A STUDY OF BACTERIAL L FORMS WITH PARTICULAR REFERENCE TO ORGANISMS OF ACTINOBACILLUS/PASTEURELLA GROUP

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Abstract

L forms of Actinobacillus and Pasteurella strains were induced on complex media. Penicillins and cycloserine induced L forms readily while vancomycin, bacitracin and novobiocin did not induce L forms or any morphological changes in the organisms tested. Glycine and lysozyme proved active against bacterial cell walls. Different cultural conditions for supporting L forms were studied. Serum was found not to be an important growth factor for L forms, and charcoal was shown to support induction and propagation of most of the L forms equally, if not better. The hypertonicity of the medium provided by sodium chloride was better than with sucrose in propagation of L forms. The morphology of L forms induced by different methods and by different inducing agents is similar and differentiation between genera or strains on L colony morphology was impossible. The susceptibility of L forms to hypertonic media was demonstrated. All L forms isolated reverted to the parental forms in the absence of the inducing agent.

The biochemical characters and fermentative activity of revertants and parents were comparable and some of the lost characters of the revertants were regained after several passages. Revertants were more resistant to cycloserine than the parents and induced to the L forms at a higher cycloserine concentration.

Bacterial variants were isolated from blood samples

of apparently healthy rats, chickens, ewes and lambs delivered by caesarian section. The use of hypertonic medium facilitated their recovery. The inability of most of the isolates to grow on agar media confirmed that these agents were not of the usual bacterial form. Other organisms isolated on agar included streptococci, micrococci and diphtheroid forms.

Part 1: Literature Review and General Introduction

Literature Review and General Introduction

History.

Filterable forms and pleomorphism in bacteria were noticed a long time ago. Fowke (1885) noticed annular bodies and corpuscles in water contaminated with cholera bacilli. Similar findings were reported by Klein (1885 a & b) who observed that comma bacilli in artificial agar media at room temperature transformed into circular corpuscles which then disintegrated into comma bacillus forms. Dowdeswell (1890) observed rounded cysts and coccoid bodies in fluid cultures of the cholera organism.

Guignard and Charrin (1887) reported morphological and physiological changes in B. pyocyaneus upon the addition of boric acid, phenol, cresol or thymol. The rods gave rise first to threads and then to spirilla which in turn produced the typical rods. The same organism produced branched forms when lithium compounds were added to the substrate (Gameleia, 1900).

Klein (1883) obtained round forms connected together or connected with typical rods when he cultured the anthrax bacillus in pork broth or pork broth gelatin kept at 20 - 25°C. Big spherical forms in chains were recorded in Bacillus megaterium, (DeBary, 1884). Matzuschita (1900) noted round forms when Bacillus anthracis was grown on salt agar. A very virulent strain of the same organism was transformed into long filaments and irregular spiral forms within 4 days when grown on agar and into pear and club-shaped cells on potato medium, (Lignières and Durrieu,

1902). They also observed irregular forms when a watery suspension was treated with ultra-violet rays.

The branched growth of Bacillus typhosus was observed on gypsum plates (Casagrandi, 1901). The addition of 0·2% saturated alcoholic solution of methyl violet to culture media caused typhoid and coli bacilli to grow into long branching threads which, in old cultures, gave rise to chains of cocci (Walker & Murray, 1904). Peju and Rajat (1906) were able to transform typhoid bacilli into long non-motile threads showing oval, spindle or pear-shaped forms when treated with potassium iodide. The effect of malachite green on a strain of colon bacillus was observed by Revis (1912). This strain produced slime and grew into long filaments or very short rods.

In cultures of the paradysentery bacillus, two types of colonies were observed (Ørskov & Larsen, 1925). Type V was made up of short bacilli while type B was highly pleomorphic, early autolysed and gave rise to type V.

Using the single cell method, Kahn and Nonidez (1932) followed the development of Mycobacterium tuberculosis. They reported that the rod broke into 3 or more ovoid units. These units broke into granules from which fine rods developed. Pleomorphism in tubercle bacillus was also reported by Pagal (1934) who observed large number of granules in slow-growing organisms and in slowly degenerating cultures. Alexander-Jackson (1947) reported the transformation to globoid forms of Mycobacterium leprae in treated cases of leprosy and of Mycobacterium tuberculosis

in cultures containing various concentrations of streptomycin.

Bacterial growth in filtrates of lysed cultures of different bacteria was detected (Bronfenbenner & Muckenfuss, 1927). Hadley, Delvis and Klimek (1931) designated the filterable forms as G forms.

In 1935 Klieneberger spotted pleuropneumonia-like colonies among Streptobacillus moniliformis colonies. She designated them as L_1 colonies, in the honour of Lister Institute. Due to similarities of L colonies with those of the organism of pleuropneumonia, Klieneberger thought that the L colonies were pleuropneumonia like organisms (PPLO) growing in symbiosis with the bacteria. Dienes (1938) obtained a pure culture of bacilli from a transplant made mainly of Streptobacillus large forms. On further experimentation with the organism (1939c) he observed its transformation into pleomorphic swellings, thick spindle-shaped forms and round forms with stem. He concluded that L colonies were derived from bacteria. Dawson and Hobby (1939) supported the view that ppl0 associated with Streptobacillus was not a symbiont but a variant phase of the organism and they obtained Streptobacillus by reconverting the PPLO. Heilman (1941), Ørskov (1942) and Dienes (1943) noticed the presence of bacillus forms of normal shape and size filling the large bodies of Streptobac. moniliformis cultures. These bacillus forms developed into bacillary colonies. This confirmed that L colonies were forms of the same organism.

Isolation of L forms from many species of bacteria indicated that it was a general property of bacteria. Investigators were in agreement that L forms were a result of bacterial transformation to soft globoid forms and granules. L colonies were isolated from Haemophilus (Dienes, 1939a; Dienes & Zamecnik, 1952). Proteus (Dienes, 1949; Medill & O'Kane, 1954; Lorkiewicz, 1957), Salmonella (Dienes, Weinberger & Madoff, 1950a; Thomas, 1976), Shigella (Weinberger, Madoff & Dienes, 1950), Streptococcus (Crawford, Frank & Sullivan, 1958; Rotta, Karakawa & Krause, 1965; Hewitt, Seligman & Deigh, 1968; Bibel & Lawson, 1972a), Mycobacterium (Mattman, Tunstall, Mathews & Gordon, 1960), Staphylococcus (Williams, 1963), Neisseria (Roberts & Wittler, 1966; Roberts, 1968; Stewart & Wright, 1969; Lawson & Douglas, 1973; Geary & Waitkins, 1977), Clostridium (Mahony, 1973), Escherichia coli (Seeberg & Brorson, 1974) and from several other bacteria.

Cell wall structure.

The cell wall comprises 25% of the cell dry weight (Work, 1957; McQuillen, 1958a). Work (1957) also reported the absence of nucleic acid, purines and pyrimidines from the cell wall. The outer layer is composed of lipoprotein, followed by lipopolysaccharide, protein and finally mucopeptide, (Weidel, Frank & Martin, 1960; Hofschneider & Martin, 1968). The mucopeptide is also referred to as the peptidoglycan, glycan or murein and it is a unique heteropolymer which, with the protein, gives strength to

the wall (Murray, 1968). The murein is made up of alternating residues of N-acetylglucosamine and N-acetylmuramic acid (Park, 1968; Blumberg & Strominger, 1974) and accounts for at least 50% of the total dry weight of the wall in Gram positive organisms being found throughout the wall, while in Gram negative bacteria it constitutes 10% of the dry weight of the cell wall and it is in the innermost layer. Pentapeptide chains are attached to the carboxyl group of the muramic acid and they are cross linked with each other (Blumberg & Stominger, 1974). Each chain consists of L-alanine, D-glutamine, D-alanine and the dibasic amino acids such as lysine, diaminopimelic acid (DAP), ornithine or 2,4 diaminobutyric acid.

Cell wall materials present in some bacterial variants cannot be detected in other variants. Most bacterial variants however have lost some of the constituents of the parent forms. Kandler, Hund and Zehender (1958) demonstrated that both the unstable L forms induced by penicillin and the parent forms of Proteus vulgaris contained the same amount of DAP. In contrast McQuillen (1958a) and Salton and Shafa (1958) noticed reduction in DAP and amino sugar content in the walls of spheroplasts of Salmonella gallinarum, Vibrio metchnikovii and E.coli. L forms of one strain of proteus contained less glucosamine, galactosamine and muramic acid compared with their vegetative forms (Sharp, 1960). Sharp (1960) pointed out that the development of L forms could be due to complete loss of

cell wall or to changes in the cell wall organisation without cell wall materials being lost. Morrison and Weibull (1962) showed that hexosamine and DAP were present in detectable amounts in all the stable L forms of Proteus they studied. Williams (1963) failed to show any muramic acid in three strains of Staphylococcus aureus L forms tested. Two strains of stable L forms of Proteus mirabilis were characterised by the loss of mucopolymers, but the stable and unstable L forms of three other strains contained mucopolymer compounds similar to those in the rod forms (Martin, 1964). Staph. aureus L forms did not contain muramic acid or glucosamine (Pratt, 1966). Weibull, Bickel, Haskin, Milner and Ribi (1967) could not detect DAP in membranes of Proteus L forms.

Examination with the electron microscope showed that lysozyme protoplasts lack the cell wall remnants (Gooder, 1968). Hofschneider and Martin (1968) reported that stability or reversion of L forms might take place regardless of the amount of cell wall present.

Cell wall synthesis.

Synthesis of the peptidoglycan can be divided into three stages (Strominger, 1968; 1973; Weinstein, 1975). The first step is the synthesis of uridine diphosphate N-acetylmuramyl pentapeptate. Following a series of reactions the N-acetylglucosamine and N-acetylmuramic acid are formed. The amino acids are then added in the proper sequence, first the L-alanine followed by D-glutamic acid

and L-lysine, the last reaction being the addition of the dipeptide D-alanyl-D-alanine as a unit. Synthesis of the dipeptide involves the conversion of L-alanine to D-alanine catalysed by a racemase and followed by the joining of the two alanine residues in adenosine triphosphate to form D-alanyl-D-alanine, this reaction being catalysed by D-alanyl-D-alanine synthetase.

During the second stage the uridine diphosphate (UDP) N-acetylmuramyl-pentapeptide and UDP N-acetylglucosamine are linked to form a polymer. The UDP N-acetylmuramyl-pentapeptide is attached by a pyrophosphate bridge to a phospholipid in the cell membrane. The N-acetylglucosamine is then added followed by the addition of five glycine residues. The disaccharide-pentapeptide unit is then separated from the lipid intermediate and the lipid pyrophosphate is generated. Elimination of one inorganic phosphate regenerates the phospholipid which enters the cycle again.

The last stage takes place outside the cell membrane. The D-alanine of the peptide chain and the free amino group of the second chain are crosslinked with the elimination of the terminal D-alanine. Transpeptidase and carboxypeptidase are involved in these two reactions.

Inhibitors of cell wall synthesis.

Cycloserine inhibits cell wall synthesis at an early stage. Bondi, Kornblum and Forte (1957) were able to inhibit the antibacterial activity of cycloserine on

staphylococci by the addition of alpha-alanine which inhibited the incorporation of lysine into the cell wall (Park, 1958). Cycloserine was found to be a competitive inhibitor of D-alanyl-D-alanine and its effect was completely reversible (Ciak & Hahn, 1959; Strominger, Threnn & Scott, 1959; Neuhaus & Lynch, 1964). Strominger (1973) and Weinstein (1975) reported that D-cycloserine inhibited both the conversion of L-alanine to D-alanine and the linking of the two alanine molecules. D-cycloserine is a structural analogue of D-alanine and hence it acts as a competitive inhibitor of the racemase and synthetase.

Jordan and Innis (1959) demonstrated complete inhibition of Staph. aureus ribonucleic acid grown in the presence of vancomycin. Incorporation of alanine, glutamic acid and lysine into the cell wall was inhibited following the addition of vancomycin (Reynolds, 1961). Since ristocetin causes an accumulation of diphosphoacetylmuramyl-pentapeptide it was considered as a cell wall inhibitor (Wallas & Strominger, 1963). Both vancomycin and ristocetin inhibited the utilization of lipid intermediates without affecting their biosynthesis (Matsuhashi, Dietrich & Strominger, 1965; Anderson, Matsuhashi, Haskin & Strominger, 1965). Strominger (1968) suggested an additional effect at the membrane affecting protoplast formation. The cleavage of the disaccharide pentapeptide units from the phospholipid was the reaction found to be sensitive to these antibiotics (Weinstein, 1975).

Paine (1951) reported that bacitracin inhibited the uptake of glutamic acid by growing staphylococci. The rate of lysine incorporation into the cell wall was decreased by 90% by 50 ug/ml bacitracin (Park, 1958). Studies suggested that the lytic action at the minimum inhibitory concentration was due to suppression of cell wall synthesis, enzyme synthesis and possibly increased permeability (Smith & Weinberg, 1962). Bacitracin caused the accumulation of uridine nucleotide (Anderson et al., 1965; Siewert & Strominger, 1967) and did not affect the synthesis or utilization of lipid intermediates (Matsuhashi et al., 1965). It interfered with cell wall synthesis by inhibiting the dephosphorylation which involves the release of inorganic phosphate and hence prevented the lipid carrier from re-entering the cell wall synthesis cycle (Siewert & Strominger, 1967; Strominger, 1973; Weinstein, 1975).

Accumulation of uridine nucleotide compounds was reported as an indication of the interference in cell wall synthesis by penicillin (Park & Strominger, 1957). Penicillin caused osmotic fragility of bacteria and in the presence of a protective agent the cell swelled (Lederberg, 1957). In Staph. aureus penicillin resulted in complete inhibition of the incorporation of lysine into the cell wall (Hancock, 1958; Park, 1958). When Staph. aureus was cultured in a medium containing penicillin the accumulated amino sugars were the same amino sugars normally found in the cell wall (Strominger, Park & Thompson, 1959).

Both penicillin and cephalosporin caused accumulation of uridine nucleotide and reduced incorporation of lysine into the cell wall (Chang & Weinstein, 1964; Anderson et al., 1965). Wise and Park (1965) demonstrated that in the presence of penicillin the product formed contained large amounts of alanine and high numbers of free amino groups. Accordingly they concluded that the transpeptidation reaction which removes the terminal D-alanine was the target. Park (1968) reported that only the cross link between lysine and D-alanine was the impaired step. It was suggested that penicillin constituted a structural analogue of the terminal D-alanyl-D-alanine. Inhibition of the transpeptidase required a concentration of penicillin similar to that which inhibits the growth of the organism and it is irreversible, while the carboxypeptidase reaction is very sensitive and reversible (Strominger, 1968; 1973).

Kubota, Montgomerie, Potter, Kalmanson and Guze (1966) obtained no growth of Streptococcus faecalis when 100,000 units/ml penicillin were used. They speculated that when the antibiotic was used in large amounts it probably exerted a toxic effect on the organism. Tipper and Strominger (1968) found that at high penicillin concentrations Staph. aureus growth was inhibited more rapidly and little or no accumulation of the uncrosslinked monomers was observed.

Brock and Brock (1959) demonstrated that novobiocin caused release of ribonucleic acid (RNA) and protein from E.coli and increased the permeability of the cell.

Treatment of Staph. aureus with this antibiotic resulted in accumulation of nucleotide (Strominger & Threnn, 1959; Anderson et al., 1965; Wishnow, Strominger, Birge & Threnn, 1965). Since its action can be reversed by the addition of magnesium ions, it was suggested that novobiocin inhibited growth by causing a magnesium deficiency (Brock, 1962). It had no effect on synthesis or utilization of lipid intermediates (Matsuhashi et al., 1965). Experiments with isotopes showed that its action was not specific to the cell wall and, in addition, it inhibited both protein-and nucleic acid-synthesis (Wishnow et al., 1965).

Maculla and Cowles (1948) demonstrated the lytic effect of glycine on bacterial cells. In Staph. aureus high concentrations of the amino acid induced accumulation of uridine nucleotide (Strominger & Birge, 1965). They postulated that the target in this process was the inhibition of the enzyme responsible for the addition of L-alanine to the muramic acid. Strominger (1968) suggested that the transformation of bacteria into spheroplasts and protoplasts was due to the reversal of the terminal cross-linking in cell wall synthesis.

Lysis of the bacterial cell by lysozyme was demonstrated by Kern, Kingkade, Kern and Behrens (1951) and Salton (1952). Lysozyme has no action on the biosynthesis of the cell wall but causes breakdown of the wall as a result of hydrolysis of the glycosidic linkage in the structure (Strominger, 1973).

Terminology.

Most of the bacterial variants can exist in one of 4 phases, protoplast, spheroplast, transitional or L phase. All the phases lack the rigid cell wall, thus they are soft, fragile and pleomorphic and preserve their structure only in the presence of stabiliser (Lederberg & St.Clair, 1958; Weibull, 1958; 1968; Little, Bosbery & Draper, 1973).

Brenner, Dark, Gerhardt, Jeynes, Kandler, Kellenberger, Klieneberger-Nobel, McQuillen, Rubio-Huertos, Salton, Strange, Tomcsik and Weibull (1958) recommended the use of the term protoplast for the spherical, osmotically fragile forms that lack those components which are exclusively found in cell walls and phage receptor sites, the specific cell wall antigen. Protoplasts are devoid of cell wall constituents and fail to absorb phage (Gooder & Maxted, 1958; Michael & Braun, 1959; Gooder, 1968; Hofschneider & Martin, 1968). Similarly Salton & Shafa (1958), Godzeski, Brier and Farran (1967) and Kagan (1968a) reserved this term for bacteria that were completely devoid of cell wall or proved negative for mucopeptide and chemical structures responsible for cell wall rigidity. Weibull (1968) described the protoplast as lacking the rigid cell wall although traces of cell wall material were present. Watanakunakorn & Hamburger (1969) used this term to describe a cell with defective cell wall without referring to the absence or presence of cell wall material.

Spheroplast is a variant that contains some of the

cell wall constituents (Michael & Braun, 1959; Chatterjee & Williams, 1965; Godzeski et al., 1967; Hofschneider & Martin, 1968; Weibull, 1968). Spheroplasts can revert or pass to the stable form (Hofschneider & Martin, 1968; McGee, Wittler, Gooder & Charache, 1971). To avoid confusion Mitchell and Moyle (1957) suggested the use of protoplast within quotation marks if complete absence of cell wall was doubtful. McQuillen (1960) preferred the use of protoplast within quotation marks when the cell wall was modified to render the organism spherical, osmotically fragile and incapable of growth on usual media.

The opinion of Bassermann, Carrere, Fasquelle, Hauduroy, Klieneberger-Nobel, Penos, Roux and Tuncman (1957) was that L form colonies had distinct classical morphology with a core growing into the agar and a superficial peripheral growth. The elements forming these colonies must not assume the parent form in the absence of inducing agent. They considered as incorrect the use of the description of L form to denote an individual morphology. Lederberg and St.Clair (1958) referred to L growth as atypical growth. Godzeski et al., (1967) referred to L form as the stable non-reverting culture of the L type and the term L phase as the non-stable L type growth. Maxted (1972) described L phase as a morphological variant of bacteria made up of pleomorphic globules which did not take Gram stain. Roberts, Ingold, Want and May (1974) used the term L form to describe a round body arising as a result of exposure of a bacillary

form to agents acting on the cell wall.

The terms stable and unstable have been used to differentiate reverting from non-reverting forms. Cultures that continued to grow as L forms and did not revert to vegetative forms in the absence of an inducing agent were the stable forms, while those that reverted were the unstable (Taubeneck & Gumpert, 1967; Gooder, 1968; Weibull, 1968; Hubert, Potter, Hensley, Cohen, Kalmanson & Guze, 1971; McGee et al., 1971; Little et al., 1973). The relatively stable forms were those that reverted under certain conditions (McGee et al., 1971).

Media used for L forms.

The production of L colonies is greatly influenced by the basal medium (Dienes & Sharp, 1956). A basal medium supplemented with the correct ingredients in an appropriate condition will support the induction and propagation of L colonies. Trypticase soy medium and pplo agar were found to be satisfactory (Dienes & Sharp, 1956; Hewitt et al., 1968; Bibel & Lawson, 1972a; Pachas & Currid, 1974). Brain heart infusion medium has been generally employed (Kubota et al., 1966; Montgomerie et al., 1966; Kagan, 1968a; Roberts, 1968; Hubert et al., 1971; Lawson & Douglas, 1973; Little et al., 1973). Hatten and Sulkin (1966) induced Brucella abortus L forms in tissue culture. Group A Streptococci L elements were obtained in human diploid cells (Schmitt-Slomska, Boue & Caravano, 1972).

Medill and O'Kane (1954), Landman, Altenbern and Ginoza (1958), Medill-Brown, Hutchinson and Cocklin (1960), Tunstall and Mattman (1961) and van Boven, Kastelein and Hijmans (1967) isolated L colonies in synthetic and semi-synthetic media. To achieve good growth in synthetic medium, van Boven et al. (1967) recommended the adaptation of the organism in semisynthetic medium.

Workers have usually used low concentrations of agar to solidify media for L forms. Soft media enables L growth to penetrate giving the characteristic morphology of the fried egg appearance. On hard agar, coagulated serum, gelatin, starch and unpenetrable media, L colonies develop without penetration (Dienes, 1947; Dienes & Bullivant, 1967). The role played by agar is physical rather than nutritional (Landman et al., 1958; Lederberg & St.Clair, 1958). Penetration was due to the tendency of the organisms to adhere to a structure, (Dienes & Bullivant, 1967). Concentration of agar determines the type of growth. In streptococci and staphylococci the L elements grew only to very large bodies when 3·5% agar was used (Dienes, 1967b). On 0·2% and up to 2·5% agar growth was a mixture of large bodies and granules. Refined agar proved better than granular agar (Mattman et al., 1960) possibly due to the presence of unidentified inhibitors in the agar and Nimmo and Blazevic (1969) suggested the use of highly purified agar.

From previous work it is obvious that the effect of agar concentration has varied with the species of organism

being investigated. A concentration of 0·8% and 1% supported growth of E.coli L forms (Lederberg & St.Clair, 1958) and 0·4% or above supported streptococcal L forms (Friemer, Krause & McCarty, 1959). Variations in agar concentration from 0·6% to 1·2% had no effect on Proteus mirabilis (Altenbern, 1961). Concentrations of 1% and 1·2% were used for Niesseria L colonies (Roberts & Wittler, 1966; Roberts, 1968; Lawson & Douglas, 1973). Pseudomonas aeruginosa continued growing as L forms on 1·5% agar (Hubert et al., 1971). Bibel and Lawson (1972a) used 1·2% for streptococci.

The optimum concentration of agar was 1% or above for E.coli (Landman et al., 1958), 0·7 - 0·8% for Proteus (Weibull & Lundin, 1961) and 1 - 1·6% for meningococci (Roberts, 1966).

Agar concentration affected colony size. Landman et al., (1958) reported that Proteus mirabilis produced medium colonies (0·5 - 1 mm diameter) at low agar concentrations, large at 1 - 1·5% and small at 2 - 2·5%. In Streptobacillus moniliformis stable L forms, the diameter of the central zone did not change significantly upon changing agar concentrations (Razin & Oliver, 1961). Razin and Oliver (1961) noted that the optimum agar concentration for the peripheral zone was 1·5% and it was inhibited with higher concentrations.

Inclusion of animal serum was necessary for growth of L forms. Sera of different sources have been tried. Ørskov (1942) found that ox serum was satisfactory.

Experiments with salmonellae and shigellae (Weinberger et al., 1950) showed that optimum results were obtained with horse serum. Rabbit serum supported growth poorly and both guinea pig and mouse serum were unsatisfactory. Horse serum prepared by defibrination of blood was superior to human serum or ascitic fluid (Dienes et al., 1950a). Mattman et al. (1960) found that guinea pig serum was as good as horse serum. Sheep serum was used with many organisms with good results (Li-Hui, Li Tien-Lin & Lei Ai-Te, 1964a). Roberts and Wittler (1966) reported that N. meningitidis L growth was equally good on media containing horse, rabbit or human serum. Swine serum at 20% was successfully used (Kang & Casida, 1967). Horse serum is usually added in concentrations of 10 - 20% (Hewitt et al., 1968; Marston, 1968; Little et al., 1973; Pachas & Currid, 1974). Hamburger and Carleton (1966a) observed that at a concentration of 20% serum L growth was decreased and at 50% serum it was suppressed.

Weibull and Lundin (1961) reported that the growth of L forms of Proteus L9 (Klieneberger-Nobel, 1956) was increased when inactivated serum was used as compared with serum which had not been inactivated. The authors also reported that the absence of serum resulted in complete disintegration of the L forms although they remained unaffected in the presence of serum. Roberts and Wittler (1966) reported that unheated serum was as good as heated serum. It was demonstrated that serum promoted L growth and inhibited reversion to the

vegetative forms (Hamburger & Carleton, 1966b; 1968; Thomas, 1976). Marston (1968) obtained Staph. aureus L growth in broth and agar media from which serum was omitted, but the yield was very low and the organisms soon died.

Lorkiewicz (1957) and Madoff and Dienes (1958) produced L forms of Proteus and pneumococci respectively when charcoal replaced serum in the culture medium. Two per cent bovine serum albumin could be used as a substitute for serum (Montgomerie et al., 1968; Hubert et al., 1971; Bertolani, Elberg & Ralston, 1975; Guze, Harwick & Kalmanson, 1976).

The addition of divalent cations was needed to stabilize L growth. Magnesium ions were required to stabilize E.coli protoplasts in liquid media (Lederberg & St.Clair, 1958). In the absence of magnesium ions pneumococci grew to large forms without having developed into L forms (Madoff & Dienes, 1958). Omission of these ions resulted in the formation of ghost cells (Muschel, Carey & Baron, 1959; Muschel, 1968). Altenbern (1961) reported that a high concentration of magnesium sulphate ($MgSO_4$) increased the total number of 3A colonies (Dienes, 1949) obtained from a constant inoculum of cells. The vegetative cell : L form ratio decreased from 593:1 to 162:1 when he increased the concentration from 0·005M to 0·04M $MgSO_4$. Concentrations as low as 0·001% $MgSO_4$ have been used (Montgomerie et al., 1966, 1968; Hubert et al., 1971; Bertolani et al., 1975). Davis, Gemsa and Wedgwood (1966) investigated the effect of magnesium and

calcium ions on spheroplasts of Gram negative rods in broth media and demonstrated that the addition of 0·01M magnesium chloride resulted in keeping the number of spheroplasts high for a long time and slowed down the appearance of ghost cells. The addition of 0·005M calcium chloride inhibited the appearance of ghost cells. Omission of magnesium ions led to the formation of ghost cells (Muschel, 1968). Nimmo and Blazevic (1969) reported that these ions were not required on solid media but the addition of 0·2% $MgSO_4$ to liquid medium was beneficial for primary isolation. Several workers obtained satisfactory results using 0·2% $MgSO_4$ (Watanakunakorn, Goldberg, Carleton & Hamburger, 1969; Watanakunakorn & Hamburger, 1969). Makemson and Darwish (1972) obtained optimum results when 0·15% calcium chloride was added to their medium together with 0·05% $MgSO_4$. They found that concentrations of $MgSO_4$ above 0·05% decreased the yield of L forms.

Hydrogen ion concentration influenced the production of L colonies. Landmann *et al*. (1958) obtained a vegetative cell : L form ratio of unity between pH 6·5 - 7·5 which increased to 1·2 at pH 5·5 and 8·0. The optimum pH for *E.coli* was 6·3 (Lederberg & St.Clair, 1958). Mattman *et al*., (1960) used media having a pH of 7·0 - 7·2 for growing *Mycobacterium* L forms but found that there was no improvement when the pH varied from 5 to 9. Nimmo and Blazevic (1969) reported that a pH between 7·6 and 7·8 was suitable for isolation of L growth from different

bacterial species. Seeberg and Brorson (1974) and Thomas (1976) in their studies found that the pH at which the organism underwent its maximum transformation to L growth depended on the strain.

Due to the absence of the rigid cell wall in bacterial L forms (Weinberger et al., 1950; Friemer et al., 1959; Taubeneck, 1962; Kagan, 1968b; Weibull, 1968) they are pleomorphic, soft and highly fragile (Lederberg & St.Clair, 1958; Friemer et al., 1959; Kagan, 1968b; Weibull, 1968). Their stability is highly dependent on the osmotic pressure and unless an osmotic counter pressure is applied the fragile membrane will be disrupted (Weibull, 1958). High osmolality was obtained by adding sugars or salts to the culture media. Hancock (1958) reported that sodium chloride, sucrose and sodium gluconate prevented the depletion of amino acid and cessation of amino acid uptake when Staph. aureus was treated with penicillin. Salts such as sodium succinate, sodium citrate, potassium chloride, sodium dihydrogen phosphate, sodium nitrate, sodium sulphate, disodium hydrogen phosphate, magnesium chloride, ammonium chloride and calcium chloride have been used (Dienes & Sharp, 1956; Landman et al., 1958; King & Gooder, 1970a). Sodium chloride which is the most commonly used was incorporated at different concentrations depending on the organism (Montgomerie et al., 1966; Kagan, 1968a; Little et al., 1973; Pachas & Currid, 1974).

Sucrose was used by Mitchell and Moyle (1956), Michael and Braun (1959), Roberts and Wittler (1966),

Roberts (1968), King and Gooder (1970a) and Hubert et al. (1971). Protoplasts of M. lysodeikticus and spheroplasts of Sarcina lutea and E.coli were not stabilized when suspended in 1·5M glycerol or D-ribose (Mitchell & Moyle, 1956; Razin & Argaman, 1963). Michael and Braun (1959) found that 10 - 20% lactose, starch, galactose or glucose did not confer protection to Shigella dysenteriae spheroplasts. Starch, raffinose and pentoses did not protect pneumococcal L forms and no growth was obtained with them, while 10% glucose and 20% maltose supported slight growth (Madoff & Dienes, 1958). Razin and Argaman (1963) did not detect lysis of the organism they tested when they used 1·5M glucose or fructose instead of sucrose.

Among the solutes tested, sucrose was the only one that provided osmotic pressure in E.coli K12 (Landman et al., 1958). Many investigators obtained satisfactory results with sodium chloride (Crawford et al., 1958; Ward & Martin, 1962; Kubota et al., 1966; Watanakunakorn, 1971a) but it proved to be inferior to sucrose for isolating Mycobacterium L forms (Mattman et al., 1960).

Requirement of high osmotic pressure was not exhibited by L forms of Salmonella typhi, Salmonella typhimurium, Proteus vulgaris, Haemophilus influenzae (Dienes & Sharp, 1956); Bacillus subtilis (Madoff, Bruke & Dienes, 1967). They all produced L forms in normal salt medium.

Inhibitory substances and growth promoting factors.

Medill and O'Kane (1954) suggested that the poor L growth in natural medium was due to the presence of inhibitors rather than the absence of growth factors. They showed that Proteus L forms grew more abundantly on defined medium than on natural medium. Their examinations of yeast extract, peptone and casein hydrolysate revealed inhibitory substances in all. Landman et al. (1958) found that casein hydrolysate made the medium satisfactory for the propagation of Proteus L forms. Lederberg and St.Clair (1958) confirmed the inhibitory effect of yeast extract with E.coli L forms but Madoff (1970) found that the addition of yeast extract enhanced production of L forms of a Streptococcus strain.

Altenbern (1961) investigated the inhibitors of 3 B type colonies (Dienes, 1949). He came to the conclusion that formic acid was the inhibitory factor. This acid was generated during the autoclave sterilization of glucose at alkaline pH in the presence of phosphates present in the medium. van Boven et al. (1967) reported that phosphate buffer in semisynthetic medium inhibited L growth of group A streptococci.

Medill and O'Kane (1954) noted that the addition of 50 mg/ml riboflavin to the medium resulted in poorer L growth of Proteus, but Tulasne, Terranova and Lavillauriex (1955) reported that riboflavin stimulated Proteus L growth. On the other hand, Lederberg and St.Clair (1958) reported that riboflavin did not stimulate E.coli L growth.

Media containing charcoal facilitated L production of Proteus and pneumococci (Lorkiewicz, 1957; Madoff & Dienes, 1958) and meat extract stimulated L growth of E.coli (Lederberg and St.Clair, 1958).

L growth inducing agents.

L forms are organisms deprived of rigid cell walls (Friemer et al., 1959; Taubeneck, 1962; Weibull, 1968; Kagan, 1968b). Thus they are produced by substances that interfere in the synthesis of the cell wall or remove the preformed cell wall (Maxted, 1972).

Pierce (1942) was the first to observe that Streptobac. moniliformis L forms were resistant to penicillin concentrations that inhibited the bacillary forms. Later this agent was tried successfully with many types of Gram positive and Gram negative bacteria. L colonies were isolated with penicillin from Staphylococcus (Youman, Williston & Simon, 1945; Kagan, 1968a); Proteus (Dienes, 1949; Taubeneck, 1962); Salmonella (Weinberger et al., 1950; Thomas, 1976); Shigella (Weinberger et al., 1950); Streptococcus (Sharp, 1954); Clostridium tetani (Scheibel & Assandri, 1959); Niesseria (Roberts, 1968) and E.coli (Makemson & Darwish, 1972).

Molander, Kagan, Weinberger, Heimlich and Busser (1964) reported the resistance of 2 strains of Staph. aureus to the action of penicillin. Braude, Siemienski and Lee (1968) were unable to induce Pseudomonas aeruginosa spheroplasts with penicillin.

Semisynthetic penicillins and cephalosporins readily induced L forms from several bacterial genera and from penicillin-resistant organisms (Williams, 1963; Molander et al., 1964; Molander, Weinberger & Kagan, 1965; Roberts, 1968; Watanakunakorn et al., 1968; Watanakunakorn & Hamburger, 1969; Bertolani et al., 1975).

Cycloserine was used successfully to induce L forms and spheroplasts of haemolytic streptococci, staphylococci, Proteus, Pseudomonas aeruginosa, Salmonella and E.coli; (Michael & Hijmans,1960; Ward & Martin, 1962; Williams, 1963; Watanakunakorn, 1971a). Neisseria L forms were induced by this antibiotic (Roberts, 1967, 1968).

Ward, Madoff and Dienes (1958) could not obtain L forms of streptococci, diphtheroid organisms, Proteus, Salmonella or Vibrio by the action of bacitracin. The authors related this result to the fact that there was no difference between concentrations inhibitory to bacteria and to the L forms. Induction of L forms was not observed when Staph. aureus was treated with bacitracin (Molander et al., 1964). In contrast it was used successfully in inducing L forms of Streptococcus (Rotta et al., 1965); and Neisseria (Roberts, 1968). Kagan (1968a) and Park (1968) reported that this antibiotic was a poor agent for inducing L forms.

Williams (1963) and Molander et al. (1964) failed to induce streptococcal L forms with vancomycin. Roberts (1967, 1968) was successful in transforming Neisseria meningitidis to its L forms by this antibiotic.

Kagan (1968a) and Park (1968) described vancomycin as a poor inducing agent of L forms. This was in agreement with Watanakunakorn (1971b) who obtained very low yields of Staph. aureus L forms with vancomycin compared to other inducers. Watanakunakorn and Bakie (1973) were able to obtain Staph. aureus L forms with this antibiotic.

Molander et al. (1964) reported that novobiocin induced pleomorphic growth in the tested strains of Staph. aureus without L transformation at a concentration inhibitory to bacterial growth. Rotta et al. (1965) and Roberts (1968) also failed to induce L forms with the same antibiotic.

Other antibiotics such as neomycin, kanamycin, streptomycin, oleandomycin, erythromycin, chloramphenicol, tetracycline and the sulpha drugs were tried unsuccessfully (Dienes & Weinberger, 1951; Molander et al., 1964; Roberts, 1968).

Bacteria of the genus Vibrio swelled when exposed to 0·25 - 1·5% glycine and growth was inhibited at 2% concentration (Gordon & Gordon, 1943). Maculla and Cowles (1948) demonstrated bacterial lysis at high concentrations of glycine. Glycine was used to induce L growth in Salm. typhi, Salm. gallinarum, Salm. typhimurium, H. influenzae and H. pleuropneumoniae, (Dienes et al., 1950a; Dienes & Zamecnik, 1952; Diena, Wallace & Greenberg, 1964a; Neil et al., 1970; Want & May, 1975; Thomas, 1976). Some organisms were reported to be resistant to the action of glycine alone. Combination of glycine with penicillin

or cycloserine resulted in the transformation of some strains of streptococci (Michel & Hijmans, 1960; Madoff, 1970). Michel and Hijmans (1960) also showed that D-isomers of serine, threonine, methionine and alanine behaved like glycine when added to penicillin. Chatterjee and Williams (1965) produced Bacillus anthracis spheroplasts when lysozyme was added to glycine treated cells. When penicillin and glycine were combined the minimum inhibitory concentration of each was reduced (Want & May, 1975).

There were many reports of the successful use of lysozyme to produce bacterial protoplasts (Weibull, 1953; Zinder & Arndt, 1956; Gilby & Few, 1960; Schockman & Lampen, 1962; Madoff et al., 1967; Gooder, 1968; King & Gooder, 1970a). Weibull (1953) demonstrated that B. megaterium treated with lysozyme in the presence of a protective agent resulted in the release of protoplasts. Egg white lysozyme was most commonly used. Friemer et al., (1959) prepared streptococcal protoplasts by phage lysin of group C streptococci, phage lysin of group D was utilized to prepare streptococcal protoplast (Gooder, 1968).

Repaske (1956) reported lysis of E.coli, Pseudomonas and Acetobacter when lysozyme was used in combination with ethylenediaminetetra-acetic acid (EDTA). Neither of them alone caused appreciable lysis. To transform Gram negative bacteria to spherical forms EDTA (Fraser & Mahler, 1957; Spicer & Spooner, 1974; Weiss, 1976), or EDTA and trypsin (Weiss & Fraser, 1973) were needed in addition to lysozyme.

Bacteria could also be transformed to spherical forms by the action of serum. Immune serum and complement proved satisfactory in isolating Salm. typhi L forms and Vibrio cholerae spheroplasts (Dienes et al., 1950a; Muschel et al., 1959; Freeman, Musteikis & Burrows, 1963). Spherical forms could also be obtained by the action of normal serum (Michael & Braun, 1959; Davis et al., 1966). Serum was inefficient when it had been heated and complement destroyed (Muschel et al., 1959).

Lysostaphin is an enzyme first described by Schindler and Schuhardt (1964) which has a bacteriolytic action against staphylococci. It was used successfully for the induction of L colonies from staphylococci (Watanakunakorn et al., 1968, 1969; Marston, 1968). Low concentrations of carboxymethoxylamine produced L colonies of Salm. typhi (Dienes, 1949; Dienes et al., 1950a). Salm. typhi was also transformed when exposed to phage (Dienes & Weinberger, 1951). Deficiency of diaminopimelic acid caused transformation of E.coli to spherical forms not distinguishable from those prepared by penicillin (McQuillen, 1958a,b; Bayer, 1967). L colonies of Mycobacterium and Streptococcus were induced in synthetic vitamin-free medium (Mattman et al. 1960; Tunstall & Mattman, 1961). Ultraviolet radiation was reported to induce L transformation in Agrobacterium tumefaciens (Rubio-Hertos & Herrera, 1966). Bacillus subtilis L forms were induced in tryptone medium containing 7% sodium chloride (Burmeister & Hesseltine, 1968). Mahony (1973) induced Clostridium perfringens L

forms with bacteriocin. Stable L forms of Listeria monocytogenes were induced with rabbit alveolar macrophages (O'Beirne & Eveland, 1974).

Pleomorphism occurred when organisms were subjected to sublethal doses of lithium, calcium, chromium and mercuric salts (Dienes & Smith, 1944). Dienes (1949) and Dienes and Sharp (1956) were able to produce large bodies with subinhibitory concentrations of lithium, cadmium and mercury salts. These bodies were similar to those produced by penicillin but failed to develop into L forms. In Proteus, refrigeration of cultures, transfer either into tap water or ascitic fluid and antagonism between strains produced large forms (Dienes, 1949).

Strain variation.

Freshly isolated strains of Salmonella not passaged in artificial media were found to be better than old strains by Weinberger et al. (1950) who could not obtain L growth from an old strain of Salmonella. Godzeski et al. (1967) reported that virulent strains were less readily transformed to the L phase than less virulent strains. In Neisseria meningitidis L transformation occurred most readily in strains recently isolated from patients (Bohnhoff & Page, 1968).

Strains differ in their susceptibility to electrolyte concentration, type and concentration of inducing agent. Dienes and Sharp (1956) noted strain differences of susceptibility to salt concentration. Hijmans and Kastelein

(1960) reported that some strains of enterococci produced L colonies when treated with 16,000 units/ml penicillin while one strain needed 32,000 units/ml of penicillin to be transformed. Li et al. (1964a) obtained L forms of one strain of Proteus out of 21 with 200 units/ml penicillin, but other strains tested could not be transformed even with 1,000 units/ml. In group D streptococci, 17 out of 20 strains produced L forms with penicillin, one with glycine and none with lysozyme (Madoff et al., 1967). Seeberg and Brorson (1974) found that within the 19 strains of E.coli they studied, 4 proved nonproductive.

Induction in vivo.

Guze and Kalmanson (1964) and Kalmanson and Guze (1964) obtained Streptococcus faecalis protoplasts from the kidneys of rats infected with the organism and treated with penicillin. Intraperitoneal infection of mice with Salm. typhimurium, Proteus mirabilis, E.coli, Strep. faecalis and Staph. aureus followed by treatment with penicillin resulted in L phase variants (Godzeski et al., 1967).

Some investigators showed that L forms were induced in vivo under normal conditions as a result of host response. In 1952 Wittler detected pleomorphic forms or granules of Haemophilus pertussis in phagocytic cells when the organism was injected intranasally or intraperitoneally into normal or immunised mice. In an experiment adopted by Mortimer (1968) streptococcal L forms were isolated when the vegetative forms were inoculated intraperitoneally into mice. Salmonella enteritidis and

Salm. typhimurium L forms were isolated from mice livers experimentally infected with these Salmonellae (Kawakami, Ishibashi, Mitsuhash, Sakaino & Fukai, 1970).

Biochemical tests and physiological properties of L forms and revertants.

Dienes et al. (1950a) observed that sugar fermentation reactions of several L forms of Salmonella were similar and similar to those of the parent. All activities can be exhibited by wall-less organisms as the bacterial protoplasm is not affected by the absence of cell wall (Weibull, 1958). L forms of P. mirabilis and its parent gave similar results with glucose and sucrose fermentation and the ability to grow on media containing streptomycin and sodium azide (Medill-Brown, Hutchinson & Cocklin, 1960). Revertants of meningococci and Klebsiella pneumoniae were biochemically identical to their parents (Stewart & Wright, 1969; Guze, Harwick & Kalmanson, 1976). Hubert et al. (1971) found that revertants of Pseudomonas aeruginosa behaved in a similar way to the parent. Salm. gallinarum revertants exhibited similar fermentation reactions to the parent but they occurred more slowly than in the parent (Thomas, 1976).

Kagan and Levashov (1957) found that revertants of Salm. typhi were divided into 3 groups. Strains reverted from typical L forms were identical to the parent. Strains reverted from spheres and granular elements were different from the parent and decomposed sucrose and arabinose,

sugars that were not decomposed by the parent. The third group were revertants from spherical and granular elements, as in the second group, but they were biochemically inert. Simon and Yin (1970) reported that mannitol fermentation varied among Staph. aureus revertants. The type of antibiotic used in induction affected the result (Watanakunakorn & Bakie, 1973). The authors reported that 11 of the 22 strains of Staph. aureus revertants failed to ferment mannitol. All cycloserine and methicillin revertants lost the ability to ferment mannitol whereas vancomycin revertants retained this property.

L forms of motile organisms were non-motile (Weibull, 1953; Lederberg, 1956; Hubert et al., 1971). Lederberg (1956) reported that spheroplasts of Salm. typhimurium which showed bacillary projections retained their motility. Medill-Brown et al. (1960) observed that Proteus mirabilis L forms were motile. Diena et al. (1964a) studied the effect of the medium on motility. They reported that Salm. typhimurium spheroplasts, induced with glycine on media containing 0·5% glucose, were non-motile and non-flagellated while those induced on 1 - 2% glucose were flagellated and motile.

Salton (1955) observed that protoplasts of spore bearing B. megaterium were able to develop spores. McQuillen (1956) reported that protoplasts divide by budding and that they are capable of synthesising amino acids and protein but incapable of producing new cell wall material. On the other hand Weibull (1958) reported that

spheroplasts and protoplasts lost the ability to undergo sporulation, division and cell wall synthesis and only L forms had the ability to divide.

Investigation by Weibull and Hammarberg (1963) indicated that Proteus mirabilis cells exhibited the same catalase activity per mg bacterial dry weight as the L forms derived from the same bacterium. The L forms of a Staph. aureus strain and a diphtheroid strain lost their catalase and cytochrome activity (Weibull & Gyllang, 1965). A third strain of Proteus mirabilis L forms contained the same cytochrome as the normal proteus bacterium. Smith and Willis (1967) found that the L forms of Staph. aureus were catalase positive. L forms of Pseudomonas aeruginosa prepared by carbenicillin lost their cytochrome activity (Hubert et al., 1971).

Williams (1963) reported that Staph. aureus L forms retained their coagulase activity. Coagulase positive staphylococci tested by Simon and Yin (1970) lost this property after reversion from the L phase. The methicillin and cycloserine revertants of Staph. aureus produced coagulase, whereas vancomycin revertants lost this ability and regained it only after several passages (Watanakunakorn & Bakie, 1973). All the 9 revertants of Staph. aureus examined by Little et al. (1973) coagulated plasma but the stable L forms failed to do so even when undiluted plasma was used.

B. subtilis lysozyme-induced protoplasts retained the ability to synthesise the induced enzyme, arabokinase,

(Wiame, Stork & Vanderwinkel, 1955). Moreover, McQuillen (1955) reported that B. megaterium protoplasts synthesised protein, amino acid, nucleic acid and the induced enzyme β-galactosidase, but were incapable of synthesising DAP. The rates of synthesis were comparable with those of intact cells. Spheroplasts of Salm. typhimurium were capable of growing and forming β-galactosidase (Lederberg, 1956). Protoplasts of P. mirabilis were able to synthesise lipopolysaccharide and lipoprotein (Hofschneider & Martin, 1968).

Wiame et al. (1955) reported that B. subtilis protoplasts respired. Weibull and Beckman (1960) studied the metabolism of the small granules (0·1 - 0·3μm) found in stable Proteus L9 (Klieneberger-Nobel, 1956) culture. They found that the granules contained the enzymes succinic dehydrogenase and catalase. They also found that the granules were able to respire. Weibull and Gyllang (1965) reported that L forms of Proteus mirabilis, Staph. aureus and species of Cornybacterium respired less than the vegetative forms. However, respiratory quotients of L forms of Gram positive organisms were more depressed.

Simon and Yin (1970) noticed reduction of penicillinase synthesis by Staph. aureus L phase. On reversion to vegetative forms most of the strains showed reduction or loss of this enzyme. Penicillinase activity was retained by Staph. aureus revertants, but it could not be demonstrated in the stable L forms (Little et al., 1973). None of the methicillin or cycloserine Staph. aureus revertants

elaborated penicillinase (Watanakunakorn & Bakie, 1973).

Freimer et al. (1959) and Neil et al. (1970) reported that L forms of group A streptococci and Haemophilus pleumopneumoniae were haemolytic. Schmidt-Slomska, Sacquet and Caravano (1967) found that revertants of group A streptococci retained their haemolytic property. Retention of this property was demonstrated to be strain-dependent (Simon & Yin, 1970). Little et al. (1973) demonstrated haemolysis in their 3 stable Staph. aureus L forms. Alkaline phosphatase activity of the parent streptococci was 5 - 10 times that of the L forms (Lynn, 1962), while the activity of inorganic pyrophosphatase of L forms was 10 times that of the parent.

Clostridium tetani L forms were toxic to mice (Scheibel & Assandri, 1959). In a study with Salm. typhi, Diena et al. (1964b) reported that the toxicity of glycine-induced spheroplasts were reduced at least 10 fold as compared with the intact cells. Czop and Bergdoll (1970) showed that production of enterotoxin by Staph. aureus revertant depended on the type of toxin produced by the parent strain. All six strains producing type A toxin continued to do so, four of five produced type B and none of the three strains that produced type C continued to produce it. K. pneumoniae lost its virulence after being passaged 109 times with penicillin. Virulence of revertants was not restored by 40 intraperitoneal passages through mice (Guze et al., 1976).

Phage susceptibility of Proteus mirabilis L phase was

lost and restored on reversion (Bloss, 1962). Similar results were obtained with Staph. aureus (Little et al., 1973). Simon and Yin (1970) found that phage type varied among revertants. Schönfeld (1959) showed that revertants of Staph. aureus attacked by phage group 1 were also attacked by group III and IV. These results were similar to Watanakunakorn and Bakie (1973), who reported that revertants of Staph. aureus regained the phage type of the parent in addition to other phage typing patterns.

L forms of Staph. aureus retained the pigment of the coccal forms (Smith & Willis, 1967). Stable L forms of Serratia marcescens lost the pigment of the parent (Hubert, Potter, Kalmanson & Guze, 1969). Similar observations were made on Pseudomonas aeruginosa (Hubert et al., 1971). In contrast Bertolani et al. (1975) reported the presence of green pigment in L forms of Pseudomonas.

Revertants of Salm. typhi (Kagan & Levashov, 1957) and L forms of Salm. gallinarum strain 9S (Thomas, 1976) did not produce hydrogen sulphide as did the parents.

Serological properties of L forms.

L forms and their parents have been found to be serologically similar. Antiserum prepared against Streptobacillus moniliformis and its L forms agglutinated both organisms (Klieneberger, 1942). Dienes (1949) reported that serum prepared against Proteus L forms or the parent agglutinated both.L forms of Salm. typhi were serologically similar to the parent (Dienes et al., 1950a).

Lederberg (1956) demonstrated that Salm. typhi spheroplasts failed to agglutinate the homologous antiflagellar serum. Gooder and Maxted (1958) reported that antisera directed against streptococcal protoplasts were devoid of antibodies against cell wall components. It was postulated that L forms of Gram positive bacteria might lack the immunologically active substances of the parent forms (Freimer et al., 1959). This was confirmed by Hijmans (1962) who found that L forms of group D streptococci lost the cell wall polysaccharide and the group specific antigens. Furthermore, complement fixation and immunodiffusion tests carried out by Weibull and Gyllang (1965) showed that there was no relationship between L forms of staphylococci and corynebacteria and their parents. However, they obtained precipitation lines with an immune serum against Proteus L forms and the extracted toxin of the parent.

Loss of serological relationship between L forms and the parents was also observed in Gram negative bacteria. Sera prepared in rabbits against both bacillary and L forms of Proteus did not agglutinate L forms at such a high titre as the corresponding bacillary forms (Dienes et al., 1950b). They could not establish a serological relationship between the third L form and its parent. Weibull et al. (1967) also failed to establish a serological relationship between one Proteus L form and its parent. A close relationship was obtained with another two strains.

Crawford (1960) showed that complement fixing antibodies

from rabbits immunised with groups A and C streptococci reacted with an extract of the L forms of group A streptococci. Furthermore, serum from a patient recently infected with Streptococcus reacted with the extract. Salm. typhi spheroplasts were less agglutinated with H and O antisera compared with the parent form (Diena, Wallace & Greenberg, 1965). With fluorescent antibody and immunodiffusion techniques, L forms of H. pleuropneumoniae and its parent were found to be identical (Neil et al., 1970). The growth to streptococcal L forms was inhibited by the homologus antiserum (Madoff, 1970). Absorption of antiserum against L forms with vegetative forms of staphylococci removed all the antibodies while the antibacterial serum absorbed with L forms still agglutinated the bacterial forms (Dannis, Sharp & Marston, 1970). The L phase of N. meningitidis contained haemagglutinin which agglutinated rabbit and chicken red blood cells but not guinea pig, sheep or human red cells (Crawford, Lytle & Nalewaik, 1970; Crawford, Nalewaik, Lytle & O'Connell, 1971).

Sensitivity of L forms to antibiotics.

Agar diffusion (Taunbeneck, 1962; Kagan, 1968a; Watanakunakorn, 1971a; Mahony, 1973), turbidimetric (Søhockman & Lampen, 1962), agar dilution (Kagan, 1968a) and broth dilution (Watanakunakorn et al., 1968; Watanakunakorn, 1971a) methods have been used to study the susceptibility of spheroplasts, protoplasts and L forms to various antibiotics.

Results have shown that the action of some antibiotics on bacteria is different from that on derived L forms. Protoplasts and spheroplasts were found to be more resistant to penicillin, cephalothin and synthetic penicillins than the parents (Dienes & Weinberger, 1951; Freimer et al., 1959; Ward & Martin, 1962; Molander et al., 1964; Montgomerie et al., 1968; Watanakunakorn et al., 1968; Little et al., 1973; Gilpin, Young & Chatterjee, 1973). Unlike the parents, L forms were not susceptible to antibiotics that interfere with cell wall synthesis (Mortimer, 1968; Spicer & Spooner, 1974).

Staphylococcal L forms were not inhibited by vancomycin or ristocetin but they were inhibited by bacitracin (Williams, 1963). Rotta et al. (1965) and Kagan (1968a) found that streptococci and staphylococci and their L forms were equally inhibited by novobiocin. Ampicillin, cloxacillin, oxacillin and methicillin had no action on Strep. faecalis protoplasts up to a concentration of 10,000 μg/ml (Montgomerie et al., 1968). The authors also found that L forms were more sensitive to novobiocin, bacitracin, vancomycin, erythromycin, chloramphenicol, oleandomycin, kanamycin, neomycin, polymyxin, tetracycline and nitrofurantoin. Watanakunakorn et al. (1968) reported similar results with bacitracin and vancomycin. In contrast bacitracin (Kagan, 1968a), vancomycin (Kagan, 1968a; Gilpin et al., 1973) were found to be more active against bacteria than against the L phase.

Dienes and Weinberger (1951), Kagan (1968a), Gilpin

et al. (1973) and Mahony (1973) reported that chloramphenicol was equally toxic to L forms and parent forms. L forms from streptomycin-resistant strains were fully resistant to the drug (Freimer et al., 1959; Medill-Brown et al., 1960; Hewitt et al., 1968).

Susceptibility of spheroplasts of Staph. aureus to antimicrobial agents was not affected by the method of induction (Dienes & Weinberger, 1951; Michael & Braun, 1959; Sharp, 1960; Watanakunakorn et al., 1968). Ward and Martin (1962) observed that L forms derived from penicillin or cycloserine were equally sensitive to both drugs. Staph. aureus spheroplasts induced by cycloserine, methicillin or lysostaphin were equally sensitive to antibiotics (Watanakunakorn, 1971a). L forms of Cl. perfringens prepared by bacteriocin were resistant to penicillin (Mahony, 1973).

L forms were more sensitive to acriflavin and crystal violet than the bacterial forms (Dienes et al., 1950a). Spheroplasts and protoplasts were sensitive to distilled water (Friemer et al., 1959; Michael & Braun, 1959). L forms of enterococci were more susceptible to heat than the vegetative forms (Hijmans& Kastelein, 1960). The difference between sensitivities of protoplasts and parent strain was influenced by the constituents of the medium. Montgomerie et al. (1968) noticed that the difference was less marked in salt medium than in a medium containing sucrose. Watanakunakorn et al. (1968) observed a significant difference in the minimum inhibitory concentration

of tetracycline, methicillin and bacitracin when tests were carried out in ordinary brain heart infusion medium than when the medium containing 5% sodium chloride and 0·2% magnesium sulphate was used.

The role of bacterial variants in disease.

It is generally considered that L forms of bacteria are not pathogenic even when derived from pathogenic organisms. The disappearance of pathogenicity has been observed in Streptobact. moniliformis L forms (Heilman, 1941; Klieneberger-Nobel, 1951; Freundt, 1956), in Salmonella (Dienes et al., 1950a), in Cl. tetani (Dienes, 1950; Dienes & Weinberger, 1951) and in Staph. aureus (Young & Dahlquist, 1967).

Experimental infection with L forms of pathogenic bacteria revealed no clinical symptoms. Two strains of coli bacilli prepared by treatment with chloramphenicol and antiserum proved non-pathogenic for mice when introduced intraperitoneally, while the vegetative forms killed all the inoculated mice (Voureka, 1951a,b). Intravenous or intraperitoneal inoculation of L phase of different bacteria failed to initiate renal infection in experimental animals (Schmitt-Slomska et al., 1967; Clasener, Engering & Hijmans, 1970; Watt, 1970). The lack of pathogenicity by the intravenous route was ascribed to the rapid destruction of L forms by the suboptimal serum osmolality or serum bactericidal activity.

Alderman and Freedman (1963) postulated that E.coli

protoplasts could initiate infection in the hypertonic renal medulla. When they injected rabbit kidney medulla with these protoplasts experimentally they found that large numbers of protoplasts produced less visible inflammation than did smaller numbers of vegetative forms. Studies with E.coli variants demonstrated that the intracardial route failed to establish an infection in the kidney (Winterbauer, Gutman, Turck, Wedgwood & Petersdorf, 1967; Gutman, Winterbauer, Turck, Wedgwood & Petersdorf, 1968). Gutman et al. (1968) ascribed this failure to the rapid killing of the variants or to the fact that the variants did not reach the kidney in sufficient numbers to cause the disease. Trials to initiate infection by inoculating E.coli directly into the renal medulla failed (Gutman et al., 1968).

Godzeski et al. (1967) reported that embryonated chicken eggs inoculated with L forms of Staph. aureus showed no evidence of infection. No positive cultures of the injected L forms were recovered when allantoic fluid, yolk sac material and homogenised embryo tissues were examined. Little and Bosberg (1973) reported that an L form isolated from a pathogenic strain of Staph. aureus lost the pathogenicity of the parent form, since injection of L forms into the bovine udder provoked no clinical mastitis. Linnemann, Watanakunakorn and Bakie (1973) obtained similar results when the same organism failed to produce the damage to the endocardium which the parent did when injected intravenously into rabbits. Direct

intramedullary inoculation of the stable L phase of the Staph. aureus failed to result in the colonization of normal or hydronephrotic rat kidney (Watanakunakorn & Bakie, 1974).

The stable L forms of N. meningitidis lost the virulence of the parent strain for mice (Bohnhoff & Page, 1968). L forms of Listeria monocytogenes proved non-pathogenic when injected into mice by various routes (Brem & Eveland, 1968) and Pseudomonas aeruginosa L forms were less virulent than the parent (Bertolani et al., 1975).

Scheibel and Assandri (1959) found that L phase variants of 4 toxigenic strains of Cl. tetani isolated using penicillin were capable of producing tetanus in mice. L forms of the Cl. tetani induced by glycine were fatal to mice and produced symptoms of tetanus in guinea pigs (Rubio-Huertos & Gonzalez-Vasquez, 1960). Administration of H. parainfluenzae protoplasts, intratracheally caused enzootic pneumonia in pigs (McKay, Abelseth & Vandreumel, 1966). Histological lesions resembled those of PPLO or viral infection rather than the bronchopneumonia type caused by the vegetative forms. Kagan,G.Y.(1968) demonstrated that intravenous or paratonsillar injection of streptococcal L forms produced angina in monkeys and some caused myocardial lesions. Pyelonephritis in rats was produced by inoculating penicillin or lysozyme induced L forms of E.coli K12 intravenously or directly into the renal medulla (Higuchi, 1969). Urease-producing Proteus L forms produced stones in the urinary bladder of rats injected

intramedullary (Braude, 1970). In primary pig kidney cell tissue culture the cytopathic effect of H. pleuropneumoniae L forms was less pronounced compared with the vegetative forms (Neil et al., 1970). Cell wall defective variants of streptococci and pneumococci produced myocarditis and endocarditis in mice (Merline, Gordon & Mattman, 1971). Orr, Tamarind, Finchman, Hawley, Quilliam and Irving (1974) found that L forms of Streptococcus faecalis were capable of producing granulomatous lesions in the rabbit intestine. Listeria monocytogenes stable L forms retained their pathogenicity to lambs when injected intravenously or intratracheally (Kotlyarova, Bakulov, Chevelev, Dymina & Prozorovskii, 1976).

Studies showed that L transformation could take place under natural conditions in vivo. These forms were demonstrated in a number of disease conditions which seemed negative by the usual bacteriological procedures. L-like cultures were isolated from the nasopharynx of rats with lung lesions (Klieneberger & Steabben, 1937). Dienes and Smith (1944) isolated L forms of Bacteroides from purulent lesions. A micro-organism with acid-fast and non-acid fast forms and with a polymorphic developmental pattern was isolated from blood and tumours from animal and human cancer patients (Alexander-Jackson, 1954). By means of fluorescent-antibody technique (Mattman & Karris, 1966) reported L variants in the spinal fluid in aseptic meningitis in all of the 8 cases tested. They isolated L forms from 4 out of the 8 cases examined,

3 cases of H. influenzae with typical and variant forms and one case with L variant colonies without classical bacterial forms. Pease (1967) isolated Listeria in a mycoplasma phase from blood of 49 healthy subjects. L forms of Pseudomonas and Staphylococcus were isolated from cases of cervical adenitis and cystic fibrosis of the pancreas respectively (Kagan, 1968b). Mattman (1968) isolated L forms of the tubercle bacillus from a case of pulmonary tuberculosis. A case of subacute bacterial endocarditis which failed to improve with penicillin therapy revealed Corynebacterium acne transitional forms (Zierdt & Wertlake, 1969). Pleomorphic organisms which gave rise to Listeria monocytogenes were isolated from brain lesions (Charache, 1970). L forms of Streptococcus agalactiae were isolated from an outbreak of mastitis in cattle (Wilson, Little & Roberts, 1970).

L forms detected in blood from disease conditions include alpha-haemolytic streptococci, staphylococci, pneumococci, E.coli, Corynebacterium and Micrococcus (Wittler, Malizia, Kramer, Tuckett, Pritchard & Baker, 1960; Godzeski, Brier, Griffith & Black, 1965; Mattman, 1968). Tunstall and Mattman (1961) failed to obtain growth in repeated cultures from blood and spinal fluid of subacute bacterial endocarditis on ordinary media. A few streptococci, large numbers of spheroplasts and pleomorphic cocci were isolated on a specially defined medium. L forms of Strep. faecalis were isolated on defined medium from two patients with septicaemia

(Mattman & Mattman, 1965). One of the patients also had the same L forms in the spinal fluid. Bacterial L forms were isolated from rheumatic heart samples (Godzeski, 1968). Barile, Francis and Graykowski (1968) and Charache (1968) reported the isolation of pleomorphic organisms from disease cases. These organisms reverted to pure streptococcal forms on subculture. Sixteen blood samples which were negative on routine examination, revealed Pneumococcus, Staph. aureus, Strep. faecalis, Diplococcus and E.coli when subcultured into L form media (Brem, 1969). Twenty blood samples from septicaemia cases were negative by routine cultures (Chattman, Mattman & Mattman, 1969). Sixteen of them proved positive for L forms demonstrated by growth in liquid medium, increased DNA content and acridine orange staining. Tedeschi, Amici and Paparelli (1969a) and Tedeschi and Amici (1972) detected mycoplasma-like forms and L forms in erythrocytes from healthy human blood. Tedeschi, Amici and Paparelli (1969b) provided evidence of metabolism of nucleosides in erythrocytes which they suggested might be related to the presence of L forms. Pease (1969, 1970) claimed the presence of L forms in the erythrocytes and joint fluids of arthritic cases. Pohlod, Mattman and Tunstall (1972) demonstrated bacterial variants in circulating erythrocytes of 57% of healthy individuals they examined.

The hypertonic environment of the urine made it a suitable medium for the survival of variants. A patient treated with penicillin developed Proteus protoplasts in

the urine (Braude, Siemienski & Jacob, 1961). Gutman, Turck, Petersdorf and Wedgwood (1965) and Swierczewski and Reyes (1970) demonstrated L forms or spheroplasts in urine from patients with chronic pyelonephritis or urinary tract infection. Gutman, Schaller and Wedgwood (1967) isolated Proteus L forms during ampicillin treatment from a case of relapsing urinary tract infection. The incidence of infected urine containing osmotically fragile forms was found to be 5% (Braude, Siemienski & Lee, 1968). Kalmanson and Guze (1968) demonstrated protoplasts in kidneys of 25 patients who had had surgical biopsies. Seventy one per cent of the patients with protoplasts had histological evidence of pyelonephritis.

Part II: Isolation of Bacterial L Forms in Vitro

Introduction

Early reports of L growth arising spontaneously from Streptobacillus moniliformis (Klieneberger, 1935) led to investigations of such growth in other bacteria. These investigations showed that incorporation of certain antibiotics that act on the cell wall in the presence of hypertonic media favoured production of L forms (Dienes & Sharp, 1956; Ward & Martin, 1962; Rotta et al., 1965; Roberts, 1967, 1968; Watanakunakorn, 1971a,b).

In addition to antibiotics other agents appeared to have L inducing properties. Gordon and Gordon (1943) described changes in bacteria arising from the action of glycine. Dienes and Zamecnik (1952) reported on the L inducing ability of glycine and other amino acids. They isolated L colonies of Salmonella typhimurium and Haemophilus influenzae with DL-methionine, L-phenylalanine and L-tryptophan. The additive effect of antibiotics and amino acids in inducing L forms has been reported (Michel & Hijmans, 1960; Madoff, 1970). Transformation of Gram positive bacteria to protoplasts by the action of lysozyme has been demonstrated (Weibull, 1953; Gooder, 1968; King & Gooder, 1970a). Gram negative bacteria were reported susceptible to lysozyme only in the presence of EDTA (Repaske, 1956; Fraser & Mahler, 1957; Spincer & Spooner, 1974; Weiss, 1976) or EDTA and trypsin (Weiss & Fraser, 1973).

The isolation of L forms requires complex media

supplemented with soft agar and serum. The osmotic fragility of the L forms leads to the use of hypertonic medium provided with a suitable stabiliser (Sharp, 1954; Dienes & Sharp, 1956; Weibull, 1958). Medill and O'Kane (1954) claimed that complex media inhibited L growth and they described a synthetic medium which they found satisfactory in isolating Proteus L forms. Mattman et al. (1960) and Tunstall and Mattman (1961) confirmed the successful use of synthetic media in isolating bacterial L forms in the absence of inducing agents. Tissue culture media was used for induction of Brucella L elements (Hatten & Sulkin, 1966) and group A streptococci (Schmitt-Slomska et al., 1972).

L forms can be induced by various methods. The inducing agent can be deposited in a trough cut into the agar to provide gradient diffusion (Sharp, 1954; Friemer et al., 1959; Hijmans& Kastelein, 1960; Roberts & Wittler, 1966; Roberts, 1968). Gradient plates have been produced also by depositing an osmotically stabilized medium on a tilted plate, followed by another layer of medium containing the inducing agent after the first layer has solidified (Hubert et al., 1971). Antibiotic paper discs placed on inoculated plates have been used (Abbate, Leonessa & Altucci, 1973). Ward and Martin (1962) deposited the inducing antibiotic in wells made in the inoculated plates. Uniform antibiotic plates have been used by a number of workers (Marston, 1968; Hubert et al., 1971; Watanakunakorn, 1971a).

Freimer et al. (1959) reported reversion of streptococcal L forms upon the removal of penicillin. L forms of a staphylococcal strain which was passaged 200 times on penicillin medium reverted after only one subculture on penicillin-free medium (Schönfeld, 1961). Other strains tested reverted after the composition of the penicillin-free medium had been changed e.g. by replacing sodium chloride by sodium or potassium phosphate or ascitic fluid by horse serum or gelatin and peptone. When streptococcal lysozyme-induced protoplasts were inoculated into a suitable medium L forms developed which converted to stable L forms shortly after isolation (Gooder, 1968).

Transformation of Actinobacillus to round forms was observed (Kim, 1976). Dienes and Weinberger (1951) reported that two strains of Pasteurella isolated from the nasal cavities of rabbits were transformed into large bodies in the presence of penicillin, but L type colonies were not observed. In this study different strains of A. equuli, one strain of A. suis and 5 strains of P. multocida were tested for their ability to transform and propagate as L growth. The action of some antibiotics on the vegetative forms and their ability to induce L growth was compared under similar environmental conditions. The L forms inducing property of glycine and lysozyme was also tested. The effect of different types of media, stabilisers, sera, different agar concentrations and the optimum temperature of induction, propagation and colony morphology were studied.

The term round form used in this study refers to the spherical forms appearing in the bacterial culture without indicating the amount of cell wall present. The use of the term L forms or L growth indicates growth composed of L elements and carries no implication of the capacity or lack of capacity of the L forms to revert to the vegetative forms.

2 - Materials and Methods

Media.

(i) Alexander-Jackson agar (AJA).

This medium was first described by Alexander-Jackson (1954). In this work a modification of the medium was used and consisted of the following:-

Myosate	(BBL - Bioquest Division)	3g
Gelysate	(BBL - Bioquest Division)	3g
Phytone	(BBL - Bioquest Division)	3g
Trypticase	(BBL - Bioquest Division)	3g
Glucose	(M & B)	5g
Glycerol	(Sigma)	40 ml
Sodium chloride		15g
Yeast extract	(Difco)	10g
$MgSO_4$ $7H_2O$	(BDH)	2·0g
Agarose	(Sigma)	4·0g
Digest broth		1 L

The pH was adjusted to 7·4. Glucose was prepared separately, Seitz-filtered and added to the rest of the mixture which had been autoclaved at 121°C for 15 minutes. Horse serum which had been inactivated at 56°C for 30 minutes was added to a final concentration of 10% prior to pouring the medium into plates.

(ii) Alexander-Jackson sucrose agar (AJSA).

This medium was as above except that 150g sucrose (BDH) replaced sodium chloride.

(iii) Alexander-Jackson broth (AJB).

This medium was prepared as (i) except that agarose was omitted.

(iv) A medium was prepared as (i) except that yeast extract was omitted.

(v) Charcoal agar (CA).

This medium was a modification of that used by Lorkiewicz (1957) and it contains the following:-

Sodium chloride		15g
Glucose)	(M & B)	5g
$MgSO_4$ $7H_2O$	(BDH)	2g
Agarose	(Sigma)	4g
Activated charcoal	(BDH)	5g
Digest broth		1 L

The pH was adjusted to 7·4 and the medium was sterilized by autoclaving at 121°C for 15 minutes. Glucose was prepared and added separately as in AJA.

(vi) Serum agar (SA).

This medium was made as in (v) with the exception that 10% inactivated horse serum replaced the charcoal.

(vii) Brain heart infusion agar (BHIA).

This has a basis of Brain heart infusion broth (Difco), a dehydrated medium which was reconstituted as directed and was supplemented with sodium chloride (1·5%), $MgSO_4$ $7H_2O$ (0·001%), yeast extract (0·5%) and agarose (0·4%). The pH was adjusted to 7·4 and the medium autoclaved at 121°C for 15 minutes. Inactivated horse serum was added as in AJA.

(viii) Synthetic medium.

This medium was used by Tunstall and Mattman (1961). It is as follows:-

Sucrose	(M & B)	10%
Dextrose	(M & B)	0·1%
Sodium lactate	(BDH)	2%
$K_2 HPO_4$		0·9%
Noble agar	(Difco)	0·8%
Salt mixture		0·2%
Vitamin free Casamino acid	(Difco)	0·5%

Salt mixture per litre

$MgSO_4 7H_2O$	40g
NaCl	2g
$FeSO_4$	2g
$MnSO_4$	8g

Agar was prepared in distilled water at double strength and autoclaved separately. All other ingredients were prepared in double strength solution with pH 7·2 and Seitz filtered. The two were mixed in equal quantities and poured into plates.

(ix) Tryptone Soy broth (Oxoid).

This medium was reconstituted as directed. pH was adjusted to 7·2 and the medium autoclaved at 121°C for 15 minutes.

Tryptone soy agar was prepared by adding sodium chloride (1·5%), $MgSO_4 7H_2O$ (0·001%), and agarose (0·4%) to the broth.

(x) Reversion media.

AJA, CA, AJB and nutrient broth free of antibiotics were used as reversion media.

Inducing agents.

(i) D-cycloserine (Sigma) was used for induction and propagation of L forms. The drug was kept desiccated at -20°C. Dilutions were made in sterile distilled water just before use.

(ii) Sodium Benzylpenicillin:(Glaxo Laboratories).

(iii) Ampicillin: injectable preparation (Beecham Research Laboratories).

(iv) Cloxacillin: injectable preparation (Beecham Research Laboratories).

(v) Bacitracin: (Sigma).

(vi) Vancomycin: (Sigma).

(vii) Novobiocin: (Sigma).

All antibiotics were in powder form. With the exception of cycloserine all other antibiotics were kept at 4°C. Dilutions were carried out in sterile distilled water before use.

(viii) Glycine: A laboratory grade (BDH-Analar) was used. Concentrations used were added to AJA before the medium was autoclaved.

(ix) Crystalline white egg lysozyme: (Sigma). This ingredient was stored at -20°C. It was prepared in 10% solution and Seitz filtered.

Other ingredients.

(i) Ethylenediaminetetra-acetic acid (Hopkins & Williams). The disodium salt was used. It was prepared as a 4% solution in distilled water, adjusted to pH 8 and sterilized by filtration.

(ii) Tris buffer (BDH). 0·1M Tris (hydroxymethyl) amino methane was prepared in distilled water. 15% (w/v) sucrose was added. pH was adjusted to 8 and the mixture was sterilized by filtration.

(iii) Serum - Horse, calf and swine serum were prepared by clotting the blood. Serum was drained, centrifuged at 3,000 r.p.m. for 20 min. to remove cells and Seitz filtered. It was dispensed in 100 ml aliquots and stored at -20°C. When needed, serum was thawed and inactivated at 56°C for 30 min. in a thermostatically controlled water bath. Serum was added to the melted medium at a temperature of 50°C.

Source of strains.

Eleven strains of Actinobacillus equuli and one strain of Actinobacillus suis were used. One strain of A. equuli was from the National Collection of Type Culture (N.C.T.C.) and designated as NCTC 8644. The other strains were from the departmental collection. These are designated B82/68, FS1144/68, M618/70, G14/3/68, DV5 outside 2, 773/68, B967/68, CCM5500, DV10 outside 3, B345/69 and the A. suis was designated P63/8/75.

Five strains of Pasteurella multocida were included.

Three strains were isolated from haemorrhagic septicaemia conditions and they were designated 17, 21 and 31. The other two were designated 6L and F. The source and site of isolation of each strain are presented in Table I.

All the strains were grown in cooked meat medium (Oxoid) for 24 hours at 37°C and then kept at 4°C. Subcultures were done every two weeks. For the tests the organisms were grown in nutrient broth for 18 - 20 hours at 37°C.

Methods.

Unless otherwise indicated, all incubations were carried out at 30°C. Agar plates showing no growth were incubated for two weeks before they were considered negative.

Induction of L growth with cycloserine.

For Actinobacillus strains different concentrations of cycloserine were added to AJA and AJSA to final concentrations of 3, 4, 5, 8 and 10 µg/ml. Concentrations of 5, 8, 10, 12 and 15 µg/ml cycloserine were added to SA and CA. The concentration which produced the maximum L transformation on induction plate and supported propagation was used in this study. Pasteurella strains were tested the same way using cycloserine concentrations of 80, 90, 100 and 110 µg/ml added to AJA, AJSA and CA.

Cycloserine was added to give a final concentration of 5 µg/ml in AJA or AJSA for A. equuli and 10 µg/ml for A.suis. In SA and CA all Actinobacillus strains were grown in 10 µg/ml.

Table I

Sources and sites of isolation of the strains used in the present study

Strain No.	Host	Site of isolation
B82/68[1]	Horse	
FS1144/68[1]	Pig	Joint
M618/70[1]	Pig	Endocardium
G14/3/68[1]	Foal	Blood
NCTC 8644[1]	Foal	Kidney abscess
DV5 outside 2[1]	Horse	Endotracheal tube
773/68[1]	Colt	Mouth swab
B967/68[1]	Horse	Endotracheal swab
CCM5500[1]	Horse	Joint
DV10 outside 3[1]	Horse	Endotracheal swab
B345/69[1]	Horse	Endotracheal swab
P63/8/75[2]	Piglet	
17[3]	Cattle	Blood
21[3]	Cattle	Blood
31[3]	Cattle	Blood
6L[3]	Pig	Lung
F[3]	Pig	Lung

1 = *Actinobacillus equuli* strain

2 = *Actinobacillus suis* strain

3 = *Pasteurella multocida* strain

Pasteurella strain 6L was grown on 90 µg/ml on AJA, AJSA and CA and the others were grown on 100 µg/ml. Plates were inoculated with 0·1 ml of 18 hour broth cultures and incubated. Subcultures were done every 48 hours by the agar block technique. A block of agar containing L colonies was placed on a fresh plate with the block facing downwards. The fresh plate was then smeared with the block which was then left on the plate.

Drug concentration was increased as subcultures continued. The antibiotic was also used in a gradient plate. Plates containing AJA were inoculated with an 18 hour broth culture of Actinobacillus or Pasteurella. 1,000 µg/ml were deposited in the trough cut into the agar to allow gradient diffusion. Plates were incubated for 48 hours and subcultures made by the agar block technique.

Induction of L growth with different osmotic stabilisers.

This experiment was done to find out the suitable stabiliser, the range and optimum stabiliser concentration necessary for isolation and propagation of Actinobacillus. Various concentrations of sodium chloride (3,2, 1·5 and 1%) and the osmotically equivalent amounts of sucrose (22, 17, 14 and 10%) were added to AJA. Plates containing 5 µg/ml were inoculated with A. equuli and plates containing 10 µg/ml were inoculated with A. suis and strain CCM5500. The plates were incubated for 48 hours. Propagation for 3 serial passages was carried out.

Effect of temperature on L growth.

The effect of temperature of incubation on L transformation was studied. AJA plates containing 5 µg/ml cycloserine were inoculated with strain FS1144/68 and DV5 outside 2. Plates of each strain were incubated at 30°C and 37°C and examined after 24 and 48 hours incubation for microscopic and macroscopic growth. Visible colonies were stained with Dienes' dry stain (see p.67). Films were stained by either Giemsa or Gram methods. Plates were subcultured on fresh antibiotic plates and each was incubated at the temperature of the first plate.

Effect of serum on L growth.

Transformation of Actinobacillus to L growth in the absence of serum and in the presence of different sera was tested. Plates were prepared as follows:-

a) AJA plates containing 5 µg/ml cycloserine and 10% inactivated calf serum.
b) AJA plates containing 5 µg/ml cycloserine and 10% inactivated pig serum.
c) AJA plates containing 5 µg/ml cycloserine and 10% inactivated horse serum.
d) AJA plates containing 5 µg/ml cycloserine and no serum was added.

All plates were incubated for 48 hours. Subcultures

were made from each plate onto similar freshly prepared plates. Growth on those plates containing no serum was subcultured onto two media, one like the induction plate and the other containing 10% inactivated horse serum.

Effect of yeast extract on L growth.

To test for the inhibitory effect of yeast extract, Actinobacillus equuli strains were induced and propagated on AJA and AJA without yeast extract containing 5 µg/ml cycloserine. Propagation was carried out to the 5th passage. The average of two experiments was taken.

Effect of different agar concentrations on L growth.

The effect of agar concentration on colony morphology was tested. Agarose was added to AJB to give final concentrations of 0·4, 0·6, 0·8, 1·0, 1·5, 2·0 and 2·5%. All the plates contained 5 µg/ml cycloserine for A. equuli FS1144/68 and 10 µg/ml for A. suis P63/8/75. The test organisms were grown in nutrient broth at 37°C for 18 hours. Growth was then diluted 1:5 with fresh nutrient broth and 0·1 ml was used as the inoculum. Plates were incubated for 48 hours.

Induction of L growth in AJB.

Eight strains of Actinobacillus and two strains of Pasteurella were tested for their L transformation in AJB. The broth was distributed in 9 ml aliquots in MacCartney bottles. 1 ml broth culture was added.

The final concentration of cycloserine was 8 µg/ml for Actinobacillus and 100 µg/ml for Pasteurella. Subcultures were attempted 48 hours later by transferring 1 ml of culture into AJB containing the same cycloserine concentration. 0·1 ml of culture was transferred to AJA containing 5 µg/ml and 8 µg/ml cycloserine for Actinobacillus and to plates containing 90 and 100 µg/ml cycloserine for Pasteurella. Plates were incubated at 37°C for 14 days.

Osmotic fragility of L forms.

The osmotic fragility of L forms was tested. Strain FS1144/68 was grown in 30 ml AJB containing 5 µg/ml cycloserine. After 48 hours incubation at 30°C the growth was shaken and 5 ml of the broth culture was deposited into each of three centrifuge tubes and centrifuged at 1,500 r.p.m. for 5 minutes. The supernatants were discarded and the deposits suspended in 5 ml respectively of AJB, nutrient broth and distilled water. Samples from each were plated on AJA containing 5 µg/ml cycloserine after 0, 5, 30 and 45 minutes at room temperature. To ensure rapid absorption of sample drops, the agar plates were previously dried, inverted with lids off for one hour at 37°C. A pasteur pipette was calibrated to deliver 0·02 ml/drop. The base of the plate was divided into 6 and labelled with the appropriate suspension used, sampling time and dilution. At each sampling time L growth was diluted with sterile saline. Dilutions were agitated before sampling. Using a dropping pipette

two separate drops of each dilution were plated. The pipette was washed out 3 times in boiling water before moving to the next suspension fluid and then 3 times in the highest dilution to be plated. Inoculated plates were allowed to dry on the bench and incubated at 30°C for 48 hours in an inverted position.

Induction of L growth with penicillin.

Penicillin was used to induce L forms of Pasteurella strains 6L and F on AJA or BHIA. Plates containing 0·2 units/ml were inoculated with 18 hour nutrient broth cultures. 1,000 units/ml penicillin were also deposited in the trough to allow gradient diffusion. Gradient plates were also prepared by another method. 5 ml of the medium containing 2 units/ml penicillin were poured on the plate in a tilted position. After the agar was solidified the plate was set flat on the bench and another 5 ml of the medium was added. The plate was inoculated with the test organism after the agar was solidified. The plate was incubated and subcultures made by the agar block technique.

Induction of L growth on synthetic medium.

All the Pasteurella and one of the Actinobacillus (FS1144/68) strains were tested for L growth induction on synthetic medium. The organisms were grown in tryptone soy broth overnight at 37°C. Growth was centrifuged at 1,500 r.p.m. for 5 min. and washed twice in sterile saline

solution. The supernatant was discarded and the deposit was suspended in 1 ml saline. Plates containing synthetic medium were inoculated with 0·1 ml culture and tilted in various directions to assure inoculum distribution. Plates were sealed to prevent drying and incubated at 37°C for 14 days. This was repeated with penicillin. 1,000 units/ml were deposited in the troughs made on the inoculated plates.

Induction of L growth with synthetic penicillins.

Ampicillin and Cloxacillin were used for all of the Actinobacillus and Pasteurella strains. The gradient technique was chosen. 5,000 µg/ml of the antibiotics were deposited in the trough in AJA.

Induction of L growth with other antibiotics.

Bacitracin, novobiocin and vancomycin were used separately as inducing agents in AJA. 0·1 ml of 5,000 µg/ml bacitracin, 0·1 ml of 10,000 µg/ml novobiocin and 0·1 ml of 150,000 µg/ml vancomycin were deposited in troughs made on inoculated plates.

Induction of L growth with glycine.

Different concentrations of glycine were added to AJA medium to final concentrations of 0·5, 0·4, 0·25, 0·15, 0·12 and 0·05% for Actinobacillus. For Pasteurella strains, 0·6, 0·5, 0·4, 0·3 and 0·15% were tested. Plates were incubated for 14 days.

Different concentrations of glycine and penicillin were added to AJA to facilitate the isolation of Pasteurella L forms. The following concentrations were tested:

0·2% glycine + 0·1 units/ml penicillin.
0·2% " + 0·01 " " "
0·1% " + 0·1 " " "
0·1% " + 0·01 " " "
0·05% " + 0·1 " " "
0·05% " + 0·01 " " "
0·025% " + 0·1 " " "
0·025% " + 0·01 " " "

Induction of L growth with lysozyme.

Actinobacillus and Pasteurella strains were subjected to the action of lysozyme. The test strains were grown in 5 ml tryptone soy broth overnight at 37°C. Cells were centrifuged at 1,500 r.p.m. for 5 min. and washed twice with 0·1M tris (15% sucrose). The sediment was then suspended in 8 ml tris (15% sucrose) at 37°C. 1 ml lysozyme containing 1,000 µg/ml was added slowly with continuous shaking. Cultures were kept at 37°C for 10 min. 1 ml EDTA was added slowly and cultures were returned to 37°C. Growth was subcultured on AJA and tryptone soy agar containing 1·5% sodium chloride.

Tests for spontaneous transformation.

Actinobacillus strains were tested for spontaneous transformation. Strains FS1144/68, DV5 outside 2 and

B967/68 were inoculated on blood agar, AJA, nutrient broth and AJB. Inoculated media were incubated overnight at 37°C, and stained preparations were examined. Plates were then left on the bench to age at room temperature. Gram stained preparations were made every 2 - 3 days. When round forms appeared growth from blood agar and AJA was transferred to fresh plates. The broth media were filtered through Millipore membrane filters 0·22 um (Millipore Ltd., U.K.). Blood agar plates and AJA were inoculated with the filtrate and they were all incubated with the filtrate at 37°C for 14 days. Revertants of the same strains were inoculated on blood agar and AJA and left on the bench to age as the parents.

Reversion of L growth to vegetative forms.

L growth was propagated on AJA, CA, AJB and nutrient broth without antibiotics or any other inducing agent. A block of AJA or CA containing large numbers of L colonies was streaked on reversion plates. Similar blocks were immersed in AJB and nutrient broth. Media were incubated at 37°C and 30°C.

Staining methods.

Dienes' Stain: This method was recommended by Dienes (1967a) for staining colonies in situ. A block of agar about 1 cm^2 containing L colonies was removed from the plate with the growth facing upward to a clean microscope slide using a clean razor blade. The agar

block was supported by a second slide held perpendicular to the first along one edge of the block. A thin layer of the block bearing the growth was removed from the surface by sliding a razor blade parallel to the first slide. This layer was then placed with the growth downward on a stained (see below) and preheated coverslip. The stained coverslip, with the piece of agar, was then placed on a microscope illuminator fitted with a 60 W lamp, the preparation covered with a strip of filter paper moistened with water and dried between two other strips and with two dry filter papers which were weighed down with microscope slides. The preparation was kept for 30 minutes on the lamp and the filter papers were then removed and the preparation mounted on a clean slide on Farrant's medium (George Curr Ltd., England). A second preparation was made after fixation of the growth by placing a filter paper with a few drops of 40% formaldehyde in the lid of the plate containing L colonies. The plate was then incubated at 37°C for one day.

Staining of coverslips.

Two loopfuls of 2% aqueous toluidine blue were mixed on a clean coverslip with two loopfuls of 20% solution of tartaric acid. After being dried the coverslip was rubbed gently with a piece of filter paper to speed crystallisation of tartaric acid. The stained coverslip was then stored in a clean glass petri dish at 50°C.

Feulgen's staining.

The method of Robinow (1956) was used. Smears of L forms were prepared on a clean microscope slide and air dried. One slide was first treated with 1NHCl for 5 minutes at 60°C and stained with Giemsa stain for 10 - 15 minutes. The slide was washed with buffer (pH 7·0) which was left on the slide for 2 minutes for the stain to differentiate. The control slide was stained in the same manner, without acid treatment.

Giemsa staining.

The rapid method was used. Smears were air dried and fixed with methyl alcohol for 3 minutes. Slides were then flooded with the stain which was a mixture of 1 part stain and 10 parts buffer (pH 7·0) for one hour. Smears were washed with the buffer, which was left on the slide for 2 minutes, and then air dried.

Gram staining.

Films were fixed by heating. Slides were flooded with methyl violet for one minute. The stain was washed with Gram's iodine which was left on the slide for one minute. The excess iodine was removed and the films were decolourised with 100% acetone. The slides were washed with water and flooded with dilute carbol fuchsin for 30 seconds. Preparations were finally washed with water, blotted and air dried.

3 - Results

Induction of L growth with cycloserine.

L growth on AJA.

When cycloserine was used in AJA, Actinobacillus strains produced marked and thick growth after 24 hours incubation. This growth adhered to the medium. Granular confluent growth with dark patches was seen under the lowest power of light microscope (X4). Plates were reincubated and subcultures were done 24 hours later.

On subculture different strains produced different types of colonies. Strains FS1144/68 and P63/8/75 produced in most cases colonies having the fried egg appearance. They have dark centres and faint peripheral zones. Such colonies are referred to as typical or classical L type colonies (plates 1 & 2). Manipulation with a loop showed that the core was growing down into the agar. The colony spreads on the surface beyond the burrowing centre. Strains G14/3/68, NCTC8644, 773/68, DV10 outside 3 and B345/69 produced dark colonies having irregular boundaries and they were not differentiated into core and periphery. These colonies are referred to as granular L type colonies (plate 3). Strain DV5 outside 2 and B967/68 produced smooth colonies having the appearance of bacterial colonies. The granular colonies were of two types. The small type measured 0·5 mm in diameter and colonies usually coalesced in heavily inoculated areas. The

large colonies reached 4 mm in diameter, were lobulated and rarely coalesced. Agar penetration was not marked or even inhibited when plates were moistened and growth was mainly confluent. Isolated well defined colonies were detected when plates were dried for a time at 37°C before being inoculated.

Strains FS1144/68, G14/3/68, DV5 outside 2, B967/68 and P63/8/75 propagated readily. With strains NCTC8644, B345/69 and 773/68, although they produced numerous L colonies on induction plates and on first subculture, the growth was lost on several attempts.

The other non-growing strains B82/68, M618/70 and DV10 outside 3 grew only on induction plates and not a single subculture was achieved. In some cases growth took place only between the two pieces of agar.

Increasing the drug concentration from 5 µg/ml to 8 µg/ml strain G14/3/68 produced typical L colonies. This growth was lost by contamination after being propagated for more than 10 times. Attempts using the same technique to reisolate these forms were unsuccessful. Strain FS1144/68 and P63/8/75 were lost after being passaged 20 times. Strain DV5 outside 2 was transferred to media containing 8 µg/ml on which it continued growing for 6 passages and was then transferred to media with 10 µg/ml. The need to increase the concentration of cycloserine continued gradually until 20 µg/ml was reached on the 150th passage. Strain B967/68 was able to grow on 40 µg/ml on the 80th passage and 120 µg/ml

on the 180th passage. The last two strains were the only strains continued on this medium.

Transfer to a higher concentration resulted in a decrease in number of L colonies and then an increase afterwards with the colonies more of the typical type. Strain CCM5500 was highly mucoid, it was resistant to 5 µg/ml cycloserine and slightly transformed when 10 µg/ml was used. Growth on AJA was irregular. On subculture bacterial growth was produced which did not continue beyond the third passage.

Four strains of Pasteurella produced granular L colonies on 100 µg/ml of cycloserine but strain 6L was able to grow on media containing 90 µg/ml. Colonies appearing after 4 days of incubation were large, having a diameter of 3 mm. Propagation was poor and did not occur beyond the third passage.

Structural elements in L colonies of all organisms in all media tested were identical when examined by phase contrast microscopy in wet preparations or by light microscopy in stained preparations. Films of L growth stained either by Gram or Giemsa showed different morphological elements.

The round forms made up the majority and appeared in different sizes. The small round forms measured 0·7 - 1·5 µm in diameter, the large round forms reached the size of 12·0 µm and the medium round forms were in between in size. The round forms were dense and deeply stained. The boundaries of the small round forms were

sharper than those of the larger forms. Inside the large round forms one could see dense structures filling the entire body. In some cases the whole peripheral zone of the round form was dark. These dense structures were not homogenous but were made up of granules which stained deep purple with Giemsa stain.

The large bodies were usually found in old cultures. They reached 12-15 µm in diameter and contained granules. Their boundaries were irregular and they were faintly stained. Non-staining areas usually appeared in these bodies. Occasionally dark round objects resembling the small round forms were present inside them (plate 4). In young cultures the number of these large bodies was small.

The internal granules were roughly circular. They were observed within the cytoplasm of the large round forms and large bodies and they were unevenly distributed. They measured 0·1-0·5 µm in diameter and sometimes reached the size of small round forms. The external granules were found outside the round forms and they varied in size and staining intensity.

The round forms of all sizes, the large bodies and the granules were referred to as L elements or L growth.

Besides the L elements other forms were also visible. Filaments with bulges or multiple swellings, branched filaments, swollen bacillus forms and irregular forms were present. Irregular strands and short filaments of variable diameter were seen. These forms were referred

to as transitional elements (plates 5, 9, 10 & 14). When the transitional elements or bacillus forms appeared with L elements the growth was referred to as heteromorphic growth. Pink thread-like structures appeared in Gram stained preparation. These threads seemed to be a result of disruption of L elements.

Actinobacillus strain NCTC8644 inoculated into AJA produced normal bacterial colonies (plate 6). Stained preparations of the colony showed small round forms and granules (plate 7). On blood agar the organisms of the same strain were highly pleomorphic showing coccobacilli, rods and short filaments in addition to the granules (plate 8).

L growth on AJSA.

Both genera produced rough lobulated colonies with irregular boundaries. On subculture the whole colony was removed leaving an impression on the old plate. Growth was poor on first passage and propagation was not successful.

L growth on gradient plates.

Surrounding the antibiotic trough there was a zone of a few centimeters of complete inhibition of growth. The L growth was identified along the border between bacterial growth and the inhibition zone. L elements were visible on Gram-stained preparations and wet preparations examined by the phase contrast microscope.

Subcultures were poor and continued to the tenth passage and L colonies were lost several times.

L growth on CA.

Induction plates of this medium contained 10 µg/ml of cycloserine. Drug concentration was subsequently increased gradually, at a rate depending on the strain. When the drug concentration was below the minimum inhibitory concentration (MIC) of the strain, transitional elements and bacillary forms emerged. Strain FS1144/68 produced very long winding filaments with terminal, subterminal or central strongly stained swellings (plates 9 & 10) with the rest of the filaments faintly stained. The ends of two adjacent rods appeared to swell and come together resulting in a fusiform structure (plate 10). In strain CCM5500 two or three organisms came together and were interconnected to make a circle or a triangle (plate 11). Circles were also made by the winding filaments, some of the loops coalescing (plates 10 & 13). The same strain produced filamentous forms with dense particles situated along the filament at irregular intervals giving the appearance of a string of beads (plate 12). Some of the filaments were broken into bacillary and coccal forms (plate 13). Branching filaments were not uncommon (plates 5 & 13) as also are multiple swellings along the filament (plate 14). On increase of the drug concentration only L elements appeared. Two to three passages on the same concentration

resulted in the appearance of transitional forms. After many passages on high cycloserine concentration, normal bacillary forms (resistant forms) appeared. This was common in haemorrhagic septicaemia strains. All the strains tested were readily induced. All the plates supported heavy growth except for strains B82/68, M618/70 and B345/69 which produced moderate growth. Colonies were minute, about 0·3 mm in diameter and usually coalesced. The growth was whitish and more abundant compared with serum plates. The charcoal medium was inconvenient for colony examination under the light microscope or for Dienes' stain. Thus only films stained with Giemsa or Gram were done. The round forms and large bodies were more abundant and larger than those on AJA. The size of the large bodies of strain B967/68, P63/8/75, F and 6L reached 20 um in diameter. Some of the round forms had small coccal forms attached to the periphery or the round form bulged on one or more sides. These structures were more clear in wet preparations examined by phase contrast than in stained preparations (plate 15). Subcultures with the agar block technique were successful. The surface growth was abundant and easily removed. Growth on the first passage was small compared with induction plates but became profuse on the second passage. Cycloserine concentration was increased following the appearance of bacillus or transitional forms with L elements. Resistance of the strain was directly proportional to the

number of passages (Fig. 1). Pasteurella strains grew on concentrations higher than those of the Actinobacillus. A comparison between AJA and CA in supporting L growth of different strains is shown in Table II.

L growth on SA.

Actinobacillus strains were transformed to thin sheets of L growth with 10 µg/ml cycloserine. Growth was poor on propagation and only rarely did isolated L colonies appear. As in CA an increased concentration of cycloserine was necessary. This medium was discontinued.

Induction of L growth with different osmotic stabilisers.

None of the Actinobacillus strains grew on 3% NaCl with the exception of strain FS1144/68 which produced a poor growth which did not grow on subculture. Strains FS1144/68, G14/3/68, NCTC8644, DV5 outside 2, B967/68 and P63/3/75 produced L growth on 2% NaCl. A concentration of 1·5% was found to be optimum for both induction and propagation. On this concentration strains M618/70 and CCM5500 gave irregular results with the absence of growth on many occasions. At 1%, with the exception of strain FS1144/68 and DV10 outside 3, growth was of the heteromorphic type Table III.

A sucrose concentration of 22% which was equivalent to 3% NaCl supported the transformation of all the strains tested. Growth on this concentration was a thin sheet of L elements. Isolated colonies were large, reaching

Strain(FS 1144/68)passaged on cycloserine charcoal agar medium.

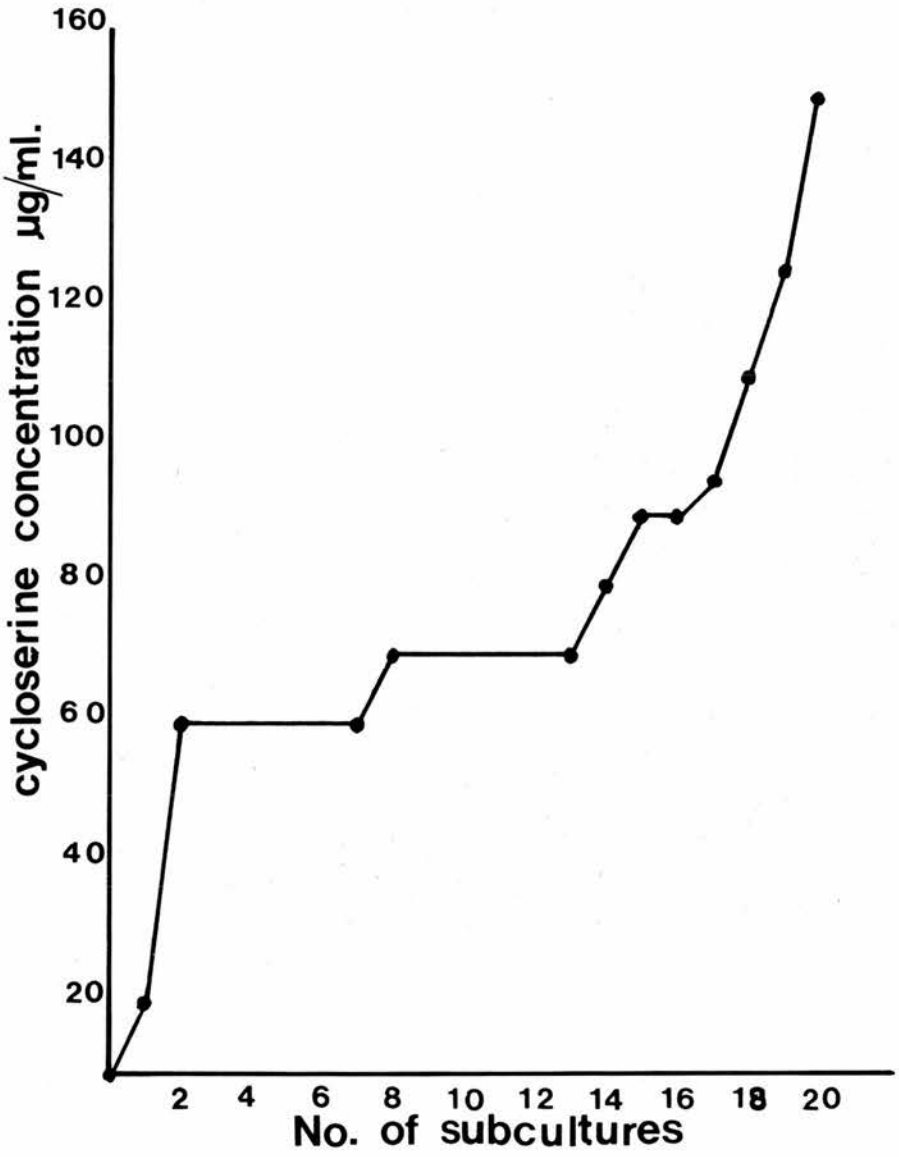

Fig.1

Correlation between cycloserine & No. of subcultures.

Table II

A comparison between Alexander-Jackson agar (AJA) and charcoal agar (CA) media in supporting Actinobacillus L growth

Strain No.	No. of subcultures on AJA	No. of subcultures on CA	Strain No.	No. of subcultures on AJA	No. of subcultures on CA
B82/68	0	> 118	773/68	2	4
FS1144/68	> 20	> 36	B967/68	> 210	> 30
M618/70	0	> 116	CCM5500	2	3
G14/3/68	> 13	> 98	DV10 outside 3	0	2
NCTC 8644	2	3	B345/69	2	2
DV5 outside 2	> 219	> 30	P63/8/75	> 20	> 35

Table III

Growth of *Actinobacillus* on Alexander-Jackson agar (AJA) containing 5 µg/ml cycloserine and different concentrations of sodium chloride

Strain No.	Concentrations of NaCl			
	3%	2%	1·5%	1%
B82/68	-	-	+L	+H
FS1144/68	+L	+++L	+++L	+++L
M618/70	-	-	++L	+++H
G14/3/68	-	+++L	+++L	+++H
NCTC 8644	-	+L	++L	+++H
DV5 outside 2	-	+++L	+++L	+++H
773/68	-	-	+++L	+++H
B967/68	-	++L	+++L	+++H
*CCM5500	-	-	++H	+++H
DV10 outside 3	-	-	+L	+++L
B345/69	-	-	+L	+++H
*P63/8/75	-	++L	+++L	+++H

\- = no growth
\+ = up to 4 colonies/plate
++ = moderate growth
+++ = good growth
L = L growth
H = heteromorphic growth.

* Strains CCM5500 and P63/8/75 were grown on AJA containing 10 ug/ml cycloserine.

the size of 4 mm diameter, and were lobated with more irregular edges than those on NaCl medium. On propagation the whole colony transferred to the fresh plate leaving only its impression on the original plate. Transitional elements appeared on 10% sucrose plates. Propagation on all concentrations was poor and never went beyond the second passage.

Effect of temperature on L growth.

Following 24 hours incubation at 37°C plates showed visible confluent growth. Examination with the X4 objective of the light microscope revealed granular growth with some dark areas. When stained films were examined, they showed round forms, large bodies and granules together with transitional elements. Long filaments, swollen rods and round forms at the ends of the rods were present. After incubation for 48 hours, growth was more pronounced and the round forms increased in size. Plates incubated at 30°C showed no visible growth after 24 hours incubation. Microscopically there were small scattered foci of granular growth. After 48 hours incubation, growth was visible being confluent and thick. Stained preparations showed round forms and granules with very few transitional elements, mainly swollen short rods. On subculture, plates incubated at 37°C produced isolated L colonies having a diameter of 2 mm. At 24 hours plates incubated at 30°C showed pin point colonies that enlarged, reaching 0·5-1·0 mm after a further 24 hours incubation.

Stained preparations again revealed more transitional elements on plates incubated at 37°C than on those at 30°C. Because of this, incubation at 30°C was chosen for subsequent experiments.

Effect of serum on L growth.

All plates containing serum supported Actinobacillus L growth. Strains B82/68, M618/70, B967/68, CCM5500 and DV10 outside 3 grew occasionally on serum-free medium.

L transformation with pig and horse serum was good but propagation with both sera was difficult. Calf serum supported a thin sheet of granular L growth. Strains FS1144/68 and P63/8/75 produced typical L colonies that grew on subculture. Gram or Dienes stained preparations of L growth from calf serum plates showed that the extracellular granules were the dominant feature (plate 16). Growth on serum-free plates did not occur when subcultured on the same medium and when subcultured on horse serum plates, growth was very poor, not more than 3 colonies/plate. As in the calf serum plates, the extracellular granules were the dominant feature.

Effect of yeast extract on L growth.

Transformation to L elements was not affected by the absence of yeast extract. On subculture growth was less attached to the medium compared to that with yeast extract. On the second passage growth was a thin sheet of L elements. The isolated colonies were 2-3 mm in diameter and of the

granular type. Some colonies of strain B967/68 showed dark patches at the periphery. The dark areas extended towards the centre and were made up of ring-like structures, some of which were incomplete (plate 17). Generally, growth was poorer on medium not containing yeast extract than when yeast extract was added. Results are presented in Table IV.

Effect of different agar concentrations on L growth.

Colony size was slightly affected by agar concentration. On 0·4% agarose L colonies were small having a diameter of 0·5-1·0 mm, while on 1·5% the size was about 1-5 mm. Examination with the light microscope X4 objective showed scattered elements at the periphery of the L colony. The core was small and very marked. Almost all of the colonies were of the classical L type. On 0·6% agarose the colonies were still showing the woolly appearance with the core less marked and slightly enlarged. The peripheral morphology was the same. On 0·8% agarose strain P63/8/75 produced smooth, glistening colonies with the core completely lost. The edges were smooth, resembling those of normal bacterial colonies. Strain FS1144/68 lost the classical appearance on 1·5% agarose and the morphology remained the same till the last concentration tested. Manipulation with a loop proved that there was more growth burrowing into the medium on 0·4% than on higher concentrations of agar.

Using Dienes' dry stain classical L colonies on 0·4%

Table IV

A comparison between Alexander-Jackson agar (AJA) with and without yeast extract in supporting Actinobacillus equuli L growth

Strain No.	No. of subcultures		Strain No.	No. of subcultures	
	with yeast extract	without yeast extract		with yeast extract	without yeast extract
B82/68	1	0	773/68	3	0
FS1144/68	5	3	B967/68	5	0
M618/70	0	0	CCM5500	2	0
G14/3/68	5	3	DV10 outside 3	1	2
NCTC 8644	3	0	B345/69	1	2
DV5 outside 2	5	3			

agarose stained very well while the smooth colonies were hard to stain. The structural elements of colonies on all concentrations were the same. They were composed of small, medium and large forms and granules. Transitional elements appeared on 1·5% and on higher concentrations. Granules were numerous on 0·4% agarose compared with other concentrations.

Induction of L growth in AJB.

Broth cultures incubated 24 hours showed thick mucoid strands at the bottom of the bottle. Phase contrast showed small round forms in the region of 1·4 µm diameter. Intracellular and extracellular granules were rarely seen. Twenty four hours later the round forms had increased in size reaching 2·4 - 3 µm. Some of them exhibited coccoid bulging at one or more sides. The extracellular granules were abundant. Strains M618/70, NCTC8644, DV10 outside 3 and G14/3/68 underwent complete transformation. In strains B82/68, DV5 outside 2, B345/69 and CCM5500 and in the Pasteurella strains 21 and 6L, transformation was not complete. Transitional elements, mainly swollen rods, were present. Growth in subcultures in broth was poor and mainly made up of transitional elements.

When broth cultures of L forms were plated on solid media different strains behaved in different ways. The heteromorphic growth of strain DV5 outside 2 failed to grow on medium containing 5 or 8 µg/ml cycloserine. Strains NCTC8644 and B345/69 did not grow on plates

containing 8 µg/ml. Strains B82/68, M618/70, G14/3/68 and DV10 outside 3 produced L growth on medium containing 8 µg/ml. With the exception of strain NCTC8644 all the strains produced heteromorphic growth on 5 µg/ml. Pasteurella strains failed to grow on both concentrations tested. The result is summarised in Table V.

Osmotic fragility of L forms.

There was a sudden drop in number of L colonies when samples from distilled water were plated on AJA containing 5 µg/ml cycloserine. The number dropped suddenly from 1,600 to 200 colonies/ml in 5 minutes. L colonies disappeared after 30 minutes. In the case of nutrient broth there was a drop followed by an increase and the number reached 150,000 colonies/ml in 45 minutes. In AJB the number of L colonies dropped after 30 minutes and increased at the end of the experiment. Stained preparations from nutrient broth cultures showed faint round or oval objects which stained only at the periphery (plate 18). Transitional elements and a few bacillary forms were present in nutrient broth and AJB at the end of the experiment. Results are presented in Table VI.

Induction of L growth with penicillin.

Pasteurella strains were transformed to L growth on AJA and BHIA both containing 0·1-0·2 units/ml penicillin. Typical L colonies were rarely seen and growth was poor and never went beyond the third transfer. On gradient

Table V

Type of growth following induction of Actinobacillus equuli L growth in Alexander-Jackson broth (AJB) and its propagation on Alexander-Jackson agar (AJA)

Strain No.	AJB containing 8 µg/ml cycloserine	AJA containing 5 µg/ml cycloserine	AJA containing 8 µg/ml cycloserine
B82/68	H	H	L
M618/70	L	H	L
G14/3/68	L	H	L
NCTC 8644	L	L	-
DV5 outside 2	H	-	-
B345/69	H	H	-
DV10 outside 3	L	H	L
CCM5500	H	H	H

L = L growth

H = heteromorphic growth.

Table VI

Osmotic fragility of Actinobacillus equuli L forms shown by the L colony count following exposure to different suspending media for varying time intervals

Suspending medium	Time of exposure			
	0 min.	5 min.	30 min.	45 min.
	No. of L colonies/ml			
AJB	100,000	100,000	1,000	50,000
Nutrient broth	1,200	1,000	2,500	150,000
Distilled water	1,600	200	No growth	No growth

Count of initial suspension = 200,000/L colonies/ml.

plates there was no growth adjacent to the trough or to the high concentration of the antibiotic. Results on trough plates were similar to those of Actinobacillus on AJA cycloserine plates. Pasteurella L growth was identified along a very narrow zone between the bacterial growth and the inhibition zone. Growth was confluent, granular and composed of L elements. On the other gradient plate L growth was confined to a narrow zone. When isolated colonies were obtained, the number decreased towards the higher concentrations. Subcultures on gradient plates were not successful.

Induction of L growth on synthetic medium.

Microscopic L colonies were present after 24 hours incubation at 37°C. Tiny macroscopic colonies appeared after 5 days made up of small peripheral zones and dark centres. Structural elements were not visible when Gram's stain was used. Dienes' dry stain was not successful. Examination of wet preparations revealed round forms of the small and medium size but large round forms and large bodies were absent. Growth on subculture was not achieved. Results were irregular and visible growth was not obtained on some occasions. No improvement resulted when 1,000 units/ml penicillin were deposited in the troughs.

Induction of L growth with synthetic penicillins.

Results on gradient plates were similar to those

obtained with penicillin. Growth on the narrow border between the inhibitory zone and the bacterial growth was made up of L elements. Growth on subculture was very poor. Pasteurella strains were more sensitive to these antibiotics than the Actinobacillus strains.

Induction of L growth with other antibiotics.

Growth on the border between the bacterial growth and the inhibition zone was thick and confluent. Growth on this border under the light microscope X4 objective resembled that of bacteria. Stained or wet preparations showed bacterial forms. Very few small round forms were seen. No transformation took place when plates were further incubated.

Induction of L growth with glycine.

Transformation to L elements was observed on low concentrations of this amino acid. Strains M618/70, 773/68, B967/68, CCM5500 and DV10 outside 3 were resistant to a 0·05% concentration. Other strains produced heteromorphic growth composed of L elements, transitional elements and bacillus forms. The bacillus forms decreased in number with increasing glycine concentration. Strains B82/68, DV5 outside 2, B967/68, CCM5500 and DV10 outside 3 did not undergo complete transformation at any of the concentrations tested. A concentration of 0·3% was bactericidal to all the Actinobacillus strains. Results are summarised in Table VII.

Table VII

Induction of *Actinobacillus* L growth on Alexander-Jackson agar (AJA) containing different concentrations of glycine

Strain No.	Concentrations of glycine				
	0·05%	0·1%	0·12%	0·15%	0·3%
B82/68	H	H	H	-	-
FS1144/68	H	H	H	L	-
M618/70	B	H	H	L	-
G14/3/68	H	L	-	-	-
NCTC 8644	H	H	L	-	-
DV5 outside 2	H	H	H	-	-
773/68	B	H	L	-	-
B967/68	B	H	H	-	-
CCM5500	B	H	H	-	-
DV10 outside 3	B	H	H	-	-
B345/69	H	H	L	-	-
P63/8/75	H	H	H	L	-

- = no growth
L = L growth
B = bacillus growth
H = heteromorphic growth

Two types of L colonies were produced. The tiny colonies measured 0·3 - 1 mm, stained well with Dienes' dry stain and their growth was mainly subsurface with little or no surface growth. The large colonies reached 2-4 mm and were difficult to stain. They had greater surface growth, irregular boundaries and were easily removed from the plate leaving pits. All the colonies were of the granular type and made up of similar elements. Propagation was attempted without success.

Pasteurella strains were transformed at a concentration higher than that of the Actinobacillus strains. Three strains underwent complete transformation on 0·5% glycine. Bacillus forms disappeared on increasing glycine concentration. Strain 6L was transformed to L elements on 0·3%, a concentration which produced little change in the other strains. The colonies appearing late (6 days) were large (4-5 mm in diameter), made up of heteromorphic growth, easily removed from the agar and difficult to stain with Dienes' dry stain. Results are summarised in Table VIII.

Different combinations of glycine and penicillin both at subinhibitory concentrations did not improve the growth or make propagation of Pasteurella L growth possible. Induction with these combinations was inferior to glycine or penicillin alone.

Table VIII

Induction of *Pasteurella multocida* L growth on Alexander-Jackson agar (AJA) containing different concentrations of glycine

Strain No.	Concentrations of glycine				
	0·15%	0·3%	0·4%	0·5%	0·6%
17	B	B	H	L	-
21	B	B	H	L	-
31	B	B	H	L	-
6L	H	L	-	-	-
F	B	H	L	-	-

- = no growth

B = bacillus growth

L = L growth

H = heteromorphic growth.

Induction of L growth with lysozyme.

The effect of lysozyme on the morphology of bacteria was clear after 30 minutes at 37°C. Examination with phase contrast showed round forms having the size of 4-6 µm, some of them in groups. Filamentous forms were the dominant feature in strain CCM5500. The resistant bacillary forms of Actinobacillus were always present. Pasteurella strains suspended in 100 µg/ml lysozyme transformed to round forms with less bacillary forms. Gram or Giemsa stain resulted in a few small round forms interconnected with thread-like material. However, the best picture was given with wet preparations examined under the phase contrast microscope X100 objective. No growth was obtained when cultures were plated on AJA or tryptone agar containing 1·5% NaCl.

Tests for spontaneous transformation.

Agar and broth cultures of Actinobacillus incubated at 37°C for 24 hours showed on Gram's stain short filaments, rods, coccobacillus forms and granules. Colonies on blood agar and AJA were sticky and difficult to remove completely. Broth cultures formed a sediment in the bottom of the tube. Smears from blood agar and AJA cultures left on the bench for some days were the same as those incubated overnight except that the organisms were faintly stained. After 10 - 12 days round forms, threads of irregular diameter, faintly stained rods and coccobacillus forms were present. Strain DV5 outside 2

gave almost pure round forms of different sizes and some of them were well demarcated and deeply stained (plate 19). Subcultures of these on blood agar or AJA failed to grow. There was no evidence of growth on blood agar or AJA inoculated with filtrates of AJB and nutrient broth cultures and the filtrates remained sterile after 14 days incubation. Revertants of the same strain left on the bench at room temperature gave rise to round forms similar to those of the parents and these did not grow on subculture.

Reversion of L growth to vegetative forms.

L growth induced on AJA or CA produced bacterial growth when subcultured on medium free of inducing agent. On AJA incubated at 37°C, reverting colonies were larger than the L colonies and the centres were less marked. The peripheral areas were large and the boundaries were made of filamentous organisms. Stained films of colonies from AJA or CA incubated at 37°C showed curved rods, short filaments and bacillary forms. Colonies on plates incubated at 30°C were smaller than those at 37°C and consisted of curved rods, transitional elements similar to those seen in L transformation and rods attached to round forms. Small round forms were also present. In broth media the round forms were enlarged and faintly stained. Curved rods and filamentous forms were present. In Pasteurella round forms with tails were seen. Some of the round forms of strain DV5 outside 2 were enlarged

and each contained between one and six brightly stained rod forms (plate 20). None of the round forms were present in broth incubated at 37°C for 48 hours, while in those cultures at 30°C the round forms persisted. Transitional elements and round forms completely disappeared on the third transfer on reversion media incubated at 30°C. Newly reverted growth did not, or hardly even grew on ordinary agar medium. At 30°C growth was very slow and colonies were attached to the medium.

Reversion was also observed in old L cultures (4 days old). The round forms at the peripheries of the colonies were replaced by normal rods and filaments. The colony size was enlarged with the centre less marked. Stained preparations showed L elements, bacillary forms and transitional elements.

Dienes' stain.

Differentiation of the L colony into core and periphery was marked in small colonies (0·5 mm). The centre was made up of small round forms and granules. Towards the periphery the round forms appeared in different sizes and differing affinities for the stain, the smaller the round form the deeper was the staining (plate 21). The edge of the colony was made up of faintly stained large bodies with the space between them greatly increased in the peripheral area. The extracellular and intracellular granules appeared as dark stained particles. Transitional elements, if present, appeared between the

round forms. Staining without fixation gave better results with more deeply stained elements and taking less time than with fixation although it was not permanent and lasted only for a short time.

Feulgen's stain.

Round forms and large bodies were visible on the control slide. They were reddish in colour with internal dark purple granules. The extracellular granules were deeply stained. Some of the round forms in the HCl treated slide stained faint pink but the majority of these forms did not take the stain. The dark purple granules were very clear especially in those round forms which did not take the stain. The granules appeared singly, in pairs, or in groups. In most cases they were together and were interconnected with thin threads of the same colour as the granules. Some of the granules were large reaching the size of the small round forms (plates 22 & 23).

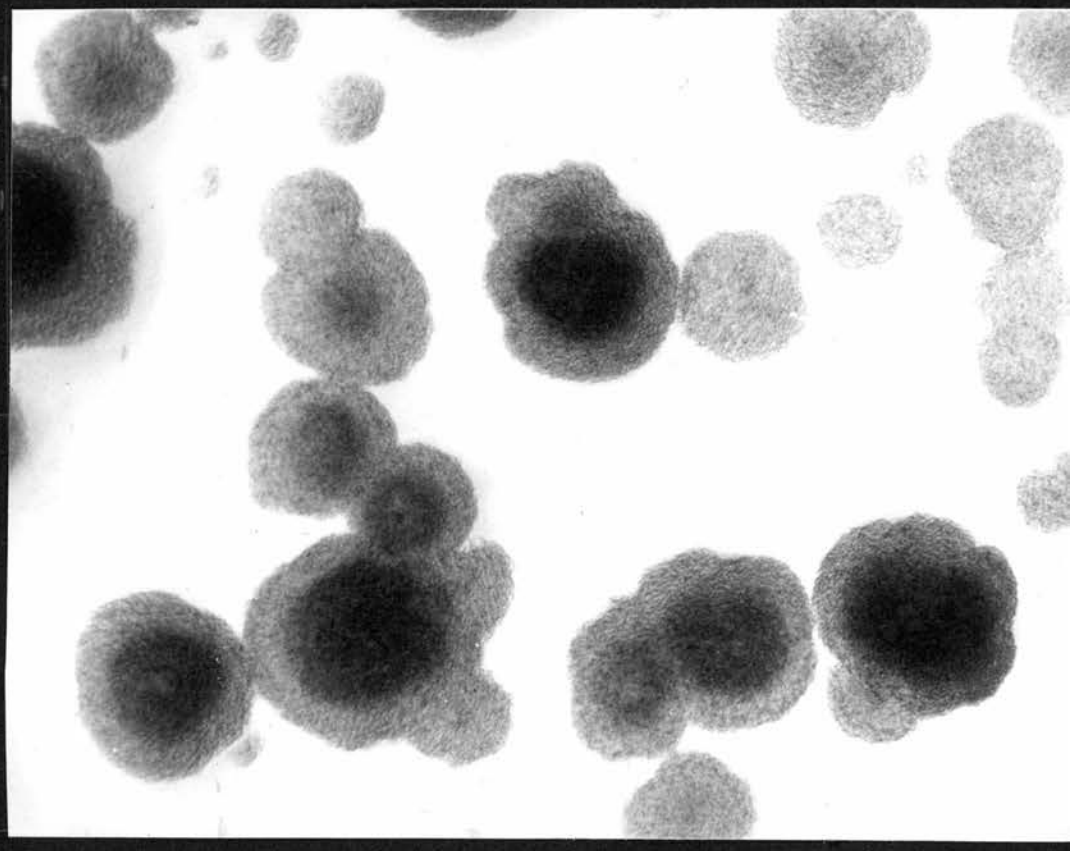

Plate 1

Actinobacillus equuli strain FS1144/68 on AJA containing 5 µg/ml cycloserine showing typical L colonies with deep centres and light peripheries X 72·5.

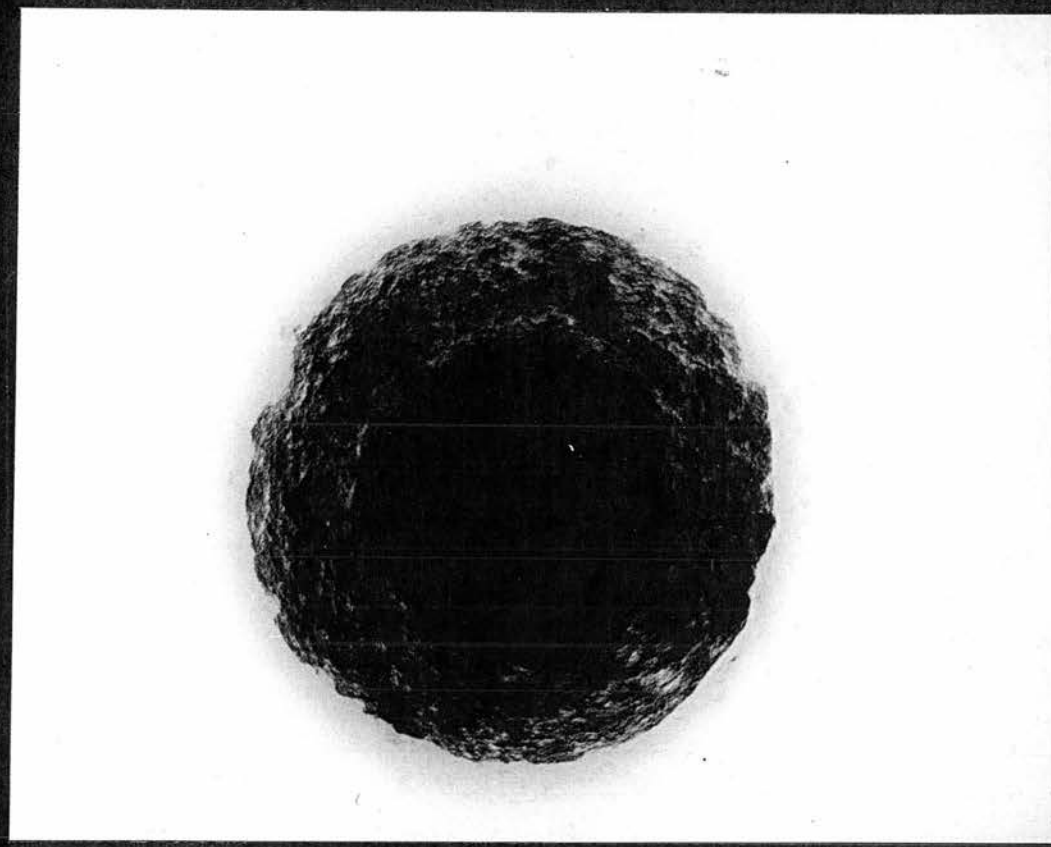

Plate 2

Four days old typical L colony of *Actinobacillus equuli* G14/3/68 on AJA containing 8 µg/ml cycloserine X 72·5.

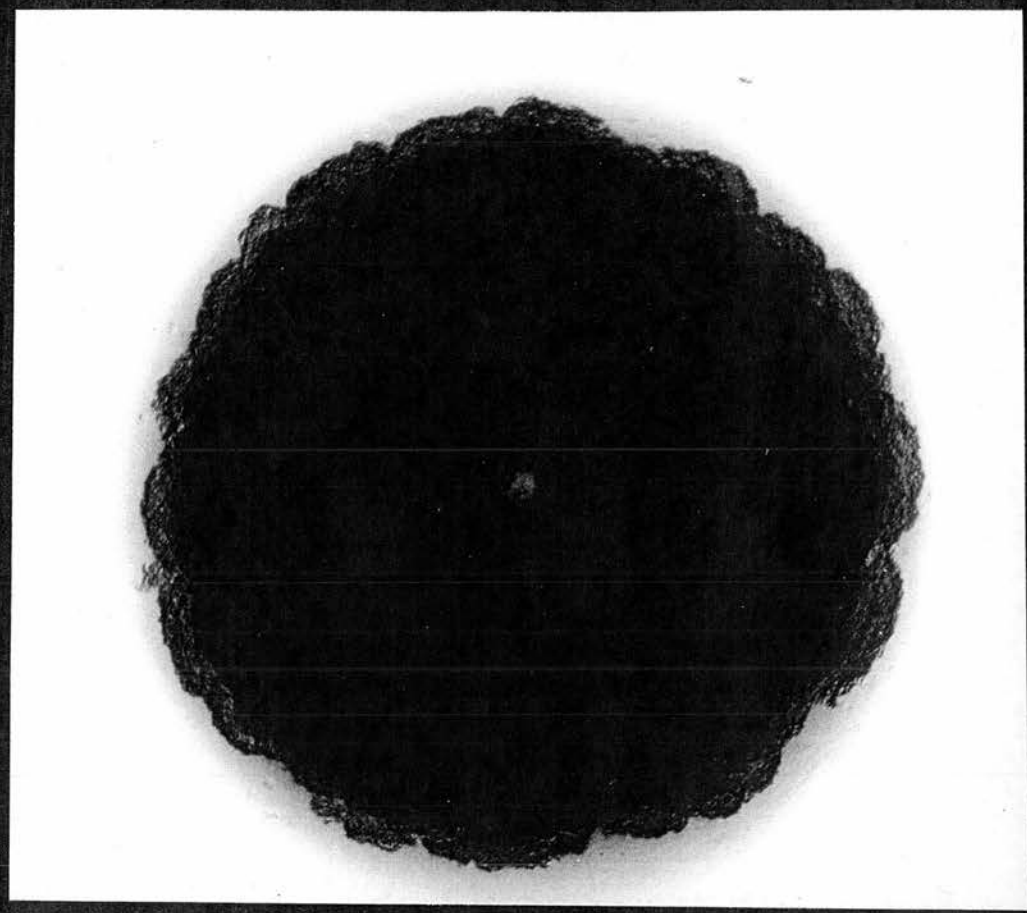

Plate 3

Four days old granular L colony of Actinobacillus equuli strain G14/3/68 on AJA containing 5 µg/ml cycloserine. Note the absence of differentiation into core and periphery X 72·5.

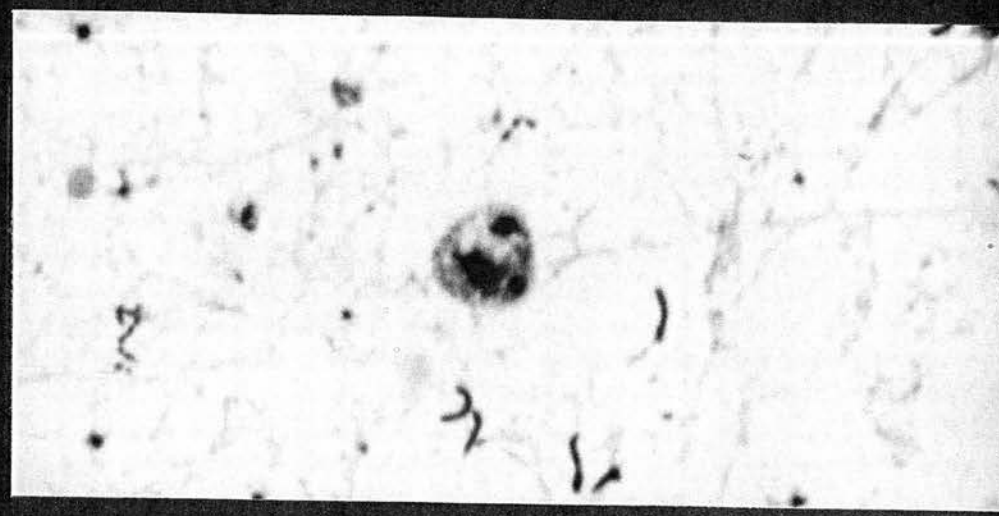

Plate 4

Actinobacillus equuli strain B345/69 on AJA containing 5 µg/ml cycloserine showing a large body with round forms and unstained areas inside. Gram X 1560.

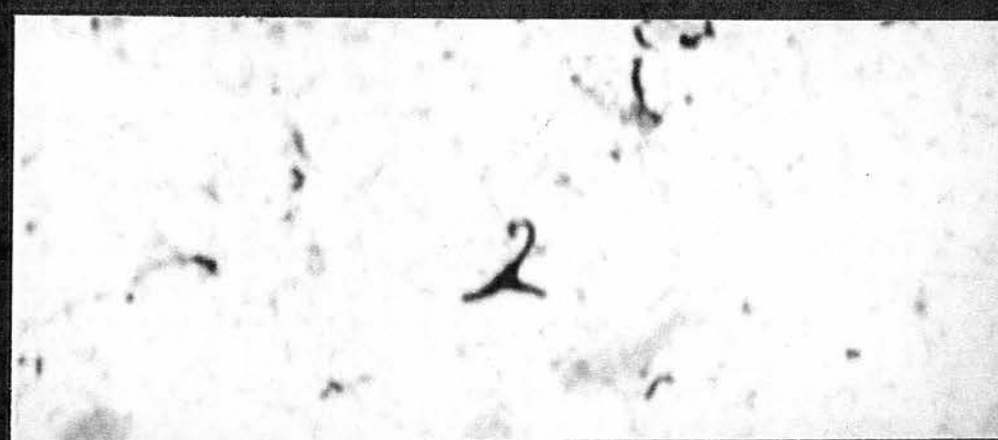

Plate 5

Actinobacillus equuli strain B345/69 on AJA containing 5 µg/ml cycloserine showing a branched filament. Gram X 1560.

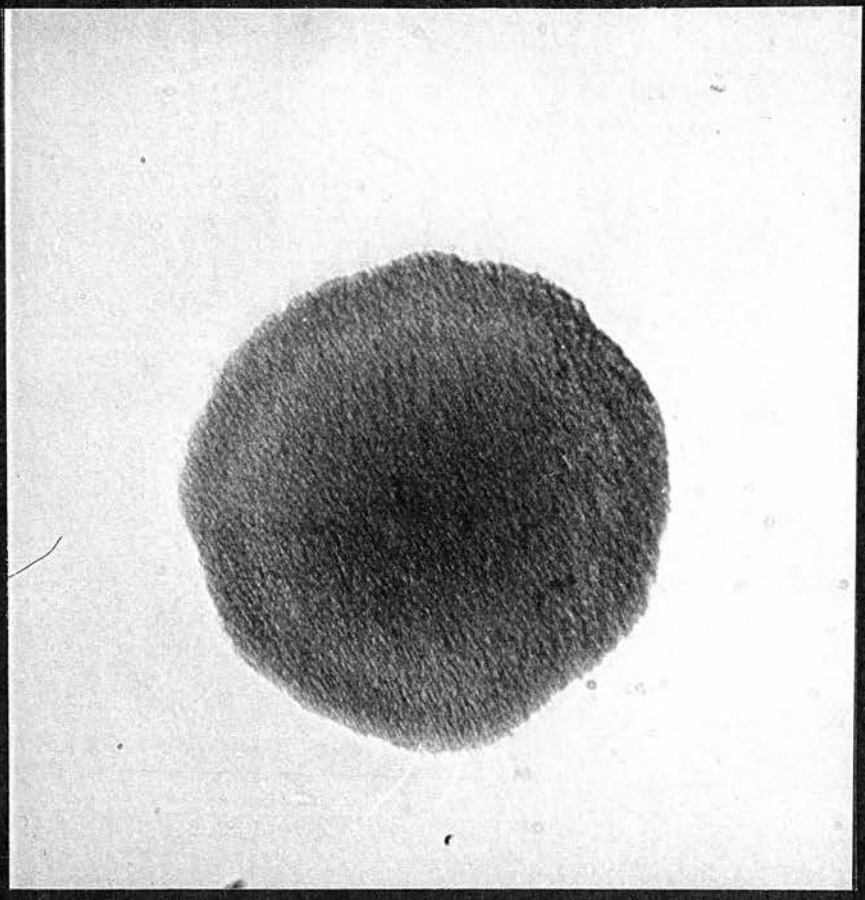

Plate 6

Bacterial colony of Actinobacillus equuli strain NCTC 8644 on AJA X 170.

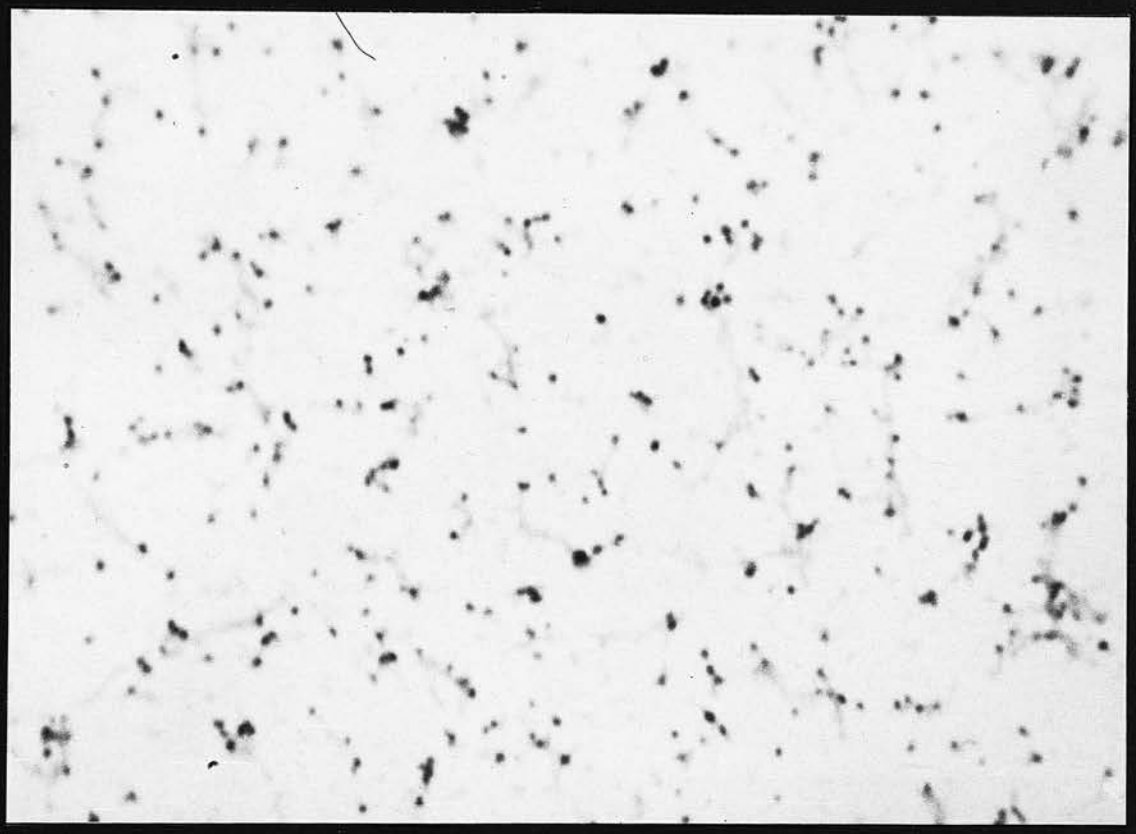

Plate 7

Actinobacillus equuli strain NCTC 8644 on AJA showing granules and small round forms. Gram X 1560.

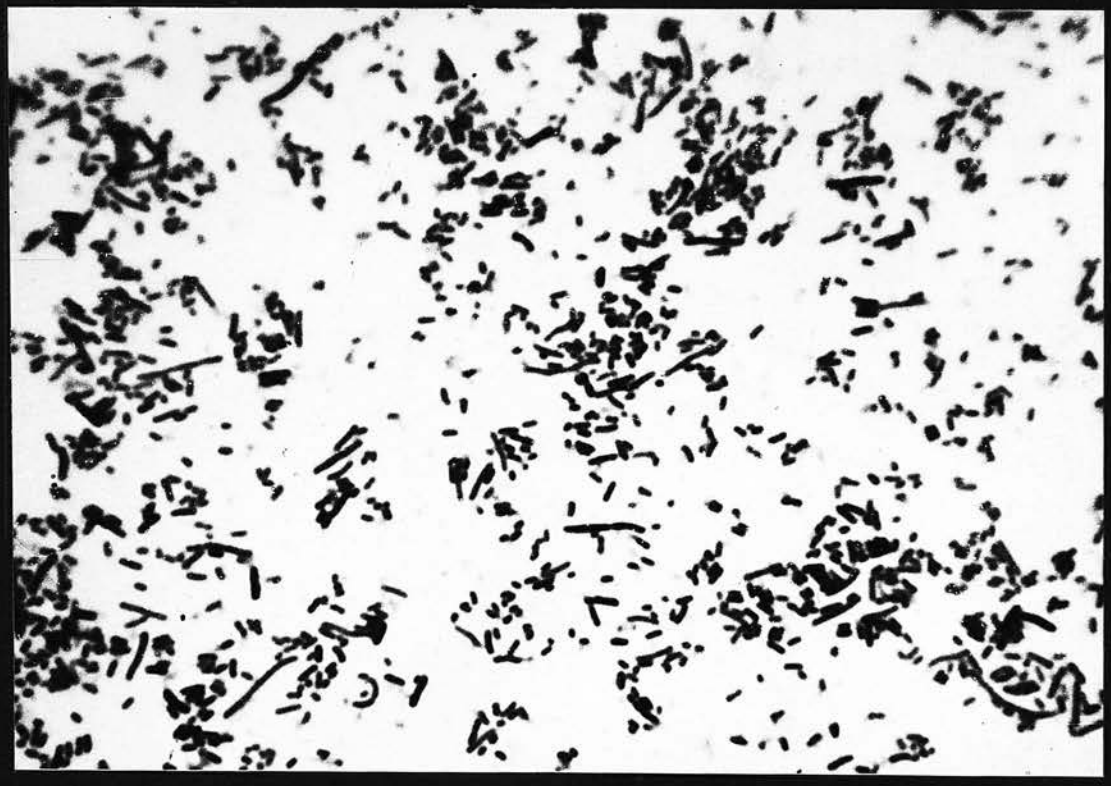

Plate 8

Actinobacillus equuli strain NCTC 8644 on blood agar showing pleomorphism of the organism. Gram X 1560.

Plate 9

Actinobacillus equuli strain FS1144/68 on CA containing 50 µg/ml cycloserine. Note the terminal swellings (a), the subterminal swelling (b), and the central swelling (c). Gram X 1560.

Plate 10

Actinobacillus equuli strain FS1144/68 on CA containing 60 µg/ml cycloserine. Note fusion of two heads of two adjacent rods (a) and winding filaments (b). Gram X 1560.

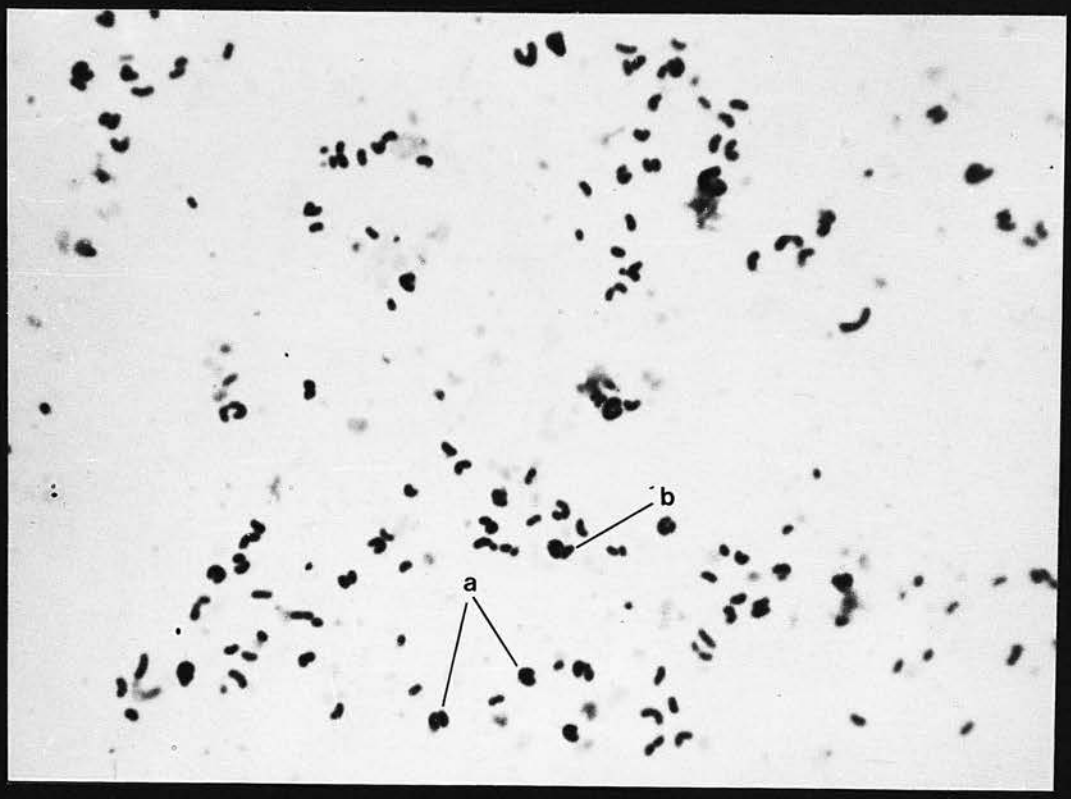

Plate 11

Actinobacillus equuli strain CCM5500 on CA containing 15 µg/ml cycloserine. Note fusion of two adjacent organisms (a) and fusion of three organisms (b). Gram X 1560.

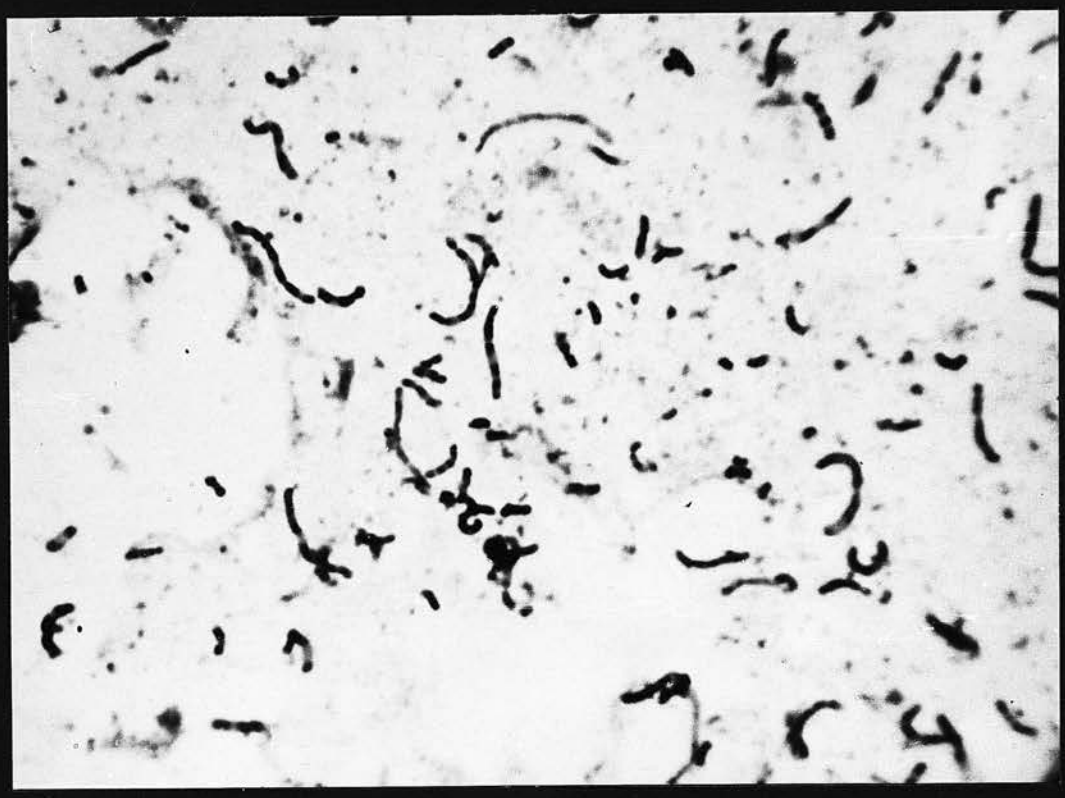

Plate 12

Actinobacillus equuli strain CCM5500 growing on CA containing 20 µg/ml cycloserine. Note the beaded appearance along the filaments. Gram X 1560.

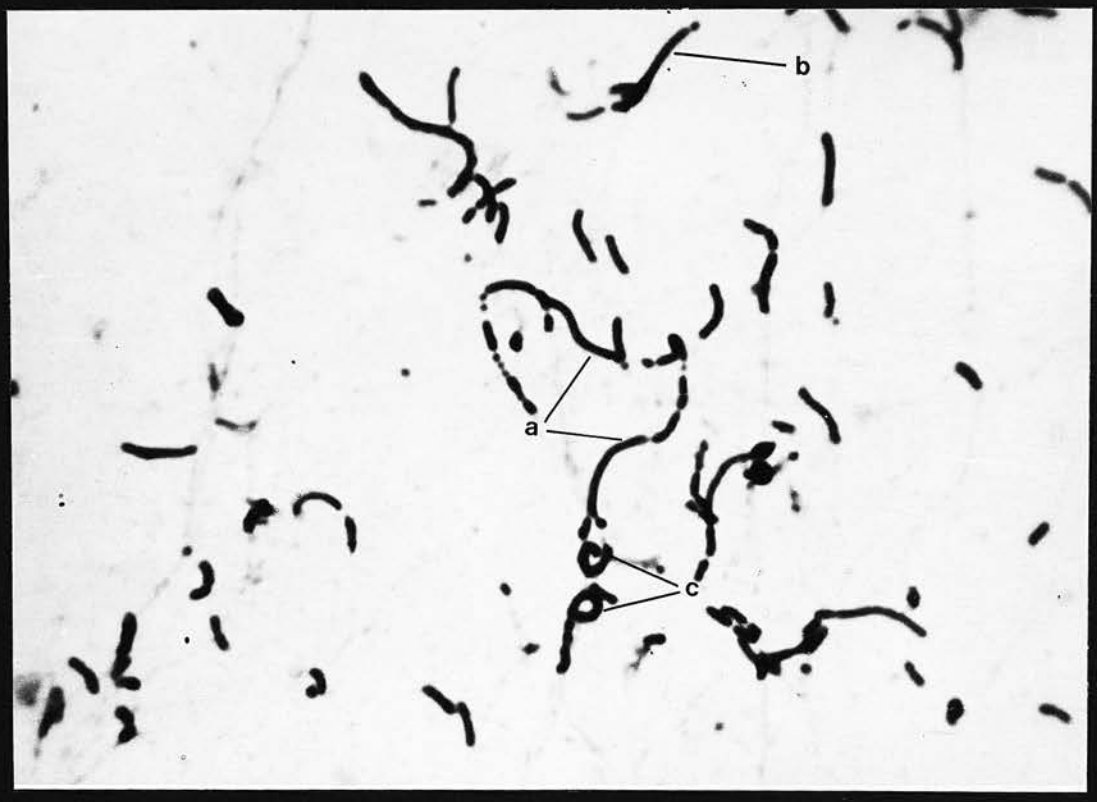

Plate 13

Actinobacillus equuli strain CCM5500 on CA containing 25 µg/ml cycloserine. Note the filamentous forms broken into bacillary forms, coccal forms and granules (a), a branching filament (b) and winding filaments (c). Gram X 1560.

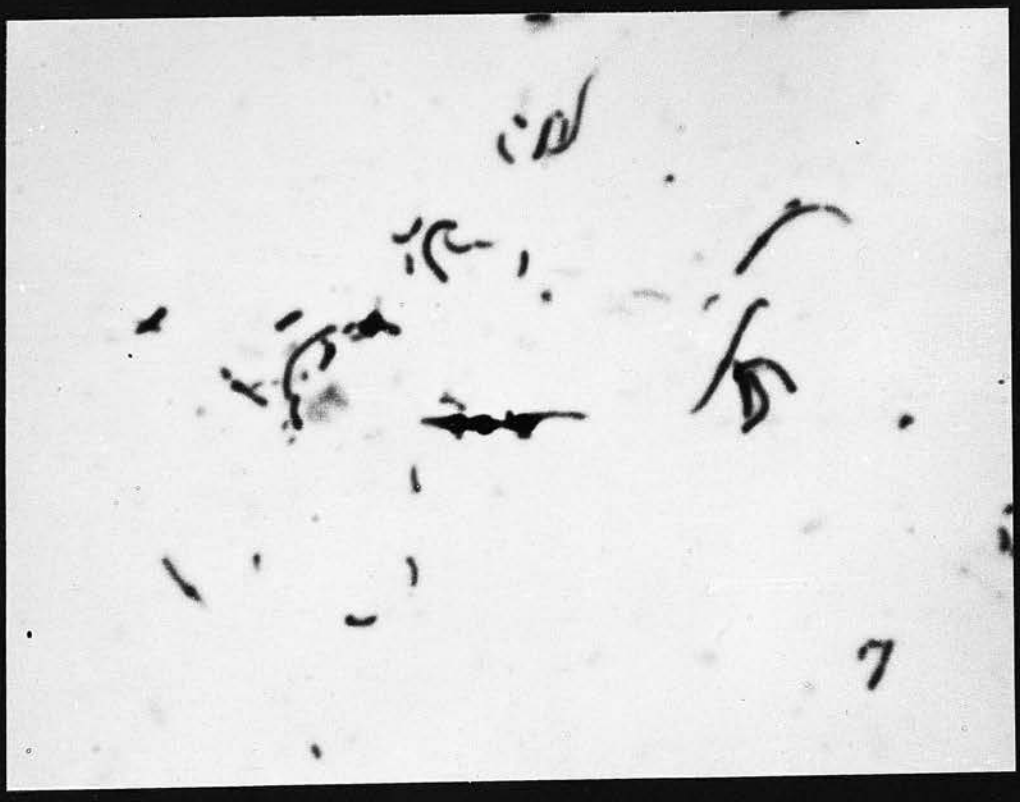

Plate 14

Actinobacillus equuli strain FS1144/68 on CA containing 6 µg/ml cycloserine showing multiple swellings along the filament. Gram X 1560.

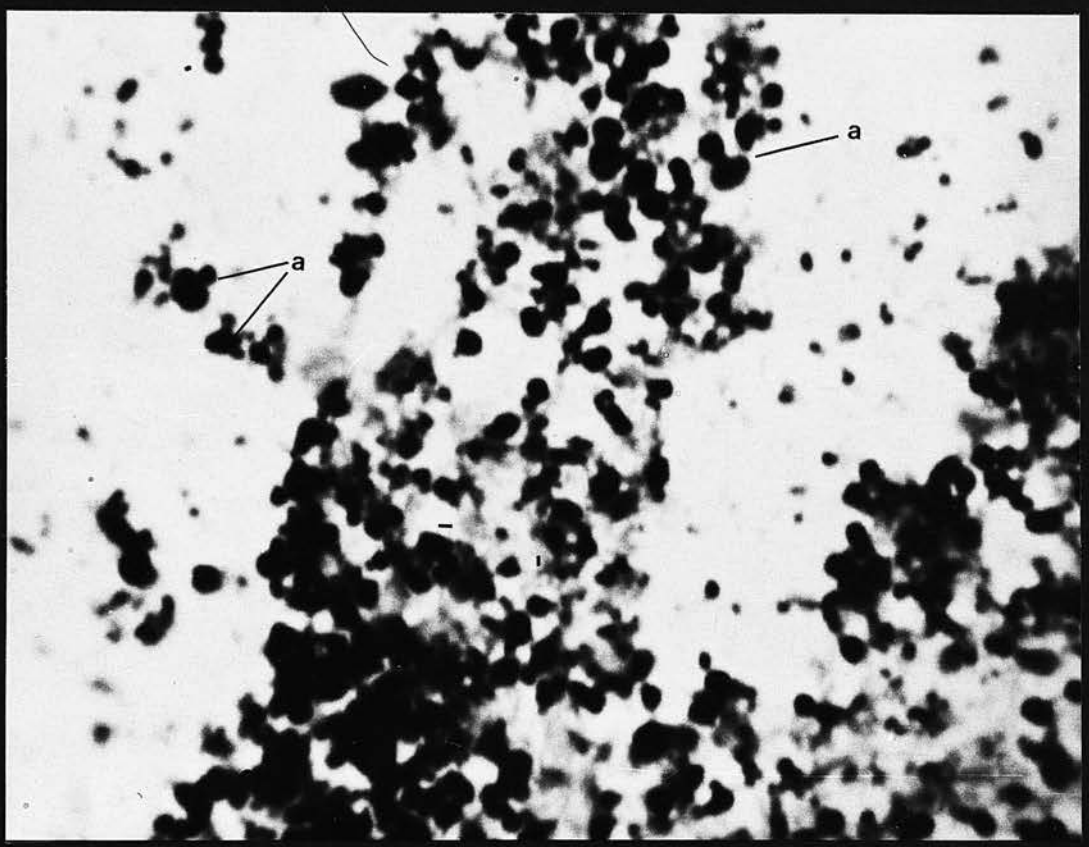

Plate 15

Actinobacillus suis strain P63/8/75 on CA containing 20 µg/ml cycloserine. Note budding of the spherical forms (a). Gram X 1560.

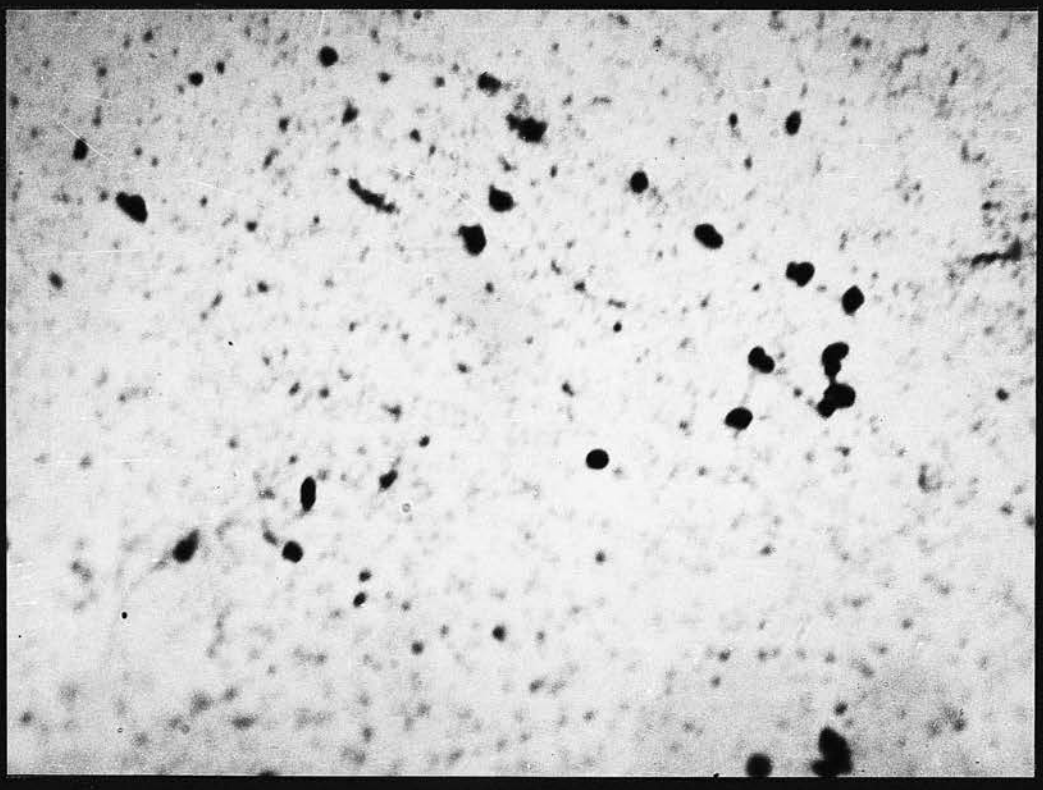

Plate 16

Actinobacillus equuli strain FS1144/68 L growth on AJA with calf serum showing a large number of extracellular granules. Gram X 1560.

Plate 17

L colony of *Actinobacillus equuli* strain B967/68 on AJA without yeast extract. Note the dark patches at the periphery X 170.

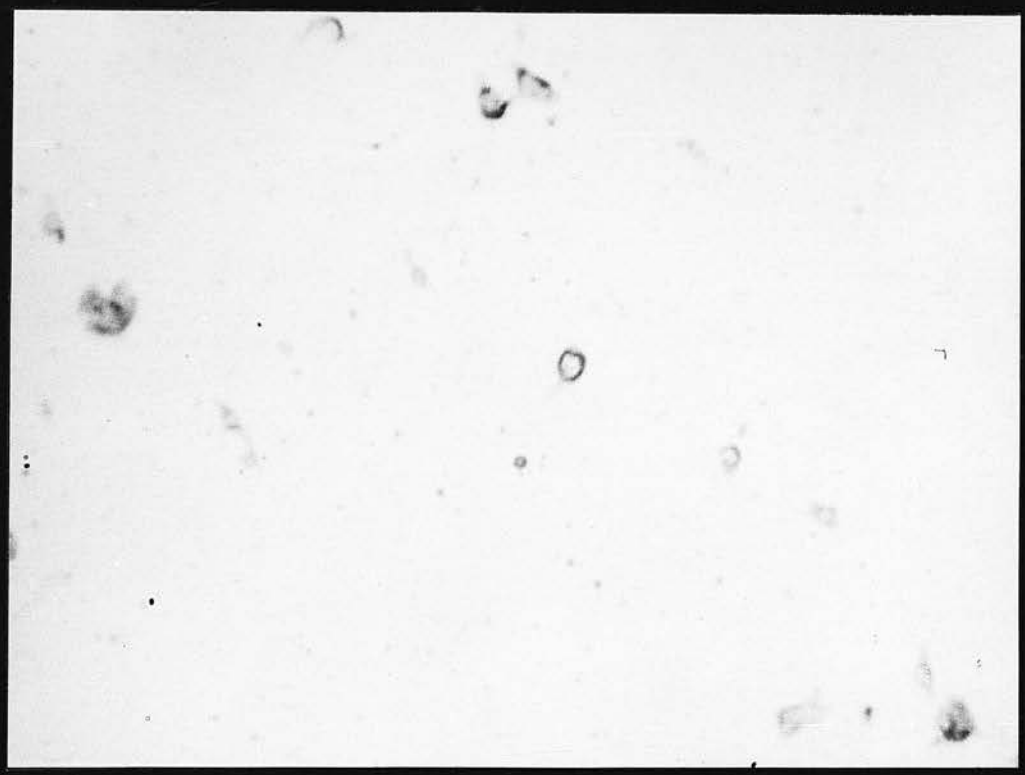

Plate 18

Actinobacillus equuli strain FS1144/68
L forms suspended in nutrient broth showing a round form that stained only at the periphery. Gram X 1560.

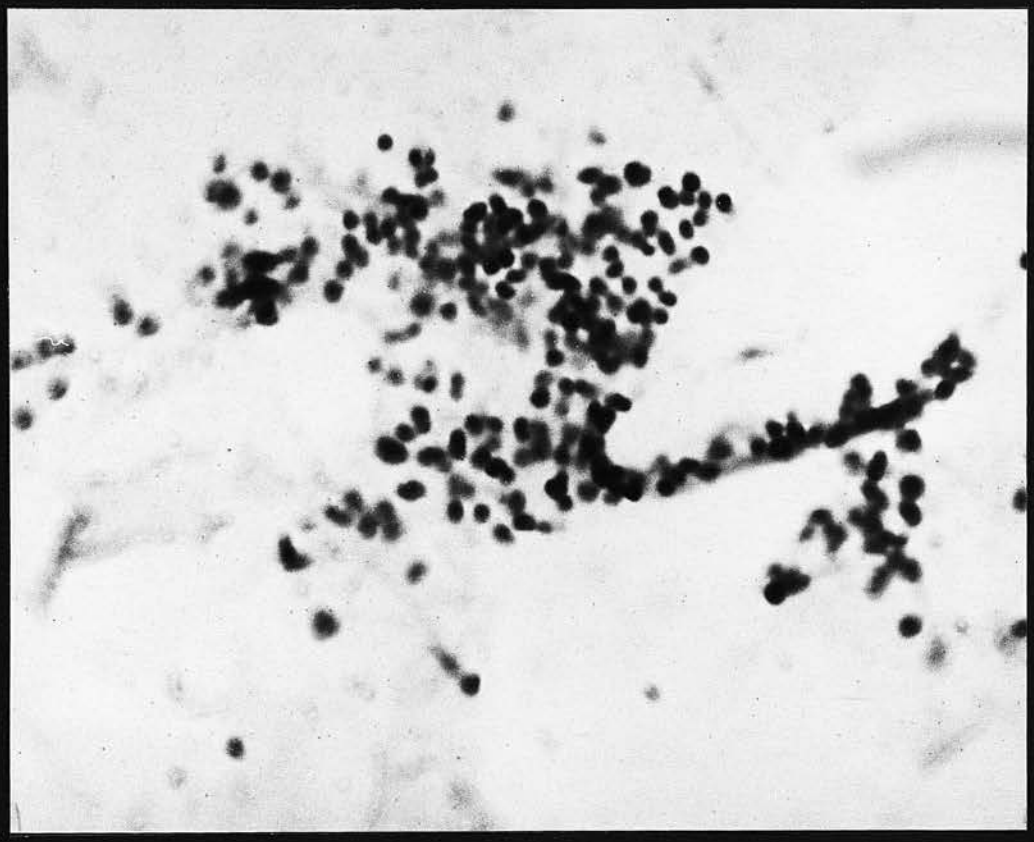

Plate 19

Actinobacillus equuli strain DV5 outside 2 on AJA showing transformation of aged culture to round forms. Gram X 2500.

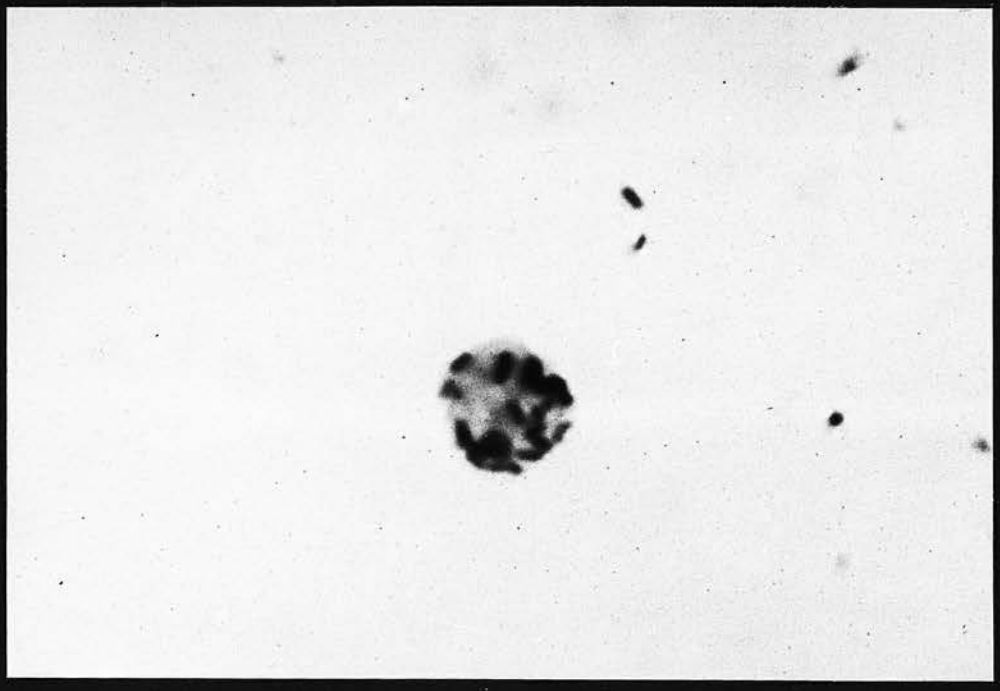

Plate 20

Actinobacillus equuli strain DV5 outside 2 on AJB reversion medium, showing enlarged faintly stained round forms with rod forms inside. Gram X 2500.

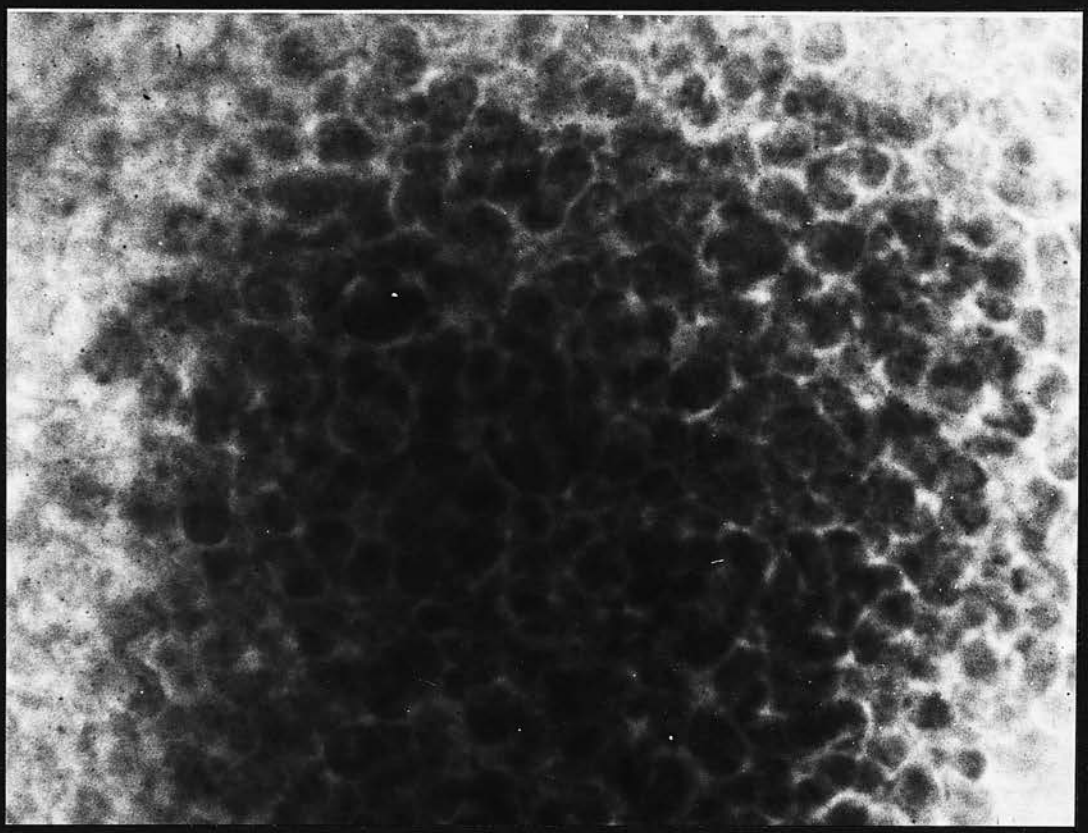

Plate 21

Actinobacillus equuli strain FS1144/68 on AJA containing 0·15% glycine. Note the darkly stained centre of the colony and the scattered faintly stained round forms at the periphery. Dienes' Stain X 2500.

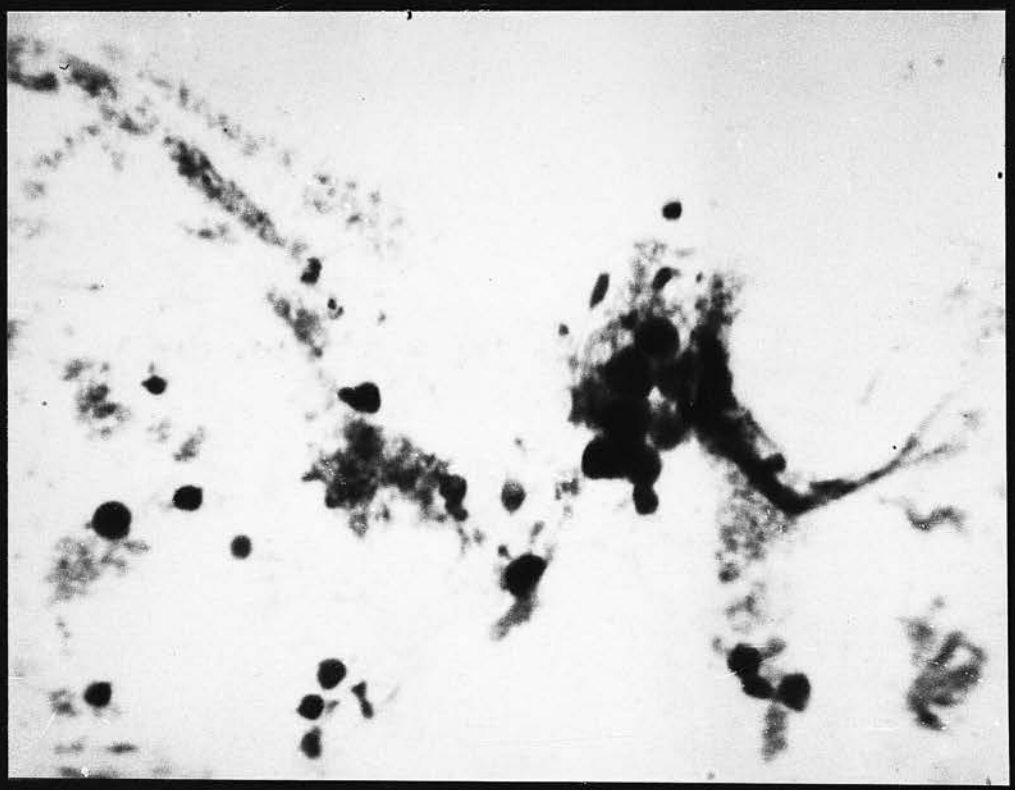

Plate 22

L growth of *Actinobacillus equuli* strain G14/3/68 showing well stained round forms. Giemsa X 1560.

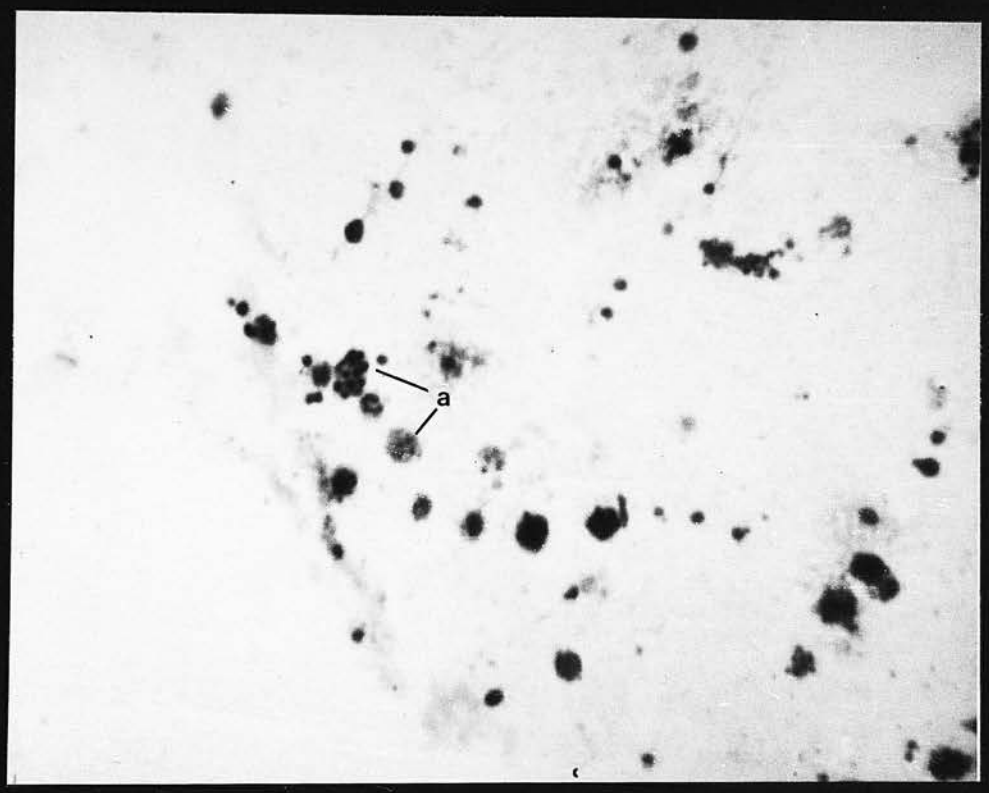

Plate 23

L growth of Actinobacillus equuli strain G14/3/68. The film was digested with 1N HCl and then stained with Giemsa. Note the faintly stained round forms and the intracellular granules (a). Gram X 1560.

4 - Discussion

From previous work it is obvious that L transformation is dependent on many factors such as the cultural conditions, the type of inducing agent and its concentration and the parent strain. In the present work some of these factors were studied.

Media with high solute concentration was shown to be essential for L growth and its stability (Weibull, 1953). Landman et al. (1958) found that L colony formation was highly dependent on high solute level. Protoplasts of B. megaterium were produced when the organism was treated with lysozyme in the presence of 0·2 M sucrose (Weibull, 1953). Landman and associates (1958) were able to achieve high osmotic pressure and a vegetative cell : L form ratio of unity when sodium succinate, sodium citrate, potassium chloride or sucrose were incorporated into the medium in an appropriate amount. Mitchell and Moyle (1956) obtained protoplasts from Micrococcocus lysodeikticus and Sarcina lutea by suspending them in sucrose or sodium chloride with an osmotic pressure of 25 atmospheres but not using glycerol. Due to this inability of glycerol to confer protoplast stability at the same atmospheric pressure, they concluded that stability of protoplasts depended on a high osmotic pressure provided by a solute which would not penetrate the cell.

Organisms differed in their requirement for salt concentration. Sodium chloride at 5% was used with

staphylococci (Montgomerie et al., 1966; Marston, 1968; Little et al., 1973) and at 3·9% for Erysipelothrix (Pachas & Currid, 1974). Salm. typhi, Salm. typhimurium, Proteus vulgaris and H. influenzae (Dienes & Sharp, 1956) and B. subtilis (Madoff et al., 1967) produced L forms in normal salt medium. In the present work the ability of one strain of Actinobacillus to grow on 3% salt and of 6 strains but not the remaining ones examined to grow on 2% salt suggested strain differences. The difference in salt concentration might be due to differences in the nature and amount of lipid in the membrane of the L form (Marston, 1968). Failure of L forms to grow on 3% sodium chloride could be due to some toxic effect of the salt or to the high external pressure exerted on the membrane in relation to the opposing internal pressure of the L elements. Failure was also probably due to plasmolysis and finally lysis of the spherical forms.

Sucrose concentration of 22% supported induction of L forms. This osmolality was equivalent to the 3% sodium chloride which failed to support growth. However, sodium chloride proved better for propagation. Among the solutes tested, sucrose was the only one that provided osmotic pressure in E.coli K12 (Landman et al., 1958). Nimmo and Blazevic (1969) found that sucrose was the best stabilizing agent. Other authors obtained satisfactory results with sodium chloride (Crawford et al., 1958; Kubota et al., 1966; Ward & Martin, 1962; Watanakunakorn, 1971a). On the other hand, sodium chloride

proved to be inferior to sucrose in isolating Mycobacterium L forms (Mattman et al., 1960).

The presence of the bacillus and transitional forms at 1% sodium chloride and not at 1·5% on the same cycloserine concentration suggested a synergistic action of the concentrations of sodium chloride and the antibiotic. Successful propagation of L forms in the presence of sodium chloride and not sucrose, the appearance of heteromorphic growth on 1% and the failure of L colonies to appear on 3% sodium chloride, emphasized the importance of the physical environment for induction and survival of L colonies.

A temperature of 30°C was chosen for incubation because an experiment with Actinobacillus showed that there was more transformation at 30°C than at 37°C. The transitional elements were rarely or not at all seen when plates were incubated at 30°C. This suggested that cycloserine was not so strongly inactivated at 30°C as at 37°C or that the temperature of 30°C was not optimum for Actinobacillus growth and this added to the unfavourable conditions that help transformation. Lower temperatures of incubation have been used by various workers with success. Thomas (1976) used a temperature of 30°C for isolating Salm. gallinarum L forms and Altenbern (1961) obtained the maximum number of 3A colonies at the same temperature together with other transforming factors. Ørskov (1942) chose a temperature of 32°C because growth was slower and easier to observe than at 37°C. Burmeister and Hesseltine

(1968) at 28°C grew B. subtilis L forms and Dienes (1949) used a temperature of 35°C for Proteus.

From other works it was obvious that serum is an important factor. Smith and Morton (1951) investigated its importance as a growth factor and Altenbern (1961) reported that serum was important for the production of 3A type L colonies and even larger amounts favoured the development of 3B colonies. Serum was not necessary for induction and development of 3A type colonies of Salm. gallinarum, although a higher yield was obtained on a serum medium than on serum-free medium (Thomas, 1976). Serum also promoted staphylococcal L colonies (Hamburger & Carleton, 1966b).

In this study the addition of serum was essential for L growth propagation in early passages. The well established L growth of strains DV5 outside 2 and B967/68 propagated on AJA serum-free medium without difficulty. Medill and O'Kane (1954) were able to induce Proteus L forms on serum-free synthetic medium. Accordingly they concluded that serum was not an essential growth factor but it might protect L forms by detoxification of inhibitory substances found in the medium. Hamburger and Carleton (1966b) and Thomas (1976) observed that serum promoted L growth and inhibited reversion to the vegetative forms.

L growth of Actinobacillus on high salt concentration without added serum was obtained. This growth could not be further transplanted in the absence of serum. The poor propagation of this growth on serum agar showed that

most of the L growth of serum-free induction plates died immediately after being isolated. The large number of external granules and the small number of spherical forms indicated the disruption of the spherical forms. Disintegration of L forms in serum-free medium was noted (Weibull & Lundin, 1961). Horse serum agar plates in which serum replaced charcoal proved to be inferior to charcoal agar. It could be argued that charcoal has a higher absorbing capacity for toxic substances than serum. Both pig and calf sera supported L growth. Two strains out of 12 produced typical L colonies and propagated for more than 3 passages in the presence of calf serum. In stained preparations the external granules were predominant. The insufficient protection afforded by calf and pig sera resulted in lysis of the fragile spherical forms. Propagation with horse serum was slightly better than with pig or calf serum.

Growth of L colonies did not improve when 15% or 20% inactivated horse serum was added to the culture media. Hamburger and Carleton (1966a) showed that there was no improvement of L growth on raising serum concentration above 10%. On the contrary L colonies were slightly suppressed at 20% and marked suppression was observed at 50%.

Growth of Actinobacillus L forms on AJA without yeast extract was poor compared with the growth on AJA with added yeast extract. The inhibitory effect of this ingredient with Proteus (Medill & O'Kane, 1954) and

with E.coli (Lederberg & St.Clair, 1958) was not shown with Actinobacillus. Growth of L forms of a Streptococcus strain was enhanced by the addition of yeast extract (Madoff, 1970).

The importance of $MgSO_4$ in stimulating L form growth caused most of the workers to include it in their culture media (Lederberg & St.Clair, 1958; Altenbern, 1961; Hamburger & Carleton, 1966a; Nimmo & Blazevic, 1969).

Although Actinobacillus and Pasteurella strains were induced on CA devoid of $MgSO_4$, the addition of 0·2% improved L growth. It could be concluded that the Mg^{++} cation was not absolutely necessary for L growth but its presence was beneficial. The addition of 0·01 M magnesium chloride or 0·005 M calcium chloride was reported to slow down the appearance of ghost cells (Davis et al., 1966). The authors suggested that the stabilization of spheroplasts by cations might be due to the formation of salt bridges which protect the cell surface protein from denaturation. Optimum results were obtained when 0·15% calcium chloride and 0·05% magnesium sulphate were added to the induction medium for E.coli (Mackemson & Darwish, 1972). When the same concentrations of calcium chloride and magnesium sulphate were added to AJA, L growth did not improve and the medium turned cloudy and was unsuitable for colony examination.

The disappearance of cores in strain P63/8/75 on 0·8% agar and of strain FS1144/68 on 1·5% agar shows that agar concentration determines L colony morphology.

On 0·4% agar the L elements of both strains penetrated either passively (Razin & Oliver, 1961) or actively (Dienes & Bullivant, 1967). On hard agar or other unpenetrable media, L colonies grew without penetration (Dienes, 1947, 1960; Dienes & Bullivant, 1967). Although the colony morphology of the strains tested was changed the elements making up the colonies remained the same. The round forms and granules persisted up to the highest concentration tested. On 3·5% agar Dienes (1967b) observed that streptococci and staphylococci grew to very large bodies only. Landman et al. (1958) reported an alteration in colony size upon changing the agar concentration. In Streptobac. moniliformis stable L forms the diameter of the central zone was not altered significantly by changing agar concentration from 0·1% to 5·0% (Razin & Oliver, 1961). The peripheral zone was largest on 1·5% agar and was reduced in size on higher concentrations of agar.

Four of the 8 Actinobacillus strains tested produced heteromorphic growth in 8 µg/ml cycloserine broth medium. This concentration was higher than that usually used on AJA induction plates on which complete transformation was brought about. Requirement of high drug concentration in broth could be due to a rapid fall of cycloserine concentration in broth. Failure of bacteria to undergo complete transformation in broth has been reported. Abbate et al. (1973) noticed slight pleomorphism in broth cultures of 2 strains of E.coli, one of Shigella flexneri

and 2 of Klebsiella-Enterobacter. Persistence of bacillary forms and transitional elements in broth cultures of Salm. gallinarum were observed by Thomas (1976). One strain of Actinobacillus and the two of the Pasteurella failed to grow when culture samples were spread on AJA containing cycloserine concentrations less than that in broth cultures. Inhibition of colony formation of 3 strains of Actinobacillus took place when broth cultures containing 8 µg/ml cycloserine were subcultured on AJA containing the same drug concentration. Although all the Actinobacillus strains received the same treatment in broth, they produced three different results on solid medium. There was an optimal concentration of cycloserine for each strain that would bring about complete transformation of the strain in broth and on solid media. Lowering the cycloserine to 5 µg/ml on the subculture medium favoured the appearance of transitional elements in strains M618/70, G14/3/68 and DV10 outside 3. These strains produced pure L elements in broth containing 8 µg/ml. This was in accord with Thomas (1976) who observed heteromorphic growth when the penicillin concentration in the propagating medium was lowered. On lowering it further he obtained bacillary type growth. The inability of some strains to grow when plated out was in agreement with the finding of Abbate et al. (1973). The authors could not obtain L colonies when appropriate plates were inoculated with penicillin-treated broth cultures of E.coli and Shigella, while bacterial growth

of Klebsiella-Enterobacter persisted. It seems that cycloserine concentrations (8 µg/ml for Actinobacillus and 100 µg/ml for Pasteurella) were toxic to the isolated L forms. Kubota et al. (1966) observed that increasing the penicillin concentration increased the yield of Strep. faecalis protoplasts. Increasing the penicillin concentration above the optimum decreased the yield and no growth was detected when 1,000,000 units/ml were used.

Using the usual method, the untreated broth cultures of Actinobacillus and Pasteurella, plated on 5 µg/ml and 100 µg/ml cycloserine respectively, underwent complete transformation. Treated broth cultures of Actinobacillus produced transitional elements when plated on 5 µg/ml. This suggested that some of the bacilli developed partial resistance to cycloserine in exposure in broth.

Poor growth in subcultures in broth and the appearance of transitional elements made propagation as pure L elements unsuccessful. The negative result can be ascribed to the difficulty in propagating L growth in liquid medium. Many workers have had difficulty in establishing L cultures in broth. This has been explained by suggesting that the L growth needs a surface to which to adhere to serve as a substitute for the mechanical support provided by the cell wall of the bacillary form (Dienes & Madoff, 1968; Dienes, 1967b; Taubeneck & Gumpert, 1967). L forms of Bacteroides and Salmonella grew in broth when 0·1-0·2% agar was added (Dienes, 1953). Meningococcal L forms were adapted to grow in broth by

transferring the L growth from agar to a diphasic medium containing a decreased amount of agar (Stewart & Wright, 1969).

L forms prepared in AJB and suspended in distilled water showed a marked drop in the number of L colonies after 5 minutes. The drop in colony count was less marked in nutrient broth than in distilled water.

After 30 minutes there was no growth from distilled water indicating that all the organisms had undergone lysis. The rise in the number of L colonies from nutrient broth after 30 minutes was due to the presence of revertants since many transitional elements were found at the end of the experiment. The hypertonic environment of AJB protected the fragile L elements whose numbers remained steady for 5 minutes and then decreased after which they increased in the same way as in nutrient broth. The high number of colonies from nutrient broth indicated that more reversion took place in the nutrient broth than in the hypertonic AJB. Fragility and disintegration of L elements when transferred from hypertonic broth to normal broth or more dilute broth have been reported (Hamburger & Carleton, 1966b). L forms of staphylococci were killed rapidly in distilled water and more slowly in nutrient broth (Williams, 1963). L forms of Staph. aureus, prepared with 5% sodium chloride, lysed when suspended in 2·5% saline (Young & Dahlquist, 1967). Bibel and Lawson (1972c) noticed a drop in the count of colony forming units when L phase elements of streptococci were transformed

from 2·5% to 1·25% sodium chloride. The osmotic fragility of Actinobacillus was further confirmed by suspending L phase cultures in ordinary nutrient broth or distilled water and observing them under the phase contrast microscope or in stained preparations. Some of the round bodies of low phase density which stained Gram negative resembled the protoplast ghosts of streptococci described by Freimer et al. (1959).

Synthetic medium with penicillin was used for the isolation of Proteus L forms by Medill and O'Kane (1964) and Tunstall and Mattman (1961) succeeded in isolating streptococcal L forms in a synthetic medium in the absence of an inducing agent. They obtained 4 out of 6 strains that had lost the ability to revert to the vegetative forms. The same medium was used in the present study but without success. Thus it became clear that methods adequate for development of streptococci were inadequate for Actinobacillus and Pasteurella. Mattman et al. (1960) obtained L growth of Mycobacterium without the addition of nicotinamide or inducing agents, although the presence of nicotinamide fostered the growth. The incomplete and irregular growth of L colonies in this work was probably due to the lack of nicotinamide, an important growth factor (Knight, 1937; Mueller, 1937; Fildes, 1938). The Actinobacillus and Pasteurella strains underwent L transformation when they were treated with the inducing agent to which they were susceptible in a suitable environment. Both AJA and CA supported the induction of

L forms. Strains G14/3/68, FS1144/68 and P63/8/75 produced typical L colonies which were readily propagated on AJA. These colonies were lost and occasionally L colonies were reisolated, but the ease with which the initial propagation of growth was brought about was never regained under similar cultural conditions. Irregularity of results was probably due to inadequacy of the medium (Dienes & Weinberger, 1951). Similarly Williams (1963) related his irregular results with Staph. aureus L forms to the absence of some growth factor or to the presence of an inhibitory factor in the medium.

Since charcoal was used successfully with Proteus (Lorkiewicz, 1957) and Pneumococcus (Madoff & Dienes, 1958) 0·5% activated charcoal was tested. This medium enabled the induction at a high antibiotic concentration compared with AJA. It also improved L growth and made propagation possible. The basal medium exerted a great influence on the growth of L forms and not all media successful in induction could support propagation of L growth. Media that were highly satisfactory for propagation were not necessarily satisfactory for induction (Hijmans, van Boven & Clasener, 1969). The morphological variation of Actinobacillus was more pronounced on blood agar and AJA than on CA. The relationship of such variation to the medium on which the organism was grown was noted by Kim (1976) who demonstrated the transformation to round bodies of A.equuli grown on glucose agar.

There was no evidence that the method of induction

was critical. L growths obtained both by the uniform plate and by the gradient plate techniques were similar.

Among the antibiotics penicillin has been the most widely used in L transformation. L colonies have been isolated with the help of penicillin from Staphylococcus (Youman et al., 1945; Little et al., 1973), Proteus (Dienes, 1949; Taubeneck, 1962), Salmonella (Dienes et al., 1950a; Weinberger et al., 1950), Shigella (Weinberger et al., 1950), Streptococcus (Sharp, 1954), Neisseria (Roberts & Wittler, 1966; Roberts, 1968), E.coli (Makemson & Darwish, 1972) and Erysipelothrix (Pachas & Currid, 1974).

In the present work Pasteurella was transformed with 0·2 units/ml penicillin. The inability of the L growth to propagate was due to the small difference between the minimum inhibitory concentration and the bactericidal concentration of the antibiotic. The narrow zone of L growth on the gradient plate confirmed the narrow range within which the organism was transformed. Formation of spheroplasts and L forms on exposure of bacteria to penicillin and synthetic penicillins indicated that the antibiotics act on the cell wall.

Cycloserine is capable of producing cell wall defective variants. These variants are similar to those obtained with penicillin, ampicillin or cloxacillin. Cycloserine has been used successfully to induce spheroplasts and L forms of many bacteria (Michel & Hijmans, 1960; Ward & Martin, 1962; Williams, 1963; Roberts, 1967, 1968; Watanakunakorn, 1971a). Pasteurella was transformed to

L growth was 90-100 µg/ml cycloserine compared to 0·2 units/ml penicillin. The amount of cycloserine needed to inhibit Staph. aureus was four times that of penicillin and the amount which caused accumulation of amino sugar was 400 times that of penicillin (Ciak & Hahn, 1959). Several passages on increased cycloserine concentration brought about an increased tolerance of the L forms to cycloserine. A similar observation with glycine as an inducing agent was shown by Neil et al. (1970).

The inability of bacitracin to induce L forms was reported by Ward, Madoff and Dienes (1958) who were unable to obtain L growth of streptococci diphtheroid organisms, Proteus, Salmonella or Vibrio. In contrast it was used successfully in inducing L forms of streptococci (Rotta et al., 1965) and Neisseria (Roberts, 1968). Kagan (1968a) and Park (1968) described bacitracin as a poor inducing agent. Failure of this antibiotic to induce L forms could be due to its additional action on the synthesis of other enzymes (Smith & Weinberg, 1962).

L growth of any strains tested was not induced with vancomycin. The result was in agreement with Williams (1963). Roberts (1967) was not able to induce L forms of 4 strains of meningococci, but one revertant underwent L transformation when treated with the antibiotic. Roberts (1968) and Watanakunakorn (1971b) reported induction and propagation of L forms with vancomycin, although the yield was very low.

The results with novobiocin were in accord with

Rotta et al. (1965) and Roberts (1967, 1968). Failure of the antibiotic to induce L transformation could be due to the release of RNA and protein from the cell (Brock & Brock, 1959). Failure of bacitracin, vancomycin and novobiocin to induce L growth or any morphological changes in the strains tested, suggested that these antibiotics behaved in a way different from penicillins or cycloserine. The inability of vancomycin to produce L growth could be due to the inhibition of RNA synthesis (Jordan & Innis, 1959). Strominger (1968) suggested an additional action of vancomycin other than its action on the cell wall. Induction of L growth in some organisms by vancomycin and bacitracin and not in others could possibly be due to differences in composition of the cell walls and the media.

The L form inducing property of glycine has been described (Dienes & Zamecnik, 1952; Dienes, Bandur & Madoff, 1964; Neil et al., 1970; Thomas, 1976). L growth was obtained from most of the strains tested in the present work. Concentrations of 0·3% and 0·6% glycine proved lethal to Actinobacillus and Pasteurella respectively. The optimum concentration at which the organisms underwent complete transformation was strain dependent. Dienes et al. (1950a) induced L growth of typhoid bacilli with 1% of glycine and H. influenzae L colonies developed at concentrations between 0·3% and 2·5% (Dienes & Zamecnik, 1952). Complete transformation when a concentration of 3% glycine was used has been

reported in H. pleuropneumoniae (Neil et al., 1970) and in Salm. gallinarum (Thomas, 1976). Want and May (1975) obtained L forms of H. influenzae when a concentration as low as 0·7% was used. The additive effect of glycine and penicillin which was useful in transformation of group A streptococci (Michel & Hijmans,1960) and a strain of streptococcus (Madoff, 1970) was not of help with Pasteurella grown on AJA treated with various concentrations of glycine and penicillin.

Induction of L colonies with the amino acid indicated that its action is on the cell wall. The inhibitory effect on growth seen in this study with high concentrations of glycine and the accumulation of uridine nucleotide in the presence of glycine in Staph. aureus (Strominger & Birge, 1965) suggests an antibiotic property.

From the results of this study it was clear that the cell walls are affected by the action of lysozyme-EDTA mixture. All tested strains were transformed to spherical forms with lysozume in the presence of tris buffer containing 15% sucrose. The appearance of filamentous and bacillus forms suggested the presence of resistant forms or that the ratio between enzyme and substrate in the reaction mixture was not favourable. The loss of the cell wall depended on the access of lysozyme to the substrate and its action at this site (Weiss, 1976).

The spherical forms did not grow into L colonies when plated out on AJA or tryptone soy agar. Some workers reported the ability of lysozyme induced protoplasts

to develop as L colonies on appropriate agar media (Madoff et al., 1967; Gooder, 1968; King & Gooder, 1970a).

L growth of some Actinobacillus strains developed in the induction plate but it did not grow in subculture or died out after a few transplants in spite of the large inoculum used. Similar results were observed with E.coli and H. influenzae (Dienes & Smith, 1944; Dienes & Weinberger, 1951). Typical L colonies of strains FS1144/68 and G14/3/68 propagated more easily than the granular colonies of strains DV5 outside 2 and B967/68 on the same medium. The subsurface growth of glycine plates rendered propagation difficult because very few or none of the L elements could be deposited on fresh plates. So the ease with which L colonies propagated was highly dependent on a) morphology of the colony, b) abundance of surface growth and c) type of medium. Seeberg (1973) observed that E.coli colonies with the typical L appearance had a high capacity to grow in subculture. Within the typical forms Roberts (1966) noticed that colonies with a small core and a wide periphery propagated well while those with a wide core and a narrow periphery propagated poorly.

On AJA 5 of the 12 Actinobacillus strains and none of the 5 strains of Pasteurella could be propagated more than 5 times. Seeberg (1973) found that in 47 strains of E.coli 9 propagated 4 times or more, 6 propagated twice and 32 could be propagated only once or not at all. Variation between strains was also revealed in the ability

of different strains to grow on different salt concentrations. One out of 12 strains tested produced L growth on 3% sodium chloride and six strains produced L growth on 2% salt. Dienes and Sharp (1956) noted strain differences with regard to salt concentration.

After repeated transfer on an antibiotic medium, strain B82/68 was resistant to 5,000 µg/ml cycloserine. On the other hand strain B967/68 produced L growth at 140 µg/ml. The antibiotic concentration at which maximum transformation was achieved depended on the strain. A strain of enterococci examined by Hijmans and Kastelein (1960) produced L colonies when 32,000 units/ml penicillin were deposited in the trough, other strains needed 16,000 units/ml. In Proteus one strain was transformed with 200 units/ml penicillin, others were not transformed even with 1,000 units/ml (Li et al., 1964b).

Some strains do not produce L growth when they are subjected to a known L form inducing agent. Three out of 20 strains of group D streptococci did not produce L forms with penicillin, one strain was transformed with glycine and none with lysozyme (Madoff et al., 1967). Four out of 9 streptococcal strains produced L forms on penicillin gradient plates (Freimer et al., 1959). Lawson and Douglas (1973) reported that 6 of the 11 strains of N. gonorrhoeae tested failed to produce L growth with penicillin. L forms with different properties from a single strain of bacterium were obtained. Strains FS1144/68 and G14/3/68 produced typical colonies that

were easily propagated but other isolates of the same strains obtained under the same cultural conditions did not propagate. Such a phenomenon was observed with other bacteria (Dienes, 1953; Dienes & Madoff, 1968; Dienes & Bullivant, 1967).

Since the discovery of L forms, numerous morphological investigations have been carried out on them. L colonies represent bacterial variants having common morphological features (Dienes, 1950, 1953; Weinberger et al., 1950; Hijmans et al., 1969) and varying in their differences from the parents (Dienes, 1953). The colonial morphology of L colonies are similar regardless of how they are induced (Dienes et al., 1950a; Dienes & Weinberger, 1951; Rotta et al., 1965; Madoff et al., 1967; Roberts, 1967, 1968; Mahony, 1973). L colonies of Actinobacillus or Pasteurella prepared with cycloserine, penicillin or glycine were similar. This was in agreement with Watanakunakorn (1971a) who obtained morphologically similar L colonies of staphylococci induced by cycloserine, methicillin or lysostaphin. The characteristic appearance of the L colony having a core embedded in the agar and a superficial peripheral growth has been described (Dienes & Bullivant, 1967; Marston, 1968; Roberts, 1968; Stewart & Wright, 1969; Little et al., 1973). Infiltration of the L colony into the agar is due to the need of the L colony for support (Weinberger et al., 1950). Young L colonies (24 hours old) resembled bacterial colonies and the growth was mainly on the surface. On

further incubation colonies were differentiated into dark centres growing into the agar and light surface growth making the periphery. This showed that infiltration of the agar was from the surface growth. This was in agreement with Bible and Lawson (1972a). On the other hand Razin and Oliver (1961) reported that growth started in the agar and from it the surface growth developed. The delay or complete inhibition of penetration of moist plates observed in this work was also reported by Bibel and Lawson (1972a).

Each of strains FS1144/68 and G14/3/68 produced typical or granular L colonies, making differentiation between strains on colony morphology impossible. Seeberg and Brorson (1974) could not divide E.coli into groups on their colonial basis because some E.coli strains produced two morphologically different types. The isolated typical L colonies of strain G14/3/68 appeared when the confluent growth on 5 µg/ml cycloserine was transferred to 8 µg/ml. This confirmed the observation of Seeberg and Brorson (1974) that production of E.coli classical L colonies was influenced by the antibiotic concentration, the homogenous L colonies being detected at the lower concentration of the antibiotic. The growing L colonies on AJA were large, did not require serum after several passages and easily reverted to the vegetative forms in the absence of cycloserine. These colonies were of the 3B type of Dienes (1949). Colonies on CA were tiny, easily propagated and usually produced

confluent growth. The difference between these colonies and the 3A type of Dienes (1949) was the ability of these colonies to grow in absence of serum and the ease with which they reverted to vegetative forms. Types 3A and 3B did not indicate stable and unstable forms since on agar both could revert to the usual bacterial forms (Dienes, 1949, 1968; Weinberger et al., 1950). Using Dienes' dry stain, large bodies at the edge of the colony were found to be scattered and less intensively stained these being the aged elements. The deeply stained centre of the colony which was made of small round forms and granules was the youngest part. Dark patches and empty rings at the periphery of the L colony of strain B967/68 on AJA without yeast extract was due to autolysis which started at the periphery and proceeded inwards. Granules and round forms present in old cultures of Actinobacillus were non viable and failed to grow on subculture. Granules produced by autolysis or irregular division did not grow in subculture (Dienes, 1946a, 1947; Hijmans et al., 1969). Dienes (1946a) suggested that round forms in old cultures could be induced by the accumulated metabolic products. Large bodies are noticed in bacterial cultures before autolysis occurs (Dienes, 1968). Unlike the Actinobacillus, the autolysed culture of Bacteroides, B. subtilis and Streptococcus developed into large bodies and then L forms (Dienes, 1949; Madoff et al., 1967; Pachas & Dienes, 1968). L forms occurred naturally in Streptobacillus moniliformis, Flavobacterium,

B. subtilis, Bacteroides, Streptococcus and Cl. tetani (Klieneberger, 1935; Dienes, 1939a & b, 1949, 1950; Dienes & Smith, 1944; Madoff et al., 1967; Pachas & Dienes, 1968; Cheng, 1973). Transformation into L forms was preceeded by changes that resulted in the development of round forms. Opinions differ in the way the round forms arise. It seems that round forms develop by more than one means. Swelling of the rod and then extension in all directions is the most commonly observed. Stempen and Hutchinson (1951) and Madoff et al. (1967) noticed swelling at one end of the rod which increased and absorbed the remainder of the rod. Actinobacillus rods swelled at one or both ends and only remnants of the rod attached to a spherical form or connecting two spherical forms was present. It was possible that the rod broke in between giving rise to two round forms. At low cycloserine concentrations strain CCM5500 developed into filamentous forms. Multiple swellings that appeared along the filament might separate later giving more than one round form per rod. On slide culture Li et al. (1964b) noticed many extrusions growing out of swollen bacteria. These extrusions grew in size and later broke off making several swellings. Some filaments of strain CCM5500 were broken into bacillary and coccal forms. Each of them had the opportunity to develop into round forms. In the same strain, two curved neighbouring rods were connected together. These sometimes formed into circle-like structures and sometimes three connected rods

produced triangular structures. The space enclosed by these forms might become decreased as a result of lateral extension of the rods. This showed that round forms could be made by fusion of two or more rods. Dienes (1946a) observed the development of large bodies from two neighbouring organisms in a strain of Bacteroides. Stempen and Hutchinson (1951) followed the development of large bodies in a single cell culture of Proteus vulgaris strain OX19. They concluded that the large body could arise from a lateral or terminal bud that increased in size and absorbed the rod, by swelling of the rod or by fusion of two adjacent cells.

Loops made by a twisted filament or curved rod were commonly seen in strain B82/68. The lumen was usually narrow but completely disappeared in others giving rise to a round form. Round forms could also be formed by protrusion of the protoplasm through the altered cell wall (Stempen, 1955). In strain FS1144/68 the appearance of swellings at the end of two adjacent rods, the presence of such rods in close contact and of structures where the dividing wall was not very apparent suggested that round forms are made by fusion of two rods. In Bacteroides fundiliformis, Smith (1944) noticed that the ends of two adjacent rods swelled, came together and the line between them disappeared resulting in a round form. Dienes (1946a) explained the fusion of two neighbouring individual bacteria that resulted in a large body as a sexual reproduction. Such type of reproduction was reported

(Mellon, 1925) when two bacterial cells conjugated giving rise to a zygospore.

Spherical bodies attached to the membrane of the round form suggested that budding was a mode of replication. This was in agreement with Bibel and Lawson (1972a). Examination of broth cultures with the phase contrast microscope showed that some of the round forms were in pairs of nearly equal size, others were large and had one or more small round forms attached to them. It could be concluded that round forms in broth culture multiplied by binary fission and by single or multiple budding. Binary fission and single budding were the mode of replication of streptococci in broth (Bibel & Lawson, 1972c). The smallest viable unit in broth was determined by van Boven, Ensering and Hijmans (1968) who reported that only elements above 0·7 µm were capable of replication. Weibull and Lundin (1962) studying Proteus L forms on slide culture noticed that elements having the size of 0·6-0·7 µm could reproduce.

Viable granules developed in vacuoles or in the cytoplasm of the large bodies (Dienes, 1947, 1967b; Roberts, 1966; Dienes & Bullivant, 1967; Bibel & Lawson, 1972a, 1975). The repeated presence of granules in large bodies suggested that these forms were a constant feature of the large bodies. Stempen and Hutchinson (1951) and Bibel and Lawson (1972a) believed that granules developed by fission or budding. The latter authors observed the

elongation of granules but they did not observe the disintegration of the central area between double granules as was seen by Dienes and Bullivant (1967) and Dienes (1967b, 1968). Dienes (1967b) noticed that granules transferred from broth culture to agar either grew into the agar or remained on the surface and grew to large bodies. Development of large bodies from expanding granules was also reported by Weinberger et al. (1950) and Madoff and Dienes (1958).

L growth films digested with HCl showed that more than one granular form was present inside the large body. Granules were in interconnected groups and were deeply stained. Some of them were large reaching the size of the small round form. These granules originated from the large body content. The first indication of their formation as described by Dienes (1967b) was the condensation of the periphery of the large body from which growth of the granules started. With Feulgen's stain Robinow (1956) demonstrated the presence of DNA which appeared deep purple within the chromatin. The digested part which did not take the stain was the RNA. Li et al. (1964a) applied this method together with acridine orange staining on Proteus L forms. The results they obtained by both methods confirmed that the protoplasmic substance of the spherical forms that was lost after digestion was RNA and it emitted red fluorescence. The granules were deeply stained with Giemsa and emitted green fluorescence with acridine orange indicating that they were DNA. In

studies with stable L forms of Proteus, Weibull and Beckman (1960) found that the small granules contained RNA and DNA.

It is suggested that the round forms which appeared in the large body were granules that had grown to the size of the round forms. The round forms could be released with some granules after disintegration of the large body and the released structures developed into large bodies containing round forms and granules and continued the cycle. The vacuole-like structures that appeared as non-stainable areas were numerous in old cultures. Klieneberger-Nobel (1960) explained that the vacuoles in the spherical forms might be filled with metabolic products. Li et al. (1964a) observed the presence of numerous vacuoles in old cultures. They claimed that some of the vacuoles emitted greenish fluorescence with acridine orange meaning that they contained chromatinic material. Dienes (1967b) reported that large bodies without fully stainable areas did not grow in subculture.

Complete transformation to L growth was obtained when overnight broth cultures were exposed to the appropriate cycloserine concentration. The same concentration of the antibiotic supported heteromorphic growth on subsequent subcultures. Hubert et al. (1969) and Guze et al. (1976) found that it was necessary to increase the penicillin concentration on subculturing Serratia marcescens and Klebsiella pneumoniae L forms. The need for increased antibiotic concentration was due to the emergence of individual resistant cells among the bacterial population.

Increase in antibiotic concentration was also necessary in Pseudomonas aeruginosa to stop the spontaneous reversion (Hubert et al., 1971; Bertolani et al., 1975). Thomas (1976) observed that heteromorphic growth of Salm. gallinarum emerged when subcultures continued in the presence of a constant amount of penicillin which was sufficient initially to induce L transformation. On the contrary Lorkiewicz (1957) and Williams (1963) induced and subcultured Proteus and Staphylococcus L forms on agar media containing a constant amount of penicillin without any signs of reversion. The emergence of transitional elements was first detected by the thick confluent surface growth which can be mistaken for bacillary growth. On increased cycloserine concentration this growth disappeared and a few isolated colonies that stick to medium appeared. A similar observation was reported by Kagan, Martin and Stewart (1964) who observed confluent growth of Staph. aureus on 500 µg/ml methicillin. This growth was replaced by discrete bacterial colonies mixed with L colonies and finally pure L colonies when the methicillin concentration was increased. Pasteurella strains were more resistant and needed a greater increase of cycloserine than that needed by the Actinobacillus. Sudden increase of cycloserine concentration resulted in discontinuation of L growth, suggesting toxicity of the antibiotic at high level. In Proteus mirabilis the number of L colonies increased with the increase of penicillin (Medill-Brown & Hutchinson, 1957). These authors observed reduction in the number

and size of the colonies towards the highest concentration and this was attributed to the inhibitory effect of the penicillin at high level. Strain DV5 outside 2 and B967/68 were stable on AJA cycloserine medium compared to CA. Transitional elements emerged after the two strains had been transferred several times on a constant cycloserine concentration. The appearance of resistant forms and the need for increased cycloserine concentration was clearly affected by the type of medium. It is of interest to note that on AJA strain DV5 outside 2 was transferred to 20 µg/ml on the 150th passage and it continued stable until the 219th passage. Growth of this strain completely disappeared on 25 µg/ml cycloserine. However, the same L isolate showed transitional elements and bacillary forms when it was transferred to 20 µg/ml on CA. On further propagation on CA it produced heteromorphic growth on 300 µg/ml and completely transformed to L elements on 350 µg/ml at the 30th passage. It seemed that the consistency of AJA had a synergistic action with cycloserine to maintain L growth at a constant antibiotic level compared with CA. The rate at which the antibiotic concentration needed to be increased was slow at low passages and increased at the later passages due to the increased resistance of the organism, and it was proportional to the number of passages. Preliminary studies with penicillin on CA showed that, as with cycloserine, increased antibiotic concentration on subculture was necessary.

Acquired resistance to glycine was not investigated as growth on subcultures was not achieved. Salm. typhi spheroplasts isolated with glycine reverted when they were subcultured on the same concentration (Diena et al., 1965). These authors described the revertants as being resistant forms.

Actinobacillus and Pasteurella L forms isolates were of the unstable type. On withdrawal of the inducing agent L forms reverted to the vegetative forms. Stabilization of L forms by propagation in reduced amounts of cycloserine, as used by Watanakunakorn (1971a) for staphylococcal L forms could not be applied in this study. Reversion to vegetative forms has been reported (Hirokawa, 1962; Hamburger & Carleton, 1966b). The ability to regain vegetative forms from the L forms depended upon cultural conditions other than the absence of the inducing agent. Increased agar concentration or a high amount of gelatin are two important factors found to favour reversion (Landman & Halle, 1963; Hamburger & Carleton, 1966b, 1968; Gooder, 1968; Landman & Forman, 1969; King & Gooder, 1970b). Furthermore, reversion was enhanced by the absence of serum (Hamburger & Carleton, 1966b, 1968; Thomas, 1976), by the addition of N-acetylglucosamine (Salowich & Mattman, 1966) and by the addition of yeast extract (Crawford et al., 1958; Kawatomari, 1958; Holmgren & Campbell, 1960; Marston, 1961; Salowich & Mattman, 1966). An L form isolated from tissue culture was converted to a Corynebacterium when grown in the presence of mucin

(Wittler, Garry & Lindberg, 1956). Dienes (1970) observed reversion of H. influenzae L forms when they were grown in the neighbourhood of colonies of large Gram positive spore-bearing bacilli or when they were immersed in the filtrate of the bacillus. Stable L forms of Salm. typhimurium reverted only when they were injected into chick embryos, rats or mice (Levina, 1972) and the stable L forms of Agromyces ramosus which died out in the absence of serum reverted to the parent forms when magnesium chloride replaced serum in the medium (Horwitz & Casida, 1978).

The appearance of bacterial forms inside the large bodies during the course of reversion has been repeatedly observed (Dienes, 1939b, 1943, 1947, 1953, 1968; Marston, 1961). Bacterial forms produced from the round body can arise both from the surface of the body as well as from within (Dienes, 1968). In the present study reversion was facilitated by agar medium and by incubation at 37°C. In liquid medium incubated at 30°C reversion was slowed down and the round forms persisted for a longer time. The rod forms which appeared inside the large body were produced by the transformation of the cytoplasm of the large body (Dienes, 1939b). This showed that one spherical form contained more than one rod form in terms of cell content. Hirokawa (1962) observed the growth of 4 rods out of each spherical form of E.coli. In H. influenzae reversion started by disintegration of the large body into granules which later

developed into bacterial forms (Dienes & Smith, 1944; Dienes, 1970). At the start of reversion Medill-Brown et al. (1960) and Taubeneck and Gumpert (1967) noted the elongation of the spheroplast which later branched and broke down into rod forms. Filamentous forms which broke down to rod forms were common in this study. Rods could also be produced by direct division of the large body (Dienes, 1946b; Freundt, 1950; Stempen & Hutchinson, 1951). Reversion of streptococcal protoplasts began by the formation of a comma shape from the tail of which the coccal forms developed and multiplied (Bibel & Lawson, 1972b). Reversion to the normal bacterial forms in the absence of inducing agent indicated that the cell retained the potential to synthesise the cell wall.

In a long incubation period of 4 days or more, the round forms present at the edge of the young L colony were replaced by filaments or rod forms. The centre was less marked, still attached to the agar and contained small round forms and granules. The start of reversion from the peripheral region was in agreement with the finding of Green, Heidger and Domingue (1974). Growth of the round form either into an L form in the presence of cycloserine or into a normal bacterial form in its absence confirmed the dual potentiality of this important structure (Dienes, 1946a, 1947).

Immediately after reversion the organisms were misshapen and pleomorphic. The highly filamentous forms and the swollen forms disappeared and resumed the parent

forms after one or two passages on reversion medium. Revertants of streptococci were identical to the parents and produced L forms with relative ease (Crawford et al., 1958; Freimer et al., 1959). The frequency of induction to L forms in revertants of N. gonorrhoeae was not significantly different from that in the original parental forms (Lawson & Douglas, 1973). Pachas and Currid (1974) found that a revertant strain of Erysipelothrix rhusiopathiae produced L forms spontaneously and at a lower osmolarity range than its parent.

The appearance of thread-like structures and distorted forms in stained films might be due to mechanical injury during preparation of the film or to the hypotonic solution used in Gram or Giemsa staining. The ability of most of the round forms to withstand the usual staining procedures demonstrated the difference in osmotic resistance between the L elements. Lysis of the lysozyme-induced round forms suggested that these forms were of a different nature than those induced by other procedures. Razin and Argaman (1963) demonstrated that spheroplasts and L; forms were more resistant to osmotic lysis than the protoplasts. They related this to the presence of a modified cell wall in L forms and spheroplasts. Dienes' dry stain preserved the structure of the colony. Better pictures were obtained when the stain was used without fixation than with fixation but the preparation was not permanent and lasted only for 1-2 days. Gram staining was mainly used and it was quick in testing for the presence or absence of L elements.

Part III: Biochemical characters and sensitivity tests of *Actinobacillus* and *Pasteurella* revertants and their parents

1 - Introduction

It has been reported that all the biochemical activities of bacterial forms may be demonstrated by their variants. In a number of studies the differences between the parents and their L forms or revertants were small. The small round forms of a stable Proteus L form studied by Weibull and Beckman (1960) contained succinic dehydrogenase, catalase and ribonuclease. Weibull and Gyllang (1965) found that, unlike the vegetative forms, the L forms of a Staphylococcus and a diphtheroid organism were devoid of catalase and cytochromes while those of a Gram negative organism Proteus mirabilis, contained the same cytochrome as normal Proteus.

Revertants of Salm. typhi (Kagan & Levashov, 1957) were not identical with the vegetative forms. On reversion none of the Staph. aureus which had reverted possessed all the characters of the original strain (Watanakunakorn & Bakie, 1973). The authors found that the type of antibiotic used in the induction of L forms affected the reactions of the revertants. The 3 revertant strains from methicillin-induced L forms and the 8 strains from cycloserine-induced L forms retained their phage type, but the remaining 4 revertants from cycloserine-induced L forms and all the 11 revertants of vancomycin-induced L forms were untypable.

All revertants of group A streptococci tested by Schmitt-Slomska et al. (1967) fermented mannitol. This

character was not stable in Staph. aureus revertants (Simon & Yin, 1970). Watanakunakorn and Bakie (1973) reported that 11 out of 22 reverted Staph. aureus were positive for fermentation of mannitol.

Schmitt-Slomska et al. (1967) inoculated streptococcal L forms intravenously and intraperitoneally into mice and found that the revertants recovered from the inoculated mice showed the same virulence as the parents. In N. meningitidis the virulence of the revertants was comparable to that of the parent (Bohnhoff & Page, 1968). Production of enterotoxin by Staph. aureus revertants depended on the type of toxin (Czop & Bergdoll, 1970). The original virulence of the parent strain of K. pneumoniae was missing when the organism reverted and was not restored by 40 intraperitoneal passages in mice (Guze et al., 1976).

Revertants were reported to be more resistant to the L form-transforming antibiotic (Marston, 1961) and they were more easily induced to L forms than the parents (Roberts & Wittler, 1966; Bohnhoff & Page, 1968; Roberts, 1968; Madoff, 1970).

In this investigation the biochemical properties and fermentative ability of the vegetative bacteria and their revertants from L forms which had been previously induced by cycloserine were studied. These properties were investigated immediately after reversion from the L forms and at intervals after passages of revertants in the laboratory. L form-inducing properties and the sensitivity

of the revertants to cycloserine were compared with those of the parents under similar cultural conditions.

2 - Materials and Methods.

L forms of seven strains of Actinobacillus and three strains of P. multocida were reverted after being passaged in cycloserine media. Reversion was carried out by propagating L growth 3 times on the same medium in the absence of the inducing agent. Then it was transferred to nutrient broth and passaged 3-4 times. Parents and revertants were grown in meat piece broth at 37°C overnight, stored at 4°C and passaged every two weeks. Overnight nutrient broth cultures of parents and revertants were used in the test. Parent and revertant of each strain were tested simultaneously under the same experimental conditions. Tests were repeated when revertants were passaged 15, 20 and 30 times in nutrient broth.

Table IX shows the number of passages, concentration of cycloserine and the type of growth at the time of reversion for each strain.

Characterisation tests.

Cultures were incubated at 37°C throughout the work. Each strain and its revertant(s) were subjected to the following tests:-

Table IX

Number of subcultures of L forms of Actinobacillus and Pasteurella strains, cycloserine concentrations and type of growth at reversion time

Strain No.	No. of subcultures on CA	cycloserine µg/ml	No. of subcultures on AJA	cycloserine µg/ml	Type of growth at time of reversion
B82/68	118	3500			H
FS1144/68	36	300			L
M618/70	116	900			L
G14/3/68	98	1300			L
DV5 outside 2	30*	350	219	20	L
B967/68	30*	130	210	120	L
P63/8/75	35	360			L
6L	116	2600			L
21	40	700			H
17	40	700			H

L = L growth. H = heteromorphic growth.

*Strains DV5 outside 2 and B967/68 were subcultured on AJA 189 and 180 times respectively before they were transferred to CA.

(i) Motility.

(ii) Catalase production.

(iii) Oxidase activity.

(iv) Asculin hydrolysis.

(v) Production of ammonia.

(vi) Liquefaction of gelatin.

(vii) Oxidation of gluconate.

(viii) Growth on MacConkey's medium.

(ix) Hydrolysis of hippurate.

(x) Production of hydrogen sulphide.

(xi) Production of indole.

(xii) Growth in KCN medium.

(xiii) Malonate utilization.

(xiv) Deamination of phenylalanine.

(xv) Methylene blue reduction test.

(xvi) Methyl red test.

(xvii) Vöges-Proskauer test.

(xviii) Nitrate reduction.

(xix) Nitrite reduction.

(xx) Phosphatase test.

(xxi) Haemolysis on blood agar.

(xxii) Hydrolysis of urea.

(xxiii) Fermentative activity.

(xxiv) β-galactosidase activity.

(i) Motility.

The activity was determined by examining a 24 hour old nutrient broth culture in a hanging drop preparation.

(ii) Catalase production.

The test organism was grown on a nutrient agar slope. 1 ml of 3% hydrogen peroxide was added to the growth. Evolution of gas immediately or within 5 minutes indicated catalase activity.

(iii) Oxidase activity.

The method used was that of Kovacs (1956) 2-3 drops of freshly prepared tetramethyl-p-phenylenediamine dihydrochloride solution in distilled water were placed on a filter paper (Whatman No.1) in a petri dish. The test organism grown on nutrient agar was removed with a glass rod and smeared on the wet filter paper. A dark purple colour within 10 seconds indicated a positive result.

(iv) Aesculin hydrolysis.

0·1% aesculin and 0·05% ferric citrate were added to peptone water. The inoculated broth was incubated and examined daily for up to 7 days. Blackening of the medium indicated hydrolysis of the aesculin. Streptococcus faecalis and Streptococcus agalactiae were included as positive and negative controls respectively.

(v) Production of ammonia.

The method used was that of Wilson and Miles (1964, p.492). Peptone water was inoculated and incubated at 37°C for 5 days. A positive reaction showed a brown colour when Nessler's reagent (B.D.H.) was added.

(vi) Liquefaction of gelatin.

Nutrient gelatin was inoculated with a straight wire and incubated for 14 days. Every 2-3 days the medium was cooled for 2 hours and examined for liquefaction.

E.coli and Staph. aureus were included as negative and positive controls. An uninoculated medium was included as a medium control.

(vii) Oxidation of gluconate.

Gluconate broth (Shaw & Clarke, 1955) was inoculated and incubated for 48 hours. 1 ml of Benedict's reagent for reducing sugars was added to 5 ml culture. The mixture was boiled for 10 minutes. Formation of a brown or orange precipitate indicated a positive reaction.

Klebsiella aerogenes served as a positive control and E.coli as a negative control.

(viii) Growth on MacConkey's medium.

MacConkey's agar (Oxoid) was inoculated with an overnight nutrient broth culture and examined for the presence of growth after 48 hours.

(ix) Hydrolysis of hippurate.

The organism was grown in hippurate broth (Hare & Colebrook, 1934) for 4 days. An uninoculated medium was set as a control. To 1 ml of the control medium varying amounts of acid ferric chloride solution (0·2, 0·3, 0·4, 0·5 ml) were added and shaken immediately. The smallest

amount of acid ferric chloride which gave a clear solution was then added to 1 ml of fluid culture. A heavy precipitate showed a positive result but with a negative test no precipitate occurred.

(x) Production of hydrogen sulphide.

Hydrogen sulphide production was detected by inserting a lead acetate paper strip between the cap and the bottle containing nutrient broth inoculated with a drop of overnight broth culture. Examination was continued for 14 days. Blackening of the paper indicated a positive reaction.

(xi) Production of indole.

Peptone water was inoculated and incubated for 48 hours. 0·5 ml Kovacs' reagent was added and the medium was shaken well and examined after 1 minute. A red colour in the top layer indicated a positive reaction.

(xii) Growth in KCN medium.

The ability of the organism to grow in the presence of cyanide was tested in KCN broth of Rogers and Taylor (1961). The KCN and the base media were inoculated with a loopful of an overnight nutrient broth culture and incubated for 48 hours. Turbidity in the KCN bottle constituted a positive reaction. A negative result with no growth in the KCN bottle was confirmed by turbidity in the basal medium. Klebsiella aerogenes and E.coli were included as positive and negative controls respectively.

(xiii) Malonate utilization.

Malonate-phenylalanine medium of Shaw and Clarke (1955) was inoculated and incubated for 24 hours. A positive reaction was indicated by a deep blue colour, a negative reaction by the unchanged greenish colour of the medium. The medium was retained for the phenylalanine deamination test.

(xiv) Deamination of phenylalanine.

After recording the malonate test, the culture was subjected to the test for the presence of phenylpyruvic acid (Shaw & Clarke, 1955). The medium was acidified with 0·2 ml of 0·1 N-HCl followed by 0·2 ml of 10% aqueous ferric chloride solution. A positive reaction was indicated by a green colour which quickly faded. A yellow colour indicated a negative result.

(xv) Methylene blue reduction test.

The organism was grown overnight in nutrient broth. A drop of 0·1% aqueous methylene blue was added to the culture which was then reincubated for 15 minutes. The absence of the blue colour indicated a positive result while its persistence indicated a negative result.

(xvi) Methyl red test.

The organism was grown in glucose phosphate peptone water (Roger & Taylor, 1961) for 48 hours. Two drops of 0·04% alcoholic solution of methyl red were added.

A red colour developed with a positive reaction, yellow colour with a negative result.

(xvii) Vöges-Proskauer test.

This is to test for acetylmethylcarbinol production from glucose. After completion of the methyl red test, 0·6 ml of 5% α-naphthol solution and 0·2 ml of 40% KOH were added to the culture. A positive test was indicated by the development of a strong red colour within 15 minutes.

Enterobacter cloacae and E.coli were tested as negative and positive controls.

(xviii) Nitrate reduction.

The medium used was that described by Cowan and Steel (1974, p.153). The test was done after 48 hours incubation. The presence of nitrite in the medium was detected by the addition of 1 ml of 0·8% solution of sulphanilic acid in 5N acetic acid and 1 ml of 0·5% solution of α-naphthylamine and 5N acetic acid. A red colour indicated a positive reaction. A negative reaction was confirmed by the addition of zinc dust (up to 5 mg per ml of culture). A red colour at this stage indicated the presence of nitrate in the medium, thus demonstrating the failure of the organism to reduce nitrate.

(xix) Nitrite reduction.

The medium used was that described by Cowan and Steel (1974, p.153). The test was done after 7-14 days of

incubation. The same reagents used in the nitrate reduction test were added to the culture. A positive reaction was indicated by the absence of a red colour while the presence of the colour indicated a negative reaction.

(xx) Phosphatase test.

Phenolphthalein phosphate agar (Cowan & Steel, 1974, p.153) was lightly inoculated to obtain isolated colonies. After 18 hours 0·1 ml ammonia (Sp. gr. 0·88) was placed in the lid of the petri dish and the medium was inverted above it. Phosphatase activity was demonstrated by the development of pink colonies.

(xxi) Haemolysis on blood agar.

Haemolytic property of the organism was demonstrated by inoculating blood agar plates prepared with horse blood (oxalated) or sheep blood (citrated). The pattern of haemolysis was recorded after 24 and 48 hours incubation.

(xxii) Hydrolysis of urea.

The medium was prepared as described by Christensen (1946). Heavily inoculated slopes were incubated and examined daily for 14 days. Development of a pink colour indicated a positive reaction.

(xxiii) Fermentative activity.

The medium consisted of peptone water to which 0·5% of the fermentable substrate was added. The indicator used was 0·2% bromothymol blue (Cruickshank, 1965, p.815). Two drops of an overnight broth culture of the test organism were inoculated into the following sugars, glucose, arabinose, lactose, maltose, mannitol, raffinose, salicin, sorbitol, sucrose, trehalose, xylose, inulin, inositol, rhamnose, dulcitol, dextrin, glycerol, galactose and mannose. Results were recorded daily for 14 days. A yellow colour indicated a positive reaction. Incomplete colour changes were recorded as negative.

(xxiv) β-galactosidase activity.

The organism was grown in the peptone medium containing O-nitrophenyl-β -D-galactopyranoside (ONPG) described by Lowe (1962) and incubated for 24 hours. β -galactosidase activity was indicated by the change of the colourless medium to yellow due to O-nitrophenol.

Sensitivity tests.

Alexander-Jackson agar (AJA), charcoal agar (CA) and cycloserine were the same as those used in part II. Parents and revertants were those used in the biochemical tests. Revertants were passaged 6-8 times in reversion medium.

The minimum inhibitory concentration (MIC) of parent and revertant(s) of each strain was determined using AJA

and CA media and cycloserine as the inducing agent. The test organism was grown in nutrient broth overnight. Cultures of all the organisms contained about $1{\cdot}6 \times 10^{10}$ viable organisms. Plates containing 10 ml of the medium were inoculated with 0·1 ml of the culture. Concentration of cycloserine started from 5 µg/ml and continued at 5 µg/ml differences. Plates were left on the bench to dry and then incubated at 30°C for 48 hours in an inverted position.

3 - Results

Characterisation tests.

All the Actinobacillus strains tested and their revertants were non-motile and showed positive reactions in the oxidase test. Neither ammonia nor indole was produced. All the strains and the revertants failed to liquefy gelatin and to oxidize gluconate. All the organisms tested were able to grow on MacConkey's agar and to hydrolyse hippurate, but none of them showed positive reactions with the malonate or phenylalanine tests. Hydrogen sulphide was produced and nitrate was reduced by all the organisms. Methylene blue was not reduced. The methyl red test was negative and the presence of acetylmethylcarbinol in the medium was not detected. Phosphatase and urease activities were shown by all the strains, with the exception of B82/68 revertant that failed to hydrolyse urea. All the Actinobacillus organisms failed to haemolyse horse blood agar. Strain P63/8/75 produced complete haemolysis on sheep blood agar. The revertant of this strain failed to do so. All the organisms induced β-galactosidase.

Glucose, raffinose, sucrose, trehalose, xylose and inulin were fermented promptly (within 24 hours) by the parents. Revertants gave positive reactions with these substrates although some of them at a slower rate than that of the parents as shown in Table X. All the parents and revertants failed to ferment inositol, rhamnose and

Table X

Biochemical characters of parents and revertants of 7 *Actinobacillus* and 3 *Pasteurella* strains

All the 7 *Actinobacillus* and the 3 *Pasteurella* strains and their revertants gave reactions listed below.

Motility	-	Vöges-Proskauer test	-
Oxidase	+	Gelatin liquifaction	-
Gluconate	-	Malonate	-
Hippurate	+	Phenyl alanine	-
Methylene blue reduction	-	Inositol	-
Nitrate	+	Phamnose	-
Phosphate	+	Dulcitol	-
Methyl red test	-	Sucrose	+
Haemolysis of horse rbc	-		

Key

+	=	positive reaction within 24 hours.
$^{+}2, ^{+}8$	=	positive reaction after 2, 7 days.
-	=	negative reaction within 14 days.
ND	=	not done
B82/68R	=	revertant of B82/68 from CA.
DV5 outside 2 AJA R	=	revertant of DV5 outside 2 from AJA.

The biochemical and fermentative reactions of any strain and its revertant(s) are given in two consecutive pages.

Table X

Strain No.	Catalase	Growth in KCN	Aesculin hydrolysis	Nitrate reduction	Growth on MacConkey	Indole production	Urea hydrolysis	H_2S production	Ammonia production	ONPG	Haemolysis of sheep rbc
B82/68	-	-	-	ND	+	-	+	+	-	+	-
B82/68R	-	-	-	ND	+	-	-	+	-	+	-
FS1144/68	-	-	-	ND	+	-	+	+	-	+	-
FS1144/68R	-	-	-	ND	+	-	+	+14	-	+	-
M618/7O	-	-	-	ND	+	-	+	+	-	+	-
M618/7OR	-	-	-	ND	+	-	+2	+	-	+	-
G14/3/68	-	-	-	ND	+	-	+	+	-	+	-
G14/3/68R	-	-	-	ND	+	-	+	+	-	+	-
DV5 outside 2	+	-	-	ND	+	-	+	+	-	+	-
DV5 outside 2R	+	-	-	ND	+	-	+	+	-	+	-
DV5 outside 2 AJA R	+	-	-	ND	+	-	+	+	-	+	-
B967/68	-	-	+	ND	+	-	+	+	-	+	-
B967/68R	-	-	-	ND	+	-	+	+	-	+	-
B967/68 AJA R	-	-	+	ND	+	-	+	+	-	+	-
P63/8/75	+	-	+	ND	+	-	+	+4	-	+	+
P63/8/75R	+	-	-	ND	+	-	+	+1O	-	+	-
17	+	+	-	+7	-	+	-	+2	+	-	-
17R	+	+	-	+7	-	+	-	+3	+	-	-
21	+	+	-	+7	-	+	-	+	+	-	-
21R	+	+	-	+7	-	+	-	+	+	-	-
6L	+	+	-	+7	-	+	-	+	+	-	-
6LR	+	+	-	+12	-	+	-	+3	+	-	-

Fermentation of

Strain No.	Glucose	Arabinose	Lactose	Maltose	Mannitol	Raffinose	Salicin	Sorbitol	Trehalose	Xylose	Inulin	Dextrin	Glycerol	Mannose	Galactose
B82/68	+	+8	+	+	+	+	-	-	+	+	+	-	+	+1	+8
B82/68R	+	+14	-	-	+	+	-	+2	+	+	+	-	-	+6	+10
FS1144/68	+	+2	+	+	+	+	+	+	+	+	+	+	+5	+4	+
FS1144/68R	+	+14	+	+	+	+	+	+2	+	+	+	+2	+14	+5	+
M618/7O	+	-	+	+	+	+	-	-	+	+	+	+4	-	+	+
M618/7OR	+8	-	+8	+	+6	+14	-	-	+6	+	+4	+4	-	-	+10
G14/3/68	+	+	+2	+2	+	+	-	-	+	+	+	+6	+4	+4	+
G14/3/68R	+	-	+4	+2	+	+11	-	+	+	+	+	+6	+6	-	+
DV5 outside 2	+	+2	+	+	+	+	-	+	+	+	+	+	-	+	+
DV5 outside 2R	+	+14	+	+	-	+	+	-	+	+	+	+	+	+14	+
DV5 outside 2	+	+14	+	+	-	+	+	-	+	+	+	+	+	+	+
AJA R															
B967/68	+	-	+	+	-	+	+	-	+	+	+	+	+3	+	+
B967/68R	+4	-	+2	+4	-	+5	+5	-	+2	+4	+9	+2	+5	+6	-
B967/68	+	-	+	+	-	+	+	-	+	+	+	+	+9	+	+
AJA R															
P63/8/75	+	+	+	+	-	+	+	-	+	+	+	-	-	+	+
P63/8/75R	+	+4	+	+	-	+	+	-	+	+4	+	-	-	-	+
17	+	-	-	-	+	-	-	+	-	+2	+	-	+6	+2	+
17R	+	-	-	-	+	-	-	+	-	+2	+	-	+6	+2	+
21	+	-	-	-	+12	-	-	+	-	+2	+	-	+5	+	+
21R	+	-	-	-	+12	-	-	+5	-	+2	+3	-	-	-	+2
6L	+	-	-	-	+	-	-	+	-	+	+	-	+6	+	+
6LR	+	-	-	-	+2	-	-	+6	-	+3	+	-	+6	+	+

dulcitol. With the remaining biochemical tests and fermentative activities, there were variations in reaction either between strains or between revertants and their parents and these are presented in Table X.

Catalase production was detected in strain DV5 outside 2 and P63/8/75 and their revertants. Aesculin was hydrolysed by strain B967/68 and its revertant from AJA and by strain P63/8/75, but the revertants of strains B967/68 and P63/8/75 failed to give positive reactions. Arabinose was not fermented by strains M618/70 and B967/68 and their revertants nor by the revertant of strain G14/3/68. Lactose and maltose were not fermented by B82/68 revertant. Revertants of strain DV5 outside 2 failed to ferment mannitol, while the parent did so. Mannitol was also not fermented by strains B967/68 and P63/8/75 and their revertants. Salicin was not fermented by either parents or revertants of strains B82/68, M618/70 and G14/3/68. Revertants of strain DV5 outside 2 gained the ability to ferment salicin, a characteristic not shown by the parent strain. Sorbitol was not fermented by strain B82/68, strain M618/70 and its revertant, strain G14/3/68, revertants of strain DV5 outside 2, strain B967/68 and its revertants and strain P63/8/75 and its revertant but it was fermented by revertants of strains B82/68 and G14/3/68. Strains B82/68 and P63/8/75 and their revertants did not ferment dextrin. Glycerol was not fermented by revertant of strain B82/68, strain P63/8/75 and its revertant, strain M618/70 and its revertant

and strain DV5 outside 2. Revertants of strains M618/70, G14/3/68 and P63/8/75 failed to ferment mannose as did their parents. Only the revertant of strain B967/68 from CA failed to ferment galactose.

The three strains of P. multocida reacted similarly. Parents and revertants were non-motile, catalase and oxidase positive. Ammonia, indole and hydrogen sulphide were produced. All the organisms failed to grow on MacConkey's agar or to convert gluconate to 2-keto gluconate. None of the organisms hydrolysed aesculin, urea or liquefied gelatin. β-galactosidase was not induced and methylene blue was not reduced. All the organisms grew in potassium cyanide and hydrolysed hippurate. Both methyl red and acetylmethylcarbinol tests were negative. None of the strains showed malonate or phenylalanine positive reactions. Nitrate and nitrite were reduced and phosphatase activity was positive.

Pasteurella strains and their revertants fermented glucose, mannitol, sucrose, sorbitol, xylose, inulin and galactose. None of them fermented arabinose, lactose, maltose, raffinose, salicin, trehalose, inositol, rhamnose, dulcitol or dextrin. All the organisms, with the exception of strain 21 revertant, fermented glycerol and mannose.

On further propagation in nutrient broth, revertants behaved more like the parents. The revertant of strain B82/68 fermented lactose, maltose and glycerol after being passaged 15 times. The ability of strain B82/68 revertant to hydrolyse urea was restored after the revertant

had been passaged 20 times. Strain G14/3/68 revertant passaged 20 times fermented arabinose within 11 days but failed to ferment mannose as also did the parent. Fermentation of mannose was shown by strain P63/8/75 revertant that had been passaged 20 times. Strain M618/70 revertant had lost the ability to ferment mannose, but this character was regained after 30 passages. Mannitol and sorbitol were not fermented by DV5 outside 2 revertants that had been subcultured 30 times in nutrient broth. Strain B967/68 revertant from CA did not ferment galactose or hydrolyse aesculin after 15 passages, but aesculin hydrolysis was regained after 20 passages. Aesculin hydrolysis and haemolysis on sheep blood agar were not shown by strain P63/8/75 revertant passaged 30 times.

Strain 21 revertant regained its ability to ferment glycerol and mannose after being subcultured 20 times.

Sensitivity tests.

The minimum inhibitory concentration of cycloserine for Actinobacillus was 5-10 µg/ml on AJA and 10-15 µg/ml on CA. Revertants were resistant to high cycloserine concentrations compared with the parents. L forms from revertants could be induced more easily on high cycloserine concentrations than those from the parents. When revertants were subcultured on CA or AJA containing the MIC for the parents, the growth obtained was confluent, thick and mainly on the surface. Stained preparations

showed that growth was composed mainly of transitional forms with some normal bacillary forms.

The parents of Pasteurella were induced on 105 µg/ml cycloserine on CA, while revertants were transformed to L elements when 150 µg/ml were used. A difference in the inducing concentration of 10 and 20 µg/ml was observed between parents and revertants when AJA was used.

The results with individual strains and their revertants are presented in Table XI.

Table XI

The minimum inhibitory concentrations of cycloserine on charcoal agar (CA) and Alexander-Jackson agar (AJA) for Actinobacillus and Pasteurella strains and their revertants

Strain No.	Cycloserine concentration µg/ml		Strain No.	Cycloserine concentration µg/ml	
	on CA	on AJA		on CA	on AJA
B82/68	10	5	B967/68	10	5
B82/68R	55	25	B967/68R	35	30
FS1144/68	15	10	B967/68R	30	50
FS1144/68R	65	25	AJA R		
M618/70	10	5	P63/8/75	15	10
M618/70R	180	25	P63/8/75R	360	80
G14/3/68	10	10	17	105	100
G14/3/68R	100	90	17R	350	110
DV5 outside 2	15	10	21	105	100
DV5 outside 2R	350	100	21R	150	110
DV5 outside 2	30	20	6L	90	90
AJA R			6LR	150	110

4 - Discussion

Characterisation tests.

Prolonged cultivation of Actinobacillus and Pasteurella on high concentrations of cycloserine resulted in some changes when the L growth was reverted and compared with the parent. Most of the revertants on agar were overgrown by contaminants because they were slow-growing organisms although they reached the same degree of growth as the parent within 24 hours in broth. Bohnhoff and Page (1968) observed that revertants of N. meningitidis grew more slowly than the parent. Growth of K. pneumoniae revertants in broth was slower than that of the parent but the same level was reached within 18 hours (Guze et al., 1976).

All revertants behaved similarly to their parents in catalase and oxidase production, growth in KCN, reduction of nitrate and nitrite, indole, ammonia and hydrogen sulphide production, gelatin liquefaction, gluconate oxidation, methylene blue reduction, hippurate hydrolysis, induction of β -galactosidase and ability to grow on MacConkey's agar. The haemolytic activity on sheep blood agar was found to vary between revertant and parent with one strain, P63/8/75, which finding is in accord with that of Simon and Yin (1970) who showed variable retention of haemolysis among different strains of staphylococci. On the contrary, revertants of group A streptococci retained their β- haemolytic ability (Schmitt-Slomska et al., 1967).

Most of the fermentation activities of the revertants occurred at a slower rate than in the parents. This observation was reported with Salm. gallinarum (Thomas, 1976)

Failure of some revertants to ferment sugars that were fermented by the parents have been reported (Kagan & Levashov, 1957; Simon & Yin, 1970, Watanakunakorn & Bakie, 1973). In the present study revertants of strain DV5 outside 2 failed to ferment mannitol unlike the parent, while revertants of other strains behaved like the parents. Simon and Yin (1970) noted that mannitol fermentation varied among Staph. aureus revertants. Watanakunakorn and Bakie (1973) reported that, of the 22 strains of Staph. aureus revertants they studied, 11 did not ferment mannitol and they ascribed this to the type of antibiotic used. They observed that all cycloserine and methicillin revertants retained the ability to ferment mannitol. These authors stated that revertants regained some of the other lost characters after several passages.

Some of the revertants in the present work were able to split sugars not decomposed by the parents. Revertants of strains B82/68 and G14/3/68 decomposed sorbitol within 48 hours and 24 hours respectively. Revertants of strain DV5 outside 2 fermented salicin and glycerol within 24 hours. Kagan and Levashov (1957) reported that Salm. typhi revertants attacked one or more sugars not attacked by the parents.

The differences between parents and revertants were not major and most of the results agreed with other investigations demonstrating the similarities between the metabolic properties of bacteria and their revertants (Little et al., 1973; Thomas, 1976). Failure of some revertants immediately after reversion to show the same reactions as the parents suggested that these organisms

and their parents were not the same. Although the transformation of the vegetative forms to the unstable L growth was not a genetic change, some revertants are biochemically different. The lost characters might return after several passages.

Sensitivity tests.

These tests were done to show whether revertants were more easily induced and more tolerant to the inducing agent or were similar to the parents. The cycloserine concentration of 5 µg/ml on AJA supported transitional elements and bacillary forms of the Actinobacillus revertants. This concentration supported pure L growth when the parents were subjected to the drug for the first time. Revertants showed a significant resistance to the inducing agent. Marston (1961), studying sensitivity of staphylococci, found that revertants retained the sensitivity to the inducing agent penicillin and sensitivity to other antibiotics tested. The ease with which revertants produced L forms has been mentioned in other revertant bacteria (Roberts & Wittler, 1966; Bohnhoff & Page, 1968; Roberts, 1968; Madoff, 1970). Roberts (1968) reported that N. meningitidis revertants produced L forms spontaneously. The increased resistance of revertants was due to the presence of resistant forms during L growth propagation.

The difference between CA and AJA in supporting revertants at different antibiotic concentrations has been shown. CA supported revertants at a higher drug concentration than the AJA. This was due to the fact that L growth, before being reverted, was passaged on CA

and on a very high cycloserine concentration. The revertant of strain M618/70 which had been passaged 116 times as L growth on medium reaching the level of 900 µg/ml of cycloserine was more resistant to the antibiotic than was the revertant of strain FS1144/68 which had been passaged as L growth 36 times on media reaching the level of 300 µg/ml of cycloserine although the MIC for the parent strain FS1144/68 was higher than that for strain M618/70. This showed that the resistance of a revertant was determined by the concentration of the antibiotic used during L growth propagation and by the number of passages.

Part IV: Isolation of bacterial variants from blood samples

1 - Introduction

The isolation of bacterial variants from disease conditions has stimulated interest in these variants in chronic diseases and in conditions of unknown aetiology. Bacterial variants have been implicated in chronic and recurrent infections. They occur spontaneously without the administration of L inducing agents, although antibiotics greatly increase the incidence. Failure of treatment was found to be due to persistant variants (Mattman & Mattman, 1965). Most investigators have focused on isolation of these variants from diseased tissues. Recently examinations were extended to healthy tissues and reports continue to appear in the literature.

Pathogenicity of bacterial variants in experimental animals was far from clear cut. Young and Dahlquist (1967) demonstrated the rapid clearance of Staph. aureus L forms injected intravenously into rabbits. Godzeski et al. (1967) reported that Staph. aureus L forms injected into embryonated chicken eggs could not initiate infection. Listeria monocytogenes stable L forms were not pathogenic upon injection into mice by various routes (Brem & Eveland, 1968). Variants of other pathogenic bacteria failed to cause disease when injected in susceptible animals (Bohnhoff & Page, 1968; Little & Bosbery, 1973; Linnmann et al., 1973; Watanakunakorn & Bakie, 1974).

Some of the variants retained the ability to be as lethal or damaging as the parents. Toxigenicity of

Cl. tetani L forms was not impaired and symptoms of tetanus were produced in mice and guinea pigs (Schiebel & Assandri, 1959; Rubio-Huertos & Gonzalez-Vazquez, 1960). Kagan,G.Y.(1968), Braude (1970), Merline et al. (1971) Orr et al. (1974) and Kotlyarova et al. (1976) reported on the pathogenicity of other bacterial variants.

In 1937 Klienberger and Steabben isolated bacterial variants from the nasopharynx of rats with lung lesions. Bacteriodes L forms were recovered from purulent lesions from man (Dienes & Smith, 1944). Following these reports the isolation of variants from disease conditions continued (Alexander-Jackson, 1954; Mattman & Karris, 1966; Kagan, 1968b; Mattman, 1968; Hessburg, Mattman, Barth & Dutcheshen, 1969; Louria, Kaminski, Grieco & Singer, 1969; Zierdt & Wertlake, 1969; Charache, 1970; Swierczewski & Reyes, 1970; Wilson et al., 1970).

Chronic infections in renal disease and difficulty of treatment could be due to the presence of variants (Braude et al., 1961; Guze & Kalmanson, 1964; Gutman et al., 1965; Turck et al., 1968). In most cases treatment with antibiotics was responsible for the emergence of these forms (Voureka, 1951a; Wittler et al., 1960; Braude et al., 1961; Guze & Kalmanson, 1964). It was the view of Lapinski and Flakas (1967) that the recurrence of infection after cessation of therapy was due to the reversion of persisting L forms to bacillary forms. Turck et al. (1968) demonstrated that patients, in whom bacterial variants could be demonstrated during treatment with

antibiotics, relapsed due to the original parent strain after completion of therapy.

Spherical forms were demonstrated attached to erythrocytes of subjects affected with scarlet fever (Mule, 1954). Godzeski *et al*. (1965) isolated staphylococcal L forms from 11 patients with recurring boils. Mattman and Mattman (1965) isolated *Strep. faecalis* L forms from the blood in septicaemic conditions and in one patient with meningitis the L forms were isolated from blood and spinal fluid. Pease (1967) reported silent infections with L forms of *Listeria*. Following her research on the blood and synovial fluids of arthritic subjects, Pease (1969, 1970) believed that the infections were due to variants of *Listeria*. L forms were also reported in blood samples of healthy persons (Tedeschi *et al*., 1969a; Tedeschi & Amici, 1972). In this work experiments were designed to test for the presence of atypical forms of bacteria in blood samples of apparently healthy animals.

2 - Materials and Methods

Media

(i) Alexander-Jackson sucrose broth without serum (AJSB-S). This medium had the same composition as AJA (p. 53) except that the sodium chloride was replaced by 150 g sucrose, the glucose was included in the basic medium before it was sterilized and agarose and horse serum were omitted.

(ii) Alexander-Jackson sucrose agar without serum (AJSA-S). This was prepared by adding 0·4% agarose (Sigma) to AJSB-S to solidify the medium.

(iii) Tryptone soy agarose broth. Tryptone soy broth (Oxoid) was reconstituted as directed. 0·05% agarose was added and the pH was adjusted to 7·2. The medium was distributed into 5 ml aliquots and sterilized by autoclaving at 121°C for 15 mins.

(iv) Routine media used were blood agar and nutrient broth, prepared by the methods of Cruickshank (1965).

Blood samples.

Twelve weeks old rats of PVG cross Campbell strain were bled by heart puncture after being anaesthetised with ether. Chicken blood was collected from white Leghorn adults from the wing vein. Blood samples were carefully collected with a sterile syringe under strict sterile measures into screw-capped bottles containing 0·5 ml of 5% sodium citrate to which about 5 ml of blood

was added. Blood samples from ewes and lambs were taken by jugular venepuncture. About 5 ml of the blood was drawn into evacuated glass containers (Vacutainers: Becton & Dickinson) each containing 7·5 mg of dried sodium ethylenediaminetetraacetic acid. Lambs were delivered by caesarian section and the blood was taken immediately. Blood from ewes was taken a day before the operation was carried out and all samples were kept at 4°C for 24 - 48 hours before use. The ewes' samples were designated V277, V289, V292, V297 and 9284. The lambs' blood had been given serial numbers, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31 and 32. Lamb number 19 was from ewe V277, lamb 18 from ewe V289, lambs 17, 29 and 32 from ewe V292.

Methods.

Blood specimens were checked for contamination. Gram and Giemsa stained preparations were examined. 1 ml of rat blood was added to 9 ml of AJSB-S or 0·5 ml blood to 4·5 ml AJSB-S. In some cases 0·5 ml blood was added to 9 ml AJSB-S. Two to five bottles were inoculated from each sample. In addition, 4·5 ml nutrient broth was inoculated with 0·5 ml blood and 0·1 ml of blood was inoculated on to the surface of blood agar and AJSA-S. Plates were sealed to prevent loss of moisture and drying. All incubation was carried out aerobically at 37°C. Uninoculated AJSB-S was incubated with the cultures as the medium control.

The experiment was repeated with fresh blood samples taken from the same rats. The inoculated AJSB-S were incubated at 37°C for 9 days, then 1 ml of broth culture was transferred to 9 ml of fresh AJSB-S and incubation was continued for a further 4 days. To the 9 ml of the original culture remaining 1 ml of fresh AJSB-S was added and reincubated.

With chicken blood the same proportions were used for inoculating broth viz 1 ml of blood to 9 ml AJSB-S. The inoculated media were incubated for 9 days at 37°C and 1 ml of the culture was transferred to 9 ml of AJSB-S. After a further 4 days of incubation 1 ml of fresh AJSB-S was added to the original culture and incubation continued. The chicken blood (1 ml) was also inoculated into 5 ml tryptone soy agarose broth. The experiment with chicken blood was repeated with fresh blood samples taken from the same chickens.

With sheep blood 1 ml was added to 9 ml AJSB-S. After 6 weeks of incubation 1 ml of AJSB-S was added to the culture which was then reincubated. Control measures were set up with chicken blood and sheep blood as in the rat blood experiment.

Stained preparations of all the blood samples and subcultures on blood agar and AJSA-S were made from the blood cultures at intervals. Cultures were recorded as positive for bacterial growth if micro-organisms appeared in stained preparations or grew on agar media. When growth was detected in broth, 1 ml was transferred to 9 ml

of AJSB-S. Blood agar and AJSA-S plates were also inoculated and incubated aerobically, anaerobically and with carbon dioxide. In chicken blood cultures showing growth 1 ml was transferred to 5 ml tryptone soy agarose broth and then after incubation subcultures were made from the tryptone soy agarose broth to blood agar and AJSA-S.

Wet preparations of all blood cultures were examined by the phase-contrast microscope.

3 - Results

Rat blood.

Rat 1.

In the first experiment two bottles were inoculated each containing 0·5 ml of blood and 4·5 ml AJSB-S. After 8 weeks of incubation one of the bottles showed round forms which appeared in groups and rarely singly. The round forms in groups fused together giving rise to large lobulated forms. All the structures were Gram positive (Plate 24). A pure culture of yellow colonies measuring 2 mm in diameter appeared within 24 hours on AJSA and blood agar. In the second experiment 5 bottles were inoculated. Three each containing 1 ml of blood and 9 ml AJSB-S and 2 bottles each contained 0·5 ml of blood and 4·5 ml AJSB-S. The bottles which contained 1 ml inoculum showed the following:-

One bottle which was incubated for 13 weeks carried tiny rods with knobs at the end. These forms were Gram positive and looked like diphtheroid organisms. Some of them were very tiny and appeared like Gram positive granules. Such forms were referred to as diphtheroids. Similar forms appeared in the second bottle incubated for 9 weeks. Growth was visible in AJSB-S when 1 ml of culture was transferred to 9 ml of fresh AJSB-S. Colonies were not formed on agar media even after the diphtheroid forms had been passaged 4 times in AJSB-S. The third bottle showed Gram positive cocci after 5 weeks incubation. The cocci were of two types, large cocci which were deeply stained and small cocci, fewer in number and faintly stained.

Diphtheroid forms were present. On agar media small white colonies composed of uniform coccal forms appeared within 24 hours. The rod forms did not produce colonies on agar. The last two samples which contained 0·5 ml of blood proved negative for bacterial growth both in stained preparations and on agar media.

Rat 2.

In the first experiment the two samples containing 0·5 ml of blood and 4·5 ml AJSB-S proved negative for bacterial growth. In the second experiment 4 bottles each containing 1 ml of blood and 9 ml of AJSB-S were incubated. Two bottles showed diphtheroids within 7-8 weeks. As in rat 1 the diphtheroids failed to produce colonies on agar media.

Rat 3.

After 2 weeks one of the two bottles containing 0·5 ml of blood and 4·5 ml AJSB-S showed Gram positive round forms and lobulated forms as in Plate 24. They produced yellow colonies on agar. Diphtheroid forms were present in the other sample which had been inoculated for 5 weeks. In the second experiment 1 ml of blood was added to 9 ml of AJSB-S and diphtheroids appeared in one out of five bottles after 7 weeks of incubation.

Rat 4.

Thin rods about 4 um in length, faintly stained diphtheroids and Gram positive coccal forms appeared within 5-6 weeks in the two bottles containing 0·5 ml of blood and 4·5 ml AJSB-S. There was no growth on agar from any of

these forms. In the second experiment 3 bottles each containing 1 ml of blood and 9 ml of AJSB-S were incubated. Gram positive irregular bacillus forms and tiny Gram positive coccal forms and granules appeared in one bottle after 2 weeks. A week later the growth was mainly diphtheroids and swollen rod forms (Plate 25). These forms did not grow on agar media. Another bottle showed a few Gram positive cocci which failed to grow on agar. On subculture to AJSB-S the cocci increased in number and produced small white colonies on agar media.

Rat 5.

In the first experiment 4 bottles each containing 0·5 ml of blood and 4·5 ml AJSB-S were incubated. No growth was detected in any one of them. In the second trial one bottle which contained 1 ml of blood and 9 ml AJSB-S and another bottle which contained 0·5 ml of blood and 9 ml AJSB-S showed diphtheroid forms within 4 and 10 weeks respectively. The other two bottles containing 1 ml of blood and 9 ml AJSB-S remained negative for bacterial growth.

Rat 6.

One bottle containing 0·5 ml inoculum and 4·5 ml AJSB-S revealed bacterial growth after 4 weeks of incubation. On AJSA-S small white colonies made up of Gram positive coccal forms of variable sizes appeared within 24 hours, while on blood agar the same colonies appeared after 3 days of incubation (Plate 26). Another bottle showed Gram positive cocci that produced small white colonies on both agar media. The last 3 bottles each containing 0·5 ml of blood and 4·5 ml AJSB-S were negative for bacterial growth. In the second

trial 4 out of 5 bottles containing 0·5 ml inoculum and 4·5 ml AJSB-S showed bacterial growth. Two bottles showed diphtheroids that appeared after 11 weeks. The other two showed diphtheroids, tiny rods and club-shaped organisms after 8 and 11 weeks respectively of incubation. Some small coccal forms were present. These forms did not develop further when 1 ml of culture was transferred to 9 ml of fresh broth. The organisms failed to grow on agar media.

Rat 7

Two of the 5 bottles which contained 0·5 ml of blood and 4·5 ml AJSB-S showed Gram positive cocci. Gram positive clumps and lobulated forms which appeared like cocci in a budding state were also present. This growth was noticed after incubation for 10 days and 6 weeks respectively. On agar media colonies like those from rat 1 were isolated. The third bottle revealed Gram positive cocci, diphtheroids and Gram positive rods. Only the Gram positive cocci grew on agar. The fourth bottle revealed large and small Gram positive cocci which failed to grow on agar media. When the experiment was repeated using 2 bottles with 1 ml of blood and 9 ml AJSB-S and another 2 bottles with 0·5 ml blood and 9 ml AJSB-S no bacterial growth was detected in stained preparations or isolated on agar media from any of these bottles.

Rat 8.

Gram positive clumps and lobulated forms were detected in 2 out of 3 bottles containing 1 ml of blood and 9 ml AJSB-S after 10 days and 4 weeks incubation respectively. Colonies similar to those isolated from rat 1 appeared on agar media. In the second experiment, 3 out of 4

bottles containing 0·5 ml blood and 9 ml AJSB-S revealed diphtheroid forms after 6-11 weeks of incubation. No colonies were obtained on agar media.

Rat 9.

Bacterial growth was detected in one out of 4 bottles containing 0·5 ml of blood and 9 ml of AJSB-S. Gram positive cocci some of which were large appeared after 3 weeks of incubation. On plating no growth was obtained on blood agar incubated for 7 days. Small colonies appeared on AJSA-S after 3 days incubation. Growth improved on subculture and grew when transferred to blood agar plates. Colonies were small, white and measured about 1-2 mm in diameter. Growth was made up of Gram positive cocci of different sizes. In the repeated experiment 3 bottles each containing 1 ml of blood and 9 ml AJSB-S and 2 each containing 0·5 ml of blood and 9 ml AJSB-S were incubated. One bottle containing 1 ml of inoculum and 2 bottles containing 0·5 ml of inoculum supported the growth of diphtheroids. No growth was obtained on agar media.

Rat 10.

One bottle out of two containing 1 ml of blood and 9 ml of AJSB-S showed marked turbidity after 7 days of incubation. Gram's or Giemsa stain showed tiny rods which were Gram positive. On further incubation the rods swelled at the ends or in the middle (Plate 27). The rods increased in length when 1 ml of culture was transferred

to 9 ml of AJSB-S and incubated for 48 hours (Plate 28). These forms did not grow on agar media. After 10 days of incubation the second bottle showed Gram positive cocci. The coccal forms failed to grow on blood agar incubated for 7 days, but small white colonies appeared on AJSA-S incubated for 3 days. Gram stained preparations showed Gram positive cocci of different sizes as in AJSB-S cultures (Plate 29). Growth on AJSA-S was improved with subculture and was able to grow on blood agar. In the second experiment 3 bottles each containing 1 ml of blood and 9 ml AJSB-S and 2 bottles each containing 0·5 ml of blood and 9 ml AJSB-S were incubated. No bacterial growth was detected in stained preparations or isolated on agar media.

Chicken blood.

Chicken 110.

Two bottles each containing 1 ml of blood and 5 ml tryptone soy agarose broth together with 2 bottles each containing 1 ml of blood and 9 ml AJSB-S were incubated. No bacterial growth was detected in any of the bottles. The experiment was repeated exactly with a further sample of blood taken several weeks later. One bottle containing 1 ml of blood and 9 ml AJSB-S showed diphtheroid forms within 2 weeks of incubation. These forms did not grow on agar media.

Chicken 111.

The blood sample was inoculated into tryptone soy agarose broth and AJSB-S bottles as in chicken 110. No bacterial growth was detected and similar results were obtained when the experiment was repeated.

Chicken 112.

Bacterial growth was not detected in the first experiment in which 2 bottles each containing 1 ml of blood and 9 ml AJSB-S and 2 bottles each containing 0·5 ml of blood and 4·5 ml AJSB-S were incubated. When the experiment was repeated 3 out of 5 bottles each containing 1 ml of blood and 9 ml AJSB-S showed bacterial growth. One bottle supported the growth of swollen rods within 9 days of incubation. When 1 ml of culture was transferred to 9 ml AJSB-S or 5 ml tryptone soy agarose broth, growth was rapid (within 24 hours) but no growth was obtained on agar media (Plate 30). The other two bottles showed diphtheroid forms within 18 and 20 days of incubation. These forms did not grow on agar media.

Chicken 113.

One bottle out of 4 containing 1 ml of blood and 9 ml AJSB-S showed bacterial growth after 4 weeks of incubation. Growth was made up of thin Gram positive rods among which Gram positive cocci appeared. On further incubation the rods increased in length and the cocci increased in number. No growth was obtained on agar media. In the second

experiment 4 bottles each containing 1 ml of blood and 4·5 ml AJSB-S were incubated. In two of the bottles growth was detected only when 1 ml of culture was transferred to 5 ml tryptone soy agarose broth. Growth in tryptone soy agarose broth appeared within 24 hours in one bottle and within 5 days in the other. Growth was composed of coccal or oval forms, sometimes of great size and attached to rudimentary rods. The coccal forms appeared in groups or singly. Swollen rods and club-shaped forms were present. Free spherical forms were rarely seen (Plate 31). When cultures were further incubated for 24 hours, the coccal forms enlarged and some of them became free. The rods developed into long broken filaments (Plate 32). When Gram's stain was applied, the growth consisted mainly of Gram positive rods with coccal forms rarely seen. With Giemsa stain, however, the coccal forms were in much greater evidence. Subcultures made on to blood agar or AJSA-S from broth immediately after evidence of growth in the broth had been seen yielded no growth, but if the broth was incubated a further 48 hours before subcultures were made on to solid media, small β-haemolytic colonies developed. These colonies were composed of Gram positive cocci. Growth was detected in the original AJSB-S 2 days later than in tryptone soy agarose broth and it could be subcultured on agar media.

Chicken 115.

Three out of 4 of the bottles containing 0·5 ml blood and 4·5 ml AJSB-S and the nutrient broth bottle showed Gram positive cocci after 2 days incubation. This group of cultures was considered contaminated and discarded. In the repeated experiment 3 out of 4 bottles containing 1 ml of blood and 4·5 ml AJSB-S supported the growth of rod forms mixed with coccal forms. This growth did not develop further when 1 ml of culture was transferred to 5 ml tryptone soy agarose broth. From one bottle of tryptone soy agarose broth, rod and coccal forms like those in chicken 113 were produced. A pure culture of Gram positive cocci was obtained when the tryptone soy agarose broth was plated out on agar media.

Chicken 116.

No bacterial growth was detected when two bottles each containing 1 ml of blood and 5 ml of tryptone soy agarose broth were incubated. Diphtheroid and coccal forms appeared in one out of two bottles each containing 1 ml of blood and 9 ml AJSB-S incubated for 20 days. From the broth showing growth 1 ml was transferred to each 5 ml tryptone soy agarose broth and 9 ml AJSB-S. Small white colonies which produced β-haemolysis on blood agar were obtained within 24 hours on agar media inoculated from the 24 hours tryptone soy agarose broth. Similar colonies appeared on agar media from AJSB-S when further incubated for 4 days. There was no growth detected when the experiment was repeated the same way.

All the isolated Gram positive coccal forms were identified as Staphylococcus epidermidis as they were oxidase negative, catalase positive and phosphatase negative, nitrate was reduced, the coagulase test was negative and both VP and DNase tests gave weak positive reactions. The large Gram positive lobulated forms were pigmented yellow. They gave positive results with oxidase and catalase and negative results with VP, nitrate reduction, DNase and coagulase production. The reaction with phosphatase was weak. These isolates were identified as a Micrococcus SPP.

Examination with the phase contrast microscope.

Cultures incubated for up to two weeks showed some morphological features which were not present in the blood before it was inoculated in the media. All blood cultures in general showed the same features but with slight differences between species of animal and the media used. Chicken blood samples inoculated into tryptone soy agarose broth and incubated for 10 days showed small number of spherical forms attached to red blood cells. Chains of 2-4 coccal forms hanging from red cells were seen. Some of the erythrocytes showed very tiny filaments, some of them were made up of streptococcal-like chains and spike-like projections sticking out from one or more sides of the red cells. Spherical forms both isolated and in chains were commonly seen freely floating. Dumb-bell shaped rods were seen occasionally.

The deformed shape of the red cells increased with increasing incubation period. More cells with spherical forms appeared after two weeks of incubation. The free spherical forms were in the majority (Plates 33, 34, 35 and 36).

Using AJSB-S these forms were very few and rarely seen within 10 days of incubation. The filaments that appeared in this medium within two weeks of incubation were very tiny and the spherical forms were very few compared to those in tryptone soy agarose broth. Rat and lamb blood inoculated into AJSB-S showed very few tiny filaments and spherical forms.

In chicken blood cultures stained with Giemsa, some spherical forms attached to the nuclei of the red cells appeared to be part of the nucleus. The other structures seen in phase contrast were not visible in stained preparations and no growth or multiplication was obtained in subcultures in broth or on agar media.

Sheep blood.

Ewes' blood samples V277 and 9284 were negative for bacterial growth. Samples V292 and V297 showed bacterial growth after 10 weeks of incubation. Tiny Gram positive rods with tiny knobs at the end similar to the diphtheroid forms reported previously were detected. On agar media these forms failed to grow. Sample V289 produced tiny short rod shaped organisms with swellings (Plate 37). They were numerous and much shorter than the organisms

in the two samples mentioned above. These forms did not grow on agar media. Cultures were reincubated for 12 weeks and subcultures were made by transferring 1 ml of culture to 9 ml AJSB-S. Within 3-5 days the organisms flourished and multiplied giving rise to large numbers of these forms. Growth only occurred in broth.

No bacterial growth was detected in lamb blood samples 17, 19, 20, 21, 22, 23, 24, 25, 26, 27, 29, 30 and 31. Gram positive cocci and a few Gram positive rods were detected in sample 18 which had been incubated for 12 weeks. Two days later the rods bulged into club-shaped forms. The tapering ends of these forms were broken into small coccal forms and granules. These forms did not grow on agar immediately after being detected in broth but, after a further 4 days of incubation in broth, pin point colonies appeared when subcultures were made and incubated for 3 days at 37°C. When films were stained with Giemsa or Gram the forms were larger than those in broth. Rods of variable diameters were present, irregular forms and club-shaped organisms joined together were seen. Coccal forms and other pleomorphic forms were present. On repeated passages on blood agar the culture was mainly made up of swollen Gram positive forms with tapering ends that disintegrated into small coccal forms and granules. When a piece of agar containing small colonies was transferred to AJSB-S or nutrient broth and incubated for 48 hours, growth started as small round forms in chains with larger round forms which further grew into club-shaped organisms

(Plates 38, 39 & 40). The morphology of the organism did not change after being passaged 15 times on blood agar. No improvement of growth was observed when plates were incubated either anaerobically or with CO_2 or when the incubation period was increased. This lamb was from the ewe number V289.

Blood sample 28 incubated for 3 weeks showed coccal forms and club-shaped forms. The morphology of all forms was similar to those isolated from sample 18 except that these forms were larger in size (Plate 41). On agar media incubated aerobically growth was very slow and tiny colonies appeared after 2-3 days of incubation. The morphology of these organisms did not change upon subculturing on blood agar. When plates were removed from the incubator and growth was left on the bench for 4-6 days, large raised glistening colonies appeared among the old colonies. Removal of these colonies with a loop indicated that these forms were burrowing in the medium. Gram stained preparations revealed filamentous forms, with swellings at the end or in the middle, and round forms of different sizes (Plate 42). Granules were faintly stained. All the structures were Gram negative and deeply stained. Similar forms appeared from several other plates that had aged on the bench. On subculture, these forms died out and the original culture suddenly failed to grow before it had been identified.

Blood sample 32 produced large coccal or oval forms

which were Gram negative and Gram positive. On agar media small colonies 1-2 mm in diameter appeared within 24 hours incubation and these enlarged and turned yellow on further incubation. The colonies were composed of Gram negative and Gram positive forms, most of which were in groups.

The isolate from lamb 18 was identified as a Corynebacterium Spp. because it was non-motile, grew aerobically and anaerobically, produced catalase but failed to produce oxidase or ferment glucose. The isolate from lamb 32 was identified as a Micrococcus Spp. as it gave similar reactions to the Micrococcus isolated from the rat blood samples.

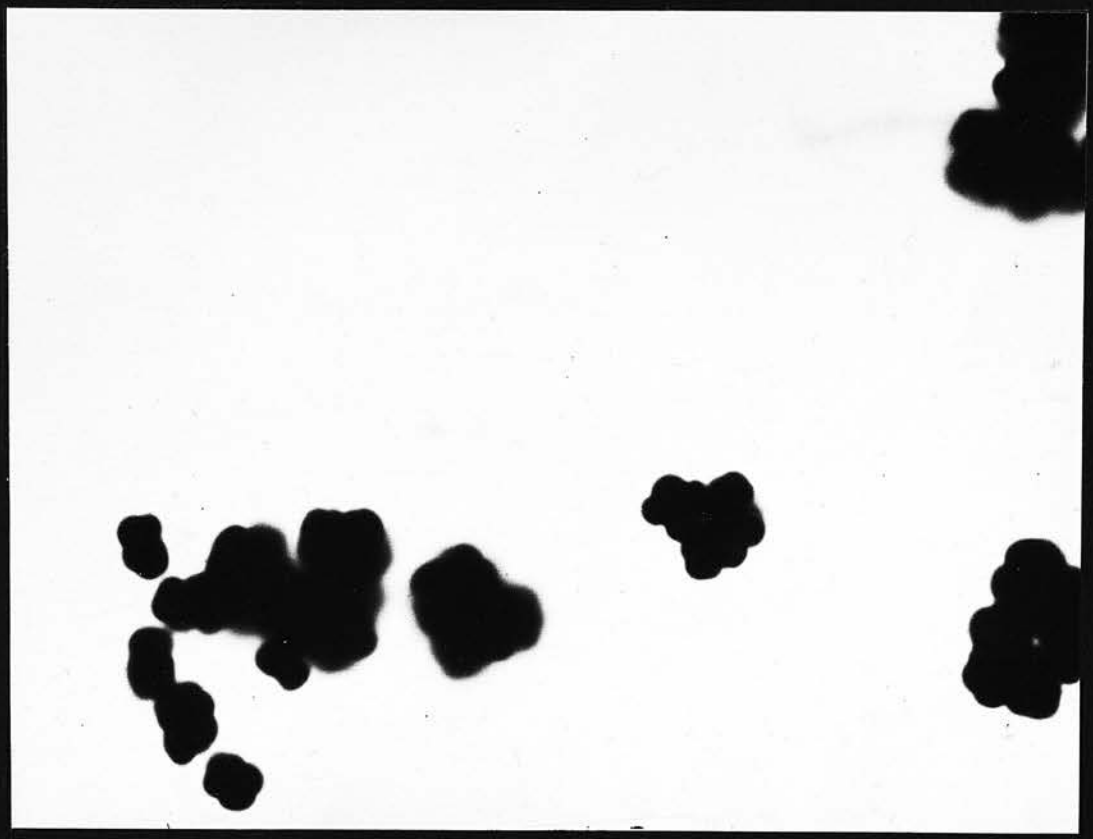

Plate 24

Blood taken from Rat No.1 on blood agar showing round forms in groups. Gram X 2500.

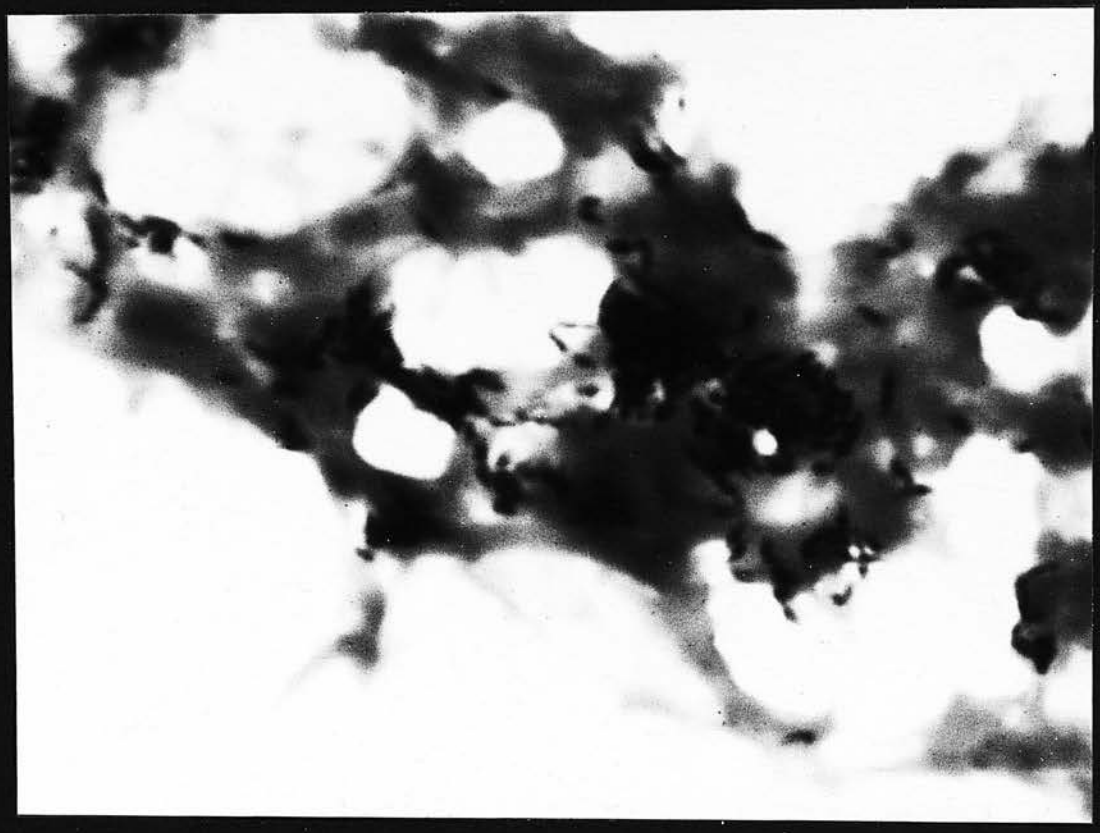

Plate 25

Blood taken from Rat No.4 in AJSB-S showing diphtheroid forms and swollen rods. Gram X 2500.

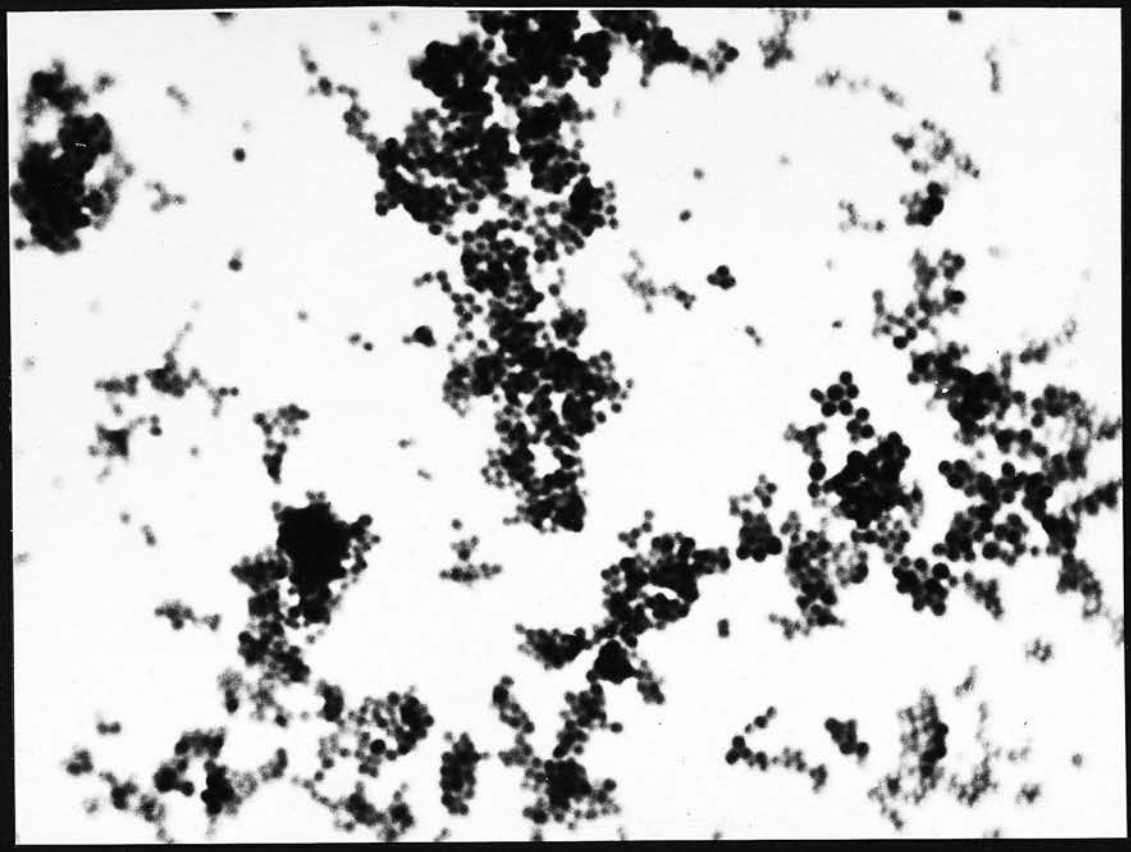

Plate 26

Blood taken from Rat No.6 on AJSA-S showing coccal forms of variable sizes. Giemsa X 2500.

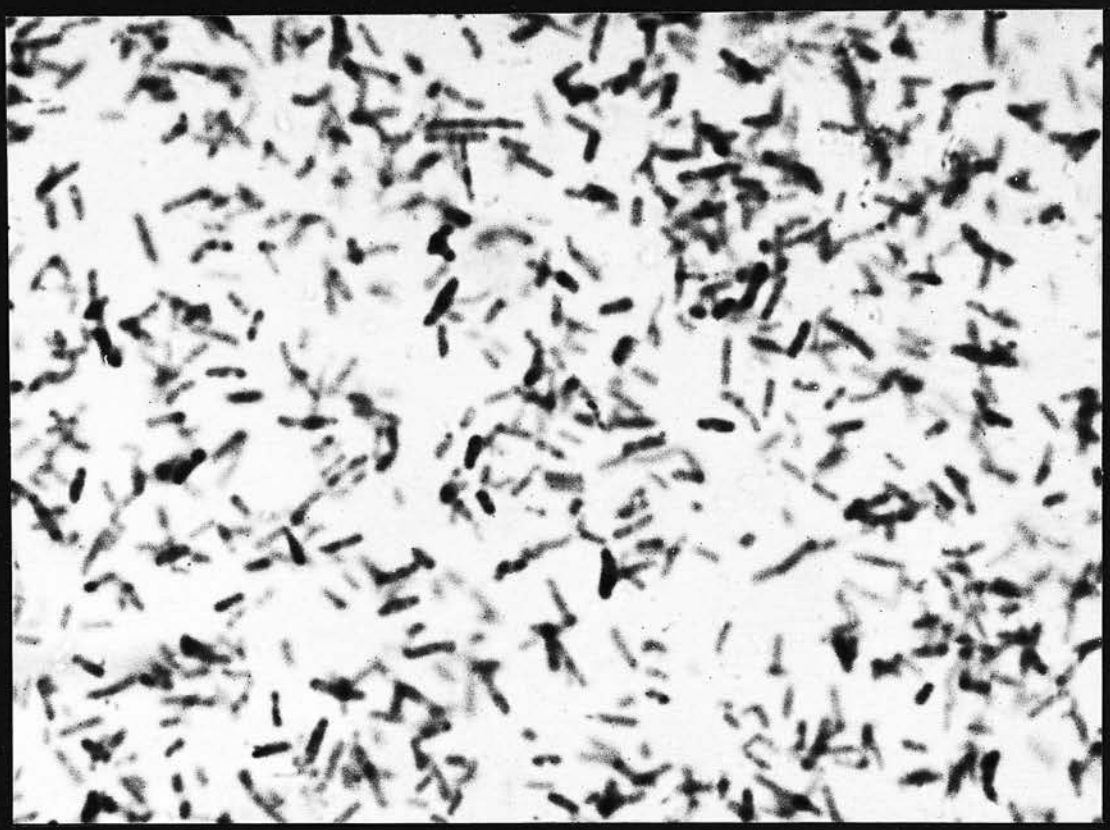

Plate 27

Blood taken from Rat No.10 in AJSB-S showing swelling of the rods at the ends or in the middle. Giemsa X 2500.

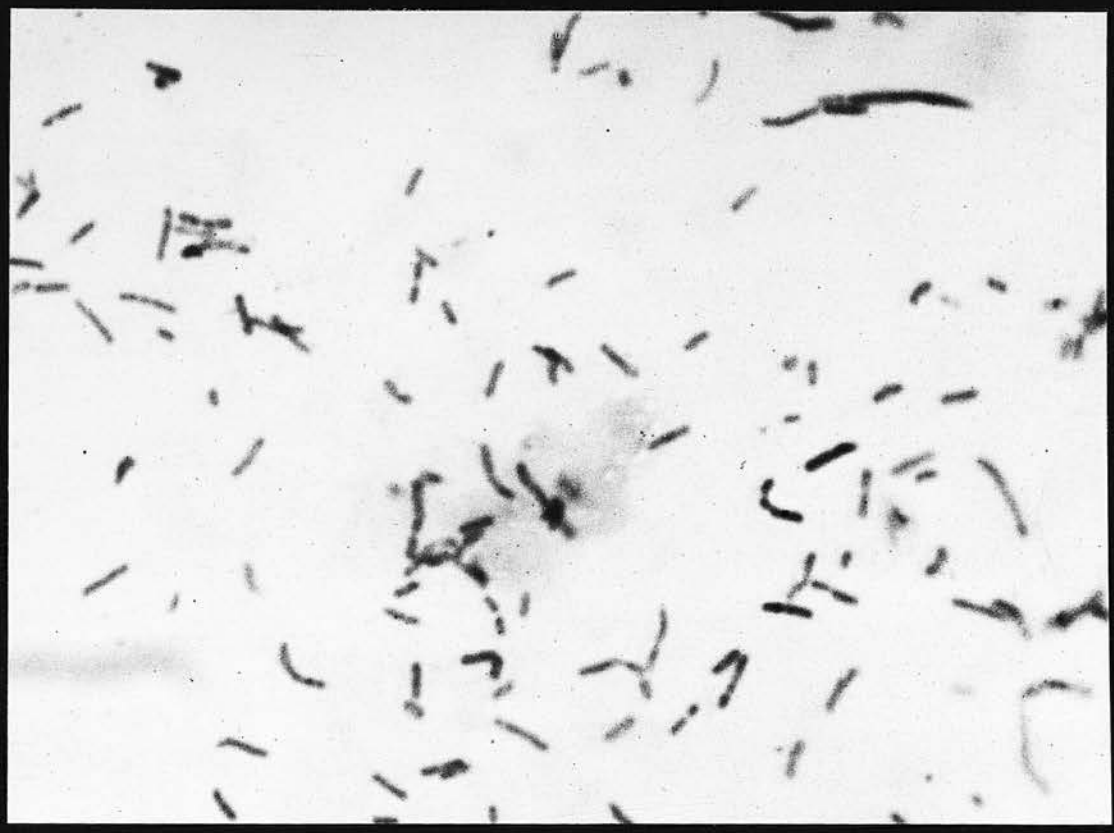

Plate 28

Blood taken from Rat No.10 showing long rods which developed when 1 ml of blood culture was transferred to 9 ml of AJSB-S and incubated for 48 hours. Giemsa X 2500.

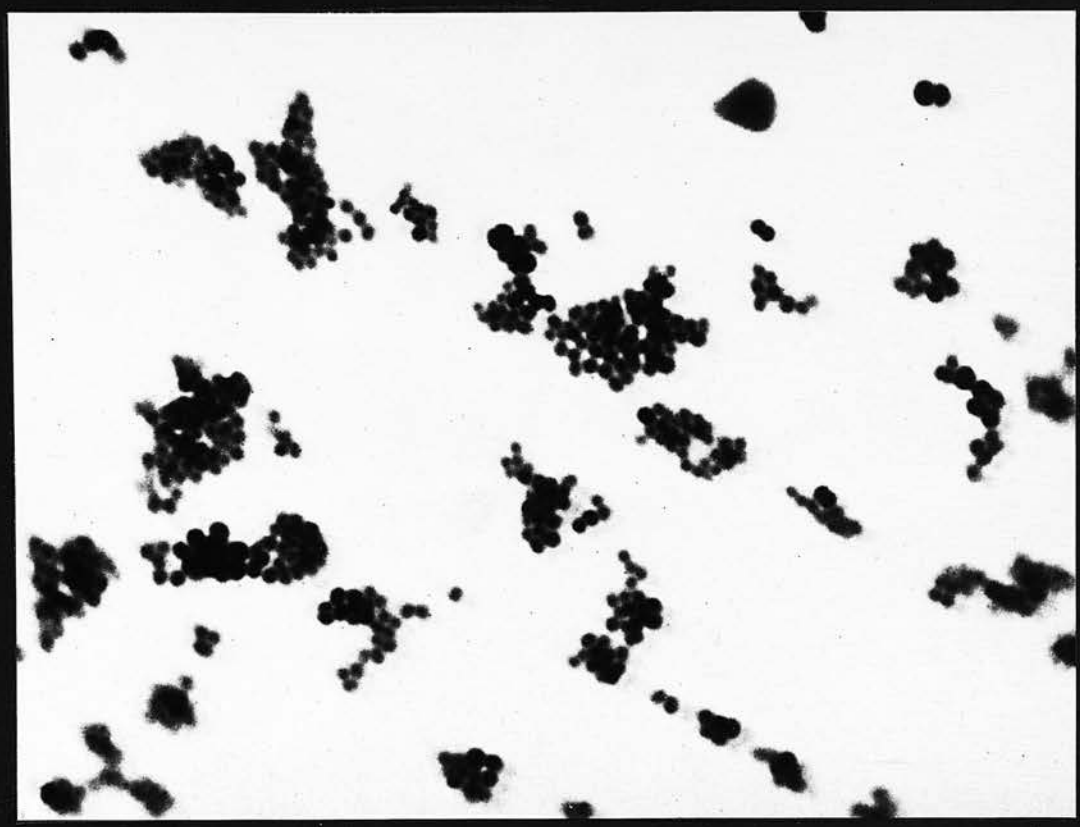

Plate 29

Blood taken from Rat No.10 on AJSA-S showing coccal forms of different sizes. Giemsa X 2500.

Plate 30

Blood taken from Chicken No.112 in AJSB-S showing swollen rods. Giemsa X 2500.

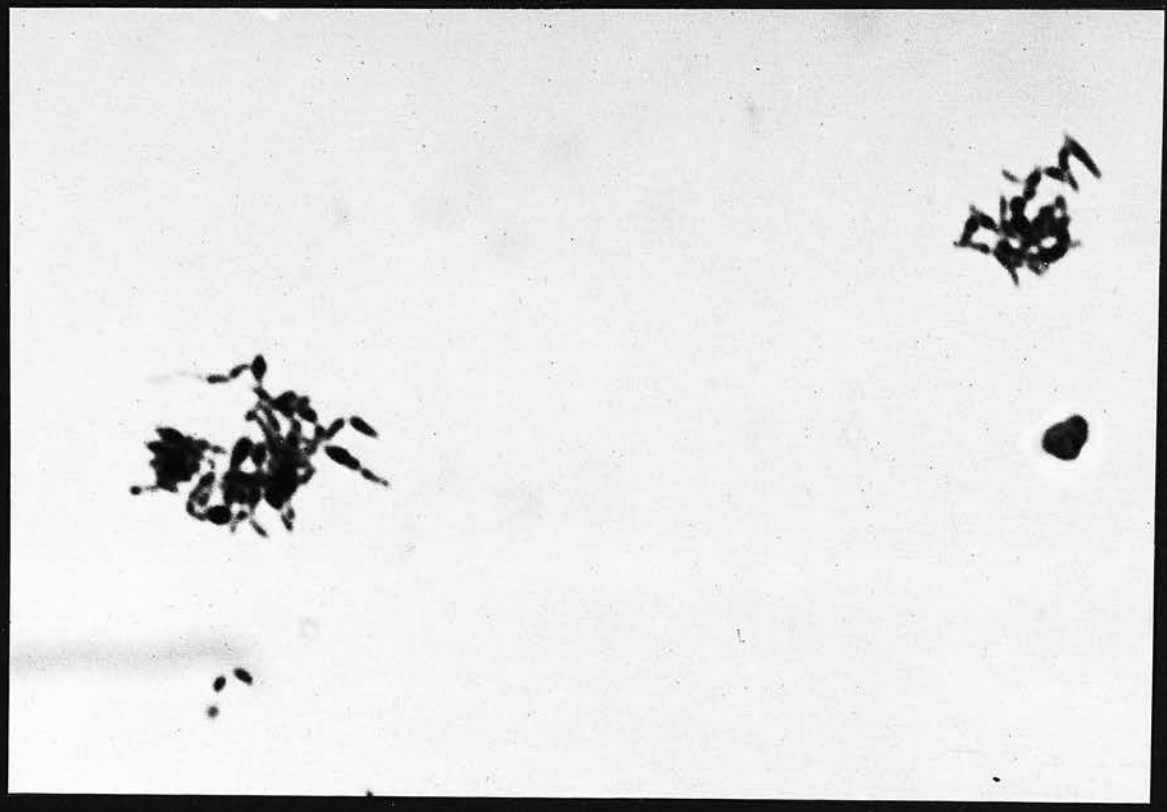

Plate 31

Blood taken from Chicken No.113 in tryptone soy agarose broth showing coccal and oval forms some of them attached to rudimentary rods. Giemsa X 2500.

Plate 32

The same culture as in Plate 31 after a further 24 hours incubation showing free large coccal forms and broken filaments. Giemsa X 2500.

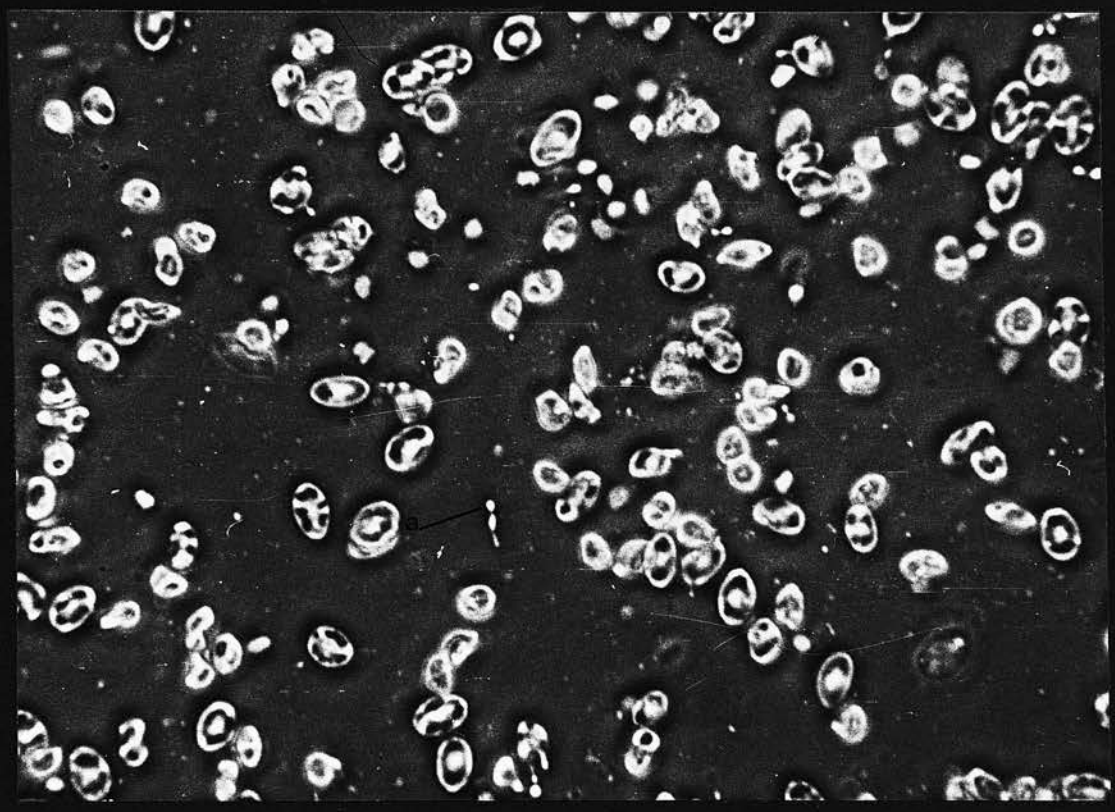

Plate 33

Chicken blood in tryptone soy agarose broth showing free spherical forms (a) and spherical forms attached to red cells. Phase contrast X 680.

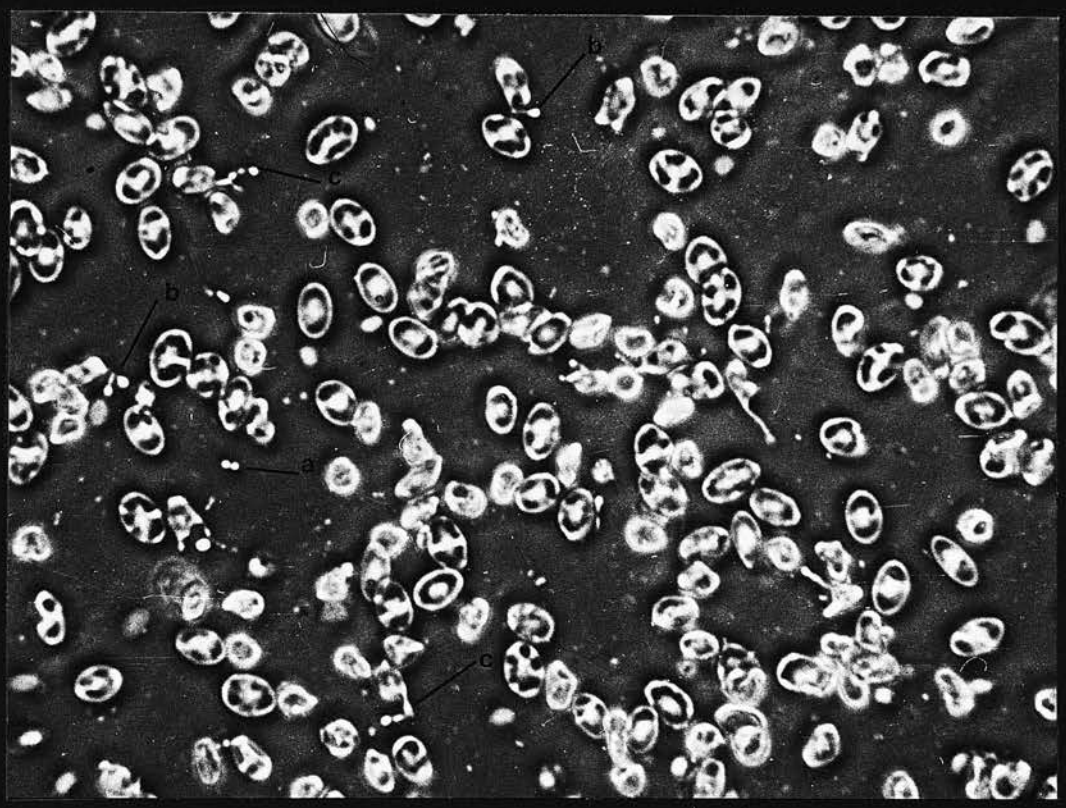

Plate 34

Chicken blood in tryptone soy agarose broth showing free spherical forms (a), round forms with stalk attached to red cells (b) and chains of cocci hanging from red cells (c). Phase contrast X 680.

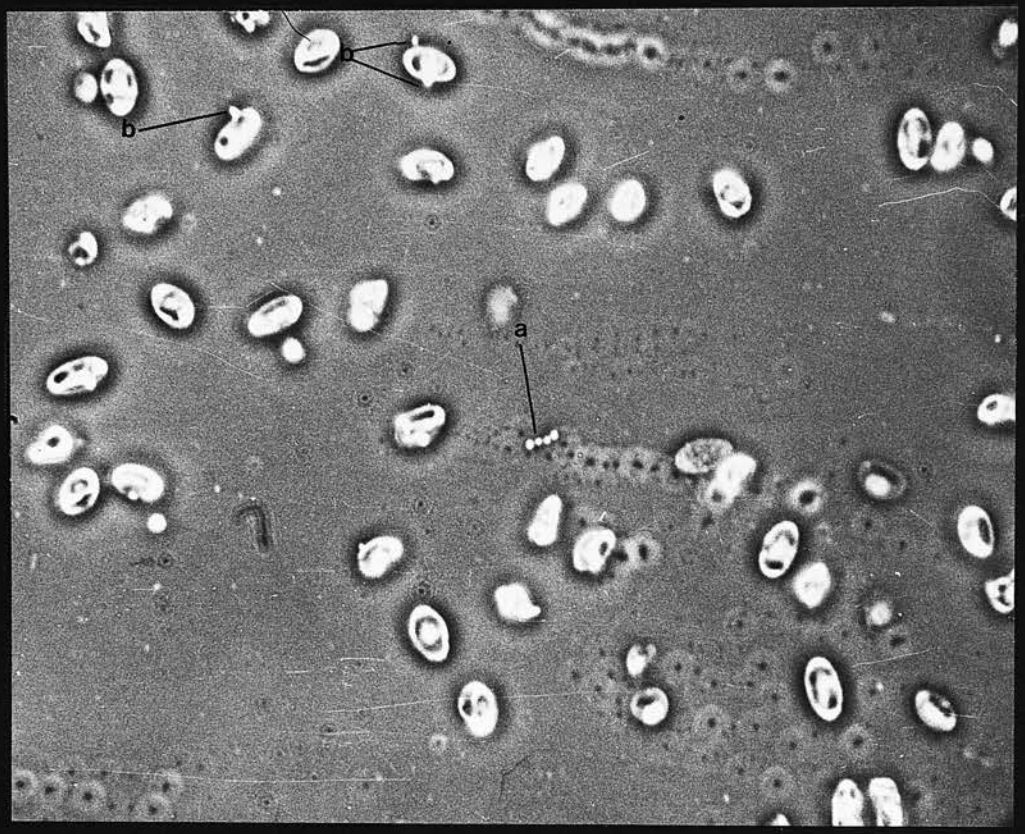

Plate 35

Chicken blood in tryptone soy agarose broth showing free coccal chain (a) and red cells with spike-like projections sticking from one or more sides (b). Phase contrast X 680.

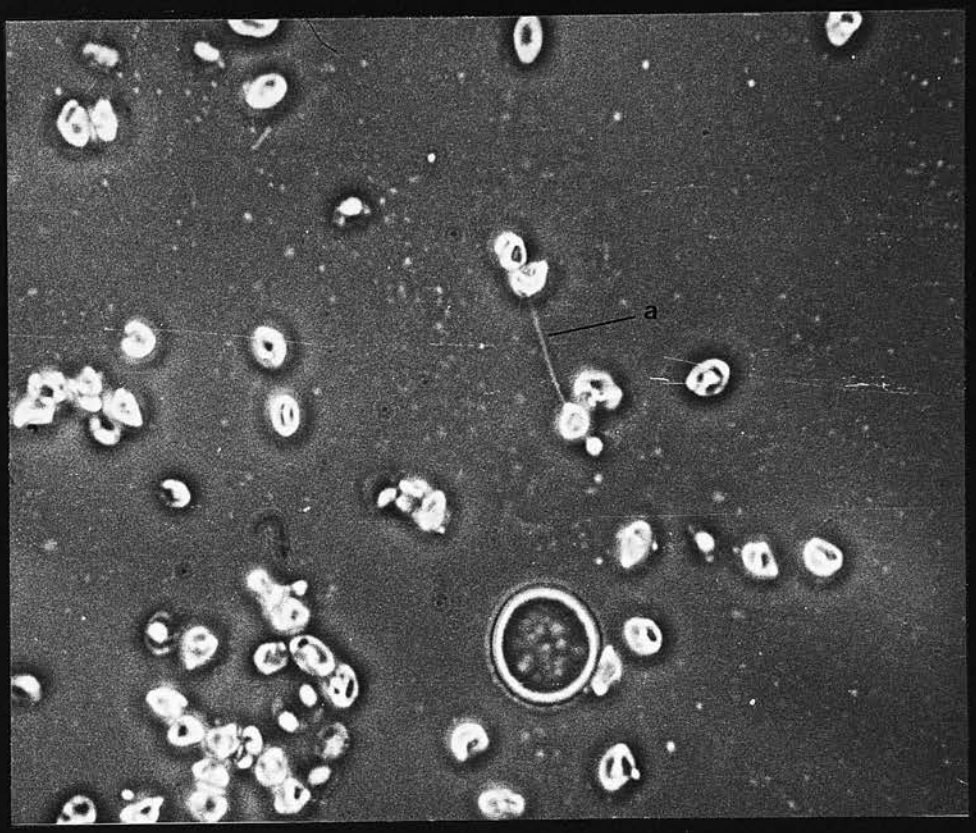

Plate 36

Chicken blood in tryptone soy agarose broth showing a red cell with a tiny filament (a) attached to another red cell. Phase contrast X 680.

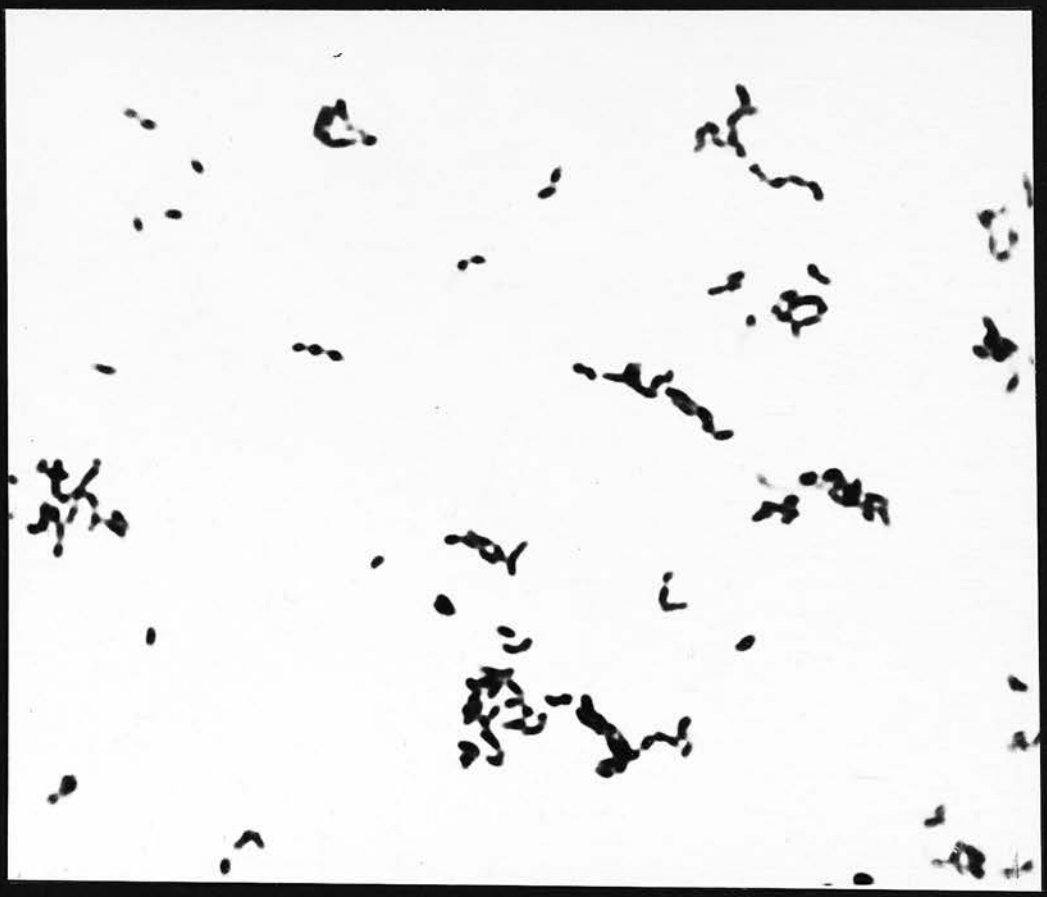

Plate 37

Blood taken from Sheep No. V289 in AJSB-S showing short swollen rods. Gram X 2500.

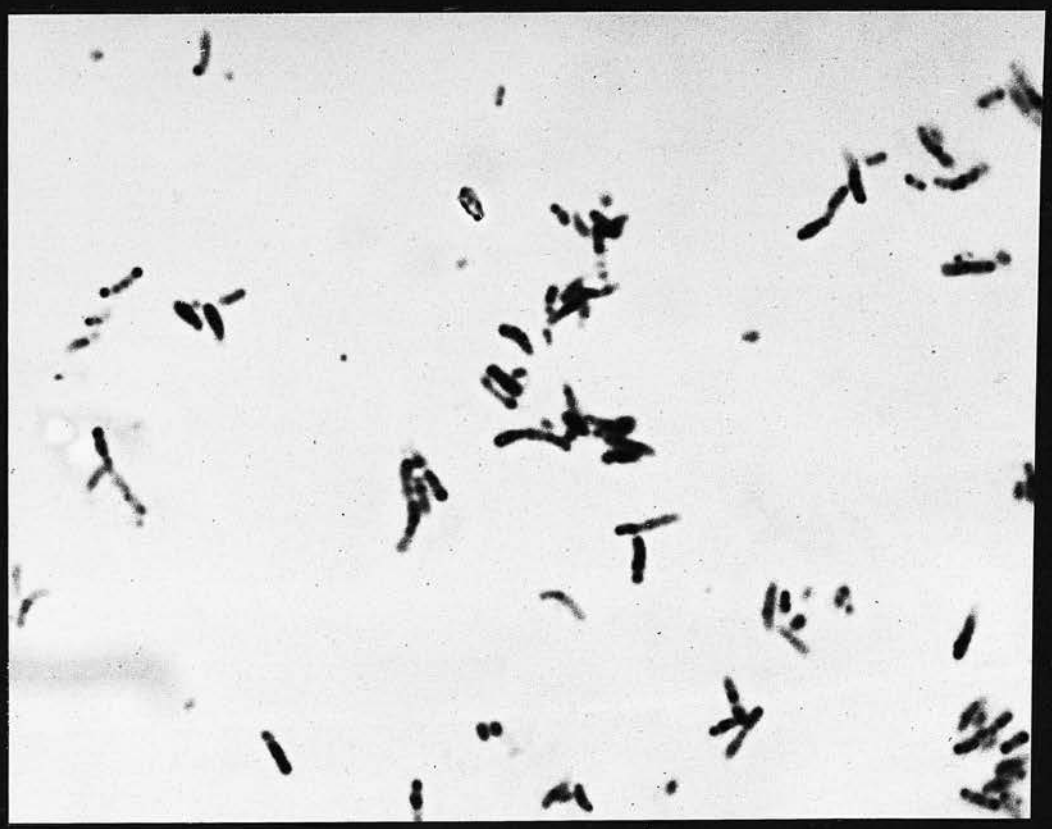

Plate 38

Blood taken from Lamb No.18 in AJSB-S showing club-shaped forms. Giemsa X 2500.

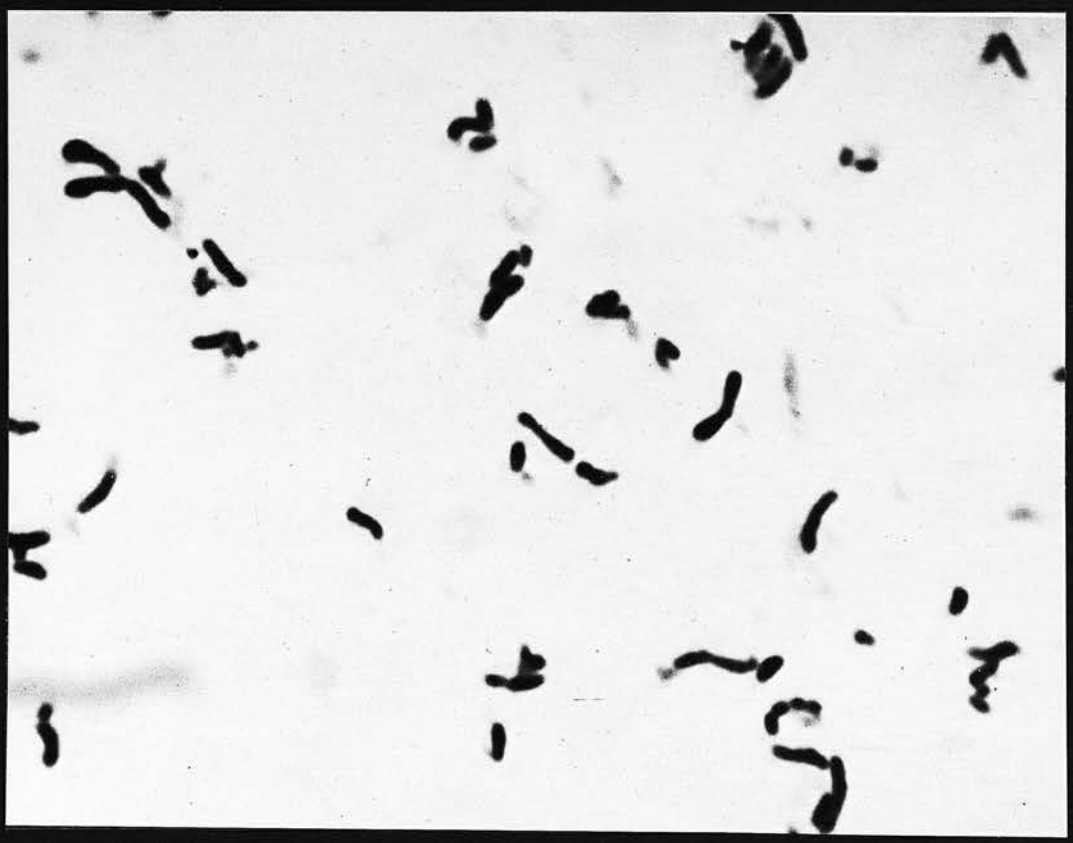

Plate 39

Blood taken from Lamb No.18 on blood agar showing rods of variable diameters and club-shaped forms. Giemsa X 2500.

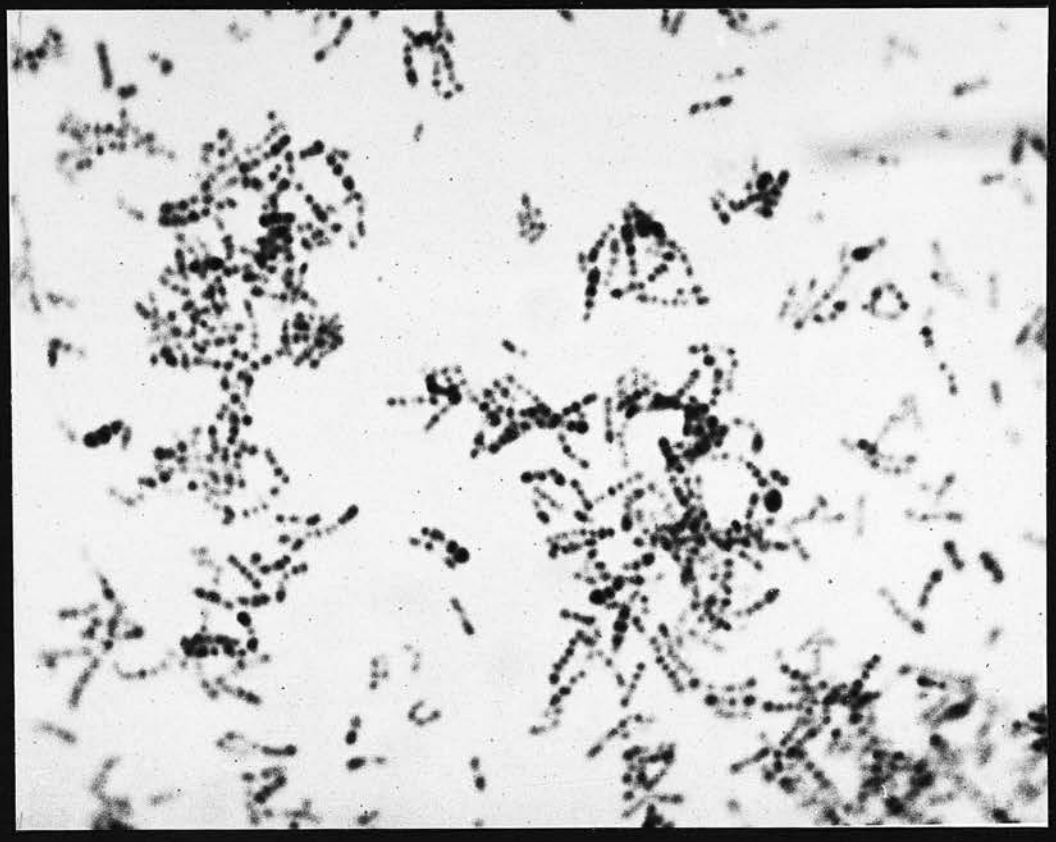

Plate 40

Lamb No.18 culture in AJSB-S inoculated from growth on blood agar obtained from the original AJSB-S culture of blood. Note the small round forms in chains. Giemsa X 2500.

Plate 41

Blood taken from Lamb No.28 on blood agar showing club-shaped organisms with the tapering ends disintegrated into small coccal forms and granules. Gram X 2500.

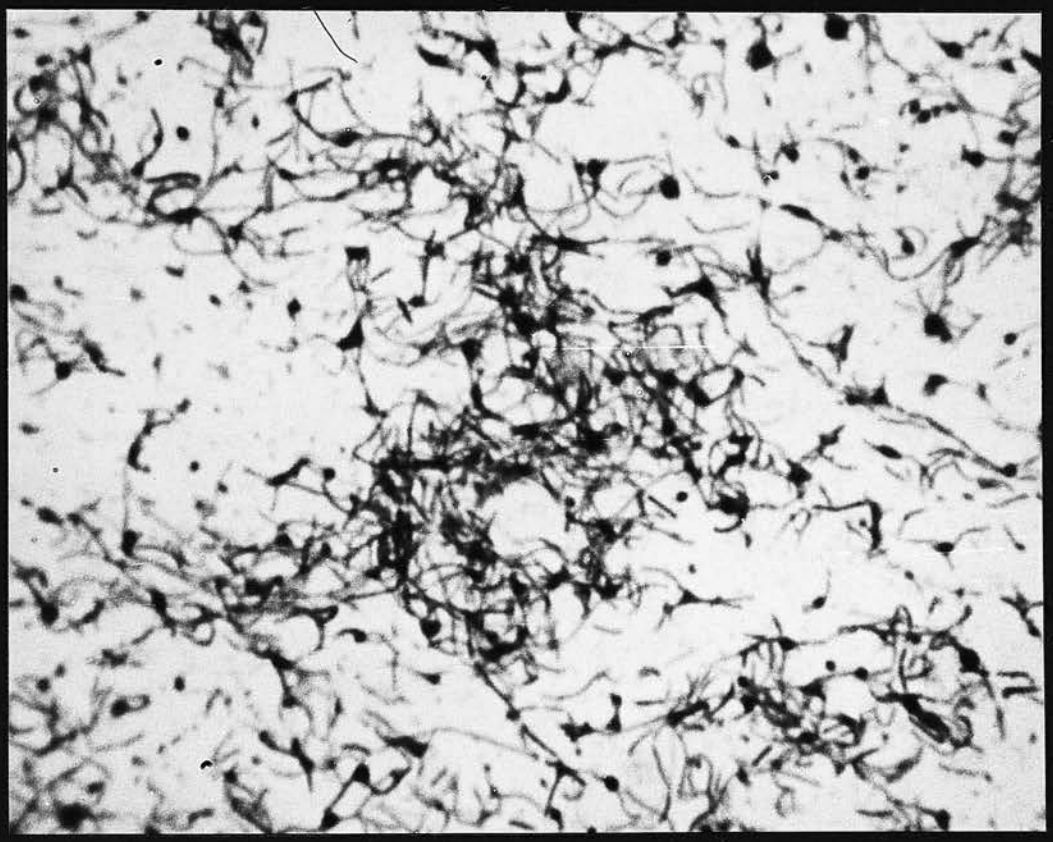

Plate 42

Smear from a large colony which appeared among colonies of blood sample 28. It shows filamentous forms with swellings and round forms of different sizes. Gram X 1560.

4 - Discussion

The techniques in the present study depended mainly on the Giemsa and Gram staining methods usually used in routine examination. In some of the rat blood small coccoid forms and tiny rods with knobs at the end were detected in stained preparations. These forms did not grow on subculture on agar. Tedeschi and Amici (1972) detected small coccoid forms and elongated cells in human blood which multiplied in primary cultures but not in subcultures. Failure of the diphtheroid forms to grow on agar confirmed that they were not contaminants. These forms might be deficient in cell wall material and they could only grow in broth. The coccal forms which appeared in the blood of rat 6 produced growth on AJSA-S within 24 hours while 3 days were needed for growth to appear on blood agar. This suggested that they were different from ordinary Gram positive cocci. Similar forms were detected in the blood of rats 9 and 10. The large and small coccal forms isolated from the blood of rats 9 and 10 produced coccal forms of uniform size after being transferred twice on agar. Charache (1968) isolated an organism with atypical morphology which reverted on subculture to classical forms.

The blood of chickens 113 and 115 each produced coccal and rod forms. The rudimentary rods attached to the coccal forms suggested that the cocci originated from rods, some of which were swollen. Very few of these

coccal forms were seen because they are vulnerable to the conventional Gram staining technique. Charache (1968) demonstrated that the fragile forms isolated from clinical materials were heat labile and only bacterial debris was visible when Gram's stain was applied. Hessburg et al. (1969) reported that the conventional staining methods destroy the morphology of the cell wall defective forms. On plating, these blood cultures yielded pure Gram positive cocci. Whether these developed from the coccal forms alone or from both types of elements seen in smears was not clear. The recovery of pure cultures from what had appeared, microscopically, to be a mixture of organisms, has been reported by Tunstall and Mattman (1961) who isolated from the blood and spinal fluids of patients with subacute bacterial endocarditis, a pleomorphic growth which gave typical streptococci after three subcultures. A similar observation was reported by Charache (1968). In the present work the presence of 0.05% agar in tryptone soy broth enhanced the growth. The time needed between the appearance of growth in broth and colony formation on agar suggested that these forms were osmotically sensitive and needed time for the development of cell wall material to enable them to grow on agar. The coccal forms which appeared in the hypertonic medium could be wall defective variants. Hypertonic medium was found to be superior to the routine media for detecting bacteraemia (Louria, Kaminski, Grieco & Singer, 1969). These authors detected L forms, cell wall defective variants

and conventional bacterial forms in hypertonic media.

The pleomorphic growth isolated from lambs' blood was of the diphtheroid type. There is strong evidence that the raised glistening colonies which appeared late in cultures from lamb 28 were variants of the earlier colonies. The Gram positive forms of the earlier colonies were spontaneously transformed to long filaments and round forms which had lost the Gram stain.

The fact that bacterial variants may be absent in one experiment but present in another is probably due to the variable presence of variants circulating in the blood at the time of sampling. The presence of variants in some bottles and not in others in the same test is a result of chance distribution of small numbers of organisms so that some of the bottles received none. It is possible that all the bottles received nearly the same number of organisms but that failure of growth in some of them was due to their death. The irregular appearance of organisms in blood has been attributed to the bactericidal activity of the blood (Wright, 1925).

Inoculation of different amounts of blood into different amounts of AJSB-S was tried to study the effect of blood dilution and the ratio of inoculum to the medium but the results were not constant. Wright (1925) in a series of blood cultures inoculated varying amounts of blood from 0·5 cc to 4 cc of each specimen to 9 cc of broth. He demonstrated that there was no tendency of growth to appear earlier in any particular dilution. He concluded

that growth was independent of the inoculum size. Buxton (personal communication) isolated spheroplasts and peculiar forms from the blood of apparently healthy animals. He found that the addition of 1 ml of blood to 9 ml of AJSB-S gave satisfactory results.

In the present work it was not infrequently found that in several bottles inoculated with the same blood sample with equal inocula growth occurred after different incubation periods. Some periods extended to several weeks. Buxton (personal communication) noted a delay in the appearance of the peculiar forms in blood primary cultures. Delayed growth of streptococci from subacute endocarditis conditions was observed by Wright (1925), who further investigated the cause of delay and found that the number of organisms in the blood had little effect on the delay period and that the delay was due to the peculiar behaviour of the organism rather than to inhibitory factors in the blood. The reason why bacterial forms could not be detected in blood smears might be due to the insufficiency of the staining techniques used. The late appearance of the organisms suggested that these forms were not free in the blood and time was needed for blood cells to disintegrate first in order to release them. Previous studies showed that microbial forms have been found within the erythrocytes. Tedeschi *et al*. (1969a) reported *Mycoplasma* or L forms of bacteria in the erythrocytes of healthy subjects. Pohlod *et al*. (1972) demonstrated intraerythrocytic forms in patients with

septicaemia. Examination of the blood of subjects affected by scarlet fever, with the electron microscope, showed round forms attached to the surface of the red blood cells (Mule, 1954). Intraerythrocytic and intraleucocytic acid-fast particles were observed in cancer blood (Seibert, Farrelly & Shepherd, 1967). These authors confirmed this observation by demonstrating in unstained fresh blood actively moving bacteria attacking the red cells. Filaments breaking into chains of spherical bodies and dumb-bell shaped rods were seen in microcultures of erythrocytes (Pease, 1970).

Variants in blood have been produced by antibiotics when patients or experimental animals are subjected to antibiotic therapy (Wittler et al., 1960; Kalmanson & Guze, 1964; Godzeski et al., 1967; Charache, 1968; Kagan, 1968b). However, stable and unstable L forms have been isolated from patients and experimental animals without there having been any prior treatment with L form-inducing substances. Wittler (1952) inoculated H. pertussis bacillary forms intraperitoneally and intranasally into normal and immunised mice. Stained preparations at necropsy showed that the bacilli had undergone transformation to L forms. Cary, Muschel and Baron (1960) injected mice intraperitoneally with Salmonella, Shigella, Proteus and Escherichia species. They noted that increased immunity and non-specific resistance in mice increased the rate of transformation of the rod forms to protoplasts. L forms of Salm. enteritidis and Salm. typhimurium were isolated

from livers of mice experimentally infected with these Salmonellae (Kawakami et al., 1970).

Atypical bacterial forms were observed in this study from blood samples of different species. The subjects from which samples were tested did not at any time receive antibiotic therapy. A possible explanation of this phenomenon is the action of antibodies or the antibody-complement system against cell wall components, since bacteria may be transformed in vitro by the action of antibody and complement (Michael & Braun, 1959; Muschel et al., 1959; Freeman et al., 1963). It is probable that the bacteria in the blood in the animal had already been phagocytosed and disintegrated by the blood cells as a result of which they were transformed to spherical and other atypical forms. The action of lysozyme in leucocytes was demonstrated on E.coli by Amano (1968). Von Haebler and Miles (1938) reported that polyanethol sulphonate (Liquoid) was effectively anticoagulant and destroyed the bactericidal activity of normal human blood for pathogenic bacteria. The authors also presented experimental evidence to show that the addition of polyanethol sulphonate in a 0·05% concentration was excellent for blood cultures and for testing for pathogenic bacteria. Later Liquoid was incorporated in blood cultures with satisfactory results (Stuart, 1948; Rosner, 1972).

The isolated variants may represent a parasite defence mechanism. Their survival in vivo suggested

that the internal environment is suitable and under favourable conditions they revert to the vegetative forms and multiply. Variant forms isolated from the blood of lambs 18 and 28 were slow-growing organisms and were usually lost on subcultures on agar. Their origin was not known but it is possible that lamb number 18 was infected *in utero* since its dam's blood showed Gram positive forms.

Most of the work done on blood variants has depended on microscopic examinations (Mule, 1954; Tedeschi *et al.*, 1969a; Pease, 1970). Chattman *et al.* (1969) demonstrated L forms in blood by growth in liquid medium, acridine orange staining and increased DNA content on incubation. In addition to staining methods, Tedeschi and Amici (1972) isolated microcolonies from healthy blood samples.

The streptococcal chains, the spherical forms with stalks and the free floating round forms seen with phase contrast microscopy resembled those reported by Pease (1970) when she examined erythrocytes cultures of arthritic subjects. She further investigated these forms with the electron microscope and obtained bacterial L forms on agar media. In the present work these forms appeared in every single blood sample examined with the phase contrast microscope with differences in the numbers of spherical forms and filaments. These forms did not appear in stained preparations or produce colonies on agar media. Such forms that could be identified only by phase contrast were not considered as bacterial forms because of the

possibility of artefacts. Failure of these forms to multiply in subcultures suggested that they were particles resulting from the disintegration of blood cells. The turbidity of the cultures was due to the increased disintegration of blood cells when the incubation period was increased. Tedeschi and Amici (1972) observed that on incubation of whole blood or washed erythrocytes, the Heinz bodies and haemoglobin passed into the medium and the haemoglobin mixed with the plasma protein resulted in particulate structures. Interpretation of results on phase contrast microscopy alone was difficult and possibly misleading because of cell debris that resembled L forms.

Gram's stain alone was confusing because in blood films well stained Gram negative spherical forms of different sizes were always present. These forms were similar to the round forms of the laboratory induced L forms. Charache (1970) reported that in hypertonic media, the haemoglobin in old sterile blood separated from the red cells and formed into Gram negative spheres. The artefacts in blood films stained with Giemsa were negligible.

The presence of bacterial variants in blood samples and tissues makes their recognition difficult. The resistance of variants to certain antibiotics presents problems in treatment and it is a possible explanation of recurrence of disease in apparently recovered patients. The negative results of blood cultures was due to the inadequacy of the medium since bacterial variants were

isolated from the same samples in hypertonic media. The slow growth of variants needs a long incubation period before cultures are considered negative. It is accepted that the use of simple media alone is unreliable for examination of clinical materials. The present results would indicate that media which will support the growth of L forms should be included in any range of routine blood culture tests.

Conclusions

1. *Actinobacillus* and *Pasteurella* strains were transformed to L forms by the action of antibiotics, glycine and lysozyme.
2. Bacitracin, novobiocin and vancomycin failed to bring about L transformation of the strains tested, although they act on the cell wall.
3. For the L form media a highly purified agar is needed in low concentrations.
4. Sucrose and sodium chloride are the agents most commonly used to stabilise L forms media.
5. The isolation of pleomorphic bacterial forms from patients who recently received antibiotics was due to the fact that the concentration of the antibiotic was below the MIC since pleomorphic organisms were isolated *in vitro* at subinhibitory concentrations of antibiotics.
6. Failure in treatment with high concentrations of antibiotics that act on the cell wall could be due to the presence of bacterial variants since these variants are resistant to such antibiotics.
7. Bacterial forms subjected to prolonged treatment with antibiotics render them biochemically different from the parent forms and they may resume the parental characters after some passages in antibiotic-free medium.

8. Bacterial variants were isolated from blood taken from healthy rats and chickens which had not received antibiotics and from healthy ewes and lambs delivered by caesarian section.
9. Because of the slow growing organisms in blood samples, long incubation periods of blood cultures are necessary.
10. In routine diagnosis a wide range of L form media should be included with media that promote good growth of bacterial forms.

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References

ABBATE, G.F., Leonessa, V. & Altucci, O. (1973). Studies on L forms of bacteria. Chemotherapy, 19, 341 - 347.

ALDERMAN, M.H. & Freedman, L.R. (1963). The direct injection of Escherichia coli protoplasts into the medulla of the rabbit kidney. Yale J. Biol. Med., 36, 157 - 164.

ALEXANDER-JACKSON, E. (1947). Non bacillary forms of Mycobacterium tuberculosis and Mycobacterium leprae. J. Bact., 54, 64 - 65.

ALEXANDER-JACKSON, E. (1954). A specific type of microorganism isolated from animal and human cancer : bacteriology of the organism. Growth, 18, 37 - 51.

ALTENBERN, R.A. (1961). Critical factors influencing growth of L forms of Proteus mirabilis. J. Bact., 81, 586 - 594.

ANDERSON, J.S., Matsuhashi, M., Haskin, M.A. & Strominger, J.L. (1965). Lipid-phosphoacetylmuramyl-pentapeptide and lipid-phosphodisaccharide-pentapeptide : presumed membrane transport intermediates in cell wall synthesis. Proc. natn. Acad. Sci. U.S.A., 53, 881 - 889.

AMANO, T. (1968). The formation of spheroplasts by leucozyme C, and the destruction of protoplasts by phospholipase A. pp. 30 - 39. In : L.B. Guze (ed). Microbial Protoplasts, Spheroplasts and L forms. The Williams and Wilkins Co., Baltimore.

BARILE, M.F., Francis, T.C. & Graykowski, E.A. (1968). Streptococcus sanguis in the pathogenesis of recurrent aphthous stomatitis. pp. 444 - 456. In : L.B. Guze (ed). Microbial Protoplasts, Spheroplasts and L forms. The Williams and Wilkins Co., Baltimore.

BASSERMANN, J.L., Carrere, L., Fasquelle, R., Hauduroy, P., Klieneberger-Nobel, E., Penso, G., Roux, J. & Tuncman, Z.M. (1957). L forms of bacteria. Nature, London, 179, 461 - 462.

BAYER, M.E. (1967). The cell wall of Escherichia coli : early effect of penicillin treatment and deprivation of diaminopimelic acid. J. gen. Microbiol., 46, 237 - 246.

BERTOLANI, R., Elberg, S.S. & Ralston, D. (1975). Variations in properties of L forms of Pseudomonas aeruginosa. Infection and Immunity, 11, 180 - 192.

BIBEL, D.J. & Lawson, J.W. (1972a). Development of streptococcal L forms colonies. J. Bact., 112, 602 - 610.

BIBEL, D.J. & Lawson, J.W. (1972b). Reversion of streptococcal protoplasts. J. Bact., 112, 611 - 614.

BIBEL, D.J. & Lawson, J.W. (1972c). Scanning electron microscopy of L phase streptococci. II Growth in broth and upon millipore filters. Can. J. Microbiol., 18, 1179 - 1184.

BIBEL, D.J. & Lawson, J.W. (1975). Morphology and viability of large bodies of streptococcal L forms. Infection and Immunity, 12, 919 - 930.

BLOSS, L. (1962). Phage resistance of a Proteus mirabilis L form. Bact. Proc., p.58.

BLUMBERG, P.M. & Strominger, J.L. (1974). Interaction of penicillin with bacterial cell : penicillin-binding proteins and penicillin-sensitive enzymes. Bact. Rev., 38, 291 - 335.

BOHNHOFF, M. & Page, M. (1968). Experimental infection with parent and L-phase variants of Neisseria meningitidis. J. Bact., 95, 2070 - 2077.

BONDI, A., Kornblum, J. & Forte, C. (1957). Inhibition of antibacterial activity of cycloserine by alpha-alanine. Proc. Soc. exp. Biol. Med., 96, 270 - 272.

van BOVEN, C.P.A., Ensering, H.J. & Hijmans, W. (1968). Size determination by the filtration method of the reproductive elements of group A streptococcal L forms. J. gen. Microbiol., 52, 403 - 412.

van BOVEN, C.P.A., Kastelein, M.J.W. & Hijmans, W. (1967). Chemically defined medium for the L-phase of group A streptococci. Ann. N.Y. Acad. Sci., 143, 749 - 754.

BRAUDE, A.I. (1970). Production of bladder stones by L-forms. Ann. N.Y. Acad. Sci., 174, 896 - 902.

BRAUDE, A.I., Siemienski, J. & Jacobs, I. (1961). Protoplast formation in human urine. Trans. Assoc. Am. physns., 74, 234 - 245.

BRAUDE, A.I., Siemienski, J. & Lee, K. (1968). Spheroplasts in human urine. pp.396 - 405. In : L.B. Guze (ed). Microbial Protoplasts, Spheroplasts and L forms. The Williams and Wilkins Co., Baltimore.

BREM, A.M. (1969). Isolation of L forms from clinical material. Am. J. med. Tech., 35, 902 - 905.

BREM, A.M. & Eveland, W.C. (1968). Induction and pathogenicity of Listeria monocytogenes L forms. Bact. Proc., P. 73.

BRENNER, S., Dark, F.A., Gerhardt, P., Jeynes, M.H., Kandler, O., Kellenberger, E., Klieneberger-Nobel, E., McQuillen, K., Rubio-Huertos, M., Salton, M.R.J., Strange, R.E., Tomcsik, J. & Weibull, C. (1958). Bacterial protoplast. Nature, London, 181, 1713 - 1715.

BROCK, T.D. (1962). The mode of action of novobiocin. Bact. Proc. P.123.

BROCK, T.D. & Brock, M.L. (1959). Effect of novobiocin on permeability of Escherichia coli. Arch. Biochem. Biophys., 85, 176 - 185.

BRONFENBENNER, J. & Muckenfuss, R. (1927). On the filterability of bacteria. Proc. Soc. exp. Biol. Med., 24, 371 - 372.

BURMEISTER, H.R. & Hesseltine, C.W. (1968). Induction and propagation of a Bacillus subtilis L form in natural and synthetic media. J. Bact., 95, 1857 - 1861.

CAREY, W.F., Muschel, L.H. & Baron, L.S. (1960). The formation of bacterial protoplasts in vivo. J. Immun., 84, 183 - 188.

CASAGRANDI, O. (1901). Sulla relazioni tra bacteri proto, meta e paratrofi, etc. Ann. d'lgiene sperim, N.S., V. 11, No.2, P.161 con tav. Abstr. in : Baumgarten's J ahrester, V.17, P.804. (Cited by Löhnis, 1922).

CHANG, T.W. & Weinstein, L. (1964). Inhibition of synthesis of the cell wall of S. aureus by cephalothin. Science, 143, 807 - 808.

CHARACHE, P. (1968). Atypical bacterial forms in human disease. pp.484 - 494. In : L.B. Guze (ed). Microbial Protoplasts, Spheroplasts and L forms. The Williams and Wilkins Co., Baltimore.

CHARACHE, P. (1970). Cell wall-defective bacterial variants in human disease. Ann. N.Y. Acad. Sci., 174, 903 - 911.

CHATTERJEE, B.R. & Williams, R.P. (1965). Formation of spheroplasts from Bacillus anthracis. J. Bact., 89, 1128 - 1133.

CHATTMAN, M., Mattman, L. & Mattman, P. (1969). L forms in blood cultures demonstrated by nucleic acid fluorescence. Am. J. clin. Path., 51, 41 - 49.

CHENG, K.J. (1973). Spheroplast formation by an anaerobic gram-negative bacteria Bacteroides ruminicola. Can. J. Microbiol., 19, 667 - 669.

CHRISTENSEN, W.B. (1946). Urea decomposition as a means of differentiating Proteus and paracolon cultures from each other and from Salmonella and Shigella. J. Bact., 52, 461 - 466.

CIAK, J. & Hahn, F.E. (1959). Mechanisms of action of antibiotics. II. Studies on the mode of action of cycloserine and its L-isomer. Antibiotics and Chemotherapy, 9, 47 - 54.

CLASENER, H.A.L., Ensering, H.L. & Hijmans, W. (1970). Persistence in mice of the L phase of three streptococcal strains adapted to physiological osmotic conditions. J. gen. Microbiol., 62, 195 - 202.

COWAN, S.T. (1974). Cowam and Steel's Manual for the Identification of Medical Bacteria, 2nd ed. Cambridge University Press.

CRAWFORD, Y.E. (1960). Studies of a complement-fixing antigen from group A streptococcal L forms. J. Immun., 84, 86 - 92.

CRAWFORD, Y.E., Frank, P.F. & Sullivan, B. (1958). Isolation and reversion of L forms of Beta haemolytic streptococci. J. infect. Dis., 102, 44 - 52.

CRAWFORD, Y.E., Lytle, R.I. & Nalewaik, R.P. (1970). Haemagglutinin from the L-form of Neisseria meningitidis group B. Bact. Proc. P.109.

CRAWFORD, Y.E., Nalewaik, R.P., Lytle, R.I. & O'Connell, J.L. (1971). Haemagglutinin from the L phase of Neisseria meningitidis group B, with observation on a serum inhibitor. Infection and Immunity, 4, 212 - 219.

CRUICKSHANK, R. (1965). Medical Microbiology - a guide to laboratory diagnosis and control of infection, 11th edition. E. & S. Livingstone Ltd., Edinburgh and London.

CZOP, J.K. & Bergdoll, M.S. (1970). Synthesis of enterotoxin by L forms of Staphylococcus aureus. Infection and Immunity, 1, 169 - 173.

DANNIS, D.C., Sharp, J.T. & Marston, J.H. (1970). Serologic studies of staphylococcal and streptococcal L-forms. Ann. N.Y. Acad. Sci., 174, 922 - 927.

DAVIS, S.D., Gemsa, D. & Wedgwood, R.J. (1966). Kinetics of the transformation of Gram negative rods to spheroplasts and ghosts by serum. J. Immun., 96, 570 - 577.

DAWSON, M.H. & Hobby, G.L. (1939). Rat bite fever. Trans. Assoc. Am. Physns., 54, 329 - 332.

DeBARY, A. (1884). Vergleichende Morpholigia und Biologie der pilze, mycetozoen und bacterien, XVI and 558, p.p.m.198. Textabb. Leipzig. (Cited by Löhnis, 1922).

DIENA, B.B., Wallace, R. & Greenberg, L. (1964a). The production and properties of Salmonella typhi spheroplasts. Can. J. Microbiol., 10, 543 - 549.

DIENA, B.B., Wallace, R. & Greenberg, L. (1964b). Immunologic studies of glycine-induced spheroplasts of Salmonella typhi. Can. J. Microbiol., 10, 555 - 560.

DIENA, B.B., Wallace, R. & Greenberg, L. (1965). Reversion and biological characteristics of Salmonella typhi spheroplasts. Can. J. Microbiol., 11, 427 - 433.

DIENES, L. (1938). Further observation of the L organism of Klieneberger. Proc. Soc. exp. Biol. Med., 39, 365 - 367.

DIENES, L. (1939a). L type variant forms in cultures of various bacteria. Proc. Soc. exp. Biol. Med., 42, 636 - 640.

DIENES, L. (1939b). A peculiar reproductive process in colon bacillus colonies. Proc. Soc. exp. Biol. Med., 42, 773 - 778.

DIENES, L. (1939c). L organism of Klieneberger and Streptobacillus moniliformis. J. infect. Dis., 65, 24 - 42.

DIENES, L. (1943). Reproduction of bacteria from the large bodies of Streptobacillus moniliformis. Proc. Soc. exp. Biol. Med., 53, 84 - 86.

DIENES, L. (1946a). Complex reproductive processes in bacteria. Cold Spring Harbor Symposia on Quantitative Biology, 11, 51 - 59.

DIENES, L. (1946b). Reproduction process in Proteus cultures. Proc. Soc. exp. Biol. Med., 63, 265 - 270.

DIENES, L. (1947). The morphology of the L_1 of Klieneberger and its relationship to Streptobacillus moniliformis. J. Bact., 54, 231 - 237.

DIENES, L. (1949). The development of Proteus cultures in the presence of penicillin. J. Bact., 57, 529 - 546.

DIENES, L. (1950). Isolation of L type cultures from clostridia. Proc. Soc. exp. Biol. Med., 75, 412 - 415.

DIENES, L. (1953). Some new observations on L forms of bacteria. J. Bact., 66, 274 - 279.

DIENES, L. (1960). Controversial aspects of the morphology of PPLO. Ann. N.Y. Acad. Sci., 79, 356 - 370.

DIENES, L. (1967a). Permanent stained agar preparation of Mycoplasma and of L forms of bacteria. J. Bact., 93, 689 - 692.

DIENES, L. (1967b). Morphology and reproductive processes of the L forms of bacteria. I. Streptococci and staphylococci. J. Bact., 93, 693 - 702.

DIENES, L. (1968). Morphology and reproductive processes of bacteria with defective cell wall. pp. 74 - 93. In : L.B. Guze (ed). Microbial Protoplasts, Spheroplasts and L forms. The Williams and Wilkins Co., Baltimore.

DIENES, L. (1970). Influence of a bacillus on the reversion of Haemophilus influenzae L form to the bacterial form. Proc. Soc. exp. Biol. Med., 135, 392 - 394.

DIENES, L., Bandur, B.M. & Madoff, S. (1964). Development of L-type growth in Neisseria gonorrhoeae cultures. J. Bact., 87, 1471 - 1476.

DIENES, L. & Bullivant, S. (1967). Comparison of morphology of PPLO and L forms of bacteria with light and electron microscopy. Ann. N.Y. Acad. Sci., 143, 719 - 733.

DIENES, L. & Madoff, S. (1968). Morphology of the L-forms of group A Streptococcus and other bacterial species. 332 - 334. In : R. Caravano (ed). Excerpta medica foundation, Amsterdam.

DIENES, L. & Sharp, J.T. (1956). The role of high electrolyte concentration in the production and growth of L forms of bacteria. J. Bact., 71, 208 - 213.

DIENES, L. & Smith, W.E. (1944). The significance of pleomorphism in Bacteroides strains. J. Bact., 48, 125 - 153.

DIENES, L. & Weinberger, H.J. (1951). L forms of bacteria. Bact. Rev., 15, 245 - 288.

DIENES, L., Weinberger, H.J. & Madoff, S. (1950a). The transformation of typhoid bacilli into L forms under various conditions. J. Bact., 59, 755 - 764.

DIENES, L., Weinberger, H.J. & Madoff, S. (1950b). Serological reactions of L type cultures isolated from Proteus. Proc. Soc. exp. Biol. Med., 75, 409 - 412.

DIENES, L. & Zamecnik, P.C. (1952). Transformation of bacteria into L forms by amino acids. J. Bact., 64, 770 - 771.

DOWDESWELL, G.F. (1890). Note on the morphology of the Cholera comma bacillus. The Lancet, 1, 1419 - 1423.

FILDES, P. (1938). The growth of Proteus on ammonium lactate plus nicotinic acid. Br. J. exp. Path., 19, 239 - 244.

FOWKE, F. (1885). On the first discovery of the comma-bacillus of cholera. Br. med. J. 1, 589 - 592.

FRASER, D. & Mahler, H.R. (1957). The effect of nucleases on the reproduction of T3 bacteriophage in protoplasts of Escherichia coli. Arch. Biochem. Biophys., 69, 166 - 177.

FREEMAN, B.A., Musteikis, G.M. & Burrows, W. (1963). Protoplast formation as the mechanism for immune lysis of Vibrio cholerae. Proc. Soc. exp. Biol. Med., 113, 675 - 680.

FREIMER, E.H., Krause, R.M. & McCarty, M. (1959). Studies on L forms and protoplasts of group A streptococci. I. Isolation, growth and bacteriologic characteristics. J. exp. Med., 110, 853 - 874.

FREUNDT, E.A. (1950). Observation of Dienes L type growth of bacteria. Acta. path. microbiol. scand., 27, 159 - 171.

FREUNDT, E.A. (1956). Experimental investigations into the pathogenicity of the L-phase variant of Streptobacillus moniliformis. Acta. path. microbiol. scand., 38, 246 - 258.

GAMALEIA, N. (1900). Elemente der allgemeinen bakteriologie. VI und 242 p.p. Berlin. (Cited by Löhnis, 1922).

GEARY, I. & Waitkins, S.A. (1977). The development of L-phase colonies of Neisseria gonorrhoeae. J. gen. Microbiol., 99, 233 - 236.

GILBY, A.R. & Few, A.V. (1960). Lysis of protoplasts of Micrococcus lysodeikticus by ionic detergents. J. gen. Microbiol., 23, 19 - 20.

GILPIN, R.W., Young, F.E. & Chatterjee, A.N. (1973). Characterisation of stable L form of Bacillus subtilis 168. J. Bact.,113, 486 - 499.

GODZESKI, C.W. (1968). *In vivo* persistence of L-phase bacteria. pp. 379 - 390. In : L.B. Guze (ed). Microbial Protoplasts, Spheroplasts and L forms. The Williams and Wilkins Co., Baltimore.

GODZESKI, C.W., Brier, G. & Farran, J.D. (1967). The interaction of antibiotics, bacteria and the bacterial L-phase *in vivo*. Ann. N.Y. Acad. Sci., 143, 760 - 777.

GODZESKI, C.W., Brier, G., Griffith, R.S. & Black, H.R. (1965). Association of bacterial L-phase organisms in chronic infections. Nature, London, 205, P. 1340.

GOODER, H. (1968). Streptococcal protoplasts and L form growth induced by muralytic enzymes. pp. 40 - 51. In : L.B. Guze (ed). Microbial Protoplasts, Spheroplasts and L forms. The Williams and Wilkins Co., Baltimore.

GOODER, H. & Maxted, W.R. (1958). Protoplast of group A beta haemolytic streptococci. Nature, London, 182, 808 - 809.

GORDON, J. & Gordon, M. (1943). Involution forms of the genus *Vibrio* produced by glycine. J. Path. Bact., 55, 63 - 68.

GREEN, M.T., Heidger, P.M. & Domingue, G. (1974). Proposed reproductive cycle for relatively stable L-phase variant of *Streptococcus faecalis*. Infection and Immunity, 10, 915 - 927.

GUIGNARD, L. & Charrin (1887). Sur les variations morphologiques des microbes. Compt. rend. Acad. Paris, 105, No.24, 1192 - 1195. (Cited by Löhnis, 1922).

GUTMAN, L.T. Schaller, J. & Wedgwood, R.J. (1967). Bacterial L-forms in relapsing urinary tract infection. The Lancet, 1, 464 - 466.

GUTMAN, L.T., Turck, M., Petersdorf, R.G. & Wedgwood, R.J. (1965). Significance of bacterial variants in urine of patients with chronic bacteriuria. J. clin. Invest., 44, 1945 - 1952.

GUTMAN, L.T., Winterbauer, R.H., Turck, M., Wedgwood, R.J. & Petersdorf, R.G. (1968). The role of bacterial variants in experimental pyelonephritis. pp. 391 - 395. In : L.B. Guze (ed). Microbial Protoplasts, Spheroplasts and L-forms. The Williams and Wilkins Co., Baltimore.

GUZE, L.B. & Kalmanson, G.M. (1964). Persistance of bacteria in protoplast form after apparent cure of pyelonephritis in rats. Science, 143, 1340 - 1341.

GUZE, L.B., Harwick, H.J. & Kalmanson, G.M. (1976). Klebsiella L forms : Effect of growth as L form on virulence of reverted Klebsiella pneumoniae. J. infect. Dis., 133, 245 - 252.

HADLEY, P., Delves, E. & Klimek, J. (1931). The filterable forms of bacteria: 1 - A filterable stage in the life history of the shiga dysentery bacillus. J. infect. Dis., 48, 1 - 159.

HAMBURGER, M. & Carleton, J. (1966a). Staphylococcal spheroplasts and L colonies: I - Population curves of vegetative staphylococci and spheroplasts in methicillin-containing broth over long periods. J. infect. Dis., 116, 221 - 230.

HAMBURGER, M. & Carleton, J. (1966b). Staphylococcal spheroplasts and L colonies: II - Conditions conducive to reversion of spheroplasts to vegetative staphylococci. J. infect. Dis., 116, 544 - 550.

HAMBURGER, M. & Carleton, J. (1968). Staphylococcal spheroplasts as persisters. Long-term population curves of L - and vegetative colonies in presence of methicillin with observation on reversion. pp. 221 - 229. In : L.B. Guze (ed). Microbial Protoplasts, Spheroplasts and L-forms. The Williams and Wilkins Co., Baltimore.

HANCOCK, R. (1958). Protection of Staphylococcus aureus from some effects of penicillin by media of high osmotic pressure. Biochem. J., 70, P.15.

HARE, R. & Colebrook, L. (1934). The biochemical reactions of haemolytic streptococci from the vagina of febrile and afebrile parturient women. J. Path. Bact., 39, 429 - 442.

HATTEN, B.A. & Sulkin, S.E. (1966). Intracellular production of Brucella L-forms: I - Recovery of L forms from tissue culture cells infected with Brucella abortus. J. Bact., 91, 285 - 296.

HEILMAN, F.R. (1941). A study of A Sterococcus muris (S. moniliformis). Morphological aspects and nomenclature. J. infect. Dis., 69, 32 - 44.

HESSBURG, P.C., Mattman, L.H., Barth, C. & Dutcheshen, L.T. (1969). Aqueous microbiology: The possible role of cell wall deficient bacteria in uveitis. Henry Ford Hosp. Med. Journal, 17, 177 - 194.

HEWITT, W.L., Seligman, S.J. & Deigh, R.A. (1968). Relationship of the antibiotic sensitivity of enterococci to acquired resistance in their L-forms. pp. 311 - 313. In : L.B. Guze (ed). Microbial Protoplasts, Spheroplasts and L-forms. The Williams and Wilkins Co., Baltimore.

HIGUCHI, M. (1969). Significance of bacterial L forms in urinary infections in particular the mechanism to cause pyelonephritis. The Kurume Medical Journal, 16, 179 - 185.

HIJMANS, W. (1962). Absence of the group specific and the cell wall polysaccharide antigen in L-phase variants of group D strepcococci. J. gen. Microbiol., 28, 177 - 179.

HIJMANS, W., van Boven, C.P.A. & Clasener, H.A. (1969). Fundamental biology of the L-phase of bacteria. pp. 67 - 143. In: L Hayflick (ed). The Mycoplasmatales and the L-phase of Bacteria. North Holland Publishing Company, Amsterdam.

HIJMANS, W. & Kastelein, M.J.W. (1960). The production of L forms of enterococci. Ann. N.Y. Acad. Sci., 79, 371 - 373.

HIROKAWA, H. (1962). Biochemical and cytological observations during the reversing process from spheroplasts to rod-form cells in Escherichia coli. J. Bact., 84, 1161 - 1168.

HOFSCHNEIDER, P.H. & Martin, H.H. (1968). Diversity of surface layers in L-forms of Proteus mirabilis. J. gen. Microbiol., 51, 23 - 33.

HOLMGREN, N.B. & Campbell, W.E.Jr.(1960). Tissue cell culture contamination in relation to bacterial pleuropneumonia-like organisms - L form conversion. J. Bact., 79, 869 - 874.

HORWITZ, A.H. & Casida, L.E. Jr. (1978). Effects of magnesium, calcium and serum on reversion of stable L forms. J. Bact., 136, 565 - 569.

HUBERT, E.G., Potter, C.S., Hensley, T.J., Cohen, M., Kalmanson, G.M. & Guze, L.B. (1971). L forms of Pseudomonas aeruginosa. Infection and Immunity, 4, 60 - 72.

HUBERT, E.G., Potter, C.S., Kalmanson, G.M. & Guze, L.B. (1969). Pigment formation in L forms of Serratia marcescens. J. gen. Microbiol., 55, 165 - 167.

JORDAN, D.C. & Inniss, W.E. (1959). Selective inhibition of ribonucleic acid synthesis in S. aureus by Vancomycin. Nature, London, 184, 1894 - 1895.

KAGAN, B.M. (1968a). Antibiotic sensitivities of staphylococcal L-forms. pp. 314 - 318. In : L.B. Guze (ed). Microbial Protoplasts, Spheroplasts and L-forms. The Williams and Wilkins Co., Baltimore.

KAGAN, B.M. (1968b). Role of L-forms in staphylococcal infection. pp. 372 - 378. In : L.B. Guze (ed). Microbial Protoplasts, Spheroplasts and L-forms. The Williams and Wilkins Co., Baltimore.

KAGAN, B.M., Martin, E.R. & Stewart, G.T. (1964). L form induction of naturally occurring methicillin-resistant strains of Staphylococcus aureus. Nature, London, 203, 1031 - 1033.

KAGAN, G.Y. (1968). Some aspects of investigations of the pathogenic potentialities of L forms of bacteria. pp. 422 - 443. In : L.B. Guze (ed). Microbial Protoplasts, Spherophasts and L-forms. The Williams and Wilkins Co., Baltimore.

KAGAN, G.Y. & Levashov, V.S. (1957). Biological properties of strains of S. typhi reverted from L forms. Izmenchivost' Mikroorganizmov, V.D. Timakova, Moscow. 2, 373 - 383.

KAHN, M.C. & Nonidez, J.F. (1932). Non acid fast rods and granules in vertical sections of Mycobacterium tuberculosis colonies. Proc. Soc. exp. Biol. Med., 30, 577 - 582.

KALMANSON, G.M. & Guze, L.B. (1964). Role of protoplasts in pathogenesis of pyelonephritis. J. Am. med. Ass., 190, 1107 - 1109.

KALMANSON, G.M. & Guze, L.B. (1968). Pyelonephritis: isolation of protoplasts from human kidney tissue. pp. 406 - 414. In : L.B. Guze (ed). Microbial Protoplasts, Spheroplasts and L-forms. The Williams and Wilkins Co., Baltimore.

KANDLER, O., Hund, A. & Zehender, C. (1958). Cell wall composition in bacterial and L forms of Proteus vulgaris. Nature, London, 181, 572 - 573.

KANG, K.S. & Casida, L.E. Jr. (1967). Large bodies of Mycoplasma and L form organisms. J. Bact., 93, 1137 - 1142.

KAWAKAMI, M., Ishibashi, H., Mitsuhash, S., Sakaino, K. & Fukai, K. (1970). Experimental salmonellosis: unstable L forms in livers of infected mice. Jap. J. Microbiol., 14, 143 - 153.

KAWATOMARI, T. (1958). Studies of the L-forms of Clostridium perfringens. J. Bact., 76, 227 - 232.

KERN, R.A., Kingkade, M.J., Kern, S.F. & Behrens, O.K. (1951). Characterisation of the action of lysozyme on Staphylococcus aureus and on Micrococcus lysodeikticus. J. Bact., 61, 171 - 178.

KIM, B.H. (1976). Studies on Actinobacillus equuli. Ph.D. Thesis, Edinburgh University.

KING, J.R. & Gooder, H. (1970a). Induction of enterococcal L-forms by the action of lysozyme. J. Bact., 103, 686 - 691.

KING, J.R. & Gooder, H. (1970b). Reversion to the streptococcal state of enterococcal protoplasts, spheroplasts and L-forms. J. Bact., 103, 692 - 696.

KLEIN, E. (1883). On a morphological variety of B. anthracis. Q. Jl. microsc. Sci., 23, 260 - 264.

KLEIN, E. (1885a). The relation of bacteria to Asiatic cholera. Br. med. J., 1, 289 - 290.

KLEIN, E. (1885b). Remarks on the etiology of Asiatic cholera. Br. med. J., 1, 650 - 652.

KLIENEBERGER, E. (1935). The natural occurrence of pleuropneumonia-like organisms in apparent symbiosis with Streptobacillus moniliformis and other bacteria. J. Path. Bact., 40, 93 - 105.

KLIENEBERGER, E. (1942). Some new observations bearing on the nature of the pleuropneumonia-like organism known as L, associated with Streptobacillus monoliformis. J. Hyg. Camb., 42, 485 - 497.

KLIENEBERGER, E. & Steabben, D. (1937). On a pleuropneumonia-like organism in lung lesions of rats, with notes on the clinical and pathological features of the under lying condition. J. Hyg., Camb., 37, 143 - 152.

KLIENEBERGER-NOBEL, E. (1951). Filterable forms of bacteria. Bact. Rev., 15, 77 - 102.

KLIENEBERGER-NOBEL, E. (1956). Ueber die wesenverschiedenheit der peripneumonieahnlichen organismen und der L-phase der bakterien. Zentr. Bakt. Parasitenk. Abt. 1, Orig., 167, 329 - 343. (Cited by Weibull & Lundin, 1961).

KLIENEBERGER-NOBEL, E. (1960). L-forms of bacteria. pp. 361 - 386. In: I.C. Gunsalus and R.Y. Stanier (eds). The Bacteria; Vol. 1: Structure. Academic Press, New York and London.

KNIGHT, B.C. (1937). The nutrition of Staphylococcus aureus; nicotinic acid and vitamin B1. Biochem. J., 31, 731 - 737.

KOTLYAROVA, G.H., Bakulov, I.A., Chevelev, S.F., Dymina, G.P. & Prozorovskii, S.V. (1976). Role of L-forms of Listeria monocytogenes in ovine listeriosis. Vestnik Sel' Skokhozyaistvennoi Nauki No.7, 84 - 88. Abst. 1369.

KOVACS, W. (1956). Identification of Pseudomonas pyocyanea by the oxidase reaction. Nature, London, 178, p.703.

KUBOTA, M.Y., Montgomerie, J.Z., Potter, C.S., Kalmanson, G.M. & Guze, L.B. (1966). Effect of penicillin concentration on "protoplast" production of Streptococcus faecalis. Antimicrobial Agents and Chemotherapy, 300 - 303.

LANDMAN, O.E., Altenbern, R.A. & Ginoza, H.S. (1958). Quantitative conversion of cells and protoplasts of Proteus mirabilis and Escherichia coli to the L-forms. J. Bact., 75, 567 - 576.

LANDMAN, O.E. & Forman, A. (1969). Gelatin induced reversion of protoplasts of Bacillus subtilis to the bacillary form : biosynthesis of macromolecules and wall during successive steps. J. Bact., 99, 576 - 589.

LANDMAN, O.E. & Halle, S. (1963). Enzymically and physically induced inheritance changes in Bacillus subtilis. J. molec. Biol., 7, 721 - 738.

LAPINSKI, E.M. & Flakas, E.D. (1967). Induction of L forms of Haemophilus influenzae in culture and their development in human bronchial secretions. J. Bact., 93, 1438 - 1445.

LAWSON, J.W. & Douglas, J.T. (1973). Induction and reversion of L forms of Neisseria gonorrhoeae. Can. J. Microbiol., 19, 1145 - 1151.

LEDERBERG, J. (1956). Bacterial protoplasts induced by penicillin. Proc. natn. Acad. Sci. U.S.A., 42, 574 - 577.

LEDERBERG, J. (1957). Mechanism of action of penicillin. J. Bact., 73, P.144.

LEDERBERG, J. & St.Clair, J. (1958). Protoplasts and L-type growth of Escherichia coli. J. Bact., 75, 143 - 160.

LEVINA, G.L. (1972). Reversion of L-forms of Salmonella typhimurium induced by penicillin. Antibiotiki, 17, 714 - 718.

LI-HUI, Li Tien-Lin & Lei Ai-Te. (1964a). The L-forms of B. Proteus vulgaris : I - A study on the dynamics of the formation of B. Proteus vulgaris with slide culture method and observations made under phase contrast microscope. Scientia Sinica, 13, 1821 - 1827.

LI-HUI, Li Tien-Lin & Lei Ai-Te (1946b). The L-forms of B. Proteus vulgaris : II - Cytological study of the L-forms of B.Proteus, using Feulgen's staining method and fluorescence microscopy with acridine orange. Scientia Sinica, 13, 1829 - 1834.

LIGNIÈRES, J. & Durrieu, H. (1902). Formes anormales de la bactèridie charbonneuse isotees dans le sang d'um taureau mort de maladie naturelle. Bull. Soc. Centr. Méd. Vét. N.S., 20, 102 - 107. (Cited by Löhnis, 1922).

LINNEMANN, C.C. Jr., Watanakunakorn, C. & Bakie, C. (1973). Pathogenicity of stable L phase variants of Staphylococcus aureus : failure to colonize experimental endocarditis in rabbits. Infection and Immunity, 7, 725 - 730.

LITTLE, T.W.A. & Bosbery, R.I. (1973). Experimental infection of the bovine udder with the L-phase of Staphylococcus aureus during lactation. Res. vet. Sci., 15, 111 - 115.

LITTLE, T.W.A., Bosbery, R.I. & Draper, W.P. (1973). The L phase of strains of Staphylococcus aureus of bovine origin. Res. vet. Sci., 15, 105 - 111.

LÖHNIS, F. (1922). Studies upon the life cycle of the bacteria part 1. Review of the literature 1838 - 1918. Mem. natn. Acad. Sci., 16, 1 - 252.

LORKIEWICZ, Z. (1957). Growth of stabilized L forms of Proteus vulgaris without the addition of serum and penicillin. Acta Microbiologica Polonica, 6, 3 - 8.

LOURIA, D.B., Kaminski, T., Grieco, M. & Singer, J. (1969). Aberrant forms of bacteria and fungi found in blood or cerebro spinal fluid. Arch. intern. Med., 124, 39 - 48.

LOWE, G.H. (1962). The rapid detection of lactose fermentation in paracolon organisms by the demonstration of β-D-galactosidase. J. med. Lab. Technol., 19, 21.

LYNN, R.J. (1962). Phosphatase activity of L-forms of group A beta haemolytic streptococci. Bact. Proc. P.58.

MACULLA, E.S. & Cowles, P.B. (1948). The use of glycine in the disruption of bacterial cells. Science, 107, 376 - 377.

MADOFF, S. (1970). L-forms from Streptococcus MG. Ann. N.Y. Acad. Sci., 174, 912 - 921.

MADOFF, S., Bruke, M.E. & Dienes, L. (1967). Induction and identification of L-forms of bacteria. Ann. N.Y. Acad. Sci., 143, 755 - 759.

MADOFF, S. & Dienes, L. (1958). L forms from pneumococci. J. Bact., 76, 245 - 250.

MAHONY, D.E. (1973). Antibiotic sensitivity of Clostridium perfringens and L-forms of Cl. perfringens induced by bacteriocin. Can. J. Microbiol., 19, 735 - 739.

MAKEMSON, J.C. & Darwish, R.Z. (1972). Calcium requirement and magnesium stimulation of Escherichia coli L form induction. Infection and Immunity, 6, 880 - 882.

MARSTON, J. (1961). Observations on L forms of streptococci. J. infect. Dis., 108, 75 - 84.

MARSTON, J.H. (1968). Production and cultivation of staphylococcal L forms. pp. 212 - 220. In : L.B. Guze (ed). Microbial Protoplasts, Spheroplasts and L forms. The Williams and Wilkins Co., Baltimore.

MARTIN, H.H. (1964). Composition of the mucopolymer in cell walls of the unstable and stable L-form of P. mirabilis. J. gen. Microbiol., 36, 441 - 450.

MATTMAN, L.H. (1968). L forms isolated from infections. pp. 472 - 483. In : L.B. Guze (ed). Microbial Protoplasts, Spheroplasts and L forms. The Williams and Wilkins Co., Baltimore.

MATTMAN, L. & Karris, G. (1966). L and transitional forms in meningitis. Bact. Proc., P.49.

MATTMAN, L.H. & Mattman, P.E. (1965). L forms of Streptococcus faecalis in septicaemia. Archs. intern. Med., 115, 315 - 321.

MATTMAN, L.H., Tunstall, L., Mathews, W. & Gordon, D. (1960). L variation in mycobacteria. Am. Rev. res. Dis., 82, 202 - 211.

MATSUHASHI, M., Dietrich, C.P. & Strominger, J.L. (1965). Incorporation of glycine into the cell wall glycopeptide in Staphylococcus aureus : role of sRNA and lipid intermediates. Proc. natn. Acad. Sci. U.S.A., 54, 587 - 594.

MATZUSCHITA, T. (1900). Die Einwirkung des Kochsalzgehaltes des Nahrbodens auf die Wuchsform der Mikroorganismen. Zeitschr. f. Hyg. 35, 495 - 510. m.6 Taf. (Cited by Löhnis, 1922).

MAXTED, W.R. (1972). Specific procedure and requirements for the isolation, growth and maintenance of the L phase of some microbial groups. pp. 423 - 449. In: J.R. Norris & D.W. Ribbons (eds). Methods in Microbiology, Volume 7A. Academic Press, London & New York.

MEDILL, M.A. & O'Kane, D.T. (1954). A synthetic medium for the L type colonies of Proteus. J. Bact., 68, 530 - 533.

MEDILL-BROWN, M. & Hutchinson, W.G. (1957). Effect of penicillin, growth phase, ultraviolet light, and ultrasonic vibration on the ability of Proteus mirabilis to form L type colonies. J. Bact., 74, 280 - 285.

MEDILL-BROWN, M., Hutchinson, W.G. & Cocklin, E. (1960). The L-forms of Proteus mirabilis. Ann. N.Y. Acad. Sci., 79, 374 - 379.

MELLON, R.R. (1925). Studies on microbic heredity: II - The sexual cycle of B.coli in relation to the origin of variants with special reference to Neisser and Massini's B. coli - mutabile. J. Bact., 10, 579 - 588.

MERLINE, J.R., Gordon, A. & Mattman, L.H. (1971). Cell wall deficient bacterial variants from man in experimental cardiopathy. Amer. J. clin. Path., 55, 212 - 220.

MICHAEL, J.G. & Braun, W. (1959). Serum spheroplasts of Shigella dysenteriae. Proc. Soc. exp. Biol. Med., 100, 422 - 425.

MICHEL, M.F. & Hijmans, W. (1960). The additive effect of glycine and other amino acids on the induction of the L-phase of group A β- haemolytic streptococci by penicillin and D - cycloserine. J. gen. Microbiol., 23, 35 - 46.

MITCHELL, P. & Moyle, J. (1956). Liberation and osmotic properties of the protoplasts of Micrococcus lysodeikticus and Sarcina lutea. J. gen. Microbiol., 15, 512 - 520.

MITCHELL, P. & Moyle, J. (1957). Autolytic release and osmotic properties of protoplasts from Staphylococcus aureus. J. gen. Microbiol., 16, 184 - 194.

MOLANDER, C.W., Kagan, B.M., Weinberger, H.J., Heimlich, E.M. & Busser, R.J. (1964). Induction by antibiotics and comparative sensitivity of L-phase variants of Staphylococcus aureus. J. Bact., 88, 591 - 594.

MOLANDER, C.W., Weinberger, H.J. & Kagan, B.M. (1965). Growth of staphylococcal L forms on and through membrane discs of various pore sizes. J. Bact., 89, p.907.

MONTGOMERIE, J.Z., Kalmanson, G.M. & Guze, L.B. (1966). The effect of antibiotics on the protoplasts and bacterial forms of Streptococcus faecalis. J. Lab. clin. Med., 68, 543 - 551.

MONTGOMERIE, J.Z., Kalmanson, G.M. & Guze, L.B. (1968). The susceptibility of protoplast and bacterial forms of Streptococcus faecalis to antibiotics. pp. 306 - 310. In : L.B. Guze (ed). Microbial Protoplasts, Spheroplasts and L-forms. The Williams and Wilkins Co., Baltimore.

MORRISON, T.H. & Weibull, C. (1962). The occurrence of cell wall constituents in stable Proteus L forms. Acta. path. microbiol. scand. 55, 475 - 482.

MORTIMER, E.A. Jr. (1968). The production of L-forms of group A streptococci in mice. pp. 345 - 351. In : L.B. Guze (ed). Microbial Protoplasts, Spheroplasts and L-forms. The Williams and Wilkins Co., Baltimore.

MUELLER, J.H. (1937). Nicotinic acid as a growth accessory substance for the diphtheria bacillus. J. Bact., 34, 429 - 441.

MULE, F. (1954). Observation by means of electron microscope on the blood of subjects affected with scarlet fever. Experientia, 10, 205 - 206.

MURRAY, R.G.E. (1968). Bacterial cell wall anatomy in relation to the formation of spheroplasts and protoplasts. pp. 1 - 18. In : L.B. Guze (ed). Microbial Protoplasts, Spheroplasts and L-forms. The Williams and Wilkins Co., Baltimore.

MUSCHEL, L.H. (1968). The formation of spheroplasts by immune substances and the reactivity of immune substances against diverse rounded forms. pp. 19 - 29. In : L.B. Guze (ed). Microbial Protoplasts, Spheroplasts and L-forms. The Williams and Wilkins Co., Baltimore.

MUSCHEL, L.H., Carey, W.F. & Baron, L.S. (1959). Formation of bacterial protoplasts by serum components. J. Immun., 82, 38 - 42.

McGEE, Z.A., Wittler, R.G., Gooder, H. & Charache, P. (1971). Wall-defective microbial variants : Terminology and experimental design. J. infect. Dis., 123, 433 - 438.

McKAY, K.A., Abelseth, M.K. & Vandreumel, A.A. (1966). Production of an enzootic-like pneumonia in pigs with "protoplasts" of Haemophilus parainfluenzae. Nature, London, 212, 359 - 360.

McQUILLEN, K. (1955). Protein, nucleic acid and adaptive enzyme formation in protoplasts of B. megaterium. J. gen. Microbiol., 13, p.IV.

McQUILLEN, K. (1956). Capabilities of bacterial protoplasts pp. 127 - 149. In: E.T.C. Spooner & B.A.O. Stocker (eds) Bacterial Anatomy. Cambridge University press.

McQUILLEN, K. (1958a). Lysis resulting from metabolic disturbance. J. gen. Microbiol., 18, 498 - 512.

McQUILLEN, K. (1958b). Bacterial "protoplasts": Effect of diaminopimetic acid deprival and penicillin addition compared in Escherichia coli. Biochim. biophys. Acta , 27, 410 - 411.

McQUILLEN, K. (1960). Bacterial protoplasts. pp. 249 - 359. In: I.C. Gunsalus & R.Y. Stanier (eds). The Bacteria. Vol.I. Structure. Academic press, New York and London.

NEIL, D.H., Garcia, M.M. & McKay, K.A. (1970). A stable L form of Haemophilus pleuropneumoniae. Can. J. comp. Med., 34, 50 - 58.

NEUHAUS, F.C. & Lynch, J.L. (1964). The enzymatic synthesis of D-alanyl-D-alanine. III - On the inhibition of D-alanyl-D-alanine synthetase by the antibiotic D-cycloserine. Biochemistry, 3, 471 - 480.

NIMMO, L.N. & Blazevic, D.J. (1969). Selection of media for the isolation of common bacterial L phase organisms from a clinical specimen. Appl. Microbiol., 18, 535 - 541.

O'BEIRNE, A.J. & Eveland, W.C. (1974). Induction of the L-phase of Listeria monocytogenes by rabbit alveolar macrophages in vitro. Can. J. Microbiol., 20, 963 - 966.

ORR, M.M., Tamarind, D.L., Cook, J., Finchman, W.J., Hawley, P.R., Quilliam, J.P. & Irving, M.H. (1974). Preliminary studies on the response of rabbit bowel to intramural injections of L-form bacteria. Br. J. Surg., 61, 921.

ØRSKOV, J. (1942). Streptobacillys moniliformis and the morphology of its variants. Acta path. microbiol. scand., 19, 575 - 585.

ØRSKOV, J. & Larsen, A. (1925). On bacterial variation. J. Bact., 10, 473 - 479.

PACHAS, W.N. & Currid, V.R. (1974). L form induction, morphology and development in two related strains of Erysipelothrix rhusiopathiae. J. Bact., 119, 576 - 582.

PACHAS, W.N. & Dienes, L. (1968). Variation of a strain of Streptococcus. Bact. Proc.,p.80.

PAGAL, W. (1934). Histological studies on the variability of the tubercle bacillus. J. Path. Bact., 39, 689 - 701.

PAINE, T.F. Jr. (1951). The similarity in action of bacitracin and penicillin on the Staphylococcus. J. Bact., 61, 259 - 260.

PARK, J.T. (1958). Inhibition of cell-wall synthesis in Staphylococcus aureus by chemicals which cause accumulation of wall precursors. Biochem. J., 70, P. 2p.

PARK, J.T. (1968). The mechanism by which penicillin causes conversion of bacterial cells to spheroplasts. pp. 52 - 54. In: L.B. Guze (ed). Microbial Protoplasts, Spheroplasts and L-forms. The Williams and Wilkins Co., Baltimore.

PARK, J.T. & Strominger, J.L. (1957). Mode of action of penicillin. Biochemical basis for the mechanism of action of penicillin and its selective toxicity. Science, 125, 99 - 101.

PEASE, P.E. (1967). Tolerated infection with the sub-bacterial phase of Listeria. Nature, London, 215, 936 - 938.

PEASE, P. (1969). Bacterial L forms in the blood and joint fluids of arthritic subjects. Ann. rheum. Dis., 28, 270 - 274.

PEASE, P. (1970). Morphological appearances of a bacterial L-form growing in association with the erythrocytes of arthritic subjects. Ann. rheum. Dis., 29, 439 - 444.

PEJU, G. & Rajat, H. (1906). Polymorphisme expérimental du bacille d'Eberth. Compt. rend. Soc. Biol., 60, 336 - 338. (Cited by Löhnis, 1922).

PIERCE, C.H. (1942). Streptobacillus moniliformis, its associated L_1 form and other PPLO. J. Bact., 43, P.780.

POHLOD, D.J., Mattman, L.H. & Tunstall, L. (1972). Structures suggesting cell-wall deficient forms detected in circulating erythrocytes by fluorochrome staining. Appl. Microbiol., 23, 262 - 267.

PRATT, B.C. (1966). Cell-wall deficiencies in L forms of Staphylococcus aureus. J. gen. Microbiol., 42, 115 - 122.

RAZIN, S. & Argaman, M. (1963). Lysis of Mycoplasma, bacterial protoplasts, spheroplasts and L-forms by various agents. J. gen. Microbiol., 30, 155 - 172.

RAZIN, S. & Oliver, O. (1961). Morphogenesis of Mycoplasma and bacterial L-forms colonies. J. gen. Microbiol., 24, 225 - 237.

REPASKE, R. (1956). Lysis of gram-negative bacteria by lysozyme. Biochim. biophys. Acta., 22, 189 - 191.

REVIS, C. (1912). The production of variation in the physiological activity of B.coli, etc. Proceed. Roy. Soc. ser. B, 85, 192 - 194. (Cited by Löhnis, 1922).

REYNOLDS, P.E. (1961). Studies on the mode of action of vancomycin. Biochim. biophys. Acta., 52, 403 - 405.

ROBERTS, R.B. (1966). L-form of Neisseria gonorrhoeae. J. Bact., 92, 1609 - 1614.

ROBERTS, R.B. (1967). Production of L-forms of Neisseria meningitidis by antibiotics. Proc. Soc. exp. Biol. Med., 124, 611 - 615.

ROBERTS, R.B. (1968). The in vitro production, cultivation and properties of L-forms of pathogenic neisseriae. pp. 230 - 238. In : L.B. Guze (ed). Microbial Protoplasts, Spheroplasts and L-forms. The Williams and Wilkins Co., Baltimore.

ROBERTS, D.E., Ingold, A., Want, S.V. & May, J.R. (1974). Osmotically stable L forms of Haemophilus influenzae and their significance in testing sensitivity of penicillins. J. clin. Path., 27, 560 - 564.

ROBERTS, R.B. & Wittler, R.G. (1966). The L form of Neisseria meningitidis. J. gen. Microbiol., 44, 139 - 148.

ROBINOW, C.F. (1956). The chromatin bodies of bacteria. pp. 181 - 214. In : E.T.C. Spooner & B.A.D. Stocker (eds). Bacterial Anatomy. Cambridge University Press.

ROGERS, K.B. & Taylor, J. (1961). Laboratory diagnosis of gastro-enteritis due to Escherichia coli. Bull. Wld. Hlth. Org., 24, 59 - 71.

ROSNER, R. (1972). A quantitative evaluation of three blood culture systems. Am. J. clin. Path., 57, 220 - 227.

ROTTA, J.W., Karakawa, W.W. & Krause, R.M. (1965). Isolation of L forms from group A streptococci exposed to bacitracin. J. Bact., 89, 1581 - 1585.

RUBIO-HUERTOS, M. & Gonzalez-Vasquez, C. (1960). Morphology and pathogenicity of L forms of Clostridium tetani induced by glycine. Ann. N.Y. Acad. Sci., 79, 626 - 631.

RUBIO-HUERTOS, M. & Herrera, E.C. (1966). Fixed L-forms of Agrobacterium tumefaciens induced by ultra light radiation. Nature, London, 209, 1262 - 1263.

SALOWICK, L. & Mattman, L. (1966). Factors in reversion of microbial L forms. Bact. Proc., p.141-142.

SALTON, M.R.J. (1952). Cell wall of Micrococcus lysodeikticus as the substrate of lysozyme. Nature, London, 170, 746 - 747.

SALTON, M.R.J. (1955). The formation of spores in protoplasts of Bacillus megaterium. J. gen. Microbiol., 13, IV - V.

SALTON, M.R.J. & Shafa, F. (1958). Some changes in the surface structure of gram-negative bacteria induced by penicillin action. Nature, London, 181, 1321 - 1324.

SCHEIBEL, I. & Assandri, J. (1959). Isolation of toxigenic L phase variants from Clostridium tetani. Acta. path. microbiol. scand., 46, 333 - 338.

SCHINDLER, C.A. & Schuhardt, V.T. (1964). Lysostaphin: A new bacteriolytic agent for the Staphylococcus. Proc. natn. Acad. Sci., U.S.A. 51, 414 - 421.

SCHMITT-SLOMSKA, J., Boue, A. & Caravano, R. (1972). Induction of L-variants in human diploid cells infected by group A streptococci. Infection and Immunity, 5, 389 - 399.

SCHMITT-SLOMSKA, J., Sacquet, E. & Caravano, R. (1967). Group A streptococcal L forms : I - Persistence among inoculated mice. J. Bact., 93, 451 - 455.

SCHÖNFELD, J.K. (1959). L forms of staphylococci, their reversibility, changes in the sensitivity pattern after several intermediary passages in the L phase. Antonie van Leeuwenhoek, 25, 325 - 331. (Cited by Watanakunakorn & Bakie, 1973).

SCHÖNFELD, J.K. (1961). L forms of Staphylococcus. Studies on the morphology of the transformation and on the reversibility. Antonie van Leeuwenhoek, 27, 139 - 150.

SEEBERG, S. (1973). Induction and surface growth of L phase variants of different Escherichia coli strains. Acta.path. microbiol. scand. Section B., 81, 703 - 706.

SEEBERG, S. & Brorson, J.E. (1974). Influence of different factors on the production of L-phase variants in individual Escherichia coli strains. Acta. path. microbiol. scand. Section B, 82, 214 - 218.

SEIBERT, F.B., Farrelly, F.K. & Shepherd, C.C. (1967). DMSO and other combatants against bacteria isolated from leukemia and cancer patients. Ann. N.Y. Acad. Sci., 141, 175 - 201.

SHARP, J.T. (1954). L colonies from hemolytic streptococci : new technic in the study of L forms of bacteria. Proc. Soc. exp. Biol. Med., 87, 94 - 97.

SHARP, J.T. (1960). The cell wall of bacterial L forms and PPLO. Ann. N.Y. Acad. Sci., 79, 344 - 355.

SHAW, C. & Clarke, P.H. (1955). Biochemical classification of Proteus and Providence cultures. J. gen. Microbiol., 13, 155 - 161.

SHOCKMAN, G.D. & Lampen, J.O. (1962). Inhibition by antibiotics of the growth of bacterial and yeast protoplasts. J. Bact., 84, 508 - 512.

SIEWERT, G. & Strominger, J.L. (1967). Bacitracin : An inhibitor of the dephosphorylation of lipid pyrophosphate, an intermediate in biosynthesis of the peptidoglycan of bacterial cell wall. Proc. natn. Acad. Sci. U.S.A., 57, 767 - 773.

SIMON, H.J. & Yin, E.J. (1970). Penicillinase studies on L phase variants, G-phase variants and reverted strains of Staphylococcus aureus. Infection and Immunity, 2, 644 - 654.

SMITH, W.E. (1944). Observations indicating a sexual mode of reproduction in a common bacterium. J. Bact., 47, 417 - 418.

SMITH, P.F. & Morton, H.E. (1951). The separation and characterization of the growth factor in serum and ascitic fluid which is required by certain PPLO. J. Bact., 61, 395 - 405.

SMITH, J.L. & Weinberg, E.D. (1962). Mechanism of antibacterial action of bacitracin. J. gen. Microbiol., 28, 559 - 569.

SMITH, J.A. & Willis, A.T. (1967). Some physiological characters of L forms of Staphylococcus aureus. J. Path. Bact., 94, 359 - 365.

SPICER, A.B. & Spooner, O.F. (1974). The inhibition of growth of Escherichia coli spheroplasts by antibacterial agents. J. gen. Microbiol., 80, 37 - 50.

STEMPEN, H. (1955). Demonstration of cell wall in the large bodies of Proteus vulgaris. J. Bact., 70, 177 - 181.

STEMPEN, H. & Hutchinson, W.G. (1951). The formation and development of large bodies in Proteus vulgaris ox - 19. 1 - Bright phase contrast observations of living bacteria. J. Bact., 61, 321 - 335.

STEWART, R.W. & Wright, D.W. (1969). Adaptation of meningococcal L form to agar-free liquid medium. J. Bact., 99, 899 - 901.

STROMINGER, J.L. (1968). Enzymatic reaction in bacterial cell wall synthesis sensitive to penicillins and other antibacterial substances. pp.55 - 61. In: L.B. Guze (ed). Microbial Protoplasts, Spheroplasts and L-forms. The Williams and Wilkins Co., Baltimore.

STROMINGER, J.L. (1973). The action of penicillin and other antibiotics on bacterial cell wall synthesis. John Hopkins Medical Journal, 133, 63 - 81.

STROMINGER, J.L. & Birge, C.H. (1965). Nucleotide accumulation induced in Staphylococcus aureus by glycine. J. Bact., 89, 1124 - 1127.

STROMINGER, J.L., Park, J.T. & Thompson, R.E. (1959). Composition of cell wall of Staphylococcus aureus. Its relation to the mechanism of action of penicillin. J. biol. Chem., 234, 3263 - 3268.

STROMINGER, J.L. & Threnn, R.H. (1959). The optical configuration of the alanine residues in a uridine nucleotide and in the cell wall of Staphylococcus aureus. Biochim. biophys. Acta., 33, 280 - 281.

STROMINGER, J.L., Threnn, R.H. & Scott, S.S. (1959). Oxamycin, a competitive antagonist of incorporation of D-alanine into a uridine nucleotide in Staphylococcus aureus. J. Am. chem. Soc., 81, 3803 - 3804.

STUART, R.D. (1948). The value of liquoid for blood culture. J. clin. Path., 1, 311 - 314.

SWIERCZEWSKI, J.A. & Reyes, K. (1970). Isolation of L-forms in a clinical microbiology laboratory. Appl. Microbiol., 20, 323 - 327.

TAUBENECK, U. (1962). Susceptibility of Proteus mirabilis and its stable L-form to erythromycin and other macrolides. Nature, London, 196, 195 - 196.

TAUBENECK, U. & Gumpert, J. (1967). Some notes on Gram-negative bacteria with experimentally altered surface structure. Folin Microbiol., 12, 258 - 263.

TEDESCHI, G.G. & Amici, D. (1972). Mycoplasma-like microorganisms probably related to L forms of bacteria in the blood of healthy persons. Annali Sclavo Rivista Di Microbiologia E Di Immunologia, 14, 430 - 442.

TEDESCHI, G.G., Amici, D. & Paparelli, M. (1969a). A study of the ultrastructure of bodies resembling Mycoplasma in cultures of erythrocytes from normal human blood. The Microscope, 17, 149 - 152.

TEDESCHI, G.G., Amici, D. & Paparelli, M. (1969b). Incorporation of nucleosides and amino acids in human erythrocyte suspensions : possible relation with a diffuse infection of Mycoplasma or bacteria in the L-forms. Nature, London, 222, 1285 - 1286.

THOMAS, J. (1976). A study of bacterial L forms. Ph.D. Thesis, Edinburgh University.

TIPPER, D.J. & Strominger, J.L. (1968). Biosynthesis of the peptidoglycan of bacterial cell wall. XII - Inhibition of cross-linking by penicillins and cephalosporins: Studies in Staphylococcus aureus in vivo. J. biol. Chem., 243, 3169 - 3179.

TULASNE, R., Terranova, T. & Lavillauriex, J. (1955). Obtention de formes l de diverses bacteries a gram negatif sur les milieux sans serum, mais contenant des vitamines du groupe B. Giorn. Microbiol. 1, 44 - 51. (Cited by Lederberg & St.Clair, 1958).

TUNSTALL, L. & Mattman, L. (1961). Growth of haemolytic streptococci in vitamin free medium. Experientia, 17, 190 - 191.

TURCK, M., Gutman, L.T., Wedgwood, R.J. & Petersdorf, R.G. (1968). Significance of bacterial variants in urinary tract infection. pp. 415 - 421. In: L.B. Guze (ed). Microbial Protoplasts, Spheroplasts and L-forms. The Williams and Wilkins Co., Baltimore.

VON HAEBLER, T. & Miles, A.A. (1938). The action of sodium polyanethol sulphonate (Liquoid) on blood cultures. J. Path. Bact., 46, 245 - 252.

VOUREKA, A. (1951a). Bacterial variants in patients treated with chloramphenicol. The Lancet, 1, 27 - 28.

VOUREKA, A. (1951b). Production of bacterial variants in vitro with chloramphenicol and specific antiserum. The Lancet, 1, 28 - 31.

WALKER, E.W.A. & Murray, W. (1904). The effect of certain dyes upon the cultural characters of the Bacillus typhosus and some other microorganisms. Br. med. J., 11, 16 - 18.

WALLAS, C.H. & Strominger, J.L. (1963). Ristocetins, inhibition of cell wall synthesis in Staphylococcus aureus. J. biol. Chem., 238, 2264 - 2266.

WANT, S.V. & May, J.R. (1975). Induction of L forms of Haemophilus influenzae in vitro. J. med. Microbiol., 8, 369 - 373.

WARD, J.R., Madoff, S. & Dienes, L. (1958). In vitro sensitivity of some bacteria, their L forms and pleuropneumonia-like organisms to antibiotics. Proc. Soc. exp. Biol. Med., 97, 132 - 135.

WARD, J.R. & Martin, C.H. (1962). Production of L phase variants of bacteria with cycloserine. Proc. Soc. exp. Biol. Med., 111, 156 - 160.

WATANAKUNAKORN, C. (1971a). Cycloserine induction, propagation and antimicrobial susceptibility of wall defective Staphylococcus aureus. Infection and Immunity, 3, 438 - 443.

WATANAKUNAKORN, C. (1971b). Vancomycin induction of L colonies of Staphylococcus aureus. Infection and Immunity, 3, 709 - 710.

WATANAKUNAKORN, C. & Bakie, C. (1973). Coagulase production, mannitol fermentation, penicillinase elaboration and phage typability of Staphylococcus aureus from L-phase variants. J. infect. Dis., 127, 571 - 575.

WATANAKUNAKORN, C. & Bakie, C. (1974). Pathogenicity of stable L phase variants of Staphylococcus aureus: failure to colonize normal and oxamide-induced hydronephrotic renal medulla of rats. Infection and Immunity, 9, 766 - 768.

WATANAKUNAKORN, C., Goldberg, L.M., Carleton, J. & Hamburger, M. (1968). Antimicrobial susceptibility of stable methicillin induced and lysostaphin-induced spheroplasts. Antimicrobial Agents and Chemotherapy, 382 - 387.

WATANAKUNAKORN, C., Goldberg, L.M., Carleton, J. & Hamburger, M. (1969). Staphylococcal spheroplasts and L colonies. III Induction by lysostaphin. J. infect. Dis., 119, 67 - 74.

WATANAKUNAKORN, C. & Hamburger, M. (1969). Induction of spheroplasts of Pseudomonas aeruginosa by carbenicillin. Appl. Microbiol., 17, 935 - 937.

WATT, P.J. (1970). Osmotic fragility and mouse virulence in enterococcal L-forms. J. med. Microbiol., 3, 209 - 217.

WEIBULL, C. (1953). The isolation of protoplasts from Bacillus megaterium by controlled treatment with lysozyme. J. Bact., 66, 688 - 695.

WEIBULL, C. (1958). Bacterial protoplasts. A. Rev. Microbiol., 12, 1 - 26.

WEIBULL, C. (1968). The morphology of protoplasts, spheroplasts and L-forms. pp. 62 - 73. In : L.B. Guze (ed). Microbial Protoplasts, Spheroplasts and L-forms. The Williams and Wilkins Co., Baltimore.

WEIBULL, C. & Beckman, H. (1960). Metabolism of small bodies isolated from stable Proteus L-form. Nature, London, 188, 428 - 429.

WEIBULL, C., Bickel, W.D., Haskins, W.T., Milner, K.C. & Ribi, E. (1967). Chemical, biological and structural properties of stable Proteus L forms and their parent bacteria. J. Bact., 93, 1143 - 1159.

WEIBULL, C. & Gyllang, H. (1965). Metabolic properties of some L-forms derived from Gram-positive and Gram-negative bacteria. J. Bact., 89, 1443 - 1447.

WEIBULL, C. & Hammarberg, K. (1963). Catalase activity of Proteus L forms and normal Proteus bacteria. J. Bact., 85, p.498.

WEIBULL, C. & Lundin, B.M. (1961). Factors influencing the growth of a stable L form of Proteus mirabilis. J. Bact., 81, 812 - 818.

WEIBULL, C. & Lundin, B.M. (1962). Growth of elements of various sizes found in cultures of stable Proteus L form. J. gen. Microbiol., 27, 241 - 248.

WEIDEL, W., Frank, H. & Martin, H.H. (1960). The rigid layer of the cell wall of Escherichia coli strain B. J. gen. Microbiol., 22, 158 - 166.

WEINBERGER, H.J., Madoff, S. & Dienes, L. (1950). The properties of L forms isolated from Salmonella and the isolation of L forms from Shigella. J. Bact., 59, 765 - 775.

WEINSTEIN, L. (1975). Mechanism of action of penicillin and related antimicrobial agents. pp. 1130 - 1166 In: L.S. Goodman and A. Gilman (eds). The Pharmacological Basis of Therapeutics. Fifth edition. Macmillan Publishing Co., Inc., New York, Collier Macmillan Canada Ltd., Toronto, Bailliere Tindall and London.

WEISS, R.L. (1976). Protoplast formation in Escherichia coli. J. Bact., 128, 668 - 670.

WEISS, R.L. & Fraser, D. (1973). Surfact structure of intact cells and spheroplasts of Pseudomonas aeruginosa. J. Bact., 113, 963 - 968.

WIAME, J.M., Storck, R. & Vanderwinkel, E. (1955). Biosynthese induite d'arabokinase dans les protoplastes de Bacillus subtilis. Biochim biophys. Acta, 18, 353 - 357.

WILLIAMS, R.E.O. (1963). L forms of Staphylococcus aureus. J. gen. Microbiol., 33, 325 - 334.

WILSON, C.D., Little, T.W.A. & Roberts, D.H. (1970). Isolation of L forms of Streptococcus agalactiae. Vet. Rec., 87, 300.

WILSON, Sir Graham S. & Miles, A.A. (1964). Topley and Wilson's principles of Bacteriology and Immunity. 5th ed. London: Edward Arnold.

WINTERBAUER, R.H., Gutman, L.T., Turck, M., Wedgwood, R.J. & Petersdorf, R.G. (1967). The role of penicillin-induced bacterial variants in experimental pyelonephritis. J. exp. Med., 125, 607 - 718.

WISE, E.M. Jr. & Park, J.T. (1965). Penicillin: its basic site of action as an inhibitor of a peptide cross-linking reaction in cell wall mucopeptide synthesis. Proc. natn. Acad. Sci. U.S.A., 54, 75 - 81.

WISHNOW, R.M., Strominger, J.L., Birge, C.H. & Threnn, R.H. (1965). Biochemical effects of novobiocin on Staphylococcus aureus. J. Bact., 89, 1117 - 1123.

WITTLER, R.G. (1952). L forms of Haemophilus pertussis in the mouse. J. gen. Microbiol., 6, 311 - 317.

WITTLER, R.G., Carry, S.G. & Lindberg, R.B. (1956). Reversion of a pleuropneumonia-like organism to a Corynebacterium during tissue culture passage. J. gen. Microbiol., 14, 763 - 774.

WITTLER, R.G., Malizia, W.F., Kramer, P.E., Tuckett, J.D., Pritchard, H.N. & Baker, H.J. (1960). Isolation of a Corynebacterium and its transitional forms from a case of subacute bacterial endocarditis treated with antibodies. J. gen. Microbiol., 23, 315 - 333.

WORK, E. (1957). Biochemistry of the bacterial cell wall. Nature, London, 179, 841 - 847.

WRIGHT, H.D. (1925). The bacteriology of subacute infective endocarditis. J. Path. Bact., 28, 541 - 578.

YOUMANS, G.P., Williston, E.H. & Simon, M. (1945). Production of small colony variants of Staphylococcus aureus by the action of penicillin. Proc. Soc. exp. Biol. Med., 58, 56 - 57.

YOUNG, R.M. & Dahlquist, E.H. (1967). Pathogenicity of L forms of Staphylococcus aureus. Am. J. clin. Path., 48, 466 - 473.

ZIERDT, C.H. & Wertlake, P.T. (1969). Transitional forms of Corynebacterium acnes in disease. J. Bact., 97, 799 - 805.

ZINDER, N.D. & Arndt, W.F. (1956). Production of protoplasts of Escherichia coli by lysozyme treatment. Proc. natn. Acad. Sci. U.S.A., 42, 586 - 590.