A PHYSICAL CHEMICAL STUDY OF THE SURFACE OF MYCOBACTERIUM BOVIS BCG

A thesis presented for the degree of
Doctor of Philosophy in the Faculty
of Science of the University of London

by

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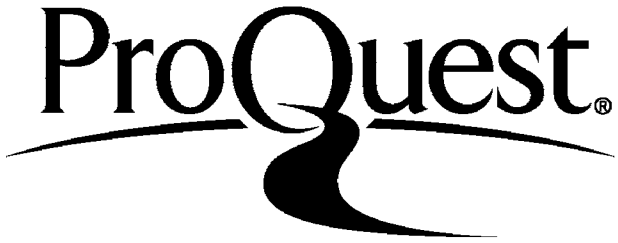

This thesis comprises a report of full-time research undertaken by the author in the Physical Chemistry Laboratories of Bedford College, University of London, from October 1976 to October 1979, and also partly in the BCG Research Unit of Glaxo-Allenbury Research, Greenford, in fulfilment of the conditions of the SRC award.

CONTENTS

ABSTRACT

The electrokinetic properties of five sub-strains of Mycobacterium bovis BCG were studied in an attempt to explain inter-strain differences and the aggregation phenomena characteristic of all strains during growth. Cells of all strains behaved as a typical macropolyanion. Studies of the variation of charge with the pH and ionic strength of the suspension medium before and after chemical and enzymic treatments revealed that the surface charge of these organisms was due solely to ionizable phosphate groups. Anion adsorption on the surface was negligible and no carboxyl, amino or sulphydryl groups could be detected. The charge on cells of all strains was constant and independent of the growth medium, the age of the cells up to 28 days and storage of the washed cells at 4 ^{o}C. The large quantity of lipid material, known to be present in the cell walls of mycobacteria, was not detectable at either the inner or outer surfaces of the cell wall. It was concluded that the lipid must be located in the middle regions of the wall.

Cells of M. phlei, M. smegmatis and M. microti exhibited surface charge properties which were identical to those of all sub-strains of BCG. The results are discussed in the light of proposed cell wall structures of mycobacteria and it is concluded that the phosphate groups, which form the predominant charging species and which therefore occur at the surface, are the phosphodiester groups linking the peptidoglycan backbone with the arabinoglactan of the cell wall.

A "slime-like" amorphous material which coats aggregates of cells, but not individual cells, was revealed by stereoscan electron microscopy. This substance, which is easily removed by manual tissue-grinding, is believed to be responsible for the aggregation of mycobacterial cells during growth; physico-chemical forces arising from the charged surface groups play no part in this aggregation phenomenon.

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SUMMARY

From the original isolate of Calmette and Guérin many sub-strains of Mycobacterium bovis BCG have arisen, owing to variations in handling and culture. These sub-strains exhibit differences in their biological and physical properties, e.g. colonial morphology, immunizing potential. However, all sub-strains are characterised by a very low growth rate and a great tendency to aggregate during growth, making the production of a homogeneous vaccine difficult. The tendency to clump can be supressed by the addition of non-ionic wetting agents to the growth medium, but this also decreases the total growth yield.

Particulate microelectrophoresis was used to examine the surface properties of five sub-strains of BCG (Glaxo, Pasteur, Japanese, Prague and Danish), in order to investigate the inter-strain differences and the aggregation phenomenon. Cells of BCG grown at 37 °C exhibited a pH-mobility curve characteristic of a surface with anionic groups only. The negative charge on the cells in suspension in buffer solutions increases rapidly up to pH 4.0 and then more slowly in the range pH 4.0 to 9.5. The surface groups had a pK of approximately 2.9 with an isopotential point (i.e. zero charge) of approximately 2.0. Positively charged surface amino groups were not revealed by the reversal of charge at low pH-values, by a further sharp increase in charge at high pH-values or as a result of chemical treatments.

Specific chemical and enzymatic modification of cells of BCG was carried out to identify the surface groups giving rise to this negative charge. The charge was due solely to ionizable phosphate groups at the cell surface. The charge was unaffected by the growth medium used, fairly vigorous chemical treatments or by storage at 4 °C and it remained constant at all ages during a 28-day growth cycle. All the

sub-strains of BCG examined, and also several other species of mycobacteria, exhibited the same electrokinetic properties. This indicated that the phosphate groups were part of the common mycobacterial cell wall skeleton. From a detailed examination of the proposed chemical structure of mycobacterial cell walls it is apparent that these phosphate groups are the phosphodiester linkages between the peptidoglycan backbone and the arabinoglactan of the cell wall.

No surface lipid was detected on the cell surface, or on the outer or inner surfaces of cell walls of BCG, implying that the large quantities of solvent-extractable lipid known to be present in mycobacterial cell walls are located in the middle regions of the structure. This conclusion was supported by the experimental results of mobility measurements made in SDS-buffer solution of decreasing ionic strength.

The aggregation of cells of BCG during growth is not the result of physico-chemical forces arising from the charged groups on the cell surface, but appears to be due to the secretion of an amorphous, "slime-like" material by the cells. Scanning electron microscopy reveals that this substance coats clumps of cells, but not single organisms, and therefore holds them together in aggregates during growth. The BCG organism prefers to grow when coated by this amorphous material, which can be easily stripped from the cells by manual tissue-grinding. Any interference with the coating of aggregates with this substance by factors such as shaking during growth or addition of wetting agent to the growth medium results in poorer growth yields. Identification and characterization of this "slime-like" material could prove to be of great importance.

CHAPTER 1

INTRODUCTION

1.1 Classification and History of Mycobacterium bovis BCG

Microorganisms are divided, on the basis of certain distinct characteristics, into eight major groups: algae, protozoa, yeasts, moulds, bacteria, pleuropneumonia-like-organisms (PPLO), rickettsia and viruses. However, the borders between these groups are diffuse and subject to much discussion.

Bacteria are usually divided into ten orders (Table 1.1). These are then divided into families on the basis of such factors as cell shape, reaction to the Gram stain, presence or absence of flagella, growth requirements, optimum growth temperature and the types of fermentation which the organisms are capable of under aerobic and anaerobic conditions.

The most useful preliminary characterisation is the Gram stain technique. Organisms may be classified as Gram-positive, Gram-negative or Gram-variable depending on their ability to retain crystal violet dye after mordanting with iodine solution. This staining technique is also useful in revealing bacterial morphology such as size and shape of cells and their spore-forming ability. In the case of the Actinomycetales another stain technique is important in classification; this is the ability of the organisms to retain carbolfuchsin dye after washing with acidified alcohol. Such organisms are called acid-fast.

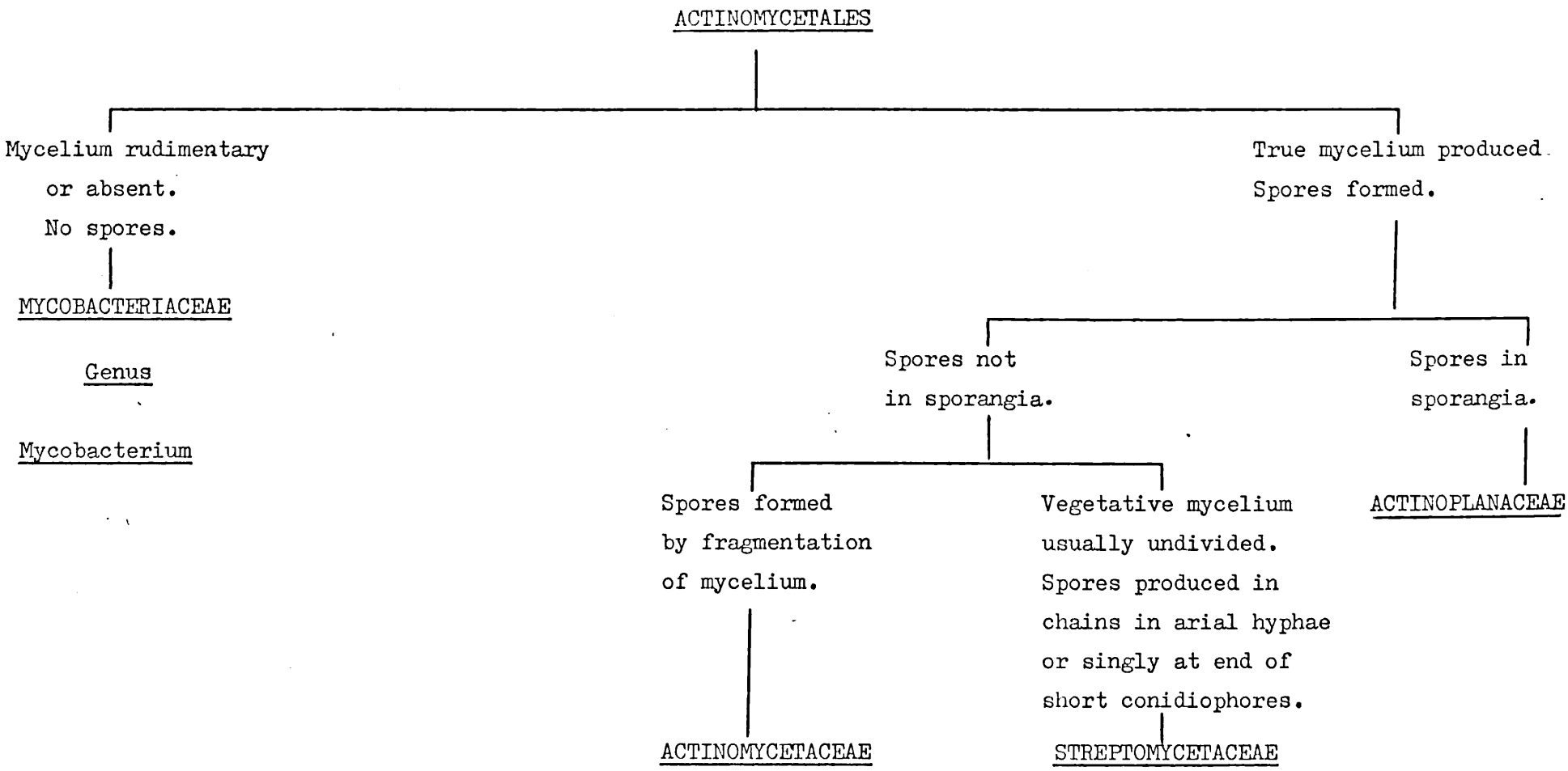
The classification of the order Actinomycetales is more confused and open to more criticism than most other bacterial orders. The order is divided into four families, Mycobacteriaceae, Actinomycetaceae, Streptomycetaceae and Actinoplanaceae (Table 1.2).

Table 1.1

Classification of bacteria with particular reference to mycobacteria.

Order	Family	Genus
Pseudomonadales		
Clamydobacteriales		
Hyphomicrobiales		
Eubacteriales		
Actinomycetales	4 including Mycobacteriaceae	1 Mycobacterium [Mycelium rudamentary or absent. No spores.]
Caryoplanales		
Beggiatoales		
Myzobacterales		
Spirochaetales		
Mycoplasmatales		

Table 1.2 Distinguishing features of the families of the order Actinomycetales



The family Mycobacteriaceae contains the non-spore-forming members of the order Actinomycetales and contains only one genus, Mycobacterium. These are Gram-positive, acid-fast, slender rods characterised particularly by their slow growth rate. Mycobacteria may be divided into two classes; slow growers, which take 5 - 7 days for appreciable growth at 37 °C and will only grow at this temperature, and fast growers, which show growth after two days at 37 °C and will grow at other temperatures.

Mycobacterium bovis BCG is one member of a group of mycobacteria which are experimentally almost identical. This contains Mycobacterium tuberculosis, M. bovis and M. bovis BCG (Bacillus of Calmette and Guérin). Calmette and Guérin cultivated the virulent M. bovis organism, which causes bovine tuberculosis and also infects man, on a medium containing a high concentration of ox-bile (Calmette and Guérin, 1908). Every 3 weeks they subcultured onto fresh bile-containing medium and 13 years later in 1921 obtained an attenuated form of M. bovis which they named BCG. Animal trials proved this organism to be stable and no longer virulent for guinea pigs. Successful human trials followed and in 1924 the Pasteur Institute was given the go ahead by the French Ministry of Public Health to begin the production of oral BCG vaccine.

The same year trials started on a new method of vaccination known as subcutaneous vaccination, the introduction of live BCG organisms below the skin. This method was soon adopted in preference to oral vaccination, but has now been largely replaced by intradermal injection (developed in Sweden) in which live organisms are placed into the epidermis layers of the skin (Guérin, 1948).

From the original BCG isolate a series of sub-strains have arisen which all have characteristic properties and show considerable morphological and biological differences. This may be due to random mutation after isolation but is most probably a result of different treatment during culture. There are four main sub-strains; the Glaxo sub-strain, which originated from the 1077^{th} passage of the original Danish sub-strain, the origins of which are unknown; the Danish strain, which comes from the 1331^{st} passage of the original Danish sub-strain; and the Pasteur and Japanese sub-strains, whose origins are not clear. These four sub-strains and the Prague sub-strain were used in this investigation; most emphasis was placed on the Glaxo sub-strain.

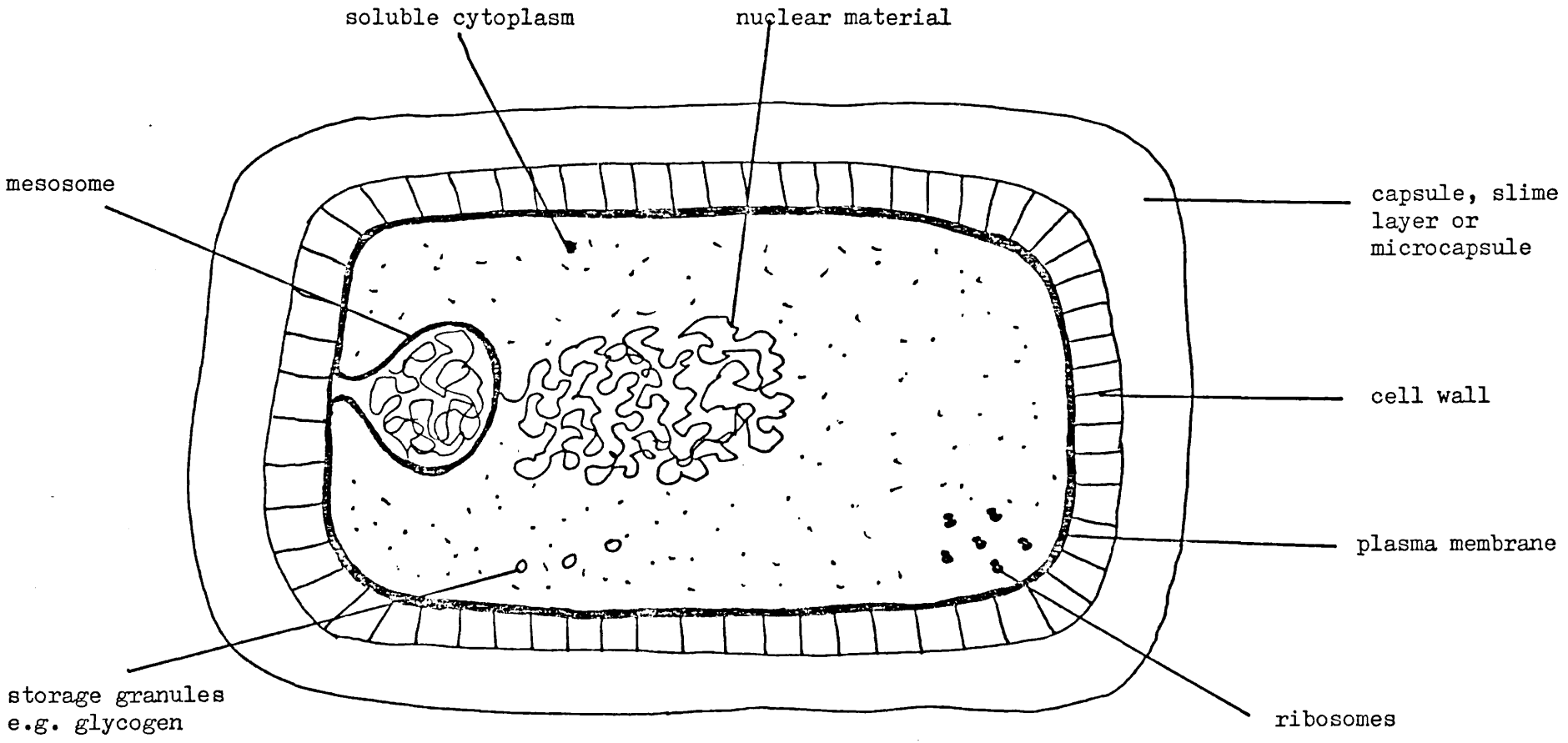
1.2. Bacterial Anatomy

The bacterial cell is prokaryotic and a cross section of a typical cell is shown in Figure 1.1. The cytoplasm is surrounded by a unit membrane, the cytoplasmic membrane, which in turn is enveloped by a rigid cell wall which determines the shape of the cell. This is sometimes surrounded by a capsule, microcapsule or slime layer.

Prokaryotic cells lack a definite membrane-bound nucleus and in bacterial cells the nuclear material, or nucleoid, lies free in the cytoplasm. On division of the nucleoid the daughter nucleoids are thought to be separated by mesosomes.

Mesosomes are extensions of the cytoplasmic membrane that reach into the cytoplasm of the cell. Electron microscopy studies show that these appear to be lamella invaginations of the cytoplasmic membrane or smaller membranous vesicles within a continuation of the membrane. These structures are the site of electron transport in the bacterial cell, and

FIGURE 1.1. : Diagrammatic representation of a bacterial cell



in nitrogen-fixing and photosynthetic bacteria, where high energy coupling reactions take place, mesosomes can reach extreme proportions of size and shape. The mesosomes, as well as being implicated in nuclear division, are also important in septation on division of the cell and possibly also in extension of the membrane.

The cytoplasm of the cell is a slightly viscous colloid complex of water, amino-acids, proteins, fats, carbohydrates and inorganic salts. Storage vesicles are not formed and any reserve material, such as glycogen, occurs free in the cytoplasm. Under the electron microscope the cytoplasm appears granular; this is due mainly to the presence of free ribosomes, which are never associated with membranes or endoplasmic reticulum.

The ribosomes of bacterial cells differ from those of eukaryotic cells in their sedimentation value, having a value of 70 S in contrast to the 80 S ribosomes found in eukaryotic cells. This difference forms the basis of specificity of many antibiotics for bacterial cells over eukaryotic cells. The ribosomes are the site of protein synthesis, and many ribosomes are often attached to one molecule of ribonucleic acid, in the form of a polysome (Schlessinger, 1964).

Motile bacteria possess flagella which protrude from the cell surface, but have their attachment site in the cytoplasmic membrane. Bacterial flagella consist almost entirely of a protein called flagellin, this is a globular protein with a relative molecular weight of 40 000. However, there is still controversy over the way these protein molecules are arranged to give the structure of the flagellum. Flagella can be either polar or peritrichous and this is often used as a basis of classification. Mycobacteria are non-motile and do not possess flagella.

Surrounding the cytoplasmic membrane is a rigid cell wall; the chemical structure of this will be dealt with in detail later. In many microorganisms a capsule or slime layer lies external to, but in close contact with, the cell wall. The amount produced depends on the nature of the growth medium and the production of these layers may be stimulated by unfavourable conditions. Capsules are often correlated with virulence in pathogenic organisms; capsulated cells are more capable of resisting phagocytosis and therefore more virulent than non-capsulated cells (Koenig, 1962).

Wilkinson (1958) classified capsules into three main groups:

1. macrocapsules, at least 0.2 μm thick, having a definite external surface;
2. microcapsules, less than 0.2 μm thick and usually detected immunologically; these are very difficult to distinguish from the cell wall;
3. slime layers, which accumulate at the surface and have little definite anatomical significance.

Capsular material consists of about 98% water but other components such as polysaccharides, hyaluronic acid and polyglutamic acid have been identified and well studied in certain species.

1.3. The Mycobacterial Cell Wall

There are marked differences between the cell walls of Gram-positive and Gram-negative bacteria (Table 1.3). This investigation is concerned with mycobacteria which are classified as Gram-positive. However, it has been held that "mycobacteria cannot be classified as Gram-positive or Gram-negative by the Gram staining technique because once they have

Table 1.3

A comparison between the cell walls of Gram-positive and Gram-negative bacteria.

	Gram-positive e.g. Staph. aureus	Gram-negative Escherichia coli
1.	The cell wall presents a unified well defined separate structure, distinct from the cytoplasmic membrane.	A very complex organisation of the outer cell layer. No clear boundary between the cell wall and adjacent structures. Cell wall possibly layered.
2.	Hydrolysis yields only a limited range of amino acids. Little protein and enzymic activity.	Hydrolysis yields up to 21 amino acids from protein units present. Much enzymic activity.
3.	Peptidoglycan may form 50% of cell wall material. Probably organised as a multilayer network.	Peptidoglycan only accounts for 5-10% of cell wall material. Probably present as a monolayer.
4.	*Contains 1-5% cell wall lipid.	Contains up to 20% cell wall lipid.
5.	If present, capsule usually consists of simple polysaccharides and occasionally polypeptide.	Capsules consist of complex polysaccharide, protein-polysaccharide and lipo-polysaccharide complexes.
6.	Capable of withstanding an osmotic pressure of up to 30 atmospheres.	Osmotic pressure across protoplasmic membrane is generally about 12 atmospheres.

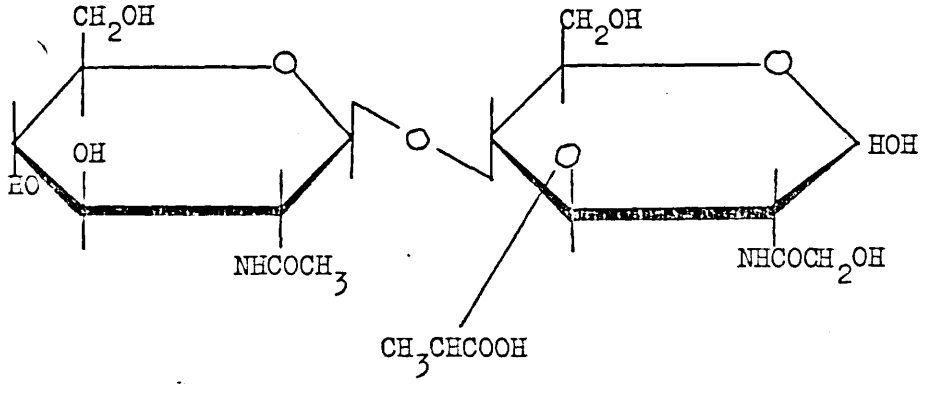
* In Mycobacteria lipid content 20-40%.

been stained by basic dyes, they cannot be decolorized by alcohol regardless of whether or not they have been treated with iodine" (Middlebrook, 1965). However, removal of the lipoidal portion of mycobacterial cell walls with alkaline ethanol (1% KOH in absolute alcohol, wt/vol) leaves an intact bacterial cell which has lost its acid-fast properties but which remains Gram-positive. As mycobacterial cell walls contain an abnormally large lipid component compared to other Gram-positive organisms it appears that the removal of these lipids (possibly responsible for acid-fastness) enables the inherent Gram-positivity of mycobacteria to be determined (Barksdale and Kim, 1977). As the bacterial cell wall of M. bovis BCG is the immediate surface component under study in this investigation, detailed consideration will be given to the structure and chemical composition of the cell walls of strains of this organism and of the cell walls of the closely related M. tuberculosis and M. bovis.

The structure of bacterial cell walls has been studied for many years and with improvements in cell wall isolation methods (Salton and Horne, 1951) and much quantitative work (Strominger, Park and Thomson, 1959; Mandelstam and Rogers, 1958; Rogers and Perkin, 1959; Hancock, 1960; Mandelstam and Strominger, 1961) the structure of a polymer which forms the framework of all Gram-positive cell walls was elucidated. This was given the trivial name "mucopeptide", but many other names have also been suggested for the polymer, including glycosaminopeptide, glycopeptide, peptidoglycan and murein. The peptidoglycan complex forms a rigid 3-D skeleton, giving mechanical support and shape to the cell, to which may be attached other short polymers, and in which may be embedded other minor wall components.

In most Gram-positive bacterial cell walls the backbone of the peptidoglycan polymer is a polysaccharide consisting of alternating β-1,4-N-acetyl-D-glucosamine (GNAc) and N-acetyl-D-muramic acid (MurNAc) units, with short peptide chains attached to the MurNAc units, these are in turn linked by penta-glycyl cross bridges. In mycobacteria most of the N-acetyl-muramic acid is replaced by N-glycolyl-muramic acid (MurNGlyc). The polysaccharide backbone consists therefore of alternating units of GNAc and MurNGlyc joined by a β 1 -> 4 linkage as shown in Figure 1.2 (Lederer, 1971, 1975; Barksdale, 1977).

FIGURE 1.2: Polysaccharide dimer forming basic unit for polysaccharide backbone of mycobacterial mucopeptide.



N-acetyl-glucosamine N-glycolyl-muramic acid

The peptide chains are attached to the MurNGlyc units and in mycobacteria are known to consist of D and L-alanine, D-glutamic acid and meso-2,6-diaminopimelic acid. A proposed structure for the mucopeptide monomer of mycobacterial cell walls is shown in Figure 1.3. Interpeptide linkages in M. bovis BCG are mediated through D-alanyl-(D)-meso-diaminopimelic acid (Lederer, 1971) and also through meso-diaminopimelyl-meso-diaminopimelic acid linkages occurring in a ratio of approximately 2:1 (Wietzerbin, 1974; Lederer, 1975).

The mucopeptide of mycobacterial cell walls is linked to a glycolipid containing mycolic acids esterified to an arabinoglactan.

Mycobacterial mycolic acids are defined as α-branched, β-hydroxy acids and range from C_{60}-C_{90}. Tuberculostearic acid (10-methyl-stearic acid) is widely distributed among the Actinomycetales but the C-alkylation reaction giving rise to branched chain mycolic acids seems to be restricted to mycobacteria. Mycolic acids occur naturally esterified with a carbohydrate; this carbohydrate is arabinose in the mycolic acids of the cell wall and Wax D, and trehalose in the substance known as "cord" factor (Asselineau, 1966; Goren, 1972).

The arabinoglactan is thought to be a branched structure consisting mainly of 1 -> 5 linked D-arabinofuranose units and 1 -> 4 linked D-galactopyranose units in approximately 5:2 molecular portions with some of the arabinose units forming non-reducing terminal ends. It is possible that the D-galactose residues are in the furanose form, but the rate of hydrolysis of BCG arabinoglactan suggests that they occur in the pyranose form (Misaki and Yukawa, 1966). Misaki, Seto and Azuma (1974) proposed a structure for mycobacterial arabinoglactan (Figure 1.4). However, this proposed structure does

FIGURE 1.3. Proposed structure for the peptidoglycan monomer of mycobacterial cell walls

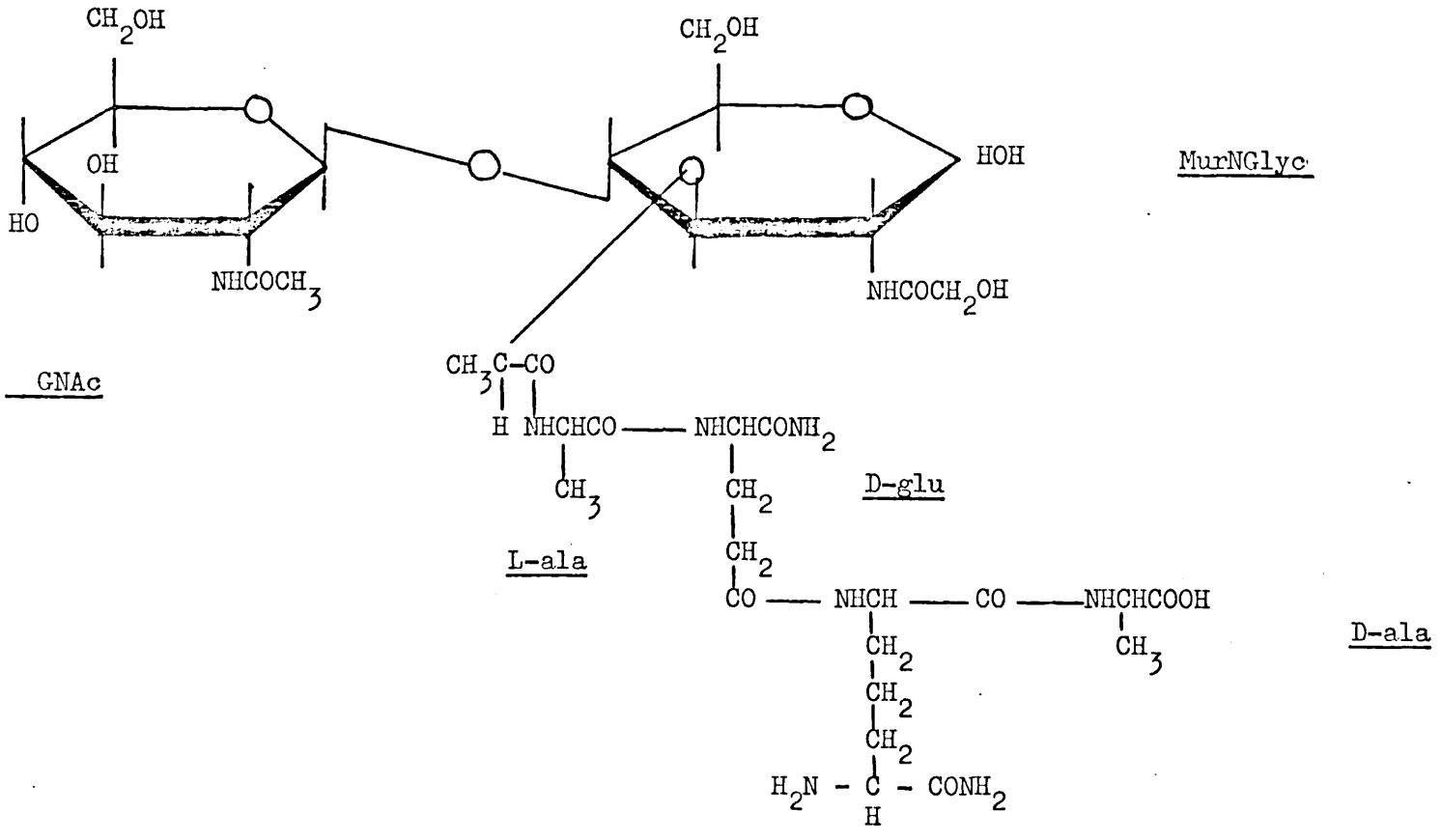
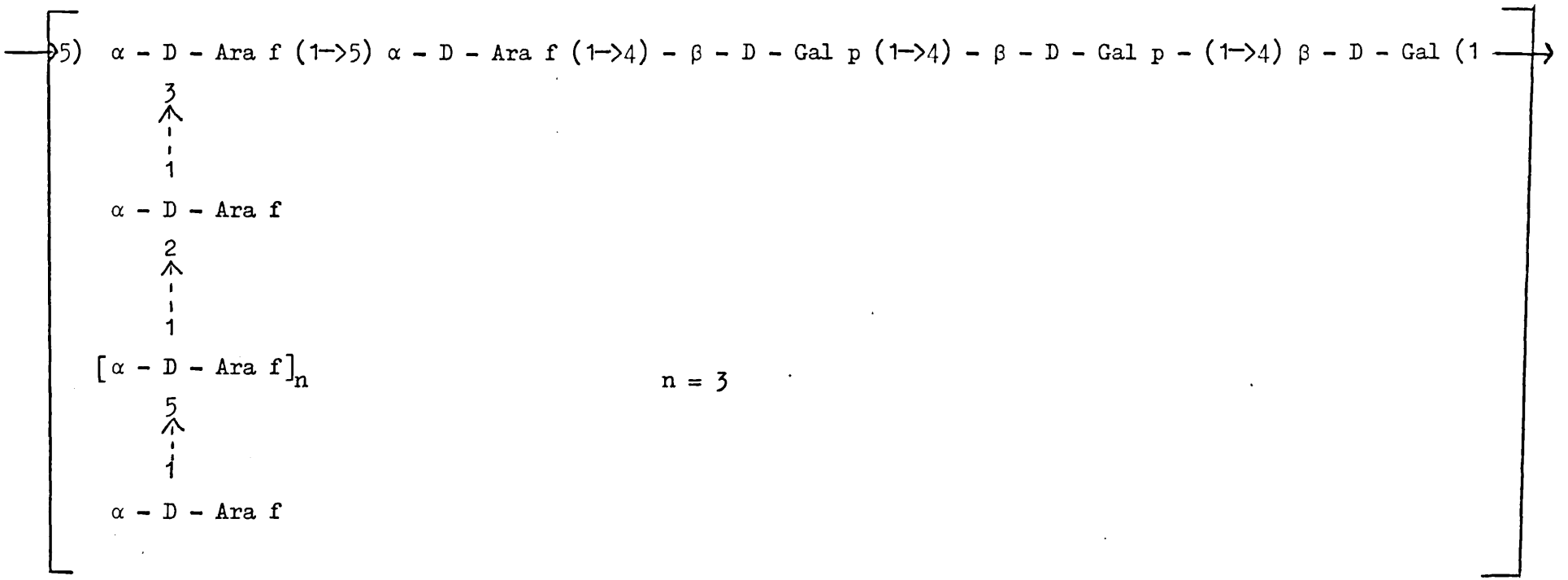


FIGURE 1.4: Proposed structure of mycobacterial arabinoglactan



Ara f = arabinose in the furanose form

Gal p = galactose in the pyranose form. If the D - galactose residues are in the furanose form linkage should be (1→5).

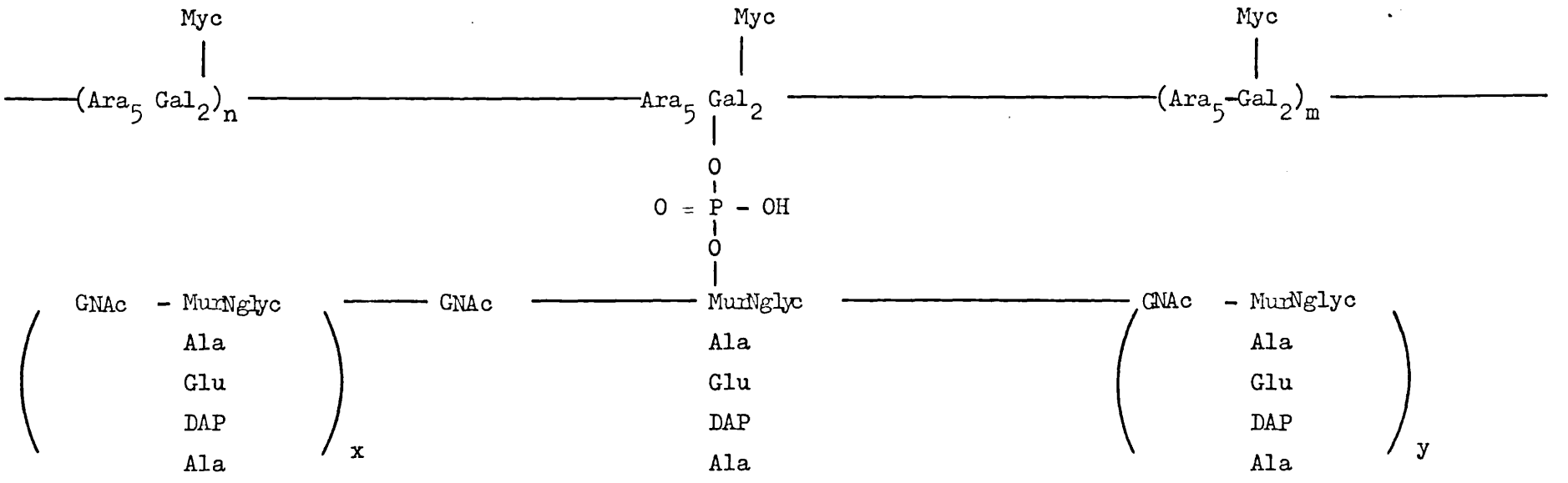
not take into account the galactofuranosyl residues reported by Vilkas et al (1973) on partial acid hydrolysis of the arabinoglactan of M. tuberculosis. Branching of the arabinoglactan may occur at the C_3 of the arabinose residues and probably at the C_6 of the galactose residues and it has been suggested that these galactofuranosyl units may arise from secondary galactosidic linkages (Misaki, Seto and Azuma, 1974).

Mycolic acid is ester-linked through its carboxyl to the 5-hydroxyl group of one of the D-arabinofuranose molecules to form the glycolipid of the mycobacterial cell wall. It is probable that the lipid moiety in these cell walls consists exclusively of mycolic acids (Lederer, 1971).

The glycolipid forms a complex with the peptidoglycan of the cell wall. The nature of the linkage is not yet perfectly clear, but the presence of muramyl phosphate suggests a possible phosphodiester linkage to the peptidoglycan backbone. There is also some evidence of a glycosidic linkage between the glycolipid and possibly the glucosamine of the peptidoglycan (Lederer, 1971). The molecular weight of the proposed monomer is approximately 3 200. The relative molecular weight of the arabinoglactan of BCG is 30 000 and 1 in every 8 to 10 molecules of muramic acid is phosphorylated; the structure of a possible "decamer", proposed by Lederer (1971), is shown in Figure 1.5.

Apart from the main peptidoglycan-glycolipid complex which forms the basis of the mycobacterial cell wall, there are several other glycolipids associated with the wall. Wax D can be isolated as the acetone-insoluble fraction of a chloroform extract of mycobacteria. The proposed structure (Figure 1.6) shows close analogy with the peptidoglycan of the cell wall, and the inhibition of synthesis of Wax D by cycloserine (a cell wall inhibitor) suggests a common biosynthetic

FIGURE 1.5: Proposed "decamer" structure for the glycolipid-mucopeptide complex of mycobacterial cell walls



n + m = 8 - 10

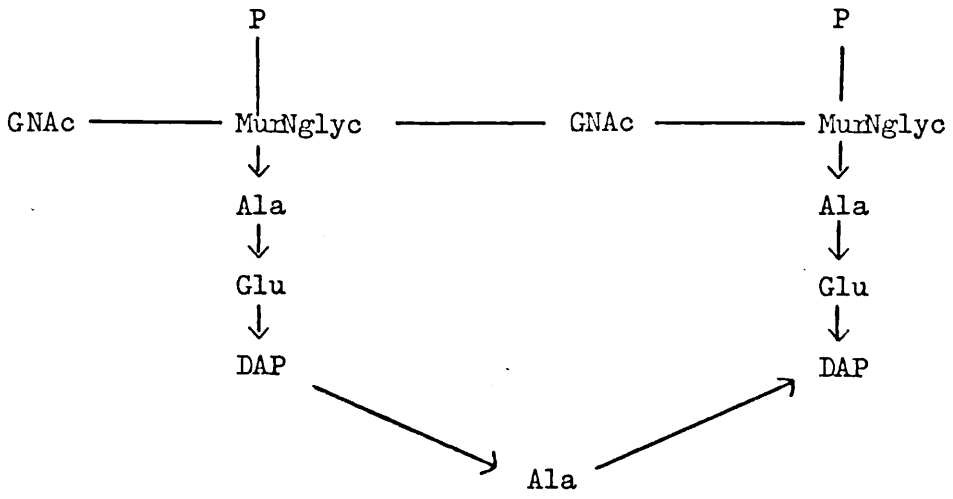
x + y = 8 - 10

Myc = mycolic acid

$Ara_5\ Gal_2$ = arabinoglactan

(GNAc - MurNglyc; Ala; Glu; DAP; Ala) = mucopeptide

FIGURE 1.6: Structure of mycobacterial Wax D



GNAc = N-acetyl-glucosamine

MurNglyc = N-glycolyl muramic acid

Ala = alanine

Glu = glutamic acid

DAP = diaminopimelic acid

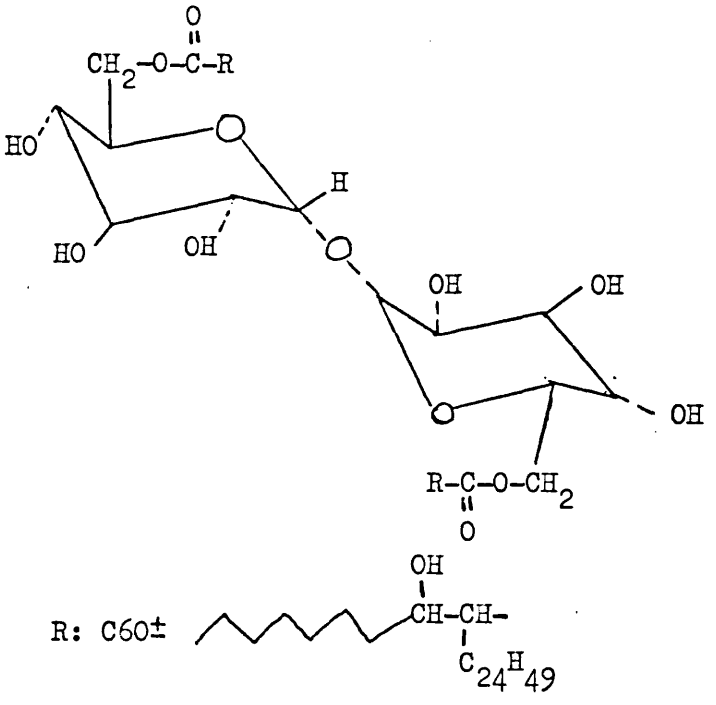
N.B. Close analogy with mucopeptide of cell wall (Fig. 1.5).

pathway for cell walls and Wax D. The proposed pathway is:

UDP ———> precursor ———> cell wall ———> Wax D.

Cord factor, as a toxic glycolipid containing trehalose, was first discovered by Bloch (1950) in petroleum ether extracts of M. tuberculosis H_{37} Rv. The structure (Figure 1.7.) was established by Noll and Bloch (1955, 1956) as a trehalose-6,6'-dimycolate, alkaline hydrolysis yielding two molecules of the disaccharide trehalose. Identical results were obtained for the cord factor of BCG.

FIGURE 1.7; 'Cord' factor: trehalose-6,6'-dimycolate (after Goren, 1972).



A whole series of natural diesters of trehalose, such as cord factor, exist and these may be essential for the transfer of mycolic acids into the mycobacterial ultrastructure. Cord factors behave as detergents and this, together with the fact that they are located about the surface of the outer cell wall, suggests that they may play a role in facilitating the absorption of molecules such as lipids into the cell (Lederer, 1971; Barksdale and Kim, 1977).

Another glycolipid species found in mycobacterial cell walls is the sulpholipids; these are anionic sulphur-containing lipids (Middlebrook et al, 1959). Goren (1972) provides a provisional structure which shows that the molecule has four acyl radicals (Figure 1.8.). At least three of these acyl radicals differ and are known as acids A, B and C. Acid B is palmitic acid and acids A and C are mixtures of homologous acids giving a new type of hydroxy acid (Figure 1.9.)

FIGURE 1.8: Sulpholipid I: provisional structure of Goren (1972)

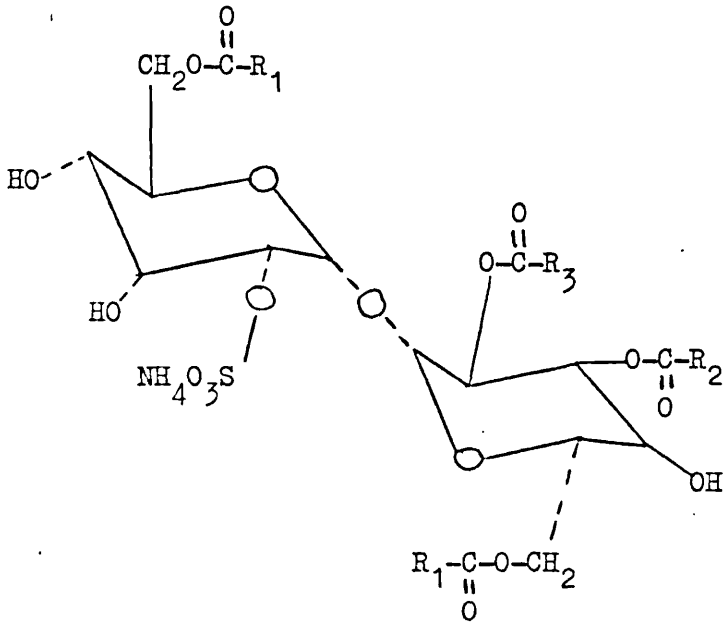
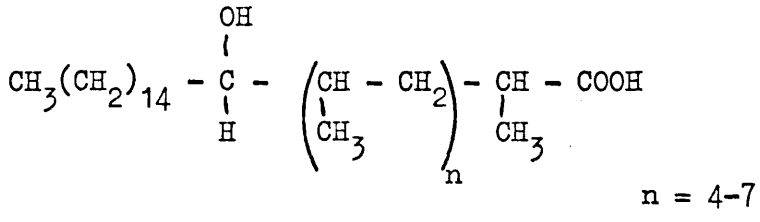


FIGURE 1.9: Acid C. (Lederer, 1971)



n = 4-7

Acid A has H at OH position

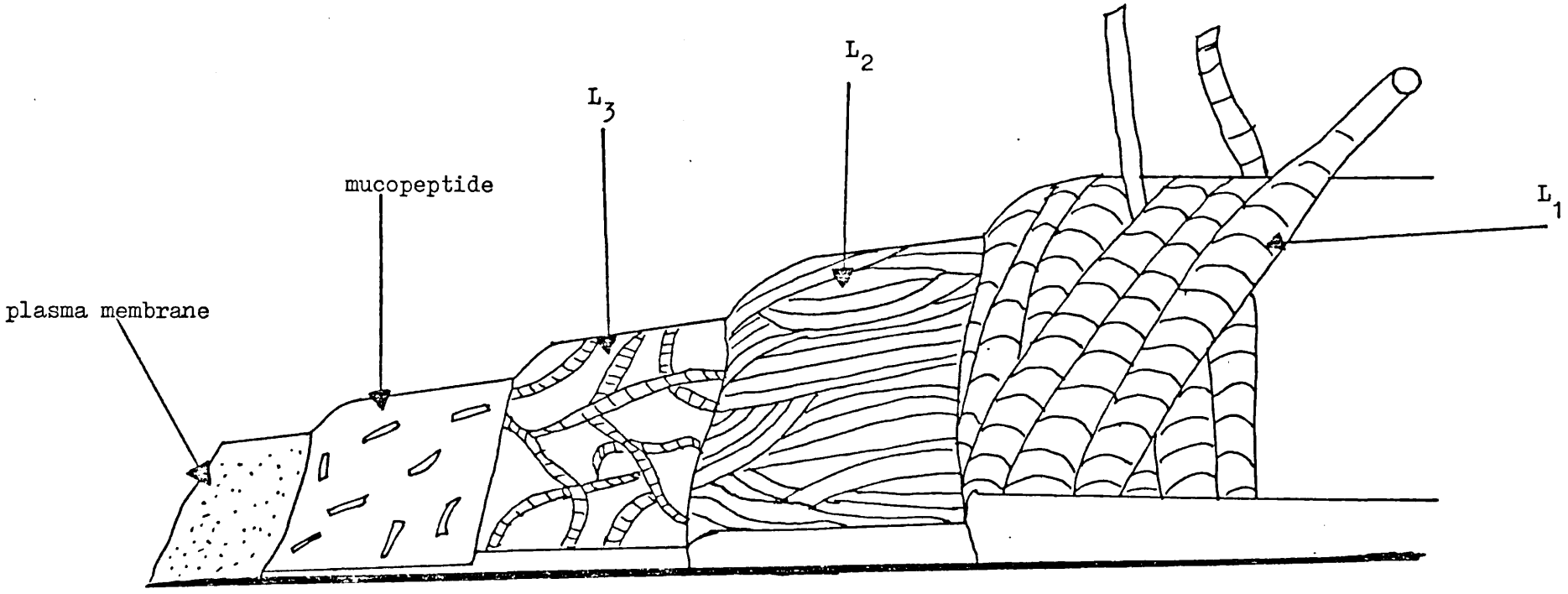
Other possible components of the cell wall are mycosides, glycolipids of mycobacterial origin. Mycoside B has been found in M. tuberculosis and contains a lipid aglycone which is phenol-glycol, the two aliphatic hydroxyl groups of which are esterified by two acyl radicals. The carbohydrate moiety is linked glycosidically to the phenolic hydroxyl group. The aliphatic moiety of the phenol-glycol is structurally closely related to phthiocerol, which is found in M. tuberculosis mostly as dimycocerosate (Asselineau, 1966; Lederer et al, 1975).

A glucan has also been located, probably on the cell wall, and several non-peptidoglycan amino-acids have been identified in hydrolysates of cell walls. These may be present in the cell wall in the form of lipopetides. No techoic acids have yet been found in cell walls of mycobacteria.

The ultrastructure of mycobacterial cell walls has been investigated by transmission electron microscopy with examinations after various chemical extractions. Imaeda (1965) proposed a hypothetical three-dimensional reconstruction of the cell wall of mycobacteria (Figure 1.10.) in which the outer surface is enclosed by a fibrillar lipid substance and the inner cell wall consists of a lipid-mucopolymer polysaccharide complex.

Both chemical elucidation of the cell wall structure and compounds, and transmission electron microscopy studies of mycobacterial cells show that mycobacterial cell walls are extremely complex and contain a large variety of substances. This is verified by immunogenic studies which reveal a large number of surface antigens, suggesting also a very complex outer surface (Harboe et al, 1977).

FIGURE 1.10. Three-dimensional reconstruction of the mycobacterial cell wall from electron micrographs



L_1 = fibrous ropelike structures merging with:

L_2 = more wrinkled and superficial layer,

L_3 = sheetlike surface glycolipid or mucopeptide.

1.4. BCG as an Immunotherapeutic Agent.

BCG has been used to protect against challenge by the virulent tuberculosis organism for over 50 years. It was noticed very early after the initial attenuation of BCG that a "para-specific" action of the organism against secondary and heterologous infections could be observed; this appeared to be of a cellular nature (Hirayama, 1930). This action has since been well confirmed in viral and bacterial infections (Freund et al, 1940; Dubos and Schaedler, 1957; Brown et al, 1968; Anderson et al, 1974).

An antagonism had long been suspected by epidemiologists and doctors between tuberculosis and cancer (Pearl, 1929; Campbell, 1961) and recently the non-specific activity of BCG was found to apply to transplanted tumours, mouse spontaneous leukemia and polyoma-induced tumours in mouse and hamster (Old et al, 1961; Lemonde and Clode-Hyde, 1962; Lemonde et al, 1971). Mathé (1972) and his co-workers obtained relatively long remissions in clinical leukemia of man; this work gave the impetus to BCG-cancer immunotherapy assays in humans. Various extractions of cells of BCG (e.g. Wax D) and cell-wall preparations also cause remission of cancers; addition of paraffin oil to cells of BCG considerably enhances the in vivo dispersion and activity. The present state of BCG as an immunotherapeutic agent in cancer has been recently reviewed (Terry and Windhorst, 1978).

Thus it can be seen that "the BCG organism behaves as an agent of both specific and non-specific cellular immunity and of homologous and heterologous antibody stimulation" (Frappier, 1976).

1.5. Electrophoresis

At any solid-liquid interface there exists an asymmetric distribution of ions such that, on a time average, ions of one sign are predominantly associated with one phase and ions of the opposite sign with the other phase. This results in the formation of an electrical double layer at the interface.

Helmholtz (1879) put forward one of the first theories of the double layer in which he predicted that two parallel layers of charges, of uniform density but of opposite sign, were held a short distance apart with one layer firmly attached to the surface and the other in the liquid; thus the double layer consituted a parallel plate condenser. Gouy (1910) suggested that thermal energy would prevent the formation of such a compact double layer and therefore modified the theory, proposing a diffuse part to this double layer. In this the potential decreased exponentially to zero over the distance $1/\kappa$, the statictical thickness of the double layer, an equilibrium being maintained in the diffuse layer between the opposing forces of the potential field, which tend to order the ions, and the forces of thermal motion, which tend to cause random redistribution.

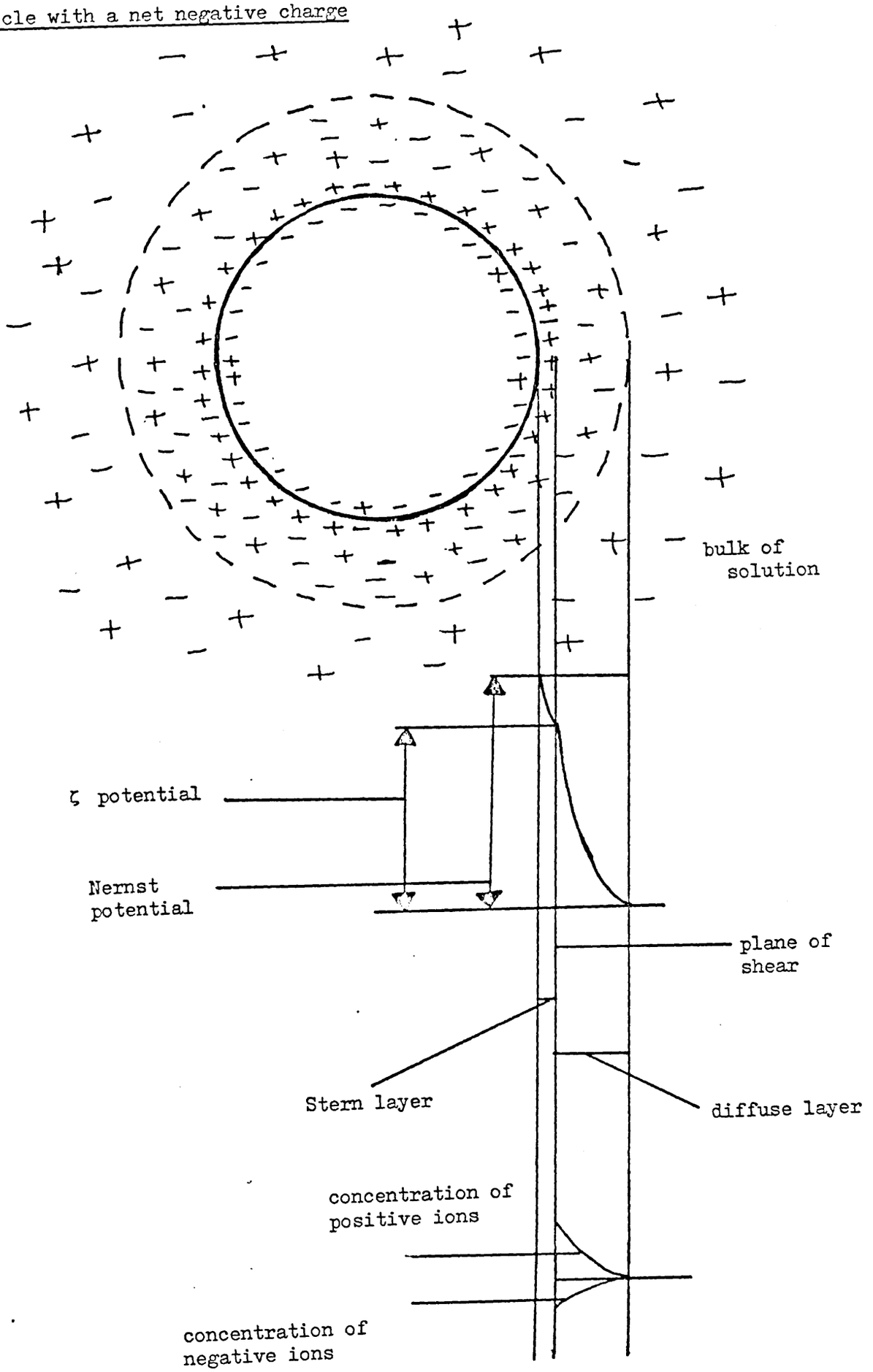
The structure of the double layer which is now widely accepted is that proposed by Stern (1924), which also allows for the finite size of ions. He showed that neither the sharp nor the diffuse double layer theories alone were adequate and developed a theory combining the two. Thus, the double layer can be divided into two parts. The first layer consists of fixed ions attached to the solid surface, partly by electrostatic and partly by van der Waals forces, strongly enough to overcome thermal forces. This layer is approximately a single ion in thickness (the Stern layer) and remains almost in contact with the surface. The second layer is a diffuse atmosphere in which the

Gouy-Chapman theory is acceptable. The fall in potential across the double layer can therefore be divided into two parts, a sharp fall in potential across the Stern layer and an exponential decrease to zero over the diffuse layer. Distribution of the positive and negative ions is not uniform as the electrostatic field at the surface results in attraction of ions of predominantly the opposite sign into the Stern layer. The potential change in the Stern layer increases with the concentration and valence type of the electrolyte, and with polyvalent counter ions it is possible to obtain reversal of charge within the Stern layer. The charge on the surface is of equal magnitude, although opposite in sign, to the total charge of the fixed and diffuse part of the double layer; hence electrical neutrality is maintained. The asymmetric distribution of ions around a negatively charged particle in suspension is shown in Figure 1.11.

Due to this asymmetric distribution of ions it follows that, on application of an electric field, the charged solid phase and the liquid phase will move relative to each other about their plane of shear. This is the phenomenon known as electrophoresis. The potential at this plane of shear is known as the zeta potential and the magnitude of this potential determines particle velocity under an applied electric field. The zeta potential is largely responsible for stabilizing hydrophobic colloids and varies markedly with the nature of the surface and the concentration and pH of the suspending electrolyte.

In suspension at pH 7 all biological cells, mammalian blood and tissue cells, and bacteria carry a net negative charge. This charge originates from the ionization of groups such as carboxyl, phosphate and amino which are located at the cell surface. Adsorption of ions does not occur on bacterial surfaces (Gittens, 1962).

FIGURE 1.11. Schematic representation of charge distribution around a particle with a net negative charge



1.6. Particulate Microelectrophoresis

In this investigation a method based on that developed by Ellis (1911) was used to measure the electrophoretic mobility of bacterial cells. An electric field is applied across a suspension of bacteria enclosed in a glass chamber. The resulting movement of the bacterial suspension is observed with a microscope and individual cells are timed moving across an eyepiece graticule.

When an electric field is applied in such a system, not only will the bacteria move relative to the suspension medium, but the suspension medium will also move relative to the glass surface of the observation chamber as a result of electroosmosis. Thus the observed velocity of the particle, v_o, is given by the expression:

$$v_o = v_L + v_P$$

where v_L is the velocity of the suspension medium relative to the glass surface and v_P the velocity of the bacteria relative to the liquid, this being constant at all depths within the chamber. As this is a closed system, the liquid flows along the two inside faces of the observation chamber towards the negative electrode and returns through the centre, causing a variation of v_L and hence of v_o with depth. As the liquid is being continuously deformed within a closed system there must be a plane at which the liquid is stationary. Thus $v_L = 0$ and $v_o = v_P$ at this level. For the flat cell of rectangular cross section used, this stationary level is observed at two levels equidistant from the cell centre. An expression for the position of these stationary levels has been derived (Komagata, 1933) for a cell with a width/thickness ratio, κ, such that:

$$\frac{s}{d} = 0.500 \pm \left(0.0833 + \frac{32}{\pi^5 \kappa}\right)^{1/2}$$

where s/d is the fractional depth measured from the inside surface. For a cell with a κ value greater than 20, the stationary levels are at 0.21 and 0.79 of the total depth from the inside face (Abramson, 1934) and the mean particle velocity, v, may be determined by the method of Ellis, thus

$$v = \frac{1}{x_1} \int_0^{x_1} v_o \, dx$$

where x_1 is the cell depth and v_o the observed velocity of the particle at that depth. For a symmetrical chamber the curve of v_o against x should be a parabola symmetrical about the centre.

The design of both cell and electrode system has been extensively reviewed (James, 1957; Seaman, 1965; James, 1979). The apparatus used in this investigation was that developed by Gittens and James (1960) in which the applied field strength, X, is best calculated from conductance and current data by the equation:

$$X = \frac{I}{q\kappa}$$

where I/A is the current, κ the conductivity of the suspension medium and q/m^2 the cross sectional area of the cell. Moyer (1936) showed that the use of applied voltage to measure the field strength may lead to errors of up to 50%, as slight changes in the electrodes can result in large changes in the field strength, without affecting the applied voltage.

1.7. Application of Microelectrophoresis to the Study of the Bacterial Cell Surface.

The absolute mobility or zeta potential of a particle in a given suspension at a certain pH-value and ionic strength does not characterize the nature of the surface. The charge on the surface is dependent on the nature and number of ionogenic groups on the surface and also on the properties of the suspending electrolyte (e.g. pH, ionic strength, presence of surfactants). It is essential that electrophoretic measurements be made on cells suspended in a solution of known and defined chemical composition, pH and ionic strength.

Variation of the pH of the suspending electrolyte, maintained at constant ionic strength, can provide valuable information about the nature of the ionizable surface groups on cells of a fixed age. For example, the ionogenic groups of a mixed amino/carboxyl-type surface will be titrated according to the equations:

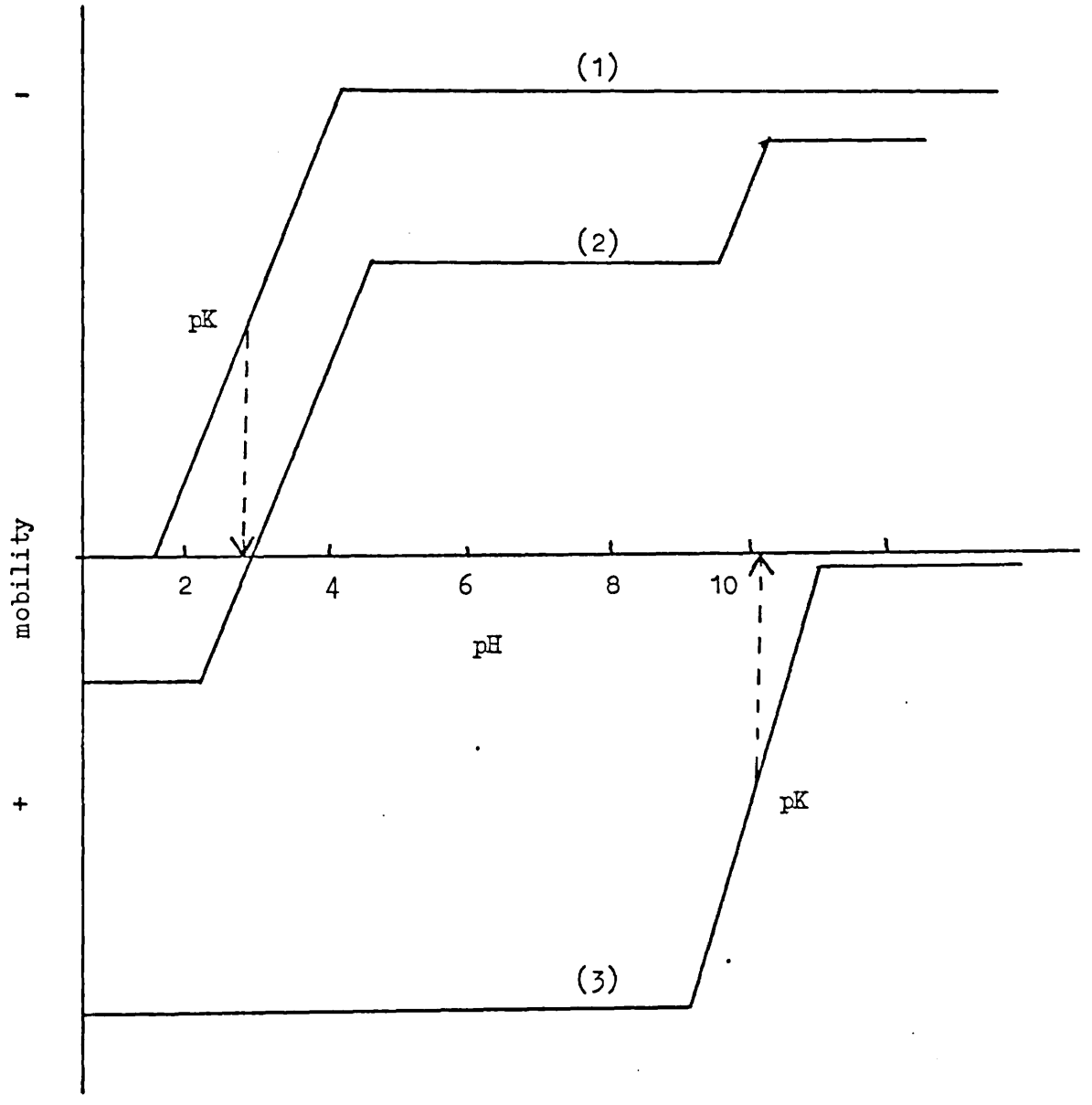
$$RCOO^- + H^+ \rightleftharpoons RCOOH$$

$$RNH_2 + H^+ \rightleftharpoons RNH_3^+$$

The predicted variation of electrophoretic mobility with pH for surfaces possessing anionic and cationic groups is shown in Figure 1.12. As all biological cells carry predominantly negative charges it is customary to plot the negative mobility values above the horizontal axis and the positive mobility values below. In solutions of low pH carboxyl groups will be protonated and so not contribute to the surface charge, at higher pH-values dissociation and ionization occurs and the carboxyl groups will contribute. Curve 1 (Figure 1.12) is a

FIGURE 1.12. Theoretical pH-mobility curves for surfaces containing the following groups:

1. carboxyl
2. carboxyl/amino
3. amino



typical titration curve for a surface with carboxyl groups only. This characteristically simple type of pH mobility curve was obtained for the polysaccharide surface of cells of Klebsiella aerogenes (Lowick and James, 1957), due to the presence of polyglucuronic acid, and for human enythrocytes (Seaman, 1965), due to the presence of neuraminic acid.

For a mixed amino-carboxyl surface a sigmoidal-type curve is obtained as in Curve 2 (Figure 1.12). At low pH-values the amino groups will be protonated and contributing to the charge, whilst the carboxyl groups will be undissociated and thereby not contributing; thus the surface charge will be positive. As pH increases (hydrogen ion concentration decreases), the positive charge becomes reduced and the negative charge increases as the carboxyl groups ionize. An isopotential point occurs between pH 4 and 5, the exact value being dependent on the relative numbers of carboxyl and amino groups present and their respective pK-values. Between pH 5 and 9 a plateau region (or regions) occurs where both the carboxyl and amino groups are fully ionized. The total charge then depends on the relative numbers of anionic and cationic species present. At pH-values higher than 9 the effective negative charge increases due to the supression of the amino group ionization. Mixed amino/carboxyl-type curves have been obtained for cells of Streptococcus pyogenes (Plummer et al, 1961; Hill et al, 1963).

Some pH-mobility curves obtained cannot be explained in terms of simple titration of the surface ionogenic groups as no distinct plateau region is observed. This has been seen for the pH-mobility curves for cells of Pseudomonas aeruginosa (Pechey and James, 1974) and can be related to gentamicin resistance of the cells, and for cells of Staph. aureus due to the presence or absence of teichoic acid

(Hill and James, 1972).

When the pH of the suspending solution is varied care must be taken that no irreversible changes to the cell surface have occurred. After suspending cells at high or low pH-values, the reversibility must be checked by rewashing and measuring the mobility of the cells at pH 7. If the mobility value does not return to the value characteristic of pH 7 then this indicates that the cell surface has undergone change which will make useful interpretation of the results difficult. In all the work reported pH-mobility curves were only plotted over a pH-range which did not cause irreversible damage of the cell surface.

The variation of mobility with pH will only give indications as to the nature and quantity of surface ionogenic groups. Specific chemical and/or enzymatic treatment of bacterial cells can be carried out to modify suspected surface groups before measurement of pH-mobility curves. This enables specific surface groups to be identified.

Some of the earliest work on chemical modification of bacterial cells was described by Cohen (1945) on cells of Bacillus proteus. Cells treated with benzene sulphonyl chloride, which was known to react with amino groups under weakly alkaline conditions, had a higher negative charge than the control cells. This indicated the presence of amino groups on the surface. Other compounds such as p-toluenyl sulphonyl chloride (PTSC) (Douglas, 1959; Gittens and James, 1963) and dinitrofluorobenzene (FDNB) (Gittens, 1962) have been used to block surface amino groups on bacterial cells.

Coddington and Perkins (1961) and Gitten (1962) treated bacterial cells with methanolic hydrogen chloride to esterify surface carboxyl groups. Cells of Klebsiella aerogenes were found to be unaffected by

FDNB, indicating no surface amino groups, but the negative surface charge was reduced to zero on treatment with methanolic HCl showing the charge to arise solely from ionized surface carboxyl groups.

Surface lipid on bacterial cells has been detected by measuring the mobility of cells in the presence of sodium dodecyl sulphate (SDS). If surface lipids are present there is an increased negative mobility due to the interaction of the hydrophobic hydrocarbon chain of the surfactant with the lipid-material, leaving the polar sulphate groups orientated into the medium. This method has been used to detect surface lipid on cells of Staph. aureus (Hugo and Stretton, 1966), K. aerogenes resistant to crystal violet (Lowick and James, 1957) and P. aeruginosa (Pechey, James and Yau, 1974).

Inorganic cations cause specific reversal of charge sequences for various colloids and this has been extended to the study of bacterial surfaces (Bugenberg de Jong, 1949). Using this method the principal ionogenic species on the surface of spores of Bacillus megatherium, B. cereus and B. subtilis was found to be carboxyl (Douglas and Parker, 1957), the surface of cells of M. phlei was found to be composed predominantly of phosphate groups (Adams and Rideal, 1958) and cells of E. coli were found to be coated with acidic polysaccharide (Davies, Haydon and Rideal, 1956).

1.8. Objects of the Present Investigation

The surface of cells of M. bovis BCG has never been studied using particulate microelectrophoresis to determine the charged species present at the cell surface. Although the chemical structure of the cell wall has been well characterised by many workers and comprehensibly

reviewed by Petit and Lederer (1978), the actual physical arrangement of the chemical components within the cell wall structure is unknown. Cells of BCG show a great tendency to aggregate during growth; this must limit the passage of nutrient and oxygen to individual cells and could be at least partially responsible for the very slow growth rates of this organism, as well as making homogeneous vaccine production difficult.

From the original BCG isolate many sub-strains have arisen which differ in their physical and biochemical properties and might possibly differ in their surface properties.

The objects of this investigation were:

1.8.1. To establish the surface properties of cells of BCG using the technique of particulate microelectrophoresis. To examine the shape and position of the pH-mobility curve for cells of BCG after growth on different media and during the growth cycle to see if changes in these surface properties were observed.

1.8.2. To carry out specific chemical and enzymatic modification of the cell surface to identify surface charged groups on cells of BCG.

1.8.3. To compare the pH-mobility curves for cells of 5 sub-strains of BCG to see whether inter-strain differences in biological and physical properties, such as colony form, are related to differences in surface charge.

1.8.4. To compare the surface properties of BCG with those of other mycobacterial species.

1.8.5. To examine the surface properties of cell wall preparations of BCG.

1.8.6. To examine the surface of cells of BCG using electron microscopy.

Most emphasis has been placed on the use of particulate electrophoresis to detect charges on the bacterial cell surface, but other techniques such as scanning and transmission electron microscopy have also been used.

CHAPTER 2

EXPERIMENTAL TECHNIQUES

2.1. Bacteriological Techniques

2.1.1. Strains

The bacterial strains used in this work are listed in Tables 2.1., 2.2. and 2.3.

Table 2.1.

Sub-strains of Mycobacterium bovis BCG

Strain	Country of origin	Usual culture maintainance
Glaxo (1077)	U.K.	Dispersed culture in the presence of wetting agent.
Pasteur (1173)	France	As surface pellicle on Sauton medium.
Danish (1331)	Denmark	"
Prague	Czechoslovakia	"
Japanese	Japan	"

Table 2.2.

Isolates of Glaxo M. bovis BCG

Code number	Description
1077 R	"Rough" isolate of 1077 BCG
1077 S	"Smooth" isolate of 1077 BCG
INDR	Isoniazid resistant strain of 1077 BCG.

Table 2.3.

Other bacterial species

Species	Source
Mycobacterium smegmatis	Glaxo Research, Greenford
Mycobacterium phlei	"
Mycobacterium microti	"
Klebsiella aerogenes (NCTC 418)	NCTC

The mycobacterial strains were all provided as freeze-dried ampoules by Glaxo Research Ltd., Greenford, Middlesex and were as close to the masterseed of each country of origin as possible.

The strain of Klebsiella aerogenes (NCTC 418) used for calibration of the electrophoretic apparatus was stored in 50 cm^3 of nutrient broth in a tightly capped 100 cm^3 medical bottle at 4 ^{o}C. This was routinely maintained by bi-monthly subculture into fresh nutrient broth.

2.1.2. Media

The compositions of the culture media used for growth of the mycobacterial strains are shown in Tables 2.4. and 2.5.

Nutrient broth was prepared by adding 13 g of Oxoid Nutrient Broth (CM1) powder to 1 dm^3 of distilled water. This was then distributed into final containers in appropriate volumes (usually 50 cm^3 of broth in a 100 cm^3 medical bottle) and sterilized by autoclaving at 15 lb $inch^{-2}$ for 20 minutes.

Table 2.4. Media designed to grow mycobacteria

Production culture medium		Dubos medium		Sauton medium (for surface culture)	
L-sodium glutamate	2 g				
L-asparagine	2 g	L-asparagine	2 g	L-asparagine	4 g
L-glutamine	1 g			Citric acid	2 g
Bactocasitone	1 g	Bactocasitone	0.5 g		
$Na_2 HPO_4$ (anhydrous)	1.4 g	$Na_2 HPO_4$	0.7 g		
$KH_2 PO_4$ (anhydrous)	5.5 g	$KH_2 PO_4$	2.75 g	$K_2H PO_4$	0.5 g
Ferric ammonium citrate	50 mg	Ferric ammonium citrate *	50 mg	Ferric ammonium citrate	50 mg
Magnesium citrate	5 mg	Magnesium sulphate	10 mg	Magnesium sulphate	0.5 g
Calcium chloride	0.5 mg	Calcium chloride	0.5 mg		
Zinc sulphate	0.1 mg	Zinc sulphate	0.1 mg		
Copper sulphate	0.1 mg	Copper sulphate	0.1 mg		
Triton 1:20 solution	5 cm^3	Tween 80	0.2 g	Glycerol	40 cm^3
Distilled water to	1000 cm^3	Distilled water to	900 cm^3	Distilled water	1000 cm^3
Sterilized by autoclaving		Sterilized by autoclaving. 100 cm^3 5% bovine albumin fraction V, 7.5% glucose added after filter sterilization.		Sterilized by autoclaving	
pH 6.3		pH 6.3		pH 7.2	

* Solution kept in dark.

Table 2.5.

Solid Dubos medium for culture of Mycobacteria

AGO complex

1. Glucose 25 g
   NaCl 5 g
   } in 500 cm^3 sterile water

2. 0.6 cm^3 oleic acid
   0.25 cm^3 NaOH (0.05 mol dm^{-3})
   } in 50 cm^3 sterile water

26.65 cm^3 of (2) were added to (1) and then 25 g of bovine serum albumin.

Sterilized by filtration through a millipore filter.

Dubos Oleic Acid Agar (DOA)

10 cm^3 AGO complex and 5 cm^3 of horse blood were added to 100 cm^3 of DOA base (Difco) which had been sterilized by autoclaving.

Nutrient agar was prepared by the addition of 28 g of powdered Oxoid Nutrient Agar (CM3) to 1 dm^3 of distilled water. The solution was heated to boiling point to ensure that all the agar was dissolved and then distributed in 350 cm^3 lots in 500 cm^3 screw top bottles and sterilized by autoclaving.

Agar plates were prepared by melting the stock agar in an autoclave and pouring appropriate volumes into sterile plastic pertri dishes. These plates were dried for 10 minutes at 55 ^{o}C before use.

Sterilization of media by autoclaving was at 15 lb $inch^{-2}$ for 20 minutes.

2.1.3. Growth of strains for experimental use.

An ampoule of a freeze-dried strain of mycobacteria was reconstituted with 1 cm^3 of sterile distilled water and cultured in one of the media; the growth characteristics are described in Table 2.6.

Table 2.6.

Growth characteristics of mycobacterial strains in different media.

Medium	Growth characteristics
Dubos	Both contain non-ionic wetting agent. Cells grow as well dispersed cultures.
Production	
Sauton	No wetting agent. Cells grow as a surface pellicle.
Dubos Oleic Acid Agar	Solidified Dubos medium. Cultures grow as clustered colonies.

All cultures were incubated for 7-21 days at 37 °C under static conditions.

Cultures grown in Dubos medium or on DOA agar were inoculated straight from the reconstituted ampoule. Cultures grown on Sauton medium were subcultured once in Dubos medium and then transferred to Sauton medium. On incubation at 37 °C the cells rose to the surface. Cells grown in Production medium were also initially subcultured in Dubos medium. Cultures in Dubos medium were maintained by bi-weekly subculture of 1 cm^3 of 14-day old culture into 20 cm^3 of fresh Dubos medium. After 10 subcultures these cultures were destroyed and new ones started by reconstitution of a new ampoule.

Cultures on Sauton medium were also maintained by transfer of some of the surface pellicle to fresh medium using a sterile platinum loop.

Cells of the Glaxo sub-strain of BCG to be examined after passage through an animal were given as a 5 mg inoculum into the thigh muscle of a guinea pig. After 6 weeks the organisms were reisolated onto DOA agar from puss in the thigh lesion and from the spleen. The DOA plates were incubated for 8 weeks at 37 °C.

Cells of Klebsiella aerogenes required for calibration of the electrophoresis apparatus were inoculated from the stock parent culture into fresh nutrient broth using a sterile platinum loop and incubated for 18 h at 37 °C.

2.1.4. Sterility checking of mycobacterial strains

The sterility of the slow-growing mycobacterial strains was checked after a few days incubation by transfer of some of the culture to nutrient broth and nutrient agar using a sterile platinum loop. Any growth observed after overnight incubation at 37 °C indicated contamination by non-mycobacterial species.

2.1.5. Cleaning and sterilization of apparatus

Contaminated disposable apparatus was immersed in 1% lysol solution before disposal. Contaminated agar plates were immersed in the lysol solution for several weeks before disposal.

Contaminated glassware was autoclaved before washing. Contaminated glass pipettes were immersed in a solution of Milton immediately after use and prior to autoclaving.

All glassware was washed and scrubbed in hot tap water, rinsed twice in distilled water and dried in an oven at 100 °C. Sometimes a small quantity of Decon was required to remove all traces of Dubos cultures after autoclaving; glassware was then rinsed very thoroughly in tap water and twice in distilled water.

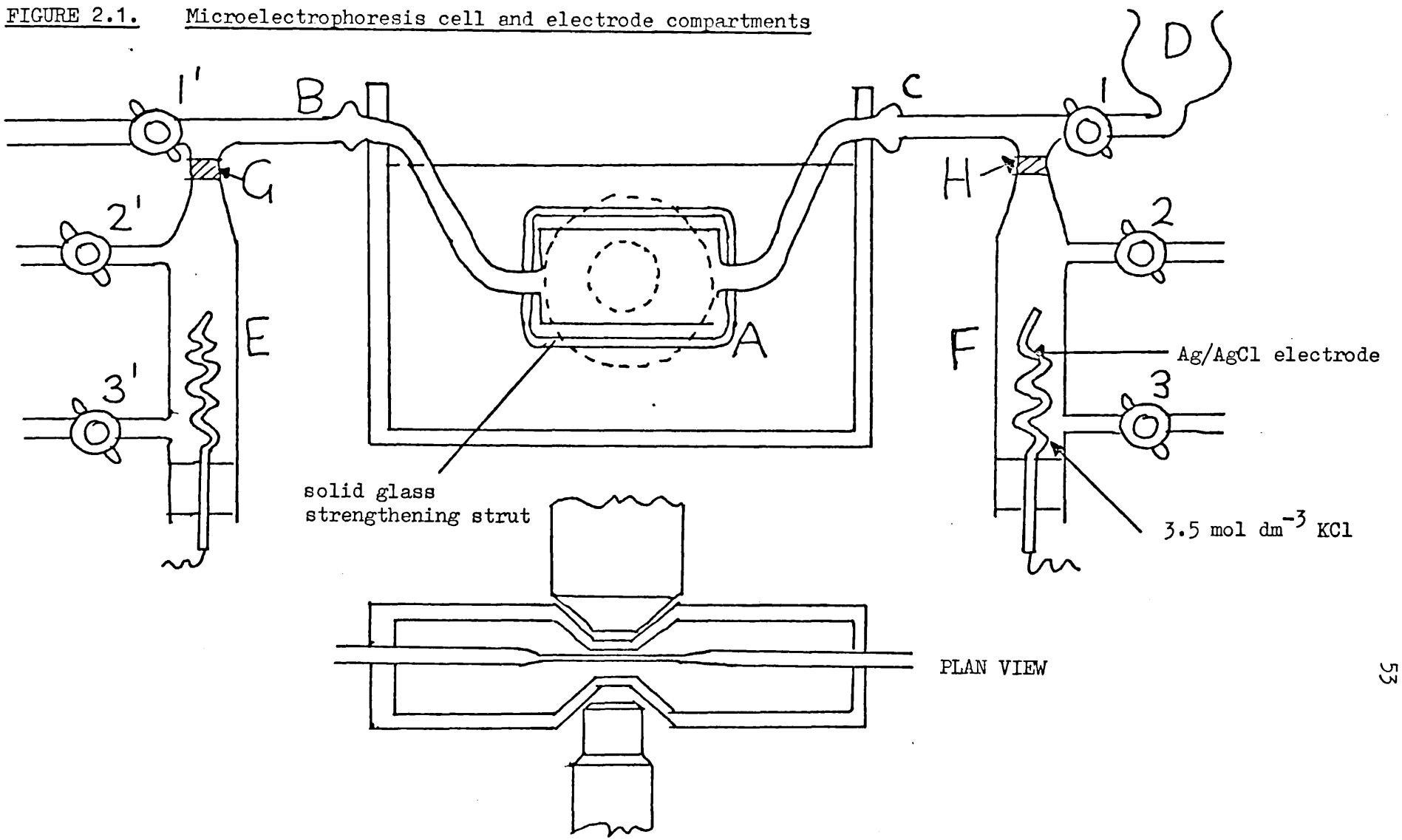
2.2. Microelectrophoretic Techniques

2.2.1. General description of apparatus

A Rank Bros. Particulate Microelectrophoresis Apparatus Mark II was used; this included a small water bath and circulating pump to maintain the electrophoretic cell at 25.0 ± 0.05 °C, an electrical circuit, a microscope unit and an electromagnetic timer. The cell and electrode compartments used were those developed by Gittens and James (1960). The apparatus is shown in Figure 2.1.

An electric field was applied across a suspension of bacterial cells contained in the glass observation chamber A. This rectangular glass chamber was made from two optically flat Hysil plates (40 x 25 x 0.5 mm) fused to give a separation of 0.5 mm. Glass rods sealed around the outside of the cell gave protection and strengthened the structure. The cells were observed under a microscope and the velocity of the cells due to the known electric field measured by the timing of

FIGURE 2.1. Microelectrophoresis cell and electrode compartments



individual cells across an eyepiece graticule.

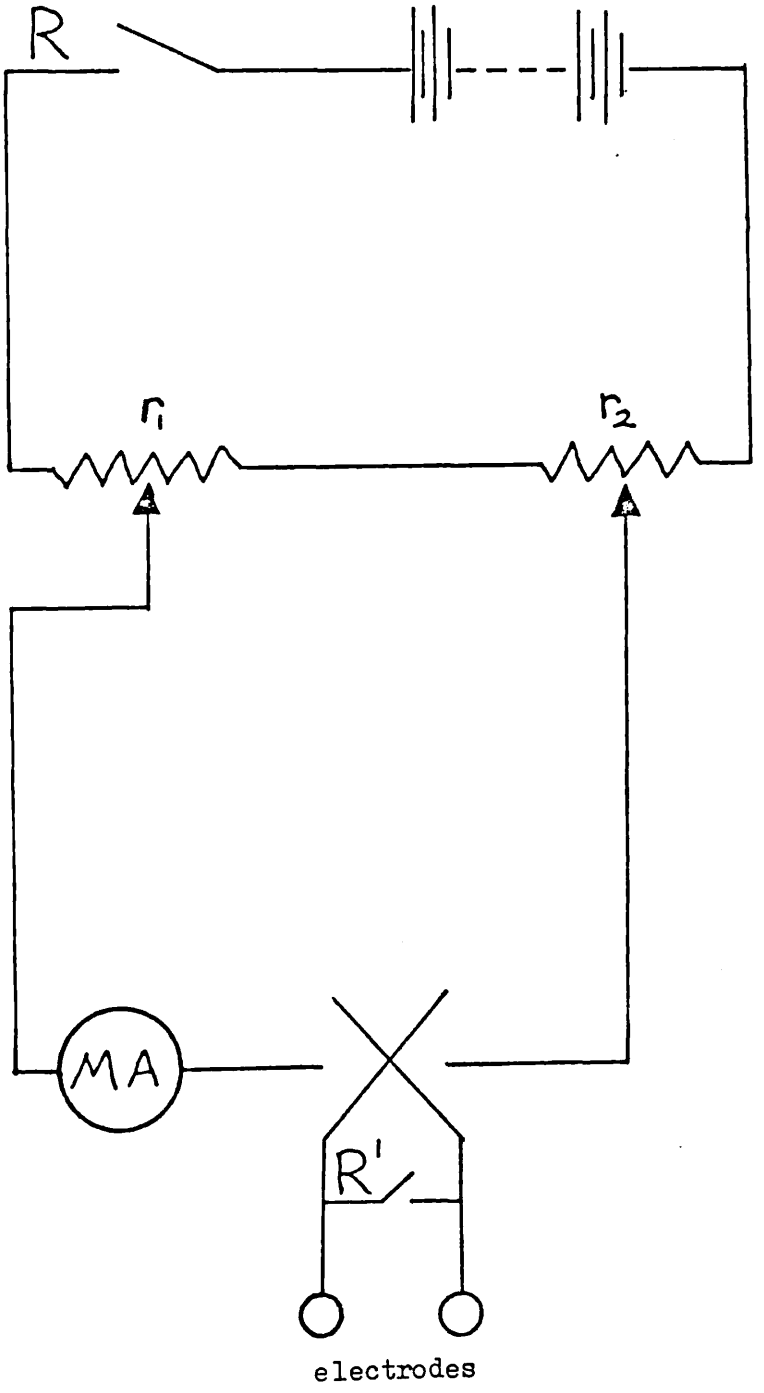
The observation chamber was connected to the electrode compartments via side arms constructed by sealing 10 mm bore Pyrex tubing directly to the chamber (A). These were bent in the plane of the cell so that the chamber could be completely immersed in the water bath. Connection of the side arms to the electrode compartments was by hemispherical glass ground joints (B and C). These were greased and metal clips attached to ensure leak-proof seals. Bacterial suspensions were introduced into the cell from the reservoir (D) by opening taps 1 and 1'.

A constant electric field was applied between the Ag. AgCl/KCl electrode systems in the compartments E and F. The electrodes consisted of 25 cm lengths of 2 mm diameter silver wire coiled and mounted in the compartments by means of rubber bungs, through which the wire passed, forming a water-tight seal. Initially the silver wire was thoroughly cleaned by abrasion with fine sandpaper, coiled and then immersed in concentrated ammonia solution and then in 50% (v/v) nitric acid. The electrodes were then anodised in series in 0.1 mol dm^{-3} hydrochloric acid using a platinum cathode, until a purple-grey coating of silver chloride was deposited on each electrode. The electrodes were then placed in their compartments (E and F) which were filled with potassium chloride solution (3.5 mol dm^{-3}). Electrical contact with the bacterial suspension was made through the sintered glass discs (G and H). The electrode compartments were refilled daily, before use, with fresh electrolyte solution from reservoirs via taps 2 and 3 and 2' and 3'.

The electrical circuit is shown diagrammatically in Figure 2.2. The mains voltage was rectified and the DC voltage controlled by a variable resistance. The applied potential could by reversed using the switch K', which also allowed the electrodes to be shorted when not

FIGURE 2.2.

The electrical circuit for the microelectrophoresis apparatus



in use, thus preventing polarisation. A milliammeter was used to measure the current flowing through the electrophoresis chamber. The time taken by a bacterium to cross a given number of squares on the eyepiece graticule of the microscope was recorded using an electromagnetic timer, operating from the mains frequency.

It is essential to maintain the bacterial suspension at a constant temperature during measurements since the viscosity and conductivity of the buffer solutions and hence the electrophoretic mobility are temperature dependent. This was achieved by immersion of the observation chamber in a small water bath which was maintained at $25^{\circ} \pm 0.5\ ^{\circ}C$ by water circulated from a large water bath. The latter was maintained at the required temperature by a Shandon heating and stirring unit. The small perspex water bath had a circular well at the front to accommodate the objective lens of the microscope. The microscope used gave an overall magnification of X 600 and a low-power dark ground illuminating condenser was employed. The light source was a 12 V, 100 watt lamp and to prevent the intense light source from causing temperature rises and convection currents in the observation chamber a quartz disc and glass heat filter were placed in front of the lamp.

The observation chamber was mounted in the water bath in the lateral position between the condenser and objective lens of the microscope. Bolts attached to perspex bars held the chamber firmly in the water bath; care was taken to ensure that the cell was horizontal when viewed from the front, vertical when viewed from the side and at right angles to the optical axis of the microscope. Protective sponge foam was wrapped around the arms before tightening the bolts. Before assembly, cells of K. aerogenes were dried onto the inner surfaces of the observation chamber providing reference particles for focusing on the inner surfaces for the location of the stationary levels of the cell.

2.2.2. Mode of operation

To ensure good and reproducible electrical connections through the sintered glass discs the following procedure was carried out each time before the use of the apparatus.

50 cm^3 of KCl solution (3.5 mol dm^{-3}) was flushed through the electrode compartments by opening taps 3 and 2 and 3' and 2'. Taps 2 and 2' were then closed and tap 1' opened causing KCl solution to be forced through the sintered glass discs so saturating them. The observation chamber was then flushed through with a large volume (50 cm^3) of distilled water to remove all electrolyte which had been forced through the discs. The observation chamber was then filled with buffer solution at the correct temperature, pH and ionic strength of the suspension to be examined. Great care was taken throughout to ensure that no air bubbles became trapped in the closed system and to prevent the introduction of grease into the observation chamber.

The microscope was then focused, by movement of the objective by coarse and fine micrometer screws, onto the reference bacteria coated on the front and back inner surfaces of the chamber. This allowed the depth of the chamber to be recorded from the calibration on the fine adjustment screw. In practice little variation in the chamber depth was observed from day to day. This scale was also used to locate bacteria in focus at the experimentally determined stationary levels within the cell; all mobility measurements were made at the front stationary level.

The cell suspension under investigation was then introduced into the observation chamber and allowed to come to thermal equilibrium. The velocity of cells selected at random and in focus at the stationary level was measured by recording the time taken for the cells to cross a given number of squares on the eyepiece graticule. A time of 2 - 4 s was considered suitable and the applied electric field was adjusted

accordingly. For each suspension at least 40 cells were timed, 20 in each direction. Change of direction was achieved by reversing the polarity of the applied field using reversing switch R'. The potential was never applied for long periods of time in either direction in order to minimise electrode polarisation and gassing during operation.

The suspension was then flushed out with distilled water through the rubber tubing, using tap 1', into a reservoir containing lysol, and the chamber was left filled with distilled water. The chamber was fed by gravity, but to remove any air bubbles a partial vacuum could also be applied.

2.2.3. Calibration of the apparatus

The symmetry of the observation chamber was checked by determining the velocity-depth curve using washed cells of K. aerogenes (2.2.5). For a symmetrical chamber the curve should be a parabola symmetrical about the centre.

Bacteria in focus were timed at various known cell depths across a fixed distance in the eyepiece graticule at a constant field strength. The fractional chamber depth from the centre of the chamber was plotted against the reciprocal of the excursion time of the bacterial cells. Typical results obtained for the cell used are shown in Figure 2.3.

The equation of the velocity-depth parabola is of the form:

$$\frac{1}{t} = a + bx + cx^2 \qquad 2.1.$$

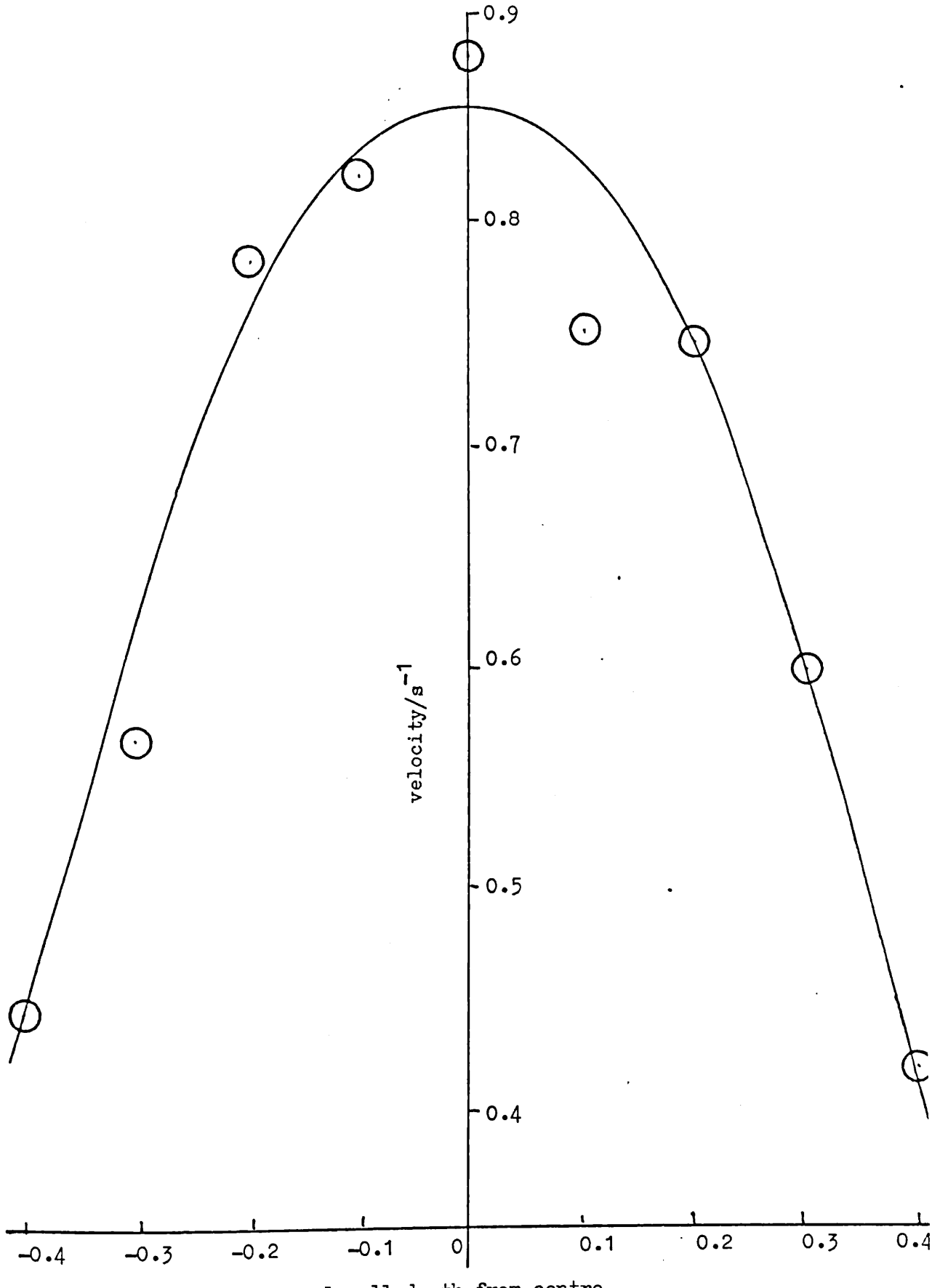
where x denotes the fractional depth from the centre of the cell and t the excursion time measured at this depth.

The equation obtained for the cell in use was:

$$\frac{1}{t} = 0.842 - 0.0188x - 2.599x^2 \qquad 2.2.$$

FIGURE 2.3.

Velocity-depth curve for cells of K. aerogenes measured at 25 °C in veronal acetate buffer solution (I = 0.02 mol dm^{-3}, pH = 7.0)



The low value of the coefficient of x indicates that the calculated and geometrical centres are very close and therefore the chamber can be accepted as symmetrical. Integration of equation 2.2. over the complete chamber depth gives the mean reciprocal time as 0.621 s^{-1}. Substitution of this time back into equation 2.2. gives values of x corresponding to the positions of the two stationary levels; in this case 0.288 and -0.295 from the centre of the chamber. Therefore the stationary levels are at fractional depths of 0.212 and 0.79 from the front inside surface of the cell; these positions are in close agreement with those predicted theoretically for a cell of the same size (Abramson, 1934) which are at fractional depths of 0.21 and 0.79 from the inside face.

The electrophoretic mobility of a particle, $\bar{\nu}/m^2\ s^{-1}\ V^{-1}$, is defined as the particle velocity, $\nu/m\ s^{-1}$, per unit potential gradient, $X/V\ m^{-1}$. It is given by the expression:

$$\bar{\nu} = \frac{\nu}{X} = \frac{n\,L}{t}\ \frac{q\,k}{I} = \frac{n\,L}{t}\ \frac{q\,J\,G}{I} \qquad 2.3.$$

where nL/m is the distance travelled (n is the number of squares of side L/m) in time t/s, q/m^{-2} is the cross sectional area of the chamber and I/A is the current flowing. $k/ohm^{-1}\ m^{-1}$ is the conductivity of the buffer solution obtained from the measured conductance, G/ohm^{-1}, and the cell constant, J/m^{-1}, of the conductance cell.

The values of G, I and t are obtained experimentally. However, since it is not possible to determine accurately the cross sectional area (q) of a rectangular observation chamber, a standard particle of known absolute mobility ($\bar{\nu}_s$) was used. Standard particles in suspension were timed and an apparatus constant κ, which included the cell constant J of the conductance cell, was obtained; κ is given by:

$$\kappa = L\,q\,J = \frac{\bar{\nu}_s\ t\ I}{n\ G} \qquad 2.4.$$

Subsequent timings (t') on bacterial cells under examination were converted to mobility values using the relationship:

$$\bar{v} = \frac{\kappa n G'}{t' I'} \qquad 2.5.$$

where the primed values are those obtained for cells in that particular suspension.

The standard particles used in this investigation were washed cells of K. aerogenes grown for 18 h in nutrient broth at 37 °C (2.2.5). Under these standard conditions the cells have an absolute mobility of -1.67×10^{-8} m^2 s^{-1} V^{-1} at 25 °C. This value was obtained from extensive calibration studies of suspensions of K. aerogenes against human erythrocytes as standard (Gittens, 1962). κ was determined before each set of electrophoretic measurements, to ensure there were no changes in mobility which could be due to changes of the apparatus constant.

In quoting mobility values, 10^{-8} and the units are not expressed and all values are negative unless prefixed by +. A value of 1.5, for example, means that the particle is negatively charged with an electrophoretic mobility of $1.5 \times 10^{-8}/m^2$ s^{-1} V^{-1}.

2.2.4. Buffer solutions

Barbiturate-acetate buffer solutions were used as the suspending electrolyte (Michaelis, 1931) for mobility determinations. AnalaR grade chemicals were used and dissolved in glass-distilled water. 2 dm^3 of stock solution (I = 0.5 mol dm^{-3}) contained:

0.15 mol dm^{-3} sodium barbitone	61.854 g
0.15 mol dm^{-3} sodium acetate (trihydrate)	40.824 g
0.20 mol dm^{-3} sodium chloride	23.376 g

The stock solution was stored at 4 °C. The buffer solutions required for electrophoretic determinations were prepared by diluting with distilled water to the required ionic strength. The pH (range 2.5 - 10.5) was adjusted by the addition of either HCl (1 mol dm^{-3}) or NaOH (1 mol dm^{-3}). A glass/calomel electrode assembly was used to measure the pH in conjunction with an E.I.L. (Model 23A) pH meter. The conductance of each buffer solution was measured in a bottle-type conductivity cell at 25 °C using a Wayne-Kerr (B8221) Universal Bridge.

Some specific chemical treatments of the cells were carried out in phosphate buffer solution (I = 0.05 mol dm^{-3}, pH = 7.4). Each dm^3 of this solution contained 6.3672 g disodium hydrogen phosphate (hydrated) and 0.9048 g potassium dihydrogen phosphate.

2.2.5. Preparation of cell suspensions for electrophoresis

As particulate microelectrophoresis had never been carried out on cells of Mycobacterium bovis BCG before, a set of standard conditions had to be established for the preparation of the cells for electrophoresis (4.1) and these were adhered to for all measurements.

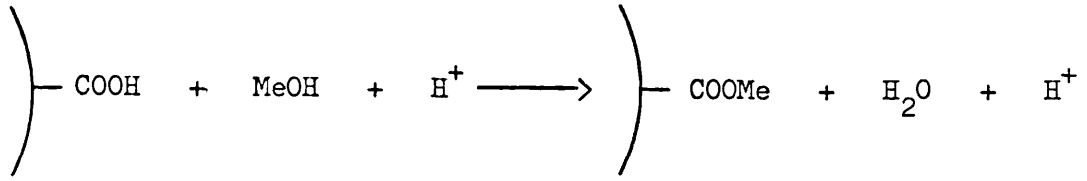
Cells of K. aerogenes grown for 18 h in nutrient broth at 37 °C were harvested by centrifugation, washed twice in barbiturate-acetate buffer solution (I = 0.02 mol dm^{-3}, pH = 7.0) and resuspended in that solution.

Mobility measurements were generally made as soon as possible after preparation of the cell suspensions. However, some longer chemical treatments required the cells to be stored overnight at 4 °C. Storage at 4 °C for up to 48 h did not have any effect on the mobility values of the cells.

2.3. Modification of the Bacterial Cell Surface to Detect Surface Groups

2.3.1. Detection of carboxyl groups

For detection of ionizable surface carboxyl groups the cells of BCG were treated with methanolic hydrogen chloride (Coddington and Perkins, 1961; Gittens, 1962). In this way free carboxyl groups were esterified and hence prevented from contributing to the total charge.



Methalonic hydrogen chloride was prepared by passing HCl gas through AnalaR methanol. The resulting solution was titrated against sodium hydroxide solution and the concentration of HCl adjusted accordingly to 0.2 mol dm^{-3} by dilution with methanol.

21-day-old cells of BCG (Glaxo sub-strain) were harvested by centrifugation, washed once in barbiturate-acetate buffer solution (I = 0.05 mol dm^{-3}, pH = 7.0) and twice in 0.85% NaCl solution (pH = 2.5) to ensure any carboxyl groups were in the protonated form. The cells were suspended, by tissue-grinding, in methanolic HCl solution (0.2 mol dm^{-3}) and shaken at 40 °C. Further samples were suspended in the methanolic HCl solution, stirred and refluxed at 60 °C. Control samples were suspended in methanol alone under the same conditions.

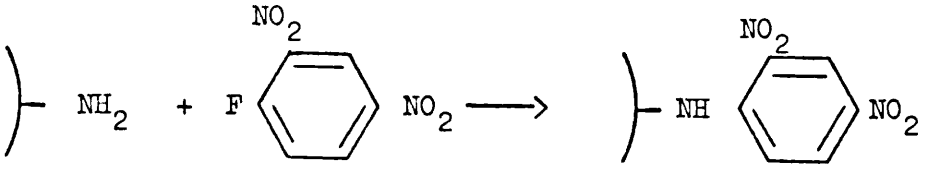
Samples were removed at various time-intervals, washed twice in barbiturate-acetate buffer solution (I = 0.05 mol dm^{-3}) and their electrophoretic mobility values measured at pH 3 and 6.

A lowering of negative surface charge compared to untreated, control samples is indicative of ionogenic surface carboxyl groups.

2.3.2. Detection of amino groups.

The presence of ionizable surface amino groups was investigated using a modified version of the Ingram and Salton method (1957) described by Gittens (1962). This method depends on the blocking of free amino

groups to give the dinitrophenyl derivatives; thus the amino groups cannot ionize and their contribution to the total charge is removed.

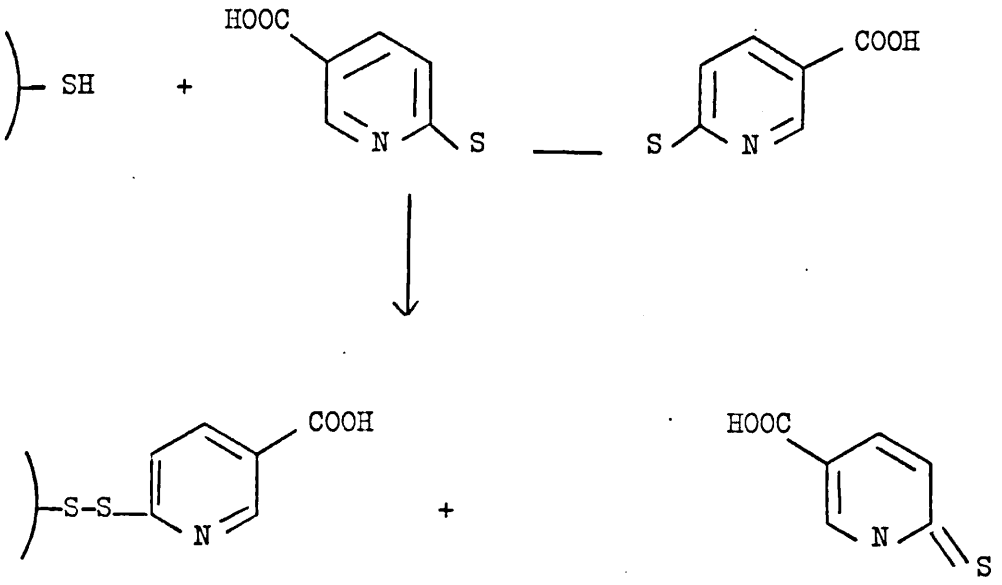


14 day old cells of BCG (Glaxo sub-strain) were harvested and washed twice in phosphate buffer solution ($I = 0.5$ mol dm^{-3}, pH = 7.0). The cells were then treated with various amounts of a 0.2% solution of ethanolic 2,4-dinitrofluorobenzene (FDNB) made up to 20 cm^3 with ethanol and mixed with 5 cm^3 of half saturated sodium bicarbonate solution. Control samples were set up using ethanol and sodium bicarbonate alone. Samples removed after 5 h at room temperature were washed twice in absolute alcohol to remove any excess FDNB and then twice in barbiturate-acetate buffer solution ($I = 0.05$ mol dm^{-3}) and their mobility values determined at pH 3 and 6.

Any increase in the negative mobility value of the cells compared to untreated control samples indicates the presence of amino groups at the surface.

2.3.3. Detection of surface sulphydryl groups.

The method for detecting surface sulphydryl groups is that described by Mehrishi and Grassetti (1969) in which the disulphide bond of dithiodinicotinic acid splits up and one half forms a disulphide bridge with the cell surface sulphydryl group; this results in the introduction of one negative charge for each thiol group reacted.



14 day old cells of BCG (Glaxo sub-strain) were harvested by centrifugation and washed twice in barbiturate-acetate buffer solution ($I = 0.05$ mol dm^{-3}, pH = 7.0). The cells were then suspended by tissue-grinding in a solution of 6,6'-dithiodinicotinic acid (carboxypyridinedisulphide, CPDS) of concentration 1.12×10^{-4} mol dm^{-3}. Samples were removed after various times, harvested and washed twice in barbiturate-acetate buffer solution ($I = 0.05$ mol dm^{-3}, pH = 7.0) and their mobility determined at pH 7.0.

Treatment of CPDS-treated cells with L-cysteine reverses the reaction with the sulphydryl groups; therefore two samples of cells were removed at different times during treatment with CPDS and treated with a solution of L-cysteine (10^{-3} mol dm^{-3}) for 15 min. The cells were then harvested, washed with barbiturate-acetate buffer solution and their mobility determined.

An increase in negative mobility compared to untreated control cells would indicate the presence of surface sulphydryl groups.

2.3.4. Detection of surface lipids.

Surface lipids on any cell can be detected by comparing the mobility of cells suspended in buffer solution with the mobility of cells of the same sample suspended in the same buffer solution but containing varying concentrations of sodium dodecyl sulphate (SDS). In the presence of low concentrations of anionic surfactants any surface lipid material will interact, probably by hydrogen-bonding, with the hydrophobic portion of the surfactant. In this way the negative charge of the surface will be increased due to the charged polar sulphate groups of the SDS, which are orientated into the medium.

The cells of BCG were harvested by centrifugation, washed in barbiturate-acetate buffer solution ($I = 0.05$ mol dm^{-3}, pH = 7.0 and 4.0) and then suspended in buffer solutions containing various concentrations of SDS. The mobility values of these cells were compared with those of control samples suspended in buffer solution in the absence of SDS.

An increase in negative mobility greater than 10% in the presence of 10^{-4} mol dm^{-3} SDS is usually considered significant and the size of the increase taken to be indicative of the amount of surface lipid present.

Detection of surface lipids was also carried out in buffer solutions of varying ionic strengths to detect any lipid located further into the cell wall structure. Cells were prepared and suspended in buffer solutions containing SDS (10^{-4} mol dm^{-3}, pH = 7) over the ionic strength-range 0.02 to 0.1 mol dm^{-3}. The mobility of these cells was compared to that of control cells in buffer solution alone.

2.3.5. Extraction of cells with cold chloroform/methanol.

Extraction of mycobacterial cells with cold chloroform/methanol is known to remove certain lipids (e.g. Wax D, cord factor) from the cell wall. These are known as "free-lipids".

Surface grown cells of BCG (Glaxo sub-strain) were harvested, washed once in phosphate buffer solution ($I = 0.05$ mol dm^{-3}, pH = 7.0) and twice in AnalaR methanol to remove all water. The cells were then tissue-ground into chloroform/methanol (2:1, v/v) and kept in suspension by shaking at room temperature for 24 h. The cells were then harvested, washed twice with barbiturate-acetate buffer solution ($I = 0.05$ mol dm^{-3}) and the mobility values determined. Control samples were shaken in buffer solution alone for 24 h before preparation for electrophoresis.

2.3.6. Enzymatic detection of phosphate groups.

Various enzymes were used in an attempt to detect phosphate groups believed to be present on the surface of cells of BCG. These were acid phosphatase (Koch/Light; ex wheat germ, freeze-dried) which hydrolyses phosphomonoesters with the liberation of orthophosphate; phosphodiesterase (BDH; freeze-dried powder from crotalus adamanteus (rattle snake) venom) which hydrolyses diesterified phosphate with the liberation of one of the alcohol groups to leave a phosphomonoester; and phospholipase C (Koch/Light; ex Clostridium perfringens, salt-free) which cleaves the phosphate diester bond in phospholipids, liberating inorganic phosphate.

Surface grown cells of BCG (Glaxo sub-strain) were harvested and washed in barbiturate-acetate buffer solutions ($I = 0.05$ mol dm^{-3}) at the optimum pH-value for the enzymic treatment; this was pH 7.3 for the phospholipase C, pH 8.8 for the phosphodiesterase and pH 4.8 for the acid phosphatase.

The cells were then suspended in a 0.01% solution of the enzyme, made up in barbiturate-acetate buffer solution ($I = 0.05$ mol dm^{-3}) of the optimum pH for that enzyme, and incubated for 24 h at 37 °C. Control samples were incubated in buffer solutions alone for the same period.

Further samples of cells were autoclaved for 15 min at 15 lb $inch^{-2}$ before preparation and suspension in the enzyme solutions.

After 24 h incubation the cells were harvested and washed twice in barbiturate-acetate buffer solutions ($I = 0.05$ mol dm^{-3}, pH = 3,5,7 and 9) and the mobility values determined.

Any change in the mobility value of the enzyme-treated cells compared to that of the control cells would indicate that the enzyme had affected the cell surface. Control samples treated with heat-inactivated enzyme preparations were included to show whether any changes were due to adsorbed enzyme on the surface or to the hydrolysis of surface phosphate groups by the active enzyme.

2.3.7. Specific sequence of reversal of charge by inorganic cations

Inorganic cations will cause the reversal of charge of various colloids, and specific patterns to the sequence of this charge reversal by a variety of cations characterise the different colloidal species. For carboxyl and sulphate colloids specific sequences among comparable ions are observed and the hexol ion shows an extremely low reversal of charge concentration, while for phosphatides the uranyl ion reverses charge at a much lower concentration than the comparable divalent cations Pb, Cu, Cd and Zn. This effect can and has been extended to bacterial cells, the sequence of charge reversal obtained being indicative of the charged groups present on the cell surface (Bugenberg de Jong, 1949).

The method used was adapted from that described by Schott and Young (1977) in which the ionic strength of the cation-containing solutions was held constant by the addition of sodium nitrate.

Solutions of copper, lead, uranyl, cadmium and zinc nitrates were used and the ionic strength of these solutions made up to

0.05 mol dm^{-3} by the addition of sodium nitrate ($NaNO_3$). A solution of $NaNO_3$ (I = 0.05 mol dm^{-3}) was used as a control.

The solutions were unbuffered, as addition of alkali caused precipitation of hydroxides. The pH of the solutions was in the range 4-5. The salts of the polyvalent cations were dissolved immediately before their addition to the bacterial cell suspension to minimise hydrolysis.

Surface grown cells of BCG (Glaxo sub-strain) were harvested by centrifugation, washed in $NaNO_3$ solution (I = 0.05 mol dm^{-3}, pH = 6.4) and resuspended in the appropriate cation solution immediately prior to electrophoretic mobility determinations.

2.4. Preparation of Cell Wall Suspensions

2.4.1. Growth of cells for cell wall preparation

A high concentration of cells was required to achieve a reasonable sample of cell walls. BCG was therefore grown on a large scale by inoculation of a Dubos-grown culture of BCG (Glaxo sub-strain) into Production medium contained in large culture vessels (bed-pans). 100 cm^3 of medium in each vessel was inoculated with 1 cm^3 of Dubos-grown culture.

The cultures were incubated at 37 °C for 14 days, harvested by centrifugation and washed once in barbiturate-acetate buffer solution (I = 0.05 mol dm^{-3}, pH = 7.0) prior to disruption by ultrasonic vibration.

2.4.2. Disruption of whole cells

The whole cell suspensions in ice-cooled buffer solution were subjected to ultrasonication for 3 min in a MSE sonicator operating at maximum output.

The resulting whole cell/cell wall suspension was centrifuged at 2 500 x *g* for 30 min to remove whole cells. The supernatant was then centrifuged at 22 000 x *g* for 30 min, when the cell walls were sedimented out. The supernatant was discarded and the cell wall pellet washed in barbiturate-acetate buffer solution ($I = 0.05$ mol dm^{-3}, pH = 7.0) and reclaimed by centrifugation at 22 000 x g.

Samples were removed at various intervals during this treatment and examined by transmission electron microscopy to check that the whole cells had been ruptured.

2.4.3. Preparation of cell walls for mobility measurements.

The cell wall suspension in barbiturate-acetate buffer solution (pH 7.0) was distributed into universal bottles and suspended in barbiturate-acetate buffer solutions ($I = 0.05$ mol dm^{-3}) of pH in the range 2.5 - 9. Control whole cell samples were grown in Production medium, harvested, washed twice in barbiturate-acetate buffer solution at pH 7.0 and prepared as for the cell wall suspensions. Mobility measurements were carried out as usual.

2.5. Preparation of Cells for Electron Microscopy

2.5.1. Scanning electron microscopy

Cells of BCG (all 5 sub-strains) were harvested by centrifugation, washed twice in distilled water and adjusted to a suitable density by suspension in distilled water.

A sterile platinum loop was used to transfer cells of each specimen to glass cover slips (10 mm diam.) which were then thoroughly air-dried. The resulting samples were mounted onto scanning electron microscope sample stubs and coated with a thin layer of gold to prevent the build up of any surface charges during scanning.

The samples were then scanned using a Cambridge Stereoscan electron microscope (S4-10) operating at 20 kV.

2.5.2. Transmission electron microscopy

Cells of BCG were harvested by centrifugation, washed twice in distilled water and resuspended to a suitable density in distilled water.

Drops of this suspension were placed onto gold electron microscope grids and thoroughly air-dried. The specimens were then negatively stained using uranyl acetate and examined under the transmission electron microscope.

Samples taken during the preparation of cell walls by ultrasonication were also prepared and examined in the same way.

CHAPTER 3

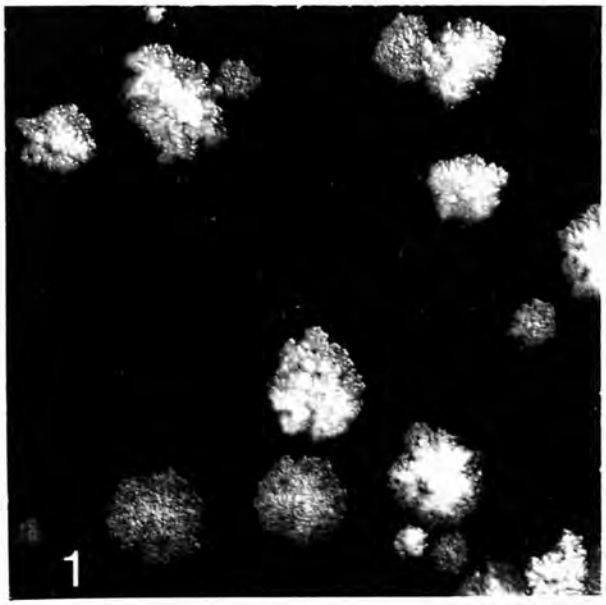
THE COLONIAL MORPHOLOGY OF SOME SUB-STRAINS OF M.BOVIS BCG

The various sub-strains of BCG, derived from the original Bacillus Calmette and Guérin and maintained in many laboratories for over 40 years, have undergone certain phenotypic and genotypic changes in biochemical activity (Boenicke, 1957; Gupta et al, 1959 and 1963), colonial morphology (Froman et al, 1955; Pierce and Dubos, 1956; Gupta, 1978) and in their ability to protect against challenge with tubercle bacilli (Murohashi et al, 1952; Dubos and Pierce, 1956; Ladefoged, Bunch-Christensen and Guld, 1970 and 1976; Frappier et al, 1971).

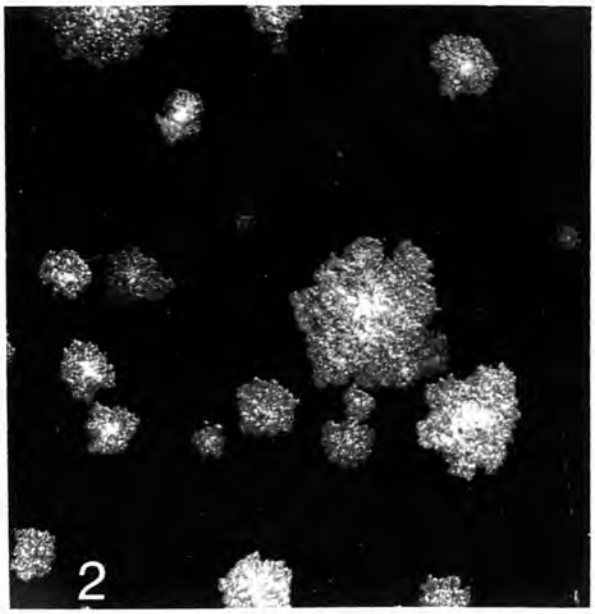
Early workers have suggested that colony form was related to surface properties (Reed and Gardiner, 1932; Choucroun and Plotz, 1934). To reexamine this, cells of the various sub-strains of BCG were plated out onto DOA agar and their colonial morphology examined. The colonial morphology for the 5 sub-strains used, Glaxo, Pasteur, Japanese, Prague and Danish, differed between strains. Two extremes of colonial morphology were displayed:
(1) the Glaxo sub-strain, which has rounded, heaped-up colonies; and
(2) the Pasteur sub-strain which has flat, spreading colonies (Figure 3.1).
Colony form such as that displayed by the Glaxo sub-strain is termed "smooth" and that displayed by the Pasteur sub-strain as "rough". The other three sub-strains exhibited colonial morphology intermediate between these two sub-strains (Figure 3.2.). The Japanese and Prague sub-strains were very similar and tended more towards "smooth" colony form than "rough", whilst the Danish sub-strain had colonies mainly intermediate between "rough" and "smooth", but was characterised by 10-20% of very "smooth" colonies.

"Rough" and "smooth" isolates of the Glaxo sub-strain of BCG were also examined (Figure 3.3). The difference between the two isolates was obvious; the "rough" isolate formed colonies closely resembling those displayed by the Pasteur sub-strain (typically "rough") and the "smooth" isolate colonies resembling normal Glaxo BCG.

FIGURE 3.1: Colonial morphology of the Glaxo and Pasteur sub-strains of BCG (x 6)

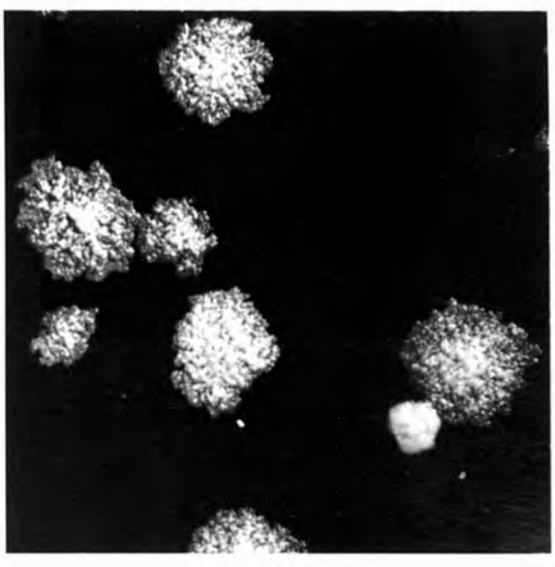


Glaxo sub-strain

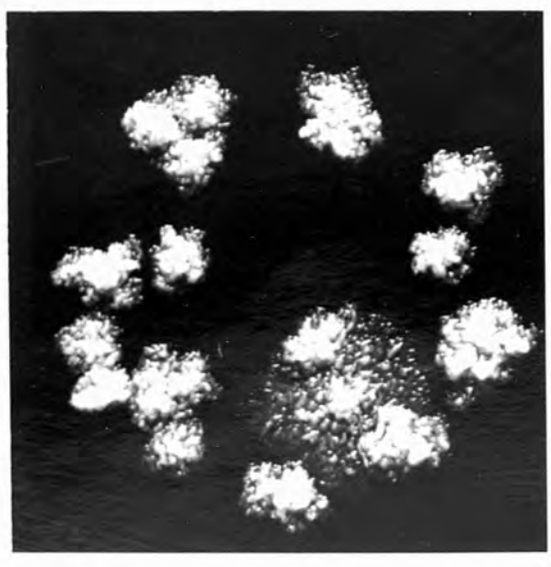


Pasteur sub-strain

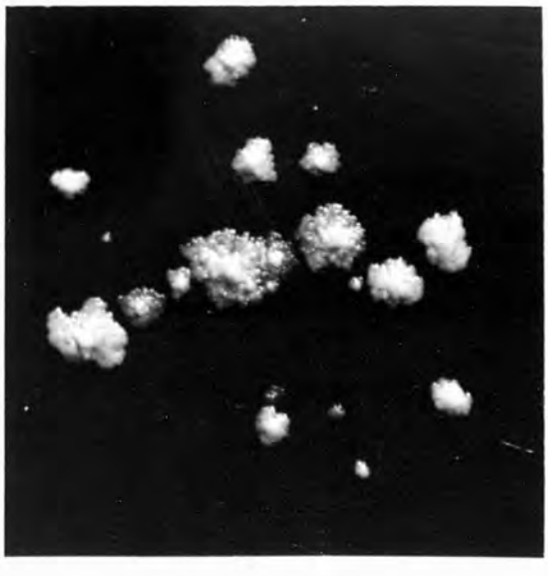
FIGURE 3.2: Colonial morphology of the Danish Japanese and Prague sub-strains of BCG (x 6)



Danish sub-strain

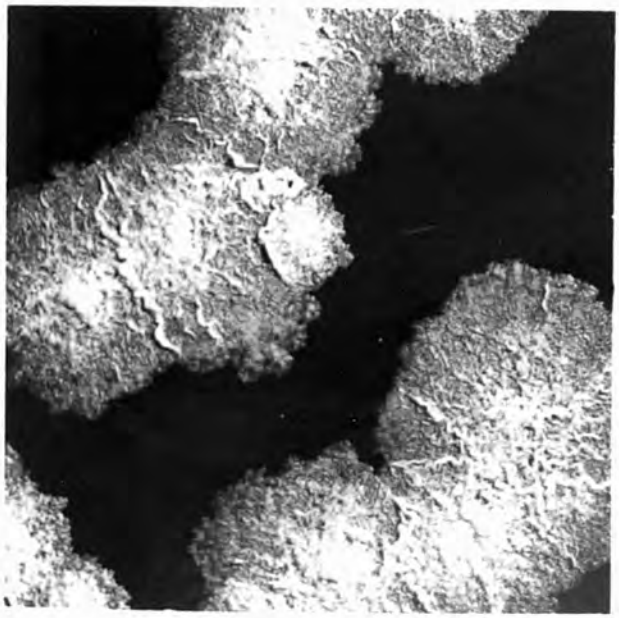


Japanese sub-strain

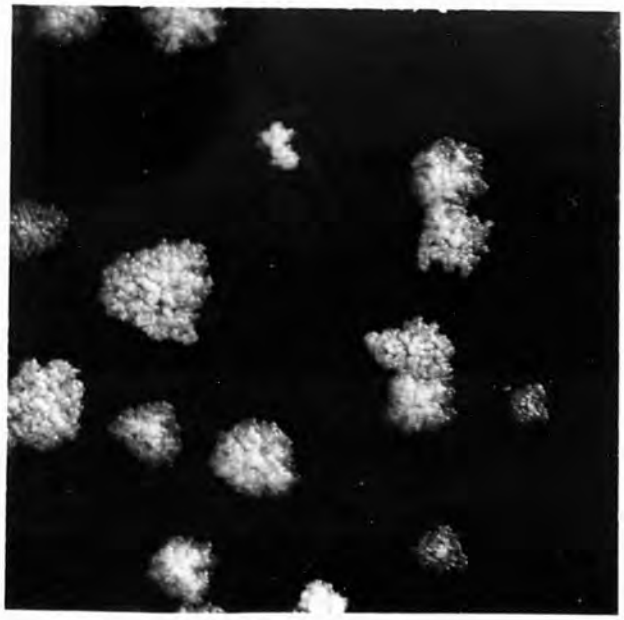


Prague sub-strain

FIGURE 3.3: Colonial morphology of "rough" and "smooth" isolates of Glaxo BCG (x 6,



"Rough" isolate



"Smooth" isolate

CHAPTER 4

THE SURFACE PROPERTIES OF SUB-STRAINS OF M.BOVIS BCG

The experiments described in this chapter were undertaken to investigate the surface properties of the 5 sub-strains of M.bovis BCG (viz Glaxo, Pasteur, Japanese, Danish and Prague) by examining the shape and position of their pH-mobility curves under a variety of conditions. Such an investigation could reveal any relationships between the different physical and biological properties exhibited by the sub-strains, e.g. colonial morphology, and their surface charge properties.

Very little work has been reported on the electrophoretic properties of mycobacterial species. Adams and Rideal (1958) examined cells of M. phlei; "rough" and "smooth" isolates of M. leprae (Reed and Gardiner, 1932), M. tuberculosis and M. bovis BCG (Choucroun and Plotz, 1934), have also been investigated. It was therefore necessary to determine the optimal conditions for the measurement of electrophoretic mobility of cells of BCG.

4.1. Establishment of Procedures for Measurement of Charge of Mycobacteria.

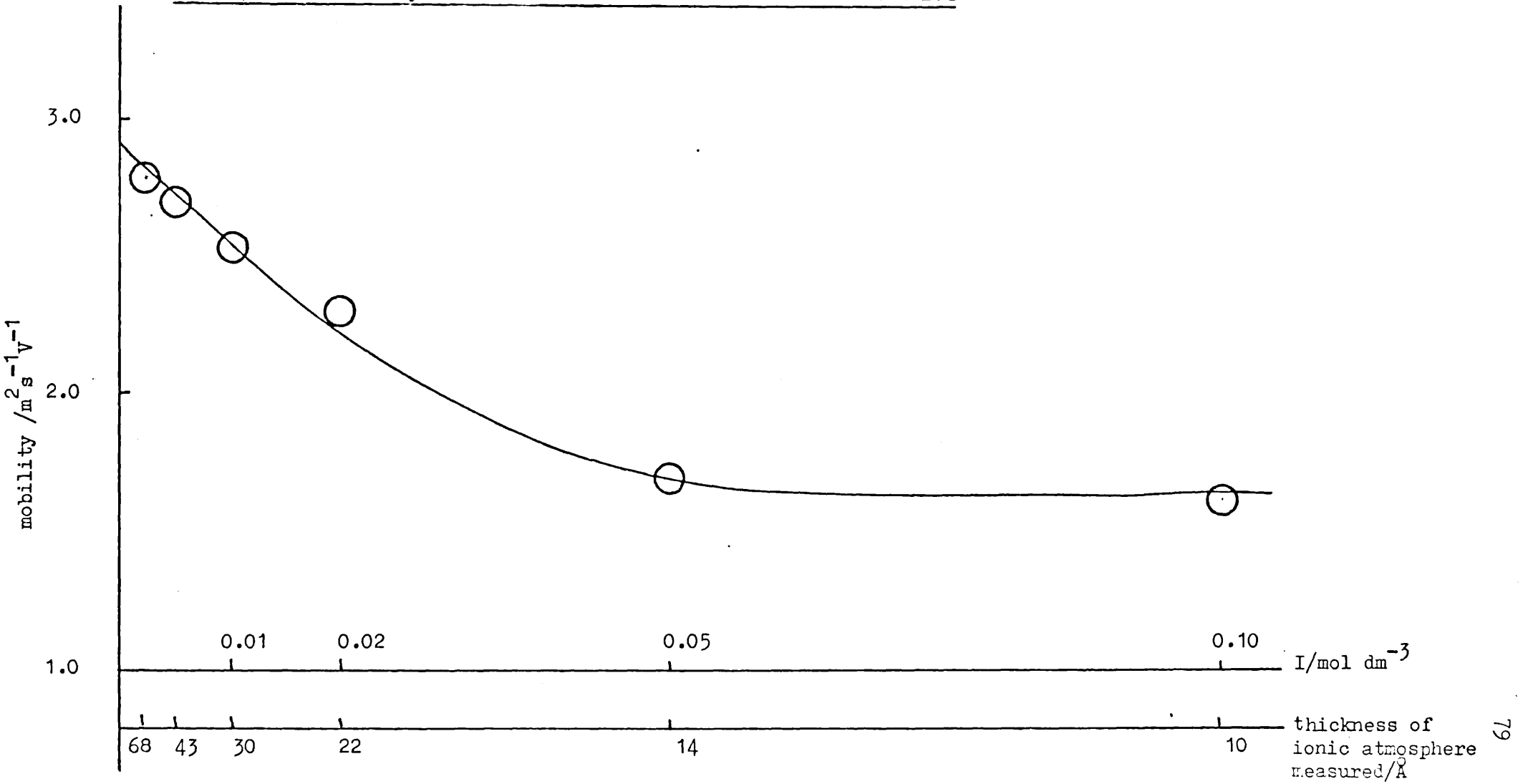
4.1.1. Ionic strength of buffer solution.

The optimum ionic strength of the barbiturate-acetate buffer solution used to determine the electrophoretic mobility of cells of BCG was determined by plotting a mobility-concentration curve for cells of BCG in buffer solutions of varying ionic strength at constant pH (7.0). The cells were grown for 14 days on Sauton medium (Table 2.4.); typical results determined on 40 measurements (2.2.2.) are shown in Figure 4.1.

As the ionic strength of the buffer solution decreases so the thickness of the double layer increases according to the equation:

$$\text{Thickness}/\text{Å} = \frac{1}{0.327 \times 10^{8} I^{1/2}} \qquad 4.1.$$

FIGURE 4.1. Mobility-concentration curve for cells of M. bovis BCG



where I is the ionic strength of the buffer solution (mol dm^{-3}). Thus the lower the ionic strength the greater will be the number of charged species which will contribute to the total charge.

From the results obtained an ionic strength of 0.05 mol dm^{-3} was chosen as most suitable for determining electrophoretic mobility values of cells of mycobacteria. This value lay on the plateau region of the mobility-concentration curve and mobility values determined at this concentration were therefore less susceptible to errors arising from any small variations in the concentration of the buffer solution.

4.1.2. Number of washings required before mobility determinations.

The ideal number of times the cells were washed in buffer solution before the examination of their mobility was determined. The number of washings has to be sufficient to remove all nutrients, salts and toxic products from the growth medium which would interfere in the determination of surface charge, while at the same time leaving the surface of the bacterial cells with all naturally arising surface components still present.

The mobility of cells of BCG was measured after varying numbers of washings in barbiturate-acetate buffer solution of constant ionic strength and pH (I = 0.05 mol dm^{-3}, pH = 7). The results are summarized in Table 4.1.

Table 4.1.

Effect of washing on the mobility of cells of M. bovis BCG.

No. of washings	10^8 x electrophoretic mobility / $m^2 s^{-1} V^{-1}$ at pH 7
1	1.78
2	1.80
3	1.77
4	1.81

To obtain an even suspension of cells it was necessary to manually grind the suspension in a glass-ground tissue-grinder between each washing. As no significant difference in mobility of the cells was observed on washing the cells several times, it was decided to wash the cells once only to minimise the amount of tissue-grinding required and thus reduce the possibility of surface disruption. However cells grown in a medium containing a non-ionic wetting agent were washed twice to ensure complete removal of the wetting agent.

From 100 mobility values measured over a period of 2 years, the day to day reproducibility at $p = 0.05$ is $\pm$ 1%. Any difference greater than 6% is therefore significant and indicates changes on the cell surface.

4.1.3. pH-range for measurement of mobility.

The pH-range for the measurement of the mobility of cells of BCG, over which the surface was not irreversibly altered, was determined by measuring the mobility of the cells at pH 7 after

suspension in buffer solutions of higher or lower pH-values. Failure to regain the original value obtained for cells suspended directly at pH 7 indicated that irreversible changes had occurred to the cell surface, and mobility values obtained at these pH-values must be considered invalid.

The pH-range for cells of BCG was 2.5 - 9.5.

4.1.4. Conditions finally adopted for the measurement of mobility of cells of mycobacteria.

The conditions finally adopted for the measurement of the electrophoretic mobility of cells of mycobacteria are summarized in Table 4.2.

The cells were manually tissue-ground between each washing and directly before mobility measurements were made.

Table 4.2.

Preparation of cells of mycobacteria for electrophoretic mobility measurements.

Ionic strength of buffer solution /mol dm^{-3}	Presence or absence of wetting agent in growth medium	No. of washings before mobility measurements	pH-range
0.05	Present	2	2.5-9.5
0.05	Absent	1	2.5-9.5

4.2. Surface Properties of Cells of BCG after Growth on Sauton Medium.

Since the yield of BCG cells growing as a surface pellicle on Sauton medium was very good, and also since there was no wetting agent present in this medium (which may have affected the surface properties of the cells), the first pH-mobility measurements were

made on cells grown on Sauton medium.

A typical pH-mobility curve obtained for cells of the Glaxo sub-strain of BCG grown on Sauton medium is shown in Figure 4.2. This pH-mobility curve is typical of a surface with only anionic surface groups. There was no indication of charge-reversal at low pH-values or a further rise in negative mobility values at higher pH, either of which would indicate the presence of cationic groups in a mixed anionic/cationic cell surface. The surface charged groups have a pK of approximately 2.9 and the charge approches zero at approximately pH = 2.

The pH-mobility curves of cells of the other four sub-strains of BCG (Figure 4.3) are all coincident with that of cells of the Glaxo sub-strain.

These results show that the total surface charge carried by cells of all five sub-strains of BCG grown on the surface of Sauton medium is identical. All five sub-strains have a negative surface charge arising from the dissociation of anionic surface species; there is no indication of any cationic species.

4.3. Surface Properties of Cells of BCG after Growth on other Media.

In other species of bacteria the electrophoretic behaviour of bacterial cells has been shown to vary with the nature of the growth medium. For example cells of Micrococcus aureus cultivated in glucose-containing medium show a higher mobility than cells cultivated in ordinary broth (Dyar, 1948); this has been attributed to a higher surface lipid content.

As cells of BCG are routinely cultured in a variety of media it was decided to investigate their surface properties after growth in these other media.

FIGURE 4.2.

pH-mobility curve for cells of the Glaxo sub-strain of BCG grown on Sauton medium

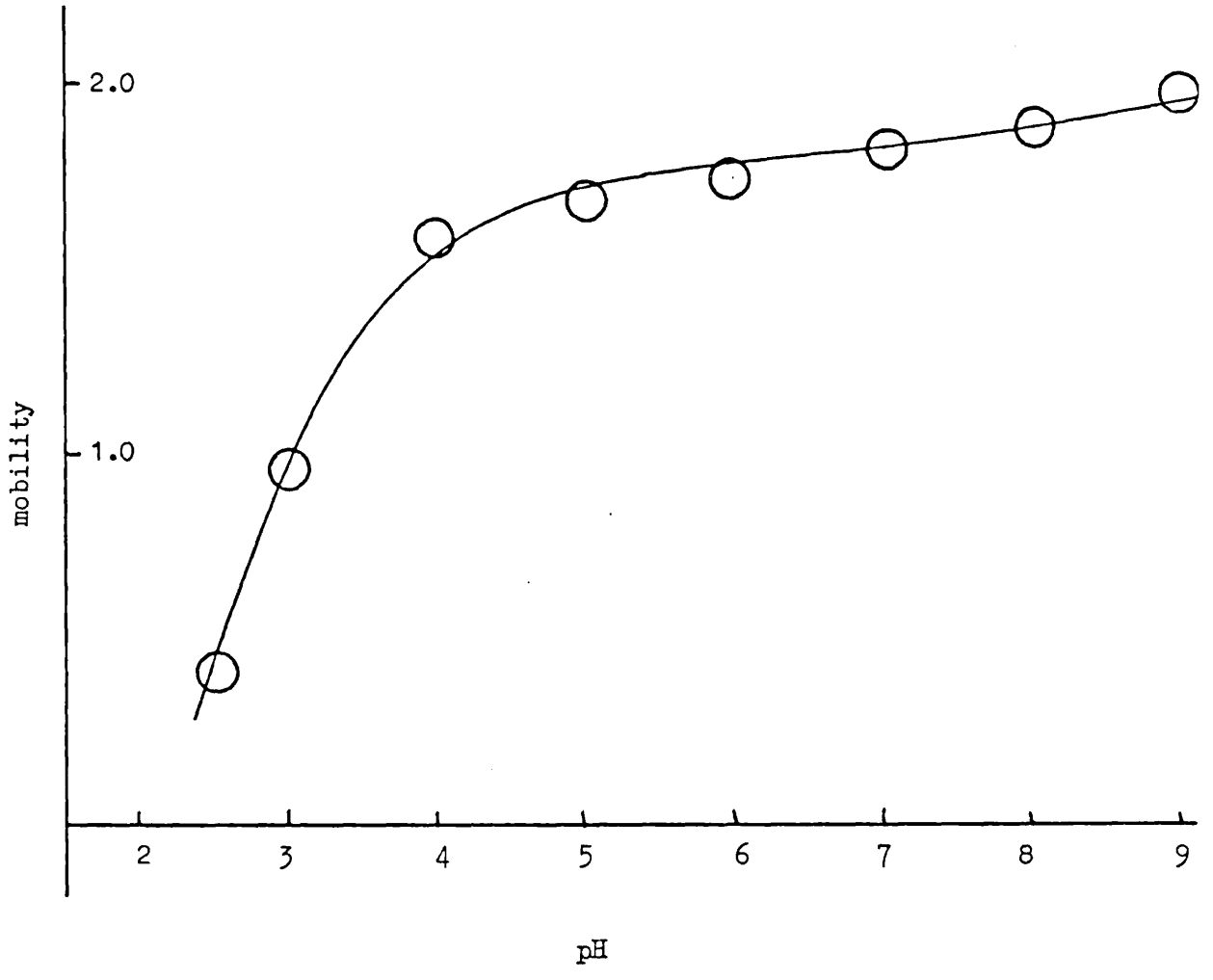
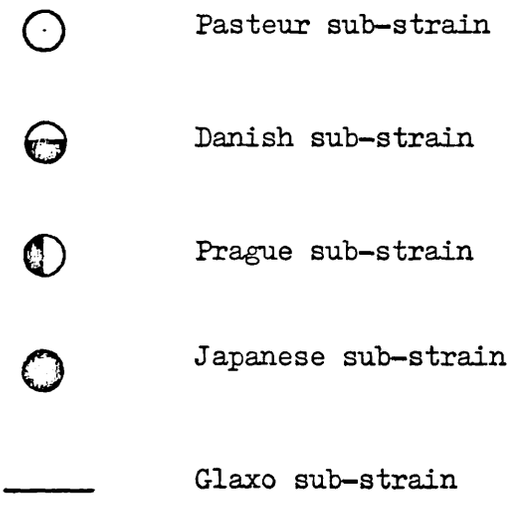
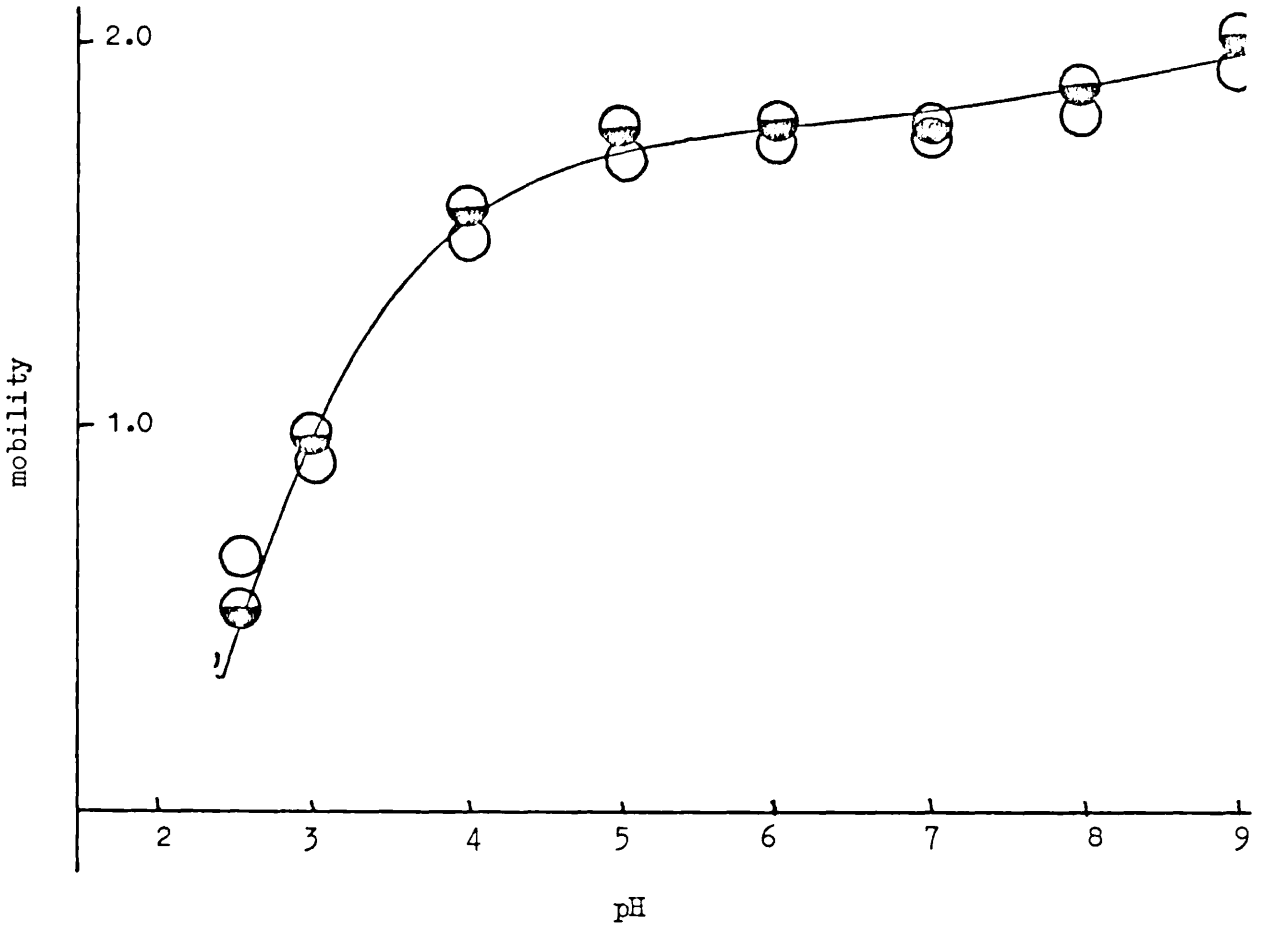
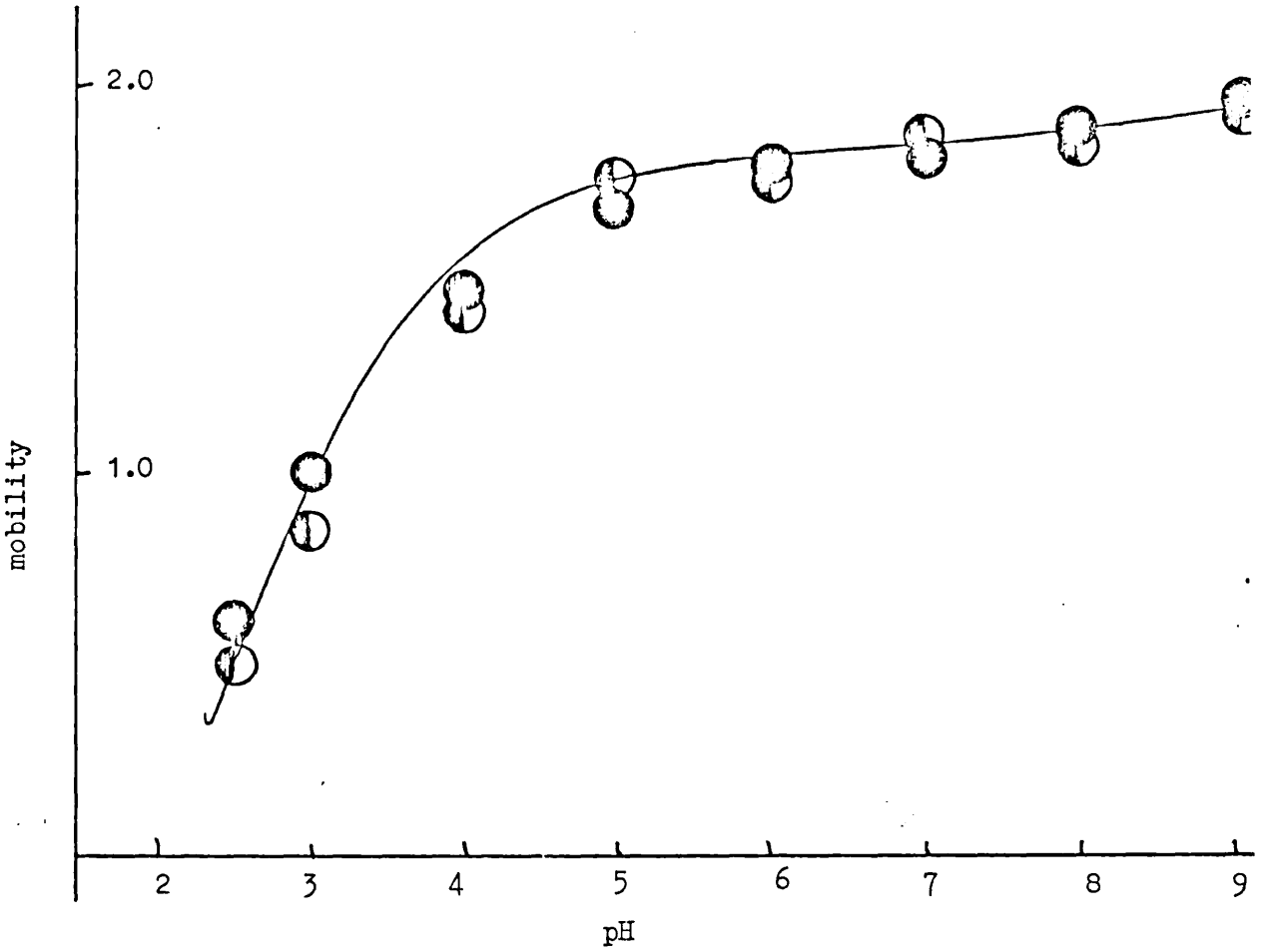


FIGURE 4.3.

pH-mobility curves for cells of the Pasteur, Danish, Japanese and Prague sub-strains of BCG grown on Sauton medium compared to that for cells of Glaxo BCG.




2.0
1.0
mobility
2
3
4
5
6
7
8
9
pH


2.0
1.0
mobility
2
3
4
5
6
7
8
9
pH

4.3.1. Surface properties after growth on Dubos Oleic Acid agar.

Typical results for the pH-mobility curves of cells of all five sub-strains of BCG grown on DOA agar (Table 2.5) are shown in Figure 4.4. There is no difference in total charge exhibited by cells of the five sub-strains of BCG grown on DOA agar, and further the shape and position of the pH-mobility curves obtained is the same as that for cells grown on Sauton medium.

4.3.2. Surface properties after growth in Dubos and Production media.

Dubos (Table 2.4.) and Production (Table 2.4.) media, used to grow cells of BCG as a dispersed culture, contain a non-ionic wetting agent to keep the cells in suspension. In Dubos medium this is Tween 80 and in Production medium Triton.

The pH-mobility curves obtained for cells of the Glaxo sub-strain of BCG grown in Dubos and Production medium are shown in Figure 4.5. There is no difference in shape or position of the pH-mobility curves obtained for cells grown in the media containing surfactants. Further the curves for cells of the Glaxo sub-strain of BCG grown in all the media used in BCG cultivation are coincident (Figure 4.6).

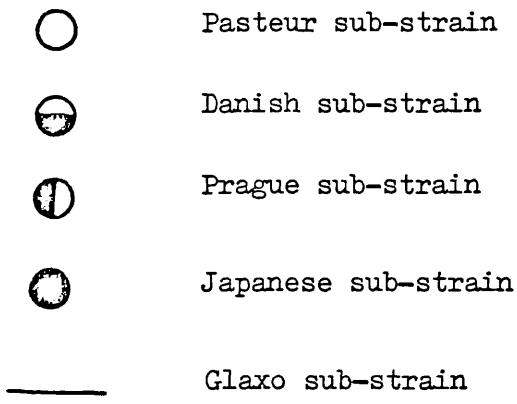
Thus it is evident that the electrophoretic mobility and the shape and position of the pH-mobility curve of cells of BCG remains constant for a variety of growth media and is also identical for all five sub-strains examined. This suggests that there is no correlation between the observed differences in colonial morphology between sub-strains and the surface charge.

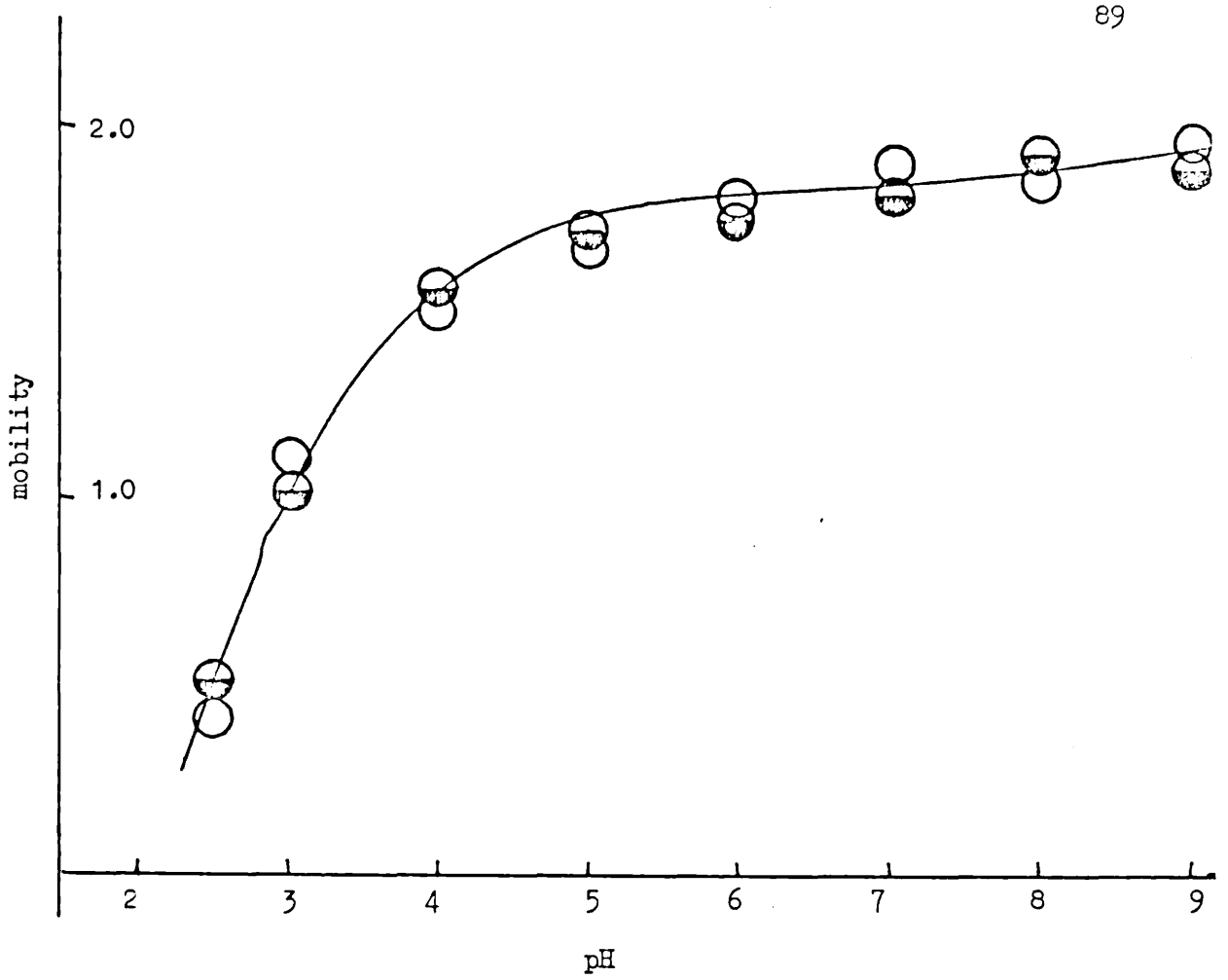
4.4. Surface Properties of "Rough" and "Smooth" Isolates of Glaxo BCG.

Earlier workers (Reed and Gardiner, 1932; Choucroun and Plotz, 1934) reported differences in electrophoretic mobility between "rough" and "smooth" isolates of mycobacterial species. The present results do not support this as far as differences in colony form between sub-strains

FIGURE 4.4.

pH-mobility curves for cells of the Glaxo, Pasteur, Danish, Japanese and Prague sub-strains of BCG grown on Dubos Oleic Acid agar




2.0
1.0
mobility
2
3
4
5
6
7
8
9
pH

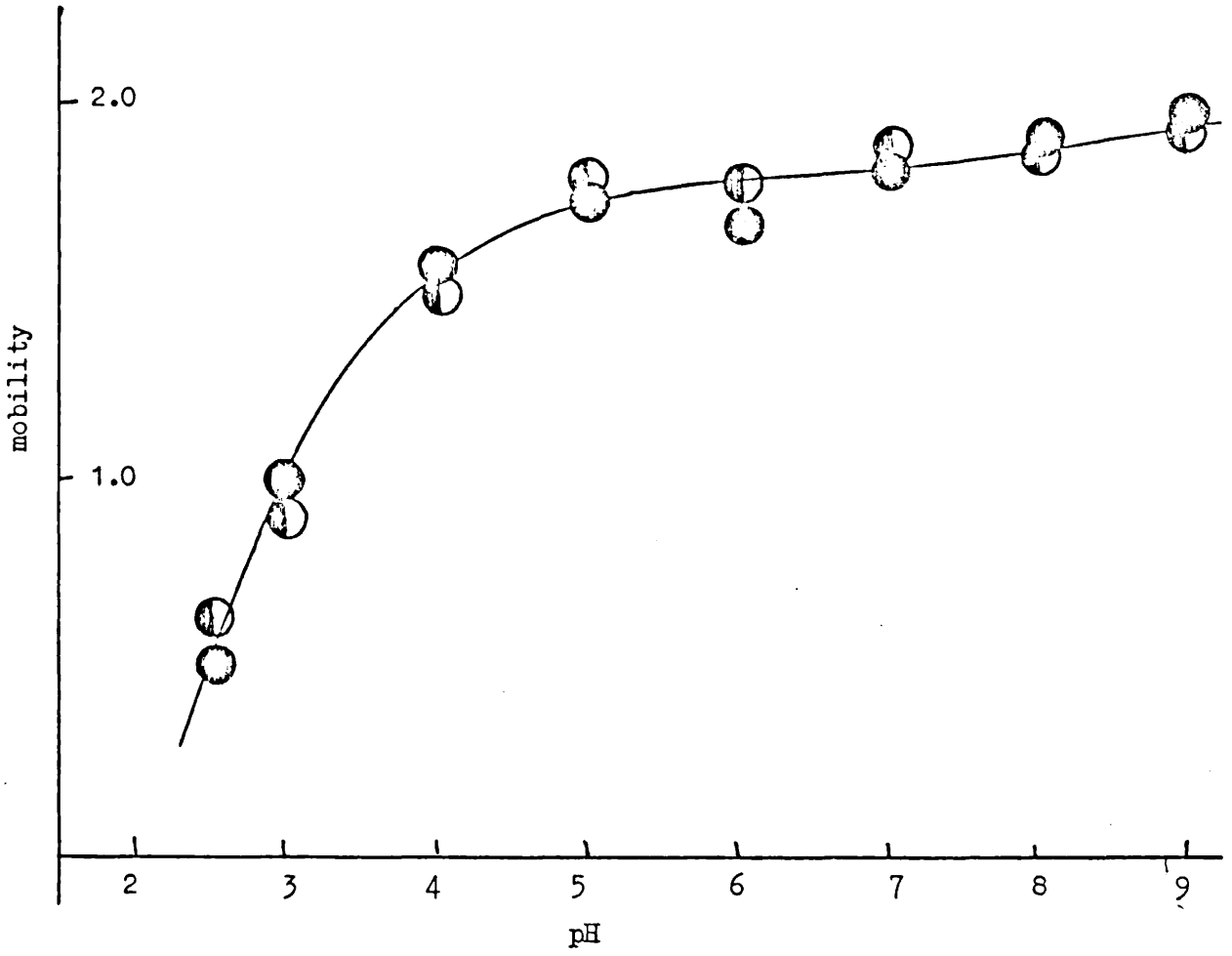

2.0
1.0
mobility
2
3
4
5
6
7
8
9
pH

FIGURE 4.5.

pH-mobility curves for cells of the Glaxo sub-strain of BCG grown in the presence of a non-ionic wetting agent

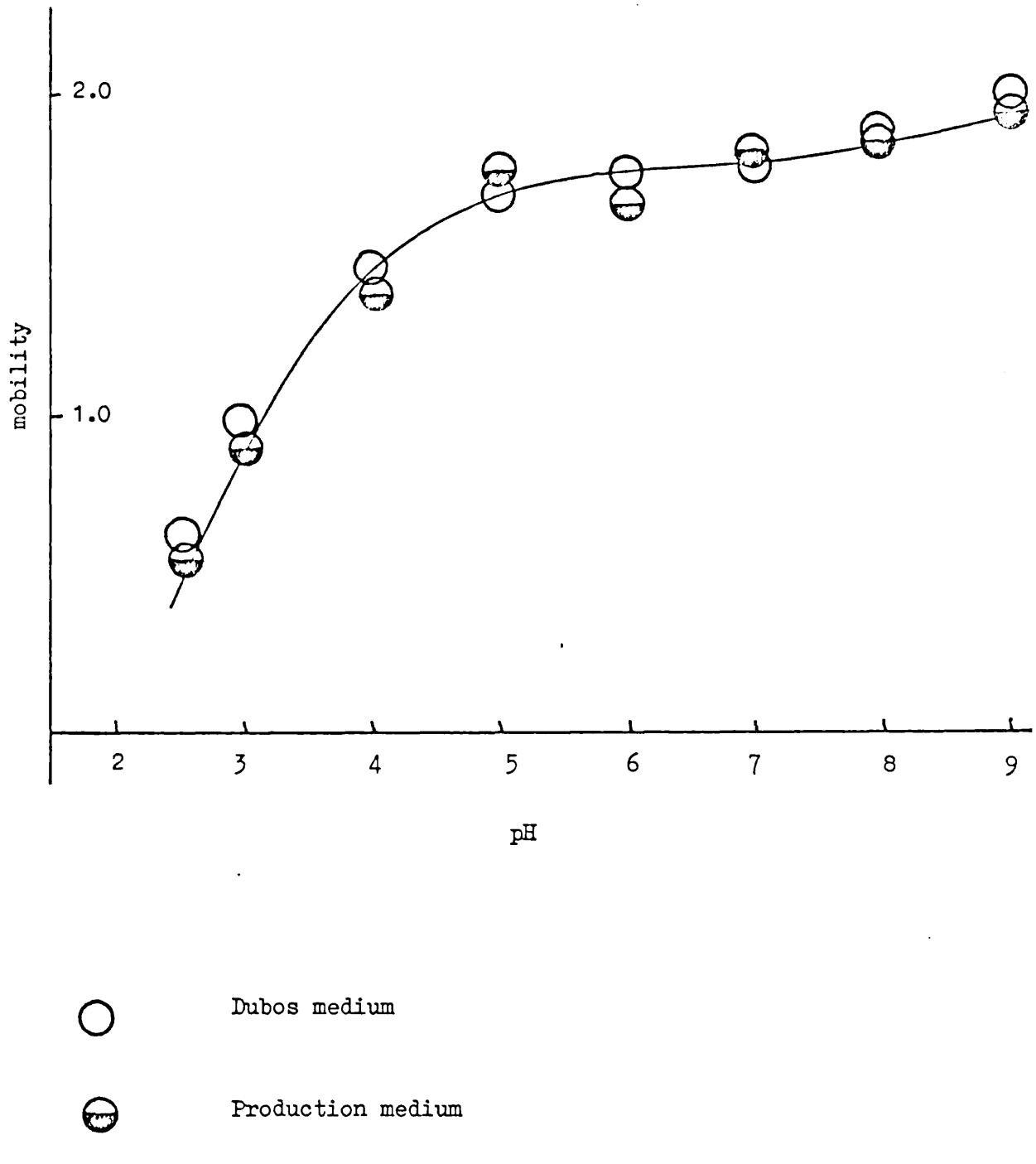
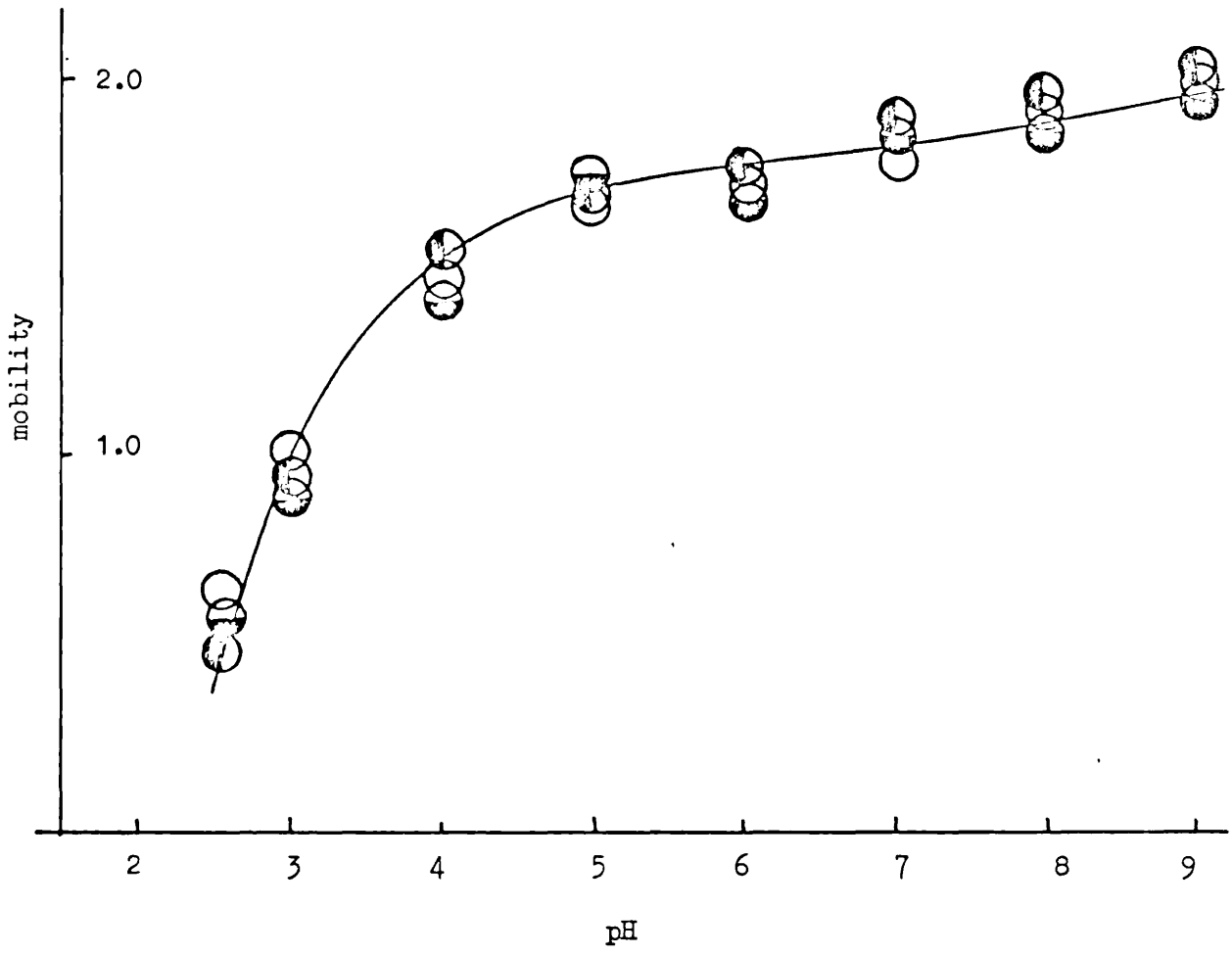


FIGURE 4.6.

pH-mobility curves for cells of the Glaxo sub-strain of BCG grown in a variety of media



Dubos medium

Production medium

DOA agar

Sauton medium

are concerned. Therefore by way of confirmation, pH-mobility curves were determined for the "rough" and "smooth" isolates of Glaxo BCG.

The results (Figure 4.7) confirm the findings that there is no relationship between electrophoretic mobility, and hence surface charge of individual cells, and the colonial morphology of cells of BCG.

It would therefore appear that the differences in colony form between sub-strains are not due to physical differences but possibly to biochemical ones, which allow the colonies to heap to give "smooth" colony forms, or prevent it to give flat, spreading, "rough" colonies.

Growth of BCG in a medium containing a non-ionic wetting agent is known to influence colonial morphology towards the "smooth" colony form, but the present results show no difference in surface properties for cells of BCG which can be related to different growth media; this again suggests that there is no relationship between colony form and surface charge.

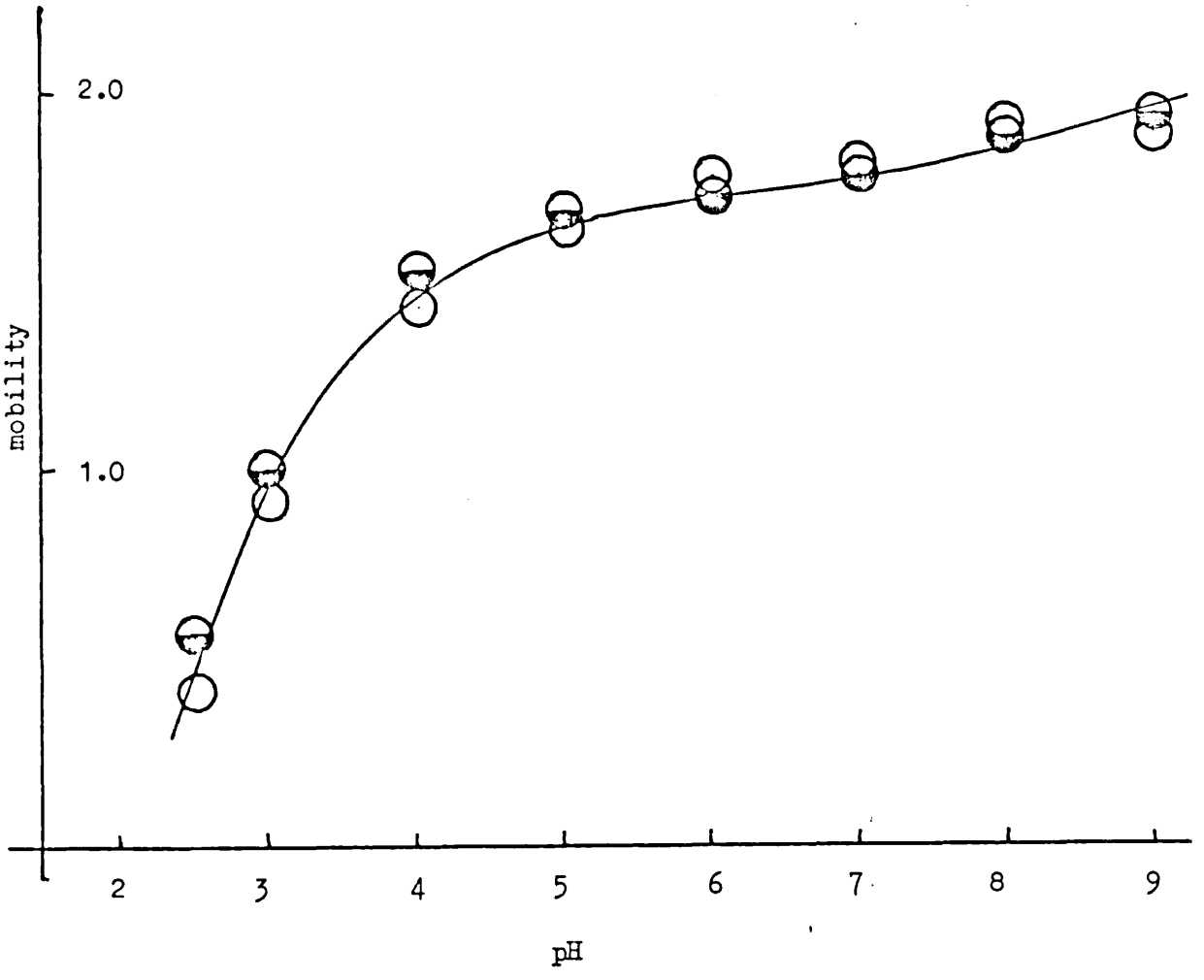
4.5. Effect of Age of a Culture on Surface Properties of Cells of BCG.

The electrophoretic behaviour of cells of some other bacterial species has been shown to vary with the age of the culture. For example, cells of E. coli exhibit a decrease in mobility during the early phase of growth, before a stable value is reached which is maintained for several days (Moyer, 1936).

Cultures of BCG show variations in their tendency to aggregate during growth. The cells are fairly well separated in the early stages and later show an increasing tendency to clump until about 14 days, after which the clumps appear to break down. It was therefore decided to investigate any changes in surface charge exhibited during growth of a BCG culture.

FIGURE 4.7.

pH-mobility curves for "rough" and "smooth" isolates of the Glaxo sub-strain of BCG



"rough" isolate

"smooth" isolate

Figure 4.8. shows the pH-mobility curves obtained for cells of BCG measured at various stages during their growth cycle. There is no variation in the total surface charge or in the distribution of charged groups during this growth of a culture of BCG.

4.6. Surface Properties of Isoniazid Resistant Cells of the Glaxo Sub-strain of BCG.

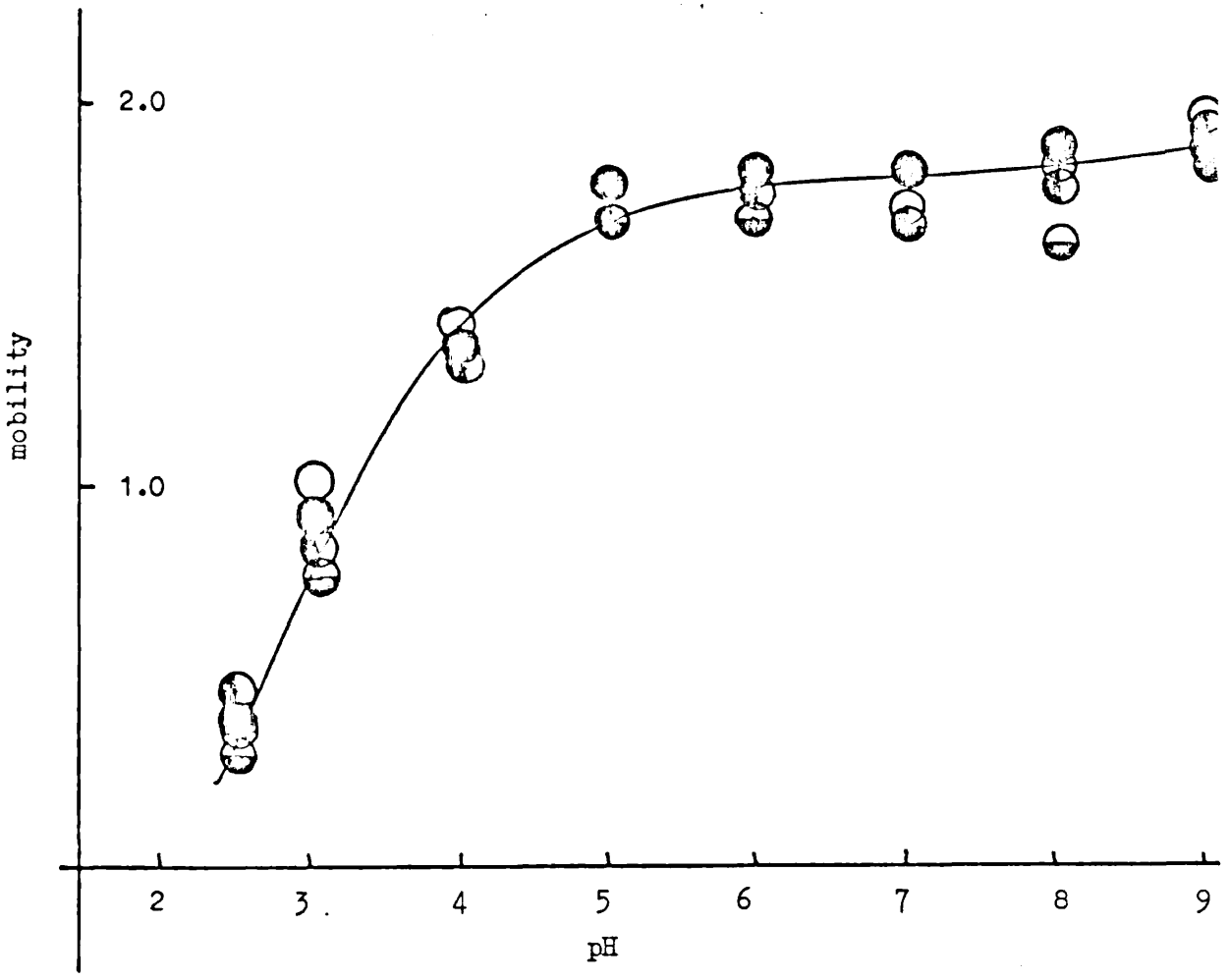
Resistance of bacterial cells to various antibiotics has, in certain species, been shown to be accompanied by changes in the surface properties. Cells of Pseudomonas aeruginosa which are sensitive to gentamicin exhibit a sinusoidal-shaped pH-mobility curve, whereas resistant cells show a curve with increasing negative mobility over the range 3 - 7.5 reaching a maximum at pH 7.5 - 8.5 (Pechey and James, 1974).

Cells of BCG are extremely resistant to most antibiotics; or chemical compounds, as are the virulent M. tuberculosis organisms. Two main compounds are used in the treatment of tuberculosis, these are streptomycin and isonicotinic acid hydrazide, isoniazid. Isoniazid is believed to interfere with mycolic acid synthesis, which eventually leads to malformation of the cell walls.

The pH-mobility curves of 14 day-old cells of isoniazid resistant and isoniazid sensitive isolates of the Glaxo sub-strain of BCG were measured (Figure 4.9). The curves for the resistant and sensitive cells are coincident showing that, in cells of BCG, isoniazid resistance is not accompanied by a change in the shape or position of the pH-mobility curve.

FIGURE 4.8.

The effect of age of culture on the pH-mobility curves of cells of Glaxo BCG



10 day

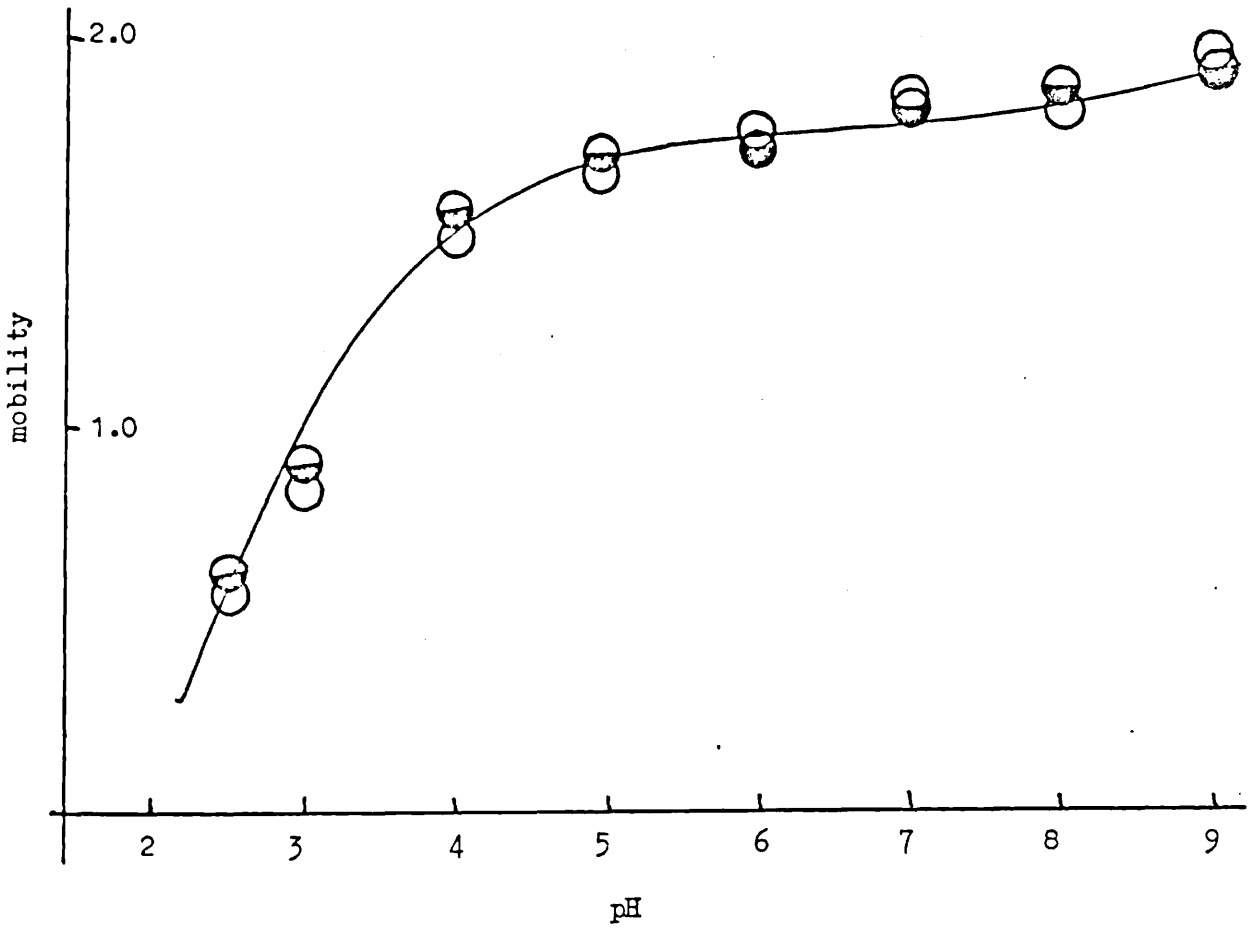
14 day

21 day

28 day

FIGURE 4.9.

pH-mobility curves for isoniazid-resistant and isoniazid-sensitive cells of the Glaxo sub-strain of BCG



isoniazid-sensitive strain

isoniazid-resistant strain

4.7. Surface Properties of Cells of Glaxo BCG after a Single Animal Passage.

When cells of BCG are passaged through a guinea pig and reisolated their colonial morphology always becomes "rough". Although no correlation between colonial morphology and pH-mobility had been observed for *in vitro* grown cells, examination of *in vivo* grown and reisolated cells was made to confirm if this still held after passage through an animal.

Cells of the Glaxo sub-strain of BCG were reisolated after one animal passage onto DOA agar (2.1.3.) and prepared for electrophoresis as usual. Cells were isolated from pus in a lesion at the sight of the inoculum (these would not have undergone true passage through the animal) and from the spleen of the infected animal (these had undergone true passage through the animal). The results are shown in Figure 4.10. The shape and position of the pH-mobility curves obtained for cells of the *in vivo* isolates are identical with those obtained for *in vitro* grown cells, confirming that changes in colonial morphology are not reflected by changes in surface charge. The surface properties of cells of BCG appear unaltered by growth *in vivo*.

4.8. Summary

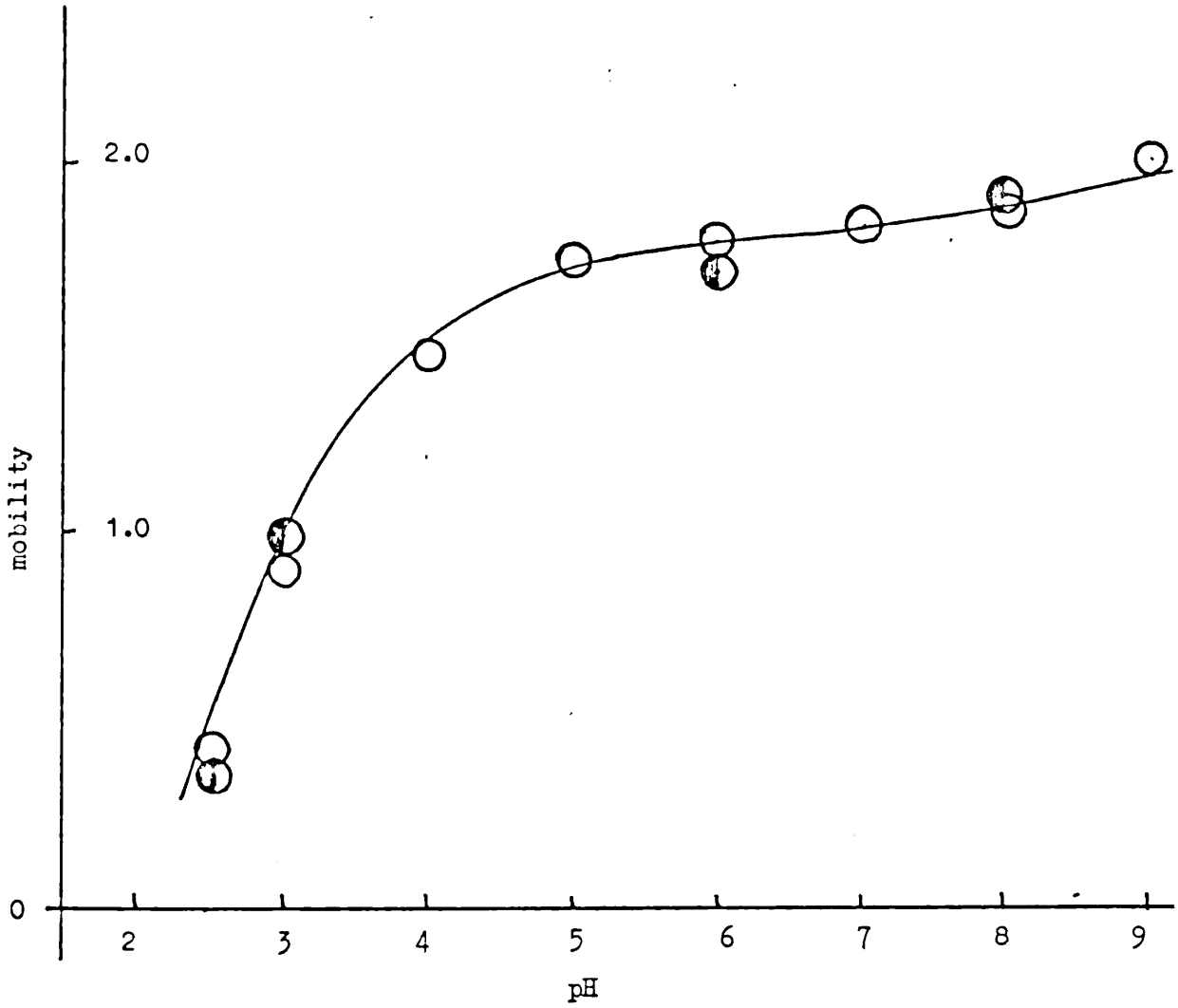
4.8.1. The optimum conditions for the measurement of electrophoretic mobility of mycobacterial cells were established.

4.8.2. The composition of the growth medium used in the culture of BCG had no effect on the surface properties of the cells.

4.8.3. The surface properties of cells of the five sub-strains of BCG examined were the same for all the strains. There was no change in the shape or position of the pH-mobility curve related to observed differences in the colonial morphology, a fact verified by examination of "rough" and "smooth" isolates of the Glaxo sub-strain of BCG.

FIGURE 4.10.

pH-mobility curves for cells of Glaxo BCG after one passage through a guinea pig



○ cells reisolated from pus in thigh lesion

◐ cells reisolated from spleen

——— cells of "in vitro" grown BCG

4.8.4. No changes in surface properties of cells of BCG were observed during 28 days growth of a culture of the organism.

4.8.5. The surface properties of cells of an isoniazid resistant strain of BCG were identical to those of an isoniazid sensitive strain.

4.8.6. *In vivo* passaged cells have identical surface properties to those of cells grown *in vitro*.

CHAPTER 5

SPECIFIC CHEMICAL AND ENZYMIC MODIFICATION OF THE SURFACE OF CELLS OF M. BOVIS BCG

5.1. Detection of Carboxyl Groups

A high proportion of the negative charge on bacterial cells often arises from the presence of ionogenic carboxyl groups at the cell surface. Some species of bacteria, for example Klebsiella aerogenes, have a surface charge composed entirely of ionized carboxyl groups (Plummer and James, 1961; Gittens, 1962).

Cells of the Glaxo sub-strain of BCG were examined for the presence of surface carboxyl groups by treatment with methanolic hydrogen chloride to esterify any surface carboxyl groups and thus to remove their contribution to the total surface charge (2.3.1.).

There was no significant decrease in the negative electrophoretic mobility of the cells even after refluxing in methanolic HCl for 28 h at 60 °C (Table 5.1) showing that there are no ionogenic carboxyl groups on the surface of cells of BCG. The observed negative charge of these cells must therefore be due to other anionic species on the cell surface.

5.2. Detection of Amino Groups.

Positively charged species on the surface of cells of BCG were not revealed by the shape of the pH-mobility curves previously reported (Figure 4.2.). However, to confirm that there were no positive amino groups present, cells of BCG were treated with ethanolic 2,4-dinitrofluorobenzene (FDNB), which blocks amino groups preventing their ionization and contribution to the surface charge (2.3.2.).

FDNB treatment had no effect on the mobility of the cells (Table 5.2) thereby confirming that there were no detectable ionogenic surface amino groups on cells of BCG.

The surface charge of cells of BCG therefore arises predominantly from ionized anionic surface groups.

Table 5.1.

Electrophoretic mobility of cells of BCG after treatment with methanolic hydrogen chloride.

Table 5.1.1. Shaken at 40 °C

Time of treatment /min	10^8 x electrophoretic mobility /$m^2\ s^{-1}\ V^{-1}$ pH 6.0	pH 3.0
0	1.79	0.70
30	1.84	0.73
60	1.78	0.68
120	1.80	0.65
240	1.71	0.70
360	1.78	-
1260	1.77	0.68
1260 (MeOH alone)	1.76	0.71

Table 5.1.2. Refluxed and shaken at 60 °C

Time of treatment /h	10^8 x electrophoretic mobility /$m^2\ s^{-1}\ V^{-1}$ pH 6.0	pH 3.0
0	1.82	0.70
2	1.83	0.79
4	1.79	0.72
9	1.62	0.69
16	1.74	0.72
28	1.83	0.82
28 (MeOH alone)	1.82	0.79

Table 5.2.

Electrophoretic mobility of cells of BCG after treatment with ethanolic 2,4-dinitrofluorobenzene.

Time of treatment /h	w/v FDNB in alcohol	10^8 x electrophoretic mobility /m^2 s^{-1} V^{-1} pH 6.0	pH 3.0
5	0	1.75	0.83
	0.10	1.77	0.87
	0.15	1.80	0.79
	0.20	1.79	0.82
24	0	1.77	-
	0.20	1.77	-

5.3. Detection of Sulphydryl Groups.

Surface sulphydryl groups do not contribute to the surface charge but can be detected by reacting the groups with dithiodinicotinic acid, resulting in the introduction of one negative charge for each sulphydryl group reacted. Cells of BCG were treated with dithiodinicotinic acid (2.3.3.); the results are summarized in Table 5.3.

There is no evidence for the presence of surface sulphydryl groups on cells of BCG.

Table 5.3.

Electrophoretic mobility of cells of BCG after treatment with dithiodinicotinic acid.

Time of treatment /min	10^8 x electrophoretic mobility /m^2 s^{-1} V^{-1} pH 7.0
0	1.89
1	1.85
5	1.87
10	1.87
15	1.87
60	1.88
60 (+ L-cysteine)	1.89

5.4. Detection of Surface Lipid.

Cell walls of mycobacteria contain very large amounts of lipid (20 - 40% of the cell wall by weight) compared to other Gram negative organisms (1-5%).

It therefore seemed highly probable that lipid material would be a component of the surface of cells of BCG.

Surface lipid on bacterial cells is detected by the method of Dyar (1948) in which the mobility of cells suspended in barbiturate-acetate buffer solution (pH = 7.0) is compared with that of cells suspended in buffer solution containing sodium dodecyl sulphate, SDS, (10^{-4} mol dm^{-3}). From this, S-values defined (Marshall, 1969) as:

$$S = \frac{\left(\bar{\nu}_{SDS} - \bar{\nu}\right) \times 100}{\bar{\nu}}$$

can be calculated, where $\bar{\nu}_{SDS}$ is the mobility value in the presence of SDS (10^{-4} mol dm^{-3}) and $\bar{\nu}$ the mobility value in barbiturate-acetate buffer solution alone. The S-value gives a measure of the amount of surface lipid present on the cell surface.

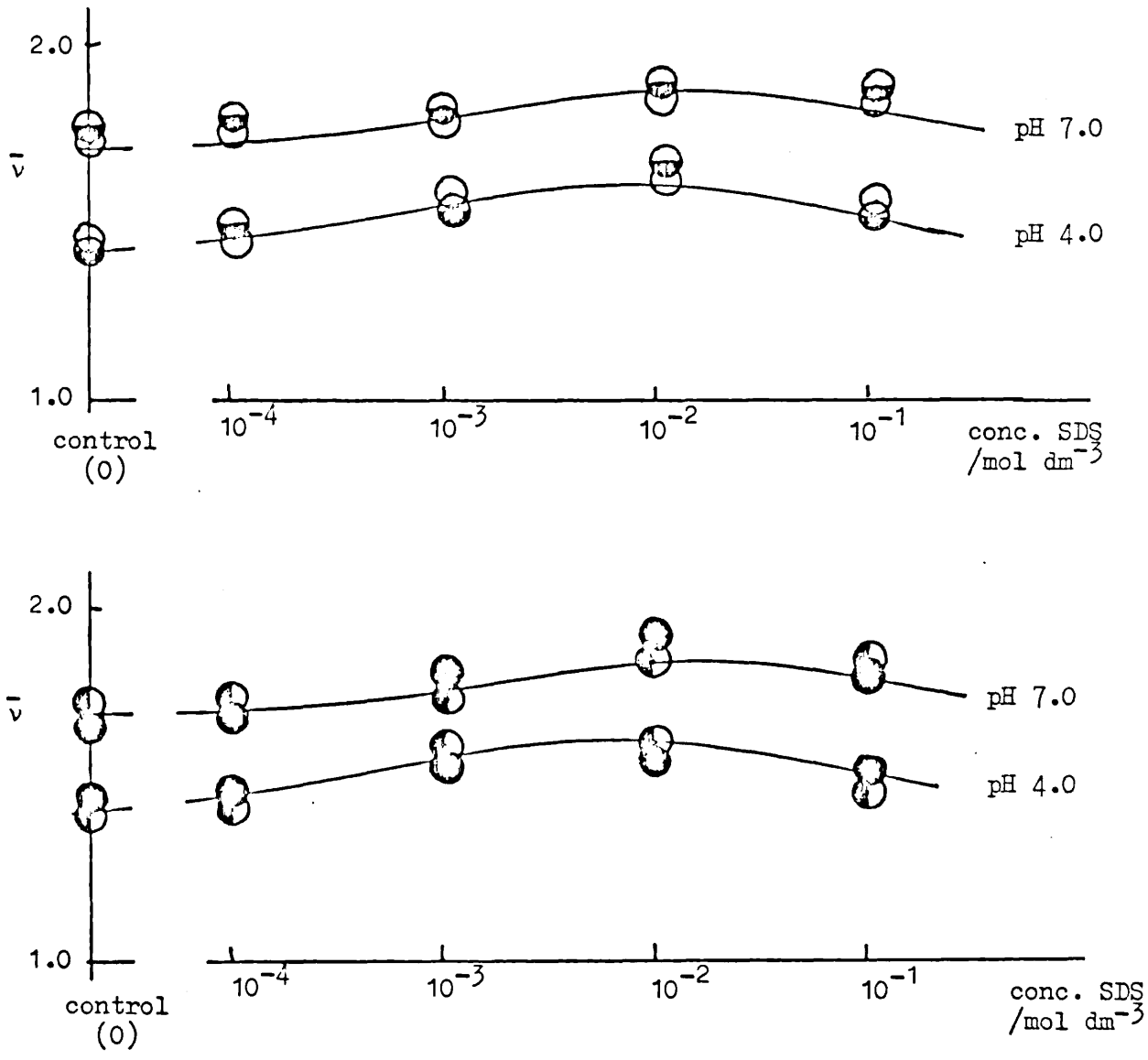
Although surface lipid has been detected by this method on other organisms (Lowick and James, 1957; Hugo and Stretton, 1966; Pechey, Yau and James, 1974), no detectable lipid was present on cells of BCG.

In view of the large quantities of lipid in mycobacterial cell walls this was surprising, therefore the mobility of cells of BCG was measured over a range of concentrations of SDS (2.3.4.). As previous workers (Dyar and Ordal, 1946) had observed an increase in negative mobility of cells of M. smegmatis and M. phlei on suspension in buffer solutions containing SDS at pH 4, but not at pH 7, mobility-concentration curves for the 5 sub-strains were determined at pH 7.0 and 4.0 (Figure 5.1). For cells of all sub-strains there was a slight increase in mobility exhibited at 10^{-2} mol dm^{-3} SDS at both pH-values. However this increase was less than 18% and therefore not indicative of large amounts of surface lipid. Further, the observed increase should be treated cautiously as it only occurs at high concentrations of SDS which could affect the surface and even extract lipid, usually resident in lower layers of the cell wall. Although concentrations of SDS higher than 10^{-4} to 10^{-3} mol dm^{-3} normally cause lysis of most bacterial cells, microscopic examination of the BCG cells revealed that the majority of cells remained intact even at a concentration of 10^{-1} mol dm^{-3} SDS.

To ascertain whether lipid was located further in the cell wall structure S-values of cells suspended in buffer solutions (I in range 0.002 to 0.1 mol dm^{-3}) containing SDS were determined. It can be seen (Figure 5.2) that as

FIGURE 5.1.

Mobility-concentration curves for cells of 5 sub-strains of BCG measured at pH 7.0 and pH 4.0 in buffer solutions containing SDS



Pasteur sub-strain

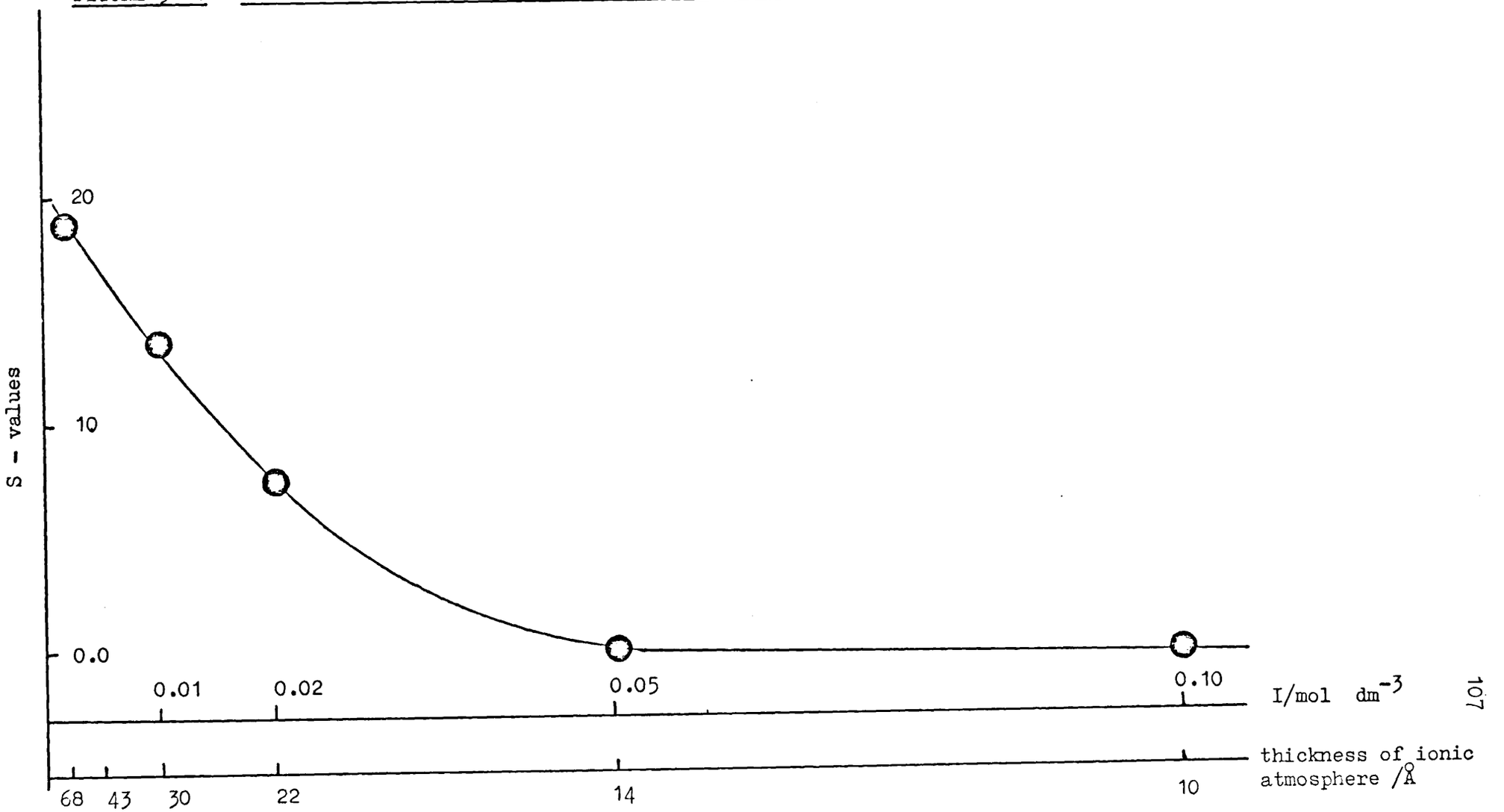
Danish sub-strain

Prague sub-strain

Japanese sub-strain

Glaxo sub-strain

FIGURE 5.2. S-values of cells of BCG over a range of ionic strength and ionic atmosphere.



the ionic atmosphere increased (i.e. as the ionic strength of the buffer decreased) so the amount of detectable lipid increased. The large quantities of cell wall lipids present in mycobacterial cells are therefore located further in the cell wall structure than the region under examination throughout this investigation. Thus lipid does not constitute a major component of the cell surface of BCG.

5.5. Extraction of Cells with Cold Chloroform/Methanol.

Certain lipids, such as Wax D and cord factor, known as "free-lipids" are removed from mycobacterial cells by extraction with cold organic solvents. To confirm the finding that surface lipids were not present at the surface of cells of BCG the cells were extracted for 24 h with cold chloroform/methanol (2.3.5.).

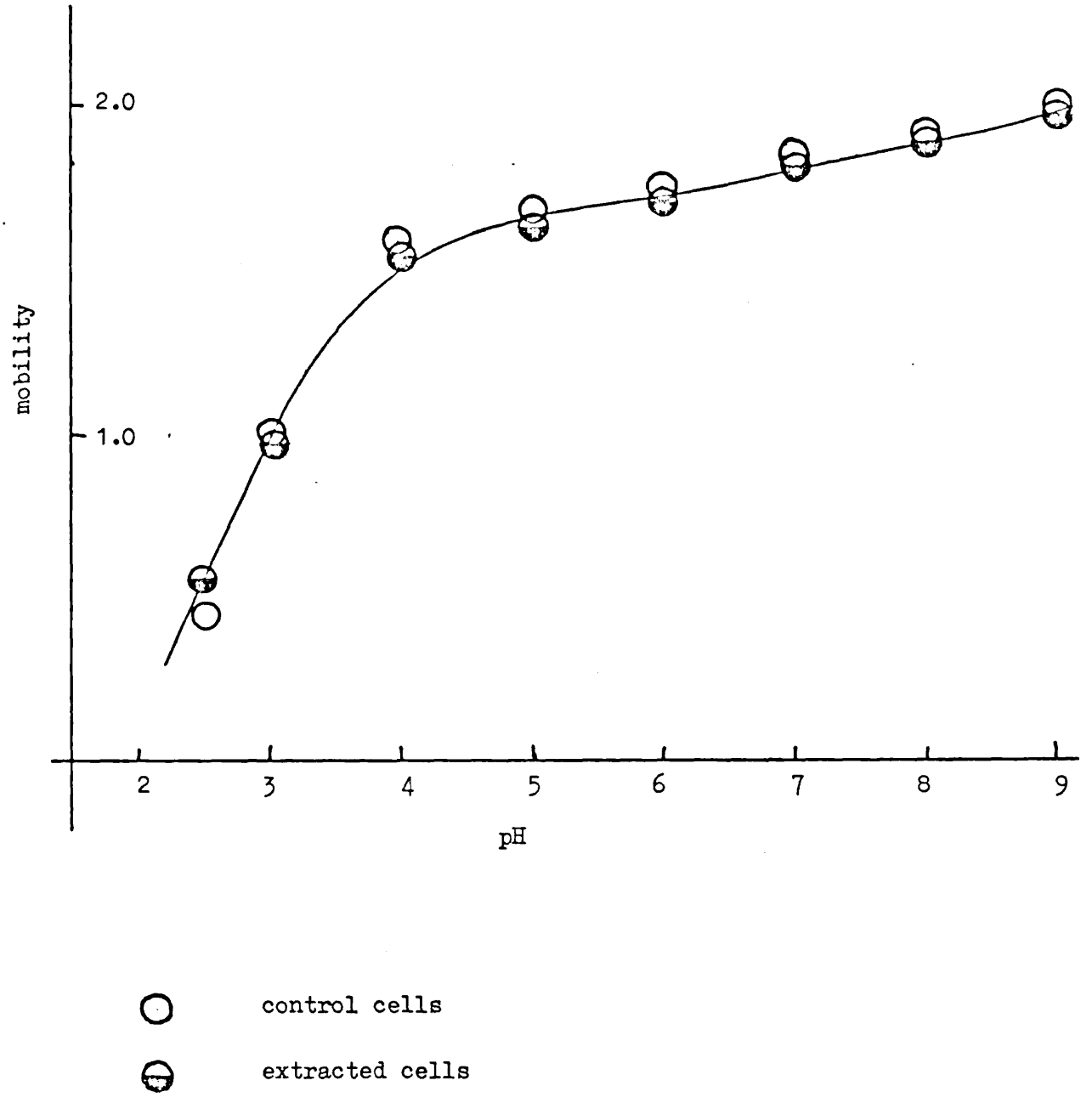
Figure 5.3. shows that removal of the "free-lipids" from cells of BCG caused no change in the shape or position of the pH-mobility curve confirming that these "free-lipid" moieties are not present at the cell surface.

5.6. Detection of Phosphate Groups.

It has already been confirmed that the negative charge of cells of BCG was not due to ionizable carboxyl groups. Sulphate groups are present in mycobacterial cells as sulpholipids in very small concentrations; these cannot be present on the cell surface as the lipid cannot be detected. The most likely anionic species which could give rise to the negative charge was therefore phosphate; this is known to be present in the cell wall of

FIGURE 5.3.

pH-mobility curves of cells of BCG before and after extraction with cold chloroform/methanol



mycobacteria. Also Adams and Rideal (1958) reported phosphate groups as the major species on the surface of cells of M. phlei.

5.6.1. Enzymatic detection of phosphate groups.

Three enzymes were used in an attempt to attack and remove phosphate groups on the surface of cells of BCG: acid phosphatase, phosphodiesterase and phospholipase C (2.3.6.). Table 5.4. shows that none of these enzymes had any effect on the surface charge of cells of BCG measured over a range of pH-values. Phosphate groups present on the surface of live cells of BCG may be immune to enzymic attack because of the presence of inhibitors which prevent the enzymic reaction. The same enzymes were therefore used to treat cells of BCG which had first been autoclaved. Autoclaving for 15 min at 15 lb $inch^{-2}$ had no effect on the value of the surface charge.

The results, summarized in Table 5.5., show again that these three enzymes have no effect on the surface charge of BCG. This means either that the ionogenic groups on the cell surface are resistant to attack by these phosphatases, on account of the highly specific nature of these enzymes or that the phosphatese esters are not presnt on the surface of cells of BCG. Cell wall phosphoesters are known to be very resistant to attack by commercially available phosphatases; these results therefore do not demonstrate conclusively the presence or absence of phosphate groups at the cell surface.

5.6.2. Specific sequence of reversal of charge by inorganic cations.

Inorganic cations cause specific reversal of charge sequences depending on the ionogenic groups present at the surface; for phosphates the uranyl ion reverses charge at a much lower concentration than the comparable divalent ions copper, lead, cadmium and zinc. These cations

Table 5.4.

Electrophoretic mobility of cells of BCG before and after enzymic treatment.

Enzyme used 0.01%	pH of measurement	10^8 x electrophoretic mobility /m^2 s^{-1} V^{-1}	
		Normal cells	Enzyme-treated cells
Acid phosphatase	9	2.24	2.20
	7	1.85	1.88
	5	1.71	1.66
	3	0.68	0.63
Phosphodiesterase	8	2.00	1.99
	6	1.82	1.80
	4	1.41	1.40
	2.5	0.73	0.73
Phospholipase	9	2.10	2.09
	7	1.70	1.75
	5	1.67	1.65
	3	0.96	0.93

Table 5.5.

Electrophoretic mobility of autoclaved cells of BCG before and after enzymic treatment.

Enzyme used 0.01%	pH of measurement	10^8 x electrophoretic mobility /m^2 s^{-1} V^{-1} Normal cells	Enzyme-heated cells
Acid phosphatase	9	2.19	2.20
	7	1.87	1.88
	5	1.70	1.66
	3	0.59	0.63
Phosphodiesterase	9	2.21	2.20
	7	1.88	1.88
	5	1.69	1.66
	3	0.62	0.63
Phospholipase C	9	2.10	2.20
	7	1.86	1.88
	5	1.70	1.66
	3	0.65	0.63

were therefore used to ascertain whether the anionic species giving rise to the negative surface charge of cells of BCG was phosphate (2.3.7.). The mobility-concentration curves (Figure 5.4.) measured at constant ionic strength show that the uranyl ion caused charge reversal at a ten fold lower concentration than did the other divalent ions.

This method of charge reversal is subject to many inacuracies. Ideally the pH used to measure all the mobility values should lie on the plateau-region of the pH-mobility curve (pH 5.0 - 9.0); however, owing to some hydrolysis it was necessary to use a pH-range of 4.0 - 5.0; this is not quite on the plateau-region and could therefore give rise to some inaccuracy. The extrapolation of the mobility-concentration curves to obtain the reversal of charge concentration is another potential source of error. However, considering these sources of error, the uranyl ion still caused charge reversal at a considerably lower concentration than did the other divalent ions used.

Figure 5.5. shows a comparison of the charge reversal spectra for cells of BCG and other phosphate biocolloids. The present results confirm the findings of Adams and Rideal (1958) that the ionogenic species giving rise to the negative surface charge on mycobacterial cells is phosphate. For all the spectra, the uranyl ion concentration for reversal is very much less than that of the nearest divalent ion, Pb, and the sequence of ions is similar.

5.7. Summary.

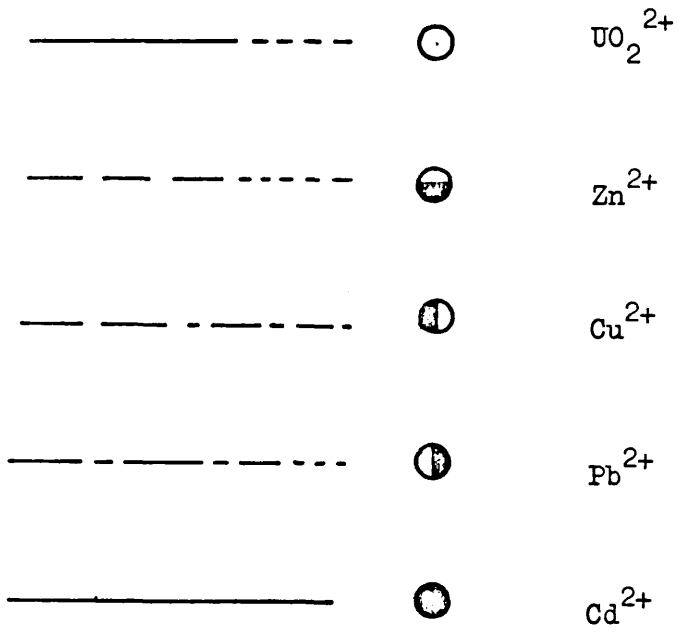
5.7.1. The negative surface charge of cells of BCG is not due to ionized carboxyl groups.

5.7.2. There are no detectable amino groups on the cell surface of BCG.

5.7.3. Lipids do not constitute a major component of the cell surface. Very little, if any, lipid is present on the surface of cells of BCG,

FIGURE 5.4.

The effect of divalent inorganic cations on the electrophoretic mobility of cells of BCG at constant ionic strength ($I = 0.05$ mol dm^{-3})



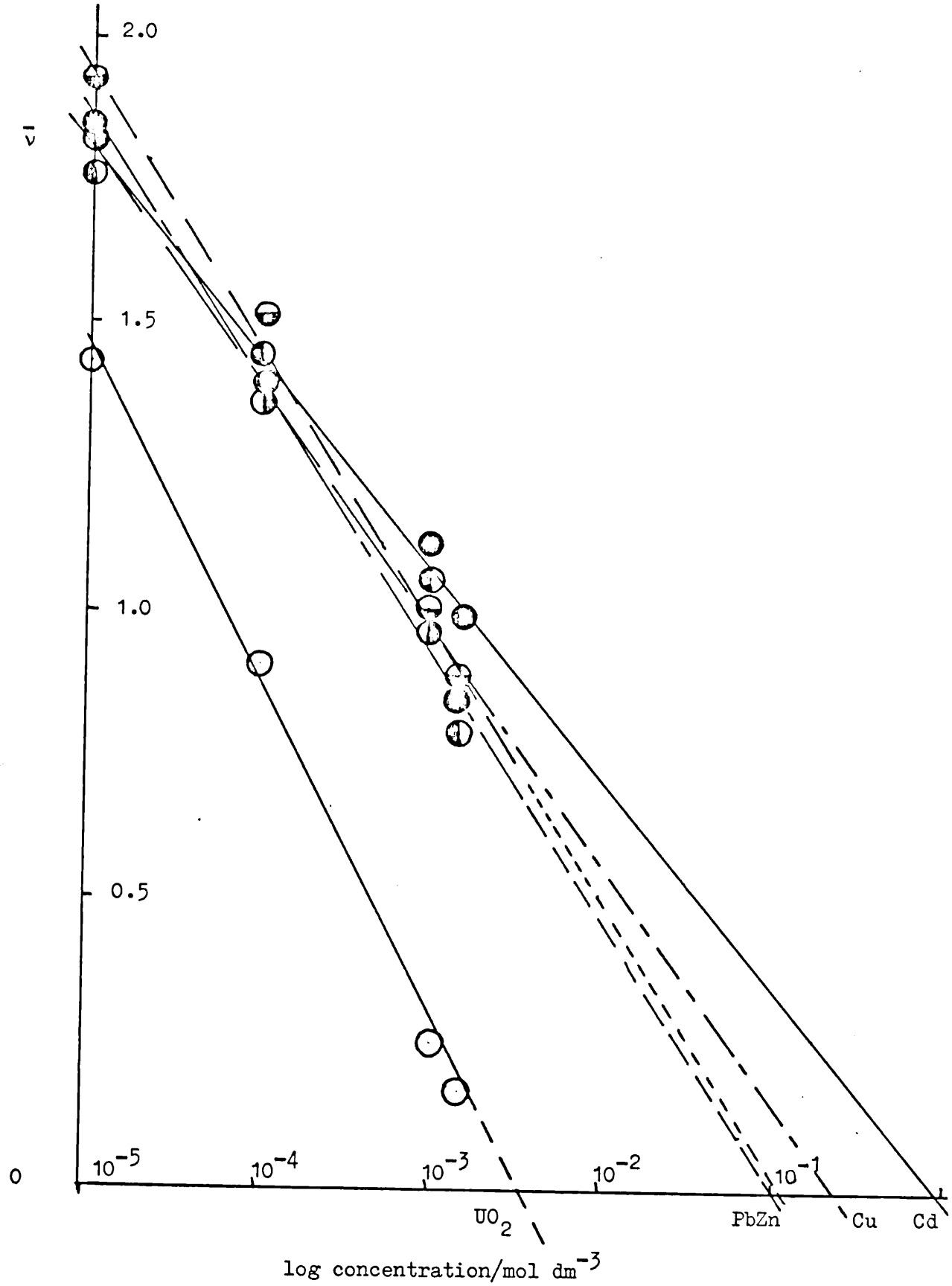
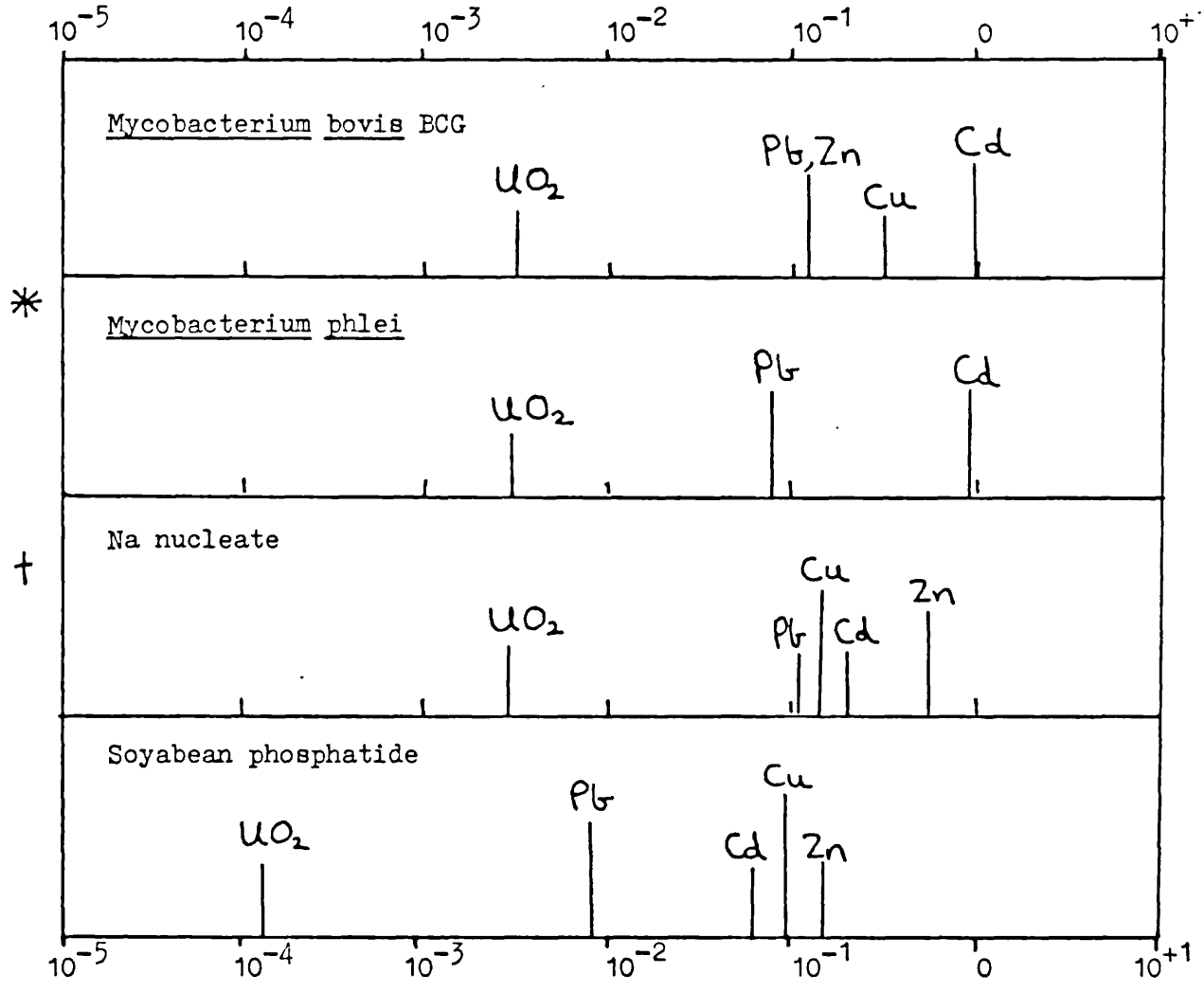

2.0
1.5
1.0
0.5
0
$\bar{\nu}$
10^{-5}
10^{-4}
10^{-3}
10^{-2}
10^{-1}
1
UO_2
PbZn
Cu
Cd
log concentration/mol dm^{-3}

FIGURE 5.5.

Comparison of the reversal of charge spectra obtained for cells of M. bovis BCG compared with those of other biocolloids



* Adams and Rideal (1958)

† Kruyt (1949)

the large quantities of cell wall lipid being located deeper into the cell wall structure.

5.7.4. There are no surface sulphydryl groups on the cells of BCG.

5.7.5. The negative surface charge of cells of BCG is due to ionizable phosphate groups, probably those providing phosphodiester linkages in the cell wall skeleton. These phosphate groups are resistant to attack by acid phosphatase, phosphodiesterase and phospholipase C.

CHAPTER 6

PREPARATION AND SURFACE PROPERTIES OF CELL WALLS OF M. BOVIS BCG

Whole cells of BCG are not only used to protect against T.B. but recently have been used as an effective immunopotentiator in the immunotherapy of cancer. Various kinds of side-effects result from such therapy, and so interest has been shown in the use of BCG cell wall preparations as immunotherapeutic agents (Yamamura, 1978) because these do not give rise to such complications. The surface properties of a cell wall preparation of the Glaxo sub-strain of BCG were examined to see if they differed from those of the intact cells.

6.1. Cell Wall Preparation.

Other workers prepare their cell walls by drastic treatments (Imaeda et al, 1968; Yamamura, 1978) so that the cell wall will be mainly fragmented. To enable electrophoretic mobility determinations to be carried out on the cell walls they were required to be broken open, but still as intact as possible. A relatively gentle method for breaking the cells was therefore required; sonication proved to be a suitable method. Washed cells suspended in ice-cooled buffer solution were subjected to ultrasonic vibrations for varying time periods and samples removed and monitored by transmission electron microscopy. Examination of the electron micrographs (Figures 6.1. and 6.2.) showed that after 1 minute of sonication approximately 65% of the cells were still intact; after 2 minutes, 35%; after 5 minutes, 2%; and after 10 minutes there were no intact cells. The control sample (Figure 6.1.) was composed entirely of intact cells.

Cell wall suspensions were therefore prepared by sonication for 3 minutes, washed in buffer solution (2.4.2.) and the washed cell walls examined by transmission electron microscopy (Figure 6.3). The samples obtained consisted of fairly intact and relatively "clean" cell walls.

FIGURE 6.1: Transmission electron micrograph of intact cells of the Glaxo sub-strain of BCG (control sample) (x 15,000)

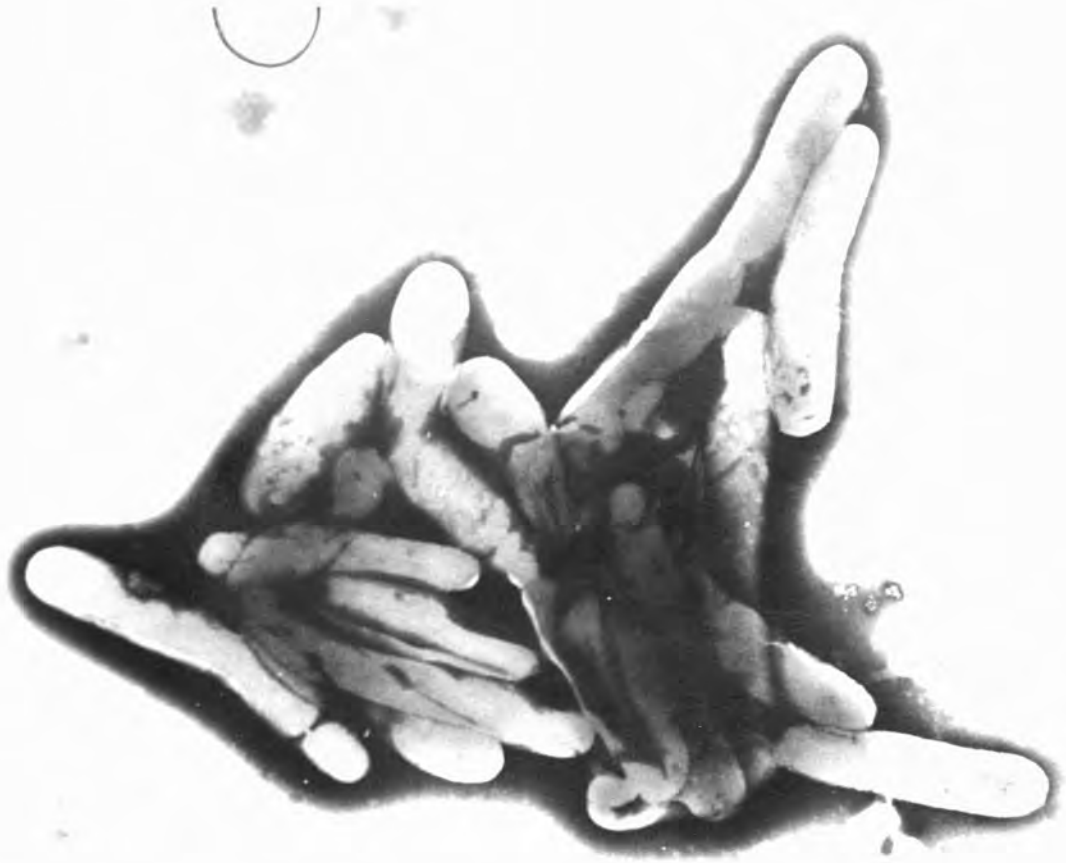


FIGURE 6.2.

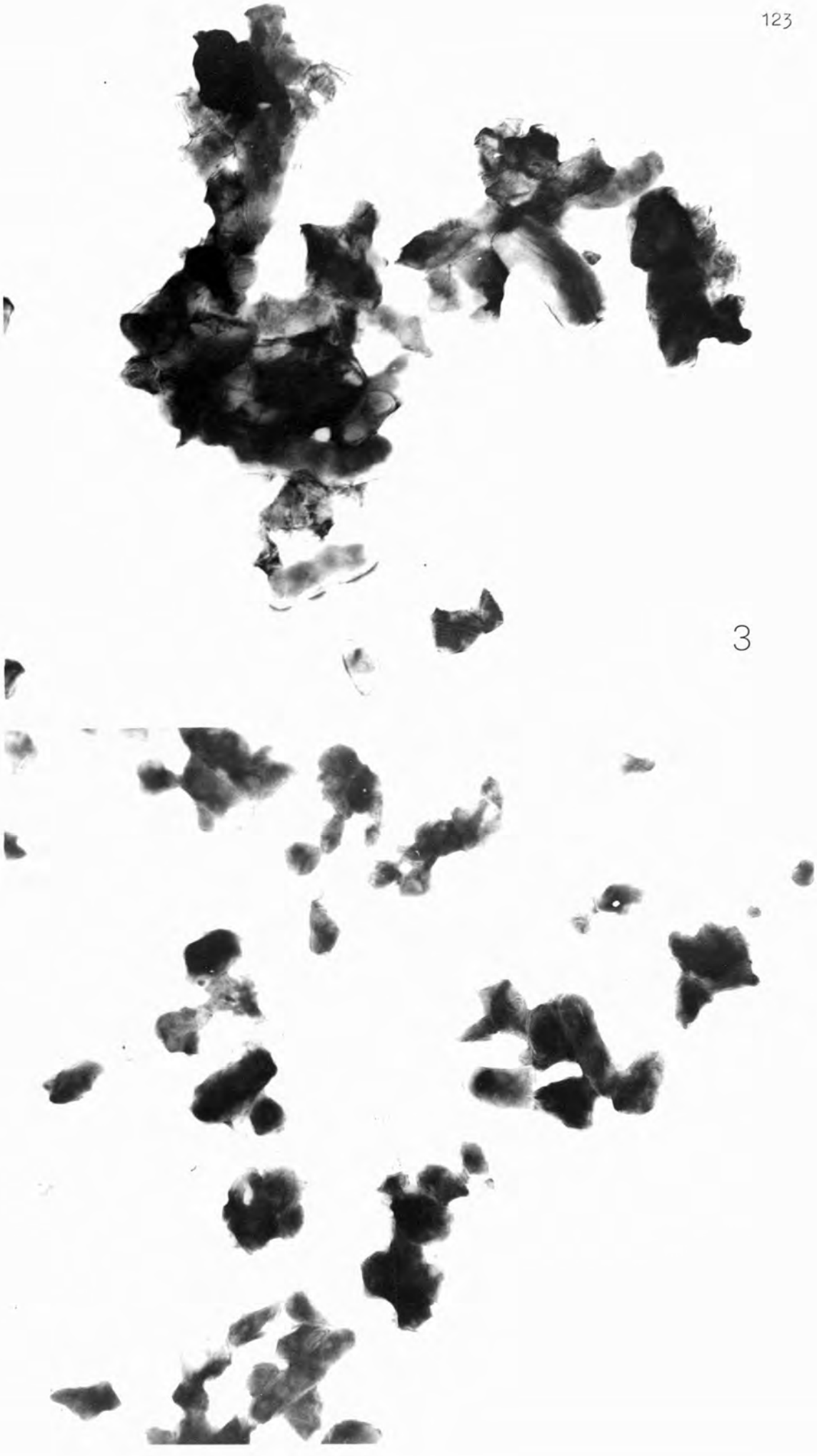
Transmission electron micrographs of cells of BCG subjected to varying periods of sonication.

1. 1 minute
2. 2 minutes
3. 5 minutes
4. 10 minutes

(magnification x 15 000)

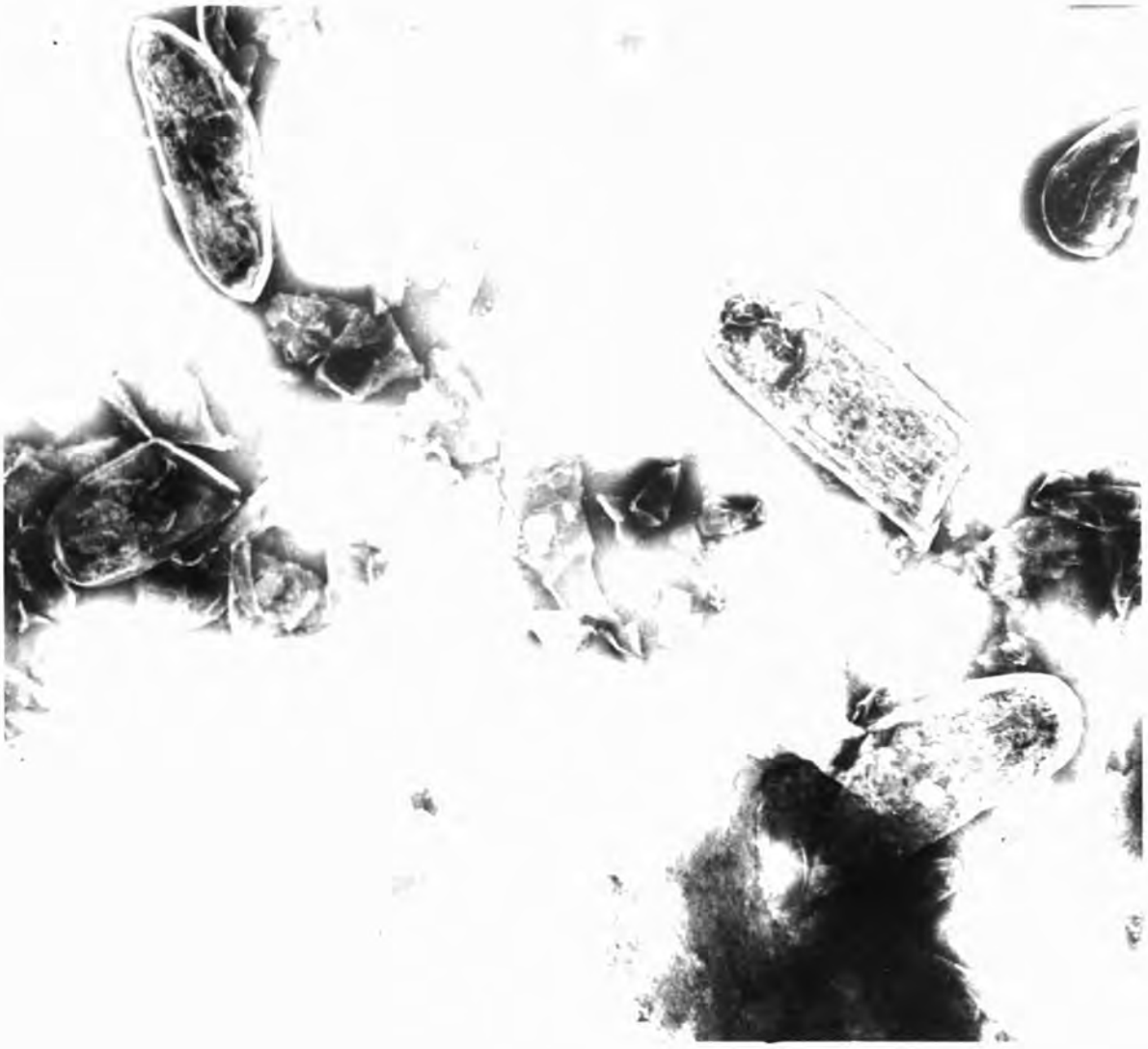
1

2



3

FIGURE 6.3: Transmission electron micrograph of a washed preparation of cell-walls of the Glaxo sub-strain of BCG prepared by sonication (x 30,000)



6.2. pH Mobility Curves and Surface Lipid Determinations.

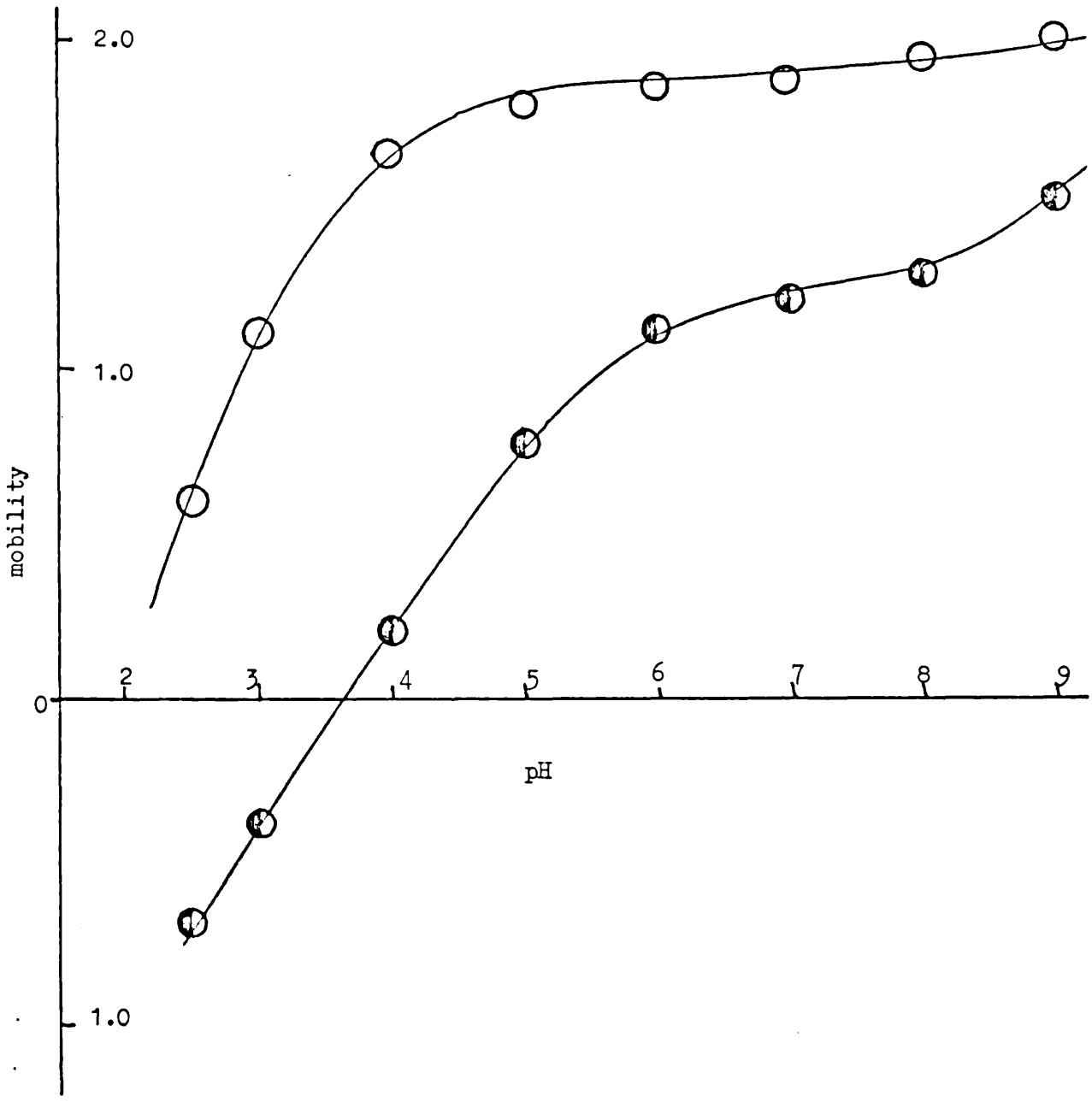
Cell walls were prepared for electrophoretic measurements as described previously (2.4.3.). The cell wall suspension exhibited a different pH-mobility curve to that obtained for whole cells (Figure 6.4.). The curve was characteristic of a mixed anionic/cationic surface, the presence of cationic (amino) surface groups was shown by the charge reversal at pH-values below 3.5 and indication of further increase in negative mobility at pH 9.0. Charges on both the inside and outside surfaces of the cell walls will be measured, due to the cells being broken. This means that the positively charged groups observed on cell walls but absent on whole cells, are most probably located on the inside surface of the cell wall structure. These are probably the ionizable amino groups present on the peptide moiety of the peptidoglycan of the cell wall. Anionic surface groups are also present and these may consist of carboxyl groups from the inner cell wall surface as well as the phosphate present on the outer surface.

Surface-lipid determination on the cell walls by suspension and measurement of mobility in buffer solutions containing 10^{-4} mol dm^{-3} SDS (2.3.4.) gave an increased mobility of 14%, indicating the presence of a small amount of surface lipid. This suggests that the large quantities of lipid material present in mycobacterial cell walls is sandwiched between the outer and inner surfaces of the cell wall structure and therefore not a component of either the inner or the outer surface. The increased surface lipid detected could be accounted for by that exposed where the cell walls were broken.

Inoculation of cell wall preparations into animals will therefore present a different ionogenic, and therefore immunogenic, surface to the challenged animal than inoculation of whole cells.

FIGURE 6.4.

pH-mobility curves of whole cells and cell walls of the Glaxo sub-strain of BCG



whole cells

cell wall preparation

6.3. Summary

6.3.1. Suitable conditions for the preparation of cell wall suspension of the Glaxo sub-strain of BCG were achieved.

6.3.2. The cell walls exhibited different surface properties to those of whole cells, possessing ionizable amino groups on the surface as well as anionic surface groups

6.3.3. Only a small amount of surface lipid was observed suggesting that the large lipid fraction of mycobacterial cell walls is sandwiched between the outer and inner surfaces.

CHAPTER 7

ELECTRON MICROSCOPY OF CELLS OF M. BOVIS BCG

The ultrastructure of mycobacteria, extensively studied by transmission electron microscopy, TEM, (Yamaguchi, 1955; Takeya et al, 1958, 1959 and 1961; Imaeda, 1965) reveals that the mycobacterial surface is enclosed by a fibrillar lipid substance; the inner cell wall is believed to consist of a lipid-mucopolmer polysaccharide complex (Takeya et al, 1963). Cell walls of mycobacteria have also been examined by TEM (Imaeda et al, 1968). Takayama et al (1973) examined cells of M. tuberculosis by scanning electron microscopy, SEM, after exposure to isoniazid, but there is no reported SEM study of normal mycobacterial cells.

7.1. Scanning Electron Microscopy of Cells of BCG.

To ensure that the cells of BCG remained as normal as possible the cells were simply air-dried onto cover-slips and mounted onto SEM stubs for examination (2.5.1.). The surface pellicle obtained after growth on Sauton medium (Figure 7.1.1.). was completely covered by an amorphous substance (Figure 7.1.2.). By sliding off the edge of the surface pellicle and focusing carefully it was possible to observe single organisms. These were sausage-shaped cells, free of the amorphous covering material (Figures 7.1.3 and 7.1.4.). There was also evidence of organisms actually within the surface pellicle, still covered by the structureless material (Figure 7.1.3., arrowed).

Cells grown as a dispersed culture in Dubos medium were observed before (Figure 7.2) and after (Figure 7.3.) manual tissue-grinding. In unground specimens large clumps of cells were present (Figure 7.2.1.), again covered by an amorphous substance with no visible structural features (Figure 7.2.2.). At the edges of these clumps single organisms were observed (Figure 7.2.3.); these were still closely associated with the amorphous material (Figure 7.2.4.).

FIGURE 7.1.

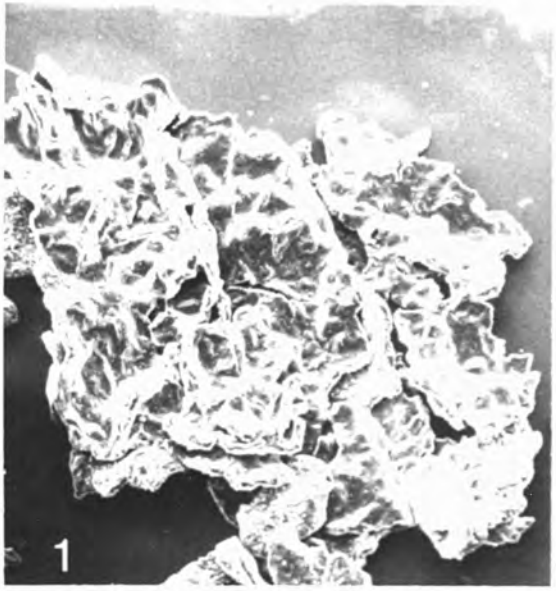
Scanning electron micrographs of BCG grown as a surface pellicle on Sauton medium.

1. Surface pellicle (x 22)

2. Surface pellicle (x 22 000)

3. Cells at the edge of the surface pellicle (x 5 500)

4. Cells (x 11 000)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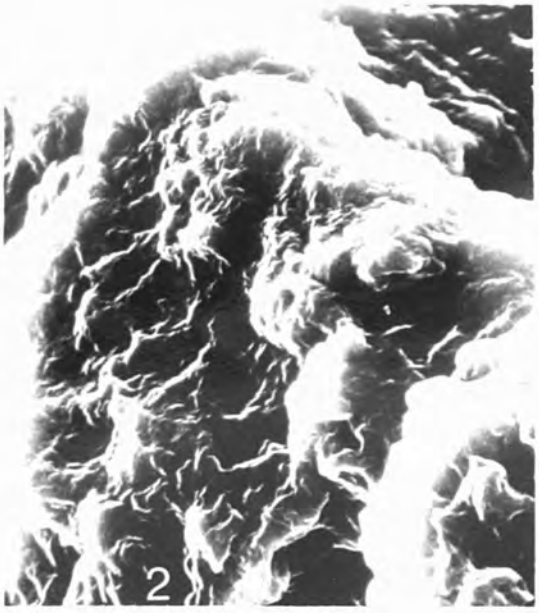
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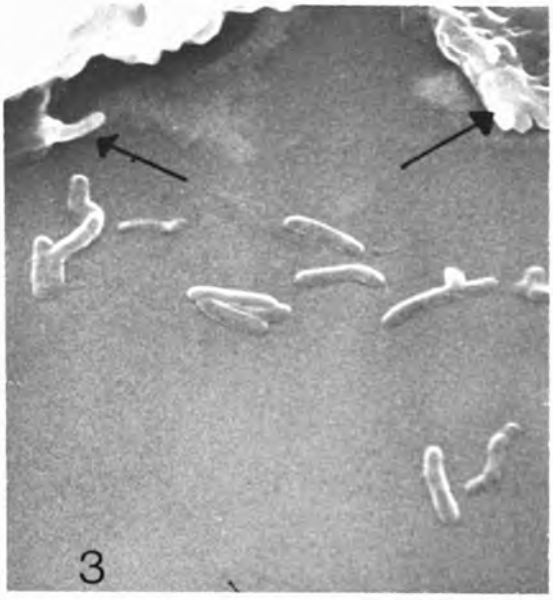
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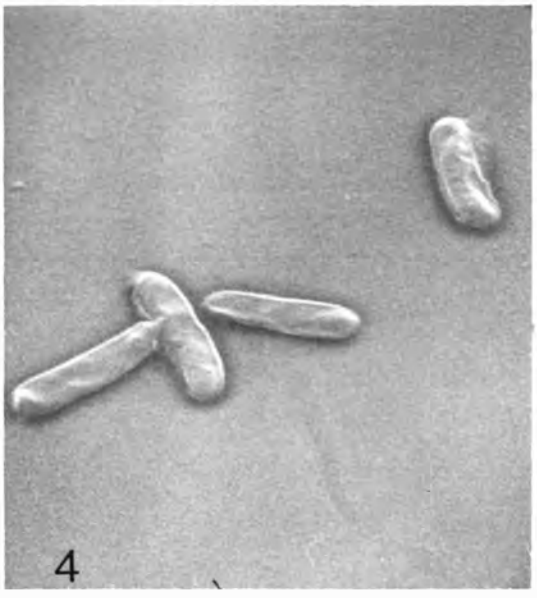

4

FIGURE 7.2.

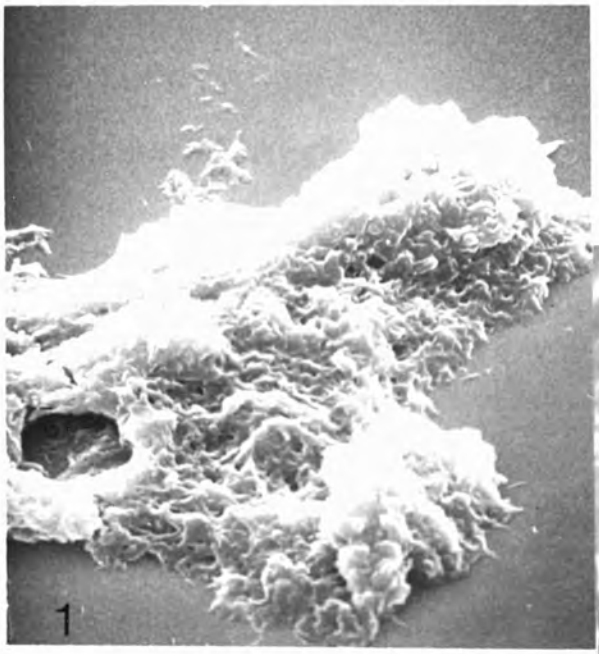
Scanning electron micrographs of BCG grown as a dispersed culture in Dubos medium, washed in distilled water.

1. "Clump" of cells (x 1 200)

2. "Clump" of cells (x 12 000)

3. Group of cells (x 6 000)

4. Group of cells (x 14 000)



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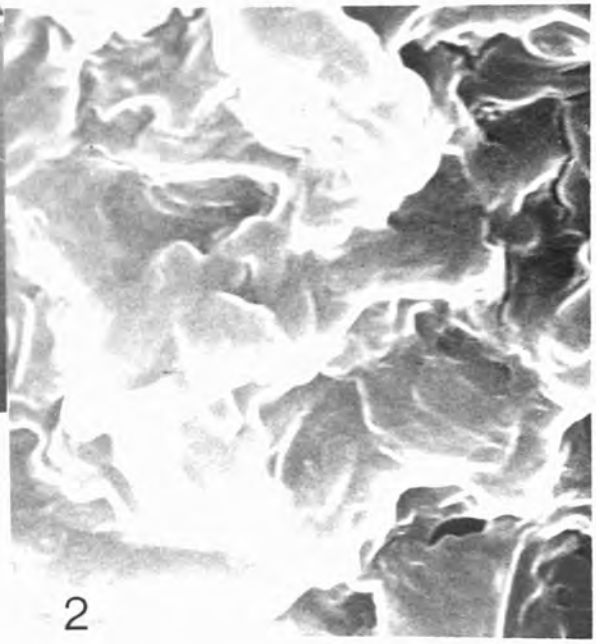

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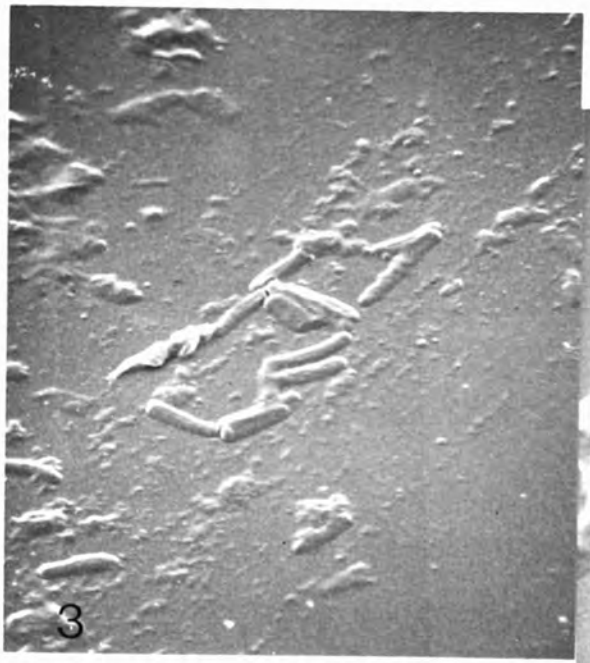

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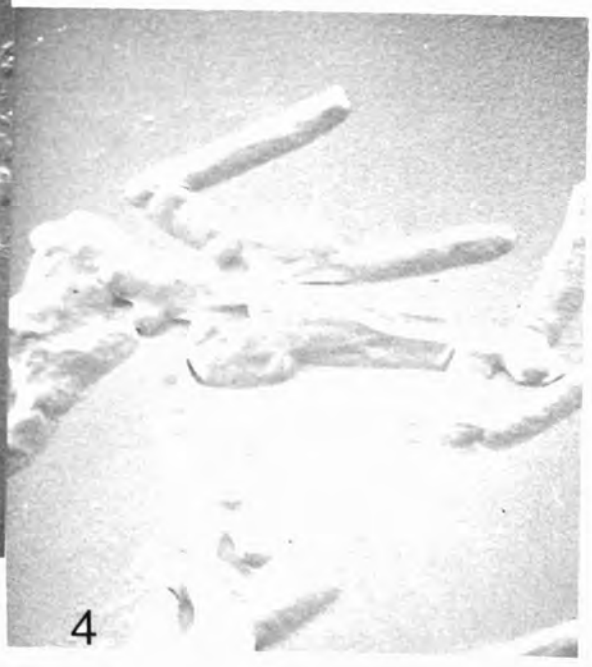


4

Examination of the tissue-ground samples showed light "patches" on the cover slip (Figure 7.3.1); magnification of these "patches" revealed globules of an amorphous substance (Figure 7.3.2.) with evidence of single organisms amongst this material (arrowed). At the edges of the "patches" were well separated, single organisms (Figure 7.3.3.). These were free of the covering material.

SEM of whole colonies of BCG (Figure 7.4.) clearly showed the difference in heaping between "smooth" colonies (Figure 7.4.1.) and "rough" colonies (Figure 7.4.3.). The whole colonies were also covered by amorphous material (Figure 7.4.4.). A section cut through a colony showed a random arrangement of many single organisms apparently free from this structureless material (Figure 7.4.2.).

It appears that cells of BCG excrete amorphous material as a covering for both colonies and clumps of cells. The material is similar in apperance to a polysaccharide-type slime layer on other organisms, but is not a true slime layer in that it does not cover each individual organism. It is also completely stripped off the cells by manual tissue-grinding to leave intact, "clean" cells free of this slime-like material.

7.2. Transmission Electron Microscopy of Cells of BCG.

Detailed examination of the ultrastructure of mycobacteria by TEM has already been reported (Barksdale and Kim, 1977). Cells of BCG were examined by TEM to obtain further evidence of the covering "slime-like" material observed in the SEM studies. Figure 7.5. shows a typical clump of BCG cells observed under TEM. The shadowing effect observed around and above the single cells is most probably due to the presence of the amorphous covering material previously observed. The fact that the single organisms constituting the clump can be clearly seen again suggests that this material covers the entire clump of cells rather than coating individual organisms.

FIGURE 7.3.

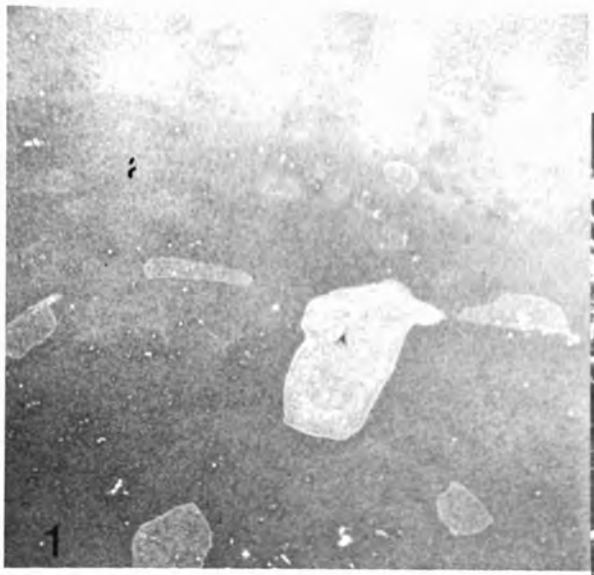
Scanning electron micrographs of BCG grown as a dispersed culture in Dubos medium, washed in distilled water and tissue-ground.

1. "Patches on the specimen cover slip (x 21)

2. Magnification of one of the "patches" (x 5 500)

3. Cells (x 12 000)

4. Single organism (x 24 000)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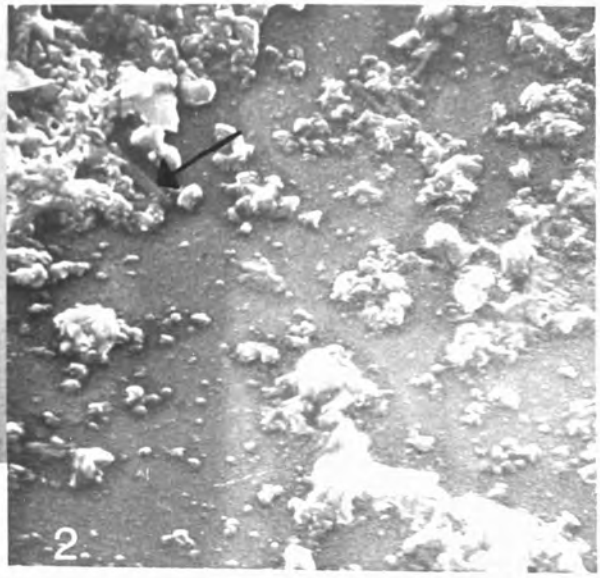
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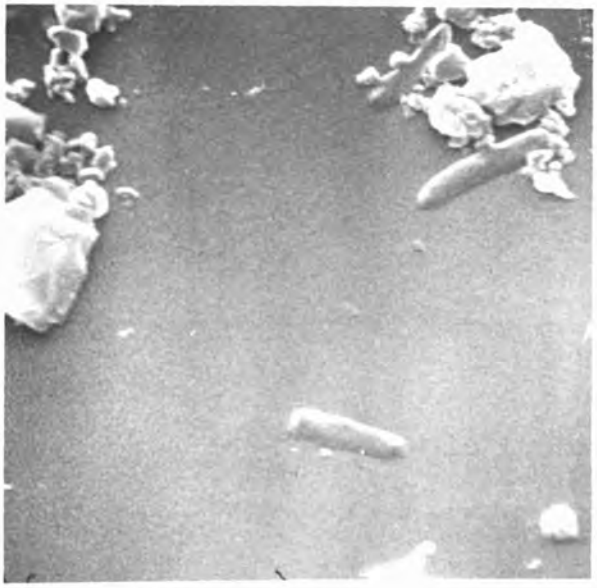


FIGURE 7.4.

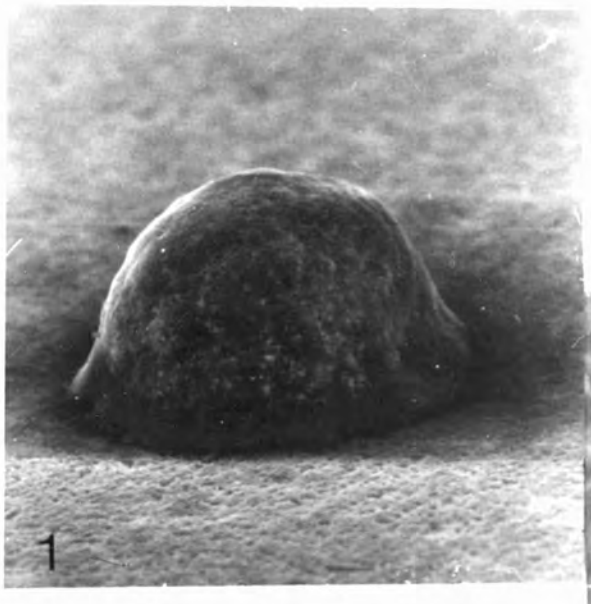
Scanning electron micrographs of colonies of BCG isolated onto filters.

1. Typical "smooth" colony of the Glaxo sub-strain of BCG (x 240).

2. Cut section of the same colony showing random organisation of organisms (x 12 000)

3. Typical "rough" colony of the Pasteur sub-strain of BCG (x 240).

4. Magnification of surface of a colony of the Pasteur sub-strain of BCG (x 22 000).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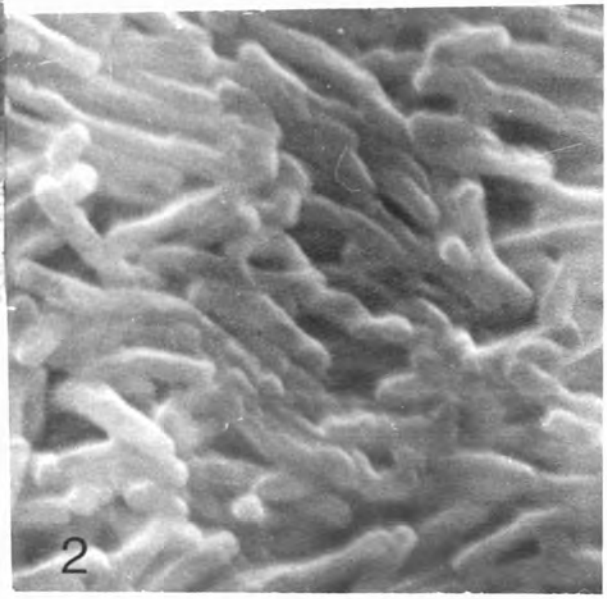
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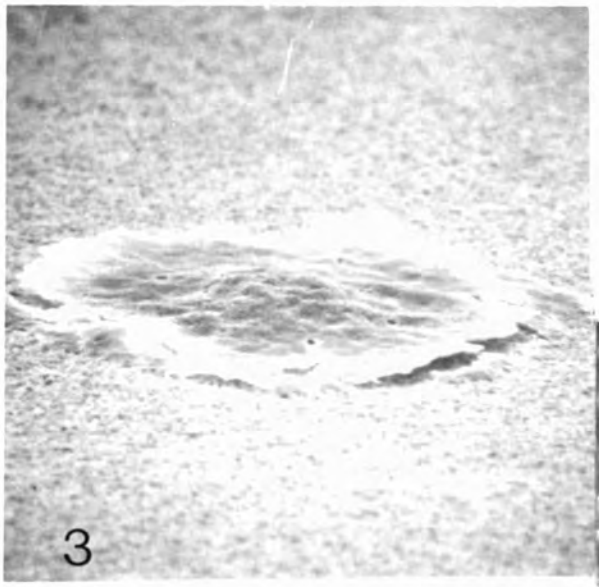
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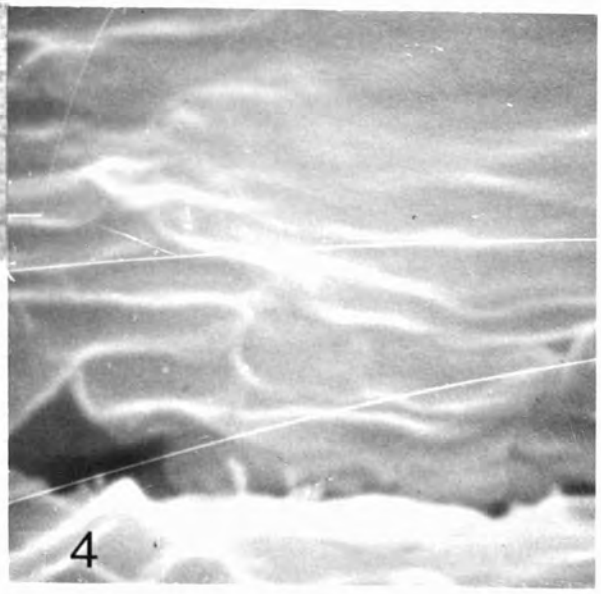
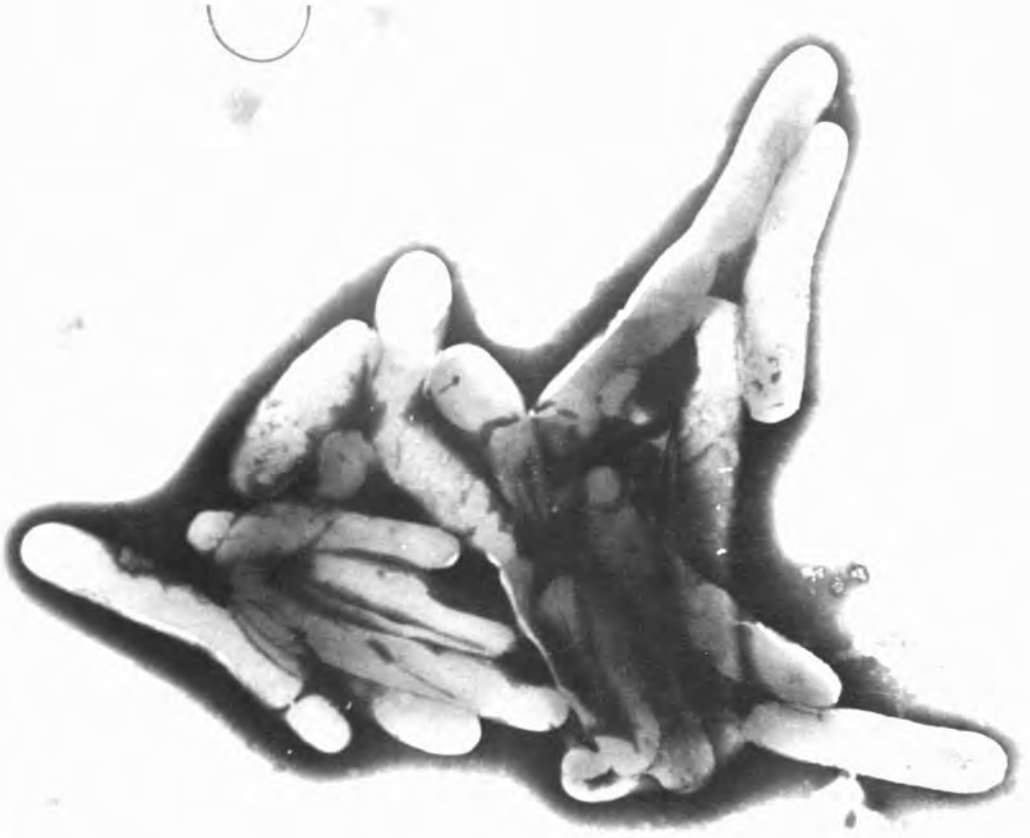

4

FIGURE 7.5: Transmission electron micrograph of the Glaxo sub-strain of BCG (x 15,000)



7.3. Electron Microscopy of Cell Wall Preparations of BCG.

Detailed examination of mycobacterial cell walls by TEM has been carried out previously (Imaeda, Kanetsuna and Galindo, 1968). SEM revealed that the cell wall preparations still clumped to give structures covered with amorphous material (Figure 7.6.1.). The broken cell wall preparations appeared very angular compared to intact cells, and some fairly intact cells which remained resembled deflated balloons (Figure 7.6.2 and 7.6.3.). Much cell debris was present. Examination of the cell-wall preparations by TEM revealed angular broken cell walls, some still fairly intact cells and again a shadowing effect giving evidence of the presence of the amorphous covering material (Figure 7.7.).

7.4. Summary.

7.4.1. Cells of BCG appear to secrete a "slime-like" substance which completely covers clumps, colonies and the surface pellicle of cells obtained after growth on Sauton medium.

7.4.2. This amorphous material does not cover individual organisms and can be stripped off cells by manual tissue-grinding.

7.4.3. This substance is secreted by cells grown on all media and by all the sub-strains examined.

7.4.4. Cell-wall preparations of BCG appear able to secrete this substance, although this could be coming from the few intact cells which still remained after sonication.

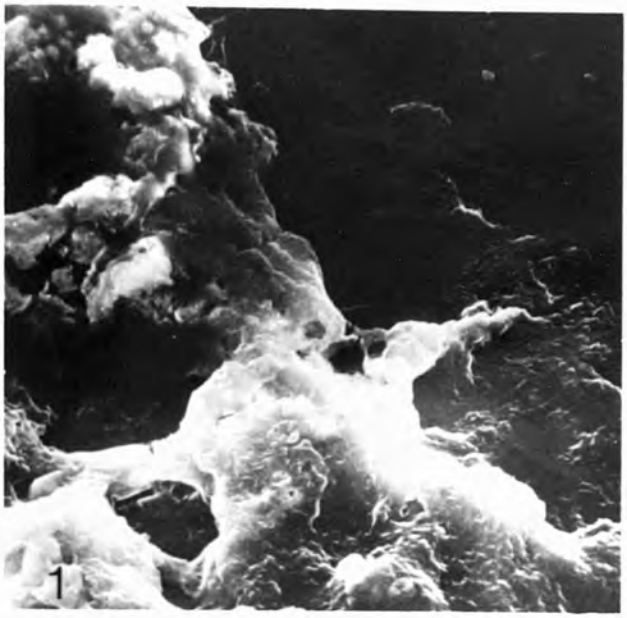
FIGURE 7.6.

Scanning electron micrograph of cell wall preparations of the Glaxo sub-strain of BCG.

1. "Clump" of cell walls (x 2 000)

2. Cell walls (x 10 000)

3. Magnification of cell walls showing deflated cells (x 21 000)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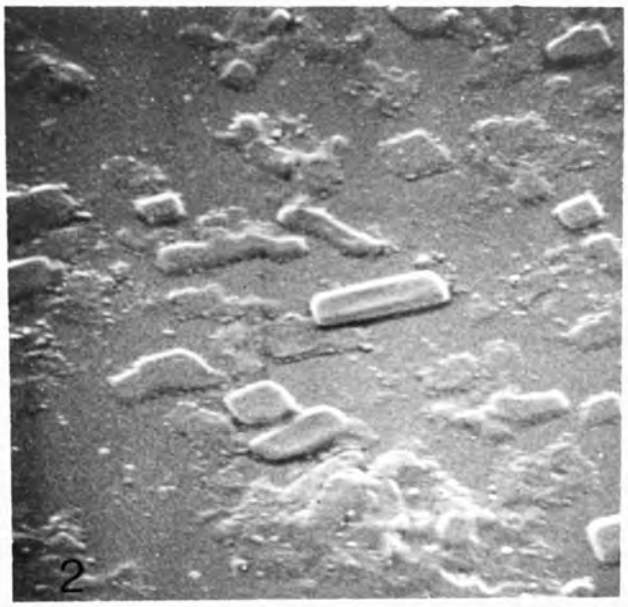
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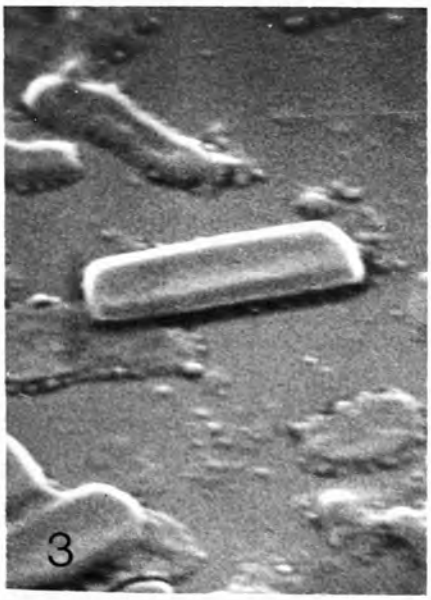
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FIGURE 7.7: Transmission electron micrograph of cell-wall preparation of the Glaxo sub-strain of BCG (x 30,000)

CHAPTER 8

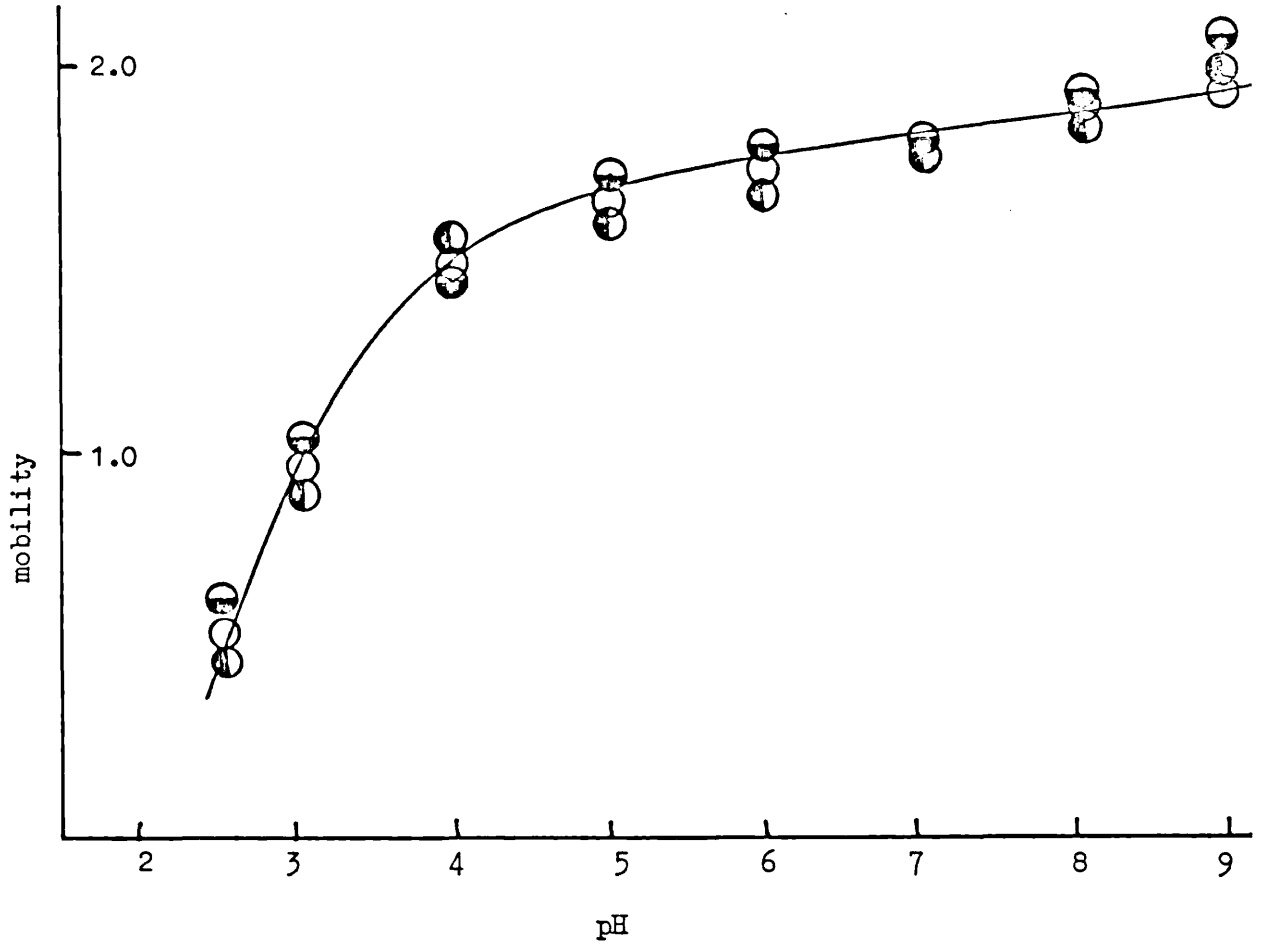
SURFACE PROPERTIES OF CELLS OF SOME OTHER MYCOBACTERIAL SPECIES

The negative charge on the surface of cells of M. bovis BCG, believed to be due to ionizable phosphate groups, was unaffected by vigorous chemical treatments, enzymatic attack or the growth media used. The charge was also common to all the sub-strains of BCG examined. It therefore seemed probable that this charge arose from phosphate groups in the covalently bound cell wall structure. As this basic covalent cell wall skeleton is common to all mycobacterial species the surface properties of some other mycobacterial species were investigated.

The pH-mobility curves for 7-day-old cells of Mycobacterium phlei, M. smegmatis and M. microti (Figure 8.1) are coincident with the pH-mobility curve of cells of BCG. This is further evidence that the surface charge on these cells is due to a common ionogenic species, possibly the phosphate groups covalently bound to the common cell wall structure. The phosphate groups probably arise from the phosphodiester linkages between the peptidoglycan backbone and the glycolipid of the mycobacterial cell wall.

FIGURE 8.1.

pH-mobility curves for cells of several species of mycobacteria



cells of BCG

cells of M. phlei

cells of M. smegmatis

cells of M. microti

CHAPTER 9

DISCUSSION

Mycobacterium bovis BCG is characterised by a very low growth rate (mean generation time 24 h) and a great tendency to aggregate during growth, which makes the production of a homogeneous vaccine extremely difficult. The aggregation of cells of BCG is markedly supressed by the addition of a non-ionic wetting agent to the growth medium, but the presence of such a surfactant causes a decrease in total yield. From the original isolate of Calmette and Guérin a whole series of sub-strains of BCG have arisen due to the different handling of these sub-strains by different laboratories. These sub-strains exhibit different biological and physical properties. It was considered that these differences and the tendency of all BCG sub-strains to aggregate could be due to physico-chemical forces arising from charges carried by the cells. As the surface properties of intact cells of BCG has never been examined in detail, an investigation of these properties was carried out.

The main technique employed was that of particulate microelectrophoresis, which enables identification of the charged groups of bacterial cell surfaces and examination of any changes in surface properties due to factors such as growth in different media. The chemical composition of mycobacterial cell walls is well established (Petit and Lederer, 1978) and suggestions as to the structure have been advanced. There is no guarantee that the molecular structure of cell wall components is the same in the free state as in the intact cell and therefore this proposed arrangement of the cell wall structure is open to discussion. The main advantage of particulate electrophoresis is that it allows the examination of undamaged, complete cells in suspension in aqueous media. The results obtained from studies using this technique have been related to the proposed cell wall structure.

Under the experimental conditions used for the measurement of the electrophoretic mobility, $\bar{\nu}$, the zeta-potential, ζ , can be calculated from the Smoluchowski equation:

$$\zeta = \frac{\bar{\nu}\ \eta}{\varepsilon_o \varepsilon_r} \qquad 9.1.$$

(where η is the coefficient of viscosity, ε_r the relative permittivity of the medium and ε_o the permittivity of free space) and hence the charge density, σ_D, in the diffuse part of the double layer from the equation for non-penetrable surfaces:

$$\sigma_D = \left(\frac{N_A\ e\ k\ T}{500\ \pi}\right)^{1/2} c^{1/2} \sinh\left(\frac{Z\ e\ \zeta}{kT}\right) \qquad 9.2.$$

(where N_A is the Avogadro constant, e the electronic charge, k the Boltzmann constant, c the concentration of the symmetrical electrolyte of valence Z).

For 1:1 electrolytes at 25 ^{o}C, inserting the values of fundamental constants, these equations become:

$$\zeta/\text{mV} = 12.85 \times \bar{\nu} \qquad 9.3.$$

and

$$\sigma_D/\text{cm}^{-2} = 3.713 \times 10^{-3}\ (\text{c/mol m}^{-3})^{1/2} \sinh\left(\frac{\zeta/\text{mV}}{51.3}\right) \qquad 9.4.$$

For penetrable surfaces, e.g. biological cells, if α is the fraction of the total space within the surface which is not available to counter ions, then the value of σ_D calculated from either equations 9.2. or 9.4. must be multiplied by the factor $[1 + (1 - \alpha)^{1/2}]$. This correction factor, first suggested by Haydon (1961), takes into account the penetrable region of the cell. For penetrable surfaces there will be free ions both inside and outside the surface; this means

that the charge density may be higher by a factor of up to twice that calculated by the original equation 9.4. Unfortunately information on the value of α within the peripheral zone of a cell and its variation with ionic strength is not available and so the calculated surface charge density and the total number of charges per cell is liable to be in error by a factor of up to 2.

Many previous workers reported their experimental results, from particulate electrophoresis, in terms of the zeta-potential at the cell-electrolyte interface. This potential is determined partly by the ionic strength and pH of the suspending electrolyte and partly by the nature and quantity of ionogenic surface groups. Calculation of the zeta-potential from the experimentally determined mobility values (equations 9.1. and 9.3.) assumes that the viscosity and relative permittivity within the electric double layer have the values appropriate to bulk water; recent work indicates that this may not be so and therefore discussion of variation of the zeta-potential is open to doubt. However, for cells of a fixed age in suspension media of fixed pH and ionic strength, changes in the experimentally measured mobility value can be interpreted in terms of changes in the nature and quantity of the charged surface groups. These conditions were adhered to in the present study.

In order to interpret the results obtained from particulate electrophoresis it is essential to use experimental and environmental conditions such that the cell surface under investigation is reproducible and free from adsorbed material, e.g. growth material, toxins and enzymes. With BCG it was not possible to remove material from the surface of the cells by repeated washings and therefore any changes observed in the mobility of cells, which had been washed once or twice in buffer solution, reflected true changes in the surface properties

of the cells. For most studies the optimum ionic strength of the suspending buffer solution was 0.05 mol dm^{-3}; at this concentration errors due to slight variations in the concentration of the buffer solution were minimized (Figure 4.1.).

The symmetry of the observation chamber was checked (Figure 2.3.) and the apparatus calibrated using 18 h old cells of Klebsiella aerogenes (Gittens and James, 1960). Cell suspensions of BCG, harvested from 10 - 14 day old cultures were prepared using the standard technique for washing and resuspension (Table 4.2.). At pH 7.0 the population was always electrokinetically homogeneous and the mobility value was very reproducible. The value was independent of the age of the culture, the nature of the growth medium and the sub-strain used. Over a period of 2 to 2 1/2 years the mobility of the cells suspended in 0.05 mol dm^{-3} acetate-barbiturate buffer solution at pH 7.0 was $1.78 \pm 0.02 \times 10^{-8}$ $m^2 s^{-1} V^{-1}$; the confidence limit for a single mean at $p = 0.05$ was $\pm$ 1%. Thus the surface of cells with mobility values differing by more than 6% can be considered to be significantly different, due either to different surface components or different amounts of the same component.

Particles or cells in suspension in electrolyte solutions aquire a charge for one or more of the following reasons:

(1) ionization of functional groups which form an integral part of the surface;

(2) ion adsorption or desorption, due to the redistribution of ions within the suspending medium at the particle/medium interface;

(3) interaction of the surface with components of the suspension medium; e.g. by specific ion adsorption.

For biological cells the charge is due to the dissociation of ionogenic groups (e.g. carboxyl, phosphate, amino); ion adsorption makes little if any contribution to the total charge (Gittens, 1962).

The nature and distribution of surface groups may be investigated by studying the dependence of the electrophoretic mobility on (a) the pH of the suspension medium; (b) the ionic strength of the suspension medium; (c) the influence of a series of polyvalent cations; (d) the presence of surfactants in the suspension medium; (e) chemical treatment of the cells and (f) treatment with enzymes.

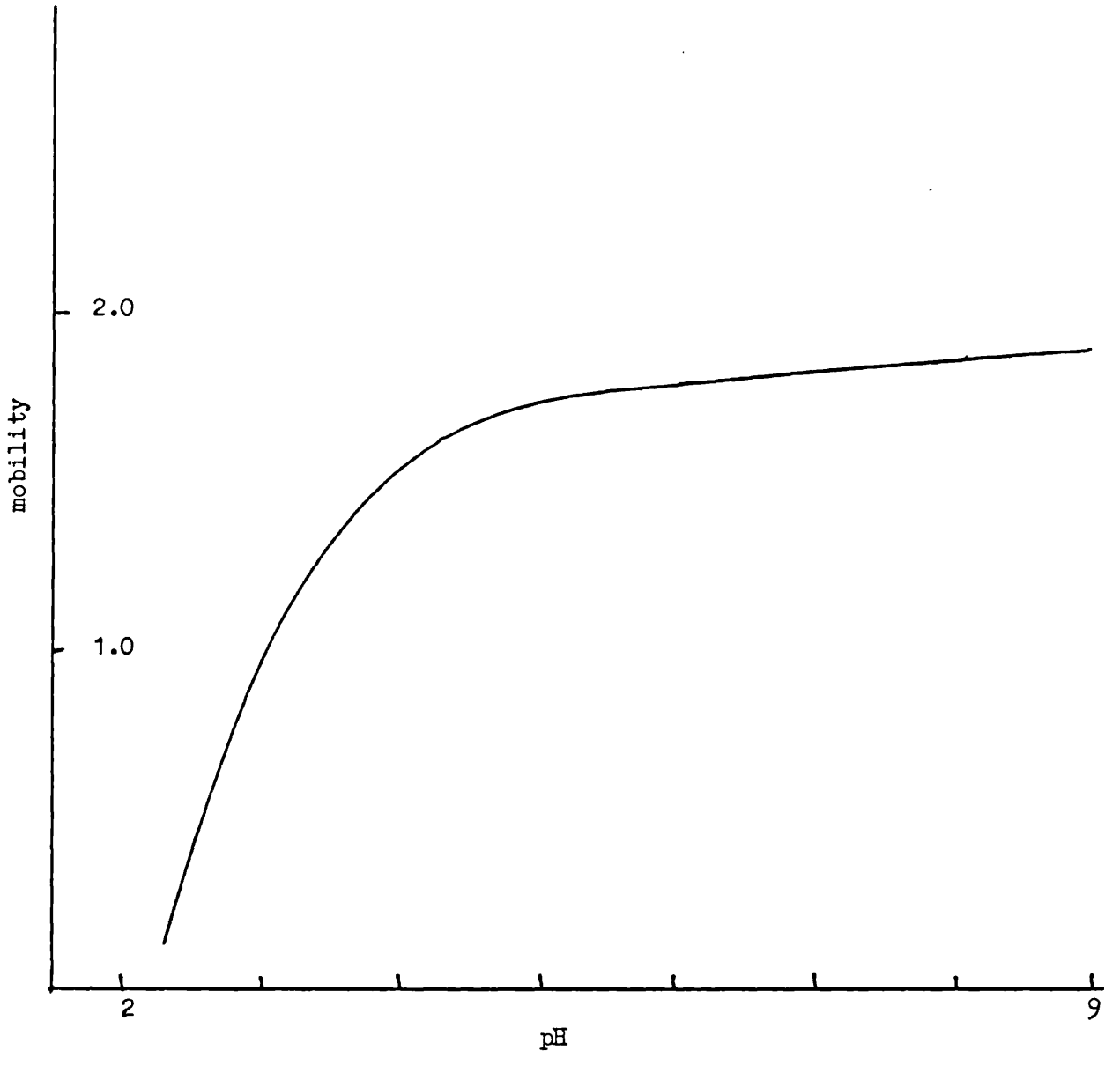
The shape and position of the pH-mobility curves were constant for cells of all ages from 7 to 28 days and independent of the nature of the growth medium and the sub-strain used. Storage of cells in buffer solution for up to 48 h at 4 ^{o}C had no effect on the surface properties of the cells. All pH-mobility curves recorded were coincident within the limits of experimental error. The pH-range 2.5 - 9.5 was adopted since over this range there were no detectable irreversible changes in the surface properties.

A typical pH-mobility curve for cells of BCG is shown in Figure 9.1. The negative charge increases sharply until pH 4.0 and then more slowly to pH 9.0. This curve is typical of a surface with anionic groups only (Figure 1.12). There is no indication of the presence of positive surface groups, either by the reversal of charge at low pH-values, or by a further sharp increase in mobility at higher pH-values. The surface charged groups have a pK of approximately 2.9 and the charge approaches zero at approximately pH = 2.0.

The pH-mobility curves obtained for cells of all five sub-strains examined were coincident, showing that observed differences in the colonial morphology displayed between sub-strains was not reflected in differences in their surface charge. Further confirmation of this observation was obtained from an examination of cells of "rough" and "smooth" isolates of the Glaxo sub-strain which gave pH-mobility curves which were again coincident with those obtained for the other cells.

FIGURE 9.1.

A typical pH-mobility curve for cells of M. bovis BCG



The shape and position of the pH-mobility curve for cells of BCG remained constant throughout a 28 day growth period, confirming the findings of Choucroun and Plotz (1934). This indicates that the nature and number of surface ionogenic groups does not depend on the time of harvesting or the age of individual cells. The surface properties of the cells were also unaffected by the growth medium used for their culture. The addition of non-ionic wetting agents to the medium had no effect on the surface charge properties, despite the fact that the presence of such surfactants supresses the aggregation of cells during growth. The clumping characteristic displayed by BCG, and other mycobacteria, during growth is therefore not due to physico-chemical forces associated with the surface charged groups.

Cells of BCG after one *in vivo* passage through a guinea pig showed identical surface properties to cells grown *in vitro*. This suggests that dramatic modification of the cell surface is not carried out by the host, although host antibodies could have been present on the cells which either had no effect on the surface charge or were removed during preparation for electrophoretic studies. Thus it can be concluded that *in vitro* culture of BCG, for vaccine production, produces an organism which, in its surface properties at least, is identical to any organism isolated from animals. Differences displayed between sub-strains in their immunizing potential are not due to differences in the outer surface of the cells, but must be due to biochemical or structural differences at deeper levels in the cell which are not reflected by changes in the surface charge. As all the sub-strains of BCG examined presented such a reproducible cell surface it is most likely that inter-strain differences arise from biochemical properties rather than from differences in the surface properties.

The general shape of the pH-mobility curves suggests that the BCG cell is a macropolyanion. At an ionic strength of 0.10 mol dm^{-3}, the thickness of the ionic atmosphere is ~ 10 Å, thus the mobility value measured in such suspensions is due to charged groups located in the top 10 - 12 Å of the peripheral region of the cell. At this ionic strength the mobility is 1.58×10^{-8} m^2 s^{-1} V^{-1}; this gives a zeta-potential of 20.3 mV (equation 9.3) and a charge density, ε_D, of 1.508×10^{-2} C m^{-2} (equation 9.4). Assuming that a typical BCG cell is 0.5 μm in diameter and 3 μm in length, the area, A, of the cell (assumed to be a solid cylinder with a hemisphere at each end and making no allowances for the porous nature of the cell) is 3.9 μm^2. Assuming that adsorbed anions make no contribution to the total charge the number of negatively charged groups per cell is given by:

$$\frac{\sigma_D \quad A}{e}$$

where e is the electronic charge, 1.602×10^{-19} C. Using the experimental data, the number of charged groups per cell is 3.7×10^5, giving an area of about 1 000 $Å^2$ per charge. This precludes charging of the surface by anion adsorption, on account of limited space and the large forces of repulsion between the negatively charged surface and negative ions approaching from solution. This conclusion is supported by the shape of the pH-mobility curve; at low pH-values, where the negative groups are completely protonated, the mobility approaches zero rather than attaining a low plateau value which could represent redidual charge from adsorbed ions.

So far the experimental results have only been interpreted in terms of unidentified negative surface groups. In an attempt to elucidate the nature of these, specific chemical and enzymatic treatments of the cell surface were carried out. At first sight the pH-mobility curve appeared to be typical of a surface containing only carboxyl groups (pK$\sim$2.9; pI$\sim$2.0); the surface was therefore extensively examined for the presence of ionizable carboxyl groups. No carboxyl groups were detected by the standard methods of esterification for short and long periods. This is not really suprising since it is well established that mycolic acids, the most likely source of carboxyl groups, do not exist in the free state in the cell walls of mycobacteria but in an esterified form.

Specific chemical treatments (5.2. and 5.3.) for the presence of amino and sulphydryl groups failed to reveal the presence of either of these as surface components. The absence, or low level, of amino groups confirmed the general shape of the pH-mobility curve. If amino groups are present then they are undetectable by the electrokinetic method; taking into account the reproducibility and accuracy of the method it can be confidently stated that the upper limit of amino groups is 4 000 - 5 000 per cell.

The only remaining anionic species which could give rise to the large negative charge on the cells are sulphate and phosphate. Since the amount of sulphate, in the form of sulpholipids, in mycobacteria is small this group was discounted. The detection of phosphate groups on the surface of bacterial cells is difficult (Neihol and Echols, 1978) unless they are susceptible to enzymic attack by certain very specific phosphatases. Cells of BCG were resistant to attack by several phosphatases. Under a wide range of conditions,

including prior autoclaving of the cell suspension to destroy any inhibitors of the enzymes, there was no change in the shape or position of the pH-mobility curve after treatment with phosphodiesterase, phospholipase C or acid phosphatase. It is well known that phosphatases are highly substrate specific; all the enzyme preparations used were shown to be active when tested against the appropriate substrate. Further, the interaction between the enzyme and the substrate on the cell is a heterogeneous reaction in which the active part of the substrate is most probably sterically protected by the complex nature of the cell wall skeleton. It is therefore not unreasonable that the enzymes had no effect on the surface groups.

Despite these negative results, phosphate was still considered to be the most likely species giving rise to the negative surface charge. Charge reversal spectra, i.e. specific sequences of cation required to give zero charge, have been used to characterise surface charged groups on bacteria and blood cells; this technique was applied to cells of BCG. The concentration for charge reversal by the uranyl ion was 10 fold lower than that for other comparable divalent ions (Figure 5.3.); this is a characteristic of a phosphate colloid (Figure 5.4.). In addition the sequence of ions in the "spectrum" of BCG is similar to that for known phosphate-type colloids. Thus the negative surface charge on cells of BCG arises solely from ionizable phosphate groups. This conclusion is supported by the pK of the surface (pK = 2.8) which is in the range of values quoted in the literature for the pK-values of organic phosphates (e.g. O-phosphoserine, pK 2.08, O-phosphorylserine methyl ester, pK 5.33).

Examination of the proposed structure of mycobacterial cell walls shows a phosphodiester linkage between the mycobacterial peptidoglycan and the arabinoglactan and it is probable that it is these linkages

which give rise to the surface phosphate groups detected electrokinetically.

Mycobacterial cell walls also contain a large amount of solvent extractable lipid (60% w/v) compared to that of the walls of other Gram-positive organisms. For many years it was thought that, because of their hydrophobic handling properties, mycobacterial cells were surrounded by a lipid layer. This has not been confirmed by electron microscopy studies and no surface lipid was detected by measuring the mobility of cells of BCG in suspension in buffer solution containing SDS. The large quantities of cell wall lipids, predominantly composed of esters of mycolic acids, must therefore be located deeper in the cell wall than the surface under examination. This was confirmed by examination of the mobility of cells in buffer solutions of decreasing ionic strength, all of which contained SDS. As the ionic strength decreases the ionic atmosphere under examination increases and charged groups deeper in the wall will contribute to the total charge. There was a clear indication of an increase in the S-value with decreasing ionic strength, showing the presence of increasing amounts of lipid as the cell wall structure was penetrated.

Isoniazid resistance of mycobacteria is known to be related to changes in their lipid composition (Alberghina, 1976), but isoniazid-resistant and sensitive cells of the Glaxo sub-strain of BCG have identical surface charge properties showing again that lipid is not located at the cell surface.

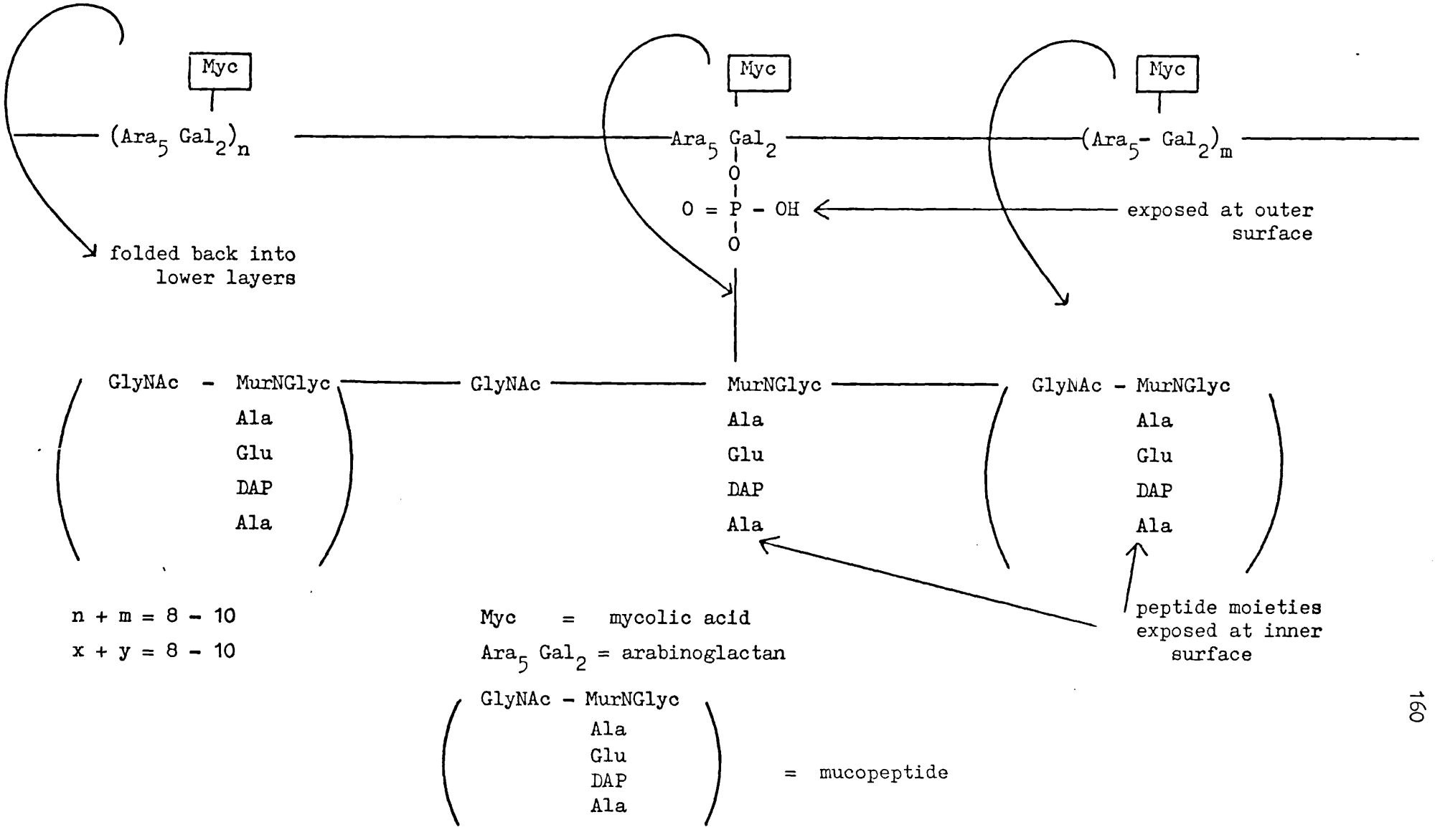
The basic chemical structure of mycobacterial cell walls is common to all species of mycobacteria and, as it was suspected that the surface phosphate groups were arising from this basic cell wall skeleton, the surface properties of three other mycobacterial species,

M. smegmatis, M. phlei and M. microti, were examined. The pH-mobility curves obtained for cells of these other species were identical to and coincident with those obtained for all sub-strains of BCG. This confirms that the charge in fact arises from a common, basic cell wall structure.

Cell wall preparations obtained by ultrasonication of cell suspensions exhibited a pH-mobility curve characteristic of a mixed anionic/cationic surface (Figure 1.12). In this case the cells were broken and therefore the charge is a mean value of charges on both the inner and outer surfaces. The negative charge is mainly due to the phosphate groups on the external surface with possibly some carboxyl groups on the inner surface contributing. The positive groups now evident from the shape of the pH-mobility curve must arise from ionizable amino groups on the inner surface, most likely those of the peptide moieties of the peptidoglycan.

Examination of these cell wall preparations for surface lipid still showed very little detectable lipid; the small amount observed probably arose from the regions where the cell-walls were broken exposing species normally resident in the middle layers of the cell wall. This lack of surface lipid when both inner and outer surfaces of the cell wall are exposed suggests that the arrangement of the cell wall components is such that the phosphodiester linkages between the peptidoglycan and arabinoglactan are at the outer surface. These phosphate groups give rise to the negative charge, while the mycolic acid esters attached to the arabinoglactan, which constitute the major lipid component of the cell wall, are folded back into the middle layers of the cell wall forming the "filling" of a "sandwich" with the peptide chains of the peptidoglycan giving rise to positive amino groups at the inner surface (Figure 9.2.).

FIGURE 9.2: Proposed "decamer" structure for the glycolipid mucopeptide complex of mycobacterial cell walls related to electrophoretic evidence.



The ultrastructure of mycobacteria has been extensively studied by transmission electron microscopy (TEM), but very little work had been reported with scanning electron microscopy (SEM). Examination of cells and colonies of BCG under SEM revealed that they secrete a "slime-like" substance which completely covers clumps of cells, colonies and the surface pellicle obtained after growth on Sauton medium (Figures 7.1., 7.2. and 7.4.). The covering material was completely amorphous with no visible structure. It did not coat individual organisms, but formed a coat around aggregates of cells from which single organisms, completely free of the covering material, could be removed by manual tissue grinding. The substance appeared to be secreted by cells after growth on all the media used and also by all the sub-strains examined, the broken cell wall preparations also appeared capable of secreting it. Detailed examination of TEM photographs also revealed the presence of this material as a shadow-effect around clumps of cells; this could easily be mistaken for an artifact of the negative strain.

It is possible that the organisms, on contact with air, secrete this material and thereby provide a barrier about themselves. It could be the secretion of this "slime-like" material that renders mycobacteria so resistant to attack by the immune system of the host and even allows division of cells within the host macrophages. Mycobacteria are notoriously resistant to attack by chemicals and antibiotics and this could be due to the secretion of this "slime-like" layer about groups of cells.

The aggregation of cells of BCG during growth is not due to electrostatic forces, but is most likely due to the secretion and presence of this amorphous material. Considering that cells of BCG grow with this coating of amorphous material around aggregates of cells

it is not surprising that problems are encountered in uptake of oxygen, nutrients etc. and this could account, at least partially, for the low growth rate characteristics of these organisms.

Cells grown in the presence of wetting-agent do not aggregate so badly. This could be due either (a) to the inhibition of production of this amorphous covering material; this is unlikely as cells grown in the presence of wetting-agent still posses this material (SEM); or (b) to a stripping effect of the detergent which stops the slime-like material from properly coating the cells. Cultures of BCG grow much better when this substance is allowed to coat the cells; cells grown in the presence of wetting-agent give poorer yields than those grown in its absence. Further, cultures do not grow well when subjected to any form of disturbance during growth, such as shaking or aeration. This could be due to interference with the process of secretion of the material and coating of the cell aggregates, since it has been established that the coating material is easily stripped off the cells by processes such as tissue-grinding. Imaeda et al (1968) reported a material which obscured their observation of fibrillar structures on the surface of cells of mycobacteria under TEM; this material could be removed by sonication and is most probably the structureless material seen so clearly under SEM.

It has been clearly demonstrated that cells of BCG are macropolyanions carrying a negative charge at their surface due entirely to ionizable phosphate groups arising from the phosphodiester linkages between the peptidoglycan and arabinoglactan of the cell wall. The considerable quantity of mycobacterial cell wall lipid is located further into the cell wall than the surface under examination and amino groups arising from the peptide chains of the peptidoglycan are located at the inner surface.

Aggregation of cells of BCG during growth is not, as was originaly suspected, due to physico-chemical forces arising from the surface charges on the cells, but is due to secretion of a "slime-like" material around cell aggregates.

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