A STUDY OF WALL-DEFECTIVE VARIANTS OF ENTEROBACTERIACEAE AND STREPTOCOCCI.

by

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DECLARATION

I declare that the detailed planning of this work and its execution were my individual responsibility and except where indicated in the acknowledgements the work was devised and carried out by me.

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SUMMARY

The growth requirements of wall-defective microbial variants were studied to devise improved culture media for these interesting organisms. The isolates from the new media were classified as vegetative bacteria or as microbial variants of the transitional or classical L-phase variant types on the basis of their morphological and cultural characteristics.

From a consideration of their nature and the clinical circumstances at the time of isolation, most of the variants were presumed to be significant pathogens. Some of the more important features of these variants and the infections they caused may be summarised as follows.

Wall-defective Enterobacteriaceae variants

Classical L-phase variants obtained from Escherichia coli by lysozyme induction in the laboratory were grown in osmotically stabilised agar-free broth without nutritional enrichment and maintained by subculture in soft agar, but attempts to isolate naturally occurring wall-defective microbial variants from blood cultures using the same methods were singularly unsuccessful. However, wall-defective microbial variants were recovered quite readily from blood cultures by the use of an osmotically stabilised nutritionally enriched medium. Despite the apparent success of this medium, it behaved somewhat erratically from batch to batch due to changes in its nutritional qualities. These changes seemed to be related to a requirement for a folate metabolite supplied by a folic acid/pABA/cytosine mixture.

From a consideration of the laboratory characteristics of the above wall-defective Enterobacteriaceae variants and the antibiotic therapy of their associated infections, a number of interesting conclusions may be drawn. First, reverted wall-defective variants were not only more often sulphonamide-resistant than vegetative bacteria, but there was a positive correlation between prior therapy with folic acid or the folic anologue sulphonamide and the development of infection. These findings support the view that pABA or folic acid is a required metabolite. Second, although the number of cases is small, the evidence suggests that infections with wall-defective variants may be more refractory to antibiotic therapy than vegetative infections. This conclusion is based on the observation that such infections required treatment with additional antibiotics rather than with aminoglycoside alone. Third, there is no evidence from the present studies that wall-defective microbial variants are induced in vivo by prior beta-lactam therapy.

Wall-defective streptococcal variants

Wall-defective forms of viridans streptococcai are known to be auxotrophs with differing nutritional requirements but most are dependent on vitamin B6 (pyridoxine) and a sulphydryl moiety which may be supplied as thioglycollate or cysteine. Two of the more outstanding features of the streptococcal variants are their diphtheroid morphology and growth in satellitism. The latter is a symbiotic type of growth around colonies of another organism which supplies the required growth factor to the nutritionally defective streptococcus. This phenomenon can make it possible to isolate variants of this type without the use of media containing specific nutritional supplements. In this study the satellitism method was used for subcultures made from a simple primary

culture medium and this was compared first, with the use of a freshly prepared, nutritionally rich primary culture medium and then with a biphasic medium. Subcultures from these two media did not involve the use of the satellitism method. Finally, a primary culture medium supplemented with vitamin B6 and cysteine was compared directly with an osmotically stabilised primary culture medium supplemented with vitamin B6 and cysteine. Subcultures from both of the latter were made to medium supplemented with pyridoxine and cysteine.

Pyridoxine-dependent variants of viridans streptococci were isolated from the blood of three patients who had bacterial endocarditis. In each case, nutritional supplementation of the primary culture medium appeared to be as important as supplementation of the subculture medium. Once again, there was no evidence to suggest that the wall-defective variants had been induced by prior beta-lactam therapy.

INTRODUCTION

The wall-defective microbial variants that are the subject of this thesis are organisms which have not only survived the normally lethal effect of cell wall damage, but have also continued to grow and multiply without the support of a structurally normal cell wall. In the past, such variants were regarded as interesting curiosities or as unimportant laboratory artefacts. However, in more recent years there has been renewed interest in these variants. This has led to a growing appreciation of their scientific and clinical importance.

From a scientific point of view, wall-defective microbial variants tell us much about normal cell wall synthesis and how it may be interrupted by enzyme defects, metabolic deficiencies, antibiotics and errors of biosynthesis. They also give much insight into the role of the cell wall in determining bacterial morphology and in its physiological function. In a more indirect way, variants which totally lack a cell wall imply much about the nature and function of the underlying plasma membrane. This structure presumably has complex regulatory mechanisms that prevent osmotic lysis and cell death in the wall-free organism.

Wall-defective microbial variants are also of considerable importance from a clinical point of view. At the present moment, there is scanty evidence that they are pathogens in the sense that Koch's postulates have been satisfied, but there is an increasing awareness of their significance as casual agents in many illnesses, of which subacute bacterial endocarditis is a prime example. Moreover, though the role of antibiotics in the laboratory induction of such variants is widely recognised, the effect of therapeutically-used antibiotics on induction

is uncertain. Similarly, the choice of antibiotics, or combinations of antibiotics in the therapy of infections due to wall deficient forms is still a matter of debate.

For all of these reasons, the study of wall defective variants is a subject of major importance, and one that is likely to be of increasing relevance to clinical medicine in coming years.

SCOPE OF THE THESIS

This thesis is concerned with clinical and laboratory aspects of infection due to wall-defective microbial variants that occurred in the Victoria Infirmary, Glasgow in the years 1973 to 1983.

Although wall-defective variants can be isolated from many types of infection, the present study was confined to septicaemic patients, partly because pure cultures of the causative organisms can be procured quite readily from the blood, and partly because the clinical significance of such isolates is seldom in doubt. As septicaemia is a complication of a wide variety of infections, this scheme was not so restrictive as might be imagined. On the contrary, it gave a comprehensive view of the subject that helped to disclose some of the factors that predispose to infection with wall-defective variants.

Particular attention was paid to wall-defective Enterobacteriaceae variants, because organisms of the Enterobacteriaceae were the commonest cause of septicaemia in the Victoria Infirmary, and it seemed reasonable to assume that wall-defective Enterobacteriaceae might also occur fairly frequently.

The laboratory section describes methods for the culture of laboratory induced wall-defective variants, and also the use of undefined and partially defined blood culture media to determine the cultural requirements of wall-defective variants from clinical material.

The clinical section is concerned with the aetiology, clinical features and treatment of infection due to wall-defective variants.

WALL-DEFECTIVE VARIANTS: THEIR CLASSIFICATION AND TERMINOLOGY

The term L-form was coined in honour of the Lister Institute where Emmy Klieneberger-Nobel made her original observations on "symbionts" found in association with Streptobacillus moniliformis (Klieneberger, 1935; Klieneberger-Nobel, 1962). Later the term 'L-form' was applied to bacteria which had sustained cell wall damage or loss, and the language of the subject became somewhat confused. Each new expert invented an elaborate nomenclature (McQuillen, 1960; Guze, 1968; McGee et al., 1971; Maxted, 1972; Mattman, 1974(a)). Applied microbiologists adopted a more sensible course and used botanical terms, calling a wall-free cell a protoplast and a wall-damaged cell a spheroplast (Lamanna, Mallette & Zimmerman, 1973 (b)).

There is a need for a general term to describe bacteria which are devoid of a cell wall or in which the cell wall is impaired or damaged. The name 'L-form' is sometimes used in this way (Williams, 1963). This has the advantage of simplicity, and it has a claim to be used on grounds of priority (Dienes, 1973). The disadvantage is that it is essentially vague, and takes no cognisance of the different guises under which such bacteria may appear in the laboratory. The classification proposed by McGee et al., (1971) is an attempt to overcome this problem. In this classification organisms with damaged or deficient cell walls are referred to as 'wall-defective microbial variants'. The term 'L-form' is reserved to describe the distinctive 'fried egg' colonial morphology sometimes seen in cultures of such bacteria. The classification of McGee et al., recognises several different types of wall-defective microbial variant. It has been adopted in its entirety in this thesis, partly because it seems the best available, but

principally because it gives a fairly clear-cut definition of the different variants. It therefore has the advantage that typical forms of the various types can be readily identified, but the disadvantage that borderline variants have to be assigned to the transitional or unclassified groups on the somewhat negative grounds that they are not typical. The various wall-defective variants may be defined briefly as follows.

Protoplasts and spheroplasts are wall-defective variants that are Gram-negative, spherical, osmotically fragile and penicillin indifferent. They may replicate serially for a few divisions but are unable to go on to colony formation. Both forms may revert to the vegetative phase or undergo further transition to the classical L-form. The major difference between these two variants is that the protoplast is totally devoid of cell wall constituents as determined by electron microscopy, whereas the spheroplast contains some cell wall material.

L-phase variants are wall-defective variants that can replicate serially on solid media to give irregularly shaped or 'fried egg' colonies. Some are osmotically fragile, but others are not. Some L-forms are very unstable in that they readily revert to the vegetative form of existence. However, other forms do not revert easily and are therefore described as being stable.

In this thesis the essential feature of an L-phase variant is therefore its ability to reproduce serially to give an irregularly shaped or 'fried egg' colony.

Another type of colony occasionally observed is described as the 'crystal' colony. Although there is some doubt about the status of 'crystal' colonies, these are presumed to be deep forms of the superficial 'fried egg' colonies, and therefore true L-forms. Certainly

they do not possess the characteristics of either vegetative bacteria or of transitional phase variants.

Transitional phase variants are wall-defective variants showing great variation in cell properties as regards shape, size, Gram-reaction and cultural behaviour. They do not fit the typical features of any of the foregoing types. As a group they probably form the majority of wall-defective isolates made from clinical specimens and although most revert rather readily to the vegetative phase, some may undergo further transition to true L-phase variants.

Unclassified wall-defective variants. The main feature of unclassified variants is that they grow as vegetative forms on first passage in hypertonic media but yield no growth in the same media without osmotic stabilisation.

Using the above classification (McGee, et al.,1971) the wall-defective variants described in this Thesis could be classified with a good deal of confidence.

SECTION 1

A REVIEW OF THE HISTORY OF THE SUBJECT,

WITH AN ACCOUNT OF CURRENT CONCEPTS

Chapter 1

Biographical Notes

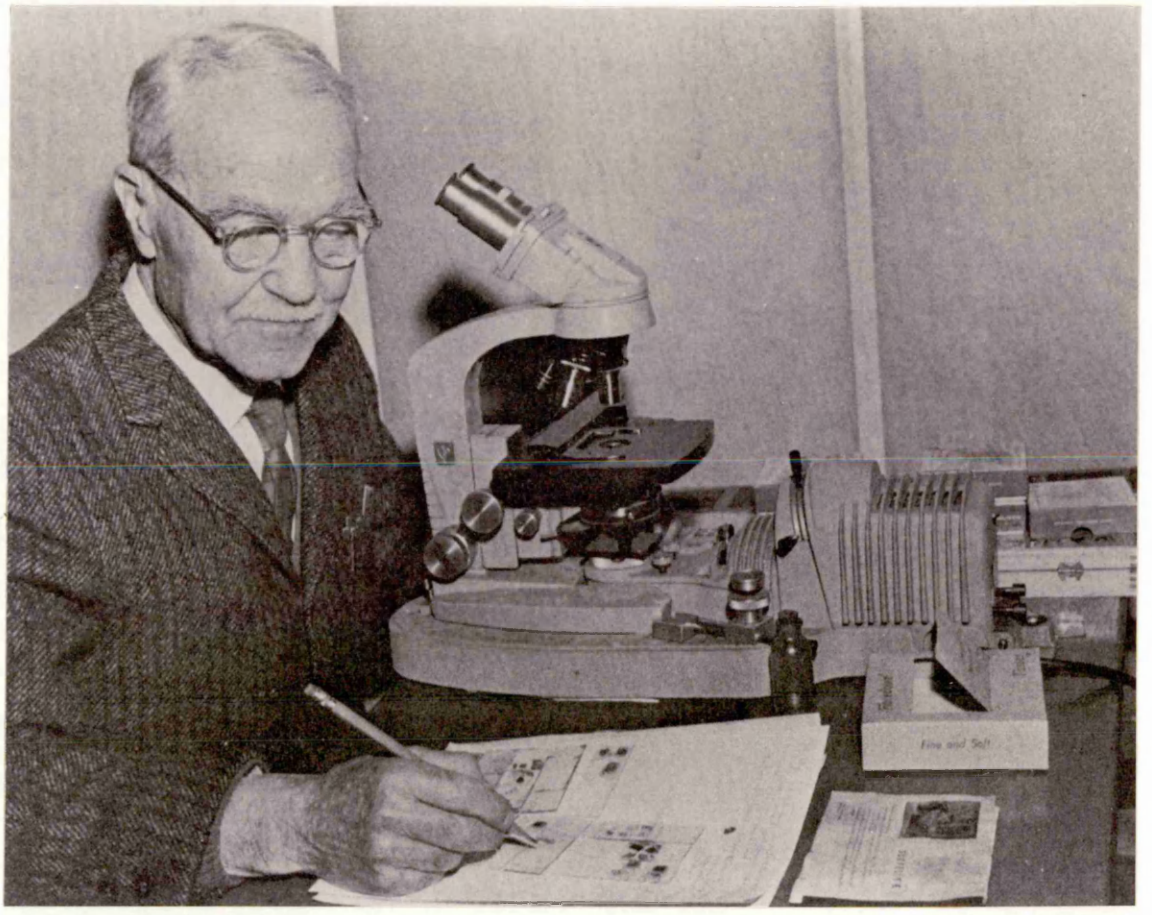


Figure 1

Louis Dienes

BIOGRAPHICAL NOTES

Emmy Klieneberger-Nobel

Emmy Klieneberger was originally a Public Health bacteriologist with Neisser in Frankfurt, and came to London in 1933 as a refugee from the anti-semitic persecution of Nazi Germany. She obtained a post at the Lister Institute, and was asked to do research on mycoplasmas, a field in which the bacteriologists of the time were intensely interested. She had a long and distinguished career in the area of mycoplasma research. Her work won international acclaim, culminating in the award of the Robert Koch medal in 1980. She was also a recipient of the Paul Ehrlich medal and was the first Honorary President of the International Organisation for Mycoplasmology. The study of wall-defective microbial variants was merely a by-product of her work on mycoplasmas (Klieneberger-Nobel, 1980; Lancet, 1985). She died in London on 11th September 1985, at the age of 93.

Louis Dienes (Figure 1)

Louis Dienes was responsible for most of the fundamental work on wall-defective microbial variants. He was an art student in Paris when he decided on a career in Medicine, graduating at the University of Budapest in 1908. Always ahead of his time, he was an immunologist whose studies with Neufeld were interrupted by the Great War. He served on the Eastern Front as Army Field Bacteriologist, and found time to collaborate with Weil on the immune response to typhus.

After the war, he left Hungary in the wake of the Bela Kun 'White Terror' regime, and emigrated to the United States. He continued his interest in immunology, specialising in research on the delayed hypersensitivity response, until research funding ceased during the

Depression. Then he turned to work on bacterial morphology, as this could be done cheaply.

In 1935 his attention was caught by L-phase variants and they became his major research interest, interspersed with work on mycoplasmas and bacterial morphology. He continued active research until 1970, and published his last paper at the age of 86, shortly before his death in 1974 (MGH. News, 1970; Swartz & Dvorak, 1974; Madoff, 1974).

Chapter 2

Review of the literature of the subject

REVIEW OF THE LITERATURE OF THE SUBJECT

1. Early Work on Wall-defective Microbial Variants.

The credit for the discovery of L-phase variants is generally given to Klieneberger (Klieneberger, 1935) but they were probably known earlier as the 'filterable forms' and pleomorphic bacteria described as part of the bacterial life cycle hypothesis current more than fifty years ago (Hadley, Delves & Klimek, 1931; Klieneberger-Nobel, 1951; Klieneberger-Nobel, 1960).

L-phase variants were described in 1935 as a result of research on mycoplasmas (pleuropneumonia-like organisms: 'PPLO'). Only two mycoplasmas were known at that time, but Klieneberger realised that other examples must exist. Her attention was drawn to the bacterium Streptobacillus moniliformis because of its associated mycoplasma-like elements. She called these 'L-organisms' in honour of the Lister Institute where she worked. She believed that the L-organisms were a new strain of mycoplasma (Klieneberger, 1935).

At first Klieneberger was unable to grow her organisms in pure culture. They required the presence of another organism for growth. Eventually Klieneberger used a filtrate of S. moniliformis as a growth supplement to obtain the L-organisms in pure culture. She concluded that the L-organisms were mycoplasma symbionts of S. moniliformis, and substantiated her opinion by comparative studies (Klieneberger, 1936).

Her findings were immediately challenged by van Rooyen (1936). He was unable to find either L-organisms or PPLO in his own S. moniliformis cultures, or in strains sent to him by Klieneberger, though he observed pleomorphic forms on microscopy of cultures grown on media containing less than the optimal amount of serum for growth, and he was able to

obtain a scanty non-symbiont growth after Berkefeld V filtration. He concluded that the L-organism found by Klieneberger was not a PPLO but a pleomorphic form of S. moniliformis. At this stage the subject was adopted by Dienes. He had already found a similar colony in a Bacillus subtilis culture (Dienes, 1932; Dienes, 1934; Pierson & Dienes, 1934) but credited Oerskov (Oerskov, 1931) with the original observation of such colonies. Dienes showed that the L-organism described by Klieneberger was a variant of S. moniliformis, not a mycoplasma (Dienes, 1938). This view found general support (Dawson & Hobby, 1939; Orskov, 1942; Warren, 1942; Freundt, 1950) and though Klieneberger made a spirited defence of her symbiosis theory (Klieneberger, 1942) she eventually recanted (Klieneberger-Nobel, 1949).

Dienes was a prolific worker, and he did an enormous amount of basic research on L-phase variants. He showed that they were not exclusive to S. moniliformis but occurred naturally in other bacterial species (Dienes, 1940a; 1940b; 1941; 1942; 1944a; 1944b; Dienes & Smith, 1944) sometimes as satellites round normal colonies (Dienes, 1939a). He devised laboratory methods for their induction, and, after Pierce (1942) had shown that L-phase variants were resistant to penicillin, he used penicillin as an inducing agent (Dienes & Smith, 1944; Dienes, 1947a; 1947b; 1948; 1949a; 1949b; Dienes, 1950; Dienes, 1953a). Thereafter he devoted himself to work on the induction of L-phase variants (Dienes, Weinberger & Madoff, 1950; Weinberger, Madoff & Dienes, 1950; Dienes & Weinberger, 1951) morphology (Dienes, 1968) electron microscopy (Dienes, 1953c; Dienes & Bullivant, 1968) and growth requirements (Dienes, Weinberger & Madoff, 1950; Dienes & Weinberger, 1951).

Much of Dienes work was repetitive, but he was responsible for three important ideas. These concerned the pathogenicity of L-phase

variants, their mode of reproduction, and their relationship to the mycoplasmas. Some of the early isolates described by Dienes came from clinical specimens (Dienes, 1940b; 1941; 1953a) and he was among the first to emphasise their potential pathogenicity (Dienes & Weinberger, 1951).

Secondly, Dienes believed that L-phase variants did not reproduce by the normal method of binary fission. Wall-damaged bacteria balloon where the cell protoplasm expands a weakened area of cell wall. The swollen areas are known as 'large bodies'. They occur spontaneously (Dienes, 1939b; 1940a; 1940b; 1941; 1942; Dienes & Smith, 1942; Dienes & Weinberger, 1951). They may also be produced by the effect of agents which interfere with cell wall synthesis, such as inorganic salts, pH changes, exposure to certain amino acids, or by deprivation of nutrients required for cell wall synthesis (Duguid & Wilkinson, 1961). Antibacterial agents also induce large bodies, and the beta-lactams are important in this respect (Dienes & Smith, 1944; Duguid, 1946; Dienes, 1948; Duguid & Wilkinson, 1961; Madoff, 1977; Glam & Waitkins, 1977).

At the time when Dienes made his observations on large bodies there were two theories on the role of pleomorphism in bacterial reproduction. The monomorphic theory, which eventually gained general acceptance, proposed that eubacteria reproduce by fission of normal cells and that pleomorphic cells are merely degenerate involution forms. The pleomorphic theory was that the pleomorphic cells were physiological stages in a bacterial life cycle, and that each abnormal form had a special function in the life cycle (Duguid & Wilkinson, 1961; Wilson & Miles, 1975a).

Dienes found that large bodies were the essential precursors of L-phase variants (Dienes, 1944b) producing normal bacteria by segmentation, or variants by germination and release of internal

granules (Dienes, 1942; Dienes & Smith, 1942; Dienes, 1943; Dienes, 1944a; Dienes & Smith, 1944; Dienes & Weinberger, 1951). This idea was verified by electron microscopy (Dienes, 1953c; Pease, 1965; Dienes & Bullivant, 1968; Bibel & Lawson, 1971). The alternative view is that L-phase variants arise by large body fission (Wilson & Miles, 1975d).

Lastly, Dienes was intrigued by the resemblance between L-phase variants and mycoplasmas (Dienes, 1945; Dienes, 1964) and suggested that both were derived from bacteria, mycoplasmas being descended from bacteria at an earlier evolutionary period (Dienes & Weinberger, 1951).

It is now recognised that the superficial colonial resemblance between mycoplasmas and L-phase variants is not genetic, but an effect of the physical milieu (Razin & Oliver, 1961; Klieneberger-Nobel, 1962). There are radical differences between them, and there is no justification for the idea that they are interrelated, of that the one proceeds from the other (Edward, 1954; Klieneberger-Nobel, 1962). Rather than regard mycoplasmas as the primitive ancestors of normal bacteria, or as derivatives of bacteria or L-phase variants, modern thought tends towards the view that mycoplasmas are the furthest evolved of prokaryotes, with a high rate of mutation (Maniloff, 1983).

2. Wall-defective Bacteria as Research Tools

Cell wall damage in wall-defective microbial variants was demonstrated by electron microscopy as early as 1948 (Smith, Hillier & Mudd, 1948) but its significance was not appreciated until the development of enzyme methods for the preparation of wall damaged bacteria in the laboratory. These were termed 'protoplasts' and 'spheroplasts' (Welshimer & Robinow, 1949; Weibull, 1956; Zinder &

Arndt, 1956; Gooder & Maxted, 1958). Numerous induction methods were developed (Salton, 1960; McQuillen, 1960; Gooder & Maxted, 1961; Duguid & Wilkinson, 1961) and the cultural requirements and reversion processes of protoplasts and spheroplasts were investigated.

The convenience afforded by protoplasts and spheroplasts for the study of bacterial cell physiology meant that interest in naturally occurring wall-defective variants waned. Wall-defective variants, spheroplasts and protoplasts were soon regarded as identical (McQuillen, 1956; Lederberg & St Clair, 1958; Freimer, Krause & McCarty, 1959; Rogers, Perkins & Ward, 1980). They were seen as bacteria which had survived wall injury and needed osmotic stabilisation, but which were otherwise metabolically normal (McQuillen, 1956; Cohen, Wittler & Faber, 1968). Both Emmy Klieneberger and Louis Dienes disagreed. Emmy Klieneberger thought that there were differences between L-phase variants and protoplasts related to the method of production (Klieneberger, 1960). Louis Dienes thought that L-phase variants were not the same as protoplasts and spheroplasts, and cited the reproductive differences between them in support of his view (Dienes, 1968). Furthermore, the new-found requirement for osmotic stabilisation in spheroplasts and protoplasts meant that the original observations of Klieneberger and Dienes were all but forgotten. Neither had used osmotic stabilisation for the culture of naturally occurring wall-defective variants (Montgomerie et al., 1972).

Reversion is a process of wall re-growth which was observed during the early work on wall-defective variants Dienes, 1939b; Dawson & Hobby, 1939; Pierce, 1942). Reversion can be effected even by protoplasts (Rosenthal & Shockman, 1975a; 1975b). It was found that the control of wall growth in bacteria involved a feed-back system (Lugtenberg, de Haas-Menger & Ruyters, 1972) and that the construction of a primer

fragment of wall was vital (Landman & Halle, 1963; Landman, 1968; Miller, Szigray & Landman, 1967). Two theories developed to explain the reversion process. In the physical theory reversion was attributed to the physical solidity of the milieu (Necas, 1961; Mattman, Tunstall & Rossmore, 1961; Gooder, 1964). The alternative was the nutritional theory which attributed reversion to stimulation by a nutrient (Crawford, Frank & Sullivan, 1958).

The question was intensively investigated by Landman and his colleagues. Adherents of the physical theory of reversion, they believed that all bacterial cells were capable of induction to the wall-defective state (Landman, Altenbern & Ginoza, 1958) and reversion therefrom (Landman, 1968; Schonfeld, 1974). They saw induction and reversion as wall damage and regeneration, not as mutation (Landman & Ginoza, 1961). They showed that the induction-reversion process was related to the action of autolysin (Landman & de Castro-Costa, 1976) a bacterial enzyme already implicated in induction (Duguid & Wilkinson, 1961; Joseph & Shockman, 1974) which is responsible for cell separation and wall remodelling during growth (Lamanna, Mallette & Zimmerman, 1973c). Landman and de Castro-Costa showed that wall damage was perpetuated by free, active autolysin in soft media. They explained that the cell built the vital 'primer fragment' of wall only when autolysin was inactive, bound to lipoteichoic acid (LTA). This occurred in solid medium, where diffusion was minimal, and dissociation from the autolysin-LTA complex was reduced (Landman & de Castro-Costa, 1976). An additional factor was that 'conditioning' to promote protein synthesis encouraged reversion (Landman & de Castro-Costa, 1976; Landman, de Castro-Costa & Bond, 1977). The three ideas conceived by Landman and his colleagues, that reversion required autolysin inhibition, the

presence of a primer fragment, and 'conditioning' were quickly accepted (Rogers, Ward & Elliot, 1976; Wyrick & Gooder, 1977) and interest in the nutritional basis of wall deficiency was lost, though it was recognised that nutritionally dependent pepidoglycan synthesis deficient mutants existed (Landman & de Castro-Costa, 1976).

Wall-defective variants are also of interest in bacterial genetics. Cell fusion has been observed after the induction of wall deficiency (Schonfeld & de Bruijn, 1977; Datta & Nugent, 1983) and recombinants have been formed by the conjugation of protoplasts and other wall-defective variants with intact vegetative bacterial cells (Wyrick, McConnell & Rogers, 1973). Finally, naturally occurring and induced wall-defective variants have unusual DNA metabolism. The naturally occurring variants have a high DNA content and the induced forms secrete such large quantities of DNA into the surrounding medium that the organisms aggregate (Chattman, Mattman & Mattman, 1969).

3. Wall-defective Microbial Variants in Medical Microbiology

Wall-defective variants were originally regarded as harmless curiosities (Klieneberger-Nobel, 1960). Apart from sporadic reports of their isolation in urogenital infection (Voureka, 1951; Moustardier, Brisou & Perrey, 1953; Barile, Yaguchi & Eveland, 1958) the subject was ignored until the introduction of the beta-lactam antibiotics. Osmotic lysis ensues when bacteria are exposed to these antibiotics, as beta-lactams interfere with the growth of new cell wall. Wall-defective variants can result from this process if the bacteria are protected from osmotic stress. Such protection may be afforded by an intracellular habitat (Mattman, 1974b; Roux, 1977), by the stabilising effect of spermine and mucine in pus (Tabor, 1962; Gooder, 1964; Mortimer et al.,

1972), or in urine by acidity and high osmolality (Braude, Siemienski & Lee, 1968; Gnarpe & Edebo, 1970).

The concept of wall defective variants as a cause of treatment failure emphasised the need to use osmotic stabilisation for their culture (Hamburger, 1968). Osmotically stabilised cultures were used successfully to isolate such variants from septicaemia (Nativelle & Deparis, 1960; Klodnitskaia, 1962; Mattman, 1968; Louria et al., 1969; Brem, 1969; Louria et al., 1976), from infected urine (Gutman Schaller & Wedgwood, 1967; Coleman & Little, 1967; Shulbt'sev et al., 1973), and from chronic osteomyelitis and other chronic staphlococcal infections (Godzeski, 1968; Kagan, 1968; Gordon, Greer & Craig, 1971).

The wall-defective variants isolated were not highly pathogenic when inoculated into experimental animals (Freundt, 1956) and there were few convincing accounts of human sepsis attributable to them (Hamburger,1968; Feingold, 1969; Clasener, 1972). It was suggested that wall-defective variants might have the capacity to lie dormant, only to cause recrudescence of infection if reversion to the normal bacterial form occurred (McDermott, 1958; Godzeski et al., 1965; Gutman et al., 1968; Lapinski & Flakas, 1967; Madoff, 1977; Roux, 1977; Timakov, 1977). This was reminiscent of the old concept of 'resting bacteria' (Kendall, Friedemann & Ishikawa, 1930; de Takats, 1932). There was little clinical evidence of a role in relapsing infection but the alleged propensity for dormant infection, coupled with the unusual antigenic composition of wall-defective variants (Klieneberger, 1942; Weibull, 1956; Freimer, 1964; Lynn & Haller, 1968; Weibull et al., 1967;

Bertolani, Elberg & Ralston, 1975) suggested a link with auto-immune disease. Claims that this occurred in rheumatic fever and acute nephritis were made (Freimer & Zabriskie, 1968; Mashkov, 1974; Iesmantaite et al., 1975; Kagan et al., 1977; De Vuono & Panos, 1978) and there were similar findings in degenerative arthritis (Klodnitskaia, Smeranov & Adamchook, 1977; Klodnitskaia, 1978; Stewart & Alexander, 1969; Cook, Fincham & Lack, 1969; Bartholomew & Nelson, 1972), but at least some of the variants isolated may have been derived from contaminating bacteria. More recently, the connection between inflammatory bowel disease and colonisation by wall-defective variants has been investigated. Though such variants were isolated from mesenteric lymph nodes in ulcerative colitis (Klodnitskaia, 1973; Burnham, Stanford & Lennard-Jones, 1977) and Crohns disease (Parent & Mitchell, 1976; 1978; Burnham, Stanford & Lennard-Jones, 1977) and from intestinal biopsy specimens in both ulcerative colitis and Crohns disease (Belsheim et al., 1983) they could not be demonstrated consistently (Bolton et al., 1973; Kagnoff, 1978; Whorwell et al., 1978; Shafi et al., 1981; Lev, 1981).

Enthusiasm soon gave way to disillusion. It became clear that wall-defective variants were of dubious pathogenicity (Fass & Barnisham, 1977. Animal inoculation showed that they were avirulent (Phair et al., 1974; Roux, 1977) and that they were easily destroyed by the immune system (Haller & Lynn, 1968; McGee et al., 1972; Montgomerie et al., 1976). Finally, the methods used for the isolation of wall-defective variants were vehemently criticised, since they often involved filtration and prolonged culture and were therefore prone to contamination (McGee et al., 1971).

The subject became the resort of the unorthodox. Such were the advocates of the 'intra-erythocyte parasite' theory. The believed that the blood was not sterile in health, but parasitised by abnormal bacterial forms, which could revert to cause disease if the host-parasite relationship altered (Mattman, 1974b; Domingue et al., 1977; Tedeschi & Santarelli, 1977; Domingue & Schegel, 1977; Bisset & Bartlett, 1978; Bisset, Tallack & Bartlett, 1979). The fact that the reverted variants invariably proved to be skin commensals did nothing to rehabilitate the subject in the eyes of conventional bacteriologists.

For all of these reasons, medical microbiologists lost interest in wall-defective variants. This was the result of well-founded criticism of the methods used for their isolation, and a general acceptance of the view that wall-defective variants were of doubtful pathogenicity. Indeed, such variants came to be regarded as laboratory artefacts or harmless contaminants not found in the natural habitat (Cruickshank et al., 1973). Paradoxically the use of hypertonic culture media is increasingly advised for the isolation of wall damaged pathogenic bacteria (Jephcott, 1981; Eng & Maeland, 1982; Crist, Amsterdam & Neter, 1982; La Scolea et al., 1983). Another area of current interest concerns the nutritionally exacting 'diphtheroid' streptococci. These streptococci are wall-deficient (Bouvet, Ryter & Acar, 1977; Peipkorn & Reichenbach, 1978; Bouvet et al., 1980). Like the variants originally described by Klieneberger and by Dienes, they may occur as symbionts and apparently do not require osmotic stabilisation. They are held to be a separate variety of streptococci, characterised by the presence of a unique red chromophore and by specific genetic markers (Bouvet et al., 1985).

It seems that there is increasing appreciation of the potential importance of wall-defective microbial variants, that isolation methods are improving, and that the time has come for a reappraisal of the subject. Appropriately, the completion of the work described in this Thesis was in 1985, a year which marked the fiftieth anniversary of the original description by Emmy Klieneberger-Nobel.

SECTION II

OBJECTIVES

Chapter 3

Problems in the Study of Wall-defective Microbial Variants and an Outline of the Purpose of the Thesis

PROBLEMS IN THE STUDY OF WALL-DEFECTIVE MICROBIAL VARIANTS AND AN OUTLINE OF THE PURPOSE OF THE THESIS

Introduction

The chief obstacle to the study of wall-defective microbial variants was that very little was known about the culture medium required. To resolve this problem, I tested established culture media with antibiotic-induced variants and subjected the best media to a trial with blood cultures. A new medium, rich in sucrose and nutrients, was evolved for blood culture work. This medium was called Victoria Medium F. It was restricted in scope, because the variants isolated were almost exclusively those of the Enterobacteriaceae (Brogan, 1976).

Purpose of the Thesis

1) Culture Medium Tests with Clinical Material: The Batch Phenomenon.

Some batches of culture medium did not grow variants. This difficulty was also encountered by Gooder and Maxted (Gooder & Maxted, 1961). The possibilities were that an inhibitor was present in some batches, or that batches varied in nutrient quality. The problem was investigated along these lines, basing assessment on the performance of different blood culture media in clinical work.

2) Culture Medium Tests in vitro: Laboratory Induction of Wall-defective Microbial Variants.

The use of clinical material to assess culture media was time-consuming, particularly since blood cultures were the area of investigation chosen. In addition, the results achieved with blood cultures were influenced by many variables. Laboratory tests on the

culture medium were needed, to improve the scope of the method and to elucidate the batch phenomenon. The problem was the lack of a stable wall-defective variant to use as a test organism.

Neither variants from clinical material nor those prepared by the antibiotic induction method were suitable because they were unstable, and reverted very easily to the vegetative state. An alternative method of induction was needed to produce a more stable test organism.

3) Identification of Wall-defective Microbial Variants.

In my preliminary work (Brogan, 1976), I complied with the experimental design of McGee et al., (1971). This comprised:-

i) demonstration of variant growth on culture medium free from known inducers.

I distinguished the growth of wall-defective variants from vegetative growth on two grounds. These were the recovery of bacteria in L-form medium only, without growth in conventional blood culture media inoculated and incubated in parallel with this medium, and the cultural appearance of the L-form colonies.

ii) proof of microbial nature given by reversion to the vegetative state.

iii) significant isolate with clinical correlation.

These criteria were not wholly satisfactory. The cultural appearance of the L-form colonies was not a reliable diagnostic feature, because some strains reverted very easily and quickly to the vegetative state. Failure to revert was also a difficulty, because the reversion method which I used was not always successful.

Factors which perpetuated the variant state, and conditions necessary for reversion were therefore investigated in an attempt to solve these problems.

4) Clinical Features

As part of my preliminary work I described a small number of patients with septicaemia. The source of the septicaemia in these patients was chronic relapsing urinary tract infection (Brogan, 1977). An extended survey of septicaemia was planned to see if this finding could be confirmed.

SECTION III

LABORATORY STUDIES ON A LYSOZYME/EDTA INDUCED WALL DEFECTIVE VARIANT OF ESCHERICHIA COLI

Chapter 4

Induction and Microscopy

INDUCTION BY THE LYSOZYME/EDTA METHOD

INTRODUCTION

A laboratory culture of wall-defective bacteria was needed for basic studies on reversion and cultural conditions. Unstable 'spheroplast' variants were chosen for this purpose, because they revert easily and are also capable of multiplication (Collee, 1978).

OBJECTIVE

The object was to prepare an induced culture of wall-defective variants of the spheroplast type.

MATERIALS & METHODS

Choice of Test Organism

The test organism was E. coli. The strain used was the National Collection of Type Cultures (NCTC) No. 10418, an organism held in most medical microbiology laboratories as a control for antibiotic sensitivity tests.

E. coli was chosen because it was a bacterium in which a partial cell wall defect could be induced to give unstable 'spheroplasts' (Murray, 1968; Birdsell & Cota-Robles, 1968) and for continuity, because the Victoria Medium F previously described by me (Brogan, 1976) on which much of the work of this Thesis is based, grew wall-defective Enterobacteriaceae variants well.

Method

Induction was performed by the method of Weiss (1976). This method used lysozyme as the inducing agent, and employed edetic acid (EDTA) to sensitise the bacteria to lysozyme by removing the surface metallic ions which interfere with lysozyme activity (Repaske, 1958; Murray, 1968). Additional refinements were the use of sucrose of low metal content as osmotic stabiliser to improve the yield of wall-defective variants (Tabor, 1962) and the use of Tris buffer as an adjunct for EDTA chelating activity (Goldschmidt & Wyss, 1967).

INDUCTION PROCEDURE (Weiss, 1976)

1) A culture of E. coli NCTC 10418 in Oxoid Brain Heart Infusion broth (BHI Oxoid) was incubated overnight with shaking at 37°C. The cells were harvested by centrifugation at 4°C (3000 rpm. for five minutes) and then washed twice with sterile Tris buffer.They were resuspended in 42.75 ml sterile 0.1M Tris buffer containing 20% sucrose, prewarmed to 37°C.

2) The suspension was transferred aseptically to a sterile 50 ml flask containing a stirring bar, prewarmed to 37°C. The flask was placed on a magnetic stirrer at 37°C. The stirrer was set to a low speed, and 2.25 ml (4500 µg) lysozyme was added, giving a final concentration of 100 µg lysozyme/ml. Incubation was continued with gentle stirring for 10 minutes.

3) Five ml of sterile EDTA, prewarmed to 37°C, was then added slowly, over a period of two to three minutes. The suspension was incubated at 37°C for 10 minutes. It rapidly settled to the bottom of the flask, and the deposit was removed without centrifugation and examined.

EXAMINATION OF A SUSPENSION OF WALL-DEFECTIVE VARIANTS BY MICROSCOPY

1) Phase Contrast Microscopy

Immediate examination of the suspension showed that 90% of the cells were spherical.

The cells were incubated for one hour at room temperature (22°C) and again examined by phase contrast microscopy. Cell aggregates had formed.

my photographs?

Both the spherical shape of the cells (Cruickshank et al., 1973) and their tendency to form aggregates after room temperature incubation (Langenfeld & Smith, 1963; Marston, 1968) were characteristic of suspensions of wall-defective microbial variants.

2) Electron Microscopy

A. Preparation of Cultures for Electron Microscopy.

The test culture was a preparation of lysozyme/EDTA induced wall-defective variants of E. coli NCTC 10418.

The control culture was a preparation of E. coli NCTC 10418 which had not been treated with lysozyme/EDTA.

An overnight culture of E. coli NCTC 10418 in Oxoid Brain Heart Infusion Broth was divided into two aliquots. The cells in both aliquots were harvested by centrifugation, washed twice in sterile Tris buffer, and resuspended in 42.75 ml sterile 0.1M Tris buffer containing 20% sucrose, which had been prewarmed to 37°C.

One cell aliquot was set aside as control material.

The other was treated with lysozyme/EDTA to induce wall defect.

This was the test material.

B. Preparation of Pour Plate Cultures.

The medium was Victoria Medium F. (Appendix I). The pour plates were made with a concentration of 0.2% agar, giving a soft medium. This was done in an attempt to prevent reversion to the vegetative state (Chapter 5).

Two plates were made, one with an inoculum of 0.25 ml of the test suspension, and one with an inoculum of 0.5 ml of the test suspension. In addition, two control plates were made, one with an inoculum of 0.25 ml of a control vegetative E. coli suspension and one with an inoculum of 0.5 ml of control vegetative E.coli suspension.

The test and control plates were incubated for 48 hours at 37°C in an atmosphere of 5% carbon dioxide in air.

They were then examined by plate microscopy, and it was confirmed that the control vegetative inoculum had yielded colonies of the vegetative type and the test inoculum had yielded colonies of the L-form type characteristic of the growth of L-phase variants.

C. Fixation Method.

The plates were flooded with 1:40 glutaraldehyde as a fixative, and then placed in the refrigerator at 4°C for 30 minutes. The excess glutaraldehyde was then removed, and the fixed cultures were submitted for electron microscopy forthwith.

Figure 2

Electron Microscopy. Control vegetative cells of E.coli NCTC 10418. Medium: 0.2% agar plate of Victoria Medium F. Fixative 1:40 glutaraldehyde. Final magnification x 45,000.

D. Electron Microscopy Procedure.

The specimens were post-fixed in 1% aqueous osmium tetroxide. Dehydration was achieved using several changes of absolute ethanol prior to clearing in propylene oxide and embedding in EM1X resin (EM scope Ltd). After overnight polymerisation at 70°C the blocks were sectioned at a thickness of 50 nm, mounted on 200 mesh copper grids and stained with saturated uranyl acetate in 50% ethanol for 10 minutes followed by five minutes staining in Reynolds lead citrate (Reynolds, 1963). This method avoids the disadvantage of precipitate formation inherent in the use of other lead compound solutions. The grids were examined at an accelerating voltage of 80 kV in a Philips EM 301 G transmission electron microscope and the images recorded on Kodak EM film, type 4489 and printed on Ilfospeed resin coated paper.

Results of Electron Microscopy

i) Control Cells. A typical example is illustrated in Figure 2. The cell contents are present, indicating potential viability. The cell has the normal cylindrical shape typical of E. coli. The cell wall outline is essentially smooth, and the cell wall and cytoplasmic membrane are closely approximated throughout most of the cell. The cell wall is therefore basically healthy. However, some bleb formation is evident, and there is a little separation of the cell wall and cytoplasmic membrane. This minor degree of cell wall injury was attributed to damage during the fixation process.

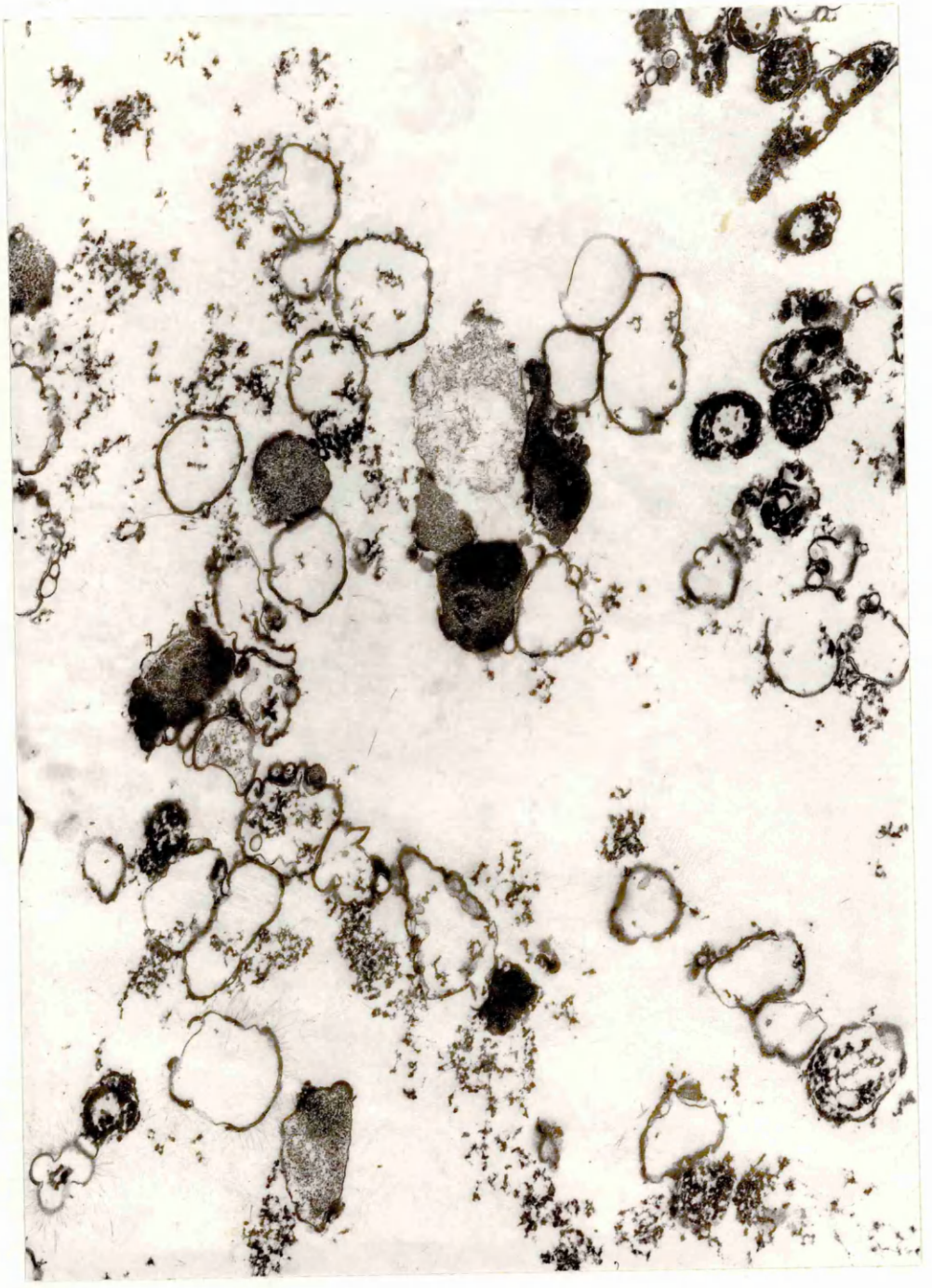


Figure 3

Electron Microscopy. Lysozyme/EDTA prepared spheroplasts of E.coli NCTC 10418. Medium 0.2% agar plate of Victoria Medium F. Fixative 1:40 glutaraldehyde. Final magnification x 15,000.

ii) Wall-defective Cells. Typical examples are illustrated in Figures 3 and 4. Many cells lack cell contents and appear as 'cell ghosts'. Other cells have retained their cell contents and are thus potentially viable.

The cells have lost the typical cylindrical shape of E. coli and are spherical. The cell walls are no longer smooth in outline, but are thrown into folds, and there is obvious separation of wall and cytoplasmic membrane in the cells. These features show that the cells have sustained wall damage.

Retention of the cell wall, albeit in a damaged condition, shows that the cells are spheroplasts rather than protoplasts.

Conclusion

It was concluded that the lysozyme/EDTA method of induction had produced wall-defective E. coli variants of the spheroplast type.

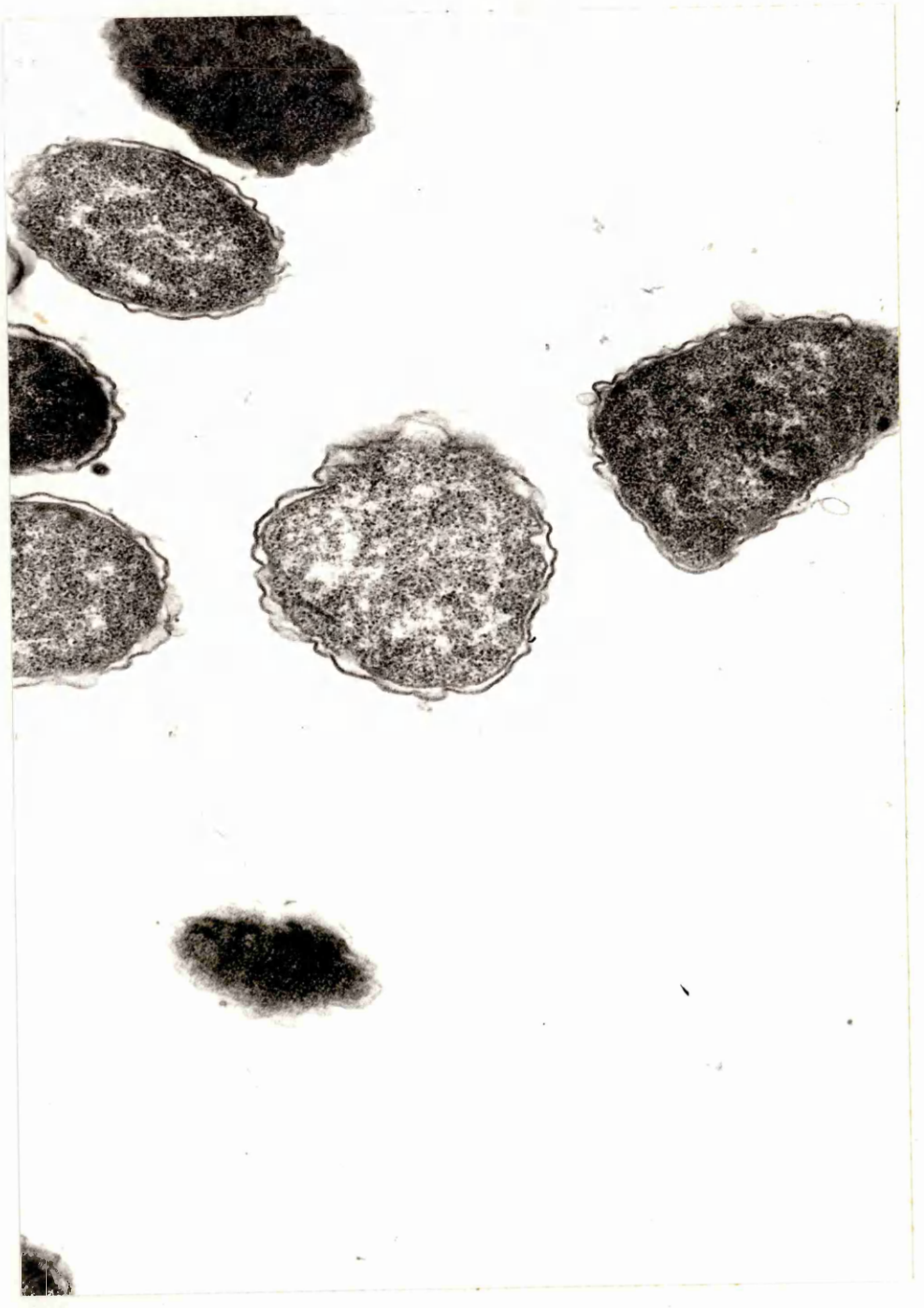


Figure 4

Electron Microscopy. Lysozyme/EDTA prepared spheroplasts of E.coli NCTC 10418. Medium 0.2% agar plate of Victoria Medium F. Fixative 1:40 glutaraldehyde. Final magnification x 44,000.

Chapter 5

Maintenance of the Wall-Defective State and its Reversion. Culture in Solid Medium.

MAINTENANCE OF THE WALL-DEFECTIVE STATE AND ITS REVERSION.

CULTURE IN SOLID MEDIUM.

INTRODUCTION

Most wall defective bacteria revert readily to the parent form as soon as they are removed from the inducing agent. Reversion can be prevented by conditions which encourage cell autolysin activity (Landman & de Castro-Costa, 1976). Autolysin is inhibited in stiff media and activated in soft media.

OBJECTIVE

The object was to find the agar concentration which maintained the wall-defective state and the agar concentration which promoted reversion.

MATERIALS & METHODS

Culture Medium

The optimal agar concentration for wall-defective growth was determined by the use of pour plates of Victoria Medium F, solidified with varying concentrations of agar (Oxoid No. 1). This agar had a setting concentration of 1%. The agar concentrations tested were 0.1%. 0.15%, 0.2%, 0.3% and 1%. Since some of these gave a very soft medium, an agar-gelatin mixture was tried (Gooder, 1968). The gelatin concentration was 5% and this was tested with 0.15% agar and with 0.2% agar.

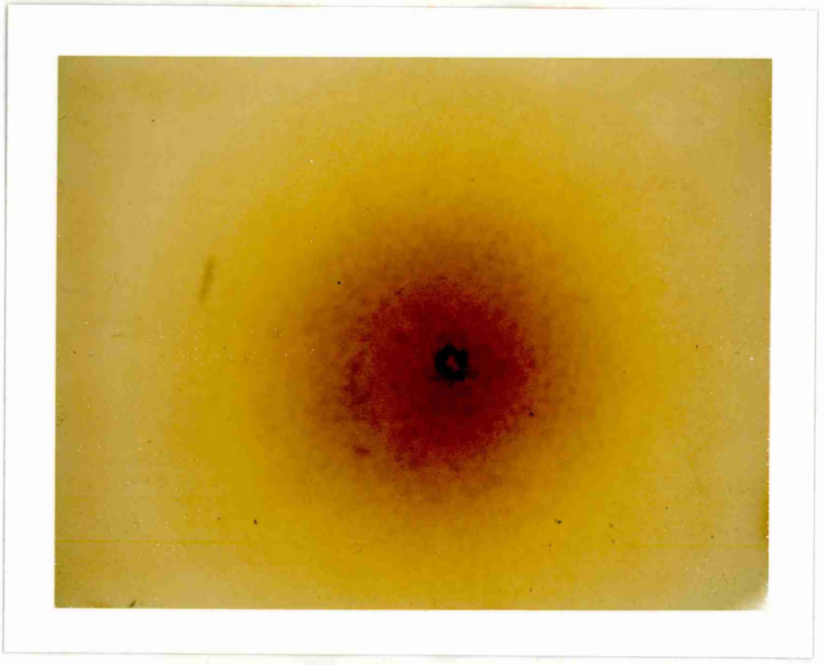


Figure 5

Culture of lysozyme/EDTA prepared spheroplasts of E.coli NCTC 10418. Medium 0.2% agar plate of Victoria Medium F. Magnification x 100. L-form 'fried egg' colony seen at agar surface.

Test Organism

A fresh suspension of wall-defective E. coli NCTC 10418 prepared by the lysozyme/EDTA induction method.

Inoculum

One ml of the suspension was inoculated to each pour plate to give a final volume of 10 ml.

Culture Conditions

The plates were incubated for three days at 37°C in an atmosphere of 5% carbon dioxide in air. They were then examined by naked eye inspection and by plate microscopy using a stereo microscope (Elvar) and a final magnification of x 25.

RESULTS

The results are shown in Tables 1 and 2.

L-form Colonies

At the lowest agar concentrations, the medium was too soft for convenient manipulation, but both the 0.2% agar and the 0.2% agar with gelatin were of suitable consistency, and gave a good yield of L-form colonies, more abundantly in the 0.2% agar medium. Naked eye inspection: There was a scarcely perceptible haze of tiny colonies.

Plate microscopy. Surface growth was minimal. Optimal conditions lay in the depths of the agar. The colonies were translucent and the colonies near the surface were the 'fried egg' type with a denser centre and a lacy periphery (Fig. 5). Knobbly 'mulberry'colonies (Brogan, 1976) and the 'crystal' colonies sometimes seen in cultures of wall-defective

Figure 6

Culture of wall-defective variants of E. coli derived from blood culture in Victoria Medium F. Medium 0.2% agar plate of Victoria Medium F. Magnification x 100. 'Crystal' colony.

variants derived from clinical material (Fig. 6) were not observed, but deep colonies were irregularly shaped.

Vegetative Colonies

The inoculum used consisted of approximately 90% spheroplast cells with 10% vegetative cells. The few vegetative colonies which were present in soft medium were probably vegetative cells from the original inoculum, which were resistant to the action of lysozyme. The vegetative colonies grown as the predominant colony type in stiff medium were presumably derived from the spheroplast cells which predominated in the original inoculum together with a much smaller number of lysozyme resistant cells. Accordingly it seemed probable that the heavy growth of vegetative colonies obtained in stiff medium was mainly attributable to reversion of the spheroplast cells from the original inoculum. Sometimes reverting colonies could be seen(Fig. 7). The best yield of vegetative colonies was at an agar concentration of 1%.

Naked eye inspection. The colonies grew well on the surface of the medium, and were of normal size.

Plate Microscopy. Colonies in the depth of the medium were opaque, smooth and symmetrical (Fig. 8).

Repetition of Experiment

The experiment was repeated twice, using 0.2% agar medium and 1% agar medium only. The results were the same as those obtained in the initial experiment. L-form colonies predominated in 0.2% agar and vegetative colonies predominated in 1% agar.

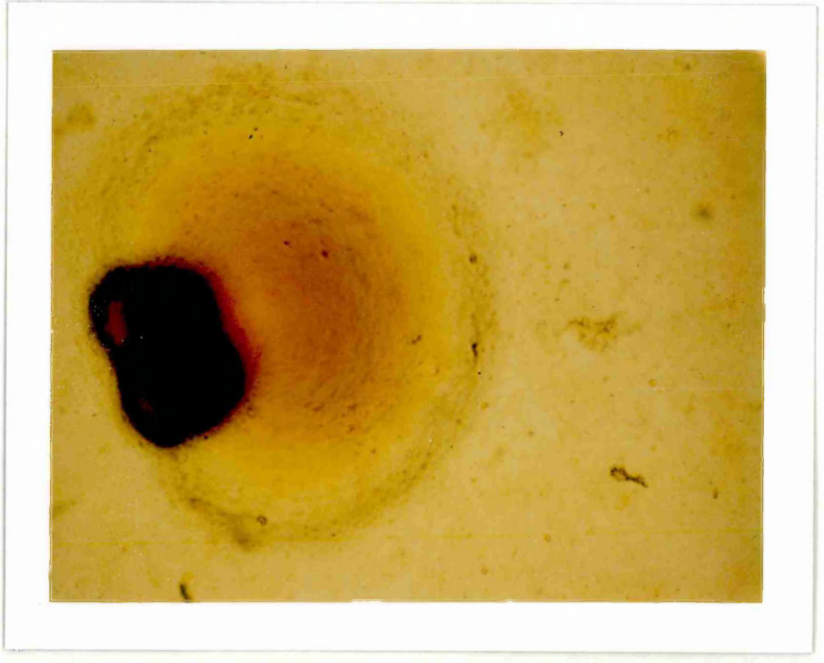


Figure 7

Culture of lysozyme/EDTA prepared spheroplasts of E.coli NCTC 10418. Medium 0.2% agar plate of Victoria Medium F. Magnification x 100. Reverting L-form colony showing area of normal vegetative growth at edge of L-form colony.

proof that these are colonies of spheroplasts?

Figure 8

Culture of lysozyme/EDTA prepared spheroplasts of E.coli NCTC 10418.

Medium 1.0% agar plate of Victoria Medium F. Magnification x 100

Reverted vegetative colonies seen in the depths of the medium.

CONCLUSIONS

1. A medium containing 0.2% agar was suitable as a culture medium for L-form colonies.

2. A medium containing 1% agar was suitable as a culture medium for vegetative colonies.

Table 1

Optimal agar concentration for the growth and reversion of lysozyme/EDTA induced L-form colonies of E.coli

Agar Conc.	Medium Consistency	L-form colonies	Vegetative colonies
0.1%	Sloppy	+++++	(+)
0.15%	Set: could not be inverted	+++++	+
0.2%	Set: could be inverted	+++++	+
0.3%	Solid	++	++++
1.0%	Solid	+	+++++

Key: +++++ = Growth confluent or semi-confluent
Colony forming units (cfu)/ml. uncountable

++++ = 1000 - 3000 cfu/ml.

+++ = 500 - 1000 cfu/ml.

++ = 100 - 500 cfu/ml.

+ = 10 - 100 cfu/ml.

(+) = less than 10 cfu/ml.

Table 2

Optimal agar-gelatin concentration for the growth and reversion of lysozyme/ EDTA induced L-form colonies of E.coli

Agar Conc. with 5% gelatin	Medium Consistency	L-form colonies	Vegetative colonies
0.15%	Set: could be inverted	++++	++
0.2%	Set: could be inverted	++++	++

Key: ++++ = 1000 - 3000 colony forming units (cfu)ml.

++ = 100 - 500 cfu/ml.

Chapter 6

Culture in Liquid Medium

(1) EFFECT OF AN AGAR-FREE MEDIUM ON THE GROWTH OF LYSOZYME/EDTA INDUCED SPHEROPLASTS OF E. COLI.

INTRODUCTION

In theory an agar-free medium should provide ideal conditions for the reversion inhibition of wall defect. Growth of wall-defective microbial variants is permitted in low agar concentrations whereas reversion to the walled state is encouraged as the agar concentration of the medium is increased.

OBJECTIVE

The object was to assess the capacity of lysozyme/EDTA induced *E. coli* spheroplasts to grow in agar-free medium.

MATERIALS & METHODS

The method chosen was a colony count in pour plates inoculated from agar-free medium seeded with a spheroplast suspension.

Materials

Test suspension. A spheroplast suspension of *E. coli* NCTC 10418 was prepared by lysozyme/EDTA method.

Culture Medium. The culture medium was Victoria Medium F (Brogan, 1976) used as an agar-free medium (Victoria Medium F broth) and also as pour plates.

Pour plates contained 0.2% agar and were prepared to a final volume of 10 ml.

Culture Conditions

Incubation was at 37°C for 24 hours in an atmosphere of 5% carbon dioxide in air.

Microscopy

Cultures were examined by plate microscopy with a stereo microscope (Elvar) using a final magnification of x 25.

Diluent for Preparation of Spheroplast Dilutions

TRIS buffered 20% sucrose pH 8.0 (Appendix I).

METHOD

Preparation of Dilutions

i) Preparation of Working Dilution. 0.1 ml of the spheroplast suspension was diluted to a final volume of 10 ml with TRIS buffered sucrose. This gave a final dilution of 1/100.

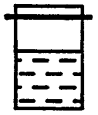
ii) Preparation of Positive Growth Control Dilutions. These were the working dilution used neat and diluted 1/10 and 1/100 with TRIS buffered sucrose. This gave final dilutions of 1/100, 1/1000 and 1/10,000. (Figure 9).

Positive Growth Controls

The positive growth controls were needed to estimate the approximate number of colony forming units/ml (cfu./ml) seeded to agar-free Victoria Medium F (Victoria Medium F broth) as the test culture.

EFFECT of a NUTRITIONALLY COMPLEX (COMPLETE) LIQUID MEDIUM

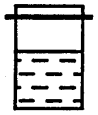
Key: CM = nutritionally complex (complete) medium



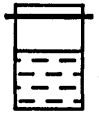
Spheroplast suspension diluted 1/100 in TRIS sucrose

TEST CULTURES

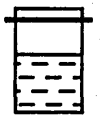
Spheroplast culture in CM broth



1 ml of 1/100 spheroplast suspension in 9 ml CM broth incubated 24 hours then diluted

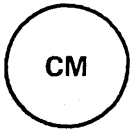


1/10



1/100

B: Pour plate cultures inoculated from neat, 1/10 and 1/100 CM broth culture



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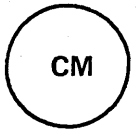

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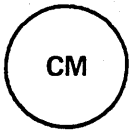


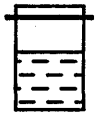
1/1,000,000

Minimum final dilution of spheroplast suspension in pour plates

POSITIVE CONTROLS

Spheroplast suspension in TRIS sucrose

1 ml of 1/100 spheroplast suspension neat and diluted 1/10 and 1/100



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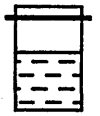

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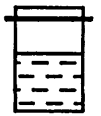

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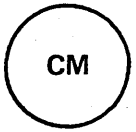
B: Pour plate cultures inoculated from 1/100, 1/1000 and 1/10,000 spheroplast suspension



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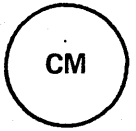

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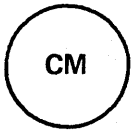


1/100,000

Opposite

Figure 9. Diagram. Effect of a nutritionally complex (complete) liquid medium on the recovery of lysozyme/EDTA induced spheroplasts of E.coli NCTC 10418. Layout of experimental procedure.

Without prior incubation, 1 ml of each growth control dilution was immediately inoculated into a pour plate of Victoria Medium F, to a final volume of 10 ml. This gave final dilutions of 1/1000, 1/10,000 and 1/100,000 (Figure 9).

The plates were incubated, examined by plate microscopy and colony counts performed.

Test Cultures

One ml of working dilution (the spheroplast suspension diluted 1/100 in TRIS buffered sucrose) was inoculated into 9 ml of agar-free Victoria Medium F, giving a final dilution of 1/1000 of the spheroplast suspension. The medium was incubated for 24 hours and then dilutions of the culture were prepared for inoculation into pour plates of Victoria Medium F. The pour plate inocula were 1 ml neat culture, 1 ml culture diluted 1/10 with TRIS buffered sucrose, and 1 ml culture diluted 1/100 with TRIS buffered sucrose.

The minimum final dilutions of the spheroplast suspension after broth passage and inoculation to the pour plates were 1/10,000, 1/100,000 and 1/1,000,000 (Figure 9).

RESULTS

The results are shown in Table 3.

The test cultures made after passage through the agar-free medium were sterile. The growth control plates showed that the neat spheroplast suspension was viable, and contained about 300,000 - 600,000 cfu/ml. The plate inoculated with the lowest growth control dilution was sterile. This was tentatively attributed to carry-over of a possible inhibitor whose effect was sufficiently weakened to permit

Table 3

Effect of passage through yeast extract supplemented agar-free medium (Victoria Medium F broth) on survival of lysozyme/EDT induced E.coli spheroplasts. Colony forming units (cfu) in pour plates of Victoria Medium F yielded after passage of test culture through Victoria Medium F broth compared with cfu yielded in pour plates of Victoria Medium F by positive growth control treated without passage through Victoria Medium F broth.

Minimal final dilution/ml. of spheroplast suspension after culture in Victoria Medium F broth followed by inoculation to Victoria Medium F pour plates as test culture	Cfu yielded in Victoria Medium F pour plate culture by spheroplast suspension after passage through Victoria Medium F broth	Dilution of spheroplast suspension inoculated to Victoria Medium F pour plates without prior culture as positive growth control	Cfu yielded in Victoria Medium F pour plate culture by sphero suspension treated with passage through Victori Medium F broth
1/1000	***	1/1000	Sterile
1/10,000	Sterile	1/10,000	69
1/100,000	Sterile	1/100,000	3
1/1,000,000	Sterile	1/1,000,000	***

Key: *** = not tested

growth at the two higher dilutions. This perhaps explained the growth failure observed in agar-free medium and could be ascribed to inadequate separation of the cell deposit from lysozyme/EDTA at the final step of preparation. Such a difficulty had not previously been encountered, and any excess of lysozyme/EDTA in the suspension presumably occurred by chance. The alternative possibility was that lysozyme/EDTA induced *E. coli* spheroplasts were unable to grow in agar-free medium.

CONCLUSIONS

Growth failure of the lysozyme/EDTA induced *E. coli* spheroplast suspension in agar-free Victoria Medium F was due to either the presence of an inhibitor or to an intrinsic inability to grow in agar-free medium.

(2) EFFECT OF A YEAST EXTRACT-FREE, AGAR-FREE MEDIUM ON THE RECOVERY OF LYSOZYME/EDTA INDUCED SPHEROPLASTS OF E.COLI.

INTRODUCTION

The possibility that lysozyme/EDTA induced spheroplasts might be unable to grow in agar-free medium was reminiscent of a similar difficulty which may be encountered in physically stressed bacteria, and which may be overcome by the use of a minimal medium for subculture (Pierson, Gomez & Martin, 1978). It was thought that this principle could be applied to spheroplast culture by the use of a nutritionally simplified subculture medium for recovery from Victoria Medium F broth.

The alternative was that growth failure in Victoria Medium F broth was due to chance carry-over of an inhibitor used in the preparation of the spheroplast suspension.

OBJECTIVE

The object was to determine whether spheroplast recovery from agar-free medium was feasible by nutritional modification of the subculture medium, or whether the difficulty of recovery from agar-free medium was merely due to the carry-over of an inhibitor from the final stage of preparation.

MATERIALS & METHODS

Materials

Culture Media. Victoria Medium F was used as an agar-free medium (Victoria Medium F broth) and as pour plates. This was 'nutritionally complete' medium (CM). The nutritionally modified medium (MM) was yeast extract-free Victoria Medium F, prepared as pour

plates. All pour plates contained 0.2% agar and were prepared to a final volume of 10ml.

The rest of the materials used were as described in Chapter 6(1).

METHOD (Figure 10)

Preparation of Dilutions

Preparation of the working dilution and positive growth control dilutions was carried out as described in Chapter 6(1).

Positive Growth Control Cultures

There were two positive growth control cultures (Figure 10).

(1) Control CM. One ml of each positive growth control dilution inoculated to CM (Victoria Medium F) pour plates to give final dilutions of 1/1000, 1/10,000 and 1/100,000.

(2) Control MM. One ml of each positive growth control dilution was inoculated to MM (yeast extract-free Victoria Medium F) pour plates to give final dilutions of 1/1000, 1/10,000 and 1/100,000.

Test Cultures

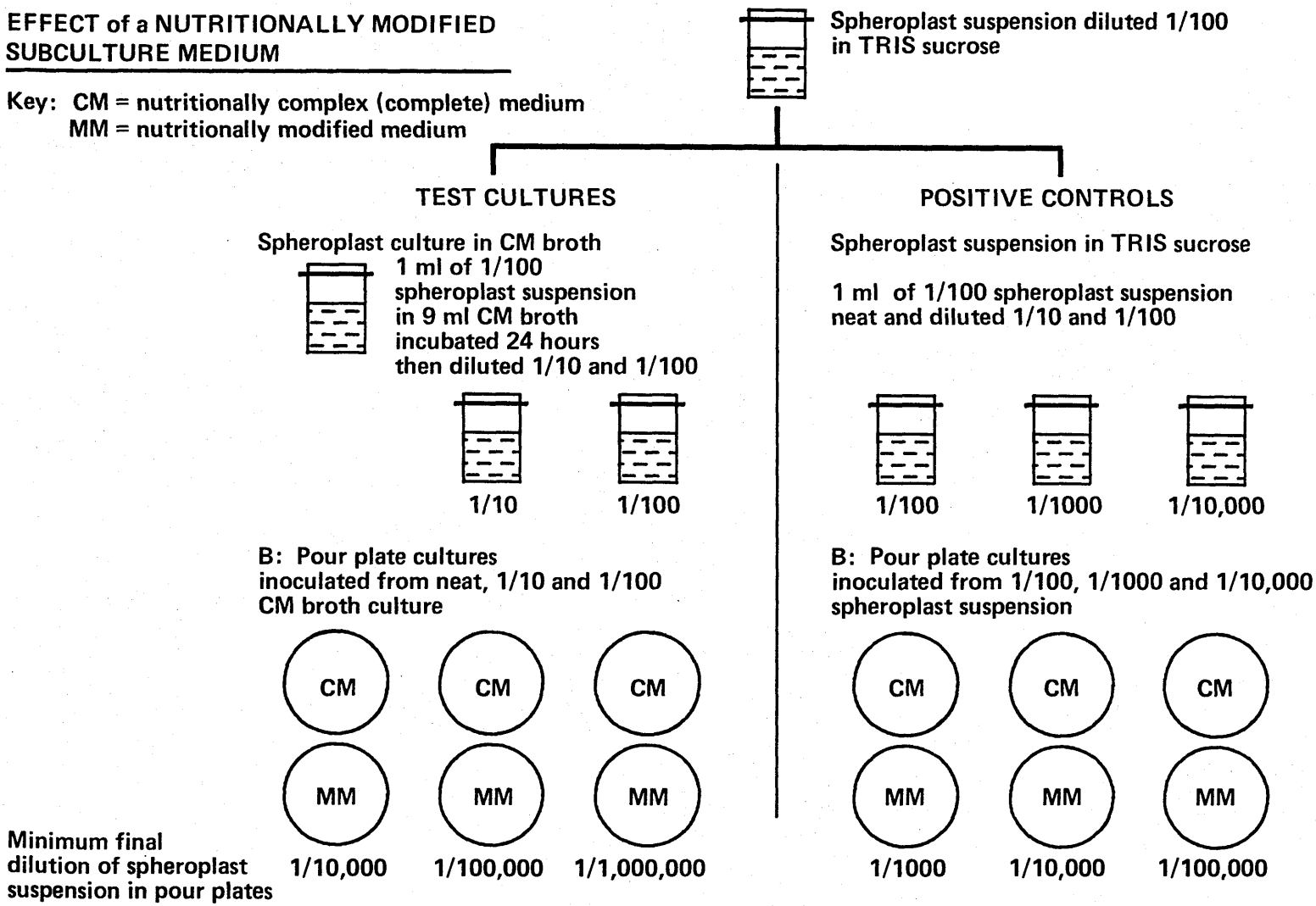
One ml of working dilution was inoculated to 9 ml of CM broth. The broth was incubated, and then dilutions were prepared for inoculation into CM and MM pour plates. The pour plate inocula were 1ml neat CM broth culture, 1 ml CM broth culture diluted 1/10 with TRIS buffered sucrose and 1 ml of CM broth culture diluted 1/100 with TRIS buffered sucrose.

These inocula gave minimum final dilutions of 1/10,000, 1/100,000 and 1/1,000,000 of the spheroplast suspension in the pour plates (Figure 10).

The plates were incubated and then examined by plate microscopy and colony counts performed.

EFFECT of a NUTRITIONALLY MODIFIED SUBCULTURE MEDIUM

Key: CM = nutritionally complex (complete) medium
MM = nutritionally modified medium



Opposite

Figure 10. Diagram. Effect of a nutritionally modified subculture medium on the recovery of lysozyme/EDTA induced spheroplasts of E.coli NCTC 10418 after passage through nutritionally complex (complete) liquid medium. Layout of experimental procedure.

RESULTS

The results are shown in Table 4.

Test Cultures

After passage through CM broth culture the organisms were recovered by subculture to pour plates of nutritionally modified medium (MM). Subcultures made to pour plates of CM were sterile.

Positive Growth Control Cultures

The positive growth control cultures yielded growth in all dilutions tested, including the 1/10 dilution, in both CM and MM pour plates.

CONCLUSIONS

The inability of lysozyme/EDTA induced spheroplasts to grow in agar-free medium was not entirely due to carry-over of an inhibitor used in the preparation of the spheroplast suspension. Growth failure seemed to be more attributable to the nutritional composition of the subculture pour plate medium used after passage through CM broth culture.

TABLE 4

Survival of lysozyme/EDTA induced E. coli spheroplasts. Effect of passage through Victoria Medium F broth followed by subc to pour plates of yeast extract supplemented medium (Victoria Medium F) and to pour plates of yeast extract-free Victoria Medium F. Colony forming units (cfu.) and colony type yielded compared with cfu. and colony type yielded from positive growth control treated without passage through Victoria Medium F broth.

Minimum final dilution/ml. of spheroplast suspension after culture in Victoria Medium F broth followed by inoculation to Victoria Medium F pour plates as test cultures	Cfu. yielded in Victoria Medium F pour plate culture by spheroplast suspension after passage through Victoria Medium F. broth		Dilution of spheroplast suspension inoculated to Victoria Medium F pour plates without prior culture as positive growth control	Cfu. yielded in Victoria Medium F pou plate culture by sphe suspension treated wi passage through Victo Medium F broth	
	Subculture to Victoria Medium F pour plate	Subculture to yeast extract-free Victoria Medium F pour plate		Subculture on Victoria Medium F pour plate	Subculture on yeast extract free Victoria Medium F pour plate
1/10,000	Sterile	Confluent; uncountable Swarming colony type	1/1000	89 L-form colony type	146 L-form colony typ
1/100,000	Sterile	Confluent; uncountable Swarming colony type	1/10,000	2 L-form colony type	10 L-form colony typ
1/1,000,000	Sterile	Confluent; uncountable Swarming colony type	1/100,000	4 L-form colony type	3 L-form colony typ

COMMENT

It is difficult to grow wall-defective microbial variants in liquid medium (Dienes, 1949a; 1953b; Gooder, 1968). The results of the experiments described indicate that it may be possible to recover lysozyme/EDTA induced E.coli of the spheroplast type from liquid medium by nutritional modification of the subculture medium effected by the omission of yeast extract from the medium. This is analogous to the 'minimal medium recovery'phenomenon which occurs in bacteria physically stressed by heat or ultraviolet irradiation. Mild stress causes DNA strand breaks (Sedgwick & Bridges, 1972), which can be repaired in suitable cultural conditions. Nutritionally complex media induce irreversible DNA strand breaks whereas a minimally nutrient medium protects against DNA strand breaks (Pierson, Gomez & Martin, 1978 ; Mossel & Van Netten, 1984). Inhibition by nutritionally complex media is more pronounced in liquid media than on agar media (Gomez & Sinskey, 1973; Gomez et al., 1973). The reason for the effect is not clear, but the similarities between the results obtained with lysozyme/EDTA induced spheroplasts and stressed bacteria suggest that perhaps similar processes may permit recovery from agar-free culture media in both groups of bacteria.

The possibility that yeast extract was in some way independently toxic to lysozyme/EDTA induced spheroplasts did not seem attractive, because yeast extract containing agar medium supported their growth satisfactorily.

Inhibition appeared to occur only in liquid medium, and seemed to be neutralised by the presence of agar.

Although the number of tests performed was not large, and although the number of colonies in the control cultures were not linear, the results may be interpreted as indicating that enriched culture in 'nutritionally complex' agar-free medium resulted in growth failure of wall-defective E.coli variants of the spheroplast type. This growth pattern seemed to be somewhat similar to that found in the 'minimal medium recovery' phenomenon.

SECTION IV

STUDIES WITH CLINICAL MATERIAL

Chapter 7

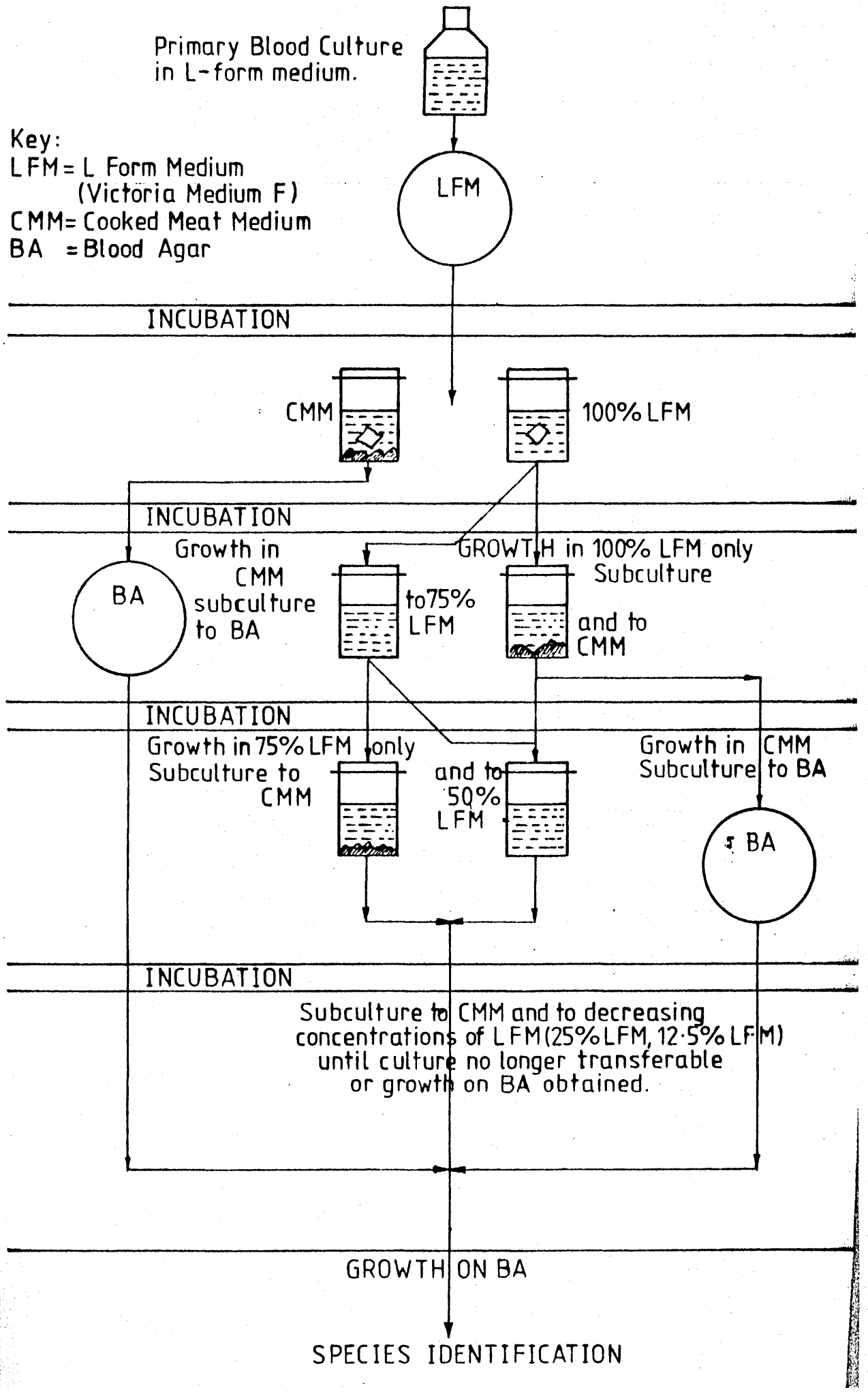
Procedures for the isolation of wall-defective microbial variants from blood cultures and for their reversion to the vegetative bacterial state.

PROCEDURES FOR THE ISOLATION OF WALL-DEFECTIVE MICROBIAL VARIANTS FROM BLOOD CULTURES AND FOR THEIR REVERSION TO THE VEGETATIVE STATE.

INTRODUCTION

In preliminary studies for this thesis (Brogan, 1976), it was sometimes difficult to distinguish true wall-defective microbial variants from mycoplasmas or artefacts in the culture medium. The problem concerned the reversion procedure, since the reversion of such variants to the vegetative state is considered an essential diagnostic feature according to the criteria of McGee et. al. (1971). Originally I used an agar block enrichment method to obtain reversion (Brogan, 1976). This was adapted from a method described by Dienes (Dienes, 1941). The method was termed a reversion series. It is illustrated in Figure 11. The reversion series consisted of five media. The 100% reversion medium was of the same composition as the primary blood culture medium. The other reversion media were media of the same base composition as the primary blood culture medium diluted with Brain Heart Infusion Broth (Oxoid) to give concentrations of 75%, 50%, 25% and 12.5% of the 100% reversion medium. Agar blocks were cut from pour plate subcultures and one inoculated to 100% reversion medium, another to Oxoid Cooked Meat Medium (CMM). The subcultures were then incubated at 37°C in an atmosphere of 5% carbon dioxide in air for a maximum of 3 days. The subcultures were inspected daily for evidence of growth. If growth occurred in CMM, subculture was made to a surface inoculated blood agar plate incubated in 5% carbon dioxide in air. If growth occurred only in reversion series medium without growth in CMM, subculture was made to 75% reversion medium. If necessary, the process was repeated until a final subculture to 12.5% reversion medium had been attempted.

REVERSION SERIES METHOD (Brogan 1976)



Opposite

Fig. 11. Diagram. Reversion procedure by reversion series method (Brogan, 1976).

The method was not satisfactory. It was theoretically prone to contamination, and did not always achieve reversion. When the method failed, the agar block subculture in 100% reversion remained sterile, or there was continued growth on the agar block, without growth in the reversion series medium. Sometimes there was granular, floccular, or even diffuse growth in the reversion series medium but this could not be subcultured to CMM or to a blood agar plate. It was usually possible to subculture such growth to 75% or 50% reversion medium but no further. Dienes also encountered this type of problem when he used his agar block subculture method (Dienes, 1941; 1949(a); 1953(b)).

I now proposed to compare a modification of the reversion series method with two other reversion methods. The modified reversion series method employed agar block subcultures to 100% reversion medium only, without subsequent use of subculture to 50%, 25% or 12.5% reversion medium. This modified reversion method was termed the agar block enrichment method. The other reversion methods compared with the agar block enrichment method were the use of a 'stiff' agar subculture medium to encourage autolysin inhibition (Chapter 5) and the use of nutritionally enriched media for the reversion of wall-defective streptococcal variants (Bouvet & Acar, 1977).

OBJECTIVE

The objective was to find a more stringent method of identifying wall-defective microbial variants and to find a better method for their reversion to the vegetative state.

MATERIALS & METHODS

Collection of Specimens and Primary Culture (Figure 12).

Clinical specimens. Blood cultures.

Primary Culture. Primary culture media were inoculated at the bedside. The blood culture set consisted of three bottles, two for routine culture and one for the culture of wall-defective microbial variants. The routine primary culture media were Oxoid Brain Heart Infusion Broth (BHI) and a cooked meat medium (CMM) consisting of Difco tryptic soy broth with Difco cooked meat (Appendix I). The media used for the culture of wall-defective microbial variants will be described in subsequent chapters. Their composition is shown in Appendix I. For the sake of brevity and simplicity they are referred to as 'L-form' media.

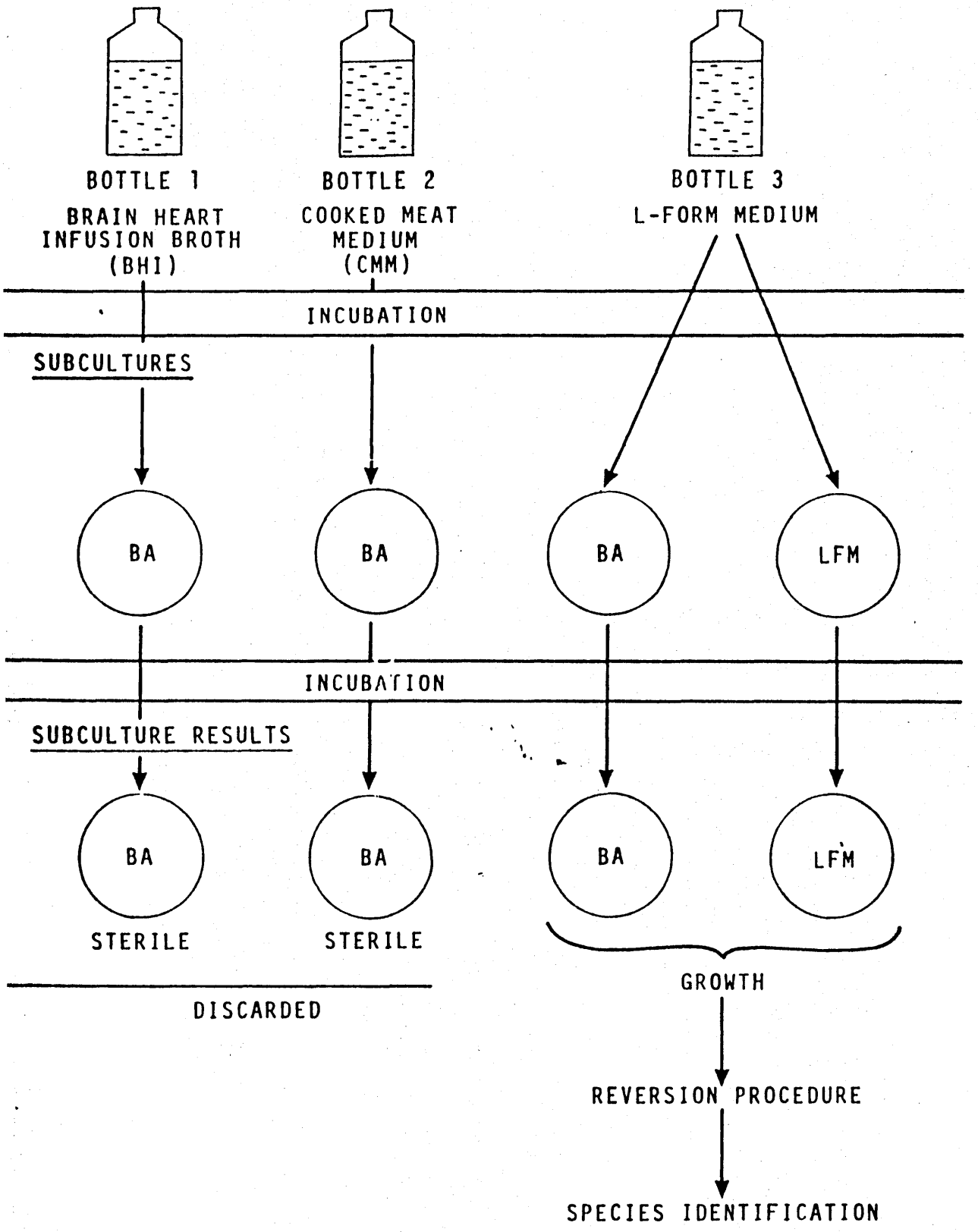
The inoculum requested was 5 ml of blood to each bottle. Incubation was aerobic in an atmosphere of 5% carbon dioxide in air at a temperature of 37°C.

Subculture

All three bottles were routinely subcultured to 5% horse blood agar plates. Two blood agar plates were subcultured from each bottle.

COLLECTION OF SPECIMENS & PRIMARY CULTURE

THREE BOTTLE BLOOD CULTURE SET INOCULATED AT THE BEDSIDE.



Key:
BA = BLOOD AGAR PLATE
LFM = L-FORM MEDIUM POUR PLATE.

Opposite

Fig. 12. Diagram. Procedure for the isolation of wall-defective microbial variants from blood cultures.

One was incubated aerobically in an atmosphere of 5% carbon dioxide in air, the other anaerobically in an atmosphere of 65% hydrogen with 5% carbon dioxide, achieved with the Baltimore Biological Laboratories (BBL) 'Gaspak' system. In addition, a pour plate of L-form medium was subcultured from the L-form primary culture medium. This was inoculated at 37°C aerobically in an atmosphere of 5% carbon dioxide in air.

The inoculum used for the blood agar plates was 10 µl from a standard loop. An inoculum of 1 ml was used for the pour plate. The final volume of the pour plate was 10 ml, i.e. the inoculum was one hundred times greater than on the blood agar plate.

Subculture Times: Subcultures were made from the primary culture bottles after 24 hours, 48 hours and 7 days incubation.

Examination of the Pour Plates. Pour plates were examined by plate microscopy with a stereo microscope (Elvar) using a final magnification of x 25.

If there was growth, the colonial morphology was recorded as vegetative, L-form, or as mixed vegetative and L-form colonies if both types were present. The colonies were counted. If more than one type of colony was present, the number of colonies of each type observed were recorded.

IDENTIFICATION OF WALL-DEFECTIVE MICROBIAL VARIANTS

The criteria used were:-

i) Isolation from L-form medium only

A culture was accepted as a possible wall-defective variant only if there was no growth from the two conventional primary blood culture media after 7 days incubation.

REVERSION PROCEDURE

1. PRIMARY BLOOD CULTURE GROWTH IN L-FORM MEDIUM ONLY SUBCULTURES FROM PRIMARY L-FORM MEDIUM YIELD GROWTH ON BLOOD AGAR (BA) PLATES & GIVE MACROSCOPICALLY VISIBLE SURFACE GROWTH ON THE L-FORM MEDIUM POUR PLATE (LFM).

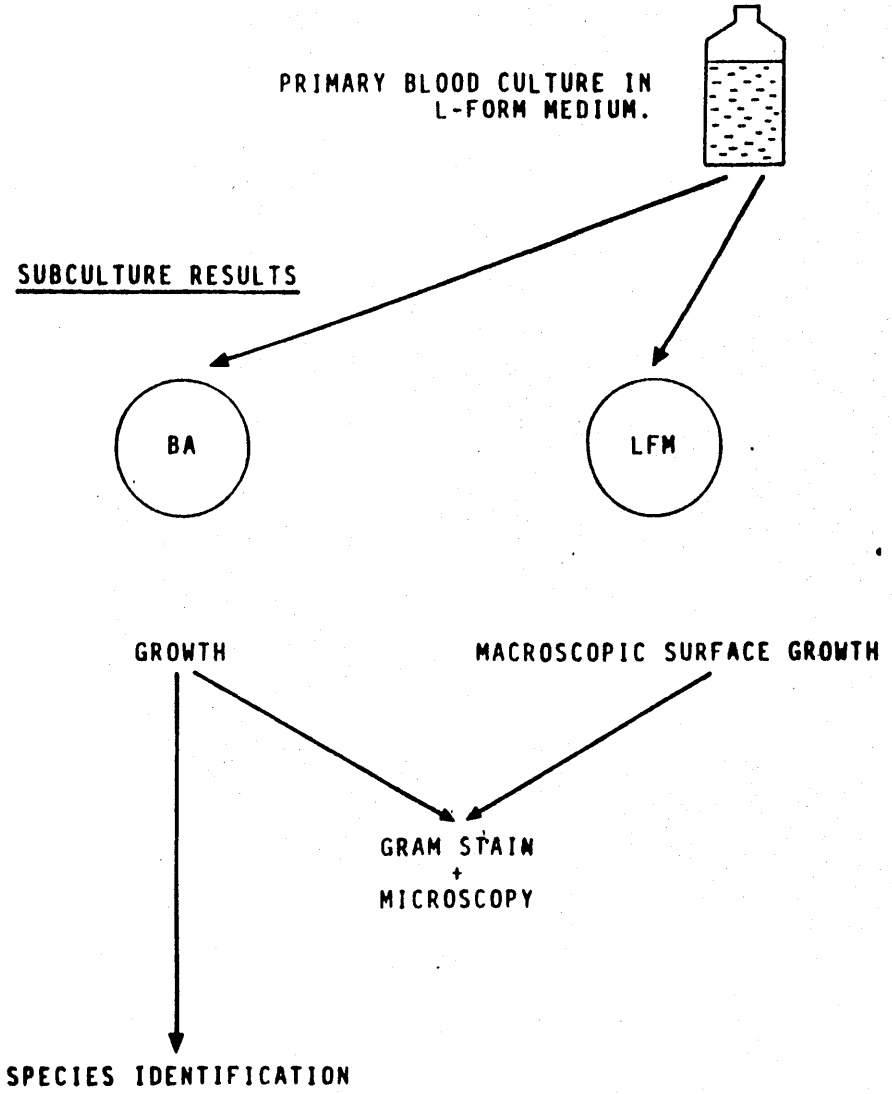


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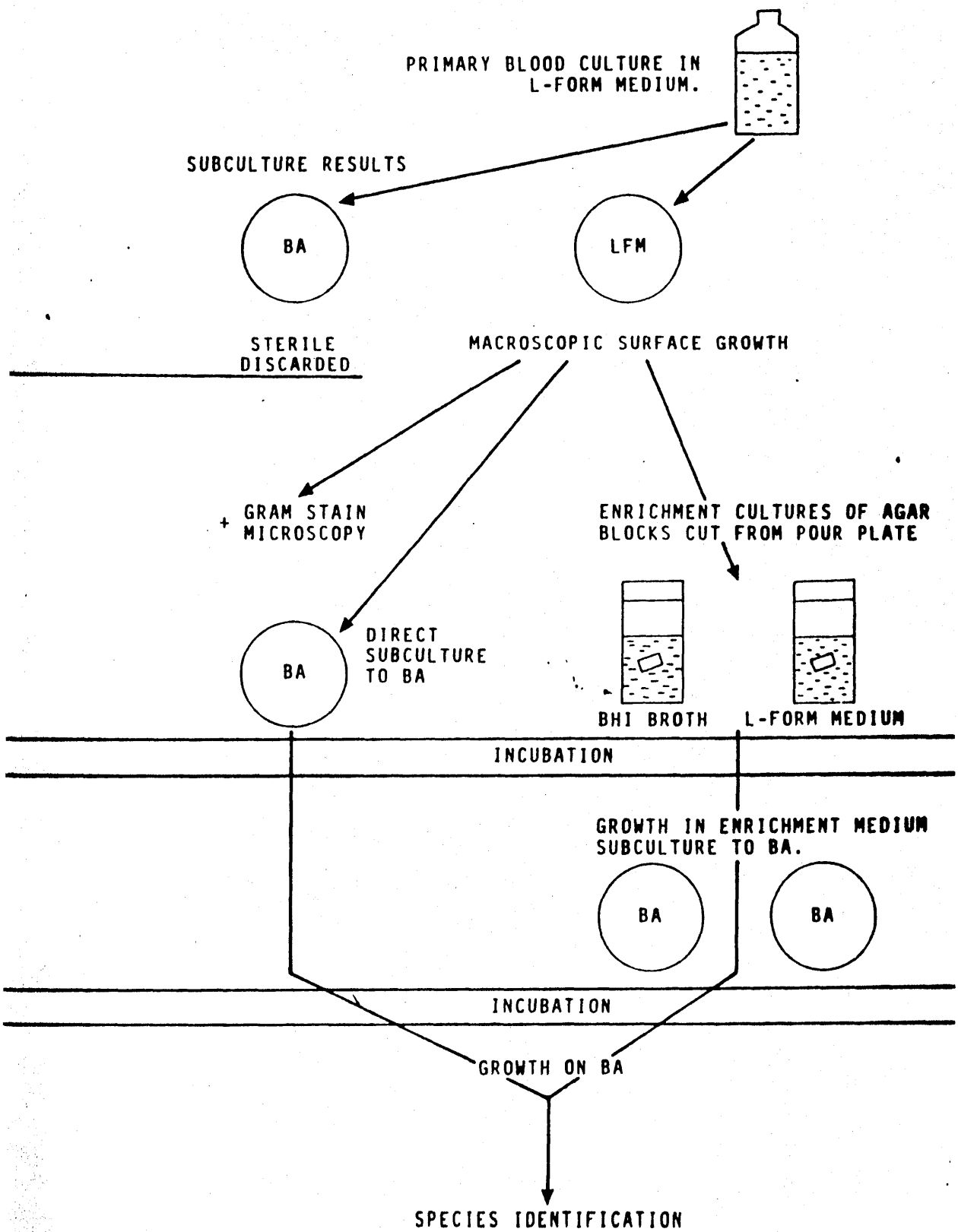
Fig. 13. Diagram. Reversion Procedure I. Primary blood culture growth in L-form medium only. Subcultures from primary L-form medium yield growth on blood agar (BA) plates and give macroscopically visible surface growth on the L-form medium pour plate (LFM).

REVERSION PROCEDURE

II. PRIMARY BLOOD CULTURE GROWTH IN L-FORM MEDIUM ONLY. SUBCULTURES FROM PRIMARY L-FORM MEDIUM STERILE ON BLOOD AGAR (BA) PLATES & GIVE MACROSCOPICALLY VISIBLE SURFACE GROWTH ON THE L-FORM MEDIUM POUR PLATE (LFM).

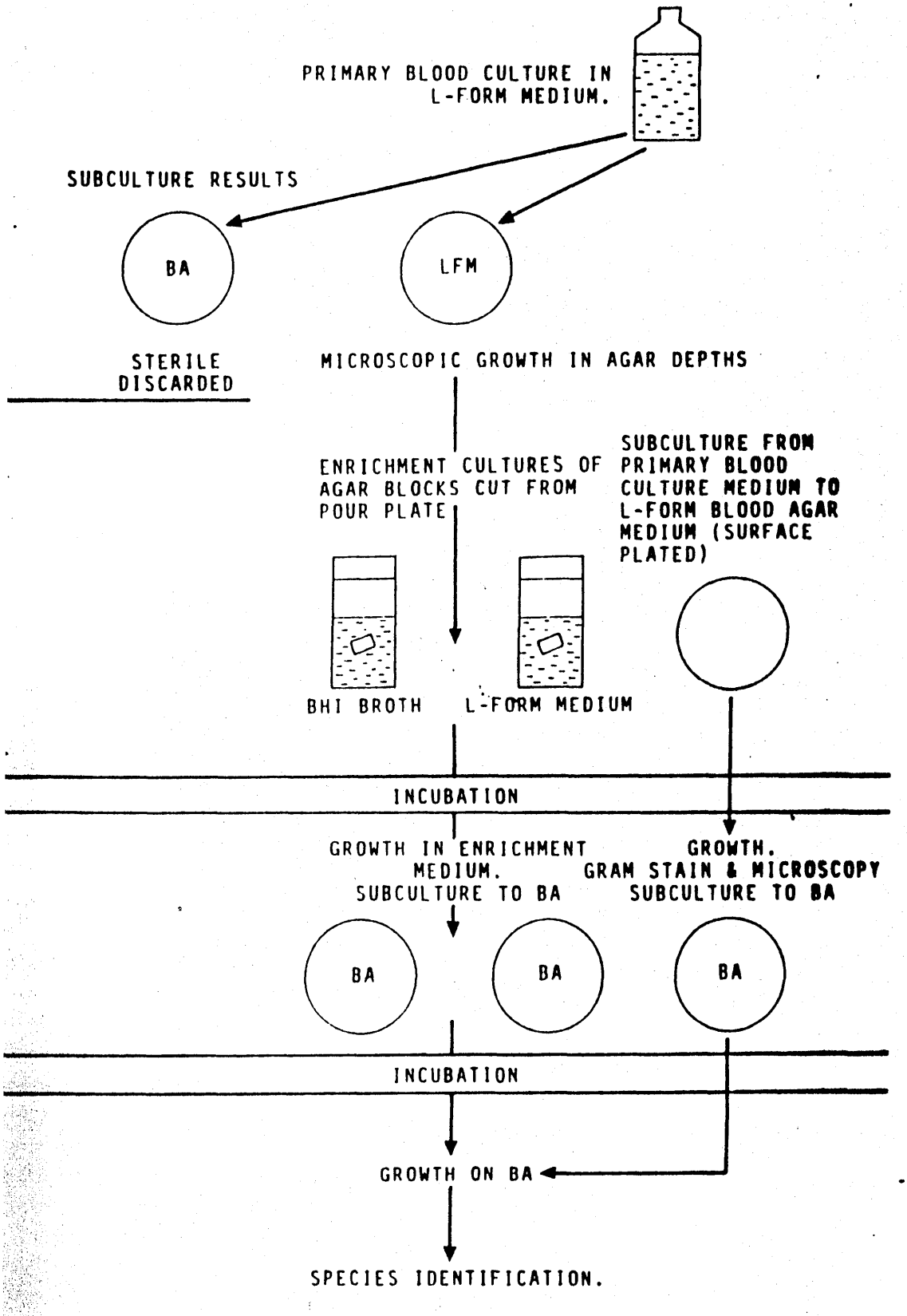


Opposite

Fig. 14. Diagram. Reversion Procedure II. Primary blood culture growth in L-form medium only. Subcultures from Primary L-form medium sterile on blood agar (BA) plates and give macroscopically visible surface growth on the L-form medium pour plate (LFM).

REVERSION PROCEDURE

III. PRIMARY BLOOD CULTURE IN L-FORM GROWTH MEDIUM ONLY SUBCULTURES FROM PRIMARY L-FORM MEDIUM STERILE ON BLOOD AGAR (BA) PLATES. MICROSCOPICALLY VISIBLE GROWTH IN DEPTHS OF L-FORM MEDIUM POUR PLATE (LFM).



Opposite

Fig. 15. Diagram. Reversion Procedure III. Primary blood culture growth in reversion medium only. Subcultures from primary L-form medium sterile on blood agar (BA) plates. Microscopically visible growth in depths of L-form medium pour plate (LFM).

ii) Proof of Microbial Nature

Proof of microbial nature was given by reversion to the vegetative stage. Reversion was deemed satisfactory if normal vegetative growth was obtained on surface plated 5% horse blood agar plates.

Reversion Methods

The reversion methods used were dependent on the subculture media on which growth was obtained from the primary L-form culture, and on the type of colonial growth, vegetative or L-form.

There were three main possibilities.

i) Growth on blood agar subcultures, with macroscopically visible vegetative surface growth on the L-form medium pour plate.

(Figure 13)

Identification of the bacterial species proceeded forthwith, using the culture grown on blood agar.

ii) No growth on blood agar subcultures, but macroscopically visible vegetative surface growth on the L-form medium pour plate.

(Figure 14)

In this situation, the vegetative surface growth was examined by Gram stain and microscopy. A subculture to a blood agar plate was also made.

In addition, agar blocks were cut from the pour plate and subcultured to both BHI broth and to L-form blood culture medium. This procedure was adapted from a method originally devised by Dienes (1941). The cultures were incubated at 37°C in an atmosphere of 5% carbon dioxide, for a maximum of 48 hours. Any growth on the blood agar plate was identified to species level. If growth in the enrichment media occurred, subcultures to blood agar were made, incubated as before, and any subsequent growth on the blood agar plates identified to species level.

iii) No growth on blood agar subcultures but microscopically visible growth in the depths of the L-form medium pour plate. (Figure 15)

In this situation, agar blocks were cut from the pour plate and subcultured to BHI broth and to L-form blood culture medium enrichment cultures as before.

In addition, the primary L-form blood culture medium was subcultured directly to L-form Blood Agar Medium (LFBA) by surface plating, and incubated at 37°C in 5% carbon dioxide in air for a maximum of 48 hours.

L-form Blood Agar Medium was L-form pour plate medium supplemented with 5% horse blood. The L-form medium was of the same base composition as the L-form medium used for primary blood culture.

Growth in the agar block enrichment cultures was subcultured to blood agar, and any growth on blood agar identified to species level.

Growth on LFBA was examined by Gram stain and microscopy, and subcultured to blood agar, and to a fresh LFBA. The procedure was repeated until growth was obtained on blood agar.

APPLICATION OF THE METHODS DESCRIBED

The methods for the recognition and reversion of wall-defective microbial variants described above were applied to the cultures grown from the L-form media described in the Chapters which follow (Chapter 8: Chapter 9).

Chapter 8

Effect of a nutritionally modified (Yeast Extract-Free) medium on the isolation of the wall-defective variants of Enterobacteriaceae from blood cultures.

EFFECT OF A NUTRITIONALLY MODIFIED (YEAST EXTRACT-FREE) MEDIUM ON THE ISOLATION OF THE WALL-DEFECTIVE VARIANTS OF ENTEROBACTERIACEAE FROM BLOOD CULTURES

INTRODUCTION

A nutritionally modified (yeast extract-free) medium (MM) permitted the growth of lysozyme/EDTA induced L-forms of E.coli by primary enrichment culture in MM broth with subculture to MM agar plates.

OBJECTIVES

The object was to use the nutritionally modified culture medium for blood culture specimens to assess its practical value in clinical work.

MATERIALS & METHODS

The materials and methods used were as described in Chapter 7. The L-form medium used for primary blood culture was Victoria Medium F without yeast extract. This was also used as the pour plate subculture medium, solidified with 0.2% agar.

RESULTS

739 culture sets were examined during a period of six months. No Enterobacteriaceae species were isolated in the L-form culture medium alone.

CONCLUSIONS

The nutritionally modified culture medium did not recover naturally occurring wall-defective Enterobacteriaceae variants from blood culture specimens, though it supported the growth of lysozyme induced

spheroplasts of E.coli. This suggested that naturally occurring wall-defective variants of the Enterobacteriaceae may differ from their lysozyme induced counterparts in their nutritional requirements.

Chapter 9

The Batch Phenomenon

THE BATCH PHENOMENON

INTRODUCTION

The usefulness of Victoria Medium F (Brogan, 1976) was limited by its erratic performance. Variants grown from blood cultures occurred as clusters of isolates during relatively short periods of time, separated by many weeks or even by months. Since the recovery of these variants was associated with particular batches of medium, this observation was termed the 'batch phenomenon'. It may be due to any of several factors such as the presence of an inhibitor (Medill & O'Kane, 1954), variation in nutritional quality, or mis-identification of wall-defective microbial variants as artefacts because of reversion failure.

OBJECTIVE

The object was to find the cause of the batch phenomenon.

MATERIALS & METHODS

The problem was investigated by comparing the isolation rates of wall-defective variants from blood culture in different batches of Victoria Medium F, by employing an improved quality control method, and by neutralising any fatty acid inhibition present in the medium with starch.

L-form Culture Medium

The medium used was based on Victoria Medium F. Its composition was essentially the same as in the original description (Brogan, 1976) but the following changes were made;-

i) Addition of calcium chloride

Oxoid No. 3 agar was no longer available and Oxoid No. 1 agar was substituted. This agar had a lower calcium content, so calcium chloride was added to substitute for the deficiency.

ii) Neutralisation of Fatty Acid Inhibitors

Inhibitory fatty acids may be present in many basic materials and may be removed with charcoal, starch, cholesterol sulphate, anion exchange resins or 10% v/v serum (Meynell & Meynell, 1965). The medium already contained cholesterol (though not cholesterol sulphate) and some serum derived from the blood specimen inoculated to the medium for culture.

As an additional measure, starch was added to the medium. The use of charcoal was considered, but rejected, because the resulting medium was too dark for plate microscopy.

iii) Nutritional Quality

An attempt to improve the nutritional quality of the medium was made by a change in sterilisation procedure. The method was changed from autoclaving at a pressure of 15lbs./sq.inch (121°C) for 20 minutes to autoclaving at a pressure of 10 lbs./sq.inch (115°C) for 20 minutes. The object was to minimise any deterioration in the vitamin content of the medium caused by overheating during the sterilisation process.

The medium was termed 'Modified Victoria Medium F'. Its composition is shown in Appendix I.

Additional Procedure Used to Identify the Cause of the Batch Phenomenon

These procedures entailed improved quality control. The batch number of all inoculated blood culture bottles was noted on receipt so that the number of bottles and isolates in each batch of medium could be determined. As an extra measure, the batch numbers of the ingredients used for the preparation of each batch of L-form medium were recorded. This was done so that any ingredient responsible for batch variation could be identified.

RESULTS

Studies were made of 1863 culture sets.

i) Nutritional Quality

The altered sterilisation procedure did not improve the recovery of wall-defective variants.

ii) Quality Control Procedures

The record of the batch numbers of the ingredients used for the preparation of each batch of L-form medium indicated that there was a possible association between the batch phenomenon and variation in batches of yeast extract and brain heart infusion broth.

iii) Inhibitors

The addition of starch to the medium did not improve the recovery of wall-defective variants.

CONCLUSIONS

1. The batch phenomenon is probably due to variation in the nutritional quality of some batches of yeast extract and brain heart infusion broth.

2. The batch phenomenon does not seem to be due to fatty acid inhibition in the culture medium as judged by the limited criterion of starch absorption.

3. The batch phenomenon does not appear to be due to heat-deterioration of nutrients during sterilisation.

Chapter 10

Identification of the growth factors required by the Wall-defective Variants of Enterobacteriaceae

IDENTIFICATION OF THE GROWTH FACTORS REQUIRED BY THE WALL-DEFECTIVE VARIANTS OF ENTEROBACTERIACEAE

INTRODUCTION

Although yeast extract is known to contain thiamine, pyridoxine, pantothenic acid, folic acid, para-aminobenzoic acid (pABA), biotin, protein and nucleic acids (Martindale, 1958) neither yeast extract nor brain heart infusion broth is nutritionally defined. In order to investigate the growth requirements of wall-defective Enterobacteriaceae variants, a basic medium was therefore supplemented with different B group vitamins and tested for its ability to support the growth of these variants.

OBJECTIVE

The objective was to identify the growth factor required by wall-defective variants of Enterobacteriaceae.

MATERIALS & METHODS

L-form culture medium

The basic medium chosen for these tests was 'Isosensitest' (Oxoid) stabilised with sucrose and magnesium sulphate. It was chosen because it contains no thymidine, folic acid, pABA, riboflavine or cysteine (Oxoid Manual, 1982) and is therefore a semi-defined medium when these Group B vitamins are added in known amounts. The following three media based on 'Isosensitest' were used in succession. Their composition is

shown in full in Appendix I, but may be summarised as follows.

Medium 1. Sucrose/magnesium sulphate stabilised 'Isosensitest' plus thymidine and riboflavin.

Medium 2. Medium 1 plus nicotinamide.

Medium 3. Medium 2 plus folic acid, pABA and cytosine.

The other materials and methods used were as described in Chapter 7 and in Chapter 9.

The three 'Isosensitest' based media were used consecutively, not in parallel. The study was therefore in three stages, a different 'Isosensitest' based medium being used for each stage. The 'Isosensitest' culture medium tested in each stage was inoculated from the same specimen of blood as the two routine blood culture media. No attempt was made to inoculate the three 'Isosensitest' based media with the same specimen of blood so as to give a direct comparison between them.

To ensure that the primary and pour plate subculture media used for each specimen contained the same nutrient supplements, overlap between the three stages of the investigation was not permitted, and inoculation of inappropriate subculture media was avoided.

The improved records described in Chapter 9 enabled identification of the type of nutrient supplement used in the primary culture medium for each inoculated blood culture received. Specimens inoculated into primary culture medium used at an earlier stage of the investigation were discarded without examination. The three stages of the investigation were therefore independent of each other.

RESULTS

Results are shown in Table 5. The strains studied seemed to require folic acid with pABA and cytosine.

CONCLUSION

Although these experiments have not been carried out using rigorously defined or completely synthetic media they do suggest that the variants under study may be auxotrophs which require a folic acid/pABA/cytosine mixture for growth and reversion.

Table 5

Semi-defined media. Effect of vitamin additives used in three separate stages on the recovery of wall defective Enterobacteriaceae variants from blood cultures in osmotically stabilised 'Isosensitest' blood culture medium.

Vitamins added to supplement osmotically stabilised 'Isosensitest' at each separate stage	Number of supplemented blood cultures examined	Number of blood cultures which yielded variants
Stage 1 Thymidine + Riboflavin	418	0
Stage 2 Thymidine + Riboflavin + Nicotinamide	519	0
Stage 3 Thymidine + Riboflavin + Nicotinamide + pABA + Folic Acid + Cytosine	822	6 (0.72%)

Stage 1 Blood cultures between 1st October 1982 - 31st January 1983.
Total: 4 months.

Stage 2 Blood cultures between 1st February 1983 - 31st May 1983.
Total 4 months.

Stage 3 Blood cultures between 1st June 1983 - 31st July 1983)Total
1st October 1983 - 1st December 1983) 6
1st February 1984 - 31st March 1984)months

Chapter 11

Evaluation of the reversion methods used and interpretation of the results of reversion.

EVALUATION OF THE REVERSION METHODS USED AND INTERPRETATION OF THE RESULTS OF REVERSION.

INTRODUCTION

The criteria described for the identification of wall-defective microbial variants in Chapter 7 were isolation from L-form medium only, and proof of microbial nature given by reversion to the vegetative state. The methods used to establish reversion, and the interpretation of the results of reversion procedures were therefore of crucial importance.

OBJECTIVE

The object was to evaluate the results given by the use of the reversion methods described in Chapter 7 and to decide how the results should be interpreted.

MATERIALS & METHODS

Records were available for 50 Enterobacteriaceae strains which grew in the primary L-form blood culture medium, but not in either of the two conventional primary blood culture media. The records were examined in order to establish the frequency with which isolates from the L-form medium grew as vegetative colonies or as L-form colonies on subculture. The interpretation of these results was also considered, in order to decide whether the isolates could properly be termed wall defective microbial variants, and to decide if they were induced or naturally occurring in type.

RESULTS

There were three types of growth. These were designated Vegetative Growth, Intermediate Growth and L-form Growth. The type of growth yielded on culture from each patient is shown in Table 6. Procedures required for the reversion of each strain are shown in Table 7, and the reversion procedures successful if obtaining growth are shown in Table 8.

Type 1 Vegetative Growth

Growth on the blood agar plate as well as in the L-form pour plate was obtained on direct subculture from the primary L-form blood culture medium in 30 blood cultures. All gave good macroscopically visible surface growth on the L-form pour plate and only vegetative colonies were observed by plate microscopy. The vegetative colonies were opaque, smooth and symmetrical (Chapter 4, Figure 8). No reversion procedure was required, and it was possible to proceed to examination by Gram stain and microscopy and species identification forthwith. Microscopy showed that all the strains were Gram negative bacilli.

All the colonies obtained were vegetative, and no L-form colonies were yielded even in the L-form medium. Following the classification of McGee et al., (1971) the members of this group were therefore presumed to be vegetative forms or transitional phase variants which had reverted so rapidly that their class could not be determined.

Type 2 Intermediate type of Growth

In 11 blood cultures there was no growth on the blood agar plate but growth in the L-form pour plate medium as macroscopically visible surface colonies. The colonies were seen to be wholly or predominantly of the vegetative type when examined by plate microscopy.

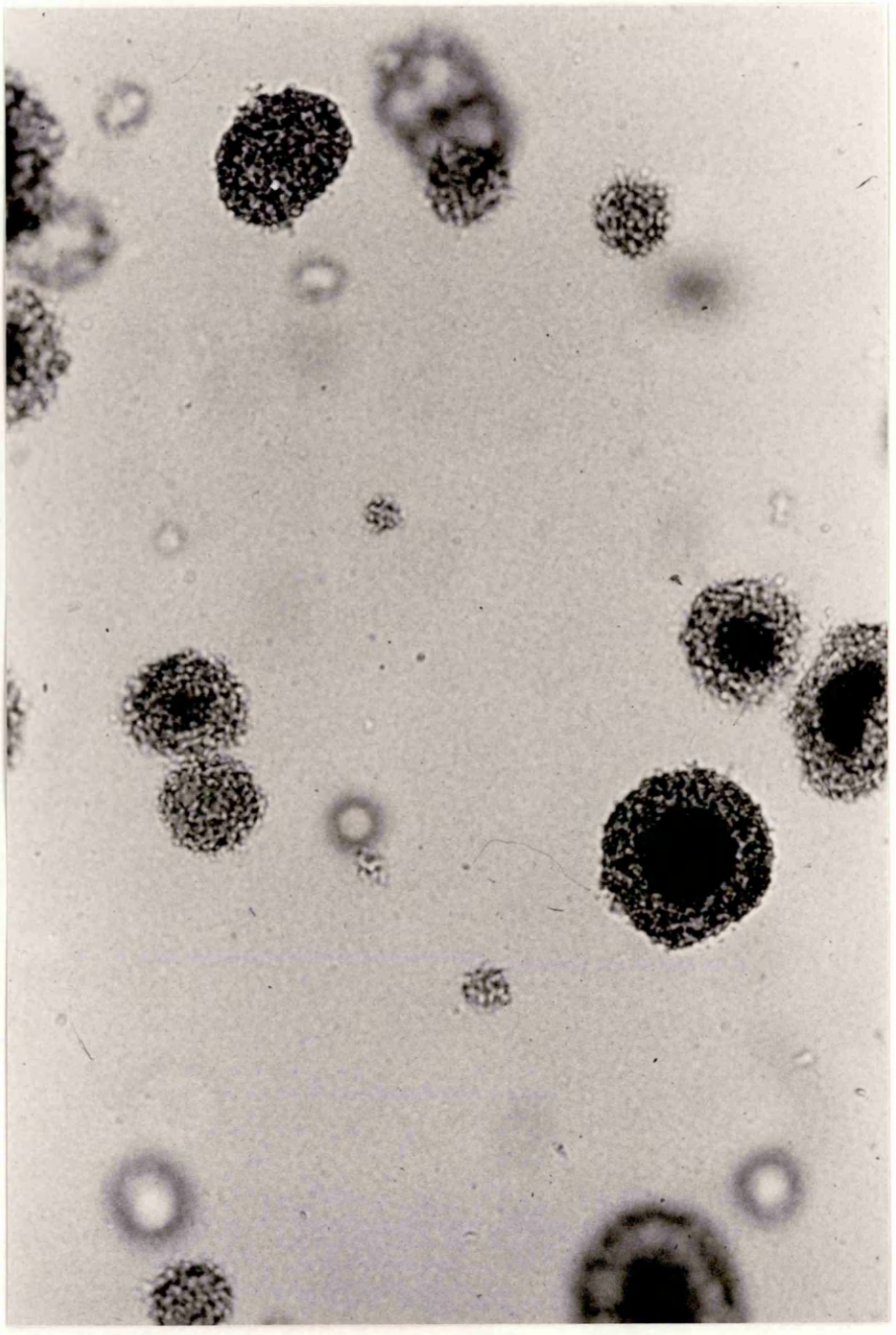


Figure 16

Culture of E.coli L-form colonies derived from blood culture in Victoria Medium F. Medium: 0.2% agar plate of Victoria Medium F. Magnification x 40.

There was heavy growth in the pour plate medium so as to give an uncountable growth of colonies. Though different inocula were used for surface inoculation of blood agar plates and for the inoculation of pour plate media, the growth density in the pour plate medium was such that inoculum differences alone could not account for growth failure on the blood agar plate. Gram stain and microscopy of the pour plate surface growth showed normal vegetative bacilli in every case, and vegetative growth was obtained by direct subculture to blood agar plates and by enrichment culture of agar blocks with subsequent inoculation to blood agar.

As all members of this group produced neither L-form colonies nor vegetative colonies on primary subculture to blood agar, they were presumed to be transitional phase variants according to the classification of McGee et al., (1971).

Type 3 L-form Growth

Blood cultures from 9 patients yielded growth of this type.

There were two groups.

Blood cultures from 7 patients grew colonies of the L-form type only. There was no growth on the blood agar plate, and no growth visible to the naked eye on the surface of the L-form pour plate medium, but growth in the pour plate medium was evident on plate microscopy. The colonies grew profusely in the depths of the pour plate, and were irregular or rhizoid in shape, with a crenated or rhizoid edge. In two instances growth occurred as 'crystal' colonies (Chapter 4, Fig. 6). The appearance of classical fried-egg colonies (Fig. 16) was observed where surface growth occurred, but not, of course, in the depths of the medium.

Reversion was obtained either by the reversion series method on by direct subculture of the primary blood culture to the surface of an LFBA plate with further subculture from the LFBA plate to blood agar to give final proof of reversion.

Blood cultures from a further 2 patients yielded L-forms as described above, except that there was also a scanty growth of vegetative colonies on the pour plate medium. Plate microscopy confirmed that both L-form and vegetative colonies were present. Gram stain and microscopy of the pour plate surface growth showed normal Gram negative bacilli. Despite the fact that there was surface growth, it was impossible to obtain reversion of the vegetative colonies either by subculture to blood agar, or by the agar block enrichment method. However, vegetative growth was obtained by subculture of a loopful of material from the primary blood culture to the surface of an LFBA plate with further subculture from the LFBA to blood agar to give final proof of reversion.

These findings provide good evidence that the 9 isolates were true L-phase variants as defined by McGee et al., (1971). The members of the first group of 7 organisms appeared to be stable whereas the two isolates of the second group were unstable as judged by the presence of vegetative forms in the pour plate medium.

Evaluation of Reversion Methods.

If no growth could be obtained by subculture to blood agar, reversion was attempted either by the reversion series method or by the agar block enrichment method together with subculture to LFBA. The agar block enrichment method failed in 4 of the 46 blood cultures in which it was attempted. LFBA subculture successfully produced reversion in all 4

of these cultures and seemed to be a better method of reversion that the agar block enrichment method.

Interpretation of Reversion Procedure Results

1. Vegetative Growth. The presence of vegetative growth on both blood agar and L-form pour plate subcultures from the primary L-form medium may be explained in the following ways.

i) Nutritionally exacting wall-defective microbial variants were able to grow in the enriched primary blood culture medium because their nutrient growth requirement was supplied. Some members of this population then acquired nutritional independence and went on to overgrow their nutritionally fastidious fellows. In these circumstances, subculture would be expected to yield a pure, or almost pure, growth of the nutritionally independent vegetative growth.

ii) Induced wall-defective microbial variants were able to re-grow wall in the absence of a wall-damaging inducer or in the presence of a low concentration of the inducer. The Enterobacteriaceae variants isolated were not like lysozyme induced L-phase variants so it was possible that they had been induced by beta-lactam antibiotics.

iii) Growth only in the primary L-form medium occurred by chance, the vegetative isolates were ordinary vegetative bacteria from the outset, and no reversion process was involved.

The existence of the batch phenomenon seemed to negate this theory, as the exclusive inoculation of the L-form blood culture medium with bacteria would be expected to occur at random, not in association with particular batches of medium.

Unfortunately, statistical analysis was not feasible, because the anticipated event (the isolation of Enterobacteriaceae from the three primary blood culture media) occurred too infrequently. Accordingly, this possibility could not be absolutely rejected.

iv) Wall-defective microbial variants of the transitional phase variant type were present in the original inoculum of blood. These required osmotic stabilisation, and therefore grew only in the osmotically stabilised L-form medium. However, they reverted very rapidly to yield only vegetative growth on subculture.

2. Intermediate type of Growth

Vegetative growth which appeared only in the L-form pour plate medium and which could in turn be subcultured to ordinary blood agar might have originated from reversion of naturally occurring or beta-lactam induced wall-defective microbial variants. The feature peculiar to this group was the occurrence of pure culture of vegetative growth in the pour plate medium, while the blood agar plate was sterile. This inferred that autolysin inhibition might be required before bacteria in this group could initiate walled growth, whatever their origin.

3. L-form Growth

The strains which refused to grow on ordinary blood agar or in enrichment media needed a substance present in the original specimen of blood in addition to the nutrients present in the L-form medium, in order to give normal vegetative growth. The nutrient requirement of these strains suggested that they might be auxotrophs. Once normal vigorous growth on the surface of a nutritionally supplemented blood agar plate (LFBA) had been obtained, it was possible to subculture the

bacteria to ordinary media. A proportion were therefore eventually capable of nutritional independence.

It was unlikely that the bacteria were beta-lactam induced wall-defective microbial variants because the LFBA inoculum was taken from the primary blood culture medium, and any inducer present should have perpetuated the wall-defective state. However, it was conceivable that deterioration of a beta-lactam inducer in the primary blood culture had reached a point where reversion was possible. Final consideration of this theory depended on evidence of beta-lactam administration before the blood cultures were taken, and this is considered in Chapter 13.

CONCLUSIONS

1. Reversion methods

Subculture to LFBA, using an inoculum taken from the primary blood culture medium, was a better method of reversion that the agar-block enrichment method.

2. Interpretation of Reversion Procedure Results

a) Vegetative Growth. Vegetative growth may have originated from the reversion of a proportion of naturally occurring wall-defective microbial variants to normal growth, or alternatively there may have been mass reversion of induced wall-defective microbial variants to the walled state. Some vegetative growth perhaps required autolysin inhibition for culture.

Some vegetative growth may have appeared exclusively in the medium by chance.

b) L-form Growth. The L-phase variants isolated were presumed to be naturally occurring rather than beta-lactam induced, but the latter possibility could not be entirely excluded.

TABLE 6

Microscopy, colonial morphology, identity and type of growth observed in 53 bacterial strains grown on subculture of 50 primary blood cultures in L-form medium to pour plates of the same L-form medium composition incubated in 5% carbon dioxide in air (LFMPP CO_2), to surface inoculated blood agar plates incubated in 5% carbon dioxide in air (BA CO_2) and to surface inoculated blood agar plates incubated in a mixture of 65% hydrogen plus 5% carbon dioxide in air (BA AnO_2).

Key: .... = not applicable

Patient	Type of Primary L-form Blood Culture Medium	Microscopy and colonial morphology of subculture growth				Identity	Type of Growth
		BA CO_2 /BA AnO_2		LFMPP CO_2			
		Growth	Microscopy	Growth	Microscopy		
1. WS.	Victoria Medium F	Sterile	.....	Deformed colonies deep in agar	Debris	Escherichia coli	L-form
2. AR.	Victoria Medium F	Sterile	.....	Deformed colonies deep in agar	Debris	Escherichia coli	L-form
3. DB.	Victoria Medium F	Sterile	.....	Deformed colonies deep in agar	Debris	Klebsiella aerogenes	L-form
4. WF.	Victoria Medium F	Sterile	.....	Mixed:	Debris	Escherichia coli	L-form
				i) Deformed colonies deep in agar			
				ii) Deformed colonies deep in agar	Debris	Citrobacter freundii	L-form
5. RC.	Victoria Medium F	Sterile	.....	Deformed colonies deep in agar	Debris	Escherichia coli	L-form

Table 6 contd.

Patient	Type of Primary L-form Blood Culture Medium	Microscopy and colonial morphology of subculture growth BA CO_2 /BA AnO_2 Growth	Microscopy	LFMPP CO_2 Growth	Microscopy	Identity	Type of Growth
6. JN.	Victoria Medium F	Sterile	.....	Vegetative with surface growth	Gram negative bacilli	Salmonella bovis-morbificans	Intermediate
7. JV.	Victoria Medium F	Vegetative	Gram negative bacilli	Vegetative with surface growth	Gram negative bacilli	Proteus mirabilis	Vegetative
8. LG.	Victoria Medium F	Vegetative	Gram negative bacilli	Vegetative with surface growth	Gram negative bacilli	Proteus mirabilis	Vegetative
9. E.McA	Victoria Medium F	Vegetative	Gram negative bacilli	Vegetative with surface growth	Gram negative bacilli	Escherichia coli	Vegetative
10. JL.	Victoria Medium F	Sterile	.....	Vegetative with surface growth	Gram negative bacilli	Escherichia coli	Intermediate
11. A.McL	Victoria Medium F	Vegetative	Gram negative bacilli	Vegetative with surface growth	Gram negative bacilli	Escherichia coli	Vegetative
12. GMcK.	Victoria Medium F	Vegetative	Gram negative bacilli	Vegetative with surface growth	Gram negative bacilli	Escherichia coli	Vegetative
13. CG.	Modified Victoria Medium F	Vegetative	Gram negative bacilli	Vegetative with surface growth	Gram negative bacilli	Escherichia coli	Vegetative
14. JB.	Modified Victoria Medium F	Vegetative	Gram negative bacilli	Vegetative with surface growth	Gram negative bacilli	Escherichia coli	Vegetative

Table 6 contd.

Patient	Type of Primary L-form Blood Culture Medium	Microscopy and colonial morphology of subculture growth BA CO_2 /BA AnO_2 Growth	Microscopy	LFMPP CO_2 Growth	Microscopy	Identity	Type of Growth
15. MB.	Modified Victoria Medium F	Vegetative	Gram negative bacilli	Vegetative with surface growth	Gram negative bacilli	Escherichia coli	Vegetative
16. KC.	Modified Victoria Medium F	Sterile	.....	Vegetative with surface growth	Gram negative bacilli	Escherichia coli	Intermedia
17. JE.	Modified Victoria Medium F	Sterile	.....	Vegetative with surface growth	Gram negative bacilli	Escherichia coli	Intermedia
18. IA.	Modified Victoria Medium F	Vegetative	Gram negative bacilli	Vegetative with surface growth	Gram negative bacilli	Proteus mirabilis	Vegetative
19. JB.	Modified Victoria Medium F	Sterile	.....	Vegetative with surface growth	Gram negative bacilli	Escherichia coli	Intermedia
20. KM.	Modified Victoria Medium F	Vegetative	Gram negative bacilli	Vegetative with surface growth	Gram negative bacilli	Escherichia coli	Vegetative
21. EJ.	Modified Victoria Medium F	Vegetative	Gram negative bacilli	Vegetative with surface growth	Gram negative bacilli	Escherichia coli	Vegetative
22. AK.	Modified Victoria Medium F	Vegetative	Gram negative bacilli	Vegetative with surface growth	Gram negative bacilli	Escherichia coli	Vegetative
23. IMcC.	Modified Victoria Medium F	Sterile	.....	Vegetative with surface growth	Gram negative bacilli	Enterobacter agglomerans	Intermedia

Table 6 contd.

Patient	Type of Primary L-form Blood Culture Medium	Microscopy and colonial morphology of subculture growth BA CO_2 /BA AnO_2 Growth	Microscopy	LFMPP CO_2 Growth	Microscopy	Identity	Type of Growth
24. AP.	Modified Victoria Medium F	Sterile	.....	Crystal colonies deep in agar	Pleomorphic Gram negative bacilli	Escherichia coli	L-form
25. CW.	Modified Victoria Medium F	Vegetative	Gram negative bacilli	Vegetative with surface growth	Gram negative bacilli	Klebsiella pneumoniae	Vegetative
26. NF.	Modified Victoria Medium F	Sterile	.....	i) Mixed: Vegetative with surface growth	Gram negative bacilli	Escherichia coli	Intermediat
				ii) Deformed colonies deep in agar	Debris	Escherichia coli	L-form
27. RD.	Modified Victoria Medium F	Vegetative	Gram negative bacilli	Vegetative with surface growth	Gram negative bacilli	Escherichia coli	Vegetative
28. CS.	Modified Victoria Medium F	Vegetative	Gram negative bacilli	Vegetative with surface growth	Gram negative bacilli	Escherichia coli	Vegetative
29. MR.	Modified Victoria Medium F	Sterile	.....	Vegetative with surface growth	Gram negative bacilli	Escherichia coli	Intermediat
30. LS.	Modified Victoria Medium F	Vegetative	Gram negative bacilli	Vegetative with surface growth	Gram negative bacilli	Escherichia coli	Vegetative
31. RT.	Modified Victoria Medium F	Sterile	.....	Vegetative with surface growth	Gram negative bacilli	Klebsiella pneumoniae	Intermediat

Table 6 contd.

Patient	Type of Primary L-form Blood Culture Medium	Microscopy and colonial morphology of subculture growth BA CO_2/BA AnO_2 Growth	Microscopy	LFMPP CO_2 Growth	Microscopy	Identity	Type of Growth
32. A.McB.	Modified Victoria Medium F	Sterile	.....	i) Mixed: Vegetative with surface growth	Gram negative bacilli	Escherichia coli	Intermedia
				ii) Deformed colonies deep in agar	Debris	Escherichia coli	L-form
33. RH.	Modified Victoria Medium F	Vegetative	Gram negative bacilli	Vegetative with surface growth	Gram negative bacilli	Escherichia coli	Vegetative
34. MMcD.	Modified Victoria Medium F	Vegetative	Gram negative bacilli	Vegetative with surface growth	Gram negative bacilli	Escherichia coli	Vegetative
35. JW.	Modified Victoria Medium F	Vegetative	Gram negative bacilli	Vegetative with surface growth	Gram negative bacilli	Proteus mirabilis	Vegetative
36. HMcC	Modified Victoria Medium F	Sterile	.....	Vegetative with surface growth	Gram negative bacilli	Escherichia coli	Intermedia
37. TG.	Modified Victoria Medium F	Vegetative	Gram negative bacilli	Vegetative with surface growth	Gram negative bacilli	Proteus mirabilis	Vegetative
38. AM.	Modified Victoria Medium F	Sterile	.....	Crystal colonies deep in agar	Pleomorphic Gram negative bacilli	Salmonella typhimurium	L-form
39. CM.	Modified Victoria Medium F	Sterile	.....	Vegetative with surface growth	Gram negative bacilli	Escherichia coli	Intermedi

Table 6 contd.

Patient	Type of Primary L-form Blood Culture Medium	Microscopy and colonial morphology of subculture growth BA CO_2/BA AnO_2 Growth	Microscopy	LFMPP CO_2 Growth	Microscopy	Identity	Type of Growth
40. SM.	Modified Victoria Medium F	Vegetative	Gram negative bacilli	Vegetative with surface growth	Gram negative bacilli	Proteus mirabilis	Vegetative
41. EMcM.	Modified Victoria Medium F	Vegetative	Gram negative bacilli	Vegetative with surface growth	Gram negative bacilli	Proteus morganii	Vegetative
42. CS.	Modified Victoria Medium F	Sterile	.....	Vegetative with surface growth	Gram negative bacilli	Escherichia coli	Intermedia
43. LF.	Modified Victoria Medium F	Vegetative	Gram negative bacilli	Vegetative with surface growth	Gram negative bacilli	Escherichia coli	Vegetative
44. AT.	Modified Victoria Medium F	Vegetative	Gram negative bacilli	Vegetative with surface growth	Gram negative bacilli	Providence stuartii	Vegetative
45. JP.	Partially Defined Medium 3	Vegetative	Gram negative bacilli	Vegetative with surface growth	Gram negative bacilli	Proteus mirabilis	Vegetative
46. ML.	Partially Defined Medium 3	Vegetative	Gram negative bacilli	Vegetative with surface growth	Gram negative bacilli	Escherichia coli	Vegetative
47. AC.	Partially Defined Medium 3	Vegetative	Gram negative bacilli	Vegetative with surface growth	Gram negative bacilli	Escherichia coli	Vegetative
48. RMcF.	Partially Defined Medium 3	Vegetative	Gram negative bacilli	Vegetative with surface growth	Gram negative bacilli	Salmonella species	Vegetative

Table 6 contd.

Patient	Type of Primary L-form Blood Culture Medium	Microscopy and colonial morphology of subculture growth				Identity	Type of Growth
		BA CO_2/BA AnO_2		LFMPP CO_2			
		Growth	Microscopy	Growth	Microscopy		
49. SK.	Partially Defined Medium 3	Vegetative	Gram negative bacilli	Vegetative with surface growth	Gram negative bacilli	Escherichia coli	Vegetative
50. JJ.	Partially Defined Medium 3	Vegetative	Gram negative bacilli	Vegetative with surface growth	Gram negative bacilla	Escherichia coli	Vegetative

TABLE 7

Procedures used for the reversion of 53 bacterial strains grown from 50 primary blood cultures in L-form medium. Subcultur growth types yielded on L-form medium pour plates incubated in 5% carbon dioxide in air (LFMPP CO_2) and use of the following methods: reversion series with final subculture to surface inoculated blood agar plates incubated in 5% carbon dioxide in air (BA CO_2), direct subculture to BA CO_2, agar block enrichment, and direct subculture of the primary blood culture in L-form medium to surface inoculated L-form blood agar plates incubated in 5% carbon dioxide in air (LFBA CO_2).

Key: + = Method used

\- = Method not used

... = Vegetative growth obtained on direct subculture of primary blood culture in L-form medium to BA CO_2. Reversion procedure not required.

Patient	Type of Primary L-form Blood Culture Medium	Subculture Growth Type On LFMPP CO_2	Reversion Method Used: Reversion series with final BA CO_2 subculture	Direct subculture to BA CO_2	Agar Block Enrichment	Direct subculture of primary blood culture in L-form medium to LFBA CO_2
1. WS.	Victoria Medium F	L-form	+	-	-	-
2. AR.	Victoria Medium F	L-form	+	-	-	-
3. DB.	Victoria Medium F	L-form	+	-	-	-
4. WF.	Victoria Medium F	Mixed:				
		i) L-form	+	-	-	+
		ii) L-form	+	-	-	+

Table 7 contd.

Patient	Type of Primary L-form Blood Culture Medium	Subculture Growth Type On LFMPP CO_2	Reversion Method Used			
			Reversion series with final BA CO_2 subculture	Direct subculture to BA CO_2	Agar Block Enrichment	Direct subculture of primary blood culture in L-form medium to LFBA CO_2
5. RC.	Victoria Medium F	L-form	+	-	-	-
6. JN.	Victoria Medium F	Intermediate	-	+	+	-
7. JV.	Victoria Medium F	Vegetative	...	...	...	...
8. LG.	Victoria Medium F	Vegetative	...	...	...	...
9. E.McA.	Victoria Medium F	Vegetative	...	...	...	...
10. JL.	Victoria Medium F	Intermediate	-	+	+	-
11. AMcL.	Victoria Medium F	Vegetative	...	...	...	...
12. G.McK.	Victoria Medium F	Vegetative	...	...	...	...
13. CG.	Modified Victoria Medium F	Vegetative	...	...	...	...
14. JB.	Modified Victoria Medium F	Vegetative	...	...	...	...
15. MB.	Modified Victoria Medium F	Vegetative	...	...	...	...
16. KC.	Modified Victoria Medium F	Intermediate	-	+	+	-

Table 7 contd.

Patient	Type of Primary L-form Blood Culture Medium	Subculture Growth Type On LFMPP CO_2	Reversion Method Used: Reversion series with final BA CO_2 subculture	Direct subculture to BA CO_2	Agar Block Enrichment	Direct subculture of primary blood culture in L-form medium to LFBA CO_2
17. JE.	Modified Victoria Medium F	Intermediate	-	+	+	-
18. IA.	Modified Victoria Medium F	Vegetative	...	...	...	...
19. JB.	Modified Victoria Medium F	Intermediate	-	+	+	-
20. KM.	Modified Victoria Medium F	Vegetative	...	...	...	...
21. EJ.	Modified Victoria Medium F	Vegetative	...	...	...	...
22. AK.	Modified Victoria Medium F	Vegetative	...	...	...	...
23. IMcC.	Modified Victoria Medium F	Intermediate	-	+	+	-
24. AP.	Modified Victoria Medium F	L-form	-	-	+	+
25. CW.	Modified Victoria Medium F	Vegetative	...	...	...	...

Table 7 contd.

Patient	Type of Primary L-form Blood Culture Medium	Subculture Growth Type On LFMPP CO_2	Reversion Method Used: Reversion series with final BA CO_2 subculture	Direct subculture to BA CO_2	Agar Block Enrichment	Direct subculture of primary blood culture in L-form medium to LFBA CO_2
26. NF.	Modified Victoria Medium F	Mixed: i) Intermediate	-	+	+	+
		ii) L-form	-	-	+	+
27. RD.	Modified Victoria Medium F	Vegetative	...	...	...	...
28. CS.	Modified Victoria Medium F	Vegetative	...	...	...	...
29. MR.	Modified Victoria Medium F	Intermediate	-	+	+	-
30. LS.	Modified Victoria Medium F	Vegetative	...	...	...	...
31. RT.	Modified Victoria Medium F	Intermediate	-	+	+	-
32. A.McB.	Modified Victoria Medium F	Mixed: i) Intermediate	-	+	+	+
		ii) L-form	-	-	+	+
33. RH.	Modified Victoria Medium F	Vegetative	...	...	...	...
34. MMcD.	Modified Victoria Medium F	Vegetative	...	...	...	...

Table 7 contd.

Patient	Type of Primary L-form Blood Culture Medium	Subculture Growth Type On LFMPP CO_2	Reversion Method Used			
			Reversion series with final BA CO_2 subculture	Direct subculture to BA CO_2	Agar Block Enrichment	Direct subculture of primary blood culture in L-form medium to LFBA CO_2
35. JW.	Modified Victoria Medium F	Vegetative	...	...	...	...
36. HMcC	Modified Victoria Medium F	Intermediate	-	+	+	-
37. TG.	Modified Victoria Medium F	Vegetative	...	...	...	...
38. AM.	Modified Victoria Medium F	L-form	-	-	+	+
39. CM.	Modified Victoria Medium F	Intermediate	-	+	+	-
40. SM.	Modified Victoria Medium F	Vegetative	...	...	...	...
41. EMcM.	Modified Victoria Medium F	Vegetative	...	...	...	...
42. CS.	Modified Victoria Medium F	Intermediate	-	+	+	-
43. LF.	Modified Victoria Medium F	Vegetative	...	...	...	...

Table 7 contd.

Patient	Type of Primary L-form Blood Culture Medium	Subculture Growth Type On LFMPP CO_2	Reversion Method Used: Reversion series with final BA CO_2 subculture	Direct subculture to BA CO_2	Agar Block Enrichment	Direct subculture of primary blood culture in L-form medium to LFBA CO_2
44. AT.	Modified Victoria Medium F	Vegetative	...	...	...	...
45. JP.	Partially Defined Medium 3	Vegetative	...	...	...	...
46. ML.	Partially Defined Medium 3	Vegetative	...	...	...	...
47. AC.	Partially Defined Medium 3	Vegetative	...	...	...	...
48. R.McF.	Partially Defined Medium 3	Vegetative	...	...	...	...
49. SK.	Partially Defined Medium 3	Vegetative	...	...	...	...
50. JJ.	Partially Defined Medium 3	Vegetative	...	...	...	...

TOTALS:		
	Strains which required a reversion procedure	23
	Strains which did not require a reversion procedure	30
	Total number of strains examined	53

TABLE 8

Reversion procedures successful in obtaining vegetative growth on surface inoculated blood agar plates incubated in 5% carbon dioxide in air (BA CO_2) in 23 bacterial strains which grew in primary blood culture from 20 patients only in L-form medium (LFM) and which grew in LFM pour plates incubated in 5% carbon dioxide in air (LFMPP CO_2) on primary subculture. Comparison of subculture to media not osmotically stabilised or nutritionally enriched ie. BA CO_2 or Brain Heart Infusion Broth (BHI), with use of osmotically stabilised nutritionally enriched subculture media ie. LFM, LFMPP CO_2.

Key: + = method successful

- = method not successful

... = method not used

Patient	Subculture growth type on LFMPP CO_2	Reversion method successful in yielding vegetative growth on final subculture to BA CO_2				
		Subculture to media not osmotically stabilised and not nutritionally enriched		Subculture to osmotically stabilised nutritionally enriched media		
		Direct subculture to BA CO_2	Agar Block subculture to BHI	Agar Block subculture to LFM	Reversion Series in LFM	Direct subculture of primary blood culture in LFM to LFBA CO_2
1. WS	L-form	...	...	...	+	...
2. AR.	L-form	...	...	...	+	...
3. DB.	L-form	...	...	...	+	...
4. WF.	Mixed:					
i)	L-form	...	...	...	+	+
ii)	L-form	...	...	...	+	+

Table 8 contd.

Patient	Subculture growth type on LFMPP CO_2	Reversion method successful in yielding vegetative growth on final subculture to BA CO_2				
		Subculture to media not osmotically stabilised and not nutritionally enriched			Subculture to osmotically stabilised nutritionally enriched media	
		Direct subculture to BA CO_2	Agar Block subculture to BHI	Agar Block subculture to LFM	Reversion Series in LFM	Direct subculture of primary blood culture in LFM to LFBA CO_2
5 RC	L-form	...	...	...	+	...
6 JN	Intermediate	+	+	+	...	...
10 JL	Intermediate	+	+	+	...	...
16 KC	Intermediate	+	+	+	...	...
17 JE	Intermediate	+	+	+	...	...
19 JB	Intermediate	+	+	+	...	...
23 IMcC	Intermediate	+	+	+	...	...
24 AP	L-form	...	-	-	...	+
26 NF	Mixed:					
i)	Intermediate	-	-	-	...	+
ii)	L-form	...	-	-	...	+
29 MR	Intermediate	+	+	+	...	...
31 RT	Intermediate	+	+	+	...	...
32 AMcB	Mixed:					
i)	Intermediate	-	-	-	...	+

Table 8 contd.

Patient	Subculture growth type on LFMPP CO_2	Reversion method successful in yielding vegetative growth on final subculture to BA CO_2				
		Subculture to media not osmotically stabilised and not nutritionally enriched		Subculture to osmotically stabilised nutritionally enriched media		
		Direct subculture to BA CO_2	Agar Block subculture to BHI	Agar Block subculture to LFM	Reversion Series in LFM	Direct subculture of primary blood culture in LFM to LFBA CO_2
36. HMcC.	Intermediate	+	+	+	...	...
38. AM.	L-form	...	-	-	...	+
39. CM.	Intermediate	+	+	+	...	...
42. CS.	Intermediate	+	+	+	...	...

Number of blood cultures in which vegetative growth was obtained by reversion on media which were not osmotically stabilised and not nutritionally enriched (Intermediate subculture growth type).....11 (11 strains isolated)

Number of blood cultures in which vegetative growth was obtained only by reversion on media which were osmotically stabilise and nutritionally enriched (L-form subculture growth type).....9 (12 strains isolated)

SECTION V

CLINICAL ASPECTS OF INFECTION DUE TO WALL-DEFECTIVE VARIANTS OF ENTEROBACTERIACEAE

Chapter 12

Clinical Features

CLINICAL ASPECTS OF INFECTION DUE TO WALL-DEFECTIVE VARIANTS OF ENTEROBACTERIACEAE

INTRODUCTION

There is little clinical information about infection due to wall-defectve microbial variants, though there may be an association with urinary tract infection of the chronic relapsing type (Guze & Kalmanson, 1964; Gutman et al., 1965; Gutman, Schaller & Wedgwood, 1967; Domingue & Schlegel, 1970; Brogan, 1977; Garrod, Lambert & O'Grady, 1981).

TERMS USED

L-form Group

In blood cultures from 50 patients, the isolation of Enterobacteriaceae strains was in L-form medium only. This group is referred to as the 'L-form group' to denote that primary isolation was from L-form blood culture medium only. It contains all 53 isolates made from the 50 blood cultures and therefore all the presumed wall-defective variants now characterised below.

Vegetative Group

In 50 patients, isolation of Enterobacteriaceae in the primary blood culture medium was in ordinary culture medium alone or in the L-form medium and also in one or both ordinary culture media. This was the vegetative group.

Acute Urinary Tract Infection

This term was applied to the first recorded episode of urinary tract infection.

Chronic Urinary Tract Infection

This term was applied when there was a history of more than one previous episode of urinary tract infection. Two types of chronic urinary tract infection could be distinguished, namely;- chronic recurrent urinary tract infection, in which repeated infections were caused by different strains of bacteria and chronic relapsing urinary tract infection in which repeated infections were caused by the same bacterial strain.

CHARACTERISATION OF WALL-DEFECTIVE VARIANTS ISOLATED FROM 50 BLOOD CULTURES.

The 53 isolates of presumed wall-defective variants isolated from 50 blood cultures using L-form media were classified by morphological and cultural studies as described in Chapters 7 and 10. As the general bacteriological characteristics of the 53 variants have already been described in these Chapters, no further description of the methods used is required here. As in Chapter 11, the 53 isolates could be grouped into 3 classes as follows:

The vegetative group contained 30 members presumed to be either unaltered vegetative forms or transitional phase variants (McGee et al., 1971) that had reverted so rapidly to the vegetative state that they could not be studied further. The intermediate group contained 13 members which never showed the true characteristics of wall-defective variants. They did not produce vegetative forms on primary subculture to blood agar plates incubated in an atmosphere of 5% carbon dioxide in air, but yielded a heavy growth of vegetative colonies in pour plates of L-form medium incubated in an atmosphere of 5% carbon dioxide in air. The true L-form group contained 10 members. These were all classical

L-phase variants according to the classification of McGee et al., (1971). They gave serial replication in a distinctive colonial form, either as typical "fried egg" colonies at the surface of the agar, or in deep agar as crystal colonies or typical deformed colonies. Of these 8 were stable, but two seemed to be unstable, as judged by the appearance of vegetative forms on primary subculture of the blood culture specimens to pour plates of L-form medium incubated in an atmosphere of 5% carbon dioxide in air.

OBJECTIVE

The object was to analyse patient data in terms of:

1. General Features

Age and sex, principal diagnosis, source of septicaemia and species of infecting bacterium.

2. Features of Urinary Tract Infection

If a urinary tract infection was the source of septicaemia, the points noted were whether the infection was acute or chronic, and if chronic, whether it was of the recurrent or relapsing type.

MATERIALS & METHODS

The clinical features of 50 patients from whom Enterobacteriaceae variants were isolated by blood culture were compared with those of 50 patients from whom ordinary vegetative Enterobacteriaceae were isolated by blood culture.

Selection of Patients

When a patient was found to have septicaemia due to wall-defective coliform variants, whether of the vegetative, intermediate or true L-form type, he was assigned to the L-form group. The next patient to have a vegetative coliform septicaemia was then assigned to the vegetative group. This was continued until there were 50 patients in each group.

RESULTS

1. General Features

Age and Sex. The hospital group from which the patients were drawn did not have a paediatric unit. Most blood cultures were received from patients over the age of 12 years. The age and sex distribution were similar in both the L-form and the vegetative group (Table 9).

Principal Diagnosis. In most patients the episode of septicaemia was incidental to another illness. Tables 10 and 11 show the principal diagnosis in each patient. In Table 12 the principal diagnoses are grouped into five broad categories. The pattern of disease was similar in both groups.

Source of Septicaemia. The urinary tract was the most common source of infection in both groups. The urinary tract was the focus of infection in 28 (56%) patients in the L-form group, but in only 19 (38%) of the vegetative group (Table 13). This difference was not significant (Chi-square test results; test statistic, 3.24; degrees of freedon, 1; significance level, 0.10).

Species of Infecting Bacterium. Most isolates were E. coli. The distribution of species between the two groups was broadly similar (Table 14).

2. Features of Urinary Tract Infection. Type of Infection: Acute or Chronic.

Most patients in both the L-form and the vegetative groups had an acute urinary tract infection, and of these a large proportion in both groups was associated with catheterisation. The number of patients who had chronic infection was small in both groups (Tables 15 and 16). Chronic relapsing infection was a feature of the L-form group rather than of the vegetative group (Tables 17 and 18). The sample numbers were too small for statistical analysis.

3. Outcome of Infection.

Three months after the septicaemic episode the proportion of patients who had recovered or died was similar in both groups (Table 19). (Chi-square test results: Test statistic, 1.09; degrees of freedom, 1; significance level, less than 0.10). Death was directly attributable to the septicaemia in only two patients, both of whom were in the L-form group.

COMMENT

Clinical features in both groups were essentially similar. There was a slight preponderance of urinary tract source septicaemias in the L-form group. Of the small number who had chronic relapsing infection of the urinary tract as a source of septicaemia, most were in the L-form group.

The results suggest that infection due to wall-defective Enterobacteriaceae variants occurs most often in the urinary tract, and that such wall-defective variants may occur in chronic relapsing urinary tract infection more often than vegetative bacteria.

Table 9

Age and sex of the patients studied

	L-form group	Vegetative group
Age		
Average	63.95 years*	64.20 years**
Median	67 years	68 years
Upper quartile	74 years	74 years
Lower quartile	57 years	54 years
Range	21-94 years	20-90 years
Sex		
Male	23 (46%)	23 (46%)
Female	27 (54%)	27 (54%)

* L-form group : age identified in 49/50 patients.

** Vegetative group : age identified in 50/50 patients.

Table 10

Nature of bacterial species isolated from blood, source of septicaemia and principal diagnosis in group of patients yielding variants.

Patient		Bacterial species	Source of septicaemia	Code*	Principal diagnosis
1. WS		Escherichia coli	Biliary tract	BT	Cholecystitis
2. AR		Escherichia coli	Urinary tract	UT	Pyelonephritis
3. DB		Klebsiella aerogenes	Urinary tract	UT	Rectal carcinoma
4. WF	(i)	Escherichia coli			
	(ii)	Citrobacter freundii	Urinary tract	UT	Prostatic carcinoma
5. RC		Escherichia coli	Urinary tract	UT	Myocardial Infarct
6. JN		Salmonella bovis-morbificans	Bowel	B	Gastro-enteritis
7. JV		Proteus mirabilis	Bowel	B	Pancreatic cyst
8. LG		Proteus mirabilis	Unknown	?	Pyrexia of unknown origin
9. E.McA		Escherichia coli	Biliary tract	BT	Cholecystitis
10. JH		Escherichia coli	Urinary tract	UT	Bacterial endocarditis
11. AMcL		Escherichia coli	Urinary tract	UT	Pyelonephritis
12. GMcK		Escherichia coli	Urinary tract	UT	Carcinoma of bronchus
[illegible]		[illegible]	[illegible]	UT	Pyelonephritis

Table 10 contd.

Patient	Bacterial species	Source of septicaemia	Code*	Principal diagnosis
14. JB	Escherichia coli	Bowel	B	Gastric carcinoma
15. MB	Escherichia coli	Urinary tract	UT	Lymphoma
16. KC	Escherichia coli	Urinary tract	UT	Pyelonephritis
17. JE	Escherichia coli	Bowel	B	Gastric carcinoma
18. IA	Proteus mirabilis	Urinary tract	UT	Pyelonephritis
19. JB	Escherichia coli	Bowel	B	Intestinal obstruction
20. KM	Escherichia coli	Respiratory tract	RT	Lung abscess
21. EJ	Escherichia coli	Urinary tract	UT	Perforated urinary bladder
22. AK	Escherichia coli	Urinary tract	UT	Pyelonephritis
23. IMcC	Enterobacter agglomerans	Urinary tract	UT	Deep venous thrombosis
24. AP	Escherichia coli	Gall bladder	BT	Cholecystitis
25. NF	Klebsiella pneumoniae	Urinary tract	UT	Benign prostatic hypertrophy
26. CW	Escherichia coli	Bowel	B	Carcinoma of colon
27. RD	Escherichia coli	Urinary tract	UT	Benign prostatic hypertrophy
28. CS	Escherichia coli	Urinary tract	UT	Carcinoma of cervix

Table 10 contd.

Patient	Bacterial species	Source of septicaemia	Code*	Principal diagnosis
29. MR	Escherichia coli	Gall bladder	BT	Cholecystitis
30. LS	Escherichia coli	Gall bladder	BT	Cholecystitis
31. RT	Klebsiella pneumoniae	Pressure sore	ST	Diabetes
32. AMcB	Escherichia coli	Urinary tract	UT	Benign prostatic hypertrophy
33. RH	Escherichia coli	Septic toe	ST	Diabetes
34. MMcD	Escherichia coli	Urinary tract	UT	Pyelonephritis
35. JW	Proteus mirabilis	Bowel	B	Anal fissure
36. HMcC	Escherichia coli	Abscess of thigh	ST	Carcinoma of colon
37. TG	Proteus mirabilis	Urinary tract	UT	Prostatic hypertrophy
38. AM	Salmonella typhimurium	Bowel	B	Non-specific arthritis (hip)
39. CM	Escherichia coli	Biliary tract	BT	Cholangitis
40. SM	Proteus mirabilis	Urinary tract	UT	Schizophrenia
41. EMcM	Proteus morganii	Urinary tract	UT	Gastric volvulus
42. CS	Escherichia coli	Urinary tract	UT	Benign prostatic hypertrophy

Table 10 contd.

Patient	Bacterial species	Source of septicaemia	Code*	Principal diagnosis
43. LF	Escherichia coli	Urinary tract	UT	Pyelonephritis
44. AT	Providence stuartii	Urinary tract	UT	Multiple sclerosis
45. JP	Proteus mirabilis	Urinary tract	UT	Carcinoma of prostate
46. ML	Escherichia coli	Urinary tract	UT	Barbiturate overdose
47. AC	Escherichia coli	Urinary tract	UT	Adenocarcinoma of ovary
48. RMcF	Salmonella species	Bowel	B	Gastric carcinoma
49. SK	Escherichia coli	Unknown	?	Carcinoma of bronchus
50. JJ	Escherichia coli	Bowel	B	Ulcerative colitis

* See Table 12

Table 11

Nature of bacterial species isolated from blood, source of septicaemia and principal diagnosis in group of patients yielding only vegetative bacteria.

Patient	Bacterial species	Source of septicaemia	Code*	Principal diagnosis
1. WB	*Escherichia coli*	Urinary tract	UT	Carcinoma of bladder
2. IS	*Proteus mirabilis*	Urinary tract	UT	Pyelonephritis
3. DM	*Serratia marcescens*	Wound	ST	Mitral valvotomy
4. DMcK	*Enterobacter aerogenes*	Urinary tract	UT	Rectal carcinoma
5. IMcM	*Klebsiella aerogenes*	Intravenous cannula	P	Caesarean section
6. PF	*Escherichia coli*	Sub-phrenic abscess	B	Gastric carcinoma
7. IMcQ	*Escherichia coli*	Lung	RT	Lymphoma
8. ED	*Escherichia coli*	Urinary tract	UT	Bacterial endocarditis
9. JW	*Escherichia coli*	Bowel	B	Strangulated hernia
10. JM	*Proteus mirabilis*	Biliary tract	BT	Cholecystitis
11. JD	*Escherichia coli*	Umbilical hernia	ST	Leukaemia
12. PO'H	*Escherichia coli*	Sub-phrenic abscess	B	Carcinoma of oesophagus
13. DH	*Proteus mirabilis*	Replacement hip joint	P	Arthritis
14. TP	*Escherichia coli*	Urinary tract	UT	Pyelonephritis
15. WS	*Salmonella bovis-morbificans*	Bowel	B	Gastro-enteritis

Table 11 contd.

Patient	Bacterial species	Source of septicaemia	Code*	Principal diagnosis
16. JC	Klebsiella aerogenes	Urinary tract	UT	Carcinoma of bladder
17. LC	Escherichia coli	Unknown	?	Pyrexia of unknown origin
18. SB	Salmonella panama	Bowel	B	Gastro-enteritis
19. DC	Escherichia coli	Urinary tract	UT	Strangulated hernia
20. AC	Proteus mirabilis	Lung	RT	Multiple injuries
21. JMcL	Aeromonas sp.	Biliary tract	BT	Cholangitis
22. IB	Salmonella panama	Bowel	B	Gastro-enteritis
23. RC	Escherichia coli	Bowel	B	Diverticulitis
24. JMcK	Escherichia coli	Bowel	B	Carcinoma of colon
25. JD	Escherichia coli	Bowel	B	Pancreatitis
26. JMcK	Escherichia coli	Urinary tract	UT	Carcinoma of rectum
27. MJ	Escherichia coli	Lung	RT	Pneumonia
28. AH	Escherichia coli	Bowel	B	Gastric carcinoma
29. JP	Escherichia coli	Bowel	B	Sub-phrenic abscess
30. TB	Klebsiella pneumoniae	Urinary tract	UT	Carcinoma of prostate
31. DF	Escherichia coli	Biliary tract	BT	Cholangitis

Table 11 contd.

Patient	Bacterial species	Source of septicaemia	Code*	Principal diagnosis
32. JM	Escherichia coli	Urinary tract	UT	Hydronephrosis
33. JS	Proteus mirabilis	Urinary tract	UT	Hydronephrosis
34. AS	Escherichia coli	Urinary tract	UT	Hydronephrosis
35. WT	Proteus mirabilis	Urinary tract	UT	Prostatic hypertrophy
36. SH	Escherichia coli	Bowel	B	Diverticulitis
37. TB	Escherichia coli	Urinary tract	UT	Pyelonephritis
38. DS	Escherichia coli	Biliary tract	BT	Cholecystitis
39. WR	Escherichia coli	Bowel	B	Carcinoma of colon
40. DS	Klebsiella pneumoniae	Pressure sores	ST	Hypothermia
41. TH	Klebsiella pneumoniae	Psoas abscess	ST	Crohns disease
42. AF	Escherichia coli	Urinary tract	UT	Carcinoma of bladder
43. AC	Klebsiella pneumoniae	Biliary tract	BT	Cholecystitis
44. BC	Proteus mirabilis	Urinary tract	UT	Prostatic hypertrophy
45. CW	Escherichia coli	Bowel	B	Carcinoma of rectum
46. DB	Escherichia coli	Bowel	B	Carcinoma of colon
47. EMcM	Klebsiella pneumoniae	Urinary tract	UT	Gastric volvulus

Table 11 contd.

Patient	Bacterial species	Source of septicaemia	Code*	Principal diagnosis
48. MMcG	Acinetobacter species	Bowel	B	Gastric carcinoma
49. MM	Escherichia coli	Urinary tract	UT	Pyelonephritis
50. JMcF	Escherichia coli	Urinary tract	UT	Intestinal obstruction

* See Table 12

Table 12

Principal diagnosis : main categories

Diagnosis	L-form group	Vegetative group
Malignancy	13 (26%)	16 (32%)
Pyelonephritis	8 (16%)	7 (14%)
Cholecystitis or Cholangitis	6 (12%)	5 (10%)
Benign Prostatic Hypertrophy	6 (12%)	2 (4%)
Miscellaneous	17 (34%)	20 (40%)
TOTAL	50 (100%)	50 (100%)

Table 13

Source of Septicaemia

Source	Code	L-form group	Vegetative group
Urinary tract	UT	28 (56%)	19 (38%)
Bowel/Alimentary tract	B	10 (20%)	16 (32%)
Biliary tract	BT	6 (12%)	5 (10%)
Soft Tissue	ST	3 (6%)	4 (8%)
Respiratory tract	RT	1 (2%)	3 (6%)
Infected prosthesis	P	0 (0%)	2 (4%)
Unknown	?	2 (4%)	1 (2%)
TOTAL		50 (100%)	50 (100%)

Table 14

Species of infecting bacterium

Species	L-form group	Vegetative group
Escherichia coli	33 (66%)	29 (58%)
Escherichia coli + Citrobacter freundii	1 (2%)	0 (0%)
Aeromonas sp.	0 (0%)	1 (2%)
Serratia marcescens	0 (0%)	1 (2%)
Klebsiella/ Enterobacter species	4 (8%)	8 (16%)
Salmonella sp.	3 (6%)	3 (6%)
Acinetobacter sp.	0 (0%)	1 (2%)
Proteus/ Providence sp.	9 (18%)	7 (14%)
TOTAL	50 (100%)	50 (100%)

Table 15

Type of urinary tract infection : acute or chronic

a) L-form group of 50 patients. Urinary tract source of infection in 28 (56%).

	ACUTE INFECTION		CHRONIC INFECTION	
	Catheter-associated	Other	Catheter-associated	Other
Number of Patients	11 (22%)	10 (20%)	1 (2%)	6 (12%)
TOTAL : 28	21 (42%)		7 (14%)	

Table 16

Type of urinary tract infection : acute or chronic

b) Vegetative group of 50 patients. Urinary tract source of infection in 19 (38%).

	ACUTE INFECTION		CHRONIC INFECTION	
	Catheter-associated	Other	Catheter-associated	Other
Number of Patients	10 (20%)	6 (12%)	1 (2%)	2 (4%)
TOTAL : 19	16 (32%)		3 (6%)	

Table 17

Type of chronic infection : recurrent or relapsing

a) L-form group of 50 patients. Chronic urinary tract infection in 7 (14%).

	Recurrent infection	Relapsing infection
Number of Patients	2 (4%)	5 (10%)
Total :	7 (14%)	

Table 18

Type of chronic infection : recurrent or relapsing

b) Vegetative group of 50 patients. Chronic urinary infection in 3 (6%).

	Recurrent infection	Relapsing infection
Number of Patients	2 (4%)	1 (2%)
Total :	3 (6%)	

Table 19

Outcome of infection

Group	Recovered	Died	No record available	Total
L-form	26 (52%)	20 (40%)	4* (8%)	50 (100%)
Vegetative	31 (62%)	19 (38%)	0	50 (100%)

* patients transferred to another hospital

Chapter 13

The influence of therapeutic drugs on the selection of wall-defective microbial variants

THE INFLUENCE OF THERAPEUTIC DRUGS ON THE SELECTION OF WALL-DEFECTIVE MICROBIAL VARIANTS

INTRODUCTION

The importance of the folic acid/pABA/cytosine content of the culture medium for the growth of wall-defective Enterobacteriaceae variants suggested firstly, that the variants which had been isolated were nutritionally exacting, and secondly, that infection caused by such organisms might be encouraged by the therapeutic administration of folic acid or its analogue cotrimoxazole. There is evidence that nutritionally exacting bacteria may be selected *in vivo* by structural analogues of essential bacterial nutrients. Some common therapeutic drugs are vitamin analogues and may have this effect. Examples are the menadione analogue Warfarin and the effect of cotrimoxazole and the barbiturates as thiamine analogues. Furthermore, gentamicin may have the potential to select nutritionally exacting strains, because they are sometimes more resistant to gentamicin than normal bacteria (Acar, Goldstein & Lagrange, 1978).

This implies that the traditional stress on the importance of beta-lactam selection of wall-defective variants may be over-emphasised, and that the possibility of selection by vitamins, vitamin analogues and gentamicin is worth consideration.

OBJECTIVE

The object was to decide whether the administration of vitamins and their analogues predisposed to infection due to wall-defective Enterobacteriaceae variants, and to determine whether either beta-lactam antibiotics or gentamicin exerted a selective pressure towards variant growth.

MATERIALS & METHODS

1. Treatment Records

The treatment records of the 50 L-form group patients and the 50 vegetative group patients were examined retrospectively. The drugs administered in the 48 hours before the withdrawal of blood cultures were recorded. In the case of nicotine and alcohol a history of excessive intake immediately before hospital admission was noted. Drugs were divided into three groups:

i) Selective Agents. Gentamicin, beta-lactams.

ii) Vitamins and Vitamin Analogues. Vitamins: folic acid, vitamin B12, vitamin B complex preparations. Vitamin analogues: barbiturates (thiamine), trimethoprim (thiamine), sulphonamide (pABA), warfarin (menadione), nicotine (nicotinamide).

iii) Miscellaneous Drugs (including alcohol).

2. Antibiotic Sensitivity Tests.

The results of antibiotic sensitivity tests on the coliforms were noted. Antibiotic sensitivity tests were performed by the disc diffusion method of the Association of Clinical Pathologists, using the Oxford Staphylococcus (NCTC 6571) as the control organism (Stokes & Waterworth, 1972). Variant strains were tested after reversion to the vegetative state.

RESULTS

1. Treatment Records.

i) Selective Agents. The results are shown in Tables 20 and 21. The number of patients who received either an aminoglycoside or a beta-lactam before blood cultures were taken was small. Aminoglycoside administration was more common in the L-form group and beta-lactam

administration in the vegetative group. The sample numbers were too small for statistical analysis.

ii) Vitamins and Vitamin Analogues. The incidence of folic acid and cotrimoxazole administration was higher in the L-form group (Table 22). The difference was statistically significant. (Chi-square test results: test statistic, 11.4; degrees of freedom. 1: significance level, 0.0001).

iii) Miscellaneous Drugs. The commonest drugs were diuretics and digoxin. Their use was more frequent in the vegetative group. The other common drugs were hormone preparations and alcohol, and their incidence of use was similar in both groups. The number of patients who received barbiturates was the same in both groups.

Antibiotic Sensitivity Tests. (Table 23)

The L-form group isolates were more often resistant to sulphonamide than the vegetative isolates. This was statistically significant (Chapter 15).

There was no significant difference in the other antibiotic sensitivity test results. The sole point of note was that the only two gentamicin resistant strains were in the L-form group.

COMMENT

1. The proportion of the patients treated with the folic acid/ cotrimoxazole in the 48 hours preceding septicaemia was greater in the L-form group than in the vegetative group. This supported the conclusion made from culture medium tests (Chapter 10, Table 5) that folic acid was required.

2. A larger proportion of the reverted variant strains were resistant to sulphonamide than the bacteria cultured directly which formed the

vegetative group. This supported the idea that sulphonamide was the important element in cotrimoxazole.

Table 20

Putative selective agents, folic acid and folic acid analogues given in the 48 hours before blood cultures were taken in the 50 L-form group patients

Selective agents		Folic acid	Folic acid analogues	Total patients receiving drug or drug combination
Beta-lactams	Aminoglycosides		Cotrimoxazole	
-	-	-	+	9 (18.00%)
-	+	-	+	1 (2.00%)
-	+	-	-	3 (6.00%)
-	+	+	-	2 (4.00%)
-	-	+	-	5 (10.00%)
+	-	+	-	1 (2.00%)
-	-	-	-	29 (58.00%)
			Total	50 (100.00%)

Table 21

Putative selective agents, folic acid and folic acid analogues given in the 48 hours before blood cultures were taken in the 50 vegetative group patients

Selective agents		Folic acid	Folic acid analogues	Total patients receiving drug or drug combination
Beta-lactams	Aminoglycosides		Cotrimoxazole	
-	-	-	+	4 (8.00%)
-	+	-	-	3 (6.00%)
+	-	-	-	4 (8.00%)
-	-	-	-	39 (78.00%)
			Total	50 (100.00%)

Table 22

Folic acid and folic acid analogue administration in the 48 hours before blood cultures were taken. A comparison of the 50 patients in the L-form group with the 50 patients in the vegetative group.

Group	Folic acid or folic acid analogue given			Neither folic acid nor folic acid analogue given	Total
	Folic acid	Cotrimoxazole	Total		
L-form patients	8 (16.00%)	10 (20.00%)	18 (36.00%)	32 (64.00%)	50 (100%)
Vegetative patients	0 (0.00%)	4 (8.00%)	4 (8.00%)	46 (92.00%)	50 (100%)

TABLE 23

Antibiotic Resistance, Results of antibiotic sensitivity tests.

1. On bacilli reverted from variants cultured from the L-form group of patients.

2. On bacilli cultured directly from patients not yielding variants who formed the vegetative group of patients.

Strains from group (Number)	Result of sensitivity test Number and % of strains resistant to: Ampicillin	Gentamicin	Sulphonamide	Trimethoprim
L-form (30 strains)*	20 (66.66%)	2 (6.66%)	14 (46.67%)	6 (20.00%)
Vegetative (21 strains)*	18 (85.71%)	0 (0.00%)	3 (14.28%)	5 (23.81%)

* Note: Complete records available for 30/50 L-form group strains.

Complete records available for 21/50 vegetative group strains.

Chapter 14

Treatment of infection due to wall-defective microbial variants.

TREATMENT OF INFECTION DUE TO WALL-DEFECTIVE MICROBIAL VARIANTS

INTRODUCTION

There are a few accounts of the treatment of infection due to wall-defective variants. It has been suggested that protein synthesis inhibitors might be effective, and erythromycin has been used with apparent success, usually in conjunction with ampicillin (Gutman, Schaller & Wedgwood, 1967; Garrod, Lambert & O'Grady, 1981; May, 1975).

OBJECTIVE

The object was to analyse patient data for the L-form group and the vegetative group to see if there was any difference in the antibiotic treatment given.

MATERIALS & METHODS

The treatment records of the 50 L-form group patients and the 50 vegetative patients were examined retrospectively. The antibiotics used to treat each episode of septicaemia were recorded.

RESULTS

Compared with the patients in the vegetative group, fewer patients in the L-form group received an aminoglycoside alone (Table 26) whereas more were given a combination of antibiotics (Tables 24 and 25). Excluding the patients who did not receive any antibiotic treatment, this difference in the two groups was statistically significant (Chapter 15).

COMMENT

Infections in the L-form group were apparently less responsive to antibiotic treatment than in the vegetative group.

Table 24

Antibiotics used for treatment of septicaemia. L-form group

Antibiotic	Antibiotic/antibiotic combination used													No treatment
	Aminoglycoside/Aminoglycoside combination									Other antibiotic treatment				
Aminoglycoside	+	+	+	+	+	+	+	+	+	-	-	-	-	-
Metronidazole	-	+	+	-	-	-	+	-	+	-	-	+	-	-
Beta-lactam	-	-	+	+	+	-	-	-	-	+	+	+	-	-
Cephalosporin	-	-	-	-	-	-	-	-	-	-	+	-	-	-
Cotrimoxazole	-	-	-	-	+	+	+	-	-	-	-	-	+	-
Macrolide	-	-	-	-	-	-	-	+	+	-	-	-	-	-
Total patients	10	6	1	3	2	2	1	1	1	8	1	1	1	12

Total patients who received aminoglycoside alone:		10 (20%)
Total patients who received aminoglycoside in combination with another antibiotic:		17 (34%)
Total patients who received other antibiotic treatment:		11 (22%)
Total patients who received no treatment:		12 (24%)
	Total:	50(100%)

Table 25

Antibiotics used for treatment of septicaemia. Vegetative group

Antibiotic	Antibiotic/antibiotic combination used								No treatment
	Aminoglycoside/Aminoglycoside combination						Other antibiotic treatment		
Aminoglycoside	+	+	+	+	+	+	-	-	-
Metronidazole	-	+	+	-	-	-	-	-	-
Beta-lactam	-	-	+	+	-	-	+	-	-
Cephalosporin	-	-	-	-	-	-	-	-	-
Cotrimoxazole	-	-	-	-	+	-	-	+	-
Macrolide	-	-	-	-	-	+	-	-	-
Total patients	21	4	1	2	1	4	4	3	10

Total patients who received aminoglycoside alone:		21 (42%))
Total patients who received aminoglycoside in combination with another antibiotic:		12 (24%)
Total patients who received other antibiotic treatment:		7 (14%)
Total patients who received no treatment:		10 (20%)
	Total:	50(100%)

Table 26

Antibiotics used for the treatment of septicaemia. Use of aminoglycosides. L-form and vegetative groups of patients compared.

Antibiotic regime	Total Patients	
	L-form group	Vegetative group
Aminoglycoside alone	10 (20%)	21 (42%)
Aminoglycoside + another antibiotic	17 (34%)	12 (24%)
Other antibiotic treatment (no aminoglycoside)	11 (22%)	7 (14%)
No antibiotic treatment	12 (24%)	10 (20%)
Total patients	50(100%)	50(100%)

Chapter 15

Statistical calculations

STATISTICAL CALCULATIONS

INTRODUCTION

Statistical analysis was used to examine the following hypotheses:

a) That the predisposing factor for infection due to wall-defective microbial variants rather than vegetative infection was prior administration of folic acid or its analogues. The assumption here was that if the wall-defective Enterobacteriaceae variants were auxotrophs dependent on a folic acid metabolite, there should be a higher incidence of prior administration of folic acid and its analogues in the L-form group.

b) That the wall-defective Enterobacteriaceae variants were more often resistance to sulphonamide than vegetative Enterobacteriaceae. The assumption here was that if the wall-defective variants of Enterobacteriaceae were nutritionally dependent on the folic acid analogue sulphonamide, there should be a higher incidence of sulphonamide resistance in the L-form group. Such a finding would support the idea that the wall-defective Enterobacteriaceae variants isolated were auxotrophs.

c) That infections due to wall-defective Enterobacteriaceae variants were more resistant to treatment with aminoglycoside alone than vegetative infections. The assumption here was that if infections due to wall-defective Enterobacteriaceae variants were more difficult to treat with aminoglycoside, it was because the wall-defective variants

displayed a special type of resistance found in nutritionally exacting bacteria. This type of resistance cannot be detected by the disc diffusion test using strains reverted to the vegetative state as test organisms (Acar, Goldstein & Lagrange, 1978).

OBJECTIVE

The object was to apply statistical analysis to help to decide whether the wall-defective Enterobacteriaceae variants isolated were auxotrophs or not.

METHOD

A two by two contingency table was used to examine the data on the hypothesis. This was to ascertain whether the breakdown of frequencies between the L-form and vegetative groups could have occurred under the null hypothesis, that is, that there was no significant difference between the two groups. The formulae used were based on the chi-square test. The chi-square test is a method of comparing observed experimental results with theoretically expected results. The larger the chi-square, the greater the probability of a real divergence of experimentally observed from expected results. The null hypothesis is the hypothesis of equal probability (P). The null hypothesis may be discarded whenever P is 0.05 or less. Additional formulae were used as well as the simple chi-square formula, because of the limitations of the chi-square test.

Limitations of the Chi-Square Test

When table entries are fairly large, chi-square (X^2) gives an estimate of divergence from the hypothesis close to that obtained by

other measures of probability. The limitations are that:-

i) X^2 is not stable when computed from a table in which any experimental frequency is less than five (e.g. the tables applied to hypotheses (a) and (b)).

ii) When the table is 2 x 2 fold (degrees of freedom = 1) X^2 is subject to a considerable error unless a correction for continuity (Yates correction) is made. The error arises because the test curve is not continuous in 2 x 2 tables, especially when the entries are small. The correction consists in subtracting 0.5 from each (observed - expected) difference. Failure to use the correction causes the probability of a given result to be underestimated. Thus the chances of its being called significant are increased.

Formulae used

i) Simple Chi-Square Formula (Garrett, 1964 ; Gilmour, 1984).

$$X^2 = \frac{\text{the sum of (observed - expected)}^2}{\text{expected}}$$

ii) Chi-Square Formula incorporating a Correction for Continuity (Garrett, 1958).

An additional formula which incorporates a correction for continuity, but which is used without computing the four expected frequencies.

The corrected formula was:-

$$X^2 = \frac{N((AD - BC) - N/2)^2}{(A+B)\ (C+D)\ (A+C)\ (B+D)}$$

HYPOTHESIS (a)

The predisposing factor for infection due to wall-defective variants of Enterobacteriaceae rather than vegetative infection is prior administration of folic acid or its analogues (e.g. cotrimoxazole).

INDEX

The frequency of prior treatment with folic acid and/or cotrimoxazole was compared for the L-form group and the vegetative group.

NUMBERS: Total patients in the L-form group = 50

Total patients in the vegetative group = 50

i) Chi-square calculation

Observed 'o'

	L-form group	Vegetative group	Total
Folic acid or analogues given	18	4	22
Folic acid or analogues not given	32	46	78
	50	50	100

Expected 'e'

Total patients given folic acid or analogues (L+V)

= 22/100 = 22%

Therefore: expected folic acid/analogues in L group

$$= \frac{22}{100} \times 50 = 11$$

expected folic acid/analogues in V group

$$= \frac{22}{100} \times 50 = 11$$

expected no folic acid/analogues in L group

$$= 50 - 11 = 39$$

expected no folic acid/analogues in V group

$$= 50 - 11 = 39$$

Calculations: sum of $\frac{(o - e)^2}{e}$

$$\frac{(18 - 11)^2}{11} + \frac{(4 - 11)^2}{11} + \frac{(32 - 39)^2}{39} + \frac{(46 - 39)^2}{39}$$

$$= \frac{49}{11} + \frac{49}{11} + \frac{49}{39} + \frac{49}{39}$$

$$= 4.45 + 4.45 + 1.25 + 1.25$$

$$= 11.5$$

Test statistic = 11.5 Degrees of freedom = 1.

P = <0.0001.

Accordingly the groups examined are significantly different.

ii) The Chi-square formula incorporating a correction for continuity used without computing the four expected frequencies.

Observed	L-form group	Vegetative group	Total
Folic acid or its analogues given	A = 18	B = 4	A+B = 22
Folic acid or its analogues not given	C = 32	D = 46	C+D = 78
	A+C = 50	B+D = 50	N = 100

$$X^2 = \frac{N((AD - BC) - N/2)^2}{(A + B) (C + D) (A + C) (B + D)}$$

$$X^2 = \frac{100((828 - 128) - 50)^2}{22 \times 78 \times 50 \times 50}$$

$$X^2 = \frac{100 (700 - 50)^2}{4290000}$$

$$= \frac{69950^2}{4290000}$$

$$= 9.84$$

Test statistic: 9.84 Degrees of freedom = 1

$P = <0.01$.

Accordingly the groups examined are significantly different.

Conclusion: The prior administration of folic acid and/or its analogues is a predisposing factor for infections due to wall-defective variants of Enterobacteriaeceae.

HYPOTHESIS (b)

Wall-defective variants of Enterobacteriaceae are more often resistant to sulphonamide than vegetative Enterobacteriaceae.

INDEX

The number of sulphonamide resistant strains in the L-form group was compared with the number of sulphonamide resistant strains in the vegetative group.

NUMBERS: Total strains in the L-form group.

50 less 20 strains for which there were

inadequate records = 30.

Total strains in the vegetative group.

50 less 29 strains for which there were

inadequate records = 21.

i) Chi-Square Calculation

Observed 'o'	L-form group	Vegetative group	Total
Sulphonamide sensitive	16	18	34
Sulphonamide resistant	14	3	17
	30	21	51

Expected 'e'

Total strains sulphonamide resistant (L+V) = 17/51

= 33.33%

Therefore: expected number of sulphonamide resistant

strains in L group = $\frac{30 \times 33.33}{100} = 9.99$

expected number of sulphonamide sensitive

strains in L group = 30 - 9.99 = 20.01

expected number of sulphonamide resistant

strains in V group = $\frac{21 \times 33.33}{100} = 6.99$

expected number of sulphonamide sensitive

strains in V group = 21 - 6.99 = 14.01

Calculation: sum of $\frac{(o - e)^2}{e}$

$$\frac{(14 - 9.99^2}{9.99} + \frac{(3 - 6.99)^2}{6.99} + \frac{(16 - 20.01)^2}{20.01} + \frac{(18 - 14.01)^2}{14.01}$$

$$= \frac{16.08}{9.99} + \frac{15.92}{6.99} + \frac{16.08}{20.01} + \frac{15.92}{14.01}$$

$= 1.60 + 2.27 + 0.80 + 1.13$

$= \underline{5.80}$

Test statistic = 5.80 Degrees of freedom = 1

$P = < 0.05$

Accordingly the groups examined are significantly different.

ii) Chi-square formula incorporating a correction for continuity used without computing the four expected frequencies.

Observed	L-form group	Vegetative group	Total
Sulphonamide sensitive	A = 16	B = 18	A+B = 34
Sulphonamide resistant	C = 14	D = 3	C+D = 17
	A+C = 30	B+D = 21	N = 51

$$X^2 = \frac{N((AD- BC) - N/2)^2}{(A+B)\ (C+D)\ (A+C)(B+D)}$$

$$X^2 = \frac{51\ (\ (252 - 48) - 51/2)^2}{17 \times 34 \times 30 \times 21}$$

$$X^2 = \frac{51 \times (178.5)^2}{364140}$$

$$X^2 = 4.4625$$

Test statistic: 4.4625 Degrees of freedom = 1

$P = < 0.05$

Accordingly the groups examined are significantly different.

Conclusion: Wall-defective variants of Enterobacteriaceae are more often resistant to sulphonamide than vegetative Enterobacteriaceae.

HYPOTHESIS (c)

Infections due to wall-defective variants of Enterobacteriaceae are more resistant to treatment with aminoglycosides alone than vegetative infections.

INDEX

The frequency with which patients were treated with aminoglycosides alone, or with another antibiotic regime was compared.

NUMBERS: Total patients in the L-form group.

50 less 12 patients who received no treatment

= 38.

Total patients in the vegetative group.

50 less 10 patients who received no treatment

= 40.

Chi-square calculation

Observed 'o'	L-form group	Vegetative group	Total
Aminoglycoside only	10	21	31
Other Antibiotic treatment	28	19	47
	38	40	78

Expected 'e'

Total patients treated with aminoglycoside only (L+V)

= 31/78 = 39.7%

Therefore: expected aminoglycoside only in L group

$$= \frac{38 \times 39.7}{100} = 15$$

expected other treatment in L group

$= 38 - 15 = 23$

expected aminoglycoside only in V group

$$= \frac{40 \times 39.7}{100} = 15.88$$

expected other treatment in V group

$= 40 - 15.88 = 24.12$

Calculation: sum of $\frac{(o - e)^2}{e}$

$$\frac{(10 - 15)^2}{15} + \frac{(21 - 15.88)^2}{15.88} + \frac{(28 - 23)^2}{23} + \frac{(19 - 24.12)^2}{24.12}$$

$= 1.66 + 1.65 + 1.08 + 1.08$

$= 5.47$

Test statistics = 5.47 Degrees of freedom = 1.

P = <0.05

Accordingly the groups examined are significantly different.

Or, by the additional formula which incorporates a correction for continuity:-

$$X^2 = \frac{N((AD - BC) - N/2)^2}{(A+B)\ (C+D)\ (A+C)\ (B+D)}$$

Observed	L-form group	Vegetative group	Total
Aminoglycoside only	A = 10	B = 21	A+B = 31
Other antibiotic treatment	C = 28	D = 19	C+D = 47
	A+C = 38	B+D = 40	N = 78

$$X^2 = \frac{78((588 - 190) - 39)^2}{31 \times 47 \times 38 \times 40}$$

$$X^2 = \frac{78\ (398 - 39)^2}{31 \times 47 \times 38 \times 40}$$

$$X^2 = \frac{78\ (359)^2}{31 \times 47 \times 38 \times 40}$$

$X^2 = 4.5$

Test statistic: 4.5 Degrees of freedom = 1.

$P = < 0.05$

Accordingly the groups examined are significantly different.

Conclusion: Infections due to wall-defective variants of Enterobacteriaceae are more resistant to treatment with aminoglycoside alone than vegetative Enterobacteriaceae infections.

CONCLUSIONS

1. The prior administration of folic acid or its analogues is a predisposing factor for infection due to wall-defective variants of Enterobacteriaceae.
2. Wall-defective variants of Enterobacteriaceae are more often resistant to sulphonamide than vegetative Enterobacteriaceae.
3. The wall-defective variants of Enterobacteriaceae are more resistant to treatment with aminoglycosides alone than vegetative Enterobacteriaceae infections.
4. The wall-defective variants of Enterobacteriaceae may be auxotrophs which are selected by folic acid or its analogues.

SECTION VI

WALL-DEFECTIVE STREPTOCOCCAL VARIANTS

Chapter 16

Introduction

INTRODUCTION

There is now a considerable body of evidence to show that wall-defective streptococcal variants are biologically and clinically a most important group of organisms. The majority of these variants are auxotrophs, pyridoxine-dependent, nutritionally-variant satelliting organisms, often of diphtheroid morphology. Although they are wall-defective, they are not analogous to the L-phase variants of McGee et al., (1971) in that they do not replicate on agar as the distinctive L-form 'fried egg' colony. The clinical importance of these wall-defective streptococci fully justifies their inclusion in this Thesis.

They occur as oral commensals (Tomley & Russell, 1978) and may be pathogenic in bacterial endocarditis (Cayeux, Acar & Chabbert, 1971; Lancet, 1977; Roberts & Sidlak, 1979; Narasimhan & Weinstein, 1980; Feder et al., 1980) in otitis media (Frenkel & Hirsch, 1961) and puerperal sepsis (McCarthy & Bottone, 1974). Some are of veterinary interest (Higgins, Biberstein & Jang, 1984).

The nutritional requirements of such viridans streptococci are extremely variable (Bouvet & Acar, 1977). In some instances the required nutrients may be supplied simply by growth in symbiosis with other bacteria (McCarthy & Bottone, 1974; Bouvet & Acar, 1977; George & Healing, 1978). In other instances specific growth factors must be supplied. The growth factor required is usually Vitamin B6, as pyridoxine, pyridoxal or pyridoxamine (George, 1974; Carey, Brause & Roberts, 1977; Carey, 1978; Cooksey, Thompson & Facklam, 1979; Peterson, Cook & Burke, 1981; Tillotson, 1981).

The pyridoxine requirement may be dispensed with under certain conditions. Bouvet, Van de Rijn and McCarty (1981) found that pyridoxine was required in a nutritionally complex medium but not in a semi-synthetic medium. Sometimes sulphydryl compounds such as cysteine or thioglycollate are needed (Frenkel & Hirsch, 1961; Cayeux, Acar & Chabbert, 1971; McCarthy & Bottone, 1974; Bouvet & Acar, 1977; George & Healing, 1978) though cysteine alone may be insufficient for isolation (Washington, Hall & Warren, 1975). The connection between Vitamin B6 and sulphydryl compounds is that pyridoxal acts as a co-enzyme in the synthesis of both the amino acid cysteine and the cell wall component D-alanine (Bouvet & Acar, 1977).

The nutritional requirement of streptococcal auxotrophs is not confined to sulphydryl/pyridoxal. Some of the strains described need yeast extract or a substance supplied by fresh blood (Bouvet & Acar, 1977; Tillotson, 1981).

Opinion on the use of nutritionally supplemented primary blood culture medium is divided. Though George (1974) found that pyridoxine had no adverse effect on growth, others emphasise that pyridoxine (Sherman & Washington, 1978) or an excess concentration of pyridoxal or cysteine (Carey, 1978) may be inhibitory, and do not recommend the use of supplemented primary media for this reason. It is held that the problem is not one of primary isolation from blood cultures, since the pyridoxal content of human blood is sufficient to support growth on solid media (Tillotson, 1981).

The problem of isolating wall-defective streptococcal variants in septicaemia is therefore threefold. Firstly, it concerns their recognition in the primary blood culture medium, and here the important procedure is the examination of a Gram stained film from the primary

blood culture. The second difficulty is to obtain growth on subculture. The third and crucial point is to decide whether the primary blood culture medium should be nutritionally supplemented.

Chapter 17

Isolation of Wall-defective Streptococcal Variants from blood cultures by the Satellitism Method

ISOLATION OF WALL-DEFECTIVE STREPTOCOCCAL VARIANTS FROM BLOOD CULTURES BY THE SATELLITISM METHOD

INTRODUCTION

Bouvet and Acar (1977) isolated wall-defective streptococcal variants from blood cultures in bacterial endocarditis patients, using streptococcal satellitism round a staphylococcus to obtain growth on plate culture. Primary culture media were either plain broth or a broth supplemented with growth factors or with saccharose.

OBJECTIVE

The object was to evaluate the satellitism method for the isolation of wall-defective streptococcal variants.

MATERIALS & METHODS

Selection of Material. Blood cultures from patients with suspected bacterial endocarditis were chosen for examination. They were selected on the basis of the clinical history supplied on the laboratory request form. A clinical diagnosis of bacterial endocarditis or history of rheumatic or congenital heart disease was sufficient for inclusion.

Blood Culture Media and Incubation Conditions. The primary blood culture medium was a single bottle of brain heart infusion broth (Oxoid) inoculated at the bedside as part of a three bottle culture set. Incubation was continued for 7 days at 37°C in an atmosphere of 5% carbon dioxide in air. A loopful was then subcultured to each of two 5% horse blood agar plates. A streak of the 'Oxford' staphylococcus (NCTC 6571) was plated over the well of the inoculum. One plate was

incubated in 5% carbon dioxide in air, the other anaerobically using the BBL 'Gaspak' system (65% hydrogen with 5% Carbon dioxide). The plates were examined after 24 hours incubation.

RESULTS

122 blood cultures from 91 patients were examined over a six month period. No wall-defective streptococcal variants were found.

COMMENT

i) Culture Media. It is possible to grow wall-defective streptococcal variants from blood cultures using simple primary media. Sometimes nutritionally supplemented media are only needed for subculture growth (McCarthy & Bottone, 1974; Bouvet & Acar, 1977; Carey, Brause & Roberts, 1977). However, it is not always clear whether the media employed for primary isolation were of the dehydrated commercial type I used, or media prepared from fresh ingredients. Freshly prepared media are held to be of a superior nutritional quality for the isolation of wall-defective streptococcal variants (Roberts et al., 1979) and this might have been a factor in the successful results obtained by others.

ii) Incubation Time. An extended primary incubation time may required for the bacteriological diagnosis of bacterial endocarditis. The time recommended is 21 days (Stokes, 1974). The incubation time I used was only seven days, and this may have been the reason for failure.

CONCLUSION

Failure to isolate wall-defective streptococcal variants was tentatively ascribed to poor nutritional quality of the primary culture medium and to an inadequate incubation time.

Chapter 18

Isolation of Wall-defective streptococcal variants from blood cultures by the use of freshly prepared Culture Medium

ISOLATION OF WALL-DEFECTIVE STREPTOCOCCAL VARIANTS FROM BLOOD CULTURES BY THE USE OF FRESHLY PREPARED CULTURE MEDIUM.

OBJECTIVE

The object was to evalute the use of a medium made with freshly prepared ingredients for the isolation of wall-defective streptococcal variants, and to compare its performance with media made with dehydrated reconstituted ingredients. The intention was also to evaluate an increased primary incubation time.

MATERIALS & METHODS

Selection of Material: All blood cultures examined over a six month period.

Inoculation of Primary Culture Media:

Media were inoculated at the bedside.

Blood Culture Media:

i) Primary Culture Media. A three bottle blood culture set was used for primary culture.

Bottle 1: Oxoid Brain Heart Infusion Broth (BHI)

Bottle 2: Cooked Meat Medium (Oxoid) reconstituted with Oxoid Brain Heart Infusion Broth. This was Cooked Meat Medium (CMM).

The media used for Bottle 1 and Bottle 2 were dehydrated reconstituted media. They were compared with the medium used for Bottle 3.

Bottle 3: Cooked Meat Medium prepared from fresh meat and Oxoid BHI (Collee, Duerden & Brown, 1977). This was Heart Fresh Medium (HFM).

ii) Subculture media. Routine subculture medium: 5% horse blood agar.

Subculture medium for wall-defective streptococcal variants: 5% horse blood agar supplemented with pyridoxine hydrochloride (BDH) 1mg/litre and 1 gram/litre.

Conditions of Incubation: Primary cultures were incubated at a temperature of 37°C in an atmosphere of 5% carbon dioxide in air. One subculture plate was incubated in an atmosphere of 5% carbon dioxide in air, and a second plate anaerobically using the BBL 'Gaspak' system. The incubation temperature was again 37°C.

Subculture Intervals. Subcultures were made after 24 hours, 48 hours, 7, 14 and 21 days incubation, from all three bottles.

Microscopy of Primary Cultures. Gram stained films were prepared at each subculture from each bottle.

Procedure for Subcultures. Subcultures were made routinely to 5% blood agar plates. If the Gram stained film from a primary culture showed chaining gram positive cocci which failed to grow on subculture, or slender diphtheroid-like streptococci, subculture to pyridoxine supplemented blood agar media was made.

RESULTS

383 culture sets were examined. A wall-defective streptococcal variant was isolated from three culture sets from one patient (S.McG), an isolation rate of 0.78%. Growth was obtained after 48 hours incubation, in HFM only. Characteristics:

i) Gram stained films made from the primary culture showed pleomorphic Gram positive bacteria. These had a diphtheroid appearance.

ii) Blood agar subcultures from the primary culture were sterile.

iii) Growth on blood agar was obtained in satellitism with the Oxford staphylococcus.

iv) Pure growth was obtained on pyridoxine supplemented blood agar. The organism required pyridoxine 1 g/litre for growth.

Clinical Features: The patient was a 42 year old woman (S.McG) with aortic valve disease following rheumatic fever at the age of 6 years. She gave a history of dental treatment about eight months before admission. The clinical diagnosis was bacterial endocarditis.

CONCLUSION

The nutritional quality of the primary culture medium was important for the isolation of the wall-defective streptococcal variant strain described.

Chapter 19

Isolation of wall-defective streptococcal variants from blood cultures by the use of a biphasic medium.

ISOLATION OF WALL-DEFECTIVE STREPTOCOCCAL VARIANTS FROM BLOOD CULTURES BY THE USE OF A BIPHASIC MEDIUM

INTRODUCTION

Biphasic medium is recommended for the isolation of wall-defective variants (Gutman et. al., 1965; Hryniewicz, 1977). It also permits prolonged culture with less risk of laboratory contamination than a medium which requires repeated subculture for growth detection.

OBJECTIVE

The object was to evaluate a biphasic medium for the isolation of wall-defective streptococcal variants.

MATERIALS & METHODS

Selection of Material: All blood cultures received over a six month period. Inoculation of Primary Culture Media:
Media were inoculated at the bedside. Blood Culture Media:
i) Primary Culture Media. Bottle 1. BHI. Bottle 2. CMM
Bottle 3. Biphasic BHI broth with agar (Gibco Biocult). Subculture media, conditions of incubation, subculture intervals and procedure were as described in Chapter 18 with the exception of the biphasic medium, which was examined by inspection only. Subcultures from the biphasic medium were not made unless growth was evident on inspection.

RESULTS

374 blood culture sets were examined during a period of six months. A wall-defective streptococcal variant was isolated from two

sets from one patient (M.H.) an isolation rate of 0.53%. Growth was obtained after 24 hours incubation in CMM only, but in each case the organism grew in symbiosis with a contaminant, Acinetobacter lwoffi in one, and Staphylococcus albus in the other. In each case the contaminant was derived from the primary CMM blood culture. The characteristics of the organism were the same as those of the wall-defective streptococcal variant described in Chapter 18 except that this strain required pyridoxine 1 mg/litre for growth.

Clinical features: The patient was a 42 year old man (M.H.) who had had a patent ductus arteriosus ligated at the age of 25. He had had two previous attacks of bacterial endocarditis, and the clinical diagnosis for his current illness was bacterial endocarditis.

CONCLUSION

The result obtained again emphasised the importance of the nutritional quality of the primary subculture medium. The biphasic medium afforded no advantage for the isolation of the wall-defective streptococcal variants.

Chapter 20

Isolation of wall-defective streptococcal variants from blood cultures by the use of nutritionally supplemented osmotically stabilised media.

ISOLATION OF WALL-DEFECTIVE STREPTOCOCCAL VARIANTS FROM BLOOD CULTURES BY THE USE OF NUTRITIONALLY SUPPLEMENTED OSMOTICALLY STABILISED MEDIA.

INTRODUCTION

Wall-defective streptococcal variants required a highly nutritious medium for primary isolation, as well as nutritionally supplemented subculture medium. Nutritional conditions required in primary culture were satisfied by a medium prepared with fresh ingredients, or by the fortuitous presence of a contaminant with which such variants could live in symbiosis. Neither of the media in which primary isolation was obtained were supplemented by osmotic or ionic stabilisation to enhance the isolation of wall damaged bacteria. Magnesium is required for the biosynthesis of cell wall peptide subunits (Lamanna, Mallette & Zimmerman, 1973a) and has a stabilising effect on the cytoplasmic membrane (McQuillen, 1960; Eisenberg & Corner, 1978) which helps to prevent osmotic stress (Marquis & Corner, 1976). In addition, the wall-defective variants of gram positive bacteria may be more susceptible to osmotic stress than those of gram negative bacteria (Maxted 1972). This suggested that ionic and osmotic stabilisation of the primary culture medium might improve the recovery of wall-defective streptococcal variants.

OBJECTIVE

The object was to compare the use of a nutritionally supplemented primary blood culture medium with a nutritionally supplemented stabilised primary blood culture medium for the isolation of wall-defective streptococcal variants.

MATERIALS & METHODS

Principles

i) Nutritional supplements. The nutritional supplements used were pyridoxine and cysteine. Wall-defective streptococcal variants are characteristically dependent on pyridoxine, though some strains require pyridoxal or pyridoxamine. Cysteine permits the growth of these strains. The use of a combination of pyridoxal and cysteine can be inhibitory and is not recommended (Carey, 1978).

ii) Osmotic stabilisation. The optimal osmolality for wall-defective streptococcal variants is 1200 milliosmoles/litre (Mortimer et al., 1972). The stabiliser chosen to achieve this osmolality was a mixture of sucrose and sodium chloride (Mortimer et al., 1972., Hryniewicz, 1977). The required osmolality can be reached by the use of 30 per cent sucrose, but preliminary tests with S. pneumoniae (NCTC 7465) and three naturally occurring strains of viridans streptococci showed that this concentration was inhibitory.

iii) The use of Agar. The yield of wall-defective streptococcal variants is improved by the use of agar. Growth is difficult to establish in liquid media (Freimer, Krause & McCarty, 1959; Gooder, 1964; Hryniewicz, 1977).

iv) Stabilisation with metallic ions. Magnesium or calcium ions stabilise wall-defective variants on primary isolation (Nimmo & Blazevic, 1969). Stabilisation with magnesium sulphate was used for the nutritionally supplemented stabilised test medium.

Culture Media. Primary Culture Media. Bottle 1. Brain Heart Infusion broth (BHI, Oxoid) supplemented with pyridoxine 1mg/litre and

cysteine 1G/litre. This was Brain Heart Infusion with pyridoxine and cysteine (BHI/PC).

Bottle 2. Cooked Meat Medium. (CMM, Oxoid).

Bottle 3. BHI/PC supplemented with sucrose 200 g/litre and sodium chloride 18 g/litre as osmotic stabiliser, and also with magnesium sulphate and agar. This was stabilised BHI/PC medium. The composition of this medium is shown in Appendix 1.

Subculture Media. Bottle 1. Subcultures were made to aerobic and anaerobic blood agar supplemented with pyridoxine 1mg/litre and cysteine 1 g/litre (BACP medium).

Bottle 2. Subcultures were made to aerobic and anaerobic blood agar.

Bottle 3. Subcultures were made to aerobic and anaerobic BACP medium, and to a pour plate of the same base composition as the primary Bottle 3 medium. The pour plate had an agar content of 0.2% Oxoid agar No. 1.

Conditions of Incubation. These were as described previously.

Subculture Intervals. Bottle 1 and 2. Subcultures were made after 24 hours, 48 hours, 7, 14 and 21 days.

Bottle 3. Subcultures were made after 7, 14 and 21 days incubation only.

Microscopy of Primary Cultures. Gram stained films were made from Bottles 1 and 2 only, after 24 hours and 48 hours incubation.

RESULTS

186 blood culture sets were examined. A wall-defective streptococcal variant was isolated from one blood culture set, an isolation rate of 0.53%. The initial isolate grew only in Bottle 1 and Bottle 3, the nutritionally supplemented media. It required pyridoxine

1mg/litre for growth and appeared after 48 hours incubation. The cultural characteristics of the organism were the same as those of the wall-defective streptococcal variants described in Chapters 17 and 18. Microscopy of a Gram stained film from both the primary pyridoxine supplemented Bottle 1 blood culture medium (BHI/PC) and from the pyridoxine supplemented culture media showed normal chaining streptococci.

Clinical Features. The patient was a woman aged 78 years (G.S.) in whom a clinical diagnosis of bacterial endocarditis had been made.

CONCLUSIONS

1. Wall-defective streptococcal variants require nutritionally enriched media for primary isolation and further subculture.

2. Such variants do not necessarily require ionic or osmotic stabilisation for primary isolation and further subculture.

Chapter 21

Antibiotic treatment of bacterial endocarditis caused by wall-defective streptococcal variants.

ANTIBIOTIC TREATMENT OF BACTERIAL ENDOCARDITIS CAUSED BY WALL-DEFECTIVE STREPTOCOCCAL VARIANTS.

INTRODUCTION

Wall-defective streptococcal variants are said to be more resistant to penicillin than ordinary vegetative streptococci (Cooksey & Swenson, 1979; Parks, Shockman & Higgins, 1980). Penicillin resistance is detectable only in nutritionally supplemented media *in vitro* or clinically manifested by treatment failure (Bouvet & Acar, 1977; Carey, Brause & Roberts, 1977). However, penicillin resistance is not always a feature *in vitro* even on supplemented media (George, 1974; McCarthy & Bottone, 1974).

The question of penicillin resistance is an important point, because penicillin is so often the initial treatment in bacterial endocarditis. The status of penicillin in the induction or selection of wall-defective streptococcal variants in bacterial endocarditis is also uncertain. Endocarditis due to wall-defective streptococcal variants appears to occur as a primary infection, but it is possible that penicillin treatment might either induce wall-defective streptococcal variants, or select penicillin resistant wall-defective variants within a mixed bacterial population.

OBJECTIVE

The objectives were:-

i) to determine whether the administration of penicillin might have initiated or selected infection due to wall-defective variants.

ii) to record the results of penicillin sensitivity tests on the wall-defective streptococcal strains isolated from bacterial

endocarditis and to decide whether penicillin resistance was an important feature.

iii) to assess the results of antibiotic treatment of the infection.

MATERIALS & METHODS

i) The records of the three bacterial endocarditis patients were examined retrospectively for evidence of penicillin administration before blood cultures were taken. The treatment records were scrutinised and the antibiotics used for treatment were noted. The result of the treatment was also noted, and in addition the three patients were followed up after six months and after two years.

ii) Antibiotic sensitivity tests were performed by the disc diffusion method of the Association of Clinical Pathologists, using the Oxford Staphylococcus (NCTC 6571) as the control organism (Stokes & Waterworth, 1972). The medium used was five per cent horse blood agar, supplemented with pyridoxine 1mg/litre. The broth medium used for the minimum bactericidal penicillin concentration test was BHI (Oxoid) supplemented with pyridoxine 1mg/litre. Pyridoxine supplemented media are acceptable for antibacterial sensitivity testing (George, 1974).

RESULTS

The results of antibiotic sensitivity tests are shown in Table 27. None of the patients received penicillin or any other antibacterial before blood cultures were taken.

All three patients made a good clinical recovery initially, and were well six months later.

After two years, patient 2 (M.H.) was in good health. Patient 1 (S.McG.) had died after cardiac surgery. Patient 3 (G.S.) could not be traced.

TABLE 27

Antibiotic sensitivity test results and treatment in bacterial endocarditis due to infection with wall-defective streptococcal variants.

Patient	Antibiotic sensitivity test result			Minimum bactericidal concentration of Penicillin (μg/ml)	Treatment
	Penicillin	Ampicillin	Erythromycin		
1. S.McG.	+	+	+	Not done	Penicillin
2. M.E.	+	+	+	0.015	Penicillin
3. G.S.	+/-	+/-	+	Not done	Erythromycin

Key:-

+ = Sensitive +/- = Moderately resistant - = Resistant

CONCLUSIONS TO SECTION VI

1. The wall-defective streptococcal variants studied needed a nutritionally supplemented medium for both primary blood culture isolation and for subculture. The pyridoxine content of the blood culture samples alone was insufficient to support primary growth of the wall-defective variants, presumably because of its dilution in the primary culture medium.

2. The isolation of wall-defective streptococcal variants was not enhanced by ionic or osmotic stabilisation.

3. The clinical association between wall-defective streptococcal variants and bacterial endocarditis was confirmed.

4. The reverted wall-defective variants of viridans streptococci vary in their susceptibility to penicillin. Some strains are demonstrably sensitive to penicillin when tested on pyridoxine supplemented media. Infections due to these strains can be successfully treated with peniciliin. This indicates that the organisms are walled in vivo.

SECTION VII

ISOLATION OF CONTAMINANTS

Chapter 22

Nature and Significance of Contaminant Wall-Defective Microbial Variants

NATURE AND SIGNIFICANCE OF CONTAMINANT WALL-DEFECTIVE MICROBIAL VARIANTS

INTRODUCTION

The reason for the disrepute which surrounds the subject of wall-defective bacteria in medical microbiology does not arise entirely from the difficulties experienced in their culture and identification. It also comes from the emphasis so often placed on the importance of commensal bacteria isolated in L-form culture medium, particularly as it is seldom clear whether these normally harmless bacteria were really significant in the disease process.

OBJECTIVE

The object was to assess the incidence of wall-defective commensal bacteria isolated as blood culture contaminants in the L-form culture media described in Section IV.

MATERIALS & METHODS

Detailed records were available for a series of 569 culture sets using Victoria Medium F as the L-form culture medium, and for a series of 310 culture sets using the partially defined culture medium supplemented with folic acid, pABA and cytosine described in Chapter 10.

Selection of Material: All isolates were deemed to be contaminants on clinical grounds. They fulfilled the following criteria:

i) Primary isolation in L-form culture medium only.

ii) Subculture growth only in the L-form pour plate, without growth on the blood agar plates subcultured in parallel. (i.e. the investigation was confined to wall-defective bacteria of the transitional phase variant and L-phase variant types).

iii) A dense and uncountable or confluent growth of colonies in the pour plate.

Analysis of Material: The following points were noted:-

i) presence or absence of surface growth on the pour plate.

ii) type of colonies - vegetative, L-form or a mixture of both.

iii) whether a reversion procedure was required or not

iv) Species identification of the isolate.

RESULTS

The isolation rate for contaminant variants was comparable in both Victoria Medium F and in the semi-defined supplemented medium (Table 28).

Most of the strains isolated proved to be coagulase negative staphylococci. They grew as vegetative colonies on primary isolation and were subcultured from the pour plate medium to blood agar without difficulty.

The remaining organisms proved to be either diphtheroids or anaerobic streptococci. They grew as L-form colonies on primary isolation, and did not grow on the surface of the pour plate, or required a reversion procedure, or both (Tables 29 and 30).

COMMENT

It is easy to disparage the work described by some authors, and to think that they deceived themselves by calling a contaminant colony an 'L-form'. I believe that these accounts may have correctly described isolates as wall-defective variants but that it was mistaken to assume that the organisms were necessarily pathogenic. The diphtheroid variants found in arthritis and cancer patients (Bisset, Tallack & Bartlett, 1979) and in autoimmune disease (Tedeschi & Santarelli, 1977) were also found in healthy subjects (Bisset & Bartlett, 1978; Domingue et al., 1977; Domingue & Schlegel, 1977). Commensal skin bacteria can be nutritionally exacting (Cove, Holland & Cunliffe, 1980) and commensal oral streptococci are sometimes wall-defective (Tomley & Russell, 1978). This supports the idea that some normal commensals may occur as wall-defective variants under conditions of relative nutrient deprivation.

CONCLUSION

Nutritionally exacting wall-defective variants can be isolated from commensal bacteria.

TABLE 28

Isolation rate of contaminant wall-defective microbial variants

Culture Medium	Wall-defective variants isolated during periods analysed		Total cultures examined for for wall-defective variants
	significant isolates	contaminant isolates	
Victoria Medium F	10 (1.75%)	14 (2.46%)	569
Semi-defined supplemented Medium	4 (0.82%)	13 (2.69%)	483

TABLE 29

Nature of contaminant wall-defective microbial variants isolated in Victoria Medium F

Species	Number isolated	Colonial type			Surface growth
		Vegetative	Vegetative + L-form	L-form	
Coagulase negative staphylococcus	9 (1.58%)	9	0	0	9/9
Diphtheroid	2 (0.35%)	1*	0	1	0/2
Anaerobic streptococcus	3 (0.52%)	1*	0	2*	0/3
Total	14 (2.46%)	11	0	3	9/14

Key:

* = reversion procedure required: the other bacteria isolated reverted spontaneously.

TABLE 30

Nature of contaminant wall-defective microbial variants isolated in semi-defined supplemented medium

Species	Number isolated	Colonial type			Surface growth
		Vegetative	Vegetative+L-form	L-form	
Coagulase negative staphylococcus	12 (2.24%)	7	5	0	12/12
Diphtheroid	1 (0.20%)	0	0	1	0/1
Total	13 (2.68%)	7	5	1	12/13

N.B.

None of the bacteria isolated required a reversion procedure.

SECTION VIII

DISCUSSION

Chapter 23

Reappraisal of Klieneberger's Symbiosis Theory.

Nutritional Factors in the Culture of Wall-Defective Microbial Variants

REAPPRAISAL OF KLIENEBERGER'S SYMBIOSIS THEORY

Some bacteria have a nutritional requirement for a particular substance in addition to the minimal nutrient requirement of normal bacteria of the same species. They may grow in symbiosis as satellite colonies round other bacteria which diffuse the required nutrient. Alternatively, they may be cultured on appropriately supplemented media, and some can be stabilised as protoplasts in an osmotically balanced medium (Lugtenberg, de Haas-Menger & Ruyters, 1972; Lamanna, Mallette & Zimmerman, 1937e). They cannot grow on ordinary media, or grow with difficulty and produce bizarre or dwarf colonies (Acar, Goldstein & Lagrange, 1978; Sparham, Lobban & Speller, 1978).

It is of historical interest that Klieneberger (1935) regarded her L-organisms as symbionts growing in mixed culture. Dienes decided that they were colonial variants within a pure culture (Dienes, 1938; 1939a; 1939b). It did not occur to him that the two theories were reconcilable and that they might be nutritionally exacting variants growing in symbiosis within a pure culture.

Klieneberger was criticised because the nutritional implications of symbiosis were not understood at the time. Vitamin disorders were a new idea in the thirties, even in clinical medicine (Low, 1930) and this aspect of bacterial nutrition was not really explored until much later (Pontecorvo, 1949; Gunsalus, 1984). Bacteriologists did not realise that a symbiont and a variant might be the same, and so L-organisms were classified as variants which were part of a bacterial life-cycle. This was the logical alternative to Klieneberger's 'symbiont' theory at the time (Klieneberger, 1936).

The bacterial life cycle hypothesis postulated genetic transfer by bacillus-coccus transformation, protoplasmic coalescence, and large bodies. Many of its manifestations were due to pleomorphism, and wall-defective microbial variants share many of the features of pleomorphic bacteria (Dienes & Weinberger, 1951). Pleomorphism is due to defective wall synthesis (Hughes, 1956; Collee, 1978). It is initiated by a number of precipitating factors which affect the integrity of the cell wall. Among these is bacterial malnutrition (Duguid & Wilkinson, 1961). This type of pleomorphism is found not only in S. moniliformis but also in Haemophilus species (Wilson & Miles, 1975g) and in nutritionally exacting viridans streptococci. In the latter cell wall damage gives a 'diphtheroid' appearance (Cayeux, Acar & Chabbert, 1971; George, 1974; Tillotson, 1981) of the bacillus-coccus type once described as a life-cycle manifestation (Mellon, 1927; Lamanna, 1944).

The Effect of Nutritional Deficiency on Cell Wall Biosynthesis

The wall-defective variants under study appeared to require specific vitamins for survival. In particular the Enterobacteriaceae variants seemed to require folate metabolites and the streptococcal variants pyridoxine and cysteine.

Nutritionally exacting viridans streptococci typically require pyridoxal, pyridoxamine or pyridoxine (George, 1974; Washington, Hall & Warren, 1975; Carey, Brause & Roberts, 1977; Carey, 1978; Cooksey, Thompson & Facklam, 1979; Peterson, Cook & Burke, 1981; Tillotson, 1981; Bouvet,Van de Rijn & McCarty, 1981) cysteine or thioglycollate (Frenkel & Hirsch, 1961; Cayeux, Acar & Chabbert, 1971; McCarthy & Bottone, 1974; Bouvet & Acar, 1977; George & Healing, 1978). In some

instances the required nutrients may be supplied by fresh blood or yeast extract, or by growth in symbiosis with other bacteria (McCarthy & Bottone, 1974; Bouvet & Acar, 1977; George & Healing, 1978).

The characteristic 'diphtheriod' morphology found on microscopy (Babes & Manolescu, 1909; Lamanna, 1944; Emmerson & Eykyn, 1977; Ahmad & Darrell, 1980) is due to aberrant growth of new wall. Cell wall growth is initiated equatorially in streptococci. The 'old' ends of the cocci are preserved intact, and are gradually pushed apart by new wall (Reynolds, 1973). If new wall growth is not possible, an elongated 'diphtheroid' cell is the result.

Electron microscopy shows that such cells are wall-defective (Bouvet, Ryter & Acar, 1977; Piepkorn & Reichenbach, 1978; Bouvet et al., 1980). This cell wall defect is possibly connected with the nutrient requirement of 'diphtheroid' streptococci for pyridoxine and related compounds, and for cysteine.

The bacterial cell wall is composed of peptidoglycan. Glycan strands of alternating N-acetylglucosamine and N-acetylmuramic acid are cross-linked by peptide bridges. Each bridge is incorporated into the cell wall from precursor Park-nucleotide which always terminates in the sequence D-alanyl-D-alanine. The terminal D-alanine residue of this dipeptide sequence is eliminated when the peptide chain is attached to the adjacent glycan strand (in Gram-negative bacilli) or to the pentaglycine bridge (in Gram-positive organisms). The peptide bridges are therefore very similar in all bacteria but may differ slightly in amino-acid composition from genus to genus or species to species (see review by Lamanna, Mallette & Zimmerman, 1973a).

The B group vitamins are important in cell wall biosynthesis because their co-enzyme forms participate in bacterial metabolism

(Mandelstam & McQuillen, 1973b). The pyridoxine co-enzyme pyridoxal phosphate acts as a transaminase in the synthesis of amino acids, including alanine (Lamanna, Mallette & Zimmerman, 1973f) and acts as an isomerase in the conversion of L-alanine to D-alanine (Mandelstam & McQuillen, 1973c).

The amino acid cysteine, like the folic acid co-enzyme tetrahydrofolate (THF), is used in methionine synthesis (Lamanna, Mallette & Zimmerman, 1973f; Mandelstam & McQuillen, 1973b). Methionine as a formyl-methionine complex (fMet) is of crucial importance in the synthesis of cell wall precursors and therefore, indirectly in the synthesis of the cell wall itself (Lamanna, Mallette & Zimmerman, 1973h). The role of THF in methionine synthesis is of particular interest since the work described in this thesis suggests that lack of folic acid and related compounds may precipitate cell wall deficiency in some Enterobacteriaceae strains. Auxotrophs of E.coli which require such substances as pABA, methionine, thymine or cysteine have been described (Bauman & Davis, 1957). This raises the possibility that some wall-defective variants of Enterobacteriaceae may be auxotrophs.

The substance required to ameliorate cell wall deficiency is not always a member of the B group of vitamins. Thus Bacteroides melaninogenicus (Fusiformis nigrescens) may require Vitamin K to prevent cell wall deficiency (Lev, 1968; Lamanna, Mallette & Zimmerman, 1973d). Sometimes a substance directly concerned in cell wall synthesis is required. For example, spheroplasts of Haemophilus influenzae may be isolated from sputum in bronchial sepsis by the use of a medium supplemented with the cell wall glycan chain component N-acetyl-glucosamine (Roberts et al., 1984). Wall deficient bacteria

thus seem to be heterogeneous in their requirements. This adds to the problem of devising a consistently successful culture medium.

The Effect of Magnesium on Cell Wall Biosynthesis

The idea that wall-defective microbial variants need nutrients ultimately concerned in wall synthesis makes it easier to understand the role of magnesium. Magnesium salts are a traditional ingredient of media used for the culture of wall-defective microbial variants, and are employed for their 'stabilising' effect (Nimmo & Blazevic, 1969; Brogan, 1976). Magnesium is an enzyme co-factor. It is utilised extensively in the biosynthesis of cell wall peptide (Lamanna, Mallette & Zimmerman, 1973a) and in the biosynthesis of the peptide bridge cross-linkages of the cell wall (Lamanna, Mallette & Zimmerman, 1973h).

Chapter 24

Physical Factors in the Culture of Wall-defective Microbial Variants

PHYSICAL FACTORS IN THE CULTURE OF WALL-DEFECTIVE MICROBIAL VARIANTS

The Osmotic Requirements of Wall-defective Microbial Variants

The degree of osmotic stabilisation required by beta-lactam induced wall-defective bacteria appears to be extremely variable. Greenwood and O'Grady (1972) found that an osmolality of 400 - 500 m.osm./kg. gave substantial protection from beta-lactam induced lysis in 'spheroplasts' of E.coli and Proteus mirabilis under laboratory conditions, whereas the 'unstable protoplasts' of Streptococcus faecalis studied by Montgomerie, Kalmanson & Guze (1967) required an osmolality of 1200 m.osm./kg for survival in the presence of penicillin. The osmolality of human blood is about 300 m.osm./kg., a level possibly insufficient for the osmotic stabilisation of at least some bacteria in which wall damage is beta-lactam induced. Lysis consequent to such injury probably occurs at the height of the exponential phase of growth when the internal osmolality of the bacterial cell may reach values as high as 1333 m.osm./kg.(Marquis & Corner 1976). This may explain the infrequent isolation of beta-lactam induced variants from the blood cultures examined.

Naturally occurring wall-defective variants of the nutritionally exacting type presumably exist in the walled state in optimal nutrient conditions, but cannot synthesise peptidoglycan adequately under conditions of relative nutrient deprivation. In such circumstances, cell components such as nucleic acids and proteins continue to be made for some time (Mandelstam & McQuillen, 1973a) and osmotic stabilisation may be needed because of the weakened condition of the cell envelope. Such variants may therefore require osmotic stabilisation on transfer to a nutritionally sub-optimal milieu. However, it is possible that the

importance of osmotic stabilisation of the primary culture medium stressed by Brogan (1976) may have been over-emphasised. Certainly, Weinstein et al. (1982) found appreciable evidence that the effect of osmotic stabilisation on blood cultures was medium-dependent. This conclusion was inferred from the results of their own survey and from a review of similar studies. Though there is some evidence that the recovery of Haemophilus species from the blood is enhanced in hypertonic media (Crist, Amsterdam & Neter, 1982; La Scolea et al., 1983) this finding may merely reflect nutritionally inadequate culture conditions. Certainly, wall-defective H. influenzae display considerable osmotic stability on appropriately enriched media (Roberts et al., 1974).

An alternative view might be that wall-defective bacteria vary in their need for osmotic stabilisation, depending on bacterial species and particularly on cell shape and the Gram staining reaction. Cell shape is important from a purely physical aspect, since small spherical objects, like cocci, withstand pressure better than cylindrical objects, like bacilli. This suggests that cocci may be less vulnerable to osmotic stress than bacilli (Mitchell & Moyle, 1956). In addition, the Gram positive cell may be less prone to osmotic stress because it is thicker and differs structurally from the Gram negative cell (Wilson & Miles, 1975b; Rogers, Ward & Burdett, 1978). The Gram positive cell wall is particularly resistant to osmotic stress because the cytoplasmic membrane is closely adherent to it (Mitchell & Moyle, 1956). The cytoplasmic membrane is the principal osmotic barrier of the bacterial cell (Mitchell & Moyle, 1956; Wilson & Miles, 1975c) so the wall-damaged Gram positive cell is better reinforced against osmotic stress than the Gram negative cell despite its higher internal osmotic pressure. This

perhaps explains why neither the pyridoxine requiring streptococci of the type described by Bouvet & Acar (1977) nor the streptococcal strains described in Section VI required osmotic stabilisation in the primary blood culture medium.

The Role of Agar

Induced wall-defective microbial variants depend on the physical solidity of the milieu for induction and reversion (Landman & Halle, 1963; Landman & de Castro-Costa, 1976; Rogers, Ward & Elliott, 1976). Induction and reversion in naturally occurring wall-defective microbial variants appear to be nutrient dependent. The role of agar is indeterminate. The naturally occurring L-phase variants studied did not revert easily in broth. They grew on the surface of agar block subcultures in broth, but not always in the broth itself, and sometimes gave granular or flocculent growth in broth after agar block subculture. Similar observations were made both by Dienes and by Klieneberger (Dienes, 1941; 1949a; Dienes & Weinberger, 1951; Klieneberger-Nobel, 1960; Klieneberger-Nobel, 1962). The explanation may be that there is gross dissociation of an autolysin/LTA complex from agar blocks in broth, so that reversion does not occur. Alternatively, reversion dependent on optimal nutrient conditions may be prevented by the diluent effect of the broth. This explanation is equally plausible.

Chapter 25

Clinical Features of Infection with Wall-Defective Organisms

CLINICAL FEATURES OF INFECTION WITH WALL-DEFECTIVE ORGANISMS

1. Infection due to Wall-defective Enterobacteriaceae.

Clinical features of the patients who formed the L-form group closely resembled those of the patients of the vegetative group. The two groups were similar in age and sex distribution, in the spectrum of major disease as evinced by the principal diagnosis, in the source and outcome of the infection, and in the range of bacterial species isolated from the blood. As far as these aspects were concerned there appeared to be no characteristic features of infection due to wall-defective Enterobacteriaceae.

Source of Infection

The source of infection in 56% of the septicaemias caused by wall-defective variants was the urinary tract. This was a higher proportion than in the vegetative group, but the difference was not statistically significant.

The urinary tract is a suitable milieu for wall-defective bacteria of both the natural and induced types. It is suitable for naturally occurring variants because it is rich in nutrients. In particular, folic acid and pABA, the nutrients required by the naturally occurring Enterobacteriaceae variants described here, are excreted in the urine.

Induced variants need osmotic stabilisation because they are wall-defective. The osmolality of the renal medulla is sufficient for stabilisation since it reaches 1200 - 1400 m.osm./kg. (Barker, 1963) and wall-defective bacteria have actually been observed in renal biopsy

specimens (Fernandes & Panos, 1977). In addition, the acid urine characteristic of infection gels bacterial cytoplasm (Brown & Corner, 1977) so that the need for osmotic stabilisation in wall damaged cells is less critical (Gnarpe & Edebo, 1970).

Incidence of Chronic Infection

Wall-defective variants may be responsible for chronic relapsing urinary tract infection (Gutman et al., 1965; Domingue & Schlegel, 1970; Brogan, 1977).

Septicaemia associated with an exacerbation of chronic urinary infection was more likely to be caused by such variant bacteria, particularly if the infection was of the relapsing type, but the numbers involved were too small for statistical analysis.

Accordingly the significance of this observation was inconclusive.

2. Influence of Antibacterials on the Induction and Growth of Wall-defective Enterobacteriaceae

Such agents might act in one of four ways:

i) Induction by beta-lactams.

ii) Selection by aminoglycosides

iii) A requirement for sulphonamide as a neutraliser of excess pABA.

iv) Growth promotion by antibacterials acting as nutrient analogues.

i) Induction by beta-lactams

The occurrence of wall-defective variants could not be attributed to antecedent treatment with beta-lactams (Table 20). This was not surprising, since the osmolality of the blood is probably too low for the stabilisation of induced variants.

The suggestion that induction by beta-lactams might occur in vivo (McDermott, 1958; Feingold, 1969), has never been satisfactorily

substantiated. It rests on the neutralisation of the effect of beta-lactams by osmotic stabilisation. It is true that osmotically stabilised media are sometimes more successful than simple conventional media in the detection of septicaemia (Louria et al., 1969; Rosner, 1972; Louria et al., 1976; Washington, 1978), but the effect may be due to nutrient stimulation (Washington, 1978; Weinstein et al., 1982) rather than to beta-lactam neutralisation.

If there is a connection between antecedent beta-lactam therapy and induction of wall-defect in vivo it may be related to the bacterial species involved. Eng and Maeland (1982) were able to show a relation between growth in hypertonic media only and antibiotic treatment in staphylococcal septicaemia, but not in Enterobacteriaceae septicaemia. Perhaps this means that wall-damaged staphylococci are better fitted to withstand an unfavourable osmotic climate than coliforms which are similarly handicapped. This may reflect an enhanced resistance to osmotic stress conferred by the shape and structure of the cell envelope in Gram positive cocci.

ii) Selection by Aminoglycosides

Nutritionally exacting bacteria are relatively resistant to the aminoglycosides and may be selected by them (Acar, Goldstein & Lagrange, 1978). More patients in the L-form group received aminoglycosides in the 48 hours before blood cultures were taken than in the vegetative group (Tables 20 and 21).

As the numbers involved were too small for statistical analysis, the significance of this observation was inconclusive.

iii) A Requirement for sulphonamide as a neutraliser of excess pABA

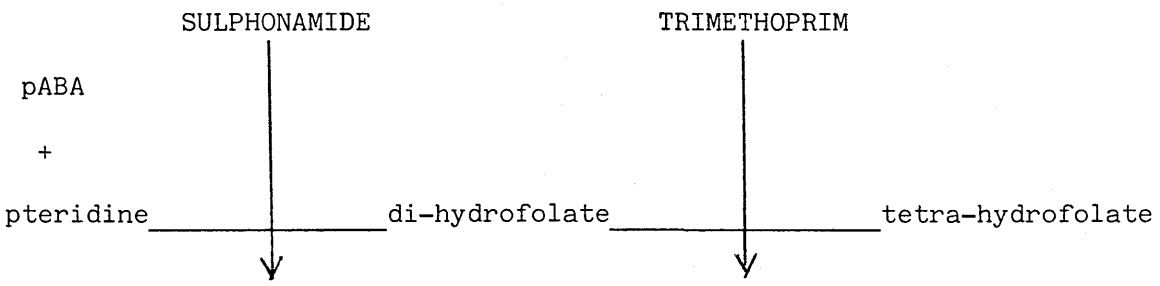
Some bacteria produce surplus pABA in toxic amounts and need sulphonamide to neutralise the excess (Lamanna, Mallette & Zimmerman, 1973d).

This seemed unlikely, as the Enterobacteriaceae variants were successfully subcultured from the primary blood culture medium without the addition of sulphonamide.

iv) L-form growth promotion by nutrients or nutrient analogues

More patients in the L-form group received folic acid or its analogue cotrimoxazole in the forty-eight hours before blood cultures were taken. This was statistically significant.

Cotrimoxazole is a combination of two antibacterials, sulphonamide and trimethoprim. They inhibit different steps in the biosynthesis of the folic acid coenzyme tetrahydrofolate in bacteria. Sulphonamide and trimethoprim inhibit bacterial growth by blocking folate mediated synthesis of the DNA precursor thymidine.



(after Wilson & Miles, 1975c)

Sulphonamide acts by competitive inhibition against pABA and is its structural analogue. Trimethoprim acts by inactivation of the enzyme dihydrofolate reductase (Wilson & Miles, 1975e).

An obligate requirement for thymidine results in mutants which are selected by exposure to trimethoprim in the presence of exogenous

thymidine (King, Shlaes & Dul, 1983). The addition of thymidine alone to the semi-defined medium was not sufficient for the growth of the Enterobacteriaceae variants I encountered. This suggested that the cotrimoxazole moiety required for selection was sulphonamide rather than trimethoprim, and also that at least some wall-defective variants were selected by sulphonamide.

Wall-Defective Variants as Metabolically Deficient Bacteria

The association with cotrimoxazole treatment suggested that the wall-defective variants might be metabolically deficient bacteria.

Metabolically deficient bacteria are nutritionally exacting bacteria selected by exposure to drugs which are structural analogues of essential nutrients. For example, trimethoprim and the barbiturates share the same pyrimidine nucleus as thiamine, and thiamine requiring bacteria have been isolated from patients receiving trimethoprim or barbiturates (Acar, Goldstein & Lagrange, 1978). Blood culture results with the partially defined medium did not show any requirement for thiamine. It was therefore unlikely that trimethoprim was a required nutrient analogue. This again suggested that the sulphonamide component selected the wall-defective variants and that the Enterobacteriaceae variants isolated were metabolically deficient bacteria dependent on a sulphonamide/pABA mediated folate pathway.

3. Influence of Antibacterials on the Induction and Growth of Wall-defective Streptococcal variants.

The three patients infected by wall-defective streptococci showed typical features of subacute bacterial endocarditis. None received antecedent treatment with antibacterials or with vitamins or vitamin

analogues. No predisposing therapeutic factor was found, but the number of patients was too small for valid comment.

Treatment of Infection due to Wall-defective Microbial Variants

The few accounts of treatment available describe the use of erythromycin combined with ampicillin (Gutman, Schaller & Wedgwood, 1967; Gnarpe, 1974) or following ampicillin as 'sequential therapy' (May, 1975; Garrod, Lambert & O'Grady, 1981) so as to destroy both variants and vegetative bacteria. However, if the nutritional requirements of naturally occurring variants are satisfied in vivo they must exist in vivo in the walled state. Infection due to such organisms should be amenable to conventional antibiotic therapy.

If some wall-defective variants are metabolically deficient another problem concerns the effect of aminoglycoside or cotrimoxazole on these bacteria. Mechanisms of bacterial resistance to aminoglycosides include bacterial dependence on aminoglycoside as a required nutrient (Wilson & Miles, 1975e). It is particularly attractive to postulate nutrient dependence as a resistance mechanism in infection caused by naturally occurring wall-defective variants. This is the disadvantage of cotrimoxazole treatment, and it is perhaps an illustration of the type of phenotypic resistance discussed by Greenwood (1985), in which bacterial environment affects the expression of phenotypic characters.

There is therefore no simple antibiotic treatment. Beta-lactam antibiotics are inappropriate as they induce wall-deficiency, yet they should be effective under optimal nutrient conditions, when nutritionally exacting variants revert to the walled state. Moreover, beta-lactam treatment was used successfully in the treatment of the streptococcal infections described in Section VI.

The important point may be the preferred habitat of the organism. Beta-lactams may be less reliably active against bacteria which colonise osmotically stabilised sites where wall deficient bacteria may survive, such as the renal medulla or the respiratory tract.

As wall-defective Enterobacteriaceae may become nutritionally dependent on aminoglycosides or cotrimoxazole, a combination of antibiotics is probably best.

SECTION IX

CONCLUSIONS

CONCLUSIONS

The original objectives of this study were the investigation of the cultural requirements, identification and pathogenicity of wall-defective microbial variants and especially those variants categorised as L-phase variants. In the course of the study, the very nature of wall-defective variants became a matter of some interest. The results of these studies and experiments may be summarised as follows:-

I. The Nature of Wall-Defective Microbial Variants.

1. Wall-defective microbial variants may occur naturally or be induced.

2. Induced variants may be produced by substances that damage the cell wall such as lysozyme or beta-lactam antibiotics. Some of these lysozyme-induced Enterobacteriaceae variants behave like stressed bacteria and may have repairable DNA strand breaks.

3. Naturally occurring wall-defective variants bear some resemblance to auxotrophs. This may mean that they too have atypical DNA.

II. Cultural Requirements of Wall-Defective Microbial Variants.

1. Natural wall-defective variants need a nutritionally-enriched medium for growth. The supplement required varies from genus to genus. Wall-defective Enterobacteriaceae require a folate metabolite and streptococcal variants pyridoxine and cysteine.

2. Induction and reversion in naturally occurring wall-defective variants are nutrient dependent, but the physical solidity of the medium is not important for reversion. In contrast, a 'stiff' medium is important for the reversion of wall-defective variants of the induced type.

III Identification of Wall-Defective Variants

The identification of these variants and their differentiation from vegetative forms depends on the demonstration of a requirement for a specific nutrient, lack of which is likely to cause cell wall defects.

IV The Pathogenicity of Wall-Defective Microbial Variants

1. Wall-defective variants may be pathogens or harmless commensals just like their parent vegetative forms.

2. Naturally occurring wall-defective variants seem to be a commoner cause of septicaemia than variants which have an induced wall defect. This may be related to the fact that induced variants require a degree of osmotic stabilisation not found in exudates and tissues, except perhaps in the renal medulla.

3. Growth promotion by folate metabolites selects the natural wall-defective variants of Enterobacteriaceae. The administration of folic acid or its analogue, sulphonamide, predisposes to infection with such variants.

4. Naturally occurring wall-defective variants of viridans streptococci may cause subacute bacterial endocarditis in the same way as normal vegetative streptococci. As in

endocarditis caused by vegetative bacteria, repeated isolation of wall-defective streptococci from patients who have clinical evidence of endocarditis is of the utmost significance and importance.

V Treatment of Infection due to Wall-Defective Microbial Variants

1. Wall-defective Enterobacteriaceae variants may be less susceptible to aminoglycosides than vegetative Enterobacteriaceae. In the treatment of infection due to such variants consideration should be given to the use of an aminoglycoside in combination with another antibiotic, or to the use of antibiotics other than aminoglycosides.

2. The apparent success of beta-lactam therapy in wall-defective streptococcal infection may be due to reversion of the organisms to the walled state in a nutritionally adequate tissue environment.

APPENDIX I

CULTURE MEDIA AND REAGENTS

LIST OF CULTURE MEDIA AND REAGENTS USED

Page

iv) Subculture Media

4. Other Culture Media

Brain Heart Infusion Broth (Oxoid)

Cooked Meat Medium (Oxoid)

Cooked Meat Medium (Difco)

Blood Agar Plates

5. Water

6. Glassware Washing Methods

1. Reagents used for Spheroplast induction by the lysozyme/EDTA method

i) 10 millimolar (10mM) tris (hydroxymethyl) aminomethane (Tris) Buffer

1.211 g of Tris was dissolved in 950ml distilled water, and sufficient N HCl added to give a pH of 8.0. The solution was made up to 1 litre in a volumetric flask, distributed in 50ml amounts and sterilised by autoclaving at 15 lbs/sq.inch (121°C) for 15 minutes.

ii) 0.1M Tris Buffer pH 8.0 containing 20% sucrose

12.114 g Tris was dissolved in 950 ml distilled water, and sufficient N HCl added to give a pH of 8.0. The solution was made up to 1 litre in a volumetric flask. 20.0 g metal free sucrose (Aristar BDH) was added for each 100 ml. The solution was distributed in 43.0 ml amounts and sterilised by autoclaving at 15 lbs/sq.inch (121°C) for 15 minutes.

iii) Lysozyme solution 2.0 mg/ml

The lysozyme (Worthington Blochemicals) had a strength of 11,770 units/mg. 0.2g was dissolved in fresh glass distilled water and made up to 100ml in a volumetric flask. It was sterilised by filtration through a Millipore 'Millex' filter pore size 0.2μ.

iv) 0.1M dipotassium EDTA. pH 7.0

4.044 g EDTA (Analar) was dissolved in 80 ml distilled water. N. NaOH was added to bring the pH to 7.0. The

solution was made up to 100 ml in a volumetric flask. After checking the pH, the solution was distributed in 7 ml amounts, and sterilised by autoclaving at 15 lbs/sq.inch (121°C) for 15 minutes.

2. Media for the Isolation of Wall-defective Enterobacteriaceae Variants.

i) VICTORIA MEDIUM F

Constituent	Amount (grams/litre)
Yeast extract (Oxoid)	25
Sucrose	300
$MgSO_4.2H_2O$	2.5*
Agar (Oxoid No. 3)	1.5
Haemin**	0.01
Cholesterol***	0.02
Sod. thioglycollate	1.0
Heparin (1000 units/ml solution)	0.5 ml
Brain Heart Infusion Broth (Oxoid)	1000 mls

Dissolve all ingredients except haemin and cholesterol.

pH to 7.5

When dissolved there will be a precipitate. Filter through a coarse filter paper (e.g. Greens '904').

When filtered, add haemin and cholesterol.

Bottle in 20 ml quantities.

Sterilise by autoclaving at 15 lbs/sq.inch (121°C) for 20 minutes. Cap, label with date and batch number. The osmolality of the culture medium should be 1100 - 1200 m.osm./kg.

NOTES

* $MgSO_4.2H_2O$. If $MgSO_4 7H_2O$ is used, the amount should be 3.2g.

** Haemin. Dissolve 0.1 g in 10 ml sodium carbonate

Then 1 ml = 0.01 g.

Add 1 ml to each litre to arrive at a final concentration of 0.01 g/litre.

*** Cholesterol. Dissolve 0.2 g in 10 ml methylated spirit.

Then 1 ml = 0.02 g.

Add 1 ml to each litre to arrive at a final concentration of 0.02 g/litre.

Pour Plate Medium for Subcultures

As Victoria Medium F, with 1% agar.

ii) MODIFIED VICTORIA MEDIUM F

Constituent	Amount (grams/litre)
Sucrose	300.00
$MgSO_4.7H_2O$ (or $MgSO_4.2H_2O$ = 2.5gm)	3.2
Yeast Extract (Oxoid)	25.00
Cholesterol	0.02
Sod. thioglycollate	1.00
Agar (Oxoid No. 1)	1.00
Soluble starch 0.15% w/v solution in water	100 mls
Brain Heart Infusion Broth (Oxoid)	900 mls
Heparin (0.5 ml of a 1000 u./ml solution)	500 units
pH	7.5

Bottle in 20 ml quantities

Sterilise by autoclaving at 10 lbs/sq.inch (115°C) for 20 minutes.

Cap, label with date and batch number. The osmolality of the medium should be 1100 - 1200 m.osm./kg.

Preparation of Starch Solution

1. Weigh out soluble starch 1.5 g.
2. Dissolve in 10 ml warm water.
3. Add 90 ml water to make a final volume of 100 ml.
4. Mix.

Pour Plate Medium for Subcultures

A Modified Victoria Medium F, but with 1% Oxoid Agar No. 1.

iii) PARTIALLY DEFINED SUPPLEMENTED MEDIA

Medium 1.

Constituents	Amount (grams/litre)
Sucrose	300.00
$MgSC_4 .7H_2 0$ (or: $MgSO_4 .2H_2 0$ = 2.5 gm)	3.2
Cholesterol	0.02
Sod. thioglycollate	1.00
$CaCl_2$	0.123
Riboflavin	0.001
Thymidine	0.01
Agar (Oxoid No. 1)	1.00
Isosensitest broth	1000 mls
Heparin (0.5 ml of a 1000 u/ml solution)	500 units
pH	7.4 approx.

Bottle in 20 ml quantities

Sterilise by autoclaving at 10 lbs/sq.inch (115°C) for 20 minutes.

Pour Plate Medium for Subcultures

As above, but substituting Isosensitest Agar for Isosensitest Broth, and omitting Oxoid Agar No. 1.

Medium 2.

Constituents	Amount (grams/litre)
Sucrose	300.00
Nicotinamide	0.005
$MgSO_4.7H_2O$ (or: $MgSO_4.2H_2O$ = 2.5 gm)	3.20
Cholesterol	0.02
Sod. thioglycollate	1.00
$CaCl_2$	0.123
Riboflavin	0.001
Thymidine	0.01
Agar (Oxoid No. 1)	1.00
Isosensitest broth	1000 mls.
Heparin (0.5 ml of a 1000 u/ml solution)	500 units
pH	7.4 approx.

Bottle in 20 ml quantities

Sterilise by autoclaving at 10 lbs/sq.inch (115°C) for 20 minutes.

Pour Plate Medium for Subcultures

As above, but substituting Isosensitest Agar for Isosensitest Broth, and omitting Oxoid Agar No. 1.

Medium 3.

Constituents	Amount (grams/litre)
Sucrose	300.00
Nicotinamide	0.005
$MgSO_4.7H_2O$ (or: $MgSO_4.2H_2O$ = 2.5 g)	3.2
Cholesterol	0.02
Sod. thioglycollate	1.00
Riboflavin	0.001
Thymidine	0.01
pABA (<u>para</u> amino benzoic acid)	0.001
Folic acid	0.001
Cytosine	0.01
$CaCl_2$	0.123
Agar (Oxoid No. 1)	1.00
Isosensitest broth (Oxoid)	1000 mls
Heparin (1000 units/ml solution)	0.5 ml
pH	7.4

Bottle in 20 ml quantities.

Sterilise by autoclaving at 10 lbs/sq.inch (115°C) for 20 minutes.

<u>Pour Plate Medium</u>

As above, but substituting Isosensitest agar (Oxoid) for Isosensitest broth, and omitting Oxoid Agar No. 1

<u>Stock Solutions Vitamins</u>

<u>Riboflavin</u> Dissolve in 0.2N Acetic Acid. Prepare a 1 mg/ml solution. Mask with foil to protect from light. Solution stable for one month at 4°C.

Thymidine Dissolve in sterile distilled water. Prepare a 10 mg/ml solution. Solution stable for one month at 4°C.

Nicotinamide Dissolve in sterile distilled water. Prepare a 5 mg/ml solution. Stable for one month at 4°C.

Cytosine Dissolve in 0.1N HCl. Stable for one month at 4°C.

pABA Dissolve in distilled water. Stable for one month at 4°C.

Folic acid Dissolve in 0.001N NaOH. Stable for one month at 4°C.

'ISO-SENSITEST' AGAR (OXOID)

FORMULA	grams per litre
Hydrolysed Casein	11.0
Peptones	3.0
Dextrose	2.0
Sodium chloride (NaCl)	3.0
Soluble starch	1.0
Disodium hydrogen phosphate	2.0
Sodium acetate	1.0
Magnesium glycerophosphate	0.2
Calcium gluconate	0.1
Cobaltous sulphate ($CoSO_4$)	0.001
Cupric sulphate ($CuSO_4$)	0.001
Zinc sulphate ($ZnSO_4$)	0.001
Ferrous sulphate ($FeSO_4$)	0.001
Manganous chloride ($MnCl_2$)	0.002
Menadione	0.001
Cyanocobalamin	0.001
L-Cysteine hydrochloride	0.02
L-Tryptophan	0.02
Pyridoxine	0.003
Panthothenate	0.003
Nicotinamide	0.003
Biotin	0.0003
Thiamine	0.00004

Adenine	0.01
Guanine	0.01
Xanthine	0.01
Uracil	0.01
Agar No. 1	8.0
Distilled water	1000 mls

pH 7.4 ± 0.2

'ISO-SENSITEST' BROTH (OXOID)

FORMULA

As 'Iso-sensitest' Agar, but without agar.

3. Media for the Isolation of Wall-defective Streptococcal Variants.

i) Biphasic Blood Culture Brain Heart Infusion Agar/Broth.

Gibco Bio-Cult Diagnostics Ltd., Sandyford Industrial Estate, Paisley PA3 4EP, Scotland.

ii) Cysteine/pyridoxine supplemented Brain Heart Infusion Broth (BHI/PC)

Constituents	Quantity (grams/litre)
Cysteine hydrochloride	1.00
Pyridoxine hydrochloride (BDH)	0.001
Brain Heart Infusion Broth (Oxoid)	1000 ml
Heparin (1000 units/ml solution)	0.5 ml
Final pH	7.5

Mix well. Bottle in 20 ml amounts and sterilise by autoclaving at 15 lbs/sq.inch (121°C) for 20 minutes.

iii) Stabilised Cysteine/pyridoxine supplemented Brain Heart Infusion Broth (stabilised BHI/PC)

Constituents	Quantity (grams/litre)
Sucrose	200.00
NaCl	18.00
Yeast autolysate	25.00
$MgSO_4.7H_2O$	3.2
Cholesterol	0.02
Cysteine hydrochloride	1.00
Pyridoxine hydrochloride (BDH)	0.001
Agar	1.5
Brain Heart Infusion Broth (Oxoid)	1000 mls

Heparin (1000 units/ml solution)	0.5 ml
Final pH	7.5

Mix well, distribute into final containers and sterilise by autoclaving at 15 lbs/sq.inch (121°C) for 20 minutes. The final osmolality of the medium should be about 1500 m.osm/kg.

iv) Subculture Media

BACP Media

a) Blood agar plates supplemented with cysteine hydrochloride 1 gram/litre plus pyridoxine hydrochloride 1 mg/litre.

b) Blood agar plates supplemented with cysteine hydrochloride 1 gram/litre plus pyridoxine hydrochloride 1 gram/litre.

Pour plates made with the same base composition as stabilised BHI/PC.

4. Other Culture Media

Brain Heart Infusion Broth (Oxoid Ltd., Wade Road, Basingstoke, Hampshire).

Cooked Meat Medium (Oxoid)

Cooked Meat Medium Granules (Oxoid)

Brain Heart Infusion Broth (Oxoid)

Heparin 500 units/litre of Brain Heart Infusion Broth

pH 7.2

Distribute granules in 1.0 gram amounts into bottles.

Add 10 ml of Brain Heart Infusion Broth to each bottle.

Sterilise by autoclaving at 15 lbs/sq.inch (121°C) for 15 minutes.

Cooked Meat Medium (Difco)

Cooked Meat Medium Granules (Difco)

Tryptic Soy Broth (Difco)

Heparin 500 units/litre Tryptic Soy Broth

pH 7.2

Distribute granules in 1.0 gram amounts into bottles.

Add 15 ml of Tryptic Soy Broth to each bottle.

Sterilise by autoclaving at 15 lbs/sq.inch. (121°C) for 15 minutes.

Blood Agar Plates

These were composed of Columbia Agar Base (Oxoid) with 5 - 6.5% horse blood.

Suspend 39 grams Columbia Agar Base in 1 litre of distilled water.

Boil to dissolve the medium completely.

Sterilise by autoclaving at 15 lbs/sq.inch (121°C) for 15 minutes.

Cool to 50°C and add 5 - 6 5% sterile defibrinated horse blood.

5. Water

Glass-distilled water was used throughout, and was distilled once.

6. Washing procedure for Glassware

New Glassware. If clean, washed and capped when received, proceed as follows. Rinse twice in glass distilled water. Dry upside down in oven set at 'high' for 1 hour. Treat bungs and caps similarly, only dry on top of oven in basket. If new glassware is 'dirty' when received, proceed as follows.

i) Rinse with tap water.

ii) Steep overnight in 1% Decon (DRI Decon, Hove) solution in tap water. Next morning, brush well and rinse 5 times in tap water, then 5 times in glass-distilled water.

iii) Dry upside down for 1 hour in drying oven set at 'high'. Treat bungs and caps similarly, only dry on top of oven in basket.

Glassware for Medium Preparation

After use, rinse with tap water. Steep in 1% Decon solution for 10-15 minutes. Brush well. Rinse thoroughly in tap water. Invert and drain dry.

LIST OF REFERENCES

Note:

Translation of Foreign Languages

Most of the references cited are in English, but material written in French, German and Russian is also included. Papers written in French were read in the original language. Papers written in German were translated into English by me. The Russian papers were not read in full, as my ability to translate Russian is extremely limited. Russian language papers were either read from the English abstract, or partly translated into English. I am most grateful to Miss Jean Nisbet of the Modern Languages Department, Craigbank Secondary School, Glasgow, who translated selected paragraphs into English for me.

In the List of References I have shown the language of origin for each foreign language paper, and I have indicated whether the material was read from the English Abstract, translated, or read in the original language.

Acar, J.F., Goldstein, F.W. & Lagrange, P. (1978) Human Infections caused by thiamine or menadione requiring Staphylococcus aureus. Journal of Clinical Microbiology, 8, 142-147.

Ahmad F.J. & Darrell, J.H. (1980) 'Diphtheroid' streptococci in Endocarditis - a reminder. Communicable Disease Report 7, 4. London: Public Health Laboratory Service, Colindale Avenue.

Alexander, H.E. (1965) The Haemophilus Group. In Bacterial and Mycotic Infections of Man, ed. Dubos, R.J. Ch. 29, pp 730-731. Philadelphia: J.B. Lippincott Company.

Babes, V. & Manolescu, D. (1909) On the presence of a pseudodiphtheroid bacillus in the vegetations of endocarditis. Berliner Klinische Wochenschrift, 46, 67 (Abstract from (1908) Romania Medicala, 12). (German: translation).

Barile, M., Yaguchi, R. & Eveland, W.C. (1958) A simplified medium for the cultivation of pleuro-pneumonia like organisms and the L-forms of bacteria. American Journal of Clinical Pathology, 30, 171 - 176.

Barker, J.N. (1963) The renal countercurrent mechanism. Medical Clinics of North America, 47, 873 - 886.

Bartholomew, L.E. & Nelson, F.R. (1972) Corynebacterium acnes in rheumatoid arthritis. II. Identification of antigen in synovial fluid leucocytes. Annals of the Rheumatic Diseases, 31, 28 - 33.

Bauman, N. & Davis, B.E. (1957) Selection of auxotrophic bacterial mutants through diaminopimelic acid or thymine deprival. Science, 126, 170.

Belsheim, M.R., Darwish, R.Z., Watson, W.C. & Schieven, B. (1983) Bacterial L-form isolation from inflammatory bowel disease patients. Gastroenterology, 85, 364 - 369.

Bertolani, R., Elberg, S.S. & Ralston, Doris. (1975) Variation in properties of L-forms of Ps. aeruginosa. Infection and Immunity, 11, 180 - 192.

Beuchat, L.R. (1978) Injury and repair of gram negative bacteria, with special consideration of the involvement of the cytoplasmic membrane. Advances in Applied Microbiology, 23, 219 - 243.

Bibel, D.J. & Lawson, J.W. (1971) Scanning-beam electron microscopy of L-form colonies of Group A and D streptococci. American Society for Microbiology, 71st Annual Meeting. Bacteriological Proceedings, p.42.

Birdsell, D.C. & Cota-Robles, E.H. (1968) Lysis of spheroplasts of Escherichia coli by a non-ionic detergent. Biochemical and Biophysical Research Communications, 31, 438 - 446.

Bisset, K.A. & Bartlett, Roberta. (1978) The isolation and characters of L-forms and reversions of Bacillus licheniformis var. endoparasiticus (Benedek) associated with the erythrocytes of clinically normal persons. Journal of Medical Microbiology, 11 335 - 349.

Bisset, K.A. Tallack, J. & Bartlett, R. (1979) Electron microscopy of the L-cycle in Bacillus licheniformis var. endoparasiticus (Benedek). Journal of Medical Microbiology, 12, 469 - 472.

Bolton, P.M., Owen, E., Heatley, R.V., Williams, W.J. & Hughes, L.E. (1973) Negative findings in laboratory animals for a transmissible agent in Crohns disease. Lancet, 2, 1122 - 1124.

Bouvet, A. & Acar, J.F. (1977) Cultural isolation and morphological study of deficient streptococci growing in satellitism. I. Isolation in bacterial endocarditis. In Spheroplasts Protoplasts and L-forms, ed. Roux, J. pp 327-338. Paris:INSERM. (French: read in original language).

Bouvet, A., Ryter, A. & Acar J.F. (1977) Cultural isolation and morphological study of deficient streptococci growing in satellitism. II. Study of optical and electron microscopy. In Spheroplasts Protoplasts and L-forms, ed. Roux, J., pp 339 - 340. Paris:INSERM. (French: read in original language).

Bouvet, A., Ryter, A., Frehel, C. & Acar, J.F. (1980) Nutritionally deficient streptococci; electron microscopic study of 14 strains isolated in bacterial endocarditis. Annales de Microbiologie, 131B, 101 - 120. (French: read in original language).

Bouvet, A., van de Rijn, I. & McCarty, M. (1981) Nutritionally variant streptococci from endocarditis: growth parameters in a semisynthetic medium and demonstration of a chromophore. Journal of Bacteriology, 146, 1075 - 1082.

Bouvet, Anne, Villeroy, Francoise, Cheng, Francoise, Lamesch, Catherine, Williamson, R. & Gutman, L. (1985) Characterisation of nutritionally variant streptococci by biochemical tests and penicillin-binding proteins. Journal of Clinical Microbiology, 22, 1030 - 1034.

Braude, A.I., Siemienski, Jennie & Lee, Kathleen. (1968) Spheroplasts in human urine. In Microbial Protoplasts, Spheroplasts and L-forms, ed. Guze, L.B. Ch. 41, pp 396 - 405. Baltimore: Williams & Wilkins Company.

Brem, Antonina M. (1969) Isolation of L-forms from clinical material. American Journal of Medical Technology, 35, 702 - 705.

Brogan, Oonagh. (1976) Isolation of L-forms by blood culture. Journal of Clinical Pathology, 29, 934 - 937.

Brogan, Oonagh. (1977) The role of the bacterial L-form in urinary tract infection. British Journal of Urology, 49, 431 - 436.

Brown, R.J. & Corner, T.R. (1977) Influence of ambient pH on the Osmotic Properties of Protoplasts from Bacillus megaterium. In Microbiology - 1977, ed. Schlessinger, D. pp 93 - 99. Washington DC: American Society for Microbiology.

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

Burnham, W.R., Stanford, J.L. & Lennard-Jones, J.E. (1977) Evidence for a mycobacterial aetiology of Crohns disease. Gut, 18, 965.

Carey, Roberta B. (1978) Culture-negative endocarditis. Lancet, 1, 278.

Carey, Roberta B., Brause, B.D. & Roberts, R.B. (1977) Antimicrobial therapy of vitamin B6-dependent streptococcal endocarditis. Annals of Internal Medicine. 87, 150 - 154.

Cayeux, P., Acar, J.F. & Chabbert, Y.A. (1971) Bacterial persistence in streptococcal endocarditis due to thiol requiring mutants. Journal of Infectious Diseases, 124, 247 - 254.

Chattman, M.S., Mattman, Lida H. & Mattman, P.E. (1969) L-forms in blood cultures demonstrated by nucleic acid fluorescence. American Journal of Clinical Pathology, 51, 41 - 50.

Clasener, H. (1972) Pathogenicity of the L-phase of bacteria. In Annual Review of Microbiology, ed. Clifton, C.E., Raffel, S. & Starr, M.P. 26, pp 55 - 84. Palo Alto, California: Annual Reviews Inc.

Cohen, R.L., Wittler, Ruth G. & Faber, J.E. (1968) Modified biochemical tests for characterisation of L-phase variants of bacteria. Applied Microbiology, 16, 1655 - 1662.

Coleman, J.C. & Little, P.J. (1967) Bacterial variants in the human kidney. Journal of Pathology and Bacteriology, 94, 213 - 215.

Collee, J.G. (1978) Morphology and nature of bacteria. In Mackie and McCartney. Medical Microbiology, ed. Duguid, J.P., Marmion, B.P. & Swain, R.H.A. 13th edition, Vol. 1, ch. 2, pp 26 - 27. Edinburgh & London: Churchill Livingstone.

Collee, J.G., Duerden, B.I. & Brown, R. (1977) Recovery of anaerobic bacteria from small inocula: a model for blood culture studies. Journal of Clinical Pathology, 30, 609 - 614.

Cook, Josephine, Fincham, W.J. & Lack, C.H. (1969) Chronic arthritis produced by streptococcal L-forms. Journal of Pathology, 99, 283 - 297.

Cooksey, R.C., Thompson F.S. & Facklam, R.R. (1979) Physiological characterisation of nutritionally variant streptococci. Journal of Clinical Microbiology, 10, 326 - 330.

Cooksey, R.C. & Swenson, Jana M. (1979) In vitro anti-microbial inhibition patterns of nutritionally variant streptococci. Antimicrobial Agents and Chemotherapy, 16, 514 - 518.

Coughlin, C.A. (1966) Spheroplast production in thymine starvation. Nature, 212, 633 - 635.

Cove J.H., Holland, K.T. & Cunliffe, W.J. (1980) The vitamin requirements of staphylococci isolated from human skin. Journal of Applied Bacteriology, 49, 29 - 37.

Crawford, Y.E., Frank, P.F. & Sullivan, Betty. (1958) Isolation and reversion of L-forms of β-haemolytic streptococci. Journal of Infectious Diseases, 102, 44 - 52.

Crist, A.E., Amsterdam, D. & Neter,E. (1982) Superiority of Hypertonic Culture Medium for Detection of Haemophilus influenzae by the BACTEC procedure. Journal of Clinical Microbiology, 15, 528 - 530.

Cruickshank, R., Duguid, J.P., Marmion, B.P. & Swain, R.H.A. (Eds.) (1973) Morphology and Nature of Bacteria. In Medical Microbiology, 12th edition, ch. 2, pp 10 - 30. Edinburgh & London: Churchill Livingstone.

Datta, Naomi & Nugent, Marilyn E. (1983) Bacterial variation. In Topley & Wilson's Principles of Bacteriology, Virology & Immunity, ed. Wilson, Sir Graham and Dick, Heather M. 7th edition, Vol. 1, Ch. 6, p 156. London: Edward Arnold.

Dawson, M.H. & Hobby, G.I. (1939) Rat-bite fever. Transactions of The Association of American Physicians, 54, 329 - 332.

de Takats, G. (1932) 'Resting Infection' in varicose veins: its diagnosis and treatment. American Journal of the Medical Sciences, 184, 57 - 66.

de Vuono, Joanna & Panos, C. (1978) Effect of L-form Streptococcus pyogenes and lipoteichoic acid on human cells in tissue culture. Infection and Immunity, 22, 255 - 265.

Dienes, L. (1932) Appearance of large amounts of non-stainable cultivable granules in bacterial cultures on saccharose media. Proceedings of the Society for Experimental Biology and Medicine, 29, 1205 - 1207.

Dienes, L. (1934) Morphologic elements in the halo of Subtilis colonies. Proceedings of the Society for Experimental Biology and Medicine, 31, 1211 - 1214.

Dienes, L. (1938) Further observations on the L organism of Klieneberger. Proceedings of the Society for Experimental Biology and Medicine, 39, 365 - 367.

Dienes, L. (1939a) L type variant forms in cultures of various bacteria. Proceedings of the Society for Experimental Biology and Medicine, 42, 636 - 664.

Dienes, L. (1939b) A peculiar reproductive process in Colon bacillus colonies. Proceedings of the Society for Experimental Biology and Medicine, 42, 773 - 778.

Dienes, L. (1940a) Origin of L-type colonies in bacterial cultures. Proceedings of the Society for Experimental Biology and Medicine, 43, 703 - 704.

Dienes, L. (1940b) L-type of growth in gonococcus cultures. Proceedings of the Society for Experimental Biology and Medicine, 44, 470 - 471.

Dienes, L. (1941) Isolation of L-type growth from a strain of Bacteroides funduliformis. Proceedings of the Society for Experimental Biology and Medicine, 47, 385 - 387.

Dienes, L. (1942) Significance of large bodies and development of the L-type of colonies in bacterial culture. Journal of Bacteriology, 44, 37 - 73.

Dienes, L. (1943) Reproduction of bacteria from the large bodies of Streptobacillus moniliformis. Proceedings of the Society for Experimental Biology and Medicine, 53, 84 - 86.

Dienes, L. (1944a) Various types of reproductive process in Bacteroides strains. Journal of Bacteriology, 47, 417.

Dienes, L. (1944b) L-type of growth in cultures of haemolytic parainfluenza bacillus. Proceedings of the Society for Experimental Biology and Medicine, 55, 142 - 144.

Dienes, L. (1945) Morphology and nature of the pleuropneumonia group of organisms. Journal of Bacteriology, 50, 441 - 458.

Dienes, L. (1946) Reproductive processes in Proteus cultures. Proceedings of the Society for Experimental Biology and Medicine, 63, 265 - 270.

Dienes, L. (1947a) Isolation of pleuropneumonia-like organisms from pathological specimens with the aid of penicillin. Proceedings of the Society for Experimental Biology and Medicine, 64, 165 - 166.

Dienes, L. (1947b) Isolation of a PPLO-like organism from H. influenzae with the aid of penicillin. Proceedings of the Society for Experimental Biology and Medicine, 64, 166 - 168.

Dienes, L. (1948) Isolation of L-type colonies from typhoid bacilli with the aid of penicillin. Proceedings of the Society for Experimental Biology and Medicine, 68, 589 - 590.

Dienes, L. (1949a) Isolation of an L-type culture from a gram positive spore bearing bacillus. Proceedings of the Society for Experimental Biology and Medicine, 71, 30 - 33.

Dienes, L. (1949b) The development of Proteus cultures in the presence of penicillin. Journal of Bacteriology, 57, 529 - 546.

Dienes, L. (1950) L-type colonies from Clostridia. Proceedings of the Society for Experimental Biology and Medicine, 75, 412 - 415.

Dienes, L. (1953a) L-type cultures isolated from streptococci. Proceedings of the Society for Experimental Biology and Medicine, 83, 579 - 583.

Dienes, L. (1953b) Some new observations on L-forms of bacteria. Journal of Bacteriology, 66, 274 - 278.

Dienes, L. (1953c) Electron micrographs made from L-forms of Proteus and two human strains of pleuropneumonia-like organisms. Journal of Bacteriology, 66, 280 - 283.

Dienes, L. (1964) Comparison of L-forms and PPLO. American Society for Microbiology, 64th Annual Meeting. Bacteriological Proceedings p 55.

Dienes, L. (1968) Morphology and reproductive processes of bacteria with defective cell wall. In Microbial Protoplasts, Spheroplasts and L-forms, ed. Guze, L.B. Ch. 8, pp 74 - 93. Baltimore: Williams & Wilkins Co.

Dienes, L. (1973) Nomenclature of bacterial L-forms and cell wall defective bacteria. Journal of Infectious Diseases, 127, 476 - 477.

Dienes, L. & Bullivant, S. (1968) Morphology and reproductive processes of the L-forms of bacteria. II. Comparative study of L-forms and Mycoplasma with the electron microscope. Journal of Bacteriology, 95, 672 - 687.

Dienes, L. & Smith, W.E. (1942) Reproduction of bacteria from the large bodies of B. funduliformis. Proceedings of the Society for Experimental Biology and Medicine, 51, 297 - 298.

Dienes, L. & Smith, W.E. (1944) The significance of pleomorphism in Bacteroides strains. Journal of Bacteriology, 48, 125 - 153.

Dienes, L. & Weinberger, H.J. (1951) The L-forms of Bacteria. Bacteriological Reviews, 15, 245 - 288.

Dienes, L., Weinberger, H.J. & Madoff, Sarabelle. (1950) The transformation of typhoid bacilli into L-forms under various conditions. Journal of Bacteriology, 59, 755 - 764.

Domingue, G.J. & Schlegel, J.U. (1970) The possible role of microbial L-forms in pyelonephritis. Journal of Urology, 104, 790 - 798.

Domingue, G.J. & Schlegel, J.U. (1977) Novel bacterial structures in human blood: cultural isolation. Infection and Immunity, 15, 621 - 627.

Domingue, G.J., Schlegel, J.U., Heidger, P.M. Jr. & Ehrlich, Melanie. (1977) Novel bacterial structures in human blood: cultural isolation, ultrastructural and biochemical evaluation. In Spheroplasts, protoplasts and L-forms of bacteria, ed. Roux, J. pp 279 - 298. Paris: INSERM.

Duguid, J.P. (1946) The sensitivity of bacteria to the action of penicillin. Edinburgh Medical Journal, 53, 401 - 412.

Duguid, J.P. & Wilkinson, J.F. (1961) Environmentally induced changes in bacterial morphology. In Microbial reaction to environment. 11th Symposium of the Society for General Microbiology. ed. Meynell, G.G. & Gooder, H. pp 69 - 99. Cambridge: University Press.

Edward D.G.ff. (1954) The pleuropneumonia group of organisms: a review, together with some new observations. Journal of General Microbiology, 10, 27 - 64.

Eisenberg, A.D. & Corner, T.R. (1978) Effects of growth temperature on protoplast membrane properties in Bacillus megaterium. Canadian Journal of Microbiology, 24, 386 - 396.

Emmerson, A.M. & Eykyn, Susannah. (1977) Streptococcus mutans endocarditis - a trap for the unwary. British Medical Journal, i, 905.

Eng. J. & Maeland, A. (1982) Correlation of growth of aerobic blood cultures in hypertonic broth with antibiotic therapy. Journal of Clinical Microbiology, 16, 890 - 894.

Fass, R.J. & Barnisham, Jean. (1977) Detection and significance of cell wall defective bacteria in the urine of bacteruric patients. In Spheroplasts, protoplasts and L-forms of bacteria, ed. Roux, J. pp 317 - 326. Paris: INSERM.

Feder, H.M., Olsen, N., McLaughlin, J.C., Bartlett, R.C. & Chameides, L. (1980) Bacterial endocarditis caused by vitamin B6 dependent viridans group streptococcus. Pediatrics, 66, 309 - 312.

Feingold, D.S. (1969) Biology and pathogenicity of microbial spheroplasts and L-forms. New England Journal of Medicine, 281, 1159 - 1170.

Fernandes, P.B. & Panos, C. (1976) Persistence, pathogenicity and morphology of an L-form of Str. pyogenes adapted to physiological isotonic conditions when in immunosuppressed mice. Infection and Immunity, 14, 1228 - 1240.

Fernandes, P.B. & Panos, C. (1977) Wall-less microbial isolate from a human renal biopsy. Journal of Clinical Microbiology, 5, 106 - 107.

Fodor, Katalin, Demiri, Elvira & Alfoldi, L. (1978) Polyethylene glycol-induced fusion of heat inactivated living protoplasts of Bacillus megaterium. Journal of Bacteriology, 135, 68 - 70.

Freimer, E.H. (1964) Streptococcal L-forms and protoplasts and the immunochemical nature of their cytoplasmic membrane. American Society for Microbiology, 64th Annual Meeting. Bacteriological Proceedings, p 60.

Freimer, E.H. & Zabriskie, J.B. (1968) An immunological relationship between the protoplast membrane of Group A streptococci and the sarcolemma, the cell membrane of cardiac muscle. In Microbial Protoplasts, Spheroplasts and L-forms, ed. Guze, L.B. Ch. 37, pp 356 - 371. Baltimore: Williams & Wilkins Company.

Freimer, E.H., Krause, R.M. & McCarty, M. (1959) Studies of L-forms and protoplasts of Group A streptococci, Journal of Experimental Medicine, 110, 853 - 874.

Frenkel, Ayala & Hirsch, W. (1961) Spontaneous development of L-forms of streptococci requiring secretions of other bacteria or sulphydryl compounds for normal growth. Nature, 191, 728 - 730.

Freundt, E.A. (1950) Observations on Dienes L-type growth of bacteria. Acta Pathologica et Microbiologica Scandinavica, 27, 159 - 171.

Freundt, E.A. (1956) Experimental investigations into the pathogenicity of the L phase variant of Streptobacillus moniliformis. Acta Pathologica et Microbiologica Scandinavica, 38, 246 - 258.

Garrett, H.E. (1964) Testing Experimental Hypotheses. In Statistics in Psychology and Education, 5th edition, Ch. 10, pp 247 - 275. London: Longmans.

Garrod, L.P., Lambert, H.P. & O'Grady, F. (1981) Urinary tract infections. In Antibiotic and Chemotherapy, 5th edition, Ch. 21, p 377. Edinburgh & London: Churchill Livingstone.

George, R.H. (1974) The isolation of symbiotic streptococci. Journal of Medical Microbiology, 7, 77 - 83.

George, R.H. & Healing, D.E. (1978) Culture negative endocarditis. Lancet, 1, 215.

Gilmour, W.H. (1984) Environment Behaviour and Health. MBChB & BDS Module 1 : Biostatistics in Medicine and Dentistry. Glasgow: University of Glasgow Departments of Community Medicine and The Dental School.

Glam, I. & Waitkins, Sheena A. (1977) The development of L-phase colonies of Neisseria gonorrhoeae. Journal of General Microbiology, 99, 233 - 236.

Gnarpe, H. (1974) Erythromycin, alkalisation and increased diuresis in the treatment of human L-phase urinary tract infections. Scandinavian Journal of Infectious Diseases, 6, 75 - 78.

Gnarpe, H. & Edebo L. (1970) Conditions affecting the viability of spheroplasts in urine. Infection and Immunity 1, 300 - 304.

Godzeski, C.W. (1968) In vivo persistence of L-phase bacteria. In Microbial Protoplasts, Spheroplasts and L-forms, ed. Guze L.B. Ch. 39, pp 379 - 390. Baltimore: Williams and Wilkins Co.

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

Goldschmidt, Millicent C. & Wyss, O. (1967) The role of Tris in EDTA toxicity and lysozyme lysis. Journal of General Microbiology, 47, 421 - 431.

Gomez, R.F. & Sinskey, A.J. (1973) Deoxyribonucleic acid breaks in heated Salmonella typhimurium LT-2 after exposure to nutritionally complex media. Journal of Bacteriology, 115, 522 - 528.

Gomez, R.F., Sinskey, A.J., Davies, R. & Labuza, T.P. (1973) Minimal medium recovery of heated Salmonella typhimurium LT-2. Journal of General Microbiology, 74, 267 - 274.

Gooder, H. (1964) L-type growth of streptococcal protoplasts. American Society for Microbiology, 64th Annual Meeting. Bacteriological Proceedings, p 60.

Gooder, H. (1968) Streptococcal protoplasts and L-form growth induced by muralytic enzymes. In Microbial Protoplasts, Spheroplasts and L-forms, ed Guze, L.B. Ch. 4, pp 40 - 51. Baltimore: Williams & Wilkins Co.

Gooder, H. & Maxted, W.R. (1958) Protoplasts of Group β-haemolytic streptococci. Nature, 182, 808 - 809.

Gooder, H. & Maxted, W.R. (1961) External factors influencing structure and activities of Streptococcus pyogenes. In Microbial Reaction to Environment. 11th Symposium of the Society for General Microbiology, ed. Meynell, G.G. & Gooder, H. pp 151 - 173. Cambridge: University Press.

Gordon, S.L., Greer, R.B. & Craig, C.P. (1971) Recurrent osteomyelitis. Report of four cases culturing L-form variants of staphylococci. Journal of Bone and Joint Surgery; American Volume, 53, 1150 - 1156.

Greenwood, D. (1985) Phenotypic resistance to antimicrobial agents. Journal of Antimicrobial Chemotherapy, 15, 653 - 658.

Greenwood, D. & O'Grady, F. (1972) The effect of osmolality on the response of Escherichia coli and Proteus mirabilis to penicillins. British Journal of Experimental Pathology, 53, 457 - 464.

Greenwood, D. & O'Grady, F. (1973) FL 1060: a new beta-lactam antibiotic with novel properties. Journal of Clinical Pathology, 26, 1 - 6.

Gunsalus, I.C. (1984) Learning. In Annual Review of Microbiology, ed. Ornston, L.N., 38, pp xviii - xxiii. Palo Alto, California: Annual Reviews Inc.

Gutman, Laura T., Schaller, Jane, Wedgwood, R.J. (1967) Bacterial L-forms in relapsing urinary tract infection. Lancet, 1, 464 - 466.

Gutman, Laura T., Turck, M., Petersdorf, R.G. & Wedgwood, R.J. (1965) Significance of bacterial variants in urine of patients with chronic bacteruria. Journal of Clinical Investigation, 44, 1945 - 1952.

Gutman, Laura T., Winterbauer, R.H., Turck, M., Wedgwood, R.J. & Petersdorf, R.G. (1968) The role of bacterial variants in experimental pyelonephritis. In Microbial Protoplasts, Spheroplasts and L-forms, ed. Guze, L.B. Ch. 40, pp 391 - 395. Baltimore: Williams & Wilkins Co.

Guze, L.B. (1968) Preface. In Microbial Protoplasts, Spheroplasts and L-forms, Preface: pp vii- viii. Baltimore: Williams & Wilkins Co.

Guze, L.B. & Kalmanson, G.M. (1964) Persistence of Bacterial 'Protoplast' form after apparent cure of pyelonephritis in rats. Science, 143, 1340 - 1341.

Hadley, P., Delves, Edna & Klimek, J. (1931) The filterable forms of bacteria. I. A filterable stage in the life history of the Shiga dysentery bacillus. Journal of Infectious Diseases, 48, 1 - 159.

Haller, G.J. & Lynn, R.J. (1968) The response observed in rabbits following implantation of diffusion chambers containing L-forms of Group A beta haemolytic streptococci. In Microbial Protoplasts, Spheroplasts and L-forms, ed. Guze, L.B. Ch. 36, pp 352 - 355. Baltimore: Williams & Wilkins Co.

Hamburger, M. (1968) Wall-defective bacteria. Archives of Internal Medicine, 122, 175 - 178.

Hancock, R.E.W. (1981) Aminoglycoside uptake and mode of action - with special reference to streptomycin and gentamicin. I. Antagonists and mutants. Journal of Antimicrobial Chemotherapy, 8, 249 - 276.

Higgins, R., Biberstein, E.L. & Jang, S.S. (1984) Nutritionally variant streptococci from corneal ulcers in horses. Journal of Clinical Microbiology, 20, 1130 - 1134.

Hughes, W.H. (1956) The structure and development of the induced long forms of bacteria. In Bacterial Anatomy, 6th Symposium of the Society for General Microbiology, ed. Spooner, E.T.C. & Stocker, B.A.D. pp 341 - 359. Cambridge University Press.

Hyryniewicz, Waleria. (1977) Streptococcal L-forms and their properties. In Spheroplasts, protoplasts and L-forms of bacteria, ed. Roux, J. pp 39 - 56. Paris:INSERM

Iesmantaite, N.A., Ptasekas, R.S., Astrauskaite, I.I., Austrauskas, V.I. & Rimkunas, A.I. (1975) Experimental study of the role of the L-forms of haemolytic streptococci in infectious pathology. Zhurnal Mikrobiologii, Epidemiologii i Immunobiologii, 2, 62 - 64. (Russian: Abstract).

Jephcott, A.E. (1981) Investigation of gonococcal infection. Association of Clinical Pathologists Broadsheet 100, p 3. London: British Medical Association.

Jones, H.E. & Park, R.W.A. (1967) The short forms and long forms of Proteus. Journal of General Microbiology, 47, 359 - 367.

Joseph, R & Shockman, G.D. (1974) Autolytic formation of protoplasts (autoplasts) of Str. faecalis 9790: release of cell wall, autolysin and formation of stable autoplasts. Journal of Bacteriology, 118, 735 - 746.

Kagan, B.M. (1968) Role of L-forms in staphylococcal infection. In Microbial protoplasts, spheroplasts and L forms, ed. Guze, L.B. Ch. 38, pp 372 - 378. Baltimore: Williams & Wilkins Co.

Kagan, G., Vulfovitch, Y., Gusman, B. & Raskova, T. (1977) Persistence and pathological effect of streptococcal L-forms in vivo. In Spheroplasts, protoplasts and L-forms of bacteria, ed. Roux, J. pp 247 - 258. Paris: INSERM.

Kagnoff, M.F. (1978) On the etiology of Crohns disease. Gastroenterology, 75, 526 - 527.

Kendall, A.I., Friedman, T.E. & Ishikawa, M. (1930) Methods for study of 'resting' bacteria: studies in bacterial metabolism. Journal of Infectious Diseases, 47, 186 - 193.

King, C.H., Shlaes, D.M. & Dul, M.J. (1983) Infection caused by thymidine-requiring, trimethoprim-resistant bacteria. Journal of Clinical Microbiology, 18, 79 - 83.

Klieneberger, Emmy. (1935) The natural occurrence of pleuropneumonia like organisms in apparent symbiosis with Streptobacillus moniliformis and other bacteria. Journal of Pathology and Bacteriology, 40, 93 - 100.

Klieneberger, Emmy. (1936) Further studies on Streptobacillus moniliformis and its symbiont. Journal of Pathology and Bacteriology, 42, 587 - 598.

Klieneberger, Emmy. (1942) Some new observations bearing on the nature of the pleuropneumonia-like organisms known as Ll associated with Streptobacillus moniliformis. Journal of Hygiene, 42, 485 - 497.

Klieneberger-Nobel, Emmy. (1949) Origin, development and significance of L-forms in bacterial cultures. Journal of General Microbiology, 3, 434 - 443.

Klieneberger-Nobel, Emmy. (1951) The L-cycle: A process of regeneration in bacteria. Journal of General Microbiology, 5, 525 - 530.

Klieneberger-Nobel, Emmy. (1960) L-forms of bacteria. In The Bacteria. A Treatise on Structure and Function, ed. Gunsalus, I.C. & Stanier, R.Y. Volume 1. Structure. Ch. 7, pp 361 - 386. New York & London: Academic Press.

Klieneberger-Nobel, Emmy. (1962) Similarities and differences between pleuropneumonia-like organisms and L-forms of bacteria. In Pleuropneumonia-like Organisms (PPLO) Mycoplasmataceae. Ch. VII, pp 75 - 150. London & New York: Academic Press.

Klieneberger-Nobel, Emmy. (1980) Memoirs. London: Academic Press.

Klodnitskaia, N.S. (1962) On the problem of the etiology of Scarlet Fever. IV. Isolation of L-forms of streptococcus from penicillin-treated scarlet fever patients and experience in the production of oohemocultures. Zhurnal Mikrobiologii, Epidemiologii Immunobiologii, 33, 31 - 35. (Russian: abstract).

Klodnitskaia, S.N. (1973) A further study of L-forms of streptococcus in blood cultures from patients with non specific ulcerative colitis, treated conservatively and by operation. Zhurnal Mikrobiologii, Epidemiologii, Immunobiologii, 50, 89 - 93. (Russian: part read in translation).

Klodnitskaia, S.N. (1978) Further study of the L-forms of streptococci in hemocultures from patients with rheumatism depending on the course of the disease and the methods of cultivation. Zhurnal Mikrobiologii, Epidermiologii, Immunobiologii, 4, 119 - 125. (Russian: abstract).

Klodnitskaia, S.N., Stepanov, P.N. & Adamchuk, V.D. (1977) L-forms of streptococci in the hemocultures from patients suffering from rheumatism at the active and inactive phase. Zhurnal Mikrobiologii, Epidemiologii, Immunobiologii, 9, 127 - 131. (Russian: abstract)

Lamanna, C. (1944) A non-life cycle explanation of the diphtheroid streptococcus from endocarditis. Journal of Bacteriology, 47, 327 - 334.

Lamanna, C., Mallette, M.F. & Zimmerman, L. (1973a) Structure of Eubacteria. The Cell Wall. In Basic Bacteriology, Its Biological and Chemical Background. 4th edition. Ch. 5, pp 183 - 196. Baltimore: Williams & Wilkins Co.

Lamanna, C., Mallette, M.F. & Zimmerman, L. (1973b) Structure of Eubacteria. Protoplast. In Basic Bacteriology. Its Biological and Chemical Background, 4th edition. Ch. 5, pp 196 - 201. Baltimore: Williams & Wilkins Co.

Lamanna, C., Mallette, M.F. & Zimmerman, L. (1973c) Growth of Bacteria. Growth of the Bacterium. In Basic Bacteriology. Its Biological and Chemical Background. 4th edition. Ch. 7, p. 348. Baltimore: Williams & Wilkins Co.

Lamanna, C., Mallette M.F. & Zimmerman, L. (1973d) Nutrition of Bacteria. In Basic Bacteriology. Its Biological and Chemical Background. 4th edition. Ch. 10 p. 565. Baltimore: Williams and Wilkins Co.

Lamanna, C., Mallette, M.F. & Zimmerman, L. (1973e) Variation and Genetics in Bacteria. Mutation. In Basic Bacteriology. Its Biological and Chemical Background. 4th edition. Ch. 12, pp 614 - 617. Baltimore: Williams & Wilkins Co.

Lamanna, C., Mallette, M.F. & Zimmerman, L. (1973f) Intermediate Metabolism in Bacteria. Amino Acids. In Basic Bacteriology. Its Biological and Chemical Background. 4th edition. Ch. 13, pp 869 - 891. Baltimore: Williams & Wilkins Co.

Lamanna, C., Mallette, M.F., Zimmerman, L. (1973g) Intermediate Metabolism in Bacteria. Metabolism of Nucelotides. In Basic Bacteriology. Its Biological and Chemical Background. 4th edition. Ch. 13, pp 912 - 913. Baltimore: Williams & Wilkins Co.

Lamanna, C., Mallette, M.F. & Zimmerman, L. (1973h) Biosynthesis of Macromolecules in Bacteria. Biosynthesis of Primary Protein Structure. In Basic Bacteriology. Its Biological and Chemical Background. 4th edition. Ch. 14, pp 981 - 995. Baltimore: Williams & Wilkins Co.

Lamanna, C., Mallette, M.F. & Zimmerman, L. (1973i) Chemical Disinfection and Chemotherapy. Chemotherapeutic Agents. In Basic Bacteriology. Its Biological and Chemical Background. 4th edition. Ch. 14, pp 1077 - 1082. Baltimore: Williams & Wilkins Co.

Lancet. (1977) Culture negative endocarditis. (Leading article). 2, 1164 - 1165.

Lancet. (1985) Emmy Klieneberger-Nobel. (Obituary). 2, 960 - 961.

Landman, O.E. (1968) Protoplasts, spheroplasts and L-forms viewed as a genetic system. In Microbial Protoplasts, Spheroplasts and L-forms, ed. Guze, L.B. Ch. 32, pp 319 - 332. Baltimore: Williams and Wilkins Co.

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-form. Journal of Bacteriology, 75, 567 - 576.

Landman, O.E. & de Castro-Costa, Maria R. (1976) Reversion of protoplasts and L-forms of bacilli. In Microbial and Plant Protoplasts, ed. Peberdy, J.F. Rose, A.H., Rogers, H.J. & Cocking, E.C. pp 201 - 217. London: Academic Press.

Landman, O.E. de Castro-Costa, Maria R. & Bond, Enriqueta, C. (1977) Mechanisms of stability and reversion of mass conversion stable L-forms of Bacillus subtilis. In Microbiology - 1977, ed. Schlessinger, D. pp 35 - 43. Washington: American Society for Microbiology.

Landman, O.E. & Ginoza, H.S. (1961) Genetic nature of stable L-forms of Salmonella paratyphi. Journal of Bacteriology, 81, 875 - 886.

Landman, O.E. & Halle, S. (1963) Enzymatically and physically induced inheritance changes in Bacillus subtilis. Journal of Molecular Biology, 7, 721 - 738.

Langenfeld, M.G. & Smith, P.F. (1963) Phosphorus distribution in pleuropneumonia-like and L-type organisms. Journal of Bacteriology, 86, 1216 - 1219.

Lapinski, E.M. & Flakas, D.E. (1967) Induction of L-forms of H. influenzae in culture and their demonstration in human bronchial secretions. Journal of Bacteriology, 93, 1438 - 1445.

Lapinski, E.M. & Flakas, E.D. (1970) Reversal of penicillin-induced L-phase growth of Haemophilus influenzae by spermine and its effects on antibiotic susceptibility. Infection and Immunity, 1, 474 - 478.

LaScolea, L.J. Sullivan, T.D., Dryja, Diane & Neter, E. (1983) Advantages of BACTEC hypertonic culture medium for detection of Haemophilus influenzae bacteremia in children. Journal of Clinical Microbiology, 17, 1177 - 1179.

Lederberg, J. & St. Clair, Jacqueline. (1958) Protoplasts and L-type growth of Escherichia coli. Journal of Bacteriology, 75, 143 - 160.

Lev, M. (1968) Vitamin K deficiency in Fusiformis nigrescens, I. Influence on whole cells and cell envelope characteristics. Journal of Bacteriology, 95, 2317 - 2324.

Lev, M. (1981) Antibody against revertant forms of cell-wall-deficient bacterial variant from patients with Crohns disease. Lancet, 2, 941.

Louria, D.B. Kaminski, Theresa, Grieco, M. & Singer, J. (1969) Aberrant forms of bacteria and fungi found in blood or cerebrospinal fluid. Archives of Internal Medicine, 124, 39 - 48.

Louria, D.N., Kaminski, Theresa, Kapila, K., Tecson, F. & Smith, L. (1976) Study on the usefulness of hypertonic culture media. Journal of Clinical Microbiology, 4, 208-213.

Low, G.C. (1930) Deficiency Diseases. Vitamins. In A Textbook of the Practice of Medicine, ed. Price, F.W. 3rd edition, Section VI. Diseases of Metabolism. pp 431 - 451. London: Humphrey Milford Oxford University Press.

Lugtenberg, E.J., de Haas-Menger, Liesbeth & Ruyters, W.H. (1972) Murein synthesis and identification of cell wall precursors of temperature sensitive lysis mutants of Escherichia coli. Journal of Bacteriology, 109, 326 - 355.

Lynn, R.J. & Haller, G.J. (1968) Bacterial L-forms as immunogenic agents. In Microbial Protoplasts, Spheroplasts and L-forms, ed. Guze, L.B. Ch. 26, pp 270 - 278. Baltimore: Williams & Wilkins Co.

Madoff, Sarabelle. (1974) Louis L. Dienes (1885-1974). Obituary. ASM News, 40, 467 - 468. Washington: American Society for Microbiology.

Madoff, Sarabelle. (1977) L-forms of Haemophilus influenzae: morphology and ultrastructure. In Spheroplasts, Protoplasts and L-forms of bacteria, ed. Roux, J. pp 15 - 26. Paris:INSERM

Mandelstam, J. & McQuillen, K. (1973a) (Eds) Growth; Cells and Populations. Unbalanced growth. In Biochemistry of Bacterial Growth. 2nd edition. Ch. 2, pp 146 - 151. Oxford: Blackwell Scientific Publications.

Mandelstam, J. & McQuillen, K. (1973b) (Eds) Class II Reactions: Synthesis of Small Molecules. The aspartate family. In Biochemistry of Bacterial Growth. 2nd edition. Ch. 4, pp 211 - 223. Oxford: Blackwell Scientific Publications.

Mandelstam, J. & McQuillen, K. (1973c) (Eds) Appendix B. Enzyme Mechanisms: Functions of Vitamins and Co-enzymes. In Biochemistry of Bacterial Growth. 2nd edition. pp 536 - 547. Oxford: Blackwell Scientific Publications.

Maniloff, J. (1983) Evolution of wall-less prokaryotes. In Annual Review of Microbiology, ed. Ornston, L. Nicholas. Ch. 37, pp 477 - 499. Palo Alto, California: Annual Reviews Inc.

Marquis, R.E. & Corner, T.R. (1976) Isolation and properties of bacterial protoplasts. In Microbial and Plant Protoplasts, ed. Peberdy, J.F., Rose, A.H., Rogers, H.J. & Cocking, E.C. pp 1 - 22. London: Academic Press.

Marston, Judith H. (1968) Production and cultivation of staphylococcal L-forms. In Microbial Protoplasts, Spheroplasts and L forms, ed. Guze, L.B. Ch. 20, pp 212 - 220. Baltimore: Williams & Wilkins Co.

Martindale. (1958) The Extra Pharmacopoeia. Published by direction of the Council of the Pharmaceutical Society of Great Britain. 24th edition, p 163. London: The Pharmaceutical Press.

Mashkov, A.V. (1974) A study of the properties of L-forms of bacteria isolated from ill children. Zhurnal Mikrobiologii, Epidemiologii i Immunobiologii, 12, 62 - 65. (Russian: part read in translation).

Maskell, Rosalind & Okubadejo, O.A. (1978) Thymidine mutants. Journal of Clinical Pathology, 31, 808.

Maskell, Rosalind, Okubadejo, O.A. Payne, R.H. & Pead, Linda. (1978) Human infections with thymidine requiring bacteria. Journal of Medical Microbiology, 11, 33 - 45.

Mattman, Lida H. (1968) L-forms isolated from infections. In Microbial Protoplasts, Spheroplasts and L-forms, ed. Guze, L.B. Ch. 47, pp 472 - 483. Baltimore: Williams & Wilkins Co.

Mattman, Lida H. (1974a) Definitions. In Cell Wall Deficient Forms. Ch. 2, pp 9 - 11. Cleveland, Ohio: CRC Press.

Mattman, Lida H. (1974b) Intracellular growth of CWD forms. In Cell Wall Deficient Forms. Ch. 19. Cleveland, Ohio: CRC Press.

Mattman, Lida H., Tunstall, L.H. & Rossmore, H.W. (1961) Induction and characteristics of staphylococcal L-forms. Canadian Journal of Microbiology, 7, 705 - 713.

Maxted, W.R. (1972) Specific procedures and requirements for the isolation, growth and maintenance of the L-phase of some microbial groups. In Methods in Microbiology, ed. Norris, J.R. & Ribbons, D.W. Vol. 7A. Ch. VIII, pp 423 - 449. London: Academic Press.

May, J.R. (1975) Antibiotics used in sequence in the treatment of chronic Haemophilus influenzae respiratory infections. In Clinical Use of Combinations of Antibiotics, ed. Klastersky, J. & Jawetz, E. Ch. 5, pp 66 - 70. London: Hodder & Stoughton.

Medill, M.A. & O'Kane, D.J. (1954) A synthetic medium for the L-type colonies of Proteus. Journal of Bacteriology, 68, 530 - 533.

Mellon, R.R. (1927) Studies in Microbic Heredity. XII. Microbic dissociation in vivo as illustrated by a case of subacute septicopyaemia. Journal of Bacteriology, 13. 99-111.

Meynell, G.G. and Meynell Elinor (1965) In Theory and Practice in Experimental Bacteriology. Ch. 2 p.50-57. Cambridge: University Press.

MGH News. (1970) 'Strange Growth' turns out to be just that. 29, No. 2. Boston: News Office Massachusetts General Hospital, Fruit Street, 02114.

Miller, I.L., Szigray, R.M. & Landman, O.E. (1967) The formation of protoplasts and quasi-spheroplasts in normal and chloramphenicol-pretreated Bacillus subtilis. Journal of General Microbiology, 49, 513 - 525.

Mitchell, P. & Moyle, Jennifer. (1956) Osmotic function and structure in bacteria. In Bacterial Anatomy. 6th Symposium of the Society for General Microbiology. ed. Spooner, E.T.C. & Stocker, B.D. pp 150 - 180. Cambridge: University Press.

Montgomerie, JZ., Kalmanson, GH., & Guze, LB. (1967) Effect of osmotic stabilisation on protoplasts and bacterial forms of Streptococcus faecalis. Journal of Laboratory and Clinical Medicine 70, 539 - 553.

Montgomerie, J.Z., Kalmanson, G.M. Hubert, E.G. & Guze, L.B. (1972) Osmotic stability and sodium and potassium content of L-forms of Streptococcus faecalis. Journal of Bacteriology, 110, 624 - 627.

Montgomerie, J.Z., Kaplan, D., Schotz, M. & Guze, L.B. (1976) Effect of serum on phospholipids of L-forms of Streptococcus faecalis. Infection and Immunity, 14, 951 - 954.

Mortimer, E.A., Cushing, Alice H., Jayanetra, P. & Bunch, G.P. (1972) Studies of streptococcal and staphylococcal L-forms in vivo. In Streptococci and Streptococcal Diseases, ed. Wannamaker, L.W. & Matsen, J.M. Ch. 25, pp 433 - 450. London: Academic Press.

Mossel, D.A.A. & van Netten, P. (1984) Harmful effects of selective media on stressed micro-organisms: nature and remedies. In The Revival of Injured Microbes, Society for Applied Microbiology Symposium Series 12. ed. Andrew, M.H.E. & Russell, A.D. pp 335 - 336. London: Academic Press.

Moustardier, G., Brisou, J. & Perrey, M. (1953) Reversion to normal bacteria of L-organisms isolated from cultures taken from non-bacterial urethritis with inclusions. Annales de l'Institut Pasteur, 85, 520 - 525. (French: read in original language).

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

McCarthy, L.R. & Bottone, E.J. (1974) Bacteremia and endocarditis caused by satelliting streptococci. American Journal of Clinical Pathology, 61, 585 - 591.

McDermott, W. (1958) Microbial Persistence. Yale Journal of Biology and Medicine, 30, 257 - 291.

McGee, Z.A., Ratner, H.B., Bryant, R.E. Rosenthal, A.S. & Koenig, M.G. (1972) An antibody complement system in human serum lethal to L-phase variants of bacteria. Journal of Infectious Diseases, 125, 231 - 242.

McGee, Z.A. Wittler, Ruth G. & Gooder, H. (1971) Wall-defective microbial variants: terminology and experimental design. Journal of Infectious Diseases, 123, 433 - 438.

McQuillen, K. (1956) Capabilities of bacterial protoplasts. In Bacterial Anatomy. 6th Symposium of the Society for General Microbiology. ed. Spooner, E.T.C. & Stocker, B.A.D. pp 127 - 149. Cambridge: University Press.

McQuillen, K. (1960) Bacterial Protoplasts. In The Bacteria, ed. Gunsalus, I.C. & Stanier, R.Y. Vol. I. Structure. Ch. 6, pp 249 - 359. New York: Academic Press.

McQuillen, K. (1973) Enzyme mechanisms: functions of vitamins and co-enzymes. In Biochemistry of Bacterial Growth, ed. Mandelstam, J. & McQuillen, K. 2nd edition, p 547. Oxford: Blackwell Scientific Publications.

Narasimhan, S.L. & Weinstein, A.J. (1980) Infective endocarditis due to a nutritionally deficient streptococcus. Journal of Pediatrics, 96, 61 - 62.

Nativelle, R. & Deparis, M. (1960) An evolving bacterial form in blood cultures. Presse Medicale, 68, 571 - 574. (French: read in original language).

Necas, O. (1961) Physical conditions as important factors for the regeneration of naked yeast protoplasts. Nature, 192, 580 - 581.

Nimmo, Lynn N. & Blazevic, Donna J. (1969) Selection of media for the isolation of common bacterial L-phase organisms from a clinical specimen. Applied Microbiology, 18, 535 - 541.

Oerskov, von J. (1931) Proof and in vitro cultivation of some presumed saprophytic virus species. Zentralblatt fur Bakteriologie, Parasitenkunde Infectionskrankenheiten und Hygiene, 120, 310 - 317. (German: read in translation).

Olivard, Joanne & Snell, E.E. (1955a) Growth and enzymatic activities of vitamin B6 analogues, I. D-alanine synthesis. Journal of Biological Chemistry, 213, 203 - 214.

Olivard, Joanne & Snell, E.E. (1955b) Growth and enzymatic activities of vitamin B6 analogues. II. Synthesis of miscellaneous amino acids. Journal of Biological Chemistry, 213, 215 - 228.

Orskov, J. (1942) Streptobacillus moniliformis and the morphology of its variants. Acta Pathologica et Microbiologica Scandinavica, 19, 575 - 578.

The Oxoid Manual of Culture Media, Ingredients and Other Laboratory Services, 5th edition. (1982) Oxoid Ltd., Wade Road, Basingstoke, Hampshire.

Parent, K. & Mitchell, P.D. (1976) Bacterial variants: etiologic agent in Crohns disease Gastroenterology, 71, 365 - 368.

Parent, K. & Mitchell, P. (1978) Cell wall-defective variants of pseudomonas-like (group Va) bacteria in Crohns disease. Gastroenterology, 75, 368 - 372.

Parks, L.C., Shockman, G.D. & Higgins, M.L. (1980) Growth of Streptococcus mutans protoplasts is not inhibited by penicillin. Journal of Bacteriology, 143, 1491 - 1497.

Pease, Phyllis E. (1965) The reproductive micro-elements of bacteria. In L-forms, Episomes, and Auto-Immune Disease, Ch. 1, pp 1 - 6. Edinburgh & London: E. & S. Livingstone.

Peirson, Octavia & Dienes L. (1934) Conditions of halo formation around subtilis colonies and growth of halo transplants. Proceedings of the Society for Experimental Biology and Medicine, 31, 1208 - 1211.

Peterson, C.E., Cook, J.L. & Burke, J.P. (1981) Media-dependent subculture of nutritionally variant streptococci. American Journal of Clinical Pathology, 75, 634 - 636.

Phair, J.P., Watakunakorn, C., Linneman, C. & Carleton, Judith. (1974) Attempts to isolate wall-defective microbial variants from clinical specimens. American Journal of Clinical Pathology, 62, 601 - 603.

Piepkorn, M.W. & Reichenbach, D.D. (1978) Infective endocarditis associated with cell wall-deficient bacteria: electron microscopic findings in four cases. Human Pathology, 9, 163 - 173.

Pierce, Cynthia H. (1942) Streptobacillus moniliformis, its associated L-form and other pleuropneumonia-like organisms. Journal of Bacteriology, 43, 780.

Pierson, M.D., Gomez, R.F. & Martin, S.E. (1978) The involvement of nucleic acids in bacterial injury. Advances in Applied Microbiology, 23, 263 - 285.

Pontecorvo, G. (1949) Auxanographic Techniques in Biochemical Genetics. Journal of General Microbiology, 3, 122 - 126.

Razin, S. & Oliver, O. (1961) Morphogenesis of mycoplasma and bacterial L-form colonies. Journal of General Microbiology, 24, 225 - 237.

Repaske, R. (1958) Lysis of gram negative organisms and the use of versene. Biochimica et Biophysica Acta, 30, 225 - 232.

Reynolds, E.S. (1963) The use of lead citrate at high pH as an electron opaque stain in electron microscopy. Journal of Cell Biology, 17, 208 - 212.

Reynolds, P.E. (1973) The bacterial cell: major structures. In Biochemistry of Bacterial Growth, ed. Mandelstam, J. & McQuillen, K. 2nd edition, Part II. Ch. 1, pp 100 - 103. Oxford: Blackwell Scientific Publications.

Roberts, D., Higgs, Elizabeth, Rutman, A. & Cole, P. (1984) Isolation of spheroplastic forms of Haemophilus influenzae from sputum in conventionally treated bronchial sepsis using selective medium supplemented with N-acetyl-D-glucosamine: possible reservoir for re-emergence of infection. British Medical Journal, 289, 1409 - 1412.

Roberts, D.E., Ingold, A., Want, Susan V. & May, R. (1974) Osmotically stable L-forms of Haemophilus influenzae and their signficance in testing sensitivity to penicillin. Journal of Clinical Pathology, 27, 560 - 564.

Roberts, K.B. & Sidlak, M.J. (1979) Satellite streptococci: a major cause of 'negative' blood cultures in bacterial endocarditis? Journal of the American Medical Association, 241, 2293 - 2294.

Roberts, R.B., Krieger, Arlene G., Schiller, N.L. & Gross, Katharine C. (1979) Viridans streptococcal endocarditis; the role of various species, including pyridoxal-dependent streptococci. Reviews of Infectious Diseases, 1, 955 - 966.

Rogers, H.J. Perkins, H.R. & Ward, J.B. (1980) Membranes of bacteria lacking peptidoglycan. In Microbial Cell Walls and Membranes. Ch. 5, pp 176 - 189. London: Chapman & Hall, Ltd.

Rogers, H.J., Ward, J.B. & Burdett, I.D. (1978) Structure and growth of the walls of Gram-positive bacteria. In Relations between structure and function in the prokaryotic cell, ed. Rogers, H.J. & Ward, J.B. pp 139 - 175. Cambridge: University Press.

Rogers, H.J., Ward, J.B. & Elliott, T.S.J. (1976) Biosynthesis of wall polymers by bacterial protoplasts. In Microbial and Plant Protoplasts, ed. Peberdy, J.F., Rose, A.H., Rogers, H.J. & Cocking, E.C. pp 219 - 235. London: Academic Press.

Rosenthal, R.S. & Shockman, G.D. (1975a) Characterisation of the presumed peptide cross links on the soluble peptidoglycan fragments synthesised by protoplasts of Str. faecalis. Journal of Bacteriology, 124, 410 - 418.

Rosenthal, R.S. & Shockman, G.D. (1975b) Synthesis of peptidoglycan in the form of soluble glycan chains by growing protoplasts (autoplasts) of Str. faecalis. Journal of Bacteriology, 124, 419 - 423.

Rosner, R. (1972) A quantitative evaluation of three blood culture systems. American Journal of Clinical Pathology, 57, 220 - 227.

Roux, J. (1977) In vivo study of L-forms, spheroplasts, protoplasts: Interaction with cells and tissues. In Spheroplasts, Protoplasts and L-forms of Bacteria, ed. Roux, J. pp 235 - 246. Paris:INSERM.

Salton, M.J.R. (1960) Surface layers of the bacterial cell. In The Bacteria, ed. Gunsalus, I.C. & Stanier, R.Y. Vol. 1, Structure. Ch. 3, pp 97 - 151. New York & London: Academic Press.

Schonfeld, J.K. (1974) Proceedings: Reversibility of the L-phase. Antonie van Leeuwenhoek, 40, 315.

Schonfeld, J.K. & de Bruijn, W.C. (1977) Ultrastructure of the transition from bacterial to L-phase and vice versa. In Spheroplasts, Protoplasts and L-forms of Bacteria. ed. Roux, J. pp 27 - 37. Paris:INSERM.

Sedgwick, S.G. & Bridges, B.A. (1972) Evidence of indirect production of DNA strand scissions during mild heating of Escherichia coli. Journal of General Microbiology, 71, 191 - 193.

Shafi, A., Sopher, S., Lev, M. & Das, K.M. (1981) An antibody against revertant forms of cell-wall deficient bacterial variants in sera from patients with Crohns disease. Lancet, 2, 332 - 334.

Sherman, S.P. & Washington, J.A. (1978) Pyridoxine inhibition of a symbiotic streptococcus. American Journal of Clinical Pathology, 70, 689 - 690.

Shulb'tsev, G.P., Burtsev, B.E., Kochemasova, Z.N., Kassterskaya, N.G. & Basanova, D.Y. (1973) Detection of L-forms of bacteria in pyelonephritis. Urologiia i Nefrologiia, 38, 14 - 16. (Russian: abstract).

Smith, W.E., Hillier, J. & Mudd, S. (1948) Electron micrograph studies of two strains of pleuropneumonia-like (L) organisms of human derivation. Journal of Bacteriology, 56, 589 - 602.

Sparham, P.D., Lobban, D.I. & Speller, D.C.E. (1978) Thymidine-requiring Staphylococcus aureus. Lancet, 1, 104 - 105.

Sparham, P.D., Lobban, D.I. & Speller, D.C.E. (1978) Isolation of Staphylococcus aureus from sputum in cystic fibrosis. Journal of Clinical Pathology, 31, 913 - 918.

Stewart, S.M., Alexander, W.R.M. & Duthie, J.J. (1969) Isolation of diphtheroid bacilli from synovial membrane and fluid in rheumatoid arthritis. Annals of the Rheumatic Diseases, 28, 477 - 487.

Stokes, E. Joan. (1974) Blood Culture Technique. Association of Clinical Pathologists Broadsheet 81. London: British Medical Association.

Stokes, E. Joan & Waterworth, Pamela M. (1972) Antibiotic sensitivity tests by diffusion methods. Association of Clinical Pathologists Broadsheet 55. London: Journal of Clinical Pathology, BMA House.

Swartz, M.N. & Dvorak, H.F. (1974) Obituary. Dr. Louis Dienes. Journal of Infectious Diseases, 130, 89 - 91.

Tabor, Celia W. (1962) Stabilisation of protoplasts and spheroplasts by spermine and other polyamines. Journal of Bacteriology, 83, 1101 - 1111.

Tedeschi, G.G. & Santarelli, I. (1977) Electron microscopical evidence of the presence of unstable L-forms of Staphylococcus epidermidis in human platelets. In Spheroplasts, Protoplasts and L-forms of Bacteria, ed. Roux, J. pp 341 - 344. Paris:INSERM.

Tillotson, G.S. (1981) Evaluation of ten blood culture systems to isolate a pyridoxal-dependent streptococcus. Journal of Clinical Pathology, 34, 930 - 934.

Timakov, V. (1977) Bacterial L-forms in Pathology. In Spheroplasts, Protoplasts and L-forms of Bacteria, ed. Roux, J. pp 357 - 364. Paris:INSERM.

Tomley, Fiona M. & Russell, C. (1978) The formation of bacillary forms by streptococci. Journal of Applied Bacteriology, 43, x - xi.

van Rooyen, C.E. (1936) The biology, pathogenesis and classification of Streptobacillus moniliformis. Journal of Pathology and Bacteriology, 43, 455 - 472.

Voureka, A. (1951) Bacterial variants in patients treated with chloramphenicol. Lancet, 1, 27 - 28.

Warren, J. (1942) Observations on some biological characteristics of organisms of the pleuropneumonia group. Journal of Bacteriology, 43, 211 - 228.

Washington, J.A. II. (1978) Conventional approaches to blood culture. In The Detection of Septicaemia, ed. Washington, J.A. II. Ch. 2, pp 41 - 87. Florida: CRC Press.

Washington, J.A. II., Hall, Marsha & Warren, E. (1975) Evaluation of blood culture media supplemented with sucrose or with cysteine. Journal of Clinical Microbiology, 1, 79 - 81.

Watakunakorn, C., Carleton, Judith, Goldberg, L.M. & Hamburger, M. (1968) Candida endocarditis surrounding a Starr-Edwards prosthetic valve. Recovery of candida in hypertonic medium during treatment. Archives of Internal Medicine, 121, 243 - 245.

Weibull, C. (1956) Bacterial protoplasts: their formation and characteristics. In Bacterial Anatomy 6th Symposium of the Society for General Microbiology. ed. Spooner, E.T.C. & Stocker, B.A.D. pp 111 - 125. Cambridge: University Press.

Weibull, C., Bicket, 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. Journal of Bacteriology, 93, 1143 - 1159.

Weinberger, H.J., Madoff, Sarabelle & Dienes, L. (1950) The properties of L-forms isolated from Salmonella and the isolation of L-forms from Shigella. Journal of Bacteriology, 59, 765 - 775.

Weinstein, M.P., Reller, L.B., Mirrett, S. & Wang, W.L.L. (1982) Controlled evaluation of hypertonic sucrose medium for detection of bacteremia and fungemia in supplemented peptone broth. Journal of Clinical Microbiology, 16, 490 - 491.

Weiss, R.L. (1976) Protoplast formation in Escherichia coli. Journal of Bacteriology, 128, 668 - 670.

Welshimer, H.J. & Robinow, C.F. (1949) The lysis of Bacillus megatherium by lysozyme. Journal of Bacteriology, 57, 489 - 499.

Whorwell, P.J., Davidson, I.W., Beeken, W.I. & Wright, R. (1978) Search by immunofluorescence for antigens of rotavirus, Pseudomonas maltophilia, and Mycobacterium kansasii in Crohns disease. Lancet. 2, 697 - 698.

Williams, R.E.O. (1963) L-forms of Staphylococcus aureus. Journal of General Microbiology, 33, 325 - 334.

Wilson, Sir Graham S. & Miles, Sir Ashley. (1975a) History. In Topley & Wilson's Principles of Bacteriology, Virology and Immunity. 6th edition, Vol. I, ch. 1, p. 14. London: Edward Arnold.

Wilson, Sir Graham S. & Miles, Sir Ashley. (1975b) Morphology. In Topley & Wilson's Principles of Bacteriology, Virology and Immunity. 6th edition. Vol. I, ch. 2, pp 33 - 37. London: Edward Arnold.

Wilson, Sir Graham S. & Miles, Sir Ashley. (1975c) Morphology. In Topley & Wilson's Principles of Bacteriology, Virology and Immunity. 6th edition. Vol. I, ch. 2, p. 39. London: Edward Arnold.

Wilson, Sir Graham S. & Miles, Sir Ashley. (1975d) Morphology. In Topley & Wilson's Principles of Bacteriology, Virology and Immunity. 6th edition. Vol. I, ch. 2, pp 48 - 53. London: Edward Arnold.

Wilson, Sir Graham S. & Miles, Sir Ashley. (1975e) Antibacterial substances used in the treatment of infections: Chemotherapy. In Topley & Wilson's Principles of Bacteriology, Virology and Immunity. 6th edition. Vol. I, ch. 6, pp 187 - 249. London: Edward Arnold.

Wilson, Sir Graham S. & Miles, Sir Ashley. (1975f) Proteus and Providence. In Topley & Wilson's Principles of Bacteriology, Virology and Immunity. 6th edition. Vol. I, ch. 27, pp 889 - 895. London: Edward Arnold.

Wilson, Sir Graham S. & Miles, Sir Ashley. (1975g) Haemophilus and Bordetella. In Topley & Wilson's Principles of Bacteriology, Virology and Immunity. 6th edition. Vol. I, ch. 32, pp 1018 - 1035. London: Edward Arnold.

Wyrick, Priscilla B. & Gooder, H. (1977) Reversion of Streptococcus faecium cell wall defective variants to the intact bacterial state. In Spheroplasts, Protoplasts and L-forms of Bacteria, ed. Roux J. pp 59 - 88. Paris:INSERM.

Wyrick, Priscilla B., McConnell, Moyra M. & Rogers, H.J. (1973) Genetic transfer of the stable L-form state to intact bacterial cells. Nature, 244, 505 - 507.

Zinder, N.D. & Arndt. W.F. (1956) Production of protoplasts of Escherichia coli by lysozyme treatment. Proceedings of the National Academy of Sciences of the United States of America, 42, 586 - 590.