SOME PHYSICAL CHEMICAL STUDIES OF ANTIBIOTIC SENSITIVE AND RESISTANT STRAINS OF STAPHYLOCOCCI

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by

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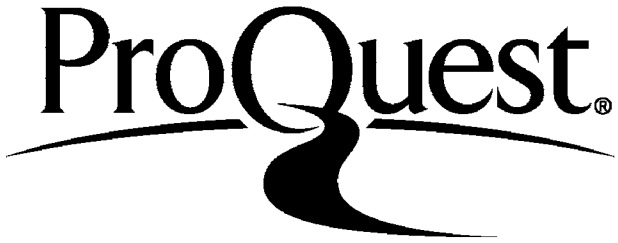

ABSTRACT

Staphylococcus aureus strains sensitive and naturally resistant to methicillin, were studied during this investigation using the technique of particulate electrophoresis. The effects of growth medium on the surface properties of the cell wall were studied.

Cells of strains of Staph. aureus which had typical characteristics of methicillin resistant strains, possessed small amounts of surface teichoic acid and showed a minimum in the negative surface charge at pH 4.5. The effect of repeated subculture at 25, 37 or 43 °C in the absence of antibiotic was studied by observing changes in the Minimum Inhibitory Concentration (MIC) and pH-mobility curves.

Cells grown repeatedly at 43 °C lost their methicillin-resistance and the ability to produce the enzyme penicillinase. The surface properties were markedly changed, the cells possessed considerable amounts of surface teichoic acid which were comparable to that of methicillin-sensitive strains. The genes controlling methicillin-resistance and penicillinase production are plasmid-borne, they were located on different plasmids, each plasmid being lost independently of the other; this was confirmed by the technique of replica plating.

Cells grown repeatedly on nutrient agar at 25 °C became more resistant to methicillin and lacked surface teichoic acid, those grown at 37 °C showed no change in resistance or surface characteristics.

Surface changes and loss of methicillin plasmid depended on the concentration of inorganic phosphate in the growth media. A method of preparing growth media containing a low concentration of inorganic phosphate was established.

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.

ACKNOWLEDGEMENTS

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Summary

Staphylococcus aureus strains, sensitive and naturally resistant to the antibiotic methicillin, were used during this work. The technique of particulate electrophoresis was used for studying properties of the bacterial cell wall. The effect of growth media and temperature of growth were studied, and some heritable properties which resulted from growth at high temperature.

The results reported during this work confirmed previous work (Annear, 1968; Hill and James, 1971 a,b) which supports the suggestion that the cells of naturally methicillin resistant strains exhibit surface properties which are dependent on the temperature of growth. When grown on nutrient agar in the absence of antibiotic, at 43 °C (a temperature at which the cells are sensitive to methicillin), teichoic acid is a surface component, the surface properties are similar to those of methicillin sensitive cells, grown at any temperature. Cells grown at 25 °C, a temperature at which they are resistant to methicillin, have no surface teichoic acid. The surface properties of cells grown at 37 °C were intermediate between those grown at 43 or 25 °C.

According to the above suggestion it is concluded that it was important to know if these changes of surface properties could be stabilised by maintaining the cells at the different temperatures. Cells resistant to methicillin (penicillinase producer and also tetracyclin, streptomycin resistant) were repeatedly subcultured in parallel on nutrient agar (Oxoid CM3 batch 8672273) in the absence of antibiotic at 25, 37 or 43 °C. After a number of subcultures at the given temperature the resistance to methicillin was determined at each temperature. Cells grown repeatedly at 43 °C became sensitive to methicillin and were penicillinase negative, while the cells grown at 25 °C became more resistant and still

produced penicillinase. On the other hand cells maintained at 37 °C were still methicillin resistant and penicillinase producers. Dornbusch et.al. (1969) found that when methicillin resistant strains were treated with acriflavin the cells became sensitive. This suggested extrachromosomal control of methicillin resistance, a process which is compatible with the present results, as high temperature is another "curing agent". Thus it is suggested that the extrachromosomal plasmid fails to replicate at the same rate as the bacteria and therefore becomes diluted out; further the plasmid does not reappear when the cells are grown at a lower temperature and even when it was left for months at room temperature.

As it is well known Staph. aureus strains produce the inducible enzyme generally known as penicillinase, this hydrolyses the β-lactam ring of penicillins and cephalosporins, with the production of the antibiotically inactive penicilloic acid, and cephalosporic acid. The structural gene for controlling this enzyme is usually borne on a plasmid (Novick, 1963); there is evidence for chromosomal location in a few strains (Ashoshov, 1966,a). This plasmid also carries the determinants of resistance to a series of inorganic ions, such as mercury, cadmium, bismuth, lead and arsenic (as arsenate). Spontaneous loss can occur from such a plasmid, but the frequency of loss can often be increased by certain physical and chemical agents notably elevated temperatures (May, et.al. 1964) and mutagens such as the acridines.

Replica plating (Lederberg et.al., 1952) was used to find out whether the two suggested plasmids in Staph. aureus strains resistant to methicillin, were independently lost or whether they are dependent on each other. Two different variants were isolated, these were methicillin sensitive but either penicillinase positive or penicillinase negative, which suggested that the genes for controlling both determinants were located on separate

plasmids. Both variants showed the same phage typing as the parent strain. Variants of penicillinase negative cells sensitive or resistant to methicillin were tested for loss of heavy metals resistance, they were all sensitive to these metals, this showed that they were located in the same plasmid as penicillinase. Variants of the methicillin sensitive, penicillinase producer were still resistant to the metals, which indicates no linkage between them.

All the studies were found to be affected by the growth medium, inorganic phosphate contained in the growth medium markedly affected the surface and resistance of the strains. Details of the preparation of a standard medium are reported with properties comparable to the good medium; this was used throughout the experimental work.

SECTION 1

Introduction

1.1 Classification, morphology and anatomy of bacteria

The majority of micro-organisms may be classified in the following large biological groups: (1) algae, (2) protozoa, (3) slime moulds, (4) fungi proper or Eumycetes, including the moulds and the yeasts, (5) bacteria or Schizomycetes, (6) Rickettsiales, (7) Mycoplasmatales and (8) viruses. The algae (except the blue-green algae), the protozoa, slime moulds and fungi include the larger and more highly developed micro-organisms; their cells have the same general type of structure and organisation, described as eucaryotic, that is found in the cells of higher plants and animals. The bacteria and the closely related blue-green algae, the organisms of mycoplasma and rikettsia groups include the smaller micro-organisms having a simpler form of cellular organisation described as procaryotic (Stanier and van Niel, 1962). The viruses are the smallest of the micro-organisms having simple structures that is not comparable with that of a cell, and their mode of reproduction is fundamentally different from that of cellular organisms.

Bacteria are small micro-organisms with a relatively simple and primitive form of cellular organisation. They are generally unicellular, but the cells may grow attached to one another in clusters, chains, rods, filaments or as in higher bacteria, a mycelium. Their cells are smaller (usually between 0.4 and 1.5 μm in short diameter) than those of protozoa and fungi; and in most cases they have relatively rigid cell walls which maintain their characteristic shape; this may be spherical (coccus) rod-shaped (bacillus), comma-shaped (vibrio), spiral (spirillum and spirochaete), or filamentous.

Microscopical examination is usually the first step taken for the identification of an unknown bacterium. The bacterium may be allocated to one or other of the major groups when its morphology and staining reactions have been observed. The morphological features of importance are the size, shape and grouping of the cells, and their possession of any distinctive structures such as endospores, flagellae, capsules and intracellular granules.

Staining reactions are observed after treatment by special procedures such as the Gram and Ziehl-Neelson stains, the different kinds of bacteria exhibiting various colours due to their permeability to certain decolourising agents.

The essential structures of the bacterial cell are shown in Fig. 1.1. The protoplast, i.e. the whole body of living material (protoplasm), is bounded peripherally by a very thin, elastic and semipermeable membrane called the plasma membrane, or cytoplasmic membrane. Outside, and closely covering this, lies the rigid, supporting cell wall, which is porous and relatively permeable. Cell division occurs by the development, from the periphery inwards, of a transverse cytoplasmic membrane and a transverse cell wall, or cross wall.

The cytoplasm, or main part of the protoplasm, consists of a watery sap packed with large numbers of small granules called ribosomes, which are visible only under an electron microscope. The nucleus, more properly called the nuclear body or chromatin body is centrally placed.

In addition to these essential structures, other intracellular and extracellular structures may be present in particular species of bacteria. In the cytoplasm, inclusion granules, consisting of volutin, lipid, glycogen or starch or membranous bodies called mesosomes. Outside the cell wall, there may be a protective gelatinous covering layer called

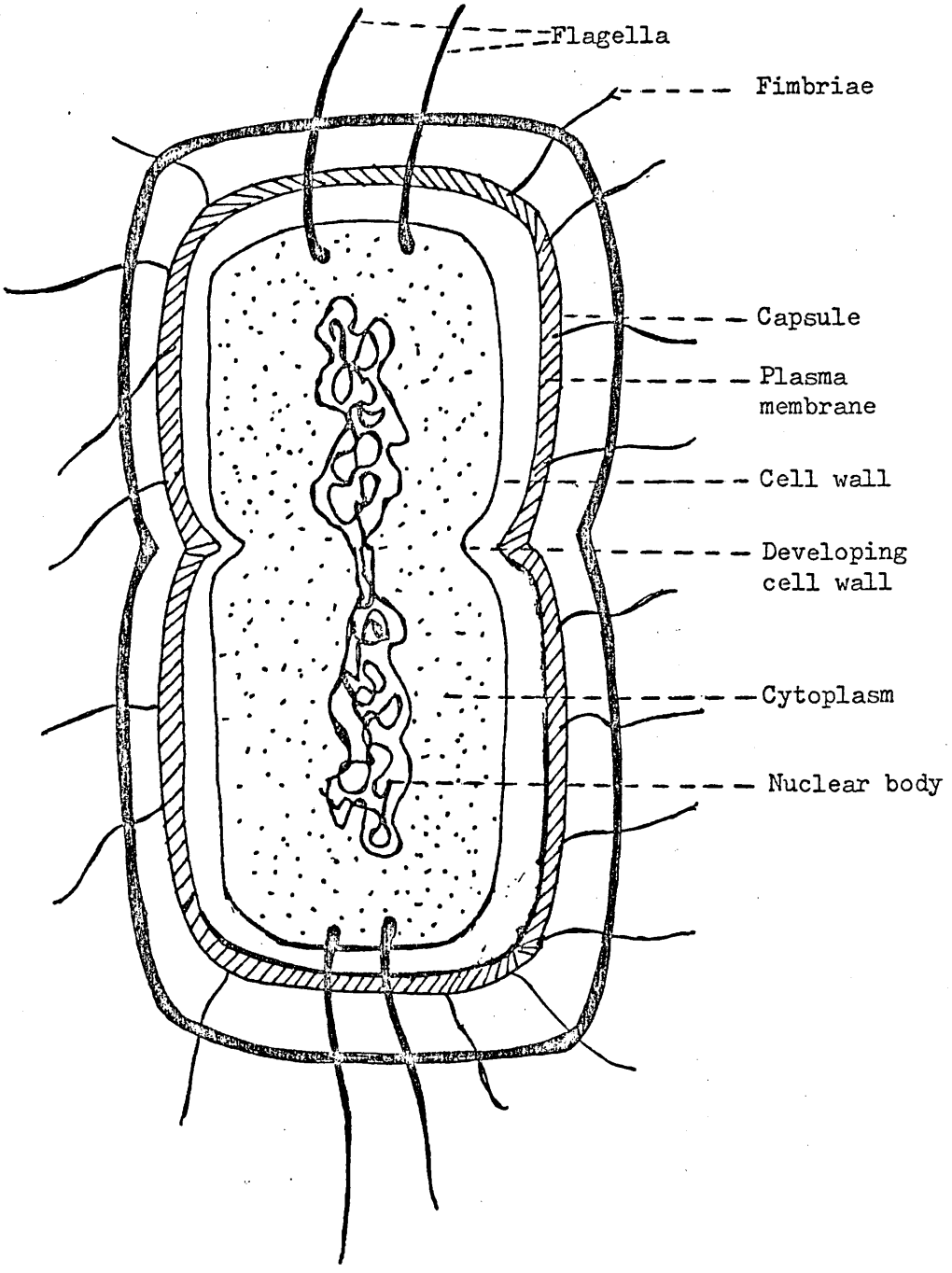


Fig. 1.1. The Structure of the Bacterial Cell

capsule or, when it is too thin to be resolved with the light microscope (< 0.2 μm) a microcapsule. Some bacteria bear, protuding outwards from the cell wall but originating in the cytoplasm (Murray and Brich-Anderson, 1963), one or both of two kinds of filamentous appendages, flagella, which are organs of locomotion, and fimbriae, which appear to be organs of adhesion. Because they are exposed to contact and interaction with the cells and humoral substances of the body of the host, it is the surface structures of bacteria, the cell wall, capsule, or microcapsule, flagella and fimbriae, that are most likely to have a special role in the processes of infection.

The bacteria are usually divided into ten orders, Pseudomonadales, Clamydobacterials, Hyphomicrobiales, Eubaceteriales, Actinomycetales, Caryoplanales, Beggiatoales, Myzobacteriales, Spirochate ales and Mycoplasmatales. The families are determined by cell shape, motility, the presence or absence of flagellae, if motile; reaction to the Gram stain, growth requirements, optimum temperature of growth, types of fermentations which the cultures are capable of carrying out under aerobic and anaerobic conditions; and other characters found to be of use in differentiation.

The morphology and staining reactions of individual organisms generally serve as a preliminary criterion, particularly for placing an unknown species in its appropriate biological group. A Gram-stained smear is first examined and this suffices to show the Gram reaction, size, shape and grouping of the bacteria, whether they possess endospores, and the shape, size and intracellular position of such spores. Furthermore, cultured characters, biochemical reactions, antigenic characters, typing of bacteria, animal pathogenicity and antibiotic sensitivity are valuable in the identification of the bacterial species.

1.2 Staphylococcus

The spherical Gram-positive cocci belonging to the family micrococcaceae and order Eubacteriales, are sub-divided into genera on the basis of the manner in which they cling together after fission. Micrococci and Staphylococci divide irregularly in more than one plane and can be in single cells or cling together in masses. Sarcina under favourable conditions, produce regular cubical packets of cells. Gaffkya divide at right angles under favourable conditions, to give rise to tetrads of cells.

The genera staphylococci and micrococci are perfect examples of ambiguity in taxonomy. Baird-Parker (1965) recommended that organisms producing acid from glucose anaerobically should be assigned to the genus of staphylococcus, and organisms which do not produce acid, or that do so oxidatively should be assigned to the genus micrococcus. This system fails, however, when many strains are considered. Cowan and Steel (1964) compared several methods for differentiating the staphylococci and micrococci and recommended that staphylococci should be divided into two groups; Staph. aureus (coagulase-positive) and Staph. epidermidis (coagulase-negative). Later it became known that there were three natural groups among Gram-positive, aerobic, catalase-positive cocci, which can be separated into three genera differing distinctly in the guanine and cytosine (GC) content of their DNA: Micrococcus (66 - 75% GC), Planococcus (39 - 51% GC) and Staphylococcus (29 - 37% GC) (Silvestri and Hill, 1965; Auletta and Kennedy, 1966; Rosypal et. al. 1966; Garrity et. al., 1969; Kocur et. al.,1971). It was found that the differences in the DNA base composition are accompanied by differences in the chemical cell wall composition of the organisms. Staphylococci differ from micrococci and planococci by the presence of glycine rich peptidoglycans in their cell wall (Schleifer and Kandler, 1970, 1972).

The existence of distinct types of teichoic acids in the cell walls of staphylococci may also be a valuable criterion for the differentiation of staphylococci (Baddiley et.al., 1968; Davison and Baddiley, 1963, 1964; Davison et.al., 1963).

Thus Staph. aureus, the organism used in this investigation, may be described as a Gram-positive, coagulase-positive, spherical cocci arranged in irregular clusters, the individual cells being approximately 1 μm in diameter. Single forms and pairs may also be noted. In films made from cultures a certain amount of breaking-up of the clusters occurs and a few short chains may be noted. Long chains are never formed. It is an aerobe and facultative anaerobe. The temperature range for growth is 10 - 43 °C; optimum 35 - 37 °C at pH 7.0 - 7.4. A uniform turbidity forms in broth cultures. Colonies are circular discs, relatively large after 24 h growth (2 - 4 mm). The colonies are opaque and convex with a shining surface and may be pigmented white, yellow, golden yellow or golden. This can be of epidemiological value as strains showing multiple antibiotic resistance and belonging to certain well-known phage-types usually produce yellow colonies, whilst strains of miscellaneous phage-types produce orange and buff colonies (Willis and Turner, 1963). The organism is non-motile, non-sporing and usually non-capsulate.

Numerous staphylococci are found in the air, dust and clothing. The relative and absolute numbers of this organism is greater in the hospital environment than in the home, and the environment reflects the rate of dissemination of the organism from human sources.

Staph. aureus occurs commonly in pyogenic lesions in the human subject. The great majority of these infections are superficial

inflammatory lesions with pus formation such as skin pustules, boils carbuncles and sticky eyes in babies. It is a common cause of wound suppuration and of mastitis in lactating mothers. It may be present in large numbers in the skin lesions of eczema and psoriasis. More serious and deep-seated infections are osteomyelitis, renal carbuncle, and localised abscesses. In a minority of cases pyaemia, septicaemia and malignant endocarditis may result from spread from a primary focus. The most pathogenic strains generally ferment mannitol, liquefy gelatin, produce coagulase, lypolytic enzymes and are β-haemolytic.

Staph. epidermidis is morphologically and culturally similar to Staph. aureus. Colonies are porcelain white or creamy in colour. It does not produce coagulase. It is much less active than Staph. aureus in its liquefaction of gelatin and fermentation of sugars. Most strains fail to produce haemolysin. It occurs as part of the normal flora of the skin. Whilst regarded as non-pathogenic, it has been reported in lesions such as acne pustules, urinary tract infections, and rarely in more serious lesions e.g. subacute bacterial endocarditis.

1.3 Phage typing

Generally speaking phages are exceedingly specific for the host bacteria in which they can multiply. Indeed, their specificity in this respect often allows a finer differentiation between types of a species of bacterium that can be obtained by any other method, e.g. serological method. This phenomenon is therefore often used to sub-divide single bacterial species into 'phage-types' according to the number and nature of the phages capable of causing their lysis, and has provided much valuable information on the spread of strains in epidemiological studies of infection in the community. If the reaction between the phage and corresponding strain of bacterium were specific the interpretation of results would be easy and allow designation of type according to the active bacteriophage. For

staphylococcoal coagulase-positive phage typing an internationally agreed set of phages has been assembled (2.1.C). Recently Dean, Williams Hall and Corse (1973) demonstrated the practicability of bacteriophage type-identification of the coagulase-negative staphylococci such as Staph. epidermidis. The typing set included 19 phages.

Wolin et.al (1966) have linked Staph. aureus phage sensitivity with chemical changes in teichoic acid complexes, and Coyette and Ghuysen (1968) have shown that phage-fixation requires 4-O-β-(N-acetyl-D-glucosaminyl) substitution in the D-ribitol units of teichoic acid, and also a definite configuration of the teichoic acid polymers. However, Rosato and Cameron (1964) have found receptor sites for phage 77 to be located within the mucopeptide.

1.4 Cell wall and surface

By cell wall is meant the thin (0.02 μm), sharply defined, relatively tough and rigid structure that can be clearly shown on electronmicrographs of cell sections. It overlies the cytoplasmic membrane and gives each bacterial species its characteristic shape. It is possible to remove the cell wall of an organism completely by muralytic enzymes; the resulting protoplast assumes a spherical shape regardless of the original form of the cell, thus demonstrating the lack of rigidity. In hypotonic solutions, osmotic pressure causes the protoplast to swell and burst.

The chemical composition of the cell wall differs considerably between different bacterial species, but in all species the main strengthening component is a peptidoglycan (glycopeptide) substance. The peptidoglycan is composed of N-acetylglucosamine and N-acetylmuramic acid molecules linked alternately in a chain, the acetylmuramic acid molecules each carrying a short peptide side-chain containing D- and L-alanine, D-glutamic acid and either L-lysine or diaminopimelic acid. The cell wall also contains some other components whose nature and amount vary with the species.

The Gram-positive cell wall, e.g. Staph. aureus, is characterised by well defined separate structures, distinct from the cytoplasmic membrane. On hydrolysis a limited number of amino acids are released, there is not very much protein and very low enzymic activity in the cell walls. Peptidoglycan may form 50% by weight of cell wall material in a multi-layer network. It contains only 1-5% cell wall lipid. If a capsule is present, this consists of simple polysaccharides and occasionally a small amount of polypeptide. The wall is very rigid capable of withstanding an osmotic pressure of up to 30 atmospheres.

On the other hand Gram-negative, e.g. Esch. coli cell walls have a very complex organisation of the outer cell layer, no clear boundary between the cell wall and adjacent structures, possibly layered. Hydrolysis yields up to 21 amino acids. The enzymic activity of the envelope is very high. Peptidoglycan formed 5-10% of cell wall material in monolayer. The envelope contains 20% by weight of cell wall lipid. The capsule, if present, is complex polysaccharide, protein-polysaccharide or lipopolysaccharide complexes. The osmotic pressure across the protoplasmic membrane generally is about 12 atmospheres.

A great interest has developed in the chemistry and biochemistry of bacterial cell walls during recent years. Walls are of interest because they frequently comprise up to 20% of the dry weight of cells and so must be regarded as important metabolites. Moreover, their chemical structure is interesting in view of their physical properties. Thus, besides being reasonably rigid structures with considerable strength they are nevertheless freely permeable towards cellular products and nutrients. Under certain conditions even large molecules such as antibodies, extra-cellular enzymes and nucleic acids can penetrate the wall. The wall is frequently the site of important antigenic material, for example, in many cases the group- or type-specific antigens are located in the outer structures of the cell, including the wall. Consequently, a better understanding of the immunological

properties of bacteria, and particularly such features as pathogenicity, require a full understanding of the structure, function and biosynthesis of wall components.

In Gram-positive cocci the cell wall is the immediate surface component. For this reason detailed consideration will be given to the structure and composition of the cell wall of strains of staphylococci.

Several methods have been used for the isolation of cell walls, most of which are based on those of Salton and Horne (1951). The bacterial cells are first ruptured by shaking cell suspensions with ballotini glass beads in a Mickle (1948) disintegrator. The rigid spherical cells of Staph. aureus are not easily broken by this technique and the Braun homogeniser, a very high energy form of the Mickle disintegrator is often used. The homogenate is then heated at 90^{o} for 10 min to destroy autolytic enzymes released from cells, which would otherwise degrade the cell walls. The walls are then separated from the undamaged cells by low speed centrifugation, and finally a pellet of crude wall preparation obtained by a high speed centrifugation. Robertson and Schwab (1960) used density gradient centrifugation to obtain homogeneous preparations. The material obtained is repeatedly washed first in either 0.1 mol dm^{-3} phosphate buffer solution, or 1.0 mol dm^{-3} sodium chloride solution and then with water. The protoplasmic membrane material is then removed from the walls by the action of enzymes such as trypsin, RNase or DNase (Cummins and Harris, 1956).

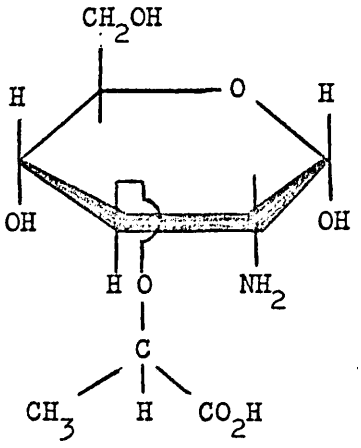
The general chemical composition of the cell of Gram-positive bacteria has been the subject of numerous investigations and the broad features of amino acid, amino sugar and monosaccharide constitution are now well known (Cummins and Harris, 1956, 1958; Work, 1957, 1961; Salton, 1953). Hydrolysis of cell walls with 6 mol dm^{-3} HCl at 120 ^{o}C of wide range of organisms for amino acids were examined. Alanine, glutamic acid and lysine or diaminopimelic acid were universally present, the last two being mutually exclusive. In

many organisms either glycine, or aspartic acid were also present. Rogers and Perkins (1968) have grouped Gram-positive species into groups, according to the amino-acids obtained from cell-wall preparations. The strains of Staph. aureus which were examined, contained only alanine, glutamic acid, lysine and much glycine. Some of amino acids showed the unusual D configuration, e.g. the alanine and glutamic acid found in Staph. aureus walls.

Cummins and Harris (1956) found that glucosamine together with another hexosamine were present; the latter was identified as 3-O-β-carboxyethyl-D-glucosamine (Fig. 1.2) (Strange and Kent, 1959). This compound since known as muramic acid, has been found in the cell walls of other eubacteriales and a few actinomycetes, blue-green algae and larger viruses.

The heteropolymer of N-acetylglucosamine and muramic acid was suggested to be a basis of cell wall in Gram-positive cell walls (Work, 1957; Salton, 1956), apparently few amino-acids combine with these two amino-sugars. Park (1952) discovered the presence of a nucleotide compound in the walls of staphylococci treated with penicillin, and showed later (Fig. 1.2) that the compound consisted of one molecule each of N-acetylglucosamine, glutamic acid, lysine and three molecules of alanine (Park and Strominger, 1957). It was suggested that the 'Park nucleotide' was a precursor for the proposed basic polymer complex. The structure of the polymer peptidoglycan (Fig. 1.3) was suggested after quantitative work (Strominger et.al, 1959; Mandelstam and Rogers, 1959; Rogers and Perkins, 1959, Hancock, 1960, Mandelstam and Strominger, 1961). Many other names have been used for this polymer, including glycosaminopeptide, glycopeptide, mucopeptide and murein. The general structure in Fig. 1.4 is an approximation intended to describe the peptidoglycan from the walls of strains of Staph. aureus. It is composed of polysaccharide chains in which N-acetylglucosamine and N-acetylmuramic acid alternate regularly. Attached to the carboxyl group of the muramic acid is

(a)



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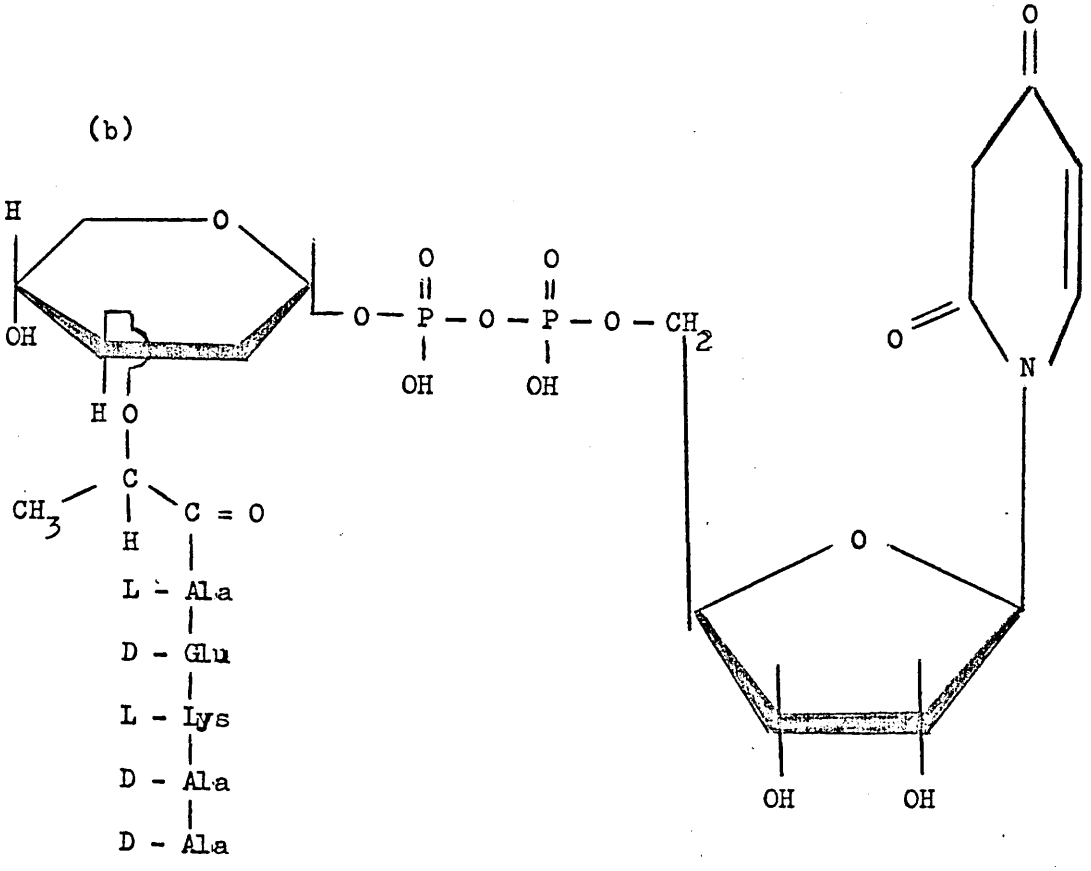


Fig. 1.2

(a) The structure of muramic acid (3-O-β-carboxyethyl-D-glucosamine).

(b) The structure of 'Park nucleotide'.

Fig. 1.3

The structure of staphylococcal peptidoglycan according to Mandelstam and Strominger (1961)

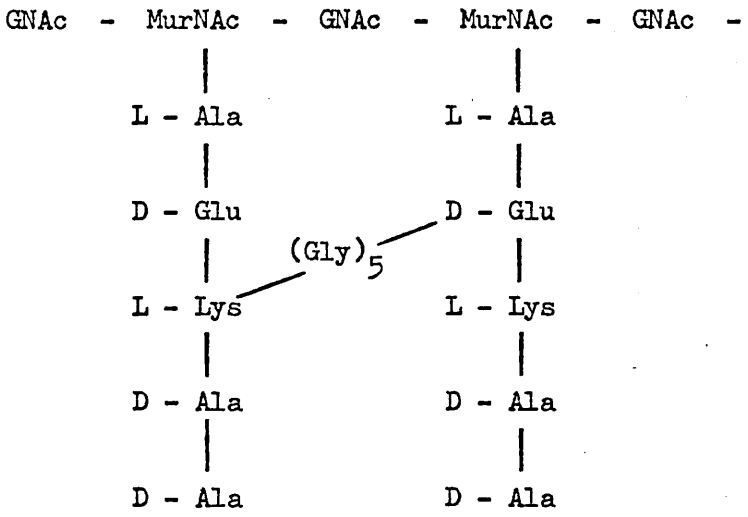
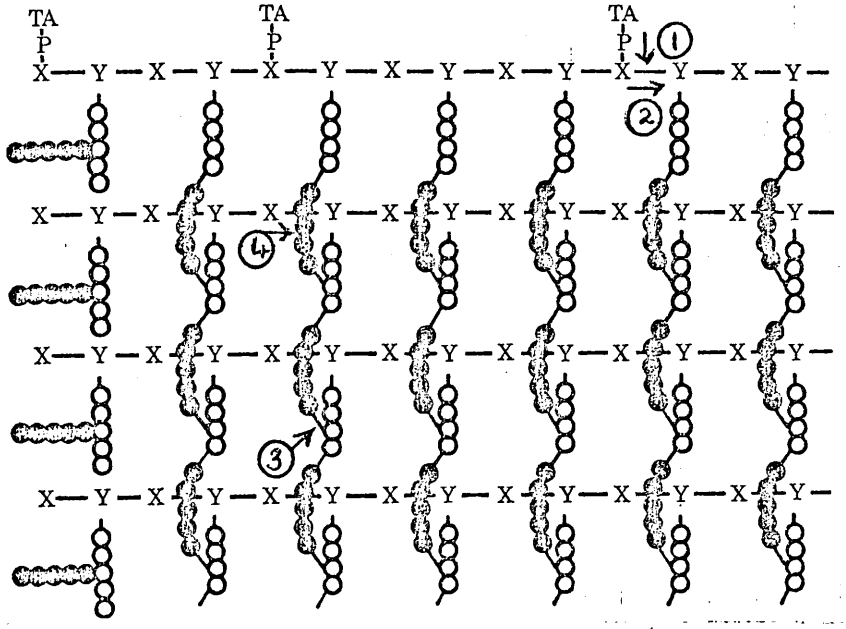


Fig. 1.4

Cell wall structure and origin of TA - peptidoglycan complex, Ghuysen et.al. (1965)



X = GNAc (N-acetylglucosamine

Y = MurAc (N-acetylmuramic acid)

⬤ = pentaglycine cross link

○ = amino acid in tetra or penta peptide

⟶ = indicates random break by hydrolysis

" 1 glycan with endo-N-acetylmuramidase

" 2 action of the N-acetylmuramyl-L-alanine amidase

" 3 opening of peptide bridges with peptidase

" 4 degradation of polyglycine " " "

a peptide composed of L-alanine, D-alanine, D-glutamic acid, L-lysine and five glycine residues. These pentaglycyl peptide chains cross-link from the ε-amino group of the lysine in one peptide chain to the free carboxyl group of the terminal alanine of the neighbouring peptide chain (Fig. 1.5), the free carboxyl group of the glutamic acid is substituted by an amide. Treatment of the wall with peptidase revealed that every tenth peptide chain on the N-acetyl muramic acid terminates with a di-D-alanyl group, the other nine having only a single terminal D-alanine (Petit, Muñoz and Ghuysen, 1966; Ghuysen, Tipper and Strominger, 1965).

Whereas it is likely that the peptidoglycan in a number of staphylococci is represented approximately by the structure (Fig. 1.4), it is now believed that in many bacteria the chemical structure of this wall component may differ markedly from the relatively regular network. For example, cross-linking may be less complete, it may occur between units in the same polysaccharide chain, and there may be variations in the nature and number of amino acids in the peptide chains. In other bacteria considerable variations in peptidoglycan structure have been noted; some lack the pentaglycine chains, whereas in others considerable numbers of muramic carboxyl groups are unsubstituted. The Staph. aureus mucopeptide is thought to be one of the most tightly linked.

Teichoic acids are known to be components of the outer layers of probably all Gram-positive bacteria; they apparently are not synthesised by any Gram-negative bacteria. Teichoic acid and structurally related sugar phosphate polymers occur covalently attached to the peptidoglycan in the walls of most Gram-positive bacteria (Archibald and Baddiley, 1966; Baddiley, 1972). Other teichoic acids, exclusively derivatives of poly (glycerol phosphate) are located in or on the surface of the cytoplasmic

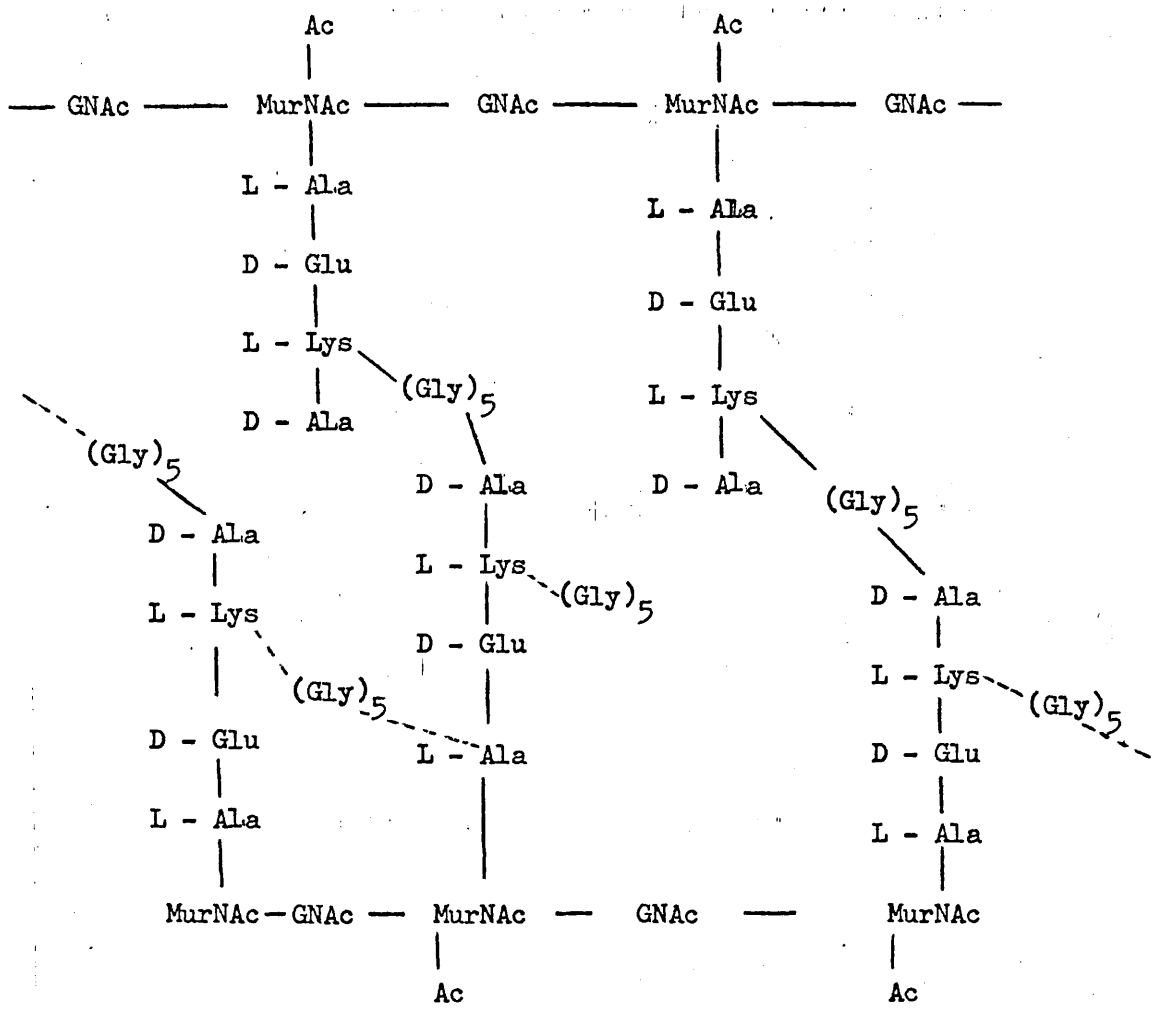


Fig. 1.5

The structure of peptidoglycan according to Rogers and Perkins (1968)

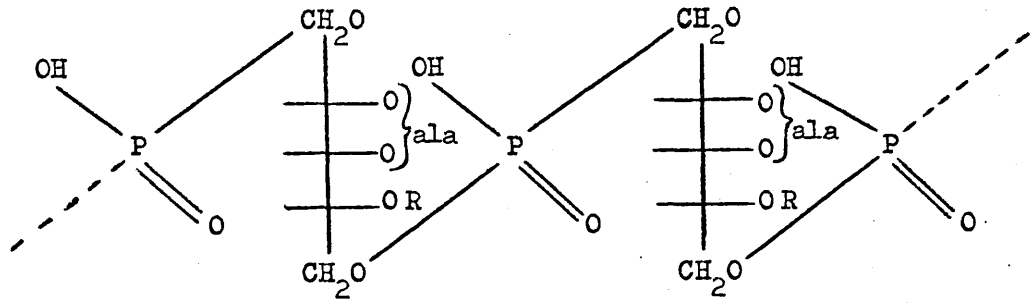
membrane of probably all Gram-positive bacteria (Hay et. al., 1963); these have been called membrane teichoic acids and are covalently attached to glycolipids or phosphatidyl-glycolipids in the membrane (Wicken and Knox, 1970; Toon et. al., 1972; Coley et. al., 1972).

The cell walls of staphylococci contain distinct teichoic acids (Davison and Baddiley, 1963). In Staph. aureus the polymer is of the ribitol type with α- and β-N-acetylglocosamine residues (Fig. 1.6,a) R, (Baddiley et. al., 1962) whereas in Staph. epidermidis it is of the glycerol type (Fig. 1.6,b), (Davison and Baddiley, 1964). There are many examples of organisms lacking these wall polymers, e.g. Lactobacillus casei and Bacillus cereus. Baddiley et. al.,(1968) also found that in all cases glycerol teichoic acids occur elsewhere in the cell. This so-called 'intracellular' teichoic acid which is found in the supernatant from disrupted cells after removal of walls. Studies with protoplasts have shown that 'intracellular' glycerol teichoic acids are located in the vicinity of the membrane, attached to it in an undefined manner. For this reason they are referred to as membrane teichoic acids.

Rogers and Garrett (1965) have shown that frequent linkages between teichoic acid and peptidoglycan do not occur in Staph. aureus and favour the view that the terminal phosphate group is involved in a linkage. Hay et. al., (1965) showed that this linkage could not be a diester linkage to an alcoholic hydroxyl group of the peptidoglycan, and proposed either the terminal reducing linkage of the peptidoglycan, or a phosphoramidate bond. All evidence so far is accumulating of teichoic acid being linked generally by its terminal residue to an amino-sugar in the peptidoglycan.

Thus the location of the teichoic acid polymer in walls of Staph. aureus is probably external to the peptidoglycan network and since it shows antigenic activity, it must be accessible to the exterior. The electron micrographs

(a)



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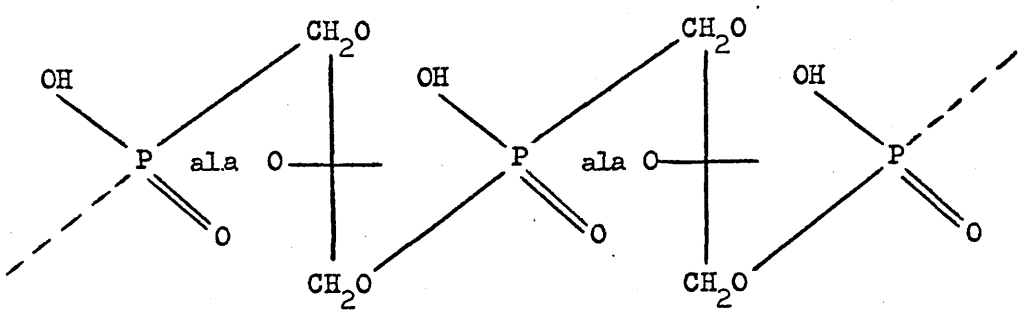


Fig. 1.6

(a) A ribitol teichoic acid.

(b) A glycerol teichoic acid.

of B. megaterium KM, shows a 100 Å thick rigid peptidoglycan layer which is surrounded by a 120 Å thick plastic layer of teichoic acid bristles (Nermut, 1967).

The role of teichoic acid in the cell is still unresolved; the most convincing data favours a role in the autolysis of Diplococcus pneumoniae cells (Mosser, and Tomasz, 1970). Mutants lacking teichoic acid have been obtained by phage selection in B. subtilis (Glaser, Ionesco and Schaeffer, 1966), Lactobacillus (Douglas and Wolin, 1971), Staph. aureus (Coyette and Ghuysen, 1968 ; Chatterjee, 1969). It has been shown that teichoic acids are associated with serological specificites (Davison et.al. 1964) receptor sites for bacteriophages (Archibald and Coapes, 1971) and ion transport (Heptinstall et.al., 1970).

The simplest and most direct study of the surface of intact bacteria involves the use of the technique of particulate electrophoresis. At any solid-liquid interface there exists a potential difference due to the asymmetrical distribution of ions. The present work is concerned with the phenomena of electrophoresis which is the movement of the solid phase relative to the liquid on the application of a potential gradient.

The concept of the double layer was first proposed by Helmhotz (1879), in this, two parallel layers of charge or ions, of uniform charge density, but opposite sign are held at a small distance apart, one firmly attached to the solid surface, and the other in the liquid. This theory, was later modified by Gouy (1910) who introduced the idea of a diffuse part of the double layer, in which the potential decreased exponentially to zero over the distance $^{1}/_{K}$, the statistical thickness of the double layer, an equilibrium in the diffuse layer being maintained between the opposing

forces of the potential field, tending to order the ions, and the forces of thermal motion tending to redistribute them randomly. Stern (1924) utilised both these hypotheses suggesting that the fall in potential at the interface can be divided into two parts; a sharp fall over the molecular condenser in contact with the surface where ions are fixed to the solid surface, followed by an exponential decrease over the diffuse outer atmosphere of the Gouy-Chapman theory (Fig. 1.7). A condition of electrical neutrality is maintained, since the surface charge is equal in magnitude, but opposite in sign to the total charge in the fixed and diffused parts of the double layer.

The phenomenon of electrophoresis arises when an electric field is applied, and particle movement relative to suspension liquid results. This causes the development of a shear plane within the double layer, and the particle velocity is governed by the magnitude of the potential at this plane, known as the zeta potential. The zeta potential is defined as the potential across the diffuse part of the double layer.

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 on to such surfaces (Gittens, 1962).

The method for the determination of the electrophoretic mobility used in this investigation is based on that of Ellis (1911). A bacterial suspension is contained in a closed glass chamber, and a potential difference is applied across the suspension from two electrodes. Using a microscope, the migration of the suspended bacteria is observed, and individual cells are timed moving across a graticule eyepiece. However, when an electric field is applied across a system, not only will the bacteria move relative to the suspension liquids, but due to electro-osmosis the suspension liquid will move relative to the glass surface of the observation chamber. Thus the observed particle velocity v_0, is given by the expression:

$$v_0 = v_L + v_P$$

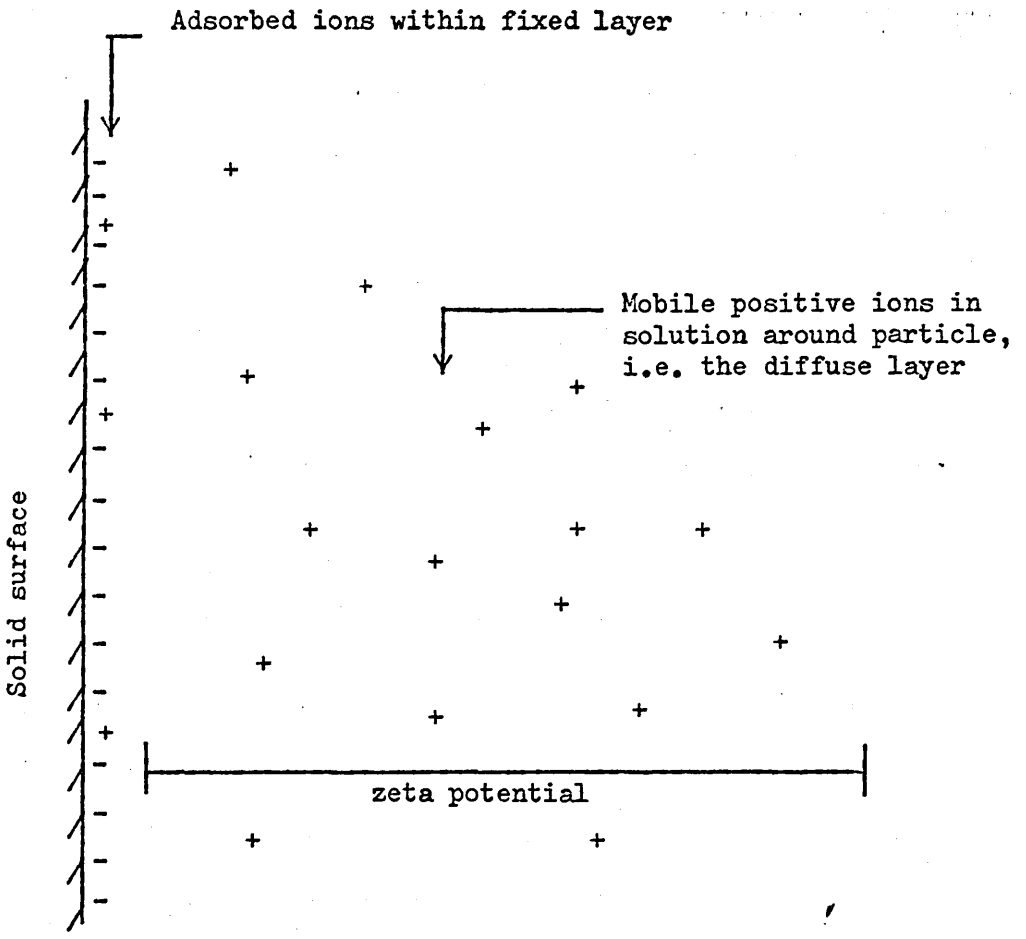


Fig. 1.7

The structure of the double layer at solid-liquid interface

where v_L is the velocity of the suspension medium relative to the glass walls and v_p the velocity of the particles relative to the liquid. In dealing with a closed system, the liquid will flow along the top and bottom faces of the chamber, and return through the centre resulting in a variation of v_L and hence v_0, with depth. As the liquid is being continuously deformed within a closed system, it follows that there must be a plane at which the liquid is stationary, at this $v_0 = v_p$. In the flat cell of rectangular cross-section used, this is equidistant from the cell centre. Komagata (1933) derived an expression for the position of the stationary levels in a cell of whose width/thickness ratio is K, such that

$$a_0 = \frac{1}{2} \pm \sqrt{\frac{1}{12} + \frac{32}{\pi^5 K}}$$

where a_0 is the fractional depth measured from the top inside surface. For a cell with K greater than 20, 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.

Cells and electrode design have been extensively reviewed by James (1957) and Seaman (1965). The cell used in this work was that described by Gittens and James (1961). With this apparatus, the applied field strength (X) is best calculated from conductance and current data, using the equation

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

where I is the current (amps), k is the specific conductance of the suspension medium, and q the cross-sectional area of the cell (m^2). Moyer (1936) showed that the use of the applied voltage to measure the field strength may lead to errors of up to 50%, since slight changes in the electrodes may result in large changes in the field strength, without affecting the applied voltage.

For the application of microelectrophoresis to the study of the bacterial cell surface, the measurements must be made on cells suspended in a medium of known chemical composition, pH and ionic strength. The nature of surface ionogenic groups on cells of a fixed age may be gained by varying the pH of the suspension medium, whilst the ionic strength is maintained at a constant value, e.g. with a carboxyl-amino type surface, the ionogenic groups will be titrated according to the equations:

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

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

At a low pH, the surface charge will be due to the positively charged amino groups, but on decreasing the hydrogen ion concentration, not only is the positive charge reduced, but the carboxyl groups ionise, giving rise to a negative charge. The Sigmoid type pH-mobility curve typical of a carboxyl-amino surface (Fig. 1.8) has an isopotential point between pH 4 and 5, the exact value being determined by the relative numbers of amino and carboxyl group and their pK values. Between 5 and 9, 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 as shown, due to supression of the ionisation of the amino group. Such curves have been obtained experimentally (Douglas, 1959; Plummer, et. al., 1962).

The pH-mobility curves characteristic of a surface with ionogenic carboxyl groups only are shown in Fig. 1.8, and were obtained for the polysaccharide surface of K. aerogenes (Lowick and James, 1957).

When varying the pH of the suspension medium, 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 re-washing and measuring the mobility at pH 7. The mobility value should not differ significantly from that of control cells at pH 7. An irreversible change indicates cell surface damage, making useful interpretation difficult. This variation of mobility with pH will only give information on the nature and quantity of surface ionogenic groups.

1.5 Antibiotic resistance and plasmids of staphylococci

The word 'antibiosis' was coined by Vuillemin (1889) to denote antagonism between living creatures in general, but the noun 'antibiotic' was first used by Waksman in 1942, who defined it as a substance produced by a micro-organism which is antagonistic to the growth or other activities of other micro-organisms in high dilution. As early as 1889, Doehle was recommending the clinical use of pyocyanase, produced by Pseudomanas pyocyaneus against the anthrax bacillus. However, these compounds were too unspecific in their toxic action to be used clinically. The outstanding feature of the penicillin group of antibiotics discovered by Fleming (1940), was their specific toxicity against bacteria, especially those of the Gram-positive species. Penicillin was first extracted from Penicillin notatum, with impressive clinical results (Abraham et.al., 1941). It had been necessary to find means of extracting a very labile substance from culture fluids, to examine its action on a wide range of bacteria, to examine its toxicity, to establish a unit of its activity and to prove its systematic efficacy in infections in mice.

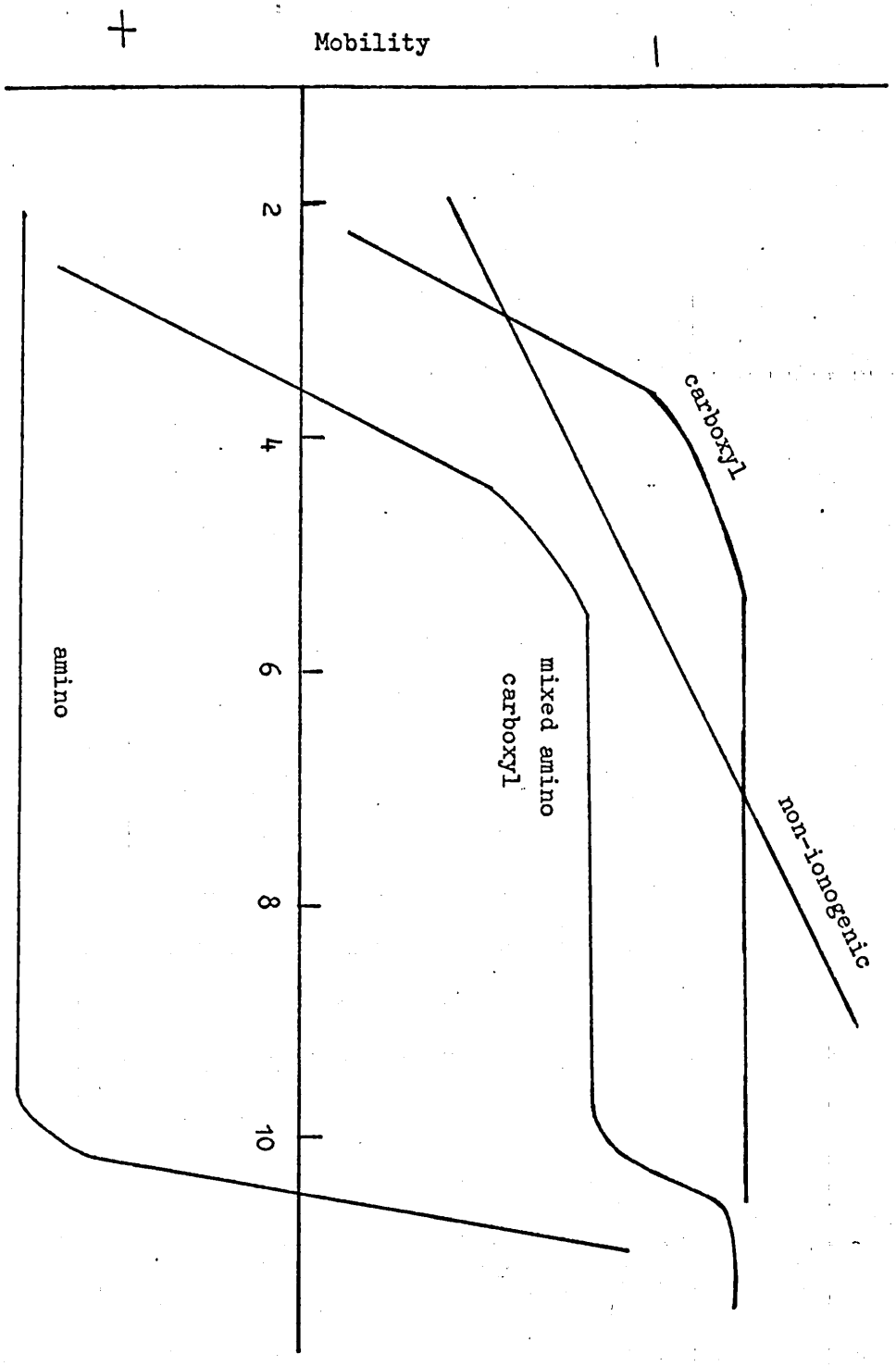


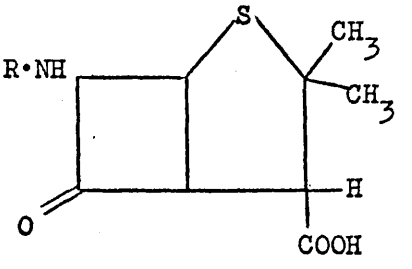
Fig. 1.8

Typical pH-mobility curves

Penicillin is the first of the antibiotics to come into general therapeutic use, and the first which was suitable for systemic use in man. It can be prepared in quantity only by the original process of cultivating a mould forming it (a high-yielding mutant of a strain of Penicillin chrysogenum is now used) in a suitable liquid medium. In early stages of large-scale production it was found that four different penicillins were formed, known as F, G, X and K. Of these G, or benzyl penicillin, had the most desirable properties, and its almost exclusive formation is ensured by adding the appropriate 'precursor', phenylacetic acid to the medium.

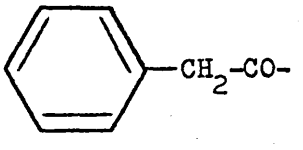
The penicillin molecule is shown in Fig. 1.9. The properties of the molecule differ according to the side-chain, R, penicillin G is the most commonly used penicillin, but the β-lactam ring in this compound is very easily opened by the action of penicillinase. The ring may be stabilised against this action by substituting different side chains; for methicillin, the penicillin of special interest in this investigation, the side group is 2-6-dimethoxybenzyl. Methicillin is readily soluble in water, but its solutions are very unstable. Neutral solutions lose 50% of their activity after 5 days at room temperature and 20% when stored at 5 °C. Acid solutions are much more unstable and at pH 2.0 half the activity is lost in 20 minutes at room temperature (Rolinson et.al., 1960).

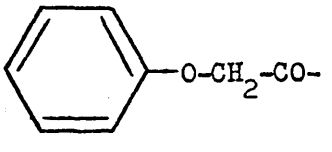
When a bacterial cell grows and divides, the final outcome is two cells where before there was one; every molecule in the parent cell has to be duplicated and inserted into its correct place during the process. Any chemical compound which hinders the cell's endogenous biosynthetic processes by restricting the building materials or catalytic enzymes or which interferes with the supply of usable energy, either directly or indirectly, will affect growth. The point at which the drug affects the cell is a single point 'target' or 'chemoreceptor'.



penicillin nucleus

The various forms differ in their side chain (R) as follows:

(1)  benzyl penicillin G

(2)  phenoxymethyl penicillin V

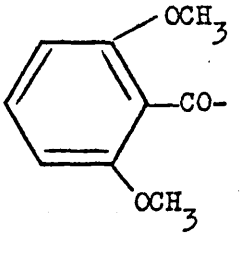
(3)  methicillin

Fig. 1.9

The naturally occurring penicillins (1),(2) and semi-synthetic penicillin (3).

The knowledge of the action of antibiotics has increased recently, it has become clear that many attack specific molecular targets, and much biochemical work has concentrated on investigating the nature of interaction of the drug with these molecules.

Antibacterial agents may be conveniently divided into four groups of which the effects centre about the synthesis of nucleic acid, of protein of cell membrane or of cell wall.

The effect of sulphonamides and diaminopyrimidines is ultimately to deprive the cell of nucleic acid, the effect of nalidixic acid is to prevent its replication. Exposure to nalidixic acid causes specific degradation of the chromosome just behind its replication point which then extends rapidly backwards to involve previously formed DNA (Ramareddy and Reiter, 1969). Rifamycins specifically inhibit the bacterial enzyme concerned with replication of RNA. This effect is brought about by the binding of one molecule of the antibiotic to one molecule of the enzyme.

It is thought that the site of antibiotic action on protein is at the initiation of polysome construction in which the first ribosome attached to the messenger is halted by streptomycin. The process of protein synthesis is essentially the same in all cells and the differential toxicity of antibacterial agents for different species depends either on differences in the ribosomes or on impermeability of the cell to the agent.

The lipoprotein layer of cell membrane controls the ingress into and of egress of material from the cells. The bundles of lipoprotein molecules are held together by magnesium ions in such a way that gaps are left through which molecules of suitable size can move. Some antibiotics unite with the membrane and function as ionophores, i.e. compounds which provide a way for abnormal movement of ions through the membrane. Therapeutically the most important agents which act on the membrane are the peptides. These consist of circular molecules, the attachment of which to the membrane modifies the ion flux and brings about lysis of the cell.

The penicillins are specific inhibitors of the biosynthesis of bacterial cell walls. This discovery was first made in 1957 and was based on two observations. First, penicillins induced the formation of protoplast or spheroplasts in bacteria in which the cell wall has been lost or weakened (Lederberg, 1957). Secondly, a uridine nucleotide accumulated in Staph. aureus and other bacteria inhibited by penicillin which had a striking relationship to composition of the cell wall (Park and Strominger, 1957). It was therefore suggested that this nucleotide was an activated precursor of the wall. A great deal of work was carried out to elucidate the structure of the bacterial cell wall and the mechanism of its biosynthesis from the uridine nucleotides and other precursors (Strominger and Ghuysen, 1967; Ghuysen, 1968). It was demonstrated that interpeptide cross-links were an important structural feature of the wall. Experiments carried out with whole cells indicated that the final step in cell wall synthesis, the cross-linking reaction catalysed by a transpeptidase, was the site of action of penicillin (Wise and Park, 1965). The cell wall polymer is weakened, and the high osmotic pressure of the cell pushes the delicate protoplasmic membrane through the weak wall, and cell lysis occurs. This is why high salt concentrations in the medium helps cells resist penicillin action. Penicillin inhibits glycopeptide biosynthesis, whereas lysozyme brings about hydrolysis of the β-1-6 linkages of the glycopeptide causing cell wall solubilisation. Penicillin can only bring about the lysis of actively growing cells, whereas lysozyme is active against resting cells.

Apart from cycloserine which interferes with the inhibition of the alanine linkage; most other antibiotics interfere with later stages of cell wall synthesis. Vancomycin interferes with the release of the phospholipid carried thus preventing further transport of new material, and bacitracin inhibits the next step in which the carrier, having delivered up its load, is regenerated in the active form.

Antibiotic resistance problems have long been recognised. In 1887 Kosaihof noted that bacteria tend to adapt to poisonous agents, and in the early days of chemotherapy, Erlich discussing the treatment of syphilis recommended the use of "massive doses" of salvarsan to guard against any resistant spirochetes which might have developed during prolonged treatment with smaller amounts. The problem became of major importance when the use of sulphonamides in the 1930's was severely limited by the development of resistant organisms. Soon after the introduction of penicillin as a therapeutic agent, there was evidence of the presence of penicillin resistant organisms (Barber, 1947; Barber and Rozwodowska-Dowzenke, 1948).

Resistance to penicillin and cephalosporins, as with other antibiotics may arise in bacteria in one of two ways. Resistance may occur as a result of chromosomal mutation or it may arise as a result of the transfer of genetic information from a resistant bacterial cell to a sensitive cell and this transfer may take place by transformation, by conjugation or by the phage mediated process of transduction. Resistant strains, once they arise, may be at a selectional advantage when the drug is used and as a result they may become more prevalent. The frequency at which antibiotic resistant strains arise, and the rate at which they subsequently increase in numbers, varies greatly with different antibiotics and different pathogens. Although penicillin was first used in 1940 some organisms are still sensitive to penicillin, e.g. β-haemolytic streptococcus. With other pathogens however, penicillin-resistant strains have become more numerous, e.g. gonococcus. Emergence of penicillin resistance was particularly marked in Staph. aureus which was established as being due to the destruction of the antibiotic by the extra-cellular enzyme penicillinase, a β-lactamase. In an attempt to overcome this resistance semisynthetic penicillins and cephalosporins, were introduced, the most important of which was methicillin. A high proportion of the penicillin-resistant strains of Staph. aureus at that time (1960) were sensitive to methicillin.

The most rapid increase in the frequency of penicillin-resistant organisms over the past years has been that of the penicillin-resistant strains of Staph. aureus. These strains owe their resistance to the formation of the enzyme penicillinase (Knox, 1962) which inactivates benzyl penicillin. The enzyme penicillinase was first found in extracts of a penicillinase-resistant strain of Escherichia coli, which destroyed the growth inhibiting properties of penicillin (Abraham and Chain, 1940). This enzyme, which is often inducible, has since been found in many Gram-positive and Gram-negative strains, the quantity formed varying from strain to strain (Pollock, 1957). The Gram-positive organisms produce the enzyme with the highest activity, and in the greatest amounts; penicillinase in some Gram-positive strains can amount to 3% of the total cell protein (Pollock, 1967).

The inactivation of penicillin by penicillinase is a hydrolysis reaction, (Fig. 1.10) forming penicilloic acid, as with the chemical break down in dilute alkali (Scudi and Woodruff, 1949). The temperature coefficient of the enzyme is low even at low temperatures while the optimum pH is 6 - 6.5.

The problem of the penicillin- resistant staphylococcus, particularly in hospital, was changed in 1960 by the introduction of methicillin, and the other semi-synthetic penicillins, which are able to exert their anti-bacterial activity against these resistant strains because they are highly stable to staphylococcal penicillinase. This stability to penicillinase has been achieved by the choice of suitable side chain structures within the penicillin molecule. The staphylococcal resistance to methicillin occurs with little or no destruction of the antibiotic. The mechanism of this type of resistance is unknown, although in a few cases it has been attributed to the presence of a penicillinase, nevertheless, this is not the sole mechanism of resistance. Organisms may produce penicillinase but at the same time may also be intrinsically insensitive to the penicillins (Sutherland, 1964).

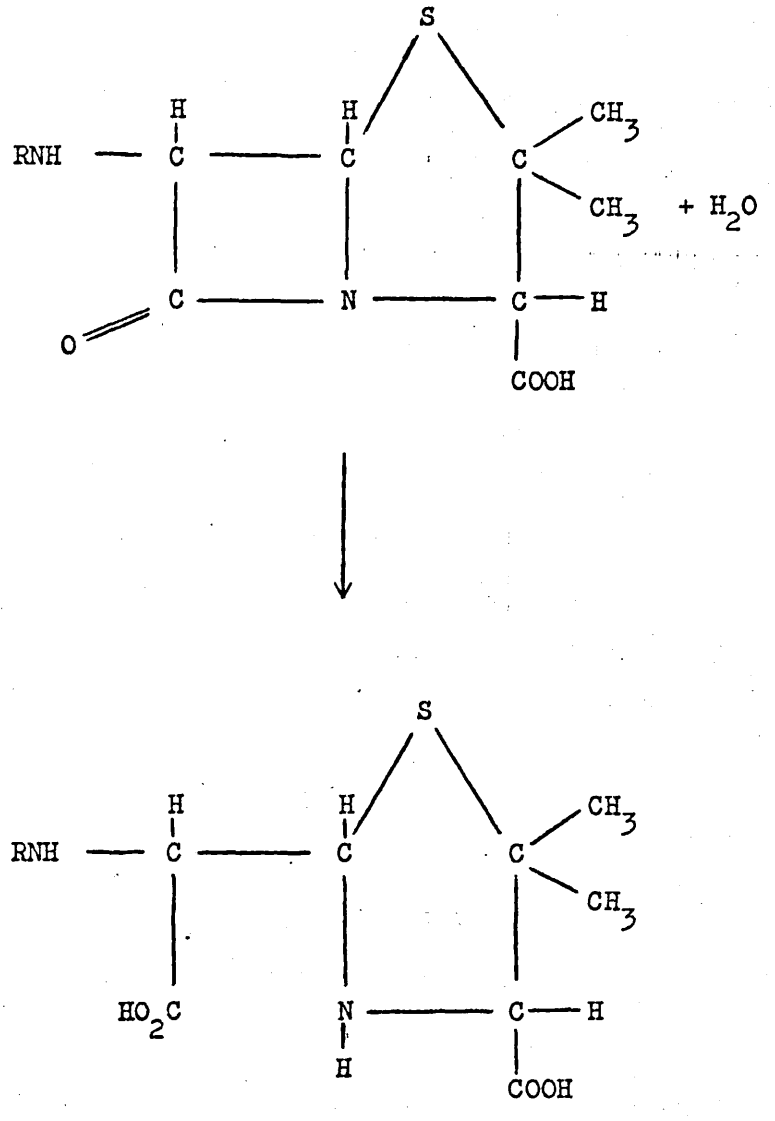


Fig. 1.10

The hydrolysis reaction which brings about the inactivation of penicillin

Jevons (1961) reported this intrinsic resistance; the incidence was very low probably less than 0.1%. Since that time the numbers of methicillin - resistant strains have increased, (Barber, 1961; Stewart and Holt, 1963; Jevons et.al., 1963). Some indication of the general incidence of methicillin-resistant staphylococci may be gained from the data of Parker and Hewitt, (1970), the percentage resistant among many thousands of strains tested at the Central Public Health Laboratory rose from 0.06 in 1960 to 0.97 in 1964 and to 4.11% in 1969. There also appears to be a difference in the incidence in different countries.

Naturally occurring methicillin-resistant strains show a heterogenous response to the antibiotic; they consist of mixed populations in which the majority of cells show a normal sensitivity to methicillin, with a slower growing minority showing a very high resistance, (Jevons, 1961; Sutherland and Rolinson, 1964; Rozgonyl and Redai, 1968). For this reason the MIC of methicillin resistant strains depends on the size of the inoculum and the length of incubation at 37 °C; therefore it is customary to use a heavy inoculum and to continue incubation for 48 h. Annear (1968) reported that enhanced and nearly uniform resistance was seen if cultures were incubated at 30 °C for 18 h. Hewitt, Coe and Parker, (1969), who investigated this problem most thoroughly, concluded that the most dependable method is that all resistant strains will grow to within 1 mm of 10 µg methicillin disc on nutrient agar, if incubated at 30 °C for 18 h. It has been recently reported that the Kirby-Bauer method gives reliable results when cultures are incubated at 35 °C (Drew et.al., 1972). Furthermore, Parker and Hewitt (1970) showed that if the resistant culture is grown at extremes of temperature (e.g. 25 and 43 °C) there is a totally different response to methicillin. Incubation at 25 °C renders all the cells highly resistant, while at 43 °C all cells in the population are very sensitive to even low concentrations of methicillin. These effects are immediately reversed when the cells are grown at different temperatures.

Methicillin-resistant strains often belong to phage group III (Stewart and Holt, 1963; Parker and Jevons, 1969; Benner and Kayser, 1968). It has been observed that when staphylococci resistant to a particular antibiotic first emerge, they often have a restricted range of phage typing pattern, predominantly group III, but later as fresh resistant strains appear, the range broadens.

The extrachromosomal elements (plasmids) in bacteria are independent replicons consisting of double-stranded deoxyribonucleic acid molecules. The genes determining the production of penicillinase by Staph. aureus are usually located extra-chromosomally on a plasmid (Novick, 1963), but there is evidence that they form part of the chromosome (Asheshov, 1966 a). Other genes that have been found on the same plasmid as the penicillinase genes are those that determine resistance to metal ions and to erythromycin (Richmond and John, 1964; Novick and Richmond, 1965; Novick and Roth, 1968).

Resistance to mercury ions (Moore, 1960) is very frequently a character of staphylococcal strains that have become established endemically in hospitals, and mercury-resistant strains usually produce large quantities of penicillinase and are resistant also to antibiotics unrelated to penicillin (Richmond et.al., 1964). There are at least three immunologically distinct staphylococcal penicillinases (Richmond, 1965); 'hospital' staphylococci generally form A-type penicillinase in considerable quantity, and a relatively large proportion of the enzyme is found extra-cellularly in such cultures. Resistance to heavy metals other than mercury, such as cadmium, arsenic (as arsenate), bismuth and lead has been observed in Staph. aureus cultures, and the determinants for these resistances are sometimes on the same plasmid as the penicillinase and mercury-resistant genes (Novick and Roth, 1968).

Barber (1949) observed that penicillinase production was spontaneously and irreversibly lost at a low frequency, and it was subsequently reported that an increased frequency of loss resulted after growth of some strains at elevated temperature (May et.al., 1964; Asheshov,1966 b)or after exposure to compounds which interfere with deoxyribonucleic acid (DNA) replication such as acridin dyes (Harmon and Baldwin, 1964 ; Hashimoto and Mitsuhashi, 1964), ethidium bromide (Bouanchand et.al., 1968) and by growth in the presence of the anionic surface-active agent sodium dodecyl sulphate (SDS) (Stephen and Baldwin, 1972). This effect is often referred to as 'curing' of the plasmid; it being implied that the plasmid is selectively inactivated or inhibited in replication.

In all methicillin-resistant clinical strains examined, methicillin resistance has a unique phenotypic nature and there is no evidence for a 'methicillinase' (Dyke, 1969). It is probable that the resistant strains have the same mode of resistance, determined by the same genes. Some methicillin-resistant strains lose their resistance in vitro (Dornbusch et.al., 1969; Lacey, 1972 ; Grubb and Annear, 1972); this evidence implies an extrachromosomal locus for the relevant genes. But Cohen and Sweeney (1970) reported that methicillin resistance is stable suggesting a chromosomal locus for the genes. Thus the genes coding for methicillin resistance have properties characteristic of both plasmid and chromosomal loci (Grinsted and Lacey, 1973 a and b) suggesting large extrachromosomal linkage group for several antibiotics and haemolysin production in strains of staphylococci.

1.6 Object of the present investigation

Earlier work (Brewer, 1966 and Marshall, 1969) has shown that the surface structure of cells of Staph. aureus, as revealed by pH-mobility studies is complex and dependent upon factors as origin and type of antibiotic

resistance. Later Hill (1971) showed the 'temperature effect' of cells of strains of Staph. aureus with natural resistance to methicillin. He suggested that the presence or absence of surface teichoic acid is intimately associated with methicillin-sensitivity or resistance respectively.

These investigations have been extended with many strains of staphylococci, to observe:

(a) changes of surface properties of cells of methicillin resistant and sensitive strains when repeatedly grown at different temperatures;

(b) the loss of the gene determinant (plasmid) of methicillin resistance and penicillinase production;

(c) the changes in the surface properties following the growth on the media (liquid and solid) containing various amounts of inorganic phosphate;

(d) the surface properties of cells of methicillin resistant and sensitive strains of Staph. epidermidis.

While most emphasis has been placed on changes in the surface structure as revealed by particulate electrophoresis, other techniques including tests for penicillinase production and antibiotic sensitivity have been used.

SECTION 2

Experimental

2.1 Bacteriological techniques

(a) Media

Standard nutrient agar was prepared by adding 28 g of Oxoid agar (code CM3 batch No. 273, 8762) into 1 dm^3 distilled water, the solution was distributed in 100 cm^3 lots in (16 oz) medical bottles and in 10 cm^3 in McCartney bottles. These were immediately sterilized by autoclaving at 15 lb in^{-2} for 20 minutes, McCartney bottles were sloped and allowed to solidify. Nutrient agar plates were prepared by melting a sufficient amount of this stock agar in a steam bath and aseptically pouring into sterile petri dishes and allowing to cool.

Oxoid nutrient agar CM3 other than batch No. 273, 8762 (standard media) was found to contain relatively large amounts of inorganic phosphate. A media preparation was established in this laboratory to remove excess inorganic phosphate as follows:

13 g of nutrient broth (Oxoid CM1, 297, 14154) were dissolved in 100 cm^3 of distilled water, and 4.7 cm^3 of a 10% solution of $Ca(OH)_2$ were added. The mixture left at room temperature for 20 minutes to allow inorganic phosphate to be precipitated. It was then filtered at the pump and immediately 3 cm^3 of 3 mol dm^{-3} HCl were added to adjust the pH to 7.2, distilled water was then added to make up to 1 dm^3. Solid agar (Oxoid No.1) was added so that the final concentration was 1%. The mixture was boiled with constant stirring and then dispensed in (8 oz) medical bottles and McCartney bottles and finally sterilized by autoclaving.

Table 2.1

Antibiotic properties of strains of Staph. aureus used.

Text 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
7	10101/67*	P (pen^+) S T M No E' N
8	Oxford	Sensitive
9	BRL 1800***	P (pen^+) S T M
10	4155/72*	P (pen^+) S T M
11	4143/72*	P (pen^+) S T M

Key: pen^+ = penicillinase producer

* = routine isolate from Staphylococcal Reference Laboratory, Colindale.

** = Dornbusch et.al. (1969)

*** = Obtained from Beecham Research Laboratories Ltd (Grubb and Annear, 1972).

Antibiotic resistance: P = Penicillin; S = Stroptomycin; T = Tetracycline; E' = Erythromycin (inducible); M = Methicillin; N = Neomycin; No = Novobiocin

Nutrient broth (Oxoid CM1, 297, 14154) treated with $Ca(OH)_2$ and untreated were also prepared. These media were distributed in 200 and 50 cm^3 amounts in medical bottles and 5 cm^3 in metal capped test tubes. These media were sterilized by autoclaving as before.

Nutrient broth (Oxoid CM1 297, 14154)solidified with 1% w/v Oxoid agar (No. 1) was prepared, and used in few experiments; this medium was not treated with $Ca(OH)_2$.

(b) Strains

The strains of Staph. aureus (Table 2.1) used in this work were supplied by the Cross Infection Reference Laboratory, Colindale; either growing on nurient agar slopes in Bijoux bottles or as freeze-dried preparations. All strains were subcultured on nutrient agar slopes in McCartney bottles the cultures were grown at 37 °C and then stored at room temperature.

The strain of Klebsiella aerogenes (NCTC 418) was maintained by regular subculturing in bottles containing 50 cm^3 of nutrient broth. When required for calibration of the electrophoresis apparatus, the cells were inoculated in nutrient broth and grown for 18 h at 37 °C with the bottle sloped and loosely capped.

(c) Growth of cells for measurements of electrophoresis mobility

After one subculture on nutrient agar from the slope, the required strain was grown on nutrient agar plates in the quantity required for examination. In some experiments nutrient broth was used as the growth media. All cultures incubated for 18 h at the required temperature.

(e) Phage typing of strains

Phage typing was used to check the purity of the strains after the culture had been used in the investigation and also after the repeated growth at the different temperatures. In addition it was used to check variants which were derived from the strain in routine use. The phage typing was carried out according to the method employed by Blair and Williams (1961).

In general, any good nutrient medium can be used for phage propogation and typing, the phages require calcium ions for adsorption to or growth on the cocci, media containing agar did not usually require additional calcium. Excess heating of the media during preparation was avoided as it may bind the Ca^{++} ions in some way making them unavailable. A deficiency of calcium was rectified by addition of calcium (400 $\mu g\ cm^{-3}$) but the resulting agar appeared slightly cloudy. Solid media for propogation and typing were kept rather soft to facilitate the recognition of small phage plaques. Care was taken not to dry the plates excessively to remove surface moisture.

Phages:

The basic set used consisted of 22 phages:

Group I	29	52	52A	79	80
II	3A	3C	55	71	
III	6	42E	47	53	54
	75	77	83A	84	85
IV	42D				
Miscellaneous	81	187			

Before each phage was used it was titrated to estimate the RTD. The RTD or routine test dilution is the highest dilution which just fails to give confluent lysis on the propogating strain. The propogating strain was identified by the same number as are used to designate the phages;

thus, 'PS 29' is used for the propogation of phage 29. A sample of dried phage was suspended in 1.0 cm^3 of broth and this fluid was diluted in tenfold steps to 10^{-6}. Separate pipettes were used for each dilution step. One drop (0.02 cm^3) of each dilution was then applied to the surface of an agar plate, previously spread with a 4 - 5 h broth culture of the propogating strain. The plate was incubated at 30 °C overnight. The following day the RTD values were established. The phages were diluted in broth to their RTD and stored at 4 °C and used for not more than 7 days.

Strains for typing should be pure and coagulase positive, they were picked up from single colonies into 5 cm^3 of broth and incubated at 37 °C for 4 - 5 h. Plates were flooded with this culture and the excess fluid pipetted off. The plates were then dried with the lids removed for about 30 minutes and the phage at the RTD was applied either with a fine pipette (care was taken not to touch the agar with the pipette) or with a multiple-loop applicator.

Reading of results:

The plates were examined by indirect transmitted light against a dark background with the aid of a hand lens of moderate magnification. Susceptibility to phages was indicated by varying degrees of lysis from a few discrete plaques to completely confluent lysis as follows:

++ 50 or more plaques

\+ 20 - 50 plaques

± 1 - 20 plaques

All lytic reactions from 50 plaques to confluent lysis were regarded as strong reactions. Lesser degrees of lysis was considered as indicative of weak reactions. The phage pattern of the strain was reported in the following form: 52/52A/80. When weak reaction of other phage was present a + sign was placed after the pattern, such as 52/52A/80 +.

(f) Antibiotic susceptibility tests for the strains:

(i) Minimum Inhibitory Concentration (MIC) of methicillin:

Methicillin is unstable at temperatures above 45 °C and it rapidly loses its activity when stored in aqueous solution. Hence freshly prepared solutions of the antibiotic were always used each time; the dilutions which were used were in the range of 0 - 200 μg cm^{-3} methicillin. The solid medium containing the antibiotic was prepared by adding suitable volume (not exceeding 2% of the final volume) of a saturated methicillin solution to a known volume of melted nutrient agar at a temperature of 45 °C. The agar was poured almost immediately to set into plates and all the plates and tubes including controls which lacked antibiotic were inoculated with approximately 10^5 bacteria from an 18 h plate culture and the plates were incubated at either or all of the temperatures: 25, 37 or 43 °C. The conventional MIC (i.e. the lowest concentration required to prevent cell growth) was recorded after 48 h at 37 °C.

(ii) Disc diffusion test:

Methicillin discs were prepared as follows. Ford blotting paper 6 mm in diameter was sterilized by autoclaving and then dried thoroughly. One drop (0.02 cm^3) of an appropriate dilution of antibiotic was placed on each with a calibrated pipette, the discs were immediately dried, and stored over anhydrous $CaCl_2$ at 4 °C. Discs were prepared containing 10 μg cm^{-3} and 20 μg cm^{-3} methicillin. Plates of nutrient agar were spread or flooded with an undiluted turbid broth culture and allowed to dry at room temperature. The discs were applied and the plates incubated 18 h at 30 °C. The width of the zones of inhibition were recorded.

Antibiotic discs other than methicillin were obtained from Mast Laboratories Limited, Liverpool, which contained concentrations of streptomycin 10 μg, erythromycin 10 μg, Tetracycline 10 μg, penicillin G one unit, Novobiocin 5 μg, Neomycin 10 μg.

Discs containing metal ions Cd^{++}, Hg^{++} and As^{+++} were also used.

Cadmium	20 µg cadmium sulphate
Mercury	0.13 µg phenyl mercuric nitrate
Arsenate	0.32 µg sodium arsenate

(g) Replica plating

The technique of replica plating was devised by Lederberg and Lederberg (1952). The idea behind it was to substitute many operations of one needle by a large multiplicity of needles attached to a base plate. In this way the entire pattern of bacterial growth on the agar surface of an initial or master plate can be sampled in a single operation and transferred as a whole to the surface of plates used for analysis.

Pile fabrics, such as velveteen, in which the pile serves as a close-set and orderly array of short flexible needles were used. A square of sterile velveteen was placed, pile upwards, over the flat end of a wooden cylindrical block of diameter slightly less than that of a standard petri dish, the material was held in position by elastic band (Fig. 2.1). The initial plate which was used, contained about 100-200 discrete colonies, which were not too close to the edge of the plate. The plate was inverted over the fabric and the agar surface pressed gently against the pile, the plate was carefully removed. In this position the pile carries a precisely positioned print of the bacterial growth on the initial plate. The replica plate (nutrient agar containing 10 µg cm^{-3} methicillin)was marked, inverted over the fabric and pressed gently against it. When it had thus been subcultured it was then removed and incubated overnight. Examination was made by putting one plate on the other to confirm if the colonies had lost methicillin-resistance or not. The colonies were marked and subcultured for further tests.

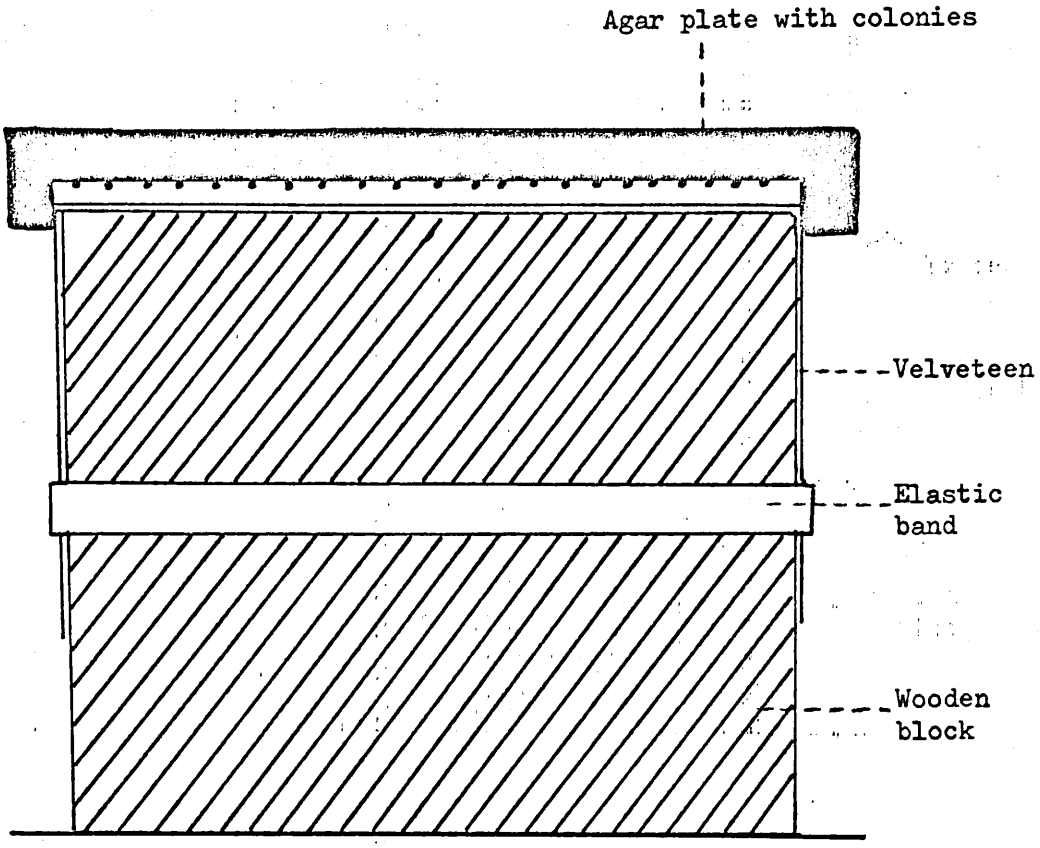


Fig. 2.1

Apparatus used for replica plating

(h) Detection of penicillinase

The starch agar method which was used is based on a modification of the method of Perret (1954). This method is the simplest method for testing for penicillinase. It is easy to perform and also it is very sensitive, allowing for the detection of as little as 0.05 units of enzyme per mg dry weight of the organism.

The strain under test was plated on starch agar (0.2% w/v soluble starch in nutrient agar). Bacteria were grown in nutrient broth for 4 - 6 h until it reached medium turbidity; then 0.1 cm^3 of a 10^5 dilution was incubated. To obtain large numbers of colonies, the bacteria were spread but again not too close to the edge of the plate. After overnight incubation at 37 °C the plates were replicated (2.1 g) and the two plates were examined and the colonies were marked. Then the colonies were developed by flooding the starch plate with a mixture containing 2 cm^3 of a solution of 0.16 mol dm^{-3} iodine dissolved in 0.32 mol dm^{-3} and 10^6 units benzyl-penicillin dissolved in 20 cm^3 of phosphate buffer solution (2.2) at pH 5.9. The agar stained a deep blue due to the reaction between starch and iodine, penicillinase producing colonies were recognised by the appearance of a white halo surrounding the colony; this is caused by the production of penicilloic acid as a breakdown product of penicillin by penicillinase. When the method was used to detect and isolate penicillinase negative mutants it was advantageous to add a small amount of inducer to the nutrient agar. Methicillin at a concentration of 0.5 µg cm^{-3} was suitable. When starch plates were flooded with the penicillin-iodine mixture penicillinase producing colonies quickly turned white and produced their characteristic halo. Penicillinase negative colonies were stained a deep yellow which gradually faded, they were recognised easily and subcultured to confirm the result.

(i) Cleaning and sterilisation of apparatus

The disposable contaminated apparatus was immersed in 1% lysol

solution prior to disposal, and glass pipettes were submerged in a 2% milton solution. Other contaminated glassware was immediately autoclaved at 15 lb in^{-2} for 20 minutes. The glassware and apparatus were then scrubbed in tap water and then rinsed once in tap water and once in distilled water. It was finally dried and sterilised in an oven at 150 °C overnight Pipettes and Pasteur pipettes were plugged with non-absorbant cotton wool, and re-sterilised in a metal container at 110 °C for two days.

2.2 Preparation of cell suspension for electrophoresis

Barbiturate-acetate buffer solutions were used as the suspending electrolyte Michaelis (1931). In all mobility determinations a pH range from 3-7 were used. 5 dm^3 of stock solution I = 0.5 mol dm^{-3} contained:

0.15 mol dm^{-3} sodium barbiturate, 154.635 g

0.15 mol dm^{-3} hydrated sodium acetate, 102.0675 g

0.20 mol dm^{-3} sodium chloride, 58.450 g

This stock solution was stored at 4 °C, the required buffer solutions were prepared by diluting the stock with distilled water to give the ionic strength, I, of 0.02 mol dm^{-3} and adjusted with HCl (1mol dm^{-3}) to the required pH. The pH was measured using a calomel and glass electrode assembly on a E.I.L. (Model 23A) pH meter. Before each measurement the pH meter was calibrated with a Sørensen phosphate buffer solution at pH 6.81 prepared by mixing equal volume of following solutions:

Solution A - 0.667 mol dm^{-3} disodium hydrogen orthophosphate dodecahydrate 23.88 g dm^{-3};

Solution B - 0.667 mol dm^{-3} potassium dihydrogen orthophosphate 9.074 g dm^{-3}.

The conductance of each buffer solution, used for mobility measurements was determined with a Wayne-Kerr (B224) Universal Bridge in a bottle type cell mounted in a thermostat maintained at the same temperature as that used for measuring the electrophoretic mobility.

The cells of K. aerogenes were harvested by centrifuging the nutrient broth culture, and washing the cells twice in the appropriate veronal-acetate buffer solution before resuspending in the same buffer solution. Mobility measurements were made on the cell suspension as soon as possible after preparation.

Cells of Staph. aureus were harvested from plates by washing with distilled water. The cells were washed twice and finally resuspended in the appropriate buffer solution for mobility measurements.

2.3 Treatment of cells with sodium metaperiodate

The method, modified after Garret (1965) and Brewer (1966); was used to destroy and remove teichoic acid from the surface of gram-positive bacteria.

Cells of 18 h growth on nutrient agar plates were harvested in distilled water, and washed once in physiological saline (0.85% w/v NaCl). The cells were 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 the aqueous ammonia. The material was then suspended in barbiturate buffer solution 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 suspension of oxidised cells was divided into the required number of aliquots, and the cells were centrifuged from the sodium metaperiodate solution and washed twice in barbiturate buffer solutions (I = 0.02 mol dm^{-3}) at suitable pH values. Mobility determinations were then made on cells in these suspensions.

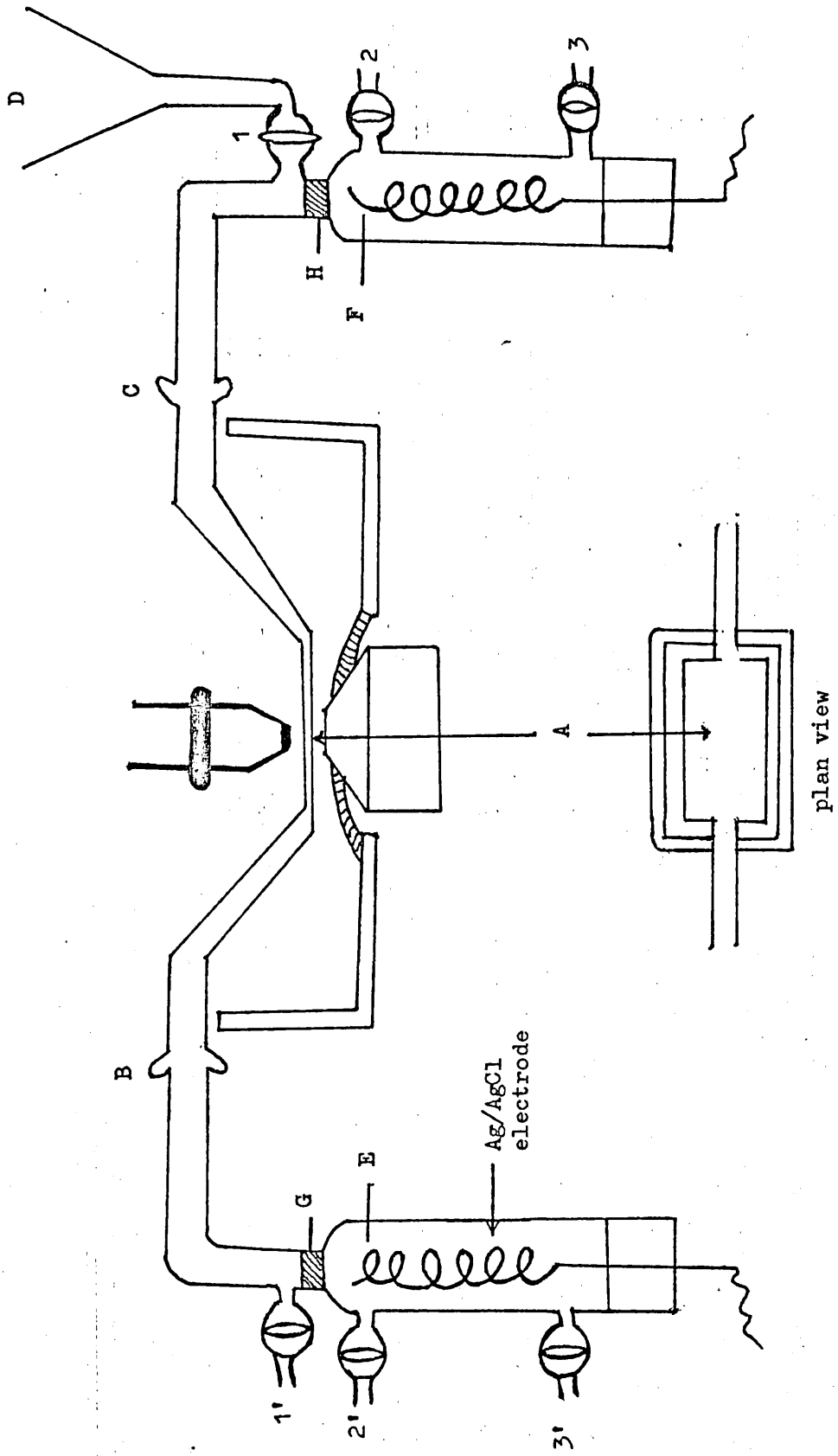


Fig. 2.2

Diagrammatic representation of the microelectrophoresis apparatus.

2.4. The Microelectrophoresis apparatus

(a) Description

The apparatus used throughout the investigation was that developed by Gittens and James (1961) and is 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 under 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 made from two optically flat Hysil Plates (40 x 25 x 0.5 mm), fused together to give a separation of 0.5 mm. This was attached to side arms fitted with hemispherical ground glass joints (B and C) and filled with suspension from the reservoir (D) by opening taps 1' and 1.

A constant electric field was applied between the Ag.AgCl/KCl electrode systems in the compartments (E and F). The electrodes consisted of spirals of silver wire (diameter 2 mm and length 14 cm) mounted in the compartments by means of rubber bungs, through which the wire passed forming a water-tight seal. The silver wire was first cleaned with dilute nitric acid and then anodised in 0.1 mol dm^{-3} HCl using a platinum cathode at a current density of 2 A m^{-2} for 20 minutes, to give a grey-purple deposit of AgCl on the electrode. The electrodes were anodised in series to ensure they were identical.

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 (G and H). The electrode compartments could be conveniently refilled at regular intervals with the electrolyte solution from reservoirs, using taps 2 and 3, 2' and 3'.

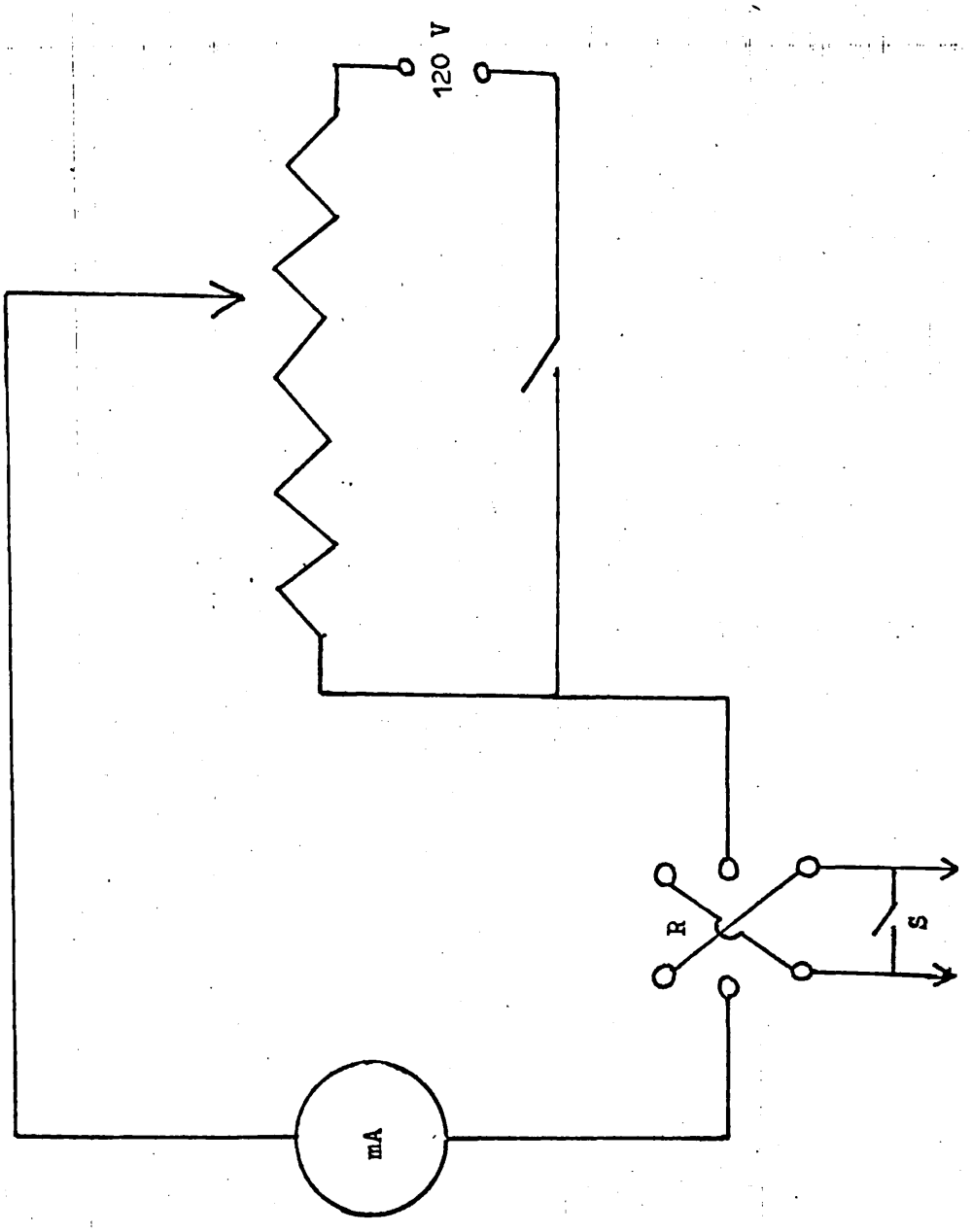


Fig. 2.3

The electrical circuit

The electrical circuit (Fig. 2.3) was based on that of Abramson et.al. (1942). The voltage was supplied by a 120 V battery, and controlled by a variable resistance, maximum resistance 10,000 ohms. The current flowing was measured by a Sangamo-Weston multirange milliammeter connected in series with the cell. The applied potential could be reversed using the switch R. The switch S, shorted the electrodes when not in use; this prevented electrode polarisation.

A Watson Service microscope was used to observe the cells in the chamber. This was fitted with an annulus phase contrast condenser and objective (x 40) and angled stem (x 1.5) and a focusing eyepiece (x 10), containing a cross -etched graticule. Illumination was provided by a Watson microscope lamp , with control unit; the light was reflected from a concave mirror below the stage.

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 made possible by immersing the observation chamber in a small water bath adapted to fit on the stage of the microscope. The water in the bath was maintained at 25.0 $\pm$ 0.5 ^{o}C by circulating water from a large thermostatic bath controlled by a Tecum "Tempunit". The water bath system was constructed of Perspex sheeting, and this was cut away beneath the observation chamber to allow the condenser lens of the microscope system to pass through. The condenser lens passed through a flexible, water-tight rubber diaphragm attached to the perspex. Both the condenser and objective lenses were waterproofed.

An additive stop-watch, reading to 0.01 s, was used to record the time taken by a bacterial cell to cross a given number of squares on the graticule.

(b) Mode of operation

Cells of K. aerogenes were dried on to the inner surface of the observation chamber before assembly. By focusing on these reference surfaces, the depth of the chamber was found in arbitrary units.

All the taps and ground glass joints were thoroughly cleaned and adequately greased. This ensured that there were no leaks which would have given rise to movement of the cells in suspension in the absence of any applied electric field.

The following procedure was carried out each day before use of the apparatus, to give reproducible electrical connections through the sintered glass plates.

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 solution through the sintered glass plates. The observation chamber was then flushed through with distilled water to remove the electrolyte which had been forced through the sintered plates. The chamber was then filled with buffer solution, at the temperature, pH and ionic strength, as the suspension to be examined. In all operations great care was taken to ensure that no air bubbles appeared in the closed system. The chamber was finally filled with bacterial suspension under observation and all the taps were finally closed. In routine use, the stationary levels were located at fractional depths of 0.21 and 0.79 from the top inside surface. All mobility measurements were made at the upper stationary level. From the known depth of the cell the microscope could be adjusted to focus at this level.

The velocity of a cell in focus at the stationary level was measured by recording the time taken for the cell to cross a given number of graticule squares under the influence of an applied electric field. A time of between 2 and 4 s was considered suitable, and the number of squares across which it was timed was adjusted accordingly. The current flowing during the timing was measured on the milliammeter. For each suspension 40 cells were timed; and by reversing the current with switch R the cells were timed in alternate directions. The potential was never applied for long periods of time in any direction, this minimised electrode polarisation during operation.

The mobility values obtained for suspension of cells of the same strain grown on different occasions were found to be reproducible to within $\pm$ 3%. If the cultures under investigation had mobility values differing by 10% or more they were considered significantly different.

(c) Calibration of apparatus

The electrophoretic mobility of a particle $\bar{v}$ m^2 s^{-1} V^{-1} is defined as the particle velocity v/m s^{-1} per unit potential gradient, X/Vm^{-1}.

It is given by the expression:

$$\bar{v} = \frac{v}{x} = \frac{nL}{t} \cdot \frac{qk}{I} = \frac{Lq}{tI} \cdot J G$$

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

The values of G, I and t were obtained experimentally. It is not possible, however, to determine the cross-sectional area (q) of a rectangular observation chamber. This difficulty was overcome by using a standard particle, which had a known absolute mobility ($\bar{v}_s$), when suspended in a buffer of a known pH and ionic strength. By timing this under the conditions previously described, a "cell constant" for the chamber K was determined, where K was given by:

$$K = L\,q\,J = \frac{\bar{v}_s\,t\,I}{n\,G}$$

The value of K also included the cell constants of the conductance cell. The subsequent timings (t') on cells under examination were converted to mobility using the relationship:

$$\bar{v} = \frac{K\,n\,G}{t'\,I'}$$

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

Cells of K. aerogenes were used as a standard particle, harvested from 18 h culture grown in nutrient broth at 37 °C (2.1.b). The standard conditions used for determining the mobility values of these standard particles are at an ionic strength of 0.02 mol dm^{-3}, a pH of 7.0 and at 25 °C; under these conditions the absolute mobility is -1.67×10^{-8} m^2 s^{-1} V^{-1}. This value was obtained as a result of extensive calibration studies of suspensions of K. aerogenes against human erythrocytes as standard (Gittens 1962). In the present work, K was determined before each set of electrophoretic measurements were made.

All mobility values are quoted without sign or units, a value 1.56 means that the particle is negatively charged with an electrophoretic mobility (towards the positive electrode) of 1.56×10^{-8} m^2 s^{-1} V^{-1}.

SECTION 3

Studies of the Surface and Biological Properties of Cells of a Naturally Occurring Methicillin-Resistant Strain of Staph. aureus. Strain 1.

Sutherland and Rolinson (1964) showed that a culture of methicillin-resistant Staph. aureus grown at 37 °C consists of a mixed population in which the majority of the cells are of normal sensitivity to methicillin but with small minority showing resistance to very high concentrations. The heterogeneous response was found in all naturally occurring resistant strains when grown at 37 °C; the temperature of incubation was found to have a marked effect on the heterogeneity of the culture(Annear (1968), Parker and Hewitt (1970)). It was found that the strains were highly resistant to the antibiotic if incubated at temperatures between 25 and 30 °C, while at 43 °C all the cells were totally sensitive.

Cells of such resistant strains exhibit surface properties which were dependent on the temperature of growth (Hill and James, 1971,b) when grown on nutrient agar in the absence of the antibiotic. After growth at 43 °C surface teichoic acid was shown to be a surface component, while there was no surface teichoic acid on cells grown at 25 °C. The surface properties of the strain grown at 37 °C although intermediate between those of cells grown at 43 °C and 25 °C, were closer to those of cells grown at 43 °C.

The determinants for methicillin resistance are believed to be in extrachromosomal elements (Dornbusch, Hallander, Lofquist, 1969), which were lost after treatment with acriflavine. Grubb and Annear (1972) reported the spontaneous loss of resistance on ageing cells at room temperature.

Therefore it was important to see how these surface properties varied and were possibly stabilised by maintenance of the cells at different temperatures. This section discusses studies on cells of Strain 1.

3.1 Effect of temperature of growth

(a) pH-mobility curves of cells grown on nutrient agar at 25, 37 or 43 °C

Standard nutrient agar (2.1.a) plates were inoculated from the parent culture grown on nutrient agar at 37 °C. The plates were incubated at 25, 37 or 43 °C for 18 h. The cells were harvested, washed (2.2) and resuspended on buffer solutions of appropriate pH. All mobility measurements were made at 25 °C.

Brewer (1966) and later workers have defined H-value as the increase of mobility (at the maximum) compared to the values at pH 7 as a percentage of the plateau mobility value. This is a valid method of classifying pH-mobility curves of cells of strains of Staph. aureus.

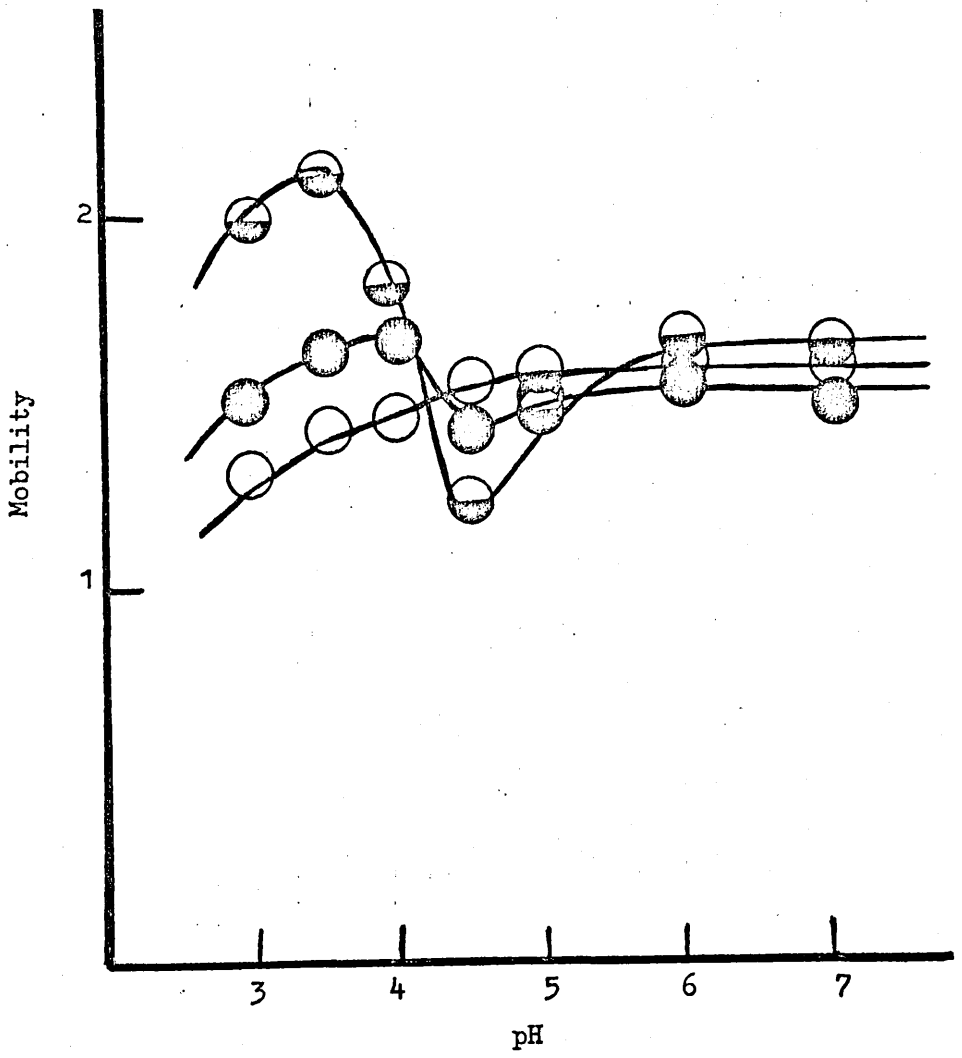
The strain used in this investigation (Fig. 3.1) showed a marked variation in surface charge with the temperature of growth. The results were very reproducible and were consistent with the results obtained by Hill and James (1971,b). When the strain was grown at 43 °C and potentially sensitive to methicillin the cells had a high H-value (30) and exhibited accentuated minimum mobility value at pH 4.5. The cells grown at 25 °C and potentially very resistant to methicillin had a low or zero H-value and there was no minimum mobility at pH 4.5. The cells grown at 37 °C had a low H-value (3) and a much less marked minimum mobility at pH 4.5.

(b) Minimum Inhibitory Concentration measured at 25, 37 and 43 °C

MIC levels of methicillin were determined by the plate method in the range 0-200 $\mu g\ cm^{-3}$ methicillin (2.1, f(i)), all plates were inoculated with approximately 10^5 bacteria from a 18 h plate culture grown at 37 °C. The

Fig. 3.1

pH-mobility curves of cells of Staph. aureus, (Strain 1) grown on standard nutrient agar at 25 °C (○); 37 °C (◉) or 43 °C (◒)



plates were incubated at each of the temperatures 25, 37 and 43 °C (Table 3.1). The conventional MIC was recorded after 18 h at 37 °C.

Table 3.1

Growth of cells of Staph. aureus (Strain 1) in the presence of methicillin

Test Temperature °C	Growth in the presence of methicillin, concentration/μg cm^{-3}						
	0	4	10	20	40	100	200
43	++++	0	0	0	0	0	0
37	++++	++++	+++	++	+	±	0
25	++++	++++	++++	+++	+++	+++	++

(The profusion of growth is denoted as ++++, +++, ++, +, ±, 0.)

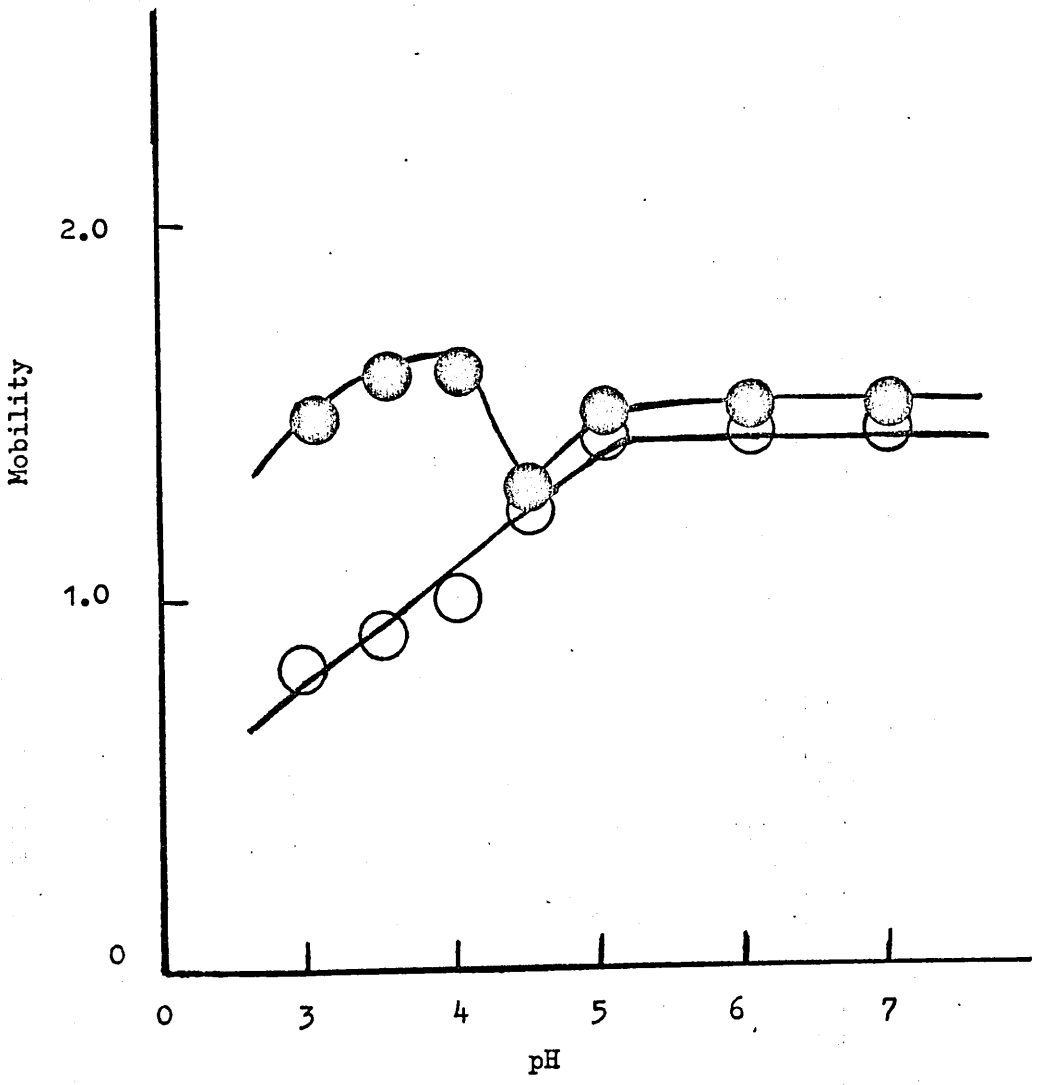
The MIC for the cells at 37 °C was 200 μg cm^{-3}, the cells were completely sensitive when incubated at 43 °C, and very resistant at 25 °C.

(c) The removal of surface teichoic acid

The oxidation of surface teichoic acid on the cells with sodium metaperiodate (2.3) was carried out. The strain was grown at 37 °C for 18 h on nutrient agar and the pH-mobility curves were determined for cells both before and after the oxidative treatment (Fig. 3.2). The peak mobility value at pH 3.5 to 4.0 was absent from the curve for the treated cells. This indicated the removal of phosphate groups of the teichoic acid, similar studies of cells grown at 43 °C showed that greater amounts of surface teichoic acid were present. In contrast no surface teichoic acid could be detected on cells grown at 25 °C.

Fig. 3.2

pH-mobility curves of cells of Staph. aureus (Strain 1) grown at 37 °C (●) and after oxidation (○)



3.2 Effect of repeated growth at 43 °C on nutrient agar

Following the previous observation (3.1) which confirmed the results of Hill and James (1971,b) it was important to know whether these changed surface properties could be stabilised by maintenance of the cells at high temperature. Cells of strain 1 were subcultured for 20 times, i.e. trained to grow at 43 °C on standard media (2.1,a) in the absence of the methicillin at 43 °C (2.1,d). The surface properties of the strain were studied and compared with the parent cells.

(a) pH-mobility curves of cells trained to grow at 43 °C and finally grown at 25, 37 or 43 °C

Cells from the culture trained on standard nutrient agar at 43 °C were inoculated on to plates and grown at 25, 37 or 43 °C for 18 h. The cells grown at each temperature were harvested and washed in the normal manner (2.2), and the electrophoretic mobility measured in buffer solution at 25 °C. The pH-mobility curves (Fig. 3.3) showed marked changes with increasing subcultures during the growth at 43 °C, the surface properties of the cells were in all respects indistinguishable from those of cells of a sensitive strain. Even the growth at 25 °C of these trained cells produced cells with all the characteristics of highly sensitive strains with a high H-value (40) in contrast to the parent cells which had a very low or zero H-value when grown at 25 °C. Growth at 37 and 43 °C, gave cells which had a high H-value (72, 73) and no minimum mobility at pH 4.5. The pH-mobility curves were independent of the temperature of final growth within the limits of experimental error.

Fig. 3.3

pH-mobility curves of cells of Staph. aureus (Strain 1) grown repeatedly at 43 °C on standard media and finally grown at 25 °C (○); 37 °C (●) or 43 °C (◒)

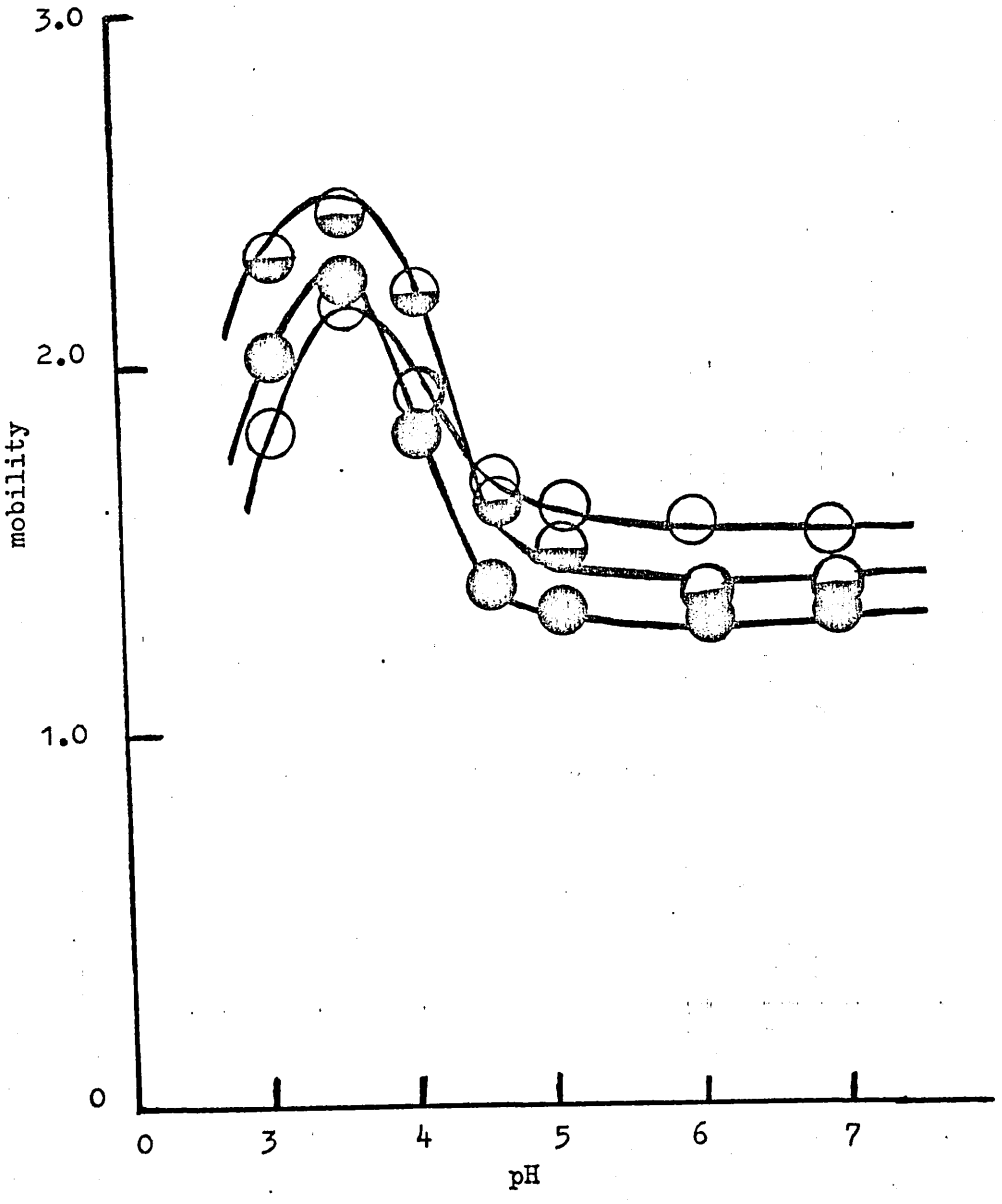
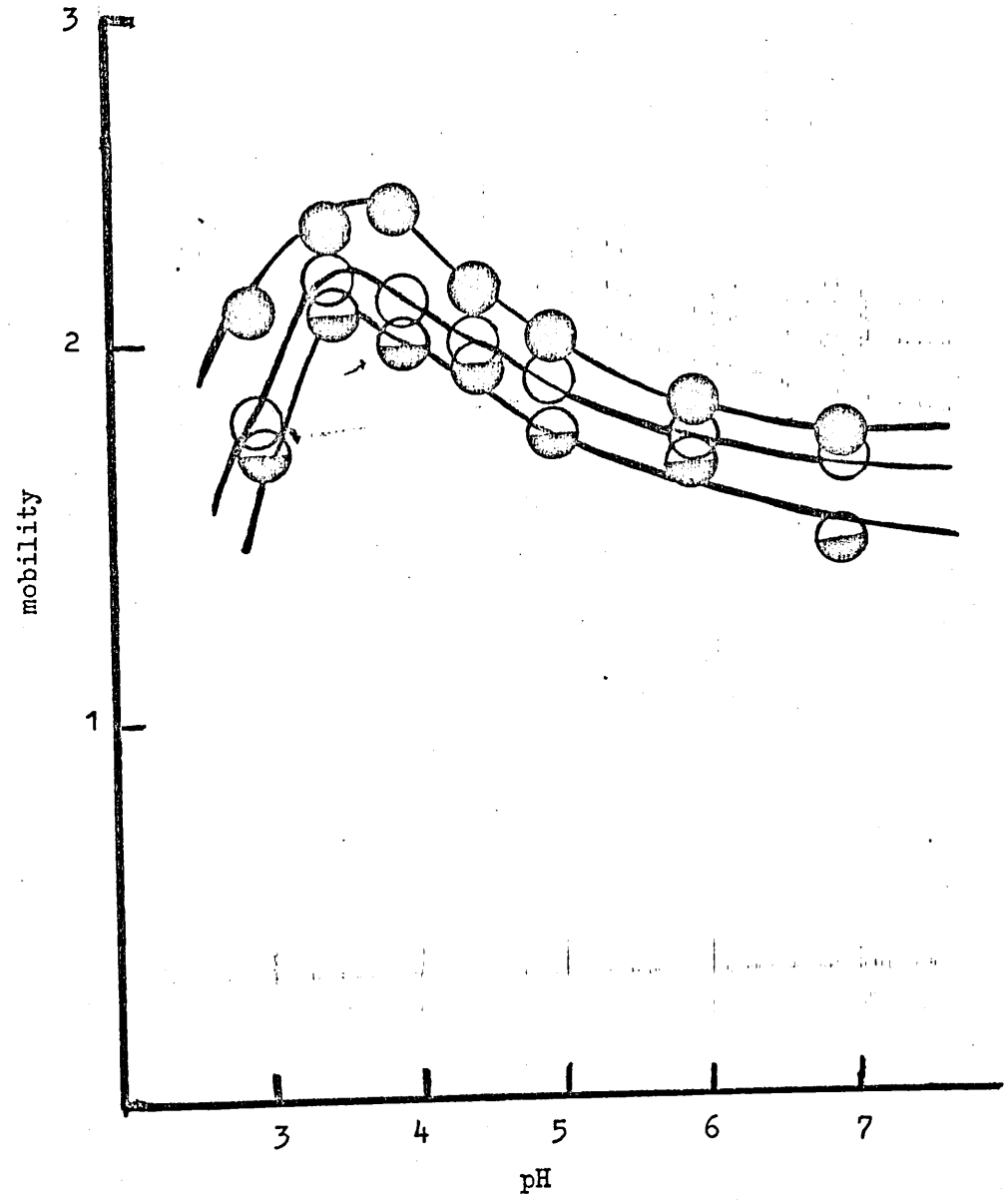


Fig. 3.4

pH-mobility curves of cells of Staph. aureus (Strain 1) grown repeatedly at 43 °C followed by 15 subcultures at 37 °C on standard nutrient agar and finally grown at 25 °C (○); (●) or 43 °C (⊖)



(b) Minimum Inhibitory Concentration measured at 25, 37 and 43 °C for cells trained at 43 °C

MIC levels of methicillin were determined by plate method on plates containing 200 μg cm^{-3} methicillin (2.1,f(i)). The plates were inoculated from 18 h culture of cells grown repeatedly on standard nutrient agar (2.1,a) at 43 °C. The plates were incubated at 25, 37 and 43 °C and the growth recorded after 48 h (Table 3.2). At the 25 °C cells were more sensitive than cells incubated at 37 °C (c.f. Table 3.1), while the MIC value at 37 °C were less than 10 μg cm^{-3}.

Table 3.2

Growth of cells of Staph. aureus (Strain 1) in the presence of methicillin after repeated growth at 43 °C in the absence of antibiotic on standard nutrient agar

Test Temperature °C	Growth in the presence of methicillin concentration/μg cm^{-3}						
	0	4	10	20	40	100	200
43	++++	0	0	0	0	0	0
37	++++	5	0	0	0	0	0
25	++++	+	10	8	3	0	0

This result was in agreement with the shape and positioning of the pH-mobility curves (Fig. 3.3); that is the cells were now sensitive to methicillin. It seemed that the determinants of methicillin were carried on a plasmid, and this was lost during the repeated growth at 43 °C and thereby gave rise to methicillin sensitive cells.

(c) pH-mobility curves after final growth at 25, 37 or 43 °C of cells after repeated growth at 43 °C and subsequent growth at 37 °C

After 35 subcultures at 43 °C the cells were repeatedly grown at 37 °C for 15 times. The cells were inoculated in parallel on to nutrient agar plates and incubated at 25, 37 or 43 °C. The pH-mobility curves (Fig. 3.4) of the different cells are very similar to the curves of cells of the parent strain kept at 43 °C after final growth at the three temperatures (c.f. Fig. 3.3). This confirmed that the change of surface properties of the resistant cells to those characteristic of sensitive cells was stabilised on repeated growth at 43 °C and was not reversible when the cells were subsequently grown at a lower temperature. The plasmid responsible for methicillin resistance did not reappear when the cells were grown at the lower temperature. The curves had all the typical characteristics of those of sensitive cells with high H-values (30, 33 and 45) at each of the three temperatures and no minimum mobility values at pH 4.5 The strain also possessed considerable amounts of surface teichoic acid.

(d) Minimum Inhibitory Concentration at 25, 37 and 43 °C of cells grown first at 43 °C and then at 37 °C

MIC levels were determined (2.1,f(i)) on 18 h cells which had been repeatedly grown at 37 °C after the training at 43 °C in the absence of antibiotic. The plates were inoculated then incubated at 25, 37 and 43 °C. The cells were completely sensitive (Table 3.3); when grown at any temperature there was not any growth even at low concentration of the antibiotic at 25°C, the temperature at which the original parent cells were very resistant.

Table 3.3

Growth of cells of Staph. aureus (Strain 1) in the presence of methicillin grown 35 times at 43 °C and then 15 times at 37 °C on standard nutrient agar

Test Temperature °C	Growth in the presence of methicillin concentration/μg cm^{-3}						
	0	4	10	20	40	100	200
43	++++	0	0	0	0	0	0
37	++++	0	0	0	0	0	0
25	++++	0	0	0	0	0	0

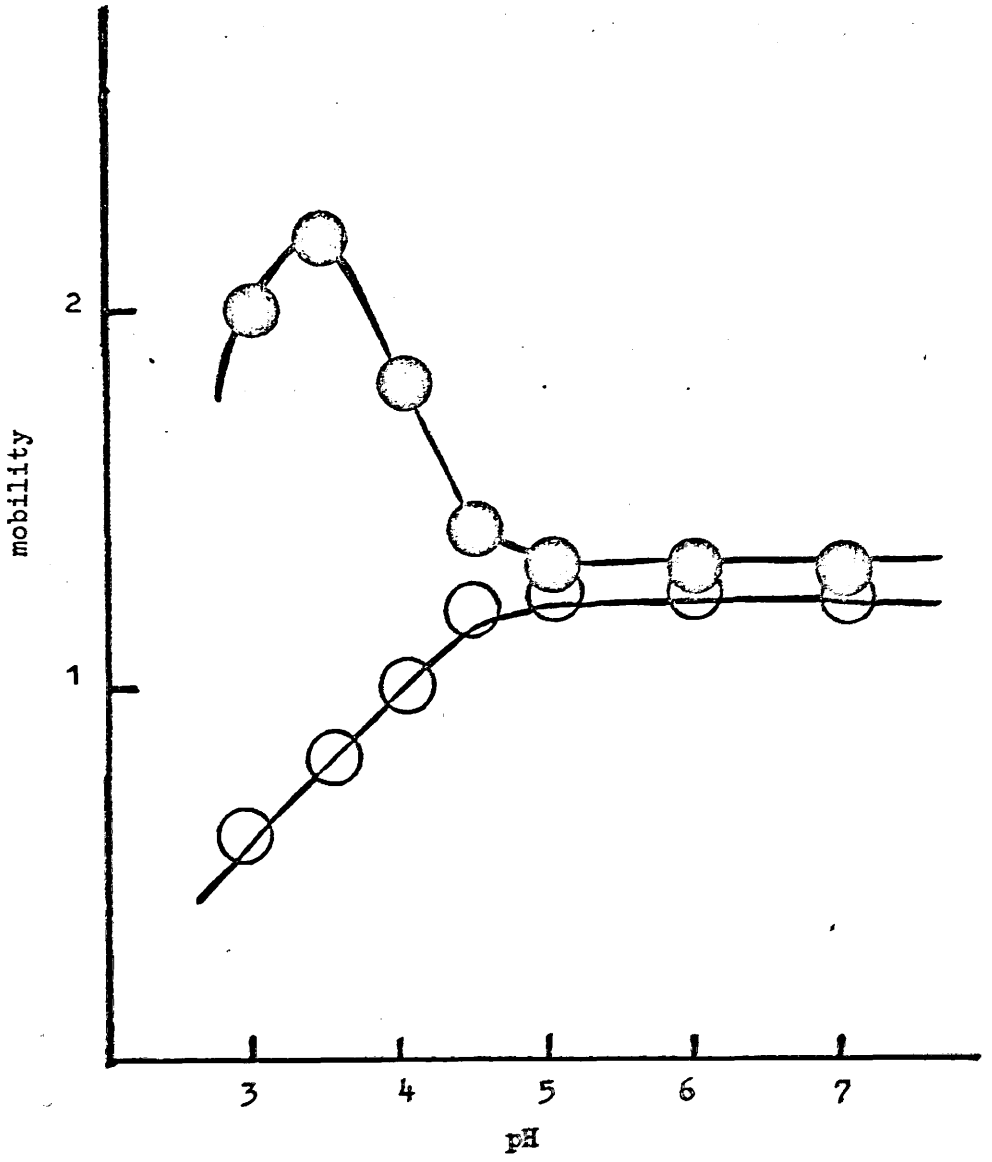
The suggestion of plasmid control of methicillin resistance became more clear, the plasmid did not reappear, when the cells were grown at a lower temperature. The cells were left for a few months on nutrient agar slope and then were examined; they were still sensitive to methicillin at each temperature.

(e) The removal of surface teichoic acid

It was apparent from the results of pH-mobility curves (Fig. 3.3) that the cells possessed a considerable amount of teichoic acid, characterised by the maximum in the mobility when suspended in solutions of low pH, i.e. the cells showed all the characteristics of methicillin-sensitive cells which James and Brewer (1968) associated with the phosphate groups of teichoic acid. The cells trained at 43 °C and finally grown at 43 °C lost their teichoic acid (Fig. 3.5) after treatment with sodium metaperiodate (2.3). Similar results were obtained for cells which had been maintained at 43 °C and finally grown at 37 °C.

Fig. 3.5

pH-mobility curves of cells of Staph. aureus (Strain 1) grown repeatedly at 43 °C on standard nutrient agar after oxidation (O); control (●)



(f) Detection of penicillinase

Staph. aureus strains produce an inducible enzyme, generally known as penicillinase, which hydrolyses the β-lactam ring of penicillin, with the production of the antibiotically inactive penicilloic acid. Evidence that the genetic elements controlling penicillinase production (and thus penicillin resistance) in staphylococci are carried on plasmid has been proved (Novick, 1963; Novick and Richmond, 1965).

The cells trained at 43 °C which lost their methicillin resistance were examined for the production of penicillinase (2.1,h). Starch plates were inoculated to obtain large numbers of colonies, after incubation of these plates at 37 °C the colonies were developed with iodine solution. The agar stained a deep blue and the colonies were stained a deep yellow; this showed that the cells had lost their ability to produce penicillinase. It was thus apparent that the plasmid-controlling penicillinase was also lost during repeated subculture at 43 °C.

The colonies were subcultured for purity and were tested again for penicillinase production. The test was carried out again after the cells had been subsequently subcultured at 37 °C and also after leaving the cells for a few months on nutrient agar slope at room temperature. There was no change; the cells were still penicillinase negative.

Loss of the ability to produce penicillinase thus appears to be irreversible and all attempts to demonstrate back mutation to the production of the enzyme have failed (Novick, 1963). The loss, therefore, resembles a deletion of the entire region controlling enzyme production rather than a point mutation. The ease with which these genetic elements can be lost irreversibly from a strain of staphylococcus without apparently affecting the viability of the cells, has led to the hypothesis that the genes are carried extrachromosomally.

3.3 Effect of repeated growth at 25 °C on standard nutrient agar

Cells grown at 25 °C and potentially very resistant to methicillin had very low or zero H-values, (Hill and James, 1971,b) as shown in Fig. 3.1. It was important to know whether these altered surface properties could be stabilised by maintenance of the cells at low temperature. Cells of strain 1 were therefore repeatedly subcultured for 20 subcultures on standard nutrient agar (2.1,a) in the absence of methicillin at 25 °C.

(a) pH-mobility curves after final growth at 25, 37 or 43 °C of cells grown repeatedly at 25 °C.

Three nutrient agar plates were inoculated in parallel from a culture which had been grown at 25 °C for 18 h; the cells were harvested and washed (2,2) and the pH-mobility curves determined (Fig. 3.6). There was no change on the shape of the curves (c.f. Fig. 3.1). The curves were displaced slightly from their original position, although it was not sufficient to be considered significant. After growth at 43 °C and 37 °C there was a minimum mobility at pH 4.5, but this was less than that shown by the parent cells; the cells also possessed surface teichoic acid. The H-values of cells grown at either 37 or 43 °C was low (14), this characteristic led to the suggestion that the cells had become more methicillin-resistant, cells grown at 25 °C had zero H-value as the parent cells.

The pH-mobility curves of cells grown at 25 °C, measured after 4, 6, 10, 15 and 51 subcultures are shown in Fig. 3.7. The shape of the pH-mobility curves was indistinguishable from that of parent cells grown once at 25 °C. There was, however, a marked increase in the negative mobility of the cells as the number of subcultures at 25 °C increased.

Fig. 3.6

pH-mobility curves of cells of Staph. aureus (Strain 1) grown 20 times at 25 °C on standard nutrient agar and finally grown at 25 °C (○); 37 °C (●) or 43 °C (⊖)

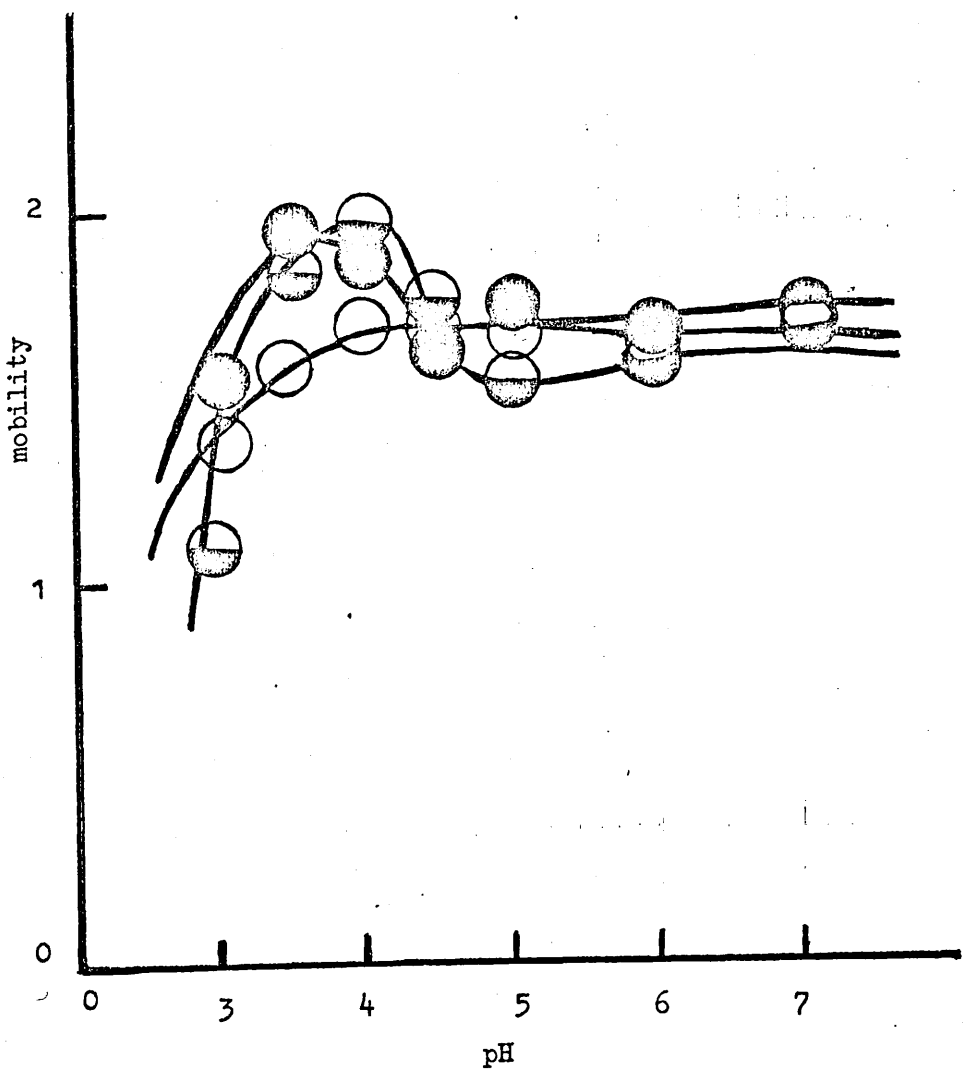
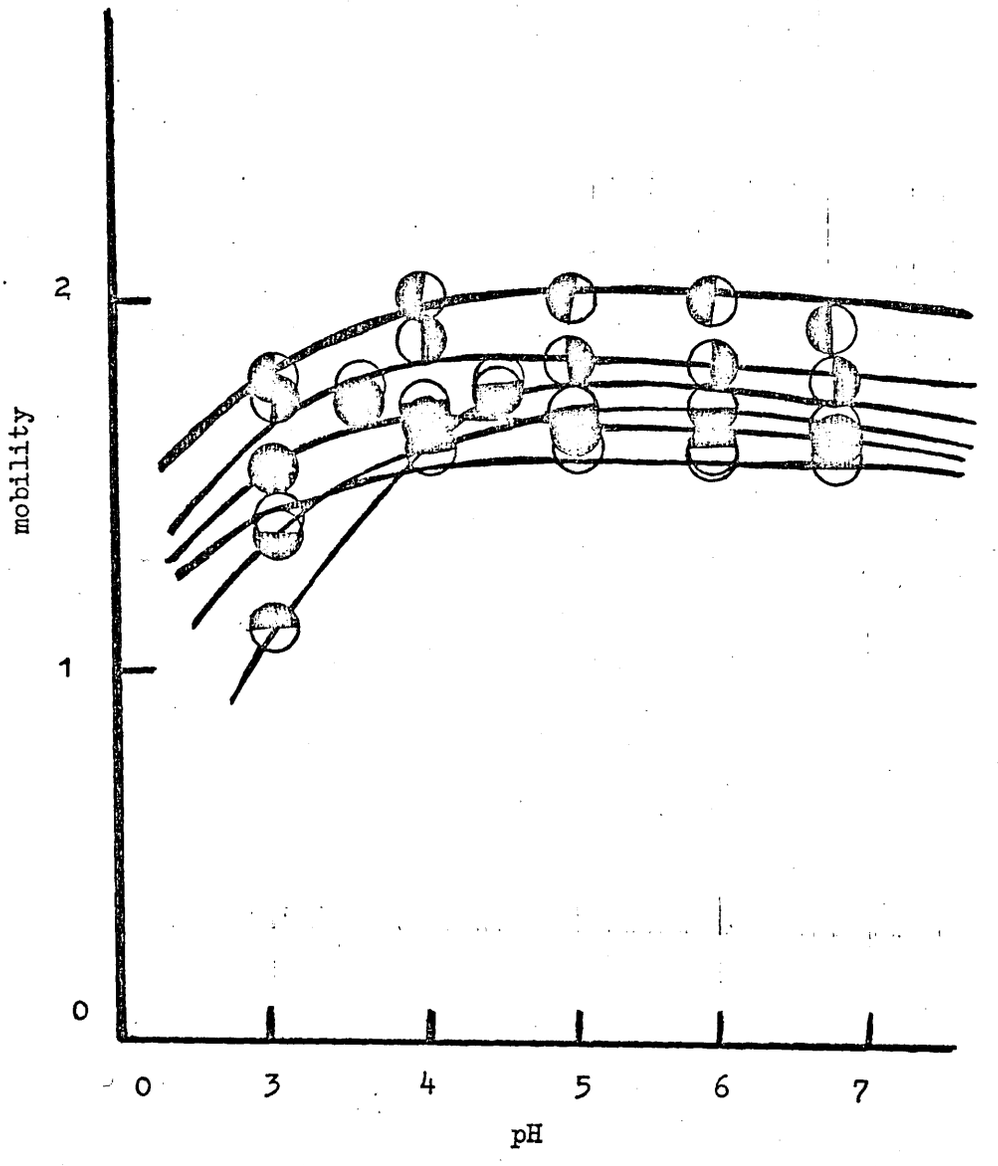


Fig. 3.7

pH-mobility curves of cells of Staph. aureus (Strain 1) grown repeatedly at 25 °C on standard nutrient agar, after 1 (0); 4 (◐); 6 (◒) 10 (◓) 15 (●) 51 (●) subcultures at 25 °C



(b) Minimum Inhibitory Concentration measured at 25, 37 or 43 °C for cells trained at 25 °C

Cells from a 18 h culture grown at 25 °C were inoculated on to the plates containing methicillin 2.1, f(i) and incubated at 25, 37 or 43 °C.

The cells became more resistant (Table 3.4) to methicillin measured both at 25 and 37 °C, the population became more homogeneous in its resistance and differed from that of the original parent strain grown at 37 °C (c.f. Table 3.1). The conventional 37 °C MIC was more than 200 μg cm^{-3}. The cells were, however, still sensitive at 43 °C.

Table 3.4

Growth of cells of Staph. aureus (Strain 1) in the presence of methicillin after repeated growth at 25 °C

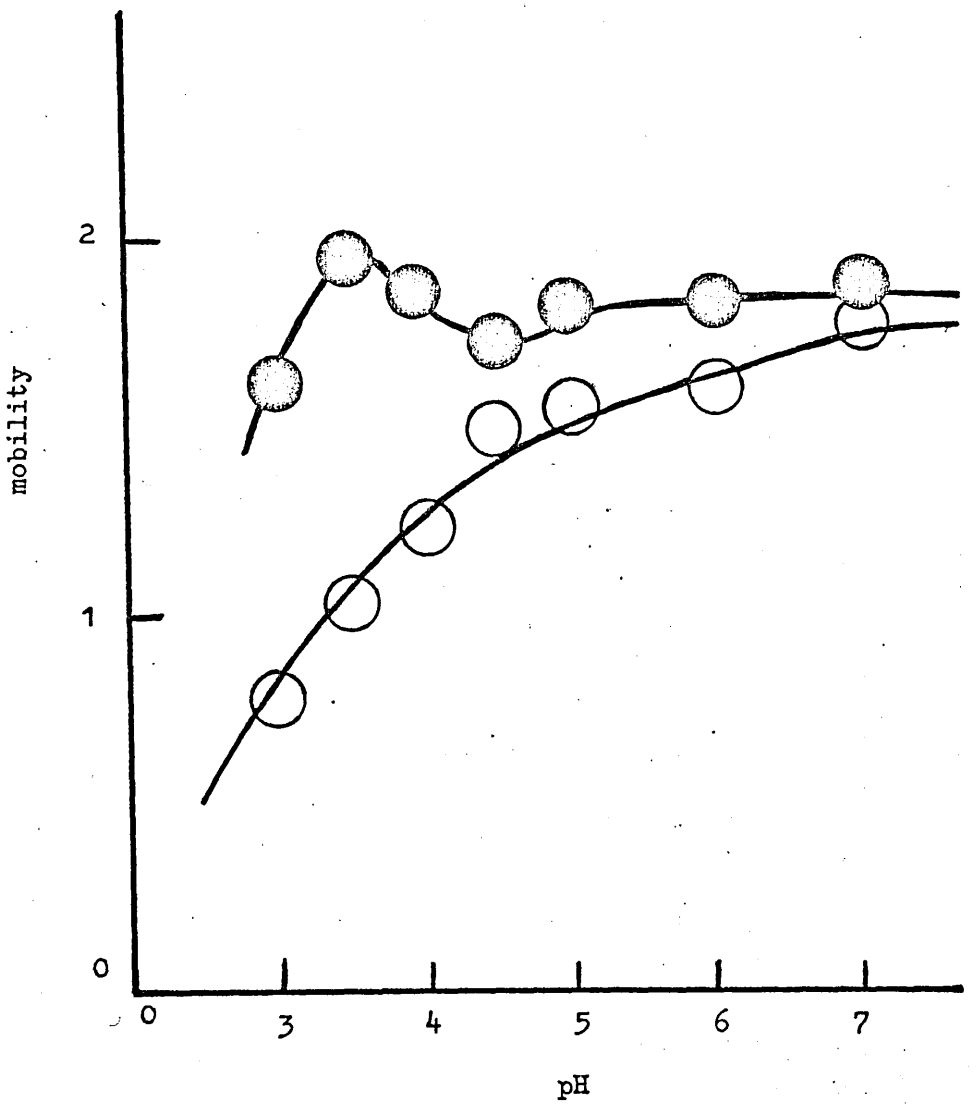
Test Temperature °C	Growth in the presence of methicillin concentration /μg cm^{-3}						
	0	4	10	20	40	100	200
43	++++	0	0	0	0	0	0
37	++++	+++	+++	+++	+++	20	10
25	++++	++++	++++	+++	+++	+++	++

(c) The removal of surface teichoic acid

Cells, grown repeatedly at 25 °C, were finally grown at 37 °C for 18 h. Cells from this culture were treated with sodium metaperiodate to remove teichoic acid, pH mobility determined before and after treatment (Fig. 3.8). The small amount of surface teichoic acid was lost from treated cells and there was no recorded maximum mobility in suspension at low pH values.

Fig. 3.8

pH-mobility curves of cells of Staph. aureus (Strain 1) grown repeatedly at 25 °C after oxidation (○) control (●)



3.4 Independent loss of methicillin and penicillinase plasmids during repeated growth at 43 °C

A number of different types of plasmids have been identified in staphylococci by genetical analysis. They include plasmids conferring resistance to antibiotics, heavy metals, toxin and pigment production. According to Dornbusch et. al., (1969) the genes controlling methicillin resistance and production of enterotoxin B appear to be associated together. Both characters were lost after treatment with acriflavine. Dornbusch and Hallander (1973) suggested that in some strains at least two plasmids were present, one of them is responsible for penicillinase production and resistance to metallic ions and the other for methicillin resistance with enterotoxin B production. The present work was undertaken to show if the plasmids for penicillinase production and methicillin resistance were lost independently or not.

(a) Isolation of mutants by replica plating

Cells which had been grown on standard nutrient agar at 43 °C for 10 subcultures were inoculated on to starch plates, and incubated 18 h at 37 °C. The colonies were replicated on to plates containing 10 $\mu g\ cm^{-3}$ methicillin (2.1,g). The master plates were kept and the replica plates were incubated for 18 h at 37 °C. The colonies which did not grow were marked on the starch plates, and then the plates were developed with iodine solution (2.1,h). Two types of penicillinase-positive colonies were recognised, one type which had grown on methicillin plates (pen^+ met-r) these are the same as the parent cells, the other type, few in number did not grow on methicillin plates (pen^+ met-s). Two types of penicillinase-negative colonies were also recognised, one type which had grown on the methicillin plates (pen^- met-r) and the other type which did not grow on the methicillin plates (pen^- met-s).

Cells from single colonies of four variants were subcultured on to other nutrient agar plates for purification. The variants were phage typed to make sure that their phage patterns resembled that of the parent cells. Tests were also carried out for loss of metal ion resistance and antibiotic resistance (Table 3.5).

(b) pH-mobility curves for cells of the variants grown at 37 °C

Cells of the four variants which were selected by replica plating were subcultured on to nutrient agar plates and incubated at 37 °C for 18 h. The cells were harvested and washed (2.2), and the mobility values of the cells measured in suspension over the normal pH-range at 25 °C. The pH-mobility curves (Fig. 3.9) of cells of the variants showed a marked variation in their surface properties. These were dependent on the resistance to methicillin but not affected at all by the production of penicillinase.

Cells of the variants, pen$^+$ met-r (penicillinase-positive, methicillin-resistant) and pen$^-$ met-r (penicillinase-negative, methicillin-resistant), (Fig. 3.9 a and b) showed a typical pH-mobility curve characteristic of resistant cells grown at 37 °C. Both had low H-values (0 and 12 respectively) and both exhibited minimum mobility values at pH 4.5, although the minimum was much less than that shown by a methicillin resistant strain grown at 43 °C.

The variants pen$^+$ met-s (penicillinase-positive, methicillin-sensitive) and pen$^-$ met-s (penicillinase-negative, methicillin-sensitive), (Fig. 3.9 c and d) showed a pH-mobility curve which was characteristic of methicillin sensitive cells grown at any temperature. Both variants had a high H-value (60 and 55 respectively) with maximum mobility values at low pH and a plateau value in suspensions of high pH values.

Thus the variants which had lost their methicillin resistance plasmid, possessed a considerable amount of surface teichoic acid.

Fig. 3.9

pH-mobility curves of cells of Staph. aureus variants grown on nutrient agar at 37 °C

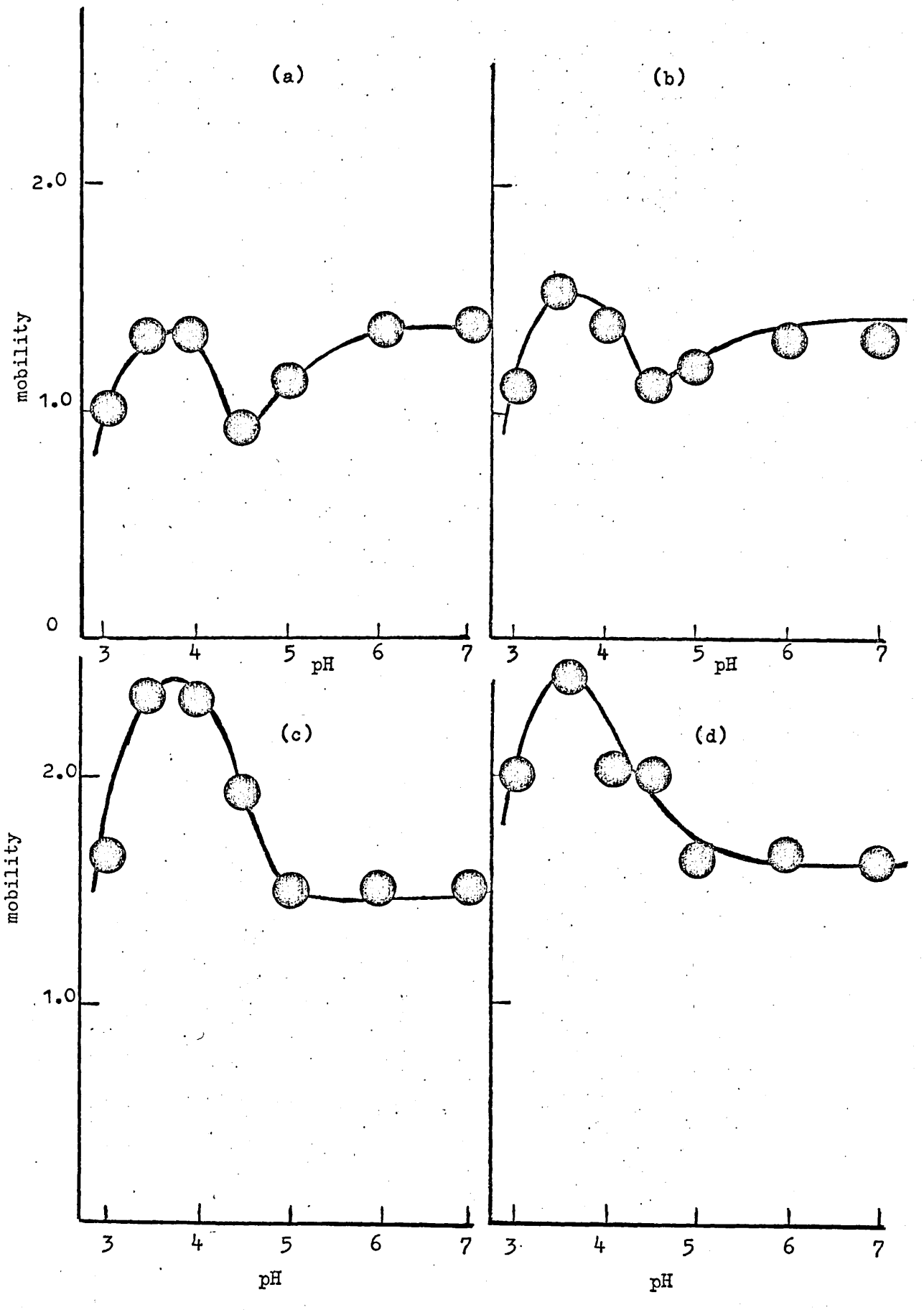
(a) pen^+ met-r (penicillinase-positive, methicillin-resistant).

(b) pen^- met-r (penicillinase-negative, methicillin-resistant).

(c) pen^+ met-s (penicillinase-positive, methicillin-sensitive).

(d) pen^- met-s (penicillinase-negative, methicillin-sensitive).

Fig. 3.9



(c) Characteristic of the mutants

Cultures of the variants were phage-typed (2.1,e) and tested for resistance to benzylpenicillin, streptomycin, tetracycline, methicillin and metal ions by paper discs containing appropriate concentrations (2.1,f(ii)). The MIC of methicillin and penicillinase production were checked for each of the variants.

The phosphatase activity of cells of each variant was assayed at 37 °C (Davies and James ,1974). All the results were summarised in Table 3.5. The variants all showed the same phage pattern as the parent cells. The variant pen$^+$ met-r which was methicillin resistant, and a penicillinase producer was resistant to tetracycline, streptomycin and the metal ions, cadmium, mercury and arsenate. This variant exhibited little surface teichoic acid, and the phosphatase activity of the cells grown at 37 °C was high. Cells of the variant pen$^-$ met-r was resistant to methicillin but had lost penicillinase plasmid together with resistance to metal ions (which are believed to be linked); they also exhibited high phosphatase activity at 37 °C. Cells of the variant pen$^+$ met-s, sensitive to methicillin and a penicillinase producer were also resistant to the metal ions and exhibited low phosphatase activity at 37 °C. Cells of variant pen$^-$ met-s, sensitive to methicillin, penicillinase negative were also sensitive to metal ions.

Irrespective of the loss of the methicillin and penicillinase plasmids, tetracycline and streptomycin resistances were not lost with the two antibiotics markers. This suggests that there is not any linkage between the genes controlling these two antibiotics and the methicillin and penicillinase plasmids.

Table 3.5

Characterisation of variants of Staph. aureus (Strain 1)

Variants	Phage typing	MIC methicillin† μg cm^{-3}	Penicillinase production	Phosphatase* activity	Disc sensitivity						
					Met	P	St	T	Hg	Cd	Ar
pen^+ met-r	47/53/54/75/77/84/85+	> 200	+	29.1	R	R	R	R	R	R	R
pen^- met-r	47/53/54/75/77/84/85+	> 200	-	59.1	R	S	R	R	S	S	S
pen^+ met-s	47/53/54/75/77/84/85	> 4	+	0	S	R	R	R	R	R	R
pen^- met-s	47/53/54/75/77/84/85	> 4	-	15.19	S	S	R	R	S	S	S

* A.L. Davies, 1974 [units: n mol min^{-1} (mg cell)$^{-1}$]

\+ Measured at 37 °C after 48 h incubation

R = resistant, S = sensitive, Met = methicillin, P = benzylpenicillin, St = streptomycin, T = tetracycline, Hg = mercury, Cd = cadmium, Ar = arsenate,

† Penicillinase producer

\- Non-penicillinase producer

3.5 Effect of repeated growth on other solidifed media at 43 °C

All the experiments were carried out on oxoid nutrient agar Code CM3 regardless of the batch numbers. The repeated growth of cells of Strain 1 on batch No. 152,13727 at 43 °C did not produce the same change of surface properties as did growth on standard media. Taking into account other experimental results in this laboratory it was suggested that this observation could be related to the content of inorganic phosphate in the media. High concentrations of inorganic phosphate in the media caused suppression of the phosphatase enzyme system of the cells; however, low concentrations of inorganic phosphate were required in growth media to give good growth of cells which exhibited a high activity (Davies and James, 1974).

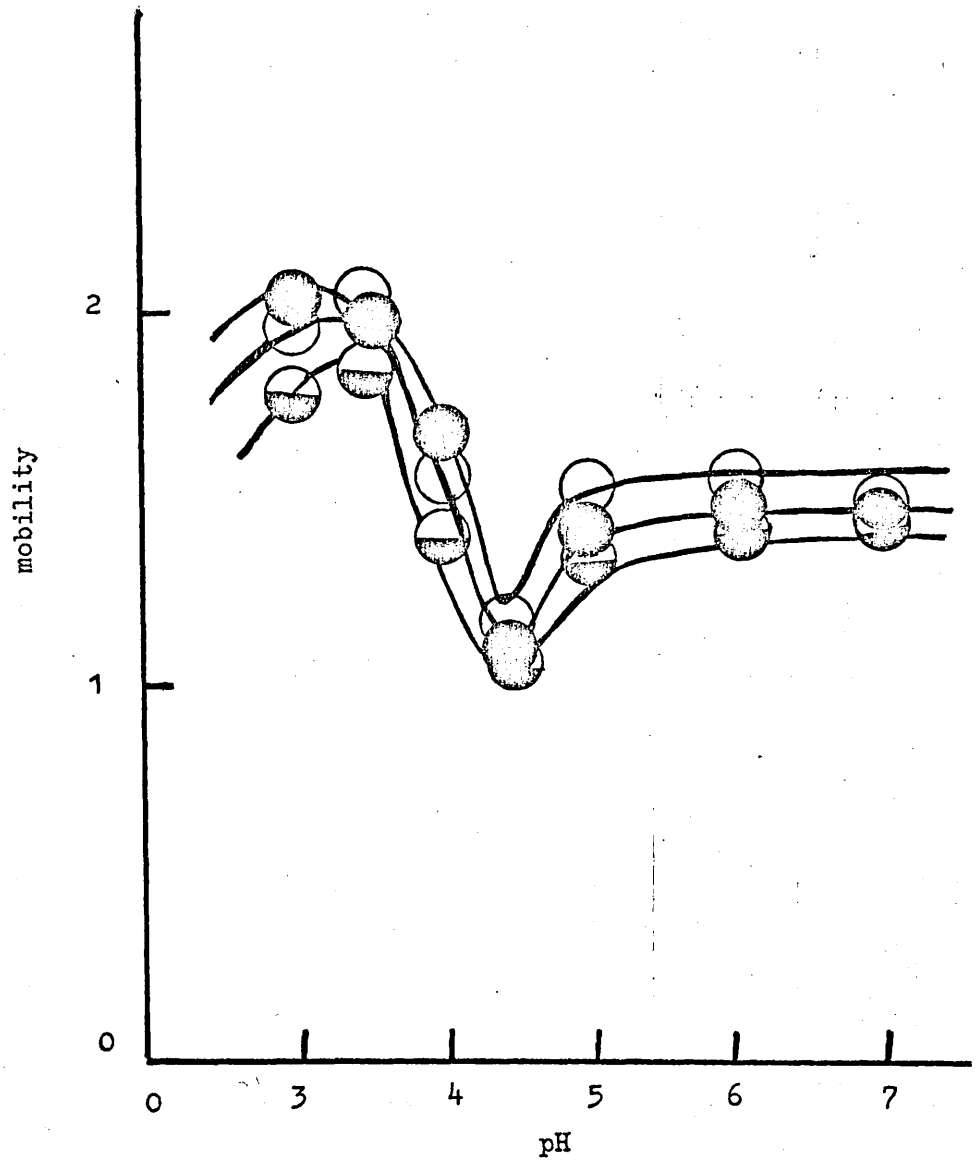
(a) pH-mobility curves of cells trained to grow at 43 °C and finally grown at 43 °C

Cells were grown repeatedly on media with high phosphate concentration (Oxoid nutrient broth Code CM1, batch No. 264,14154, solidified with Oxoid agar No.1) in the absence of antibiotic at 43 °C. After 20 subcultures cells of 18 h growth were inoculated in parallel on to 3 plates, and incubated at 25, 37 or 43 °C.

The pH-mobility curves of the cells grown at all of the temperatures were coincident (Fig. 3.10) and independent of the temperature of growth (c.f. Fig. 3.1). The typical characteristic of a marked minimum mobility at pH 4.5 was observed for cells grown at each temperature. The cells had also lost the ability to produce penicillinase.

Fig. 3.10

pH-mobility curves of Staph. aureus (Strain 1)
grown at 43 °C on nutrient agar batch No. 297, 14154
after growth at 25 °C ○ ; 37 ◐ or 43 °C ◒



(b) Minimum Inhibitory Concentration measured at 25, 37 and 43 °C of cells trained to grow at 43 °C

Nutrient agar plates containing methicillin in the range 0 - 200 μg cm^{-3} were inoculated from 18 h growth of cells grown repeatedly at 43 °C on solidified nutrient medium (297,14154). MIC determinations were carried out after 10 and 20 subcultures (Table 3.6). At 25 °C the growth was very homogeneous at all concentrations and the strain appeared very resistant. The MIC at 37 °C after 48 h was > 200 μg cm^{-3} while at 43 °C the cells were sensitive to methicillin.

Table 3.6

Growth of cells of Staph. aureus (Strain 1) in the presence of methicillin after repeated growth at 43 °C on nutrient agar batch No. 297,14154

Subcultures at 43 °C	Test temperature / °C	Growth in the presence of methicillin concentration/μg cm^{-3}						
		0	4	10	20	40	100	200
10	43	++++	0	0	0	0	0	0
	37	++++	+++	+	50	30	9	
	25	++++	++++	+++	++++	+++	+++	++
20	43	++++	0	0	0	0	0	0
	37	++++	+++	+++	++	++	30	10
	25	++++	++++	++++	+++	+++	+++	+++

3.6 Effect of growth media on pH-mobility curves of cells after one subculture from standard nutrient agar

It was apparent that the presence of a high inorganic phosphate level in the growth medium prevented the loss of methicillin resistance of cells of strain 1 when grown repeatedly at 43 °C. A more detailed study of the effect of inorganic phosphate on the MIC and the surface properties was undertaken.

The quantitative analysis of different batches of Oxoid nutrient agar and broth showed that there was a marked variation of content of inorganic phosphate. The presence of inorganic phosphate has a marked effect on the phosphatase activity of methicillin-resistant cells of Staph. aureus (Davies and James, 1974).

Nutrient agar plates or nutrient broth bottles were inoculated from parent cells grown 18 h at 37 °C which were maintained on standard medium. The cells were grown for 18 h at 37 °C and the pH-mobility curves were determined (Fig. 3.11). The cells were electrokinetically very homogeneous after growth on all media. The pH-mobility curves after growth on different media (Table 3.7) were characterised by minimum mobility values at 4.5, with varying H-values and phosphatase activity (Davies and James, 1974) depending on the growth medium.

Fig. 3.11

pH-mobility curves of cells of Staph. aureus (Strain 1) grown only at 37 °C

(a) On solidified calcium hydroxide treated medium.

(b) On Oxoid nutrient agar, Code CM3, 152,13727.

(c) In Oxoid nutrient broth, Code CM1, 297,14154.

(d) In Oxoid nutrient broth, Code CM1, 297,14154, treated with calcium hydroxide.

------ (Standard medium) Oxoid nutrient agar Code CM3, 273,8762.

Fig. 3.11

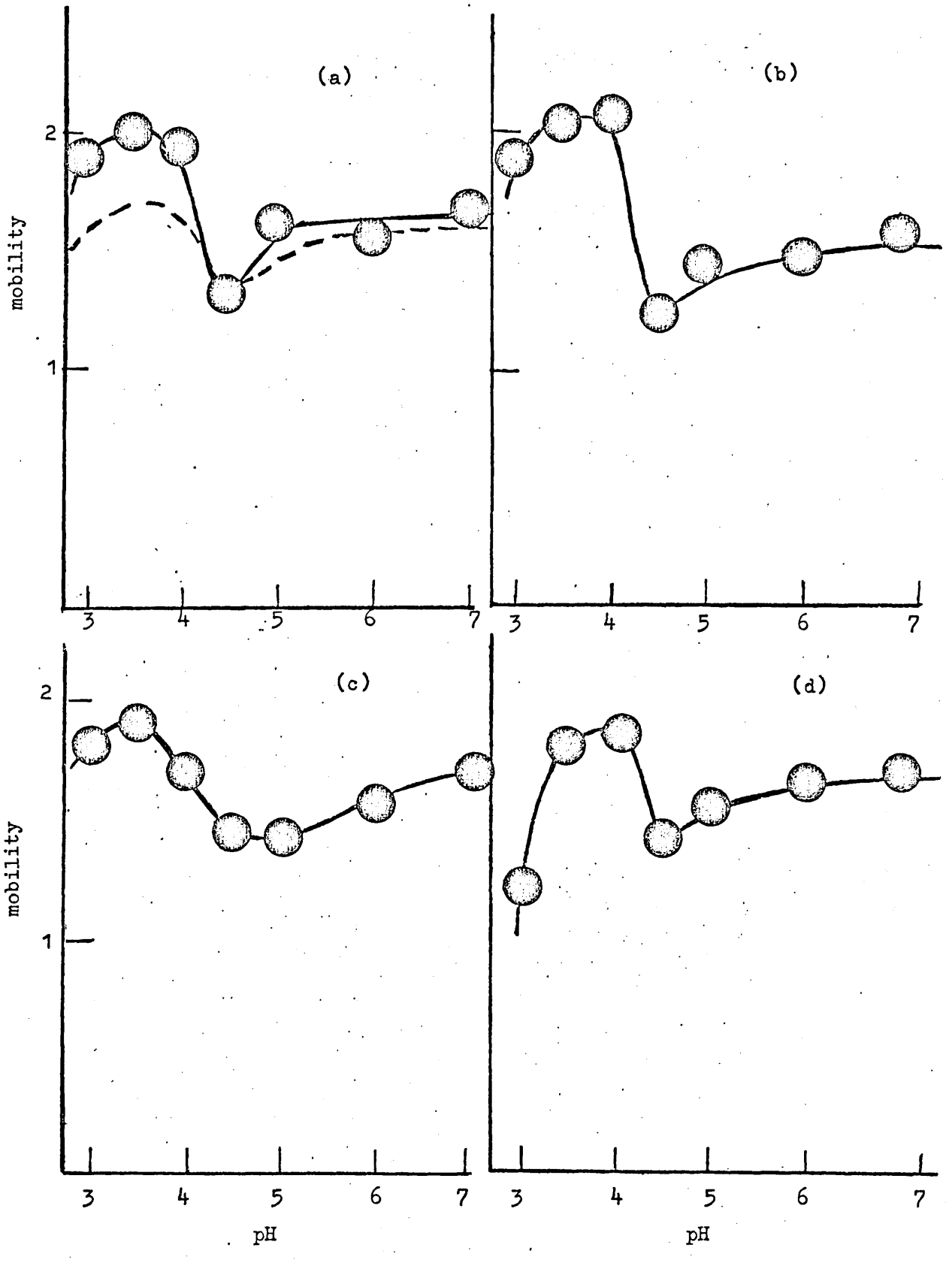


Table 3.7

Variation of H-values of cells of Staph. aureus (Strain 1) with amount of inorganic phosphate in the media

	Growth media	Concentration of inorganic phosphate* $\mu g\ cm^{-3}$	Phosphatase activity %* (Relative to standard)	H-values
	(Standard medium) Oxoid CM3 nutrient agar 273,8762	10	100	5
(a)	Oxoid CM1 nutrient broth 297,14154 treated with $Ca(OH)_2$ + agar	7	57	20
(b)	Oxoid CM3 nutrient agar 152,13727	35	0	35
(c)	Oxoid CM1 nutrient broth 297,14514	58	0	19
(d)	Oxoid CM1 nutrient broth 297,14154 treated with $Ca(OH)_2$	4	6	19

* Davies and James, 1974

3.7 Effect of repeated growth at 43 °C on calcium hydroxide treated medium

It was obvious from the results of repeated growth at 43 °C on standard medium with a low concentration inorganic phosphate (Fig. 3.2), that the surface properties of the cells of methicillin-resistant strain 1 were changed markedly; the cells also became sensitive. Repeated growth at 43 °C on media containing larger amounts of phosphate (Fig. 3.10) did not produce any change in the surface properties of the resistance of the population.

Therefore a method was established (2.1,b) to remove excess inorganic phosphate from the commercial media. This treated medium was used instead of the standard media to see the effect on the change of the surface properties of resistant cells when repeatedly grown at 43 °C.

(a) pH-mobility curves of cells trained at 43 °C after growth at 37 °C

Cells from the parent strain which was maintained on standard medium were inoculated on to calcium hydroxide treated medium. The cells were repeatedly grown both at 37 °C and 43 °C, pH-mobility curves were plotted for cells grown 18 h at 37 °C at regular intervals.

The MIC determined at 37 °C after 30-40 subcultures at 43 °C was reduced from an original of > 200 μg cm^{-3} of methicillin to about 40 μg cm^{-3} and after a further 10 subcultures there was further reduction to < 20 μg cm^{-3} of methicillin. The curves (Fig. 3.12 a,b and c) for cells subcultured 40 times at 43 °C were characterised by a minimum mobility value at 4.5. The pH-mobility curves of cells which had been subcultured for 45 times (Fig. 3.12, d), exhibited no minimum mobility value at 4.5, the shape of the curve was now indistinguishable from that shown by methicillin-sensitive cells.

Fig. 3.12

pH-mobility curves of cells of Staph. aureus (Strain 1) grown at 37 °C after repeatedly subculture at 43 °C on calcium hydroxide treated medium

(a) 30 subcultures at 43 °C

(b) 33 subcultures at 43 °C

(c) 40 subcultures at 43 °C

(d) 45 subcultures at 43 °C

Fig. 3.12

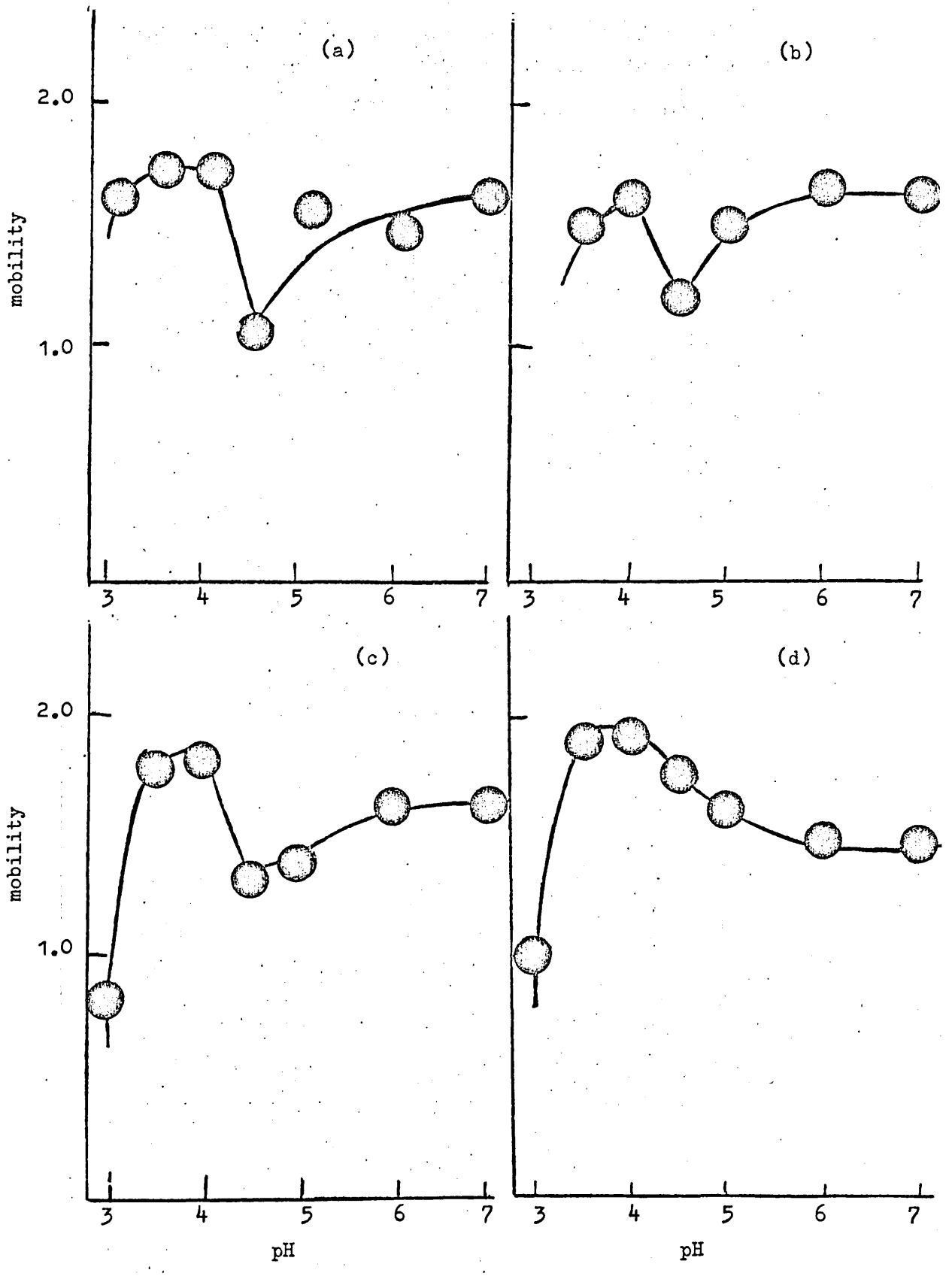
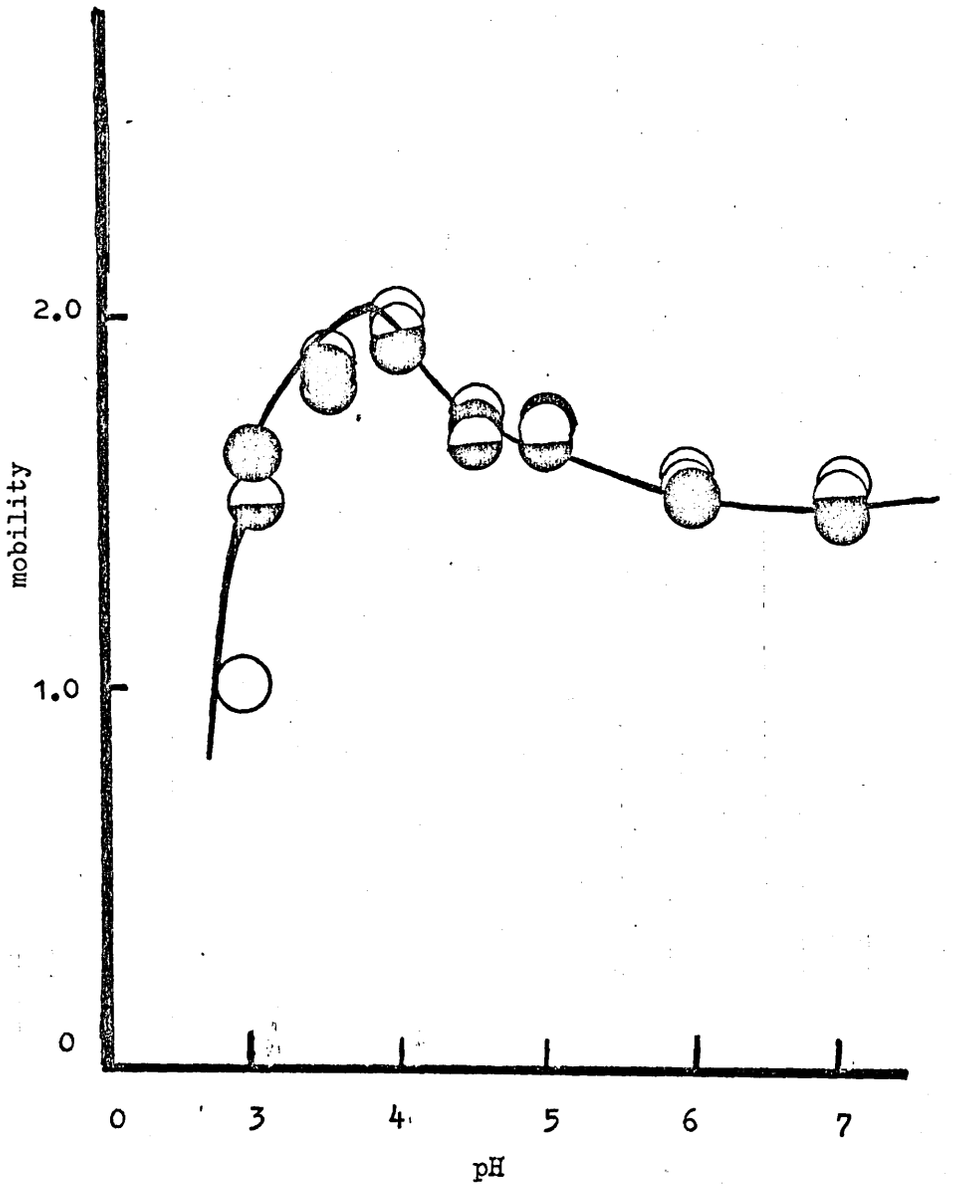


Fig. 3.13

pH-mobility curves of cells of Staph. aureus (Strain 1) grown repeatedly at 43 °C on calcium hydroxide treated media after growth at 25 °C (○); 37 °C (◍) or 43 °C (◉)



pH-mobility curves were also determined for cells which had 56 subcultures at 43 °C followed by final growth at 25, 37 or 43 °C (Fig. 3.13). The curves for cells grown at all three temperatures were coincident with no minimum mobility value at 4.5 and a H-value of 33. Even when these trained cells were grown at 25 °C, a temperature at which the cells are potentially resistant to methicillin, they possessed the surface properties of methicillin sensitive cells.

The cells had also lost the ability to produce penicillinase at a frequency of about 82.3% when repeatedly grown at 43 °C.

(b) Minimum Inhibitory Concentration at 37 °C of cells grown repeatedly at 43 °C on calcium hydroxide treated medium

MICs at 37 °C after 48 h incubation were tested regularly during the training at both 37 and 43 °C. After 8 and 18 subcultures there was no significant difference between the growth at all concentrations at both temperatures. After 32 subcultures the growth of cells, grown repeatedly at 43 °C, showed a marked change, there was no growth at 40 μg cm^{-3} methicillin, while for the cells maintained at 37 °C there was growth of cells up to 200 μg cm^{-3}. After 56 subcultures at 43 °C there was only sparse growth at 4 μg cm^{-3}; in contrast cells grown repeatedly at 37 °C still grew at concentration of methicillin up to 200 μg cm^{-3}.

It is now clear that the surface properties and MIC level changed in parallel after subsequent growth at 43 °C on the prepared medium. The change in these properties was much slower and H-values were not so high as in the case of repeated growth on standard media at 43 °C. It might be suggested that the standard media contained some constituents which helped the curing of the plasmid but that were lacking in the prepared media; alternatively, the presence of higher concentrations of inorganic phosphate causes the retention of the plasmid.

Table 3.8

Growth of cells of Staph. aureus (Strain 1) at 37 °C in the presence of methicillin after repeated growth at 37 or 43 °C in the absence of antibiotic on calcium hydroxide treated medium

No. of subcultures	Temperature of repeated growth / °C	Growth in the presence of methicillin concentration/μg cm^{-3}						
		0	4	10	20	40	100	200
One (parent)	37	++++	++++	+++	++	+	±	20
8	37	++++	+++	++	+	±	30	12
	43	++++	+++	++	+	20	12	9
18	37	++++	+++	++	+	±	20	10
	43	++++	++	±	±	40	10	3
32	37	++++	+++	++	++	±	25	15
	43	++++	+	±	50	0	0	0
56	37	++++	+++	+++	++	±	20	10
	43	++++	±	20	5	0	0	0

3.8 Summary

(a) Cells of the methicillin-resistant strain of Staph. aureus (Strain 1) showed a marked variation in surface properties with the temperature of growth. After growth at 37 °C for many subcultures the cells had a low H-value and there was a minimum mobility value at pH 4.5.

(b) Cells trained to grow at 43 °C on standard nutrient agar (low inorganic phosphate), lost their methicillin resistance and ability to produce penicillinase; then surface properties became characteristic of those of methicillin sensitive strains. These changes were not reversible when the cells were subsequently grown at a lower temperature.

(c) Cells trained to grow at 25 °C or 37 °C on standard nutrient agar did not show change in the surface properties. At 25 °C the cell became more resistant.

(d) The genes which control methicillin resistance and penicillinase production are most probably carried by different plasmids. These were lost independent of each other during growth at high temperature.

(e) Methicillin sensitive variants showed typical surface properties of methicillin sensitive cells irrespective of the loss of penicillinase plasmid.

(f) Resistance to metal ions was linked with the penicillinase plasmid.

(g) Cells trained to grow at 43 °C on nutrient agar containing high amounts of inorganic phosphate did not lose their resistant to methicillin, however, the cells lost the ability to produce penicillinase.

(h) pH-mobility curves after one subculture from standard media on to different media were characterised by minimum mobility value at 4.5, with varying H-values depending on the growth medium.

SECTION 4

Studies of the Surface and Biological Properties of Cells of Methicillin Sensitive Strains of Staph. aureus

The surface properties of the fully antibiotic (including methicillin) sensitive strains and methicillin sensitive, penicillin resistant strains were studied. The cells were electrokinetically homogeneous when studied at all pH values in the range 3 - 7. The pH-mobility curves of cells of these strains were independent of antibiotic sensitivity, growth media, and the temperature of growth.

4.1 Cells of fully antibiotic sensitive strain (Strain 2)

(a) Effect of temperature of growth on pH-mobility curves of cells grown on nutrient agar at 25, 37 or 43 °C

The pH-mobility curves (Fig. 4.1) were typical of cells of methicillin sensitive strains of Staph. aureus. The curves were characterised by having a sharp increase in negative mobility value in the pH range 3.5 - 4.0 and a plateau value from 5.0 - 7.0. This was in accord with the results of Hill and James (1971, b.). The curves showed no dependence on the temperature of growth; within the limits of experimental error the curves of cells grown at 25, 37 or 43 °C were coincident. The strain possessed considerable amount of surface teichoic acid with a high H-value (56).

(b) Effect of growth media on pH-mobility curves of cells after one subculture from standard nutrient agar

Nutrient agar plates or nutrient broth bottles were inoculated from parent cells grown for 18 h at 37 °C maintained on standard media. The cells were grown for 18 h at 37 °C and the pH-mobility curves after growth on

Fig. 4.1

pH-mobility curves of cells of Staph. aureus (Strain 2) grown on nutrient agar at 25 °C (○), 37 °C (●) or 43 °C (◒)

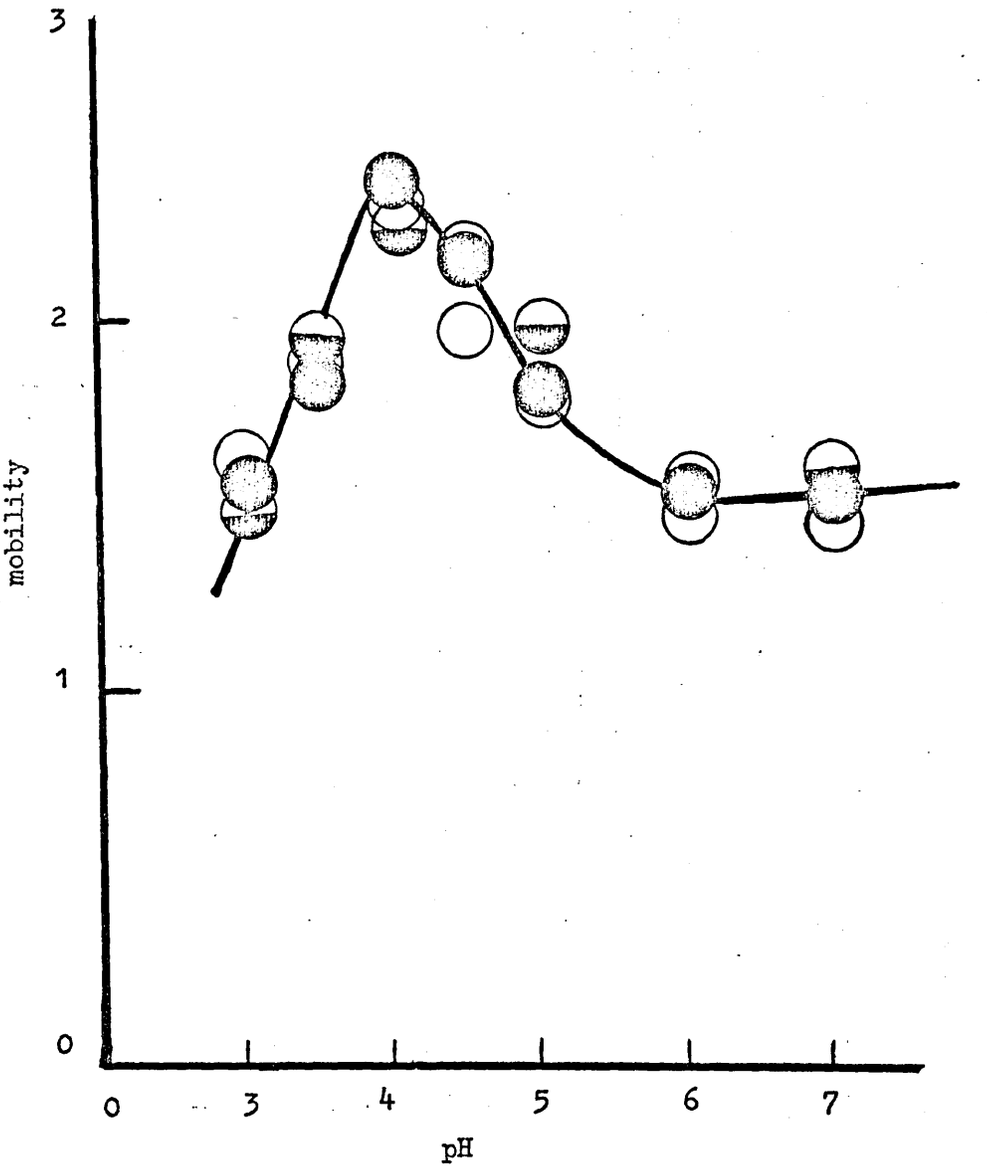


Fig. 4.2

pH-mobility curves of cells of Staph. aureus (Strain 2) grown once at 37 °C

(a) On solidified calcium hydroxide treated medium.

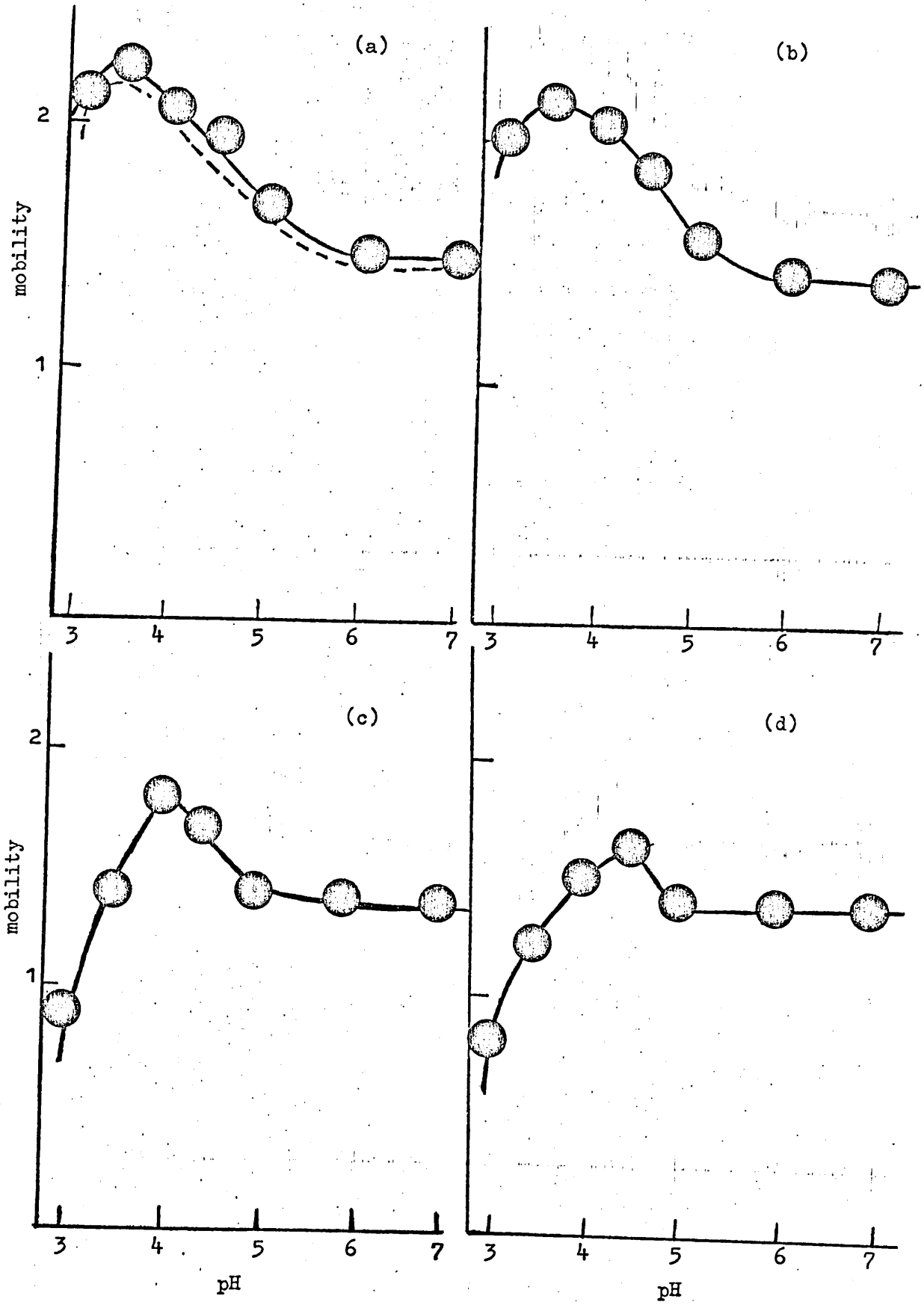
(b) On Oxoid nutrient agar, Code CM3, 152,13727.

(c) In Oxoid nutrient broth, Code CM1, 297,14154.

(d) In Oxoid nutrient broth, Code CM1, 297,14514 treated with calcium hydroxide.

--------- (Standard medium) Oxoid nutrient agar Code CM3, 273,8762.

Fig. 4.2



solid media (Fig. 4.2, a and b) were characterised by high H-values (50 and 51) at pH 3.5 - 4 and a plateau value from 5.0 to 8.0, this was independent of the amount of inorganic phosphate in the medium. On the other hand the pH-mobility curves of cells grown in liquid medium (Fig. 4.2, c and d) showed lower H-values (32, 21) than the H-values of the same strain grown on nutrient agar (c.f. Fig. 4.2, c and d). Allowing for experimental error, the curves a and b, and c and d) were coincident and were characteristic of methicillin sensitive staphylococci. This was in accord with the results obtained by Marshall (1969) and Hill (1971) which showed that growth on solid medium enhanced the H-value compared with that for cells grown in broth.

(c) Effect of repeated growth at 25 and 43 °C on nutrient agar on pH-mobility curves

Cells from the culture subcultured 20 times on standard media (3.1,a) in the absence of antibiotic at 43 or 25 °C were inoculated on to plates and grown at 37 °C. There was no change on the shape of the pH-mobility curves (Fig. 4.3) with increasing subcultures at either temperature (c.f. Fig. 4.1).

4.2. Cells of a methicillin sensitive, penicillin resistant strain (Strain 3)

(a) Effect of temperature of growth on pH-mobility curves of cells grown on nutrient agar at 25 or 43 °C

The pH-mobility curves (Fig. 4.4) of cells of methicillin sensitive strain of Staph. aureus grown at 25 or 43 °C, were coincident and showed no dependence on the temperature of growth. The curves are characterised by a sharp maximum negative mobility value in pH range 3.5 - 4 and plateau values from 5 - 7 with a very high H-value (115). This characteristic was independent of the resistance to penicillin i.e. penicillinase producer.

Fig. 4.3

pH-mobility curves of cells of Staph. aureus (Strain 2) grown repeatedly on nutrient agar at 43 °C(●) or 25 °C(○) and finally at 37 °C, ----- control

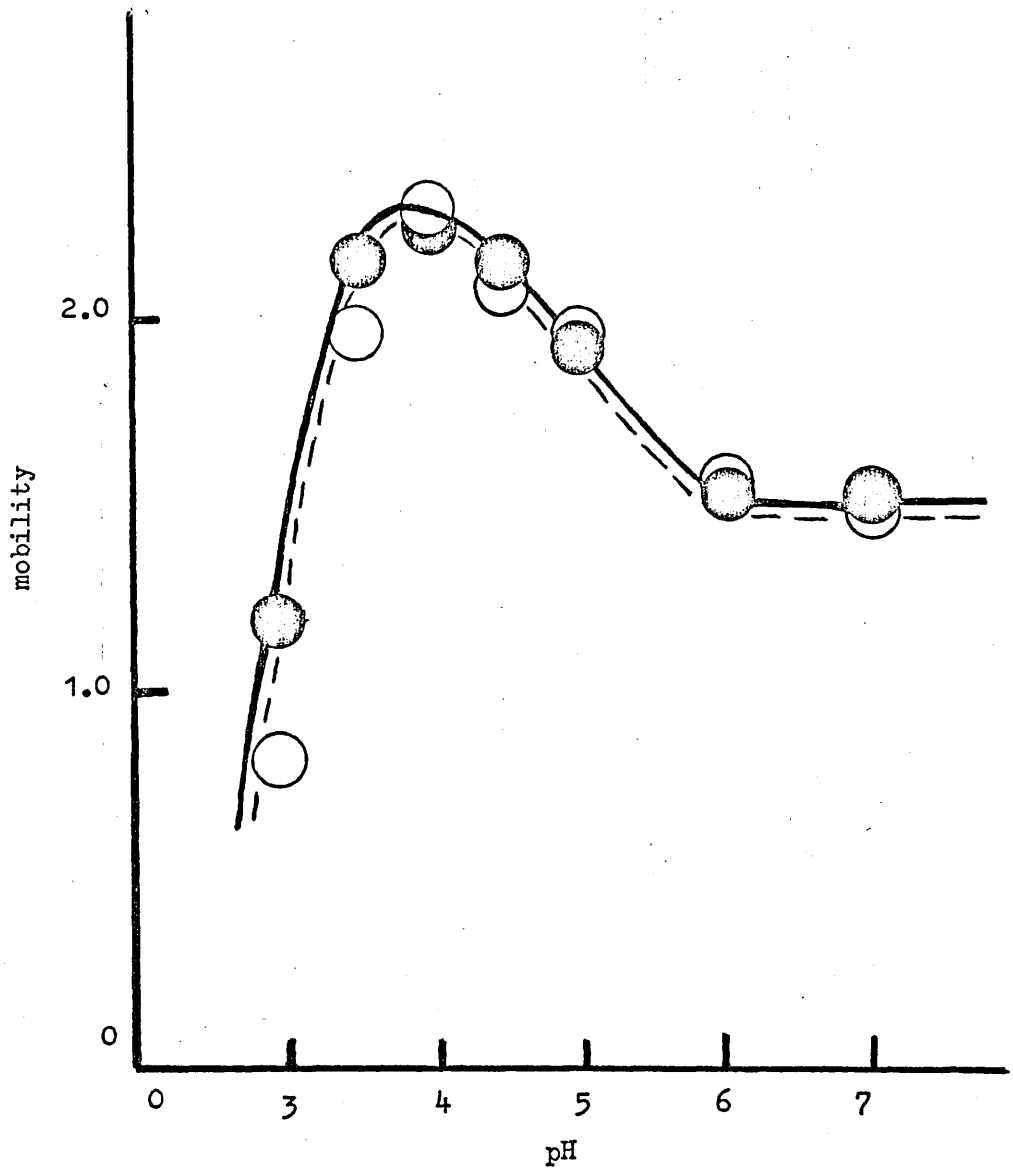


Fig. 4.4

pH-mobility curves of cells of Staph. aureus (Strain 3) grown on nutrient agar at 43 °C(⊖); 37 °C(○)

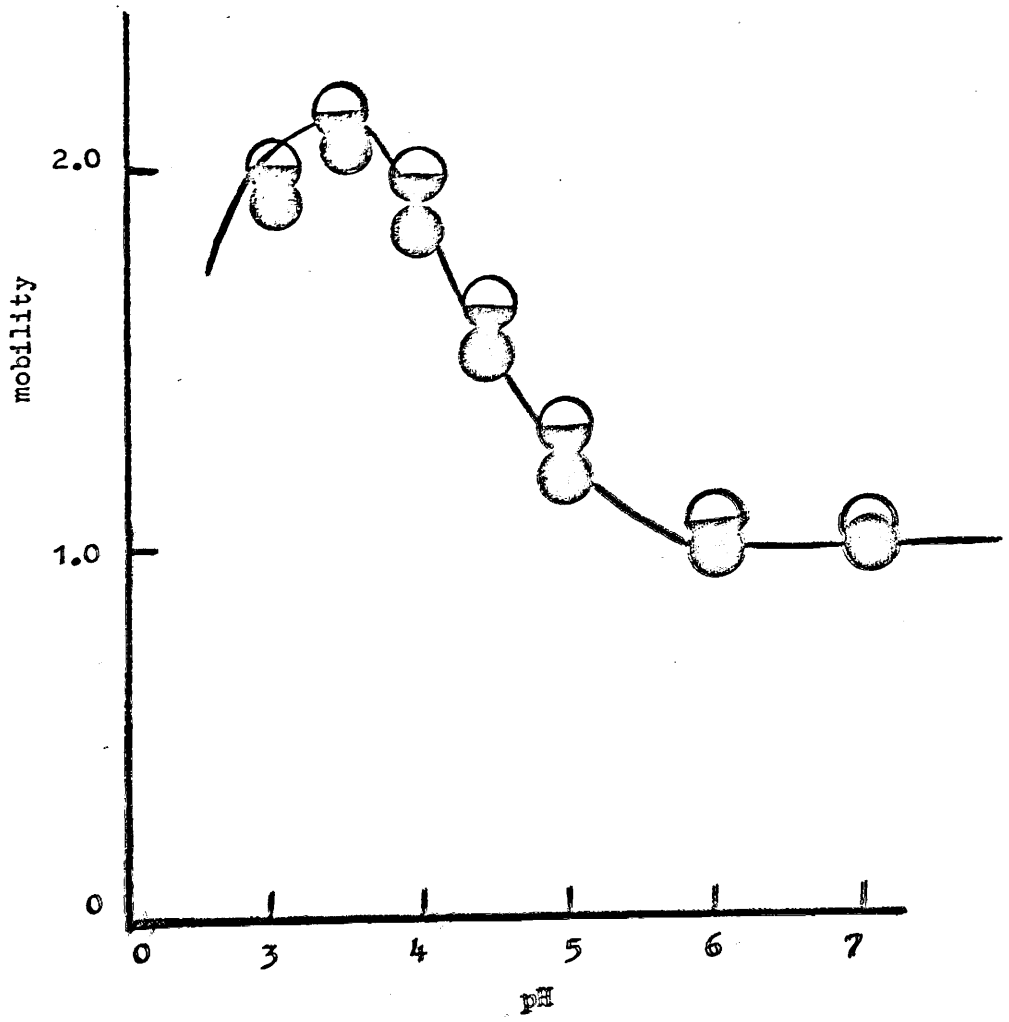
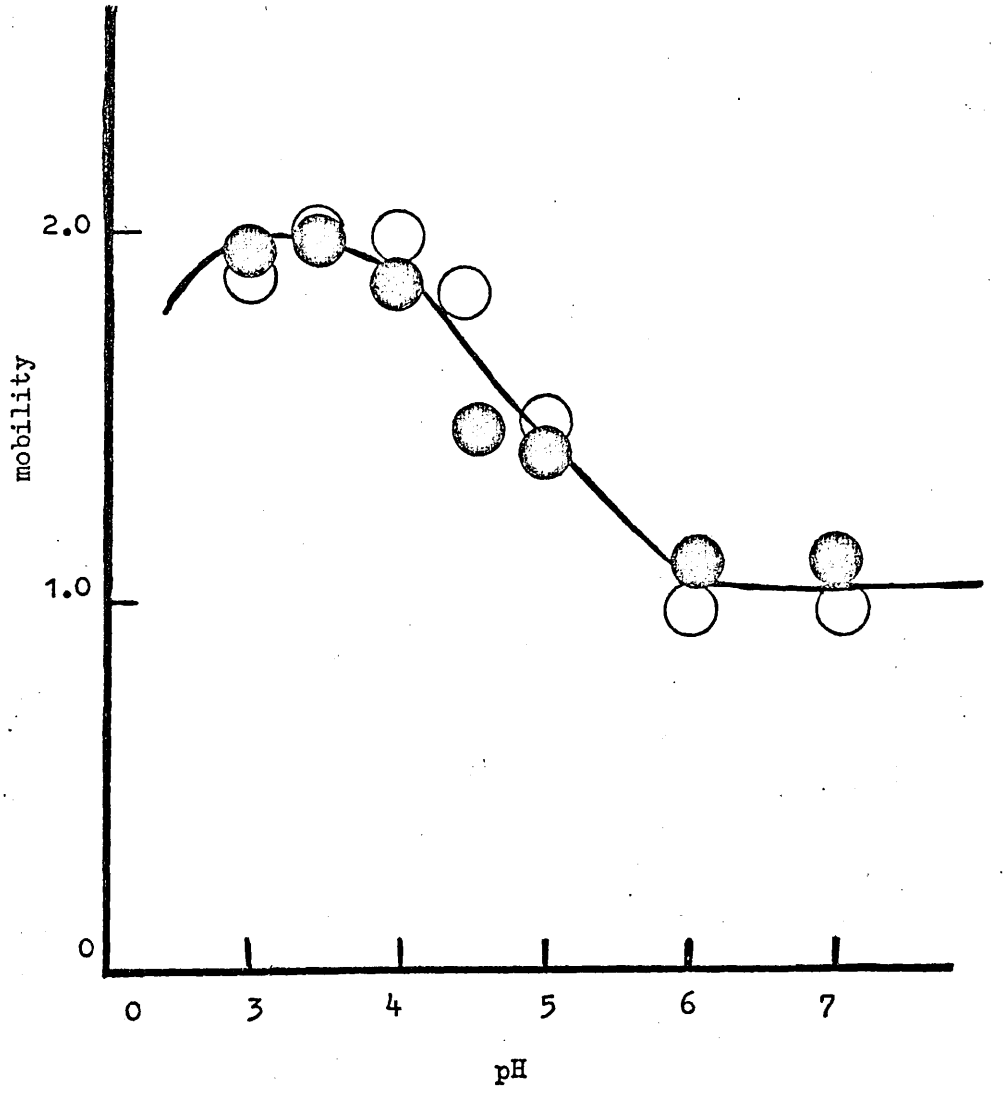


Fig. 4.5

pH-mobility curves of cells of Staph. aureus (Strain 3) grown repeatedly at 37 °C(○)or 43 °C (●) and finally at 37 °C



(b) Effect of repeated growth at 37 or 43 °C on nutrient agar on pH-mobility curves and penicillinase production

Cells from the culture subcultured 20 times on nutrient agar at 37 or 43 °C were inoculated on to plates and grown 18 h at 37 °C. There was no change in the shape or position of the pH-mobility curves (Fig. 4.5). They were typical of a methicillin sensitive strain (c.f. Fig. 4.4) with high H-values (100 and 88).

The tests for penicillinase production (2.1, h) were carried out for cells repeatedly grown at 37 and 43 °C; 1230 and 1076 colonies were screened respectively. All the colonies of cells grown at both temperatures were still penicillinase positive; 758 colonies from parent cells were also included as control. This was in contrast to cells of strain 1 which lost their ability to produce penicillinase at high frequency when repeatedly grown at 43 °C. This suggests a chromosomal control of penicillinase production in such strains.

4.3 Summary

(a) pH-mobility curves of fully antibiotic sensitive strains were characterised by maximum mobility value between 3.5 and 4.0 and a plateau value from pH 5.0 - 7.0. The shape and position of the curves were independent of the growth temperature.

(b) A typical pH-mobility curve of fully sensitive cells was obtained after one subculture in media containing various amounts of inorganic phosphate. The H-value of cells grown in liquid media were less than that of cells grown on solid media.

(c) Cells of fully antibiotic sensitive strains trained to grow at 25 or 43 °C showed no change on the shape or position of pH-mobility curves.

(d) Methicillin sensitive, penicillin resistant cells exhibited a typical pH-mobility curve of a methicillin sensitive strain before and after repeated growth at 37 or 43 °C. The strain did not lose the ability to produce the enzyme penicillinase, after repeated growth at 43 °C.

(e) Thus the surface properties of methicillin sensitive cells are constant independent of the ability to produce penicillinase, and independent of the growth temperature.

SECTION 5

Studies of Surface and Biological Properties of Cells of other Strains of Staph. aureus with Natural Resistance to Methicillin

5.1 Recently isolated methicillin resistant strains of Staph aureus (Strains 10 and 11)

(a) pH-mobility curves of cells grown on nutrient agar at 37 °C

These two recently isolated strains were studied to find out if they exhibited the same characteristics as the other methicillin resistant strains. The pH-mobility curves (Fig. 5.1) of 18h cells grown at 37 °C showed the typical characteristic features of methicillin resistant strains with low H-values (21, 0).

(b) Minimum Inhibitory Concentration measured at 25, 37 and 43 °C

MIC values were determined by the plate method, the plates were inoculated with 18 h cells grown at 37 °C. The results were recorded after 48 h growth at 25, 37 and 43 °C (Table 5.1); the conventional MIC at 37 °C exceeded 200 $\mu g\ cm^{-3}$ for both strains.

5.2 A strain of methicillin resistant Staph. aureus which shows the surface characteristic of sensitive strains (Strain 9)

(a) pH-mobility curve of cells grown on nutrient agar at 37 °C

The strain, obtained from Beecham Research Laboratories, was that used previously by Grubb and Annear (1972). The shape of the pH-mobility curve (Fig. 5.2) was indistinguishable from that shown by cells of methicillin sensitive strain grown at 37 °C (c.f. Fig. 4.1) with H-value of 42.

Fig. 5.1

pH-mobility curves of cells of methicillin resistant strains of Staph. aureus for Strain 10 (◎) and 11(○) grown on nutrient agar at 37 °C

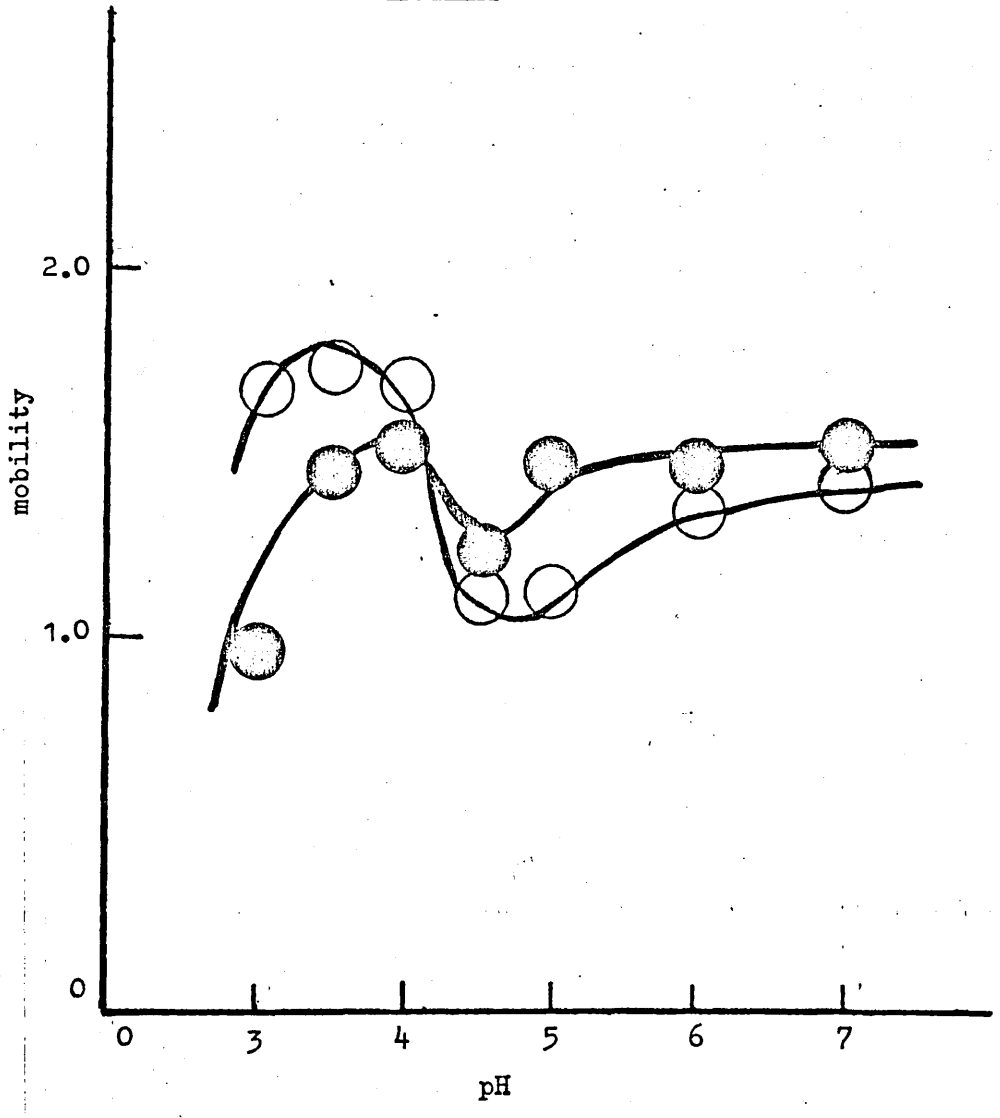


Table 5.1

Growth of cells of Staph. aureus (Strains 10 and 11) in the presence of methicillin

Strain No.	Test temperature / °C	Growth in presence of methicillin concentration $\mu g/cm^{-3}$						
		0	4	10	20	40	100	200
10	25	++++	+++	+++	+++	++	++	++
	37	++++	++++	+++	++	++	±	few
	43	++++	0	0	0	0	0	0
11	25	++++	+++	+++	+++	+++	++	+
	37	++++	++	+	±	30	18	10
	43	++++	0	0	0	0	0	0

Fig. 5.2

pH-mobility curves of cells of Staph. aureus (Strain 9) grown at 37 °C (●) and after oxidation (○)

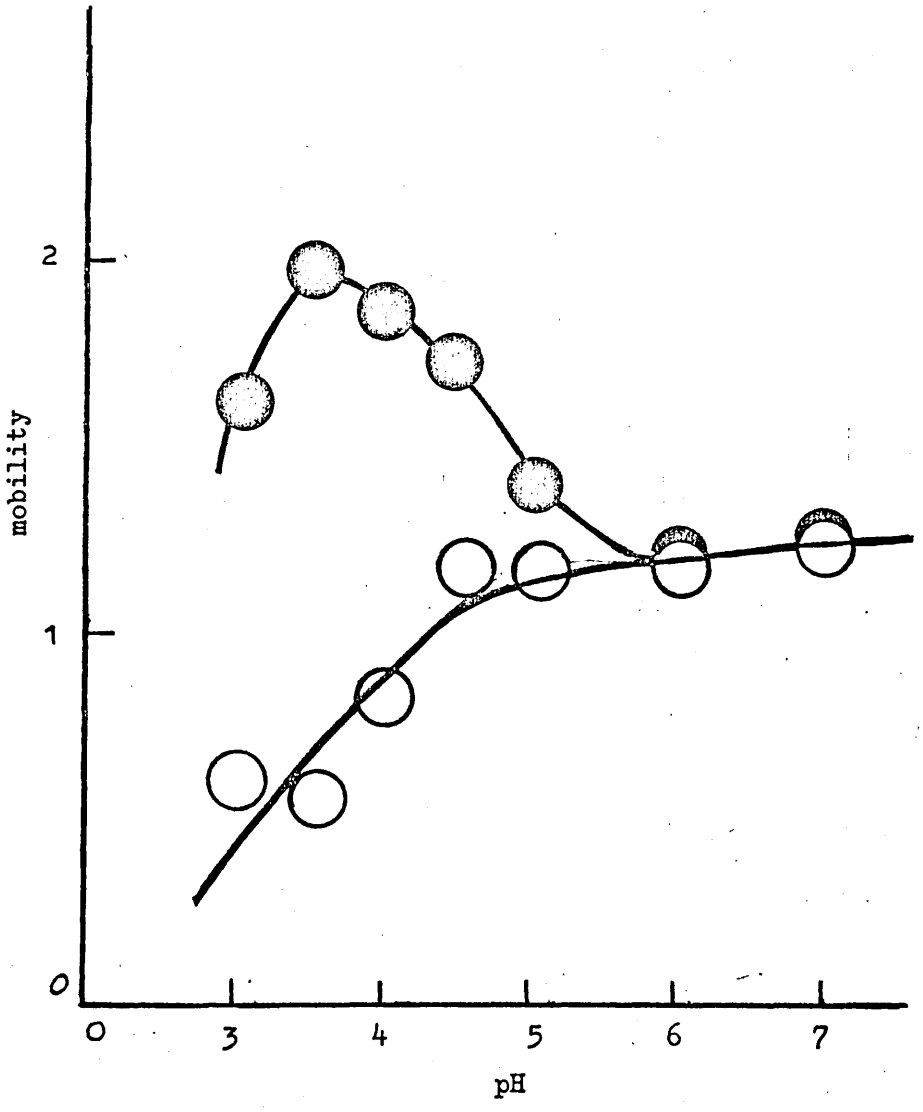
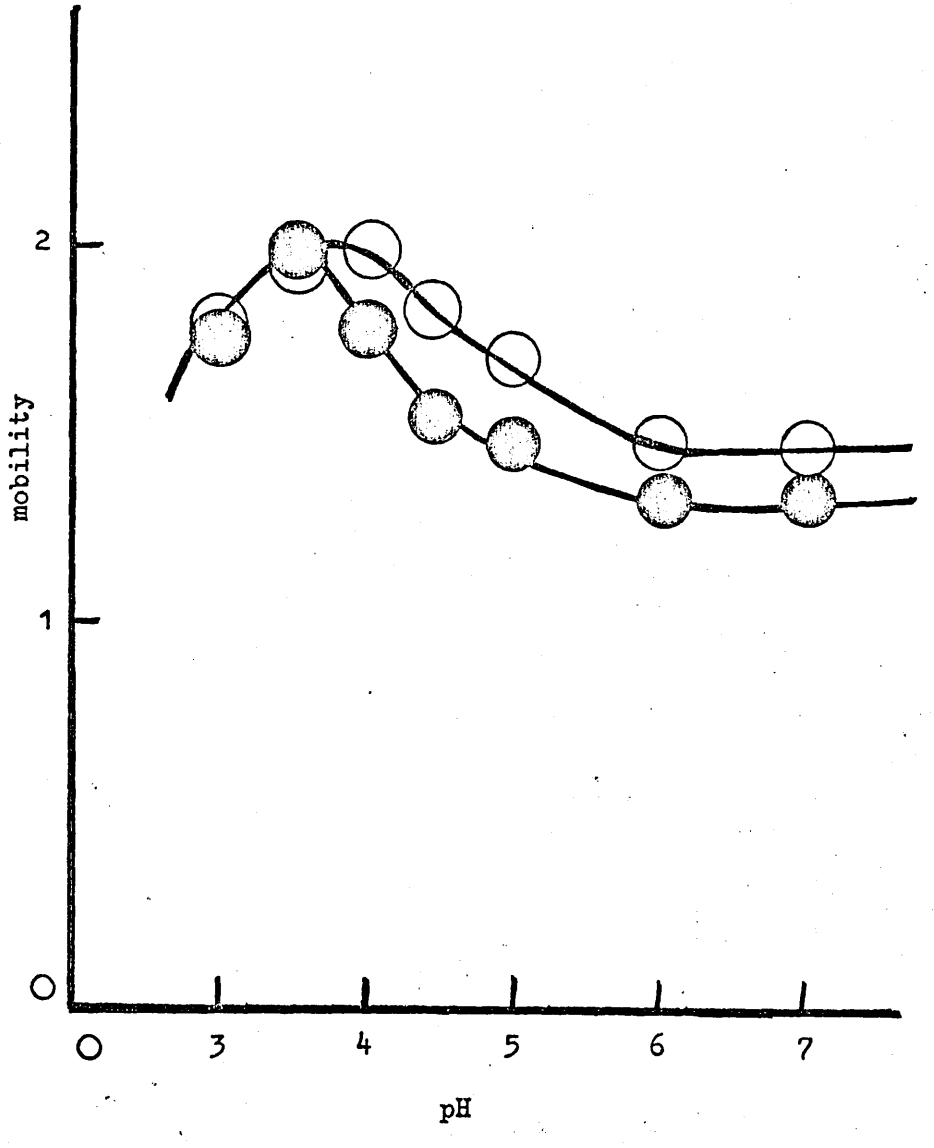


Fig. 5.3

pH-mobility curves of cells of Staph. aureus (Strain 9) grown repeatedly on nutrient agar at 43 °C and finally grown at 43 °C (○) and 37 °C (◎)



The surface teichoic acid was removed by oxidation with sodium metaperiodate (2.3). The pH-mobility curve of the treated cells was compared with that of the control (Fig. 5.2). The peak mobility value was absent from the curve of the treated cells. This indicated the removal of the phosphate group of the teichoic acid.

(b) pH-mobility curves of cells trained to grow at 37 and 43 °C and finally grown at 37 or 43 °C

Cells from the parent strain were inoculated on to two nutrient agar plates; one plate was incubated at 37 °C and the other at 43 °C. The cells in these two series were grown 20 times at either 37 °C or 43 °C. The conventional MIC at 37 °C for parent and trained cells were determined (Table 5.2). The population of the cells trained to grow at 43 °C became more sensitive with a MIC of 10 μg cm^{-3}, whereas MIC for parent cells and cells grown repeatedly at 37 °C exceeded 200 μg cm^{-3}.

Table 5.2

Growth of cells of Staph. aureus (Strain 1) in the presence of methicillin after repeated growth at 43 and 37 °C in the absence of antibiotic

Temperature of repeated growth / °C	Test temperature / °C	Growth in presence of methicillin concentration/μg cm^{-3}						
		0	4	10	20	40	100	200
Parent	37	++++	++++	+++	++	+	±	few
37 (20 subcultures)	37	++++	++++	+++	++	+	+	±
43 (20 subcultures)	37	++++	few	0	0	0	0	0

pH-mobility curves for cells trained to grow at 43 °C were plotted after 18 h growth on nutrient agar at 37 and 43 °C (Fig. 5.3). The curves of cells repeatedly grown at 43 °C showed that the same features as that of the parent cells (c.f. Fig. 5.2) and they were all indistinguishable from the pH-mobility curves of cells of typical methicillin sensitive strains.

(c) Effect of ageing on the MIC

Nutrient broth and nutrient agar containing various amounts of inorganic phosphate were used in the ageing experiment. Cells from parent slopes of strain 9 and strain 1 (as control) were inoculated in duplicate in these media. After overnight incubation at 37 °C, the cap of each culture was screwed down tightly and the cultures were stored at room temperature.

At intervals of 2, 5, and 10 months the MIC values were determined by inoculating one drop of 18 h culture from both strains on to methicillin plates. The results were recorded after 48 h incubation at 37 °C, (Table 5.4). The table only lists the MIC values after 10 months, over this period there was no change in the MIC level as a result of leaving the cultures at room temperature.

Table 5.3

Growth of cells of Staph. aureus strain 1 and 9 in presence of methicillin after leaving the cultures at room temperature for 10 months in different media

Growth medium	MIC at 37 °C/μg cm^{-3} methicillin	
	Strain 1	Strain 9
Oxoid CM3 nutrient agar 273,8962	> 200	200
Oxoid CM3 nutrient agar 152,13727	200	> 200
Oxoid CM1 nutrient broth 297,14154	> 200	> 200
Oxoid CM1 nutrient broth 297,14154 (treated with $Ca(OH)_2$)	200	200
Oxoid CM1 nutrient broth 297,14154 (treated with $Ca(OH)_2$ + agar)	200	> 200
Oxoid No.2 nutrient broth	> 200	> 200

5.3 Summary

(a) Recently isolated methicillin resistant Staph. aureus strains exhibited the same surface characteristics as the other methicillin resistant strains, with low H-values and a minimum mobility value at pH 4.5.

(b) Strain 9, which is methicillin resistant shows the surface characteristics of methicillin sensitive strains. When the strain was trained to grow at 43 ^{o}C in the absence of antibiotic the population became more sensitive, with MIC of 10 $\mu g\ cm^{-3}$.

(c) There was no change in the MIC level as a result of leaving the cultures of strain 9 and strain 1 at room temperature for long periods of time in media containing various amounts of inorganic phosphate.

SECTION 6

Studies of the Surface and Biological Properties of Cells of Staph. epidermidis

The strains were obtained from the blood cultures at the Cross Infection Reference Laboratory, and had been tested for coagulase reaction, antibiotic sensitivity and phage typing. Coagulase-negative strains which were sensitive or resistant to methicillin were selected for study. The numbers refer to the blood culture number of the Cross Infection Reference Laboratory.

6.1 Methicillin sensitive strains (Strains 8 and 70)

The pH-mobility curves (Fig. 6.1) of 18 h cells grown at 37 °C have the typical shape of methicillin sensitive cells of Staph. aureus (c.f. Fig. 4.1). The curves were characterised by maximum mobility values at 3.5 - 4.0 with no minimum negative mobility and H-values of 40 and 63 respectively.

Cells of strain 70 were incubated at each of the temperatures 25, 37 or 43 °C on nutrient agar for 18 h. The pH-mobility curves (Fig. 6.2) were coincident and independent of the temperature of growth. At all temperatures the maximum mobility occurred in the pH range 3.5 - 4.0, with a plateau value from pH 5 - 7. The curves indicated that the cells possessed considerable amounts of surface teichoic acid. The pH-mobility curves of cells (Fig. 6.3) before and after treatment with sodium metaperiodate confirmed this. The peak mobility value at pH 3.5 - 4 was absent from the curve of treated cells, this indicates loss of phosphate groups of teichoic acid.

The MIC of methicillin for such strains were < 4 μg cm^{-3} methicillin and an inhibition zone of 22 mm occurred around a disc containing 10 μg cm^{-3} methicillin.

Fig. 6.1

pH-mobility curves of cells of Staph. epidermidis grown 18 h at 37 °C on nutrient agar.

Strain 8 (○) and Strain 70 (◉)

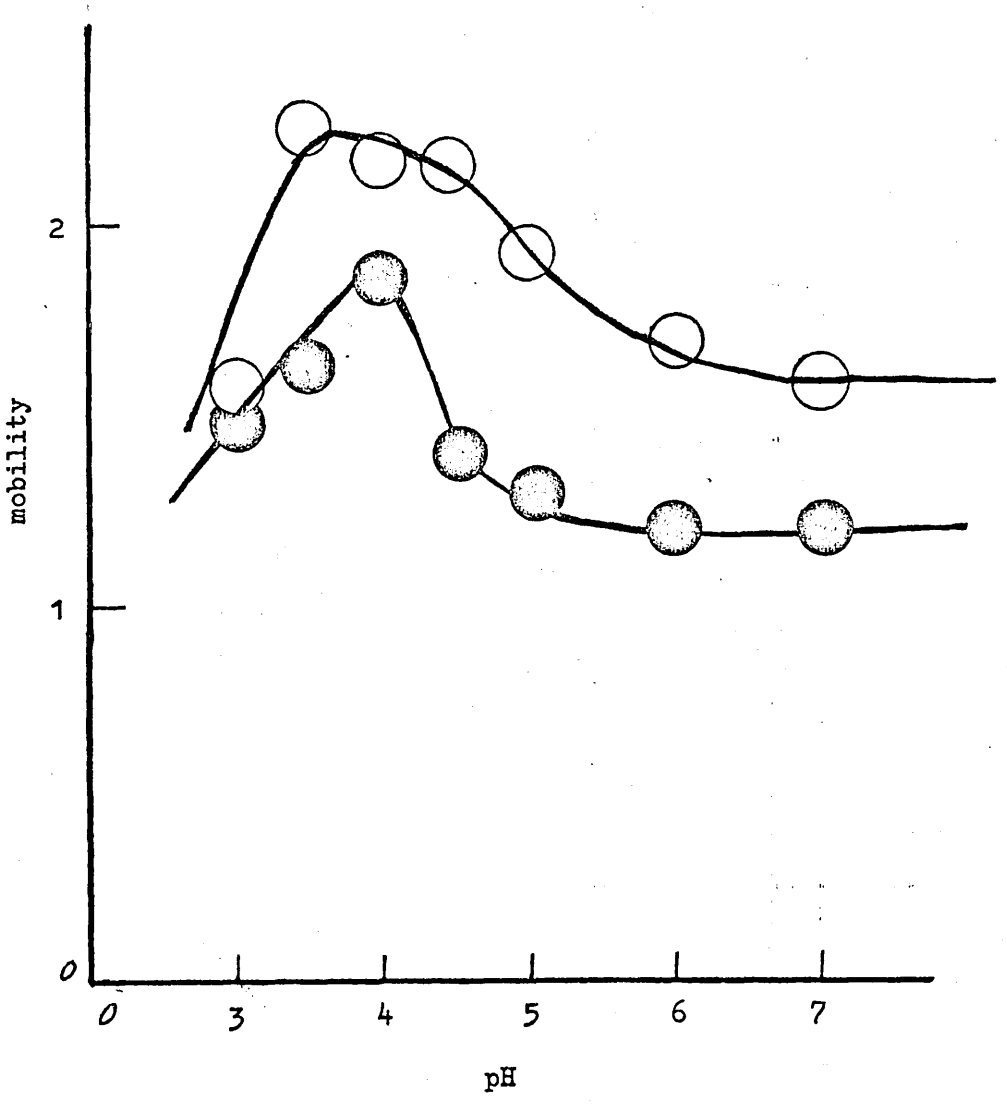


Fig. 6.2

pH-mobility curves of cell of Staph. epidermidis (Strain 70) grown on nutrient agar at 25 °C ○, 37 °C (◑) or 43 °C (●)

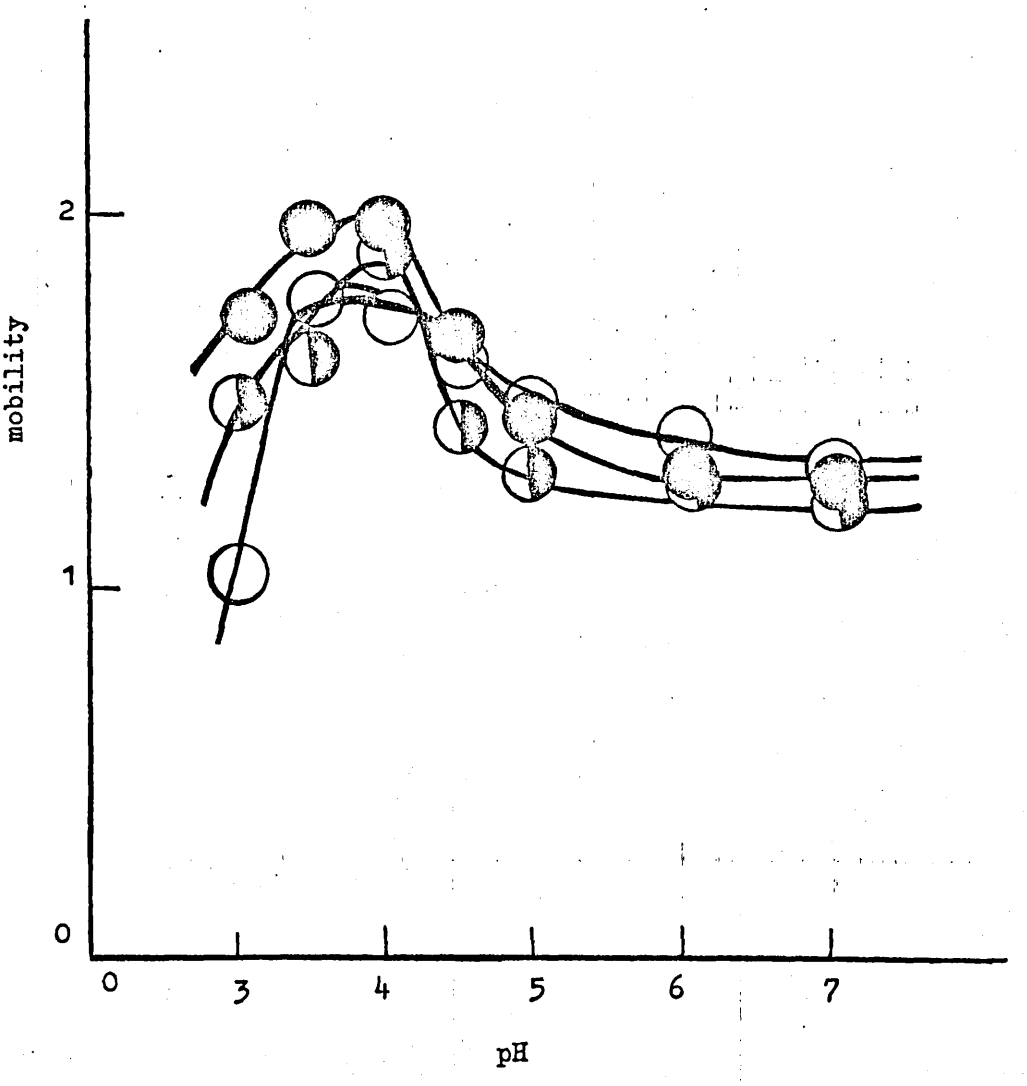
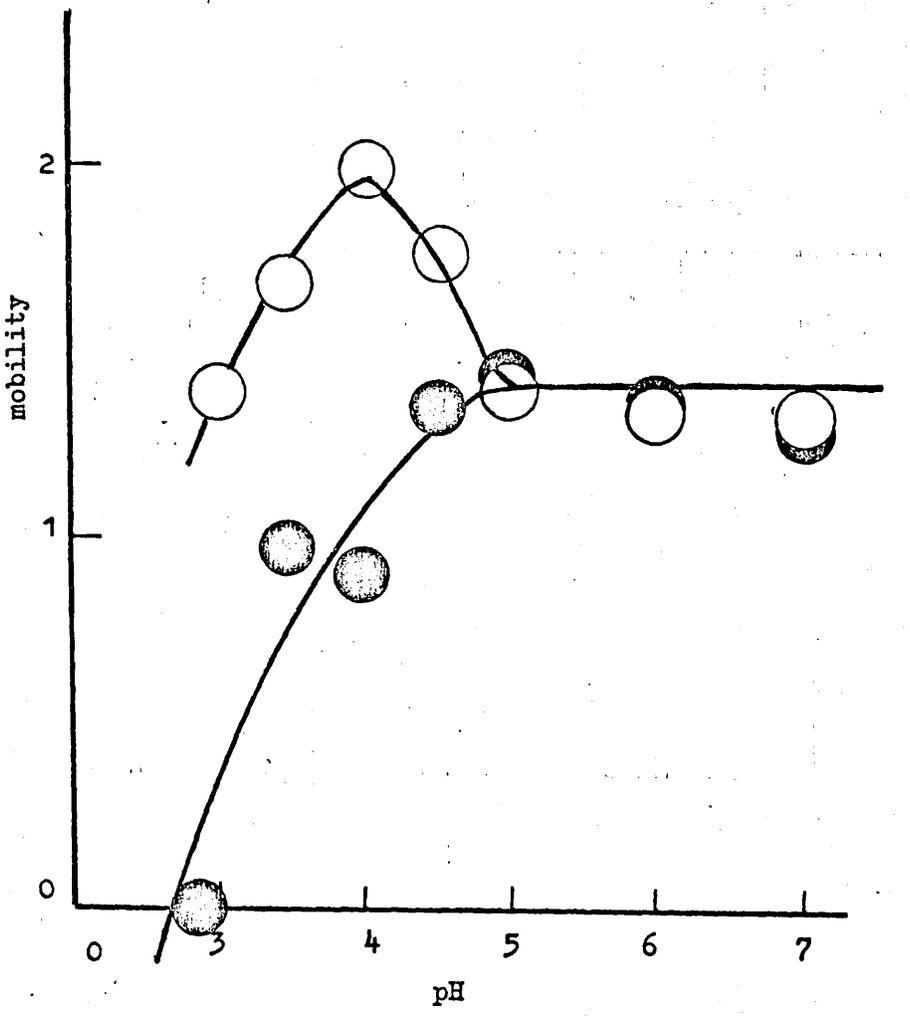


Fig. 6.3

pH-mobility curves of cells of Staph. epidermidis (Strain 70) grown on nutrient agar at 37 °C(○) and after oxidation(●)



6.2 Methicillin resistant strains (Strains 15 and 42)

The pH-mobility curves (Fig. 6.4) for cells of strain 15 show marked variation in surface charge with the temperature of growth. The cells grown at 25 °C had low H-value of 6, while cells grown at 37 °C or 43 °C exhibited an accentuated minimum mobility value at pH 4.5 with higher H-values (38 and 36 respectively). The MIC of methicillin of this strain was > 200 μg cm^{-3} and there was no inhibition zone around discs containing 10 μg cm^{-3} methicillin.

The pH-mobility curves (Fig. 6.5) for cells of strain 42 grown at 25, 37 or 43 °C are almost coincident and are typical of the curves of cells of methicillin resistant strain 15 and methicillin resistant Staph. aureus when grown at 25 °C. The curves are typical of those shown by a carboxyl surface.

The MIC of methicillin for strain 42 was > 200 μg cm^{-3}; growth occurred at all concentrations. This strain showed an abnormal phenomenon in which optimal zones were found around methicillin discs containing 10 μg cm^{-3}; around each disc were three zones; on immediate contact with the disc, there was good growth over a region of 3 mm; in the next zone (3 mm wide) there was very sparse growth, and beyond this there was luxuriant growth. Cells from the zone immediately surrounding the disc and from the outer zone, beyond the sparse zone were inoculated on to plates and incubated for 18 h at 37 °C. The pH-mobility curves (Fig. 6.6) show the same shape and positioning as did cells of the parent cells grown at any temperature (c.f. Fig. 6.5).

The pH-mobility curve of cells of the parent strain treated with sodium metaperiodate (Fig. 6.6) was coincident, within the limits of experimental error with those of parent cells grown at any temperature.

Fig. 6.4

pH-mobility curves of cells of Staph. epidermidis (Strain 15) grown on nutrient agar at 25 °C O ; 37 °C(●) or 43 °C(⊖)

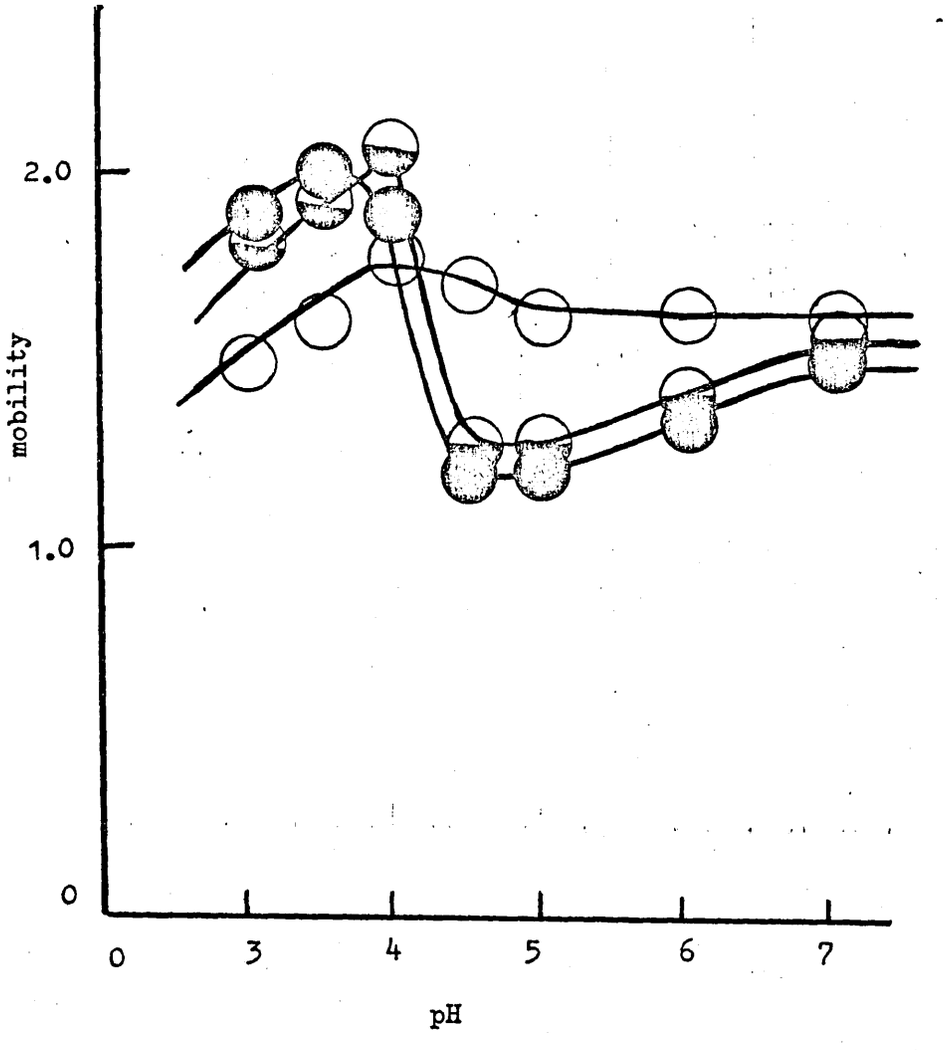


Fig. 6.5

pH-mobility curves of cells of Staph. epidermidis (Strain 42) grown on nutrient agar at 25 °C ○ ; 37 °C (◉) or 43 °C (⊖)

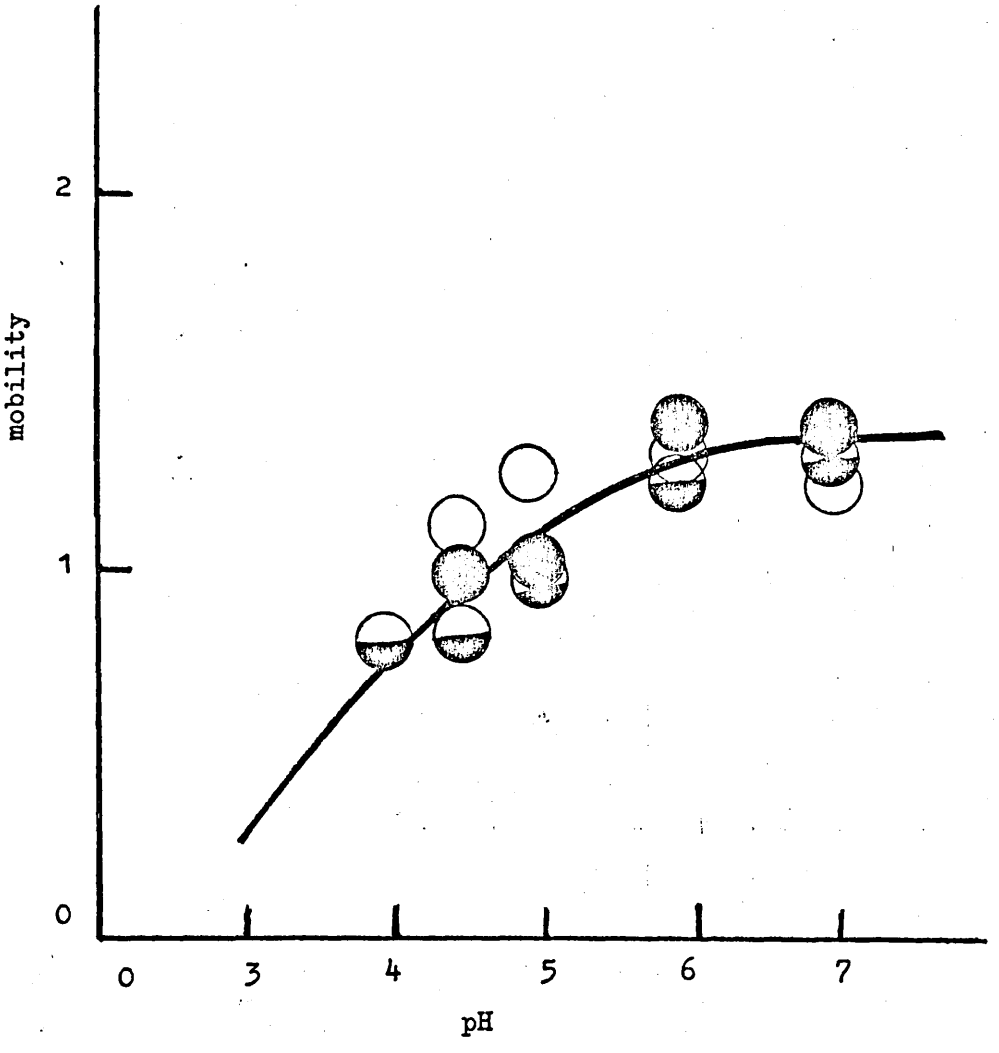
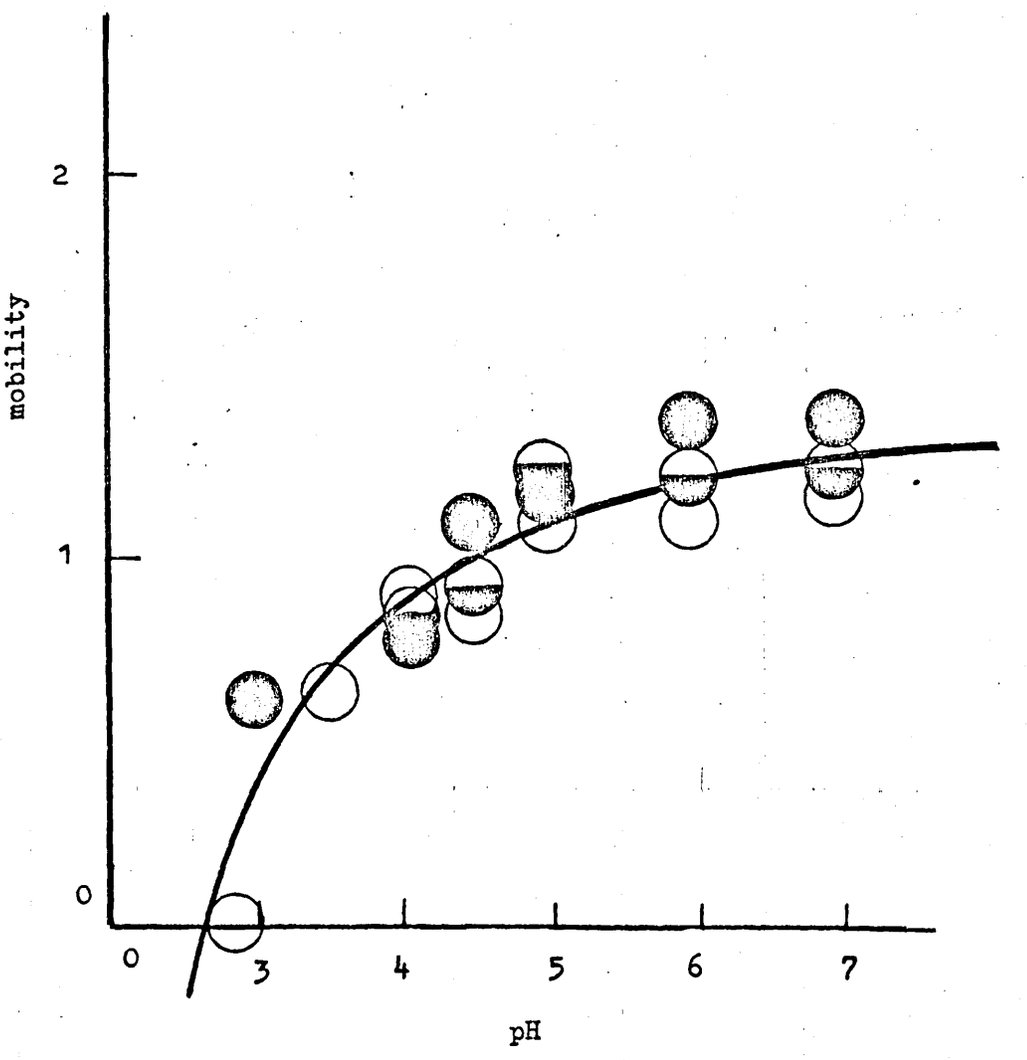


Fig. 6.6

pH-mobility curves of cells of Staph. epidermidis grown on nutrient agar at 37 °C. Outer layer growth (◉) inner layer growth (○) and oxidised (◒)



6.3 Summary

(a) The pH-mobility curves of methicillin sensitive cells of Staph. epidermidis show a maximum mobility value between 3.5 - 4 and a plateau value from pH 5 - 7. The shape and position of the curves were independent of the growth temperature.

(b) The pH-mobility curves of methicillin resistant cells of strain 15 showed a marked variation in surface properties with the temperature of growth, i.e. similar to the curves of methicillin resistant Staph. aureus.

(c) The pH-mobility curves of methicillin resistant cells of strain 42 were independent of the temperature of growth; the curves were typical of a carboxyl type surface.

SECTION 7

DISCUSSION

Any information which is obtained by analysis of cell wall components after acid hydrolysis, gives no indication of the actual molecular structure which exists in the cell wall. Ideally an attempt should be made to make studies on intact cells, but at the moment this is impossible; the best which can be achieved is a study of cell surfaces on intact cells. The study of the surface of cell walls is important for the following reasons:

(a) The surface is the only part of the cell which is in equilibrium and in contact with the environment.

(b) Through the cell surface, passes food stuffs and metabolites inward and toxins and other waste products outwards.

(c) The surface is the first and often the final site of attack of drugs and antibiotics on the cell.

Thus the information about structure and nature of components at the surface and any variation of these during growth in different media or as a result of bacteria becoming resistant to antibiotics will be a great interest. Such information should be of use in the design of molecular structure of drugs or antibiotics. In such physical studies, it is essential to know how such changes can be correlated with known biological properties such as MIC, enzyme activity etc.

Studies of the surface of bacteria must be carried out on undamaged surfaces. The technique chosen was that of particulate electrophoresis, in which the cells under study are suspended in buffer solutions. Under these conditions of correct pH and ionic strength, they suffer no serious surface or cellular denaturation. This is in marked

contrast to electron microscopy or light microscopy in which the cells must be stained and fixed on a grid or glass sheet before observation. The surface studied in particulate electrophoresis is not the same surface as revealed by light or electron microscopy. It is a region within a distance of about 5 Å from the cell surface in which the ions are held, where ionogenic surface groups contribute to the total charge and where food and toxins are about to either enter or leave the cell. Under the correct conditions of washing and age, this is found to be a reproducible region; reproducible results were obtained throughout the investigation.

The zeta-potential at the cell/electrolyte interface is determined partly by the nature and quantity of the ionogenic groups at the surface and partly by the pH and ionic strength of the suspending medium. Although the viscosity and relative permittivity within the electrical double layer are generally assumed to have the value of bulk water, recent work indicates that this may not be so. Thus any conversion of the mobility value ($\bar{v}$) to a zeta-potential using the Smoluchowski equation:

$$\bar{v} = \frac{\varepsilon \zeta}{4\pi\eta} = \frac{\varepsilon_o \varepsilon_R \zeta}{\eta}$$

(where η is coeff of viscosity, ε_o the permittivity of free space and ε_R the relative permittivity of the medium), and the subsequent discussion of the variation of the zeta-potential is open to criticism. However, if the pH and ionic strength of the suspending electrolyte are kept constant, then it is permissible to discuss changes in the experimentally determined mobility in terms of changes in the nature and quantity of surface charged groups. This is the experimental situation obtaining in this work.

For the interpretation of the electrokinetic results it is essential that the surface under study is free from adsorbed material, e.g. components of the growth medium or antibiotics, and further that it is reproducible from day to day. Cells washed three times by centrifugation before suspension for mobility measurements possessed no material that could be subsequently removed by washing; this was the adopted washing procedure. Under these conditions any variation of mobility represents true changes in the surface properties of the cells.

Cells of all strains were electrokinetically homogeneous. The homogeneity of a given population was independent of the antibiotic sensitivity or resistance of the cells, the temperature of growth, the physical state of the growth medium and the pH and ionic strength of the suspending electrolyte. Earlier workers (Marshall, 1969; Hill, 1971) showed that although cells of naturally occurring methicillin resistant strains of Staph. aureus were biologically heterogeneous, nevertheless, they are electrokinetically homogeneous.

The observation chamber in the electrophoresis apparatus was rectangular in cross-section and it was therefore necessary to calibrate with a particle of known mobility. The accepted standard reference particles are human erythrocytes suspended in 0.667 mol dm^{-3} phosphate buffer solution at pH 7.35. The sub-standard reference (Gittens and James, 1961) 18 h cells of K. aerogenes suspended in acetate-barbiturate buffer solution, was used. In confirmation of previous work, cells of a given strain of Staph. aureus after 18 h growth on standard nutrient agar medium had a reproducible surface. The day to day reproducibility of the measurement (including possible cell variation) was $\pm$ 3%; thus cells with mobility values differing by more than 10% have significantly different surfaces.

Care was taken to ensure that denaturation of the cell surface did not occur at the pH values used to determine the mobility. The influence of extreme pH conditions was estimated by the reversibility of thesurface after exposure. After the cells had been suspended in a buffer solution of extreme pH, they were centrifuged out of that solution and resuspended in buffer solution at pH 7; if these cells did not have a mobility value which agreed to within 3% of that of the control cells suspended immediately in buffer solution at pH 7, the surface was considered to be denatured and the results discarded. The cells were reversible when suspended in buffer solutions in the pH range 3 - 9, without loss of reversibility and denaturation; most work, however, was limited to the pH range 3 - 7.

The experimental results indicated that the nature (solid or liquid) and the composition (particularly inorganic phosphate content) of the medium had a marked effect on the surface compounds of these cells. Initially the results of the work using the standard medium will be discussed.

The shapes of the pH-mobility curves for methicillin sensitive cells of Staph. aureus (Fig. 7.1,) were confirmed (Hill and James, 1972,a; Marshall and James, 1971,). The curve is characterised by a maximum value in the negative mobility value in the region pH 3 - 4 with a plateau mobility in the region pH 6 - 8. The maximum mobility value, and hence the H-value are reduced by the removal of cell surface teichoic acid on oxidation with sodium metaperiodate; the plateau mobility at pH 7 was not affected by this treatment. The maximum in the pH-mobility curve (Fig. 7.1,c) is attributed to the presence of charged phosphate groups from teichoic acid. As the pH of the suspension is reduced below pH 5 the surface carboxyl groups are no longer fully ionised; this change in the electrostatic environment results in a new conformation of the surface teichoic acid molecules whereby phosphate groups from the teichoic acid are oriented into the liquid which then contribute to the total charge.

Fig. 7.1

Typical pH-mobility curves for cells of Staph aureus

A Periodate treated cell.

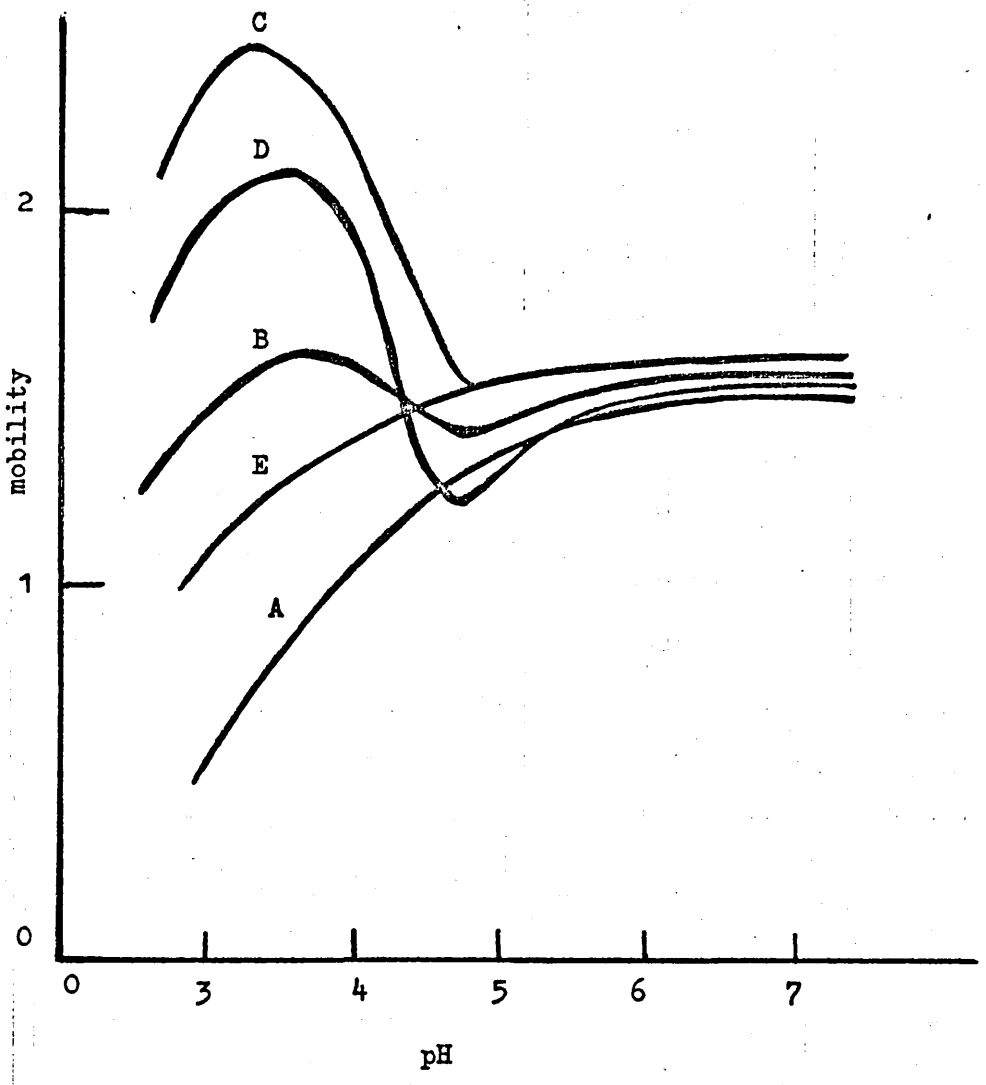
B Methicillin resistant cells grown at 37 °C.

C Methicillin sensitive cells grown at 25, 37 or 43 °C.

D Methicillin resistant cells grown at 43 °C.

E Methicillin resistant cells grown at 25 °C.

Fig. 7.1



At pH 3.5 these groups are fully negatively charged and so give an increased value and finally a maximum value of the surface charge. At lower pH values, however, the phosphate groups also take on a proton and the mobility then decreases from the maximum. These general conclusions are consistent with all the experimental facts. Cells of methicillin sensitive strains grown at 25, 37 or 43 °C had coincident curves; the shape and positioning of the pH-mobility curves were independent of resistance or sensitivity to other antibiotics including penicillin.

The shape of the pH-mobility curves for methicillin resistant cells of Staph. aureus were confirmed (Fig. 7.1, B; see also Figs. 3.1 and 5.1), (Hill and James, 1972,a; Marshall and James, 1971,). The curve for cells grown at 37 °C is characterised by a minimum mobility at pH 4.5 and a plateau mobility in the region pH 6 - 8. Such cells have a low H-value which is defined as the difference between the maximum mobility at pH 4 and that at pH 7.0 expressed as a percentage of the value of the mobility at pH 7 (James and Bewer, 1968). The feature of low H-values of these cells was first noted by Marshall and Hill, as a characteristic of all clinicallymethicillin resistant strains grown at 37 °C. After treatment of the cells with sodium metaperiodate, which removes surface teichoic acid (Hill and James, 1972,a) the pH-mobility curve (Fig. 7.1 A) is typical of a simple carboxyl type surface.

The essential difference between the pH-mobility curves of methicillin sensitive and resistant cells are in the much lower mobility curves at pH values below 5, and in particular the minimum mobility observed for resistant cells at pH 4.5. This difference is due to the different amounts of surface teichoic acid at the surface on this type of cell.

Since the pH-mobility curve of the oxidised cells is independent of the methicllin resistance of the original cell, the difference of mobility at pH 3.5 - 4 gives a measure of the relative amounts of teichoic acid. At pH 3.5 the charge on the normal cells is due to the presence of both carboxyl and phosphate (from teichoic acid) while that on oxidised cells is due solely to carboxyl groups. It is possible to calculate the contribution of the surface charge densities at these points. From the charge densities for the normal and oxidised cells a value of PO_4^{---}/COO^- ratio can be obtained, using the equation:

$$\sigma/C\ m^{-2} = 3.713 \times 10^{-3} \sqrt{(I/mol\ m^{-3})} \sinh (e\zeta/2kT)$$

The values of this ratio for sensitive and resistant cells grown at 37 °C are 2.25 and 0.75 respectively. It is thus obvious that there is considerably less surface teichoic acid on the surface of the resistant cells and in consequence the charge in conformation of these molecules does not have such a drastic effect on the electrokinetic properties.

One of the first methicillin resistant strains of Staph. aureus was isolated in 1961 (Jevons, 1961). Strain 1 used in this investigation is one of the original strains. Rolinson (1961) and Knox (1961) noted a peculiarity in the type of resistance shown. On incubation at 37 °C large numbers of cells grew in the presence of low concentrations of methicillin and a few cells in the population were capable of growing in the presence of high concentrations of the antibiotic. Broth cultures grew well in the presence of the antibiotic up to the MIC value, but after 18 h incubation, growth could be detected in broth containing much higher concentrations of methicillin. The terms 'resistant' and 'sensitive' can be misleading using only a simple sensitivity test with heavy inocula.

Sutherland and Rolinson (1964) concluded that cultures of naturally occurring methicillin resistant staphylococci consisted of mixed populations in which the majority of cells were of normal sensitivity to methicillin with a minority showing methicillin resistance. The resistant members also differed from the rest of the population in that they grew more slowly even in the absence of methicillin. Pure cultures of the resistant minority were obtained, but on repeated transfer in the absence of methicillin, the cultures reverted to a mixed population characteristic of that original naturally occurring parent strain.

Annear (1968) and Dyke (1969) showed that the state of heterogeneity exists only at relatively high temperatures. Annear showed that a culture of a clinically resistant strain grown at 30 °C had a much greater tolerance to methicillin than the same strain grown at 37 °C. A more detailed investigation was carried out by Parker and Hewitt (1970); cells were grown on solid medium in the presence of varying amounts of methicillin and surface counts determined, and these were expressed as percentage viability of the original inoculum after incubation for 48 h at 25, 30, 37 or 43 °C. At 37 °C the whole inoculum grew as normal staphylococcal colonies on up to 3.12 μg cm^{-3} of methicillin, but on higher concentrations progressively fewer cells formed colonies, i.e. the culture was heterogeneous. At 30 °C the whole inoculum formed normal colonies on media containing up to 50 μg cm^{-3} of methicillin in 24 h, with heterogeneous growth at 100 μg cm^{-3}, whereas at 25 °C the whole inoculum grew well in the presence of 100 μg cm^{-3} of methicillin. At 43 °C only a very small part of the inoculum was capable of growing in the presence of a concentration of antibiotic 8 times less than the conventional MIC for the sensitive cells. Thus methicillin resistance and sensitivity can be clearly distinguished by growth at 25 or at 43 °C.

In an extension of this 'temperature effect' the shape and position of the pH-mobility curves of cells with natural resistance to methicillin have been studied (Hill and James, 1972,b); and these also proved to be dependent on the temperature of growth. In contrast, the shape and position of the mobility curves of cells of methicillin sensitive and of cells of strains trained in vitro to methicillin were unaffected by growth at different temperatures.

The large differences in the pH-mobility curves of cells grown at 25, 37 or 43 °C occur only when the cells are in suspension at the pH values below 6. Thus the surface components contributing to the charge at higher pH values are unaffected by the temperature of growth. Hill and James (1972,b) showed the differences in the surface properties evident at pH values below 5 can be associated with a variation of the surface teichoic acid. Cells grown at 43 °C have a high H-value more characteristic of cells of a sensitive strain (Fig. 7.1,D) but they differ from truly sensitive cells (Fig. 7.1,C) in that the pH-mobility curve passes through a minimum value at pH 4.5.

Table 7.1

The contribution of the surface charge density of carboxyl and phosphate groups on cells of naturally resistant Strain 1.

Temperature of growth °C	10^3 x charge density/C m^{-2}			
	carboxyl + phosphate	carboxyl	phosphate	phosphate/ carboxyl
25	5.7	3.77	1.93	0.4
37	6.6	3.8	2.83	0.75
43	9.40	3.8	3.8	1.0

The teichoic acid which is a surface component is easily removed by mild oxidation (Fig. 7.1,A). Cells grown at 25 °C (Fig. 7.1,E) have a zero H-value with no minimum mobility at pH 4.5, these are characteristic of cells known to be resistant to methicillin. Teichoic acid on such cells was very much reduced, often to zero. Cells grown at 37 °C with a low H-value and a minimum mobility at pH 4.5 have surface properties more like those of cells grown at 43 °C. The surface teichoic acid was also easily removed by mild oxidation. Although cells grown on nutrient agar at 37 °C are known to be biologically heterogeneous, there was no evidence of heterogeneity in their electrokinetic properties. In marked contrast the surface properties of methicillin sensitive cells were affected by the temperature of growth.

The indication of a temperature dependent enzyme system associated with methicillin resistance (Annear, 1968) was supported by the data of Hill and James (1972,b) who suggested that the enzyme system could be a phosphatase. Recent studies (Davies and James, 1974) have demonstrated the presence of an alkaline phosphatase system in cells of methicillin resistant strains.

Following these previous observations of the change in surface properties of methicillin resistant cells as a result of growing the cells once at different temperatures, the probability of stabilising these changes was coincident Within this aim, methicillin resistant and methicillin sensitive cells (maintained at 37 °C) were repeatedly subcultured on standard nutrient agar in the absence of antibiotic at each of the temperatures 25, 37 and 43 °C. In addition to the following changes in the shape and position of the pH-mobility curves, changes in biological properties (e.g. MIC of methicillin and penicillinase production) were also assayed.

Cells of a fully antibiotic sensitive strain (2) were grown repeatedly at 25 or 43 °C for 20 subcultures on standard nutrient agar, the shape and positioning of the pH-mobility curves were unchanged, and were undistinguishable from those of the parent cells grown at any temperature.

Cells of the methicillin sensitive, penicillinase producer organisms (3) exhibited typical and coincident pH-mobility curves similar to those of cells of fully sensitive Staph. aureus strain (2). When such cells were repeatedly grown at 25 or 43 °C for 20 subcultures, the shape and positioning of the curve did not change. The ability to produce the enzyme penicillinase after repeated growth at 43 °C was not lost; a result which confirmed the suggestion of the chromosomal as distinct from extra chromosomal control of penicillinase production in such strains. Ashoshov,(1966 a) has also shown such kind of stability of the gene controlling penicillinase production in some strains of penicillinase producer Staph. aureus. It would be interesting to speculate whether the penicillinase control is located chromosomally on methicillin sensitive penicillinase producers, but extra chromosomally (or in a plasmid) when the penicillinase producing organisms are resistant to methicillin. It will be necessary to study further strains before a firm conclusion can be arrived at.

It was thus apparent that the surface properties of methicillin sensitive cells are consistent and independent of the ability to produce pencillinase, and independent of growth temperature. However, it was confirmed that cells of methicillin sensitive strains which may or may not be resistant to other antibiotics carry large amounts of teichoic acid; cells of these strains exhibit no phosphatase activity when grown at any temperature. (Davies and James, 1974).

In contrast, cells of methicillin resistant strains of Staph. aureus have a minimum mobility value at pH 4.5 and the shape of pH-mobility curves was dependent on the temperature of growth. Cells grown once at 43 °C have large amounts of teichoic acid (Fig. 7.1,D) (as revealed by sodium meta-periodate treatment) and exhibit no phosphatase activity; when grown once at 25 °C (Fig. 7.1,A) or 37 °C (Fig. 7.1,B), however, the cells which in general are

resistant to a wide range of antibiotics, have little or no surface teichoic acid, but exhibit high phosphatase activity. Recently isolated methicillin resistant cells exhibited the same surface characteristics as cells of other and older methicillin resistant strains.

When the resistant cells were repeatedly grown for 20 subcultures on standard nutrient agar at 25 °C or 37 °C there was no change in the surface properties. At 25 °C the cells became more resistant, the MIC for methicillin at 37 °C for such cells were exceeding 200 $\mu g\ cm^{-3}$ (Table 3.4), the population became more homogeneous in its resistance and different from that of the original parent strain grown at 37 °C (c.f. Table 3.1). The pH-mobility curves were characteristic of resistant cells. After one subculture at 43 °C the pH-mobility curve had a very low H-value (14) compared to the value of 30 for parent cells. The minimum mobility value at pH 4.5 for cells grown at 43 °C was less than that of parent cells. These characteristics provide more support to the suggestion that the cells had become more resistant to methicillin.

The shape of the pH-mobility curve measured at 25 °C did not change with increasing numbers of subcultures at 25 °C. There was, however, a marked increase in the negative mobility of the cells (measured at pH 6.0) as the number of subcultures at 25 °C (Table 7.2, see also Fig. 3.7).

This might be due to slight changes in the orientation of cell surface peptidoglycan, whereby more negatively charged groups or less positively charged groups are exposed at the surface. Since there has been no massive change in the shape of the pH-mobility curves it is obvious that there has been no massive alteration of surface components.

Table 7.2

Variation of the mobility, measured at pH 6.0 with number of subcultures at 25 °C of cells of a methicillin resistant strain (1) of Staph. aureus

Number of subcultures at 25 °C	Mobility	% Increase of negative mobility
1	1.5	0
4	1.55	3
6	1.55	3
10	1.6	7
15-20	1.65	10
51	2.0	33

When cells of a resistant strain were repeatedly grown at 43 °C for 20 subcultures, they lost their methicillin resistance and the surface properties of the cells became in all respects indistinguishable from those of cells of a sensitive strain (i.e. the pH-mobility curve changed from typical of curve D to typical curve C. When these trained cells were subsequently grown at 25 °C the shape of the pH-mobility curve still had all the characteristics of highly sensitive strains; with high H-values. When grown at 37 or at 43 °C the cells exhibited no minimum mobility at pH 4.5 and all showed high H-value. This independence of the pH-mobility curves of the temperature of final growth suggested that the cells had become homogeneous with all the surface characteristics of sensitive cells, in which teichoic acid is a surface component.

When the cells of such sensitive population were grown repeatedly at 37 °C the pH-mobility curves (of cells grown at any temperatures) were identical to that of the parent strain grown at 43 °C. Thus the change of the surface properties of the resistant cells to those characteristic of sensitive cells was stabilised by repeated growth at 43 °C and was not reversible when the cells were subsequently grown at a lower temperature. These trained cells have the same phage typing as the parent grown at 37 °C, thus the change of surface properties is not due to any change of phage typing pattern.

The cells also became penicillin sensitive, they did not produce penicillinase and the strain was classified as a penicillinase negative variant. Therefore, the plasmids which were responsible for the resistance of methicillin and penicillinase were lost, during repeated growth at the higher temperatures. It is apparent that this is an irreversible phenomenon resulting in complete loss of the plasmid rather than slight thermal damage which could be repaired after growth at lower temperatures.

At this stage of the experimental work the batch of Oxoid nutrient agar medium used became depleted and another batch was purchased. Cells grown on this medium exhibited many different properties to those grown on the original standard medium, e.g. cells of methicillin resistant strains exhibited little if any phosphatase activity. According to the manufacturer there had been changes whereby the nutrient broth (hence nutrient agar) was not treated with calcium hydroxide to precipitate out excess phosphate. Chemical analysis of this medium revealed the presence of large amounts of inorganic phosphate compared with the relatively small inorganic phosphate content of the original medium.

Repeated growth at 43 °C of methicillin-resistant strains on this nutrient agar containing high inorganic phosphate did not produce the same changes of surface properties as did growth on standard medium. It is an established fact (Davies and James, 1974) that high concentrations of inorganic phosphate in the media suppressed the formation of the phosphatase enzyme system of the cells and thus permitted the formation of surface teichoic acid.

After 20 subcultures (on the high phosphate agar) at 43 °C, the pH-mobility curves of the cells grown once at each growth temperature (Fig. 3.9) were still characterised by a marked minimum mobility at pH 4.5. The MIC at 37 °C for such a strain was > 200 μg cm^{-3} while at 43 °C the cells were sensitive to methicillin. These results suggest that high concentrations of inorganic phosphate in the media suppressed the curing of methicillin genes which are probably carried by a plasmid. The strain, however, lost the ability to produce penicillinase, that is the plasmid loss was independent of the inorganic phosphate content of the medium. It is well known that this plasmid is lost spontaneously (Barber, 1949) and after growth at high temperature (May, et.al., 1964; Ashoshov, 1966 b).

In an attempt to test the importance of the presence of inorganic phosphate, various batches of nutrient broth and nutrient agar were treated with calcium hydroxide, as recommended by the manufacturers. After repeated subcultures on this treated media at 43 °C, cells of the methicillin resistant strains became more sensitive and the pH mobility curves showed gradual changes of surface properties. The pH-mobility curves of cells which had been subcultured for 45 times now exhibited no minimum mobility value at 4.5 and the shape of the curve was indistinguishable from that shown by methicillin sensitive cells. The pH-mobility curves of such cells grown finally at either 25, 37 or 43 °C were coincident with no minimum mobility value. The surface properties and MIC level changed in

parallel after growth at 43 °C. In contrast to the changes observed during growth on the standard medium the changes in surface properties and MIC occurred more slowly. Further, the H-values of the resultant strain were not so high as in the case of repeated growth on standard medium at 43 °C.

It is, of course, probable that the standard medium contained some constituents which helped the curing of the plasmid but which were lacking in the prepared medium, this could arise during the precipitation of the calcium phosphate which may absorb some essential metabolite and remove it from the medium. This is not very likely because the cells grew well in the treated medium. It is most likely that the presence of higher concentration of inorganic phosphate causes the retention of the methicillin plasmid although the cells lost the penicillinase plasmid independent of content of inorganic phosphate.

The alkaline phosphatase activity of cells with natural resistance to methicillin is very dependent on the nature and composition of the growth medium (Davies and James, 1974). Although cells grown on standard nutrient agar exhibited a high activity, nevertheless, when they were grown on media containing large amounts of inorganic phosphate, they exhibited little, if any activity. However, when grown on relatively low phosphate containing media, there was a high alkaline phosphatase activity and in consequence few surface phosphate groups. This relationship between phosphatase activity and surface phosphate groups is further supported by the typical pH-mobility curves. It is obvious that cells of sensitive strains possess considerable amounts of surface teichoic acid and hence phosphate, but exhibit no phosphatase activity. While cells of strains with natural resistance to methicillin show a parallel temperature dependence of surface teichoic acid and alkaline phosphatase activity.

It is of interest to recall that when methicillin resistant cells grown at 37 °C are subcultured at 43 °C they immediately lose their phosphatase activity. In marked contrast many subcultures at 43 °C are required for the cells to become truly sensitive as revealed by the MIC and the pH-mobility curve.

On the other hand methicillin resistant cells grown repeatedly at 25 °C (the temperature at which the cells exhibit high phosphatase activity and no detectable surface teichoic acid) again stabilise their surface properties, the curves had all the typical characteristics of methicillin resistant cells.

The experimental data for teichoic acid, H-value and phosphatase activity are all listed in Table 7.3. There is a correlation between the sensitivity to methicillin, phosphatase activity and H-value of strains of Staph. aureus.

Teichoic acid is a known cell wall component of Staph. aureus and from the available experimental evidence it seems that it is also a surface component on cells of methicillin sensitive strains, or cells of resistant strains which have been repeatedly grown at higher temperatures. There is evidence that it is not a major surface component of methicillin resistant cells, but at present it is not known whether this polymer is replaced by teichuronic acid (1:1 N-acetylglucosamine-glucuronic acid polymer not containing phosphate groups) or whether the teichoic acid is only present at lower levels in the cell wall. Previous work (Hill and James, 1971) has shown that if resistant cells grown at 25 °C showing no surface teichoic acid, are grown at 43 °C then teichoic acid becomes a predominant surface component; the converse is also true.

Table 7.3

Summary of H-value, phosphatase activity and phosphate/carboxyl ratio of some strains of Staph. aureus

Strain No.	Temp ^{o}C of Growth	Sensitivity to methicillin	PO_4^{---}/COO^-	H-value	Phosphatase activity % *
3	37	S	-	115	0
2	25	S	2.10	56	0
2	37	S	2.25	56	0
2	43	S	2.2	56	0
1	25	R	0.4	0	102
1	37	R	0.75	3	100
1	43	S	1.0	30	0
9	37	R	3.8	42	15-20
1 (pen^+ met-r)	37	R	0.6	0	29.1
1 (pen^+ met-r)	37	R	0.6	12	59.1
1 (pen^+ met-s)	37	S	1.4	60	0
1 (pen^- met-s)	37	S	1.45	55	15.2

* Davies, (1974).

However, after repeated growth at 43 °C the cells lose their surface teichoic acid even when grown at lower temperatures. Thus some mechanism has been interfered with such that the teichoic acid polymer has become stabilised and independent of temperature. At the same time these cells have lost their phosphatase activity and hence become sensitive to methicillin, these facts probably all derive from a common site within the cells. It is not likely that these associated properties are cause and effect.

The genes which control resistance and penicillinase production are most probably carried by different plasmids. They were lost independently of each other during growth at higher temperature on standard medium. To study this further the different variants were isolated by replica-plating at 37 °C of a methicillin resistant penicillinase organism. Penicillinase positive and negative variants were recognised. Each of them were either sensitive or resistant to methicillin (Table 3.5). Cells of the methicillin sensitive variants showed typical surface properties of methicillin sensitive cells irrespective of the loss of the penicillinase plasmid. The phosphatase activity of variant pen$^-$ met-s was low, while there was no activity for variant pen$^+$ met-s (Table 7.3). Methicillin resistant variants showed typical characteristics for resistant cells regardless of penicillinase production. Phosphatase activity for the variant pen$^-$ met-r was very high as the parent cells (variant pen$^+$ met-r).

It was concluded that the genes for both characters were located in different plasmids. The cells became cured in the presence of a low concentration of inorganic phosphate in the media as well as certain constituents in the standard medium which may have helped the curing of the plasmid, but which could be lacking in the precipitated medium. In contrast to the loss of methicillin resistance the plasmid responsible for pencillinase production was also lost when the cells were grown on media

containing high inorganic phosphate. Apparently the loss of this marker is not dependent on the phosphate content of the media. The resistance to the metal ions was linked with the penicillinase plasmid; pencillinase negative variants were sensitive to the ions. But irrespective of the loss of the methicillin and penicillinase plasmids, tetracycline and streptomycin resistance were not lost. This suggests that there is no linkage between the genes controlling these two antibiotics and methicillin and pencillinase plasmids. Lacey (1972 b) reported that in some strains of methicillin resistant staphylococci, the genes that determine methicillin resistance are not linked to those specifying other antibiotic resistance or hamolysin or pigment production. Dornbusch and Hallander (1973) and Dornbusch (1973) suggested that a number of markers can be linked in Staph. aureus such as metal ions with penicillinase plasmid and methicillin resistance with enterotoxin β production. The present work confirms these conclusions.

Further studies on the effect of phosphate on the surface properties of methicillin resistant cells revealed that, although the general shape of the pH-mobility curve was unchanged with a minimum mobility curve at pH 4.5, nevertheless there was a variation of H-value when the cells were grown at each different temperature. Repeated growth on medium containing high concentration of phosphate at 43 ^{o}C resulted in the cells having a higher negative charge at pH 3 - 4 as compared to that of cells after growth on standard medium at the same temperature.

The phosphatase activity in the cells of resistant strain was very dependent on the nature and composition of the growth medium. In contrast to the high phosphatase activity the low H-value shown by cells grown on standard nutrient agar and calcium hydroxide treated (low inorganic phosphate),

no phosphatase activity and high H-values were shown when the cells were grown on nutrient agar containing large amounts of inorganic phosphate.

Cells of methicillin sensitive or resistant strains were grown once on solid or liquid media containing varying amounts of inorganic phosphate. The general shape of the pH-mobility curves were independent of the amount of inorganic phosphate in the medium. However, cells of methicillin sensitive strains grown in nutrient broth had low H-values (32 and 21) compared with higher values (55, 50 and 51) for cells grown on nutrient agar. Marshall (1969) and Hill (1971) have previously shown that growth on solid medium enhanced the H-value. The H-value for cells grown in nutrient broth containing lower amounts of inorganic phosphate was lower than that for cells grown in nutrient broth containing higher amounts of inorganic phosphate. It thus appears that the presence of inorganic phosphate in the growth medium has a greater effect when the medium is liquid, on solid medium the pH-mobility curves in the presence of small or large concentrations of inorganic phosphate are coincident. In contrast the phosphate does not play an important role in determining the surface properties of methicillin resistant cells on solid medium, or in liquid medium.

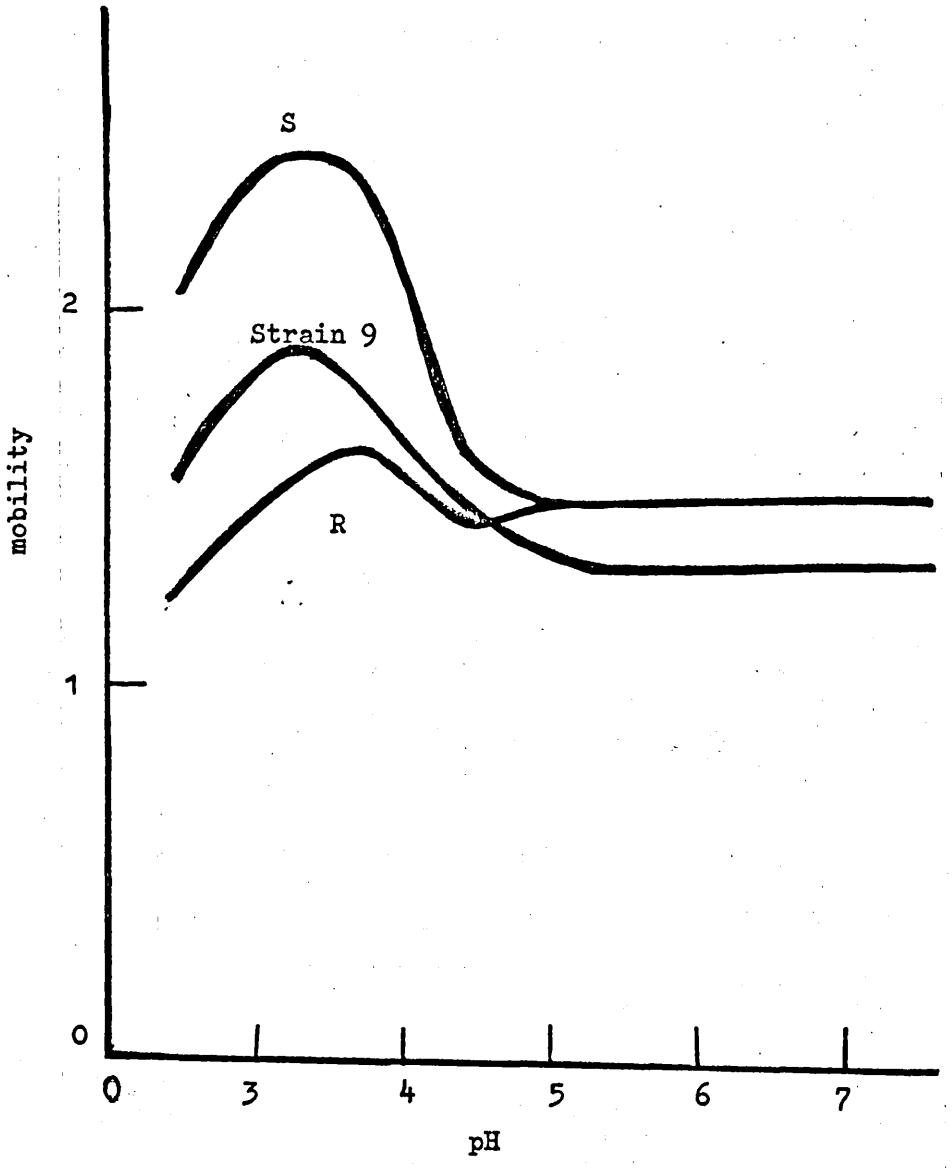
During the course of this work, Grubb and Annear (1972) published a paper on the loss of methicillin resistance by a strain (9) of Staph. aureus under a range of different experimental conditions. The surface properties of cells of this strain were unlike any reported previously for naturally occurring methicillin resistant strains of Staph. aureus (Fig. 7.2) and were more typical of cells of a sensitive strain. The surface teichoic acid was removed easily by oxidation with sodium metaperiodate. This strain has a MIC at 37 ^{o}C in excess 200 $\mu g\ cm^{-3}$. When the strain was

trained to grow at 43 °C in the absence of antibiotic the population became more sensitive, with MIC of 10 μg cm^{-3}. The pH-mobility curve was still typical of that shown by sensitive strains. Grubb and Annear report that when cells of this strain are kept for several months at room temperature they lose their methicillin plasmid. It was hoped that this would provide an alternative way of losing methicillin resistance and in consequence cells were left at room temperature in and on the media containing various amounts of inorganic phosphate. Cells of other methicillin-resistant strains (strain 1) were left in the same way. The MIC values of all strains determined at intervals of 2, 5, and 10 months showed no change with storage. Cells of the strain 9 had also a very low phosphatase activity (Davies and James, 1974) and were thus more characteristic of cells of a sensitive strain, in agreement with the shape of the pH-mobility curve. This strain is therefore most anomalous and it was impossible to compare the results of Grubb and Annear. It is of course not surprising that strains exist which do not fit into a common pattern of behaviour.

Strains of the coagulase negative Staph. epidermidis were also studied. Cells of methicillin sensitive and methicillin resistant strains of this organism had very similar surface properties to strains of Staph. aureus. The pH-mobility curve of methicillin sensitive strains show a maximum mobility at 3.4 - 4; the shape and position of the curves were independent of growth temperature. The surface teichoic acid was easily removed with mild oxidation. The pH-mobility curve, and hence the surface properties of cells of one methicillin resistant (strain 15) strain were markedly dependent on the temperature of growth (Fig. 6.4). In contrast the pH-mobility curves of cells of strain 42, which is also resistant to

Fig. 7.2

pH-mobility curves for strains of Staph. aureus (resistant, sensitive and 9) grown at 37 °C



methicillin were almost coincident and independent of the growth temperature. The shape was typical to that obtained for cells of methicillin resistant strain 15 and methicillin resistant Staph. aureus when grown at 25 °C; the curve was typical of a carboxyl surface. The strain showed the abnormal phenomenon in that an optimal zone was formed around methicillin discs containing 20 μg cm^{-3}. The pH-mobility curves were determined for cells isolated from the zone which is in immediate contact with the disc and from the layer beyond the sparse growth. The curves (Fig. 6.6) show the same shape and positioning as did cells of parent cells grown at any temperature. There was no detectable surface teichoic acid on cells of this strain grown at any temperature. These results suggested that a very homogeneous population were present although the strains showed the abnormal phenomena of 'optimal zone'.

The surface charge differences between hospital isolated methicillin resistant and methicillin sensitive strains have now been conclusively confirmed. Using particulate microelectrophoresis, it is possible to recognise cells of methicillin sensitive, clinically resistant, strains of Staph. aureus grown at 37 °C from the general shape of the pH-mobility curve (Fig. 7.1).

The methicillin marker was dominant in determining both the surface properties and the phosphatase activity. Cells resistant to penicillin (i.e. penicillinase producer) or to other antibiotics do not produce phosphatase or show abnormal surface properties. This confirmed the proposal that separate plasmids control methicillin resistance and penicillinase production. The shape of pH-mobility curves for the variants isolated for their ability to produce penicillinase and their resistance or sensitivity to methicillin and their phosphatase activity strengthen the proposal that there is a link between phosphatase production, the

lack of surface teichoic acid and methicillin resistance. 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.

In conclusion the plasmid responsible for the methicillin resistance is lost during growth at high temperatures in the presence of low concentrations of phosphate , while in contrast it is not lost during growth in the presence of high concentrations of inorganic phosphate. When cells are grown at 25 °C, the cells become competely resistant to methicillin and exhibit a high phosphatase activity and no teichoic acid. Davies (1974) recently concluded that, 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. Therefore it seems that the same plasmid is controlling methicillin resistance, alkaline phosphatase production and either directly or indirectly the formation of teichoic acid.

Thus, in conclusion it is evident that the suggested gene is located on a separate plasmid. The penicillinase plasmid carries genes controlling metal ions resistance which are lost at the same time as penicillinase plasmid. The loss of the methiciilin plasmid depends on the growth medium, in particular inorganic phosphate, and on the growth temperature.

SECTION 8

BIBLIOGRAPHY

1. Abraham, E.P., and Chain, N., (1940), Nature, Lond., 146, 837

2. Abraham, E.P., Chain, C.M., Flory, H.W., Gardner, A.D., Heatley, N.G., and Jennings, M.A., (1941), Lancet, 2, 177.

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

4. Abramson, H.A., Mayer, L.S., and Gorin, M.H., (1942), "Electrophoresis of Protein".

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

6. Archibald, A.R., and Baddiley, J., (1966), Advan. Carbohy. Chem., 26, 323.

7. Archibald, A.R., and Coapes, H.E., (1971), Biochem. J., 123, 665

8. Asheshov, E., (1966,a), Nature, Lond. 210, 804.

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

10. Auletta, A.E., and Kennedy, E.R., (1966), J. Bact., 92, 28

11. Baddiley, J., (1972), Essays Biochem., 8, 35.

12. Baddiley, J., Buchanan, J.G., Martin, R.O., and Rajbhandary, U.L., (1962), Biochem.J., 82, 439.

13. Baddiley, J., Brock, J.H., Davison, A.L. and Partridge, M.D., (1968), J. Gen. Microbiol., 54, 393.

14. Baird-Parker, A.C., (1965), J. Gen. Microbiol. 38, 363.

15. Barber, M., (1947), Brit. Med. J. II, 863.

16. Barber, M., and Rozowodowska-Dowzenka, M., (1948), Lancet II, 641.

17. Barber, M., (1949), J. Gen. Microbiol., 3, 274.

18. Barber, M., (1961), J. Clin. Path., 14, 385.

19. Benner, E.J., and Kayser, F.H., (1968), Lancet II, 741.

20. Blair, J.E., and Williams, R.E.O., (1961), But. Wld. Hlth. Org., 24, 771.

21. Bouanchand, D.H., Scanvizzi, M.R. and Chabbert, Y.A., (1968), J. Gen. Microbiol, 54, 417.

22. Brewer, J.E., (1966), Ph.D. thesis, London.

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

24. Chatterjee, A.H., (1969), J. Bact., 98, 519.

25. Cohen, S, and Sweeny, H.M., (1970), J. Bact., 104, 1153

26. Coley, J., Duckworth, M., and Baddiley, J., J. Gen. Microbiol, (1972), 73, 587.

27. Cowan, S.J., and Steel, K.J., (1964), J. Bact. 88, 864.

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

29. Cummins, C.S., and Harris , H., (1956), J. Gen. Microbiol., 14, 583.

30. Cummins, C.S., and Harris, H., (1958), J. Gen. Microbiol., 18, 173.

31. Davies, A.L., (1974), Ph.D. thesis, London.

32. Davies, A.L., and James, A.M., (1974), Microbios., 10, 257.

33. Davison, A.L., and Baddiley, J., (1963), J. Gen. Microbiol., 32, 271.

34. Davison, A.L., Baddiley, J., Hofstand, T., Losnegard, N., and Oeding, P., (1963), Acta. Path. Microbiol. Scand., 58, 482.

35. Davison, A.L., and Baddiley, J., (1964), Nature, Lond., 202, 874.

36. Dean, A.B., Williams, R.E.,O., Hall, F., and Corse, J., (1973), J. Hyg., 71, 261.

37. Douglas, L.J., (1959), Trans. Faraday Soc., 55, 850.

38. Douglas, L.J., and Wolin, M.J., (1971), Biochemistry, 10, 1151

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

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

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

42. Drew, W.L., Barry, A.L., O'Toole, R., and Sherris, J.C., (1972), Appl. Microbiol., 24, 240.

43. Dyke, K.G.H., (1969), J. Med. Microbiol. 2, 261.

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

45. Garret, A.J., (1965), Biochem. J., 95, 6c

46. Garrity, F.L., Detrick, B., and Kennedy, E.R., (1969), J. Bact., 97, 557

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

48. Gittens, C.J., (1962), Ph.D., thesis London.

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

50. Ghuysen, J.M., (1968), Bact. Rev., 32, 425

51. Glaser, L., Ionesco, H., and Schæffer,P., (1966), B.B.A. 124, 415

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

53. Grinsted,Jand Lacey, (1973a), J. Med. Microbiol., 6, 351

54. Grinsted J, and Lacey, R.W., (1973,b), J. Gen. Microbiol., 75, 259.

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

56. Hancock, R., (1960), Biochem. Biophys. Acta. 37, 42.

57. Harmon, S.A., and Baldwin, J.M., (1964), J. Bact., 87, 593

58. Hay, J.B., Wicken, A.J., and Baddiley, J., (1963), B.B.A., 71, 188.

59. Hay, J.B., Davey, N.B., Archibald, A.R., and Baddiley, J., (1965), Biochem. J., 94, 7c

60. Hashimoto, H., Kono, K., and Mitsuhash, S., (1964), J. Bact., 88, 261

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

62. Heptinstall, S., Archibald, A.B., and Baddiley, J., (1970), Nature, Lond., 225, 519

63. Hewitt, J.H., Coe, A.W, and Parker, M.T., (1969), J. Med. Microbiol., 2, 443.

64. Hill, A.W,, (1971), Ph.D. thesis, London

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

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

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

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

69. Jevons, M.P., (1961), Brit. Med. J., 1, 124

70. Jevons, M.P., Coe, A.W., and Parker, M.T., (1963), Lancet, 1, 904

71. Knox, R., (1961), Brit. Med. J., 1, 126

72. Knox, R., (1962), "Resistance of Bacteria to the Pencillin CIBA Foundation Study Group 13.

73. Kocur, M., Bergan, T., Mortenson, N., (1971), J. Gen. Microbiol, 69, 167

74. Komagata, S., (1933), Res. Electrock. Tokyo, No. 348

75. Lacey, R.W., (1972, a), J. Med. Microbiol., 5, 497

76. Lacey, R.W., (1972, b), J. Gen. Microbiol, 71, 399

77. Lederberg, J., (1957), J. Bact. 73, 166

78. Lederberg, J., and Lederberg, E.M., (1952), J. Bact., 63, 399

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

80. Mandelstam, J., and Strominger, J.L., (1961), Biochemical Biophysical Res. Comm., 5, 466

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

82. Marshall, N.J., (1969), Ph.D. thesis, London.

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

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

85. Michaeles, L., (1931), Biochem. J., 234, 139

86. Mickle, H., (1948), J.R., Micro., Sco., 68, 10

87. Moore, B., (1960), Lancet, 2, 453

88. Mosser, J.L., and Tomasz, A., (1970), J, Biol. Chem., 24, 287

89. Moyer, L.S., (1936), J. Bact. 31, 531

90. Murray, R.G.E., and Brich-Anderson, A, (1963), Canad. J. Microbiol., 9, 393.

91. Nermut, N.V., (1967), J. Gen. Microbiol., 49, 503.

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

93. Novick, R.P., and Roth, C., (1968), J. Bact., 95, 1335.

94. Novick, R.P., and Richmond, M.H., (1965), J. Bact. 90, 467

95. Park, J.T., (1952), J. Biol. Chem., 194, 877

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

97. Parker, M.T., and Jevons, M.P., (1969), Post. Grd. Med. J., 40, 170

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

99. Perret, C.J., (1954), Nature, 174, 1012

100. Petit, J.F., and Muñoz, E., and Ghuysen, J.M., (1966), Biochemistry, N.Y., 5, 2765

101. Plummer, D.T., James, A.M., Gooder, H., and Maxted, W.R., (1962), B.B.A., 60, 595

102. Pollock, M.R., (1957), CIBA Foundation Symposium, "Drug Resistance in Microorganism", p.78

103. Pollock, M.R. (1967), Brit. Med. J. 2, 71

104. Ramareddy, G., and Reiter, H., (1969), J. Bact., 100, 729

105. Richmond, M.H., and John, M., (1964), Nature, Lond., 202, 1360

106. Richmond, M.H., Parker, M.T., Jevons, M.P., and John,M., (1964), Lancet, 1, 293

107. Richmond, M.H., (1965), Biochem. J., 94, 467

108. Robertson, D.S., and Schwab, J.H., (1960), B.B.A., 44, 436

109. Rolinson, G.N., Stevens, S., Batchelor, F.R, Wood, J.C., and Chain, E.B., (1960), Lancet, 2, 564

110. Rolinson, G.N., (1961), Brit. Med. J., 1, 125

111. Rogers, H.J., and Garrett, A.J., (1965), Biochem. J., 96, 231

112. Rogers, H.J., and Perkins, H.R., (1959), Nature, Lond., 184, 520

113. Rogers, H.J., and Perkins, H.R., (1968), Pub. Spon. London.

114. Rosato, R.R., and Cameron, J.A., (1964), B.B.A., 83, 113.

115. Rosypal, S., Rosypalova, A., and Horejs, J., (1966), J. Gen. Microbiol., 44, 281

116. Rozgongl, F., and Redai, I., (1968), Acta. Microbiol. Acad. Sci., Hung., 15, 253

117. Salton, M.R.J., and Horne, R.W., (1951), B.B.A., 7, 177

118. Salton, M.R.J., (1953), B.B.A., 10, 512

119. Salton, M.R.J., (1956), Symp. Soc. Gen. Microbiol., 6, 81

120. Scudi, J.V., and Woodruff, H.B., (1949), "The Chemistry of Penicillin" Chap. 29, p.1026.

121. Schleifer, K.H., Kandler, O., (1970), J. Bact. 103, 587.

122. Schleifer, K.H., and Kandler, O., (1972), Bact. Rev., 36, 407

123. Schmerson, A.H., (1964), Antibiotiki, 9, 323

124. Seaman, G.V.F., (1965), "Cell Electrophoresis"Ed. Abrose, Pub. Churchill.

125. Silvestri, L.G., and Hill, L.R., (1965), J. Bact. 90, 136

126. Stanier, R.Y., and Van Niel, L.B., (1962), Arch. Mikrobiol., 42, 17

127. Stephen, A.S., and Baldwin, J.N., (1972), J. Bact., 109, 602

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

129. Stewart, R.E., and Holt, R.H., (1963), Brit. Med. J., 1, 308

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

131. Strominger, J.L., and Park, J.L., and Thomson, R.E., (1959), J. Biol. Chem., 234, 3263

132. Strominger, J.L., and Ghuysen, J.M., (1967), Science, N.Y., 156, 213

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

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

135. Toon, P., Brown, P.E., and Baddiley, J., (1972), Biochem. J., 127, 394

136. Wicken, A.J., and Knox, K.W., (1970), J. Gen. Microbiol. 60, 293

137. Willis, A.T., and Turner, G.C., (1963), J. Path. Bact., 85, 395

138. Wise, E.M., and Park, J.T., (1965), Proc. Ntr. Acad. Sci. U.S.A., 54, 75.

139. Wolin, N.I., Archibald, A.R., and Baddiley, J., (1960), Nature, Lond. 209., 484.

140. Work, E., (1957), Nature, Lond. 179, 89

141. Work, E. (1961), J. Gen. Microbiol. 25, 167