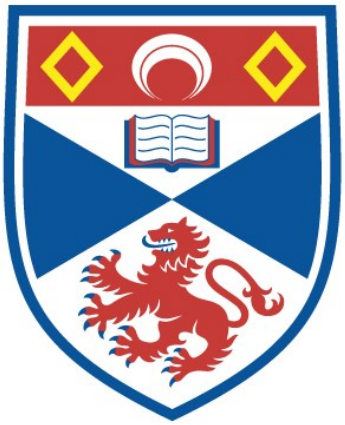
# CELL WALL COMPOSITION AND ULTRASTRUCTURE OF THE EXTREMELY HALOPHILIC COCCUS, SARCINA MARINA

Stephen John Wilfrid Millar

A Thesis Submitted for the Degree of PhD
at the
University of St Andrews



1979

"Cell Wall Composition and Ultrastructure of the Extremely Halophilic Coccus, Sarcina marina."

being a thesis presented by Stephen John Wilfrid Millar to the University of St. Andrews in application for the degree of Doctor of Philosophy.

August, 1979

# Cell Wall Composition and Ultrastructure of the Extremely Halophilic Coccus, Sarcina marina

Stephen John Wilfrid Millar, 1979

## ABSTRACT

Cells of S. marina (N.C.M.B. 778) were disrupted using a Hughes press and a purified cell wall fraction obtained using a previously reported method for halococcal wall isolation. This procedure was monitored by examination of thin sections in the electron microscope and the final wall preparation was seen to be relatively free of cytoplasmic and membranous contaminants. However, treatment of the wall fraction with crude trypsin did appear to remove particulate surface components.

The total ninhydrin-positive components detectable accounted for only about 14% of the cell wall dry weight. The major amino acids present were glycine, alanine, glutamic acid and aspartic acid although very small amounts of others were detected. The amino sugar components included glucosamine and galactosamine although these only accounted for some 60% of the total amino sugars. The remainder was probably made up of one or more of four unidentified, acid-labile components detected on amino acid analysis and by paper chromatography. This is in accord with the finding of unusual, labile amino sugars in the cell walls of other halococcal species.

Approximately 37% of the cell wall dry weight was made up of the neutral sugars, glucose, galactose and mannose which were present in equimolar amounts.

In addition, the wall was found to contain a negligible lipid (0.1% dry weight) and a high ash (9.2% dry weight) content. The poor recovery of organic material after analysis is almost certainly due to the lability of some of the more unusual (and in this work unidentified) components.

Attempts to selectively solubilise the wall material with a view to identifying discrete polymers met with some success. In particular, treatment with trichloroacetic acid (TCA) at 35° extracted all of the glactosamine from the wall (in addition to other components) but none of the unknown component, $X_2$. Further treatment with TCA at 60° extracted all of another unknown component, $X_1$. These results suggest that some degree of resolution of different polymers constituting the wall may be possible and may have been achieved here.

Treatment of S. marina with the antibiotics, D-cycloserine, novobiocin, bacitracin, penicillin G and vancomycin, known to affect cell wall biosynthesis in other bacteria, was carried out. Possible effects of the antibiotics were monitored by electron microscopy and turbidimetric estimation of bacterial growth. Only novobiocin and bacitracin had any effect on growth but this was marked; in both cases growth was prevented by addition of the antibiotic. The other three antibiotics all lost their antibiotic activity (against appropriate indicator organisms) when incubated over a period of a few hours in Dundas medium. It is suggested that this may be a significant consideration when explaining the antibiotic insensitivity of microorganisms, such as S. marina whose doubling times are of the same order of magnitude as that necessary for antibiotic inactivation.

Thin sections of control and antibiotic-treated cells showed interesting ultrastructural features comparable with those seen in more conventional halophilic cocci. Some minor ultrastructural changes were seen in some of the antibiotic-treated cells, the most notable being extensive plasmolysis in the case of novobiocin. However, none of the antibiotics tested appeared to cause cell lysis or osmotic fragility which may preclude their use as agents for the non-destructive removal of the cell wall.

To Dad,

for much more than I can ever repay.

Acknowledgements.

I would like to express my gratitude to my supervisor, Dr. M.I.S. Hunter, for his continuous support, encouragement and advice throughout the project. I would also like to thank Mr. J. Hunter for his technical assistance in operating the Amino Acid Analyser, and my brother, Peter, for typing the thesis.

I am indebted to the Science Research Council for the grant that made this work possible and also to the Eli Lilly Company, Basingstoke, England, for their donation of a sample of pure D cycloserine.

Declaration.

I hereby declare that the following thesis is based on work carried out by me, that the thesis is my own composition, and that no part of it has been previously presented for a higher degree.

The research was conducted in the Department of Biochemistry, St. Andrews University, under the direction of Dr. M. I. S. Hunter.

Signed _____

Academic Record.

I first matriculated at the University of St. Andrews in October, 1968, and graduated with the degree of Bachelor of Science, Second Class Honours (Upper Division) in Biochemistry in June, 1973. I matriculated as a research student in the Department of Biochemistry, St. Andrews University, in October, 1973.

Certificate.

I hereby certify that Stephen John Wilfrid Millar has spent 10 terms engaged in research work under my direction, and that he has fulfilled the conditions of Ordinance No. 12 (St. Andrews) and that he is qualified to submit the accompanying thesis for the degree of Doctor of Philosophy.

Signed _____

CONTENTS.

Methods and Materials (ctd). Page.

Methods and Materials (ctd). Page.



Results.

Results (ctd). Page.

Results (ctd). Page.

## Abbreviations.

### Amino Acids.

Asp: asparate
Gly: glycine
Glu: glutamate
Thr: threonine
Ala: alanine
Val: valine
Lys: lysine
Ile: isoleucine
Ser: serine
Pro: proline
Met: methionine
Leu: leucine
Tyr: tyrosine
His: histidine
Arg: arginine
DAP: diaminopimelic acid

### Peptides.

Pentagly: pentaglycine
Gly-gly: glycyl glycine
Gly-ala: glycyl alanine
Ala-gly: alanyl glycine

### Monosaccharides.

Gal: galactose
Glc: glucose
Man: mannose

### Amino Sugars.

$GlcNH_2$ : glucosamine
$GalNH_2$ : galactosamine

$GlcNH_2HCl$: glucosamine hydrogen chloride (standard)
$GalNH_2HCl$: galactosamine hydrogen chloride (standard)

Mur : muramic acid

FIGURES.

Results. Page.

Tables. Page.

ELECTRON MICROGRAPHS.

Cell Wall Isolation.

| Plate No. | Description. | Magnification. |
|---|---|---|
| 1 | 2 x Hughes-pressed cells. | 16,857 |
| 2 | 2 x Hughes-pressed cells. | 54,833 |
| 3 | 2 x Hughes-pressed cells. | 54,833 |
| 4 | Washed, broken cells prior to trypsin treatment. | 24,062 |
| 5 | Washed, broken cells prior to trypsin treatment. | 74,666 |
| 6 | Washed, broken cells prior to trypsin treatment. | 74,666 |
| 7 | Purified trypsin-treated (Reistad) wall. | 17,187 |
| 8 | Purified trypsin-treated (Reistad) wall. | 40,833 |
| 9 | Purified trypsin-treated (Reistad) wall. | 50,166 |

Antibiotics.

| Plate No. | Description. | Magnification. |
|---|---|---|
| 10 | Control cells. | 89,833 |
| 11 | Control cells. | 39,666 |
| 12 | Control cells. | 39,666 |
| 13 | D Cycloserine (300ug/ml) - Method 1. | 72,333 |
| 14 | D Cycloserine (300ug/ml) - Method 1. | 72,333 |
| 15 | D Cycloserine (300ug/ml) - Method 1. | 73,500 |

Antibiotics (ctd).

| Plate No. | Description. | Magnification. |
|---|---|---|
| 16 | D Cycloserine (300ug/ml) - Method 1. | 60,666 |
| 17 | D Cycloserine (600ug/ml) - Method 1. | 73,500 |
| 18 | D Cycloserine (600ug/ml) - Method 1. | 73,500 |
| 19 | D Cycloserine (600ug/ml) - Method 1. | 84,000 |
| 20 | D Cycloserine (256ug/ml) - Method 2. | 22,500 |
| 21 | D Cycloserine (256ug/ml) - Method 2. | 80,000 |
| 22 | D Cycloserine (256ug/ml) - Method 2. | 16,256 |
| 23 | Bacitracin (256ug/ml). | 40,625 |
| 24 | Bacitracin (256ug/ml). | 60,666 |
| 25 | Bacitracin (256ug/ml). | 33,833 |
| 26 | Bacitracin (64ug/ml). | 63,000 |
| 27 | Vancomycin (256ug/ml). | 15,937 |
| 28 | Vancomycin (256ug/ml). | 73,500 |
| 29 | Vancomycin (256ug/ml). | 59,500 |
| 30 | Vancomycin (256ug/ml). | 54,833 |
| 31 | Penicillin G (256ug/ml). | 16,875 |
| 32 | Penicillin G (256ug/ml). | 49,000 |
| 33 | Penicillin G (256ug/ml). | 49,000 |
| 34 | Novobiocin (256ug/ml). | 25,313 |
| 35 | Novobiocin (256ug/ml). | 51,333 |
| 36 | Novobiocin (256ug/ml). | 38,500 |

# Introduction.

Halophiles are microorganisms that require more than the usual physiological concentration of salt of 0.9% (w/v) and represent a most unusual and mysterious form of life. Sodium chloride has been widely used as a preservative against microbial deterioration of food and other similar perishable materials and it was realised that the red coloured bacteria found living in the presence of high salt concentrations were very specialised organisms (1,2). These bacteria, which seem in many ways to have evolved differently from other organisms, represent extreme examples of adaptation to a severe enviroment, involving many interesting biochemical changes in such a concentrated brine.

The word 'Halophile' has been used to describe many marine organisms which normally live in 3% (w/v) salt brine as well as to bacteria that live in stronger brines, up to the saturation point of sodium chloride. A classification of these organisms has been described (3,4) based on their salt requirement without regard for underlying metabolic mechanisms.

The term 'Halotolerant' or 'Facultative Halophile' is applied to those organisms that apparently require no salt for growth, but may proliferate in salt concentrations of up to 10% (w/v) or, in some cases, even higher.

'Moderate Halophile' refers to bacteria that can grow in a salt brine of 3-20% (w/v). Probably many species require some salt for growth and can replicate in higher concentrations of salt. Several distinct species of such bacteria have been isolated (5,6). Their chemical properties are less unusual than the extreme halophiles, growing over a wide range of salt concentrations. Few studies regarding their enzymes have been made and, although many need some salt for their activity, in general they are less salt tolerant than the enzymes of the extreme halophiles. In fact, some of

the enzymes were partially inhibited by the salt concentration that the microorganisms grew best in (4). Relatively few of this class of organism were pigmented (5) and the presence of both muramic acid and diaminopimelic acid (DAP) in the cell envelope has been demonstrated, unlike the extreme halophiles, most of which contain carotenoid pigment (7,8) and which lack these two characteristic cell wall components (9,10,11,12).

The classification 'Extreme Halophile' is applied to those organisms that will only grow if the salt concentration is 15% (w/v) or greater. For many organisms sodium chloride is an indispensable nutrient and some bacteria grow best in a medium that is almost saturated with salt. Not only do they live and proliferate in a concentrated salt brine, but they also require these unusually adverse conditions for their best maintenance and growth. This unusual dependence on the presence of salt was a most confusing characteristic for biologists and led to the extreme halophiles being described as representing 'the borderland of physiological possibilities' (13). This unique ability is found only in the bacterial kingdom and is confined to two distinct types, the halobacteria (3) and the halococci (14).

Extreme Halophiles.

All organisms that have been shown with certainty to grow best in sodium chloride concentrations of 20% (w/v), or greater, have been assigned to one of the two types of bacteria mentioned. Both types are found all over the world where the salt concentration is very high, especially in the solar evaporation ponds of salt works where they frequently occur in such large numbers that they tinge the brine red. They are also commonly found on heavily salted protein products such as fish and hides, in salt lakes and in meat curing brines, although in the latter case the organisms are

generally of the moderate variety and may have a role in the curing process itself. The primary source of the extreme halophiles is solar salt (5).

The halobacteria belong to the genus Halobacterium and the Pseudomonadaceae family (3). These rod-shaped bacteria are highly pleomorphic, obligate halophiles requiring a minimum of 12-20% (w/v) sodium chloride for growth, the optimum salt concentration being dependent on the specific strain. When motile, these Gram-negative, non-spore forming organisms are lophotrichously flagellated and, on exposure to hypotonic solution, lyse dramatically below a critical level of salt of around 12% (w/v)(5).

The halococci, which occur singly, in pairs, in irregular clusters or in sarcina-like packages, belong in part to the genus micrococcaceae and in part to the sarcinae (3), although an individual classification, halococci, has also been proposed (14). These non-spore forming, non-motile organisms are obligate halophiles with a minimum requirement of 5-15% (w/v) for sodium chloride and an optimum of 20-25% (w/v). If a smear is prepared from a suspension of these organisms in distilled water they appear Gram-variable, but a smear from a saline suspension stains characteristically Gram-positive (9). The morphology of the cell varies between strains and is also dependent on the condition of the culture. On exposure to hypotonic solution they do not display the lysis phenomenon characteristic of the halobacteria (12).

The extreme halophiles are obligate aerobes and divide very slowly, even under the most favourable of conditions. Halobacteria have a characteristic doubling time of 7 hours and the halococci 15 hours. Because of the time necessary to grow these organisms, it is ideally desirable to optimise yield. For H. salinarium, 8-10g wet weight of cells per litre were obtained in a yeast extract-trypticase medium with a 25% inoculum (15).

The strong orange-red pigmentation of these organisms led to them being described as the 'red-halophiles'(5). This colouration is due to the presence of carotenoids which are situated mainly in the cell envelope and constitute in the order of 0.1% of the dry weight of the cell envelope (12). It is not related to their halophilic character, since colourless extreme halophiles exist, but, where present, may serve to protect the cells from the detrimental effects of photochemical damage as their natural habitat includes solar evaporation ponds where they are inevitably exposed to strong sunlight (7,8). However, the discovery of a rhodopsin-like protein from the 'purple membrane' of H. halobium (16), has suggested that pigmentation may have an extremely important functional significance for the cell. This component utilises light to create ion gradients, particularly a proton gradient, across the membrane, comparable to the chemiosmotic theory (17), and this can drive ATP synthesis or the uptake of other components, e.g. amino acids (18). Non-pigmented or colourless cells are very rare, although no survey of or attempt to locate these organisms in environments without the presence of strong sunlight, such as the soil of salt marshes, has been made.

Electron microscopy studies of the cells of the extreme halophiles have revealed the presence of gas vacuoles. In high salt concentrations, the solubility of oxygen is markedly decreased. As all the extreme halophiles are obligate aerobes, it has been postulated (19) that the cells must come to the surface of the salt brine to obtain the necessary oxygen and that the gas vacuoles have a role to perform in this respect.

Necessary Ions.

The extreme halophiles have a specific requirement for sodium chloride

for growth. Experiments replacing the sodium chloride with other ions have, in general, been unsuccessful (5), although in some cases a small fraction of the salt could be replaced by potassium chloride (20,21). The stability of the cell can be maintained by high concentrations of salts other than sodium, but only sodium chloride supported normal growth (12).

Not only is there a specific requirement for the sodium cation, but also for the chloride anion. Replacement with other anions did not support growth (5). The external sodium ion has also been shown to be required in active transport into the cells of the halobacteria and marine organisms. In H. salinarium (22), the uptake of glutamate, an active transport process, required a high external sodium ion concentration. This phenomenon, and its interrelationship with the 'purple membrane' is currently under investigation

Magnesium is needed at the unusually high concentration of 0.1-0.5M in the medium for best growth (20). This is 1-2 orders of magnitude higher than the normal level for bacteria in general. However, at lower magnesium ion concentrations, the normally rod-shaped cells of the halobacteria adapt by becoming coccoid and may even retain this conformation after being transferred back to their normal environment.

The optimum requirement of about 25mM for potassium (20) is comparable to other bacteria, although the intracellular concentration is extremely high in the extreme halophiles (23). Ferrous ions at 10 ppm increased the yield of halobacterial cells and allowed more normal growth at the lower limit of the sodium chloride requirement. At 0.05ppm, manganese ions stimulated growth, the halobacterial cells showing a marked increase in pigmentation (24).

Internal Salt Concentration.

The requirement for high levels of both sodium and potassium for the intracellular enzymes (12), coupled with the low freezing point of extreme halophiles (21,25), suggests that they have a very concentrated internal environment. In fact, it is as high as, or possibly even a somewhat higher ionic strength, than the external environment, though the cells may actively select the ions found inside as the ionic composition is different. Besides the sodium and chloride ions, potassium is also a dominant ion of the internal salt (20,21,23) which is unexpected because of its low content in the growth medium. Both the halobacteria and the halococci appear to be equipped with a mechanism that concentrates potassium ions intracellularly against a steep gradient to such an extent that, in H. salinarium (23), the potassium content inside the cell becomes close to its solubility limit in water. This potassium uptake is achieved at the expense of sodium and the apparent concentration of potassium ions is considerably higher than sodium ions inside the cell, although the combined intracellular concentration of sodium and potassium ions exceeds the sodium ion concentration in the medium. The dominant anion in the cell is chloride, but its intracellular concentration is lower than in the medium (23).

An important discovery regarding this high intracellular concentration of potassium ions has been the correlation between the internal potassium ion content and the tolerance to sodium chloride in a number of non-halophilic bacteria (26). As the concentration of sodium chloride is increased in the medium, there is a corresponding increment in the intracellular concentration of potassium ions; i.e. the higher the tolerance to sodium chloride then the higher the internal potassium ion concentration. This increased potassium

content seems to be a general characteristic of bacteria that possess the ability to live in a strong sodium chloride brine, or just a potential to do so. It has also been suggested (26) that this phenomenon in non-extreme halophiles imparts resistance to plasmolysis and dehydration by overcoming the detrimental effects of the external salt. In the extreme halophiles, the potassium ion possibly has a similar function.

Enzymes.

About 30 different enzymes from both the halobacteria and the halococci have been examined (27). In each case, they were found to function optimally at concentrations of sodium chloride approaching saturation and most of them needed considerable concentrations of salt to operate efficiently. All fit the general concept (4,28,29) that the enzymes from the extreme halophiles are extremely halotolerant and, in most cases, even strikingly halophilic in character, having adapted to act catalytically in a concentrated salt brine In H. salinarium and S. morrhuae, although the various types of enzymes differed quantitatively from each other in their salt responses, enzymes of the same type from the two bacteria displayed similar salt responses (5).

In normal extremely halophilic cells, reducing the sodium chloride concentration produced drastic effects. The proteins denatured, the enzymes became inactivated and the whole enzymic machinery of the cell ceased to function. Initially, the loss of enzymic activity was thought to be irreversible (28,29,30) as, by restoring the salt rapidly, the enzymic activity was, at best, only erratically recovered. However, by replacing the salt slowly, by dialysis for example, a number of enzymes from H. salinarium reproducibly regained an activity that was a constant fraction of the original activity before the removal of the salt (31,32). In general, this was of

the order of 50-70%, alanine dehydrogenase regaining 70% and malate dehydrogenase 60% of their original activity. However, some enzymes, such as ethanol dehydrogenase, did not recover their activity and this implies that, in this case, the enzyme was completely and irreversibly denatured.

The exact chemical nature of this difference in salt response of the enzymes from the extreme halophiles and non-halophilic organisms is not fully understood. It has been postulated (30,32) that as halophilic enzymes contain more ionised groups at their catalytic centre, the salt might act as counter-ions to reduce repulsive forces of an electrostatic nature. This would allow the enzyme molecule or complex to attain the correct conformation for it to be catalytically active. As yet, no pure enzyme preparation from an extreme halophile has been isolated and the amino acid composition studied. However, proteins from both halobacterial and halococcal cells have proved to be characteristically acidic in nature (33,34,35,36) and this may also prove to be the case for the enzymes. This would be in agreement with and lend support to the proposed theory.

A relationship between the salt concentration and the pH has been demonstrated in H. salinarium with the enzyme malate dehydrogenase (32) which indicates that the ionised groups within the molecule do indeed play an important role in its activity and reaction in a salt free environment. Thus, the idea that the salt acts by shielding mutually repulsive groups on the protein molecule is basic to the studies of extreme halophiles, although the picture is probably not so simple (37,38,39). This aspect is discussed in the Protein section below.

The potassium ion has also been found to be an exceptionally potent activator of enzymes which correlates with potassium being one of the dominant

intracellular cations. Mole for mole potassium was, in H. salinarium (4,28,29) and S. morrhuae (12), at least as effective as sodium in activating the halophilic enzymes and, in most cases, produced twice the activity. It is proposed (12) to be not only an enzyme activator but, in conjunction with sodium, to also be a stabiliser of the enzyme complex.

Proteins.

The 'bulk' protein is representative of the 'average' protein in the cell and in the extremely halophilic bacteria this is strikingly acidic in nature (36). The 'average' protein from H. halobium had an excess of acidic to basic amino acids of 10.5 mole% compared to the corresponding non-halophilic pseudomonad value of about neutral tending toward basic. Similarly, two halococci had ratios of acidic to basic amino acids of 10.8 and 10.0 mole%, comparable to the halobacteria. The corresponding non-halophilic micrococcus had a ratio of about neutral tending toward acidic.

Proteins from the cell envelope of the halobacteria are also strikingly acidic in nature (33,34) and constitute as much as 75% of the cell envelope. In H. cutirubrum, the protein was of the order 45-57% (40) and in H. halobium and H. salinarium 65 and 75% respectively (41) of the cell envelope. In H. halobium, at physiological pH values, the intact envelope has been shown (42), by titrimetric and electrophoretic studies, to be strongly negatively charged at, or near, the surface caused by the dominance of ionised carboxyl groups. This acidic nature of their amino acids has been proposed (33) as the key to understanding the molecular basis for extreme halophilism.

The proteins are so acidic in nature that it is generally agreed (12,27, 43) that the cation of the salt is necessary to neutralise the excess of negative charges on the protein molecule to maintain its proper conformational

state to be functional. If the salt was removed, the electrostatically repulsive forces would denature the proteins and the structural proteins might even disperse and go into solution. However, the charge screening theory to explain salt requirement does not account for the very high salt concentrations required when simple charge neutralisation would be achieved with much lower concentrations of salt, nor the specificity for the sodium cation. The picture is probably not so clear and simple (37,38,39).

The dependence of proteins and enzymes for sodium and/or potassium chloride may reflect not only the importance of ionic interactions, but also, possibly to an even greater extent, the importance of hydrophobic interactions within the halophilic protein molecule. In the presence of high concentrations of sodium chloride, the non-polar side chains are envisaged as being positioned in the interior of the structure where they hydrophobically bond, enhancing the stable conformation of the protein molecules necessary for their structural and catalytic functions (37,38,39).

Nucleic Acids.

Several pieces of evidence have led to the conclusion that the nucleic acids of these organisms do not differ radically in their 3-D structure from those of non-halophilic bacteria. The total salt free dry weight of the nucleic acids from H. salinarium was 15%, of which 4.3% was DNA (44). These figures are comparable to those commonly found for other non-halophilic bacteria. The base composition of the bulk DNA and RNA was also comparable to the general situation in pseudomonads (45) and the elution patterns for DNA and RNA from H. salinarium on two different ion-exchange resins were identical to non-halophilic pseudomonads. Even the Tm values for the DNA

preparations were in agreement (12).

The Cell Envelope.

In bacteria the cell adopts its shape according to the biosynthetic pattern of the cell wall. The peptidoglycan was envisaged as a 'bag-shaped' macromolecule (46) which could be formed more spherically or cylindrically shaped as directed by the genetic apparatus. It not only confers shape but also rigidity and strength to the cell.

A clear concept of the common features of the cell wall structure and its biosynthesis exists and has been extensively reviewed (46,47,48,49,50, 51). The peptidoglycan, a common feature of both Gram-positive and most Gram-negative bacteria, is a rigid structural framework comprising of a complex polymer of acetamido sugars and a small number of characteristic amino acids covalently interlinked. The degree of rigidity is a variable factor between bacteria and is dependent on the nature and extent of cross-bridging between peptide sub-units, the frequency of muramic acid residues not substituted by tetrapeptide chains, the presence and nature of substituents on the carboxyl group of glutamic acid and the nature of the dibasic amino acids.

Current evidence suggests that most of the amino sugars in the peptidoglycan are present as the N-acyl, probably the N-acetyl, derivative. The major amino sugars of bacterial envelopes have been identified as glucosamine, galactosamine, Muramic acid and an amino uronic acid. Muramic acid is the most distinctive of these having the capability of a dual function, linking both peptides and amino sugars through peptide and glycosidic bonds.

As few as three or four characteristic amino acids, identified as glycine, alanine, lysine or diaminopimelic acid (DAP) and glutamic acid,

were identified in the peptidoglycan. One of the unusual features of these amino acids has been the widespread occurance of alanine and glutamic acid as their D isomer.

Muramic acid, which is present in almost all Gram-positive and Gram-negative bacteria, is located exclusively in the peptidoglycan and its nucleotide precursors, and its detection is an indication of the presence of peptidoglycan. DAP is also used as an indicator of the peptidoglycan component, but it is not so universally found as muramic acid.

Electron microscopy studies of both intact cells and isolated cell envelopes (40,52,53) have shown that a characteristic of the halobacteria is a textured surface with a regular hexagonal pattern, comparable to that found on the surface of other Gram-negative bacteria (48). Sectioning of both whole cells and isolated envelopes of H. halobium and H. salinarium (41,54) revealed one three layered envelope resembling the 'unit membrane', although a five layered structure was also observed in some sections of the whole cells of H. halobium and in all sections from H. salinarium. However, the total thickness of this three layered structure was somewhat larger than the common unit membrane, namely 110 A (41,54) compared to 75-80A.

There has been a distinct lack of success in any investigation in demonstrating the presence of two clearly separable membrane structures, the outer three layered structure referred to as the outer membrane in other Gram-negative bacteria and the three layered structure referred to as the 'cytoplasmic membrane'. Possibly the envelope layers of the halobacteria are of a chemically different nature to those of other Gram-negative bacteria (12) so that they are not equally well revealed by the common staining techniques. It has been suggested (41) that the envelope has a simpler

structure than normally found and that this may be a feature having a bearing on the life of these organisms.

Similar studies on the extremely halophilic coccus S. morrhuae initially showed that this bacterium resembled the non-halophilic sarcinae and micrococci in section (9). However, a more detailed study of Halococcus morrhuae (10) indicated that it lacked the characteristic dark-light-dark tribanded layer of the Gram-positive micrococci and also the double layered cell wall characteristic of the Gram-negative cocci. In this respect, the wall of Halococcus morrhuae is very similar to that of the halobacteria, although the wall of the latter is thinner.

Halococcus morrhuae had a single layered wall 50-60 nm thick with fuzzy material attached to the outer surface and a three layered cytoplasmic membrane 18nm thick. There were also gas vacuoles, 200-300nm in size with walls 2-3nm thick, present and also polyphosphate granules.

'Halophilic coccus' strains 24 and 46 had relatively thick cell envelopes 300-600nm thick, resembling the micrococcaceae with the appearance of a typical 'micrococcal' wall. There was also evidence of the formation of cross-walls prior to cell division (55). Although mesosomes were seen frequently in the former and occasionally in the latter strain, no cytoplasmic membrane was detected in the thin sections of cells fixed directly from the growing culture.

When the cells of these two strains were dialysed against pure water prior to fixation, the walls showed no fundamental differences to cells fixed directly from the growing culture. However, the cytoplasmic membrane was now visible in the thin sections. When the cells were fragmented in an homogeniser the overall contour of the cell was maintained, although little

cytoplasmic material was left in the cell. The internal pressure seemed to have forced most of the cytoplasmic material out when the wall was ruptured. The membrane was easily detectable and had formed closed vesicles, resealing after releasing the cytoplasmic material. Membrane synthesis also appeared to precede wall biosynthesis in the cell wall division process.

The envelopes of the halobacteria are of particular interest with regard to their unusual chemical properties and the effect of salt on their stability. The envelope is predominantly lipoprotein (44), the lipid in H. halobium constituting 17-23% (42) and the protein in H. halobium and H. salinarium 65 and 75% respectively (41). Sugars were shown to be present (12) but at much lower levels than commonly detected in other Gram-negative bacteria (48) and, although amino sugars were also detected, their content was only of the order of 0.5-1.5% of the envelope (12). In H. salinarium, the amino sugar was 1.5% of the envelope content, but only half of this was glucosamine, the remainder being composed of a 2-amino sugar derivative. This derivative may constitute a part of a peptidoglycan-like polymer bearing some resemblance to, but different from, the classical picture. However, it is present in such small amounts that it is hard to envisage it playing a significant role. There was also a lack of teichoic acids in the envelope of H. cutirubrum (56).

Muramic acid was not detected in the envelopes of H. cutirubrum (40), H. salinarium (41), H. halobium (41) or any other halobacterium studied (27). There was also no evidence for, or detection of, diaminopimelic acid (DAP) and, in H. cutirubrum, there were no D-isomers of amino acids (56). Thus, this genus lacks some of the components essential for the peptidoglycan normally present and indicates that this rigid layer found in all other Gram-negative bacteria, save the pleuropneumonia-like organisms (48), is

absent. This is highly significant as it appears to be a general characteristic of extreme halophilism.

The thick walled halococci are very difficult to rupture and relatively few studies on this genus have been made. In all the strains investigated, however, the chemical composition of the wall appears fundamentally different to those of non-halophilic organisms (9,11,57).

The walls lacked both DAP and muramic acid and the characteristic amino acids of the peptidoglycan were either absent or present in only trace amounts. In 'Halophilic coccus' strain 24 glutamate was present in equimolar amounts with glycine, but 87% was in the L form (11). Thus, as in the halobacteria, components essential for the formation of a peptidoglycan structure were absent and this was further substantiated by the almost complete solubility of the wall in hot formamide (11). This lack of a peptidoglycan structure and, more specifically, the lack of muramic acid, explains the failure of lysozyme, an N-acetyl muramidase, to cleave the wall (9).

The amino sugars glucosamine and galactosamine were detected in the walls of both S. littoralis and S. marrhuae in approximately equimolar amounts (9). The amounts varied between strains from 5.2% of the dry weight of wall in S. littoralis (9) to 10% in 'Halophilic coccus' strain 24 (11), where three additional unidentified ninhydrin-positive components $X_1$, $X_2$, and $X_3$ were found. $X_2$ has subsequentially been identified as gulosaminuronic acid (57) and has been detected in all strains studied. It is estimated as being present in quantities at least as great as the other hexosamines and has tentatively been proposed as being an obligatory constituent of the halococcal wall (57).

Glycine is also a common component of the wall (57), although the

common amino acids were detected in trace amounts (9,11). These, however, were assumed to be contaminants from plasma membrane still attached to the wall after isolation, as the wall preparations were tinged faintly pink even after trypsin treatment.

Hot and cold TCA extractions on 'Halophilic coccus' strain 24 (11) showed that there were no teichoic acids present and that the wall may be composed of two polymers. The first, extracted in warm TCA, contained glucosamine and the unidentified component $X_1$, the second, the non-extracted fraction, amino acids, $X_3$, and a small amount of $X_1$. As $X_1$ and $X_3$ remain unidentified, this finding could reflect the presence of repeating units, each involving a relatively small number of the unidentified ninhydrin-positive compounds.

The monosaccharides, glucose, galactose and mannose were detected in the wall of S. littoralis and S. morrhuae in approximately equimolar amounts (9). Glucuronic acid was also tentatively identified and the total hexose contents of these two bacteria were 21 and 16% respectively. The total reducing substance content was, as expected, higher at 30-36 and 20-31% respectively.

Although the ash content of the wall was unusually high, 11.9% in S. littoralis (9), only 15% of the wall could be accounted for as ninhydrin-positive material (11). This value is very low when compared to other Gram-positive non-halophilic cocci (48) and by far the majority of the wall is composed of ninhydrin-negative compounds. Since peptidoglycan of a type similar to that found in Gram-positive cocci is lacking, something else must confer rigidity and strength to the halococcal walls.

Neutral and cationic polymers play no major role in the cell wall of

Halococcus morrhuae (58) and the inability to extract single polymers suggested that the purified wall may consist of a single complex heteroglycan. A sulphated heteroglycan has been postulated as being the major cell wall polymer of Halococcus morrhuae (58). This polymer would represent the supporting structure of the wall and, in this respect, fulfill the function of the bacterial peptidoglycan, although its chemical structure is quite different. There is also evidence that a sulphated polysaccharide may be present in the non-rigid cell envelope of H. halobium (98). Sulphated heteropolymers have hitherto not been found as structural components of bacterial walls although the walls of marine algae very often contain similar sulphated structural polysaccharides (99). This may indicate a phylogenetic relationship between the cell walls of the extreme halophiles, procaryotic cocci, and the eucaryotic marine algae.

## Antibiotics

Studies on the effect of antibiotics on halobacterial cell envelopes have not been extensive. Proliferating cells of H. salinarium were about equally sensitive to penicillin, a compound known to interfere with peptidoglycan synthesis (59,60,61), as those of other Gram-negative bacteria, displaying a similar pattern of structural transformations and lysis (52). However, in the absence of a peptidoglycan component, penicillin sensitivity may reflect interference with the formation of some other envelope compound (12).

D-cycloserine, a compound that also acts at the level of wall synthesis in both Gram-positive and Gram-negative bacteria (60,61,62), had no effect on H. salinarium cells (12). However, bacitracin inhibited the growth of the halobacteria (63).

Envelope Lysis and the role of Sodium.

On exposure to hypotonic solution the halobacteria show a dramatic lysis effect. If the concentration of sodium chloride is gradually reduced the bacteria undergo a change in shape and, below a critical salt concentration, experience cellular lysis. Originally, this phenomenon was thought to be due to osmosis alone, but subsequent investigations have revealed that other factors are involved (43).

Although the envelopes disintegrate very rapidly, the process does not go to completion. The speed of dissolution suggests that there is no involvement of peptide bond splitting, no release of some low molecular weight substance and no cleavage of protein-lipid bonds (43). Several metabolic inhibitors do not prevent this disintegration process and led to the conclusion that the process is non-enzymic (33,40,52,64). It seems to involve at least two separate processes, one being more rapid than the other in the complete absence of salt (40).

Although osmotic forces play an important role in this phenomenon, the effect of different solutes in preventing lysis of intact walls cannot be explained purely on the basis of osmotic pressure. In extreme halophiles, as well as marine organisms, magnesium salts are more effective than the corresponding sodium salt in preserving the integrity and preventing lysis in isolated envelopes. Apparently lower magnesium ion concentrations are sufficient to prevent dissolution, but not to overcome the physical forces of osmotic pressure in intact cells (43). Thus the salt has a dual role in not only maintaining a balance of osmotic forces but also in preserving envelope structure.

The first postulations for the function of sodium chloride in preserving

the envelope as an integral unit (65,66) was that hydrogen bonds, coulomb forces or 'salt'linkages held the envelope together, although very loosely. In the presence of high concentrations of salt the electrostatic forces would be screened and the bonds would hold the organism in its usual conformation.

This theory of excessive negative charges causing the dispersion of the cell envelope and the dependance on sodium chloride in the environment to neutralise them (33) is supported by the excess of acidic to basic amino acids in the envelope (33,34). The masked basic groups accentuate the effect and accounts for the ability of various cations to prevent envelope dispersion. In this respect, the sodium cation seems to be of primary importance.

The rod shape of the bacterium is dependent on the presence of high sodium and potassium ions (52). Lowering their ionic concentration or replacing them with other mono or divalent cations caused a loss of rigidity and the spontaneous conversion to spheres. It is argued (67) that the sodium ions confer sufficient rigidity to the structure, stiffening the rod-shaped orientation, by orientating an atmosphere of sodium ions along the envelope surface. There would, therefore, be no requirement for a peptidoglycan layer or polysaccharide component to maintain cell rigidity and would account for the lack of some of the essential substances for this structure.

The removal of both protein and lipid components from the envelope of H. cutirubrum left fractions rich in carbohydrate retaining the same shape as untreated cells (34). The function of carbohydrates in preserving the cell shape is not fully understood and requires further investigation. However, the removal of lipid greatly increased the magnesium ion concentration necessary to maintain stability (68). This implies that the lipids

are involved as a potential binding site for magnesium. The lipids could be hydrophobically bound to one group of proteins and polarly bound to another . The magnesium ions could then chelate between the aspartate and glutamate moities on the protein and the lipid diphosphate head groups. A further ionic link could then exist between the terminal lipid phosphate and an arginyl residue on the protein.

Halophilic Lipids.

A survey of the lipids of these bacteria (69) has revealed two major differences from the normal situation in other bacteria. They have much higher levels of unsaponifiable material caused by the dominance of diether linkages and only trace amounts, if any at all, of ester linked fatty acids. These two features are characteristically striking for these bacteria and of great taxonomic and evolutionary importance.

Most of the lipids are derivatives of a glycerol-diether type, analogous to the glycerol phosphatides save they contain ether rather than the normal ester link, and long chain alkyl groups, generally dihydrophytol derivatives, instead of fatty acids or straight chain aliphatic alcohols (70). These differences have been detected in both the halobacteria and the halococci, but are absent in moderate halophiles, even when grown in high salt concentrations (71).

In H. cutirubrum the total lipid content of the envelope was 22% and the ether component, which dominated the lipids of the whole cell, was concentrated here (40). Elucidation of the structure and chemical composition of the major polar lipid (72) showed it to be a diether analogue of phosphatidyl glycerophosphate, accounting for 13% of the salt-free dry weight of the envelope.

Lipid metabolism also differed from other microorganisms in that it was directed toward the production of isoprenoid compounds. The malonyl CoA system was found to be inhibited in the presence of high concentrations of salt and a mevalonate system was utilised instead, producing the isoprenoid chains (72).

The ether link and the isoprenoid nature of the side chains have selective advantages over the commonly found ester links and fatty acids. Ether links are more stable to pH extremes and high temperatures than the ester bond and the dihydrophytol side chains are more resistant to oxidative conditions found in the extreme halophiles natural enviroment than fatty acids.

On the Phenomenon of Extreme Halophilism.

One of the fundamental questions is how did these specialised organisms arise in nature? They are found all over the world where the concentration of sodium chloride is high and it is speculated that they developed spontaneously from non-halophilic and marine organisms, selectively developing in a strongly saline enviroment (5).

There has been no success in attempts to derive a non-halophilic bacterium, nor even a less halotolerant form, from an extreme halophile. Conversely, attempts to produce an extreme halophile from a non-halophile, or a halotolerant organism from one that is halosensitive, have also proved unsuccessful. Thus, halophilism is a characteristic not easily acquired or lost (12). In conjunction with the basic differences in the composition of the cell envelope, the presence of diether containing phospholipids, the absence of fatty acids and the dependence on high concentrations of salt for all physiological functions, suggest that evolutionarily the

extreme halophiles diverged long ago from other bacteria.

Apart from the unique lipid composition which appears to be a signature of extremely halophilic character, probably the most notable biochemical feature related to this phenomenon of halophilism is the acidic nature of the proteins (27). Remarkable also is that these unusual bacteria are confined to only two distantly related bacterial types, the pseudomonads and the sarcinae and micrococcaceae.

Obviously a full understanding of these unique and highly specialised organisms is far from complete and they still represent a most mysterious and confusing form of life.

Aims of this Project.

The extent of current knowledge and understanding of the phenomenon of extreme halophilism have been mainly derived from studies on the Gram-negative halobacteria because they lyse so dramatically on dilution. This has greatly facilitated more detailed investigations of these bacteria and their dependence on sodium chloride.

Although at the present time there is no evidence to suggest that the general characteristics of extreme halophilism are radically different in the Gram-positive halococci, relatively few studies on these species have been made. The halococcal cells do not display the lysis phenomenon on dilution and are very difficult to rupture. The conditions for the removal of the thicker, very rigid cell wall are so severe that they cause the destruction of the remainder of the cell.

This investigation has been concerned with an attempt to develop a non-destructive method for wall removal so that studies on the membrane and its permeability properties might be possible. Two main approaches were followed.

A chemical analysis of the wall from S. marina was made to identify and quantitate the components with the intention of constructing a model of its structure. This picture would then possibly suggest chemical or enzymatic agents that would cleave bonds holding the wall in so rigid a conformation.

The effects of five different antibiotics, penicillin G, novobiocin, D cycloserine, vancomycin and bacitracin that are reported to exert their influence at the level of the cell wall synthesis (60,61), were tested on proliferating S. marina cells. It was hoped that one or more of these

antimicrobial compounds would interfere with wall synthesis, weakening the wall sufficiently to facilitate removal by less destructive means.

## Methods and Materials.

## 2.1 Maintenance and Growth.

### 2.1.1 Maintenance Culture.

Maintenance cultures of the extreme halophiles were grown on a solid medium (73) of the following composition: sodium chloride, 25%; magnesium sulphate, 1%; calcium chloride, 0.02%; potassium chloride, 0.5%; yeast extract (Oxoid), 1%; tryptone (Oxoid), 0.5%; glucose, 1%; Agar No. 3 (Oxoid), 2.5%. All percentages are expressed as w/v.

Although it is reported that the extreme halophiles do not metabolise carbohydrates (5), it has been shown that 1% (w/v) glucose in the medium enhanced the growth of S. morrhuae (74) and was included in the growth medium.

The medium was sterilised by autoclaving at 15 lb/sq. in. pressure for 20 minutes. The bacteria were subcultured every three to four weeks, and the petri dishes sealed in a polythene bag with a moist cotton wool plug to prevent the loss of moisture and salt crystal formation. The bacteria were incubated at 34°C.

### 2.1.2 Liquid Culture.

100-500ml. batch cultures were grown on a New Brunswick G-25 orbital incubator shaker at 34°C with a rotation speed of 350rpm. The medium was of the same composition as section 2.1.1, but without the Agar No. 3, and will be referred to as Dundas medium. It was sterilised by autoclaving at 15 lb/sq.in. for 20 minutes. These cultures were used for all the growth experiments and as inocula for large scale batch cultures.

### 2.1.3 Growth Determination.

The degree of bacterial growth in a batch culture at any given time was determined by absorbance measurements (turbidimetry) on a Unicam SP 600 series spectrophotometer at 610nm.

From the turbidity-dry weight relationship (section 2.1.5) it was sometimes found necessary to perform suitable dilutions using sterile medium to ensure final O.D.s of 0.1-0.4.

2.1.4 Halococcal Growth Comparison.

An inoculum culture of each of the following obligate extremely halophilic cocci, all obtained from the National Collection of Marine Bacteria, Torry Research Station, Aberdeen, was grown in 100ml of sterile Dundas medium at 34°C to mid-logarithmic phase:

S. marina (NCMB 778)

S. littoralis (NCMB 757)

S. morrhuae (NCMB 761)

S. sreenivasani (NCMB 776)

A 5ml aliquot of each culture was transferred in duplicate to 100ml of Dundas medium and the growth of the bacteria recorded turbidimetrically at 610nm.

2.1.5 Absorbance-Dry Weight Relationship.

The absorbance values at 610nm were related to the dry weight of S. marina (NCMB 778) cells as follows:

A crucible was scrupulously cleaned in chromic acid, rinsed in distilled water and dried to constant weight at 100°C. A logarithmic phase culture of S. marina (50ml) was harvested by centrifugation and the cells washed five times in distilled water to remove the sodium chloride. The washed cells were suspended in distilled water to a final volume of 25ml and a 5ml aliquot was taken to dryness and constant weight at 100°C in the tared crucible. Dilutions were performed on the cells to give turbidity readings in the range 0.1-1.0. The O.D.s at 610nm were plotted against the

calculated dry weight of cells. The assay was conducted in triplicate.

2.1.6 Salt Tolerance of S. marina.

Duplicate 100ml batches of Dundas medium were prepared with varying concentrations of sodium chloride, 0-25% (w/v). These were inoculated with 2ml aliquots of an homogeneous logarithmic phase culture of S. marina and growth curves plotted for the different concentrations of sodium chloride.

2.1.7 Large Scale Batch Culture.

Batch cultures (3 litres) were grown in a New Brunswick 3x5 litre Fermentor in Dundas medium, sterilised by autoclaving at 15 lb/sq.in. for 40 minutes. The medium was agitated at 100rpm, aerated at 5l/min. and maintained at 34°C. Each batch was inoculated with a 300ml logarithmic phase culture of S. marina, grown in an identical medium, and the bacterial growth was recorded turbidimetrically at 610nm. Any loss of water due to evaporation was corrected for by the addition of sterile water.

The cells were harvested in late logarithmic phase on a Sharples High Speed Centrifuge, the rate of flow being adjusted to give the fastest flow compatible with a clear effluent. The cells were washed three times in Dundas Basal Salts (DBS) and stored at -15°C in a sealed plastic container.

2.2 Cell Wall Isolation.

The cell wall from S.marina was isolated following the method of Reistad (11) which is outlined in Figure M1.

The washed whole cells were prepared as a thick suspension in distilled water and added dropwise to liquid nitrogen. The frozen bacterial globules were passed through the Hughes Press twice at -45°C. This device (75) which operates on the 'ice-shear' principle for cellular disruption, is generally

Figure M.1

Cell Wall Isolation.

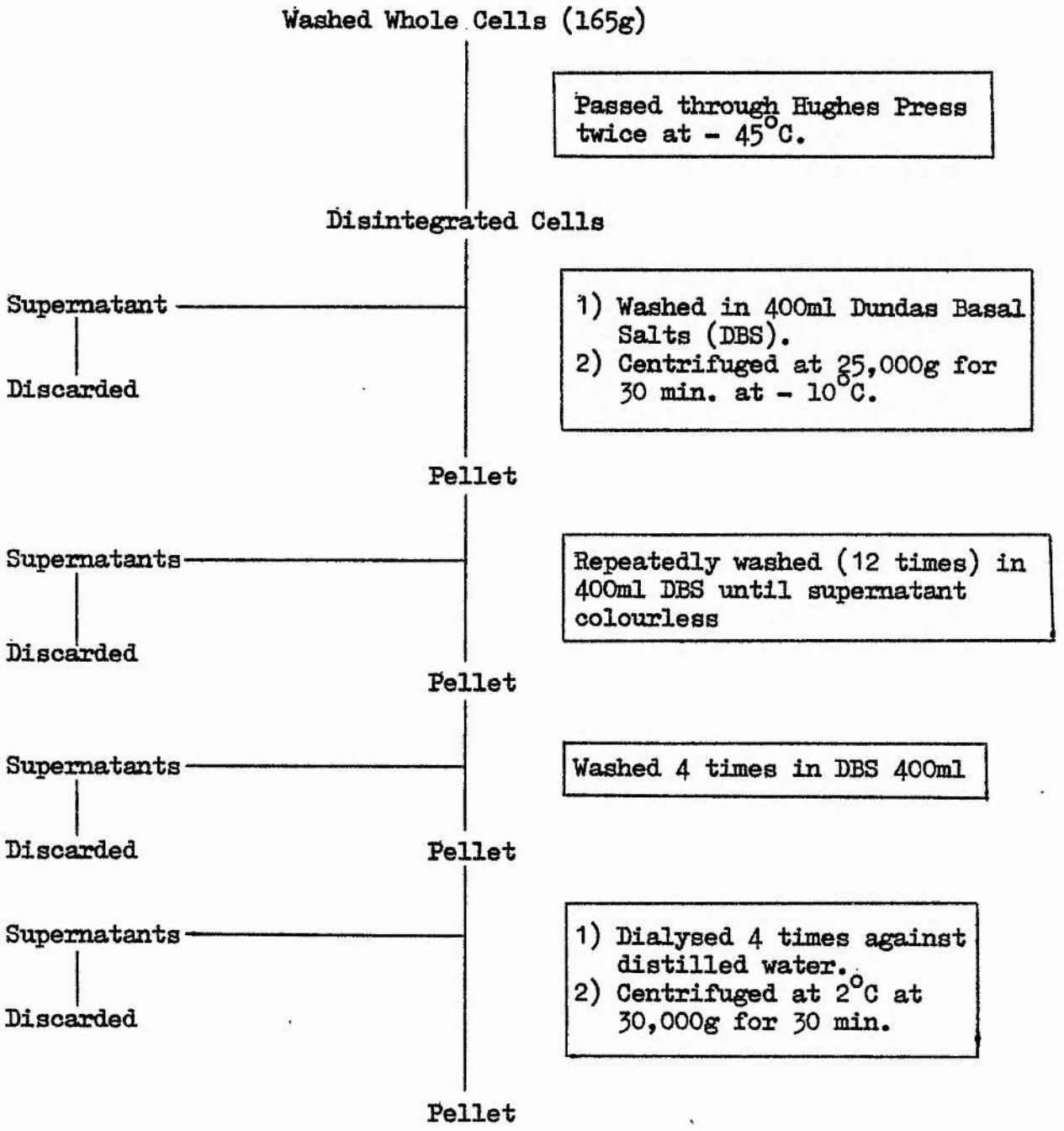
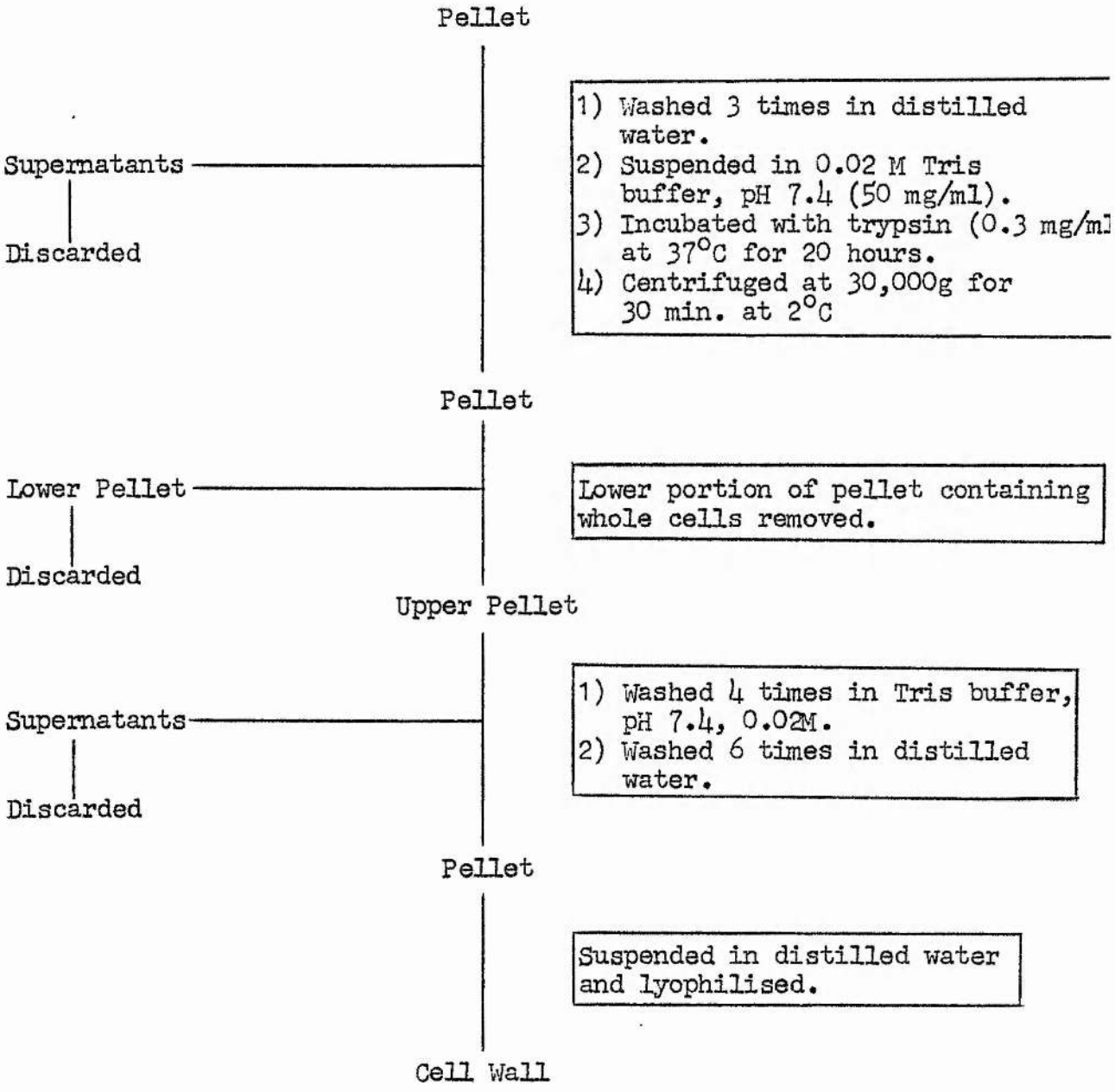


Figure M.1 (ctd.)



used for cell destruction in enzyme isolation (48).

## 2.3 Cell Wall Analysis.

### 2.3.1 Lipid Extraction of the Wall.

The isolated wall was subjected to lipid extraction to determine whether the membrane had been effectively removed. A modification of the Bligh and Dyer method (76), specific for halophiles, was used (77). The procedure is shown in Figure M.2.

### 2.3.2 Dry Weight and Ash Determination.

A crucible and lid were thoroughly cleaned in chromic acid, rinsed in distilled water and taken to constant dry weight at 100°C. Wall (100mg) was accurately weighed into the tared crucible and dried to constant weight at 100°C. The crucible and contents were heated in a furnace to 450°C for three hours. After cooling, one drop of concentrated sulphuric acid was added and the crucible was heated to 700°C for three hours. The process was repeated until a constant weight was recorded. The assay was conducted in triplicate.

### 2.3.3 Amino Acid Analysis.

#### 2.3.3.1 N Terminal Analysis.

A qualitative identification of the N terminal amino acid in the wall was determined by the method of Gros and Labousse (78).

Reagents:

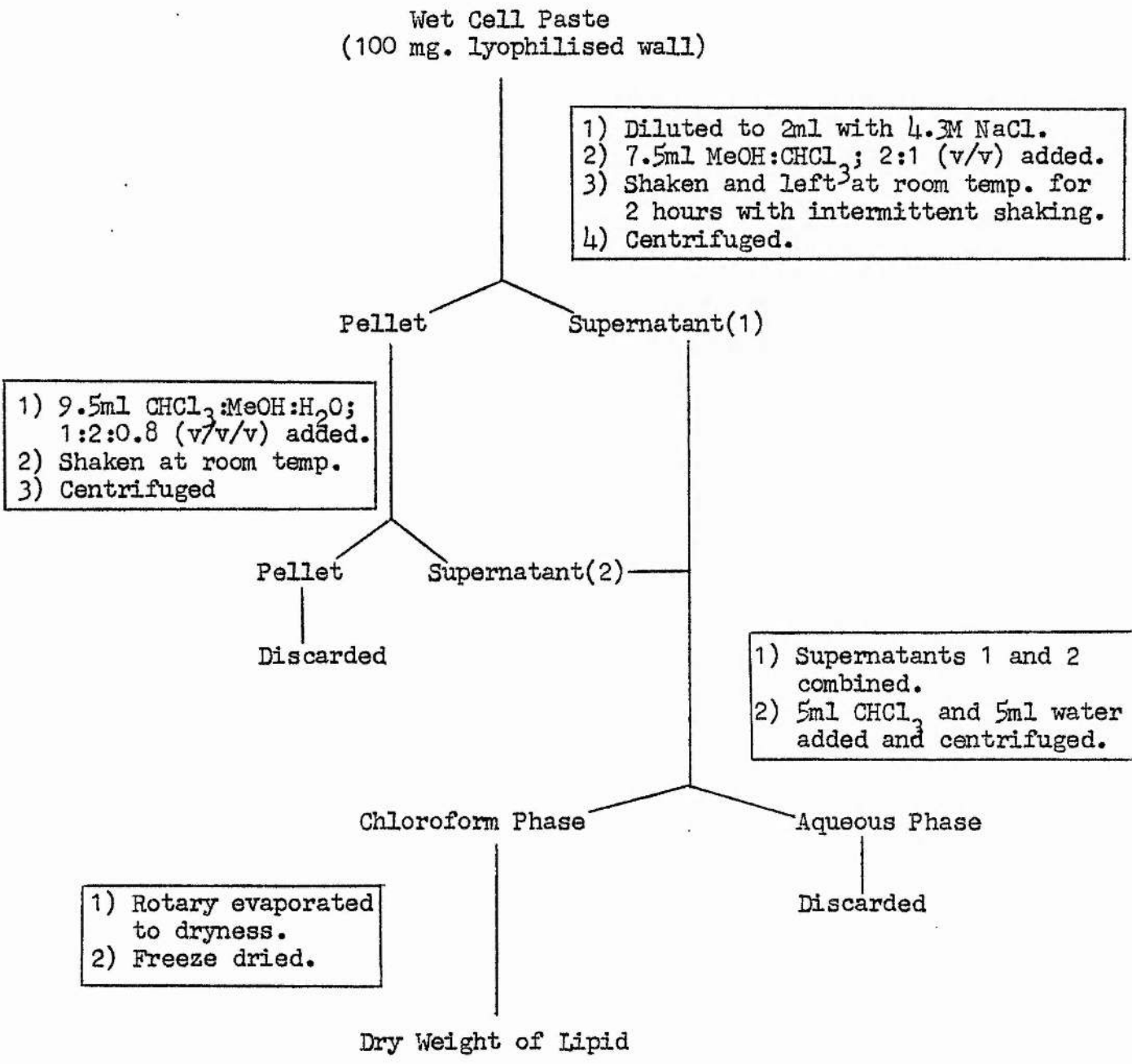
50mM sodium bicarbonate

6N HCL

50% (v/v) aqueous pyridine

Figure M.2

Lipid Extraction.



Dansyl Chloride: 2mg of dansyl chloride was dissolved in dry redistilled acetone and made up to a final volume of 5ml. The solution was stored at -15°C.

Method: freeze dried wall (10mg.) was suspended in 5ml of distilled water by sonication and a 10ul aliquot removed for the assay. 50mM sodium bicarbonate (20ul) was added and the pH checked. It should be greater than 7.5; if not, more bicarbonate was added. Dansyl chloride (30ul) was added and the reaction allowed to proceed at 37°C for 30 minutes. After drying in vacuo, 6N HCl (100ul) was added, the tubes sealed and hydrolysis conducted overnight at 105°C. The excess acid was removed in vacuo and the solid residue extracted twice in the top phase of an ethyl acetate: water, (1:1 v/v) mixture (100ul). The extracts were dried and dissolved in 50% (v/v) aqueous pyridine (15-20ul). The samples were examined by two dimensional TLC on polyamide layer sheets (BDH) in the following solvent systems:

Solvent 1: 1.5% aqueous formic acid (v/v)

Solvent 2: benzene: acetic acid; 9:1 (v/v)

Identification of the amino acids was made by comparison to authentic amino acid standards.

2.3.3.2 Acid Hydrolysis.

Duplicate 10mg. samples of lyophilised wall were hydrolysed in an atmosphere of nitrogen at a concentration of 2mg/ml in 6N HCl at 105°C for 2,4,8,12,24 and 48 hours. The hydrolysates were filtered to remove the 'humin' and repeatedly washed and dried in vacuo until neutral to remove the excess acid. The samples were freeze dried and suspended in distilled water to a final volume of 5ml for analysis.

2.3.3.3 Quantitative Ninhydrin Estimation.

The Moore and Stein method (79) was used to determine the level of the ninhydrin-positive components in the hydrolysates.

Range: 0-50ug/ml

Reagents:

Acetate Buffer pH 5.5: 544g of sodium acetate was dissolved in 400ml of warm water. After cooling, 100ml glacial acetic acid was added and the final volume made up to 1 litre.

Ninhydrin: 1g of ninhydrin and 150mg of hydantoin were dissolved in 37.5ml of methoxyethanol and 2.5ml of acetate buffer.

Samples: the hydrolysates were diluted in distilled water to give a final concentration of 200ug hydrolysed wall material per ml for the assay.

Method: ninhydrin reagent (1ml) was added to the sample (1ml) containing between 0-50ug of ninhydrin-positive material, and thoroughly mixed to ensure complete oxidation. The tubes were capped, placed in a boiling water bath for 15 minutes, cooled and diluted with 50% (v/v) ethanol (5ml). After thorough mixing for 30 seconds, the absorbance of the purple colour was measured at 570nm against a distilled water-reagent blank. A standard calibration curve was prepared using leucine as standard.

2.3.3.4 Paper Chromatography.

This technique was used to provide a qualitative identification of the amino acids and amino sugars in the hydrolysates. It was conducted by one dimensional descending paper chromatography in the machine direction on Whatman 3MM paper at room temperature. Identification of the components was made by comparison to authentic amino acid and amino sugar standards.

The following solvent systems were used:

I) n butanol: pyridine: water; 6:4:3; (v/v/v) 1x30 hours

II) n butanol: acetic acid: water; 5:1:2; (v/v/v) 1x24 hours

III) isopropanol: acetic acid: water; 75:10:15; (v/v/v) 1x24 hours

Sample: 100ug of hydrolysed wall material was applied to the paper for analysis.

Detection Reagent: 0.25% (w/v) ninhydrin was dissolved in acetone: collidine: water; 95:1:5; (v/v/v). The chromatogram was dipped through the detection reagent, dried and heated at 100°C for five minutes to develop the colour.

This reagent produced characteristic colours for certain of the amino acids and stained the amino sugars a light green. This allowed identification on the basis of both Rala values and colour.

2.3.3.5 Ion Exchange Chromatography.

Quantitation and additional qualitative identification of the amino acids in the hydrolysates were obtained from analysis on a Locarte Automatic Amino Acid Analyser, using correction values from and by comparison to authentic amino acid standards.

Sample: 1mg. of hydrolysed wall material was applied to the analyser.

2.3.4 Carbohydrate Analysis.

2.3.4.1 Acid Hydrolysis.

Duplicate samples of lyophilised wall (10mg) were hydrolysed in an atmosphere of nitrogen at a concentration of 2mg/ml in 0.5N HCl at 105°C for 4,8,12,18,24, and 48 hours. The hydrolysates were filtered to remove the 'humin', repeatedly washed in distilled water and dried in vacuo until neutral to remove the excess acid. The samples were freeze dried and

resuspended in distilled water to a final volume of 5ml for analysis.

2.3.4.2 Phenol-Sulphuric Acid Colourimetric Test.

This method (80) was used to determine the total carbohydrate content of the hydrolysates.

Range: 0-100ug/ml total carbohydrate (measured as glucose).

Reagents:

Phenol (5%): 50g of redistilled phenol was dissolved in distilled water and the final volume made up to 1 litre.

Sulphuric Acid: 96% Reagent Grade

Sample: the hydrolysates were diluted with distilled water to a final concentration of 100ug hydrolysed wall material/ml for the assay.

Method: 5% phenol (1ml) and aqueous sample (1ml), containing between 10-70ug of total carbohydrate, were thoroughly mixed. Sulphuric acid (5ml) was rapidly added from a fast flowing pipette so that the stream hit the surface of the liquid directly to produce good mixing and even heat distribution each tube being shaken during the operation. After standing at room temperature for 10 minutes, the tubes were shaken and incubated at 25-30°C for 20 minutes. The absorption of the yellow-orange colour was measured at 490nm against a distilled water-reagent blank.

A standard calibration curve was prepared using D glucose.

2.3.4.3 Total Reducing Substances.

The release of reducing groups by acid hydrolysis was measured by the Park and Johnson method (81).

Range: 1-9ug reducing substances (measured as glucose).

Reagents:

Carbonate-Cyanide Solution: 5.3g sodium carbonate and 0.65g of potassium cyanide were dissolved in distilled water to a final volume of 1 litre.

Ferricyanide Solution: 0.5g potassium ferricyanide was dissolved in distilled water and the final volume made up to 1 litre. The solution was stored in a brown bottle.

Ferric Ion Solution: 1.5g ferric ammonium sulphate and 1g Dupanol were dissolved in 0.025M sulphuric acid to a final volume of 1 litre.

Sample: the hydrolysates were diluted in distilled water to a final concentration of 20ug wall/ml for the test.

Method: 1ml of carbonate-cyanide reagent, 1ml of ferricyanide solution and 1ml of aqueous sample were thoroughly mixed. After heating in a boiling water bath for 15 minutes, the samples were cooled and 5ml of ferric ion solution was added. The blue colour was read at 690nm after standing for 15 minutes to allow complete colour development.

A calibration curve was prepared with D glucose and the samples were read against a distilled water-reagent blank.

2.3.4.4 Paper Chromatography.

A qualitative identification of the carbohydrate components in the wall was achieved by this technique by comparison to authentic standards. It was conducted by one dimensional descending paper chromatography on Whatman 3MM paper in the machine direction at room temperature in:

n butanol: pyridine: water; 6:4:3; (v/v/v). 1x24 hours.

Detection Reagent - Ammoniacal Silver Nitrate.

0.1ml of a saturated silver nitrate solution was added to 19.9ml of acetone, and water was added dropwise until the white precipitate was dissolved.

The chromatogram was sprayed with this solution and dried in a stream of warm air. The paper was then sprayed with 0.5M sodium hydroxide in ethanol, and the excess silver oxide was removed by immersion in 6M ammonium hydroxide for a few moments. The chromatogram was washed in a continuous flow of water for 1 hour to remove the excess ammonia, and dried. The sugar residues developed as brown spots on a light background.

Sample: 100ug of hydrolysed wall material was applied to the paper for analysis.

2.3.4.5 Gas Liquid Chromatography.

The quantitative amount of each of the carbohydrate components present in the acid hydrolysates was determined by gas chromatography (82).

Reagents:

Trimethylsilylating Agent (TMS): a mixture of pyridine, trimethylchlorosilane and hexamethyldisilazane (5:1:1; v/v/v) was prepared freshly every 3 days.

Sample: 0.1ml of hydrolysate containing 200ug wall material.

Method: 0.05umoles of mannitol was added to 0.1ml of the hydrolysate to act as an internal standard. The samples were dried in a vacuum desiccator over calcium oxide for at least 12 hours. TMS (0.05ml) was added and, after thorough trituration, the tube was stoppered and allowed to stand at room temperature for at least 30 minutes. A sample (1-5ul) was injected into the gas chromatograph under the following conditions:

| | |
|---|---|
| Gas chromatograph | Pye 104 Series Gas Chromatograph |
| Column | Glass columns, internal diameter 0.25in., 9ft. in length. |
| Column Packing | 3% SE-30 on Diatomite CQ support. |

| | |
|---|---|
| Injection Volume | 1-5ul |
| Detector Oven Temperature | 250°C |
| Attenuation | $2x10^2$ |
| Nitrogen Flow | 45ml/min. |
| Hydrogen Flow | 45ml/min. |
| Air Flow | 600ml/min. |
| Chart Speed | 5mm/min. |

Temperature Programme.

| | |
|---|---|
| Starting Temperature | 140°C |
| Hold Time | 1min. |
| Increment | 0.5°C/min. |
| Finishing Temperature | 200°C |

Calculation of Results.

Quantitative results were obtained by the technique of internal standardisation in which a known weight of mannitol, the internal standard, was added to each sample before analysis. The ratio of the peak area of each component to that of mannitol was then a direct measure of the amount of that component in the original sample. However, different classes of compounds produce different responses in the detection system and it was necessary to determine the 'response factor' for each component.

Method.

A standard solution containing the monosaccharides of interest (glucose, galactose, mannose) was pipetted into ampoules to give a final concentration of 0.025-0.1umole for each monosaccharide. 0.05umole of mannitol was added and after thorough drying, the samples were subjected to the procedure

described above. Peak areas were measured by triangulation.

The total peak area for each monosaccharide was obtained by summing the peak areas of the various isomers. The ratio of the total peak area to the peak area of mannitol, the internal standard, (Total Peak Area Ratio) was plotted against the mole ratio of the monosaccharide to mannitol. The Molar Relative Response Factor was then given by the slope of the graph, and the micromoles of each monosaccharide was calculated from the equation:

$$\text{umole monosaccharide} = \frac{(\text{total peak area ratio})(\text{umole int. std.})}{(\text{molar relative response factor})}$$

2.3.5 Amino Sugar Analysis.

2.3.5.1 Acid Hydrolysis.

Duplicate 10mg samples of lyophilised wall were hydrolysed in an atmosphere of nitrogen at a concentration of 2mg/ml in 4N HCl at 105°C for 2,4,8,12,24 and 48 hours. The hydrolysates were filtered to remove the 'humin' and repeatedly washed with distilled water and dried to remove the excess acid until neutral. The samples were freeze dried and suspended in distilled water to a final volume of 5ml for analysis.

2.3.5.2 Hexosamine Determination.

The total quantity of hexosamine present in each of the hydrolysates was determined using a modified Elson-Morgan reaction (83).

Reagents:

Saturated sodium bicarbonate solution.

5% (v/v) Acetic anhydride solution: prepared freshly immediately prior to use.

5% (w/v) Sodium tetraborate solution.

Colour Reagent: 16g p-dimethylaminobenzaldehyde was dissolved in glacial acetic acid to a final volume of 95ml, and 5ml of concentrated HCl was added. Two volumes of this stock solution were diluted with five volumes of glacial acetic acid for the assay.

Sample: 0.3ml of hydrolysate containing 600ug of wall was used in the assay.

Method: 0.3ml of aqueous sample containing 0-60ug of total hexosamine was added to 0.1ml of saturated sodium bicarbonate and the solution thoroughly mixed. After standing at room temperature for 10 minutes to allow N-acetylation, the sample was placed in a boiling water bath for exactly three minutes and then cooled. Sodium tetraborate (0.5ml) was added, mixed and the sample again heated in a boiling water bath for 7 minutes. Colour reagent (7ml) was added and, after mixing, the sample was incubated at 37°C for 20 minutes. The red-yellow colour was read against a distilled water-reagent blank at 585nm.

A standard calibration curve was prepared using glucosamine HCl as a standard.

2.3.5.3 Gas Liquid Chromatography.

Identification and quantitation of each hexosamine component in the hydrolysates was attempted by this method (82).

Direct silylation, the method employed in the analysis of the carbohydrate components, did not produce a sufficiently effective resolution of the hexosamines. Consequently, the hexosamines were modified to a more volatile form prior to silylation.

Reagents:

TMS: see section 2.3.4.5

Gas chromatography conditions: see section 2.3.4.5

Methanolic HCl: to 5 volumes of dry redistilled methanol was added 1 volume of acetyl chloride: this reagent was prepared immediately prior to use.
Method: 0.05umole of mannitol as an internal standard was added to 0.1ml of the hydrolysate (200ug of wall) and dried in a vacuum oven overnight at 30°C. Methanolic HCl (0.5ml) was added to each ampoule and a steady stream of nitrogen bubbled in for 30 seconds. The ampoules were immediately sealed and placed in an oven at 90°C for 24 hours, at which time the acid was neutralised by the addition of solid silver carbonate. Acetic anhydride (0.05ml) was added and the ampoule covered with parafilm (Gallenkemp, London). After standing at room temperature for at least 6 hours and, following thorough trituration, each ampoule was centrifuged. The supernatant was transferred to another ampoule and the trituration and centrifugation steps were repeated three times. The pooled supernatants were evaporated to dryness and placed in a vacuum desiccator over calcium oxide for at least 12 hours. TMS (0.05ml) was added to the dried material and, after thorough trituration, the ampoule was stoppered and allowed to stand at room temperature for at least 30 minutes. After centrifugation, 1-5ul of the supernatant was injected into the gas chromatograph for analysis.

The results were calculated as before (section 2.3.4.5).

## 2.4 Fractionation Studies on the Wall.

The following studies were undertaken in an attempt to resolve the total wall material into any major polymeric components, if indeed the wall is heterogeneous. Although it was recognised that the more usual wall components, e.g. teichoic acids, might not be present, the accepted methods for extracting and isolating the more conventional wall polymers were used in the hope that analogous structures in the halophiles might behave in a

similar manner and be thus fractionated and identified.

2.4.1 Extraction Techniques.

2.4.1.1 Teichoic Acids.

TCA Extraction at 0°C: an outline of the method (84) is shown in Figure M.3.

TCA Extraction at 35°C: this procedure (85), which reportedly yields higher levels of teichoic acids extracted although the product is more degraded, was used. The method is shown in Figure M.4.

TCA Extraction at 60°C: this method was conducted on TCA extracted walls at 35°C to attempt further separation of the wall layers. The procedure (11) is outlined in Figure M.5.

2.4.1.2 Hot Formamide Extraction.

Extraction with hot formamide, although a somewhat drastic procedure, can be utilised to solubilise the polysaccharide containing material from microbial wall. The peptidoglycan behaves as a large, highly crossed-linked macromolecule and remains insoluble to this treatment (86). The method (87) is shown in Figure M.6.

2.4.1.3 Alkali Extraction.

As there was evidence for the presence of both glucose and mannose in the wall, this method (88) was used since it has been reported to separate glucan and mannan polymers. An outline is shown in Figure M.7.

2.4.1.4 Acetic Acid Extraction.

This extraction is another procedure that separates the polysaccharide fraction from the wall. The method (89) is shown in Figure M.8.

Figure M.3

TCA Extraction of Wall at 0°C.

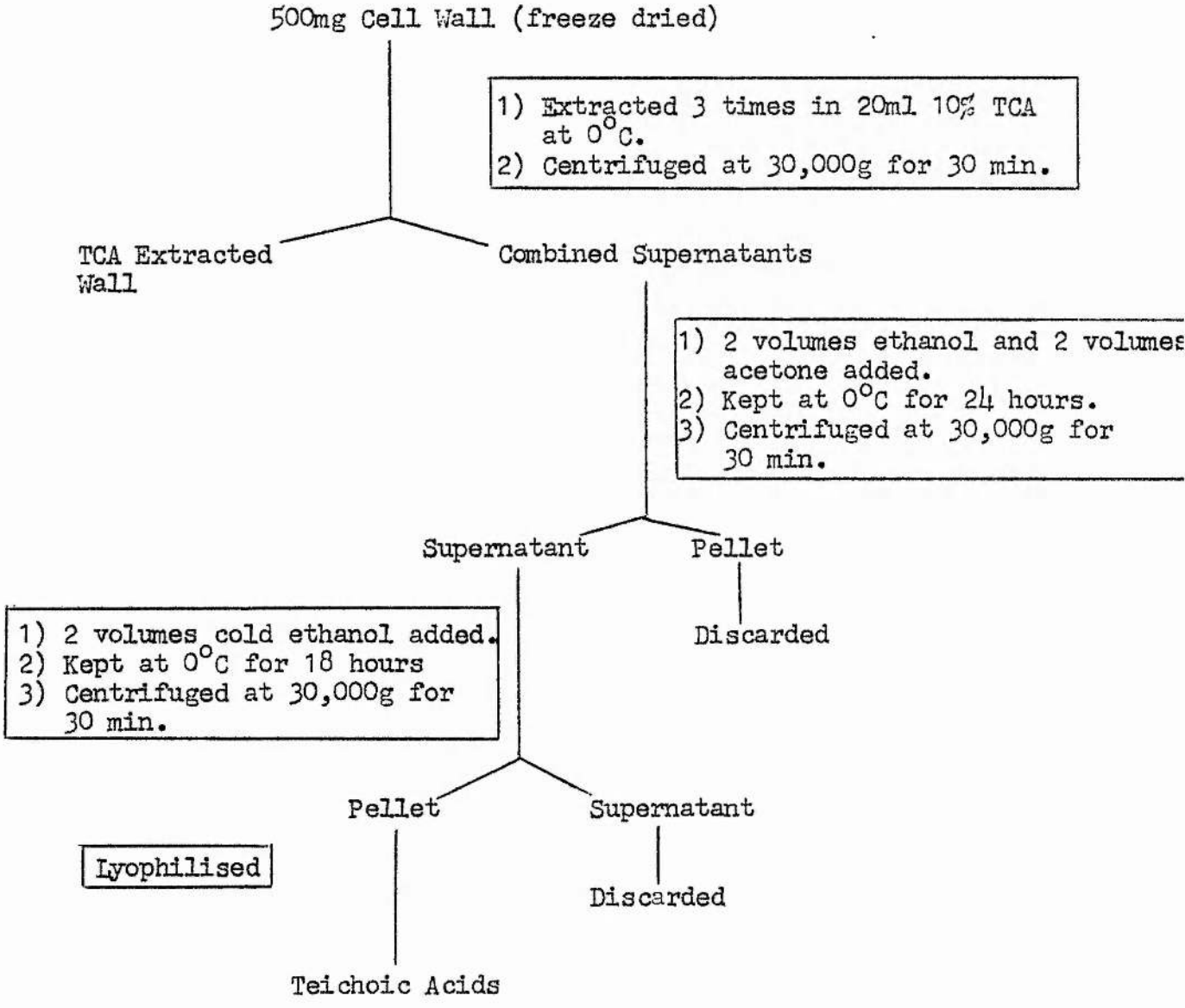


Figure M.4

TCA Extraction at 35°C.

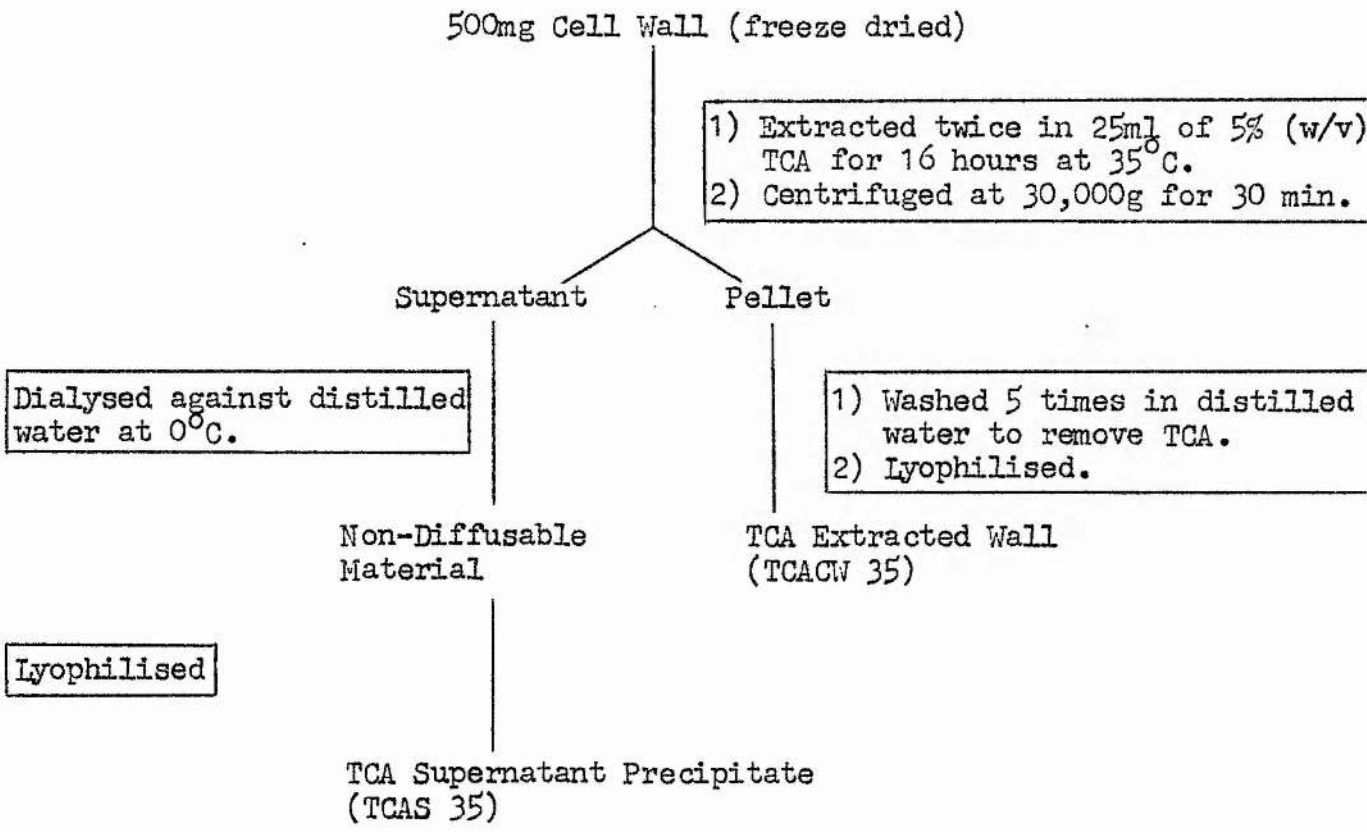


Figure M.5

TCA Extraction at 60°C.

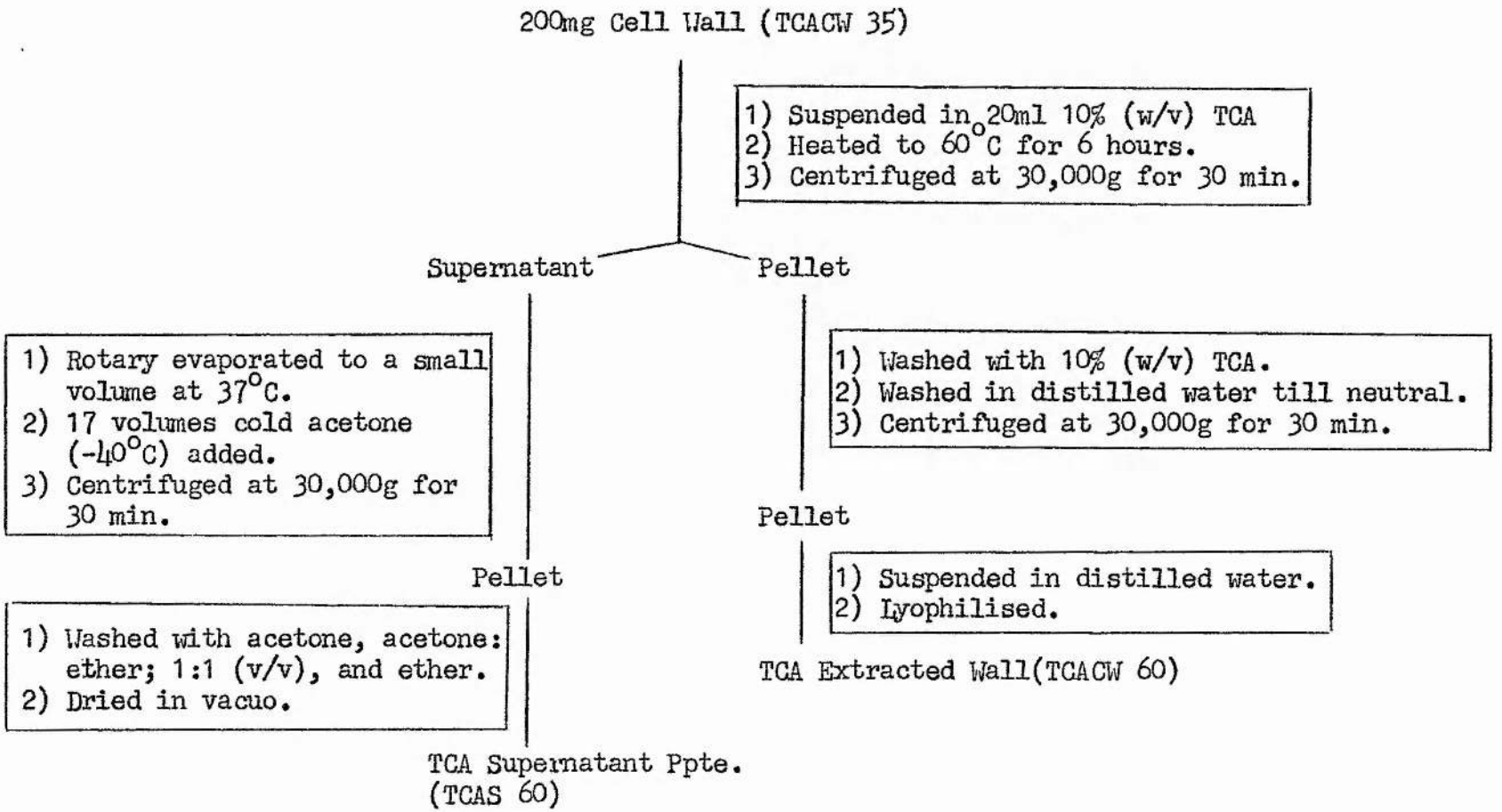


Figure M.6

Hot Formamide Extraction.

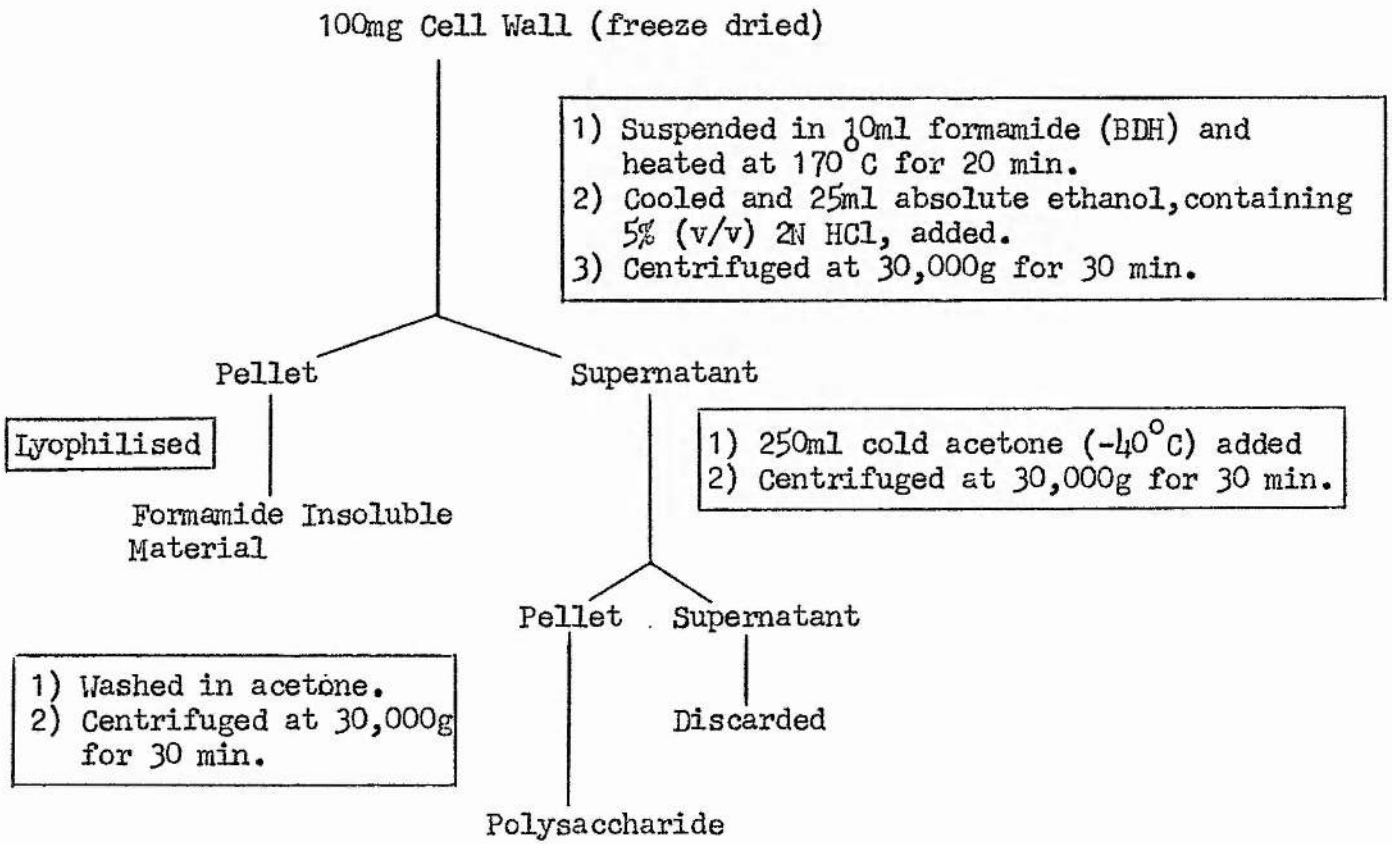


Figure M.7

Alkali Extraction.

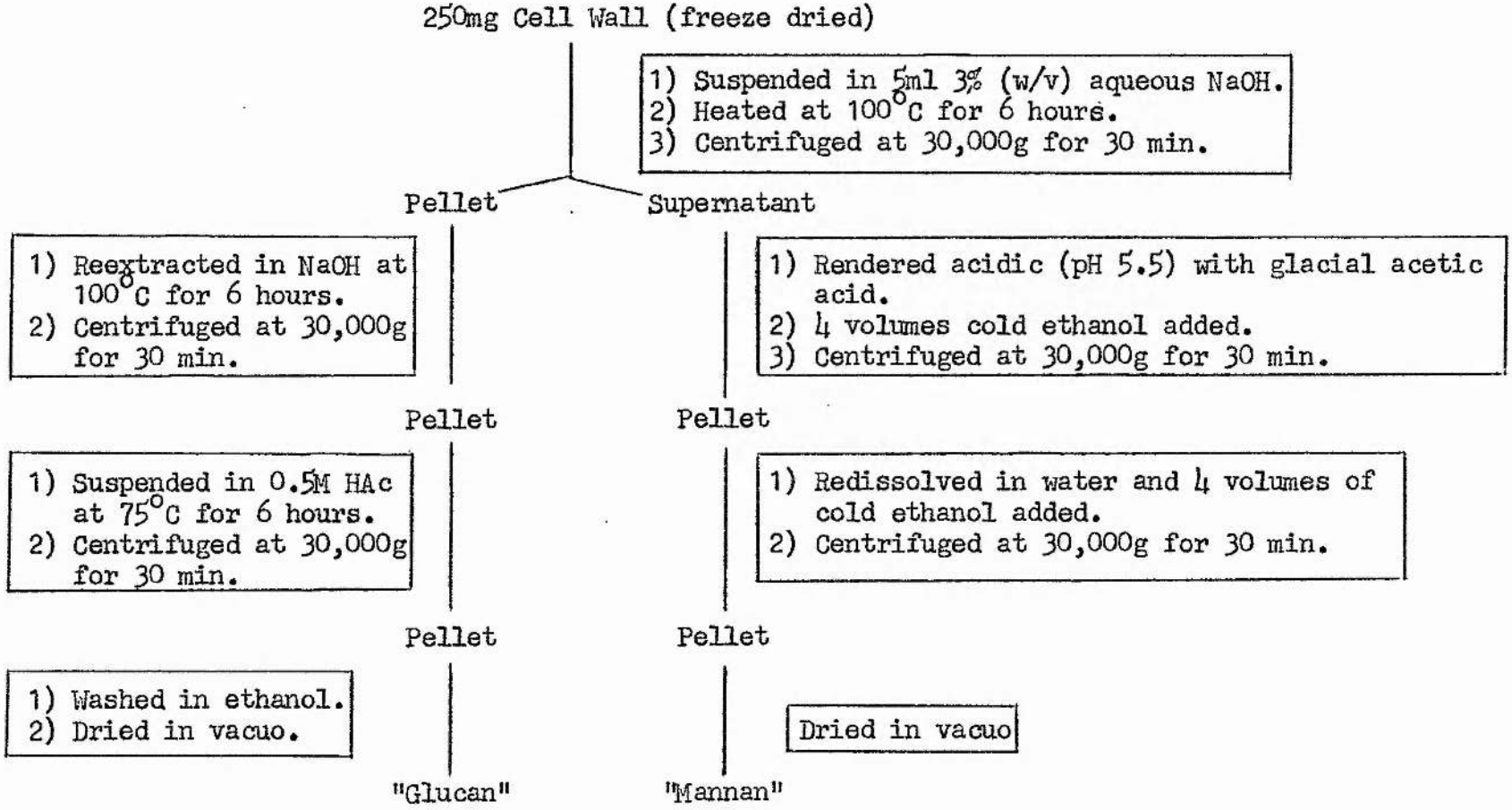
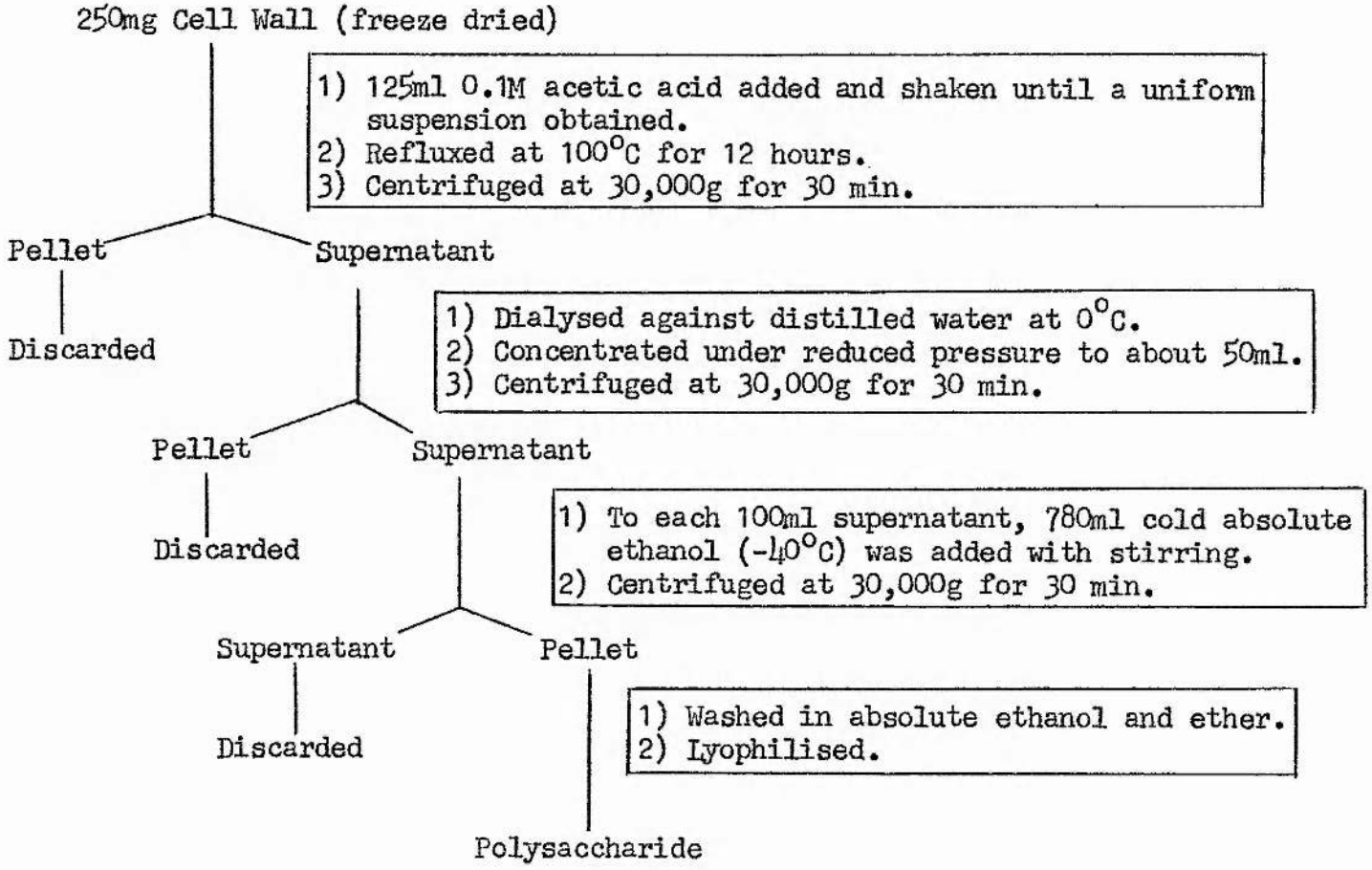


Figure M.8

Acetic Acid Extraction.



## 2.4.2 Analysis of the Fractionated Material.

### 2.4.2.1 Amino Acid Analysis.

Samples of each of the fractionated precipitates were hydrolysed for 12 hours as described in Section 2.3.3.2. Qualitative identification and quantitative analysis of the amino acids were achieved by ion exchange chromatography, Section 2.3.3.5, of the hydrolysates.

### 2.4.2.2 Carbohydrate Analysis.

Samples of each of the fractionated precipitates were hydrolysed for 18 hours as described in Section 2.3.4.1. The total monosaccharide content of the hydrolysates was evaluated by the Phenol-Sulphuric Acid Test, Section 2.3.4.2. Monosaccharide components present in the hydrolysates were determined by GLC, Section 2.3.4.5.

## 2.5 Antibiotics.

The antibiotics listed below, which are reported as exerting their influence at the level of wall synthesis, were used in this investigation.

D cycloserine (Lilly)
Vancomycin (Lilly)
Penicillin G.(Glaxo)
Bacitracin A (Sigma)
Novobiocin (Sigma)

### 2.5.1 Antibiotic Effect on S. marina Growth.

An inoculum of S. marina was grown to mid-logarithmic phase and 5ml aliquots were transferred to 100ml of Dundas medium. The cultures were grown to early exponential phase and the antibiotics added to give final concentrations of:

| | |
|---|---|
| D cycloserine: | 16,64 and 256ug/ml |
| Vancomycin: | 256ug/ml |
| Novobiocin: | 16,64 and 256ug/ml |
| Bacitracin A: | 16,64 and 256ug/ml |
| Penicillin G: | 256ug/ml |

The growth of the bacteria was recorded turbidimetrically at 610nm and compared to a control containing no antibiotic. In late logarithmic phase, 10ml aliquots were removed from each culture and prepared for electron microscopy, Section 2.6.

2.5.2 Detection of Antibiotic in Dundas Medium.

As bacitracin A and novobiocin inhibited the growth of S. marina, further investigation was only required on D-cycloserine, vancomycin and penicillin G to determine whether:

1) these antibiotics were taken up into the cell.

2) these antibiotics retain their activity in Dundas medium.

2.5.2.1 U.V. Scanning Spectroscopy.

To determine whether there were characteristic absorption peaks for the antibiotics in Dundas medium, each was tested by U.V. scanning spectroscopy. As a control, each antibiotic was dissolved in sterile water at the following concentrations:

| | |
|---|---|
| D-cycloserine | 15ug/ml |
| Vancomycin | 20ug/ml |
| Penicillin G. | 30ug/ml |

U.V. profiles were prepared on a Unicam SP 800 Series Spectrophotometer over the range 200-450nm against a sterile water blank. The procedure was

repeated with the same concentration of antibiotic in Dundas medium and the profiles were measured against a Dundas medium blank.

2.5.2.2 Bioassay Technique - Agar Diffusion.

The theory and precautions for this technique have been fully reported (90).

Test Organisms.

The test organism, Staphylococcus aureus (NCIB 8625) and the inhibitory range of the antibiotics, were obtained from the National Collection of Industrial Bacteria.

| Antibiotic | Test Organism | Range |
|---|---|---|
| Vancomycin | S. aureus (NCIB 8625) | 20-200 units |
| Penicillin G. | S. aureus (NCIB 8625) | 0.5-4 units |

Reagents:

Plates: 30ml of nutrient agar (Oxoid)

Inoculum: 0.1ml of S. aureus (NCIB 8625), grown in nutrient broth (Oxoid) in a bijou bottle overnight at 34°C.

Sterile Pads: Oxoid.

Method: the antibiotics were dissolved in sterile water to produce concentrations, in 10ul, in the range of sensitivity of the test organism shown above. S. aureus (NCIB 8625), 0.1ml, was uniformly flood cultured on the nutrient agar plate and a sterile pad placed in the centre of the plate. The antibiotic solution (10ul) was pipetted on the sterile pad and the plate incubated overnight at 34°C. The diameter of the zone of inhibition was measured in millimeters and the logarithm of the antibiotic concentration plotted against the diameter squared. Each bioassay was conducted in

duplicate and a control of sterile water was also prepared.

The procedure was repeated exactly as described, but with the antibiotics prepared in Dundas medium and the control of Dundas medium.

2.5.2.3 Detection of Cycloserine.

This colourimetric test (91) is designed to quantitatively determine the concentration of cycloserine in the growth medium.

Reagents:

1M Acetic acid

Colour Reagent: sodium nitroprusside, 0.5% aqueous solution, was prepared freshly every two weeks and stored in a brown glass bottle. Equal volumes of sodium nitroprusside and 1M NaOH were mixed immediately prior to use.

Method: 3ml of 1M acetic acid were added to 1ml of sample containing 0-100ug of cycloserine and thoroughly mixed. 1ml of colour reagent was added and, after mixing, the solution was left at room temperature for 10 minutes. The intensity of the blue colour was measured at 625nm against a medium-reagent blank.

Standard calibration curves were prepared in 0.1M NaOH and Dundas medium to ensure that the salt did not interfere with the colour reaction.

2.5.3 S. marina-active uptake of antibiotics and stability in Dundas medium.

An inoculum of S. marina was grown to mid logarithmic phase and 5ml aliquots were transferred to 100ml of Dundas medium. The cultures were grown to early logarithmic phase and cycloserine, vancomycin and penicillin G were added to give final concentrations of:

| | |
|---|---|
| Cycloserine | 100ug/ml |
| Vancomycin | 2,500 units/ml |

Penicillin G 100 units/ml

A control flask, containing no antibiotic, was set up at the same time. Control flasks, containing the same concentration of antibiotics as above, but with no bacteria present, were also prepared. The growth of the bacteria was recorded turbidimetrically at 610nm and compared to the control with no antibiotic. At the same time an aliquot of the culture was centrifuged to remove the bacteria and the supernatant was tested for the level of antibiotic activity by:

Cycloserine - colour test (Section 2.5.2.3)

Vancomycin - bioassay technique (Section 2.5.2.2)

Penicillin G - bioassay technique (Section 2.5.2.2)

The antibiotic concentration was compared to the level in the control flasks, containing no bacteria, all measurements being made in duplicate.

## 2.6 Electron Microscopy.

The effectiveness of the wall isolation procedure was investigated by viewing sections prepared from various stages of the isolation method in the electron microscope. The effect of the antibiotics on the wall (Section 2.5.1) was also viewed in the electron microscope.

The samples were processed for electron microscopy as shown in Figures M. 9 (Method 1) and M. 10 (Method 2). The sections were cut on an LKB Ultratome I ultramicrotome, collected on carbon-coated grids and viewed in an AE1 EM6B electron microscope.

### 2.6.1 Preparation of Sections.

Method 1.

Reagents:

Fixative Buffer Solution: A buffer solution containing 25ml 0.3M sodium cacodylate (6.42g in 100ml) and 1.4ml of 0.3M HCl was made up in 100ml distilled water at pH 7.4. The Fixative Buffer Solution of the following composition was prepared in 100ml of this buffer: magnesium sulphate $7H_2O$, 1%; calcium chloride $6H_2O$, 0.02%; potassium chloride, 0.5%; sodium chloride, 25%; potassium cyanide, 0.26%: (all % were w/v).

Fixative: 0.1g of Osmium tetroxide was dissolved in 10ml of Fixative Buffer Solution.

Tryptone Medium: Bacto-tryptone, 1%; magnesium sulphate $7H_2O$, 1%; calcium chloride $6H_2O$, 0.02%; potassium chloride, 0.5%; sodium chloride, 25%, were dissolved in distilled water. All % were expressed as w/v.

Agar: 2% Agar No. 3 (Oxoid) was prepared in Fixative Buffer Solution.

Uranyl Acetate: 0.5g uranyl acetate was shaken overnight at room temperature in Fixative Buffer Solution and filtered. This reagent is essential for membrane preservation.

Resin: TAAB resin (10ml), D.D.S.A. (9.5ml) and M.N.A. (0.5ml) were mixed in a universal bottle on a roller shaker for one hour. The DMP-30 (0.4ml) was added and the resin mixed for a further 30 minutes.

Abbreviations: D.D.S.A. - Dodecenyl Succinic Anhydride
M.N.A. - Methyl Nadic Anhydride
DMP-30 - Trimethylaminomethylphenol.

Method 2.

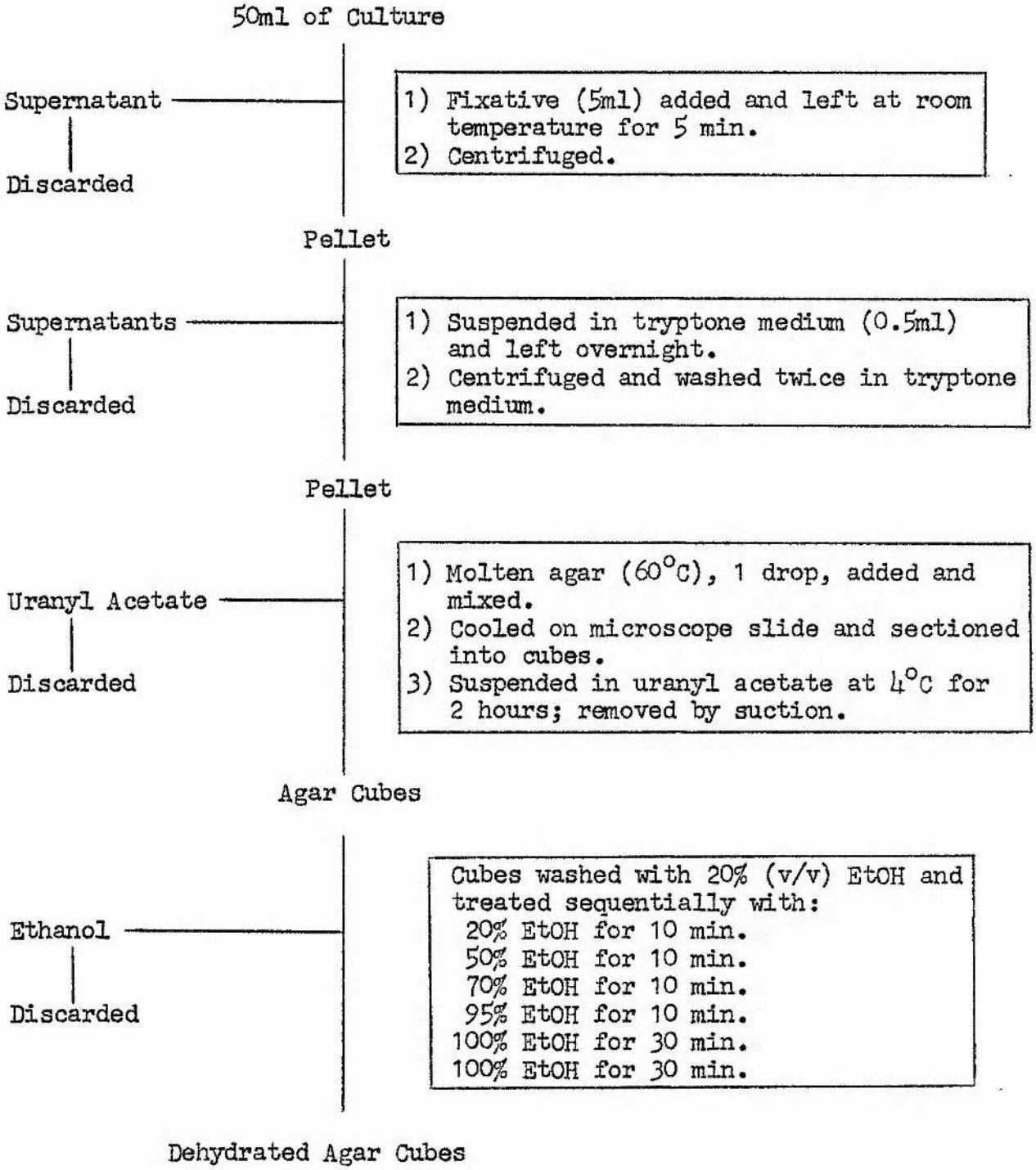
Reagents:

Dundas Basal Salts (DBS): a solution of DBS of the following composition was prepared: sodium chloride, 25%; magnesium sulphate $7H_2O$, 1%; calcium chloride $6H_2O$, 0.02%; potassium chloride, 0.5%; (all % were w/v).

Figure M.9

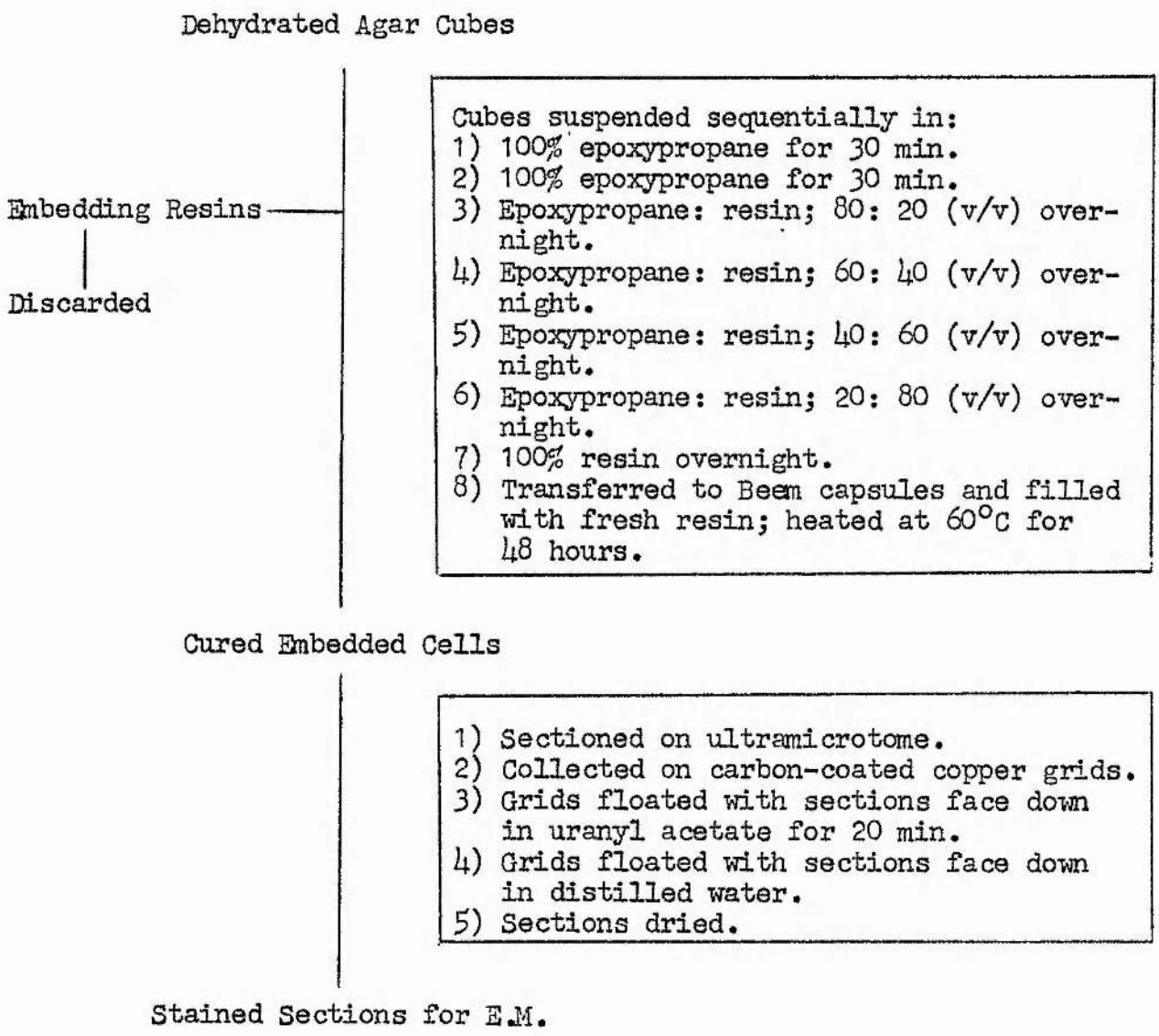
Preparation of Sections for Electron Microscopy.

Method 1.




PTO

Figure M.9 (ctd)



Fixative: 2% osmium tetroxide (5ml), DBS (5ml) and 4M KCN (0.1ml of a solution of 2.6g of KCN dissolved in 10ml of distilled water and filtered) were mixed and the pH of the final solution adjusted to pH 6 with 1M HCl.

Tryptone: bacto-tryptone (1g) and sodium chloride (0.5g) were dissolved and made up to a final volume of 100ml in distilled water containing a few drops of formaldehyde.

Agar: 2% Agar No. 3 (Oxoid) was prepared in DBS.

Uranyl Acetate: 0.5% (w/v) uranyl acetate was prepared in DBS. This reagent is essential for membrane preservation.

Dry Acetone: redistilled acetone was dried over anhydrous sodium sulphate.

Resins: araldite of the following composition was prepared by heating the components to 60°C to mix them.

Araldite: resin: hardener: plasticiser; 10:10:1; (v/v/v)

Resin: CY212

Hardener: DDSA964

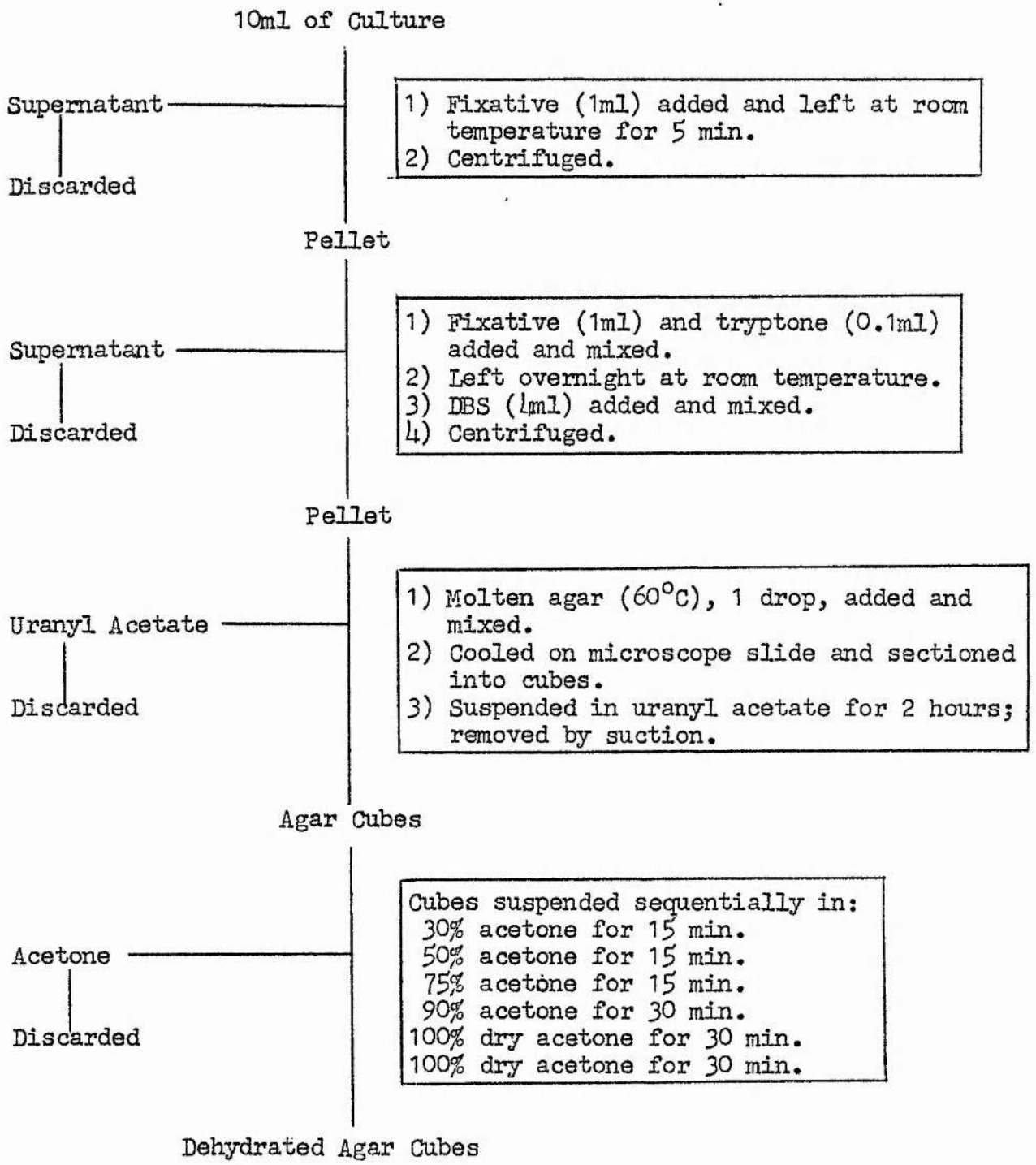
Plasticiser: Dibutyl Phthalate

Accelerator: DMP-30.

Figure M.10

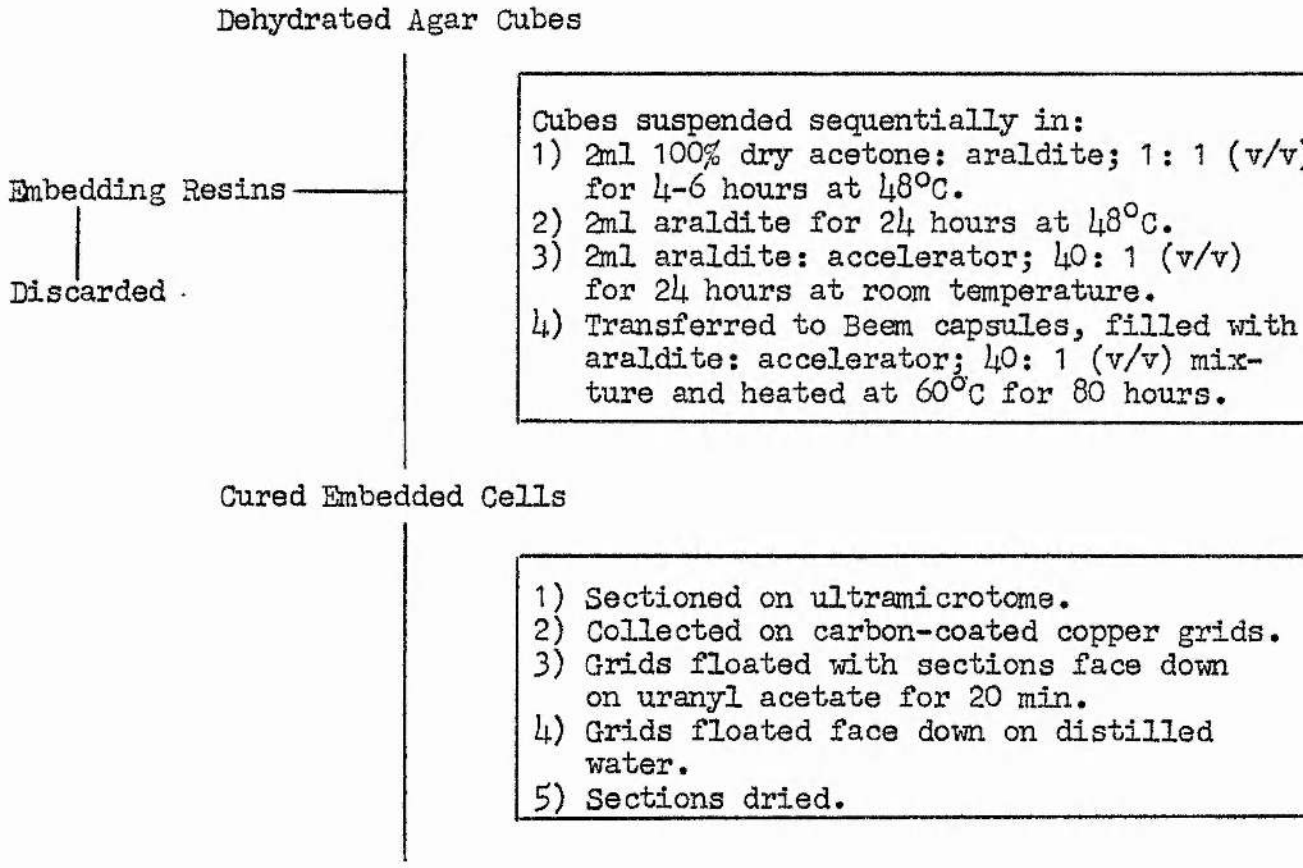
Preparation of Sections for Electron Microscopy.

Method 2.




PTO.

Figure M.10 (ctd)



Results.

## 3.1 Growth Experiments.

### 3.1.1 Halococcal Growth Comparison.

The halococci divide very slowly even under the most favourable of conditions. In terms of ease of culture and large scale batch fermentation and production of bacterial cells, the most appropriate microorganism for the research was the one that had the minimum lag phase, the shortest doubling time and the highest yield (measured as dry weight of cells per litre).

A comparison of these parameters for the available halococci was obtained and the results are shown in Table TR. 1. From consideration of these variables, S. marina (NCMB 778) was chosen for the research.

### 3.1.2 Salt Tolerance.

The effect of different sodium chloride concentrations in the medium on the growth of S. marina were recorded using the same parameters as Section 3.1.1, and the results are shown in Table TR. 2.

Below 15% (w/v) sodium chloride in the medium there was no significant growth. As the lag phase and doubling time for 20 and 25% (w/v) salt were identical, the choice of 25% (w/v) salt in the medium was based on the fractionally higher yield produced.

### 3.1.3 Turbidity-Dry Weight Relationship.

The relationship between turbidimetric values, measured at 610nm, and the corresponding dry weight of cells is shown in Figure R. 1. The linear relationship between these two variables is only valid up to an O.D. of 0.4 units. Consequently, all measurements were made in the range 0-0.4 units and all bacterial concentrations outwith this range were suitably

Table TR. 1

Halococcal Growth Comparison.

| Organism | Lag Phase (hr) | D.T. (hr)* | Yield** |
|---|---|---|---|
| NCMB 757 | 43 | 27.5 | 1.45 |
| NCMB 761 | 58 | 23.5 | 1.6 |
| NCMB 776 | 147 | - | 0.66 |
| NCMB 778 | 51 | 21.5 | 2.31 |
| S. morrhuae | 62 | 33.5 | 1.65 |

*D.T. (Doubling Time): measured as the time taken for an increase in turbidity from 1 to 2 units.

**Yield: expressed as g. dry weight of cells per litre.

Table TR. 2

Salt Tolerance of S. marina.

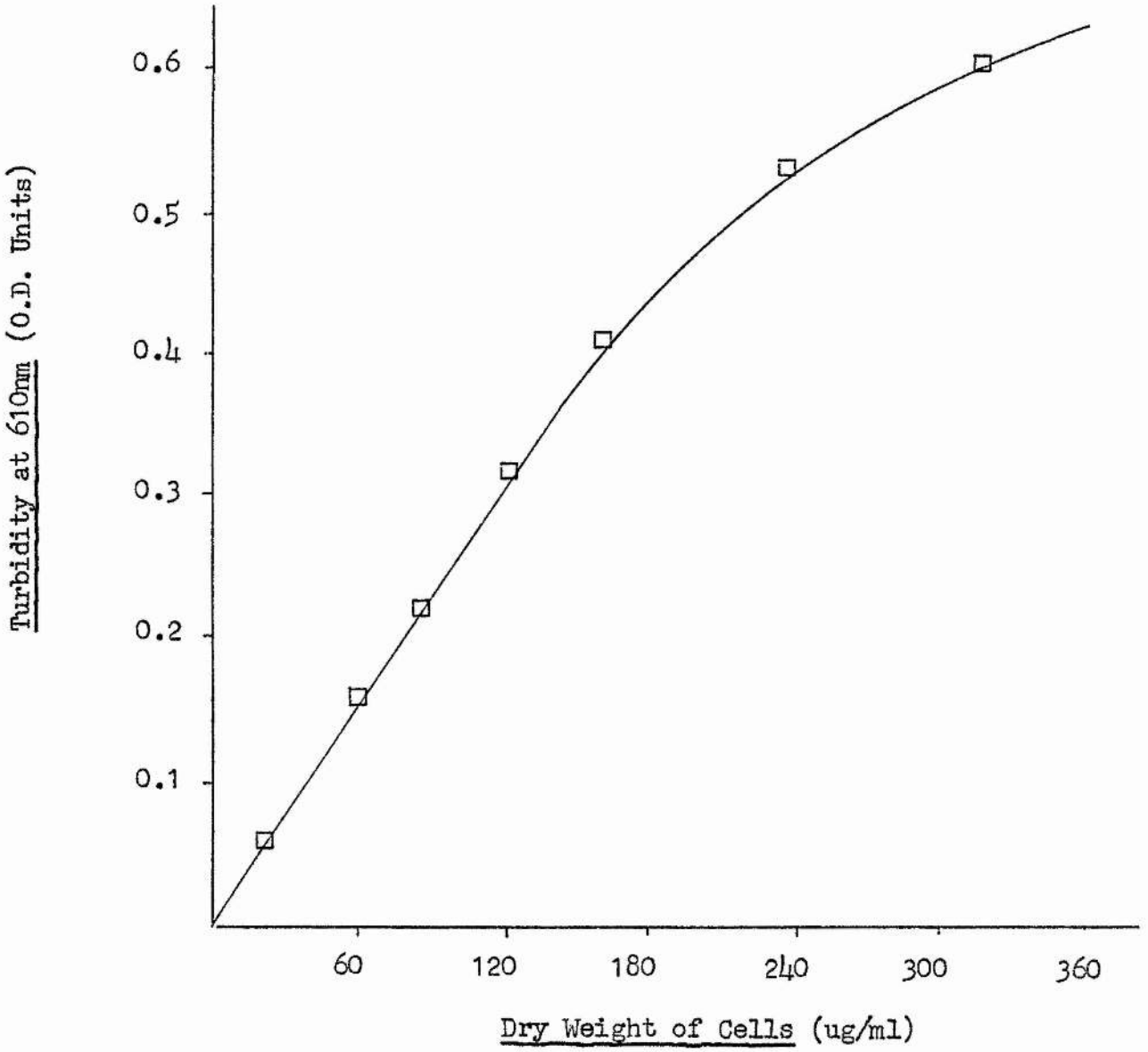
| Salt % (w/v) | Lag Phase (hr) | D.T. (hr)* | Yield** |
|---|---|---|---|
| 0 | - | - | - |
| 10 | - | - | - |
| 15 | 80 | 40 | 0.57 |
| 20 | 68 | 16 | 0.99 |
| 25 | 68 | 16 | 1.04 |

*D.T. (Doubling Time): measured as the time taken for an increase in turbidity from 0.5 to 1.0 units.

**Yield: expressed as g. dry weight of cells per litre.

Figure R. 1

Turbidity - Dry Weight Relationship.



diluted in sterile Dundas medium.

3.2 Cell Wall Isolation.

S. marina cells were grown and harvested (Section 2.1.7) from two runs on the 3x5 litre fermentor. The cells were harvested in mid to late logarithmic phase and combined prior to the wall isolation to average any minor deviations in the wall composition caused by the different fermentor runs.

The effectiveness of the wall isolation procedure, with particular reference to the extent of cell breakage in the Hughes Press and the purity of the isolated wall material, was assessed by electron microscopy (Section 3.6.1), and the results are discussed later.

The pressed cells were tacky, adopting a chewing gum-like texture which was probably due to the release of nucleic acids from the broken cells. The supernatants from the washing of the pressed cells gradually changed from deep red to almost colourless. Although the pressed cells were washed in Dundas Basal Salts (DBS), the deep red colour of the initial supernatant may reflect membrane instability. As potassium is the dominant intracellular cation in the extreme halophiles (20,21,23), washing with a solution with a high concentration of this cation might have been more effective in maintaining membrane stability.

The final wall material was of two types, distinguished by their colour. The less dense, upper portion of the final pellet was white and the lower, denser segment pink. This pink colour is probably due to residual membrane contaminants adhering to the wall even after trypsin treatment. Apart from the N terminal amino acid analysis (Section 3.3.3.1), the two types of wall material were combined prior to analysis.

The yield of S. marina from the fermentor runs and of wall from the

isolation procedure are shown below in Table TR. 3.

Table TR. 3

Yield of Bacteria and Wall from S. marina.

| | |
|---|---|
| Yield of Bacteria (g. dry wt. cells/litre)(1) | 1.782g |
| Yield freeze dried wall (g. fr. dried wall/g. dry bact.) | 0.126g |
| Yield dry wall (g. dry wall/g. dry bact.)(2) | 0.121g |

1) Determined from the turbidity-dry weight relationship (Section 3.1.3)

2) Evaluated from the dry weight determination (Section 3.3.2)

The wall constituted only 12.1% of the dry weight of the cell. However, this is probably a gross underestimate because of the wall losses incurred in the isolation. The exhaustive washings and the removal of the lower portion of the pellet, constituting intact cells, account for some loss of wall material. One other possible source that was investigated, but not reported here, was autolysis (92). The level of autolysin activity, if there was any at all, was very low and autolysis does not, therefore, represent a significant source of wall loss.

## 3.3 Cell Wall Analysis.

### 3.3.1 Lipid Extraction.

The lipid extract from the wall, constituting less than 0.1% of the dry weight of the wall, was red in colour, probably due to the carotenoid content of contaminating residual membrane fragments. However, as it is a very minor contaminant, lipid extraction of the wall material prior to analysis was not deemed necessary.

3.3.2 Dry Weight-Ash Determination.

The moisture and ash content of the freeze dried wall are shown below in Table TR. 4.

Table TR. 4

Dry Weight-Ash Determination of Wall.

| Content | %(w/w) Wall |
|---|---|
| Moisture | 4.3 |
| Ash | 9.2 |
| Organic Material | 86.5 |

The ash content, 9.2% of the dried wall material, is unusually high compared to non-halophilic, Gram-positive micrococci but, with the probability of salt bound to the wall, not unexpected.

All subsequent analytical results will be expressed as functions of the organic content of the wall material.

3.3.3 Amino Acid Analysis.

3.3.3.1 N Terminal Analysis.

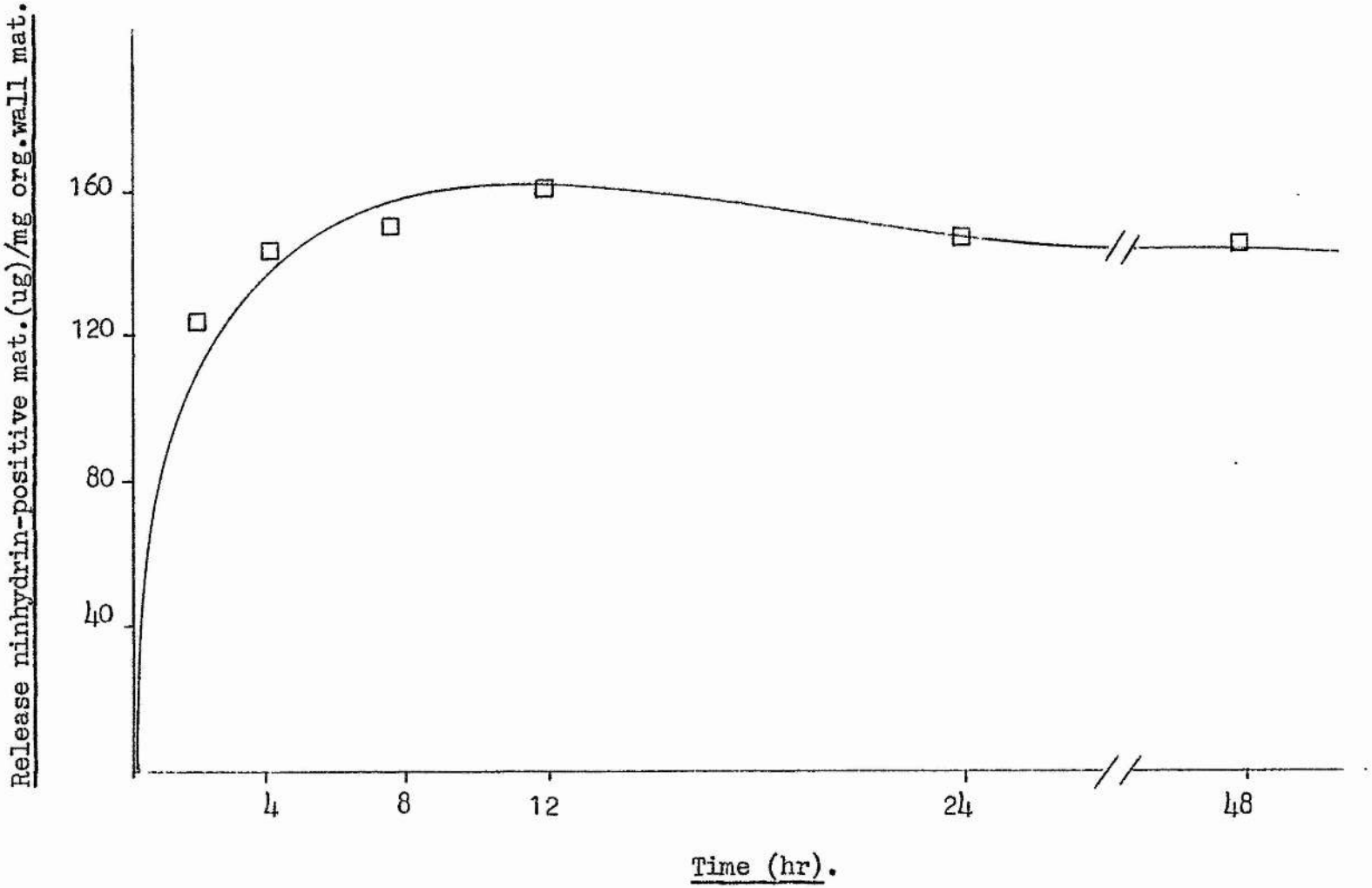
The major N terminal amino acid of both the white and the pink cell wall material was identified as alanine. In the pink wall material, however, a trace of glycine was also present.

3.3.3.2 Estimation of Optimum Hydrolysis Time.

Quantitative ninhydrin estimation (Section 2.3.3.3) on the wall 6N HCl hydrolysates are shown in Figure R. 2. The maximum release of ninhydrin-positive material, measured as leucine, occurred after 12 hours of hydrolysis at 100°C and constituted 15.8% of the organic content of the wall material.

Figure R. 2

Release of Ninhydrin-Positive Components from 6N HCl Hydrolysates.



3.3.3.3 Paper Chromatography.

Diagrammatic representations of the paper chromatograms from the 6N HCl hydrolysates are shown in Figures R.3-R.7. Several amino acid and two amino sugar components were identified by comparison of their Rala values to authentic standards. As the ninhydrin detection reagent produced characteristic colours for certain of the amino acids and stained the amino sugars a light green, this allowed additional identification and verification of certain of the ninhydrin-positive components in the 6N HCl hydrolysates.

There were minor deviations in the Rala values for the authentic amino acid and amino sugar standards between different chromatograms run in the same solvent system. This was probably due to the fluctuations in temperature, as all the chromatograms were run at room temperature. The problem was resolved by running a mixture of the standards on each chromatogram for direct comparison.

Figure R. 3

The amino acids and amino sugars identified in this solvent system are shown in the figure. Some brown residual material, probably 'humin' from the hydrolysis, was left at the origin and there was no evidence for either lysine (Spot 8: Rala 0.31) or muramic acid (Spot 9: Rala 1.12) being present in the hydrolysates.

However, three unidentified ninhydrin-positive components, Spots 11, 12, 13 with Rala values of 0.05, 0.17, 0.27 respectively, were detected close to the origin. After 48 hours of hydrolysis, Spot 13 became very faint and Spot 11 was no longer detected. This suggests that prolonged acid hydrolysis causes either a degradation or a breakdown into simpler components

Figure R. 3

Key.

| Spot. | Component. | Rala. | Colour. |
|---|---|---|---|
| 1 | $GalNH_2HCl$ | 0.40 | Green |
| 2 | $GlcNH_2HCl$ | 0.51 | Green |
| 3 | Asp | 0.59 | Blue |
| 4 | Gly | 0.66 | Red-brown |
| 5 | Glu/Thr | 0.77 | Purple |
| 6 | Ala | 1.00 | Purple |
| 7 | Val | 1.74 | Purple |
| 8 | Lys | 0.31 | Purple |
| 9 | Mur | 1.12 | Purple |
| 10 | Ile | 2.12 | Purple |
| 11 | ? | 0.05 | Purple |
| 12 | ? | 0.17 | Purple |
| 13 | ? | 0.27 | Purple |

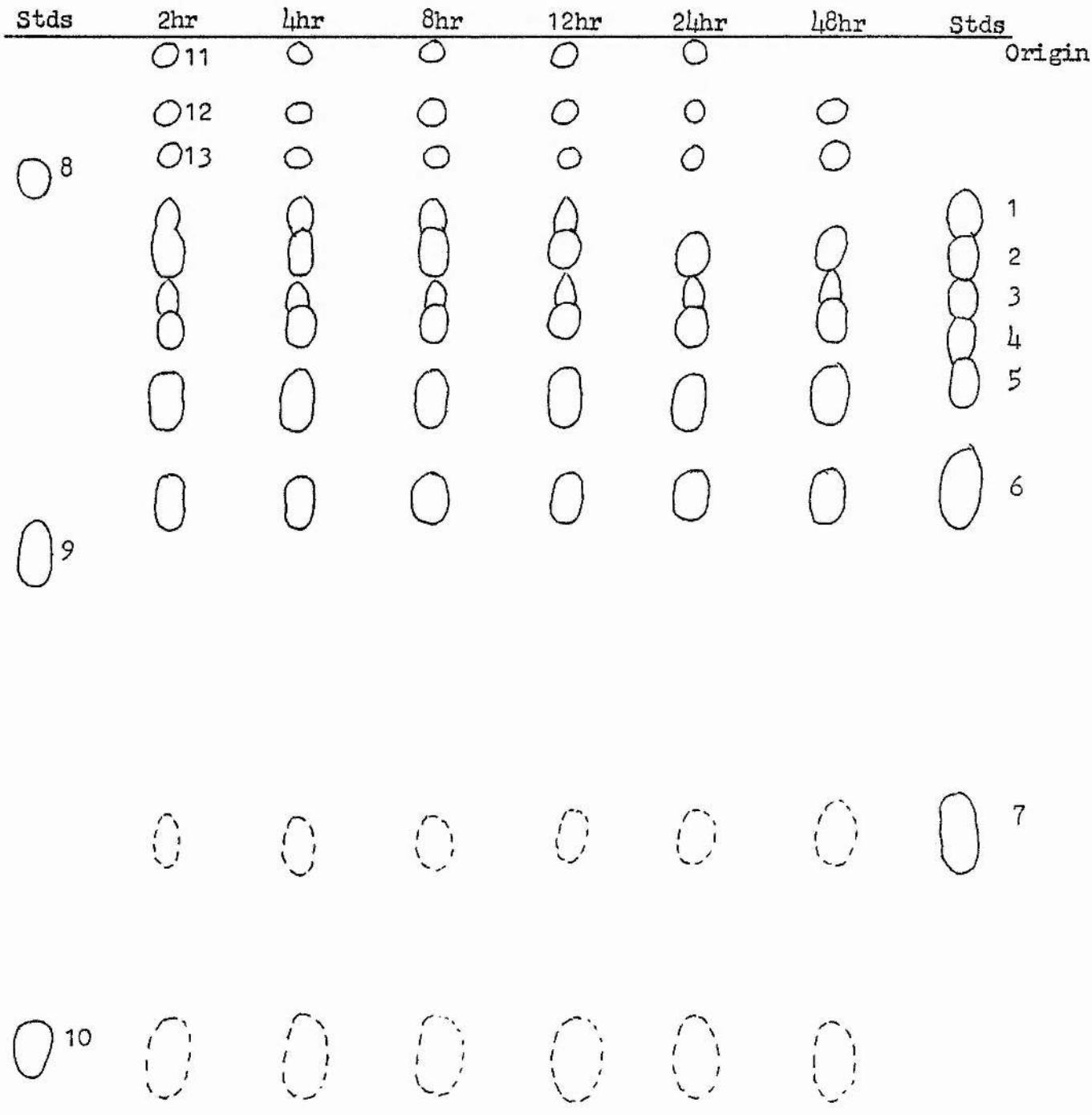
Figure R. 3

Paper Chromatography - 6N HCl Hydrolysis.

Solvent: n butanol: acetic acid: water; 5: 1: 2 (v/v/v).

Stain: Ninhydrin.

Whatman 3 MM, 1x24 hours in the machine direction.



of these ninhydrin-positive compounds.

Figure R. 4

The amino acids and amino sugars identified in this solvent system are shown in the figure. Two unidentified ninhydrin-positive components, Spots 10,11 with Rala values of 0.05, 0.3 respectively, were detected. The latter spot was yellow and could possibly be a peptide with glycine as the N terminal amino acid since, with this ninhydrin detection reagent, gly-terminal peptides also stain yellow. This aspect was investigated further (Figures 5,6), particularly as this yellow spot disappeared after 12 hours of hydrolysis, suggesting either degradation of or a breakdown into simpler components by this compound.

Figures R. 5 and R. 6

As shown in Figure R. 5, the yellow unidentified ninhydrin-positive component (Spot 8), in this solvent system, did indeed correspond, in both colour and Rala value of 0.35, to the pentaglycine standard (Spot 1). However, in a different solvent system (Figure R. 6), there was no correlation between the pentaglycine standard (Spot 1) and any of the hydrolysate components. There was, in fact, no yellow spot even detected and this was possibly due to the component being left at the origin where it would be masked by the brown residual 'humin' material. In addition, there was no correlation between any of the hydrolysate components and the pentaglycine standard on ion exchange chromatography (Section 3.3.3.4).

Thus, the yellow ninhydrin-positive component detected, (Figures R. 4 and R. 5), is not pentaglycine nor any of the other peptide standards tested, although its mobility and lability suggest that it may indeed be a peptide.

Figure R. 4

Key.

| Spot. | Component. | Rala. | Colour. |
|---|---|---|---|
| 1 | Asp | 0.21 | Blue |
| 2 | Glu | 0.25 | Purple |
| 3 | Gly | 0.61 | Red-brown |
| 4 | Ser | 0.71 | Purple |
| 5 | Thr/Ala | 1.00 | Purple |
| 6 | $GalNH_2HCl$ | 1.46 | Green |
| 7 | $GlcNH_2HCl$ | 1.63 | Green |
| 8 | Val | 1.88 | Purple |
| 9 | Ile | 2.47 | Purple |
| 10 | ? | 0.05 | Purple |
| 11 | ? | 0.3 | Yellow |

Figure R. 4

Paper Chromatography - 6N HCl Hydrolysis.

Solvent: n butanol: pyridine: water; 6: 4: 3 (v/v/v).

Stain: Ninhydrin.

Whatman 3 MM, 1x30 hours in the machine direction.

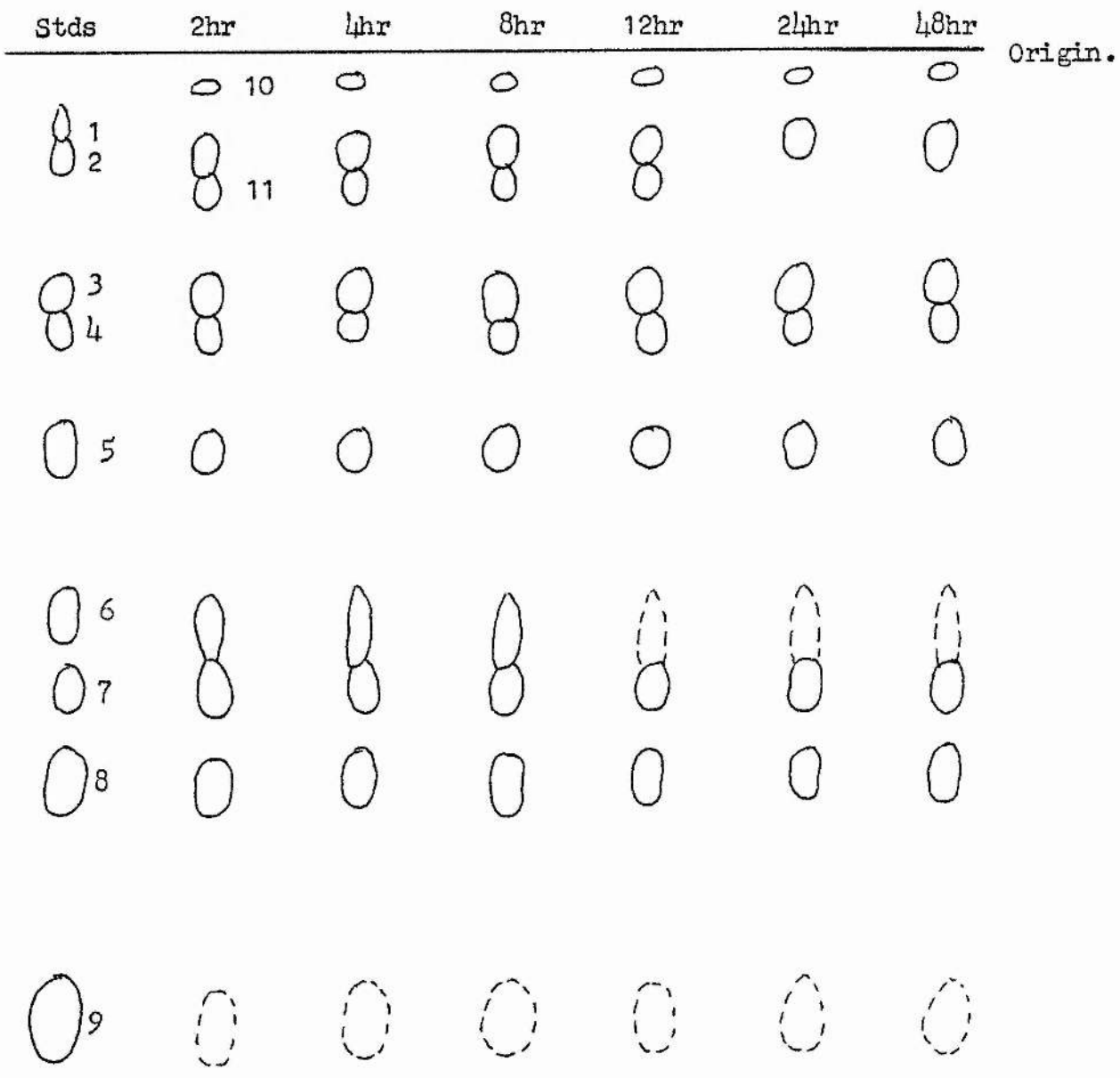


Figure R. 5

Key.

| Spot. | Component. | Rala. | Colour. |
|---|---|---|---|
| 1 | Pentagly | 0.35 | Yellow |
| 2 | Gly-gly | 0.48 | Yellow |
| 3 | Gly-ala | 0.69 | Yellow |
| 4 | Ala-gly | 0.93 | Purple |
| 5 | ? | 0.05 | Purple |
| 6 | Asp | 0.23 | Blue |
| 7 | Glu | 0.28 | Purple |
| 8 | ? | 0.35 | Yellow |
| 9 | Gly/Ser | 0.60 | Red-brown |
| 10 | Thr/Ala | 1.00 | Purple |
| 11 | $GalNH_2HCl$ | 1.45 | Green |
| 12 | $GlcNH_2HCl$ | 1.63 | Green |
| 13 | Val | 1.92 | Purple |
| 14 | Ile | 2.60 | Purple |

Figure R. 5

Paper Chromatography - 6N HCl Hydrolysis.

Solvent: n butanol: pyridine: water; 6: 4: 3 (v/v/v).

Stain: Ninhydrin.

Whatman 3 MM, 1x24 hours in the machine direction.

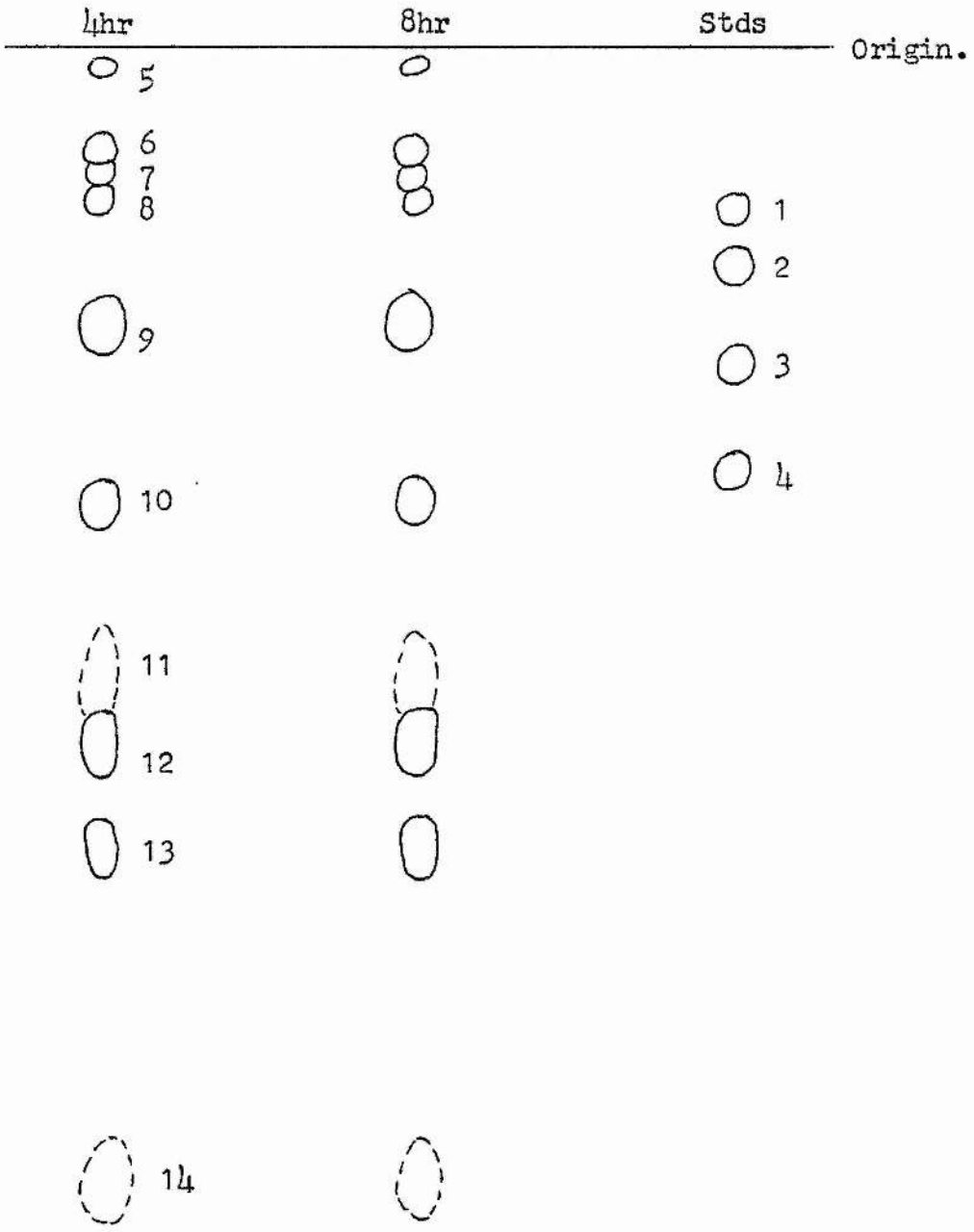


Figure R. 6

Key.

| Spot. | Component. | Rala. | Colour. |
|---|---|---|---|
| 1 | Pentagly | 0.41 | Yellow |
| 2 | Gly-gly | 0.73 | Yellow |
| 3 | Gly-ala | 1.10 | Yellow |
| 4 | Ala-gly | 1.45 | Purple |
| 5 | ? | 0.04 | Purple |
| 6 | ? | 0.10 | Purple |
| 7 | ? | 0.24 | Purple |
| 8 | $GalNH_2HCl$ | 0.45 | Green |
| 9 | $GlcNH_2HCl$ | 0.56 | Green |
| 10 | Asp/Gly | 0.71 | Blue-brown |
| 11 | Glu/Thr | 0.83 | Purple |
| 12 | Ala | 1.00 | Purple |
| 13 | Val | 1.73 | Purple |

Figure R. 6

Paper Chromatography - 6N HCl Hydrolysis.

Solvent: n butanol: acetic acid: water; 5: 1: 2 (v/v/v).

Stain: Ninhydrin.

Whatman 3 MM, 1x24 hours in the machine direction.

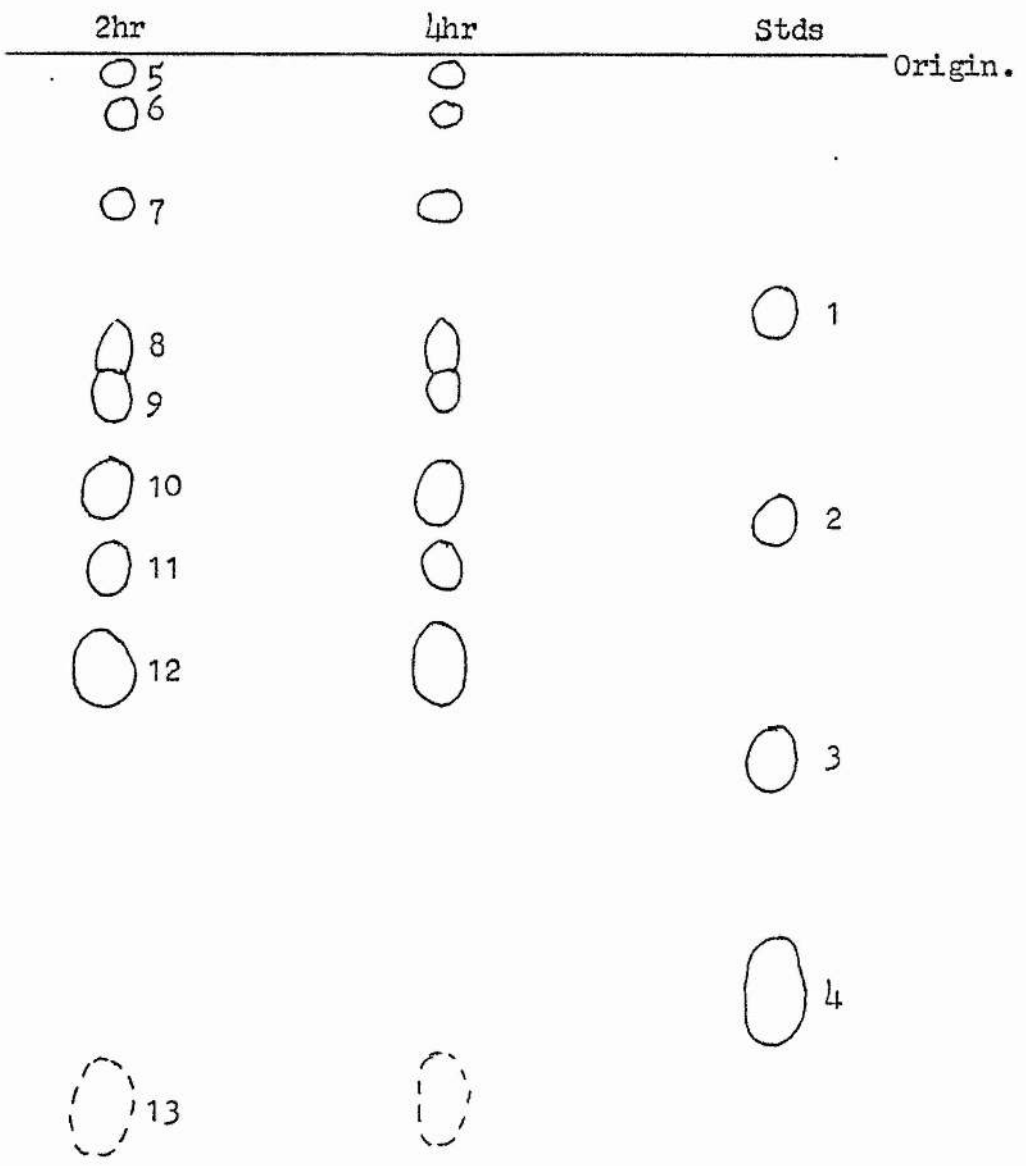


Figure R. 7

None of the hydrolysate components and, in particular, the three unidentified components, Spots 10, 11 and 12 with Rala values of 0.08, 0.18 and 0.21 respectively, corresponded to the diaminopimelic acid (DAP) standard with an Rala value of 0.26. It was therefore concluded that there was no DAP in the wall material.

Summary.

The amino acids aspartate, glycine, glutamate, serine, threonine, alanine, valine and isoleucine, and the amino sugars glucosamine and galactosamine were identified as components of S. marina wall. However, four unidentified ninhydrin-positive components, one of which stained yellow in the ninhydrin detection reagent, were also present.

The yellow component was not pentaglycine or any of the other peptide standards tested, but its mobility and lability to acid hydrolysis suggest that it may indeed be a peptide. As a sulphated heteropolymer has been postulated as a structural component of the wall from Halococcus morrhuae (58), one or more of the unknown spots detected may be a sulphated amino sugar.

Neither of the components generally associated with, and indicating the presence of, peptidoglycan: muramic acid and diaminopimelic acid, was detected in the wall. This suggests that, as for other extreme halophiles (9, 11,57), a peptidoglycan structure similar to that in most Gram-positive and Gram-negative microorganisms, is absent.

3.3.3.4 Ion Exchange Chromatography.

A diagrammatic representation of a typical ion exchange chromatography trace, from the 12 hour, 6N HCl hydrolysate, is shown in Figure R. 8. The

Figure R. 7

Key.

| Spot. | Component. | Rala. | Colour. |
|---|---|---|---|
| 1 | Gal$NH_2HCl$ | 0.44 | Green |
| 2 | Glc$NH_2HCl$ | 0.52 | Green |
| 3 | Asp | 0.63 | Blue |
| 4 | Gly | 0.68 | Brown-red |
| 5 | Glu/Thr | 0.82 | Purple |
| 6 | Ala | 1.00 | Purple |
| 7 | Val | 1.68 | Purple |
| 8 | Ile | 2.10 | Purple |
| 9 | DAP | 0.26 | Purple |
| 10 | ? | 0.08 | Purple |
| 11 | ? | 0.18 | Purple |
| 12 | ? | 0.21 | Purple |

Figure R. 7

Paper Chromatography - 6N HCl Hydrolysis.

Solvent: n butanol: acetic acid: water; 5: 1: 2 (v/v/v).

Stain: Ninhydrin.

Whatman 3 MM, 1x24 hours in the machine direction.

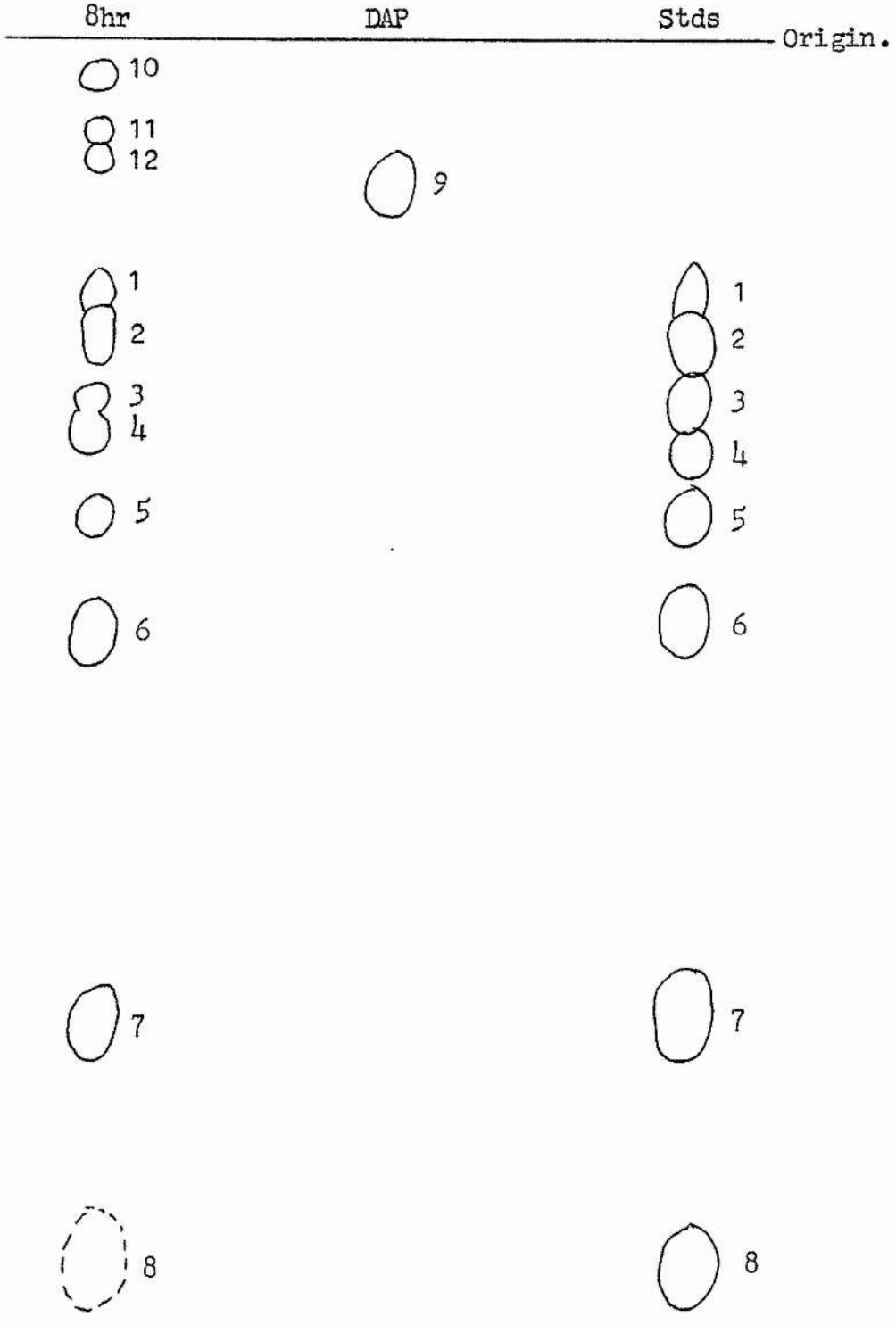
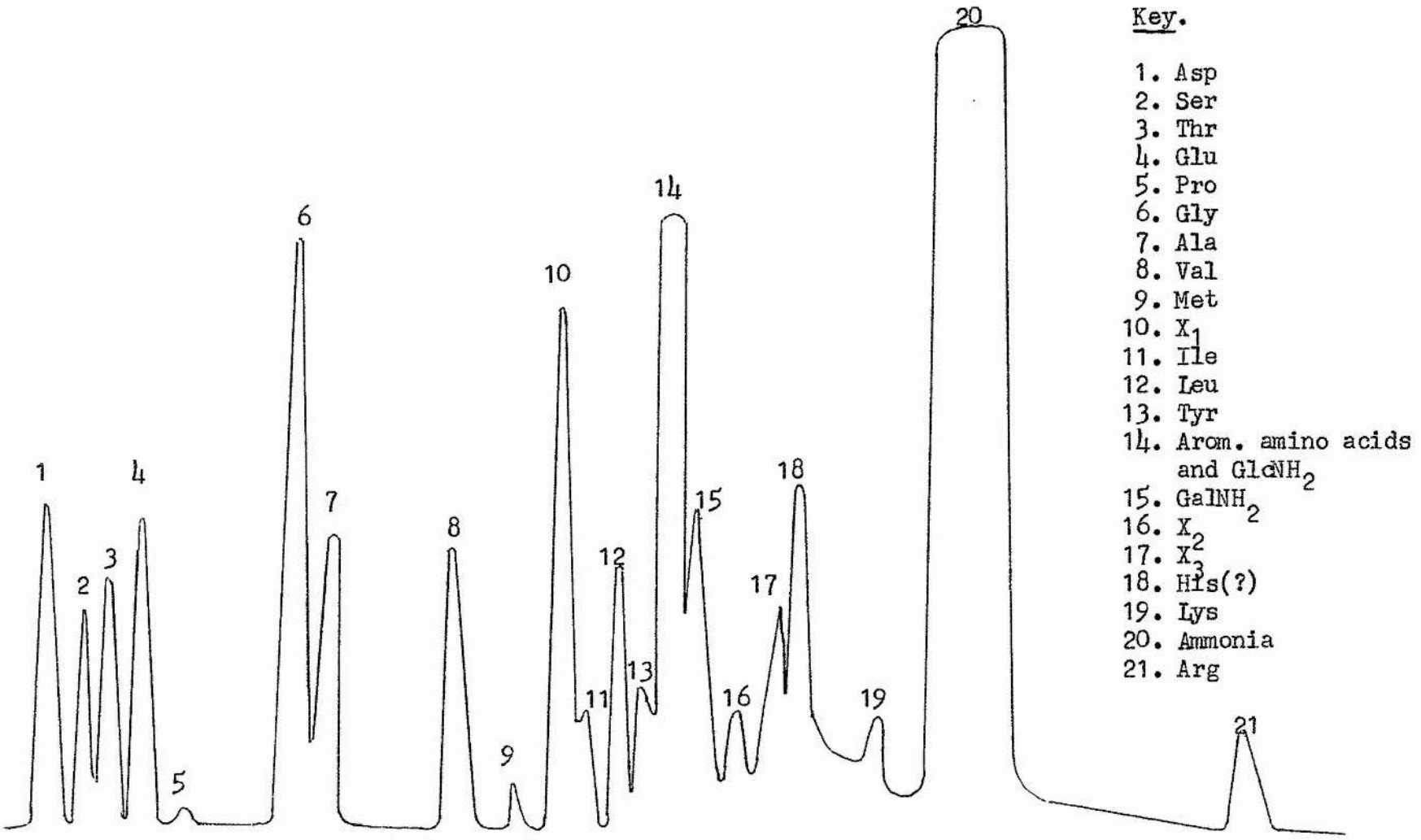


Figure R. 8

Ion Exchange Chromatography Trace of 6N HCl Hydrolysate (12 hr).



amino acid and amino sugar components were identified by comparison to authentic standards, which also provided colour values and allowed quantitative analysis of these ninhydrin-positive components.

The total amino acid content of the 6N HCl hydrolysates are shown in Figure R. 9. The maximum release of amino acids occurred after 12 hours of hydrolysis, which is the same as the optimum release of ninhydrin-positive components (Section 3.3.3.2), but they constituted only 4.3% of the organic wall material. The amino acid analysis of this 12 hour 6N HCl hydrolysate is shown in Table TR. 5. It is probable that a single homogeneous protein is not being analysed as the molar ratios of the amino acids are not integers, within the limits of experimental error. In fact, as all the common amino acids from protein are present, the adhering contaminant membrane fragments, that give the wall its pink tinge, are probably being assessed, in addition to genuine wall components.

The amino acids identified by paper chromatography (Section 3.3.3.3) were also detected by ion exchange chromatography. However, this more sensitive analysis identified 7 additional amino acids, arginine, lysine, histidine, leucine, proline, methionine and tyrosine, the last three in only trace amounts. Three unidentified ninhydrin-positive components, $X_1$, $X_2$, and $X_3$, were also detected, but none of these corresponded to either muramic acid or diaminopimelic acid.

The major amino acid components in the wall were, in order of magnitude, glycine, histidine, alanine, glutamate, and aspartate and their content in the 6N HCl hydrolysates are shown in Figure R. 10. They separated into two groups with different optimum hydrolysis times for their maximum release, alanine and glycine after 4 hours, and histidine, glutamate and

Figure R. 9

Release of Amino Acids from 6N HCl Hydrolysates.

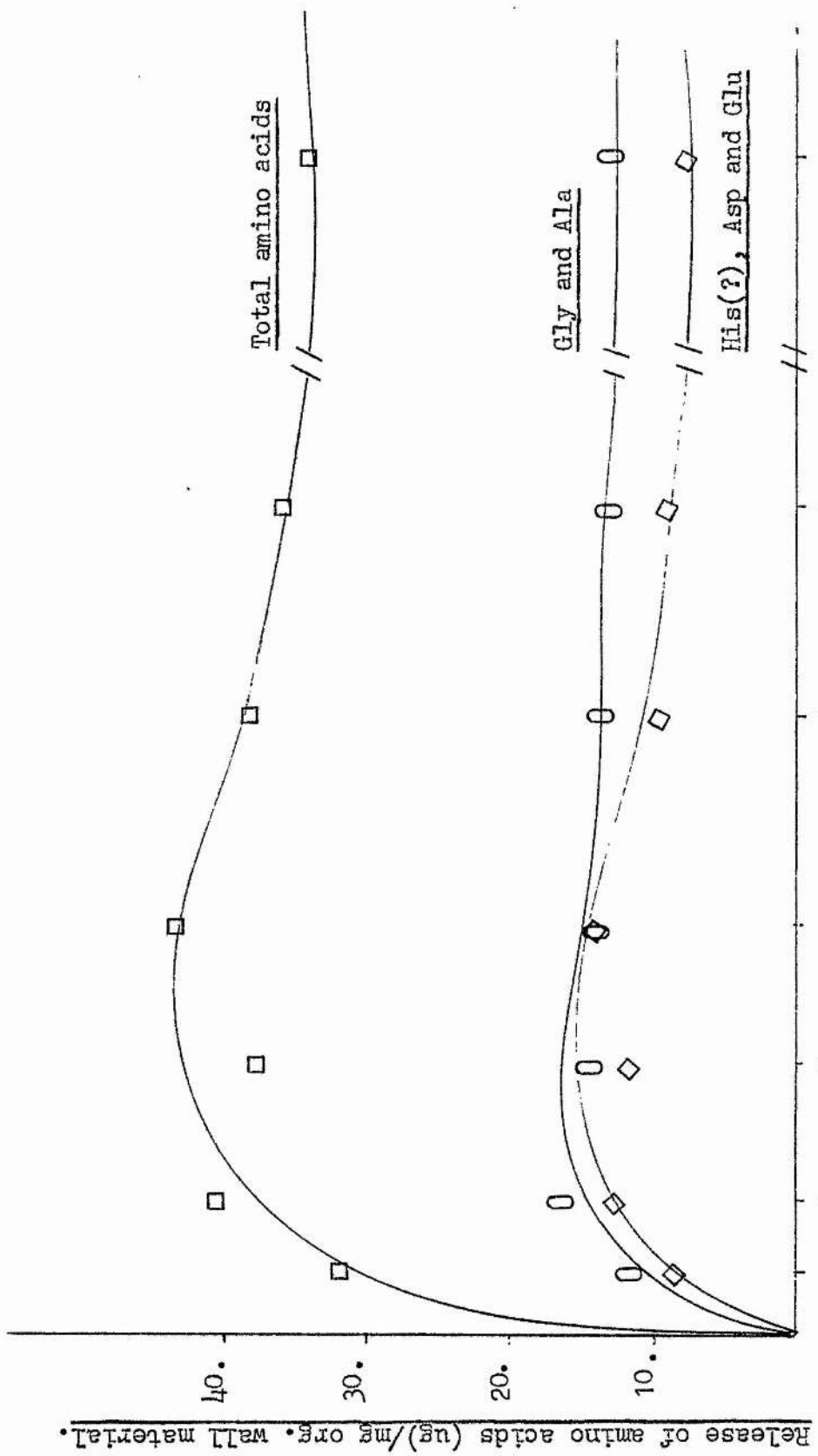


Table TR. 5

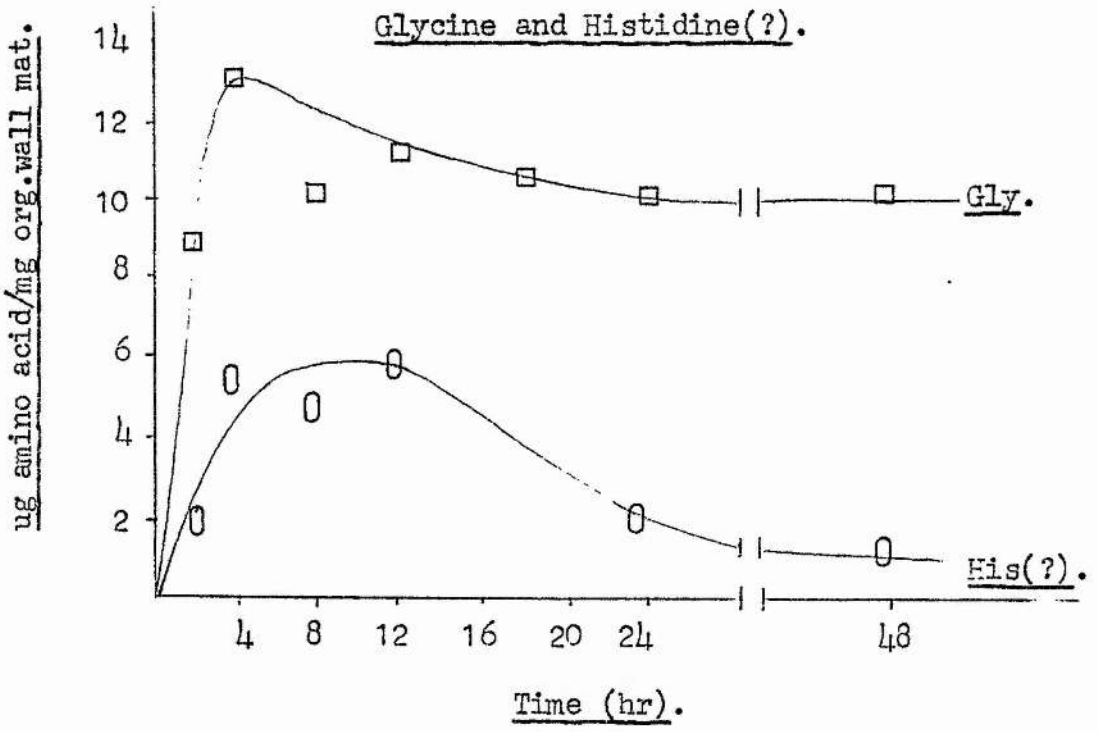
Amino Acids released from 12 hour, 6N HCl Hydrolysates.

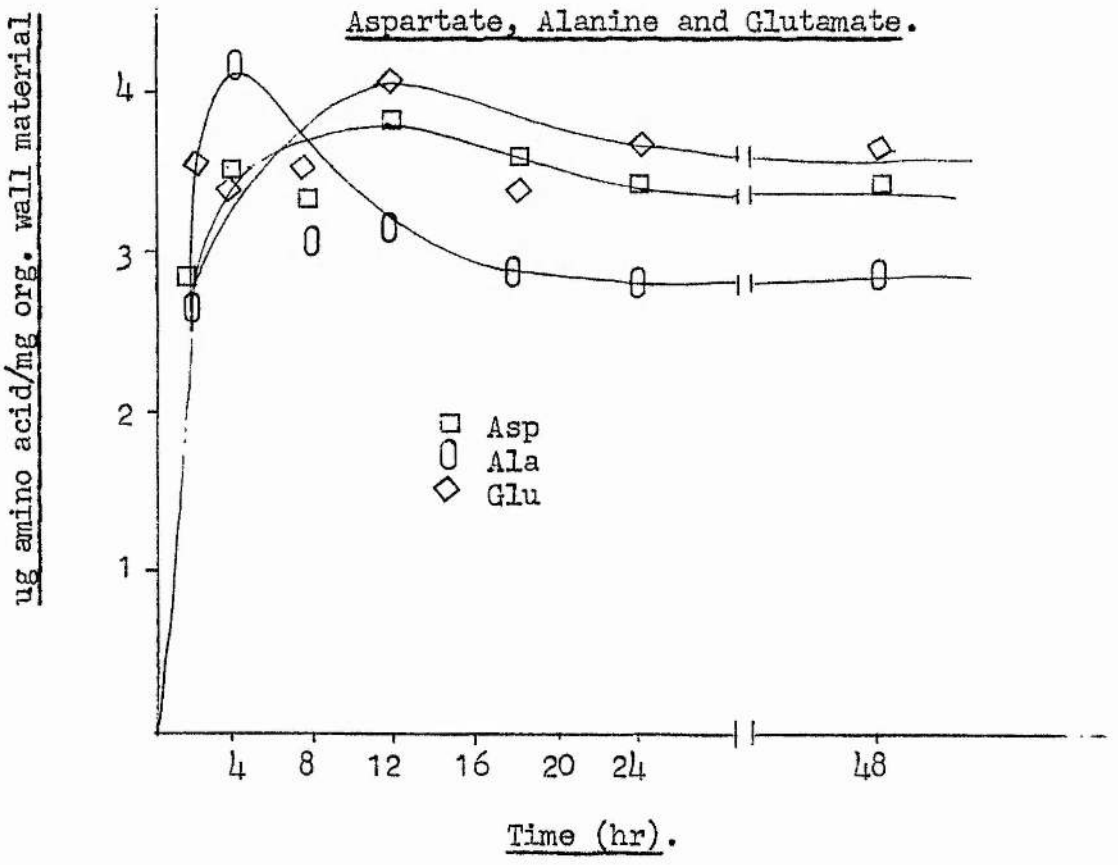
| Amino Acid. | ug/mg wall.* | umoles/mg wall.* | moles/$10^3$ moles. | MR.** |
|---|---|---|---|---|
| Aspartate | 3.8 | 0.0286 | 75 | 3.2 |
| Threonine | 2.1 | 0.0174 | 45.6 | 1.9 |
| Serine | 2.0 | 0.0187 | 49 | 2.1 |
| Glutamate | 4.0 | 0.0275 | 72 | 3.0 |
| Proline | trace | - | - | - |
| Glycine | 11.0 | 0.146 | 383 | 16.2 |
| Alanine | 3.1 | 0.034 | 89 | 3.8 |
| Valine | 1.7 | 0.014 | 37 | 1.6 |
| Methionine | trace | - | - | - |
| Isoleucine | 1.6 | 0.0124 | 32.5 | 1.4 |
| Leucine | 2.8 | 0.0216 | 56.7 | 2.4 |
| Lysine | 1.4 | 0.009 | 23.6 | 1.0 |
| Histidine(?) | 6.2 | 0.040 | 105 | 4.4 |
| Arginine | 2.1 | 0.012 | 31.5 | 1.3 |
| Tyrosine | trace | - | - | - |

*mg wall: the results are expressed per mg of organic material in the wall.
**MR: Molar Ratio.

Figure R. 10

Release of Major Amino Acids from 6N HCl Hydrolysates.



aspartate after 12 hours. The individual maximum content of each amino acid in the 6N HCl hydrolysates are shown below in Table TR. 6, and, as a group, in Figure R. 9.

Table TR. 6

Maximum Release of Major Amino Acids from 6N HCl Hydrolysates.

| Amino Acid. | Max.Release (hr). | ug/mg org. wall. |
|---|---|---|
| Glycine | 4 | 12.3 |
| Alanine | 4 | 4.0 |
| Histidine(?) | 12 | 6.2 |
| Aspartate | 12 | 3.8 |
| Glutamate | 12 | 4.0 |

Although histidine has been identified as a major amino acid component of S. marina wall, it is not a major constituent of many proteins and certainly not of halococcal wall (11,57,58). Histidine is also not noted as being especially unstable to hydrolysis conditions, contrary to the data in Figure R. 10, in which it shows significant degradation after 12 hours of hydrolysis. Consequently, this component may not in fact be histidine, but something else, possibly an amino sugar, which happens to run in the same place.

The content of the three unidentified components $X_1$, $X_2$ and $X_3$ in the 6N HCl hydrolysates were assessed and the results are shown below in Table TR. 7. In the absence of colour values, the level of these wall constituents is represented by their area on the ion exchange chromatography trace of a 1mg sample of hydrolysed wall material.

Table TR. 7

Release of $X_1$, $X_2$ and $X_3$ from 6N HCl Hydrolysis.

| Component. | Hydrolysis Time (hr). | | | | | | |
|---|---|---|---|---|---|---|---|
| | 2. | 4. | 8. | 12. | 18. | 24. | 48. |
| $X_1$ | 2318 | 22767 | 17192 | 15201 | 6556 | 2478 | - |
| $X_2$ | 927 | 4111 | 2926 | 1419 | 1777 | 1500 | 1490 |
| $X_3$ | 2316 | 786 | 1535 | 1751 | 1632 | 1741 | 1020 |

The maximum release of both $X_1$ and $X_2$ occurred after 4 hours of hydrolysis and they showed significant degradation thereafter. In fact, $X_1$ was completely destroyed by the hydrolysis conditions after 48 hours. The third unidentified component, however, had double optima for release, after 2 and 12-24 hours. This suggests that there are possibly two polymers in the wall with $X_3$ as a constituent of both. Alternatively, part of the $X_3$ component, in a single polymer, may be protected by some other wall constituent/constituents that have to be removed before the remainder of the $X_3$ can be released. In view of the extended optimum release time of 12 hours, this is probably more likely. However, like the $X_1$ and $X_2$ constituents, $X_3$ also showed significant degradation immediately after both its optima, at 4 and 48 hours.

Summary.

All the common amino acids from protein and the amino sugars, glucosamine and galactosamine, were identified as components of S. marina wall, although some were present in only trace amounts. The total amino acids released constituted only 4.3% of the organic wall material which, although very low compared to Gram-positive non-halophilic cocci (48), is consistent

with other available data on the halococci (9,11,57,58).

The major amino acids were, in order of magnitude, glycine, histidine(?), alanine, glutamate and aspartate, although there is some doubt as to the validity of the histidine identification. They separated into two groups with different optimum hydrolysis times for their maximum release, alanine and glycine after 4 hours, and histidine(?), glutamate and aspartate after 12 hours.

The three unidentified components $X_1$, $X_2$ and $X_3$ and the component initially identified as histidine, are all acid labile. Some, or all, of these wall constituents may correspond to the 4 unknown components from the paper chromatograms (Section 3.3.3.3). In particular, $X_1$, Spot 11 (Figure R. 3) and Spot 11 (Figure R. 4),the yellow spot, were not detected after 48 hours of hydrolysis on either paper chromatography or ion exchange chromatography.

### 3.3.4 Carbohydrate Analysis.

#### 3.3.4.1 Total Carbohydrate.

The total carbohydrate released from wall 6N HCl hydrolysates (Section 2.3.4.2) are shown below in Table TR. 8. The maximum carbohydrate content of the wall, measured as D glucose, occurred after 2 hours of hydrolysis at 100°C and constituted only 11.2% of the organic wall material. However, as would be expected, the carbohydrate content showed a marked decrease with the hydrolysis time and is probably due to the destruction of carbohydrate by the hydrolysis conditions.

Consequently, the total carbohydrate content of the wall was assessed by milder acid hydrolysis conditions, 0.5N HCl at 100°C (Section 2.3.4.2)

and the results are shown in Figure R. 11. The maximum release of total carbohydrate, measured as D glucose, occurred after 18 hours of hydrolysis and constituted 36.5% of the organic wall material.

Table TR. 8

Release of Total Carbohydrate from 6N HCl Hydrolysis.

| Hydrolysis Time (hr). | Carbohydrate* Released. |
|---|---|
| 2 | 112.2 |
| 4 | 50.2 |
| 8 | 22.5 |
| 12 | 20.4 |
| 24 | 11.2 |
| 48 | 10.5 |

*Carbohydrate Released: results expressed as ug carbohydrate released/mg org. wall material.

3.3.4.2 Total Reducing Substances.

The total reducing substances released from the wall 0.5N HCl hydrolysates (Section 2.3.4.3) are shown in Figure R. 11. The maximum released, measured as D glucose, occurred after 18 hours of hydrolysis, the same as the hydrolysis time for maximum total carbohydrate content, and constituted 39.25% of the organic wall material.

3.3.4.3 Paper Chromatography.

A diagrammatic representation of the paper chromatogram from the 0.5N HCl hydrolysate is shown in Figure R. 12. Several monosaccharide components were identified by comparison of their Rglc values to authentic standards. As before (Section 3.3.3.3), a mixture of the standards was run on each chromatogram for direct comparison to resolve the problem of minor devia-

Figure R. 11

Release of Carbohydrate and Total Reducing Substances from 0.5N HCl Hydrolysates.

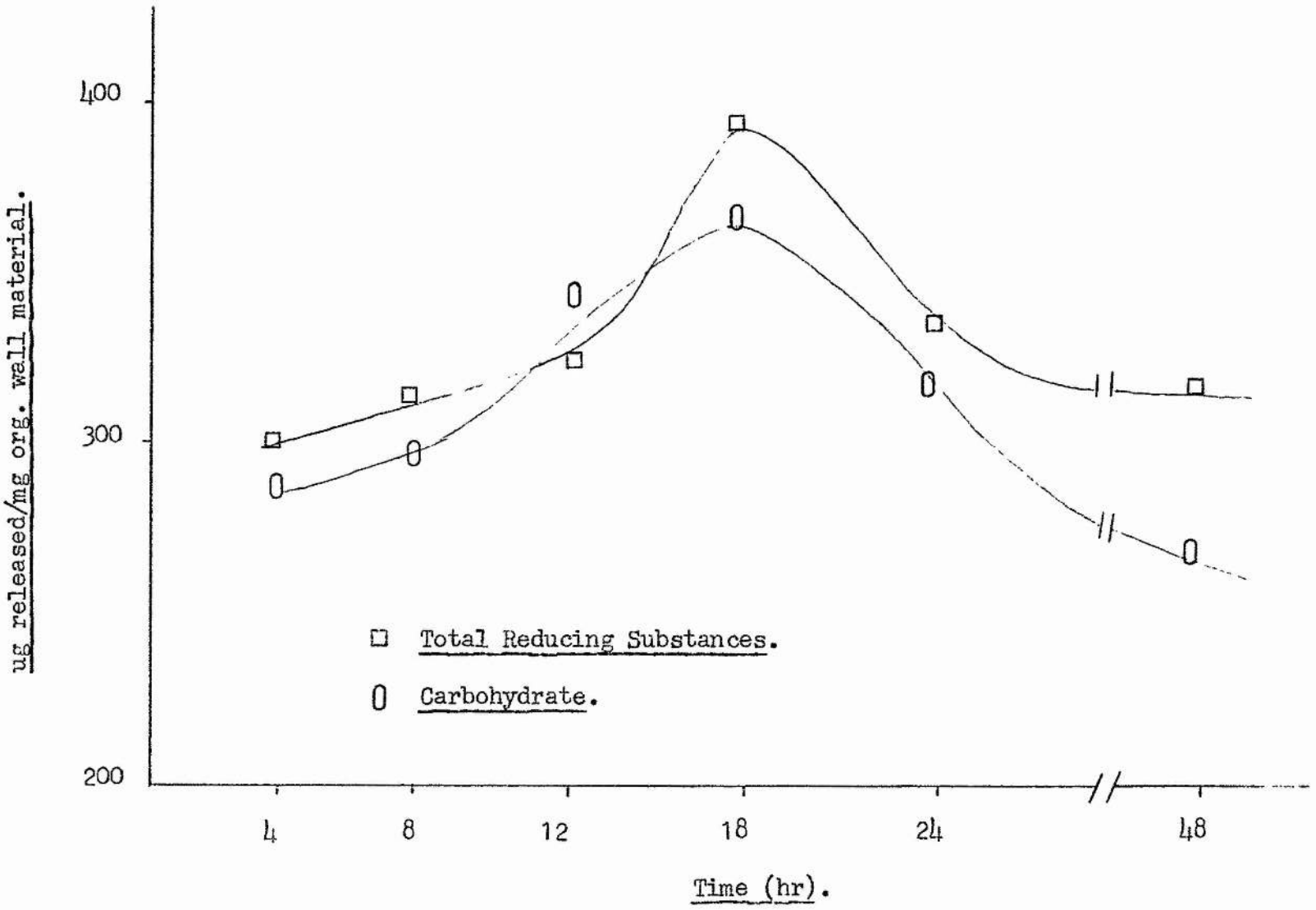


Figure R. 12

Key.

| Spot. | Component. | Rglc. |
|---|---|---|
| 1 | Galactose | 0.89 |
| 2 | Glucose | 1.00 |
| 3 | Mannose | 1.13 |
| 4 | Rhamnose | 1.49 |
| 5 | $GalNH_2HCl$ | 0.74 |
| 6 | $GlcNH_2HCl$ | 0.81 |
| 7 | ? | 0.28 |
| 8 | ? | 0.48 |
| 9 | ? | 0.55 |

Additional Standards (not shown).

| Monosaccharide. | Rglc. |
|---|---|
| Muramic acid | 0.19 |
| Fucose | 1.23 |
| Ribose | 1.32 |
| Glucose | 1.00 |

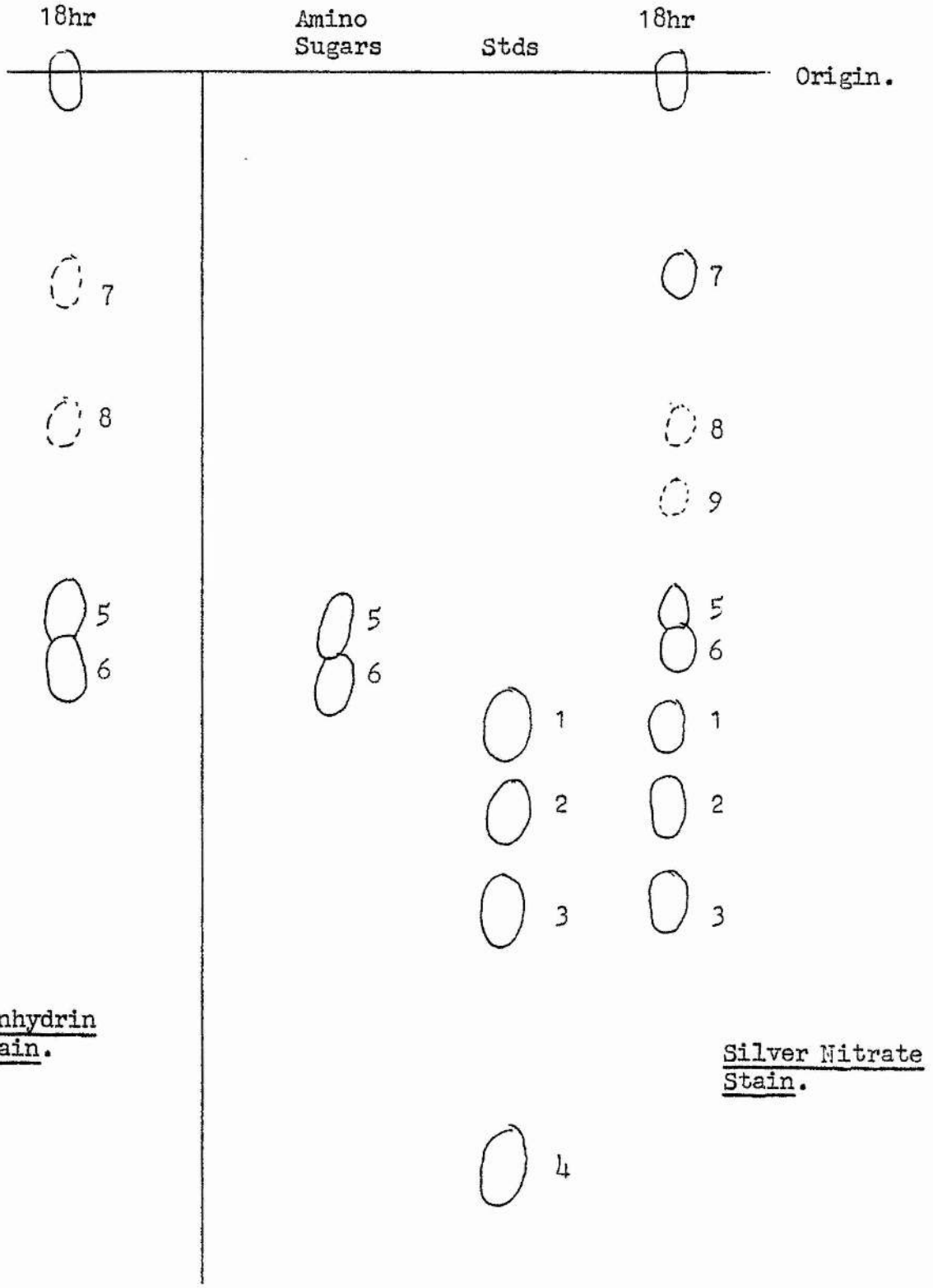
Figure R. 12

Paper Chromatography - 0.5N HCl Hydrolysis.

Solvent: n butanol: pyridine: water; 6: 4: 3 (v/v/v).

Stain: Ninhydrin and Silver Nitrate.

Whatman 3 MM, 1x24 hours in the machine direction.



tions in Rglc values caused by temperature fluctuations.

The monosaccharides glucose, galactose and mannose, and the amino sugars glucosamine and galactosamine, were identified as components of the wall. Some brown residual material, probably 'humin' from the hydrolysis, was left at the origin and there was no evidence for muramic acid (Rglc 0.19) being present in the hydrolysates. However, three other components, Spots 7, 8 and 9 with Rglc values of 0.28, 0.48 and 0.55 respectively, which did not correspond to any of the monosaccharide and amino sugar standards tested, were detected. Two of these unidentified components, Spots 7 and 8, contained reducing and amino groups, as they were stained by both the ninhydrin and silver nitrate detection reagents. However, the third component, Spot 9, had only reducing groups.

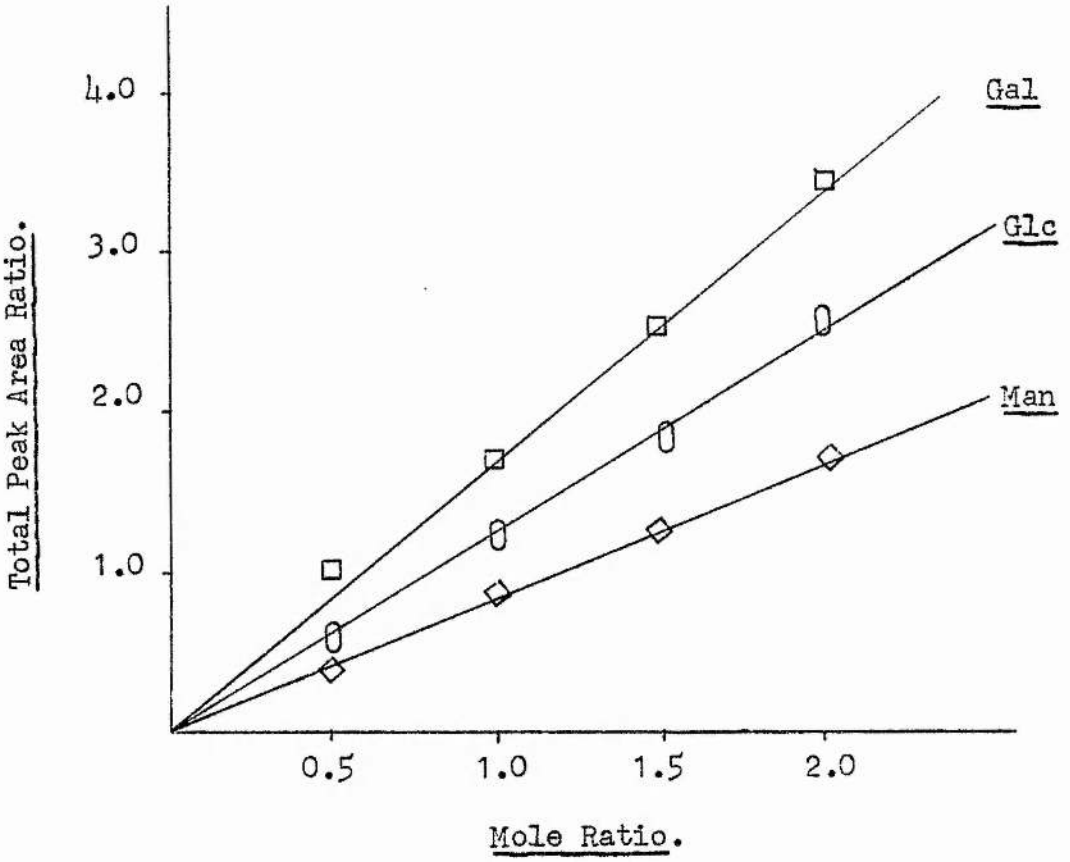
3.3.4.4 Gas Liquid Chromatography.

The Relative Molar Response Factor (RMRF) for the monosaccharides glucose, galactose and mannose were evaluated (Section 2.3.4.5) and the results are shown in Figure R. 13. The content of these monosaccharides in each hydrolysate was assessed and the values are shown in Table TR. 9.

As for the total carbohydrate (Section 3.3.4.1) and the total reducing substances (Section 3.3.4.2), the maximum release of these three monosaccharides occurred after 18 hours of hydrolysis. They were present in approximately equimolar amounts, the molar ratio being galactose: glucose: mannose; 1: 1.19: 1.14, and together constituted 24.7% of the organic wall material. A diagrammatic representation of the GLC trace from the 0.5N HCl, 18 hour hydrolysate is shown in Figure R. 14. There was no evidence for the presence of any other significant component/components in the wall hydrolysate from this analysis.

Figure R. 13

Determination of Relative Molar Response Factor (RMRF) for Monosaccharides.




| Monosaccharide. | RMRF. |
|---|---|
| Glucose | 1.264 |
| Galactose | 1.618 |
| Mannose | 0.826 |

Table TR. 9

Release of Monosaccharides from 0.5N HCl Hydrolysis.

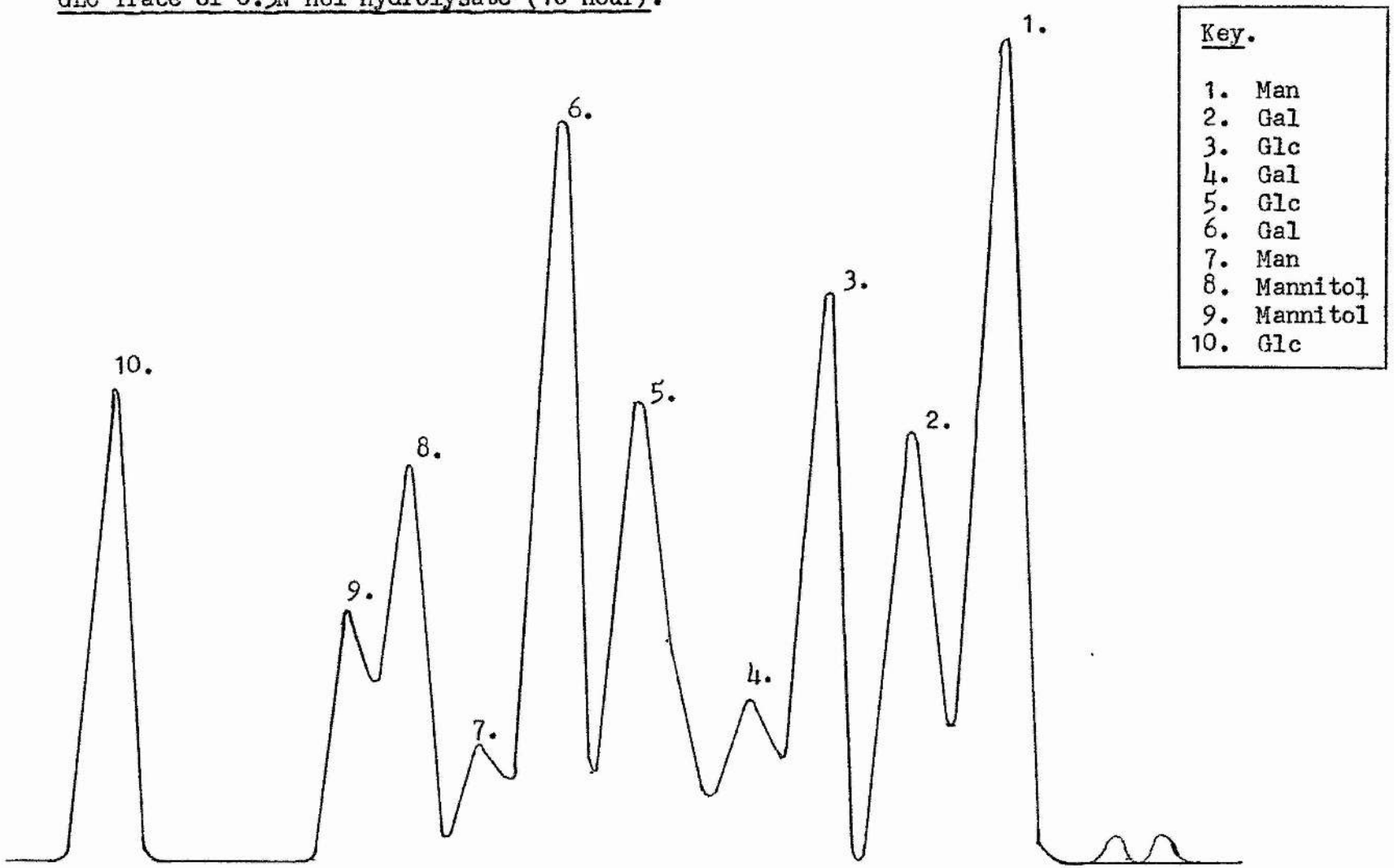
| Hydrolysis Time (hr). | Mannose | | Glucose | | Galactose | | Total |
|---|---|---|---|---|---|---|---|
| | ug* | M.R. | ug* | M.R. | ug* | M.R. | ug* |
| 4 | 50.6 | 1.00 | 65.4 | 1.29 | 57.0 | 1.13 | 173 |
| 8 | 59.7 | 1.09 | 54.6 | 1.00 | 62.0 | 1.14 | 176.3 |
| 12 | 55.4 | 1.13 | 75.6 | 1.54 | 49.0 | 1.00 | 180 |
| 18 | 84.4 | 1.14 | 88.3 | 1.19 | 74.0 | 1.00 | 246.7 |
| 24 | 55.2 | 1.42 | 67.4 | 1.73 | 39.0 | 1.00 | 161.6 |
| 48 | 45.2 | 1.09 | 58.9 | 1.43 | 41.3 | 1.00 | 145.4 |

* ug: results expressed as ug monosaccharide/mg organic wall material.

M.R: Molar Ratio.

Figure R. 14

GLC Trace of 0.5N HCl Hydrolysate (18 hour).



### 3.3.5 Amino Sugar Analysis.

#### 3.3.5.1 Hexosamine Determination.

The hexosamine content of the 0.5N HCl hydrolysates was assessed (Section 2.3.5.2) and the values are shown below in Table TR. 10.

Table TR. 10

Release of Hexosamines from 0.5N HCl Hydrolysates.

| Hydrolysis Time (hr). | ug hexosamine/ mg org. wall |
|---|---|
| 4 | 17.3 |
| 8 | 17.6 |
| 12 | 18.3 |
| 18 | 19.8 |
| 24 | 20.1 |
| 48 | 24.0 |

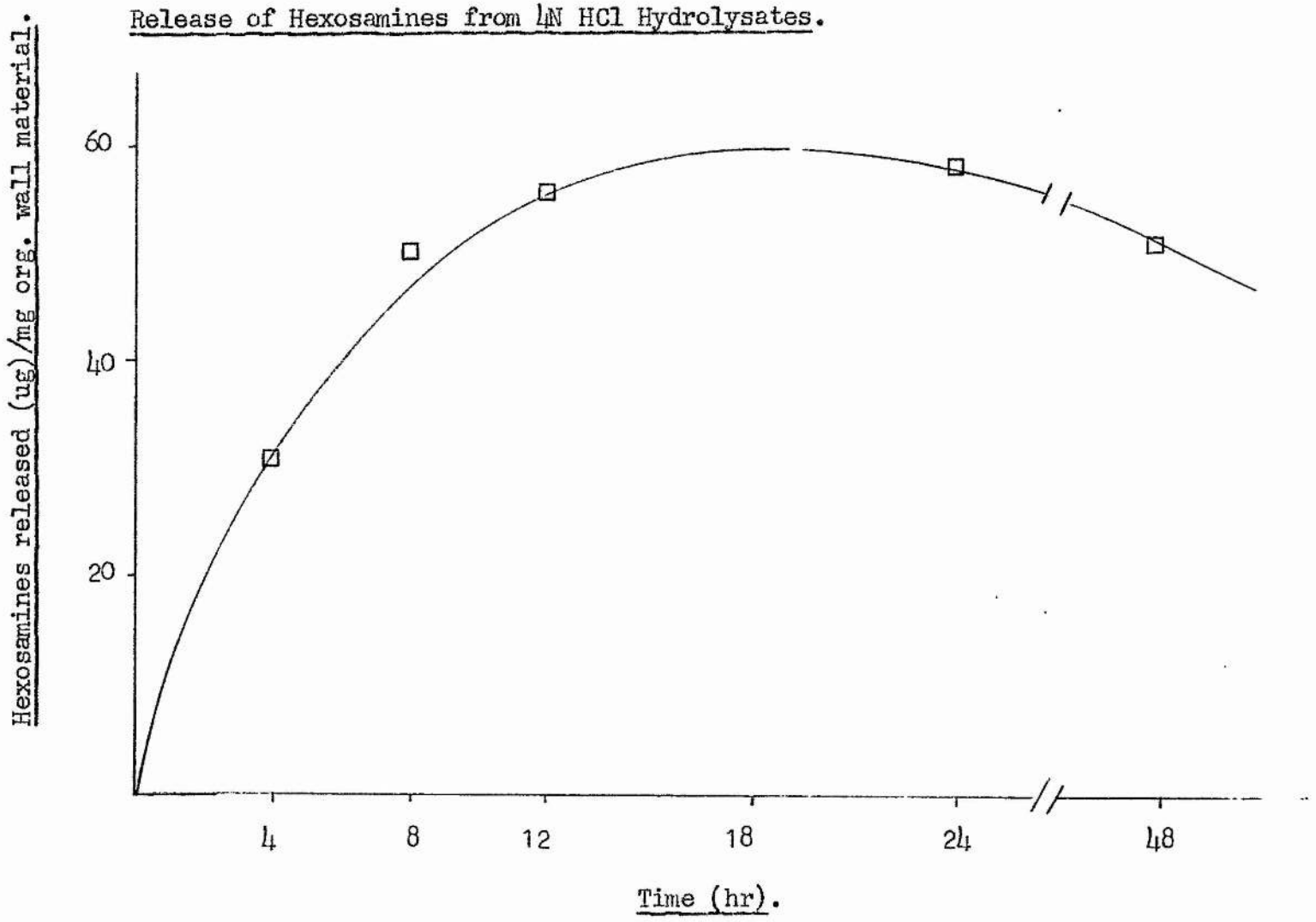
The hexosamine content of the hydrolysates, measured as glucosamine HCl, showed a steady increase, up to and including 48 hours of hydrolysis. This suggests that the maximum release of hexosamine from the wall had not been attained with the mild acid hydrolysis conditions used, even after 48 hours. Thus, stronger hydrolysis conditions, 4N HCl at 100°C, were used and the hexosamine content of each hydrolysate is shown in Figure R. 15. The maximum release of hexosamines occurred after 24 hours and constituted 5.8% of the organic material in the wall.

#### 3.3.5.2 GLC Analysis of Hexosamines.

An attempt to quantitate the levels of glucosamine and galactosamine in 4N HCl hydrolysates was made by GLC. However, despite a report (82) to the contrary, this method did not resolve the hexosamines sufficiently for such analysis. Even the hexosamine standards produced a variety of peaks, which

Figure R. 15

Release of Hexosamines from 4N HCl Hydrolysates.



may be caused by unknown derivatives of the amino sugars.

3.3.5.3 Ion Exchange Chromatography of Hexosamines.

The glucosamine and galactosamine levels in the wall were assessed by ion exchange chromatography of 6N HCl wall hydrolysates. Although galactosamine was isolated as a single peak on the trace, glucosamine formed a composite peak with phenylalanine and could only be approximately estimated. The results are shown in Figure R. 16.

The maximum release of both of these amino sugars occurred after 4 hours of hydrolysis. Together they constituted 3.5% of the organic wall material, which is low compared to the 5.8% for total hexosamine released from the 4N HCl hydrolysis (Section 3.3.5.1). However, part of this difference is probably due to the increased degradation of these hexosamines in the stronger acid hydrolysis conditions. Another important consideration is that, although glucosamine and galactosamine constitute the detected hexosamine components, only these two hexosamines were quantitated. Other unidentified components, which may be hexosamines, particularly $X_1$ and $X_2$ which could not be quantitated in the absence of their colour values, were also present in the wall. There is also evidence for other hexosamines and/or their derivatives, particularly gulosaminuronic acid, in halococcal wall (57,58).

3.3.6 Summary of Cell Wall Analysis.

The identified constituents of S. marina wall and their maximum levels are shown in Table TR. 11.

The yield of wall from the cell is probably a gross underestimate because of the losses of wall material incurred in the isolation procedure. The

Figure R. 16

Release of Hexosamines from 6N HCl Hydrolysates.

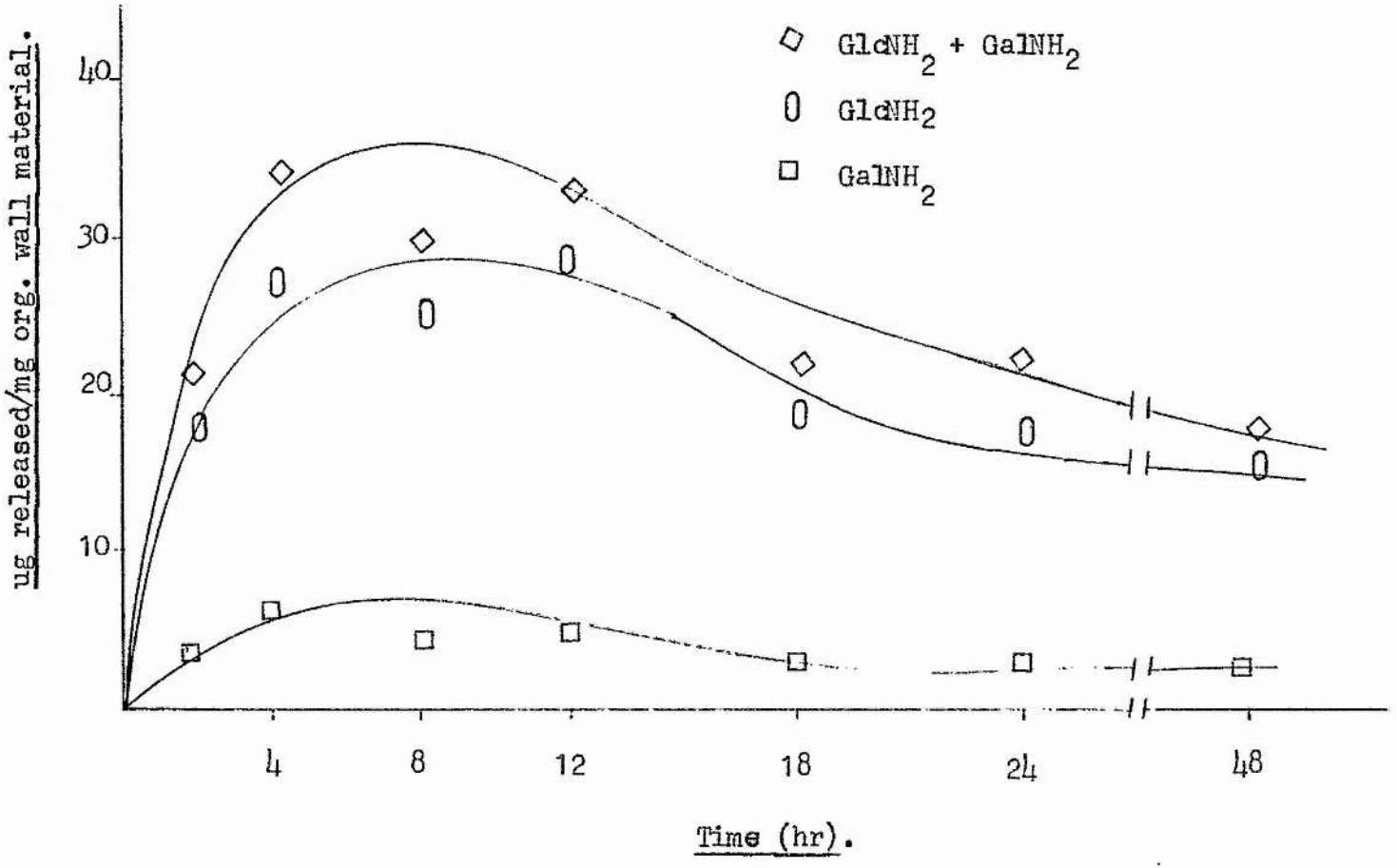


Table TR. 11

Cell Wall Analysis - Summary.

| Component. | Content. |
|---|---|
| Wall | 12.1% freeze dried cells |
| Lipid | 0.1% freeze dried wall |
| Moisture | 4.3% " " " " |
| Ash | 9.2% " " " " |
| Organic Material | 86.5% " " " " |
| Ninhydrin-positive (1) | 15.8% org. wall material |
| Total Amino Acids (2) | 4.3% " " " " |
| Glycine | 1.23% " " " " |
| Histidine(?) | 0.6% " " " " |
| Alanine | 0.4% " " " " |
| Glutamate | 0.4% " " " " |
| Aspartate | 0.38% " " " " |
| Total Hexosamines (3) | 5.8% " " " " |
| Glucosamine (2) | 2.8% " " " " |
| Galactosamine (2) | 0.7% " " " " |
| Ninhydrin-negative | |
| Total Carbohydrate (4) | 36.5% org. wall material |
| Total Reducing Substances (5) | 39.3% " " " " |
| Glucose (6) | 8.83% " " " " |
| Galactose (6) | 7.4% " " " " |
| Mannose (6) | 8.44% " " " " |

1) Section 3.3.3.2
2) Section 3.3.3.4
3) Section 3.3.5.1
4) Section 3.3.4.1
5) Section 3.3.4.2
6) Section 3.3.4.4

lipid extract, a deep red in colour, is probably not a genuine wall component and was probably extracted from the residual contaminating membrane fragments adhering to the wall representing, possibly, carotenoid pigmentation. The high ash content is typical of the halococci (9,58) and, in view of the doubt concerning its chemical composition (9,58), may reflect either salt bound to the wall components or the presence of sulphated heteropolymers.

All the common amino acids from protein and the amino sugars glucosamine and galactosamine were identified as components of S. marina wall, although some were present in only trace amounts. The major amino acid constituents of the wall were, in order of magnitude, glycine, histidine(?), alanine, glutamate and aspartate, although there is some doubt as to the validity of the histidine identification. It may, in fact, be something else that co-chromatographs with histidine, possibly an amino sugar.

Several additional ninhydrin-positive components, $X_1$, $X_2$ and $X_3$ and the component initially identified as histidine, were detected in the wall (Section 3.3.3.4) and were all acid labile. Some, or all, of these unidentified wall constituents may correspond to the 4 unknown components from the paper chromatograms (Section 3.3.3.3).

The monosaccharides glucose, galactose and mannose were identified as wall components and occurred in approximately equimolar amounts although, together, they constituted only 67.6% of the total carbohydrate content and 62.8% of the total reducing substances detected in the wall. Paper chromatography (Section 3.3.4.3), however, detected 3 additional unidentified components, two of which contained both amino and reducing groups, the third only reducing groups. None of these unidentified components, however, were detected by GLC analysis (Section 3.3.4.4).

## 3.4 Fractionation Studies.

### 3.4.1 Extraction Techniques.

#### 3.4.1.1 Teichoic Acids.

TCA Extraction at 0°C: as there was no precipitate on the addition of cold ethanol (Figure M. 3), it was concluded that there were no conventional teichoic acids in the wall.

TCA Extractions at 35°C and 60°C: The extent of separation of wall material constituents caused by these extractions is shown in Table TR. 12. At 35°C, the majority of the wall was insoluble but at 60°C the converse was true. The amino acid, amino sugar and carbohydrate content of these wall "fractions" was analysed (Section 2.4.2) and the results are reported later (Section 3.4.2).

#### 3.4.1.2 Hot Formamide Extraction.

Hot formamide treatment produced little resolution of the wall material. The majority of the wall was solubilised and only a minor, almost insignificant fraction (less than 5%), remained as an insoluble residue.

As this method has been used to isolate peptidoglycan from cell wall, it was concluded that a conventional peptidoglycan was not a significant, if even present, constituent of S. marina wall. In view of the almost complete solubility of the wall material in hot formamide, further analysis was not conducted.

#### 3.4.1.3 Hot Alkali Extraction

The majority of the hot alkali treated wall material was recovered as the soluble residue, the 'mannan' component, but it only constituted 43.7% of

Table TR. 12

TCA Extractions: Separation of Wall Material.

| Extraction Method. | Initial Wt. of Wall (mg). | Insoluble Residue (mg). | %* | Soluble Residue (mg). | %* | %Recovered |
|---|---|---|---|---|---|---|
| TCA at 35°C | 500 | 365.3 | 73.1 | 44.6 | 8.9 | 82 |
| TCA at 60°C | 200** | 37.6 | 18.8 | 141.8 | 70.9 | 89.7 |

*%: percentage of the initial weight of wall extracted.
**200: wall had previously been extracted with TCA at 35°C.

the initial weight of wall material. The recovery of the insoluble residue, the 'glucan' component, was very low, only 4%. However, the wall material is very sensitive to hot acetic acid treatment (Section 3.4.1.4) and the 'glucan' fraction is exposed to similar conditions (Figure M. 8). This could account for the low recovery of both the 'glucan' fraction and the total fractionated wall material, less than 50% (47.7%) of the initial weight of wall material treated.

The ninhydrin-positive and monosaccharide components of the soluble residue, the 'mannan', were analysed (Section 3.4.2) but, because of the low recovery, further analysis of the insoluble residue, the 'glucan', was not conducted.

3.4.1.4 Acetic Acid Extraction.

Although the wall was almost completely solubilised by refluxing in acetic acid, no soluble residue was recovered when absolute ethanol at -40°C was added. A negligible insoluble residue was left in the acid conditions used (Section 2.4.1.4).

The extreme solubility of the wall in acetic acid underlines the fundamental difference of S. marina wall from conventional walls. The lack of precipitate with ethanol suggests that the usual polysaccharide components, for example capsular polysaccharides, are absent.

3.4.2 Analysis of Fractionated Wall Material.

As the recovery of fractionated wall material was not 100%, the analytical results could not be related to a constant weight of organic wall material as before. Consequently, the ninhydrin-positive components and the monosaccharides were expressed as residues/1000 residues and umoles/mg, respec-

tively, of the fractionated wall material hydrolysed. Only the general trends in the fractionated material could be assessed.

3.4.2.1 Ninhydrin-Positive Components.

Only the major ninhydrin-positive components, evaluated by ion exchange chromatography of the extracted wall fractions (Section 2.4.2.1), are shown in Table TR. 13. Some loss of the amino sugars, the unidentified components $X_1$, $X_2$ and $X_3$, and the histidine(?) component could be incurred in both the acid hydrolysis and extraction conditions, but their levels and detection in the fractions are still useful for comparative purposes.

Almost all the minor amino acids previously detected in the wall (Section 3.3.3.4) were present in both the soluble and insoluble residues, although in varying amounts. However, as these components were attributed to contaminating membrane fragments, they probably do not represent a significant aspect of wall fraction composition. The major amino acid components (Table TR. 13), with the exception of histidine(?), were also present in the residues with, in many cases, notable differences in their levels between fraction The significance of this distribution of the major amino acids, and especially glycine which appears to be a common constituent of halococcal wall(57) is not known but may indicate the presence of more than one polymer in the wall.

As histidine is not noted as being especially unstable to either the hydrolysis or extraction conditions, the lack of significant amounts of this component in both the soluble and insoluble residues is suprising. However, some doubt about the identification of this component as being histidine has already been suggested (Section 3.3.3.4). This data here supports the contention that this ninhydrin-positive component may be something else,

Table TR. 13

Analysis of Ninhydrin-Positive Constituents of Fractionated Wall.

| | Ninhydrin-Positive Constituent. | | | | | | | | | %* |
|---|---|---|---|---|---|---|---|---|---|---|
| Extraction. | Gly. | His. | Ala. | Glu. | Asp. | $GlcNH_2$. | $GalNH_2$. | $X_1$ | $X_2$ | |
| TCACW 35°C | 250.0 | trace | 60.6 | 50.2 | 49.8 | 384.5 | n.d. | + | + | 97.9 |
| TCAS 35°C | 111.3 | n.d. | 54.5 | 38.4 | 32.3 | 414.7 | 239.7 | + | n.d. | 109.6 |
| TCACW 60°C | 132.7 | 13.0 | 130.8 | 99.0 | 91.8 | 58.0 | n.d. | n.d. | n.d. | 65.3 |
| TCAS 60°C | 206.7 | trace | 10.9 | 8.4 | 10.3 | 475.2 | n.d. | + | n.d. | 87.6 |
| Alkali (1) | 163.4 | n.d. | 16.4 | 20.6 | 15.0 | 580.5 | 96.4 | + | + | 109.9 |
| Wall (2) | 260.0 | 71.4 | 60.6 | 49.1 | 51.0 | 272.4 | 47.6 | + | + | 100 |

The values in the table are expressed as residues/1000 residues in the fraction.

TCACW: the insoluble residue left after TCA extraction.
TCAS: the soluble residue from TCA extraction.

1) the 'mannan' fraction from the hot alkali extraction (Section 3.4.1.3).
2) from the analysis of the ninhydrin-positive components in S. marina wall (Section 3.3.3.4)

n.d.: not detected.
+: detected.

%*: **total major ninhydrin-positive constituents of fractionated wall expressed as a percentage of the unfractionated wall.**

possibly an amino sugar, that co-chromatographs with histidine.

As galactosamine was completely solubilised but all the $X_2$ component was left in the insoluble residue by TCA extraction at 35°C, and all the $X_1$ component was solubilised by TCA extraction at 60°C, some division of the wall into separate wall polymers may have, in fact, been achieved. However, one anomalous result was noted. Although the $X_2$ component was detected in the insoluble TCACW 35°C residue, none was present in either the soluble or insoluble residues from the TCA extraction at 60°C. It may be that $X_2$ is extremely sensitive to the latter extraction conditions.

3.4.2.2 Monosaccharide Components.

Only the identified monosaccharides glucose, galactose and mannose, evaluated by GLC analysis of the extracted wall fractions (Section 2.4.2.2), are shown in Table TR. 14. The lower levels of the monosaccharides in the fractionated wall material compared to untreated wall may reflect the sensitivity of wall polymers to the extraction conditions.

Although none of the TCA extraction methods showed integral molar ratios, within the limits of experimental error, for these three monosaccharides, the 'mannan' component contained them in an approximately equimolar ratio, similar to untreated wall. As TCA extraction at both 35°C and 60°C produced a significant difference in the relative content (Molar Ratio) of glucose between the soluble and insoluble residues, a preferential extraction of a glucose-rich wall polymer, possibly glucan-like, may have been achieved.

Summary.

All efforts to extract single polymers, e.g. 'mannan', peptidoglycan or teichoic acids were unsuccessful. The possibility of a preferentially

Figure TR. 14

Analysis of Monosaccharides in Fractionated Wall Material.

| Extraction. | Mannose umoles/mg. | M.R. | Glucose umoles/mg. | M.R. | Galactose umoles/mg. | M.R. |
|---|---|---|---|---|---|---|
| TCACW 35°C | 0.11 | 1.33 | 0.19 | 2.29 | 0.083 | 1.0 |
| TCAS 35°C | 0.16 | 1.14 | 0.21 | 1.5 | 0.14 | 1.0 |
| TCACW 60°C | 0.15 | 1.0 | 0.29 | 1.93 | 0.20 | 1.33 |
| TCAS 60°C | 0.03 | 1.0 | 0.16 | 5.3 | 0.07 | 2.3 |
| Alkali (1) | 0.15 | 1.15 | 0.13 | 1.0 | 0.14 | 1.08 |
| Wall (2) | 0.47 | 1.14 | 0.49 | 1.19 | 0.41 | 1.0 |

Results are expressed as umoles/mg of fractionated wall material hydrolysed.

TCACW: the insoluble residue after TCA extraction.
TCAS: the soluble residue from TCA extraction.

1) the 'mannan' fraction from the hot alkali extraction (Section 3.4.1.3).
2) from the analysis of the carbohydrate content of S. marina wall (Section 3.3.4.4).

extracted glucose-rich wall polymer and the division of galactosamine, $X_1$ and $X_2$ between the soluble and insoluble residues, suggests that some division of the wall into separate wall polymers may have been achieved. However, apart from these exceptions, the soluble and insoluble residues contained, at least qualitatively, the same constituents. The significance of the distribution of these constituents is not known.

## 3.5 Antibiotics.

### 3.5.1 Antibiotic Solubility in Dundas Medium.

D cycloserine, vancomycin and penicillin G were all readily soluble in both sterile water and Dundas medium. Novobiocin and bacitracin, although soluble in sterile water, were insoluble in Dundas medium. Novobiocin formed white insoluble globules that floated on the surface of Dundas medium, and bacitracin became yellow, adopting a chewing-gum like texture. However, if both of these antibiotics were initially dissolved in a small volume of sterile water, when added to Dundas medium they both remained in solution.

### 3.5.2 Antibiotic Effect on S. marina Growth.

A comparison of the growth curves of S. marina in the presence of each of the antibiotics to a control with no antibiotic were obtained as described (Section 2.5.1). The results are shown in Figures R.17-R.21.

All three concentrations of D cycloserine and the single concentrations of penicillin G and vancomycin had no turbidimetrically detectable effect on the halococcal growth. Novobiocin and bacitracin, however, showed a marked inhibition to growth at all three concentrations tested.

Each of the antibiotic treated cultures and the control cells were sub-

Figure R. 17

Effect of D Cycloserine on S. marina Growth.

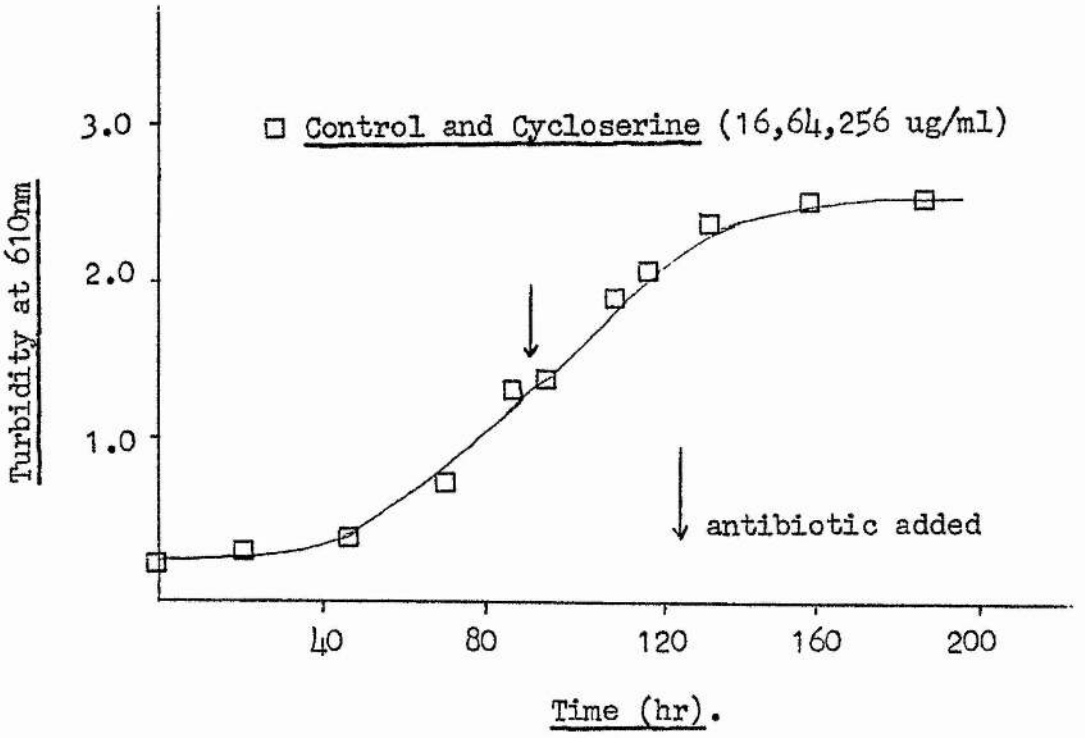



Figure R. 18

Effect of Novobiocin on S. marina Growth.

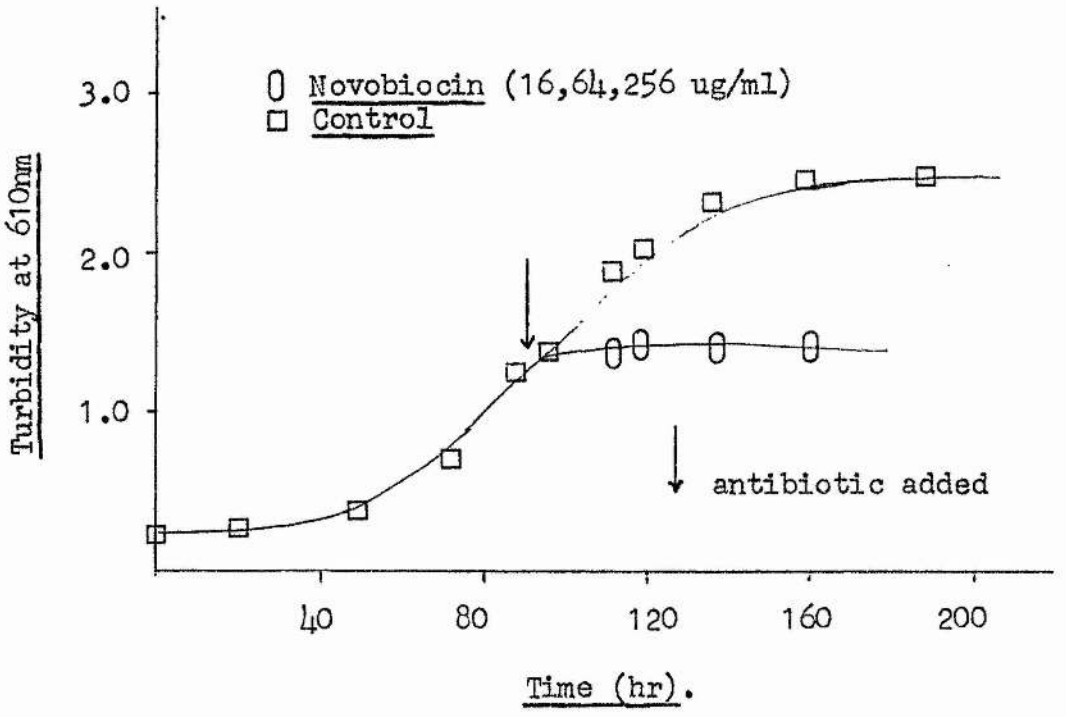


Figure R. 19

Effect of Bacitracin on S. marina Growth.

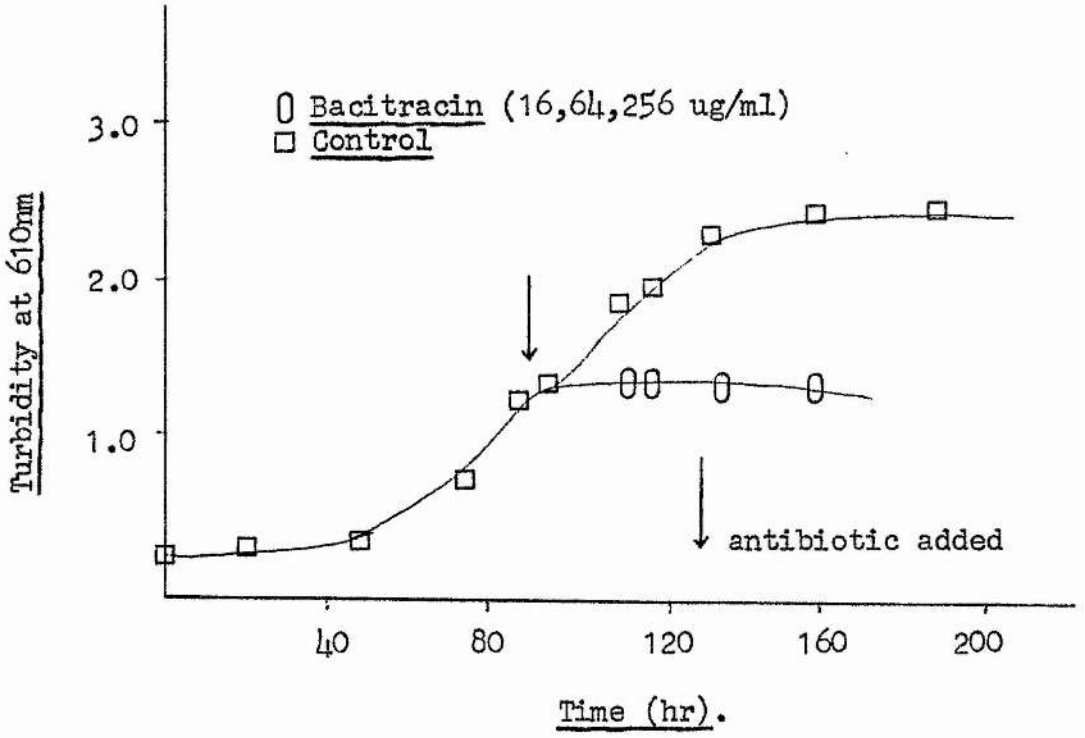



Figure R. 20

Effect of Penicillin G on S. marina Growth.

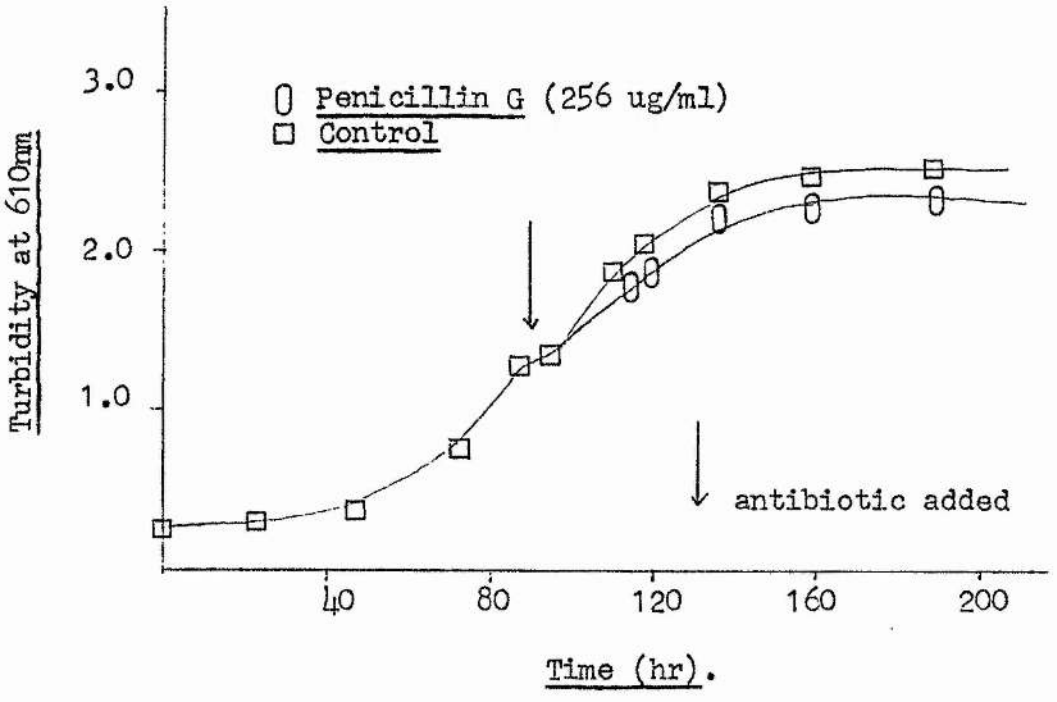
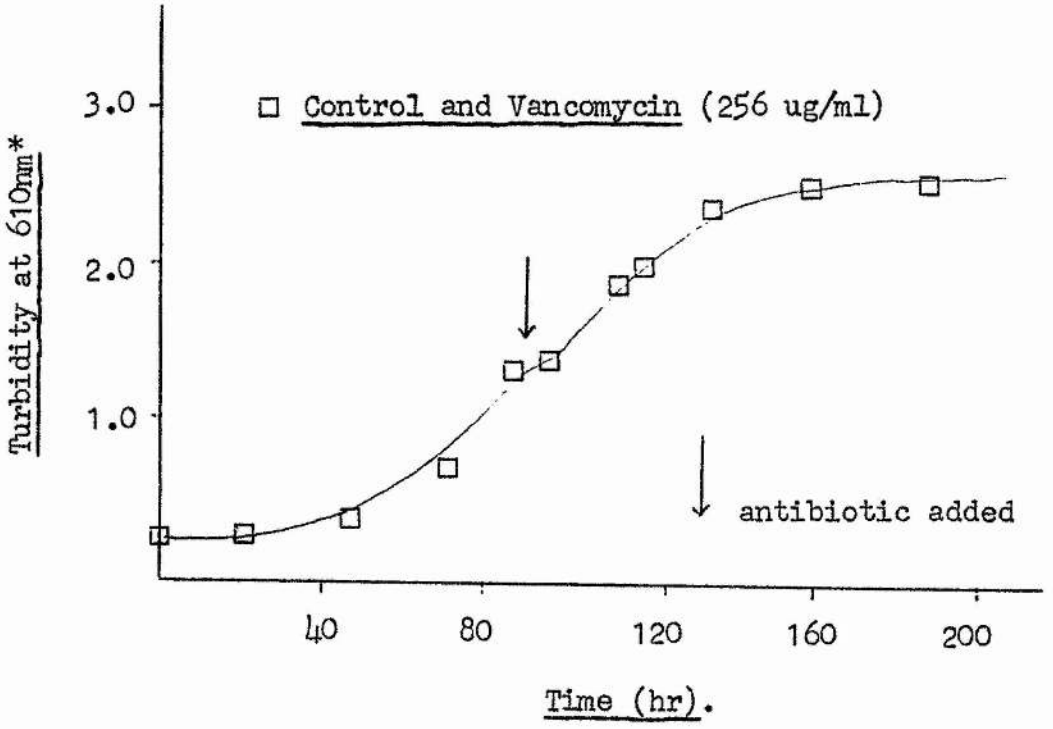


Figure R. 21

Effect of Vancomycin on S. marina Growth.




* Figures R.17 - R.21: Turbidity at 610nm was measured in optical density units.

mitted to further analysis in the electron microscope. The electron micrographs are discussed later (Section 3.6.2).

3.5.3 Detection of Antibiotics in Dundas Medium.

Because of the significant inhibitory effect of novobiocin and bacitracin on S. marina growth, further investigation was only required on D cycloserine, vancomycin and penicillin G to determine whether:

1) these antibiotics were actively taken up into the cell.
2) these antibiotics retained their activity in the extremely adverse conditions of Dundas medium.

It was, therefore, necessary to find methods for quantitatively detecting each of these antibiotics in Dundas medium.

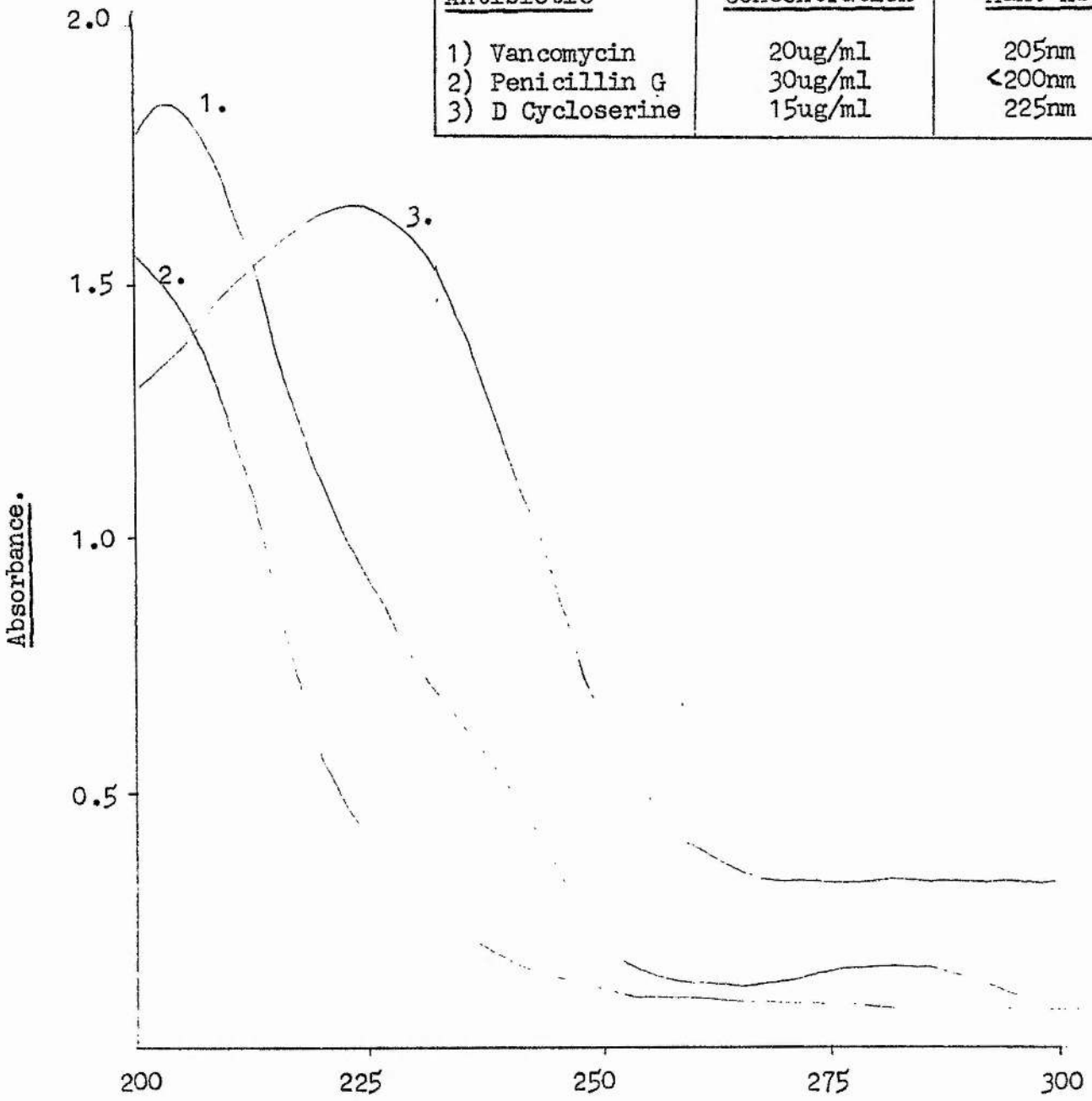
3.5.3.1 U.V. Scanning Spectroscopy.

The absorption spectra of D cycloserine, vancomycin and penicillin G were measured in sterile water, with sterile water as blank. All three antibiotics had some characteristic absorption peaks and these are shown in Figure R. 22. However, Dundas medium also demonstrated strong absorption in the same region. When the spectra of the antibiotics were rerun, with the antibiotics dissolved in Dundas medium and with a Dundas medium blank, these characteristic absorption peaks disappeared. It was concluded that absorption of the antibiotics at a specific wavelength could not be used as a means of detecting them in Dundas medium.

Figure R. 22

U.V. Scanning Spectroscopy of Antibiotics.

| Antibiotic | Concentration | Max. Abs |
|---|---|---|
| 1) Vancomycin | 20ug/ml | 205nm |
| 2) Penicillin G | 30ug/ml | <200nm |
| 3) D Cycloserine | 15ug/ml | 225nm |




| | |
|---|---|
| Reference: | Sterile Water |
| Path Length: | 10mm |
| Scan Speed: | Fast |

3.5.3.2 Bio-assay Technique - Agar Diffusion.

Calibration curves for both vancomycin and penicillin G were prepared as described (Section 2.5.2.2) and are shown in Figure R. 23. This technique showed a linear relationship between the logarithm of the antibiotic concentration and the square of the area of the zone of inhibition, measured in $mm^2$., for both vancomycin and penicillin G. Consequently, this method was used for the quantitative determination of these two antibiotics in Dundas medium.

The range of linearity was 20-65 units for vancomycin and 0.5-2.0 units for penicillin G.

3.5.3.3 D Cycloserine Determination in Dundas Medium.

A calibration curve for cycloserine was prepared, as described (Section 2.5.2.3), in both 0.1M NaOH and Dundas medium and is shown in Figure R. 24. Although the intensity of the blue colour, measured at 625 nm, was reduced in the Dundas medium, it still retained the linear relationship to the D cycloserine concentration. This method was, therefore, used for the quantitative determination of D cycloserine in Dundas medium.

The range of linearity for D cycloserine was 0-100 ug.

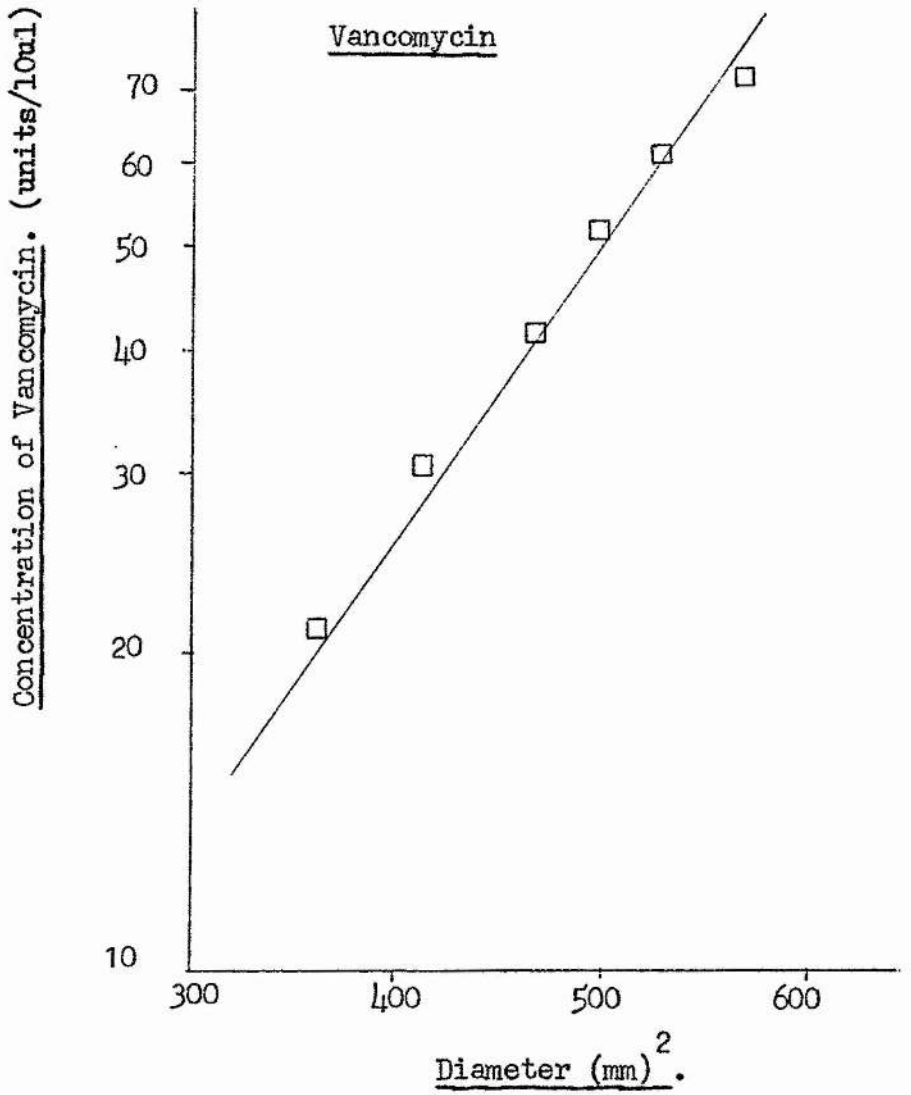
3.5.4 S. marina - Uptake of Antibiotics and Stability in Dundas Medium.

The experiment was conducted as described (Section 2.5.3) and the results are shown in Figure R. 25 and Table TR. 15.

Penicillin G and vancomycin both slowly decreased in their level of antibiotic activity over a period of hours in Dundas medium with proliferating cells. However, the corresponding control, with no S. marina cells present, had an identical loss of antibiotic activity. It was concluded that there was no, or very little, active uptake of the antibiotics. In fact, the loss

Figure R. 23

Calibration Curves of Vancomycin and Penicillin G (plotted on semi-logarithmic paper).



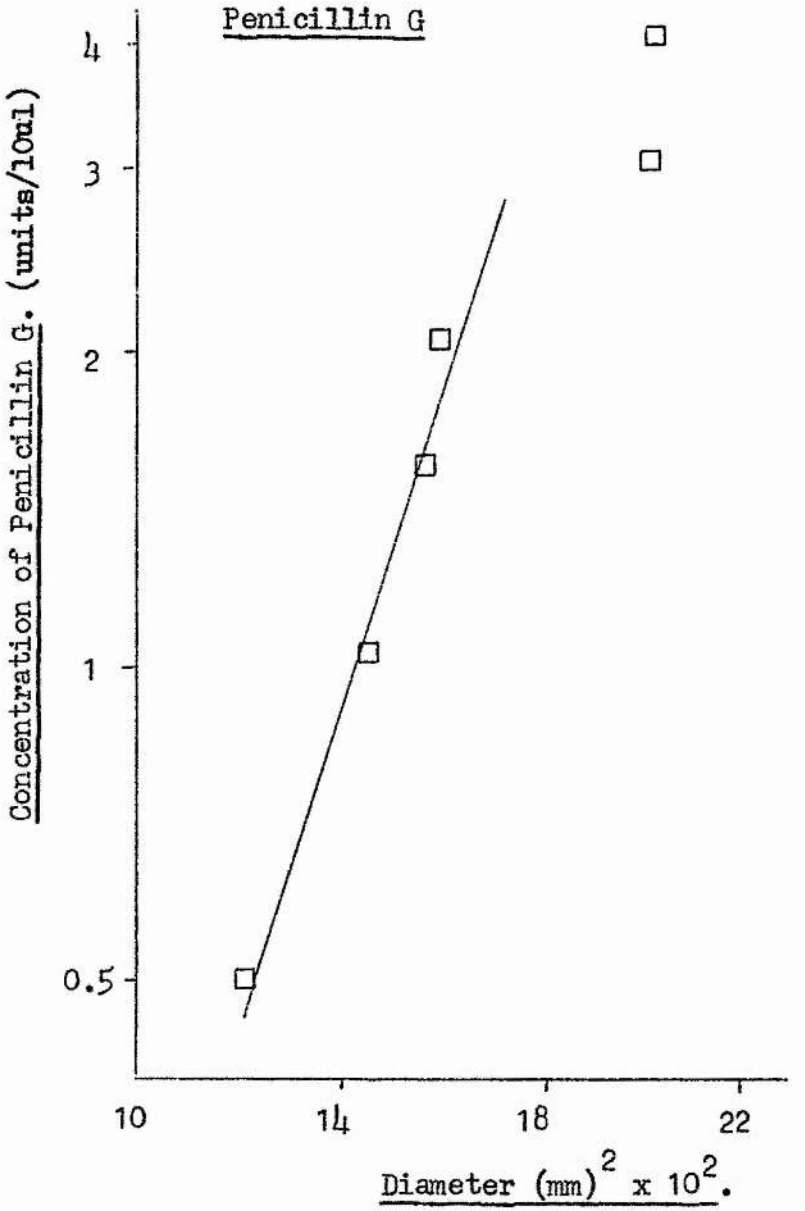
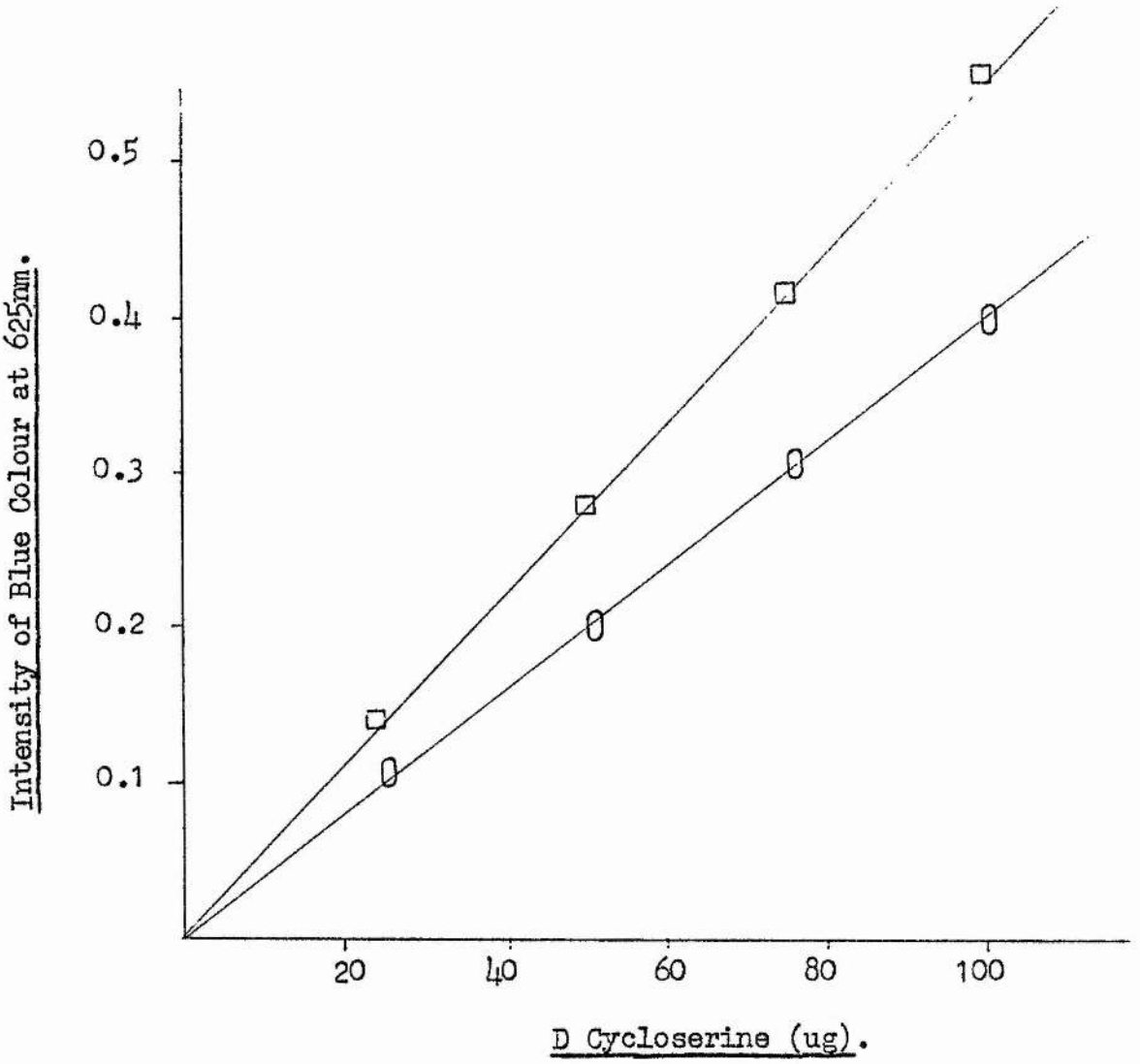



Figure R. 24

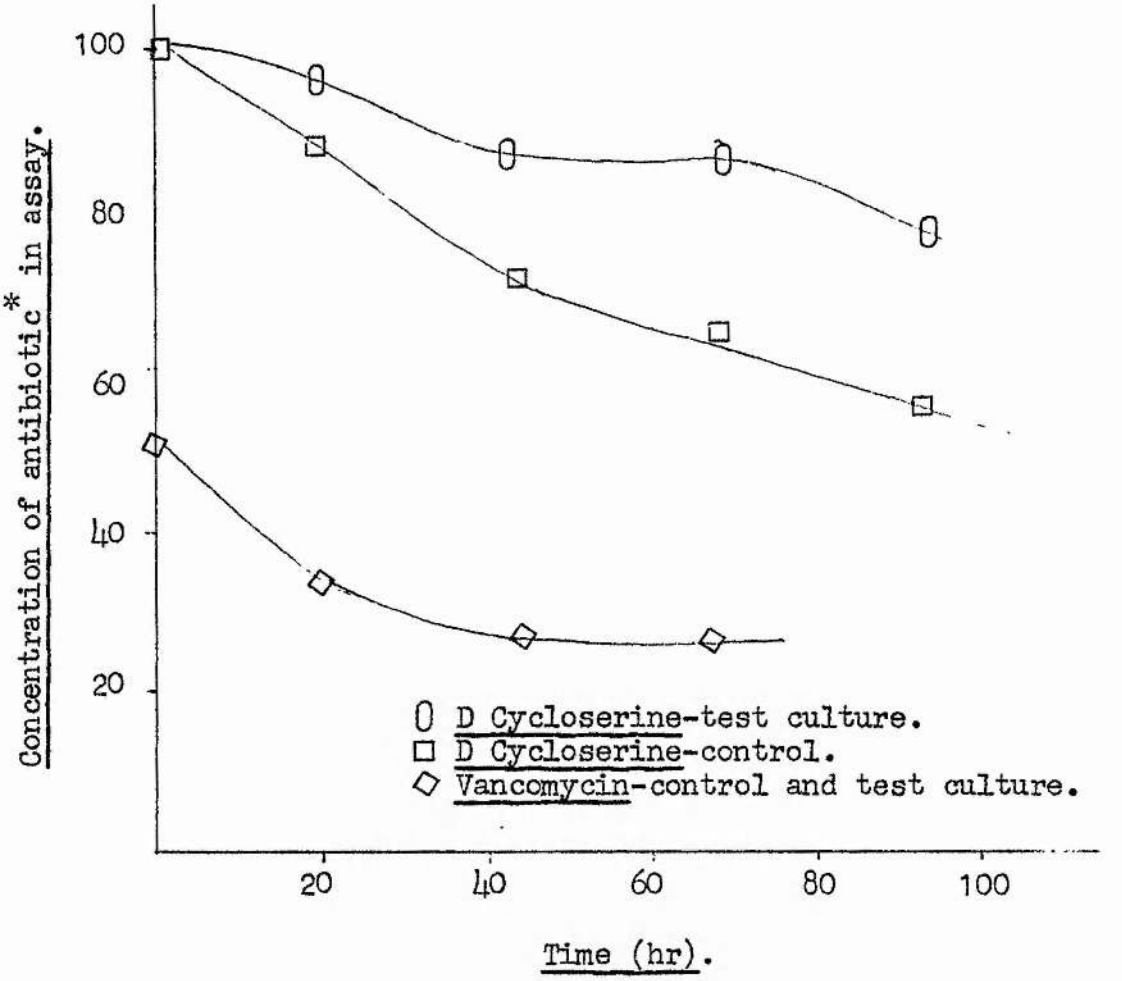
D Cycloserine Calibration Curves.




□ Calibration curve in 0.1N NaOH.
0 Calibration curve in Dundas medium.

Figure R. 25

Variation of Antibiotic Activity with Time in S. marina Cultures and Controls.




*D Cycloserine: 100ug/assay
Vancomycin: 50 units/assay

Table TR. 15

Variation in Penicillin G Activity with Time in Cultures of S. marina and Controls.

| Time (hr). | Sample (units). | Control (units). |
|---|---|---|
| 0 | 1.00 | 1.00 |
| 1 | 0.95 | 0.93 |
| 20.5 | 0.64 | 0.63 |
| 44.5 | - | - |

- below the range of linearity.

of activity is probably caused by the detrimental effect of the adverse conditions in Dundas medium on the antibiotics themselves.

Cycloserine, on the other hand, showed a marked difference in the loss of activity, which again occurred over a period of hours, between the control and the test culture. As the cycloserine activity was more rapidly lost in the control than in the test culture, this suggested that the cells, in some way, served to maintain the activity of the antibiotic. There are several possible explanations of this phenomenon and they are discussed later (see Discussion). However, as for penicillin G and vancomycin, there is no evidence of cycloserine being actively taken up by the cells.

The turbidity of each culture in the presence of the antibiotics was compared to a control containing no antibiotic. As before (Section 3.5.2), there was no turbidimetrically detectable difference in growth.

## 3.6 Electron Microscopy.

### 3.6.1 Cell Wall Isolation.

The effectiveness of the wall isolation method was assessed by electron microscopy. Sections from several different stages in the isolation were prepared using Method 2 (Section 2.6.1) and the electron micrographs are shown in Plates 1-9.

### Plates 1-3

The sections were prepared after two passes through the Hughes Press. Extensive breakage of the cells had occurred, the sections (Plate 1) being densely covered with cellular debris. However, despite the extreme conditions of temperature and pressure used, some intact cells still remained; but these were effectively removed at a later stage (Plate 7). At higher

magnification (Plates 2,3), the electron micrographs showed sections containing intact wall. Because of the low density of the cell contents compared to electron micrographs of whole cells (Plates 10,11), it is highly likely that some lysis had occurred in another plane.

Plates 4-6

The sections were prepared immediately prior to trypsin treatment. The exhaustive washings in Dundas basal salts removed the majority of the cytoplasmic debris, released when the cells were broken. Some of this contaminating material is, however, still visible although the wall fragments have become more prevalent in the section (Plate 4). Wall fragments, at higher magnification (Plates 5,6), had a regular particle-like arrangement on both the inner and outer surfaces.

Plates 7-9

The sections were prepared from the final isolated wall material. The breakage of the wall is restricted to only a few sites per cell. This is suprising in view of the extreme pressure and temperature conditions used in the Hughes Press and the sharp crack that normally accompanied disintegration. However, it is difficult to envisage a mechanism for increasing the surface area of the bacterial cell wall that does not involve structural weakness at the areas of active wall synthesis (92). These limited sites of wall breakage may be such areas or points of incipient weakness in the growing wall structure.

The proteolytic enzyme trypsin removed the majority of the remaining debris, including the cytoplasmic membrane (Plate 7). However, in the final wall preparation, there was still some contaminating material adhering

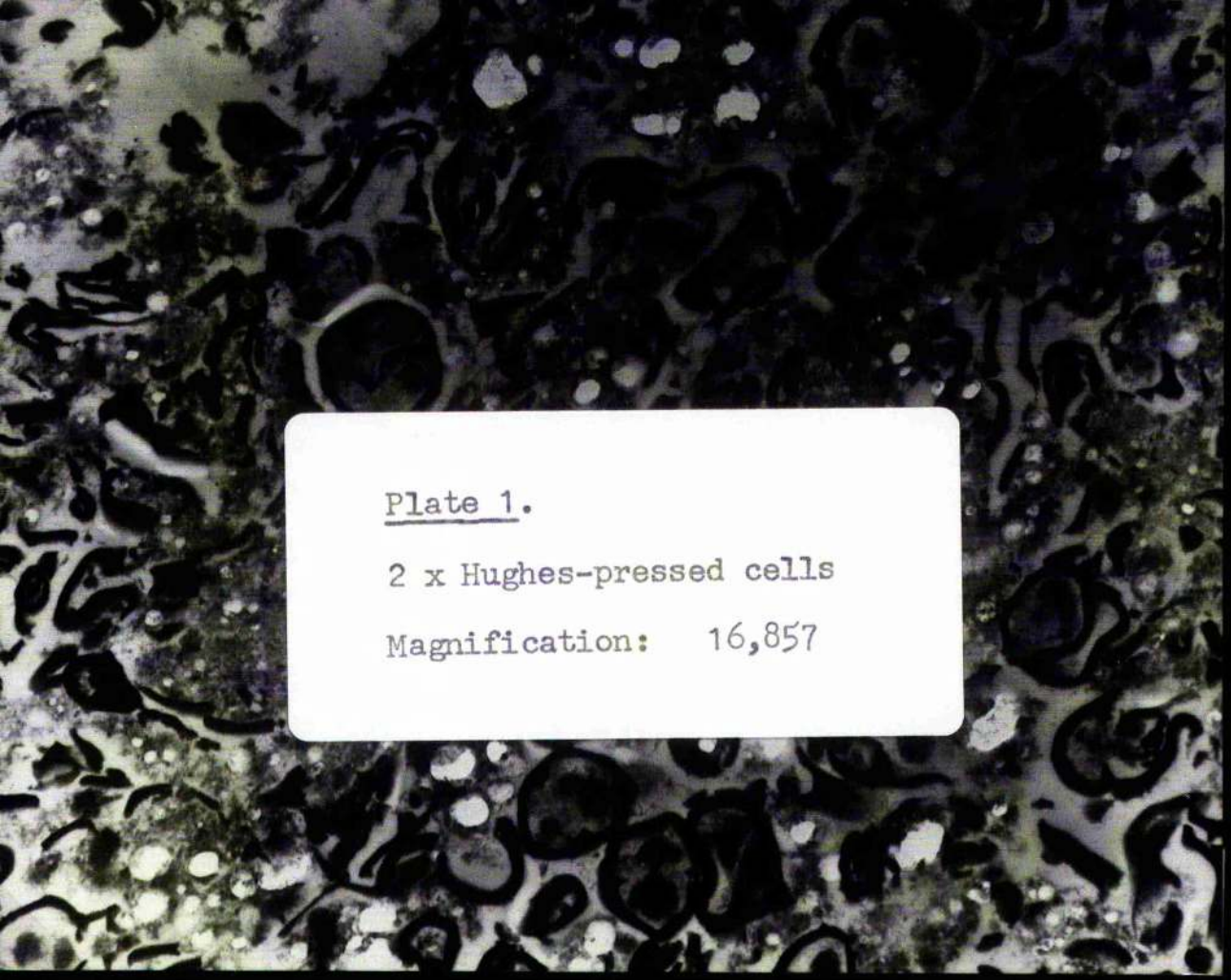


Plate 1.

2 x Hughes-pressed cells

Magnification: 16,857

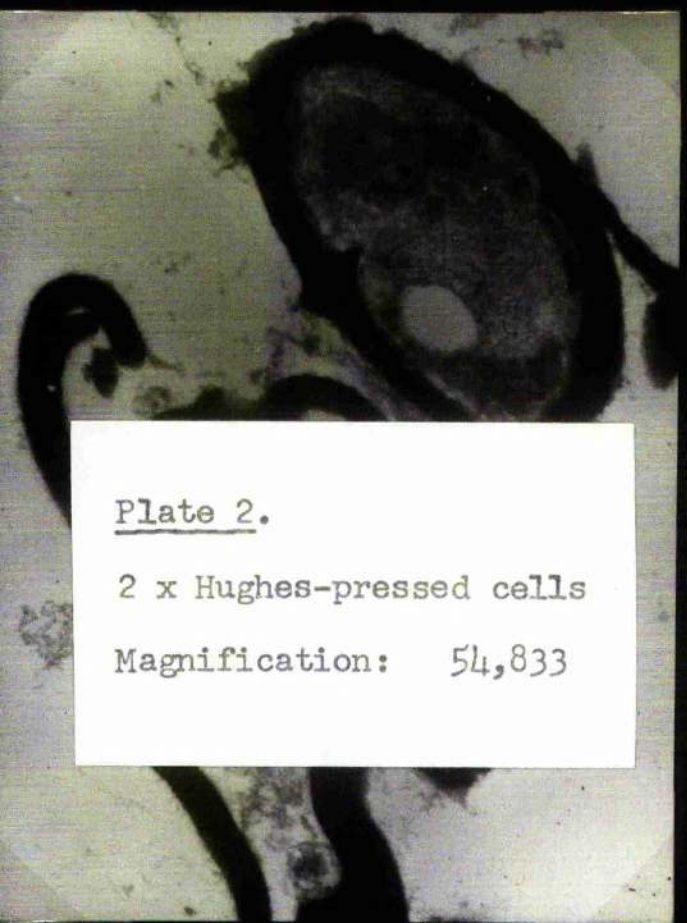


Plate 2.

2 x Hughes-pressed cells

Magnification: 54,833

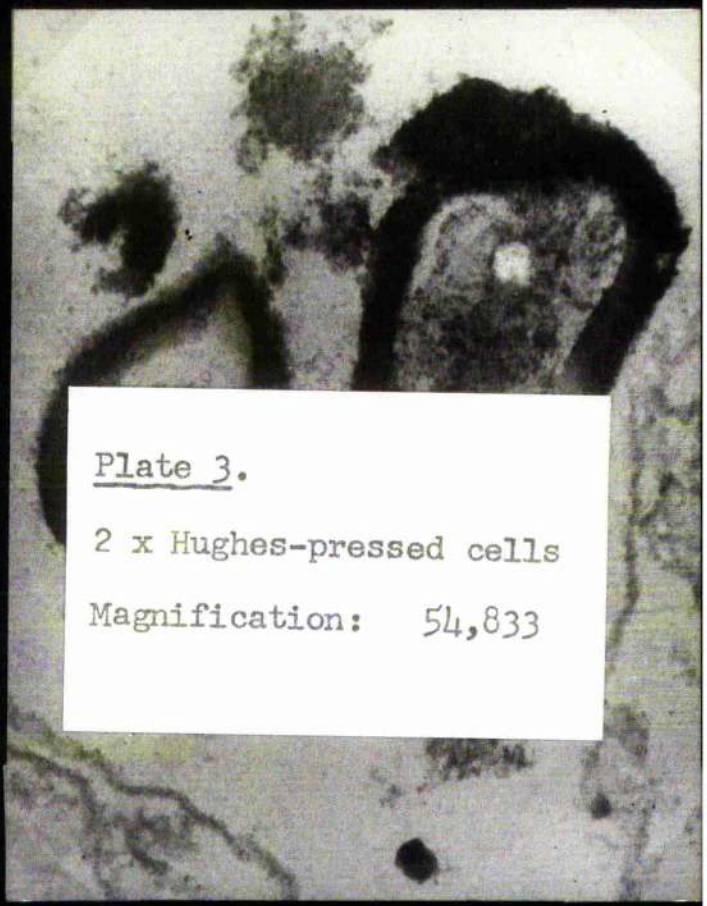
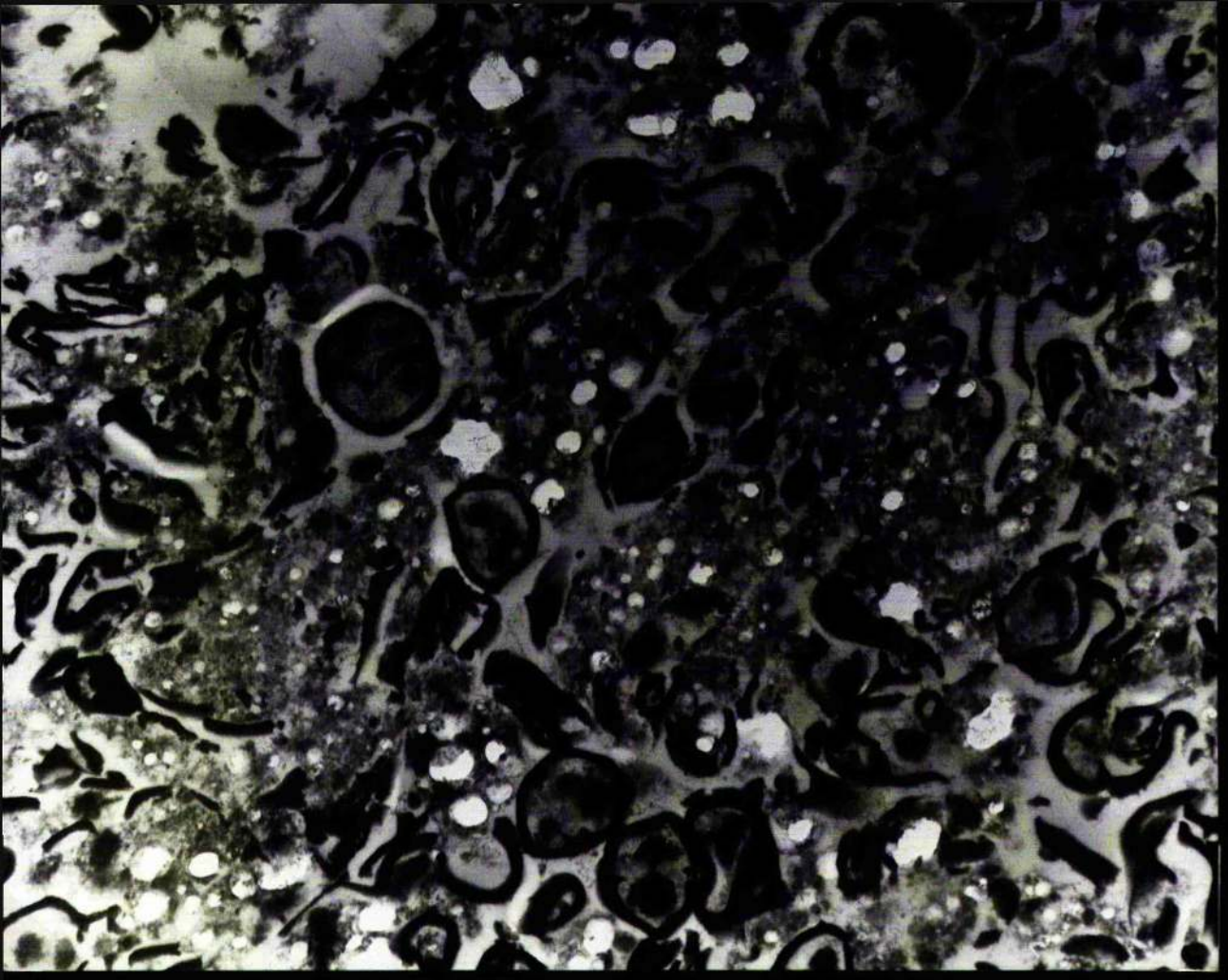
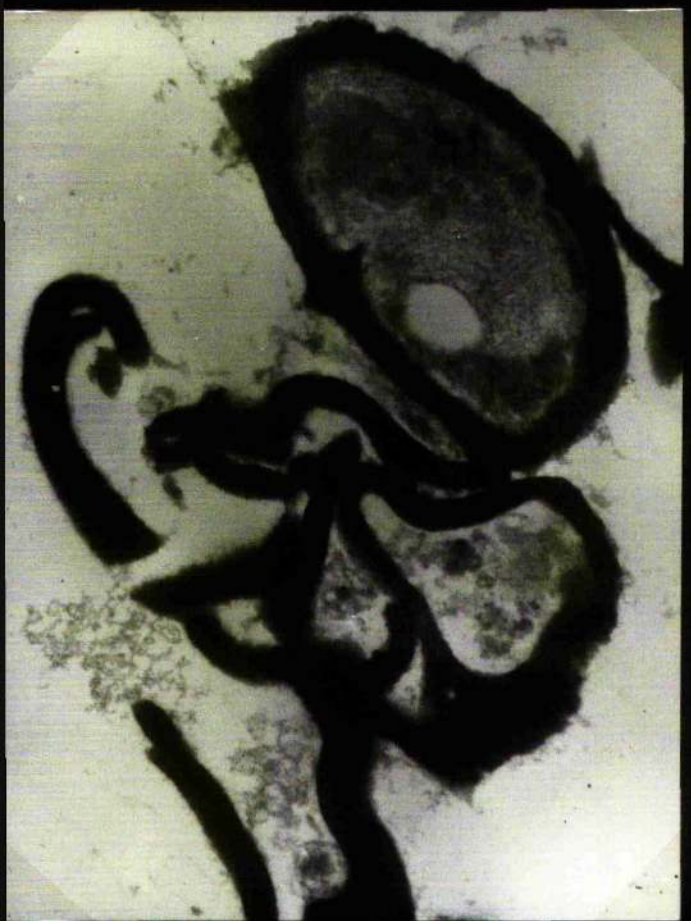


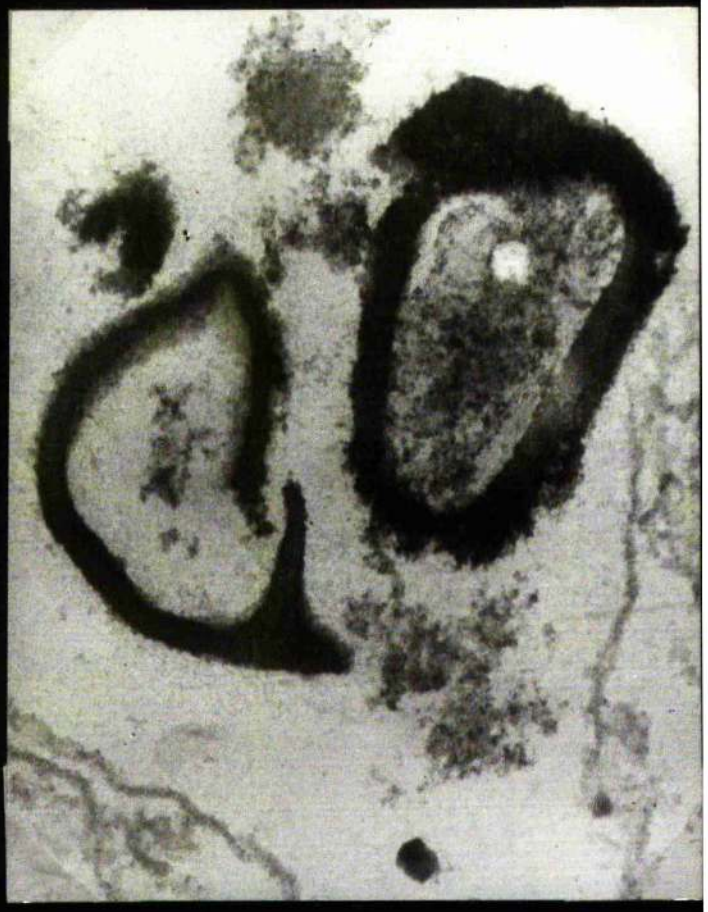
Plate 3.

2 x Hughes-pressed cells

Magnification: 54,833







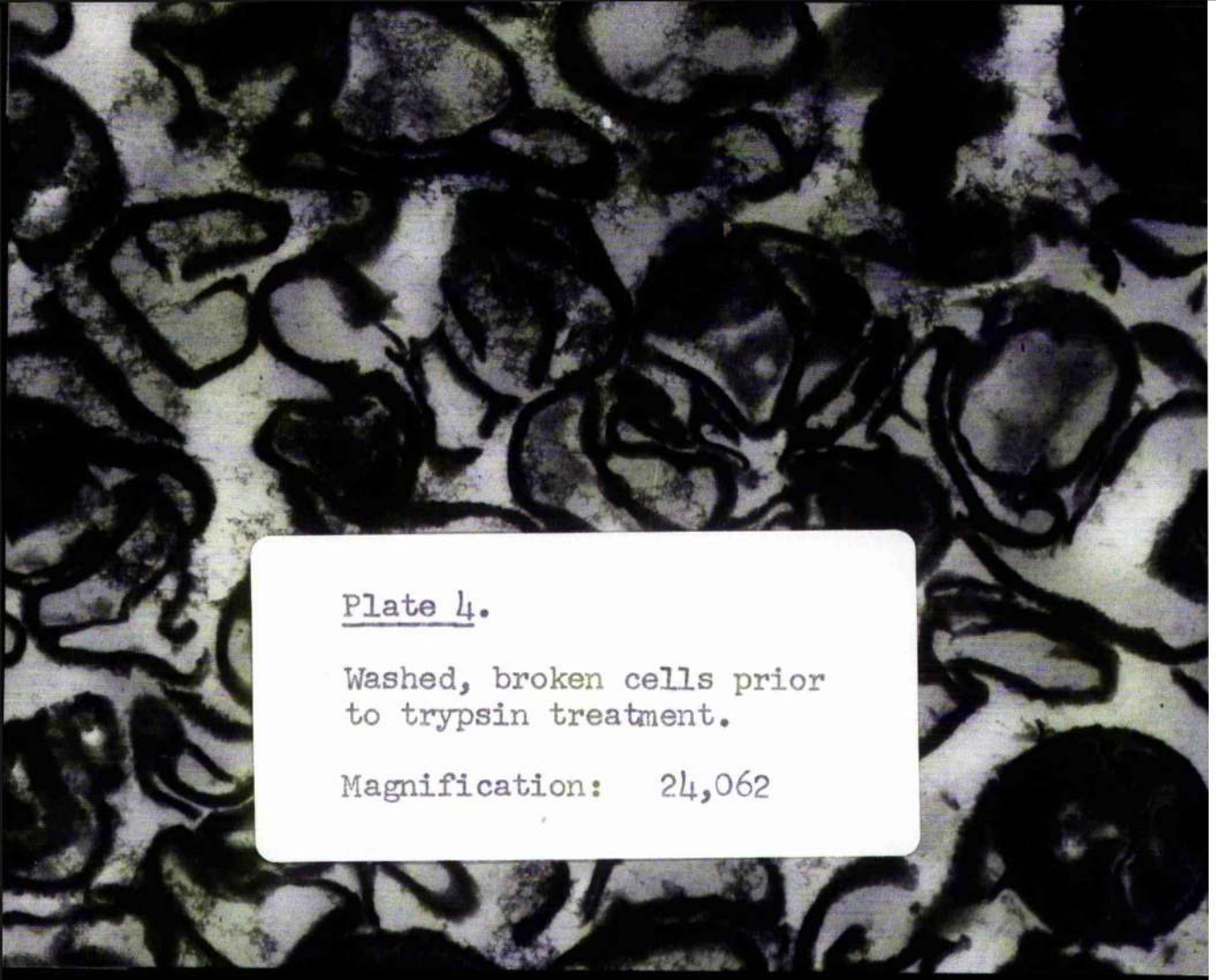


Plate 4.

Washed, broken cells prior to trypsin treatment.

Magnification: 24,062

Plate 5.

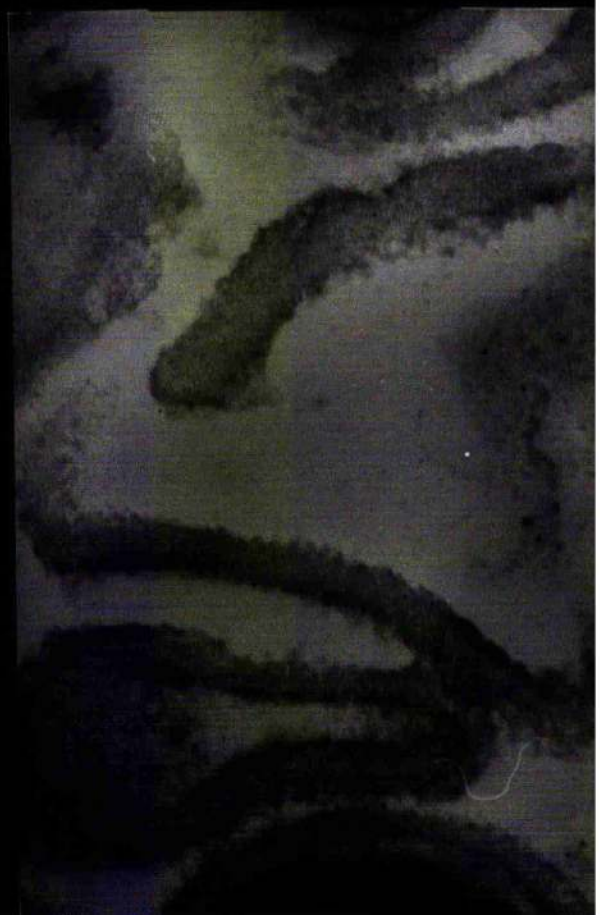
Washed, broken cells prior to trypsin treatment.

Magnification: 74,666

Plate 6.

Washed, broken cells prior to trypsin treatment.

Magnification: 74,666



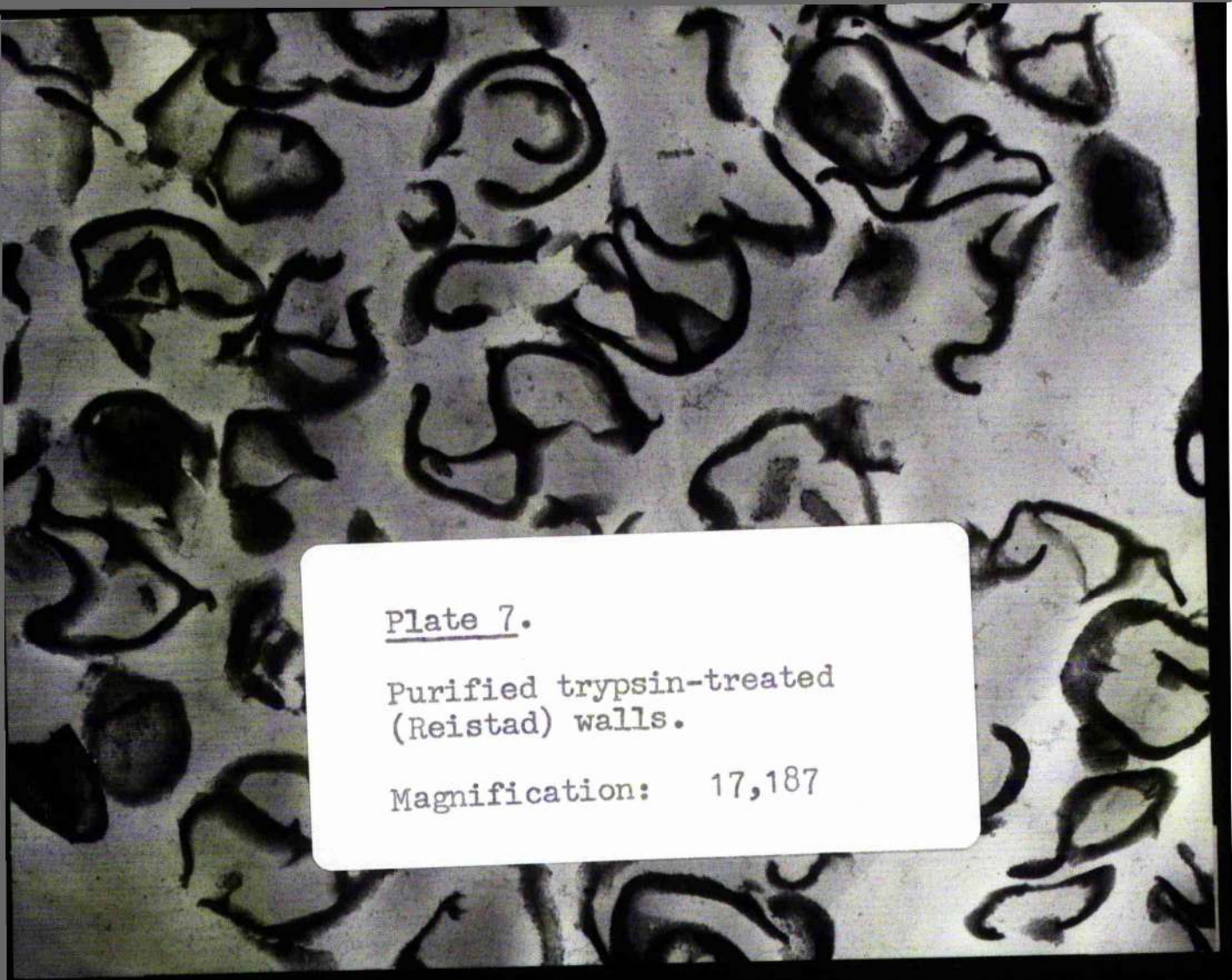


Plate 7.

Purified trypsin-treated (Reistad) walls.

Magnification: 17,187

Plate 8.

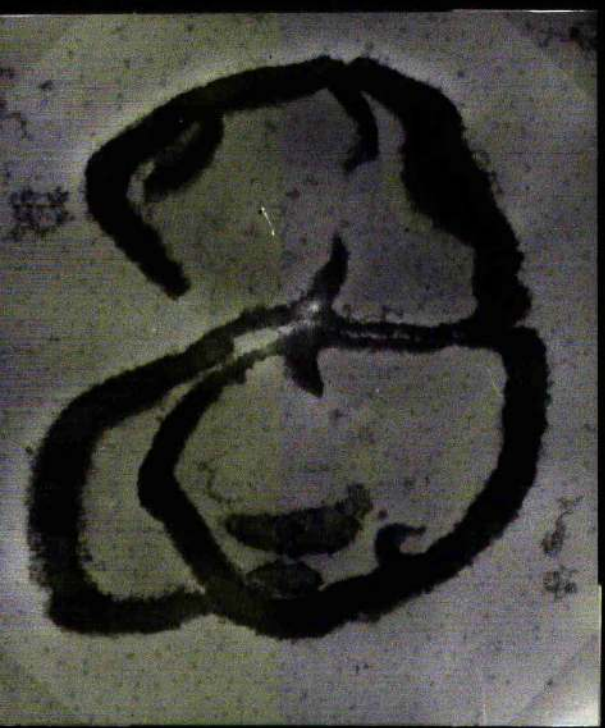
Purified trypsin-treated (Reistad) walls.

Magnification: 40,833

Plate 9.

Purified trypsin-treated (Reistad) walls.

Magnification: 50,166



to the wall (Plates 8,9). This residual contaminant is probably membrane fragments (9,11) and could be responsible for the pink colour of the wall. One significant feature, the regular particle-like arrangement seen on both the inner and outer surfaces of the wall (plates 5,6), was not visible after trypsin treatment, and they may be proteins.

3.6.2 Antibiotics.

The effect of the antibiotics on proliferating cells of S. marina was assessed both turbidimetrically (Section 3.5.2) and by electron microscopy. Sections from each of the antibiotically treated cells and control cells, with no antibiotic present, were prepared and the electron micrographs are shown in Plates 10-36.

3.6.2.1 Antibiotically Treated Cultures - Stationary Phase.

In the preliminary investigation, the antibiotics were added to a mid-logarithmic phase culture of S. marina, but the cells were not harvested till stationary phase. Sections were prepared using Method 1 (Section 2.6.1) but only the cycloserine treated cells embedded sufficiently for adequate preservation of fine structure (Plates 13-19).

The control cells (Plates 10-12) were from a late logarithmic phase culture of S. marina, and sections were prepared using Method 2 (Section 2.6.1), because it produced better structural preservation in the cells than Method 1. However, the lack of extensive fine structure preservation in the cells is probably due to penetration problems of the embedding resin. The wall and/or membrane of halobacteria are known to be very impermeable to, e.g. potassium ions (23), and this characteristic could account for the poor penetration of the embedding resin.

Although the control electron micrographs are from a late logarithmic phase culture of S. marina, and not stationary phase, they were included in this section for the purposes of comparison.

Plates 10-12

The sections were prepared from a control culture of S. marina in late logarithmic phase containing no antibiotic. The relatively thick cell envelope stained evenly and the cell wall consists of a single layer with a fuzzy appearance to the surface (Plates 10,11). Part of the cytoplasmic membrane is visible (Plate 10) and has the characteristic double striation of the classical 'unit' membrane. Structures resembling mesosomes can be faintly discerned and, although not clearly visible, the nuclear region appears in the sections (Plate 10) as fibrillar. The cytoplasm also contains gas vacuoles which may have a similar function to that of the halobacteria or other bacteria, allowing the cells to ascend giving them access to air in a water enviroment (19). The cells form cross walls prior to cell division (Plates 11,12) and this characteristic distinguishes the micrococci from the Gram-negative, rod-shaped bacteria which divide by a simple 'pinching off' process (55).

The anatomical features of S. marina, in thin section, are similar to those observed in other extremely halophilic cocci (9,10,55) and bear much resemblance to non-halophilic members of the family Micrococcaceae. This applies, in particular, to the cytoplasmic membrane, mesosome structures and the formation of cross walls prior to cell division. However, although the uniformly staining thick wall and envelope have the appearance of a typical micrococcal 'cell wall' (9,55), it has been observed (10) that both the dark-light-dark tribanded appearance to the wall, characteristic for

most of the Gram-positive cocci, and the double layered wall, characteristic for the Gram-negative cocci, were not present. In this respect, the cell wall of S. marina is very similar to the halobacteria which have, however, thinner walls (10).

Plates 13-16

The sections were prepared from a stationary phase culture of S. marina containing D cycloserine at 300ug/ml. There is a pronounced thickening of the cell wall (Plates 13,14,15) of 4-5 times that of the control (Plates 10, 11) and the D cycloserine cells have separated into individual entities rather than the pairs and tetrads in the control. However, although the antibiotically treated cell walls still have a fuzzy surface, these observations probably reflect the phase of growth rather than any antibiotic effect of D cycloserine.

Structures resembling mesosomes, with the characteristic onion-like cross section, are easily discernable (Plates 13,14,15) and the nuclear region, now clearly visible (Plate 16), is indeed fibrillar. The triad of cells (Plate 16) are comparable in section to the controls although the cross walls appear somewhat thicker. The slight invagination (Plate 16) may be the site of new cross wall initiation.

Plates 17-19

The sections were prepared from a stationary phase culture of S. marina containing D cycloserine at 600ug/ml. Although the cell has been lysed (plate 17), but at only a limited number of sites, the overall contour of the wall has been maintained. As before (Plates 13-16) this is probably a natural process caused by the phase of growth rather than by any antibiotic

effect of D cycloserine. In fact, segments of cells with pronounced thickening of cell wall, similar to those observed before (Plates 13-15), are visible (Plate 18). In the lysed cell (Plate 17) there is little cytoplasmic material left in the cell, the internal pressure of the intact cell forcing most of the cytoplasm out when the wall was ruptured.

A system of membranes is easily detectable in the broken cells (Plate 17) and these membranes may stem in part from the mesosomes and in part, as they are localised along the cell wall, from the cytoplasmic membrane. In the lysed cell (Plate 17), one of the membranes has formed a closed vesicle indicating that it resealed after releasing the cytoplasmic material.

An extracellular closed vesicle (Plate 19), however, shows an increase in the size of the membrane bound structure compared to intact cells (Plate 10), although, as before, the loss of the majority of the cytoplasmic material indicates that the membrane has resealed. The increased membrane surface area could be due to the unfolding of the mesosomes or the combination of membrane material from more than one cell.

The membrane is clearly visible in the closed vesicle (Plate 19) and the unsealed membrane (Plate 18) and, with its double striated structure, has the appearance of the classical 'unit' membrane. In the unsealed membrane (Plate 18), although the majority of the cytoplasm has been lost, a fibrous network, which is almost certainly DNA, is in the process of being extruded. The increased size of the membrane is also clearly evident as segments of intact cells are visible at two corners in this section (Plate 18).

3.6.2.2 Antibiotic Treated Cultures - Late Logarithmic Phase.

The antibiotics were added to early logarithmic phase cultures of S. marina and the cells harvested in late logarithmic phase (Section 3.5.2). Sections were prepared for electron microscopy using Method 2 (Section 2.6.1) because, as mentioned before, it produced better structural preservation in the cells than Method 1.

The electron micrographs of the antibiotically treated cells are shown in Plates 20-36. The sections from the control cells (Plates 10-12) were included in Section 3.6.2.1 for the purpose of comparison.

Plates 20-22

The sections were prepared from D cycloserine treated, late logarithmic phase cultures of S. marina. As the antibiotic had no turbidimetrically detectable effect on growth (Figure R. 17), the sections are from the culture containing the maximum concentration of D cycloserine tested, 256ug/ml.

The D cycloserine treated cells appear, in section (Plates 20,22), very similar to the controls (Plates 11,12). The cell wall consists of a single layer with a fuzzy appearance to the surface and there is extensive evidence of cross wall formation. However, there might be a slight plasmolysis, with the membrane coming away from the wall, particularly the cross walls. A closed vesicle (Plate 21) was also seen. Although the vesicle has lost most of the cytoplasmic material, the membrane structure is not clearly visible, unlike the previous observations (Plates 18,19).

Plates 23-26

The sections were prepared from bacitracin treated, late logarithmic phase cultures of S. marina. As the antibiotic significantly inhibited

growth (Figure R. 19), the sections are from two concentrations of bacitracin, 64ug/ml (Plate 26) and 256ug/ml (Plates 23-25).

The anatomical features of the bacitracin treated cells, at both concentrations, show no visually detectable differences to the controls (Plates 10-12).

Plates 27-30

The sections were prepared from vancomycin treated, late logarithmic phase cultures of S. marina. The antibiotic had no turbidimetrically detectable effect on growth (Figure R. 21) and the sections are from the single concentration of vancomycin tested, 256ug/ml.

There are several structural aspects of the cells that are easily detected. The cells occur in sarcina-like packages (Plate 27) and higher magnification of this section reveals both a partially completed cross wall (Plate 30) and cross wall formation shortly after its initiation (Plate 29). However, the anatomical features of the vancomycin treated cells, like bacitracin, show no visually detectable differences to the controls (Plates 10,11,12).

Plates 31-33

The sections were prepared from penicillin G treated, late logarithmic phase cultures of S. marina. The antibiotic had only slight, if any, effect on growth (Figure R. 20) and the sections are from the single concentration of penicillin G tested, 256ug/ml.

The penicillin G treated cells stain less heavily which, in conjunction with better embedding, may imply a real effect of the antibiotic. There are also several interesting features in these cells. The dark line at the

centre of the septum (Plate 32) may represent the hydrophilic channel thought to exist at the centre of common septa in Gram-positive microorganisms. This is possibly the site of teichoic acids and/or autolysins. There are also 4-5 distinct regions of probably nuclear material visible in the sections (Plates 31,33), indicating either multiple nuclei or a rather unusual 3 D configuration for a single nucleus to produce this type of image on sectioning.

Plates 34-36

The sections were prepared from novobiocin treated, late logarithmic phase cultures of S. marina. The antibiotic significantly inhibited growth (Figure R. 18) and the sections are from the maximum concentration of novobiocin tested, 256ug/ml.

The anatomical features of the novobiocin treated cells appear similar, in section, to the controls (Plates 11,12). However, there may be a slight plasmolytic effect (Plate 35) similar to the one already observed in D cycloserine treated cells (Plates 20,22), with the cytoplasm coming away from the cell wall.

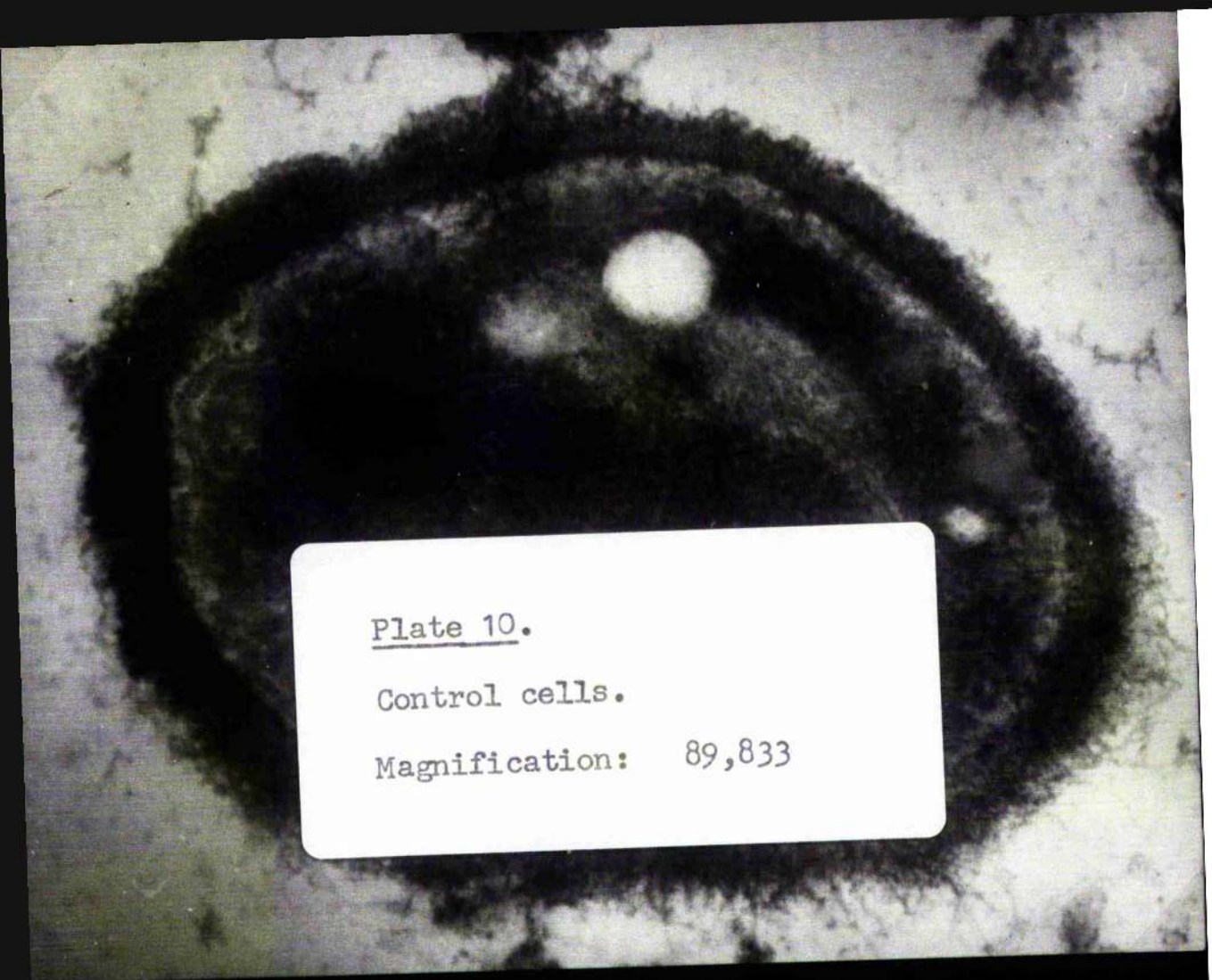


Plate 10.

Control cells.

Magnification: 89,833

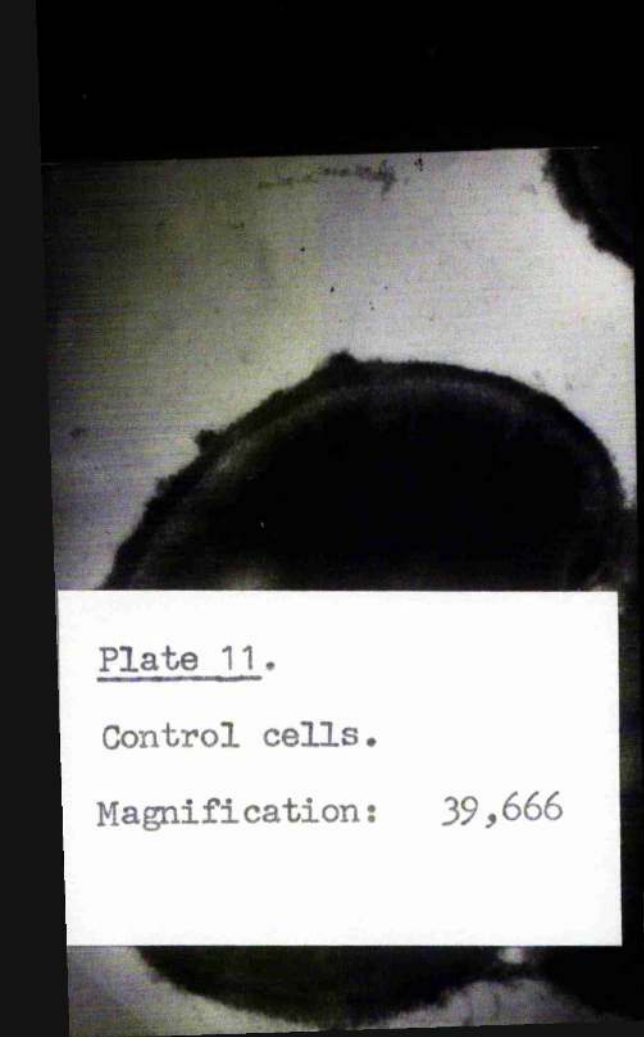


Plate 11.

Control cells.

Magnification: 39,666

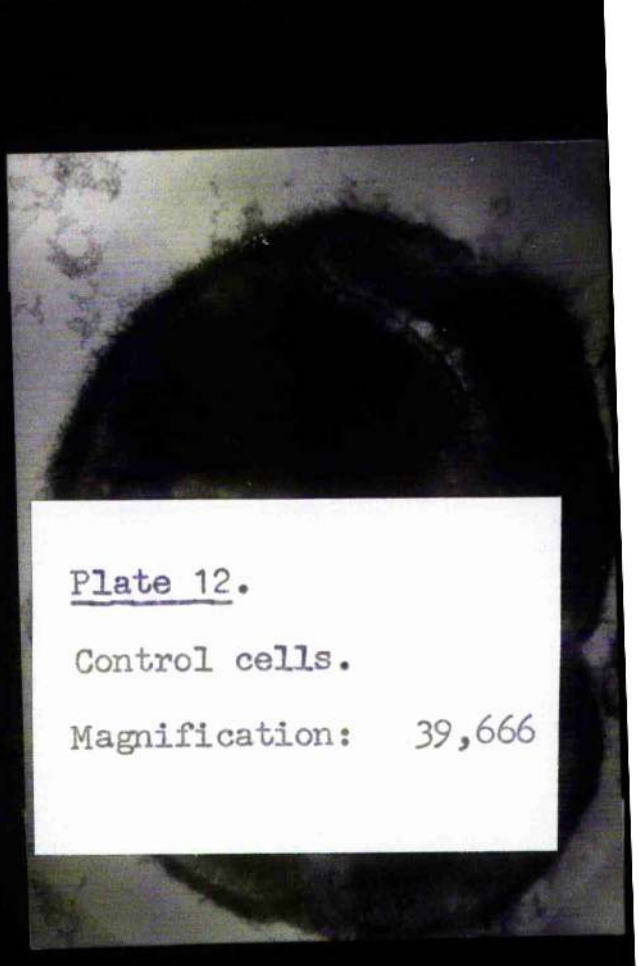


Plate 12.

Control cells.

Magnification: 39,666

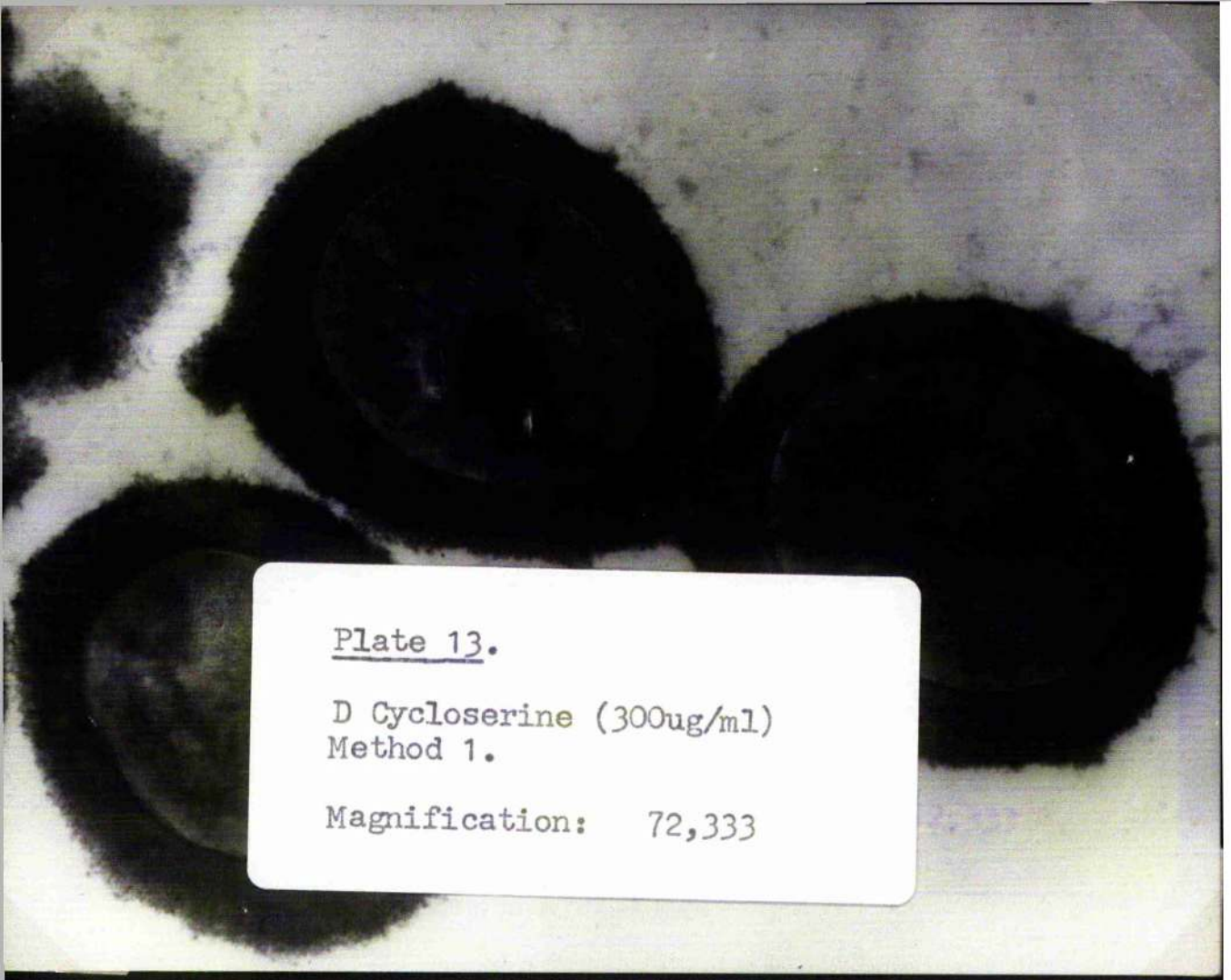


Plate 13.

D Cycloserine (300ug/ml)
Method 1.

Magnification: 72,333

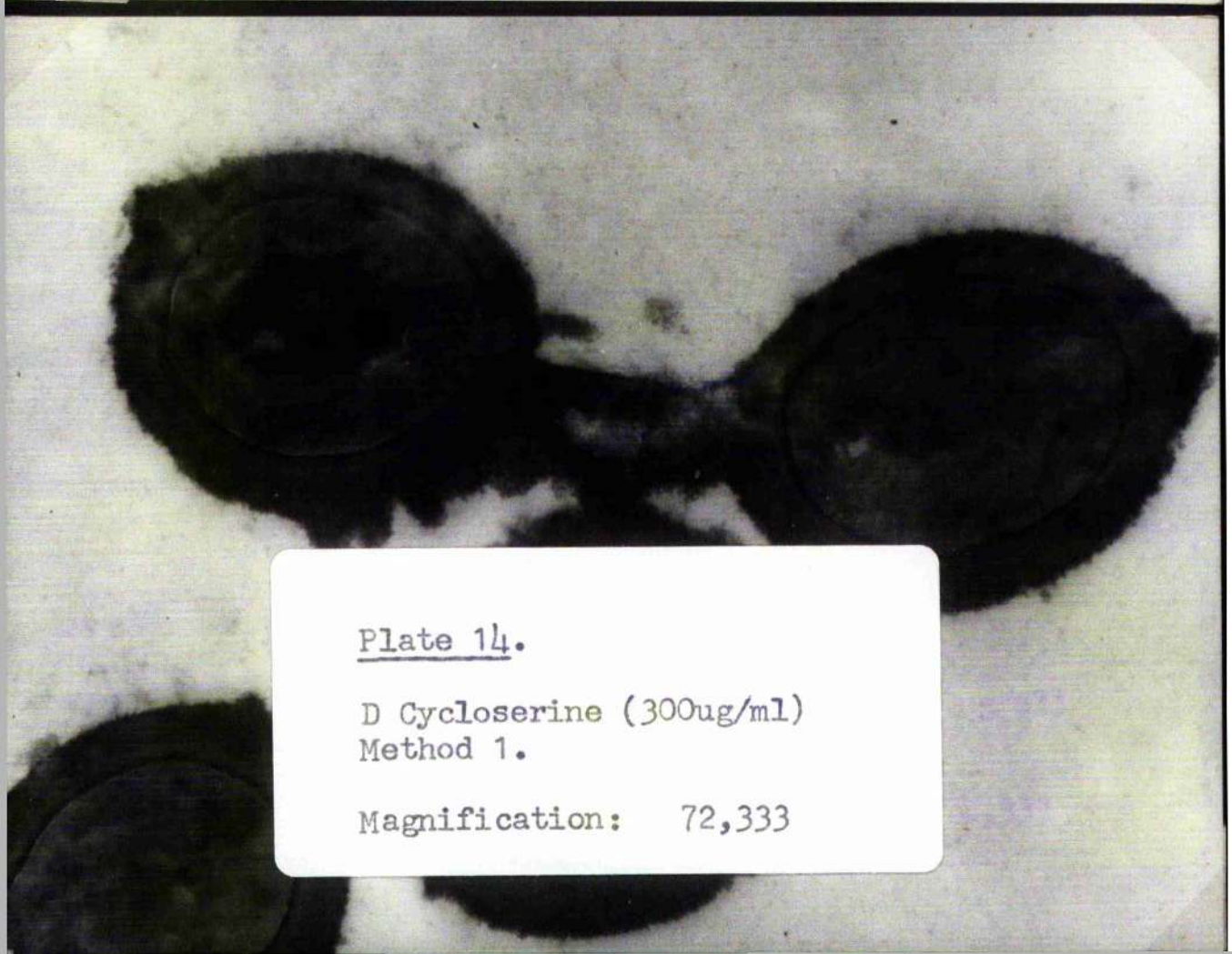
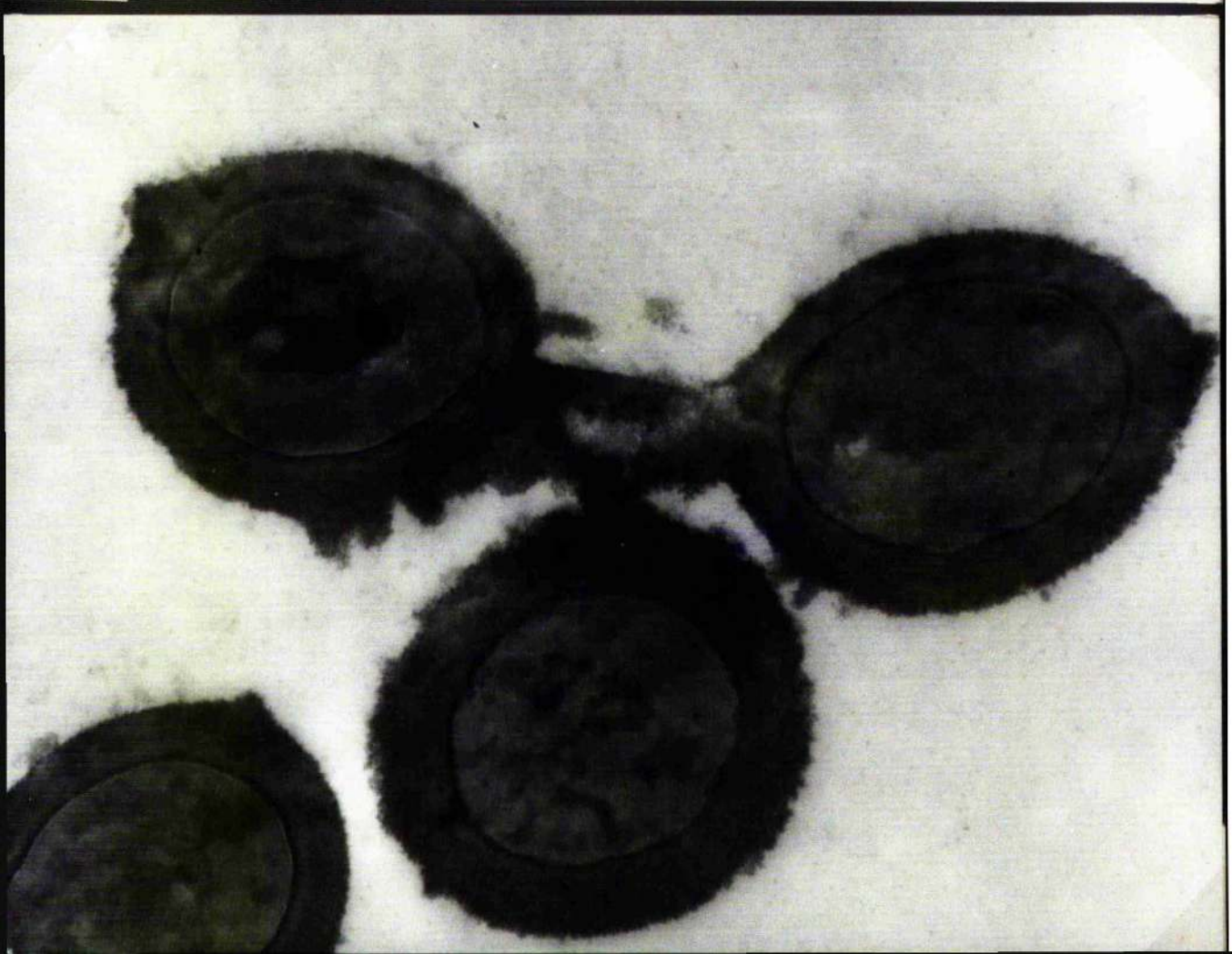


Plate 14.

D Cycloserine (300ug/ml)
Method 1.

Magnification: 72,333



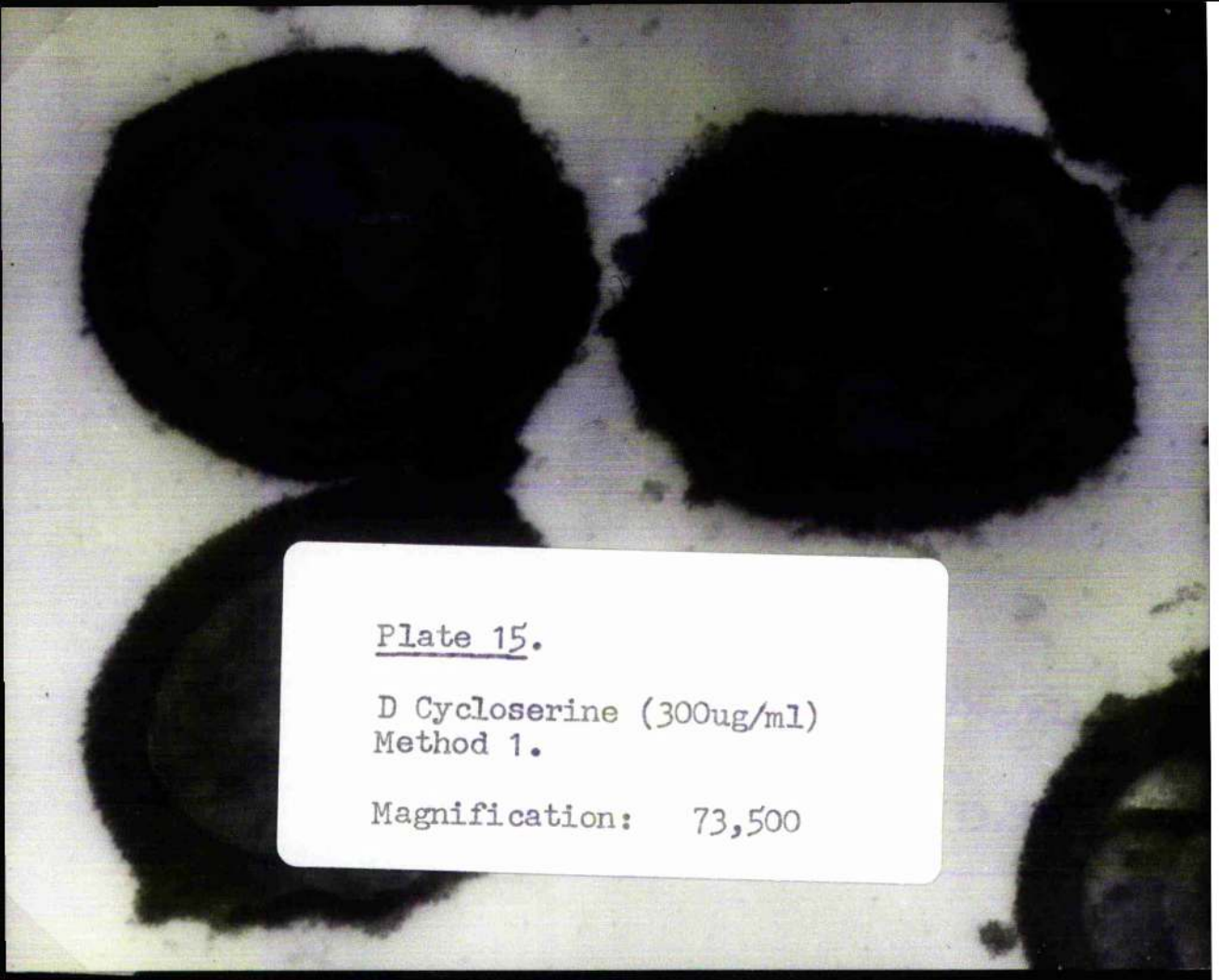


Plate 15.

D Cycloserine (300ug/ml)
Method 1.

Magnification: 73,500

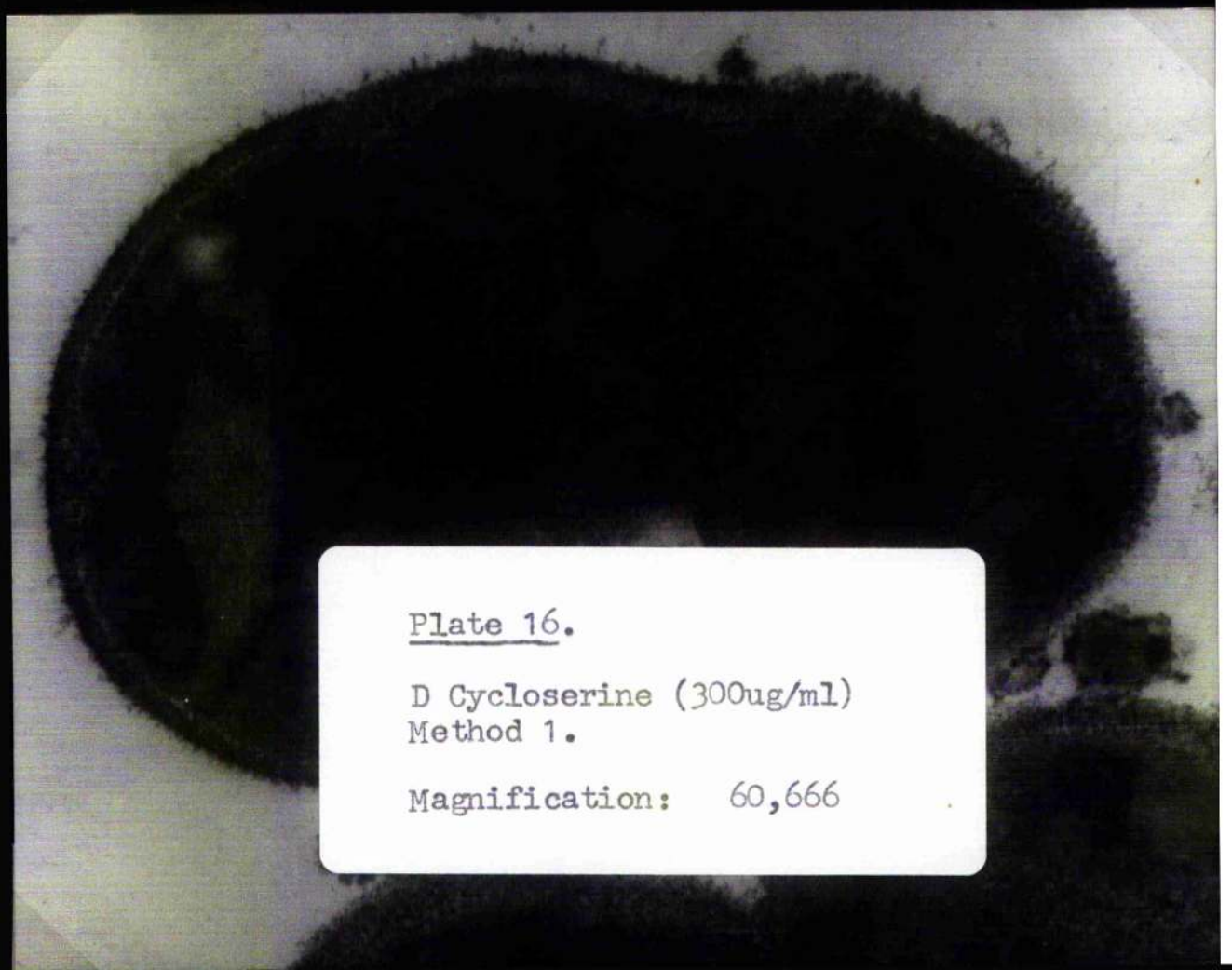
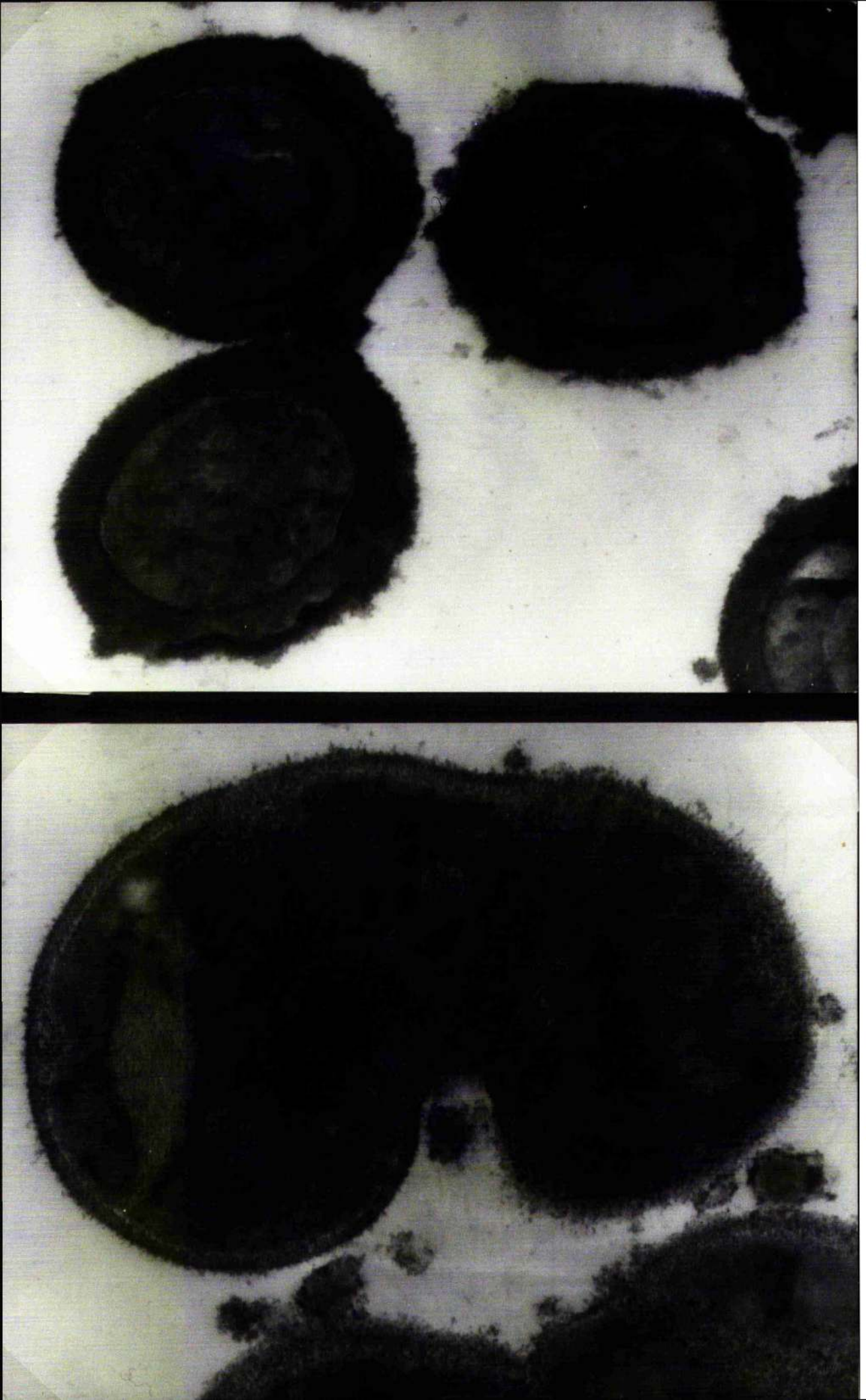


Plate 16.

D Cycloserine (300ug/ml)
Method 1.

Magnification: 60,666



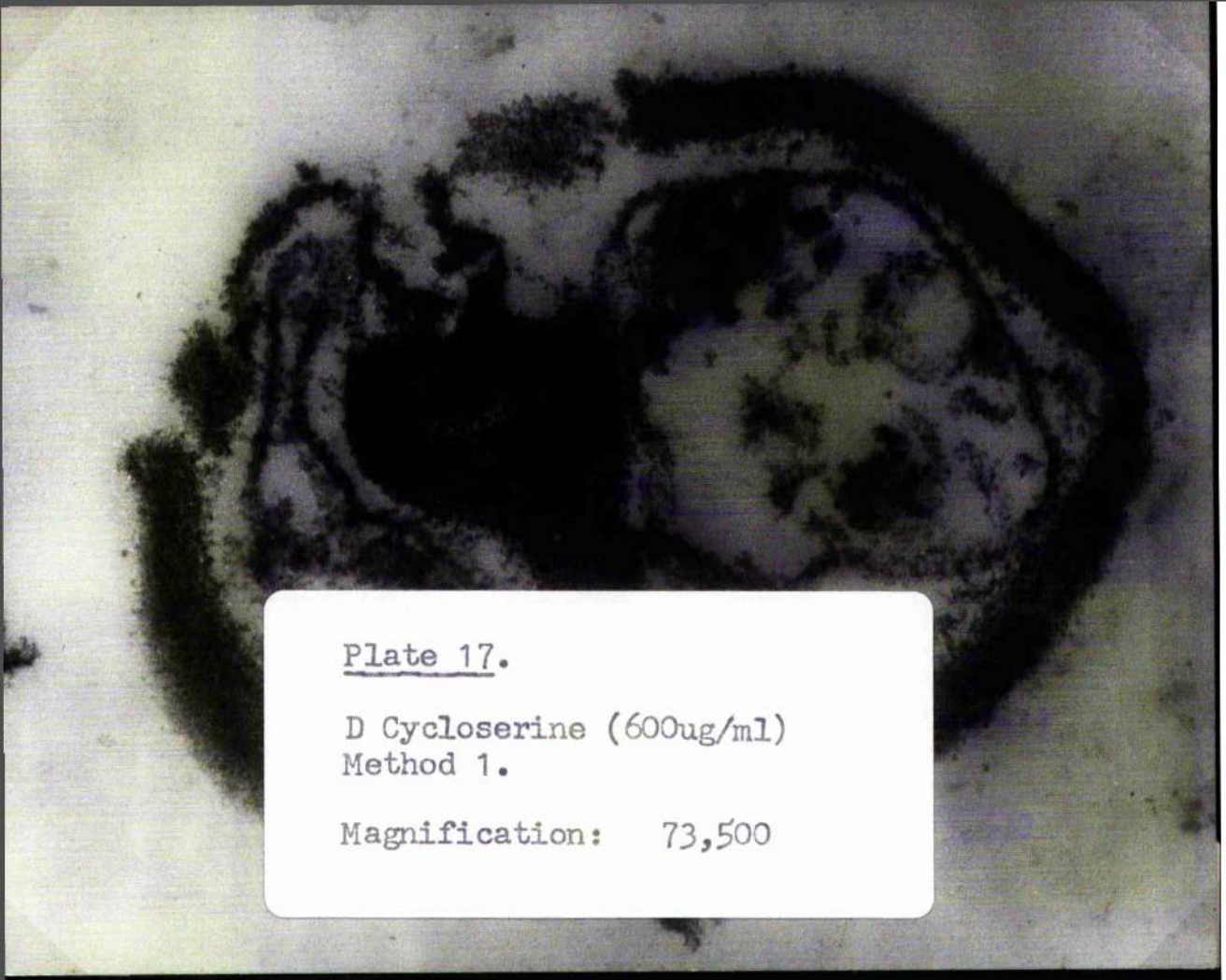


Plate 17.

D Cycloserine (600ug/ml)
Method 1.

Magnification: 73,500

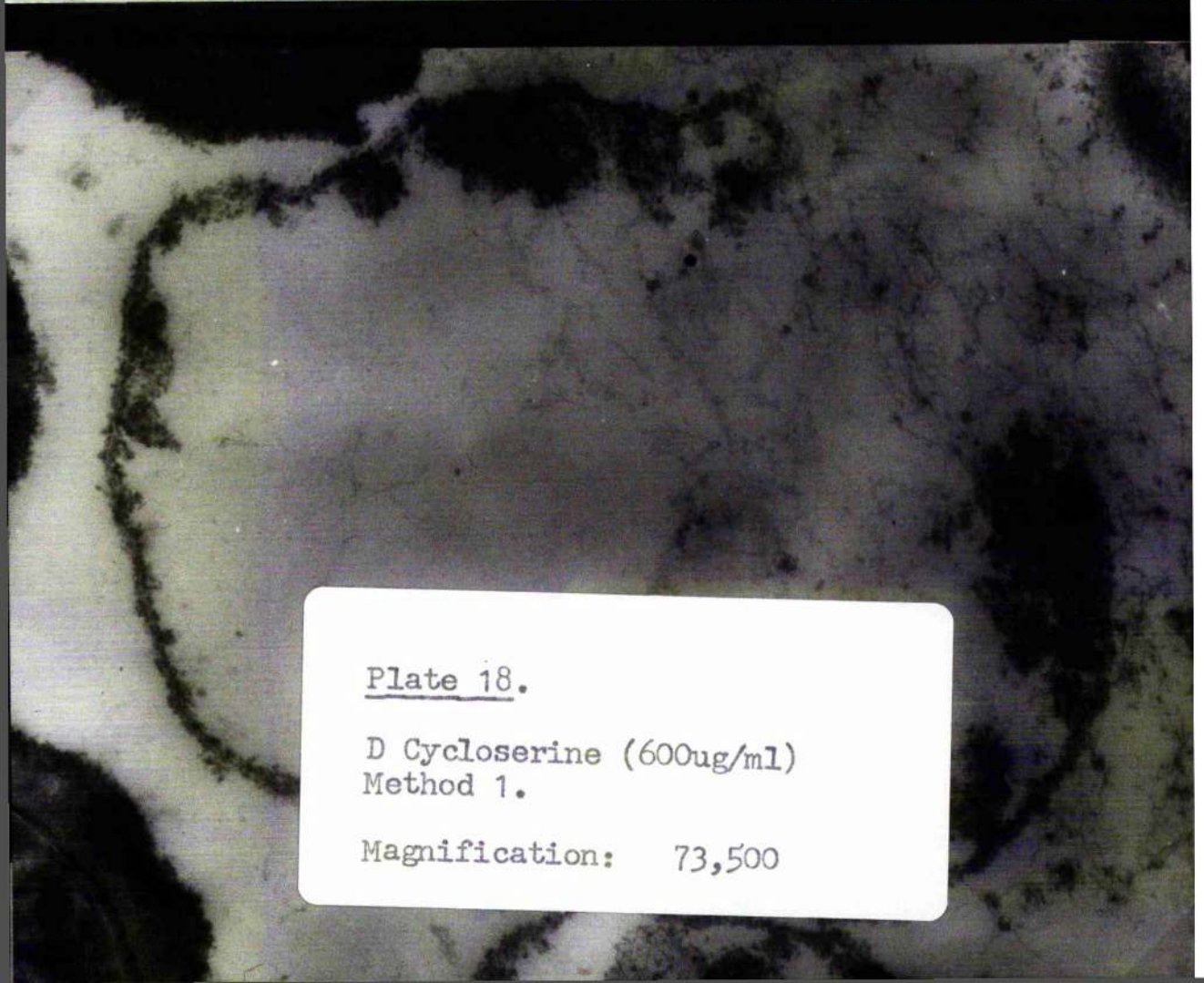
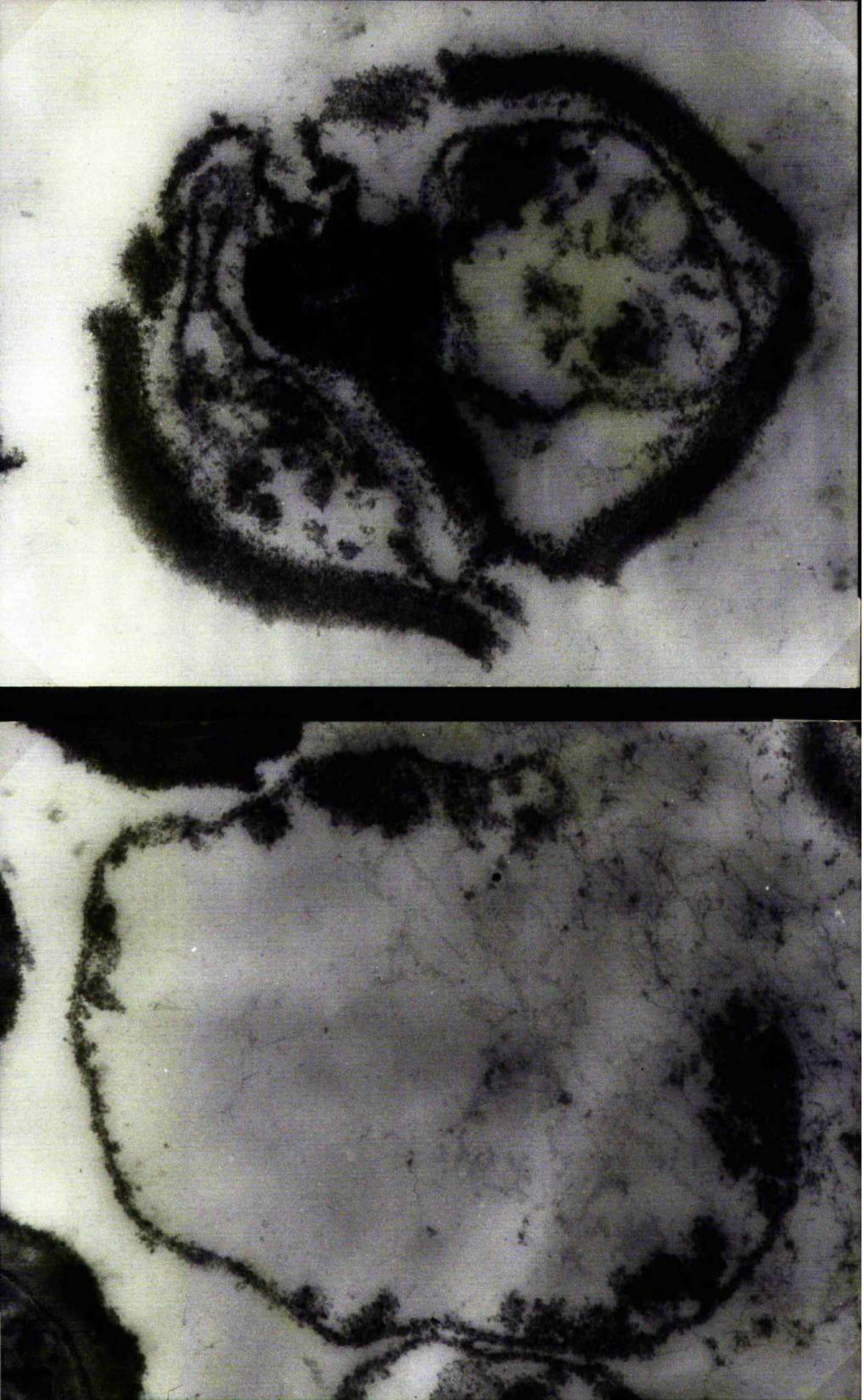


Plate 18.

D Cycloserine (600ug/ml)
Method 1.

Magnification: 73,500



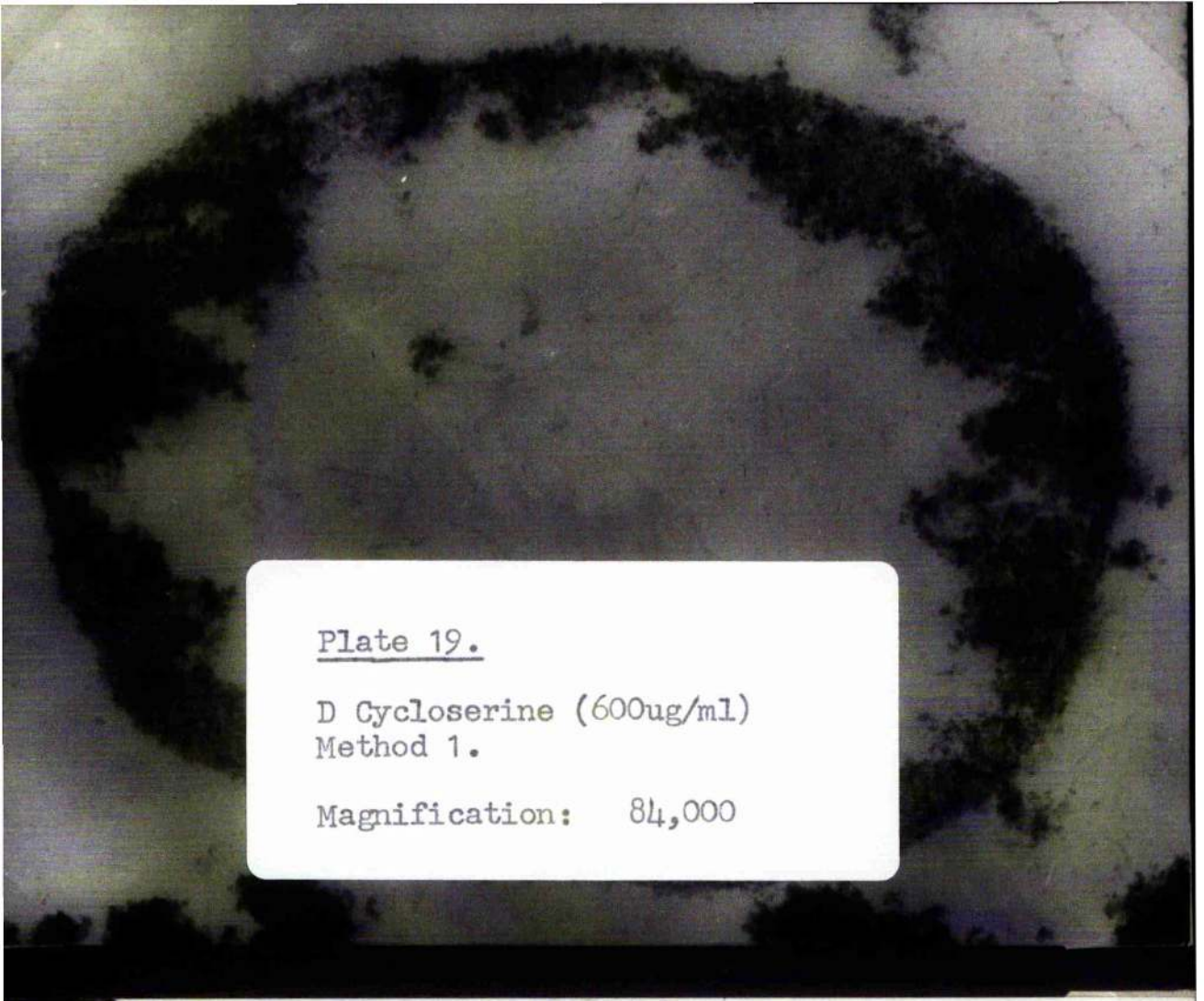


Plate 19.

D Cycloserine (600ug/ml)
Method 1.

Magnification: 84,000

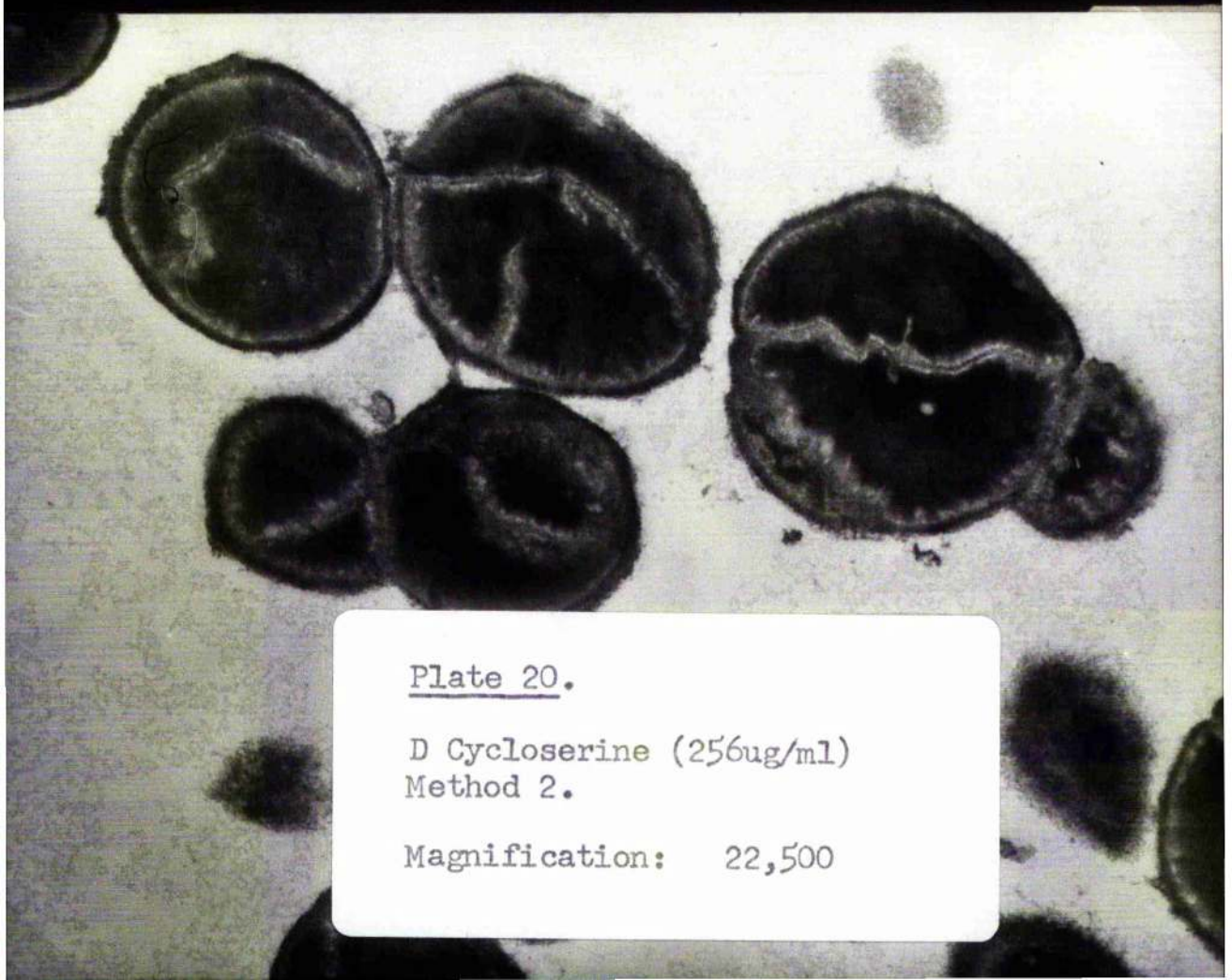
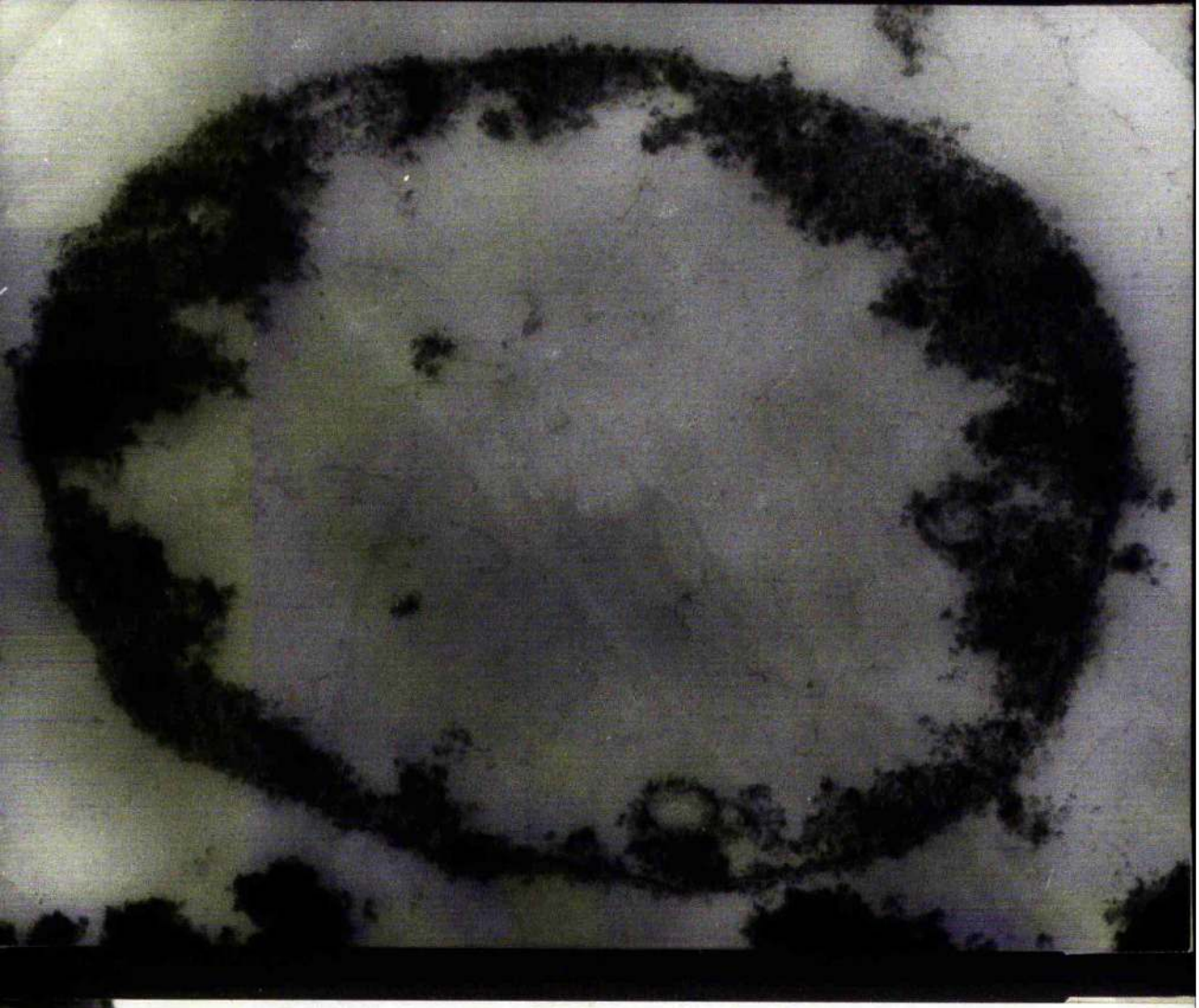
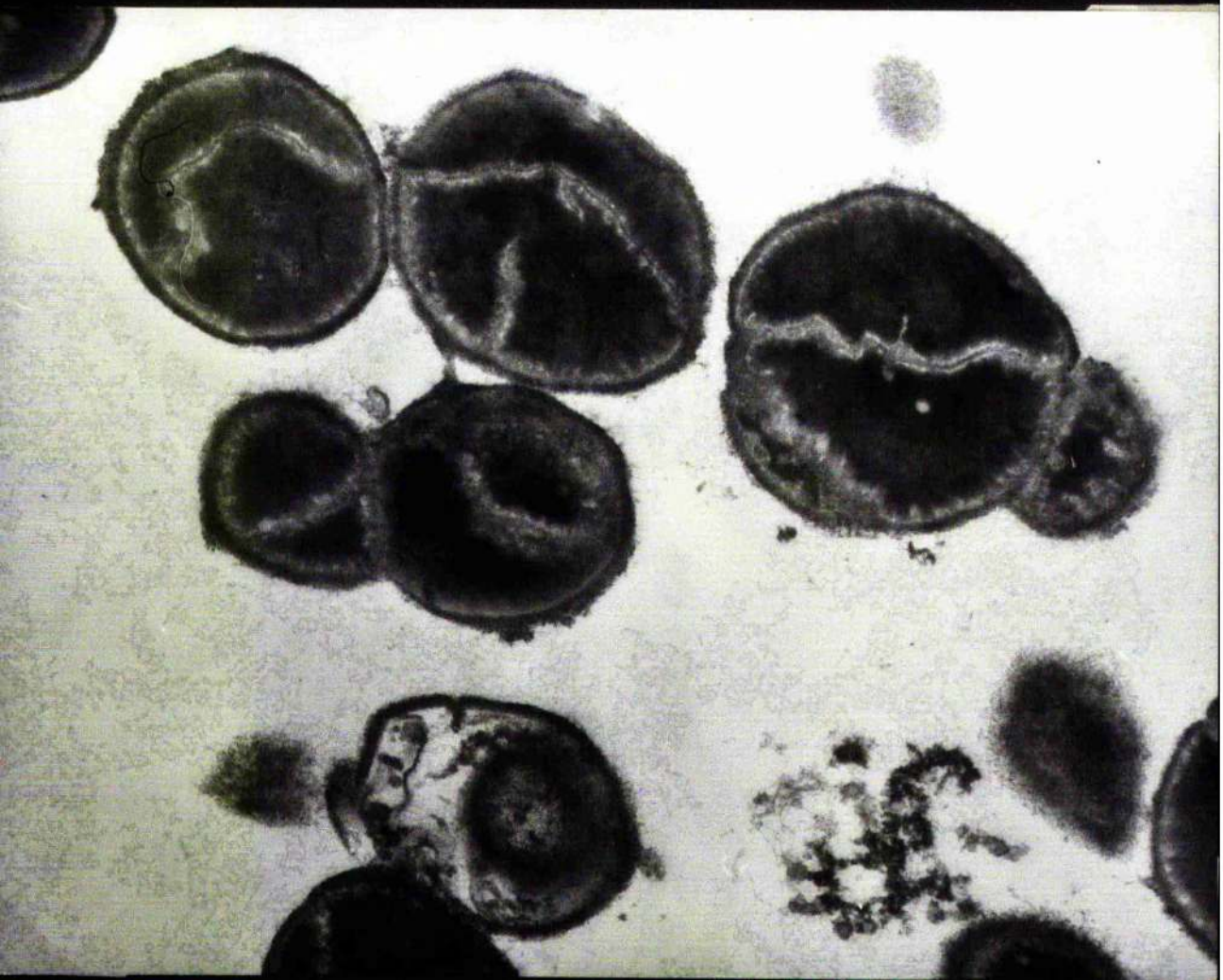


Plate 20.

D Cycloserine (256ug/ml)
Method 2.

Magnification: 22,500





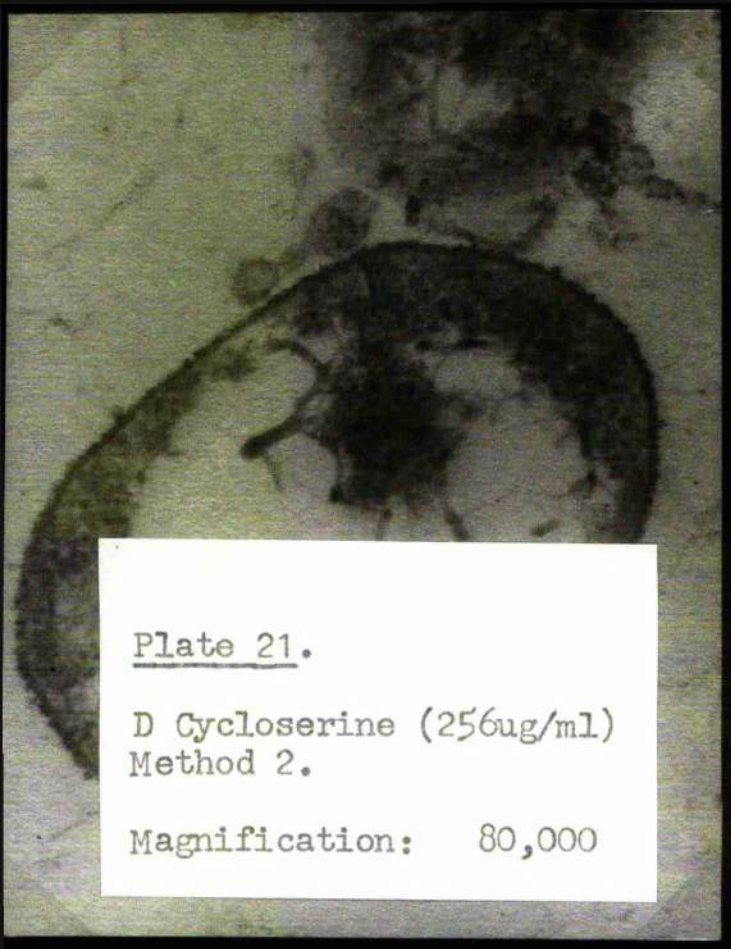


Plate 21.

D Cycloserine (256ug/ml)
Method 2.

Magnification: 80,000

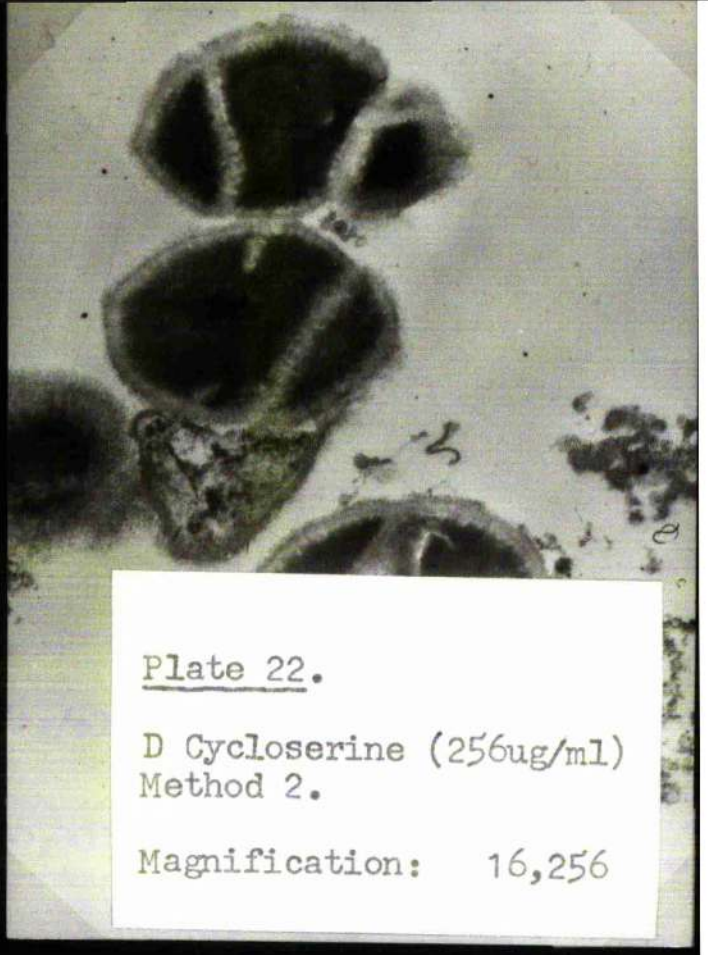


Plate 22.

D Cycloserine (256ug/ml)
Method 2.

Magnification: 16,256

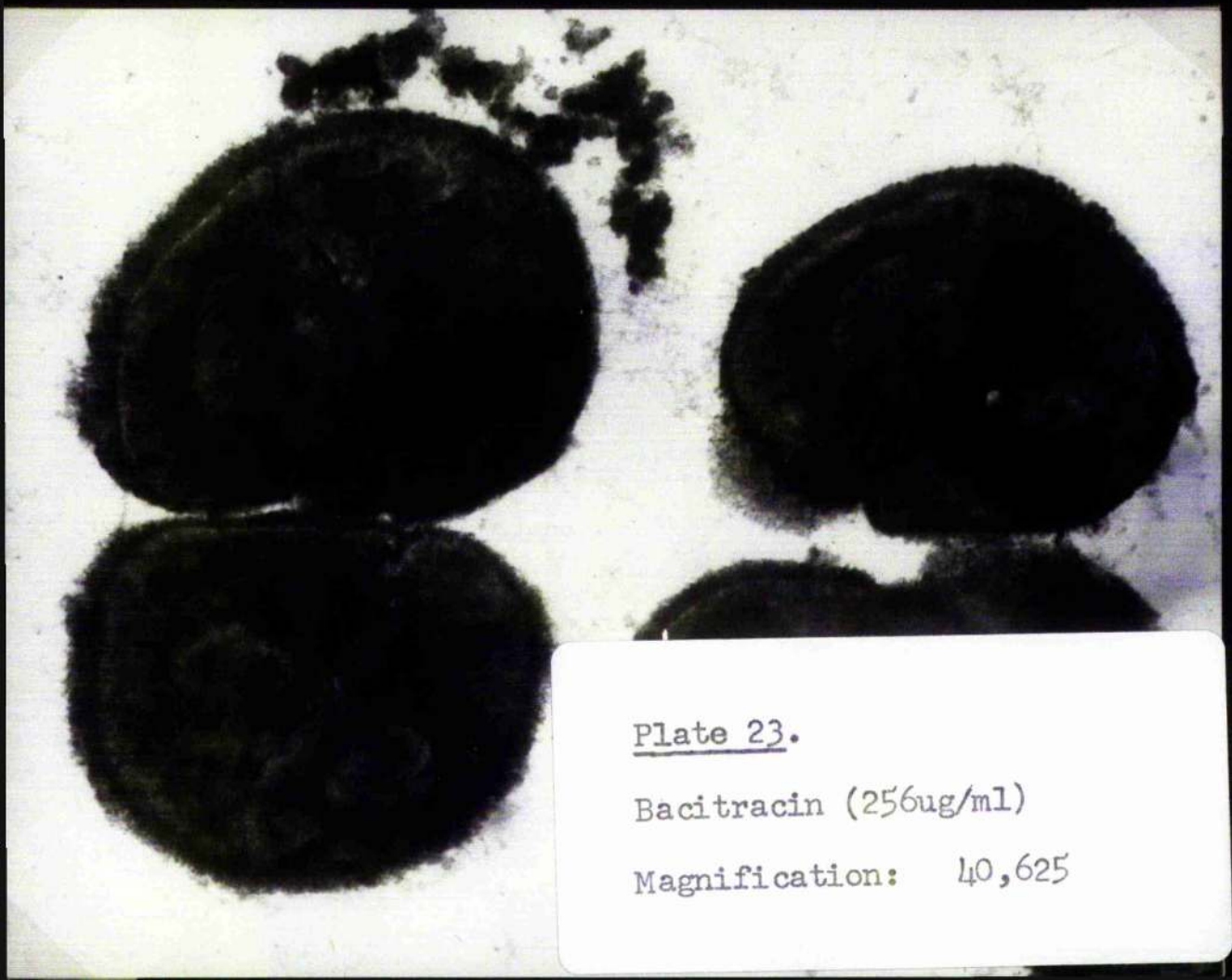
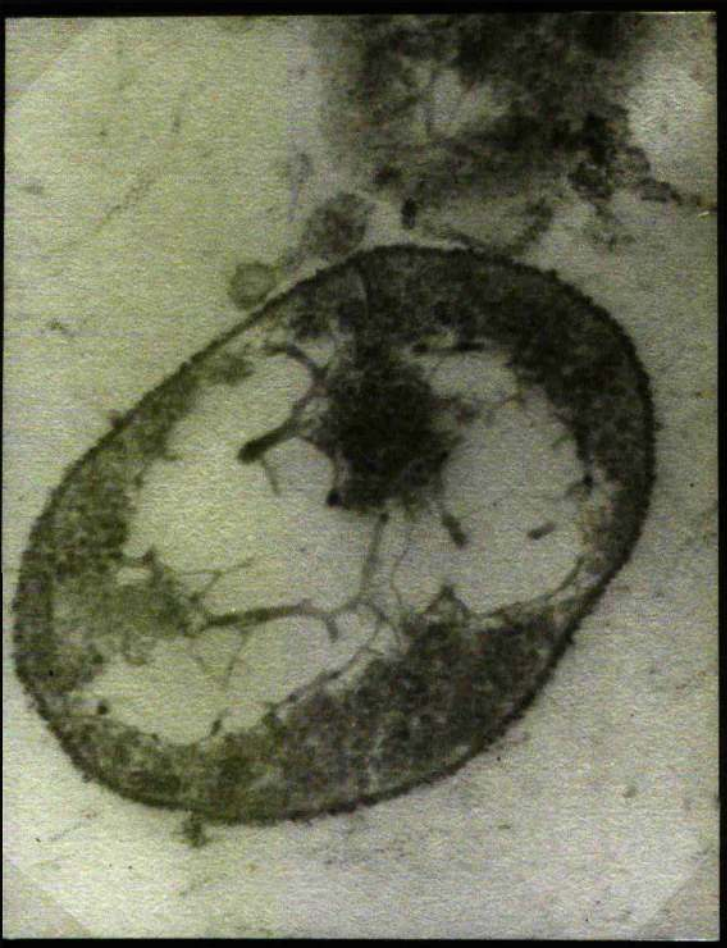
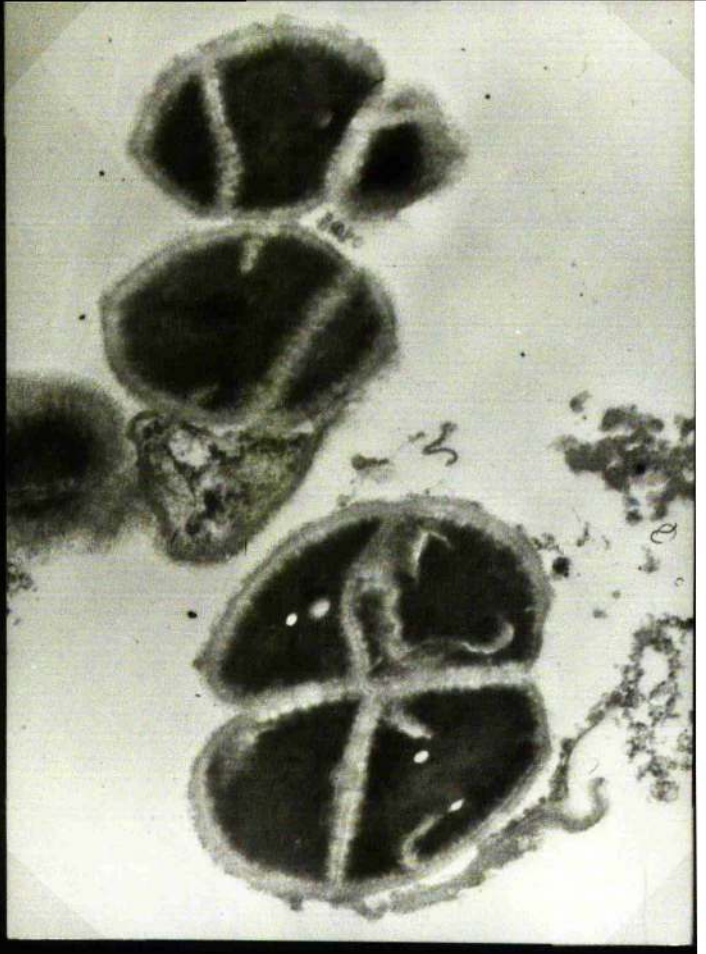


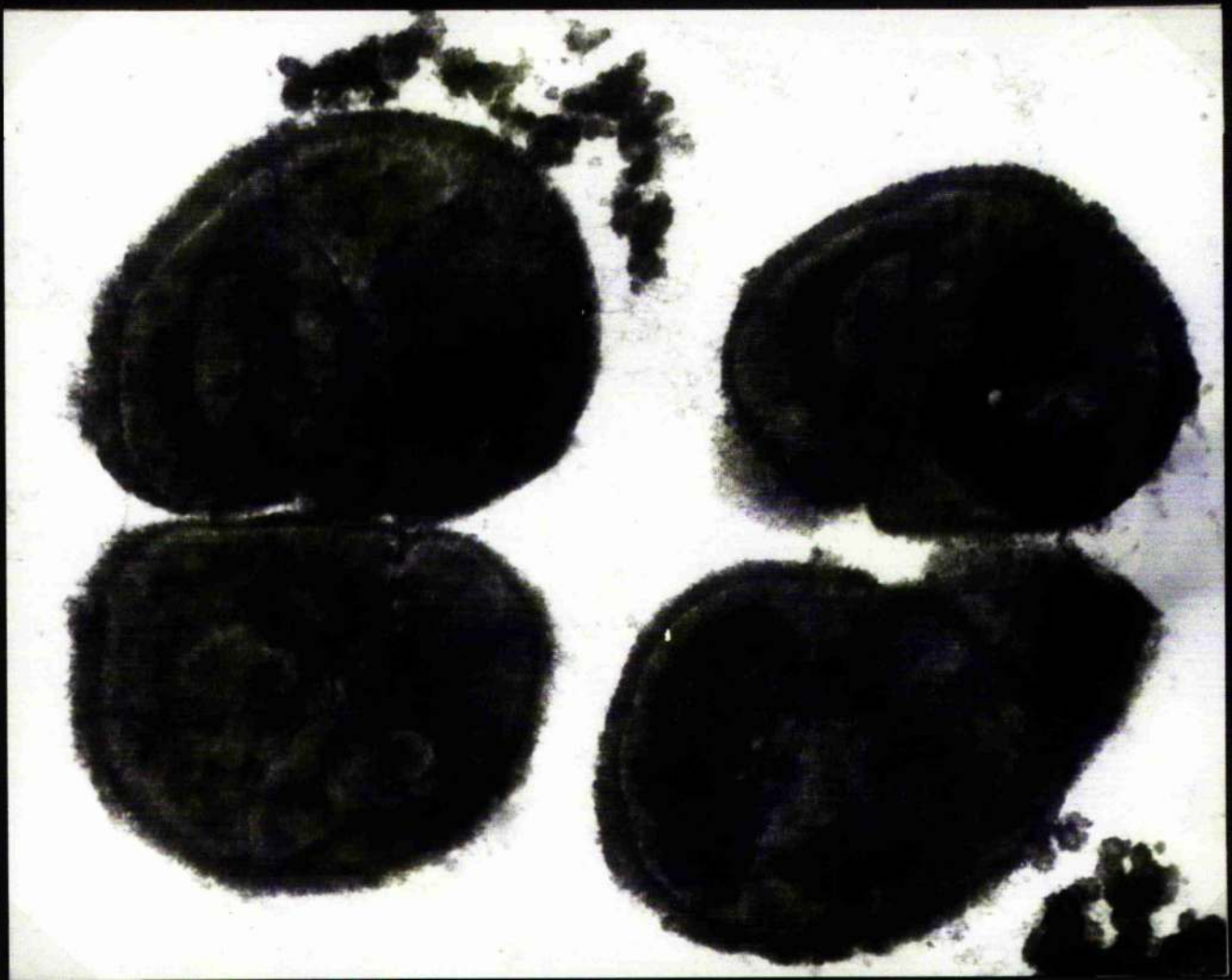
Plate 23.

Bacitracin (256ug/ml)

Magnification: 40,625







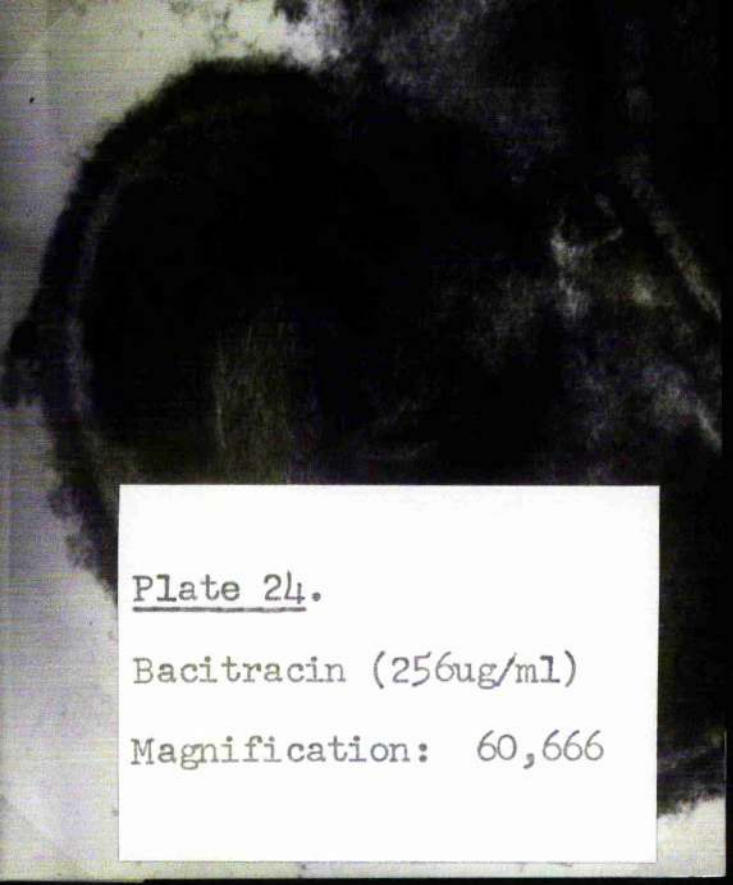


Plate 24.

Bacitracin (256ug/ml)

Magnification: 60,666

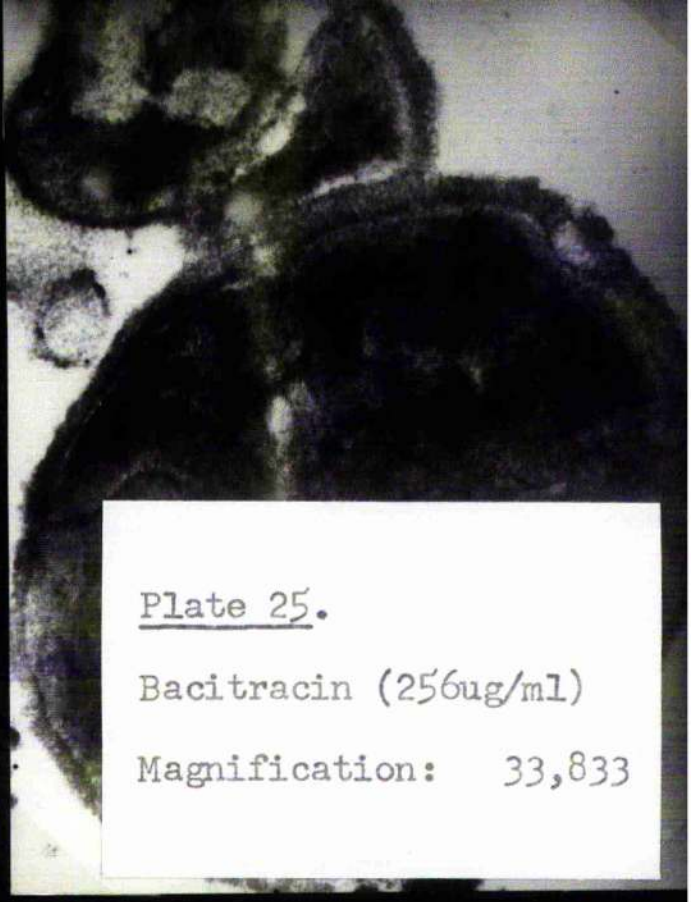


Plate 25.

Bacitracin (256ug/ml)

Magnification: 33,833

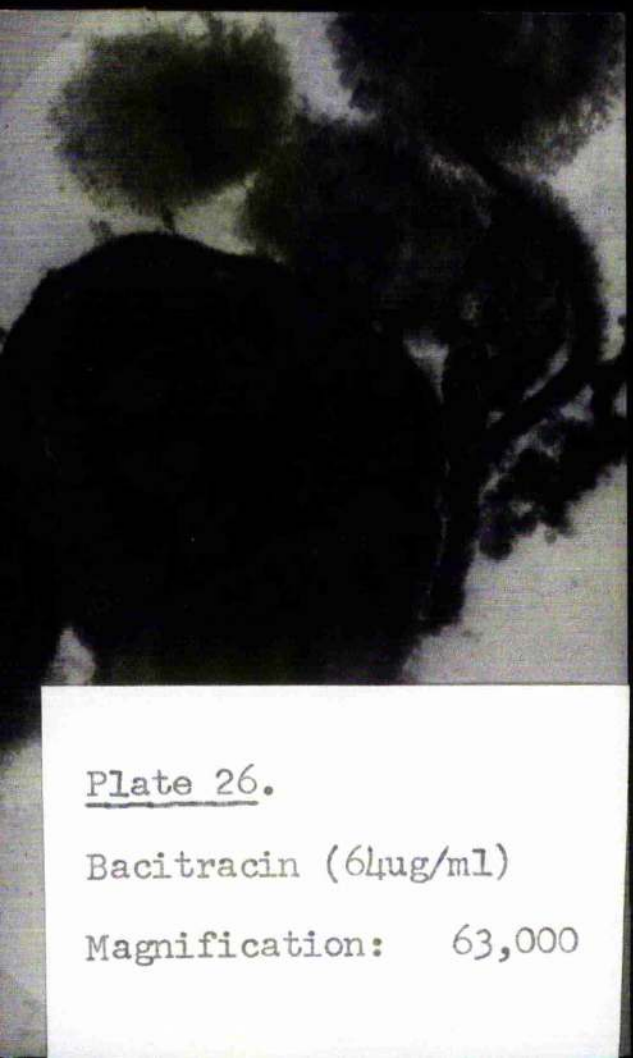


Plate 26.

Bacitracin (64ug/ml)

Magnification: 63,000

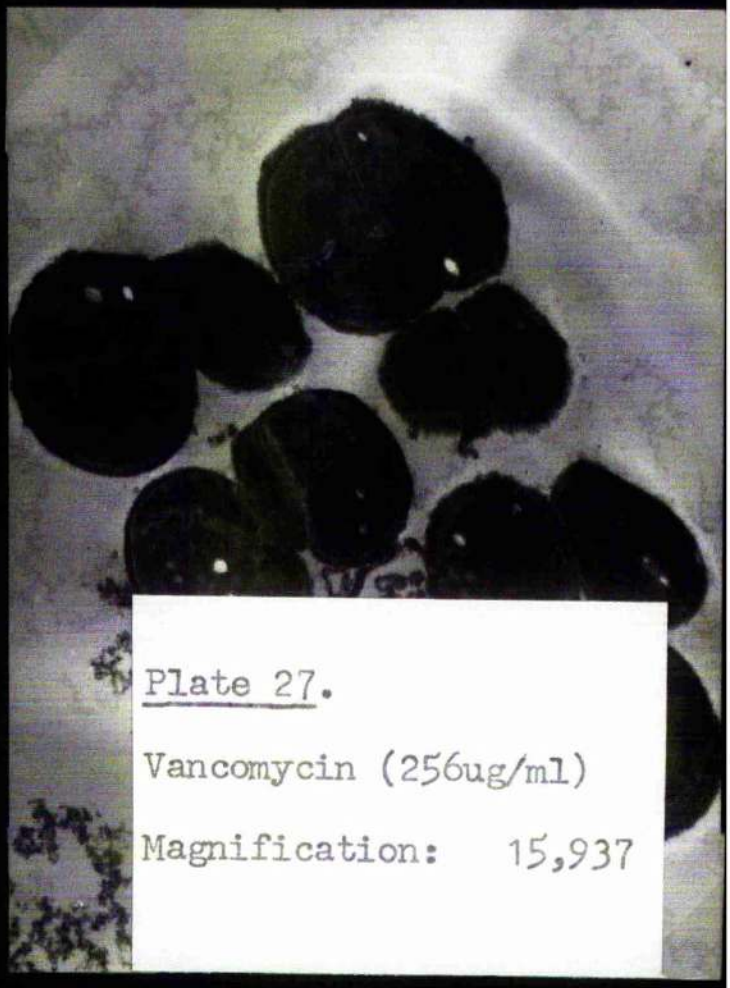
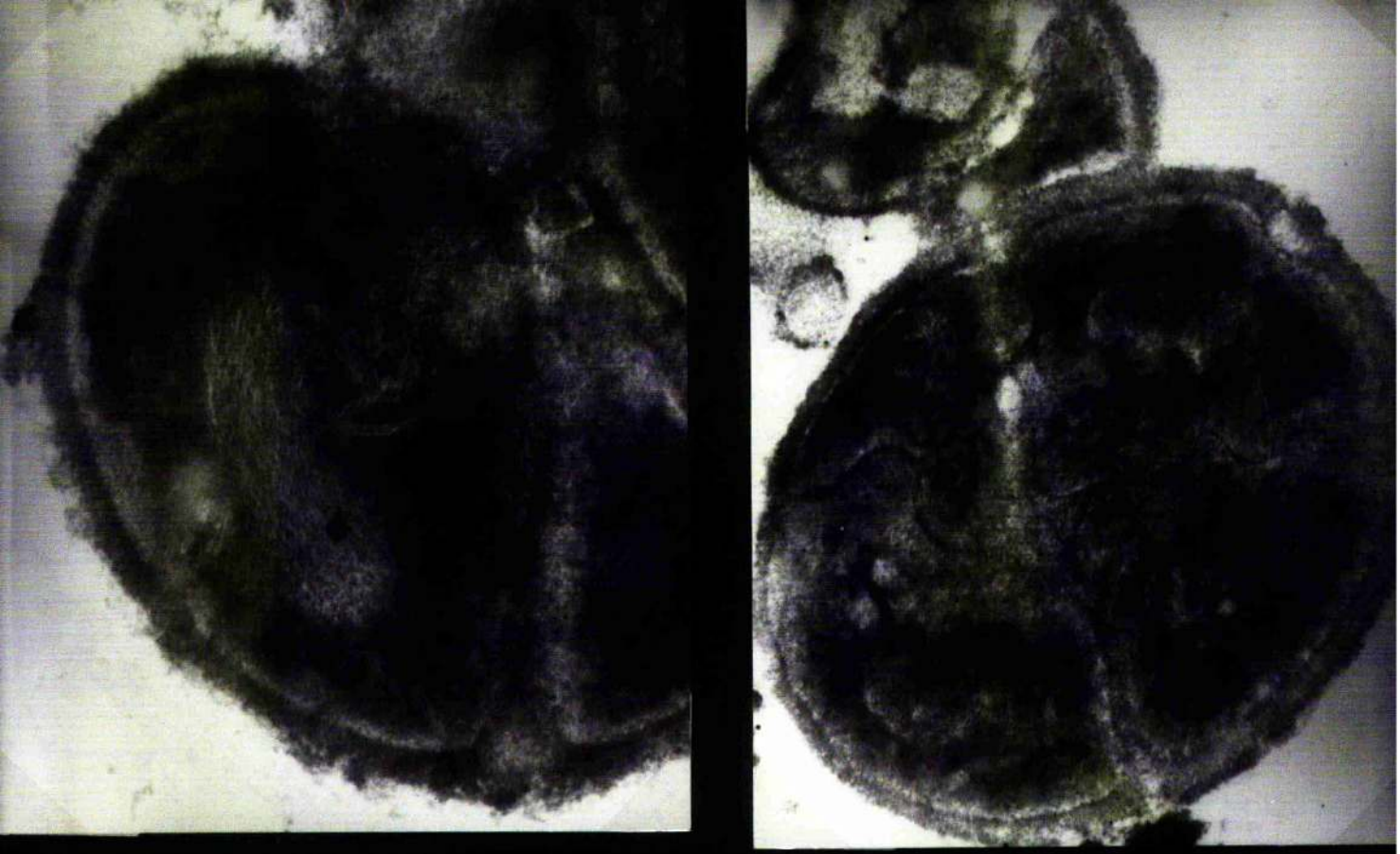


Plate 27.

Vancomycin (256ug/ml)

Magnification: 15,937



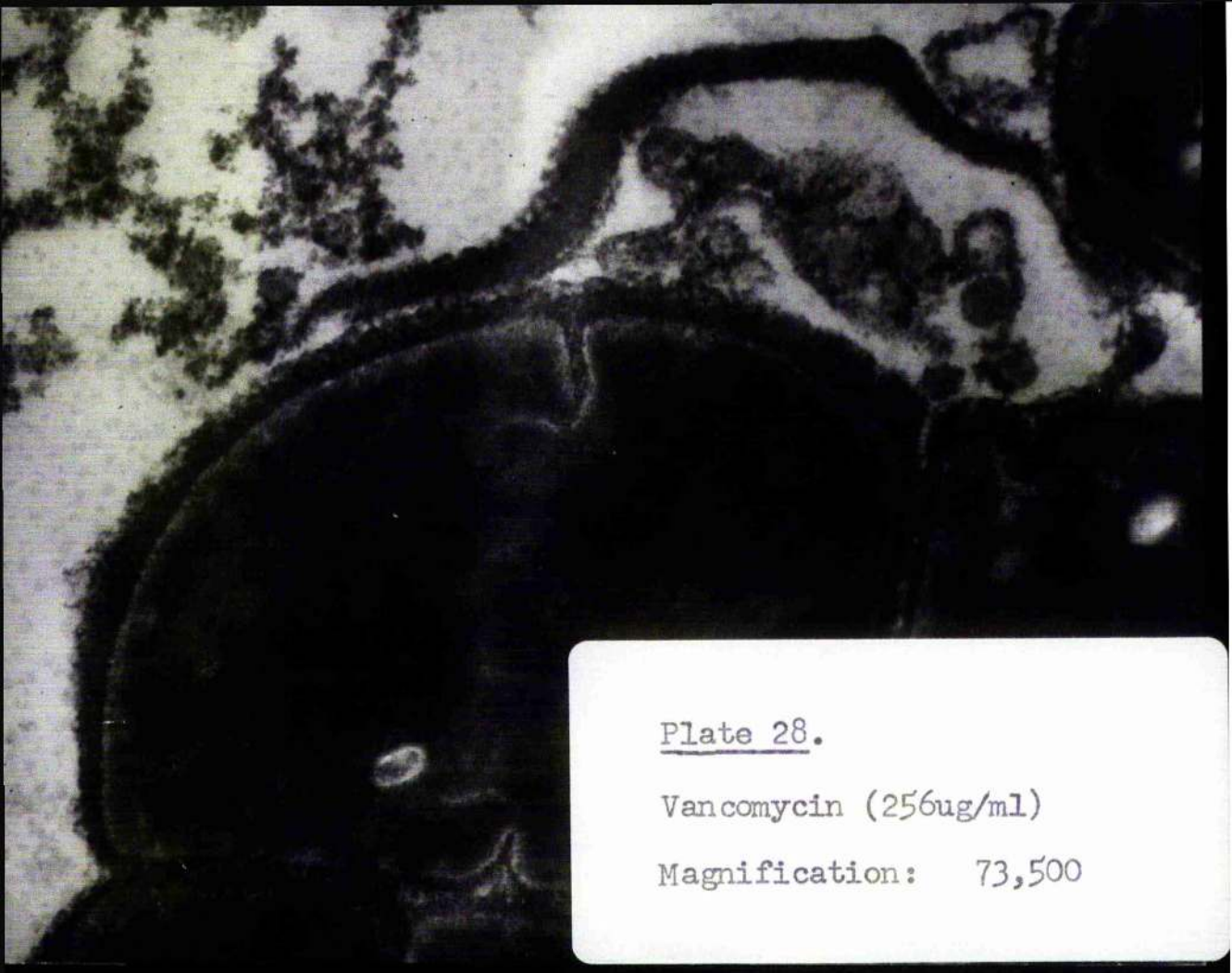


Plate 28.

Vancomycin (256ug/ml)

Magnification: 73,500

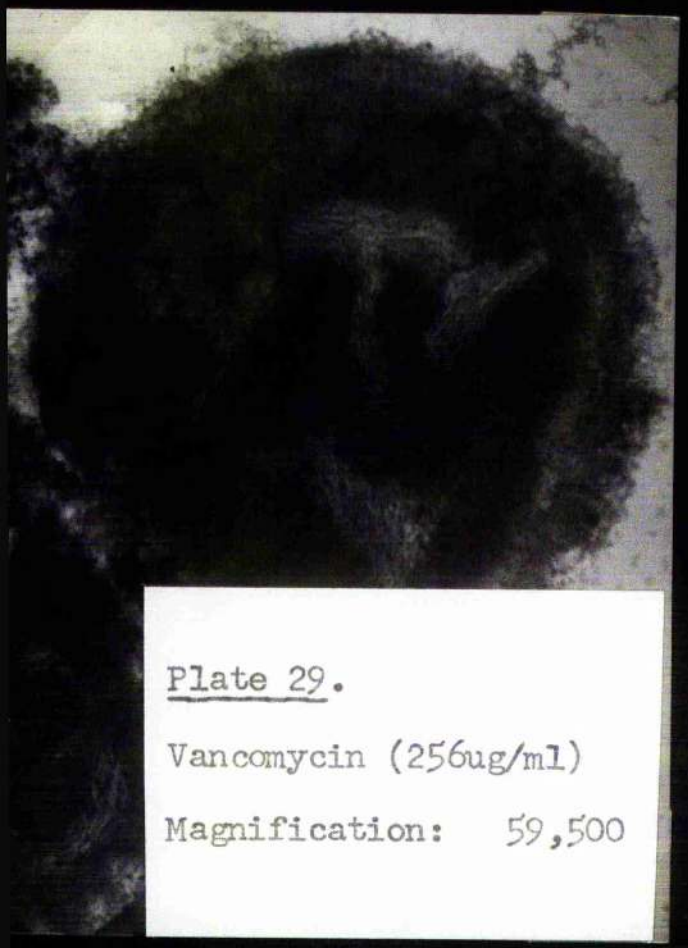


Plate 29.

Vancomycin (256ug/ml)

Magnification: 59,500

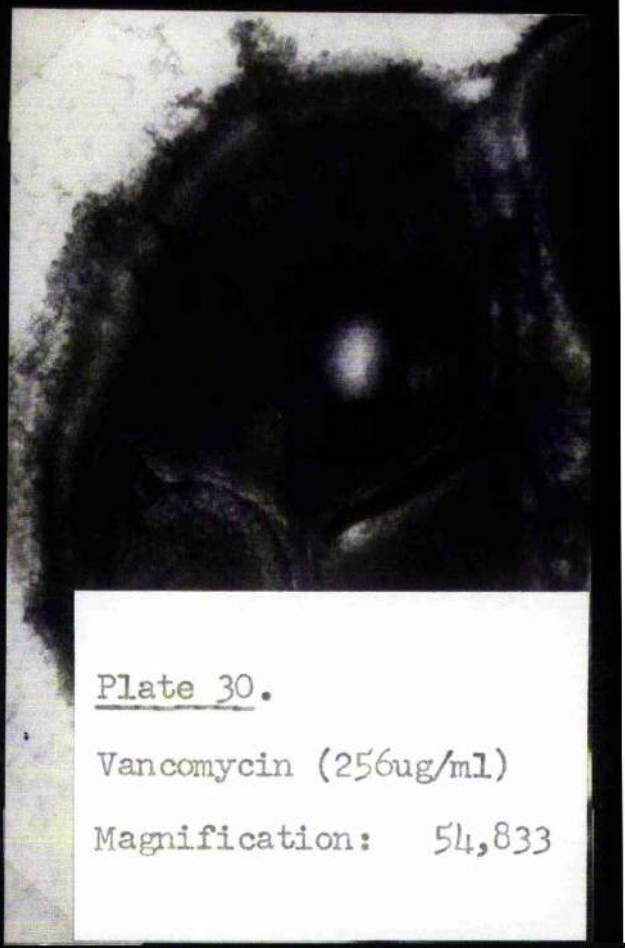
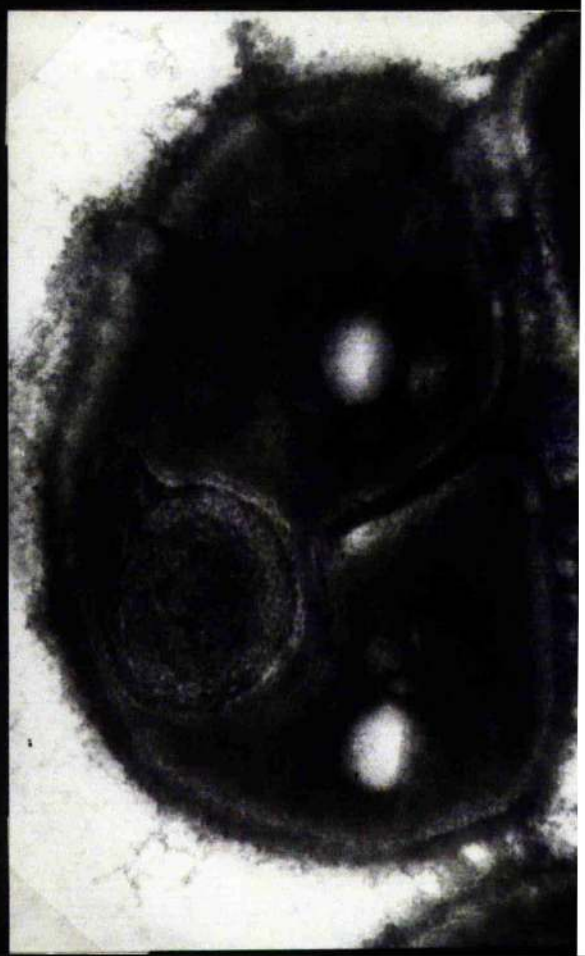


Plate 30.

Vancomycin (256ug/ml)

Magnification: 54,833



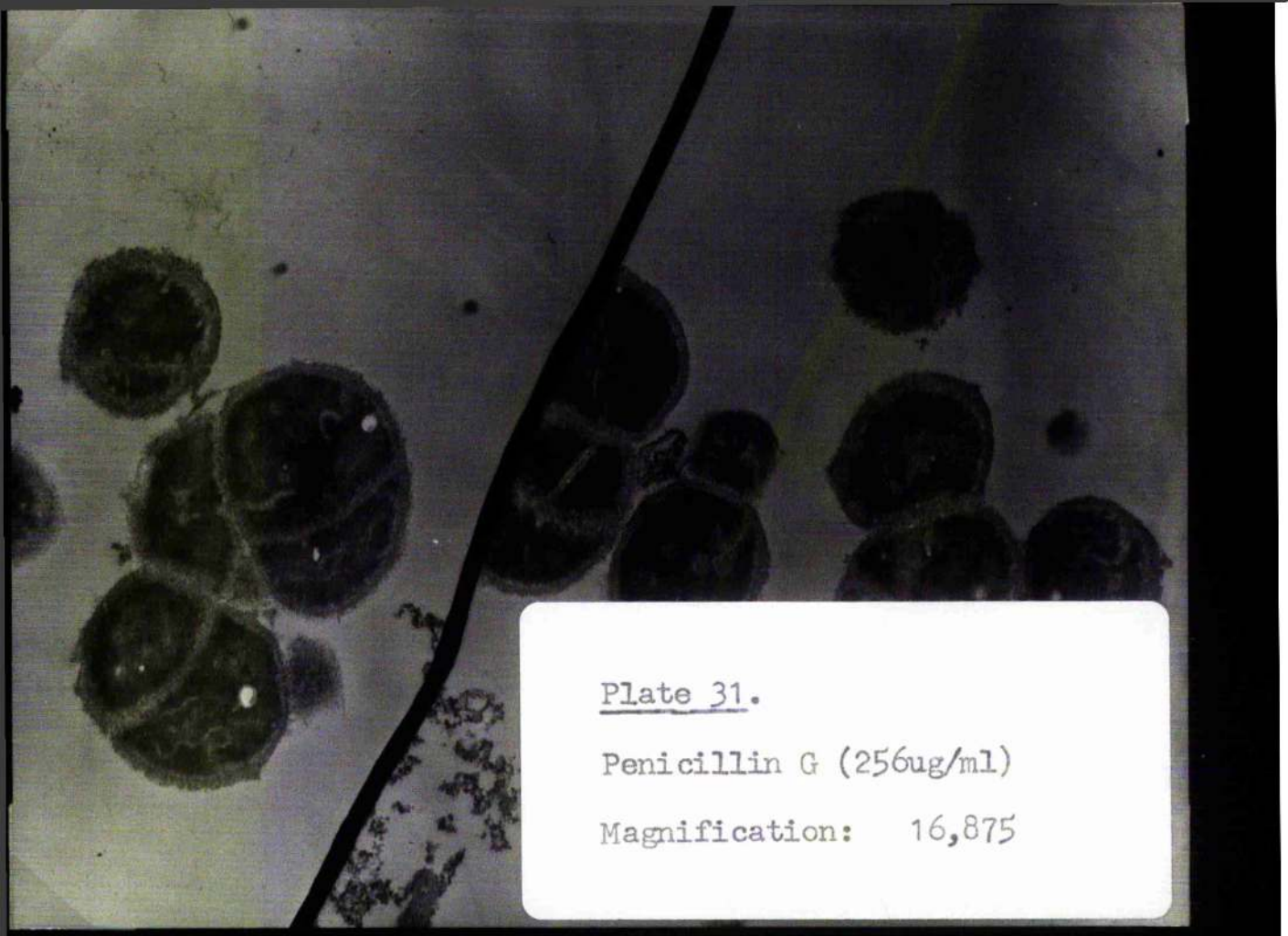


Plate 31.

Penicillin G (256ug/ml)

Magnification: 16,875

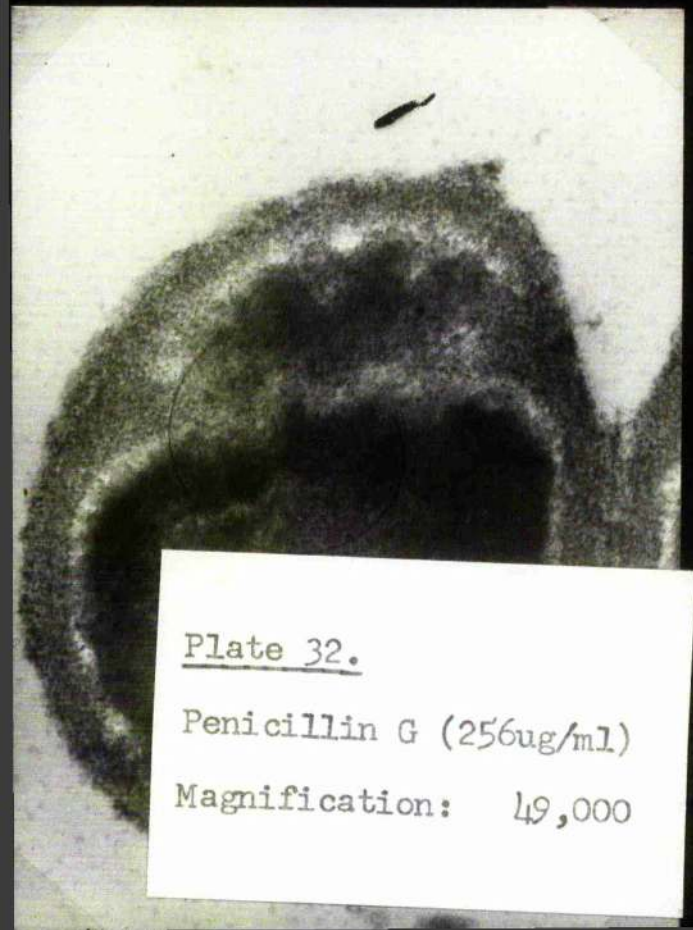


Plate 32.

Penicillin G (256ug/ml)

Magnification: 49,000

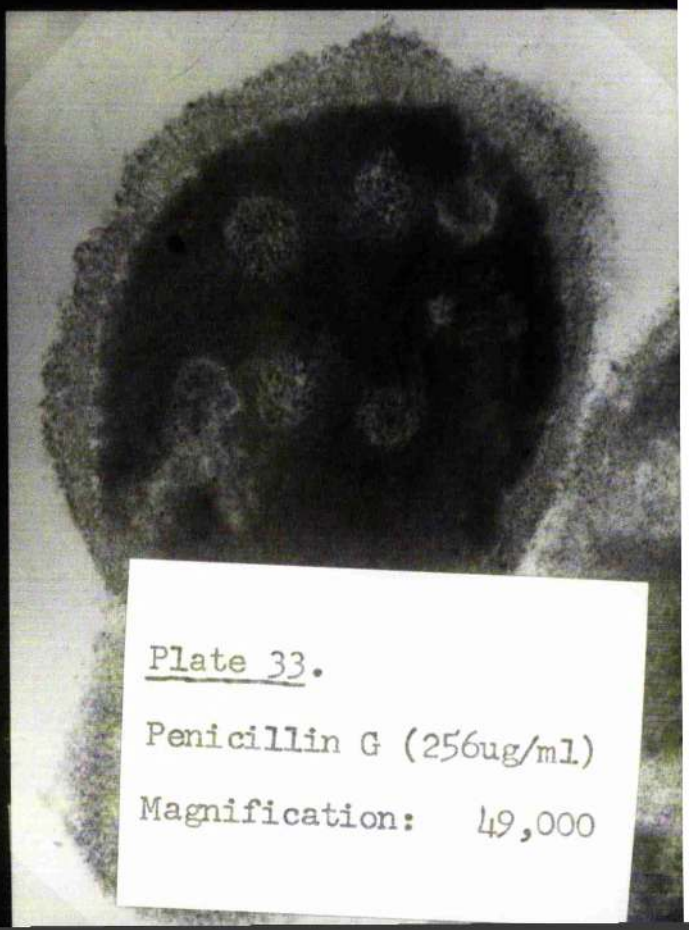
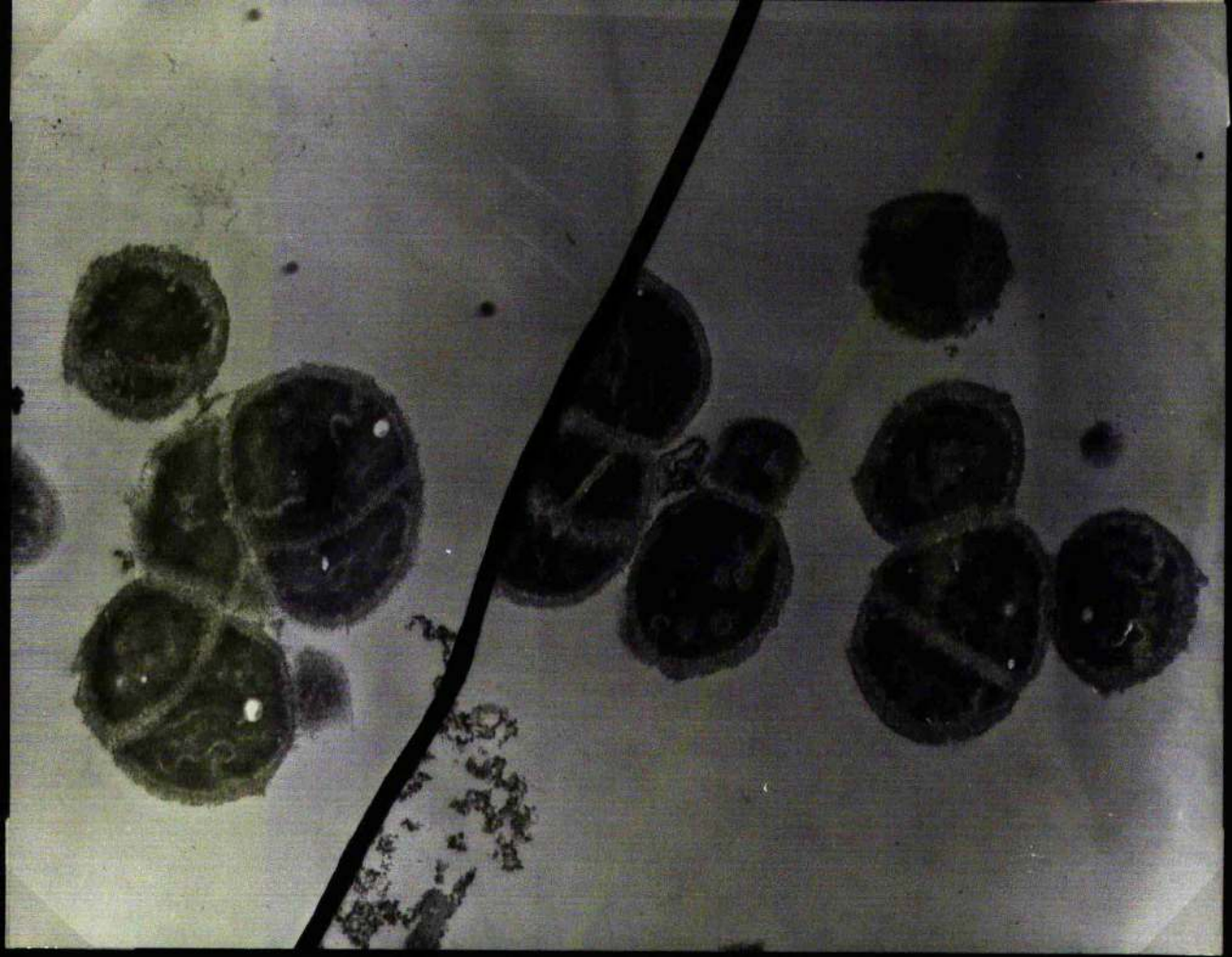
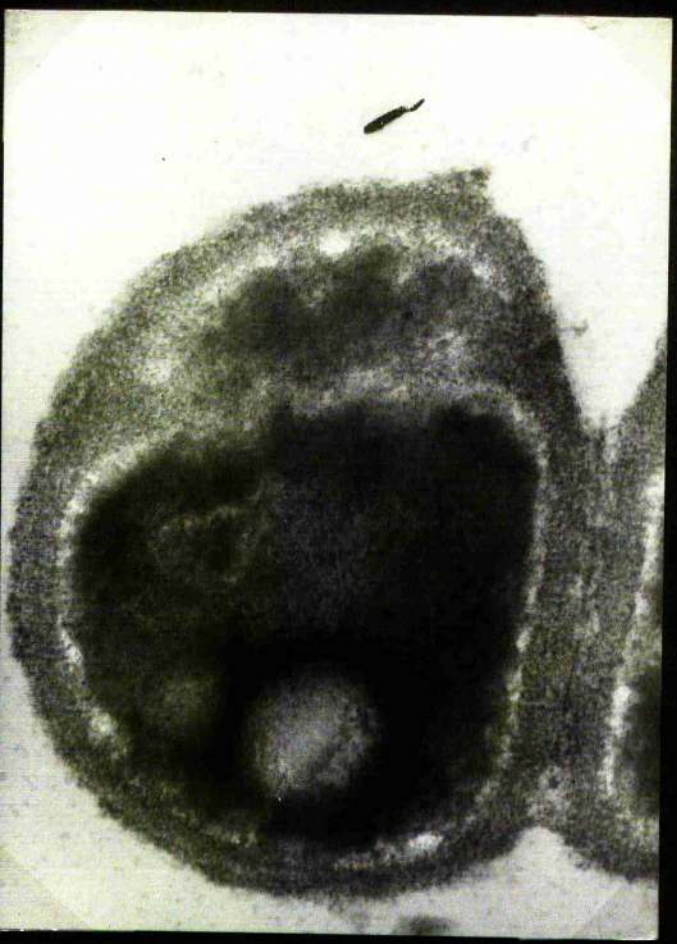


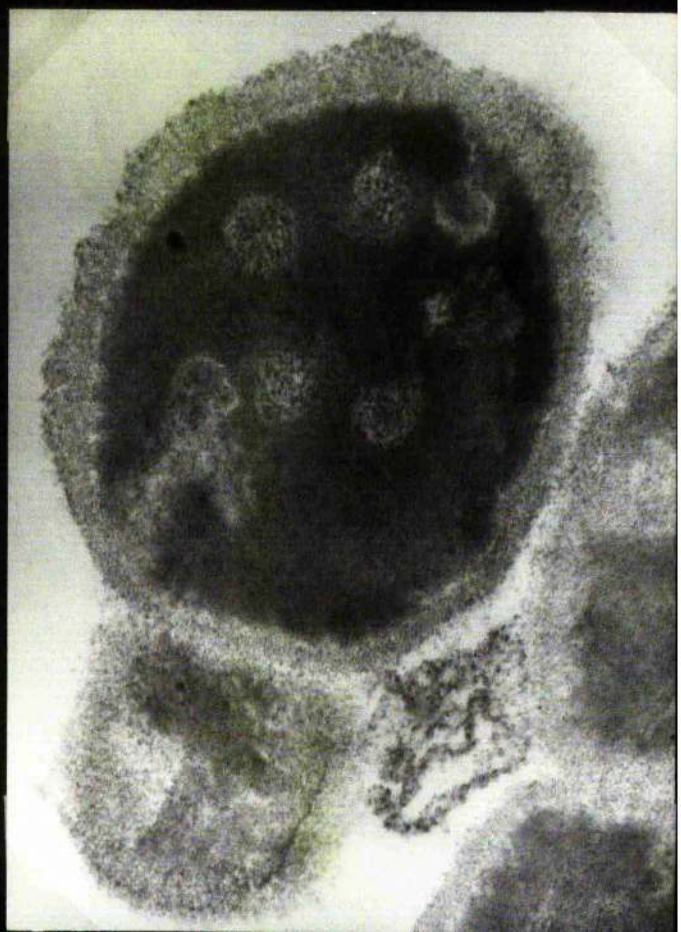
Plate 33.

Penicillin G (256ug/ml)

Magnification: 49,000







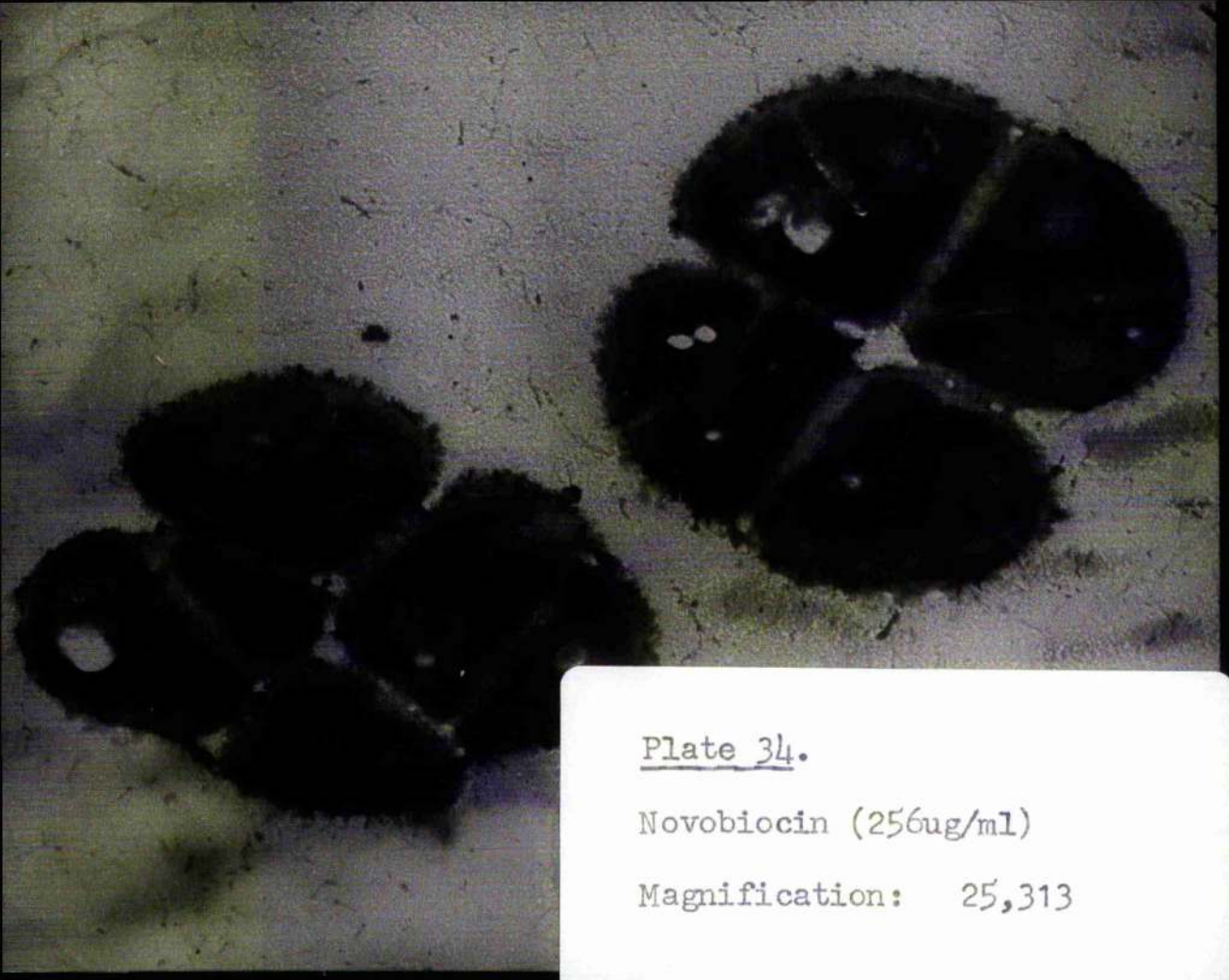


Plate 34.

Novobiocin (256ug/ml)

Magnification: 25,313

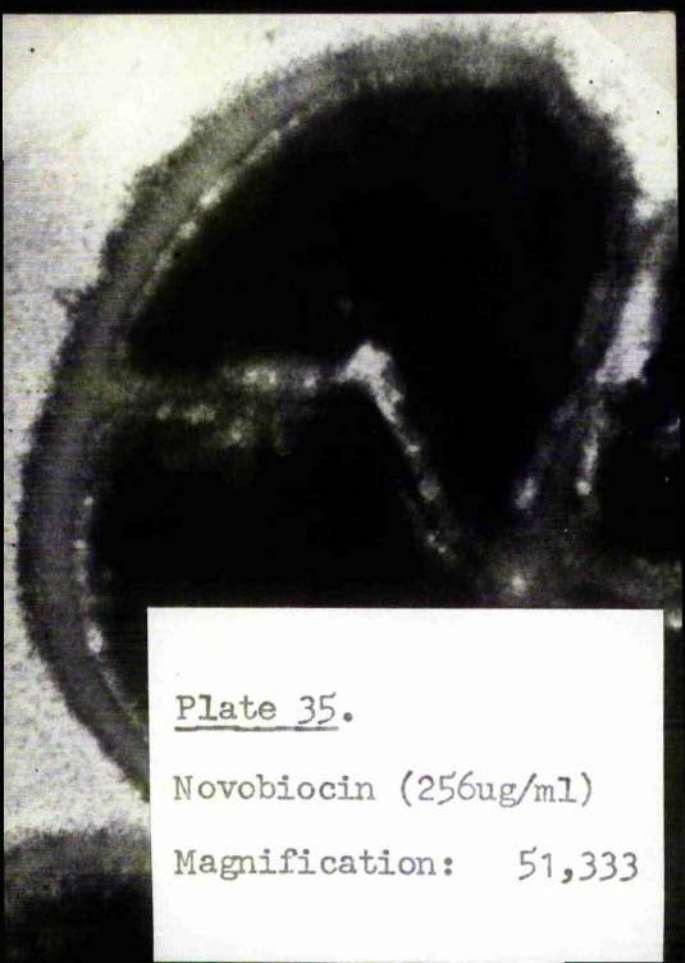


Plate 35.

Novobiocin (256ug/ml)

Magnification: 51,333

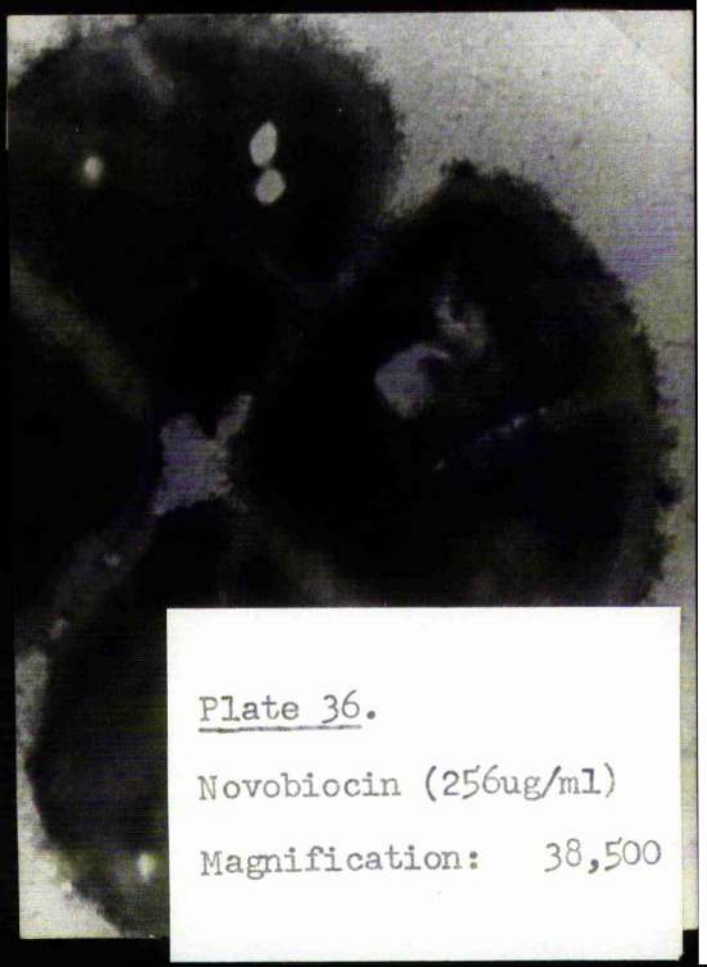
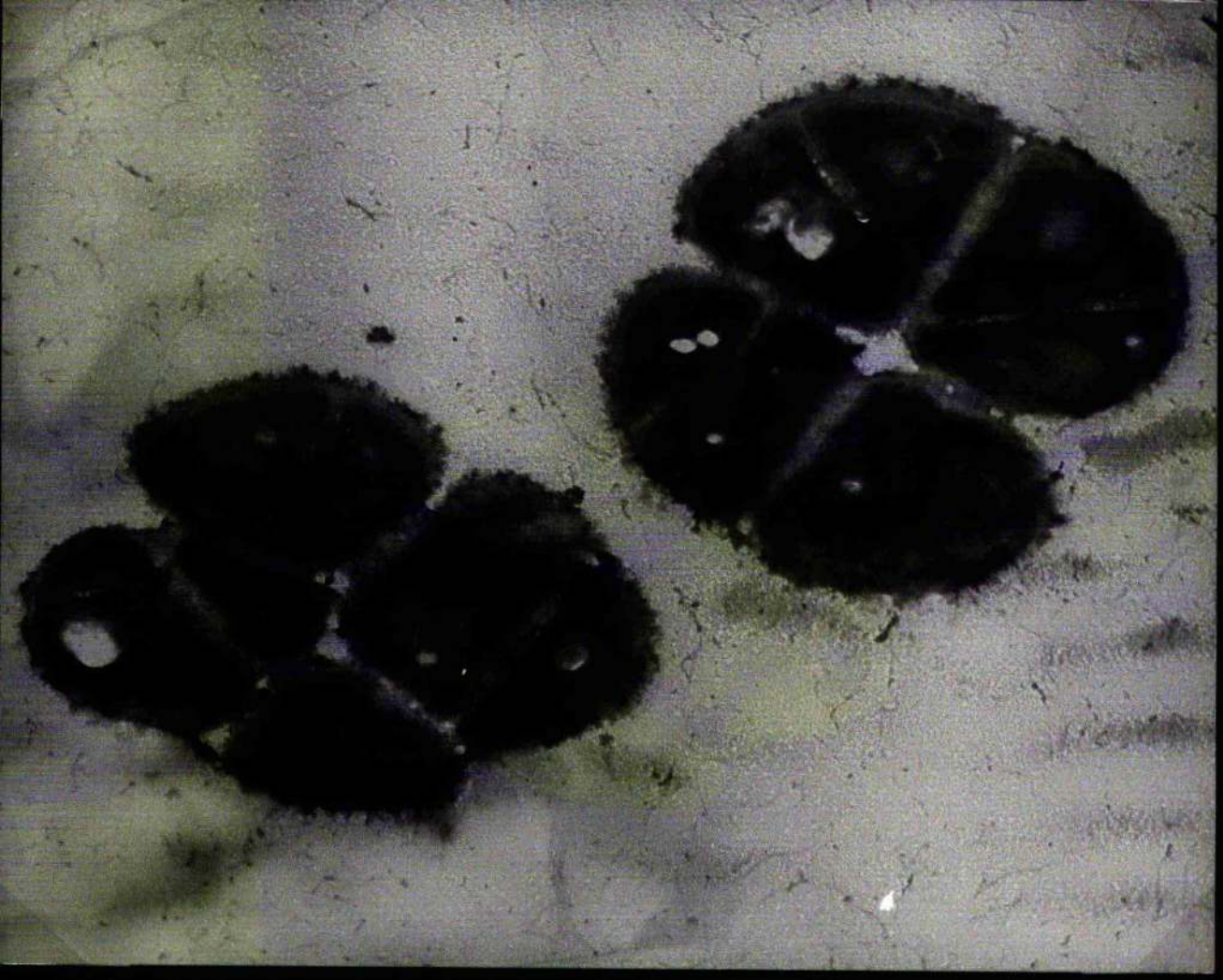
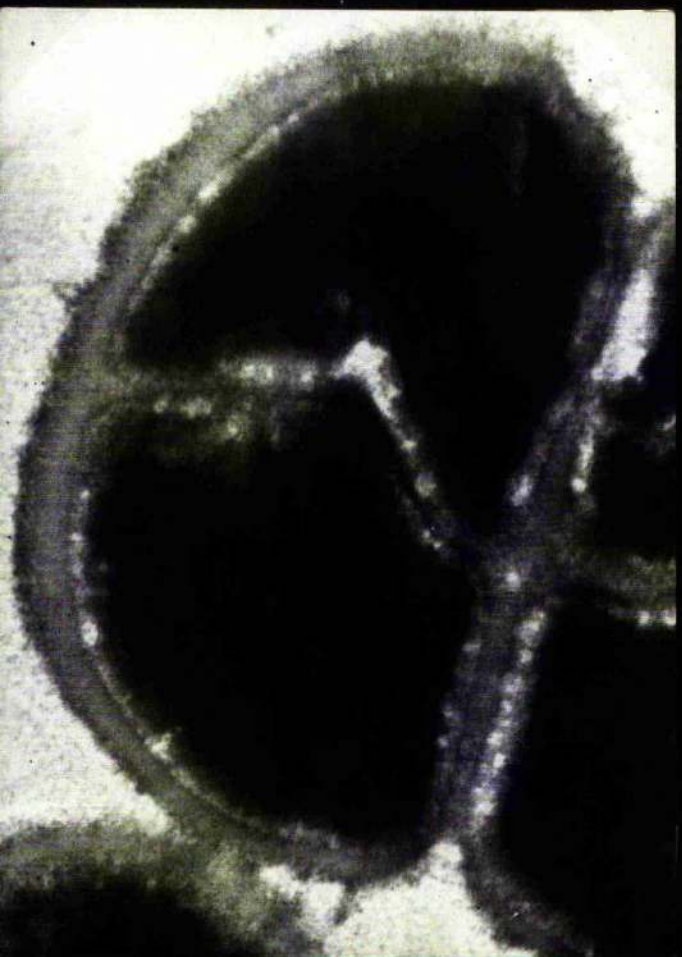


Plate 36.

Novobiocin (256ug/ml)

Magnification: 38,500





Discussion.

S. marina (NCMB 778) demonstrated the characteristic salt dependence of the extremely halophilic cocci. The bacterium had no detectable growth at concentrations of less than 15% (w/v) and grew optimally at 20-25% (w/v) sodium chloride in the medium. The choice of this bacterium over the other strains tested was based on the parameters of length of lag phase, doubling time and highest yield. In Dundas medium 9g wet weight of cells/litre were obtained comparable to the 8-10g wet weight of cells/litre achieved for H. salinarium in a yeast extract-trypticase medium (15).

Halococcal cells are very resistant to disrupture by either low ionic environments or mechanical means. S. marina cells showed no lysis on exposure to hypotonic solution and extensive sonication had little effect. Cell cleavage was achieved by means of a Hughes Press (75), a very severe method for splitting cells, which operates on the 'ice-shear' principle. Because of the high internal salt concentration of the cell, the cells were frozen in liquid nitrogen and the temperature of the press lowered to -45°C to maximise the effect.

Various stages of the wall isolation procedure and the final wall material were viewed in the electron microscope. The electron micrographs are discussed later. The pressed cells were tacky, adopting a chewing gum-like texture which was probably due to the release of nucleic acids from the cleaved cells. The final wall material was of two types distinguished by their colour. The less dense, upper portion of the final pellet was white and the denser, lower segment pink. This pink colour was attributed to residual adhering membrane fragment contaminants remaining even after trypsin treatment.

The yield of wall material, 12.1% of the dry weight of the cell, is

very low compared to non-halophilic Gram-positive micrococci (48). This figure is probably a gross underestimate because of the wall losses incurred in the isolation procedure. The exhaustive washings and the removal of the lower portion of the pellet, constituting intact cells, account for some loss of wall material. Electron microscopy of the various stages in the isolation procedure indicated that trypsin, in addition to solubilising contaminating membrane material, may also remove genuine protein components, the particles seen in the electron micrographs (Plates 5,6) but not visible after trypsin treatment (Plates 8,9), from the wall.

One other possible source of wall loss that was investigated was autolysis. Autolysins are lytic enzymes synthesised by the cell and it has been proposed (92) that they play some role in wall growth and cell division. They probably break existing bonds in the matrix of wall polymers so that a new piece of synthesised wall precursor can be inserted. However, as even a small amount of damage to the wall generally results in osmotic fragility or cell lysis (93), the new bit of wall polymer must be inserted into the existing polymeric chain immediately after the action of the lytic enzyme at that site. Thus, the autolytic enzyme systems are closely associated with and probably controlled by the cell division process. Autolysis and autolytic enzyme systems have been observed in a variety of Gram-positive and Gram-negative bacteria (92).

Whole cells and both trypsin treated and untreated wall fragments of S. marina were suspended by sonication in either buffer solutions of differing pH and molarities, or sodium chloride concentrations of between 1-4.3M, in the temperature range 30-50°C. The loss of turbidity with time was measured at 610nm. The release of reducing sugars, with time, was also assessed (94). The level of autolysin activity, if there was any

at all, was outwith the sensitivity of the assays. However, as the autolytic enzyme systems are closely associated with the cell division process and as S. marina has an extremely long doubling time, the lack of any significant autolytic enzyme activity was not unexpected. Thus, autolysis does not represent a significant source of wall loss, nor a potential non-destructive method for wall removal.

The ash content of the wall from S. marina, 9.2% of the dried wall material, was unusually high compared to non-halophilic, Gram-positive micrococci (48). However, the result is consistent with the findings in other halococci (9,58), although there is some doubt as to the chemical composition of the ash. In S. morrhuae (9), 11.9% of the dried wall was ash, 89% of which was sodium chloride, and probably reflects salt bound to wall components. In Halococcus morrhuae CCM 859 (58) however, 20% of the wall was ash, 50% of which was identified as sulphate, and the sodium chloride content was very low. If the wall is sulphated in S. marina, then the bulk of the ash may not be sodium chloride but sulphate.

Sulphated heteropolymers have hitherto not been found as structural components of bacterial walls, although the walls of marine algae very often contain similar sulphated structural polysaccharides. This may indicate a phylogenetic relationship between the cell walls of the extreme halophiles, procaryotic cocci, and the eucaryotic marine algae (58).

The lipid extract from S. marina wall, constituting less than 0.1% of the dry weight of the wall, was deep red in colour and is probably due to residual contaminating membrane fragments, possibly carotenoid pigmentation. This figure is significantly lower than the 2% lipid content of wall in Halococcus morrhuae (58) and the 22% lipid in the envelope of the halobacterium H. cutirubrum (40). However, the higher lipid content in Halococcus

morrhuae may not indicate a genuine lipid component in the wall, but, more likely, less efficient removal of membrane in the isolation of the wall than achieved in S. marina.

The major N terminal amino acid in the wall was alanine, although a trace of glycine was also present in the 'pink' wall material. This may reflect a classical type of wall structure with alanine as the N terminal of the tetrapeptide side chain and glycine as the N terminal of the cross-bridging peptide. However, as these amino acids were present in such low amounts in the wall, it is hard to envisage them playing such a significant role in the wall structure, particularly as there is strong evidence pointing to a lack of peptidoglycan. Another possible explanation of this observation is the presence of a teichoic acid-like polymer where alanine is esterified as a single amino acid to the carbohydrate residues in the chain. A common substituent attached in this fashion to both ribitol and glycerol teichoic acids in many Gram-positive microorganisms is D alanine.

The major amino acid constituents of S. marina wall were, in order of magnitude, glycine, histidine, alanine, glutamic acid and aspartate, although there is some doubt as to the validity of the histidine identification. There were also traces of most of the other common amino acids, but they were assumed to be contaminants from residual membrane fragments as the wall material was faintly tinged pink even after trypsin treatment, similar to other investigations (9,11).

Glycine was the main amino acid in the wall and was present in only very low levels compared to non-halophilic, Gram-positive micrococci. However, similar glycine results were obtained from wall analysis of other halococci (11,57,58), although some quantitative variations were observed from strain to strain (57).

Although histidine has been identified as a major amino acid component of S. marina wall, it is not a major constituent of many proteins and certainly not of halococcal wall (11,57,58). Histidine is also not noted as being especially unstable to hydrolysis conditions, but this component is, in fact, acid labile. Consequently, this constituent may not be histidine but something else, possibly an amino sugar, which co-chromatographs with histidine.

In a survey of several halococci (57) the total amount of amino acids and hexosamines constituted 7-15% of the weight of the cell walls, and glucosamine and galactosamine were the main amino sugars identified. These two amino sugars were present in S. marina wall and the amino acids and total hexosamines combined, at 8.7% of the weight of the cell wall, were within the above range. However, the total hexosamines only represented 5% of the wall weight, which is very low compared to the 12.5% in Halococcus morrhuae (58) and the glucosamine and galactosamine content of 3% is also very low compared to the 10% in Halophilic coccus strain 24 (11) and the 10.4% in Halococcus morrhuae (58). Although rather large quantitative variations in the major cell wall constituents were observed from strain to strain within each group displaying the same qualitative pattern in the survey (57), the increased degradation of hexosamines and, in particular, glucosamine and galactosamine, in the strong acid hydrolysis conditions may also, in part, be responsible for their low content in S. marina wall.

The difference in the total hexosamines, assessed in 4N HCl at 100°C, and the glucosamine and galactosamine combined content, assessed in 6N HCl at 100°C, is probably partly due to the increased degradation of these two amino sugars in the stronger acid hydrolysis conditions. Another important consideration is that, although glucosamine and galactosamine constitute

the detected hexosamine components, only these two hexosamines were quantitated. Other unidentified components which may be hexosamines, particularly $X_1$ and $X_2$ in view of their acid lability, were also present in the wall. There is evidence for other hexosamines and/or their derivatives, particularly gulosaminuronic acid, in halococcal wall (57,58).

The three unidentified ninhydrin-positive components $X_1$, $X_2$ and $X_3$ and the component initially identified as histidine are all acid labile. Some, or all, of these wall constituents detected by ion exchange chromatography may correspond to the 4 unknown components from paper chromatography, one of which stained yellow in the ninhydrin detection reagent. However, this yellow component was not pentaglycine or any of the other peptides tested, although its mobility and acid lability suggest that it may indeed by a peptide. Although the identities of $X_1$, $X_2$ and $X_3$ are unresolved, they may correspond to the ninhydrin-positive unidentified components detected in Halophilic coccus strain 24 (11), and, because of their acid lability, could also be unidentified amino sugars or peptides.

Peptide analysis of partial acid hydrolysates from S. marina wall, however, met with virtually no success. In view of the low levels of the amino acids and the lack of a peptidoglycan in the wall, this was not unexpected. One possible peptide, tentatively identified as glycyl alanine, was present but in such low amounts that the chromatograms had to be grossly overloaded for its detection and it was consequently partially obscured by the other amino acids.

One of the unidentified ninhydrin-positive components is probably gulosaminuronic acid which may be an obligatory constituent of halococcal walls (57). As a sulphated heteropolymer has been postulated as a structural component of the wall from Halococcus morrhuae (58), one of these unknown

components may be a sulphated amino sugar.

Neither muramic acid nor diaminopimelic acid, which are indicative of a peptidoglycan, were components in the wall material. This is typical of the extreme halophiles (9,57,58) and, although the characteristic amino acids in normal wall were present, they occurred in very low amounts. Thus, some of the essential components of a normal peptidoglycan were absent in S. marina wall. The lack of peptidoglycan, which is characteristic of extreme halophilism (57), in S. marina was further substantiated, as in Halophilic coccus strain 24 (11), by the almost complete solubility of the wall material in hot formamide, which has been utilised to solubilise the polysaccharide containing material from microbial wall and leave the insoluble peptidoglycan intact (86).

The ninhydrin-positive constituents of the wall account for only about 14% of the dry weight of wall which, although similar to other halococci (11), is significantly lower than the amounts present in the common micrococci. Although the unidentified ninhydrin-positive components could account for a few percentage, by far the majority of the wall is composed of ninhydrin-negative compounds.

One of the major ninhydrin-negative components is carbohydrate. As in Halococcus morrhuae (58) and both S. littoralis and S. morrhuae (9), the three monosaccharides glucose, galactose and mannose were present in approximately equimolar amounts. However, although they did represent 21.3% of the weight of the wall, very similar to the 19.1% in Halococcus morrhuae (58), they constituted only 67.6% of the total carbohydrate and 62.8% of the total reducing substances detected in S. marina wall.

The difference between the monosaccharides and the total carbohydrate

and total reducing substances is significant and probably reflects the presence of unidentified components. Only these three monosaccharides, glucose, galactose and mannose, were detected by GLC analysis although three additional components were found by paper chromatography. Two of these components contained both amino and reducing groups, the third only reducing groups. With the direct silylation conditions employed in the analysis of the carbohydrate components, these unidentified components may not be resolved for analysis, possibly analogous to the situation for hexosamines. They may be amino sugars or their derivatives or possibly di-, tri- or even oligosaccharides incompletely hydrolysed into their individual monosaccharide units. There is also evidence (9,58) for the acidic carbohydrate constituents glucuronic and galacturonic acids which were not assessed in S. marina wall.

As in Halococcus morrhuae (58), all efforts to extract single polymers, e.g. 'mannan', teichoic acids and peptidoglycan, were relatively unsuccessful. Cell wall material could be extracted under various mild acidic or alkaline conditions, but in almost every case the extracted material and the insoluble residue contained, at least qualitatively, the same constituents.

The major amino acid components, with the exception of histidine(?), were present in both the extracted material and the insoluble residues with, in many cases, notable differences in their levels between fractions. The significance of this distribution of the major amino acids, and especially glycine, is not known.

The lack of significant amounts of histidine(?) in both the extracted material and the insoluble residues is suprising. However, some doubt about the identification of this component as being histidine has already been expressed. This data supports the contention that this ninhydrin-positive

component may, in fact, not be histidine but something else, possibly an amino sugar that co-chromatographs with histidine.

However, as TCA extraction at both 35 and 60°C produced a significant difference in the relative content (Molar Ratio) of glucose between the soluble and insoluble residues, a preferential extraction of a glucose-rich wall polymer, possibly glucan-like, may have been achieved. The division of galactosamine, $X_1$ and $X_2$ between the insoluble residues and the extracted material in TCA extractions at both 35 and 60°C may also indicate the separation of wall polymers. As the unidentified ninhydrin-positive component $X_3$ has double optima for its release in acid hydrolysis, this suggests that there are possibly two polymers in the wall with $X_3$ as a constituent of both. However, part of the $X_3$ component, in a single polymer, may be protected by some other wall constituent/constituents that have to be removed before the remainder of the $X_3$ can be released.

The lower levels of the monosaccharides in the fractionated wall material compared to the untreated wall may reflect the sensitivity of the wall polymers to the extraction conditions. Only the 'mannan' residue had the monosaccharides in an approximately equimolar ratio similar to the untreated wall.

The soluble and insoluble residues, with the few possible exceptions mentioned, contain at least qualitatively the same constituents. The significance of the distribution of these components between the extracted material and the insoluble residues is not known.

Thus, the walls of S. marina are fundamentally different to the common micrococci but bear marked similarities to the other halococci. Compounds other than amino acids and hexosamines are present in the structure

conferring shape, rigidity and integrity to the wall, the major part of which is composed of ninhydrin-negative components. It is not known whether the ninhydrin-positive compounds are linked directly to each other, or with ninhydrin-negative compounds intercalated in the polymer or polymers. At this point there are too many unknown factors to suggest a structure for the wall polymers although the fractionation studies do suggest the possible existence of more than one polymer.

Several general problems were encountered in obtaining satisfactory electron micrographs, particularly with whole cells. The most notable was the presence of 'holes' in the sections. The best sections were obtained from broken cells and isolated wall, suggesting a problem with penetration of either the fixative or, more likely, the embedding resin. Two different water-soluble resins were also tried before reasonable sections for the whole cells were obtained. Because of the high internal salt concentration of the cell, a water-soluble resin was potentially more effective than the water-insoluble systems. However, the sections from this system were, in fact, even worse.

The effectiveness of the wall isolation procedure was assessed by electron microscopy. The Hughes Press caused extensive cellular disruption, although cell breakage was restricted to only a few sites per cell. This was suprising in view of the extreme conditions of temperature and pressure used. However, these may reflect weaker parts of the wall, possibly sites for incorporation of new wall material where the incorporation has not yet been completed. Some intact, whole cells still remained even after two passes through the press, but these were effectively removed as there was no evidence of them in the final isolated wall material.

Exhaustive washing of the pressed cells removed the majority of the cytoplasmic material, with the wall fragments becoming more predominant. Higher magnification of these wall fragments revealed a regular, particle-like arrangement on both surfaces of the wall material (Plates 5 and 6), which were not clearly visible following trypsin treatment (Plates 8 and 9). These particles may represent genuine protein components of the wall which would not be analysed because of their release on trypsin treatment. Trypsin, a proteolytic enzyme, removed not only this particle-like arrangement on the wall surface, but also adhering cytoplasmic material, membrane and protein. The removal of protein did not diminish the rigidity of the walls, indicating that the proteins are not essential for the structural integrity of the walls.

The wall preparation was relatively clean and free from contaminants, particularly whole cells. However, there was evidence of minor residual material still adhering to the wall. This was probably membrane fragments which could be responsible for the pink colour of the wall material since the carotenoids in this microorganism are probably associated exclusively with the cytoplasmic membrane, as has been shown for other pigmented species (95).

Several antibiotics, active in interfering with peptidoglycan synthesis, were studied. Insensitivity of S. marina toward the antibiotics was to be expected as peptidoglycan is not a constituent of its wall. However, cycloserine and penicillin G are dependent on the terminal D-alanyl-D-alanine dipeptide of the pentapeptide side chain in the classical peptidoglycan structure as the site of their inhibition. As the major N terminal amino acid was alanine, further investigations were conducted.

The lack of any significant turbidimetrically detectable effect on S. marina cell growth by cycloserine, penicillin G and vancomycin was as expected in the absence of a classical peptidoglycan layer. Electron micrographs of these antibiotic treated cells, however, showed some noticeable differences to control cells with no antibiotic present (see below). In the control, the wall was a single layered structure with a fuzzy appearance to the surface and, in section, closely resembled the electron micrographs of previously studied extremely halophilic cocci (10,55). S. marina demonstrated the formation of cross-walls for cell division, typical of the non-halophilic, Gram-positive micrococcaceae and sarcinae. However, the lack of fine structure preservation in these and other unaffected antibiotic treated cells was probably due to penetration problems of either the fixative or, more likely, embedding resin. In view of the extremely high concentration gradients for sodium and potassium ions (23), the envelope is probably highly passively impermeable to these ions and this characteristic may account for, in part, the penetration problems.

In the preliminary antibiotic experiments, the antibiotic treated cells were harvested in stationary phase. Only the cycloserine treated cells embedded sufficiently well for satisfactory sectioning and this may reflect some modification in the wall, facilitating the entry of the embedding resin. The cells appeared as single entities and the walls were significantly thicker than both the cycloserine treated and control cells, harvested in late logarithmic phase. These observations probably reflect the phase of growth rather than any antibiotic effect. For cells harvested in late logarithmic phase, the walls of cycloserine treated and control cells were of similar thickness. However, there was evidence of slight plasmolysis in the

cycloserine treated cells, with the membrane pulling away from the wall.

Penicillin G treated cells stained less heavily and had better embedding than the control cells. This suggests a genuine effect of the antibiotic and, again, may reflect some modification of the wall. The nuclear material appeared as 4-5 distinct packages, suggesting either multiple nuclei or a rather unusual 3-D configuration. This, also, may reflect some genuine antibiotic effect.

Thus, both cycloserine and penicillin G may have had some slight effect on S. marina wall. It has been suggested (12) that, in the absence of a peptidoglycan, penicillin may exert its influence on some other function in the halobacteria. This concept has, however, not been fully investigated and is very speculative. Cells treated with vancomycin showed no detectable differences to the control cells, either turbidimetrically or in the electron microscope.

The few studies on the effect of different antibiotics on the extreme halophiles have primarily been concerned with the halobacteria (63). There have been no reports on the adverse effect of the extreme growth conditions on antibiotic activity. This becomes an important factor in the case of microorganisms like the halophiles where the extremely long doubling time is of the same order as the half-life of the antibiotic in solution.

The loss of activity of both penicillin G and vancomycin in Dundas medium, with proliferating cells, occurred slowly over a period of hours. However, the corresponding controls had an identical loss of activity and it was concluded that there was no or very little active uptake of the antibiotics. It should be noted, however, that because of the limits of vancomycin detection, this antibiotic was present in the test culture at extremely high concentrations. Consequently, even saturated binding to

S. marina cells would probably not cause a sufficient loss of vancomycin in the medium to be detectable. Cycloserine, on the other hand, showed a marked difference in the loss of activity, which again occurred over a period of hours, between the control and the test culture. As the cycloserine activity was more rapidly lost in the control than in the test culture, this suggested that the cells, in some way, served to maintain the activity of the antibiotic. There are several possible explanations of this phenomenon. The cells could produce some compound extracellularly which interferes with the colour reaction, causing anomalously high readings. Alternatively, the compound could stabilise the antibiotic or prevent cycloserine dimerisation (91), which would cause a lack of detection by the colour assay.

Thus, cycloserine, vancomycin and penicillin G lost their activity over a period of hours in Dundas medium. As already pointed out above, this may indeed be a significant factor in explaining the insensitivity of microorganisms, like S. marina, whose doubling times are also of this order.

Unlike cycloserine, vancomycin and penicillin G, both novobiocin and bacitracin showed, turbidimetrically at least, a marked inhibition of S. marina growth, even at low antibiotic concentrations. However, although sections of novobiocin treated cells showed notable differences to the control, bacitracin treated cells appeared identical, in section, to the control, with no detectable variation in the cell ultrastructure.

The site of novobiocin action on wall synthesis is not known, but one theory for its antimicrobial effect (97) is that the antibiotic complexes with metal ions, particularly magnesium. In the extreme halophiles, magnesium is needed at unusually high concentrations (20), 1-2 orders of magnitude higher than the normal level for bacteria in general, for best growth. Magnesium has also been shown to have an important function in

maintaining the integrity and shape of the halobacterial envelope (34,68), with lipid as a potential binding site for this metal ion.

As mentioned above, the electron micrographs of novobiocin treated cells had significant differences to the sections from the controls. The most notable of these was the evidence of extensive plasmolysis, which was much more pronounced than in the cycloserine treated cells, with the membrane again pulling away from the wall. Although there was material adhering to the outer surface of the novobiocin treated cells, it was of similar appearance to the controls.

Thus, the antimicrobial effect of novobiocin on S. marina cells might not reflect the inhibition of the synthesis of wall polymers or their precursors. By complexing with the magnesium ions, the antibiotic could deprive the cells of an important metal ion, necessary for maintaining the integrity and stability of the cell envelope.

The antimicrobial effect of bacitracin, a polypeptide antibiotic, is its inhibition of the biosynthesis of peptidoglycan and, possibly, other wall polymers by preventing the dephosphorylation of the membrane bound C 55 isoprenoid alcohol carrier molecule. The antibiotic irreversibly binds directly, through chelation reinforced by hydrophobic bonding steric factors, to this molecule, not only inhibiting peptidoglycan synthesis but also blocking other synthetic processes dependent on the regeneration of the lipid moiety through the dephosphorylation reaction (60). Alternatively, and possibly of greater significance, bacitracin may inhibit the synthesis of polyisopreniod quinones, important components in the bacterial electron transport chain.

As mentioned, although bacitracin showed, turbidimetrically, a marked inhibition of S. marina growth at concentrations comparable to those required for normal bacteria, the bacitracin treated cells had, in section, no

detectable differences in ultrastructure to the control. If it is really wall synthesis in S. marina which is affected by bacitracin, this must imply that an analogous carrier system to the bactoprenol one in peptidoglycan synthesis must be operating. It is certainly reasonable to assume that the cells have the capacity to make an isoprenoid carrier since their lipid-synthesising enzymes are directed solely toward the production of isoprenoid compounds, like the carotenoids and dihydrophytol side chains of their polar lipids (72).

The only comprehensive report dealing with the effects of antibiotics on any halophiles, albeit the halobacteria (63), showed that all the antibiotics tested, with the exception of bacitracin, either caused no inhibition at the maximum concentrations tested or caused inhibition at concentrations 100-10,000 times greater than those required for inhibition of bacteria possessing peptidoglycan. The survey included all the antibiotics used in this research with the notable exception of novobiocin. Unlike S. marina, however, bacitracin caused a morphological change in the halobacteria, the organisms gradually changing from the normal rod shape to spherical forms. Complete conversion to spherical forms occurred only after growth in bacitracin for approximately one doubling time. This bacitracin induced conversion to spherical forms could be caused by inhibiting the synthesis of glycoproteins, particularly in the light of evidence from H. salinarium (100,101). All the nonlipid carbohydrate was covalently bound to a single protein which accounted for 40-50% of the total envelope protein. This envelope glycoprotein was the only major cell surface component and formed a relatively thick external layer. Removal by proteolytic enzymes or alteration of its structure by growth in bacitracin caused morphological changes,

e.g. conversion of the rods to spheres. This strongly suggests that it forms a rigid matrix at the cell surface, a structural component responsible for maintaining normal cell morphology, the characteristic rod shape. Alternatively, bacitracin could change the lipid composition of the membrane by inhibiting the synthesis of saturated isoprenoid chains of the type known to be present in at least one of the lipids of H cutirubrum (63). Thus, because of the nature of the components in the cell envelope of the extreme halophiles, the mode of action of bacitracin in inhibiting growth in S. marina and the halobacteria may to some extent be similar to its mode of action in bacteria possessing peptidoglycan.

Although novobiocin and bacitracin had significant inhibitory effects on S. marina growth, neither of these antibiotics appear to have weakened the wall sufficiently to cause cell lysis or osmotic fragility. Consequently, they do not represent a potential non-destructive method for wall removal. It has been suggested (63) that the inhibition observed in the halobacteria by antibiotics at extremely high concentrations is probably non-specific. However, although D cycloserine and penicillin G did not affect the growth of S. marina turbidimetrically, they did show some evidence of causing slight plasmolysis in the cell. The loss of activity of both D cycloserine and penicillin G over a period of hours in Dundas medium may be a significant factor in explaining the insensitivity of S. marina, whose doubling time is also of the order of hours. The slight plasmolytic effects of these two antibiotics may be unrelated to their specific effects on peptidoglycan synthesis in normal bacteria.

Summary.

S. marina (NCMB 778) demonstrated characteristics of the extreme halophiles. Although the cell wall bears marked similarities to other halococci, it is fundamentally different to the common Gram-positive micrococci.

The final wall material was relatively clean and free of contaminants, particularly whole cells, although there was some residual membrane still adhering to the wall. This contaminant was minor as the lipid content of the wall was negligible although sufficient to colour the wall pink.

The ninhydrin-positive constituents accounted for only about 14% of the dry weight of the wall. Only a limited number were found in significant amounts, the amino acids, in order of magnitude, glycine, histidine(?), alanine, glutamate and aspartate, although there is some doubt as to the validity of the identification of histidine(?), and the amino sugars glucosamine and galactosamine. In addition, three other components $X_1$, $X_2$ and $X_3$ from ion exchange chromatography and four components from paper chromatography were detected. Some, or all, of these unidentified components from ion exchange may correspond to the four unknown components from paper chromatography. Neither muramic acid nor diaminopimelic acid were detected in the wall which, in conjunction with its complete solubility in hot formamide, indicates the lack of a typical peptidoglycan. Although the unidentified ninhydrin-positive components could account for a few percent, by far the majority of the wall is composed of ninhydrin-negative compounds.

The major ninhydrin-negative components were the monosaccharides glucose, galactose and mannose which were present in approximately equimolar amounts. Three additional components were detected by paper chromatography, two of which had both amino and reducing groups, the third only reducing groups.

All efforts to extract single polymers were relatively unsuccessful.

The soluble and insoluble residues contained, at least qualitatively, the same constituents. Although the significance of their quantitative distribution is not known, there is some evidence to suggest the possible existance of more than one polymer in the wall.

The antibiotics D cycloserine, penicillin G and vancomycin thought to specifically affect peptidoglycan synthesis at several different points in the biosynthetic pathway do not inhibit the growth of S. marina. However, sections of D cycloserine and penicillin G treated cells in the electron microscope showed some differences to the control cells suggesting some slight effect of the antibiotic. Vancomycin treated cells showed no differences to the controls. However, the apparent non-inhibitory effect on S. marina by these antibiotics may not be entirely due to a lack of their uptake and action. D cycloserine, penicillin G and vancomycin lost their antibiotic activity over a period of hours in Dundas medium. This may be a significant factor in explaining the insensitivity of S. marina where the doubling time is also of the same order of magnitude.

Both novobiocin and bacitracin, however, had marked inhibitory effects on S. marina growth. Novobiocin treated cell sections appeared to have suffered extensive plasmolysis but the bacitracin treated cell sections were identical to the controls.

Thus, none of the antibiotics tested have weakened the wall sufficiently to cause cell lysis or osmotic fragility and do not represent a potential non-destructive method for wall removal.

The walls of S. marina are basically different to Gram-positive micrococci. Compounds other than amino acids and hexosamines are present and confer shape, rigidity and integrity to the wall, the major part of which is composed of ninhydrin-negative components. At this point there are too many

unknown factors to suggest a structure for the wall polymer/polymers.

Bibliography.

1) Klebahn, H. (1919). Mitt. Inst. allgem. Botan. Hamburg 4, 11-69.

2) Harrison, F.C. and Kennedy, M.E. (1922). Trans Roy. Soc. Canada V, 16, 101-152

3) In: "Bergey's Manual of Determinative Bacteriology", 7th. edition. (1957); R.S. Breed, E.G.D. Murray and N.R. Smith, eds.; Williams and Wilkins Co., Baltimore, Md.

4) Baxter, R.M. and Gibbons, N.E. (1956). Canad. J. Microbiol. 2, 599-606

5) Larsen, H. "Halophilism" in the 'Bacteria', vol. IV, pp. 297-342; I.C. Gunsalus and R.Y. Stanier, eds.; New York Academic Press, 1962

6) Kushner, D.J. (1964). Expl. Chemotherap. 2, 113-168

7) Dundas, I.D. and Larsen, H. (1962). Arch. Mikrobiol. 44, 233-239

8) Dundas, I.D. and Larsen, H. (1963). Arch. Mikrobiol. 46, 19-28

9) Brown, A.D. and Cho, K.Y. (1970). J. Gen. Microbiol. 62, 267-270

10) Kocur, M., Smid, B. and Martinec, T. (1972). Microbios 5, 101-107

11) Reistad, R. (1972). Arch. Mikrobiol. 82, 24-30

12) Larsen, H. (1967). Adv. Microbial Physiol. 1, 97-132

13) Baas Becking, L.G.M. (1928). Tijdschr. Ned. Dierk. Ver. (Ser. III D). 1, 6-9

14) Schoop, G. (1935). Dtsch. tierarztl. Wschr. 43, 817-820

15) Holmes, P.K., Dundas, I.E.D. and Halvorson, H.O. (1965). J. Bact. 90, 1159-1160

16) Blaurock, A.E. and Stockenius, W. (1971). Nature, New Biology, 223, 149-154

17) Mitchell, P. (1969). In: "Molecular Basis of Membrane Functions": D.C. Tosteston, ed.: p. 483. Prentice Hall Inc., Englewood Cliffs, N.J.

18) Symposium: "Light Energy Transduction by the Purple Membrane of Halophilic Bacteria". Fed. Proc. (1977). 36, 1797-1839

19) Larsen, H., Omang, S. and Steensland, H. (1967). Arch. Mikrobiol. 59, 197-203

20) Brown, H.J. and Gibbons, N.E. (1955). Canad. J. Microbiol. 1, 486-494

21) Christian, J.H.B. (1956). Doctoral Thesis, University of Cambridge.

22) Stevenson, J. (1966). Biochem. J. 99, 257-260

23) Christian, J.H.B. and Waltho, J. (1962). Biochim. Biophys. Acta. 65, 506-508

24) Sehgal, S.N. and Gibbons, N.E. (1960). Canad. J. Microbiol. 6, 165-169

25) Christian, J.H.B. and Ingram, M. (1959). J. Gen. Microbiol. 20, 27-31

26) Christian, J.H.B. and Waltho, J. (1961). J. Gen. Microbiol. 25, 97-102

27) Larsen, H. (1973). Antonie van Leeuwenhoeck. 39, 383-396

28) Baxter, R.M. and Gibbons, N.E. (1954). Canad. J. Biochem. Physiol. 32, 206-217

29) Baxter, R.M. and Gibbons, N.E. (1957). Canad. J. Microbiol. 3, 461-465

30) Baxter, R.M. (1959). Canad. J. Microbiol. 5, 47-57

31) Holmes, P.K. and Halvorson, H.O. (1963). Canad. J. Microbiol. 9, 904-906

32) Holmes, P.K. and Halvorson, H.O. (1965). J. Bact. 90, 316-326

33) Brown, A.D. (1963). Biochim. Biophs. Acta 75, 425-435

34) Kushner, D.J. and Onishi, H. (1966). J. Bact. 91, 653-660

35) Stoeckenius, W. and Kunau, W.H. (1968). J. Cell Biol. 38, 337-357

36) Reistad, R. (1970). Arch. Mikrobiol. 71, 353-360

37) Lanyi, J.K. and Stevenson, J. (1970). J. Biol. Chem. 245, 4074-4080

38) Liebermann, M.M. and Lanyi, J.K. (1971). Biochim. Biophys. Acta 245, 21-33

39) Lanyi, J.K. (1971). J. Biol. Chem. 246, 4552-4559

40) Kushner, D.J., Bayley, S.T., Boring, J., Kates, M. and Gibbons, N.E. (1964). Canad. J. Microbiol. 10, 483-497

41) Brown, A.D. and Shorey, C.D. (1963). J. Cell Biol. 18, 681-689

42) Brown, A.D. (1965). J. molec. Biol. 12, 491-508

43) Kushner, D.J. (1968). Advances appl Microbiol. 10, 73-99

44) Smithies, W.R., Gibbons, N.E. and Bayley, S.T. (1955). Canad. J. Microbiol. 1, 605-613

45) Belozersky, A.N. and Spirin, A.S. (1960). In: "The Nucleic Acids", 3, p. 147; E. Chargaff and J.N. Davidson, eds.: Academic Press, New York

46) Weidel, W. and Pelzer, H. (1964). Adv. Enzymol. 26, 193-232

47) Rogers, H.J. and Perkins, H.R. (1968). In: "Cell Walls and Membranes"; E. and F.N. Spon Ltd., London

48) Salton, M.R.J. (1964). In: "The Bacterial Cell Wall". Elsevier Publishing Co., Amsterdam

49) Osborn, M.J. (1969). Ann. Review Biochem. 38, 501-538

50) Glaser, L. (1973). Ann. Review Biochem. 42, 91-112

51) Braun, V. and Hanthe, K. (1974). Ann. Review Biochem. 43, 89-121

52) Mohr, V. and Larsen, H. (1963). J. Gen. Microbiol. 31, 267-280

53) Kushner, D.J. and Bayley, S.T. (1963). Canad. J. Microbiol. 9, 53-63

54) Brown, A.D. and Shorey, C.D. (1962). Biochim. Biophys. Acta. 59, 258-260

55) Steensland, H. and Larsen, H. (1971). K. norske Vidensk. Selsk. Skr. 8, 1-5

56) Kushner, D.J. and Onishi, H. (1968). Canad. J. Biochem. 46, 997-998

57) Reistad, R. (1975). Arch. Microbiol. 102, 71-73

58) Steber, J. and Schleifer, K.H. (1975). Arch. Microbiol. 105, 173-177

59) Strominger, J.L., Blumberg, P.M., Suginaka, H., Umbreit, J. and Wickus, G.G. (1971). Proc. Roy. Soc. London, Series B, 179, 369-383

60) In: "Molecular Basis of Antibiotic Action", Ch. 3: E. Gale, E. Cundliffe, P.E. Reynolds, M.H. Richmond and M.J. Waring, eds.; John Wiley & Sons, 1972

61) In: "Biochemistry of Antimicrobial Action", p. 35: Franklin and Snow, eds.; Chapman and Hall, 1973

62) Plapp, R. and Kandler, O. (1965). Arch. Mikrobiol. 50, 282-297

63) Mescher, M.F. and Strominger, J.L. (1975). J. Gen. Microbiol. 89, 375-378

64) Kushner, D.J. (1964). J. Bact. 87, 1147-1156

65) Abram, D. and Gibbons, N.E. (1960). Canad. J. Microbiol. 6, 535-543

66) Abram, D. and Gibbons, N.E. (1961). Canad. J. Microbiol. 7, 741-750

67) Brown, A.D. (1964). Bacteriol. Rev. 28, 296-329

68) McClare, C.W.F. (1967). Nature, New Biology, 216, 766-771

69) Kates, M., Palameta, B., Joo, C.N., Kushner, D.J. and Gibbons, N.E. (1966). Biochemistry 5, 4092-4099

70) Sehgal, S.N., Kates, M. and Gibbons, N.E. (1962). Canad. J. Biochem. Physiol. 40, 69-81

71) Kushner, D.J. (1968). Adv. App. Microbiol. 10, 73-99

72) Kates, M., Yengoyan, L.S. and Sastry, P.S. (1965). Biochim. Biophys. Acta 98, 252-268

73) Dundas, I.D., Srinivasan, V.R. and Halvorson, H.O. (1963). Canad. J. Microbiol. 9, 619-624

74) Clark, B. (1971). B.Sc. (Hons.) thesis, University of St. Andrews.

75) Scully, D.B. and Wimpenny, J.W.T. (1974). Biotechnol. Bioeng. 16, 675-687

76) Bligh, E.G. and Dyer, W.J. (1959). Canad. J. Biochem. Physiol. 37, 911-917

77) Kates, M. (1972). In: "Techniques in Lipidology; isolation and identification of lipids", p. 351; Amsterdam, North Holland Publishing Co.

78) Gros, C. and Labousse, B. (1969). Europ. J. Biochem. 7, 463-470

79) Moore, S. and Stein, W.H. (1948). J. Biol. Chem. 176, 367-388

80) Dubois, M., Gilles, K., Hamilton, J.K., Rebers, P.A. and Smith, F. (1951). Nature 168, 167

81) Park, J.T. and Johnson, M.J. (1949). J. Biol. Chem. 181, 149-151

82) Bhatti, T., Chambers, R.E. and Clamp, J.R. (1970). Biochim. Biophys. Acta 222, 339-347

83) Ghuysen, J.M., Tipper, D.J. and Strominger, J.L. (1966). Methods in Enzymol. 8, 692-693

84) Armstrong, J.J., Baddiley, J. and Buchanan, J.G. (1960). Biochem. J. 76, 610-621

85) Hughes, R.C. (1965). Biochem. J. 96, 700-709

86) Krause, R.M. and McCarty, M. (1961). J. exp. Med. 114, 127-140

87) Perkins, H.R. (1965). Biochem. J. 95, 876-882

88) Northcote, D.H. and Horne, R.W. (1952). Biochem. J. 51, 232-236

89) Freeman, G.G. (1942). Biochem. J. 36, 340-356

90) Linton, A.H. (1958). J. Bact. 76, 94-103

91) Jones, L.R. (1956). Anal. Chem. 28, 39-41

92) Shockman, G.D. (1965). Bacteriol. Rev. 29, 345-358

93) Martin, H.H. (1963). J. Theoret. Biol. 5, 1-34

94) Thompson, J.S. (1968). Anal. Biochem. 22, 260-268

95) Salton, M.R.J. and Ehtisham-ud-din, A.F.M. (1965). Austr. J. Exp. Biol. Med. Sci. 43, 255-264

96) Craig, G.H. and Harned, R.L. (1972). In: " Analytical Microbiology ", Vol. 2, pp. 255-263; Kavanagh, F, ed.: Academic Press

97) Brock, T.D. (1962). J. Bact. 84, 679-682

98) Koncewicz, M. (1972). Biochem.J. 130, 40p-41p

99) Percival, E. (1970). In: "The Carbohydrates", Vol. 2B, pp 537-587; W. Pigman and D. Horton, eds; New York - London Academic Press

100) Mescher, M.F., Strominger, J.L. and Watson, W. (1974). J. Bacteriol 120, 945-954

101) Mescher, M.F. and Strominger, J.L. (1976). Proc. Nat. Acad. Sci. U.S.A. 73, 2687-2691.