OF THE LIPIDS IN THE CELL MEMBRANE OF B. AMYLOLIQUEFACIENS.

Diagram depicting physical state of the lipid which exists in both the exterior (EXT) and interior (INT) halves or lipid monolayers of the cell membrane of B. amyloliquefaciens as a function of temperature. T_s and T_f refer to the temperatures for the initiation and termination respectively of each lipid phase transition. Lipid phases are referred to as F (fluidus or liquid-crystalline), S (solidus or crystalline) and F+S (mixed liquid-crystalline and crystalline). The temperatures are the average obtained by spin-labelling, enzyme kinetics and fluorescent probe studies and confirmed by thermal analysis.


OUTER
T_f
T_s
EXT.
F
F
38°
F
F+S
21°
F+S
F+S
15°
F+S
S
7°
S
S
INT.
T_f
T_s
INNER

of the bilayer. Multiple thermotropic phase transitions have also been observed in E. coli (Morrisett et al., (1975). These examples perhaps lend support to the asymmetry hypothesis in B. amyloliquefaciens. However this hypothesis is based on a number of unproven assumptions and these are as follows:

1. It is assumed that O.L. vesicles are the same orientation as cells while F.P. vesicles are reversed. No direct attempt was made in these studies to determine vesicle orientation and thus the assumption is based on the results obtained by other workers on other bacterial systems. The argument that O.L. vesicles have a membrane orientation equivalent to that in intact cells has been supported by the findings of Konings et al., (1973); Short et al., (1974); Altendorf and Staehelin (1974); Futai and Tanaka (1975) and Konings (1975). That F.P. vesicles have an opposite orientation is supported by the findings of Hertzberg and Hinkle (1974); Rosen and McClees (1974); Tsuchiya and Rosen (1975) and Futai and Tanaka (1975). If the two types of membrane vesicles are of opposite orientation, the finding that O.L. vesicles exhibit the higher rates of succinate and NADH oxidation, is difficult to reconcile with the proposal that in these vesicles, the respective dehydrogenases are believed to be located on the inner half of the membrane and as such, not directly accessible to their respective substrates (Futai, 1974). Thus in O.L. vesicles, respiratory activity may be due to the permeation of the substrate across the membrane or alternatively some rearrangement of the respiratory enzymes within the membrane may occur at lysis. Nevertheless B. amyloliquefaciens O.L. vesicles were capable of oxidizing externally added respiratory substrates and were also capable of the active transport of amino acids (results not shown), which is in agreement with the results obtained for osmotically-

prepared vesicles of B. subtilis (Konings and Freese, 1972; Bisschop et al., 1975) and E. coli (Kaback, 1972). The lower rate of respiration in F.P. vesicles may be due to the loss of some membrane protein as was observed in Chapter 5.

2. It is also assumed that Tween 80 when added to intact vesicles cannot completely penetrate through the membrane because of its polar sorbitan group, and therefore it is only free to interact with the exposed half of the membrane. Although this results in an inhibition of respiration, it is assumed that the remaining activity is still valid for the purpose of determining transitions in the membrane.

The results obtained with the isolated phospholipid vesicles are at first sight contrary to the hypothesis of vertical lipid asymmetry. It would be expected that upon isolation of the phospholipids, lipid asymmetry would be destroyed and only one transition (2 characteristic temperatures) would be observed as demonstrated by Wisnieski et al. (1974a,b) for phospholipids isolated from the plasma membrane of cultured animal cells. However in this study, the findings suggest that asymmetry may be re-established in the sonicated phospholipid vesicles, (and therefore give rise to two phase transitions). There is evidence that this phenomenon does occur due to the effects of curvature in phospholipid vesicles. Litman (1974) and Berden et al. (1975) have shown that mixtures of phosphatidyl ethanolamine and phosphatidyl choline give rise to vesicles whose inner layer is enriched in phosphatidyl ethanolamine. Michaelson et al. (1973) have similarly demonstrated an asymmetric distribution of phosphatidyl glycerol and phosphatidyl choline in phospholipid vesicles. The major phospholipids of B. amyloliquefaciens are cardiolipin and phosphatidyl ethanolamine (Chapter 5). Considered in terms of the different molecular

volume of the acyl chains and the different head group charge, these phospholipids may lend themselves particularly well to the generation of asymmetric vesicles.

It is possible that differences in lipid fluidity in each half of the membrane may also be due to differences in the lipid-to-protein ratio as evident from the effects of altered lipid-to-protein ratios on the fluidity of Mycoplasma hominis membranes (Rottem and Greenberg, 1975). Differences in the lipid-to-protein ratio in each half of the membrane could possibly be brought about by the 8:1 asymmetry in intramembranous particle distribution (Chapter 3). However the results obtained with the isolated phospholipids would indicate that differences in the fluidity between each half of the membrane, are most likely the result of an asymmetry in acyl fatty acid distribution, rather than to the effects of protein.

In summary, the hypothesis of lipid bilayer asymmetry is compatible with the observations in this chapter, however, it has the disadvantage of resting on certain unproven, if reasonable, assumptions.

At this stage a decision was faced concerning the best policy for future work. One alternative was to attempt to obtain evidence on vesicle orientation, penetration of Tween 80 etc., but this was rejected on the grounds that evidence obtained from phase transition studies alone could not conclusively prove or disprove lipid asymmetry. The more attractive alternative was to study directly the phospholipid distribution in the membrane using techniques such as chemical labelling and selective phospholipase action on the membrane phospholipids. To this end studies on the membrane lipids of B. amyloliquefaciens were commenced; however the effects of lipid on the secretion of extracellular enzymes became of great interest and as a result,

there was insufficient time to continue the work on membrane phospholipid distribution. However this work is now in progress in our research group.

CHAPTER 5

STUDIES ON THE LIPID COMPOSITION OF *B. AMYLOLIQUEFACIENS* CYTOPLASMIC MEMBRANES

A. INTRODUCTION

The cytoplasmic membrane of bacteria participates in a variety of biochemical functions including transport (Kaback, 1972), electron transport reactions (Machtiger and Fox, 1973), lipid synthesis (Carter, 1968), cell wall synthesis (Pardee, 1968) and reactions involving RNA (Schlessinger, 1963; Schelssinger et al., 1965) and DNA (Ganesan and Lederberg, 1965; Sueoka and Hammers, 1974). Although proteins catalyse such processes, lipid plays not only a structural role but also may govern the activities of membrane-associated enzymes. In this regard the observations made in Chapter 4 clearly demonstrate that the physical properties of the lipid can influence membrane-associated enzymes. In view of these results, a study was undertaken principally on the fatty acid composition of the cell membrane. Initially this was done as a prerequisite for a direct chemical attack on the problem of lipid asymmetry raised in the previous chapter. However it was also hoped that a means could be found to alter the fatty acid composition of the membrane and thereby influence by changes in fluidity, activities associated with the membrane, including extracellular enzyme secretion. This latter aspect became the major part of this work and is described in Chapter 6.

The possibility that lipid may be actively involved in the process of extracellular enzyme secretion in B. amyloliquefaciens was also investigated in the work described in this chapter.

B. RESULTS

1. Chemical composition of B. amyloliquefaciens cytoplasmic membranes

The average lipid, protein and RNA content of well-washed B. amyloliquefaciens cytoplasmic membranes, prepared by either osmotic lysis (O.L. vesicles) or by the French pressure cell (F.P. vesicles) is shown in Table 5.1. Extraction of lipid material from both types of vesicle was essentially complete after the first chloroform/methanol extraction step and no additional lipid material was released from lipid-depleted membranes after hydrolysis in 6N HCl. The two types of vesicle exhibit markedly different lipid to protein ratios (O.L. vesicles, 0.3; F.P. vesicles, 2.0) which may be due to loss of protein from the F.P. vesicles during preparation. The lipid content of whole cells isolated from late-log phase culture was about 5% of the total dry weight. The RNA content of either type of vesicle did not exceed 3.6% of the total dry weight.

After fractionation of the total lipid extract from O.L. vesicles by silicic acid chromatography, three classes of lipid were evident, the phospholipid class being the major species (Table 5.2). The phospholipid content of O.L. vesicles (67 to 75% by weight of the total lipid) was identical to that obtained for phospholipids isolated from total lipid extracts of whole cells by the acetone precipitation procedure (Table 5.3). Thus essentially all of the cellular phospholipid must be associated with the cytoplasmic membrane.

TABLE 5.1

(% of Dry weight)

	LIPID	PROTEIN	RNA
O.L. Vesicles	19-25	60	1.7-3.6
F.P. Vesicles	55	28	1.1

CHEMICAL COMPOSITION OF CYTOPLASMIC MEMBRANES OF B. AMYLOLIQUEFACIENS

Membrane vesicles were prepared either by the osmotic lysis of protoplasts or by the French pressure cell disruption of cells. Lipid was extracted from both types of vesicles by the procedure described in Chapter 2 and the dry weight determined after the removal of non-lipid contaminants. Protein was determined by the procedure of Lowry et al. (1951) and RNA by the orcinol procedure after hydrolysis of the sample in 5% (w/v) trichloroacetic acid at 90^{o} for 15 minutes.

TABLE 5.2

(% Dry Wt. of total lipid)

NEUTRAL LIPID	GLYCOLIPID	PHOSPHOLIPIDS
14 - 21	11 - 17	67 - 75

LIPID COMPOSITION OF CYTOPLASMIC MEMBRANES OF B. AMYLOLIQUEFACIENS, (O.L. VESICLES).

Osmotically-prepared membrane vesicles were lipid extracted as described in Chapter 2 and the purified lipid extract was fractionated on a column of silicic acid as described. Neutral lipids were eluted with chloroform, glycosyl glycerides with acetone and phospholipids with methanol followed by chloroform-methanol-water (10:10:1, v/v/v), as described in Chapter 2.

2. Studies on the fatty acid composition of B. amyloliquefaciens

It has been shown that the lipids of many Gram-positive bacteria are essentially all located in the cytoplasmic membrane (Macfarlane, 1961; Kolb et al., 1963; Vorbeck and Marinetti, 1965; Bishop et al., 1967). For this reason it was initially decided to determine the fatty acid composition of whole cells rather than isolated membranes as this should reflect the fatty acid composition of the cell membrane.

Lipids extracted from well-washed B. amyloliquefaciens LF^- cells were processed as described in Chapter 2 and the fatty acid methyl esters analysed by G.L.C. The chromatograms obtained when samples were analysed on either a non-polar 3% Apiezon L or a polar DEGS column, are shown in Figures 5.1 and 5.2. Peaks were identified by comparing their R_f values (relative to methyl palmitate) to known fatty acid methyl ester standards. Their identities are shown accompanying each figure; (names of these fatty acids are presented in Chapter 2). On both columns the branched-chain fatty acids preceeded their corresponding straight-chain homologue, the Apiezon column giving the greater resolution. Complete separation of iso and anteiso fatty acids of equivalent carbon number was not possible using these columns, but some degree of separation was achieved when using the EGSS-X column. When this column was operated at 115° with a carrier-gas flow rate of 36 ml/minute, it was clear that the branched-chain $C_{15:0}$ peak was comprised of a shoulder which eluted slightly ahead of the main peak. This gave a ratio of anteiso $C_{15:0}$: iso $C_{15:0}$ of approximately 2.7:1. Under these same conditions only one symmetric peak was evident for the branched-chain $C_{14:0}$, and this corresponded to the iso form. Increasing the temperature to 135° and maintaining the same flow

FIGURE 5.1. CHROMATOGRAM OF TOTAL FATTY ACIDS OF B. AMYLOLIQUEFACIENS RUN ON A NON-POLAR COLUMN

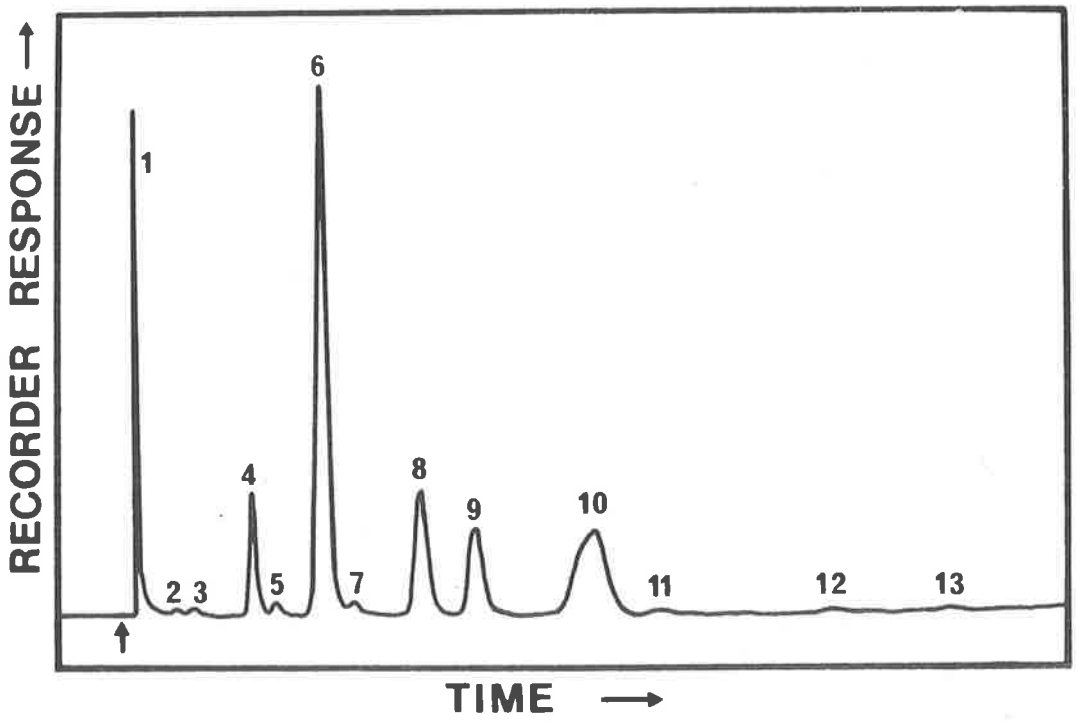
Fatty acid methyl esters were prepared from the total lipid extract of B. amyloliquefaciens cells, and analysed by G.L.C. using a 3% Apiezon column. (Temperature 190°; Carrier gas, 50 ml/minute).
Identity of peaks is as follows:

1. Injection/Solvent peak
2. Unknown
3. C_{12} - C_{13} normal and branched-chain
4. iso $C_{14:0}$
5. normal $C_{14:0}$
6. iso and anteiso $C_{15:0}$
7. normal $C_{15:0}$
8. iso $C_{16:0}$
9. normal $C_{16:0}$
10. iso and anteiso $C_{17:0}$
11. normal $C_{17:0}$
12. normal $C_{18:1}$
13. normal $C_{18:0}$

FIGURE 5.2. CHROMATOGRAM OF TOTAL FATTY ACIDS OF B. AMYLOLIQUEFACIENS RUN ON A POLAR COLUMN

Fatty acid methyl esters were prepared from the total lipid extract of B. amyloliquefaciens, and was identical to that used for the previous figure. A DEGS column was used for the G.L.C. analysis. (Temperature 165°; Carrier gas 40 ml/minute).
Identity of peaks is as follows:

1. Injection/Solvent peak
2. Not present using DEGS column
3. C_{12}-C_{13} normal and branched-chain
4. iso $C_{14:0}$
5. normal $C_{14:0}$
6. iso and anteiso $C_{15:0}$
7. normal $C_{15:0}$
8. iso $C_{16:0}$
9. normal $C_{16:0}$
10. iso and anteiso $C_{17:0}$
11. normal $C_{17:0}$
12. normal $C_{18:1}$
13. normal $C_{18:0}$


RECORDER RESPONSE
1
2 3
4
5
6
7
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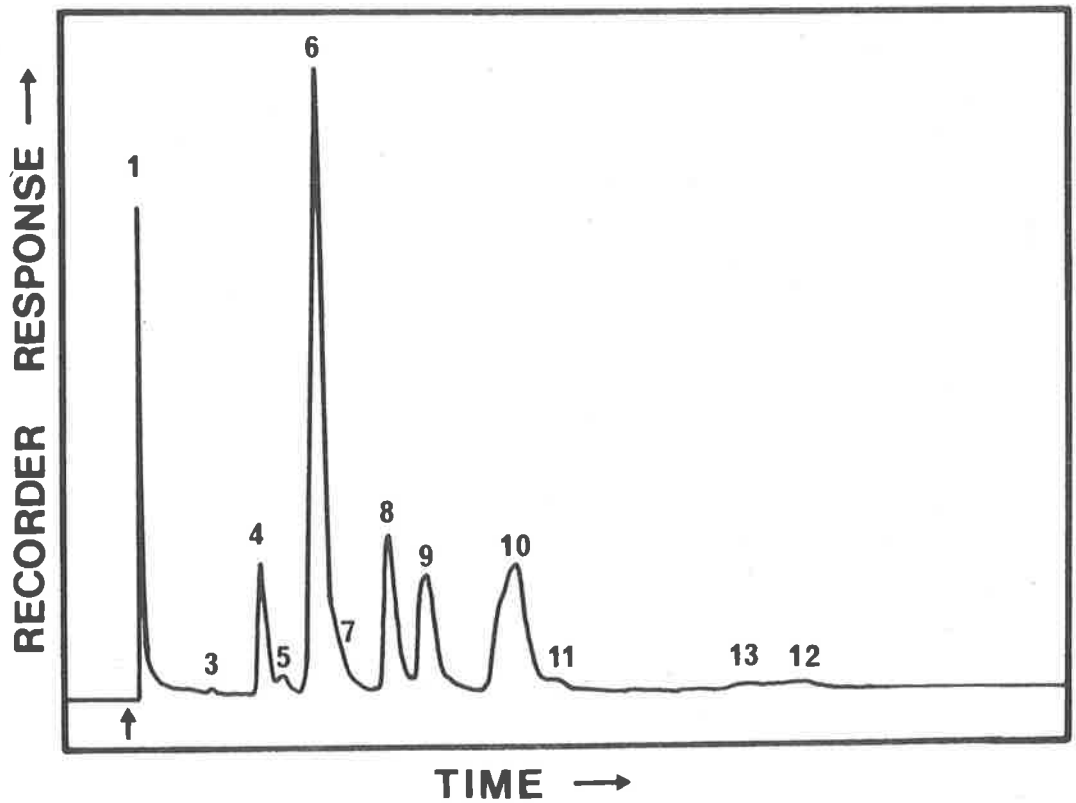
9
10
11
12
13
TIME


RECORDER RESPONSE
1
3
4
5
6
7
8
9
10
11
13 12
TIME

rate permitted some resolution of the branched-chain $C_{17:0}$ peak. Again a leading shoulder was observed associated with the main peak. This shoulder is also evident in the chromatograms shown in Figures 5.1 and 5.2. The ratio of anteiso to iso $C_{17:0}$ was approximately 2.1:1. Only one symmetric peak was evident for the branched-chain $C_{16:0}$ peak and this represented the iso form. The presence of only the iso form of $C_{14:0}$ and $C_{16:0}$ and the ratios obtained for anteiso : iso $C_{15:0}$ and $C_{17:0}$ are in good agreement with those obtained for B. subtilis (168) by Bishop et al. (1967) in which only the iso $C_{14:0}$ and $C_{16:0}$ forms were present and the anteiso : iso ratios for $C_{15:0}$ and $C_{17:0}$ were 2.8 and 1.6 respectively. Due to the difficulty in separating iso and anteiso forms, the branched-chain $C_{15:0}$ and $C_{17:0}$ fatty acids, will each be expressed as the total of the two isomeric forms.

The only unsaturated fatty acid present was $C_{18.1}$ and this represented 0.17% of the total fatty acids. Its identity as an unsaturated fatty acid was indicated by its different behaviour on the two types of column; on the non-polar column the unsaturated fatty acid preceeded its corresponding saturated homologue, whilst the opposite was the case when using a polar column. Its identity was confirmed when it was not separated from $C_{18:0}$ after the samples were hydrogenated and rerun on both types of column. Although the position of the double bond was not confirmed, this fatty acid is likely to be either cis-vaccenic ($C_{18:1}^{\Delta 11}$) or oleic ($C_{18:1}^{\Delta 9}$) as the presence of both has been shown in other bacteria (Kates, 1972). The peak of unknown identity on the Apiezon column (Peak No. 2), eluted well after $C_{18:1}$ on the DEGS column. Thus it may represent some form of polar fatty acid such as a hydroxy fatty acid.

The fatty acid composition of the total cellular lipids

of B. amyloliquefaciens is shown in the histogram presented in Figure 5.3 A. The predominant fatty acids are branched-chain and they comprise 84% of the total fatty acids. Of these, branched-chain $C_{15:0}$ and $C_{17:0}$ comprise about 43 and 20% whilst the even-carbon numbered iso-branched fatty acids, $iC_{14:0}$ and $iC_{16:0}$ are present at about 7 and 13%, respectively. The straight-chain fatty acids make up the bulk of the remaining fatty acids and of these $nC_{16:0}$ is predominant. Quantitative rather than qualitative differences were observed when comparing the total cellular fatty acids (Figure 5.3 A), to the total cellular phospholipids (Figure 5.3 B), and to the isolated-membrane phospholipid fatty acids (Figure 5.3 C). In all instances the predominant branched-chain fatty acid was $C_{15:0}$ and the predominant straight-chain was $nC_{16:0}$. The main contributing factor to the different proportion of branched-chain to straight-chain fatty acids in these three samples, is the difference in the amount of the four predominant fatty acids, ($bcC_{15:0}$, $bcC_{17:0}$, $iC_{16:0}$ and $nC_{16:0}$). Nevertheless from this comparison it can be seen that the total cellular fatty acids approximate well to the fatty acid composition of the cytoplasmic membrane phospholipids.

3. Studies on the fatty acid composition of cells and culture supernatants of B. subtilis 168 and B. amyloliquefaciens LF^- and WT

The LF^- mutant strain of B. amyloliquefaciens routinely used in this work was derived from a wild type strain (WT) of B. amyloliquefaciens by mutagenesis (Sanders and May, 1975). It is characterized by the inability to produce an active surfactin-like lytic factor which is capable of lysing protoplasts from this and a variety of other bacteria (May and Elliott, 1970).

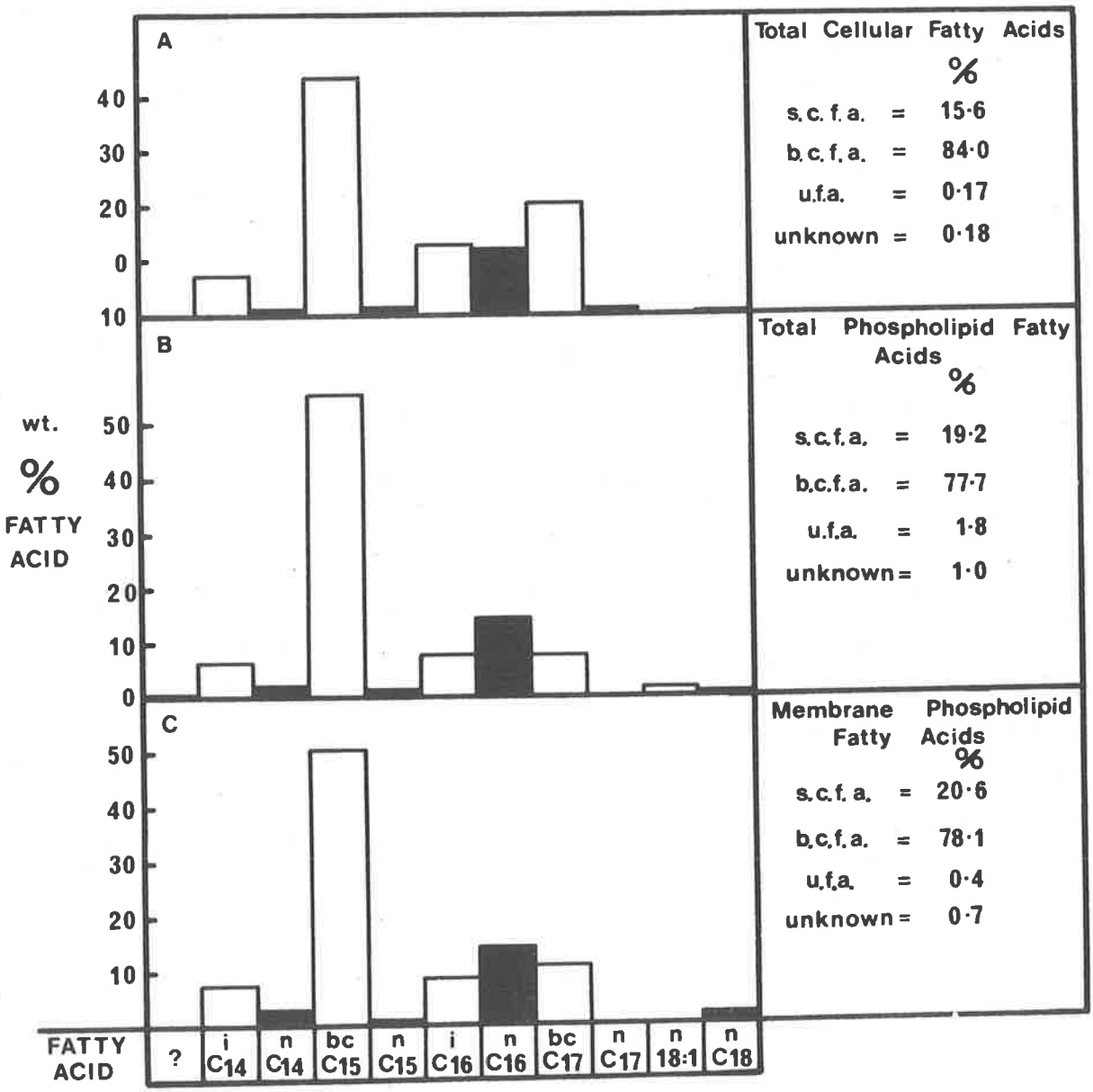
FIGURE 5.3. FATTY ACID COMPOSITION OF TOTAL CELLULAR LIPIDS, TOTAL CELLULAR PHOSPHOLIPIDS AND MEMBRANE PHOSPHOLIPIDS OF B. AMYLOLIQUEFACIENS (LF^-).

Histograms showing the fatty acid composition of the total cell lipids (A), total cell phospholipids (B) and isolated membrane phospholipids (C).

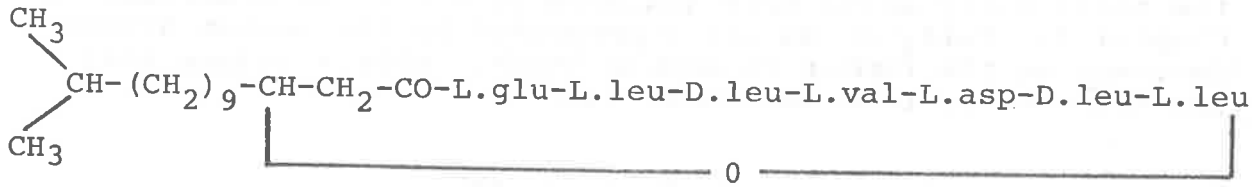
Late-log phase cells grown at 30^{o} were harvested, washed and the lipids extracted. Phospholipids were isolated from the total lipid extract by acetone precipitation.

Membranes were prepared by the osmotic lysis of protoplasts and the lipid extracted as described. Phospholipids were fractionated by silicic acid column chromatography as described in Chapter 2.

Fatty acid methyl esters were prepared as described and analysed by G.L.C. Abbreviations used are: i, (iso); n (normal); bc, (branched-chain, iso plus anteiso forms); s.c.f.a., (straight-chain fatty acids); b.c.f.a., (branched-chain fatty acids) and u.f.a., (unsaturated fatty acids). Fatty acids are represented by the carbon number and for unsaturated fatty acids, by both carbon number and number of double bonds. Branched-chain fatty acids are shown in the clear regions and straight-chain (normal) fatty acids are shown in the shaded regions.


A
B
C
wt.
%
FATTY
ACID
40
30
20
0
10
50
40
30
20
10
0
50
40
30
20
10
FATTY
ACID
?
i C14
n C14
bc C15
n C15
i C16
n C16
bc C17
n C17
n 18:1
n C18
Total Cellular Fatty Acids
%
s.c.f.a. = 15·6
b.c.f.a. = 84·0
u.f.a. = 0·17
unknown = 0·18
Total Phospholipid Fatty Acids
%
s.c.f.a. = 19·2
b.c.f.a. = 77·7
u.f.a. = 1·8
unknown = 1·0
Membrane Phospholipid Fatty Acids
%
s.c.f.a. = 20·6
b.c.f.a. = 78·1
u.f.a. = 0·4
unknown = 0·7

On the basis of extensive structural studies on the lytic factor produced by B. amyloliquefaciens (Gould et al., 1971) and on the lytic factor produced by B. subtilis (Arima et al., 1968; Kakinuma et al., 1969), the following structure for the lytic factor has been proposed (Scheme 1).



Scheme 1

The fatty acid moiety of the surfactin which consists of an iso C_{15} β-hydroxy fatty acid could easily be derived from the fatty acids present in the membrane as it was previously shown that the branched-chain C_{15} fatty acids were the predominant fatty acids in B. amyloliquefaciens. Thus a comparison of the fatty acid composition of LF^- and WT B. amyloliquefaciens may possibly provide some information on the relation between fatty acid composition and synthesis of the lytic factor.

A comparison of the fatty acid composition of LF^- and WT B. amyloliquefaciens cells and of B. subtilis (168) cells, all taken from late-log phase culture, is shown in Figure 5.4. A marked similarity exists between LF^- (Figure 5.4A) and WT (Figure 5.4B) B. amyloliquefaciens cells with regard to the type and amount of fatty acid present, with both exhibiting similar levels of branched and straight-chain fatty acids. The major difference between the two strains is the 10% greater level of branched-chain $C_{15:0}$ in the WT strain which appears to be solely at the expense of the branched-chain $C_{17:0}$ fatty acids. Thus it is possible that the comparatively lower level of branched-chain $C_{15:0}$ in the LF^- cells may have a bearing on the inability of this strain to produce the lytic factor; (the lesion

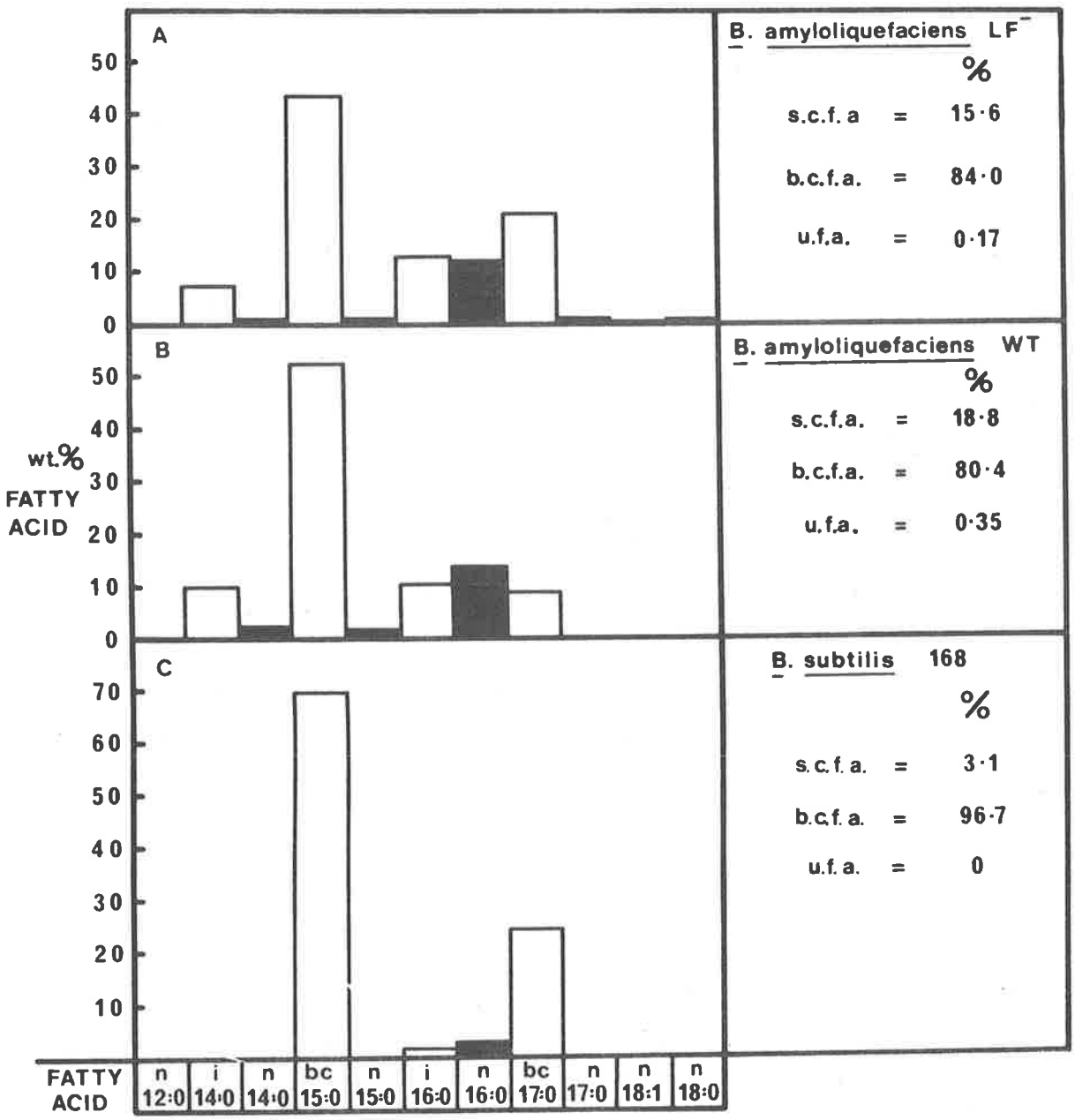
FIGURE 5.4. FATTY ACID COMPOSITION OF B. AMYLOLIQUEFACIENS (LF^- AND WT) AND B. SUBTILIS 168 ($TRYP^-$) CELLS.

Lipids were extracted from late-log phase cells and the total fatty acids were analysed by G.L.C. as described in Chapter 2. Fatty acids are represented by the carbon number followed by the number of double bonds. Abbreviations used are the same as used in Figure 5.3.

A. B. amyloliquefaciens LF^-

B. B. amyloliquefaciens WT

C. B. subtilis 168 ($Tryp^-$)


A
B
C
wt.% FATTY ACID
0
10
20
30
40
50
60
70
B. amyloliquefaciens LF⁻
%
s.c.f.a = 15·6
b.c.f.a. = 84·0
u.f.a. = 0·17
B. amyloliquefaciens WT
%
s.c.f.a. = 18·8
b.c.f.a. = 80·4
u.f.a. = 0·35
B. subtilis 168
%
s.c.f.a. = 3·1
b.c.f.a. = 96·7
u.f.a. = 0
FATTY ACID
n 12:0
i 14:0
n 14:0
bc 15:0
n 15:0
i 16:0
n 16:0
bc 17:0
n 17:0
n 18:1
n 18:0

which results in the lack of surfactin production is unknown). The fatty acid composition of B. subtilis (168) differs from both strains of B. amyloliquefaciens by exhibiting far less compositional variation. Together the branched-chain $C_{15:0}$ and $C_{17:0}$ fatty acids comprise about 94% of the total, whilst in both strains of B. amyloliquefaciens these fatty acids comprise only about 62% of the total. About 97% of the total fatty acids of B. subtilis are of the branched-chain type. In contrast to both strains of B. amyloliquefaciens which contained small amounts of $C_{18:1}$, no unsaturated fatty acid was detected in B. subtilis, in agreement with the data of Bishop et al. (1967).

All samples contained a minor peak at an R_f relative to methyl palmitate of 3.6 when analysed on the DEGS column, however this peak was not present when samples were run on the non-polar Apiezon column (data not shown). This peak may represent the iso $C_{15:0}$ ω-hydroxy fatty acid, a possible precursor of the surfactin molecule. It would be indistinguishable from the branched-chain $C_{15:0}$ peak when analysis was performed on the Apiezon column.

No differences were evident in the level of phospholipid from B. subtilis (168) and from both strains of B. amyloliquefaciens, the average phospholipid content of all three being about 70% (w/w) of the total lipid (data not shown). Phospholipid was detected in the lipid extracted from late-log phase culture supernatants from all of the above, though the level was considerably less than was present in cells (see Table 5.3).

Fatty acid analyses of the total lipid extract from late-log phase culture supernatants of B. amyloliquefaciens (LF^-) and B. subtilis (168) are shown in Figure 5.5. In marked contrast

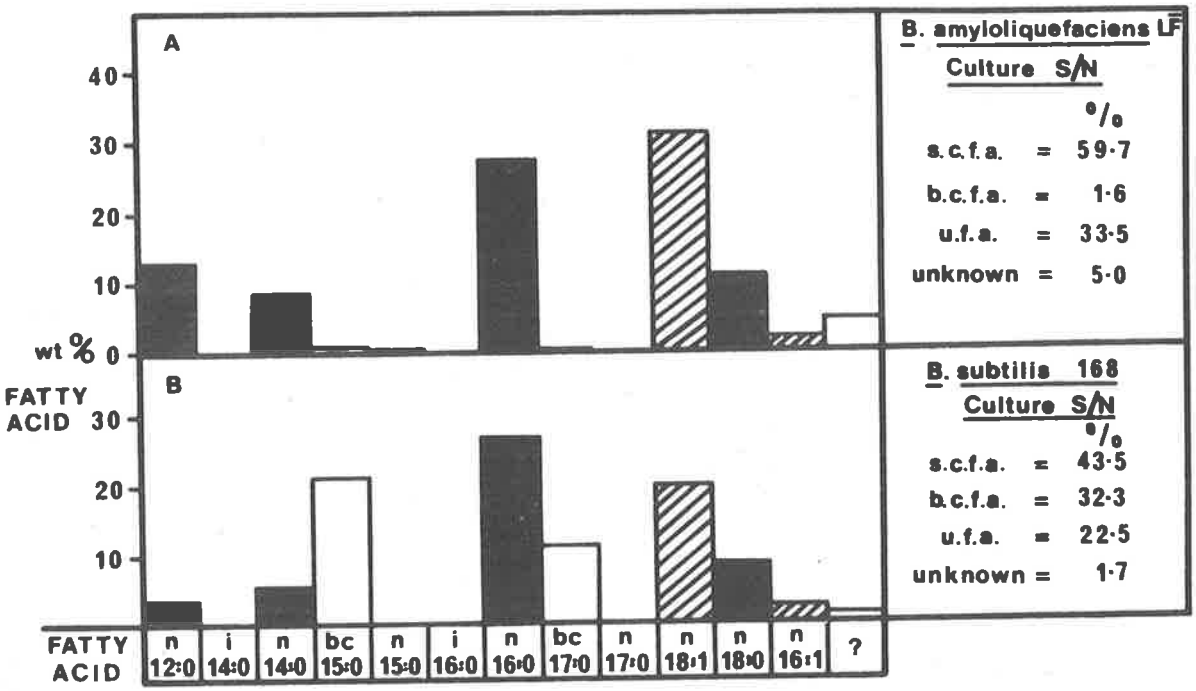
to the cellular and membrane fatty acid composition shown previously, both supernatants exhibited relatively high levels of even-carbon numbered straight-chain fatty acids, together with high levels of $C_{18:1}$ and to a lesser extent $C_{16:1}$. Although the major fatty acids of B. amyloliquefaciens (LF^-) cells are the branched-chain $C_{15:0}$ and $C_{17:0}$ fatty acids, these account for only 1.6% of the total fatty acids of the supernatant from these cells (Figure 5.5A). Because of this and the relatively lower phospholipid level in the supernatant, the fatty acids present in the culture supernatant are unlikely to be derived from the lysis of cells. Indeed in washed-cell supernatants there was a time-dependent accumulation of these same fatty acids, indicating that they were derived from some selective secretory process. The culture supernatant of B. subtilis (168) also showed this active accumulation of lipid material however in this instance the branched-chain fatty acids accounted for 31% of the total fatty acids (Figure 5.5B). The proportion of branched-chain $C_{15:0}$ to branched-chain $C_{17:0}$ fatty acids in B. subtilis culture supernatant was approximately the same as was present in B. subtilis cells. As these two fatty acid types were the most predominant fatty acid species in cells, it is likely that their presence in culture and washed-cell supernatants is due to (lytic factor induced?) cellular lysis and subsequent release of membrane fatty acids into the medium. The fatty acid composition of the culture supernatant of B. amyloliquefaciens WT cells was exceedingly complex, and as identification of all fatty acids was difficult, this result is not shown. In addition all supernatants contained the late-running peak of R_f = 3.6 (relative to methyl palmitate) on the DEGS column, which was not evident on the Apiezon column. In the LF^- supernatant, this peak accounted for about 5% of the total fatty acid.

FIGURE 5.5. FATTY ACID COMPOSITION OF THE CULTURE SUPERNATANTS OF B. AMYLOLIQUEFACIENS LF^- AND B. SUBTILIS 168 ($TRYP^-$).

Lipids were extracted from late-log phase culture supernatants and the total fatty acids were analysed by G.L.C., as described in Chapter 2. Abbreviations are the same as used in Figure 5.3. Branched-chain fatty acids are shown in the clear regions, straight-chain fatty acids in the shaded regions and unsaturated fatty acids in the hatched regions.

A. B. amyloliquefaciens LF^- culture supernatant

B. B. subtilis 168 ($Tryp^-$) culture supernatant


A
B
40
30
20
10
0
wt %
FATTY ACID
B. amyloliquefaciens LF
Culture S/N
% s.c.f.a. = 59·7
b.c.f.a. = 1·6
u.f.a. = 33·5
unknown = 5·0
B. subtilis 168
Culture S/N
% s.c.f.a. = 43·5
b.c.f.a. = 32·3
u.f.a. = 22·5
unknown = 1·7
FATTY ACID
n 12:0
i 14:0
n 14:0
bc 15:0
n 15:0
i 16:0
n 16:0
bc 17:0
n 17:0
n 18:1
n 18:0
n 16:1
?

Lipid material has previously been shown to accumulate in both culture and washed-cell supernatants of B. amyloliquefaciens WT cells (Glenn and Gould, 1973). In an attempt to determine whether the secretion of lipid in the LF^- strain was linked to the process of extracellular enzyme secretion, the effect of chloramphenicol on the lipid levels in both cells and washed-cell supernatants was investigated. Table 5.3 shows the results of two experiments in which cells were incubated for 90 minutes with 40μg/ml chloramphenicol, a level which inhibits general cellular protein synthesis by 95 to 98% and inhibits the secretion of the three major extracellular enzymes by about 85 to 90% (Sanders and May, 1975). The cellular lipid content decreased in the presence of chloramphenicol, but the proportion of phospholipid within experimental error, remained constant. A far greater amount of lipid was extracted from the washed-cell supernatant derived from cells incubated in the presence of chloramphenicol, and a significant decrease in the proportion of phospholipid in the supernatant was observed after chloramphenicol treatment (Experiment 1 and 2, Table 5.3). The fatty acid composition of both cells and washed-cell supernatants was not affected by chloramphenicol treatment, and appeared identical to those shown previously for cells (Figure 5.4A) and culture supernatant (Figure 5.5A). The only difference observed in the washed-cell supernatants was a slightly elevated level of branched-chain $C_{15:0}$ and $C_{17:0}$ fatty acids (in the same proportion as in the membrane), in the supernatant derived from chloramphenicol-treated cells. Thus it is likely that the decreased level of cellular lipid together with the elevated level of lipid in the supernatant of chloramphenicol-treated cells was reflecting some chloramphenicol-induced lysis of cells during the incubation period. Thus lipid was subsequently

TABLE 5.3

	Lipid Content (µg dry wt/ml of culture strength cells or washed-cell supernatant)	Phospholipid (%wt of total lipid)
EXPT. 1		
CELLS	96	74
CELLS + CAP	79	67
W.C. S/N	23	27
W.C. S/N + CAP	66	13
EXPT. 2		
W.C. S/N	-	18
W.C. S/N + CAP	-	5

EFFECT OF CHLORAMPHENICOL ON THE LIPID AND PHOSPHOLIPID CONTENT OF B. AMYLOLIQUEFACIENS (LF^-) CELLS AND WASHED-CELL SUPERNATANTS.

Cells were harvested from late-log phase cultures, twice washed in W.C.S.M. and resuspended in this medium at a dilution equivalent to that in culture. Cells were incubated for 90 minutes at 30° with and without 40 µg/ml chloramphenicol. After this time, cells were isolated by centrifugation and lipid extracted. The washed-cell supernatant was lipid extracted after the removal of cells. For control cells and supernatants, an equivalent amount of chloramphenicol was added just prior to lipid extraction. Phospholipids were isolated by acetone precipitation as described in Chapter 2.

Abbreviations used are: CAP, (chloramphenicol); W.C. S/N, (washed-cell supernatant).

extracted from membrane fragments remaining in the supernatant, after the removal of cells.

4. Influence of growth temperature on the fatty acid composition of B. amyloliquefaciens

In addition to determining the lipid composition of B. amyloliquefaciens one of the aims of this study was to attempt to alter the fatty acid composition of this organism and to determine whether such a change influenced various activities associated with the cell membrane, including extracellular enzyme secretion. Numerous unsaturated fatty acid auxotrophs of E. coli with lesions in the fab A (Cronan and Gelmann, 1973) or fab B (Rosenfield et al., 1973) genes have been obtained and such mutants have proven extremely useful in the study of processes influenced by changes in membrane fatty acid composition and hence fluidity (Shaw and Ingraham, 1965; Esfahani et al., 1971; Overath and Träuble, 1973; Sackman et al., 1973; Linden et al., 1973; Haest et al., 1974). However due to the fact that B. amyloliquefaciens grows in chains difficulty in separating cells makes mutant isolation difficult. It was therefore decided not to attempt to obtain fatty acid auxotrophs of B. amyloliquefaciens for the purposes of altering the membrane fatty acid composition.

The fatty acid composition of a wide variety of organisms has been shown to change as a result of environmental conditions, particularly temperature (Marr and Ingraham, 1967; Fulco, 1974). In E. coli lowering the growth temperature typically results in a higher proportion of unsaturated fatty acid being incorporated into the phospholipids of the cell membrane (Shaw and Ingraham, 1965), whilst for growth at increased temperature there is a tendency toward the incorporation of

longer and more saturated fatty acids, (Sinensky, 1971). The nett result of such changes appears to be the production of membranes whose lipids have a constant fluidity at the temperature of growth; a process termed "homeoviscous adaptation", (Sinensky, 1974). Changes in growth temperature have been reported to affect only the acyl fatty acid composition rather than the membrane phospholipid headgroup composition (Haest et al., 1969; Cullen et al., 1971).

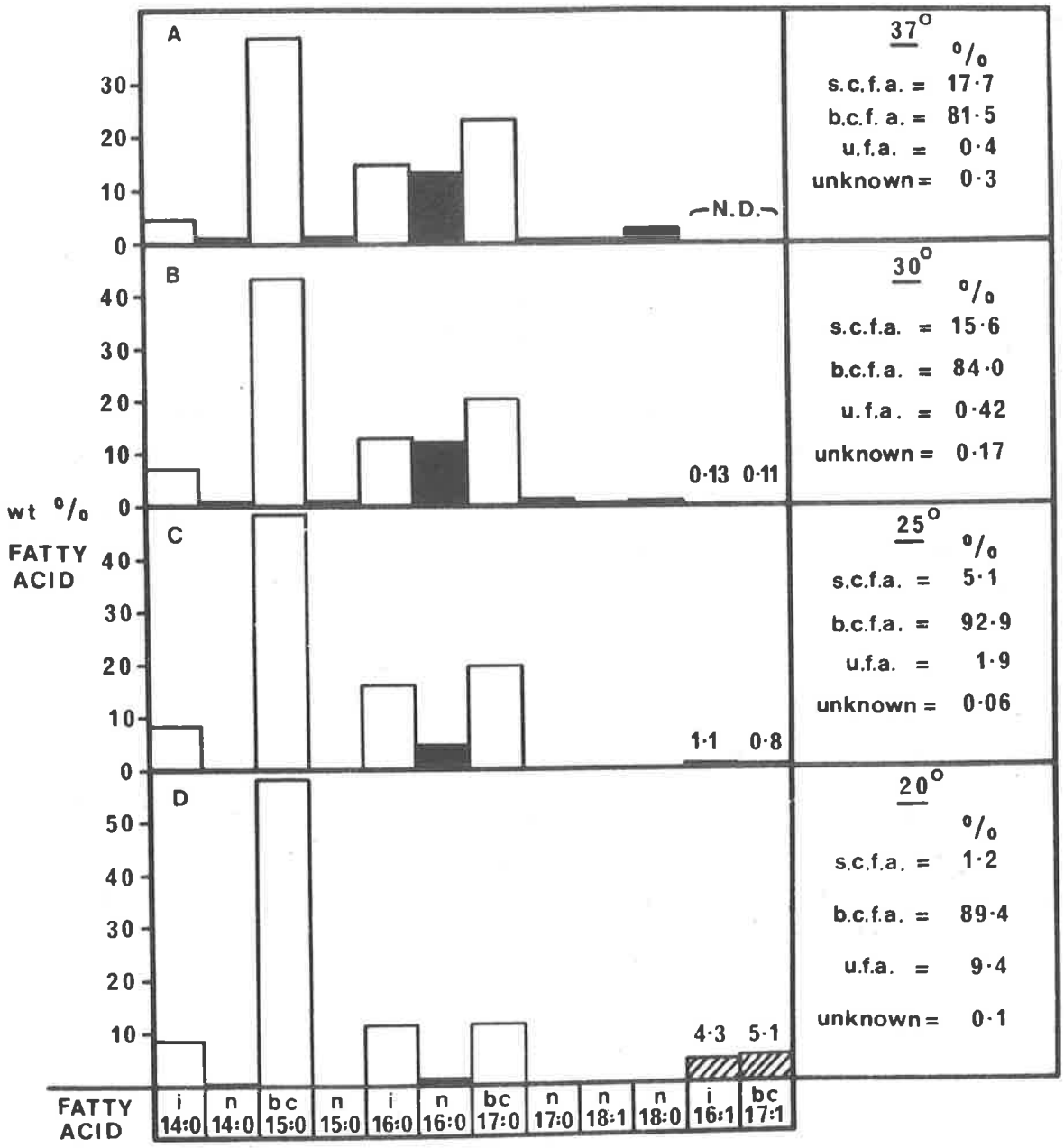
B. amyloliquefaciens LF^- cells were grown from spores at temperatures of 37^o, 30^o, 25^o and 20^o and lipids were extracted from cells after growth reached late-log phase; the time taken for growth varied between 12.5 hr for cells grown at 37^o to about 70 hours for cells grown at 20^o (see Chapter 6, Plate 6.1). The fatty acid composition of cells grown at these temperatures is shown in Figure 5.6. On lowering the growth temperature, the proportion of straight-chain fatty acids decreased from 17.7% at 37^o to 1.2% at 20^o. This was accompanied by an increase in the amount of branched-chain fatty acids (mainly $C_{15:0}$), from 81.5% at 37^o to a maximum of 92.9% at 25^o. Both of these changes would tend to bring about an increase in the fluidity of the membrane. Of particular interest however was the finding that the total level of unsaturated fatty acids rose from 0.4% at 37^o, to 9.4% at 20^o, a 23-fold increase. This change would also be expected to lead to an increase in the membrane fluidity. This increased unsaturated fatty acid content was not due to an elevation in the level of pre-existing $C_{18:1}$. Rather, on lowering the growth temperature, this unsaturated fatty acid disappeared and was replaced by other unsaturated fatty acids some of which were identified as branched-chain unsaturated fatty acids. Due to the rather unusual nature of these fatty acids, some detail on their identification is warranted.

FIGURE 5.6. FATTY ACID COMPOSITION OF B. AMYLOLIQUEFACIENS CELLS GROWN AT DIFFERENT TEMPERATURES.

Histograms showing the fatty acid composition of the total lipid extracted from B. amyloliquefaciens cells grown at 37^{o} (A), 30^{o} (B), 25^{o} (C) and 20^{o} (D). Cells were grown until late-log phase was reached, the lipid extracted and the fatty acids analysed by G.L.C. as described in Chapter 2.

Branched-chain fatty acids are shown in the clear regions, straight-chain fatty acids in the shaded regions and branched-chain unsaturated fatty acids in the hatched regions.

Abbreviations are the same as used in Figure 5.3; N.D., (not detectable). Fatty acids are represented by their carbon number followed by the number of double bonds present. Levels of $iC_{16:1}$ and $itaC_{17:1}$ are included in the histograms.


A
B
C
D
wt %
FATTY ACID
30
20
10
0
40
50
N.D.
0·13 0·11
1·1 0·8
4·3 5·1
37° %
s.c.f.a. = 17·7
b.c.f. a. = 81·5
u.f.a. = 0·4
unknown = 0·3
30° %
s.c.f.a. = 15·6
b.c.f.a. = 84·0
u.f.a. = 0·42
unknown = 0·17
25° %
s.c.f.a. = 5·1
b.c.f.a. = 92·9
u.f.a. = 1·9
unknown = 0·06
20° %
s.c.f.a. = 1·2
b.c.f.a. = 89·4
u.f.a. = 9·4
unknown = 0·1
FATTY ACID
i 14:0
n 14:0
bc 15:0
n 15:0
i 16:0
n 16:0
bc 17:0
n 17:0
n 18:1
n 18:0
i 16:1
bc 17:1

Shown in Figure 5.7 are chromatograms obtained for fatty acids extracted from 20° grown cells which were run before and after hydrogenation on a DEGS (Trace A and B) and on an Apiezon column (Trace C and D). In Trace A, four extra peaks were observed in the positions marked and these were not observed after hydrogenation (Trace B). The position of these peaks, their removal after hydrogenation and the concomitant increase in the level of iso $C_{16:0}$, $nC_{16:0}$ and the branched-chain $C_{17:0}$ peaks after hydrogenation, suggested that they were the following unsaturated fatty acids: (i) iso $C_{16:1}$; (ii) $nC_{16:1}$; (iii) iso $C_{17:1}$; (iv) anteiso $C_{17:1}$. This is supported by their position on the Apiezon column, in which unsaturated fatty acids elute before their corresponding saturated homologue. However when using this column (Trace C), peaks (iii) iso $C_{17:1}$ and (iv) anteiso $C_{17:1}$ ran as one broad peak (as do the corresponding saturated homologues). The fatty acid represented by peak (ii) $nC_{16:1}$ was not observed in this trace as it most likely co-eluted with the iso $C_{16:0}$ peak. Hydrogenation resulted in the disappearance of all three peaks (i, iii and iv), together with an increase in the relative levels of iso $C_{16:0}$, $nC_{16:0}$ and the iso/anteiso $C_{17:0}$ peaks, as would be expected if these peaks represented the unsaturated fatty acids described.

These four branched-chain unsaturated fatty acids were not detectable in cells grown at 37°, however $C_{18:1}$ was present at a level of 0.4%. They became observable in samples of 30° grown cells only if sample overloading conditions and high sensitivity settings were employed. At this temperature they were present at a level of less than 0.1%. For cells grown at 25°, the individual levels of the branched-chain unsaturated fatty acids rose by a factor of 10 and $C_{18:1}$ disappeared. At

FIGURE 5.7. IDENTIFICATION OF UNSATURATED FATTY ACIDS OF B. AMYLOLIQUEFACIENS CELLS GROWN AT 20°.

Chromatograms of fatty acid methyl esters of 20° grown cells obtained after G.L.C. on polar (DEGS) and non-polar (Apiezon) columns, before and after hydrogenation, as described in Chapter 2.

A. DEGS column - before hydrogenation

B. DEGS column - after hydrogenation

C. Apiezon column - before hydrogenation

D. Apiezon column - after hydrogenation

Numbered peaks are positively identified as follows:

1. Solvent/Injection peak
2. iso $C_{14:0}$
3. iso and anteiso $C_{15:0}$
4. iso $C_{16:0}$
5. normal $C_{16:0}$
6. iso and anteiso $C_{17:0}$

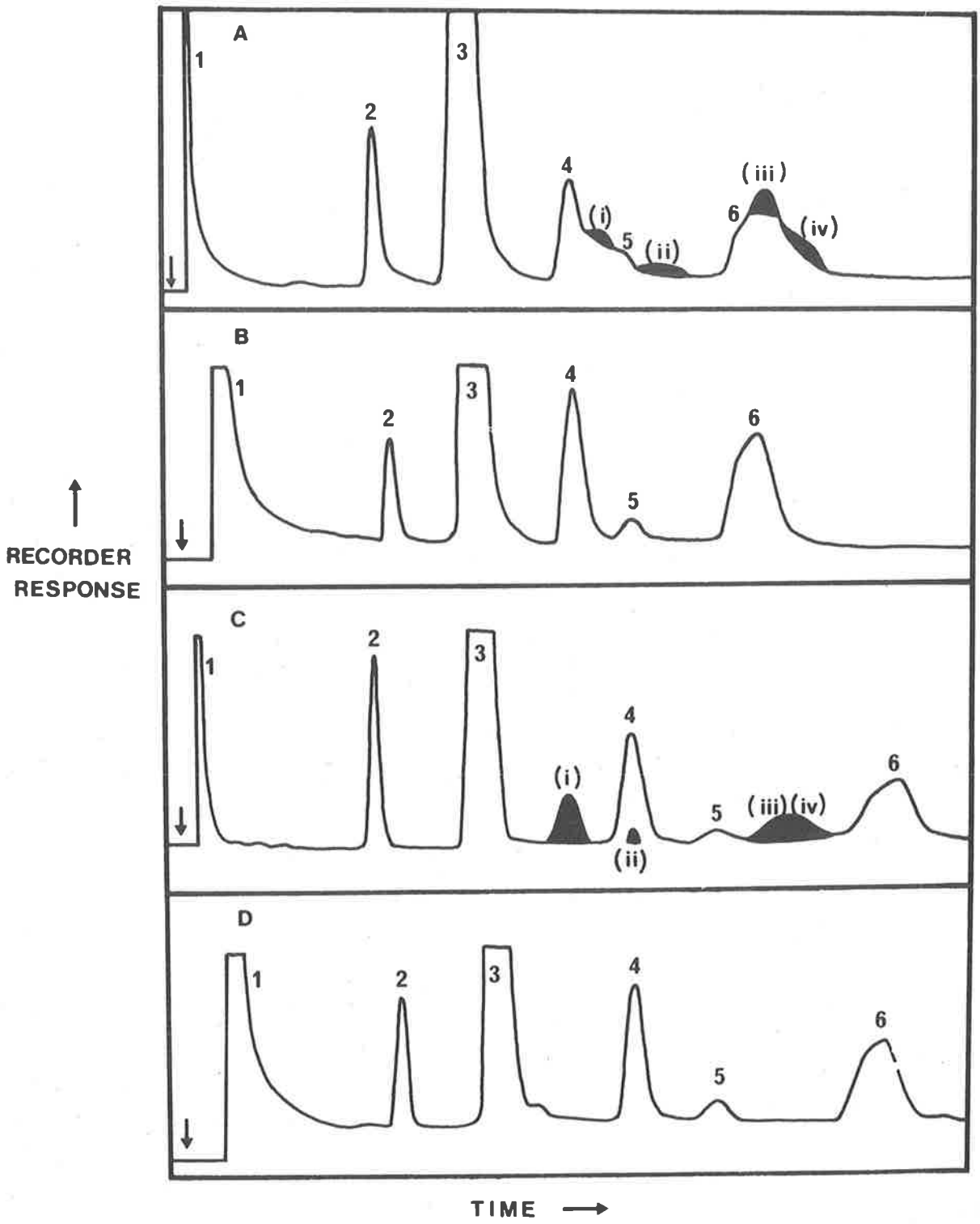
Other peaks are tentatively identified as follows:

(i) iso $C_{16:1}$

(ii) normal $C_{16:1}$

(iii) iso $C_{17:1}$

(iv) anteiso $C_{17:1}$


A
1
2
3
4
(i)
5
(ii)
(iii)
6
(iv)
B
1
2
3
4
5
6
C
1
2
3
(i)
4
(ii)
5
(iii)(iv)
6
D
1
2
3
4
5
6
RECORDER RESPONSE
TIME

a growth temperature of 20°, a 50-fold increase in the individual levels of these branched-chain unsaturated fatty acids was observed in comparison to the levels present in 30° grown cells.

The total fatty acid composition of the supernatants from late-log phase cultures of B. amyloliquefaciens (LF^-) grown at 37°, 30°, 25° and 20° are shown in Figure 5.8. In contrast to the dramatic changes observed in the cells, only slight changes were observed in the culture supernatants. The most significant of these was the 5% rise in the level of unsaturated fatty acids (principally $C_{18:1}$), in cells grown at 20°. Again there was a marked contrast between the types and amounts of fatty acids in the cells and in the culture supernatants and this was most striking in the cells grown at 20° (Figure 5.8D). For example $C_{18:1}$ was present at a level of 35%, however it was undetectable in the cells; $nC_{16:0}$ was present at 28% yet in cells it represented only 1% of the total; and $nC_{12:0}$ and $nC_{14:0}$ were present at a combined level of about 21% yet in cells only $nC_{14:0}$ was detected and this was at a level of less than 1%.

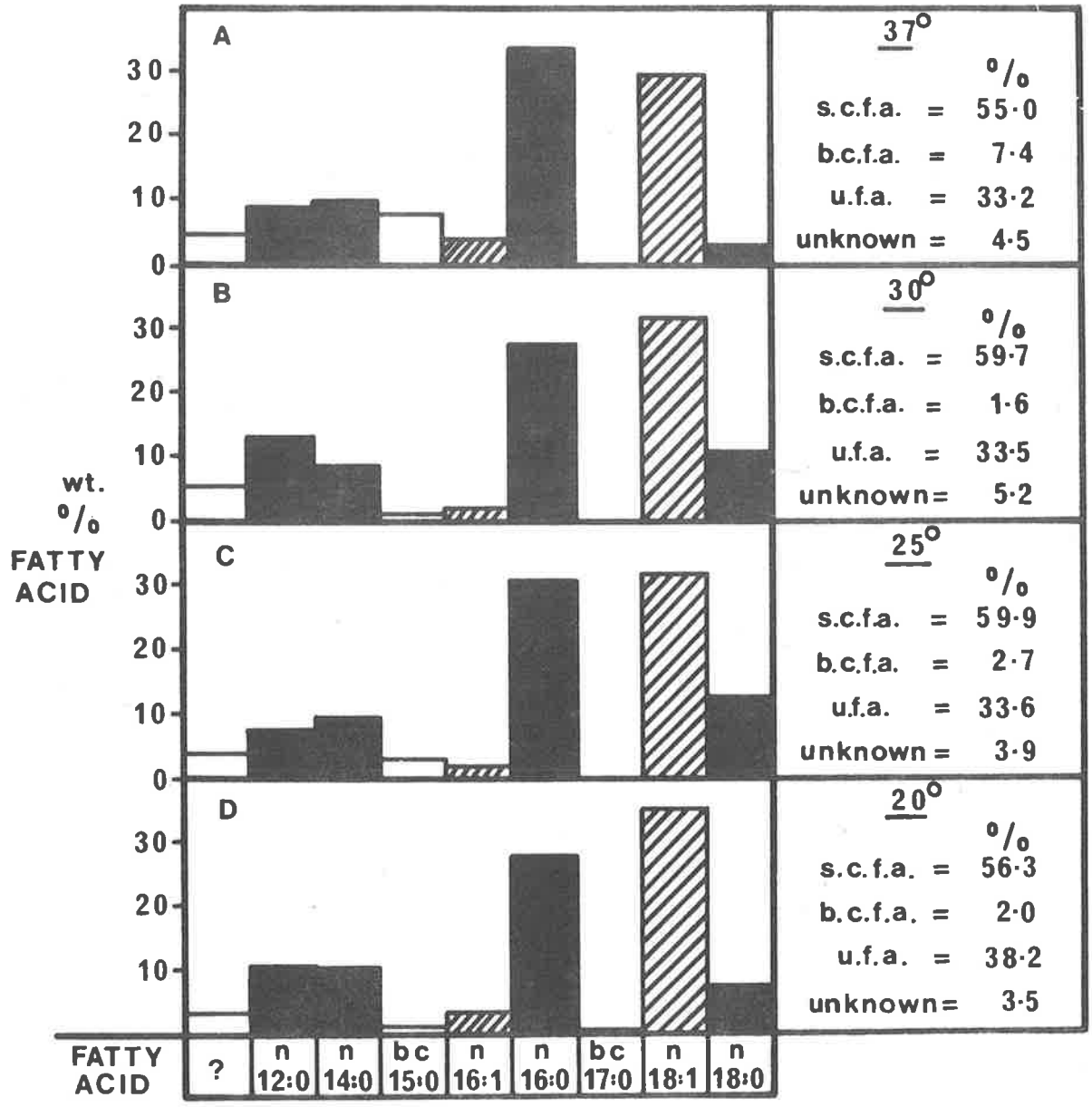
5. Alteration of B. amyloliquefaciens fatty acid composition by growth in various alcohols

Alcohols, local anaesthetics and other lipophilic agents are capable of interacting directly with membrane lipids to bring about changes in the fluidity or molecular freedom of the acyl fatty acids of the membrane (Paterson et al., 1972; Sheetz and Singer, 1974; Trudell et al., 1975; Lee, 1976). The change in membrane fluidity which results from the interaction of various alcohols with membranes and phospholipid bilayers (Hubbell et al., 1970; Hui and Barton, 1973; Grisham and Barnett, 1973),

FIGURE 5.8. TOTAL FATTY ACID COMPOSITION OF THE CULTURE SUPERNATANTS FROM *B. AMYLOLIQUEFACIENS* (LF^-) CELLS GROWN AT DIFFERENT TEMPERATURES.

Fatty acids from the total lipid extract of culture supernatants of *B. amyloliquefaciens* LF^- cells at the late-log phase of growth, were prepared as described in Chapter 2. Histograms show the composition of culture supernatants from cells grown at 37^{o} (A), 30^{o} (B), 25^{o} (C) and 20^{o} (D). The shaded regions represent the saturated straight-chain fatty acids, the clear regions the branched-chain saturated fatty acids and any unidentified fatty acids, whilst the hatched regions represent the unsaturated fatty acids.

Abbreviations are the same as used in Figure 5.3.


A
B
C
D
wt. % FATTY ACID
30
20
10
0
37°
% s.c.f.a. = 55·0 b.c.f.a. = 7·4 u.f.a. = 33·2 unknown = 4·5
30°
% s.c.f.a. = 59·7 b.c.f.a. = 1·6 u.f.a. = 33·5 unknown = 5·2
25°
% s.c.f.a. = 59·9 b.c.f.a. = 2·7 u.f.a. = 33·6 unknown = 3·9
20°
% s.c.f.a. = 56·3 b.c.f.a. = 2·0 u.f.a. = 38·2 unknown = 3·5
FATTY ACID
?
n 12:0
n 14:0
bc 15:0
n 16:1
n 16:0
bc 17:0
n 18:1
n 18:0

can be considered analogous to those induced by changes in growth temperature. E. coli has been shown to alter its membrane fatty acid composition in response to growth in different alcohols and this change is believed to represent an adaptive membrane alteration which compensates for the direct physicochemical interaction of alcohols with the membrane (Ingram, 1976). This change was found to be unique to the fatty acids, no change being observed in the phospholipid composition of the membrane under these conditions (Ingram, 1976). It was decided to attempt to alter the fatty acid composition of B. amyloliquefaciens by growth in different alcohols.

The selection of both the chain length of the alcohol and their relative concentrations was made on the basis of the effect of alcohols on the fatty acid composition of E. coli, as described by Ingram (1976). Growth of E. coli in C_1 to C_5 alcohols resulted in greater fatty acid unsaturation and presumably increased membrane fluidity, while the opposite was the case for growth in alcohols of chain length C_5 to C_{10}. It was decided to grow B. amyloliquefaciens in methanol, ethanol and pentanol, as these alcohols caused the greatest shift in the fatty acid composition in E. coli. As the molar effectiveness of alcohols in eliciting an adaptive change in fatty acids composition was found to be directly related to their lipid solubility (indicative of their direct membrane interaction) (Ingram, 1976), various concentrations of the above alcohols were tested. The concentrations of alcohols which retarded growth of B. amyloliquefaciens to only a minor extent were found to be, 3% (v/v) methanol, 2% (v/v) ethanol and 0.2% (v/v) pentanol. In comparison to the 18 hours required for control cells to reach the late-log phase of growth, these alcohols caused a lag of 2 hr, 5 hr and 3½ hr respectively.

B. amyloliquefaciens cells were grown from spores at 30° in normal medium containing methanol, ethanol and pentanol at the above concentrations, harvested at the late-log phase of growth and twice washed with buffer (not containing the alcohols) prior to the extraction of lipid. The fatty acid composition of these cells is shown in Figure 5.9. Those grown in medium containing methanol or ethanol exhibited changes in the fatty acid composition which would be expected to bring about an increase in the membrane fluidity. The most marked changes were an increase in the level of branched-chain $C_{15:0}$ and $C_{17:0}$ fatty acids, and a decrease in the $nC_{16:0}$ and to a lesser extent, $nC_{14:0}$, $nC_{15:0}$ and $nC_{17:0}$ levels; changes in the levels of $C_{18:1}$ and the trace amounts of branched-chain unsaturated fatty acids, were insignificant. In direct contrast, cells grown in pentanol exhibited changes consistent with a decrease in membrane fluidity although the change in comparison to control cells, was slight. Significant among these changes was an increase in the level of the longer chain saturated fatty acids, $nC_{15:0}$ and $nC_{17:0}$. Again changes in the level of unsaturated fatty acids were insignificant. Thus it is clear that the changes in the fatty acid composition (and presumably membrane fluidity) are brought about by growth in these alcohols. These changes are primarily achieved by an alteration in the proportion of branched-chain to straight-chain fatty acids. (The effect of altered fatty acid composition on extracellular enzyme secretion, is examined in Chapter 6).

Analysis of the culture supernanant of the alcohol grown cells showed no significant change in the amount of extractable lipid or in the amount of phospholipid present. Fatty acid analysis of the total culture supernatant fatty acids showed variable levels of straight-chain, branched-chain and unsaturated

FIGURE 5.9. FATTY ACID COMPOSITION OF B. AMYLOLIQUEFACIENS CELLS GROWN IN VARIOUS ALCOHOLS.

Histograms showing the fatty acid composition of the total lipid extracted from B. amyloliquefaciens cells after growth in media supplemented with the alcohols indicated. Cells were grown at 30° until late-log phase and the lipid extracted and the methyl esters of the fatty acids analysed by G.L.C. as described in Chapter 2.

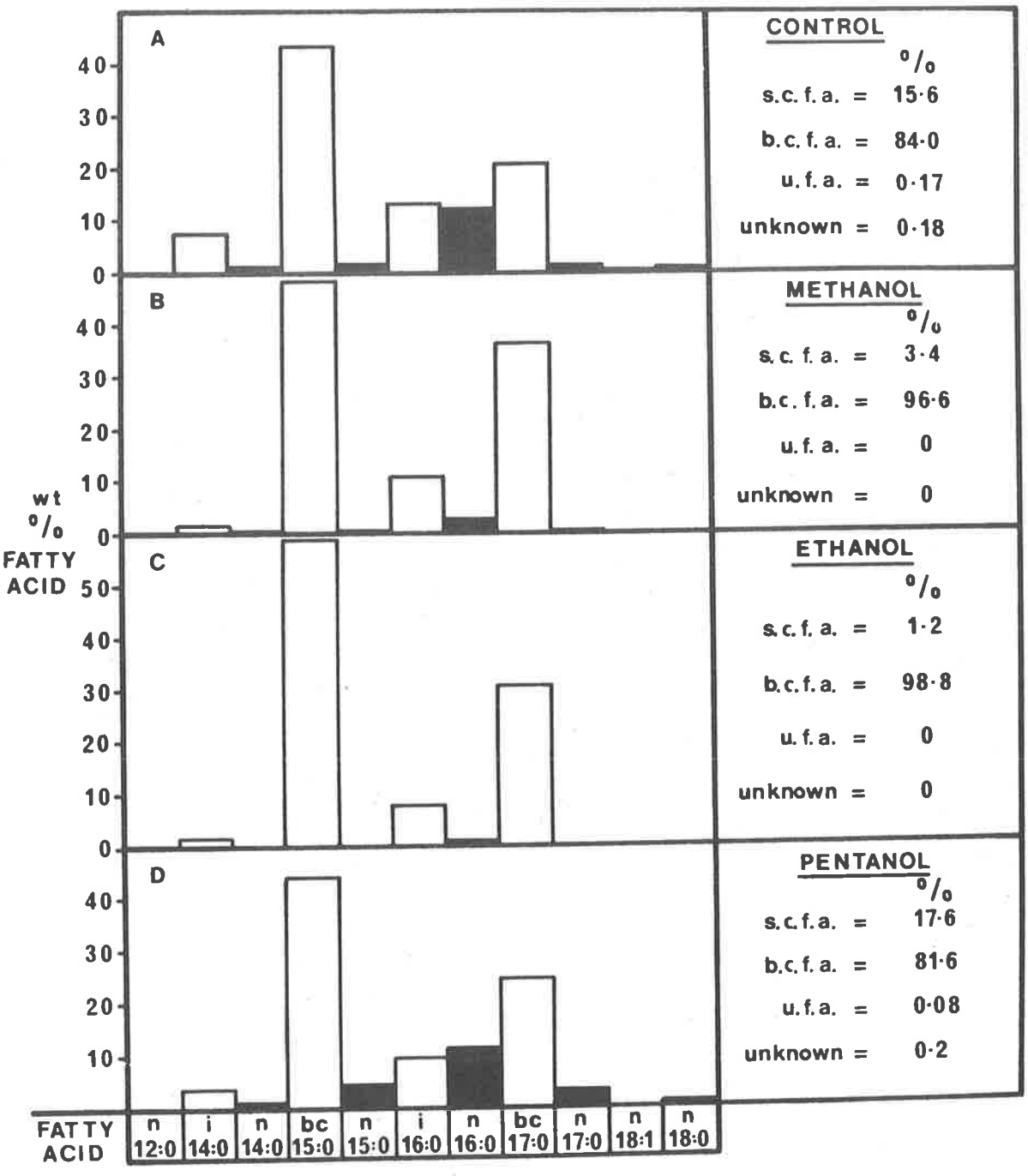
A. Control

B. 3% (v/v) Methanol

C. 2% (v/v) Ethanol

D. 0.2% (v/v) Pentanol

Abbreviations are the same as used in Figure 5.3. Branched-chain fatty acids are shown in the clear regions and straight-chain fatty acids in the shaded regions.


A
B
C
D
wt %/o FATTY ACID
0
10
20
30
40
50
CONTROL
%/o
s.c.f.a. = 15·6
b.c.f.a. = 84·0
u.f.a. = 0·17
unknown = 0·18
METHANOL
%/o
s.c.f.a. = 3·4
b.c.f.a. = 96·6
u.f.a. = 0
unknown = 0
ETHANOL
%/o
s.c.f.a. = 1·2
b.c.f.a. = 98·8
u.f.a. = 0
unknown = 0
PENTANOL
%/o
s.c.f.a. = 17·6
b.c.f.a. = 81·6
u.f.a. = 0·08
unknown = 0·2
FATTY ACID
n 12:0
i 14:0
n 14:0
bc 15:0
n 15:0
i 16:0
n 16:0
bc 17:0
n 17:0
n 18:1
n 18:0

fatty acids however no discernable trend relating these to the fatty acid levels in the membrane was observed, (results not shown). Only the unknown fatty acid peak of R_f = 3.6 on the DEGS column which was previously described (isoω-hydroxy $C_{15:0}$?), was influenced by growth in the various alcohols and in this instance its level in the supernatant was inversely proportional to the bcfa:scfa* ratio present in the cells. It is of interest to note that upon fractionation of the supernatant lipids into phospholipid and acetone-soluble lipid, all fatty acids were present in the acetone-soluble fraction however only $nC_{16:0}$, and to a lesser extent $nC_{12:0}$, were associated with the phospholipid fraction.

C. DISCUSSION

The composition of the cytoplasmic membrane of the genus Bacillus has been extensively studied. For B. subtilis it has been shown that the cytoplasmic membrane is composed primarily of protein (62%), RNA (22%) and lipid (16%), and contains essentially all of the lipid material of the cell (Bishop et al., 1967). The lipid/protein ratio may be altered if the medium contains glucose, as has been shown for B. subtilis and B. cereus (Kusaka, 1974). Cell membranes of B. amyloliquefaciens, prepared by osmotic lysis were comparable to the cell membrane of B. subtilis (Bishop et al., 1967) in regard to the lipid/protein ratio and the absence of additional lipid material extractable after acid hydrolysis of membranes. In comparison to osmotically-prepared membranes, the preparation of B. amyloliquefaciens membranes by the French press, resulted in a loss of protein. This loss of protein may correlate with the

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* bcfa (branched-chain fatty acid); scfa (straight-chain fatty acid).

absence of stalked particles (ATPases?), and the relatively lower rates of oxidative activity associated with this type of membrane vesicle.

Wide variations in the amount of RNA associated with bacterial membrane preparations have been reported and these variations have been attributed to such parameters as, (a) age of the culture (b) method of cell breakage (c) Mg^{2+} concentration and (d) bacterial species (De Ley, 1964). Reported values of RNA associated with membrane preparations of various B. megaterium strains range from 1 to 25% (Yudkin and Davis, 1965; Mizushima et al., 1966) to over 50% (of the total cellular RNA for B. megaterium (KM) (Schlessinger, 1963; Schlessinger et al., 1965). The average RNA content of osmotically-prepared B. subtilis (168) membranes after extensive washing was 20% (Bishop et al., 1967). The finding that membrane vesicles of B. amyloliquefaciens exhibited very low levels of associated RNA together with the absence of ribosomes in these preparations as judged by morphological methods, implies that previously determined levels of RNA in various bacterial membrane preparations, may be influenced by ribosome entrapment within the membrane vesicles, which was clearly seen in Chapter 3. The successive washing of membranes leading to a constant level of RNA, protein and lipid cannot be taken as unequivocal evidence for membrane-bound ribosomes. Indeed for E. coli, although membrane-associated ribosomes are observed after lysozyme digestion, no significant numbers of ribosomes are observed in membranes prepared by sonication and this has been taken as evidence for ribosome entrapment and adventitious binding of ribosomes to the membrane, possibly mediated by lysozyme (Patterson et al., 1970; Machtiger and Fox, 1973). The small amount of RNA associated with B. amyloliquefaciens membrane preparations may represent either

entrapped ribosomes, membrane-bound ribosomes (although none were seen) or some other RNA species (m-RNA?) associated with the cytoplasmic membrane.

No detailed analysis was undertaken on the composition of the neutral and glycolipid classes of B. amyloliquefaciens. However for B. subtilis it has been shown that the neutral lipid class contains diglyceride together with small amounts of free fatty acid and the glycolipid class contains diglucosyl diglyceride (Bishop et al., 1967). Diglucosyl diglycerides have also been identified in the glycolipids of B. cereus (Lang and Lundgren, 1970) and B. licheniformis (Button and Hemmings, 1976). Analysis of the phospholipid class by thin layer chromatography has previously shown that cardiolipin (diphosphatidyl glycerol, CLP), phosphatidyl ethanolamine (PE) and phosphatidyl glycerol (PG) are the major species of phospholipid in B. amyloliquefaciens (Glenn and Gould, 1973). Results obtained in this laboratory by Mr. J. Paton have shown that PE is present at about 20%, PG at about 11% and CLP at about 60% of the total phospholipids. Minor amounts of lysophosphatidyl ethanolamine (7%) and a trace amount of lysophosphatidyl glycerol were also detected. Thus the major phospholipid species of B. amyloliquefaciens are qualitatively and to some extent quantitatively the same as that present in B. subtilis cytoplasmic membranes (Bishop et al., 1967) and B. stearothermophilus membranes (Oo and Lee, 1972).

The fatty acid composition of B. amyloliquefaciens cells, cellular phospholipids and membrane phospholipids were similar in type and amount of fatty acids present. In general for Gram-positive vegetative bacteria, 90 to 95% of the total fatty acids are accounted for by the cytoplasmic membrane (Bishop et al., 1967; Daniels, 1969). The major fatty acids of B. amyloliquefaciens (both LF^- and WT) are the branched-chain fatty

acids of $C_{15:0}$, $C_{16:0}$ and $C_{17:0}$ which account for over 80% of the total. The bulk of the remainder are made up from straight-chain saturated fatty acids (predominantly $C_{16:0}$), there being only a trace (<0.4%) of unsaturated fatty acids ($C_{18:1}$) in cells grown at 30°. The phospholipids of Bacillus species have been shown to contain a variety of branched-chain fatty acids comprising between 80 to 95% of the total fatty acids (Bishop et al., 1967; Kaneda, 1968a, 1972a,b; Weekamp and Heinen 1972a,b). Branched-chain fatty acids have also been shown to be the major type of fatty acid in other Gram-positive bacteria including Micrococcus lysodeikticus and Staphylococcus aureus (Macfarlane, 1961; Kates, 1964; Kaneda, 1967). The ratios of the anteiso/iso $C_{15:0}$ and $C_{17:0}$ fatty acids are variable and depend on the particular species. The ratios obtained for B. amyloliquefaciens are similar to those obtained for B. subtilis (Kaneda, 1963; Bishop et al., 1967), but differ from those of B. subtilis as reported by Saito (1960; 66), B. pumilis, B. licheniformis and B. stearothermophilus (Shen, et al., 1970) and B. cereus (Kaneda, 1968b) where the iso $C_{15:0}$ and $C_{17:0}$ fatty acids are the predominant branched-chain odd-numbered fatty acids. Equal amounts of anteiso and iso $C_{15:0}$ fatty acids were detected in B. thuringiensis (Nickerson et al., 1975).

The synthesis of branched-chain fatty acids has been studied in a variety of bacteria including B. subtilis (Kaneda, 1963), M. lysodeikticus (Lennarz, 1961), Ruminococci sp. (Alison et al., 1962) and Bacteriodes succinogens (Wegner and Foster, 1963). The precursors of the anteiso acids containing 15 and 17 carbons have been shown to be 2-methylbutyrate or iso-leucine, whilst chain elongation of iso-valerate or leucine gives rise to odd-numbered iso acids and iso-butyrate or valine serves as the precursor of the iso acids containing 14 and 16 carbon atoms.

In B. subtilis (Kaneda, 1967) and in the extreme thermophiles B. caldolyticus and B. caldotenax (Weerkamp and Heinan, 1972b; Lauwers and Heinan, 1973), changes in the availability of the respective precursors has been shown to influence the relative abundance of the branched-chain fatty acids.

Although trace levels of unsaturated fatty acids were detected in both LF^- and WT strains of B. amyloliquefaciens grown at 30°, no unsaturated fatty acids were detected in B. subtilis (168), in confirmation of Bishop et al. (1967). Small amounts of unsaturated fatty acids have been detected in B. pumilis, B. licheniformis and B. stearothermophilus (Shen et al., 1970), while in B. cereus grown at 21°, the level of monounsaturated fatty acids was as high as 27% of the total fatty acids (Kaneda, 1972b). Polyunsaturated fatty acids have not been detected among the Bacillus species (Kaneda 1967,68a).

Dramatic changes in the fatty acid composition of B. amyloliquefaciens were observed when cells were grown at different temperatures or in the presence of various alcohols. In the absence of sterols in these membranes, an increase in the lipid fluidity of the membrane could be brought about by one or more of the following changes: (1) An increase in branched-chain fatty acids at the expense of straight-chain saturated fatty acids, (2) an increase in the proportion of anteiso to iso branched-chain fatty acids, the anteiso fatty acids having the lower melting point in comparison to the iso homologue (Sobar, 1968), (3) a decrease in the carbon chain length, (4) an increase in the level of unsaturated fatty acids and (5) an increase in the lipid to protein ratio. The polar head groups may also influence fluidity (Träuble and Eibl, 1975).

A decrease in the growth temperature of B. amyloliquefaciens leads initially to an increase in the level of

branched-chain fatty acids at the expense of the straight-chain saturated fatty acids. It was not determined whether the proportion of anteiso to iso branched-chain fatty acids increased on lowering the growth temperature, however such a change has been demonstrated for both mesophilic and thermophilic species of Bacillus (Shen et al., 1970; Chan et al., 1971). Further lowering of the growth temperature to 20°, resulted in the appearance in the membrane of the branched-chain mono-unsaturated fatty acids, anteiso $C_{17:1}$, iso $C_{17:1}$ and iso $C_{16:1}$. Although B. amyloliquefaciens can grow at 30° when 99% of its fatty acid complement is of the branched-chain type (cells grown in 2% (v/v) ethanol), at 20° the addition of cis-unsaturated fatty acids to the membrane may be essential for the requisite fluidity; branched-chain fatty acids may not provide for a sufficient fluidity increase. In B. cereus growth at 21° has been shown to result in an increase in (mono) unsaturated fatty acids rather than by changes in the level of branched-chain fatty acids or in the anteiso/iso ratio (Kaneda, 1972). With the exception of iso $C_{16:1}$, the unsaturated fatty acids produced in B. amyloliquefaciens (normal $C_{16:1}$; iso $C_{17:1}$ and anteiso $C_{17:1}$), were identical to those produced by B. cereus under similar conditions. In B. cereus the branched-chain unsaturated fatty acids have been shown to occur as the Δ^5 and Δ^{10} isomers and these have a marked preference for the 1 position of the phospholipids (Kaneda, 1972). The desaturating enzymes of Bacillus sp. are of two types, one a Δ^5-desaturase is temperature sensitive, whilst the other a Δ^9-desaturase is relatively temperature insensitive (Fulco, 1969). In B. cereus, lowered growth temperature increases the proportion of Δ^{10}-unsaturated fatty acids (relative to the total fatty acids) and

also induces the synthesis of Δ^5-isomers which do not occur at higher temperature (Kaneda, 1972). Although the double bond position for the branched-chain mono unsaturated-fatty acids of B. amyloliquefaciens were not determined, the production of similar types of unsaturated fatty acids suggests such a mechanism is most likely operating in this organism. Changes in the fatty acid composition consistent with a change in membrane fluidity were also evident when cells were grown in various alcohols, but in this instance changes were observed only in the relative proportions of the branched-chain and straight-chain fatty acids rather than in the level of unsaturation. (For a complete discussion on the proposed mechanism by which alcohols ellicit their effects by directly influencing membrane fluidity, the reader is referred to the article by Ingram, 1976). Thus in B. amyloliquefaciens it would appear that the preservation of appropriate membrane fluidity may be attained by a variety of means, the choice of which depends on the growth conditions.

The alterations in the fatty acid composition which were observed in response to the changes described above, would be expected to alter the boundary temperatures of the lipid phase transition occurring in these membranes and thus may affect the temperature limits for growth as well as a number of membrane-associated physiological processes. Such changes have previously been shown for a variety of organisms (Esser and Souza, 1974; McElhaney, 1974; Cronan and Gelmann, 1975; Fox, 1975; Finne and Matches, 1976). An examination of some of the effects of altered fatty acid composition and presumably therefore of membrane fluidity, on the physiology of B. amyloliquefaciens is dealt with in the following chapter.

In B. amyloliquefaciens there appears to be a selective secretion of fatty acids into the medium since the

extracellular lipids do not correspond with those expected from lysis, however there is no direct evidence associating the secretion of fatty acids with the process of extracellular enzyme secretion. It is of interest to note that the fatty acid component of the phospholipopeptide of B. licheniformis membrane penicillinase consists of $^{n}C_{16:0}$; $^{n}C_{18:0}$ and $^{n}C_{18:1}$ (Yamamoto and Lampen, 1975), and these fatty acids are similar to those secreted by B. amyloliquefaciens. The possibility still exists that lipid covalently associated with the extracellular enzymes (and therefore not directly extractable by the procedures used), may be involved in the secretion process, as is the case for B. licheniformis penicillinase (Yamamoto and Lampen, 1975). However after the addition of radioactively labelled glycerol and acetate to cells, the purified extracellular enzymes did not possess associated radioactivity. Thus the question of a direct involvement of lipid in the process of secretion in this organism remains open.

The unknown polar fatty acid (R_f = 3.6 on DEGS, relative to methyl palmitate) appearing in both the cell and the culture supernatants, may have been the iso $C_{15:0}$ β-hydroxy fatty acid of the lytic factor described previously. However as all attempts to isolate the fatty acid moiety from purified lytic factor by established procedures were unsuccessful and no standard could be prepared for identification purposes, no further work on this aspect was done.

The possibility that the phospholipids of B. amyloliquefaciens are arranged asymmetrically in the cell membrane and give rise to two independent phase transitions, was discussed in the preceeding chapter. Although the phospholipid species associated with each half of the membrane

have yet to be determined, it may be expected that the acyl fatty acids of the outer half of the cell membrane, would be the more fluid, as the boundary temperatures for the phase transition in this half of the membrane were considered to be the lower.

CHAPTER 6

INFLUENCE OF CHANGES IN THE LIPID COMPONENTS OF THE CELL MEMBRANE OF *B. AMYLOLIQUEFACIENS* ON EXTRACELLULAR ENZYME SECRETION AND COLD SHOCK

A. INTRODUCTION

The mechanism of extracellular enzyme secretion in bacteria is far from clear. As already mentioned May and Elliott (1968), have proposed a translational-extrusion mechanism for secretion in B. amyloliquefaciens in which nascent peptides, synthesized on membrane-associated ribosomes, are vectorially extruded through the cell membrane. Other models for extracellular enzyme secretion in bacteria such as that proposed for penicillinase secretion in B. licheniformis indicate the involvement of a phospholipopeptide form of the exoenzyme which becomes membrane associated during secretion (Sargent and Lampen, 1970; Sawai et al., 1973; Dancer and Lampen, 1975). In addition for the secretion of α-amylase in B. amyloliquefaciens, it has been proposed that this exoenzyme becomes associated with the cell membrane (Fernández-Rivera Río and Arroyo-Begovich, 1975).

Irrespective of the mechanism for extracellular enzyme secretion in bacteria, the possibility exists that changes in the physical state of the membrane lipids may influence secretion. As it was found possible to alter the physical state and the fatty acid composition of the cell membrane of B. amyloliquefaciens (Chapters 4 and 5), it seemed worthwhile to investigate whether such changes affect extracellular enzyme secretion. This chapter is concerned with such studies.

There was also an additional purpose for these studies. Smeaton and Elliott (1967a) have shown that B. amyloliquefaciens cells undergo "cold shock" when rapidly cooled over a narrow critical range of temperature (16° to 14°). Cells subjected to cold shock become permeable and cellular protein synthesis ceases. Protoplasts also undergo instantaneous lysis as a result of such treatment. One of the aims of this work was to investigate

whether the cold shock phenomenon could be modulated in any way. It was anticipated that if the membrane lipids were involved in cold shock, the ability to alter the membrane fatty acid composition might influence both the magnitude of the cold shock and the temperature at which it occurs. This may also aid in elucidating the mechanism of cold shock, however there was also a practical application for these studies. For cell-free work in bacteria involving the isolation of polysomes etc., it is essential that cells be rapidly cooled to temperatures near 0^{o}. This in B. amyloliquefaciens leads to the cessation of protein synthesis and for protoplasts results in their instantaneous lysis. Therefore it clearly would be an advantage for proposed cell-free work to eliminate if at all possible, the cold shock phenomenon.

B. RESULTS

1. Effect of temperature on extracellular enzyme secretion in cells

As previously described in Chapter 4, a combination of physical and enzymic techniques has shown that the cell membrane of B. amyloliquefaciens apparently undergoes four changes at temperatures of about 38^{o}, 21^{o}, 15^{o} and 7^{o}, which have been interpreted as arising from changes in the molecular ordering of the membrane lipids (Chapter 4). To investigate whether such changes in the cell membrane influence the secretion of extracellular enzymes in B. amyloliquefaciens, rates of enzyme secretion were determined at different temperatures and graphed as Arrhenius plots. Changes in the slope of Arrhenius plots for other membrane-associated processes, have previously been taken as evidence for the occurrence of some temperature-induced

change in the membrane directly influencing the process under study (Kumamoto, et al., 1971; Raison 1973a,b; McMurchie et al., 1973).

The rates of production of protease and ribonuclease in washed-cell suspensions are sufficiently linear to permit the determination of initial rates of secretion over a 15 minute period. However, for ∝-amylase, a lag in secretion which increased at lower temperatures prevented this. Data is presented only for the rates of secretion of protease and ribonuclease in the 17^{o} to 30^{o} temperature range. Below 17^{o} rates of exoenzyme secretion were extremely low while above 40^{o} enzyme denaturation occurred.

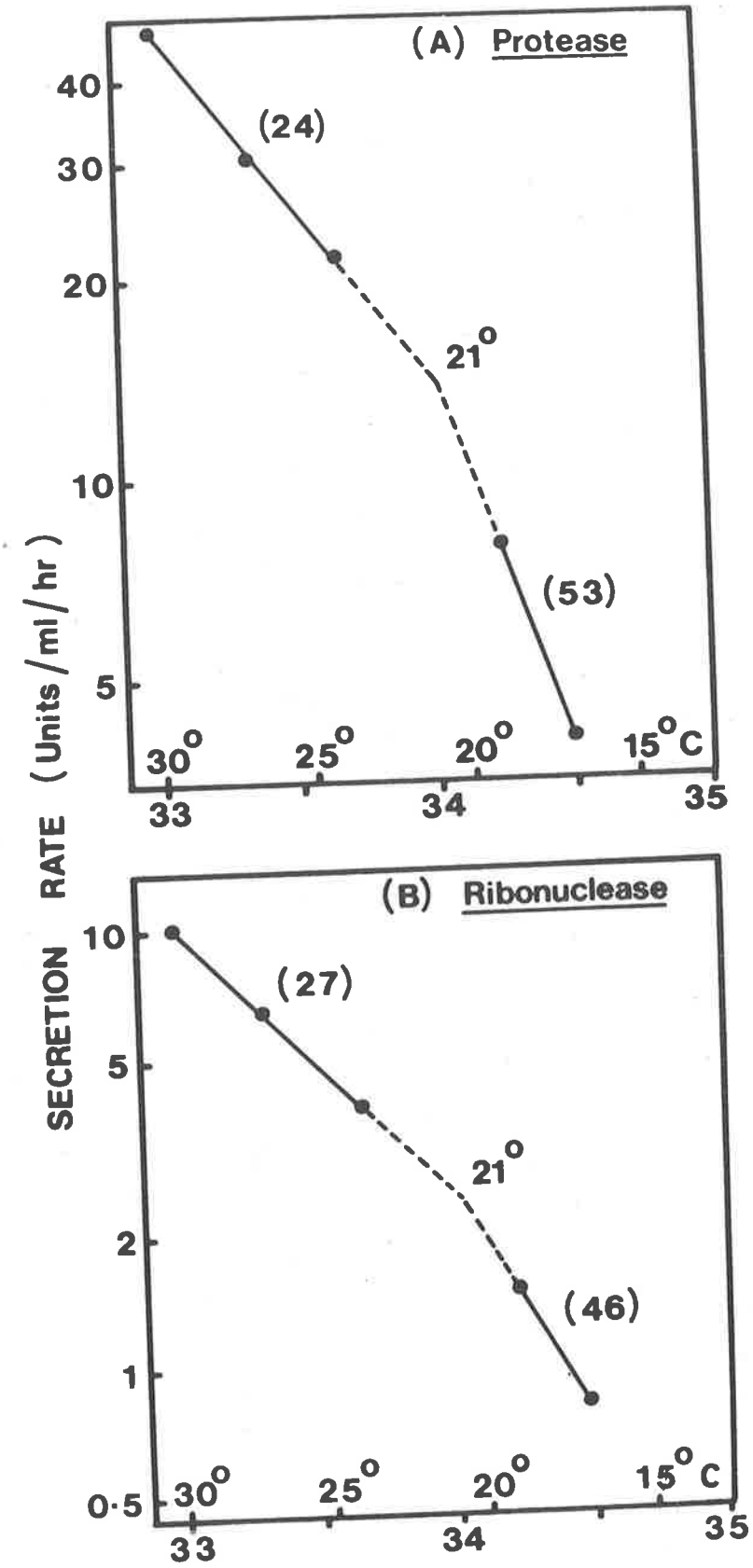
Figure 6.1 shows Arrhenius plots of the initial rates of both protease (A) and ribonuclease (B) secretion in washed-cell suspensions of late-log phase B. amyloliquefaciens cells grown at 30^{o}. A change in slope is evident in both plots, and after extrapolation the intersecting temperature was 21^{o}. This temperature is coincident with one of the four previously determined phase transition temperatures. (In terms of the tentative hypothesis offered in Chapter 4, this would correspond to the termination of the lipid phase transition in the outer half of the cell membrane). The temperature range within which secretion was studied covered only this one characteristic transition temperature. The Arrhenius activation energy for secretion of each extracellular enzyme increased at temperatures below 21^{o}, and the values for the two exoenzymes were similar above (24 to 27 kcal/mole) and below (53 to 46 kcal/mole) the transition point. The Arrhenius activation energy for the incorporation of ^{14}C-leucine into T.C.A.-precipitable material measured in whole cells over the temperature range 35^{o} to 25^{o}, was linear and of value 14 kcal/mole (data now shown).

FIGURE 6.1. ARRHENIUS PLOTS OF THE RATES OF SECRETION OF PROTEASE AND RIBONUCLEASE IN B. AMYLOLIQUEFACIENS CELLS

Cells were harvested from late-log phase culture, grown at 30°, and after washing were finally resuspended in WCSM. The initial rates of secretion of the extracellular enzymes over a 15 minute period were measured at the temperatures indicated. Figures in brackets are the Arrhenius activation energy in kcal/mole.

A. Protease - Units/ml/Hr

B. Ribonuclease - " " "


(A) Protease
40
30
20
10
5
(24)
21°
(53)
30°
25°
20°
15°C
33
34
35
SECRETION RATE (Units/ml/hr)
(B) Ribonuclease
10
5
2
1
0·5
(27)
21°
(46)
30°
25°
20°
15°C
33
34
35

2. Effect of altered growth temperature on extracellular enzyme secretion and the protein-synthesizing ability of whole cells

The observation in Chapter 5 that the membrane lipid composition could be altered by growth at different temperatures, prompted an investigation as to whether these changes influence the secretion of extracellular enzymes by B. amyloliquefaciens. Although no previous investigation of this aspect has been undertaken in this organism, for B. caldolyticus it was reported that alteration of the membrane fatty acid composition by changes in growth temperature (Weerkamp and Heinen, 1972a) or composition of the growth medium (Weerkamp and Heinen, 1972b; Grootegoed et al., 1973; Lauwers and Heinen, 1973), altered extracellular enzyme activity. However, in these studies no clear discrimination was made as to whether the altered levels of extracellular enzymes were due to the changed permeability properties of the cytoplasmic membrane or to indirect causes such as alteration in the metabolism of such cells. Using E. coli fatty acid auxotrophs supplemented with various fatty acids, it has been shown that the induction of alkaline phosphatase is sensitive to changes in the fatty acid composition of the membrane (Kimura and Izui, 1976).

Changes in the fatty acid composition of B. amyloliquefaciens as a result of altered growth temperature were examined in Chapter 5 and the results are summarized in Figure 6.2 for cells grown at 20^{o}, 25^{o}, 30^{o} and 37^{o}; (generation times for these cells are noted in Plate 6.1). A decrease in the growth temperature results in changes consistent with an increase in membrane fluidity. The ratio of straight-chain to branched-chain fatty acids decrease while a dramatic increase in the level of

FIGURE 6.2. EFFECT OF GROWTH TEMPERATURE ON THE LEVEL OF BRANCHED-CHAIN, STRAIGHT-CHAIN AND UNSATURATED FATTY ACIDS IN B. AMYLOLIQUEFACIENS

Summary of the results obtained for the type and amount of fatty acids present in cells of B. amyloliquefaciens grown at different temperatures. The unsaturated fatty acid in cells grown at 37^{o} was $C_{18:1}$, however this was not produced at lower growth temperatures. The elevated unsaturated fatty acid level at low temperatures was due to the production of $iC_{16:1}$ and bc (anteiso and iso) $C_{17:1}$.

(•) BCFA, (branched-chain fatty acid)

(■) SCFA, (straight-chain fatty acid)

(o) UFA, (unsaturated fatty acid)

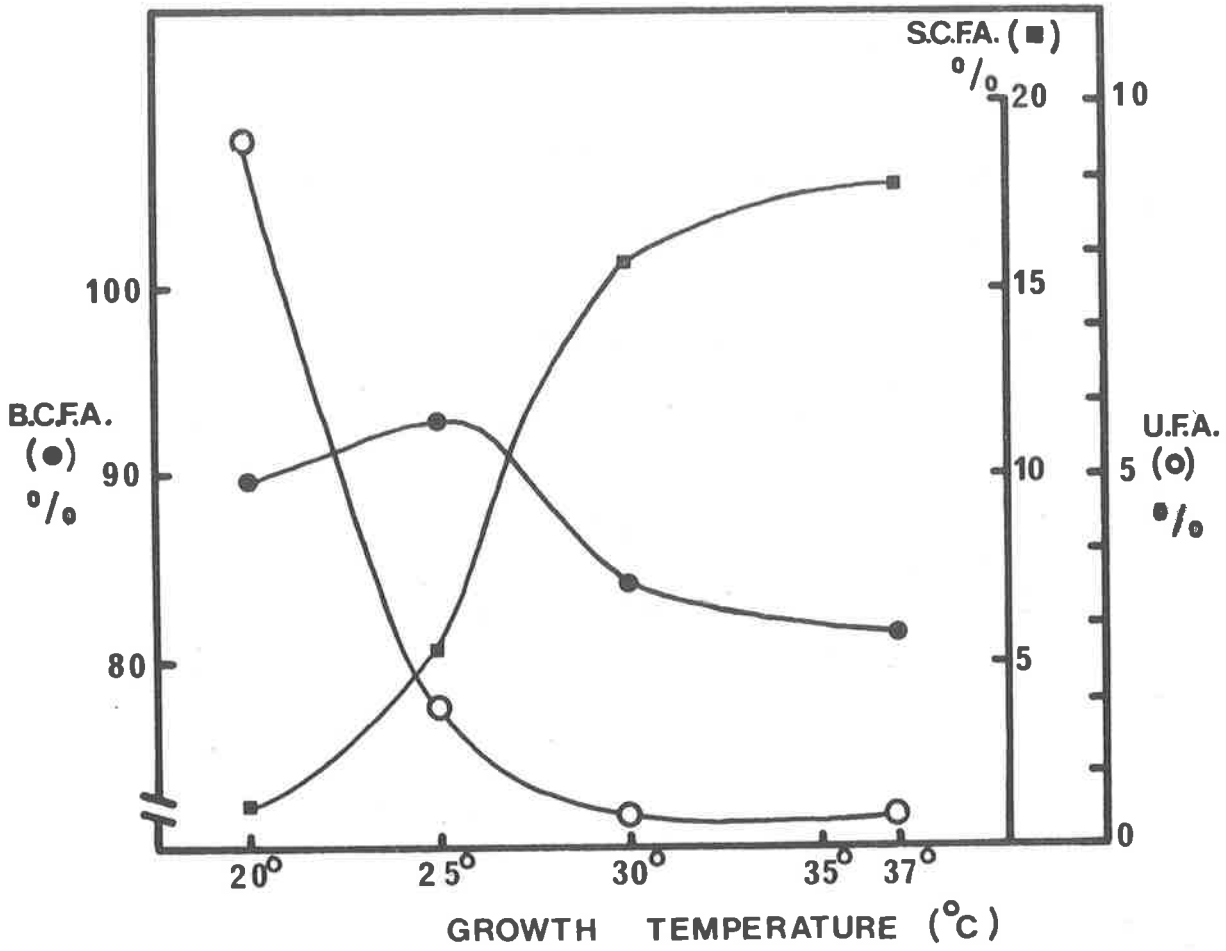
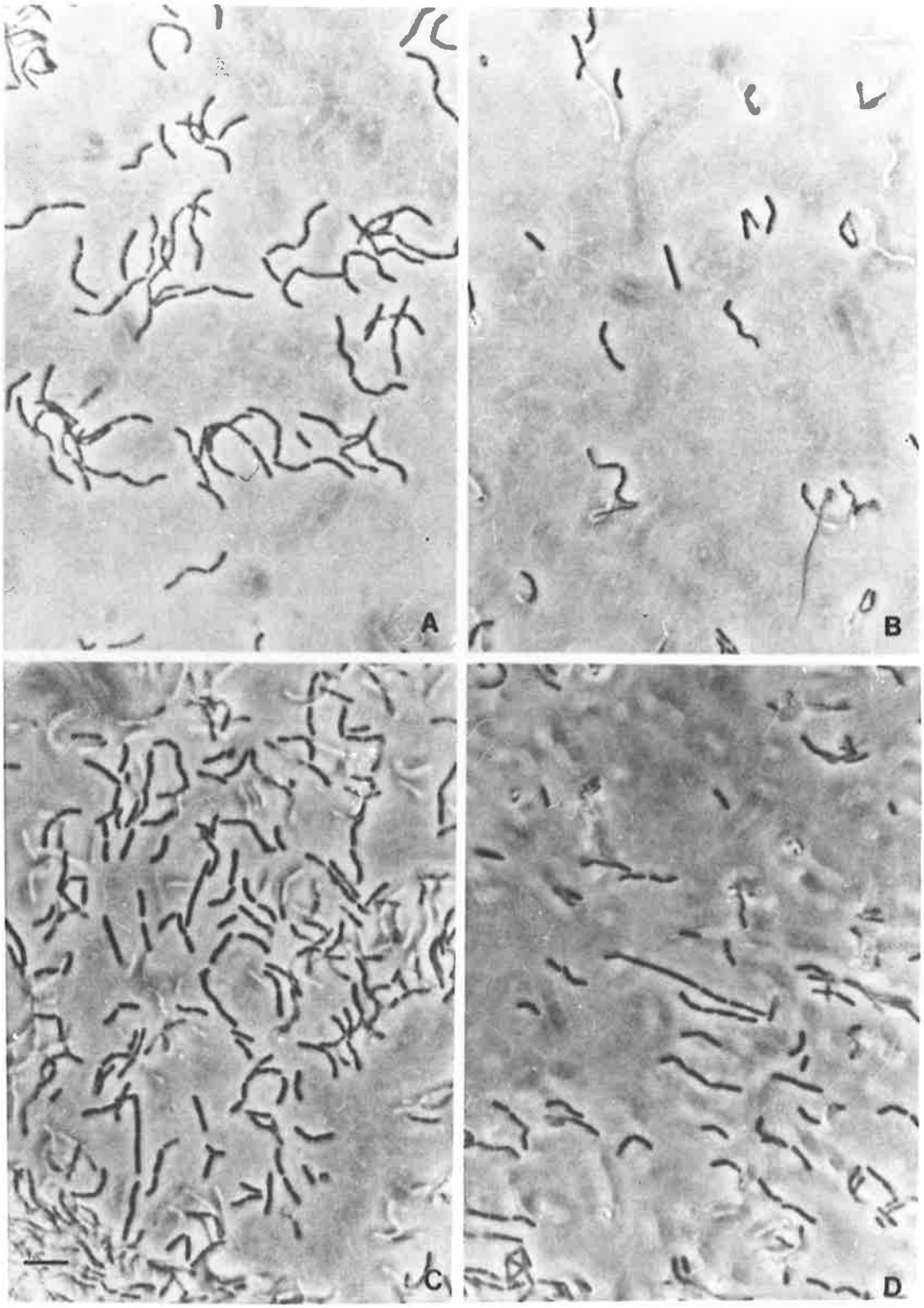

S.C.F.A. (■)
%
20
10
100
15
B.C.F.A.
(●)
%
90
10
U.F.A.
5 (○)
●/o
80
5
0
20°
25°
30°
35°
37°
GROWTH TEMPERATURE (°C)

PLATE 6.1. PHASE-CONTRAST PHOTOMICROGRAPHS OF *B. AMYLOLIQUEFACIENS* CELLS GROWN AT DIFFERENT TEMPERATURES.

Cells were grown from spores at the temperatures indicated, and were harvested when growth reached late-log phase. In this series of photomicrographs cells were at different dilutions. Bar marker = 5.0μ

	Time to reach late-log phase (Hr.)	Generation Time (Hr.)
A. Cells grown at 30°	12.5	2
B. " " " 37°	18	2
C. " " " 25°	34	4.5
D. " " " 20°	65-78	7.5


A
B
C
D

unsaturated fatty acids (principally branched-chain mono-unsaturated fatty acids) occurs. Attempts to further alter the fatty acid composition by growth at temperatures lower than 20^{o} failed because although growth occurred at temperatures as low as 15^{o}, a considerable degree of cell lysis took place. For such cells the fatty acid composition was virtually identical to that of cells grown at 20^{o}.

Some peripheral observations were made on cells grown at different temperatures. There was a reduction in the number of cells per chain when grown at 37^{o} or 20^{o}, relative to the number in chains at 30^{o} and 25^{o} (Plate 6.1). At 20^{o}, some elongated cells were observed (Plate 6.2D) but in all instances these were empty cell walls. Cell mobility increased with lower growth temperature down to 20^{o}. In addition, on lowering the growth temperature, cultures became progressively redder due to the accumulation of material in the culture supernatant which was not soluble in chloroform/methanol.

To examine the influence of altered lipid composition on the secretion of extracellular enzymes and on the protein-synthetic capacity of cells, the following experiment was performed. Cultures were inoculated at different times and grown at 37^{o}, 30^{o} and 25^{o} such that all reached the late-log phase of growth at approximately the same time; (cells grown at 20^{o} were not used in this experiment due to the variation in time taken to reach the required cell density). Dry weights of cells grown at 37^{o}, 30^{o} and 25^{o} were identical. After harvest of the cells grown at 25^{o}, 30^{o} and 37^{o}, the rates of secretion of protease and amylase and the rate of ^{14}C-leucine incorporation into T.C.A.-precipitable material were determined for each cell sample at temperatures of 25^{o}, 30^{o} and 37^{o} (incubation temperatures), using washed-cell suspensions. For protease synthesis and ^{14}C-amino

acid incorporation, the initial rates were taken, whilst for amylase, the rate taken was that obtained after a linear rate of secretion was established. The results of this experiment are shown in Figure 6.3.

For protease synthesis, the rate of secretion per unit mass of cells (at any incubation temperature) increases as the growth temperature decreases. The same is true for amylase synthesis (with the exception that 25^{o} and 30^{o} grown cells are similar) and for ^{14}C-amino acid incorporation. This gives rise to the interesting situation in which the rate of protease secretion in cells grown at 25^{o} and assayed at 25^{o} is greater than if cells grown at 37^{o} are assayed at 37^{o}. In many instances the rate of exoenzyme secretion and ^{14}C-amino acid incorporation in cells grown at 25^{o} was as much as 3-times that of cells grown at 37^{o}, at similar incubation temperatures.

3. Influence of growth temperature on the phenomenon of cold shock

The rapid cooling of some micro-organisms results in a phenomenon termed cold shock characterized by a breakdown in membrane permeability and a decrease in cell viability (Gorrill and McNeil, 1960; Strange and Ness, 1963; Ring, 1965; Farrell and Rose, 1967; Sato and Takahashi, 1968,69). The rapid cooling of *B. amyloliquefaciens* late-log phase cells through 15^{o}, results in a release of U.V.-absorbing material, an increase in the permeability to both the fluorescent dye *n*-tolyl-α-napthylamine-8-sulphonic acid and exogenously added *B. amyloliquefaciens* ribonuclease, and the release of an intracellular ribonuclease inhibitor (Smeaton and Elliott, 1967a). U.V.-absorbing material is also released from *B. subtilis* cells subjected to sudden chilling (Henneberry and Freese, 1973). Although the final

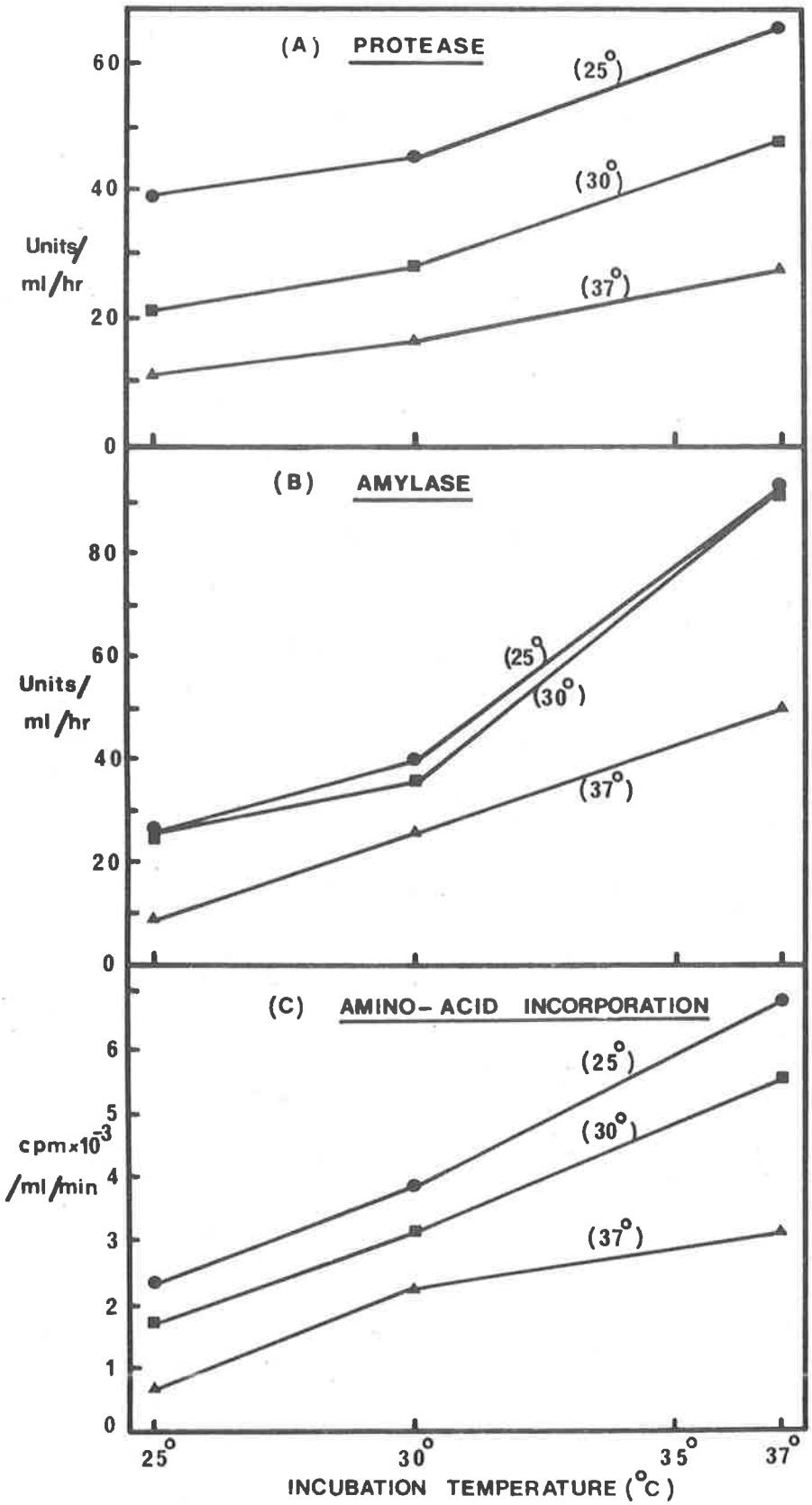
FIGURE 6.3. RATES OF PRODUCTION OF PROTEASE AND AMYLASE AND THE RATES OF GENERAL PROTEIN SYNTHESIS AT DIFFERENT TEMPERATURES FOR B. AMYLOLIQUEFACIENS CELLS GROWN AT DIFFERENT TEMPERATURES.

Cells were grown at 25^{o}, 30^{o} and 37^{o} and were harvested when growth reached late-log phase. Cell densities for cells grown at different temperatures were the same throughout the experiment. The rates of extracellular enzyme secretion and ^{14}C-leucine incorporation for cells grown at different temperatures (indicated by the figures within brackets) were determined at the (incubation) temperatures indicated.

A. Protease

B. Amylase

C. ^{14}C-leucine incorporation


(A) PROTEASE
(25°)
(30°)
(37°)
Units/ ml/hr
0
20
40
60
(B) AMYLASE
(25°)
(30°)
(37°)
Units/ ml/hr
0
20
40
60
80
(C) AMINO-ACID INCORPORATION
(25°)
(30°)
(37°)
cpm×10³ /ml/min
0
1
2
3
4
5
6
25°
30°
35°
37°
INCUBATION TEMPERATURE (°C)

results of cold shock are similar to those observed as a result of chilling injury, cold shock is considered distinct from chilling in that rapid cooling is essential for the effects to be observed.

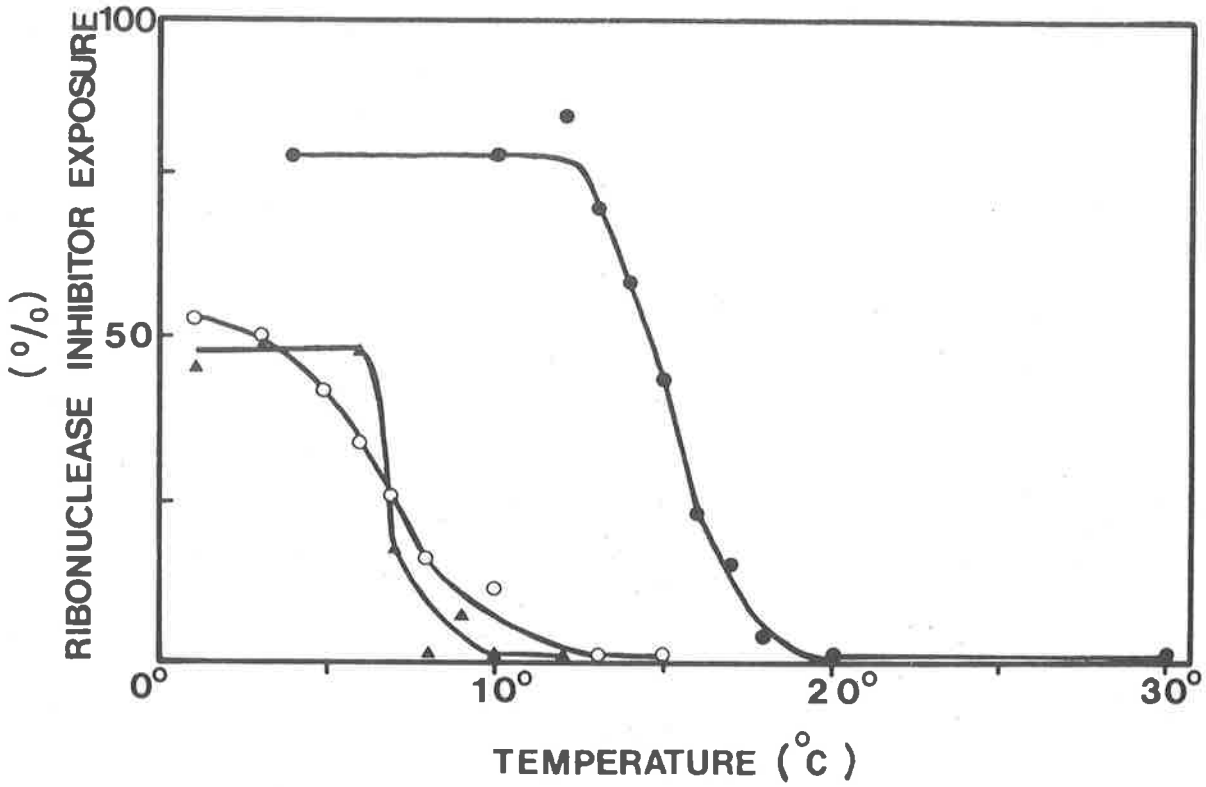
An explanation for the cold shock phenomenon has been offered by Haest et al., (1972) and Leder, (1972), suggesting that cold shock is due to the crystallization of membrane lipids. Chilling injury in plants is also believed to be the result of a temperature-induced phase transition in the membrane lipids (Raison, 1973a,b). It was of interest to determine whether the incidence of cold shock in B. amyloliquefaciens correlates which changes in the physical state of the cell membrane. Since the lipid composition (and hence the fluidity) of the membrane can be altered by changing the growth temperature (Chapter 5), it was anticipated that such changes might affect the cold shock phenomenon due to a shift in the membrane phase transition temperatures.

Several procedures for assaying cold shock can be employed, one of which was exposure of intracellular ribonuclease inhibitor to added B. amyloliquefaciens ribonuclease as described in Chapter 2. Procedures for cold shocking are also described in this chapter.

For cells rapidly chilled through the temperature range 13^{o} to 17^{o}, there was an 80% exposure of intracellular ribonuclease inhibitor (Figure 6.4). The midpoint of this range, (the temperature to which cells must be cooled to obtain 50% of maximum observed inhibitor exposure) was 15^{o}. Rapid (virtually instantaneous) cooling was essential for this phenomenon to occur; slow cooling (2^{o} per minute) from 30^{o} to 12^{o} gave no effect (Figure 6.4). Starting temperature had no effect as cells rapidly cooled from either 42^{o}, 30^{o} or 19^{o} all

FIGURE 6.4. EFFECT OF MODE OF COOLING ON THE EXPOSURE OF INTRACELLULAR RIBONUCLEASE INHIBITOR IN *B. AMYLOLIQUEFACIENS* CELLS.

Cells were either rapidly cooled from 30° to the indicated temperature (●); slowly cooled from 30° to the indicated temperature (o); or slowly cooled from 30° to 12° and then rapidly cooled to the indicated temperature (▲). The amount of inhibitor exposed is expressed as a percentage of the total level of intracellular inhibitor.


RIBONUCLEASE INHIBITOR EXPOSURE (%)
100
50
0°
10°
20°
30°
TEMPERATURE (°C)

showed the same levels of inhibitor exposed when chilled through the range 13° to 17°.

A second critical temperature zone for RNAase inhibitor exposure was observed when cells were slowly or rapidly cooled through a temperature range of 5° to 9° (Figure 6.4). This was evident when cells were slowly cooled from 30°, or rapidly cooled from 12° (after initial slow cooling from 30° to 12° to prevent inhibitor exposure). Depending on the treatment, the ranges of the critical temperature zones differed slightly, however both exhibited a midpoint at about 6.5°. Chilling through this temperature range, irrespective of the rate, resulted in the exposure of approximately 50% of total intracellular inhibitor. This limited exposure was not due to a slower rate of entry of RNAase into the cells as incubation for 240 minutes rather than 60 minutes during the exposure assay resulted in no more than 50% exposure.

An alternative method for the measurement of cold shock which involved assaying the protein-synthetic capacity of cold-shocked cells, also verified the results obtained using the above method, (results not shown). Rapid cooling of cells through 15° resulted in a total loss of their protein - synthetic capacity (measured by ^{14}C-amino acid incorporation), whilst either slow or rapid cooling through 6° resulted in only about a 35% decrease in the level of incorporation. In addition the rapid cooling of cells through 15° also resulted in the complete loss of cell viability, whilst slow or rapid cooling through 6° resulted in only an 18% loss in cell viability (results not shown).

The effect of cooling cells in the presence of Tween 80 was investigated using the ribonuclease inhibitor exposure assay for the measurement of cold shock. For cells rapidly cooled from 30° to various temperatures in buffer containing 0.01% or 1.0% Tween 80,

the mid-point for inhibitor exposure decreased from a temperature of 15^{o} for the control to 11.5^{o} and 8^{o}, respectively in the presence of Tween 80, (Figure 6.5 A). The levels of intracellular inhibitor exposed were similar in all cases (80% of the total intracellular inhibitor). The slow cooling of cells from 30^{o} to various temperatures in buffer containing Tween 80 results in the exposure of intracellular inhibitor being reduced to 19% and 10% (of the intracellular level) for buffer containing either 0.01% or 1.0% Tween 80 respectively, in comparison to the 50% exposure of inhibitor in control cells (Figure 6.5 B). The rapid cooling of cells from 12^{o} (after prior slow cooling from 30^{o}) in buffer containing the above concentrations of Tween 80, resulted in almost identical levels of inhibitor being exposed as was observed when using a slow cooling mode, (results not shown).

The possibility that the phenomenon of cold shock relates to the physical events occurring in the membrane lipid as a function of temperature, prompted an investigation of the cold shock phenomenon in cells exhibiting an altered fatty acid composition. The changes in fatty acid composition as a result of altered growth temperature are summarized in Figure 6.2. The changes observed on lowering the growth temperature are consistent with an increase in membrane fluidity and as such may result in a lowering of the membrane phase transition temperature as has been shown for both artificial lipid systems (Shimshick and McConnell, 1973; Chapman, 1975) and fatty acid auxotrophs (Overath et al., 1970,71; Wilson and Fox, 1971; Linden et al., 1973a,b; Overath and Träuble, 1973; Fox, 1975).

The results of this study are shown in Figure 6.6. The assay for cold shock in this instance was the secretion of protease as described in Chapter 2. For late-log phase cells

FIGURE 6.5. THE EFFECT OF TWEEN 80 ON THE COLD-SHOCK INDUCED EXPOSURE OF RIBONUCLEASE INHIBITOR IN B. AMYLOLIQUEFACIENS.

Cells were either rapidly cooled (A) or slowly cooled (B) from 30^{o} to the indicated temperature in Tris buffer without Tween 80 (●); Tris buffer + 0.1% (v/v) Tween 80 (■); or Tris buffer + 1.0% (v/v) Tween 80 (▲). The amount of RNAase inhibitor exposed is expressed as a percentage of the total increcellular level. Tween 80 was included in both washing and treatment buffers.

Buffer = 0.05M tris, pH8.0

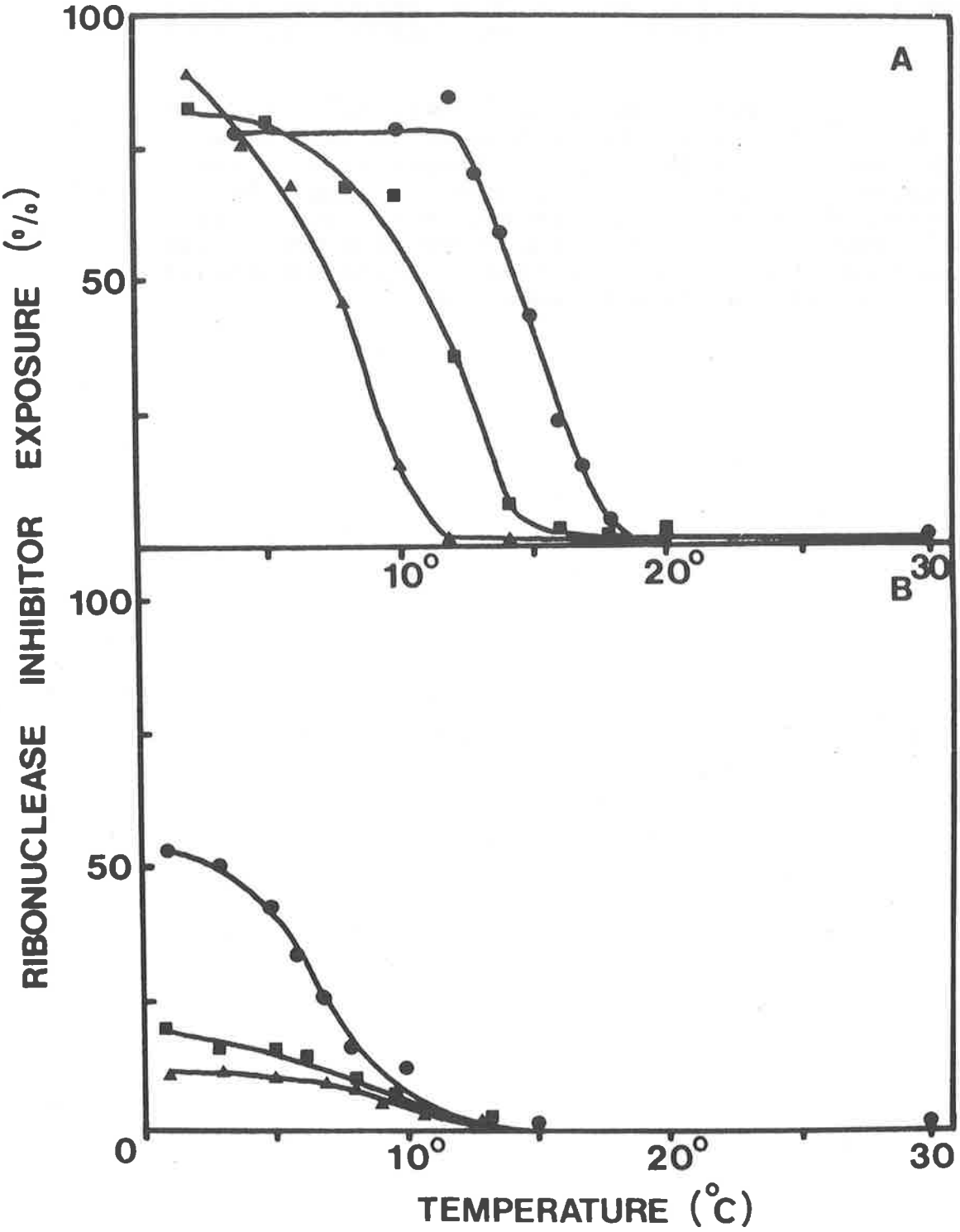
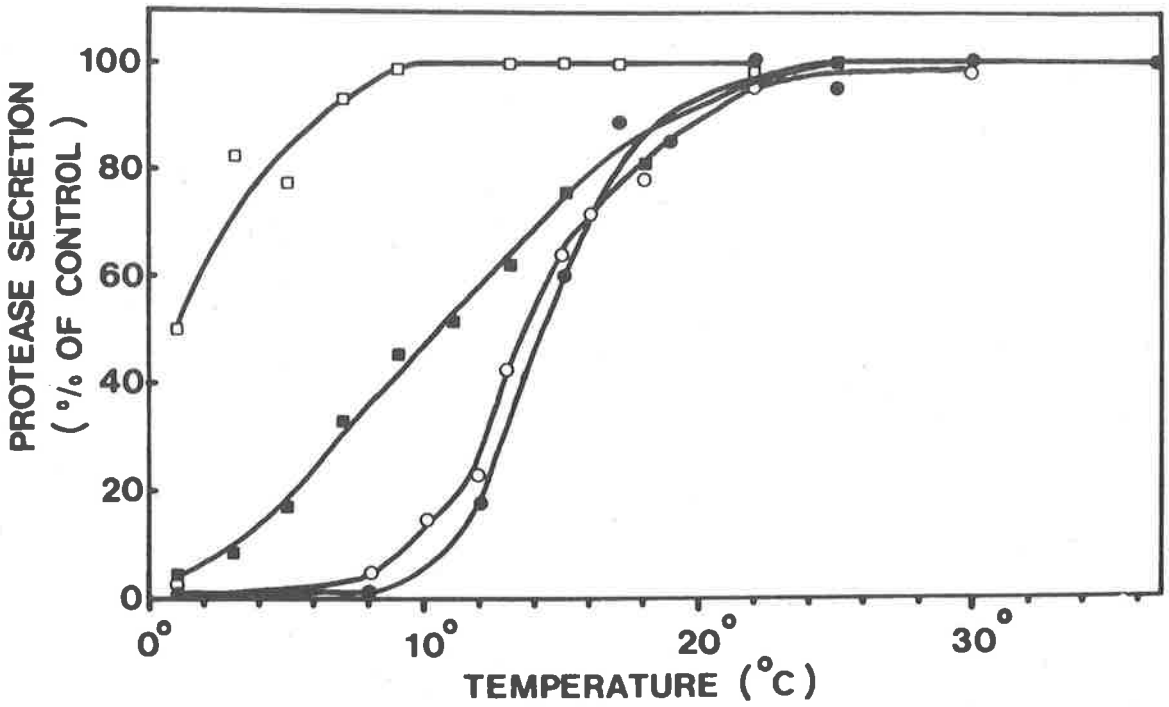

100
50
A
RIBONUCLEASE INHIBITOR EXPOSURE (%)
10°
20°
30
B
100
50
0
10°
20°
30
TEMPERATURE (°C)

FIGURE 6.6. EFFECT OF GROWTH OF B. AMYLOLIQUEFACIENS AT DIFFERENT TEMPERATURES ON COLD SHOCK.

Cells were grown at 37° (●), 30° (○), 25° (■) and 20° (□) and after resuspension in WCSM were rapidly cooled from their growth temperature to the temperatures indicated. After cold shocking, the secretion of protease was measured at the individual growth temperatures, and is expressed as a percentage of the rate of protease secretion in cells maintained at their particular growth temperature.


PROTEASE SECRETION
(% OF CONTROL)
100
80
60
40
20
0
0°
10°
20°
30°
TEMPERATURE (°C)

grown at 37°, 30°, 25° and 20° and cold shocked in WCSM, the mid-point temperatures for the cold shock, (i.e. the temperature corresponding to a 50% inhibition of the rate of protease secretion in comparison to the rate in cells maintained at their particular growth temperature), were 14.5°, 14°, 11° and 1°, respectively.

4. Effect of growth of cells in alcohols on extracellular enzyme secretion and protein-synthesizing ability of whole cells

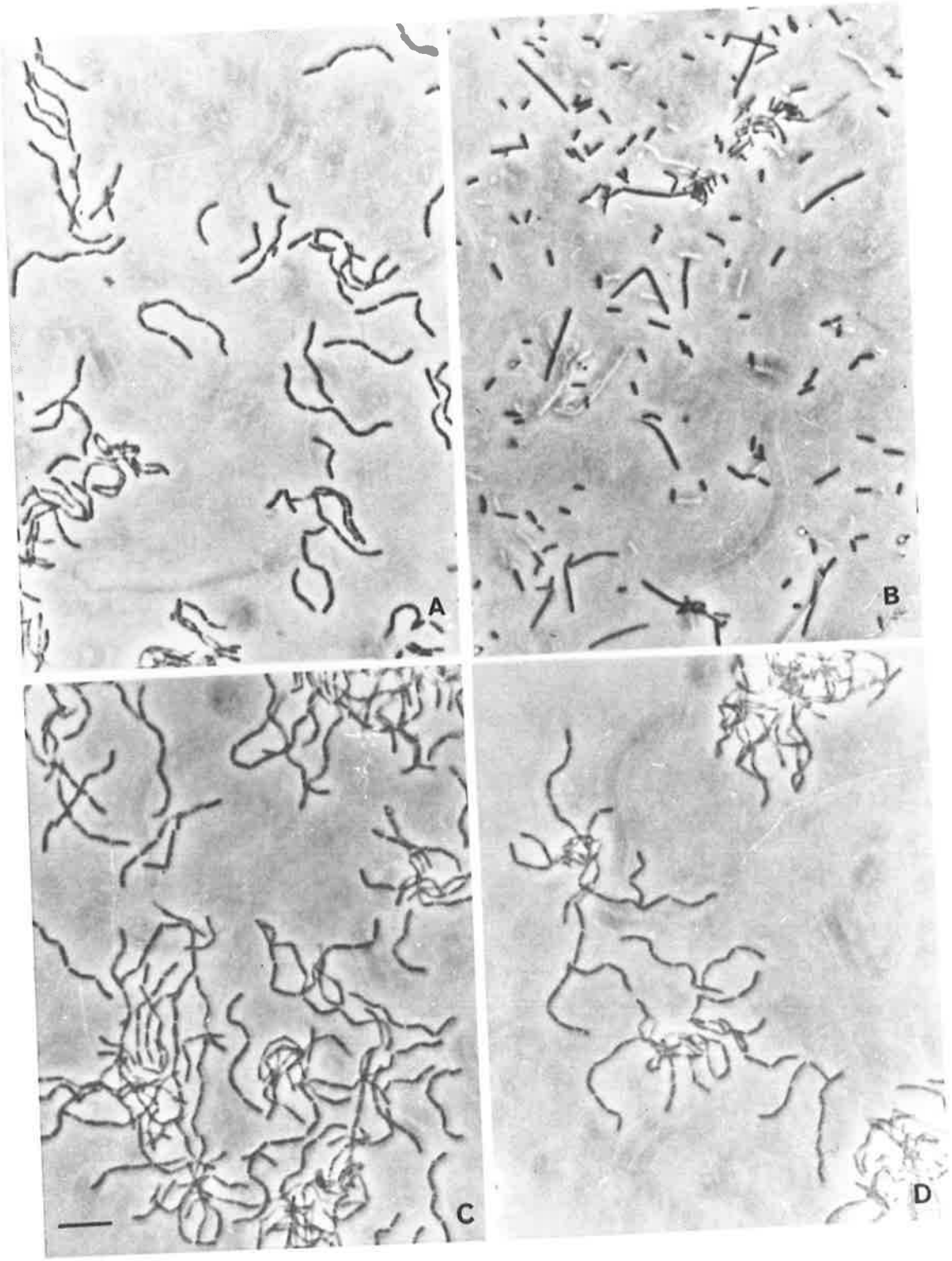
An alternate method for altering the fatty acid composition of B. amyloliquefaciens and presumably the fluidity of the cell membrane, was achieved by growing cells in media containing various alcohols. The possible mechanism whereby these cells alter their fatty acid composition in response to alcohols, and the type of changes which are observed in the fatty acid composition of B. amyloliquefaciens, have been presented fully in Chapter 5. These changes which were obtained for cells grown at 30°, involved an alteration in only the straight-chain to branched-chain fatty acid ratio; there was no increase in the levels of unsaturated fatty acids as was observed with cells grown at lowered temperatures.

The selection of the types of alcohols and their respective concentrations were made on the basis of the particular alcohols' toxicity and influence on the generation time, as discussed in Chapter 5. The morphology of cells grown in the selected alcohols is shown in Plate 6.2. Cells grown in the presence of pentanol existed primarily as dimers or single cells together with a few elongated cells, the latter being similar in appearance to cells grown at 20°. Cells grown in the presence of pentanol were motile in contrast to those grown in medium

PLATE 6.2. PHASE-CONTRAST PHOTOMICROGRAPHS OF *B. AMYLOLIQUEFACIENS* CELLS GROWN IN MEDIUM SUPPLEMENTED WITH VARIOUS ALCOHOLS.

Cells were grown at 30° in liquid growth medium containing the alcohols indicated and harvested when growth reached late-log phase. Bar marker = 6.0μ

		Time to reach late-log phase (Hr.)
A.	Control	18
B.	0.2% (v/v) Pentanol	21 to 22.5
C.	3.0% (v/v) Methanol	19 to 20.5
D.	2.0% (v/v) Ethanol	22.5 to 24


A
B
C
D

containing the other alcohols.

The fatty acid composition of cells grown in the presence of the various alcohols expressed as the % level of straight-chain and branched-chain fatty acids were as follows: Control (15.6;84.0); Methanol (3.4; 96.6); Ethanol (1.2; 98.8); Pentanol (17.6; 81.3), (Chapter 5). To examine whether altered fatty acid composition influences extracellular enzyme secretion, the following experiment was performed. Cells were grown in medium containing either methanol (3.0%), ethanol (2.0%) or pentanol (0.2%) and harvested when growth reached late-log phase; (dry weights for these and control cells were identical at harvest). The cells were washed by centrifugation and resuspended in (alcohol-free) WCSM. The secretion of protease and amylase and ^{14}C-leucine incorporation into TCA-precipitable material were measured in washed-cell suspensions as described in Chapter 2. The results are shown in Figure 6.7.

The secretion of protease was reduced by about 90% in ethanol grown cells and by about 72% in methanol grown cells. Cells grown in medium containing pentanol, after a slight lag in the rate of protease secretion, exhibited a level of protease production which was identical to that of control cells after 40 minutes (Figure 6.7 A). The secretion of amylase showed that growth in all three alcohols resulted in a 50% or greater inhibition in the rate of secretion of this exoenzyme, greatest inhibition (83%) was observed in methanol grown cells. Amylase secretion in pentanol grown cells exhibits an increase in rate with time (Figure 6.7 B). Protein synthesis (^{14}C-amino acid incorporation) by cells grown in the presence of the various alcohols was only slightly reduced in comparison to control (Figure 6.7 C).

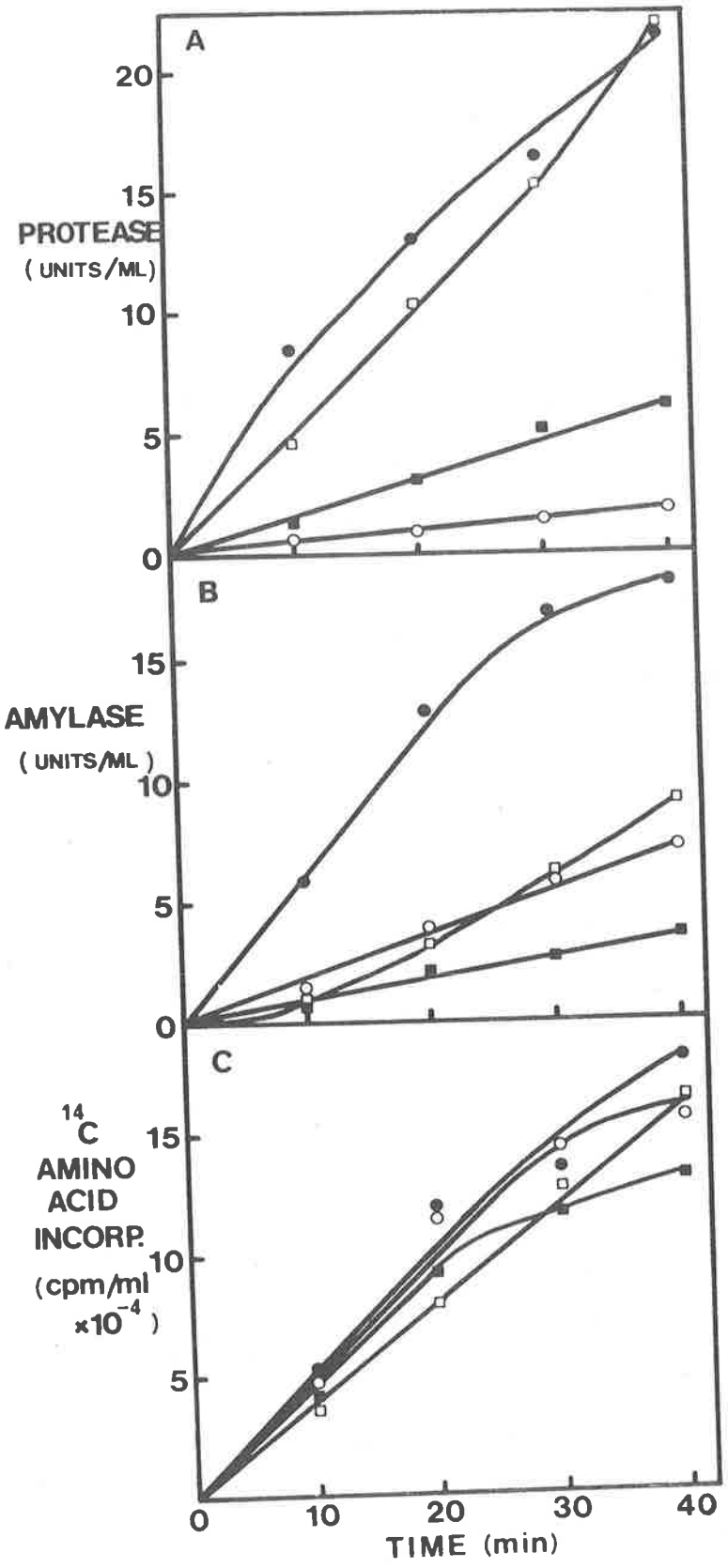
The levels of amylase and protease present in the

FIGURE 6.7. EFFECT OF GROWTH OF B. AMYLOLIQUEFACIENS IN MEDIUM CONTAINING VARIOUS ALCOHOLS ON THE SECRETION OF PROTEASE AND AMYLASE AND ON GENERAL PROTEIN SYNTHESIS.

Cells were grown at 30^{o} in liquid growth medium (●), or liquid growth medium containing 3% (v/v) Methanol (■), 2% (v/v) Ethanol (○), or 0.2% (v/v) Pentanol (□). Cells were harvested from late-log phase culture and resuspended in WCSM (not containing alcohol).

Secretion of protease (A) and amylase (B) and ^{14}C-leucine incorporation into TCA-precipitable material (C) was followed for 40 minutes at a temperature of 30^{o}.

Cells grown in medium not containing alcohol, and which after harvest were exposed to the above alcohols for a short period, exhibited identical rates of protease and amylase secretion and of ^{14}C-amino acid incorporation, to that of control cells.


A
B
C
PROTEASE
(UNITS/ML)
AMYLASE
(UNITS/ML)
^{14}C
AMINO
ACID
INCORP.
(cpm/ml
$\times 10^{-4}$)
0
5
10
15
20
0
10
20
30
40
TIME (min)

culture supernatant after growth of cells in medium containing the various alcohols, is shown in Table 6.1. In contrast to the washed-cell experiments where cells were resuspended into medium lacking alcohol, this table represents the level of amylase and protease in culture medium containing the particular alcohol. Thus even in the presence of the alcohol, the reduction in the levels for both enzymes were similar to those observed in washed-cell experiments. This reduction was not due to the effect of these alcohols on the activity of the enzymes (Table 6.2).

To investigate whether the alcohols selectively inhibited the secretion of the enzymes rather than their synthesis and caused a build up of the intracellular form of the enzyme, the level of amylase and protease in lysates of cells grown in the presence of the various alcohols was determined (Table 6.3). Very little intracellular (or membrane-associated) amylase or protease were detected in such lysates. The level of protease in cells grown in the presence of methanol or pentanol was raised, but the actual increase was quantitively small and may not be significant. All other levels were equal to or less than the control level even though a considerable reduction in the extracellular level was observed (Figure 6.7, Table 6.1). It is of interest to note that the amount of intracellular amylase associated with control cells is much higher than the level of intracellular protease in comparison to the level of each in the culture supernatant. Cell membrane associated forms of amylase have previously been observed in B. amyloliquefaciens by Fernández-Rivera Río and Arroyo-Begovich (1975).

TABLE 6.1. LEVEL OF AMYLASE AND PROTEASE IN CULTURE SUPERNATANTS OF B. AMYLOLIQUEFACIENS GROWN IN VARIOUS ALCOHOLS.

Cells were grown at 30^{o} in liquid-growth medium containing the alcohols shown, and the amylase and protease activities in the supernatant from late-log phase cultures was determined. Enzyme levels are expressed as % of control, which is shown as Units/ml of culture supernatant. Although the culture supernatant contains the alcohols at the concentrations shown, the effect of these alcohols on enzyme activities per se, was not great.

TABLE 6.2. EFFECT OF ALCOHOLS ON THE ACTIVITY OF B. AMYLOLIQUEFACIENS AMYLASE AND PROTEASE.

The activity of extracellular amylase and protease in the presence of various alcohols, was assayed as described in Chapter 2.

TABLE 6.3. LEVEL OF AMYLASE AND PROTEASE IN THE LYSATES OF B. AMYLOLIQUEFACIENS CELLS GROWN IN VARIOUS ALCOHOLS.

Cells were grown at 30^{o} in liquid-growth medium containing the alcohols shown. Cells were harvested from late-log phase culture, twice washed and resuspended in WCSM (not containing alcohol) to a dilution equivalent to that in the culture. Cells were immediately lysed by single passage through a French-pressure cell at 12,000 p.s.i. and after centrifugation (35,000g, 30 minutes), the supernatant was assayed for amylase and protease activity, as described in Chapter 2. Enzyme levels are expressed as % of control, which is shown as Units/ml of lysate of culture-strength cells.

TABLE 6.1

CULTURE SUPERNATANT

TREATMENT	AMYLASE (% of Control)	PROTEASE (% of Control)
Control	100 (94) **	100 (164) **
3% Methanol	31	9
2% Ethanol	57	9
0.2% Pentanol	40	80

** Units/ml culture supernatant

TABLE 6.2

EFFECT OF ALCOHOLS ON ENZYME ACTIVITY

TREATMENT	AMYLASE (% of Control)	PROTEASE (% of Control)
Control	100	100
3% Methanol	102	83
2% Ethanol	95	82
0.2% Pentanol	98	111

TABLE 6.3

LYSATE

TREATMENT	AMYLASE (% of Control)	PROTEASE (% of Control)
Control	100 (1.20) *	100 (0.05) *
3% Methanol	42	200
2% Ethanol	97	100
0.2% Pentanol	48	340

C. DISCUSSION

The conditions of growth which cause alterations in the lipid composition of B. amyloliquefaciens and the changes in the physical properties of the membrane as a function of temperature, also alter several physiological parameters which may be associated with the cell membrane. However, there is no direct proof of a causal relation between changes in the membrane lipids and the physiological observations made in this chapter. Therefore for the purpose of discussion it will be assumed that the two are associated in some manner, subject to the above qualification.

With regard to extracellular enzyme secretion, it appears from the Arrhenius plots of the rates of protease and ribonuclease secretion that some process(es) associated with the secretion of both enzymes may be sensitive to the physical state of the membrane. Both enzymes exhibit a marked increase in activation energy at a temperature corresponding to one of the previously determined characteristic temperatures (Chapter 4). However from this data it is impossible to decide whether the increased activation energy for secretion below 21^{o} is the result of an altered permeability of the membrane to the extracellular enzyme or to some other rate-limiting event which is affected by the altered physical state of the membrane. Such other processes could include the entry into the cell of metabolites, or the synthesis of extracellular enzymes by a class of membrane-bound ribosomes, the functioning of which is influenced by the physical state of the membrane to which they are bound. Indeed evidence obtained from protein-synthesis experiments using rat liver endoplasmic reticular membranes has shown that the physical state of the membrane influences the functioning of membrane bound, but

not free, ribosomes in protein synthesis (Towers et al., 1972). Arrhenius plots of ^{14}C-amino acid incorporation exhibited a "break" at a temperature corresponding to that which induced a change in the physical state of the membrane. Arrhenius plots for the incorporation by free ribosomes did not show this (Towers et al., 1972).

The similarities in the activation energies for protease and ribonuclease secretion in the temperature regions both above and below the temperature at which a change in the physical state of the membrane occurs, indicates that both are experiencing similar energy barriers to their permeation through the cell membrane. From the Arrhenius plots, the activation energy for the secretion of both extracellular enzymes at the growth temperature of 30^{o}, is 24 to 27 kcal/mole which is considerably higher than the 14kcal/mole observed for ^{14}C-amino acid incorporation. In this regard the secretion of these two extracellular enzymes in B. amyloliquefaciens is not unlike the secretion of extracellular alkaline phosphatase in Micrococcus sodonensis as studied by Glew and Heath (1971), although comparatively lower values for both processes were observed in this study. Thus in terms of the events which must occur for the secretion of extracellular enzymes, i.e., (1) entry of amino acids into the cell, (2) protein synthesis and, (3) secretion of the extracellular enzyme through the cell membrane, only (1) and (2) can be considered as common events to both amino acid incorporation and extracellular enzyme secretion. Thus the relatively higher activation energy observed for the secretion of protease and ribonuclease may be a reflection of some rate-limiting process involving the passage of these hydrophilic enzymes through the hydrophobic barrier of the membrane.

The growth of cells at different temperatures results

in altered fatty acid composition (Chapter 5) and associated changes in the cell morphology, particularly when growth is at low temperatures. However, the effect of altered fatty acid composition is difficult to interpret. Since both bulk protein synthesis and extracellular enzyme secretion increase in concert with the assumed increase in membrane fluidity, one cannot deduce that membrane composition specifically affects secretion. The explanation as to why cells grown at the lower temperatures have a greater capacity for protein synthesis is not known.

It seems reasonable to assume that the inability of cells to grow at lowered temperatures and the phenomenon of cold shock relate to the temperature-induced changes in the physical state of the membrane lipids. For cells grown initially at 30^{o}, and then slowly transferred to lowered temperatures, the lower temperature limit for growth was 15^{o}, a temperature which corresponds to what is considered the point of complete crystallization or "gelling" of the membrane lipids (Chapter 4). The consequences of such a change in the membrane would be numerous and would include the greatly restricted functioning of membrane-associated processes such as energy production and transport. The change in the physical state of the membrane which occurs at 21^{o} is not presumed to result in complete crystallization of the membrane lipid, and as such does not restrict the growth of B. amyloliquefaciens, although it does influence the functioning of the membrane-associated respiratory enzymes (Chapter 4). Thus growth is most likely limited to that temperature range in which the membrane lipids are not in a state of complete crystallization. In this regard the results obtained for B. amyloliquefaciens are in agreement with those obtained for other micro-organisms including B. stearothermophilus (Esser and Souza, 1974) and Acholeplasma laidlawii B

(McElhaney, 1974a,b).

Two critical temperatures for cold shock in B. amyloliquefaciens were detected on the basis of ribonuclease inhibitor exposure, ^{14}C-amino acid incorporation and cell viability. Only rapid cooling to temperatures below 15^{o} leads to irreversible changes in these parameters being apparent. In addition neither the initial physical state of the membrane (starting temperature) nor the magnitude of the temperature decrease, affected the cold shock phenomenon. This is in contrast to the work of Sato and Takahashi (1968,69) in which although two critical temperatures for cold shock were observed in E. coli, B. subtilis and Pseudomonas fluorescens, the magnitude of the temperature decrease was considered of critical importance. As rapid cooling is essential only for the 15^{o} cold shock, this temperature must be considered as the only cold shock temperature in B. amyloliquefaciens. In contrast, as the effects seen at 6^{o} are not dependent on rapid cooling, the effect of cooling through this temperature might be considered analogous to the chilling injury reported for chill-sensitive plants (Raison, 1973a,b).

The explanation for the difference in the levels of intracellular RNAase inhibitor exposure at these critical temperatures is not known, however it must relate to the degree of damage sustained by the cells as a result of the cooling process. As partial recovery from the effects of cold shock were observed under conditions where washed-cell suspension medium was employed, it is probable that small molecular weight components (similar to those present in this medium), are lost from the cell due to the associated changes in the permeability of the membrane.

The two critical temperatures for cold shock and chilling injury respectively in B. amyloliquefaciens correspond to two of the four critical temperatures observed in the cell membrane using a combination of physical and physiological techniques (Chapter 4). On the basis of the hypothesis presented in Chapter 4, the temperatures 15° and 6° are each considered to represent one of the critical temperatures (Ts_{IN} and Ts_{OUT}, respectively) for two separate phase transitions which result in the complete crystallization of the membrane lipid when the temperature is lowered. Thus the correspondence between the physical events occurring in the membrane lipid and the incidence of cold shock confirms the observations of Haest et al., (1972) and Leder (1972). However, the effects associated with each cold shock temperature, i.e. the magnitude of the cellular damage and the respective requirement for fast or slow cooling, may relate to events occurring in both halves of the cell membrane.

Further evidence for the relation between cold shock and physical state of the membrane lipids is provided by the results obtained using the non-ionic detergent, Tween 80. Due to its amphipathic nature, Tween 80 is believed to perturbate only the lipid comprising the outer half of the membrane (when using membrane vesicles), and as a result abolish the co-operative phase transition associated with this half of the membrane. Thus only the changes in the physical state of the membrane at 39° and 15° are observed (Chapter 4).

The effect of Tween 80 on the 15° cold shock is difficult to reconcile with its presumed effects only on the outer (exposed) half of the membrane. However the explanation may be dependent on the interaction of both halves of the membrane during the cold shock. Thus Tween 80, by modulating

in some manner the physical properties of the lipid in the outer half of the membrane, may influence the expression of the cold shock phenomenon which for this temperature (based on the asymmetric hypothesis in Chapter 4) is most likely caused by the crystallization of the membrane lipids in the inner half of the membrane (Ts_{IN}). The cooling of cells through 6^{o} in the presence of Tween 80 results in only a relatively small amount of ribonuclease inhibitor exposure. Thus the abolition of the change in the physical state of the membrane lipids which occurs at 6^{o} (Ts_{OUT}), results in virtual abolition of the 6^{o} cold shock.

The ability to reduce both the temperature and the magnitude of the cold shock by growing cells at lowered temperatures could prove useful for studies on cell-free protein synthesis and secretion where rapid cooling and lack of membrane damage are required. The lowering of the temperatures for the cold shock in these cells may relate to the changes in fatty acid composition. Although time did not permit the determination of the membrane phase transition temperatures in cells grown at different temperatures, it seems possible that the cells grown at 25^{o} and 20^{o} would exhibit a lowered phase transition temperature(s) as a consequence of the increased membrane fluidity. If this were the case, with reference to the fatty acid composition of these cells as outlined in Figure 6.2, the slight decrease in the cold shock temperature for the 30^{o} grown cells in comparison to the 37^{o} grown cells, most likely reflects the decrease in the ratio of the straight-chain to branched-chain fatty acids, which was the only significant change in the fatty acid composition observed in these cells. However the dramatic decrease in the cold shock temperatures for cells grown at 25^{o} and 20^{o} in comparison to the other two growth temperatures, most likely reflects a combination of a decrease in the straight-

chain to branched-chain fatty acid ratio, and an increase in the levels of unsaturated fatty acids. For cells grown at 20°, the further decrease in the temperature of the cold shock in comparison to the 25° grown cells, could primarily be due to the higher proportion of unsaturated fatty acids, as only a slightly lower straight-chain to branched-chain fatty acid ratio was observed. However it must be pointed out that it is difficult, if not impossible at the present time to ascertain the relative contributions of the different classes of fatty acids (and indeed the individual fatty acids) on the phase transitions without knowledge of the packing and molecular arrangement of the fatty acids within the membrane and how these parameters influence the occurrence of lipid phase transitions.

The results obtained for protease and amylase secretion and for amino acid incorporation in cells grown in medium containing various alcohols indicate that the greatly reduced levels of extracellular enzyme secretion are probably not the result of a reduction in protein synthesis. Nevertheless the possibility remains that as ^{14}C-amino acid incorpoartion is a measure of the total cellular protein synthesis, any selective inhibition which occurred to those ribosomes responsible for the synthesis of extracellular enzymes (membrane-bound ribosomes?), may not be observable by this assay method. Alternatively, if secretion rather than synthesis has been affected by altered lipid composition, the results imply that protease secretion is sensitive to an increase in membrane fluidity and amylase secretion is sensitive to an increase or a decrease in membrane fluidity. Under such conditions, the presence of an intracellular form of the extracellular enzyme may accumulate if synthesis and secretion are not tightly coupled events. Indeed for B. caldolyticus a marked increase in the level of intracellular protease has been

observed under conditions where the permeability properties of the cell membrane have been altered by changes in its fatty acid composition (Lauwers and Heinen, 1973). Attempts to locate such a pool in B. amyloliquefaciens on the basis of activity alone were unsuccessful. However the accumulation of inactive precursors is not excluded and a search for these by immunological means is at present progressing in this laboratory. In addition, the observation that in medium containing pentanol B. amyloliquefaciens grows as single cells rather than in chains, could prove useful for mutagenesis work on this organism.

CHAPTER 7

FINAL SUMMARY AND DISCUSSION

FINAL SUMMARY AND DISCUSSION

The results in this thesis do not greatly increase our knowledge of the mechanism of extracellular enzyme secretion in B. amyloliquefaciens, however they are of considerable interest in terms of basic membrane biology and as such may be important in our overall understanding of the secretory process in the future.

The behaviour of the membrane lipids as a function of temperature is interesting in view of the possibility that the phospholipids are asymmetrically distributed in the cell membrane, as has been shown for a number of other membranes. Although the significance of this in the secretion process is not known, it does appear that such physical properties as the fluidity and physical state of the membrane lipids may influence physiological processes such as cold shock, extracellular enzyme secretion and growth. As a consequence of these studies, the ability to modulate cold shock in this organism by altering the membrane lipid composition may prove extremely useful in proposed cell-free studies on extracellular enzyme synthesis.

At the present time it is unclear as to whether the cell membrane is involved in both synthesis and secretion of extracellular enzymes. The fact that no membrane-bound ribosomes were observed in this study does not disprove their existence. However, until such time that the in vitro synthesis of extracellular enzymes is achieved, possibly by the use of cell-free protein synthesizing systems in combination with exoenzyme specific m-RNA, the theory that the synthesis and secretion of extracellular enzymes are simultaneous events occurring at the membrane, remains essentially unproven.

Irrespective of the site of synthesis of extracellular enzymes, the mechanism of their secretion across the permeability

barrier of the cell membrane is an important and interesting problem, the answer to which may well come from a greater understanding of the architecture of biological membranes in general.

CHAPTER 8

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