A thesis titled

AN ENZYMIC AND PHYSICAL CHEMICAL STUDY OF ANTIBIOTIC SENSITIVE AND RESISTANT STAPHYLOCOCCI.

by

ANNE LLOYD DAVIES.

Submitted to the Faculty of Science for the Degree of Doctor of Philosophy in the University of London.

Department of Chemistry,
Bedford College,
Regent's Park,
London N.W.1

July, 1974.

ABSTRACT

Microelectrophoretic and enzyme assay techniques were used to investigate the surface properties of cells of strains of Staphylococcus aureus which were sensitive or resistant to methicillin.

An alkaline phosphatase enzyme system was found in cells with natural resistance to methicillin; cells sensitive to the antibiotic or which had been repeatedly grown in the presence of the antibiotic showed no phosphatase activity. This heat labile enzyme system had an optimum activity at pH 10.00 - 10.20 and 37°C and was firmly attached to the cell. The alkaline phosphatase was not inhibited by inorganic phosphate, although excess phosphate in the growth medium repressed its formation.

The production of the enzyme was sensitive to the te mperature of growth of the cells; cells grown at 27° and 37°C exhibited a high phosphatase activity where as cells grown at 42°C showed little or no activity.

There was a correlation between the production of the enzyme system, the amount of surface teichoic acid associated with the cells and methicillin resistance. It was concluded that this alkaline phosphatase enzyme system was the temperature dependent enzyme suggested previously to account for the temperature response of resistant cells of Staph. aureus to methicillin.

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 1971 to July 1974.

Part of this work has been accepted for publication by Microbios.

ACKNOWLEDGEMENTS

I would like to express my sincere thanks to Professor A.M. James for his constant supervision and encouragement, which has made this work most enjoyable.

I am indebted to the Science Research Council for financial support, which made this work possible.

Thanks are also due to the academic and technical staff of Bedford College for their help and co-operation, and to Miss C.M. Morgan for typing the manuscript.

CONTENTS

CHAPTER THREE

CHAPTER FOUR

CHAPTER FIVE

SUMMARY

A technique was established for the assay of the phosphatase activity of cells of methicillin resistant and sensitive strains of Staph. aureus using 4 - nitrophenyl disodium orthophosphate as the substrate. The presence of an alkaline phosphatase enzyme system with maximum activity at pH 10.00 - 10.20 and 37°C was demonstrated in cells of strains of Staph. aureus with natural resistance to methicillin, when grown on "Standard" Nutrient Agar Medium. This heat labile enzyme system was easily accessible to the cell surface, firmly attached to the cell. Cells of strains sensitive to methicillin, including completely sensitive and penicillin resistant strains exhibited no such phosphatase activity.

The phosphatase activity in naturally occurring methicillin resistant cells was very dependent on the nature and composition of the growth medium. In contrast to the high activity shown by cells grown on standard nutrient agar, a very low activity was shown when the cells were grown in liquid nutrient broth; this difference in activity was attributed to the different environments of the cells in liquid medium and on agar. Although the enzyme was not inhibited by the presence of inorganic phosphate in the assay mixture, the production of the enzyme system was repressed by the presence of excess phosphate in the growth medium. However, a very low concentration of inorganic phosphate was essential for good growth and high phosphatase activity. Ellwood (1970) has reported that when cells of a strain of Bacillus subtilis were grown under conditions of limiting inorganic phosphate in a chemostat, the cell wall teichoic acids were replaced by teichuronic acids. Growth of the cells in medium containing excess phosphate produced cells which contained a relatively large amount of phosphate in their walls.

Thus it was postulated that since cells of Staph. aureus with natural resistance to methicillin after growth on media containing a low concentration of inorganic phosphate showed a high alkaline phosphatase activity, their cell walls had a low phosphate content; and conversely after growth on media containing a high concentration of phosphate the cells exhibited no phosphatase activity and possessed a high cell wall phosphate content. This relationship between the phosphatase activity and surface phosphate groups was supported by an investigation of the surface properties of the cells, using microelectrophoretic techniques. Hill and James (1972a), using this technique, showed that there was a correlation between the phosphate groups on the cell surface and the teichoic acid content of the cell walls. It has now been established that cells grown in the presence of excess inorganic phosphate have a higher amount of surface phosphate groups and hence teichoic acid compared to cells grown on the standard medium (low inorganic phosphate content).

Cells of methicillin sensitive and resistant strains grown in the presence of inosine had a common electrokinetic surface and exhibited a very high phosphatase activity. However, the change in surface properties brought about by growth in the presence of inosine had no effect on the resistance or sensitivity of the cells to methicillin.

Hill and James (1972b) also showed that the nature and quantity of the surface components of cells of strains with natural resistance to methicillin were dependent on the growth temperature. These differences were also reflected in the phosphatase activity of the cells. Cells grown at 37°C exhibited a high phosphatase activity and possessed a small amount of surface teichoic acid; at 27°C the cells had no teichoic acid but exhibited a high phosphatase activity, while at 43°C the cells had a large amount of teichoic acid but no demonstrable phosphatase activity.

A relationship between the surface properties of the cells, phosphatase activity and methicillin resistance and sensitivity was also apparent. Cells of sensitive strains had a large amount of surface teichoic acid but no phosphatase. Naturally occurring methicillin resistant cells grown at 37°C showed a heterogeneous response to the antibiotic where as cells grown at 25°C were very resistant and those grown at 43°C were sensitive to even low concentrations of antibiotic and after repeated growth became completely sensitive (Al Salihy and James, 1972).

Although cells selected from a culture showing a heterogeneous response to methicillin exhibited a spectrum of surface properties depending on the concentration of antibiotic on which they were originally isolated, they all exhibited the same phosphatase activity. Cells of a sensitive strain repeatedly grown in the presence of methicillin until they became resistant exhibited no phosphatase activity; this type of resistance was thus obtained by a different mechanism to natural resistance.

It was concluded that this phosphatase enzyme system was the temperature-dependent enzyme first suggested by Annear (1968) to account for the temperature response of resistant cells of Staph. aureus to methicillin.

The experimental results reported in this thesis give an indication of the structural differences associated with the surfaces of cells resistant or sensitive to methicillin. The temperature-dependent enzyme system (the phosphatase) controls or influences the surface structure of the cells. All evidence suggests that the presence or absence of surface teichoic acid and hence the absence or presence of the alkaline phosphatase enzyme system respectively are intimately associated with methicillin sensitivity or resistance.

CHAPTER ONE

INTRODUCTION

1.1 Classification of Staphylococcus Aureus

Microorganisms may be divided into eight major groups : algae, protozoa, yeasts, moulds, bacteria, pleuropneumonia - like - organisms (PPLO.), rickettsia and viruses. Members of each group possess distinct characteristics essential for inclusion into the group. The borders between the groups, however, are diffuse and are subject to much discussion.

Bacteria are usually divided into ten orders (Table 1.1).

The families are determined by such factors as cell shape; motility, the presence or absence of flagella if motile; reaction to the Gram stain; growth requirements; optimum temperature of growth; and types of fermentations which the cultures are capable of carrying out under aerobic and anaerobic conditions.

The Gram stain is very useful as a preliminary characterisation technique; the organism being described as Gram - positive, Gram - negative or Gram - variable, depending on their ability to retain the crystal violet dye after mordanting with iodine solution. This test reveals their morphology and their ability to form spores.

The spherical cocci of the family Micrococcaceae are sub-divided into genera depending on the way in which they cling together after fission. Micrococci and staphylococci divide irregularly in more than one plane and can exist either as single cells or clusters. Sarcina produce regular cubical packets of cells by division in three perpendicular planes and gaffkya divide at right angles, forming tetrads of cells.

Staph. aureus, the organism used in this investigation, is a Gram - positive, coagulase - positive coccus, capable of producing acid from glucose under anaerobic conditions. Growth at 37°C, in liquid medium (pH 7.4 - 7.6) gives small groups, and short chains of non - pigmented cocci, while growth on solid medium gives cells

Table 1.1

Classification of bacteria with particular reference to staphylococci

Order	Family	Genus
Pseudomonadales	13	Micrococcus
Clamydobacterials	including	Staphylococcus
Hyphomicrobiales	Micrococcaceae	Gaffkya
Eubacteriales		Sarcina
Actinomycetales		
Caryoplanales		
Beggiatoales		
Myzobacterales		
Spirochaetales		
Mycoplasmatales		

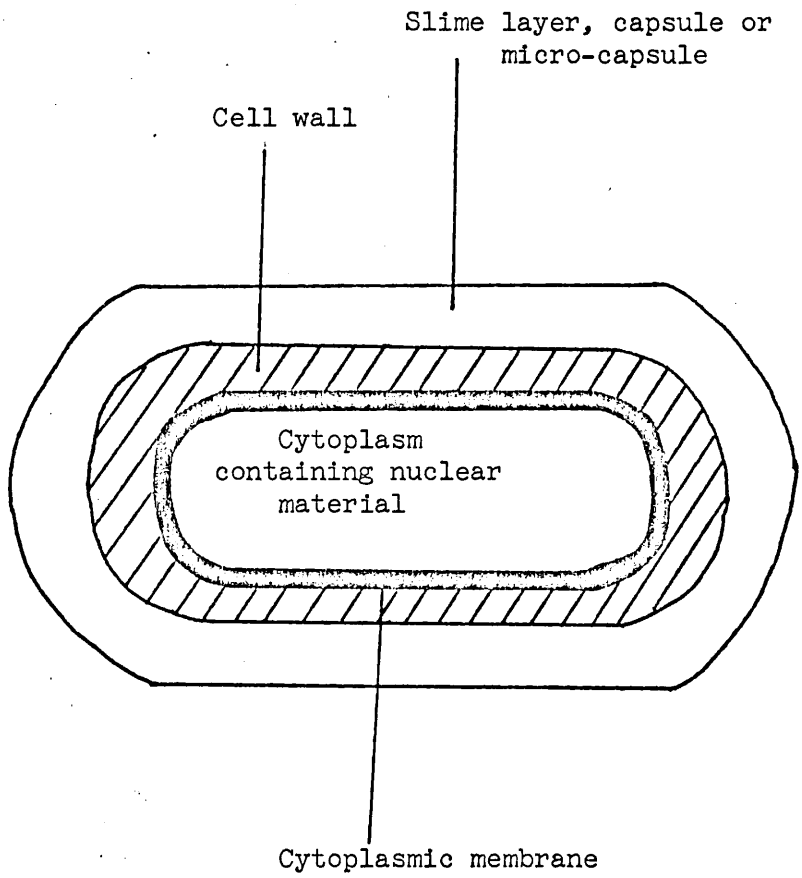
arranged in grape - like clusters, producing colonies usually 1 to 3 mm in diameter with an entire edge and smooth glistening surface. Each coccus is 0.8 to 1.0 μm in diameter, non - motile, non - sporing and usually non - capsulate.
Staphylococci are usually found on the skin or mucus membranes of the animal body, especially in the nose and throat.
Staph. aureus is notorious as the cause of such conditions as mastitis of women and cows, boils, carbuncles, infantile impetigo and internal abscesses in man. The most pathogenic strains of staphylococci generally ferment mannitol, liquefy gelatin and produce coagulase, a golden yellow pigment, lipolytic enzymes and wide zones of β-haemolysis on blood agar. Coagulase may be a virulence factor, by coagulating blood plasma and preventing the natural defensive action of the host body.

1.2 Bacterial Anatomy

Fig. 1.1 shows the basic cellular organisation of a typical bacterial cell. The cytoplasm is surrounded by a membrane, which is enveloped by a cell wall and possibly a capsule or slime layer.
The cytoplasm is a slightly viscous colloidal complex of water, amino acids, proteins, fats, carbohydrates and inorganic matter, often stored in the nutrients and oils suspended in it. The granular appearance of the cytoplasm is due to the storage particles and the ribosomes. The ribosomes are the cytoplasmic site of protein synthesis and many ribosomes are often attached to one molecule of ribonucleic acid. The nuclear material, or nucleoid, lies in the cytoplasm and is not enclosed in a nuclear membrane.

Fig. 1.1

Diagramatic representation of the anatomical relationship between the outer layers of a bacterial cell.



Some cells are motile, although very few, if any cocci have this property. Motility of cells is brought about by flagella which are thread - like structures protruding from the cell surface of bacteria, but which originate in the cytoplasm. The flagella are almost entirely made up of a protein called flagellin.

Capsules and slime layers surround many microorganisms lying external to but in close contact with the rigid wall. The amount produced depends on the nature of the growth medium; the production of such layers may be stimulated by unfavourable growth conditions.

1.3 The Bacterial Cell Wall

The successful adaptation of bacteria to a wide variety of physical - chemical environments during the course of their evolution can be attributed in part to the development of suitable surface structures. The mechanical strength of the cell wall membrane structures and the ability to withstand the physical - chemical effects of various natural and synthetic lytic agents are factors of great importance to the continued survival of the bacterial cell.

The walls of Gram - positive organisms have a high enough tensile strength to protect the cells against osmotic explosion when the salt and sugar content of the external environment becomes very dilute. The chemical component responsible for the rigidity of the walls of both Gram - positive and Gram - negative bacteria is the peptidoglycan which also contributes a great deal to the stability of the cell and confers a number of other general properties on the bacterial walls.

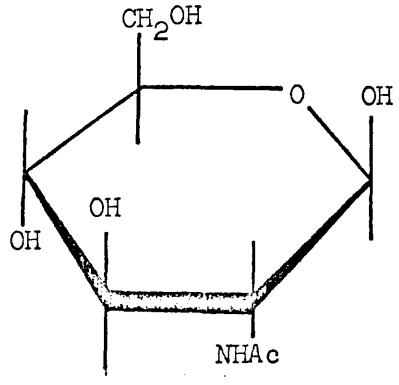
Table 1.2

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

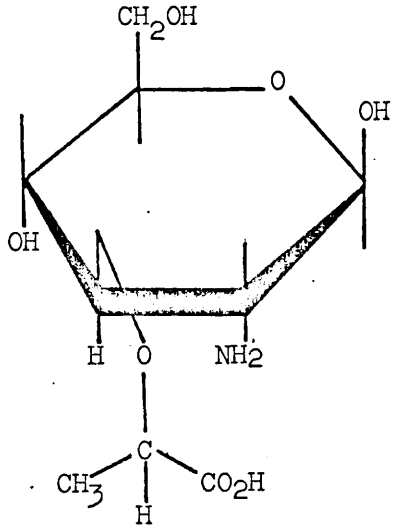
	Gram - positive e.g. Staph. aureus	Gram - negative Escherichia coli
1.	The cell wall presents a unified, well defined separate structure, distinct from the cytoplasmic membrane.	A very complex organisation of the outer cell layer. No clear boundary between the cell wall and adjacent structures. Cell 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 poly-saccharides 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.

There are, however, marked differences between the cell walls of Gram - positive and Gram - negative bacteria (Table 1.2). The cell wall is the thin (0.02 μm), sharply defined, relatively tough and rigid structure underlying any slime layer or capsule which may be present. The wall envelops the protoplast of the organism, giving a particular microbial cell its characteristic shape, and provides the structural support for the physically fragile, osmotically susceptible cytoplasmic membrane.

The major chemical component which is responsible for the rigidity of the bacterial cell wall is the peptidoglycan, a complex of amino acids and amino sugars. Analysis of the peptidoglycan isolated from cells of Staph. aureus shows that the main amino acid components are lysine, glycine, glutamic acid and alanine (Mandelstam and Rogers, 1959). Glucosamine and muramic acid (first isolated by Strange and Kent, 1959) are also present. Mandelstam and Strominger (1961) showed that the cell wall of Staph. aureus strain Copenhagen is made up of three polymer threads : a glycopeptide backbone, a pentaglycine component which appears to cross-link peptide chains and the ribitol phosphate polymer. This is in agreement with the work of Salton (1962). More recent work (Coyette and Ghysen, 1968; Strominger et al, 1971) confirm that the walls of bacteria consist of glycan strands in which two sugars acetylglucosamine,



and acetyl muramic acid



strictly alternate. The acetyl muramic acid residues of the polymer are substituted by a tetrapeptide. The peptidoglycan strands are linked to each other by means of the interpeptide bridge (Fig. 1.2).

In Staph. aureus the interpeptide bridge is a pentaglycine chain which extends from the carboxyl group on the terminal D - alanine residue of the tetrapeptide to the ε-amino group of the lysine, the third amino acid in the tetrapeptide chain (Fig. 1.3). The wall of Staph. aureus is a very tightly knit structure in that virtually every peptide subunit is cross-linked to another subunit by means of this interpeptide bridge. About 50% of the cell wall is made up of peptidoglycan.

Three dimensional molecular models of peptidoglycan show that extensive hydrogen bonding between peptides is a reasonable possibility (Rogers, 1970). In staphylococcal peptidoglycan, the degree of hydrogen bonding between contiguous peptide chains can be as high as 80%. Thus some of the physical properties of

Fig. 1.2

The structure of staphylococcal peptidoglycan (Mandelstam and Strominger, 1961).

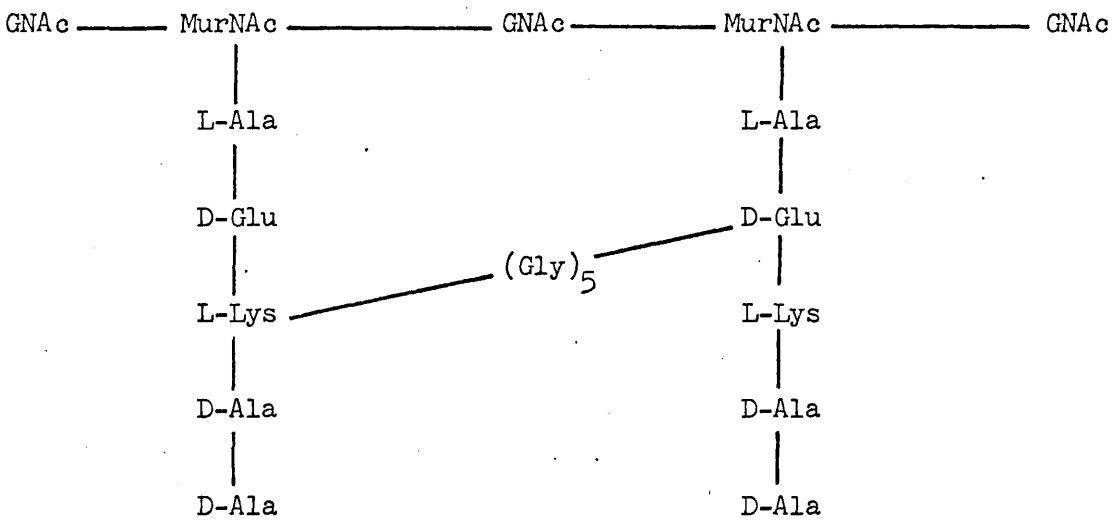
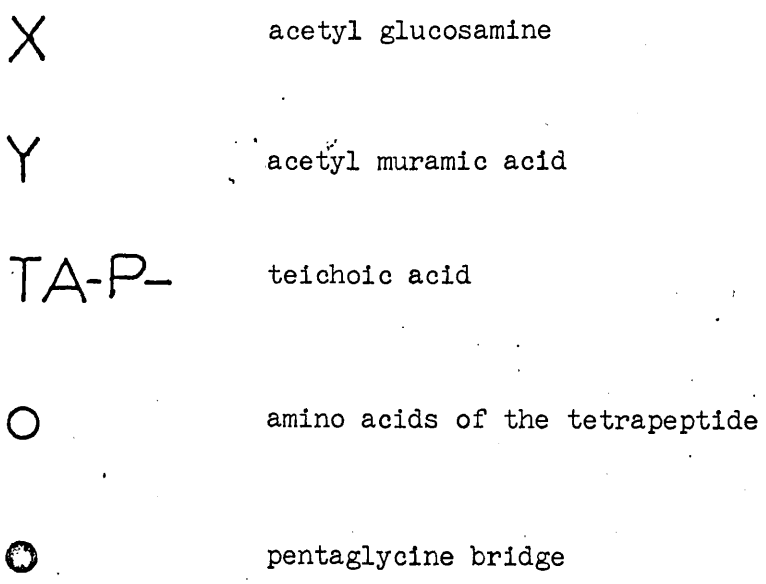
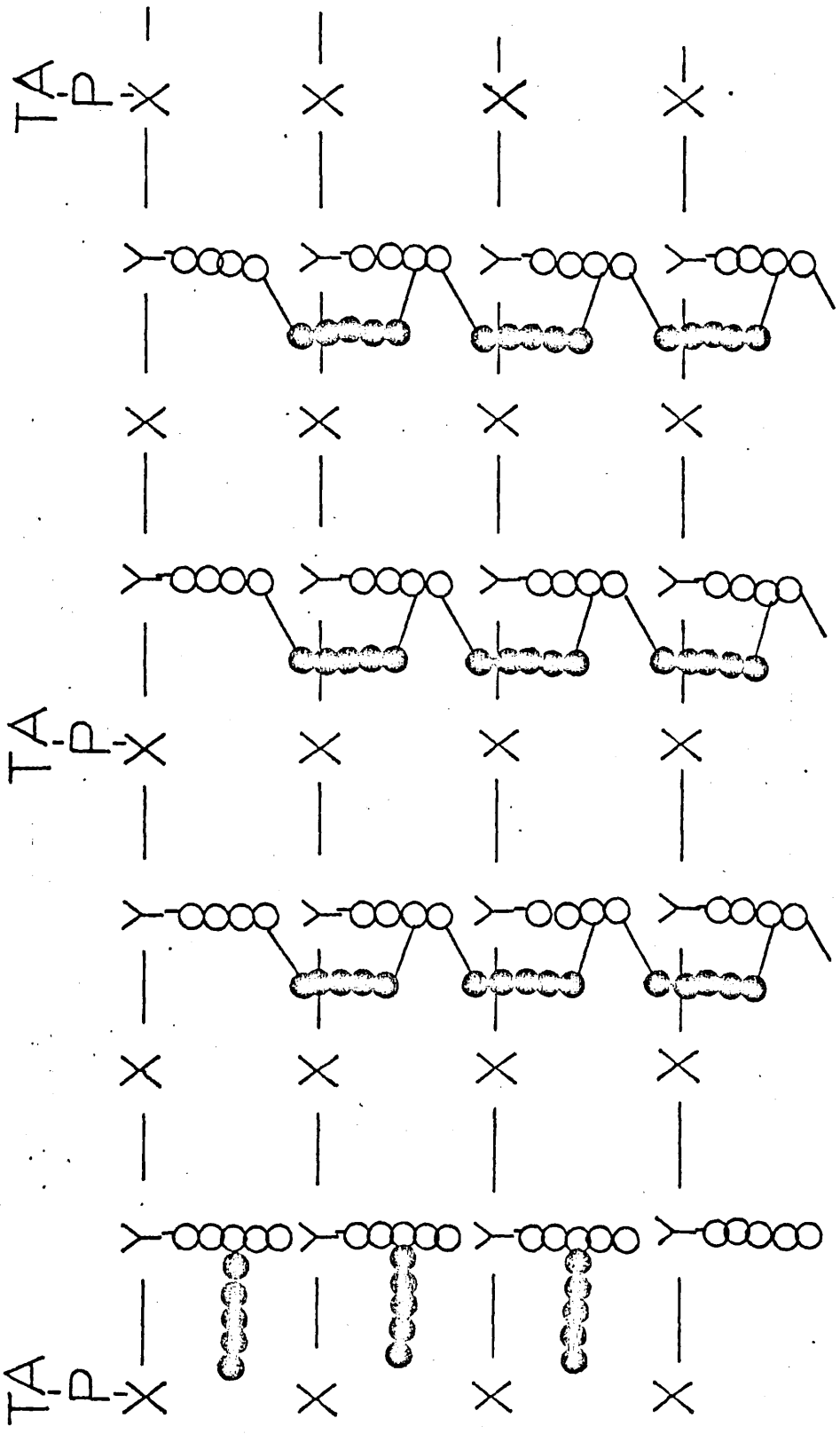


Fig. 1.3

The structure of the cell wall peptidoglycan of Staph. aureus




TA-P
TA-P
TA-P

bacterial cell walls may come from co-operative hydrogen bonding of one part or another.

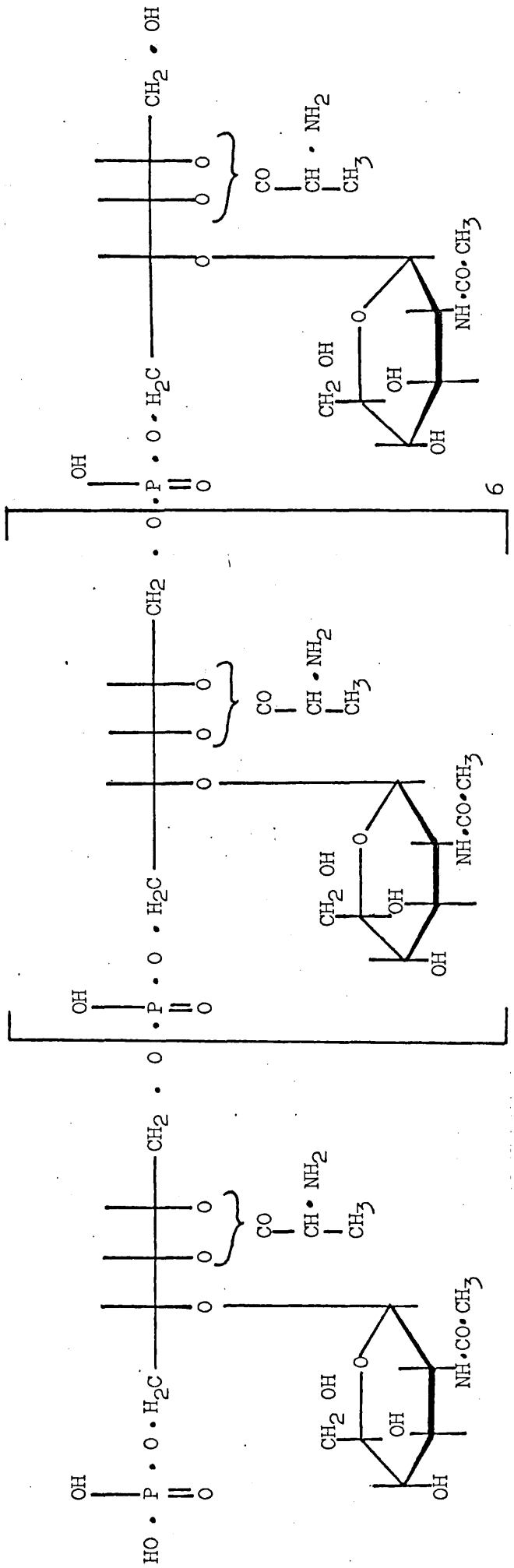
The cell wall of many Gram - positive bacteria, grown under normal conditions have a high phosphate content.. This is attributed to the presence of surface or wall teichoic acid which is attached to the cell wall peptidoglycan. Teichoic acids are regarded as either cell wall or cell membrane components, unlike peptidoglycan which is peculiar to cell walls. Although the occurrence of wall teichoic acids (first discovered by Armstrong et al, 1958 a) is variable between different genera, membrane teichoic acids appear to be virtually ubiquitous in Gram - positive bacteria. Membrane - associated teichoic acids are characterised by their uniformity of structure while wall-associated teichoic acids are remarkable for their structural diversity.

The simplest wall teichoic acids are either glycerol teichoic acid or ribitol teichoic acid in which ribitol replaces glycerol as the backbone polyol unit. These polymers contain only a restricted array of sugar substituents. The teichoic acid associated with the walls of Staph. aureus is the ribitol type, although the walls of some staphylococci contain a glycerol teichoic acid (Armstrong et al, 1959; Armstrong et al, 1960). Schleifer and Kocur (1973) have suggested a classification for staphylococci based on the type of teichoic acid associated with the cell walls.

The wall teichoic acid of Staph. aureus strain "H" consists of ribitol units joined by phosphate residues and substituted by N-acetyl glucosamyl residues (Baddiley et al, 1962). Each repeating unit is further substituted with alanine by an exceptionally alkali - labile ester linkage. The structure of Staph. aureus teichoic acid is shown in Fig. 1.4. The teichoic acid of Staph. aureus strain "Copenhagen" is a linear polymer of 4-O-β and 4-O-α N-acetyl-D-glucosamyl-D-ribitol units bridged by 1, 5 phosphodiester linkages (Coyette and Ghuysen, 1968).

Fig. 1.4

Structure of cell wall ribitol teichoic acid from Staph. aureus.



The linkage of teichoic acid to the peptidoglycan complex has been the subject of much speculation. Armstrong et al (1958b) first suggested that the teichoic acid could be held to the wall peptidoglycan by salt linkages and Archibald et al (1961) put forward the idea of ionic linkages. Rogers and Garrett (1961) decided that the alanine of the teichoic acid was not involved in linkages with the peptidoglycan but that end linkages are involved, either through the primary alcohol group at one end of the polymer or the phosphate group at the other end. Another suggestion was that the ribitol teichoic acid was mostly entangled in the peptidoglycan fibres or held by hydrogen bonding (Rogers, 1963). Archibald and Baddiley (1965) proposed phosphoramidate bonds as being the type of linkage between teichoic acid and amino sugars. Work on Staph. lactis (Button et al, 1966) led to the suggestion of a phosphodiester linkage to a muramic acid residue in the glycan moiety of the peptidoglycan, although the position of attachment to the muramic acid residue in the glycan chain is not known. Covalent bonding between the teichoic acid and the structural peptidoglycan of the cell wall is another possibility (Wolin et al, 1966) which is supported by Coyette and Ghuysen (1968). Thus evidence seems to be accumulating to favour a linkage (possibly a covalent bond) between the phosphate group of the teichoic acid and an amino sugar on the peptidoglycan.

The location of the teichoic acid polymer is also uncertain. Most evidence suggests that the teichoic acid polymer is external to the peptidoglycan network. Balyuzi et al (1972) who do not support the idea of three-dimensional models in which the peptidoglycan is arranged so as to achieve maximum hydrogen bonding, suggest that since wall synthesis is an ordered process, the location of the teichoic acids on the peptidoglycan chains should be non-random. Two models were found for the distribution of peptidoglycan and teichoic acid in the walls of Staph. lactis

(Archibald et al, 1973; Bauer et al, 1974) where (i) the glycan strands are lying radially and 40% of these were attached to all the teichoic acid molecules on an outside layer or (ii) the glycan strands are lying parallel to the surface and then the teichoic acid would be fairly uniformly distributed throughout the walls. When grown under favourable conditions, cells of Staph. aureus, in common with other Gram - positive organisms have surface teichoic acid, however, under unfavourable conditions acidic polysaccharides possessing uronic acid residues are present in the cell walls (Hepinstall et al, 1970). It is unlikely that these compounds serve the same purpose as teichoic acids. Cells of Bacillus subtilis, for example, can dispense with wall teichoic acid provided that they can substitute another acidic polymer for it (Ellwood and Tempest, 1967). Even under conditions of phosphate limitation which causes teichuronic acid to be formed, membrane teichoic acid continues to be synthesised, thus showing that there must be different processes for the production of membrane and wall teichoic acids (Ellwood and Tempest, 1968). It is also evident that, unlike wall teichoic acid, membrane teichoic acid must have a vital and indispensible role. Ellwood (1970) found that the wall content of cells of Bacillus Subtilis grown in a chemostat varies with both the growth rate and phosphate limitation. When grown under Mg^{++} limitation the cells contained a glycerol teichoic acid which was totally replaced by teichuronic acid (a polymer containing equimolar amounts of N-acetyl glucosamine and glucuronic acid, Janczura et al, 1961) under phosphate limitation, an increased growth rate resulted in an increase in teichoic or teichuronic acid synthesis. Isolated bacterial walls bind cations, especially bivalent cations; this is possibly a function of teichoic acid and the binding property may be due to the phosphate groups of the polymer.

It has been proposed that the major function of wall and membrane teichoic acid is to maintain a high concentration of bivalent cations in the region of the membrane; wall teichoic acid is functionally less important and could be substituted by other acidic polymers under unfavourable growth conditions. The reason for the large amount of wall teichoic acid produced under Mg^{++} limitation in a chemostat could be to scavenge the limited supply of bivalent cations present. Thus the main function of teichoic acid appears to be to provide the correct ionic environment for the membrane. The maintenance of a repulsive charge by wall teichoic acids may be a desirable property enabling cell populations to disperse in the growth medium and, therefore, use up the nutrients efficiently. These findings are in agreement with the work of Hughes et al (1973) and Archibald et al (1973).

Another important function of teichoic acids may be in relation to expansion of the wall matrix; since teichoic acids have a net negative charge, they act to expand the interstices of the wall, but the significance of this has yet to be determined (Ou et al, 1973).

Recent work suggests serological functions, receptor sites for bacteriophages as well as ion transport as being the purpose of wall teichoic acids, but as yet no conclusive evidence has been obtained for the true function of wall teichoic acids.

1.4 Antibiotics

Antibiotics were originally defined as compounds produced by microorganisms, yeasts etc., which at low concentrations inhibit growth and other activities of other microorganisms.

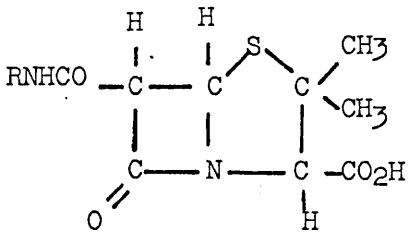
The properties of the penicillin molecule (Fig. 1.5) differ according to the side chain R; penicillin G is the most commonly used penicillin but the β-lactam ring in this compound is very easily opened by the action of an enzyme penicillinase, (a β-lactamase) to give the biologically inactive penicilloic acid (Fig. 1.6).

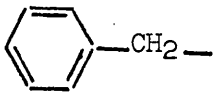
An important class of semisynthetic penicillins is that in which the carbonyl group of the amide function is attached directly to a benzene or heterocyclic ring. The introduction of ortho substituents in the benzene ring leads to steric hindrance around the amide link; the resulting change in conformation reduces the affinity for the active site of β-lactamase-producing staphylococci if the substituent is large enough. The best results are obtained with smaller substituents in both ortho positions. 2, 6-dimethoxyphenyl penicillin (methicillin) was the first penicillin to show good activity against penicillin-resistant staphylococci (i.e. penicillinase producers) in vivo as well as in vitro (Rolinson et al, 1960).

The key observations which led to a theory to account for the action of penicillin on bacteria where (i) the structural changes in the wall believed to accompany morphological abnormalities of bacteria grown in the presence of the antibiotic and (ii) the recognition of the biochemical significance of the nucleotides accumulating in penicillin-treated cells of Staph. aureus (Park and Strominger, 1957). The inhibition by penicillin of a step or series of steps in the biosyntheseis of the cell wall glycosaminopeptide could account for the principal structural and biochemical changes observed in the cells.

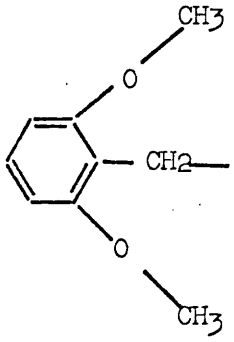
Fig. 1.5

The molecular structure of two forms of penicillin.





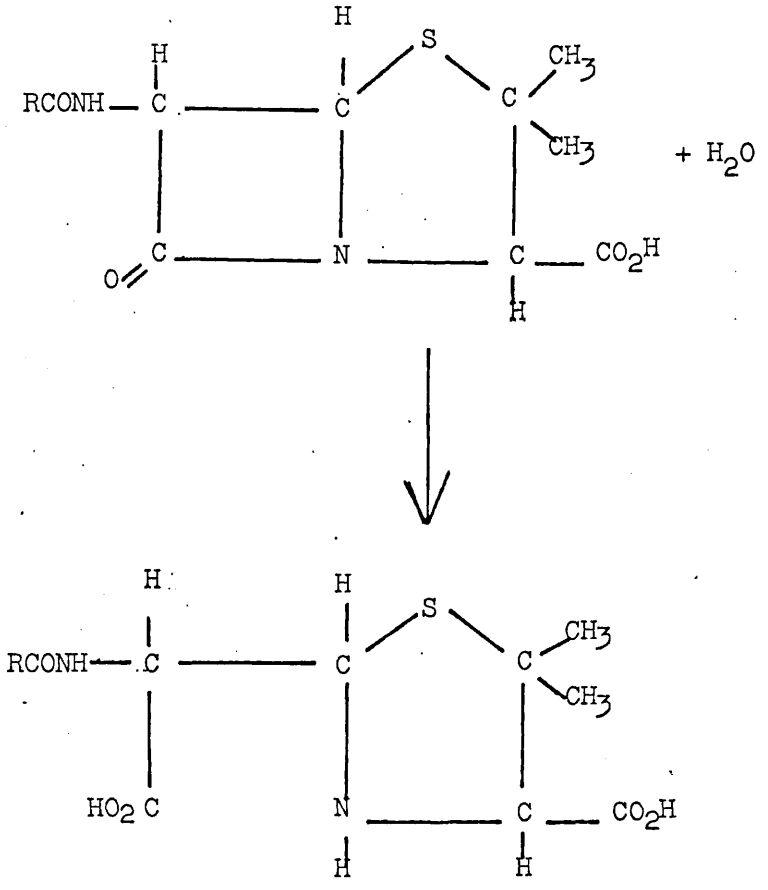
Benzyl penicillin (G)



Methicillin

Fig. 1.6

The hydrolysis reaction which brings about the inactivation of penicillin.



Although penicillin has now been shown to inhibit glycosaminopeptide formation in "resting" washed cells (Mandelstam and Rogers, 1959), the structural consequences of the action of the penicillins are dramatically seen in bacteria grown in the presence of these antibiotics.

Penicillin acts by inhibiting the formation of the penta-glycyl peptide cross-linkages in Staph. aureus (Fig. 1.3), thus weakening the cell wall polymer. The high osmotic pressure of the cell forces the delicate protoplasmic membrane through the weak wall and cell lysis occurs. Penicillin can only bring about lysis of actively growing cells.

Mechanisms of resistance to antibiotics can be divided into two broad classes : (a) those which alter some cellular component such that the antibiotic either does not reach or does not interact normally with its target site within the cell and, (b) those which lead to chemical modification and subsequent inactivation of the antibiotic. The development of resistance in bacteria by these differing mechanisms is controlled by environmental factors. Resistances of type (a) which arise by a mutational alteration of a cellular component, are very common in laboratory derived resistant strains, but clinical isolates of this class have also been reported. Resistances of type (b) are generally found only in clinical isolates of resistant bacteria and are normally associated with an extrachromosomal element or plasmid.

Penicillin resistance in Staph. aureus was influenced significantly by the introduction of certain semi-synthetic penicillins and cephalosporins in the early 1960's, one of the first of which was methicillin.

A high proportion of the penicillin resistant strains of Staph. aureus at that time were sensitive to methicillin. With further passage of time, however, staphylococci resistant to the semi-synthetic penicillins and cephalosporins have become evident. These strains which owe their resistance to penicillin to the formation of the enzyme penicillinase, were present in significant numbers at the time that penicillin was first introduced into medicine and this mechanism of resistance, accentuated by the fact that benzyl penicillin induces the formation of penicillinase in this organism, allowed such strains to survive penicillin therapy and largely replace the sensitive strains.

Since the semisynthetic penicillins and cephalosporins were highly stable to the action of penicillinase, the problem of penicillin resistant staphylococci was changed significantly by their introduction in 1960. However, an indication of the general incidence of methicillin resistant staphylococci may be gained form data reported by Parker and Hewitt (1970).

Novick (1963) found that the genes for penicillinase synthesis in Staph. aureus were located extrachromosomally on a plasmid. The penicillinases on plasmids are inducible enzymes; induction is usually performed with low concentrations of a penicillinase - resistant penicillin e.g. methicillin (Benveniste and Davies, 1973). The various natural and semisynthetic β-lactam antibiotics differ in their ability to induce the enzymes in Staph. aureus. Although various models have been proposed to account for the regulation of staphylococcal penicillinase synthesis (Imsande, 1970; Imsande et al, 1972) the biochemical basis of induction has not been explained.

β-lactamase production is not the sole mechanism of resistance; bacteria may be intrinsically insensitive to penicillins (Sutherland, 1964) and still produce β-lactamase or they may be intrinsically resistant and not produce the enzyme. Intrinsic resistance to penicillins may be due to a diminished rate of

reaction with the ultimate target site. Slight changes in the configuration of the target enzyme might result in a decreased affinity for a given penicillin with the result that a higher concentration of drug would be required to effect the requisite rate of reaction and the cell would accordingly show a degree of resistance (Rolinson, 1971).

The locus for methicillin resistance may be on a plasmid in some strains, but the evidence is not clear since a plasmid cannot be transduced from all strains (Seligman, 1966; Dyke et al, 1966). The biochemical basis for methicillin resistance in Staph. aureus is unknown, but it is independent of β-lactamase production. In all clinical methicillin resistant strains, methicillin resistance has a unique phenotypic nature and there is no evidence for a "methicillinase". Methicillin resistant strains of Staph. aureus differ from methicillin sensitive strains in that they grow more slowly, have an altered cell surface (Sabath et al, 1970) and tend to be coresistant to a number of other antibiotics e.g. the cephalosporins but they are still sensitive to other antibiotics, e.g. fusidic acid, lincomycin, gentamicin. Resistance may thus be due to impermeability to the antibiotic.

Naturally occurring methicillin resistant strains show a heterogeneous response to methicillin; they consist of mixed populations in which the majority of cells show a normal sensitivity to methicillin, with a slow growing minority showing a very high resistance (Sutherland and Rolinson, 1964). Annear (1968) and Parker and Hewitt (1970) have shown that the heterogeneous population of methicillin resistant Staph. aureus only occurs on incubation at relatively high temperatures (37°C).

Incubation at 25°C renders all the cells of a methicillin resistant strain highly resistant where as on incubation at 43°C the cells in the population are very sensitive to the antibiotic. There have been recent reports of methicillin resistant strains of Staph. aureus which lose their methicillin resistance and/or their ability to produce penicillinase in vitro when grown at high temperatures (May et al, 1964; Asheshov, 1966; Dornbusch et al, 1969; Johnston and Dyke, 1971; Al Salihy and James, 1972) and lose their methicillin resistance at room temperature (Grubb and Annear, 1972; Noble, 1972; Annear and Grubb, 1973a and b). This evidence implies an extrachromosomal locus for the methicillin resistant genes, but according to Stiffler et al (1973), the locus of methicillin resistant determinants still remains elusive.

1.5 The Phosphatase Enzyme Systems

Intact bacterial cells and crude cell free extracts show a widespread ability to split phosphate esters. This phosphomonoesterase activity has been demonstrated over a considerable pH range (2.0 - 10.0) and towards numerous substrates.

Initial observations on phosphatases indicated that they could generally be divided into two groups on the basis of pH optima :

(a) "Acid phosphatases" which display an optimum activity between pH 3.0 and 6.0. The synthesis of these enzymes is influenced by the carbon source of the growth medium but is independent of the inorganic phosphate content of the medium.

(b) "Alkaline phosphatases" which are most active in the pH range 8.0 to 10.0. These are synthesised only when the inorganic phosphate in the medium becomes exhausted or when growth occurs on an organic phosphate as the sole phosphorus source.

These two categories do not include all types of activity that are observed. Some enzyme fractions show very broad pH optima and are active at both acid and alkaline pH.

In the following discussion, the properties of the alkaline phosphatase enzyme systems will be reviewed.

The metabolic function of the alkaline phosphatase has not been fully established but it may be to provide a supply of inorganic phosphate from phosphate esters inside the cell, when this essential nutrient is in restricted supply in the growth medium.

The alkaline phosphatase enzyme system associated with cells of Escherischia coli has been extensively studied. The influence of the inorganic phosphate content of the growth medium on enzyme synthesis was initially observed in E. coli (Horiuchi et al, 1959; Levinthal, 1959) coincident with the exhaustion of phosphate from the medium, alkaline phosphatase activity appeared. The enzyme was synthesised at a high differential rate, accounting for 6% of the total protein. Addition of inorganic phosphate to the culture medium caused an instant cessation of further enzyme synthesis. Torriani (1960) observed that inorganic phosphate not only repressed enzyme synthesis in growing cultures but also inhibited the action of the enzyme in broken cell preparations; both these properties distinguished the enzyme from the acid phosphatases.

The phenomenon of phosphate - repression of alkaline phosphatase, although widespread, is not a general effect (Kuo and Blumenthal, 1961). Synthesis of the enzyme by different strains of E. coli varies in its sensitivity to excess phosphate and with one strain the presence of phosphate actually stimulated the enzyme synthesis. Gallant and Stapelton (1964) found that in a mutant strain of E. coli, repression of the enzyme synthesis by inorganic phosphate was inversely proportional to the temperature of cultivation. Restoration of the enzyme proceeded more rapidly the lower the temperature of cultivation, thus suggesting that the repressor is a protein with a rate of synthesis which is inversely proportional to temperature in the mutant strain.

Several workers have reported methods for overcoming the repressive influence of excess inorganic phosphate in the growth medium. Bowne et al (1966) showed that the addition of various nucleosides, e.g. inosine, guanosine and adenosine, to the growth medium stimulated normal enzyme production and reduced the time required for recovery from repression by the excess phosphate. The results were more pronounced for inosine than for the other two compounds. This is further supported by the work of Csopak et al (1972) who showed that a low concentration of inosine in the growth medium had no effect on the rate of growth of cells of E. coli but resulted in a 10% increase in the alkaline phosphatase activity of the cells. Wilkins (1972) also observed that the removal of orthophosphate was not necessary for the induction of the alkaline phosphatase. The enzyme can be induced by starvation of pyrimidines or guanine in the growth medium, e.g. the starvation of a thymine or uracyl auxotroph in the presence of excess phosphate, although the starvation of thymine produced the better results. These starvation treatments are thought to affect the nucleotide metabolism in the cells.

Garrahan et al (1969) reported that a concentration of magnesium ions was required to give optimal activation of the enzyme in human red blood cells, independent of the substrate concentration. The presence of potassium ions in addition to Mg^{++} ions produces a greater activity. This suggests that Mg^{++} combines with the enzyme at a site independent of and non interacting with the substrate or K^{+} sites. The presence of divalent ions is an absolute requirement for the activity of inorganic pyrophosphatase from baker's yeast (Baykov et al, 1972).

Arsenates and orthophosphates have the highest affinity (3 times that of nitrophenyl phosphate) as competitive inhibitors for the enzyme in cells of E. coli (Garren and Levinthal, 1960)

The alkaline phosphatase of E. coli appears to interact specifically with monosubstituted phosphates; other structural features of the substrate play only a minor role (Heppel et al, 1962). Snyder and Wilson (1972) have shown that when phosphoramidic acid derivatives are used as substrates e.g. mono amido phosphates, $RNHPO_3H_2$, no differences in rates of hydrolyses are detected whether R is an alkyl, aryl or acyl group.

The alkaline phosphatase enzyme contains firmly bound zinc at a high concentration; the zinc is essential to enzyme activity (Plocke et al, 1962); Halford et al (1972) have shown that E. coli alkaline phosphatase exists as a dimer made up of two identical subunits containing two Zn^{++} ions. On addition of excess Zn^{++} ions, the molecular weight of the enzyme increases as a function of pH until at pH 8.0, the molecular weight is doubled, due to the formation of a tetramer. Bloch and Schlesinger (1973) suggest that the enzyme contains 2 - 4 tightly bound Zn^{++} ions which are essential for catalytic action. The enzyme is also reported to bind inorganic phosphate, a product of substrate hydrolysis

such that 1 mole phosphate per mole of enzyme is firmly bound and 1 or more moles held more loosely.

The inactivation of E. coli alkaline phosphatase at high pH values is accompanied by the dissociation of the native dimeric enzyme to inactive monomers (Csopak, 1972). This process is time dependent and is partially reversible; the reactivation of the inactivated enzyme is affected by the presence of a Zn^{++} ion.

The alkaline phosphatase enzyme systems associated with other Gram-negative bacteria have been recently studied. The release of the enzyme from whole cells of Pseudomonas aeruginosa as a function of magnesium chloride concentration is proportional to the release of lipopolysaccharide from the cells (Ingram et al, 1973). The enzyme is located in the periplasmic region of the cells and exists in subunit form (Day and Ingram, 1974). The P. aeruginosa alkaline phosphatase contains 4 moles of zinc per mole of enzyme, compared to 2 for E. coli. Alkaline phosphatase is associated with the cell walls of a number of Gram-negative bacteria (Cheng and Costerton, 1973). In some species, cell wall-associated enzymes were confined to the periplasmic space (i.e. the area between the cytoplasmic membrane and the double-track layer of the cell wall), in others they were found in the cell surface but in cells of most species they were present in both places. The binding of the enzyme by a structural component of the cell wall anchors the enzyme and provides it with a protected ionic environment due to the effect exerted by the bound anions of the structural polymers.

It is an established fact that cells of Staph. aureus give a positive test for phosphatase (Baird - Parker, 1963). However, the properties of this enzyme have not been as extensively studied as the enzyme associated with E. coli. More work has been done on the acid phosphatase of cells of Staph. aureus.

Kuo and Blumenthal (1961) correlated the amount of acid phosphatase produced by cells of Staph. aureus with coagulase production in these cells as a biochemical index of pathogenicity. They showed that the average amount of acid phosphatase produced by coagulase - positive strains was approximately 4 times greater than that produced by coagulase - negative strains. No phosphate repressible enzyme system was detected in any of the strains studied. Cannon and Hawn (1963), however, disagreed with these results, stating that all coagualse - positive strains did not produce larger amounts of acid phosphatase than coagulase - negative strains. Further they could find no correlation between high phosphatase production and penicillin resistance in the cells.

Other workers (Shah and Blobel, 1967; Malveaux and San Clemente, 1969 a) have shown that strains of Staph. aureus can produce an alkaline phosphatase enzyme system, which is repressible by the addition of excess inorganic phosphate to the growth medium, as well as the acid phosphatase which is not affected by the inorganic phosphate content of the medium.

1.6 Particulate Microelectrophoresis

At a solid/electrolyte interface there is an electrical double layer due to an excess of ions on the solid phase and an equivalent amount of ionic charge of opposite sign distributed in the liquid phase near the interface. There are two different potentials operating at a charged surface - the zeta potential (ζ) and the thermodynamic potential (E). The zeta potential is a function of the ionic strength and is very sensitive to traces of electrolyte ions especially those of polyvalent electrolytes. At constant pH, the thermodynamic potential is independent of the concentration of dissimilar ions up to relatively high concentrations. Any theory of the double layer must take these two potentials into account.

One of the first theories of the double layer was due to Helmholtz (1879) who assumed that the charge on a particle in an electrolyte solution was caused by the adsorption of ions onto the surface. These charges would be firmly attached to the surface and would move with it. To maintain electrical neutrality, he suggested that in the immediate vicinity, in the liquid phase there is a movable, oppositely charged layer; this is equivalent to an electrical condenser of constant capacity.

This theory, which did not explain all the experimental facts, was later modified by Gouy (1910) who allowed for the randomising thermal forces and considered that in the region away from the surface there is an excess of oppositely charge ions. Stern (1924) put forward a model for the double layer which while combining the essential characteristics of the Helmholtz and Gouy theories (i.e. the opposing electrical and thermal forces) also allows for the finite size of ions.

In the vicinity of the surface there is a higher concentration of oppositely charged ions, which gradually tails out into the bulk of the solution until there is no such excess. This theory also postulates that some ions (gegen ions) of the opposite charge to the charge-determining ions (i.e. those in excess near the surface) are adsorbed onto the surface and move with the surface (Fig. 1.7). These ions are adsorbed because of the very strong electrical forces operating or because of ionic hydration.

Under the influence of an applied electric field, the charged solid phase and the liquid phase move relative to one another. This phenomenon is known as electrophoresis. The position at which the double layer shears is known as the shearing plane and the potential at this plane is the zeta potential.

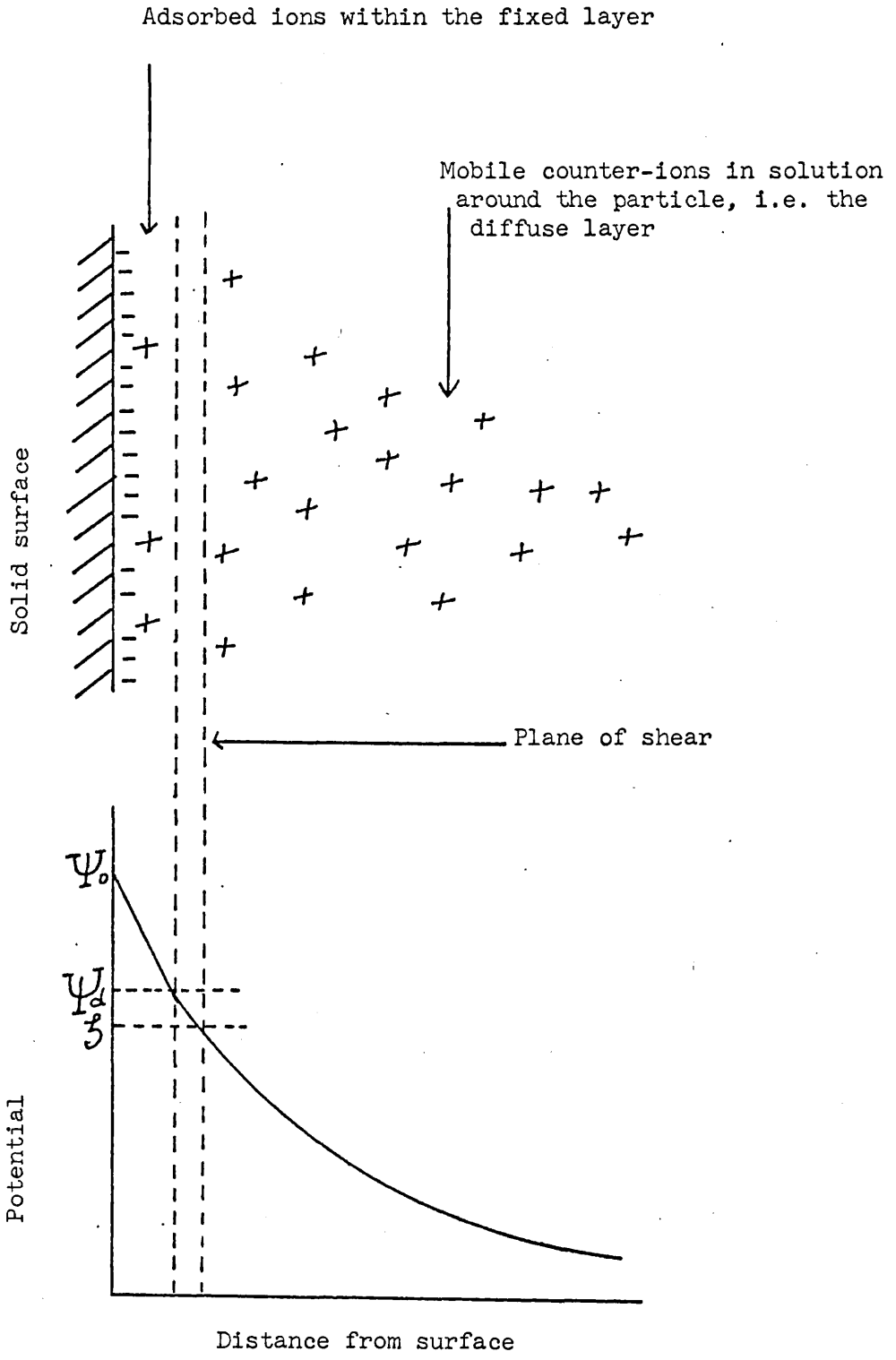
In biological cells, the charge originates from ionogenic groups, e.g. amino, carboxyl and phosphate, located at the surface. There is no charge due to ion adsorption onto such a surface (Gittens, 1962). Unlike hydrophobic sols, biological cell suspensions are stable even when the zeta potential is very low; this is because of the large amounts of water associated with the macromolecules which make up biological structures.

(a) Particulate electrophoresis

A method based on that of Ellis (1911) was used throughout this investigation to determine the electrophoretic mobility of bacterial cells. A potential difference is applied across a bacterial suspension contained in a closed glass chamber. The migration of the suspended bacteria is observed with a microscope and individual cells are timed moving across a graticule in the eyepiece.

Fig. 1.7

The structure of, and the electrical potentials associated with, the double layer at a solid-electrolyte interface.



When an electric field is applied across a system, the bacteria will move relative to the suspension medium and the suspension medium will move relative to the glass surface of the observation chamber. The latter effect is due to electroosmosis of the suspension medium. Thus the observed particle velocity, V_0, is given by

$$V_0 = V_L + V_P$$

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 is constant at all depths within the chamber). In a closed system, the liquid flows along the two inside faces of the chamber, towards the negative electrode and returns through the centre, resulting in a variation of V_L and hence V_0 with depth. Since the liquid is continuously deformed within a closed system, there must be a plane at which the liquid is stationary i.e. $V_L = 0$, at this level $V_0 = V_P$.

In a flat cell of rectangular cross section, this plane is observed at two levels equidistant from the cell centre. Komagata (1933) derived an expression for the position of the stationary levels in a cell with a width/thickness ratio, K, such that :

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

Where s/d is the fractional depth measured from the inside surface. For a cell with a K value greater than 20, the stationary levels are at 0.21 and 0.79 of the total depth

from an inside face (Abramson, 1934), and the mean particle velocity, V, may be determined by the method of Ellis, whereby

$$V = \frac{1}{x_1} \int_0^{x_1} V_0 \, dx$$

where x_1 is the cell depth, and V_0 the velocity of the particle observed at depth x. For a symmetrical cell the curve of V_0 against x should be a parabola symmetrical about the centre.

Cell and electrode design has been extensively reviewed by James (1957) and Seaman (1965). With the apparatus used in this work (Gittens and James, 1961) the applied field strength (X) is best calculated from conductance and current data, using the equation :

$$X = \frac{I}{qk}$$

Where I/A is the current, k the conductivity of the suspension medium, and q/m^2 the cross sectional area of the cell.

(b) Application of microelectrophoresis to the study of the bacterial cell surface

Electrophoretic measurements must be made on cells suspended in a medium of known chemical composition, pH and ionic strength. The ionogenic groups of a carboxyl-amino type surface commonly found on bacterial surfaces will be titrated according to the equations :

$$COO^- + H^+ \rightleftharpoons COOH$$

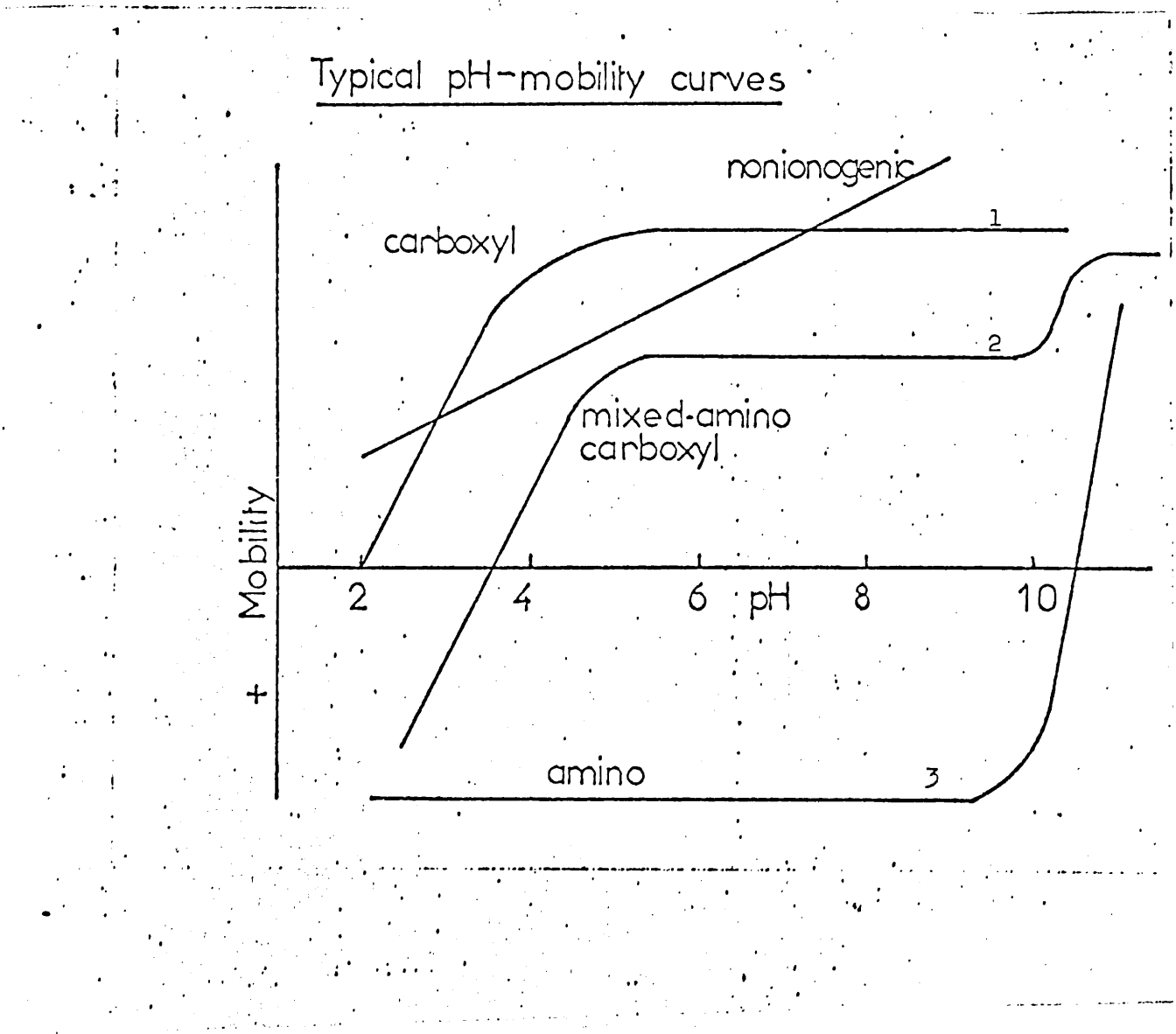
$$-NH_2 + H^+ \rightleftharpoons NH_3^+$$

Typical pH - mobility curves for this type of surface are shown in Fig. 1.8.

Curves of these shapes have been obtained for "model surfaces". However, when varying the pH of the suspension medium of any biological material, care must be taken not to cause irreversible changes in the surface by using extreme pH values. After suspending cells at a high or low pH, the reversibility must be checked by rewashing and measuring the mobility of the cells at pH 7.0. The mobility value should not differ significantly from that of control cells at pH 7.0. An irreversible change indicates cell surface and maybe cell wall damage, making useful interpretation of the results difficult. This variation of mobility with pH gives information about the nature and quantity of surface ionogenic groups. In all the work reported, pH - mobility curves were plotted over a range where no irreversible disruption of the surface occurred.

Although the curves (Fig. 1.8) are those obtained for a model surface, similar curves have been obtained for bacterial and other biological cells. Curve 1 shows a typical titration curve for a carboxyl group where, at low pH values the carboxyl group is undissociated and as the pH of the suspension is increased, the negative charge associated with the particles increases owing to the ionisation of these carboxyl groups.

Fig. 1.8


Typical pH-mobility curves
nonionogenic
carboxyl
1
2
mixed-amino
carboxyl
amino
3
Mobility
+
2
4
6
pH
8
10

This is the simplest type of surface associated with bacterial cells e.g. Klebsiella aerogenes (Lowick and James, 1957), and human erythrocytes (Seaman, 1965).

Curve 2 is that obtained for a mixed amino/carboxyl surface where there is an excess of carboxyl groups; at low pH values the surface charge will be due to the positively charge amino groups, but on decreasing the hydrogen ion concentration, the positive charge is reduced because initially the carboxyl groups ionise, giving rise to an overall negative charge. An isopotential point occurs in suspension at low pH, the exact value being determined by the relative numbers of amino and carboxyl groups and their pK values. At higher pH values (up to pH 9.0) a plateau region occurs where both amino and carboxyl groups are fully ionised, the mobility then depending on the relative numbers of carboxyl and amino groups. The effective negative charge increases beyond pH 9.0, as shown, due to the suppression of the amino group ionisation. This sigmoid - type pH - mobility curve is typical of that of the carboxyl - amino surface of cells of Streptococcus pyogenes (Hill et al, 1963).

The pH - mobility curves of cells of Staph. aureus are unlike any of the "model surfaces". Typical curves obtained for cells of methicillin sensitive strains of Staph. aureus are characterised by having a sharp increase in the negative mobility value in the pH range 3.5 to 4.0 and a plateau value from pH 5.0 to 8.0 (James and Brewer, 1968).

Naturally occurring methicillin-resistant cells of Staph. aureus grown at 37°C have pH - mobility curves which show a minimum at pH 4.5 and a maximum in the pH range 3.5 to 4.0 (Marshall and James, 1971; Hill and James, 1972 a). The maximum in these curves is attributed to the presence of charged groups of the surface teichoic acid. As the pH is reduced from pH 6.0, the surface carboxyl groups tend to become less fully ionised and it is suggested that the change in the electrostatic environment results in a new conformation of the teichoic acid molecules which exposes phosphate groups to the cell surface; since these are fully ionised at pH 3.5 - 4.0 there will be an increased negative charge (James and Brewer, 1968).

1.7 Object Of the Present Investigation

Previous work (Hill, 1971) has shown that cells of Staph. aureus which are sensitive or resistant to methicillin have different surface components, in particular it has been suggested that there are different amounts of teichoic acid on the cell surface. Particulate electrophoresis studies have shown that these differences could be a reflection of the varying amounts of phosphate at the cell surfaces.

The present investigation includes :

(a) the establishment of a technique for studying the alkaline phosphatase activity of cells of Staph. aureus.

(b) a study of the alkaline phosphatase activity of cells of strains of Staph. aureus which are sensitive or resistant to methicillin.

(c) a study of the surface properties of cells of methicillin resistant and sensitive strains of Staph. aureus, in particular, the effect of different growth conditions on the surface properties of these cells.

The objects of these studies were to establish :

(i) the effect of temperature of growth and nature of the growth medium on the alkaline phosphatase activity, surface teichoic acid and methicillin resistance or sensitivity of cells of Staph. aureus.

(ii) any correlation between the alkaline phosphatase activity, absence or presence of surface teichoic acid and methicillin resistance or sensitivity of these cells.

CHAPTER TWO

EXPERIMENTAL TECHNIQUES

2.1 Bacteriological Techniques

(a) Strains

The strains (Table 2.1) used in this investigation were supplied by the Cross Infection Reference Laboratory, Colindale, growing on nutrient agar slopes in Bijoux bottles.

All strains were stored in the laboratory in tightly sealed universal bottles on nutrient agar slopes at room temperature. The type of growth medium used will be defined later.

(b) Growth of strains for mobility observations

All strains of Staph. aureus were grown once on nutrient agar, after removal from the storage culture, before being subcultured onto nutrient agar plates in the quantities required for further examination. Unless stated, all cultures were incubated for 18h. at the required temperature.

The strain of Klebsiella aerogenes (NCTC 418) was maintained by regular subculturing at 37°C in nutrient broth in 100 cm^3 medical flats. When used as a standard for calibration of the electrophoresis apparatus, cells from the parent culture were inoculated into about 50 cm^3 of Nutrient Broth (Oxoid Code CM1) in a loosely capped medical flat and incubated at 37°C for 18h.

(c) Growth of cells during training to methicillin

Cells of Staph. aureus, strains 2 and 3, were trained to develop a resistance to methicillin, using a method described by Rolinson et al (1960).

Table 2.1

Biological Properties of strains of Staphylococcus aureus

Code Number	Strain Number	Antibiotic Characteristics
1	13136/60*	P(pen +), S, T, M
2	9341/67*	Sensitive
3	9322/67*	P(pen +)
4	4916**	P(Pen +), S, T, M
5	9300/67*	Sensitive
7	10101/67*	P(pen +), S, T, M, E', No, N
8	Oxford	Sensitive
9	BRL 1800***	P(pen +), S, T, M

P - penicillin, S - streptomiycin, T - tetracycline, M - methicillin, No - novobiocin, N - neomiycin, E' - erythromiycin (inducible), pen + - penicillinase producer.

* - Routine isolate from Staphylococcal Reference Laboratory, Colindale.

** - See Dornbusch et al (1969).

*** - Obtained from Beecham Research Laboratories Ltd. (Used by Grubb and Annear, 1972).

The strain was initially grown on nutrient agar containing a very low concentration of methicillin (1.25μg cm^{-3}) at 37°C. Initially, very little growth was obtained but after further subcultures at the same concentration of antibiotic better growth was obtained. When healthy growth had been obtained at this concentration of antibiotic, the culture was inoculated onto a plate containing a slightly higher concentration of antibiotic. This procedure was repeated until the resulting culture had a minimum inhibitory concentration (MIC.) of 200μg cm^{-3}. Methicillin is unstable above a temperature of 45°C and in aqueous solution. For these reasons, certain precautions were taken in the preparation of the agar plates used. The stock solution of methicillin was made up freshly each time by aseptically weighing the antibiotic and dissolving it in sterile distilled water. The correct volume of this solution was added aseptically to the molten agar, cooled to 40-45°C to give the required concentration of antibiotic.

(d) Measurement of the minimum inhibitory concentration

Nutrient agar plates containing different concentrations of methicillin in the range 0-200μg cm^{-3} were prepared as described above (2.1 (c)). The culture to be examined was grown on a nutrient agar plate, containing no antibiotic, at the required temperature for 18h. The cells were harvested in sterile nutrient broth and 2 drops of this suspension were inoculated onto each antibiotic - containing plate. The plates were incubated at 25, 37 and 43°C and were read after 24 and 48h growth, to determine the inhibitory concentration. The MIC. of methicillin is the lowest concentration of antibiotic on which the cells will not grow.

(e) Cleaning and sterilisation of apparatus

All glassware was washed thoroughly in hot water, rinsed twice in tap water, once in distilled water and dried in an oven at 170°C.

All solutions and growth media required in a sterile state were autoclaved at 15 lb. per sq. in. for 15 minutes. Contaminated disposable apparatus was immersed in a 1% lysol solution. All other contaminated glassware was autoclaved before washing.

2.2 Determination of Inorganic Phosphate Present in the Growth Media

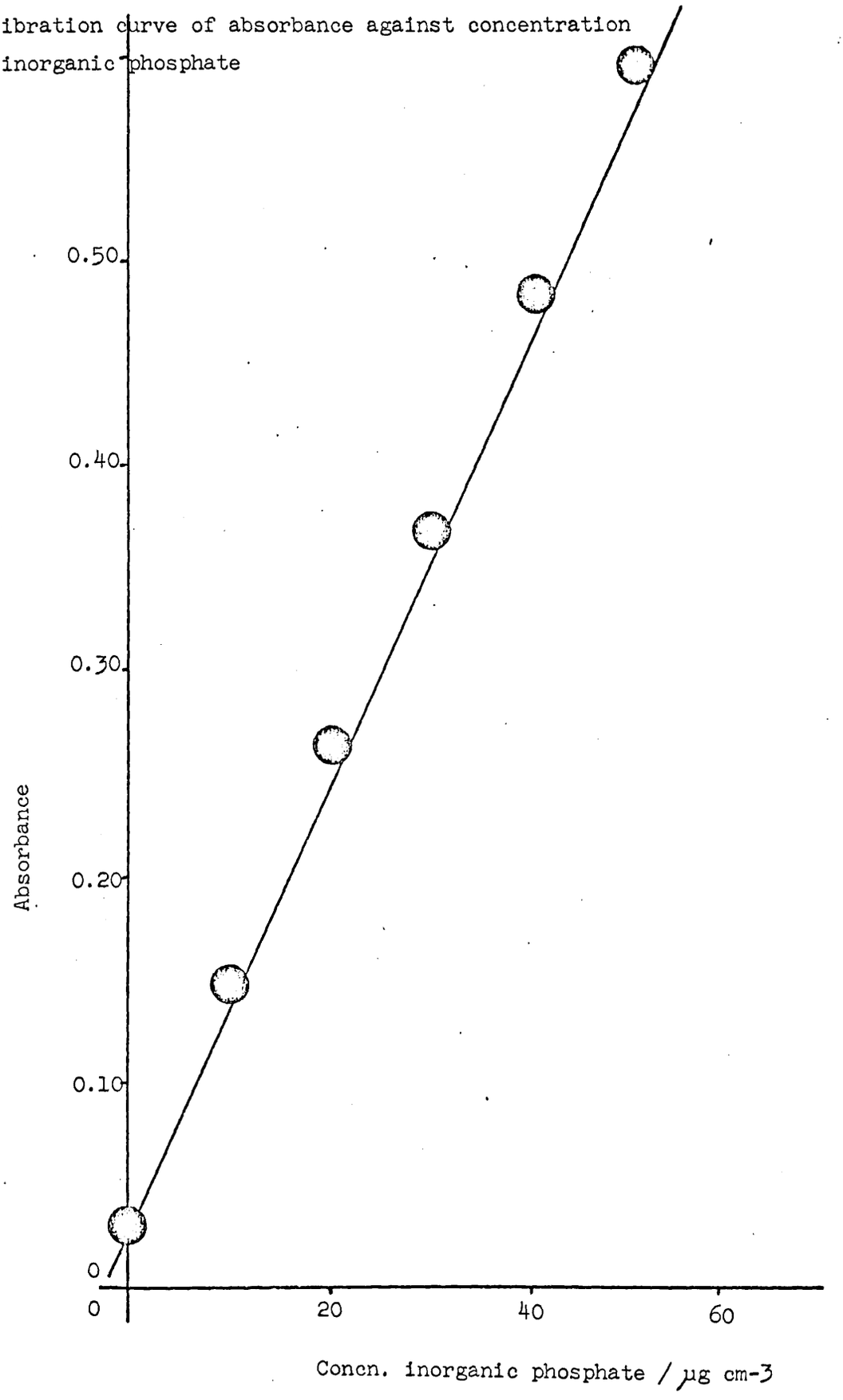
A quantitative analysis of the free inorganic phosphate present in the various growth media was carried out using a spectrophotometric method (James, 1967).

Standard solutions containing 10, 20, 30, 40 and 50 μg P/cm^3 were prepared from AnalaR KH_2PO_4 dried at 110°C. Each solution was treated as follows : to 2 cm^3 phosphate solution, 2 cm^3 60% perchloric acid was added followed by 2 cm^3 amidol reagent and 1 cm^3 ammonium molybdate solution. This solution was made up to 25 cm^3 with water and the absorbance reading was measured at 730 nm. Distilled water also contained a detectable amount of inorganic phosphate and, therefore, untreated water was used as the blank and the absorbance of 2 cm^3 of distilled water, treated as above was also measured.

A calibration curve of absorbance against concentration of inorganic phosphate was plotted (Fig. 2.1). The inorganic phosphate content of both the liquid and solid media was measured. The broth and solid media were prepared in the normal way. 2 cm^3 samples of each were diluted to 10 cm^3 and 2 cm^3 of this solution were treated as previously described. (This dilution was sufficient to dilute the agar so that the resulting solution could be pipetted).

Fig. 2.1

Calibration curve of absorbance against concentration of inorganic phosphate



From the absorbance reading obtained and the calibration curve, the concentration of free inorganic phosphate in the growth media was calculated.

Since the rate of development of the colour varied with atmospheric conditions it was always necessary to construct the calibration curve and determine the inorganic phosphate content of the media at the same time.

2.3 Micro-electrophoresis Techniques

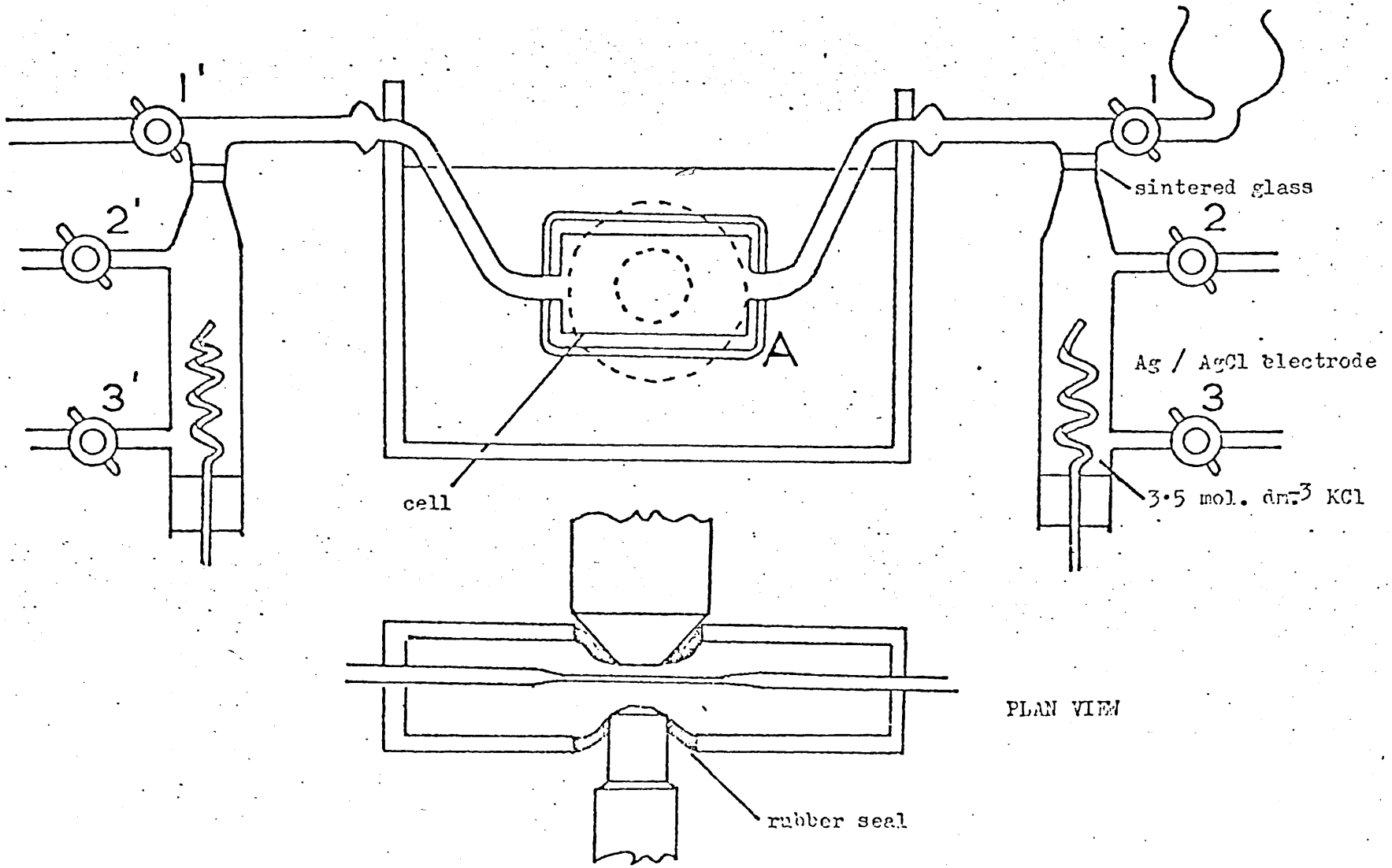
(a) General description

The cell and electrode compartments used were those developed by Gittens and James (1961), the observation chamber was mounted in the lateral position as shown in Fig. 2.2.

The electric field was applied across a suspension of bacterial cells contained in the glass observation chamber (A). Observing the cells with a microscope, the velocity due to a known applied field was determined by timing individual cells across a given number of squares in an eyepiece graticule. The rectangular chamber (A) was constucted by the fusion of two optically flat Hysil glass plates (40 x 25 x 0.5 mm) such that the depth of the chamber was 0.5 mm. The side arms were constructed of 10 mm bore pyrex tubing, this being sealed directly into the chamber and bent in the plane of the cell so that the chamber could be immersed completely in a water bath. Glass rods around the chamber gave structural rigidity. Spherical quick fit joints were used to attach the side arms to the electrode compartments and clips attached to these ensured leak proof seals.

A constant electric field was applied between the Ag. Ag Cl/ KCl electrode systems in the compartments which were constructed with high quality vacuum taps.

Fig. 2.2. MICROELECTROPHORESIS CELL AND ELECTRODE COMPARTMENTS



The No. 2 sintered glass discs were sealed as near the side arms as possible, thus eliminating dead space, easing the removal of air bubbles and assisting cleaning.
Each electrode was made by coiling 25 cm of 2 mm diameter silver wire and sealing into a rubber bung which provided a water tight seal. The silver wire was first cleaned with 50% nitric acid and then the electrodes were anodised in series in 0.1 mol dm^{-3} hydrochloric acid using a platinum cathode until a grey-purple coating of Ag Cl was deposited on each electrode. The electrodes were then placed in the compartments, 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. The electrode compartments could be conveniently refilled at regular intervals with the electrolyte solution from reservoirs, using taps 2 and 3, 2' and 3'.
Since the viscosity and conductivity of the buffer solution and hence the cell electrophoretic mobility were temperature dependent, it was essential to maintain the bacterial suspension at a constant temperature. This was achieved by immersing the observation chamber in a small water bath which was maintained at $25.0 \pm 0.5^{\circ}C$ by circulating water from a large thermostatically controlled bath.
The small water bath, electrical circuit, microscope unit and the timing device were part of the Rank Bros. Particle Micro-Electrophoresis Apparatus Mark II. The small water bath was constructed of perspex sheeting with a circular indentation at the front to accommodate the microscope objective lens.
The chamber was mounted in a lateral position between the condenser and objective lens of the microscope by means of bolts attached to perspex clamps.

Sponge foam was wrapped around the side arms before tightening the bolts. The microscope system employed gave an overall magnification of x 600.

Illumination of the chamber was provided by a 12v, 100 watt lamp of standard design. For long life, the "quartz iodine" illuminator was switched on at low intensity and then turned up. To assist focusing and observations, a low power dark ground illuminating condenser was provided. Temperature rises and convection currents in the observation chamber arising from the heating effect of the intense illumination were eliminated by placing a quartz disc and glass heat filter in the beam, in front of the lamp unit.

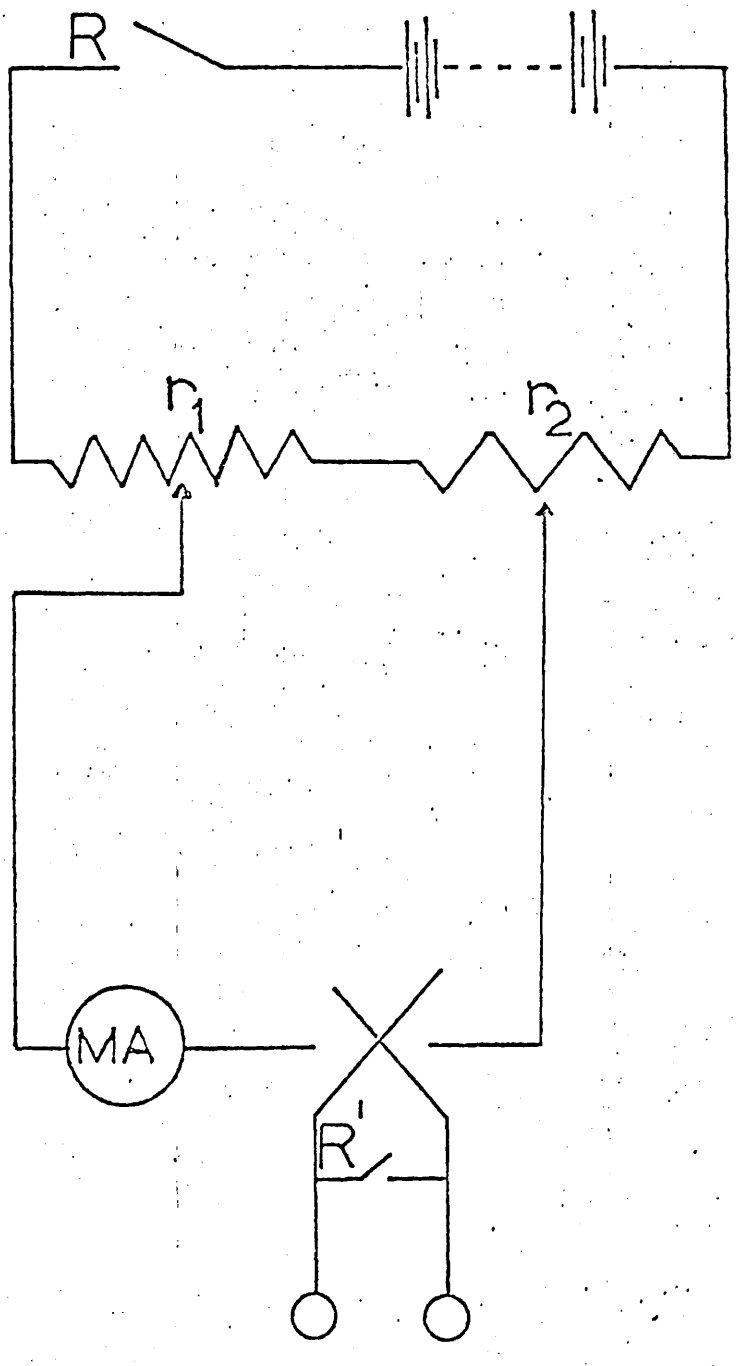
The electrical circuit used is shown in Fig. 2.3.

The voltage was supplied by the mains frequency and controlled by a variable resistance. The current flowing through the electrophoresis chamber was measured with a milliammeter. The applied potential could be reversed using the switch R'. This switch also allowed the electrodes to be shorted when not in use to prevent polarisation.

An electromagnetic timer, operating from the mains frequency, was used to record the time taken by a bacterial cell to cross a given number of squares on the graticule.

The suspension under investigation was introduced through the funnel attached to the right hand side of the apparatus and flushed out, through rubber tubing, into a reservoir containing lysol below the bench. The chamber was fed by gravity, but a partial vacuum was applied when required to remove any air bubbles. The microscope was focused by movement of the objective, the fine adjustment screw, which was calibrated over the whole of its range, was used to determine the depth of the chamber and to focus at required positions within the cell.

Fig.2.3. THE ELECTRICAL CIRCUIT FOR THE MICROELECTROPHORESIS APPARATUS.



(b) Assembly of the observation chamber

To aid location of the positions of the inner front and back surfaces of the chamber, these were coated with bacteria before assembly of the apparatus cells of *Klebsiella aerogenes* were harvested and suspended in ethanol and the resulting suspension pipetted into the chamber; the ethanol was evaporated at 43°C in an oven, with suitable manipulations of the chamber to ensure that both surfaces were coated. Subsequent washing of the chamber with distilled water removed the vast majority of the adhering bacteria but left sufficient cells for easy location of the inner surfaces.

The chamber was mounted firmly in the perspex clamps, the spherical ground joints lightly greased and the electrode compartments attached. Care was taken that the chamber was truly horizontal when viewed from the front and also square to the microscope eye piece and the condenser (i.e. vertical when viewed from the side).

Water was flushed through the apparatus and all air bubbles were removed.

(c) Mode of operation

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

About 50 cm^3 of KCl solution were flushed through each electrode compartment by opening taps 3 and 2, and 3' and 2'; then taps 2 and 2' were closed and tap 1' opened, forcing the solution through the sintered glass discs.

Distilled water was flushed through the observation chamber to remove the electrolyte which had been forced through the discs.

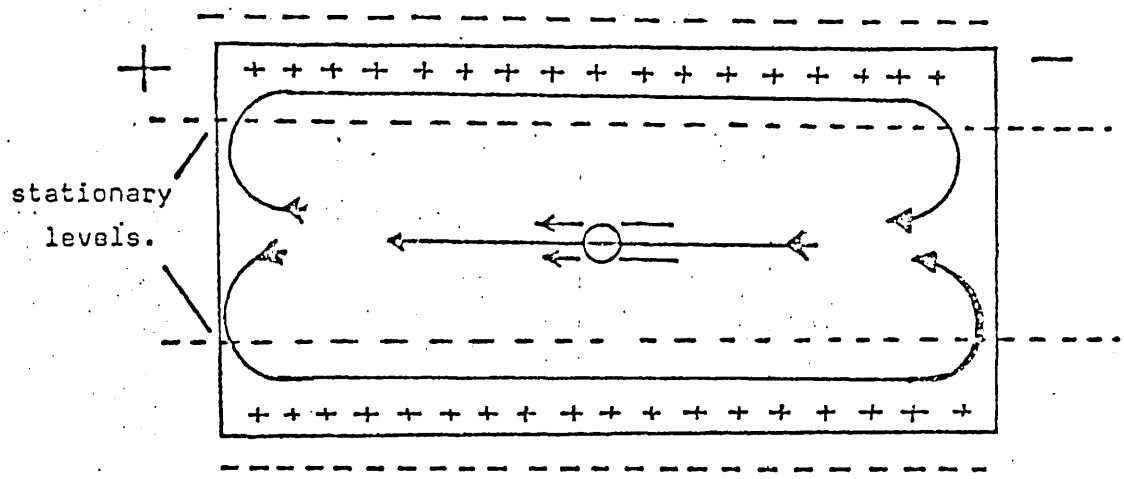
The chamber was finally filled with buffer solution at the temperature, pH and ionic strength of the suspension to be examined. Great care was always taken to ensure that no air bubbles were trapped in the closed system.
The microscope was focused on bacteria on the front inner surface using the fine adjustment and then racked in the required amount to the calculated position of the front stationary level (Fig. 2.4) All mobility measurements were made at this stationary level.
The cells were allowed to come to thermal equilibrium and after establishing that they exhibited only Brownian motion (i.e. no systematic drift due to various causes) the current was switched on and adjusted to give a suitable transit time (2-4 seconds) across a known number of squares of the graticule.
The time for one particle to traverse a fixed distance of the graticule was then determined, using the electromagnetic timer. The polarity of the electrodes was reversed and the process repeated. A minimum of 20 cells were timed for each suspension and the value of the current recorded for each measurement.
The cell depth was measured before each experiment, but was found to be effectively constant.

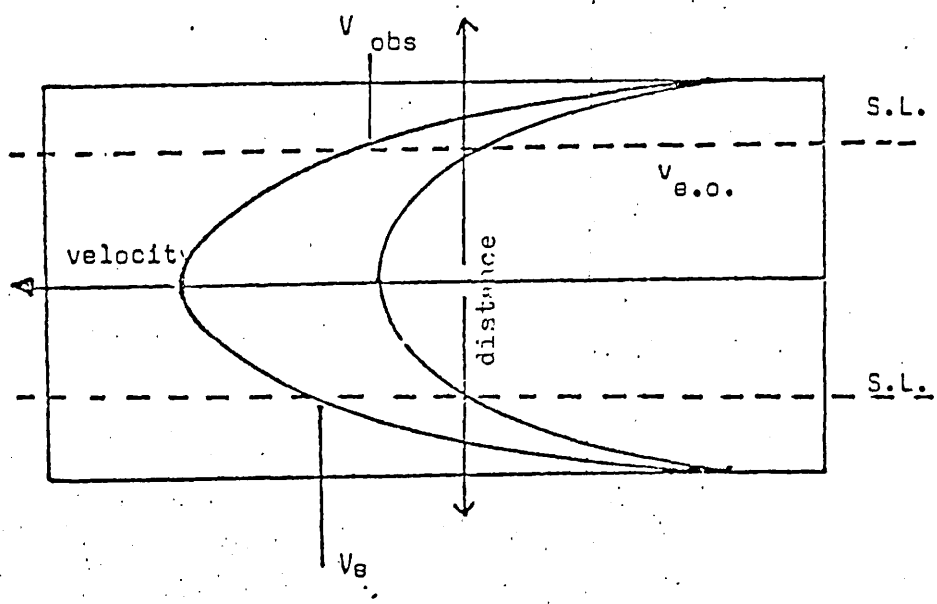
(d) Calibration and theory of the apparatus

Since the internal surfaces of the chamber are charged, the applied electric field causes not only electrophoretic migration of the cells but also an electro-osmotic flow of liquid near to the inner surfaces. Because the chamber is closed there is a compensating return flow of liquid, with maximum velocity at the centre of the chamber (Fig. 2.4). This results in a parabolic distribution of liquid velocity with depth (Fig. 2.5) and the true electrophoretic velocity of the cells, i.e. the cell velocity relative to the

Fig. 2.4

(a) ELECTROOSMOTIC FLOW IN MICROELECTROPHORESIS CELL.

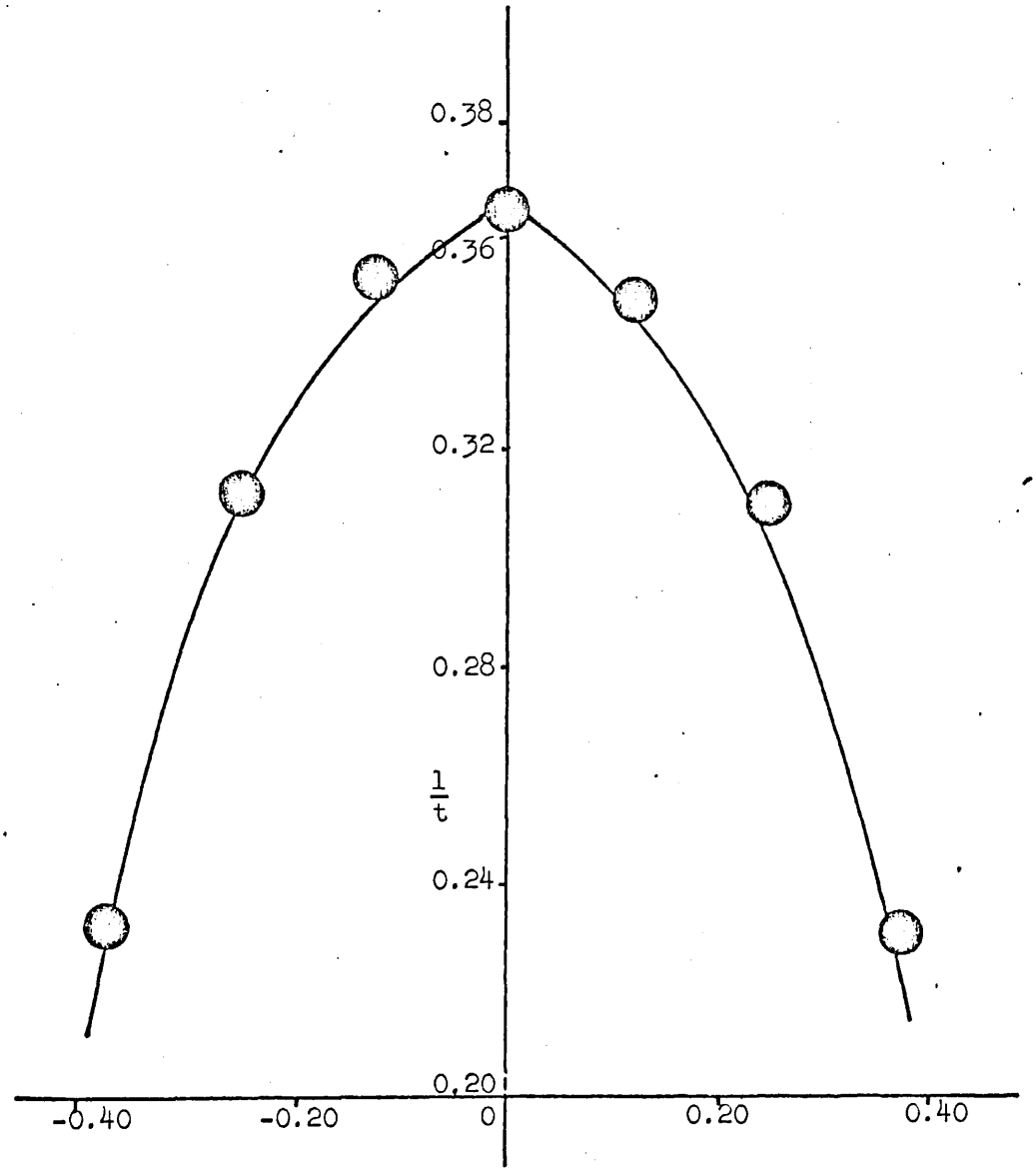




(b) VELOCITY GRADIENT IN MICROELECTROPHORESIS CELL.

Fig. 2.5

Velocity - depth curve of the microelectrophoresis chamber



stationary liquid, is only observed at the levels in the chamber where the electro-osmotic and return flow of liquid cancel each other. These are known as the stationary levels and in a rectangular chamber are flat levels within the chamber parallel to the sides of the chamber.

The symmetry of the chamber was examined by determining the velocity - depth curve using cells of K. aerogenes. The cells were harvested from 18h. cultures grown in nutrient broth at 37°C, centrifuged out of the broth, washed twice in barbiturate buffer solution (pH=7.0, I=0.02 mol dm^{-3}) and resuspended in the buffer solution. The time for cells in focus at a known cell depth to cross a known number of squares of the graticule at a constant electric field strength was determined and the reciprocal time plotted against the cell depth, expressed as fractional depth measured from the centre of the cell (Fig. 2.5).

The equation of the parabola was determined as follows : Let x denote the fractional depth from the centre of the cell and y the velocity (i.e. reciprocal time measured at this depth). The equation of the velocity-depth parabola is of the form :

$$y = a + bx + cx^2 \qquad 2.1$$

The experimental equation for the chamber under study was :

$$y = 0.3664 - 0.002563x - 0.9584x^2 \qquad 2.2$$

The small value of "b" indicates that the calculated centre of the parabola is only very slightly displaced from the geometrical centre and that the chamber can be accepted as symmetrical. Integration of equation 2.2 between $x = \pm 0.5$ (i.e. over the complete depth) gives the mean electrophoretic velocity i.e. 0.2859 s^{-1}. Substitution of this valve back into equation 2.2 gives valves for x corresponding to the stationary levels of +0.289 or -0.291 from the centre of the chamber, indicating that the stationary levels are at 0.211

and 0.791 of the depth of the chamber from the front inside surface. These positions are in close agreement with those predicted theoretically for a cell of the size used.

The electrophoretic mobility, $\bar{v}$ / $m^2 s^{-1} V^{-1}$, defined as the particle velocity v/ms^{-1} per unit potential gradient in the stationary level, is given by

$$\bar{v} = \frac{v}{\bar{x}} = \frac{nL}{r}\frac{qKs}{I} = \frac{nL}{r}\frac{qJG}{I} \qquad 2.3$$

where nL/m is the distance travelled (n being the number of squares of side L) in time t/s; q/m^2 the area of cross section of the chamber; and I/A the current flowing. $K_s/ohm^{-1}m^{-1}$ is the conductivity of the suspension obtained from the measured conductance G/ohm^{-1} and the cell constant J/m^{-1} of the conductance cell. The values of G, I, t are obtained experimentally. Since it was not possible to measure the cross sectional area of the chamber, a standard particle of known mobility $\bar{v}_s$ was timed in the apparatus and an apparatus constant K, which included the cell constant J of the conductance cell was determined. K is given by

$$K = LqJ = \frac{\bar{v}_s\, tI}{nG} \qquad 2.4$$

where t, I, G refer to values of time, current and conductance of suspension for the standard particle. Subsequent timings (t') on cells under examination were converted to mobility valves using the relationship :

$$\bar{v} = \frac{KnG'}{t'I'} \qquad 2.5$$

where t' I' and G' are the corresponding values obtained for cells in that particular suspension.

The standard particles (cells of K. aerogenes) suspended in barbituarate buffer solution pH = 7.0; I = 0.02 mol dm^{-3} at 25°C have a mobility value of - 1.67 x 10^{-8} m^2 s^{-1} V^{-1} relative to red blood cells (Gittens, 1962). K was determined before each set of electrophoretic measurements was made.

All mobility values will be quoted as a single number. A value of 1.67 indicates that the particle is negatively charged with an electrophoretic mobility (towards the positive electrode) of 1.67 x $10^{-8} m^2 s^{-1}$. In some instances, particles having positive mobility values were encountered; the mobility values for such particles will be prefixed by + .

(e) Buffer solutions

In all mobility determinations barbituarate - acetate buffer solutions with a pH range of 2.5 - 7.0 were used as the suspending electrolyte (Michaelis, 1931).

The stock solution was prepared from :

Sodium barbitone	154.64g
Sodium acetate trihydrate	102.07g
Sodium chloride	58.4g

dissolved in 5 dm^3 distilled water to give I = 0.5 mol dm^{-3}. This stock solution was kept at 4°C to prevent bacterial growth and diluted to give a final ionic strength of 0.02 mol dm^{-3} with distilled water. The buffer solutions were prepared by the addition of 1.0 mol dm^{-3} HCl to the diluted solution to give the required pH values (measured on an ElL (Model 32A) pH meter). The conductivity of each buffer solution was determined in a bottle type conductance cell at 25.0°C using a Wayne Kerr (B221) Universal Bridge.

(f) Preparation of cells for electrophoresis

Cells of Staph. aureus grown on the required growth medium were harvested and washed off the solid surface with distilled water, divided into the required number of aliquots and centrifuged. The cells were then resuspended in the appropriate buffer solution, of known pH, centrifuged and finally resuspended in the buffer solutions to give approximately 10^7 cells cm^{-3}.

The cells of K. aerogenes were harvested by centrifuging the nutrient broth suspensions and washing the cells twice in the buffer solution of pH = 7.0 before resuspending them in this buffer solution.

(g) Treatment of cells with sodium metaperiodate

Teichoic acid was destroyed and removed from the cell surfaces by a method modified after Garrett (1965) and Brewer (1966).

Cells of 18h cultures grown on nutrient agar plates were harvested in distilled water, washed once in water and then once in physiological saline (0.85% w/v, NaCl). The cells were then suspended in 0.1 mol dm^{-3} aqueous ammonia to remove any ester-linked alanine and then washed in distilled water to remove any alanine and aqueous ammonia. The material was then suspended in barbiturate - acetate buffer solution at pH = 6.0 (I = 0.02 mol dm^{-3}) containing sodium metaperiodate (0.05 mol dm^{-3}) for 30 minutes in a water bath at 37°C.

The oxidised cells were divided into the required number of aliquots, centrifuged from the sodium metaperiodate and washed twice in barbiturate - acetate buffer solutions at the appropriate pH values. Mobility determinations were then made on the cells in these suspensions.

CHAPTER THREE

PHOSPHATASE ACTIVITY OF CELLS OF STRAIN 1.

3.1 Establishment of Technique

The method, based on the procedure of Ohmori (1937) and Bessey et al (1946), uses a 4-nitrophenyl phosphate salt as the substrate. The bacterial suspension, in a buffer solution, was thermally equilibrated in a water bath at 37°C and the enzyme reaction started by adding the substrate, a solution of 4-nitrophenyl disodium orthophosphate. At suitable intervals, samples of the reaction mixture were removed and immediately mixed with 4.0 cm^3 of 0.20 mol dm^{-3} sodium hydroxide solution, this concentration of alkali was sufficient to stop further enzyme action and to develop fully the colour of the 4-nitrophenol liberated. The absorbance of each solution was measured at 400 nm against a blank taken at zero time.

The relationship between the absorbance and the 4-nitrophenol concentration under the conditions of assay, established in a separate experiment, is shown in Fig. 3.1.

The equation of this line is :-

$$x = \frac{y - 0.0108}{0.0222} \qquad 3.1$$

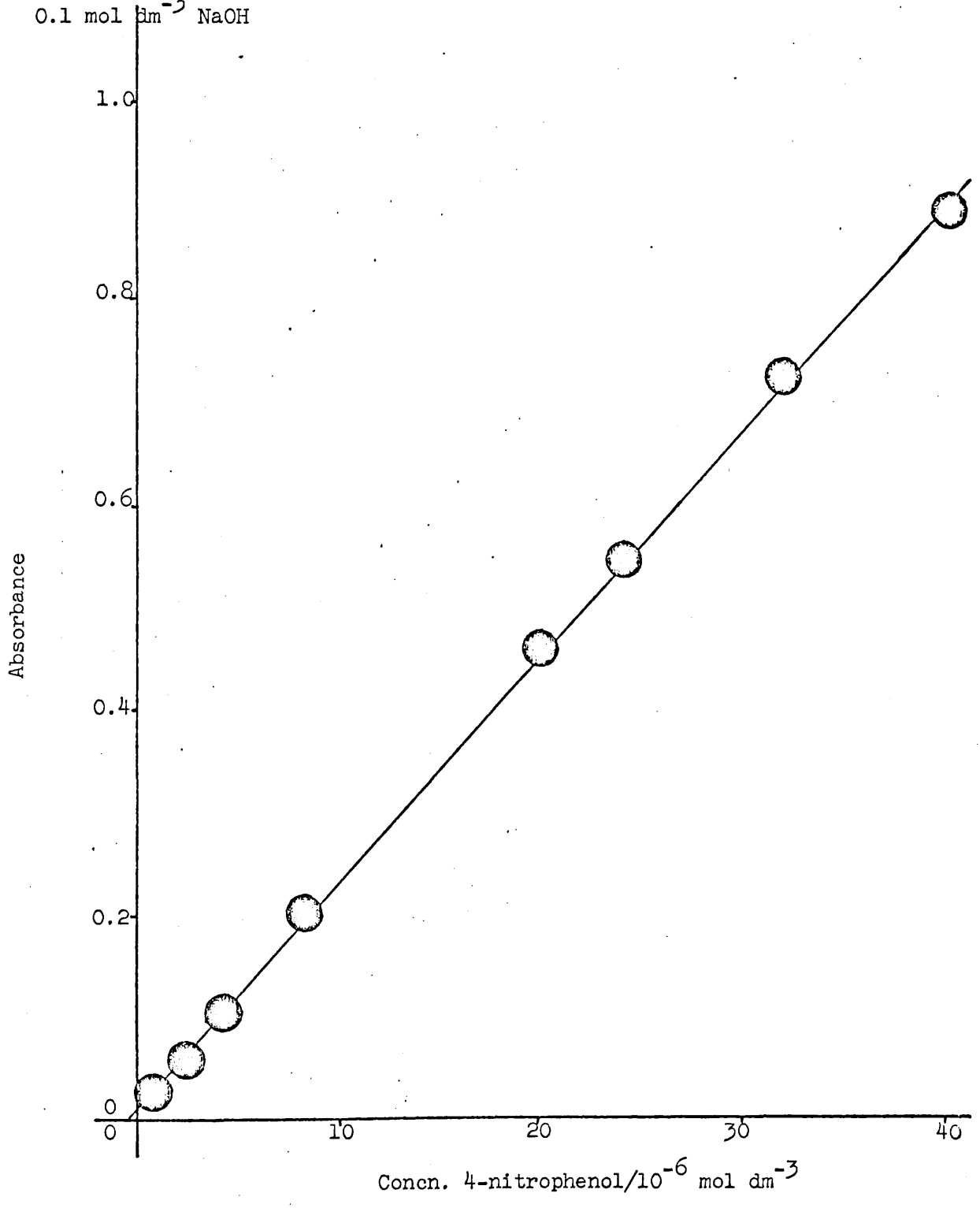
where the concentration of 4-nitrophenol is $x \cdot 10^{-6}$ mol dm^{-3} and y is the absorbance reading.

(a) Relationship between absorbance and dry weight of cell for strain 5 grown at 37°C.

Cells of Staph. aureus strain 5 were used as the standard for all the experiments. The cells were grown on standard nutrient agar plates for 18h. at 37°C, harvested in distilled water and washed twice with water. The opacity of the suspension was adjusted to give an absorbance reading of 0.45 - 0.50 at 620nm. 10.0 cm^3 of this suspension was measured accurately into 10 beakers

Fig. 3.1

Variation of absorbance with concentration of 4-nitrophenol in 0.1 mol dm^{-3} NaOH



which had been dried to constant weight. The suspensions were allowed to dry in the oven at 105°C to constant weight.

A suspension of cells of strain 5 with an absorbance reading of 0.500 at 620 nm contained 0.259 mg cm^{-3} of dry cell. The dry weight of cells of all other strains in suspension were obtained from absorbance measurements and the use of this factor - i.e. dry weights are all relative to the standard cells.

(b) Relationship between absorbance and concentration of cell suspension for strain 5.

The suspension of cells in the absorbance range 0.45 - 0.50 at 620 nm was prepared as described above. This suspension was then diluted with distilled water and the absorbance reading of the different concentrations of cell suspensions was measured at 620 nm on the spectrophotometer. The variation of absorbance with concentration of cell shows a linear relationship (Fig. 3.2).

The equation of this line is :-

$$a = \frac{b - 0.0106}{1.9175} \qquad 3.2$$

where a/mg cm^{-3} is the concentration of cells and b the absorbance reading.

(c) Growth and preparation of cells for the phosphatase activity assay.

The cells of Staph. aureus strain 1, used throughout the following experiments since they showed a high phosphatase activity, were grown on Standard Growth Medium for 18h. at the required temperature.

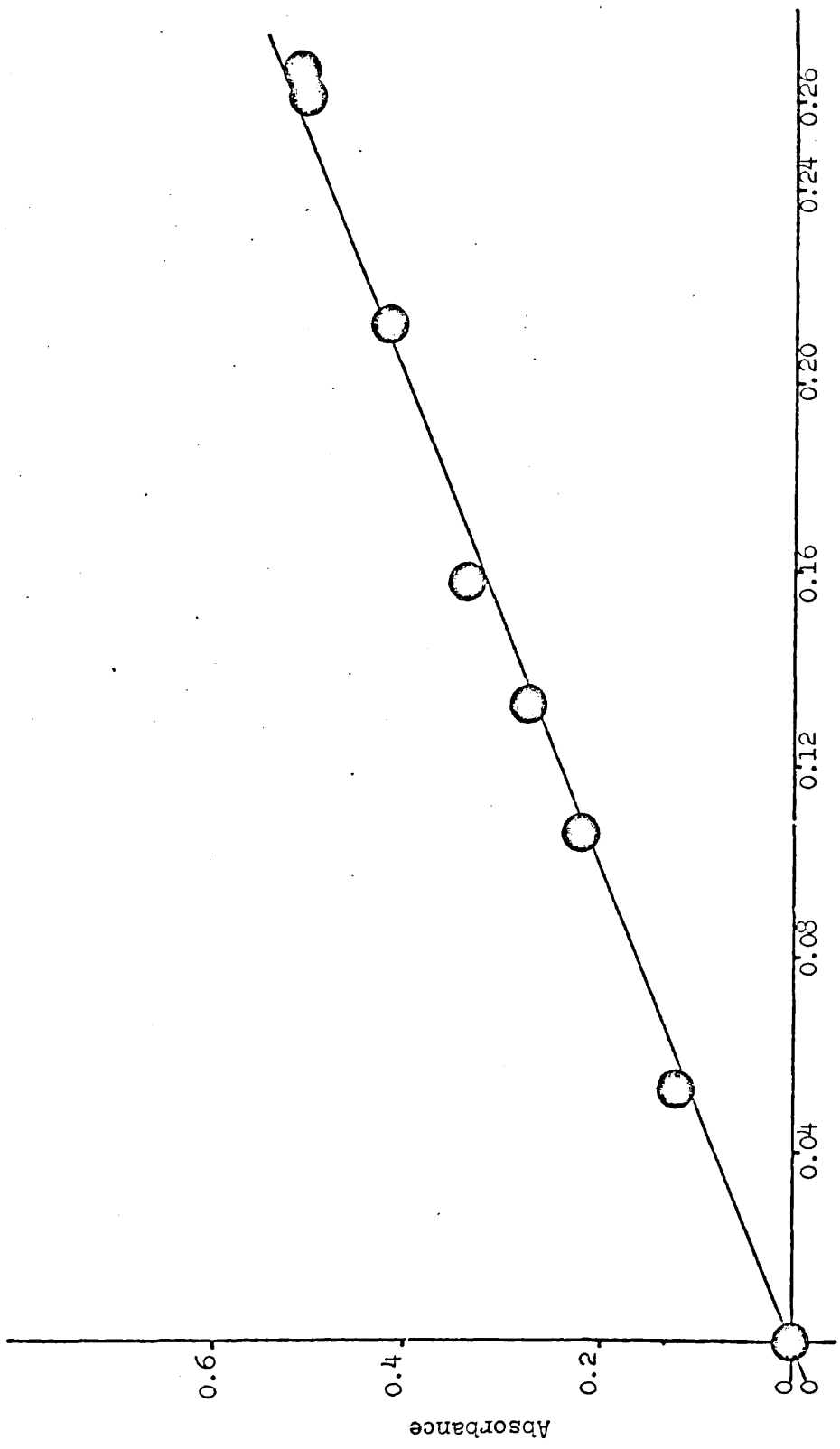


Fig. 3.2

Variation of absorbance with concentration of cell

The cells were harvested from the plates with distilled water and then washed once in water. A suspension was made up in distilled water and its opacity adjusted to give an absorbance reading in the range 0.45-0.50 when measured at 620 nm against a blank of distilled water. A 10.0 cm^3 sample of this suspension was measured accurately into a tube and centrifuged for 25-30 minutes to ensure complete separation of the cells from the supernatant, which was then decanted. The cells were resuspended in 10.0 cm^3 of the appropriate buffer solution. This suspension was put in a thermostat bath at 37°C and allowed to equilibrate for 10-15 minutes before the addition of the substrate solution to start the enzyme reaction.

(d) Buffer Solutions

The buffer solutions used for the enzyme assay are shown in Table 3.1

(e) Variation of the concentration of liberated 4-nitrophenol with time at different pH values

When the cell suspension had attained thermal equilibrium at 37°C, the enzyme reaction was started by the addition of 2.0 cm^3 disodium 4-nitrophenyl phosphate solution (12mg cm^{-3} distilled water). At suitable intervals, 2.0 cm^3 aliquots of the reaction mixture were withdrawn and added to 4.0 cm^3 of 0.20 mol dm^{-3} NaOH solution. The absorbance reading of each sample was measured at 400 nm against a blank taken at zero time.

The graphs (Fig. 3.3) do not pass through the origin because on addition of the substrate to the bacterial suspension, a reaction occurs causing a large amount of 4-nitrophenol to be liberated.

Table 3.1

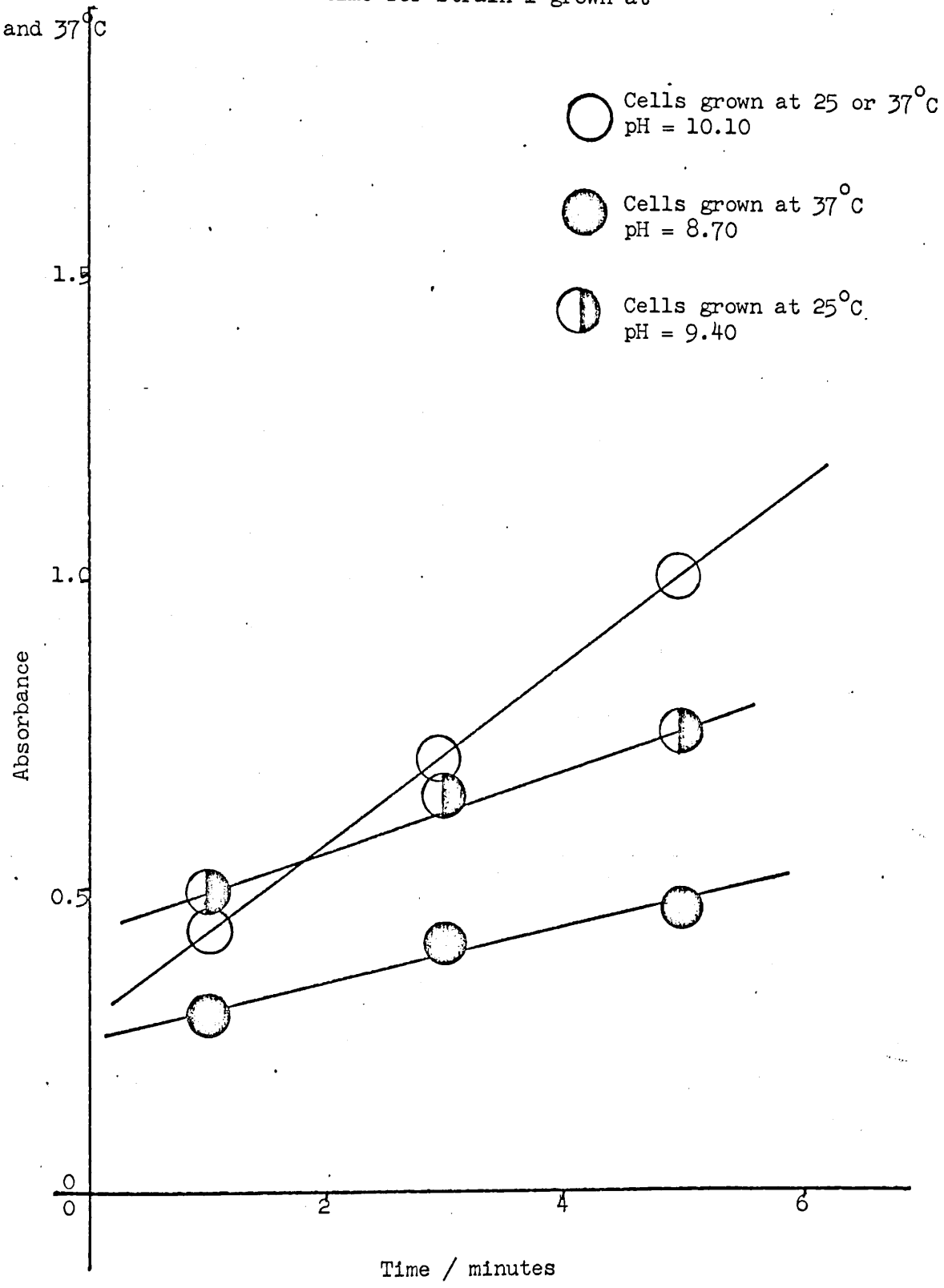
Buffer solutions for phosphatase activity assays

Buffer Solution			pH of buffer solution	pH at end of reaction
0.10 mol dm^{-3} AcOH	0.10 mol dm^{-3} Na OAc			
164 cm^3	36 cm^3		3.80	4.70
103 cm^3	97 cm^3		4.45	5.30
42 cm^3	158 cm^3		4.90	6.35
0.20 mol dm^{-3} Tris	0.10 mol dm^{-3} HCl	H_2O		
50 cm^3	91 cm^3	59 cm^3	7.10	7.85
50 cm^3	55.6 cm^3	94.4 cm^3	8.00	8.55
0.10 mol dm^{-3} Na_2CO_3	0.10 mol dm^{-3} $NaHCO_3$			
14 cm^3	186 cm^3		8.85	9.30
36 cm^3	164 cm^3		9.15	9.50
124 cm^3	76 cm^3		10.00	10.20
164 cm^3	36 cm^3		10.30	10.40
Approx. 10^{-3} mol dm^{-3} NaOH solution			11.70	11.10

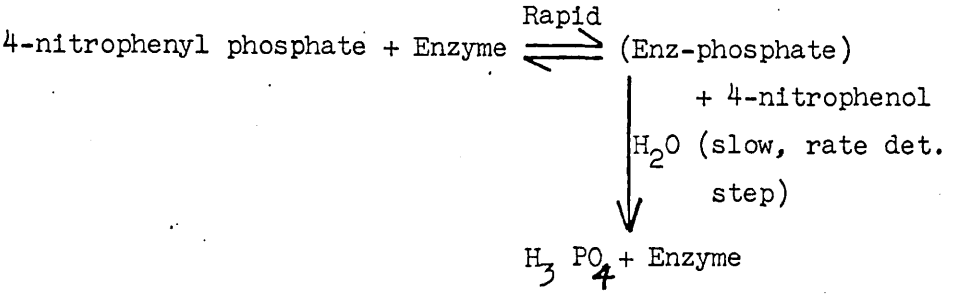
Tris = tris (hydroxymethyl) aminomethane

Fig. 3.3

Variation of absorbance with time for strain 1 grown at 25° and 37°C



After the initial burst of activity, the rate of liberation of 4-nitrophenol falls appreciably and a slower linear increase with time is attained. The intercept is the position where one molecule of 4-nitrophenol is equivalent to one molecule of enzyme. The reaction occurring is of the form :



From Fig. 3.3 it is seen that after an interval of 5 minutes some absorbance valves are greater than 0.90 and are, therefore, beyond the accuracy of the instrument; also, the Beer - Lambert Law is not valid in this region. For these reasons it was decided to calculate the activity from readings taken after intervals of 1 and 3 minutes from the start of the reaction.

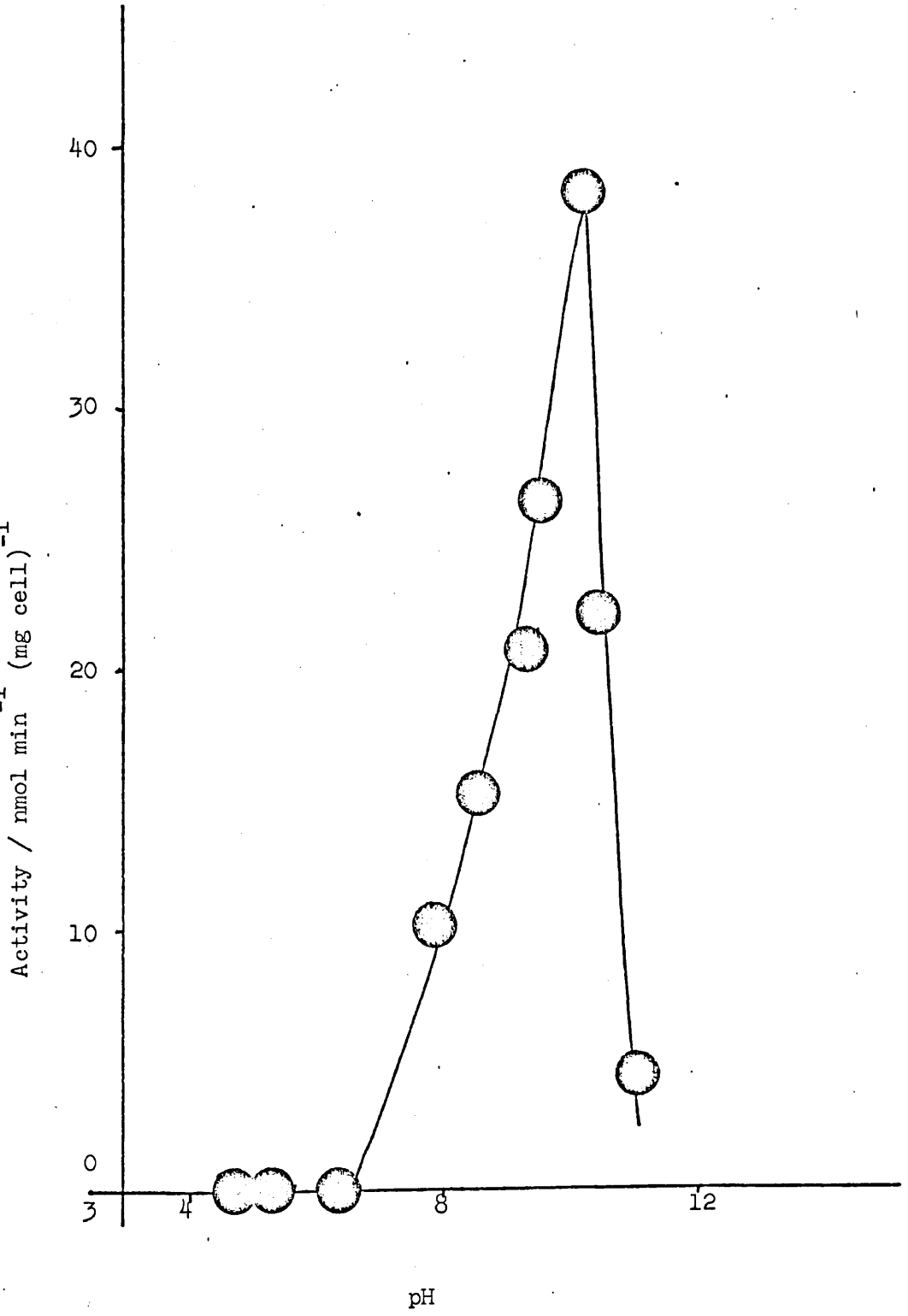
(f) Variation of activity with pH for cells of strain 1 grown for 18h at 37°C

The cells were prepared and the enzyme assays carried out as described previously over the pH range 4.0 - 11.0. The results are shown graphically in Fig. 3.4. The units of the enzyme activity are mol 4-nitrophenol min^{-1} $(mg\ cell)^{-1}$ (See 3.3).

The optimum pH lies in the range 10.00 - 10.20. It was also established that there was a linear relationship between the activity and enzyme concentration (i.e. concentration of cells).

Fig. 3.4

Variation of activity with pH for cells of strain 1 grown at 37°C for 18 h



(g) Variation of activity with concentration of substrate

Cells of strain 1 grown at 25°C for 18h were used. A bacterial suspension in the absorbance range 0.45 - 0.50 at 620 nm was prepared with a buffer solution of pH = 10.10; 10.0 cm^3 samples of this suspension were measured accurately into Universal bottles and thermostated at 37°C. A solution of 4-nitrophenyl disodium orthophosphate containing 3.0g in 100 cm^3 of buffer solution of pH = 10.10 was prepared. This solution was diluted using the same buffer solution to obtain concentrations in the range 0.6 to 3.0g in 100 cm^3. The enzyme reactions were carried out in the normal way and the results are shown in Fig. 3.5. The phosphatase activity remained constant for concentrations of substrate greater than 1.2g in 100 cm^3. A concentration of 2.0g in 100 cm^3 was selected for use in all subsequent experiments. Since the substrate is unstable at room temperature, the solid was kept in the refrigerator until required; made up in buffer solution pH = 10.10 and this solution kept in ice until required.

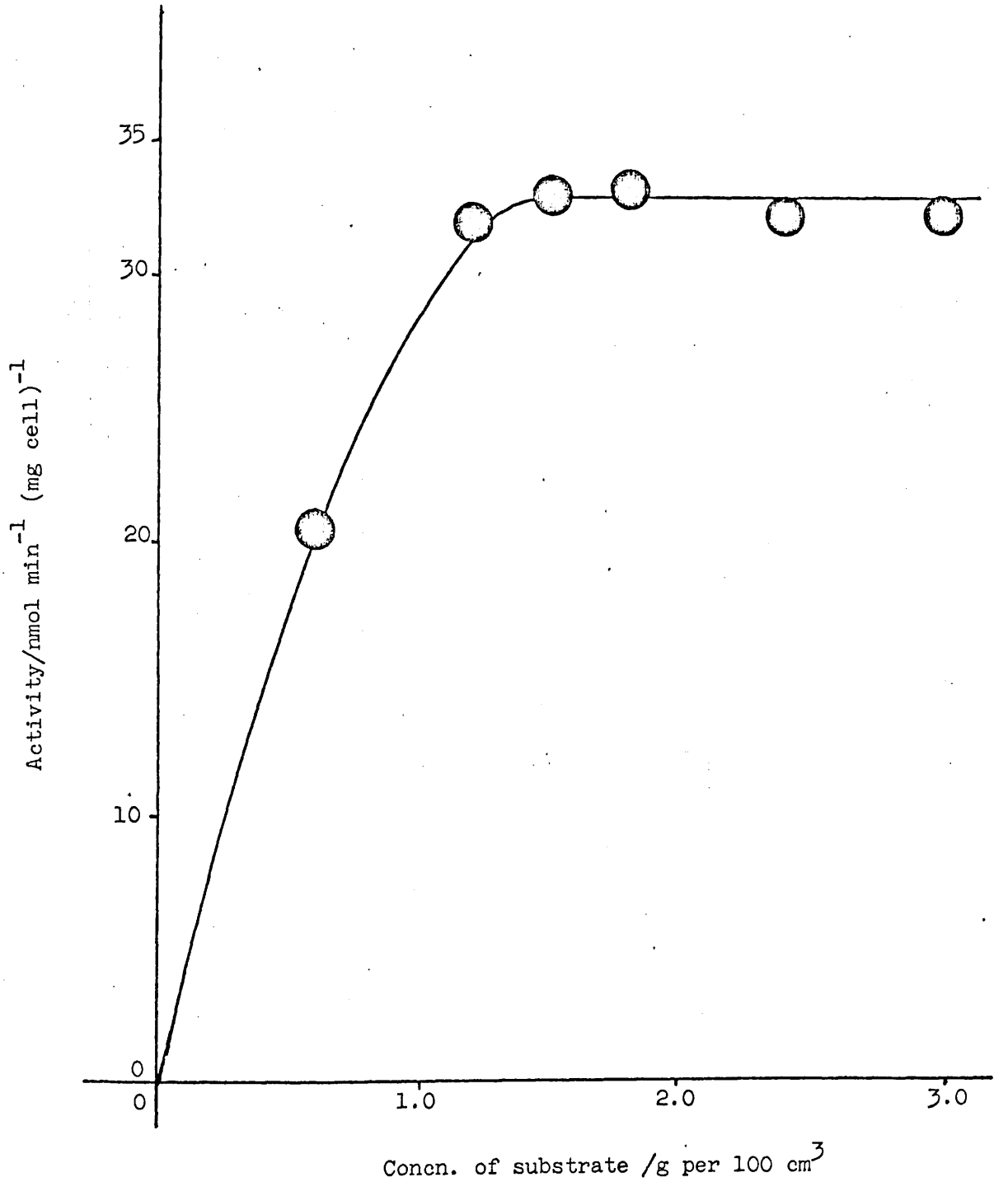
(h) Effect of the addition of magnesium ions to the reaction mixture

It has been shown by Engstrom (1961) and Garrahan et al (1969) that the presence of magnesium ions in the reaction mixture enhances the phosphatase activity.

A solution of magnesium chloride was prepared and different volumes of this solution were added to the suspension of cells before addition of the substrate to start the reactions. The reactions were carried out at pH = 10.20 and 37°C on cells of strain 1 grown at 25°, 37° and 43°C for 18h.

Fig. 3.5

Variation of activity with substrate concentration for strain 1 grown at 25°C for 18 h



The graphs (Fig. 3.6) show that there was a marked increase in activity for the cells grown at the three temperatures after the addition of a low concentration of $MgCl_2$. There was a plateau region at 0.2 - 0.7 mg cm^{-3} Mg^{++} ions in the reaction mixture. At higher concentrations, a precipitate was formed and this interfered with the measurement of absorbance.

Since the reactions occurred very rapidly on addition of magnesium ions, the concentration of the bacterial suspension was halved for all subsequent studies.

(i) Variation of activity with the temperature of reaction for cells of strain 1 grown at 25° and 37°C for 18 h

The cells were grown at 25° and 37°C for 18 h, and the enzyme activities measured at five different temperatures. The graphs of activity against temperature of reaction (Fig. 3.7) showed that the greatest activity occurred when the reaction was carried out at 37°C.

From a graph of $\log_{10}$ (activity) against T^{-1}, the ΔH value for the reaction (strain 1 grown at 37°C) was calculated using the Clausius Clapeyron equation in the form :

$$\frac{d \ln (\text{activity})}{dT} = \frac{\Delta H}{RT^2}$$

From the gradient of the graph in Fig. 3.8

$\Delta H = 16.3$ kJ mol^{-1}

Fig. 3.6

Variation of activity with concn. of Mg^{++} ions in the reaction mixture for strain 1 grown at 27°, 37° and 42°C

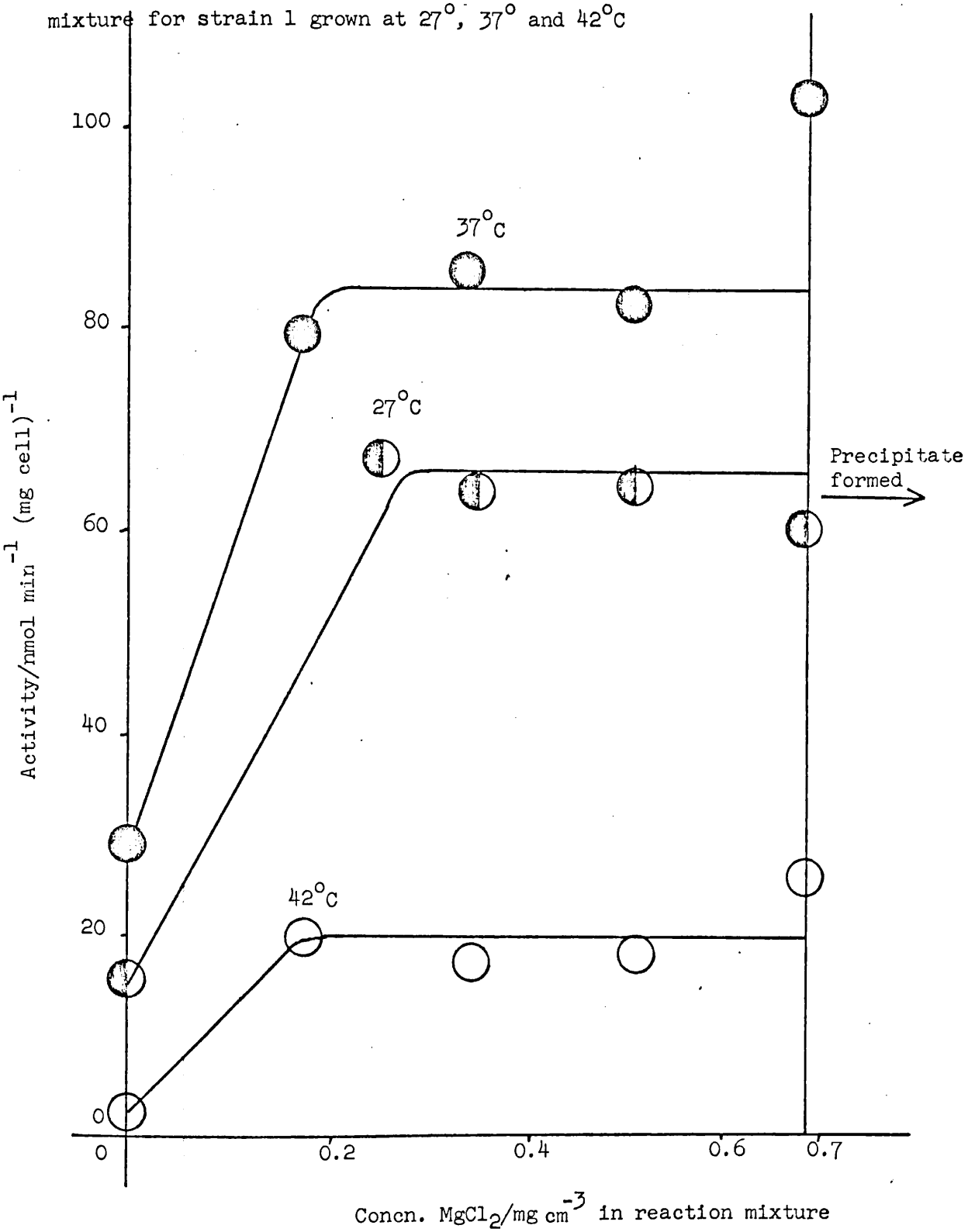


Fig. 3.7

Variation of activity with temperature of reaction for strain 1 grown at 25° and 37°C

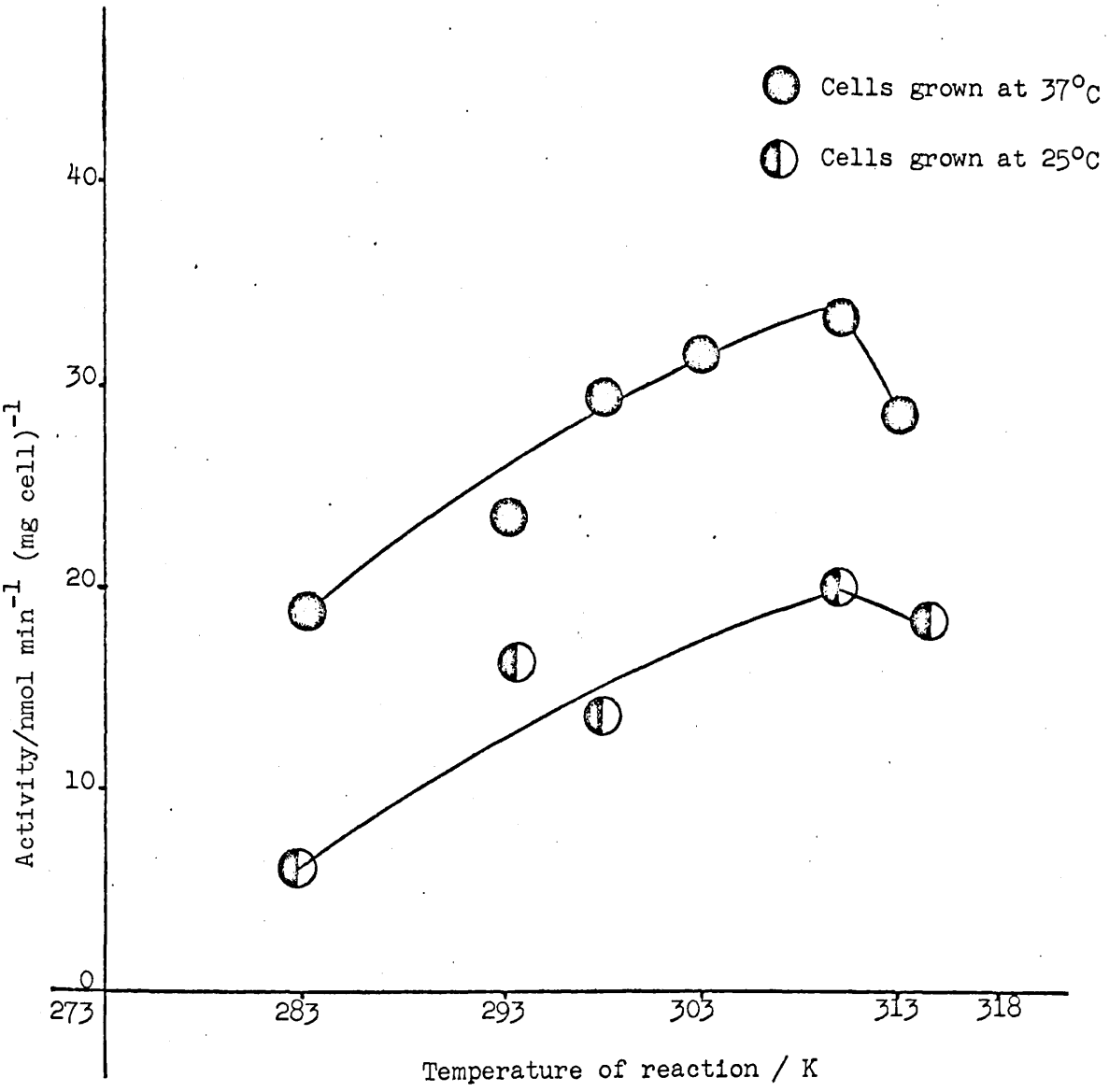
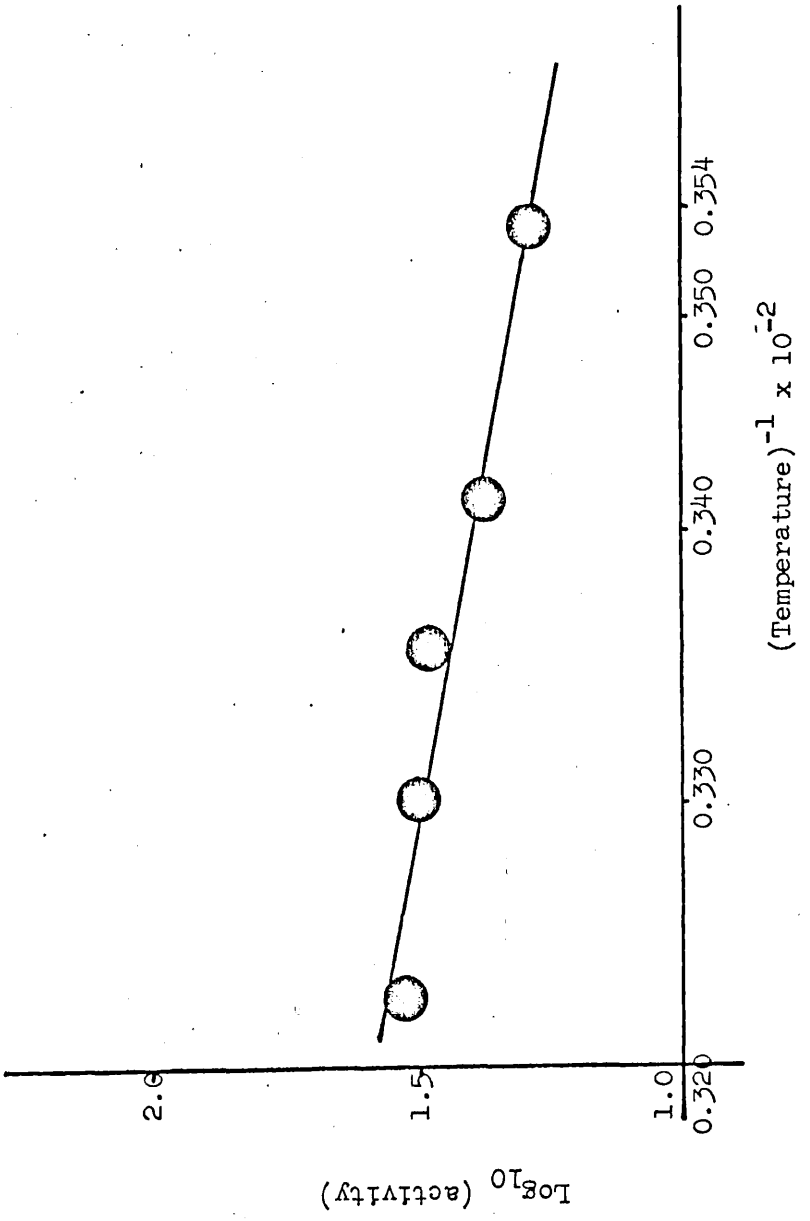


Fig. 3.8

Graph of $\log_{10}$ (activity) against (temperature)$^{-1}$ for strain 1 grown at 37°C



3.2 Reproducibility of Results

Analysis of replicate determinations on cells of the same strain grown and tested at different times revealed that the confidence limit of a single mean at P = 0.05 is ± 2%. Repeated washing of the cells had no effect on the phosphatase activity, suggesting that the enzyme is firmly located in the cell surface.

(a) Variation of activity with age for cells of strain 1 grown at 27°, 37° and 42°C

The cells were grown on a slope from the parent strain and then subcultured onto agar plates (standard medium) and incubated at 37°C for 18 h. These cells were then subcultured onto other plates and incubated at 27°, 37° and 42°C for 6, 24 and 48 h. (Batch A). Cells were also subcultured from the parent plates 6 and 9.5 h later than the first batch (Batches B and C respectively) and grown for 18 and 15 and 39 h respectively.

The phosphatase activity of the parent cells (grown at 37°C) and the cells grown for the different periods of time were measured at pH = 10.10 and 37°C.

It appears from the results (Table 3.2) that when grown at 37°C, the activities of batches subcultured at the same time were almost constant. Cells grown at 27°C showed an initial decrease in activity, increasing again after 24 h and decreasing after 48 h. The batches subcultured at 27°C, 6 and 9.5 h later showed a constant activity. At 42°C the activity decreased with age to zero activity.

Therefore, for a given parent inoculum, the activity of the daughter cells grown at either 27° or 37°C was independent of the age of the cells when harvested up to 48 h later.

Table 3.2

Variation of phosphatase activity with age of cells

Period of growth/h.		Activity/n mol min^{-1} (mg cell)$^{-1}$ at		
		27°C	37°C	42°C
Parent		-	55.5	-
6		5.1	39.0	2.3
24	Batch A	65.7	39.7	0
48		33.2	32.3	0
18	Batch B	32.1	27.7	0
15	Batch C	28.9	22.6	3.4
39		22.8	25.1	0

However, the activity of the daughter cells depended on the age of the cells used for inoculum.
Owing to the difficulty of establishing the exact age of cells grown on solid media no further study was made of this effect of age of the parent cells when subcultured on the activity of the daughter cells.

3.3 Summary of test conditions for the assay of alkaline phosphatase activity

To 10.0 cm^3 of bacterial suspension (0.1 - 0.15mg cm^3) in the appropriate buffer solution at 37°C were added 2.0 cm^3 of a freshly prepared substrate solution, made up in buffer solution, pH = 10.10, containing added magnesium chloride (20 mg cm^{-3} 4-nitrophenyl disodium orthophosphate, 0.25 mg cm^{-3} $MgCl_2$). A 2.0 cm^3 sample was removed after 0, 1 and 3 minutes and mixed with 4.0 cm^3 of 0.20 mol dm^{-3} NaOH solution. The absorbance of each solution was measured at 400 nm against the blank removed at zero time. From the initial slope of the absorbance - time plot and the known dry weight of cells, the activity of the enzyme was expressed as mol of 4 - nitrophenol formed per minute per mg dry weight of cells (mol min^{-1} (mg $cell^{-1}$), using equation 3.3 :

$$\text{Activity} = \frac{(A_3 - A_1) - 0.0108}{0.0222} \times \frac{1}{\text{dry wt. of cell}}$$

Where A_3 and A_1 are the absorbance readings after 3 and 1 minutes respectively.

CHAPTER FOUR

EFFECT OF GROWTH MEDIA ON THE PHOSPHATASE ACTIVITY OF CELLS OF METHICILLIN RESISTANT STRAIN 1

4.1 Loss of Activity

During the course of these studies, the original batch of Oxoid nutrient agar became depleted and was replaced by a later batch of "allegedly identical medium". The phosphatase activity of 18h. cells of strain 1 grown on this medium was zero. According to the manufacturers, the preparation of the medium had been changed between the two batches; the practice of treating the medium with calcium hydroxide to precipitate out inorganic phosphate has now been discontinued. It was, therefore, necessary to find another type of nutrient agar medium which did not have this repressive effect on the phosphatase enzyme system. Enzyme assays were carried out on cells grown on different types of commercially available and synthetic media. Table 4.1 lists some of the different growth media used.

(a) Variation of phosphatase activity of cells grown on different types of commercially available media

Cells of strain 1 grown once at 37°C for 18h on a plate of "Standard" medium A, were inoculated in parallel onto plates of medium A and various other Oxoid media and a Difco medium and incubated at 37°C for 18h. The cells were harvested (3.1 (c)) and the enzyme assays were carried out at pH = 10.10 and 37°C (3.3). The results (Table 4.2) are presented as a percentage of the activity of the same cells grown in parallel on medium A; this method was necessary to allow for the variation of the age of the parent cells used for the inoculum.

Table 4.1

Commercial and treated growth media used.

Growth medium	Batch No.	Code in text
Oxoid CM3 Agar	273 8762 (Standard)	A
Oxoid CM1 Broth	297 14154	B
Oxoid CM1 Broth treated with $Ca(OH)_2$	297 14154	C
Oxoid CM3 Agar	272 11382	D
Oxoid CM3 Agar	152 13727	E
Oxoid CM1 Broth Solidified with agar*	297 14154	F
Oxoid CM1 Broth treated with $Ca(OH)_2$ for 20 minutes, solidified with agar*	297 14154	G
Oxoid CM1 Broth treated with $Ca(OH)_2$ for 20 minutes + 10^{-2} mol dm^{-3} inosine + agar*	297 14154	H
Difco Agar	55463	J

* The agar used to solidify liquid media was Oxoid No.1 agar.

Table 4.2

Relative phosphatase activity of cells of Staph. aureus strain 1 grown on commercially available media.

Growth medium	Cell growth	Phosphatase activity (relative to standard cells)
A	+ + +	100
D	+ + +	0
E	+ + +	0
E diluted x 2 + agar to solidify	+ + +	0
F	+ + +	0
J	+ + +	0

The medium E was prepared at half the recommended concentration to establish whether the concentration of any repressive factor(s) could be diluted sufficiently so that the cells could exhibit phosphatase activity.
Although the growth of the organisms was good on each type of medium, there was no observable phosphatase activity at all.

(b) Variation of the activity of cells grown on some synthetic grown media

A semisynthetic growth medium containing a low concentration of inorganic phosphate, yeast, peptone and sodium chloride (Haest et al, 1972) was prepared and solidified with 1% Oxoid No. 1 agar. A similar medium was prepared in which the inorganic phosphate was replaced by tris (hydroxymethyl) aminomethane.

Two fully synthetic media were prepared according to the details of Garen and Levinthal (1960) and Evans (1948). The cells were grown once on each of these media for 18h at 37°C and the enzyme was assayed in the usual way.

Table 4.3

Phosphatase activity of cells grown on synthetic media

Growth medium	Cell growth	Phosphatase activity (relative to cells grown on Medium A,%)
Synthetic medium (Garen & Levinthal, 1960)	+ -	0
Synthetic medium (Evans, 1948)	+ -	0
Semisynthetic medium (low concn. phosphate)	+ + +	0
Semisynthetic medium (phosphate replaced by Tris)	+ + +	0

The synthetic media were unsuitable because the cell growth was very poor (Table 4.3). Although the growth on the semisynthetic media was comparable to that on Medium A, there was no detectable phosphatase activity in the cell suspensions.

4.2 Investigation of the Inorganic Phosphate Content of the Commercial Media

Shah and Blobel (1967) have shown that Staph. aureus can produce an alkaline phosphatase, the formation of which is repressed by the presence of excess inorganic phosphate in the growth medium. The results obtained suggest that an excess of inorganic phosphate in the medium could be acting as a repressor to the formation of the alkaline phosphatase enzyme system.

(a) Determination of the inorganic phosphate content of some of the media used.

To confirm the effect of the presence of inorganic phosphate in the growth medium on the phosphatase activity of the cells, a quantitative assay of the inorganic phosphate content was carried out (2.2) Table 4.4 shows that some of the media do contain a large amount of inorganic phosphate. This phosphate is obviously exerting a repressing effect on the alkaline phosphatase enzyme system, in agreement with the conclusions of Shah and Blobel (1967).

(b) Addition of inorganic phosphate to Medium A

To confirm that the presence of excess inorganic phosphate in the growth medium represses the formation of the phosphatase enzyme system, known concentrations of ammonium dihydrogen orthophosphate were added to Medium A and the pH of the resulting medium was adjusted as necessary to pH = 7.0 The cells of strain 1 were grown once on each of the media containing added phosphate before the enzyme was assayed.

Table 4.4

Concentration of free inorganic phosphate present in the various media and its effect on the alkaline phosphatase activity of cells of Staph. aureus strain 1.

Growth medium	Concn. inorganic phosphate/μgcm-3	Phosphatase activity/%
A	10	100
B	58	0
C	4	6
D	27	0
E	35	0
F	58	0
G ($Ca(OH)_2$ treated)	7	57
H ($Ca(OH)_2$ treated + inosine)	7	80

Table 4.5

Phosphatase activity of cells of Staph. aureus strain 1 after growth on Standard Medium A containing added inorganic phosphate

Concn. of added ammonium phosphate / mol dm^{-3}	Actual concn. free inorganic phosphate in medium / mol dm^{-3}	Phosphatase activity (relative to cells grown on standard Medium A)
0 (Control cells)	10^{-4}	100
10^{-7}	10^{-4}	93
5×10^{-7}	10^{-4}	91
10^{-6}	10^{-4}	127
5×10^{-6}	10^{-4}	103
10^{-5}	1.1×10^{-4}	95
5×10^{-5}	1.5×10^{-4}	65
10^{-4}	2.0×10^{-4}	4
10^{-3}	1.1×10^{-3}	0
10^{-2}	1.01×10^{-2}	0

The presence of a low concentration of inorganic phosphate is necessary for good growth and high phosphatase activity (Table 4.5). An added concentration of phosphate greater than 5×10^{-6} mol dm^{-3} has a repressive effect on the formation of the phosphatase enzyme system.

(c) Preparation of growth media from commercial media by the precipitation of inorganic phosphate

13g of Oxoid CM1 Broth was dissolved in 100 cm^3 distilled water. A suspension of 10% calcium hydroxide was added to the broth solution to give a pH of 9.5 - 10.0 (4.7 cm^3 $Ca(OH)_2$ suspension). The resulting suspension was left for defined periods of time and the precipitate was rapidly filtered off on Whatman No. 5 filter paper.

The pH of the supernatant was adjusted to 7.0 by the addition of 3.0 cm^3 dilute hydrochloric acid. The solution was made up to 1 dm^3 and 10g Oxoid No. 1 agar added. The growth medium was autoclaved for 15 minutes at 15 lb. per sq. in.
Cells of strain 1 were subcultured onto this medium for enzyme assay.

Table 4.6

Variation of phosphatase activity with time of contact of broth with calcium hydroxide.

Time of contact with $Ca(OH)_2$ / minutes	Activity relative to standard cells /%
0	0
5	2
10	26
15	29
20	57
30	19

The results (Table 4.6) show that the time of contact of the calcium hydroxide with the broth is an important factor. The cell growth on all the different preparations was very good, but the maximum enzyme activity was exhibited by cells grown on the media obtained after 20 minutes contact of broth with calcium hydroxide.

Thus the removal of inorganic phosphate by precipitation as calcium phosphate improved the phosphatase activity considerably but the activity was always less than that of cells grown on medium A. It was considered possible that the precipitate was removing some other essential component from the growth medium.

Varying concentrations of magnesium and iron III salts were added to the growth medium but this did not improve the activity.

Since this medium gave the best results for the phosphatase activity and showed no adverse effects on the cell surfaces (6.1), it was decided that further batches of nutrient medium would be prepared in this way, leaving the calcium hydroxide in contact with the broth for 20 minutes before filtering off the precipitate (medium G)

The parent cultures and all cells required for phosphatase activity measurements were grown on medium A. Cells were also grown on medium G for electrophoretic measurements.

(d) Effect of pH of the growth medium on the phosphatase activity

Medium G was prepared in the normal way except that different volumes of dilute hydrochloric acid were added to give media with pH values in the range 5.0 - 10.0. The pH of each batch was measured before autoclaving. At lower pH values (4.0) the agar would not solidify. Cells of strain 1 were grown on each batch of the media at 37°C for 18h for alkaline phosphatase assay.

Table 4.7

Variation of phosphatase activity with the pH of the growth medium.

pH of growth medium	Activity/n mol min^{-1} $(mg\ cell)^{-1}$
9.70	8.8
8.80	21.2
8.00	39.3
7.50	32.0
6.90	52.2
6.60	55.5
5.55	43.1

The greatest activity occurred when the pH of the growth medium was in the region pH = 6.60 - 6.90. Extremes of acidity or alkilinity resulted in a repression of the enzyme system (Table 4.7).

(e) Addition of inosine to the growth medium

Bowne et al (1966) have shown that the ribonucleoside inosine is an effective phosphate acceptor. Csopak et al (1972) observed that although the growth of cells of E. coli was independent of the presence or absence of inosine in the growth medium, there was an increase of 10% in the alkaline phosphatase enzyme production when the cells were grown in the presence of inosine.

Varying concentrations of inosine were added to media A, E and G and cells of strain 1 were grown on each batch for the enzyme assay.

Table 4.8

Effect of varying concentrations of inosine in the growth medium on the alkaline phosphatase activity.

Growth medium	Concentration of inosine/mol dm^{-3}	Activity relative to standard cells /%
A	10^{-4}	128
E	0	0
	10^{-4}	0
	5×10^{-3}	33
	10^{-2}	86
	5×10^{-2}	82
	10^{-1}	49
G	0	57
	10^{-4}	53
	5×10^{-4}	56
	10^{-3}	50
	5×10^{-3}	25
	10^{-2}	80
	2×10^{-2}	68
	5×10^{-2}	44

There was a slight increase in activity for cells grown on medium A containing 10^{-4} mol dm^{-3} inosine but this concentration had no effect on the activity exhibited by cells grown on the other two media (Table 4.8).
Good growth was observed on all media containing the different concentrations of inosine but the greatest enzyme activity was exhibited by cells grown on media E and G containing a concentration of 10^{-2} mol dm^{-3} inosine. Since the inorganic phosphate has been precipitated out of medium G using $Ca(OH)_2$, these results suggest that inosine may have some effect other than the removal of inorganic phosphate from the medium.
The effect of inosine on the surface properties of the cells was investigated using micro-electrophoretic techniques (6.3).

4.3 Phosphatase Activity of Cells Grown in Liquid Media

Cells of methicillin resistant strain 1 were grown in parallel on plates of medium A and in each of three batches of broth : B, C and Oxoid No. 2 broth for 18h. at 37°C. The phosphatase activity of 18h. cells was measured in the usual way (3.3).

Table 4.9
Phosphatase activity of cells of Staph. aureus strain 1 grown in broth for 18h. at 37°C.

Growth medium	Cell growth	Activity relative to cells grown on standard medium /%
A	+ + +	100
B	+ +	0
C	+	6
Oxoid Nutrient Broth No. 2 (Batch No. 272 13986)	+ + +	5

Compared to the activity obtained for cells grown on medium A, the values obtained for the activity of cells grown in broth are negligible (Table 4.9).

Although the liquid media contained varying concentrations of inorganic phosphate (Table 4.4) the phosphatase activity of the cells grown in these media was very low.

4.4 Summary

(a) It has been established that certain growth conditions can repress the formation of the alkaline phosphatase enzyme system in methicillin resistant cells of Staph. aureus under investigation.

(b) Phosphatase activity of cells grown on solid media was dependent on the concentration of inorganic phosphate present in the media. A high concentration of inorganic phosphate had a repressive effect on the enzyme system where as a low concentration of phosphate was required for good growth and high phosphatase activity (4.2 (b)). The batches of commercial media tested contained large amounts of inorganic phosphate (Table 4.4).

(c) Growth media prepared from commercial media by precipitation of inorganic phosphate using $Ca(OH)_2$ gave good growth and a much improved phosphatase activity.

(d) Cells grown in the presence of a nucleoside, inosine, exhibited a very high phosphatase activity, regardless of the initial concentration of inorganic phosphate present in the medium (Table 4.8).

(e) The highest enzyme activity was shown by cells grown on media containing small amounts of inorganic phosphate and maintained between pH 6.60 and 6.90.

(f) Cells grown in liquid medium exhibited no phosphatase activity - this is due to the state of the media rather than to the presence of excess phosphate.

CHAPTER FIVE

PHOSPHATASE ACTIVITY OF CELLS OF STAPH. AUREUS

5.1 Variation of the Phosphatase Activity of Cells of Strain 1 (Methicillin Resistant)

(a) Effect of temperature of growth

The cells were grown once for 18h. on plates at 27°, 37° and 42°C and the phosphatase activity of cells grown at each temperature was measured over a range of pH values at 37°C.

The phosphatase activity of the cells grown at 27° and 37° were the same at all pH values (Fig. 5.1). There was no detectable activity for cells grown at 42°C. Recent work has shown that cells of a methicillin-resistant strain have varying amounts of surface teichoic acid, depending on the temperature of growth; cells grown at 43°C have surface teichoic acid, less at 37° and none at 25°C (Hill and James, 1972 a). It was concluded that these results were consistent with the presence of a temperature-dependent enzyme system, previously suggested by Annear (1968) to explain methicillin resistance in Staph. aureus.

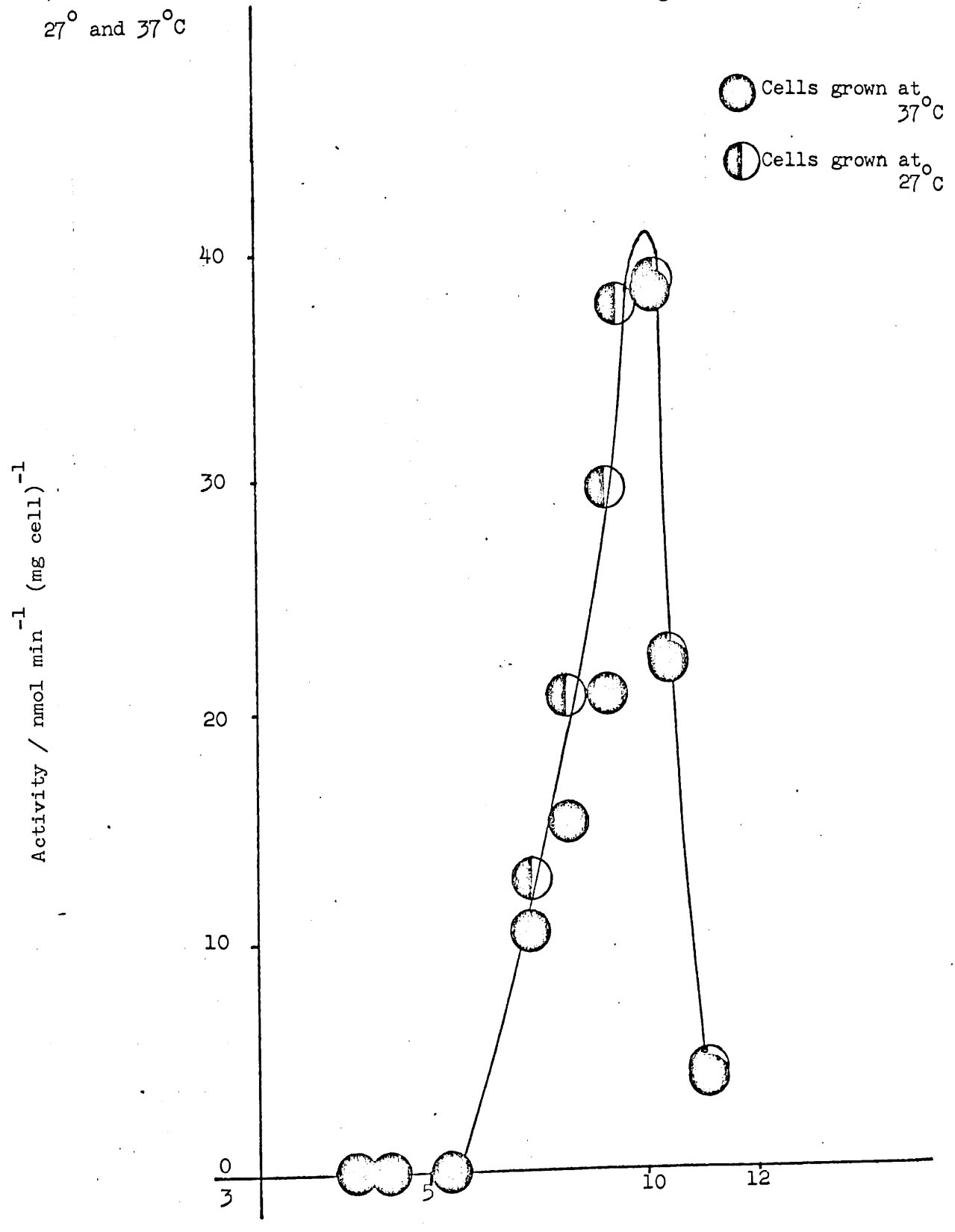
These differences found in the phosphatase activity of cells of a methicillin resistant strain grown at different temperatures could, therefore, be an indication of the amount of teichoic acid on the cell surface.

(b) Effect of repeated subculture at 27°, 37° and 42°C

The cells were repeatedly subcultured on plates at each of the three temperatures and the phosphatase activity of the cells was measured at pH = 10.10 after different numbers of subcultures.

Fig. 5.1

Variation of activity with pH for cells of strain 1 grown at 27° and 37°C



There was no activity at all for the cells grown at 42°C. The activity of the cells grown at 27° and 37°C remained constant throughout the training series at the different temperatures.

It has been shown (Sutherland and Rolinson, 1964) that cells of a naturally occurring methicillin resistant strain of Staph. aureus show a heterogeneous response to methicillin when grown at relatively high temperatures, i.e. 37°C. These cells consist of mixed populations in which the vast majority are sensitive to methicillin with a slow growing minority showing very high resistance to the antibiotic. Growth at extremes of temperature produces cells which show a totally different response to the antibiotic; on incubation at 25°C the cells of a methicillin resistant strain are highly resistant. On incubation at 43°C, however, all the cells in the population appear to be very sensitive to even low concentrations of the antibiotic and after repeated growth at this higher temperature the cells become completely sensitive (Al Salihy and James, 1972).

(c) Effect of repeated washing of the cells

The cells were usually washed twice in distilled water before adjusting the opacity of the suspension. In order to determine whether or not the enzyme was firmly bound to the cell, the cells were thoroughly washed more times and the activity assayed after each additional washing. There was no change in the phosphatase activity after repeated washing and no activity was detected in the washing water. This strongly suggests that the enzyme is firmly bound to the cell.

(d) Effect of boiling the cells at 100°C

Cells of strain 1 were harvested after 18h. growth at 37°C and washed once in water. The cells were resuspended in water and this suspension was boiled at 100°C for 10-15 minutes. These cells were centrifuged and the phosphatase activity was measured in the usual way (3.3).
There was no detectable phosphatase activity for these cells. Thus the measured activity is caused by a heat labile enzyme and not by the cells themselves.

(e) Effect of "Aeration" during growth

Occasionally, due to some moisture inside the plates, some plates appeared to become more airtight than others.
The effect of "aeration" during growth was, therefore, briefly investigated.
Cells of strain 1 were subcultured onto 3 plates of medium A. One plate was left unsealed, one sealed with "parafilm", and the third arranged so that the top fitted very loosely, in this way the cells received a greater amount of air during growth.
The cells grown on the sealed plate were very easy to harvest where as those grown on the open plate were more difficult to harvest. This was probably due to slime formation by cells grown on the open plate.

Table 5.1

Effect of "aeration" during growth on the phosphatase activity of cells of strain 1

Condition of growth	Activity /n mol min^{-1} $(mg\ cell)^{-1}$
unsealed (normal)	77.8
sealed	87.7
open	85.3

There was no significant difference between the activities of the cells grown on an "open", sealed or unsealed plate (Table 5.1). Thus "aeration" during growth has no marked effect on the phosphatase activity.

(f) Effect of the addition of inorganic phosphate to the assay mixture

Varying amounts of ammonium dihydrogen orthophosphate were added to the washed bacterial suspensions immediately before the addition of the substrate to start the reactions.
The final concentration of inorganic phosphate was in the range 10^{-3} - 10^{-5} mol dm $^{-3}$. Because of possible precipitation effects due to the phosphate, no magnesium chloride was added to the substrate solution. The pH of each reaction mixture was measured at the end of the reaction.
No change in activity was found after the addition of fairly low concentrations of inorganic phosphate (0 - 10^{-3} mol dm^{-3}). Higher concentrations of phosphate had the effect of lowering the pH values. It was, therefore, concluded that the addition of inorganic phosphate to the reaction mixture caused no significant change in the phosphatase activity of the cells, i.e. inorganic phosphate is not an enzyme inhibitor.

(g) The addition of different concentrations of methicillin to the assay mixture

Methicillin was added to the bacterial suspensions, to give concentrations in the range 0 - 200 μg cm^{-3}, immediately before the addition of the substrate solution.
The phosphatase activity was measured in the usual way (3.3) at pH = 10.10 and 37°C.
There was no change in the phosphatase activity of the cells of strain 1 even in the presence of the highest concentration of methicillin.

The experiment was repeated but now the methicillin was left in contact with the cells for 10-15 minutes before the start of the reaction, again there was no change in activity. Thus, methicillin is not an enzyme inhibitor.

(h) The addition of inosine to the assay mixture

Inosine was added to the assay mixture, to give a final concentration of 10^{-2} mol dm^{-3}, immediately before the addition of the substrate solution. The phosphatase activity was measured at pH = 10.10 and 37°C.
The activity of cells in the presence of inosine was slightly less than that of the control cells (from 39.0 to 34.7 nmol min^{-1} $(mg\ cell)^{-1}$). This change was, however, negligible compared to the increase in phosphatase activity when inosine was added to the growth medium (4.2 e).

(i) Selection of methicillin - resistant mutants at 37°C

Cells of methicillin resistant strain 1 were subcultured in parallel on plates of medium A containing varying concentrations of methicillin (0 - 100 $\mu g\ cm^{-3}$). After 18 h. growth at 37°C, the colonies which had grown on each plate were picked off and subcultured onto plates of medium A containing no methicillin and incubated at 37°C for 18 h. The cells on these plates were harvested and their phosphatase activity measured.

Table 5.2

Phosphatase activity of cells of Staph. aureus strain 1 after growth and selection in the presence of different concentrations of methicillin.

Concn. methicillin in plate/μg cm^{-3}	Phosphatase activity/ n mol min^{-1} (mg cell)$^{-1}$
0	54.90
5	71.5
10	56.2
20	55.0
50	36.7
100	52.6
Average	54.5

All cells selected from such a heterogeneous culture produce cultures in which the cells have very similar phosphatase activities (Table 5.2), irrespective of the individual resistance to methicillin. This is in marked contrast to the surface properties of the mutants. Hill and James (1972 b) obtained a spectrum of pH - mobility curves for cells selected in this way, ranging from the curve typical of a naturally occurring resistant strain grown at 37°C to a curve characteristic of a simple carboxyl type surface for cells isolated from a 100 μg cm^{-3} methicillin plate.

It is, therefore, apparent that although the cells exhibit a range of surface properties depending on the concentration of antibiotic on which they were originally isolated, they all possess the same phosphatase activity independent of the antibiotic concentration on which they were selected.

(j) Phosphatase activity of variants of strain 1

From the methicillin resistant, penicillinase - producing cells of strain 1, cells of variant strains were selected, depending on their ability to produce penicillinase and their resistance or sensitivity to methicillin.
Cells of these four variants were grown on plates of medium G for 18h. at 37°C and their phosphatase activity was measured at pH = 10.10 and 37°C.

Table 5.3

Phosphatase activity of variants of strain 1 grown on medium G for 18h at 37°C.

Methicillin	Penicillinase	Phosphatase activity/ n mol min^{-1} $(mg\ cell)^{-1}$
Resistant	-	59.1
Resistant	+	29.1
Sensitive	-	15.4
Sensitive	+	0

The results are shown in Table 5.3. Although there was a considerable difference in the phosphatase activity of the variants it is apparent that the methicillin resistant cells exhibit a much greater phosphatase activity than that shown by the sensitive cells. Further the presence of the penicillinase marker seems to have a depressant effect on the phosphatase activity of the cells, independent of their methicillin sensitivity. These results do not present a clear cut story on their own; however, taken in conjunction with the surface properties of the 4 variants (Al Salihy, 1974, personal communication) it is apparent that the dominant factor in deciding the properties of the cells is their resistance to methicillin.

5.2 Phosphatase Activity of Cells of other Methicillin Resistant Strains

Strain 1 which was used for all the previous studies was resistant to methicillin; it was also a penicillinase producer. Cells of other strains (4, 7 and 9) with mulitple antibiotic resistance were grown on medium A at 27°, 37° and 42°C and the phosphatase activity of the various cells was measured at pH 10.10 and at 37°C.

Table 5.4

Phosphatase activity of cells of methicillin resistant strains of Staph. aureus (MIC > 200μg cm^{-3} at 37°C) grown on medium A

Strain	Phosphatase activity at pH 10.10 and 37°C/n mol min^{-1} $(mg\ cell)^{-1}$		
Growth temperature :	27°C	37°C	42°C
1	38.9	38.4	0
4	44.0	45.2	5.1
7	30.2	39.5	4.7
9	-	5.9	-

Cells of strains 4 and 7 had similar phosphatase activity to cells of strain 1 when grown at all temperatures (Table 5.4). Cells of strain 9 (MIC.$>$ 200μg cm^{-3}) exhibited a much lower phosphatase activity than that of cells of the other resistant strains (15% of the activity of cells of strain 1 grown at 37°C); cells of this strain also exhibited anomalous surface properties (6.2).

Thus it appears that all methicillin resistant strains exhibit phosphatase activity, irrespective of their other antibiotic characteristics.

5.3 Phosphatase Activity of Cells of Methicillin Sensitive Strains

Cells of strains 2, 5, 3 and 8 were grown on medium A for 18h. at the three temperatures and the activity was measured at 37°C and at pH = 10.10.

There was no detectable activity for cells grown at any temperature or tested at any pH value for strains 2, 5 and 3.

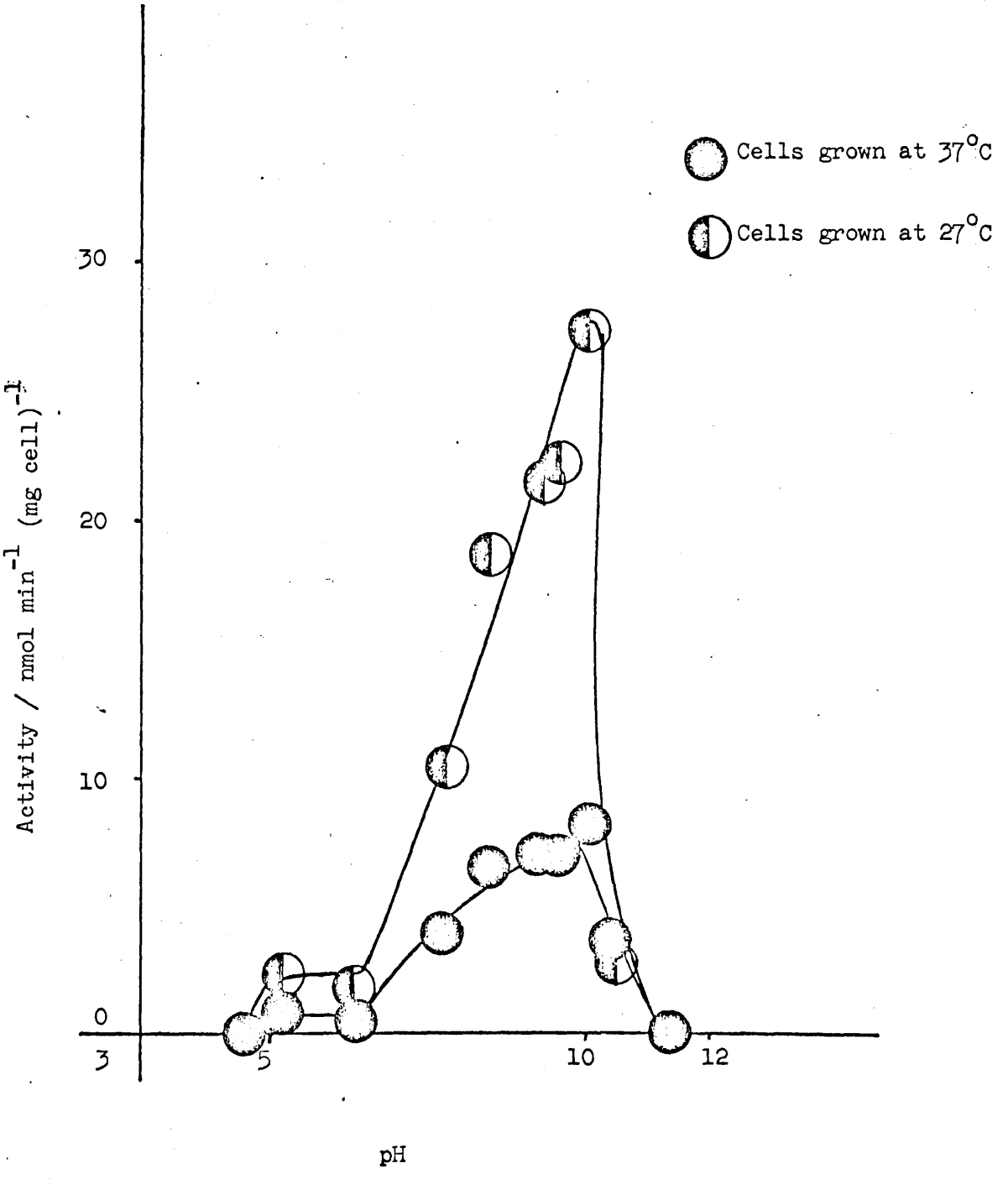
The strain of Staph. aureus, Oxford, strain 8, showed anomalous behaviour in that it is a methicillin sensitive strain which exhibited alkaline phosphatase activity when grown at 27° and 37°C but none at 42°C (Fig. 5.2). Cells of this strain are known to be anomalous in many other respects (Marshall, 1969; Hugo and Stretton, 1966).

Although strains 2, 5 and 3 are sensitive to methicillin, strain 3 is resistant to penicillin and is a penicillinase producer.

These results indicate that methicillin resistance is a determining factor for cells of Staph. aureus to exhibit phosphatase activity.

Fig. 5.2

Variation of activity with pH for cells of strain 8 grown at 27° and 37°C



5.4 Phosphatase Activity of Sensitive Strains After "Training" to Methicillin

Cells of strains 2 and 3 were "trained" to become resistant to methicillin (2.1c). When the MIC. was greater than 200 $\mu g\ cm^{-3}$, the phosphatase activity of the cells was measured.
There was no phosphatase activity for these cells even though they were highly resistant.
This is yet another example of different properties exhibited by cells with natural resistance to methicillin and those with induced resistance to methicillin. This suggests that resistance to methicillin can be acquired by at least two alternative mechanisms.

5.5 Summary

(a) Cells of naturally occurring methicillin resistant strains of Staph. aureus exhibit, alkaline phosphatase activity where as methicillin sensitive cells do not.

(b) The production of the phosphatase enzyme system exhibited by resistant cells is temperature dependent i.e. cells grown at 27° and 37°C have a high phosphatase activity where as cells grown at 42°C have a very low activity.

(c) Phosphatase activity is only exhibited by viable cells and the enzyme system appears to be firmly bound to the cell.

(d) The phosphatase enzyme system is not inhibited by the presence of inorganic phosphate, methicillin or inosine in the assay medium.

(e) Methicillin - resistant cells obtained by "training" the cells to become resistant to the antibiotic exhibited no phosphatase activity, thus suggesting that there may be alternative mechanisms for methicillin resistance.

(f) All cells which are resistant to methicillin exhibit the same phosphatase activity irrespective of their individual antibiotic resistance.

(g) The dominant factor in deciding the properties of cells is their resistance to methicillin.

CHAPTER SIX

THE SURFACE PROPERTIES OF CELLS OF *STAPHYLOCOCCUS AUREUS*

Valuable information about the nature of surface ionogenic groups on cells of a fixed age may be obtained by measuring the electrophoretic mobility of cells in suspension at different pH values whilst the ionic strength is maintained at a constant value.

6.1 Effect of Different Growth Media on the pH - Mobility Curves of Resistant and Sensitive Strains of Staph. aureus

(a). Cells of strain 1

Cells of strain 1 (resistant to methicillin) were grown on media A, E and G for 18h. at 37°C and their mobility values were measured in suspension over the pH range 3.0 - 7.0 (at pH values less than 2.5 irreversible changes occur at the surface).

Figs. 6.1 and 6.2 show the typical shape and positioning of the pH - mobility curve of cells of the resistant strain of Staph. aureus grown at 37°C. The curve is characterised by a minimum mobility value at pH 4.5 (cf. Marshall and James, 1971; Hill and James, 1972 a), the maximum in the curve has been attributed to the presence of charged groups of the surface teichoic acid (James and Brewer, 1968).

As the pH is reduced below pH 6.0, the surface carboxyl groups will no longer be fully ionised and it was suggested that this change in the electrostatic environment results in a new conformation of the teichoic acid molecule, which exposed phosphate groups on the cell surface. At pH 4.0 - 5.0 these were fully negatively charged and so contributed to give an increased value of the surface charge. At lower pH values, the phosphate dissociation also decreased giving rise to a maximum in the pH - mobility curve.

The graphs (Figs 6.1 and 6.2) show that the difference in the concentration of inorganic phosphate present in the growth media has an effect on the negative charge on the cells at lower pH values; an increase in the phosphate

Fig. 6.1

pH - mobility curves of strain 1 grown on media A and G for 18 h at 37°C

Cells grown on medium A

Cells grown on medium G

Fig. 6.2

pH - mobility curves of strain 1 grown on media A and E

Cells grown on medium A

Cells grown on medium E

Fig. 6.1

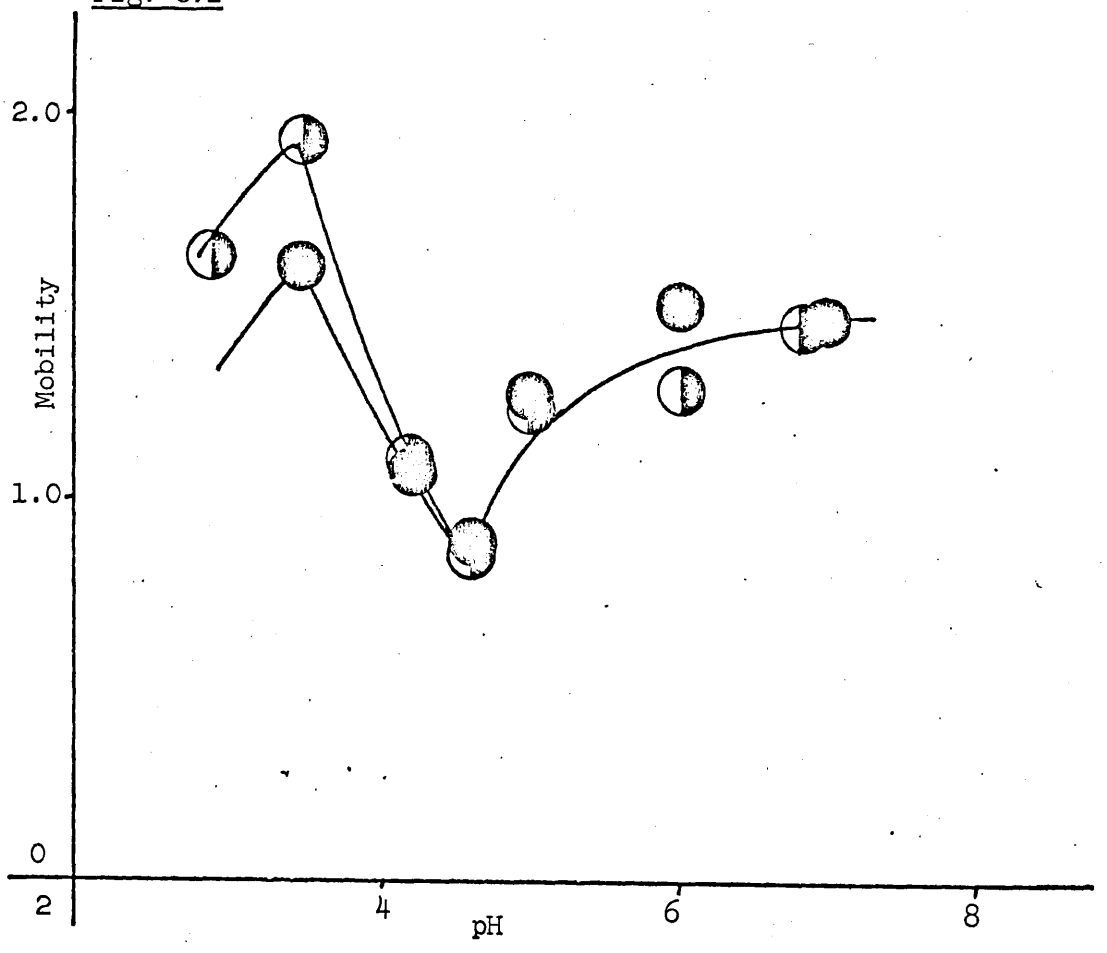
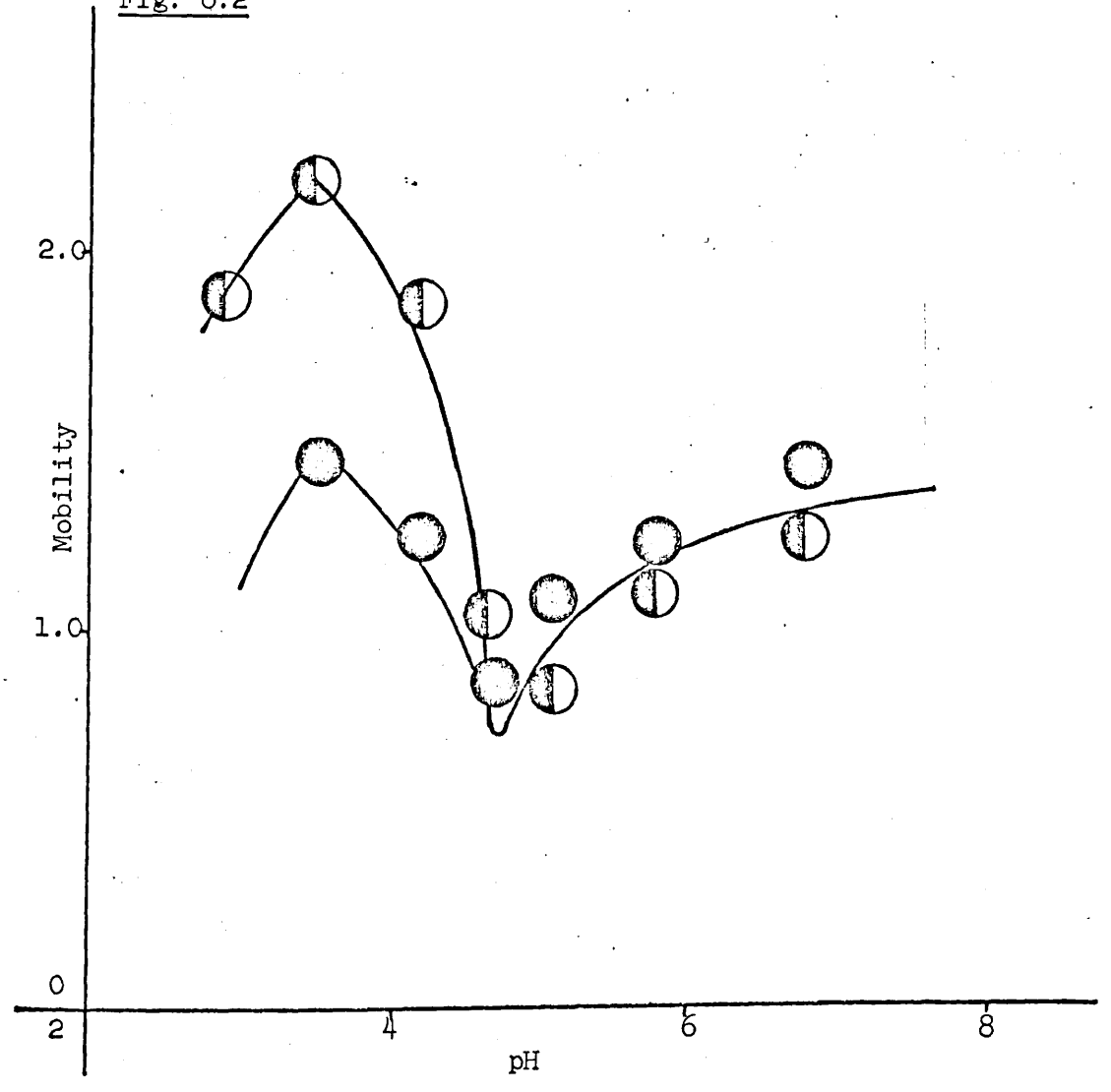


Fig. 6.2



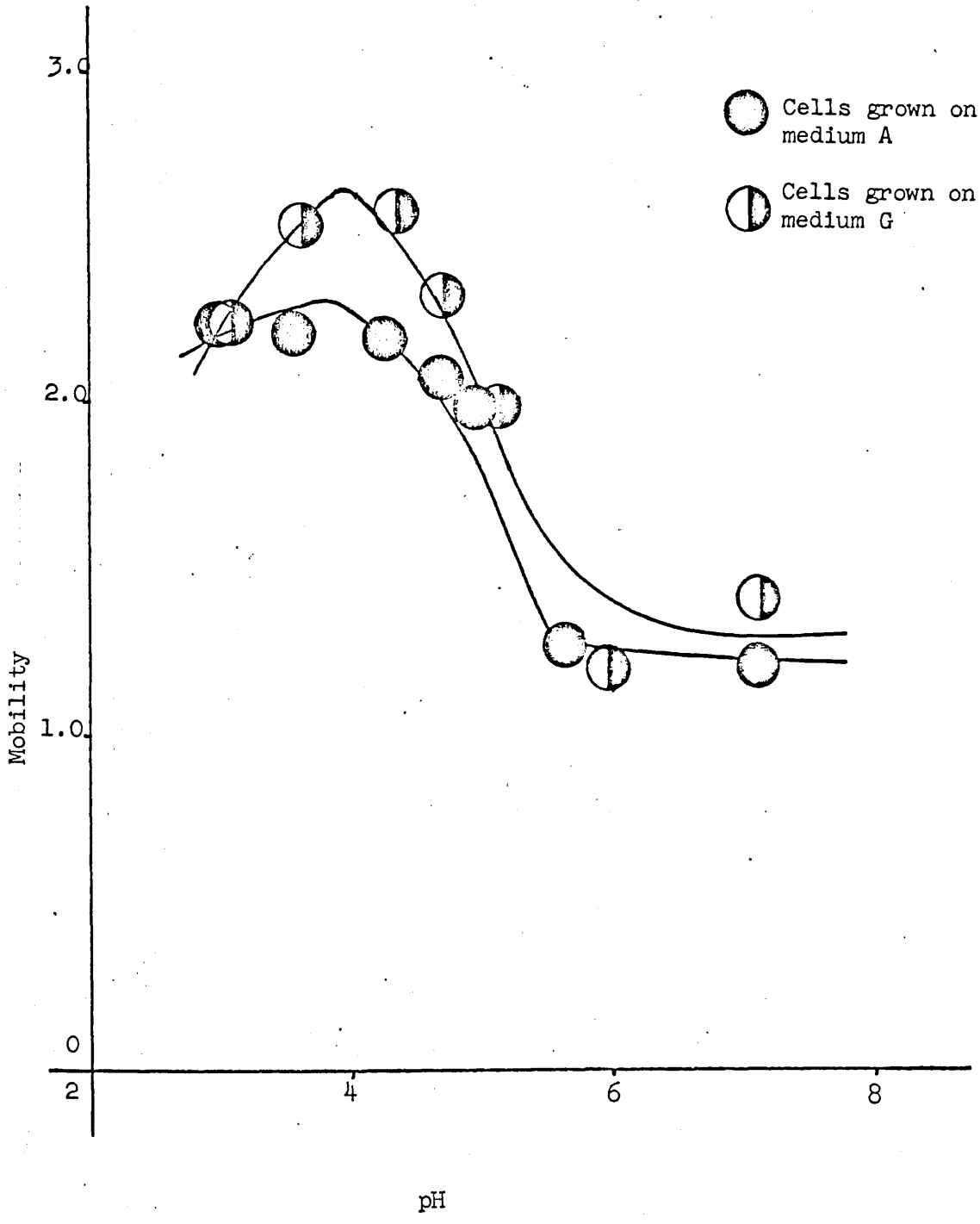
concentration resulted in a higher negative charge at pH 3.0 - 4.0. Since the maximum in this pH range has been attributed to a change in conformation of the surface teichoic acid molecules, exposing the phosphate groups to the surface, these results suggest that cells grown in the presence of a high concentration of inorganic phosphate (medium E Fig. 6.2) contain a higher concentration of phosphate groups on the surface than cells grown on a low inorganic medium (media A and G; Table 4.4).
It is of interest to note that at higher pH values the pH - mobility curves of cells grown on any of the media are coincident, within the limits of experimental error.
Thus the presence of a high concentration of inorganic phosphate is affecting only the amount of surface teichoic acid and is without effect on the amounts of other inorganic groups present on the cell surface.

(b) Cells of strain 2

Cells of the fully antibiotic sensitive strain 2 were grown on media A and G for 18h at 37°C and their pH - mobility curves determined (Fig. 6.3).
These curves have the typical shape of pH - mobility curves of cells of a sensitive strain of Staph. aureus (James and Brewer, 1968). Unlike resistant cells, methicillin sensitive cells exhibit a maximum mobility in suspension in the pH range 3.0 - 5.0. Thus, the teichoic acid conformation associated with methicillin sensitive cells in this pH region must be such that the ionised carboxyl groups on the surface areout-weighed by the phosphate groups at low pH.

Fig. 6.3

pH - mobility curves of cells of strain 2 after 18 h growth at 37°C on media A and G



Cells grown on medium G have a slightly higher negative charge in the pH region 3.0 - 4.0 than cells grown on the standard medium A. This again suggests a possible increase in the concentration of phosphate groups of cells grown on medium G.

6.2 pH - Mobility Curves of Cells of Staph. aureus, Strain 9

Cells of strain 9 (used by Grubb and Annear, 1972) showed anomalous results when the alkaline phosphatase activity was studied (5.2). A much lower phosphatase activity was exhibited by this strain than was observed for other resistant strains; the cells were, however, very resistant.
The pH - mobility curves of cells of this strain grown at 37° and 42°C on medium A (Figs. 6.4 and 6.5) appeared to be more typical of cells of a sensitive strain than those for cells of a resistant strain. The shapes of the two curves at the different temperatures are quite unlike any that have been reported previously from this laboratory.

6.3 The Effect of Inosine on the Surface Properties of Resistant and Sensitive Strains of Staph. aureus.

When the ribonucleoside , inosine, was added to the growth medium, cells of strain 1 showed a marked increase in phosphatase activity (4.2 e). It was, therefore, necessary to investigate the effect of inosine on the surface properties of the cells.

(a) The addition of inosine to the growth medium

Inosine was added to medium G (calcium hydroxide-treated medium) to give a concentration of 10^{-2} mol dm^{-3} inosine (i.e. medium H).

Fig. 6.4

pH - mobility curves of cells of strains 1 and 9 after 18 h growth on medium A at 37°C

Cells of strain 1

Cells of strain 9

Fig. 6.5

pH - mobility curves of cells of strains 1 and 9 after 18 h growth on medium A at 42°C

Cells of strain 1

Cells of strain 9

Fig. 6.4

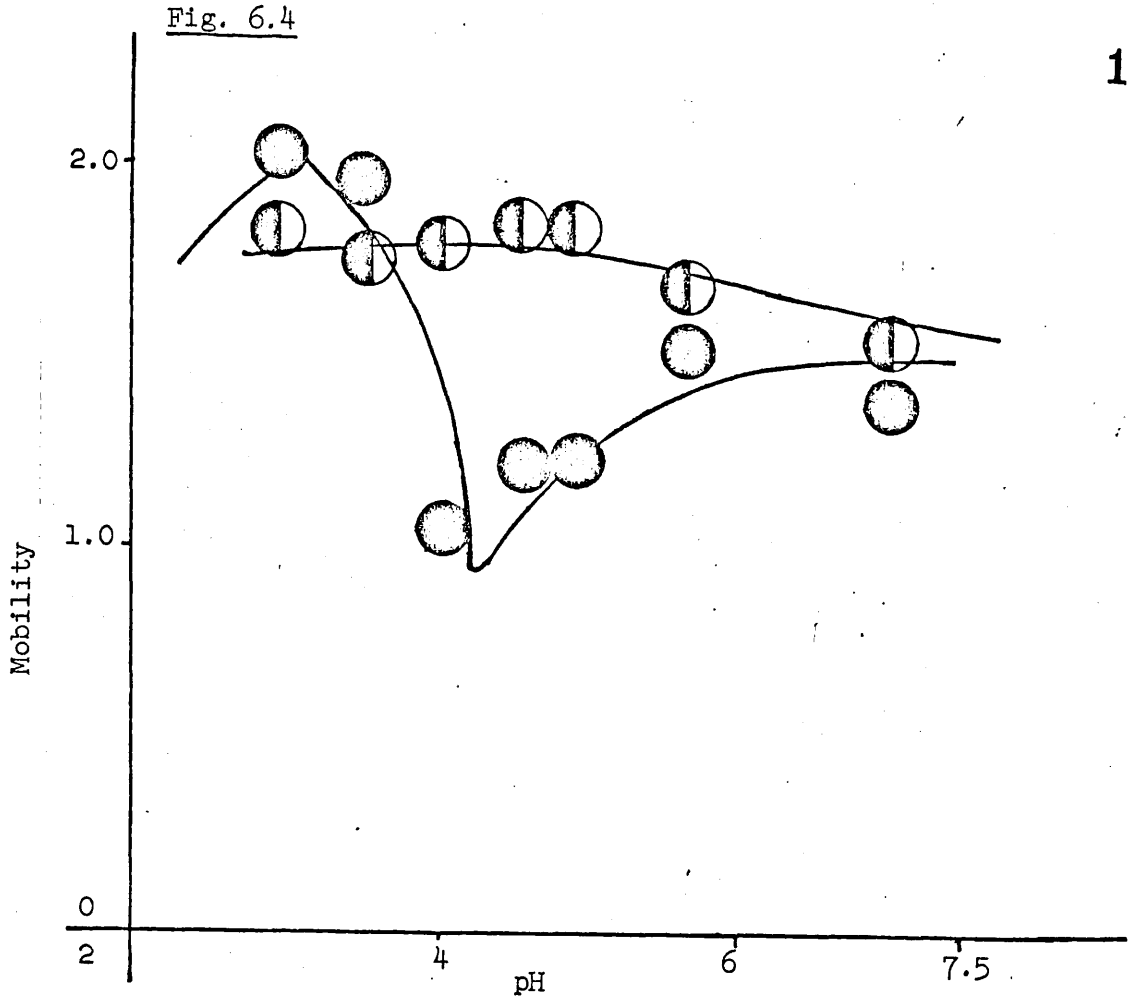
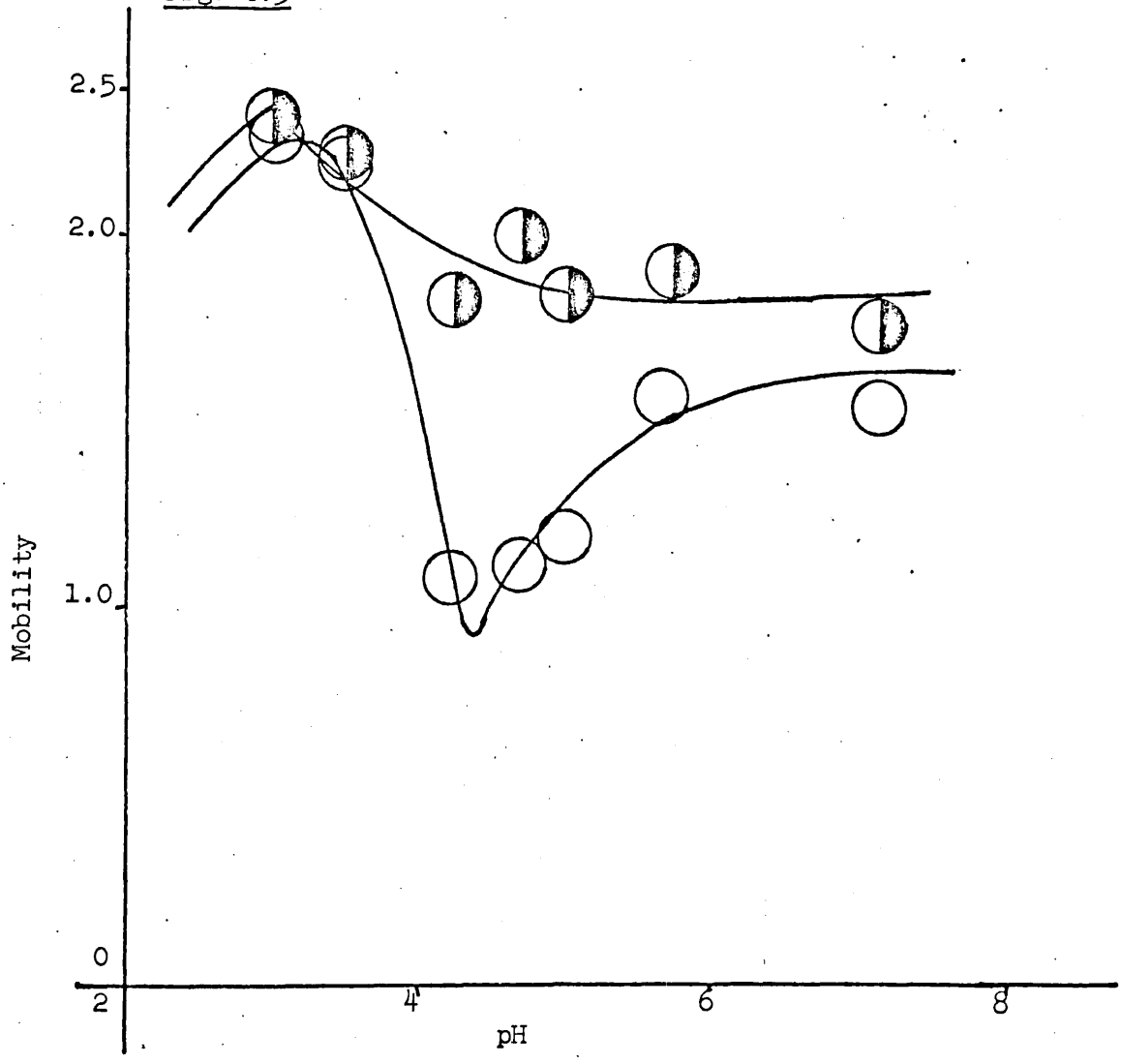


Fig. 6.5



This was then autoclaved and the required number of plates were poured.

Cells of both methicillin sensitive and resistant strains (2, 3, 1 and 9) were grown on this medium for a minimum of 4 subcultures at 37°C and their pH - mobility curves and phosphatase activity were determined after final growth in the presence of inosine. The washed cells were also treated with sodium metaperiodate (2.3g) to remove any teichoic acid from the cell surface.

The pH - mobility curves for these strains are shown in Figs. 6.6, 6.7, 6.8 and 6.9 and the phosphatase activity in Table 6.1

Table 6.1

Effect of inosine in the growth medium on the phosphatase activity of cells of Staph. aureus after 5 subcultures

Strain	Phosphatase activity/n mol min^{-1} $(mg\ cell)^{-1}$	
	Medium G	Medium H
2	13	119
3	0.6	92
9	9	65
1	63	147

The phosphatase activity of both the resistant and sensitive cells showed a very large increase after growth in the presence of inosine.

Fig. 6.6

pH - mobility curves of Staph. aureus strain 2 after growth in the presence of inosine

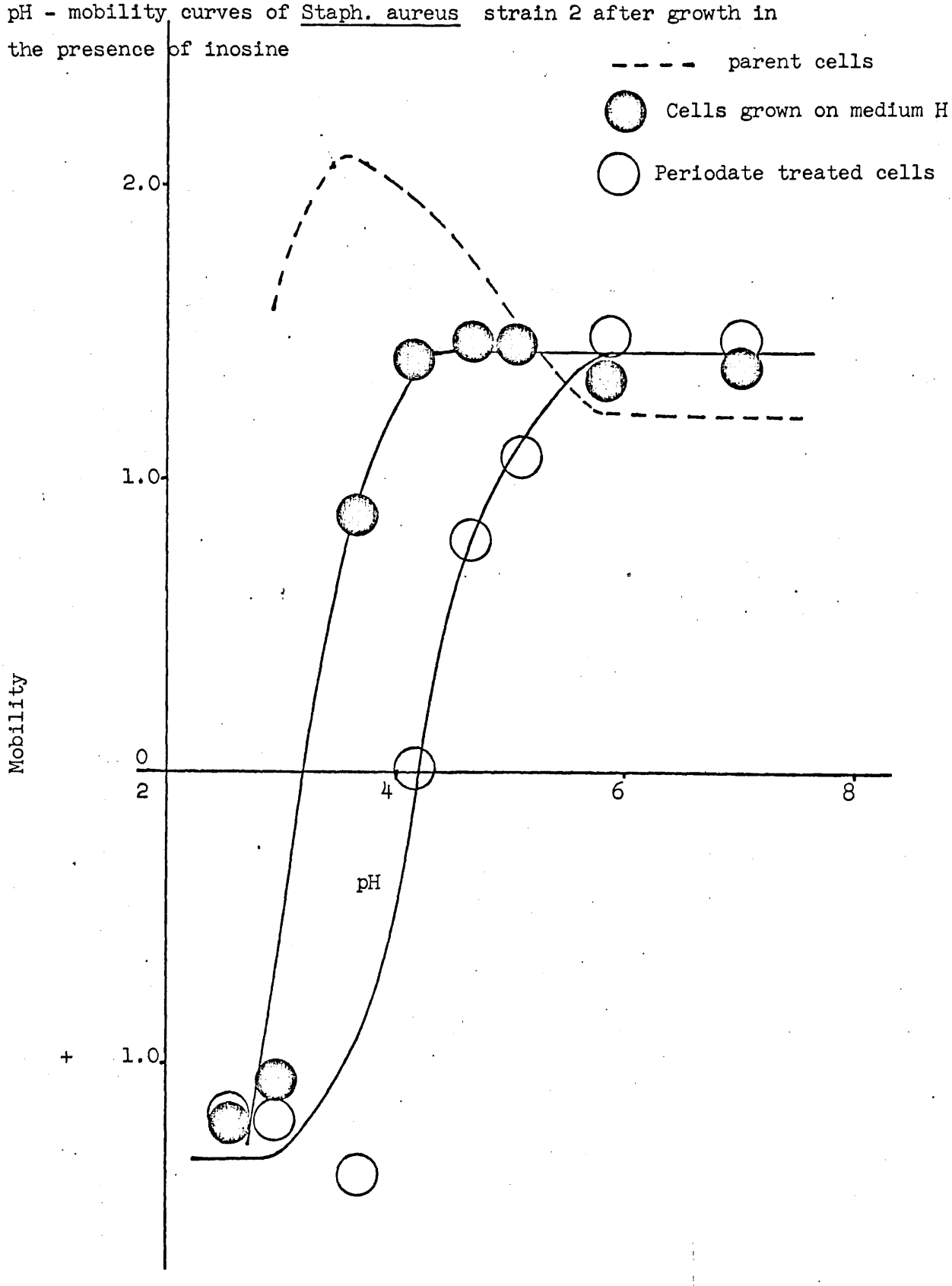


Fig. 6.7

pH - mobility curves of Staph. aureus strain 3 after growth in the presence of inosine

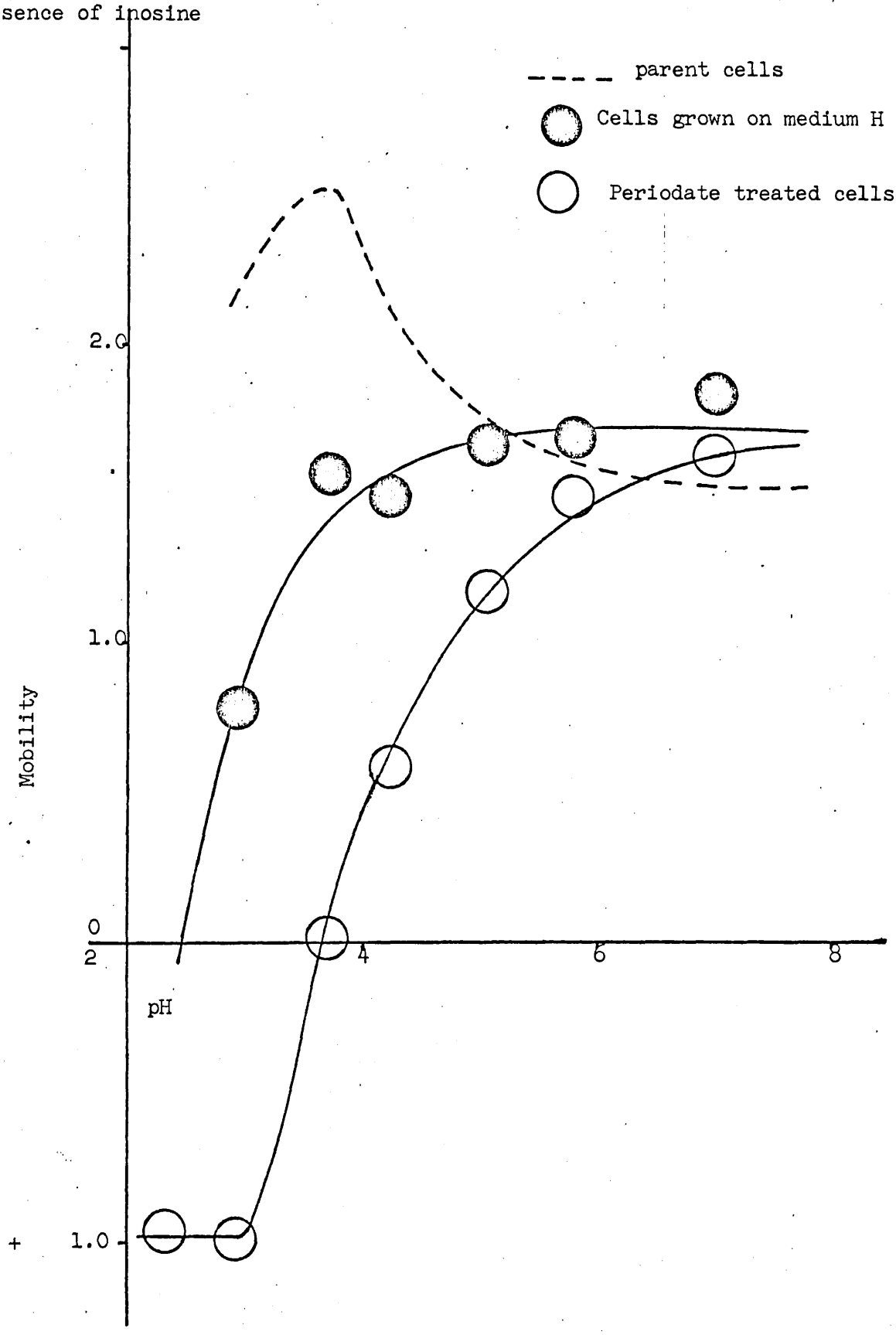


Fig. 6.8

pH - mobility curves of Staph. aureus strain 1 after growth in the presence of inosine

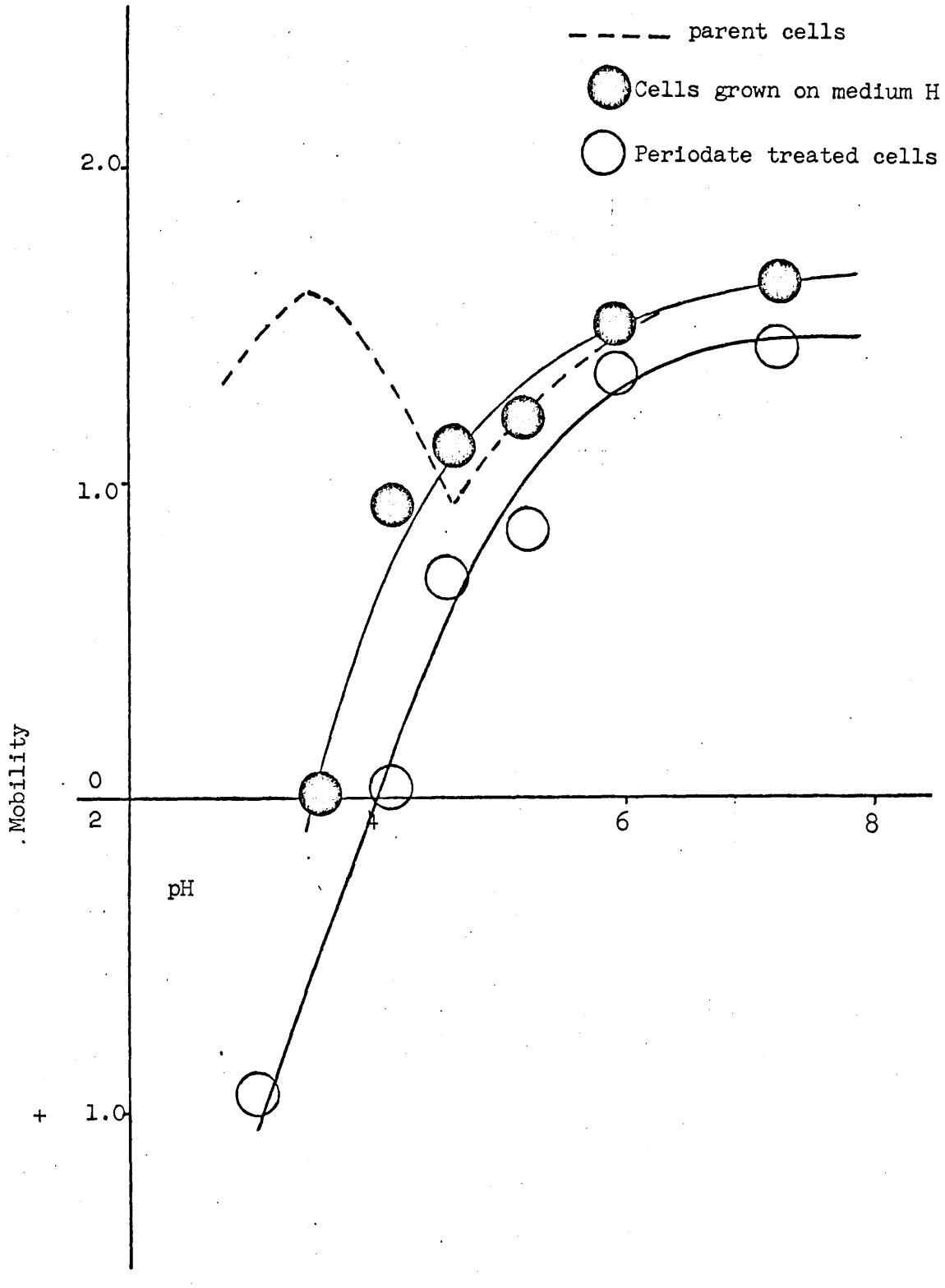
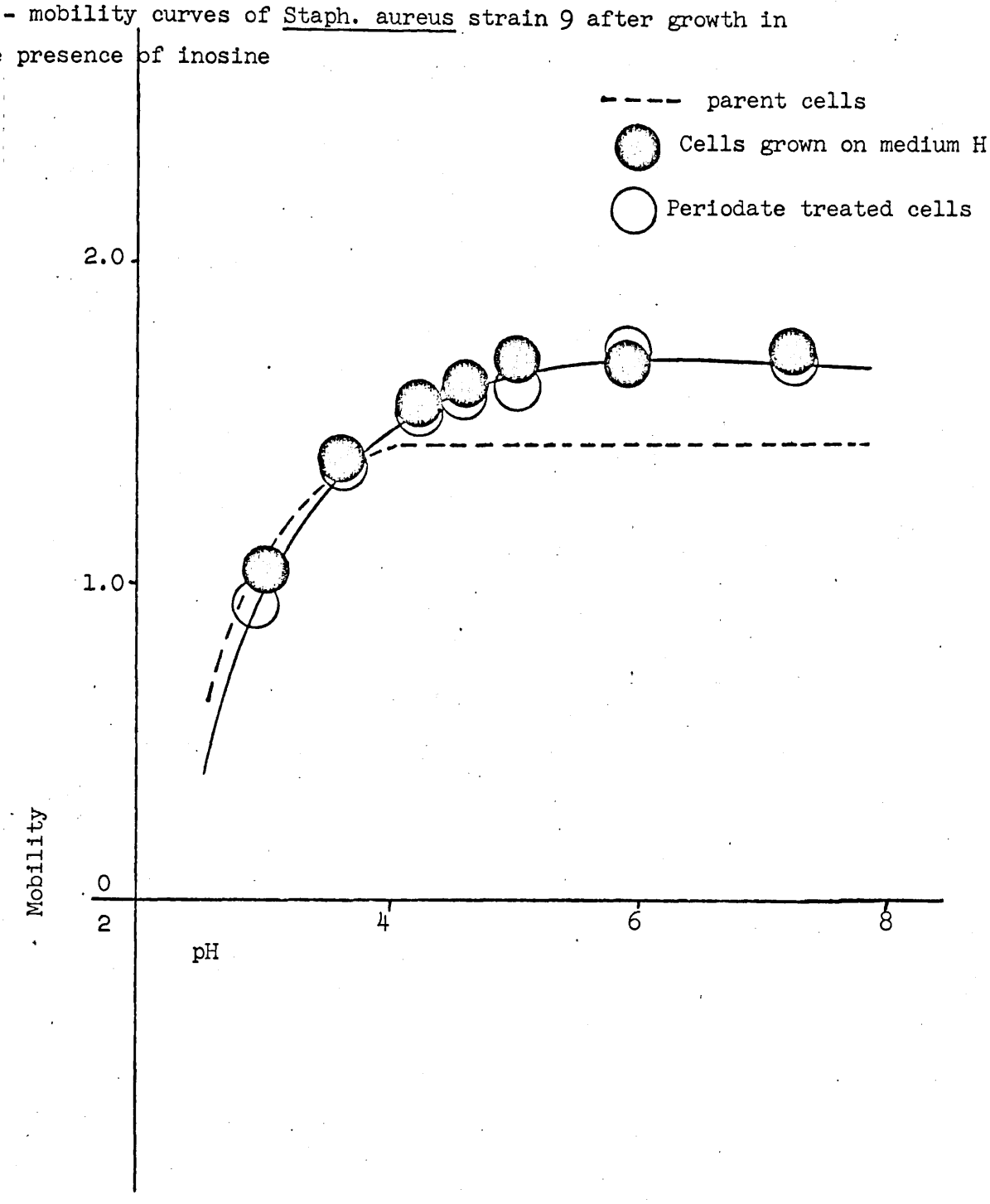


Fig. 6.9

pH - mobility curves of Staph. aureus strain 9 after growth in the presence of inosine



The pH - mobility curves of cells of each strain grown in the presence of inosine now had the same shape, i.e. similar to the curve obtained for cells of a resistant strain grown at 25°C on normal growth medium (a carboxyl - type curve) Fig. 6.10 shows the curves obtained for cells of strain 1 grown on media G and H at 29°C. The mobility values measured at pH 6.0 of cells of the parent strain (i.e. grown in the absence of inosine) and of cells grown in the presence of inosine were the same within the limits of experimental error.

Oxidation of the cells grown on medium H with sodium metaperiodate resulted in very little change of the pH - mobility curve from that obtained before treatment.

The results indicate that growth on media containing inosine either (i) has the effect of changing the configuration of the surface teichoic acid so that the phosphate groups are no longer accessible to the surface, or (ii) preventing the formation of teichoic acid and enhancing the production of the alkaline phosphatase enzyme system.

It is, however, important to note that although the cells were brought to a common surface when grown in the presence of inosine, their resistance or sensitivity to methicillin remained unchanged.

(b) <u>The effect of repeated growth of "inosine-grown cells" in the absence of inosine</u>

Cells of strain 1 were first grown on medium H for 4 subcultures and then for 5 subcultures on medium G, containing no inosine. The pH - mobility curves of the "parent cells", inosine-grown cells and reverted cells were determined (Fig. 6.11).

Fig. 6.10

pH - mobility curves of cells of strain 1 after growth on medium G and medium H at 29°C for 5 subcultures

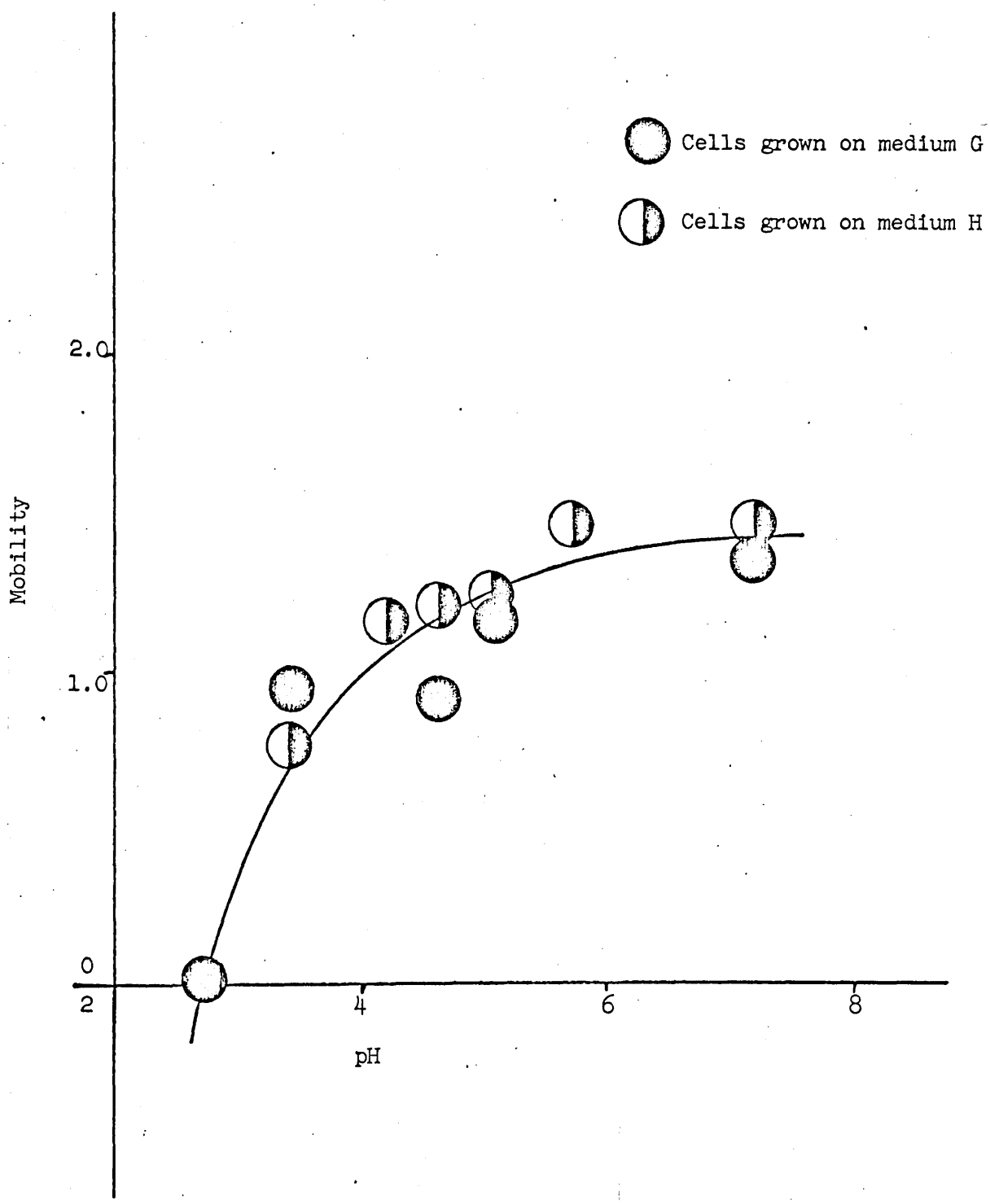
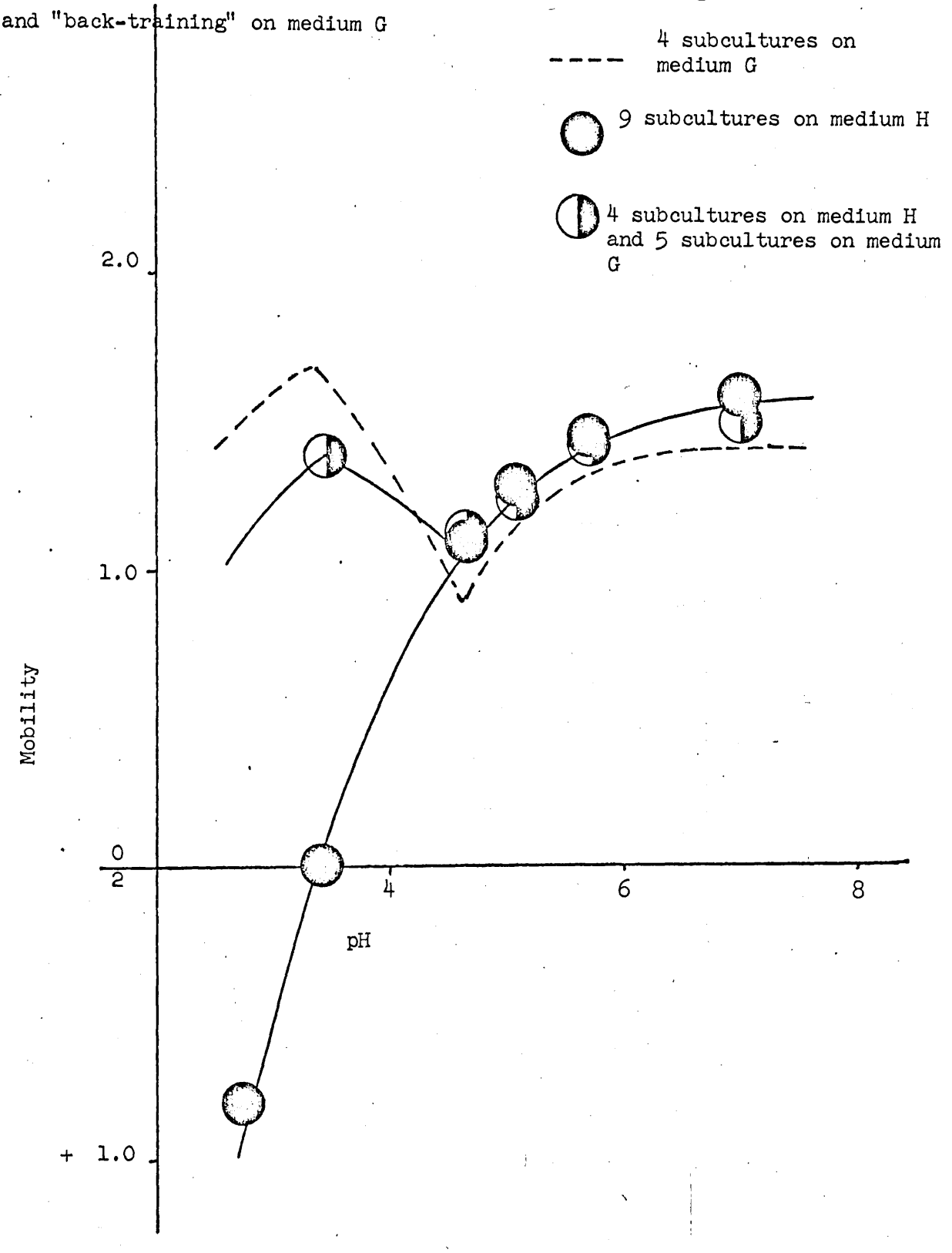


Fig. 6.11

pH - mobility curves of cells of strain 1 after "training" on medium H and "back-training" on medium G



After growth on inosine medium, the cells of resistant strain 1 had a typical carboxyl-type surface where as growth of these cells on medium containing no inosine for 5 subcultures showed that the surface of the cells had, once again, reverted to that typical of a resistant strain. Thus, it may be concluded that the effect of inosine on these cells is a reversible process.

(c) The effect of inosine in the buffer solution on the mobility of cells of Staph. aureus

To establish whether or not inosine was binding onto the surface of the bacterial cells and thus preventing the phosphate groups of the teichoic acid molecules becoming accessible to the surface, inosine was added to the barbitu.rate -acetate buffer solutions to give a final concentration of 10^{-2} mol dm^{-3}. A pH - mobility curve was determined for cells of strain 1 grown on medium G in these solutions.

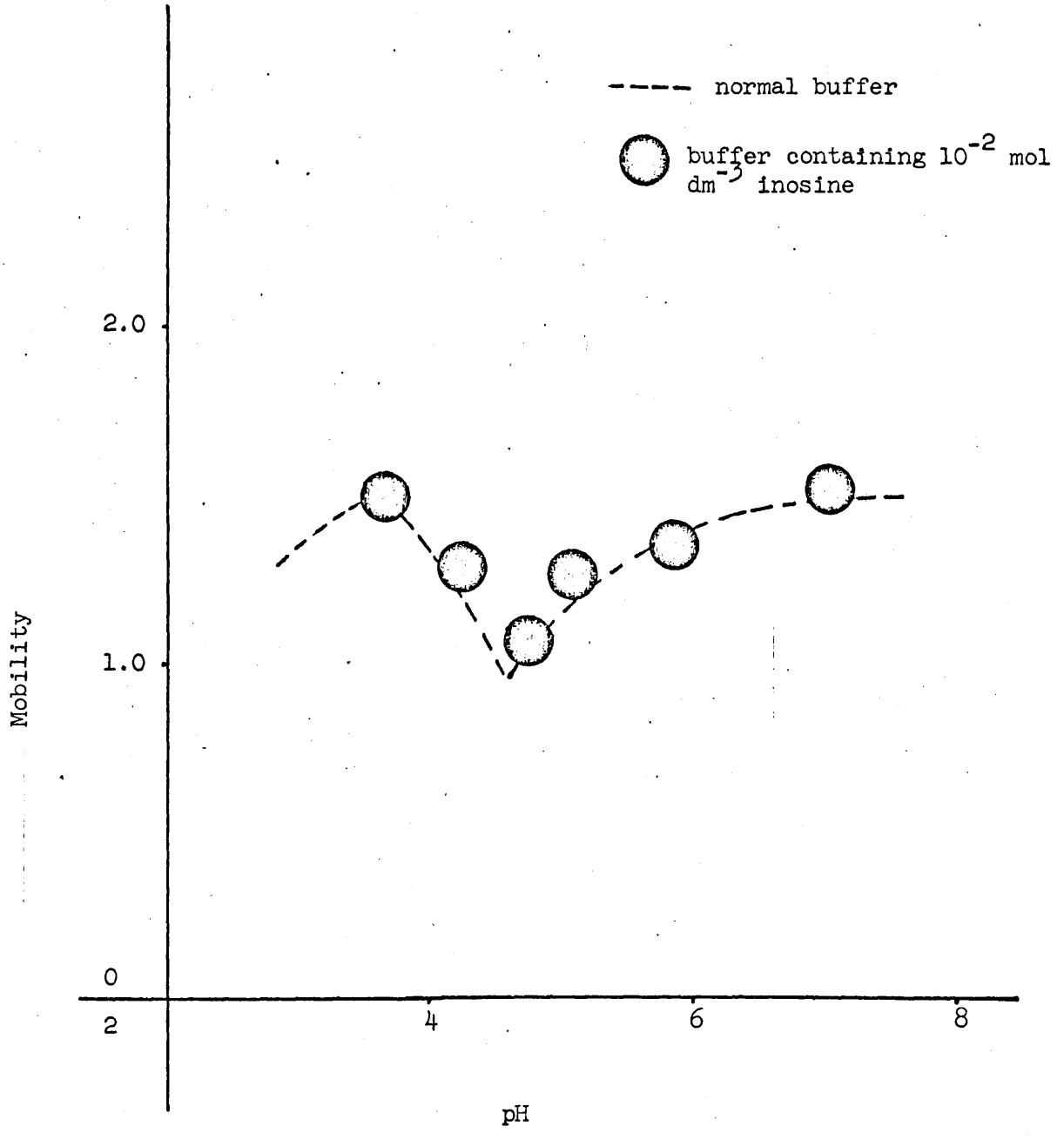
This concentration of inosine had no effect on either the pH or conductance value of any of the buffer solutions used. Further the addition of inosine to the buffer solutions had no effect on the mobility values (Fig. 6.12) and hence confirms that inosine does not bind onto the cell surface.

6.4 Effect of the Growth of Cells Under Limiting Phosphate Conditions in a Chemostat

Ellwood (1970) has reported that in a strain of Bacillus Subtilis, the cell wall composition varied with the inorganic phosphate content of the growth medium.

Fig. 6.12

pH - mobility curves of cells of strain 1 grown on medium G at 37°C for 18 h, measured in normal buffer solutions and buffer solutions containing 10^{-2} mol dm^{-3} inosine



In particular, when the cells were grown under conditions of limiting phosphate, the cell wall teichoic acids were replaced by teichuronic acids. When the cells were grown in medium with excess phosphate, the cell walls contained a relatively large amount of phosphate.

Cells of Staph. aureus (strain H) grown under limiting phosphate conditions in a chemostat were kindly provided by Dr. D.C. Ellwood, (M.R.E., Porton). The pH -- mobility curves and phosphatase activity of these cells were determined. These cells were also grown on nutrient agar plates containing a high concentration of inorganic phosphate (medium E) and the surface properties of these cells were investigated.

The pH - mobility curve of the cells grown under phosphate limitation (Fig. 6.13) lies between that expected for cells of a methicillin sensitive strain and cells of methicillin - resistant strain grown at 37°C. Oxidation of these cells with sodium metaperiodate indicated that there was still some teichoic acid present on the surface of these cells.

Growth of these cells on medium E produced cells which had a pH - mobility curve typical of a methicillin sensitive strain (Fig. 6.14) i.e. with more surface teichoic acid than cells grown under phosphate limitation. These conclusions were further supported by the alkaline phosphatase assays (7.7 n mol min^{-1} $(mg\ cell)^{-1}$ for cells grown under limiting phosphate and zero for cells grown on medium E.) Cells which had no surface teichoic acid would be expected to have a very much higher phosphatase activity.

Fig. 6.13

pH - mobility curves of cells of Staph. aureus strain H

Normal cells

Periodate treated cells

Fig. 6.14

pH - mobility curves of cells of Staph. aureus strain H after growth at 37°C on medium E

Cells grown on medium E

Periodate treated cells

Fig. 6.13

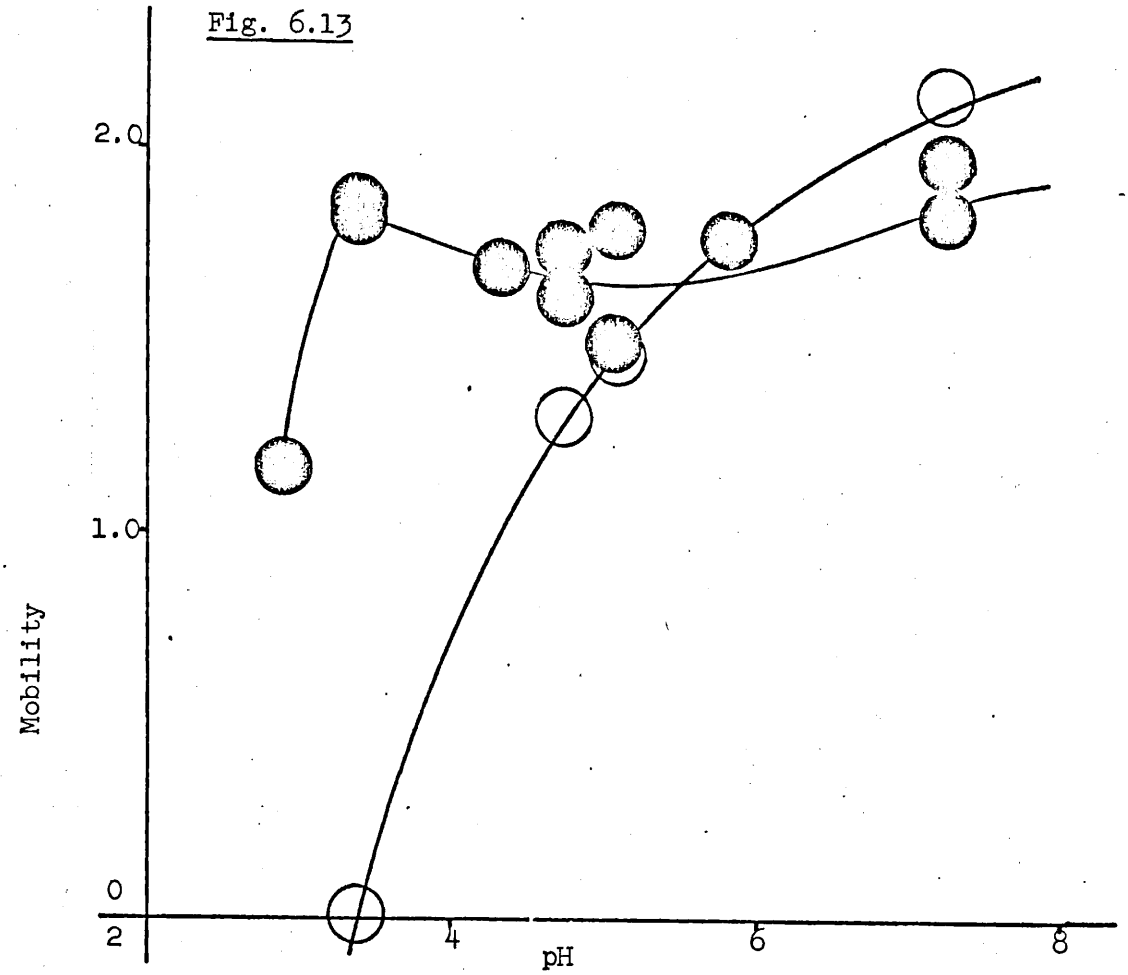
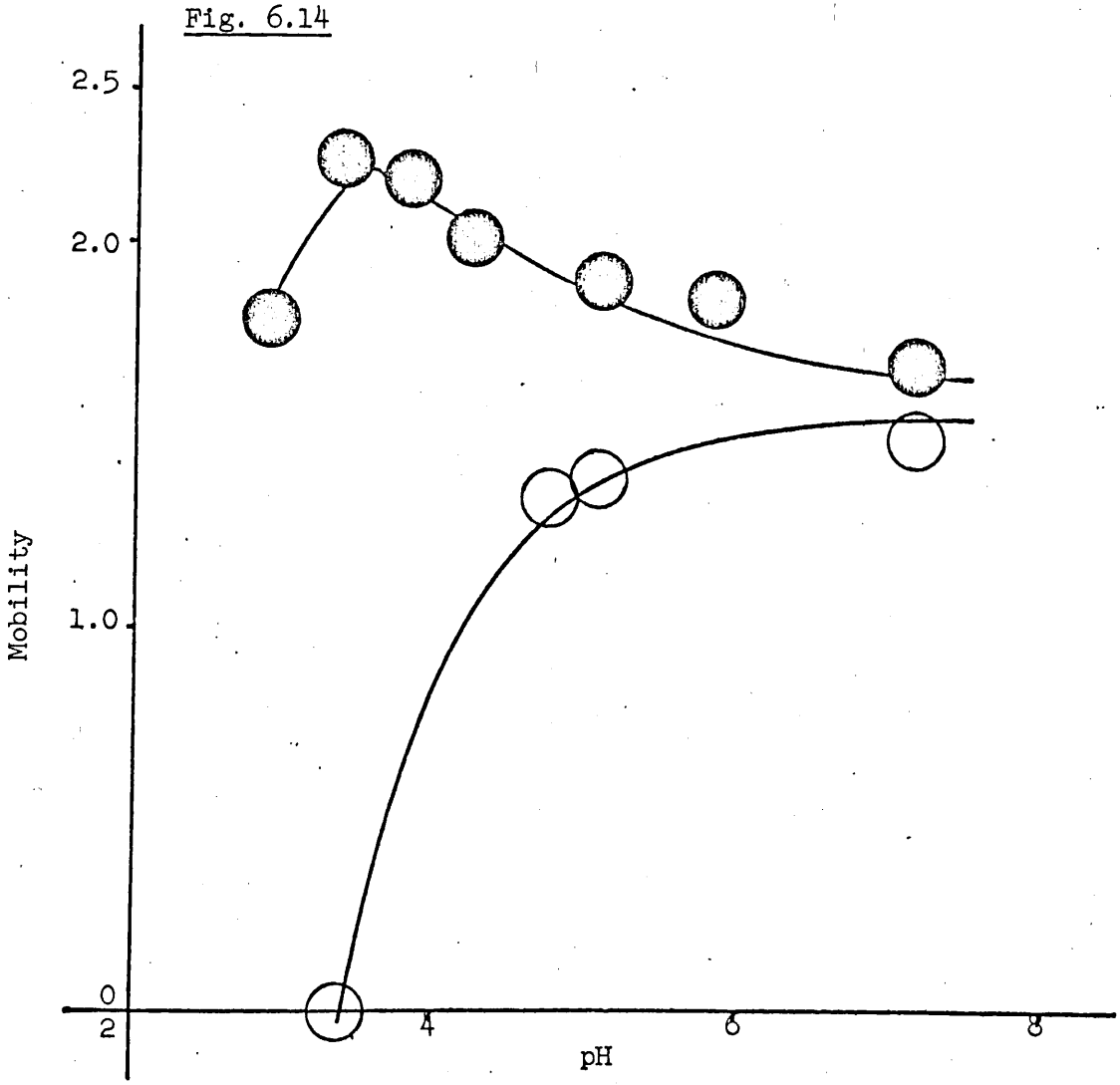


Fig. 6.14



6.5 Summary

(a) Cells of both methicillin sensitive and resistant strains grown on media containing a high concentration of inorganic phosphate have a higher negative charge at lower pH values than cells grown on media containing smaller amounts of phosphate. This is attributed to the number of ionised phosphate groups of the surface teichoic acid exposed to the surface. The charge carried by organisms in suspension at higher pH values is independent of the presence of phosphate in the growth medium.

(b) Cells of methicillin resistant, strain 9, which showed anomalous results for the alkaline phosphatase assays (5.2) also showed anomalous surface characteristics.

(c) The presence of inosine in the growth medium had the effect of bringing cells of both the methicillin sensitive and resistant strains to a common surface. This change was, however, reversible when the cells were grown in the absence of inosine. The resistance or sensitivity of the cells remained unchanged after treatment with inosine.

(d) The mobility value of cells suspended in buffer solutions containing inosine were identical to the control value, showing that inosine did not bind onto the cell surface.

(e) Growth of another methicillin sensitive strain of Staph. aureus under limiting phosphate conditions in a chemostat produced cells which still contained some surface teichoic acid, although when grown on media containing a high concentration of inorganic phosphate, more teichoic acid was produced by these cells.

CHAPTER SEVEN

DISCUSSION

Any meaningful interpretation of results obtained from both the phosphatase assay and particulate electrophoresis experiments was dependent on obtaining cells which gave reproducible results.

For the particulate electrophoresis experiments, the surface under study had to be reproducible and free from adsorbed material.

To ensure that changes in mobility represented true changes in the surface properties of the cells, the cells were washed twice before a suspension was prepared for mobility measurements. This was sufficient to remove adsorbed material on the cell surfaces, e.g. components of the growth medium or antibiotics, and not so violent that any actual surface components were removed. All the mobility measurements were made in buffer solutions in the pH range 2.5 - 7.0; in this region no irreversible damage was caused to the cell surface. The populations of cells of all the strains studied were electrokinetically homogeneous despite the fact that many of the populations were biologically heterogeneous, e.g. strains resistant to methicillin (Sutherland and Rolinson, 1964).

The rectangular - cross - section observation chamber was calibrated with particles of known mobility under defined conditions. This was necessary because it was not possible to measure the cross-sectional area of the observation chamber, which was required for the calculation of the absolute mobility value (2.3d). Thus the electrophoretic mobility values are relative values and not absolute values. This is not a serious drawback since any discussion of the experimental results does not require a knowledge of an absolute value. The main interest lies in the changes of surface properties which occur as a result of change of growth medium, variation of antibiotic spectrum, etc.

Cells of an 18 h culture of K. aerogenes suspended in acetate-barbiturate buffer solution at pH 7.0 were used for the calibration of the chamber. This is a secondary reference, the primary accepted reference is the human erythrocyte suspended in phosphate buffer solution at pH 7.35 (Gittens and James, 1961; Seaman, 1965).

In common with previous workers, it was found that there was a slight day-to-day variation of the "cell constant". Although the real cause of such changes have never been established, it may be that cell wall debris accumulates on the surface of the observation chamber and in consequence affects the electroosmotic flow within the cell or changes the cross-sectional area. To eliminate the possibility of such errors interfering with the data obtained, the electrophoresis apparatus was calibrated daily.

The zeta potential at the cell-electrolyte interface is determined partly by the nature and quantity of ionogenic groups at the surface and partly by the pH and ionic strength of the suspending medium. The conversion of mobility values to zeta potentials using the Smoluchowski equation :

$$\bar{v} = \frac{\epsilon \zeta}{4\pi\eta} = \frac{\epsilon_0 \epsilon_R \zeta}{\eta}$$

(where η is the coefficient of viscosity, ϵ_0 the permitivity of free space and ϵ_R the relative permitivity of the medium) is open to critisism since the viscosity and relative permitivity within the electrical double layer, which are unknown, are normally assumed to have the same values as bulk water. If the pH and ionic strength of the suspending electrolyte are kept constant, it is possible to discuss changes in the experimentally determined mobility in terms of changes in the nature and quantity of surface charged groups. It is for these reasons that the electrokinetic results will be discussed in terms of the changes in the shape and/or positioning of the pH - mobility curves which occur (i) with a change in the antibiotic spectrum of the

organisms, (ii) with a change of growth medium or (iii) with chemical treatments of the cells.

Cells of all strains of Staph. aureus contain a phosphatase enzyme system; this fact is commonly used as a test for the organism (Baird - Parker, 1963). Therefore, when cells of Staph. aureus are left in contact with a suitable substrate (a phenolphthalein phosphate or 4-nitrophenyl phosphate salt) for a long period of time they will all give a positive test for the presence of the enzyme. The cells of all the strains studied in this investigation gave a positive test for the presence of the enzyme if they were left in contact with the substrate for a few hours. This would suggest that this type of enzyme is present in the bulk of the cell and can diffuse to the cell surface. The work described here, however, is concerned with the presence of an enzyme system which can be assayed within a very short period of time (up to 5 minutes from the time of addition of the substrate). This enzyme system must, therefore, be easily accessible to the substrate and thus possibly located at the cell surface. Cells of all the strains studied did not exhibit this type of enzyme activity.

To ensure reproducible results, the cells were washed twice with distilled water before they were prepared for the enzyme assay.

A technique was established to measure the phosphatase activity of suspensions of cells of Staph. aureus using 4-nitrophenyl disodium orthophosphate as the substrate. Analysis of replicate determinations on cells of the same methicillin resistant strain grown and tested at different times revealed that the confidence limit of a single mean at $P = 0.05$ is $\pm$ 2%. As is apparent from the experimental results, the differences in activity between cells of various strains, particularly those between methicillin sensitive and resistant cells, are highly significant.

The production of this enzyme system in methicillin resistant cells was, however, very sensitive to the age of the parent cells used for inocculum. It was observed that daughter cells derived from an aged culture on agar possessed a higher phosphatase activity. This considerably affected experiments designed to study the variation of activity with age. This observation is difficult to reconcile with the fact that in general, enzyme activities decrease with increasing age of cells. It is commonly believed that the metabolic function of the alkaline phosphatase is the conversion of phosphate esters into inorganic phosphates. When this inorganic phosphate, an essential nutrient, is in restricted supply in the growth medium, as may be the case in aged cultures, then it is feasible that older cells will exhibit a higher phosphatase activity. Hence any daughter cells will also exhibit a higher phosphatase activity. Recent work (Bosmann et al, 1974) has shown that the surface electrokinetic parameters of Staph. aureus differ dramatically over the growth cycle.

Allowing more air than usual to circulate over the plates during the growth of the cells enhanced slime formation in the cells but there was no significant difference in the phosphatase activity of these and the "normal" cells exposed to less air. Thus any day to day variation in the phosphatase activity of cells of Staph. aureus can be attributed to differences in age of the parent cells. In an attempt to control this age variation a standard procedure was adopted. 18 h cells grown on nutrient agar were used to study the phosphatase activity; these cells were harvested after 18 h growth.

There are various reports in the literature (Shah and Blobel, 1967; Malveaux and San Clemente, 1969 a) of the alkaline phosphatase activity of Staph. aureus, but there has been no systematic study of the relation of this activity to antibiotic resistance. The phosphatase enzyme system associated with cells of strains of Staph. aureus with natural resistance to methicillin was an alkaline phosphatase with an optimum pH in the range 10.00 - 10.20.

Under normal assay conditions, no acid phosphatase was detected, this is in contrast to reports from other workers (Kuo and Blumenthal, 1961; Cannon and Hawn, 1963; Malveaux and San Clemente, 1969 b). However, if the cells had been left in contact with the substrate solution for long periods of time, then it may have been possible to detect the presence of an acid phosphatase enzyme system. In common with most enzyme systems, the optimum temperature for maximum activity was 37°C, and $\Delta H = 16.3$ kJ mol^{-1} for cells of strain 1 grown at 37°C. The presence of Mg^{++} ions in the assay mixture greatly enhanced the phosphatase activity of methicillin resistant cells grown at any temperature; this is in agreement with the report of Ingram et al (1973) that the release of alkaline phosphatase of whole cells of P. aeruginosa is a function of the concentration of magnesium chloride. For the cells of Staph. aureus it is not certain whether the Mg^{++} ions cause the release of the alkaline phosphatase or whether they are acting as co-enzymes.

The alkaline phosphatase of naturally occurring methicillin resistant cells of Staph. aureus is firmly bound to the cells, accessible to the surface and is not easily washed off. The measured activity is caused by a heat labile enzyme system and not by the cells themselves. The production of this enzyme system is very dependent on the temperature of growth; cells grown at 25° and 37°C exhibited high alkaline phosphatase activity whereas those grown at 43°C had no measurable activity. Repeated growth at any of these temperatures had no subsequent effect on the phosphatase activity of these cells.

Although the production of the enzyme was repressed by the presence of excess inorganic phosphate in the growth medium, the presence of inorganic phosphate in the assay medium had no inhibitory effect, as was found for the phosphatase activity of cells of E. coli (Garen and Levinthal, 1960).

Neither the presence of methicillin nor inosine in the assay medium had an inhibitory effect on the phosphatase activity of the cells. The production of the alkaline phosphatase enzyme system was very dependent on the type of growth medium used. Very low phosphatase activity was exhibited by cells which had been grown in liquid medium, irrespective of its inorganic phosphate content. When the broth containing a low concentration of inorganic phosphate was solidified, the cells grown on the resulting medium exhibited a very much higher phosphatase activity. Thus, the production of the enzyme is more dependent on the state of the growth medium than the presence of excess inorganic phosphate.

Cells grown in liquid medium are in a different environment from those grown on solid medium. In liquid medium the cells are continually in contact with both nutrients and toxins while on solid medium, the toxins can diffuse away from the cells and phosphates can diffuse to the cells. It is possible that in liquid medium the phosphatase is removed from the cells to give an extracellular enzyme system which could operate to the advantage of the cell, while on nutrient agar the enzyme remains firmly attached to the cell.

The phosphatase activity of cells grown on solid media, however, was very dependent on the concentration of inorganic phosphate present in the medium. Although a high concentration of inorganic phosphate had a repressive effect on the formation of the enzyme system, nevertheless a low minimal concentration of phosphate was required for good growth and high phosphatase activity (4.2 b). Since the metabolic purpose of the alkaline phosphatase enzyme is to convert phosphate esters (e.g. teichoic acid) into inorganic phosphates when these have been exhausted from the growth medium it can be postulated that if the medium contains excess inorganic phosphate then no phosphatase will be produced and this will result in the accumulation of teichoic acid on the cell surface.

If, however, the growth medium contains a low concentration of inorganic phosphate, it will be necessary for the phosphate esters to be converted into inorganic phosphates and thus the cells will exhibit phosphatase activity and have little or no surface teichoic acid. On this basis, therefore, it is possible that there is a relationship between high and low or zero phosphatase activity and the absence or presence of surface teichoic acid respectively.
The surface properties of methicillin resistant and sensitive cells grown on different media are in agreement with this postulate.
When the cells of either a methicillin resistant or methicillin sensitive strain were grown on media containing varying concentrations of inorganic phosphate, the general shape and positioning of the pH - mobility curve was unchanged (i.e. resistant strains showed a minimum mobility at lower pH values; the sensitive strains showed a maximum at pH 3.5). There was, however, a change in the actual value of the mobility for both types of cells in suspension at lower pH values.
A high concentration of phosphate in the medium resulted in a higher negative charge at pH 3.0 - 4.0 (Figs. 6.1; 6.2; 6.3). This is confirmed by a comparison of H - values for cells of strain 1 grown on the three different media (Table 7.1).

Table 7.1

Variation of H - values for cells of strain 1 grown on different media

Growth medium	Concn. inorganic phosphate/$\mu g\ cm^{-3}$	H - Value
A (standard)	10	4
E (high phosphate)	35	75
G ($Ca(OH)_2$ treated)	7	28

The H - value, first defined by Marshall (1969), is the percentage increase of the mobility above the plateau value and is, therefore, an indication of the amount of phosphate groups associated with the cell surface. There is a marked increase in the H - value obtained for cells grown on medium containing a high concentration of inorganic phosphate, compared to the standard medium. However, cells grown on medium G also have a fairly high H - value although the medium contains a very low concentration of inorganic phosphate; this again suggests that the calcium hydroxide treatment has some effect on the medium other than the simple removal of the inorganic phosphate. It is thus apparent that during growth in the presence of a high phosphate concentration there has been an increase in the concentration of phosphate groups on the cell surface and thus an increase in the amount of surface teichoic acid associated with the cells. It is important to note that in suspension at higher pH values there was no change in the surface charge of the cells and, therefore, the increase in inorganic phosphate concentration in the growth medium only affects the concentration of phosphate groups on the surface. Previous workers (Hill and James 1972 a) have postulated that phosphate groups do not contribute to the charge in suspension at pH 7.0 and above.

Ellwood has reported that when cells of a strain of Bacillus subtilis were grown under conditions of limiting inorganic phosphate in a chemostat, the cell wall teichoic acids were replaced by teichuronic acids (phosphate free polymers of N-acetyl-galactosamine and glucuronic acid); when grown in media containing excess phosphate, the cell walls contained a large amount of phosphate.

The surface properties of Staph. aureus (strain H) grown under similar conditions of limiting phosphate in a chemostat lie between those expected for cells of a methicillin sensitive strain and cells of a resistant strain grown at 37°C (Fig. 6.13) i.e. there was still some teichoic acid present on the surface. Growth of these cells on media containing inorganic phosphate produced cells with the surface characteristics of a methicillin sensitive strain, containing more teichoic acid than cells grown under phosphate limitation (Fig. 6.14).

From a calculation of the charge densities of the normal and periodate treated cells measured at pH 4.0, an indication of the amount of phosphate and carboxyl groups associated with the cells of the same strain grown under different conditions can be observed (Table 7.2).

Table 7.2

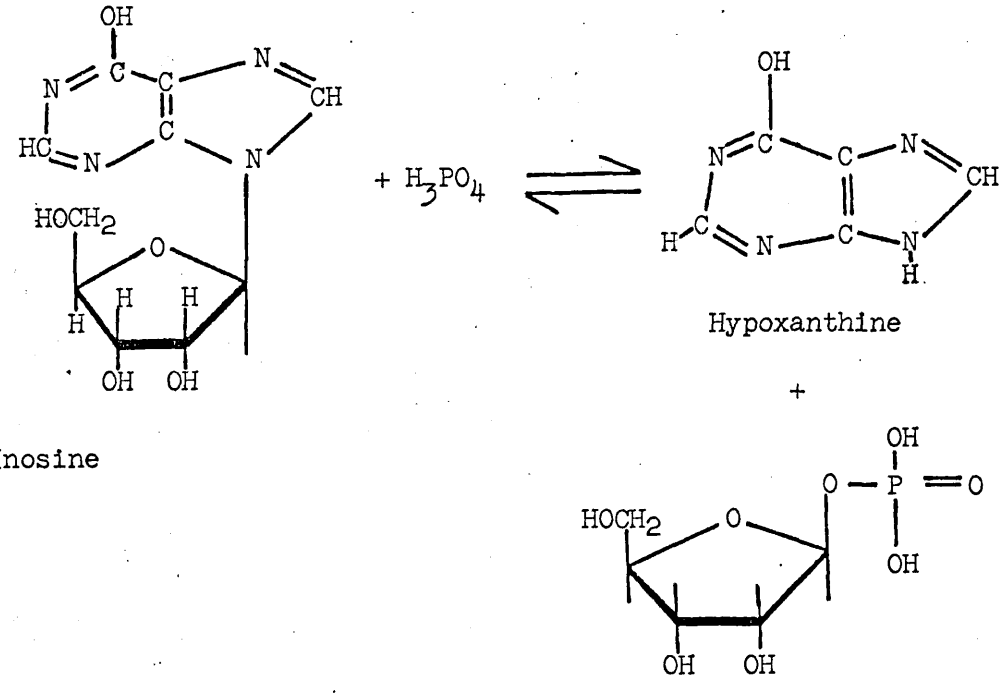
The contribution to the surface charge density of carboxyl and phosphate groups on cells of Staph. aureus, strain H grown under conditions of excess and limiting inorganic phosphate (cf. Fig. 7.2)

		Strain H limiting phosphate	Strain H excess phosphate
$COO^- + PO_4^{---}$	10^3 x charge density / $C\ m^{-2}$	7.5	9.2
COO^-		2.7	3.5
PO_4^{---}		4.8	5.7
$\frac{PO_4^{---}}{COO^-}$		1.8	1.6

The values of the phosphate/carboxyl ratios for these cells are very similar and are intermediate between the values expected for sensitive and resistant strains (see Fig. 7.2). Although a higher value of the phosphate/carboxyl ratio would have been expected for the cells grown on excess phosphate, the total charge density associated with these cells is 23% higher than that associated with the cells grown on limiting phosphate.

The cells grown under limiting phosphate conditions exhibited a low phosphatase activity (7.7 nmol min^{-1} (mg cell)$^{-1}$), quite unlike methicillin sensitive cells, while when these cells were grown on media containing inorganic phosphate they showed no activity. Since cells with no surface teichoic acid should have a much higher phosphatase activity, it is concluded that these cells grown under phosphate limitation do have some residual surface teichoic acid but less than on the parent cells.

Csopak et al (1972) observed that cells of E. coli grown in medium containing inosine exhibited a higher phosphatase activity than that of cells grown in the absence of inosine. Inosine is a ribonucleoside which is an effective phosphate acceptor (Bowne et al, 1966). The position of the equilibrium between inosine and an inorganic phosphate :



Inosine

Ribose - 1 - phosphate

lies towards the left hand side i.e. towards the synthesis of the nucleoside. In the presence of excess phosphate, this equilibrium will be displaced and some of the phosphate removed (Kalckar, 1947).
Cells of methicillin resistant or sensitive strains grown on medium E (high phosphate content) containing added inosine or G ($Ca(OH)_2$ treated) containing inosine (10^{-2} mol dm^{-3}) exhibited a very high phosphatase activity. Irrespective of their sensitivity to methicillin, cells of all strains when grown in the presence of inosine had a common electrokinetic surface, as revealed by the pH - mobility curves. The pH - mobility curves (Figs. 6.6; 6.7; 6.8; 6.9) were characteristic of a simple carboxyl surface and were quite unlike those obtained for cells of either the resistant or sensitive strains. There was very little change in the surface charge at higher pH values, indicating a change affecting the amount of surface teichoic acid only. Despite the change of surface properties, the cells retained their original sensitivity or resistance to methicillin. This means that growth of the cells in the presence of inosine does not interferewith the resistance mechanism. Growth in the presence of inosine does not confer a permanently altered surface on the cells, for when subsequently grown in its absence, the cell surface reverted to that characteristic of the parent sensitive or resistant cells.
The presence of a high concentration of inosine in the buffer solutions had no effect on the pH - mobility curves of untreated cells, showing that inosine did not bind onto the cell surface, thus preventing the phosphate groups from being accessible to the surface. It is thus apparent that the changed surface characteristics brought about by growth in the presence of inosine are not due to adsorbed inosine on the cell surface, but reflect actual changes in the growth pattern brought about by the reduced concentration of phosphate in the growth medium.

The phosphatase activity exhibited by both sensitive and resistant cells was greatly increased when the cells were grown in the presence of inosine. It is apparent that the presence of inosine in the growth medium, which reduces the phosphate content, enhances the phosphatase activity of the cells in an attempt to increase the required phosphate concentration by the breakdown of the cell surface teichoic acid. In doing this the teichoic acid is removed from the cell surface as demonstrated electrokinetically.

Cells of naturally resistant strains of Staph. aureus have different surface characteristics, depending on the temperature of growth (Hill and James, 1972 a). These authors attributed the differences to varying amounts of teichoic acid on the cell surface; the phosphate/carboxyl ratio for the cells grown at the different temperatures was used as a measure of surface teichoic acid. Cells grown at 43°C have considerable amounts of surface teichoic acid; at 37°C they have less; while cells grown at 25°C have no detectable surface teichoic acid. When grown at 37°C, these cells also show a heterogeneous response to methicillin (Sutherland and Rolinson, 1964), in which the vast majority of the cells are sensitive to methicillin with a slow growing minority, showing very high resistance to the antibiotic. Incubation of such resistant strains at 25°C produces cells which are uniformly methicillin resistant at a high level (Parker and Hewitt, 1970). In contrast, after growth at 43°C, the population appears to be very sensitive to even low concentrations of the antibiotic and repeated growth at this high temperature gives rise to completely sensitive cells (Al Salihy and James, 1972).

The first indication that a temperature-dependent enzyme system was associated with methicillin resistance (Annear, 1968) was supported by the results of other workers (Hill and James, 1972 b), who suggested that the enzyme system could be a phosphatase.

The results obtained here strengthen the proposal that there is a link between phosphatase production, the lack of surface teichoic acid and methicillin resistance.

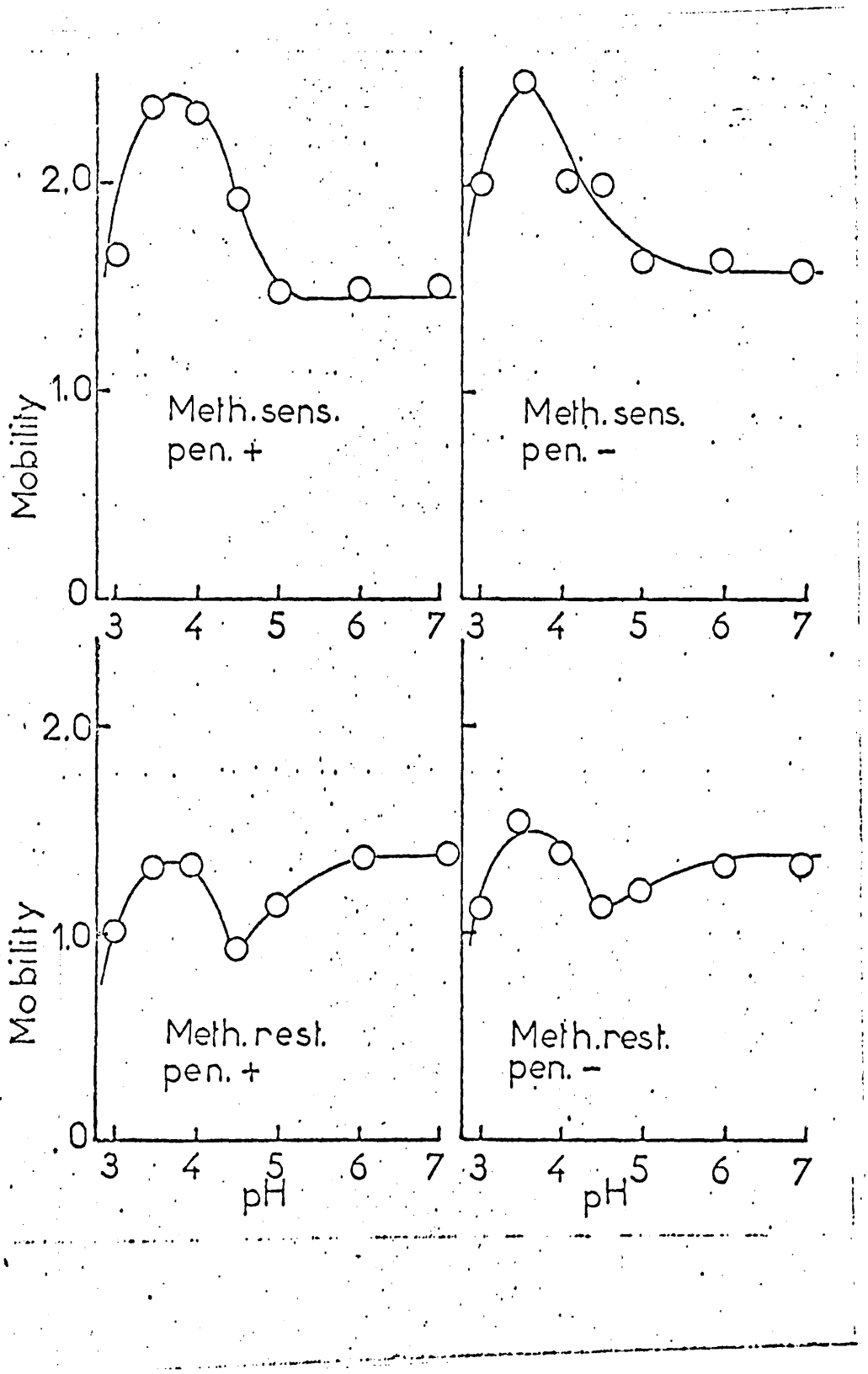
It was confirmed that the methicillin marker was dominant in determining both the surface properties and the phosphatase activity; cells resistant to penicillin (i.e. penicillinase producers), or to other antibiotics do not produce phosphatase or show abnormal surface properties. This suggests that separate plasmids control methicillin resistance and penicillinase production; there is evidence, however, (Al Salihy, 1974, Personal communication) that when cells of resistant strains are grown at high temperatures both plasmids are lost.

More conclusive evidence was provided from a study of the variants of cells of strain 1 which were selected for their ability to produce penicillinase and their resistance or sensitivity to methicillin (Table 5.3). The shape of the pH - mobility curves of these variants are shown in Fig. 7.1 (Al Salihy, 1974 Personal communication). There was a considerable difference in the phosphatase activity of the variants, the methicillin resistant cells exhibited a greater phosphatase activity than did the sensitive cells. The penicillinase positive cells have a lower phosphatase activity, irrespective of their methicillin resistance. These results, together with the surface properties of the cells, show that the dominant factor in deciding the properties of the cells is their resistance to methicillin. This is in agreement with Cannon and Hawn (1963) who found no correlation between high phosphatase production and penicillin resistance in cells of Staph. aureus.

Cells of methicillin sensitive strains which may or may not be resistant to other antibiotics carry large amounts of surface teichoic acid and exhibit no phosphatase activity. In contrast, cells of methicillin resistant strains, which in general are resistant to a wide range of antibiotics, have little or no surface teichoic acid but exhibit high phosphatase activity when grown at 25° or 37°C.

Fig. 7.1

pH - mobility curves of variants of Staph. aureus strain 1



Cells of methicillin resistant strains grown at 43°C have large amounts of surface teichoic acid and exhibit no phosphatase activity; at this temperature the cells are very sensitive to methicillin (Parker and Hewitt, 1970). Further more, repeated growth of these resistant cells at 43°C stabilizes this sensitivity, so that eventually the cells are sensitive even on repeated growth at 37°C (Al Salihy, 1974, Personal communication); these cells have large amounts of surface teichoic acid but no phosphatase activity.

Although the surface properties and phosphatase activities of other methicillin resistant and sensitive strains were very similar to those already discussed, nevertheless anomalous results were obtained for two strains of Staph. aureus. Cells of methicillin resistant strain 9 had a very low phosphatase activity compared to other resistant strains. The surface properties of cells of this strain were unlike any reported previously for naturally occurring methicillin resistant strains of Staph. aureus (Figs. 6.4 and 6.5) and were more typical of cells of a sensitive strain. This strain which has been extensively used by Grubb and Annear (1972) and Annear and Grubb (1973) has a MIC at 37°C in excess of 200 $\mu g\ cm^{-3}$ methicillin. In contrast, cells of the methicillin sensitive strain 8 (Oxford) exhibited low alkaline phosphatase activity when grown at 27° and 37°C, although their surface properties were typical of cells of a sensitive strain. There have been several reports in the literature of other anomalous properties shown by this strain; e.g. Hugo and Stretton (1966) observed that these cells had a very high lipid content; Marshall (1969) demonstrated a higher surface lipid content than that of normal strains of Staph. aureus. It is thus apparent and perhaps not surprising that within a series of sensitive or resistant strains there is a gradation of properties with inevitably some overlap.

Cells of methicillin sensitive strains after repeated growth in the presence of low concentrations of methicillin at 37°C until they became resistant still exhibited no phosphatase activity. This type of induced resistance is, therefore, quite unlike natural resistance and is most probably attained by a different mechanism. Hill (1971) has already shown that the surface properties of such cells are not really comparable to those of resistant cells, except perhaps when resistant cells are grown at 25°C in the absence of methicillin. It is of significance that cells selected from a biologically heterogeneous culture (strain 1 grown at 37°C) produced cells which had the same phosphatase activity irrespective of the individual resistance to methicillin (Table 5.2). In contrast, Hill and James (1972 b) obtained a spectrum of pH - mobility curves for cells selected in this way, ranging from the typical curve for a naturally occurring resistant strain grown at 37°C to a curve characteristic of a simple carboxyl type surface for cells isolated from a 100 μg cm^{-3} methicillin plate, thus showing the absence of teichoic acid. Thus although the cells exhibited a range of surface properties and hence a range of amounts of surface teichoic acid (albeit relatively small) depending on the concentration of antibiotic on which they were originally isolated, nevertheless they all possessed the same phosphatase activity, independent of the antibiotic concentration on which they were selected. The surface properties and phosphatase activity exhibited by cells of methicillin sensitive and resistant strains of Staph. aureus under different growth conditions are summarised in Fig. 7.2. Sensitivity or resistance to methicillin is the important factor in determining the surface properties of the cells, not their resistance to other antibiotics, or their ability to produce penicillinase.

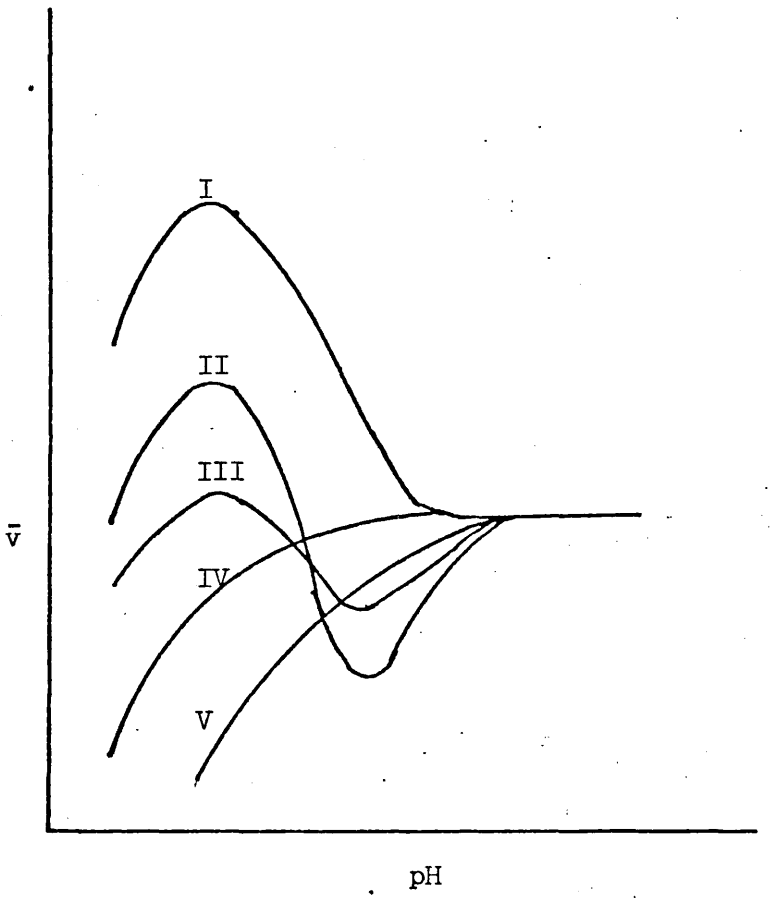
Fig. 7.2

Summary of the surface properties of cells of methicillin resistant and sensitive strains of Staph. aureus.

Key

Meth. Sens. : methicillin sensitivity
Phos. Act. : alkaline phosphatase activity
Surf. T.A. : surface teichoic acid
R. : resistant cells
S. : sensitive cells
P. : periodate treated cells
T. : "trained" resistant cells
A : standard growth medium
G : calcium hydroxide treated medium
H : calcium hydroxide treated medium + inosine

Meth. Sens.	Growth Med.	Growth Tp/°C	pH-$\bar{v}$ curve	Phos. Act.	$\frac{PO_4^{---}}{COO^-}$	Surf. TA.
S		43	I	0	4.0	+++
S		37	I	0	4.0	+++
S		25	I	0	4.0	+++
R		43	II	0	0.8	+++
R	A or G	37	III	++	0.4	+
R		25	IV	++	0	+ -
P R/S		25,37,43	V			
T		37	IV	0	0.8	+
R/S	H	37	IV	+++	0	0



Cells of Staph. aureus with natural resistance to methicillin (e.g. strain 1) grown on relatively low phosphate media exhibit a high alkaline phosphatase activity and, therefore, presumably have a low cell wall phosphate content; conversely growth on media containing a high concentration of phosphate produces cells with no phosphatase activity, indicating a high phosphate content in the cell wall. This relationship between phosphatase activity and surface phosphate groups is supported by the shape of the pH - mobility curves of various strains of Staph. aureus previously reported by Hill and James (1972 a). Curve V is representative of the curve of cells (irrespective of methicillin sensitivity) after treatment with sodium metaperiodate (i.e. no surface teichoic acid), this is typical of a carboxyl surface. The decrease of mobility at pH 6.0 - 4.5 on curve III for resistant cells grown at 37°C is most probably due to the partial decrease in the ionisation of carboxyl groups; this minimum occurring because of a change in the conformation of the surface teichoic acid, which brings ionised phosphate groups to the surface. It is these groups which subsequently result in the increase of the negative mobility between pH 3.0 and 4.0. The teichoic acid conformation associated with methicillin sensitive cells grown at any temperature in the pH region 3.0 to 5.0 (curve I) must be such that the number of ionised carboxyl groups on the surface are out-weighed by the number of phosphate groups arising from the teichoic acid. Cells of a resistant strain grown at 43°C (curve II) exhibit surface properties intermediate between those of cells of a resistant strain grown at 37°C and those of cells of a sensitive strain. However, on repeated growth of resistant cells at 43°C the pH - mobility curve approaches that typical of a sensitive strain (curve I). Cells of a resistant strain grown at 25°C were unaffected by periodate treatment and had a pH - mobility curve similar to IV. From a study of the charge densities of the normal and periodate treated cells at pH 4.0, the ratio of phosphate/ carboxyl groups at the surface was determined.

This ratio is an indication of the amount of surface teichoic acid. Although the growth of cells of both methicillin resistant and sensitive strains in the presence of inosine brought the cells to a common surface (curve IV), there was no change in the resistance or sensitivity of the cells; all cells showed a very high phosphatase activity. Marquis (1968) has reported that cells of Bacillus megaterium contracted in volume when transferred from water into unbuffered, nonplasmolyzing sodium chloride solutions. A difference in volume was also obtained for cells suspended in buffer solutions of varying ionic strengths. It was concluded that environmental pH and ionic strength were important factors affecting wall structure in bacterial cells; the primary process in salt induced contraction of nonplasmolyzed B. megaterium cells was due to electrostatic wall contraction rather than any osmotic response of the cells. Such changes in cell volumes have been postulated as the reason for the differences in mobility values (Marquis and Carstensen, 1973).
The charge density associated with some methicillin sensitive and resistant cells at pH 4.0 and 7.0 are shown in Table 7.3.

Table 7.3

The charge density of some methicillin sensitive and resistant cells after growth on different media at 37°C

Strain	Growth medium	10^3 x charge density /C m^{-2}	
		pH = 4.0	pH = 7.0
Resistant	A (standard)	5.1	6.5
Resistant	E (high phosphate)	8.5	6.1
Resistant	H (inosine)	2.7	7.0
Sensitive	G ($Ca(OH)_2$ treated)	11.8	5.6
Sensitive	A	10.0	5.1
Sensitive	H	5.4	5.9

An indication of the change of surface area and, therefore, cell volume, assuming a solid spherical model particle can be obtained from the charge density data. Although small changes in the surface area and hence in volume to accommodate a decrease in charge density from 5.9 to 5.4 $C\,m^{-2}$ on change of pH (sensitive strain grown on medium H) would be feasible, larger changes (e.g. 5.6 to 11.8 $C\,m^{-2}$) requiring a doubling of the cell area would be very unlikely, unless the cell surface became very pitted for example by molecules of teichoic acid undergoing rearrangement at the surface. On the basis of solid spheres, such large changes which could amount to a factor of 2 to 3 in volume would be immediately obvious during electrophoresis experiments; no such changes in cell size were observed. This postulate, therefore, seems to be an unlikely explanation for the changes in electrophoretic mobility obtained in this study. It, therefore, appears that the various changes in the cell characteristics are due to cellular changes rather than to changes in environmental conditions.

In most penicillin resistant strains of Staph. aureus, penicillinase, the structural gene responsible for this resistance is carried on a plasmid (Novick, 1963). Methicillin resistance is independent of penicillinase production (Seligman, 1966; Dyke et al, 1966) and is, therefore, associated with different plasmids. Cohen et al (1972) found no linkage between the penicillinase and methicillin resistant plasmids, although other reports (Dornbusch et al, 1969; Dornbusch and Hallander, 1973; Dornbusch, 1973) indicate that in some strains of Staph. aureus there is a linkage between the plasmids. The penicillinase plasmid is temperature sensitive (May et al, 1964; Asheshov, 1966 a and b) as is the methicillin resistant plasmid (Al Salihy and James, 1972; Grubb and Annear, 1972). Present results show that methicillin resistance is an important factor in deciding the properties of the cells irrespective of their ability to produce penicillinase.

How can this methicillin resistant plasmid be used to explain the results obtained in this investigation ?
When grown at 37°C on standard growth medium, the cells carrying this plasmid exhibit a high alkaline phosphatase activity; the possible purpose of this enzyme system could be to remove surface teichoic acid from the cells. The question of the purpose of removing the teichoic acid from resistant but not from sensitive cells then arises. These resistant cells have little or no detectable surface teichoic acid; teichoic acid may be completely absent from the cell surface or walls or it may be replaced by another polymer i.e. teichuronic acid. There appears to be no real need for an additional cell wall polymer such as teichoic or teichuronic acid since these are presumably on the outside of the wall and are not, therefore, important structural features.
An additional or alternative possibility is that the plasmid responsible for methicillin resistance is also responsible for the formation of teichuronic acid. When the resistant cells are grown on media containing excess inorganic phosphate, the phosphatase activity is repressed and more surface teichoic acid is produced. This is a reversible phenomenon over relatively few subcultures, therefore, the action of the plasmid is not irreversibly changed, whether extensive culturing in the presence of high concentrations of inorganic phosphate will eventually bring about the irreversible loss of phosphatase production is not known. This could be some kind of blocking procedure with alternative pathways becoming operative. Growth of resistant cells in the presence of excess inorganic phosphate had no effect on the resistance of the cells to methicillin. Growth on inosine resulted in a complete loss of teichoic acid and a large increase in phosphatase activity.

This phenomenon is, however, reversible, the cell surface reverting to the normal surface characteristics of a methicillin resistant cell when the cells were grown in the absence of inosine. The methicillin resistance of the cells is not affected by growth in the presence of inosine.

Repeated growth at 43°C on standard medium, however, caused irreversible loss of the methicillin resistant plasmid, thus resulting in methicillin sensitive cells. These cells exhibited no phosphatase activity but now possessed a large amount of surface teichoic acid. In contrast, when methicillin resistant cells were subcultured at 43°C on high phosphate medium for the same number of times as on standard medium, there was no change in either the surface properties or resistance to methicillin. Thus, it would seem that the plasmid is not so readily lost at high temperatures in the presence of excess phosphate as in the presence of low concentrations of inorganic phosphate.

When grown at 25°C, the cells became completely resistant to methicillin, had a high phosphatase activity and no surface teichoic acid. Growth in the presence of inosine produced no change in the surface properties of the cells, thus indicating that this plasmid is not lost during growth at 25°C.

In addition to controlling the resistance of the cells, the methicillin resistant plasmid could also control the formation of the phosphatase enzyme system which could, in turn, prevent the formation of teichoic acid at the surface, or at least, never let it accumulate.

Alternatively, it could be responsible for causing a change in the metabolic pathways of the methicillin resistant cells, as distinct from the methicillin sensitive cells, whereby teichuronic acid is formed at the cell surface and not teichoic acid.

The surface properties and phosphatase activity of methicillin sensitive cells grown in the presence of inosine were very similar

to those of the resistant cells but their sensitivity to methicillin remained unchanged. Growth medium containing inosine will have a very low concentration of free inorganic phosphate, therefore, these observations can be understood by considering the need for the enzyme to break down the phosphate esters to provide the inorganic phosphate which is necessary for the normal metabolic purposes of the cells.

Pechey and James (1973) found that there was a direct linkage between the lipid production at the cell surface and gentamicin resistance in cells of P. aeruginosa. Thus, in a similar way, the methicillin resistant plasmid could be controlling the formation of the alkaline phosphatase enzyme system which could in turn be attempting to put up a barrier against the antibiotic in such a way that teichuronic acid is more impermeable to methicillin than is teichoic acid; in this respect, easy access of the antibiotic to the target site could be prevented. It is apparent, however, that this barrier is not impermeable to benzyl penicillin since the production of penicillinase is independent of both the phosphatase enzyme system and the surface teichoic acid; this could be due to the stereochemistry of the antibiotics, since the methicillin molecule is very much bigger than the benzyl penicillin molecule. Resistance to benzyl penicillin is known to be due to the production of an extracellular enzyme - penicillinase; this mechanism of resistance is thus different from that of intrinsic methicillin resistance.

It is, therefore, concluded that the same plasmid is controlling methicillin resistance, alkaline phosphatase production and either directly or indirectly the formation of teichoic or teichuronic acid.

BIBLIOGRAPHY

Abramson, H.A., (1934) "Electrokinetic Phenomena" (The Chemical Catalog. Co. N. Y.)

Al Salihy, S.M.S. and James, A.M., (1972) Lancet 2 331

Annear, D.I., (1968) Med. J. Aust. 1 444

Annear, D.I., and Grubb, W.B., (1973 a) Lancet 1 110

Annear, D.I., and Grubb, W.B., (1973 b) Lancet 1 664

Archibald, A.R., Armstrong, J.J., Baddiley, J. and Hay, J.B., (1961) Nature 191 570

Archibald, A.R., and Baddiley, J., (1965) Biochem. J. 95 19C

Archibald, A.R., Baddiley, J. and Heckels, J.E., (1973) Nature 241 29

Armstrong, J.J., Baddiley, J., Buchanan, J.G., Carss, B., and Greenberg, G.R., (1958 a) J. Chem. Soc. Pt. IV 4344

Armstrong, J.J., Baddiley, J., Buchanan, J.G. and Carss, B., (1958 b) Nature 181 1692

Armstrong, J.J., Baddiley, J., Buchanan, J.G., Davison, A.L., Kelemen, M.V. and Neuhaus, F.C., (1959) Nature 184 247

Armstrong, J.J., Baddiley, J. and Buchanan, J.G., (1960) Biochem. J. 76 610

Asheshov, E.H., (1966 a) Nature 210 804

Asheshov, E.H., (1966 b) J. Gen. Microbiol. 42 403

Baddiley, J., Buchanan, J.G., Rajbhandary, U.L. and Sanderson, A.R., (1962) Biochem. J. 82 439

Baird - Parker, A.C., (1963) J. Gen. Microbiol. 30 409

Balyuzi, H.H.M., Reaveley, D.A. and Burge, R.E., (1972) Nature New Biology 235 252

Bauer, H., Farr, D.R. and Horisberger, M., (1974) Arch. Microbiol. 97 17

Baykov, A.A., Braga, E.A., Avaeva, S.M., (1972) Febs. Letters 21 80

Benveniste, R. and Davies, J., (1973) Ann. Rev. Biochem. 42 471

Bessey, O.A., Lowry, O.L. and Brock, M.J., (1946) J. Biol. Chem. 164 321

Bloch, W. and Schlesinger, M.J., (1973) J. Biol. Chem. 248 5794

Bosmann, A.B., Case, K.R., Young F.E. and Chatterjee, A.N., (1974) Biochem. Biophys. Res. Commun. 57 1175

Bowne, S.W., Oudrey, J.A. and Wise, J.L., (1966) Nature 211 980

Brewer, J.E., (1966) Ph.D. Thesis, London

Button, D., Archibald, A.R. and Baddiley, J., (1966) Biochem. J. 99 11C

Cannon, F.D. and Hawn, C.V.Z., (1963) J. Bact. 86 1052

Cheng, K.J. and Costerton, J.W., (1973) J. Bact. 116 424

Cohen, S., Gibson, C.J. and Sweeney, H.M., (1972) J. Bact. 112 682

Coyette, J. and Ghuysen, J-M., (1968) Biochem. 7 2385

Csopak, H., (1972) Acta. Chem. Scand. 26 2417

Csopak, H., Garellick, G. and Hallberg, B., (1972) Acta. Chem. Scand. 26 2401

Day, D.F. and Ingram, J.M., (1974) Can. J. Microbiol. 19 1225

Dornbusch, K., Hallander, H.O. and Lofquist, F., (1969) J. Bact. 98 351

Dornbusch, K., (1973) J. Gen. Microbiol.76 13

Dornbusch, K. and Hallander, H.O., (1973) J. Gen. Microbiol. 76 1

Dyke, K.G.H., Jevons, P. and Parker, M.T., (1966) Lancet 1 835

Ellis, R., (1911) Z. Phys. Chem. 78 321

Ellwood, D.C., (1970) Biochem. J. 118 367

Ellwood, D.C. and Tempest, D.W., (1967) Biochem. J. 104 69P

Ellwood, D.C. and Tempest, D.W., (1968) Biochem. J. 108 40P

Engstrom, L., (1961) Biochim. Biophys. Acta. 52 36

Evans, A.E., (1948) J. Bact. 55 793

Gallant, J. and Stapelton, R., (1964) J. Mol. Biol. 8 431

Garen, A. and Levinthal, C., (1960) Biochim. Biophys. Acta. 38 470

Garrahan, P.J., Pouchan, M.I. and Rega, A.F., (1969), J. Physiol. 202 305

Garrett, A.J., (1965) Biochem. J. 95 60

Ghuysen, J-M., Tipper, D.J. and Strominger, J.L., (1965) Biochem. 4 474

Gittens, G.J., (1962) Ph.D. Thesis, London

Gittens, G.J. and James, A.M., (1961) Analyt. Biochem. 1 478

Gouy, G., (1910) J. Phys. Radium 9 457

Grubb, W.B. and Annear, D.I., (1972) Lancet 2 1257

Haest, C.W.M., De Gier, J., Op Den Kamp, J.A.F., Bartels, P. and Van Deenen, L.L.M., (1972) Biochim. Biophys. Acta. 255 720

Halford, S.E., Schlesinger, M.J. and Gutfreund, H., (1972) Biochem. J. 126 1081

Helmholtz, H., (1879) Ann. Phys. 7 237

Hepinstall, S., Archibald, A.R. and Baddiley, J., (1970) Nature 225 519

Heppel, L.A., Harkness, D.R. and Hilmore, R.J., (1962) J. Biol. Chem. 237 841

Hill, A.W., (1971) Ph.D. Thesis, London

Hill, A.W. and James, A.M., (1972 a) Microbios 6 157

Hill, A.W. and James, A.M. (1972 b) Microbios 6 169

Hill, M.J., James, A.M. and Maxted, W.R., (1963) Biochim. Biophys. Acta. 75 414

Horiuchi, T., Horiuchi, S. and Mizuno, D., (1959) Nature 183 1529

Hughes, A.H., Hancock, I.C. and Baddiley, J., (1973) Biochem. J. 132 83

Hugo, W.B. and Stretton, R.J., (1966) J. Gen. Microbiol. 42 133.

Ingram, J.M., Cheng, K.J. and Costerton, J.W., (1973) Can. J. Microbiol. 19 1407

Imsande, J., (1970) J. Bact. 101 173

Imsande, J., Zyshinol, J.W. and Mile, I., (1972) J. Bact. 109 122

Janczura, E., Perkins, H.R. and Rogers, H.J., (1961) Biochem. J. 80 82

Hepinstall, S., Archibald, A.R. and Baddiley, J., (1970) Nature 225 519

Heppel, L.A., Harkness, D.R. and Hilmore, R.J., (1962) J. Biol. Chem. 237 841

Hill, A.W., (1971) Ph.D. Thesis, London

Hill, A.W. and James, A.M., (1972 a) Microbios 6 157

Hill, A.W. and James, A.M. (1972 b) Microbios 6 169

Hill, M.J., James, A.M. and Maxted, W.R., (1963) Biochim. Biophys. Acta. 75 414

Horiuchi, T., Horiuchi, S. and Mizuno, D., (1959) Nature 183 1529

Hughes, A.H., Hancock, I.C. and Baddiley, J., (1973) Biochem. J. 132 83

Hugo, W.B. and Stretton, R.J., (1966) J. Gen. Microbiol. 42 133.

Ingram, J.M., Cheng, K.J. and Costerton, J.W., (1973) Can. J. Microbiol. 19 1407

Imsande, J., (1970) J. Bact. 101 173

Imsande, J., Zyshinol, J.W. and Mile, I., (1972) J. Bact. 109 122

Janczura, E., Perkins, H.R. and Rogers, H.J., (1961) Biochem. J. 80 82

James, A.M., (1957) Progr. Biophys. Biophys. Chem. 8 95

James, A.M., (1967)"Practical Physical Chemistry", 2nd Ed. Churchill Ltd., London, p. 144

James, A.M. and Brewer, J.E., (1968) Biochem. J. 107 817

Johnston, L.H. and Dyke, K.G.H. (1971) J. Bact. 107 68

Kalckar, H.M., (1947) J. Biol. Chem. 167 477

Komagata, S., (1933) Res. Electroteck. Lab. Tokyo No. 348

Kuo, M.H. and Blumenthal, H.J., (1961) Nature 190 29

Levinthal, C., (1959) Brookhaven Symp. Biol. 12 76

Lowick, J.H.B. and James, A.M., (1957) Biochem. J. 65 654

Malveaux, F.J. and San Clemente, C.L., (1969 a) J. Bact. 97 1209

Malveaux, F.J. and San Clemente, C.L., (1969 b) J. Bact. 97 1215

Mandelstam, J. and Rogers, H.J., (1959) Biochem. J. 72 654

Mandelstam, J. and Strominger, J.L., (1961) Biochem. Biophys. Res. Commun. 5 466

Marquis, R.E., (1968) J. Bact. 95 775

Marquis, R.E. and Carstensen, E.L., (1973) J. Bact. 113 1198

Marshall, N.J., (1969) Ph.D. Thesis, London

Marshall, N.J. and James A.M., (1971) Microbios 4 217

May, J.W., Houghton, R.H. and Perret, C.J., (1964) J. Gen. Microbiol. 37 157

Michaelis, L., (1931) Biochem. Z. 234 139

Noble, W.C., (1972) Lancet 1 929

Novick, R.P., (1963) J. Gen. Microbiol. 33 121

Ohmori, Y., (1937) Enzymologia IV 217

Ou, L., Chatterjee, A.N., Young, F.E., Marquis, R.E., (1973) Can. J. Microbiol. 19 1393

Park, J.T. and Strominger, J.L., (1957) Science N.Y. 125 99

Parker, M.T. and Hewitt, J.H., (1970) Lancet 1 800

Pechey, D.T. and James, A.M., (1973) Biomedicine Express 19 127

Plocke, D.J., Levinthal, C. and Vallee, B.L., (1962) Biochem. 1 373

Rogers, H.J., (1963) J. Gen. Microbiol. 32 19

Rogers, H.J., (1970) Bact. Rev. 34 194

Rogers, H.J. and Garrett, A.J., (1961) Biochem. J. 88 6P

Rolinson, G.N., (1971) Proc. R. Soc. Lond. 179 403

Rolinson, G.N., Batchelor, F.R., Stevens, S., Cameron-Wood, J. and Chain, E.B., (1960) Lancet 2 56

Sabath, L.D., Leaf, C.D., Gerstein, D.A. and Finland, M., (1970) Nature 225 1074

Salton, M.R.J., (1962) J. Gen. Microbiol. 29 15

Schleifer, K.H. and Kocur, M., (1973) Arch. Mikrobiol. 93 65

Seaman, G.V.F., (1965) "Cell Electrophoresis" Ed. E.J. Ambrose, J. and A. Churchill, p.4

Seligman, S.J., (1966) Nature 209 994

Shah, D.B. and Blobel, H., (1967) J. Bact. 94 780

Snyder, S.L. and Wilson, I.B., (1972) Biochem. 11 1616

Stern, O., (1924) Z. Electrochem. 30 508

Stiffler, P.W., Sweeney, H.M. and Cohen, S., (1973) J. Bact. 116 771

Strange, R.E. and Kent, L.H., (1959) Biochem. J. 71 333

Strominger, J.L., Blumberg, P.M., Suginaka, H., Umbreit, J. and Wickus, G.G., (1971) Proc. R. Soc. Lond. 179 369

Sutherland, R., (1964) J. Gen. Microbiol. 34 85

Sutherland, R. and Rolinson, G.N., (1964) J. Bact. 87 887

Torriani, A., (1960) Biochim. Biophys. Acta. 38 460

Wilkins, A.S., (1972) J. Bact. 110 616

Wolin, M.J., Archibald, A.R. and Baddiley, J., (1966) Nature, 209 484

Phosphatase activity of methicillin-resistant and methicillin-sensitive cells of *Staphylococcus aureus*

Anne L. Davies and Arthur M. James

Department of Chemistry, Bedford College (University of London), Regent's Park, London NW1 4NS, Great Britain

Abstract

Cells of naturally occurring methicillin-resistant strains of *Staphylococcus aureus* produce an alkaline phosphatase enzyme system when grown at 27 or 37 °C. In contrast cells of methicillin-sensitive strains grown at 27, 37 or 42 °C or resistant strains grown at 42 °C do not show any phosphatase activity. The production of the alkaline phosphatase is repressed by the presence of excess inorganic phosphate in the growth medium, although a low concentration of phosphate is necessary for good growth and high phosphatase activity. All the evidence suggests that the presence or absence of the alkaline phosphatase is intimately associated with the absence or presence of surface teichoic acid and with methicillin resistance or sensitivity respectively.

Introduction

Naturally occurring methicillin-resistant strains of *Staph. aureus* show a heterogeneous response to methicillin. They consist of mixed populations in which the vast majority of cells have a normal sensitivity to methicillin, with a slow-growing minority showing very high resistance. This heterogeneous population only occurs on growth at relatively high temperatures, *i.e.* 37°C (Sutherland and Rolinson, 1964), and when grown at extremes of temperature the cells show a totally different response to the antibiotic. On incubation at 25°C all the cells of a methicillin-resistant strain are highly resistant. However, on incubation at 43°C, all the cells in the population are very sensitive to even low concentrations of the antibiotic and after repeated growth at this higher temperature the cells become completely sensitive (Al Salihy and James, 1972).

Recent work has shown that cells of methicillin-sensitive strains of *Staph. aureus* possess surface teichoic acid when grown at 25, 37 or 43°C, whereas cells of resistant strains have surface teichoic acid when grown at 43°C, less at 37 and none at 25°C. Cells of a sensitive strain which have been repeatedly grown in the presence of methicillin have no surface teichoic acid when grown at any temperature (Hill and James, 1972a). These results were, it was concluded, consistent with the presence of a temperature-dependent enzyme system, previously suggested by Annear (1968) to explain methicillin resistance in *Staph. aureus*.

Since the differences in the amounts of surface teichoic acid on the cells of *Staph. aureus*, shown by particulate electrophoresis, could be a reflection of the varying amounts of phosphate at the cell surfaces, an investigation of the phosphatase activity of suspensions of antibiotic resistant and sensitive cells was carried out.

Materials and methods

A range of organisms with different antibiotic resistance was used (Table 1). The cells were maintained on nutrient agar medium (Oxoid CM3, batch num-

Table 1 Biological properties of the strains of *Staphylococcus aureus*

Code number	Strain number	Antibiotic characteristics
1	13136/60*	P(pen +), S, T, M
2	9341/67*	Sensitive
3	9322/67*	P (pen +)
4	4916**	P (pen +), S, T, M
5	9300/67*	Sensitive
7	10101/67*	P (pen +), S, T, M, E', No, N
8	Oxford	Sensitive
9	BRL 1800***	P (pen +), S, T, M

P = penicillin. S = Streptomycin. T = Tetracycline. M = Methicillin. No = Novobiocin. N = Neomycin. E' = Erythromycin (inducible). pen + = Penicillinase producer.
* Routine isolate from the Staphylococcal Reference Laboratory, Colindale, England.
** See Dornbusch *et al.* (1969).
*** Obtained from Beecham Research Laboratories Ltd (Used by Grubb and Annear, 1972).

ber 273 8762); this will be referred to as standard medium. The effect of various other growth media (defined later) on the phosphatase activity was studied. Organisms required for enzyme assay were obtained from cultures grown on nutrient agar medium, in the absence of any antibiotic, for 18 hr at the required temperature of 27, 37 or 42°C. The cells were harvested in distilled water, washed twice with water and finally resuspended in the appropriate buffer solution to a known concentration (0·2 to 0·3 mg organisms per cm^3). The concentration of the organisms in suspension was obtained from the measured absorbance at 620 nm and the previously determined calibration curve (absorbance — bacterial mass per cm^3).

Tris-HCl buffer solutions were used in the range pH 4·70 to 8·55 and sodium carbonate-bicarbonate buffer solutions in the range pH 9·30 to 10·40. At pH 11·10 a dilute solution (10^{-3} mol dm^{-3}) of sodium hydroxide was used.

Estimation of phosphatase activity

The method, based on the procedure of Ohmori (1937) and Bessey *et al.* (1946), uses 4-nitrophenyl phosphate as substrate. The bacterial suspension was thermally equilibrated in a water bath at 37°C and the enzyme reaction was started by adding the substrate, a solution of 4-nitrophenyl disodium orthophosphate. At suitable intervals, samples of the reaction mixture were removed and immediately mixed with 4·0 cm^3 of 0·20 mol dm^{-3} sodium hydroxide solution. This concentration of alkali was sufficient to stop further enzyme action and to develop fully the colour of the 4-nitrophenol liberated. The absorbance of each solution was measured at 400 nm against a blank taken at zero time. The linear Beer-Lambert plot of absorbance against concentration of 4-nitrophenol measured at 400 nm was used to determine the extent of the reaction.

The experimental procedure was established with cells of strain 1 grown on the standard medium at 37°C for 18 hr. To 10·0 cm^3 of a suspension of washed cells (0·2–0·3 mg cm^{-3}) at a thermal equilibrium of 37°C were added 2·0 cm^3 of an aqueous solution of 4-nitrophenyl disodium orthophosphate (12 mg cm^{-3}). A 2·0

cm^3 sample was removed from this mixture at regular intervals and added to 4·0 cm^3 of sodium hydroxide solution, and the absorbance of each solution was measured at 400 nm against the blank. After 5 min incubation the absorbance exceeded 0·9, it was therefore necessary to determine the initial enzyme activity from readings taken at 1, 3 and 5 min.

Maximum activity was observed when the pH of the reaction mixture was 10·10. The effect of concentration of substrate over the concentration range 0·6 to 30 mg cm^{-3} (Figure 1) was as expected for a normal enzyme system. The activity increased linearly with bacterial concentrations over the range 0 to 0·3 mg cm^{-3}.

The presence of magnesium ions (Engstrom, 1961; Garrahan *et al.*, 1969), in the assay mixture greatly enhanced the phosphatase activity of the cells (Figure 2) grown at the different temperatures. At high concentrations a precipitate formed, and this interfered with the measurement of the absorbance. The activity was measured over the temperature range 10 to 43°C, and the temperature of maximum activity was 37°C; for the enzyme reaction $\Delta H = 16{\cdot}3\ kJ\ mol^{-1}$.

As a result of extensive studies, the optimum conditions for the assay were established. To 10·0 cm^3 of bacterial suspension (0·1 to 0·15 mg cm^{-3}) in the appropriate buffer solution at 37°C were added 2·0 cm^3 of a freshly prepared substrate solution, made up in buffer solution pH 10·10, containing added magnesium chloride (20 mg cm^{-3} 4-nitrophenyl disodium orthophosphate, 0·25 mg cm^{-3} $MgCl_2$). A 2·0 cm^3 sample was removed after 0, 1 and 3 min and mixed with 4·0 cm^3 of 0·2 mol dm^{-3} NaOH solution. The absorbance of each

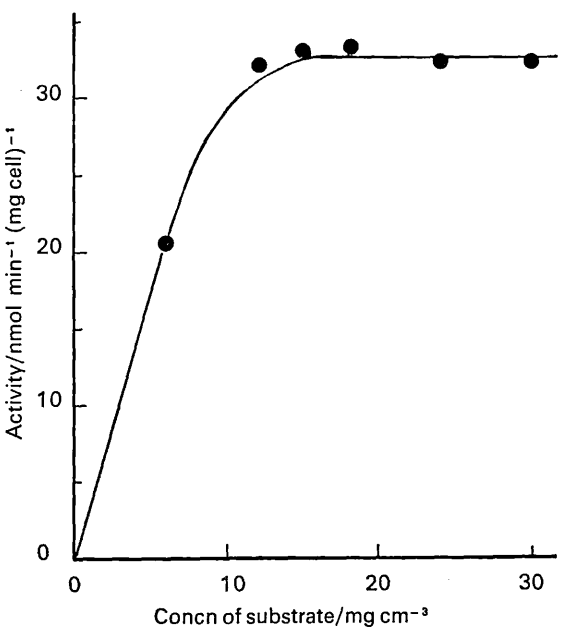


Figure 1 Variation of phosphatase activity with concentration of substrate for cells of *Staph. aureus* (strain 1) grown at 25 °C for 24 hr.

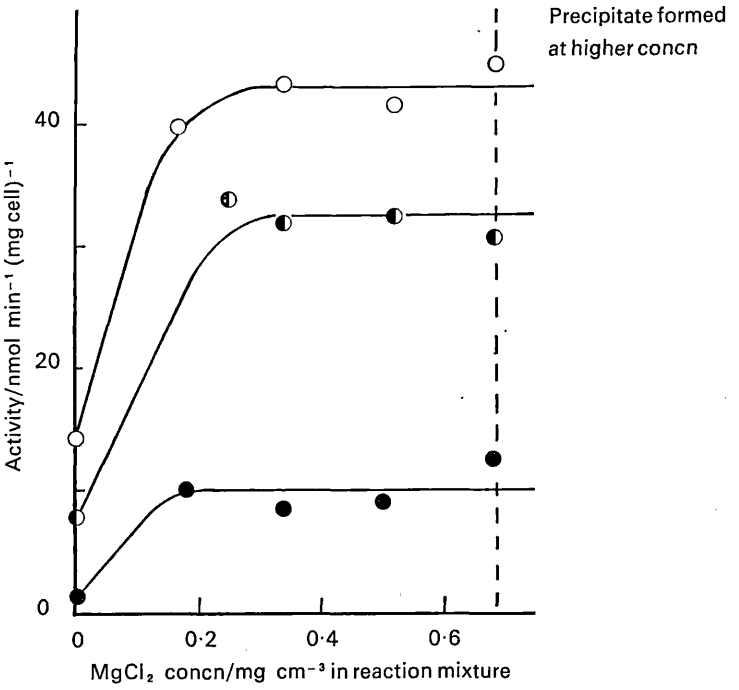


Figure 2 Variation of phosphatase activity with concentration of magnesium ions in the assay mixture for cells of *Staph. aureus* strain 1 grown at: ◐ 27 °C, ○ 37 °C, ● 42 °C. The broken line indicates precipitate formed at higher concentration.

solution was measured at 400 nm against the blank removed at zero time. From the initial slope of the absorbance-time plot and the known dry weight of cells, the activity of the enzyme was expressed as mol 4-nitrophenol formed per min per mg dry weight of cells [mol min^{-1} (mg cell)$^{-1}$]. Analysis of replicate determinations on the same strain revealed that the confidence limit for a single mean at $P = 0{\cdot}05$ is $\pm$ 2%. Repeated washing of the cells had no effect on the phosphatase activity, suggesting that the enzyme is firmly located in the cell surface.

Results

During the course of these studies, the original batch of Oxoid nutrient agar became depleted and it was replaced by a later batch of 'allegedly identical medium', Oxoid CM3, batch number 272 11382. The phosphatase activity of cells of strain 1 grown on this medium was zero. According to the manufacturers the preparation of the medium had been changed between the two batches, and the practice of treating the medium with calcium hydroxide to precipitate out inorganic phosphate has now been discontinued. There was only a trace of phosphate in the original batch of medium, while later batches contained large amounts of phosphate; this inorganic phosphate was obviously inhibiting the formation of the phosphatase enzyme system. Studies were therefore made on

cells grown on: (a) the original medium to which known amounts of ammonium phosphate had been added; (b) various batches of other commercially available media; (c) media prepared from Oxoid CM1 broth, treated for varying periods of time with calcium hydroxide (13 g of broth powder dissolved in 100 cm^3 of distilled water were stirred with 4·7 cm^3 of 10% $Ca(OH)_2$ suspension for a fixed period of time. The precipitate was filtered off and the supernatant neutralized to pH 7·0 and solidified with 1% agar. A pH of 7·0 was chosen as this produced good growth and high enzyme activity); and (d) various batches of media containing different amounts of a nucleoside, inosine (Csopak *et al.*, 1972).

Cells of strain 1 were grown for 18 hr at 37°C on each medium and the phosphatase activity was measured at pH 10·10. The results (Table 2) are presented as a percentage of the activity of the same cells grown in parallel on the standard Oxoid medium. This method of expressing the activity was necessary to allow for the variation of the age of the parent used for the inoculum. High concentrations of phosphate in the growth medium inhibited the formation of the phosphatase enzyme system and so gave rise to a reduced activity. There was, however, a low minimum concentration of inorganic phosphate that was required for good growth and high activity. In contrast, the addition of ammonium

Table 2 Phosphatase activity of cells of methicillin-resistant *Staph. aureus* after growth on different media

Basic medium	Treatment	Comments	Activity (%) relative to standard cells
Oxoid CM3 agar 273 8762	Ammonium phosphate added	Control cells	100
		10^{-7} mol dm^{-3}	93
		5×10^{-7}	91
		10^{-6}	127
		5×10^{-6}	103
		10^{-5}	95
		5×10^{-5}	65
		10^{-4}	4
		10^{-3}	0
		10^{-2}	0
Oxoid CM3 agar 272 11382			0
Difco agar 55 463			0
Oxoid CM3 agar 152 13727			0
Oxoid CM1 broth 297 14154	Treated with $Ca(OH)_2$ and solidified	Time of contact with $Ca(OH)_2$	
		5 min	2
		10	26
		15	29
		20	57
		30	19
Oxoid CM3 agar 273 8762	Inosine added	Concn 5×10^{-4} mol dm^{-3}	128
Oxoid CM3 agar 152 13727	Inosine added	5×10^{-3} mol dm^{-3}	33
		10^{-2}	86
		10^{-1}	49
Oxoid CM1 broth 297 14154	Treated with $Ca(OH)_2$ for 20 min, inosine added, solidified	Concn of inosine	
		5×10^{-4} mol dm^{-3}	56
		10^{-2}	80
		5×10^{-2}	44

phosphate to the assay medium has no effect on the phosphatase activity. Growth on commercially available media, which contained large amounts of phosphate again produced cells which had no phosphatase activity. Similar results were obtained when the cells were grown on synthetic media containing tris or phosphate or varying amounts of bactopeptone.

The removal of inorganic phosphate by precipitation as calcium phosphate improved the phosphatase activity considerably, but the activity was always less than that of cells grown on the standard Oxoid medium. It was concluded that the precipitate was removing some other essential component from the growth medium; the addition of magnesium or iron(III) salts did not improve the activity. The activity of cells grown on Oxoid agar (152 13727) media containing different concentrations of inosine increased with the concentration of the nucleoside, the greatest activity occurring at 10^{-2} mol dm^{-3} inosine. The phosphatase activity of cells grown on media, pretreated with $Ca(OH)_2$ and containing inosine also increased with increasing inosine concentration. The results, however, suggested that the inosine may have some effect on the media or the cells, other than the removal of inorganic phosphate. The effect of inosine on the surface and other properties of the cells is a subject of current investigations.

A quantitative analysis of the free inorganic phosphate present in the various growth media was carried out using the spectrophotometric method (James, 1967). The results (Table 3) confirm the fact that high concentrations of phosphate inhibit the formation of the phosphatase enzyme system.

In marked contrast to the high enzyme activity of cells grown on the standard solidified medium, there was little or no phosphatase activity for cells of strain 1 grown in broth for 18 hr (Table 4).

The phosphatase activity of cells of the methicillin-resistant strain 1 depended on the temperature of growth. Cells grown on agar at 37°C were used as an inoculum for plates which were grown for 18 hr at 27, 37 and 42°C. The pH-

Table 3 Concentration of free inorganic phosphate present in the various batches of media and its effect on the phosphatase activity of cells of *Staph. aureus* (strain 1)

Growth medium	Concentration of inorganic phosphate/μg cm^{-3}	Phosphatase activity %
Oxoid CM3 agar (273 8762)	10	100 (Control)
Oxoid CM1 broth (297 14154)	58	0
Oxoid CM1 broth (297 14154) treated with $Ca(OH)_2$	4	6
Oxoid CM3 agar (272 11382)	27	0
Oxoid CM3 agar (152 13727)	35	0
Oxoid CM1 broth (297 14154) solidified with agar	58	0
Oxoid CM1 broth (297 14154) treated with $Ca(OH)_2$ and solidified with agar	7	57
Oxoid CM1 broth (297 14154) treated with $Ca(OH)_2$ + 10^{-2} mol dm^{-3} inosine and solidified with agar	7	80

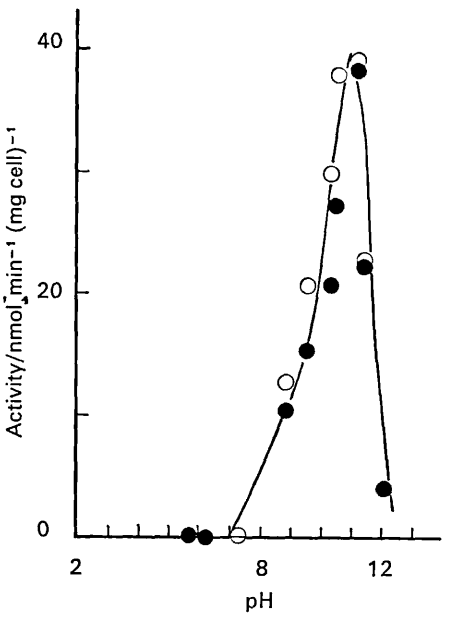


Figure 3 Variation of phosphatase activity with pH of assay medium for cells of *Staph. aureus* (strain 1) grown for 18 hr at: ○ 27 °C, ● 37 °C.

activity curves for the cells grown at 27 and 37°C were coincident, and growth at 42°C produced cells which had no detectable phosphatase activity over the range pH 4–11 (Figure 3). Repeated growth on agar at each of these 3 temperatures brought about no further change in the activity of 18 hr cells.

For a given parent inoculum, the activity of the daughter cells, grown at either 27 or 37°C, was independent of the age of the cells when harvested up to 48 hr later. However, the activity of the daughter cells depended upon the age of the cells used for the inoculum; cells originating from aged parent cells had a considerably reduced activity.

The strain used for these earlier studies was resistant to methicillin; it was also a penicillinase-producing organism. Cells of other strains (4 and 7) with multiple antibiotic resistance patterns (including methicillin) exhibited similar phosphatase activity when grown on the standard solid medium in the absence of any antibiotic (Table 5).

Table 4 Phosphatase activity of cells of *Staphylococcus aureus* (strain 1) grown in broth for 18 hr at 37 °C

Growth medium	Activity relative to that of cells grown on standard medium
Oxoid CM3 agar (273 8762)	100 (Control)
Oxoid CM1 broth (297 14154)	0
Oxoid CM1 broth treated with $Ca(OH)_2$	6
Oxoid nutrient broth no. 2, (272 13986)	5

Table 5 Phosphatase activity of cells of methicillin-resistant and -sensitive strains of *Staph. aureus* grown on standard medium for 18 hr

Strain	Methicillin	Phosphatase activity at pH 10·10/nmol min^{-1} (mg cell)$^{-1}$ at: 27°C	37°C	42°C
1	Resistant	38·91	38·40	0
4	Resistant	44·04	45·20	5·14
7	Resistant	30·22	39·53	4·72
2	Sensitive	0	0	0
5	Sensitive	0	0	0
3	Sensitive (pen +)	0	0	0
8	Sensitive	27·22	8·09	0

In marked contrast, cells of methicillin-sensitive strains (irrespective of other antibiotic resistance) showed no phosphatase activity, measured at any pH value, after 18 hr growth at any of the three temperatures. Cells of strain 8 (Oxford) were anomalous, in that although they were sensitive to methicillin, they exhibited phosphatase activity when grown at 27 or 37°C.

Cells of two sensitive strains (2 and 3) which had been trained to become resistant to methicillin by repeated growth on nutrient agar plates containing gradually increasing concentrations of methicillin (Rolinson *et al.*, 1960) had no phosphatase activity even though the MIC of cells at 37°C exceeded 200 μg cm^{-3}. The addition of methicillin (at concentrations up to 200 μg cm^{-3}) to the assay medium was without effect on the phosphatase activity of cells of methicillin-resistant strains.

It is an established fact that cultures of methicillin-resistant cells are biologically heterogeneous, and while the large majority of the cells are sensitive to the antibiotic, smaller numbers of cells will grow in the presence of up to 200 μg cm^{-3} of methicillin. Cells of the resistant strain 1 were subcultured in parallel on agar plates containing varying concentrations of methicillin. After 18 hr growth at 37°C, the colonies which had grown on each plate were picked off and subcultured on to standard nutrient agar plates (containing no antibiotic) and incubated at 37°C for a further 18 hr. The cells from each plate were harvested and the phosphatase activity measured (Table 6). Thus all cells selected from

Table 6 Phosphatase activity of cells of *Staph. aureus* (strain 1) after growth and selection in the presence of different concentrations of methicillin

Concn of methicillin in plate/μg cm^{-3}	Phosphatase activity at pH 10·10/nmol min^{-1} (mg cell)$^{-1}$
0	54·90
5	71·47
10	56·17
20	54·97
50	36·72
100	52·63
Average	54·5

such a heterogeneous culture produce cultures in which the cells have very similar phosphatase activities, irrespective of the individual resistance to methicillin.

Discussion

There are various reports in the literature of the alkaline phosphatase activity of cells of *Staph. aureus*, but there has been no systematic study of the relation of this activity to antibiotic resistance. The first indication that a temperature-dependent enzyme system was associated with methicillin resistance (Annear, 1968) was supported by the results of other workers (Hill and James, 1972b), who suggested that the enzyme system could be a phosphatase.

Using 4-nitrophenyl phosphate as substrate, the presence of an alkaline phosphatase enzyme system, with maximum activity at pH 10·10 and at 37°C, has been demonstrated in cells of strains of *Staph. aureus* with natural resistance to methicillin, when grown on standard nutrient agar medium. Cells of strains sensitive to methicillin (including completely sensitive and penicillin-resistant strains) had no such phosphatase activity.

The phosphatase activity in cells with natural resistance to methicillin was very dependent on the nature and composition of the growth medium. In contrast to the high activity shown by cells grown on standard nutrient agar, a very low activity was shown when the cells were grown in liquid nutrient broth. The lack of activity is probably related to the difference in the surface properties of cells grown in broth and on nutrient agar (Marshall, 1969). Enzyme action of some extracellular products may be responsible for the change in surface properties. On nutrient agar, extracellular enzymes can diffuse away from a colony into the agar, but in broth the cells are continuously immersed in a dilute enzyme solution and hence more susceptible to enzyme attack.

Inorganic phosphate in the enzyme assay mixture had no effect on the phosphatase activity, but when phosphate was present in excess in the growth medium, the formation of the enzyme system was repressed. Shah and Blobel (1967) have shown that *Staph. aureus* can produce an alkaline phosphatase, the formation of which is repressed by the presence of inorganic phosphate in the growth medium. However, a very low concentration of inorganic phosphate is essential for good growth and high phosphatase activity. Ellwood (1970) has reported that in a strain of *Bacillus subtilis*, the cell wall composition varied with the inorganic phosphorus content of the growth medium. In particular, when the cells were grown under conditions of limiting inorganic phosphorus the cell wall teichoic acids were replaced by teichuronic acids (phosphate-free polymers of N-acetylgalactosamine and glucuronic acid). When the cells were grown in medium with excess phosphorus, the cell walls contained a relatively large amount of phosphate.

It is possible to generalize these results for cells of *Staph. aureus* with natural resistance to methicillin (*e.g.* strain 1), since when the cells were grown on relatively low phosphate media, there was a high alkaline phosphatase activity and therefore, presumably, low phosphate content. Conversely, when grown on media containing a high concentration of phosphate, there was no activity,

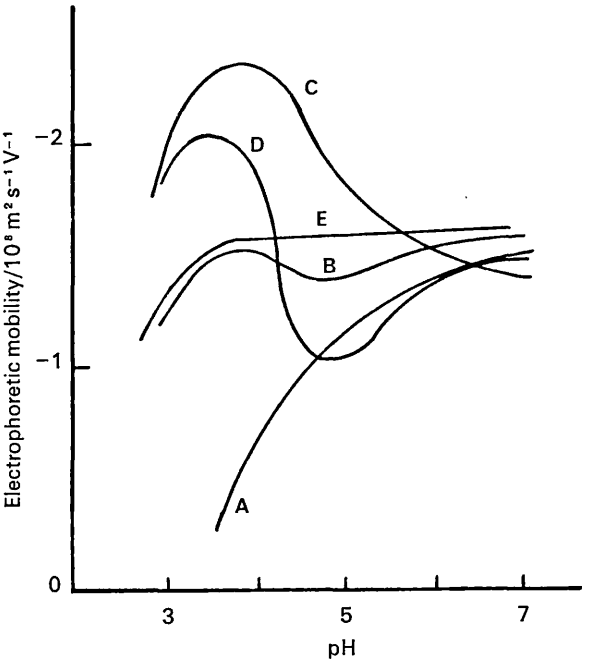


Figure 4 Typical pH-mobility curves for cells of *Staph. aureus* (after Hill and James, 1972a). A — periodate-treated cells. B — methicillin-resistant cells grown at 37 °C. C — methicillin-sensitive cells grown at 27, 37 or 43 °C. D — methicillin-resistant cells grown at 43 °C. E — methicillin-resistant cells grown at 25 °C.

indicating a high phosphate content in the cell wall. This relationship between phosphatase activity and surface phosphate groups is further supported by the shape of the pH-mobility curves of various strains of *Staph. aureus* (Figure 4), previously reported by Hill and James (1972a). Curve A is representative of cells after treatment with sodium metaperiodate (*i.e.* no surface teichoic acid) and this is typical of a carboxyl surface. The minimum mobility at pH 4·5, on curve B for resistant cells, is most probably due to the carboxyl groups exerting an effect before the change in conformation of the surface teichoic acid brings ionized phosphate groups to the surface. It is these groups which produce the increase of the negative mobility between pH 3 and 4.

The teichoic acid conformation associated with methicillin-sensitive cells and resistant cells grown at 43°C in the pH region 3 to 5 (curves C and D) must be such that the ionized carboxyl groups on the surface are outweighed by the phosphate groups at low pH. Cells of a resistant strain grown at 25°C were unaffected by periodate treatment and had a pH-mobility curve similar to E. From a study of the charge densities of the normal and periodate treated cells, at pH 4·0, the ratio of phosphate/carboxyl groups at the surface was determined (Table 7). This ratio is a measure of the amount of surface teichoic acid.

It is thus apparent that cells of sensitive strains possess considerable amounts of surface teichoic acid and hence phosphate, but exhibit no phosphatase activity. In contrast, cells of strains with natural resistance to methicillin show a parallel temperature-dependence of surface teichoic acid and alkaline phos-

phatase activity. Methicillin-resistant cells grown at 42°C have considerable amounts of surface teichoic acid but no alkaline phosphatase activity. At 37°C there is a smaller amount of surface teichoic acid and high phosphatase activity, while at 27°C there is no detectable teichoic acid but high phosphatase activity. It is interesting to speculate on the correlation between these two factors. Since there is no phosphatase activity in cells of sensitive strains (grown at any temperature) or in methicillin-resistant cells grown at 42°C (at which temperature they are sensitive to the antibiotic), teichoic acid accumulates and is evident at the surface. Cells of methicillin-resistant strains grown at lower temperatures produce a phosphatase enzyme system which is responsible for the removal of the phosphate groups from the teichoic acid, leaving behind teichuronic acids as the cell wall polymers. This phosphatase enzyme system is the temperature-dependent system associated with the temperature response of resistant cells of *Staph. aureus* to methicillin.

Two apparent exceptions have been observed. The antibiotic sensitive *Staph. aureus* (Oxford), strain 8, exhibited alkaline phosphatase activity when grown at 27 and 37°C, but no activity when grown at 42°C. This strain is, however, anomalous in many other respects. Strain 9 (MIC > 200 μg cm^{-3}) had a much lower phosphatase activity than that found in the other resistant strains studied (15% of the activity of strain 1 when grown at 37°C). The pH-mobility curve of cells of this strain grown at 37°C was more typical of a sensitive than of a resistant strain, and the ratio phosphate/carboxyl (Table 7) was 1·36 (Al Salihy, private communication). This value confirms the presence of considerable amounts of teichoic acid and is comparable with the value for cells of strain 1 grown at 43°C (sensitive to methicillin at this temperature), but is considerably lower than that for cells of a completely sensitive strain.

It is of significance that cells selected from a heterogeneous culture (strain 1 grown at 37°C) produced cells which had the same phosphatase activity irrespective of the individual resistance to methicillin. Hill and James (1972b) obtained a spectrum of pH-mobility curves for cells selected in this way, ranging from the typical curve for a naturally occurring resistant strain grown at 37°C to a curve characteristic of a simple carboxyl type surface for cells isolated from a 100 μg cm^{-3} methicillin plate thus showing the absence of teichoic acid. It is

Table 7 Ratio of phosphate/carboxyl groups on the surface of some strains of *Staph. aureus*, and its relationship to the phosphatase activity

Strain	Phosphate/carboxyl	Phosphatase activity %
3 (Sensitive)	4*	0
1 Grown at 25°C	0*	102
1 Grown at 37°C	0·4*	100 (control)
1 Grown at 43°C	1·2*	0
9 Grown at 37°C	1·36**	15–20

* Data of Hill and James (1972b).
**Al Salihy — private communication.

therefore apparent that although the cells exhibit a range of surface properties depending on the concentration of antibiotic on which they were originally isolated, they all possess the same phosphatase activity independent of the antibiotic concentration on which they were selected.

The work reported here gives an indication of the possible structural differences associated with the surfaces of cells which are resistant or sensitive to methicillin. The surface structure is controlled or influenced by a temperature-dependent enzyme system, and since the differences were found in the surface teichoic acid, the phosphatase group of enzymes seems to be the influencing factor. All the evidence suggests that the presence or absence of surface teichoic acid is intimately associated with methicillin sensitivity or resistance and this is further supported by the results of the study of the alkaline phosphatase activity of the cells. It is, however, impossible to say whether the presence or absence of such an enzyme system confers resistance or sensitivity to methicillin on the cells.

Acknowledgements

One of us (A.L.D.) is indebted to the Science Research Council for financial support.

References

Al Sahily S. M. S. and **James A. M.** 1972. Loss of methicillin-resistance from strains of *Staphylococcus aureus*. *Lancet* **ii** 331–2.

Annear D. I. 1968. The effect of temperature on resistance of *Staph. aureus* to methicillin and some other antibiotics. *Med. J. Aust.* **1** 444–6.

Bessey O. A., Lowry O. L. and **Brock M. J.** 1946. A method for the rapid determination of alkaline phosphatase with five cubic millimeters of serum. *J. biol. Chem.* **164** 321–9.

Csopak H., Garellick G. and **Hallberg B.** 1972. Purification of *Escherichia coli* alkaline phosphatase. *Acta Chem. Scand.* **26** 2401–11.

Dornbusch K., Hallander H. O. and **Lofguist F.** 1969. Extrachromosomal control of methicillin resistance and toxin production in *Staph aureus*. *J. Bact.* **98** 351–8.

Ellwood D. C. 1970. The wall content and composition of *Bacillus subtilis* var *niger* grown in a chemostat. *Biochem. J.* **118** 367–73.

Engstrom L. 1961. Studies on calf-intestinal alkaline phosphatase. *Biochim. biophys. Acta* **52** 36–48.

Garrahan P. J., Pouchan M. I. and **Rega A. F.** 1969. Potassium activated phosphatase from human red blood cells. The mechanism of potassium activation. *J. Physiol.* **202** 305–27.

Grubb W. B. and **Annear D. I.** 1972. Spontaneous loss of methicillin resistance in *Staph. aureus* at room temperature. *Lancet* **ii** 1257.

Hill A. W. and **James A. M.** 1972a. Surface properties of cells of methicillin-sensitive and -resistant strains of *Staphylococcus aureus* grown at 37 °C. *Microbios* **6** 157–67.

Hill A. W. and **James A. M.** 1972b. Effect of growth temperature on the surface properties of cells of *Staphylococcus aureus* with particular reference to methicillin-resistance. *Microbios* **6** 169–78.

James A. M 1967. *Practical Physical Chemistry*. 2nd Edn. Churchill Ltd, London, p. 144.

Marshall N. J. 1969. A correlation of changes in the cell walls of *Staph. aureus* with methicillin-resistance. Ph.D thesis. University of London.

Ohmori Y. 1937. Über die Phosphomonoesterase. *Enzymologia* **IV** 217–31.

Rolinson G. N., Batchelor F. R., Stevens S., Cameron-Wood J. and **Chain E. B.** 1960. Bacteriological studies on a new penicillin BRL 1241. *Lancet* **ii** 564–7.

Shah D. B. and **Blobel H.** 1967. Repressible alkaline phosphatase of *Staphylococcus aureus*. *J. Bact.* **94** 780–1.

Sutherland R. and **Rolinson G. N.** 1964. Characteristics of methicillin-resistant staphylococci. *J. Bact.* **87** 887–99.

Accepted 24 November 1973