

STUDIES ON THE L-FORMS
OF STAPHYLOCOCCI

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ABSTRACT.

The biological, chemical and immunological properties of four strains of Staphylococcus aureus L-forms have been investigated. Three of the L-form strains used were induced into the L-phase by the action of penicillin and the fourth strain by the action of D-cycloserine. All four L-forms produced typical colonies on agar with added sodium chloride and serum protein. Growth in liquid medium was accompanied by production of large amounts of extracellular DNA.

The L-forms were osmotically sensitive and susceptible to surface active agents. Chemical analyses revealed a major cell wall deficiency in that no muramic acid could be detected, thus indicating the lack of cell wall mucopeptide. The L-forms lacked not only the rigid layer of the cell wall but were also deficient in cell wall ribitol teichoic acid; and there were indications of the lack of glycerol teichoic acid.

Although the normal means of identification of the L-forms as being derived from Staph. aureus had been lost, immunological identity was established between the bounding membrane of the L-form and the cell membrane of the staphylococcus. Acridine orange fluorescent staining, which showed the L-form cultures to be heterogeneous not only in regard to size of elements but also in nucleic acid content,

provided an insight into the development of cultures; and indicated the existence of a possible growth cycle when combined with separation techniques. Granular elements isolated from L-form cultures proved to be viable and to be capable of reproducing the normal L-form culture.

CONTENTS.

	<u>Page.</u>
ABSTRACT.	2
INTRODUCTION.	8
Section 1. Historical Survey.	
A. First isolations of L-form.	9
B. Methods of induction.	11
C. Morphology and development.	19
D. The terms "L-phase" and "L-form".	23
2. General Properties of L-forms.	
A. Chemical composition.	27
B. Nutrition and metabolism.	32
C. Immunological properties.	34
D. Pathogenicity and "persistor state".	36
3. The Bacterial Cell Wall.	
A. Isolation and composition of cell walls.	40
B. The cell wall of <u>Staphylococcus aureus</u> .	48
C. Action of antibiotics on cell wall.	51
D. Cell wall antigens in <u>Staphylococcus aureus</u> .	52
4. Aims of the Present Investigations.	54
METHOD AND MATERIALS.	
Section 1. Induction and Growth.	
A. Bacterial strains and media.	57
B. Method of induction into L-phase.	59
C. Propagation and growth in L-phase.	60

D. Harvesting of L-form cultures.	61
2. Lytic Action of Hypotonic Solutions and Surface Active Agents.	
A. Preparation of L-form cells.	63
B. Preparation of protoplasts of staphylococci.	63
C. Lysis of staphylococci by <u>Chalaropsis B</u> enzyme.	64
D. Lysis in salt and sucrose solutions.	64
E. Lysis by alcohols.	65
3. Isolation of Cell fractions.	
A. Preparation of cell walls.	67
B. Preparation of cell membranes.	69
C. Isolation of intracellular contents.	71
D. Isolation of mucopeptide.	71
E. Extraction of teichoic acids.	73
F. Isolation of polysaccharide A and antigen A (Jensen).	75
4. Analytical Methods.	
A. Hydrolysis conditions.	78
B. Paper chromatography.	78
C. Separation and quantitative estimation of hexosamines.	81
D. Extraction and estimation of nucleic acids.	90
5. Acridine Orange Staining.	
A. Staining procedure.	94
B. Fixation.	94

	<u>Page.</u>
C. Specificity of staining.	96
D. Optical systems.	96
6. Separation of Elements from Liquid Culture.	
A. Gradient Centrifugation.	98
B. Differential centrifugation.	99
7. Immunological Methods.	
A. Preparation of antisera.	101
B. Agar gel diffusion.	102
C. Preparation of antigens.	104

RESULTS.

Section 1. Induction and Growth in the L-phase.	
A. Induction and subculture on solid medium.	106
B. Characteristics of L-form colonies.	108
C. Growth in liquid culture.	109
D. Harvesting of growth from liquid culture.	111
E. The effect of protein and other growth supplements.	111
2. Susceptibility to Lysis.	
A. Suspension in hypotonic solutions.	115
B. Lysis by alcohols.	124
3. Chemical Evidence for the Lack of Normal Cell Wall in L-form Cells.	
A. Mucopeptide.	129
B. Teichoic acid.	143
4. Immunological Properties.	148
5. Acridine Orange Staining.	

	<u>Page.</u>
A. Staining of agar colonies.	156
B. Staining of cells from liquid culture.	160
6. Separation of Elements in Liquid Culture.	
A. Staining of fractions.	164
B. Growth of isolated culture fractions.	171
DISCUSSION.	176
ACKNOWLEDGMENTS.	192
REFERENCES.	193

INTRODUCTION.

Section 1

HISTORICAL SURVEY

A. First Isolations of L-form

During studies on the organism Streptobacillus moniliformis, Klieneberger (1935) discovered a variant in normal cultures. The variant organism would grow only in the presence of large quantities of serum, but was consistently isolated from the original culture. It could be freed from contamination with the normal bacterial form by repeated subculture on to high serum medium. The morphology and growth characteristics of this variant differed markedly from those of the bacterial form. The variant growth did not contain bacterial forms but consisted entirely of soft protoplasmic elements and minute granules and for this reason, was regarded as being entirely unrelated to the bacterial form.

The variant, called Ll, was assumed by Klieneberger to be a symbiont of the normal bacterial form. The possibility that this Ll organism was a growth phase of S. moniliformis was considered but "seemed very audacious at the time and full of far-reaching implications" (Klieneberger-Nobel, 1951). Similar growth forms were also isolated by Klieneberger (1936) from other strains of Streptobacillus and all the variants isolated bred true and showed no tendency to revert to a bacterial form on subculture.

Not all of the later workers in this field accepted the symbiosis explanation for the L1 organism and evidence to the contrary was presented notably by Dienes (1939,1943, 1947) and Orskov (1942). It was shown that, far from being an entirely separate organism from S. moniliformis, the L1 was very closely related. Reversion of some of the big L bodies to bacilli was shown by Dienes (1943) and Dienes & Smith (1942,1944). These findings were later confirmed by Klieneberger-Nobel (1949) and on the strength of these findings, the symbiosis theory was abandoned in favour of the view that the two forms were different phases in the growth of one organism. Since these early observations, other reports have appeared confirming the relationship between members of various bacterial species and their derived L-forms. L-forms have been defined generally as that growth, derived from bacteria, which consists of soft protoplasmic elements with no defined morphology (Klieneberger-Nobel,1960). The L-forms should be stable on agar and should no longer contain rigid bacterial forms or revert to them. The colonies produced on appropriate media are characteristic, having a dark centre embedded in the medium and a lighter periphery of large round cells.

B. Methods of Induction

Early methods used by Klieneberger (1935,1936) and Dienes (1947) for the isolation of L-forms from Streptobacillus spp., Bacteroides spp. and a Flavobacterium sp. employed simply serum enriched media. Purification of the isolated L-form was achieved by repeated subculture on to the media, which provided growth conditions advantageous to the L-form. The L-forms produced remained stable on solid media but subculture to liquid media initiated reversion to the bacterial form. Dienes (1948) showed that the L-forms of some Bacteroides spp. could be subcultured and maintained in Brewer's. medium supplemented with ascitic fluid and containing 0.2% agar. The importance of agar in the growth of the Ll organism has been stressed by Dienes & Weinberger (1951).

In 1942 Pierce observed that the Ll organism was penicillin resistant whereas the parent bacterium was penicillin sensitive; this offered another method by which L-forms could be isolated. Subsequently, L-forms of many Gram-negative and some Gram-positive bacteria were produced by cultivating the bacteria in the presence of penicillin. The L-forms most comprehensively studied apart from those of Streptobacillus spp. and Bacteroides spp. were mainly derived from Gram-negative bacteria.

Exposure of S. moniliformis to penicillin (Pierce, 1942) produced L-form similar to the spontaneously occurring Ll. Both of the L-forms are extremely resistant to penicillin, up to 10,000 units/ml., whereas the parent bacillus is inhibited by only 0.02 units/ml. The pleomorphism of S. moniliformis is less apparent in broth than it is on agar, and conversion of the bacilli to the L-form has not been observed in broth, even with penicillin.

Members of the genus Bacteroides have been grown in the L-form, which was produced both spontaneously (Dienes & Smith, 1944) and by growth in the presence of penicillin (Klieneberger-Nobel, 1947; Dienes, 1948). Morphologically, the L-forms of Bacteroides spp. resemble very closely the Ll isolated from S. moniliformis. Studies of these Bacteroides L-forms were undertaken by Dienes (1941, 1948) Dienes & Smith (1944), Smith (1944), Klieneberger-Nobel (1947) and Smith, Mudd & Hillier (1948). The L-forms were found to have several points in common, such as growth only under anaerobic conditions, production of gas in Brewer's medium and the characteristic faecal odour indicating similarities in their metabolic processes.

As with the L1 organism, L-forms of Bacteroides are produced only on agar, prolonged incubations or subculture in broth containing no agar yielding bacilli. Whether the L-forms were isolated spontaneously or in the presence of penicillin, the phenomenon of reversion to bacilli when subculture to broth was observed. Some irregularity in L-form production was noted in Bacteroides even under the influence of penicillin and in general, the more pleomorphic the original strain, the more likely it is to produce L-forms (Dienes, 1948). The observation of the reversion of the L-form to bacilli, which was well documented supports the relationship between the L-form and the bacilli in Bacteroides.

Proteus sp. L-forms have been thoroughly studied by Dienes (1946,1949), Tulasne (1949) and Freundt (1950). Some spontaneously pleomorphic cells are often seen in young cultures of Proteus strains; and various stimuli, such as transfer to tap water or ascitic fluid, are said to increase the proportion of these pleomorphic cells. These large cells are viable and on subculture can produce either bacilli or, in the presence of penicillin, small L-type colonies. Exposure of Proteus strains to penicillin in broth results in swelling of the viable cells, but no further growth occurs. On transfer to agar, the large swollen cells produce either normal bacterial colonies or, in the presence of penicillin, L-form colonies.

Development on solid media follows the same course except that after swelling of the bacterial cells, further growth occurs and small L-colonies are produced. However, in addition to the very small colonies, a few larger (1 to 2 m.m.) colonies also appear. Dienes (1949a) designated the small colonies 3A and the larger ones 3B. The form of the two types of colony is similar and both resemble the colonies of L-forms from other bacteria. The potential development of the two types of colony is different; the 3A colony is capable of being subcultured in the L-form even on penicillin-free media, but reversion to bacilli does occur on prolonged incubation. The larger 3B colonies, on the other hand, when transferred to a penicillin-free media, revert to bacilli within a few hours. This rapid reversion occurs even after prolonged cultivation in the presence of penicillin.

Similar properties have been noted for the penicillin-produced L-form of Salmonella sp. The two types of colony have been produced from S. typhimurium and S. enteritidis, but only the 3A type was produced from S. typhi (Dienes & Weinberger, 1951).

The colonies from these organisms showed the same characteristics, in respect to reversion, as the L-forms of Proteus. However, bacilli were not recovered, either on agar or in broth from the 3A L-colonies of Salmonella. An interesting aspect of this work on Salmonella strains was the ability to produce L-forms from S. typhi under the influence of antibody to the bacilli. Although a smaller proportion of the bacilli produced L-forms than was found with penicillin, typical L-type colonies resulted. Amongst other agents also capable of inducing the growth of L-form in Salmonella were glycine, DL-methionine, L-phenylalanine and bacteriophage (Dienes, Weinberger & Madoff, 1950).

Strains of Shigella have also been reported (Weinberger, Dienes & Madoff, 1950) to produce L-forms on penicillin, but although typical colonies were produced, they failed to grow on subculture. Lederberg & St. Clair (1958), using penicillin, were able to produce L-forms of Escherichia coli and to propagate them in subculture. An earlier report of Dienes & Weinberger (1951) had described the production of spontaneous L-forms of Escherichia, but subculture was not possible. The production of L-form colonies from Pasteurella, Neisseria and Haemophilus strains has also been reported (Dienes, 1951), but little further study on their propagation has been done.

Relatively few reports have appeared until recently on production of L-forms of Gram-positive organisms. The earliest reports were made by Dienes (1949b, 1950) on the production of L-form from Clostridium tetani and other Clostridium spp. and two organisms belonging to the genus Bacillus. Some of these organisms produced, on penicillin agar, L-form colonies similar to the 3A type produced by various Gram-negative bacteria, but in none of these cases was reversion to bacilli observed. During later work on L-form production, Dienes (1951) observed that the morphology of penicillin-sensitive staphylococci, streptococci and diphtheroids was unchanged in the presence of penicillin and that no L-forms were produced.

Sharp (1954) provided a stimulus to the interest in L-form of Gram-positive bacteria by his introduction of a medium which proved successful for the production of L-forms from Group A streptococci. The medium contained a high concentration of sodium chloride together with serum and penicillin. On this medium, the colonies of the streptococcal L-form resembled closely those of L-forms produced from Gram-negative species.

A slight modification of this medium was suggested by Hijmans & Dienes (1955) who were able, by substituting phosphate for chloride in the original Sharp medium, to produce L-forms from one in four of the α -haemolytic streptococci examined. A total of 29 strains of streptococci, all belonging to Lancefield Group D, were studied by Hijmans & Kastelein (1960) using Sharp's medium with phosphate. L-form growth was observed in 19 of the strains tested and production of L-growth found to be reproducible. None of the L-forms proved to be stable in the absence of penicillin and even after 12 subcultures in the L-form, reversion to cocci was seen in penicillin-free media.

An enzyme isolated from a phage lysate of Group C streptococci was used by Gooder & Maxted (1958) and later by Freimer, Krause & McCarty (1959) to induce L-form growth in Group A streptococci. This enzyme was shown to affect the cell wall of Group A streptococci causing lysis of the cells. When the enzyme was allowed to act in an osmotically protective medium the resulting protoplasts were capable of producing L-form colonies on suitable media containing serum and a high salt concentration.

Penicillin was found unnecessary for the production of the L-form colonies, but when penicillin was incorporated in the medium, the colony morphology remained the same. The Group A streptococcal L-form was also obtained in broth culture by inoculation of agar blocks containing L-colonies, and remained stable (Gooder & Maxted, 1961). Induction of L-form growth of Group A streptococci has also been achieved with D-cycloserine (Michel & Hijmans, 1960) and by bacitracin (Gooder & Maxted, 1961).

The first reports of growth of staphylococci in the L-form were provided by Dienes & Sharp (1956) who described briefly the production of staphylococcal L-form by penicillin. More comprehensive reports were produced by Schönfeld (1959, 1961) giving details of production of the L-form with penicillin and subsequent reversion to cocci on antibiotic-free media. The L-forms were found to revert to cocci even after more than 20 passages in the L-phase and one strain, described in the second of the two reports, reverted even after 200 passages in the L-form. The formation of staphylococcal L-forms under the influence of penicillin was also described by Marston (1961a,b), who succeeded in growing the L-form in a liquid medium.

Reversion to cocci was shown by these L-forms even after 13 passages on penicillin-containing medium. No reversion to cocci occurred after growth in liquid medium, even on transfer to penicillin-free medium containing a slightly lower salt concentration than that used for induction.

Spontaneously occurring L-forms from staphylococci were described by Mattman, Tunstall & Rossmore (1961) who established L-growth on specially purified media. The transformation, by penicillin, of 21 strains of Staphylococcus aureus into the L-form was described by Williams (1963). It was found that many of the early foci of swollen cocci on the primary transforming medium failed to develop and produce typical L-colonies. The ratio L colonies/coccus proved very variable at least on the medium used, and prevented accurate quantitative analysis of transformation.

C. Morphology and Development.

One of the striking characteristics of all L-forms so far isolated from many different bacteria, is the morphology of the colony. All reports on the growth of L-form colonies have emphasised the characteristic colony form (Dienes & Weinberger, 1951; Klieneberger-Nobel, 1960).

The L-forms, growing on suitable solid media, produce small round colonies having a pronounced dark centre and a lighter periphery. Microscopic study shows the dark centre portion of the colony to consist of masses of granular material, which by differential focussing can be shown to penetrate the surface of the agar. The peripheral zone of the colony, which consists mainly of large globular cells, remains as a surface growth.

Razin & Oliver (1961) studied the development of colonies of various Mycoplasma spp. and of L-forms of bacteria. Their results suggested that the unique type of colony produced by these organisms is a consequence of the small size of some of the viable elements present in inocula. It was suggested that these small particles were drawn into the agar matrix by capillary attraction and that development within the interstices of the agar gel produces the dense granular centre portion of the colonies. When this growth reaches the agar surface continued reproduction in the thin free water film on the surface of the agar, produced the peripheral zone typical of mature colonies.

Growth in liquid medium has not been achieved for L-forms isolated from all bacteria, but where it has been possible, the growth produced is usually granular in appearance, either adhering to the walls of the culture vessel or sinking to the bottom.

There are exceptions to this, particularly in the case of the L-forms of Proteus vulgaris (Dienes, 1953), which form a pellicle on the surface.

The microscopic appearance of liquid cultures of all L-forms so far studied is similar. The elements in these cultures range in size from large globular bodies, sometimes up to 15 or 20 μ in diameter, to very small granules near the limit of visibility in the light microscope. Between these two extremes, the elements form a more or less continuous size spectrum. Some of the globular cells contain inclusions and very often vacuoles. Phase contrast microscopy shows the granular elements to be very numerous in these cultures; they are phase dense as are the inclusions in the large bodies.

This marked pleomorphism of L-forms in agar cultures and ~~more~~ especially in liquid cultures, has given rise to two main theories concerning reproduction in these organisms. Controversy has arisen over the role taken by the very small granules produced in cultures, not only of L-forms, but also of pleuropneumonia-like organisms. The presence of the granular inclusions of the large globular bodies has been confirmed in the case of Proteus L-form (Thorsson & Weibull, 1958) and in Streptococcus L-form (van der Hoof & Hijmans, 1959) by the use of ultrathin sections viewed in the electron microscope.

There seems to be general agreement that lysis of such large bodies releases numerous granules into the culture medium. What is not agreed is whether the release of these granules plays a part in the continued reproduction of the organism.

Klieneberger-Nobel (1960) and Dienes (1960) hold the view that the granules released by lysis of the large cells are viable and reproduce the large cells, under favourable conditions, by swelling. They have proposed a growth cycle involving alternate stages of granules and large bodies. The main evidence available for the viability of the small granules is from filtration experiments. One criticism which might apply to such experiments, particularly when performed on cells as plastic as those found in L-form cultures, is the possibility of contamination of the filtrates by large cells being forced through the pores of the filter.

An alternative view, held by Roux (1960) and Liebermeister (1960), is that the granules released in the culture medium are "chondrioids" or cellular debris and that reproduction of the L-form takes place by a process of sprouting from the large bodies.

The granules are regarded as having no part in the reproductive process in the L-form. Various morphological evidence has been produced in support of this theory, particularly by Roux (1960) who, with the aid of micromanipulation, isolated both the large bodies and granules from cultures of Proteus L-form. On transfer to solid medium, colonies were produced by one out of two large bodies, but in no case was growth observed by subculture of granules released from large bodies. The conclusion drawn by Roux was that multiplication takes place exclusively by division of large bodies.

D. The Terms "L-phase" and "L-form".

Confusion has arisen over the precise meaning which should be attached to the term "L-form" (L for Lister Institute), which was originally used to describe the morphologically distinct organisms derived from cultures of Streptobacillus moniliformis (Klieneberger, 1935). The term was restricted to describing ^{the} characteristic colony produced by these organisms and was not intended to describe the individual elements present in cultures of bacteria growing in the L-form.

The "Réunion sur les formes L et sur les formes évolutives des bactéries" (1957) stated that the term "L-form" should be reserved for the growth form arising, either spontaneously or by stimulation, from bacteria and having a characteristic colony with dense centre and lighter periphery on media not containing the stimulatory substances. It was further suggested that the term be restricted to those cultures which do not assume the morphology of the parent bacterium.

This suggested definition serves to describe all the stable L-form as far isolated from both Gram-negative and Gram-positive bacteria. Within this definition, however, there are differences between L-forms. The spontaneously arising L-form of S. moniliformis was propagated in media containing salt concentrations normal for bacterial growth; whereas penicillin induced L-forms of other bacteria required, at least on primary isolation, some form of osmotic protection usually provided by increased salt concentration. Growth of some of these L-forms has subsequently been obtained in media, containing more normal salt concentration by serially decreasing the osmotic protection available. The result is that L-form such as Ll and some Proteus strains, can be subcultured in low salt concentration media and are described as salt non-requiring L-forms.

Many other L-forms, although capable of growth in lower salt concentrations than were required for transformation into the L-form, still require some measure of osmotic protection. These salt-requiring L-forms can be propagated only in media containing a high salt or sucrose concentration.

L-forms of bacteria have some properties in common with protoplasts, spheroplasts and also pleuropneumonia-like organisms (PPLO). The first two of these terms have been used rather loosely by some workers, necessitating the definition of these terms.

It was agreed in 1958 (Brenner et al.) that the term "protoplast" be used only for structures derived from vegetative bacterial cells by the removal of the entire cell wall. A list of criteria, such as absence of cell wall components, antigens, phage receptors etc., was drawn up and it was recommended that only structures conforming to these criteria should be regarded as true protoplasts. True protoplasts, although capable of limited growth in liquid media, do not produce colonies even on osmotically protective solid medium (McQuillen, 1960).

Both L-forms and PPLO produce a similar type of colony with a central dark and a lighter peripheral part. This similarity between the two forms applies also to growth in liquid culture; although the macroscopic picture of growth is somewhat different, the elements seen in the cultures are strikingly similar in appearance. Both cultures consist of a range of cells from small granules to large bodies, although in general the large bodies of the L-form are bigger than those of the PPLO. The granules found in L-forms are said to be twice as large as those of the PPLO (c.150 μ m.), (Klieneberger-Nobel, 1960).

Section 2.

GENERAL PROPERTIES OF L-FORMS.

A. Chemical composition.

(1) Protein, Amino acids and Sugars.

Investigations into the sugar and amino acid content of L-forms have been almost wholly concerned with those sugars, amino sugars and amino acids commonly found in the bacterial cell wall. Weibull (1958) and Weibull & Beckman (1960a) found the L-form Proteus L9 to contain α , ϵ diaminopimelic acid, an amino acid associated with the cell wall, but to contain smaller amounts than in the parent bacterium. The general non-cell wall amino acid composition of the salt-requiring streptococcal L-form, AED-L was found to differ in several respects from that of the parent coccus (Panos, Barkulis & Hayashi, 1959), but more striking differences were found in the contents of amino acids common in the cell wall. The sugar and amino sugar composition of these streptococcal L-forms was also investigated by Panos et al., who confirmed the earlier findings of Sharp, Hijmans & Dienes (1957) that the L-form contained little or no glucosamine and rhamnose, and no group-specific polysaccharide.

McCarty (1952) had demonstrated that this group-specific substance constituted a significant part of the cell wall in this organism. The observation that the L-form lacked this material provided good evidence of a major structural alteration resulting from the transformation to the L-form. The L-forms of Proteus spp. did not provide such a clear cut distinction, since they were found still to possess significant amounts of somatic antigen although reduced when compared to the amounts found in the bacterial form (Sharp & Dienes, 1959).

More direct evidence concerning the amino sugar contents of L-forms of various bacteria was provided by Sharp (1963). It was found that, whereas the L-forms derived from streptococci and staphylococci contained virtually no muramic acid, those derived from Proteus sp. did contain this unique cell wall amino sugar. Whether or not the muramic acid was present as a structural component or as a precursor in the cytoplasm was not established.

(11) Lipids.

Smith & Rothblat (1962) in a survey of the lipid contents of both salt-requiring and salt non-requiring L-forms, found a greatly increased lipid content in the L-forms when compared to their parent bacteria.

They concluded that in the salt-requiring L-forms, the increased lipid content, two to three times that of the parent bacterium, reflected the loss of cell wall material, thereby reducing the total solid content of the L-form compared to the bacterial form. The salt non-requiring L-form, L1, had at least five times more total lipid than its parent bacterium Streptobacillus moniliformis. The difference between total lipid content in the other salt non-requiring L-form, 18-L and its parent bacterium, Proteus 18 was only two fold, similar to the salt-requiring strains. Langenfeld & Smith (1963) found in their investigations that in general, salt non-requiring L-forms possessed appreciably greater lipid phosphorus contents than did the salt-requiring strains.

(111) Nucleic Acids.

Kandler, Zehender & Müller (1956), investigating the salt non-requiring L-forms of Proteus and Streptobacillus, found the total nucleic acid contents of these organisms to fall in the stationary phase, due to a reduction in the ribonucleic acid (RNA) contents of the cells. Weibull (1958) found a total nucleic acid content of 14 to 19% of dry weight in these organisms and attributed the low values of Kandler et al. to losses of nucleic acid during manipulation.

Langenfeld & Smith (1963) found the strain 18L, derived from a Proteus sp, to contain 8% and L1, derived from S. moniliformis, 10% total nucleic acid.

Panos, Barkulis & Hayashi (1959) presented results on the salt-requiring L-form, AED-L, derived from a β -haemolytic streptococcus. Total nucleic acid accounted for 12% of the dry weight of the L-form. The values for other salt-requiring L-forms, derived from streptococci, diphtheroids and a staphylococcus, were considerably lower, only 0.6 to 3.4% (Langenfeld & Smith, 1963).

Many of these apparently diverging results could be explained not only by strain variation but also by the age of the culture used. The decrease in RNA in the stationary phase (Kandler et al, 1956) and the possibility of cellular disruption could account for such discrepancies. Another, possibly more important, reason^{was} suggested by the results of Mandel, Sensenbrenner, DeGregorio & Bader, (1959), who showed a wide range in the ratios of RNA to deoxyribonucleic acid (DNA) in different size elements in a culture of the L-form of Proteus 18.

The highest RNA:DNA ratio was found in the bodies ranging from 1.9 to 3.8 μ diameter and it was concluded that these bodies represent the actively growing part of the culture, since they also showed the highest respiration related to DNA content (Mandel, Terranova, Sensenbrenner & Feo, 1959). The fraction containing the very small granular bodies (0.1-0.3 μ . diameter) was also studied by Weibull & Beckman (1960b). Whereas Mandel, Terranova et al. (1959) found these small bodies to contain substantial amounts of DNA, Weibull & Beckman showed their DNA content to vary considerably and to be very low. For this reason, the granules were regarded as being cellular debris, although they exhibited catalase and succinic dehydrogenase activities comparable to the unfractionated cultures. However, mechanically disintegrated L-form showed at best only very low respiration.

Deoxyribonucleic acid represents between 1.5% and 4.0% of the dry weight of L-forms (Kandler, Zehender & Müller, 1956; Weibull, 1958; Panos, Barkulis & Hayashi, 1959) and as in bacteria remains relatively constant throughout the growth cycle (Kandler et al., 1956; Weibull & Beckman 1960a).

B. Nutrition and Metabolism.

Studies on the nutrition and metabolism of L-forms have been difficult since there has yet to be devised a strictly defined medium which will support adequate growth of any of the L-forms currently studied.

Medill & O'Kane (1954) described a defined basal solid medium for Proteus L-forms, which was reported to support better growth of the organism than crude media. Abrams (1955) further improved this medium and obtained good growth of the Proteus L-form in a liquid medium containing amino acids, salts, vitamin-free casamino acids and nicotinamide.

Even less well-defined media have been described for other possibly more fastidious L-forms. Edward (1953) was able to grow the Ll organism in media supplemented with bovine serum albumin or an acetone-insoluble lipid fraction of egg-yolk. This same organism is also capable of growth on the semi-defined medium of Razin & Knight (1960), containing vitamin-free casamino acids, cystine and tryptophan; but growth was obtained only in the presence of serum.

In the media of Abrams (1955) and Razin & Knight (1960), glucose fulfilled the energy requirements of the L-forms concerned. Vitamin requirements in the Proteus L-form were satisfied by nicotinamide in Abrams' medium.

No reports have appeared on the optimal temperature for growth of L-forms and it has generally been assumed that the optimum is the same as that of the parent bacterium. Much the same situation exists with regard to optimum pH of growth media for L-forms, although it has been stated that L-forms require alkaline pH to multiply (Dienes & Weinberger, 1951; Sharp, 1954). A more recent report by Banville (1964) on growth of L-forms of Staphylococcus aureus, recommends an optimal pH on solid media of 6.5.

The osmotic requirements of L-forms appear to vary with the particular organism but in general growth occurs over a range of sodium chloride concentrations of 0.25 to 1.1 M (Sharp, 1954). Various other salts have been used in place of sodium chloride to provide osmotic protection, e.g. potassium, ammonium, calcium and magnesium chlorides or potassium dihydrogen and disodium phosphates.

Panos & Barkulis (1959) concluded that the protective effect of high salt concentrations is not merely an osmotic one but may involve also stabilisation of the cell membrane by cation binding on to phospholipid residues.

All the L-forms so far investigated for their oxidative properties have been shown to possess essentially the same properties as the parent bacterium. This is true of Proteus L-forms (Kandler, Zehender & Müller, 1956; Mandel, Terranova, Sensenbrenner & Feo, 1959) and Weibull & Hammarberg (1962;1963) were able to show that the catalase activity of various Proteus L-forms was equivalent to that in the parent bacteria. The one salt-requiring L-form studied, that derived from a streptococcus, showed some differences from the parent bacterium (Panos, 1962). The L-form showed greater metabolic activity with galactose rhamnose and various compounds of the tricarboxylic acid cycle than did the parent coccus; but lower activity with glucose, glucosamine or N-acetyl glucosamine as substrates.

C. Immunological Properties

The immunological properties of the L-form of Streptobacillus moniliformis were first investigated by Klieneberger (1942).

It was found that an antiserum to the bacilli, when absorbed with the L-form was still capable of agglutinating the bacilli, although to a lower titre than the unabsorbed serum. All agglutinins were removed from an L-form antiserum absorbed with either L-form or bacilli. The results indicated the presence of an antigen in the bacilli, which was absent from the L-form; the significance of this finding was not at the time realised. Similar results were reported by Dienes, Weinberger & Madoff (1950) for Proteus L-forms and parent bacilli and the main differences between antisera to the two forms was in agglutination titres. The agglutinin titres to the L-form were found to be lower in most cases than those to the bacilli. Neither of these studies gave any indication of any structural changes in the L-form.

The precise location of the group-specific polysaccharide in the cell wall of Group A streptococci by McCarty (1952) provided a criterion in the study of the L-forms derived from these organisms. Sharp, Hijmans & Dienes (1957) found that Group A streptococcal L-forms lacked the group specific polysaccharide, indicating the changed nature of the cell envelope.

Because the group-specific polysaccharide constitutes only a part of the cell wall in these streptococci, it was not possible to distinguish between total loss of cell wall and loss of only a part of the structure. The L-form of Proteus 18 was found by Sharp & Dienes (1959) to contain a somatic antigen which could normally be found associated with the cell wall of the bacterial form. There was some doubt, however, as to whether this antigen was present on the surface of the L-form or in the cytoplasm. Group D streptococcal L-forms have been studied by Hijmans (1962), who failed to find serological evidence for the presence of either cell wall antigen or the group D antigen. The group D antigen, which has been identified with the so-called 'intracellular' glycerol teichoic acid (Elliot, 1962; Wicken, Elliot & Baddiley, 1963), has been said to be located between the cytoplasmic membrane and the cell wall (Shattock & Smith, 1963).

D. Pathogenicity and the "Persistor State".

None of the L-forms so far isolated has been shown to be pathogenic in its own right, although reports have appeared on infections caused by reversion of L-forms to bacilli in vivo (Klieneberger-Nobel, 1960).

One feature used to distinguish between L-forms and Mycoplasma organisms is the lack of pathogenicity of the former. However, some L-forms appear to possess potential pathogenicity at least, in that they are capable of producing substances which, in the parent bacterium, are associated with pathogenic processes. One example of this is the finding of coagulase in L-forms of staphylococci (Mattman, Tunstall & Rossmore, 1961; Williams, 1963). Group A streptococcal L-forms continue to produce M-protein, which is released into the culture medium (Sharp, Hijmans & Dienes, 1957; Freimer, Krause & McCarty, 1959; Gooder & Maxted, 1961). The presence of neuraminidase has been reported in the L-forms of Vibrio cholerae (Madoff, Annenberg & Weinstein, 1961); and toxigenic L-forms have been described from Clostridium tetani (Rubio-Huertos & Gonzalez-Vazquez, 1960).

Much interest has centred on the so-called "persistor state" in bacterial infection; and the L-forms of bacteria have been mentioned in this connection (McDermott, 1958). It is not uncommon, with many different bacteria for apparent cure of an infective lesion to be effected with antibiotics, but for a recurrence of infection to develop almost immediately the treatment is discontinued.

No difference in drug resistance or other characteristics can be found between the organisms isolated in the primary infection and those isolated in the recurrent infection (Gunnison, Fraher & Jawetz, 1964). Guze & Kalmanson (1964) studying experimental enterococcal pyelonephritis in rats, were able to show an apparent cure of the infection by administration of large doses of penicillin. Bacterial counts on normal media showed the kidneys to be clear of infection after 3 weeks of treatment. Counts produced on osmotically protective media (containing 0.3M sucrose) however, demonstrated the presence of organisms even 13 weeks after antibiotic therapy. The conclusion drawn was that the bacteria were persisting in the rat kidneys in the "protoplast" form and so required the presence of an osmotic stabilizer for their demonstration.

Gunnison et al. (1964) reported the persistence of Staphylococcus aureus in penicillin in vitro. They found 0.1% of the exposed bacteria survived treatment and showed the same sensitivity as the original population. The persistors were judged not to be spheroplasts, since they were not osmotically fragile; or L-forms, since they produced normal staphylococcal colonies within 24 hr. after the removal of the penicillin.

Since penicillin, in common with other antibiotics, is effective only against actively proliferating bacteria, it was concluded that persistors arise in a population because at the time of exposure to the antibiotic, they were in a non-susceptible state.

Section 3.

THE BACTERIAL CELL WALL.

Studies on the cell walls of bacteria and other related organisms date back only to 1950. At that time, interest was growing in the morphology and chemical structure of the outer layers of bacterial cells. Since then the development of new techniques particularly in the field of biochemistry has increased the knowledge of the structure and also the biosynthesis of the bacterial cell wall.

A. Isolation and Composition of Cell Walls.

Various methods, both chemical and mechanical, have been devised for the isolation of cell walls of bacteria. Among the mechanical methods available, disruption of the cells with glass beads first studied by Dawson (1949) and Salton & Horne (1951), has been widely used. Disintegration of cells by such treatment or with abrasives and shearing forces, as in the Hughes press have also been used for preparation of cell wall fractions. One of the end products of all these methods is an insoluble residue, which under the electron microscope can be shown to consist of structures having the same shape as the original cells.

The residue immediately after disruption contains the cell wall contaminated to some degree with various cellular proteins, some of which can be removed by centrifugation. Other contaminating materials can be removed by washing of the crude cell walls with M sodium chloride solution or phosphate buffers, and subsequent treatment with trypsin to remove protein (Salton & Horne, 1951). Cummins & Harris (1956) employed more extensive enzymatic treatment of the cell walls in an effort to remove nucleic acids and proteins; the crude cell walls being treated with ribonuclease, trypsin and pepsin. Cell walls prepared by either of these two methods can be shown by electron microscopy to be free of any gross contamination. Other criteria for the purity of cell wall preparations consist of ultraviolet absorption at 260mp. for nucleic acids and, at least in the case of certain Gram-positive organisms, the finding of a limited range of amino acids on acid hydrolysis of the walls.

Cell walls of Gram-negative organisms, prepared by disruption methods, prove rather more difficult to purify, since large amounts of protein have been shown to occur in the cell wall itself (Salton, 1953d).

Consequently, removal of contaminating intracellular proteins presents a problem, and no general criterion of purity exists for these preparations. Studies on the walls of Gram-negative bacteria have been largely concerned with preparations of particular parts of the cell rather than with the whole structures.

Chemical methods of extraction of wall material have been employed more for Gram-negative organisms than for Gram-positive, but of the methods available for Gram-positive organisms, the extraction procedure of Park & Hancock (1960) provides wall material admirably suited to comparative study. The final residue from the procedure represents the hot trichloroacetic acid and trypsin-insoluble portions of the whole cell. This insoluble wall material has been shown, at least in Staphylococcus aureus, to consist chiefly of the mucopeptide, the other components having been removed during acid treatment.

Most of the wall isolation procedures for Gram-negative organisms have been based on chemical rather than mechanical methods.

The various layers associated with the cell wall in Gram-negative species have been isolated free from other components by Weidel & Primosigh (1958); Weidel, Frank & Martin (1960) and Mandelstam (1961, 1962). All of these methods produce isolated structures from the cells and, combined with electron microscope studies, have yielded valuable data on the structural relationships existing in the complex cell envelope of Gram-negative bacteria.

In both Gram-positive and Gram-negative bacteria the cell wall serves the important function of maintaining the structural integrity of the cell. This implies the property of rigidity of the wall, or at least a part of it. This is borne out by electron microscopic studies of isolated cell walls, which even after sometimes rigorous mechanical or chemical treatment, retain to some degree the shape and dimensions normally associated with the whole cell (Salton & Horne, 1951). Extensive extraction procedures carried out on cell walls from Gram-positive or Gram-negative cells leave a residue which is insoluble in a variety of solvents such as hot formamide, 90% phenol and sodium dodecyl sulphate (Salton, 1964).

However, examination of such a residue in the electron microscope shows it still to possess the shape of the organism from which it was prepared. Chemical analyses of such residues have shown them to consist of a restricted number of amino acids and amino sugars. This new class of structural polymers, called mucocomplexes (Salton, 1956) or mucopeptides (Mandelstam & Rogers, 1959) has been found in all Gram-positive and in most Gram-negative bacteria so far examined. The exceptions are the Mycoplasma organisms (Plackett, 1959) and Halobacterium halobium (Brown & Shorey, 1962).

All of the mucopeptides so far examined have been found to contain the two amino acids alanine and glutamic acid, both in the D-configuration, together with either L-lysine or α, ϵ -diaminopimelic acid (DAP) (Work, 1949, 1951). In mucopeptides isolated from certain organisms other amino acids such as glycine, serine or aspartic acid appear. Although qualitative identification of amino acids has been performed on mucopeptides from a wide range of Gram-positive species, the data available for Gram-negative bacteria have been limited by the difficulties met with in preparing mucopeptide free from wall or membrane proteins.

One very important finding, following the discovery of large amounts of hexosamine in Gram-positive cell walls and lesser, though still substantial, amounts in Gram-negative cell walls, was the detection of an unknown amino sugar in spore peptides by Strange & Powell (1954). A similar compound, with identical properties had been described by Cummins & Harris (1956) in hydrolysates of cell walls. Subsequently, Strange & Dark (1956) were able to isolate this unknown amino sugar from both spore peptides and cell walls; and its structure was determined as 3-O-carboxyethyl glucosamine (Strange, 1956). The name 'muramic acid' was proposed for this new sugar. Since then various methods of synthesis of muramic acid have been evolved, among them those of Strange & Kent (1959) and Gigg & Carroll (1961). Park & Strominger (1957) identified the previously unknown amino sugar reported in nucleotides accumulated by Staphylococcus aureus treated with penicillin (Park, 1952), as muramic acid.

Muramic acid has to date been found only in mucopeptide fractions of cell walls or in nucleotide structures isolated from cells treated with certain antibiotics.

This amino sugar has been shown to be a constituent of cell walls of all Gram-positive and of most Gram-negative bacteria so far examined and is also present in cell walls of blue-green algae (Salton, 1960) and Rickettsiae (Perkins & Allison, 1963). Muramic acid has therefore come to be regarded as a distinctive indicator, and its presence in any cell is taken to be evidence of the presence of cell wall mucopeptide.

In addition to muramic acid, cell wall mucopeptides also contain glucosamine, which unlike muramic acid may also be present in cell wall polymers other than mucopeptides. Enzymatic studies on isolated mucopeptides have indicated that most of the amino sugar in these structures is present in the form of N-acetyl compounds.

The other major cell wall polymers discovered, in addition to mucopeptides, were the teichoic acids (Baddiley, Buchanan & Carss, 1958). In Gram-positive organisms, these polymers can represent a large proportion of the total cell wall material. The teichoic acids, however, do not apparently contribute to the rigidity of the wall structure since their removal from the wall leaves the rigid mucopeptide intact (Archibald, Armstrong, Baddiley & Hay, 1961).

Since the first reports of these compounds were made in Gram-positive organisms, most of the subsequent work by Armstrong, Haddiley, Buchanan, Davison, Kelemen & Neuhaus (1959); Salton & Pavlik (1960); Haddiley (1961) and Ikawa (1961) has been done within this group. Only one report by Lilly (1962) has appeared on teichoic acid isolated from cell walls of a Gram-negative species, Escherichia coli, strain 26-26.

Two forms of the polymer have been found to exist in cell walls, one containing ribitol, phosphate, alanine and a sugar or amino sugar; the other form of the polymer containing glycerol in place of ribitol. Both of these types have been found in cell walls, but only in Streptococcus faecalis has evidence been found that both might be present in the wall of the same organism, (Armstrong et al., 1959; Ikawa, 1961). A great deal of structural data has been produced on teichoic acids from various organisms and some of the proposed structures confirmed by chemical synthesis. The exact functions of these polymers in the cell wall are not clearly understood, although a number of theories, such as regulation of passage of ions across the cell surface (Archibald et al., 1961), have been put forward.

The more complex cell envelopes of the Gram-negative bacteria possess, in addition to mucopeptide, other complexes mainly accounted for by protein, lipid and polysaccharide. Large polymers belonging to all three classes, alone and in combination as lipopolysaccharides, lipoproteins etc., have been isolated from surface structures of a large number of Gram-negative organisms (Weidel & Primosigh, 1958; Weidel,^{Frank} & Martin, 1960; Salton, 1960). Gradually, more information regarding the inter-relationships of the layers going to make up the whole cell wall is being accumulated. The position at present, however, is far from clear and much information is needed to reach the stage existing in the study of walls from Gram-positive species.

B. The Cell Wall of Staphylococcus aureus

Much of the information gained on cell wall structure in Gram-positive bacteria has been the result of studies on isolated cell walls from strains of Staphylococcus aureus (Salton, 1964).

(1) Mucopeptide

The mucopeptide of Staph. aureus cell walls has been shown to consist of a relatively few amino acids, glutamic acid, alanine, lysine and glycine; and two amino sugars,

glucosamine and muramic acid (Hancock & Park, 1958; Rogers & Perkins, 1959; Strominger, Park & Thompson 1959), although small amounts of serine have also been reported (Salton & Pavlik, 1960) in some strains. The presence of large amounts of glycine in the isolated mucopeptide from Staph. aureus Copenhagen led Mandelstam & Strominger (1961) to the view that the normal short peptide chains found in mucopeptide are, in the case of Staph. aureus, cross-linked by bridges of polyglycine. Some support for this view was afforded by Perkins & Rogers (quoted by Rogers, 1963) who found glycylglycine in strong acid hydrolysates of cell walls of strain 524/SC Staph. aureus. The polyglycine cross-linking is said to occur between the free amino group of lysine in one peptide chain and the carboxyl group of glutamic acid in the next chain.

Although the complete cell wall of Staph. aureus is resistant to lysozyme, the isolated mucopeptide, free from teichoic acid, is solubilized by the enzyme. Analysis of the lysozyme digestion products (Ghuysen & Strominger, 1963) showed that most of the muramic acid residues present in the mucopeptide carry peptide chains. It would appear from these results that there is a high degree of cross-linking in the mucopeptide of Staph. aureus and Rogers (1963)

has proposed a net-like arrangement of mucopeptide forming a strong rigid framework.

(11) Teichoic Acid.

The teichoic acid isolated from cell walls of Staph. aureus H was shown by Armstrong, Baddiley, Buchanan, Carss & Greenberg (1958) to be a ribitol phosphate polymer with side chains of N-acetyl glucosamine and D-alanine. The more detailed structure of this teichoic acid was studied by Baddiley, Buchanan, Martin & RajBhandary (1962) who showed the N-acetyl glucosamine side chains to be attached to the ribitol phosphate 'backbone' at the 4-position of the ribitol. The ester-linked D-alanine was assigned to the hydroxyl groups at positions 2- or 3- of the ribitol units. In teichoic acid from cell walls of Staph. aureus. Copenhagen, on the other hand, Sanderson, Juergens & Strominger (1961) suggested that the D-alanine residues were to be found on the N-acetyl glucosamine side chains and not on the ribitol. Similar differences between teichoic acids from cell walls of other strains of Staph. aureus might also exist although no detailed studies have yet been carried out. The so-called 'intracellular' teichoic acid of Staph. aureus H is a glycerol phosphate polymer containing D-alanine and N-acetyl glucosamine.

Detailed structural work has not been carried out on glycerol teichoic acids from other strains of Staph. aureus apart from strain H (RajBandhary & Baddiley, 1963).

The precise relationships of these teichoic acids in the cell is not known with certainty.

C. Action of Antibiotics on Cell Wall.

Park (1952) isolated and characterised several nucleotides accumulated in Staph. aureus under the influence of penicillin. At that time, the identity of the 'unknown amino sugar' was in doubt, but the work of Strange & Powell (1954) and Cummins & Harris (1956) led to its recognition as muramic acid, (Park & Strominger, 1957). It was subsequently found that these nucleotides consisted of uridine diphosphate (UDP) molecules to which were attached N-acetyl muramic acid and a short peptide chain of amino acids normally found in cell wall mucopeptides. These findings offered strong circumstantial evidence that these uridine compounds might be precursors of cell wall mucopeptide. Strominger & Threnn (1959) showed that the quantitative as well as the qualitative analyses of these peptides agreed with figures produced for cell wall preparations.

The accumulation of these UDP compounds and of cytidine diphosphate (CDP) compounds has been reported in bacteria exposed to a variety of agents such as penicillin, bacitracin, novobiocin, vancomycin, D-cycloserine, gentian violet and 5-fluorouracil. Saukkonen (1961) was able to show a massive accumulation of nucleotides in penicillin-treated Staph. aureus, with large amounts of CDP-ribitol being produced.

If current theories on the nature of these nucleotide compounds as precursors of cell wall structures are true, then it seems certain that the agents which cause the accumulation of these compounds are acting by interfering with some stage in the biosynthesis of the whole cell wall.

D. Cell Wall Antigens in Staphylococcus aureus.

In 1934, an antigenic 'polysaccharide A' was isolated from pathogenic staphylococci by Julianelle & Wiegand. During later work on this polysaccharide, Haukenes (1962) found it to consist of a complex of two components, cell wall mucopeptide and cell wall ribitol teichoic acid. The cell wall teichoic acid formed the greater part of the purified 'Polysaccharide A'. The inhibition of the agglutination of cell walls of Staph. aureus Copenhagen had been reported by Sanderson, Juergens & Strominger (1961) by the addition of known parts of the teichoic acid structure.

The results indicated that in this particular organism, the serological specificity was due to the presence of α -N-acetylglucosaminyl ribitol residues in the cell wall. This finding was confirmed by Haukenes (1962), although in the strain used, no α -linked residues could be detected in one of the preparations of teichoic acid.

Section 4

AIMS OF THE PRESENT INVESTIGATIONS

Since little previous work has been done on L-forms of Staphylococcus aureus, apart from studies of induction and growth morphology, it is thought important to attempt a more general investigation of the properties of these forms. It is proposed that this investigation should include both chemical and immunological aspects of these L-forms together with further morphological studies. In this way it may be possible to establish a relationship between the organism in the L-phase and in the bacterial phase.

1. The main interest in the chemical composition of the L-form is stimulated by reports that in L-forms derived from other bacteria, especially Gram-positive species, at least part of the normal cell wall is believed to be absent. The projected chemical comparison between the L-form and the staphylococcus will serve to elucidate this possibility with regard to the staphylococcal L-form.
2. In Staphylococcus aureus, the immunological properties are to a large extent related to the chemical composition of the cell wall. Consequently, any deficiencies in cell wall structure in the L-form should also be evident in results from immunological studies.

3. The L-form of Staphylococcus aureus possesses an unusual morphology, not only in the macroscopic colony form but also microscopically. With the use of various separation and staining techniques, it is hoped to be able to investigate aspects of the growth and reproduction of the L-form.

METHODS AND

MATERIALS

Section 1

INDUCTION AND GROWTH

A. Bacterial Strains and Media.

Four strains of Staphylococcus aureus were used in this investigation. Three of these strains, 1444, 6 and 42E were sensitive to penicillin; strain 1444 (phage type 3B/+) was a routine isolate from clinical material and strains PS6 (NCTC 8403) and PS42E (NCTC 8418) were propagating strains from the routine phage-typing set (Blair & Williams, 1961). The fourth strain, Staphylococcus aureus strain Rogers (phage type 42E) was isolated from clinical material and was a penicillinase-producer.

All four strains were induced to grow in the L-phase, strains 1444, 6 and 42E by exposure to penicillin and strain Rogers by exposure to D-cycloserine.

L-form colonies were produced on a solid medium having the following composition:

Difco brain-heart infusion broth (containing 0.5% (w/v) NaCl).	37 g.
Sodium chloride	30 g.
Sterile normal horse serum	100 ml.
Agar (Noble)	13 g.
Distilled water	1000 ml.

27
The basal medium, containing all the above materials, except the serum, was prepared, sterilised and allowed to cool to 50°. The sterile horse serum was added and the medium mixed by gentle agitation. For induction of penicillin-sensitive staphylococci, penicillin G (sodium benzyl penicillin, Glaxo Laboratories Ltd.) was added to the warm medium to a concentration of 60 µg/ml. The now complete medium was gently mixed and dispensed in 20 ml amounts in sterile plastic Petri dishes. Solid medium for the induction of strain Rogers was prepared in the same way except that 40g of sodium chloride were added per litre and penicillin was replaced by D-cycloserine (E. Lilly & Co. Ltd) at a final concentration of 500 µg/ml.

Liquid medium for the growth of the established L-form strains consisted of the above brain-heart infusion medium, but without the added agar.

In later experiments, the horse serum was replaced by 0.5 (v/v) Difco PPLO serum fraction, 0.5% (v/v) bovine serum albumen fraction V (Armour) or 1% (w/v) granular charcoal (British Drug Houses). The first two supplements were added as sterile solutions to the sterile basal medium. The granular charcoal was sterilised in the liquid medium.

B. Method of Induction into L-phase.

All the strains of Staph. aureus were obtained in the L-phase as described by Williams (1963). Overnight cultures of the staphylococci in glucose broth were centrifuged and the organisms washed three times with physiological saline. The washed cells were resuspended in fresh broth at a concentration 10 x that of the original culture and 0.5 ml. of this suspension spread on the surface of a plate of the complete medium. The inoculum was allowed to absorb into the surface and the plate incubated at 37°. All plates were contained in plastic bags to lessen moisture loss by evaporation. Plates were examined daily under a plate microscope until typical L-colonies could be seen, usually after 2 to 6 days incubation.

The L-colonies were examined by cutting blocks of agar from the plates and placing them, colonies uppermost, on microscope slides. A coverslip was spread with a mixture of 1% (w/v) ethanolic methylene blue and 1% (w/v) azure II and allowed to dry. The coverslip was placed over the L-colonies, which took up and retained the dye. The L-colonies were then viewed under low-power magnification in the microscope.

C. Propagation and Growth in L-phase.

Suitable L-form cultures on primary plates were transferred to fresh solid medium using an agar-block method. Blocks of agar, containing fully developed L-colonies, were cut from the primary plates and inverted on to fresh medium. The plates were incubated for 18 hr. at 37° and then the blocks moved over the surface of the agar. Where the blocks had been originally placed, small foci of growth could be seen. Further incubation for 18 hr. at 37° yielded typical L-colonies. This method of propagation was used in all preliminary growth experiments.

Establishment of L-form growth in the liquid medium was achieved by transfer of agar blocks containing colonies to small volumes of complete liquid medium. Growth in broth could be established only with difficulty, and the results obtained were irregular. Disintegration of the agar blocks and inoculation of broths with a suspension of colonies and agar produced the same irregular results. Eventually, all four strains were obtained in broth culture. Incubation of the broth for 2 to 3 days at 37° produced 'stringy' growth of the L-form adhering to the agar block. Gentle agitation freed the growth from the agar and thereafter subculture could be performed using normal methods. Large scale liquid

culture of the L-form at first proved difficult but by serially increasing the culture volume, half-litre cultures were eventually obtained.

D. Harvesting of L-form Cultures.

L-form cells were harvested from liquid culture by high-speed centrifugation. Although the 'stringy' growth could be sedimented from the broth at only 1,000 x g for 15 min., the supernatant from such treatment still contained appreciable numbers of cells. It was found that centrifugation at 27,000 x g for 10 min. was required to sediment these cells which were then added to the pellet from the low speed centrifugation. The harvested cells were washed with sodium chloride solution, 4.5% (w/v) for L-Rogers and 3.5% (w/v) for the other strains, and resedimented at 27,000 x g for 10 min.

The washed L-form was resuspended in 0.5M sucrose solution (0.75M in the case of L-Rogers) containing 0.2 mg/ml. of deoxyribonuclease (grade B, California Biochemicals) and 0.005M $MgCl_2$. The suspension was incubated at 37° for 1 hr. and the cells recovered by centrifuging at 70,000 x g for 30 min. The pellets from the high speed centrifugation were drained, resuspended in distilled water and dialysed overnight in a Visking membrane sac.

The dialysed suspensions were freeze-dried and the dry material transferred to tightly stoppered containers.

When preparations of L-form were required for growth experiments, washing of the deposited cells and deoxyribonuclease treatment were carried out in fresh sterile culture medium. The enzyme-treated cells were recovered at 70,000 x g for 30 min. and resuspended in liquid medium.

Section 2

LYTIC ACTION OF HYPOTONIC SOLUTIONS AND SURFACE-ACTIVE AGENTS.

A. Preparations of L-form cells.

Broth cultures of the L-forms were harvested and treated with deoxyribonuclease as described in Section 1.D. The suspension of evenly dispersed cells in 0.5M sucrose was centrifuged at 70,000 x g for 30 mins. and the pellet resuspended, with gentle agitation, in 0.5M sucrose solution. The density of the suspension was adjusted so that 0.5 ml., when diluted with 4 ml. of 0.5M sucrose, gave a reading of approximately 0.5 optical density units at 500 mμ, using 1 cm. cells in a Unicam SP.500 spectrophotometer. Suspensions of washed bacterial cells were also prepared to give the same optical density.

B. Preparation of Protoplasts of Staphylococci.

Staphylococci were grown overnight in glucose broth at 37°, harvested at 1,000 x g for 15 mins. and washed three times with physiological salt solution. After the final washing, the cells were resuspended in 0.5M sucrose containing 0.01M acetate buffer pH 5.7 (Hash, Wishnick & Miller, 1964). Chalaropsis B enzyme (Lederle Laboratories) was added to give a final concentration of approximately 50 μg/ml. The suspensions were incubated at room temperature overnight to effect conversion of the bacterial cells to

osmotically sensitive forms, which were collected at 25,000 x g for 30 min. The protoplasts were resuspended in 0.5M sucrose solution at a density such that 0.1 ml. of the suspension diluted with 4 ml. of 0.5M sucrose gave an optical density reading of approximately 0.5 at 500 mμ.

C. Lysis of Staphylococci by Chalaropsis B enzyme

Suspensions of washed staphylococci were prepared both in 0.5M sucrose solution containing 0.01M acetate buffer pH 5.7 and in buffer alone. The initial optical densities were adjusted to approximately 0.5 at 610 mμ in a 1 cm. glass cell. Four ml. volumes of each suspension were pipetted into glass spectrophotometer cells and Chalaropsis B enzyme added to each to give a final concentration of 50 μg/ml. A control suspension of staphylococci in buffer containing no enzyme was also prepared. Optical density readings at 610 mμ (Hash et al., 1964) were taken of the control and the two test samples at intervals. All readings were made using a buffer blank.

D. Lysis in Salt and Sucrose Solutions.

A series of solutions of sodium chloride (British Drug Houses) were made to give a range of concentrations from 0 to 4.5% (w/v). Four ml. quantities of each of these solutions were dispensed into each of two tubes, and 0.1 ml. of the L-form suspension added to one set and 0.1 ml. of the

bacterial suspension added to the second set. The contents of the tubes were gently mixed and allowed to stand at room temperature for 30 min. Optical densities of the suspensions were read at 500 m μ using 1 cm. glass cells in the Unicam SP 500 spectrophotometer.

Similar procedures were used for determining the extent of lysis in sucrose solutions of varying concentration. Sucrose (Kerfoot & Co) solutions were prepared to give a range of concentrations from 0 to 0.75M. Each of the solutions was dispensed in 4 ml. amounts into each of three test tubes. At timed intervals, 0.1 ml. of the suspension of L-form was added to one set of tubes, 0.1 ml. of the protoplast suspension added to a second set and to the third set, 0.1 ml of bacterial suspension. All the tubes were allowed to stand at room temperature for 30 min. after the addition of the suspensions and then the optical densities of the mixtures read at 500 m μ in 1 cm. glass cells using a water blank.

E. Lysis by Alcohols.

Solutions of each of the primary alcohols, ethanol, n-propanol and n-butanol (British Drug Houses) were made in 0.5M sucrose to give a range of concentration. The solutions of ethanol ranged from 0 to 7.5M, those of n-propanol from 0 to 3.0M and of n-butanol from 0 to 0.5M

alcohol. Each of the alcohol solutions was dispensed in 4 ml. quantities into each of three test tubes. At timed intervals, 0.1 ml. quantities of suspensions of L-form, staphylococcal protoplasts and bacterial cells were added to each set of tubes. After 30 min. incubation at room temperature, optical densities were read at 500 m μ in 1 cm. cells.

Section 3.

ISOLATION OF CELL FRACTIONS.

A. Preparation of Cell Walls.

Cell walls were isolated from staphylococci by the method of Salton & Horne (1951), using a Mickle tissue disintegrator and No.12 Ballotini glass beads (Chance Glass Co.Ltd.).

Staphylococci were grown overnight at 37° in Nutrient Broth No.2 (Oxoid) containing 0.1% (w./v.) glucose. The cells were harvested at 1,000 x g for 15 min. washed once with physiological saline and once with distilled water. The pellet of cells was resuspended in distilled water at a density equivalent to approximately 16 mg. dry weight/ml. and 6 ml. portions mixed with an equal volume of glass beads in the glass Mickle cups. Rubber bungs, covered with thin cellophane were used to seal the cups, which were vibrated at maximum amplitude. In preliminary preparations, disruption of the cells was followed by Gram staining of smears made at intervals. Vibration for 15 min. was found sufficient to produce over 90% disruption of the cells in the suspension, as judged by loss of Gram-positive staining property. Suspensions of disrupted cells were freed from glass beads by filtration through a grade 0

sintered-glass filter (Scientific Supplies, Ltd) and the beads washed several times with distilled water. The thick suspensions, containing cell walls, membranes, intracellular contents and some whole cells, were centrifuged at 5,000 x g for 10 min. in a Servall 'Superspeed' centrifuge (I. Sorvall & Co.Ltd.) Supernatant fluid was removed and stored in the refrigerator for future use.

The pellets, consisting of two distinct layers, were washed three times with cold M sodium chloride solution, and then three times with cold distilled water. Washing with sodium chloride removed much of the adhering cellular protein from the cell walls. The crude cell walls and whole cells were resuspended in $M/15$ phosphate buffer pH 7.4 containing 1 mg./ml. of trypsin (British Drug Houses). A few drops of toluene were added to the suspension, which was incubated overnight at 37°.

The trypsin-digested cell walls and any whole cells were sedimented at 12,000 x g for 10 min. and washed three times on the centrifuge with cold M sodium chloride solution. Whole cells and cell walls were separated by repeated centrifugation from distilled water at 1,000 x g for 10 min. After each centrifugation, a pellet consisting of two layers was obtained. The lower, grey-coloured layer consisted of whole cells and the upper, white layer of cell walls.

Each time, the layer of cell walls was carefully removed from the whole cells and recentrifuged. This process was repeated until no whole cells were detected as a separate layer. The cell walls were washed thoroughly with cold distilled water to remove sodium chloride, the supernatant fluid being tested for the presence of chloride by the addition of silver nitrate. Three washes in distilled water were usually sufficient to remove all traces of salt. The purified cell walls were resuspended in the smallest possible volume of distilled water and freeze-dried.

B. Preparation of Cell Membranes.

The supernatant fluid from the first 5,000 x g centrifugation of the cell walls described in Section 3A was recentrifuged at 5,000 x g for 30 min. to remove any particles of cell wall. The resulting supernatant was then centrifuged at 20,000 x g for 20 min. in the Servall 'Superspeed' centrifuge and the pellet containing the membrane fraction recovered, the supernatant being stored in the refrigerator for future use.

The pellet was washed three times on the centrifuge with cold physiological saline and incubated at 37° for 30 min. in $M/100$ phosphate buffer, pH 7.4 containing 0.04M NaCl, 0.005 M $MgCl_2$ and 0.02 mg./ml. of both deoxyribonuclease (grade B, California Biochemicals) and ribonuclease (2 x cryst., California Biochemicals) as described by Freimer (1963).

The insoluble membrane fraction was collected by centrifugation, washed twice in cold $M/100$ phosphate buffered saline pH 7.4, twice with cold distilled water and freeze-dried from distilled water. The final product consisted of a buff-coloured powder.

Membrane fractions from L-form cells were prepared by a modification of the above method. The L-forms, previously harvested and washed once with the appropriate strength sodium chloride solution, were lysed by suspension in $M/60$ phosphate buffered saline ($0.9\%w/v$) pH 7.4. To allow time for completion of lysis, suspensions were stored at 4° for approximately 16 hr. Deoxyribonuclease, 0.02 mg./ml. and 0.005M $MgCl_2$ were added to the lysed suspension, which was incubated at 37° for 30 min. to reduce the viscosity caused by the release of deoxyribonucleic acid.

Particulate material was sedimented at $20,000 \times g$ for 10 min., resuspended in the buffered saline and again centrifuged. The supernatants from these two centrifugations were pooled and stored for future use. The pellets were resuspended in $M/60$ phosphate buffered saline pH 7.4, containing 0.005M $MgCl_2$ and 0.02 mg./ml. of both deoxyribonuclease and ribonuclease. Suspensions were incubated at 37° for 30 min. and the insoluble residue sedimented at $20,000 \times g$ for 10 min. The pellets were washed once with cold buffered saline, resuspended in

buffered saline and incubated for a further 30 min. at 37° with nucleases. The insoluble material was again sedimented at 20,000 x g for 10 min., washed several times with cold buffered saline, then with distilled water and freeze-dried. The residual powders from various preparations were light brown to almost white in colour.

C. Isolation of Intracellular contents.

Supernatant fluids, after removal of cell walls and membranes from suspensions of disrupted cocci, and cell membranes from lysed L-form, were centrifuged at 100,000 x g for 1 hr. (Davison & Baddiley, 1963) in the M.S.E. 'Superspeed 40' centrifuge. The sediment from this treatment consisted of a small pellet of clear gel, which was freeze-dried from distilled water.

D. Isolation of Mucopeptide.

A mucopeptide fraction was isolated from coccal and L-form cells according to the method of Park & Hancock (1960), which allows the preparation of cell wall mucopeptide contaminated with less than 5% of cellular protein.

Cells were harvested as described and washed once with the appropriate sodium chloride solution. The washed cells (approximately 2.5 - 3.0 m.g. dry weight) were resuspended in 2 ml. of distilled water and sufficient cold 25% (w./v.) trichloroacetic acid added to give a final concentration of 5% (w./v.). The mixture was allowed

to stand for 10 min. at 4° and then centrifuged at 4,000 x g for 10 min. The supernatant, which contained the acid-soluble cell constituents, was carefully decanted.

The residue from the cold acid treatment was resuspended in 2.5 ml. of 75% (v./v.) ethanol in water, thoroughly mixed by shaking and allowed to stand for 10 min. at room temperature. Insoluble material was centrifuged and the supernatant, containing ethanol-soluble lipid and 'protein' decanted.

The nucleic and teichoic acids were removed from the ethanol-treated cells by suspension in 2.5 ml. of 5% (w./v.) trichloroacetic acid and heating for 6 min. at 90°. The extract was cooled, centrifuged at 4,000 x g for 15 min. and the supernatant decanted.

The residue was suspended in 0.95 ml. of 0.05M NH_4HCO_3 containing 0.005N NH_4OH and 0.05 ml. of a solution of 1mg./ml. crystalline trypsin (L. Light & Co.) in the same buffer added. The mixture was incubated at 37° for 2 hr., or until digestion was complete and then centrifuged at 4,000 x g for 15 min. The extract of trypsin-soluble proteins was decanted.

According to Park & Hancock (1960), any residue remaining after this treatment consists of cell wall mucopeptide, which is resistant to digestion with hot trichloroacetic acid and with trypsin (Weidel, 1951;

McCarty, 1952; Salton, 1953b, 1956; Cummins & Harris, 1956).

Because of the extremely small yields obtained from L-forms, the above extraction procedure was also carried out on larger samples of L-forms. The volumes of the extractants used were adjusted accordingly.

Mucopeptide was also isolated with the hot formamide method originally introduced by Fuller (1938) for the extraction of polysaccharide from bacteria. Complete solution of the bacteria had been assumed by Fuller, but it has been shown by Krause & McCarthy (1961) that the mucopeptide of the cell wall is resistant to digestion by this treatment.

Samples of whole cocci, L-form and cell walls were extracted by the Fuller procedure. The cells or cell walls were suspended in 2 ml. of formamide (British Drug Houses) and heated, with occasional stirring, at 170° in an oil bath for 20 min. The extract was cooled and 2 volumes of acid alcohol (95 parts absolute ethanol + 5 parts 2 N HCL) added. Although it was found that the residue could be recovered by direct centrifugation, the addition of the acid alcohol facilitated the separation. The residues were washed once in cold distilled water and freeze-dried.

E. Extraction of Teichoic Acids.

Isolation of teichoic acids from whole cells, cell walls and other cell fractions was performed according to methods

described by Armstrong, Baddiley, Buchanan, Carss & Greenberg (1958); Davison & Baddiley (1963) and RajBandhary & Baddiley (1963).

Whole cocci and L-form cells were defatted by successive treatment with acetone, ethanol and ether, each for a period of 10 min. The resulting fat-free cells were stirred for 16 hr. with cold 10% (w./v.) trichloroacetic acid. Cell debris was centrifuged at 20,000 x g for 10 min. and resuspended in fresh, cold trichloroacetic acid and again stirred for 16 hr. at 4°. The supernatants from both these treatments were pooled and mixed with 5 volumes of ethanol. After overnight standing at 4°, the resulting precipitate was centrifuged, triturated with a small volume of trichloroacetic acid and the insoluble material removed. Ethanol was added to the acid solution and the precipitate collected after 16 hr. at 4°. The final precipitate was washed with acetone, ethanol and ether and dried in a vacuum desiccator.

Teichoic acid was extracted from cell walls by essentially the same procedure involving treatment with cold trichloroacetic acid and precipitation from ethanol.

Treatment of the freeze-dried intracellular gel by stirring with 10% (w./v.) trichloroacetic acid for 48 hr. at 4° extracted considerable amounts of protein and nucleic acids, as shown by paper chromatography, together with any

teichoic acid present. Accordingly, attempts were made to remove the contaminating protein and nucleic acid by triturating the alcohol precipitate with cold 10% (w./v.) trichloroacetic acid.

The crude precipitate from the initial alcohol treatment was stirred with 1 ml. of cold trichloroacetic acid for 2 hr. and the grey insoluble residue centrifuged at 10,000 x g for 15 min. Five volumes of ethanol were added to the supernatant and then allowed to stand for 16 hr. at 4°. Precipitated material was removed by centrifugation at 10,000 x g for 15 min. Further trituration of the precipitate with 1 ml. of cold 10% (w./v.) trichloroacetic acid, reprecipitation with ethanol and drying of the precipitate with ethanol and ether, yielded less than 5 mg. of a white powder from approximately 0.2g of freeze-dried gel. Because the yields of material were so low, further purification by passage through a column of Sephadex G75 (RajBandhary & Baddiley, 1963) was not attempted. The final products from the above procedure still contained appreciable amounts of ribonucleic acid, but acid hydrolysis and chromatography showed that all the contaminating protein had been removed.

F. Isolation of Polysaccharide A and Antigen A (Jensen)

The extraction of both polysaccharide A and antigen A (Jensen

from whole cells was performed by the method described by Haukenes, Losnegard & Oeding (1961).

Bacterial and L-form cells were harvested and washed once with salt solution of the appropriate strength. The cells were resuspended in the smallest possible volume of $M/15$ phosphate buffer pH 7.0 to give a thick paste. The paste was transferred to the chamber of an 'X-press' (AB Biox, Sweden), frozen at -25° and the cells disrupted by three passages through the orifice of the press. The paste was removed from the press and allowed to thaw; two volumes of $M/15$ phosphate buffer pH 7.0 were added and the suspension allowed to stand for 24 hr. at 4° . The extract was adjusted to pH 4.2 by the addition of dilute ($N/1$) hydrochloric acid and centrifuged at $12,000 \times g$ for 20 min. The supernatant was readjusted to pH 5.2 with sodium hydroxide and 4 volumes of ethanol added. After overnight standing at 4° , the precipitate was collected at $12,000 \times g$ for 10 min. and redissolved in a small volume of distilled water.

This solution was again adjusted to pH 4.2 and the precipitate collected. The supernatant was readjusted to pH 5.2 and after standing at 4° , the precipitate collected as before. This procedure was repeated twice, each time the acid precipitates being collected and pooled.

The final alcohol precipitate was freeze-dried from

distilled water. Gel diffusion showed this precipitate to consist of polysaccharide A contaminated to some extent with antigen A (Jensen).

The pooled acid precipitates were dissolved in distilled water and freeze-dried. The preparation produced strong antigen A (Jensen) lines in gel diffusion reactions, with evidence of some slight contamination with 'cytoplasmic' antigen.

Section 4

ANALYTICAL METHODS.

A. Hydrolysis Conditions.

All hydrolyses for the detection and estimation of sugars, sugar alcohols and amino acids were carried out in hydrochloric acid at 100°. Samples for the chromatographic detection of amino acids and amino sugars were hydrolysed in 4N HCl for 16 hr.

Samples for the chromatographic detection of sugars and sugar alcohols were hydrolysed in 2N HCl for 3 hr.

The samples were sealed in hard glass test tubes with hydrochloric acid of the appropriate strength and heated in a thermostatically controlled hot air oven (Baird & Tatlock Ltd) for the required length of time. After cooling, the hydrolysates were filtered through Whatman No.52 paper and the papers washed with distilled water. The washings were added to the hydrolysate and hydrochloric acid removed by drying in a vacuum desiccator over concentrated sulphuric acid and potassium hydroxide pellets. The dried hydrolysates were reconstituted once with distilled water and redried.

B. Paper Chromatography.

All paper chromatography, both ascending and descending, was performed on Whatman No.1 paper at a temperature of 26°

Amino acids and amino sugars were separated by a two-dimensional ascending technique, using 10" square papers in the Aimer Universal Apparatus (Aimer Products Ltd.). The papers were marked with an origin in the bottom right-hand corner, 1" from each edge. The dried hydrolysates were dissolved in a small quantity of distilled water and applied to the papers in the frame with a wire loop and dried with a hair drier. The chromatographic run in the first dimension was made with n-butanol: acetic acid: water (120:30:50 v./v.) for 16 hr. The papers were dried with a cold air fan and then developed in the second dimension with 2:4/2:5 lutidine: water (130:70 v./v.) for 16 hr. The papers were again dried with a fan.

Both amino acids and amino sugars were detected by dipping the papers in 0.2% (w./v.) ninhydrin (British Drug Houses) in acetone. The acetone was allowed to evaporate and the papers heated in a hot air oven at 90° for 5 min. The colours produced with this reagent fade with time and spots were preserved by dipping the papers through a $M/2$ nickel sulphate solution in water. The blue-purple ninhydrin colours were changed to red, which remained stable for many months.

Amino sugars were detected more specifically using the Elson-Morgan reaction on paper (Smith, 1960). The following reagents were used:-

- | | |
|--|---------|
| a). KOH 5g. in 20 ml. water plus 80 ml. ethanol | 1 vol. |
| b). Acetylacetone (British Drug Houses) 1%
(v./v.) in ethanol. | 10 vol. |
| c). P-dimethylamino benzaldehyde ('Analar',
British Drug Houses) 10% (w./v.) in conc.
HCl (sp.gr. 1.18, British Drug Houses) | 1 vol. |
| d). Ethanol | 9 vol. |

Dried chromatograms were dipped through a fresh mixture of reagents a). and b). and heated for 5 min. at 110° . The papers were then dipped through a mixture of c). and d). and dried with a warm air fan. The amino sugars yielded a transient purple colour, reaching a maximum after 1 to 2 min., when the papers were reheated to 80° for 3 to 4 min. After this treatment, glucosamine yielded a permanent red colour and muramic acid orange-red.

Reducing sugars were separated by descending chromatography using n-butanol: pyridine: water (6:4:3 v./v.) for 16 hr. (Block, Durrum & Zweig, 1958). Chromatograms were dried and the reducing sugars detected using a dipping reagent of 3% (w./v.) p-anisidine hydrochloride (British Drug Houses) in n-butanol. The papers were heated at 90° for 5 to 10 min. when aldohexoses yielded red-brown spots and ketohexoses yellow spots.

Sugar alcohols were separated by descending chromatography in n-butanol: acetic acid: Water (120:30:50 v./v.) for 40 hr. The detection agent used was periodic acid-benzidine (Smith, 1960) consisting of:-

- a). Periodic acid (British Drug Houses)
2.28g. in 100 ml. water 1 vol.
- b). Acetone 19 vol.
- c). Benzadine (British Drug Houses)
184 mg. in 0.6 ml. acetic acid,
4.4 ml. water and 95 ml. acetone
added

The dried chromatograms were dipped through a fresh mixture of a). and b). and allowed to stand for 3 to 4 min. The papers were then dipped through reagent c). when white spots, corresponding to sugar alcohols and sugars, appeared on a blue background.

C. Separation and Quantitative Estimation of Hexosamines.

The method used for the estimation of amino sugars was one modified by Rondle & Morgan (1955) from Elson & Morgan (1933).

Hexosamines present in hydrolysates were freed from interfering chromogens by the ion-exchange method of Boas (1953):-

Dowex 50 resin (200-400 mesh, X-8, V.A. Howe & Co.Ltd) was washed slowly on a Buchner funnel with 2N sodium hydroxide followed by 2N hydrochloric acid, and then washed with distilled water until chloride-free by the silver nitrate test. A 1:1 suspension of the resin was made in distilled water and after vigorous shaking, 5 ml. of the suspension pipetted into a glass column having an internal diameter of 10 m.m. A filter paper circle was placed on

on top of the resin. Hydrolysates were diluted to 1N with respect to hydrochloric acid and applied to the column. After the hydrolysate had passed through, the column was washed with 10 ml. of distilled water. The hexosamines were eluted from the column with 2N hydrochloric acid and collected to the mark in a 5 ml. volumetric flask. This solution represented the 'total hexosamine' fraction.

The individual hexosamines were separated by the method of J.T. Park as described by Perkins & Rogers (1959). A slurry containing 5g. Norit A (Pfanstiehl Chemical Co.) and 5g. Celite 545 (Johns-Manville Products) was prepared in 100 ml. distilled water. After thorough mixing, 2 ml. of this slurry was pipetted into a glass column having an internal diameter of 5 m.m. A filter paper disc was placed on top of the charcoal: celite, and the column washed with 10 ml. of 2N hydrochloric acid. The hydrolysate was made 2N with respect to hydrochloric acid and a sample equivalent to 4 mole or less of total hexosamine (as glucosamine base) added to the column. The sample was allowed to absorb to the charcoal: celite and then glucosamine eluted with 10 ml. of distilled water. Further treatment of the column with 10 ml. of 5% (v/v.) ethanol in water eluted muramic acid. Where necessary, eluates suspected of containing muramic acid were evaporated to dryness and reconstituted with distilled water at a suitable volume.

Estimations of 'total hexosamine', glucosamine and muramic acid were made using the Rondle & Morgan (1955) modification of the Elson-Morgan test. The reagents used in the test were prepared as follows:-

- a). Acetylacetone reagent - Fresh acetylacetone (British Drug Houses), 1 ml., was dissolved in 50 ml. of 0.5N sodium carbonate solution.. Since this reagent is stable at room temperature for only 2 to 3 hr., it was prepared immediately before use.
- b). Ethanol - The ethanol used was dried over calcium oxide and distilled.
- c). Ehrlich's reagent - 'Analar' grade p-dimethylamino-benzaldehyde (British Drug Houses) was recrystallized from warm ethanol by the addition of 50% (v/v.) distilled water, collected by filtration and dried in a vacuum desiccator. The aldehyde was considered suitable for use in the test only if colourless or very pale yellow. The reagent was prepared by dissolving 0.6g. of the aldehyde in 30 ml. of ethanol and adding 30 ml. of concentrated hydrochloric acid ('Analar' sp.gr. 1.18, British Drug Houses). The correctly prepared reagent was free from insoluble material and pale yellow in colour. It was stored at -10°

Standard solutions for use in the test were prepared in distilled water and stored frozen at -10° . D-glucosamine.

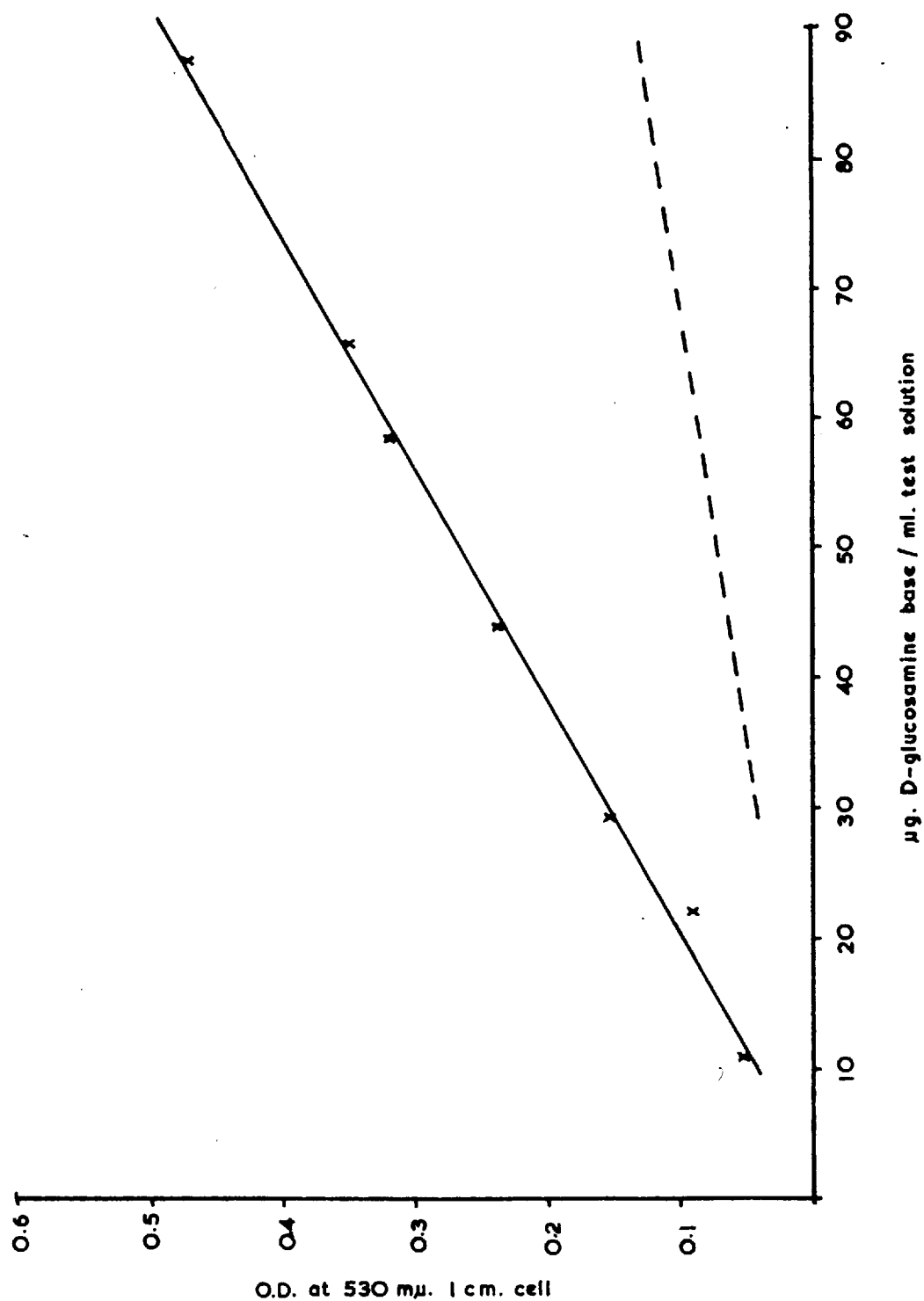


FIGURE 1. Calibration curve for glucosamine in Rondle & Morgan test. The calculated curve for muramic acid is shown by the broken line.

hydrochloride was obtained from British Drug Houses. The muramic acid used was a synthetic product prepared by the method of Gigg & Carroll (1961) and obtained from Dr. H.R. Perkins.

Estimations were performed in stoppered, graduated test tubes. To 1 ml. of the test solution in the test tube, 1 ml. of acetylacetone reagent was added, and the contents gently mixed by shaking. The walls of the tube were washed down with 1 ml. of distilled water. In cases where the original test solution was acid, 1 drop of phenolphthalein indicator was added before the addition of the acetylacetone reagent. A minimum quantity of N NaOH was added to give a pink colour, which was then just discharged by the addition of dilute HCL. After the addition of the acetylacetone reagent, the tube was washed down with water to give a final volume of 3 ml. The mouths of the tubes were closed with small glass condensers containing approximately 3 ml. of distilled water, and the reaction mixtures heated on a vigorously boiling water bath for 20 min. The tubes were cooled to room temperature and 5 ml. of ethanol added, followed by 1 ml. of Ehrlich's reagent. The final volume was adjusted to 10 ml. by the addition of ethanol. Tube contents were thoroughly mixed and carbon dioxide liberated by heating at 65° for 10 min. After cooling to room temperature, the tubes were allowed to stand for 2 hr.

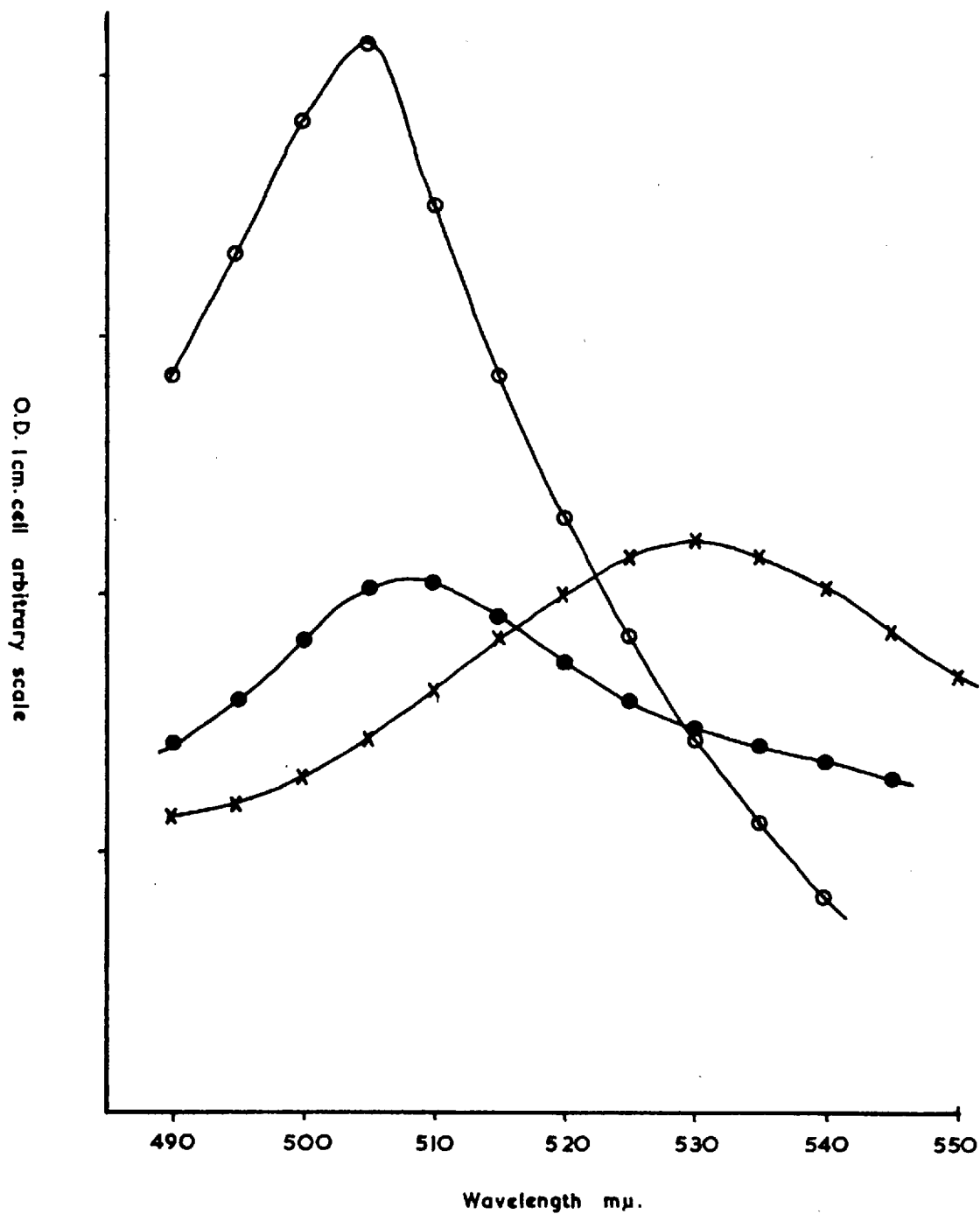


FIGURE 2. Absorption spectra of glucosamine and muramic acid in Rondle & Morgan test.

x - glucosamine 2hr

o -muramic acid 2hr

o -muramic acid 24hr

before optical densities were read at 530 m μ . in a Unicam S.P. 500 spectrophotometer, using 1 cm. glass cells.

The calibration curve obtained for glucosamine in this test is presented in Fig.1. A calculated calibration ^{curve} for muramic acid was constructed using the observation of Crumpton (1959) that 1 mg. of muramic acid produces only 27.1% of the colour at 530 m μ . given by 1 mg. of glucosamine base.

The subsequent acquisition of authentic muramic acid permitted the reduction of the level of estimation of this amino sugar in the test. Muramic acid produces a characteristic spectrum in the Randle & Morgan test with a peak of absorption at 510 m μ ., which shifts to 505 m μ . on standing overnight (Fig.2.). Optical density readings were very much higher at 505 m μ . than at 530 m μ ., the maximum for glucosamine, and greater sensitivity could therefore be gained by reading the optical densities of the test samples after 24 hr. rather than immediately and at a wavelength of 505 m μ ., rather than 530 m μ .

The estimation procedure was further refined by the use of a micromethod based on the Randle & Morgan modification. The final volume used in the microtest was only 1.5 ml. as compared to one of 10 ml. in the original test. All reagents and procedures were as used in the full scale test, except that the reagent volumes were scaled down, and optical densities were read using glass microcells with a path length of 2 cm. The calibration curve for authentic muramic

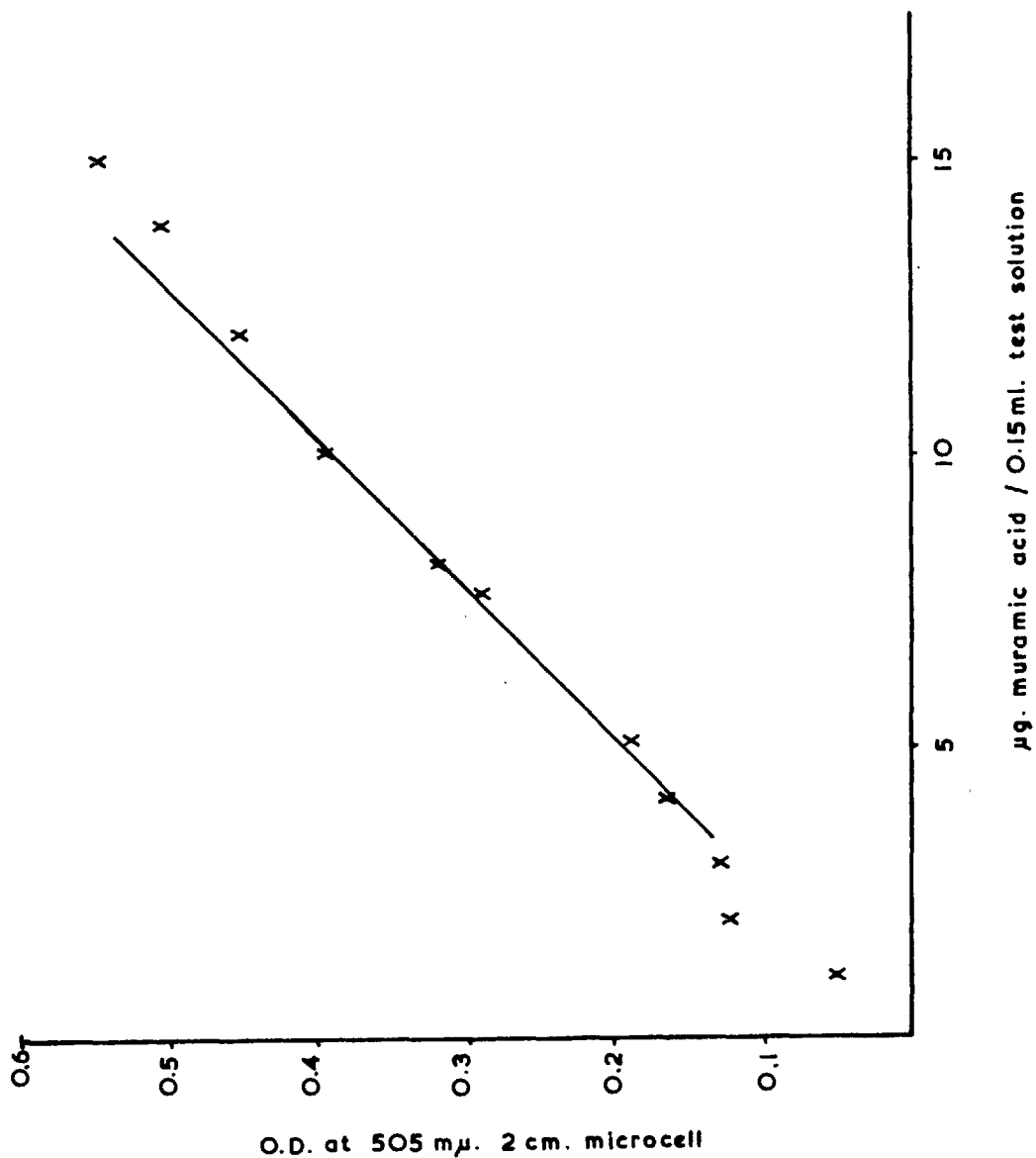


FIGURE 3. Calibration curve for muramic acid in the micromodification of the Rondle & Morgan test.

acid in the micro-test is shown in Fig. 3. The lower level of estimation of muramic acid in the micromodification was taken as 4 μ g., this being the lower limit of the range over which Beer's Law was obeyed. Amounts of muramic acid below this level could be detected, by virtue of the characteristic spectrum, but could not be quantitated with certainty.

N-acetyl hexosamines were estimated using the Strominger (1957) micromodification of the method of Reissig, Strominger & Leloir (1955). The reagents were prepared according to Strominger (1957), but the volumes used in the test were adjusted to give a final volume of 1.1 ml. (Michel, 1961).

- a). Sodium tetraborate reagent - A solution of 2% (w./v.) sodium tetraborate ('Analar', British Drug Houses) prepared in 0.125 N sodium hydroxide.
- b). Ehrlich's reagent - 'Analar' grade p-dimethylamino benzaldehyde was recrystallized from ethanol as described above. A solution containing 16% (w./v.) p-dimethylamino benzaldehyde was prepared in 95% (v./v.) glacial acetic acid, 5% (v./v.) concentrated hydrochloric acid ('Analar' sp.gr. 1.18, British Drug Houses).

Standard solutions were prepared from N-acetyl glucosamine (British Drug Houses) to cover the range 0.02 to 0.09 μ .mole/0.05 ml. distilled water. The solutions were

stored frozen at -10° .

The test solution (0.05 ml.) containing 'bound' N-acetyl amino sugar, was heated on a boiling water bath for 5 min. with 0.05 ml. of 0.25N hydrochloric acid. The resulting hydrolysate was mixed with 0.1 ml. of the sodium tetraborate reagent and again heated on a boiling water bath for 7 min. The tubes were cooled and 0.8 ml. of glacial acetic acid added, followed by 0.1 ml. of Ehrlich's reagent. The contents of the tubes were mixed and incubated at 37° for 20 min. Optical densities of the solutions were read at 550 m μ . in 2 cm. glass microcells.

The following tubes were prepared for the test:-

1. Internal N-acetyl glucosamine standard.
2. Reagent blank, consisting of the test sample subjected to the estimation procedure, but omitting both heating steps. This blank measured the presence of compounds other than N-acetyl amino sugars, which react in the test.
3. Test sample subjected to the whole procedure.

Tube 2 was used as a blank for tube 3.

D. Extraction and Estimation of Nucleic Acids.

Ribonucleic and deoxyribonucleic acids were extracted and estimated by the procedure of Ogur & Rosen (1950).

Samples were treated with 3 ml. of 70% (v./v.) ethanol in water, centrifuged at 4,000 x g for 10 mins. and then resuspended in 70% ethanol containing 0.1% (w./v.) perchloric acid (May & Baker, Limited). After treatment for 10 min.

at room temperature, the suspension was centrifuged at 4,000 for 10 min.

The residue from the acid alcohol extraction was resuspended in 5 ml. of 3:1 (v./v.) ethanol and ether and boiled gently for 3 min. on a water bath. The mixture was centrifuged and the insoluble material extracted a second time with boiling ethanol:ether.

The ethanol:ether insoluble material was extracted twice with 5 ml. quantities of cold 0.2N perchloric acid, each time for a period of 5 min. The residue was centrifuged at 4,000 x g for 10 min.

Ribonucleic acid (RNA) was extracted from the 0.2N perchloric acid residue by prolonged treatment with 5 ml. of 1N perchloric acid at 4° for 18 hr. The suspension was centrifuged and extracted twice more with 5 ml. volumes of 1N perchloric acid for periods of 2 hr. at 4°. Insoluble material was removed each time by centrifugation at 4,000 x g for 10 min. and the extracts combined and made up to 15 ml.

Deoxyribonucleic acid (DNA) was extracted from the remaining material by heating at 70° in a water bath for 20 min. with 5 ml. of 0.5N perchloric acid. This process was repeated, the extracts combined and made up to 10 ml.

Estimations of nucleic acids were made by ultraviolet absorption measurements of the appropriate perchloric acid.

extracts at 260 m μ . for RNA and at 268 m μ . for DNA.

Standards were prepared from commercial preparations of RNA (Na salt from yeast, British Drug Houses) and DNA (Na salt from thymus gland, British Drug Houses). Standard solutions of RNA were made in 1N perchloric acid; DNA standard solutions were prepared in 0.5N perchloric acid and heated at 70° for 20 min. Extinctions of these standard nucleic acid preparations were related to phosphorus content and the resulting values:

0.347/ μ g.P./ml. for RNA at 260 m μ .,

and 0.279/ μ g.P./ml. for DNA at 268 m μ .

used to calculate the nucleic acid phosphorus contents of the test samples.

Phosphorus estimations were made using the method of Allen (1940), with the following reagents:-

- a). Ammonium molybdate - An 8.3% (w./v.) solution of ammonium molybdate ('Analar', British Drug Houses) in distilled water.
- b). Amidol - Solution containing 2g. amidol (British Drug Houses) and 40g. of sodium metabisulphite (British Drug Houses) in 200 ml. distilled water. This reagent was kept in a well-stoppered, dark bottle and discarded after a period of 2 days.

The material under test was placed in a hard glass test tube with a small piece of porous pot, and 2.2 ml. of

60% (w./v.) perchloric acid ('R' grade, sp.gr. 1.54, May & Baker Ltd.) added. The tube was heated over a small flame until the contents became colourless, the combustion being accelerated by the addition of two drops of 30% (v./v.) hydrogen peroxide (British Drug Houses). The tube was cooled and the contents mixed and rinsed with distilled water into a 25 ml. volumetric flask; 2 ml. of amidol reagent and 1 ml. of ammonium molybdate reagent were added. The total volume was adjusted to 20 ml. with distilled water. Optical densities at 650 mμ. were read after standing for 15 min. at room temperature. Comparisons were made with standard solutions of potassium dihydrogen phosphate ('Analar', British Drug Houses).

Section 5

ACRIDINE ORANGE STAINING.

A. Staining Procedure.

The acridine orange used in this investigation was obtained direct from Imperial Chemical Industries Ltd, Dyestuffs Division and was designated Acridine Orange R150. All staining solutions were prepared in 0.2M acetate buffers at a concentration of 0.05% (w./v.) of the dye. Solutions were stable for many weeks if stored at 4°, but for routine use, fresh staining solutions were made each week. The staining procedure for fixed preparations was as follows:- The slide or coverslip, containing the fixed material, was equilibrated for 10 min. in acetate buffer of the same pH as that used in the preparation of the staining solution. The slide or coverslip was then stained in acridine orange solution for 10 min. Excess stain was washed off the specimen with acetate buffer and the stained preparation washed in acetate buffer for 10 min. After one more wash in the buffer for 10 min. the slide was gently blotted dry and mounted with buffer, the edges of the coverslip being sealed with paraffin wax.

B. Fixation.

Colony impression preparations were made by inverting blocks of agar containing the colonies on to coverslips and then immersing in the fixative. After fixation, the agar block was peeled off the coverslip leaving the colonies

fixed to the glass surface. The preparations were then easily handled for staining.

The fixatives used were:-

- a). Carnoy's solution - consisting of absolute ethanol, 6 parts; chloroform, 3 parts and glacial acetic acid, 1 part (v./v.). After fixation, preparations were washed quickly in 95% (v./v.), then 50% (v./v.) ethanol and finally acetate buffer.
- b). Bouin's fluid - saturated aqueous solution of picric acid, 75 parts; formalin, 25 parts and glacial acetic acid, 5 parts (v./v.). The fixative was washed from preparations with 50% (v./v.) ethanol and acetate buffer.
- c). Zenker's fluid - containing 5g. mercuric chloride, 2.5g. potassium bichromate and 1g. sodium sulphate in 100 ml. distilled water. Immediately before use, 5% (v./v.) of glacial acetic was added. Fixed material was washed in 50% (v./v.) ethanol and acetate buffer.
- d). Formalin - 10% (v./v.) formalin in physiological saline. After fixation, the material was washed in acetate buffer only.

The efficiency of fixation of the agar colonies was judged by staining the preparations with acridine orange at pH3 and viewing under ultraviolet/blue light. Of the fixatives used only Bouin's fluid showed sufficient

penetrating power to fix the whole colony structure. The other fixatives failed to fix the granular centres of the colonies and with Zenker's fluid and formalin, some distortion of cells was noted.

C. Specificity of Staining.

The specificity of the acridine orange staining at various pH values was investigated using agar colony preparations, fixed in Bouin's fluid. Staining solutions, containing 0.05% (w./v.) acridine orange were prepared in acetate buffers with pH values ranging from 2.8 to 5.6. Specimens of fixed colonies were stained with the dye solutions of varying pH and mounted in the appropriate buffer. A second set of preparations was treated with $M/15$ phosphate buffer pH 7.4 containing 0.02 mg./ml. of ribonuclease (2 x cryst, California Biochemicals) at 37° for 2 hr. and then stained with the acridine orange solutions. A third set of preparations was treated with deoxyribonuclease (grade B, California Biochemicals) 0.02 mg./ml. in $M/15$ phosphate buffer pH 7.4, containing 0.005M $MgCl_2$, at 37° for 2 hr. The enzyme treated preparations were then stained with acridine orange at various pH values.

All the specimens were examined under ultraviolet/blue light and the staining reactions noted for each pH value.

D. Optical Systems.

Two optical systems were used in this investigation, an optical bench apparatus and a commercial 'Zetopan' Binolux

fluorescence microscope (Reichert AG.).

The optical bench instrument consisted of a high-pressure mercury lamp (200W. ME/D. Mazda Ltd.) with a heat absorbing filter cell (20 m.m. width) containing a solution of 15% (w./v.) aqueous copper sulphate. The emission filter consisted of a Wratten 50 gelatin (Kodak Ltd) filter sandwiched between two plates of thin glass, and the secondary filter used was a 2 m.m. gelatin Minus Blue (Ilford Ltd). The secondary filter was incorporated in the eye piece of a horizontal Zeiss microscope, fitted with a Watson apochromatic X90 oil immersion objective with adjustable iris diaphragm.

The 'Zetopan' fluorescent microscope was provided with a HBO 200W high-pressure mercury lamp (Osram, W. Germany) giving a bright light intensity. For ultraviolet blue illumination, a BG12/6 m.m. glass filter (Schott u. Gen.) was used in conjunction with a secondary ultraviolet/blue-stopping filter (GG9/1 m.m. + OG1/1.5 m.m., Schott u. Gen.).

Section 6

SEPARATION OF ELEMENTS FROM LIQUID CULTURE.

A. Gradient Centrifugation.

Gradients were prepared with sterile sucrose solutions. The following solutions of sucrose (Kerfoot & Co.) were prepared and sterilised at 10 lbs/sq. in. for 10 min. in the autoclave - 2M, 1.5M, 1M and 0.5M. Descending gradients were prepared by carefully pipetting 0.75 ml. of each of the sucrose solutions into a sterilised polypropylene centrifuge tube of total capacity 5 ml. Discontinuous gradients were used within 30 min. of preparation, before diffusion of the sucrose layers had taken place. Continuous gradients were prepared as described above, but were allowed to equilibrate for 24 hr. at 4° before use.

A suspension of L-form in 0.5M sucrose solution was prepared and treated with deoxyribonuclease as described in Section 1D, and 0.75 ml. of this suspension carefully layered over the sucrose gradient. The centrifuge tubes were then placed in the 3 x 5 ml. swing-out rotor (No.29314) of the 'Superspeed 40' centrifuge (M.S.E. Ltd.) and centrifuged at 15,000 r.p.m. (average R.C.F. 20,000 x g) for varying time

After centrifugation, the tubes were examined in front of a strong microscope light to detect the 'bands' formed. Any 'bands' of cells were collected aseptically with fine Pasteur pipettes and transferred to sterile tubes. The parts

of the gradients between the bands were also collected. Each gradient cut was examined under phase-contrast and inoculated on to L-form agar and into L-form broth. Fixed preparations for acridine orange staining were made by placing drops of the fractions on agar blocks, inverting the blocks on coverslips and fixing in Bouin's fluid overnight.

B. Differential Centrifugation.

Differential centrifugation of L-form cultures was carried out on cells previously harvested and treated with deoxyribonuclease as described in Section 1D. The enzyme-treated cells were resuspended in growth medium for centrifugation.

Centrifuges used in the procedure were the 'Superspeed 40' (M.S.E. Ltd.) and the Servall 'Superspeed' (I. Sorvall & Co.Ltd). Autoclavable polypropylene tubes with tightly fitting caps were used in both these machines, and aseptic precautions were taken in all the manipulations during the centrifuging process.

The isolated fractions were examined under phase-contrast and inoculated on to solid and into liquid growth media. Preparations for acridine orange staining were prepared by the agar fixation method, although it was subsequently found that identical staining results could be obtained with unfixed cells. For rapid monitoring of

the centrifugation process, unfixed fractions were stained by the addition of approximately 1% (v./v.) of the acridine orange staining solution to the liquid fractions. Drops of these mixtures were mounted under coverslips, sealed with wax and examined under the fluorescence microscope. Final preparations of the fractions, however, were prepared by fixation and the specificity of staining verified by the use of nucleases.

Section 7

IMMUNOLOGICAL METHODS.

A. Preparation of Antisera.

Antigen suspensions for injection were prepared from whole staphylococci and L-forms, previously killed by the addition of 1% (v./v.) formalin to broth cultures. The cells were harvested by centrifugation, washed once in the appropriate salt solution, and resuspended in physiological saline containing $1/10,000$ (v./v.) 'Merthiolate' (Lilly & Co.Ltd) at a density of approximately 5 mg. dry weight/ml. Similar suspensions were also prepared from purified cell walls and cell membranes.

Young rabbits, between $1\frac{1}{2}$ and 2 Kg., were used for the production of antisera. The rabbits were bled from the ear vein before commencing the injection schedule. Injections of the antigen suspensions were administered one each day for periods of 4 days with a rest period of 3 days between each course. Preliminary injections consisted of 0.5 ml. of antigen suspension intravenously; at the second course, the volume was increased to 0.75 ml., and at the third course to 1 ml. Thereafter, throughout the immunisation procedure, 1 ml. volumes of antigen suspensions were injected.

The schedule was continued for 4 weeks, when the rabbits were rested for 1 week following the last injection and then bled from the ear vein. The titres of the sera obtained were

tested by agglutination and if satisfactory, the animals were bled to obtain a larger volume of serum. In cases where the titre was found to be low, the injection schedule was continued until on testing, satisfactory titres were obtained. The rabbits were finally bled by cardiac puncture. All sera were preserved by the addition of $1/10,000$ (v./v.) 'Merthiolate' or 1% (w./v.) sodium azide (British Drug Houses), and stored at -10° .

B. Agar Gel Diffusion.

Agar gel double diffusion techniques used were based on those of Ouchterlony (1958).

The agar gel was prepared as a 2% (w./v.) aqueous solution of 'Ionagar' No.2 (Oxoid) containing 1% (w./v.) sodium azide. The hot agar was mixed with an equal volume of $M/15$ phosphate buffer pH 7.2, and layers of this mixture made on clean glass plates (2" x 2") or standard microscope slides. The volume of agar gel used for the glass plates was 3.5 ml. and for the microscope slides, 2.6 ml. The agar mixture was prevented from flowing off the glass by surface tension and when set formed layers, approximately 2-3 m.m. thick. Various patterns of wells were cut in the agar layers using a No.4 cork borer in conjunction with a Perspex template. It was not found necessary to seal the bottoms of the wells with agar, since no diffusion between the agar layers and the glass was seen. The agar plates were stored in air-tight

boxes, containing wet filter paper, until required.

The wells were filled with the appropriate antigen suspensions and antisera, which were allowed to diffuse against one another, the agar plates being kept moist to prevent drying and distortion of the agar. The plates were examined after 18 hr. and at regular intervals until all precipitin lines were judged to have formed. Unprecipitated protein was removed from the agar layers by washing in physiological saline, and the slides preserved by drying of the agar layer under filter paper in an incubator at 37°. When the paper was removed, the agar remained attached to the glass as a very thin layer. At this stage, the precipitin lines were not visible, but because of their protein nature, were easily stained with conventional protein stains.

The glass slides with the dried agar layers, were placed in a 3% (v./v.) solution of acetic acid for 10 min. to fix protein and then transferred to a staining solution of 150 mg. Ponceau S (G.T. Gurr & Co.) in 100 ml. of distilled water containing 3% (w./v.) trichloroacetic acid. The plates were stained in this solution for 30 min. or until the agar was overstained, and then differentiated in 3% (v./v.) acetic acid. Washing was continued in acetic acid until all the excess dye had been removed, when the plates were given one wash of 5 min. in 3% (v./v.) acetic acid containing 10% (v./v.) glycerol. The glycerol was quickly washed off the slides with acetic acid and the slides set, face downward, to dry at 37°.

When dry, the lines of precipitation appeared red in the thin layer of almost colourless agar adhering to the glass.

C. Preparation of Antigens.

Suspensions of particulate antigens were prepared in $M/30$ buffer pH 7.2 at a concentration of 20 mg./ml. Cell suspensions in $M/30$ phosphate buffer pH 7.2 were crushed in the 'X-press' and the resulting extract diluted to contain the equivalent of 20 mg. dry weight of whole cells/ml. The soluble antigens, teichoic acid and polysaccharide A were dissolved in $M/30$ phosphate buffer pH 7.2 at a concentration of 2mg./ml.

Cell membranes were prepared for gel diffusion tests by trypsin digestion as described by Freimer (1963). Freeze dried cell membranes were suspended at 10 mg./ml. in $M/15$ phosphate buffer pH 7.4 and mixed with an equal volume of physiological saline containing 20 μ g. of crystalline trypsin (L.Light & Co.)/ml. The mixture was incubated at 37° for 6 hr. and the insoluble residue removed by centrifugation at 20,000 x g for 20 min. The supernatant was used in precipitin tests.

All antigen suspensions or solutions were kept frozen at -10° .

RESULTS

Section 1

INDUCTION AND GROWTH IN THE L-PHASE.

Following the observation by Pierce (1942) that Streptobacillus moniliformis could be induced to grow in the L-phase by treatment with penicillin, many bacteria have been produced in the L-form. In many cases, L-form growth required the presence of a high osmotic environment and a relatively complex medium. The present investigation deals with the induction and growth of L-forms from strains of Staphylococcus aureus.

A. Induction and Subculture on Solid Medium.

Penicillin-sensitive strains of Staphylococcus aureus were induced to grow in the L-phase by plating on medium containing serum, a high sodium chloride concentration and penicillin, (see Methods). For the strains used in this investigation, large inocula of staphylococci were required to produce L-colonies regularly; even 0.5 ml. of a 10x concentration suspension of an overnight culture of staphylococci, plated on the complete medium commonly gave only small numbers of L-colonies, (Williams, 1963). Incubation of the plates for 2 to 6 days was usually necessary before typical L-colonies were seen.

The L-colonies from primary plates were subcultured by inversion of blocks of agar containing fully developed colonies on to fresh medium. After 18 hr. incubation at 37°

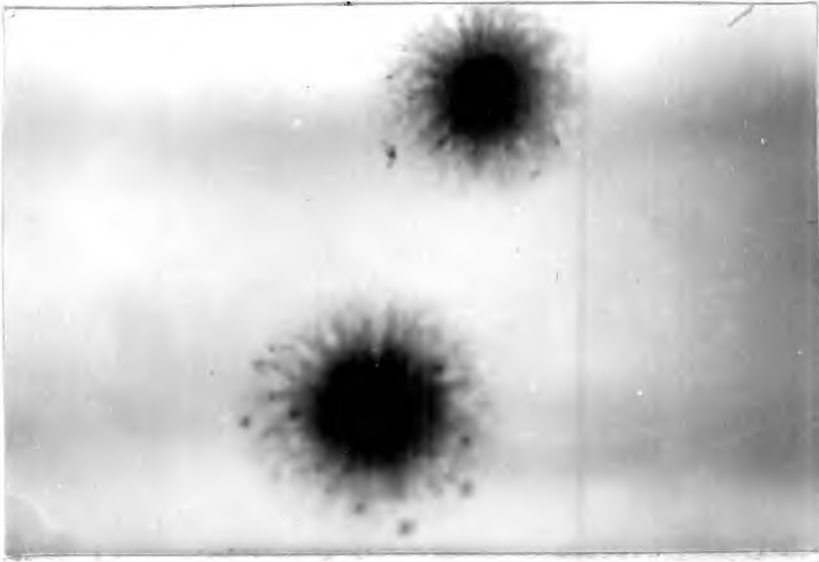


FIGURE 4. Staphylococcal L-form colonies.
Agar block mount, stained with
methylene blue and Azure II
mixed stain. x125

the blocks were moved over the surface of the agar, leaving areas of new growth. Further incubation produced fully developed colonies where the agar block had been originally placed.

Various attempts to induce L-colony formation on anything but the complete medium proved unsuccessful. When serum was omitted from the medium, or when the salt concentration was below 3%, no typical L- colonies were produced. When penicillin was not present, in an otherwise complete medium, confluent growth of staphylococci occurred.

The isolation and subculture of three of the L-form strains used in this work have been described previously by Williams (1963). They were isolated from penicillin-sensitive cultures of Staph. aureus, PS 42E, PS6, and 1444 (phage type 3B/+). The fourth L-form culture was isolated by a similar method, with D-cycloserine in place of penicillin, from a penicillinase-producing strain of Staph. aureus, Rogers (phage type 42E). All four L-form strains produced identical colony forms on the protective medium.

B. Characteristics of L-form Colonies.

Staphylococci growing in the L-form produce characteristic colonies similar to those described for L-forms of other bacteria and for pleuropneumonia-like organisms (Fig.4).

The centres of the developed colonies are dense, granular structures which are hard to the touch and very

difficult to remove from the agar. Examination of the colonies under the microscope shows the hard centre portion to be embedded in the agar to a depth of $\frac{1}{2}$ to 1 m.m. The peripheral growth consists of large round or oval structures varying in size from 2 to 10 u., and appears to be associated with a slime-like substance. The globular cells immediately adjacent to the granular centre can be seen to contain granular inclusions, when stained with a mixture of methylene blue and azure II. In young colonies, the cells on the edge of the colony are clear and homogeneous, but in older colonies some of these cells show similar granular inclusions. Very old colonies show only a narrow peripheral growth of large cells, the greater part of the colony consisting of granular material.

C. Growth in Liquid Culture.

The establishment of L-form growth in liquid medium was difficult, although eventually all the strains were obtained in liquid culture. This was achieved by subculturing blocks of agar containing developed colonies into small volumes of liquid medium. In successful cases, 'strings' of slime-like growth could be seen adhering to the agar blocks after incubation for 48 hr. When these cultures were gently shaken, some of the thick growth was released into the liquid medium. Inoculation of some of this slime into fresh medium resulted in a liquid culture capable of producing typical L-colonies when transferred back to solid medium. Liquid cultures could

be propagated by normal methods.

The liquid cultures consistently produced the thick, slimy growth with little or no turbidity in the supernatant broth. Microscope examination of the growth under phase-contrast revealed a general background of slightly phase-dense amorphous material, embedded in which were numerous elements of varying size. The cells ranged in size from large vesicles (15 to 20 u.) to very small granules, which were just visible. The supernatant broth, although showing very little turbidity, also contained this range of elements but in very much smaller numbers.

Various enzymes were used in an attempt to remove the amorphous material and so give a uniform turbidity to the cultures. Purified deoxyribonuclease (DNAase) proved extremely effective in this respect (see Methods). On examination of DNAase-treated cultures, the cells were found to be evenly dispersed throughout the medium. Such treated cultures were still capable of growth in liquid and on solid medium.

The efficiency of pure deoxyribonuclease in removing the slime-like material from the cultures suggests that this material is highly polymerised deoxyribonucleic acid. This was later confirmed by both chemical and staining procedures. The deoxyribonucleic acid is presumably released either as extracellular DNA during the growth of the organism, or by

lysis of cells. The high sodium chloride concentration of the medium probably contributes to the accumulation of DNA by inhibiting the action of any cellular deoxyribonuclease which may be produced.

D. Harvesting of Growth from Liquid Culture.

As a consequence of the large amounts of DNA produced, growth from a broth culture could be centrifuged and deposited at relatively low speed, 1,000 x g for 15 min. However, the supernatant fluid after such treatment was faintly turbid and on examination was found to contain numbers of large, globular cells and small granules. Centrifugation at 70,000 x g for 30 min. was necessary to remove these large cells and granules from the culture medium, leaving it virtually sterile.

Pretreatment of the broth cultures with deoxyribonuclease produced a uniform turbidity in the broth. The large globular cells could be sedimented by centrifugation at 27,000 x g for 10 min., leaving an almost clear supernatant, which still contained large amounts of granular material. The granules were sedimented by centrifugation at 70,000 x g for 30 min.

E. The Effect of Protein and Other Growth Supplements.

In all preliminary experiments on growth in liquid medium, 10% (v./v.) horse serum was used as a growth supplement. The whole serum was replaced later by 0.5% (v./v.) PPLO serum fraction (Difco).

TABLE 1. Growth responses of L-form 1414 with protein
and other supplements.

<u>Supplement</u>	<u>Concentration</u>	<u>Growth Response*</u>
Whole horse serum	10 %(v/v)	100
"	5 %(v/v)	25-30
PPL0 serum fraction	0.5%(v/v)	90-110
"	0.4%(v/v)	80-85
"	0.3%(v/v)	20-25
Bovine serum albumin	0.5%(w/v)	60-65
"	0.4%(w/v)	60
Granular charcoal	5 %(w/v)	5-10
"	1 %(w/v)	5-10
None	-	0

*Turbidity of enzyme-treated cultures compared
with medium + 10%(v/v) horse serum.

Turbidities were read in a Hilger 'Spekker'
absorptiometer with H508 neutral filters.

The function of the serum or serum fraction in the growth of the L-form was unknown, but it has been suggested that detoxification of the medium is the main function (Klieneberger-Nobel, 1960). Accordingly, it was decided to test the effect of various known detoxifying agents, such as bovine serum albumin (Davies & Dubos, 1947; Razin & Knight, 1960) and charcoal (Pollock, 1949; Smith, 1955) on the growth of the organism.

The supplements were incorporated in the liquid medium and the complete media inoculated with 0.1 ml. of a concentrated suspension of enzyme-dispersed L-form. Incubation was for 48 hr. at 37°. The 'stringy' growths were dispersed by deoxyribonuclease treatment and the resulting turbidities compared with that produced by growth in whole serum-supplemented medium (TABLE 1).

Difco PPLO serum fraction, at 0.5% (v./v.), gave good growth of the L-form and compared favourably with 10% (v./v.) whole horse serum as a growth supplement, although at concentrations less than 0.5%, growth response was reduced. Bovine serum albumin Fraction V (Armour) also permitted good growth, but at 0.5% (w./v.), the amount of growth was only just over half that produced by whole serum. Comparable results were obtained with 0.4% (w./v.) serum albumin. Some growth of the L-form was obtained when the serum was replaced by 5% (w./v.) granular activated charcoal. In this case, growth was seen as a surface coating on the pieces of charcoal in the

medium, but was poor when compared with that produced in protein supplemented media. Reduction of the concentration of charcoal to 1% (w./v.) did not alter the growth response.

Section 2.

SUSCEPTIBILITY TO LYSIS.

In preliminary experiments on harvesting of L-form from broth culture, the organisms showed a marked sensitivity to lysis in hypotonic solutions. Suspension of the harvested growth in water or physiological salt solution resulted in a fall in optical density and loss of viability when plated on protective medium. This is in direct contrast to staphylococci which remain viable after suspension in salt solutions having a wide range of concentration. The 1444 strain of Staph. aureus and its L-form were tested for their reactions to suspension in salt solutions of varying concentration and to treatment with surface-active agents.

A. Suspension in Hypotonic Solution.

The L-form, grown in medium containing a total of 3.5% (w./v.) NaCl, were harvested and treated to remove extracellular DNA. The cells were then suspended in salt solutions of varying strength and incubated at room temperature for 30 min. Staphylococcal cells of the same strain were also suspended in similar salt solutions and incubated. The optical densities of the suspensions were read at 500 mu. in the spectrophotometer. The results are presented in FIG.5.

Suspensions of staphylococcal cells exhibited no marked changes in optical density over the range of salt concentrations, except for a slight decrease at high

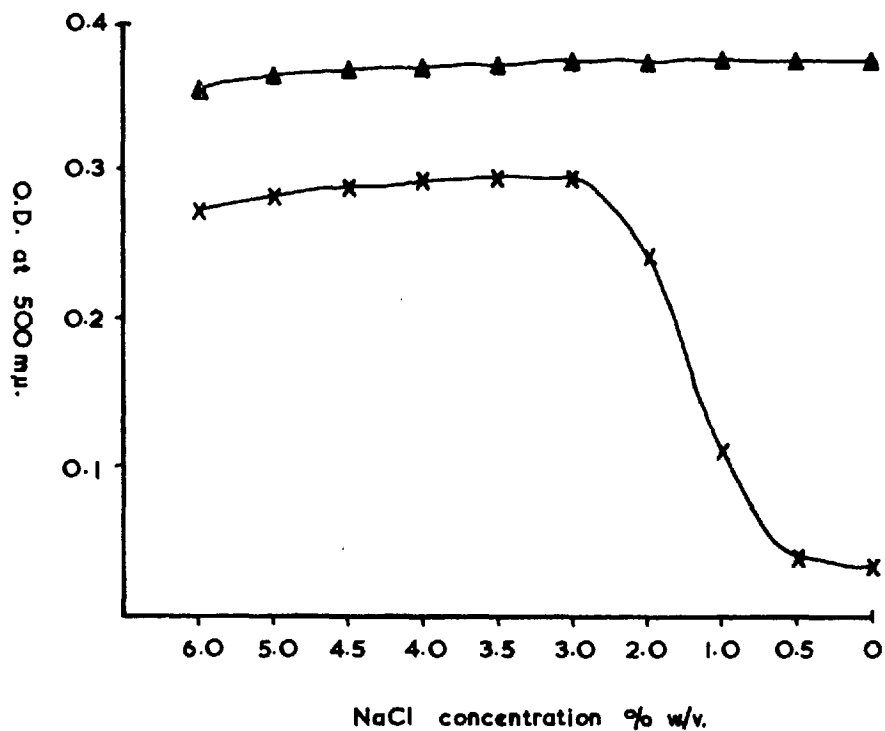


FIGURE 5. Effect of suspension of staphylococcal L-form 1444 in salt solutions of varying concentration.

▲ - suspension of staphylococcal cells

x - suspension of L-form 1444

Readings taken after 30min incubation at R.T.

concentrations. This might have been due to depression of the light-scattering of the organisms caused by the high refractive index of the suspending solution (Gilby & Few, 1959). The suspensions of the L-form showed little change in optical density over the range 4.5% to 3.0% NaCl, but below this level optical density fell to reach at 0% NaCl only 12% of the optical density shown in 3.0% NaCl solution. The optical density level in low salt concentrations presumably represents a suspension of cell debris.

The behaviour of the L-form in this test showed similarities to that of bacterial protoplasts suspended in solutions of decreasing tonicity. Bacterial protoplasts are stabilized by sucrose or sodium chloride solutions only above a concentration of the solute representing high osmotic protection levels (Gilby & Few, 1959). At solute concentrations below the 'lytic threshold', the protoplasts imbibe water from the medium and because they lack a rigid cell envelope, lysis occurs.

It was of interest therefore to compare the osmotic properties of the L-form with those of protoplasts prepared from the parent staphylococcus.

The staphylococcal cell wall is normally resistant to enzymes, such as lysozyme, which have been used in the preparation of protoplasts from other organisms. Various other methods have been suggested for the preparation from staphylococci, among them the autolytic method of Mitchell &

Moyle (1957).. With the discovery by Hash (1963) of an enzyme isolated from a fungus, capable of lysing Staph. aureus cells, a new and more easily controlled method of producing protoplasts presented itself.

Later work by Hash, Wishnick & Miller (1964) described the production of "protoplasts" from Staph. aureus H using the fungal N-acetyl hexosaminidase of Chalaropsis sp. It was shown that during incubation with the enzyme, the rigid part of the cell wall of the staphylococcus was removed completely, leading to lysis of the cells or to the production of "protoplasts" if the incubation was carried out in protective medium. The resulting "protoplasts" were shown to contain no rigid cell wall or any of the amino sugars associated with this structure. The fate of the other major cell wall component, teichoic acid was not determined hence the use of the qualified term "protoplast" (Brenner et al, 1958).

Some of the purified Chalaropsis B N-acetyl hexosaminidase was obtained from Dr. J. Hash and used for the preparation of "protoplasts" from the 1444 strain of Staph. aureus and from Staph. aureus H. The effect of the enzyme was also tested on the L-form cells.

Staphylococcus aureus 1444 cells were suspended in acetate buffer (0.01 M, pH 5.7) and in 0.5M sucrose containing the same buffer as recommended by Hash, Wishnick & Miller (1964). Chalaropsis B enzyme was added to each

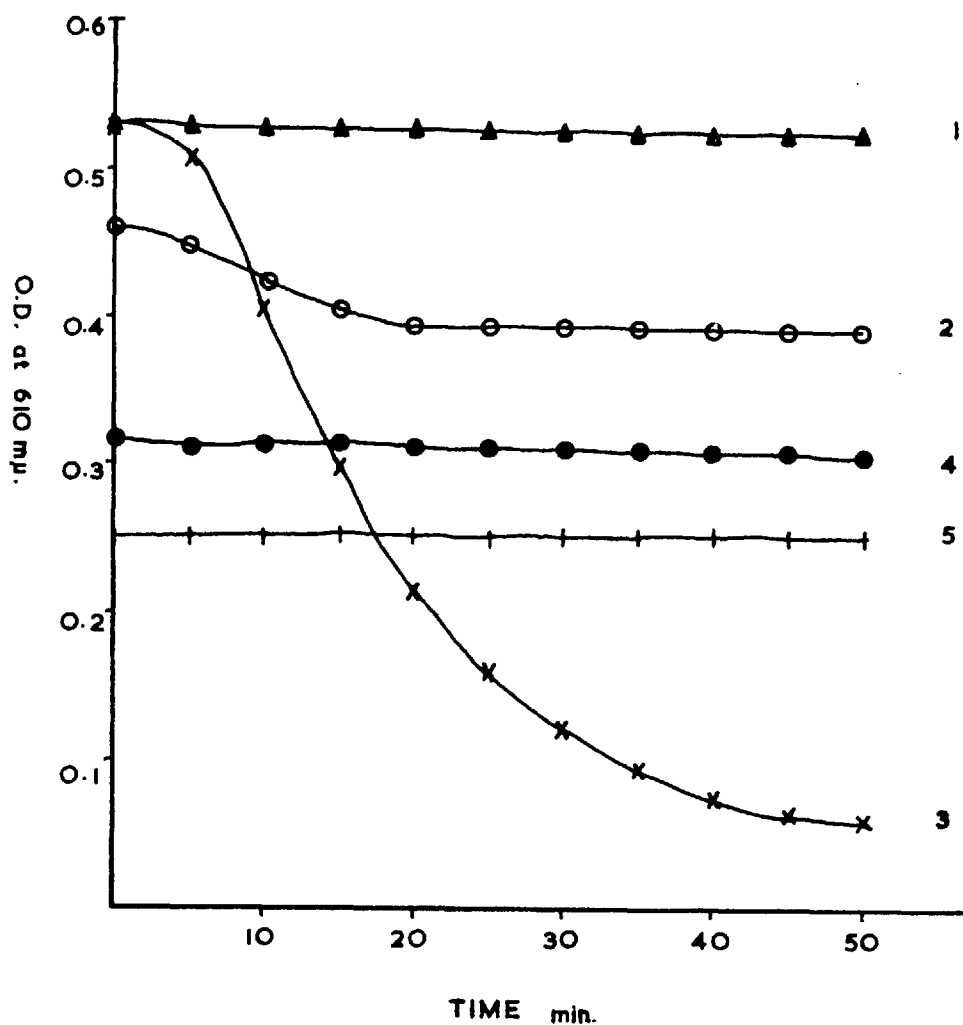


FIGURE 6. Action of Chalaropsis B N-acetylhexosaminidase.

1. staphylococci 1444 - control
2. staphylococci 1444 in 0.5M sucrose+enzyme
3. staphylococci 1444+enzyme (absence of sucrose)
4. L-form 1444 in 0.5M sucrose+enzyme
5. L-form 1444(lysed)+enzyme (absence of sucrose)

suspension to a concentration of approximately 30 $\mu\text{g./ml.}$ Control suspensions of cells without enzyme were also prepared. Changes in optical density were followed in a spectrophotometer at 610 m μ using 1 cm. glass cells. The resulting curves are presented in FIG.6.

Control cells, incubated in acetate buffer but without the enzyme showed no fall in optical density after 1 hr. incubation at room temperature. A similar preparation of cells but containing the enzyme, showed a slight loss of optical density during the first 5 min., after which the rate of clearing rapidly increased. At the end of 1 hr. at room temperature, the optical density had reached a more stable level but some lysis still occurred.

The staphylococcal cells suspended in the sucrose buffer solution and treated with the enzyme showed a small initial drop in optical density, which after a period of 20 min. remained virtually constant. Preparations of Staph. aureus H, the organism used by Hash, gave similar results at the same enzyme concentration except that the initial lag in lysis was less marked. Samples of the "protoplast" preparations in 0.5M sucrose showed rapid clearing when diluted with 1 vol. of water or physiological saline; this was not shown by the central cells when similarly treated.

This enzyme has been characterised by Hash (1963)

as an N-acetyl hexosaminidase capable of dissolving 80 to 85% of S. aureus cell wall mucopeptide fractions prepared by the method of Park & Hancock (1960). It was of interest to discover whether the L-form cells contained any material which might act as a substrate for this enzyme, this indicating the continued presence of cell-wall material.

Suspensions of the L-form were made in the acetate buffer and also in the buffer with protective sucrose. These suspensions were allowed to equilibrate overnight to facilitate the completion of any lysis which might occur. Chalaropsis B enzyme was added and any changes in optical density followed as before at 610 mμ. These curves are shown also in FIG. 6.

The suspensions of the L-form prepared in the protective medium remained at a constant optical density throughout the period of the test, except for very small variations. Buffer suspensions of the L-form did not show any fall in optical density apart from minor changes, which were also shown by suspensions of control cells.

With the Chalaropsis B enzyme as a means of producing "protoplasts", it was possible to undertake a comparative study of the effect of various surface-active and other agents on staphylococcal cells, L-form cells and "protoplasts". The L-form cells were prepared as previously described, and the "protoplasts" prepared by overnight incubation at

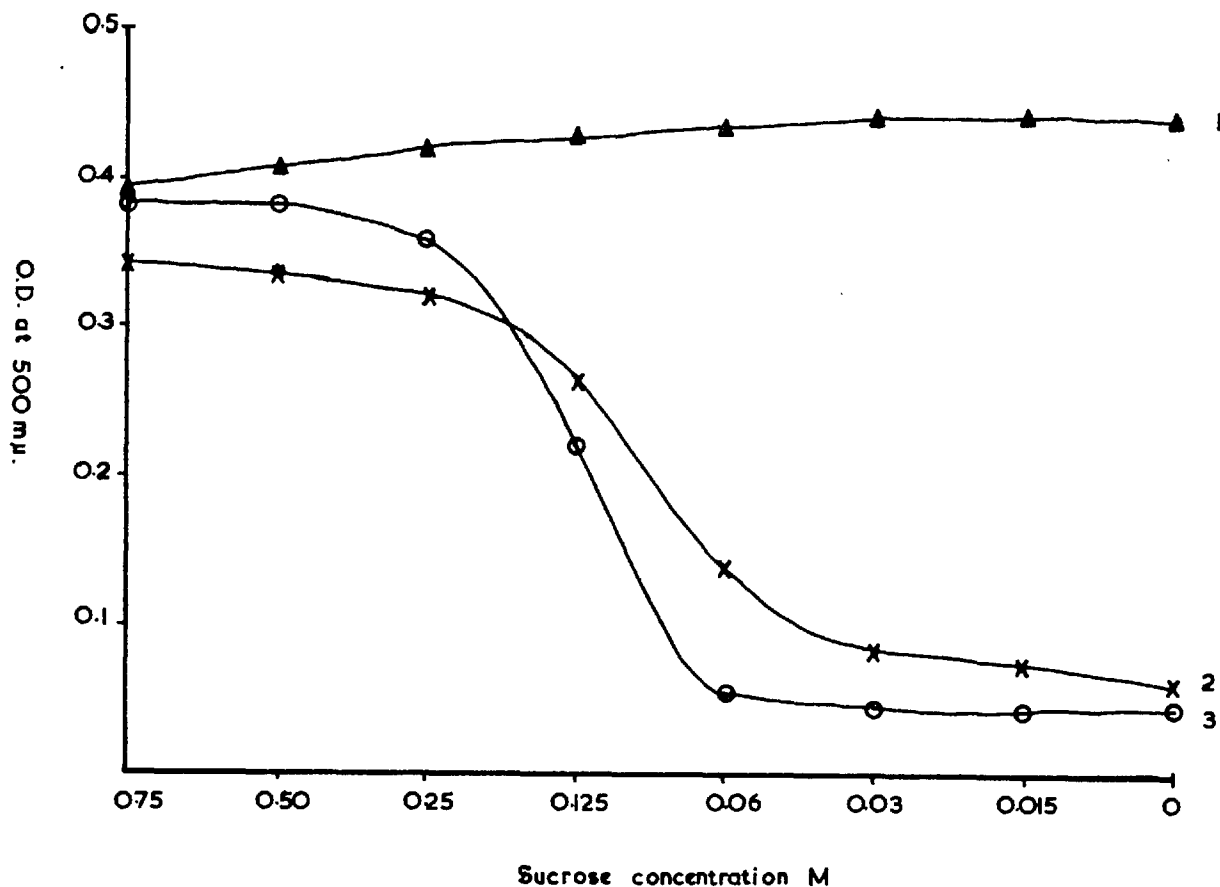


FIGURE 7. Lysis of L-form 1444 and protoplasts 1444
in sucrose solutions.

1. staphylococci 1444 - control

2. L-form 1444

3. staphylococcal protoplasts 1444

Readings taken after 30min incubation at R.T.

4° of staphylococcal cells suspended in 0.5M sucrose containing 0.01M acetate pH 5.7 and 30 $\mu\text{g./ml.}$ of Chalaropsis B enzyme. The "protoplasts" were collected by centrifugation at 25,000 x g for 30 min. and then resuspended in 0.5M sucrose containing 0.05M NaCl. This suspending medium was used throughout the tests except where solutions of decreasing tonicity were needed.

A solution of sucrose (1M) containing 0.05M NaCl was diluted with 0.05M NaCl solution to give a series of solutions with decreasing tonicity. To 4 ml. quantities of these solutions was added 0.1 ml. of the suspension of organisms and the mixtures were incubated at room temperature for 30 min. Optical densities were then read at 500 m μ . using 1 cm. cells. The results are presented in FIG.7.

The suspensions of control staphylococcal cells maintained a constant optical density over the range of tonicity, except for the optical effect at high sucrose concentrations. The preparations of "protoplasts" showed evidence of lysis below 0.5M sucrose and a marked drop in optical density was seen below 0.25M sucrose. Lysis of the "protoplasts" continued at lower concentrations, finally reaching equilibrium between 0.06M and 0.03M. Below 0.03M sucrose, the optical density was constant and probably represented debris from the lysed cells.

The lysis of the L-form is represented by a much flatter curve, although lysis of the cells commenced at

approximately the same sucrose concentration as lysis of the "protoplasts". At lower concentrations of sucrose, the optical density of the L-form preparations fell steadily but did not reach a constant level. The less rapid and more prolonged lysis of the L-form is thought to be due to the great variation in size of the cells, the larger ones being possibly more easily lysed by low tonicity than smaller cells. The very small cells, because of their reduced surfaced area, might be capable of withstanding lower osmotic pressures, for a longer time.

B. Lysis by Alcohols.

The work of Gilby & Few (1960), Razin & Argaman (1963) and other investigators on the action of various surface-active agents on the cell envelopes of Mycoplasma cells and bacterial protoplasts suggested that such studies might prove informative in the case of the staphylococcal L-form. The effects of various primary alcohols were tested on the L-form and staphylococcal cells and the results compared with those obtained for the enzyme-produced "protoplasts".

Solutions of the alcohols were made in the normal suspending medium and suspensions of the organisms added. Optical densities were read at 500 mμ. after 30 min. incubation at room temperature. The results obtained with ethanol, propanol and n-butanol are shown in FIG. 8.

Both the L-form and the "protoplasts" appeared very sensitive to primary alcohols and were lysed by all three.

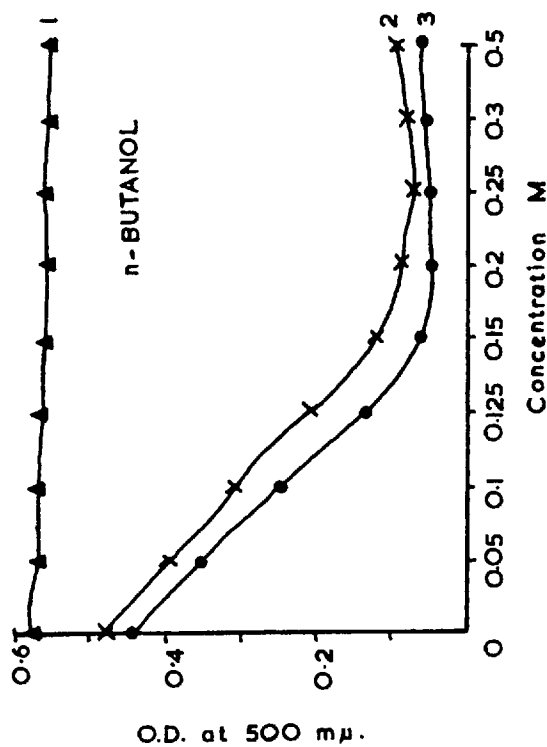
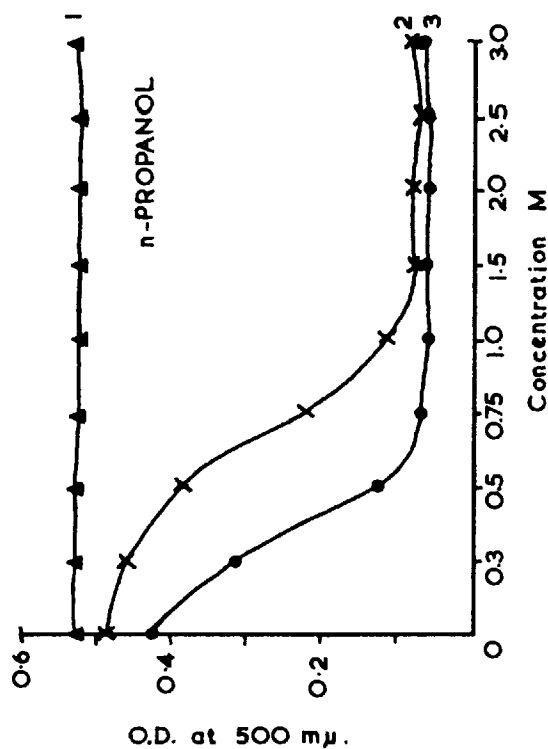


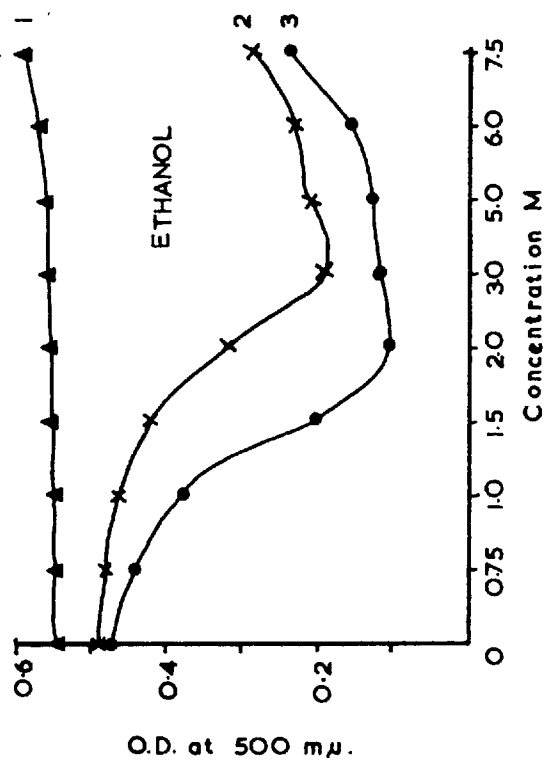
FIGURE 8. Lysis of L-form 1444 and 1444 protoplasts in primary alcohols.

1. staphylococci 1444

2. L-form 1444

3. staphylococcal protoplasts

1444



Readings taken after 30min incubation at R.T.

Lysis of the "protoplasts" by ethanol was initiated between 1M and 2M alcohol, a sensitivity similar to that of the L-form. Lysis of both cells was complete at concentrations of ethanol below 3M; at higher concentrations the optical density increased slightly. This increase in optical density was probably due to precipitation of cell protein in the high alcohol concentration (Razin & Argaman, 1963). Control suspensions of staphylococci showed slight variations in optical density over the range of the test. Susceptibility of the cells to lysis by propanol and n-butanol closely resembled that shown to ethanol. The concentration of propanol required to initiate lysis of the L-form and "protoplast" cells was approximately one third that of ethanol. Similarly, the concentration of n-butanol to effect the same degree of lysis in both types of cell was one third to one quarter that of propanol.

Section 3

CHEMICAL EVIDENCE FOR THE LACK OF NORMAL CELL WALL IN L-FORM CELLS.

The results obtained in the experiments described in the previous section have indicated some similarity in osmotic sensitivity between the L-form and "protoplasts". Since penicillin has been found to exert some inhibitory action in cell wall synthesis (Salton, 1964), it was of interest to find the extent to which the L-form organism resembled the parent staphylococci in the structure of the cell envelope.

The presence of Staphylococcus aureus cell walls of two distinct polymers, mucopeptide and ribitol teichoic acid, affords a useful criterion in investigations into the nature of the surface of the L-form organisms (Salton, 1964). Mucopeptide, which forms the rigid layer in Staphylococcal cell walls, has been shown to yield, on acid hydrolysis, D-glucosamine, muramic acid, D- and L- alanine, D-glutamic acid, L-lysine and glycine. In the case of walls from the cocci under study, small amounts of leucine (or isoleucine) and valine have also been found (FIG.9).

Muramic acid (3-O-carboxy ethyl D-glucosamine) has been found only in members of the Eubacteriales, Rickettsia and some blue-green algae. Its presence within the individual organism is also restricted, mainly to the cell wall mucopeptide or as a biosynthetic intermediate in the

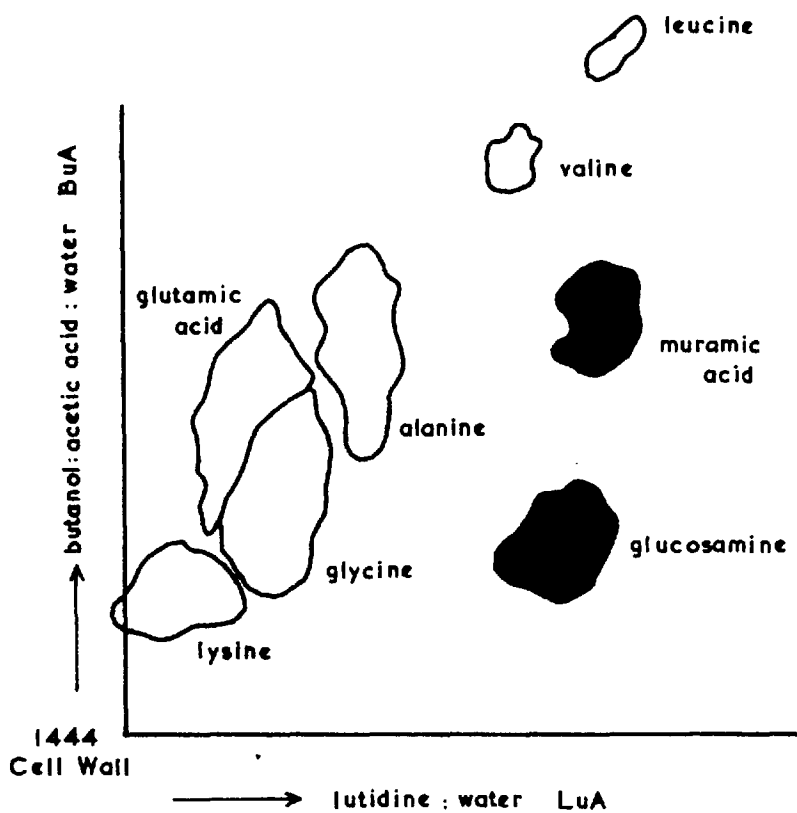


FIGURE 9. Acid hydrolysate of Staphylococcus aureus 1444
cell wall.

The shaded areas represent spots giving positive
reactions with both ninhydrin and Elson-Morgan
reagents.

interior of the cell. Failure to detect this unique sugar in hydrolysates of bacterial cells would provide strong evidence for the absence of normal cell wall structure.

Ribitol teichoic acid does not appear to contribute to the rigidity of the cell wall, but is found in close proximity to the mucopeptide, (Archibald, Armstrong, Baddiley & Hay, 1961). Acid hydrolysis of the isolated teichoic acid or of whole cell walls produces a variety of phosphate compounds, glucosamine and alanine and 1:4 anhydroribitol, which appears as an acid decomposition product of ribitol phosphates. The detection of anhydroribitol provides an easily recognised marker for the presence of ribitol teichoic acid. As in the case of muramic acid, the location of ribitol phosphate is limited to the cell wall, except in a transitory state as an intracellular precursor.

Both muramic acid and anhydroribitol may thus be used as indicators of the existence of what has come to be regarded as the normal cell wall in Staphylococcus aureus.

A. Mucopeptide.

(1) Cells.

Preliminary experiments involving acid hydrolysis of whole L-form and staphylococcal cells and subsequent chromatography of the products, revealed a striking difference between the L- and bacterial form (FIG.10). The acid hydrolysates (See Methods) of the bacterial form showed in addition to the

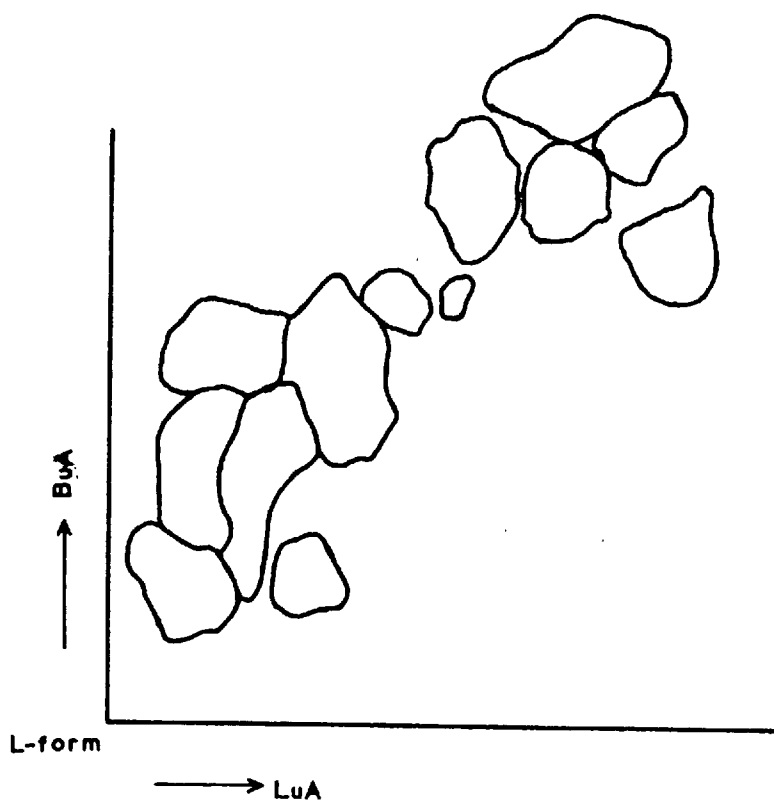
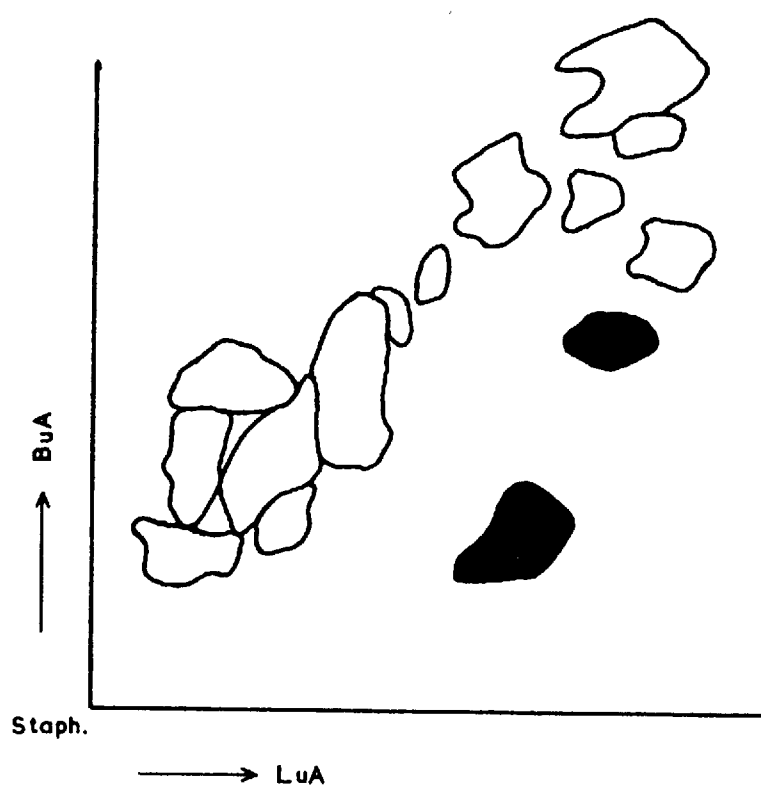


FIGURE 10. Acid hydrolysates of *Staphylococcus aureus*

1144 and L-form 1144.

Shaded areas as in FIG.9.

"protein amino acids", two spots corresponding to amino sugars. Neither of these spots was present in chromatograms of hydrolysates of L-form cells, which showed only the amino acids. Comparison of these chromatograms with others of hydrolysates of cell walls and reference to published Rf values, showed the two spots to be glucosamine and muramic acid.

Quantities of whole L-form up to 10 mg. dry weight failed to show any chromatographic spot corresponding to muramic acid, although at this level, a faint trace of glucosamine could be detected. However, even this faint trace was absent in hydrolysates of cells which had been extracted for a short time with cold trichloroacetic acid prior to hydrolysis. The removal of glucosamine with such mild treatment indicates its presence in the intracellular pool rather than as a part of any cell structure.

Quantitative studies of the contribution of amino sugars to the structure of the L-form cells, were undertaken using the methods of Elson & Morgan (1933), Randle & Morgan (1955) and Boas (1953). Since it was believed that only very small quantities of amino sugar would be found in these organisms, it was essential to eliminate the possibility of loss of any amino sugar during manipulation. The most likely point at which amino sugar could be lost, through degradation, is in hydrolysis. The most favourable conditions for hydrolysis were therefore determined by following the

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The second is the *New England Journal of Medicine*.

The third is the *British Medical Journal*.

The fourth is the *Lancet*.

The fifth is the *Annals of the New York Academy of Sciences*.

The sixth is the *Journal of the Royal Society of Medicine*.

The seventh is the *Journal of the American Society of Nephrology*.

The eighth is the *Journal of the American Society of Hypertension*.

The ninth is the *Journal of the American Society of Gerontology*.

The tenth is the *Journal of the American Society of Geriatric Medicine*.

The eleventh is the *Journal of the American Society of Geriatric Medicine*.

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TABLE 2. Release of total hexosamine during hydrolysis
of Staph. aureus 1444 cells.

Normality of HCl	Time (hr) at 100°				
	2	3	4	6	16
1	-	-	-	19.0	23.8
2	20.4	24.4	24.4	-	25.7
3	21.5	25.0	25.0	26.8	25.5
4	26.0	26.5	27.9	27.3	24.6

Results expressed as μg hexosamine/mg. dry
weight bacterial cells.

TABLE 3. Total hexosamine content of staphylococci
and L-forms.

	Sample	Hexosamine*	Hexosamine as
	(mg)	content(μg)	% dry weight
1444 cocci	10.18	284	2.79
L-form 1	12.20	< 10	< 0.082
2	26.50	< 10	< 0.038
PS6 cocci	10.62	309	2.91
L-form 1	7.10	< 10	< 0.141
2	31.20	< 10	< 0.032
PS42E cocci	9.65	279	2.89
L-form 1	6.70	< 10	< 0.149
2	28.80	< 10	< 0.035
Rogers cocci	2.50	95	3.80
L-form 1	6.70	< 10	< 0.149
2	12.50	< 10	< 0.080

*Material eluted by 2N HCl from Dowex 50 H⁺,
estimated in Rondle & Morgan (1955) test at
530 mμ and expressed as glucosamine base.

release of total hexosamine from samples of whole bacterial cells, (TABLE 2). Within the range tested, the maximum amounts of hexosamine (as glucosamine base) were released from whole cells when hydrolysis was carried out in 4N HCl for 4 hr. at 100°. Hydrolysis for longer periods showed evidence of degradation of amino sugar. The figures presented represent the resultant of amino sugar release and degradation of free amino sugar. Since the greatest amount of hexosamine was detected in 4N HCl hydrolysates, all subsequent samples for hexosamine estimation were treated in this way.

Using whole bacterial and L-form, results of total hexosamine content showed a very much higher level of amino sugar in the bacterial cells than in the L-form, (TABLE 3).

Whereas the extracts from the whole bacterial cells gave readings in the test equivalent to 2.79-3.80% dry weight of amino sugar, extracts from the L-form consistently gave very low readings. The results presented in the table indicate the maximum possible level of amino sugar which could be present under the conditions used in the quantitative test. Although the hexosamine contents of the L-form cells are very low, they do not of necessity indicate the complete absence of amino sugar from the cells. It is possible that hexosamine is present in these cells but reduced in quantity to such an extent as to be undetectable at the level of sensitivity employed. The use of larger samples of cells

TABLE 4. Glucosamine and muramic acid contents of
staphylococci and L-forms.

		Sample	Glucos.	Muramic	%*	%*
		(mg)	(μ g)	(μ g)	Glucos.	Muramic
		-----	-----	-----	-----	-----
1444	cocci	7.4	202	120	2.73	1.62
	L-form	86.3	<10	<30	<0.011	<0.033
PS6	cocci	6.9	173	96	2.51	1.39
	L-form	84.0	<10	<30	<0.012	<0.036
PS42E	cocci	7.2	199	104	2.76	1.45
	L-form	78.5	<10	<30	<0.013	<0.039
Rogers	cocci	5.5	151	95	2.75	1.73
	L-form	67.7	<10	<30	<0.015	<0.044

* Amino sugar as % dry weight sample.

TABLE 5. Muramic acid contents of staphylococci and L-forms estimated by a micromodification of Rondle & Morgan procedure.

		Sample (mg)	Muramic acid content, μ g	Muramic acid as % dry wt. sample
1444	cocci	10.1	230	2.28
		12.3	284	2.31
	L-form	66.0	< 4	< 0.0061
PS6	cocci	15.4	323	2.10
		8.6	200	2.33
	L-form	79.5	< 4	< 0.0050
PS42E	cocci	10.6	243	2.30
		13.7	314	2.29
	L-form	70.4	< 4	< 0.0057
Rogers	cocci	9.0	202	2.25
		11.4	276	2.42
	L-form	51.4	< 4	< 0.0077

nevertheless failed to demonstrate the presence of any appreciable amount of hexosamine.

In the above experiments, total amino sugar was estimated; separation of the component amino sugars, glucosamine and muramic acid, yielded the results shown in TABLE 4.

The acquisition of a sample of authentic muramic acid permitted the use of a more sensitive test for the presence of muramic acid in cell hydrolysates. Hydrolysates of whole cells were treated to separate the individual amino sugars and the amount of muramic acid estimated, (TABLE.5).

Amounts of L-form cells in excess of 60 mg. dry weight failed to show the presence of any muramic acid, even with the increased sensitivity introduced by the micromethod. Extracts of whole bacterial cells gave results for muramic acid content within the range to be expected.

Attempts were made to concentrate further any cell wall material present by isolation of fractions of the cells which were most likely to contain constituents of the cell wall (see Methods). 'Wall' fractions from the L-form cells were isolated by lysis of the osmotically fragile cells and treated according to the procedures used in the isolation of wall material from normal cells. 'Mucopeptide' fractions were isolated from bacterial and L-form cells using the method of Park & Hancock (1960). This involves treatment of the

TABLE 6. Yields of fractions isolated from whole
staphylococci and L-forms.

	Sample (mg)	Ext.	Fraction (mg)			Fraction as % sample
			'Wall'	Muco.	Form res.	
1444 cocci L-form	45.5	S+H	8.2			18.00
	63.8	P+H		15.7		24.60
	14.2	Form.			2.0	14.10
	67.2	S+H	4.8			6.82
	113.0	P+H		2.9		2.57
	56.1	Form.			3.4	6.06
PS6 cocci L-form	50.3	S+H	7.6			15.11
	17.8	P+H		2.4		13.48
	79.0	S+H	5.4			6.84
	55.6	P+H		1.9		3.42
PS42E cocci L-form	43.3	S+H	7.1			16.78
	34.3	P+H		4.3		12.54
	47.9	P+H		1.7		3.55
	33.3	Form.			4.3	12.91
Rogers cocci L-form	31.4	S+H	5.4			17.24
	20.5	P+H		3.3		16.00
	14.7	Form.		2.8	—	19.04
	53.4	S+H	1.6			3.00
	55.5	P+H		1.0		1.80

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TABLE 7. Muramic acid analyses of fractions from
whole cells.

		Fraction Sample	Eq.wt.	Mur.acid	%Mur.acid	%Mur.acid	
		(mg)	cells	(μ g)	fraction	cells	
Cocci	1444	'Wall'	2.0	11.1	278	13.9	2.50
	PS6	"	2.0	13.2	363	18.1	2.75
	PS42E	"	2.0	12.2	327	16.3	2.68
	Rogers	"	2.0	11.6	322	16.1	2.78
L-form	1444	'Wall'	4.8	67.2	< 4	< 0.08	< 0.006
	PS6	"	5.4	79.0	< 4	< 0.07	< 0.005
	Rogers	"	1.6	53.4	< 4	< 0.25	< 0.007
Cocci	1444	'Muco'	2.0	8.1	260	13.0	3.20
	PS6	"	2.4	17.8	593	24.7	3.33
	PS42E	"	2.0	15.9	459	22.4	2.89
	Rogers	"	2.0	12.5	406	20.3	3.25
L-form	1444	'Muco'	2.9	113.0	< 4	< 0.14	< 0.004
	PS6	"	1.9	55.6	< 4	< 0.21	< 0.007
	PS42E	"	1.7	47.9	< 4	< 0.24	< 0.008
	Rogers	"	1.0	55.5	< 4	< 0.44	< 0.007
Cocci	1444	From.res.	2.0	14.2	415	20.7	2.92
	Rogers	"	2.0	10.5	317	15.9	3.02
L-form	1444	"	3.4	56.1	< 4	< 0.12	< 0.007
	PS42E	"	4.3	33.3	< 4	< 0.09	< 0.012

cells with hot trichloroacetic acid and digestion with trypsin, leaving as a final product, cell wall mucopeptide contaminated by less than 5% of cell proteins. Cells were also extracted with hot formamide (Fuller, 1938) and the residues collected. The yields of material obtained from these fractionation procedures are shown in TABLE 6.

In all three procedures, the amounts of material obtained from L-form cells were far less than the comparable fractions from normal cells. Hydrolysis and chromatographic analysis of the residues from bacterial cells showed only a limited number of amino acids to be present in any amount, although faint traces of 'protein' amino acids could be found. In contrast, hydrolysates of residues from L-form cells showed evidence of large amounts of all the 'protein' amino acids. Estimation of the muramic acid contents of these residues was carried out and the results are presented in TABLE 7.

In none of the L-form residues could any evidence of muramic acid be found and consequently the lower limit of sensitivity of the test was taken as the maximum level of this amino sugar present in any sample. Residues from the normal cells gave expected results. These results show that L-form cells cannot contain more than 0.012% of their dry weight of muramic acid.

11. Supernatant Medium.

In view of the observations by Rogers & Perkins (1962) that under suitable conditions protoplasts are capable of

synthesising soluble mucopeptides, the possibility that the staphylococcal L-forms might also produce these compounds was investigated.

Supernatant medium from large volume cultures of the L-form, grown in the presence and in the absence of penicillin, was freeze-dried and the dried material hydrolysed at 100° for 4 hr. in 4N HCl. 'Hexosamine' peaks were isolated by chromatography on Dowex 50 (H⁺) resin, and separated on charcoal:celite to yield an extract expected to contain any muramic acid which might have been present. The extracts were then assayed for muramic acid.

No trace of the amino sugar could be found in the culture supernatants from any of the L-forms, even when the starting material represented culture volumes in excess of 4 litres.

The data presented serve to show that the organisms under study appear to be devoid of any cell wall mucopeptides and are apparently unable to synthesise the compound even in the absence of penicillin.

Although the results above indicate the absence of muramic acid as a structural component in the L-forms, the possibility remains that the organisms might be capable of producing precursors of the cell wall structure. To test this possibility, the acid-soluble constituents were extracted from the L-forms growing under various conditions.

Harvested cells from L-form liquid cultures were washed once in ice-cold sodium chloride solution, yielding a gelatinous mass consisting of cells and extracellular deoxyribonucleic acid. This cell mass was then extracted by grinding for 5 min. in a glass tube with 3 ml. ice-cold 10% trichloroacetic acid. The insoluble precipitate was recovered by centrifugation and re-extracted with a further 3 ml. ice-cold 10% TCA. The extracts were combined and the TCA removed by shaking 5 times with equal volumes of ether. After adjustment of the aqueous extract to pH 8, residual ether was removed using a jet of cold air, the extract evaporated to dryness and then made up to 1 ml. of distilled water. Aliquots of extracts made from L-form cultures of various ages, with and without penicillin were analysed for the presence of N-acetyl amino sugars by a modification of the method of Strominger (1957) (see Methods).

None of the extracts showed evidence of containing N-acetyl hexosamine since no colour could be detected above that produced by controls. From these results, it appears that the Staph. aureus L-forms, whether grown in the presence or absence of penicillin, do not accumulate acid-soluble N-acetyl sugar compounds.

B. Teichoic Acid.

There is at present little evidence on the precise structural relationship between mucopeptide and ribitol teichoic acid in the cell wall. It is, therefore, conceivable that although mucopeptide production has ceased in the L-form, production of teichoic acid might proceed if only at reduced efficiency. Penicillin has been shown to cause increased accumulation of cytidine diphosphate ribitol precursors of teichoic acid in staphylococci (Saukkonen, 1961). It is not known whether this accumulation is the result of a direct action of the antibiotic or is the consequence of reduced mucopeptide synthesis.

If the L-forms, which were induced under the influence of penicillin, are still capable of producing wall teichoic acid, then it might be found in one of two sites. The polymer might still be associated with the cell, indicating that the presence of mucopeptide is not necessary for the continued attachment of teichoic acid; or it might be released into the medium as it was produced.

If the latter were the case it would be analagous to the continued production and release of M protein by L-forms of Gp. A streptococci, (Sharp, Hijmans & Dienes, 1957; Freimer, Krause & McCarty, 1959 and Gooder & Maxted, 1961).

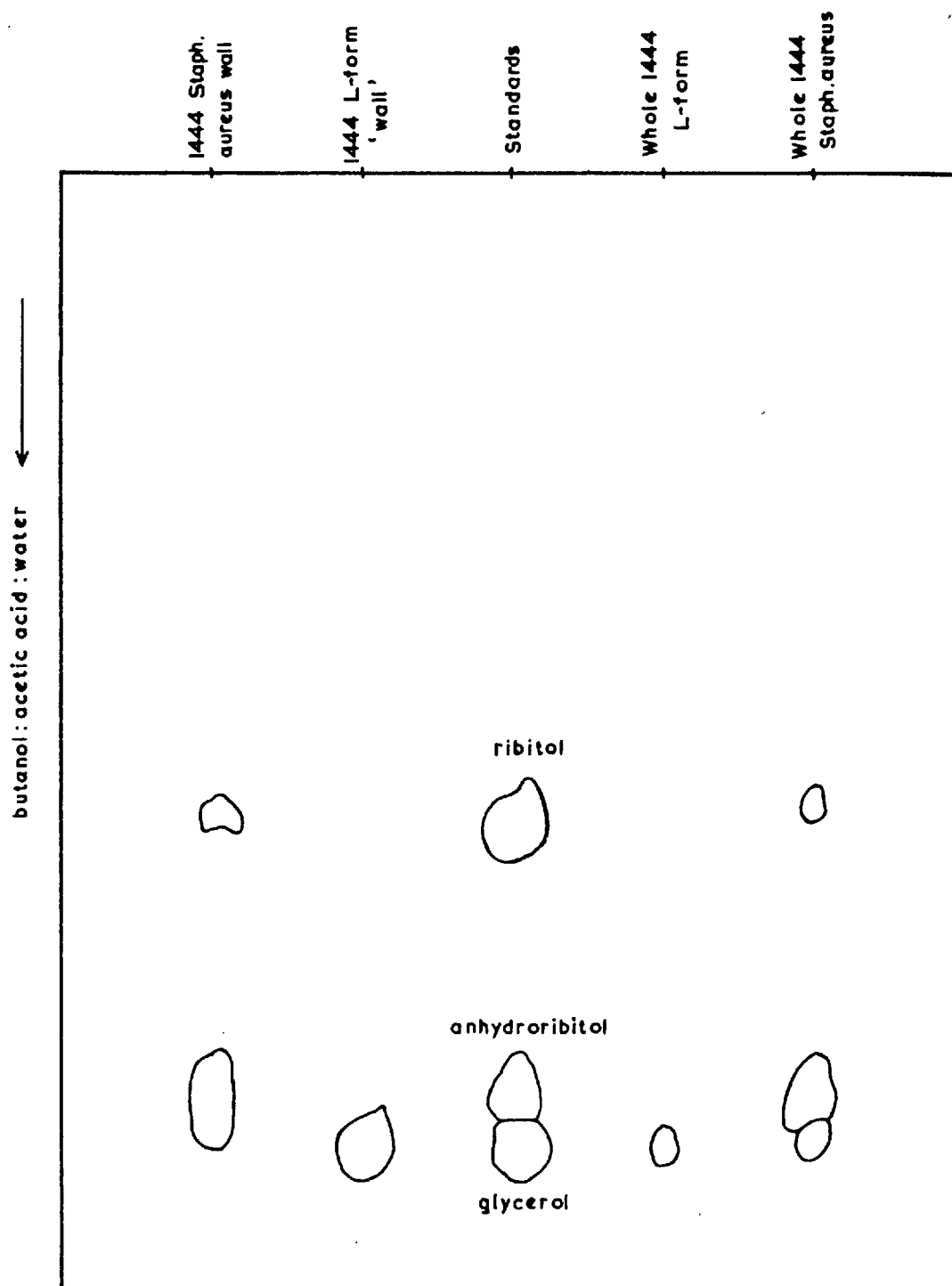


FIGURE 11. Separation of sugar alcohols from acid hydrolysates of Staphylococcus aureus 1444, L-form 1444 and wall preparations.

(1) Cells.

As with the investigations for amino sugars, preliminary hydrolysates of whole bacterial and L-form cells were prepared and the components separated by paper chromatography. In the solvent used for irrigation, glycerol and anhydroribitol have similar migrations but ribitol is well separated. Where difficulty might be experienced in distinguishing between glycerol and anhydroribitol, reliance was placed on co-chromatography and on the difference in colour produced with the two substances by the detection agent, periodate-benzidine.

As expected, anhydroribitol was evident in preparations of bacterial cells, but no such spot was detected in hydrolysates of L-forms. Both of these preparations showed faint traces of glycerol. 'Wall' preparations from L-form cells showed no anhydroribitol or ribitol but did contain traces of glycerol. Isolated cell walls from the parent staphylococcus on hydrolysis yielded only anhydroribitol and no glycerol. (FIG.11)

Large samples (up to 500 mg. dry weight) of L-form cells were extracted with cold 10% trichloroacetic acid in an attempt to isolate teichoic acids. The alcohol-precipitable materials from the TCA extracts consisted mainly of nucleic acids, which were partly removed by further treatment of the precipitate with cold TCA. The final reprecipitated material, although still containing some

nucleic acid, was hydrolysed but yielded no trace of anhydroribitol on subsequent chromatography. Similar treatment of bacterial cells indicated the presence of quite large amounts of anhydroribitol in the final precipitate. The extracted material from whole bacterial cells showed faint traces of glycerol, which was taken to indicate the presence of intracellular glycerol teichoic acids, (Davison & Baddiley, 1963). Not even faint traces of glycerol were found in hydrolysates of extracted material from L-form cells. The glycerol that was detected in hydrolysates of whole L-form cells and from isolated 'wall' preparations was undoubtedly the product of acid hydrolysis of cell lipids.

(11) Supernatant Medium.

Although no evidence for the existence of ribitol or glycerol teichoic acids could be found in the L-form cells, the possibility remained that these substances might have been produced and liberated into the medium. To resolve this possibility, supernatant culture medium from growth of the organisms was freeze-dried and subjected to the extraction procedures described for the cells.

Hydrolysates of the freeze-dried material showed no spots corresponding to ribitol or anhydroribitol, but did contain faint traces of glycerol. The trichloroacetic acid extracts of supernatant medium showed no ribitol, anhydroribitol or glycerol on hydrolysis. Since the original medium contained serum, the traces of glycerol seen in

hydrolysates of the dried medium may well be accounted for by the presence of lipids. More prolonged extraction of the medium with cold TCA yielded only traces of glycerol and no other sugar alcohol, even when freeze-dried residue from four litres or more of medium was used.

It is evident, therefore, that the L-form cells have lost the ability to synthesise not only the mucopeptide polymer of the cell wall but also the other major cell wall polymer, the ribitol teichoic acid. There is some evidence also of the loss of intracellular glycerol teichoic acid from these organisms.

Section 4.

IMMUNOLOGICAL PROPERTIES.

Knowledge of the antigenic components associated with the cell wall of Staphylococcus aureus has in recent years been greatly extended (Oeding, 1960). A major contribution in this field has been the isolation and chemical characterisation of the group-specific antigen which was first described in 1934 by Julianelle & Wieghard as 'polysaccharide A'. Recent work by Sanderson, Juergens & Strominger (1961) and Haukenes (1962) has shown that the greater part of purified 'polysaccharide A' consists of the cell wall ribitol teichoic acid, and that the immunological specificity can be traced to the presence of N-acetylglucosaminyl-ribitol residues.

Another polysaccharide antigen associated with the cell wall of coagulase-positive staphylococci is that described by Jensen (1958). This 'antigen A' reacts with normal human and rabbit sera to produce specific precipitates said to be due to the presence of 'normally occurring antibody' to the polysaccharide.

Since both of these polysaccharide antigens are described as being closely associated with the cell wall, failure to detect either or both in preparations of the L-form would provide further evidence of loss of cell wall material.

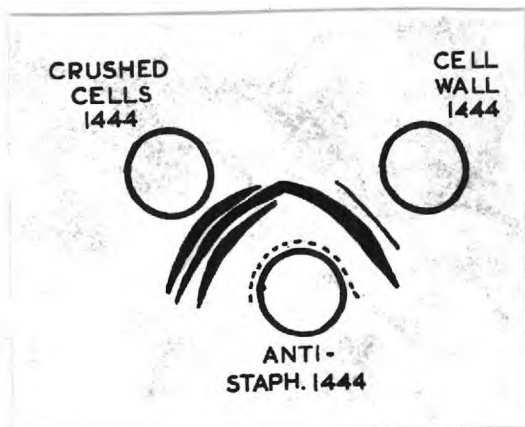


FIGURE 12. Agar gel diffusion of Staph. aureus 1444 antiserum against crushed 1444 staphylococci and 1444 cell walls.

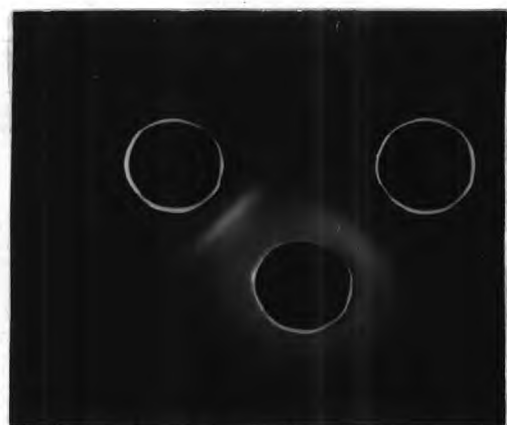
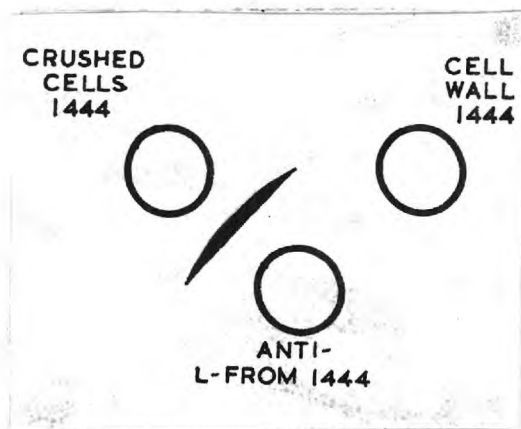


FIGURE 13. Agar gel diffusion of L-form 1444 antiserum against crushed 1444 staphylococci and 1444 cell walls.

Antisera were prepared against staphylococcal cells, cell walls and L-form by injection of the materials into rabbits. Both whole staphylococcal cells and isolated cell walls were agglutinated to high titre by antisera prepared against whole cells and cell walls. Suspensions of L-form, however, showed little or no reaction with either of these antisera. Antisera prepared against L-form agglutinated suspensions of the L-form but suspensions of bacterial cells and cell walls were not agglutinated to any degree. This indicated that the surface of the L-form differed from that of the bacterium.

Agar gel precipitation tests, in which buffered saline suspensions of crushed bacteria and purified cell walls were diffused against antiserum to whole cells, showed the formation of a precipitation line close to the serum well (FIG.12.). Comparison of this line with one produced by diffusion of crude 'polysaccharide A' gave a reaction of identity. No line corresponding to 'polysaccharide A' was produced by either of these sera when diffused against suspensions of the L-form, or by L-form antiserum when diffused against crushed bacterial cells or cell walls (FIG.13).

A precipitation line was formed midway between the antiserum and antigen wells by crushed bacterial cells with antiserum to whole staphylococci and with antiserum to the L-form, (FIGS. 12 & 13). A similar line was produced



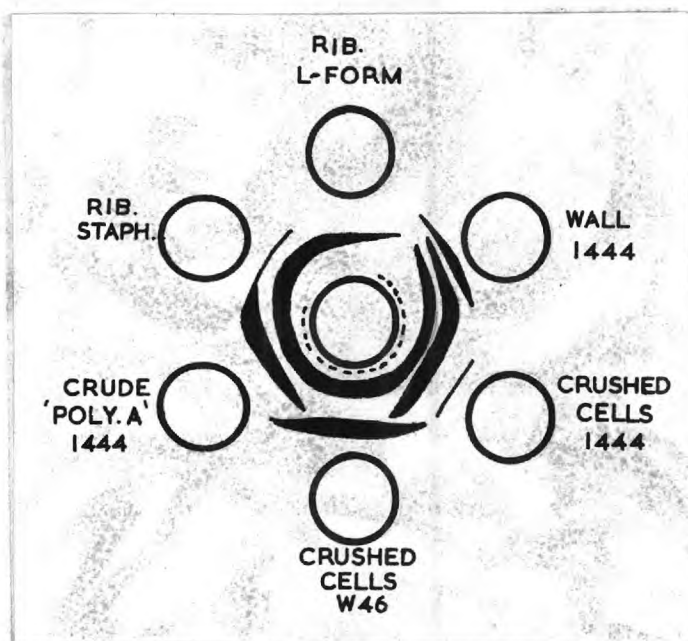
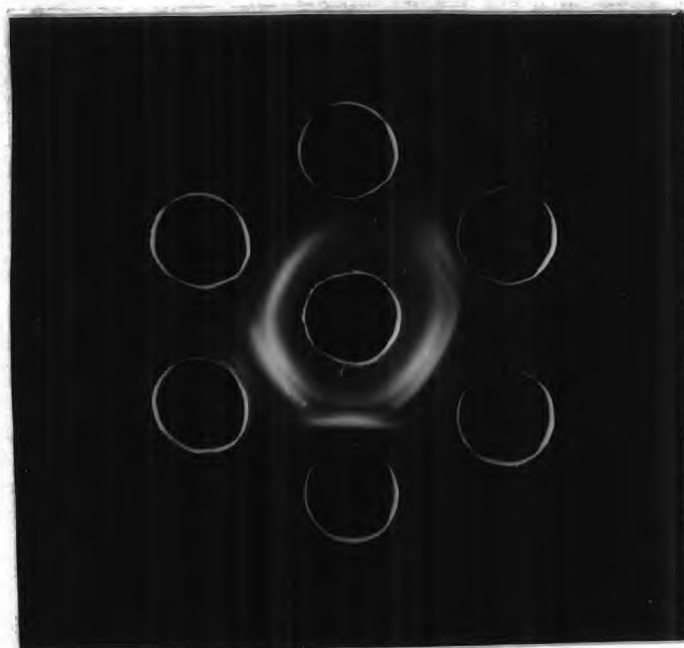


FIGURE 14. Agar gel diffusion of Staph. aureus 1444 antiserum against crushed 1444 staphylococci, 1444 cell walls and various cell fractions.

by diffusion of L-form suspension against its antiserum, but no such line was produced by the cell wall suspension with either antiserum. This line was designated the 'cytoplasmic' line.

The other major precipitation line shown in FIG.12, was identified as being produced by Jensen's 'antigen A'. The identity of this line was confirmed by the use of a preparation of isolated 'antigen A' and by the fact reported by Haukenes (1962) that Staph. aureus strain Wood 46 does not produce this antigen.

Suspensions of crushed bacterial cells and of cell walls also produced another line close to the antigen well, with antisera to staphylococci and cell walls. This was thought to represent a type-specific antigen, and although no further study of it was made, it was noted that no similar line was produced by the L-form and that antiserum to the L-form appeared not to contain antibodies to this antigen.

FIGURE 14 illustrates the result obtained by diffusion of antiserum to staphylococci with preparations of crushed cells, cell walls and various cell fractions. Crushed bacterial cells and cell walls show the formation of all the precipitation lines described above, except that no 'cytoplasmic' line has been produced by the cell walls. The preparation of crushed Wood 46 cells contains no line corresponding to 'antigen A'. Crude 'polysaccharide A', on the other hand, shows, not only a line for 'polysaccharide A',

but also lines for 'antigen A' and cytoplasmic contents, indicating contamination of the preparation with these antigens. The ribosomal or cytoplasmic fractions from the L-form and parent staphylococci show the formation of a fused cytoplasmic line, indicating the similar nature of the preparations.

The absence of 'polysaccharide A' in the L-form has been shown by the failure of suspensions of the organism to produce characteristic 'polysaccharide A' precipitation lines when diffused with antisera known to contain antibodies to the antigen. The situation with regard to 'antigen A' is not quite so clear since faint lines possibly corresponding to this antigen could sometimes be found in tests using L-form antiserum. In such cases, repeated absorption of the antiserum with whole L-form failed to remove the antibodies responsible.

In the above experiments, only one strong precipitation line was evident in reactions between the L-form and its antiserum, and this line was shown to represent 'cytoplasmic' constituents.

Reference to work on immunological properties of cell membranes (Vennes & Gerhardt, 1956; Freimer, 1963), indicated the possible existence of antibodies not previously detected by the methods used. Consequently, cell membranes from staphylococci and from L-forms were prepared as described under Methods and rendered suitable for use in

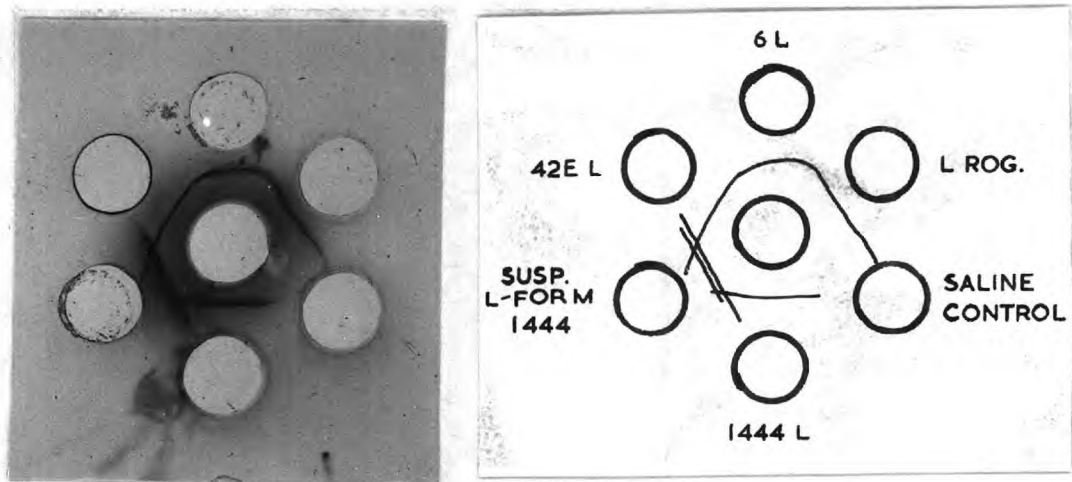


FIGURE 15. Diffusion of L-form 1444 antiserum against trypsinized extracts of L-form cell membranes. Preparation dried and stained.

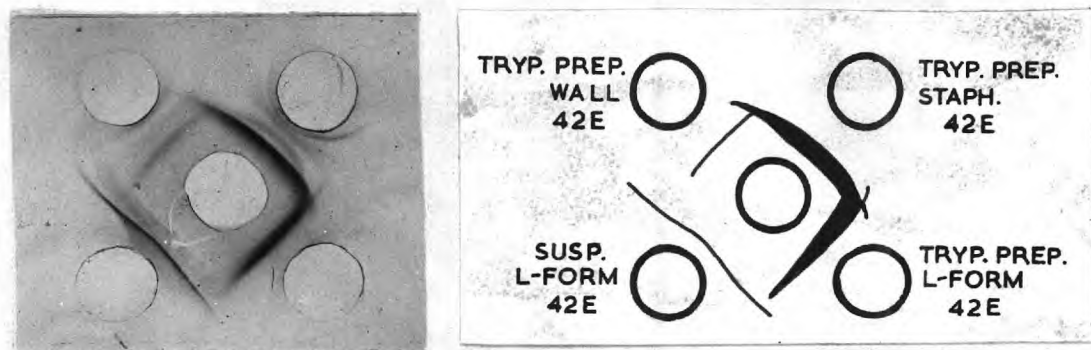


FIGURE 16. Diffusion of L-form 42E antiserum against L-form 42E and membrane extract, and Staph. aureus 42E membrane and cell wall extracts.

Preparation dried and stained.

immunodiffusion tests by treatment with trypsin. Agar diffusion of these trypsin extracts against L-form antiserum (FIG.15), showed the presence of precipitation lines from extracts of all the L-forms having complete identity with each other and partial identity with the 'cytoplasmic' line produced by a suspension of the L-form. This indicated the multiple nature of the 'cytoplasmic' line.

Antiserum against isolated L-form membranes showed no precipitation with suspensions of cell walls of the parent organism or with cytoplasmic contents. Trypsin extracts of some early cell wall preparations, known to be contaminated with cellular protein, produced precipitation lines with the antiserum. These lines showed fusion with the L-form membrane lines and showed a possible antigenic relationship between the L-form membrane and that of the parent bacterium. To test this further, extracts of 42E L-form and 42E staphylococcal cell membranes were diffused against the L-form antiserum. The results are shown in FIG.16. Complete fusion resulted between precipitation lines produced against both L-form and staphylococcal membranes, clearly demonstrating their antigenic identity. Similar identity was also demonstrated between membrane preparations from the other L-forms and their corresponding parent staphylococci.

Section 5.

ACRIDINE ORANGE STAINING.

Acridine orange has been described as specific stain for nucleic acids. It forms complexes with deoxyribonucleic and ribonucleic acids giving distinct fluorescent colours. The specificity of the reaction is dependant on the particular material used, on pH conditions and the method of fixation employed. The fluorescent colours produced by the nucleic acid complexes at acid pH value have been described as red-orange for ribonucleic acid and yellow-green for deoxyribonucleic acid. However, the production of these colours is greatly influenced by the optical system used to view the specimens. It is therefore necessary to test the specificity of any system.

A. Staining of Agar Colonies.

In the case of the L-form of the staphylococcus, staining was at first carried out on fixed preparations of agar colonies. Various fixatives were used, and all showed some degree of distortion of the soft protoplasmic growth of the colony periphery. Least distortion was shown by fixation of the colonies in Bouin's fluid. After fixation, the colonies were treated with specific nucleases, stained with acridine orange at various pH values and examined under ultraviolet light. The results are shown in TABLE 8.

At pH values below 3.6, complete removal of red-staining material was achieved by prior treatment of the

TABLE 8. Acridine orange staining reactions of L-form

1444 colonies fixed and stained at various

pH values.

pH value	Colony staining reaction		Fluorescent colours abolished by pretreatment with:-	
	granular	centre periphery	RNAase	DNAase
5.6	green	red+green	green	red (Incomplete)
5.0	green	red+green	green	red (Incomplete)
4.6	green+red	red+green	green	red (Incomplete)
4.0	red+green	green+a little red	green+red	green+red
3.6	red+green	green+a little red	red (Incomplete)	green (Incomplete)
3.0	red	green+red	red	green
2.8	red	green+red	red	green

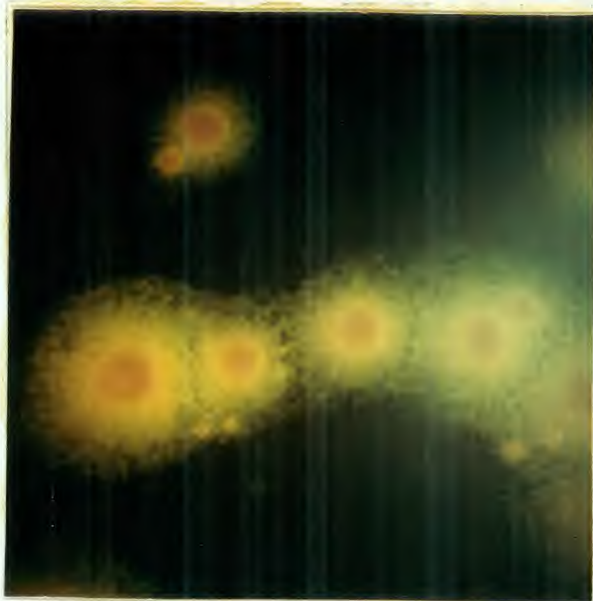


FIGURE 17. Fully developed staphylococcal L-form colonies.

Impression preparation, fixed in Bouin's
fluid and stained with acridine orange

at pH 3.0.

x125.

fixed colonies with ribonuclease. Likewise, pretreatment of the colonies with deoxyribonuclease resulted in removal of green-staining material. This abolition of green fluorescent colour was more effective when the staining was carried out at pH 3.0 than at highervalue. The results for both ribonucleic and deoxyribonucleic acids indicated the use of the dye at pH 3.0.

The fully developed L-form colonies, when stained at this pH value yielded the result shown in FIG.17.

The dense, granular centres of the colonies show a strong staining reaction for RNA, whereas the peripheral growth shows variation from RNA-staining vesicular bodies adjacent to the centre to cells staining wholly for DNA at the extreme edge of the colony. The red-staining (RNA) material is present in some cells as localized areas on an otherwise green (DNA) background. Those cells nearer the centre of the colony appear from their staining reaction to contain only RNA.

Pretreatment of the colony with RNAase and subsequent staining showed the RNA to be completely removed, leaving only the colony periphery have a DNA-staining reaction. The dense centre of the colony had been completely removed by the enzyme treatment. When the fixed colonies were treated with DNAase and then stained, the red granular centres of the colonies were intact, but in place of the peripheral cells only ghost-like debris remained.

The development of the typical colony form was followed by preparation of serial samples taken from an agar plate seeded with a 24 hr. broth culture. The early stages of colony formation consisted of clumps of large, vesicular bodies the majority of which contained little or no RNA as judged by acridine orange staining. Later samples showed the central cells in these clumps to contain granules of RNA material. The RNA in these cells increased in amount and staining intensity, until finally the central area took on the granular appearance seen in the fully developed colonies. Colonies which had been allowed to develop for longer than 72 hrs. showed staining almost wholly for RNA, only the cells at the extreme periphery of the colonies showing any evidence of DNA-staining material.

B. Staining of Cells from Liquid Culture.

Liquid cultures of the L-form, when stained with the dye gave essentially the same reaction as colonies, except that the individual elements were separated and their structure more easily seen. Also, since there is less restriction on the growth of the cells in liquid medium, a much wider range in size was seen in these cultures. The majority of cells in a 48 hr. liquid culture were within the size range 1μ - 5μ , although much larger cells, up to 15μ in diameter, were also seen.

Phase-contrast examination of liquid cultures had

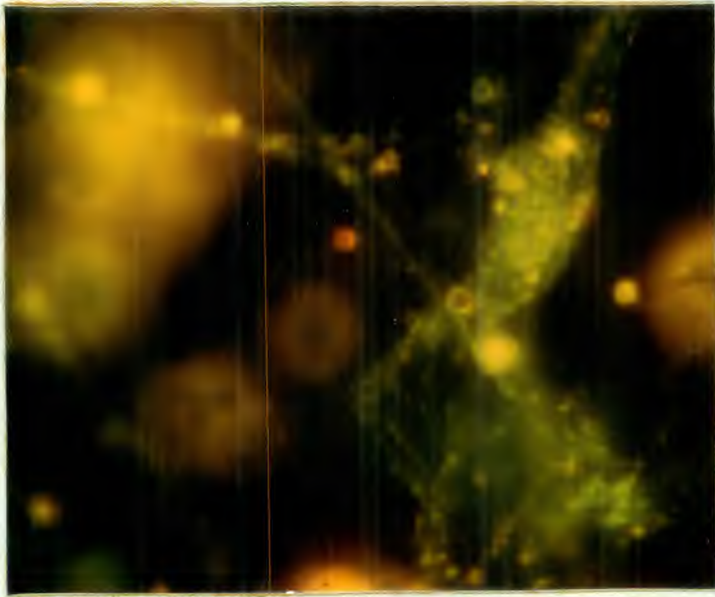


FIGURE 18. Staphylococcal L-form from liquid culture
stained with acridine orange at pH 3.0.
x250.

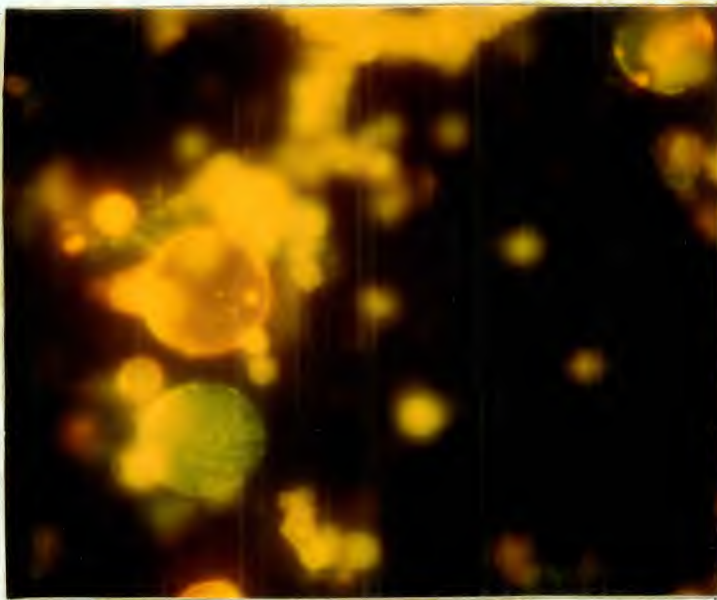


FIGURE 19. Staphylococcal L-form from liquid culture,
treated with DNAase. and stained with
acridine orange at pH 3.0.
x1,100.

revealed the presence of small granular elements having an apparent size of only approximately 0.3μ diameter. When stained with acridine orange, however, the cultures showed many more of these granules due to the increased sensitivity of the method, which rendered the granules fluorescent against a black background.

The cells from these liquid cultures could be fixed by suspension in 3.5% sodium chloride solution containing 10% formaldehyde. The fixed cells were then treated with nucleases and stained with the dye to confirm the staining reactions.

Untreated cultures showed the presence of a veil-like amorphous material staining strongly for DNA (FIG.18). The individual cells could be seen embedded in this matrix and could be dispersed by pretreatment of the unfixed culture with deoxyribonuclease. Enzyme treated cultures no longer showed evidence of this extracellular DNA matrix and the individual cells were quite separate (FIG.19).

Superimposed on the size range of cells in liquid cultures was the apparent complexity of nucleic acid content. Some cells gave staining reactions only for DNA (green), there being no trace of any red-staining material (RNA). Other cells exhibited RNA staining to varying degrees, the

RNA being present in some cases as only isolated small patches on the cell surface. Still other cells in the cultures gave staining reactions only for RNA. Between these extremes, there was a distribution of RNA material.

The liquid cultures of the L-form exhibited a complexity not seen in cultures of any normal bacterial cells.

Section 6.

SEPARATION OF ELEMENTS IN LIQUID CULTURE.

The complexity of growth form of the L-phase staphylococci, as seen by phase-contrast and fluorescence microscopy, made any attempts to gain information on the mode of reproduction very difficult. Before any studies on this aspect of the biology of the organisms could be undertaken, it was essential to obtain at least one homogeneous preparation from the heterogeneous cultures. Centrifugation presented a possible means of separation, and various methods were investigated.

A. Staining of Fractions.

Centrifugation of complex mixtures over gradients of varying density have been widely used in separation procedures involving cell structures, nucleic acids and proteins. Attempts were made to separate the elements of the cultures by centrifuging over gradients prepared from solutions of sucrose of different densities.

It proved difficult with any of the gradients used to separate the constituent cells into distinct groups. The very small granules, because of their marked difference in size from the rest of the cells in the cultures, were fairly well separated in all the gradients. The remaining cells, however, formed either a continuous spectrum in both size and nucleic acid content in the continuous gradients,

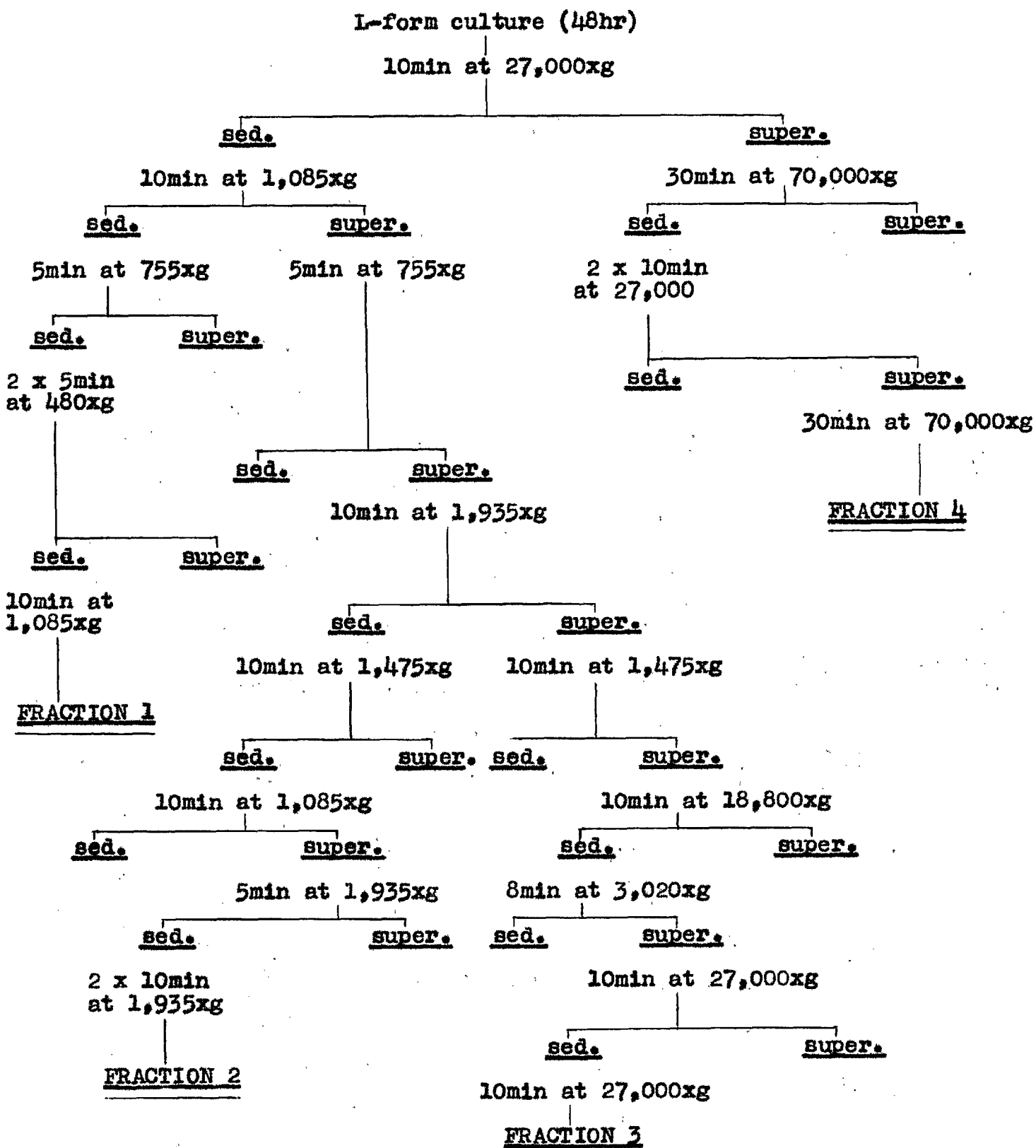


FIG. 20 Differential centrifugation scheme for fractionation of staphylococcal L-form liquid cultures.

TABLE 9. Stages in the separation of Fraction 2 from whole
L-form culture in terms of acridine orange staining.

Separation stage	Containing fractions:-	Microscopic appearance
Sup.1,085xg 10'	2+3	Mixed red- and green-staining cells 1-5 μ ; green-staining cells 1-10 μ .
Sup. 755xg 5'	2+3	Green and red-and-green cells 1-5 μ .
Sed. 755xg 5'	1*	Few large 5-10 μ green-staining cells.
Sup.1,935xg 10'	3*	Cells 1-10 μ mixed red and green.
Sed.1,935xg 10'	2	Green cells, majority 1-5 μ some >5 μ ; few mixed-staining cells.
Sup.1,475xg 10'	3*	Red- and green- and red-staining cells.
Sed.1,475xg 10'	2	Majority of cells green-staining 1-3 μ ; some red-staining material.
Sup.1,085xg 10'	2	Cells 1-3 μ , green-staining; few large green cells and few red cells.
Sed.1,085xg 10'	1*	Some large green cells >3 μ .
Sup.1,935xg 5'	3*	Few mixed-staining cells.
Sed.1,935xg 5'		<u>FRACTION 2.</u> Green-staining globular cells, size range 1-3 μ .

*-fractions discarded.

or heterogeneous bands in discontinuous gradients. No doubt further manipulation of the gradients and centrifugation speeds and times could produce well defined homogeneous bands.

More success was achieved using differential centrifugation of the organisms from culture fluid. Using a small number of manipulations, it was found possible to obtain suspensions of the large (3 μ -5 μ) globular cells, which on staining with acridine orange showed a reaction for DNA only. Extension of this method led to the formulation of a scheme resembling closely that used by Mandel, Terranova, Sensenbrenner & Feo (1958) to separate sphaeroplasts of Proteus sp.

The two systems differed slightly at various points and because of the osmotic instability of the staphylococcus L-form, washing of the fractions in 0.9% saline, as used by Mandel et al, was omitted.

The centrifugation scheme as finally used is set out in FIG.20.

The products of each manipulation were stained with acridine orange and viewed under ultraviolet light. In this way, the progress and efficiency of separation could be judged. The results of the separation of one of the fractions, in terms of acridine orange staining reaction, are presented in TABLE.9.

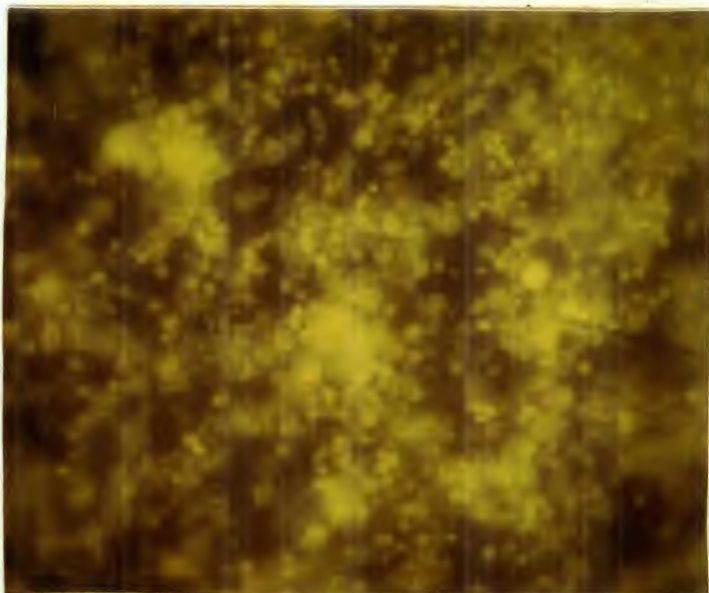


FIGURE 21. Fraction 1 from L-form liquid culture.
Acridine orange stain.

x250



FIGURE 22. Fraction 2 from L-form liquid culture.
Acridine orange stain.

x1,100.

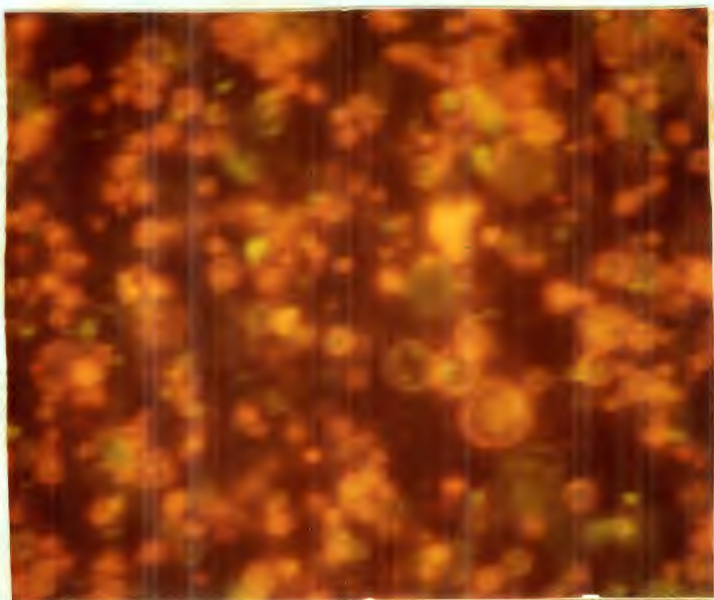


FIGURE 23. Fraction 3 from L-form liquid culture.

Acridine orange stain.

x1,100.

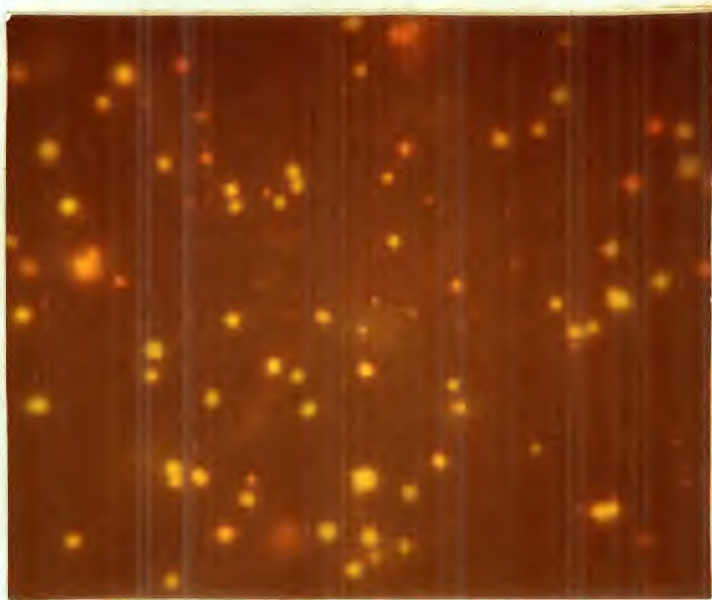


FIGURE 24. Fraction 4 from L-form liquid culture.

Acridine orange stain.

(represented by the supernatant from the
first 27,000xg centrifugation, see FIG.20)

x1,100.

That the method is reproducible is shown by the similarity of the isolated fractions, obtained from different separation procedures performed on cultures of similar age.

Cultures of less than 48 hr. incubation contained relatively larger amounts of cells falling into fractions 1 and 2 and smaller amounts of fractions 3 and 4. Older cultures incubated for approximately 72 hr. gave very small yields of fractions 1 and 2, most of the cells present being found in fractions 3 and especially 4.

Photomicrographs of acridine orange stained fractions obtained from a 48 hr. culture are shown in FIGS 21, 22, 23 & 24. Each of these isolated fractions proved capable of reproduction and produced growth on solid and in liquid media. Some difficulty was experienced in initiating growth from some preparations of fraction 4, particularly on solid medium. It was found that reproducible growth of these fractions could be obtained by the use of a decreased (0.9% (w./v.)) agar concentration in the medium (Razin & Oliver, 1961),

Because of the small size (app. 0.3 μ) of the elements of fraction 4, it was felt that growth of this fraction might be due to contamination with cells from other fractions of the original culture. Extensive microscopical examination of acridine orange stained specimens of fraction 4 from various preparations showed the majority of the specimens to be free of cells associated with other fractions or to

contain very small numbers of these cells. In none of the preparations of fraction 4 was the level of contamination sufficient to account for the growth produced by that fraction.

B. Growth of Isolated Culture Fractions.

When whole unfractionated cultures were used as inocula for solid medium, the plates at any one time contained colonies at all stages of development. In contrast, the great majority of colonies grown from the fractions were all at a similar stage, although small numbers of colonies at earlier or later stages of growth could be found. This provided a further indication of the homogeneity of the fractions, at least in respect to growth capabilities.

The isolated fractions grew in the form of a layer on the glass surfaces of the culture vessel and subsequently it was found possible to grow them on cover-slips. The fractions were therefore inoculated into Leighton tubes containing a narrow cover-slip immersed in a small amount of the medium. After incubation for 1 hr. the cover-slips could be carefully removed from the tubes and were found to have a layer of the L-form cells adhering to one side of the glass. Such specimens were easily handled for fixing and staining in acridine orange. It was possible, using this technique, to follow the course of development of a given inoculum of cells by removing and examining samples

at various times. The method was applied to each of the fractions.

Fractions 1 and 2, when examined by this method, showed essentially the same growth characteristics. The inocula contained almost wholly large globular cells staining only for DNA. When the growth sequence was followed, the first detectable changes in the cells was the gradual development of small areas of red-staining (RNA) material on the cell surface. Further samples from this series showed increases in the amount of RNA in the cells until they gave an appearance similar to that shown by fraction 3 from the original culture. It was noted that not all the cells developed RNA inclusions, particularly some of the larger cells i.e. those over 5μ in diameter. Still later samples showed evidence of lysis of some of these cells with release of orange-red staining granular material similar to that seen in fraction 4.

Fraction 3 preparations also showed some of the above changes, although the inocula contained cells staining both for DNA and RNA. Serial samples taken from these tubes showed there to be a general increase in the amount of RNA-staining. As with preparations from fractions 1 and 2, some lysis and release of RNA granules was seen at later stages. In still later preparations, some very small globular cells staining wholly for DNA were seen. These cells were smaller (0.75μ to 1μ diameter) than those seen in

fraction 2 but gave the same staining reaction.

Fraction 4, which consisted of the small, granular elements seen in great numbers in whose cultures, also produced growth on the cover-slips. The inocula, after periods varying from 10 to 16 hr., showed the production of small globular cells similar to those produced during growth of fraction 3. The conversion of the granular elements to the small globular cells was not complete, some of the granules appeared to be incapable of growth. Even after periods of incubation exceeding 24 hrs. the preparations contained in addition to the small DNA-staining cells, appreciable numbers of granules.

Using the cover-slip method of observation, it was not possible to culture the organisms for periods longer than 24 hr., due to the small volume of medium used and to crowding of the cells. By some manipulation of culture volumes and size of inocula it was possible to observe the growth of the organisms for a further hour or two. However, the short extension of incubation time did not contribute much more information on the growth process. When the fractions were grown in relatively large culture, all yielded after 48 hr. organisms showing the full range of size and nucleic acid staining reactions previously seen in whole cultures. This suggests that on prolonged incubation with less restricted nutrient, the initially homogeneous inocula become more asynchronous. Some evidence of this was seen in

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TABLE 10. Average RNA phosphorus/DNA phosphorus values
in Staph. aureus 1444 L-form and culture fractions

Sample	Size Range	Average* RNA P/DNA P
Whole culture	approx. 0.2-10.0 μ	1.56
Fractions 1	3.0-10.0 μ	2.75
2	1.0- 3.0 μ	2.24
3	1.0- 4.0 μ	7.89
4	approx. 0.2- 0.5 μ	2.28

* Each value represents the average of
at least four determinations.

the growth on cover-slips where a few cells in the inocula failed to develop or perhaps developed later than the other cells in the culture.

Analysis of the isolated fractions for nucleic acid content provided some measure of confirmation of the observed staining reactions, (TABLE 10).

It can be seen from TABLE 10, that the nucleic acid ratios of fractions 1 and 2 are very similar, as were their staining reactions. Both fractions actually contain appreciable amounts of RNA by analysis, although no evidence of its presence could be seen in stained cells. Analysis of cells of fraction 3 show them to have an increased RNA phosphorus:DNA phosphorus, which again appears to be in agreement with the observed staining reactions. The ratio produced from fraction 4 indicates the presence of DNA which was not detectable by staining alone. In view of the fact that some of the material in this fraction appeared incapable of growth, at least under the conditions employed, it seems reasonable to assume that the figures obtained might include ribonucleic acids from cell debris.

DISCUSSION

DISCUSSION.

The use of penicillin to induce the production of L-phase variants in Staphylococcus aureus has been described by Williams (1963). Three of the L-form strains produced in that study were used in the present investigation. Their general growth properties were similar to those of the L-forms described by Schonfeld (1959, 1961) and Marston (1961a), in that they produced a characteristic type of colony and required the presence of serum and high levels of sodium chloride for growth. A fourth L-form, derived from a penicillin-resistant strain of Staph. aureus was also induced into the L-phase, but by D-cycloserine. Whereas Schonfeld (1961) found that the number of L-form colonies produced was proportional to the square of the number of staphylococci in the inoculum, no such relationship was found with the penicillin-induced strains (Williams, 1963) or the D-cycloserine-induced strain in the present study.

L-Rogers, derived with D-cycloserine, on primary isolation required a higher sodium chloride concentration than the three penicillin-induced strains which could after varying periods, be grown in the absence of the inducing agent; but this stage was reached with L-Rogers only after much longer periods of subculture in the presence of D-cycloserine. The L-form Rogers remained throughout the greater part of this study in the 'unstable' state and was used as such in the

various chemical and immunological investigations. Eventually, all the L-form strains were stabilised and no evidence of reversion to cocci was seen during subsequent growth. This is in direct contrast to the reports by Schönfeld (1959, 1961) and Marston (1961a) describing the ease with which reversion to staphylococci takes place even after prolonged growth in the L-phase. Marston (1961b) reported the absence of reversion of L-form strains which had been grown in liquid culture.

The technique of initiating growth in liquid medium by inoculation with agar blocks yielded all four strains in liquid culture. Great variation was encountered in the ease with which growth could be established, but once established the liquid cultures were propagated in the normal way. In broth culture, the L-forms produced thick, rope-like growth which could not readily be dispersed by mechanical means without seriously reducing the culture viability. The effectiveness of deoxyribonuclease treatment in dispersing growth indicated the presence of large amounts of extracellular DNA. This finding would be in agreement with that of Catlin (1956), who found that staphylococci also produce extracellular DNA, particularly when grown in brain-heart infusion medium. The presence of sodium chloride in the L-form medium tends to further inhibit depolymerisation of any DNA released, leading to appreciable accumulation.

The osmotic fragility of the L-forms could also contribute to this accumulation by lysis of cells and release of DNA.

Replacement of the whole serum in L-form growth medium with various other substances indicated the detoxifying nature of the serum protein. It was found that whereas the serum could be wholly replaced by an equivalent concentration of Difco serum fraction, known detoxifying agents (Davies & Dubos, 1947; Pollock, 1949) provided only partial replacement. The growth response obtained with charcoal was very low and although bovine serum albumin gave better results, the response was still far below that obtained with either whole serum or serum fraction. It is evident from these results that the function of the serum in staphylococcal L-form medium is not solely of a detoxifying nature. There have been other indications that complex protein supplements are not necessary for the growth of staphylococcal L-forms, which have been grown in a semi-defined medium without the addition of serum protein (Banville, 1964).

The marked osmotic fragility of L-phase organisms has been described repeatedly in other genera and the staphylococcal L-forms proved similar in this respect. Lysis of the L-form in decreasing sodium chloride concentration was gradual, there being no dramatic loss of optical density such as might be found with suspensions of protoplasts (McQuillen, 1960). Comparison of the lytic spectrum of the L-form with that of

enzyme-produced 'protoplasts' of the parent staphylococci showed certain similarities between the two forms. The suspensions of 'protoplasts' produced the expected sudden fall in optical density, due probably to the more uniform size of the organisms. The suspensions of the L-form, on the other hand, containing cells of a wide size range, showed a more gradual loss of optical density. Although the responses of the L-form and 'protoplasts' to lysis by alcohols did not agree entirely, there were similarities in the lytic patterns. Both types of cell were lysed at approximately similar concentrations of the three alcohols, indicating to some degree the similarities in their surfaces (Razin & Argaman, 1962).

The osmotic fragility of the staphylococcal L-form indicated that the surface of the bacterial cell had been radically changed during induction into the L-phase. Investigation of the gross chemical properties of the coccus and the L-form further supported this belief in that the L-form was found to have a much reduced amino-sugar content. The amino sugars concerned were glucosamine and muramic acid. Whereas glucosamine has been reported to occur in various parts of bacterial cells, muramic acid appears to be unique to the bacterial cell wall or to its precursors (Salton, 1964). This uniqueness of muramic acid makes it a convenient marker, the demonstration of which provides irrefutable

evidence of the presence of a cell wall structure or of the ability to produce such a structure.

The results of the early chromatographic experiments in the present study showed that acid-washed staphylococcal L-forms, from which any UDP-precursors would have been removed, contained no detectable muramic acid or glucosamine. Although large samples of L-form had been used, it could not be said at this stage that nothing even approximately to an organised cell wall existed in these organisms. Later quantitative analyses of the L-forms demonstrated with certainty that such a case did exist.

However, reports on L-forms derived from Gram-negative bacteria (Sharp, 1963) have shown that while the amount of wall material was much reduced, the organisms still retained remnants of a cell wall. It was considered essential to determine whether a similar situation existed in the case of the staphylococcal L-forms.

The various methods used were designed to concentrate to a more easily detectable level, any cell wall material which might be present in the L-form. None of the methods used yielded preparations containing muramic acid and it was judged that the maximum amino sugar present in the L-form could not exceed 0.008% dry weight. These results provided conclusive evidence that no organised cell wall existed in the L-forms derived from staphylococci either by penicillin

or by D-cycloserine. Sharp (1963) had reported the failure to find muramic acid in one other staphylococcal L-form strain, that of Staph. aureus H.

The other major polymer associated with the cell wall of normal Staphylococcus aureus is the ribitol teichoic acid. Ribitol has some points in common with muramic acid, notably its unique position within the bacterial cell, and to date has not been demonstrated in bacterial cells except in the cell wall teichoic acid or as a CDP precursor. Here again, an excellent marker compound was available, and added to this was the production on acid hydrolysis of ribitol teichoic acid, of an unusual product, anhydroribitol (Salton, 1964). Both of these sugars could be used as indicators of the presence of a ribitol phosphate polymer. No previous reports have been made regarding the fate of cell wall teichoic acid during induction of staphylococci into the L-phase.

The results of the chromatographic analyses of acid hydrolysates of staphylococci and their derived L-forms showed that while the bacterial cells yielded large quantities of both ribitol and anhydroribitol on hydrolysis, neither of these compounds could be detected in the L-forms. Even very large samples of the L-forms failed to yield any evidence of either of these two sugars.

There remained the possibility that teichoic acid was being produced by the L-form, but that in the absence of a

rigid cell wall, it was being released into the surrounding medium. The L-forms of group A streptococci provide an example of this phenomenon (Sharp, Hijmans & Dienes, 1957), since they continue to produce M protein in the absence of the rigid cell wall. However, examination of supernatant broth from cultures of the staphylococcal L-forms showed no evidence that ribitol teichoic acid was being released.

Hijmans (1962) has reported the loss of the group antigen, which has been identified as a glycerol teichoic acid, as well as the cell wall antigen, from group D streptococci during induction into the L-phase. Although traces of glycerol were found in hydrolysates of the staphylococcal L-form, no glycerol acid could be extracted from the cells using the normal procedures. It is believed that the traces of glycerol were the result of hydrolysis of cell or medium lipid rather than teichoic acid. It would appear then that the L-form contains neither glycerol nor ribitol teichoic acids.

It seems therefore that the induction of staphylococci into the L-phase involves not only the loss of the rigid mucopeptide part of the cell wall but also loss of teichoic acids. The precise relationship between these polymers in the cell wall is still a matter of speculation. The question then arises whether the inhibition of mucopeptide synthesis known to occur with penicillin and D-cycloserine contributes to the loss of teichoic acids or whether this loss is a more

direct consequence of antibiotic action.

Cytidine diphosphate ribitol and glycerol are known to accumulate, in addition to the UDP-muramic acid compounds, when staphylococci are treated with penicillin (Saukkonen, 1961). Attempts to extract uridine or cytidine diphosphate precursors of mucopeptide and teichoic acid from the L-forms failed. In view of the report on extraction of UDP compounds from a streptococcal L-form (Edwards & Panos, 1962), it is surprising that these compounds were not found in the staphylococcal L-forms, particularly in L-Rogers. Whereas the three penicillin-induced L-forms had been cultured for a number of years and had been 'stabilised' in the L-phase, L-Rogers was still considered 'unstable'. For this reason, it might have been expected to contain UDP precursors to cell wall mucopeptide and also perhaps CDP compounds.

The chemical demonstration of the loss of the cell wall also provides an explanation for the results of antibiotic sensitivities of these L-forms reported by Williams (1963).

The reaction of the nucleic acids RNA and DNA with acridine orange produces complexes which fluoresce with characteristic colours when irradiated with short wavelength blue light. The complex formed with RNA shows red-orange fluorescence and that with DNA green fluorescence, the specificity of the colour reactions being highly dependant on pH value. These reactions have been used as a cytological

tool to investigate the development of animal (Furness, Henderson, Csonka & Fraser, 1962) and bacterial (Anderson, Armstrong & Niven, 1959) viruses. The application of this technique to the study of the staphylococcal L-forms revealed a striking difference in staining reaction from that shown by the normal bacterial form. The optimal pH for staining of bacteria with this dye is 5.0 to 5.5 (Anderson et al., 1959), but in the case of the L-form, pH 3.0 was found to be the optimum. This is nearer the level described for animal cells by Furness et al. (1962).

Using the dye at this pH, a wide range in apparent nucleic acid contents was found in the cells from L-form cultures. As was found in the earlier results of enzyme treatment, the amorphous background present in liquid cultures of the L-form showed a reaction with the dye typical of DNA, i.e. a green fluorescence. Pretreatment of unfixed cultures with DNAase abolished the general green-staining of this background, and the cells remaining after removal of the extracellular DNA continued to show the extensive range of staining seen in the untreated cultures.

Attempts to separate the individual elements from these cultures by gradient centrifugation yielded varying results, and with none of the gradients used, either continuous or discontinuous, was effective separation achieved. The development of a separation scheme similar to that used by

Mandel, Terranova, Sensenbrenner & Feo (1959) for *Proteus* L18, led to the separation of the total culture into four homogeneous fractions. There appeared to be some little overlap between fractions 1, 2 and 3, but none between fraction 4 and the other three. The sizes of the individual elements in each fraction agreed fairly closely with the results obtained by Mandel, Terranova et al. and could be identified with the sizes of the elements in the original cultures. The fractions, however, appeared to lack the very large (15u) globules seen in the unfractionated cultures. This could be explained by mechanical lysis of these large globules during the centrifuging and resuspending operations.

Analysis of these isolated fractions for nucleic acid content yielded results which to some extent, agreed with the observed staining reactions. These results were similar to those obtained for *Proteus* L18 (Mandel, Sensenbrenner, DeGregorio & Bader, 1959) except that the ratio RNA phosphorus: DNA phosphorus in fraction 3 was not as high. Since Mandel, Sensenbrenner et al. performed the final centrifugation of each fraction from 0.9% saline, their result might possibly be explained by lysis of cells and consequent loss of DNA. In the case of the staphylococcal L-form, all centrifugations and final resuspensions were made in growth medium, to preclude as far as possible, lysis of cells.

Growth of all four fractions yielded cultures similar to that from which the fractions had been isolated, thus indicating the possible existence of some form of growth sequence. The results obtained from growth experiments conducted on the individual fractions confirmed this indication and illustrated the relationships between the fractions. The gradual development in the large globular cells of fractions 1 and 2 of 'patches' of RNA gave rise to cells similar in appearance to those seen in fraction 3. Further incubation of these cultures showed the liberation of small RNA granules from the larger cells; but this was more clearly seen when the inoculum consisted of cells isolated as fraction 3. The release of granules from these cells was very marked.

A significant result of these studies concerned the growth of the RNA granules. When cultured, these granules produced a similar heterogeneous mixture of elements seen by acridine orange staining of whole cultures. In view of the conflicting reports on the viability of such granules in other L-form strains (Roux, 1960; Liebermeister, 1960), it is interesting to discover that in L-forms of Staphylococcus aureus at least, some of the granules are viable. The use of the cover-slip technique enabled a little of the early development of the granules to be studied. By no means all of the granules proved capable of growth, but the percentage

viability was not measured. The fact that not all the granules were viable, together with possible damage during micro-manipulation, might account for the failure of Roux (1960) to grow the granules isolated in his experiments on *Proteus* L-form. The growth of the granules from staphylococcal L-forms supports the report by Panos, Barkulis & Hayashi (1960) on viability of similar elements released by sonic treatment of a streptococcal L-form. Weibull & Beckmann (1960) studying the metabolism, in growth medium, of small bodies from a stable *Proteus* L-form found them to contain succinic dehydrogenase, catalase and ribonucleic acid, and to respire at 50% of that of the whole culture. Similar results were obtained by Panos (1962) for small bodies of a streptococcal L-form, which respired at 46% of that of the whole culture. In the present case, analysis for nucleic acids in the granules demonstrated the presence not only of ribonucleic acid, already shown by staining, but also deoxyribonucleic acid. The amount of ribonucleic acid found undoubtedly reflects the presence of non-viable particles (the 'chondrioids' of Roux and Liebermeister?).

The cell wall antigens of *Staphylococcus aureus* which have been studied in greatest detail are polysaccharide A and antigen A. The former antigen has been the subject of extensive research by Haukenes (1962) who found it to consist

mainly of ribitol teichoic acid, thus confirming the report of Sanderson, Juergens & Strominger (1961). Polysaccharide A was shown to be a potent hapten, precipitating at high dilution with homologous antiserum. Gel diffusion tests with suspensions of Staphylococcus aureus L-forms confirmed the chemical analyses in that no polysaccharide A and so no ribitol teichoic acid could be detected. Extraction procedures designed to isolate polysaccharide A failed to extract any of the antigen from the L-forms.

Antigen A was first described by Jensen (1958) as a carbohydrate antigen associated with the cell wall in Staph. aureus. More recently, Yoshida, Mudd & Lenhart (1963) have characterised this antigen as a protein agglutinin. All the parent staphylococci used in this study were found to contain antigen A, but the situation in the L-forms was not clear from the results of gel diffusion tests. Faint lines corresponding to antigen A were sometimes found with antisera to L-forms, but repeated absorption of these antisera with L-form cells failed to remove the antibody. Jensen (1958) reported the occurrence in normal rabbit serum of antibodies to this antigen and this could explain the present result. In view of the report by Yoshida et al. on the sensitivity of the isolated antigen to digestion by proteolytic enzymes, it is difficult to explain the presence of the antigen in trypsin-digested staphylococcal cell walls. One possible

explanation is that the trypsin-resistant protein described by Park & Hancock (1960) in cell wall mucopeptide preparations contains antigen A. This possibility was not resolved in the present study, although none of the purified cell wall preparations showed evidence of protein contamination by the conventional tests. On hydrolysis, however, they did show the presence of leucine (or isoleucine) and valine.

In the absence of reversion to the bacterial form, there might exist some doubt as to whether the L-forms are actually derived from Staphylococcus aureus. The loss of the cell wall brings with it loss of such identifying characters as cell wall associated antigens, phage susceptibility, Gram-staining properties and typical morphology. Some evidence such as continued coagulase production and lysogenicity was presented by Williams (1963) in support of the origin of the three penicillin-induced L-forms. The present finding that the surface membrane of the L-form bears a close serological relationship to the cell membrane of the staphylococcus provides further evidence on the origin of these L-form strains.

It had been hoped that the present investigation would throw some light on the role, if any, of L-forms in disease. The osmotic requirements of the organisms raise some doubts as to whether they could exist in vivo. Two possible sites where the osmotic requirements might be met in the animal

body are synovial fluid and kidneys. At the time of writing, several attempts had been made to establish staphylococcal L-forms in vivo in mice, and although the organisms could be detected, after varying periods in the animal, by fluorescent antibody methods, they could not be reisolated. By serial subculture, the L-forms were obtained growing in medium offering lower osmotic protection, but even organisms growing in only 1.2% total NaCl, when injected into the mice, could not be recovered in a viable state. It was felt that more success might be achieved if the L-form could be grown at physiological salt levels before attempting animal experiments.

It is obvious from the results presented that the L-forms of Staphylococcus aureus and of other organisms offer some problems of fundamental importance. These organisms could prove invaluable tools in such fields as membrane structure and function, having advantages not possessed by non-viable protoplasts. However, more information needs to be gathered on the possible physiological changes concomitant with removal of the cell wall.

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