The University of Glasgow

# SOME STUDIES OF BACTERIAL LYSIS AND OF THE BACTERIAL CELL-WALL

by

HAROLD STERN, M.B., Ch.B.
(Medical Research Council Scholar)

Thesis submitted for the degree of Ph.D.
in the Faculty of Science.

Department of Bacteriology
and Department of Chemistry.

October, 1953

# INDEX

# PREFACE

During the past few decades considerable advances have been made in the understanding of the physiological and metabolic activities of bacteria but the details of bacterial structure are still remarkably scanty. Thus, for instance, the question of whether bacteria possess discrete nuclei is still unsettled. This lack of knowledge is due mainly to the fact that bacteria lie at the limits of resolution of the light microscope. The advent of the electron microscope raised high hopes that, at last, the internal details of bacterial structure would be revealed. Unfortunately, these hopes have not yet been realised because, although bacterial structure is well within the limits of resolution of the electron microscope, the bacterial body has proved to be, in most cases, too thick and dense to allow of ready penetration of the electron beam. Direct examination of bacterial structure must, therefore, await microscopes with a higher voltage acceleration of electrons although a second, very promising solution lies in the technical perfection, already rapidly approaching, of section cutting for the electron microscope. At present one is compelled to carry out controlled treatments of bacteria which render the structural details more readily visible in the electron microscope.

Bacteria undergoing autolytic disintegration, with resulting reduction in the thickness and density of the cells, should be ideal subjects for electron microscopy and the present study is partly concerned with an investigation of the morphological changes undergone by the bacterial cells during this process. Detergents are well known to influence markedly autolysis and have been shown to produce certain characteristic

morphological effects on the cells. The action of the cationic detergent, cetyl-trimethyl-ammonium bromide, has, therefore, been examined in detail in the hope that information as to the mechanism and site of action of these compounds might be obtained.

Any discussion of lysis must inevitably involve the surface membrane of the cell. It has recently become possible to isolate the bacterial surface membrane, namely the bacterial cell-wall, from the internal contents of the cell. This membrane is almost ideal for electron microscopy and an attempt has been made to elucidate both the intimate structure of the cell-wall and its functional importance both with regard to bacterial lysis and cell division.

Major parts of this work have been accepted for publication by the Journal of Pathology and Bacteriology and by Biochimica et Biophysica Acta.

# INTRODUCTION

# INTRODUCTION

## The mechanism of autolysis

The spontaneous autolysis of micro-organisms is a very old observation. In 1874, Schutzenberger observed that beer yeast, incubated in the absence of nutrient material for 12 to 15 hours, lost into solution 8 to 9 per cent of its solid matter. This loss he attributed to an action exercised by the yeast on its own substance. Emmerich and Löw (1899) pointed out that there occur in the cultures of many bacteria enzyme-like substances which are produced by the bacteria and which eventually tend to dissolve them. Albert and Albert (1901) observed that yeast cells suspended in water became Gram-negative and that this change was closely associated with the autolytic disintegration of the cells. Trommsdorf (1902) showed that this phenomenon did not occur in the ice-box or after heating the cells to a high temperature and concluded that the autolysis and loss of Gram reaction were due not to simple solution but to the action of intracellular enzymes. Rettger (1904) demonstrated that the autolysis of yeasts and bacteria was associated with the liberation of intracellular protein material which was subsequently digested to various simpler products. He found that autolysis occurred best at 37°C. in a neutral or slightly alkaline medium and that autolysis was prevented by heating the organisms to 58° to 60°C. He also recognised that certain weak antiseptics in low concentrations, such as chloroform or toluol, accelerated the rate of autolysis and that higher concentrations of these antiseptics might inhibit the process. Flexner (1907), in his extensive investigations of meningococci, which are highly susceptible

to autolysis, similarly noted that autolysis was inhibited by heating to over 60°C. and that it was accelerated by the presence of toluol. Moreover, potassium cyanide, a well-known enzyme poison, was found to kill the organisms and to inhibit autolysis; after removal of the cyanide by washing partial autolysis occurred. Working with pneumococci Lord and Nye (1919,1922a) confirmed that the maximum rate of autolysis required an optimum temperature and an optimum pH. They showed that autolysis was inhibited by heating and by the presence of human serum and that it was accelerated by suspending the organisms in a solution in which large numbers of pneumococci had previously dissolved. They considered that these facts were consistent with the activity of an endocellular enzyme.

A series of investigations carried out by Avery and Cullen (1920a,1920b,1920c) demonstrated the presence in autolysed broth cultures of pneumococci of intracellular, proteolytic, lipolytic and carbohydrate-splitting enzymes. These enzymes were shown to be readily destroyed by heating to 100°C. and to possess similar pH optima. Subsequently, (Avery and Cullen, 1923), the autolysate was shown to contain an enzyme or group of enzymes capable of lysing suspensions of heat-killed pneumococci. This enzyme was not type-specific and attacked, although to a lesser degree, the closely related Streptococcus viridans. It was inactive against other streptococci and against Staphylococcus aureus. This bacteriolytic enzyme closely corresponded in pH optimum and heat stability to the other enzymes present in the autolysate and Avery and Cullen were unable to decide whether the bacteriolytic enzyme was identical or not with these other enzymes. Neil and Avery (1924) were, however, able to differentiate the bacteriolytic enzyme from the proteolytic,

lipolytic and carbohydrate-splitting enzymes by their sensitivity to destruction by oxidation. Whereas the proteolytic and lipolytic enzymes were completely unaffected by oxidation the bacteriolytic enzyme and, even more readily, the carbohydrate-splitting enzyme were progressively destroyed. Extension of these studies by Goebel and Avery (1929) showed that autolysis of pneumococci was accompanied by proteolysis, resulting in an increase of amino-acid and non-coagulable nitrogen, and by lipolysis, resulting in an increase of ether-soluble fatty acids. A comparable proteolysis and lipolysis occurred when heat-killed cells were lysed by autolysates containing the intracellular enzymes. The separation of bacteriolytic from proteolytic and lipolytic activity, achieved by Neil and Avery, suggested that proteolysis and lipolysis were but the final stages of bacteriolysis and probably also of spontaneous autolysis and that the bacteriolytic enzyme or group of enzymes was responsible for the primary stages which preceded proteolysis and lipolysis. Thus, Goebel and Avery were able to demonstrate that suspensions of pneumococci at 0°C. could be readily dissolved by sodium desoxycholate without the occurrence of either proteolysis or lipolysis. Although Goebel and Avery suggested that bile solution of pneumococci differed from that of normal autolysis it was subsequently shown, as will be described in the following section, that bile solution is but an acceleration of normal autolysis.

Dubos (1937a,1938) also using pneumococci confirmed much of the previous work and made the important observation that before disintegration of the cells occurred they became Gram-negative and that this change in staining property was not necessarily followed by actual disintegration of the cells. He showed that formaldehyde in very low concentrations increased the rate of lysis of pneumococci but in high concentrations

completely inhibited lysis. So long as the excess of formaldehyde was present the organisms remained Gram-positive and morphologically intact. When the organisms were washed free of formaldehyde and suspended in physiological saline at pH 7.0 a limited autolysis occurred; the cells slowly became Gram-negative and smaller in size but further disintegration of the cells did not take place. The subsequent addition of pneumococcal autolysate to these Gram-negative cells resulted in complete disintegration. The formaldehyde had, therefore, caused a reversible inactivation of the factor responsible for the change in Gram staining and an irreversible inactivation of the enzymes responsible for cellular disintegration. Dubos concluded from his experiments that the enzyme system which caused autolysis was composed of many enzymes of which the one responsible for the change of the bacterial cell from the Gram-positive to the Gram-negative state could remain active under conditions where the enzymes responsible for final disintegration were inactivated.

The nature of the factor responsible for the change of Gram staining was suggested by the work of Dubos and MacLeod (1938) who isolated from polymorphonuclear leucocytes an enzyme which was capable of converting heat-killed, Gram-positive pneumococci to the Gram-negative state. It was unable to accomplish disintegration of the cells. The enzyme was shown to be apparently identical with the ribonucleinase of the pancreas and similarly to decompose yeast nucleic acid. The confirmation that a nucleic acid substrate was involved during the change in Gram staining of bacterial autolysis was supplied by Thompson and Dubos (1938); by a process of controlled autolysis which did not involve disintegration of the cell they were able to recover nucleic acid and nucleoprotein from the material released from the cell. Bartholomew and Umbreit

(1944) subsequently demonstrated that crystalline ribonuclease prepared by Kunitz's (1940) method could change the Gram staining of a variety of heat-killed, Gram-positive organisms.

However, Dubos, himself, showed caution in interpreting these various findings and stressed the fact that most animal tissues possess other enzymes, such as lysozyme, which possess very similar physico-chemical properties to ribonucleinase and are also capable of affecting the staining properties of bacterial cells. Lysozyme (Fleming, 1922) is an enzyme with the same heat resistance and solubility properties as ribonucleinase and may contaminate even crystalline preparations of the latter. This enzyme depolymerises and hydrolyses specifically acetyl-amino polysaccharides (Epstein and Chain, 1940), which polysaccharides have been shown to be present in many bacteria and the discovery that a certain bacterium is susceptible to lysozyme can be used as evidence that this organism possesses as an essential component of its structure the lysozyme substrate, acetyl-amino polysaccharide. Dubos (1937a) had already demonstrated that pneumococcal autolysate, which attacked heat-killed pneumococci, was capable of liberating reducing sugars from various acetyl-amino-glucose glucosonides of animal and bacterial origin, and that the action of the autolytic enzymes on the heat-killed cells could be inhibited by previous incubation with sufficient amounts of the amino-polysaccharides. There was, however, evidence that the bacterial polysaccharidase was not identical with lysozyme; Meyer, Dubos and Smyth (1937) showed that a polysaccharide acid obtainable from the vitreous humour, the umbilical cord or haemolytic streptococci was hydrolysed by the enzymes of pneumococcal autolysate but was not attacked by lysozyme. The non-identity of lysozyme with the polysaccharidase of pneumococcal autolysate was further shown

its resistance to inactivation by alcohol and acetone. Although it was thus undeniable that a specific polysaccharidase was involved in the pneumococcal autolytic process the precise site of action of this enzyme was not clear; the polysaccharidase was not type-specific whereas the pneumococcal polysaccharides were highly type-specific. This problem was elucidated by subsequent investigations of the antigenic behaviour of pneumococci.

From the work of Dubos (1937b,1945) a close correlation emerged between the Gram-positive state of an organism and its ability to stimulate the formation in animals of type-specific antibodies. Any procedure which caused the cell to lose its Gram-positivity also caused loss of antigenicity. Complete disintegration of the cell was not necessary for this loss to occur; treatment of pneumococci with tissue enzymes (ribonucleinase?) or with formaldehyde, which destroyed the disintegrative enzymes but not the factor responsible for change of Gram staining, resulted in loss of antigenicity. However, these procedures seemed to inactivate the capsular, polysaccharide antigen complex without destroying the capsular polysaccharide itself; the latter could be isolated intact from the cell. The capsular polysaccharide, freed of all protein, was not antigenic. Further, heat-killed pneumococci treated with type-specific polysaccharidases, which hydrolysed the capsular polysaccharides, lost neither their Gram-positivity nor their specific, antigenic properties. Again, treatment of heat-killed pneumococci with trypsin caused a rapid clearing of the suspension leaving an insoluble residue which contained only 22 per cent of the original cell weight. Microscopic examination revealed that the residue consisted of Gram-positive cocci of very small size. These cocci could still give rise to type-specific antibodies when injected into

rabbits; destruction of the greater part of the organism which left intact the surface structures responsible for Gram-staining did not, therefore, interfere with the antigenicity of the cell. These various experiments indicated that the capsular polysaccharide was, on the pneumococcus, linked to a second substance and it seemed likely that this second component was intimately connected with the Gram staining property of the cell. It, therefore, became highly probable that it was the non-type-specific linkage between this second component of the antigenic complex and the type-specific polysaccharide which was attacked by the non-type-specific polysaccharidase of the pneumococcal autolytic enzyme system.

Further elucidation of the enzyme composition of the autolytic system was achieved by Stacey and Webb (1948). They confirmed that the autolysis of Gram-positive organisms occurred in two stages; first, the conversion of the Gram-positive cell to the Gram-negative form, and second, the disintegration of the Gram-negative cytoskeleton. Under reducing conditions only the first stage of autolysis was found to occur and it thereby proved possible to isolate the enzymes responsible for the first stage free from the enzymes of the second stage. The preparation was capable of hydrolysing free ribonucleic acid but could be differentiated from the ribonucleinase of animal tissues. Stacey and Webb worked principally with strains of Clostridium welchii and Staphylococcus citreus and they found that although the first stage or ribonucleinase preparations from both these organisms possessed similar properties they were species-specific and even to some extent strain-specific. Thus, the enzyme from Cl. welchii readily converted heat-killed, Gram-positive Cl. welchii to Gram-negative forms but had no action on heat-killed, Gram-positive Staph. citreus or Staph.

aureus. Similarly, the enzyme from Staph. citreus exhibited no action against heat-killed, Gram-positive Cl. welchii or Staph. aureus but it was found to be active against a rough variant isolated from an aged culture of a strain of Staph. aureus. Since the rough strains of most bacteria are believed to differ from the smooth strains by the absence of a surface carbohydrate the latter observation suggested that the specificity of these bacterial enzymes was in some way determined by the specific carbohydrates of the cell. During autolysis the change of the Gram-positive cells to the Gram-negative forms was shown to be associated with the liberation of the specific carbohydrates in an apparently undegraded state, a finding already noted by Dubos for pneumococci. These polysaccharides could be hydrolysed to a variable extent by lysozyme but were completely unaffected by the bacterial ribonucleinase preparations. The release of the specific carbohydrates during the change in Gram staining of the bacterial cell is accompanied by the release of nucleic acid and nucleoprotein (Thompson and Dubos, 1938). Bile extraction at 60°C. has also been shown to change the Gram reaction of organisms with the release of carbohydrates and magnesium ribonucleinate (Henry and Stacey, 1946). Stacey and Webb, therefore, concluded that the ribonucleic acid of the bacterial surface was linked to the specific polysaccharide and that before the ribonucleinase could attack the nucleic acid another enzyme, which they called the "polysaccharide-releasing enzyme", must first split the combining linkages and release the protective, specific polysaccharide. In confirmation of the presence of a polysaccharide-nucleic acid linkage at the bacterial surface they were able by a process involving complete disintegration of the cellular structure of various pneumococci to isolate specific polysaccharides

which contained as much as 5 per cent of ribonucleic acid in firm combination. The high specificity of the autolytic enzymes was said to be due to this "polysaccharide-releasing enzyme". The carbohydrate-nucleic acid linkage is presumably present in the same form in all types of pneumococci but differs in some way in other organisms. The animal preparations of ribonucleinase probably also possess a similar enzyme as a contaminant as Dubos suspected. Since these tissue preparations are not species-specific their polysaccharide-releasing enzyme is probably closely related to or even identical with the relatively non-specific lysozyme; Fleming (1932) has shown that lysozyme is widely distributed in animal tissues and Webb (1948) has demonstrated that Gram-positive organisms are rendered Gram-negative, with the release of specific polysaccharides and ribonucleic acid, by lysozyme. The bacterial ribonucleinase, itself, was apparently non-specific since the ribonucleic acids liberated from different, Gram-positive organisms were not found by Stacey and Webb to be fundamentally different from one another. Further, the ribonucleic acid extracted by bile salts from one Gram-positive organism could be readily replated onto the cytoskeleton of another organism rendered Gram-negative by bile extraction.

The second stage of autolysis Stacey and Webb found to be non-specific. From the Gram-negative cytoskeletons they isolated a casein-hydrolysing and a peptone-hydrolysing enzyme. The isolated enzymes had no action on living organisms or heat-killed, Gram-positive organisms but readily dissolved not only the Gram-negative cytoskeletons of all Gram-positive organisms but also heat-killed, true Gram-negative organisms. Since ribonucleinase-free proteolytic enzymes, such as trypsin, were also unable to lyse heat-killed, Gram-positive cells but readily dissolved the Gram-

negative forms of these cells, it was concluded that the disintegration of the Gram-negative cytoskeletons in autolysis is the direct result of the action of these proteolytic enzymes.

Although the detailed investigations of the mechanism of autolysis have been concerned almost exclusively with Gram-positive organisms sufficient information is available that similar processes probably operate with Gram-negative bacteria. Autolysis of meningococci (Flexner, 1907) and gonococci (Wollstein, 1907) has been shown to be due to an intracellular autolytic enzyme but whether a two stage process is involved as with Gram-positive organisms is unknown. However, since Gram-negative organisms, when killed by heating, are readily attacked by proteolytic enzymes, such as trypsin, whereas Gram-positive organisms, heat-killed, are not susceptible until rendered Gram-negative it seems likely that the process is a very much simpler one. Probably, only the enzymes of the second stage of Gram-positive cell autolysis are involved (Stacey and Webb, 1948). It is interesting that the autolysis of acid-fast bacteria has been shown to occur in two stages, the first involving loss of acid-fastness and the second cellular disintegration; it is possible to produce loss of acid-fastness without subsequent disintegration of the cells (Baisden and Yegian, 1943; Dubos, 1945).

## The influence of surface-active agents on autolysis

Robert Koch, while investigating Rinderpest, noted that the bile of animals dead from the disease conferred immunity when injected into healthy animals. While conducting experiments to determine whether bile might influence the course of other bacterial infections Neufeld (1900) discovered that pneumococci possessed the remarkable property of being soluble in bile. Pneumococci which had been killed by heat, streptococci, staphylococci, corynebacteria and various Gram-negative bacteria were found to be insoluble in bile. This property of bile solubility Neufeld attributed to swelling and precipitation of the cell-wall with consequent loss of cytoplasm. The apparent uniqueness of the pneumococcus he believed to be due to the greater porosity of the pneumococcal cell-wall. Schultze (1907) and Mandelbaum (1907) subsequently showed that although bile did not dissolve streptococci it nevertheless killed them; 10 per cent bile killed all streptococci in a suspension within 24 hours. Bacillus coli, on the other hand, was unaffected. More complete comparative studies were carried out by Nicolle and Adil-Bey (1907) who showed that B. avisepticus, B. mallei and B. pestis were dissolved by bile but not so dramatically as the pneumococcus. The salts of alkaline earths, such as magnesium sulphate, favoured the occurrence of lysis. V. cholerae, B. typhosus, B. coli, B. anthracis and B. pyocyaneus were more resistant but the resistance could be partly overcome by the presence of magnesium sulphate. Staphylococci and streptococci were completely resistant to bile lysis.

Lord and Nye (1922b), who had recognised the enzymatic nature of bacterial autolysis, rejected the theories that bile solubility was directly due to the solvent action of the bile salts and suggested that the bile affected the cell so as

to allow a more rapid action of the normal process of autolysis. Atkin (1926) observed that pneumococcal colonies, grown on serum-agar plates, underwent autolysis within 2 to 3 days and that within the areas of autolysis there developed papillae of organisms which did not autolyse even after 3 weeks' incubation; the organisms of the papillae were Gram-positive and showed normal morphology. These organisms were found by Atkin to be insoluble in bile. When they were subcultured to a fresh medium the organisms recovered their ability to autolyse and at the same time their bile solubility. Since subculture to a fresh medium resulted in return of normal susceptibility to autolysis and bile solubility it would seem that an enzyme or coenzyme was deficient, probably as a result of exhaustion of certain nutrient materials in the medium. These results demonstrated the close relationship between spontaneous autolysis and bile solution. The suggestion of Lord and Nye that bile acts by hastening the normal autolytic process was supported by Mair (1930) who showed that the conditions necessary for autolysis of pneumococci and for bile solubility were apparently identical. The close relationship between autolysis and bile solution was further demonstrated by Downie, Stent and White (1931) who investigated the relative efficiency of various bile salts with regard to solution of pneumococci. They noted that both smooth and rough strains of pneumococci were equally susceptible to bile solution but obtained a single rough strain which was completely resistant to autolysis even after the addition of autolysate from a normal strain. This organism was also bile resistant. Further proof of the enzymic nature of bile solution was provided by Wollman and Averbusch (1932) who showed that pneumococci rendered insoluble in bile by heating were dissolved by bile if a few drops of a suspension of

living pneumococci, soluble in bile, were added.

Dubos (1937b) demonstrated conclusively that pneumococci, living or dead, were soluble in bile only when a) the autolytic enzymes were present in a potentially active form and b) conditions were favourable for enzyme action. Pneumococci could be killed by dilute iodine, by acetic acid of pH 4.4 or by M/20 sodium hydroxide without dstroying the autolytic enzymes. Such organisms, when washed free from the above substances and resuspended in buffer at pH 7.0, underwent spontaneous autolysis and the rate of autolysis could be markedly accelerated by the addition of bile salts. On the other hand, if the dead organisms were boiled, after washing, to destroy the autolytic enzymes then neither autolysis nor bile solution occurred. As with spontaneous autolysis the rate of bile solution increased up to about 50°C. At 60°C. bile solution ceased due to inactivation of enzymes. In the ice-box both spontaneous autolysis and bile solution were markedly slowed. Dubos considered that his various experiments proved the dependence of bile solution on normal autolysis. Moreover, he recognised that the clarification of suspensions of pneumococci by bile probably required only the initial stages of autolysis since, as Goebel and Avery (1929) had shown, bile solution could still take place when proteolysis, which is responsible for the completion of autolytic disintegration, was inhibited by high concentrations of bile salts or by a temperature of 0°C.

Sturdza (1938) demonstrated that ultraviolet irradiation destroyed the bile solubility of pneumococci. Since inactivation by ultraviolet light is a general characteristic of enzymes this provided further support for the enzymatic nature of bile solution.

Although the investigations detailed above were concerned

exclusively with the solvent properties of bile or the various bile salts it was early recognised that other surface-active agents were as potent as or even more potent than the bile salts in dissolving bacteria. Sodium oleate (Lamar, 1911) and sodium ricinoleate (Kozlowski, 1925) were found to resemble bile in their actions against pneumococci and Lamar suggested that the oleate caused dissolution by attacking lipoid constituents of the cell including, possibly, similar constituents in the cell membrane. In 1937, Bayliss first introduced a synthetic surface-active agent, the anionic detergent, sodium lauryl sulphate, as a substitute for bile in testing for the bile solubility of pneumococci. He showed that it was active in relatively high dilutions and cleared suspensions of not only pneumococci but also, although less dramatically, many Gram-negative organisms. Apart from the pneumococcus and a Bacillus laterosporus, Gram-positive organisms were apparently unaffected. Cationic detergents, such as the polypeptide, tyrocidine, were subsequently found to be even more potent lytic agents and to be readily active also against various Gram-positive organisms, such as staphylococci and streptococci (Hotchkiss, 1944; Gale and Taylor, 1947).

## The mechanism of the bactericidal action of surface-active agents

Although it is now well established that the dissolution of bacteria by surface-active agents is due to the activation of cellular autolytic enzymes there is still the problem of the mechanism whereby the surface-active compound causes death of the bacterial cell with resulting activation of the autolytic enzymes. Work on this subject has been concerned almost entirely with synthetic, surface-active compounds but it seems logical to assume that the bile salts act by a mechanism similar to that of the synthetic substances.

It has long been recognised that the bactericidal and lytic properties of surface-active compounds cannot be definitely related to any one of their measurable physical properties, such as surface tension depression, wetting action, detergency, dispersing or emulsifying ability. This is not surprising in view of the fact that these properties are all indirect measurements of surface-activity at arbitrarily chosen interfaces. Similarly, no single, specific, chemical structure has been recognised; the bile salts vary widely in their chemical structure from the synthetic detergents. Anson (1939), however, suggested that the surface-active and the bactericidal properties of both bile salts and synthetic detergents depend on the same general type of hydrophobic-hydrophilic structure, a large hydrophobic group with a small hydrophilic group attached to it. Indeed, he suggested that "bile salts are simply biological detergent molecules whose specific structures are of secondary importance".

The first process involved in the interaction of surface-active agents is the adsorption of the ions of these compounds onto the bacterial surface. The water insolubility

of the hydrophobic portion of the ion is the driving force which tends to crowd even minute amounts of the substance out to the boundaries of the aqueous phase and it is by virtue of this property that surface-active agents are enabled to act on bacteria in such high dilutions. The specific affinities of the hydrophobic group are responsible for the firm anchoring of the compound onto the bacterial surface. The complex, so formed, must not, to be harmful, dissociate readily. Albert (1942) has shown that the high molecular weight of the most potent surface-active agents is an important factor in the formation of weakly-dissociable complexes. Although the large hydrophobic group is responsible for the formation of a stable complex with the bacterial surface it is the polar, hydrophilic group which enables the detergent to become initially linked to the susceptible groupings of the bacterial surface structure. McCalla (1940,1941) has shown that bacteria can absorb hydrogen ions and other cations from solution and can enter into ionic exchange. Further evidence of the ability of bacteria to act as ion exchangers is derived from the fact that the bactericidal activity of surface-active agents is dependent on hydrogen ion concentration, which operates to increase the activity of the detergent when the production of oppositely charged ions in the bacterial cell is favoured. More direct evidence of the specific reaction of surface-active ions with oppositely charged groups in the bacterium was afforded by the demonstration by Valko and Dubois (1944) that the killing action of surface-active cations on bacteria could be reversed, under certain conditions, by the addition of anionic agents: the anionic compound had to be added within 5 to 10 minutes of the cationic and they interpreted the "reversal" effect as the interaction of the oppositely charged surface-active ions with desorption of the cationic

agent from the negatively charged groups of the cell. The authors also showed that harmless cations can protect against toxic cations presumably by competition for the reactive sites in the bacterium. The "reversal" mechanism has been challenged by Klein and Kardon (1947) who found that anionic agents could protect the cell only if added before or simultaneously with the toxic cationic agents. The anionic agents could then react with and neutralise the cationic and thus prevent the attack of the latter against the bacteria. Whether the adsorption is reversible or irreversible it can be considered as only the first stage in the bactericidal process; certain non-toxic, surface-active agents are known which can be firmly adsorbed to the bacterial surface. Therefore, in the case of the toxic agents other processes set in which finally cause death of the organism.

Anson (1939) first noted that bile salts and synthetic detergents readily denature proteins. He found that the concentrations of detergents required for denaturation were proportional to the amounts of protein present and concluded that a combination of the detergent with the protein occurred in the process. Kuhn and Bielig (1940) were further able to show that synthetic detergents were capable of splitting certain protein conjugates. They suggested that the capacity of surface-active agents to denature proteins and dissociate protein conjugates brings about the inactivation of essential components of living bacteria and thus causes the death of the organisms.

Baker, Harrison and Miller (1941a,b) examined the effect of a wide range of anionic and cationic detergents upon the metabolism and viability of various Gram-positive and Gram-negative bacteria. They showed that, in general, there was a correlation between bactericidal activity and the ability to inhibit bacterial metabolism, the latter estimated in terms of

glycolysis and respiration. The antibacterial effect and the inhibition of metabolism could be prevented by the prior or simultaneous addition of small quantities of phospholipoids. These compounds also possess a polar-non-polar structure and presumably have a similar affinity as the detergents for attractive groups on the bacterial surface. Since the phospholipoids did not themselves affect bacterial metabolism or viability and were not removed by washing the bacteria they were considered to so alter the structure of the bacterial surface as to prevent adsorption and penetration of the toxic detergent molecules. On the basis of these experimental results Baker, Harrison and Miller proposed a hypothesis of bactericidal action of detergents involving two stages: first, disorganisation of the bacterial cell membrane by virtue of the great surface-activity of these compounds; and second, denaturation of certain enzymes essential for metabolism and growth. Ordal and Borg (1942), using the oxidation of lactate as an index of metabolic activity, confirmed that there seemed to be a general relationship between metabolic inhibition and bacterial death following the addition of surface-active compounds.

On the other hand, there has been a considerable amount of evidence conflicting with the view that the bactericidal effect is the result of metabolic inhibition. Baker, Harrison and Miller, themselves, noted an exception to this view; the anionic detergent, Tergitol T, was found to depress markedly the respiration of Proteus vulgaris without having any apparent bactericidal effect. Sykes (1939) had already shown that, although a relationship existed between the effect of various alcoholic and phenolic germicides on bacterial metabolism, measured as succinic dehydrogenase activity, and the effect on the viability of the cells, in general the viability of the

cells was affected at concentrations of germicide lower than those which inhibited bacterial metabolism. Ely (1939) also noted discrepancies in effect on bacterial respiration and lethal effect with different germicides. Bucca (1943) examined the action of many germicides on various enzyme activities of the gonococcus and showed that death of cells occurred before significant enzyme inhibition.

These discrepancies might well be due, as Hotchkiss (1946) noted, to the comparison of quantitative metabolic measurements with the essentially qualitative determinations of viability by subculture, employed by all the above workers. Subculture will still detect viable cells after more than 99.9 per cent of the cells are dead. The unusually large amount of detergent required to kill the last few cells (Dubois and Dibblee, 1947), which may be protected by clumping (McCulloch, 1947), heightens the discrepancy. Roberts and Rahn (1946) pointed out that if cell death and metabolic inhibition are demonstrated to occur together with the same amount of detergent, an investigation limited to intact cells leaves one unable to decide which of the results is cause and which effect.

As opposed to the view that detergents exert their bactericidal effects on detergent-sensitive, metabolic enzymes Valko (1946) believes that the detergents act by a process of general protein denaturation. He considers that surface-active ions are readily capable of penetrating into the bacterial body and that it is unnecessary to regard the interaction of a hypothetical, bacterial membrane with the surface-active ions as important in the first phase of antibacterial action. Valko used as an argument the work of Kuhn and Jerchel (1941a,b) to support his view. These workers showed that micro-organisms, suspended in nutrient medium containing the colourless salts of the surface-active tetrazolium compounds, became red-coloured

and this they claimed to be due to reduction of the tetrazolium ion within the cells. In view of the extraordinary, intrinsic affinity of surface-active ions for proteins they combine readily with proteins and this Valko believes causes "a disturbance of the intermolecular structure of the proteins by upsetting the balance of electrostatic forces, as well as that of the non-coulombic adhesion in the molecule. At the same time the interaction of the proteins with the solvent molecules may undergo profound changes and, as a further consequence, the bonds between the components of the conjugated proteins may be disrupted". These changes were said to result in protein denaturation, enzyme inactivation and cell death.

That detergents should exert their bactericidal effect by protein denaturation was challenged by Hotchkiss (1946) on the grounds that the concentrations of detergents which denature most ordinary proteins are in a higher range than those necessary for killing bacteria. Proteins, in general, require for denaturation from about one-fifth to more than equal their own weight of detergents (Anson, Kuhn and Bielig). Bacteria, which have combining weights similar to those of pure proteins (Stearn and Stearn, 1924; McCalla, 1941; Valko and Dibblee, 1944), are killed by as little as one-fiftieth or even one-hundredth their weight of suitable cationic detergents and at most one-tenth to one-fifteenth their weight of anionic detergents. One would therefore have to conclude that either the enzyme proteins or some other vulnerable element of the bacterial cell have a higher sensitivity to surface-active agents than do other proteins in general, including even the other proteins of the bacterial cell itself. Hotchkiss showed that the inhibition of bacterial metabolism produced by bactericidal concentrations of detergents rarely exceeded 80 to 90 per cent and that increasing concentrations of the detergents had little

further effect. He stated that these facts were not in agreement with the hypothesis that a specific, detergent-sensitive enzyme exists, whose inactivation is responsible for metabolic inhibition and cell death, since it would be expected that increase of the concentration of the detergent would soon give an excess capable of completely blocking metabolic activity. Since these high rates of metabolic inhibition were associated with sterilisation of the bacterial suspension the small residual metabolism could not be due to a few surviving, detergent-resistant organisms. Hotchkiss himself admitted that the possibility could not be excluded that a specifically-sensitive enzyme existed and in addition a small, residual metabolism operating through an alternative and subsidiary, detergent-resistant pathway. He offered, however, the alternative hypothesis that surface-active agents acted not on enzyme proteins but on the bacterial cell membrane. Damage to this membrane, with increased permeability, would allow intracellular components such as enzymes, coenzymes and essential ions to diffuse into the surrounding medium, a situation obviously incompatible with life. The resulting enormous dilution of intracellular constituents would, in itself, reduce metabolic activity to a small fraction of its original value since full activity is dependent on the maintenance of these multifarious components within the very small volume of the intact cell. Once this dilution has occurred the addition of more detergent cannot influence the degree of dilution and further depression of metabolic activity will not occur, at least until sufficient agent has been added to cause possible denaturation of enzymes. In confirmation of this hypothesis that the complex enzyme systems of micro-organisms, treated with detergents, were more commonly inactivated by dilution than by denaturation he showed that fermentation by yeast extract, in

which the active components are already "diluted" away from the cell, was not further affected by those concentrations of detergents that reduced the fermentation of intact cells to a small fraction of the normal rate.

Hotchkiss produced experimental evidence for the theory of cytolytic damage. When various organisms, including staphylococci, streptococci, B. coli and yeast cells, were treated with various cationic, anionic and non-ionic detergents death of the organism was associated with the simultaneous release of trichloracetic acid-soluble phosphorus and nitrogen compounds into the medium. The various detergents killed the bacteria only when they also initiated this release. On the other hand, this effect was not observed when other antiseptics, such as hydrogen peroxide, potassium permanganate, formaldehyde, mercuric chloride, iodine, acridine dyes and gentian violet, killed staphylococci. He did not believe that surface-active compounds could penetrate into the living cell and claimed that Kuhn and Jerchel, in their tetrazolium experiment, failed to demonstrate that the compounds had not been reduced on the surface of the cells without actual penetration.

In his theory of the bactericidal action of surface-active agents Hotchkiss pictures first, interaction of the surface-active ions with oppositely charged groups on the bacterial surface. If the hydrophobic groups have the appropriate affinity adsorption is followed by irreverible damage to the cellular membrane. The increased permeability allows the loss of the soluble, cytoplasmic constituents of the cell. At this stage metabolic activity is low and the cell is dead but morphologically unchanged. Cold or certain enzyme poisons may keep the cell in this state for a limited time. Otherwise, following the cytolytic injury, the autolytic enzymes of the dead cell are responsible for the further break-up of cell constituents with,

in certain species, complete structural disintegration and clearing of the suspension.

Support for the cytolytic mechanism of detergent action was offered by Gale and Taylor (1947) who showed that treatment of Strept. faecalis, containing high internal concentrations of lysine and glutamic acid, with various surface-active compounds, such as tyrocidine, CTAB, aerosol and phenol, resulted in loss of the internal amino-acids into the surrounding medium. They considered that the lytic effect was sufficient to account for the bactericidal action of these compounds.

Knox and his collaborators (1949) substituted detailed viability counts for the "all or none" tests of viability by simple subculture in their investigations of the action of detergents on bacteria and also examined the effect of the detergents on cell-free, enzyme extracts. Using intact cells they showed that there was a direct relationship between the percentage inhibition of bacterial metabolism, estimated in terms of glucose oxidation, and the percentage death rate caused by a given amount of detergent: an amount of detergent which caused 50 per cent metabolic inhibition caused death of 50 per cent of the cells. On the other hand, inhibition of other reactions, such as those of arginine decarboxylase and catalase, occurred only with large amounts of detergent, of the order able to denature the cell proteins. Indeed, with bactericidal amounts of detergent there occurred an increase in arginine decarboxylase activity, apparently due to the increased cell permeability to the substrate. Since this enzyme requires pyridoxyl phosphate as coenzyme Knox et al. used this as an argument against Hotchkiss's theory that the diffusion of coenzymes and metabolites from the dead cell accounts for the inhibition of bacterial metabolism. They then treated cell-free enzyme systems of the bacteria with detergents and showed that although many of these were resistant certain

enzymes and particularly lactic acid oxidase were inhibited by bactericidal amounts of detergent. They, therefore, concluded that the surface-active agents were bactericidal not because they primarily alter the cell structure but because they affect a specific, detergent-sensitive enzyme such as lactic acid oxidase since this enzyme possesses, in the absence of the cell structure, the intact cell's pattern of sensitivity to the detergent. They suggested that such enzymes may be necessary to maintain the dynamic integrity of the cell membranes and their inhibition would then account for observed permeability phenomena and cell death.

Salton (1951) demonstrated that there was a parallel relationship between the amounts of purines, pyrimidines, glutamic acid and phosphorus compounds lost from both Gram-positive and Gram-negative bacteria when treated with a cationic detergent. He showed that the amounts of these constituents released in the initial phase of lysis were similar to those released from the cells by boiling water and that autolysis, therefore, played no part at this early stage. It was concluded that there was little to disagree with the hypothesis of cell membrane damage as the cause of the bactericidal action of detergents and that it was unnecessary to invoke the specific inhibition of detergent-sensitive enzymes. Indeed, if there were a chain of reactions initiated by a primary metabolic inhibition one would expect a greater time lag before the occurrence of secondary effects such as the release of cellular constituents, whereas this leakage of cell solutes seems to take place simultaneously with cell death.

The hypothesis that the lytic effect of surface-active compounds is primarily due to the disorganisation of a surface membrane receives strong support from experiments carried out by Schulman and Rideal (1937) on model systems. They showed that

compounds with surface-active properties readily penetrate lipoprotein monolayers and by markedly increasing surface pressure cause disruption of the lipoprotein film. It has long been accepted that the osmotic barrier of living cells is lipoprotein in nature (Overton, 1895; Traube, 1904; Schmitt et al., 1936,1938). Staining reactions (Knaysi, 1938,1946; Burdon, 1946) and electrophoretic studies (Dyar, 1948) have confirmed the presence of lipoprotein membranes in bacteria. The original observation of Klein and Stone (1931) that the lysis of pneumococci by the relatively weakly surface-active compound, saponin, was dependent upon sensitisation of the cells with cholesterol is also highly suggestive of a surface penetration effect similar to that described by Schulman and Rideal. It would also appear to oppose the arguments of Valko that surface-active compounds can readily penetrate into living cells without previous disorganisation of the surface membrane.

## Microscopical evidence of structural changes in the bacterial body treated with surface-active agents

Direct visual evidence that detergents may damage the bacterial surface was first provided by Mitchell and and Crowe (1947). these workers were able to show, with the electron microscope, that streptococci treated with tyrocidine were stripped of their cell-walls and that this change appeared to occur concomitantly with the leakage of internal amino-acids. They suggested that either the cell-wall itself was the amino-acid retaining barrier or, being closely associated with the cytoplasmic membrane, was ruptured together with that membrane.

Dyar (1947) and Meisel and Umansksya (1949) noted that bacterial and yeast cells, treated with detergents, underwent a shrinkage of the protoplast away from the cell-wall.

Kriss and Biryuzova (1950) examined, with the electron microscope, the spontaneous autolysis of the spore-bearing bacillus, B. mycoides, and the effects produced by the action of tyrocidine. During normal autolysis the cells showed fragmentation of the cytoplasm into a number of parts which then rapidly disintegrated leaving, apparently intact, the cell-wall membrane. With the addition of small concentrations of tyrocidine the autolytic process occurred as before but, on prolonged incubation, the cell-wall itself also underwent disintegration. With higher concentrations of tyrocidine autolysis did not occur due to inactivation of the autolytic enzymes but a contraction of the protoplast from the rigid cell-wall took place. Cell-wall stripping was apparently not observed although the authors were able to separate by centrifugation the contracted protoplasts of cells, treated with high concentrations of tyrocidine, from the preserved cell-walls.

Salton, Horne and Cosslett (1951) suspended various Gram-positive and Gram-negative organisms in solutions of the cationic detergent, CTAB, and observed that, with low concentrations, a marked contraction of the cytoplasm from the cell-wall occurred and they were able to demonstrate that the proportion of cells showing such contraction could be correlated with the proportion of cells killed by the treatment. Higher concentrations of the detergent or longer exposure to the lower concentrations appeared to cause stripping of the cell-walls.

Chaplin (1952) treated the cells of Bacillus megatherium with low concentrations of a cationic detergent and followed the cell-wall changes in the optical microscope after staining with the cell-wall stain, Victoria blue B. He noted three stages of damage: first, degeneration of the cytoplasmic membrane; second, brittleness of the cell-wall; and third, fragmentation of the cell-wall with release of the cytoplasmic material. Although the second and third stages are quite obvious in Chaplin's published photographs the first stage cannot be considered as convincingly demonstrated. Indeed, one cannot be convinced that a cytoplasmic membrane, distinct from the cell-wall, is even present.

## The cellular anti-autolytic factor

In the living bacterial cell the autolytic enzymes are apparently inactive and only after death of the cell do they attack the cell structure to cause dissolution of the cell. All available evidence would appear to indicate that lipoids are intimately concerned with this inhibition of autolysis in the living cell.

Jobling and Petersen (1914) extracted with organic solvents lipoids from various bacteria and showed that these lipoids exhibited "antiferment" activity against trypsin. From a comparison of the antiferment index of the organisms with their normal content of non-coagulable nitrogen (presumably non-protein nitrogen) they were able to demonstrate that the lipoids influenced intracellular proteolytic activity. (The antiferment index was calculated by multiplying the lipoid content of a bacterium with the latter's iodine value). Thus, diphtheria bacilli with a high antiferment index possessed only 5 to 7 per cent of its total nitrogen in a non-coagulable form whereas Bacillus subtilis with a low antiferment index had 33 to 35 per cent of non-coagulable nitrogen. The degree of tryptic digestion of ground, dried bacteria was also found to vary inversely with the antiferment index of the cells; diphtheria bacilli were most resistant and B. subtilis least resistant. Staphylococci and B. coli were found to occupy intermediate positions. These workers considered that with death of the cell there occurred increased intracellular acidity due to failure of removal of acid waste products and that this altered the state of dispersion of the lipoids with loss of antiferment activity. They recognised the resistance to tryptic digestion of intact, heat-killed, Gram-positive organisms as compared with the ready susceptibility of heat-killed, Gram-negative organisms and explained this by a

more intimate association of the lipoids with the protein of the Gram-positive cells; they found it more difficult to extract lipoids from Gram-positive than from Gram-negative bacteria.

It has become increasingly evident that bound lipoids, and particularly lipoproteins, are of primary importance in preventing the indiscriminate activity of intracellular enzymes. The bound lipoids, which may be present in bacteria in amounts equal to or even higher than free lipoids, can be readily extracted by fat solvents only after hydrolysis with 1 per cent hydrochloric acid (Smedley-MacLean, 1922; Terroine and Lobstein, 1923; Anderson and Roberts, 1930; Starkey, 1946). Since most of the available data on the lipoid content of bacteria does not include the bound portion it is not possible to relate conclusively the resistance to autolysis of the various bacterial species to the lipoid content. Burdon (1946), however, examined the lipoid content of many bacteria with the stain, Sudan black B, which is believed to detect both free and bound lipoid. He showed that lipoids are present in large amounts in the acid-fast bacteria, the corynebacteria, the clostridia and the yeasts. Lipoid was absent or present only in small amount, in B. subtilis, pneumococci, Staph. aureus and in the majority of Gram-negative organisms. These results agree well with the greater susceptibility to autolytic disintegration of the second group of organisms.

In developing the conclusion that the lipoid content of bacteria is correlated with the susceptibility of the organisms to autolysis it is important to consider the various views on the structural composition of bacteria with regard to lipoids. The enormous literature on the chemistry of cytoplasm was reviewed by Bensley (1942) who believes that cytoplasm, which has no ultimate structural unit, consists of several, perhaps many, different types of unit, all cooperating in an orderly

fashion to produce that ensemble of properties which we call life. The following category of units was recognised:-
(1) Those upon which the integrity of the cell as a unit of structure and organisation depends. Fibrous proteins and nucleoproteins with associated lipoids play an important part.
(2) Particulates, microscopic and submicroscopic, of highly complex composition mediating special chemical processes.
(3) Interparticulate lipoid menstruum, also of complex composition but at present little understood.

In this conception lipoids play an important part in the framework and organisation of the cytoplasm and might well be responsible for the localisation within the cell of the varied enzyme activities. A similar view was suggested by Dubos (1937b,1945) that in the living cell the intracellular enzymes are separated from their respective substrates by membranes which require energy for their maintenance; the membranes break down after death, allowing a variety of enzyme reactions, which are held in abeyance during life, the sum total of which constitutes autolysis. That lipoproteins can form such membranes was shown by Palmer, Schmitt and Chargaff (1941), who carried out X-ray analyses of the structure of various tissue lipoproteins. The results indicated that these consist of units composed of a thin protein layer inserted between lipoid leaflets and that these units are arranged in a regular manner to form large complexes.

The conception that lipoids are the anti-autolytic agents within the cell would explain the long-recognised ability of lipoid solvents such as toluol, chloroform and acetone to stimulate the autolytic dissolution of bacteria (Rettger, 1904; Flexner, 1907; Murray, 1929). Lamar (1911) suggested that the solution of pneumococci by sodium oleate was due to its

ability to disorganise lipoid-containing substances. Macheboeuf and Tayeau (1938) and Tayeau (1939) demonstrated that detergents are capable of splitting lipoprotein molecules which might well be the explanation for the stimulating effect on autolysis of these compounds.

Gram-negative organisms, in general, possess very much less total lipoid than Gram-positive organisms and are readily subject to spontaneous autolysis. On the other hand, they tend to be much more highly resistant to the bactericidal action of detergents than are Gram-positive bacteria. It has been suggested (Dubos, 1945) that this resistance may be due to the presence of lipoid complexes (phospholipoids) at the surface of Gram-negative bacteria which tend to prevent the penetration of the detergent molecules. Indeed, Boivin and his coworkers (1933, 1938) isolated from a wide variety of Gram-negative organisms a toxic O antigen which appeared to consist of a phospholipoid-containing carbohydrate; they were unable to isolate a similar compound from Gram-positive bacteria. This work has been confirmed by many workers and the antigen proved to be a phospholipoid-carbohydrate-polypeptide complex (Raistrick and Topley, 1934; Topley et al.,1937; Miles and Pirie, 1939; Morgan and Partridge, 1940; Freeman and Anderson, 1941; Goebel et al., 1945). Morgan (1949) has shown by detailed antigenic analysis that the complex is situated on the surface of the cell and possesses a specific orientation. Almost conclusive evidence that it is the phospholipoid on the surface that protects against surface-active agents was produced by Miller et al. (1942) who showed that Gram-negative organisms could be sensitised even to anionic detergents by pretreatment with protamines which are known to precipitate phospholipoids (Chargaff and Ziff, 1939). Moreover, the adsorption of phospholipoids onto the surface of Gram-positive organisms protects these normally susceptible cells

against the action of detergents (Baker, Harrison and Miller, 1941b, Hotchkiss, 1944). Finally Salton (1952b) has, by direct chemical analysis of the isolated bacterial cell-walls, shown that whereas the cell-walls of Gram-positive bacteria, in general, possess only a few per cent of lipoid those of Gram-negative bacteria may possess as much as 22 per cent.

## The bacterial cell-wall

Cohn (1872) was apparently the first worker to suggest that bacteria possess a surface membrane similar to that of plants and fungi. He noted that bacteria were remarkably resistant to solution by dilute acids and alkalis and he attributed this resistance not to some special property of the cytoplasm but to the presence of a discrete, resistant membrane around the cytoplasm. In view of the resistance of the cell to dilute alkali he suggested that the cell membrane was composed of cellulose or some other carbohydrate. Cohn believed he could see the membrane by careful focussing of his microscope as a slightly yellowish band around the cell. The presence of an outer "skin" was confirmed by Fischer (1891) who treated various bacteria, such as Spirillum volutans, with hypertonic solutions of salt. The retraction of the protoplast rendered visible the bounding, rigid membrane.

Zettnow (1896) was unable to confirm Fischer's work on Spirillum volutans or to demonstrate a bounding membrane on other bacteria by either plasmolysis or staining. He was also unable to demonstrate the presence of cellulose in bacteria by specific staining techniques. He was, however, able to show by plasmolysis that Chromatum okinii, a sulphur bacterium, did possess a surface skin, a finding originally communicated to Zettnow by Butschlii. Subsequently, a cuticular layer was described by Schaudinn (1902) in the very large cockroach bacillus, B. butschlii, and surface membranes were soon observed in many bacteria by numerous workers both by plasmolytic and by staining techniques. The term ectoplasm was used to describe this membrane by Preisz (1904) and subsequently by Eisenberg (1909) and by Meyer (1912). The term cell-wall was used by Dobell (1908,1911) who examined the large bacillus, B. flexilis

(from the large intestine of frogs and toads) both unstained and stained with Giemsa.

Gutstein (1924a,b) developed the first differential cell-wall staining technique using tannic acid as a mordant and was able to differentiate the surface membrane from the underlying cytoplasm. Some confusion of terminology was introduced by Churchman (1927,1929) who attributed the Gram-positivity of bacteria to the presence of a "cortex" surrounding a Gram-negative "medulla". He considered that the cortex, which could apparently be destroyed by dilute acriviolet, was different from the ectoplasmic layer of Gutstein.

Cell-walls were observed by St.John-Brooks (1930) and by Barnard (1930) on living organisms, as a dense layer on the lighter cytoplasm, by means of dark-ground, ultraviolet illumination.

That the bacterial cell-wall is a discrete structure was conclusively proved by Wamoscher (1930) who carried out ingenious, needle microdissection experiments. He worked chiefly with a large, aerobic, spore-bearing bacillus, Bacillus mazun, but supported his conclusions with work on the smaller cells of the coli-typhoid-dysentery group. He showed that bacteria possess a discrete cell-wall which is extremely tough and yet remarkably elastic and extensile.

In the same year Knaysi (1930) presented evidence that the cells of B. subtilis possessed not only an outer cell-wall but also a cytoplasmic membrane immediately surrounding the cytoplasmic body. Cells stained with 0.1 per cent crystal violet showed a purple cell-wall surrounding a dark violet membrane which, in turn, enclosed a deeply stained cytoplasm of a much lighter hue. The two membranes were even more obvious after the cells had been plasmolysed with 25 per cent sodium chloride.

With the advent of the electron microscope it became

possible to observe readily the cell-wall in the unstained state. The cell-wall is clearly recognisable in the electron micrographs of bacteria published by the early workers, Piekarski and Ruska (1939) and Jakob and Mahl (1940) , although they incorrectly interpreted these surface membranes as hitherto unrecognised capsules. The differentiation of the cell-wall from the underlying cytoplasm has since been clearly demonstrated in electron micrographs of streptococci (Mudd and Lackman, 1941) in which the continuity of the cell-wall accounts for the chain formation, of bacteria of the genus Bacillus (Mudd et al., 1941), of Thiobacillus thio-oxidans (Bartholomew and Umbreit, 1942) and of a strain of Fusobacterium (Mudd et al., 1942).

So-called "ghost" cells, staining very faintly with the usual stains, are well known to bacteriologists. Burke and Barnes (1929) were able to produce such cells by crushing bacteria between glass slides and recognised that they were the empty cell-walls of the bacteria. Empty cell-walls can also be produced by sonic vibrations (Mudd et al., 1941), by phage lysis (Wyckoff, 1949; Hillier et al., 1949) and by heating (Salton and Horne, 1951a). A method of obtaining quantitative yields of separated bacterial cell-walls in a clean, practically intact state was first described by Dawson (1949) who utilised a technique originally used by Curran and Evans (1942) for the mechanical destruction of spores and later similarly used by King and Alexander (1948) for killing bacteria. The method consists of shaking bacteria with minute glass beads in buffer at high speeds. This causes puncturing of the cell-wall of the bacterium with loss of internal contents and the cell-walls are then isolated by differential centrifugation (Salton and Horne, 1951b; Hotchin, Dawson and Elford, 1952).

## The cytoplasmic membrane

The presence of a membrane immediately enclosing the cytoplasm and distinct from the outer cell-wall was first clearly illustrated by Knaysi (1930) by staining with crystal violet the plasmolysed cells of B. subtilis. Subsequently (1946), he demonstrated, using his own differential cell-wall staining technique (1941), a similar internal membrane in the "ghost" cells of B. megatherium. By histochemical methods, he showed that it was composed mainly of lipoprotein and was the site of the Gram-positive and acid-fast staining reactions. Burdon (1946), using Sudan black B, was also able to demonstrate a surface, lipoid-containing membrane on many bacteria.

The cytoplasmic membrane has not proved to be readily visible in the electron microscope, presumably due to low electron scattering power, but Knaysi and his coworkers claim to have demonstrated the membrane by using special techniques: by growing B. mycoides in a nitrogen-free medium to lower the ribonucleic acid content of the cytoplasm and thus improve the electron penetrating power (Knaysi and Hillier, 1949); and by subjecting the cells of an avian strain of Mycobacterium tuberculosis to an electron beam of high intensity which causes a more rapid disintegration of the cytoplasm than the cytoplasmic membrane (Knaysi, Hillier and Fabricant, 1950). On the other hand, an internal membrane, discrete from the cell-wall, has been clearly demonstrated with the electron microscope in the autolysed cells of the Gram-negative bacillus, B. proteus (Houwink and Van Iterson, 1950). Although not commented upon by the authors, an appearance very suggestive of a cytoplasmic membrane can be seen in the micrograph of a bacteriophage-lysed cell of Clostridium welchii (Elford et al., 1952).

## The structure of the bacterial cell-wall

Exact investigations of the detailed structure of the bacterial cell-wall has become possible only with the advent of the electron microscope with its very high resolving power.

Johnson, Zworykin and Warren (1943) examined the process of plasmolysis of certain, marine luminous bacteria. Treatment of these bacteria with distilled water resulted in the separation of cell-wall fragments in various stages of disintegration. The membranes showed circular and slit-like areas of transparency, the smallest of which were about 5 mμ in diameter, the larger being probably due to the confluence of the smaller. They suggested that the cell-walls were built up as a mosaic of such small circular units. The separation of small circular and elliptical segments from the cell-wall "ghosts" of bacteriophage-lysed cells of B. coli was noted by Hillier et al. (1949) but they were unable to decide whether the segments pre-existed in the intact cell-wall or were artefacts resulting from phagolytic disintegration.

Knaysi and Hillier (1949) examined the cell-wall of the spore of B. megatherium and demonstrated that it was composed of parallel, beaded fibres or threads cemented together by a ground substance. The threads were invisible in the intact cell-wall and became visible only after dissolution of the cementing, ground substance during spontaneous disintegration of the shed spore coat. The length of the individual fibres varied from 60 to 120 mμ with a diameter of about 9 mμ. The beads on the fibres had a diameter of about 12 mμ and tended to unite laterally with each other to form a network. A similar structure was claimed for the cell-wall of the vegetative cell but the photographs are very much less convincing.

An electron microscope with a very short focal length

objective lens was applied to the study of the isolated cell-wall of Spirillum serpens by Houwink and LePoole (1952). The cell-wall was shown to consist of at least two membranes, the outer one containing or consisting of a single layer of spherical macromolecules of 120 to 140 Å diameter; these were arranged in a regular, hexagonal pattern. The inner membrane showed no obvious structure. In B. coli or B. subtilis the cell-walls could not be shown to be double nor could any evidence of structure be detected.

The structure of the yeast cell-wall was investigated by Houwink and Kreger (1953); apart from bud scars, the untreated cell-walls possessed a smooth outer surface although the inner surface showed a network of very thin fibrils which were all but concealed by an amorphous ground substance. Boiling with dilute acid dissolved the amorphous substance revealing throughout the cell-wall a network of fibrils of about 50 Å diameter which aggregated on continued boiling into coarser strands. Electron diffraction studies, however, appeared to indicate that the acid-insoluble material was present in the normal cell-wall in a very small crystallite form and did not possess the fibrillar appearance which was an artefact produced by acid treatment. The amorphous, acid-soluble material accounted for about 75 per cent of the weight of the cell-wall.

These few investigations serve to indicate the diversity of structure which might be expected in the cell-walls of the different species of microorganisms.

## The chemical composition of the cell-wall

Until comparatively recently the results of investigations of the chemical composition of the bacterial cell-wall have been inconclusive and contradictory. Both histochemical staining techniques and direct chemical analyses have been employed. The latter method has been particularly unreliable due to the original methods of obtaining cell-wall material by extracting the intact cells with caustic alkalis. This has been shown by electron microscopy (Salton and Horne, 1951b) to leave behind much cytoplasmic material and also seriously to alter the cell-wall itself.

The earliest investigators seem to have been prejudiced towards the detection in bacteria of the carbohydrate compounds already well known as the surface components of the plant and animal kingdoms. Cellulose was detected in Sarcina ventriculi by histochemical reactions (Suringar, 1866; de Bary, 1887; Migula, 1897) but apart from this one possible exception cellulose would not appear to be a constituent of the bacterial cell-wall. Knaysi (1951) apparently believes that the pellicle which forms in cultures of Acetobacter xylinum, and which is largely composed of cellulose (Brown, 1885; van Wisselingh, 1925; Hibbert, 1930), consists mainly of cell-wall material. That the pellicle consists of cellulose fibrils separate from the contained cells and arising extracellularly from a slimy material secreted by the cells was demonstrated by Aschner and Hestrin (1946) and by Muhlethaler (1949). The assumption that the cell-wall of Acetobacter xylinum is also composed of cellulose would thus appear to be premature and unwarranted.

Nishimura (1893) and Meyer (1912) suggested that hemicellulose may be the cell-wall component of bacteria. The term hemicellulose is a comprehensive one used to designate complex carbohydrates distinct from but giving some of the colour reaction

of cellulose and therefore the statements of these workers means little more than that a complex carbohydrate, of unknown nature, is present.

The recognition that nitrogen or glucosamine may be present in cell-wall preparations in about the same proportions as in chitin was used as an argument in favour of this compound (Vincenzi, 1887; Iwanoff, 1902). This was, however, denied by van Wisselingh (1898,1925) who applied his specific, chitosan, colour reaction to bacteria. The positive colour reactions reported by Viehoever (1912) have been shown to have been incorrectly controlled (von Wettstein, 1921).

That carbohydrates are indeed present in the cell-walls of many bacteria was recently demonstrated by Pennington (1949) who used the highly reliable periodate reaction as a histochemical test; the test proved positive for various yeasts and aerobic, spore-bearing bacilli. Knaysi (1951) considers that Pennington's results apply to the cytoplasmic membrane but Pennington, himself, by staining broken yeast cells and plasmolysed cells of B. cereus, showed that the cell-wall itself stained red.

The presence of protein as a cell-wall constituent was demonstrated by Murray (1930) who analysed a cell-wall preparation of C. diphtheriae, obtained by plasmolysis followed by centrifugation. However, the high figure of 88 per cent protein was undoubtedly due to cytoplasmic contamination.

More recently a stimulus has been given to the carrying out of detailed chemical analyses of the bacterial cell-wall by the facility of obtaining pure cell-wall preparations by shaking or grinding with glass beads. Mitchell and Moyle (1951) have shown that the cell-wall of Staph. aureus consists mainly of a glycerophosphoprotein complex. Lipoid is also present but apparently very little nucleic acid, the latter finding being at variance with the work of Stacey (1949). On the other hand, Salton (1952a)

showed that the cell-wall of Strept. faecalis was mainly muco-polysaccharide with less than 25 per cent of protein. The rapid disintegration of the isolated cell-wall of Micrococcus lysodeikticus by lysozyme (Salton, 1952c) would appear to indicate that it also is basically mucopolysaccharide. A carbohydrate-protein complex was demonstrated by Holdsworth (1952) in the cell-wall of C. diphtheriae. Houwink and Kreger (1953) showed by chemical treatment and electron diffraction that the cell-walls of various yeast cells contain the polysaccharide glucan (about 20 per cent) and a small amount of chitin but the principal component remained unidentified.

While there would seem to be a wide variation of chemical composition among the cell-walls of even the organisms within the Gram-positive group Salton (1952b) has shown that certain basic differences exist between the cell-walls of Gram-positive and Gram-negative bacteria. Whereas Gram-positive bacteria have, in general, only a few per cent of lipoid, Gram-negative bacteria may have as much as 22 per cent. The cell-walls of Gram-positive bacteria were also shown to have a more limited complement of amino-acids, aromatic and sulphur-containing amino-acids being absent; Strept. pyogenes being an exception because of the presence of the M protein. This latter compound, probably a nucleoprotein, is almost certainly a component of the cell-wall of Strept. pyogenes (Zittle and Mudd, 1942) although the polysaccharide C substance would appear to be a more important structural unit (Maxted, 1948; Salton, 1952b).

Henry and Stacey (1946), in their now classical experiments, demonstrated that the Gram-positivity of a bacterial cell is intimately related to the presence of magnesium ribonucleoprotein. The ease of removal of the ribonucleic acid leaving an apparently intact cytoplasmic body suggested that the nucleoprotein was a component of the cell-wall; ultraviolet microscopy indicated an

ordered arrangement of the compound at the bacterial surface. Stacey (1949) postulated a layered structure for the cell-wall of protein and nucleic acid with lesser amounts of lipoid and carbohydrate also being present. He believes that nucleoprotein is also present in the cell-wall of Gram-negative organisms but that the absence of the characteristic basic proteins of the Gram-positive bacteria accounts for the negative Gram reaction. Lamanna and Mallette (1950) found it possible to stain by Gram's method, decolourise and then apply a cell-wall stain to one and the same smear of organisms. Their results indicated that the Gram stain and the cell-wall stain acted on the same structure. Mitchell and Moyle (1951), on the other hand, were unable to detect significant amounts of nucleic acid in the isolated cell-walls of Staph. aureus by ultraviolet absorption. Knaysi (1951), who has shown that the stain affinity of the bacterial cell is most markedly possessed by the cytoplasmic membrane, considers that the nucleic acid responsible for the Gram reaction is present in the cytoplasmic membrane and not in the cell-wall. Harris (1953) has examined by electrophoresis bacterial cells before and after treatment with ribonuclease and his results appear to support the view that ribonucleic acid is present not at the surface of the cell but rather in the deeper cytoplasmic membrane.

Electrophoresis was also used by Dyar (1948) to detect components of the bacterial surface and was shown to be capable of providing a quantitative result. He showed that the cell surface of Staph. aureus possesses about 10 per cent lipoid and also an amphoteric substance. Since both these compounds could be removed without affecting the viability of the cells they would not appear to be important structural compounds; most of the lipoid would seem to be external to the cell-wall. The electrophoretic reactions of the cell after the removal of the above substances suggested the presence in the cell-wall of a

carbohydrate which was different from cellulose and streptococcal dextran. Both the lipoid and the amphoteric substance could be detected at the surface of M. smegmatis but could not be removed by the methods effective for Staph. aureus. Lipoid was not detected at the surface of B. subtilis or B. cereus.

Hurst (1952) carried out electron diffraction studies on the structure and orientation of lipoids at the surface of yeast cells, of Staph. aureus and of B. coli. His results indicated a high proportion of bound lipoids in the cell-walls and that these were oriented normal to the surface. He suggested, on the basis of his work, that the cell-wall of these organisms consists of free lipoids embedded in a lipoprotein network and that the free lipoids were possibly stabilised by carbohydrate components of the cell-wall. He also considers that the cell-wall lipoids are at least partly responsible for surface permeability.

Immunochemical analysis has also proved a valuable tool in cell-wall study. This technique has shown that the cell-wall of many Gram-negative bacteria contains a polysaccharide-protein-phospholipoid complex and Morgan (1949) has presented evidence that this polymolecular unit is arranged as a regular mosaic in the cell-wall. This complex is apparently absent from Gram-positive bacteria (Boivin et al., 1933).

The extensive literature which has already accumulated on the chemical composition of the bacterial cell-wall indicates the complexity of the subject. Instead of a single structural component the cell-wall appears to contain carbohydrates, proteins and lipoids and probably other compounds all of which possibly possess both structural and functional importance. Moreover, it is now evident that, as in the case of the structural formation of the cell-wall, there is no single formula for the chemical composition of the cell-wall of bacteria in general. Indeed, there appear to be wide qualitative and quantitative differences

among even the various species of a single genus of organisms.

## The cell-wall in cell division

It has long been recognised that the cell-wall plays an important part in the division of the bacterial cell but there is much controversy as to the method of intervention of the cell-wall.

The presence of transverse septa in the rod-shaped bacteria was recognised by the early bacteriologists and they are illustrated in their published diagrams (de Bary, 1884; Klein, 1889; Migula, 1894). Migula (1897) was probably the first to give a detailed description of cell division; he observed the process in a filamentous bacterium, Bacillus oxalaticus (now B. megatherium). He noted a central sap vacuole in the cell and as the cell elongated a ring-shaped bulge appeared in the peripheral cytoplasm and grew inwards to split the vacuole. These "plasma rings" were formed at several places along the length of the cell. Considerably later annular ridges appeared at the bases of the plasma rings and grew centripetally to form transverse cell-walls. The final separation of the daughter cells was apparently slow to occur and the method of separation was not described. Schaudinn (1902) described a different, centrifugal form of division in Bacillus butschlii. A brightly refracting granule appeared in the centre of the cell and extended laterally to form a transverse septum. A split then appeared in the middle of the septum and extended peripherally to divide the daughter cells. This apparently unique process has never been described in other bacteria.

As opposed to the method of division by septum formation Schaudinn (1903) described the division of B. sporonema as due to simple constriction of the cell-wall. Swellengrebel (1907) noted both forms of division; Spirillum giganteum and Spirillum buccale divided by constriction and Spirochaeta balbianii by

septum formation. According to Guilliermond (1908) cell division in various, aerobic, spore-forming bacilli commenced with the formation of two granules at opposite sides of the cell which grew inwards to form a transverse disc which then split by the formation of an internal hyaline zone. Dobell (1908,1911) observed division by simple constriction in B. flexilis and in various, unidentified micrococci. A similar claim was made for Myxococcus ruber (now M. fulvus) by Vahle (1909). Often conflicting claims were made for the division of a single organism by different workers; the division of Spirillum volutans was described as due to transverse septum formation by Migula (1904) and to simple constriction by Guilliermond (1908), Swellengrebel (1909) and Ellis (1922), the latter worker later reversing his opinion (1932).

Knaysi (1929a,b) outlined the process of cell division in Mycobacterium tuberculosis and in Proteus vulgaris. In the former organism there apparently occurred a retraction of the cytoplasm to the opposite ends of the cell followed by the formation of two distinct cell-wall septa; separation of the daughter cells followed withering of the connecting portions of cell-wall. In Proteus cytoplasmic retraction did not occur but cytoplasmic division was due to the formation of a transverse septum; immediately after the septum had formed or even before it was complete it began to split from the edges inwards. This latter process Knaysi believed might easily be mistaken for division by simple constriction. Gardner (1930) and Ellis (1932) both apparently agreed that cell division in all bacteria is preceded by septum formation. Henrici (1934), on the other hand, stated that division both by septum formation and by simple constriction could be observed in a single culture, the age of the culture being mainly responsible for the predominant method of division.

The next step in the elucidation of the dividing mechanism

was achieved by Knaysi (1930) when he recognised in B. subtilis the presence of a cytoplasmic membrane which closely enveloped the cytoplasm and lay internal to and distinct from the true cell-wall of the organism. The first stage in the cell division of the organism was described by Knaysi as the appearance of small bulges on the cytoplasmic membrane at opposite sides of the cell, and which then grew inwards to unite with the opposite bulges to form transverse septa. Several of these might form in a single cell. Later a line of division appeared through the septa resulting in cell division. Subsequent studies by Knaysi (1941,1951) led him to amend his original conclusions and to propose a new scheme of cell division which he considers to apply to all bacteria. There first occurs division of the cytoplasm by the inward growth from the cytoplasmic membrane of a transverse septum. This is followed by the inward growth from the cell-wall of a cell-wall septum which splits the cytoplasmic membrane septum in two. The cell-wall septum is at first single but very soon differentiates into two layers, one for each daughter cell. The formation of the cell-wall septum and its early differentiation were confirmed by electron microscope studies of both rough and smooth variants of Bacillus mycoides (Knaysi and Hillier, 1951). The speed of separation of the daughter cells Knaysi believes to depend on the rate of growth and on the physico-chemical properties of the cell-wall. He considers that the narrow ring of lateral wall from which the cell-wall septum develops is subjected to pulling forces by the growing daughter cells. Smooth strains of organisms are said by him to have soft cell-walls with the result that separation readily occurs; this separation which occurs centripetally through the weakened central layer of the transverse cell-wall septum usually begins before the formation of the latter is completed and thus may give the impression of

division by constriction. Cell-wall septum formation, however, always precedes this apparently simple constriction. In rough strains of organisms which are said to have tough cell-walls separation of daughter cells does not occur until chemical disintegrative changes have partly withered the connecting portions of cell-wall. This late separation accounts for the chain formation characteristic of rough strains.

Robinow (1945), who studied the mechanism of cell division by means of acid-Giemsa and cell-wall staining techniques, agrees largely with the views of Knaysi. He believes that transverse septa arising from the cytoplasmic membrane precede the formation of transverse cell-wall septa, the latter growing centripetally from the lateral cell-wall. He showed that in organisms such as the aerobic, spore-forming bacilli (generally forming rough colonies) several transverse cell-wall septa may form before cell fission occurs whereas in organisms such as coliforms, Proteus and Salmonella (generally forming smooth colonies) fission of the cell closely follows or even coincides with the formation of the first transverse cell-wall.

On the other hand, Bissett (1939,1948a,b,1950) considers that bacteria divide either by transverse cell-wall septum formation or by simple constriction, the method of division being characteristic respectively of rough and smooth strains of bacteria. The terms rough and smooth are used by Bissett in relation to colonial morphology which depends on the bacterial arrangement within the colony; bacteria which form long chains of cells give rise to rough, "Medusa-head" colonies whereas bacteria which lie singly form smooth, structureless colonies. Bissett agrees with Knaysi and Robinow that cell division in all bacteria commences with the formation of transverse septa, originating from the cytoplasmic membrane. He further agrees that in rough strains of bacteria a cell-wall septum forms in

the middle of the cytoplasmic membrane septum but he differs in believing that the cell-wall septum does not grow inwards from the lateral cell-wall but rather that it is secreted uniformly across the width of the cell. The septum is said then to split to complete division of the cell. In the case of smooth strains of bacteria he is opposed to the views of Knaysi and Robinow in that he believes that division of the cell is effected by a constriction of the lateral cell-wall through the line of the cytoplasmic membrane septum without the prior formation of a cell-wall septum. He claims to have demonstrated that, in their mode of division, the long chained-streptococci and the majority of pathogenic staphylococci and pathogenic neisseriae are related to the rough strains of the rod-shaped bacteria and the short-chained streptococci and most non-pathogenic staphylococci and neisseriae to the smooth strains of bacilli. Corynebacteria and mycobacteria apparently divide by transverse cell-wall septum formation (Bissett, 1949).

# EXPERIMENTAL WORK

Previous investigations of bacterial autolysis and lysis have been largely concerned with the chemical and enzymatic processes involved in the disintegration of the cell. While a great deal has been discovered of these processes very little is known concerning the details of the associated structural changes undergone by the cell. For example, it is unknown whether the bacterial cell-wall ruptures during autolysis, as does the red cell membrane in distilled water, or simply becomes more permeable. The use of an electron microscope has enabled such a study to be carried out. An organism relatively resistant to autolysis, Staphylococcus aureus, and one highly susceptible, Bacillus subtilis, have been investigated and the following work will show that the speed of autolysis depends to a large extent on the behaviour of the cell-wall.

The effect of a surface-active agent, cetyl-trimethyl-ammonium bromide (CTAB), on the structural changes of autolysis has also been studied and it has been found that the detergent can cause not only acceleration of lysis but also cell fixation and an intermediate state between lysis and fixation; the precise effect depends entirely on the cell to detergent weight ratio. A comparison of the structural details of disintegration occurring in spontaneous autolysis with those of CTAB lysis and again after extraction of the cells with lipoid solvents has enabled an hypothesis to be formulated of the mechanism of action of detergents. In view of the controversy on whether or not detergents have a primarily cytolytic action on bacteria the isolated cell-walls have been examined during treatment with autolysate and with CTAB; the results would appear to indicate that the cell-wall is indeed actively involved in the mechanism of lysis.

Some investigations have been carried out of the intimate structure of the staphylococcal cell-wall by means of chemical and enzymatic degradation procedures. The results, while not conclusive, tend to confirm the view of staphylococcal cell-wall structure proposed by previous workers on the basis of chemical analyses.

The study of certain, equatorial structures on the isolated cell-walls of various organisms has resulted in a simple and valuable method of investigating the stages of bacterial cell division.

## MATERIALS AND METHODS

Organisms. The organisms examined in the present work were a coagulase-positive strain of Staphylococcus aureus, a strain of Bacillus subtilis and a strain of non-haemolytic, short-chained Streptococcus faecalis. Unless otherwise stated the organisms were grown for 16 to 18 hours at 37°C. in 200 ml. quantities of horse-heart, digest broth contained in 500 ml. conical flasks. In the case of the staphylococcus the broth was continuously aerated by suction. The cells were collected by centrifugation at 3000 r.p.m. and washed twice with distilled water.

Cell-wall suspensions. The organisms were washed a further two times with phosphate-citrate buffer, pH 7.2, and finally suspended in buffer to give a concentration of about $2 \times 10^{10}$ cells per ml. in the case of Staph. aureus and equivalent opacities in the case of the other organisms. Quantities of 5 ml. were shaken with 4 g. of "ballotini" glass beads in a Mickle microshaker at 3000 r.p.m. for half-an-hour. The suspension was then centrifuged at 3000 r.p.m. for 10 minutes to deposit beads and intact cells. The resulting supernatant was centrifuged at 10,000 r.p.m. for half-an-hour to separate the cell-walls. These were washed twice with buffer and twice with distilled water to yield a clean, practically pure suspension of isolated cell-walls. All stages of the preparation were carried out under sterile conditions and the suspensions were used immediately after preparation; it was found that the membranes stored, even in the refrigerator, for longer than 24 hours began to undergo disintegrative changes. In the case of B. subtilis it was more difficult to obtain completely intact cell-walls since the long, cylindrical membranes tended to fracture under bead bombardment and better preparations were

often obtained by reducing the time of shaking to 15 minutes.

Autolytic enzymes. The washed organisms were suspended in 5 ml. quantities of distilled water to give milky suspensions (at least $4 \times 10^{10}$ organisms per ml. in the case of Staph. aureus). The suspensions were incubated at 37°C. for 24 hours; with this concentration about 80 per cent lysis or even higher occurs. The contents of the tubes were then centrifuged at 10,000 r.p.m. for half-an-hour to remove intact cells and debris. The supernatants were used immediately for lysis experiments. The potency of each batch of autolysate was checked against autoclaved cells. In the case of the staphylococcal autolysate the autoclaved cells were suspended in the autolysate to give a cell concentration of $5 \times 10^9$ cells per ml. If, on incubation, 75 per cent of the organisms became Gram-negative within 24 hours the potency of the autolysate was considered adequate. In the case of B. subtilis autolysate a concentration of autoclaved cells of B. subtilis giving a similar tube opacity to the staphylococci was used and a change of 75 per cent of organisms to the Gram-negative state within 1 hour was taken as satisfactory.

Cetyl-trimethyl-ammonium bromide ("Cetrimide"), a synthetic, cationic detergent, was obtained from Imperial Chemicals (pharmaceuticals), Ltd. Solutions were sterilised by autoclaving at 15 lbs. per sq.in.

Estimation of lysis. This was carried out by comparing the opacity of the suspensions of organisms with the standard opacity tubes issued by the Wellcome Laboratories.

Dry weight determinations. This was carried out by centrifuging a given number of cells in distilled water and exposing the deposit to a vacuum of about $10^{-3}$ mm. Hg., in the presence of phosphorus pentoxide, until a constant dry weight was reached.

Electron microscopy. Specimens for examination were washed

twice with distilled water in the centrifuge (at 3000 r.p.m. for intact organisms, and 10,000 r.p.m. for cell-walls). The specimens were not fixed. The final suspensions were diluted with distilled water to near transparency and droplets were then deposited on standard Philips' specimen holders covered with "formvar" film (polyvinyl formal). They were allowed to dry down at room temperature and then shadowcast with palladium metal at an angle of from 15 to 20° from the plane of the film (Williams and Wyckoff, 1946). This method of preparation was used for all specimens examined; the effect of drying on the morphology of the specimens, although impossible to eliminate, was thus standardised. Examination was carried out in the Philips' electron microscope.

## A. AUTOLYSIS IN DISTILLED WATER

The changes associated with death and autolysis of bacteria have been studied using distilled water as the suspending medium. This not only gives cleaner preparations for electron microscopy but allows of a more exact quantitative study of the action of detergents on the autolytic process because of the elimination of the various substances present in a more complex medium, such as nutrient broth or buffer solutions, which would also react with the added detergents. Some studies have been carried out of the morphological changes undergone by the bacteria during autolysis in nutrient broth and in buffer solutions and these have not been found to differ significantly from those occurring in distilled water.

### The autolysis of intact bacteria

The organisms were suspended in tubes each containing 5 ml. of distilled water and incubated at 37°C. The concentration of both Staph. aureus and B. subtilis was kept constant at about 2.8 mgm. dry weight of cells per ml. (in the case of Staph. aureus this is equivalent to $8 \times 10^9$ cells per ml.). At intervals specimens were removed for opacity estimations, staining by Gram's method and electron microscope examinations. A typical set of results is shown in table I.

Staph. aureus. Examination after 1 hour and again after 4 hours revealed no change in the opacity of the suspensions and all the organisms remained Gram-positive. After 24 hours, however, opacity readings indicated that about 35 per cent lysis had occurred and over 50 per cent of the organisms were now found to be Gram-negative. Only after 96 hours did lysis reach 90 per cent, the maximum degree of lysis, with 99.9 per cent of the organisms Gram-negative; at this stage the majority

Table I

Rate of autolysis and change of Gram staining of *Staph. aureus* and *B. subtilis* in distilled water

| Time (hrs.) | Staph. aureus | B. subtilis |
|---|---|---|
| 0 | 0<br>(0) | 0<br>(0) |
| 1 | 0<br>(0) | 35<br>(50) |
| 4 | 0<br>(0) | 65<br>(95) |
| 24 | 35<br>(50) | 85<br>(99·9) |
| 48 | 65<br>(90) | 95<br>(99·9) |
| 96 | 90<br>(99·9) | 95<br>(99·9) |

The upper figure is lysis expressed as percentage clearing of the original suspension opacity. The figure in brackets is the percentage of Gram-negative cells.

of the organisms were very faintly staining.

Electron microscope examination of freshly collected, untreated cells of Staph. aureus showed a roughly spherical, dense and apparently structureless cytoplasmic body surrounded by a distinct cell-wall (fig.1). Since the preparations were dried down the latter appears as a flattened rim. Despite the dehydration to which the specimen was subjected by the high vacuum conditions in the microscope the cytoplasm remains in close contact with the cell-wall, to the very edge of which it can be seen to extend as a whitish haze. After 4 hours' incubation in distilled water no obvious morphological changes had occurred. After 24 hours many cells now showed marked retraction of the cytoplasm from the cell-wall (fig.2). The contracted body of the cell remained uniformly dense. Cell counts, at this stage, indicated that the proportion of cells with retracted cytoplasm was about equal to the proportion of Gram-negative cells in the specimen. The possibility that the cytoplasmic retraction was a purely osmotic phenomenon, although one would expect swelling rather than retraction in distilled water, was investigated by incubating the cells in an isotonic, phosphate-citrate medium; significant differences in electron microscope appearances were not noted, the retraction being as marked as in distilled water. After 48 hours in distilled water a slow disintegration became evident; this involved the cytoplasm unevenly with the result that the cytoplasmic body eventually became fragmented into large, unequal particles (fig.3). The cell-wall showed thinning but remained intact. The cytoplasmic particles underwent further fragmentation and disintegration until, after about 10 days, many cells could be seen in which there had occurred almost complete solution of the cytoplasm, leaving behind empty cell-walls, themselves in various states of disintegration (fig.4).

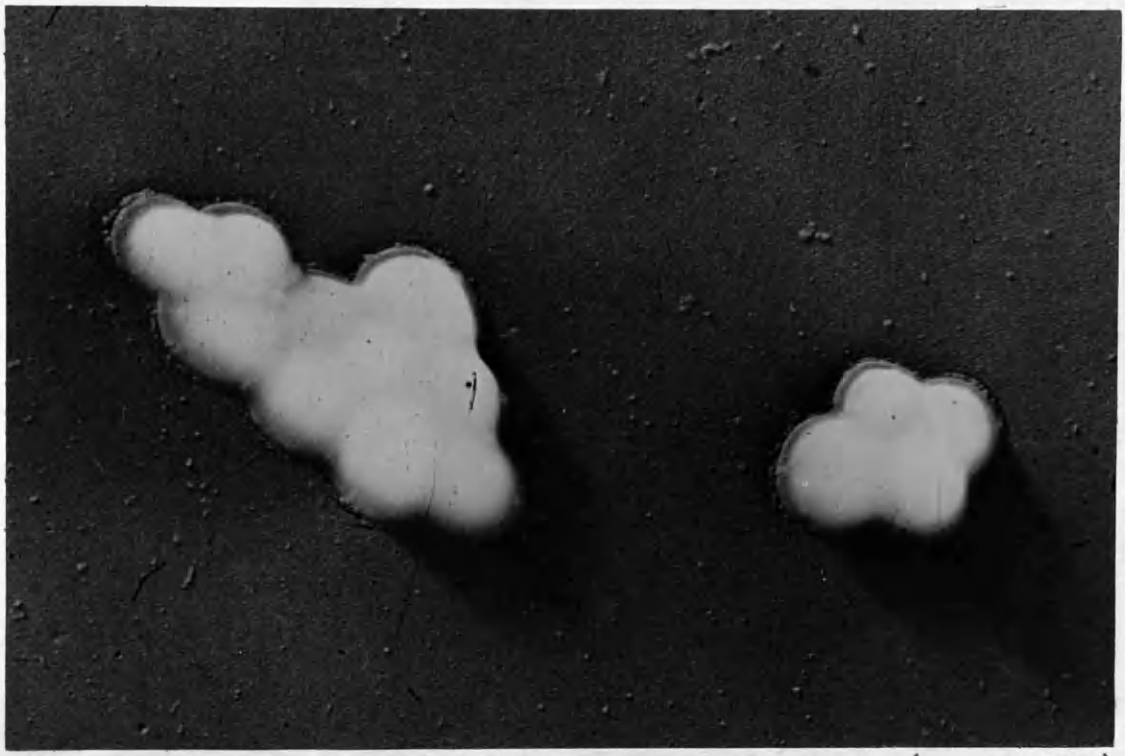


Fig. 1. Staph. aureus. Untreated controls. ( × 15,000).

Fig. 2. Staph. aureus suspended in distilled water for 24 hours showing retraction of the cytoplasm from the cell-wall. ( × 15,000).

Fig. 3. Staph. aureus suspended in distilled water for 5 days showing the irregular fragmentation of cytoplasm. ( × 15,000).

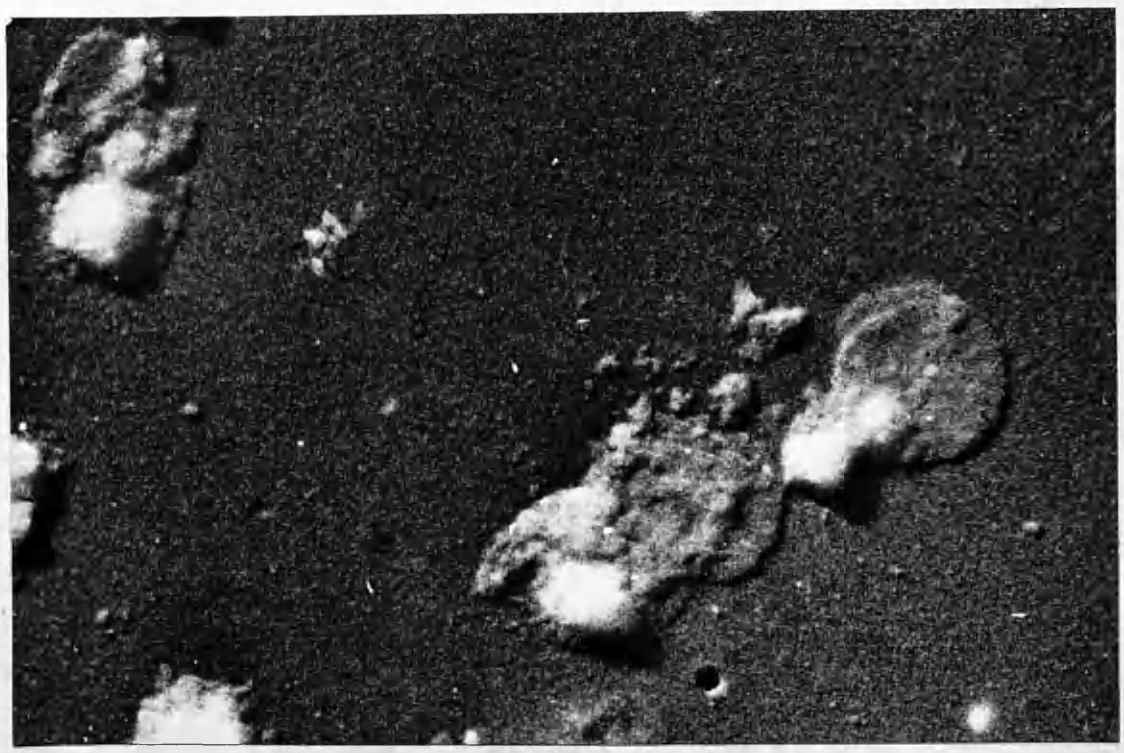


Fig. 4. Staph. aureus suspended in distilled water for 10 days. Final disintegration of cytoplasm leaving empty cell-walls. ( × 15,000).

The cell-wall was obviously more resistant to autolytic disintegration than the contained cytoplasm. A primary rupture of the cell-wall, with evacuation of cytoplasmic content, did not occur; cytoplasmic autolysis progressed to completion within the cell-wall.

B. subtilis. Lysis of this organism in distilled water was extremely rapid, as compared with Staph. aureus. Within 1 hour opacity readings showed as much as 35 per cent lysis, which reached over 65 per cent in 4 hours and over 85 per cent in 24 hours. Gram staining indicated an even more rapid rate of disintegration: untreated suspensions of B. subtilis normally show as many as 5 per cent of Gram-negative forms but within 1 hour at least 50 per cent of the organisms were Gram-negative and very faintly staining; at 15 and 30 minutes well-stained, Gram-negative cells were seen. By 4 hours only scanty, Gram-positive cells could be seen amidst masses of faintly staining "ghost" cells and debris.

Electron microscope examination of the freshly collected, untreated organisms showed that they resembled Staph. aureus in possessing a dense cytoplasmic body in close contact with the surrounding cell-wall (fig.5). The first stage of autolysis was also the retraction of the cytoplasm from the cell-wall and after 1 hours' incubation in distilled water the majority of the cells were in this condition (fig.6). Many cells, even at this early stage, already showed evidence of cytoplasmic fragmentation and disintegration (fig.7). By 4 hours the great majority of the organisms were in advanced states of disintegration (figs.8 to 12). In some cases the cytoplasm, as in Staph. aureus, completely disintegrated within a still intact cell-wall but in the great majority of cases the cell-wall underwent early rupture and fragmentation with the evacuation and rapid disintegration of cytoplasmic material.

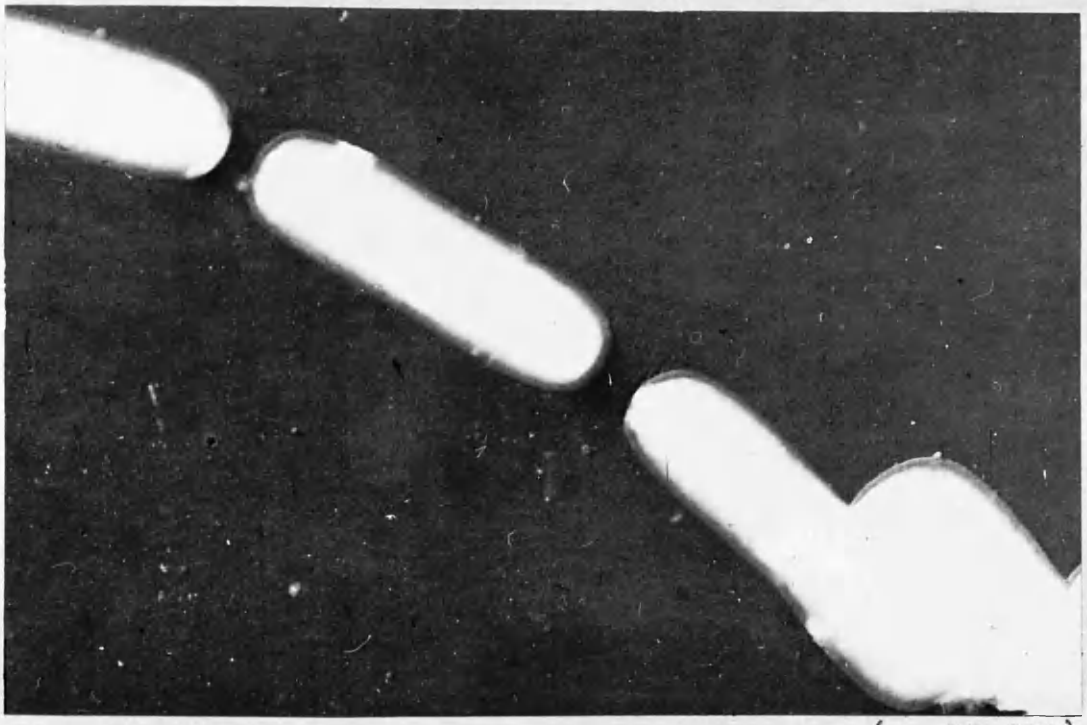


Fig. 5. B. subtilis. Untreated controls. ( x 15,000).

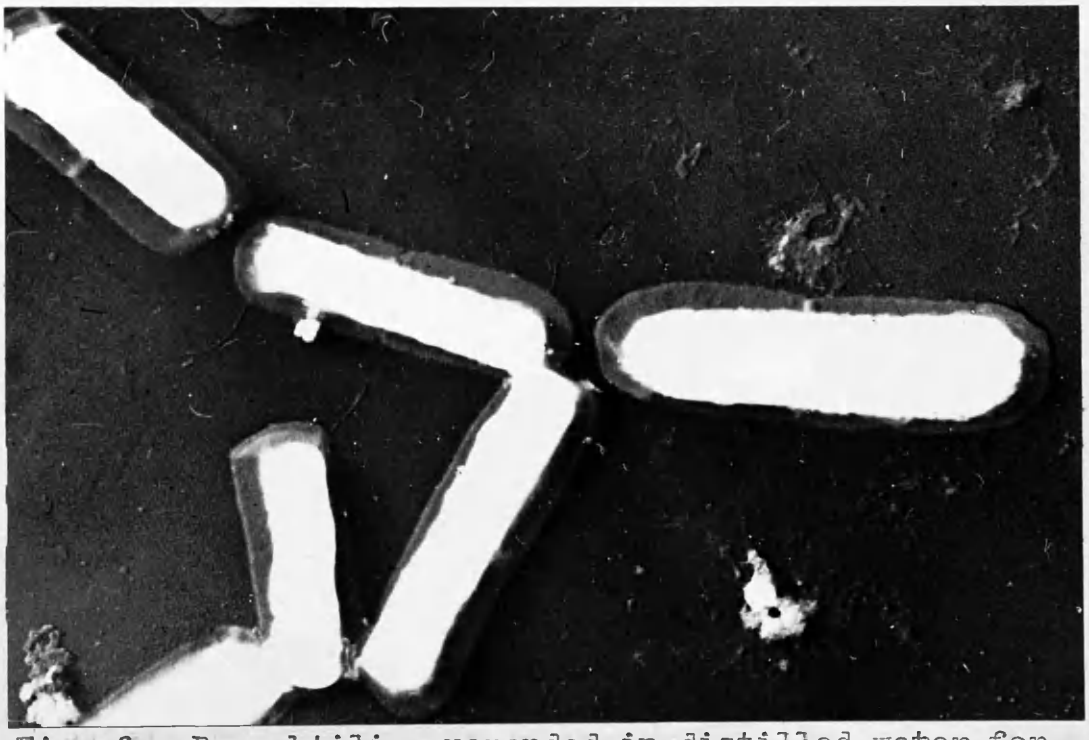


Fig. 6. B. subtilis suspended in distilled water for 1 hour showing cytoplasmic retraction. ( x 15,000).

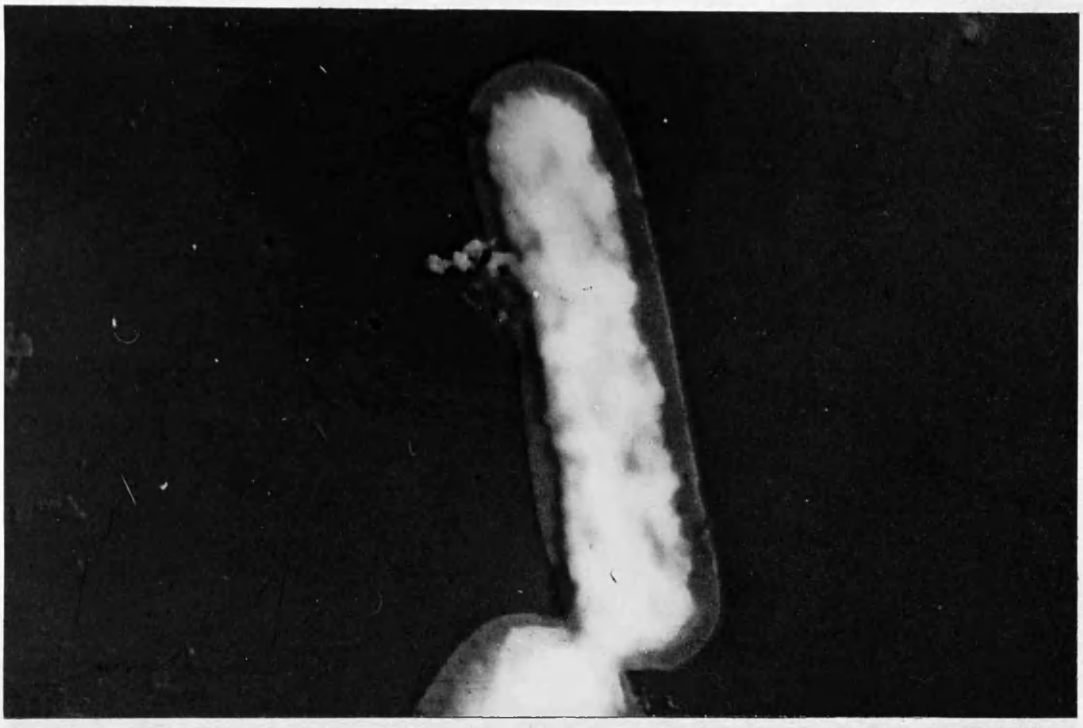


Fig. 7. B. subtilis suspended in distilled water for 1 hour showing commencing cytoplasmic fragmentation. ( × 25,000).

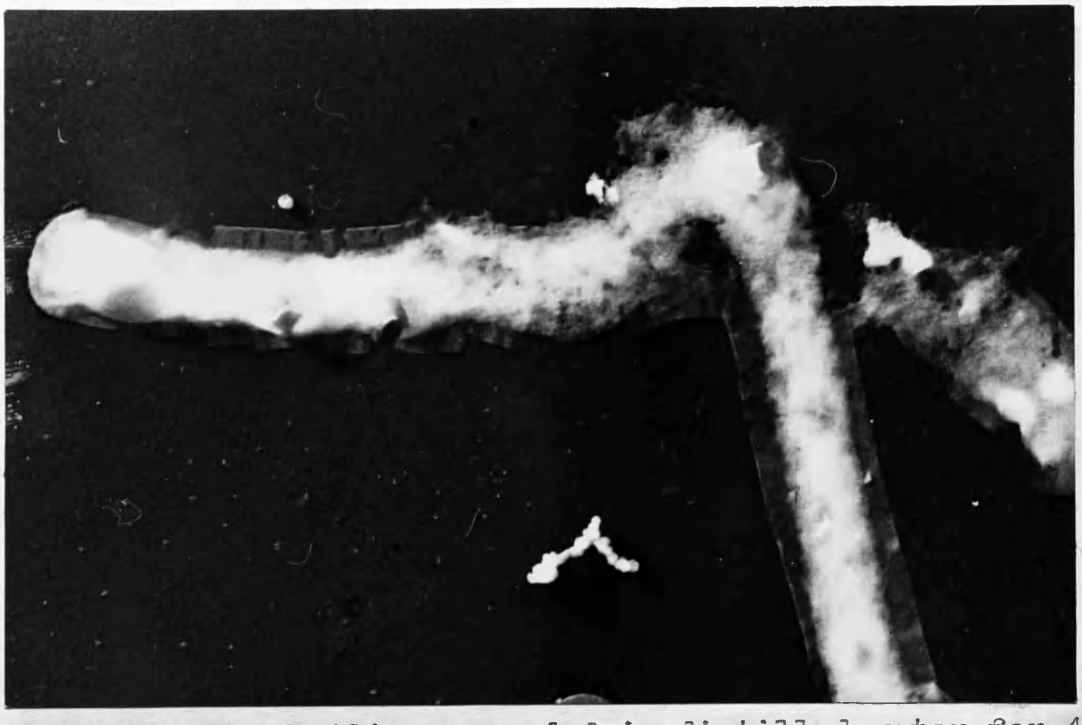


Fig. 8. B. subtilis suspended in distilled water for 4 hours. Both cell-wall and cytoplasm are disintegrating. ( × 15,000).

Fig. 9. B. subtilis suspended in distilled water for 4 hours. The cell-wall has ruptured allowing evacuation of the cytoplasm. (× 15,000).

Fig. 10. B. subtilis suspended in distilled water for 4 hours. Rupture of cell-wall with loss of cytoplasm. ( × 15,000).

Fig. 11. B. subtilis suspended in distilled water for 4 hours. The evacuated cytoplasm has a somewhat fibrous appearance. ( x 22,000).

Fig. 12. B. subtilis suspended in distilled water for 4 hours. The evacuated cytoplasm has a finely granular appearance. ( x 22,000).

The cell-walls then underwent rapid, complete disintegration although they appeared to be somewhat more resistant to autolysis than the cytoplasm. The possibility that the rupture of the cell-wall was due to osmotic swelling was checked by incubating the organisms in isotonic, buffer solutions but without significant difference; indeed the process of autolysis was somewhat faster in the buffer than in the distilled water.

The detailed structure of the cytoplasm cannot be resolved while still within the cell envelope but after its evacuation from the broken cell-wall it can be seen to consist of spherical particles of a markedly homogeneous size (figs.10 and 12). The particles , probably macromolecules, appear to be linked together in a highly organised state similar to that described by Wyckoff (1949) in B. coli, lysed by bacteriophage. In many cells, probably older cells, a more fibrous structure is apparent (fig.11).

## The autolysis of isolated cell-walls

The ease with which the cell-walls of bacteria can be isolated in a practically intact state enables a more detailed examination with the electron microscope to be carried out of the progressive changes undergone by the cell-wall during the autolytic process. The washed cell-walls were suspended in both distilled water and in the homologous bacterial autolysate. Washed cell-walls probably do not contain within them more than minimal quantities of the autolytic enzyme system and a suspension of these membranes in distilled water can be used as controls for comparison with the cell-walls suspended in autolysate. The cell-wall concentration in all tubes was equivalent to about 0.75 mgm. dry weight of cell-wall per ml. The suspensions were incubated at 37°C. and examined at intervals.

Staph. aureus. In distilled water the cell-walls disintegrated

very slowly (fig.13). Within 24 hours the membranes had become flattened in comparison with freshly prepared, control cell-walls. After 48 hours they assumed an appearance which suggested loss of rigidity; these "plastic" membranes showed irregular, spiky outgrowths at their edges and thinning gradually took place, probably owing to loss of material via the outgrowths. Although examinations were continued for 3 weeks the majority of the cell-walls were still found to be in this state without evidence of further disintegration.

In autolysate the process of disintegration followed the same path as in distilled water but progressed more rapidly to completion (fig.14). Within 48 hours the typical plastic appearance with irregularity of the edges was present; thinning and roughening of the surface was also evident. Roughening and grooving of the surface became more marked and by about the sixth day fragmentation of the cell-wall took place. The large fragments of cell-wall broke up still further and within about about 10 to 12 days little remained but debris. The isolated cell-walls, treated with autolysate, thus underwent the same fragmentary type of disintegration as do the whole cells (which have their autolytic enzymes within them) suspended in distilled water. Moreover, the time necessary for the disintegration of the isolated cell-walls agreed well with that of the cell-walls on the intact cells.

B. subtilis. The isolated cell-walls of B. subtilis were found to disintegrate very rapidly even in distilled water, possibly due to the greater difficulty of removing cytoplasmic material completely from the elongated cell-walls. The freshly prepared membrane is a fairly robust-looking structure with a smooth, apparently structureless surface (fig.15). After as little as 1 hour in distilled water it became thinner (fig.16) and localised loss of material occurred with the production of

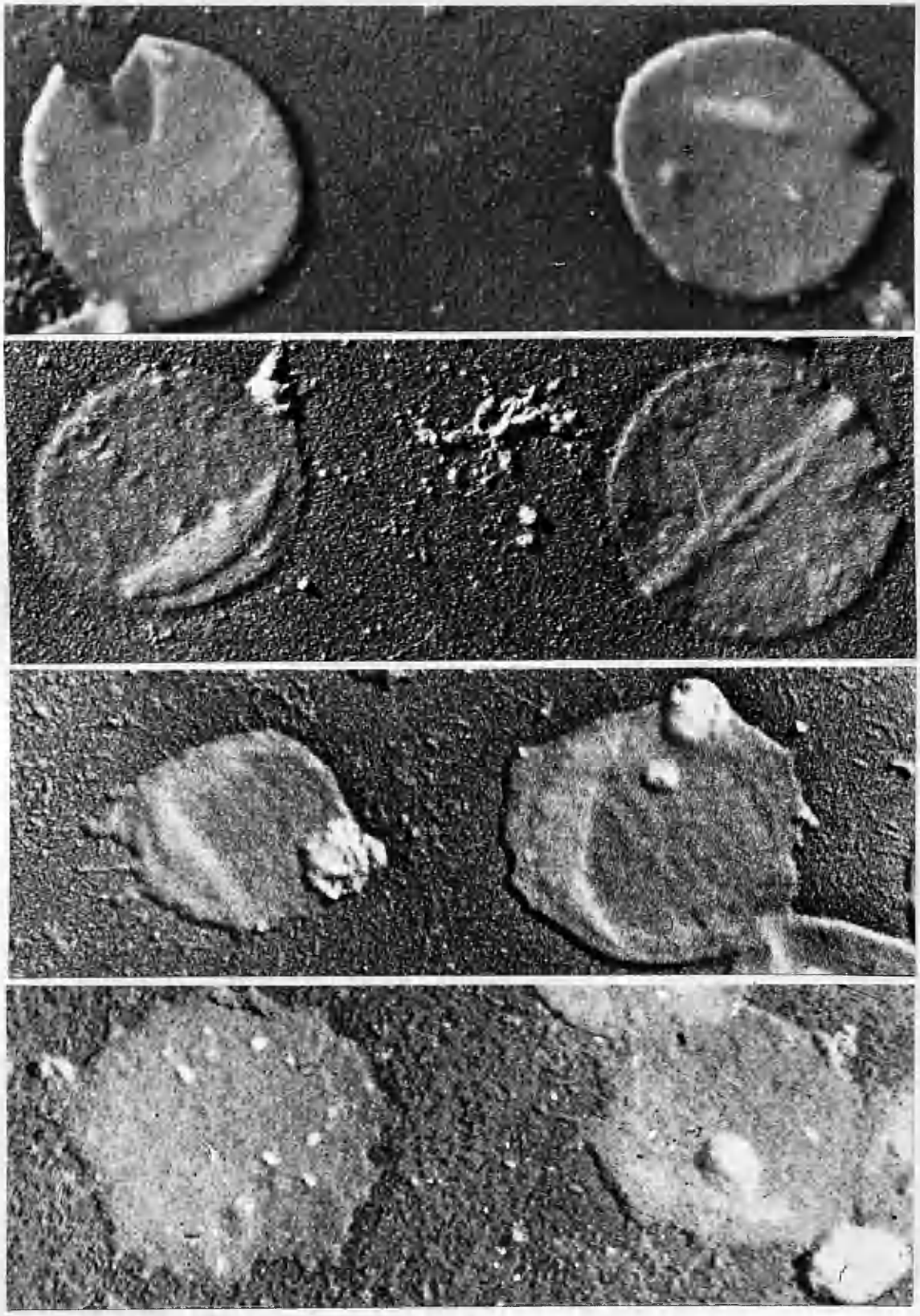



Fig. 13. Isolated cell-walls of Staph. aureus suspended in distilled water a) after 0 hr., untreated b) after 24 hr. c) after 48 hr. d) after 21 days. ( × 35,000).

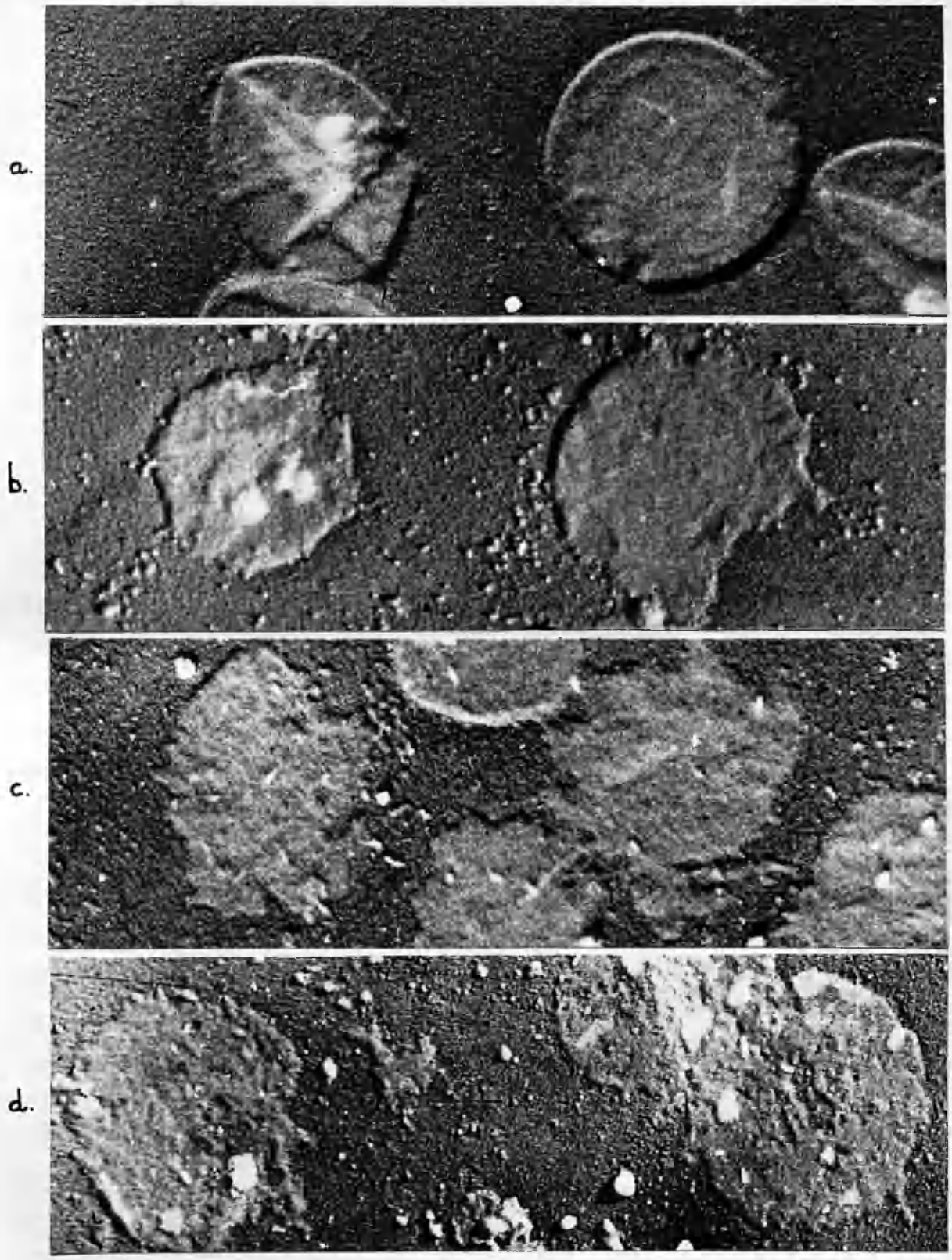



Fig. 14. Isolated cell-walls of Staph. aureus treated with autolysate a) after 24 hr. b) after 48 hr. c) after 6 days d) after 12 days. ( × 35,000).

punched-out, circular holes (fig.17). These became more numerous and the small holes fused to form larger holes (fig.18). The smaller holes tended to appear in linear rows running around the organism at right angles to its long axis, indicating a possible organised structure for the cell-wall. Within 24 hours little remained but debris.

Suspension of the cell-walls in autolysate did not appreciably alter the process or the time of disintegration.

The lysis of autoclaved cells and cell-walls

Suspensions of Staph. aureus and B. subtilis were autoclaved at 15 lb. per sq.in. for 15 minutes in order to destroy the autolytic enzymes. The cells, so treated, remained Gram-positive and when examined in the electron microscope they differed very little from non-autoclaved cells apart from some slight crenation of the surface of the cytoplasmic body; the cell-walls remained intact on the cells. Such cells, when incubated in distilled water, showed no evidence of autolysis or change of Gram staining even after a period of several weeks.

However, when the cells were suspended in autolysate lysis did take place but at a relatively slow rate. The staphylococcal suspensions began to show visible decrease in opacity only after 48 hours and even after 6 days had reached only 50 per cent of the original opacity. On the other hand, the majority of the cells were Gram-negative within 24 hours. The autoclaved cells thus became Gram-negative as rapidly as, or even more rapidly than, non-autoclaved cells in distilled water but differed from the latter in that the suspension showed little loss of opacity and the cells, at 24 hours, did not show any morphological changes detectable with the electron microscope apart possibly from some thinning of the cell-walls.

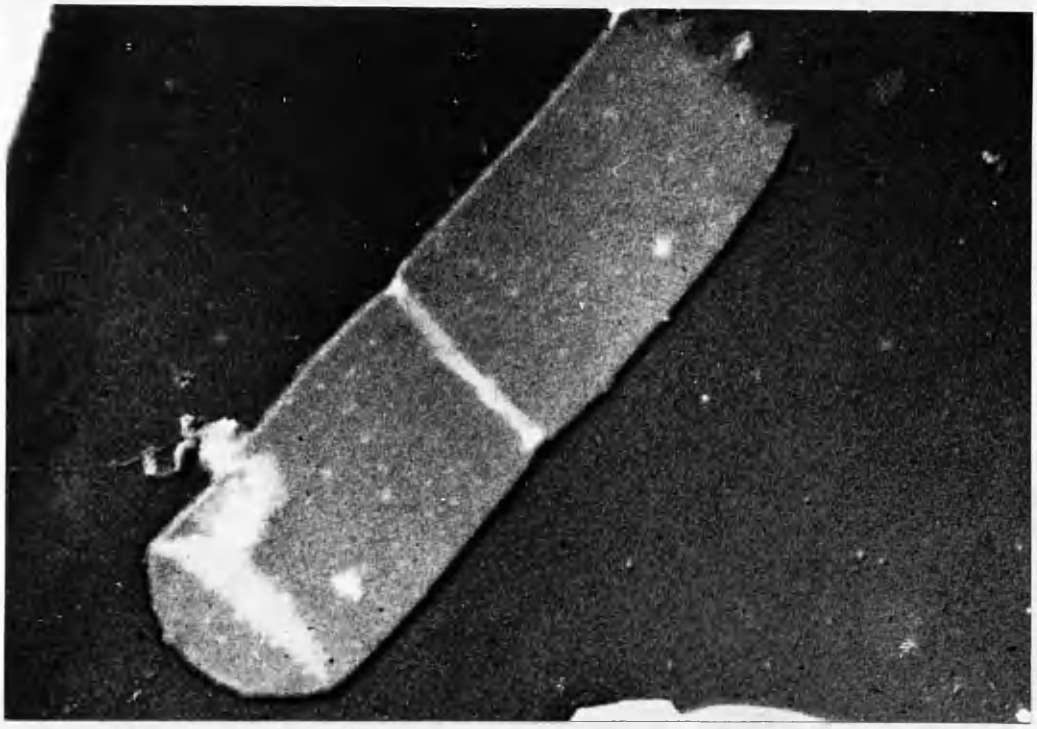


Fig. 15. Isolated cell-wall of B. subtilis. Untreated control. ( x 22,000).

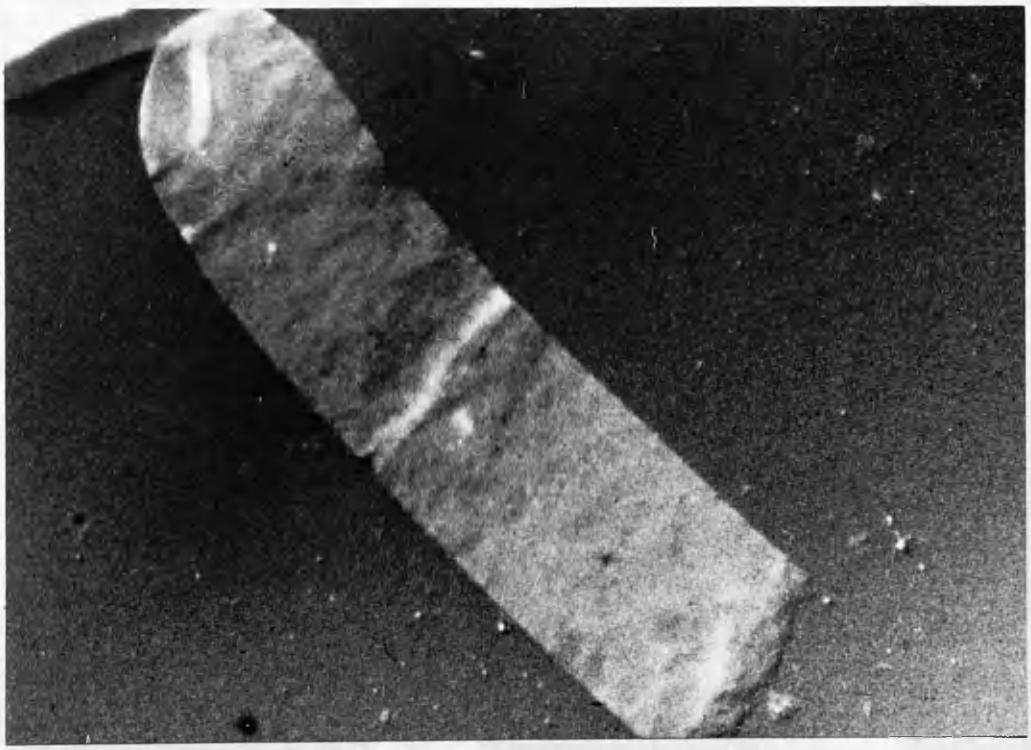


Fig. 16. Isolated cell-wall of B. subtilis treated with distilled water for 1 hour. The membrane shows thinning with early, localised loss of material. ( x 22,000).

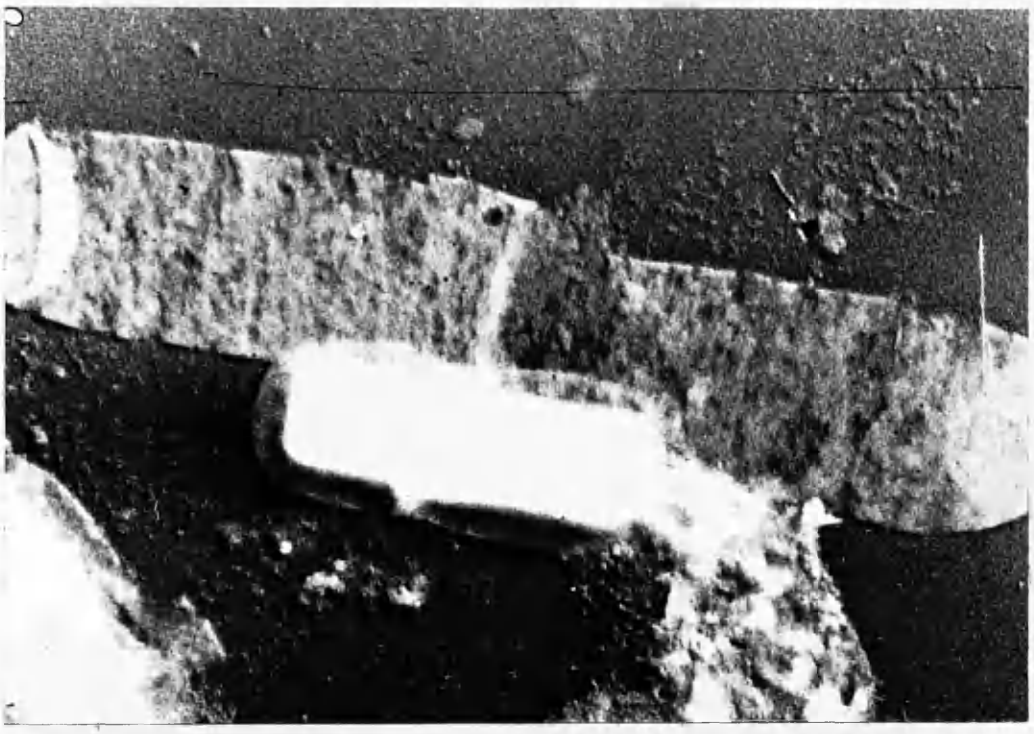


Fig. 17. Isolated cell-wall of B. subtilis treated with distilled water for 4 hours. Localised loss of material has produced regular formations of small, circular and oval holes. ( × 22,000).

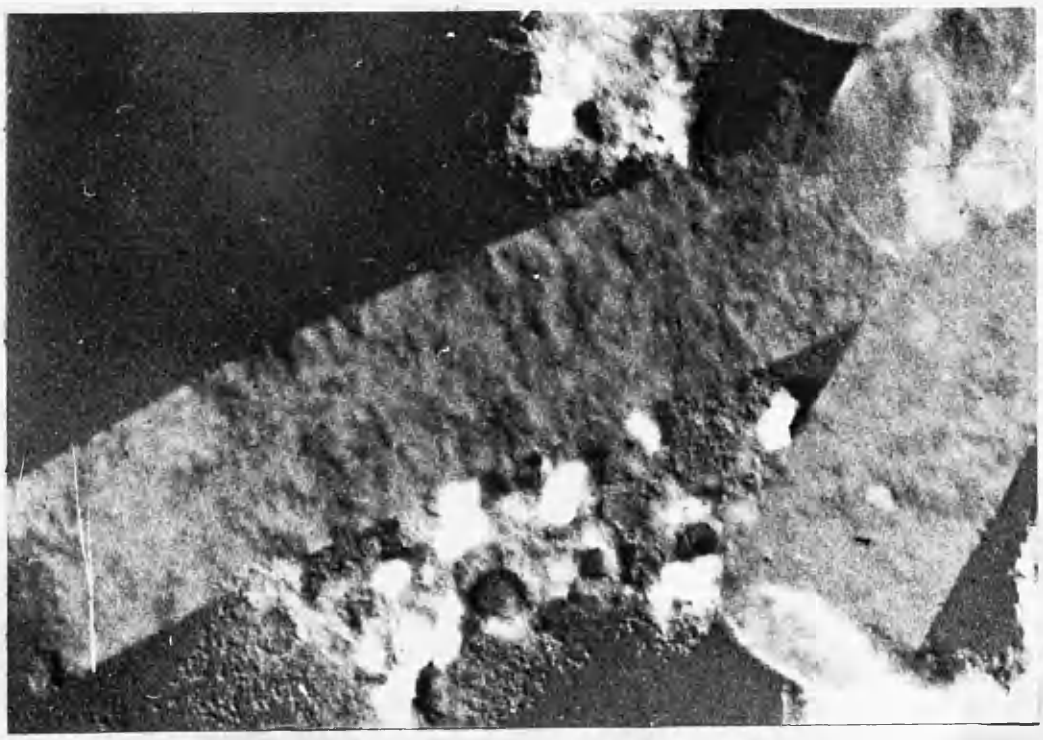


Fig. 18. Isolated cell-wall of B. subtilis treated with distilled water for 4 hours and showing the final stage of disintegration. ( × 22,000).

After 48 hours the cells showed an increasingly "moth-eaten" appearance with small, localised areas of digestion obviously proceeding from the surface inwards (fig.19); cytoplasmic retraction was absent but the cell-wall showed obvious thinning with disappearance at an early stage. Digestion of the cytoplasm was still incomplete in many cells after 10 days.

The suspensions of autoclaved cells of B. subtilis showed a similarly slow lysis in autolysate as compared with non-autoclaved cells. Within 4 hours the opacity had decreased by no more than 15 to 20 per cent but 90 per cent of the cells were already Gram-negative. By 24 hours a localised, surface digestion was obvious (fig.20); cytoplasmic retraction was insignificant but again there appeared to be early loss of the cell-wall.

Autoclaved cell-wall suspensions. Distilled water suspensions of washed cell-walls were autoclaved and incubated in distilled water and in autolysate. The autoclaving, itself, produced no obvious morphological changes in the membranes.

The cell-walls, incubated in distilled water, underwent no changes even after 21 days.

In autolysate, disintegration took place and the process, both with Staph. aureus and B. subtilis, was identical with that of non-autoclaved cell-walls in autolysate.

## Conditions influencing rate of autolysis in distilled water

The opacity of a bacterial suspension depends mainly on the number of bacteria present, on their state of aggregation and on the size and opacity of the individual cells. The strains of organisms used in the present investigation showed no tendency to aggregate in distilled water, indeed they became progressively more evenly dispersed as incubation proceeded. The loss of opacity of a bacterial suspension in distilled

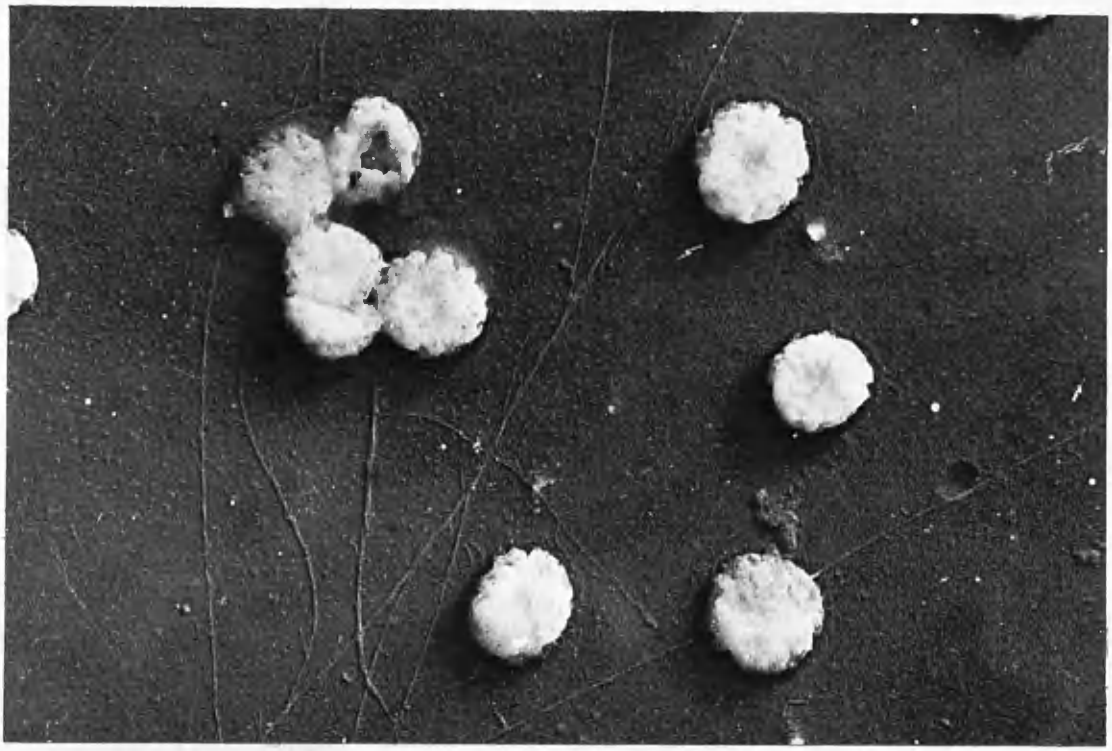


Fig. 19. Autoclaved cells of Staph. aureus treated with autolysate for 6 days. Localised digestion from the surface. ( × 15,000).

Fig. 20. Autoclaved cells of B. subtilis treated with autolysate for 24 hours and showing surface digestion. ( × 15,000).

water must, therefore, be due to decrease in either the size or the opacity, or both, of the individual cells, that is to changes associated with disintegration or autolysis of the organisms. The loss of opacity of a bacterial suspension can thus be used as an indication of the total degree of autolysis of the organisms composing the suspension. Using this as an index, it became obvious that the rate of autolysis could be markedly influenced by various conditions which, in order to obtain comparable results in different experiments, must be standardised. This was of especial importance when studying the influence on autolysis of specific conditions such as the presence of detergents.

The following conditions were found to be important:-

(1) The concentration of organisms. When the organisms were suspended in distilled water and allowed to autolyse it was found that the rate of lysis increased with increasing concentrations of cells. This is illustrated for Staph. aureus in table II. Dubos (1937a) has shown that one pneumococcus contains sufficient autolytic enzyme to disintegrate 100 dead pneumococci. Since the reaction is enzymatic the increased rate of autolysis can be explained by the increasing substrate concentration and by the enormously increasing concentration of autolytic enzymes which are liberated by autolysis and then assist in the disintegration of still intact cells. Electron microscopy showed that, although the rate of lysis of the staphylococci was faster with high concentrations of cells, the morphology of lysis remained unaltered.

(2) Age of cells. The cells used in all the present experiments were grown for 16 to 18 hours in broth. It was found that younger cells underwent a more rapid lysis and older cells a slower lysis than the 16 to 18-hour cells. This might possibly be explained on a structural basis: Wyckoff

Table II

The effect of cell concentration on the rate of autolysis of Staph. aureus in distilled water

| No. of organisms ($\times 10^9$) | Percentage lysis after | | |
|---|---|---|---|
| | 1 hr. | 24 hrs. | 72 hrs. |
| 57 | 30 | 75 | 90 |
| 16·8 | 0 | 60 | 90 |
| 8·4 | 0 | 35 | 75 |
| 4 | 0 | 20 | 70 |

Lysis expressed as percentage clearing of original suspension opacity.

(1949) has shown by electron microscope examinations of bacteriophage-lysed, young and old cells of B. coli that in young cells the cytoplasm is fluid and homogeneous, being composed of spherical macromolecules whereas in older cells the cytoplasm becomes increasingly fibrous and viscous. This latter morphological change might well be responsible for increased difficulty of autolytic disintegration.

(3) Washing of cells. When the organisms were washed 4 times instead of the usual twice before incubation with distilled water it was found that the rate of lysis was distinctly slower. The rate of lysis could often be reduced by one half, but could not be completely prevented by more thorough washing. The original rate of lysis could be almost completely restored by adding the first washings. It seems likely that washing might remove ions or molecules essential for autolysis (Born, 1952). Magnesium ions, known to be essential for the action of ribonuclease, were not found to be effective in restoring original rates of lysis.

(4) Hydrogen ion concentration. The maximum rate of lysis of both Staph. aureus and B. subtilis occurs at a restricted range of pH, namely 6.5 to 7.2. Deviations beyond this range produce marked slowing of the rate of lysis. Table III illustrates the effect of iso-osmotic, phosphate-citrate buffer of various hydrogen ion concentrations on autolysis. An incidental finding was that in the tubes containing buffer of alkaline pH considerable amounts of a mucinous substance were formed. This substance was not obvious in the tubes of acid pH although staining with dilute carbol-fuchsin showed its presence in small quantities; in the alkaline tubes large masses were visible and staining showed it surrounding the cells and forming string-like formations. The mucin could be dissolved by dilute mineral acids but not by dilute alkalis.

Table III

The effect of hydrogen ion concentration on the rate of autolysis of Staph. aureus

| Time (Hrs.) | Percentage lysis at hydrogen ion concentrations of | | | |
|---|---|---|---|---|
| | 5•8 | 6•5 | 7•2 | 8 |
| 0 | 0 | 0 | 0 | 0 |
| 24 | 25 | 38 | 35 | 20 |
| 48 | 50 | 68 | 65 | 40 |

Lysis expressed as percentage clearing of original suspension opacity.

(5) Temperature of incubation. The rate of lysis at 30°C. and at room temperature (approximately 18°C.) was markedly slower than at 37°C. Thus at room temperature 100 per cent of the cells in a suspension of Staph. aureus remained Gram-positive after 24 hours and evidence of lysis became apparent only after as long as 36 to 48 hours. Higher temperatures than 37°C. were not investigated.

## B. THE INFLUENCE ON AUTOLYSIS OF CTAB

### The lysis of intact organisms

The possibility was discussedin the Introduction that the effects on bacteria of detergents may vary with concentration. It was, therefore, decided to study the influence of a wide range of CTAB concentrations on a fixed mass of organisms. In the present experiments the concentration of both Staph. aureus and B. subtilis was kept constant at about 2.8 mgm. dry weight of cells per ml. (in the case of Staph. aureus this is equivalent to $8 \times 10^9$ cells per ml.). The concentration of CTAB ranged from 0.003 per cent to 1 per cent (30 to 10,000 µg. per ml.). The suspensions of cells in the CTAB solutions were incubated at 37°C. and examined at intervals by opacity estimation, staining by Gram's method and electron microscopy. Typical sets of results are shown in tables IV and V.

Staph. aureus. Concentrations of CTAB of 0.003 per cent (cell to CTAB weight ratio of about 84 to 1) and less showed little difference in rate of lysis over that in distilled water. With concentrations of 0.01 per cent (cell to CTAB weight ratio of about 28 to 1) and 0.03 per cent (cell to CTAB weight ratio of about 9 to 1) the rate of lysis during the first 24 hours was greatly accelerated. Marked lysis was observable by 1 hour, and by 24 hours about 70 per cent lysis had occurred with all the cells Gram-negative. However, there were striking differences between the subsequent results with these two concentrations. With 0.01 per cent CTAB the suspension opacity, beyond 24 hours, continued to diminish rapidly and the cells showed a rapid loss of ability to retain the carbol-fuchsin of the Gram stain. With 0.03 per cent CTAB lysis apparently ceased after 24 hours and even after prolonged incubation the

Table IV

Rate of lysis and change of Gram staining of Staph. aureus in distilled water and in various concentrations of CTAB

| Time (hrs.) | Distilled water | CTAB in concentrations, per cent, of | | | | |
|---|---|---|---|---|---|---|
| | | 0·003 | 0·01 | 0·03 | 0·1 | 1·0 |
| 0 | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) |
| 1 | 0 (0) | 0 (0) | 35 (50) | 35 (50) | 0 (0) | 0 (0) |
| 4 | 0 (0) | 0 (0) | 40 (75) | 45 (75) | 0 (0) | 0 (0) |
| 24 | 35 (50) | 40 (50) | 70 (100) | 70 (100) | 0 (0) | 0 (0) |
| 48 | 65 (90) | 70 (90) | 90 (100) | 70 (100) | 0 (0) | 0 (0) |
| 96 | 90 (99·9) | 90 (100) | 90 (100) | 70 (100) | 0 (0) | 0 (0) |

The upper figure is lysis expressed as percentage clearing of original suspension opacity. The figure in brackets is the percentage of Gram-negative cells.

Table V

Rate of lysis and change of Gram staining of B. subtilis in distilled water and in various concentrations of CTAB

| Time (hrs.) | Distilled water | CTAB in concentrations, per cent, of | | | | | |
|---|---|---|---|---|---|---|---|
| | | 0·003 | 0·01 | 0·03 | 0·1 | 0·3 | 1·0 |
| 0 | 0 (5) | 0 (5) | 0 (5) | 0 (5) | 0 (5) | 0 (5) | 0 (5) |
| 1 | 35 (50) | 40 (60) | 65 (90) | 30 (100) | 30 (100) | 0 (5) | 0 (5) |
| 4 | 65 (95) | 65 (100) | 80 (100) | 30 (100) | 30 (100) | 0 (6) | 0 (5) |
| 24 | 85 (99·9) | 85 (100) | 90 (100) | 30 (100) | 30 (100) | 0 (5) | 0 (5) |
| 48 | 95 (99·9) | 95 (100) | 95 (100) | 30 (100) | 30 (100) | 0 (5) | 0 (5) |

The upper figure is lysis expressed as percentage clearing of original suspension opacity. The figure in brackets is the percentage of Gram-negative cells.

to stain brilliantly with carbol-fuchsin. With concentrations of 0.1 per cent (cell to CTAB weight ratio of about 3 to 1) and higher lysis did not occur and the cells remained Gram-positive. With these latter concentrations marked agglutination of the cells was observed; with the lower concentrations of 0.003 to 0.03 per cent it was absent.

Electron microscope examination revealed that the stimulation of lysis produced by 0.01 per cent CTAB was associated with striking morphological differences compared with those occurring in distilled water. Within 1 hour the majority of the cells showed cytoplasmic retraction (fig.21); again the proportion of cells with retracted cytoplasm equalled the proportion of Gram-negative cells. By 24 hours the retraction was present in practically every cell. Within 36 to 48 hours there appeared evidence of a fine granulation involving the entire cytoplasmic body of the cell (fig.22). This became progressively more marked and was associated with a rapid thinning of the cell-wall. Within 5 days the entire cell, including the cell-wall, had been reduced to a mass of granular debris (fig.23). This process of disintegration differed from that in distilled water not only in being faster but also in the uniformity with which it involved the entire cell, contrasting with the irregular, fragmentary process in distilled water. With 0.03 per cent CTAB cytoplasmic retraction appeared within the first hour as with 0.01 per cent CTAB (fig.24) but thereafter a striking difference became evident. At 24 hours the cell-walls had been stripped from the cytoplasmic bodies in irregular fragments (fig.25). Although the cell-walls had been thus fragmented they seemed to be of about normal thickness. The stripped bodies of the cells underwent no further disintegration and even after several

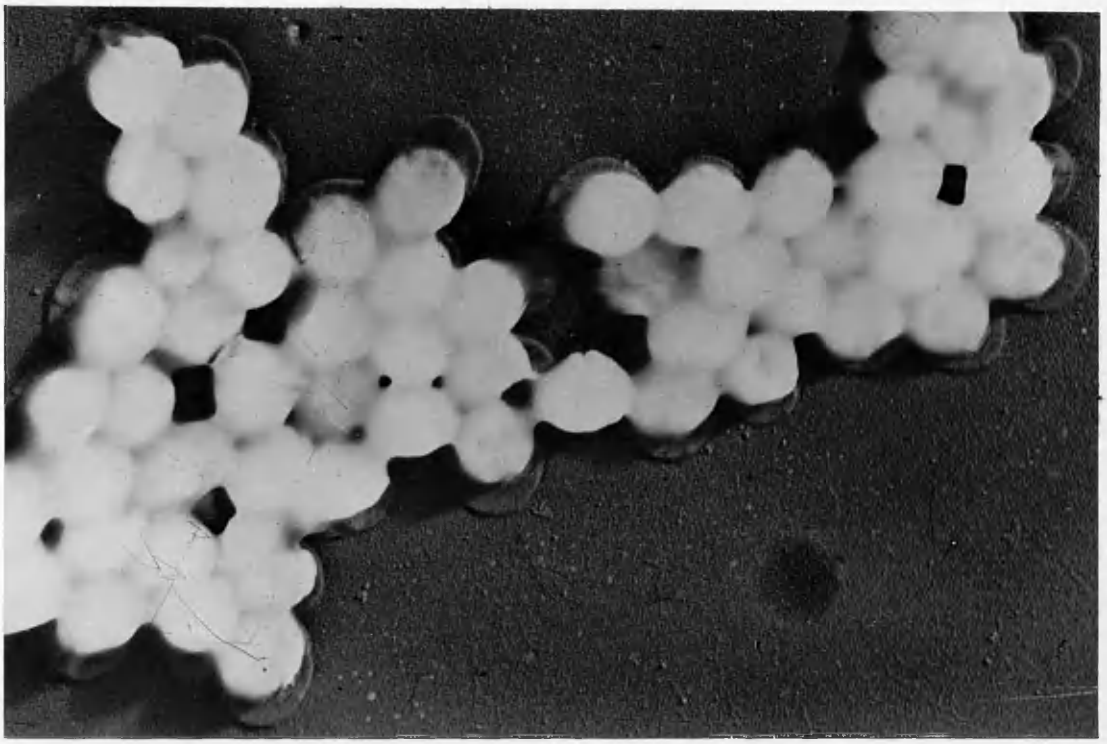


Fig. 21. Staph. aureus treated with 0·01 per cent C.T.A.B. for 1 hour. Many cells already show cytoplasmic retraction. ( × 15,000).

Fig. 22. Staph. aureus treated with 0·01 per cent C.T.A.B. for 48 hours. A fine granulation of the cytoplasm is obvious. ( × 15,000).

Fig. 23. Staph. aureus treated with 0·01 per cent C.T.A.B. for 5 days. Rapid, uniform disintegration of cytoplasm and cell-wall. ( x 25,000).

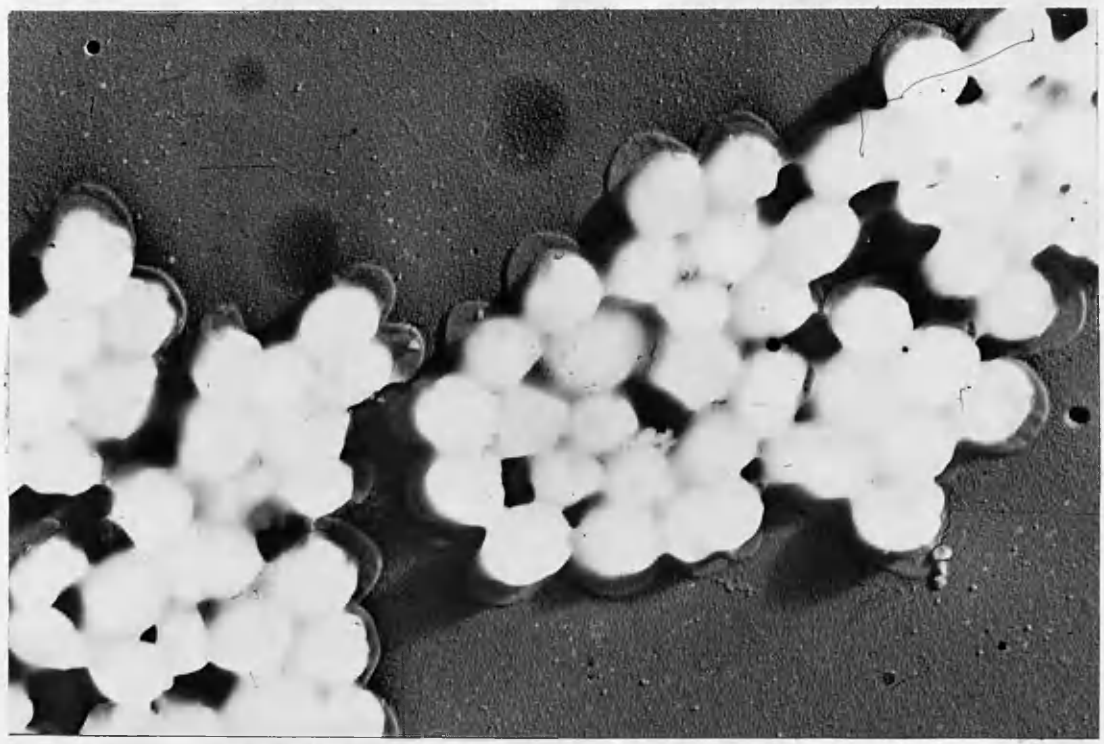


Fig. 24. Staph. aureus treated with 0·03 per cent C.T.A.B. for 1 hour. Marked cytoplasmic retraction is present. ( x 15,000).

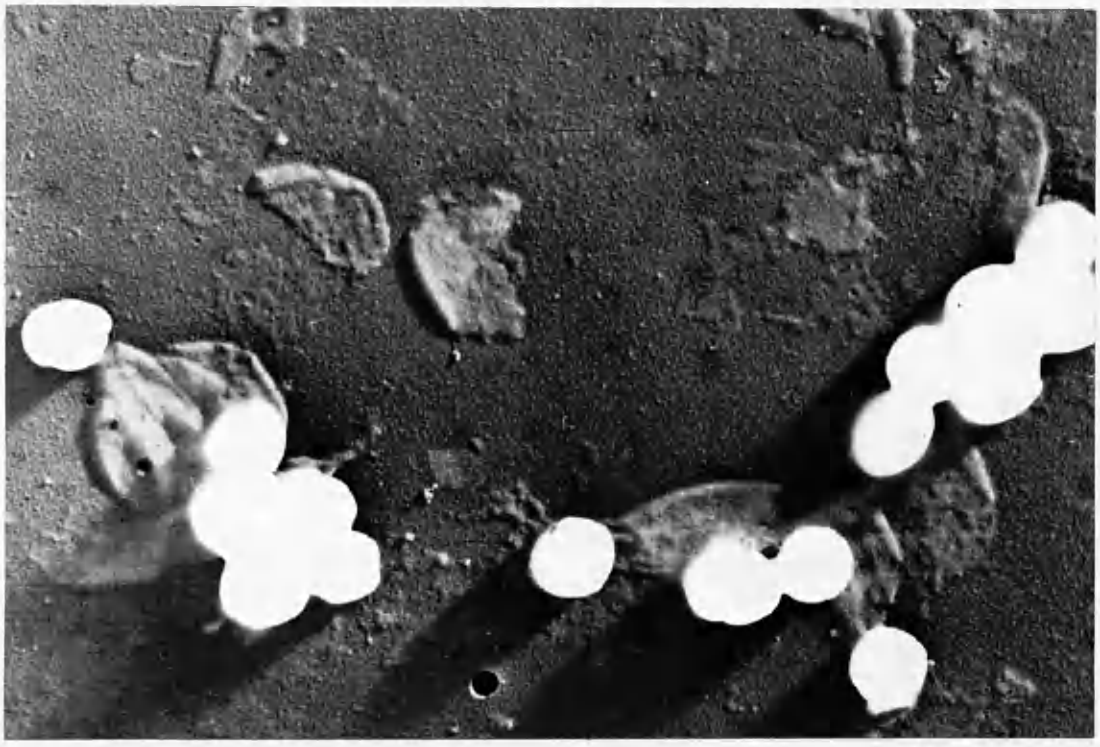


Fig. 25. Staph. aureus treated with 0·03 per cent C.T.A.B. for 24 hours. The cell-walls have been stripped from the organisms. (× 15,000).

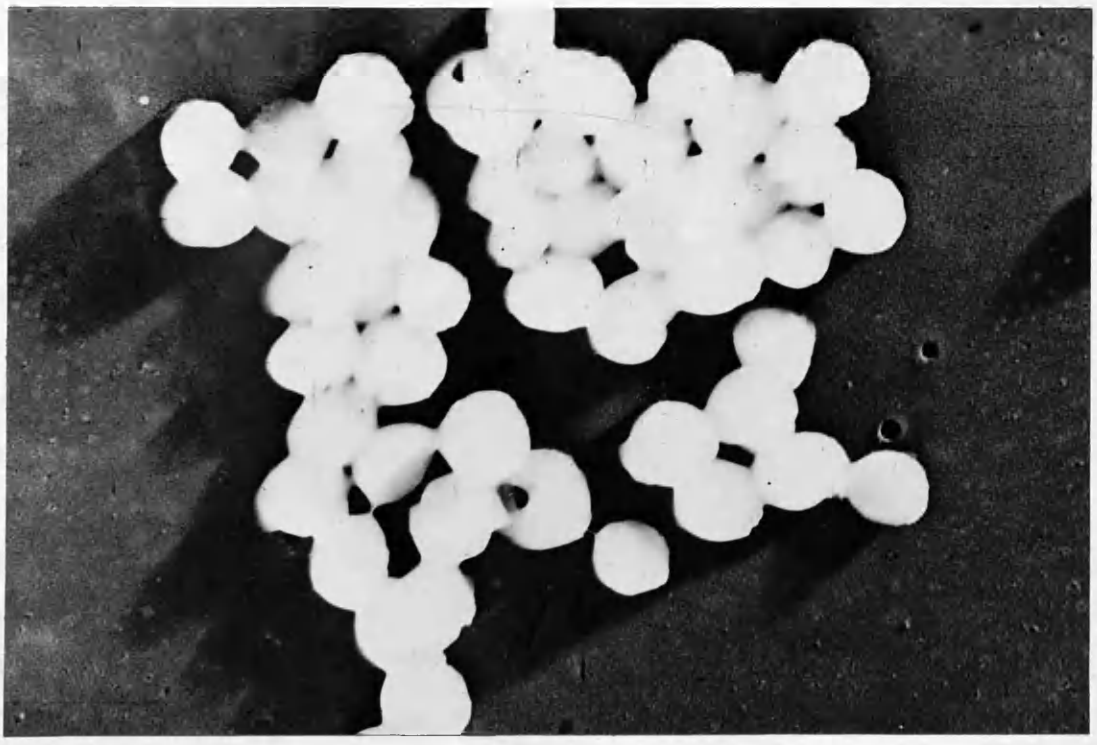


Fig. 26. Staph. aureus treated with 0·03 per cent C.T.A.B. for 10 days. No advance in the disintegration of the cytoplasmic bodies of the cells compared with fig. 25. ( × 15,000).

weeks remained unchanged (fig. 26). At 0.1 per cent and higher concentrations, CTAB had a fixative action; cytoplasmic retraction did not take place and the cells remained apparently unaltered even after prolonged incubation (fig.27).

B. subtilis. The effects produced on this organism varied with the concentration of CTAB in a manner similar to that of Staph. aureus. The maximum rate of lysis was reached with 0.01 per cent CTAB (cell to CTAB weight ratio of about 28 to 1). Within 1 hour the majority of the cells were Gram-negative and were already showing loss of staining reaction with carbol-fuchsin. Electron microscopy demonstrated, however, that, unlike the staphylococcus, the method of disintegration did not differ from that in distilled water. The entire process was obviously speeded up, since within 1 hour the majority of the cells were already in advanced states of disintegration, but the already diffuse, uniform process of water autolysis was not found to be significantly altered from the morphological point of view, even when examinations were carried out at 10 minute intervals during the first hour. Cell-wall rupture with cytoplasmic evacuation was a conspicuous feature as in distilled water.

With both 0.03 per cent (cell to CTAB weight ratio of about 9 to 1) and 0.1 per cent (cell to CTAB weight ratio of about 3 to 1) CTAB only partial loss of opacity occurred but all the cells were Gram-negative within 1 hour; the cells continued to stain brilliantly with carbol-fuchsin, even after prolonged incubation. Electron microscopy revealed the remarkable cell-wall stripping action, as with Staph. aureus, obvious within 1 hour (fig.28).

A higher concentration of CTAB, namely 0.3 per cent (cell to CTAB weight ratio of about 1 to 1), than for Staph. aureus was required to cause cell fixation.

Fig. 27. Staph. aureus treated with 0·1 per cent C.T.A.B. for 10 days. The cells are fixed. ( × 15,000).

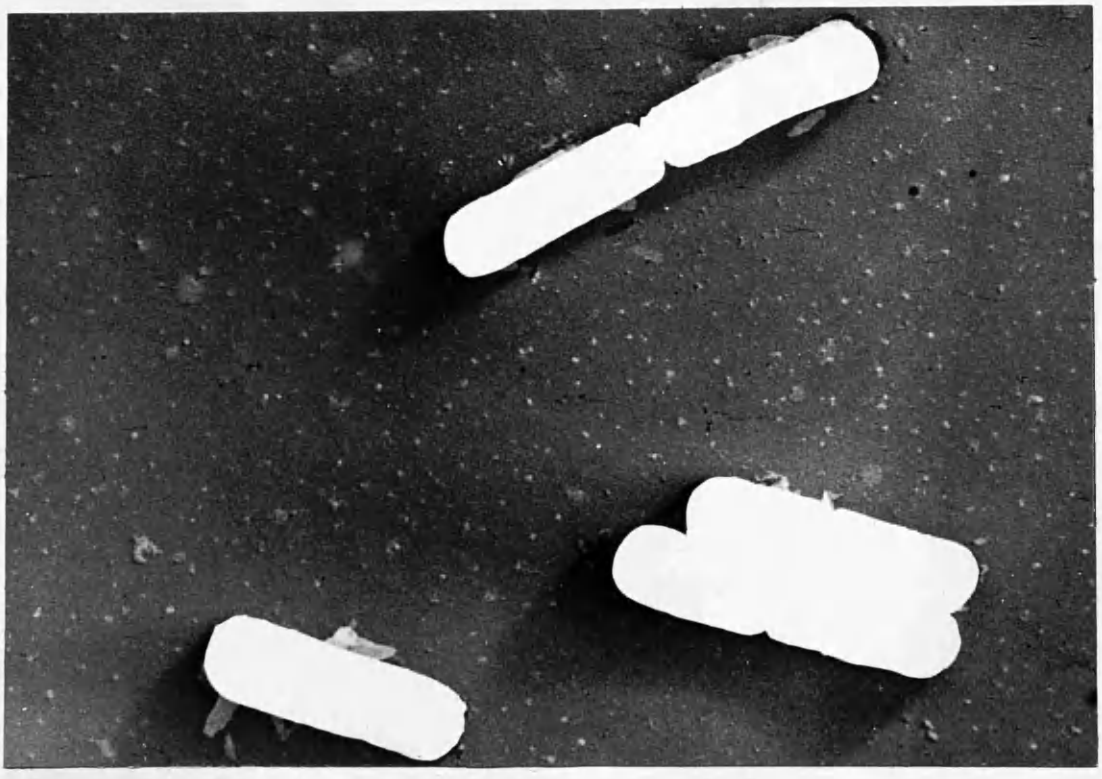


Fig. 28. B. subtilis treated with 0·03 per cent C.T.A.B. for 1 hour. Cell-wall stripping has occurred. ( × 15,000).

## The lysis of isolated cell-walls

The cell-walls were suspended in solutions of CTAB ranging from 0.003 to 1 per cent (30 to 10,000 µg. per ml.). The cell-wall concentration in all tubes was equivalent to about 0.75 mgm. dry weight of cell-wall per ml. The tubes were incubated at 37°C. and specimens removed at intervals for electron microscope examination.

Staph. aureus. In 0.003 per cent CTAB a process of disintegration occurred quite different from that in distilled water (fig.29). After 48 hours the cell-wall showed a fine surface roughness, quite distinct from the coarse roughening of autolysate-treated cell-walls. Loss of finely granular material into the background is obvious in the micrographs. The plastic stage of water autolysis did not occur. The loss of material continued and by about 4 days the membrane had an appearance suggesting the presence of a network of interlacing fibres. By about 6 to 7 days the cell-wall had become completely granular and rapidly dispersed into the background. The process was thus not only faster than in distilled water but also differed from it in the absence of fragmentation of the membrane; instead there occurred a diffuse, even disintegration to the final granular stage. The isolated cell-walls, treated with low concentrations of CTAB (cell-wall to CTAB weight ratio of about 25 to 1), thus disintegrated in an identical manner to intact cells treated with CTAB at a similar cell to CTAB weight ratio.

In 0.01 per cent CTAB (cell-wall to CTAB weight ratio of about 8 to 1) and higher concentrations the cell-walls did not disintegrate but remained completely preserved.

B. subtilis. The disintegration of the isolated cell-walls of this organism was not appreciably affected by the presence

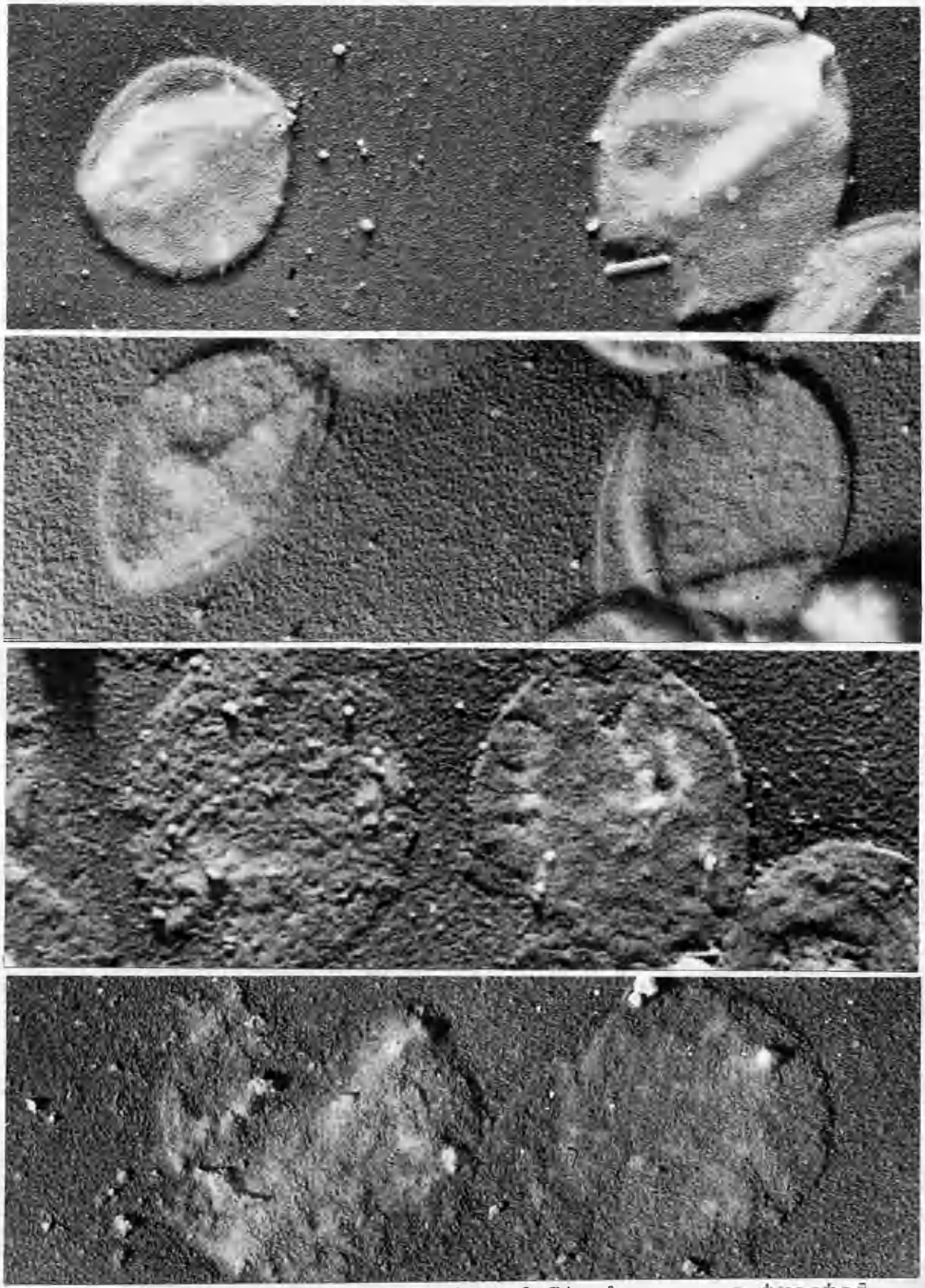



Fig. 29. Isolated cell-walls of Staph. aureus treated with 0·003 per cent C.T.A.B. a) after 24 hr. b) after 48 hr. c) after 4 days d) after 7 days. ( × 35,000).

of 0.003 per cent CTAB. The rate of disintegration was possibly slighter faster than in distilled water but, as with the intact cells, the morphology of disintegration was not obviously altered. Higher concentrations of CTAB caused fixation, as with the cell-walls of Staph. aureus.

The lysis of autoclaved cells and cell-walls

The intact cells and the isolated cell-walls of Staph. aureus and B. subtilis, autoclaved as described previously, were suspended in concentrations of CTAB ranging from 0.003 to 1 per cent, incubated at 37°C. and examined at intervals by opacity estimation, staining by Gram's method and electron microscopy. The cell and cell-wall concentrations were as in previous experiments.

Even after several weeks' incubation neither the intact cell suspensions nor those of the isolated cell-walls showed any change of opacity. The intact cells remained Gram-positive. Electron microscopy showed that both the intact cells and the isolated cell-walls remained completely unaltered and apparently preserved.

When the autoclaved cells were suspended in autolysate containing the lower concentrations of CTAB lysis did occur. Thus, the intact cells, in autolysate containing 0.003 per cent CTAB, showed the same "moth-eaten" form of disintegration as autoclaved cells in autolysate alone; the CTAB produced little or no alteration in either the rate or the form of autolysis. When higher concentrations of CTAB were present in the autolysate the latter was inactivated and lysis did not occur. With no concentration of CTAB could stripping of cell-walls from autoclaved cells be achieved.

On the other hand, when the isolated cell-walls of Staph. aureus were suspended in autolysate plus 0.003 per cent CTAB

the form of disintegration was that of non-autoclaved cell-walls in 0.003 per cent CTAB, namely the rapid, diffuse form of disintegration. With higher concentrations of CTAB the autolysate was inactivated. The autoclaved cell-walls of B. subtilis behaved in the same manner as those of Staph. aureus.

## The relationship between cell concentration and CTAB concentration

In the preceeding experiments the effect of varying concentrations of CTAB against a constant concentration of organisms has been studied. It seemed important to investigate whether the critical concentrations causing stimulation of lysis, cell-wall stripping and fixation are absolute in their effects and independent of cell concentration or whether the effects produced by a given concentration of detergent vary with the concentrations of organisms. An experiment was, therefore, carried out in which the concentration of Staph. aureus and the CTAB concentration were both varied. The degree of lysis, as estimated by opacity measurement, was noted in each tube after 48 hours. The morphological changes produced on the individual cells was determined by Gram staining. The results are illustrated in table VI.

The table shows that the effects produced by CTAB depend both on its own and on the cell concentration. To obtain a fixed rate of lysis a constant weight ratio of cells to CTAB is necessary. Thus, to maintain the original rate of lysis and the original type of action, doubling of the cell concentration requires doubling of the CTAB concentration. There, therefore, exists between cell concentration and CTAB concentration a directly proportional or stoichiometric relationship.

These results were used to determine the part played by detergent micelles in bacterial lysis. Detergents, being

Table VI

The influence of cell concentration on the lytic effects of various concentrations of CTAB on Staph. aureus

| Concentration of cells ($\times 10^9$) | Concentration of CTAB (per cent) | | | |
|---|---|---|---|---|
| | 0·01 | 0·02 | 0·03 | 0·1 |
| 60 | 80 D | 80 D | 80 D | 85 D |
| 16 | 75 D | 90 D | 80 D | 70 S |
| 8 | 90 D | 75 D | 70 S | 0 F |
| 4 | 75 D | 65 S | 70 S | 0 F |
| 2 | 65 S | 0 F | 0 F | 0 F |
| 1 | 0 F | 0 F | 0 F | 0 F |

The numbers are percentage lysis of cell suspension after 48 hours as estimated by decrease of original suspension opacity. The letters refer to the morphological effect on the cells; D is cellular disintegration, S is cell-wall stripping and F is cell fixation.

members of the group of substances termed colloidal electrolytes, show the spontaneous formation of colloidal aggregates or micelles when the concentration exceeds a certain critical limit. The micelle consists of a large number of detergent ions, usually about 50 in the case of a $C_{16}$ straight-chain soap such as CTAB (Trim and Alexander, 1949); the ions are arranged so that the hydrocarbon chains are aggregated in a fluid state with the ionised, hydrophilic groups in contact with the outer aqueous phase. The micelles have the remarkable property of solubilising otherwise insoluble substances. Some experiments by Trim and Alexander suggested that the free ions of a detergent and the micelles might possess different biological properties. Using the roundworm, Ascaris, they found that CTAB had little influence upon the rate of phosphate leakage until micelles were present in the solution (at about 0.01 per cent CTAB); the sudden increase in leakage they ascribed to the micelles "deterging" lipoid constituents of the surface membrane and thus removing the principal barrier to ionic diffusion. These workers, however, did not investigate whether the concentration of 0.01 per cent CTAB is absolute or will be ineffective with increasing weights of Ascaris. Yet, it seemed possible that the different effects of low and high concentrations of CTAB on bacteria might be due respectively to the absence or presence of micelle formation and that the latter might be responsible for the cell-wall stripping. It was, therefore, decided to determine the critical concentration of CTAB for micelle formation and to attempt to relate this to the effects produced on the bacterial cell.

The critical concentration for micelle formation can be calculated by plotting equivalent conductivity against concentration. In very dilute solution the conductivity approximates to that of an ordinary electrolyte but on reaching

C. T. A. B.

| CONC. % | MOLAR CONC. | $\sqrt{C}$ | $\Delta$ |
|---|---|---|---|
| 0·01 | 0·00025 | 0·011 | 101·92 |
| 0·05 | 0·00125 | 0·035 | 96·6 |
| 0·1 | 0·0025 | 0·05 | 89·4 |
| 0·2 | 0·005 | 0·07 | 81·5 |
| 1·0 | 0·025 | 0·158 | 67·5 |
| 2·0 | 0·05 | 0·233 | 67 |

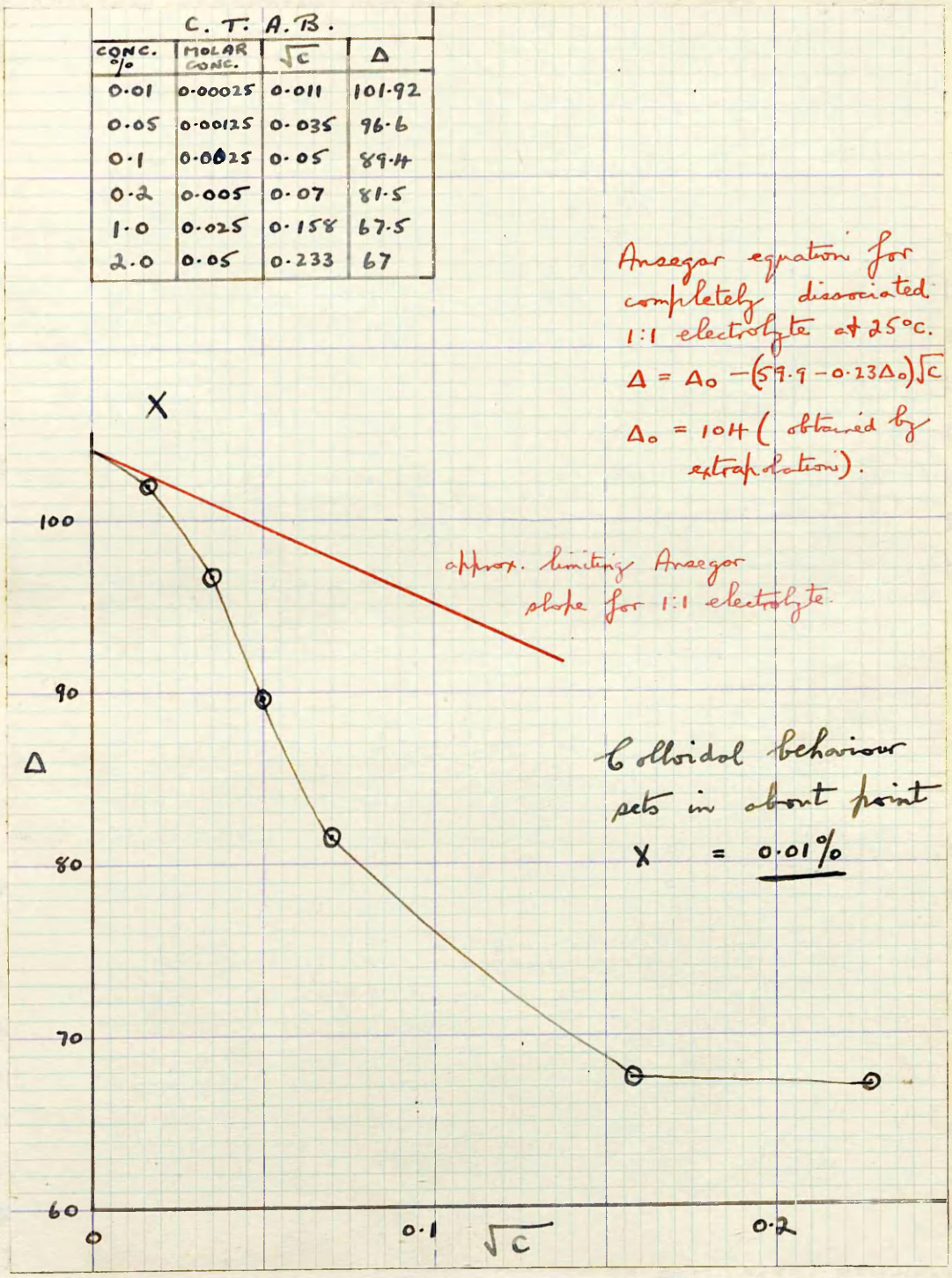



Fig. 30. Graph demonstrating the concentration of C. T. A. B. at which micelle formation commences.

the critical concentration the conductivity falls off sharply, indicating the disappearance of free ions and the formation of colloidal micelles. The conductivity measurements were carried out as described by Findlay (1941) and the results plotted in graph form (fig.30).

Micelle formation would appear to occur at about 0.01 per cent, the figure quoted by Trim and Alexander. From this result one might interpret the effects produced by concentrations of CTAB above 0.01 per cent as due to the action of micelles. However, table VI shows that by increasing the cell concentration both cell-wall stripping and fixation can be made to occur with 0.01 per cent and complete lysis with the higher concentrations of 0.03 and 0.1 per cent CTAB. These results indicate that in the action of detergents on bacteria the biologically active unit is not the micelle but the free ion.

## The influence of lipoid extraction on autolysis

In view of the current theories that detergents act on a lipoid-containing structure in the bacterial cell it was thought desirable to compare the effect of lipoid extraction by the usual organic solvents with the effect of CTAB on the course of autolytic disintegration. It was necessary to employ a method which would disrupt the lipoid-containing complexes of the cell and yet, like dilute CTAB, leave the autolytic enzymes intact. Chargaff and Bendich (1944) showed that freezing to very low temperatures in the presence of organic lipoid solvents, such as alcohol-ether, causes splitting of lipoprotein molecules while Neurath et al. (1944) demonstrated that at low temperatures organic solvents do not cause protein denaturation. It was, therefore, decided to use an alcohol-ether mixture at low temperature in studying the effect of extraction by organic solvents on Staph. aureus.

The organisms were washed twice and suspended in an equal parts alcohol-ether mixture. For a period of 2 hours the suspension was maintained below -25°C. by a solid carbon dioxide-acetone freezing mixture. The organisms were then washed twice with distilled water to remove organic solvent. Cells, so treated, were still Gram-positive and electron microscopy revealed no morphological changes apart from possibly some slight cytoplasmic retraction. On culture the suspension proved sterile. The cells were suspended in distilled water and in 0.01 per cent CTAB to give a concentration of about 2.8 mgm. dry weight of cells per ml. ($8 \times 10^9$ cells per ml). The suspensions were incubated at 37°C. and opacity estimations (table VII), staining by Gram's method and electron microscopy were carried out at intervals.

In distilled water the cells underwent a process of autolysis almost identical with that of non-extracted cells in 0.01 per cent CTAB. In 1 hour there was 30 per cent lysis with over 50 per cent of the cells Gram-negative. Within 24 hours there was over 60 per cent lysis with 100 per cent of the cells Gram-negative. Electron microscopy revealed the rapid, diffuse form of disintegration, complete within 5 to 6 days, that occurred in non-extracted cells treated with low concentrations of CTAB.

In 0.01 per cent CTAB the rate of lysis, change of Gram reaction or morphological disintegration of the extracted cells could not be further increased over that in distilled water. Indeed, the CTAB actually depressed the rate of lysis.

Table VII

Rate of lysis of alcohol-ether extracted cells of *Staph. aureus* in distilled water and in CTAB

| Time (hrs.) | Percentage lysis in | |
|---|---|---|
| | distilled water | 0·01 per cent CTAB |
| 0 | 0<br>(0) | 0<br>(0) |
| 1 | 30<br>(55) | 15<br>(50) |
| 4 | 45<br>(80) | 30<br>(65) |
| 24 | 65<br>(100) | 60<br>(100) |

The upper figure is lysis expressed as percentage clearing of original suspension opacity. The figure in brackets is the percentage of Gram-negative cells.

## C. BACTERIAL CELL-WALL STRUCTURE

The cell-wall of Staph. aureus is most readily obtained in a clean, practically intact condition and has, therefore, been used, almost exclusively, in the following investigations. The staphylococcal cell-wall, when examined in the electron microscope, shows an apparently smooth, structureless surface (fig.31) apart from the presence in certain cells of various, equatorial, ridge and band-like structures (these are considered in the next section). It was decided to subject the cell-wall to various forms of treatment in an attempt to cause a gradual degradation of some of its structural components and possibly in this way to reveal the details of its intimate, morphological structure.

The methods employed included enzymic attack, alkaline and acid hydrolysis and lipoid extraction.

### Treatment with enzymes

Recent evidence in the literature has suggested a protein basis for the cell-wall structure of Staph. aureus and, therefore, the effect on the membrane of proteolytic enzymes was tested.

The cell-walls were treated with crystalline preparations of pepsin and trypsin (Armour); concentrations as high as 0.5 per cent in N/10 hydrochloric acid and N/10 sodium carbonate respectively were used. However, even after incubation at 37°C. for up to 7 days in the case of the pepsin and 28 days in the case of the trypsin, no evidence of digestion could be obtained. Treatment with pepsin followed by trypsin also had no obvious effect.

Nucleases. The possibility that the cell-wall may contain

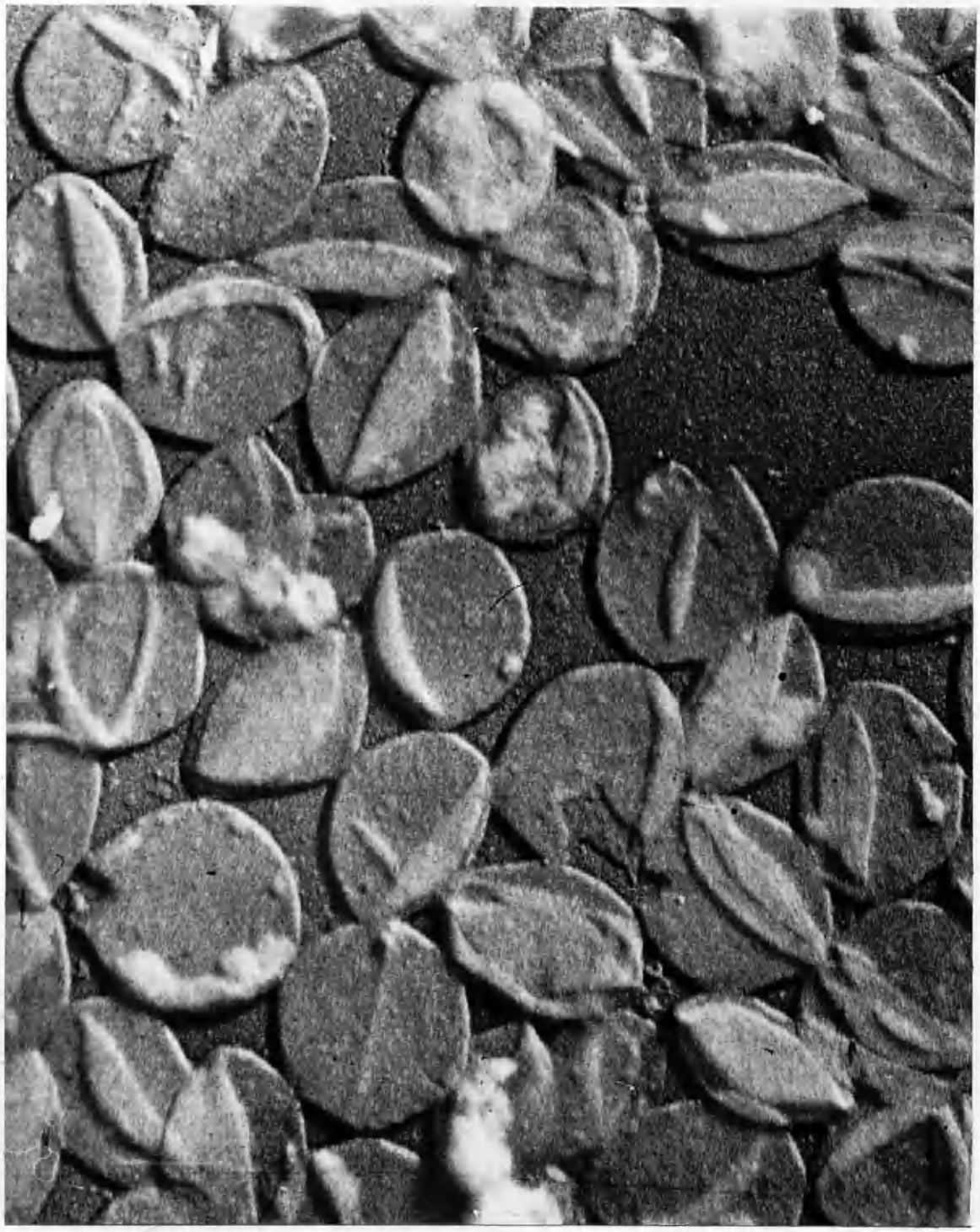


Fig. 31. Isolated cell-walls of Staph. aureus. Untreated controls. ( × 20,000).

nucleic acid in quantities sufficient to allow its loss, following treatment with nucleases, to be detected by electron microscopy was investigated.

A well-washed batch of Staph. aureus was shaken, in the usual manner, with glass beads and then centrifuged at 10,000 r.p.m. for half-an-hour to separate the cell-walls from the cytoplasmic content (a preliminary spinning at a lower speed to remove beads and still-intact cells was first carried out). The supernatant and the washed cell-walls were each split into two equal fractions, one of which was submitted to Professor J. N. Davidson of the Biochemistry Department who very kindly carried out nucleic acid estimations; this was done chemically interms of organic, non-lipoid phosphorus. The second half of each fraction was dried and weighed. Table VIII shows the results of three such estimations. The different proportions of the two nucleic acid components in the cell-wall and cytoplasmic fractions would appear to indicate that the ribonucleic acid content of the cell-wall fraction is not derived from the cytoplasm of the cell. The low content of desoxyribonucleic acid in the cell-wall further indicates that the cytoplasmic contamination of the cell-wall preparation was low. It appears that the cell-walls contain from 10 to 15 per cent of ribonucleic acid.

The cell-walls were treated with crystalline preparations of ribonuclease or desoxyribonuclease prepared respectively by Kunitz's and McCarty's method and supplied by Professor Davidson. Concentrations up to 0.1 per cent were used in veronal-acetate buffer of pH 6.0 for ribonuclease and pH 6.8 for desoxyribonuclease; a trace of magnesium sulphate was also added to the solutions. The suspensions were incubated at 37°C.

Even after 48 hours' incubation the membranes were

Table VIII

Nucleic acid content of separated cell-walls and cytoplasm of Staph. aureus

| | Experiment | | |
|---|---|---|---|
| | 1 | 2 | 3 |
| Cell-walls | | | |
| RNA | 483µg. | 409µg. | 212µg. |
| DNA | 18µg. | 9µg. | |
| Cytoplasm | | | |
| RNA | 1021µg. | 1184µg. | |
| DNA | 387µg. | 341µg. | |
| Cell-walls dry-weight | 2.5mg. | 3.2mg. | 1.4mg. |

The content of RNA and DNA was determined chemically in terms of organic, non-lipoid phosphorus.

apparently completely unaffected by either of the enzymes when examined in the electron microscope.

Staphylococcal autolysate. The cell-wall of Staph. aureus, when suspended in the cellular autolysate, readily undergoes disintegration (as described in Section A). However, the method of disintegration is fragmentary and irregular and yields no evidence as to the intimate structural details of the membrane. If the autolysate contains a low concentration of CTAB the disintegration becomes not only more rapid but also involves the cell-wall in a regular and diffuse manner. At an intermediate stage of this disintegration structural details become visible; a close network of fibres can be observed. Figs. 32 to 35 illustrate such intermediate stages. The fibres are fairly regular in diameter, about 50 to 150 Å, but of no readily discernible standard length. The fibres eventually disintegrate into fine granules.

## Alkaline hydrolysis

The cell-walls were suspended, to give distinctly cloudy suspensions, in 50 ml. amounts of sodium hydroxide of concentrations varying from 0.25 to 10 per cent (N/16 to 2.5N). The suspensions were boiled, under reflux condensers, and specimens removed at 15-minute intervals. They were washed three times with distilled water and examined in the electron microscope. Suspensions were also kept at room temperature for 12 hours and then similarly washed and examined.

The suspensions boiled with the higher concentrations of sodium hydroxide cleared within as little as 15 minutes and a scanty, somewhat fibrinous-looking deposit appeared. This deposit, when examined, was found to consist of agglutinated cell-walls in the final stages of disintegration. The supernatant yielded nothing of value when examined in the

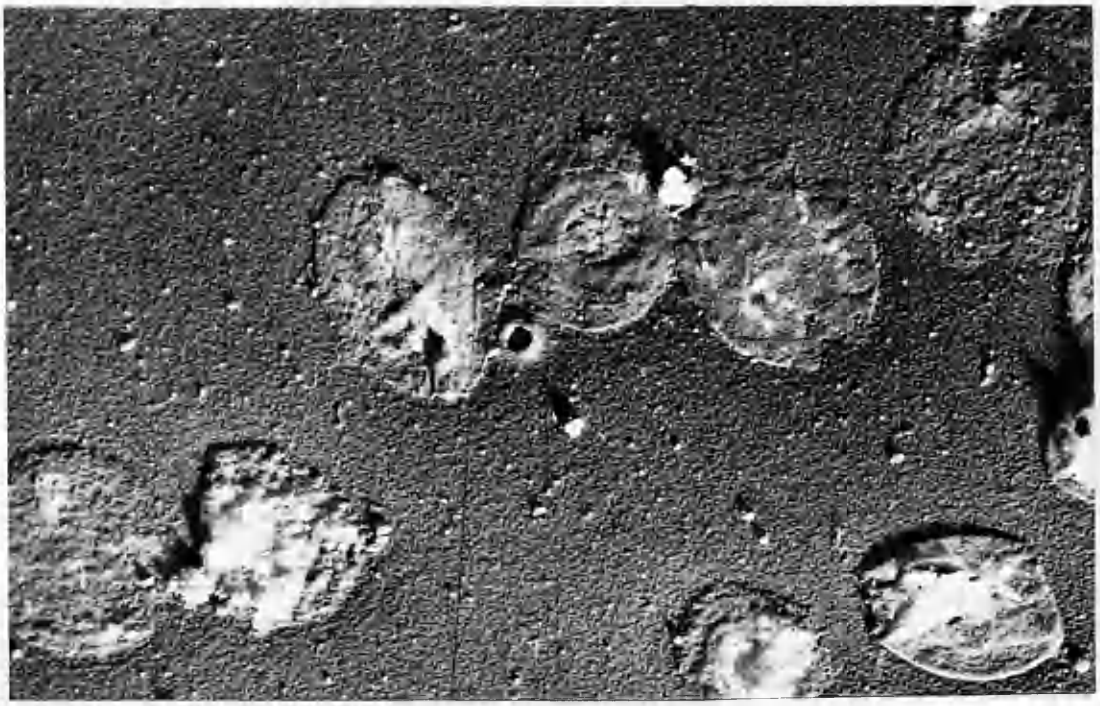


Fig. 32. Cell-walls of Staph. aureus treated 0·03 per cent C.T.A.B. Early stage of disintegration. ( × 20,000).

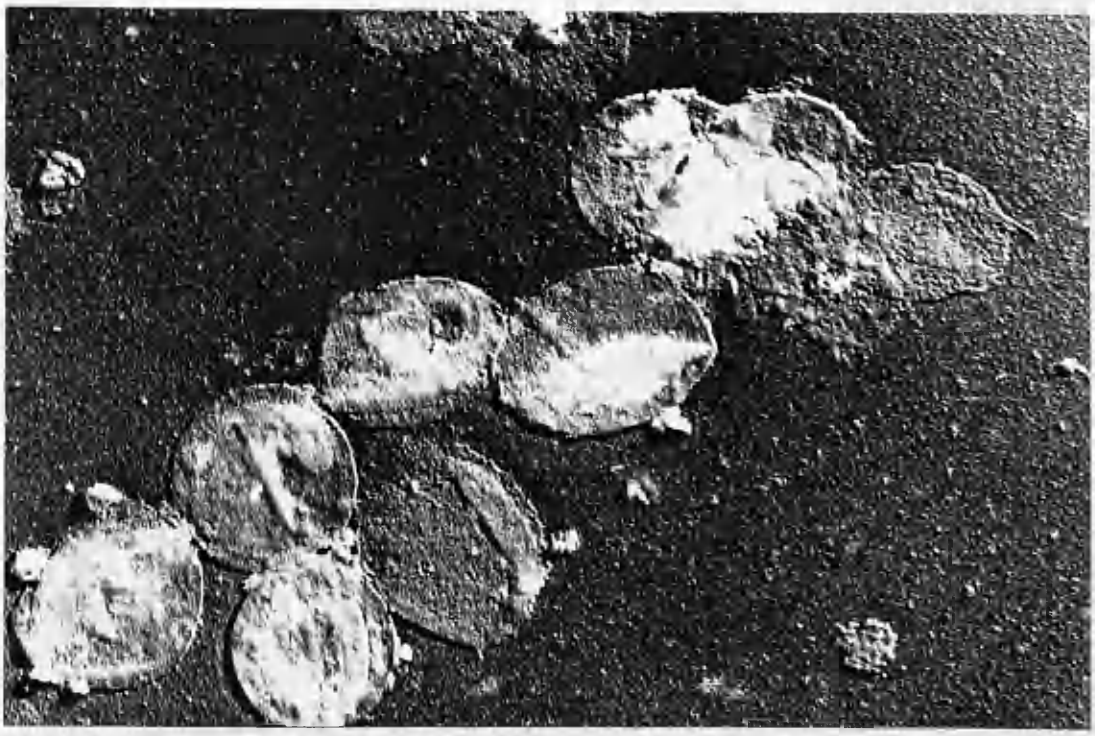


Fig. 33. Cell-walls of Staph. aureus treated with 0·03 per cent C.T.A.B. showing intermediate stage of fibrous appearance. ( × 20,000).

Fig. 34. Cell-walls of Staph. aureus treated with 0.03 per cent C.T.A.B. Fibrous appearance is marked. ( × 20,000).

Fig. 35. Cell-walls of Staph. aureus treated with 0.03 per cent C.T.A.B. Well-marked fibrous appearance. ( × 20,000).

microscope. With 0.5 per cent sodium hydroxide the suspension cleared more slowly and did not become transparent until after about 2 hours. The 15-minute specimen showed distinct thinning of the cell-walls but no evidence of break-up. After half-an-hour irregular loss of material from the membranes had occurred as illustrated in fig. 36. The appearances are somewhat similar to those resulting from treatment with low concentrations of CTAB but the presence of distinct fibres cannot be conclusively recognised. After 1 hours' treatment only debris remained.

At room temperature the lowest concentrations of sodium hydroxide had no obvious effect. With 3 per cent the majority of the cell-walls were, after 12 hours, in various stages of thinning, including the apparently final stage in which there remained what appeared to be a single layer of fine granules. Although the membrane retained its original shape until the final stage no intermediate stage such as that obtained with the boiling alkali was detected. With higher concentrations the suspension cleared more rapidly but no further information was obtained.

## Acid hydrolysis

The cell-walls were treated with concentrations of hydrochloric acid ranging from 0.25 to 10 per cent (N/15 to 2.8N) in a similar manner to the sodium hydroxide. Boiling with the acid caused a much slower loss of suspension opacity than did the alkali; with 2 per cent hydrochloric acid clearing was complete within about 3 hours. Examination at intervals showed that the membranes underwent a progressive thinning to a finely granular stage (fig.37). An intermediate stage in the disintegrative process such as that found with the sodium hydroxide was not detected. Fragmentation of the cell-wall

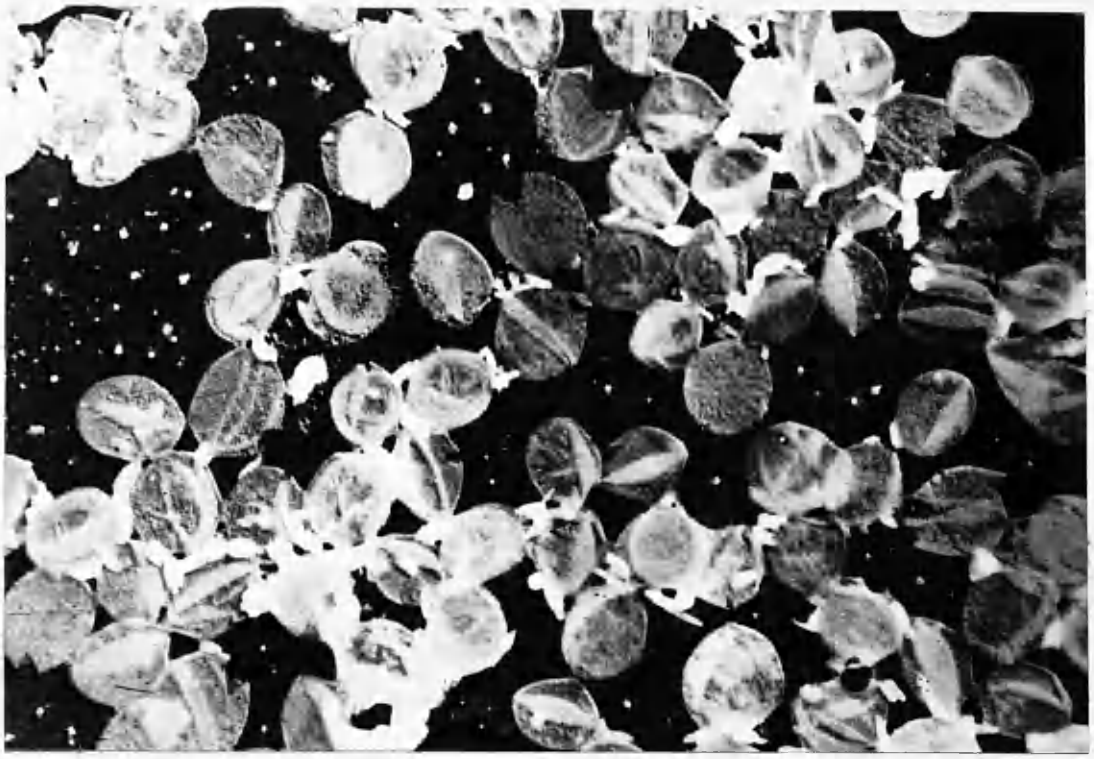


Fig. 36. Cell-walls of Staph. aureus treated with 0·5 per cent sodium hydroxide at 100°C. for half-an-hour. ( × 10,000).

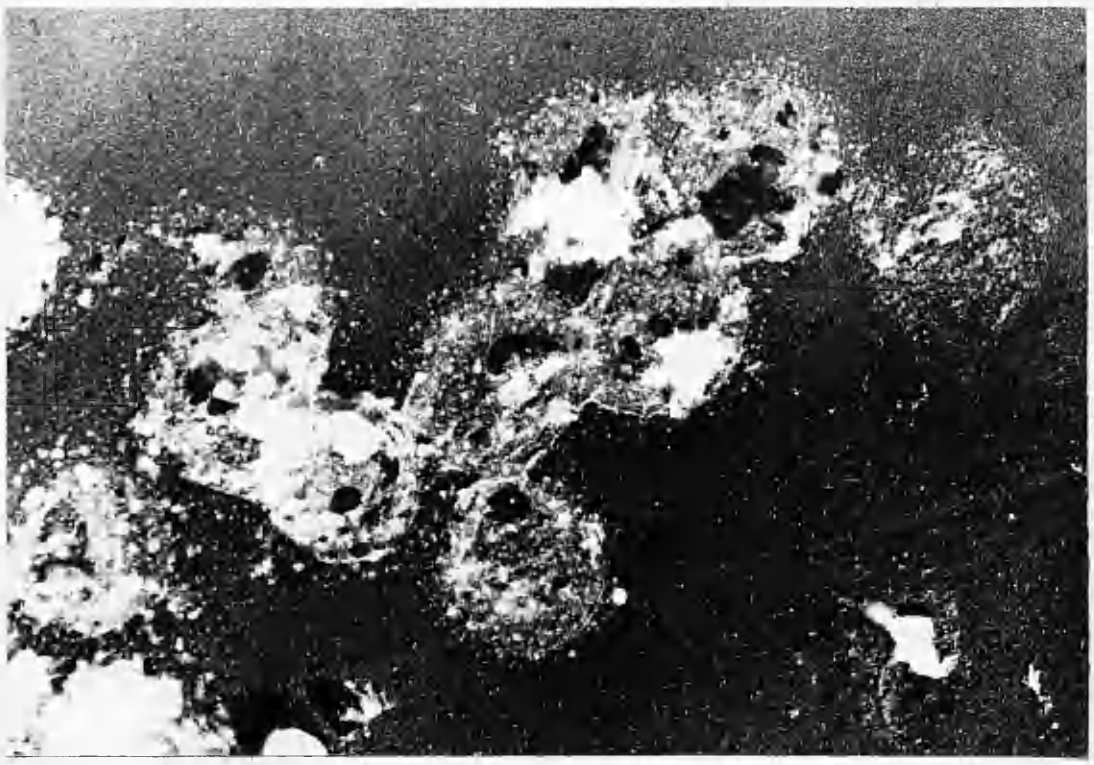


Fig. 37. Cell-walls of Staph. aureus treated with 2 per cent hydrochloric acid at 100°C. for 2 hours. ( × 20,000).

did not occur and the shape of the cell-wall was retained till the final disappearance. At room temperature the cell-walls were apparently not attacked, at least within 12 hours.

## Lipoid extraction

The cell-walls were suspended in an equal parts alcohol-ether mixture and also in a similar mixture but containing 1 per cent hydrochloric acid. The suspensions were boiled under reflux and examined at intervals. A cell-wall suspension in alcohol-ether was also kept at -25°C. by means of a solid carbon dioxide-acetone freezing mixture for 6 hours and then examined, after washing, in the microscope.

The cell-walls which were boiled with alcohol-ether remained intact even after 6 hours. With alcohol-ether-acid clearing of the suspension occurred within about 2 hours. The cell-walls underwent a process of disintegration apparently identical with that which occurred with hydrochloric acid alone.

Alcohol-ether at low temperature had no obvious effect on the cell-walls, even after 6 hours.

## D. THE BACTERIAL CELL-WALL IN CELL DIVISION

The part played by the bacterial cell-wall in cell division has, until recently, been studied almost entirely with the light microscope by means of various staining techniques. Since the cell-wall and the septa arising from it are at the lower limits of resolution of the light microscope the results obtained by different workers have not been wholly convincing and have led to controversy. For example, there has been some recent controversy based on photographs of stained Bacillus megatherium in which certain appearances have been alternatively interpreted as either mitotic figures (DeLamater and Mudd, 1951) or as cell-wall transverse septa (Bissett, 1953). A detailed examination with the electron microscope of large numbers of cell-walls, isolated from cells of actively growing cultures, was, therefore, undertaken in an attempt to elucidate the role played by the cell-wall during the course of cell division. The organisms investigated were Staph. aureus, Strept. faecalis and B. subtilis. These organisms were grown, unless otherwise stated, for 16 to 18 hours in digest broth and the cell-walls isolated by the method already described.

The observations on the cell-walls of these organisms show that the structural changes in them tend to follow the same general pattern although the details of these changes have been easier to observe in Strept. faecalis. Fig. 38 shows a typical preparation of isolated cell-walls of Strept. faecalis; while some of the cells have a smooth surface others show the presence of equatorial ridge and band-like structures. The following investigations indicate that these structures are not due to random folding in a spherical membrane which has collapsed flat on the supporting film but that they are the result of various stages of cell division.

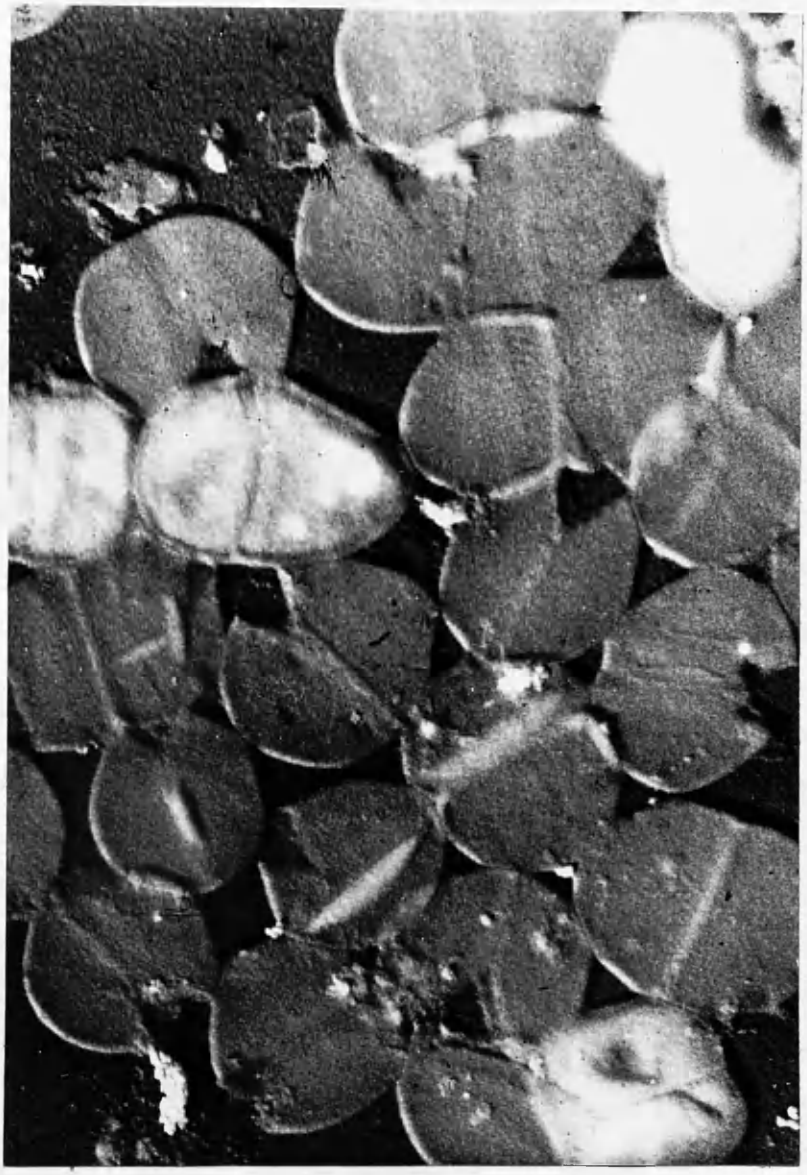


Fig. 38. Isolated cell-walls of Strept. faecalis. ( × 20,000).

Staph. aureus. Figs. 39 to 44 illustrate the various types of these structures in the isolated cell-walls of Staph. aureus. In fig. 39 the cell-wall is quite smooth apart from the presence of a small fold in the membrane. In fig. 40 there is a single, slender ridge of thickening across the diameter, which in fig. 41 is distinctly double. In fig. 42 the two ridges are quite separate from each other. Instead of a ridge the cell-wall surface in fig. 43 possesses a wide, band-like area of thickening. In fig. 44 the band lies between the points of constriction of a dividing cell.

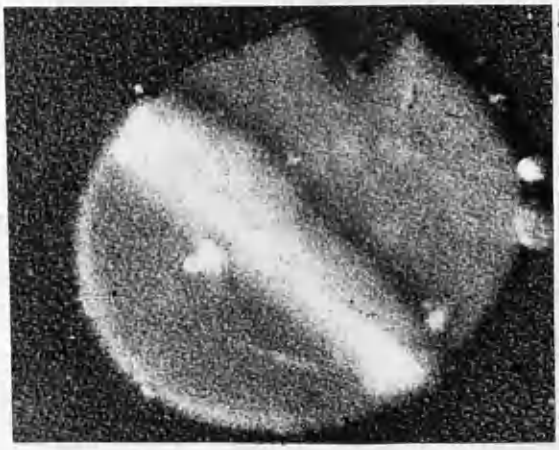
A careful study of undisrupted staphylococci, both by examination of intact cells in the electron microscope (fig. 45) and by the use of surface-replica techniques, failed to reveal the presence of similar structures on the surfaces of the intact organisms. However, if the intact cells are treated with the appropriate concentration of CTAB (cell to CTAB weight ratio of 28 to 1) they assume the appearances shown in fig. 46; the cells show cytoplasmic retraction and many of them, marked X in the micrograph, can now be seen to consist of two hemispherical sections. In some cells this is indicated only by a depression running across the equator of the cell but in others there has occurred a partial separation of the two halves of the cell; the arrow in the micrograph depicts a cell in which the two halves of the cell have almost completely separated exposing the flat inner surface of one of the halves. Fig. 47 shows similarly treated cells at a higher magnification and it can be seen that opposite the line of cytoplasmic division there is present on the flattened rim of cell-wall a distinct, raised ridge. These appearances are almost certainly due to the presence of a septum stretching across the centre of the intact cell. The septum, which is formed at an early stage of cell division, is not readily seen in normal, intact cells: it

39

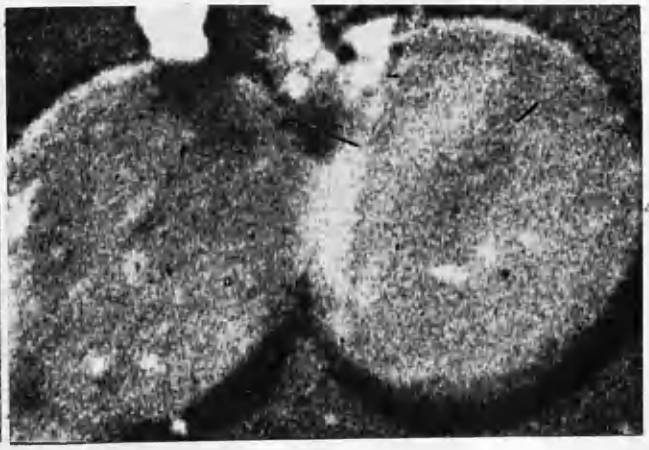
40

41

42



43



44

Figs. 39 to 44. Cell-walls of Staph. aureus showing the successive stages of transverse cell-wall septum formation during cell division. ( × 45,000).

Fig. 45. Intact cells of Staph. aureus. The cell surface shows no obvious evidence of structure. ( x 10,000).

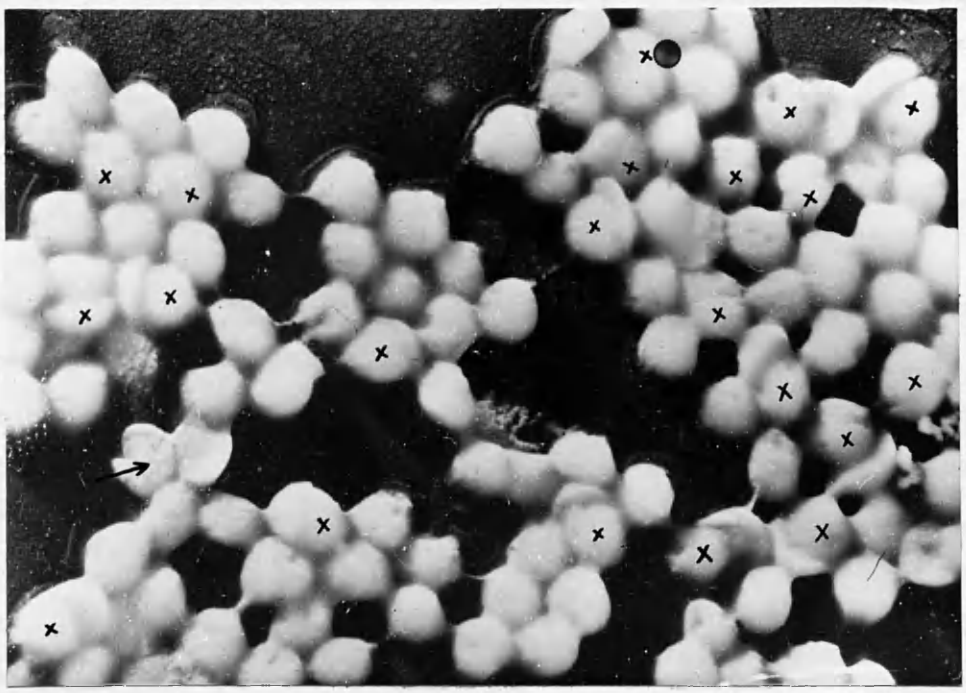


Fig. 46. Intact cells of Staph. aureus treated with C.T.A.B. The letter X indicates the cells which appear to consist of two sections. The arrow depicts a cell in which the two halves of the cell have almost completely separated. ( x 10,000).

becomes visible following treatment with CTAB which brings about firstly, a retraction of the cytoplasm away from the cell-wall and from the septum and secondly, a loss of the normal rigidity of the cell-wall thus allowing the varying degrees of separation of the two cytoplasmic sections of the cell to occur. In order to confirm that the appearances observed in the whole cells, treated with CTAB, and also the equatorial structures observed in the isolated cell-walls were related to cell division, counts of the proportions of cells and cell-walls showing these appearances in cultures at various stages of growth were carried out (table IX). This table shows that the younger and more actively growing a culture the higher the proportion of cells and cell-walls showing the appearances described above. In a 6 hours' culture as many as 50 to 60 per cent of the cells, after treatment with CTAB, showed the double structure of the cytoplasmic body and an even higher proportion of the isolated cell-walls the various forms of equatorial differentiation; after 48 hours as few as 10 per cent of cells and cell-walls showed these appearances.

An attempt can now be made to reconstruct the stages in the formation of the cell-wall septum, using the structural appearances on the isolated, collapsed cell-walls. In fig. 39 the septum has not yet commenced to form. The first sign of its formation is the presence of a ridge of thickening on the cell-wall (fig.40). As the ridge, which, of course, encircles the originally spherical cell-wall, becomes more developed, its part on the lower layer of the collapsed cell-wall will show through the upper layer (figs.41 and 42); the degree of separation of the two ridges depends on the angle of the line of septum formation to the plane of the film at the moment of collapse of the originally spherical cell-wall membrane. As the septum grows inwards to form a membrane the collapse of the septum

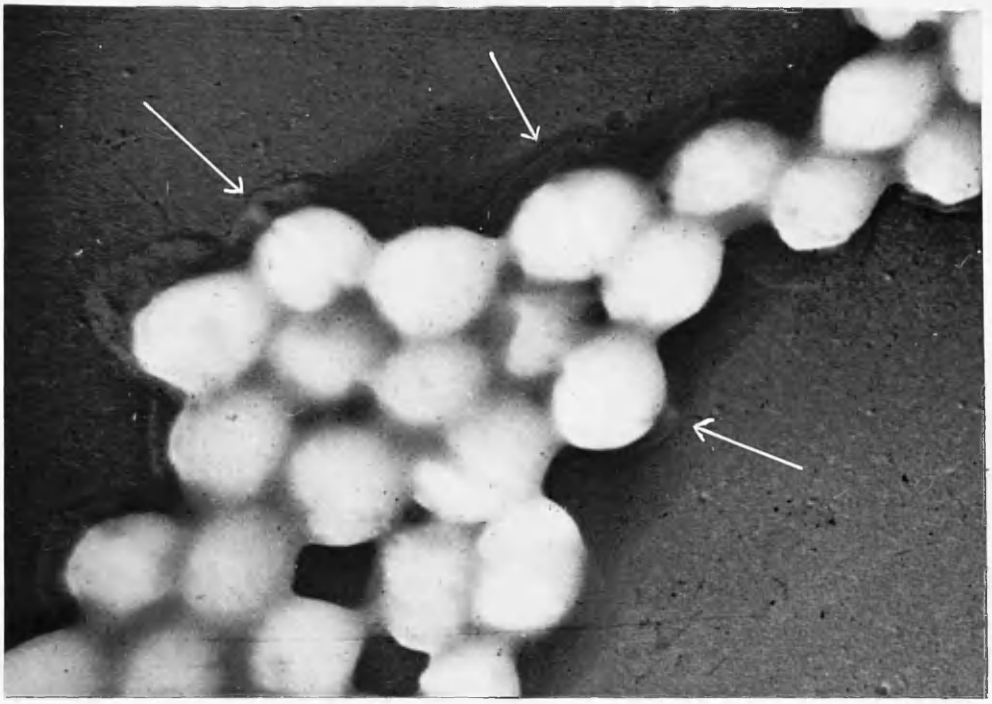


Fig. 47. Intact cells of Staph. aureus treated with C.T.A.B. The arrows indicate cell-wall ridging opposite the cytoplasmic constrictions. ( x 18,000).

Fig. 48. Cell-wall of Staph. aureus showing the growing edge of the cell-wall septum in a horizontal plane. ( x 45,000).

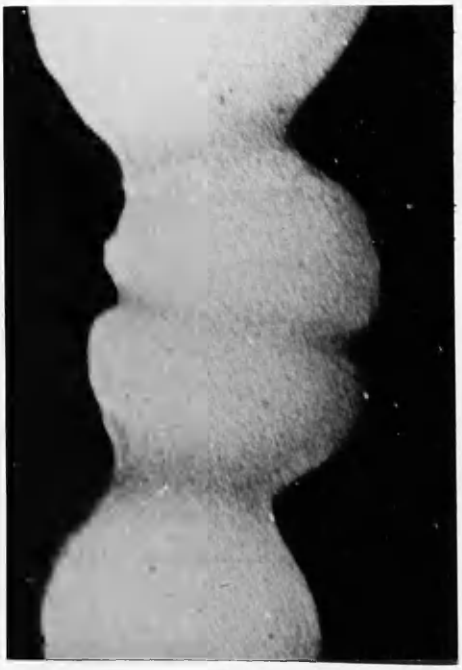


Fig. 49. Dividing cells of Staph. aureus, untreated. ( x 15,000).

Table IX

Variation in proportions of whole cells showing cytoplasmic splitting after treatment with CTAB and of isolated cell-walls showing equatorial structure with age of culture

| Time (hrs.) | Whole cells | | | Isolated cell-walls | | |
|---|---|---|---|---|---|---|
| | Intact cytoplasm | Split cytoplasm | Ratio | Without structure | With structure | Ratio |
| 6 | 156 | 144 | c.1:1 | 184 | 116 | c.1·5:1 |
| 12 | 128 | 172 | c.1:1·5 | 140 | 160 | c.1:1 |
| 18 | 80 | 220 | c.1:3 | 108 | 192 | c.1:2 |
| 24 | 51 | 249 | c.1:5 | 92 | 208 | c.1:2 |
| 48 | 36 | 264 | c.1:8 | 56 | 244 | c.1:5 |

A total of 300 cells or cell-walls was counted in each instance.

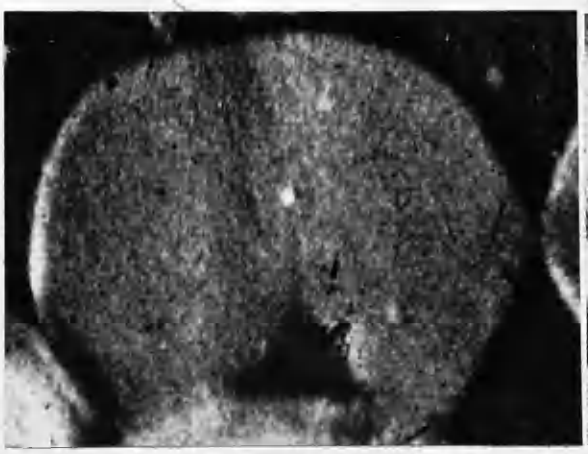
within the collapsed cell-wall will give rise to the band-like appearance shown in fig. 43. After septum formation is complete the cell constricts through the line of the septum to become two daughter cells. Fig. 48 shows a cell-wall which has collapsed with the plane of the septum in the horizontal position. The growing edge of the septum can be seen as a concentric ridge within the periphery. The final stage of constriction is the earliest visible stage in the electron microscope of cell division in whole, untreated cells (fig.49).

Strept. faecalis. In this organism the process of division is basically similar to that of the staphylococcus but the early structural changes in the cell-wall are more pronounced.

The first evidence of the process of division in the cell-wall is the appearance of parallel striations across the centre of the membrane (fig.50). These seem to coalesce to form a band of diminished density to electron penetration (figs.51 and 52). In the centre of this band there forms a slender line of increased density (fig.53). This line becomes progressively more prominent to form a distinct ridge on the surface of the collapsed cell-wall (figs.54 and 55). Fig. 56 shows the double-ridge appearance due to the ridge on the under surface showing through the upper. The inward growth of the septum then results in the band-like structure of fig. 57. Fig. 58 suggests that the septum has now split to form a double septum with collapse of the two layers on either side of the mid-line. Finally, the cell-wall constricts through the line of the septum (fig.59). Figs. 60 to 62 illustrate the formation of septa in the two daughter cells before the latter have themselves completely separated. Mostly, septum formation gives rise to two or four daughter cells (fig. 63), but occasional exceptions are found in which three daughter

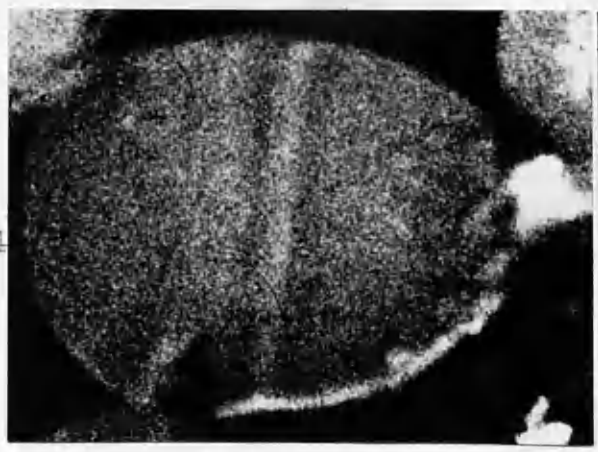
50

51

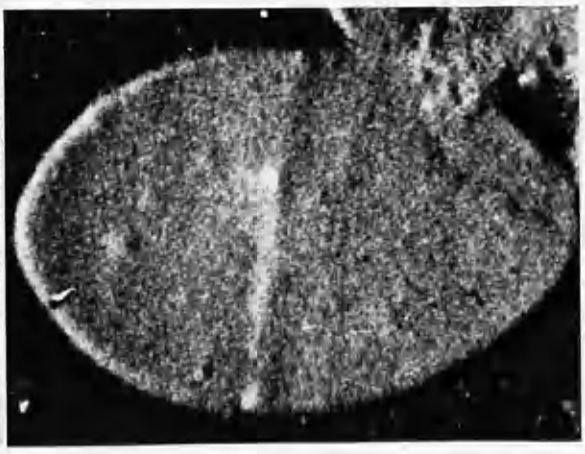


52

53

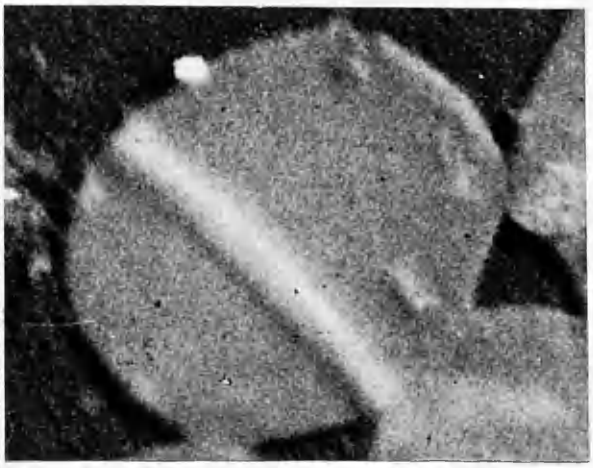


54

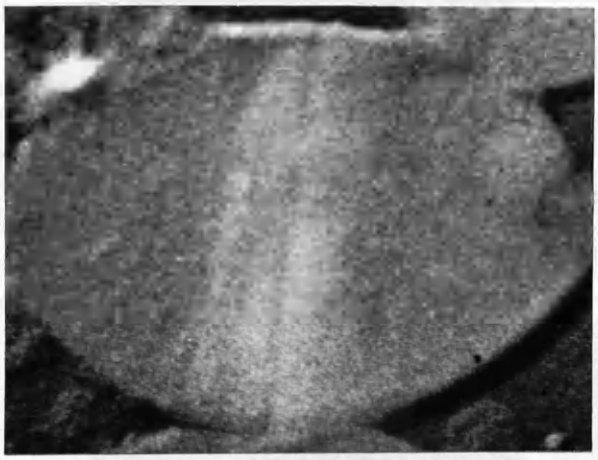


55

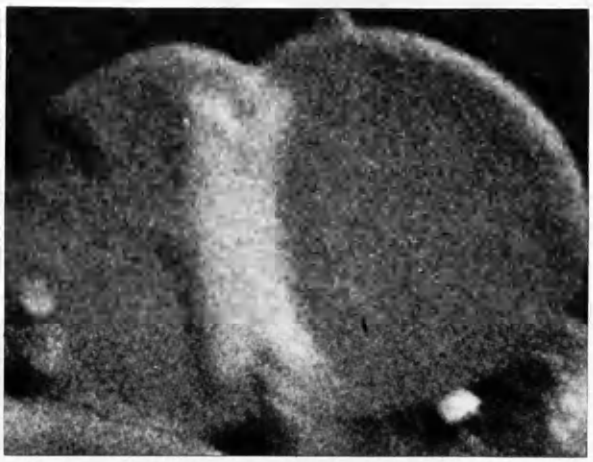
56



57



58



59

Figs. 50 to 59. Cell-walls of Strept. faecalis showing the successive stages of transverse cell-wall septum formation during cell division. ( x 45,000).

Fig. 60. Cell-wall of Strept. faecalis. The daughter cells show commencing septum formation before their own separation. ( x 30,000).

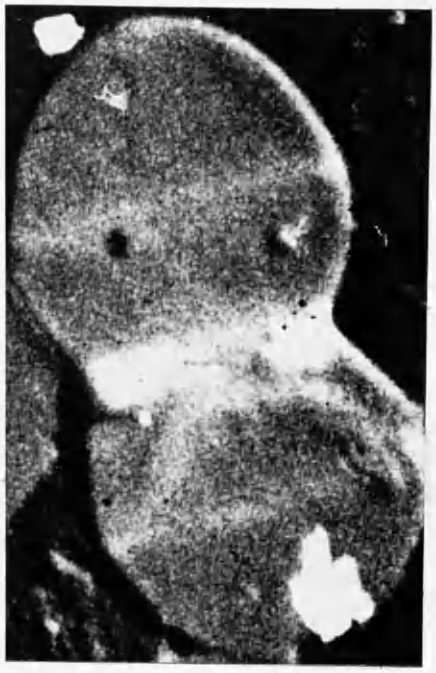


Fig. 61. Later stage of fig. 60. ( x 30,000).

Fig. 62. Still later stage of fig. 60. ( x 30,000).

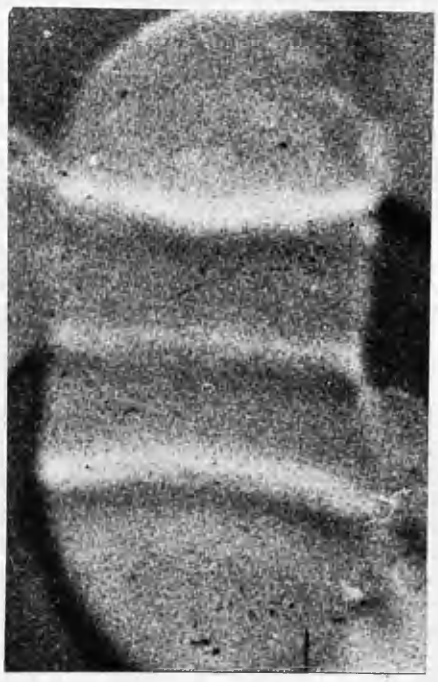


Fig. 63. Usual method of division of Strept. faecalis into 2 or 4 units. ( x 30,000).

cells are formed as in fig. 64.

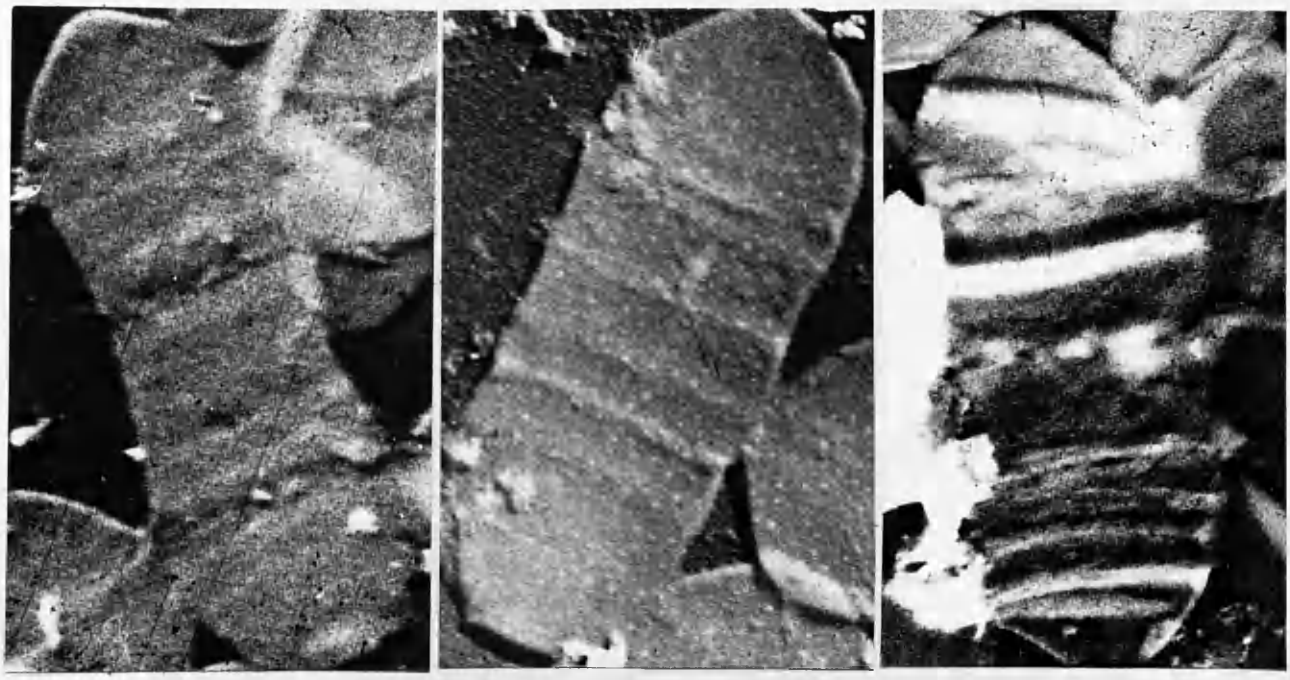
Thus, the ususal method of division in Strept. faecalis is the formation of a transverse cell-wall septum followed by constriction of the cell-wall through the line of the septum. However, there are interesting but relatively uncommon exceptions. Fig. 65 shows cell division by simple constriction without preliminary septum formation. On the other hand, figs. 66 to 68 illustrate the successive stages of septum formation but with failure of the final process of constriction. As a result the cell elongates to form a bacillus with an extraordinary large number of close-packed, completed septa. Similar irregularities were not found in Staph. aureus.

B. subtilis. This organism appears to undergo basically the same cell-wall changes as the above two organisms. Because of its cylindrical shape the cell-wall, on collapsing flat, can rotate only in the direction of its smaller diameter and thus the primary ridge rarely shows the double appearance so common with spherical organisms. Fig. 69, however, does show such an example of collapse which has allowed of separation of the ridges on the upper and lower layers of the cell-wall. Fig. 70 demonstrates a short chain of cell-walls in various stages of division. When the septum has completed its formation, appearing as a wide, dense band across the collapsed cell-wall, an inward constriction occurs and finally separates the daughter cells. With this organism the constricting process tends to lag considerably behind the completion of septum formation, thus allowing chain formation. The septum, itself, seems to split at a relatively early stage, possibly commencing even before completion of inward septal growth; fig. 16 shows a cell-wall in which a rupture has readily split the septal ridge into two layers.

The cytoplasmic membrane. The great majority of workers seem

Fig. 64. Unusual division of Strept. faecalis into 3 units. ( x 30,000).

Fig. 65. Cell-wall of Strept. faecalis showing division by constriction without preceding septum formation. ( x 30,000).



Figs. 66 to 68. Cell-walls of Strept. faecalis showing multiple cell-wall septum formation. The final dividing stage of constriction appears to have failed. ( x 25,000).

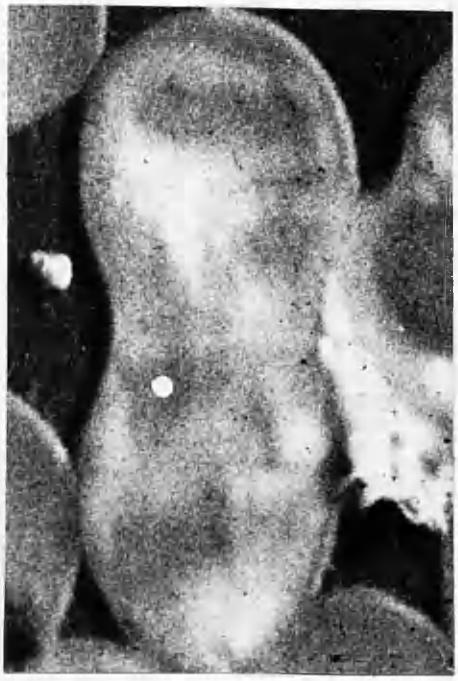
Fig. 69. Cell-walls of B. subtilis showing ridge and band formations. ( x 22,000).

Fig. 70. Chain of cell-walls of B. subtilis with cell-wall septa. ( x 10,000).

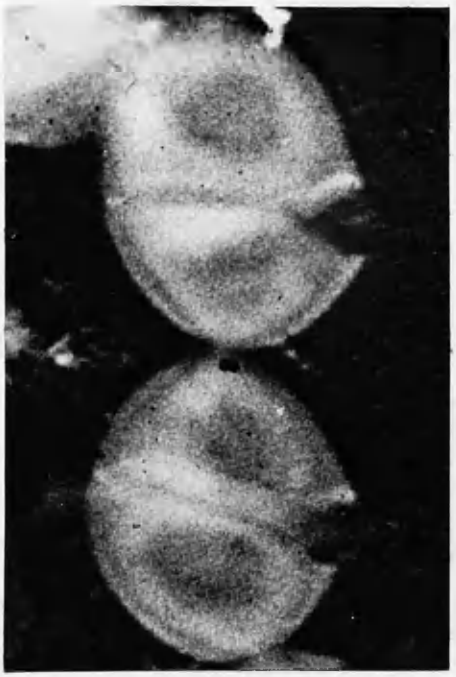
to be agreed that the primary stage of cell division is the formation of a transverse septum derived from the cytoplasmic membrane. Examination of many thousands of whole cells, both before and during lysis, and of isolated cell-walls has failed to reveal conclusive evidence of a cytoplasmic membrane in either Staph. aureus or B. subtilis. In the case of Strept. faecalis, however, appearances highly suggestive of such a membrane have been obtained (figs.71 to 74) in as many as 5 to 10 per cent of the isolated cell-walls. The internal membranes in these micrographs often show transverse septum formation. These septa have invariably been found to be double in structure and between the two layers of which the cell-wall transverse septum grows. Whether these septa are formed initially as double layers or as a single layer which later splits it has not yet proved possible to determine.

71

72



73



74

Figs. 71 to 74. Cell-walls of Strept. faecalis with cytoplasmic membranes within them. Transverse septa originating from the cytoplasmic membrane are obvious. ( fig. 71 x 45,000; figs. 72 to 74 x 25,000).

## DISCUSSION

Since bacterial cells or cell-walls, which have been autoclaved in order to destroy the enzymes contained in them, do not disintegrate unless fresh autolysate is added it must be accepted that whatever the primary cause of bacterial death the actual morphological disintegration is due to the action of the cellular autolytic enzymes. These various enzymes, although readily capable of digesting the substrate of a cell killed by autoclaving, are apparently inactive when present within the living cell. Dubos (1937b,1945) has suggested that in the living cell the structural components are separated from cellular enzymic activity by vital, lipoprotein complexes or membranes. The disruption of these protective complexes after death would be expected to be a fairly slow and irregular process throughout the cell. This appears to be supported by the electron micrographs showing the slow, fragmentary disintegration of Staph. aureus in distilled water. This slow rate of disintegration of individual cells together with the relatively slow death rate of cells in distilled water accounts for the slow rate of lysis as estimated by suspension opacity. The isolated cell-walls of Staph. aureus show a correspondingly slow and irregular form of disintegration when exposed to the action of autolysate thus indicating that the processes involved in the initiation and course of autolysis of the isolated cell-walls are probably similar to those of the whole cell. The extremely slow and incomplete lysis of the cell-walls in distilled water is presumably due to the low content of autolytic enzymes within the washed membranes.

Bacillus subtilis shows in distilled water both a higher death rate and a very much faster rate of disintegration of individual cells than Staph. aureus. The higher death rate

is not due to greater sensitivity to osmotic effects since the substitution of isotonic buffer solutions for distilled water makes little difference to the rate of autolysis. B. subtilis is possibly more sensitive to the absence of nutrient material or has a shorter life-span than Staph. aureus. This problem, however, requires detailed investigations of viability and relative rates of metabolism under various environmental conditions. The very rapid rate of autolysis of the cells can be, at least partly, explained by the early rupture of the cell-wall with evacuation of the cytoplasm which occurs in the majority of the cells; in the staphylococcus cytoplasmic digestion is completed within the cell-wall which remains intact until a relatively late stage. The early rupture of the cell-wall in B. subtilis is apparently not an osmotic phenomenon and the most likely explanation is that following early death of the cell with activation of autolytic enzymes there occurs early disorganisation and weakening of the molecular structure of the cell-wall; the mechanical strains in the weakened, cylindrical membrane of B. subtilis would be much greater than those in the correspondingly weakened, spherical cell-wall of Staph. aureus and a gross rupture of the cell-wall would thus be more likely to occur. This early molecular disorganisation is soon followed by a remarkably uniform and regular disintegration of the cell-wall. The entire process of autolysis differs strikingly from the slow, irregular fragmentation of Staph. aureus.

The marked differences in the rates of autolysis of these two organisms could also be explained, on the basis of Dubos's theory, by the relative stabilities or quantities of protective lipoid complexes. If these anti-autolytic barriers are very unstable or few in number, their disruption after death of the cell would allow of a more rapid and uniform action

of the autolytic enzymes throughout the cell. The necessary confirmative chemical data are not available in the literature although the early work of Jobling and Petersen (1914) indicated that B. subtilis possessed very much less unsaturated (? bound) lipoid than Staph. aureus and these workers were able to relate this difference to the relative susceptibilities of the two organisms to proteolytic digestion. A more complete understanding of the factors controlling the relative rates of autolysis of different organisms must await accurate and complete determinations of chemical structure and of the localisation within the cell of the various structural and enzymatic units which compose the bacterial cell.

## The action on the cell of CTAB

The concentration of CTAB which produces maximum lysis of Staph. aureus (cell to CTAB weight ratio of about 28 to 1) causes death of 100 per cent of the organisms within 1 hour. This rapid death rate with the simultaneous onset of autolytic disintegration in practically all the cells in the suspension would, in itself, account for the marked stimulation of lysis, as estimated by decrease in suspension opacity, over that in distilled water. However, electron microscopy shows that the disintegration of the individual cell follows a different pattern from that in distilled water; it takes the form of a very rapid, uniform, granular disintegration. This finding cannot be accounted for simply by the more rapid rate of lysis with rapid increase in concentration of extracellular autolytic enzyme, since the rate of lysis can be markedly accelerated in distilled water by increasing the cell concentration (table II) without any electron microscope evidence of alteration in the manner of disintegration of individual cells. Nor can it be satisfactorily accounted for by the current theories that CTAB

acts only by disorganising a cytoplasmic surface membrane or by inactivating essential metabolic enzymes. Both the cytoplasmic membrane and the metabolic enzymes are considered to lie inside the cell-wall in the intact cell; if these are the only points of attack of CTAB in the cell then the rate and manner of disintegration of the isolated cell-wall in distilled water should not differ from that in low concentrations of CTAB. In fact the differences are pronounced. It is evident that CTAB not only kills the organism but must affect the structural components of both the cytoplasm and the cell-wall in such a way as to account for the characteristically rapid and diffuse manner of disintegration. However, the experiments with autoclaved cells and cell-walls have shown that CTAB is, in itself, incapable of disintegrating the cell or cell-wall. It must, therefore, be inferred that the CTAB brings about an alteration in the molecular structure of the cell or cell-wall which enables the autolytic enzymes to act not only more rapidly but also more uniformly throughout the cell. The action of the CTAB must yet be sufficiently gentle to leave intact the autolytic enzymes themselves; that this is possible was demonstrated by Hotchkiss (1946) who found that the concentrations of detergents which are bactericidal may be well below the concentrations necessary for protein denaturation. On the other hand, detergents are extremely lipophilic and have been shown to be capable of splitting lipoprotein complexes (Macheboeuf and Tayeau,1938; Tayeau,1939) which Dubos has suggested protect the cell from the action of its autolytic enzymes. The hypothesis is, therefore, forwarded that CTAB acts by splitting bacterial lipoid complexes, probably lipoproteins, rapidly and uniformly throughout the entire cell and thus allows the autolytic enzymes to bring about a rapid, diffuse disintegration. Since the cell-wall is equally involved with the cytoplasm in

the stimulating action of CTAB the cell-wall would also be expected to possess lipoprotein complexes in its structure; chemical analysis of the isolated cell-wall of Staph. aureus (Mitchell and Moyle,1951) has shown it to contain about 60 per cent protein and over 7 per cent lipoid of which more than half is bound lipoid, extractable only after hydrolysis.

This hypothesis is supported by the alcohol-ether extraction experiment. These liposolvents, at low temperatures, are also capable of splitting lipoproteins without protein denaturation (Chargaff and Bendich,1944; Neurath et al.,1944). The extracted cells disintegrate in distilled water in an apparently identical manner to non-extracted cells treated with low concentrations of CTAB. Also, the rate and manner of disintegration cannot now be further stimulated by low concentrations of CTAB. This suggests that the alcohol-ether and the CTAB have a common site of action in the cell.

The destruction by CTAB of a lipoprotein cytoplasmic membrane (Baker, Harrison and Miller,1941; Hotchkiss,1946) would, according to the above hypothesis, be merely a part of a more general process of lipoprotein destruction throughout the cell although the disruption of a surface lipoprotein layer, with loss of the osmotic barrier, might well be the immediate cause of death. On the other hand, the results of the action of CTAB on the cell-wall have suggested that the cell-wall itself possesses a lipoprotein structure which is generally accepted to be the basis of osmotic barriers in living organisms. It is thus possible that the cell-wall of Staph. aureus possesses, in addition to its mechanical properties, at least some of the osmotic functions which have been attributed to a discrete, cytoplasmic membrane. This view has already been suggested by Mitchell (1949) on the basis of osmotic reactions, by Mitchell and Moyle (1951) on the basis of chemical analysis and by

Hurst (1952) on the basis of electron diffraction studies.

Since it is proposed that CTAB acts by splitting or disrupting lipoprotein complexes and since there is evidence that B. subtilis possesses either a low content of lipoprotein or lipoproteins of high instability which readily break down following death of the cell in distilled water, CTAB would be expected to have a less striking effect on this organism than on Staph. aureus. The experimental results do indeed show that although low concentrations of CTAB have some stimulating effect on the rate of lysis, as estimated by suspension opacity, the manner of disintegration of the individual cell is not significantly different from that occurring in distilled water. On the other hand, a given weight of cells of B. subtilis requires, for maximum stimulation of lysis, the same concentration of CTAB as does an equal weight of cells of Staph. aureus. If CTAB, at the lowest concentrations, does indeed attack mainly lipoid it would follow that the two organisms possess equal proportions of lipoids. In contradistinction, Jobling and Petersen showed that B. subtilis possesses much less lipoid than Staph. aureus. However, these two authors did not estimate bound lipoids which other workers have shown to be all-important with regard to anti-autolysis. On the other hand, the protein content of the cell may also be involved in the action of low concentrations of CTAB on the cell. This argument gains strength from the observations of Putnam (1948) which are discussed in the following pages (see p.96).

Intermediate concentrations of CTAB (cell to CTAB weight ratio of about 9 to 1) cause cytoplasmic retraction and a change to the Gram-negative state, as with the lower concentrations of CTAB. However, this is not followed by the complete disintegration of the cell; instead, there occurs stripping of the cell-wall from the cytoplasmic body which undergoes no further

alteration and remains apparently, indefinitely intact. At these concentrations protein denaturation becomes an important factor (Hotchkiss,1946) and would account for inactivation of the autolytic enzymes with absence of disintegration of the cytoplasmic body. The stripping of the cell-wall from the retracted and relatively rigid cytoplasm, which is such a striking feature with intermediate concentrations of CTAB, cannot be satisfactorily explained without detailed knowledge of the alterations produced in the molecular structure of the cell-wall by the CTAB. The following tentative explanation is offered. Stripping would appear to take place only when protein denaturation and cytoplasmic retraction are present together; at higher and lower concentrations of CTAB only one or the other occurs. The molecular derangements resulting from protein denaturation might well cause weakening of the structure of the cell-wall and Neurath et al. (1944) have shown that, as a result of denaturation, the surface area of a protein molecule is increased, due to the unfolding of the normally condensed polypeptide chains. This, in itself, is insufficient to produce fragmentation of the cell-wall since treatment of the isolated cell-walls with corresponding concentrations of CTAB (cell to CTAB weight ratio of about 8 to 1) causes no obvious change. With retraction of the cytoplasm from the cell-wall there occurs a loss of underlying support which produces additional strains on the weakened and expanded membrane. The result is fragmentation and stripping of the cell-wall.

The cytoplasmic retraction of the cells which occurs at an early stage of both water autolysis and CTAB lysis cannot be explained as an osmotic phenomenon since it occurs also and as readily in isotonic buffer solutions. Indeed, it seems likely that many of the examples of plasmolysis in the literature were, in fact, examples of cytoplasmic retraction following

death of the cell. Cytoplasmic retraction offers an explanation of the so-called cortex, described by Churchman (1927,1929) as responsible for the Gram-staining reactions of Gram-positive bacteria. He claimed that the cortex was destroyed by such treatments as incubation with acriviolet, hydrolysis with water or even sometimes spontaneously, leaving behind a Gram-negative medulla, smaller in size than the original cell. In reality, Churchman was producing cytoplasmic retraction in his cells with corresponding loss of Gram-positivity. Salton (1951) showed that in cells treated with CTAB the loss of the originally soluble cytoplasmic components was complete in about 4 hours and the present experiments have demonstrated that cytoplasmic retraction practically reaches a maximum within this period of time. A similar relationship can be shown to hold for autolysis in distilled water. Cytoplasmic retraction can therefore be related to the loss of soluble cytoplasmic constituents which occurs as a result of death of the cell and loss of the lipoprotein osmotic barriers.

The change in the Gram staining of the cells might be explained on the basis of the work of Dubos (1938,1945) who showed that bacteria possess a ribonuclease and that it is more resistant to chemical destruction than the autolytic enzymes. However, although this is almost certainly the explanation of the change to the Gram-negative state of autoclaved cells treated with autolysate, an alternative explanation is possible in the case of non-autoclaved cells. It has been possible to relate the proportion of cells showing cytoplasmic retraction at various stages of incubation, both in distilled water and in CTAB, to the proportion of Gram-negative cells. These two factors could be intimately related. Neither the freshly isolated cell-walls nor the separated cytoplasmic material of

Staph. aureus or B. subtilis have proved to be Gram-positive, a result which was originally demonstrated with crushed yeast cells by Benians (1912,1920), and which might well suggest that Gram-positivity is determined by the close association of cytoplasmic and cell-wall components. Marked retraction of the cytoplasm, due to loss of soluble cytoplasmic material, by breaking the cytoplasmic-cell-wall association, would thus cause loss of Gram-positivity.

When the concentration of CTAB is increased to give a cell to CTAB weight ratio of about 3 to 1 in the case of Staph. aureus (which Salton (1951) has shown to be the level of cell saturation) and of about 1 to 1 in the case of B. subtilis the cells become completely "fixed". Cytoplasmic retraction and change of Gram staining do not occur. It would be tempting to explain this by assuming the formation on the surface of the saturated cell of concentric layers of CTAB molecules which would prevent the leakage of soluble cytoplasmic material and the subsequent cytoplasmic retraction. However, McQuillen's (1950) work on the elctrophoretic mobilities of CTAB-treated staphylococci does not support this view and Salton (1951) has shown that maximum loss of material still occurs in cells suspended in high concentrations of CTAB. It seems more likely, therefore, that with high concentrations of CTAB protein denaturation is followed by a rapid coagulation of the cytoplasm. This, while allowing leakage of soluble material, would prevent cytoplasmic retraction from the cell-wall. The cell thus remains Gram-positive and apparently unaltered. Clearly, cytoplasmic coagulation must also occur with the intermediate concentrations of CTAB since, otherwise, the stripped cells would hardly retain their spherical or cylindrical shapes on drying down. The occurrence of cytoplasmic retraction, however, suggests that the coagulation at these lower concentrations is

a much slower process. Bacterial cells which have been autoclaved, a process which also results in loss of soluble cytoplasmic material (Salton,1951), undergo heat coagulation; such cells are also apparently fixed. The failure of the low concentrations of CTAB to stimulate the digestion of autoclaved cells by autolysate is possibly due to the difficulty of penetration of the large CTAB molecules into the heat coagulated cytoplasm.

The strictly stoichiometric relationship which exists between cell concentration and CTAB concentration in producing the three types of CTAB action indicates that the action of CTAB is not a purely surface phenomenon but that the detergent readily penetrates the cell to react with the various lipoid and protein constituents. This relationship has also shown that, in the case of CTAB, the biologically active unit is the free ion and not the micelle. The maximum detergent-binding capacity seems to vary with different and even with closely related proteins (Hotchkiss,1946; Putnam,1948): Salton (1951) has shown that the CTAB saturation level for a small number of bacterial species varies from 3 to 1 (cell to CTAB parts by weight) for Staph. aureus to 1.7 to 1 for B. pumilus (vel B. mesentericus). The different cell to CTAB weight ratios required for fixation (? saturation) of Staph. aureus and B. subtilis are probably due to their possession of different proteins or different proportions of similar proteins with correspondingly different total numbers of reactive groups.

Studies of the interactions of various, native protein solutions and detergents (Putnam,1948) have shown that the combinations of these substances occur in strict stoichiometric ratios which are within the range observed for whole bacteria and CTAB. Moreover, Putnam showed that detergents form various complexes with the proteins the structure of which vary with the protein to detergent weight ratio. In the complex with

the highest weight ratio the protein is still apparently in its native state; in the second complex, with a higher proportion of detergent, structural disorder of the protein with resulting denaturation ensues; finally, a third complex is formed, in which the maximum detergent-binding capacity of the protein is reached and which is accompanied by further disorientation of the protein leading to a large viscosity increase in the suspension. Putnam's observations may be used for a physico-chemical explanation of the action of detergents on bacteria: the formation of the first protein-detergent complex leaves the protein undenatured, yet the changes caused by the combination of the detergent with the protein molecules may well be sufficient for the disruption of lipoprotein conjugates (Kuhn and Bielig,1940; Macheboeuf and Tayeau,1938), with resulting acceleration of lysis; with the formation of the higher complexes protein denaturation and increased viscosity occur and would thus be responsible for enzyme inactivation and cytoplasmic coagulation.

The paradoxical action of low and high concentrations of CTAB on bacterial lysis is not an isolated phenomenon. Wollman and Wollman (1936) and Pulvertaft and Lumb (1948) made similar observations with various bactericidal agents other than detergents. Indeed, this phenomenon of opposite effects produced by low and high concentrations of a compound upon living cells is widespread throughout the biological field. That there is an intermediate stage between lysis and fixation, in the case of bacteria, namely cell-wall stripping, has not been previously recognised. Some experiments of Dubos (1938) suggest that this intermediate stage may also occur with non-detergent, bactericidal agents; pneumococci which had been treated with high concentrations of formaldehyde and then washed

became, on incubation in saline, Gram-negative and smaller in size but did not undergo subsequent, complete disintegration. In the case of CTAB the various effects produced on the bacterial cell would appear to be the result of the different sensitivities of certain constituents of the cell towards the detergent molecules. Whether the same mechanism applies to other bactericidal agents is a matter for further experimentation.

## The structure of the bacterial cell-wall

The experimental work on the structure of the staphylococcal cell-wall has not yielded conclusive evidence as to its detailed structural formation and any deductions must, therefore, be largely tentative.

The ready solution of the cell-wall by alkali suggests that it is largely composed of protein. The resistance to digestion by pepsin and trypsin, a fact already long known from experiments with whole, heat-killed cells, would place the protein in the resistant, keratin or fibrous group. Mitchell and Moyle (1951) have, indeed, shown that the cell-wall of Staph. aureus is mainly protein and have remarked on its close chemical similarity to the highly resistant protein of silk fibroin. On the other hand, the protein of the cell-wall can be hydrolysed by the autolytic enzymes of the cell, which Stacey and Webb (1948) have shown to contain proteolytic enzymes. These latter workers also demonstrated that before the autolytic enzymes can act on the cell, killed by heating, a polysaccharide-releasing enzyme must disrupt what is probably a polysaccharide-protein complex to expose the protein of the cell to enzyme attack. Mitchell and Moyle showed that the staphylococcal cell-wall contains as much as 12 per cent of a glycero-phosphoric acid compound which is closely bound to the

protein framework of the cell-wall. This glycero-phosphoric acid compound, according to Mitchell and Moyle, is apparently the principal carbohydrate present in the cell-wall of Staph. aureus and might therefore be the substrate, in this organism, for the carbohydrate-releasing enzyme of Stacey and Webb. Mitchell and Moyle indicated that this compound is normally mistaken for ribonucleic acid when the latter is estimated in terms of organic, non-lipoid phosphorus; using ultraviolet spectroscopy they found that the staphylococcal cell-wall contained little or no nucleic acid. Their figure of 12 per cent for the glycero-phosphate corresponds to the 10 to 15 per cent nucleic acid content, estimated in the present investigations by the usual method of chemical analysis. If one accepts the work of Mitchell and Moyle there is an explanation for the apparent lack of action of ribonuclease on the cell-wall; on the other hand, the loss of as little as 10 per cent of uniformly distributed material from the cell-wall might not be readily detected with the electron microscope.

Treatment of the isolated cell-wall with very low concentrations of CTAB has produced appearances suggestive of a fibrous network. This appearance together with the results of the treatment of whole cells and isolated cell-walls with CTAB and liposolvents allows the formulation of a hypothesis for the structure of the staphylococcal cell-wall. This can be considered to consist of a protein fibrous network embedded within a finely granular matrix containing lipoid and carbohydrate components in close combination with the protein molecules. The CTAB would act by disrupting the important lipoprotein complexes and thus allow the autolytic enzymes to come into contact with their substrates. The primary destruction or release of the carbohydrate component of the

of the matrix will expose the protein network to enzymic disintegration. Mitchell and Moyle (1951) and Hurst (1952) have, as previously described, suggested similar structures on the basis respectively of chemical analysis and electron diffraction studies.

The failure to demonstrate this protein network by acid or alkaline hydrolysis might be due to a primary attack by the acid or alkali on the protein molecules which are, therefore, more rapidly disintegrated than by the autolytic enzymes. However, the possibility exists that these fibres, demonstrated by a single technique only, may be artefacts and the result of chemical transformation. In the same way, Houwink and Kreger (1953) demonstrated a carbohydrate fibrous network in yeast cell-walls by acid hydrolysis but concluded from electron diffraction studies that the fibres did not exist in the untreated cell-wall. The final elucidation of the problem must await more refined degradation techniques, an electron microscope of greater resolving power or possibly a detailed investigation by means of electron or X-ray diffraction analysis.

The method of disintegration of the cell-wall of B. subtilis during autolysis closely resembles that shown by Johnson, Zworykin and Warren (1943) to occur in autolysing cell-walls of certain marine, luminous bacteria; the cell-walls disintegrated by the loss of small circular and elliptical segments. In B. subtilis these small units seem to be arranged in linear patterns. The structure of these cell-walls as mosaic complexes of small circular units is radically different from that of Staph. aureus. However, recent studies on this subject have indicated that wide differences can be expected both chemically and physically in the structure of the cell-walls of different species of bacteria.

## The cell-wall in cell division

The examination of the isolated cell-walls has proved to be of considerable value in following the successive stages of cell division. The results, in general, confirm the three-stage process of cell division described by Knaysi and by Robinow: transverse cytoplasmic membrane septum formation, then the growth of a cell-wall septum and finally cell-wall constriction through the line of the septa to complete division.

The electron micrographs have yielded convincing evidence for the presence of a cytoplasmic membrane, which forms transverse septa, in Strept. faecalis; but examination of large numbers of specimens have failed to detect, conclusively, a similar membrane in either Staph. aureus or B. subtilis. In Gram-negative bacteria it is relatively simple to demonstrate, with the electron microscope, a discrete cytoplasmic membrane within the cell-wall, the latter isolated by shaking with glass beads; fig. 75 shows such a membrane in the isolated cell-wall of Bacillus coli. Mitchell (1949) has, indeed, from consideration of the osmotic properties of bacteria, suggested that Gram-positive bacteria differ from Gram-negative bacteria in not possessing a cytoplasmic membrane, the cell-wall itself being the osmotic barrier of the cell. The same technique as is successful in demonstrating a cytoplasmic membrane in B. coli was unsuccessful with Staph. aureus and B. subtilis. On the other hand, Knaysi's demonstration of a cytoplasmic membrane in B. subtilis and B. megatherium by differential staining techniques (1930,1946) is not unconvincing and, in the present work, Strept. faecalis certainly seems to possess a cytoplasmic membrane. There would appear to be no reason why the cytoplasmic membrane should not vary somewhat in structure and in chemical composition among different bacteria as does the cell-wall itself and thus vary in its resistance to

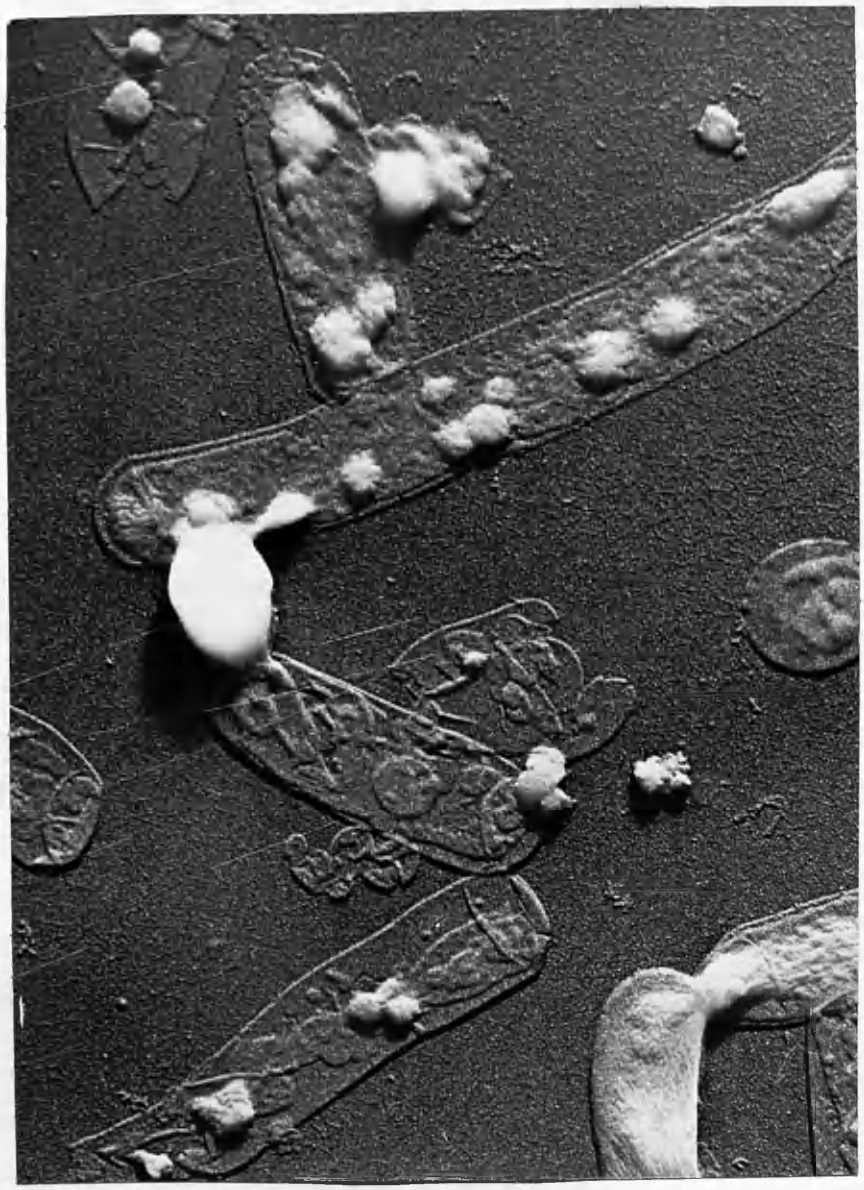


Fig. 75. Cell-walls of B. coli containing cytoplasmic membranes. ( × 15,000).

the destructive action of shaking with glass beads.

The successive stages in the formation of the cell-wall septum, illustrated in the micrographs, indicate that it originates as an equatorial thickening of the cell-wall and then grows inwards to divide the cytoplasm. There would appear to be no basis for the view of Bissett that the cell-wall does not grow inwards but is secreted uniformly across the width of the cell. The nature of the early structural changes in the cell-wall of Strept. faecalis, namely the band of decreased density, is obscure unless it is due to the mobilisation of material for the synthesis of the septum.

Bissett's statements that cell-wall septum formation does not occur in smooth strains of bacteria, in which group he includes the short-chained streptococci, is not supported by the present work on Strept. faecalis. This short-chained streptococcus definitely forms transverse cell-wall septa preliminary to the final process of constriction. The latter stage, in the three organisms studied, does not, in general, commence until the cell-wall septum has completed its formation, but is not thereafter long delayed; neither the Strept. faecalis nor the B. subtilis, used in this study, were conspicuous for chain formation. A preliminary study of a smooth strain of B. coli (not reported in this thesis) has indicated that its cells also form transverse cell-wall septa. Knaysi's view that the only difference in the process of cell division in rough and smooth strains of organisms is in the interval of time elapsing between the commencement of cell-wall septum formation and cell-wall constriction would thus appear to be confirmed. On the other hand, examples of cell-wall division of Strept. faecalis by constriction without cell-wall septum formation, although not common, has been demonstrated (fig.65). The possibility must be considered that bacteria are capable

of both methods of division (Henrici,1934), the predominant method depending on so far, undetermined environmental factors. Before dogmatic views can be formulated a detailed investigation of the cell division of both rough and smooth strains of a wide variety of organisms, using the isolated cell-wall technique, is required. The demonstration in Strept. faecalis of multiple septum formation without subsequent constriction and division and of constriction without previous cell-wall septum formation seems to indicate that the two processes, although obviously closely associated, are yet the result of discrete and dissociable mechanisms. The nature of these mechanisms is obscure but by growing the organisms under various controlled conditions it might be possible to interfere with one or other of these processes and thus to elucidate the underlying physico-chemical principles.

## SUMMARY

Investigations involving the use of the electron microscope have been carried out of the structural changes involved in bacterial autolysis, of the action on bacteria of a cationic detergent (cetyl-trimethyl-ammonium bromide), of the structure of the bacterial cell-wall and of the part played by the bacterial cell-wall in cell division.

Spontaneous autolysis was studied in Staph. aureus and in B. subtilis, using both the intact cells and the isolated cell-walls. In both organisms the earliest visible change is a retraction of the cytoplasm from the cell-wall and this is associated with a change to the Gram-negative state. In the case of Staph. aureus this is followed by a slow, irregular disintegration of the cytoplasm within the cell-wall which also undergoes a slow, irregular disintegration but remains undisrupted until a relatively late stage. In B. subtilis the rate of autolysis is very much more rapid and this is due mainly to the early rupture of the cell-wall allowing rapid evacuation and dispersal of the cytoplasm; the cell-wall, itself, also undergoes a more rapid and uniform disintegration than the cell-wall of Staph. aureus. It is suggested that the loss of Gram-positivity during spontaneous autolysis is due not to enzymic activity but to the disruption of cytoplasmic-cell-wall contact, the latter being associated with loss of soluble cytoplasmic constituents. Certain conditions (cell concentration, age of cells, washing of the cells, pH and temperature) influencing the rate of autolysis of Staph. aureus have also been studied.

The action of CTAB on Staph. aureus and B. subtilis, again using both intact cells and isolated cell-walls for the investigation, has been found to follow stoichiometric

proportions and to vary with the cell to detergent weight ratio. A high ratio results in a marked stimulation of autolytic disintegration of the cell. A low ratio results in cell fixation. Intermediate ratios cause stripping of the cell-wall but the cytoplasmic body, contracted and Gram-negative, undergoes no further disintegration. From these results together with a comparison of the effects on autolysis of treatment of the bacterial cell with CTAB and with lipoid solvents an hypothesis has been formulated that detergents cause splitting of lipoprotein complexes throughout the cell: where the concentration of the detergent is relatively low this allows an acceleration of normal autolysis; with higher concentrations of detergent protein denaturation predominates and is responsible for inactivation of autolytic enzymes, cell-wall stripping and cell fixation. The quantitative results obtained show that the active unit of CTAB in producing the above effects is the free ion and not the micelle.

The investigation of the intimate structure of the cell-wall of Staph. aureus tends to support the view that the cell-wall of this organism contains a protein, fibrous network. The cell-wall of B. subtilis, on the other hand, seems to consist of a mosaic of small, circular or oval units.

The stages of bacterial cell division have been followed by examinations of the isolated cell-walls of Staph. aureus, Strept. faecalis and B. subtilis. In all three organisms a transverse cell-wall septum is formed which originates in and grows centripetally from the cell-wall. The cell-wall septum splits into two layers and division is completed by constriction of the cell-wall through the line of the double septum. Cell-wall septum formation and cell-wall constriction appear to be dissociable phenomena. A cytoplasmic membrane, forming transverse septa before the

formation of the cell-wall septum, has been demonstrated in Strept. faecalis.

# BIBLIOGRAPHY

The title abbreviations are those described in the World List of Scientific Publications, 1952, 3rd ed.


Albert, A. 1942 Lancet, 633

Albert, R. and Albert, W. 1901 Zbl.Bakt., Abt.II,7,737

Anderson, R. J. and Roberts, E.G. 1930 J.biol.Chem., 85,529

Anson, M. L. 1939 J.gen.Physiol., 23,239

Aschner, M. and Hestrin, S. 1946 Nature, 157,659

Atkin, E.E. 1926 Brit.J.exp.Path., 7,167

Avery, O.T. and Cullen, G.E. 1920a J.exp.Med., 32,547

1920b ibid., 32,571

1920c ibid., 32,583

1923 ibid., 38,199

Baisden, L. and Yegian, D. 1943 J.Bact., 45,163

Baker, Z., Harrison, R.W. and Miller, B.F. 1941a J.exp.Med., 74,611

1941b ibid., 74,621

Barnard, J.E. 1930 A System of Bacteriology, Med.Res.Coun., 1,115

Bartholomew, J.W. and Umbreit, W.W. 1942 J.Bact., 44,317

1944 ibid., 47,415

Bayliss, M. 1937 J.Lab.clin.Med., 22,700

Benians, T.H.C. 1912 J.Path.Bact., 17,199

1920 ibid., 23,411

Bensley, R.R. 1942 Science, 96,389

Binkley, F., Goebel, W.F. and Perlman, E. 1945a J.exp.Med., 81,315

1945b ibid., 81,331

Bissett, K.A. 1939 J.Path.Bact., 48,427

1948a J.gen.Microbiol., 2,83

1948b ibid., 2,126

1949 ibid., 3,93

1950 The Cytology and Life-History of Bacteria, Livingstone, Edinb.

1953 J.gen.Microbiol., 8,50

Boivin, A., Mesrobineau, I. and Mesrobineau, L. 1933 C.R. Soc.Biol., 114,307

Boivin, A. and Mesrobineau, L. 1938 Ann.Inst.Pasteur, 61,426

Born, C.V.R. 1952 J.gen.Microbiol., 6,344

Brown, A.J. 1886a J.chem.Soc., 49,172

1886b ibid., 49,432

Burdon, K.L. 1946 J.Bact., 52,665

Burke, V. and Barnes, M.W. 1929 J.Path.Bact., 18,69

Bucca, M.A. 1943 J.Bact., 46,151

Chaplin, C.E. 1952 ibid., 64,805

Chargaff, E. and Bendich, A. 1944 Science, 99,147

and Ziff, M. 1939 J.biol.Chem., 131,25

Churchman, J.W. 1927 Proc.Soc.exp.Biol., N.Y., 24,736

1929 J.Bact., 18,413

Cohn, F. 1872 Beitr.Biol.Pfl., I,Heft 2,127

Curran, H.R. and Evans, F.R. 1942 J.Bact., 43,125

Dawson, I.M. 1949 in The Nature of the Bacterial Surface, Blackwell Sci. Publ., Oxford, p.119

de Bary, A. 1884 Vergl.morphol.u.biol.der pilze, Leipzig.

1887 Vorlesungen uber Bakterien, Engelman, Leipzig.

DeLamater, E.D. and Mudd, S. 1951 Exp.Cell Res., 2,499

Dobell, C.C. 1908 Quart.J.micr.Sci., 52,121

1911 ibid., 56,395

Downie, A.W., Stent, L. and White, S.M. 1931 Brit.J.exp. Path., 12,1

Dubois, A.S. and Dibblee, D. 1947 Science, 103,734

Dubos, R.J. 1937a J.exp.Med., 65,877

1937b ibid., 66,101

1938 ibid., 67,389

1945 The Bacterial Cell, Harvard Univ. Press, Cambr., Mass.

and MacLeod, C.M. 1938 J.exp.Med., 67,791

Dyar, M.T. 1947 J.Bact., 53,498

1948 ibid., 56,821

Eisenberg, P. 1909 Zbl.Bakt., I,49,465

Elford, W.J., Guelin, A.M., Hotchin, J.E. and Challice, C.E. 1953 Ann.Inst.Pasteur, 84,319

Ellis, D. 1922 Brit.Med.J., 2,731

1932 Sulphur Bacteria, Longmans, Green and Co., London.

Ely, J.O. 1939 J.Bact., 38,391

Emmerich, R. and Low, O. 1899 Z.Hyg.InfectKr., 31,1

Epstein, L.A. and Chain, E. 1940 Brit.J.exp.Path., 21,339

Findlay, A. 1941 Practical Physical Chemistry, Longmans, Green and Co., London.

Fischer, A. 1891 Ber.sachs.Ges.Akad.Wiss., 43,52

Fleming, A. 1922 Pro. roy. Soc. B, 93,306

1932 Proc. R. Soc. Med., 26,71

Flexner, S. 1907 J. exp. Med., 9,105

Freeman, G.G. and Anderson, T.H. 1941 Biochem. J., 35,564

Gale, E.F. and Taylor, E.S. 1947 J. gen. Microbiol., 1,77

Gardner, A.D. 1930 A System of Bacteriology, Med. Res. Coun., 1,159

Goebel, W.F. and Avery, O.T. 1929 J. exp. Med., 49,267

Guilliermond, A. 1908 Arch. Protistenk., 12,9

Gutstein, M. 1924a Zbl. Bakt., I,93,233

1924b ibid., I,93,393

Harris, J.O. 1953 J. Bact., 65,518

Henrici, A.T. 1934 The Biology of Bacteria, Heath and Co., New York.

Henry, H. and Stacey, M. 1946 Proc. roy. Soc. B, 133,391

Hibbert, H. 1930 Science, 71,419

Hillier, J., Mudd, S. and Smith, A.G. 1949 J. Bact., 57,319

Holdsworth, E.S. 1952 Biochim. biophys. Acta, 8,110

Hotchin, J.E., Dawson, I.M. and Elford, W.J. 1952 Brit. J. exp. Path., 33,177

Hotchkiss, R.D. 1946 Ann. N.Y. Acad. Sci., 46,479

1944 Advanc. Enzymol., 4,153

Houwink, A.L. and Kreger, D.R. 1953 Leeuwenhoek ned. Tijdschr., 19,1

and LePoole, J.B. 1952 Phys. Verh., Mosbach, 5,98

and Van Iterson, W. 1950 Biochim. biophys. Acta, 5,10

Hurst, H. 1952 J. exp. Biol., 29,30

Iwanoff, K.S. 1902 Beitr. chem. Physiol. Path., 1,524

Jobling, J.W. and Petersen, W. 1914 J.exp.Med., 20,452

Johnson, F.H., Zworykin, N. and Warren, G. 1943 J.Bact., 46,167

Jakob, A. and Mahl, H. 1940 Arch.exp.Zellforsch., 29,97

King, H.K. and Alexander, H. 1948 J.gen.Microbiol., 2,315

Klein, L. 1889 Zbl.Bakt., I,6,377

Klein, M. and Kardon, Z.G. 1947 J.Bact., 54,245

Klein, S.J. and Stone, F.M. 1931 ibid., 22,387

Knaysi, G. 1929a J.infect.Dis., 45,13

1929b J.Bact., 17,4

1930 ibid., 19,113

1938 Bot.revs., 4,83

1941 J.Bact., 41,141

1946 ibid., 51,113

1951 Elements of Bacterial Cytology, Comstock Publ. Co., Ithaca, N.Y.

and Hillier, J. 1949 J.Bact., 57,23

1951 micrographs in Knaysi's Elements of Bacterial Cytology.

and Fabricant, C. 1950 J.Bact., 60,423

Knox, W.E., Auerbach, V.H., Zarudnaya, K. and Spirtes, M. 1949 ibid., 58,443

Kozlowski, A. 1928 ibid., 16,203

Kriss, A.E. and Biryuzova, V.N. 1950 J.gen.Biol., Moscow.

Kuhn, R. and Bielig, H.J. 1940 Ber.dtsch.chem.Ges., 73,1080

and Jerchel, D. 1941a ibid., 74,941

1941b ibid., 74,949

Kunitz, M. 1940 J.gen.Physiol., 24,15

Lamanna, C. and Mallette, M.F. 1950 J.Bact., 60,499

Lamar, R.V. 1911 J.exp.Med., 13,1

Lord, F.T. and Nye, R.N. 1919 J.exp.Med., 30,389

1922a ibid., 35,689

1922b ibid., 35,703

McCalla, T.M. 1940 J.Bact., 40,23

1941 ibid., 41,775

McQuillen, K. 1950 Biochim.biophys.Acta, 5,463

Macheboeuf, M. and Tayeau, F. 1938a Compt.rend., 206,860

1938b C.R.Soc.Biol., 129,1181

1938c ibid., 129,1184

Mair, W. 1929 A System of Bacteriology, Med.Res.Coun., 2,166

Mandelbaum, M. 1907 Munch.med.Wochr., 54,1431

Maxted, W.R. 1948 Lancet, 255

Meisel, M.N. and Umanskaya, V.P. 1949 Microbiology, Moscow, 18,11

Meyer, A. 1912 Die Zelle der Bakterien, Fischer, Jena.

Meyer, K., Dubos, R.J. and Smyth, E.M. 1937 J.biol.Chem., 118,71

Migula, W. 1894 Arb.bakt.Inst.Karlsruhe, 1,139

1897 System der Bakterien, I, Fischer, Jena.

1904 in Lafar's Handb.tech.mykol., I,29

Miles, A.A. and Pirie, N.W. 1939 Brit.J.exp.Path., 20,278

Miller, B.F., Abrams, R., Dorfman, A. and Klein, M. 1942 Science, 96,428

Mitchell, P. 1949 in The Nature of the Bacterial Surface, Blackwell Sci. Publ., Oxford, p.55

Mitchell, P.D. and Crowe, G.R. 1947 J.gen.Microbiol., 1,85

Mitchell, W.T.J. and Partridge, S.M. 1940 Biochem.J., 34,169

Mudd, S. and Lackman, D.B. 1941 J.Bact., 41,415

, Polevitzky, K., Anderson, T.F. and Chambers, L.A. 1941 ibid., 42,251

and Kast, C.C. 1942 ibid., 44,361

Murray 1930 cited by Rideal, E.K. in A System of Bacteriology, Med.Res.Coun., 1,122

Murray, E.G.D. 1929 Sp.Rep.Ser., Med.Res.Coun., Lond., No.124

Muhlethaler, K. 1949 Biochim.biophys.Acta, 3,527

Neil, J.M. and Avery, O.T. 1924 J.exp.Med., 40,405

Neufeld, F. 1900 Z.Hyg.InfectKr., 34,54

Neurath, H., Greenstein, J.P., Putnam, F.W. and Erikson, J.O. 1944 Chem.revs., 34,157

Nicolle, M. and Adil-Bey 1907 Ann.Inst.Pasteur, 21,20

Nishimura, T. 1893 Arch.Hyg.,Berl., 16,318

1894 ibid., 21,52

Ordal, E.J. and Borg, A.F. 1942 Proc.Soc.exp.Biol.,N.Y., 50,332

Overton, E. 1895 Vjschr.naturf.Ges.Zurich, 40,159

Palmer, K.J., Schmitt, F.O. and Chargaff, E. 1941 J.cell. comp.Physiol., 18,43

Pennington, D. 1949 J.Bact., 57,163

Perlman, E., Brinkley, F. and Goebel, W.F. 1945 J.exp.Med., 81,349

Piekarski, G. and Ruska, H. 1939 Arch.Mikrobiol., 10,302

Preisz, H. 1904 Zbl.Bakt., I, 35,280,416,537,657

Pulvertaft, R.J.V. and Lumb, G.D. 1948 J.Hyg.,Camb., 46,62

Putnam, F.W. 1948 Advanc.Protein Chem., 4,79

Raistrick, H. and Topley, W.C.C. 1934 Brit.J.exp.Path., 15,113

Rettger, L.F. 1904 J.med.Res., 13,79

Roberts, M.H. and Rahn, O. 1946 J.Bact., 52,639

Robinow, C.F. 1945 Addendum to Dubos' The Bacterial Cell.

Salton, M.R.J. 1951 J.gen.Microbiol., 5,391

1952a Biochim.biophys.Acta, 8,510

1952b ibid., 9,334

1952c Nature, 170,746

and Horne, R.W. 1951a Biochim.biophys.Acta, 7,19

1951b ibid., 7,177

and Cosslett, V.E. 1951 J. gen.Microbiol., 5,405

Schaudinn, F. 1902 Arch.Protistenk., 1,306

1903 ibid., 2,416

Schmitt, F.O., Bear, R.S. and Ponder, E. 1936 J.cell.comp. Physiol., 9,89

1938 ibid., 11,309

Schulman, J.H. and Rideal, E.K. 1937 Proc.roy.Soc.B, 122,46

Schultze, W.H. 1907 Munch.med.Wochr., 54,1167

Schutzenberger, P. 1874 Bull.Soc.chim.Paris, 21,204

Smedley-MacLean, I. 1922 Biochem.J., 26,370

St.John-Brookes, R. 1930 A System of Bacteriology, Med.Res. Coun., 1,104

Stacey, M. 1949 in The Nature of the Bacterial Surface, Blackwell Sci. Publ., Oxford, p.29

and Webb, M. 1948 Nature, 162,11

Starkey, R.L. 1946 J.Bact., 51,33

Stearn, A.E. and Stearn, E.W. 1924 ibid., 9,479

Sturdza, S.A. 1938 C.R.Soc.Biol., 129,410

Suringar 1866 cited in Meyer's Die Zelle der Bakterien

Swellengrebel, N.H. 1907a Ann.Inst.Pasteur, 21,448

1907b ibid., 21,562

1909 Zbl.Bakt., I,49,529

Sykes, G. 1939 J.Hyg.,Camb., 39,463

Tayeau, F. 1939 C.R.Soc.Biol., 130,1027

Terroine, E.F. and Lobstein, J.E. 1923 cited in W.R. Bloor's Biochemistry of Fatty acids and their compounds, The Lipids, Reinhold Publ. Co., N.Y.,1943

Thompson, R.H.S. and Dubos, R.J. 1938 J.biol.Chem., 125,65

Topley, W.C.C., Raistrick, H., Wilson, J., Stacey, M., Challinor, S.W. and Clark, J. 1937 Lancet, 252

Traube, I. 1904 Pflug.Arch.ges.Physiol., 105,54

Trim, A.R. and Alexander, A.E. 1949 Symp.Soc.exp.Biol.,N.Y., 3,111

Trommsdorf, R. 1902 Zbl.Bakt., II,8,82

Vahle, C. 1909 Zbl.Bakt., II,25,178

Valko, E.I. 1946 Ann.N.Y.Acad.Sci., 46,451

and Dibblee, D. 1944 cited in Valko, E.I., 1946 Ann.N.Y.Acad.Sci., 46,451

and Dubois, A.S. 1944 J.Bact., 47,15

van Wisselingh, C. 1898 Jb.wiss.Bot., 31,619

1925 in K. Linsbauer's Handb.PflAnat., Berl., 3,1

Viehoever, A. 1912 Ber.dtsch.bot.Ges., 30,443

Vincenzi, L. 1887 Hoppe-Seyle Z., 11,181

von Wettstein, F. 1921 S.B.Akad.Wiss.Wien., I,130,3

Wamoscher, L. 1930 Z.Hyg.InfectKr., 111,422

Webb, M. 1948 J.gen.Microbiol., 2,260

Williams, R.C. and Wyckoff, R.W.G. 1946 J.appl.Phys., 17,23

Wollman, E. and Averbusch, M. 1932 C.R.Soc.Biol., 110,623

and Wollman, E. 1936 Ann.Inst.Pasteur, 56,137

Wollstein, M. 1907 J.exp.Med., 9,588

Wyckoff, R.W.G. 1949 Electron Microscopy, Interscience Publ., Inc., N.Y.

Zettnow, E. 1896 Z.Hyg.InfectKr., 24,72

Zittle, C.A. and Mudd, S. 1942 Ann.N.Y.Acad.Sci., 43,47

## ACKNOWLEDGEMENTS

I wish to express my indebtedness to the Medical Research Council for their generosity in providing me with a scholarship during the tenure of which the present work was carried out.

Thanks are also due to the Rockefeller Foundation, the Medical Research Council and the Rankin Research Fund for the generous provision of grants towards the purchase of equipment.

My deepest gratitude is due to Dr. Iwo Lominski of the Bacteriology Department and to Dr. Ian M. Dawson of the Chemistry Department for their constant advice, criticism and encouragement. I am especially indebted to Dr. Lominski for introducing me to and teaching me the subject of Bacteriology and without whom the present work would never have been carried out. I am similarly indebted to Dr. Dawson for teaching me the techniques of Electron Microscopy and for indicating to me their value in investigating bacteriological problems.

I am extremely grateful to Professor J. M. Robertson, F.R.S. for acting as my sponsor in obtaining my scholarship and to both Professor Robertson and Professor J. W. Howie, M.D. for allowing me to work in their respective departments and for their continued interest in my work.

It is with pleasure that I express my appreciation of the help given to me throughout my work by the technical staffs of both the Bacteriology and Chemistry Departments.