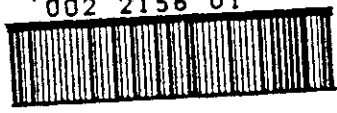
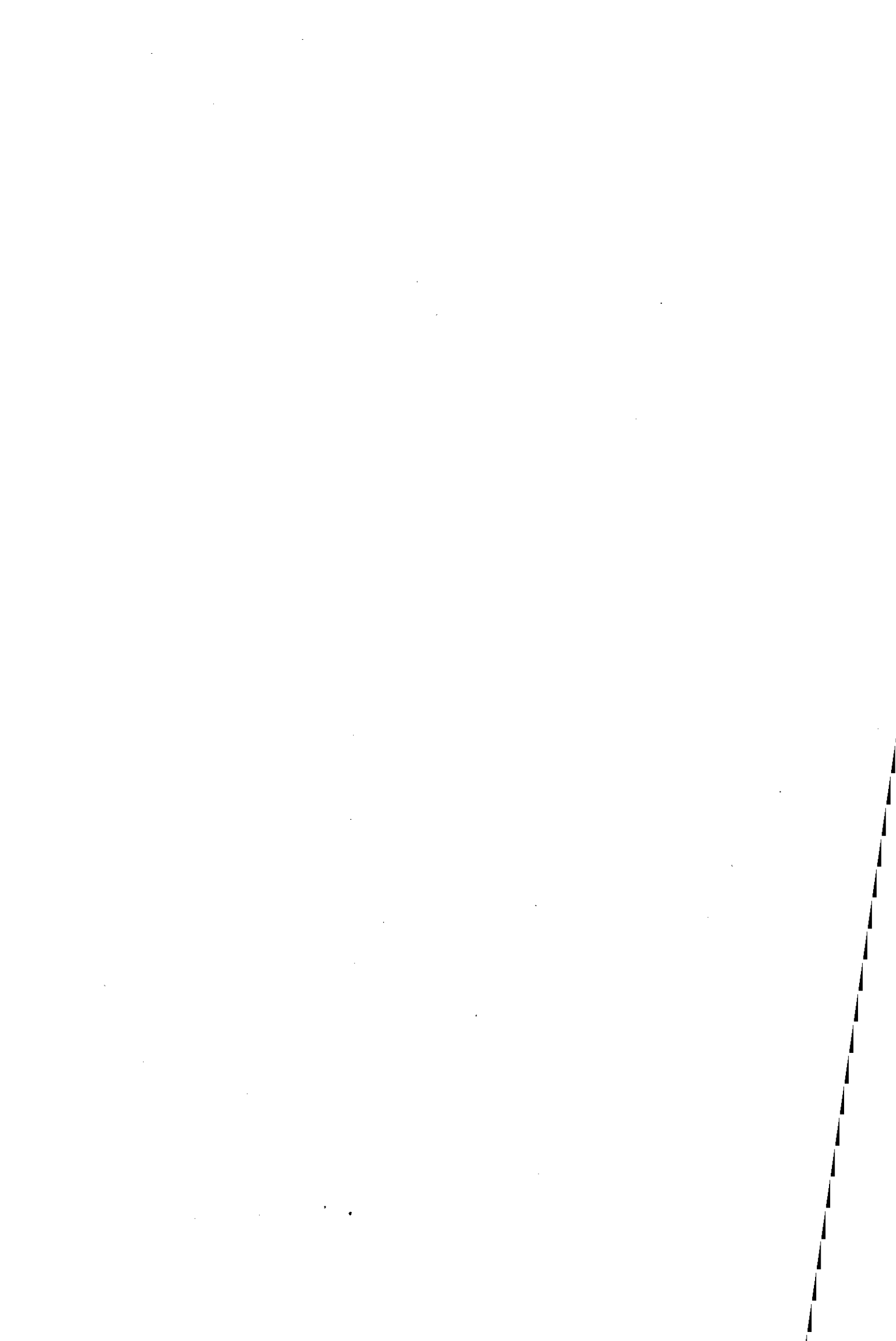


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ASPECTS OF THE CHEMISTRY OF DIFFERENTIATION
IN SOME PROTISTA

by

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Erratum.

Pages 21 and 22 have been deleted.

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Summary

Several species of Actinomyces viscosus were examined using the scanning electron microscope and considerable morphological heterogeneity was seen and one species, Actinomyces viscosus WVU 398B, was observed to undergo a form of differentiation, for which the term 'vacuolation' has been introduced. This form of differentiation can be regulated by inhibitors of peptidyl transferase or translocase.

Further examples of the regulation of differentiation were provided by an examination of the interaction of antibiotics and antimetabolites with a species of Bacillus cereus isolated from laboratory dust.

3-Chloropropane-1,2-diol exhibited an ability to impair cell division in Staphylococcus aureus and Escherichia coli and this action may be due to the glycerol antagonism of 3-chloropropane-1,2-diol. An examination of the lipid extracts from microorganisms cultured in the presence of 3-chloropropane-1,2-diol revealed that increasing concentrations of the chemical impaired the synthesis of glycolipids and lipoaminoacids. The fluorescence probe, 1-anilino-naphthalene-8-sulphonate (ANS), demonstrated that microorganisms cultured in the presence of increasing concentrations of 3-chloropropane-1,2-diol possessed an increasingly more hydrophobic cell surface. This increase in hydrophobicity was paralleled by an increase in sensitivity to lipophilic antimetabolites and a decrease in sensitivity to hydrophilic antimetabolites.

In an examination of the role of inorganic cations in the differentiation of microorganisms it was demonstrated that lanthanum and neodymium could replace calcium in the development of an alga, Scenedesmus sp., and in the sexual development of the fungus, Phytophthora cactorum. However, neither lanthanum nor neodymium could replace calcium in the development of heat resistance in bacterial spores. It is suggested that calcium interacts with the phospholipids of cell membranes and so induces conformational forms in the membrane which regulate enzymatic activity. In the case of bacterial spores it is suggested that calcium interaction with the phospholipids of the spore protoplast could induce a dehydrated state which contributes to heat resistance.

Microorganisms implicated in extrinsic allergic alveolitis have been shown to be potential pathogens. In the case of the actinomycetes involved in extrinsic allergic alveolitis they can produce infected nodules resembling actinomycotic mycetoma. As a control an isolate from laboratory dust, Bacillus cereus SV-1, was examined and was shown to be a potential pathogen.

- - - - -

PART I.

ASPECTS OF THE CHEMISTRY DIFFERENTIATION IN
SOME PROTISTA.

Chapter 1.

Some Aspects of Microbial Differentiation.

A very satisfactory account of microbial differentiation is contained in the 23rd Symposium of the Society for General Microbiology (1).

Cell differentiation is frequently assumed to be a characteristic of higher organisms, however, it is not their sole prerogative as many microorganisms exhibit complex specialisation.

Cell division is related to chromosomal replication which in bacteria can occur within 20 mins. In the simplest cases of bacterial division (Bacillus subtilis, Escherichia coli and Streptococcus faecalis) cell division occurs successively within the same plane while others (Micrococcus radiodurans, Gaffyka and Sarcina) resemble some eukaryotes at early cleavage stages by dividing at right angles to produce colonies which are the result of ordered growth in either two or three dimensions. Cell growth (that is, cell mass increasing with time) should not be confused with cell division. Normally, cell division and cell growth are in close correspondence but it is possible to grow cells in the absence of cell division and so produce very long cells. DNA and RNA synthesis can be inhibited while cell mass continues to increase in which case the DNA protein ratio falls. Such unbalanced growth permits a study of the contribution to growth from various cell components. An analysis of chemostatic growth reveals that the rate of RNA synthesis varies first and that protein and DNA synthesis commences only when RNA synthesis

has reached steady state kinetics. Evidently, RNA must be considered a candidate for the role of growth regulator.

While eukaryotes have much in common with bacterial growth, they are much more complex. Bacteria and other microorganisms evolved under conditions that selected for maximum growth under a wide range of environmental situations. Multicellular organisms do not require a rapid growth mechanism which is, in fact, deleterious, for example, in the case of neoplastic tissue. Eukaryotic growth is complicated by the existence of energy producing organelles such as mitochondria and chloroplasts.

While prokaryotes undergo cell constriction at the centre during wall and membrane synthesis, the eukaryotes divide by 'mitosis', a five step process, which involves partition of DNA between the parent cell and the daughter. In addition to mitosis, an energy requiring process which can be inhibited by cyanide, carbon monoxide and colchicine, eukaryotic cells can undergo 'meiosis' which permits the reduction of DNA since nucleic divide twice while chromosomes divide once.

It is now quite widely accepted that cell organelles such as chloroplasts and mitochondria have considerable genetic independence of the nucleus. Evidence indicates that yeast mitochondria contain sufficient genetic information to control partially their development. Such autonomy must be accepted since obvious problems would arise during the synthesis of protein inside the mitochondrial membrane if the only locus of protein synthesis was at the nucleus. Thus, there may be more than one pathway for information transfer within cells. Recent evidence indicates that

some mitochondrial proteins are synthesised from nuclear mRNA while others originate from mitochondrial mRNA. For example, outer mitochondrial membrane enzymes and intramitochondrial enzymes are synthesised in the cytoplasm under nuclear control and then transferred to their mitochondrial locus while succinic acid dehydrogenase and other insoluble inner membrane systems are produced by the mitochondrion from its own DNA.

The protista are characterised by the requirement that the prokaryotic cell must be able to carry out all the metabolic events within one cell. The higher cells, such as animal cells, do not possess this ability and various cells perform different roles within an integrated system. Initially, one parent cell must carry all the information for the progeny. Cell division continues until cells are produced which perform only one cellular task - "differentiation".

The simplest form of cell differentiation is bacterial sporulation, which has been the subject of much research (1-4).

An important concept of genetic regulation is the idea of 'commitment' which implies 'point of non-return' and this occurs when the biochemical and morphological events of the developmental system are directed towards the differentiated forms. The **irreversibility** idea was developed by Hardwick and Foster in 1952 when it was observed that the vegetative cells of several Bacillus species sporulated two hours after transfer to distilled water from synthetic medium (5). However, there has been some dispute regarding the classification of bacterial sporulation as true differentiation (6-8). If the process was true differentiation

then the spore should contain only those constituents present in the vegetative cell. However, this is not the case since there is no appreciable calcium in the vegetative cell and this would indicate that sporulation is not the endogenous metabolic process demanded of true commitment (9).

Although a much studied subject, bacterial sporulation still remains very much a mystery. It has been widely accepted that the bacterial spore is a response to the depletion of vital nutrients (10) although other work has disputed such a hypothesis (11). However, Stewart and Weisman has shown that amoebal encystment may occur when the amoeba are transferred to non-nutrient media (11a). The observed dramatic changes in the pH of sporulation medium may result from major metabolic shifts (12, 13), although as yet pH variation of the medium has not been demonstrated to be a prerequisite of sporulation (14). When the culture medium pH falls below pH 5.0 there is an inhibition of sporulation which has been attributed to the existence of conditions which inhibit the tricarboxylic acid cycle enzymes (11, 15). In a complex medium containing glucose (conditions which may produce an acidic medium) the Bacillus species suffer a repression of the tricarboxylic acid (TCA) cycle enzymes during vegetative growth (16, 17). It has been suggested that sporulation may be initiated on de-repression of the TCA cycle (18).

The events which occur during the transition from vegetative cell to bacterial spore include the following: (i) alkaline phosphatase production, (ii) onset of refractility, (iii) initiation of dipicolinic acid synthesis, (iv) development of heat

resistance (18). Each of these steps has been studied in great detail. These steps are regarded as the major events in the production of the eubacterial spore which may be regarded as being produced ~~where refractility has developed.~~ The synthesis of dipicolinic acid together with the subsequent accumulation of calcium and development of heat resistance are secondary characteristics which need not be typical characteristics of the eubacterial spore. The work of Luedemann and Casmer has shown the refractile spores of the actinomycete, Micromonospora chalcea, lack the typical internal spore structure (19).

In recent years there has been an improvement in the understanding of cellular changes and several groups of workers have begun to characterise such changes in terms of regulation of protein synthesis at the transcription and translation loci. Several reports have considered cryptobiotic stages, such as sporulation, to be due to a block ^{at the} level of transcription (20-22), or translation (23, 24).

The primitive adaptation exhibited by Escherichia coli when it changes its protein pattern under conditions of starvation may represent regulation at the transcription or translation level (25). Proteolysis of cellular constituents is an important event in more evolved cells and has been observed in sporulating yeasts (26) and in the early stages of development of the slime mould, Dictyostellium discoideum (27). Similar proteolytic events apparently occur in the alga, Acetabularia, which exhibits developmental changes several weeks after the removal of the nucleus (28). Such long term changes in Acetabularia may be due to stable mRNA with a long biological half-life. In the past it has been assumed that

mRNA exists for a short period, although recent evidence has indicated the existence of stable mRNA in sporulating bacteria (29), developing slime moulds (27), developing sea-urchin eggs (30) and in amphibian eggs (31).

With the increase in knowledge of microbial genetics and the availability of a variety of antibiotics capable of perturbing the cells it has become possible to study microbial differentiation to a greater depth. Bacteria which possess several morphological forms through their developmental cycle are the most suitable organisms for studying the interaction of antibiotics and differentiation. In a study of the role of transcription in the temporal control of development in Caulobacter crescentus it was shown that rifampicin addition could inhibit each step in development up to a critical point in the cell cycle (32). The evidence indicated that development in Caulobacter was controlled partly at the transcription level. However, the wide variation in timing between the transcriptional event and the execution of the event implied that other levels of control exist. Other examples of transcriptional control include the inhibition of RNA synthesis by the peptide antibiotic, tyrothricin, in growing cultures of Bacillus brevis (33). Tyrothricin may regulate gene transcription from vegetative growth to sporulation. The inhibition of mRNA synthesis by 6-methylpurine in the imperfect fungus Monacrosporium doehycoides enhances the development of the perithecial initials (34).

An important form of differentiation is represented by cell - cell interactions which are normally regarded as of little consequence to microbiologists. However, such cell - cell interactions

are important events in the life cycle of myxobacteria which go through a process involving both cellular and colonial morphogenesis. In the cellular form myxobacteria are typical Gram-negative organisms until they are transferred to a solid medium lacking specific nutrients where they aggregate in response to chemotactic stimuli and form fruiting bodies consisting of myxospores which are round, heat resistant, refractile and metabolically quiescent cells. Exponentially growing vegetative cells can be converted to myxospores by polyhydric compounds within 100 minutes of addition. Such cell - cell interactions constitute an important adaptation. As yet no significant study has been made of the membranes of such cells (39). Mammalian cell - cell interactions require a variety of medium constituents at low cell density but not at higher populations (35). Other forms of cell culture exhibit a regulation phenomenon as a result of release of auto-inhibitors (36). In comparison, bacteria regulate cell population levels by toxic end product inhibition but this process does not have the same teleonomic implications as the inhibition of multicellular organisms.

The stimulus responsible for production of multicellular structures is not understood. Cyclic AMP diffusion induces aggregation of slime moulds (37). Aggregation of Dictyostelium discoideum results in the transcription of the gene coding for UDP-galactose: polysaccharide transferase, an enzyme which is required for the synthesis of a mucopolysaccharide found specifically in spore capsules (38). In Dictyostelium discoideum prolonged cell contact is necessary for translation of certain mRNA molecules (39).

Investigations of differentiation have included studies of the blue-green algae, a group of organisms which may be representatives of an old evolutionary order and which represent the most complex form of prokaryotic differentiation. The blue-green algal body, the akinete, possesses heat and desiccation resistance which differentiates it from the endospore produced by this group of organisms (40). The akinete has been regarded as an enlarged vegetative cell with modifications, whereas the heterocyst should be thought of as a distinct body formed by vegetative cell differentiation (41) and this is borne out by the greater DNA content of the Anabaena akinetes (42). Akinete production is favoured by a high calcium concentration and low phosphate (43). Additional features which distinguish the heterocyst from the akinete are the cell wall constituents: the akinete possesses less polysaccharide but more amino compounds than the heterocyst, although the akinete and heterocyst possess a carbohydrate composition which is untypical of the vegetative cell wall (44).

Significant variations in lipid composition differentiate the heterocyst from the vegetative cells of Anabaena cylindrica (45). The vegetative cells are characterised by the possession of four unsaturated galactosylglycerides, phosphatidyl glycerol and a sulpholipid - all of which are absent from heterocysts which possess a glycoside and an acyl lipid (45).

The parasitic flagellate protozoa Trypanosomatidae are characterised by the marked alterations in structure and metabolism (46) and many of these characteristic forms are reflected in their variations in lipid and sterol composition (47). When the trypano-

some are cultured on mammalian cells they reflect the lipid composition of the host cell (48).

Although fungi have now become the subject of extensive research the amount of research on the cell membrane does not compare with that conducted on the bacterial cell membrane. The various morphological forms exhibited by fungi has prompted extensive research into the genetics of such development. Wolf and Mirocha have reviewed the hormonal regulation of fungal reproduction (49).

The life cycle of the heterothallic yeasts makes them an ideal group to study in terms of eukaryotic cell division at the single cell level and in this respect they have been studied extensively by Tingle et al (50). The ascospores can be readily differentiated from the vegetative cells by the greater RNA content while the vegetative cells contain much more protein which is unusual in that it contains so very little proline. It has been observed by Illingworth et al that sporulating cells have four times the lipid content of vegetative cells (51). The increased lipid content was represented by triglycerides, sterol esters and phospholipids. There was a significant increase in the unsaturated fatty acid content. In general the ascospore walls, which are thicker and stronger than the vegetative cell walls, contain more polysaccharide than the vegetative cells.

Viral infections induce changes in cell function which can be considered to be a form of differentiation. Infected cells which are continuously releasing parainfluenza virus suffer an inhibition of sphingomyelin synthesis and a stimulation of

glycosphingolipid synthesis (51). The infection of non-permissive E. coli by amber mutants of a specific phage results in the development of aberrant membranous structures in the cytoplasm which coincides with an increase in cardiolipin content (4% to 20%) and a compensating decline in phosphatidylethanolamine (53).

Chapter 2.

The Membrane : Differentiation and Regulation of Uptake of Antimicrobial Agents.

In the early nineteenth century it was proposed by Scheiden and Schwann that the cell membrane/wall played a part in the organisation of cells (54). However, with the discovery of chromosomal genes this idea fell out of favour. In recent years evidence has been obtained which indicates that the physical structure serves to some extent as a template for further growth and thus may determine the pattern of that growth (55, 56). Whitaker produced a cylindrical shaped Fucus cell by drawing the freshly fertilised cell into a narrow capillary tube where the naked cell secreted a thin mucilage which eventually hardened. When the cell was blown out of the tube it retained its deformed shape, and the rhizoid which grew out of it always developed at one end of the cylinder (57).

Bacterial cells deprived of the rigid mucopeptide become spherical in liquid while the isolated mucopeptide retains the shape of the intact cell (58). However, the mucopeptide itself does not solely determine the shape of the progeny cells since partial or complete removal of this layer does not change the morphology of progeny cells derived from the altered cells (59). Point mutations are known which alter the shape of the cell which indicates some direct genetic control of cell shape (60).

It has been suggested that the cell surface membrane may regulate cell growth (61) and animal cell division (62). Many attempts have been made to correlate chemical changes of the

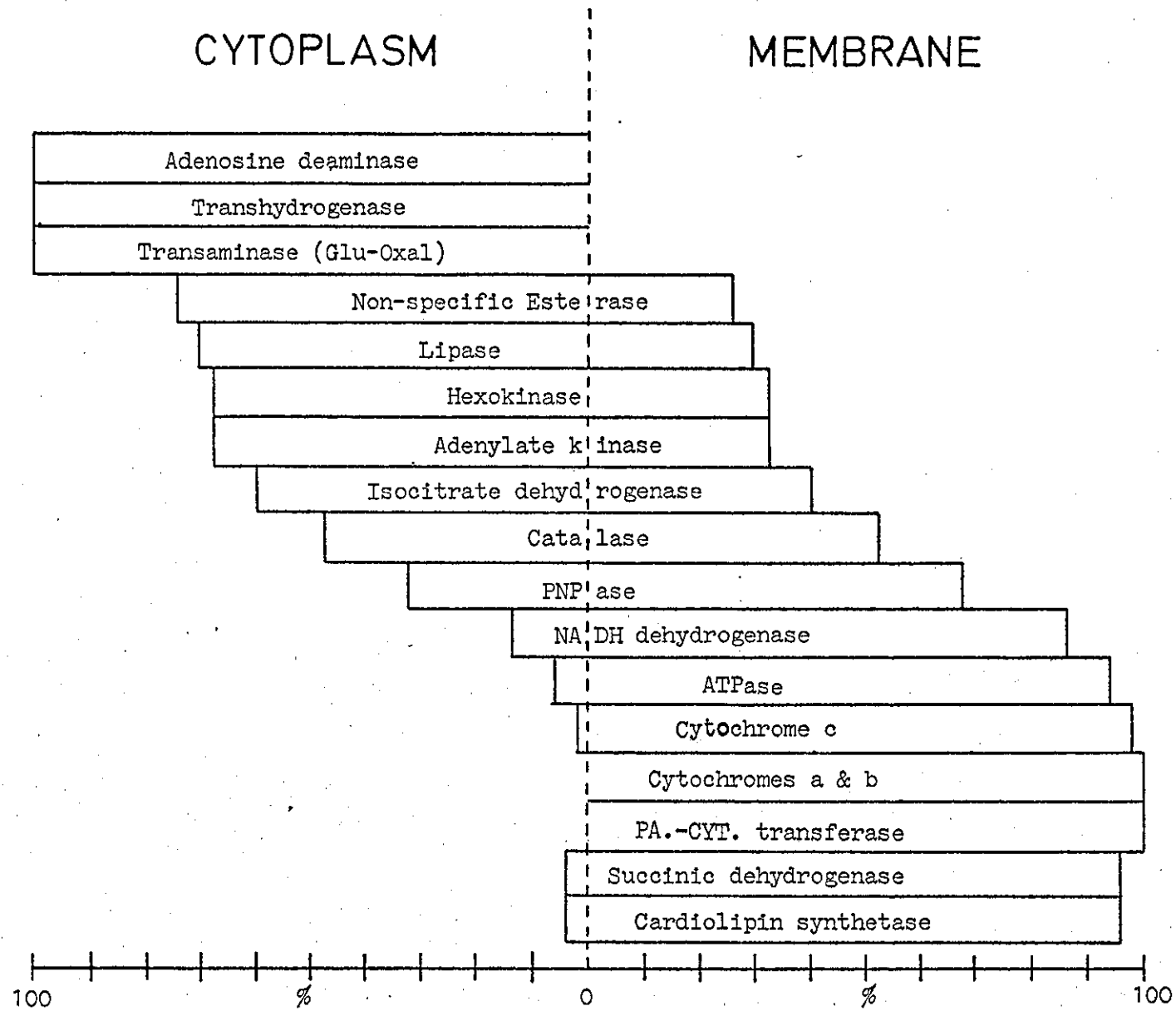


Fig. 1.

surface membrane with alterations in cell growth (63-65). The lectin, concanavalin A, has been found to interact with various carbohydrates of cell surface and has demonstrated that configurational differences of the cell surface may regulate cell growth (66). Transformed cells produced by a chemical or viral technique interact more strongly with the lectin than the parent normal cells. It is believed that the agglutin site is present in the surface of the normal cell but that it is in a configuration which prevents interaction with the lectin. Some species of transformed cells exhibit normal characteristics at a non-permissive temperature while at the permissive temperature they display agglutinability (67). Plainly membranes are susceptible to changes of 2-3°.

In studies of cultured rat muscle cells it has been observed that lysolecithin modified the cell membranes during differentiation (68).

Variations in the physical properties of membranes could have quite extensive consequences on the chemistry of the cell as a result of altering membrane bound enzymes. Salton has produced a histogram (Fig 1) which shows the distribution of enzymes of Micrococcus lysodeikticus (69).

Sterols and Membrane Composition.

To the chemist sterols are generally regarded as rather unreactive molecules and it is possible that at first consideration there could be a tendency to play down their contribution to membrane structures. However, the work of van Deenen and his colleagues has contributed a great deal to elucidating their role in membranes (70-74).

It would appear that two roles are possible for sterols. One role could involve their incorporation into the membrane, and in this respect could regulate cell differentiation in fungi such as the Pythiaceae which are unable to synthesise sterols and consequently exhibit sterol dependence. In media which lack sterols the growth of Pythium spp and Phytophthora spp is generally vegetative but when the media is supplemented with suitable sterols vegetative growth is increased and sexual organs (oogonia and antheridia) develop and oospores are formed (75-80). Elliot et al (81) have examined many sterols and steroids and concluded that active compounds must possess a β -3-hydroxy with methyl groups at C-10 and C-13, and a side-chain containing 8-10 carbons at C-17. The biosynthetic precursors of cholesterol, squalene and lanosterol, were inactive. Similar sterol requirements have been observed for Paramecium aurelia (82). When the sterols were replaced by bile acids or sterols containing hydroxy groups or ketone groups in the side-chain the sterols were no longer active (83).

A protozoan of questioned taxonomy, Labyrithula, exhibits a sterol dependence (84) while in some cases parasitic amoebae have exhibited a similar dependence which is not characteristic of free-living amoeba (85). In addition to being incorporated into cell membranes sterols may interact more directly with cell metabolism and this subject has been reviewed by Buetow and Levedahl (85).

With the exception of the Mycoplasmatales sterols have not been isolated from the prokaryotes. Some workers maintain that they have isolated large quantities of sterols from prokaryotes

(86). However, it is generally agreed that eubacteriales are devoid of sterols. The eubacteriales are able to conduct the common metabolic sequence of reactions acetate → farnesol → sequalene → C₅₅-isoprenyl pyrophosphate but apparently are unable to undergo the oxidative cyclisation required to produce sterols. It is possible that in some cases the antibacterial action of sterols and steroids could be due to interaction with the synthesis of C₅₅-isoprenyl pyrophosphate.

The response to steroid supplementation is varied: Tetrahymena pyriformis S exhibited stimulated growth while T. pyriformis W suffered partial to total inhibition in the presence of steroids (87). A variety of androgenic, oestrogenic and adrenal steroid hormones at concentrations as low as 10⁻⁷M-10⁻⁹M accelerated the exponential growth rate of a streptomycin-bleached Euglena sp. (88). Low concentrations of oestradiol stimulated E. coli growth while higher concentrations were inhibitory (89). Deoxycorticosterone (10⁻⁴M) inhibited the growth of a large number of Gram-positive and Gram-negative bacteria as well as inhibiting several moulds and yeasts (90). Hydrocortisone (10⁻⁶-10⁻⁷M) stimulated growth of Salmonella typhosa while cortisone (10⁻⁴M) inhibited growth and caused marked cytoplasmic changes (91).

Conner and Longobardi demonstrated that colchicine inhibition of T. pyriformis could be prevented by stigmasterol (92) which together with cholesterol could annul the effects of 2,4-dinitrophenol on this organism, however, this protective action was exerted only during growth (93). Sterol glycosides afforded greater protection against purine analogue inhibition than the parent sterols (94).

The natural and synthetic oestrogens are bactericidal to Gram-positive organisms only (95, 96) and the following activity order has been assigned: stilbenes > deoxydiethylstiboestrol > oestrone > oestradiol (108). 21,21-Dimethoxyprogesterone exhibited fungistatic and bacteriostatic activity which was reversed by squalene and deoxycorticosterone in submerged culture only (97). Respiration rate studies have indicated deoxycorticosterone could function by uncoupling phosphorylation from oxidation (98). Oxidation of triphosphopyridine nucleotide by the enzyme preparations from mammalian and bacterial cells was inhibited by low concentrations of certain steroids (99).

Saccharomyces cerevisiae under aerobic metabolism synthesises ergosterol (100) and regardless of the medium used anaerobic cells always contain less sterol and less fatty acid than aerobic cells (101). A close correlation between suppression of sterol synthesis and the loss of respiratory capacity in S. cerevisiae has been observed (102). In addition, it has been observed that cytochromes a and b are not synthesised (103). The anaerobic culture of yeasts is characterised by the absence of mitochondria, and mitochondria are absent from yeasts cultured in the presence of sterols (104). This is yet another means of regulating differentiation.

Membranes and Non-Sterol Components.

Membranes contain a large proportion of lipids and any variation of such constituents could influence cellular functions which are dependent upon the integrity of the membrane. Such variations are observed in the case of Haemophilus parainfluenzae which exhibits a perturbation of the membrane-bound electron

transport system (105). Staphylococcus aureus exhibits similar changes as a result of membrane labilisation due to an inhibition of carotenoid and phospholipid synthesis (106, 107). Mindich has observed the maintenance of nucleic acid synthesis in Bacillus subtilis glycerol auxotrophs which although no longer able to synthesise phospholipids do continue to maintain membrane development through protein synthesis (108). Although there was no net increase in phospholipids there was active metabolism of the phospholipids which resulted in a shift in the proportions of the phospholipids (109).

In the case of glycerol deprivation of a S. aureus glycerol auxotroph it was observed that fatty acid synthesis continued at the normal rate with an accumulation of unesterified fatty acids while there was a complete cessation of phospholipid and isoprenologue biosynthesis. While there was a conservation of the glycerol esters, complex phospholipids and glucolipids other constituents suffered an immediate fall in the synthesis rate: monoglucosyldiglyceride (30%), diglucosyldiglyceride (60%), and polar and non-polar carotenoids (50%). Although the non-specific membrane proteins were synthesised other functions such as glycine transport halted (110). These experiments complement Overath's demonstration that there is a complete randomisation of the lipid phase during the synthesis of E. coli membranes (111).

Over the years there have been a variety of investigations of inhibition and stimulation of lipid synthesis. Fatty acid biosynthesis has been impaired by using flavin mononucleotide (10^{-4} M) and acriflavine (10^{-5} M) to block the sulphhydryl groups of dehydrogenases (112). While biotin supplementation of Lacto-

Bacillus plantarum lowered the cis-vaccenic and lactobacillic content in favour of an increase in palmitic acid (113); a biotin limitation in S. aureus resulted in a protoplast lipid decrease of 20% and a protein decrease of 30% (114). The lysphosphatidyl glycerol content increased by 100% with a small increase in teichoic acid content. These lipid deficient cells were more sensitive to a series of phenols which contained long chain alkyl substituents (115).

Mycobacterium tuberculosis cultured on a glycerol-free medium contained 8-13% lipid but 23-45% lipid on a glycerol medium (116). Other experiments on lipid depletion of M. tuberculosis included iron salt supplementation and replacement of glycerol by glucose (117-121). Although sodium acetate supplementation increased the yield by 180% the cells contained less lipid (122). Pantothenate deficiency reduces the lipid content of Acetobacter suboxydans (123). The influence of the medium composition on the lipid content of Corynebacterium diphtheriae (124), Agrobacterium tumefaciens (125), B. mucosus and B. megaterium (126) has been studied.

Other studies of lipid depletion have involved the action of streptomycin (30 μ /ml) on Serratia marcescens which exhibited depsipeptide depletion but an increase in the phospholipid content (127). The resultant leakage of nucleotides from E. coli cultured in the presence of streptomycin may have been due to either lipid or protein loss from the membrane (128). Penicillin has been reported as failing to inhibit the incorporation of glycerol into the lipid fraction of S. aureus and B. megaterium (129, 130). However, low doses of penicillin produced

filamentous E. coli and while not influencing the total rate of phospholipid formation did succeed in altering the ratios of individual phospholipids. Filamentous forms contained more cardiolipin and less phosphatidyl glycerol than normal exponentially dividing cells. U.V. induced filaments showed similar changes in the phospholipid ratios (131).

The anti-protozoal drug, pentamidine, inhibits lipid metabolism in Crithidia fasciculata and also inhibits S. aureus at 30 µg/ml when it suppresses nucleic acid and phospholipid synthesis. The incorporation of glycerol was more insensitive than the phosphate incorporation, and displayed a resistant fraction which corresponded to neutral fat (132). Other inhibitors of glycerol metabolism include N-iodoacetyl-glucosamine which acted upon E. coli (133).

No detailed study has been made of the interrelationship of glycerol transport and the subsequent incorporation into lipids. It has been generally accepted that cells are freely permeable to glycerol (134) and that glycerol was captured by glycerol kinase (135). Subsequent research revealed that the glycerol uptake in E. coli K12 was mediated by an inducible facilitated diffusion process controlled by a specific gene product (136) while an inducible binding protein specific for glycerol was demonstrated in Pseudomonas aeruginosa. It was concluded that glycerol entry into the cell occurred by either facilitated diffusion or active transport (137). More recent work with Nocardia asteroides has demonstrated that a saturable and non-saturable mechanism operate and that the enzyme systems have the characteristics of facilitated diffusion or active

transport and free diffusion, respectively (138).

In some cases antibiotics have demonstrated the possibility of studying the most elementary form of cell differentiation - dividing bacterial cells. Low concentrations of penicillin induce giant forms of E. coli (139), filamentous Proteus vulgaris (200 μ) (140), and elongated forms of Streptococcus lactis, S. thermophilus and Leuconstoc dextranicum (141). Since a wide number of enzymes are located on the cell membrane it is possible that these cells fail to divide because of some membrane perturbations. Electron microscopy of filamentous E. coli K12, produced by irradiation, reveals an intricate network of intracellular membranes (142). Other forms of filamentous E. coli have included heat induced forms which were shown by electron microscopy to lack a septum (143). Ultrasonication failed to break up the filaments. Other morphological changes have included coccal forms of B. subtilis which were induced by salt curing (144). It is unfortunate that none of these studies included an investigation of the lipid contents.

Over the years yeasts have continued to remain one of the preferred cellular forms for those workers investigating cell chemistry. Schopfer et al attributed morphological variation in Schizosaccharomyces pombe to induced inositol deficiency (145) while Dawson et al isolated a variety of inositol lipids from Kloeckera brevis (inositol⁻⁻⁻) (146). An inositol deficient strain of S. cerevisiae accumulated acetone-soluble lipids (147). When S. carlesbergensis was grown under conditions of inositol deficiency the cells exhibited the unusual phenomenon of aggregation which was attributed to a defect in the cell wall synthesising

mechanism since the cells were found to contain greater amounts of glucan than the normal cells (148, 149). The internal structure of the inositol-deficient S. carlesbergensis was deranged and lipid globules were present (150). A similar accumulation of lipids occurs in inositol deficient Neurospora crassa (151).

Other studies of yeast lipid metabolism have involved choline supplementation of S. cerevisiae medium which stimulated phosphatidyl-choline synthesis (152) and in a similar manner Ratcliffe et al promoted S. cerevisiae phosphatidyl ethanolamine synthesis by ethanolamine supplementation (153).

Chapter 3.

Factors Affecting Thermophily.

A thermophile is generally regarded as an organism that grows at temperatures above those considered to be the maximum limits for most forms of life. In the case of bacteria and fungi they are considered to be thermophiles if they grow at 45° and above. The thermophilic yeasts are not discussed here since they are considered to be thermophilic if they grow at 37° and above.

Since Tsiklinsky's discourse in 1899 on the thermophilic fungus, Thermomyces (Humicola) lanuginosa (154) there have been many reviews on the subject and the principal authors have included : Gaughran, who lists twenty five theories of thermophily, (155), Wood (156), Koffler (157), Farrell and Rose (158), Farrell and Campbell (159), Brock (160, 161), Christophersen (162), and Langridge and McWilliam (163). The most recent review on this subject is that of Crisan (164).

In this review the heat resistant prokaryotic spores are included.

The primary factor that determines the ultimate temperature limits for the growth of an organism is its genetic constitution. There is evidence that the ability to grow at high temperatures can be transmitted from the thermophilic to mesophilic bacteria by genetic transformation (165-167). As yet technology is not able to probe the gene to greater depths.

Some of the principal theories of heat resistance are detailed below.

Cell Size.

Slender cell size is a characteristic of thermophilic variants of B. cereus, B. megaterium (168), hot spring blue-green algae (169) and thermophilic actinomycetes which are much smaller than their mesophilic counterparts (170). The large surface to volume ratio of small diameter cells may favour thermophiles since there will be a rapid inflow of substrates and a correspondingly high excretion rate (171). However, this theory breaks down in the case of the thermophilic fungi which have a diameter in excess of 10 μ (172).

Nutritional Control.

The idea that nutrition may contribute to heat resistance is based upon the observations that thermophiles tend to require a complex medium and this was interpreted as a requirement for ready made "building-blocks" (173). However, this theory is not entirely inviolate as it has proved possible to grow the thermophile Thielavia thermophilum on a highly defined medium (174).

However, subtle variations in the medium composition such as oxygen content may explain some observations (175). McFeters and Ulrich observed that the extreme thermophilic bacterium Thermacus aquaticus has an oxygen uptake four times greater at 70° than at 50° (176). This increased respiratory rate could indicate a difference between the electron transport of this thermophile and that of mesophiles. The decrease in oxygen content of a medium with increase in temperature has been shown to influence yield and growth rate. Streptococcus lactis grown at 34° (9° above optimum) exhibited a normal growth rate only

when the medium was rapidly aerated (177). The dissolved oxygen content of water falls by 50% when the temperature is raised from 37° to 55°. Other responses to medium composition have been demonstrated in the case of Saccharomyces cerevisiae which did not grow above 42° in a medium lacking ergosterol and oleic acid (178).

A factor closely identified with nutritional control is that of growth rate. It has been observed that Bacillus stearothermophilus exhibits a more rapid turnover of labile RNA than E. coli (179). Similar variations in protein turnover have been observed for a bacillus thermophile which exhibited a maximum synthesis rate at 37° and 75° while the minimum occurred at 45°-55° (180). In the case of thermophilic Bacillus subtilis there is greater heat resistance when the organism is cultured under rapid growth conditions (181).

Stability of Cellular Constituents.

An attractive theory of thermophily is that essential macromolecules such as enzymes and other proteins possess an unusual degree of thermostability. However, contradictory results have been obtained regarding the heat stability of such proteins. Some reports indicated the proteins of a thermophile were no more stable than those from mesophilic counterparts (182), while other reports were the opposite (183). An early report indicated that protein coagulation did not parallel the thermal death of the organism (184). Ray has summarised the conflicting data concerning heat stability of proteins (186). (At this point it is sufficient to comment

that a solution of protein contained in a beaker is in an environment totally different to that of a cell). However, some studies of isolated protein have shown that there is an intrinsic stability to heat as demonstrated by flagellin isolated from a Bacillus thermophile where the viscosity decrease occurred at 70° in comparison to that of E. coli which occurred at 50°-60° (186, 187). Other similar studies have indicated that the heat stability of nucleic acids could be attributed to the base pair composition (188).

The response of proteins to heat could influence allosterism and some reports indicate that allosteric enzymes lose their affinity for the effector molecule with temperature elevation (189).

The Role of Ordered Water in Thermophily.

Over the years water has often been considered an important controlling factor in the life of a cell. In a great number of publications the cell has been presented as a minute sack containing macromolecules haphazardly dissolved in water.

In 1892 Roentgen proposed the existence of ordered water to explain ice formation as an equilibrium between a more dense species and a bulkier species of water. As knowledge of the structure of water increased attempts were made to correlate the temperatures of microbial growth optima (11°, 25° and 39°) and growth minima (16°, 31° and 43°) with the known physical changes of water which occur over a narrow range ($\pm 2^\circ$) near 15°, 30°, 45° and 60° (190). Similar dramatic changes in

physical properties occur below 0° and these changes are interpreted in terms of the molar Gibbs free energy (191). Ice-formation in water is accompanied by an abrupt fall in density of water, a halving of the specific heat, an increase in viscosity ($10^{18} \times$) and a drop in the vapour pressure, which is lower than that of supercooled water.

Direct evidence of ordered water can be observed as a result of slow cooling gelatin-water (12:88) gel which continues to produce ice at the gel surface until the water content reaches 34% and finally fails to freeze even at -70° (192). It is possible that living cells could exhibit similar characteristics (193, 194). When water molecules within biological membranes are in close proximity to the protein and phospholipids that comprise the membrane it is possible that such propinquity to charged groups could result in an acquisition of unusual properties by the water molecules. Chemical evidence of ordered water has been obtained by studying the exchange rate of labile protons for deuterons from deuterium oxide in $>CONH_2$ groups where a very slow rate is obtained when the amide is attached to a polymeric matrix and a rapid rate when the amide is part of a small molecule (195).

This interaction between water and cells could have a consequence in protection against heat since the ordered water structures could act as a heat sink. In an examination of cell water of spray-dried active Saccharomyces cerevisiae Koga, Echigo and Nonamura identified four regions of cell water : the solution region, the gel region, the mobile adsorption region and the localised region (196). The

"solution region" water functions as a continuous medium so that the biochemical reactions may proceed at retarded rates. The gel region water molecules no longer function as a continuous solvent for biochemical reactions. The "localised water region" and the "mobile adsorbed region" represent the water which has lost its various modes of molecular motion. The "localised water" molecules have a strong interaction with the organic cell constituents with an estimated heat of adsorption of 20 kcal/mole. The absence of dielectric polarisation would suggest that the water is irrotationally bound to some strongly ionisable sites. The "mobile adsorbed region" is characterised by a heat of adsorption which varies from 20 to 10 kcal/mole, and an N.M.R. line width adsorption of 10^{-2} - 10^{-1} gauss, indicating that molecular exchange might possibly take place in a limited way between adjacent adsorption sites. The molecules of the gel region are highly mobile and cluster around non-ionisable polar sites.

The interaction of water with membranes could have a much more far reaching consequence than merely producing ordered water structures. Over the last few years many instrument techniques, such as N.M.R., have shown that lipid methylene groups exist in several solid phases which lie between rigid-like matrices similar to those of low temperature environments and the fully mobile liquid system. The addition of water to such systems decreases the rigidity of the matrix and the methylene protons enter a more fluid environment (197, 198). N.M.R. spectra also show protein protons to exist in

various forms from a solid state through to a liquid state (199). Such increases in molecular motion in the presence of water have been recorded for other biopolymers such as cellulose (200). Hanstein, Davis and Hatefi have demonstrated that certain anions (haloacetates, SCN^- , ClO_4^- , I^- , NO_3^- , Br^-) destabilise biomembranes and multiprotein complexes and increase the water solubility of a variety of non-electrolytes (201).

(The interaction of water with lecithin-cholesterol systems and with Ca^{2+} -phospholipid systems is discussed in Chapter 5).

The Cell Membrane in Heat Resistance.

In many cases cells respond to high temperatures by variations in the lipid content and composition. Henriques and Hensen were the first to suggest a relationship between lipid character and high temperatures when they observed an inverse correlation between melting point of animal fats and the temperature at which it lived (202). Since then many intermittent reports suggested that the protoplasmic lipids determined the heat resistance of the organism (203-208). High temperatures have been shown to favour an increase in saturated fatty acids in the lipids from bacteria and animals (203, 205, 208) and such variations in membrane composition could be interpreted as a need to maintain a certain protoplasmic viscosity. This specificity for protoplasmic viscosity is exemplified in the case of Serratia marcescens (209, 210), which when grown at 10° rather than at 30° exhibits a decrease in cyclopropane and palmitic acids in favour of an increase in the hexadecanoic and octadecanoic acids. The specificity of fatty acid structure has been succinctly demonstrated by the use of an E. coli fatty acid auxotroph which exhibited normal growth

at 37° by incorporation of trans-octadecanoic acid but underwent lysis at 27° (211). This variation in fatty acid saturation with temperature has been rationalised by James in terms of an increased oxygen content of the medium as the temperature is lowered (212).

Based upon many of these early reports Brock has updated the early reports and has become the principal supporter of the theory that life at high temperatures is due to a specific membrane composition (160, 161). Ray has studied the effect of temperature on the protoplasts of mesophilic Sarcina lutea and Streptococcus faecalis and on the protoplast of Thermacus aquaticus (185). Temperature sensitivity was monitored in terms of loss of intracellular ¹⁴C-glycine and decrease in transmission of cell suspensions. Both techniques were in good agreement at 60° while the thermophile showed no comparable lysis until the temperature was increased to 90°. Mesophilic protoplasts could be stabilised against lysis at 60° by addition of the polyamines, spermine and cadavarine. Additional evidence of the significance of protoplast stability has been presented by Golovacheva who showed that protoplasts of Bacillus stearothermophilus were resistant to heat shock and to osmotic shock (213).

In addition to variations in fatty acid composition with temperature some organisms such as the thermophilic alga Cyanadia caldarium show major increases in glycolipid content (90% increase) when it is cultured at 45° instead of 20° (214). In addition to linoleic acid replacing linolenic acid, the predominant acid at 20°, there was 40% more phospholipid present.

While some cells respond to temperature changes by variations in lipid content other cells have possibly adapted themselves to very hostile environments by synthesis of very unusual lipids. Bu'Lock et al have observed that an acidophilic thermophilic bacillus has unusual lipid constituents such as pentacyclic triterpene hydrocarbons and cyclohexane fatty acids (215) while Langworthy et al (216) have shown that Thermoplasma acidophilum possesses the usual lipid constituents as well as the unusual long chain isoprenol glycerol diether molecules. Oshima and Yamakawa have complemented the work of the Langworthy group by identifying similar lipids in an extreme thermophile, Flavobacterium thermophilum (217).

Some early work by Dyer attempted to explain the heat resistance of vegetative cells of B. stearotherophilus 2184 in terms of high melting point sphingomyelins (218). However, Gard et al (219) showed that the principal phospholipids were diphosphatidyl glycerol, phosphatidyl glycerol and phosphatidyl ethanolamine and in this claim they have been supported by Oo and Lee who observed similar phospholipids in B. stearotherophilus B65 (220). Other investigations of heat resistance of eubacteriales were conducted by Sugiyama who showed that fatty acids present in the medium of Clostridium botulinum produced heat resistant spores (221) although the extraction of surface lipids from the spores did not decrease heat resistance (222, 223).

Some attempts have been made to rationalise optimum temperature of growth with phase transitions of membranes. Raison et al used spin labelled fatty acids to demonstrate that

the mitochondria of a chill-resistant plant and a poikilothermic animal displayed no membrane phase transitions similar to those observed for a chill-sensitive plant and a homeothermic animal (224). These phase transitions occurred at 12° and 23°, respectively, and coincided with the temperatures at which the plant and animal suffered injury upon exposure to adverse temperatures. Such results may be taken to indicate that some membrane-bound enzymes are sensitive to the physical state of membrane lipids.

Although many investigators of thermophily have concentrated their effects upon an investigation of the organic constituents of cells a few other investigators have noted the ability of divalent cations, such as Mg²⁺ and Ca²⁺, to increase the thermostability of enzymes, ribosomes, membranes and other cellular components (155, 225-231). Thermophilic bacteria appear to have a higher requirement for Ca²⁺ than do mesophiles (232) and this calcium requirement for resistance to heat is a characteristic of the heat resistant prokaryotic spore. A detailed review of the membrane bioinorganic chemistry of calcium appears in Chapters 5 and 6.

The Bacterial Spore as a Special Case of Thermophily.

A considerable number of research publications have been concerned with the heat resistance of the eubacterial spore and it is very interesting that these publications have failed to include references to other heat resistant cells such as the akinetes of blue-green algae which exhibit desiccation and heat resistance (233). A detailed spore bibliography is contained in several books (1-4).

The extraordinary properties of bacterial spores increasingly appear to be related not only to peculiar biochemical components and processes but also to a distinctive anatomy. The very inert character of the spore has prevented any extensive chemical investigation. Bacterial spores are optically refractile and resistant to microbiological stains and cell poisons. X-ray diffraction studies reveal that spores are unusual in that they contain keratins (234) which are synthesised from sulphur-containing, water-soluble substances contained within the original vegetative cell. Keratins, such as wool, are characterised by their ability to take up large quantities of water (35% by weight). There have been many investigations of the water content of spores as it has been suggested that the specific water content may be an index of the heat resistance of the spore. (It is possible that the adsorbed water may serve as a heat sink).

Black and Gerhardt (235) and others (235-238) used heavy water (D_2O) to investigate the nature of spore water. These various workers calculated that the spore contained ca 60% water and that 85% of its volume was available for water uptake. These experiments indicated that the spore contained no water which was not readily exchangeable with external water. However, these studies did not indicate if the spore contained any region which was impermeable to water. Lewis et al proposed that the spore possibly achieved a measure of impermeability by contraction of the spore cortex during its maturation (239). Other workers have suggested that the difference in water content of the spore and vegetative cell was insufficient to contribute to heat resistance and they suggested that the form of the bound water

may be more significant (240, 241).

In an evaluation of bound water in B. megaterium Koga and his co-workers extended their studies on yeast and they concluded that there was no major difference in terms of thermodynamic properties in an intermediate humidity range (242). On the basis of dielectric constant studies and nuclear magnetic resonance measurements they concluded that spore water was less mobile than that in vegetative cells.

Black and Gerhardt (235, 243) have considered that the heat resistance of the bacterial spore might not entirely be due to its impermeability since 67% of the spore can be penetrated by exogenous water and 40% by exogenous glucose. Experiments with tritiated water showed that the internal and external water of the spore is virtually in complete equilibrium and thus tends to refute the idea of an anhydrous core (243). A larger number of ionic and non-ionic compounds exhibited restricted spore germinability which may be due to lipid insolubility, electrolyte dissociation and molecular weight (243). On the basis of such experiments it has been suggested that the core of the dormant spore exists as an insoluble and heat stable gel, in which cross-linking between macromolecules occurs through stable but reversible bonds so as to form a high polymer matrix including entrapped 'free' water.

Murrell has objected to this technique of Black and Gerhardt on the grounds that solutes could be concentrated by adsorption and in addition there are the inherent errors due to the non-mixing of water (244). As yet it has not been

entirely disproved that there may be an impermeable region which could be anhydrous and responsible for the heat resistance of the spore (243).

In addition to the contribution of bound water to the heat resistance of spores it has been suggested that dipicolinic acid and calcium may be agents which control spore heat resistance. Since the discovery by Curran et al (245) in 1943 of the high concentration of calcium in the aerobic bacillus spore many workers have demonstrated that both aerobic and anaerobic bacillus spores with a calcium deficiency possessed a reduced heat resistance (245, 246). To date no cation, under a wide variety of conditions, has been found to confer the same degree of heat resistance as calcium although strontium, barium, nickel and zinc gave a better degree of heat resistance than no metal at all (247, 248). Manganese(II) gave spore crops that germinated extensively during harvesting (249).

Since its discovery by Powell in 1953 (250) dipicolinic acid (DPA, pyridine, 2,6-dicarboxylic acid) has in various ways been related to heat resistance of bacterial spores. Spores are unique in that they contain large amounts of DPA : 5 to 15% of their dry weight (251-253). The appearance of heat resistance during formation and maturation of spores has been correlated with the synthesis of DPA (254-256).

An intimate relationship between DPA and calcium has appeared. The biosynthesis of DPA takes place at a late stage of sporulation when forespore formation appears to

be completed (256, 257). Calcium uptake, decrease of stainability, increase of refractility and appearance of heat resistance of endospores occur in parallel with DPA formation (256, 258). Vinter has postulated that calcium accumulation to a certain level might include DPA synthesis (259). When sporulation takes place in absence of calcium then the spores are not heat resistant and there is a decrease in refractility together with a fall in DPA content (248, 260).

When calcium is added after the initiation of DPA synthesis it does not facilitate a total recovery in the DPA synthesis, which normally reaches a level proportional to the calcium concentration (157, 167, 260). It would appear that calcium is involved in the induction of/or regulation of DPA synthesis. Calcium and DPA frequently exist in a 1:1 molar ratio (260, 261). A maximum concentration of either component does not confer a maximum heat resistance (262).

Exchange studies with ^{45}Ca indicate that two types of calcium exist : a freely exchangeable species (33%) and a tightly bound form (261). Electron spin resonance studies have indicated that DPA may bind Mn^{2+} and that there is an 85% loss of manganese on spore germination (263). Electron spin resonance studies indicate that Cu^{2+} in spores is tightly bound to proteins (264).

Young found evidence of association of CaDPA with peptide complexes recovered from germination exudates (265). The complex observed masked the α -amino groups since they were unable to react with ninhydrin. Tang et al found that cysteine, alanine and glycine formed stronger complexes with DPA in the presence of DPA than in its absence (266). In addition to

DPA there appear to be other molecules, such as proteins, which can sequester calcium (267).

Rode and Foster succeeded in releasing by electro-dialysis large amounts of DPA from B. megaterium spores suspended in the anode compartment (268) and these spores behaved as if negatively charged. However, when the spores were separated from extremes of pH and temperature there was no DPA loss (269). The release of DPA from B. stearrowthermophilus spores at pH 4.0 in the range of 80° to 100° corresponded to an activation energy of 46,000 calories (270).

Studies on the replacement of DPA by other organic molecules has centred around the synthesis and use of its analogues. The requirements for a CaDPA-like germination include a molecular shape essentially unaltered from that of CaDPA and a metal associating strength somewhat similar to that of CaDPA. 4H-Pyran-2,6-dicarboxylate (PDC), with a pKCa (log of the dissociation constant for the Ca^{2+} chelate) of 3.6 vs 4.2 for DPA, and a near-identical geometry, is fully effective (271). 4-Methyl-DPA, with pKCa 4.3, is ineffective. Isophthalate and pyridine-3,5-dicarboxylate, with shapes nearly identical to that of DPA but with low Ca^{2+} binding strength, are ineffective. Smaller molecules with suitable association constants, such as diglycolate, are ineffective.

In addition to DPA other small molecules, such as sulpholactic acid, have been isolated from B. subtilis spores by Kornberg's group (272). The thorough and systematic work of the Kornberg group showed that 3-phospho-D-glyceric acid

(PGA) became the principal acid-soluble phosphate on the appearance of refractility in B. megaterium, B. subtilis and B. cereus spores. The spore bound PGA is not readily exchangeable with external PGA (273). Rhaese et al have discovered two unknown and unusual polyphosphate compounds which were secreted during sporulation (274). Other small molecules include large quantities of firmly bound glutamic acid as well as lesser quantities of other amino acids (275).

Chapter 4.

Membrane Models.

Many models have been proposed for cell membranes since Carl Nägeli in 1855 conceived the idea of a 'plasmamembrane' to explain the inability of dissolved dyes to enter unicellular plants, algae and fungi. The first postulate on membrane structure was by Gorter and Grendel who used Langmuir trough measurements to show that the erythrocyte possessed just sufficient lipid to permit a bimolecular leaflet to cover the cell (276). The Davson and Danielli model extended the concept of the bimolecular leaflet (277) and it appeared to have joined many other theories in the ranks of undisputable fact when Robertson produced his electron microscopic and X-ray evidence for the Davson-Danielli-Robertson unit model (278). This model implies that the polar head groups of the phospholipids are largely not in contact with the bulk aqueous phase, but in contact with the polar and ionic groups of the proteins. However, thermodynamic considerations rule out this model since it necessitates the exposure of large portions of the hydrophobic groups to water. The electrostatic stabilisation results in the burial of a high percentage of ionic groups in a non-aqueous environment and this requires the input of large quantities of free energy.

The Benson model (279), the successor to the Davson-Danielli-Robertson model, amalgamated several features but was soon redundant since, although, it did have a lower free energy requirement than the Davson-Danielli-Robertson model the free energy level was still not low enough. The Benson model

proposed that hydrophobic interactions were maximised by placing the protein, now assumed to be globular, within the membrane interior. The principal grounds for the unacceptability of this model is that it requires non-fatty acid groups to intercalate with the polypeptide which will result in suppression of the maximum number of interpeptide hydrogen bonds (278).

The X-ray evidence accumulated by Luzzatti and Hussan (279a) and Stoeckenius (279b) from observations of phospholipids have been complemented with theoretical studies. Together with these results, and the Lucy and Glauert work there evolved the thesis that phospholipids existed in small globular micelles (280, 281). Lucy proposed a theoretical model which considered the lipoprotein membrane to be in dynamic equilibrium between leaflet lipids and globular micelle lipids (282). This model of Lucy's is one of the forerunners of the 'Lipid-Globular-Protein-Mosaic' model (LGPM) derived independently by Lenard and Singer (283) and Wallach and Zahler (284).

A considerable amount of instrumental evidence (optical rotary dispersion, infrared spectroscopy and fluorescence techniques) indicated a more intimate interaction between membrane proteins and lipids than had been expounded by previous models. Such evidence of strong interactions weighed strongly in favour of the proponents of the LGPM who cited other earlier isolated reports of such evidence (285-288).

The essential features of the LGPM require interactions between the hydrophobic portions of the lipids and a large fraction of the non-polar amino acids of the proteins. These interactions are said to occur within the membrane interior

which is free from water molecules while the ionic groups are in contact with water. An essential difference between the LGPM and Davson-Danielli-Robertson models is the nature of the protein which in the LGPM presents globular proteins intercalated into the membrane in a thermodynamic sense and not an ad hoc sense. The extent to which the protein molecule may penetrate into the interior of the membrane and whether or not it completely spans the membrane from one surface to the other is dependent upon the size and structural properties of the membrane. As the proteins comprise a large percentage of the dry weight of the membrane and are almost certainly responsible for the specific permeability and enzymatic characteristics of the membrane, then the two models are not only structurally quite different but possess different functional characteristics.

Several membrane systems have been investigated and these have indicated that the lipid of the LGPM is a fluid matrix (289). Experiments with fusion hybrids showed that membrane components on the fused plasma membrane interdiffused on a time scale of minutes (290) and experiments with ferritin-labelled plant agglutins (concanavalin A and ricin) revealed that the lysed red blood cell possessed saccharide moieties on the outside only, with little or no significant rotation of glycoproteins from the outside to the inside under physiological conditions (291). Such results confirmed earlier studies using spin-labelled zwitterionic species, such as phospholipids, which revealed only very slow inside-outside transistions in

synthetic phospholipid vesicles (292).

Singer used these ideas of lipid fluidity to explain contact inhibition of normal cells and the agglutinability of transformed cells by lectins (293). Mild proteolysis can induce normal cells to agglutinate like transformed cells and this phenomenon Singer interprets as an increased aggregation of lectin binding sites which are forced together by the increased hydrophobicity of the membrane which in turn arises from loss of glycoprotein units. Support for such ideas is strong since malignant transformed cells exhibit distinct chemical changes in the glycolipids and glycoproteins of the cell membrane (294, 295). It is evident that if such fluid regions do exist in membranes then they could afford a delicate mechanism of control over many cellular events, and such controls have been proposed by Changeaux's cooperative interactions which he terms cis and trans-effects (296). The Changeaux postulates consider trans-effects to be cooperative (allosteric) changes which operate at some localised part of the membrane surface and transmit an effect from one side of the membrane to the other. It is possible that an integral protein may exist in the membrane as an aggregate of two (or more) subunits, one exposed to the aqueous solution at the outer membrane surface while the other subunit is in contact with the cytoplasm. A specific interaction by a molecule on the outer integral subunit could bring about a conformational change which could transmit the effect to the cell interior. The cis-effects are similar cooperative changes dependent upon the allosteric phenomenon but these effects differ from the trans-effects in

Table 1.

Membranes from Gram-positive bacteria (308).

| Organism | Protein | RNA | Lipid | Hexoses | Nature of hex. |
|---|---------|-----|-------|---------|----------------|
| Bacillus subtilis | 63 | 20 | 16 | + | glc* |
| Bacillus licheniformis | 75 | 0.8 | 28 | + | glc, gal |
| Bacillus stearothermophilus | 74 | 11 | 18 | + | glc |
| Bacillus megaterium | 75 | 12 | 7 | 1.5 | glc |
| Bacillus megaterium M | 67 | 1.3 | 19 | 4.8 | glc |
| Bacillus megaterium KM | 65 | 5.1 | 20 | 8.0 | glc |
| Micrococcus lysodeikticus | 68 | 2.3 | 23 | ++ | glc, gal, man |
| Sarcina lutea | 57 | 5.4 | 23 | | glc, gal, man |
| Staphylococcus aureus H | 67 | 4.6 | 23 | 0.9 | glc |
| Streptococcus faecalis | 46 | 2.7 | 32 | + | glc, gal |
| Streptococcus group A | 68 | 2.0 | 25 | 2.1 | glc |
| Streptococcus pyogenes | 68 | - | 15 | 1.7 | glc |
| Bifidobacterium bifidum var. pennsylvanicus | 70 | 8.3 | 8 | 12 | glc, gal |

*Abbreviations: glc, glucose; gal, galactose, man, mannose; hex, hexose.

Table 2.

Lipid composition of animal and bacterial plasma membranes (308).

| Membrane | Animal | Bacterial |
|--------------------------------|----------------------|----------------------------|
| Ratio protein/lipid | 0.25-4 | 2-10 |
| Cholesterol | present ₁ | absent ₂ |
| Phosphatidylcholine | present | absent ₂ |
| Sphingolipids | present | absent ₃ |
| Polyglycerolphospholipids | very little | important |
| Aminoacyl-phosphatidylglycerol | absent | often present ₄ |
| Glycosyldiglycerides | very little | present |
| Charge of polar head groups | slightly negative | negative |
| Polyunsaturated fatty acids | present | absent |
| Branched or cyclopropane acids | absent | present |

1. except in erythrocytes of ruminants
2. except in the genera Agrobacterium and Hyphomicrobium and in photosynthetic bacteria
3. except in Bacteroides melaninogenicus
4. in most Gram-positive organisms and Pseudomonas strains

Table 3.

Lipid composition of plasma membranes (308).

| Origin | Major polar lipids | Major fatty acids |
|-------------------------|----------------------------|---|
| Myelin | Cerebrosides, PE, PC, S | 18:0, 18:1, long-chain, α -hydroxy |
| Human erythrocyte | PE, PC, S | 16:0, 18:0, 18:1, 18:2, 20:4 |
| Rat liver | PC, PE, S | 16:0, 18:0, 18:1, 18:2, 20:4 |
| Ehrlich ascites cell | PC, PE, S | 16:0, 18:0, 18:1, 18:2, 20:4 |
| Bacillus megaterium | PE, PG, Lys-PG, Gluc-PG | br 14:0, br 15:0 |
| Streptococcus pyogenes | DPG, Glc-DG | 16:0, 16:1, 18:1 |
| Bifidobacterium bifidum | X, PG, Gal-DG | 16:0, 18:0, 18:1 |
| Lactobacillus lactis | PG, Lys-PG, Glycolipids | 16:0, 18:1, cyclo 19:0 |
| Escherichia coli | PE, PG | 16:0, 18:1, cyclo 17:0, cyclo 19:0 |
| Mycoplasma laidlawii | PG, DPG, Glc-DG | 16:0, 18:0, (18:1) |

Abbreviations: PE, phosphatidyl ethanolamine; PC, phosphatidyl choline; S, sphingomyelin; PG, phosphatidyl glycerol; Lys-PG, lysyl phosphatidyl glycerol; Glc-PG, glucosaminyl phosphatidyl; DPG, diphosphatidyl glycerol; Glc-Dg, glucosyldiglycerides; X, phosphogalactolipid; Gal-DG; galactosyldiglycerides. Fatty acid designation is by number of carbon atoms, plus the number of double bonds while prefixes "br" and "cyclo" stand for branched and cyclopropane, respectively.

Table 4.

Protein and lipid content of membrane (309).

| Membrane | Protein lipid (wt/wt) | Cholesterol polar lipid (mole/mole) |
|----------------------------------|-----------------------------|---|
| Myelin | 0.25 | 0.70-1.2 |
| Chloroplast lamellae | 0.80 | 0 |
| Endoplasmic reticulum | 0.70-1.2 | 0.03-0.08 |
| Plasma (liver cell) | 1.0 -1.4 | 0.3 -0.5 |
| Bacteria (Gram-positive) | 2.0 -4.0 | 0 |
| Mitochondria (inner membrane) | 3.6 | 0.02-0.04 |

Table 5.

Amino acid distributing in some membrane (310).

| Membrane | Acidic amino acids Glu, Asp | Basic amino acids His, Lys, Arg | Non-polar amino acids Gly, Ala, Leu, iLeu, Val, Phe, Met, Pro, Tyr | Sulphydryl cysteine cystine |
|--|---|--|--|-----------------------------------|
| Myelin, basic protein | 12.4 | 22.2 | 44.6 | 0.3 |
| Red cell mem- brane | 20.2 | 12.0 | 53.4 | 1.4 |
| Mitochondria integral pro- tein | 18.0 | 12.7 | 53.8 | 1.9 |
| Spinach chlo- roplast, lamellar pro- tein | 16.6 | 8.9 | 50.9 | 0.33 |
| Spinach, chlo- roplast, integral protein | 27.0 | 12.3 | 51.1 | 1.31 |
| Algal (Duna- liella) mem- brane | 21.4 | 7.4 | 56.3 | 0 |
| <u>Halobacterium</u> <u>cutirubrum</u> envelope li- poprotein | 25.6 | 5.9 | 48.5 | 0.3 |

Quantities are quoted in mole percent. The average molecular weights are about 23,000, figures are approximately half the number of amino acids per mole of protein.

that they are held to be cooperative changes which occur throughout the entire membrane, or large areas of it. Some examples of long range cis-effects include the action of colicin E upon intact E. coli (297) and the interaction of human growth hormone with isolated erythrocyte membranes (298, 299).

Membrane Composition.

It is the possession of specific membrane constituents which enables the organism to perform its characteristic functions and such specificity has been demonstrated by Esfahani, Crowfoot and Wakil who used aqueous acetone to extract phospholipids from an unsaturated fatty acid auxotroph of E. coli which recovered its 70% loss of succinic-dichloroindophenol reductase activity when the phospholipids were restored (300). The work of Frerman and White has demonstrated that the organism must also undergo changes in lipid composition during the development of an electron transport system in S. aureus when it is shifted from anaerobic to aerobic growth (301).

Over the last fifteen years there has been a rapid expansion in research in lipid chemistry and in the last few years many reviews have appeared:

Kates (302), Goldfine (303), Shaw (304) and Lennarz (305) have all reviewed bacterial lipids, while Smith has reviewed mycoplasma lipids (306) and Thompson and Nozawa have reviewed the protozoal lipids (307). Other articles worth consulting include those of Veerkamp (308), Korn (309) and Benson (310).

Chapter 5.

Phospholipid Monolayers as Model Membranes.

1. Interactions with Water.

Cholesterol is a common constituent of cell membranes and in certain cases it is present in the same proportions as phospholipids (311). Many investigations have been conducted in order to understand the role of the membrane cholesterol.

Some of the earliest studies of membranes included investigations of the polymorphism and thermotropic mesomorphism of dry phospholipids (312, 313). In general those phospholipids which possess long hydrocarbon chains can exist in more than one crystalline form (313), although such compounds usually crystallise so that the molecules are arranged in layers with saturated portions of the chains in the trans configuration. Ladbroke and Chapman have summarised the various thermal transitions of phospholipids (314). The addition of water to phospholipids results in several new structures which are a result of the phenomenon known as 'lyotropic mesomorphism' which is dependent upon temperature, water content and lipid polarity (315, 316). These lyotropic phases can be characterised by the 'Krafft point' (T_c) which is recognised as the minimum temperature below which water cannot penetrate into the crystal lattice of polar lipids (317). When the Krafft point is reached the hydrocarbon chains melt and water diffuses throughout the polar region of the crystal lattice. The Kraft point is a very characteristic property dependent upon the hydrocarbon chain, the polar region of the molecule, the amount of water present

and the specific solutes of the water. When the water has entered the phospholipids and the temperature of the sample falls below T_c there is a rearrangement of the hydrocarbon chains to give an orderly crystalline lattice, however, the water is not necessarily expelled from the system.

Chapman and co-workers have studied the phase properties of various phospholipids such as lecithin which dispersed readily in water while phosphatidylethanolamine did not disperse so readily, and a ready dispersion of phosphatidylserine could be achieved only by using monoalkaline salts of phosphatidylserine (318, 319). In contrast to lecithin the phosphatidylserines can incorporate large amounts of water. The calorimetric studies of phospholipid water systems have revealed interesting facts: the first 20% of added water does not freeze at 0° (319). Of the ten molecules of water bound to each lecithin group, five comprise the "primary" hydration layer which consists of bound and partially ordered water with the result that the polar group lattice is loosened and the hydrocarbon chain-packing density reduced (320, 321). The phospholipids absorb additional water, up to 40%, which then forms a free water layer between the polar groups (322). A more detailed account of such interactions has been given by Phillips (323) who has reviewed the ability of various spectroscopic techniques (infrared, nuclear magnetic resonance, X-ray diffraction and electron spin resonance) to probe the ordered structure of the hydrocarbon chains. Such techniques have shown that the terminal methyl group of the hydrocarbon chain is significantly more mobile and that the motion of the molecules in the bilayer is tightest in the region of the glycerol backbone (323). Electron spin resonance studies of spin-labelled phospholipids in water have revealed that the

hydrophobic regions of the phospholipid bilayers become more and more "fluid" towards the terminal methyl groups (324).

The interaction of phospholipids with water is dependent upon the side chain. Theoretical considerations indicate that lecithins with less than 8 carbon atoms would not form gels (313) and this was confirmed by Van Deenen et al who showed that diheptoyl lecithin will not form an insoluble monolayer at the air-water interface (325). In contrast to the non-polar region there is very little known about the interaction of the polar portion with water. While the phosphatidylethanolamine head group appears to be curled up in contact with water (326) the phosphatidylcholine head group unfolds into an extended conformation as the water content increases so that the $-N(CH_3)_3$ group is able to undergo rapid motions when in excess water (327). The disruption of the internal linkage between $-N(CH_3)_3$ and phosphate on addition of water to lecithin frees the $-N(CH_3)_3$ group for reaction with base (328).

2. Interactions with Cholesterol.

The initial studies of Van Deenen et al (325) into cholesterol induced phospholipid condensation were expanded by Chapman and coworkers who used a variety of unsaturated fatty acids (326, 327). A variety of other molecules such as 5α -androstan- 3β -ol, stigmaterol, cholesterol acetate, vitamin D_3 and abietic acid condense phospholipid monolayers (326) while cholesterol esters with a long hydrocarbon chains fail to condense such monolayers (328). Very polar steroids exhibit little ability to interact with phospholipids (329).

As yet very little is known about the monolayer condensation mechanism although the current consensus of opinion accepts that van der Waals forces are involved (330). Infra-red spectroscopy indicates that the 3β -OH group of cholesterol and the phosphate group are associated via a hydrogen bond (331).

X-ray diffraction studies have helped in understanding the interactions of cholesterol-lecithin-water systems (332). The evidence indicates that the lamellar phase always contains a maximum of one molecule of cholesterol per molecule of lecithin. In addition to controlling the maximum thickness of the lipid and aqueous layers at a maximum of 33⁰ mole % cholesterol (331), the cholesterol was also able to regulate the T_c of such ternary mixtures and control the fluidity of the hydrocarbon chains by inhibiting the motions of chains in the liquid crystal phase (332-336). Nuclear magnetic resonance studies of these ternary mixtures have shown that methylene groups in direct contact with the sterol nucleus are more immobilised than those methylene groups near the end of the chains. Molecular models indicate that the cholesterol hydroxyl group is located adjacent to the phosphate group of a lecithin molecule while the remainder of the sterol molecule reaches to about the tenth carbon atom along the hydrocarbon. In the presence of cholesterol the glycerol backbone of the lecithin exhibits more freedom than when the phospholipid-water is free of cholesterol (323).

The addition of cholesterol to lipid monolayers can alter membrane properties in a variety of ways: black lipid membranes are more stable; the capacitance of lecithin bilayers increases;

membranes show a decrease in permeability to macrolide antibiotics (336, 337); lecithin interaction with osmium tetroxide is increased (338). It is possible to explain all these observations in terms of a more "viscous" bilayer.

3. Interactions with Calcium.

In 1911 Koch and Todd suggested that the phosphatides may be able to bind Group IA and IIA cations (339). Over the years there have been a few investigations of such interactions. In 1927 Chibnall and Channon isolated in ether extracts calcium mono- and diglycerophosphates from cabbage leaves (340) while Hakamori et al isolated a metal-bound complex from ox brain (341). Cations such as Ca^{2+} and Mg^{2+} alter the solubilities and ionic properties of brain phosphoinositides (342).

The interaction of Ca^{2+} with phospholipid monolayers results in an increase in the surface potential of 100 mV in the case of species such as diacetyl phosphate (343, 344). The surface potential of a monolayer is defined as

$$\Delta V = V_f - V_o$$

where V_f and V_o are the interfacial potential in the presence and absence of the monolayer, respectively.

The surface potential can also be influenced by the position of the double bond in the hydrocarbon side chain. Oleic acid possesses a surface dipole moment which is half that of its isomer, Δ^2 -octadecenoic acid, in which the carboxyl group is vicinal to the double bond. When the double bond is more than 5-(C-C)-distance away from the carboxyl group the double bond remains uninfluenced. However, in the case of plasmalogens and spingomyelins the polar groups exhibit strong influences over

the adjacent double bonds with the result that these two classes of lipids possess large surface potentials (343, 345).

All the available evidence indicates that the membrane constituents which bind calcium are the phospholipids (346). Variations in the phospholipid content could have a significant bearing upon the degree of cation binding since in the case of gangliosides the $\text{Ca}^{2+}/\text{Mg}^{2+}$ ratio is 4.3 while that of the triphosphoinositides is 21 (347). Calcium ions on either side of phosphatidylserine membranes influence the membrane stability over a wide range of pH (2-9). However, if there is a Ca^{2+} or pH gradient across the membrane then it becomes much more permeable to ions due to the difference in surface charge density and eventually the membrane ruptures when the Ca^{2+} concentration reaches 1 to 4 mM (348). In contrast, phosphatidylcholine membranes did not exhibit this phenomenon.

In a study of pH regulation of calcium uptake by diacetyl phosphoric acid and phosphatidylinositol monolayers Quinn and Dawson showed that the uptake was minimal at pH 3 and that there was a rapid uptake at pH 7 to 8 (349).

However, the possession of a phosphate group at the specific pH is not necessarily the sole requirement for calcium binding since yeast lecithins are unable to bind Ca^{2+} as the unsaturated hydrocarbon side chains are in a configuration which produces a lecithin with a large cross-sectional area (350). Molecular models of the ionic structures of dioleoyl-, and dipalmitoyl-lecithin monolayers showed that the trimethylammonium group was a counter-ion to the negatively charged phosphate group in

the same molecule. In models where there was a decrease in the unsaturation the polar groups interacted to promote an increase in repulsion of adjacent phosphates with a resulting decrease in interaction between the trimethylammonium group and the phosphate group (351).

Cardiolipin monolayers undergo a marked decrease in surface area as a result of interaction with divalent cations (343).

From a consideration of the above facts it may be possible to rationalise many of the intrinsic properties of cells in terms of specific cell membranes whose constituents can be varied by nutrition and environmental factors. Many of the membrane constituents can influence the pH on surfaces and since most enzymes function in a gel-like environment or when adsorbed on solid-state assemblages, such as appear to exist in mitochondria or other organelles, then a consideration of surface pH is necessary. Danielli has indicated that the pH of a surface phase is different from that of the bulk by up to 2 pH units (352). The spatial range of these pH effects will be small, extending for a mere 50-100 nm from the physical interface. A protein could have a diameter of around 250 nm so if its active centres are to fall within the region of abnormal pH it must be adsorbed with the correct orientation. Chambers and Chambers demonstrated the existence of pH domains in cells by the microirgic injection of a variety of indicators (353). The existence of E. coli periplasmic enzymes with an optimum at pH 2 is further proof of such domains (354). Weiss

demonstrated that such domains are not restricted to the cell envelope of Gram-negative organisms when he succeeded in isolating a cell bound penicillinase from B. subtilis which required an acidic pH for maximum activity (355).

The variations in surface pH and surface viscosity offer a fine control over cellular activity. Bangham and Dawson, and Quarles and Dawson have demonstrated that hydrolysis of phospholipid monolayers by the phospholipases is reduced considerably at surface pressures below 10 dynes/cm (356, 357). Rideal demonstrated that the phospholipase hydrolysis of lecithin was greater (180 x) when the lecithin molecular area was $900 \text{ nm}^2/\text{molecule}$ and not $4.7 \text{ nm}^2/\text{molecule}$ (358).

Table 6.

| Calcium Requirement/Action | Reference |
|---|-----------|
| Stabilisation of bacterial protoplasts - can be replaced by polyamines | 359 |
| Ca (and Mg) stabilise extreme halophiles; lipid extraction reduced this action | 360 |
| Promotes growth of thermophilic fungi | 361 |
| Barotolerance of streptococci enhanced by Ca and Mg (Sr and Mn inactive) | 362 |
| Loss of rapid phage transfer in absence of Ca | 363 |
| Ca (and Mg) stimulate L-form induction of <u>E. coli</u> | 364 |
| Regulates <u>Lactobacillus bifidus</u> pleomorphism | 365 |
| Increase in Ca and Mg concentration decreased heat resistance of heat - acclimatized animals and increased cold resistance of animals | 366 |
| Reseals human erythrocyte ghosts after hypotonic haemolysis | 367 |
| Ca and Mg promote maintenance of avian kidney cells in culture | 368 |
| Promotes stability of some bacterial proteases | 369-371 |
| Retards lysis of <u>Streptomyces fradiae</u> and thermophilic actinomycetes | 372,373 |
| Regulates yeast spore formation, very few asci formed in absence of Ca | 374 |
| Slime mould aggregation dependent upon Ca | 375 |
| Ca accumulates during myxomycete sporulation | 376 |
| Ca regulates differentiation of the water mould <u>Achlya</u> | 377,378 |
| Ca inhibits oat coleoptile elongation; La, Pr and Nd show similar but greater inhibition | 379 |

Table continued/

| Calcium Requirement/Action | Reference |
|--|-----------|
| Rabbit gallbladder becomes preferentially permeable to anions at low pH or in presence of Ca, Ba, La and Th (IV) | 380 |
| Thermal resistance of leaf epidermis increased by treatment with Ca, Mg and Mn. Ca effective at 0.01M and at 0.06M reversed resistance | 381 |
| Stabilises marine red alga to hypotonicity | 382 |
| <u>Chlorella fusca</u> autospore release facilitated by Ca (3.4×10^{-5} g-atom/l). Sr could replace Ca for autospore release but did not facilitate growth | 383 |
| Washed gametes of <u>Chlamydomonas moewusii</u> mated only in presence of Ca | 384 |
| Ca interaction with pectic acids promoted heat and desiccation resistance of green alga | 385 |
| A low pCa blocks amoebal pinocytosis | 386 |
| Regulates cell propagation of <u>Scenedesmus</u> , controls synthesis of chlorophyll, carotene, nucleic acid and protein | 387,388 |
| High concentration of Ca in soil facilitates growth of thermophiles | 389 |

Chapter 6.

The Specificity of Calcium Interactions.

The ubiquity of calcium lends to the tendency to be somewhat sceptical about the specific requirements for calcium in a variety of biological systems: such ubiquity suppresses the feeling that calcium should be considered a trace element. The essential bio-inorganic requirements for calcium are now well documented (Table 6).

The biochemical function of the alkali and alkaline earth cations (in particular, Na^+ , K^+ , Ca^{2+} and Mg^{2+}) has not been studied in as much detail, nor with as much success, as has been the case with the d-transition cations. This situation has developed simply because the physical and chemical properties of these ions are difficult to utilise in order to obtain information about their complexes.

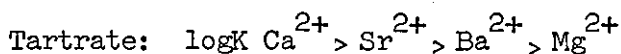
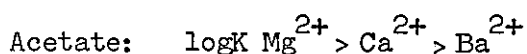
A consideration of the so-called diagonal relationship of inorganic chemistry, based upon the relationship of ionic potential (ion charge/ion radius) and charge increase of cation, reveals that Li^+ and Mg^{2+} , and Na^+ and Ca^{2+} are related. Since isomorphous replacement may occur when the radius of two ions is equal within 10% it is found that Li^+ can replace Mg^{2+} , and Na^+ may replace Ca^{2+} (in both cases with the necessary charge adjustment). In spite of this diagonal relationship quite dramatic differences in behaviour are exhibited by sodium (and potassium) and calcium ions in their interactions with an oil-water emulsion. Sodium (a small highly charged ion, coordination number six) remains partially hydrated in its interactions with the anions of the emulsion while calcium with a coordination

number of eight is able to interact with several large anions with a loss of its water of hydration and so breaks the emulsion through precipitation of calcium salts (390).

In some cases magnesium can replace calcium but there is a greater tendency for magnesium to replace manganese(II) rather than calcium. Magnesium has a ligand binding preference for carbonate, phosphate and nitrogen donors while calcium has a tendency to bind with similar ligands it forms weaker complexes with nitrogen donors, phosphate and other multidentate anions (391).

In studies of model systems and metalloenzymes it has been observed that the rate of replacement of water in octohedral systems is $\text{Ca}^{2+} > \text{Cu}^{2+} > \text{Zn}^{2+} > \text{Mn}^{2+} > \text{Fe}^{2+} > \text{Co}^{2+} > \text{Mg}^{2+} > \text{Ni}^{2+}$. In model systems the rate determining step involves metal substrate complex rearrangement in which the metal ion assists in orientating the substrate for reaction. The utility of calcium in particular enzyme systems is possibly a result of the greater rate of interaction of Ca^{2+} with water which may result from the capacity of Ca^{2+} to vary its coordination number. The rate constant for the formation of a calcium complex is one thousand times larger than the magnesium complex which means that calcium complexes have a shorter life-time.

In the case of simple ligands such as weak acids the binding is determined by the polarising power of the cation and therefore decreases with increase in size of the cation. However, this order can be changed by varying the complexity of the ligand.



While calcium and strontium form stable complexes with saccharic acid magnesium does not complex.

Table 6a

Formation Constant Data for Groups IA and IIA Cations (392).

| | Mg ²⁺ | Ca ²⁺ | Sr ²⁺ | Ba ²⁺ | Eu ²⁺ | Nd ³⁺ |
|------------------------|------------------|------------------|------------------|------------------|------------------|------------------|
| Acetate | 0.82 | 0.77 | 0.44 | 0.4 | | |
| Nitriloacetate | 4.4 | 6.4 | 5.0 | 4.8 | | |
| EDTA | 5.3 | 10.7 | 8.8 | 7.9 | 7.7 | 6.7 |
| Sulphate | 2.23 | 2.28 | | | | 3.64 |
| DPA (271) | | 4.2 | | | | |
| 4-CH ₃ -DPA | | 4.3 | | | | |
| PDC | | 3.6 | | | | |

Pauling Ionic Radii (nm)

| | $\times 10^{-2}$ |
|------------------|------------------|
| Mg ²⁺ | 6.5 |
| Ca ²⁺ | 9.4 |
| Sr ²⁺ | 11.0 |
| Ba ²⁺ | 12.9 |
| Eu ²⁺ | 11.8 |
| La ³⁺ | 10.61 |
| Nd ³⁺ | 9.95 |

The size of the cations is the most important factor in determining the structure, solubility and stability of the compound. Small cations solvate readily and the energy of hydration is usually sufficiently high to overcome the lattice energy. The solubility of a series is usually decreased as the series is descended while the solubility can be increased by the presence of large anions which do not contribute to strong lattice energies.

The replacement of one d-transition element by another is a common practice in the elucidation of their bio-inorganic chemistry. However, since calcium is unable to utilise the 3 d orbitals in bonding, as these energy levels are too high, then it is impossible to use d-transition elements in place of calcium. In addition, the alkaline earth cations form different complexes to the d-transition cations. The latter normally form strong octahedral or tetrahedral complexes with sulphur, nitrogen and oxygen donor ligands while the former complex primarily with anionic oxygen donor ligands of uncertain geometry (393). Other than the remaining alkaline earths there are few species which can be considered suitable to replace calcium. An examination of the ionic radii reveals that Mg^{2+} is much too small while Ba^{2+} is much too large. In particular it is unlikely that they could satisfactorily replace Ca^{2+} in cases of ligands with rigid geometry such as pyridine-2,6-dicarboxylic acid.

As probes for calcium binding sites of proteins, Birnbaum et al have initiated the use of rare earth ions such as Nd^{3+} (394).

The rare earth ions resemble the alkaline earth ions in that they have essentially no crystal field stabilisation energy, no significant covalent bonding and negligible f-orbital directing steric forces in their complexes (395). These lanthanide ions, like calcium, form complexes primarily with oxygen donor ligands which exhibit fast exchange kinetics (396). The effect of the higher ion charge is to increase the stability of the coordination compounds relative to Ca^{2+} , as reflected by larger stability constants, rather than to change the kind of complexes formed. Comparative biological studies are rare (397, 398); however, Takata et al have referred to lanthanum itself as "supercalcium" in describing its effect on ionic conductance in the lobster axon membrane (399) while Lehninger and Carfoli have demonstrated that La^{3+} could interact with the respiration coupled Ca^{2+} transport (400). La^{3+} ions were found to inhibit the Ca^{2+} carrier but were not carried by it and were, instead, bound to a large number of mitochondrial external sites for which Ca^{2+} was not a strong competitor. Pickard has demonstrated that La^{3+} , Pr^{3+} and Nd^{3+} can inhibit oat coleoptile elongation to a greater extent than Ca^{2+} (379).

As yet no one has applied the lanthanides to a study of the calcium induced heat stabilisation of bacterial spores. As the bacterial spores contain large amounts of divalent cations such as Ca^{2+} , Co^{2+} , Cu^{2+} , Mg^{2+} , Ni^{2+} and Zn^{2+} (10, 245, 402-403), many workers have investigated divalent cations for ability to replace calcium in the production of heat resistant bacterial spores (402-405).

Although no one has found cations which can completely replace Ca^{2+} in heat resistant bacteria, Davies has observed that Sr^{2+} can replace Ca^{2+} in stimulating growth of Phytophthora fragariae (406) while Ingraham and Emerson (407) and Basu have found that Ba^{2+} , as well as Sr^{2+} , can replace Ca^{2+} (408). Walker (409, 410) and Kylin and Das (411) have demonstrated that Sr^{2+} can replace Ca^{2+} in the morphogenetic production of Scenedesmus. A calcium dependence has been demonstrated for many fungi (Phytophthora cactorum (412), Pythium irregulare (413), Penicillium glaucum (414), Allomyces spp. (413), Penicillium griseofulvum (415), Coprinus ephernerus (416), Phytophthora fragariae (417), Trichophyton interdigitale (418), Chaetomium spp. (408, 419), Saprolegnia (420), Trichoderma viride (421) and Ceratocystis fimbriata (422)). However, the possibility of using the calcium dependence of fungal sporulation as a model for the interaction of calcium with bacterial spores has not been investigated.

Strong evidence indicates that calcium interacts with bacterial protoplasts can be inferred from Salton's observations that protoplasts of Micrococcus lysodeikticus are not readily lysed in the presence of Ca^{2+} (69).

MATERIALS and METHODS

Organisms

The following organisms were generous gifts: Actinobifida dichotomica CUB 339, Streptomyces coelicolor CUB 108 (Dr. T. Cross, Bradford University); Actinomyces viscosus species: WVU 745 (ATCC 15987, type strain, Howell T6) hamster plaque isolate, WVU 440 (Howell H-S-2) hamster plaque isolate, WVU 371 (ATCC 19246, serotype 2) actinomycotic lesion isolate, WVU 627 and WVU 398B human plaque isolates (Dr. Mary A. Gerencser, West Virginia University); Thermoactinomyces sacchari A978 (J. Lacey, Rothamsted Experimental Station); Actinoplanes missouriense (L.G. Willoughby, Freshwater Biological Laboratory, Ambleside).

Bacillus subtilis (B⁴2 glycerol auxotroph); (Dr. L. Mindich, Public Health Research Inst., N.Y.); Bacillus insolitus ATCC 23299 (Professor W.E. Innis, Waterloo University, Ontario); Scenedesmus sp. (Anders Kylin, Botanical Institute, Stockholm); Candida albicans A39 (The Boots Drug Co. Ltd., Nottingham).

The following organisms were obtained from the respective collections:

Actinomyces bovis NCTC 4500, Actinomyces humiferus ACTC 25174, Agromyces ramosus ACTC 25173, Bacillus cereus NCIB 8849, Bacillus megaterium NCIB 9521, Bacillus megaterium NCIB 8291, Bacillus subtilis NCIB 3610, Escherichia, irregular Type 1, NCIB 8667 (a streptomycin dependent strain), Escherichia coli NCTC 9001, Klebsiella aerogenes NCIB 8267, Pseudomonas aeruginosa NCIB 8295, Proteus vulgaris NCIB 8067, Salmonella abortus-ovis NCTC 10241, Micromonospora chalcea NCIB 9599, Streptomyces griseus NCIB 8232, Streptomyces pelletieri NCTC 3026,

Staphylococcus aureus NCIB 8625, Thermoactinomyces vulgaris NCIB 9780, Saccharomyces cerevisiae CMI 19391, Aureobasidium (Pullaria) pullulans CMI 62456, Cladosporium herbarum CMI 131128, Paramecium caudatum NCAP 1660/X.

Cell Cultures.

Dr. A. Ladner, Fisons Ltd., Pharmaceutical Division, Loughborough kindly supplied HEp-2 cells - (a cell line established by Moore et al who obtained it from irradiated-cortisonised weanling rats which had been injected with epidermoid carcinoma tissue obtained from the larynx (423)) and HeLaS₃ (the first aneuploid, epithelial - like cell line to be derived from human tissue and maintained continuously by serial cell culture, and was isolated originally from a carcinoma of the cervix (424)).

Chemicals.

The Sigma Chemical Co. Ltd., London, supplied Gramicidin J, β-phenethyl alcohol, wheat germ lipase, glyoxal bis-8-hydroxy anil, dicoumarol, calcium cyclamate.

Koch-Light, Ltd., Colnbrook supplied 2,3,5-tri-iodobenzoic acid, 3-chloropropane-1,2-diol, dithiothreitol, aesculin, thiophene 2,5-dicarboxylate sodium salt, hexylamine, dipicolinic acid, picolinic acid, Nd(NO₃)₃·6H₂O, La(NO₃)₃·7H₂O, L-lecithin (1,2-dipalmitoyl-L-3-glycerylphosphorycholine), L-phosphatidylethanolamine, cholesterol, cholesterylchloride and 5α-cholestan-3β-ol.

B.D.H, Ltd., Poole supplied 2,4-dinitrophenol, cetyltrimethyl ammonium bromide (CTAB), acridine orange, magdala red (phloxine, colour index 45410), phenyl mercuric nitrate and 8-hydroxyquinoline. 5-α-Androstan-

3β -ol, 5-cholesten- 3α -ol and 5β -cholestan- 3α -ol were obtained from Micro-Biol Laboratories, London. All other chemicals were of the highest grade possible.

Antibiotics and Antimetabolites.

The following antibiotics and antimetabolites were generous gifts: zinc bacitracin, neomycin sulphate, chloramphenicol, polymyxin B sulphate (Riker Laboratories, Loughborough), phosphonomycin as the calcium salt (Merck, Sharp & Dohme Ltd., Hoddesdon), chlorhexidine diacetate and chloroquine phosphate (I.C.I. Pharmaceuticals Ltd., Macclesfield) nystatin (E.R. Squibb & Sons Ltd., Twickenham), D-cycloserine, vancomycin hydrochloride (Eli Lilly & Co. Ltd., Basingstoke) gentamycin (Nicholas Laboratories Ltd., Slough) clindamycin and lincomycin (Upjohn Ltd., Crawley) sodium fusidate (Leo Laboratories, Hayes), phanquone (4,7-phenathroline-5,6-quinone, Ciba Laboratories Ltd., Horsham), ethane-1-hydroxy-1,1-diphosphonic acid and sodium methane-dichlorodiphosphonate (B.H. Wiers, Procter & Gamble Co., Cincinnati) pentamidine isethionate and griseofulvin (May & Baker Ltd., Dagenham) rifampicin (Lepetit Pharmaceuticals Ltd., Maidenhead) methicillin (Beecham Research Laboratories, Brentford) benzyl-penicillin and 'alphaxalone' (3α -hydroxy- 5α -pregnane-11,20-dione (Glaxo, Greenford, Middx.), bronopol, quinacillin, streptomycin sulphate, ethidium bromide, 21-desoxyprednisolone- 17α -propionate (The Boots Drug Co. Ltd., Nottingham) colchicine (Arthur H. Cox Ltd., Brighton) calcium ionophore X-537A (Julius Berger, Hoffman-La Roche Inc, New Jersey) pimaruficin (natamycin, Brocades Ltd., Brighton) trichomycin (Fujisawa, Japan).

Cell Wall Preparation.

Cells were suspended in distilled water and fractured in the 'X-press' (LKB Instruments) by repeated passage. They were estimated to be more than 95% disrupted as judged by methylene blue staining and to be

free of cytoplasmic material. The broken cells were lyophilised after they had been washed several times with saline and finally distilled water.

Cell Wall Hydrolysis.

The ruptured cells were hydrolysed for 2 h in sealed evacuated ampoules at 98° by 2N-hydrochloric acid (2 mg/ml). The cooled contents were clarified by centrifugation (4500 x g) and the ^{liquid} supernatant lyophilised until required when it was reconstituted and applied semi-quantitatively to Whatman No.1 chromatograms which were developed by descending elution with n-butanol:pyridine: water (6:4:3, ^v/v) (425). The chromatograms were viewed with a 'Mineralite' lamp (254 m μ) to locate ultraviolet absorbing zones, aniline hydrogen phthalate (426) was used to detect reducing sugars, polyols were detected with silver nitrate (427), amino sugars with the ninhydrin spray (428), phosphoric esters with the molybdate reagent (429) and α -glycols with the periodate-Schiff's reagent (430).

Lipid Analysis.

Lipids were extracted for 4 days by the method of Shaw and Dinglinger using chloroform-methanol (2:1, ^v/v) (431). A preliminary examination of lipid degradation by this solvent system indicated that the isolation method of Letters was not necessary (432). The lipids were concentrated in vacuo at 37° and applied quantitatively to silica gel G thin layer chromatograms (0.2 mm) which were developed in chloroform:methanol:water (65:25:4, ^v/v) to separate lipids (433) while chloroform:acetone:formic acid (74:25:1, ^v/v) was used to separate sterols (434). Developed chromatograms were visualised for glycolipids with the periodate Schiff's reagent (430) for free amino groups with ninhydrin (438) and for phospholipids with the molybdate reagent (435). Chromato-

grams were charred by the method of Jones et al (436) which involved placing the plate for 2 mins in a sealed jar containing sulphuryl chloride (1 ml). After the plates had been steamed for 30 secs they were placed on a hot plate at 200°. The Lifschutz reagent (conc. sulphuric acid: acetic acid, 1:1, ^v/v) was used to detect sterols as red or purple spots (437).

Determination of Dehydrogenase Activity.

The dehydrogenase activity of a cell suspension was estimated, under aerobic conditions, by the triphenyl-tetrazolium chloride (TTC) method of Hugo (438) using a system containing, in 5 ml, 0.5 mg dry weight of cells, 200 µg/ml TTC, glucose 0.05M and phosphate buffer 0.01M pH 7.2. The resulting formazan was estimated at 525 nm using an SP500 (Cambridge Instruments Co. Ltd., Cambridge, England).

Determination of Respiration Competence.

The method of Horn and Wilkie (439) was used to assess the variation in respiration competence of yeasts when cultured under conditions such as steroid supplementation of media which could influence lipid composition. Yeasts were cultured on Magdala Red medium which contained a range of steroids.

Gradient-Plate Techniques for Antimetabolite Sensitivity Studies.

The response of cells to the presence of antimetabolites is regulated by the ratio, cells : moles of antimetabolite. By careful optimisation of this ratio it should be possible to study very subtle changes in events occurring during cell growth and differentiation. The method of Szybalski (440) was used with petri dishes 10 cm x 10 cm (Sterilin, Richmond, Surrey). The quantity of antimetabolite to give the required concentration was dissolved in 50 ml of nutrient agar and this constituted the lower layer which was allowed to solidify as a wedge-shaped layer before it was covered by a further 50 ml of nutrient agar. The antimetabolite was allowed to diffuse for 8 h at 4° to establish

the gradient of antimetabolite concentration. A loopful of an 18 h broth culture of the organism was streaked on to the surface from the region of minimum to maximum concentration. The plates were incubated at the optimum temperature for 20 hours and the cells removed from various concentration zones for examination on the microscope.

Media.

The following media (g/l) were used and were sterilised at 15 lb/in² for 15 mins (unless otherwise specified). When required solidified media were obtained by adding Oxoid No.3 agar to a concentration of 1.5%. All media were prepared from 'Oxoid' products unless stated otherwise.

Actinobifida dichotomica media.

Nutrient broth, 6.5; corn steep liquor solids, 1.0; starch, 10.0; pH 7.3.

Streptomyces coelicolor media.

Malt extract, 3; yeast extract, 3; bacteriological peptone, 5; dextrose, 10; pH 6.8.

Nutrient Broth.

Beef extract, 10; neutralised peptone, 10; NaCl, 5; pH 7.2.

Nutrient Soya Broth.

Beef extract, 10; neutralised soya peptone, 10; NaCl, 5; pH 7.2.

Tryptone Soya Broth.

Neutralised tryptone, 10; neutralised soya peptone, 5; NaCl, 5; pH 7.2.

Magdala Red Medium.

Ammonium sulphate, 1.5; potassium dihydrogen phosphate, 1.5; magnesium sulphate, 1.0; yeast extract, 1.5; glucose, 20; magdala red (Phloxine B, colour index 45,410, G.T. Gurr, Ltd.,) 10 ppm; pH 5.4.

Sterol Deficient Medium.

KH_2PO_4 , 5.4; $(\text{NH}_4)_2\text{SO}_4$, 12; sterol free yeast extract, 18; pH 7.0 (bacteria) and pH 5.4 (yeasts). Sterol free yeast extract was prepared by soxhlet extraction of yeast extract with chloroform-methanol (1:1, v/v) for 2 days.

Phytophthora cactorum Medium

The basal medium contained sucrose, 10; L-asparagine, 1.0; KH_2PO_4 , 0.5; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.25; trace element solution, 1 ml; thiamine hydrochloride, 0.001. The trace-element solution contained (mg/l) $\text{Na}_2\text{B}_4\text{O}_7 \cdot 10\text{H}_2\text{O}$, 88; $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 393; $\text{Fe}_2(\text{SO}_4)_3 \cdot 9\text{H}_2\text{O}$, 910; $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 72; $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, 50; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 4403; ethylenediaminetetraacetic acid disodium salt, 5000. Glass distilled (triple) water was used to prepare the medium.

Algae Medium.

KNO_3 , 2.5; MgSO_4 , 0.51; KH_2PO_4 , 0.135; $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$, 0.0236; trace elements, 2 ml; iron salts solution, 2 ml. The trace elements solution contained (mg/l) boric acid, 143; $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 90.5; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 111; $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 40; $(\text{NH}_4)_2\text{MoO}_4$, 1.5; NH_4NO_3 , 1.1; $\text{KCrSO}_4 \cdot 12\text{H}_2\text{O}$, 5; $\text{NiSO}_4 \cdot 6\text{H}_2\text{O}$, 2.25; $\text{Co}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$, 2.5; $\text{Na}_2\text{WO}_4 \cdot 2\text{H}_2\text{O}$, 8.0; TiCl_3 , 1.1. The trace elements were dissolved in 0.1N H_2SO_4 (30 ml) and diluted to 1000 ml. Glass distilled (triple) water was used to prepare the medium.

Actinoplanes Medium.

Neutralised tryptone, 3; neutralised soya peptone, 1; beef extract, 2; NH_4NO_3 , 3; NaCl, 2; humic acid 0.2; pH 7.1. Humic acid was prepared by the method of Willoughby *et al* (441).

Maltose Peptone Medium.

Maltose, 38; yeast extract, 2.5; mycological peptone, 8; malt extract, 2 g; agar, 20. pH 5.5.

Glutamic Acid Salts Medium.

Na_2HPO_4 , 1.15; $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$, 0.3; $(\text{NH}_4)_2\text{FeSO}_4 \cdot 6\text{H}_2\text{O}$, 0.005; KNO_3 , 0.5; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.25; glutamic acid, 10; pH 6.8. Glucose was added as required.

Potato Dextrose Agar.

Potato extract, 4; glucose, 20; agar No.3, 15.

Sabouraud's Agar.

Mycological peptone, 10; dextrose, 40; agar No.3, 15.

Defined Medium.

Fumaric acid, 1.5; sodium pyruvate, 1.0; D-glucose, 0.5; $K_2HPO_4 \cdot 3H_2O$, 0.5; L-asparagine, 1.0; L-proline, 1.0; L-cysteine, 1.0; glycine, 1.0; uracil, 0.025; histidine, 0.0002; arginine, 0.0004; lysine, 0.0004; tyrosine, 0.0006; tryptophan, 0.0002; methionine, 0.0002; 1 ml of salts solution and 20 ml of vitamins solution, pH 7.2. The salts solution contained (g/100 ml) $MgSO_4 \cdot 7H_2O$, 5.0; $FeSO_4 \cdot 7H_2O$, 0.5; $ZnSO_4 \cdot 7H_2O$, 0.08 and $MnSO_4 \cdot 4H_2O$, 0.036. The vitamins solution contained (mg/100 ml) biotin, 100; folic acid, 100; choline chloride, 100; nicotinamide, 100; D-calcium pantothenate, 100; pyridoxal hydrochloride, 100; thiamine hydrochloride, 100; riboflavine, 10 and i-inositol, 180 and the resulting solution sterilised by filtration using membrane filters of 0.2 μm porosity (Sartorius, V.A. Howe & Co. Ltd., London).

Human Cell Culture Medium (10X) TC25

Eagle's medium (M.E.M.) 10% v/v ; foetal bovine serum, 10%; L-glutamine (200 mM), 1% v/v , sodium bicarbonate (1.4% w/v) 10% v/v ; antibiotics solution (100X) 1%; glass distilled water to volume.

Eagle's medium contained (mg/100 ml) NaCl, 6800; KCl, 400; $CaCl_2$, 200; $MgSO_4 \cdot 7H_2O$, 200; $NaH_2PO_4 \cdot 2H_2O$, 150; L-arginine hydrochloride, 125; L-cystine, DL-isoleucine, 104; DL-leucine, 104; L-lysine hydrochloride 70; DL-methionine, 30; DL-phenylalanine, 64; DL-threonine, 96; DL-tryptophan, 20; L-tyrosine, 36; DL-valine, 92; aneurine hydrochloride, 1.0; choline chloride, 1.0; folic acid, 1.0; inositol, 2.0; nicotinamide, 1.0; calcium pantothenate, 1.0; pyridoxal hydrochloride, 1.0; riboflavin, 0.1.

The antibiotics solution was added to the medium so that the antibiotics were present in the final concentrations: penicillin, 100 I.U/ml; streptomycin, 100 µg/ml; neomycin, 25 µg/ml; amphotericin B, 5 µg/ml.

Microscopy.

Scanning Electron Microscopy.

Glutaraldehyde (G.T. Gurr, Bucks) was added to broth cultures of the strains of cell suspensions to give a final concentration of 1.5% W/v. After 2 mins contact the cells were removed by centrifugation at 3,000 x g at 4°C, for 15 mins and resuspended in 2 ml of glutaraldehyde (5% W/v) for 16 h at 4°C. The cells were then removed by centrifugation (3,000 x g for 15 mins), washed three times with distilled water and re-suspended to give the required density. One drop of this suspension was allowed to air-dry on a microscope cover-slip and then dehydrated over CaCl₂ under a partial vacuum. The samples were then coated with gold-palladium in a high vacuum unit to obtain a coating of approximately 10 nm thickness. These samples were examined in a Cambridge Stereoscan (Mark II A, Cambridge Instruments Co. Ltd., Cambridge) with a beam specimen angle of 45°, using a voltage of 30 KV. Photographs were obtained using FP4 film (Ilford).

On some occasions it was necessary to culture the organisms in contact with a cover-slip (1.3 cm diam) which was placed on an elevated nutrient agar block, 6 mm diam, previously inoculated. After a suitable period of culture the cover-slip was immersed for 16 h in glutaraldehyde (5% V/v) at 4°C, washed 3 times in distilled water, dehydrated and subsequently treated by the above procedure.

Light Microscopy.

The routine examination of samples and phase contrast microscopy was carried using a Vickers M25 series microscope. Dried and fixed films were stained with the following:

Burdon's Sudan Black Lipid Stain.

Sudan black B (0.3 g) was dissolved in 70% ethanol (100/ml), shaken thoroughly at repeated intervals and allowed to stand overnight. The slide was flooded with the solution and allowed to stand at room temperature for 15 mins. Excess stain was removed, air-dried, rinsed thoroughly with xylene and dried. Aqueous safranin (0.5%) was used to counterstain the cells, the slide washed with tap water and blotted dry.

Cell Wall Stain.

The cell wall mordant was prepared from 20% tannic acid solution (30 ml) and saturated potassium aluminium sulphate solution (70 ml). After the mordant had been applied for 10 mins and washed with water it was counterstained with carbol fuchsin for 2 to 3 mins and dried.

Spore Stains.

Bacterial spores were stained by the method of Lechtman et al (442) and Asby's modification of the Schaeffer and Fulton stain (443).

Glyoxal-bis-8-hydroxy-anil Calcium Stain.

Alcoholic glyoxal-bis-8-hydroxy anil (GBHA) solution (2 ml), prepared by dissolving GBHA (400 mg) in absolute ethanol (100 ml), was diluted to 100 ml with distilled water together with 0.3 ml of sodium hydroxide solution (5%) and this solution was applied to a smear of cells which had been washed three times with distilled water to remove any contaminating calcium salts. After 3 mins the stain was removed and washed with 70% ethanol and the slide immersed for 15 mins in alcoholic sodium carbonate-potassium cyanide solution (90% ethanol saturated with sodium carbonate and potassium cyanide). The slide was rinsed twice with 95% ethanol and covered with glycerol.

This procedure will stain for Ca, Ba, Sr, Cd, Cu, Co and Ni forming red or blue-purple precipitates. However, the sodium carbonate-potassium cyanide decolourises all precipitates except the Ca - ones. As a control cells grown in calcium free medium were briefly immersed in the calcium containing medium and carried through the procedure. In no case was it possible to observe any significant quantities of calcium in these controls. This procedure was adapted from that of Shida (444).

Light micrographs were obtained using a Leitz Photoplan. Black and white photographs were obtained with FP4 (Ilford) film and colour ones with Kodachrome-X (Kodak).

Chemical and Enzymatic Treatment of Actinomyces viscosus 398B.

Cells (2 mg dry weight/ml) were shaken with dithiothreitol (100 µg/ml) for 90 mins or CTAB (2 mg/ml) for 60 mins or DMSO (5 mg/ml) for 24 h at 37°.

The enzymatic digestion involved shaking the cells (50 mg) for 12 h with wheat germ lipase (500 µg/ml) in 0.01M phosphate buffer (25 ml), pH 7.4. A similar cell suspension was shaken with lysozyme under the same conditions.

Variation in Culture Conditions of Actinomyces viscosus 398B.

In order to assess influence of culture conditions on Actinomyces viscosus 398B the organism was grown at 37° in shake-culture for 12 h in either tryptone-soya broth (TS) or in nutrient broth (NB). The cells were harvested, washed 3 times with sterile saline (0.85% w/v) and the cell masses divided into two portions in both cases. One portion of each was stored for 98 days at 2° after which it was restored to its original medium while the remaining fraction was incubated in the other, alternative medium for a further 14 h.

Determination of Heat Resistance.

Bacillus cereus SV-1 spores were cultured for 8 days on nutrient agar at 30° in Roux flasks. This line of B. cereus was established by repeated subculture on nutrient agar until microscopy indicated the production of large quantity of spores. The spores harvested from Roux flasks were washed with 0.85% saline (4X) and the spores were found to be sufficiently pure as to not warrant the customary lysozyme treatment. Native spores (150 mg) were stirred for 6 h with 0.01M HNO₃ (200 ml) to produce acid-spores (H-spores) which were converted to the appropriate metal-spore (Ca-spore, La-spore and Nd-spore) by stirring for 6 h the H-spores with 0.025M metal nitrate solution. The metal-spores were washed several times with saline and stored at 0° until required.

Spore suspensions (ca 1.5 x 10⁹ cells/ml) were prepared and added to pre-heated glass containers which were held at 98° in a water bath. Spore suspensions (1 ml) were removed at regular intervals, adjusted to a suitable dilution and one ml plated out on pre-poured nutrient agar plates (triplicate) which were incubated for 20 h at 30°

Actinomyces viscosus 398B (vacuolated and non-vacuolated cells) were adjusted to similar cell suspensions and held at 98° and 82°. Similar dilutions were prepared, plated out and the nutrient agar plates incubated at 37° for 14 h.

Fluorescence Studies.

1-Anilino-8-naphthalene sulphonate (ANS), Serva Chemicals, London, was used to investigate the polar nature of the membrane.

Cells which had been grown in various concentrations of 3-chloro-propane-1,2-diol or alpha-xalone were suspended in a final concentration of 3 x 10⁻⁵ M ANS dissolved in 1M NaCl. The final cell concentration

was adjusted, in all cases, to give an optical density of 0.55 at 420 nm. These systems were examined at an excitation wavelength of 372 nm (Baird-Atomic 'Fluoripoint' Fluorimeter) and the maximum emission wavelength determined.

Arsenate-Resistant *Saccharomyces cerevisiae*.

The method of Cerbon was used to produce arsenate resistant *Saccharomyces cerevisiae* (445). A large quantity of cells were harvested aseptically from nutrient broth supplemented with yeast extract (1%) and maltose (1%) and transferred to the fresh medium containing sodium arsenate (10^{-5} M). The cells were harvested aseptically after 8 days static culture at 28° and transferred to fresh medium containing sodium arsenate (10^{-4} M). After a further 8 days the cells were transferred to a medium containing sodium arsenate (10^{-3} M) and the process repeated until the cells could be cultured in (10^{-2} M) sodium arsenate. This arsenate resistant (ASR) *Saccharomyces cerevisiae* was examined for sensitivity to antimetabolites.

Determination of Minimum Inhibitory Concentration (MIC) values.

The MIC values were determined, in nutrient broth using a tube dilution method. An estimate of antibacterial activity was made with a filter paper disc method using Whatman (13 mm) discs impregnated with the required compound placed on seeded nutrient agar. In addition, some antimicrobial compounds were tested by the 'Multodisk' (Oxoid) method.

Growth Rates.

The rate of growth of shake cultures was followed by determining the optical density at 420 nm or 660 nm (SP500, Cambridge Instruments, Cambridge).

Growth in the presence of 3-Chloro-propane-1,2-diol.

The effect of 3-chloro-propane-1,2-diol on a streptomycin dependent Escherichia spp. was determined by preparing a series of concentrations ranging from 0 to 3,600 $\mu\text{g/ml}$ of the compound in nutrient broth containing streptomycin (1,000 $\mu\text{g/ml}$). Cells from an 18 h culture were added to give 10^6 cells/ml. After overnight culture at 37° the optical density (420 nm) was determined.

Bacillus subtilis (B⁴² glycerol⁻) was cultured on the synthetic medium supplemented with glycerol (20,000 $\times 10^4$ $\mu\text{g/ml}$). 3-Chloro-propane-1,2-diol was added at concentrations of 0, 200 $\times 10^2$, 2,000 $\times 10^3$ and 5,000 $\times 10^3$ $\mu\text{g/ml}$.

Response of Scenedesmus sp. to Calcium, Lanthanum and Neodymium salts.

Pyrex glassware was washed with dilute hydrochloric acid and stored for 3 days full of Na_2 EDTA solution. The glassware was rinsed six times with triple distilled water. This preparation procedure was used on other occasions when experiments were conducted on the cellular role of calcium.

Scenedesmus sp. was cultured on the algae medium under the absence of calcium salts, in the presence of CaCl_2 (0.1 mM), $\text{La}(\text{NO}_3)_3 \cdot 7\text{H}_2\text{O}$ (0.075mM) and $\text{Nd}(\text{NO}_3)_3 \cdot 6\text{H}_2\text{O}$ (0.075 mM). The flasks were shaken for 10 days at room temperature under a constant light source of 4 x 60 watt tungsten bulbs. The intensity at the level of the flasks was estimated to be 12,000-20,000 ergs per second per cm^2 . However, in the case of flasks containing the lanthanum and neodymium salts it was difficult to predict the intensity on the cells since these solutions were slightly turbid as a result of the rare earth phosphate precipitates.

The calcium ionophore, X537-A, was sterilised by filtration using membrane filters of 0.2 μm porosity ('Sartorius', V.A. Howe & Co. Ltd., London) and added to give a final concentration of 3.5 $\mu\text{g/ml}$.

The size of the unicellular algae was determined with the 'Model T' Coulter Counter (Coulter Electronics Inc., Hialeh, Florida). Cells were harvested by centrifugation, washed with NaCl (30 mM) and resuspended in the saline solution to give suspensions of approximately 15×10^6 cells per ml.

The Contribution of Sterols, and Divalent and Trivalent Salts to Sporulation of *Phytophthora cactorum*.

Phytophthora cactorum medium (10 ml) was added to calcium-free 100 ml Pyrex conical flasks. Divalent and trivalent salts were added to give a final concentration of 0.075 mM. Sterols were dissolved in diethyl ether and added so that each flask contained 200 μ g of sterol. The cells were recovered after 10 days, washed and lyophilised. Other samples were prepared for calcium staining, light microscopy and SEM. Experiments were conducted in triplicate on two separate occasions.

Complex Formation of Dipicolinic Acid: Interaction with Calcium, Lanthanum and Neodymium Cations.

Triple distilled water was used throughout, boiled before use and maintained in an atmosphere of nitrogen. A 0.01M metal-DPA solution was prepared by dissolving DPA and metal nitrate in 1M KNO_3 and keeping the resulting solution under an atmosphere of nitrogen. The metal-DPA solution was titrated potentiometrically with standard, carbonate-free, sodium hydroxide.

Chemical Composition of Spores.

Calcium was determined by the method of Bronner et al (446) and dipicolinic acid by the method of Jansenn et al (447).

Lipo some Formation

Lipo some of calcium lecithinate and calcium salt of phosphatidylethanolamine were prepared and examined by the method of Katzman et al (448). A weighed amount of CaCl_2 was added to triple distilled water which contained a sonicated dispersion (prepared at 0°) of either phosphatidylethanolamine or lecithin (cholesterol was added when necessary to a concentration of 33 mole %). The solution was sonicated for 60 sec and allowed to stand for 10 mins and the process repeated twice. The solution was concentrated in vacuo, dissolved in CHCl_3 : MeOH (2:1, v/v) and used as stock. Thin layer chromatography showed no evidence of degradation. Known quantities of the lipid preparations were dissolved in buffer (0.05M) so that the calcium lipid salt had a concentration of 2×10^{-4} M. The sonicated suspensions were filtered through 0.8 μ millipore filter and adjusted to give a series of solutions containing 0 to 5 mM dipicolinic acid, ethane-1-hydroxy-1,1-diphosphonic acid, picolinic acid, sodium cyclamate, Na_2 EDTA and aesculin. The optical density was determined at 546 nm at 28° . Lipo some containing lanthanum were prepared in a similar manner.

Germination of *Bacillus cereus* SV-1 spores.

Washed native spores were maintained at 37° and suspended in buffer solutions containing the following: DPA, EHDP, sodium cyclamate, Na_2 EDTA, aesculin and hexylamine (all at 0.5 mM). The rate of germination was followed by recording the O.D. change at 546 nm.

RESULTS

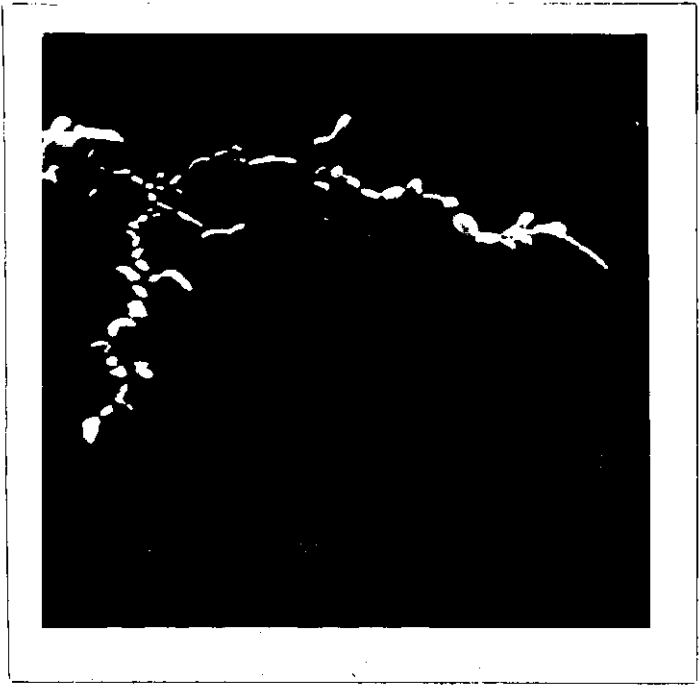


Plate 1. - S.E.M. of a 26 h nutrient broth culture of A. viscosus WVU 745 showing the arrangement of the cocco-bacilli. x 3,600.

Plate 2. - S.E.M. of a 48 h nutrient broth, static culture of A. viscosus WVU 745 showing the filamentous form and the development of cocco-bacilli. x 8,000.

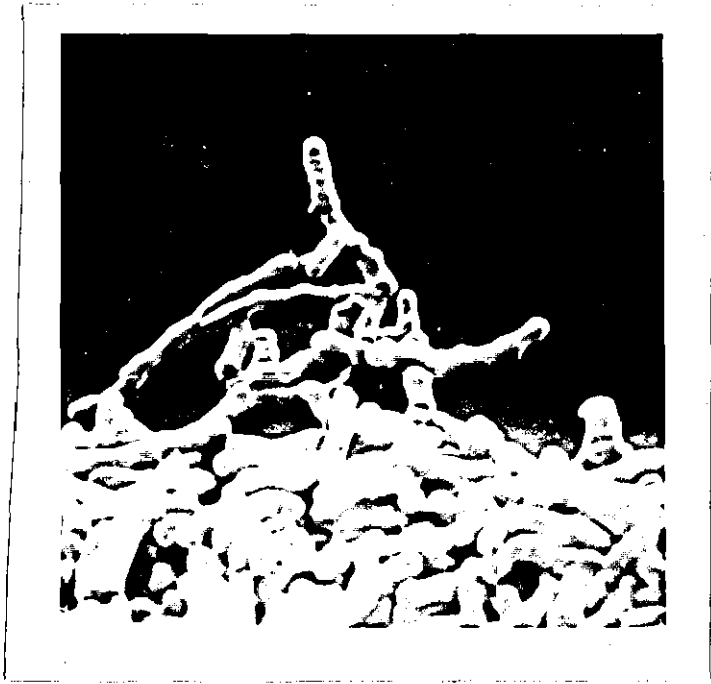


Plate 3. - S.E.M. of a 60 h nutrient broth of A. viscosus
WVU 371 showing the filamentous arrangement.
x 4,000.



Plate 4. - S.E.M. of a 24 h nutrient broth culture of A. viscosus WVU 440 showing branching filaments. x 16,000.

Plate 5. - S.E.M. of 48 h nutrient broth of A. viscosus WVU 440 showing the typical coccus. x 16,000.



Plate 6. - S.E.M. of a 24 h nutrient broth culture of A. viscosus
WVU 627 showing the typical filamentous form. x
4,800.

Plate 7. - S.E.M. of a 72 h nutrient broth culture of A. viscosus
WVU 627 showing the formation of cocco-bacilli. x
4,000.

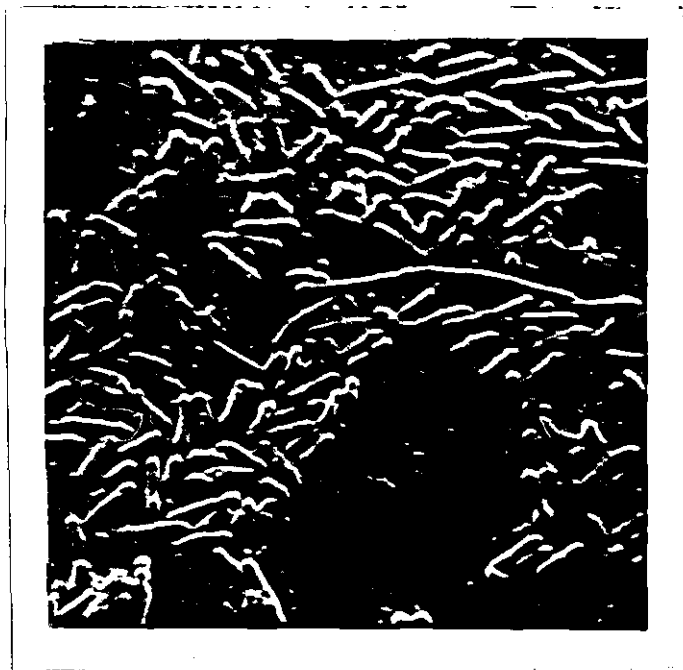


Plate 8. - S.E.M. of a 36 h blood-agar culture of A. bovis
showing the filamentous and rod-forms. x 8,000.

A. Morphological Variation in some selected Prokaryotes.

(i) Actinomyces viscosus.

Scanning electron microscopy revealed that the strains of Actinomyces viscosus studied are far from being a homogeneous group. Actinomyces viscosus WVU 745 grew best in shake cultures and the 24 h cells from nutrient broth showed that filamentous and cocco-bacilli forms were present (Plate 1). The cocco-bacilli forms appear to be formed from within fragmenting filamentous forms (Plate 2). In comparison, the rate of growth of A. viscosus WVU 371 was less than that observed with A. viscosus WVU 745 and the filamentous form was the only distinguishable morphological form in either static or shake culture (Plate 3) even after 200 h. While, in contrast, A. viscosus 440 showed the typical actinomycotic morphology, branching filamentous forms (Plate 4), after 24 h growth which was followed by fragmentation to produce cocco-bacilli after 48 h (Plate 5).

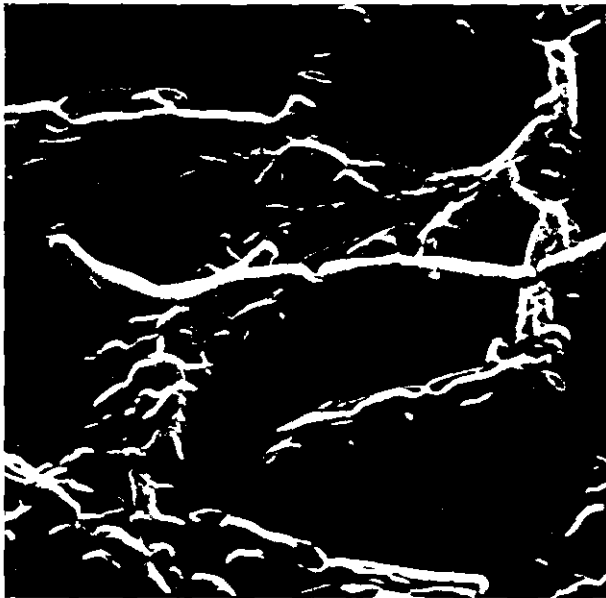
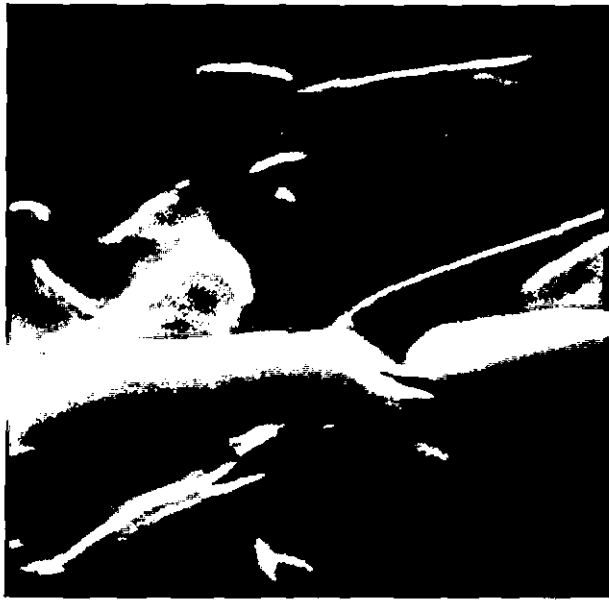
Although not as aerophilic as A. viscosus 398B A. viscosus 627 exhibited better growth under shake culture than the other three organisms. SEM revealed that A. viscosus 627 existed as a typical filamentous organism with some branching (Plate 6) but as the culture entered the stationary growth phase some cocco-bacilli were evident (Plate 7). Filamentous forms, with little or no branching, but some short rods, were typical of the growth of A. viscosus 398B.

SEM showed A. bovis to exist in fine filaments which fragmented into short rods (Plate 8).



Plate 9. - Light microscopy of A. viscosus WVU 398B grown in tryptone-soya broth for 24 h showing clear areas in the cytoplasm. Methylene blue stain. x 1,250.

Plate 10. - S.E.M. of A. viscosus WVU 398B grown in nutrient broth for 40 h showing collapse of the mycelial form to give a sheath like appearance of coccobacilli forms. x 7,600.



Plates 11 & 12. - S.E.M. of A. viscosus WVU 398B grown in nutrient broth for 40 h, showing collapse of the mycelial form to give a sheath like appearance with cocco-bacilli forms. x 7,600.

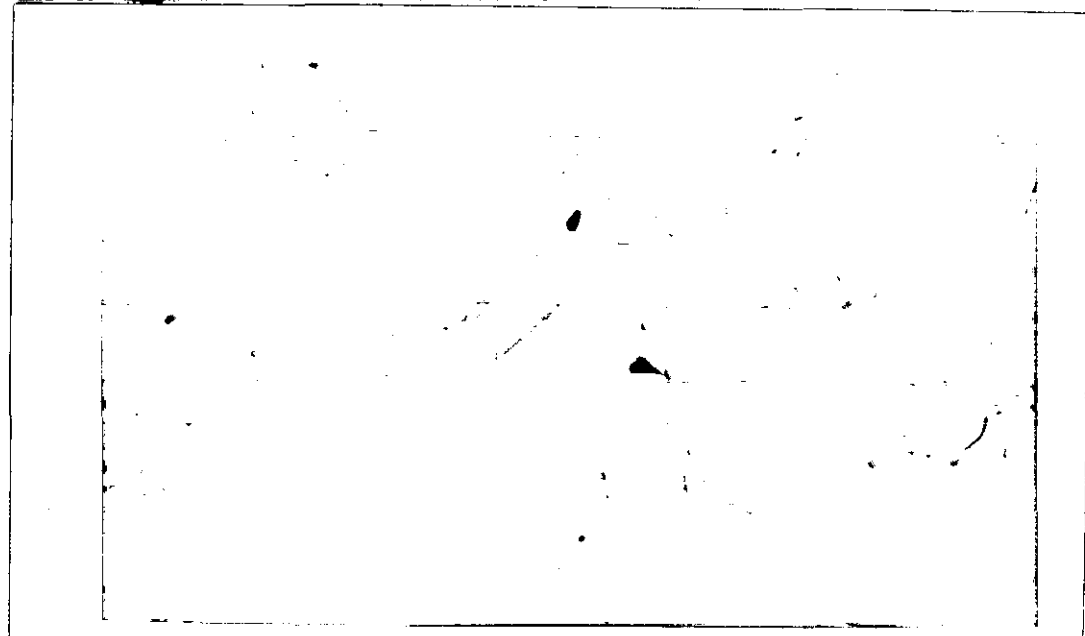
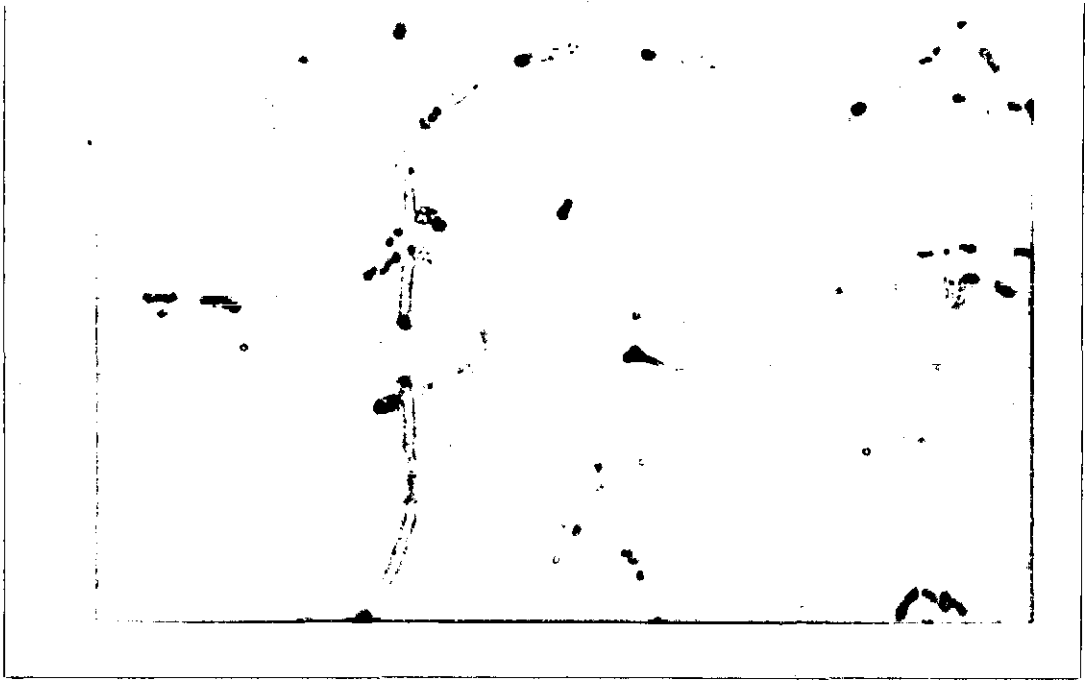


Plate 13. - A. viscosus WVU 398B in the vacuolate form stained for lipid. x 1,250.

Plate 14. - A. viscosus WVU 398B in the vacuolate form stained to reveal the cell wall. x 1,250.

Plate 15. - A. viscosus WVU 398B in the vacuolate form stained with the Schaeffer-Fulton spore stain. x 1,250.

Fig 2.

Growth of A. viscosus in tryptone soya broth (o — o) and nutrient broth (x — x). 'A' indicates the onset of vacuolation and 'B' when 90% of the cells had vacuolate appearance.

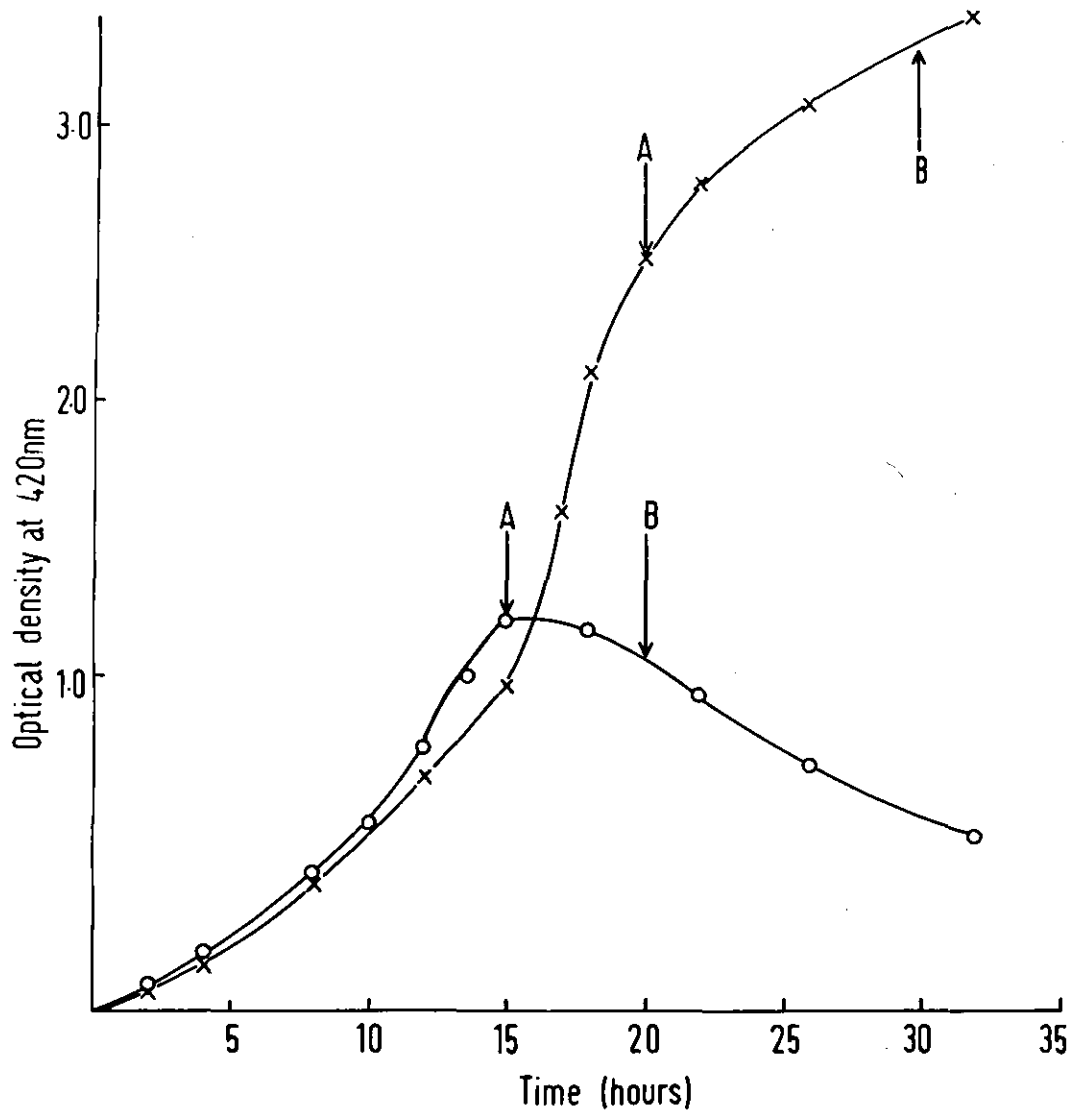
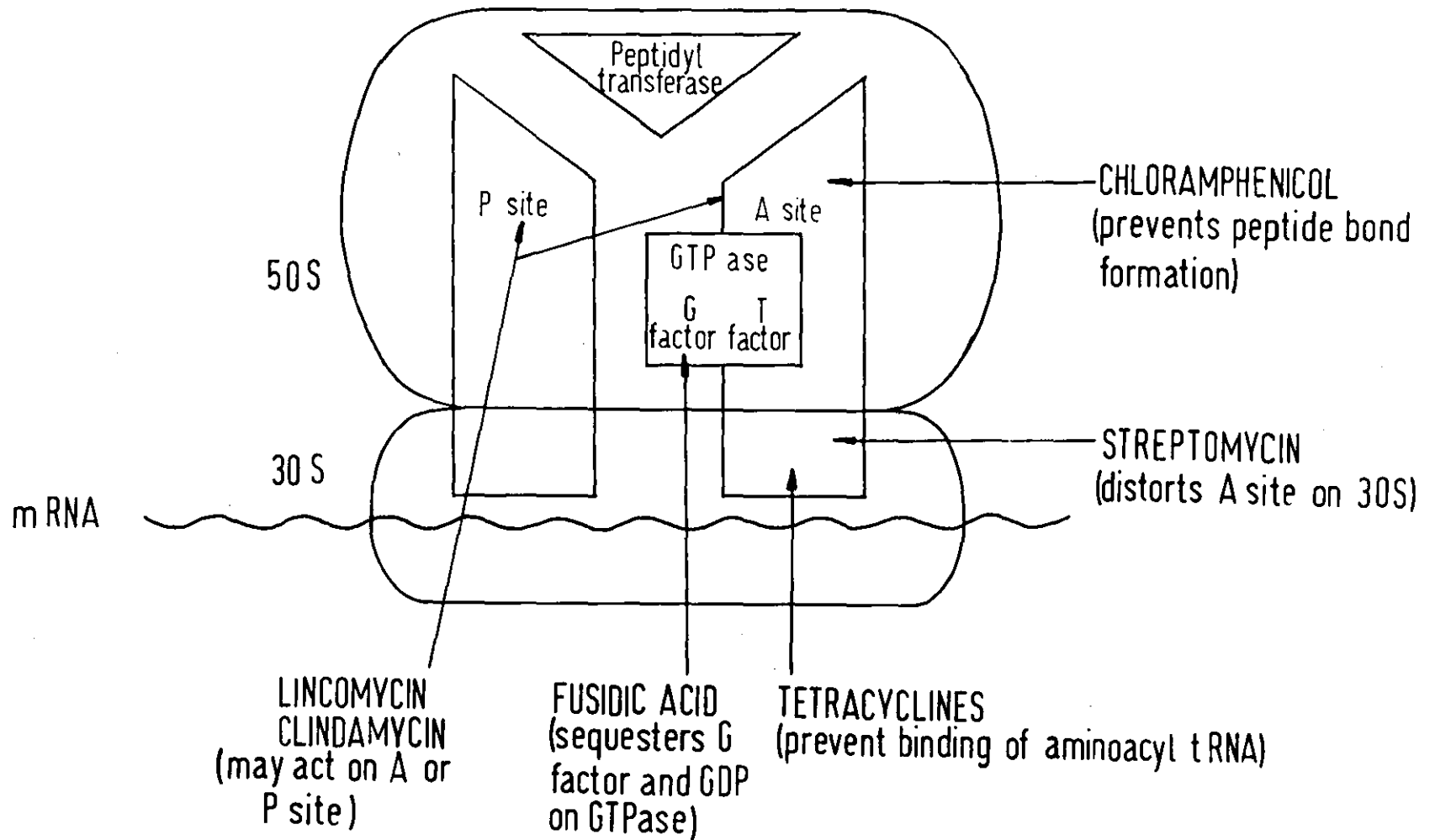


Fig 3.

Diagrammatic representation of the binding sites of the inhibitors of ribosome function.



The large diameter (0.9 μ) of A. viscosus 398B enabled the organism to be examined without any difficulty by light microscopy. The other organisms, with the exception of A. viscosus 627, could not be examined in any detail by light microscopy. This large diameter of A. viscosus 398B permitted a ready recognition of an unusual cellular event which resulted in a partial clearing of the cytoplasm (Plate 9) and to a collapse of part of the filament wall (Plates 10-12). This event for the sake of brevity is termed 'vacuolation'.

Vacuolation was observed to occur after 14 h in nutrient broth or 20 h in tryptone-soya broth. In addition, a rapid vacuolation was observed in the synthetic medium within 14 h, although the yield, as judged by cell mass, was low. Vacuolation occurred whether the organism was grown aerobically or in an atmosphere of CO₂/N₂ (5:95%). When the growth rate was followed it became apparent that vacuolation started at the end of active growth in both nutrient broth and tryptone-soya broth and was followed by a fall in dry weight (Fig 2).

No positive results were obtained with the lipid stain although some lipid staining material apparently existed (Plate 13). However, these lipid staining areas did not correspond to the vacuoles. Similarly, the cell wall stain revealed no unusual features (Plate 14). However, the spore staining procedure of Lechtman et al removed a large number of the vacuoles, but small vacuoles remained and no structure stained with any spore stain. The spore stain of Schaeffer and Fulton left a large number of the vacuoles intact (Plate 15).

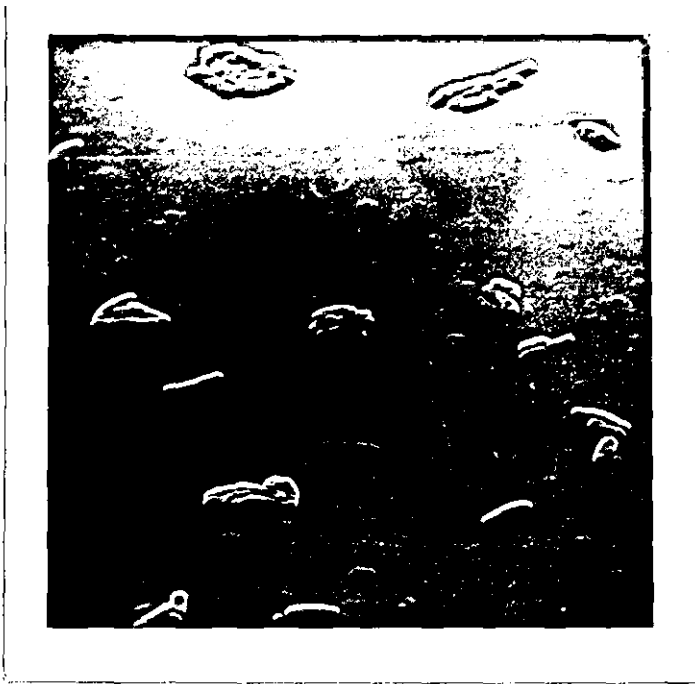


Plate 16. - S.E.M. of A. humiferus grown in nutrient broth
for 4 days. x 8,000.

Plate 17. - S.E.M. of A. ramosus grown in nutrient broth
for 4 days. x 8,000.

Effect of Antimetabolites upon Vacuolation.

The cells exhibited no alteration in appearance when exposed under the gradient plate conditions to certain inhibitors of nucleic acid and protein synthesis, namely, chloroquine, colchicine, chloramphenicol, acridine orange, lincomycin, neomycin, gentamycin, ethidium bromide, oxytetracycline or phenylethyl alcohol. In addition, these antimetabolites failed to inhibit vacuolation. On the other hand, sodium fusidate, chlorotetracycline, clindamycin and rifampicin, which do inhibit protein synthesis, did prevent vacuolation taking place either completely or to a significant extent.

The inhibition of peptidoglycan synthesis by benzylpenicillin, D-cycloserine, gramicidin J, bacitracin, vancomycin or phosphomycin did not affect vacuolation. In some instances methicillin did inhibit vacuolation, although when the cells were shaken in nutrient or tryptone-soya broth, for 5 h, and methicillin added to give a final concentration ($\mu\text{g/ml}$) of 20, 40, or 170 partial vacuolation occurred.

Actinomyces humiferus and Agromyces ramosus existed as short rods with little or no evidence of filamentous forms (Plates 16 & 17).

(ii) Fermentation and Characterisation of the Organism Designated as Bacillus cereus SV-1 (spore variable-1).

Phenol peptone water:

Glucose, salicin and sucrose, acid but no gas; mannitol, xylose, arabinose, no acid; glycerol, perhaps a small quantity of gas.

There was no evidence of citrate utilisation on Simmons citrate agar, neither was any hydrogen sulphide produced from "Kliger iron agar ('Oxoid' CM 33)".



Plate 18. - Phase contrast microscopy of B. cereus SV-1 repeatedly sub-cultured in nutrient-broth-tryptone soya medium. The phase dark particles exhibited Brownian motion. x 1,250.

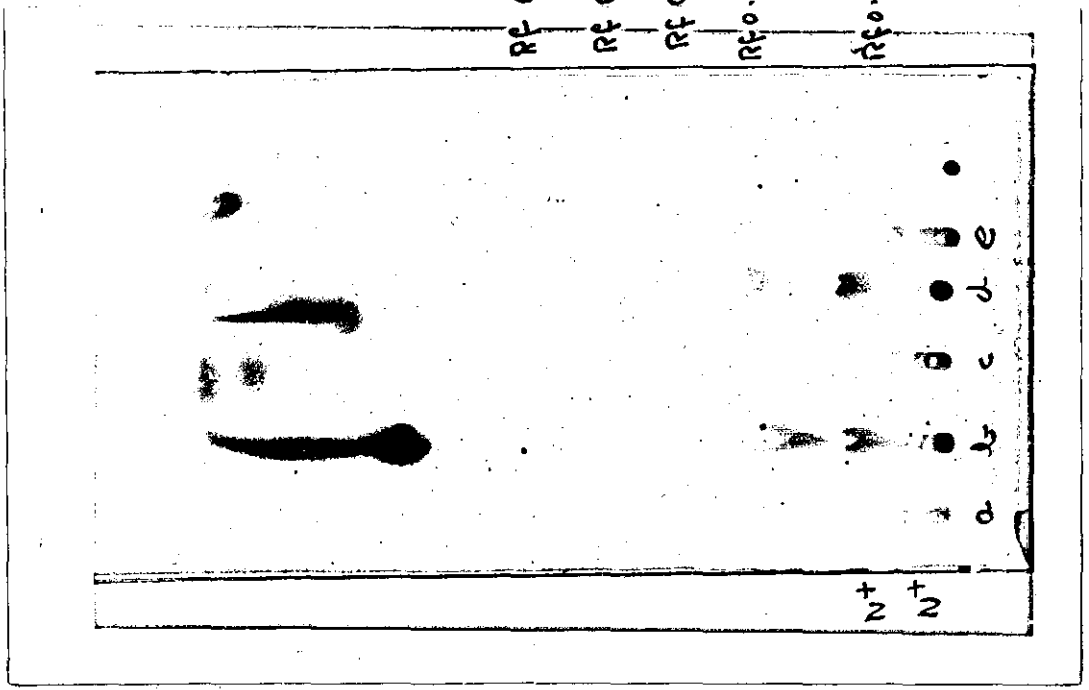
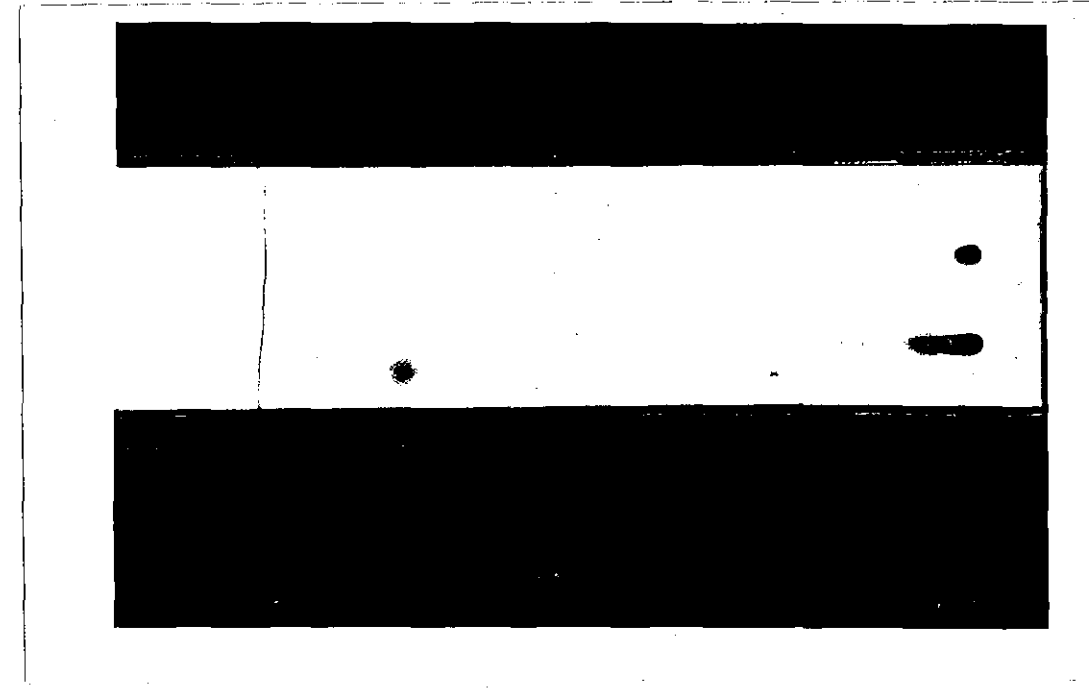


Plate 19. - Thin layer chromatogram of lipids from B. cereusSV-1.
Left to right:

- a) 18 h. surface culture at 26°.
Ninhydrin positive lipids near origin with traces of glycolipid.
- b) 26° broth culture.
One ninhydrin positive lipid on origin with ninhydrin positive lipids at Rf 0.11 and 0.22. Rf 0.11 was positive to the phospholipid spray while Rf 0.53 was positive to periodic acid Schiff's (PAS) reagent.
- c) 31° broth culture.
4 ninhydrin positive lipids were detected on the origin.
Only a trace of ninhydrin positive lipid at Rf 0.22 was observed. No PAS positive component was observed.
- d) 37° broth culture.
Ninhydrin positive component occurred on origin with a phospholipid at Rf 0.15.
PAS positive components occurred at Rf. 0.27 (yellow), Rf 0.32 (purple), Rf 0.62 (green).
- e) 45° broth culture.
Ninhydrin positive components occurred on the origin with phospholipids close to the origin.
Component Rf 0.27 (yellow) was PAS positive.

Plate 20. - Thin layer chromatogram of lipids from B. insolitus cultured at 20° and 30°. Lipids were visualized with ninhydrin.

Table 6b.

Amino-acid composition of bacterial cell walls
(g amino-acid per 100 g sample)

| | 26° | 37° | 26° |
|---|----------------|-----------------|------------------|
| | I ⁺ | II ⁺ | III ⁺ |
| Aspartic Acid | 2.5 | 4.1 | 1.4 |
| Threonine | 1.4 | 1.6 | 1.0 |
| Serine | 1.3 | 0.7 | 0.9 |
| *Glutamic Acid (Muramic Acid) | 7.9 | 6.9 | 6.7 |
| Glycine | 1.3 | 1.3 | 1.2 |
| Alanine | 5.3 | 5.4 | 5.6 |
| Valine | 1.3 | 1.0 | 0.04 |
| Isoleucine | 1.8 | 0.8 | 1.5 |
| Leucine | 2.6 | 1.5 | 2.5 |
| *Tyrosine (Glucosamine gamma-aminobutyric acid) | 14.1 | 7.7 | 6.8 |
| *Phenylalanine (Galactosamine) | 6.8 | 1.9 | 1.8 |
| Histidine | 0.9 | 0.4 | 0.6 |
| Lysine | 1.4 | 2.5 | 1.0 |
| Ammonia | 1.6 | 1.6 | 1.3 |
| Arginine | 5.4 | 0.6 | 1.5 |
| Diaminopimelic Acid | 7.2 | 5.2 | 6.5 |

*Tentative identification in area where other components, in parenthesis, are eluted.

I⁺, Surface culture (26°)

II⁺, Broth culture (37°)

III⁺, Broth culture (26°)

Spores could be induced from a broth culture by repeated sub-culturing on nutrient agar slopes at 30°. When this sporulating culture was transferred to nutrient broth it gradually lost its ability to produce spores on repeated subculture.

(iii) Morphology and Differentiation of *Bacillus cereus* SV-1.

Examination of *B. cereus* SV-1 under phase contrast microscopy revealed that the cells exhibited variations in morphology at different temperatures.

An established sporulating culture produced spores in nutrient broth quite readily at 31° after 5 days while at 37° very few spores were produced in the same period of time and an inoculum at 42° produced even fewer spores. A phenomenon characteristic of growth at 37° and 42° was the production of small phase dark bodies exhibiting Brownian motion within the cell (Plate 18), and in some cases these phase dark bodies were observed outside the cell.

A *B. cereus* culture adapted to a liquid medium exhibited morphological diversity when grown at 31°, 37° and 42°. After growing for 10 h at 42° the cells formed long thin filaments (20 μ) while those grown at 31° were much shorter and broader and those grown at 37° exhibited intermediate shape. When the same stock culture was used to inoculate an agar surface in Roux flasks maintained at 26° the cells recovered were somewhat similar to those of a broth culture at 26°.

The cells obtained from these cultures grown for 15 h under broth shake culture conditions and on an agar surface exhibited quite different lipid contents as demonstrated by thin layer chromatography (Plate 19). As well as exhibiting quite different lipid compositions the cell walls contained amino acids in varying proportions (Table 6b). (Courtesy of Dr. C. Hitchcock, Unilever, Sharnbrook, Bedfordshire).

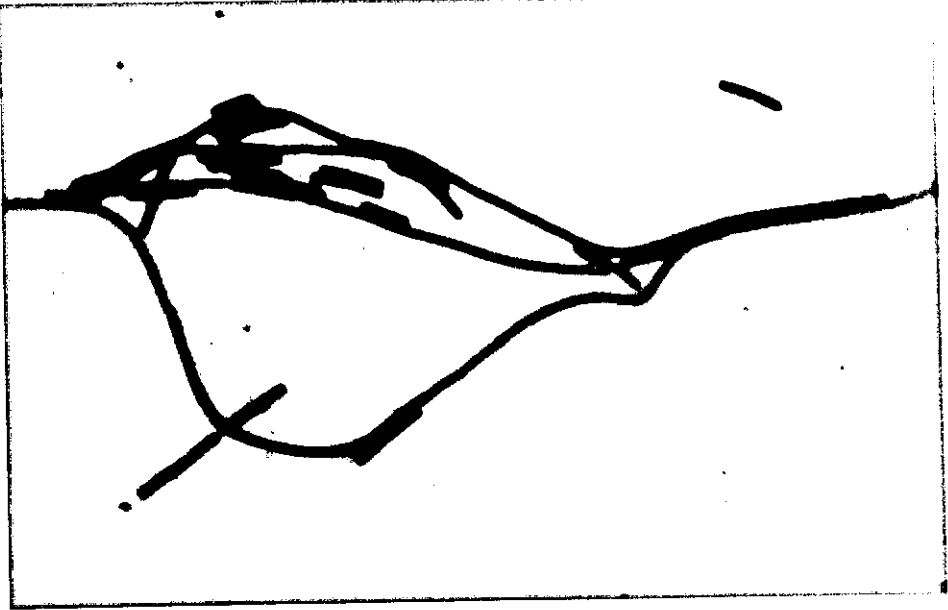


Plate 21. - Filamentous forms of B. cereus SV-1 cultured in the presence of streptomycin. Methylene blue.
x 1,250.

Plate 22. - Filamentous forms of B. cereus SV-1 cultured in the presence of acridine orange. Methylene blue.
x 1,250.

Plate 23. - Filamentous forms of B. cereus SV-1 cultured in the presence of clindamycin. Methylene blue.
x 1,250.

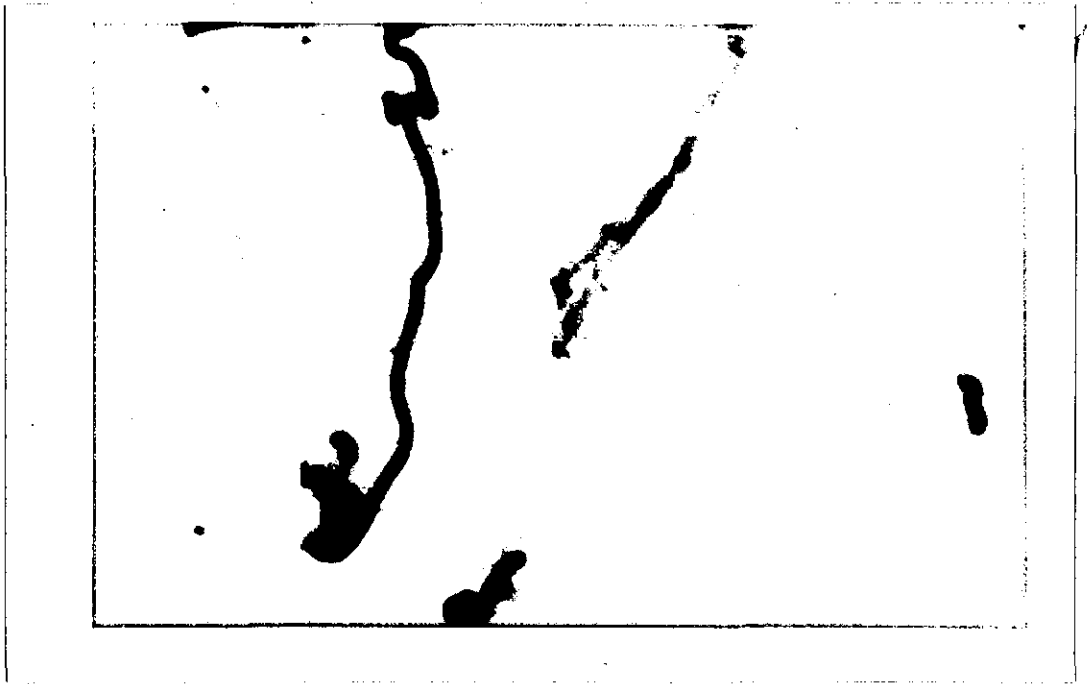


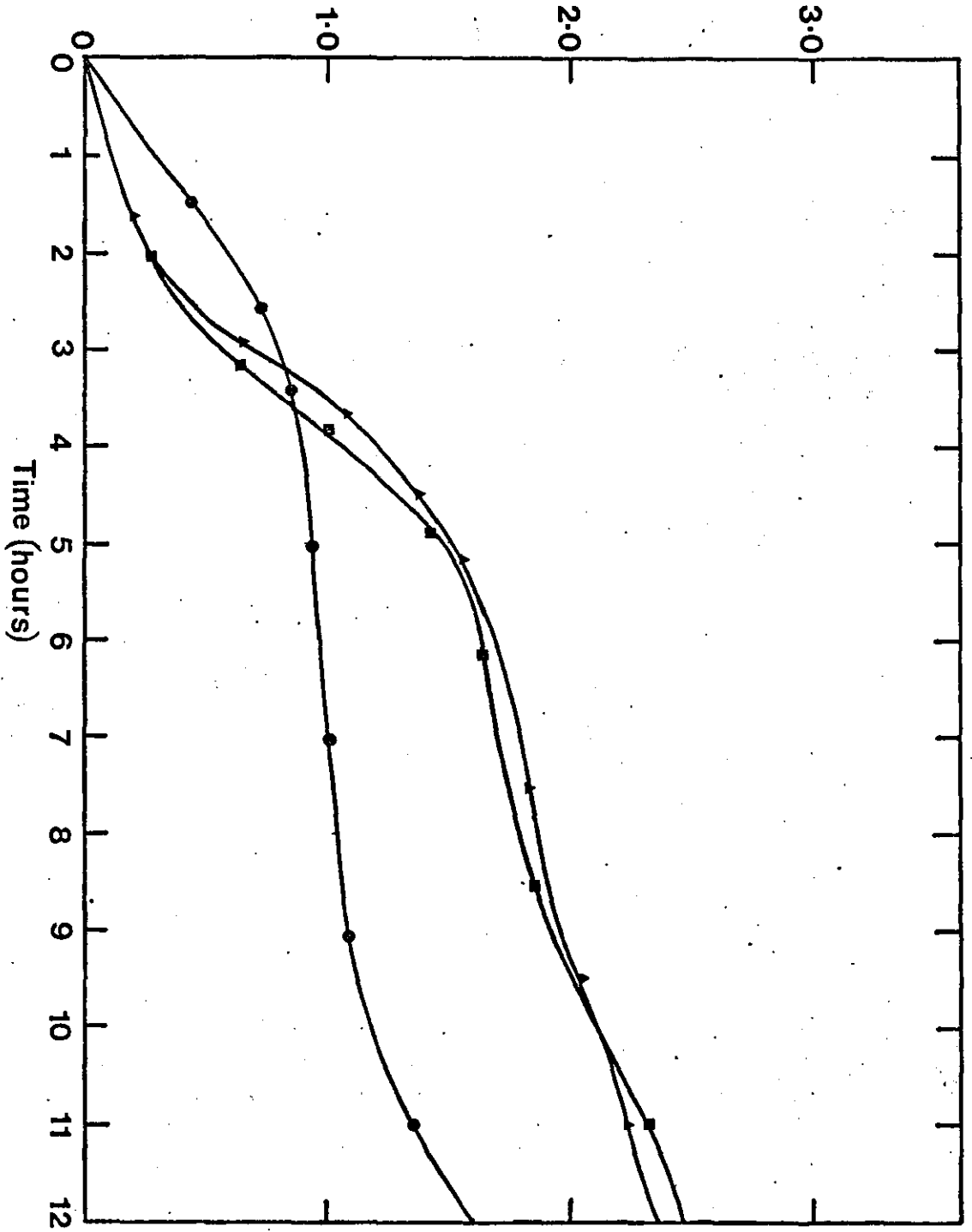
Plate 23a. - Long twirling forms of B. cereus SV-1
characteristic of ethidium bromide action.
Methylene blue. x 1,250.

Fig 4.

Growth of B. cereus SV-1 as response to temperature.

- 42°
- 37°
- ▲ 31°

O.D. 420 nm.



The psychrophile B. insolitus exhibited different lipid composition at 26° and 30° (Plate 20).

The growth rate of B. cereus SV-1 decreased with increase in temperature (Fig 4).

Response of Bacillus cereus SV-1 to Antimetabolites.

Bacillus cereus showed no morphological variation when cultured on a nutrient agar gradient plate at 30° in the presence of methicillin, gentamycin, rifampicin, sodium fusidate or chlorotetracycline.

However, when B. cereus was cultured at 30° on a gradient plate in the presence of streptomycin and acridine orange very long filaments were produced (Plates 21 & 22). Clindamycin produced a few long filaments but they were not as long as those produced by streptomycin or acridine orange (Plate 23). Lincomycin produced filaments of a similar size to those of clindamycin. Ethidium bromide produced long filaments but these were characterised by the large number of twirling structures (Plate 23a) and in some cases shorter rods were produced which were extensively curled. Unfortunately, these cells did not exhibit detailed morphology when stained with methylene blue or any other nucleic acid staining dye. Chloroquine phosphate produced elongated cells which had a slightly greater diameter than those observed under normal conditions of growth. Colchicine produced cells of similar diameter to those of normal cells but they were twice as long as normal and were characterised by the larger number of very small vacuoles in the cell. None of these vacuoles retained the spore stains.

In all of the cases the organisms exhibited typical morphology at the area where the antimetabolite concentration was very low and in every case the organism exhibited normal growth when the pseudo-filamentous cells were restored to normal culture conditions. In

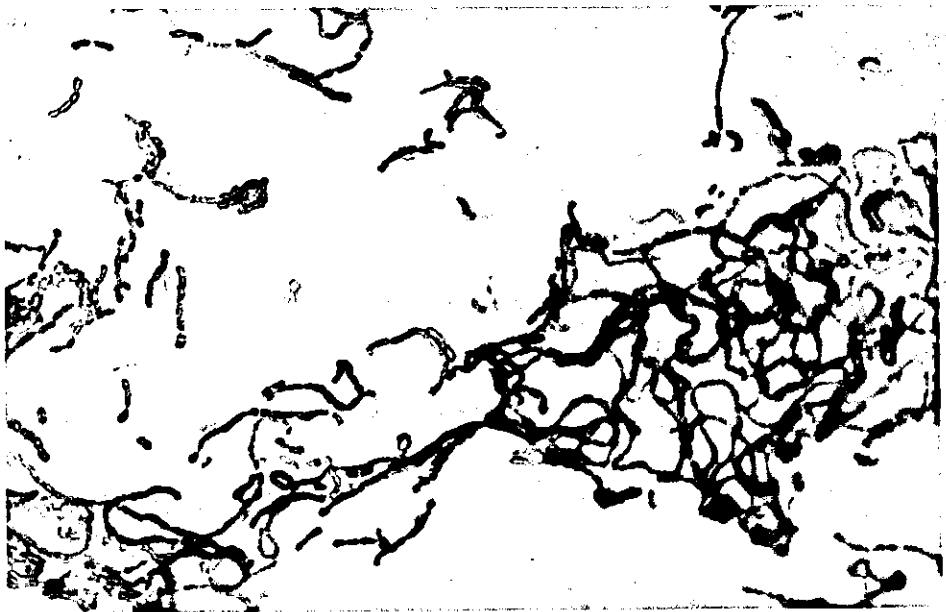


Plate 24. - Phase contrast microscopy of A. missouriense
cultured on Actinoplanes medium for 7 days.
x 1,250.

Plate 25. - S.E.M. of A. missouriense cultured on
Actinoplanes medium for 7 days. x 4,000.

Plate 26. - Filamentous forms of A. missouriense obtained
in the presence of clindamycin. x 1,250.

addition to producing morphological diversity in B. cereus the anti-metabolites severely impaired sporulation in a strain adapted to producing spores on an agar surface. In some cases sporulation occurred 5 days after it was observed in the low concentration area.

In no cases did the cells contain any lipid droplets.

No filamentous forms could be induced for E. coli NCTC 9001, B. megaterium KM NCIB 9521, B. subtilis NCIB 3610, B. cereus NCIB 8849, P. vulgaris NCTC 8067 and Ps. aeruginosa NCIB 8295.

Scanning electron microscopy of B. cereus revealed no features of particular interest. The very low yields of cells at the high concentration points of the antimetabolites made it very difficult to prepare representative samples for SEM. It proved impossible to produce similar morphological forms from broth culture.

(iv) Morphological Variation of Actinoplanes missouriense.

When Actinoplanes missouriense was cultured under static conditions in the Actinoplanes medium it produced growth after 6-8 days over the bottom of the flask and with little or no turbidity in the remainder of the medium. Examination of the cells by phase contrast microscopy revealed large ellipsoid cells (Plate 24). The lipid stain showed the presence of a few lipid droplets while the cell wall stain showed a very thick wall. Scanning electron microscopy showed the large ellipsoid bodies to be surrounded by diplococcus like cells which were possibly produced by a budding mechanism (Plate 25).

Actinoplanes missouriense when cultured on gradient plates containing clindamycin produced long curling filaments and these reverted to the ellipsoid forms on subculture (Plate 26). Similar filaments were produced by lincomycin. None of the other antimetabolites produced morphological variants. It was estimated that

clindamycin was at a concentration of 5 $\mu\text{g/ml}$ at the point where the filaments were induced.

As in the case of B. cereus it was not possible to produce any similar filaments in a broth culture containing clindamycin and lincomycin.

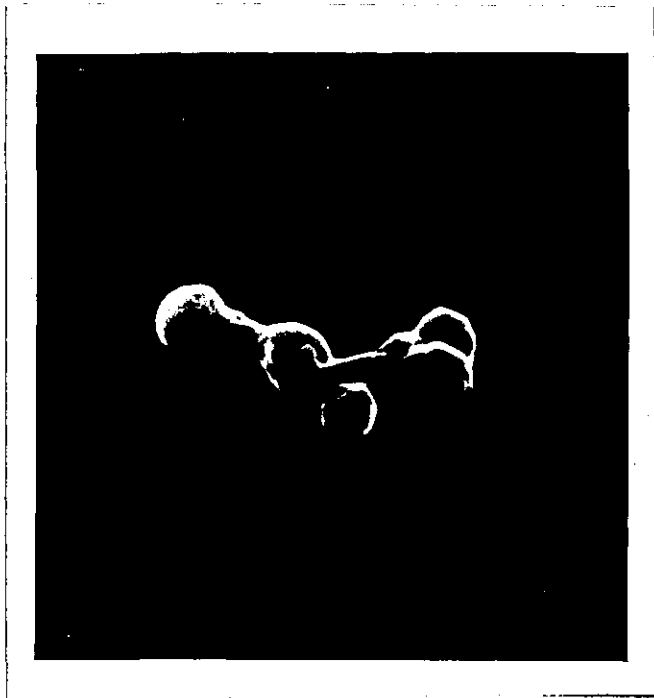


Plate 27. - Scanning electronmicrograph of E. coli grown for 24 h in the presence of 2,500 $\mu\text{g/ml}$ of 3-CPD showing incomplete separation of the cells. x 16,000.

Plate 28. - Scanning electronmicrograph of S. aureus grown for 30 h in the presence of 3,000 $\mu\text{g/ml}$ of 3-CPD showing lack of separation of the cocci. x 7,200.

Plate 29. - Scanning electronmicrograph of E. coli grown for 24 h in a nutrient medium containing 4,800 $\mu\text{g/ml}$ of 3-CPD showing filamentous form exhibiting surface damage and debris from disintegrated cells. x 8,000.

Table 7.

Inhibitory effect of 3-chloropropane-1,2-diol using a filter-paper disc method (Whatman 3 mm) impregnated with 12500 μ g of the compound.

| Organism | Diameter of the zone of inhibition (mm) |
|-------------------------|---|
| <i>E. coli</i> | 25 |
| <i>Sal. abortusovis</i> | 40 |
| <i>Pr. vulgaris</i> | 31 |
| <i>K. aeruginosa</i> | 0 |
| <i>S. aureus</i> | 12 |
| <i>S. cerevisiae</i> | 15 |

B. The Response of Microorganisms to Variations in Lipid Composition.

(i) Supplementation of Medium with 3-Chloropropane-1, 2-diol.

Escherichia coli NCTC 9000, Sal. abortusovis NCTC 10241, Pr. vulgaris NCIB 8067, K. aerogenes NCIB 8267 and S. aureus NCIB 8625, were cultured on tryptone soya broth and S. cerevisiae was cultured on maltose peptone medium. The response of all these organisms to 3-chloropropane-1, 2-diol (3-CPD) was determined by the filter disc assay method (Table 7). None of the organisms studied was able to utilise 3-CPD as its sole carbon source. 3-CPD did not inhibit growth as it proved impossible to obtain a minimum inhibitory concentration value for either E. coli or S. aureus.

When E. coli was cultured under static conditions for 20 h on tryptone soya broth supplemented with 3-CPD (3,000 µg/ml), the cells were demonstrated by methylene blue staining to contain a proportion (ca 15%) of filaments which averaged a length of 10-50 cells. Similar results could be obtained from shake cultures when the culture time was extended to 30 h. When S. aureus was cultured in the medium supplemented with 3-CPD (2,000 µg/ml) light microscopy of the methylene blue stained sample demonstrated the presence of tightly associated cocci. Further details of these cells were demonstrated by scanning electron microscopy which showed filaments of E. coli (Plate 27). When 3-CPD was used at a concentration of 4,500 µg/ml the filamentous forms exhibited evidence of damage at the cell surface (Plate 29). In the case of S. aureus the cocci were compact (Plate 28). The available evidence indicated that 3-CPD was impairing cell division.

After 12 h static culture in the presence of 3-CPD (1,500 µg/ml) only a few filamentous forms of K. aerogenes could be found, however, the staining reaction indicated that there was some loss of cellular constituents. When the 3-CPD concentration was doubled the cell

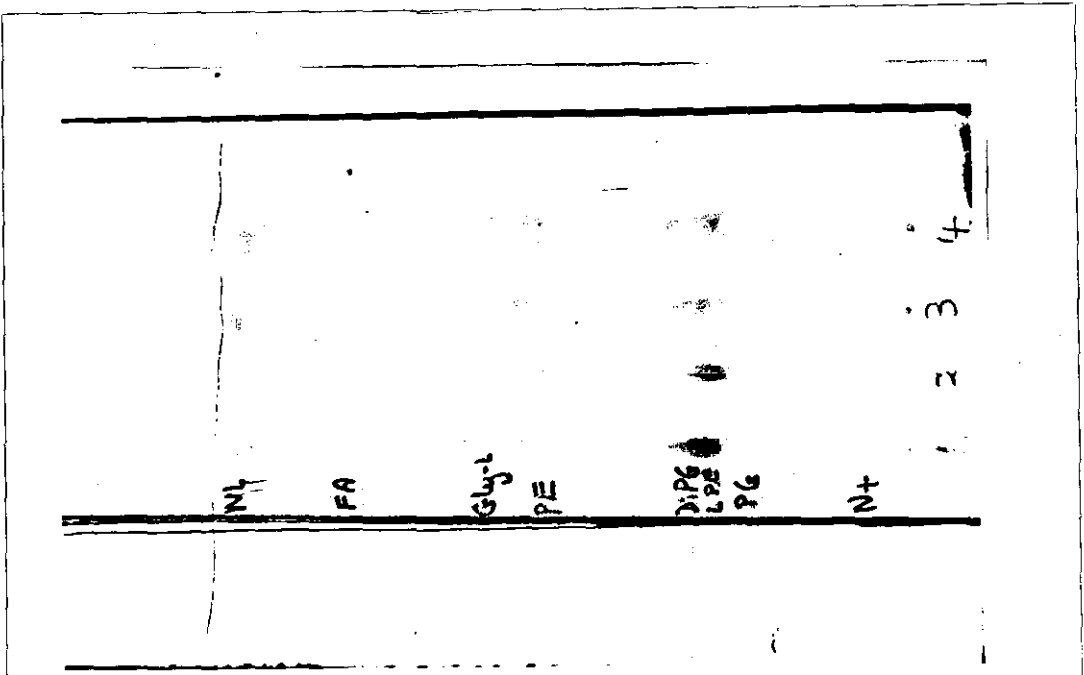
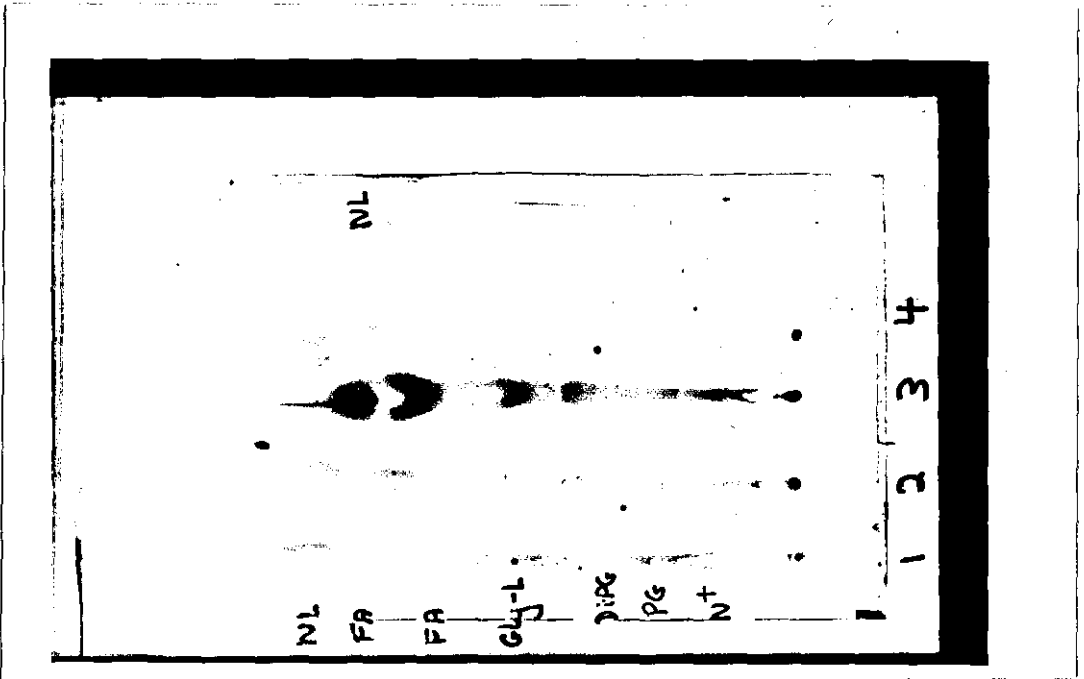
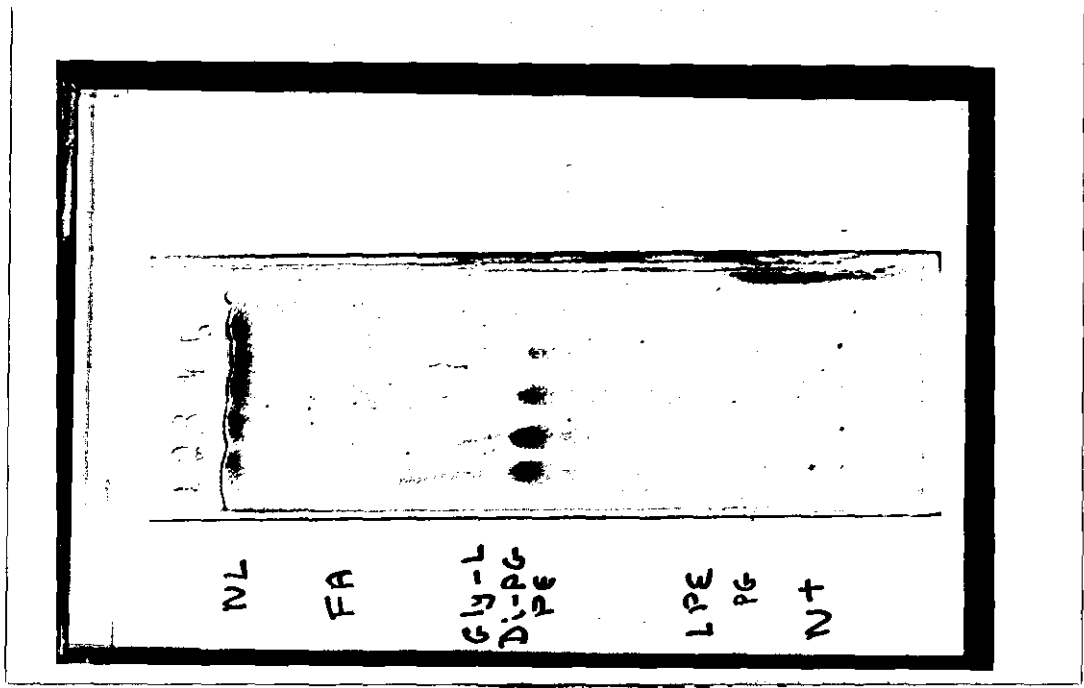


Plate 30. - Thin layer chromatogram charred of the chloroform: methanol extractable lipids of (1) E. coli (2) E. coli grown in presence of 750 $\mu\text{g/ml}$ 3-CPD (3) E. coli grown in the presence of 1,200 $\mu\text{g/ml}$ 3-CPD (4) E. coli grown in the presence of 2,500 $\mu\text{g/ml}$ 3-CPD and (5) E. coli grown in the presence of 3,500 $\mu\text{g/ml}$ 3-CPD. The abbreviations are: LPE, lysophosphatidylethanolamine; PG, phosphatidylglycerol; PE, phosphatidylethanolamine; Di-PG, diphosphatidylglycerol; FA, fatty acid; NL, neutral lipid; N^+ , ninhydrin positive lipid, Gly-L, glycolipid.

Plate 31. - Thin layer chromatogram charred of the chloroform: methanol extractable lipids of (1) S. aureus (2) S. aureus grown in the presence of 1,200 $\mu\text{g/ml}$ of 3-CPD (3) S. aureus grown in the presence of 2,500 $\mu\text{g/ml}$ of 3-CPD (4) S. aureus grown in the presence of 3,500 $\mu\text{g/ml}$ of 3-CPD. The abbreviations are: LPE, lysophosphatidylethanolamine; PG, phosphatidylglycerol; PE, phosphatidylethanolamine; Di-PG, diphosphatidylglycerol; FA, fatty acid; NL, neutral lipid; N^+ , ninhydrin positive lipid; Gly-L, glycolipid. There are two neutral lipid components appearing in (4).

Plate 32. - Thin layer chromatograms charred of the chloroform: methanol extractable lipids of (1) S. cerevisiae (2) S. cerevisiae grown in the presence of 1,200 $\mu\text{g/ml}$ of 3-CPD (3) S. cerevisiae grown in the presence of 2,500 $\mu\text{g/ml}$ 3-CPD (4) S. cerevisiae grown in the presence of 3,500 $\mu\text{g/ml}$ of 3-CPD. The abbreviations are: LPE, lysophosphatidylethanolamine; PG, phosphatidylglycerol; PE, phosphatidylethanolamine; Di-PG, diphosphatidylglycerol; FA, fatty acid; NL, neutral lipid; N^+ ninhydrin positive lipid; Gly-L, glycolipid. Samples 3 and 4 show a decrease in ninhydrin positive lipid.

Table 8.

The effect of 3-chloropropane-1,2-diol on antibacterial action assessed by the filter paper disc method (Whatman 13 mm) and measuring the zone of inhibition (mm). produced.

| Antimetabolite | E. coli in the presence of 3-chloropropane-1,2-diol ($\mu\text{g/ml}$) | | | | | | S. aureus in the presence of 3-chloropropane-1,2-diol ($\mu\text{g/ml}$) | | | | | |
|---|---|-----|------|------|------|------|---|-----|------|------|------|------|
| | 0 | 550 | 1300 | 2650 | 3950 | 5230 | 0 | 550 | 1300 | 2650 | 3950 | 5250 |
| Chloramphenicol | 22 | 19 | 22 | 21 | 23 | 26 | 24 | 24 | 24 | 26 | 30 | 30 |
| Tetracycline | 16 | 17 | 19 | 22 | 23 | 29 | 25 | 27 | 34 | 34 | 41 | 39 |
| Streptomycin | 24 | 24 | 18 | 12 | 8 | 0 | 25 | 20 | 19 | 0 | 0 | 0 |
| Erythromycin | 14 | 0 | 0 | 0 | 0 | 0 | 32 | 28 | 27 | 26 | 22 | 22 |
| Clindamycin | 0 | 0 | 0 | 0 | 0 | 0 | 35 | 35 | 35 | 33 | 32 | 36 |
| Lincomycin | 0 | 0 | 0 | 0 | 0 | 0 | 38 | 33 | 25 | 25 | 26 | 26 |
| Sodium fusidate | 0 | 0 | 0 | 0 | 0 | 0 | 38 | 35 | 39 | 38 | 41 | 45 |
| Gentamycin | 24 | 21 | 18 | 18 | 17 | 18 | 32 | 28 | 28 | 28 | 28 | 28 |
| Pencillin G | 0 | 0 | 0 | 0 | 0 | 0 | 36 | 37 | 38 | 38 | 43 | 44 |
| Vancomycin hydrochloride | 0 | 0 | 0 | 0 | 0 | 0 | 19 | 21 | 20 | 21 | 23 | 24 |
| Quinacillin | 0 | 0 | 0 | 0 | 0 | 0 | 33 | 36 | 40 | 40 | 46 | — |
| Phosphonomycin | 22 | 25 | 23 | 23 | 23 | — | 30 | 30 | 25 | 21 | 21 | 22 |
| Chlorhexidine | 22 | 22 | 22 | 22 | 24 | 25 | 25 | 24 | 25 | 29 | 29 | 29 |
| Diphenyliodonium chloride bis 2,2'-biphenylene iodonium tetrafluoroborate | 24 | 20 | 18 | 18 | 0 | 0 | 27 | 24 | 20 | 0 | 0 | 0 |
| Bronopol | 30 | 27 | 27 | 28 | 33 | 37 | 30 | 29 | 30 | 30 | 30 | 30 |
| Phenyl mercuric nitrate | 30 | 32 | 32 | 34 | 36 | 40 | 36 | 36 | 37 | 36 | 43 | 42 |
| 4,7-Phenanthroline-5,6- quinone | 20 | 20 | 20 | 14 | 20 | 25 | 42 | 42 | 42 | 44 | 44 | 48 |
| 2,4-dinitrophenol | 28 | 30 | 28 | 28 | 30 | 33 | 27 | 28 | 28 | 29 | 28 | 29 |
| | 0 | 0 | 17 | 18 | 19 | 22 | 12 | 33 | 33 | 33 | 44 | 46 |

Table 9.

Antibacterial effect of bacitracin (100 μg) in the presence of various concentrations of 3-chloropropane-1,2-diol, estimated by the filter-paper disc (Whatman 3 mm) method.

| | Diameter of the zone of inhibition (mm) produced by 100 μg of bacitracin | | | |
|---|---|----------------|---------------------|-------------------------|
| Concentration of 3-chloropropane-1,2-diol ($\mu\text{g}/\text{ml}$) | <i>K. aerogenes</i> | <i>E. coli</i> | <i>Pr. vulgaris</i> | <i>Sal. abortusovis</i> |
| 0 | 15 | 14 | 20 | 30 |
| 550 | 16 | 14 | 29 | 48 |
| 1300 | 16 | 20 | 30 | 48 |
| 2600 | 20 | 24 | 38 | + |
| 4000 | 20 | 24 | + | + |
| 5200 | 22 | 26 | + | + |

+ Organism so sensitive no growth on plate.

Table 10.

The effect of various concentrations of 3-chloropropane-1,2-diol on the antibacterial activity of polymyxin B sulphate (100 µg) estimated by the filter-paper disc method.

| Concentrations of 3-chloropropane-1,2-diol (µg/ml) | Diameter of the zone of inhibition (mm) produced by 100 µg of polymyxin | | | | |
|--|---|----------------|---------------------|-------------------------|-----------------------|
| | <i>K. aerogenes</i> | <i>E. coli</i> | <i>Pr. vulgaris</i> | <i>Sal. abortusovis</i> | <i>Ps. aeruginosa</i> |
| 0 | 15 | 22 | 23 | 24 | 18 |
| 550 | 18 | 23 | 23 | 27 | 18 |
| 1300 | 20 | 24 | 24 | 24 | 18 |
| 2600 | 20 | 24 | 24 | + | 18 |
| 4000 | 20 | 25 | + | + | 18 |
| 5200 | 20 | 26 | + | + | 21 |

+ Organism is so sensitive no growth on plate.

Fig 5.

Effect of 3-chloropropane-1,2-diol on the growth of E. coli in nutrient broth.

- o — o control culture
- — ● 1,330 $\mu\text{g/ml}$ 3-chloropropane-1,2-diol
- — ● 2,650 $\mu\text{g/ml}$ 3-chloropropane-1,2-diol
- x — x 4,740 $\mu\text{g/ml}$ 3-chloropropane-1,2-diol

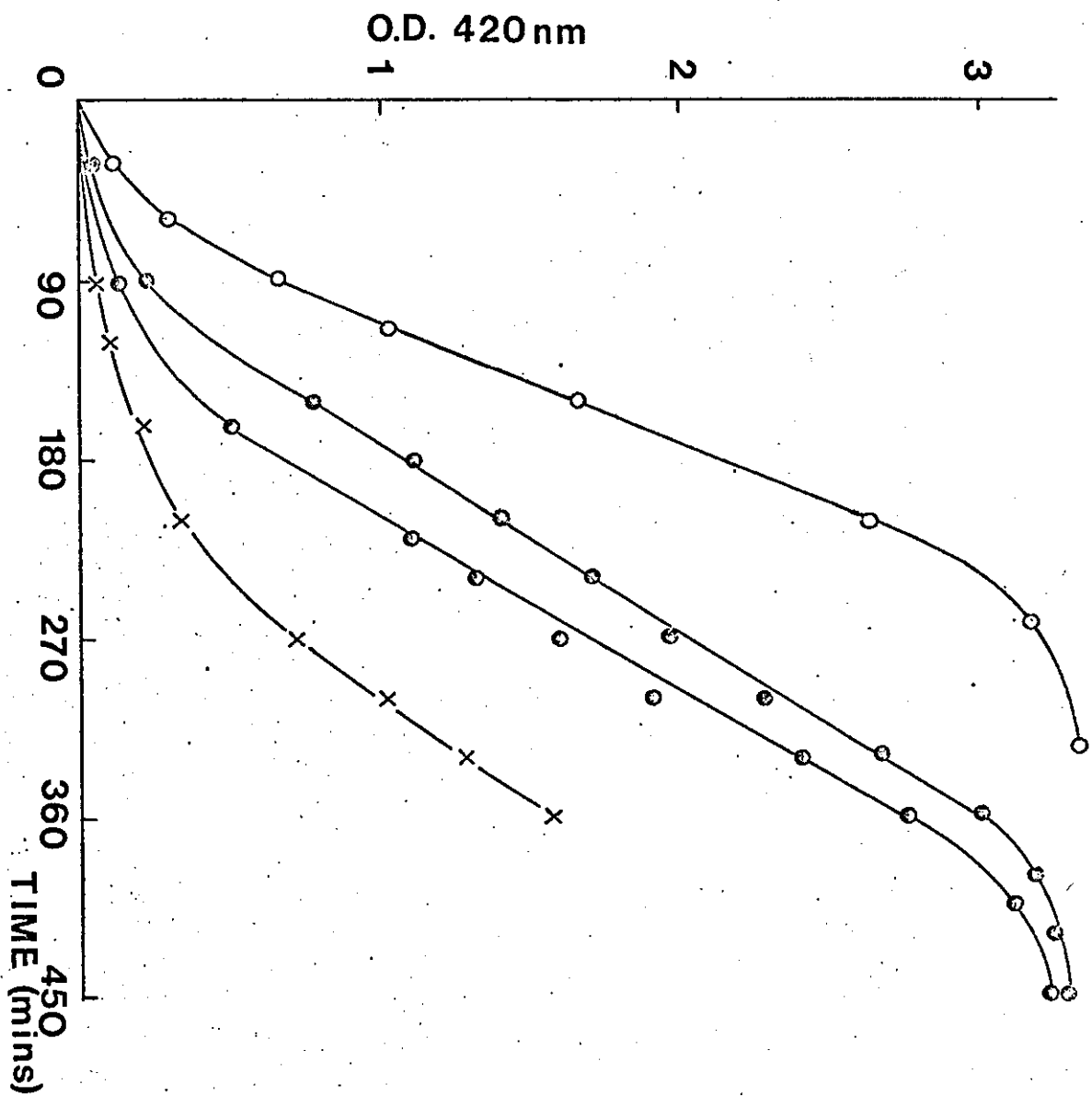


Fig 6.

Effect of 3-chloropropane-1,2-diol on the growth of
S. aureus in nutrient broth.

- o — o control culture
- — ● 1,330 µg/ml 3-chloropropane-1,2-diol
- ◐ — ◐ 2,650 µg/ml 3-chloropropane-1,2-diol
- x — x 4,740 µg/ml 3-chloropropane-1,2-diol

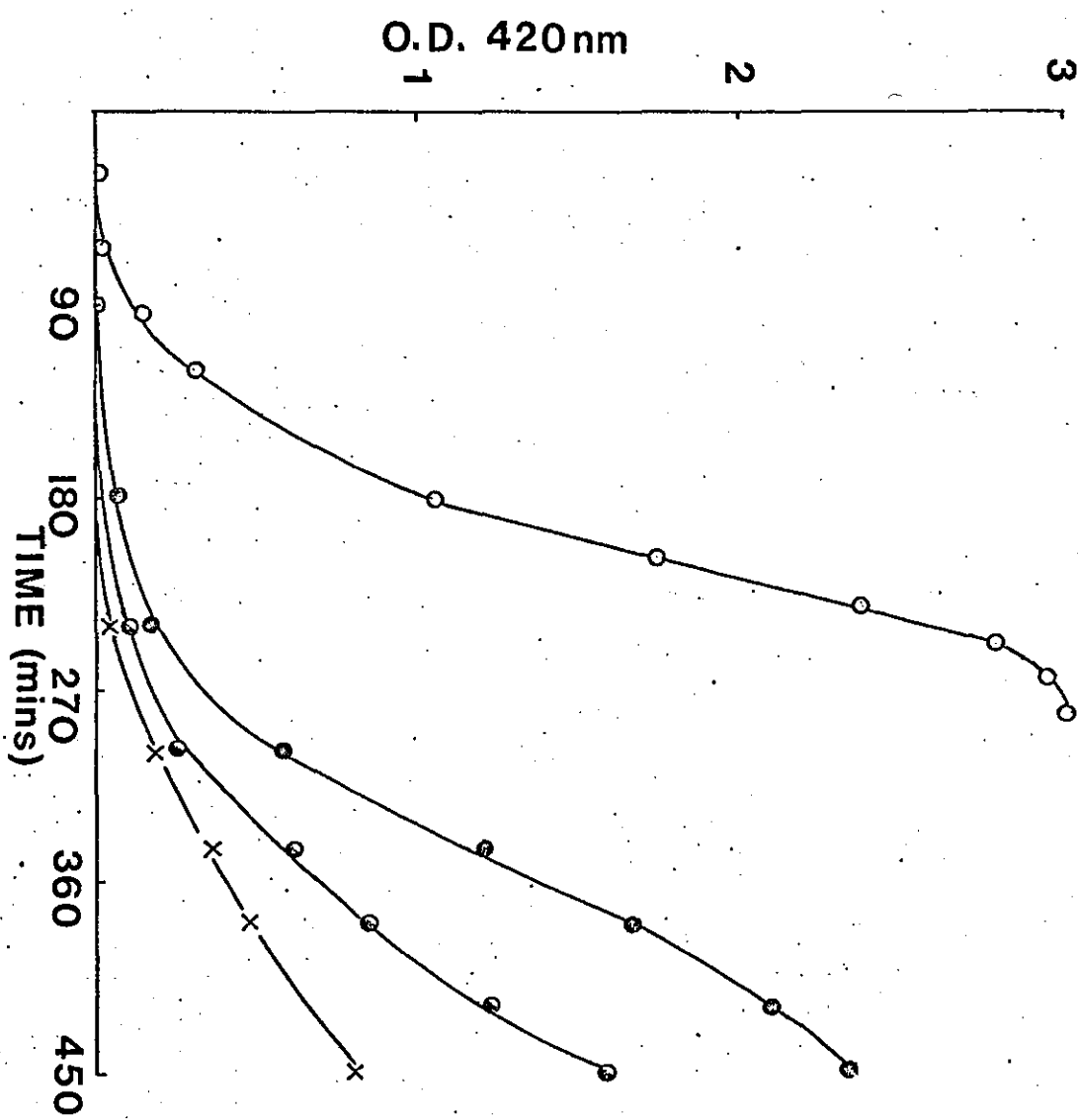


Fig 7.

Effect of 3-chloropropane-1,2-diol on the growth of a glycerol dependant mutant of B. subtilis in the synthetic medium containing 20,000 $\mu\text{g/ml}$ of glycerol.

- o — o control culture
- x — x 200 $\mu\text{g/ml}$ of 3-chloropropane-1,2-diol
- — ● 2,000 $\mu\text{g/ml}$ of 3-chloropropane-1,2-diol

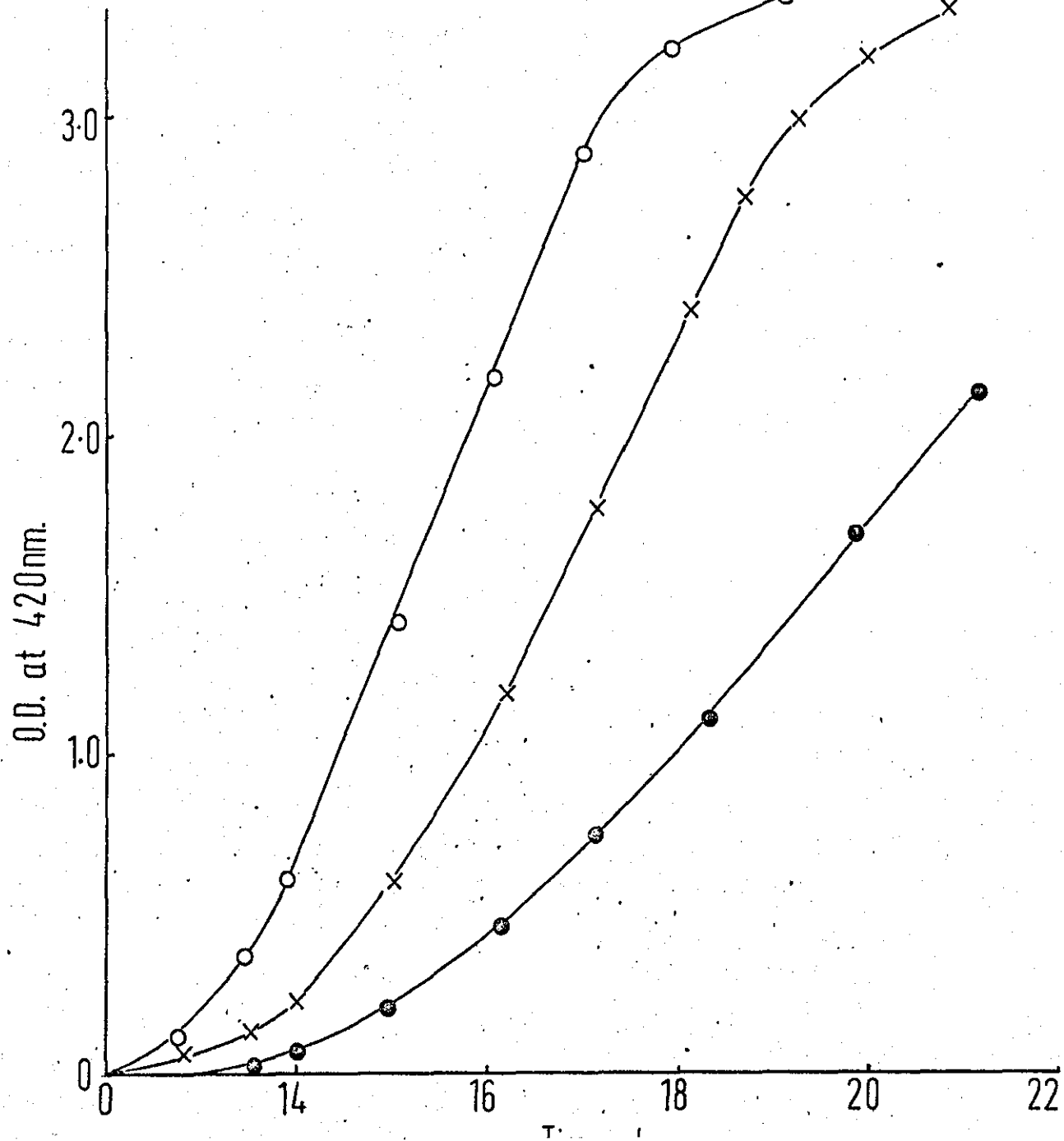
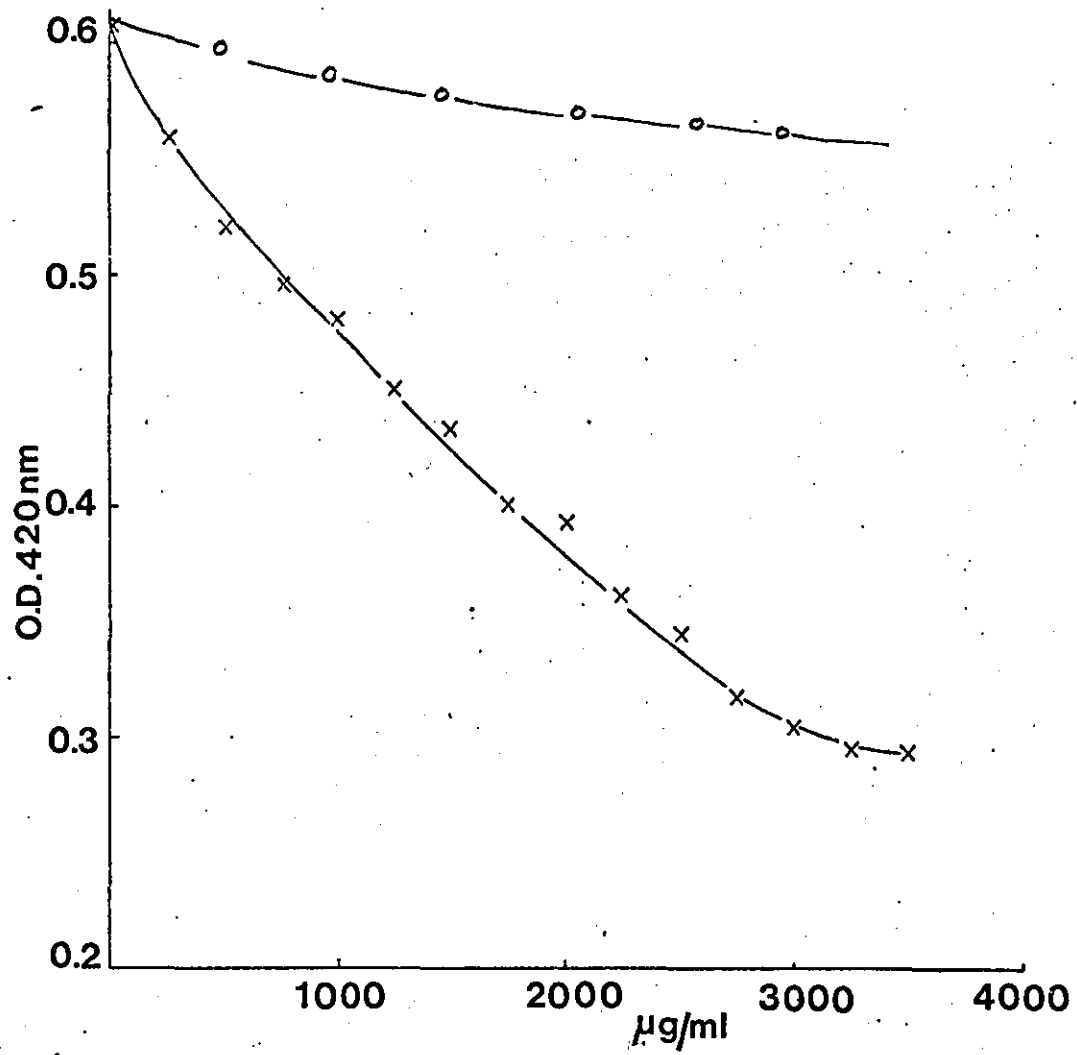


Fig 8.

Effect of 3-chloropropane-1,2-diol on the growth of
a streptomycin dependant mutant of E. coli in
nutrient broth containing 1,000 $\mu\text{g/ml}$
streptomycin.

- o — o control culture, streptomycin resistant
E. coli
- x — x growth produced by the streptomycin dependant
mutant after 18 h incubation



length increased by a factor of two.

Analysis of growth curves indicated that 3-CPD supplementation of media prolonged the lag phase and slowed the growth rate of E. coli (Fig 5) and S. aureus (Fig 6). The glycerol auxotroph, B. subtilis B42, when cultured on the defined medium supplemented with $20,000 \times 10^4$ $\mu\text{g/ml}$ of glycerol exhibited a prolonged growth phase in the presence of 200×10^2 and $2,000 \times 10^3$ $\mu\text{g/ml}$ of 3-CPD which became a complete inhibition of growth at $5,000 \times 10^3$ $\mu\text{g/ml}$ (Fig 7). The limitation in growth of the streptomycin dependent mutant when in contact with increasing concentrations of 3-CPD provided further evidence of the response of organisms to 3-CPD (Fig 8).

When the sensitivities of E. coli and S. aureus to the antibiotics and other antibacterial agents were considered (Table 8) it was apparent that the presence of 3-CPD increased sensitivity to certain compounds but had little effect in other cases while in other instances the organisms became more resistant. The response of the organisms to the agent in question was in no way related to the mode of action of the compound but instead it appeared that the effect produced was related to the hydrophilic or hydrophobic nature of the compound in question.

Polymyxin and bacitracin, antibiotics which act on the cytoplasmic membrane of Gram-negative organisms, exhibit increased action in the presence of 3-CPD (Tables 9 & 10).

The response of S. cerevisiae to nystatin, a polyene which acts upon sterols, remained unchanged in the presence of 3-CPD.

Thin layer chromatographic analysis of the lipids obtained from E. coli showed (Plate 30) that as the concentration of 3-CPD in the growth medium was increased from 0 to 5000 $\mu\text{g/ml}$ there was a reduction in certain lipid components, namely, phosphatidylglycerol, phosphatidyl ethanolamine and in the amounts of lipoamino acids. There was no

alteration in the level of fatty acids. Staphylococcus aureus and S. cerevisiae exhibited a similar variation in lipid composition (Plates 31 & 32).

Lyophilised E. coli and S. aureus had lipid contents of $10 \pm 2\%$ and $8 \pm 2\%$, respectively, when the organisms were cultured in medium containing 3-CPD ranging from 0 to 4,800 $\mu\text{g/ml}$. The analysis indicated that there was no relationship between lipid content and concentration of 3-CPD but instead the only variation observable was that of lipid composition.

The ANS-fluorescence studies showed that the emission maximum was 474 nm in all cases and that the relative emissions increased as the concentration of 3-CPD increased. In the case of S. aureus the emission maxima was 0.75, 0.91 and 1.45 at 3-CPD concentrations of 0, 1,500 and 3,500 $\mu\text{g/ml}$, respectively. Both E. coli and S. aureus exhibited similar variations.

(ii) Effect of Sterol/Steroid Supplementation.

The absence of any variation in the zone of inhibition of those compounds listed (below) suggested that the membranes of E. coli and S. aureus had remained unchanged when the organisms were cultured in sterol deficient medium supplemented (0-600 $\mu\text{g/ml}$) with a variety of sterols and steroids (cholesterol, 5 α -cholesten-3 β -ol, 5-cholesten-3 β -chloride, 5 β -cholestan-3 α -ol, 5 α -androstan-3 β -ol, 3 α -hydroxy-5 α -pregnane-11,20-dione (alpha-xalone) and 21-desoxyprednisolone-17 α -propionate). Neither S. aureus nor E. coli responded to the presence of the sterols by a variation in lipid composition.

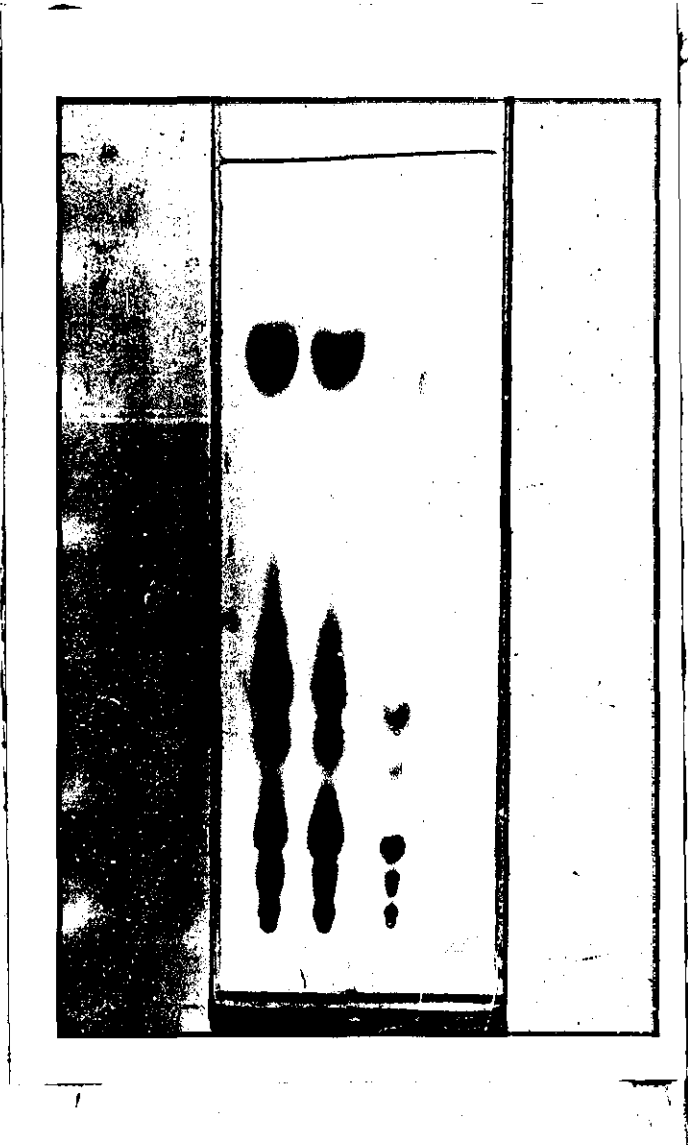


Plate 33. - Thin layer chromatograms (visualised with ninhydrin) of the chloroform-methanol extractable lipids of (1) S. cerevisiae (2) S. cerevisiae grown in the presence of 90 $\mu\text{g/ml}$ of alphaxalone (3) S. cerevisiae grown in the presence of 350 $\mu\text{g/ml}$ of alphaxalone.

Table 11.

Response of S. cerevisiae and C. albicans to antimetabolites when cultured in the presence of alphaxalone.

| Alphaxalone μg/ml | <u>S. cerevisiae</u> | | <u>C. albicans</u> | |
|----------------------|--------------------------------|--|--------------------------------|--|
| | 8-hydroxy-* quinoline mm | Phenylmercuric nitrate ⁺ mm | 8-hydroxy-* quinoline mm | Phenylmercuric ⁺ nitrate mm |
| 0 | 32 | 28 | 51 | 36 |
| 30 | 30 | 32 | 45 | 38 |
| 90 | 28 | 37 | 38 | 44 |
| 150 | 23 | 45 | Limited | Growth |
| 300 | 15 | 46 | Limited | Growth |

*Whatman 13 mm disc air dried after immersion in ethanolic solution (50 μg/ml).

+ " " " " " " " " aqueous solution (50 μg/ml).

Table 12.

Response of *C. albicans* to antimetabolites in the presence of various sterols.

| | 1 | 2 | 3 | 4 |
|-----------------------------------|------|-------|-------|-------|
| Bronopol* | 0 mm | 26 mm | 20 mm | 17 mm |
| Phenylmercurinitrate ⁺ | 43 | 50 | 52 | 30 |

1. 5 α -androstan-3 β -ol 300 μ g/ml

2. 5 β -cholestan-3 α -ol 300 μ g/ml

3. 5-cholesten-3 α -ol 300 μ g/ml

4. Control, no sterols

*Whatman 13 mm disc air dried after immersion in ethanolic solution (500 μ g/ml).

⁺Whatman 13 disc air dried after immersion in aqueous solution (50 μ g/ml).

In the case of S. cerevisiae and C. albicans the action of alphaxalone (0 to 600 µg/ml) resulted in an inhibition of the synthesis of the ninhydrin-positive lipids as demonstrated by thin layer chromatography (Plate 33). These yeasts also exhibited a varying response to 8-hydroxyquinoline and phenyl-mercuric-nitrate in the presence of increasing concentrations of alphaxalone (Table 11). However, neither organism exhibited any variation in sensitivity to nystatin and natamycin in the presence of alphaxalone.

While alphaxalone was the only compound out of the above list which could modify the synthesis of the yeast lipids some of the sterols (5-androstan-3β-ol, 5β-cholestan-3α-ol and 5-cholesten-3α-ol) did modify the response of C. albicans to the thiol-active compounds, bronopol and phenyl-mercuric-nitrate (Table 12).

When the yeasts were cultured on the magdala red medium there was no change in the colour of the medium and it would appear that the steroid supplementation of the medium failed to induce impairment of the respiratory enzymes.

Response of Saccharomyces cerevisiae (Arsenate-Resistant) to Antimetabolites.

The zone of inhibition of trichomycin (Whatman 13 mm disc immersed in aqueous solution, 500 µg/ml) against S. cerevisiae ASR increased from 18 mm (arsenate, 0M) to 36 mm (arsenate, 10^{-2} M), but the sensitivity to 8-hydroxyquinoline (Whatman 13 mm disc immersed in aqueous solution, 500 µg/ml) remained unchanged at 22 mm.

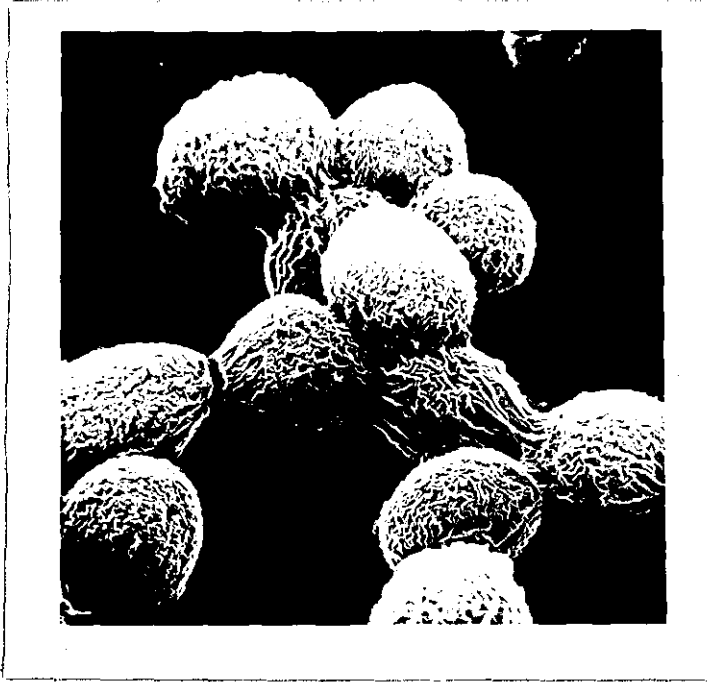


Plate 34. - Scanning electron microscopy of Scenedesmus sp. grown for 5 days in a calcium free medium. x 4,000.

Plate 35. - Scanning electron microscopy of Scenedesmus sp. grown for 5 days in a medium containing 0.1mM calcium. x 8,000.

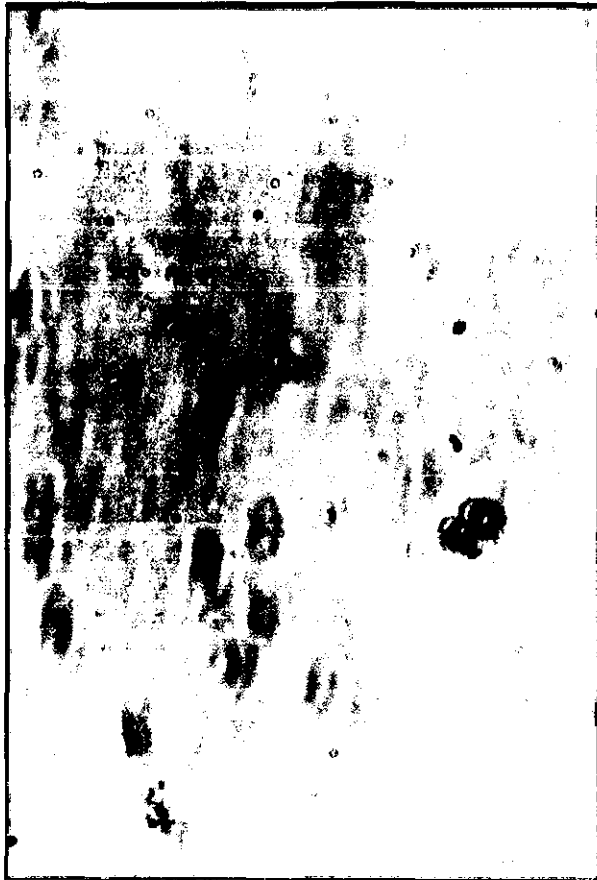
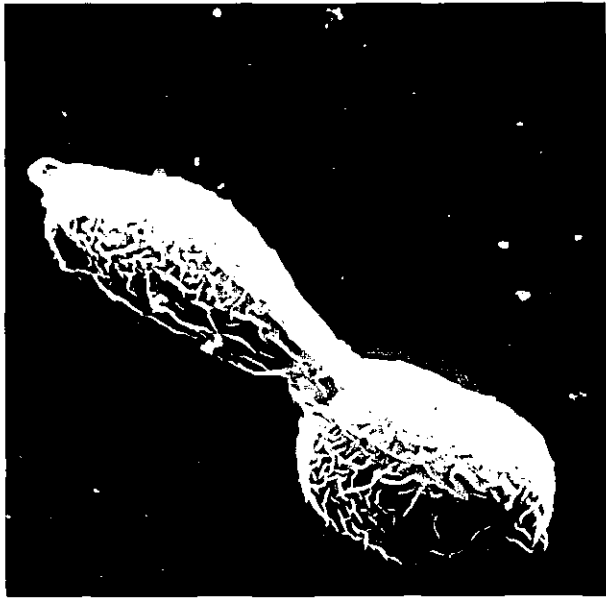


Plate 36. - Scanning electron microscopy of Scenedesmus sp. grown for 5 days in a medium containing 0.075mM lanthanum. x 8,000.

Plate 37. - Location of calcium in Scenedesmus sp. following growth in a medium containing 0.1mM calcium. x 1,250.

Fig 9.

Size distribution of Scenedesmus sp. in the range 1.5 to 11 μm when grown in medium with calcium 0.01mM (A), without calcium (B), with lanthanum 0.075mM (C), with neodymium 0.075mM (D).

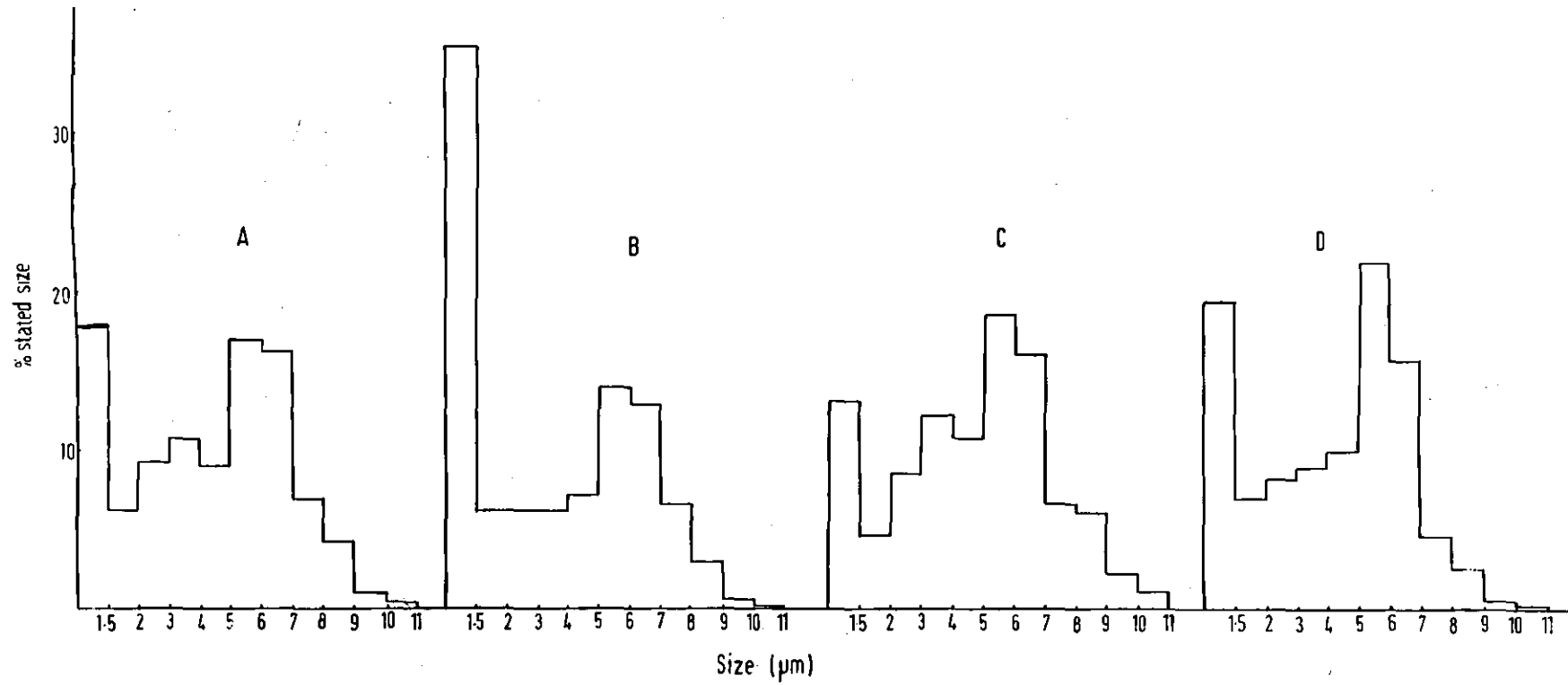


Fig 10.

Size distribution in the range 8 to 14 μm of Scenedesmus sp. grown in the presence of calcium 0.1mM (A), without calcium (B) lanthanum 0.075mM (C) neodymium 0.75mM (D).

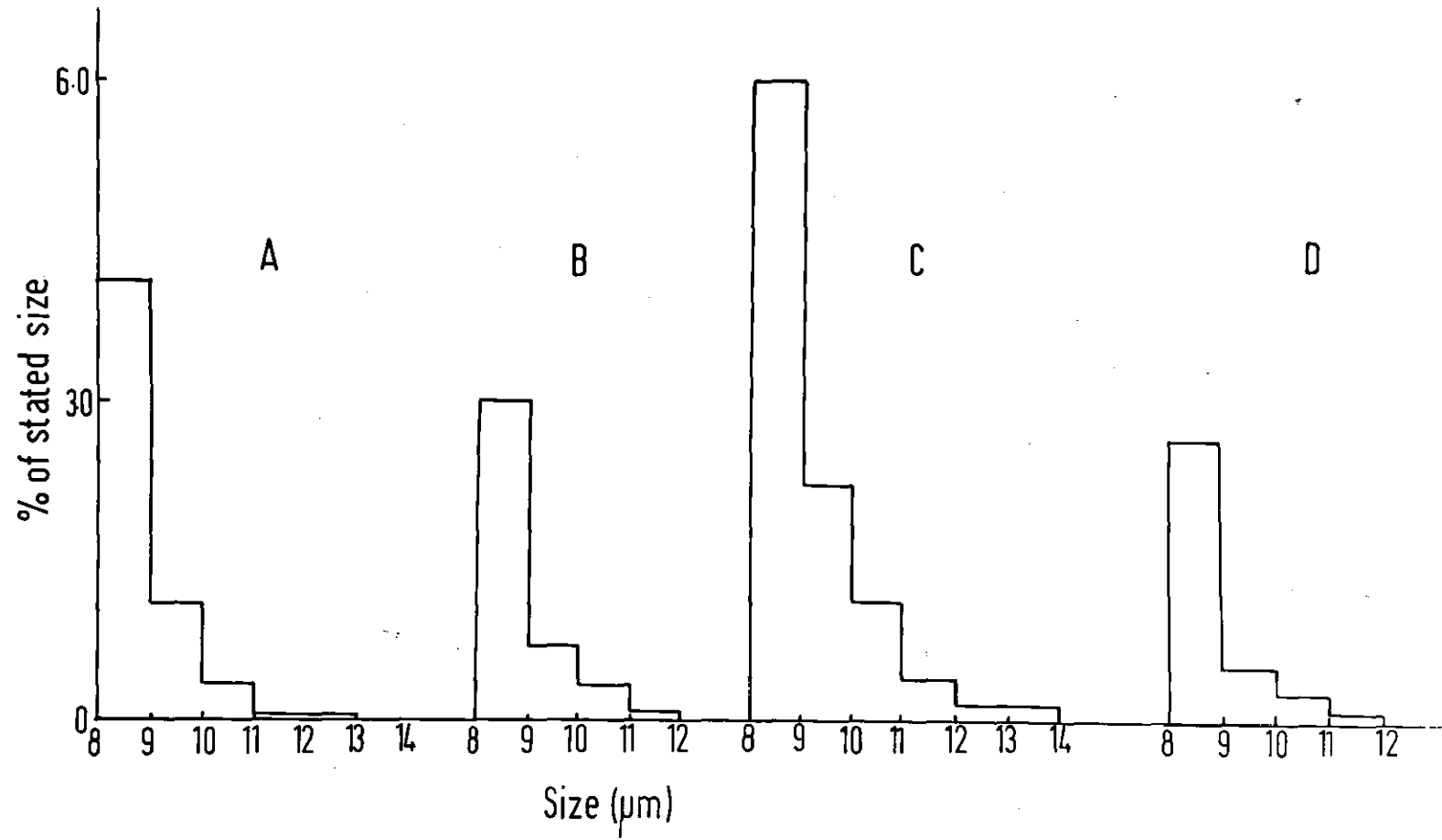
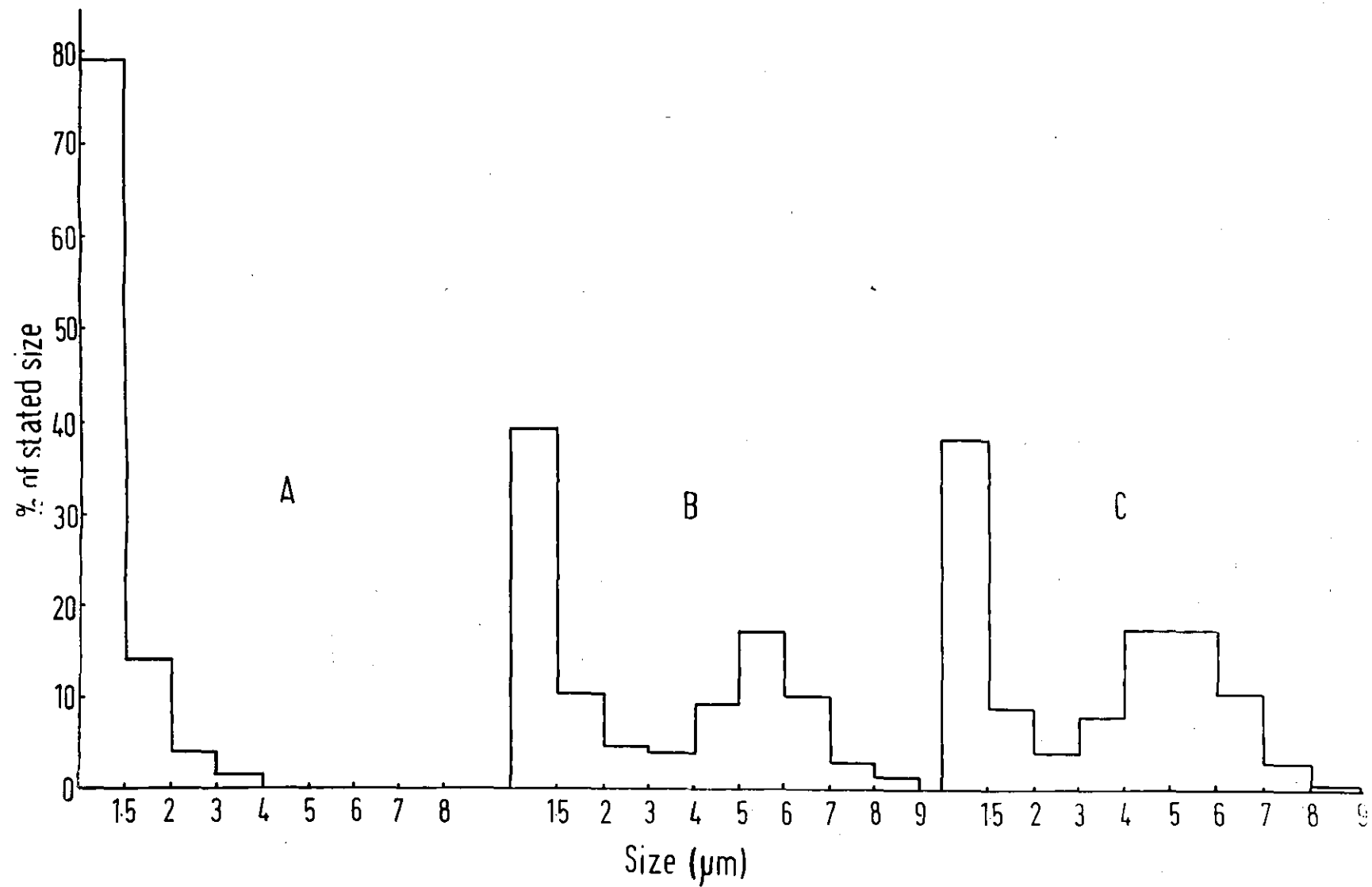


Fig 11.

Size distribution in the range 1.5 to 9 μm of Scenedesmus sp. grown in a medium without calcium and X-537A (A) with 0.1mM calcium and X-537A 3.5 $\mu\text{g/ml}$ (B) or without calcium (C).



C. The Ability of the Lanthanons, Lanthanum and Neodymium, to replace Calcium in some Cellular Processes.

(i) The Effects of Lanthanum and Neodymium on the Morphology and Size of Scenedesmus sp.

Scenedesmus sp. responded to a deficiency of Ca^{2+} by producing very few spindle-like forms, however, the organism produced many more such forms in the presence of La^{3+} and Nd^{3+} . A convoluted appearance, demonstrated by SEM, characterised the cells (Plates 34-36) and there was no significant variation in the topography of the cells when grown in the presence of La^{3+} or Nd^{3+} or Ca^{2+} .

The GBHA stain demonstrated the localisation of Ca^{2+} in Scene desmus (Plate 37). No red colour was observed when the cells were grown without calcium, even when the cells were washed with the calcium - containing medium. The red colour was also absent from cells which had been cultured in the calcium - containing medium which was supplemented with the calcium ionophore X-537A (3.5 $\mu\text{g}/\text{ml}$). The GBHA stain did not demonstrate the loci of lanthanum and neodymium in the cells.

The organism responded to the absence of calcium from the medium by producing a large number of small cells (Fig 9). When lanthanum or neodymium replaced calcium in the medium the size distribution of the cells was very similar to that obtained with calcium in the size range 1.5 to 8 μm (Fig 9). However, the most significant differences were noticed in the extreme of the size range 8-14 μm (Fig 10). In particular it was observed that many more large cells were produced in the presence of lanthanum than were produced in the presence of neodymium and in the presence or absence of calcium (Fig 10).

The addition of X-537A to the culture medium impaired the morphogenetic action of calcium and when incorporated into a medium containing no calcium it increased by 100% the number of cells in the size range of 1.5 μm (Fig 11).

There was no significant variation in cell yield, by dry weight determinations, irrespective of whether the organism was grown in the presence of calcium, lanthanum or neodymium or without calcium.

(ii) Phytophthora cactorum: Morphological Variation as a Response to Lanthanum and Neodymium, and to sterol supplementation.

With the exception of Ba^{2+} all the cations supported growth of P. cactorum. However, the dry weight analysis did not show any direct relationship between the cation supplement and cell yield. The mean yield was 38 ± 5 mg/10 ml, although when Pb^{2+} was present the yield was 29 ± 3 mg/10 ml. In addition, it was not possible to recognise any stimulation or inhibition of growth in the presence of the various sterols when added to the calcium supplemented medium.

An examination of the various cultures on the light microscope showed quite significant variations in the development and morphology of P. cactorum. In the presence of calcium and cholesterol the organism exhibited all stages of development and oospores were abundant (Plate 37a). The GBHA stain showed that calcium was localised in discrete areas throughout the sporangia (Plate 37b) and in the filament, but was less abundant in the latter. In medium supplemented with cholesterol it was observed that individual addition of Pb^{2+} , Sn^{2+} and Cu^{2+} resulted in irregularly shaped filaments. In all three cases there was little evidence of formation and development of sporangia. Although manganese supported development of sporangia these were few and very irregular in shape. In the case of strontium there was development of sporangia and the production of oospores, however, the numbers were markedly less than when the medium was supplemented with calcium (25 ± 3 and 65 ± 8 sporangia/field, respectively). The addition of lanthanum and neodymium salts gave an opaque precipitate and these cations were toxic to the

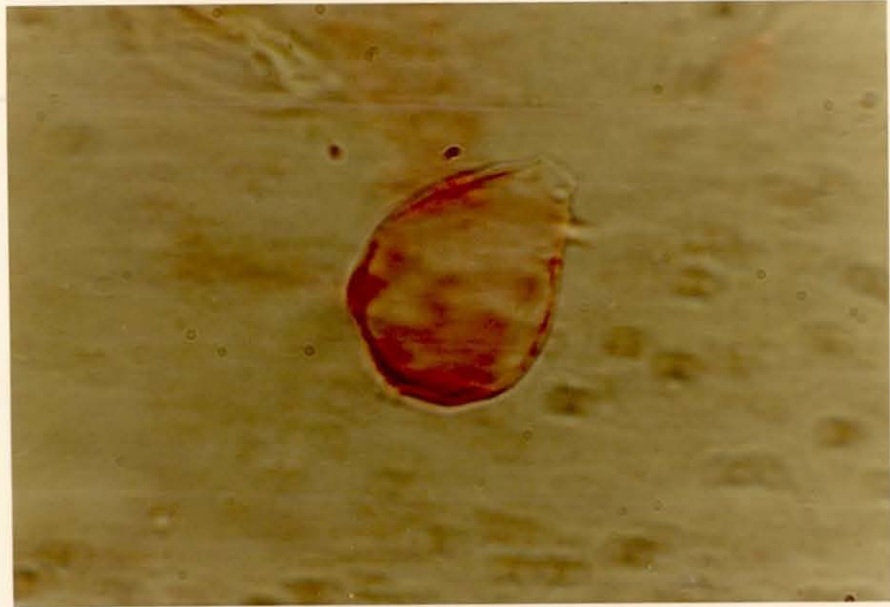
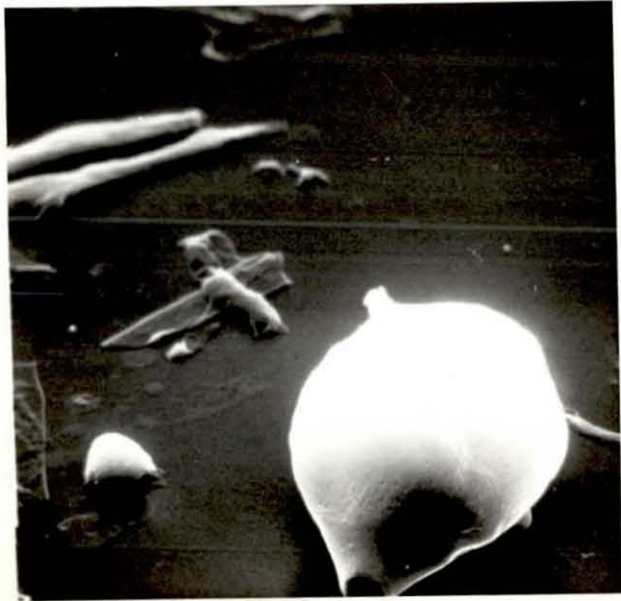


Plate 37a. - S.E.M. of sporangia of P. cactorum produced in the presence of Ca^{2+} . x 1,600.

Plate 37b. - Localisation of Ca^{2+} in sporangia as revealed by GBHA. x 1,250.

Plate 37c. - S.E.M. of oospore of P. cactorum produced in the presence of Nd^{3+} . x 4,000.

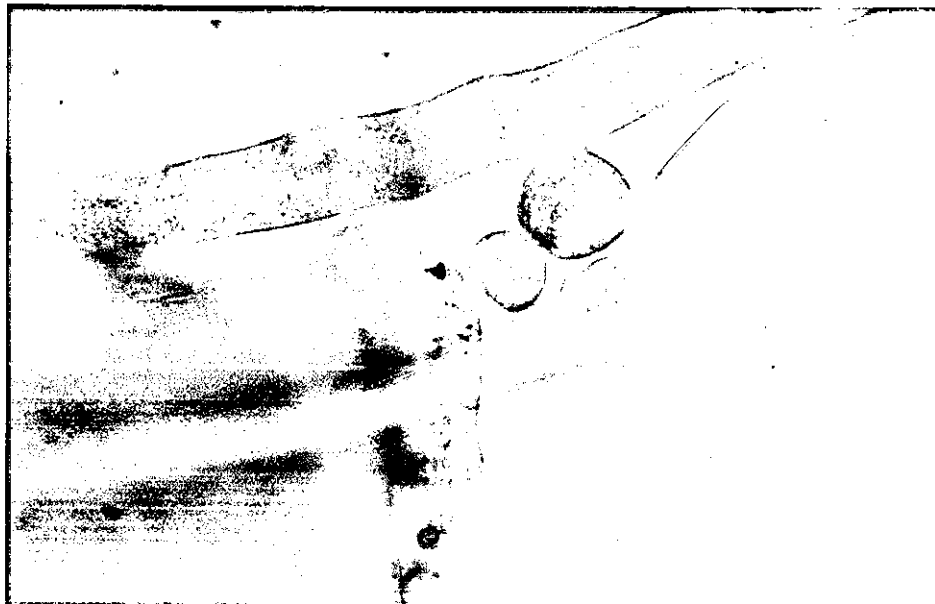
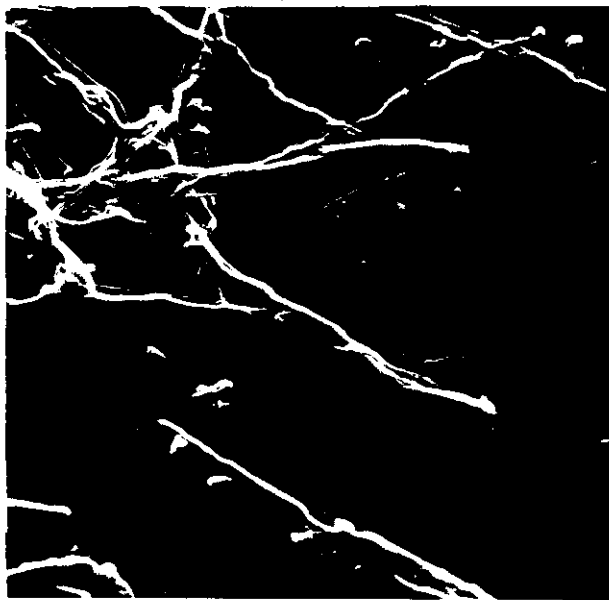


Plate 38. - Phase contrast microscopy of irregular shaped sporangia produced by P. cactorum when cultured in the presence of 5β -cholestan- 3α -ol. x 1,250.

Plate 39. - SEM of P. cactorum cultured in the presence of 5β -cholestan- 3α -ol. There is evidence of a sheath around the hypae. x 1,600.

Plate 40. - Contorted forms of P. cactorum produced by 5α -androstan- 3β -ol as observed by phase contrast microscopy. x 1,250.

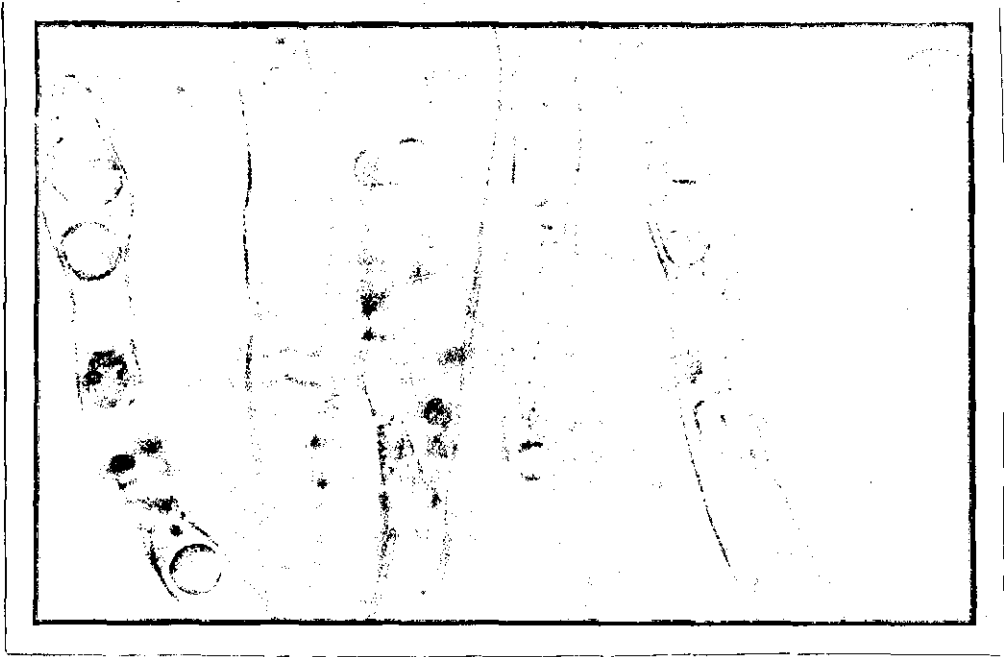


Plate 41. - Phase contrast microscopy of P. cactorum reveals globose forms induced by 3β -cholesteryl chloride. x 1,250.

Plate 42. - SEM of P. cactorum grown in the presence of 3β -cholesteryl chloride. x 4,000.



Plate 43. - Phase contrast microscopy of P. cactorum reveals contorted filaments induced by alphaxalone. x 1,250.

Plate 44. - Globose, fat-like bodies induced in P. cactorum by alphaxalone as revealed by phase contrast microscopy. x 1,250.

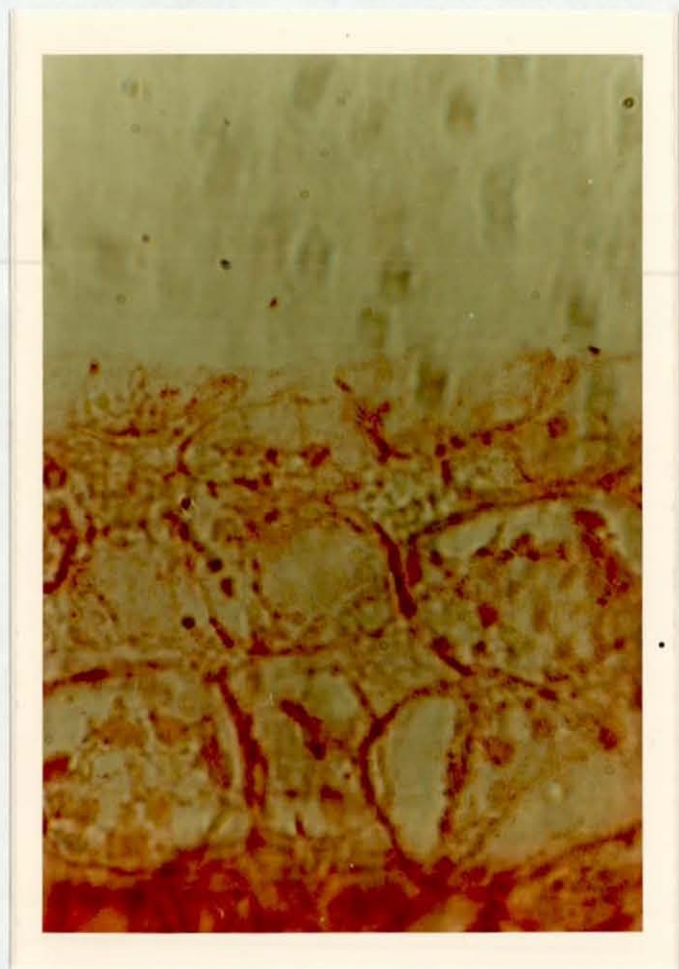


Plate 45. - Calcium deposition in the cell membranes of
grapefruit seeds. x 1,250.

organism at 0.15 mg/ml and 0.25 mg/ml, respectively. These cations supported the development of sporangia (40 ± 8 sporangia/field), many of the sporangia matured to oospores (Plate 37c).

When the cholesterol of the growth medium was replaced by 5β -cholestan- 3α -ol only a very few mature oospores were observed but there were several irregular sporangia (Plate 38). Light microscopy suggested the presence of a sheath about the aerial filament and this was confirmed by SEM (Plate 39). In the case of 5α -cholesten- 3α -ol no sporangia were observed and the filaments appeared slightly swollen and contorted. When 5α -androstan- 3β -ol replaced cholesterol contorted forms were produced and in some cases these were within the filaments. Some of these forms resembled sporangia (Plate 40). In the case of 5α -cholestan- 3β -ol malformed filaments and sporangia were present, and it is possible that some sporangia had matured sufficiently to produce oospores. In the presence of 3β -cholesteryl chloride only asexual growth occurred and this was characterised by slightly enlarged filaments containing numerous globose forms (Plate 41). SEM showed that there may have been some outgrowth from the filaments and it is possible that this outgrowth represented immature sporangia (Plate 42). Growth of *P. cactorum* in the presence of 21-desoxyprednisolone- 17α -propionate and alphaxalone resulted in the production of contorted filaments (Plate 43). Many of the filaments possessed collapsed outgrowths, similar to those produced by 3β -cholesteryl chloride (Plate 44).

An examination of the extracted lipids showed no variation in the distribution of ninhydrin-positive lipids nor in the overall lipid or sterol composition.

Fig 12.

Response of 'metal'-spores of B. cereus SV-1 to heat.

- Ca²⁺ - spores
- ▲ La³⁺ - spores
- Nd³⁺ - spores

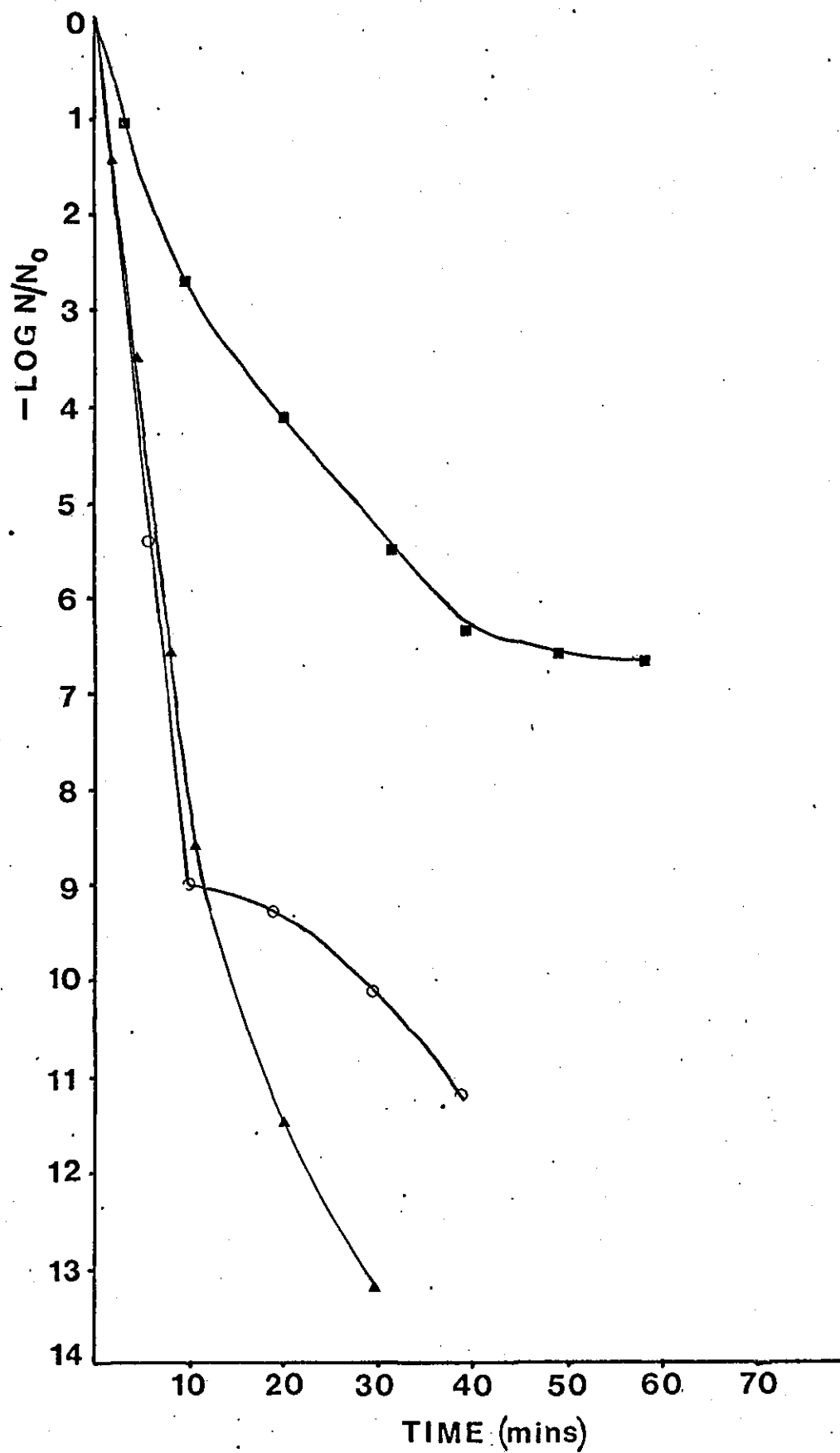
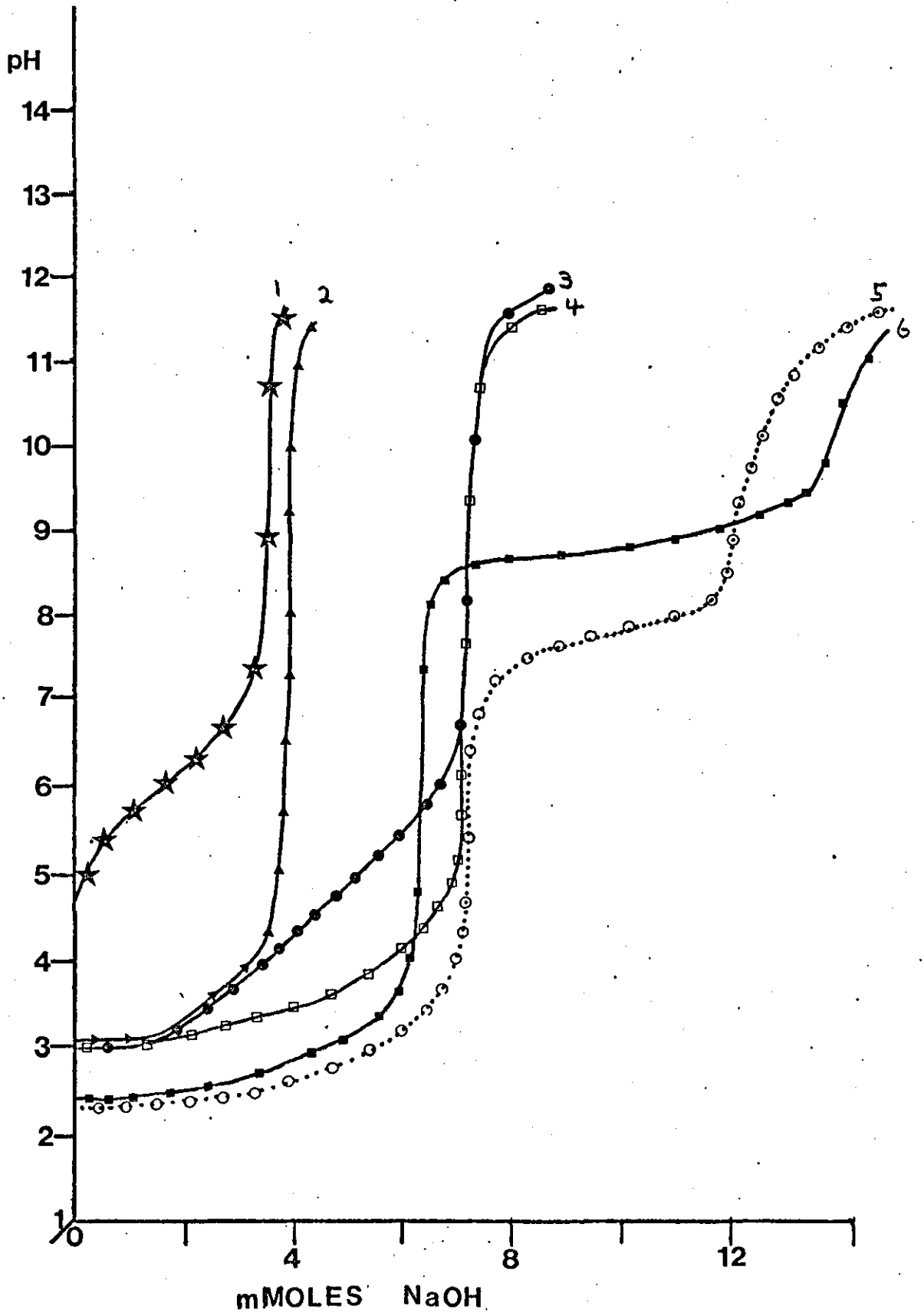


Fig 13.

Potentiometric analysis

1. Ca^{2+} - Pyridine - 2 -carboxylic acid.
2. Ca^{2+} - 2-methyl-pyridine-6-sulphonic acid.
3. DPA.
4. Ca^{2+} - DPA.
5. Nd^{3+} - DPA.
6. La^{3+} - DPA.



(ii) The Contribution of Lanthanum and Neodymium to the Heat Resistance of *Bacillus cereus* spores.

After the acid treatment the spores contained no detectable calcium and all spores contained about 10% (dry weight) of DPA.

The 'acid'-spores exhibited no heat resistant properties and were all killed within 2 mins. However, the calcium-spores were heat resistant and survived exposure to 98° for over 60 mins whilst the lanthanide spores had little heat resistance (Fig 12). None of the spores after acid-treatment and reconstitution were as heat resistant as the untreated native spores.

The potentiometric analysis showed that calcium reacted with DPA to form a 1:1 chelate (Fig 13). The ligand curve showed a slight inflection at 1 mole of base : 1 mole of ligand with a more pronounced inflection at 2 moles of base : 1 mole of ligand. This potentiometric curve indicates the dissociation in turn of a proton from each of the two carboxyl groups of DPA. Similarly, the inflections in the 1 mole of ligand represent the combination of calcium ions with DPA through the dissociation of two protons from the carboxyl groups. In the case of the lanthanides the interactions between these cations and DPA is much stronger than the calcium as the cations bear a higher charge.

The fluorescence studies showed that the emission wavelengths and maxima were not significantly different from the control, untreated spores and those which had been acid treated or autoclaved. In the latter cases, however, there was a consistent increase in fluorescence and a shift down of 3 nm of the emission peak. When the acid spores were reconstituted with Ca^{2+} , La^{3+} or Nd^{3+} the emission maximum shifted from 478 nm to $468 \text{ nm} \pm 2 \text{ nm}$ and there was a threefold increase in fluorescence.

Table 13

Liposome Stability.

| O.D. $\lambda 546\text{m}\mu$ | | | | |
|----------------------------------|--------------------|---------------------|---------------------|-------------------------|
| "Membrane Disrupting Agent" (mM) | DPA ¹ , | EHDP ¹ , | EDTA ¹ , | Aesculin ² . |
| 5 | 0.152 | 0.138 | 0.136 | 0.138 |
| 1 | 0.155 | 0.142 | 0.145 | 0.142 |
| 0.1 | 0.155 | 0.150 | 0.143 | 0.145 |
| 0.01 | 0.155 | 0.150 | 0.148 | 0.145 |
| 0.005 | 0.155 | 0.150 | 0.150 | 0.150 |
| — | 0.162 | 0.152 | 0.150 | 0.155 |

1 : No cholesterol, 2 : supplemented with cholesterol.

Liposome Stability

The action of the membrane disrupting agents indicated that they were possibly acting by removing the cations from the liposomes (Table 3). It appeared that very low concentrations could induce a 'once-and-for-all' disruption and that increasing concentrations of the selected agent was no more effective disrupting the liposome. There was no significant variation in the stability of the lanthanum liposomes. Picolinic acid and sodium cyclamate exerted no effect on the liposomes.

With the exception of DPA none of the agents examined could induce germination of the B. cereus spores.

DISCUSSION

A. Morphological Variation in some selected Prokaryotes.

The form taken by cells is characteristic of a regular series of metabolic events and any impairment of these events can produce non-typical forms. Such morphological impairment can facilitate an understanding of normal growth.

(i) Actinomyces viscosus.

Through the optical microscope the vast majority of the Actinomyces appear as filaments and these cells are so small it is difficult to characterise the exact sequence of events of the cells. In one of the few studies of morphology of the Actinomyces spp. Gerencser and Slack studied nineteen isolates of A. viscosus, all of human origin, and described them as Gram-positive rods existing as diphtheroid cells and filaments (449). The rods exhibited X, Y and V forms with palisading. These authors found it impossible to distinguish these strains morphologically from those isolated from hamsters. A few studies have demonstrated that scanning electron microscopy can reveal a great detail of the ultrastructure of actinomycetes (450). Boyde and Williams have studied the morphological differences of A. naeslundii and A. odontolyticus (451).

In this study it has been demonstrated that there is little uniformity of appearance within the organisms classified as A. viscosus.

While light microscopy of A. viscosus WVU 745, WVU 371 and WVU 440 revealed very little detail, it was possible to examine A. viscosus WVU 627 and WVU 398B by the conventional methods, but in all cases the use of SEM, with its greater resolution, permitted greater morphological detail of external form to be observed.

The fixative procedure developed in this study preserved the structure without excessive vacuum damage and was, in this respect, superior to the techniques which rely on coating unfixed specimens, which produce extensive

collapsing under vacuum. There was no significant difference in appearance if the dehydration was by the method described or using ethanol, although the latter technique causes a loss of lipid material from bacterial specimens (452).

Morphological variation in Actinomyces sp. is limited although Morris (453) reported the existence of complicated life cycles for A. bovis. However, Erikson (454) considered that facultative anaerobic cocci of various types can be carried for several generations within the entangled filamentous mass and subsequently Erikson and Porteus (455) have shown this to be the case. Erikson (453) has demonstrated that the diphtheroid form becomes predominant when A. israelii is cultured aerobically while a diphtheroid form predominates in some species of A. viscosus.

The pattern of growth observed for these species of Actinomyces viscosus is not entirely unique, since Csillag has observed that species of Nocardia and Mycobacterium fragment from the pseudomycelial form to the cocco-bacillus form (456). In addition, Nikitina and Kalakoutskii (457) have shown that this type of growth can be induced when Streptomyces spp. are grown on media containing D-fructose.

In this study it has been shown that A. viscosus WVU 745, WVU 371, and WVU 627, resemble A. naeslundii and A. odontolyticus as demonstrated by Boyde and Williams (451) using SEM, and in all cases the cells are of similar dimensions. The morphological appearance of A. viscosus WVU 440 was somewhat different from the other strains as it presented initially a typical branched filamentous form which eventually underwent fragmentation to coccoid elements.

An entirely different morphological pattern was presented by A. bovis which existed as fine filaments fragmenting into small rods.

The morphological forms of A. humiferus and Agromyces ramosus were similar and SEM showed no evidence of the cells existing as filaments

and this study suggests that these two organisms must be re-examined since there is very little evidence of any filamentous forms as initially reported by Gledhill and Casida (458).

Of all the species of Actinomyces examined A. viscosus WVU 398B proved to be the most interesting. Light microscopy showed that the organism underwent a morphological variation which resulted in a clearing of cytoplasm and a collapse of part of the pseudo-filament wall and in this respect the organism may resemble the morphological forms observed by Csillag in a study of Nocardia and Mycobacteria. The sheath observed by SEM (Plates 10-12) bears some resemblance to that observed by Locci and Baldan in Planobispora longispora (459). However, Locci and Baldan failed to attribute any specific role to the sheath, although it is possible that the sheath served as some form of sporangiospore.

The production of vacuoles may be a process of cell differentiation and could be a stage in the development of the cocco-bacilli form, as it occurs at a late stage in growth, which is seen in Actinomyces sp. growing in vitro but is rarely seen in the in vivo infections of A. israelii (460). The transition from a mycelial form to the cocco-bacilli form would demand a reorganisation of the cytoplasm and at this stage of growth it is significant that cell metabolism, as judged by dehydrogenase activity, is low. As indicated above, morphological variations can occur in some Streptomyces sp. when cultured on D-fructose (457) while Csillag has shown that both species of Mycobacterium and Nocardia pass from a pseudomycelial form to a cocco-bacilli form (456, 461).

The process which occurs in A. viscosus does not lead to the production of a heat resistant form and it is this lack of heat resistance in the differentiated form which indicates the demarcation line between the Actinomyces and those other actinomycetes which undergo differentiation to produce heat resistant endospores (462).

Over a period of several hours there is a gradual appearance of vacuoles and their appearance in large numbers at a definite time in the growth cycle suggests a process under enzyme control. In addition, it would appear that there is a commitment to vacuolation and it is possible that the process may be similar to that observed in spore-forming bacteria when they continue to sporulate, or germinate, on being transferred to an appropriate nutrient medium (463). If this was so, then the cells would be genetically committed to vacuolation and this particular aspect was examined, to some extent, by determining the effect on this event of antimetabolites whose specific mode of action is known. This use of antibiotics in studies of cell differentiation is not in isolation as several other groups of workers have initiated morphological variation in cells exposed to a variety of antibiotics. Smith et al obtained filamentous Gram-negative organisms on exposure to novobiocin (464) while Greenwood and O'Grady obtained chains forms of S. aureus (465). Of the more recent studies the most interesting are those of Newton who regulated transcription in Caulobacter crescentus with rifampicin (32) while Chater used it to block the development of aerial mycelium in Streptomyces coelicolor (466).

Some inhibitors of nucleic acid and protein synthesis (ethidium bromide, colchicine, chloroquine, acridine orange and β -phenylethyl alcohol) did not suppress vacuolation. However, it was possible to obtain partial suppression of vacuolation by some antibiotics (neomycin, streptomycin and gentamycin) in high concentration. These antibiotics have the common function of inhibiting ribosome function (Fig 3). Streptomycin, in the appropriate system, inhibits all aspects of protein synthesis i.e. initiation, elongation, and termination. Streptomycin enhances ambiguity of translation of mRNA, as do neomycin and gentamycin, and it is possible that such effects could impair the function of the 30S moiety, and in

particular the ribosomal A site (467). Further anomalies in the action of streptomycin have been revealed by McMeekin who found that streptomycin could inhibit the spore germination and outgrowth of the obligate parasite Peronospora parasitica (468). This inhibition by streptomycin could be reversed by calcium and manganese and it is significant that Brazil and Corrado, examining its neuromuscular blocking activity, have demonstrated a curariform action for streptomycin which may be due to the binding of streptomycin to phospholipids (469).

Chloramphenicol failed to suppress vacuolation and since it inhibits peptide chain elongation and the movement of ribosomes along the mRNA it is unlikely that vacuolation is controlled at this point. Vacquez considers that chloramphenicol may block interaction between the enzyme and the substrate at the 50S moiety of the ribosomal A site (470).

Other inhibitors of protein synthesis at the ribosomal level, lincomycin and clindamycin had interesting effects. Lincomycin showed only limited inhibition of vacuolation but clindamycin, the deoxy-7(S)-chloro derivative of lincomycin hydrochloride, suppressed vacuolation. The biological activity of lincomycin and clindamycin is dependent upon the alkyl substituents of the sulphur atom and the ring nitrogen atom. Lincomycin inhibits bacterial protein synthesis (particularly in Gram-positive bacteria) without inhibiting RNA or DNA synthesis (471). The wider spectrum of activity of clindamycin has been attributed to its increased ease of penetration into cells (467). The precise mode of action of these two antibiotics is in dispute. It is possible that they act on the peptidyl "transferase" which is an integral part of the 50S subunit (472), this is also the site for aminoacyl t-RNA. An alternative site for interaction may include the "translocase" factor which promotes the transfer of peptidyl t-RNA from the acceptor site to the donor site (473). The translocase function is dependent upon the G-factor catalytic hydrolysis of guanosine triphosphate (GTP). On the

other hand Mielck and Garret (474) on the basis of microbial kinetics suggest that lincomycin possesses two modes of action which have been attributed to (i) an impairment of t-RNA by the binding of the drug to the 50S subunit and (ii) a possible interference in the synthesis and utilization of a stored metabolite. Fusidic acid appears to act indirectly with the G-factor (and subsequently with the 50S subunit) and translocation is suppressed by prevention of repeated hydrolysis of GTP (467). As vacuolation was readily suppressed by fusidic acid it appears that the event could be sensitive to inhibitors of transferase or translocase.

In the case of rifampicin, a semi-synthetic macrolide antibiotic, an anomalous result was obtained. Rifampicin exerts its action by binding to the β -subunit of RNA polymerase, without any suppression of DNA polymerase (475). Cells appear to have great sensitivity to rifampicin as observed by Hartmann et al who obtained 50 per cent inhibition of RNA polymerase with a concentration of 2×10^{-8} M (476). The macrocyclic ring of rifampicin binds the molecule to the RNA polymerase whilst the other parts of the molecule may modify the permeability of the organism (477). In this case the inhibition of vacuolation may be suppressed by a similar mechanism as that invoked by Newton (32) who explained the inhibition of stalk formation and cell division in C. crescentus in terms of an inhibition of transcription.

It is possible that the tetracycline induced partial suppression of vacuolation may be due to an impairment of membrane permeability as a result of chelation of divalent cations (478), although Gale et al have suggested that tetracyclines may act elsewhere on the ribosomal site as well acting against the 30S moiety (467).

Other metabolic events investigated were those involving the cell membrane directly because this might be directly implicated when the

actions of tetracycline or rifampicin were considered. However, the compounds studied, namely 2,4-dinitrophenol, an uncoupler of oxidative phosphorylation (479); 3-chloropropane-1,2-diol, a glycerol antagonist which may impair synthesis of the cell membrane (discussed below) or pentamidine; which suppresses nucleic acid and phospholipid synthesis in Staphylococcus aureus (132) were without effect. It seems probable that the cell membrane metabolism and composition has no primary role in this process of differentiation. Although this study has shown that vacuolation is unlikely to be influenced significantly by anti-metabolites which act on the cell membrane other organisms are known where such a regulation is critical. Brown and Reda have shown that enzyme activity and morphological changes in Nocardia corallina occur as a result of development of impermeability of the membrane after growth for 8 h (480). This impermeability block can be relaxed by addition of cetyltrimethyl ammonium bromide.

Several inhibitors of cell wall synthesis (benzyl penicillin, gramicidin J, bacitracin, phosphonomycin, vancomycin and D-cycloserine) all of which act at various stages were without effect on vacuolation. However, methicillin in high concentrations (10x MIC) did inhibit vacuolation and this may be a secondary effect as there was no evidence of cell wall damage at this concentration, as judged by SEM.

This work suggests that vacuolation is an event mainly dependent on protein synthesis at the ribosomal level and that it occurs at the end of log phase growth. As the process of vacuolation may be under enzymatic control it is pertinent to consider similar events in other bacteria such as bacterial sporulation which is due to the production or activation of a proteolytic enzyme. However, such an event is not just specifically concerned with sporulation but it also occurs at the more elementary level of E. coli when the culture is starved and stops

growing. Mandelstam has considered such events to be primitive adaptation mechanisms which permit organisms to alter their development conditions where net synthesis is impossible (25). Proteolytic changes are important in more evolved cells such as sporulating yeasts (26) and at an early stage in the development of the slime mould (27). Similar developmental changes exist in the alga Acetabularia which continues to undergo developmental changes several weeks after the nucleus has been removed (28). In the slime moulds and sporulating Bacilli it appears that regulation exists both at the level of transcription and of translation and that a similar phenomenon may control vacuolation in Actinomyces viscosus. While this study has shown that some antibiotics can regulate differentiation in A. viscosus 398B the work is not in isolation as some of these antimicrobial substances were found to be capable of inducing morphological variation in Bacillus cereus SV-1 and Actinoplanes missouriense.

(ii) Morphological Variation in Bacillus cereus SV-1.

Just as in the case of A. viscosus 398B in which vacuolation is regulated at the level of transcription it appears that the morphological diversity of B. cereus can also be regulated at the level of transcription. However, unlike A. viscosus 398B, B. cereus SV-1 responded to a wider range of antimicrobial agents.

The morphological diversity exhibited by B. cereus SV-1 has not been reported for any other B. cereus variants yet. However, it is possible that the absence of lecithinase activity may be the key to an understanding of the unusual characteristics of the organism.

The induction of filaments in B. cereus SV-1 as a response to variation in temperature is not without precedent as Ferroni and Inniss have obtained filaments of the psychrophile B. insolitus when cultured at 30° instead of the optimum, 20° (481) and these workers later used

antibiotics in a manner similar to that utilized in my investigation. They showed that filaments obtained at 30° did not undergo resumption of cross-wall formation when cultured at 20° in the presence of chloramphenicol, or when D-cyclo-serine, vancomycin, or penicillin was present (482). Other research groups, have obtained morphological variants as a result of departure from an optimum temperature : Hoffman and Frank obtained filamentous E. coli (483), Strange and Shon observed formation of filamentous Aerobacter aerogenes as a response to thermal stress (484) while Siebruth transformed a Gram-negative filamentous marine Arthrobacter sp. into a Gram-positive coryneform Arthrobacter by raising the temperature from 20° to 26° (485). Hughes has suggested that there are three main classes of agents which can cause aberrant long forms to appear in culture: (i) certain poisons, including antibiotics, (ii) starvation, and (iii) physical trauma (486).

Variations in lipid composition as a response to changes in temperature have received only cursory attention. In particular these studies have been concerned primarily with alterations in the degree of saturation of the fatty acids (487). However, De Siervo has observed significant variations in the phospholipid composition of E. coli B - at 27° diphosphatidyl glycerol replaced phosphatidic acid (488). In this study it has been demonstrated that there are variations in the lipid composition of B. cereus SV-1 and it is possible that these variations may regulate the morphological diversity induced by variations in the temperature of growth. It is possible that the variations in lipid composition may regulate the variations in the zone of inhibition of the antibiotics examined. Additional factors which may regulate this variation in sensitivity to antibiotic may be the significant variations in amino acid content of the cell-wall which must be taken to indicate that the cells contain different polypeptides when grown at different temperatures.

The variations in composition of cell membranes could regulate the enzymatic activities of the cells inducing conformational changes in the enzymes. The modern theories of membrane structure, as propounded by Singer and his associates, permit such regulation. Ferroni and Inniss suggest that the filamentous form of B. insolitus is due to the suppression of septum formation (481), and Hoffman and Frank used electron microscopy to demonstrate the absence of septa in the filamentous forms of E. coli which were classified as mechanically strong (483). In this study the cell wall stain indicated the absence of septa while the nucleic acid stain showed that B. cereus filaments were multinucleate.

The production of filamentous B. cereus SV-1 as a response to antibiotics is not paralleled by the induction of filaments in the other species of Bacilli examined in this study. However, other workers have reported that elongated bacteria can be induced by mitomycin (489), nalidixic acid (490), bleomycin (491), cephalixin (492) and rifamycin-SV (493).

Unlike A. viscosus 398B, B. cereus SV-1 responded to inhibitors of nucleic acid synthesis and protein synthesis (colchicine, chloroquine phosphate, acridine orange and ethidium bromide) by producing morphologically diverse forms. However, these inhibitors exhibited a wide range of responses with acridine orange being the most effective antimetabolite of this group. Streptomycin sulphate was particularly effective at inducing filaments. Clindamycin and lincomycin were also effective in this respect but sodium fusidate was not and it would appear that there may be several loci which can regulate induction of filaments in B. cereus SV-1. It is unfortunate that this study could not be completed in sufficient depth and so include an examination of the filaments by transmission electron microscopy. The failure to obtain large quantities of filaments was a setback in this study.

(iii) Morphological Diversity of Actinoplanes missouriense.

In comparison of the morphological forms of Actinoplanes missouriense observed in this study it is apparent that there is an absence of agreement between this work and that reported by Willoughby (441). In this study it has not been possible to identify any forms which can be considered to represent mycelia. Morphological forms identifiable as sporangia (Willoughby's term) abounded. Unlike the sporangia described by Willoughby the bodies did not undergo the characteristic dehiscence, however, large numbers of non-motile spores could be observed surrounding sporangia. Many of these spores were diplococcus-like. Wide differences exist between the studies reported here and those reported by Willoughby. However, the conditions under which the organism was cultured are quite different. The forms observed by Willoughby were normally on solid media which was of very low nutritional value and was held to be similar to the aquatic environment from which the isolation was made. The conditions used in this study involved a high nitrogen content medium supplemented with various vitamins and growth factors. While this nutritionally rich medium may have little resemblance to the environment from which the organism was isolated it does facilitate the production of large numbers of sporangia from a strain which has lost the ability to fruit (Willoughby, personal communication).

B. The Response of Microorganisms to Variations in Lipid Composition.

In the preceding section some consideration has been given to the role of antibiotics and other antimicrobial substances in the initiation of morphological diversity. Even though it is sometime since Monod suggested that the membrane may serve as a matrix for nucleic acid (494) there appears to have been very little research on the response of cells in which membrane synthesis has been impaired. The other methods of impairing membrane synthesis (for details see Chapter 2) are not very discriminating and it was thought that an analogue of glycerol, 3-chloro-propane-1,2-diol (3-CPD), may impair membrane development by inhibiting the synthesis of glycolipids.

An examination of the growth curves obtained from the glycerol-dependent mutant of B. subtilis, in the presence of increasing concentrations of 3-CPD, suggests that the compound may act as an inhibitor of glycerol metabolism. This finding is in agreement with those of Eisenthal et al (495) who demonstrated that fluoro-analogues of glycerol can act either as weak substrates or as competitive inhibitors of glycerol or glycerol kinase.

The apparent inhibition of glycerol metabolism is accompanied by an alteration in the lipid composition of E. coli, S. aureus and S. cerevisiae, although the total amount of lipid material remains constant in any organism. The change is most noticeable in the phospholipid fraction. In the case of phospholipid bilayers there have been few studies of the permeability characteristics although Nikaido and Nakae (496) have shown that such bilayers are permeable to small hydrophilic molecules and that the lipopolysaccharide component from Gram-negative organisms fails to modify the permeability characteristics. However, some care must be taken in interpreting these results of Nikaido and

Nakae as numerous other groups have shown that the presence of lipopolysaccharide can regulate EDTA-controlled sensitivity to antimicrobials as diverse as polymyxin, benzylpenicillin and dicyclohexyl-carbodiimide (497-500).

This change in lipid composition was accompanied by an increase in the ANS-fluorescence which would imply that the environment of the cell has become more hydrophobic in nature (501).

Any dramatic change in lipid composition would be expected to be reflected most obviously in the cell membrane. In the presence of 3-CPD both E. coli and S. aureus show a dramatic alteration in sensitivity to streptomycin and gentamycin. These molecules are very hydrophilic and would be expected to be less likely to pass across a hydrophobic membrane. The increased inability of streptomycin to pass through hydrophobic membranes was indicated by the decrease in growth of the streptomycin-requiring mutant when it was cultured in the presence of increasing concentrations of 3-CPD. The inability to obtain a complete suppression of growth could have been due to the presence of streptomycin-permeases which have been reported by Hurwitz and Rosano (502). This failure to acquire sufficient streptomycin to support normal growth would suggest that it became progressively more difficult for streptomycin to diffuse through the membrane.

3-CPD has exhibited the ability to modify the microbial sensitivity of a wide range of antibiotics and antimetabolites, all with differing modes of action, and this would indicate that the modification of sensitivity was due either to an increase or to a decrease in the permeability of the outer layers of the cell to the specific compound and not to any specific competition at the active site of action.

Those compounds which act on membrane components exhibited a range of effects. Bronopol, a very polar molecule, oxidises sensitive thiols and is very active against enzymes located within the cell

membrane (503). The increase in activity of bronopol could be accounted for if the membrane proteins were more exposed following a change in lipid composition as result of growth in the presence of 3-CPD. Phenylmercuric nitrate, a thiol seeking compound, exhibited a similar response against the various cells. Chlorohexidine exhibited an unusual pattern of activity as the compound exhibited a pronounced zone of inhibition in the presence of high concentrations of 3-CPD within 10 h. However, lower concentrations exhibited no antibacterial action for a further 10 h. This variation in sensitivity may be due to its having a greater affinity for hydrophobic membranes, since it has been shown by Hugo and Longworth to exert its antibacterial action by a surface active effect (504).

Polymyxin, a polypeptide active against Gram-negative bacteria (505), possesses a hydrophilic peptide moiety and a branched fatty acid residue which penetrates the non-polar region of the phospholipid layer (506). The increased sensitivity of the Gram-negative bacteria to polymyxin when in the presence of 3-CPD can be explained by this hydrophobic interaction.

Stretton has shown that the antimicrobial action of diphenyliodonium chloride is dependent upon action on membrane-bound enzymes (private communication). The very low lipid solubility of diphenyliodonium chloride will explain the complete abolition of the antibacterial action when in the presence of high concentrations of 3-CPD. However, the related compound 2,2'-biphenyleneiodonium chloride, which alters the permeability of mitochondrial and chloroplast membranes (507), increases in activity against cells grown in the presence of 3-CPD. It is significant that this compound is much more lipophilic than diphenyliodonium chloride.

The mode of action of vancomycin has been well documented (467) and it would appear that the antibiotic acts by binding rapidly to the outer layers of the cell and so affects the synthesis and functioning of the

protoplast membrane. Vancomycin, it acts without disrupting the membrane, blocks the cycle of reactions involving the C₅₅-isoprenoid alcohol at the stage where the disaccharide-pentapeptide is transferred to the growing point of the peptidoglycan. In this study it has been demonstrated that the uptake and binding of vancomycin is independent of the lipid composition of S. aureus.

The peptide antibiotic, bacitracin inhibits peptidoglycan synthesis and is surface active (467). In vitro studies have shown that it can disrupt artificial membranes when present in concentrations which suppress dephosphorylation of C₅₅-lipid pyrophosphate (467). Such properties as those described above may explain the increased activity of the compound when it acts on the abnormal envelopes produced in the presence of 3-CPD.

The response of the abnormal cells to 2,4-dinitrophenol is a strong indicator of the properties of the abnormal membranes. Mitchel has presented 2,4-dinitrophenol as a weak lipophilic acid which dissociates to an ionic base whose π -bonded structure permits the anion to be relatively soluble in a non-polar system without a charge neutralising partner (508). Therefore, dinitrophenol will be able to sit in the hydrophobic portion of the membrane and act as a proton conductor, thus, the more hydrophobic the membrane the greater its antibacterial activity, as seen with both E. coli and S. aureus.

The activity of phanquone, a lipophilic molecule, which acts on membrane bound enzymes (R.J. Stretton, private communication) is unaffected by the lipid composition of the cell.

The compounds which act only on cell wall synthesis are also influenced by growth of the organisms in the presence of 3-CPD. The small polar molecule, phosphonomycin has its activity lowered when S. aureus is grown in the presence of 3-CPD and it is possible that this response is due to an impairment in the transport of the

compound. Escherichia coli did not respond to phosphonomycin for reasons which remain obscure.

The increase in sensitivity of S. aureus to penicillins can be rationalised in terms of the variation in the phospholipid fraction and this finding is in agreement with the observations of Dunnick and O'Leary (509) who showed that a decrease in the level of phosphatidyl glycerol leads to increased sensitivity. Padfield and Kellaway have shown that binding of penicillins with phospholipids occurs at the oil-water interface which influences the further transport of the antibiotics through the non-aqueous phase (510).

The studies of Wolf-Watz and Normark on the antibiotic supersensitive mutant (envA) of E. coli have facilitated an understanding of the action of 3-CPD on the organisms (511). The envA mutant is characterised by an increase in permeability which is accompanied by a decrease in phosphatidyl glycerol. The envA mutation also mediates a cell division abnormality causing inadequate separation of dividing cells. By interfering with glycerol metabolism and so with the synthesis of phosphatidyl glycerol a similar effect to the mutation is produced indirectly which manifests itself as an increase in antibiotic sensitivity and an impairment of cell division.

The response of bacteria, cultured in the presence of 3-CPD, to inhibitors of protein synthesis was interesting and followed the predicted pattern that one could expect in light of an examination of current literature. Thus, fusidic acid, a steroidal antibiotic, possessing all the structural attributes of a lipophilic molecule, has increased activity against cells when grown in the presence of 3-CPD. In a similar manner it is possible to rationalise the differences in activity between lincomycin and the 7-deoxy-7(S)-chloro derivative, clindamycin which is probably due to the increased ease of penetration of the latter into cells (467). Resistance to the tetracyclines has been attributed to a

decreased permeability of the drug (512-514). However, after growth in the presence of 3-CPD both E. coli and S. aureus became more sensitive to tetracycline. On first consideration this is a result which goes contrary to expectations as tetracycline is a very hydrophilic molecule. This result, however, is not in isolation since Villani et al (515) showed that tetracycline sensitivity increased in a lipophilic environment.

The resistance to tetracyclines and presumably other antibiotics does not result from the development of an extra molecular layer to prevent the inhibitor reaching its target within cell, if this were the case, entry of metabolites would also be prevented. It has been shown by Franklin that the accumulation of tetracycline within the cell is a complex process (516). Sensitive cells can accumulate tetracycline, however, the tetracycline can diffuse out again. Sensitive cells respond to low concentrations of tetracycline by undergoing a variation in membrane permeability which inhibits outflow without suppressing the inflow. The resistant cells do not have a permeability barrier to facilitate the accumulation of tetracycline and consequently the internal concentration never rises to a lethal level (517). It is possible that cells undergo an enhanced accumulation of tetracycline when they are cultured in the presence of 3-CPD.

The increase in sensitivity of S. aureus to chloramphenicol agrees with the observations of Vaczi et al who showed that those cells with a low phospholipid content were the most sensitive to the antibiotic (518).

Nystatin and related polyenes are known to interact with sterols (519-521) and since 3-CPD fails to increase the sensitivity of yeasts to nystatin it would appear that membranes depleted of phospholipids retain a considerable measure of stability.

In this investigation of the action of 3-CPD on microorganisms it

has been shown that 3-CPD in high concentration can induce aberrant forms as well as regulating membrane permeability. As a result of this ability to induce aberrant forms it must be considered with many other compounds such as novobiocin (464), actinobolin (522), bleomycin (491) and mitomycin (489) which exert their action through an impairment of nucleic acid synthesis, and in this respect it is interesting that glycerol auxotrophs undergo a cessation of RNA synthesis on depletion of glycerol (523). However, 3-CPD, unlike the antibiotics, must be present in moderately high concentrations before there is a marked impairment in the membrane permeability and before there is a disruption of morphological integrity as shown in Plate 29. It is possible to explain the action of 3-CPD in inducing aberrant forms in terms of the working model of cell division propounded recently by Huff et al (524). These workers suggested that membrane-bound DNA sequesters Mg^{2+} and so activates the adjacent plasma membrane to synthesize lipoteichoic acid which in turn sequesters more Mg^{2+} . At a specific concentration of Mg^{2+} the membrane-bound enzymes synthesize new membrane with elevated phospholipids and lipoteichoic acid. The resulting mesosomal vesicle acts as the locus of cell wall synthesis.

Further research into the action of 3-CPD must include a study of the binding of Mg^{2+} at various concentrations of 3-CPD. The role of divalent cations in cell development will be returned to later.

As indicated earlier there have been many investigations of lipid composition in microorganisms and many of these studies have been concerned with the lipid composition of yeasts. Principally these studies have been concerned with boosting the level of lipid components by supplementing the medium with either choline (525, 526) or ethanolamine (153). There appear to have been few attempts at depleting yeasts of lipid constituents.

Whilst examining various steroids and sterols for ability to inhibit cholesterol synthesis it was found that alphaxalone inhibited the synthesis of ninhydrin-positive lipids in yeasts. Doorbenbos and his associates have investigated the structure-activity relationships of azasteroids and found that the cyclopentanphenanthrene nucleus was not necessary for antimicrobial activity (527). Antimicrobial activity was maximum when the azasteroids possessed both hydrophilic and hydrophobic groups. However, there appear to have been few other studies on the structure-activity relationship of steroids and in this study it has not proved possible to recognise any essential feature of the steroid nucleus which could contribute to suppression of ninhydrin-positive lipids.

The mechanism by which 5α -androstan- 3β -ol, 5β -cholestan- 3α -ol and 5-cholesten- 3α -ol potentiate the action of the thiol active compounds is obscure in view of the fact that none of these sterols induce lipid depletion.

The suppression of the activity of 8-hydroxyquinoline when in the presence of alphaxalone could be due to the inability of copper 8-hydroxyquinolate (the active principal of 8-hydroxyquinoline) to penetrate hydrophobic membranes. In the case of phenylmercuric nitrate, the slightly lipophilic molecule will be able to penetrate hydrophobic barriers. It is unlikely that the interaction of the thiol-active compounds will be with sulphhydryl groups of any structural protein of the cell membrane as there appears to be little or no sulphur containing amino acid in the microbial cell membrane (528).

The mechanism by which alphaxalone impairs the synthesis of ninhydrin positive lipids in S. cerevisiae only is obscure, however, Smith et al have shown that alphaxalone prevents ATP synthesis in rabbit brain mitochondria in two ways : (i) inhibition of oxidation

of NAD^+ - linked substrates and (ii) acts an uncoupling agent (529).
Chattaway et al have observed that deoxycortisone exerts an anti-microbial action by uncoupling oxidative phosphorylation (530).
Other groups have indicated that the mitochondrion may regulate lipid synthesis : Lester has indicated that cardiolipin synthesis in yeasts is controlled at this level during the transistion of the cells from anaerobic to aerobic states (531). In a study of cholesterol synthesis in yeasts Klein et al showed that the cyclo-oxidation of squalene occurred at the membrane (532). Some of the evidence indicated that lipid synthesis may occur outside the mitochondria and that preformed units built up the new mitochondria.

The specificity of the eukaryotic membrane has been demonstrated by Medoff et al who showed that the action of rifampicin and cytosine can be potentiated by the sterol disrupting polyene, amphotericin B (533), while Capek et al have shown that resistance to the antimycotic, 2-methyl-thio-5-(3-iodopropargyloxy) pyrimidine by C. albicans was paralleled by a decrease in ergosterol (534). These results of Capek et al can be interpreted in terms of preservation of membrane integrity and it is interesting that Sherr has demonstrated that lysine uptake into Neurospora crassa was dependent upon lecithin in the cell membrane (535).

Cerbon has shown a variation in lipid composition in Saccharomyces carlsbergensis when cultured in the presence of arsenate (458, 536). In his adapted cells there was 50 to 100% increase in phosphatidyl-inositol with no detectable turnover in either phosphatidylcholine or phosphatidyl ethanolamine. In this examination it has been shown that when the arsenate resistant strain of S. cerevisiae was cultured in the presence of arsenate (10^{-2}M) the cells were more sensitive to trichomycin (a water-soluble antifungal polyene), than either the sensitive or resistant strain when cultured in the absence of arsenate. Cerbon has indicated that arsenate

forms a complex with phospholipids (536), such a complex could suppress interaction between the phosphate moiety of the phospholipids and the 3- β -hydroxy group of cholesterol (331).

Although the change in permeability to the compounds studied has been attributed directly to the lipid content the actual situation may only be indirectly related to this component of the membrane. Ling (537) has suggested that it is not lipid, but water polarised in multilayers by cellular proteins which provides the cell with its selective barrier. It may be that the membrane lipid components regulate the size of the pores in the membrane and their frequency and so indirectly determine the amount of ordered water available for the diffusion of the antimicrobial agents, unless these effect the membrane directly.

In addition to those chemicals discussed above others were examined for ability to produce morphological variants. Santos-Mota et al induced depletion of phospholipids in Streptococcus faecalis when cultured in the presence of dicumarol, an inhibitor of oxidative phosphorylation (538). In this study both dicumarol and 2, 3, 5-tri-iodobenzoic acid, an inhibitor of oxidative phosphorylation in plants (539), failed to exert any control over all stages of development in the endospore producing actinomycetes. While these studies did not present any opportunity of studying the synthesis of the membrane in dividing cells other workers have been more successful in demonstrating some possible loci of membrane development.

Sargent has demonstrated that phospholipid is synthesised throughout the cell in B. subtilis (540) and Kusaka has shown that some species of Bacilli regulate lipid composition at the cytochrome level (541). In E. coli two loci may be possible : Merlie and Pizer showed that guanosine tetraphosphate may regulate phospholipid synthesis at a step which was believed to involve the glycerol-3-phosphate acyl transferase step (542),

while Munro and Bell have shown that an E. coli polyamine auxotroph was unable to synthesis phospholipids under conditions of polyamine deficiency (543). Polyamines are known to interact with the phosphate groups of nucleic acids and it could be that this is the mechanism which regulates phospholipid synthesis.

The ability of the cell membrane to regulate cell function has been examined by many workers. Allison and Malucci have shown that stabilisation of lysosomal membranes could retard cell division (544) while Pardee has shown that the cell surface regulates animal cell division (545). With the aid of freeze fracture electron microscopy Tewari and Mahotra have demonstrated alterations in the membranes of germinating Phycomyces spores, differentiating hepatocytes and several other types of cells (546).

As the membrane is presumed to regulate chromosome segregation and cell division (547) interest in membrane synthesis is almost certainly going to continue to expand and this interest will continue to bring together the various sciences in order to sustain the continued expansion of cell surface science.

C. The Response of Cells to Lanthanum and Neodymium as
Substitutes for Calcium.

In sub-section A it has been established that control of cell development can result from interactions of specific antimetabolites with enzymes involved in nucleic acid synthesis while in sub-section B it has been shown that a gross depletion of the cell lipids can impair the mechanism which regulates cell division. In this section it will be established that in a wide variety of cells calcium can exercise a diverse series of controls from controlling a dormant, heat resistant state in bacterial spores to promoting sexual growth of P. cactorum.

As yet no evidence has been obtained which demonstrates that the lanthanides fulfil any essential biological role. However, Robinson et al (548) have observed an accumulation in Carya sp. of several lanthanides (ppm): La(370), Lu(4.5), Gd(70), Ce(320), Er(46), Nd(440) and Pr(46). Bowen and Robinson (549) have reported that C. albicans accumulates La while Servigne and Tchakirian (550) have reported an accumulation of Nd in calcareous red algae.

The response of Scenedesmus sp. to lanthanum and neodymium affords an opportunity of studying the biochemistry of the stable trivalent state.

A number of in vitro studies have indicated that the lanthanides can replace calcium in certain processes, such as, the activation of enzymes (summarised by Bulman and Stretton (401)). Pickard, in one of the few in vivo studies of the lanthanides,

showed that neodymium, lanthanum and praseodymium exert an inhibition of the elongation of oat coleoptile which is slightly greater than that exerted by calcium (379). In this study it has been shown that lanthanum and neodymium, like calcium, do not favour the production of large numbers of cells in the size range 1.5-2.0 μ . However, this replacement is not total as small variations in cell size distribution were observed, particularly in the case of lanthanum, which promoted very large cells or aggregates of cells.

In the absence of calcium the medium promoted development of a large number of small cells. The distribution of cell sizes when the cells are grown without calcium or in a calcium-containing medium supplemented with X-537A are very similar, supporting the observations that X-537A is a calcium ionophore (401). (In the first eight months of 1974 there were no fewer than eight reports concerning the interactions of ionophores (X-537A and A23187) with calcium in biological systems (551-556). Such reports have detailed the action of the ionophores on calcium-induced exocytosis of mast-cells (552, 553), the regulation of contractility of frog eggs (551) and the control of cell division of Schizo saccharomyces pombé (556)). When X-537A was added to a theoretically calcium free medium the percentage of cells less than 1.5 μ m increased dramatically and cells over 4 μ m were completely absent. This result suggests that even though rigorous steps were taken to ensure freedom from calcium it was still possible for a sufficient number of atoms to be present and so exert a slight action. The addition of X-537A

suppressed completely the action of the extraneous ions. X-ray crystallographic studies have shown the barium-X-537A complex to consist of two molecules of X-537A wrapped around Ba^{2+} so that the whole complex is electrically neutral with a lipophilic outer sphere (557). A calcium-X-537A complex will have a similar structure and will so facilitate the diffusion of Ca^{2+} through cell membranes.

The precise role of calcium in the biochemistry of algae is unknown, and in only a few cases has it been successfully replaced by the closely related strontium (387), which is usually toxic or without effect (409). Calcium is recognised as an essential trace element for microalgae (387) and has been shown to regulate cell shape and possibly cell division in Scenedesmus (411). Das shown that calcium promoted phosphate assimilation and decreased the ATP level in synchronously cultured cells whilst the synthesis of chlorophyll, carotene, nucleic acid and protein was increased (388). Nilshammer et al have suggested that calcium may be required for a balanced transport of primary wall material (558).

As in the case of Scenedesmus, lanthanum and neodymium can replace calcium in supporting the growth of P. cactorum, although there is a decrease in the number of sporangia which develop. The ability of strontium to support sexual growth of P. cactorum is in agreement with the findings for other Phycomyces (559).

The GBHA stain revealed that calcium was concentrated in the membrane region of HeLa cells, Hep cells, Paramecium (Plate 45) caudatum, grapefruit seeds and the sporangia of A. missouriense.

However, within the limits of detection, it was impossible to detect calcium in S. cerevisiae.

Although lanthanum and neodymium can replace calcium in the sexual growth of P. cactorum and Scenedesmus sp. these lanthanides fail to replace calcium in the production of heat resistant eubacterial spores and it would appear that these lanthanides are able to replace calcium only in specific cases. However, this inability of the lanthanides to replace calcium in the case of heat resistant bacterial spores does not necessarily imply that calcium has one specific biochemical role in P. cactorum and Scenedesmus sp. and another role in bacterial spores. Although many workers have noted a requirement for calcium for algae and fungi few of them have discussed its biochemical role. On the other hand spore workers have deliberated for many years on the role of calcium in heat resistant bacterial spores.

The two principal theories concerning the heat resistant properties of bacterial spores are, firstly, based on the concept of a low water content, or the hydrophobic nature of the spore (560). Or, secondly, the stabilisation of spore components, either by cross-linking between the cell biopolymers (235) or by "calcification" of the spore interior involving chelation of divalent ions by spore ligands (250, 561-563). However, Grecz and Smith (564) have suggested that the two concepts may not be mutually exclusive because the hydrophobic nature of the spore may result from their high content of coordinated complexes.

The spores produced in the presence of lanthanum and neodymium do have heat resistant properties, but they are not as heat resistant as the calcium containing spores. However, the acid treatment does alter the composition of the spore in some way because the heat resistance of the spores after the addition of calcium is not as great as the untreated spores.

It would appear, on the basis of the ANS study, that heating or acid treating the spores does not significantly alter the hydrophobic nature of the spore. However, the addition of calcium or the lanthanides resulted in a dramatic increase in fluorescence combined with a shift of maximum emission to shorter wavelengths. Such dramatic changes in fluorescence intensity and a shift in the wavelength of emission are normally interpreted as evidence of a hydrophobic environment (565, 566) and it has been suggested that ANS binds to phospholipids (567).

As the acid treatment affects the nature of the spore, as judged by the heat resistance, it is probably better to consider spores after acid treatment and these spores when the cations have been added. On the basis of the fluorescence results it would appear that addition of the cations produces a more hydrophobic spore. It is possible that the lack of any significant fluorescence from untreated spores suspended in ANS may result from the spore coat sieving out the relatively large ANS molecule. Black and Gerhardt obtained no TNS fluorescence from spores until the spore germinated (568). However, some

care must be taken in interpreting the sieving ability of spores because the free space measurements of Gerhardt and Black (243) indicate that most of the spore is permeable to neutral solutes whose molecular weight does not exceed 500. Lewis considers that the inability of ethylenediamine tetraacetic acid (disodium salt) to induce germination may be due to its anionic nature which prevents penetration to the active site (569). The specificity of molecular architecture appears to be very precise since it has been demonstrated by Lewis that 4-methyl-pyridine-2,6-dicarboxylic acid (which has pCa of 4.3 compared to pCa of 4.6 for DPA) cannot germinate spores (569).

An examination of the titration curves of the metal-DPA complexes shows that both lanthanum and neodymium form complexes with DPA which are similar to the calcium complex. However, it is possible that the lanthanide $3+$ state interacts with DPA to produce complexes which are more stable than the calcium complex and such a stable complex would prevent DPA from exerting its ion exchange properties. The preservation of the ion exchange characteristics of DPA could be an essential requirement for the production of heat resistant spores as it has been shown by Stretton and Kempson (private communication) that there is some correlation between metal-ligand stability and heat resistance. These workers showed that the lowest chelate stability (calcium) conferred the greatest heat resistance. In the bacterial spore the ion exchange system may require a cation with the same ionic radius as calcium, and in this respect it is of interest to note that Germaine and Murrell (570) and Leanz and Gilvarg (571) have demonstrated that DPA is located within the

spore protoplast. The exact form of the protoplast DPA is still uncertain, however, Woodruff et al have established that DPA does not exist as a simple complex with Ca^{2+} , or the acid form and neither does it exist as the di-anion (572). Instead the Raman spectra obtained by these workers shows that it is an amalgam of all three forms. It is quite possible that this intermediate form could represent some interaction of DPA with phospholipids or membrane proteins and in this respect it is interesting that Young has shown an association of DPA with peptides obtained from spores exudates (265). However, Stretton and Kempson have found that divalent cations with ionic radii similar to calcium cannot replace calcium in producing heat resistant spores, even though the metal-DPA titration curves are similar in all cases. It would appear that an important criterion may well be the ionic charge/ionic radius which should differ from calcium by no more than 10% for the isoelectronic species. This requirement will be met in full by La^{3+} and partially by Nd^{3+} as these cations have ionic configurations of 2:8:18:18 and 2:8:18:21, respectively, while that of Ca^{2+} is 2:8:8.

Many theories have been advanced to explain the dependence of the heat resistance of the spore on calcium and/or DPA. It has been suggested by Kalakoutskii et al that the heat resistance of actinomycetes spores was proportional to the concentration of Ca^{2+} , Mg^{2+} and DPA (573) while Riemann (574), and Black and Gerhardt (235) have suggested that the calcium-DPA complex may stabilise enzymes through a cross-linking of macromolecules. In vitro studies of Hachisuka et al have shown that

calcium-DPA and calcium 2,5-dipicolinate can stabilise glucose dehydrogenase (575). On the basis of the work of Levinson et al (576), who showed that lanthanum can complex with proteins, it is possible to rationalise the inability of lanthanum and neodymium to produce heat resistant bacterial spores as being a result of the peptides being locked in a stable configuration by the interacting lanthanides.

Up to now I have discussed the interactions which may occur between various cations and cells. At the moment technology is not sufficiently advanced in order to demonstrate the precise biochemical events which occur in the cell as a result of these cationic interactions. Consequently, it becomes necessary to turn to in vitro studies to explain such cellular events. In Chapter 5 I have reviewed the interactions of calcium with phospholipids while in Chapter 6 I have recounted the ability of the lanthanides to replace calcium. It is the contention of this thesis that calcium exerts its specific cytochemical role by complexing with the phospholipids of the membranes. Numerous spectroscopic techniques have established that cations can interact with membranes or membrane constituents and in this respect it is interesting that Chan et al may well have contributed to an understanding of the resistance to heat stress of some microorganisms (577). These workers were able to demonstrate with the aid of ESR signals from fatty acids incorporated into the membrane of a facultative thermophilic Bacillus sp that cells cultured at 55° possessed a membrane which was much more fluid than the membranes in cells cultured at 37°. In a study which is complementary to the work of Chan et al, Lussan and Faucon

demonstrated, by use of fluorescent-labelled phospholipids, that exposure of phospholipids to a series of cations resulted in an increase in the ordering of phospholipids and this order was $\text{La}^{3+} > \text{Ca}^{2+} > \text{Na}^{+}$ (578). Ehrström et al (579) have shown, using cytoplasmic membranes from B. subtilis, that calcium and magnesium are structure promoting whilst monovalent ions disorganise membrane structures. Also Tocanne et al (580, 581) have demonstrated that calcium causes phospholipids to condense and their freeze-etching studies have shown that such in vitro interactions produce membrane-like structures. There are, however, additional factors which can contribute to the interactions between cations and phospholipids. It has been shown that calcium interactions with yeast lecithins were weak since the unsaturated fatty acids induced too large a cross-sectional area in the lecithin molecule (582). Other factors which might well regulate calcium binding could be the specific pH of the phospholipid which might be influenced by the presence of local polypeptides (the subject is discussed in detail in Chapter 5). Variations in polypeptide composition may well be induced by temperature of culture, as shown by the variation in amino acid composition of the cell wall preparations of B. cereus SV-1, (Table 6b), such factors could contribute to the variation in the heat resistant properties of bacterial spores obtained from various media. Variations in the culture conditions could also influence the availability of free amino acids and other small molecules within the spore. The role of such molecules is considered below.

Many microbiologists who have studied the heat resistant properties of bacterial spores have devoted a considerable effort to examining the role of the water adsorbed by the spore. (A detailed review is presented in Chapter 3). In a study of the physical state of electrolytes and water within B. cereus spores Carstensen et al concluded, on the basis of dielectric measurements, that while the cell water was tightly bound it was not as tightly bound as in clathrate hydrates or hexagonal ice (583). In addition, they showed that there was no evidence to suggest that any water was in any unusual state in the dormant spore. However, these workers were able to refute a model of the dormant spore as involving freely mobile, ionised electrolytes and instead they presented a model involving electrostatically bound electrolytes. Such a model is in good agreement with the view of Nelson and Kornberg who suggested that the many small organic molecules (L-glutamic acid, sulpholactic acid, amino acids and 3-phospho-D-glyceric acid) of spores may be associated as a whole net-work of complexes within the spores (584). Black and Gerhardt have suggested that spore polymers might be cross-linked to form a gel (235). Such polymers as suggested by Black and Gerhardt might well be the acetic acid soluble low molecular weight proteins isolated by Setlow from dormant spores (585). Setlow showed that these proteins (7-12,000 Daltons) represented 40% of the protoplast membrane and could not be isolated from any state of growth other than the spore.

The development of a gel within the spore could be facilitated by the presence of Ca^{2+} which might well aid dehydration

in a manner similar to that observed by Ikegami and Imai who showed that Ca^{2+} interactions with polymers aided exclusion of water (586). As Ca^{2+} is regarded as a structure promoting cation (587) it might have the additional advantage of contracting flexible polymers such as peptidoglycan (584).

A possible mechanism for dehydration of the spore protoplast has been the hypothesis of the 'contractile cortex', a mechanism which squeezes all water out of the cell (239). However, the work of Fitz-James (588) has shown that a spore protoplast can be produced by digestion of coatless spores with lysozyme. In the presence of calcium and the absence of magnesium the structures which are formed are spore protoplasts and these are partly refractile, indicating that the protoplast is maintained in a dehydrated state even after cortex digestion. On the basis of this evidence the 'contractile cortex' does not provide an adequate explanation for the dehydrating mechanism. It may be that the specific role of calcium in the mechanism of heat resistance is the dehydration of the spore protoplast. It is interesting that Palmer and Schmidt as long ago as 1941 demonstrated, by X-ray diffraction studies, that the addition of calcium salts to a phospholipid dispersion resulted in an extrusion of water from the phospholipid bilayers (589). A similar phenomenon has been demonstrated by Rendi (590). If calcium was to be the agent which promotes dehydration of the spore protoplast then it must be present within the protoplast and this has been demonstrated by Scherrer and Gerhardt by use of electron probe X-ray microanalysis (591).

With the aid of data obtained from light scattering studies on bacterial spores, Wyatt suggested that DPA acted as a space filler in the protoplast and so prevented free water penetration (592). The ability of calcium to cause an extrusion of water from macromolecules could contribute to the stabilisation of membranes in view of the observations of Clifford et al (197), and Chapman and Salsburg (198) who showed that the addition of water to lipids resulted in increased mobility of methylene protons as the lipid matrix became less rigid. (See Chapter 3 for an account of water-lipid interactions). The crystal structure of the phospholipids could be instrumental in producing heat resistant spores. Rand and SenGupta (593) have shown that calcium cardiolipinate can exist as the hexagonal phase while magnesium cardiolipinate exists either as the hexagonal or lamellar phase, the latter being favoured by low temperature and a low Mg^{2+} concentration. The magnesium cardiolipinate differs from the calcium cardiolipinate in that it contains much more water. The demonstration that calcium is located within the membrane region of citrus fruit seeds could well indicate another case of calcium regulating dormancy and in this respect the interaction of calcium with macromolecules may be one of the few mechanisms universally available. The possibility that DPA may be exerting its action at the membrane is strengthened by my examinations of liposomes. In my examination of the interactions of DPA, EDTA, ethane 1-hydroxy-1,1-diphosphonic acid and aesculin with the calcium-lecithin liposomes, I have shown that all these substances disrupted the liposomes.

However, the greatest disruption occurred in the presence of DPA. The increase in O.D at 0.01M EDTA and H_4 EHDP are anomalous but it is possible that the polyanionicity of these species may be causing an aggregation of the phospholipids. In the case of aesculin the ability to disrupt the phospholipid liposomes is in agreement with Olsen's observations that aesculin can de-stabilise fungal membranes and result in the release of UV-absorbing substances (594). In a similar manner the ability of DPA to interact with the the phospholipid liposomes is in accordance with the observations of Fields who showed that DPA can induce leakiness in the vegetative cells of a Bacillus sp. (595). This ability of DPA to interact with phospholipid-bound Ca^{2+} could explain the ability of DPA to induce germination and the fact that DPA is a much more powerful germinating agent than any other chelator. The recognised lipophilicity of DPA could facilitate its penetration to the apolar sites propounded by Gitler and Montal (596). These authors suggested that ionic lipid-cation-protein interactions could stabilise membrane proteolipids in apolar environments. In view of the well-recognised structure promoting activity of Ca^{2+} it is quite possible that Ca^{2+} is stabilising such apolar sites.

This hypothesis that the membrane contributes to the heat resistance properties of bacterial spores is not in isolation as Brock (160) has suggested that membrane composition may contribute to the thermophilic character of microorganisms and in this respect it is significant that Mindich (personal

communication) has observed very few heat resistant spores in his B. subtilis glycerol-auxotroph.

The ideas advanced in this thesis may well remain a hypothesis for a long time before they can be translated into scientific fact. However, the necessary experiments might not be too far off. If spores can be made available with spin-labelled fatty acids incorporated into the protoplast lipids it might be possible to correlate the ESR signal with a particular membrane conformation which might only be obtained in the presence of calcium. A similar technique has been used by Raison et al (225) who showed that the phase transitions of mitochondria from a chill-sensitive plant and a homeothermic animal coincided with the optimum temperature for growth. Improvements in the technology of differential thermal analysis balances might demonstrate the occurrence of phase transitions in the spore.

The ability of calcium to regulate cellular activity through a possible dehydration mechanism need not be the sole mechanism of cell regulation. In the case of Scenedesmus sp and P. cactorum calcium could exert its action by cis and/or trans effects upon the cell membrane (see Chapter 4 for a discussion of these effects). Such variations in conformation could regulate enzymatic activity and so influence the development of the large Scenedesmus cells. Kirkpatrick and Sandberg (597) have shown that the chaotropic action of Ca^{2+} results in conformational changes of proteins associated with erythrocyte membranes. The cell shape and size could result from such an

interaction with the cell membrane or membrane-bound enzymes. This is to some extent confirmed by the similarity of effects of calcium absence or the addition of X-537A which could be preventing the accumulation of calcium ions in a lipophilic site or is promoting calcium outflow from an essential locus. Other factors which could influence cellular activity have been discussed in detail in Chapter 5 and it is sufficient to indicate that a gradient of surface pH and surface viscosity could regulate the activity of membrane-bound enzymes (356, 357).

The dependence of the sexual growth of P. cactorum upon the structure of the sterol nucleus is a strong indication of the mechanism which controls the sexual growth. Van Deenen and his associates have shown that the 3- β -hydroxy group and a C-17 side-chain are essential for condensation of phospholipid-sterol monolayers (71). Such interactions could be essential for the cis and trans effects of the lipid-globular-protein mosaic membrane (296). Phillips (323) has shown that such cis and trans effects would be accompanied by decreases in mobility of the methylene protons which are in close proximity to the sterol nucleus while the glycerol moiety exhibits increased mobility. As I have observed in Chapter 5, it is quite possible that such variations in the membrane topography could act as a fine and subtle control over enzymatic activity.

In the last fifteen years there has been a rapid advance in our understanding of the regulation of cell development as controlled by events on the ribosome. Perhaps the next fifteen

years will see a similar advance in our understanding of the cell membrane, for only then will we be in the position of understanding such mysteries as neoplastic growth.

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APPENDIX

Synthesis of Pyridine-2,6-dicarboxylic acid Analogues.

With the exception of Lewis's work there have been very few investigations of the replacement of spore dipicolinic acid (DPA) by other analogues. The compounds synthesised by Lewis included 4-amino-pyridine-2,6-dicarboxylic acid, 4-chloro-pyridine-2,6-dicarboxylic acid, 4-methyl-pyridine-2,6-dicarboxylic acid, pyrimidine-2,4-dicarboxylic acid, pyrazine-2,6-dicarboxylic acid, 4H-4-methyl-pyran-2,6-dicarboxylic acid and 4H-pyran-2,6-dicarboxylic acid (1).

This investigation was undertaken with the intention of synthesising pyridine-2,6-disulphonic acid and pyridine-2-carboxylic-6-sulphonic acid.

By far the largest amount of pyridine chemistry involving the synthesis of 2,6-disubstituted analogues was conducted in the late nineteenth and early twentieth century and in some cases it is possible that the claimed product may have been anything but the intended product. Some procedures of synthesis were regarded as impractical since they required the use of high pressure apparatus.

Pyridine-2,6-disulphonic acid.

It was intended to synthesise pyridine-2,6-disulphonic acid by the reaction of 2,6-dihalopyridines with potassium hydrosulphide and then oxidising the dimercaptopyridine with nitric acid to produce the disulphonic acid derivative. Thirtle had reported the synthesis of 2-mercaptopyridine from 2-bromopyridine by refluxing it with potassium hydrosulphide using propane-1,2-diol to provide a boiling point sufficiently high to obviate the use of an autoclave or the bomb-tube method (2).

2,6-Dichloropyridine (25 g, Koch-Light) was dissolved in propane-1,2-diol (60 ml, Fisons) and added in drops to a mixture of refluxing propane-1,2-diol (120 ml) and freshly prepared potassium hydrosulphide (70 g). Potassium hydrosulphide was prepared by passing hydrogen sulphide into potassium hydroxide (70%).

The solution was refluxed for 24 h at 150-175° and the cooled flask was allowed to cool and stored at room temperature for 96 h. The potassium chloride was removed by filtration and washed with ethanol (200 ml). The filtrates were bulked and concentrated in vacuo. The flask contents appeared purple at the end of the reaction and this colour could be removed readily on addition of a small quantity of acid. The thick viscous residue produced in vacuo was taken up with distilled water and adjusted to pH 5.0 with glacial acetic acid to produce brown-orange oil which developed on the surface of the liquid. After the flask had been chilled overnight the oil-like deposit was removed by filtration and re-crystallised from benzene to give a yellow product (1 g) which was subsequently identified as sulphur. Further attempts were made to concentrate the reaction products but these yielded a purple residue which defied identification.

The reaction was repeated on several occasions using various refluxing temperatures which were obtained by adding higher boiling alcohols to the propane-1,2-diol. These modifications were not successful. Further attempts were made at synthesising the dimercaptopyridine by replacing 2,6-dichloropyridine with 2,6-dibromopyridine (Ralph N. Emmanuel) and these experiments also failed.

There are conflicting reports on the synthesis of 2,6-dimercaptopyridine. A successful patent has been granted for the synthesis of 2,6-dimercaptopyridine (3). However, the essential procedure did not vary from the above process. Marckwald, Klemm and Trabert in 1900 reported the synthesis of 4-chloro-2,6-dimercaptopyridine (4). A personal communication from Unilever Ltd., Port Sunlight reported the failure to synthesise the 2,6-dimercaptopyridine and a very low yield of 4-hydroxy-2,6-dimercaptopyridine.

Phillips and Shapiro reported the successful synthesis of 2-mercaptopyridine (5) using thiourea to produce the isothiuronium salt which was decomposed subsequently by ammonium hydroxide.

2,6-Dibromopyridine (5 g), thiourea (5 g) and ethanol (40 ml) were refluxed for 1 h; after cooling, aqueous ammonia (d 0.880; 30 ml) was added, the mixture kept for five days at room temperature, the ammonia removed under reduced pressure, and the residue acidified with acetic acid and extracted with chloroform. Removal of the solvent gave a residue which was identified as the starting material. The experiment was modified by raising the reflux temperature by the addition of n-butanol and extending the time of reflux, but these modifications were unsuccessful.

No more attempts were made to synthesise 2,6-dimercaptopyridine.

Pyridine-2-carboxylic-6-sulphonic acid.

A reaction procedure similar to the thiourea method was adopted to produce 2-methyl-pyridine-6-sulphonic acid which

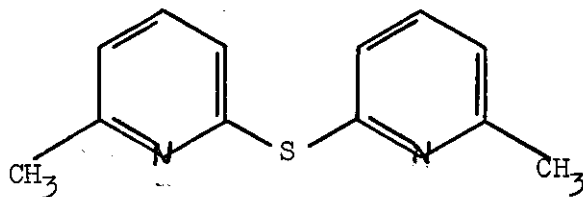
was to be subjected to potassium permanganate oxidation to pyridine-2-carboxylic-6-sulphonic acid.

2-Bromo-6-methyl-pyridine was produced by diazotisation of 2-amino-6-methyl-pyridine by a variant of the Craig process which has been used to synthesise successfully 2-bromo-pyridine from 2-amino-pyridine. Some dispute exists as to the diazotisability of 2-amino-pyridines.

2-Amino-6-methyl-pyridine (60 g, Koch-Light) in hydrobromic acid (24%) was added to hydrobromic acid (48%, 250 ml) which was cooled previously to 5°. The temperature was lowered to -5° and bromine (80 ml) added in small portions over 20 mins. Sodium nitrite (90 g) was added over 3 h with the temperature maintained at -5°. Dilute sodium hydroxide (20 ml, 2M) was added to the "perbromide" complex, the flask contents distilled in vacu and the fraction which came off at 88-118° collected. A heavy brown tar-like residue remained. This purified fraction distilled at 202-206° at 750 mm. Yield 70 g, picrate m.p. 113 (literature m.p. 115-116° (6)).

2-Methyl-6-mercaptopyridine was prepared by the thiourea method of Phillips and Shapiro (5). 2-Bromo-6-methyl-pyridine (8.6 g) and thiourea (3.8 g) were refluxed for 1 h in ethanol (25 ml), cooled and aqueous ammonia (30 ml, 0.880) added. After the flask had stood at room temperature for five days the solvent was evaporated off, the residue was acidified with glacial acetic acid and extracted with chloroform which was removed in vacu. Glacial acetic acid was added to the residue, filtered and neutralised by the addition of ammonia. The aqueous layer was extracted with chloroform and the product re-crystallised from chloroform three times. The yield was 3.1 g, m.p. 139°.

Mass spectral analysis (Physico-Chemical Measurements Unit, Harwell, Didcot) indicated a molecular species compatible with



Presumably this species had been produced by elimination of hydrogen sulphide.

2-Methyl-pyridine-6-sulphonic acid was produced by the nitric acid oxidation procedure of Evans and Brown (7).

The product (25 g) obtained by thiolation of 2-bromo-6-methyl-pyridine was oxidised with nitric acid ($d = 1.42$, 74 ml) in distilled water (200 ml) for 45 mins on a steam bath. The solvent was removed in vacuo and the residue re-crystallised six times from ethanol to yield a white crystalline compound, yield, 10 g. The compound melted with decomposition at 250° . Mass spectral analysis indicated the presence of a methyl group and sulphonic acid group. Infrared spectroscopy indicated a sulphonic acid group.

2-Methyl-pyridine-6-sulphonic acid was subjected to oxidation with potassium permanganate using a procedure similar to that of Vogel (8). 2-Methyl-pyridine-6-sulphonic acid (5 g) was suspended in distilled water (50 ml) heated to 70° and potassium permanganate was added in 10 x 2.5 g quantities over 3-4 h. The temperature was raised to $85-90^{\circ}$ for the last five additions and a small quantity of water (2 ml) was used to washdown the oxidant which was only added when the previous sample had been consumed.

The isolation of the oxidised product was conducted according to Vogel. However, no product could be obtained which could be characterised as pyridine-2-carboxylic-6-sulphonic acid. Analysis of the reaction mixture indicated the presence of large quantities of sulphate which was presumably produced as a result of degradation of the pyridine nucleus. The reaction was repeated several times on a similar scale (1.25 g) but the reaction still failed to yield the required product. Estimation of the sulphate by the gravimetric barium sulphate procedure gave a near quantitative yield.

The failure to obtain the pyridine-2-carboxylic-6-sulphonic acid may be due to a labilisation of the pyridine nucleus by the sulphonic acid moiety. Further attempts to synthesize the acid must use milder oxidation procedures.

An examination of the titration curve of 2-methyl-pyridine-6-sulphonic acid (Fig 13) showed that the acid was much stronger than picolinic acid. On the basis of this stronger acidity of 2-methyl-pyridine-6-sulphonic acid it is possible that pyridine-2-carboxylic-6-sulphonic acid may form too strong a complex with calcium, for as, Stretton and Kemps-on (private communication) have observed DPA-chelate complexes which are more stable than Ca-DPA fail to contribute to the heat resistant properties of spores. However, it is possible that the possession of such strong chelating properties may well make the compound an ideal germinating agent. Lewis has shown that of all his chelating agents the only compound which could replace DPA as a germinating agent was 4-pyran-2,6-dicarboxylic (9).

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PART II.

AN INVESTIGATION OF THE PATHOGENICITY OF MICROORGANISMS
IMPLICATED IN EXTRINSIC ALLERGIC ALVEOLITIS.

Chapter 1.

The Actinomycetes.

The actinomycetes possess a variety of biological properties which make them an ideal group of organisms to study in terms of the cytological chemistry of sporulation and morphological diversity.

The nomenclature of the Actinomycetales order is complicated partly by their disputed fungal and bacterial characteristics. The taxonomic difficulties of these organisms adds an additional complication and this is exacerbated by the differences in nomenclature adopted in Europe, U.S.A. and U.S.S.R. However, research over the last fifteen years has assisted in the classification.

Many investigators have placed the Actinomycetales in the Fungi Imperfecti and in terms of morphology these investigators may be correct. Strong fungal characteristics (aerial mycelium and spores borne in the typical fungal manner) are displayed by the genera Streptomyces, Micromonospora, Streptosporangium and Thermopolyspora. Pridham, Heseltine and Benedict classified the Streptomyces as fungi (1).

Vuillemin placed Nocardia farcinia in the fungal family Microsiphonales within the Arthrospora order (2). However, others have indicated that the Actinomycetales should be considered as a transitional group between fungi and bacteria (3) while Krassilnikov has stressed most strongly the morphological characteristics common to the Actinomycetales and Fungi Imperfecti (4).

However, in spite of their filamentous and morphological nature there is a strong case for considering the actinomycetes to be closer to bacteria than fungi. The actinomycetes have bacterial dimensions (ca 0.5-1.0 μ) and they are normally Gram-positive. They contain acid fast species such as the Nocardia and cell wall analysis reveals the absence of chitin and cellulose. Muramic and teichoic acids are present, they are sensitive

to the typical bacterial antibiotics and to phage. The absence of mitochondria places them in the prokaryotic division of the protista.

The variation in the morphology of the Actinomycetales (from the Chinese letter like Corynebacteria through the thermophilic spore bearers to the aquatic Actinoplanes) presents an opportunity of studying cell differentiation without the added complexities of the fungi, which contain mitochondria.

The Actinomycetales spores are many and varied and include several species which produce heat resistant spores (5). The spores produced by the genus Streptomyces have been considered 'conidia' - a mycological term used to describe dispersible asexual spores, or more specifically, those spores pinched off from the tip of an actively proliferating conidiophore. Electron microscopy has revealed that the Streptomyces spores are formed by the ingrowth of specialised cross walls in existing hyphae (6-8). The mature spores are held in chains by a thin outer sheath which forms the hairy or spiny spore appendages characteristic of certain species. Such spores should be called arthrospores since they correspond to the arthrospores of fungi described by Madelin (9). Conclusive proof that the catenate spores of the genera Actinomadura, Microbispora, Micropolyspora and Microtetraspora are arthrospores is still awaited. The term 'fragmentation spore' (10) has been used to describe the propagation mode of the genus Nocardia which fragments into coccoid or bacillary elements. These are basically exogenous arthrospores arising by the disarticulation of hyphal elements at the position of preformed cross walls.

It is possible to describe the passively liberated spores of the Micromonospora spp. and the Thermomonospora spp. as "aleuriospores" (11). Lysis liberates the supporting hyphae or the point of attachment of the spore wall.

Actinomycetes spores are formed also within spore vesicles which have been termed 'sporangia' (a term introduced originally to describe

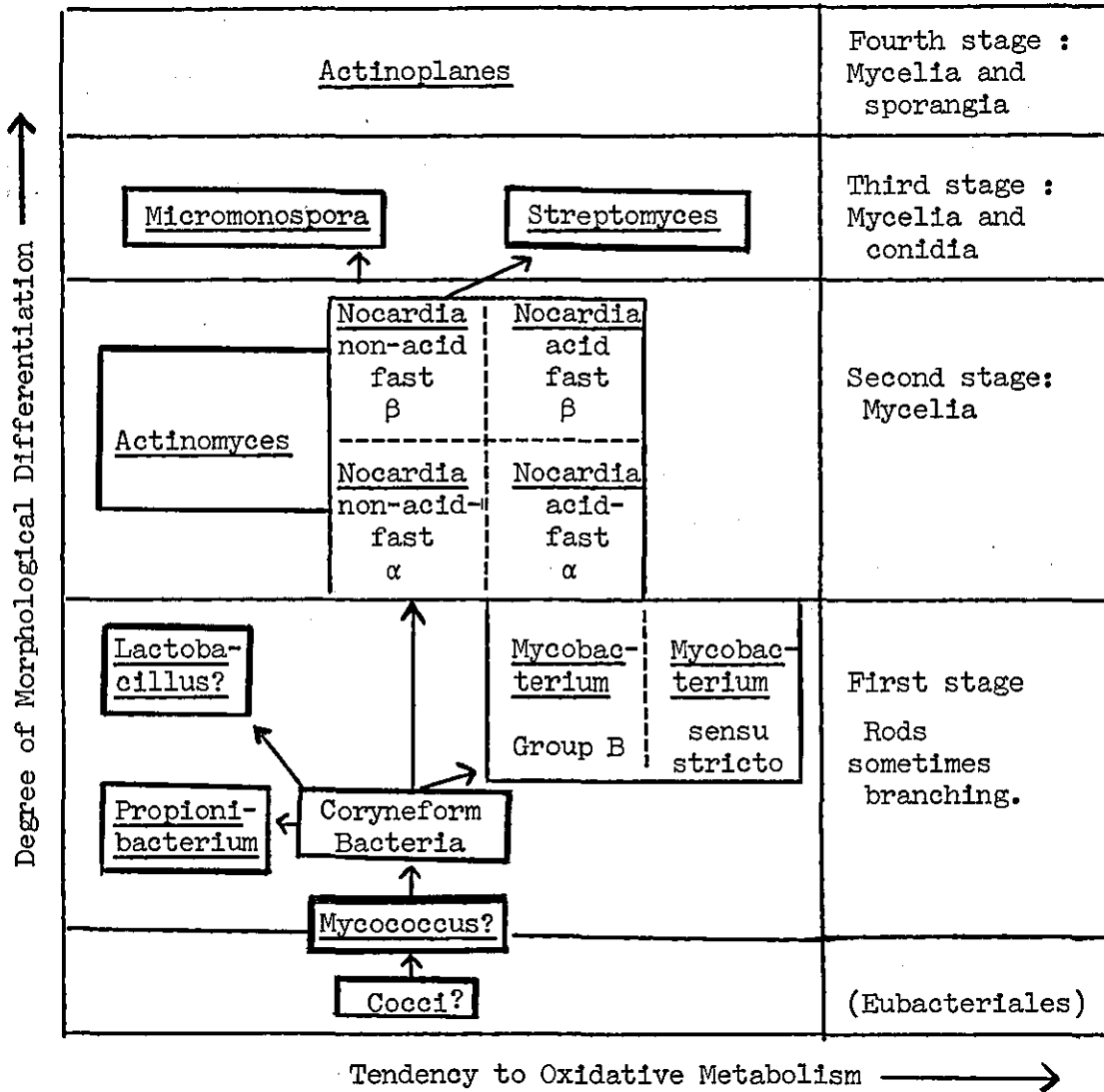


Fig. 1

the vegetative cell surrounding the bacterial spore (12), but understood by the mycologist as the container of asexual spores formed as a result of nuclear division and cytoplasmic cleavage). The Actinoplanes, Strepto-
sporangium and Ampullaria form spores by septation of existing hyphae within a protecting envelope (13, 14).

Spores produced endogenously (15).

Endospores sensu stricto

Bacillus
Clostridium
Thermoactinomyces vulgaris
Actinobifida dichotomica
Sarcina ureae
Sporolactobacillus inulinus
Methanobacterium omelianskii

Spores of hyphal origin:

Arthrospores

Streptomyces
Streptoverticillium
Actinomadura
Microbispora
Micropolyspora
Microtetrastora

Fragmentation spores:

Nocardia

Aleuriospores

Micromonospora
Thermomonospora

Spores formed within spore vesicles:

Zoospores

Actinoplanes
Ampullariella
Spirillospora
Planomonospora
Planobispora
Dactylsporarium
Dermatophilus
Geodermatophilus

Alanospores

Streptosporarium
Microellobosporia

The significance of thermophily in the classification of actinomycetes has been adequately covered (16). The spores of the thermophilic Actinobifida chromogena possess the typical ultrastructure of the Bacillus and Clostridia spores and also contain significant quantities of dipicolinic acid (17). The phenomenon of heat resistance of the actinomycete spores has been investigated (18).

The phylogenetic relationships of the actinomycetes is illustrated in Fig. 1.

The Actinomycetales have also been classified according to cell wall composition (15).

| <u>Cell Wall Type</u> | <u>Major constituents</u> |
|----------------------------------|---|
| <u>Streptomyces</u> or Type I | L-Diaminopimelic acid (DAP), glycine. |
| <u>Micromonospora</u> or Type II | meso-DAP, glycine; hydroxy DAP may also be present. |
| <u>Actinomadura</u> or Type III | meso-DAP |
| <u>Nocardia</u> or Type IV | meso-DAP, arabinose, galactose |
| <u>Oerskovia</u> | Lysine, aspartic, galactose |
| <u>Actinomyces bovis</u> | Lysine, aspartic |
| <u>Actinomyces israelii</u> | Lysine, ornithine |
| <u>Agromyces</u> | Diaminobutyric acid, glycine |
| <u>Mycoplana</u> | meso-DAP + numerous others. |

Cell wall types and Whole-Cell Sugar Patterns of Aerobic Actinomycetes containing meso-DAP.

| <u>Cell Wall</u> | <u>Whole-Cell Sugar Pattern</u> |
|---------------------------------|---------------------------------|
| Type Principal constituents (+) | Type Diagnostic sugars |
| I Glycine | D Xylose, arabinose |
| II None | B, C 3-O-Methyl-D-galactose |
| III Arabinose, galactose | A Arabinose, galactose |

(+)

All cell wall preparations contain major amounts of alanine, glutamic acid, glucosamine, and muramic acid.

| <u>Morphology</u> | <u>Possible generic assignment</u> |
|--|---|
| I. No sporangia formed. | |
| 1. Mycelium dividing in more than one plane | <u>Dermatophilus</u> , <u>Geodermatophilus</u> . |
| 2. Mycelium dividing perpendicularly to main hyphal axis | |
| a. Aerial mycelia only | <u>Sporichthya</u> |
| b. Mycelium (primary) | |
| Conidia absent, no motility | <u>Mycobacterium</u> , <u>Agromyces</u> , <u>Nocardiodes</u> , <u>Nocardia</u> |
| Conidia absent, motility | <u>Oerskovia</u> , <u>Mycoplana</u> . |

| | | |
|----|--|--|
| c. | Primary and secondary mycelia No spores | <u>Mycobacteria</u> , <u>Nocardiodes</u> , <u>Nocardia</u> . |
| | Single spores on aerial mycelia | <u>Thermomonospora</u> |
| | Single spores on both mycelia | <u>Thermoactinomyces</u> |
| | Longitudinal pairs of spores on aerial mycelia | <u>Actinomadura</u> <u>Streptomyces</u> , <u>Nocardia</u> |
| | Pairs of spores (longitudinal) on aerial mycelium | <u>Microbispora</u> |
| | Short conidial on aerial mycelia | <u>Actinomadura</u> , <u>Streptomyces</u> <u>Nocardia</u> . |
| | Conidial short chains on both mycelia | <u>Micropolyspora</u> , <u>Nocardia</u> |
| | Conidial long chains on aerial mycelia | <u>Streptomyces</u> , <u>Streptoverticillium</u> <u>Nocardia</u> , <u>Actinomadura</u> , <u>Pseudonocardia</u> . |
| | Conidial long chains on both mycelia | <u>Streptomyces</u> , <u>Micropolyspora</u> |
| II | <u>Sporangia formed.</u> | |
| | Sporangia each with one single spore | <u>Planomonospora</u> |
| | Sporangia each with a longitudinal pair of spores | <u>Planobispora</u> |
| | Sporangia with one single chain of spores | |
| | Nonmotile sporangiospores | <u>Microcellobosporia</u> |
| | Motile sporangiospores | <u>Dactylosporangium</u> |
| | Sporangia with many spores | |
| | Sporangia globose to lageniform, motile spores | <u>Actinoplanes</u> , <u>Ampullariella</u> <u>Amphosporangium</u> , <u>Spirillosporia</u> |
| | Sporangia globose, spores nonmotile | <u>Streptosporangium</u> |

**Fig. 2. Interaction of small particles with the bronchio-
respiratory system.**

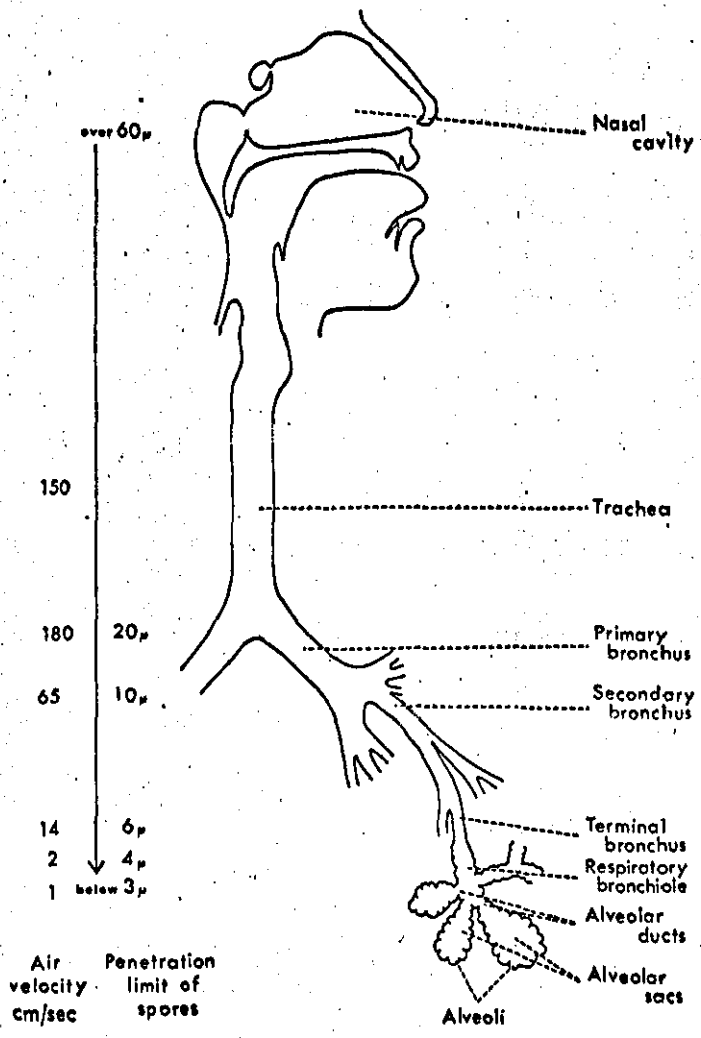


Table 1.

Extrinsic Allergic Alveolitis

| <u>Disease</u> | <u>Inhalant Involved</u> | <u>Probable Antigens</u> |
|-------------------------|--|--|
| Farmers lung | Mouldy hay | <u>Microsporysora faeni</u> (20) <u>Thermomonospora vulgaris</u> (21, 22) |
| Bagassosis | Mouldy bagasse | <u>T. vulgaris</u> , <u>T. sacchari</u> (23, 24) |
| Mushroom Workers lung | Mushroom compost | <u>M. faeni</u> , <u>T. vulgaris</u> (25, 26) |
| Bovine lung | Mouldy hay | <u>M. faeni</u> (27, 28) |
| Fog fever/Broken wind | Mouldy hay | Hay dust (29, 30) |
| Suberosis | Mouldy oak bark | Mouldy cork dust (31) |
| 'New Guinea' lung | Dried grass and leaves | Thatch of huts (32) <u>Streptomyces olivaceous</u> (33) |
| Malt Workers lung | Mouldy barley | <u>Aspergillus clavatus</u> <u>Aspergillus fumigatus</u> (34, 35) |
| Bird fanciers lung | Pigeon/buderigar/ parrot/hen droppings | Sera (36-39) |
| Wheat weevil disease | Infested wheat flour | <u>Sitophilus granarius</u> (40) |
| Sesquiosis | Mouldy sawdust | <u>Aureobasidium pullulans</u> (41) |
| Maple-bark pneumonitis | Mouldy maple bark | <u>Cryptostroma corticale</u> (42) |
| Air conditioner disease | Dustor water from air-conditioning systems | <u>T. vulgaris</u> (43-45) |

The history of the actinomycetes is a history of their isolation from man and animals. The role of the Nocardia, Mycobacteria and Corynebacteria will not be discussed. Those organisms which will be considered appear to be saprophytes and commensals. In many cases these organisms produce infections which have resulted in the actinomycetes being classified as opportunistic pathogens (19). In many cases these opportunistic properties are also reflected in fungal infections.

The sporulating actinomycetes are frequently identified as the casual agents of a group of diseases termed 'extrinsic allergic alveolitis' (Table 1). Extrinsic allergic alveolitis is a lung condition due to the inhalation of 'organic' dust which may include the spores of certain fungi and actinomycetes. Pepys has reviewed the various conditions described as due to extrinsic allergic alveolitis (46).

Austwick (47) has indicated that only those particles with a diameter ca 1μ will be able to penetrate respiratory tract and eventually reach the alveoli (Fig.2). The largest organic material is normally retained by the nasal hairs with slightly smaller material being removed by the mucoid lining of the respiratory tract and this control is dependent upon the inertial changes exerted upon the foreign bodies as a result of constant changes in airflow direction. Even smaller particles are removed as a result of gravitation forces which cause the foreign bodies to sediment out of the still air at a speed dependent upon size and density.

Although a great deal of research has established the casual agents of extrinsic allergic alveolitis there has been no investigation of the pathogenic potential of the actinomycetes and fungi implicated in the syndrome.

One group of the actinomycetes, the Actinomyces, are oral commensals and are well characterised as agents of actinomycotic mycetoma (actinomycosis).

Actinomycosis was first reported by Lebert in 1857 and in 1874 he observed Streptothrix foersti in the lachrymal canal. In 1887 Bollinger described "lumpy jaw" in cattle and observed mycelia in the mandible isolates. Bollinger's findings were confirmed by Hartz. In 1885 J. Israel published a report of 38 similar clinical cases in humans. Such studies remained controversial (48, 49) until it was demonstrated that at least two Actinomyces species existed (50, 51). There have been many reports that the casual agent of bovine lumpy jaw, A. bovis, has been isolated from human actinomycosis (52). However, the fluorescent labelled antibody (FA) technique failed to detect A. bovis in dental calculus which on average contained 2-4 species of Actinomyces with A. israelii, the casual agent of human actinomycosis, being present in nearly all cases (53).

Originally the Actinomyces genus comprised anaerobic to micro-aerophilic, non-acid fast species which did not reproduce by sporulation. This genus was restricted to the catalase negative species but was later modified to include the catalase positive A. viscosus (originally isolated as Odontolyticus viscosus, (54). Now several other Actinomyces spp. have been isolated from various teeth (A. odontolyticus, naeslundii, Arachnia propionicus and Bifidobacter eriksonii, formerly Actinomyces eriksonii). A more detailed account of the aetiological nature of the Actinomyces exists (55). These organisms are not only agent of actinomycosis but also complicate the diagnosis of other diseases. Cell wall deficient forms of A. naeslundii and A. odontolyticus have been isolated from patients with leukaemia (56).

The location of actinomycotic infections has been recognised predominantly as cervicofacial (57, 58). However, the predominance of the case reports is possibly a result of the ease of diagnosing the infection rather than actual incidence. Gruner has reviewed the difficulties in diagnosing actinomycosis (59). Many cases of

misdiagnosis have been recorded before (60, 63), while actinomycosis of the colon has been described as "mock cancer" (63). Actinomycotic infections have been reported in the abdomen (64), the spine (65), the anorectal region (66) the testes (67) and in a case of actinomycotic empyema (a rare form of thoracic actinomycosis) (68). An unusual actinomycotic infection due to an organism which possessed a typical Actinomyces properties has been reported (69). Actinomyces viscosus was isolated from an infected lung after penetration by a knife (70). Actinomyces viscosus isolates from an infected dog had morphological, metabolic and serologic characteristics which were similar to those from goats, pigs, hamsters and humans (71).

For many, many years the consensus of opinion has accepted that the Actinomyces are opportunistic invaders. In 1900 Ruhrah compiled a detailed account of the incidents which may have precipitated actinomycoses (72) and in 1925 Sandford and Voelker compiled a detailed account of the infected organs of cases reported in the U.S.A., including those reported by Ruhrah (73). Both accounts are of considerable historical interest.

Five pathways of entry in the development of pleuropulmonary actinomycosis have been considered: (i) cutaneous, through penetration, (ii) digestive, the organism is ingested and spreads through the pharynx and oesophagus into the mediastinum and pleura, (iii) contiguous, from cervicofacial or intrabdominal through lesions, (iv) inhalant, by aspiration through the tracheobronchial tree, and (v) lymphohematogenous, the most common (74). Wangenstein considered that the oral commensal Actinomyces could possibly pass through the gastrointestinal tract uninfluenced by the stomach acid (75). Grässner found osseous involvement in 15% of 486 patients with the following distribution: spine, 37%; mandible, 25%; ribs, 10%; maxilla, 8% (76).

Considerable interest in the Actinomyces arises as a result of their possible involvement in dental caries. The relationship of oral bacteria to periodontal disease and dental plaque has been reviewed (77, 78). The isolation of A. viscosus from periodontal plaque in hamsters has added a new stimulus to the investigation of the Actinomyces. Actinomyces viscosus has been implicated in the transmission of periodontal disease syndrome (79). The distinguishing features of the human and animal strains of A. viscosus have been reviewed (80). Other oral strains have been examined (81, 82) and Georg and Coleman have demonstrated the pathogenic potential of some species in mice (83).

Actinomyces viscosus has been shown to produce an extracellular levan (84) which has been rediscovered recently (85). It has been observed that the intracellular cementing substance for A. naeslundii may be a hyaluronic acid since it was susceptible to commercial hyaluronidase (86).

Considerable controversy surrounds the isolation of Micromonospora spp. from animal hosts. The anaerobic Micromonospora propionici and M. acetiformis have been isolated from the intestinal tracts of worker termites (87, 88). An isolate from equine mycetoma, A. caballi, has been considered by some workers as a Micromonospora spp. (89). Erikson considered the organism, A. gallicus, isolated originally from human mycetoma, to be devoid of sufficient sporulation characteristics to place it in the Micromonospora (89). This organism, designated NCTC 4582 (Lister Institute) was listed originally as Streptomyces gallicus (6th. Edition Bergey, 1948) but has been re-classified recently as M. narashinoensis (90). Baldacci has studied an organism isolated from a mycetoma of the knee (92, 93) which may be accepted as a Micromonospora spp. (94).

Extensive confusion exists as to the aetiology of infections due to certain Streptomyces spp. Cross lists several species which were isolated from keratolytic infections (95). In addition, he lists a S. albus sp. as an isolate from the vagina of a heifer while another species was isolated from a blood sample. Even an examination of stained infected tissue by light microscopy does not permit an accurate classification since it has been observed that there are organisms other than Actinomyces which can form granules which may contain Gram-positive or periodic-acid-Schiffs-positive filaments (96). Heymer (97) reported the isolation of S. coelicolor from tonsils, however, this should not be taken as proof of pathogenicity since it is a common air-borne organism and may have lodged at the tonsillar site during respiration. Some pseudofilamentous organisms described as Lactomyces have been isolated from tonsils (98).

Infections due to Streptomyces pelletieri have been reported in human lymphocutaneous mycetomas in Africa and the Americas (99). In the past this organism has been called Nocardia pelletieri and A. pelletieri while recently it has been suggested that it should be called Actinomadura pelletieri (100). The first report of S. pelletieri in neonatal infections occurred recently (101).

Very little is known about the distribution of the Actinomyces in environments other than animals with the exception of a reported isolation of A. israelii from farm yard manure and from artesian wells (102). Waksman and Purvis isolated from peat an Actinomyces spp. which grew at 40° but they failed to make any detailed examination of the organism (103). Gledhill and Casida have reported the isolation of catalase negative species from soil and which are intermediate between Actinomyces and Nocardia (104, 105). The optimum temperature was 30° and not 37°.

Fungal Infections

Fungal infections are far too numerous to be comprehensively reviewed here. A detailed account of such infections has appeared (106). The only fungi which will be considered are those which have been considered as potentiating agents of extrinsic allergic alveolitis.

The genus Cladosporium is an assemblage of over 160 species, many of which are quite possibly non-congeneric. Cladosporium herbarum Link ex Fr. is found the world over in dead organic material and apparently occurs as a plant parasite. Bernton and Thorn (107) and Tomiskova, Dura and Novackova (108) have associated it with allergic syndromes, although it has not been implicated in local infections.

Other Cladosporium spp. have been identified as casual agents of the mycosis "cladosporiosis". However, in many cases the fungi have been re-classified. The condition "chromomycosis" caused by C. carrionii is not considered as an example of cladosporiosis since C. carrionii has characteristics similar to Phialophora pedrosoi (106). Cladosporium trichoides is the casual agent of cladosporiosis. Patients infected by C. trichoides present, generally, a history of brain abscess and very occasionally the organism is isolated from cutaneous skin ulcers (106, 109).

The epidermal fungal infection 'tinea nigra' has been attributed to C. werneckii which is confused with Aureobasidium pullulans. The young epidermal colonies are yeast-like on first isolating them but after a few days they become olivaceous or black. The fungus produces a dark olive mycelium with the morphology of Cladosporium and when sporulation occurs it is of the Cladosporium type (106).

Aureobasidium pullulans may be the causal agent of sequiosis (41). An examination of the lungs of a sequiosis patient revealed some unusual birefringent lamellar deposits. Some confusion over the nature of these

particles has resulted. Particle shape indicated that they were possibly redwood dust which acted as niduses about which larger lamellar deposits could have formed. Similar particles were said to have been observed in cases of farmers lung disease. Biopsy of the lung failed to produce any fungus spores, however, the patient had not been exposed to redwood sawdust for two months. Aureobasidium pullulans has been isolated from the lymph nodes of a man (110) while Wynne and Gott have reported the isolation of A. pullulans from granuloma in patients with Hodgkin's disease (111) and also, it has been isolated from patients with rheumatoid arthritis (cited by Merdinger (112)).

Aspergilli have been reported as the casual agents of a variety of conditions. Aspergillosis is a granulomatous, necrotising and cavitiary disease of the lungs, often with hematogenous spread to other organs. Aspergillus fumigatus is the usual causal agent of aspergillosis (113).

Aspergillus niger may be the causal agent of otomycosis. ~~However, this diagnosis~~
of
A fungal infection of the ear is often made erroneously since bacteria usually cause the disease. Mycetomas caused by A. amstelodami together with the usual A. glaucus and A. nidulans may have little aetologic relationship with the usual species of Aspergilli (106). Aspergillus terreus and A. clavatus are isolated occasionally from the sputum under conditions suggesting secondary invasion of pulmonary lesions. Aspergillus restrictus, which resembles A. glaucus, has resulted in a fatal infection as a result of its wide dissemination (106). Aspergillus fumigatus and A. flavus may invade the central nervous system in patients with diabetes mellitus or other conditions such as corticosteroid therapy which may reduce resistance (114).

It has been suggested that A. clavatus may be the source of antigens in malt workers lung (34), Aspergillus clavatus was reported to have been isolated from the air sac of a chicken. However, the Commonwealth Mycological Institute have failed to obtain growth ~~of this organism~~ (personal communication).

Cryptostroma corticale has been identified as the casual agent of maple bark strippers lung. Spores of C. corticale have been recovered by lung biopsy of affected workers (42). Histological examinations of lung tissue revealed numerous zones of cellular infiltration of the alveolar walls and disruption of the bronchiolar walls. Extensive fibrosis of the alveolar walls was observed together with granulomas. Spores (diameter 3-5 μ) were seen in these samples. There was no evidence of budding of the spores and neither could mycelia be observed. A white growth was obtained after 72 hours on Sabouraud's agar. Preliminary reports indicated no growth at 37°C.

Bacillus cereus Infections.

Although Bacillus cereus is not implicated in extrinsic allergic alveolitis it is a common saprophyte which has received little attention in terms of its general pathogenic properties. However, its toxins are casual agents of food poisoning (115).

It is well recognised that B. cereus has many morphological and biochemical similarities to the more virulent Bacillus anthracis (116, 117) but it is generally dismissed as a laboratory contaminant when isolated from blood culture or clinical material (118, 119). However, B. cereus has been shown to cause serious infections, including pneumonia (120, 121) bacteraemia (122, 123) and meningitis (124).

Lammana and Jones have shown that B. cereus was lethal on injection in the mouse when high inocula were used (125). In addition, Burdon Davis and Wende (126) produced local infections in mice following subcutaneous or intraperitoneal injection of rapidly growing cultures of B. cereus, the infection resulted in death of the mice.

Bacillus cereus has been implicated by Jasper et al (127) in infections of the bovine udder resulting in gangrenous mastitis while others have demonstrated that experimentally induced B. cereus infections are fatal to the ewe and cause pregnant ewes and heifers to abort (128).

EXPERIMENTAL

Materials and Methods.

Organisms.

Thermoactinomyces vulgaris NCIB 9780, Micromonospora chalcea NCIB 9599 and Thermoactinomyces sacchari A978 (generously supplied by Dr. J. Lacey, Rothamsted Experimental Station) were the representative aerobic actinomycetes used. Actinomyces viscosus WVU 398B (kindly supplied by Dr. Mary A. Gerencser, University of West Virginia) was used as a control aerobic actinomycete whose animal pathogenicity is under investigation. Streptomyces pelletieri NCTC 3026 was used as a control of known pathogenicity. An alternative control, S. griseus NCIB 8232, failed to produce nodules in rabbits. However, the maximum temperature permitting growth in vitro was 30°. Streptomyces coelicolor CUB 108, a thermophilic species isolated from cheese, (129) was obtained from Dr. T. Cross of Bradford University.

Cryptostroma corticale was isolated from infected maple bark generously supplied by F.I. Wenzel, Marshfield Clinic Foundation, Wis. The morphological characteristics were the same as those described by Gregory and Waller who described the organism as the casual agent of sooty bark disease in sycamores (130). Cladosporium herbarum ex Fries CMI 131128 and Aureobasidium pullulans (Pullularia pullulans) CMI 2456 were used as fungi representative of those implicated in the described lung conditions.

Thielavia thermophila CMI 145136, originally isolated from wheat straw compost was used as a control thermophilic organism which occurs in a similar environment to the thermophiles implicated in extrinsic allergic alveolitis.

Bacillus cereus (spore-variable) SV-1 was isolated from the laboratory atmosphere. Dr. Ruth E. Gordon of Rutgers Institute of Microbiology has confirmed the organism to be Bacillus cereus (personal communication).

Media.

The actinomycetes and B. cereus SV1 were maintained on nutrient broth, nutrient agar (prepared by the addition of 1.5% ^W/v 'Oxoid' agar No.3) and blood agar which was supplemented with 5% ^V/v defibrinated horse blood ('Oxoid'). Prior to the preparation of the suspension required for injection M. chalcea was cultured in the glucose-asparagine medium. Cryptostroma corticale, A. pullulans and C. herbarum were maintained on Sabouraud's agar while T. thermophilum was cultured on potato dextrose agar.

Enzyme Studies

The enzymatic properties of B. cereus were investigated:

Phospholipase activity was indicated by the production of a clear halo surrounding the colonies when cultured on 'Oxoid' egg yolk emulsion (10%) dispersed in nutrient agar.

Proteinase activity was detected by the ability to digest autoclaved milk (10%) dispersed in nutrient agar. The proteolytic activity was demonstrated by flooding a test plate with 10% acetic acid. A positive result was indicated by the production of a clear zone around the colonies.

Lipase activity was demonstrated by the method of Jones and Richards (131). The cells were incubated for 4 days on glyceryl tributyrate (1%) dispersed in nutrient agar and the acid salt of Victoria Blue B, maintained at pH 7.8. Positive activity was indicated when a blue zone appeared in the pink agar which resulted from the absorption of the Victoria Blue B into the fat layer.

Morphological and Biochemical Characterisation.

Prior to injection, and after isolation from the excised tissue, the organisms were examined for purity. Streptomyces pelletieri, M. chalcea, T. vulgaris, S. coelicolor, and S. griseus were examined by the methods described in Bergey's Manual (118). T. saccharii was examined according to Lacey (24) and A. viscosus according to Howell et al (55). Cryptostroma corticale was examined according to Gregory and Waller (130). Cladosporium

herbarum and A. pullulans were examined on the light microscope.

Bacillus cereus was characterised on the basis^{of} Bergey's Manual (118) Burdon and Wende (116) and Wolf and Barker (117).

Preparation of suspensions.

One ml of a broth culture of M. chalybeata was used to inoculate 250 ml of glucose asparagine medium and a similar inoculum of A. viscosus and S. pelletieri was added to nutrient broth (250 ml). The inoculated flasks (A. viscosus, 24 h, S. pelletieri, 76 h, and M. chalybeata, 144 h) were shaken at 37°C for the indicated times. The cells were removed by centrifugation at 6,000 x g at 4°, washed four times with sterile normal saline solution and stored, if necessary, at 4° until required. Thermoactinomyces vulgaris and T. sacchari were added in two ml of nutrient broth to 200 ml of nutrient agar contained in a Roux flask and then incubated for 72 h at 55°. Cells were recovered with sterile distilled water and centrifuged at 1000 x g to remove any contaminating agar particles. The agar-free liquid was centrifuged four times at 6,000 x g and the washed cells stored in sterile normal saline until required.

The hyphae of T. thermophilum were obtained after culturing for 18 h on potato dextrose agar while the spores were washed off the agar surface after 90 h.

Cladosporium herbarum (3 days, 28°), A. pullulans (3 days, 30°) and C. corticale (vegetative cells : 30 h, 30° and spores : 4 days, 30°) were cultured on Sabouraud's agar under the conditions indicated in parenthesis. These cells were recovered and processed in the same manner as was T. sacchari and T. vulgaris.

Bacillus cereus SV-1 was cultured on nutrient agar at 37°. Vegetative cells were recovered after 18 h and spores after 8 days from an inoculum repeatedly subcultured on nutrient agar. The cells were processed in the same manner as the other cells which were recovered from an agar surface.

In all cases the deposited cells were resuspended prior to injection in sterile normal saline solution, using a glass tissue homogeniser (Jencons (Scientific) Ltd., Herts) and the cell density adjusted to give 10^3 cells/ml using a Thoma counting chamber. Filamentous cells were adjusted to Brown's Opacity Tube No.1 (Burrough's Wellcome & Co., London). For one suspension of T. sacchari which was used for subcutaneous injection, the suspension was adjusted to correspond to Opacity Tube No.9. The number of viable units/ml was also checked by carrying out pour plate counts before injection, in all cases this was around 10^3 /ml. The suspensions were also plated out and were found to be free from organisms other than the required species. Suspensions of killed cells were prepared by dividing the above suspensions adding sodium thiomersalate to give a final concentration of 0.01%^{w/v} and leaving for 72 h at 4° or by heating in an autoclave at 120° for 20 mins. These treated suspensions were used to inoculate the necessary medium which was incubated for the required time at the appropriate temperature. Examination of the inoculated medium revealed that the treated suspensions were sterile. The remaining viable suspensions were retained together with the killed suspensions for injection into the rabbits.

Experimental Animals and Injection Procedure.

Male Dutch RHS rabbits weighing 1.8 ± 0.2 kg were used throughout. When the intramuscular route was employed 0.2 or 0.5 ml of the required suspension were injected into the flank and for the subcutaneous route 0.3 ml. Rectal temperatures were recorded daily following injection. Rabbits were sacrificed at intervals of 7, 10 or 14 days and 6 months after injection.

Necropsy was performed and samples of kidney, liver, spleen, lung and any obviously infected tissue were removed and samples inoculated

onto the required solid media and onto the solid media containing 5% ^W/v of defibrinated blood. All the inoculated plates were incubated aerobically and in a N₂/CO₂ (95:5%) atmosphere at 37°. In addition plates inoculated with fungi were incubated at 28° and the T. vulgaris and T. sacchari were incubated at 55°.

Representative samples of the excised tissue were fixed immediately for 18-24 h in buffered-formal sublimate solution (saturated mercuric chloride solution : formalin, 9:1, ^V/v). The fixed tissue was washed repeatedly in distilled water and then repeatedly immersed in aqueous ethanol (50%) solution containing dissolved iodine. The tissue was judged to be free of mercuric chloride when the iodine solution was no longer decolourised. The tissue was stored in 70% ethanol and then processed on the Shandon-Elliot tissue processor as follows: absolute ethanol (3 x 1 h), paraffin (1 h) paraffin/chloroform 50:50, 1 h), paraffin wax (56°, 2 x 1 h) and embedded in wax.

Sections were cut at 5 µm thickness and stained with eosin haemoloxylin and by the Gram-stain (132). The von Kossa and the alizarin red S method were used to detect deposits of calcium (133). The periodic-acid-Schiffs-stain was used to detect fungi (106). Samples of pus were smeared and Gram-stained for fungi, bacteria and actinomycetes. In addition, fungi were detected with the PAS stain and with methylene blue. Samples of pus were diluted with saline and the pour plate technique used for determination of viable counts where necessary.

Artificial digestive juices (British Pharmacopoeia, 1973) were used to determine the sensitivity of A. viscosus and T. vulgaris to digestion along the alimentary tract. The organisms were suspended in artificial gastric juice, at 37°, agitated and samples removed at appropriate periods and plated out on nutrient-agar and then incubated at 37°. After 3 h the artificial gastric juice was removed by centrifugation and the deposited cells resuspended, washed with

sterile distilled water, resuspended in artificial pancreatic juice and their viability assessed after suitable time intervals. In addition, these cells were also suspended in reconstituted rumen fluid (a gift from Dr. M. Elizabeth Sharp, N.I.R.D., Reading) and the viability examined. Organisms were suspended in sterile distilled water or sterile normal saline and used as controls.

Antibiotic Sensitivity.

The actinomycetes were examined for susceptibility to the chemotherapeutic agents which are normally active against the typical bacteria. The sensitivity was examined using the 'Multodisk' (Oxoid) method. Other antibiotics, not covered by the commercially available discs, namely, clindamycin, lincomycin (a gift from Upjohn Ltd., Crawley, Sussex) and sodium fusidate (a gift from Leo Laboratories, Hayes, Middlesex) were examined using the filter paper disc method, at a concentration of 10 µg per disc. Since the A. viscosus strain appeared to be resistant to penicillin G its sensitivity to some semi-synthetic penicillins was determined. Methicillin and ampicillin were donated by Beecham Research Labs., Brentford and quinacillin was donated by The Boots Co. Ltd., Nottingham. The seeded nutrient agar plates were incubated at the optimum temperature for growth for 24 h and the resulting zones of inhibition were measured.

Preparation of Antisera.

Cell suspensions (Brown's Opacity Tube No.1) of M. chalcea, T. vulgaris and A. viscosus were prepared from the thiomersalate treated cells and aliquots (1 ml) were injected into each flank of a rabbit. Micromonospora chalcea vegetative cells were used in addition to a suspension of cells which consisted principally of spores. The spores were obtained from a nutrient agar surface culture which had been incubated for 18 days at 30°. Thermoactinomyces vulgaris vegetative

cells were obtained from a 20 h culture while the spores were obtained from a 100 h culture. Ten days later the process was repeated. The regime was repeated on four subsequent occasions at intervals of seven days. Blood (25 ml) was collected from the ear. At first the blood was collected in a polystyrene tube but subsequent experience showed that lysis could be suppressed by collecting the blood in a glass tube. After collection sodium thiomersalate (0.04%) was added to the tube and the contents incubated at 37° for 90 mins. The clotted blood was freed from the surface of the tube with a fine stick and the tube and its contents kept at 4° for 18 h. The pale orange serum was obtained by centrifuging the tube for 5 mins at 300 x g. The serum was divided into several portions and lyophilised. Serum was also prepared against the vegetative cells of Thielavia thermophilum and against a preparation which was predominantly spores.

Isolation of Antigens.

Pyridine-acetic acid buffer (pH 5.0) was stirred for 48 h at 18° with the spore and vegetative cell preparation of the following: T. vulgaris, M. chalcea, T. thermophilum and C. corticale. Antigens were also extracted from A. viscosus. All cell preparations were prepared under conditions identical to those used to prepare the samples for serum production.

Immunodiffusion Studies.

Ouchterlony's double diffusion technique was used (134, 135). Preliminary studies indicated the unsuitability of a gel prepared from 'Oxoid ionagar' No.2 (0.85%^w/v in 0.85% NaCl) since an extensive halo which surrounded the antiserum well masked the precipitin lines. The deposition of the lipoprotein halo was satisfactorily suppressed by using a gel preparation modified as follows:

Ionagar No.2 (0.7%), glycine (1M), sodium barbital (M/25) and sodium

chloride (0.85%), pH 7.4 (136). The cutters (Shandon) produced wells with a capacity of around 0.06 ml. The loaded gels were allowed to diffuse for 18 h at 18°. In many cases the precipitin lines observed were very close to the antiserum well. Attempts were made to modify the point of deposition of the precipitin by allowing the antiserum to diffuse for various periods of time before addition of the antigen but these were unsuccessful since the precipitin lines were too faint to photograph in the normal state a variety of protein stains were used. However, these stains were abandoned since they stained an extensive area surrounding the antiserum well. No suitable de-staining procedure could be found (137).

The methylene-blue reaction of McFadyean (133) was utilised to confirm that the organism used was B. cereus and not B. cereus var. anthracis. The material so examined was obtained from the abscesses and blood films from infected animals, and from the organism growing on blood agar.

Isolation of a Polymer from the Culture Media of Actinomyces viscosus WVU 398B

Actinomyces viscosus was cultured in tryptone soya broth plus sucrose (2%) for 60 h at 37° in 10 l. flasks aerated at 1 l. per min. The broth (10 l) was centrifuged at 2,500 x g for 30 mins. Quantities of 2 litres were concentrated in vacuo at 65° to give a viscous brown fluid. The concentrates were bulked and dialysed for five days at 15°. No growth was observed in the dialysis sack during this period. The contents of the dialysis tube were stirred overnight with chloroform-butanol (4:1, ^v/v, 100 ml) to precipitate the protein. The recovered aqueous layer was again stirred with chloroform-butanol for 2 h to ensure complete loss of protein. The recovered aqueous layer was centrifuged and stored overnight at pH 9.0. The flocculent precipitate was recovered by decantation and centrifugation. Addition of acetone to the liquor did not produce any more precipitate and was

subsequently discarded. The precipitate was dissolved in the minimum volume of 1N hydrochloric acid and the alkaline precipitation repeated. This process was repeated three times and the final precipitation was repeated by adjusting the solution to pH 7.5 followed by the addition of acetone until the solution become opalescent. The solution was stored at 6° for 48 h. The recovered solution produced no further precipitate on addition of further alkali and was discarded. The grey precipitate was dissolved in dilute hydrochloric acid after it had been washed with aqueous acetone. The acid solution was dialysed for five days against twice daily changes of distilled water (4 l). The solution was lyophilised to yield a browish-white solid (1.5 g). Previous attempts to isolate this substance produced a gum on lyophilisation which could not be dehydrated any further.

Structural Analysis.

The polymer (10 mg) was hydrolysed in a sealed tube at 100° for 2.5 h with 2N HCl (3 ml). The hydrolysate was lyophilised and dissolved in the minimum amount of distilled water and applied to a paper chromatogram (Whatman No.1) and developed by descending elution (138). When dry the chromatogram was examined for polyols, hexoses and hexosamines.

The action of alkali was investigated by heating the polymer (10 mg) in 1N sodium hydroxide (20 ml) at 100° in a micro reflux flask agitated with N₂. The solute was passed through Dowex-50 (NH₄⁺) and the eluate lyophilised to give a brown-orange residue (26%). The alkali-insoluble fraction was insoluble in acid and could not be further resolved. The lyophilisate was applied to a paper chromatogram and developed by descending elution (138) and when dry it was sprayed with periodate-Schiff's spray (139). The acid hydrolysate was examined on a paper chromatogram for the presence of phosphoric esters (140, 141).

Infrared spectroscopy was performed in a potassium bromide disc on an Unicam SP200. The ultraviolet absorption spectrum was examined on a 0.1N hydrochloric acid solution with a Pye Unicam SP8000. The optical rotation was determined on a 0.1N hydrochloric acid solution with a Bendix polarimeter.

The total carbohydrate content was determined by the phenol-sulphuric acid method (142).

The acid-soluble polymer (100 mg) was examined by chromatography on a column (25 x 2.5 cm) of water expanded DEAE-cellulose-32 (Whatman). The polymer was eluted with water (500 ml) and 0.1N hydrochloric acid (500 ml) which were mixed so as to produce an increase in acidity with time. The eluate was collected in fractions of 5 ml.

Periodate oxidation of the polymer (200 mg) was achieved by stirring it in the absence of light at 20° for 4 days in acetate buffer (100 ml, pH 4.0) which contained sodium metaperiodate (0.5 mg). Excess periodate was destroyed by the addition of excess sodium borohydride and after reduction for 24 h acetic acid was added dropwise (4-5 drops) and the solution passed through Dowex-50 (H⁺) resin (8 ml). The eluate (50 ml) was evaporated in vacuo and the residue evaporated with methanol (3 times) on a hot plate. After no more than a few drops (4-5) had been evaporated the residue was partitioned: one fraction was acid hydrolysed (2N HCl, 100° 2 h) and examined by paper chromatography after lyophilisation while the other fraction was examined by the Ouchterlony immunodiffusion technique against the antisera produced by injecting A. viscosus cells into rabbits.

Barium chloride (2N) solution was added to an acidified solution of the polymer (2 mg/ml) to determine the presence of any group, such as sulphate, which possibly contributed to the solubility properties.

Methanolic hydrogen chloride solution (0.15 M) was added to the polymer and the solution (2 mg/ml) stirred for 48 h at 20°. The

recovered hydrolysate was examined for serological activity.

The polymer (2 mg/ml) was dissolved in acetate buffer (pH 4.6) and digested with "glusulase" (Endo Laboratories, Inc., N.Y.). The protein digestion solution (0.2 ml of "glusulase" per 5 ml of buffer) was stirred with chloroform-butanol at the end of 18 h to remove the protein. Glucosidase and cellulase (both from Koch-Light) were used in quantities and at the same pH as in the "glusulase" experiment.

A sterile aqueous suspension (2 ml) of the polymer (25 mg/ml) was injected intramuscularly and subcutaneously into a rabbit which was subjected to a post mortem examination after seven days.

RESULTS

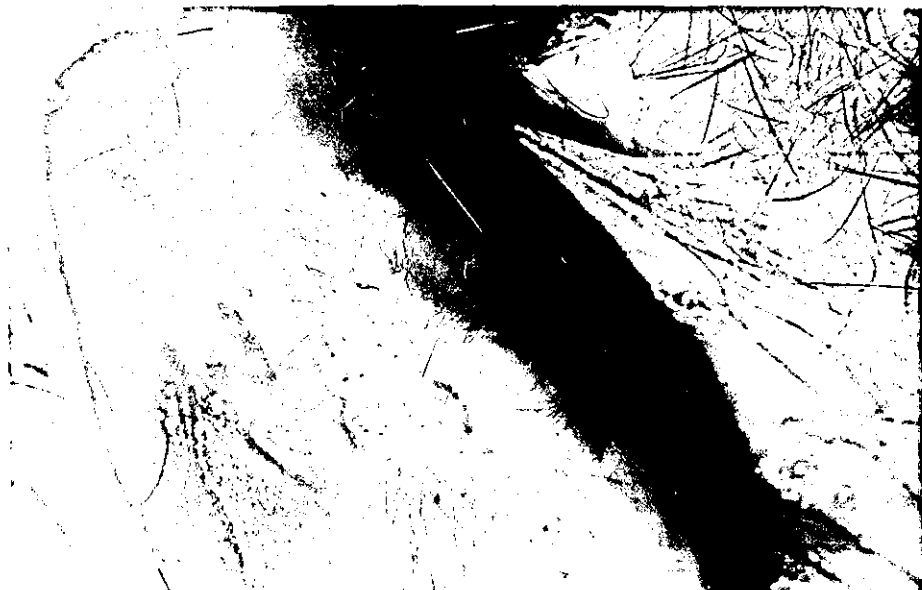
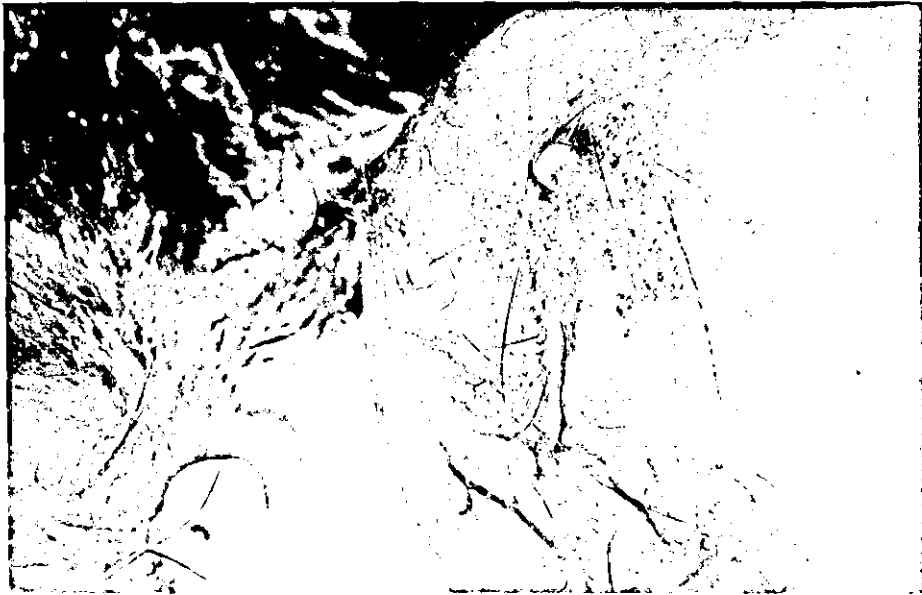


Plate 1. - Nodules produced on the lateral aspect of the hamstring muscles 7 days after a single i.m. injection of M. chalcea x 2.

Plate 2. - A subcutaneous nodule at 14 days, produced by injection of T. vulgaris and showing the increased vascularity of the cutaneous vessels x 2.

Plate 3. - A nodule produced within 7 days after s.c. injection of A. viscosus. It occurred in the muscle of the quadriceps femoris block x 2.



- Plate 4. - A nodule occurring on m. adductor magnus produced within 7 days after i.m. injection of A. viscosus x 2.
- Plate 5. - An intramuscular nodule due to M. chalcea observed after 7 days, occurring on m. adductor magnus x 2.
- Plate 6. - A nodule which occurred on m. adductor magnus following i.m. injection of 0.5 ml of S. pelletieri x 1.5.

Actinomycetes.

All the organisms grew well on the media except T. sacchari which gave moderate growth on nutrient agar. Thermoactinomyces vulgaris gave good growth at 55° but only moderate at 37°.

Seven days after intramuscular or subcutaneous injection it was possible to feel small nodules beneath the skin and these persisted for several weeks - in 80% of the cases they persisted for six months. The infection caused no apparent discomfort, except for a slight sensitivity in some cases when the nodules were probed. There was no detectable rise in body temperature, except for T. vulgaris where a slight rise persisted for 48 h.

Necropsy revealed abscesses just beneath the skin or on the muscle and in many cases in the spaces between the muscle blocks. A frequent site of infection was the space surrounding the sciatic nerve. In many cases the area of infection was extensive and appeared avascular, except at the periphery which exhibited extensive vascularity (Plates 1 to 6). With the exception of S. pelletieri, the pus was white to pale yellow and had a granular cheese-like consistency. Abscesses infected by S. pelletieri contained a liquid but viscous pus which was yellow with pink granules dispersed through it. Infections due to S. pelletieri were much more wide-spread than the other ones. Intramuscular injection of S. pelletieri produced extensive spreading involving the regional lymph nodes of the abdominal wall. This condition was not observed in any of the other animals infected with other organisms. Injection of heat or chemically killed cells produced no similar abscesses or histological change.

The site of injection of the killed organisms was without any reaction. Examination of the excised tissue exudates showed that no

organisms other than those injected could be observed. In all cases there was a very low number of viable units, the maximum was estimated by pour plate at 35 per ml for the S. pelletieri.

Subcutaneous injection of M. chalcea produced small nodules (0.5 cm in diameter) while intramuscular injection produced much larger nodules (1.5 x 1.0 cm) which were seen frequently on the m. adductor magnus (Plate 5). On some occasions nodules were located on the lateral part of the hamstring block in an area which became markedly hyperaemic. Infections due to T. vulgaris were similar to the M. chalcea ones, although there were many more nodules (Plate 2). Similar results were obtained for T. sacchari. In addition, there was some evidence of spreading and invasion of the muscle to a depth of ca 1 cm in several instances following subcutaneous injection.

Actinomyces viscosus produced infections of a slightly different character. Subcutaneous injection produced lesions within ten days of the initial injection at sites which were approximately 3 cm away from the injection point (Plate 3). Other infected sites were located 5 cm away on the surface of and penetrating the intermediate quadriceps block. This phenomenon was observed in several cases and it would suggest that the organism had some invasive properties. Intramuscular injection produced nodules at the site of injection and on the m. adductor magnus (Plate 4) in contact with the sciatic nerve. Similar nodules were observed for T. vulgaris, T. sacchari and M. chalcea (Plates 4, 5 and 6). As expected S. griseus produced no infected sites. In addition, S. coelicolor which can reproduce at 37° did not produce any infected sites.

The post mortem examination showed that the lungs, kidney, liver and spleen had no recognisable evidence of infection. The histopathological examination of the excised organs revealed the absence of any actinomyceete.

The inoculated blood-agar plates were examined every 8 h. An abundant and rapid growth was observed when the plates were incubated in the N_2/CO_2 atmosphere. The only organisms recovered were those injected initially. These re-isolated organisms were identical with the original species in terms of morphology and biochemical properties. In all cases the excised organs showed the absence of any of the organisms under investigation.

The growth of M. chalcea after recovery from infected animals was quite variable. On some occasions abundant growth was seen on the plate within 36 h, while on other occasions no growth was observed for 72 h. Extensive haemolysis, after animal passage, was apparent even at the micro-colony stage for M. chalcea, T. vulgaris and T. sacchari (Plate 18). Even after animal passage S. pelletieri remained non-haemolytic. The haemolytic properties of the other four actinomycetes decreased rapidly with subsequent subculturing on blood agar plates until they were judged to be non haemolytic like the parent species. Thermoactinomyces vulgaris, on isolation, gave rapid and abundant growth at 55° after 12 h incubation. At 37° growth was abundant but required 36 h to reach a level comparable to that obtained at 55°. Extensive haemolysis was observed at both temperatures. Thermoactinomyces sacchari exhibited similar behaviour.

Re-isolation of A. viscosus was more difficult since two out of three subcultures from nodules failed to produce colonies. Where growth was obtained it was very rapid, for example, from a point inoculation onto an overdried blood-agar plate 24 cm² were covered after 36 h incubation in the N_2/CO_2 atmosphere. The organism was without haemolytic action.

Microscopic examination of the pus smears revealed the presence of all five actinomycetes including a few branched forms. However, the

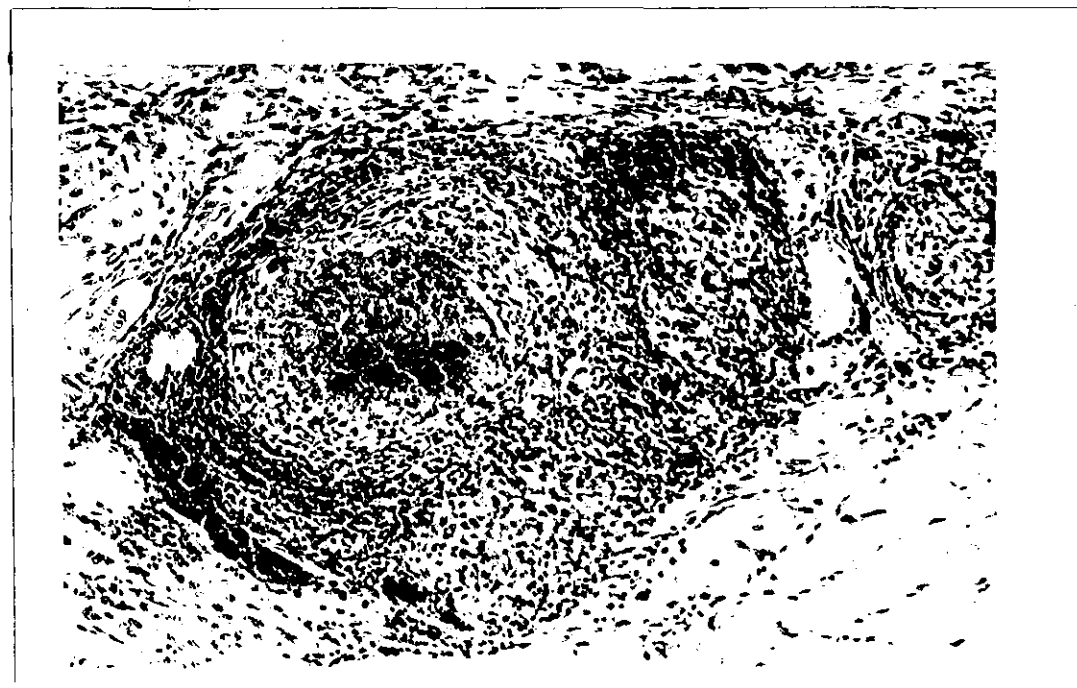
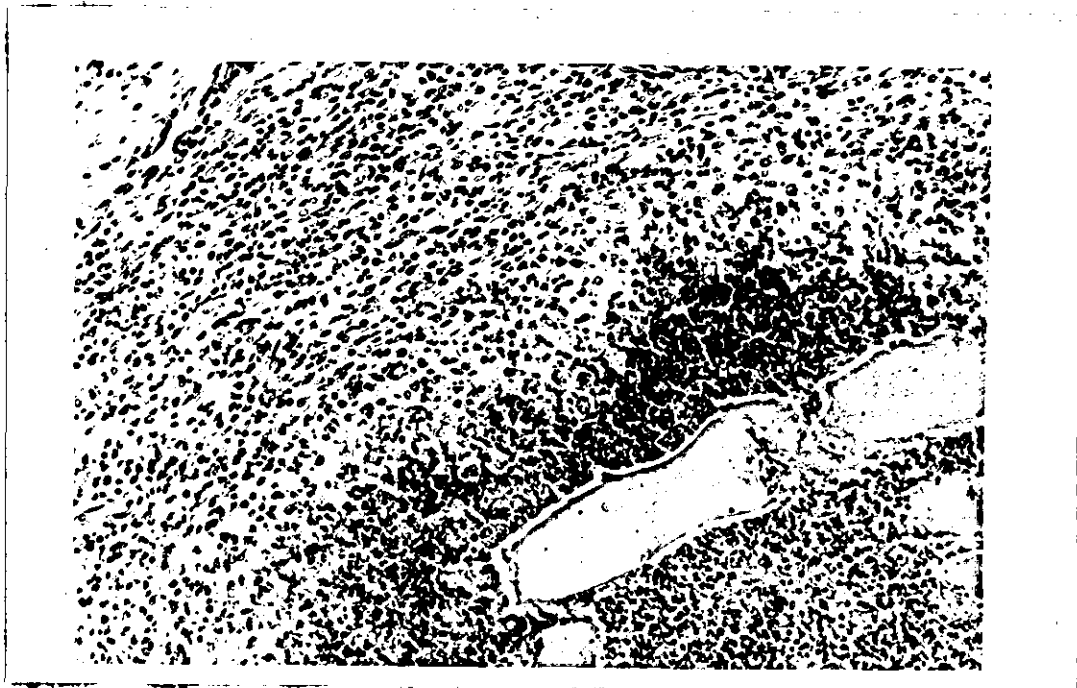
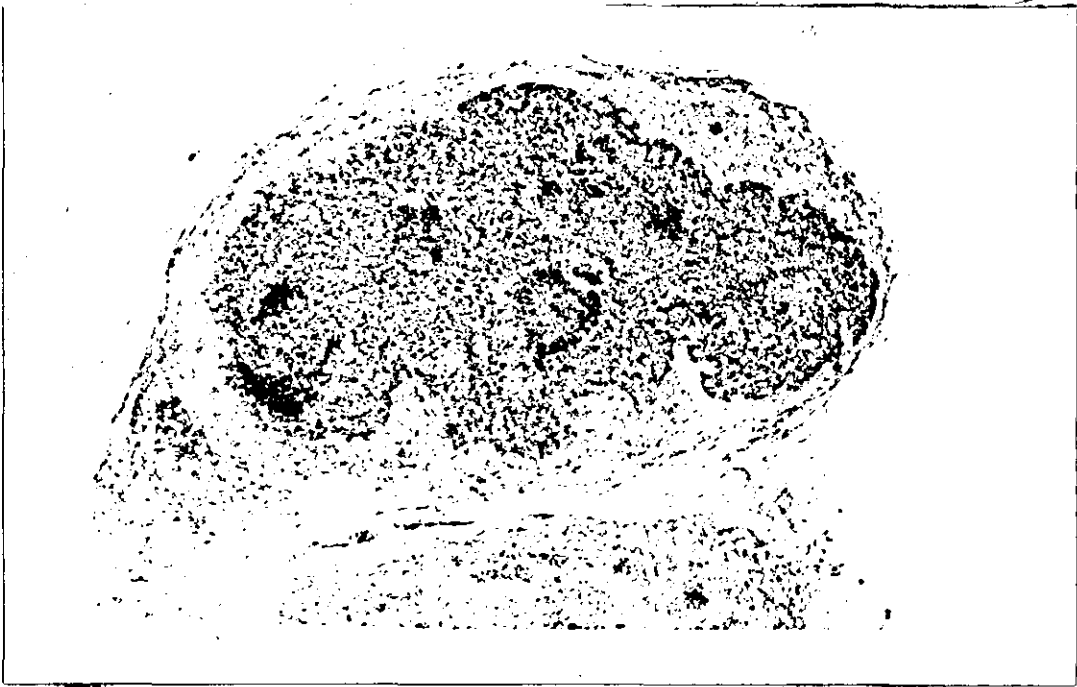


Plate 7. - Microabscess of M. chalcea in muscle nodule from infected rabbit. H & E stain x 50.

Plate 8. - A typical actinomycotic granule induced by A. viscosus. Fibrosis is apparent around the nodule periphery. H & E stain x 16.

Plate 9. - Microabscess of A. viscosus in rabbit muscle. H & E stain x 125.

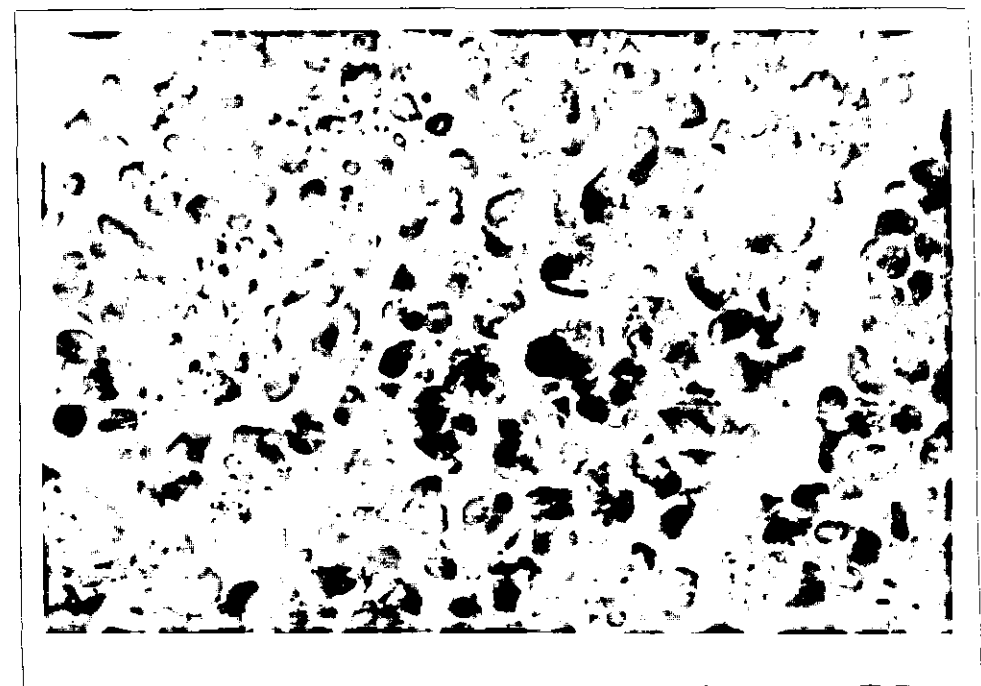
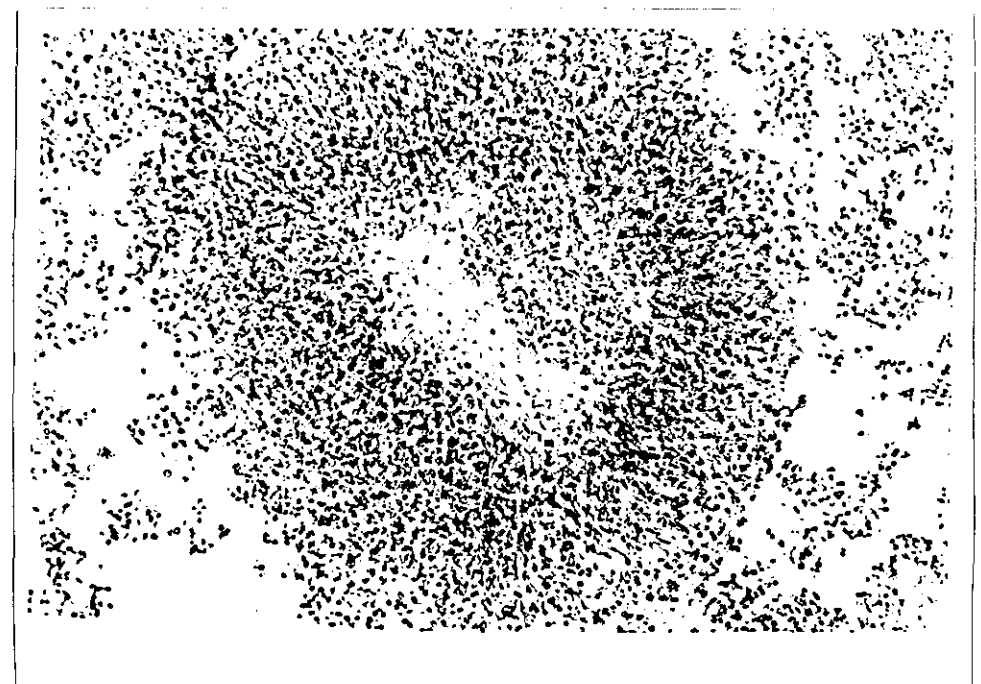
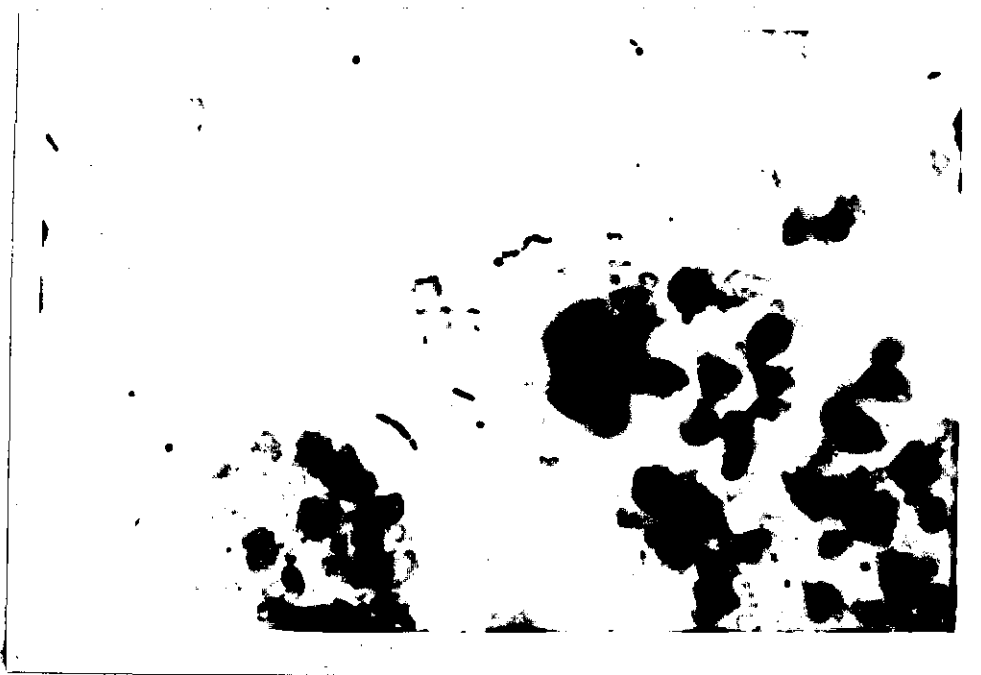


Plate 10. - Coccoid and diphtheroid forms in granule of M. chalcea, from muscle nodule. Gram stain x 1,250.

Plate 11. - Granule of M. chalcea from muscle nodule, typical actinomycotic sulphur granule. H & E stain x 200.

Plate 12. - Coccoid and diphtheroid forms in granule of A. viscosus. Gram stain x 1,250.

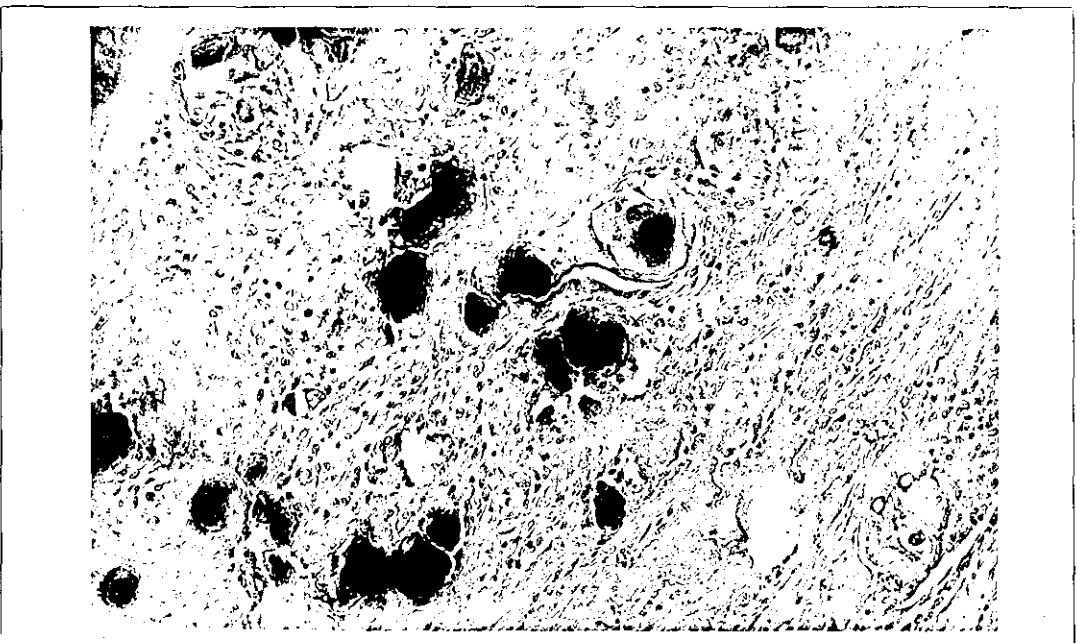
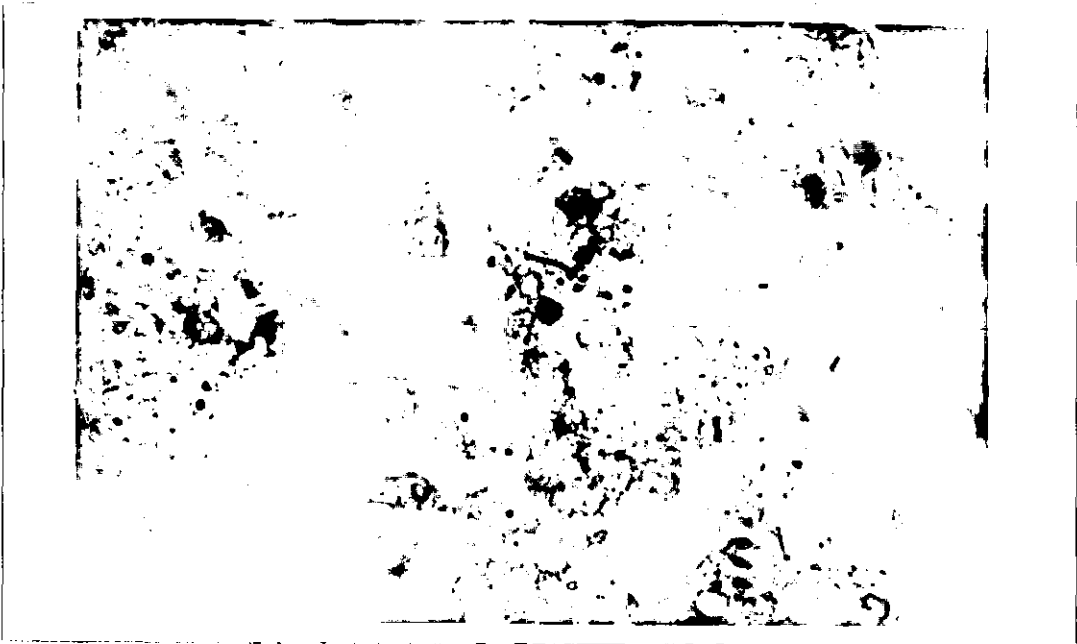
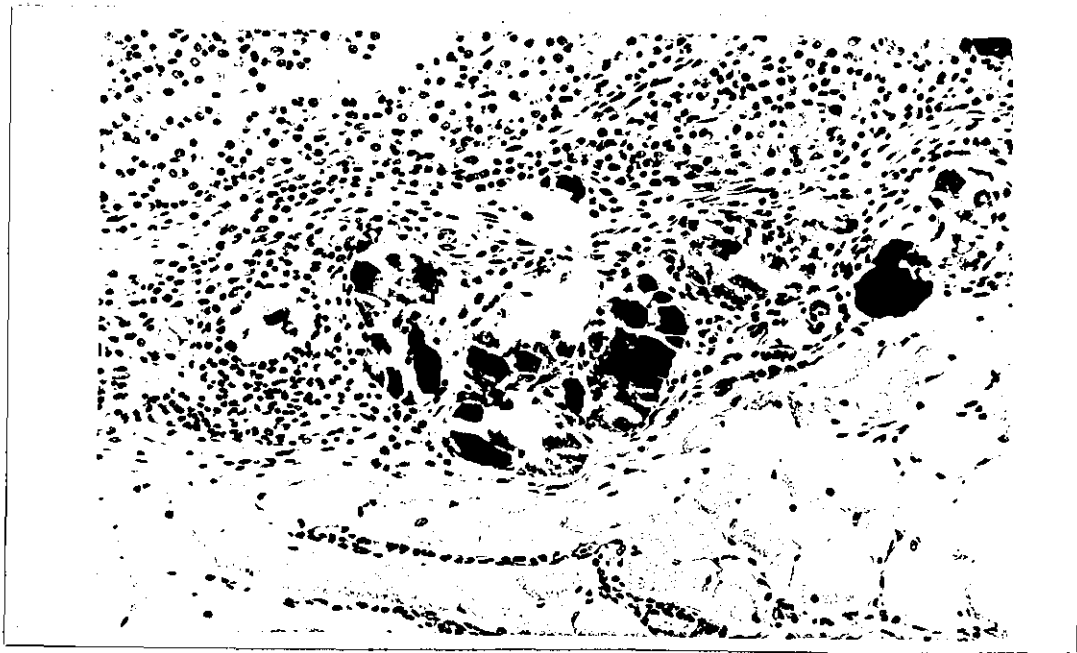


Plate 13. - Microabscess of S. pelletieri in muscle nodule from experimentally infected rabbit. H & E stain x 125.

Plate 14. - Diphtheroid and coccoid forms in a granule of S. pelletieri from muscle nodule. Gram stain x 1,250.

Plate 15. - Calcium in nodule of A. viscosus. Alizarin red S stain x 200.

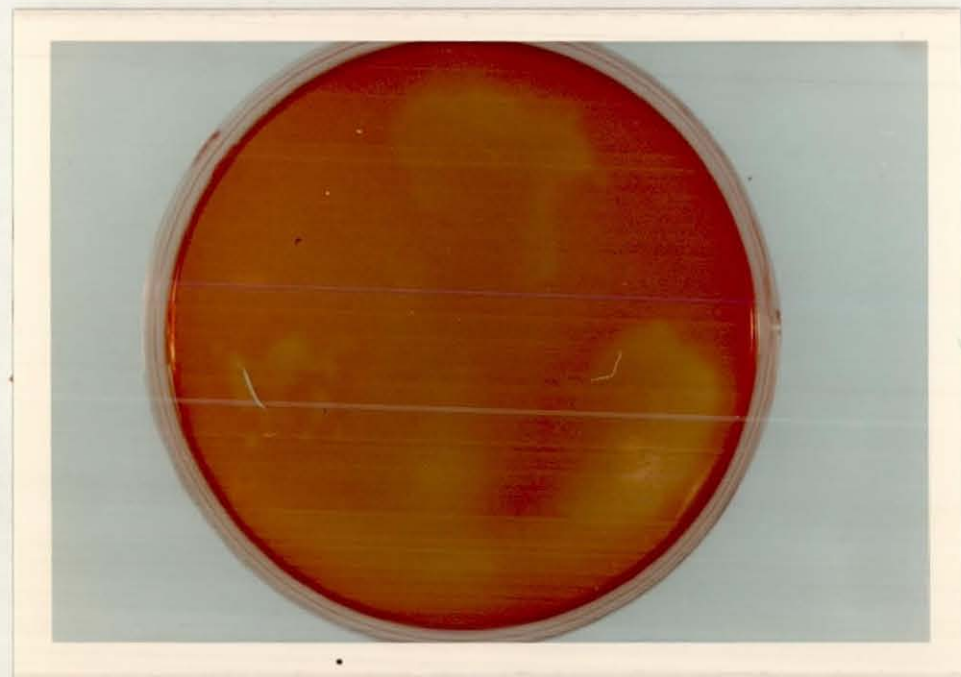


Plate 16. - Calcium in necrotic muscle fibre, M. chalcea infection. Alizarin red S stain x 200.

Plate 17. - Calcium in nodule of S. pelletieri Von Kossa-stain x 125.

Plate 18. - Haemolysis of blood agar plate with T. vulgaris at the microcolony stage.

Table 2.

Sensitivity of Actinomycetes to Chemotherapeutic Agents

| Organism | Diameter of the zone of inhibition in mm. | | | | | | | | |
|----------------------|---|------------------------|------------------------|--------------------------------|-------------------------------|------------------------|-----------------------------|--------------------------|-----------------------|
| | Penicillin* 1.5 units | Streptomycin* 10 µg | Tetracycline* 10 µg | Chloram- phenicol* 10 µg | Sulpha- furazole* 10 µg | Erythromycin* 10 µg | Clinda- mycin** 10 µg | Fusidic acid 10 µg | Lincomycin** 10 µg |
| <i>M. chalcea</i> | 14 | 18 | 34 | 20 | 30 | 30 | 42 | 30 | 30 |
| <i>T. vulgaris</i> | 34 | 28 | 34 | 26 | 22 | 32 | 24 | 22 | 14 |
| <i>T. sacchari</i> | 0 | 28 | 32 | 16 | 22 | 28 | 26 | 20 | 24 |
| <i>A. viscosus</i> | 0 | 32 | 14 | 24 | 0 | 38 | 31 | 25 | 21 |
| <i>S. pelletieri</i> | 0 | 0 | 29 | 20 | 10 | 21 | 39 | 34 | 35 |

*Oxoid Multodisks

**Filter paper discs (Whatman, 13 mm).

incidence was very low. The histopathological investigation showed that typical actinomycotic granules, surrounded by polymorphonuclear leucocytes (Plates 7, 8, 9 & 11) differing from the characteristic granule form of S. pelletieri (Plate 13) and with the actinomycetes penetrating between the muscle fibres (Plates 7 & 11). There was no obvious degradation although some necrotic fibres were observed (Plate 11) together with some fibrosis surrounding the nodule periphery (Plates 7 & 11). The test actinomycetes were present mainly in the diphtheroid form, (Plates 10 & 12) as was S. pelletieri (Plate 14), although some filamentous and coccoid forms were seen occasionally. Similar morphological forms were also characteristic of nodules produced by injection of spore suspensions.

Thermoactinomyces vulgaris and T. sacchari showed a similar histological appearance to those presented in the plates. No histological changes were observed at the sites of injection of the heat or chemically killed cells.

Calcium deposition was observed in the actinomycotic nodules (Plates 15, 16 & 17) and within the necrotic muscle fibres (Plate 16). Calcium deposits surrounded the S. pelletieri granule (Plate 17). Both the von Kossa and alizarin red S methods showed a similar deposition of calcium.

Exposure of A. viscosus and T. vulgaris to the artificial gastric juice or reconstituted rumen fluid resulted in viable numbers which were comparable with the sterilised saline controls. There was a decrease in viability when the cells were transferred from the gastric juice to the pancreatic juice.

The in vitro sensitivity tests (Table 2) showed that the antibiotics clindamycin, erythromycin and tetracycline were effective inhibitory agents.

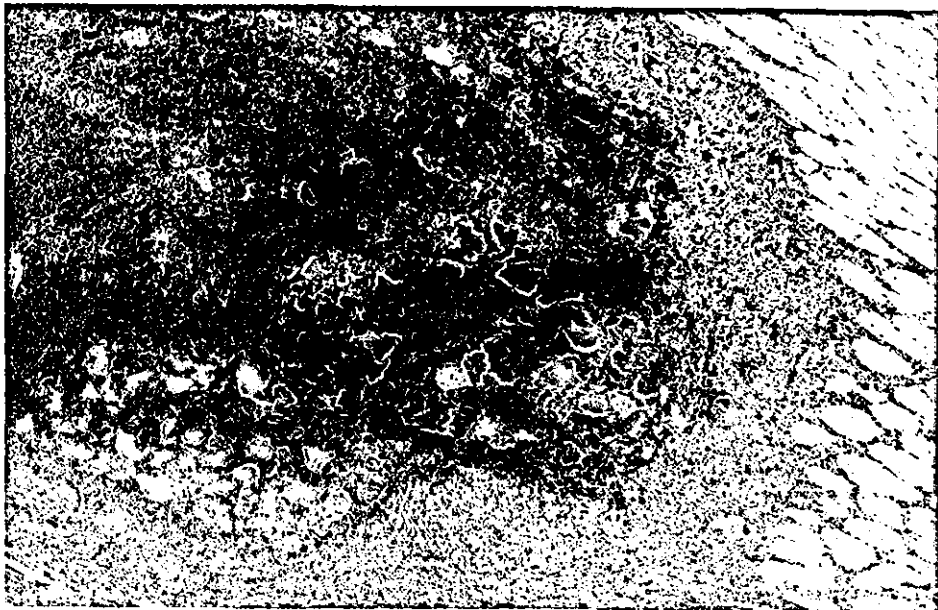


Plate 19. - Nodules produced after 7 days following i.m. infection with A. pullulans as a spore suspension located on the m. semimembranosus x 1.

Plate 20. - Nodules produced after 7 days following i.m. infection with C. corticale as a spore suspension, located on the m. semimembranosus x 0.5.

Plate 21. - Nodule produced by A. pullulans after 7 days in the m. semimembranosus showing necrosis of muscle fibres and limited fibrosis. H & E x 400.

Penicillin G was without useful action against T. sacchari, A. viscosus and S. pelletieri. The semi-synthetic penicillins, quinacillin, methicillin and ampicillin were without action at 10 µg and 100 µg per disc on these strains of T. sacchari and A. viscosus, although at 1,000 µg per disc a zone of inhibition of 22 mm was produced. Sulphafurazole was not particularly effective against any organism.

Fungi

Animals injected with all three fungi exhibited no discomfort on examination and the rectal temperature remained unchanged.

At necropsy nodules were found for only C. corticale and A. pullulans following injection of either hyphae or spores. In the case of A. pullulans these nodules were always small and were located in the m. semimembranosus (Plate 19). The periphery of the nodule was avascular.

In the case of C. corticale larger nodules ca 3.0 cm were observed and were located typically on the m. semimembranosus (Plate 20). In contrast to the A. pullulans these nodules exhibited considerable vascularity. Nodules were produced when either hyphae or spores were injected in 80% of the animals by either the subcutaneous or intramuscular routes.

The organisms exhibited no evidence of spreading and the post mortem examination showed the absence of nodules from the kidney, liver and spleen, nor could the organisms be recovered from these organs.

In all cases where killed cells were injected it was impossible to find any nodules, even at the site of the injection. The histopathological examination confirmed these findings.

The only organisms which could be recovered from the pus were those injected initially, as judged by morphology and colonial appearance. The pus from C. corticale contained approximately 100 viable units/ml while A. pullulans contained approximately 50 units/ml.

The histopathological examination showed the presence of abscesses showing a typical inflammatory response. Both organisms exhibited evidence

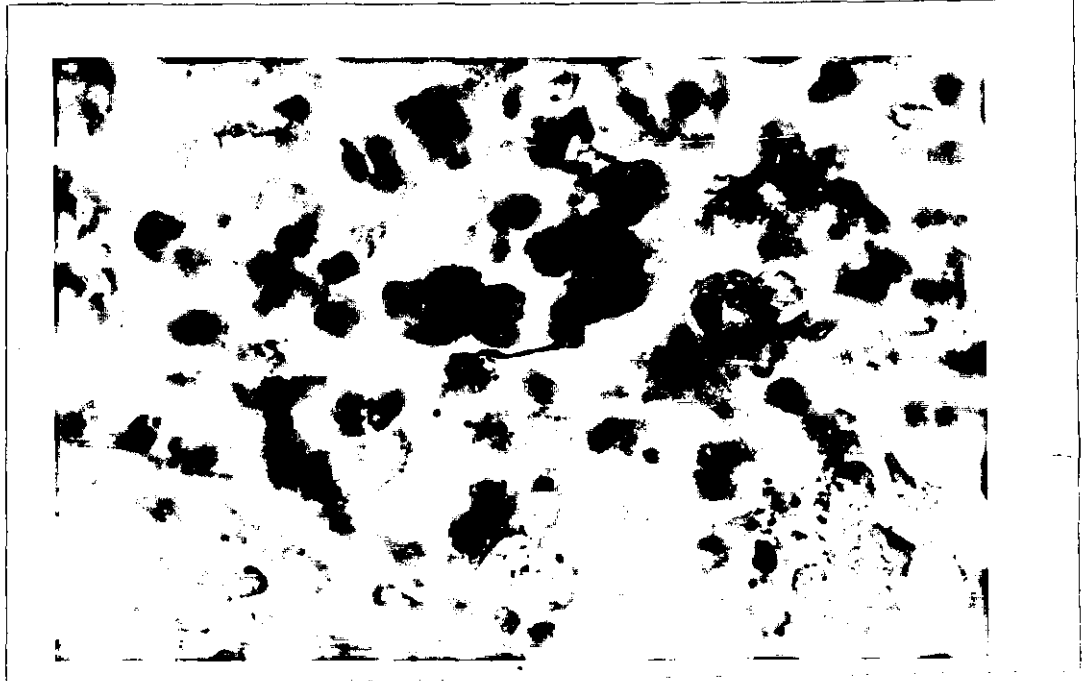
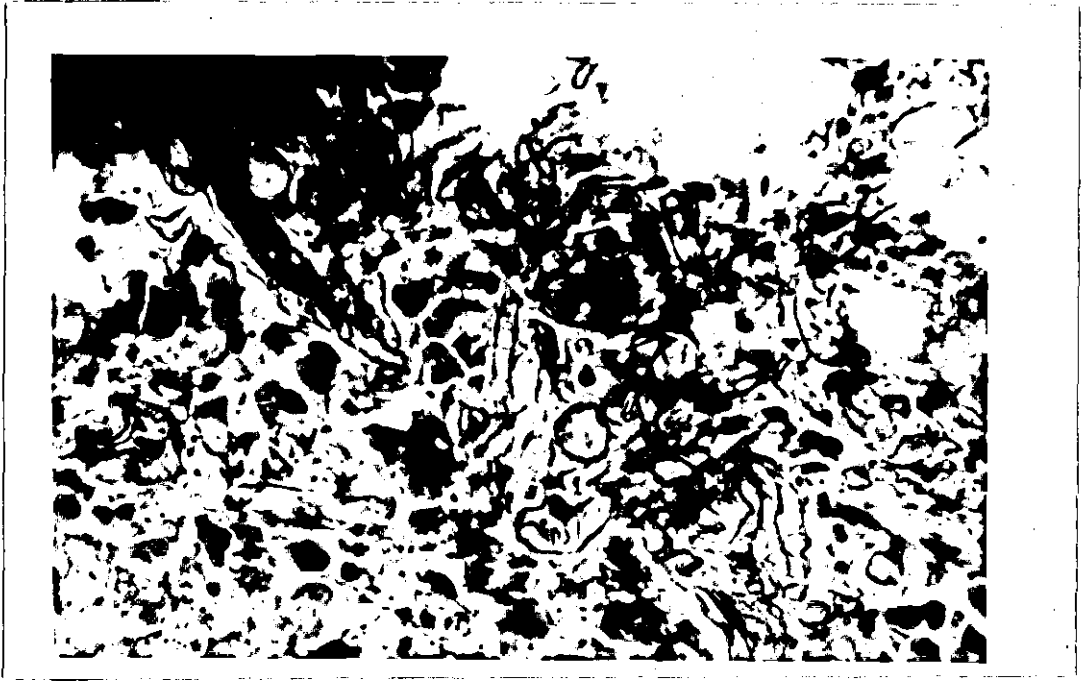
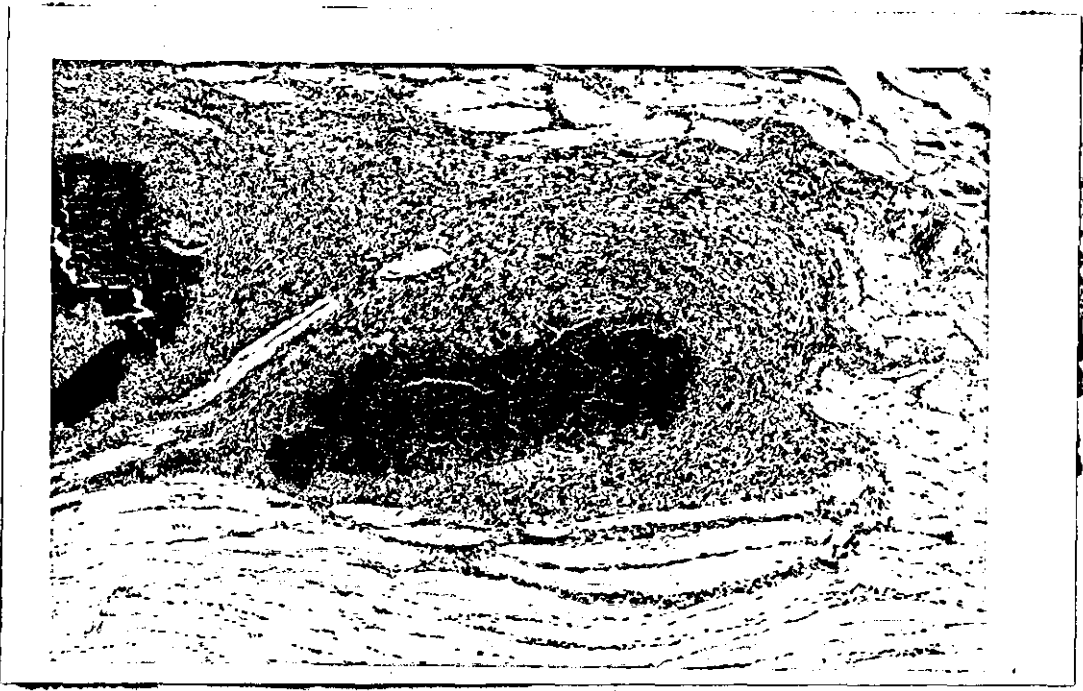


Plate 22. - Nodule produced by C. corticale after 7 days in the m. semimembranosus showing necrotic muscle fibres, fibrosis and inflammatory response. H & E x 160.

Plate 23. - Hyphae and spores present in module produced by C. corticale. PAS x 1,250.

Plate 24. - Short hyphae present in nodule produced by A. pullullans. Gram x 1,250.

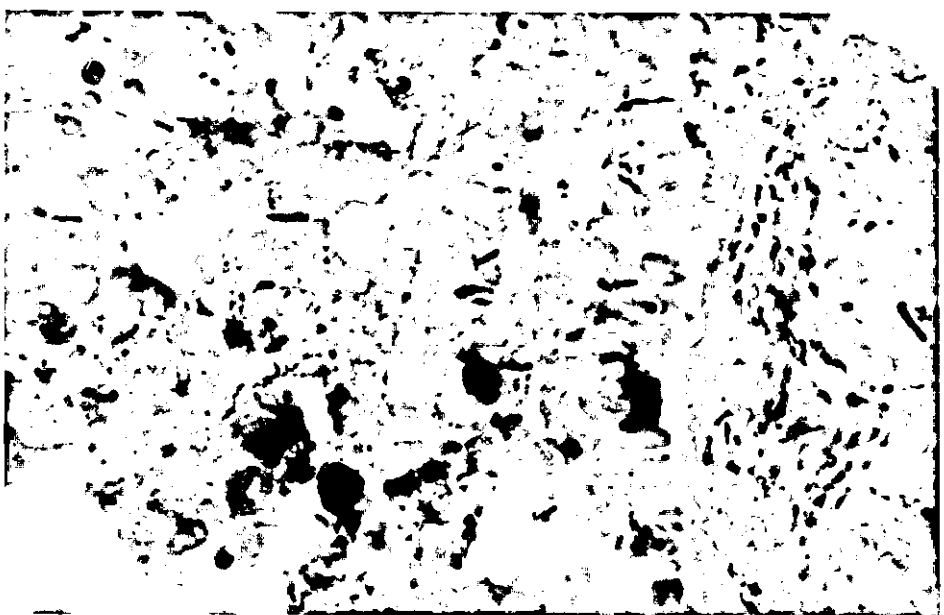
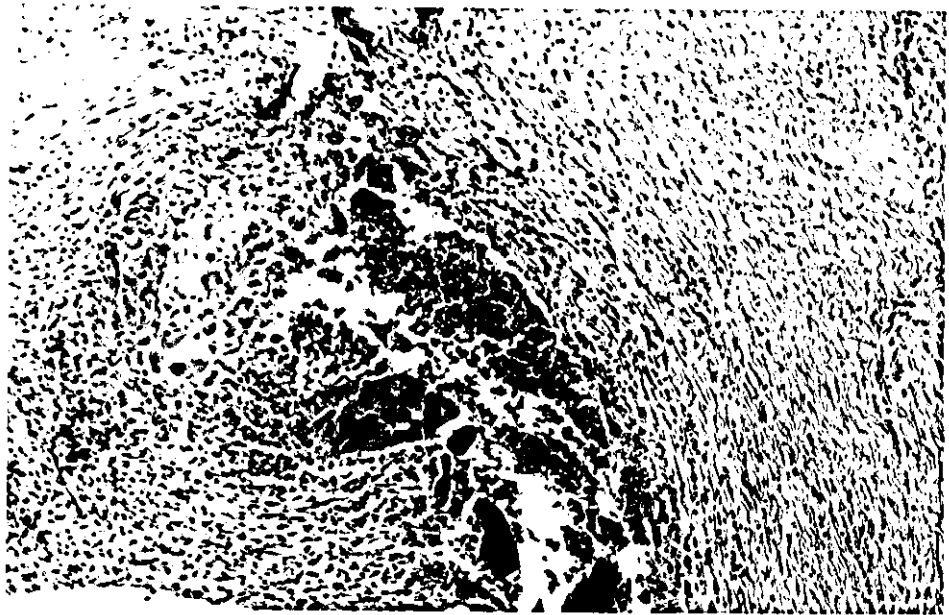


Plate 25. - Nodule produced after 7 days on the m. adductor magnus; following intramuscular injection of B. cereus SV-1 spores; natural size.

Plate 26. - Nodule of B. cereus SV-1 infection showing inflammation and fibrosis. H & E x 100.

Plate 27. - Gram stain nodule of i.m. infection due to B. cereus SV-1 x 1,000.

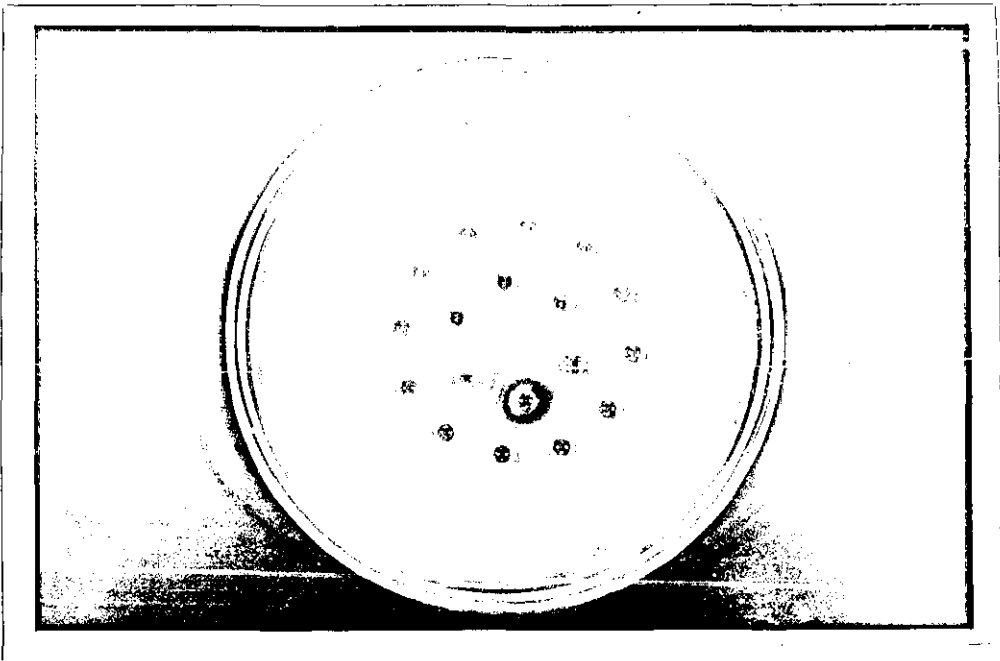
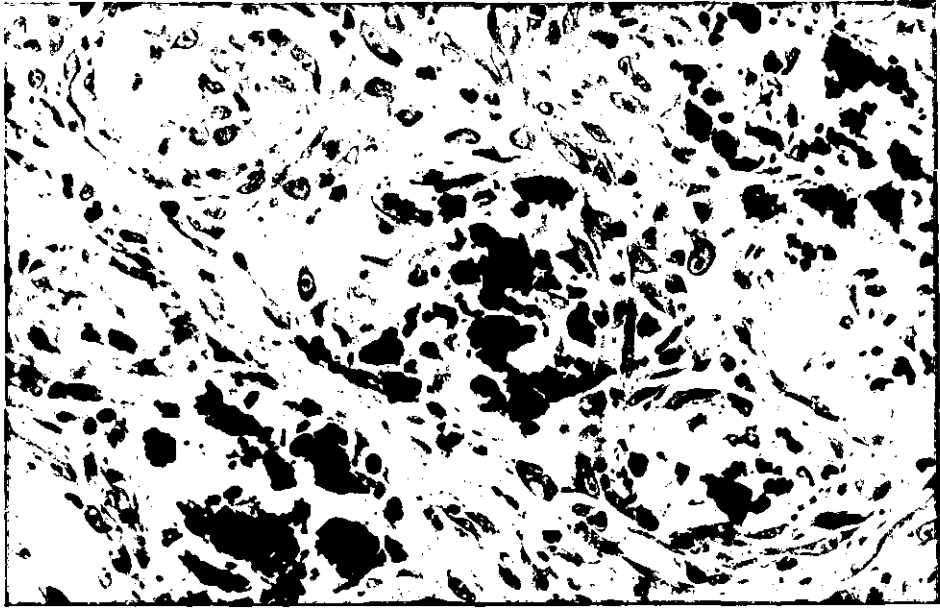


Plate 28. - Nodule produced by i.m. injection of B. cereus SV-1 with the onset of necrosis of muscle fibres and calcification. Alizarin red S x 400.

Plate 29. - Precipitin reaction between antiserum prepared against A. viscosus and the periodate oxidized polymer obtained from culture medium of A. viscosus.

Table 3.

| Antibacterial Agent ($\mu\text{g}/\text{disc}$). | Diameter of zone of inhibition - mm. |
|---|--------------------------------------|
| Penicillin G (1.5 units) | 0 |
| Sulphafurazole (100) | 0 |
| Streptomycin (10) | 0 |
| Erythromycin (10) | 24 |
| Chloramphenicol (10) | 19 |
| Tetracycline (10) | 15 |
| Clindamycin (50) | 41 |
| Fusidic acid (50) | 35 |
| Chlorhexidine (50) | 28 |
| Vancomycin (50) | 28 |

In vitro sensitivity of Bacillus cereus SV1 to antibacterial agents.

The organisms responsible for the infection were present in large numbers and exhibited pleomorphism (Plate 27). Calcification was observed (Plate 28) in 80% of the animals after the infection had progressed for 7 days, but was not seen after 3 days.

The strain of B. cereus used was insensitive to penicillin G, sulphafurazole and streptomycin (Table 3) but was sensitive to the other antibacterial agents examined, clindamycin, in vitro being very affective.

The enzyme studies indicated the absence of phospholipase and lipase but indicated the presence of large quantities of proteolytic activity.

MacFadyean's stain showed the absence of any methylene blue staining material.

Polymer

Infra-red spectroscopy indicated the absence of any carbon-hydrogen bonds and failed to detect any other functional groups. The ultra-violet absorption spectrum showed that the polymer solution (17 mg/ml) had an absorption maximum at 213 n.m. (absorbance 1.2) and a shoulder at 275 n.m. (absorbance 0.3). The optical rotation of the solution was $[\alpha]_D^{26} + 1.77^\circ$.

The carbon-hydrogen-nitrogen analysis (Manchester University Commercial Service) gave the following composition : 10.4%C, 3.1%H, 2.1%N and 0.0%S. The amino acid analysis (courtesy of Dr. C. Hitchcock, Unilever, Sharnbrook) showed that the content was not significant.

The acid hydrolysis indicated the presence of small quantities of galactose and a pentose. An ultra-violet absorbing substance (275Å) occurred at $R_{\text{glucose}} 0.12$ and which was not silver nitrate positive. Phosphoric esters, hexosamines, uronic acids and polyols could not be detected in any significant quantity. The total carbohydrate content of the polymer was found to be 23% hexose and 10% pentose. The sodium hydroxide extract contained 10% hexose and 5% pentose while the residue contained 1-2% of both hexose and pentose. Paper chromatography of the

alkaline soluble fraction showed the absence of periodate-Schiffs-positive substances which would have been indicative of saccharinic acids produced by alkaline degradation 1,2-, 1,3- or 1,4- linked sugars (143). However, there was a periodate-Schiffs-positive purple spot a $R_{\text{glucose}} 0.17$.

The Smith periodate oxidation study indicated the absence of degradation products typical of degraded polysaccharides.

The DEAE - cellulose chromatography indicated that the polymer was homogeneous and could be recovered in 96% yield in fractions 99-103.

The serological analysis indicated that the polymer cross-reacted with the prepared antiserum to produce a precipitin line close to the antiserum well. Methanolic hydrogen chloride hydrolysis and alkaline hydrolysis resulted in loss of the precipitin activity. Enzymatic digestion did not alter the precipitin activity. However, the Smith periodate oxidation study produced a modification of the precipitin activity which resulted in the production of an intense precipitin line midway between the wells (Plate 29). This new precipitin line was accompanied by a much fainter line just behind it in the direction of the antiserum well. A faint precipitin line was observed in the original position. A pyridine-acetic acid extract of A. viscosus cells was treated to a similar periodate oxidation. The resultant material when examined by immunodiffusion exhibited no characteristics similar to the isolated polymer.

Barium chloride did not precipitate the polymer.

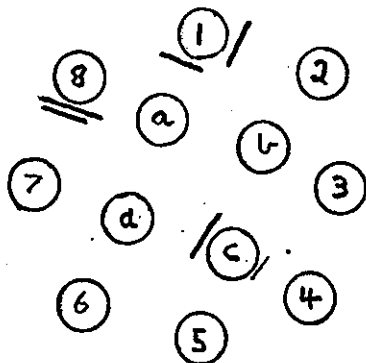
The rabbit showed no signs of ill-health following the injection of the polymer. No lesions could be observed at the site of injection.

It is possible that the initial difficulty in obtaining a material which was not a gum was a result of residual impurities.

Immunodiffusion Studies.

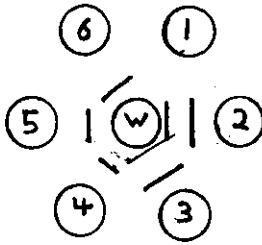
The precipitin lines were very faint and were restricted, in many cases, to areas close to the serum wells. The following patterns were observed :

Immunodiffuion 1.



- | | | | |
|----|------------------------|----------------------|------------|
| 1. | <u>T. thermophilum</u> | (principally spores) | antisera. |
| a. | <u>T. thermophilum</u> | " " | antigens. |
| 2. | <u>T. vulgaris</u> | " " | antigens. |
| 3. | Empty | | |
| 4. | <u>T. vulgaris</u> | " " | antisera. |
| b. | Empty | | |
| 5. | Empty | | |
| 6. | <u>A. pullulans</u> | mixed | antigens. |
| c. | <u>T. thermophilum</u> | " " | antisera. |
| 7. | <u>M. faeni</u> | " " | antigens. |
| 8. | <u>T. thermophilum</u> | | antiserum. |
| d. | <u>C. corticale</u> | | antigens. |

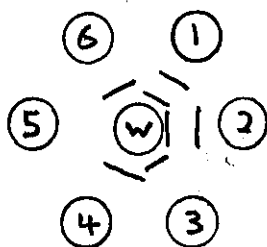
Immunodiffusion 2.



- w. T. vulgaris (principally spores) antisera.
- | | | | |
|----|---------------------|----------------------|-------------|
| 1. | <u>M. chalcea</u> | mixed | antigens. |
| 2. | <u>A. pullulans</u> | mixed | antigens. |
| 3. | <u>C. corticale</u> | (principally spores) | antigens. |
| 4. | <u>M. faeni</u> | " | " antigens. |
| 5. | <u>T. vulgaris</u> | " | " antigens. |
| 6. | <u>M. faeni</u> | " | " antigens. |

None of the precipitin lines appeared identical.

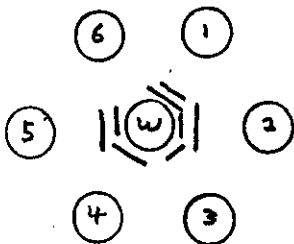
Immunodiffusion 3.



- w. T. vulgaris (principally spores) antisera.
 1. C. corticale " " antigens.
 2. A. pullulans mixed antigens.
 3. T. thermophilum " " antigens.
 4. T. vulgaris " " antigens.
 5. Empty
 6. M. faeni " " antigens.

None of the precipitin lines were identical.

Immunodiffusion 4.



- w. T. polyspora antiserum.
 1. T. polyspora antigens.
 2. C. corticale antigens.
 3. T. thielavia antigens.
 4. T. vulgaris antigens.
 5. A. pullulans antigens.
 6. Empty

DISCUSSION

Actinomycetes and Fungi.

The production of lesions of A. viscosus in the rabbit is further evidence for the classification of this organism as a potential pathogen and this finding supports the observation of Georg and Coleman who used other strains to induce experimental lesions in mice (83). Georg et al have observed actinomycotic infections in dogs, pigs, and goats and they attributed these infections to A. viscosus (71). Actinomycotic infections in man due to A. viscosus ATCC 19246 (formely A. discofolius) were first observed over forty years ago (144). More recently A. viscosus was isolated from a lung infection which had been at first classified as a tubercle condition (70). Some workers have observed nodular lesions at the site of intradermal injections of the cell walls of other strains (145). In the case of A. viscosus WVU 398B no such lesions could be observed after subcutaneous or intradermal injection of killed cells. It is possible that intradermal injections are more irritating to the animal.

Actinomycotic infections have been classified as opportunistic infections (19). Several workers have associated actinomycotic mycetoma with carious teeth, or following traumatic shock such as may accompany dental procedures or a blow to the jaw (53, 71, 146).

Oral commensals such as A. israeli (147) and A. viscosus may be involved in caries or periodontal disease. The ability of A. viscosus and T. vulgaris to survive the digestive juices would suggest that they could be the causative agent of actinomycetoma along the alimentary tract. It is significant that the vast majority of the reported cases of actinomycetoma have involved this region of the body. Wagenstein suggested that resistance to gastric juice could be a contributory factor (75).

In the case of A. viscosus, T. vulgaris, T. sacchari and M. chalcea histopathology revealed 'sulphur' granules, typical of actinomycetoma. Histopathology, in the case of S. pelletieri, revealed that the infection did not produce a 'sulphur' granule. A distinguishing feature of actino-

mycetoma caused by A. israeli are the concentric layers of eosin-haemotoxylin-positive tissue (35, 71) and these concentric layers are absent from the S. pelletieri infection (Plate 13). However, this tissue arrangement was found in the case of the other four actinomycetes.

Shah has described the actinomycetoma as giving rise to multiple cutaneous fistulas, which are discrete and separated by dense fibrosis (53). The fibrosis helps to localise the infection but forms a barrier to the penetration of any chemotherapeutic agent and the low vascularity within the nodule aids in this, as does the calcification. Other workers have reported calcification in other infections (148) and in carcinoma (149, 150). Perhaps it is significant to draw attention again to the frequency with which actinomycosis of the colon has been mistaken for cancer of the colon (64). In the case of S. pelletieri calcification was observed in spite of the fact that the organism showed definite evidence of spreading and invasiveness. It would appear that calcification may contribute to localisation of infection but does not prevent spreading of an active pathogen as in the case of Mycobacterium tuberculosis which is effectively localised by fibrosis and calcification until immunosuppressive therapy is initiated (151). However, Binford has reported the calcification does not occur in actinomycosis (152).

Actinomycosis of the jaw is accompanied by an osseous condition which involves a loss of bone at the infected site (60). Very recent investigations of the sulphur granule have shown that the granule contains hydroxyapatite (160). Further studies of these experimentally induced actinomycetomas must involve an elemental analysis of the excised 'sulphur' granule. As yet no elemental analysis has been performed on the nodular deposits observed by X-rays in the lungs of patients suffering from chronic bagassosis (154).

These experimentally induced actinomycotic infections were not complicated by the presence of other organisms such as Staphylococcus

aureus, Pseudomonas aeruginosa and other Gram-negative organisms which have been observed many times in clinical conditions (114). From the histological examination the organisms do not appear to produce tissue destroying enzymes and A. viscosus has been shown to be deficient in chondroitin sulphatase (155). However, cell-free culture filtrates of M. chalcea have been shown to have a high proteolytic and $\beta(1-3)$ -glucanase activity, which if produced in vivo, could favour tissue destruction (156). In clinical actinomycosis it would be highly possible that the presence of organisms such as S. aureus and Ps. aeruginosa would produce an environment of high proteolytic activity. Any tissue destruction, to allow the observed spread and penetration could result from a release of degranulating leucocytes around the nodule (157). However, this type of inflammatory response could result from a non-specific response to the injection of foreign material although the absence of a reaction following the injection of the killed cells argues against this being the case. The organisms certainly survive within the host and the presence of diphtheroid and filamentous forms following the injection of spores suspensions suggests limited growth and differentiation.

The absence of tissue destruction and spreading along the tissue planes differentiates the infections due to T. sacchari, T. vulgaris, M. chalcea and A. viscosus from that seen with the Streptomyces species, such as S. pelletieri, which are not limited to the fascia but the lesions penetrate the muscles (106). The isolated actinomycetes showed the expected morphological and biochemical characteristics. However, M. chalcea, T. vulgaris, and to a lesser extent T. sacchari showed marked haemolytic properties which were lost on repeated subculture. This particular property has not been described before in M. chalcea and T. vulgaris (118). The acquisition of this haemolytic property suggests that enzymes favouring invasion may be induced in vivo which are not demonstratable in vitro. Such an acquisition would suggest that some

growth, at least, must have occurred to permit such induction.

During the production of antisera to the vegetative forms of the actinomycetes it was observed that the injection of viable organisms at two or more sites, at weekly intervals, then nodules were obtained at all the sites. It would appear that the defence mechanism is unable to significantly alter the nature of the local infection. Such an ineffective response may be due to the poor vascularity and penetration to the region of active growth. Huppert has observed similar evidence in the case of fungal infections where the tissue exhibited a decrease in the ability to resist infection as the distance from the affected area increased (158). In addition to the immune response it is possible for other factors to contribute to the host resistance to actinomycosis, for example, leucocyte myeloperoxidase could be involved (159). However, as myeloperoxidase has a requirement for peroxide it may become inactive once an anaerobic or microaerophilic environment is established within an avascular abscess.

Several other workers have experienced difficulty in isolating the causal agent from a mycetoma (160, 161) and a similar difficulty has been encountered in isolation of the actinomycotic agent (106). The pleomorphism as observed in this study confirms the pleomorphic descriptions of Georg et al in their clinical cases of actinomycetoma (71) and such observations are not uncommon in fungal mycetoma (162) where the form seen in vivo may differ significantly from that seen typically in vitro.

The germination and outgrowth of spores of T. vulgaris and T. sacchari in tissues, as well as the survival of vegetative forms, shows that the normal body temperature is adequate and that they could cause human or animal local infections. Also, the spores or vegetative cells could be introduced into abrasions, or into the alimentary tract, of man or animals, and give rise to local infections.

In experimental studies of farmers lung disease Zaidi et al were unable to recover viable M. faeni from the lungs of experimentally infected guinea pigs after seven days (162), whereas this study revealed that viable organisms could be recovered from the actinomycotic infections up to 3 months after infection. However, it is possible that the lung may have properties which contribute to the destruction of the spore. The organisms used in this study, except A. viscosus and S. pelletieri, are all present in large numbers in certain natural environments. Lacey and Lacey reported 1.6×10^9 spores/m³ in moderately dusty hay (163).

The role of both spore and vegetative forms in local infection assumes some importance in view of recent evidence which indicates that the antigenic stimulus in farmers lung disease may in part be attributable to the germination and possible limited development of M. faeni spores in the lung (164). Some recent investigations have indicated the absence of serologically active materials from the spores of M. faeni and it has been suggested that the hypersensitivity, in farmers lung disease, may arise from mycelial fragments carried to the lung on the spores or that the spores germinate rapidly with elimination of the mycelium on the lung surface (165).

The resistance of this particular strain of A. viscosus to the low concentrations of penicillins was surprising in view of the sensitivity reported by Jordan and Howell (166). However, A. viscosus and T. sacchari were sensitive to higher concentrations of semi-synthetic penicillins, although, this may not represent useful in vivo sensitivity. Werder and Sonnabend reported that their S. pelletieri strain, isolated from a neonatal infection, was sensitive to penicillin (101). In this study S. pelletieri, and the non-pathogenic S. coelicolor, were found to be resistant to penicillin. In clinical cases of farmers lung disease corticosteroid therapy has been used (154). In view of this study which has demonstrated that T. sacchari and T. vulgaris can produce an experimental mycetoma and

the isolation of viable spores of T. vulgaris from the lung (20, 46) it would be wise to avoid the use of such cytostatic drug therapy. Corticosteroid therapy has been reported to potentiate the spread of sporotrichosis (167). Other workers (101, 168) have reported that their clinical isolates responded to sulphonamide therapy. However, S. pelletieri NCTC 3026 was insensitive in the test system adopted.

The ability of C. corticale and A. pullulans to produce local infections was rather unexpected in view of their apparent difficulty in growing at 37° in vitro. In fact Emanuel et al reported that C. corticale did not grow at 37° (42). However, Wenzel (personal communication) has confirmed that the organism does grow at 37°. The observation that A. pullulans and the plant pathogen C. corticale, can produce local infection supports the evidence of Lie-Kian-Joe et al (169) who observed that a plant pathogen Cercospora apii can also cause mycetoma of the thigh and thorax as well as being the causal agent of leaf-spot disease.

The lack of growth of C. herbarum in vivo was not surprising in view of its lack of growth at 37° in vitro. Its inability to grow in vivo enabled it to be used as a control representing a commonly saprophytic group with species which are occasionally pathogenic (106).

The organisms did not produce any tissue destroying enzymes, as judged from the histopathological examination and they were certainly deficient in haemolysin. The tissue destruction necessary for limited growth could result from the release of proteolytic enzymes from the degranulating leukocytes which surrounded the periphery of the nodule (157).

This investigation of fungal pathogenicity could profit from the use of a simultaneous regime of cystostatic drugs which may facilitate an understanding of fungal pathogenicity.

Bacillus cereus.

The invasive properties of the two groups of organisms implicated in extrinsic allergic alveolitis are dissimilar in that actinomycosis

is characterised by calcification which is absent from the fungal mycetoma. As both groups of organisms are present in dust it was considered that the study may profit from an investigation of the pathogenicity of other dust borne organisms which are not implicated in extrinsic allergic alveolitis. The sites of B. cereus infection resemble actinomycosis only in that calcification occurs. Actinomycotic infections can be differentiated from fungal mycetoma and B. cereus infections by the presence of concentric tissue layer (Plate 9).

The production of lesions by B. cereus in the rabbit indicates that it may possess pathogenic properties even when present in relatively small numbers. Lamman and Jones demonstrated the toxic properties of 10^7 cells in the mouse (125) while others have demonstrated that even fewer cells were toxic if young rapidly dividing cultures were employed (126).

The presence of considerable proteolytic activity would facilitate the spread of the organism.

As in the case of the actinomycetes and fungi, killed cells did not produce similar nodules. This study has demonstrated that following injection of small quantities of spores a sufficient number can survive phagocytosis to produce a local infection.

The vegetative cells within the nodule showed a degree of pleomorphism which is more characteristic of actinomycotic and fungal infections (106). These pleomorphic forms were common in the in vitro studies. Pleomorphism has been observed in many cases such as the reversion of an urethral exudate strain of a pleuropneumonia - like organism to corynebacterium during tissue culture passage (170). The factors which contribute to such in vivo pleomorphism are unknown. In vitro pleomorphism of Lactobacillus bifidus can be induced by a high salt concentration which is reversed by calcium ions (171). In Part I of this thesis it has been demonstrated that morphological variation can be induced and regulated by chemotherapeutic substances. Other results have indicated

that morphological variation is accompanied by alterations in the cell membrane constituents. Jordan and Howell observed morphological variation in A. viscosus when it was cultured in an atmosphere of carbon dioxide (166). It is possible that growth of an organism within an avascular abscess may possibly give similar results.

It is possible that the morphological change is an environmental factor which contributes little to successful invasive spread. In which case, it is difficult to understand the adaptive significance of such a phenomenon. In the case of L-form bacteria they are usually isolated from environments of high osmotic strength, such as urine, and it could be that the cell no longer requires the mechanical aid of the cell wall in preserving its integrity against its own internal osmotic pressure. As yet there has been no successful demonstration that the morphological variant is any more pathogenic than the ordinary cell. There is some evidence that surface active polyoxyethylene ethers such as 'Triton' WR 1339 protect the mouse against infection by Mycobacterium tuberculosis (172, 173). Maximum protective activity was exhibited by compounds having an ethylene oxide chain length of 15-20 units while those with 45 units became protuberculous. These ethers exhibit very little effect in vitro. Mackaness showed that the growth of such bacilli in cultures of macrophages is limited in the presence of the polyoxyethylene (174). Wattiaux Wibo and Baudhuin have demonstrated that the 'tritons' are concentrated in the lysosomes, the cell organelles which exhibited lytic activity against foreign bodies (175). Sterols such as cortisol are known to stabilise lysosomal membranes and in some cases they suppress intracellular digestion of foreign bodies (176). As yet no role has been established for the sulpholipids which are said to be characteristic of the virulent strains of M. tuberculosis (177),

however, these lipids could inactivate the enzymes of the lysosomal membranes and so potentiate successful infection.

The research reported in this thesis has demonstrated that cell membranes may play a key role in the regulation of cell differentiation and the control of uptake of antimicrobial substances. In view of the evidence that the membrane structure and composition may regulate invasive properties and cellular interactions, there is justification in recommending a continued investigation into cell membranes with the hope that such research may provide an insight into invasive properties.

The structure of the isolated polymer (the non-dialysability of the substance is the sole basis for terming it a 'polymer') remains undetermined. The homogeneity of it has been established. The periodate oxidation and alkali degradation evidence indicates the absence of any major polysaccharide linkage for the 33% total carbohydrate content and no evidence has been obtained which would indicate the nature of the residual 67% of the polymer. Analysis has indicated the absence of phosphorus and sulphur. The absence of any significant quantities of hexosamines and amino acids presents difficulty in attributing the relatively high nitrogen content of 2.1% to any group.

The absence of any carbon-hydrogen stretching frequency in the infra-red spectrum could be due to highly charged groups in the polymer. The group absorbing at 274 nm cannot be attributed. The very slight optical rotation of + 1.77 could be due to the presence of a small percentage of β -anomeric linkages.

The serological activity is unusual and there appears to be no doubt that the origin of the polymer is A. viscosus. The absence of a levan partially compares with the work of Stone et al (180) who found no evidence for levan production by A. naeslundii when cultured on a sucrose containing medium. The low yield (0.1 mg/ml) obtained is similar to the polysaccharide yield obtained by Stone et al. It

is possible that these extracellular polymers may be important constituents of dental plaque (78) and perhaps facilitate the agglutination of certain bacterial species present in plaque (181, 182). In addition to serving as a plaque constituent (183, 184) and an energy source it is possible that the dextrans produced by the cariogenic Streptococcus spp. may also concentrate ions (185).

The recent publications by van der Hoeven (186) and Rosan and Hammond (187) have detailed the chemical composition of extracellular polysaccharides produced by two different strains of A. viscosus. Van der Hoeven found glucosamine and glucose to be the predominant sugar components and he interpreted the high acetyl content to be entirely N-acetyl since he found no evidence of the O-acetyl function. There was no fructose, glycerol, pyruvate, sulphate or uronic acid present. Although van der Hoeven prepared his polysaccharide material in a completely different way to the method used in this study and to that used by Rosan and Hammond all three investigations are in agreement in that they failed to obtain anything other than a trace of serologically active polymer. The polysaccharide(s) produced by Rosan and Hammond were characterised by the following composition : N-acetyl glucosamine (62%), galactose (7%), glucose (4%), uronic acid (3%), and smaller amounts of glycerol, rhamnose, arabinose and xylose (187).

The problems encountered in the study of the serology of the polymers of A. viscosus were encountered also in the studies of the organisms implicated in extrinsic allergic alveolitis. The immunodiffusion studies demonstrate the considerable difficulty which can be encountered in obtaining active antisera to the actinomycetes. The results obtained in immunodiffusions 1-4 indicate considerable heterogeneity in the cross reactions. The abundance of precipitin lines obtained between the various antigens and the antisera was unexpected. The weakness of many of these

precipitin lines would possibly indicate the possession of one or two common specific groups of sufficient character to interact with the antisera and so produce a precipitin line. In addition, to the weakness of the precipitin lines many were nearer to the antiserum well than was expected. In both cases varying reports exist concerning the intensity and position of the precipitin lines. Fletcher et al reported very sharp and intense precipitin lines midway between the M. faeni antigens and the antisera obtained from patients with farmers lung disease (178). In contrast, Salvaggio and Buechner have observed much weaker precipitin lines nearer to the antiserum well which contained the antiserum from a bagassosis patient (154). Barboriak et al observed that C. corticale antigenic determinants reacted with the antiserum from patients suffering from farmers lung disease, bagassosis, maple bark strippers disease and pigeon breeders disease (179). In addition these workers observed that all the antisera, except from maple bark strippers disease, cross-reacted with the majority of the antigen extracts from T. vulgaris, pigeon droppings, mouldy hay and C. corticale.

Although his study has not contributed anything to an understanding of the role of A. viscosus in producing plaque other workers will soon resolve such mysteries. Although the Actinomyces spp. were among the first microorganisms to be recognised as the aetiological agents of disease the frontiers of their mysterious biological processes have contracted dramatically over the last few years. Mankind has been fortunate that these agents of incapacitation have been counterbalanced by other actinomycetes which have provided numerous antibiotics and antitumour agents.

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Publications arising from this thesis.

1. Sabouraudia, 12, 245-257 (1974).
Experimentally induced actinomycotic mycetoma.
2. J. Hygiene (Cambridge) 73, 369-374 (1974).
Lesions experimentally produced by fungi implicated in extrinsic allergic alveolitis.
3. Zentrabl. Bakt. Hyg., I Abt. Orig A, 232, 83-87 (1975).
Experimental infection of rabbits with Bacillus cereus.
4. Cytobios, 10, 103-111 (1974).
A study of the morphology of some strains of Actinomyces viscosus using scanning electron microscopy.
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Effect of antimetabolites on differentiation in Actinomyces viscosus wvu 398B.
6. Microbios 11, 183-191 (1974).
Effects of the lanthanides, lanthanum and neodymium, on the morphology and size of Scenedesmus sp.
7. Microbios 12, 1-21 (1974). Influence of 3-chloro-propane-1, 2-diol on the microbial lipid composition and its effects on the activity of some antibacterial agents.
8. Microbios (in press). The heat resistant properties of Bacillus cereus sv-1 spores treated with lanthanum and neodymium.

Marshfield Clinic Foundation

FOR

MEDICAL RESEARCH AND EDUCATION

March 5, 1975

510 N. ST. JOSEPH AVE.

MARSHFIELD, WIS. 54449

Dr. Robert A. Bulman
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Harwell, Didcot, Berks. OX11 0RQ
England

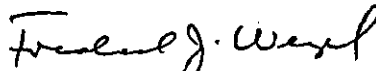
Dear Doctor Bulman:

Thank you very much for your letter of January 16, 1975 with the enclosed copies of your two papers on extrinsic allergic alveolitis. I enjoyed reading them very much and I also gave them to Dr. Jim Marx of our research staff. We were quite interested in the proposition that farmer's lung could be the result of a bacterial infection rather than a hypersensitivity disease. We have talked about this hypothesis before but have not seriously pursued the matter. With the idea of hemolysis production and the elicitation of these lesions by viable but not heat or chemically killed organism, the relative virulence of the different species would seem to correlate with the incidence of antibodies, at least in this area of the country. The difference that was shown with the streptomyces may also explain why this genus has not been demonstrated to cause farmer's lung syndrome.

These results could also be interpreted to suggest a Type II reaction which, of course, we have been arguing against for many years.

Again, I appreciate your sending the papers and I hope that we can keep in further communication.

Sincerely yours,



FREDERICK J. WENZEL
Executive Director
Marshfield Medical Foundation

FJW:ad

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1975-04-29

Dr. Robert A. Bulman
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Dear Dr. Bulman,

Many thanks for your letter and the reprint with your interesting results! As you can see, I have transferred to Copenhagen, Denmark; but the work on Scenedesmus morphology as influenced by mineral nutrition is pursued in Stockholm by Monica Holmwall. (née Nilshammar, so you know her by that name!). I think she would appreciate a reprint of your communication!

As for your hypothesis, it is well in line with modern biological thinking. To prove it is another question - both as a scientist and as editor of "Physiologia Plantarum" I realize more and more how the need to prove interpretations takes us deeper and deeper into biochemistry and biophysics; at the same time as the need to coordinate and evaluate our information takes us higher and higher into the spheres of ecology and applied biology. I can only say that I enjoyed reading your paper and wish you every success in pursuing your problem further!

Technically, I feel that especially the calcium ionophore X-537A would be highly useful also for Monica Holmwall.

Best wishes! - Yours sincerely,

Anders Kylin
(Anders Kylin)

EXPERIMENTALLY INDUCED ACTINOMYCOTIC MYCETOMA

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The ability of *Thermoactinomyces vulgaris*, *T. sacchari* and *Micromonospora chalybeata* to produce local actinomycotic mycetoma was compared with that in *Actinomyces viscosus* and *Streptomyces pelletieri*. Nodules were produced following subcutaneous or intramuscular injection by all the organisms studied and typical 'sulphur granules' were seen histopathologically, when *T. vulgaris*, *M. chalybeata*, *T. sacchari* and *A. viscosus* were involved. All the organisms were sensitive to antibiotics *in vitro*.

The principal causal agents of human actinomycosis were summarised by Georg (1970). These are, mainly, species of *Actinomyces* or *Nocardia* which usually respond to treatment with penicillin or sulphonamides respectively. Mycetoma are also produced by several *Streptomyces* spp. (Emmons, Binford & Utz, 1970), although Cross & Goodfellow (1973) have referred the pathogenic *S. madurae* and *S. pelletieri* to the genus *Actinomadura*.

Actinomyces viscosus has been isolated frequently from the human mouth, the teeth of hamsters and from various human and animal clinical materials (Jordan & Howell, 1965; Jordan, Fitzgerald & Stanley, 1965; Jordan, 1971; Georg *et al.*, 1972; Jordan & Hammond, 1972; Loesche, Hockett & Syed, 1972). Five strains of *A. viscosus* were shown by Georg & Coleman (1970) to produce lesions in mice, following intraperitoneal injection, and it has also been involved in actinomycotic infection in the dog (Georg, *et al.*, 1972). Negroni (1932) isolated *A. discoloratus* (now *A. viscosus* ATCC 19246) from a human actinomycotic infection and Scharfen (1971) considers that it may play a primary role in pathogenesis and it has recently been isolated from a lesion following a stab wound (Lewis & Gorbach, 1972).

The thermophilic actinomycetes have been implicated in extrinsic allergic alveolitis (Pepys, 1969) and the aetiological agents of farmer's lung disease have been identified as *Micropolyspora faeni* and *Thermoactinomyces vulgaris* (Pepys *et al.*, 1963, Wenzel, Emanuel & Lawton, 1964, and Wenzel *et al.*, 1967). These organisms have also been implicated in mushroom worker's lung disease (Fergus, 1964 and Sakula, 1967).

Thermoactinomyces vulgaris and *M. faeni* were isolated from a lung biopsy by Wenzel *et al.* (1964, 1967), from patients suffering from farmer's lung. In view of the high temperature requirements (55°C) of *T. vulgaris*, Wenzel *et al.* (1967) considered that it would be unlikely to multiply in tissue, however, Lacey (1971) has shown that it could grow at 35°C on nutrient agar. *Thermoactinomyces sacchari*, and *T. vulgaris*, have been identified by Lacey (1971) as the antigenic material in bagassosis. Salvaggio & Buechner (1971) have reported, as a result of X-ray examination, the presence of small nodular deposits throughout the lungs of patients with bagassosis. Similar

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nodular patterns have been reported for extrinsic allergic alveolitis including those caused by the mesophilic fungi (Salvaggio & Buechner, 1971).

Recently Banaszak, Thiede & Fink (1970) have shown that *T. vulgaris* is also responsible for air conditioner's disease and thermophilic actinomycetes have been isolated from heating ducts by Barboriak, Fink & Scribner (1972).

The pathogenicity of *Micromonospora* spp. has not been studied in detail, although Luedemann (1969) in discussing the *Micromonospora* describes *M. caballi* and *M. melanospora* and states that they were isolated from equine and human mycetoma respectively.

This study examines the ability of selected aerobic actinomycetes to produce localised infections, apart from the granulomatous nodules occurring in the lung.

MATERIALS AND METHODS

Organisms

Thermoactinomyces vulgaris NCIB 9780, *Micromonospora chalcea* NCIB 9599 and *T. sacchari* A978 (kindly provided by J. Lacey, Rothamsted Experimental Station) were used as representative aerobic actinomycetes. *Actinomyces viscosus* WVU 398B (kindly provided by Mary A. Gerencser, University of West Virginia) was used as a control organism whose pathogenicity in certain animals is being established and *Streptomyces pelletieri* NCTC 3026 as an aerobic actinomycete which is a proven pathogen.

Media

The organisms were maintained in a nutrient broth containing (g/litre) Oxoid beef extract, 10; Oxoid neutralised peptone, 10; sodium chloride, 5. Solid media were prepared by the addition of 1.5% W/V Oxoid agar No. 3 and blood agar by the further addition of 5% V/V defibrinated horse blood. Prior to the preparation of the suspension required for injection, *M. chalcea* was grown in a glucose-asparagine medium (g/litre) glucose, 10; asparagine, 0.5; dipotassium hydrogen phosphate, 0.5; Oxoid beef extract, 1. The pH of all media was adjusted to 7.2 before use.

Morphological and Biochemical Characterisation

The organisms used were examined for purity, including the presence of contaminants, before injection and after isolation from the experimentally infected animals. The examination in the cases of *S. pelletieri*, *M. chalcea* and *T. vulgaris* was by the methods described in Bergey's Manual (1957) and *T. sacchari* was examined according to the description of Lacey (1971) and *A. viscosus* according to Howell, Jordan, Georg & Pine (1965).

Preparation of suspensions

One ml of a broth culture of *M. chalcea* or *A. viscosus* or *S. pelletieri* was inoculated into 250 ml of glucose-asparagine medium or nutrient broth, respectively. The flasks were shaken at 37°C for 24 h in the case of *A. viscosus* or 76 h for *S. pelletieri* or 144 h for *M. chalcea*. The cells were removed by centrifugation at 6000 g at 4°C, washed 4 times with sterile normal saline (0.9% W/V) solution and stored, if necessary at 4°C until required. Two ml of a nutrient broth culture of *T. vulgaris* or *T. sacchari* were inoculated on 200 ml of nutrient agar in a Roux flask and then incubated for 72 h at 55°C. The cells were then removed by washing with sterile distilled water,

centrifuged at 1000 g to remove contaminating agar particles. This agar-free liquid was then centrifuged 4 times at 6000 g and the cells washed with sterile normal saline solution. In all cases the deposited cells were resuspended prior to injection in sterile normal saline solution, using a glass tissue homogeniser (Jencons (Scientific) Ltd., Herts) and the cell density adjusted to give approximately 10^8 cells/ml using a Thoma counting chamber. The number of viable units/ml was also checked by carrying out pour plate counts before injection, in all cases this was *ca* 10^8 /ml. The suspensions were also plated out and were found to be free from organisms other than the required actinomycetes. Suspensions of killed cells were prepared by dividing the above suspensions and adding thiomersalate to give a final concentration of 0.01% w/v and leaving for 76 h at 4°C or by heating in an autoclave at 120°C for 20 min. The absence of viable organisms from these suspensions was checked by streaking out and by the inoculation of 1 ml of the suspension into 10 ml of nutrient broth and incubating at the optimum temperature.

Suspensions were also prepared, in normal saline solution, which contained 100 spores/ml (where this was applicable). Such suspensions were divided and a control portion heat killed and the remainder used as viable organisms for injection. Both suspensions were checked for the presence of viable organisms before use.

Experimental Animals and Injection Procedure

Male Dutch rabbits weighing 1.8 ± 0.2 kg were used throughout. When the intramuscular (i.m.) route was employed, 0.2 or 0.5 ml of the required suspension was injected into the flank and for the subcutaneous (s.c.) route, 0.3 ml. The rectal temperature was taken daily following injection. Rabbits were killed at intervals of 7, 10 or 14 days and 6 months after inoculation.

At necropsy, samples of kidney, liver, spleen, lung and any obviously infected tissue were removed and inoculated on nutrient and blood agar and all samples were incubated aerobically and in a N_2/CO_2 (95:5%) atmosphere at 37°C. The samples taken from rabbits inoculated with *T. vulgaris* or *T. sacchari* were treated in the same way but were incubated also at 55°C.

Representative samples of tissue, including obvious lesions, were excised, fixed immediately in mercuric chloride - formalin solution, dehydrated and embedded in paraffin, sections of 5 μ m thickness cut and stained with H & E and by Gram (Cruickshank, 1970). The calcium staining techniques of Von Kossa and the alizarin red S method were also used (Pearse, 1972). Smears prepared from the pus were also examined by Gram immediately following necropsy. Samples of the pus were checked, by streaking out, for the presence of organisms other than actinomycetes and, after dilution with normal saline, viable counts were carried out by the pour plate technique.

The effects of exposing *A. viscosus* and *T. vulgaris* to the gastrointestinal tract were examined using artificial digestive juices (British Pharmacopoeia, 1963). The organisms were suspended in artificial gastric juice, at 37°C, agitated and samples withdrawn at 15 min intervals and viability assessed by plating out on nutrient agar and incubating at 37°C. Also, after the organism had been suspended for 3 h in artificial gastric juice the cells were removed by centrifugation, washed with sterile distilled water and resuspended in artificial pancreatic juice. Their viability was assessed at 15 min. intervals. These organisms were also suspended in reconstituted rumen fluid (Dr. M. Elizabeth Sharp, N.I.R.D. Reading) and the viability examined. Control systems were examined with the organisms suspended in either sterile normal saline solution or sterile distilled water.

Antibiotic Sensitivity

The *in vitro* susceptibility of the organisms to selected chemotherapeutic agents was examined, using the 'Multodisk' (Oxoid) method. Other antibiotics, not covered by the commercially available discs, viz: clindamycin, lincomycin (from Upjohn Ltd., Crawley, Sussex) and sodium fusidate (from Leo Laboratories, Hayes, Middlesex) were examined using the filter paper disc method, at a concentration of 10 µg per disc. In view of its apparent resistance to penicillin G, the sensitivity of this strain of *A. viscosus* to some of the semi-synthetic penicillins, e.g., methicillin, ampicillin (Beecham Research Labs, Brentford) and quinacillin (The Boots Co. Ltd., Nottingham) was examined. The seeded nutrient agar plates were incubated at the optimum temperature for growth for 24 h and the resulting zones of inhibition measured.

RESULTS

All the organisms grew well on the media described except *T. sacchari* which gave moderate to poor growth on nutrient agar. *Thermoactinomyces vulgaris* grew well at 55°C but only moderately at 37°C.

After s.c. or i.m. injections nodules were produced which could be felt beneath the skin within 7 days and persisted for at least several weeks and in 80% of the cases for over 6 months. At *post mortem* examination circumscribed abscesses were seen either just beneath the skin or on muscle and invading the space between muscle blocks. The area of the nodule was usually extensive and relatively avascular, except at the periphery where the increased vascularity was marked (fig. 1-4). The pus present in all the nodules, except those caused by *S. pelletieri*, was white to pale yellow, granular and cheesy. That from nodules where *S. pelletieri* was implicated was viscous and pale yellow with a few pink granules. When either heat or chemically killed cells were injected there was no reaction at the site of injection nor any development of nodules. There were no organisms other than actinomycetes present in the nodule. The actual numbers of viable units present was, in all cases, very low, being a maximum of 35 per ml of pus (including *S. pelletieri*).

The organisms caused no detectable rise in body temperature, except for *T. vulgaris* where a slight increase persisted for 48 h. No other adverse symptoms were noticed, apart from obvious discomfort on touching the nodules. Fourteen days after injection with *S. pelletieri* there was involvement of the lymph nodes but this was not observed with any of the other actinomycetes.

The nodules produced by *M. chalcea* were relatively small *ca* 0.5 cm in diameter following s.c. injection but much larger (*ca* 1.5 × 1.0 cm) after i.m. injection and located typically on the adductor magnus muscle (fig. 4). However, nodules were seen occasionally on the lateral part of the intermediate quadriceps block (fig. 1) and the whole area became markedly hyperaemic. The situation in *T. vulgaris* infections was very similar to that obtained with *M. chalcea* although several nodules were frequently observed (fig. 2).

Again, *T. sacchari* gave almost identical results, but there was some indication of spreading and invasion of the muscle to a depth of *ca* 1 cm in several instances following subcutaneous injection.

The results obtained using *A. viscosus* were somewhat different from the above, because s.c. injection gave rise to lesions 10 days after the injection at the site of injection, *ca* 3 cm (fig. 3) and *ca* 5 cm away on the surface of and penetrating the intermediate quadriceps block. This phenomenon was observed in several cases suggesting that the organism has some invasive properties. Intramuscular injection

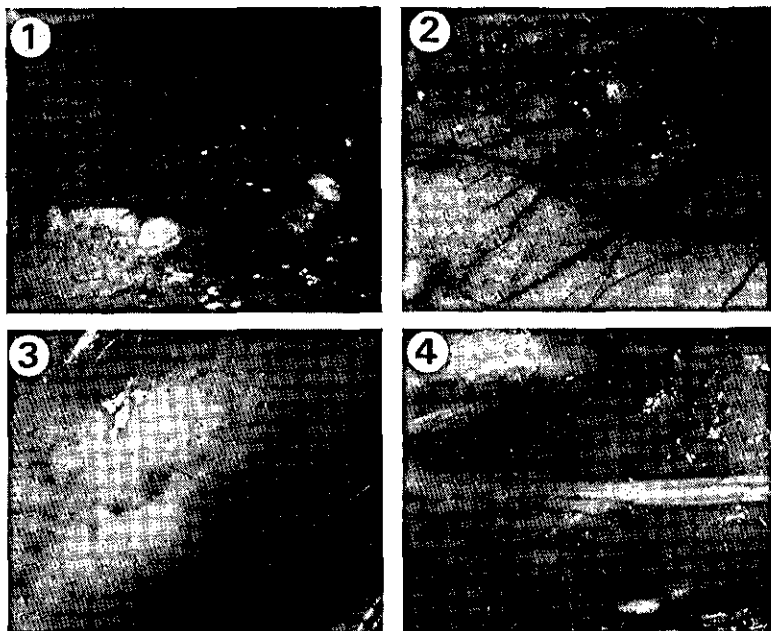


Figure 1.—Nodules on the lateral aspect of the hamstring muscles 7 days, following a single i.m. injection of *Micromonospora chalcea*.

Figure 2.—Subcutaneous produced, 14 days, after injection of *Thermoactinomyces vulgaris* showing the increased vascularity of the cutaneous vessels [$\times 2$].

Figure 3.—Nodule 7 days after s.c. injection of *Actinomyces viscosus*. It occurred in the muscle of the quadriceps femoris block [$\times 2$].

Figure 4.—Nodule produced, after 14 days, on the surface of m. adductor magnus following i.m. injection of 0.5 ml of a suspension of *Streptomyces pelletieri* [$\times 1.5$].

typically produced nodules at the site of injection and on the adductor magnus muscle (fig. 4) in contact with the sciatic nerve. Similar nodules were formed by all the organisms studied.

At *post mortem* examination there were no nodules on the lungs, liver or spleen of any animal infected with the actinomycetes. Organisms were not demonstrated in any of these organs.

The blood-agar plates obtained after inoculation with material obtained from nodules from the infected animals were examined every 8 h. The most rapid and abundant growth was observed when the plates were incubated in a N_2/CO_2 atmosphere (95:5%). The only organisms recovered were those injected initially. Growth was not observed from the kidney, spleen, lung or liver obtained from animals infected with any of the 5 organisms.

Growth of *M. chalcea* after recovery from the infected animals, by plating out, was very variable. On some occasions abundant growth was observed after 36 h, whilst with other samples no growth was detectable until after 72 h. Also, haemolysis, after animal passage, was very extensive and could be seen at the micro-colony stage. *Thermoactinomyces vulgaris*, on isolation, gave rapid and abundant growth at 55°C

after 12 h incubation. Growth was also abundant at 37°C but required 35 h to reach a similar amount to that obtained at 55°C; in both instances haemolysis was extensive. A similar pattern was obtained with *T. sacchari* with moderate growth producing extensive haemolysis, at 37°C after 12 h. The haemolytic properties of all organisms diminished considerably after 2 sub-cultures on blood-agar. *Streptomyces pelletieri* was non-haemolytic even after re-isolation from the rabbit. None of the organisms exhibited haemolytic properties before injection into the animal.

It proved more difficult to re-isolate *A. viscosus*; in general 2 of every 3 subcultures from nodules failed to produce colonies. Where growth was obtained it was rapid, e.g. from a point inoculation onto an overdried blood-agar plate 24 cm² were covered after 36 h incubation in a N₂/CO₂ (95:5) atmosphere, but no haemolytic action was observed.

Microscopic examination of the smears of pus revealed the presence of organisms including a very small percentage of branched mycelial forms from all lesions examined. Histopathological examination revealed the presence of typical actinomycotic granules, surrounded by poly-morphonuclear leucocytes, (fig. 5 & 9) but *S. pelletieri* produced the characteristic granule form (fig. 8). The actinomycetes were penetrating between the muscle fibres (fig. 6 & 9). There was no obvious alteration to or digestion of the muscle fibres although some necrotic fibres were observed (fig. 9) and fibrosis was noted around the periphery of the nodule (fig. 6 & 9). The test actinomycetes were present mainly in the diphtheroid form, (fig. 7) as was *S. pelletieri* (fig. 10), although some filamentous and coccoid forms were seen occasionally these forms were also characteristic of nodules produced by injection of spore suspensions.

There were no histological changes at the sites of injection of the heat or chemically killed cells.

The calcium stains showed that it was being deposited in the nodules (fig. 11 & 12) and that its presence was most noticeable in necrotic muscle fibres (fig. 11). It was deposited in a similar manner at the locus of infection with *S. pelletieri* (fig. 13). Both methods of calcium staining gave identical positive results.

Following exposure to artificial gastric juice or reconstituted rumen fluid, the numbers of viable *A. viscosus* and *T. vulgaris* obtained after 7 h contact were similar to those obtained in the control systems. Similarly, viable organisms were recovered after pre-treatment with gastric juice and subsequent treatment, up to 8 h, with artificial pancreatic juice, although the viable numbers were greatly reduced on transfer to this latter solution compared with the control system.

The *in vitro* tests for sensitivity to chemotherapeutic agents (Table) showed that the antibiotics were effective inhibitory agents, in particular clindamycin, erythromycin and tetracycline, although penicillin was without useful action against *T. sacchari* and *A. viscosus*, and sulphafurazole was not particularly effective against any organism studied. The semi-synthetic penicillins: quinacillin, methicillin and ampicillin were also without action at 10 µg and 100 µg per disc on these strains of *A. viscosus* and *T. sacchari*, although at 1000 µg per disc a zone of inhibition of 22 mm was produced.

DISCUSSION

The production of lesions by *A. viscosus* in the rabbit is further evidence for the potential pathogenicity of this organism to another host animal. Schuster, Hayashi & Bahn (1967) observed the production of small nodular lesions at the site of infection following intradermal injection, in rabbits of both cells and cell walls of *A. viscosus*.

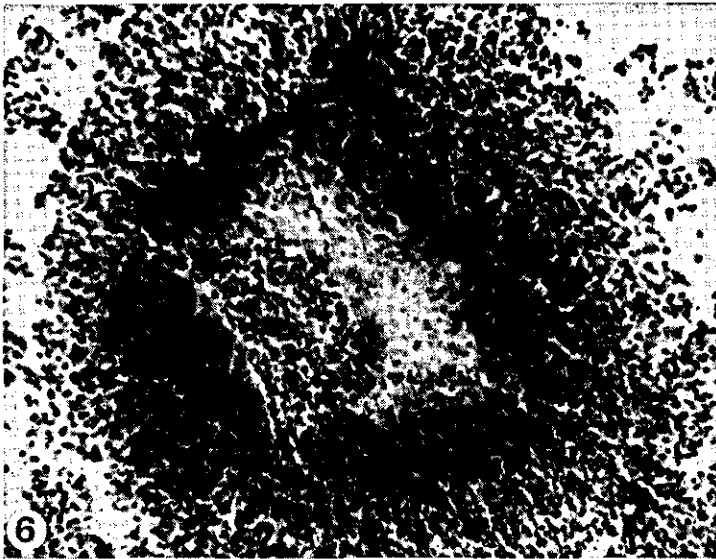
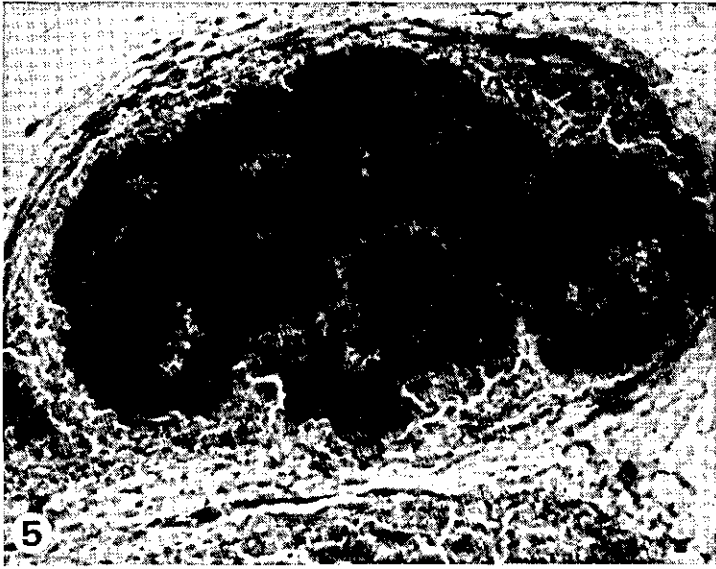


Figure 5.—Microabscess of *M. chalcea* in muscle nodule from infected rabbit. H & E stain $\times 50$.

Figure 6.—Granule of *M. chalcea* from muscle nodule, typical actinomycotic sulphur granule. H & E stain $\times 200$.

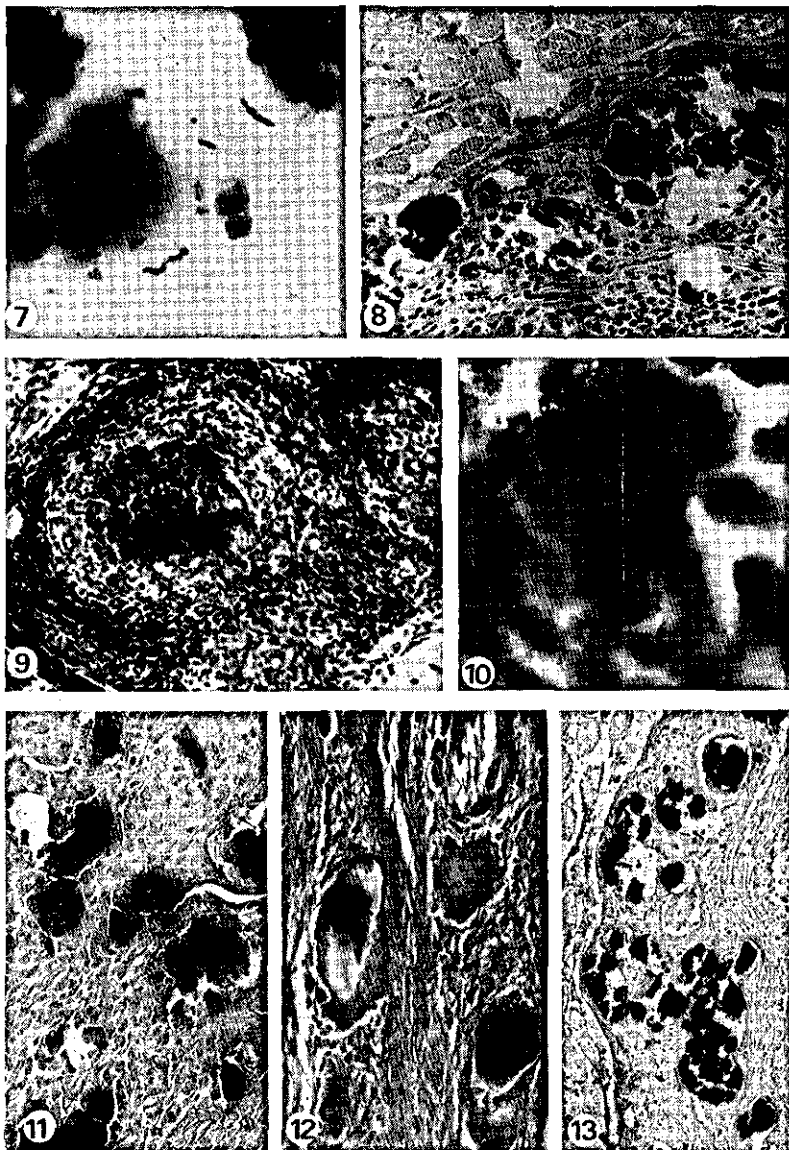


Figure 7.—Coccoid and diphtheroid forms in granule of *M. chalcea* from muscle nodule. Gram stain $\times 1250$.

Figure 8.—Microabscess of *S. pelletieri* in muscle nodule from experimentally infected rabbit. H & E stain $\times 125$.

Figure 9.—Microabscess of *A. viscosus* in rabbit muscle. H & E stain $\times 125$.

Figure 10.—Diphtheroid and coccoid forms in a granule of *S. pelletieri* from muscle nodule. Gram stain $\times 1250$.

Figure 11.—Calcium in nodule of *A. viscosus*. Alizarin red S stain $\times 200$.

Figure 12.—Calcium in necrotic muscle fibre, *M. chalcea* infection. Alizarin red S stain $\times 200$.

Figure 13.—Calcium in nodule of *S. pelletieri*. Von Kossa stain $\times 125$.

TABLE.—SENSITIVITY OF ACTINOMYCETES TO CHEMOTHERAPEUTIC AGENTS

| Organism | Diameter of the zone of inhibition in mm. | | | | | | | | |
|----------------------|---|------------------------|------------------------|--------------------------------|-------------------------------|------------------------|-----------------------------|----------------------------|-----------------------|
| | Penicillin* 1.5 units | Streptomycin* 10 µg | Tetracycline* 10 µg | Chloram- phenicol* 10 µg | Sulpha- furazole* 10 µg | Erythromycin* 10 µg | Clinda- mycin** 10 µg | Fusidic acid** 10 µg | Lincomycin** 10 µg |
| <i>M. chalcea</i> | 14 | 18 | 34 | 20 | 30 | 30 | 42 | 30 | 30 |
| <i>T. vulgaris</i> | 34 | 28 | 34 | 26 | 22 | 32 | 24 | 22 | 14 |
| <i>T. sacchari</i> | 0 | 28 | 32 | 16 | 22 | 28 | 26 | 20 | 24 |
| <i>A. viscosus</i> | 0 | 32 | 14 | 24 | 0 | 38 | 31 | 25 | 21 |
| <i>S. pelletieri</i> | 0 | 0 | 29 | 20 | 10 | 21 | 39 | 34 | 35 |

*Oxoid Multodisks

**Filter paper discs (Whatman, 13 mm).

However, we saw no lesions developing after injection of killed cells of this particular strain.

Actinomyces viscosus, like *A. israelii*, is an oral commensal (Burnett & Scherp, 1957) and may be involved in caries or periodontal disease and as it is unaffected by gastric juice or rumen fluid and can survive to a limited extent in pancreatic juice it could be considered as a possible pathogenic agent for human or animal disease along the alimentary tract, as could *T. vulgaris*. The resistance to gastric juice has been suggested by Wagenstein (1936) for other actinomycetes which cause lesions.

The nodules produced, by all the organisms used, are similar to those described for typical actinomycotic mycetoma. They give rise to multiple cutaneous fistulas, which remain discrete and separated by dense fibrosis. The fibrosis helps to localise the infection but forms a barrier to the penetration of any chemotherapeutic agent and the low vascularity within the nodule aids in this, as does the calcification. The calcification is similar to that seen in other infections (Auerbach, 1966; and El-Gazayerli, Koraitim & El-Shazly, 1970) and in carcinoma (Anghileri & Miller, 1971A, B; and Ward, 1966). The calcification was very similar in the case of *S. pelletieri* where there was definite evidence of spreading and invasiveness, so the calcification would not appear to play a major part in localising infection by actinomycetes.

Leucocyte myeloperoxidase (Klebanoff, 1968) would be expected to contribute to the host resistance to actinomycetes. However, because myeloperoxidase has a requirement for peroxide it may become inactive once an anaerobic or microaerophilic environment is established within an avascular abscess.

The resistance of this particular strain of *A. viscosus* to the low concentration of penicillins was surprising in view of the sensitivity reported by Jordan & Howell (1965). However, *A. viscosus* and *T. sacchari* were sensitive to higher concentrations of the semi-synthetic penicillins, although, this may not represent useful *in vivo* sensitivity.

From the histological examination the organisms do not appear to produce tissue destroying enzymes and *A. viscosus* has been shown by Lederman, Hayashi & Bahn (1972) to be deficient in chondroitin sulphatase. However, cell-free culture filtrates of *M. chalcea* have been shown by Gacto & Villanueva (1971) to have a high proteolytic and β (1-3) glucanase activity, which if produced *in vivo*, could favour tissue destruction. Any such destruction, to allow the observed spread and penetration could result from a release of proteolytic enzymes from degranulating leucocytes around the nodule (Cline, 1970). However, this type of inflammatory response could result from a non-specific response to the injection of foreign material, although the absence of a reaction following the injection of killed cells argues against this being the case. The organisms certainly survive within the host and the presence of diphtheroid and filamentous forms following injection of spore suspensions suggests limited growth and differentiation.

The absence of tissue destruction and spreading along the tissue planes differentiates the type of infection produced from that seen with species of *Streptomyces*, e.g. *S. pelletieri*, which are not limited to the fascia but penetrate the muscles (Emmons, Binford & Utz, 1970). Following isolation from the infected areas the actinomycetes showed the expected morphological and biochemical characteristics, except that *M. chalcea* and *T. vulgaris* showed marked haemolytic properties. However, neither organism has been described as possessing this particular property (Bergey, 1957) and none of the organisms studied showed this property before injection. This suggests that enzymes favouring invasion may be induced *in vivo* which are not demonstrable

in vitro and that some limited growth, at least, must have occurred to permit such induction.

The problem encountered in isolating *A. viscosus* from nodules is not uncommon (Howard, 1961; Hanshaw, Griffith & Samitz, 1972; Emmons, Binford & Utz, 1970). The histopathological appearance resembles typical actinomycotic mycetoma in the presence of 'sulphur granules'. The pleomorphism observed is similar to that seen by Georg *et al.* (1972) and is not uncommon in fungal mycetoma (Howard, 1961) where the form seen *in vivo* may differ significantly from that seen typically *in vitro*.

The germination and outgrowth of spores of *T. vulgaris* and *T. sacchari* in tissues, as well as the survival of vegetative forms, show that the normal body temperature is adequate and that they could cause local infections in humans or animals. Also, the spores or vegetative cells of any of the actinomycetes studied, could be introduced into abrasions, or into the alimentary tract, of man or animals, and give rise to local infections.

T. vulgaris and *T. sacchari* are present in large numbers in certain natural environments (Lacey & Lacey, 1964 and Buechner, 1967). As well as being involved, in extrinsic allergic alveolitis they should be considered, along with other actinomycetes, whenever actinomycotic mycetoma are found.

ZUSAMMENFASSUNG

In Tierversuchen am Kaninchen wurde die Fähigkeit von *Thermoactinomyces vulgaris*, *T. sacchari* und *Micromonospora chalcea*, lokale Mycetome zu bilden, mit derjenigen von *Actinomyces viscosus* und *Streptomyces pelletieri* verglichen. Die subkutane oder intramuskuläre Injektion aller dieser Mikroorganismen führte zur Ausbildung von Knoten; histopathologisch fanden sich bei *T. vulgaris*, *M. chalcea*, *T. sacchari* und *A. viscosus* typische Drusen. Alle Mikroorganismen zeigten *in vitro* Sensibilität gegenüber Antibiotica.

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Lesions experimentally produced by fungi implicated in extrinsic allergic alveolitis

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SUMMARY

The ability of *Aureobasidium pullulans*, *Cladosporium herbarum* and *Cryptostroma corticale* to produce local lesions in the rabbit was examined. Both *C. corticale* and *A. pullulans* can survive *in vivo* and produce the inflammatory response typical of mycetoma. *C. herbarum* failed to grow or survive *in vivo*.

INTRODUCTION

The disease condition of extrinsic allergic alveolitis can be caused by several organisms, including both fungi and actinomycetes (Salvaggio & Buechner, 1971; Pepys, 1969). The actinomycetes which have been implicated, namely, *Thermoactinomyces vulgaris* and *Thermoactinomyces sacchari* have been shown to be capable of causing local mycetoma (Stretton & Bulman, 1974).

Cladosporium herbarum and *Aureobasidium (Pullularia) pullulans* are common airborne fungi which occur frequently on plants and other objects (Bernstein & Feinberg, 1942; Gregory & Lacey, 1963). Though *Aureobasidium pullulans* has been implicated by Cohen, Merigan, Kosek & Eldridge (1967) in sequoiosis, and *C. herbarum* has been associated by Bernton & Thorn (1937) and Tomsikova, Dura & Novackova (1973) with causing allergic syndromes, neither organism has been considered to be capable of causing local infection. Wynne & Gott (1956) have reported the isolation of *A. pullulans* from granulomas in patients with Hodgkin's disease and it has also been isolated from patients with rheumatoid arthritis (Arthritis & Rheumatism Council, 1966). However, it has been suggested by Emmons, Binford & Utz (1970) that *A. pullulans* can be equated with *Cladosporium werneckii*, an epidermal pathogen, although Cooke (1959) accepted them as different species.

Cryptostroma corticale is the causal agent of maple-bark disease (Towey, Sweany & Huron, 1932) and the organism was obtained by Emanuel, Wenzel & Lawton (1966) from the granulomatous nodules by lung biopsy.

This study examines the ability of these organisms as hyphae or spores to survive and produce local lesions, apart from the granulomatous nodules which occur in the lung.

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MATERIALS AND METHODS

Organisms

Cryptostroma corticale was isolated from infected maple bark, kindly provided by F. J. Wenzel, Marshfield Clinic, U.S.A. *Aureobasidium pullulans* CMI2456 (isolated originally from lymph node mycetoma) and *Cladosporium herbarum* CMI131128 were used throughout.

Media

The organisms were maintained on a medium containing (g./l.) mycological peptone, 10; and dextrose, 40. Solidified media were prepared by the addition of Oxoid Agar no. 3, 1.5%. The ability to grow on this medium was examined at 28° and 37° C.

Morphological characteristics

The organisms were examined for purity, including the presence of contaminants, before injection and after isolation from experimentally infected animals. *Cryptostroma corticale* was examined according to the description of Emanuel, Wenzel & Lawton (1966), *A. pullulans* according to the description of Wynne & Gott (1956) and *C. herbarum* by its morphological character. They were also examined for haemolytic activity on the nutrient medium with an added 5% v/v defibrinated horse blood.

Preparation of suspensions

Two ml. of a spore suspension, containing 10^6 spores/ml., were inoculated into 200 ml. of liquid medium for the production of the hyphal form and all organisms were incubated for 48 hr. at 28° C. The hyphae were removed by centrifugation at 4,000 g and washed 4 times with sterile normal saline (0.9% w/v) solution. On examination by phase-contrast microscopy these suspensions were free from spores.

Spore suspensions were prepared after inoculation on the surface of 200 ml. of solidified medium in Roux flasks and incubating for 6 days at 28° C. The spores were removed from the surface of the agar by washing with sterile normal saline and then depositing the spores by centrifugation at 4,000 g and washing 4 times with sterile normal saline.

The deposited cells were resuspended in sterile normal saline using a glass tissue homogenizer (Jencons (Scientific) Ltd., Herts) and the cell density adjusted to give *ca.* 10^2 spores/ml., or 10^2 hyphal fragments/ml. using a Thoma counting chamber. The cell density was also checked to be of the same order in each case by measuring the optical density at 420 nm. (SP500, Pye Unicam, Cambridge). The number of viable units was also checked by carrying out pour-plate counts before injection; in all cases this was *ca.* 10^2 /ml.

The suspensions were also plated out on the solidified medium and were found to be free from organisms other than the required fungi.

Suspensions of killed cells were prepared by dividing the above suspensions and heating in an autoclave at 120° C. for 20 min. The absence of viable organisms

from these suspensions was checked by streaking out and by the inoculation of 1 ml. of the suspension into nutrient medium and incubating at 28° C.

Experimental animals and injection procedure

Male Dutch rabbits weighing 2.0 ± 0.4 kg. were used throughout. When the intramuscular (i.m.) route was employed 0.3 ml. of the required suspension was injected into the flank and for the subcutaneous (s.c.) route, 0.2 ml. The rectal temperature was measured daily after injection. Rabbits were killed at intervals of 7 and 14 days after injection.

After necropsy samples of kidney, liver, spleen and any obviously infected tissue were inoculated on nutrient medium and incubated aerobically. Also, representative samples of tissue, including obvious lesions, were excised, fixed immediately in mercuric chloride-formalin solution, dehydrated and embedded in paraffin. Sections of 5 μ m. thickness were cut and stained with haematoxylin and eosin, Gram stain, periodic acid Schiff (PAS) and for calcification by the alizarin red S method (Pearse, 1972). Smears prepared from the pus were also examined immediately after necropsy using the PAS stain.

Samples of the pus were checked, by streaking out on the maintenance medium and nutrient agar, for the presence of organisms other than the appropriate organism.

RESULTS

All the organisms grew well on the medium described at 28° C. *C. corticale* grew slowly at 37°. *Aureobasidium pullulans* gave very scanty growth at 37° and *C. herbarum* failed to grow at all at 37°. There was no detectable haemolysis, before or after isolation, of any organism from the rabbit.

There was no significant alteration in the body temperature following injection of any of the organisms.

In the case of *C. corticale* and *A. pullulans* nodules were detected on necropsy when either hyphae or spores had been injected. There were no nodules present when *C. herbarum* was implicated.

The nodules produced by *A. pullulans* were always small (ca. 0.5 cm.) and located at the site of injection, typically in the m. semimembranosus (Pl. 1, fig. 1). The periphery of the nodule was avascular.

C. corticale produced larger nodules, ca. 3 cm. diameter, located typically on the m. semimembranosus (fig. 2). The periphery of the nodule was marked by an increased vascularity. The pus obtained from the nodule was pale yellow in colour, of a cream-like texture, and contained hyphae. Nodules were produced when either hyphae or spores were injected in 80 % of the animals, by the s.c. or i.m. routes.

In no case were the lesions seen to be spreading, and the nodules were all located at or near the site of initial infection. The *post mortem* examination showed no nodule present on the liver, spleen or kidney, nor were any of the organisms recovered from these organs.

In all cases where the killed cells were injected there was no reaction at the site

of injection nor was there any development of nodules, even at the site of the injection.

There were no organisms present in the samples of pus removed from the nodules other than those initially injected, as judged by their morphology and colonial appearance. Viable organisms were recovered from the nodules produced by *A. pullulans* and *C. corticale*; this included those occurring just at the site of injection. The pus from *C. corticale* contained *ca.* 100 viable units/ml. and *A. pullulans* 50/ml.

The histopathological examination revealed the presence of abscesses showing a typical inflammatory response. The type of abscess produced was similar when either *A. pullulans* or *C. corticale* was the cause (Pl. 1, fig. 3 and Pl. 2, fig. 4). There was evidence of necrosis of muscle fibres, particularly in the case of *C. corticale*, and fibrosis was noted around the periphery of the nodule (figs. 3 and 4). The organisms which produced the limited infections were present predominantly as hyphae, even after the injection of spore suspensions. In the nodules of *C. corticale* some spores remained ungerminated (fig. 5) and normal hyphae could be detected. There were fewer and shorter hyphae present when *A. pullulans* was seen in nodules (fig. 6). There was no evidence of calcification in any of the nodules, even after 14 days.

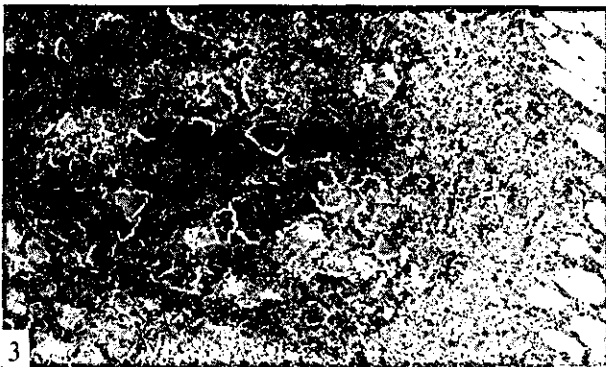
No organisms were detected in any of the organs examined histopathologically, only in the nodules themselves. There were no histological changes at the site of injection when killed cells had been injected.

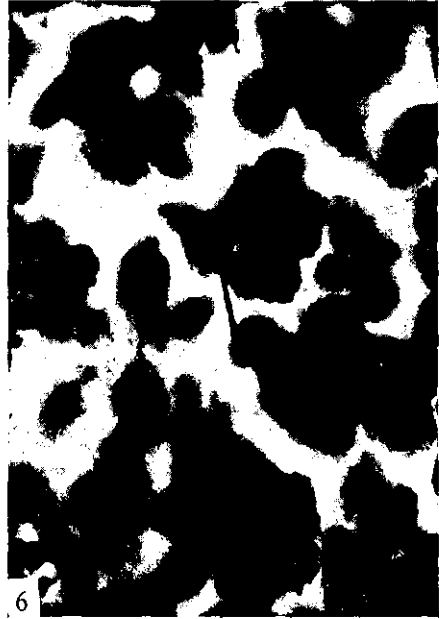
DISCUSSION

The ability of *C. corticale* and *A. pullulans* to produce local infections was somewhat unexpected in view of their apparent difficulty in growing at 37° C. *in vitro*. In fact Emanuel *et al.* (1966) stated that *C. corticale* did not grow at 37° C. We found that, although growth was slow, it did occur and subsequently F. J. Wenzel (personal communication) has confirmed this observation. The observation that *A. pullulans* and the plant pathogen *C. corticale* can produce local infection supports the evidence of Lie-Kian-Joe, Tjoei Eng, Kertopati & Emmons (1957) that a plant pathogen *Cercospora apii* can also cause mycetoma of the thigh and thorax as well as being the causal agent of leaf-spot disease.

The lack of growth of *C. herbarum*, *in vivo*, was not surprising in view of its lack of growth at 37° C. *in vitro*, and in this work it served as a suitable control representing a commonly saprophytic group with species which are occasionally pathogenic (Emmons *et al.* 1970).

Both *C. corticale* and *A. pullulans* can survive *in vivo*. The presence of hyphae in the nodules following injection of spore suspensions suggests that limited growth and some differentiation had taken place. Nodules similar to those produced in this instance can be produced by the injection of foreign material which gives a non-specific inflammatory response. However, this is unlikely to have happened, as the injection of heat-killed cells failed to produce nodules or elicit any response, as seen by histological examination.





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The organisms did not appear to produce any tissue-destroying enzymes, as judged from the histopathological examination. They were certainly deficient in haemolysin. The tissue destruction which would be necessary to allow even limited spreading could come from a release of proteolytic enzymes from the degranulating leucocytes (Cline, 1970) which are present around the periphery of the nodule.

The spread of infection is limited by fibrosis around the nodule, and similar fibrosis is common in cases of deposition of agricultural dusts in the lung (Rankin, Kobayashi, Barbee & Dickie, 1965). A feature of the nodules produced by these organisms is the absence of calcification, which in other infections may help to limit the spread of the infecting organisms.

The low numbers of organisms present in the nodule are not unexpected in view of the difficulty frequently encountered in obtaining the causal organism from such a lesion (Emmons *et al.* 1970). The pleomorphism exhibited in the case of *A. pullulans* is typical of several fungi where the form seen *in vivo* can differ significantly from that seen *in vitro*. This is seen in sporotrichosis (Howard, 1961) or in actinomycotic mycetoma (Georg *et al.* 1972; Stretton & Bulman, 1974).

With the organisms being present in large numbers in certain environments (Gregory & Lacey, 1963) and *A. pullulans*, in particular, being ubiquitous, the role of these fungi as possible opportunistic invaders may need reappraisal. Emmons (1962) considered the situation of several pathogenic fungi and concluded that so long as the environmental conditions were favourable they were vigorous and self-sufficient saprophytes and were parasites by accident. This is probably true of saprophytic fungi which are opportunistic invaders.

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EXPLANATION OF PLATES

PLATE 1

- Fig. 1. Nodules produced after 7 days following i.m. infection with *Aureobasidium pullulans* as a spore suspension located on the m. semimembranosus. $\times 1$.
- Fig. 2. Nodule produced after 7 days following i.m. infection with *Cryptosporium corticale* as a spore suspension, located on the m. semimembranosus. $\times 0.5$.
- Fig. 3. Nodule produced by *A. pullulans* after 7 days in the m. semimembranosus showing necrosis of muscle fibres and limited fibrosis. H. & E. $\times 400$.

PLATE 2

- Fig. 4. Nodule produced by *C. corticale* after 7 days in the m. semimembranosus showing necrotic muscle fibres, fibrosis and inflammatory response. H. & E. $\times 160$.
- Fig. 5. Hyphae and spores present in nodule produced by *C. corticale*. PAS, $\times 1000$.
- Fig. 6. Short hyphae present in nodule produced by *A. pullulans*. Gram $\times 1000$.

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A study of the morphology of some strains of *Actinomyces viscosus* using scanning electron microscopy

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Abstract

The morphology of five strains of *Actinomyces viscosus*, originally isolated from different sources, were examined using scanning electron microscopy and compared to *A. bovis*. A wide variation was observed in the form and arrangement of the cells.

Introduction

Negrani (1932) reported the isolation of an organism from human actinomycotic infection which he named *Actinomyces discofoliatus*. This has been reclassified as *A. viscosus* serotype 2 (Georg, Pine and Gerencser, 1969). Howell *et al.* (1959) described the occurrence of a variety of actinomycete-like organisms in the human oral activity. A number of Gram-positive, filamentous bacteria, isolated from rodents, were designated *Odontomyces viscosus* by Howell *et al.* (1959) and Howell *et al.* (1965); however, Georg *et al.* (1969) proposed that the organism be named *A. viscosus*. This organism has been implicated in periodontal infections in animal model experiments by Jordan and Keyes (1964), and Jordan, Fitzgerald and Stanley (1965). *A. viscosus* has been shown by Georg, Brown, Baker and Cassell (1972), Stretton and Bulman (1974), to be capable of producing localized actinomycetic mycetoma in animals.

The cellular morphology of 19 isolates, of human origin, of *A. viscosus*, was studied using light microscopy by Gerencser and Slack (1969) which they described as Gram-positive rods existing as diphtheroid cells and filaments. The rods exhibited X, Y and V forms with palisading. It was not possible to distinguish these strains, morphologically, from those isolated from hamsters.

The usefulness of scanning electron microscopy (SEM) for studying the morphology of actinomycetes has been demonstrated by Baldan, Locci and Baldan (1971), Williams and Davies (1967), and Williams, Hatfield and Mayfield (1970). The morphology of *A. naeslundii* and *A. odontolyticus* was studied by Boyd and Williams (1971) using this technique.

This study examines the differences in morphology of strains of *A. viscosus* originally isolated from different sources.

Materials and methods

The following cultures of *A. viscosus* kindly supplied by Dr M. A. Gerencser, West Virginia University, were used: WVU 745 (ATCC 15987, type strain, Howell T6) isolated from hamster plaque; WVU 440 (Howell HS-2) from hamster plaque; WVU 371 (ATCC 19246, serotype 2) from actinomycotic lesion; WVU 627 and 398B from human plaque. *A. bovis* NCTC 4500 was used.

Media

The strains of *A. viscosus* were maintained in a nutrient broth containing Oxoid beef extract, 10 g/l; Oxoid neutralized peptone, 10 g/l; sodium chloride, 5 g/l. For comparative purposes a tryptone-soya broth was also employed, containing Oxoid neutralized tryptone, 10 g/l; soya peptone, 5 g/l; sodium chloride, 5 g/l. Solidified media were prepared by the addition of 1.5% w/v Oxoid agar number 3. All media were adjusted to pH 7.2 prior to use.

All the strains of *A. viscosus* were grown aerobically in both static and shake culture, at 150 rpm, incubated at 37°C and samples removed at half-hourly intervals and examined by SEM.

Actinomyces bovis was grown on nutrient agar containing 5% v/v defibrinated horse blood in an atmosphere of CO₂:N₂ (5%:95%). When samples were required for SEM an elevated block of 6 mm diameter, of the blood agar was inoculated and covered with a sterile microscope cover-slip, incubated at 37°C and examined at hourly intervals.

Scanning electron microscopy

Glutaraldehyde (G. T. Gurr, Buckinghamshire, England) was added to broth cultures of the strains of *A. viscosus* to give a final concentration of 1.5% w/v. After 2 min contact the cells were removed by centrifugation at 3,000 × *g* at 4°C, for 15 min and resuspended in 2 ml of glutaraldehyde (5% w/v) in 0.25 strength Ringer's solution for 16 hr at 4°C. The cells were then removed by centrifugation (3,000 × *g* for 15 min) washed with distilled water and resuspended to give the required density. One drop of this suspension was allowed to air-dry on a microscope cover-slip and then dehydrated over CaCl₂ under partial vacuum. Several methods and times of drying were tried and this technique gave the optimum results. The samples were then coated with gold-palladium in a high vacuum unit to obtain a coating of ca. 10 nm thickness. The samples were examined in a Cambridge Stereoscan (Mark IIA, Cambridge Instrument Co. Ltd, Cambridge) with a beam-specimen angle of 45°, using a voltage of 30 kV. Photographs were obtained using FP4 film (Ilford).

The microscope cover-slip carrying the samples of *A. bovis* were immersed for 16 hr in glutaraldehyde (5% w/v) at 4°C, washed 3 times in distilled water, dehydrated and subsequently treated by the above procedure.

Results

A. viscosus WVU 745 grew best in shake culture and the 24 hr cells from nutrient broth showed that filamentous and cocco-bacilli forms were present (Figure 1). The development of cocco-bacilli appears to be from within fragmenting filamentous forms (Figure 2).

The rate of growth of *A. viscosus* WVU 371 was less than that observed with *A. viscosus* WVU 745. The filamentous form was the only distinguishable morphological form in either static or shake culture (Figure 3) even after 200 hr.

A. viscosus 440 showed typical actinomycotic morphology, having clearly branching filamentous forms (Figure 4) after 24 hr growth and fragmenting to cocco-bacilli after 48 hr (Figure 5).

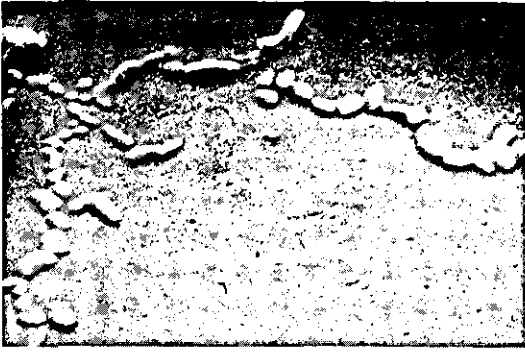


Figure 1 A 26 hr nutrient broth culture of *A. viscosus* WWU 745 showing the arrangement of the cocco-bacilli. $\times 4,500$.

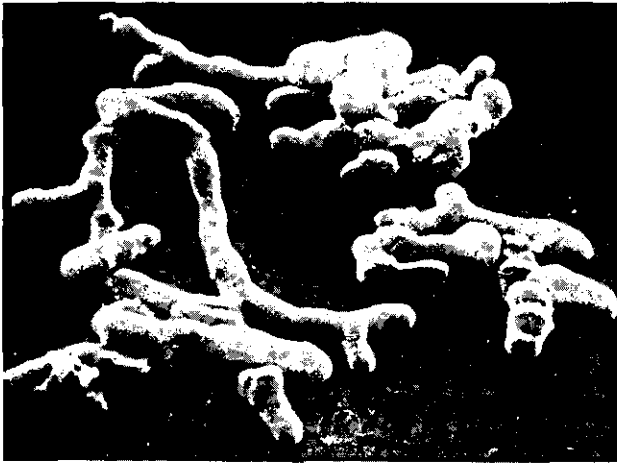


Figure 2 A 48 hr nutrient broth, static, culture of *A. viscosus* WWU 745 showing the filamentous form and the development of cocco-bacilli. $\times 10,000$.

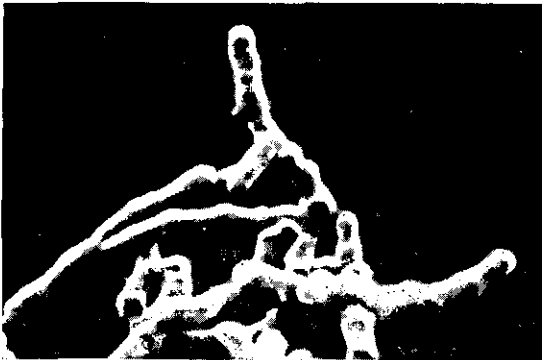


Figure 3 A 60 hr nutrient broth culture of *A. viscosus* WWU 371 showing the filamentous arrangement. $\times 5,000$.

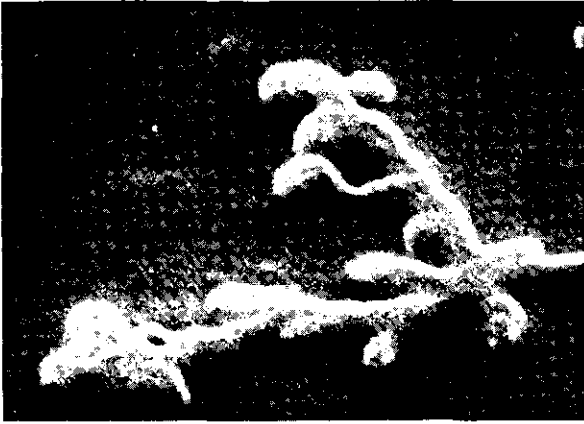


Figure 4 A 24 hr nutrient broth shake culture of *A. viscosus* WVU 440 showing branching filaments. $\times 20,000$.



Figure 5 A 48 hr nutrient broth culture of *A. viscosus* WVU 440 showing the typical coccus form. $\times 20,000$.

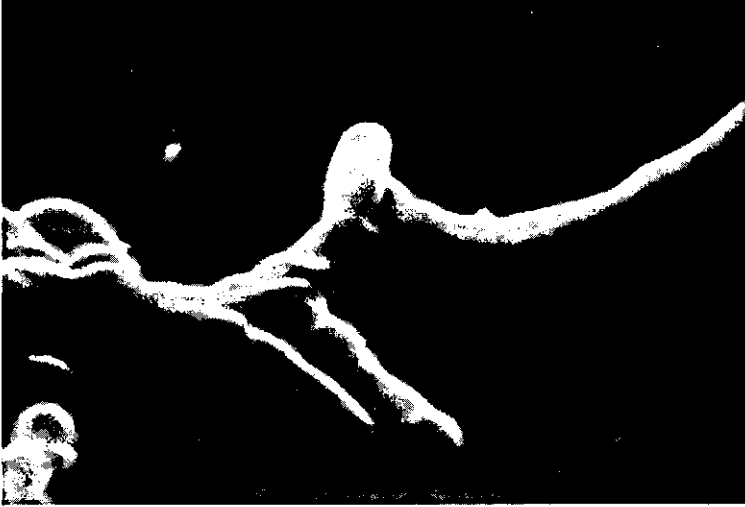


Figure 6 A 24 hr nutrient broth culture of *A. viscosus* WVU 627 showing the typical filamentous form. $\times 5,000$.

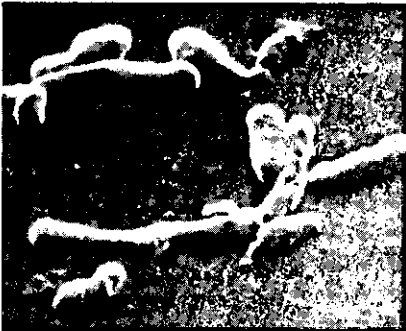


Figure 7 A 72 hr nutrient broth culture of *A. viscosus* WVU 627 showing the formation of cocco-bacilli. $\times 5,000$.



Figure 8 A 24 hr nutrient broth culture of *A. viscosus* WWU 398B showing the arrangement of short rods joined by what may be a sheath. $\times 10,000$.

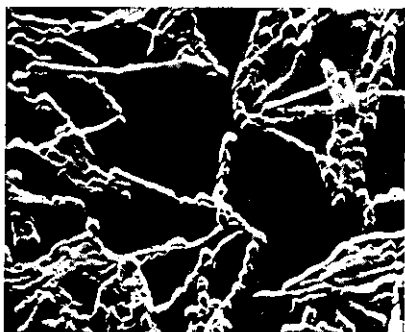


Figure 9 A 132 hr tryptone-soya broth culture of *A. viscosus* WWU 398B showing a fragmenting mycelial form. $\times 2,000$.



Figure 10 A 36 hr blood-agar culture of *A. bovis* showing the filamentous and rod-forms. $\times 10,000$.

A. viscosus WVU 627 was typically seen as filamentous organisms, with some evidence of branching (Figure 6) and grew well in shake culture. As the culture entered the stationary phase of growth some cocco-bacilli were observed (Figure 7).

The most aerophilic organism examined was *A. viscosus* WVU 398B which grew rapidly in nutrient broth in shake culture. The organism was filamentous in form (Figure 8) with little evidence of branching, although short rods were present which may be produced by breakdown of the filaments (Figure 9).

The form of *A. bovis* was very different being arranged in fine filaments which fragmented into short rods (Figure 10). There was a wide range in the diameters of the filaments from *A. viscosus* WVU 371, 0.4 μm and *A. viscosus* 398B, 0.9 μm ; all other strains being between these extremes.

Discussion

The morphological studies show that there is little uniformity of appearance within the organisms classified as *A. viscosus*.

Studies of the morphology of *A. viscosus* WVU 745, WVU 371 and WVU 440 using light microscopy were very difficult because the cells were so small. However, it was possible to examine *A. viscosus* WVU 627 and WVU 398B by the conventional methods, but in all cases the use of SEM, with its greater resolution, permitted greater morphological detail to be observed. The technique used preserved the structure without excessive vacuum damage and was, in this respect, superior to the techniques which rely on coating unfixed specimens, which produced extensive collapsing under vacuum. There was no significant difference in appearance if the dehydration was by the method described or using ethanol, although the latter technique causes a loss of lipid material from bacterial specimens. (McGee-Russell and de Bruyan, 1968; Silva *et al.*, 1971.)

The observed pattern of growth has also been seen in the species of *Nocardia* and *Mycobacterium* which fragment from the pseudomycelial form to the cocco-bacilli form (Csillag 1963, 1970). In addition, Nikitina and Kalakoutskii (1971) have shown that this type of growth can be induced when *Streptomyces* spp. are grown on media containing D-fructose.

In morphological appearance *A. viscosus* WVU 745, WVU 371 and WVU 627 resemble *A. naestlundii* and *A. odontolyticus* as demonstrated by Boyde and Williams (1971) using SEM and the cell dimensions are approximately equal in all cases. *Actinomyces viscosus* WVU 398B showed a similar arrangement of filaments but the overall cellular dimensions were greater than the preceding strains and ultimately rods were produced.

The morphological appearance of *A. viscosus* WVU 440 was somewhat different from the above strains as it presented initially a typical branched filamentous structure but fragmented ultimately to coccoid elements.

Actinomyces bovis, having an arrangement of fine filaments fragmenting into small rods, differed from all the strains of *A. viscosus* examined.

From a consideration of the morphology as revealed by SEM, of the organisms, classified as *A. viscosus* it may appear that the strains are very heterogenous and that on morphological grounds there may be a case for further classification.

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Effect of antimetabolites on differentiation in *Actinomyces viscosus* WVU 398B

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Abstract

The differentiation of *Actinomyces viscosus* WVU 398B from the mycelial form to the cocco-bacilli form can be prevented by inhibitors of peptidyl transferase or translocase. The cocco-bacilli form is produced at the end of active growth and does not have properties of heat resistance.

Introduction

In addition to being aetiological agents of actinomycosis (Georg and Coleman, 1970) *Actinomyces* sp. have been isolated from dental plaque (Jordan, Fitzgerald and Stanley, 1965) and caries (Howell, 1963). *Actinomyces viscosus* was formerly classified as *Odontomyces viscosus*, but, in view of its being catalase positive it has been reclassified (Howell, Jordan, Georg and Pine, 1965). It was originally isolated from the gingival plaque from hamsters with spontaneous periodontal disease, but now strains have been isolated from human sources (Gerencser and Slack, 1967). *Actinomyces viscosus* differs from other *Actinomyces* sp. in giving maximum growth under aerobic conditions. Apart from its involvement in periodontal disease *A. viscosus* has not been generally implicated in other human or animal infections although Georg and Coleman (1970) have shown it to be capable of producing suppurative lesions when inoculated into mice, dogs (Georg, Brown, Baker and Cassell, 1972) or rabbits (Stretton and Bulman, 1974).

Morphological variation in *Actinomyces* sp. is limited although Morris (1951) reported the existence of complicated life cycles for *A. bovis*. However, Erikson (1953) considered that facultative anaerobic cocci of various types can be carried for several generations within the entangled filamentous mass, and Erikson and Porteus (1953) have shown this to be the case. The diphtheroid form is said to become predominant, for example, when *A. israelii* is cultured aerobically (Erikson, 1953) and the existence of such a diphtheroid form has also been demonstrated by Jordan and Howell (1965) in the case of *A. viscosus*.

The effects of antibiotics on cell differentiation, as expressed by bacterial cell division, have been studied in several instances. The formation of filaments in Gram-negative bacteria exposed to novobiocin was observed by Smith, Dietz, Sokolski and Savage (1956), and chain formation in *Staphylococcus aureus* was seen by Greenwood and O'Grady (1972). Also, Newton (1972) examined the development of *Caulobacter crescentus* using rifampicin to prevent initiation of transcription.

The work reported here describes the action of antibiotics, whose mode of action is known, on the presence of differentiation from the mycelial to the cocco-bacilli form in *A. viscosus*.

Materials and methods

Organism

Actinomyces viscosus WVU 398B, kindly provided by Dr Mary A. Gerencser of West Virginia University, was used throughout this study.

Media

A nutrient broth containing 10 g of Oxoid beef extract, 10 g of Oxoid neutralized peptone, 5 g of sodium chloride and water to 1 litre, was used for the maintenance of the organism. In addition the synthetic medium of Hoeprich, Barry and Gay (1971) and tryptone-soya medium, containing 15 g of Oxoid neutralized tryptone, 5 g of soya peptone (Oxoid), 5 g of sodium chloride, and water to 1 litre, were used. Solidified media were prepared by the addition of 1.5% (w/v) agar (Oxoid number 3). The final pH of all media was adjusted to 7.2.

Chemicals

The antimetabolites used were: 2,4-dinitrophenol, cetyltrimethyl ammonium bromide (CTAB) and acridine orange (BDH Ltd, Poole, England), bacitracin neomycin sulphate and chloramphenicol (Riker Laboratories, Loughborough, England), phosphonomycin disodium salt (Merck, Sharp and Dohme, Ltd, Rahway, New Jersey, U.S.A.) rifampicin (Lepetit Pharmaceuticals Ltd, Maidenhead, England), streptomycin sulphate and ethidium bromide (Boots Ltd, Nottingham, England) D-cycloserine and vancomycin hydrochloride (Eli Lilly Co. Ltd, Basingstoke, England) sodium salt of fusidic acid (Leo Laboratories, Hayes, England), gentamicin sulphate (British Schering Ltd, Slough, England) β -phenylethyl alcohol and gramicidin J (Sigma Chemical Co., London, England) pentamidine and griseofulvin (May and Baker Ltd, Dagenham, England) lincomycin and clindamycin (Upjohn Ltd, Crawley, England) oxytetracycline and chlorotetracycline (Pfizer Ltd, Sandwich, England) colchicine (Arthur H. Cox Co. Ltd, England) 3-chloropropane-1,2-diol and dithiothreitol (Koch-Light Laboratories Ltd, Colnbrook, England), methicillin (Beecham Research Laboratories, Brentford, England) and benzylpenicillin (Glaxo Laboratories, Greenford, England), and chloroquine phosphate (Imperial Chemical Industries Ltd, Pharmaceutical Division).

The enzyme lysozyme (Boehringer Corporation (London) Ltd, England) and wheat germ lipase (Sigma Chemical Co., London, England) were used. All other chemicals used were of analytical reagent grade.

Preparation of *Actinomyces* suspensions

The organisms were obtained either by shake culture at 200 rpm or by aerating 5 litres of medium, at a rate of 2 litres of air per min. Incubation in all cases was at 37°C. The cells were removed by centrifugation at $5,000 \times g$ for 15 min at 4°C, or $2,500 \times g$ for 30 min at 4°C.

Examination of the influence of various factors on growth and morphology

The effects of several treatments of growing cells of *A. viscosus* were examined to determine the events occurring during cell growth and differentiation.

(a) Gradient-plate technique for sensitivity to antimetabolites

The method of Szybalski and Bryson (1952) was used with Petri dishes 10 cm \times 10 cm (Sterilin, Richmond). The quality of antimetabolite to give the required

concentration was dissolved in 50 ml of nutrient agar and this constituted the lower layer. When this wedge-shaped layer had solidified a further 50 ml of nutrient agar was added and the antimetabolite allowed to diffuse for 8 hr, at 4°C, to establish the gradient of antimetabolite concentration. Then a loopful of an 18 broth culture of *A. viscosus* was streaked on to the surface from the region of minimum to maximum concentration of antimetabolite. The plates were then incubated at 37°C for 20 hr.

(b) Chemical treatment

Actinomyces viscosus cells from a 24 hr broth culture were suspended to give a final concentration of 2 mg dry weight/ml and shaken with dithiothreitol (100 µg/ml) for 90 min or CTAB (2 mg/ml) for 60 min or DMSO (5 mg/ml) for 24 hr, at 37°C.

(c) Enzymatic treatment

Wheat germ lipase (500 µg/ml) was used to 50 mg of *A. viscosus* cells, from a 24 hr broth culture, in 25 ml of phosphate buffer 0.01 M, pH 7.4, and the resulting suspension shaken for 12 hr. A similar cell suspension was shaken with lysozyme under the same conditions.

(d) Nature of vacuolation

Actinomyces viscosus was grown in shakeculture for 12 hr in either tryptone-soya broth (TS) or in nutrient broth (NB). The cells were harvested and washed 3 times with sterile saline (0.85% w/v) and the cell masses so obtained were divided into two portions (in both cases) and one sample stored for 98 days at 20°C. The remaining fraction was incubated in the other, alternative, medium for a further 14 hr.

(e) Estimation of heat resistance

Cells from a 24 hr TS broth culture were washed and suspended in distilled water to give a cell density of 2.0 OD at 420 nm. The suspension was heated, in a thin-walled glass vessel, at 98°C and 1 ml samples were removed at intervals of 2, 5, 7 and 10 min then plated out onto nutrient agar and incubated at 30°C. The heat resistance was also examined at 80°C.

Microscopy

The routine examination of samples and phase contrast microscopy was carried out using a Vickers M25 series microscope. Dried and fixed bacterial films were stained with Loeffler's methylene blue or the cell wall stain of Robinow (1949), or Burdon's Sudan black stain (1946), or the spore stains of Lechtman *et al.* (1965) and Ashby's (1968) modification of the Schaeffer and Fulton stain. Light micrographs were obtained using a Leitz Photoplan and FP 4 (Ilford) film.

Scanning electron microscopy (SEM) was performed by the method of Bulman and Stretton (1974a).

Determination of dehydrogenase activity

The dehydrogenase activity of cell suspensions were estimated, under aerobic conditions, by the triphenyltetrazolium chloride (TTC) method of Hugo (1954) using a system containing, in 5 ml, 0.5 mg dry weight of cells, 200 µg/ml TTC, glucose 0.05 M and phosphate buffer 0.01 M, pH 7.2. The resulting formazan was estimated at 525 nm using an SP500 (Cambridge Instruments Co. Ltd, Cambridge, England).

Growth curves

The patterns of growth in both nutrient broth and tryptone-soya broth were determined by removing the cells from 100 ml of the culture by centrifugation and lyophilizing the washed cells. The dried cell mass was then weighed.

Results

Microscopic appearance

From the series of both light and scanning electron micrographs it became apparent that after 14 hr in nutrient broth or 20 hr in tryptone-soya broth a cellular event was taking place which resulted in the clearing of the cytoplasm (Figure 1) and to a collapse of part of the filament wall (Figure 2). This event for the sake of brevity, we termed vacuolation.

Vacuolation occurred rapidly in nutrient broth and in synthetic medium, in all cases within 20 hr, however, in the synthetic medium low yield was obtained, as judged by the cell mass. Vacuolation occurred whether the organism was grown aerobically or in an atmosphere of CO₂/N₂. When the growth rate was followed it became apparent that vacuolation started at the end of active growth in nutrient broth and was followed by a fall in dry weight (Figure 4).

No positive results were obtained with the lipid stain, suggesting that the clear regions seen by phase contrast microscopy areas are not fat inclusions. Similarly, the cell-wall stain revealed no unusual features. However, the spore staining procedure of Lechtmann *et al.* (1965) removed a large number of the vacuoles, but small vacuoles remained and no structure stained with any spore stain. The spore stain of Schaeffer and Fulton left a large number of the vacuoles intact.

Effect of antimetabolites

There was no alteration in the appearance of the cells particularly of vacuolation when certain inhibitors of nucleic acid and protein synthesis were used, namely, chloroquine, chloramphenicol, acridine orange, lincomycin, neomycin, gentamicin, ethidium bromide, oxytetracycline or phenylethyl alcohol. On the other hand, sodium fusidate, chlorotetracycline, clindamycin and rifampicin, which do inhibit protein synthesis, did prevent vacuolation taking place either completely or to a significant extent.

The inhibition of peptidoglycan synthesis by benzylpenicillin, D-cycloserine, gramicidin J, bacitracin, vancomycin or phosphonomycin did not affect vacuolation. In some instances methicillin inhibited vacuolation, although when the cells were shaken in nutrient or tryptone-soya broth, for 5 hr, and methicillin added to give a final concentration ($\mu\text{g/ml}$) of 20, 40 or 170 no detectable vacuolation occurred.

There was no suppression of vacuolation when griseofulvin, 2,4-dinitrophenol, pentamidine or 3-chloropropane-1,2-diol were used.

Effect of chemical treatment

Treatment with dithiothreitol solubilized a major portion of the material comprising the vacuole region, although viable cells were recovered even after 5 hr of this treatment. Many of the cells which were in long filaments still retained vacuoles but the diameter of the filament was reduced. However, the large vacuoles were completely solubilized.

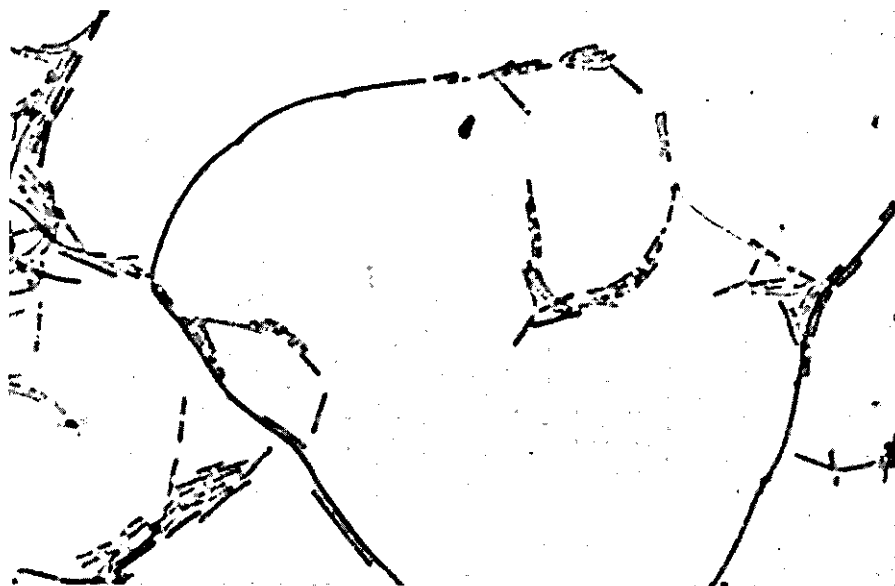


Figure 1 Light microscopy of *Actinomyces viscosus* grown in tryptone-soya broth for 24 hr showing clear areas in the cytoplasm. Methylene blue stain. $\times 1,400$.

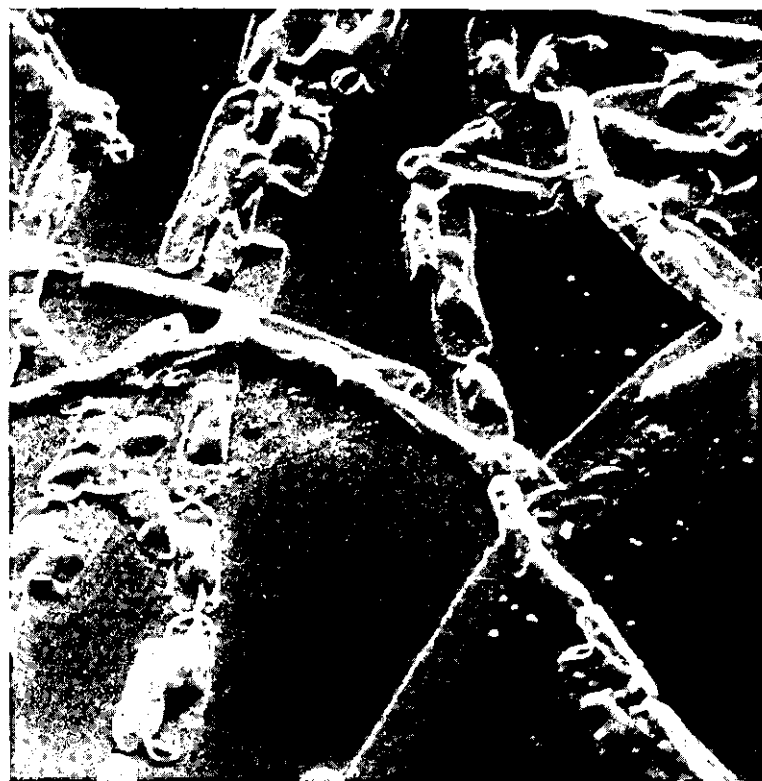


Figure 2 Scanning electron microscopy of *Actinomyces viscosus* grown in nutrient broth for 40 hr, showing collapse of the mycelial form to give a sheath like appearance with cocco-bacilli forms. $\times 9,500$.

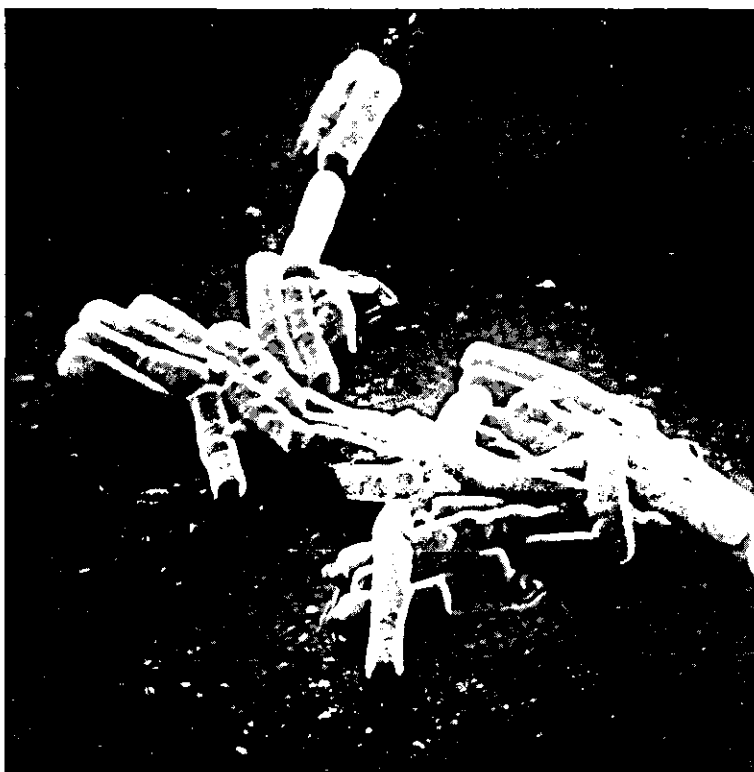


Figure 3 Scanning electron microscopy of *Actinomyces viscosus* grown in nutrient broth for 30 hr and treated with GAB (2 mg/ml) showing the presence of cocco-bacilli forms and little evidence of cell collapse. $\times 5,000$.

In addition, CTAB removed much of the vacuole region, this was readily detectable by SEM (Figure 5). Treatment with DMSO had no detectable effect.

Heat resistance

The mycelial form, was completely inactivated after 10 min at 98°C and after 15 min at 80°C . The vacuolate cells had 50% survivors at 2 min, 20% at 5 min, 1.0% at 7 min and 0% at 10 min. When survival at 80°C was examined there was no significant difference from the results obtained at 98°C .

Effect of enzymatic treatment

When the cells were treated with wheat germ lipase the large empty vacuoles disappeared and the remaining cell stained very lightly, if at all, with Loeffler's methylene blue. The large vacuoles were removed when the cells were treated with lysozyme although the filaments stained normally with Loeffler's methylene blue. In this case, a number of small vacuoles were observed although these may be produced by digestion of the filaments.

Nature of vacuolation

The cells subjected to the medium exchange TS to NB were composed of strings of partially vacuolated cells of very small diameter. After 2 hr a few filaments

characteristic of cells grown in NB were seen and after 18 hr the cells appeared as cocci-bacilli with only a few filamentous forms. When the medium change was NB to TS the cells were in short filaments containing a few vacuoles. The large vacuoles characteristic of cells grown for 24 hr in NB were absent. After 48 hr the cells assumed the form characteristic of growth in TS, although some filaments with small vacuoles were present, and after 60 hr the coccobacilli form predominated.

When the cells stored at -20°C for 98 days were returned to the freshly prepared appropriate broth (NB to NB, and TS to TS) growth was normal.

Dehydrogenase activity

The dehydrogenase activity, as shown by TTC reduction, remained constant during the lag and early log phase growth but was reduced by 80% in nutrient broth cultures after 14 hr and by 70% in tryptone-soya broth cultures after 20 hr.

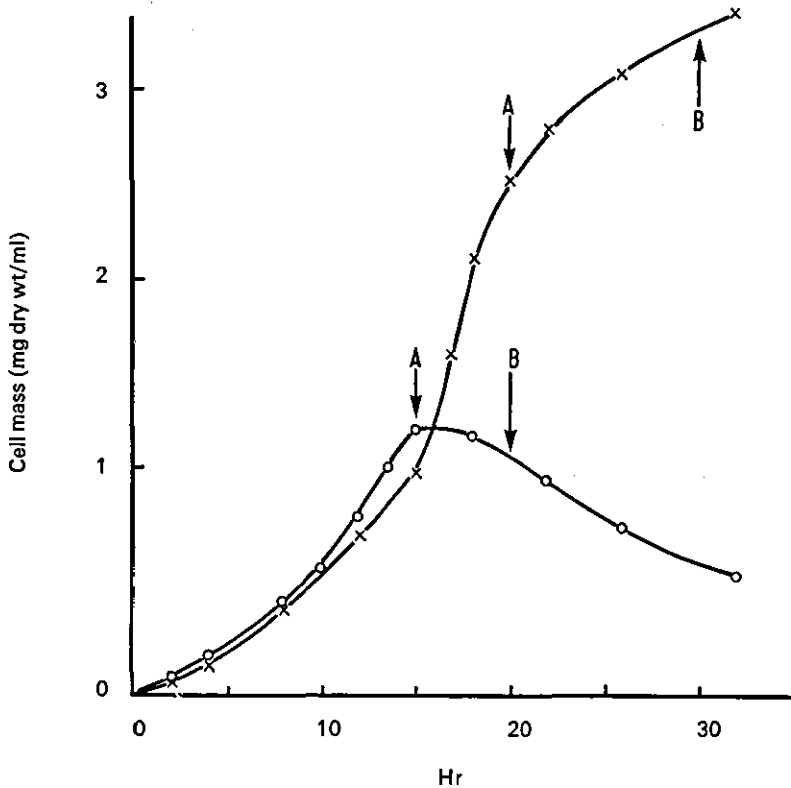


Figure 4 Growth of *Actinomyces viscosus* in tryptone soya broth (O—O) and nutrient broth (X—X). A indicates the onset of vacuolation and B when 90% of the cells had a vacuolate appearance.

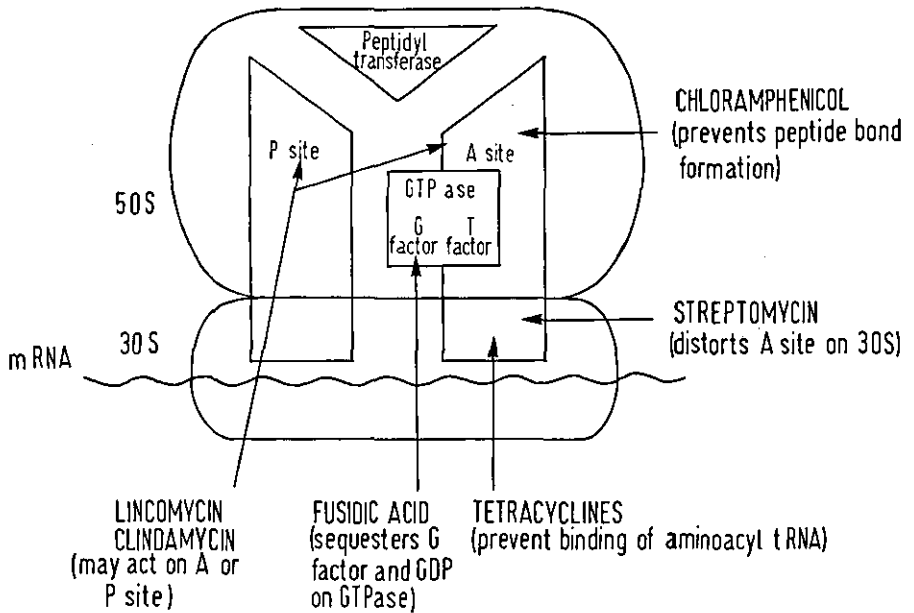


Figure 5 Diagrammatic representation of the binding sites of the inhibitors of ribosome function.

Occurrence of vacuolation and growth phase

Vacuolation occurred at a late stage in the growth cycle, in nutrient broth less than 25% of the cells were vacuolate after 15 hr, but 50% were vacuolate after 20 hr and extensive vacuolation *ca.* 90% had occurred after 21 hr (Figure 4). A similar pattern was observed (Figure 1) in tryptone-soya broth cultures but the onset of vacuolation was delayed until the stationary phase at *ca.* 15 hr and 90% vacuolation occurred after 20 hr growth.

Discussion

The sheath, seen by SEM, surrounding the bacillus form and which gives a filamentous appearance when seen by light microscopy, resembles that observed by Locci and Baldan (1971) in *Planobispora longispora*. This particular feature was not observed in 4 other strains of *A. viscosus* (Bulman and Stretton, 1974a) nor in *A. naeslundii* or *A. odontolyticus* (Boyd and Williams, 1971).

When the organism was treated with a variety of compounds, dithiothreitol, lipase, lysozyme or CTAB some of the surface layer was removed in all cases, but it was never removed completely. This indicates that the outer layer is not a simple compound but must be a complex material.

The presence of vacuoles which were readily detectable by light microscopy was a distinct feature of this strain of *A. viscosus*. When 4 other strains of this organism and *A. bovis* were examined vacuoles may have been present but it proved difficult to be certain because of the small diameter of the cells and their presence was not confirmed by SEM (Bulman and Stretton, 1974a).

The production of vacuoles is probably a process of cell differentiation and may be a stage in the development of the cocco-bacilli form, as it occurs at a late stage in growth, which is seen in *Actinomyces* sp. growing *in vitro* but is rarely seen in the *in vivo* infections of *A. israelii* (Cruikshank, 1970). The transition from a mycelial to the cocco-bacilli form probably demands a reorganization of the cytoplasm and for cross walls to form and it occurs at a stage when cell metabolism, as judged to dehydrogenase activity, is low. In *Streptomyces* sp. this form can be produced by alteration of nutrients in the growth medium (Nikitina and Kalakoutskii, 1971). Species of *Mycobacterium* and *Nocardia* have also been shown by Csillag (1963, 1970) to fragment readily from a pseudomycelial form to the cocco-bacilli form.

The process which occurs in *A. viscosus* does not lead to the production of a form which is more heat resistant than the original mycelial form. This is a difference from other actinomycetes which differentiate to form spores or with bacterial endospores where the differentiated spore form is more heat resistant than the vegetative state.

The gradual development of vacuoles over several hours and their appearance in large numbers at a definite time in the growth cycle suggests a process under enzyme control. In addition, the results obtained when the commitment to vacuolation was studied indicates that the process may be similar to that observed in spore-forming bacteria when they continue to sporulate, or germinate, on being transferred to an appropriate nutrient medium (Jordan and Murrell, 1967). If this was so, then the cells would be already genetically committed to vacuolation and this particular aspect was examined, to some extent, by determining the effect, on this event, of antimetabolites whose specific mode of action is known.

Inhibitors of nucleic acid and protein synthesis, at all levels, namely, ethidium bromide, colchicine, chloroquine, acridine orange and β -phenylethyl alcohol did not suppress vacuolation at all. However, partial suppression of vacuolation was observed by compounds in high concentration, which inhibit ribosome function (Figure 5). The compounds in question being neomycin, streptomycin and gentamicin. Streptomycin, in the appropriate system, inhibits all aspects of protein synthesis, *i.e.* initiation, elongation and termination. It can enhance ambiguity of translation of m-RNA as do neomycin and gentamicin. Such effects could reflect an impairment of functions associated with the 30S moiety and in particular with ribosomal A site (Gale *et al.*, 1972).

Chloramphenicol did not suppress vacuolation, and, because it inhibits peptide chain elongation and the movement of ribosomes along the m-RNA it is unlikely that vacuolation is controlled at this point. The mode of action of chloramphenicol is possibly the result of blocking interaction between enzyme and substrate at the 50S moiety of the ribosomal A site (Vazquez, 1964, 1966).

Other inhibitors of protein synthesis at the ribosomal level, lincomycin and clindamycin had interesting effects. Lincomycin showed little or no inhibition of vacuolation but clindamycin, the 7-deoxy-7(S)-chloro derivative of lincomycin hydrochloride, suppressed vacuolation. The biological activity of lincomycin and clindamycin is dependent upon the alkyl substituents of the sulphur atom and the ring nitrogen atom. Lincomycin inhibits bacterial protein synthesis

(particularly in Gram-positive cells) without inhibiting RNA or DNA synthesis (Josten and Allen, 1964). The wider spectrum of activity of clindamycin has been attributed to its increased ease of penetration into cells (Gale *et al.*, 1972). There is some dispute regarding the precise mode of action of lincomycin. It may act on the peptidyl 'transferase' which is an integral part of the 50S subunit (Chang, Sih and Weisblum, 1966; Vazquez and Monro, 1967; Wilhelm, Oleinick and Corcoran, 1967) which is also the site for aminoacyl t-RNA. Or, a site of interaction may include the 'translocase' factor which promotes the transfer of peptidyl t-RNA from the acceptor site to the donor site (Monro and Vazquez, 1967). The translocase is dependent upon the G-factor catalytic hydrolysis of guanosine triphosphate (GTP). On the other hand, microbial kinetics suggest (Mielk and Garret, 1969) that lincomycin possesses two modes of action which have been attributed to (1) an impairment of t-RNA by binding of the drug to the 50S subunit, and (2) a possible interference in the synthesis and utilization of a stored metabolite. Fusidic acid appears to act indirectly with the G-factor (and subsequently with the 50S subunit) and translocation is suppressed by prevention of repeated hydrolysis of GTP (Gale *et al.*, 1972). As vacuolation was readily suppressed by fusidic acid, it appears that the event could be most sensitive to inhibitors of transferase or translocase.

An apparently anomalous result was seen with rifampicin, a semi-synthetic macrolide antibiotic, which markedly suppressed vacuolation. Rifampicin acts by binding to the β -subunit of RNA polymerase, without suppressing DNA polymerase (Wehrli and Staehelin, 1970). The bacterial RNA polymerase is inhibited 50% by 2×10^{-8} M rifampicin (Hartmann *et al.*, 1967). The macrocyclic ring of rifampicin binds it to the RNA polymerase whilst the other parts of the molecule may modify the permeability of the organism (Wehrli and Staehelin 1968). The situation where vacuolation is suppressed may be similar to the inhibition of stalk formation and cell division in *C. crescentus* observed by Newton (1972). He attributed the action of rifampicin in this case primarily to the inhibition of transcription (Wehrli *et al.*, 1968) although other levels of control could be operating.

The partial suppression of vacuolation seen with tetracycline may be due to an impairment of membrane permeability by chelation with divalent cations (Laskin, 1967). However, Gale *et al.* (1972) have suggested that although tetracyclines act against the 30S moiety of the ribosomal A site they may bind elsewhere on the ribosome also and it may be that this latter effect could prevent vacuolation to some extent.

Other metabolic events investigated were those involving the cell membrane because this might be directly implicated when the action of tetracycline or rifampicin were considered. However, the compounds studied, namely 2,4-dinitrophenol, an uncoupler of oxidative phosphorylation (Harris, Hofer and Pressman, 1967); 3-chloropropane-1,2-diol; a glycerol antagonist which impairs synthesis of the cell membrane (Bulman and Stretton, to be published) or pentamidine; which suppresses nucleic acid and phospholipid synthesis in *Staphylococcus aureus* (Gale and Folkes, 1967), were without effect. It seems probable that the cell membrane metabolism and composition has no primary role in this process of differentiation.

The inhibitors of cell wall synthesis, at all stages, namely, benzylpenicillin, gramicidin J, bacitracin, phosphonomycin, vancomycin and D-cycloserine were without effect on vacuolation. However, methicillin, in high concentrations ($10 \times \text{MIC}$) did inhibit vacuolation, this may be a secondary effect as there was no evidence for cell wall damage, at this concentration of methicillin, as judged by SEM.

As vacuolation seems to be mainly dependent on protein synthesis, at the ribosome level, and occurs at the end of log phase growth and may be enzymatically controlled it is significant that an early event in bacterial sporulation is the production or activation of a proteolytic enzyme. However, such an event is not just specifically concerned with sporulation but it also occurs at the more elementary level of *Escherichia coli* when the culture is starved and stops growing, it is possibly a primitive adaptation mechanism which allows an organism to change its pattern under conditions where net synthesis is impossible (Mandelstam, 1960). Also proteolysis is important in differentiation higher in the evolutionary scale, e.g. during sporulation of yeast (Meller and Hofmann-Ostenhof, 1964) and at a stage in the development of the slime mould (Sussman, 1966; Sussman and Sussman, 1969; Wright, 1966). Similar developmental changes exist in the alga *Acetabularia* in which extensive developmental changes can occur some weeks after removal of the nucleus (Harris, 1968). In the slime moulds and sporulating *Bacilli* it appears that regulation exists both at the level of transcription and of translation and that a similar phenomenon may control vacuolation in *Actinomyces viscosus*.

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Effects of the lanthanides, lanthanum and neodymium, on the morphology and size of *Scenedesmus* sp.

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Abstract

The lanthanides, lanthanum and neodymium, can replace calcium in the growth medium for *Scenedesmus* as judged by cell size and morphology. The replacement is not absolute as the spherical cells characteristic of calcium-free medium are also seen when the lanthanides are used. The addition of the calcium ionophore X-537A leads to an increase in the number of cells less than 1.5 μm in size.

Introduction

Calcium is a ubiquitous element and is involved in a wide variety of essential cellular reactions in both plants and animals. It is a well known trace element for micro-algae (Stegman, 1940; and Walker, 1956) and has been shown to control cell shape and possibly cell division in *Scenedesmus* (Kylin and Das, 1967).

Snellen and Raj (1970) have shown that if calcium is absent from the culture medium of *Leucothrix mucor*, a colourless derivative of a marine blue-green alga, filamentous growth is produced with an unusual cord-like appearance.

The biochemical studies of Das (1968) showed that calcium promoted phosphate assimilation and decreased the ATP level in synchronously cultured cells of *Scenedesmus* whilst the synthesis of chlorophyll, carotene, nucleic acid and protein was increased (Das, 1973).

Walker (1956) and Kylin and Das (1967) have shown that in some algae calcium can be replaced by strontium, but in other strains this is either toxic or without effect.

This study examines the ability of the lanthanides, lanthanum and neodymium to replace calcium in *Scenedesmus* sp.

Materials and methods

Organism

The *Scenedesmus* sp. used was kindly provided by Anders Kylin (Botanical Institute, Stockholm University, Sweden).

Cleaning of apparatus

All glassware was of Pyrex quality and was soaked in EDTA solution (3% w/v) for 2 periods of 48 hr and was then rinsed 5 times with triple glass-distilled water.

Medium and cultural conditions

The organism was grown at 25°C in a medium containing KNO_3 , 25 mM; MgSO_4 , 2.13 mM; KH_2PO_4 , 1 mM; sodium edetate, 0.15 mM; to this was added the iron and trace elements solution of Wintermans (1958). Lanthanum nitrate

(Koch-Light Ltd) or neodymium nitrate (Hopkins and Williams Ltd) were added to give a final concentration of 0.075 mM, this being non toxic to the culture. Calcium nitrate was added to give a final concentration of 0.1 mM. When the calcium ionophore X-537A (kindly provided by Dr J. Berger, Hoffman-La Roche, New Jersey, U.S.A.) was used it was sterilized by membrane filtration (Sartorius Membranfilter, V. A. Howe and Co. Ltd, London, England) 0.2 μ m porosity; and added to give a final concentration of 3.5 μ g/ml. Triple-glass distilled water was used for the preparation of all solutions and media. The flasks were shaken at 100 rpm under illumination from daylight fluorescent tubes and tungsten lamps, from above and from the sides. The intensity at the level of the flasks was estimated to be between 12,000 and 20,000 ergs/sec/cm². Growth continued for 10 days, the volume of medium being kept constant by the addition of sterile water.

Analysis of cell size

The algae were harvested after 10 days by centrifugation, washed with NaCl, 30 mM; and resuspended in the saline solution to give a cell density of *ca* 1.5×10^7 cells/ml. The cell size was then determined on a calibrated 14 channel Model T (Coulter Electronics Inc., Florida, U.S.A.).

Scanning electron microscopy (SEM)

Glutaraldehyde (G. T. Gurr, Buckinghamshire, England) was added to the culture to give a final concentration of 1.5% w/v. The cells were removed, after 2 min contact, by centrifugation at $3,000 \times g$, for 15 min at 4°C, then resuspended, in 2 ml of glutaraldehyde (5% w/v) in 0.25 strength Ringers solution, for 16 hr at 4°C. The cells were removed by centrifugation, washed 3 times with distilled water and resuspended to give the required cell density. One drop of this suspension was allowed to air dry on a microscope cover slip and then dehydrated over CaCl₂ under partial vacuum. The samples were coated, in a high vacuum unit, with gold-palladium to give a coating of *ca.* 10 nm thickness and examined in a Cambridge Stereoscan (Mark IIA, Cambridge Instruments Co. Ltd, Cambridge, England) with a beam specimen angle of 45° and a voltage of 30 kV. Photographs were obtained using FP4 film (Ilford).

Calcium staining

The technique was adapted from Shida (1970). Alcoholic glyoxal *bis*-8-hydroxy-anil (GBHA, Sigma Ltd) solution was prepared by dissolving GBHA, 400 mg; in absolute ethanol, 100 ml. Two ml of this solution was diluted to 100 ml with 0.3 ml of 5% w/v NaOH solution and distilled water. This latter solution was applied to a smear of cells which had been washed 3 times with distilled water to remove any contaminating calcium salts. After 3 min contact the excess stain was removed by washing with 70% ethanol and the slide immersed for 15 min in alcoholic sodium carbonate-potassium cyanide (90% ethanol saturated with sodium carbonate and potassium cyanide). The slide was rinsed twice with 95% ethanol and mounted in glycerol.

This procedure stains for calcium, barium, strontium, cadmium, copper, cobalt and nickel forming red or blue-purple precipitates. However, the sodium carbonate-potassium cyanide decolorizes all precipitates except those with calcium. As a control, cells which grew in a calcium-free medium were

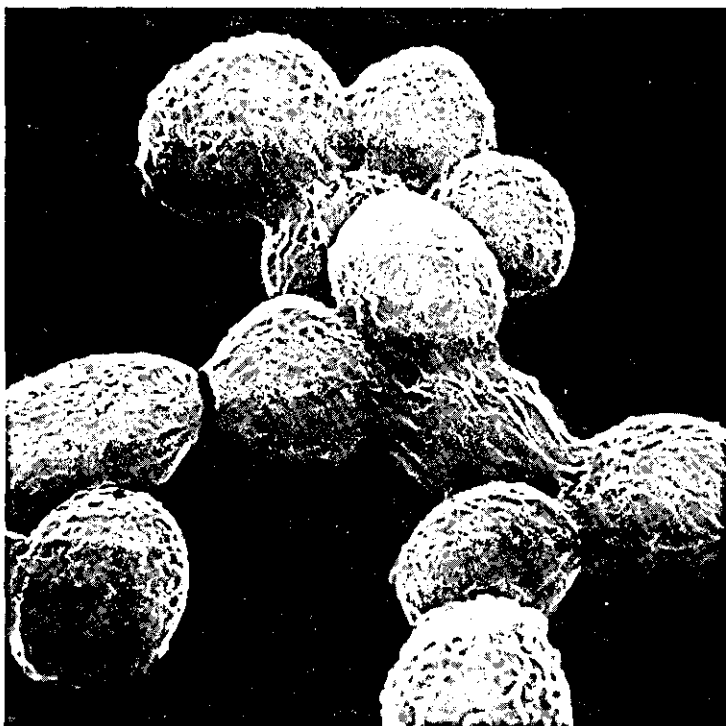


Figure 1 Scanning electron microscopy of *Scenedesmus* sp. grown for 5 days in a calcium free medium, $\times 5,000$.



Figure 2 Scanning electron microscopy of *Scenedesmus* sp. grown for 5 days in a medium containing 0.1 mM calcium, $\times 5,000$.

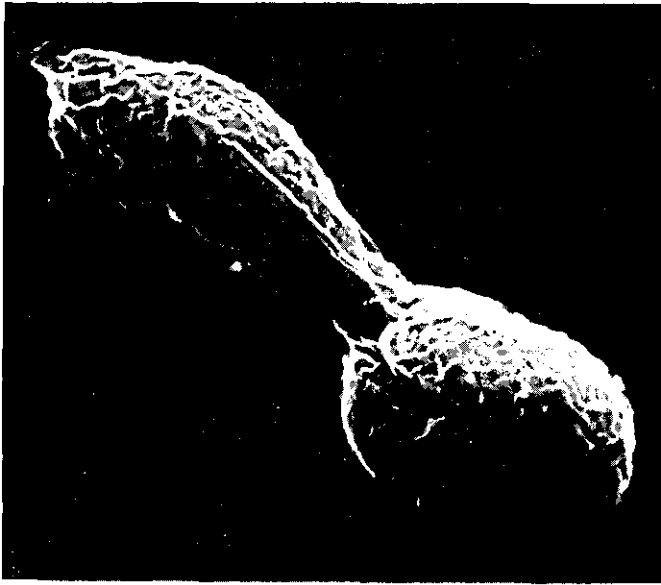


Figure 3 Scanning electron microscopy of *Scenedesmus* sp. grown for 5 days in a medium containing 0.075 mM lanthanum. $\times 10,000$.

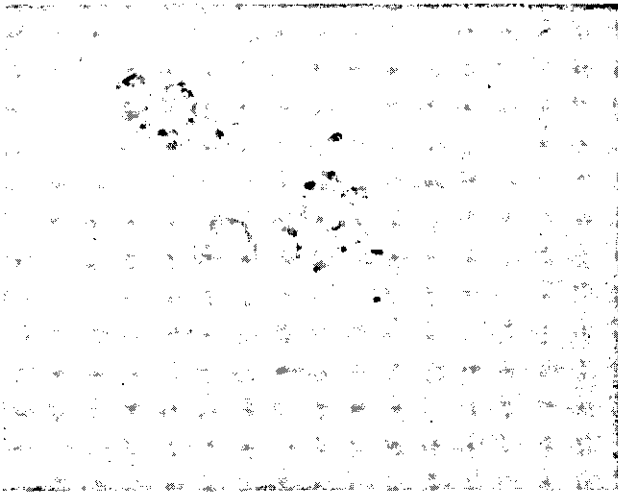


Figure 4 Location of calcium in *Scenedesmus* sp. following growth in a medium containing 0.1 mM calcium. $\times 1,250$.

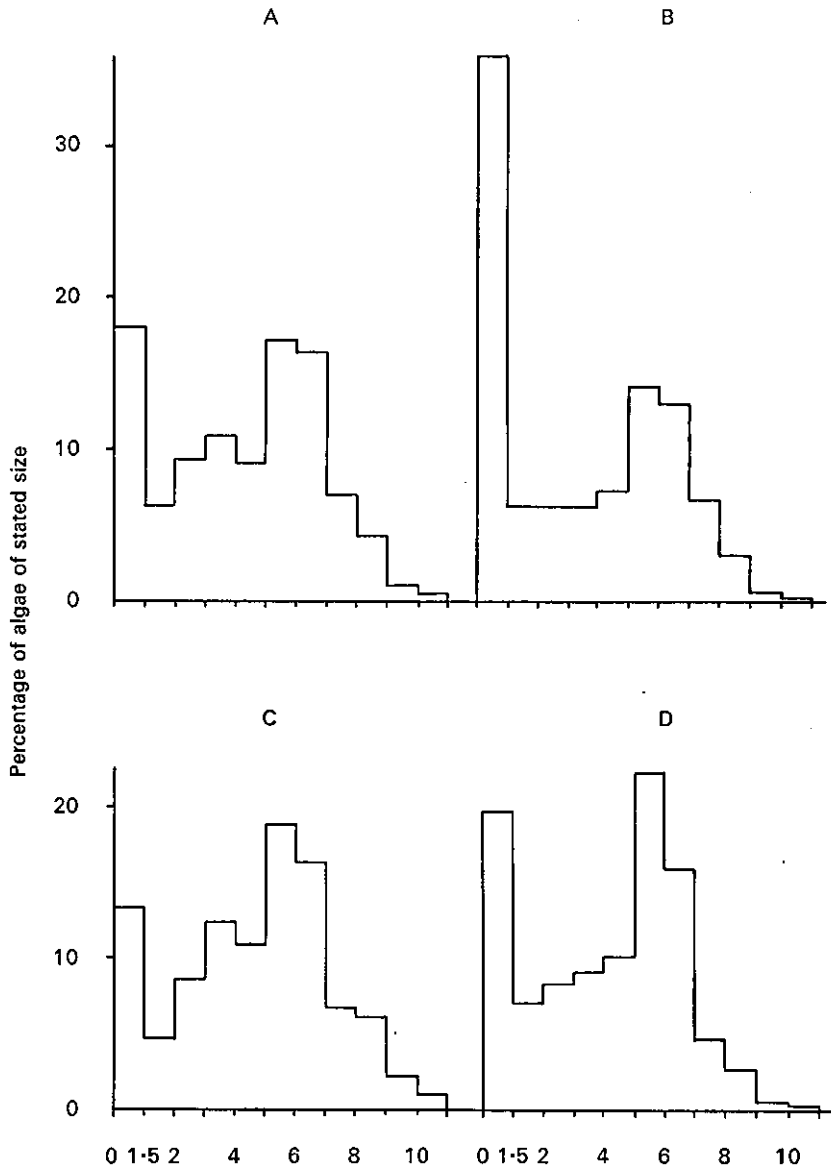


Figure 5 Size distribution of *Scenedesmus* sp. in the range 1.5 to 11 μm when grown in (A) medium without calcium, (B) with calcium 0.1 mM, (C) lanthanum 0.075 mM, (D) neodymium 0.075 mM.

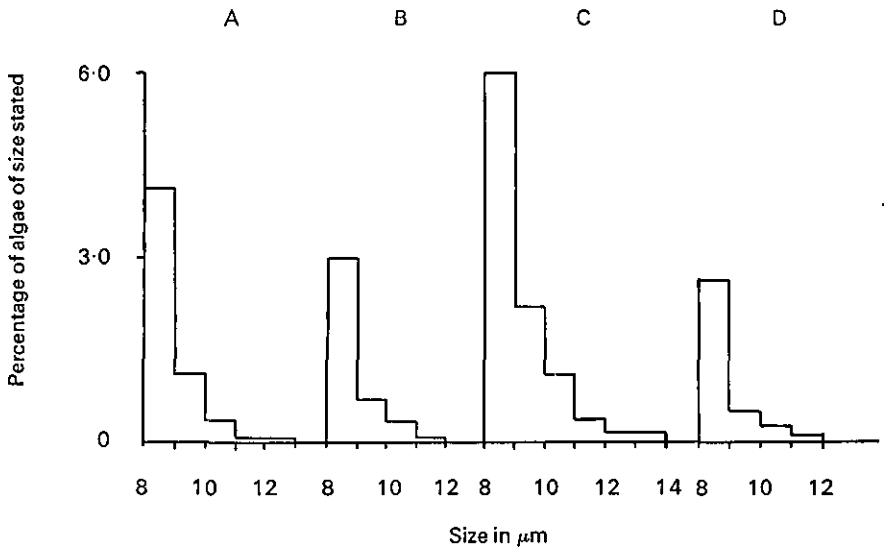


Figure 6 Size distribution in the range 8 to 14 μm of *Scenedesmus* sp. grown (A) in the presence of calcium 0.1 mM, (B) without calcium, (C) lanthanum 0.075 mM, (D) neodymium 0.075 mM.

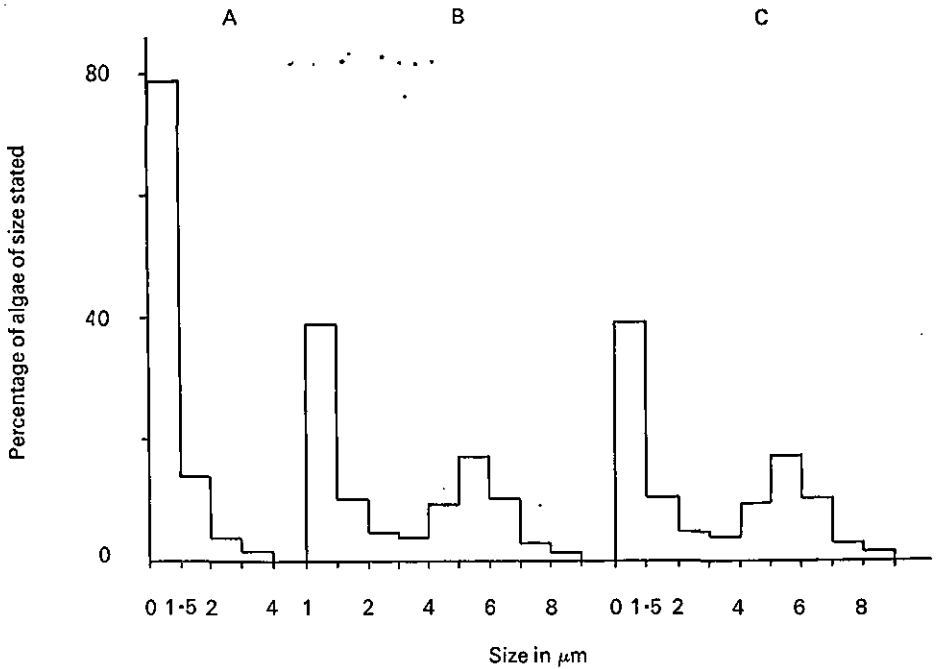


Figure 7 Size distribution in the range 1.5 to 9 μm of *Scenedesmus* sp. grown in (A) a medium plus X-537A and without calcium, (B) with 0.1 mM calcium and X-537A 3.5 $\mu\text{g/ml}$, or (C) without calcium.

briefly immersed in a calcium containing medium and carried through the procedure. Photomicrographs were obtained using Leitz Photoplan and Kodachrome-X (Kodak) film.

Results

Cell morphology

In the case of lanthanum and neodymium more spindle-like forms were present than when the cells were produced in the absence of Ca^{2+} . The cells were characterized by their convoluted appearance as seen by SEM (Figures 1, 2 and 3) there was no significant difference between the cells whether grown in the presence of Ca^{2+} , lanthanum or neodymium as judged by the surface morphology, but both spindle shaped and spherical cells were present when lanthanum and neodymium were involved.

Cellular calcium

The calcium was localized in the cells (Figure 4) which were grown in the calcium medium. No red colour was observed when cells were without calcium even when these cells were washed in the calcium-containing medium. When the algae were grown in the presence of calcium and X-537A no calcium was detectable. The GBHA stain gave no reaction with neodymium or lanthanum so it was not possible to demonstrate any loci for these in the cell.

Cell size distribution

The cell size distribution was affected by the presence of calcium, the number of small cells of size less than $1.5 \mu\text{m}$ ^{decreased} (Figure 5). In the presence of lanthanum and neodymium the distribution of the cell sizes was very similar to that obtained with calcium in the size range 1.5 to $8 \mu\text{m}$ (Figure 5). The most significant differences were noticed at the extreme of the size range 8 – $14 \mu\text{m}$ (Figure 6) when growth in the presence of lanthanum increased the percentage of cells in this range when compared to the distribution of cells grown in media without calcium, or with calcium or neodymium.

The addition of X-537A to the culture medium impaired the morphogenetic action of calcium and when incorporated into a medium containing no calcium it increased by 100% the number of cells in the size range of $1.5 \mu\text{m}$ (Figure 7).

There was no significant variation in cell yield, by dry weight determinations, irrespective of whether the algae were grown in the presence of Ca^{2+} , lanthanum or neodymium or without calcium.

Discussion

It is extremely difficult to free glassware from contaminating divalent cations (Kylin and Das, 1967) but we consider that our technique reduced the level of contaminants to a level where it was no longer significant and that the results obtained are due to the action of lanthanum and neodymium.

A number of *in vitro* studies have suggested that the lanthanides can replace calcium in certain biochemical processes, such as, the activation of enzymes

(Darnall and Birnbaum, 1970; 1973; Williams, 1970; Smolka, Birnbaum and Darnall, 1971; and Furie *et al.*, 1973). The lanthanons exhibit some similarity to calcium as Pickard (1970) has demonstrated that neodymium, lanthanum and praseodymium exert an inhibition of elongation of oat coleoptile which is slightly greater than that exerted by calcium. This study has shown that they can largely replace calcium *in vivo* in the regulation of cell size and shape in algae. This replacement is not total as small variations in cell size distribution were observed, particularly in the presence of large cells or aggregates when lanthanum was involved.

The removal of calcium from the growth medium resulted in an increase in the numbers of small cells as did the addition of X-537A to either medium, *i.e.* with or without calcium. The distribution of cell sizes when the cells are grown without calcium or with X-537A in a calcium containing medium are very similar, supporting the observations that X-537A is a calcium ionophore (Estrada, Céspedes and Calderon, 1972). When X-537A was used in a theoretically calcium free medium the percentage of cells less than $1.5 \mu\text{m}$ increased dramatically and cells over $4 \mu\text{m}$ were completely absent. This suggests that the medium was not completely free from calcium, which could be present, as a trace, in the chemicals used and that this trace was removed by X-537A.

The precise biochemical role of calcium in the biochemistry of algae is unknown. Nilshammer, Walles and Kylin (1972) have suggested that calcium may be required for a balanced transport of primary wall material to the cell plate in algae. Shah (1966, 1973) and Triggler (1972) have shown, *in vitro*, that calcium interacts with the phosphate moiety of phospholipids and in these interactions the phospholipid micelles are re-ordered.

Butler *et al.* (1970) have demonstrated that lanthanum ions are more effective in ordering phospholipid molecules than calcium, and Bangham and Dawson (1959) showed that lanthanum ions were 100 times more effective in reversing the negative charge of phospholipid micelles. Lanthanum ions also decrease membrane permeability to cations (Takata *et al.*, 1967; Van Breemen, 1969) and prevent membrane leakiness induced by metabolic depletion (Casteels *et al.*, 1972). As lanthanum ions also interact with cell proteins (Levinson, Mikiten and Smith, 1972) these ions may cause alterations in membrane permeability as well as altering the capacity to actively transport ions, particularly since lanthanum displaces calcium from membrane transport sites (Mela, 1968; Van Breemen and de Weer, 1970).

It is possible that lanthanum and neodymium exert a similar biochemical action to calcium on *Scenedesmus* either by a direct interaction with a protein or by re-ordering the phospholipid molecules which in turn activate a membrane protein. Kirkpatrick and Sandberg (1973) have shown that the chaotropic action of calcium ions results in conformational changes of proteins associated with erythrocyte membranes. The cell shape and size could result from such an interaction with the cell membrane or membrane-bound enzymes. This is to some extent confirmed by the similarity of effects of calcium absence or the addition of X-537A which could be preventing the accumulation of calcium ions in a lipophilic site or is promoting calcium outflow from an essential locus.

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