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ENDOGENOUS METABOLISM OF NOCARDIA CORALLINA

A thesis presented in partial fulfilment of the requirements for  
the degree of Doctor of Philosophy in Biochemistry  
at Massey University.

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## ABSTRACT

The endogenous metabolism of the soil microorganism N. corallina has been studied with special reference to physiological and structural changes in starvation conditions.

When N. corallina was grown on the surface of nutrient agar growth was characterised by the development of branched hyphae 8-12  $\mu$ . long, while in liquid medium bacilli approximately 4  $\mu$ . long were produced. Clumping of cells in liquid medium was reduced by growing the organism in cleated flasks on a rotary shaker.

For studies of endogenous metabolism and survival, suspensions of N. corallina were prepared from cultures harvested at full growth and resuspended in phosphate buffer containing magnesium ions. Analyses of total and viable cell counts were affected by clustering of cells and detergents were used to reduce the size of clusters. The cell viability, was estimated using formulae, from the cluster viability and cluster size distribution which were determined using the slide culture technique. The viability of starved cells fell from 99% to 90% over a period of 7 days and subsequently to 50% after a further period of 13 days. A rise in total cell count of 13% was recorded over a 5 day period of starvation. During the first 48 hr. of starvation the bacterial dry wt. fell by 30-40%, and at the same time the initial  $Q_{O_2}$  of approximately 10 fell to a value of approximately one. The initial fall in dry wt. was due largely to a decrease in the level of cell polysaccharide from 25% to 5-10% of the cell dry wt. Following this drop in polysaccharide, ammonia was released at a relatively constant rate and at the same time there was a fall in the level of cell protein.

There was a fall in the levels of intracellular free nitrogenous compounds at the onset of starvation but no corresponding release of these substances into the supernatant occurred. Ribonucleic acid appeared to be broken down during starvation. The contribution of the individual cell fractions to the total fall in dry wt. on prolonged starvation were; polysaccharide, 40%; protein, 25%; RNA, 6% and total fatty acids, 5%. The decrease in viability of starved organisms could not be directly correlated with the utilization of any of these cellular components.

Hydrolysis of the total unbound lipid which constituted 15% of the cell dry wt. yielded trehalose, mannose, inositol and glycerol as the water soluble components. Triglycerides were isolated from the total lipids by silicic acid column and thin-layer chromatography. Evidence from thin-layer chromatograms indicated that triglycerides were not major constituents of the total lipids. Incubation of N. corallina with U-<sup>14</sup>C-palmitate resulted in a large proportion of the radioactivity being incorporated into the triglycerides.

Total fatty acids constituted approximately 12% of the cell dry wt. and contained 3 fractions: (a) C<sub>10</sub>-C<sub>20</sub> fatty acids, (b) nocardic acids and (c) a minor unidentified fraction which was more polar than the nocardic acids. Trimethylsilyl derivatives of the methyl nocardates were separated by gas chromatography on the basis of molecular wt. Mass spectrometry of methyl nocardates and TMS derivatives, indicated that the structures of the nocardic acids could vary in 3 ways: in carbon number from C<sub>36</sub>-C<sub>48</sub>, in degree of saturation (saturated, mono-unsaturated and diunsaturated acids occurred), and in their isomeric configurations.

Studies on N. corallina using both light and electron microscopy showed clearly the pleomorphic nature of the organism. Parts of the cell surface were covered with fibrous material which appeared to be distinct from the cell wall. Cell division occurred by the formation of septa which were generally associated with extensive cytoplasmic membrane systems. Polyphosphate granules, ribosomes and either polyribosomes or glycogen granules appeared in the cytoplasm during growth. Use of the freeze-etch technique illustrated the granular nature of the cytoplasm, cell wall and membrane surfaces. Starvation of the cells appeared to be associated with (a) a thickening of the cell wall, (b) an increase of the amount of fibrous material per cell, (c) an increase in the size of the polyphosphate granules and (d) the disappearance of large cytoplasmic granules.

Possible implications of the present findings have been considered in relation to previous investigations with this organism and to studies of the endogenous metabolism and survival characteristics of other microbial species.

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SECTION I

INTRODUCTION

## CHAPTER 1

## INTRODUCTION

Endogenous Metabolism of Microorganisms with Special Reference to  
Nocardia corallina

Endogenous metabolism has been defined as the total metabolic reactions that occur in a living cell when it is held in the absence of compounds or elements which may serve as specific exogenous substrates (Dawes and Ribbons, 1964). The present study of endogenous metabolism and survival of the soil microorganism N. corallina was undertaken as an extension of previous investigations which were initiated in 1951 by Batt and Woods as a study of microbial pyrimidine catabolism. Subsequently, studies were carried out with this organism involving growth requirements and propionic acid oxidation (Martin and Batt, 1957a, 1957b), endogenous respiration and oxidative assimilation (Midwinter and Batt, 1960), induction of enzymes for pyrimidine catabolism (Batt, 1961), and polyol oxidations (Maurer and Batt, 1962). In 1962, Midwinter, in a further study, examined some aspects of metabolic versatility and endogenous metabolism in N. corallina and concluded that both lipids and proteins were degraded during periods of starvation. Robertson (1964) extended the lipid studies by examining oxidative assimilation of long chain fatty acids, detecting 10-methylstearic acid as a cellular constituent and defining aspects concerning the biosynthesis of this acid. Although no tests for survival potential were carried out, the impression was gained that this organism was remarkably stable. Webb and Clark (1957)

also working with N. corallina made a similar observation. For the present study it was proposed to examine the survival potential of the organism with special reference to the possible role of lipids as endogenous reserves.

The importance of high survival capacity under natural conditions was also to be considered. N. corallina is typical of the autochthonous or secondary group of soil microorganisms (Alexander, 1964; Conn, 1948; Clark, 1967). Characteristically this group includes organisms which grow relatively slowly and appear at a late stage in the ecological succession which occurs during the decomposition of plant residues (Alexander, 1964). The autochthonous group includes species of actinomycetes (Alexander, 1964; Conn, 1948; Waksman, 1959) some of which show exceptional catabolic abilities by degrading a wide range of complex materials typical of those produced during animal or plant decay. The autochthonous group of soil microorganisms complements the zymogenous or primary soil microorganisms (Bacillus, Pseudomonas and fast growing species of fungi, Alexander, 1964) which grow rapidly following the addition of fresh plant material to the soil. Zymogenous organisms show large fluctuations in numbers in soil disappearing rapidly after depletion of readily available nutrient (Clark, 1967). By contrast the numbers of autochthonous organisms, including many species of actinomycetes, show comparatively small variations. Although Clark (1967) considers that the terms autochthonous and zymogenous do not have a strict taxonomic basis it is quite possible that there is a relationship between the filamentous habit of the autochthonous group (which is also the taxonomic parameter) and their late development in the ecological succession (Alexander, 1964).

Periodically, soil microorganisms will be faced, in their natural environment, with nutrient deficiencies and the survival of the species could depend on the colonisation of a new ecological niche. Brock, (1966) has listed "resistance to starvation in dilute medium" as one possible morphological or physiological adaptation which may have evolved to aid dispersal. Clark (1967) has suggested that many cells occur in the soil in a resting or dormant condition and it is possible that they survive by utilizing endogenous reserves (Dawes and Ribbons, 1962; Lamanna, 1963). However, apart from an increased survival capacity in soil, which can be related to sporogenesis (Sneath, 1962) or possibly to desiccation or low temperature (Erock, 1966), there is little direct evidence to support the suggestion that soil bacteria can exist for long periods in a resting state, (Park, 1965) even although approximately 75% of soil bacteria (excluding the actinomycetes) are non-spore formers (Clark, 1967). Early studies with pathogenic bacteria showed that unless these organisms grew (Clostridium tetanii, Bacillus anthracis) they died out very quickly after being added to soil, which was therefore not considered to be a continuing source of infection (Waksman, 1947). Many workers consider that vegetative bacterial cells do not have a "resting state" and that the term "resting cell" used by Quastel and Whetham (1924) to distinguish spores from non growing cells presents an incorrect implication (Dawes, 1963; Dawes and Ribbons, 1964; Clifton, 1966a). There have been only a small number of studies of the survival capacity of microorganisms reported and very few of these have been concerned with organisms which could be described as members of the autochthonous group. In this connection a recent study by Zevenhuizen (1966) with Arthrobacter species which may be considered as autochthonous

(Conn, 1948) suggested that these organisms may in fact have a higher survival capacity than most organisms examined (Table 1). The present study was planned as an investigation of the survival capacity of a typical autochthonous, non-sporulating microorganism with the aim of defining more clearly the nature of the endogenous metabolism during periods of prolonged starvation.

#### The Genus Nocardia

The genus Nocardia (Breed, Murray and Smith, 1957; McClung, 1956) occurs in the order Actinomycetales which is described as a transitional group between true bacteria (Eubacteriales) and fungi, (Bessey, 1961; Lechevalier and Lechevalier, 1967; Waksman, 1959). The Order includes those organisms with a tendency to form a mycelium of branched hyphae (0.5-1.0  $\mu$ . in diameter) and spores. The mycelium may be rudimentary with no spores formed or well developed with the formation of segmentation spores formed by transverse division of the hyphae (Breed, Murray and Smith, 1957). The Order has been divided into four families Mycobacteriaceae, Actinomycetaceae, Streptomycetaceae and Actinoplanaceae between which only broad distinctions can be made, based on the degree of hyphal formation and fragmentation and the formation of spores. Classification of actinomycetes is made difficult by the extreme variability of their morphological and physiological characteristics (McClung, 1949, 1954, 1955). New methods based on factors other than morphology are being used to facilitate classification (Lechevalier and Lechevalier, 1967). The taxonomic position of the Nocardia in the family Actinomycetaceae, based on the formation of mycelium which subsequently fragments into coccoid and bacillary elements, has been contested (Gordon, 1966; Lechevalier and

Table 1  
Survival Characteristics of Bacteria

Organism	Carbon growth substrate	Source of cells for starvation experiments	Time interval for change in viability (hr.)	Change* in viability following resuspension (%)	Reference
<u>Aerobacter aerogenes</u>	glycerol limiting	chemostat	0-10	100-20	Postgate and Hunter (1962)
<u>Arthrobacter</u> (strains)	glucose	late log phase cells suspended in 1) phosphate buffer	0-5 (days) 5-19 "	100-85 85-70	Zevenhuizen (1966)
		2) phosphate buffer containing $\text{NH}_4^+$	0-3 " 3-13 "	100-130 130-10	
<u>Azotobacter agilis</u>	glucose	log phase	0-72	100-30	Sobek, Charba and Foust (1966)
	succinate	"	0-48	100-5	
	glucose	stationary phase	0-24 24-72	100-100 100-85	
	succinate	"	0-48 48-72	100-100 100-80	
<u>Escherichia coli</u>	glucose	stationary phase	0-12 12-48	100-100 100-15	Dawes and Ribbons (1965)
<u>Sarcina lutea</u>	peptone	stationary phase	0-30 30-96	100-85 85-15	Burleigh and Dawes (1967)
	peptone glucose	mid log phase	0-22	100-60	
<u>Streptococcus lactis</u>	lactose	late log phase	0-20 20-30	100-95 95-40	Thomas and Batt (1968)

\*Change in viability in given time interval was approximately linear.

Lechevalier, 1967). Gordon and Mihm (1957) suggested that aerobic actinomycetes, which form a mycelium capable of fragmentation into coccoid and bacillary elements but which do not produce aerial hyphae should be included in one genus Mycobacterium rhodochrous. Gordon (1966) refers to the Nocardia as a catch all genus but believes that any generic assignment of the species, Mycobacterium rhodochrous, should only be made following clearer delineation of the genera Corynebacterium, Mycobacterium, Nocardia and Arthrobacter.

Nocardia species occur widely in soils (Breed, Murray and Smith, 1957; Lechevalier and Lechevalier, 1967; Waksman, 1959; Gordon, 1967) although numerically they do not appear to be as common as the Streptomyces (Lechevalier, 1964; Kuster, 1967; Waksman, 1959). However the close morphological similarities of species of Nocardia with species of Corynebacterium and Arthrobacter, which also commonly occur in soils (Conn, 1948; Gordon, 1967) make estimations of the numerical importance of soil Nocardia species difficult.

Members of the genus Nocardia are capable of metabolising a wide range of carbon compounds including; cellulose, proteins, flavones, phenols and polyphenols, lipids and hydrocarbons (Waksman, 1959). Presumably these substrates also supply nutrient for these organisms in their natural environment. Some of these compounds are typical of the material remaining after the initial bacterial explosion (Clark, 1967) which occurs following the addition of plant or animal matter to the soil. The nature of these compounds together with their occurrence in the soil at levels which do not fluctuate widely over long periods, may perhaps be related to the autochthonous nature of microorganisms such as N. corallina.

There have been few studies on starvation resistance with mycelia of actinomycetes (Waksman, 1959; Erikson, 1949). Many Nocardia species do not form spores comparable with those produced by the Streptomyces. However the coccoid bodies that are formed by fragmentation of Nocardia mycelia could conceivably have durable properties and have been referred to as segmentation spores by Breed, Murray and Smith (1957). Hagedorn (1959a) has described small coccoid cells of N. corallina as "durable" but no physiological evidence was presented to suggest any special resistance property. The coccoid cells which occur in old cultures of Nocardia species resemble the so-called "arthrospores" of Arthrobacter species but there appear to be no reports showing that these entities have characteristic spore like properties (Breed, Murray and Smith, 1957). In a recent study on the survival of Arthrobacter species, Zevenhuizen (1966) reported that under some conditions these organisms have a resistance to starvation which appears to be greater than that for most organisms so far studied (Table 1).

#### The Species N. corallina

Nocardia corallina is described (Breed, Murray and Smith, 1957) as a soil microorganism producing branching rods 1.0-1.5  $\mu$ . by 10  $\mu$ . in young cultures grown on the surface of agar and characterised by the presence of short rods and cocci in old cultures. The organism is non-motile, non-acid fast, gram positive and does not form spores or aerial hyphae. It has an optimum growth temperature of 22-25<sup>o</sup>. Colonies on agar are generally coral pink in colour and either shiny or matt in texture with filamentous edges. The moisture content of

the solid medium influences colony morphology to a considerable extent (Webb and Clark, 1957). When grown on nutrient agar at 28-29° over a period of 40 hr. N. corallina produced coenocytic hyphae which subsequently fragmented. The coccoidal cells so formed grew by swelling followed by the formation of an outgrowth from one or both ends of each cell, (Webb and Clark, 1957). Hyphal elongation seemed to occur by insertion of new material at hyphal tips and not by intercalary growth except at branching points (Brown and Clark, 1966). Nuclear division accompanied hyphal elongation and gave rise to nuclei spaced regularly along the hyphae (Webb and Clark, 1957). With cells grown on agar the fragmentation process is said by Brown and Clark (1966) to be initiated by agents which accumulate in broth cultures towards the end of growth and subsequently disappear. These authors have claimed that when cells were grown in close proximity on membrane filters, fragmentation of hyphae occurred earlier which suggested the existence of a diffusible fragmentation initiator. A study of fragmentation with N. corallina grown in liquid medium (Webb and Clark, 1957) showed that coccoid cells developed to form coenocytic hyphae 12-16  $\mu$ . in length which fragmented after 15-20 hr. to yield binucleate bacillary cells. After a further 10-15 hr. coccoidal cells were formed. Webb and Clark (1957) have emphasised the need for using standardised inocula since fragmentation was delayed and hyphae were much longer when a very small inoculum was used. In this connection the amount of aeration was also very important in liquid cultures, since the fragmentation process could be greatly delayed or even absent with reduced oxygen tensions. The addition of fructose or glucose to the medium has been shown to reduce the hyphal stage by shortening the average

length of the hyphae formed (Webb and Clark, 1957). Clark and Frady (1957) and Webb and Clark (1957) have reported that the division of coccoidal cells in liquid culture could continue without hyphal formation in a manner similar to binary fission. The transfer of actively dividing cocci to fresh media or even distilled water caused the production of hyphae. The growth of coccoids on the surface of nutrient agar using plate count methods (Webb and Clark, 1957) was shown to approach 100% even from cultures which were several weeks old.

Webb and Clark (1957) and Clark (1958) reported that suspensions of N. corallina former large tenacious clumps which were considered to be caused by a secretion or slime layer on the outsides of the cells. This could be removed in part by washing with distilled water although various salt solutions, dilute acids, bases and organic solvents were ineffectual in this respect. The technical problems of clumping could be partially overcome by shaking suspensions with glass beads and removing clumps by low speed centrifugation to provide suspensions which contained at least 90% of the cells as singles.

Studies on the ultrastructure of N. corallina have been reported by Hagedorn (1959a, 1959b) and these have been discussed in more detail in a later section of the thesis. The general morphology of the organism as reported by Hagedorn was similar to that described by Clark et al. N. corallina shows a high degree of biochemical versatility in the range of organic compounds which it can oxidise as substrates under manometric conditions (Midwinter, 1962). Tepper and Karyagina (1966) have grown this organism on abietic acid, humic acid, lignin, vanillin, quercetin and quercetrin which are present in soils containing decaying plant and animal matter.

### The Chemical Composition of Nocardia Species

A detailed chemical examination of the cellular constituents of Nocardia species has not been reported. Most analytical studies have been related either to taxonomy or were concerned with the isolation and analysis of the cellular lipids. These investigations have been reviewed in detail (Lechevalier and Lechevalier, 1967; Asselineau, 1966, Salton, 1964).

The cell walls of actinomycete species represent approximately 25% of the cellular dry wt. (Work, 1957). The basic unit which confers rigidity to the cell wall consists of a glycoaminopeptide (mucopolysaccharide) made up from a small number of amino acids, amino sugars and other sugar components (Salton, 1964). In Nocardia species these include characteristically; glucosamine, muramic acid, alanine, glutamic acid, meso-diaminopimelic acid and the following monosaccharides; arabinose, glucose, galactose and mannose (Lechevalier and Lechevalier, 1967). With a large number of gram-positive organisms the mucopolysaccharide may represent most of the cell wall material (Salton, 1964). However in actinomycete species (which are also gram-positive) lipid has been shown to account for up to 60% of the dry wt. of cell walls (Kotani, Kitura, Hirano and Tanaka, 1959). Total lipids isolated from N. asteroides have been studied by Michel, (reported by Asselineau, 1966) who found that lipid represented 43% of the dry cell wt. and contained a high proportion of fats. Total lipids from the related species of Mycobacterium, commonly contain glycerides, esters of trehalose, fatty acids and phosphatidylinositolpolymannosides

(Asselineau, 1966). Total lipid content figures of 10% have been obtained for N. opaca (Webley, Duff and Anderson, 1962) and 15% for N. corallina (Midwinter, 1962). It is noteworthy that when N. corallina was grown in an acetate medium that the lipid content increased to 30% of the dry cell wt. (Midwinter and Batt, 1960). Webb and Clark (1957) found that glycerol increased fat formation in N. corallina. Michel, Bordet and Lederer (1960) isolated an unsaturated hydroxy acid of empirical formulae  $C_{50}H_{96}O_3$  ( $\pm CH_2$ ) from lipid fractions of N. asteroides and identical or closely related nocardomycolic acids (nocardic acids) have been isolated from N. pellegrino, M. rhodochrous and two strains of N. asteroides (Lanele, Asselineau and Castelnuovo, 1965). N. asteroides was found to contain a high proportion of a di-unsaturated branched chain alcohol, (nocardol) of molecular formulae  $C_{49}H_{96}O$  ( $\pm CH_2$ ) (Michel and Lederer, 1962). It has been pointed out by Etamadi (1965, 1967) and Lechevalier and Lechevalier (1967) that differences in molecular wt. of the mycolic acids could be of assistance in microbial classification. Characteristically, lipid skeletons of about 80 carbon atoms (mycolic acids) are found in Mycobacterium species whereas in species of Nocardia the carbon skeleton contains approximately 50 carbon atoms (nocardic or nocardomycolic acids) and for Corynebacterium species chain skeletons containing about 32 carbon atoms are found.

The composition of the  $C_{10}$ - $C_{20}$  fatty acids of several Nocardia species has been determined by gas chromatographic analysis (Bordet and Michel, 1963). Straight chain saturated, monounsaturated, iso and anteiso acids in addition to 10-methylstearic acid were detected. Similar results were also obtained for N. corallina (Midwinter, 1962; Robertson, 1964). Raymond and Davis (1960) and Davis (1964) have reported that a species of Nocardia, grown on long chain n-alkanes accumulated aliphatic waxes.

When the organism was grown on propane, infrared evidence suggested the formation of poly- $\beta$ -hydroxybutyrate and when grown on n-butane a copolymer of  $\beta$ -hydroxybutyric and  $\beta$ -hydroxybutenoic acids was formed. These polymers represented approximately 10% of the cell dry wt. while the remainder of the lipid made up almost 40% of the cell dry wt. and primarily consisted of glycerides. The presence of glycerides in M. rhodochrous has been demonstrated by Laneele, Asselineau and Castelnuovo (1965). Midwinter (1962) was unable to detect poly- $\beta$ -hydroxybutyric acid in N. corallina. There have been few reports on constituents, other than lipids in species of Nocardia. Webley, Duff and Anderson (1962) reported that N. opaca was capable of storing a reserve carbohydrate, probably glycogen, in amounts ranging from 5-40% of the cell dry wt. The RNA and DNA contents of this organism were each of the order of 4% of the cell dry wt. Protein, determined as total nitrogen, ranged between 30-70% of the cell dry wt. depending on whether carbohydrate contents were low or high respectively. Polyphosphate granules have been frequently observed in species of actinomycetes. Arai, Kuroda and Koyama (1961) isolated granules from cells of N. leishmanii and characterised them as mixtures of polyphosphate and nucleic acid. Such granules have also been shown to occur regularly in the tips of bacillary cells of N. rubra, (Adams and McClung, 1962).

#### Endogenous Metabolism and Bacterial Survival

Studies of endogenous metabolism and survival with microorganisms have been reviewed repeatedly (Dawes and Ribbons, 1962; Dawes and Ribbons, 1964; Duguid and Wilkinson, 1961; Herbert, 1961; Neidhart, 1963; Postgate, 1967; Wilkinson, 1958, 1959; Wilkinson and Duguid, 1960). The investigations may be divided into four categories, namely: (a) the effect of the

environment on the chemical composition of microorganisms, (b) studies relating endogenous metabolism to survival capacity, (c) factors affecting the survival of microorganisms and (d) the functional significance of endogenous metabolism.

(a) The effect of the environment on the chemical composition of microorganisms. Studies on the chemical composition of microorganisms have shown marked differences in the levels of different cellular constituents depending on the conditions of growth and the time of harvest (Herbert, 1961). Studies on the levels of protein, RNA and DNA, and on cell size suggest that the chemical composition of a cell is primarily dependent on the rate at which it is growing, and is affected by the chemical composition of the medium only so far as it effects the growth rate (Herbert, 1961; Neidhardt, 1963). During the growth of bacteria in batch culture there is a rapid increase in individual cell mass and in the percentage content of ribosomal RNA (Ecker and Schaechter, 1963). The DNA percentage content of each cell decreases although the absolute amount in each cell increases two or three fold. During the log phase of growth RNA and DNA percentage contents, and cell mass remain relatively constant but towards the end of the log phase when the growth rate decreases, the cell mass and the RNA percentage content fall and the DNA percentage content rises to the pre log phase level (Herbert, 1961; Strange, Dark and Ness, 1961). Changes in the percentage composition of protein, RNA and DNA in the organisms Salmonella typhimurium, Aerobacter aerogenes and Bacillus megaterium grown in continuous culture at increasing growth rate levels were of the order of: protein, 65-55%, RNA, 7-20% and DNA, 5-3% (Herbert, 1961; Maaløe, 1960). An examination of changes in morphology or chemical composition relative to growth for Nocardia species has not been reported. Webley, Duff and Anderson (1962) have given an

RNA content figure for N. opaca of 3-4%, estimated towards the end of growth.

Compounds which are commonly found in bacterial species and referred to as reserves, include lipids, polysaccharides and volutin (Wilkinson, 1959). Evidence suggesting that these compounds are truly reserves in that they prolong survival of starving cells tends to be inconclusive (Wilkinson and Munro, 1967). Intracellular polysaccharide, (starch or glycogen) and poly- $\beta$ -hydroxybutyrate generally accumulate in cells under conditions in which phosphorus, nitrogen, potassium or sulphur are the growth rate limiting nutrients. Poly- $\beta$ -hydroxybutyrate may accumulate under conditions in which a carbon compound is limiting growth and therefore may have some specific function in some cells (Wilkinson and Munro, 1967). The amounts of the carbon polymers which accumulate under any particular condition appears to be related in a complex way to the rate of growth and the growth limiting nutrient. In general there is a tendency for the proportion of the polymers to be high at low growth rates (Wilkinson and Munro, 1967; Herbert, 1961). Polyphosphate (volutin) generally accumulates with nitrogen or sulphur deficiency or under acid conditions (Wilkinson and Munro, 1967; Wilkinson and Duguid, 1960).

Whereas intracellular polysaccharides are generally degraded and reutilized under starvation conditions, extracellular polysaccharides are not (Wilkinson, 1958). As exceptions some strains of streptococci can hydrolyse the hyaluronic acid capsules by producing extracellular hyaluronidase (MacLennan, 1956) and cells of Sarcina lutea have been found incapable of utilizing intracellular polysaccharides (Dawes and Holms, 1958). Wilkinson and Duguid (1960) have reviewed evidence

suggesting that polysaccharide production in aerobes may be affected by oxygen tension, pH and the temperature of the medium. Dagely and Johnson (1953), working with E. coli showed that the nature of the substrate influenced the nature of the polymer accumulated: e.g. acetate stimulated PHB synthesis whereas glucose promoted polysaccharide synthesis. In studies on the metabolism of iron, zinc and manganese deficient N. opaca (Webley, Duff and Anderson, 1962) it was shown that glycogen accumulated in cells (35% of the cell dry wt.) towards the end of growth; with cells deprived of zinc, glycogen accumulated earlier and disappeared later.

Poly- $\beta$ -hydroxybutyrate occurs in a large number of bacteria in the form of intracellular granules which may represent up to 40% of the cell dry wt. (Wilkinson and Duguid, 1960; Stockdale, Ribbons and Dawes, 1968). Like glycogen, PHB may be found in large amounts in cells supplied with glucose or substrates such as pyruvate, acetate or butyrate, in the absence of a nitrogen source (Macrae and Wilkinson, 1958; Doudoroff and Stanier, 1959). It is well established that PHB is broken down rapidly in stationary phase cells (Norris and Greenstreet, 1958; Macrae and Wilkinson, 1958). Although glycerides are commonly found in the lipids of Mycobacterium species (Asselineau, 1966), factors which might affect the levels of these compounds specifically do not appear to have been reported. Asselineau (1966) has reviewed evidence showing that glycerol by comparison with glucose stimulates the deposition of total lipid in Mycobacterium species. The effect of acetate on the deposition of lipid varies widely even with individual strains of Mycobacterium. Schaefer and Lewis (1965) have provided evidence, based on an electron microscopy study, for the accumulation of "fat" by M. kansasii incubated with oleic acid or Tween 80.

(b) Studies relating endogenous metabolism to survival capacity.

Normally studies on endogenous metabolism involve the harvesting of bacterial cells followed by resuspension in non-nutrient buffer systems. The pattern of metabolism obtained under such conditions is generally similar to that observed where cells are left to incubate in spent medium after reaching full growth. In a study of endogenous metabolism with E. coli (Dawes and Ribbons, 1965) it was found that glycogen accumulated in the later stages of growth and was degraded in 1 to 2 hr. when cells were resuspended in a buffer solution. The fall in glycogen content corresponded to a fall in  $Q_{O_2}$ . Protein turnover was found to continue at a linear rate throughout the period of glycogen utilization and then ammonia was released from the cells. Dawes and Ribbons (1965) concluded that glycogen utilisation prevented the net degradation of nitrogenous material but not protein turnover. Clifton (1966b) also working with E. coli reached a similar conclusion. Zevenhuizen (1966) using strains of Arthrobacter showed that intracellular glycogen was utilized as a source of carbon for protein synthesis during endogenous respiration. In addition the Arthrobacter strains were found to accumulate both extra and intracellular polysaccharide although only the intracellular glycogen was degraded during endogenous metabolism. Zevenhuizen (1966) has claimed that the presence of intracellular glycogen promoted cell viability during endogenous respiration and that if ammonium ions were added to the incubation system the glycogen was utilized faster and viability decreased more rapidly (Table 1). Dawes and Ribbons (1965) have not claimed any direct relationship between cell viability and changes in the levels of cellular constituents in their study of the endogenous metabolism of E. coli. From a study of the endogenous

metabolism of Azotobacter agilis, Sobek, Charba and Foust (1966) suggested a time relationship between the complete utilization of PHB and the onset of cell death, while Burleigh and Dawes (1967) working with starved suspensions of Sarcina lutea found that cells containing a high proportion of polyglucose depleted this constituent during starvation and died more rapidly than cells which did not contain the polyglucose. Postgate and Hunter (1962) in a study of A. aerogenes, reported that the death of cells in the population began immediately after resuspension (Table 1) and that cells catabolised RNA and to a lesser extent polysaccharide and protein. In 1960, Midwinter and Batt concluded that carbohydrate could act as an endogenous reserve in suspensions of N. corallina respiring endogenously in phosphate buffer. However Midwinter in a later study reported that carbohydrate was not a major reserve constituent. Instead lipids and proteins were considered to be utilized under conditions of endogenous respiration. Stephenson and Whetham (1922, 1924) have reported that non-phosphatidic lipids acted as endogenous reserves in M. phlei. By contrast Frouin and Guillaumie (1928) and Cassagne (1939) reported that the percentage lipid of tubercle and diphtheria bacilli increased with age. With N. rugosa, loss of protein was said to account for most of the dry wt. loss during endogenous respiration (Bardi and Boretti, 1958) while lipid, RNA and DNA were not utilized. However for other systems the breakdown of RNA during starvation of bacteria has been well documented. RNA is broken down almost immediately following resuspension of E. coli (Dawes and Ribbons, 1965) and for S. lutea (Burleigh and Dawes, 1967) grown on peptone salts, the cellular content of RNA falls during the decline in growth rate that occurs towards the end of the log phase. RNA was also

degraded under conditions of resuspension in buffer. Strange, Dark and Ness, (1961) have reported that RNA was broken down in starved suspensions of A. aerogenes and a similar report has been made with respect to Pseudomonas aeruginosa by Gronlund and Campbell (1965). In several of these reports the breakdown of RNA was shown to be accompanied by the release of pentose and ultraviolet material into the supernatant and in some cases, ammonia was produced in amounts greater than what was expected from the breakdown of proteins or amino acids alone (Dawes and Ribbons, 1965; Burleigh and Dawes, 1967; Sobek, Charba and Foust, 1966). Starved cells may also release amino acids into the extracellular fluid (Dawes and Ribbons, 1965). Burleigh and Dawes (1967) showed that the level of the free intracellular amino acid pool fell when S. lutea was starved and there was a concomitant release of ammonia. In addition Bardi and Boretti (1958) have noted that amino acids were utilized by N. rugosa under starvation conditions.

(c) Factors effecting the survival of microorganisms. In addition to the possibility that the utilization of intracellular reserves may promote cell survival a number of other factors have been shown to have marked effects on the viability of starving cells. These effects have been reviewed by Postgate (1967). The survival capacity of starving cells of A. aerogenes has been shown to be related to the growth rate at the time of harvesting for the starvation experiments. Cells grown in continuous culture at rapid growth rates died more slowly than those harvested from continuous cultures in which growth rate was slow. The effect on A. aerogenes of low temperature during harvesting has been examined by Strange and Dark (1962) who found that cells subjected to temperatures of 0° lost viability during the subsequent period of resuspension more rapidly than those held at 20°. This effect has been referred to as cold shock and may be partly due to interference with a

permeability control mechanism. Postgate and Hunter (1962) found that A. aerogenes had an optimal survival temperature of 20° under conditions of resuspension while Thomas and Batt (1968) reported that survival of S. lactis was extended with temperatures down to 3°.

Tris-saline and phosphate buffers prepared from highly purified reagents may contain impurities which permit limited growth (Postgate and Hunter, 1962; Strange, Dark and Ness, 1961; Garvie, 1955) and during starvation non-viable organisms can loose material into the supernatant which may be subsequently utilized for growth by viable organisms; an effect known as cryptic growth (Postgate, 1967). Clifton and Cherry (1966) have also pointed out that viable organisms may leak substances which could be reutilized. By contrast laboratory glassware, reagents and distilled water may contain materials which are actively toxic to bacteria. Postgate and Hunter (1962) found that some batches of buffer contained appreciable amounts of copper at toxic levels. Inclusion of EDTA removed the toxic effect but an excess of EDTA can itself become toxic to cells (Thomas and Batt, 1968). The inclusion of magnesium in buffer used for experiments with starved cells prolongs survival, an effect which may be due to a stabilizing action on either, bacterial ribosomes or on permeability (Strange and Hunter, 1967). The concentration at which cells of A. aerogenes were resuspended affected their viabilities and Harrison (1960) has shown that there was an optimum concentration for survival of cells in suspension. Thomas and Batt, (1968) working with S. lactis have suggested that the effect of cell concentration, known as the population effect, (Postgate and Hunter, 1962) may be due to the leakage of magnesium from the cells to a level which subsequently affords protection against loss of viability.

(d) The functional importance of endogenous metabolism.

The two extremes regarding the possible significance of endogenous metabolism have been outlined by Dawes and Ribbons (1964) "Endogenous metabolism occurs either because the organism cannot help it and it therefore bears no relationship to survival, or survival characteristics are related directly to the endogenous metabolic activities of the cells." Dawes and Ribbons (1964) suggested that the truth lay somewhere between these extremes "some reactions may occur because they are essential to the viability of the cell and others may occur simply because substrate and enzymes are in contact." Lamanna (1963) postulated that endogenous metabolism was indicative of a maintenance energy (Mallette, 1963; McGrew and Mallette, 1962) that existed at all times for sustaining viability. Marr, Nilson and Clark (1963) in developing the concept of a maintenance energy requirement in E. coli postulated that the metabolism of a carbon source provides energy for various processes which occur independently of growth. Such processes might include motility, solute accumulation, maintenance of higher intracellular to extracellular concentrations of some cell constituents, and protein and RNA turnover. Marr, Nilson and Clark (1963) have defined the specific maintenance as a measure of the carbon and energy diverted for purposes other than growth. Claims for the demonstration of specific maintenance have been made from studies with continuous cultures. When the infusion rate of glucose was decreased the yield of cells from a defined amount of glucose was decreased. Postgate (1967) has criticised these experiments and others on maintenance (Bohinski and Mallette, 1965; Mallette, Cowan and Campbell, 1964; McGrew and Mallette, 1965) on the grounds that cryptic

growth might have occurred. However it seems that the concept of a "maintenance energy" is now generally accepted although yet to be experimentally confirmed (Postgate, 1967).

It was mentioned earlier in this discussion that some authors do not recognise a "resting state" for vegetative cells (Dawes and Ribbons, 1963; Dawes, 1963; Clifton, 1966a). From the summary of results on cell survival reported in the literature (Table 1) it is clear that bacteria do not survive for very long. Rather than a "resting state" the starving vegetative cell is considered to be in a state of negative growth or growth rate decline leading to a finite minimum below which cells do not survive (Ecker and Schaechter, 1963). Tempest, Herbert and Phipps (1967) have investigated the concept of a finite minimum growth rate for A. aerogenes grown in continuous culture and have shown that the doubling time of viable organisms tended to a maximum value of about 80 hr. where growth was limited by glycerol. When growth was limited by levels of  $\text{NH}_4^+$ , the maximum doubling time for the organism was about 100 hr. When the growth temperature was decreased the yield of cells was increased suggesting that the minimum growth rate may be lowered by lowering the temperature.

## CHAPTER 2

## THE AIM OF THE PRESENT INVESTIGATION

Following a study of the endogenous metabolism of N. corallina, Midwinter (1962) concluded that the catabolism of lipid and protein components provided energy for cell maintenance in the absence of exogenous substrate. Little information was obtained on either the nature or the turnover of the cellular lipids and this aspect was taken up for further study. It was also proposed to investigate the survival capacity of N. corallina in relation to the depletion of cellular lipids.

Earlier studies (Batt, unpublished results; Robertson, 1964) had demonstrated that when cells of N. corallina were incubated with the potassium salts of either palmitic, stearic or oleic acid, approximately 80% of the exogenous  $^{14}\text{C}$ -labelled fatty acid was oxidatively assimilated. A large proportion of the assimilated fatty acid was incorporated into a neutral lipid fraction assumed to be triglyceride. Accordingly it was proposed to positively identify the triglyceride fraction in lipids isolated from N. corallina cells, to label this fraction by oxidative assimilation from  $^{14}\text{C}$ -palmitic acid and to determine the subsequent rate of turnover of the fraction in cells incubated under starvation conditions.

The ability of N. corallina to incorporate fatty acids almost quantitatively into cellular lipids presented the possibility of localising the "reserve triglyceride pool" by means of autoradiography and electron microscopy. However as a preliminary study it was necessary to obtain information on the ultrastructure of the organism during growth and also under conditions of starvation.

The conditions for growth and incubation of N. corallina had been

selected previously from a limited amount of experimental information. To carry out effective studies with suspensions of starved cells it was recognised that experimental conditions would have to be carefully controlled and initially the investigation was directed to a study of defined physiological conditions for growth and survival. It was clear that conditions should be sought which prevented the cells clumping, since total and viable cell counts would be used for estimating survival potential. Although the results reported by Midwinter (1962) gave a general picture of metabolic changes during starvation, the levels of intracellular constituents were re-examined, specifically in relation to viability estimates.

From the variety of possible experimental approaches to this study of cell survival, the investigation developed as a more detailed re-examination of cellular changes under growth and starvation conditions with concurrent investigations into the ultra structure of the cells and structural analysis of fatty acids.

Although turnover studies are still considered essential for better understanding of the metabolic changes which occur during endogenous metabolism, emphasis should perhaps be directed more to protein rather than lipid constituents, in the light of the findings from the present study.

SECTION II

EXPERIMENTAL

## CHAPTER 1

## METHODS

Bacteriological ProceduresOrganism

The strain of N. corallina used in the present investigation was isolated by Batt and Woods (1951). Throughout the investigation the organism was maintained at 2° on glucose yeast-extract, agar slopes (Appendix 1). Slopes were subcultured at monthly intervals on to fresh slopes which were incubated at 30° for 48 hr. and then stored at 2°.

The original isolate was identified by Professor Jensen as a strain of N. corallina (Batt and Woods, 1961). In 1965 the organism was examined by Dr. Ruth Gordon who described it as Mycobacterium rhodochrous. However Dr. Gordon pointed out that it was quite reasonable to retain the name N. corallina until the taxonomic problems associated with the species are settled (pers. comm.).

Growth in Liquid Medium

Cells from a slope which had been stored for no longer than 2 days were transferred, using a sterile loop, into 300 ml. of liquid medium held in a clefted conical flask (1L.) or a standard conical flask (1L.). The 0.1M phosphate buffer medium (Appendix 1) contained per L.: Vitamin B<sub>1</sub>, 0.025 g.; (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 3.0 g.; MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.10 g.; KH<sub>2</sub>PO<sub>4</sub>, 13.6 g.; adjusted to pH 7.00 ± 0.05 by addition of 5N NaOH. Glucose (0.75% final conc.) was added after autoclaving.

The flask containing the inoculated medium (referred to as the primary flask) was incubated at 30° with shaking at 140 rev./min. on a New Brunswick gyrotary shaker (Model G-25). After a period of approximately 40 hr., growth of the primary culture had reached the mid log phase as defined by the OD measurement at 660 m $\mu$ . Aliquots (10.0 ml.) were then transferred to secondary flasks after carrying out a brief examination of the morphology of the organisms in the primary culture using light microscopy and Gram staining. The secondary flasks contained 300 ml. of the same medium as the primary flasks and were incubated under identical conditions. Full growth, as demonstrated by the establishment of maximum OD at 660 m $\mu$ , occurred in the secondary flasks after a period of approximately 50 hr.

All experiments described in the subsequent sections of this report were carried out on cultures grown in secondary flasks.

#### Special Growth Flasks

Cultures were grown in standard conical flasks (1L.) or cleated conical flasks (1L.). The cleated conical flasks were standard conical flasks indented on the lower part of the wall with 6 equidistant cleats approximately 1½" high and ½" deep. The mouths of flasks were plugged with muslin coated cotton wool or covered with 2 milk filters ("Cestra" embossed, 10 1/4" D.) folded in quarters and held around the neck of a flask by a rubber band. Cleated flasks and milk filters were used to improve the aeration efficiency of culture systems (Corman et. al. 1957). In cases where milk filters were used the flasks also had a side arm (1.5 cm. I.D.), which was plugged with muslin coated cotton wool.

### Preparation and Incubation of Cell Suspensions

In a typical experiment requiring the incubation of suspensions cells were harvested from secondary cultures by centrifugation (Servall, Model RC-2) at 2000 g. for 7 min. at 2° in screw-capped polypropylene cups (250 ml. Nalgene) which had been sterilised by autoclaving (15 min., 121°). The supernatant fluid was discarded and the pellets washed twice by resuspension and recentrifugation in 100 ml. (per wash) of sterile, icecold, sodium potassium phosphate buffer (0.1M pH 7.00 ± 0.05) containing  $\text{MgSO}_4$  ( $4 \times 10^{-4}$ M) hereafter referred to as phosphate buffer. Although complete resuspension of the pellets was difficult, dispersion was obtained if pellets were agitated in approximately 15 ml. of phosphate buffer, using a cyclo-mixer, (Clay Adams Inc.) before the main portion of the buffer was added. Pellets from the same secondary flask were combined during the first wash so that the cellular contents of each flask were contained in separate centrifuge cups. The pellets from individual secondary flasks were finally resuspended in a total volume of 300 ml. of phosphate buffer (room temperature), in autoclaved, standard or cleated conical flasks (1L.). Mouths of flasks were closed with muslin coated cotton wool or milk filters. Where milk filters were used the suspensions were introduced to the flasks through the side arm. Finally, all flasks were weighed and then incubated at 30° with shaking at 140 rev./min. on the gyrotary shaker (New Brunswick). Samples were withdrawn aseptically as required.

During prolonged incubation loss of water of approximately 2ml./day occurred by evaporation. This was corrected for by weighing the flasks at intervals and adding sterile, glass-distilled water.

### Total Cell Counts

Total cell counts were made using a Thoma bacteriological counting chamber (Hawksley, England) and optically-plane coverslips. Samples (0.5 ml.) of flask cultures containing approximately  $4.0 \times 10^9$  cells/ml. were diluted to 10.0 ml. with phosphate buffer containing 0.5% Teepol. The diluted suspension was shaken for 30 sec. on the cyclomixer before assembling the chamber and observing the grid under bright field illumination at a total magnification of x 500. Cells in the same four groups of quintets (five 0.05 mm. squares) were counted each time the chamber (depth, 0.02 mm.) was assembled. The chamber was filled and counted 8 times for each diluted sample. Cells often occurred in clusters containing two, three and four cells, however the number in each cluster could usually be determined without difficulty.

### Viable Counts

The viability of resuspended cultures was estimated by the slide culture method of Postgate, Crumpton and Hunter (1961). Aliquots (0.5 ml.) of cell suspensions were diluted to 10 ml. with sterile phosphate buffer containing 0.5% Tween 80. Following vigorous mixing on a cyclomixer (Clay-Adams Inc.) 0.5 ml. of the first dilution were diluted to 5 ml. with the same buffer containing 0.5% Tween 80. This too was mixed vigorously. Drops of the second dilution were spread on nutrient agar held in stainless steel rings (1.5 mm. x 20 mm. (I.D.) x 22 mm. (O.D.)) on microscope slides, (2 rings/slide), according to the methods of Postgate, Crumpton and Hunter (1961).

Agar media for the slide cultures (Appendix 1) was prepared in

test tubes (12 cm. x 1.5 cm.) tightly plugged with muslin coated cotton wool. Immediately after autoclaving, the test tubes containing the molten agar, were spun at 2,500g for 15 min. in an angle head bench model centrifuge (MSE) in which the metal cup holders had been heated to 80° just before centrifugation. Although agar media subjected to this treatment was not entirely free from particulate material, it provided an adequate layer on which cells of N. corallina could be recognised, unambiguously, under the microscope.

Duplicate slides were inoculated from each suspension for which a viable estimate was required. One slide was incubated at 30° and the other at 0°. Coverslips were not used to cover the agar held in the rings, drying being avoided during incubation at 30° by placing Petri dishes holding the slides in a large filter paper lined desiccator, with an inch of water in the bottom. The desiccator was held in a 30° warm room and the slides were incubated for 16 to 20 hr. They were then examined to determine numbers of live and dead clusters. Slides stored at 0° were examined to determine the proportion of clusters of different sizes. Slides were examined with a Leitz Ortholux microscope under phase contrast or bright field illumination at a magnification of x 500. An eye piece graticule ruled in 100 one mm. squares was used to facilitate counting. All cells appearing within the grid were counted; the area on the agar which appeared in the grid was 0.04 mm<sup>2</sup>.

With slide cultures prepared from dilutions of suspensions containing  $4 \times 10^9$  cells per ml., 20 to 40 cells or clusters of cells occurred in each grid. Dead cells or clusters of cells could easily be distinguished from live clusters after a period of incubation at

30°. Dead clusters were observed as several cocci 2 to 4  $\mu$  in length and 0.5 to 1.0  $\mu$  in width, adhering to each other in a random arrangement. Clusters which had developed by growth from a live cell consisted of cells 8 to 12  $\mu$  in length grouped on the surface of the plate in a typical pattern of branching hyphae.

Formulae for Estimating Cell Viability from Cluster Viability

The cell viability was estimated from the cluster viability and cluster size distribution using the following formulae (see Appendix 2 for derivation).

$$C = \sum_{j=3}^{j_{\max}} n_j (1-p)^j - (1-P) \quad \dots\dots\dots(1)$$

$$1-p = \frac{\sqrt{(n_1^2 - 4n_2C)} - n_1}{2n_2} \quad \dots\dots\dots(2)$$

Where P = The cluster viability (number of live clusters/number of live clusters plus number of dead clusters).

p = The cell viability (number of live cells/number of live cells plus number of dead cells).

j = The cluster size, determined by the number of cells in a cluster.

$n_j$  = The number of clusters of size j expressed as a proportion of the total number of clusters of all sizes.

C = A common term dependent on p, which includes all terms for clusters containing three or more cells.

The cell viability was determined by:-

(1) Assuming an approximate initial value  $p_1$  for  $p$ , by setting  $p_1$  equal to  $P$ , and substituting  $p_1$  for  $p$  in equation (1) giving  $C_1$ .

(2) Substituting  $C_1$  into equation (2) giving  $p_2$  the second approximation for  $p$ .

(3) Substituting  $p_2$  for  $p$  in equation (1) giving  $C_2$  which in turn was substituted into equation (2). This process of iteration was repeated until successive values,  $p_n$  and  $p_{n+1}$  were identical.

An example of the determination of the cell viability from the cluster viability and cluster size distribution is presented in Appendix 2.

#### Photography of Cells on Agar Slides

Photographs of cells on agar slides were taken under bright field illumination (x 340) on Agfa L. Agepan FF film using a Leica camera and a Leitz Mikas attachment and extension tube on the Leitz microscope. A neutral density heat filter was placed between light source and condenser.

#### Analytical Methods

##### Spectrophotometric Equipment

Colorimetric estimations were carried out using a Beckman DU Spectrophotometer. Ultraviolet and Infrared Spectra were studied in a Unicam SP 800 Ultraviolet Spectrophotometer, and a Perkin-Elmer 137 Sodium Chloride Spectrophotometer respectively.

##### Dry Weight

The dry weight content of cell suspensions was determined from a

standard curve calibrated in terms of dry weight against optical density (OD) at 660 m $\mu$ . The standard curve was constructed using log and stationary phase (3 days after full growth) cells of N. corallina cultured in glucose media and harvested separately by centrifugation (2000 g , 7 min.). The pellets were washed twice with glass distilled water and finally resuspended in glass distilled water using a 10 ml. Teflon Pestle tissue homogeniser to ensure complete dispersion. Aliquots of the final suspension of both log and stationary phase cells were dried to constant weight in preheated (120<sup>o</sup>) preweighed aluminium milk bottle caps (0.330 g. approx.). Drying was carried out by heating at 105<sup>o</sup> in an electric oven and cooling over P<sub>2</sub>O<sub>5</sub> before weighing.

Aliquots of the same suspensions were diluted to 10 ml. or 50 ml. with glass distilled water for OD determinations at 660 m $\mu$  using a Bausch and Lomb Spectrophotometer (Spectronic 20). The graph of OD against dry weight was linear up to a maximum OD of 0.4 and there was no detectable difference in the slopes of graphs obtained for log and stationary phase cells. The slope of the graph and standard error for combined results was 0.4263  $\pm$  0.0035. Throughout the experimental work the following relationship was used.

$$\text{Dry wt. (mg./ml. of solution in the Bausch and Lomb tube) =} \\ (0.43) \times (\text{OD at } 660 \text{ m}\mu.)$$

For routine dry weight estimations aliquots of cultures or cell suspensions were diluted to 10 ml. or 50 ml. with glass distilled water and thoroughly dispersed using the tissue homogeniser (A.H. Thomas Ltd.). The dry weight was determined from the standard curve after measuring the optical density at 660 m $\mu$  in the Bausch and Lomb Spectrophotometer.

### Respiratory Quotients

Respiratory Quotients were determined by direct Warburg respirometry using Braun manometers and single side arm flasks (Umbreit, Burris and Stauffer, 196+). Each flask (vol. fluid 2.2 ml. vol. gas 15 ml.) contained 2.0 ml. of bacterial suspension and either 0.2 ml. of 20% KOH in the centre well or 0.2 ml. 3N H<sub>2</sub>SO<sub>4</sub> in the side arm. A piece of folded Whatman no. 1 filter paper was placed in the centre well of all flasks. All manometry was carried out at 30°.

### Oxygen Partial Pressure Estimations

Partial pressures of oxygen in liquid and gas phases were determined using an oxygen analyser (Bekman Model 777) coupled to a chart recorder (Sargeant Model SR). For gas phase estimations the analyser was set at 100% in air and 0% in oxygen free nitrogen (N.Z. Industrial Gases). For liquid phase estimations the settings were 100% in phosphate buffer equilibrated with air and 0% in buffer equilibrated with oxygen-free nitrogen. During estimations of oxygen levels in liquid and gas phases in culture flasks, the analyser probe was bound to a stainless steel rod (3 mm. D.) fitted into the main aperture of the flask and steadied with a curved metal collar around the neck of the flask. The arrangement was such that milk filters could still be placed over the flask opening; rubber bands held both filters and metal collar in place. A splash guard which did not prevent free gaseous diffusion was placed over the electrode during gas phase determinations.

Aeration efficiency rates were determined for flasks with various combinations of smooth or cleated walls and cotton wool bungs or milk filters. Each flask contained 300 ml. of phosphate buffer and was incubated at 30° with shaking at 140 rev./min. on the New

Brunswick gyrotary shaker. The flasks were flushed with oxygen-free nitrogen through the side arm until a zero oxygen partial pressure was obtained. The nitrogen supply was then disconnected, the side arm stoppered as required and the rate of diffusion of air into the flask determined by recording the increase with time in the partial pressure of oxygen.

#### Total Unbound Lipids

Total unbound lipids were isolated from freeze dried cells (dried to constant weight over silica gel) by extracting three times with chloroform methanol (2:1 v/v) over a period of three days at room temperature. The lipid extract was drawn off from the residue and filtered through Whatman paper (no. 542) which had been refluxed with chloroform: methanol (2:1 v/v) in a Soxhlet extractor. The filtered unwashed extract was dried by rotary evaporation at 25° in a pre-weighed round-bottom flask and then dried to constant weight over silica gel in a vacuum desiccator.

#### Triglycerides

Triglycerides, isolated from thin layer plates after fractionation of the neutral lipid fraction from silicic acid columns, were analysed for the presence of esters and glycerol.

Ester Determination was carried out in glass Servall centrifuge tubes (15 ml.) according to the method of Vioque and Holman (1962). The tubes were centrifuged at 4,500 g for 10 min. at room temperature just before measuring the absorbance. Methyl palmitate (Sigma 99+%) was used as a standard.

Glycerol Determination was carried out by the method of Van Handel and Zilversmit (1957). Tripalmitin (Eastman Organic Chemicals)

was used as the standard and analyses were carried out in 15 ml. glass stoppered Quickfit test tubes.

Alkaline Hydrolysis: samples of triglycerides were hydrolysed by the KOH isopropanol method of Noll and Bloch (1955). After refluxing for 1 hr. the solution was cooled, neutralised with 1N HCL and the organic soluble components exhaustively extracted with diethyl ether. Components of the ether soluble fraction were analysed by gas chromatography. The water soluble fraction was analysed (after desalting) by paper chromatography.

#### Total Fatty Acids

Alkaline Hydrolysis of Total Cells - Total fatty acids were extracted from freeze dried or wet cells by refluxing for 3 hr. with 10% KOH in 50% aqueous methanol (Hulanicka, Erwin and Bloch, 1964). After acidification with 5N HCL and dilution with glass distilled water, the fatty acids were exhaustively extracted with redistilled di-ethyl ether. The ether extract, even after washing with distilled water, always showed some slight opalescence.

Acid Hydrolysis of Total Cells. As an alternative procedure to the one described above, wet cells were hydrolysed in 6N HCL in sealed glass tubes at 100° for 6 hr. (Salton, 1953; Midwinter, 1962). The di-ethyl ether extract of the acid hydrolysate was washed with glass distilled water to yield a perfectly clear solution. For quantitative analysis of levels of total fatty acids in cells the washed ether extract was dried by rotary evaporation and made up to 25 ml. with redistilled di-ethyl ether. Aliquots of this solution were taken for total carbon analysis by the chromate method.

Estimation by Weight. Total fatty acids obtained after hydrolysis of total cells were determined by drying down the washed

ether extract in preweighed round bottom flasks on a rotary evaporator (Buchi). The weight of total fatty acids was determined by drying the flask to constant weight over silica gel in a vacuum desiccator.

Estimation by Chromate Oxidation. Quantitative analysis of total fatty acids in ether extracts was carried out using chromate reagent (Amenta, 1964). Aliquots of the ethereal solutions were dried under partial vacuum in a desiccator placed under a heat lamp. Further drying was carried out by overnight heating at 30° under high vacuum in a desiccator. Beakers containing fresh chromic acid solution (Vogel, 1964) and KOH pellets were included in the desiccator. After the addition of chromate reagent (2 ml.) each tube was incubated at 100° for 45 mins. The contents of the tubes were each diluted to 50 ml. with glass distilled water and read at 350 m $\mu$  using distilled water blanks. Palmitic acid (Sigma 99+%) was used as the standard.

#### Total Nitrogen

The total Nitrogen content of cells or phosphate buffer supernatants was determined by the micro-Kjeldahl procedure. Nitrogenous material was digested overnight with conc. H<sub>2</sub>SO<sub>4</sub> and a catalyst consisting of a mixture of CuSO<sub>4</sub>; K<sub>2</sub>SO<sub>4</sub>; SeO<sub>2</sub>; 1:8:0.1 (w/w/w) (Bryant, A., pers. comm.). The total digest was washed into a steam distillation apparatus (Markham, 1942) and the ammonia liberated with NaOH. The ammonia was trapped in a boric acid solution (2%) containing a few drops of a methyl red-methylene blue indicator (McKenzie and Wallace, 1954), and titrated with 0.01N HCL.

#### Protein

Cellular Protein. Total cellular protein was determined by the

Biuret procedure described by Stickland (1951). Times required for the complete extraction of cellular protein were examined. With cells suspended in glass distilled water and heated at  $100^{\circ}$  under alkaline conditions, complete extraction took 40 min. When cells were suspended in phosphate buffer the time for complete protein extraction was 60 min. If protein estimations were carried out in glass test tubes the alkali seemed to extract some component from the glass which complexed with  $\text{CuSO}_4$  and gave rise to a high and variable blank. To overcome this problem total cellular protein estimations were routinely carried out in thin walled polypropylene centrifuge tubes (20 ml.). Cell suspensions (3.3 ml.) were mixed with 20% NaOH (0.6 ml.) in the tubes which were covered by glass marbles and incubated for 60 min. at  $100^{\circ}$ . The contents of the tubes after cooling were mixed with 0.1 ml. of a solution of 25%  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  (w/v). After 20 min. the reaction system was centrifuged at 4,500 g. for 10 min. at room temperature (Servall Model SS-3); If the tubes were centrifuged at  $2^{\circ}$  a fine suspension, possibly caused by salts of fatty acids, appeared and this interfered with the subsequent estimation. Standards containing crystalline bovine plasma albumin (A grade Calbiochem), dissolved in either distilled water or phosphate buffer, were carried through all extraction and estimation procedures. The standards showed a decrease in colour response of 5 to 10% when they were heated at  $100^{\circ}$  in alkali for an hour.

Protein in Solution. Soluble protein, in supernatants and in  $\text{HClO}_4$  extracts of cells, was determined by the Folin method of Lowry, Rosebrough, Farr and Randall (1951). The Folin reagent (EDH) gave a white precipitate if samples containing more than 0.1 ml. of 0.1M phosphate buffer were used in the estimations.

### Amino Acids

Estimation. The method of Rosen (1957) as modified by Krishnaswamy et al (1965) was used to determine levels of amino acids in distilled water or 0.2N HClO<sub>4</sub> extracts of cells and in phosphate buffer supernatants from cell suspensions. Ammonia standards were not included in amino acid analyses, however, corrections were made for ammonia where it had been shown to be present by independent analyses using nesslerisation. The colorimetric response of ammonia in the amino acid estimation procedure was found to be 56% of that for glycine on a molar basis. Rosen (1957) quoted the molar response of ammonia compared with glycine at approximately 60%.

Extraction of Intracellular Amino Acids. Intracellular free amino acids can be extracted from bacteria with boiling water (Holden, 1962). When this procedure was examined with N. corallina cells it was found that the release of ninhydrin-positive material, determined by the Rosen (1957) method, reached a maximum after 15 min. The release of material absorbing at 260 m $\mu$  also reached a maximum after 15 min. It was apparent that proteinaceous material was being extracted by longer boiling times since at 15 min. the 260/230 and 260/280 wavelength ratios were 1.76 and 2.74 respectively whereas after 1 hr. heating the ratios were 1.52 and 2.53.

For routine estimations of intracellular amino acid levels 5 ml. samples (from resuspended cultures) in 15 ml. polypropylene tubes were centrifuged at 12,000 g for 7 min. at 2° (Servall RC-2 Centrifuge). The pellet was washed twice by resuspension in 5 ml. glass distilled water. Air condensers were inserted in the centrifuge tubes which were placed in a boiling water bath for 15 min., removed, cooled and centrifuged at 12,000 g for 10 min. Supernatants were stored at -25° pending amino acid analyses.

### Ammonia

In early experiments ammonia analyses were carried out according to the microdiffusion method of Conway (1947). In later experiments, samples containing dissolved ammonia were pipetted into the outer chamber of 20 ml. centre well flasks containing 0.5 ml. of 1N H<sub>2</sub>SO<sub>4</sub> in the centre well. The opening of each flask was plugged by a Suba Seal (no. +1) and 0.2 ml. of saturated potassium carbonate solution (Conway, 1947) was injected, through the seal and into the outer chamber, using a long hypodermic needle and syringe. Flasks were shaken gently at 30° overnight. The centre well contents were transferred with 1.5 ml. of glass distilled water into test tubes and the ammonia concentrations estimated by nesslerisation (Johnson, 1941) as described by Umbreit, Burris and Stauffer (1964).

### Total Carbohydrates

Total hexose content of cells and supernatants was determined by direct analysis of aliquots using the anthrone method (Dreywood, 1946). Samples (0.1 ml.) were diluted to 1 ml. with glass distilled water in large test tubes (2 cm. x 20 cm.). The tubes were placed in a water bath at 1 - 2° and agitated during the addition of the anthrone reagent. Air condensers were attached to the tubes which were heated at 100° for exactly 7 min. After cooling (20 min.) the colour absorption at 625 mμ was measured. Glucose (H and W Analytical Reagent) dissolved in phosphate buffer was used as the standard.

Total reducing sugars were estimated on acid hydrolysed samples by the method of Nelson (1944). An examination of the rate of release of reducing sugars from cellular polysaccharides by hydrolysis in 1N H<sub>2</sub>SO<sub>4</sub> showed that complete liberation occurred within 2 hr. For the routine analysis of polysaccharides as total reducing sugars,

5 ml. aliquots of cell suspensions were centrifuged at 4,500 g for 7 min. in glass centrifuge tubes (15 ml. Servall). The supernatant was discarded, 5 ml. of 1N  $H_2SO_4$  was added, the tubes were then fitted with an air condenser and heated in a boiling water bath for 2 hr. The acid digests were subsequently washed into 50 ml. polypropylene centrifuge tubes, neutralised with solid  $BaCO_3$  and centrifuged (3,000 g, 7 min.). The supernatant was poured off and the pellet resuspended in distilled water and recentrifuged at 12,000 g for 7 min. The combined supernatants were made up to 20 ml. in a volumetric flask. The solution was typically turbid and required filtering through a membrane filter (0.8  $\mu$  HA Millipore) before aliquots were taken for reducing sugar analysis. Addition of Nelson Reagent to test samples gave a light white precipitate (probably  $BaSO_4$ ) which was removed by centrifugation (12,000 g, 7 min.). Standard solutions were not affected by the formation and subsequent removal of the white precipitate.

The use of  $BaCO_3$  to neutralise samples after acid hydrolysis provided a convenient method of desalting samples which would also be used in paper chromatographic separations.

### Ribose

Pentose estimations using ribose (Calbiochem. A grade) as the standard were carried out by the orcinol method of Kerr and Seraidarian (1945) with a heating time for colour development of 30 min. (Munro, Hutchison, Ramaiah and Neilson, 1962). When these estimations were carried out on supernatant from cell suspensions in phosphate buffer a fine deep purple precipitate often formed during colour development. This precipitate was possibly caused by the presence of

peptides and could be removed by filtration through a membrane filter (0.45  $\mu$  HA Millipore).

### Deoxyribose

The diphenylamine method of Burton (1956) was used to detect deoxyribose in HClO<sub>4</sub> (0.1 and 0.2N) extracts of cells using deoxyribose (Calbiochem. A grade) as standard.

### RNA

Intracellular RNA was estimated by the method of Munro and Fleck (1966). Cell suspensions (10 ml.) of N. corallina containing approximately 2 mg. dry wt. of cells per ml. were centrifuged at 4,500 g for 7 min. in Servall glass centrifuge tubes (15 ml.). The phosphate buffered supernatants were transferred to test tubes and frozen at -25<sup>o</sup>. Pellets were suspended in 5 ml. ice cold 0.2N HClO<sub>4</sub> for 10 min. centrifuged (4,500 g , 7 min.) and washed twice more by resuspension in 0.2N HClO<sub>4</sub>. All of the washing solutions from a single pellet were combined to yield the 0.2N HClO<sub>4</sub> wash fraction which was stored at -25<sup>o</sup> for subsequent examination of the UV absorbance.

The washed pellet was incubated for 2 hr. at 37<sup>o</sup> with 3N KOH and the so called RNA fraction containing the products of RNA hydrolysis in 100 ml. of 0.1N HClO<sub>4</sub> was prepared by the method of Munro and Fleck (1966) with one modification. Combined supernatants and washes containing the products of RNA hydrolysis were filtered through a membrane filter (0.45  $\mu$  HA Millipore) at 2<sup>o</sup> into a test tube (50 ml.) held in a Buchner flask. Filtration of the extract through a membrane filter was required for two reasons. Firstly,

after 2 hr. incubation with 0.5N KOH at 37° the final 0.1N HClO<sub>4</sub> extract always showed a faint pinkish opalescence. On standing overnight at 2° this tended to aggregate. Secondly, after centrifuging the 15 ml. glass centrifuge tubes, a white precipitate collected at the surface of the supernatant at the end of the 2 hr. incubation period. Both pink opalescence and white precipitate (possibly lipid) were removed by filtration to yield a perfectly clear solution. The filter was washed with 0.6N HClO<sub>4</sub> and the filtrate was transferred to a 100 ml. volumetric flask and made up to the mark with distilled water.

The absorbance of the phosphate buffered supernatant from the original cell suspension, the 0.2N HClO<sub>4</sub> wash and the 0.1N HClO<sub>4</sub> solution containing the products of alkaline hydrolysis of RNA, was measured at 260 mμ. using the Beckman D.U. Spectrophotometer or between 200 and 400 mμ. using a Unicam SP-800 Ultraviolet Spectrometer.

### Preparative Methods

#### Freeze Dried Cells

Cells were freeze dried (Virtis freeze dry apparatus) as thick suspensions (5-10 gm. wet wt.) spread over the inside of a 500 ml. freeze dry flask. Drying was considered to be complete when ice could no longer be observed in the flask (10 hr., approx.).

#### Organic Solvents

All organic solvents used were either analytical grade or purified and redistilled according to the methods of Vogel (1964).

### Potassium Palmitate Solutions

Palmitate solutions were prepared by neutralising palmitic acid dissolved in ethanol with 1N KOH. After removal of the ethanol by rotary evaporation (Buchi Rotavapor) the potassium palmitate was dissolved by warming in phosphate buffer. Aliquots of the warm solution were transferred to manometer flasks for R.Q. estimations.

Where experiments were carried out with  $^{14}\text{C}$ -labelled palmitic acid, aliquots of [ $^{14}\text{C}$ ] palmitic acid (100  $\mu\text{C}/\mu\text{mole}$ , Amersham) were added to the ethanolic solution of carrier fatty acid. The solution was then neutralised, taken to dryness and dissolved in phosphate buffer as described above.

### Methylation of Fatty Acids

Methyl esters were prepared either by refluxing the free acids for 2 hr. in 1%  $\text{H}_2\text{SO}_4$  in super dry methanol (Vogel, 1964) or more commonly by the method of Schlenk and Gellerman (1960) using diazomethane generated from N-Methyl-N-nitroso-p-toluenesulfonamide (Diazald. Aldrich Chemical Co.). In both cases excellent conversions of acids to esters were obtained but the diazomethane was much more rapid and convenient.

### Silylation of Hydroxy Esters

Silylation of hydroxy esters was carried out using TRI-SIL DMF (Pierce Chemical Co.). Methyl esters of hydroxy acids, dissolved in diethyl ether, were taken to dryness in 10 ml. rotary evaporator tubes. A small volume (0.5 ml.) of a mixture of TRI-SIL 1 and TRI-SIL 2 (1:1 v/v) was prepared and added.

Trimethylsilyl ethers were isolated from the reaction mixture after addition of distilled water by extraction with diethyl ether.

## Chromatography

### Paper Chromatography of Polyols and Sugars

Preparation of Samples. The water soluble fraction obtained after alkaline hydrolysis of triglycerides was desalted, by passing through a column (1.5 cm. x 10 cm.) of a mixed bed resin (Biodeminrolite, Hopkin and Williams Ltd.) which had been thoroughly washed with distilled water. The eluate was concentrated by rotary evaporation at 30°. Aliquots of this solution were applied directly to paper chromatograms.

Analysis of Sugars in Perchloric Acid Solutions Obtained During RNA Estimations. The  $\text{HClO}_4$  solutions were neutralised with 1N KOH, cooled to 2°, filtered through a membrane filter (0.45  $\mu$  HA Millipore) at 2°, reduced in volume by rotary evaporation at 30° and finally acidified to give a 1N  $\text{H}_2\text{SO}_4$  solution. This solution was refluxed for 2 hr. at 100°, cooled, transferred to a 50 ml. polypropylene centrifuge tube and neutralised with solid  $\text{BaCO}_3$ . After centrifuging at 12,000 g for 10 min. the supernatant was passed directly through a de-ionising column of Biodeminrolite. The eluate was taken almost to dryness at 30° and then the concentrate was made to 5 ml. with glass distilled water. Aliquots were applied directly to paper chromatograms.

Solvent Systems and Detection of Components. Samples containing sugars were chromatographed on Whatman no. 1 paper by descending chromatography in either, ethyl acetate, acetic acid, formic acid, water, 18:3:1:4, or ethyl acetate, pyridine, water, 2:1:2 (upper phase in trough, lower phase in tank). Papers were developed by dipping in the silver nitrate reagent of Smith (1960). Quantitative analysis of the sugars on paper chromatograms was carried out by the

method of Wilson (1959). Papers were sprayed with phthalate reagent and the absorbance of the eluted spots measured at 350 m $\mu$ . for pentoses and 390 m $\mu$ . for hexoses.

#### Silicic Acid Column Chromatography

Total Unbound Lipids. Total chloroform methanol (2:1 v/v) soluble lipids dissolved in 5 ml. of chloroform methanol 2:1 were run onto a column of 100 mesh Mallinckrodt silicic acid slurried in chloroform. The silicic acid had been previously activated at 110<sup>o</sup> for 5 hr. The neutral lipid fraction was eluted with 200 ml. chloroform and the polar lipids with 200 ml. methanol.

Total Fatty Acids. Silicic acid columns using 325 mesh silicic acid prepared by Bio-Rad laboratories according to the method of Hirsch and Ahrens (1958) were used to separate classes of fatty acids. The size of column required for any particular separation was estimated using the ratio 100 gm. of silicic acid to 1 gm. of fatty acid (Cashmore A.R., pers. comm.). A slurry of silicic acid in light petroleum (57<sup>o</sup>-59<sup>o</sup>) was poured into a glass column fitted with a teflon tap and a hydrophobic polyethylene sinter (Bel-Art Products) to support the packing. About 1 cm. (depth) of Celite 545 was placed on the sinter before pouring in the slurry of acid. Care was taken to prevent the packing from running dry at any stage.

Fatty acid samples were dissolved in redistilled diethyl ether and added slowly to a few grams of silicic acid in a beaker, so that the ether evaporated between each addition. The mixture of fatty acids and the silicic acid was spread on a large watch glass, placed under vacuum in a desiccator for 15 min. and finally poured as a very fine stream into the light petroleum at the top of the silicic acid

column. When the added material had settled, an additional 1 gm. of silicic acid was added, followed by a circle of chloroform methanol (2:1) extracted filter paper. Different classes of fatty acids were eluted at a flow rate of 2.5 ml./min., using mixtures of light petroleum (57°-59°) and diethyl ether. Fractions (50 ml.) were collected manually or with a Gilson Fraction collector and the solvent removed by rotary evaporation in preweighed round bottom flasks.

#### Thin Layer Chromatography

Preparation of Thin Layer Plates. Analytical (250  $\mu$  thick) and preparative (750  $\mu$  thick) thin layer plates were prepared with Adsorbosil 1 (Applied Science Labs.) slurried in glass distilled water, using a Shandon Unoplan thin layer applicator. The plates were allowed to dry at room temperature for at least 12 hr. before running solvent mixture through the thin layer as a washing procedure. The washing procedure involved placing the plates in a tank containing an appropriate solvent, for 2 to 3 hr. during which time the solvent moved up the plate and was absorbed by a layer of Whatman no. 3 filter paper (2 cm. x 0.5 cm. x the width of the plate) attached to the top. Plates were generally washed in the same solvent used in the subsequent chromatographic separation, although washing in more polar solvents was desirable when small amounts of compounds were being purified prior to Mass Spectrometry. After washing, plates were dried for approximately 15 min. at room temperature before the application of the samples.

Analytical silver nitrate plates were prepared using Adsorbosil 1 mixed with 7% (w/w) silver nitrate. These plates were dried at

room temperature in the dark, activated at  $110^{\circ}$  for 1 hr. cooled to room temperature and the samples applied without prior washing.

Application of Samples. Samples dissolved in an organic solvent were applied with micro pipettes or capillary tubes. The best resolutions were obtained if samples were applied using disposable applicators similar in basic design to the applicator described by Monteiro (1965). A draught of air from a fan was used to evaporate solvents during sample application to the plates which were never warmed.

Solvent Systems. Solvent systems used for separating neutral lipid fractions were mixtures of petroleum ether ( $57^{\circ}$ - $59^{\circ}$ ), diethyl ether and acetic acid. For more polar lipids mixtures of chloroform, methanol and acetic acid were used.

Identification and Isolation of Components. Thin layer plates were developed to a height of approximately 10 cm. and components were identified by spraying with an ethanolic solution of phosphomolybdic acid, (15% w/v) followed by heating ( $150^{\circ}$  15 min.) and subsequent exposure of the cooled plates to 0.880 ammonia vapour.

To localise components on preparative plates the centre was covered and the edges were sprayed with a solution of phosphomolybdic acid. Gentle heating of the sprayed areas using a fishtail burner effectively revealed the positions of the components without heating the whole plate. Components in the unsprayed section were removed from the plate using a square, hollow, stainless steel rod (6.5 cm. x 0.7 cm. x 0.7 cm.). The inside edges at one end were sharpened and by pushing the rod across a plate, held almost vertically, a very clean removal of a band into the hollow rod was effected. Material

from the plate fell through the rod into a test tube held under the lower end.

Autoradiography of Thin Layer Plates. Prior to autoradiography, thin layer plates which had been developed to separate radioactive compounds, were dried at room temperature for several hours and then lightly sprayed with 1% collodion (w/v) dissolved in amyl acetate: acetone (1:1 w/v) to harden the surface. The dried plates were placed in close contact with X-ray film (Kodak Royal Blue Medical X-ray Film) and left in the dark for 2 to 3 weeks. The film was developed and the plates were sprayed with 15% phosphomolybdate and heated. The presence of collodion on the plates increased background staining but the components were still visible.

#### Gas Chromatography

Gas Chromatography of Methyl Esters of Fatty Acids Derived from Triglyceride. The methyl esters of fatty acids derived from triglycerides were chromatographed using a Gas Chromatograph (Packard Series 800) fitted with a tritium foil ionisation detector. A 4 ft. glass column (4 mm. I.D.) packed with 15% Apiezon L on 100/120 Chromosorb W was maintained at 200° and argon (K.Z. Industrial Gases) was used as the carrier gas at a flow rate of 40 ml./min.

Silyloxy Derivatives of Methyl Nocardates. Gas chromatography of the silyloxy derivatives of methyl nocardates was carried out using an Aerograph Model 660 gas chromatograph with a hydrogen flow ionisation detector. Column and temperature conditions were similar to those reported by Kuksis and Breckenridge (1966) for the separation of triglycerides. The injector block temperature was maintained at

280<sup>o</sup>, the detector and gas outlet at 300<sup>o</sup> and the column temperature was manually programmed from 250<sup>o</sup> to 300<sup>o</sup>. An 80 cm. stainless steel column (0.4 cm. I.D. 0.6 cm. O.D.), the inside surface of which had been deactivated with HMDS, was filled with 3.65 g. of HMDS Chromosorb W (Wilkins) coated with 2% SE-30 (Wilkins). After conditioning the column for 2 days at 280<sup>o</sup> using nitrogen as a carrier gas it was connected to the flame ionisation detector by a 1 to 4 stream splitter. The column was considered well conditioned when no base line rise could be detected on programming to temperatures up to 300<sup>o</sup>. The nitrogen flow through the column was adjusted to 120 ml./min. and the flow through the flow ionisation detector (2+ ml./min.) balanced 1 to 1 with the flow of hydrogen from a hydrogen generator (Aerograph Model 650).

Fractions corresponding to peaks detected by the flame ionisation detector were cut at the major gas outlet using sections of glass tubing. The silyloxy derivatives condensed on the surface of the glass within a few centimeters of the heated outlet.

Pyrolysis of Methyl Nocardates. Pyrolysis was carried out using an unmodified Aerograph 660 gas-chromatograph. Using the same column and detection system as described for separating silyloxy derivatives (no stream splitter) and a column gas flow rate of 25ml./min. the injector temperature was adjusted to 280<sup>o</sup> and the column temperature to 90<sup>o</sup>. The column gas flow was turned off using the fine control and the methyl esters of the nocardic acids injected. After 12 min. a gas flow of 25 ml. was re-established and the products of pyrolysis were separated with temperature programming of the column from 90<sup>o</sup> to 260<sup>o</sup> on the SE-30 column. Components in the effluent gas stream were detected by a hydrogen flameionisation system.

## CHAPTER 2

GROWTH CHARACTERISTICS OF N. CORALLINADevelopment of a Defined Medium

In previous investigations with N. corallina (Robertson, 1964), the organism was grown in a liquid medium containing:- glucose (0.75%), yeast extract, thiamine,  $(\text{NH}_4)_2\text{SO}_4$ , phosphate buffer (0.1M approx.) and salts ( $\text{MgSO}_4$ ,  $\text{CaCl}_2$  and  $\text{NaCl}$ ). The reaction of the final medium was pH 6.8. The preparation of the medium involved both heating and filtration steps which changed the final salt concentrations. Midwinter (1962) cultured N. corallina in a similar medium but yeast extract was not included.

Experiments were carried out to produce a defined medium capable of (a) producing a high yield of cells and (b) maintaining a constant pH during growth. The medium finally adopted (Appendix 1) contained per litre: thiamine, 0.025 g.;  $(\text{NH}_4)_2\text{SO}_4$ , 3.0 g.;  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.1 g. and  $\text{KH}_2\text{PO}_4$ , 13.6 g. The medium was adjusted to pH  $7.00 \pm 0.05$  by addition of 5N NaOH. Glucose (0.75% final conc.) was added after autoclaving.

The yield of cells at full growth (approx. 50 hr. after inoculation) from 300 ml. of this medium (contained in a cleated flask fitted with a milk filter) was approximately 0.90 g. dry wt. A change of reaction from pH 7.0 to 6.7 was recorded during growth.

Aeration Efficiency of Culture Flasks

Considerable variation was observed in the yield of cells and rate of flocculation on standing, with cultures grown in standard flasks (conical 1L) plugged with cotton wool. As a possible explanation

for these variations it was considered that the cultures might not be adequately aerated during growth. An investigation was carried out using the sulphite method (Corman et. al., 1957; Ecker and Lockhart, 1959) for determining the rate oxygen supply to standard flasks fitted with cotton wool plugs. The results suggested that the maximum rate of oxygen diffusion into the solution contained in the flasks was less than the maximum rate of oxygen uptake (determined manometrically) in late log phase cultures. Sparging as a method for improving aeration presented difficulties because of foaming and the adherence of clumps to the sparger. The method of Corman et. al. (1957) using cleated flasks and milk filters to provide rapid and reproducible rates of aeration was tested. Standard flasks (conical 1L.) were indented in the lower part of the wall with six equidistant cleats approximately  $1\frac{1}{2}$ " high and  $\frac{1}{2}$ " deep. Tests with these flasks showed that for contents of 300 ml. a shaking rate of 140 rev./min. on the New Brunswick gyrotary shaker could not be exceeded without splashing.

A comparative study of aeration efficiencies in standard and cleated flasks was carried out using an oxygen electrode. Although the results (Table 2) showed that the cleats increased the rate of solution of oxygen, the major factor which affected the efficiency of aeration was the type of plug used. Cotton wool plugs, compared with milk filters decreased the rate of oxygen diffusion into flasks threefold.

#### Oxygen Demand in Liquid Cultures

Measurement of  $pO_2$  values in flask cultures was made by inserting an oxygen electrode directly into actively growing cultures. Meaningful  $pO_2$  values could only be obtained over short periods (2-3 hr.) since the electrode membrane became coated with organisms, and frequent washing

Table 2

Rates of Diffusion of Oxygen into Gas and Liquid Phases  
of Cleated and Standard Flasks.

Flask*	Time (min.) for $pO_2$ to reach 50% from 0% of saturation.**	
	Liquid phase	Gas phase
Cleated; milk filter; cotton wool plug in side arm.	8	5
Standard; milk filter; cotton wool plug in side arm.	11	5
Cleated; cotton wool plug; cotton wool plug in side arm.	23	19
Standard; cotton wool plug; cotton wool plug in side arm.	30	22
Cleated; milk filter; air pumped into side arm (1.5 L./min.).	1	0.5
Standard; milk filter; air pumped into side arm (1.5 L./min.).	3	0.5

\* Flasks (conical 1 L.) containing phosphate buffer (300 ml.) were incubated at  $30^{\circ}$  with shaking at 140 rev./min. Flasks were stoppered as described in the left hand column.

\*\* The response of the oxygen analyser was very nearly linear between 0 and 50% of saturation.

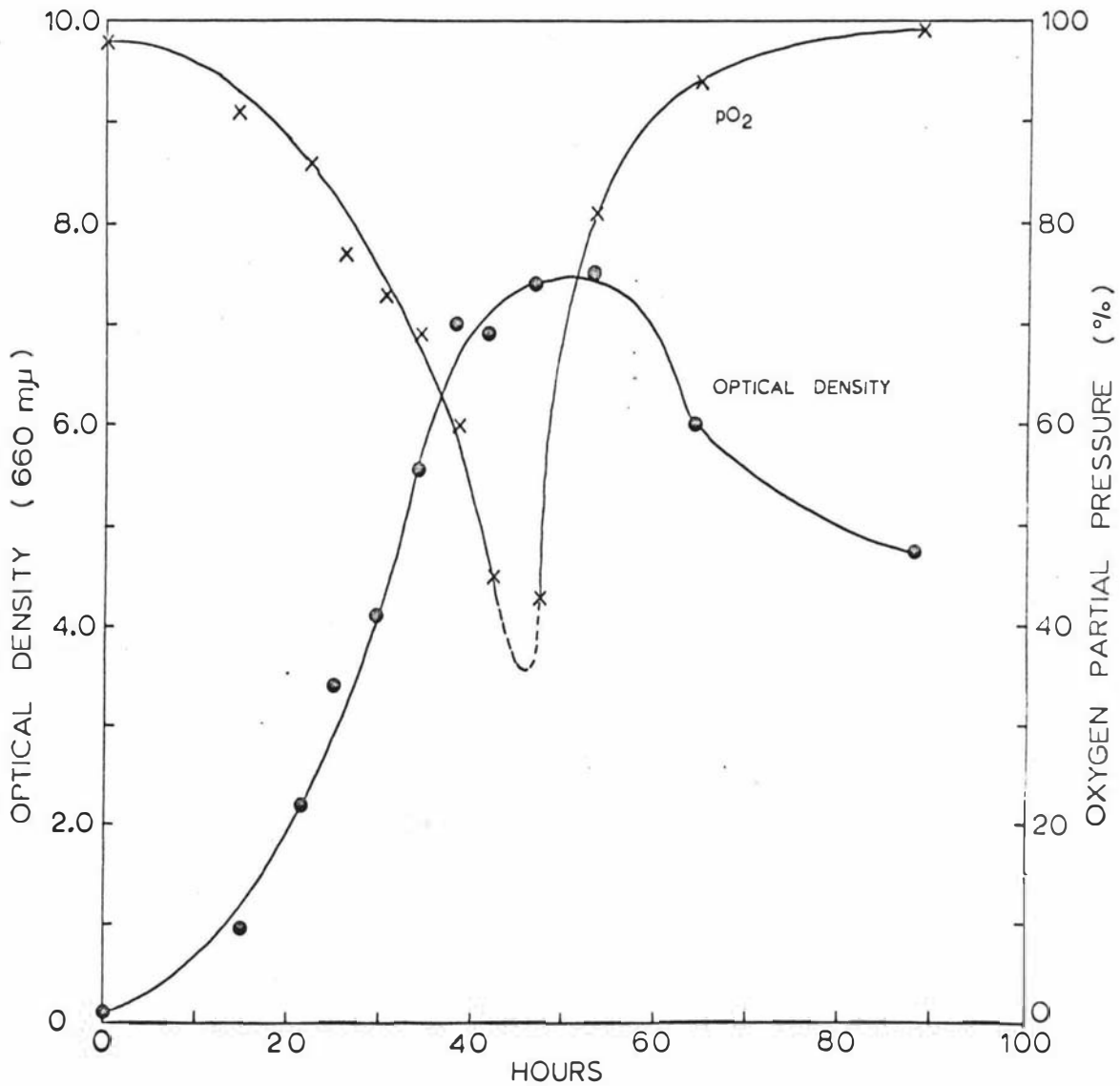
of the electrode followed by reinsertion into the same flask greatly increased the possibilities for contamination. To some extent this problem could be overcome by transferring the electrode, washed by a jet of sterile distilled water from a wash bottle, into a new culture flask after an interval of several hours. The results of an experiment in which the electrode was transferred from flask to flask as growth progressed are shown diagrammatically in Fig. 1. Two  $pO_2$  determinations were obtained for each of the 6 clefted flask cultures which had all been inoculated at the same time from the same primary flask (see Methods). Optical density estimations were also carried out on the contents of the flasks during the experiment. The results showed that the  $O_2$  demand was greatest in the late log phase and that the partial pressure of oxygen in solution could fall to 35-40% of saturation.

The respiratory rate-limiting  $pO_2$  for a phosphate buffer suspension of cells (3.0 mg./ml.) incubated with glucose (0.5 mg./ml.) at  $30^\circ$  was determined using the oxygen electrode. After flushing the suspension with air, inlets and outlets were sealed off and the oxygen uptake recorded. The consumption became dependent on the rate of oxygen supply (as determined by the  $pO_2$  at which the oxygen uptake curve became non linear) at a  $pO_2$  of 15-20% of saturation. It seemed reasonable to conclude that since the  $pO_2$  of growing cultures fell to 35-40% of saturation these cultures were not seriously restricted by deficiency of oxygen although there was a possibility that the rate of consumption could approach the rate of supply in the late log phase.

The observation that growth curves were identical, for cultures grown in clefted flasks with milk filters with or without humidified air

Figure 1

Change in Oxygen Partial Pressure in Cultures of N. corallina Grown in Liquid Medium.



Cultures were grown in cleaned flasks (conical 1L, fitted with milk filters) containing 300 ml. of glucose medium, by incubating at 30° with shaking at 140 rev./min. After two pO<sub>2</sub> values had been obtained for the contents of a single flask the oxygen electrode was removed and inserted into a fresh flask.

Optical density estimations, corrected for dilution, were not carried out on flasks after they had been used for more than two pO<sub>2</sub> estimations.

pumped into the side arm, also indicated that oxygen was not limiting growth in this system.

### Clumping of Cultures

Comparative studies of the rates of growth of N. corallina in standard and cleated flasks fitted with milk filters showed marked differences. Firstly, the growth rate of cultures in cleated flasks always appeared more rapid than in standard flasks, and secondly, a fall in optical density always occurred after full growth had been reached in cleated flask cultures, whereas no, or only a small decrease was observed with cultures in standard flasks (Fig. 2). Differences were also observed in the shape of growth curves of cultures grown in cleated flasks possibly because the cleats were not uniform. Where cleats were sharp and deep, cultures were evenly suspended. Where cleats were shallow and rounded the cells tended to stick together in clumps of up to 250  $\mu$  in diameter. These clumps flocculated rapidly on standing.

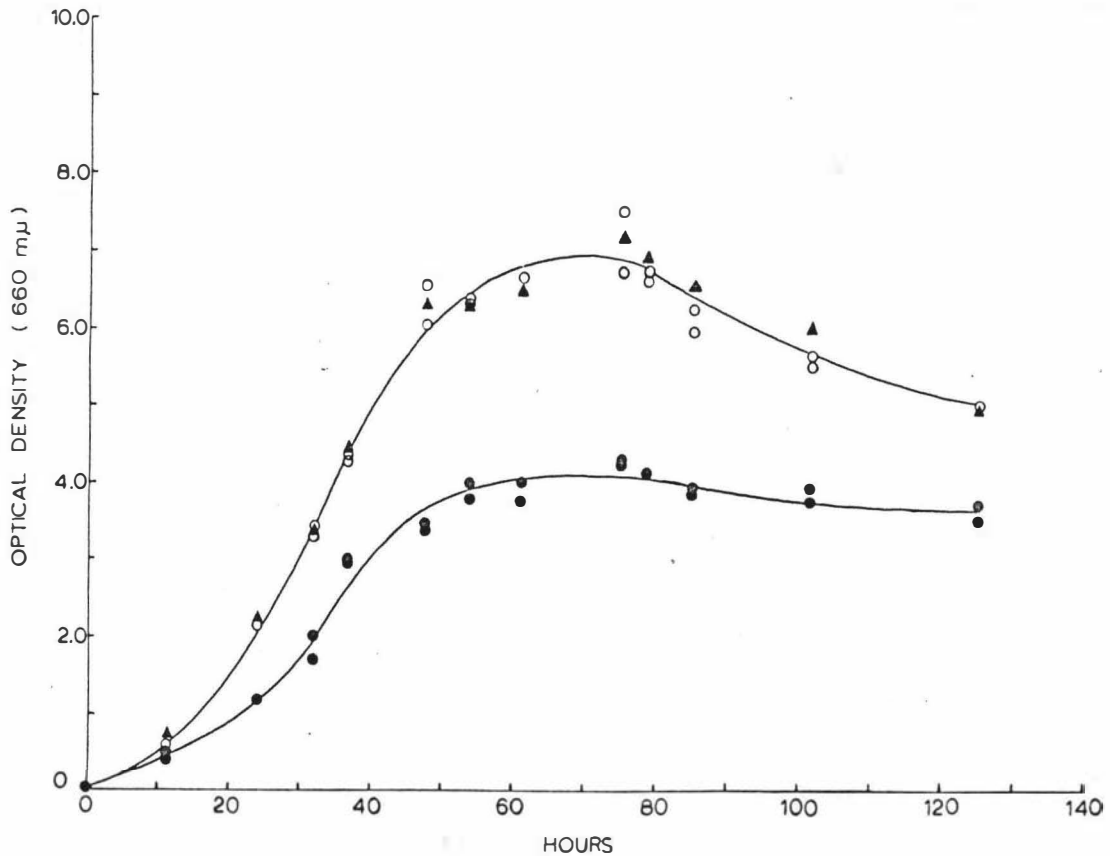
The effect on the optical density estimations of homogenising samples taken from cultures grown in standard flasks was examined (Fig. 2). If samples were homogenised prior to reading at 660 m $\mu$  the resulting growth curve was found to be identical with that observed in sharply cleated flasks. A fall in optical density was also observed in standard flask cultures following full growth providing samples were thoroughly homogenised (Fig. 2).

### Culture Pigmentation

Cultures grown in standard flasks with cotton wool plugs showed a considerable variation in culture pigmentation. Some cultures were deep pink at the end of growth whereas others were cream or yellow in

Figure 2

The Effect on Optical Density Estimations of Homogenising Samples from Cultures of N.corallina Grown in Standard Flasks.



Cultures were grown in cleated and standard flasks (fitted with milk filters) containing 300 ml. of medium, by incubating at 30° with shaking at 140 rev./min. In this experiment full growth was not reached for a period of 60 hr. after inoculation because the primary culture used to inoculate the medium in the secondary flasks had not reached the mid-log stage before the secondary flasks were inoculated. (see Methods)

●: Optical density of samples from 2 standard flasks. Samples were not homogenised prior to optical density estimations.

○: Optical density of samples from the same standard flask cultures. Samples were homogenised prior to optical density estimations.

▲: Optical density of samples from a single cleated flask culture. Samples were not homogenised prior to optical density estimations.

colour. Although pellets obtained after centrifuging indicated that cells from late stationary phase cultures were a deeper pink than those from log phase cultures, the differences in culture pigmentation were largely due to an optical effect related to the concentration of organisms in liquid cultures. At low concentrations the cultures appeared cream, at high concentrations they were pink. Where cultures were grown in cleated flasks with milk filters the colour change from cream to coral pink during growth was consistently reproducible. Pellets obtained from cultures grown in a poorly buffered acetate media (pH rose to 8.0 during growth) showed a peach colouration.

#### Variations in the Size of Individual Cells

Although cells were routinely cultured in cleated flasks with milk filters, it was observed that some batches tended to flocculate more rapidly than others. Although this was partly due to cleat variations in different flasks, it was also noted that there were variations in the length of cells. Cultures containing a high proportion of cells, 5-8  $\mu$  in length tended to flocculate more rapidly on standing than those containing cells predominantly 2-4  $\mu$  in length. Cell length was determined microscopically after Gram staining. Reasons for differences in cell lengths have not been explained but they could be related to the ability of N. corallina to grow in hyphae, which subsequently fragment into cocci, 2-4  $\mu$  in length. It was possible that the cleats promoted fragmentation. There also seemed to be a relationship between the age of the slope used to inoculate the primary culture and the average length of cells in the secondary cultures. Cultures grown from fresh slopes which had been stored for no longer than 2 days at 2<sup>o</sup>, generally contained cells 2-4  $\mu$  in length. All cells were between 0.8-1.2  $\mu$  in width.

### Clusters in Suspensions of *N. corallina*. Their Effect on Total and Viable Cell Counts

A study of a sample of a suspension of *N. corallina* in phosphate buffer (contained in a cleaned flask) using the Leitz microscope and Thoma counting chamber, showed that although large clumps were not present, clusters occurred. Some of these consisted of aggregations of several hundred cells sticking together in what appeared to be a random fashion. Many of these clusters adhered to the glass walls of the counting chamber making total cell counts very difficult. It had been noted previously that where cell suspensions were spread on agar contained in Petri dishes and subsequently incubated at 30° that some colonies appeared as pin point colonies after 2 days whereas others were as large as 2 mm. in diameter. These variations could be due to the presence of different sizes of clusters in the inoculum.

### The Effect of Detergents on Cluster Size

The effect of detergents on the dispersion of clusters was examined by the method for total cell counts. The distribution of cells within clusters of different sizes was determined for a phosphate buffer suspension of *N. corallina* containing approximately  $4 \times 10^8$  cells/ml. in the presence and absence of detergent. Approximately 300 cells were counted in each determination. It was found that both Teepol and Tween 80 facilitated the dispersion of the larger clusters and prevented cells sticking to the glass surface of the counting chamber.

Detergent	Distribution of cells (%) in clusters of different sizes in phosphate buffer suspensions of <u>N. corallina</u> .			
	Cells per cluster			
	1	2	3	4 >>
- Teepol	24	35	14	27
+ Teepol	59	34	3	4
- Tween 80	23	24	12	41
+ Tween 80	46	28	9	17

Teepol seemed to be slightly more efficient than Tween 80, but since Teepol completely inhibited the growth of cells on agar slides, Tween 80 was used for viable counts and Teepol for total counts.

The effect of the detergents in both decreasing the adherence of cells to glass, and facilitating the dispersion of clusters, made it feasible to use the counting chamber method for total cell count estimations. Some clustering still existed but more drastic methods for dispersion were not used in case extensive cell damage occurred. Accordingly, for viable counts, Tween 80 was added to suspensions and an estimate of the degree of clustering was made for each cell viability determination.

The method for determining cell viability as described by Postgate, Crumpton and Hunter (1961) gave a rapid procedure for determining both cluster size distribution and cluster viability. From these values the cell viability was determined using formulae (see Methods). Using the formulae on results obtained from suspensions where the majority of cells

were in clusters of ones, twos and threes, it was found that the cluster viability could be up to 10% greater than the cell viability (Appendix 2). At high cluster viabilities however the effect of the clustering was small (3-5%).

#### Growth of *N. corallina* on Agar Slides

In a study of the slide culture method of Postgate, Crumpton and Hunter (1961) using suspensions of *N. corallina* in phosphate buffer containing 0.5% Tween 80 a sequence study of the growth of a single cell on the surface of the agar was made by examining and photographing the same cell at time intervals under bright field light microscopy (Fig. 3). The growth pattern was similar to that described for *N. corallina* by Brown and Clark (1966). Fragmentation into cocci on agar slides was not generally observed since the hyphae formed a dense mat over the entire surface of the agar. If slides, on which small non-fragmented colonies had developed after incubation for 18 hr. at 30°, were stored at 2° then fragmentation into cocci, 2-4  $\mu$  in length, did occur.

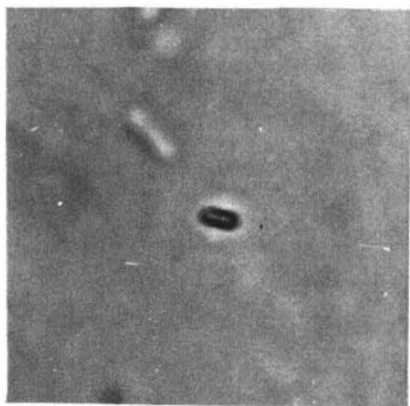
Considerable variation was noted in the times taken for the initiation of growth of individual cells on the agar surface. These variations were particularly pronounced with cells which had been resuspended in phosphate buffer for some days.

#### Summary

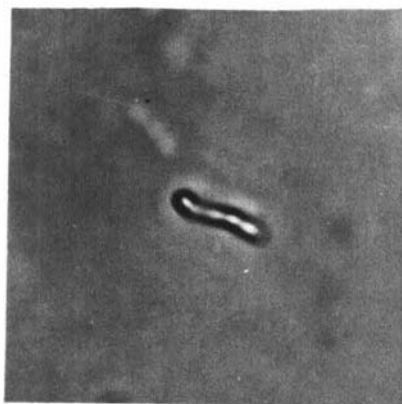
Cultures grown in standard flasks (conical 1L.) plugged with cotton wool, showed heavy clumping. Cultures grown in cleated flasks with milk filters, were evenly suspended, showed reproducible growth rates and were adequately aerated throughout growth. Detergents were used to reduce the size of cell clusters in suspensions for viable and total cell counts. Growth of *N. corallina* in liquid medium was characterised by division of bacilli approximately 4  $\mu$ . long and growth on agar was characterised by the formation of hyphae 8-12  $\mu$ . long.

Figure 3

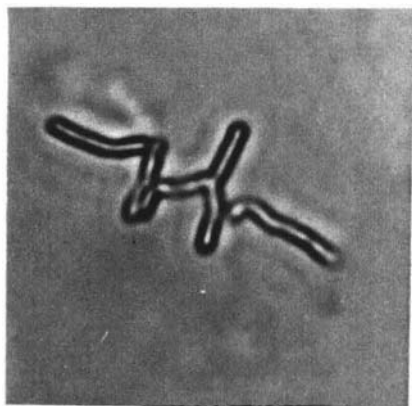
Growth of a Single Colony (or Cluster) from a Single Cell of N.corallina on the Surface of an Agar Slide



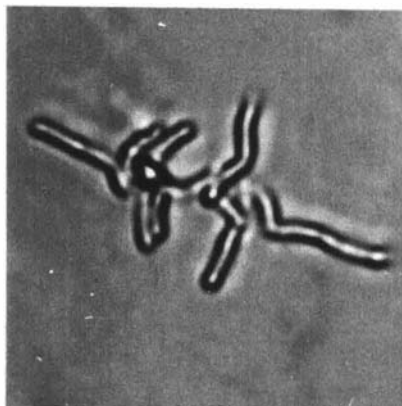
6½ hr.



13½ hr.



19½ hr.



21½ hr.

The agar slide was incubated at 30° for 21½ hr after inoculation.

The growth of a single cell was studied by photographing the same cell at time intervals during the incubation period. Scale. 1cm.: 5.88μ.

## CHAPTER 3

TRIGLYCERIDES IN N. CORALLINA

In 1962 Midwinter had shown that total cellular fatty acids of N. corallina decreased from approximately 17 to 14% of the cell dry weight. Midwinter (1962) concluded that this fall would account for as much as 50% of the total oxygen uptake by cell suspensions respiring endogenously. Other studies, (Batt, unpublished results; Robertson, 1964) had shown that when cells were incubated with the potassium salts of either  $^{14}\text{C}$ -labelled palmitic, stearic or oleic acid, these acids were rapidly incorporated into a neutral lipid fraction, assumed to be triglyceride. Such findings led to the proposition that triglycerides might be important endogenous reserves. It was therefore decided to confirm the presence or absence of a triglyceride fraction in the total lipids extracted from N. corallina and to determine whether  $^{14}\text{C}$ -labelled palmitic acid was incorporated into such a fraction.

Extraction and Fractionation of Total Lipids

Earlier studies had used hot chloroform-methanol (2:1) to extract total unbound lipid from cells dried by rotary evaporation at  $30^{\circ}$  (Robertson, 1964). This method had a number of disadvantages. Firstly, the extracted lipid was very difficult to wash by the Folch method (Sperry, 1952) due to the formation of stable emulsions. Secondly, the dried lipid would not completely redissolve in cold chloroform-methanol (2:1). Further, it was found that when tripalmitin was refluxed with chloroform-methanol (2:1) for 4 hr., a mixture of products was

obtained including a component which chromatographed on thin-layer plates with methyl palmitate. It seemed likely that some transesterification was occurring and it was found that this could be avoided if cells were extracted with cold chloroform-methanol (2:1). For this reason, cold solvent extractions were used for routine extraction of total unbound cellular lipids. Fractionation of total unbound lipids extracted with cold chloroform-methanol (2:1) into neutral and polar fractions was carried out using silicic acid column chromatography (Borgstrom and Olivecrona, 1961). Neutral lipids were eluted with chloroform and then polar lipids with methanol. This procedure, used previously by Robertson (1964) and again in the present investigation presented difficulties through the tendency of the total lipids, extracted from N. corallina to form a gel in chloroform. The gel zone that formed at the top of a silicic acid column caused the flow rate to be very slow. In part this problem could be overcome by applying high pressures to the solvent at the top of the column. In later studies it was observed that the gel formation in both chloroform and carbon tetrachloride solutions was a property of long chain hydroxy acids. Such acids were found in the lipids of N. corallina.

#### Thin-Layer Chromatography of Lipids of N. corallina

As an alternative procedure to column chromatography total lipids were fractionated by thin-layer chromatography. This technique was also used to fractionate the neutral lipids obtained after silicic acid column chromatography of total lipids.

Initial separation of mono, di and triglycerides was carried out on silica gel plates which had been activated for 2 hr. at 90-95°, (Levin and Head, 1965). Samples which had been applied to plates

were chromatographed in petroleum ether: diethyl ether: acetic acid mixtures and components were detected using phosphomolybdate spray reagent. Using these techniques, it was regularly noted that a dark irregular line occurred across the plates approximately mid-way between the solvent front and the origin. This dark line was considered to be caused by impurities in the silica gel which collected at a secondary solvent front that developed during chromatography. This problem could be avoided by pre-washing the plates with appropriate solvent mixtures for a period of two to three hours prior to applying the samples. With pre-washing, excellent separations of glyceride classes were obtained (Fig. 5).

#### Identification of Triglycerides in the Lipids of *N. corallina*

Thin-layer chromatographic analyses of total lipids using phosphomolybdic acid spray reagent to detect components, indicated that the triglyceride fraction represented no more than 5% of the total lipid. Only in cases where total lipid samples were heavily loaded onto plates could a component chromatographing with tripalmitin be detected. If *N. corallina* cells were incubated with long chain fatty acids prior to lipid extraction, then an analysis of total lipids by thin-layer chromatography did show the presence of a component running with the tripalmitin (Fig. 5). The incubation of cells with fatty acids seemed to lead to a large increase in the level of the triglyceride fraction in the cells.

Initial tests to determine the level of glycerides in total lipids were carried out by direct analysis for glycerol (van Handel and Zilversmit, 1957). Tests on total lipids extracted from cells which had not been incubated with a long chain fatty acid but which had been

Figure 4

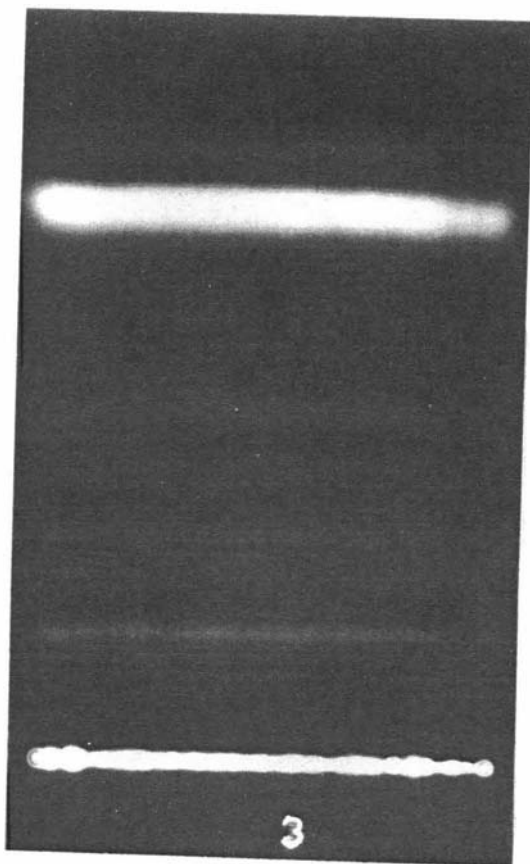


Figure 5

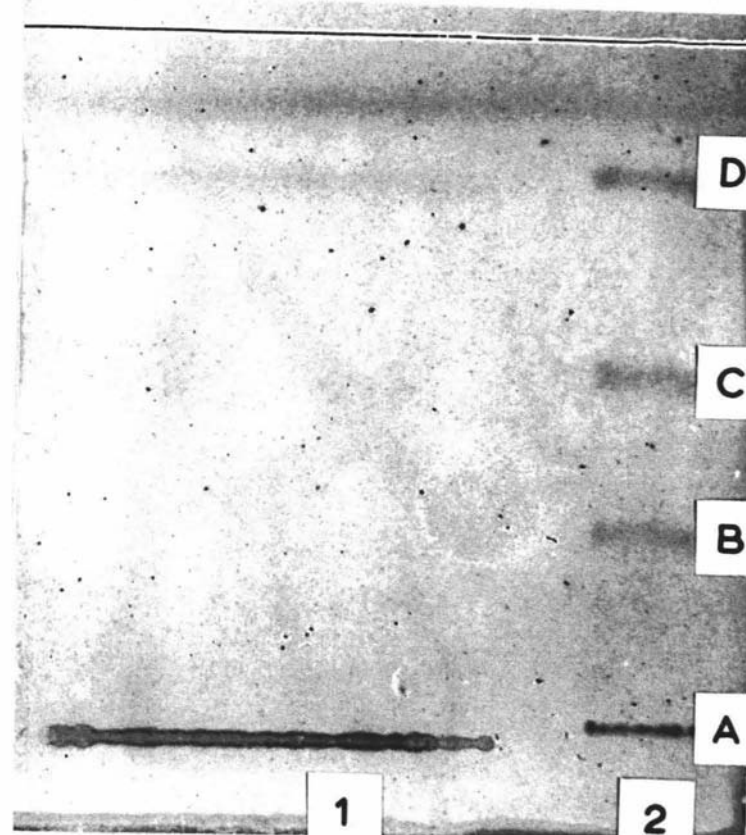


Figure 4) An autoradiograph of the thin-layer separation described in Fig. 5.

Figure 5) Thin-layer separation of  $^{14}\text{C}$ -labelled total unbound lipids extracted with cold chloroform-methanol (2:1) from *N.corallina* cells previously incubated with U- $^{14}\text{C}$  palmitate. Thin layer chromatography of the lipids was carried out on Adsorbosil 1 using the solvent system; pet.ether (57-59 $^{\circ}$ ):diethyl ether:acetic acid.(70/30/1) Components were detected with phosphomolybdic acid spray reagent following autoradiography (see Fig.4) The line ruled across the plate marks the solvent front. 1) Total unbound lipids. 2A) monostearin. 2B) distearin. 2C) Stearic acid. 2) Tripalmitin.

freeze dried immediately after harvesting from a late log phase culture, gave results which appeared unrealistically high (34  $\mu\text{g}$ . glycerol/mg. total unbound lipid). A subsequent paper chromatographic analysis (ethyl acetate: pyridine: water, 2:1:2, silver nitrate dip) of the water soluble fraction obtained after alkaline hydrolysis (Noll and Bloch, 1955) of a sample of total unbound lipid (40 mg.) showed five components. Three ran with the standards, trehalose, inositol and glycerol, one chromatographed just behind inositol and could have been mannose and the fifth ran very slightly ahead of glucose. It seemed likely therefore that the high glycerol analyses were partly caused by the presence of inositol.

Alkaline hydrolysis of a sample of neutral lipid (separated by silicic acid column chromatography) followed by paper chromatographic analysis of the water soluble fraction showed the presence of three components. One chromatographed with glycerol and the other two, which were only just detectable with the silver nitrate reagent, did not move far from the origin. These two unknowns did not chromatograph with glucose, trehalose or inositol. Although silicic acid column chromatography presented difficulties because of gel formation the procedure clearly separated a fraction containing glycerides from more polar lipids.

Further separation of the neutral lipid fraction was carried out on preparative thin-layer plates. The component which ran with tripalmitin in the solvent system, petroleum ether: diethyl ether: acetic acid (90:10:1, v/v/v) was isolated. The infrared spectrum ( $\text{CS}_2$ ) of the isolated material was very similar to that of tripalmitin. Determination of the ester to glycerol molar ratio by quantitative analysis for esters by the method of Vioque and Holman (1962) and for glycerol by the method of van Handel and Zilversmit (1957) gave ratios

of 2.8 and 3.3. Alkaline hydrolysis (Noll and Bloch, 1955) followed by extraction of the water-soluble components, and subsequent analysis by paper chromatography (solvents, ethyl acetate: pyridine: water, 2:1:2 and ethyl acetate: acetic acid: formic acid: water, 18:3:1:4) yielded only one component which ran with glycerol in the two solvents. The  $R_{\text{glucose}}$  values for the unknown and the glycerol standard were 1.52 in the pyridine solvent and 3.85 in the acid solvent.

The infrared spectra ( $\text{CS}_2$ ) of the petroleum ether soluble fraction obtained after alkaline hydrolysis of the material isolated from preparative thin-layer plates and considered to be triglyceride was very similar to that of palmitic acid. Esterification of this fraction using 1%  $\text{H}_2\text{SO}_4$  in methanol followed by gas chromatography on 15% Apiezon L revealed a spectrum typical of fatty acids isolated from N. corallina (Robertson, 1964). Identification of the components from retention volumes relative to methyl palmitate followed by an estimation of the different esters, revealed that the  $\text{C}_{14}$  -  $\text{C}_{18}$  esters constituted over 75% of the trace area and that the ratio of saturated to unsaturated esters in this group was very nearly 2:1. Tuberculostearic acid, identified from its retention volume, constituted only 2% of the total esters. No tests were carried out to determine the relative response of the detector to different esters.

#### The Incorporation of U - $^{14}\text{C}$ Palmitic Acid into the Triglyceride Fraction

Following the demonstration that triglycerides were present in the lipids of N. corallina, it was proposed to determine whether the  $^{14}\text{C}$ -labelled long chain fatty acids were incorporated into triglycerides as earlier work had suggested (Batt, unpublished results; Robertson, 1964).

A suspension of cells (81 mg. dry wt.) in phosphate buffer was incubated with potassium U-<sup>14</sup>C palmitate (palmitic acid, 10 mg. 5  $\mu$ c.) in a large manometer flask (Vf. 16 ml., Vg. 159.5 ml.). After 5 hr. at 30°, by which time the rate of oxygen uptake had decreased considerably, the cells were harvested by centrifugation, washed twice with distilled water and freeze dried. Total unbound lipids were extracted with cold chloroform methanol (2:1) and aliquots of the extract were applied to thin-layer plates on which the lipids were chromatographed using the solvent systems, petroleum ether: diethyl ether: acetic acid or chloroform: methanol: acetic acid. Dried plates were autoradiographed and components located using phosphomolybdate spray reagent. In each case, a highly active component, detected by autoradiography, ran with the tripalmitin standard (Fig. 4, 5). There was no high activity component in the free fatty acid region suggesting either (a) that fatty acids occurred in the cells as salts which were not extracted with solvent, or (b) that the level of free fatty acids in the cell was low in comparison with the triglycerides.

Separation of the lipids which remained at the origin with the solvent petroleum ether: diethyl ether: acetic acid (70:30:1), using chloroform: methanol: acetic acid (90:10:1) showed that this lipid consisted of three or four highly active components together with a similar number of components which were only just detectable on the autoradiograph. None of these components were investigated further.

Scanning thin-layer plates, on which separations of total unbound <sup>14</sup>C-labelled lipid had been carried out using a Packard (Model 7200) thin-layer chromatogram scanner, followed by triangulation of the peak areas, revealed that the triglycerides contained approximately 60% of

the total radioactivity on the plate.

Summary

These experiments showed that,

1) Triglycerides were components of the total lipids of N. corallina.

2) Long chain fatty acids were incorporated into the triglyceride fraction during oxidative assimilation.

## CHAPTER 4

FATTY ACIDS IN N. CORALLINA

The results of studies described in Chapter 3 suggested that although a triglyceride fraction was present in N. corallina it did not constitute a major portion of the total unbound lipid. Since Midwinter (1962) had studied changes in the levels of total fatty acids in starved cells it was considered advisable, before continuing with studies on triglycerides, to determine whether total fatty acids were degraded when cells, cultured in cleated flasks, were subsequently starved. It was recognised however, that changes in the amounts of material extracted with diethyl ether following acid or alkaline hydrolysis of total cells, could lead to erroneous conclusions as to the relative importance of fatty acids as endogenous reserves. It was possible that the diethyl ether extract could contain many components not all of which would be  $C_{10} - C_{20}$  fatty acids. Accordingly, an analysis of the components of the diethyl ether extract was carried out using thin layer chromatography, gas chromatography and infrared and mass spectrometry. While this work was progressing it became clear from studies reported in a subsequent chapter that the fall in total fatty acids represented only a small proportion of the total loss in dry weight during endogenous respiration. However a considerable body of information had accumulated on the nature of the fatty acid components in the diethyl ether extract and this is presented.

Extraction, Fractionation and Identification of Fatty Acids fromN. corallina

Total fatty acids together with the non-saponifiable lipid constituents (approx. 12% of the dry wt.) were isolated from N. corallina after alkaline hydrolysis of freeze dried cells by the method of Hulanika, Erwin and Bloch (1964). The non-saponifiable compounds were not extracted with petroleum ether prior to acidification since it was found that the petroleum ether extract contained an appreciable proportion of the salts of fatty acids and these were extremely difficult to remove by shaking with water.

Thin-layer chromatographic analysis (Fig. 6) of aliquots of the diethyl-ether extract obtained after acidification of the alkaline hydrolysate showed the presence of three major components. On esterification (diazomethane) these three components disappeared and three new components were observed (Fig. 6). The least polar of these components ran with methyl palmitate. It was concluded that the diethyl ether extract contained three classes of fatty acids, one corresponded to the  $C_{10} - C_{20}$  class previously studied by gas chromatography (Robertson, 1964). The other two classes were of unknown structure.

A sample (6.5 mg.) of the fraction running immediately behind the  $C_{10} - C_{20}$  class of fatty acids (Fig. 6) was isolated from preparative thin-layer chromatograms. The infrared spectrum of the isolated material was consistent with the compounds being fatty acids. Mass spectrometric analysis of the products obtained after esterification (diazomethane) suggested that the isolated material was a mixture of



Figure 6 Thin-layer chromatography of total fatty acids (and corresponding methyl esters) obtained by diethyl ether extraction of an alkaline hydrolysate of freeze dried cells. Chromatography was carried out on Adsorbosil 1 using the solvent system: pet.ether; diethyl ether; acetic acid.(70;30;1.) Components were detected with phosphomolybdic acid spray reagent.

- 1) Stearic acid
- 2) Total fatty acids extracted from N.corallina
- 3) Methyl ester of total fatty acids
- 4) Methyl palmitate

Figure 7 Thin-layer chromatography of total fatty acids, and fractions from a silicic acid column separation of the total fatty acids, from N.corallina. Conditions of thin layer chromatography were identical to those described for Fig.6.

F) Total fatty acids. 1-28) Fractions from a silicic acid column separation of total fatty acids. Fractions 11 to 18 contained the nocardic acids. The component at the solvent front in fractions 18-28 was caused by an impurity in a batch of diethyl ether.

Figure 8 Thin-layer chromatography of methyl esters and trimethylsilyl derivatives of methyl nocardates. The separation was carried out on Adsorbosil 1 using the solvent; petroleum ether; diethyl ether. 100;1. Components were detected with phosphomolybdic acid spray reagent.

- 1) Methyl palmitate
- 2) Methyl esters of nocardic acids derived from silicic acid column Fraction 11 (see Fig.7)
- 3) Diethyl ether extract of a silylation reaction mixture containing the silylation products of the methyl esters of silicic acid column Fraction 11.
- 4) The diethyl ether extract of a silylation mixture to which no esters had been added.
- 5) Methyl 12-hydroxystearate.

Figure 6

Thin-layer chromatography of total fatty acids and corresponding methyl esters of total fatty acids.

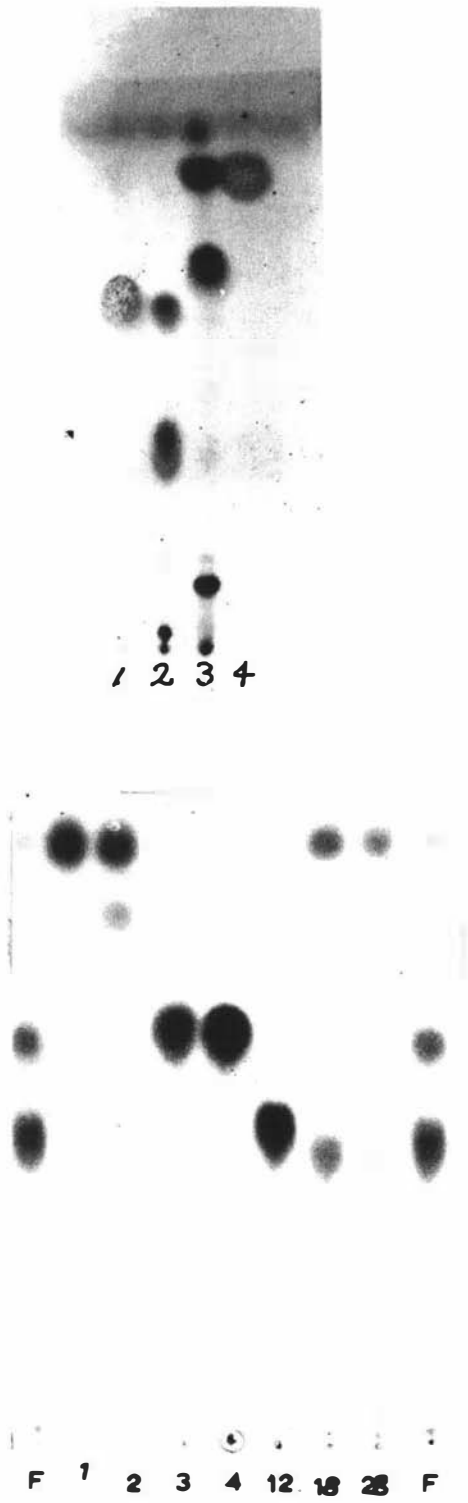


Figure 8

Thin-layer chromatography of putative methyl nocardates and trimethylsilyl derivatives of methyl nocardates.



Figure 7

Thin-layer chromatography of total fatty acids, and fractions from a silicic acid column separation of total fatty acids, from N. corallina.

saturated and unsaturated acids of carbon number  $C_{37} - C_{45}$  containing three oxygen atoms per molecule. From comparisons with studies by the French workers Etemadi, Lederer, Asselineau et. al, it seemed likely that these compounds were nocardic acids.

Silicic acid column chromatography of 130 mg. of total fatty acids using the solvent system, petroleum ether: diethyl ether: (95:5) yielded 55 mg. of the  $C_{10} - C_{20}$  fatty acids and 60 mg. of putative nocardic acids. Aliquots of the fractions from the silicic acid column were assayed by thin-layer chromatography (Fig. 7) using the solvent system, petroleum ether: diethyl ether: acetic acid (70:30:1). A slight tendency for fraction 12 to run ahead of fraction 18 was observed (Fig. 7) indicating that some separation of components within the fraction had been effected. Fractions 11 to 18 (fraction 11 was identical with fraction 12 on thin-layer chromatograms) were soluble in warm petroleum ether ( $57^{\circ} - 59^{\circ}$ ) but tended to precipitate on standing. All fractions were very soluble in diethyl ether. At concentrations of approximately 10 mg./ml. fractions 11 to 18 tended to form gels with both chloroform and carbon tetrachloride. It was noted that gel formation also occurred with 12 hydroxystearic acid in chloroform or carbon tetrachloride solutions.

The mass spectrum of the methyl esters of fraction 11 was consistent with an identification of the compounds as methyl nocardates. The two most intense peaks (of approximately equal intensity) with m/e ratios of 658 and 630 had the formulae  $C_{45}H_{86}O_2$  and  $C_{43}H_{82}O_2$  respectively. Besides these two major peaks, there were weaker ones at m/e 676 and 648 corresponding to  $C_{45}H_{88}O_3$  and  $C_{43}H_{84}O_3$ . It seemed likely that the peaks at 658 and 630 had arisen from those at 676 and 648 by loss of water and this was confirmed by the presence of metastable peaks. In addition there were four other very intense peaks in the spectrum at 299, 271,

270 and 242 which corresponded to the formulae  $C_{18}H_{35}O_3$ ,  $C_{16}H_{31}O_3$ ,  $C_{17}H_{34}O_2$  and  $C_{15}H_{30}O_2$ . If the parent compounds were methyl esters of nocardic acids of formulae  $C_{47}H_{88}O_3$  and  $C_{43}H_{84}O_3$  respectively, it was logical to suppose that the fragment ions containing three oxygen atoms arose by cleavage,  $\alpha$  to the hydroxyl group. The empirical formulae, tentative structural formulae and mass to charge ratios of the molecular and fragment ions are presented in Table 3.

If the ion radical at  $m/e$  270(242) (Table 3) had arisen from the molecular ions 676 (648) by a McLafferty rearrangement (Table 3) involving the migration of a hydrogen atom ( $R = H$ ), then the structure of the parent compounds as  $\alpha$  branched  $\beta$  hydroxy acids would be confirmed. However, the methyl ester of 12 hydroxystearic acid yields ions at  $m/e$  200 [ $CH_3 - (CH_2)_9 - COOCH_3$ ] $^+$  analogous to those at  $m/e$  270 (242). The corresponding ion  $m/e$  144 [ $CH_3 - (CH_2)_5 - COOCH_3$ ] $^+$  also occurs in the spectrum for the methyl ester of 8 hydroxystearic acid (Ryhage and Stenhagen, 1960). These results indicate that the position of the carboxyl group in the parent acid does not have to be situated  $\alpha$  to the hydroxyl group for cleavage  $\alpha$  to the hydroxyl group to occur.

The occurrence of molecular ion peaks at  $m/e$  676 (648) and fragmentation peaks at  $m/e$  299 (271) indicated that pyrolysis of the molecule (in the mass spectrometer) between the ester and hydroxyl group to yield an ester and an aldehyde (Etemadi, 1964) had not occurred to any great extent. No significant peaks corresponding to aldehydes were detected.

The mass spectrum of the methyl esters from fraction 18 from the silicic acid column separation represented in Fig. 7 showed molecular ion peaks at 592 and 564 corresponding to the mono unsaturated compounds

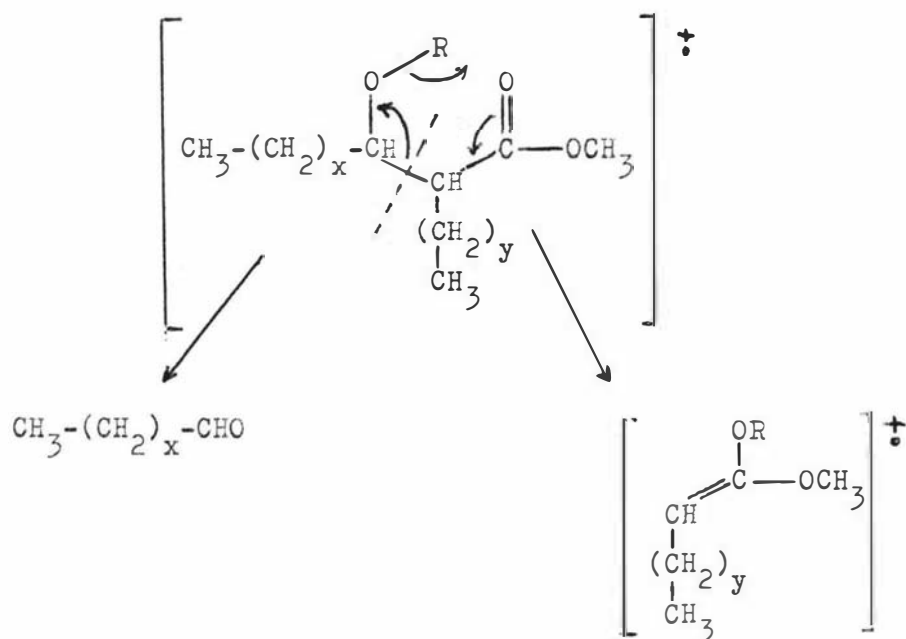
Table 3

Significant Peaks in the Mass Spectrum of the Methyl Esters of  
Nocardic Acids Derived from Silicic Acid Column Fraction 11

(see Fig. 7)

m/e	Empirical formulae	Structural formulae (Tentative)
676(648) molecular ions	$C_{45(43)}H_{88(84)}O_3$	$[CH_3-(CH_2)_x-CH=CH-(CH_2)_y-\overset{OH}{\underset{ }{CH}}-\overset{COOCH_3}{\underset{ }{CH}}-(CH_2)_{13(11)}-CH_3]^\dagger$ $x+y=24$
299 (271) fragment ions	$C_{18(16)}H_{35(31)}O_3$	$H\overset{+}{O}=CH-\overset{COOCH_3}{\underset{ }{CH}}-(CH_2)_{13(11)}-CH_3$
270 (242) fragment ions	$C_{17(15)}H_{34(30)}O_2$	$[CH_3O-\overset{OH}{\underset{ }{C}}=CH-(CH_2)_{13(11)}-CH_3]^\dagger$

McLafferty Rearrangement



[R=H, or  $Si(CH_3)_3$ ]

$C_{39}H_{76}O_3$ ,  $C_{37}H_{72}O_3$  and at 590 and 562, corresponding to the diunsaturated compounds  $C_{39}H_{74}O_3$  and  $C_{37}H_{70}O_3$ . The relative intensities of the ions 592, 564, 590, 562 were in the ratio 4:5:1:1.

Comparison of the mol. wt. of components in fractions 11 and 18 showed that the lower mol. wt. compounds were more firmly bound to the silicic acid column.

#### Silylation of Methyl Nocardates

The evidence so far reported suggested that the compounds were possibly  $C_{36}$ - $C_{44}$  branched, hydroxy acids in the mol. wt. range 500-700. Reaction of the methyl esters of the acids (silicic acid column fraction 12, see Fig. 7) with Tri-Sil to form the trimethylsilyl ethers gave, on thin-layer chromatographic analysis (Fig. 8) products which were less polar than methyl palmitate. Retention values for fatty acid and ester standards, nocardic acids and esters, and TMS derivatives are presented in Table 4.

Samples (approximately 2 mg.) of the TMS derivatives of methyl 12-hydroxystearate and putative methyl nocardates were isolated by preparative thin-layer chromatography using solvent systems petroleum ether: diethyl ether (100:2.5) and petroleum ether: diethyl ether (97.5:2.5) respectively.

#### Gas Chromatography of the TMS Derivatives of Methyl Nocardates

The method of Kuksis and Breckenridge (1966) for separating triglycerides by gas chromatography was adopted as a possible method for fractionating the TMS derivatives. The capacity of the column to fractionate triglycerides was thoroughly tested with a water washed, diethyl ether extract of butter before the TMS derivatives were

Table 4

Relative Retention Values\* of Acids, Esters and Trimethylsilyl Derivatives of Hydroxy Esters on Silica Gel Thin-Layer Chromatograms.

LIPID	Solvent Petroleum ether (57°-59°); diethyl ether; acetic acid						
	70:30:1	85:15:1	90:10:1	95:5:1	100:0:1	97.5:2.5:0	100:2.5:0
Methyl palmitate	0.75	0.69			0.52		
Stearic acid	0.55						
Methyl 12-hydroxystearate	0.35	0.18			0.03		0.02
12-hydroxystearic acid	0.17						
TMS derivative of methyl 12-hydroxystearate		0.85			0.78		0.37
Fraction 11 acids** (Nocardic acids)			0.10				
Fraction 11 methyl esters (Methyl nocardates)		0.52	0.27	0.16	0.10	0.07	
Fraction 11 TMS derivatives of methyl esters (Methyl TMS nocardates)		0.80	0.78	0.73	0.71	0.45	

\* Relative retention value =  $\frac{\text{Distance of component from origin}}{\text{Distance of solvent front from origin}}$

\*\* Fraction 11 from silicic acid column separation of total lipids (see Fig. 7)

applied. Excellent separation of triglycerides was obtained up to mol. wt. of 806 (Tripalmitin). Chromatography of the TMS derivatives of the methyl nocardates showed the presence of five components (Fig. 9). Fractions were collected at the column outlet and subsequently analysed by mass spectrometry.

#### Mass Spectrometry of the TMS Derivatives of Methyl Nocardates

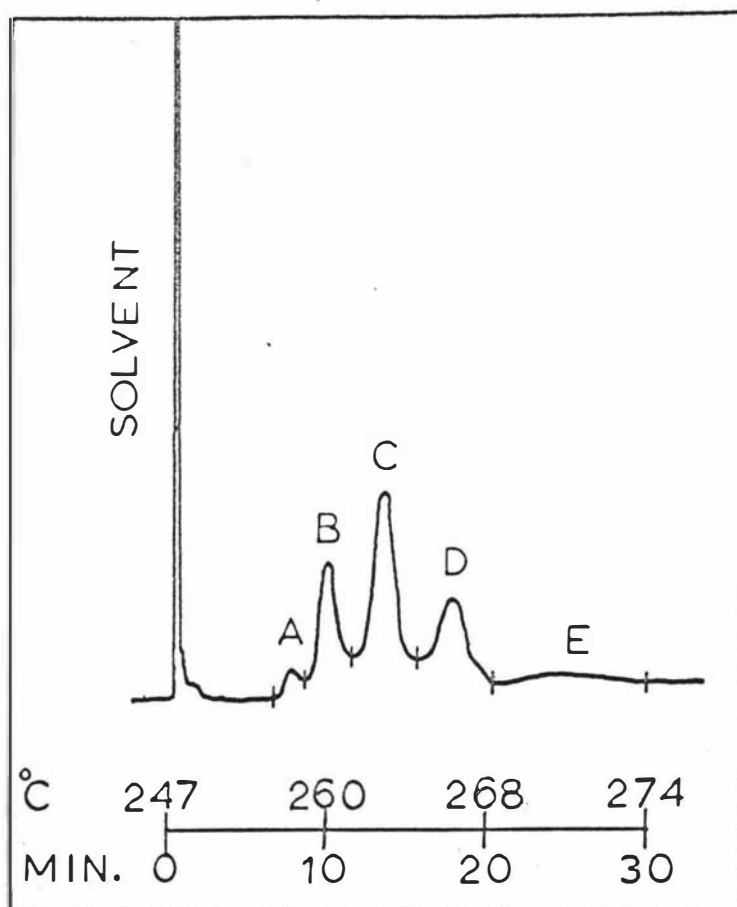
The mass spectrum of the gas chromatograph fraction C (Fig. 9) indicated that it was still a mixture. The main peaks of mass greater than 200 in the spectrum of fraction C together with their intensities are listed in Table 5. An interpretation of the spectrum was based on the following;

- 1) The fraction was assumed to be a mixture of homologues.
- 2) Intense fragment peaks were assumed to arise from one intense molecular ion of  $m/e$  720.
- 3) All ions less than 5% as intense as the base peak were ignored.

The spectrum could be rationalised if fraction C contained three isomeric compounds (I, II, III, Table 5) of empirical formulae  $C_{46}H_{92}O_3Si$ . The ions of mass 705, 689, 688 and 630 could clearly arise from the molecular ion by loss of  $CH_3$ ,  $CH_3O$ ,  $CH_3OH$ ,  $(CH_3)_3SiOH$ , respectively. Metastable peaks confirmed the transitions 720 - 689 and 720 - 630. The ions of mass 451 and 371 were considered to arise by cleavage of  $I^+$   $\alpha$  to the trimethylsilyloxy group giving ions of structures IV and V (Table 5). These peaks defined the position of the trimethylsilyloxy group. The ion radical of  $m/e$  342 could have arisen from the molecular ion by a McLafferty rearrangement

Figure 9

Separation of Trimethylsilyl Derivatives of Methyl Nocardates by Gas Chromatography.



Trimethylsilyl derivatives of methyl nocardates were prepared from fraction 12 from a silicic acid column fractionation of total fatty acids from N.corallina (see Fig. 7). Separation was achieved using 2% SE-30 on HMDS Chrom W with nitrogen carrier gas (125 ml./min.), and with manual temperature programming from 250° to 280°. Components were detected with a flame ionisation detector and fractions(A-E) were cut from the major gas outlet of a 1:4 stream splitter placed between the column and the flame ionisation detector.

Table 5

Significant Peaks in the Mass Spectrum of Gas Chromatogram Fraction C  
(see Fig. 9) containing the TMS Derivatives of Methyl Nocardates

m/e	Intensity (% of base peak)		Empirical Formulae		
			I	II	III
720	75		$C_{46}H_{92}O_3Si$	$C_{46}H_{92}O_3Si$	$C_{46}H_{92}O_3Si$
705	81		$C_{45}H_{89}O_3Si$	$C_{45}H_{89}O_3Si$	$C_{45}H_{89}O_3Si$
689	8		$C_{45}H_{89}O_2Si$	$C_{45}H_{89}O_2Si$	$C_{45}H_{89}O_2Si$
688	13		$C_{45}H_{88}O_2Si$	$C_{45}H_{88}O_2Si$	$C_{45}H_{88}O_2Si$
630	33		$C_{43}H_{82}O_2$	$C_{43}H_{82}O_2$	$C_{43}H_{82}O_2$
507	17				$C_{33}H_{67}OSi$
479	36			$C_{31}H_{63}OSi$	
451	24		$C_{29}H_{59}OSi$		
371	18		$C_{21}H_{43}O_3Si$		
343	30			$C_{19}H_{39}O_3Si$	
342	75		$C_{20}H_{42}O_2Si$		
315	23				$C_{17}H_{35}O_3Si$
314	100			$C_{18}H_{38}O_2Si$	
286	54				$C_{16}H_{34}O_2Si$
m/e	Fragment	x+y	z	Structural Formulae (Tentative)	
720	I	22	13	$\left[ \begin{array}{c} OSi(CH_3)_3 \\   \\ CH_3-(CH_2)_x-CH=CH-(CH_2)_y-CH-CH-COOCH_3 \\   \\ (CH_2)_z-CH_3 \end{array} \right]^+$	
720	II	24	11		
720	III	26	9		
451	IV			$C_{29}H_{49}-CH=O^+-Si(CH_3)_3$	
371	V			$C_{14}H_{29}-\overset{COOCH_3}{\underset{ }{CH}}-CH=O^+-Si(CH_3)_3$	
342	VI			$\left[ \begin{array}{c} OSi(CH_3)_3 \\   \\ C_{14}H_{29}-CH=C-OCH_3 \end{array} \right]^+$	

(Table 3,  $R=Si(CH_3)_3$ ) involving the migration of a trimethylsilyl group yielding fragment VI (Table 5). If this were so, it would provide confirmation of the structures of the parent compounds as  $\alpha$  branched  $\beta$  hydroxy acids. However methyl 12-trimethylsilyloxystearate yielded analogous ions at  $m/e$  272 [ $(CH_3)_3 - Si - (CH_2)_{10} - COOCH_3$ ]<sup>+</sup> and at  $m/e$  200 [ $CH_3 - (CH_2)_4 - COOCH_3$ ]<sup>+</sup> which demonstrated that cleavage  $\alpha$  to the hydroxyl group could occur with transfer either of the  $Si(CH_3)_3$ -group or by transfer of a hydrogen atom without the necessity of a carbonyl group in the position  $\alpha$  to the silyloxy group.

The results from mass spectrometric analysis of the other fractions obtained from gas chromatography of the TMS derivatives (Fig. 9) are presented in Table 6 as the structures of the parent acids. The interpretation of the spectra was based on the same reasoning as for fraction C. The results showed that gas chromatography brought about a separation on the basis of carbon number, the range separated being between  $C_{38} - C_{48}$ . Saturated, mono-unsaturated and di-unsaturated components were present with a tendency for the degree of unsaturation to increase with the chain length. Within each fraction, isomers of the same empirical formulae but different side chain lengths occurred. The results also demonstrated that the predominant acids contained even numbers of carbon atoms.

#### Pyrolysis of Nocardic Acids

Further evidence for the structure of the components isolated from the total fatty acids of N. corallina and tentatively designated nocardic acids was obtained by pyrolysis and subsequent gas chromatography of the products. Gas chromatograms (Fig. 10A) showed that

Table 6

Nocardic Acids Corresponding to the Trimethylsilyl Derivatives of the Methyl Nocardates Separated by Gas Chromatography (see Fig. 9)

General formulæ of nocardic acid									
Saturated	$C_m H_{2m+1} - CHOH - CHCOOH - C_n H_{2n+1}$								
Monounsaturated	$C_p H_{2p-1} - CHOH - CHCOOH - C_n H_{2n+1}$								
Diunsaturated	$C_q H_{2q-3} - CHOH - CHCOOH - C_n H_{2n+1}$								
Gas chromatography fraction number (Fig. 9)	m + n	m	n	p + n	p	n	q + n	q	n
A	$\left. \begin{matrix} 35 \\ 35 \end{matrix} \right\} (100)^*$	23 21	12 14	$\left. \begin{matrix} 36 (3) \\ 37 (5) \end{matrix} \right\}$					
B				$\left. \begin{matrix} 37 \\ 37 \\ 38 (15) \end{matrix} \right\} (100)$	23 25	14 12			
C				$\left. \begin{matrix} 39 \\ 39 \\ 39 \end{matrix} \right\} 100$	25 27 29	14 12 10			
D				$\left. \begin{matrix} 39 (60) \\ 41 (100) \end{matrix} \right\}$			41 (90)		
E				$\left. \begin{matrix} 43 \\ 43 \end{matrix} \right\} (70)$	29 31	14 12	$\left. \begin{matrix} 43 \\ 43 \\ 44 (15) \\ 45 (10) \end{matrix} \right\} (100)$	29 31	14 12

\* Figures in brackets represent the relative intensities of the molecular ion for any fraction.

Figure 10

Separation of the Products of Pyrolysis of Methyl Nocardates by Gas Chromatography.

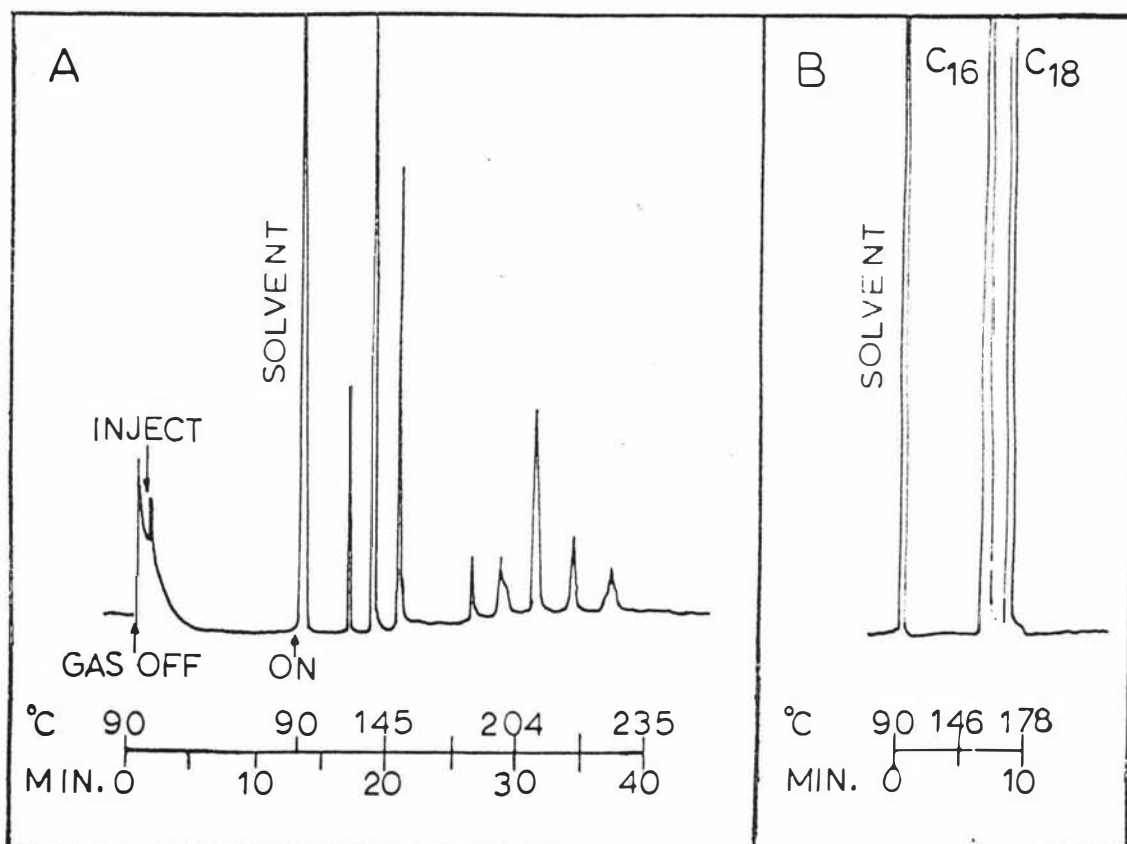


Fig. 10A Pyrolysis followed by gas chromatography of the methyl esters of nocardic acids derived from silicic acid column fraction 12. (see Fig. 7) Gas chromatography was carried out on 2% SE-30 (HMDS Chrom W) with nitrogen as carrier gas (25 ml./min.). The gas was switched off prior to injection of the esters onto the column which extended into the heated (280°) injector block. The gas flow to the column was turned on 12 min. after the injection of the esters and the column temperature was programmed manually from 90°-250°. The products of pyrolysis were detected using the flame ionisation detector.

Fig. 10B Separation of the methyl esters of palmitic stearic acids on 2% SE-30 (HMDS Chrom W) using nitrogen gas (25 ml./min.) and manual temperature programming from 90°-200°.

after heating for 12 min. (no gas flowing) with subsequent temperature-programmed chromatography, a series of peaks were obtained which fell into two groups. Although further analysis of the products was not carried out, gas chromatography under almost identical conditions of standards, methyl palmitate and methyl stearate (Fig. 10B) showed that these components had approximately the same retention volumes as the components in the first group of peaks (Fig. 10A). It was concluded in view of the results of the mass spectrometric analysis of the TMS derivatives (Table 6) that the first group of peaks (Fig. 10A) corresponded to the methyl esters of the  $C_{12}$ ,  $C_{14}$  and  $C_{16}$  fatty acids and the second group, corresponded to a range of even carbon numbered aldehydes containing 22 to 32 carbon atoms. This interpretation was consistent with similar studies on the pyrolysis of nocardic acids reported by Etemadi (1964) and Laneelle, Asselineau and Castelnuovo (1965).

#### Oxidation of Nocardic Acids

Nocardic acids were isolated by silicic acid column chromatography from total fatty acids, which had been extracted with diethyl ether after acid hydrolysis (6N HCl 100°) of wet cells under nitrogen. A sample of the putative nocardic acids (5 mg.) was dissolved in acetone, and titrated with chromate reagent (Bowers, Halsall, Jones and Lemin, 1953) until the solution showed a slight orange tint. The reaction mixture was diluted with water, extracted with diethyl ether and the ether extract washed with 1N NaOH solution. Mass spectrometric analysis of the ether-soluble neutral products showed three major peaks which were considered to be the molecular ions  $C_{37}H_{74}O$ ,  $C_{39}H_{76}O$  and  $C_{41}H_{80}O$ . These structures could correspond to ketones formed by

oxidation of the parent acids  $C_{38}H_{74}O_3$ ,  $C_{40}H_{76}O_3$  and  $C_{42}H_{80}O_3$ . The  $C_{37}$  compound was assumed to be a saturated ketone and this was confirmed by the presence in the spectrum of the fragment ions,  $C_{23}H_{47}C\equiv O^+$ ,  $C_{21}H_{43}C\equiv O^+$ ,  $C_{15}H_{31}C\equiv O^+$  and  $C_{13}H_{27}C\equiv O^+$ . These ions could be formed by a fission  $\alpha$  to the carbonyl group in the two possible isomeric structures  $C_{23}H_{47}COC_{13}H_{27}$  and  $C_{21}H_{43}COC_{15}H_{31}$ . No prominent fragment ions could be ascribed to the two unsaturated ketones  $C_{39}H_{76}O$  and  $C_{41}H_{80}O$ . The effect of the double bonds on the fragmentation of these compounds is not known.

The facile formation of ketones by oxidation of the parent acids was confirmatory evidence that these compounds were in fact  $\alpha$  branched  $\beta$  hydroxy acids.

#### Fractionation of Methyl Nocardates on Silver Nitrate Impregnated Plates

Preliminary experiments in which the methyl esters of the nocardic acids (fraction 13 from the silicic acid column separation represented in Fig. 7) were chromatographed on silver nitrate impregnated thin-layer plates showed three components after 3 hr. continuous chromatographing. These components occurred 3.3 cm., 2.2 cm. and 0.6 cm. from the origin.

Hydrogenation of a sample of total esters using Adams catalyst yielded only one component which ran with the foremost of the components in the non-hydrogenated esters. It would appear that the esters consist of three groups based on the degree of saturation, one of which is fully saturated. This result is consistent with mass spectrometric evidence (Table 6).

Summary

In summary, thin-layer chromatography of the total fatty acids obtained by diethyl ether extraction of the acid or alkaline hydrolysate of total cells, showed the presence of three fractions. The least polar fraction corresponded to the  $C_{10} - C_{20}$  class of fatty acids. The fraction running immediately behind these compounds was positively identified as a complex mixture of  $\alpha$  branched  $\beta$  hydroxy fatty acids (nocardic acids). A third minor fraction observed in thin-layer separations of total fatty acids was more polar than the nocardic acids and was not identified.

Separation of the TMS derivatives of methyl nocardates by gas chromatography followed by mass spectrometric analysis of the fractions cut from the column effluent revealed that the structures of the nocardic acids could vary in three ways.

Carbon number A range of predominantly even numbered nocardic acids occurred containing between 38 and 48 carbon atoms.

Degree of unsaturation Saturated, mono-unsaturated and di-unsaturated nocardic acids occurred with a tendency for the acids of higher carbon number to be more unsaturated. The unsaturated bonds always occurred in the longest hydrocarbon side chain.

Structural isomerism Variations in the position of the carboxyl group within a single molecular weight species gave rise to structural isomers.

## CHAPTER 5

VARIATIONS IN DRY WEIGHT, TOTAL AND VIABLE COUNTS AND RESPIRATORY  
ACTIVITY IN STARVED SUSPENSIONS OF N. CORALLINA

By following optical density changes in cultures of N. corallina through the growth phase and afterwards it had been shown that there was a significant fall in optical density after growth had ceased (Fig. 1). It had been demonstrated that the relationship between dry wt. and optical density was the same for cells harvested during the log phase and after several days of endogenous incubation (see Methods). Presumably the observed fall in optical density following full growth was due to a net decrease in the dry wt. of the total cell population. It was proposed to examine this assumption using starved cell suspensions. It was also proposed to correlate changes in dry wt. with changes in total and viable cell counts and respiratory activity.

As a general procedure, cultures which had reached full growth (determined by maximum optical density, Fig. 1) were harvested by centrifugation and pellets were resuspended in phosphate buffer (300 ml.) in standard or cleated flasks (milk filters). Cell suspensions were incubated at 30° with shaking at 140 rev./min. for varying periods during which time flasks were sampled aseptically. Complete resuspension of pellets in phosphate buffer was always difficult due to the tendency of cells to clump. The use of cleated flasks overcame this problem in that small pieces of pellet were dispersed in the first hour after resuspension. However where cleated flasks were used a faint pink film of dried organisms could be seen on the inside walls of the flasks

after a period of 4-5 days. This film did not occur where suspensions were contained in standard flasks but complete dispersion of small pieces of pellet required as long as 12 hr. shaking at 140 rev./min.

#### Variations in Viable and Total Cell Counts and in Dry Weight

In an experiment lasting 131 hr. changes in viable and total cell counts and in dry wt. were studied in starved cell suspensions contained in cleaned flasks. Five replicates of the test system were analysed simultaneously. The results of viability estimates (Table 7) are presented as normalised cluster size distributions, cluster viabilities and cell viabilities. Cell viabilities were determined from cluster viabilities and the normalised cluster size distributions using formulae given on p. 28. Total cell counts for each flask are presented in Table 8 (total cell count/ml. of flask suspension = total grid count  $\times 2.5 \times 10^6$ ). Optical density estimations, carried out on 1:50 dilutions of cell suspensions, were corrected for dilution to give optical density values for the original suspensions (Table 9). The dry wt. mg./ml. of the original suspensions was calculated from the relationship; optical density  $\times 0.43$  = dry wt. mg./ml. of suspension.

Means of analyses were obtained by averaging the results for the 6 flasks at each sampling time and these are presented with standard errors in Fig. 11.

Estimates of viability showed that over 90% of the cells remained viable through the 131 hr. period. Cell viability values calculated from cluster viabilities using the formulae given on p. 28 showed that for the degree of clustering observed (Table 7) the difference between cell and cluster viabilities was only 3%.

Table 7

Cluster Size Distribution and Viable Estimates Determined by the Slide Culture Method on Starved Suspensions of N. corallina

Hours	Flask	Normalised cluster size distribution. Clusters of different sizes expressed as a proportion of the total number of clusters.						Cluster viability P	Cell viability p
		n <sub>1</sub>	n <sub>2</sub>	n <sub>3</sub>	n <sub>4</sub>	n <sub>5</sub>	n <sub>6</sub>		
7.5	1	0.4824	0.4253	0.0708	0.0108	0.0108	0.0000	0.969	0.940
	2	0.4602	0.3720	0.0902	0.0777	0.0000	0.0161	0.957	0.913
	3	0.3542	0.4583	0.1458	0.0417	0.0000	0.0000	0.966	0.913
	4	0.4602	0.5398	0.0000	0.0000	0.0000	0.0000	0.964	0.927
	5	0.3750	0.4464	0.1339	0.0208	0.0000	0.0238	0.990	0.975
	6	0.5827	0.2328	0.0975	0.0652	0.0000	0.0217	0.980	0.966
								Mean S.E.M.	0.971 ±0.005
46.0	1	0.5393	0.4001	0.0455	0.0152	0.0000	0.0000	0.951	0.915
	2	0.6004	0.3193	0.0553	0.0250	0.0000	0.0000	0.979	0.966
	3	0.5463	0.3890	0.0646	0.0000	0.0000	0.0000	0.942	0.901
	4	0.5667	0.3792	0.0542	0.0000	0.0000	0.0000	0.925	0.878
	5	0.6795	0.2424	0.0781	0.0000	0.0000	0.0000	0.934	0.906
	6	0.7247	0.2601	0.0152	0.0000	0.0000	0.0000	0.963	0.950
								Mean S.E.M.	0.949 ±0.008
129.0	1	0.5833	0.3364	0.0250	0.0000	0.0553	0.0000	0.941	0.903
	2	0.6107	0.3036	0.0857	0.0000	0.0000	0.0000	0.928	0.889
	3	0.7064	0.2268	0.0669	0.0000	0.0000	0.0000	0.914	0.883
	4	0.6733	0.2650	0.0617	0.0000	0.0000	0.0000	0.963	0.947
	5	0.6865	0.2680	0.0455	0.0000	0.0000	0.0000	0.938	0.913
	6	0.6576	0.2772	0.0652	0.0000	0.0000	0.0000	0.917	0.880
								Mean S.E.M.	0.939 ±0.008

Suspensions of cells in phosphate buffer were incubated at 30° in cleated flasks for 129 hr. with shaking at 140 rev./min. Samples were taken at time intervals after resuspension.

The normalised cluster size distribution for each flask was a mean value determined from counts of the numbers of clusters of different sizes on 2 grids on 1 ring (approx. 25 clusters/grid).

The cluster viability for each flask was a mean value obtained from counts of numbers of live and dead clusters on 8 grids, 4 from each of 2 rings (approx. 25 clusters/grid).

The cell viability for each flask was determined by formulae from values for the normalised cluster distribution and cluster viability.

The mean flask cluster and cell viability and standard error was determined from the individual flask estimates from each sampling time treated separately.

Table 8

Total Cell Counts of Starved Suspensions of N. corallina

Hours	Flask grid count totals*						Mean grid count and S.E.M.
	Flask number						
	1	2	3	4	5	6	
1.5	1622	1599	1610	1587	1521	1455	1566 ± 33
57.15	1685	1782	1645	1670	1687	1794	1711 ± 33
131.0	1717	1749	1942	1784	1829	1717	1790 ± 33
Detectable difference							
0.05							104
(0.01)							148

The results of the analysis of variance were:-

Source of variation	Degrees of freedom	Mean square and results of F tests
Flasks	5	2,299 n.s.
Days	2	77,420 p=0.01 **
Residual	10	6,567
Total	17	

Suspensions of cells in phosphate buffer were incubated in cleaned flasks at 30° for 131 hr. Samples were taken at time intervals after resuspension.

\* Grid count total x 2.5 x 10<sup>6</sup> = total cell count/ml. of suspension contained in each flask.

Counting scheme

6 cleaned flasks

1 sample (0.5 ml.) / flask, diluted to 10.0 ml. with sterile phosphate buffer containing 0.5% Teepol

8 grids/sample

4 quintets/grid

Table 9

Optical Density of Starved Suspensions of N. corallina

Hours	Optical density (660 m $\mu$ )						Mean optical density and S.E.M.
	Flask number						
	1	2	3	4	5	6	
0.5	5.50	6.00	5.90	5.95	5.50	5.45	5.717 $\pm$ 0.048
46.0	3.90	3.90	4.00	3.95	3.95	3.70	3.900 $\pm$ 0.048
131.0	3.45	3.60	3.70	3.60	3.50	3.35	3.533 $\pm$ 0.048
Detectable difference							
0.05							0.152
(0.01)							0.216

The results of the analysis of variance were:-

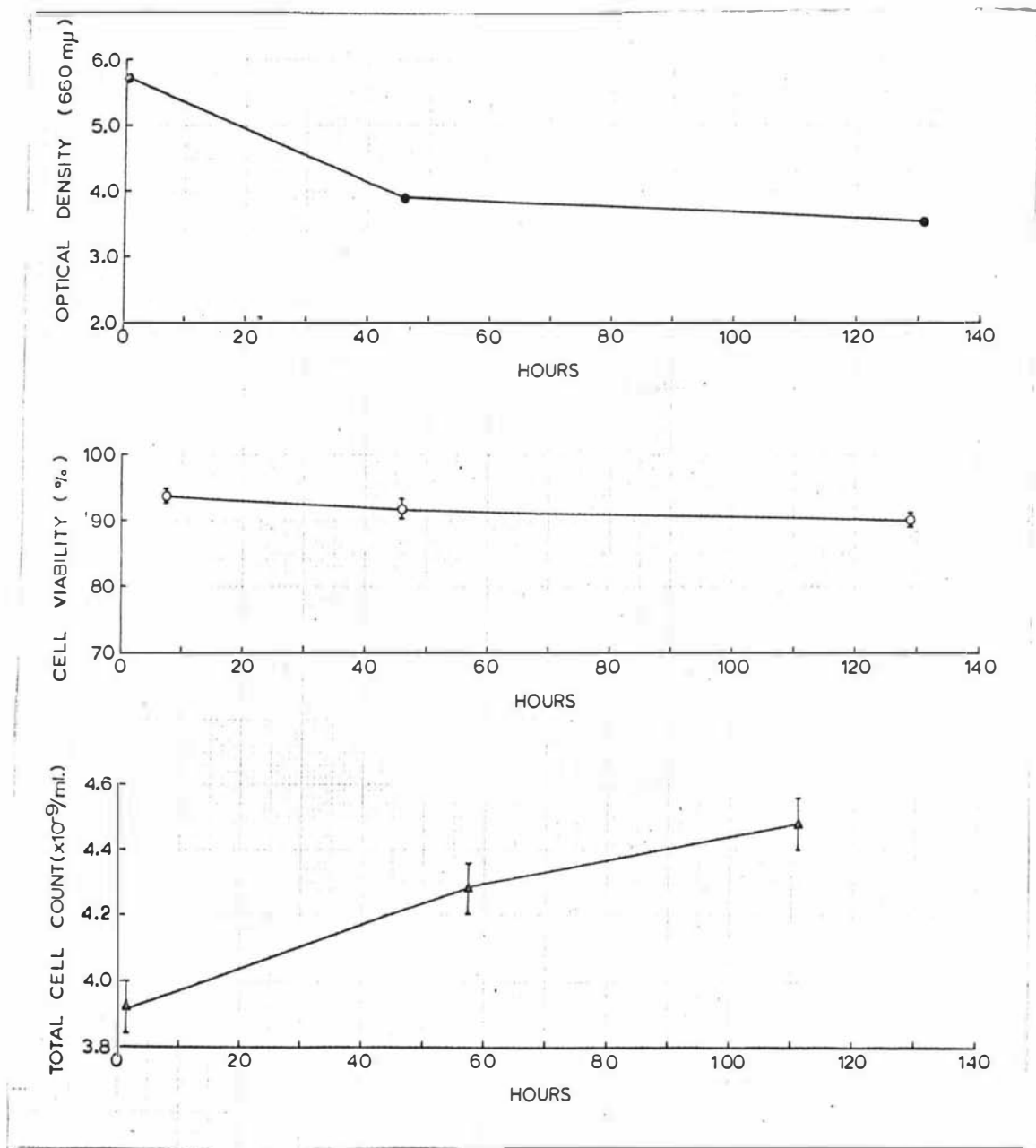
Source of variation	Degrees of freedom	Mean square and results of F. tests
Flasks	5	0.066 p = 0.01 **
Days	2	8.20 p = 0.01 **
Residual	10	0.014
Total	17	

Suspensions of cells in phosphate buffer were incubated in cleaned flasks for 131 hr. at 30<sup>o</sup> with shaking at 140 rev./min.. Samples were taken at time intervals after resuspension.

Optical density estimations were corrected for 1:50 dilution.  
Optical density x 0.43 = dry wt. mg./ml. of suspension.

Figure 11

Changes in Optical Density, Cell Viability and Total Cell Counts in Starved Suspensions of N.corallina



Cells suspended in phosphate buffer (cleated flasks; conical 1L.) were incubated for 5 days at 30° with shaking at 140 rev./min. Samples were taken for optical density, viability and total cell count estimates at time intervals after resuspension. The mean and S.E.M. of results from 6 flasks are presented (see Tables 7, 8, 9.).

During the 131 hr. incubation period there was a significant fall ( $p = 0.01$ ) in the optical density readings (Fig. 11) corresponding to a decrease in dry wt. of 0.77 mg./ml. of suspension (31% of the original dry wt.) in the first 46 hr. This decrease was consistent with previous observations on cultures incubated after full growth had been reached (Fig. 1).

There was a rise ( $p = 0.05$ ) in total cell count in the first 57 hr. (Fig. 11) while no significant rise occurred in the period 57-131 hr. The total rise in the 131 hr. period was however highly significant ( $p = 0.01$ ).

These results could indicate either a) that cell division continued after resuspension or b) that fragmentation of cells already divided by septa occurred after resuspension.

Since cell numbers did not decrease it seemed unlikely that cell lysis was contributing to the observed decrease in dry wt. Presumably the fall in optical density corresponded to the catabolism of some cellular component. If this interpretation was correct then clearly the utilization of the cellular component did not effect viability.

In a second experiment, the results of which are presented in Table 10 and Fig. 12, cell suspensions contained in standard flasks were incubated endogenously for a period of 483 hr. (20 days). The results of viability tests over the first 5 days were identical to those obtained in the previous 131 hr. incubation experiment. After the 8th day the viability values decreased more rapidly and concurrently the effects of clustering on cell viability estimations became more pronounced.

Table 10

Cluster Size Distribution and Viable Estimates Determined by the Slide Culture Method on Starved Suspensions of N.corallina

Viable Estimates

Hours	Flask	Flask cluster viability	Flask cell viability	Mean flask cluster viability, S.E.M. (cell viability, S.E.M.)
27.5	1	0.996	0.992	0.993 $\pm$ 0.004 (0.987 $\pm$ 0.004)
	2	0.992	0.987	
	3	0.991	0.983	
76.0	1	0.973	0.957	0.977 $\pm$ 0.004 (0.963 $\pm$ 0.004)
	2	0.978	0.966	
	3	0.980	0.966	
123.0	1	0.955	0.927	0.960 $\pm$ 0.004 (0.934 $\pm$ 0.004)
	2	0.965	0.943	
	3	0.961	0.933	
170.5	1	0.954	0.920	0.932 $\pm$ 0.011 (0.895 $\pm$ 0.014)
	2	0.915	0.871	
	3	0.927	0.893	
219.0	1	0.884	0.820	0.863 $\pm$ 0.012 (0.777 $\pm$ 0.022)
	2	0.843	0.761	
	3	0.862	0.751	
267.0	1	0.800	0.697	0.799 $\pm$ 0.004 (0.711 $\pm$ 0.010)
	2	0.801	0.732	
	3	0.795	0.705	
339.0	1	0.735	0.642	0.689 $\pm$ 0.024 (0.595 $\pm$ 0.025)
	2	0.679	0.582	
	3	0.653	0.560	
386.0	1	0.677	0.589	0.697 $\pm$ 0.014 (0.603 $\pm$ 0.010)
	2	0.689	0.598	
	3	0.724	0.622	
435.0	1	0.624	0.548	0.647 $\pm$ 0.012 (0.562 $\pm$ 0.011)
	2	0.664	0.585	
	3	0.654	0.552	
483.0	1	0.603	0.525	0.586 $\pm$ 0.008 (0.491 $\pm$ 0.017)
	2	0.575	0.465	
	3	0.580	0.482	

The cluster viability for each flask was a mean value obtained from counts of numbers of live and dead clusters on 10 grids 5 from each of 2 rings. (approx 25 clusters/grid)

The cell viability for each flask was determined using formulae from values of the normalised cluster size distribution (see Table 10 continued over page) and cluster viability.

The mean flask, cluster and cell viabilities and standard errors were determined from the individual flask estimates for each sampling time treated separately.

Continued over page.

Table 10 (continued)

Cluster Size Distribution and Viable Estimates Determined by the Slide Culture Method on Starved Suspensions of N. corallina

Cluster Size Distribution

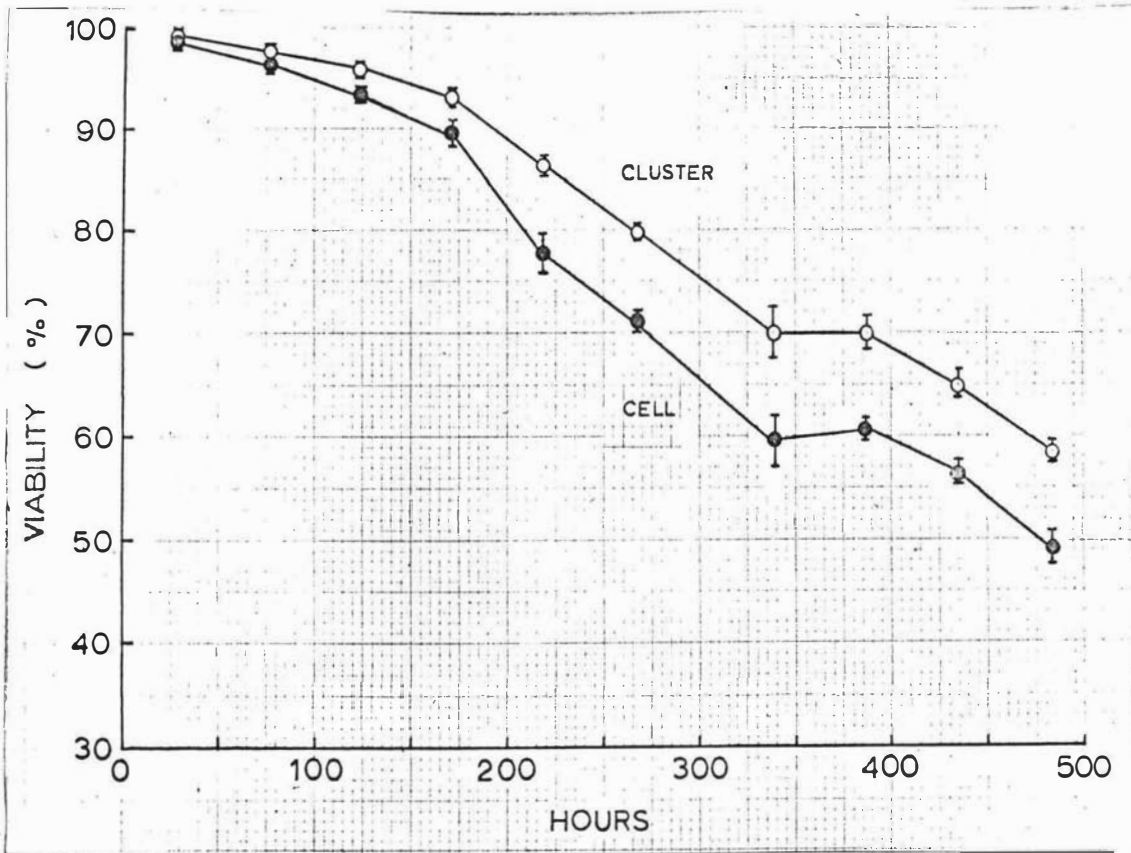
Hours	Flask	Normalised cluster size distribution (Clusters of different sizes expressed as a proportion of the total number of clusters.)							
		n <sub>1</sub>	n <sub>2</sub>	n <sub>3</sub>	n <sub>4</sub>	n <sub>5</sub>	n <sub>6</sub>	n <sub>7</sub>	n <sub>8</sub>
27.5	1	0.5762	0.3398	0.0841	0.0	0.0	0.0	0.0	0.0
	2	0.6246	0.2725	0.0884	0.0145	0.0	0.0	0.0	0.0
	3	0.5586	0.3268	0.0729	0.0295	0.0080	0.0042	0.0	0.0
76.0	1	0.6194	0.3237	0.0337	0.0122	0.0087	0.0023	0.0	0.0
	2	0.6348	0.2567	0.0463	0.0527	0.0048	0.0048	0.0	0.0
	3	0.5712	0.3289	0.0616	0.0320	0.0062	0.0	0.0	0.0
123.0	1	0.5922	0.3106	0.0664	0.0173	0.0059	0.0077	0.0	0.0
	2	0.5953	0.3041	0.0658	0.0264	0.0083	0.0	0.0	0.0
	3	0.5648	0.3153	0.0838	0.0074	0.0245	0.0043	0.0	0.0
170.5	1	0.5520	0.3338	0.0847	0.0077	0.0127	0.0091	0.0	0.0
	2	0.6277	0.2426	0.0817	0.0216	0.0217	0.0048	0.0	0.0
	3	0.6519	0.2300	0.0853	0.0245	0.0	0.0083	0.0	0.0
219.0	1	0.5858	0.3052	0.0972	0.0	0.0042	0.0077	0.0	0.0
	2	0.5967	0.2357	0.0852	0.0604	0.0111	0.0050	0.0	0.0059
	3	0.4610	0.3450	0.0790	0.0605	0.0198	0.0300	0.0	0.0048
267.0	1	0.5537	0.3176	0.0854	0.0348	0.0045	0.0040	0.0	0.0
	2	0.6753	0.2410	0.0522	0.0316	0.0	0.0	0.0	0.0
	3	0.6174	0.2386	0.0661	0.0374	0.0123	0.0282	0.0	0.0
339.0	1	0.6387	0.2462	0.0897	0.0255	0.0	0.0	0.0	0.0
	2	0.6475	0.2533	0.0711	0.0233	0.0	0.0048	0.0	0.0
	3	0.6604	0.2603	0.0661	0.0132	0.0	0.0	0.0	0.0
386.0	1	0.6936	0.1885	0.0834	0.0154	0.0145	0.0045	0.0	0.0
	2	0.6640	0.2439	0.0610	0.0122	0.0	0.0189	0.0	0.0
	3	0.6139	0.2682	0.0875	0.0136	0.0168	0.0	0.0	0.0
435.0	1	0.7113	0.2558	0.0226	0.0105	0.0	0.0	0.0	0.0
	2	0.7220	0.1732	0.0964	0.0053	0.0	0.0030	0.0	0.0
	3	0.6309	0.2698	0.0993	0.0	0.0	0.0	0.0	0.0
483.0	1	0.7137	0.2347	0.0460	0.0056	0.0	0.0	0.0	0.0
	2	0.6326	0.2619	0.0530	0.0392	0.0062	0.0071	0.0	0.0
	3	0.6637	0.2416	0.0681	0.0266	0.0	0.0	0.0	0.0

The normalised cluster size distribution for each flask was a mean determined from counts of the numbers of clusters on 10 grids 5 from each of 2 rings. (approx. 25 clusters / grid)

Suspensions of cells in phosphate buffer were incubated (standard flasks; conical 1L.) at 30° for 20 days with shaking at 140 rev./min. Samples were taken at time intervals after resuspension.

Figure 12

Changes in Cluster and Cell Viability in Starved Suspensions of  
N. corallina



Cells suspended in phosphate buffer (standard flasks; conical 1L.) were incubated for 20 days at 30° with shaking at 140 rev./min. Samples were taken for cluster viability determinations at time intervals after resuspension. The mean and S.E.M. of results from 3 flasks are presented. (see Table 10)

Respiration Rates for Cell Suspensions of *N. corallina*

It seemed likely that the fall in dry wt. of the cell suspensions would be reflected in changes in respiratory activity. Midwinter (1962) had examined changes in RQ values for acetate and propionate grown cells of *N. corallina*. He stated that when cells were first resuspended the RQ values were very nearly equal to 1 and that these values fell to approximately 0.7 after a period of incubation at 30°. Although RQ values for glucose grown cells were not reported the relative amount of lipid degraded in these cells was comparable to the amounts catabolised in acetate and propionate grown cells (Midwinter, 1962).

It was decided to study again the respiratory changes of *N. corallina* incubated under endogenous conditions with an emphasis on the respiratory activity of glucose grown cells.

In an initial experiment the change in respiratory activity of nonresuspended cultures was examined. Two cultures grown in cleated flasks were incubated endogenously and aliquots were withdrawn at intervals for RQ determinations in Warburg flasks. Samples were also taken for dry wt. estimations from optical density measurements. The results (Table 11, Fig. 13) showed a rapid decrease in the rate of respiration from the end of the growth phase followed by a levelling out to an almost constant low value (1  $\mu$ l. O<sub>2</sub>/mg./hr.). In no instance did the RQ approach 0.7; the average figure for all determinations was 1.15 (Table 11).

In a subsequent experiment changes in the RQ of cells resuspended in phosphate buffer and incubated (with shaking) for a period of 118 hr. were followed. Samples were taken and transferred directly into Warburg flasks for RQ estimations and aliquots were used to determine the dry wt. content per ml. of suspension (Table 12, Fig. 14). The

Table 11

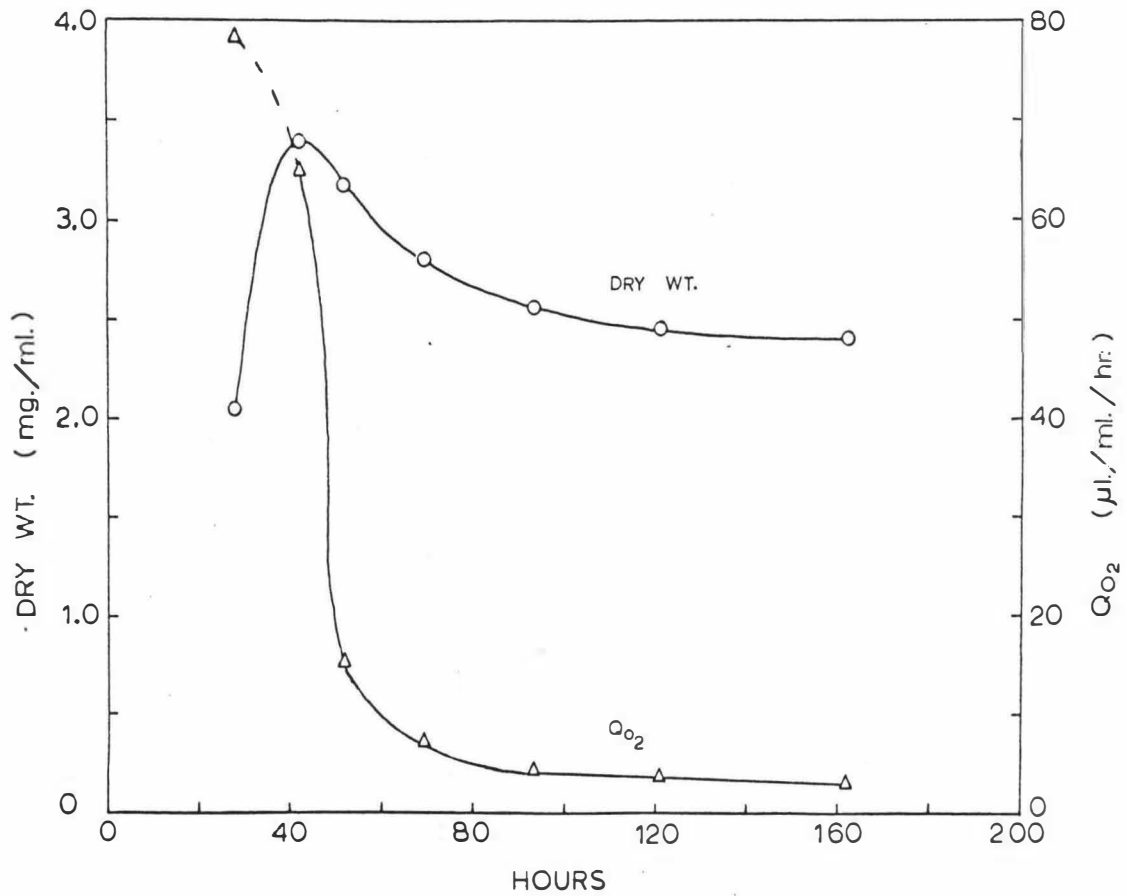
Respiration Studies with Cultures of N. corallina

Hours	Flask	$Q_{O_2}$ $\mu\text{l./ml./hr.}$	$Q_{CO_2}$ $\mu\text{l./ml./hr.}$	RQ	OD (660 $m\mu$ ) (dry wt.mg./ml.)
28	1	81.8	100.0	1.22	4.75 (2.04)
	2	75.5	94.8	1.25	4.75 (2.04)
42	1	61.4	72.0	1.17	7.90 (3.40)
	2	68.2	80.0	1.17	7.90 (3.40)
51.30	1	15.0	14.2	0.95	7.25 (3.12)
	2	15.3	17.0	1.11	7.50 (3.22)
69.45	1	6.9	8.5	1.11	6.50 (2.80)
	2	7.5	8.4	1.11	6.50 (2.80)
93.45	1	3.9	4.6	1.18	5.90 (2.54)
	2	4.3	5.2	1.20	6.0 (2.58)
121.00	1	3.7	4.5	1.22	5.90 (2.54)
	2	4.0	5.0	1.30	5.60 (2.41)
162.00	1	2.7	2.5	0.95	5.70 (2.45)
	2	3.0	3.5	1.17	5.50 (2.36)

Cells cultured in cleated flasks were left to incubate at 30° with shaking at 140 rev./min. for 120 hr. after full growth had been reached. Samples were removed directly from the flasks into Warburg manometer cups for estimations of O<sub>2</sub> and CO<sub>2</sub> exchange rates. Samples were also taken for optical density determinations.

Figure 13

Changes in  $Q_{O_2}$  and Dry Weight Content of Cultures of N. corallina



Cultures were grown in cleated flasks incubated at  $30^{\circ}$  with shaking at 140 rev./min. Full growth was reached at approximately 40 hr. after inoculating the flasks. Samples were taken at time intervals after inoculation for estimation of respiratory quotients and dry weight. (see Table 11)

Table 12

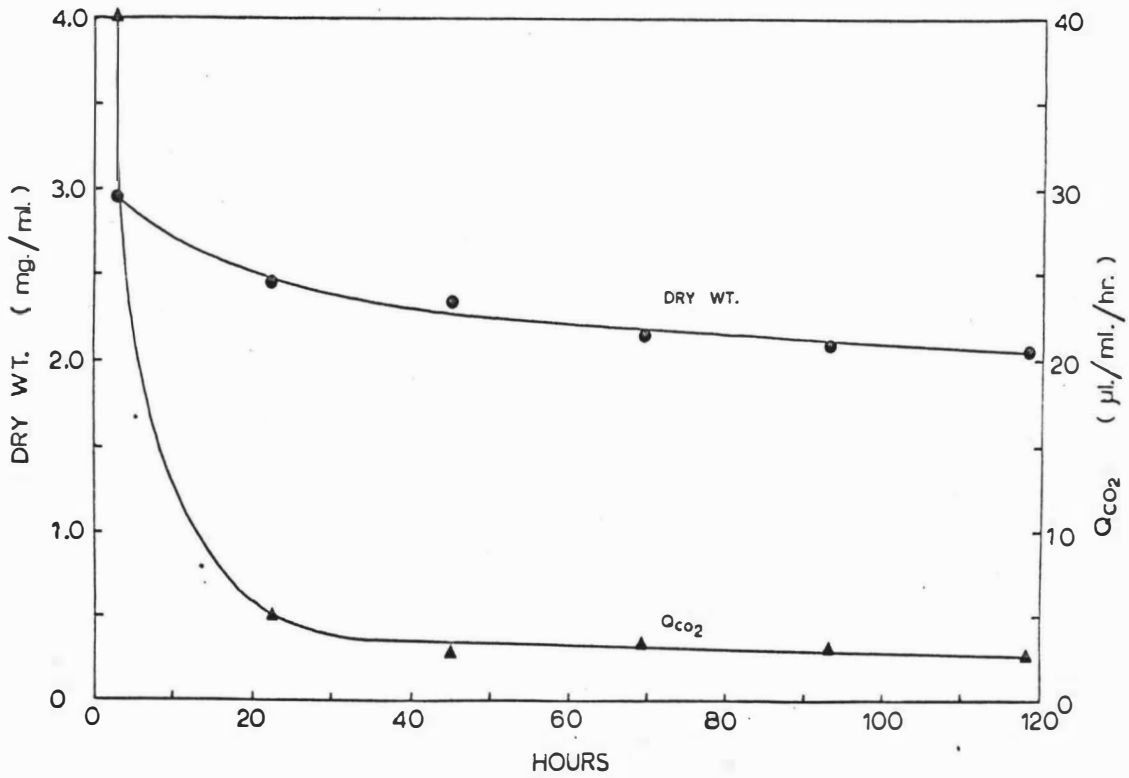
Respiration Studies with Starved Suspensions of N. corallina

Hours	Flask	$Q_{O_2}$ $\mu\text{l./ml./hr.}$	$Q_{CO_2}$ $\mu\text{l./ml./hr.}$	RQ	OD (660 m $\mu$ ) (dry wt. mg./ml.)
2.45	1	33.0	38.0	1.15	6.50 (2.80)
	2	32.6	42.1	1.29	7.25 (3.12)
22.15	1	4.45	4.87	1.09	5.45 (2.34)
	2	4.60	5.13	1.05	6.00 (2.58)
45.00	1	2.53	2.65	1.05	5.20 (2.24)
	2	2.97	3.17	1.08	5.75 (2.47)
69.45	1	2.87	3.26	1.15	4.90 (2.11)
	2	2.83	3.64	1.34	5.10 (2.19)
93.00	1	2.75	3.04	1.10	4.75 (2.04)
	2	3.02	3.26	1.07	5.00 (2.15)
118.15	1	2.24	3.06	1.36	4.70 (2.02)
	2	1.61	2.15	1.33	4.90 (2.11)

Cells cultured in clefted flasks were resuspended in phosphate buffer in clefted flasks and incubated at 30° with shaking at 140 rev./min. Samples were removed at time intervals directly from the flasks into Warburg manometer cups for estimations of O<sub>2</sub> and CO<sub>2</sub> exchange rates. Samples were also taken for optical density determinations.

Figure 1.

Changes in  $Q_{CO_2}$  and Dry Weight Content of Starved Suspensions of N. corallina



Cells suspended in phosphate buffer (cleated flasks) were incubated for 5 days at  $30^{\circ}$  with shaking at 140 rev./min. Samples were taken at time intervals for estimation of respiratory quotients and dry wt. (see Table 12).

results were almost identical with those from the first experiment. Again no RQ values approaching the characteristic lipid values of about 0.7 were recorded. The average value for all estimations was 1.17.

#### Respiration Experiments with Acetate Grown Cells

The high RQ values obtained with glucose grown cells could not be correlated with the findings of Midwinter (1962) which had led to the proposition that lipid was catabolised during prolonged endogenous respiration. However since Midwinter had studied acetate and propionate grown cells it was proposed to repeat the previous experiment with cells grown on acetate. Cells were cultured (cleated flasks, milk filters) in the standard liquid medium (Appendix 1) with the exception that acetate (0.02M final conc.) was used in place of glucose and the initial pH was adjusted to pH 6.8. The yield of cells at full growth, (determined using the relationship dry wt. mg./ml. = OD x 0.43) was 0.3 mg./ml. Cells from cultures (4) harvested 12 hr. after full growth had been reached were resuspended (0.4 mg./ml.) in phosphate buffer in two cleated flasks and incubated at 30° for 40½ hr. Prior to carrying out RQ estimations samples from the suspensions were centrifuged (2,000 g , 7 min.) and washed twice by suspending in phosphate buffer and recentrifuging. Aliquots (2 ml.) of the final phosphate buffer suspension (2.5 mg. dry wt./ml.) were incubated in Warburg manometers for gas exchange determinations. The results (Table 13) showed the same high RQ values observed previously with glucose grown cells.

#### Respiration Experiments with Cells Incubated with Palmitate

It was considered possible that there was a basic fault in methodology which lead to the absence of low RQ values reported by Midwinter (1962) in endogenous respiration experiments with N. corallina.

Table 13

## Respiration Studies with Starved Suspensions of Acetate Grown

N. corallina

Hours	Flask	$Q_{O_2}$ μl./ml./hr.	$Q_{CO_2}$ μl./ml./hr.	RQ	OD (660 mμ) (dry wt.mg./ml.)
1.0	1	2.80	2.92	1.04	0.88 (0.38)
	2	2.85	3.30	1.28	0.97 (0.42)
12.30	1	2.43	2.34	0.96	0.90 (0.39)
	2	2.56	3.09	1.19	0.96 (0.41)
40.30	1	1.58	1.64	1.04	0.80 (0.35)
	2	1.40	1.74	1.24	0.85 (0.37)

Cells grown in cleated flasks in medium (Appendix 1) in which acetate (0.02 M) replaced glucose, were resuspended in phosphate buffer in cleated flasks and incubated at 30° with shaking at 140 rev./min. Samples of the cell suspensions taken at time intervals after resuspension were concentrated by centrifugation immediately before respiratory activity estimations were carried out. Estimations of respiratory activity were corrected to give values for suspensions contained in the cleated flasks. Optical density estimations (dry wt.) also refer to the suspensions in cleated flasks.

To examine this possibility glucose grown cells were incubated with potassium palmitate in Warburg manometers. Cells from cultures, harvested 3 days after growth had ceased and showing a low endogenous respiration rate (Fig. 13) were resuspended (4.8 mg. dry wt./ml.) in phosphate buffer. Aliquots (1 ml.) of this suspension were incubated in Warburg flasks containing potassium palmitate (palmitic acid, 0.3 mg.) suspended in 2 ml. phosphate buffer. The RQ value of the cells incubated in the absence of palmitate was 1.08, in agreement with values obtained previously. The RQ values for cells incubated in the presence of palmitate, determined in 3 separate experiments were 0.62, 0.68 and 0.66. The theoretical RQ for palmitate is 0.69.

#### Summary

In summary, experiments carried out to determine variations in dry wt., total and viable cell counts and respiratory activity of cells suspended in phosphate buffer established the following.

1) A fall in dry wt. (mg./ml. of suspension) of 31% occurred during the first 46 hr. following resuspension. Thereafter the decrease in dry wt. was slight.

2) The respiratory activity decreased markedly during the first 24 hr. following resuspension to a low and relatively constant level of approximately 1  $\mu$ l./mg./hr. The period of highest respiratory activity corresponded to the period in which the fall in dry wt. was most pronounced.

3) An increase in total cell count of 12% occurred over a 131 hr. period following resuspension.

4) Viable estimates of over 90% were obtained during a period of 8 days endogenous incubation. Following this period the viability declined more rapidly, reaching 50% 20 days after resuspension.

## CHAPTER 6

CHANGES IN THE LEVELS OF LIPID, CARBOHYDRATE AND PROTEIN IN STARVED  
SUSPENSIONS OF N. CORALLINA

Analyses of the levels of cellular components were initiated in an attempt to determine the reasons for the sharp decrease in dry wt. which occurred in the first few days of endogenous incubation. From a consideration of the results of Midwinter (1962) attention was directed initially towards quantitative changes in lipid levels.

Lipid Content of Cells of N. corallina

In a preliminary experiment to determine the cellular level of total unbound lipid, cultures were grown in cleated flasks and incubated for 4 days after growth had ceased. The pellets obtained by centrifuging the cultures were twice resuspended in distilled water and centrifuged prior to freeze drying. The coral pink powder was extracted with cold chloroform-methanol (2:1) giving yields, from duplicate analyses, of 15.3 and 17.2% of the dry wt. of the cells. These percentage compositions for lipid were similar to those reported by Midwinter (1962) and Robertson (1964) for N. corallina grown on glucose medium.

In a subsequent experiment samples for total unbound lipid estimations were removed from cultures at the end of the growth phase and again 5 days after the end of growth. Total unbound lipids were extracted from the freeze dried cells using chloroform-methanol (2:1). During the 5 day period of endogenous incubation there was an increase in lipid (calculated as % dry wt.) from 10.8 and 11.3 (duplicates) to 13.9 and 14.3. A percent

increase in cellular lipid with no net increase could arise as a result of a large decrease in some other cellular constituent during endogenous incubation. Therefore correct interpretations of the results of analyses could only be made if due account was taken of the total change in dry wt. during the period of endogenous incubation.

Since the chloroform-methanol extract could contain components other than unbound lipids and since thin-layer chromatographic analysis suggested that triglycerides did not represent a large proportion of the total unbound lipid (Chapter 3) it was decided to examine changes in lipid levels in terms of total fatty acids. Midwinter (1962) had previously reported a decrease in the total cellular fatty acid fraction, from glucose grown cells, from 17.2 to 14.5% of the total cell dry wt. during a 33 hr. period of endogenous incubation.

#### Fatty Acid Levels in *N. corallina*

Three cultures in clefted flasks were sampled (150 ml.) at the end of full growth and again after 5 days endogenous incubation. The cells were washed with distilled water by centrifugation and freeze dried prior to alkaline hydrolysis and extraction of the fatty acids with diethyl ether. The fatty acid content of the cells, (% dry wt.) determined after drying the ether extract to constant weight, showed an increase over the 5 day period of endogenous incubation (Table 14). The fall in total dry wt. (28%) was determined in a separate but identical culture which was sampled for dry wt. estimations at full growth and again after 5 days. The estimate of the fall in dry wt. (28%) was used to calculate the percent dry wt. of lipid in the 5 day sample had there been no fall in dry wt. over the 5 day period. A net fall in fatty acid content from 11.7 to 10.4% of the initial dry wt. was deduced from these calculations.

Table 14

Fatty Acid Content of Cells of N. corallina

Flask	Total fatty acids (% dry wt.)		Total fatty acids from 5 day sample corrected using factor of 28% to give % dry wt. at full growth
	Full growth	5 days after full growth	
1	11.6	14.3	10.4
2	11.4	14.3	10.4
3	12.0	14.3	10.4
Dry wt. (mg./ml.) of culture contained in flask 4			
	Full growth	5 days after full growth	Loss in dry wt. (%)
	3.2	2.3	28%

Cells cultured in cleated flasks were left to incubate (30°, 140 rev./min.) for 5 days after reaching full growth as determined by maximum optical density (660 mp.). Total fatty acids were obtained from freeze dried cells by ether extraction following alkaline hydrolysis.

Although the statistical significance of this result was not assessed the contribution of the fall in lipid to the total fall in dry wt. was of the order of 4%. From this finding attention was drawn towards other cellular constituents.

Total Nitrogen, Ammonia, Protein and Carbohydrate Levels Related to Changes in Dry Weight

To correlate dry wt. changes with variations in cellular composition, cultures were harvested at the end of the growth phase and the cells were resuspended in phosphate buffer in cleaned flasks for subsequent endogenous incubation. At intervals, duplicate samples (25 ml.) were withdrawn from each flask. One sample was centrifuged and the supernatant was stored at  $-25^{\circ}$  for subsequent analysis of total hexose and total nitrogen. The packed cells were washed twice with distilled water by recentrifugation before being resuspended in distilled water. Aliquots of the distilled water suspension were taken for dry wt. and for total protein estimations. Results were corrected for appropriate dilutions to give an estimate of the level of protein in the phosphate buffer suspension. The second sample (25 ml.) was used directly for total hexose, total nitrogen and dry wt. estimations. All analyses except those for dry wt. were carried out in duplicate. The results of the experiment are shown in Table 15.

The results of an analysis of variance showed that there was no significant change in the level of total nitrogen in the suspensions. There was however a significant rise ( $p = 0.01$ ) in supernatant total nitrogen which indicated that there had been a considerable release of nitrogenous material from cells over the 5 day incubation period. The release of total nitrogen could be equated to protein breakdown if the nitrogen equivalent of the decrease in cellular protein (0.375 mg./ml. Table 15) was estimated using the factor 6.25. Although the protein estimations were subject to appreciable errors

Table 15

Changes in the Levels of Fractions in Starved Suspensions of  
N. corallina

Fraction (analytical method)	Flask	Level of fraction (mg./ml. of suspension)			
		Time after resuspending (hr.)			
		0		118	
Total nitrogen (Kjeldahl)	1	0.276	0.272	0.272	0.276
	2	0.284	0.288	0.288	0.274
	3	0.292	0.290	0.290	0.292
	Mean S.E.M.	0.284 0.004		0.282 0.004	
Total supernatant nitrogen (Kjeldahl)	1	0.003	0.005	0.044	0.042
	2	0.007	0.009	0.040	0.043
	3	0.005	0.004	0.045	0.049
	Mean S.E.M.	0.005 0.001		0.044 0.002	
Cellular protein (biuret)	1	1.82	1.90	1.51	1.59
	2	1.84	1.72	1.43	1.57
	3	1.93	2.09	1.47	1.54
	Mean S.E.M.	1.883 0.051		1.508 0.022	
Supernatant ammonia nitrogen (Conway)	1	0.0	0.0	0.0335	0.0313
	2	0.0	0.0	0.0325	0.0350
	3	0.0012	0.0016	0.0363	0.0350
	Mean S.E.M.	0.0005 0.0003		0.0339 0.0008	
Total carbohydrate (anthrone)	1	0.66	0.66	0.15	0.14
	2	0.71	0.73	0.13	0.13
	3	0.79	0.77	0.14	0.17
	Mean S.E.M.	0.720 0.023		0.143 0.006	
Supernatant carbohydrate (anthrone)	1	0.02	0.02	0.02	0.02
	2	0.01	0.01	0.03	0.02
	3	0.01	0.01	0.01	0.01
	Mean S.E.M.	0.013 0.002		0.018 0.003	
Dry wt. (OD <sub>660</sub> )	1	3.35		1.94	
	2	3.40		1.94	
	3	3.55		1.95	
	Mean S.E.M.	3.43 0.05		1.94 0.01	

Cells suspended in phosphate buffer were incubated (cleated flasks, 30°, 140 rev./ min.) for 118 hr.

(Table 15) the results were consistent with the supposition that cellular protein was being catabolised to release all of its nitrogen as ammonia. An important feature of the results was the highly significant ( $p = 0.01$ ) fall in the level of cellular carbohydrate (0.58 mg./ml.) from 21% of the cell dry wt. to 4% after 5 days endogenous incubation. This decrease represented 39% of the total fall in dry wt. (1.49mg./ml.). The total contribution of carbohydrate and protein accounted for 64% of the total fall in dry wt.

The experiment clearly demonstrated that a large proportion of the fall in dry wt. could be attributed to the catabolism of cellular constituents. The results also provided an explanation for the apparent rise in lipid content in the cells during endogenous metabolism.

#### Changes in Levels of Total Fatty Acids, Protein, Carbohydrate, Dry Weight and Cluster Viability During Endogenous Incubation

A further experiment was carried out to determine whether the fall in carbohydrate coincided with the fall in dry wt. which had been observed immediately after cells were resuspended from cultures harvested at the end of the growth phase. Three cultures grown in clefted flasks were resuspended in clefted flasks. Immediately after resuspending, samples (15 ml.) were withdrawn from each flask into sterile, cotton wool plugged test tubes. The samples were used for estimations of total carbohydrate, total fatty acids (acid hydrolysis, chromate oxidation), total protein and cluster viability. The results (Table 16, Fig. 15) showed the typical fall in dry wt. which occurred when cultures were first resuspended. The fall in dry wt. was observed to coincide with an initial fall in the total hexose content from 24% to 5.5% of

Table 16

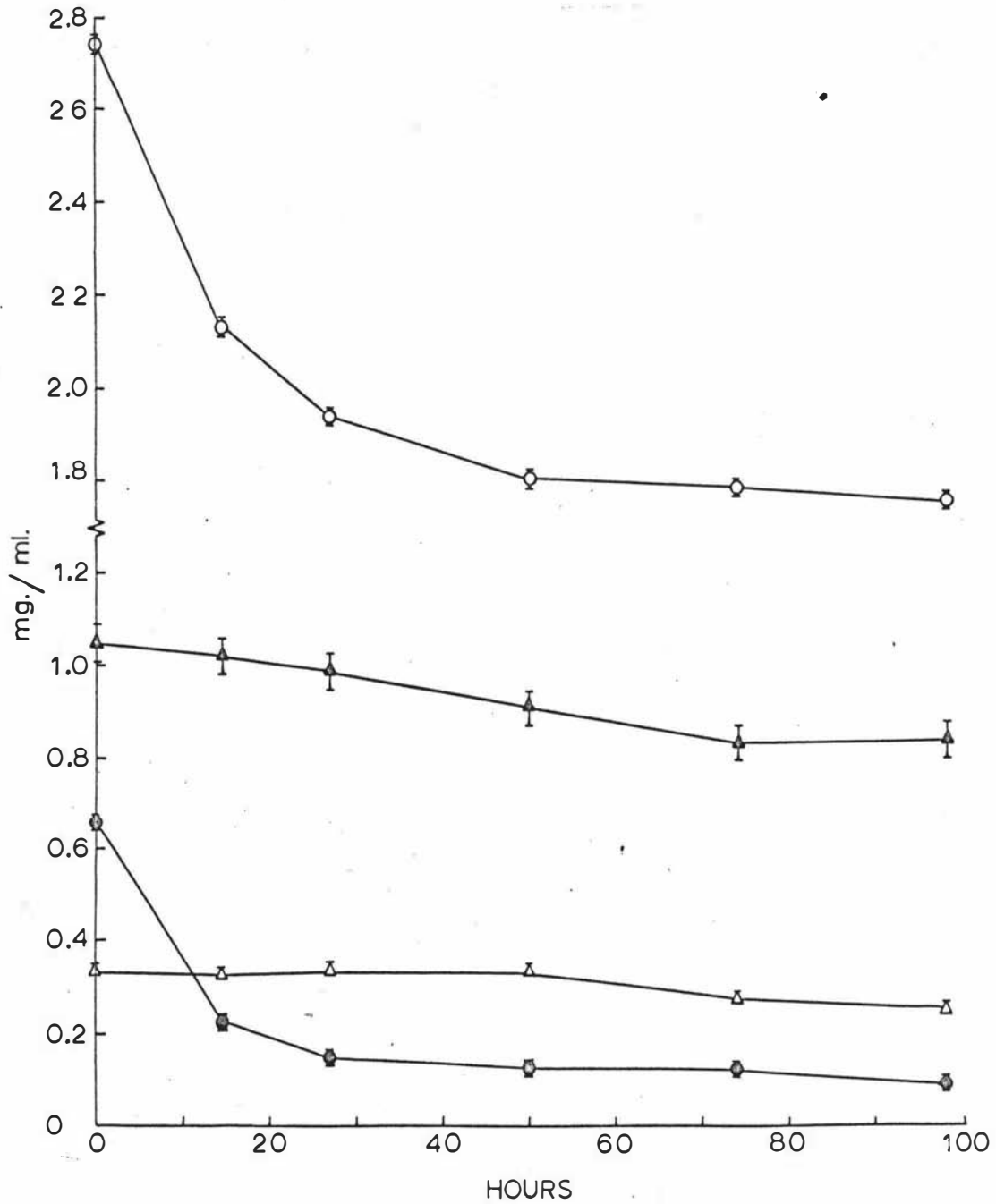
Changes in the Levels of Fractions in Starved Suspensions of  
N. corallina

Fraction (analytical method)	Flask	Level of fraction (mg./ml. of suspension)						
		Time after resuspension (hr.)						
		0.0	15.0	27.0	50.0	74.0	98.0	S.E.M.
Total carbohydrate (anthrone)	1	0.605	0.197	0.149	0.121	0.143	0.103	0.018
	2	0.665	0.288	0.152	0.139	0.127	0.097	
	3	0.710	0.200	0.149	0.127	0.106	0.097	
	Mean	0.660	0.228	0.150	0.129	0.125	0.099	
Supernatant carbohydrate (anthrone)	1	0.002	0.020	0.027	0.037	0.009	0.015	0.003
	2	0.002	0.023	0.023	0.024	0.018	0.018	
	3	0.001	0.026	0.027	0.026	0.021	0.021	
	Mean	0.002	0.023	0.026	0.029	0.016	0.019	
Total cellular fatty acids (chromate)	1	0.334	0.316	0.382	0.318	0.290	0.250	0.015
	2	0.334	0.316	0.316	0.306	0.280	0.244	
	3	0.334	0.350	0.296	0.366	0.250	0.266	
	Mean	0.334	0.327	0.331	0.330	0.273	0.253	
Total protein (biuret)	1	1.21	1.04	0.96	0.92	0.86	0.82	0.04
	2	1.04	1.04	1.03	0.92	0.82	0.82	
	3	0.89	0.99	0.99	0.88	0.81	0.88	
	Mean	1.05	1.02	0.99	0.91	0.83	0.84	
Supernatant protein (biuret)	1	0.0	0.114	0.057	0.091	0.057	0.114	0.039
	2	0.125	0.080	0.320	0.149	0.068	0.068	
	3	0.091	0.068	0.057	0.125	0.068	0.136	
	Mean	0.072	0.087	0.144	0.121	0.064	0.106	
Dry weight (OD <sub>660</sub> )	1	2.75	2.13	1.92	1.83	1.83	1.75	0.02
	2	2.77	2.17	2.00	1.79	1.75	1.72	
	3	2.74	2.08	1.90	1.79	1.77	1.77	
	Mean	2.75	2.13	1.94	1.80	1.78	1.75	
Cluster viability (%)	1	96				94		
	2	94				97		
	3	96				96		

Cells, suspended in phosphate buffer, were incubated (cleated flasks, 30°, 140 rev./min.) for 98 hr. Samples were taken at time intervals after resuspension.

Figure 15

Changes in Levels of Fractions in Starved Suspensions of N. corallina



Cells suspended in phosphate buffer (cleated flasks) were incubated at 30° with shaking at 140 rev./min. for 98 hr. Samples were taken at time intervals. The mean and S.E.M. of results from 3 flasks are presented (see Table 16).

- : Total dry wt.
- ▲: Total cellular protein.
- △: Total cellular fatty acids.
- : Total hexose.

the initial dry wt. in a period of 27 hr. Protein was broken down ( $p = 0.01$ ) throughout the duration of the experiment (98 hr.). The large errors in protein estimations were due to difficulties encountered in the analysis, discussed previously (see Methods). There was a significant ( $p = 0.01$ ) fall in the level of total fatty acids from 12.1 to 9.2% calculated with reference to the initial dry wt. The breakdown in total fatty acids contributed only 6.5% of the total loss in dry wt. (1.0mg./ml.) of the cells during starvation, carbohydrate contributed 56% and protein 21%.

Changes in Levels of Total Fatty Acids and Correlation of Protein Breakdown With Ammonia Production

To examine protein breakdown in relation to ammonia production and concurrently repeat the analysis of lipid levels in starving cells two cultures at the end of the growth phase were resuspended in cleaned flasks, shaken at  $30^{\circ}$  and sampled at intervals. Analyses were carried out for dry wt. total fatty acids (acid hydrolysis, chromate oxidation), total protein, supernatant protein, supernatant ammonia and supernatant amino acids. The results (Table 17, Fig. 16) showed a fall in total fatty acids from 14.2 to 9.5% (of the initial dry wt.) which was only just significant ( $p = 0.05$ ), a significant fall ( $p = 0.01$ ) in the level of cellular protein and a significant rise in the level of supernatant ammonia nitrogen. The amount of ammonia nitrogen ( $37 \mu\text{gm}$ ) excreted into the supernatant during the period of incubation (176 hr.) can be compared directly with a theoretical yield of ammonia nitrogen ( $30 \mu\text{gm}$ ) if it is assumed that all the protein degraded released its nitrogen into the supernatant. Of interest was the finding that there was no

Table 17

Changes in the Levels of Fractions in Starved Suspensions of

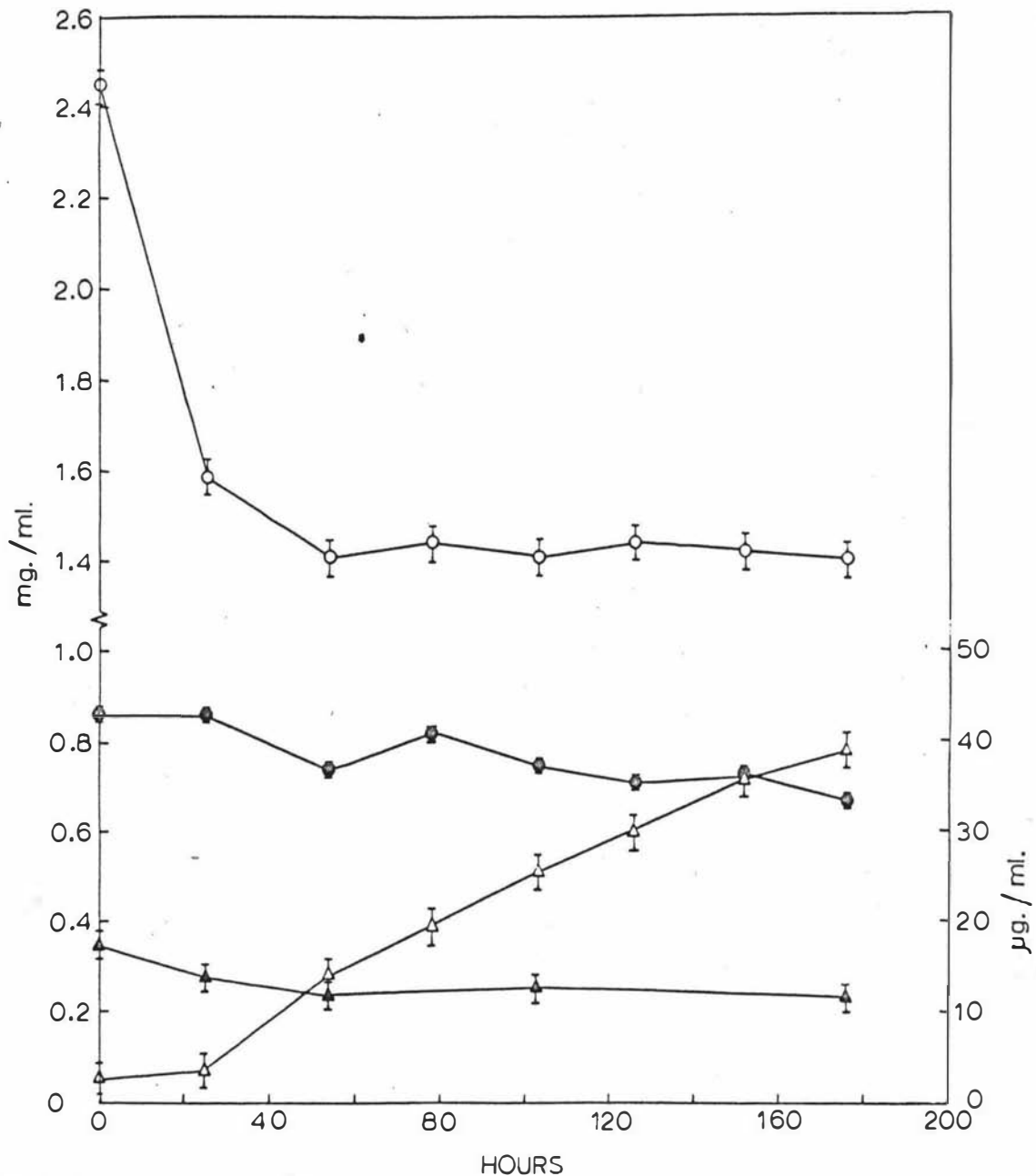
N. corallina

Fraction (analytical method)	Flask	Level of fraction (mg./ml. of suspension)							
		Time after resuspending (hr.)							
		0	25	54	78	103	126	152	176
Total protein (biuret)	1	0.85	0.87	0.77	0.82	0.75	0.72	0.77	0.70
	2	0.87	0.85	0.72	0.82	0.75	0.70	0.70	0.65
	Mean	0.86	0.86	0.74	0.82	0.75	0.71	0.73	0.67
	S.E.M.	±0.02							
Supernatant protein (biuret)	1	0							0.05
	2	0							0.05
Supernatant ammonia nitrogen (Nessler)	1	0.0046	0.0061	0.0178	0.0243	0.0315	0.0357	0.0436	0.0461
	2	0.0006	0.0011	0.0106	0.0149	0.0189	0.0245	0.0284	0.0319
	Mean	0.0026	0.0036	0.0142	0.0196	0.0252	0.0301	0.0360	0.0390
	S.E.M.	±0.0021							
Supernatant amino acids (Rosen). Not corrected for ammonia	1	0.0205	0.0168	0.0480	0.0678	0.0838	0.1008	0.1063	0.1140
	2	0.0186	0.0132	0.0341	0.0441	0.0627	0.0689	0.0831	0.0905
	Mean	0.0195	0.0150	0.0410	0.0559	0.0732	0.0848	0.0947	0.1022
	S.E.M.	±0.0053							
Total cellular fatty acids (chromate)	1	0.365	0.315	0.250		0.225			0.210
	2	0.330	0.230	0.220		0.280			0.225
	Mean	0.347	0.272	0.235		0.252			0.232
	S.E.M.	±0.029							
Dry wt. (OD <sub>660</sub> )	1	2.43	1.68	1.51	1.51	1.46	1.48	1.48	1.48
	2	2.47	1.51	1.31	1.38	1.36	1.40	1.36	1.33
	Mean	2.45	1.59	1.41	1.44	1.41	1.44	1.42	1.40
	S.E.M.	±0.04							
Cluster viability (%)	1			100					95
	2			100					96

Cells suspended in phosphate buffer were incubated (cleated flasks, 30°, 140 rev./min.) for 176 hr. Samples were taken at time intervals after resuspension.

Figure 16

Changes in Levels of Fractions in Starved Suspensions of N.corallina



Cells suspended in phosphate buffer (cleated flasks) were incubated at 30° with shaking at 140 rev./min. for 176 hr. Samples were taken at time intervals. The mean and S.E.M. of results from 2 flasks are presented.

(see Table 17)

- : Total dry wt. (mg./ml.)
- : Total protein (mg./ml.)
- ▲: Total cellular fatty acids (mg./ml.)
- △: Supernatant ammonia-nitrogen (µg./ml.)

significant release of ammonia from the cells during the first 25 hr. i.e. during the period which corresponded to a phase of rapid carbohydrate catabolism. During the same period there was no significant breakdown of protein.

The results of amino acid analyses (Table 17) were not corrected for ammonia since ammonia standards were not included in the amino acid analyses. It was determined however that the molar response of ammonia in the Rosen method for amino acids was 56% of that of glycine. Using this correction factor and knowing the level of ammonia in the solutions (Table 17) it was deduced that the quantities of amino acids in the samples were negligible.

#### Correlation of Breakdown of Cellular Carbohydrate With Production of Ammonia

The observation that ammonia was not released from cells during the first 24 hr. following resuspension of cultures was restudied in an experiment in which analyses were carried out for extracellular ammonia, total carbohydrate as reducing sugar, intracellular free reducing sugar, intracellular and extracellular amino acids and supernatant protein. The results presented in Table 18 and Fig. 17 demonstrated that ammonia was not released from the cells until after the major portion of the carbohydrate had been catabolised. Total cellular carbohydrate constituted 35% of the dry wt. when cells were first resuspended in comparison with 24% determined by the anthrone method for total hexoses (Table 16). The total reducing sugar content of the cells fell to approximately 5% of the initial dry wt. after a period of 195 hr. Analysis of the levels of intracellular free reducing sugar (Table 18) demonstrated that the bulk of the total reducing sugar occurred in cells

Table 18

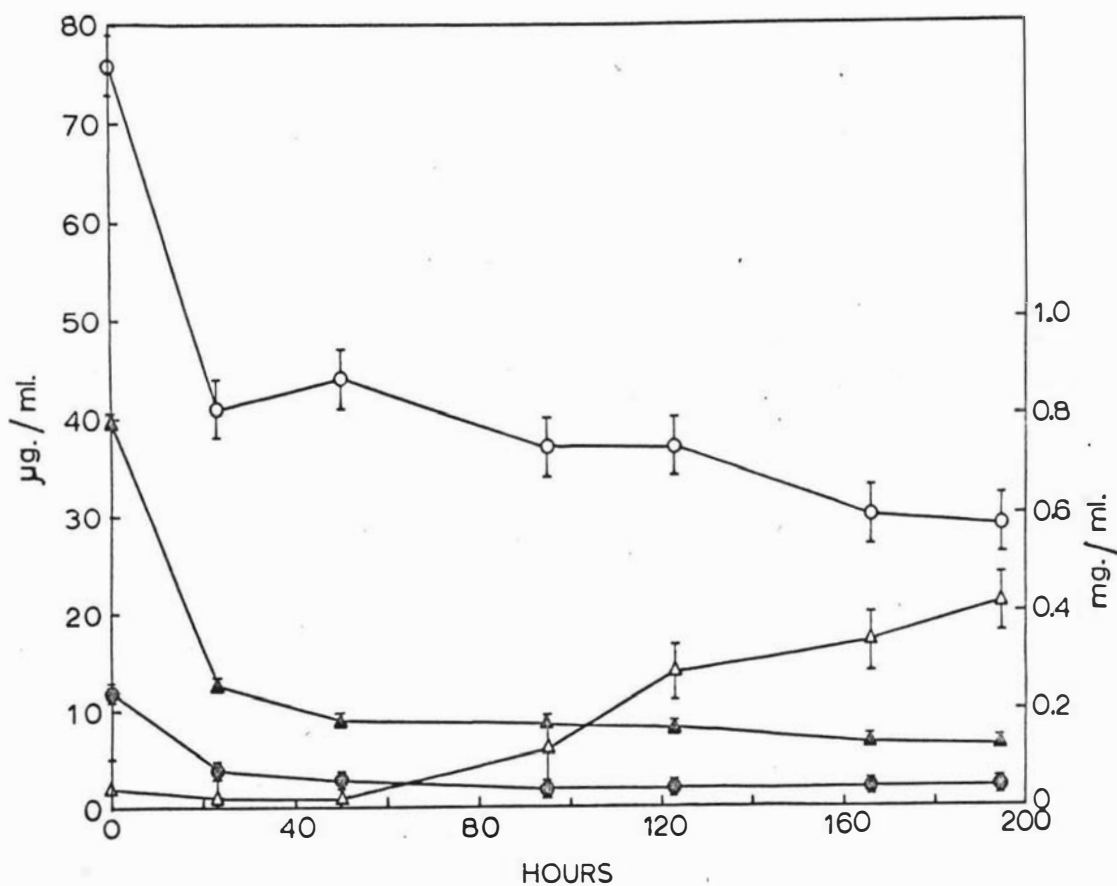
Changes in the Levels of Fractions in Starved Suspensions of N.corallina

Fraction (Analytical Method)	Flask	Level of fraction (mg./ml. of suspension)							
		Time after resuspension (hr.)							
		0	23	50	95	123	166	195	S.E.M.
Total reducing sugar (Nelson)	1 2 3 Mean	0.789 0.709 0.875 0.791	0.230 0.251 0.275 0.252	0.184 0.161 0.195 0.180	0.183 0.138 0.196 0.172	0.185 0.130 0.186 0.167	0.132 0.113 0.154 0.133	0.121 0.101 0.153 0.125	$\pm 0.014$
Intra- cellular free reducing sugar (Nelson)	1 2 3 Mean	0.014 0.010 0.012 0.012	0.004 0.004 0.004 0.004	0.002 0.004 0.002 0.003	0.002 0.002 0.002 0.002	0.002 0.002 0.002 0.002	0.002 0.003 0.002 0.002	0.002 0.001 0.002 0.002	$\pm 0.001$
Supernatant ammonia nitrogen (Nessler)	1 2 3 Mean	0.002 0.001 0.004 0.002	0.001 0.001 0.003 0.001	0.0 0.001 0.002 0.001	0.008 0.001 0.011 0.006	0.019 0.003 0.019 0.014	0.022 0.004 0.025 0.017	0.026 0.005 0.032 0.021	$\pm 0.003$
Supernatant amino acids (Rosen) Not corrected for ammonia.	1 2 3 Mean	0.013 0.013 0.013 0.013	0.010 0.003 0.003 0.006	0.010 0.003 0.017 0.010	0.030 0.007 0.053 0.030	0.050 0.017 0.089 0.045	0.073 0.036 0.086 0.065	0.093 0.059 0.112 0.088	$\pm 0.007$
Intra- cellular amino acids (Rosen) Not corrected for ammonia.	1 2 3 Mean	0.080 0.059 0.088 0.076	0.042 0.031 0.050 0.041	0.051 0.035 0.048 0.044	0.034 0.034 0.043 0.037	0.037 0.034 0.041 0.037	0.030 0.027 0.034 0.030	0.028 0.026 0.033 0.029	$\pm 0.003$
Supernatant protein (Folin)	1 2 3 Mean	0.0 0.0 0.0 0.0	0.025 0.013 0.030 0.023	0.052 0.023 0.048 0.041	0.045 0.025 0.053 0.041	0.045 0.045 0.073 0.054	0.060 0.048 0.058 0.055	0.070 0.049 0.063 0.061	$\pm 0.007$
Dry wt. (O.D. 660)	1 2 3	2.36 1.83 2.57	1.15 1.10 1.62						

Cells suspended in phosphate buffer were incubated (cleated flasks, 30°, 140 rev./min.) for 195 hr. Samples were taken at time intervals after resuspension.

Figure 17

Changes in Levels of Fractions in Starved Suspensions of N. corallina.



Cells suspended in phosphate buffer (cleated flasks) were incubated at 30° with shaking at 140 rev./min. for 195 hr. Samples were taken at time intervals. The mean and S.E.M. of results from 3 flasks are presented (see Table 18).

- : Intracellular amino acids and ammonia (µg./ml.)
- : Intracellular free reducing sugar (µg./ml.)
- △: Supernatant ammonia-nitrogen (µg./ml.)
- ▲: Total reducing sugar (mg./ml.)

in the form of a polysaccharide. By correcting amino acid analyses for the presence of ammonia using the factor of 56% (see Methods) it was deduced that the levels of supernatant amino acids were negligible in 2 of the 3 flasks studied. In the third flask however (Table 18, Flask 2) release of amino acids was apparent. Most significant was the fall in intracellular amino acids plus ammonia determined by the Rosen method. This result suggested that the free nitrogenous components in the cells were very rapidly incorporated into more complex materials since there was no corresponding rise in the extracellular levels of either ammonia or amino acids during the period immediately following resuspension (Fig. 17). Protein determinations using the Folin method on supernatant samples showed that some protein had been released during the period of endogenous incubation as suggested in an earlier experiment (Table 16) using the biuret test.

#### Summary

The results of studies on changes in levels of components in cell suspensions of N. corallina may be summarised as follows.

- 1) The initial fall in dry wt. which occurred when cells were resuspended from cultures which had reached full growth appeared related primarily to the breakdown of cellular polysaccharide. Analyses of total carbohydrate showed levels of 20 to 35% of the total cell dry wt. falling to 5 to 10% after periods of 24 to 36 hr.
- 2) Ammonia accumulated in the supernatant following the breakdown of polysaccharide.
- 3) A fall in the level of intracellular free nitrogenous components (ammonia and amino acids) occurred almost immediately after the cells were suspended but no corresponding rise was observed in the supernatant. This suggested that these components were incorporated into other cellular constituents.

4) The fatty acid content of the cells decreased from approximately 13 to 9% of the initial total cell dry wt. during endogenous incubation.

5) The contribution of the decreases in cell fractions, to the total decrease in cell dry wt. which occurred when cells were resuspended was of the order of 40% for carbohydrate, 25% for protein and 5% for total fatty acids.

## CHAPTER 7

CHANGES IN THE LEVEL OF RNA IN STARVED SUSPENSIONS OF N. CORALLINA

The decrease in dry wt. of cells respiring endogenously could not be accounted for solely in terms of protein, carbohydrate and lipid. Since RNA is commonly degraded in starving microbial cells (Herbert, 1961) changes in the level of this cellular constituent were examined. The method of Munro and Fleck (1966) was used for the RNA estimations. The procedure applied to cell suspensions in phosphate buffer gave three fractions for analysis.

a) The phosphate buffer supernatant obtained by centrifuging the cell suspension.

b) The 0.2N HClO<sub>4</sub> fraction (20 ml.) obtained by washing the pellets in 0.2N HClO<sub>4</sub> (ice cold). This fraction contained the free intracellular ultraviolet absorbing material.

c) The 0.1N HClO<sub>4</sub> fraction (100 ml.) containing the products of alkaline hydrolysis of RNA.

Phosphate Buffer Supernatants

Studies of absorption spectra of supernatants showed a strong absorbance in the 260 m $\mu$ . region. The 260/230 and 260/280 ratios for supernatants from two experiments were (1) 1.20 and 2.17 and (2) 0.58 and 1.90. Strong absorption in the 230 m $\mu$ . region indicated the presence of proteins, previously detected by both Folin and biuret estimations (Table 17, 18). The degree of absorption in the 230 m $\mu$ . region varied markedly between flasks both in the same and different experiments.

The nature of the material absorbing the 260 mu. region was examined in an aliquot (40 ml.) of supernatant from a 10 day resuspension experiment. The solution was passed through a charcoal column used for desalting solutions of nucleotides, (Petersen, 1963) and samples of UV absorbing eluate were chromatographed on two dimensional paper chromatograms (Wyatt, 1955). Although loadings were low seven components were observed under ultraviolet light. Three of these moved in isopropanol-HCl solvent but not in butanol-NH<sub>3</sub> and they were tentatively identified (from Rf values and from spectra in solution, after elution from the paper with 0.1N HCl) as; (1) a mixture of guanylic and adenylic acids (2) cytidylic acid (3) uridylic acid. A component with an Rf of 0.51 in isopropanol-HCl and 0.07 in butanol-NH<sub>3</sub> was identified as barbituric acid by cochromatography with barbituric acid (BDH lab. reagent) applied on the two dimensional chromatogram, and by comparison of the spectrum of the unknown component with barbituric acid after both standard and unknown had been eluted from the paper chromatogram with distilled water.

These results suggested (a) that the products of RNA breakdown were liberated into the extracellular solution when cultures were resuspended in phosphate buffer and (b) that starving cells were capable of metabolising pyrimidines, since barbituric acid has been reported as a product of both cytosine and uracil catabolism for N. corallina (Batt and Woods, 1961).

#### 0.2N HClO<sub>4</sub> Fraction

Studies carried out to determine the time for complete extraction of free intracellular UV-absorbing material using 0.2N HClO<sub>4</sub> at 2° showed that a period of 4 hr. was required before extraction was

essentially complete. After the 4 hr. period there was no further increase in the amounts extracted, of ribose (determined by the orcinol method) or amino acids (determined by the Rosen method). No deoxyribose could be detected in the extracts. A study of UV spectra of the 0.2N HClO<sub>4</sub> solutions showed 260/230 and 260/280 wavelength ratios of 2.00 and 2.17 respectively. Continued extraction with 0.2N HClO<sub>4</sub> for periods up to 8 hr. after the initial 4 hr. extraction showed a small but constant rate of increase in the absorption at 260 mμ.

A study of the 0.2N HClO<sub>4</sub> extracts using the orcinol reagent for pentoses and OD<sub>260</sub> for nucleic acid material showed that the ribose content, determined by the orcinol method, was always greater than that estimated from the absorption at 260 mμ. on the basis that OD<sub>260</sub>=1 is equivalent to 32 μg. of RNA/ml. (Munro and Fleck, 1966). This could be explained if the purine to pyrimidine ratio in the free nucleotide material was > 1 or if some other orcinol positive component was being extracted by the 0.2N HClO<sub>4</sub>. Paper chromatographic analysis was carried out to determine the sugars present in a 3½ hr. 0.2N HClO<sub>4</sub> extract of cells from a culture which had been left incubating, (cleated flask, 30°, 140 rev./min.) without harvesting and resuspension, for 10 days after reaching full growth. The ribose content of the 0.2N HClO<sub>4</sub> extract determined from the optical density at 260 mμ. was 5.2 μg. ribose/ml. whereas the orcinol test gave 8.9 μg. ribose/ml. Material contained in aliquots of the 3½ hr. 0.2N HClO<sub>4</sub> extract of cells was either hydrolysed using 1N H<sub>2</sub>SO<sub>4</sub> desalted and chromatographed, or desalted and chromatographed directly, without acid hydrolysis (see Methods). Using the method of Wilson (1959) to determine the amounts of sugars on paper chromatograms it was shown that glucose; mannose;

ribose and arabinose occurred in the 1N H<sub>2</sub>SO<sub>4</sub> hydrolysed extracts in the molar ratio; 14:3:9:1. Although arabinose was found to give a 90% response relative to ribose in the orcinol reaction (whereas glucose and mannose gave no response) the amount of ribose and arabinose together, detected by the method of Wilson (1959), accounted for only 75% of the pentose shown to be present (by the orcinol test) in the original 0.2N HClO<sub>4</sub> extract. No further tests were carried out to determine whether any pentose was being lost during acid hydrolysis or during the subsequent deionization steps. Quantitative paper chromatography of an equivalent amount of non acid-hydrolysed material from the same 3½ hr. 0.2N HClO<sub>4</sub> extract of cells did not reveal the presence of any sugars. It was concluded that the four sugars mentioned above occurred in a form which was soluble in 0.2N HClO<sub>4</sub> but which could be hydrolysed with 1N H<sub>2</sub>SO<sub>4</sub> at 100° for 2 hr. to yield the free sugars.

#### 0.1N HClO<sub>4</sub> Fraction

Studies carried out to determine the time for complete hydrolysis of intracellular RNA using 0.3N KOH at 37° (followed by acidification to give a final solution 0.1N with respect to HClO<sub>4</sub>) showed that complete extraction of both OD<sub>260</sub> and orcinol positive material occurred after a period of 2 hours incubation with 0.3N KOH. Peptide material (15 µg./ml.) was detected (Folin reagent) in the final 0.1N HClO<sub>4</sub> extract but no deoxyribose could be detected. A study of the UV spectra of the 0.1N HClO<sub>4</sub> fraction containing the products of alkaline hydrolysis of RNA showed 260/230 and 260/280 wavelength ratios of 2.82 and 1.65. These values may be compared with those reported by Munro and Fleck (1966) for rat liver RNA of 3.25 and 1.38 respectively.

A study of the 0.1N HClO<sub>4</sub> fraction using the orcinol reagent for

pentoses and  $OD_{260}$  for nucleic acid material showed that the ribose content, determined by the orcinol method, was greater than that estimated from the absorption at 260  $m\mu$ . on the basis that  $OD_{260}=1$  is equivalent to 32  $\mu\text{g.}$  of RNA/ml. (Munro and Fleck, 1966). There was however better agreement than was found for the 0.2N  $\text{HClO}_4$  fraction. Analysis of the sugars present in the 0.1N  $\text{HClO}_4$  extracts before and after hydrolysis with 1N  $\text{H}_2\text{SO}_4$  showed the presence of glucose in the non-hydrolysed samples and glucose, mannose, ribose and arabinose in the hydrolysed samples.

#### Levels of RNA in Cell Suspensions Incubated under Endogenous Conditions

To determine changes in RNA levels under endogenous conditions, cultures grown in clefted flasks were resuspended at full growth in phosphate buffer and incubated (standard flasks,  $30^\circ$ , 140 rev./min.) for 262 hr. Samples of the suspensions, taken at time intervals, were analysed for RNA (see Methods) using a 10 min. washing period in 0.2N  $\text{HClO}_4$  as recommended by Munro and Fleck (1966) instead of the required 4 hr. mentioned previously. It was estimated that the use of a 10 min. wash followed by two rinses in 0.2N  $\text{HClO}_4$  would have removed approximately 40-50% of the free intracellular UV absorbing material. The remaining free intracellular UV absorbing material, not extracted, could constitute approximately 20% of the total UV absorbing material contained in the 0.1N  $\text{HClO}_4$  extract (products of alkaline hydrolysis of RNA). Taking this into account the results of an experiment are presented (Table 19) as evidence for the breakdown of RNA in starved suspensions of N. corallina. On the basis that  $OD_{260}=1$  is equivalent to 32  $\mu\text{g.}$  of RNA/ml. of the 0.1N  $\text{HClO}_4$  extract

Table 19

RNA Estimations on Starved Suspensions of N.corallina

Fraction	Flask	Optical densities (260 m $\mu$ ) of fractions Obtained During RNA Estimations.							
		Time after resuspending (hr.)							
		0	22	46	70	94	166	190	262
0.1N HC10 <sub>4</sub> (100 ml) Products of alkaline hydrolysis of RNA*	1	0.272	0.235	0.178	0.149	0.157	0.130	0.111	0.085
	2	0.284	0.214	0.180	0.148	0.162	0.115	0.102	0.088
	3	0.228	0.198	0.137	0.135	0.143	0.113	0.091	0.073
0.2N HC10 <sub>4</sub> (20 ml.) Free intracellular ultra-violet absorbing material	1	0.040	0.106	0.103	0.100	0.148	0.153	0.154	0.185
	2	0.053	0.090	0.124	0.123	0.123	0.154	0.157	0.189
	3	0.081	0.117	0.178	0.168	0.108	0.205	0.171	0.217
Phosphate buffer supernatants	1	0.012	0.314	0.362	0.335	0.396	0.452	0.486	0.576
	2	0.028	0.317	0.368	0.366	0.420	0.459	0.499	0.561
	3	0.008	0.249	0.312	0.298	0.334	0.388	0.431	0.490
Total cell dry wt. (mg./ml.) determined from OD <sub>660</sub>	1	1.94	1.10	1.08	1.06	1.08	1.02	0.88	0.99
	2	2.13	1.18	1.18	1.10	1.05	1.05	1.03	0.99
	3	1.94	1.10	1.03	0.97	0.90	0.90	0.99	0.92

Cells suspended in phosphate buffer were incubated (standard flask, 30°, 140 rev./min.) for 262 hr. Samples were taken at time intervals after resuspension.

\*RNA ( $\mu$ g./ml.) in 0.1N HC10<sub>4</sub> extract = OD<sub>260</sub> x 32 (Munro and Fleck, 1966)

(Munro and Fleck, 1966) the level of the mean for RNA estimations from three flasks fell from 83  $\mu\text{g./ml.}$  to 26  $\mu\text{g./ml.}$  of bacterial suspension over the 262 hr. period, or expressed as a percentage of the initial dry wt., from 4.1% to 1.3%. The results (Table 19) show a rise in the level of intracellular free UV absorbing material during starvation. This was difficult to explain without further studies involving a longer period of washing with 0.2N  $\text{HClO}_4$  at  $2^\circ$ . It was possible that during RNA breakdown there was a rise in the level of free UV absorbing material in the cells. If the absorptions of both the 0.1N and 0.2N  $\text{HClO}_4$  fractions are combined and allowance is made for dilutions, a net loss of UV absorbing material from the cells is clearly indicated. The rise in optical density at 260  $\text{m}\mu$ . in the phosphate buffer supernatants (Table 19) could support this view.

#### Summary

The results indicate a fall in the level of RNA during endogenous respiration from 4% to 1% of the initial cell dry wt. in a period of 11 days. The fall in RNA would account for approximately 6% of the total fall in dry wt. Ultraviolet absorbing compounds were released into the suspending medium and some of these could be accounted for as degradation products of RNA. On acid hydrolysis of the material contained in the  $\text{HClO}_4$  fractions, glucose, mannose, ribose and arabinose were released. Presumably the ribose is derived from ribotides and the other sugars from a cell wall polysaccharide. Cell walls of Nocardia species characteristically yield on acid hydrolysis, the monosaccharides, arabinose, glucose, mannose and galactose (Salton, 1964).

## CHAPTER 8

STUDIES ON THE ULTRASTRUCTURE OF N. CORALLINA

As a concurrent study it was decided to examine the ultrastructure of N. corallina with special reference to changes which might occur during periods of endogenous incubation. Accordingly an investigation was initiated using techniques of negative staining, shadowing, thin sectioning and freeze etching. Although originally intended, autoradiography was not attempted. However the information presented in this chapter should provide a sound basis for future studies involving such techniques as autoradiography.

Methods and materials for electron microscopyThe Organism

The organism was cultured in a glucose medium (Appendix 1) in standard or clefted flasks (conical 1L.) with milk filters. In one experiment cells were grown in medium (Appendix 1) in which acetate (0.02 M final conc.) replaced glucose as the carbon substrate. Samples for electron microscopy (Phillips EM 200) were withdrawn at intervals from cultures or suspensions used in studies already described (Chapters 6 and 7).

Negative-stained Preparations

a) Samples from cultures were dialysed against distilled water for 24 hr. at 4<sup>o</sup> and then centrifuged (3,000 g) at room temperature

prior to resuspension in 0.2% phosphotungstic acid (adjusted to pH 5.7 with 1N KOH) at a concentration suitable for spraying onto grids. Grids were made of copper, coated with collodion and stabilised with carbon.

b) Samples from cultures were centrifuged (3,000 g) at room temperature and the cells resuspended in 0.3% phosphotungstic acid (pH 5.7) containing approximately 0.03% bovine serum albumin and polystyrene latex spheres, (264  $\mu$ .) prior to spraying onto grids.

#### Shadowed Preparations

Samples of cell suspensions were centrifuged (3,000 g) at room temperature, resuspended in distilled water, sprayed onto grids and shadowed with platinum at an angle of  $\tan^{-1} 1/4$ .

#### Thin Sections

a) Pellets obtained by centrifugation (3,000 g) were fixed for 4 hr. (without resuspension) in 0.08M phosphate buffer (pH 7.0) containing 3% glutaraldehyde. The pellets were rinsed twice, (without resuspension) in 0.1M phosphate buffer and fixed a second time for 5 hr. in a 0.1M solution of phosphate buffer containing 2%  $\text{OsO}_4$  (pH 7.0). Finally the pellets were dehydrated using ethyl alcohol (graded steps) and embedded in "Araldite". Sections were cut with a glass knife (LKB microtome) and stained for 20 min. with lead hydroxide (Millonig, 1961).

b) As an alternative procedure pellets were centrifuged and fixed by resuspending in 1% glutaraldehyde in 0.1M phosphate buffer

(pH 6.1) containing 0.01M  $Mg^{++}$ . After a period of 3 hr. the cells were centrifuged (1,000 g) and fixed by resuspension in 0.1M phosphate buffer (pH 6.1) containing 1.0%  $OsO_4$ . The pellets obtained by subsequent centrifugation (1,000 g) were washed twice by resuspending in phosphate buffer (0.1M), embedded in 2% agar, dehydrated using ethyl alcohol (graded steps) and embedded in "Araldite" by the method of Luft (1961).

#### Freeze-etched Preparations

Freeze-etching was carried out in a commercial plant (Balzers) using the technique described by Moor and Muhlethaler (1963). Cells were centrifuged (3,000 g) and suspended in 20% glycerol prior to freezing.

### Results and Discussion

#### General Morphology and Growth Characteristics in Liquid Medium

A study of a large number of cells which had been negatively stained gave the following mean dimensions for the organism:

	Length $\pm$ S.E.M.	Breadth $\pm$ S.E.M.
Cells (67) from a mid-log phase culture	2.38 $\pm$ 0.07 $\mu$	0.95 $\pm$ 0.02 $\mu$
Cells (67) from a culture left to incubate for 7 days after reaching full growth (Fig. 18).	2.88 $\pm$ 0.07 $\mu$	1.04 $\pm$ 0.02 $\mu$

Considerable shrinkage was observed with negatively stained preparations (Fig. 18, 19, 20, 21) and accordingly the sizes of the cells were deduced from the dimensions of the casts. A transverse septum was observed in many cells and in some cases two or three septa appeared in the same organism. Where this occurred the septa were characteristically very close together. As a general feature the cells tended to be irregular in shape with a large proportion being more pointed at one end than the other (Fig. 18). It was concluded that the flattened end had resulted from the separation of two daughter cells at a transverse septum. From the growth characteristics of the organism on agar (Fig. 3), the pointed end was interpreted as corresponding to the growing tip. Growth in liquid medium seemed to occur from both ends of newly formed daughter cells (approx. 2  $\mu$ . in length) giving rise to cells 4 to 5  $\mu$ . in length. During elongation the nuclear area divided by constriction and transverse septa appeared (one and sometimes two or three) forming daughter cells which subsequently separated from each other. The growing tips appeared to be more electron transparent in negatively stained preparations (Fig. 19) in comparison with the rest of the cell. In addition the newly formed growing tips were often found to be smaller in diameter (0.7  $\mu$ .) in comparison with other regions of the cells (1.2  $\mu$ .). Such differences in diameter appeared to be partly associated with the accumulation of a fibrous material around the older cell sections (Fig. 19).

In 1959 Hagedorn referred to durable cells in cultures of N. corallina grown on agar or broth media. They were described as coccoidal in shape, slightly thicker in diameter than the average cell (1.2  $\mu$ . in comparison

with 0.7  $\mu$ .), and coated by a thick envelope or slime layer. Frequently the durable cells were divided by a transverse septum and two nuclear areas could occur in at least one of the daughter cells. Although similar cells were observed in the present investigation the variations in shape, size and degree of outer wall coating on individual cells were such that it would be difficult to claim that any one group of cells was clearly different from the general population. Small cells with thick outer coats may well have special durable properties but no evidence has been obtained in this investigation to support such a proposal. Hagedorn (1959a) did not present evidence to confirm special durable properties in the cells he described.

True branching was never observed in cultures grown in clefted flasks although some cells showed rounded protrusions which may have represented the initiation of branching (Fig. 19). A typical branched hyphal system was observed in only one sample from a standard flask (conical 1L.) in which heavy clumping was observed. It was concluded that hyphal growth typical of N. corallina grown on agar (Fig. 3) did not occur to the same extent in liquid medium possibly because of continual agitation. Webb and Clark, (1957) have observed that the degree of hyphal formation in N. corallina grown in liquid medium is highly variable and depends largely on growth conditions.

#### Cell Coat

From negatively stained preparations it was noted that the outer coating of the cells appeared as a fibrous material which could cover the entire cellular surface or be partially or completely absent (Fig. 19, 20, 21). Variations in the amount of fibrous material were considerable with cells from different cultures. In general however,

there was a tendency for the growing tips to be free of the material, while the surfaces of older sections were heavily coated (Fig. 19). Cells from cultures which had been incubated for several days after full growth had been reached (Fig. 21) appeared to be more heavily coated over the entire surface than did cells harvested from the log phase (Fig. 19, 20). With shadowed preparations the outer coat had the appearance of being loosely associated with the cell walls (Fig. 22) and when cells were heated for 20 min. at  $100^{\circ}$ , what appeared to be the outer coat, came away from the surface of the cell in long fibres which seemed to consist of a number of coiled strands (Fig. 23). These strands occurred on cells grown in both acetate and glucose media. A freeze-etched preparation (Fig. 28) of the outer surface of what was presumed to be two cells separated by a septum showed that the exterior surface could be either fibrous or granular. The granular section was considered to correspond to the electron transparent region of the cell observed after negative staining (Fig. 19, 20) and the fibrous region (Fig. 28) to the fibrous or electron dense regions observed after negative staining (Fig. 19, 21).

The thickness of the cell coat was difficult to assess. Cross sections (Fig. 25, 26) showed a diffuse zone of up to 75  $\mu$ . in thickness on the surface of the cell wall. This could correspond to the fibrous material observed with negative-stained and freeze-etched preparations. Sections of cells subjected to periods of endogenous incubation (Fig. 25) often showed a thick wall immediately outside the membrane with beyond this again an electron dense layer. It is not clear whether the diffuse material is essentially different from the material constituting the electron dense layer. In some cases the electron dense layer appeared

to make up most of the material covering the cell membrane while in other cases it was less prominent (Fig. 25). It was considered possible that the fibrous material might contribute to the excessive clumping observed with cultures of N. corallina (Chapter 2). However, pellets obtained by centrifugation always seemed most difficult to resuspend when cells had been harvested from cultures at full growth. If the fibrous material contributed to the clumping then it might have been expected that older cells would be most difficult to resuspend. In 1958 Clark suggested that clumping in N. corallina was due to a sticky secretion which could be washed off with distilled water. The fibrous material observed in the present study although not strongly associated with the cells (Fig. 22) could not be easily removed by washing with water. Kawata and Inoue (1965) described for N. asteroides, a cell outer coat or envelope which appeared to contain amorphous material dispersed with fine granules of 1.5 to 3.0  $\mu$ m. in diameter. Possibly these granules correspond to cross sections of fibres similar to those observed in this study (Fig. 23). Kawata and Inoue (1965) were unable to establish the origin of the cell envelope in N. asteroides. Working with the organism Dermatophilus congolensis, a holocarpic actinomycete, Gordon and Edwards (1963) found that the cells were surrounded by a thick capsular material which they claimed was a product of cell wall degradation. Examination of freeze-etched preparations of N. corallina (Fig. 28, 31) showed the appearance of fibre formation on the granular surface. The gross differences in morphology between the fibrous and granular sections appeared so great that it seemed unlikely that one structural form was the degradation product of the other.

### Cell Wall

The cell wall occurs between the membrane and the cell coat. In log phase cultures the thickness of the wall was approximately 15  $\mu$ . (Fig. 24) whereas in older stationary phase cultures it varied between 25 and 30  $\mu$  (Fig. 25). Approximately the same dimensions were noted in specimens examined after freeze-etching (Fig. 30, 31). The outer surface of the cell wall observed in freeze-etched specimens had a granular appearance (Fig. 28, 29) and in some cases quite large particles of up to 35  $\mu$ . in diameter were embedded in the wall material (Fig. 30).

### Cytoplasmic Membrane

The cytoplasmic membrane, clearly observed in thin sections, occurred immediately beneath the cell wall (Fig. 24, 25, 26, 27). The membrane occurred as a trilayered structure in many thin-section preparations (Fig. 25). However in others the three layers of the membrane were difficult to distinguish (Fig. 24, 27). Kawata and Inoue (1965) observed a trilayered membrane for N. asteroides in contrast to the report of Hagedorn (1959a) who described only a single layered membrane for N. corallina.

A freeze-etch study of the layers lying immediately below the cell wall (Fig. 29, 32) showed two different types of surface. In one, particles of approximately 10  $\mu$ . in diameter occurred very close together (Fig. 29) while in the other, particles of approximately the same size occurred more sparsely distributed (Fig. 32). In addition there were regions where no particles occurred (Fig. 29, 32). Such areas were partially covered with plaques. The particulate layer observed in Fig. 29 was considered to correspond

to the outside surface of the membrane while the particulate layer in Fig. 32 could correspond to the inside surface of the membrane. The areas covered with plaques could represent regions in the membrane where no particles occurred. Alternatively the membrane itself may have divided between lipid monolayers in which case the plaques could represent small pieces of one of the lipid monolayers. This interpretation is based on the studies of Staehelin (1968).

### Intracytoplasmic Membrane Systems

Intracytoplasmic membrane systems were observed in many cells. Some were associated with septa while others occurred randomly distributed on the inside surface of the cell membrane (Fig. 20). These systems consisted of concentric lamellae or clusters of vesicles which were observed to be continuous with the cell membrane (Fig. 27). Small structures, which were observed associated with the cell membrane in freeze-etched preparations, (Fig. 31) could be identical with the intracytoplasmic membrane systems observed in sections. Hagedorn (1959a) was unable to detect intracytoplasmic membrane systems in N. corallina although the possibility that these occurred was not excluded. The membrane systems observed in the present investigation were very similar to those described by Kawata and Inoue (1965) for N. asteroides and to other membrane systems reported for species of the Eubacteria and Actinomycetales.

Figure 25 and other sections of aged cells showed vesicular bodies which have been described as intracytoplasmic membrane systems. Although N. corallina is not reported to be a spore former many actinomycete species do form spores and the possibility that these bodies are in some way related to spore formation cannot be discounted.

Certainly such bodies bear a resemblance to those observed in the early stages of spore formation (Murrell, 1967).

It has been reported for N. corallina (Hagedorn, 1959a; Clark and Aldridge, 1960) and for N. rubra (Adams and McLung, 1962) that inclusions which stain with sudan black are lipid droplets. However, since membranes contain a high proportion of lipid, intracytoplasmic membrane systems could possibly show as sudanophilic granules using light microscopy. Such a possibility might explain why Clark and Aldridge (1960) found difficulty in extracting the sudanophilic inclusions.

### Cytoplasm

Cytoplasm was observed in both thin sections (Fig. 26) and freeze-etched preparations (Fig. 33) to contain granules (approx. 15  $\mu$ . in diameter) which were tentatively identified as ribosomes by comparison with particles of a similar size which have been observed in many other microorganisms. Kawata and Inoue (1965) have described particles 15  $\mu$ . in diameter as ribosomes in N. asteroides while Schaefer and Lewis (1965) have described particles 30  $\mu$ . in diameter as ribosomes and polyribosomes in Mycobacterium kansasii. There appeared to be more ribosomes (15  $\mu$ .) in N. corallina cells from cultures which had just reached full growth as distinct from cultures which had been subjected to periods of endogenous incubation. There were however considerable variations in the numbers of ribosomes in cells which had been starved for any period and in some cells no ribosomes appeared, e.g. the cell shown in Fig. 25. In this case, the cell could in fact be non viable. In many sections there appeared to be a band of cytoplasm just inside the cell membrane in which no ribosomes occurred (Fig. 26).

Granules of approximately 75  $\mu$ . in diameter occurred in the cytoplasm of log phase cells (Fig. 24). These granules, which showed a tendency to aggregate in the terminal positions of the cells, were not observed in late stationary phase cells (Fig. 25) nor were they observed in cells resuspended in phosphate buffer for 24 hr. (Fig. 26). It was considered possible that these granules might be polyribosomes and their occurrence in the growing tip of the cell could support this proposition. Certainly their appearance was very similar to that described for ribosomes by Schaefer and Lewis (1965). However there is an alternative explanation concerning the nature of these granules (75  $\mu$ . in diameter)(Fig. 24). Holme and Palmstierna (1956a, 1956b) and Cedergren and Holme (1959) in a study of thin sections of E. coli B found that the cytoplasm of glycogen-rich cells included many particles which they described as glycogen granules (50 to 100  $\mu$ . in diameter). Polysaccharide estimations on cells harvested from cultures of N. corallina at full growth (Chapter 7) showed that this organism contained a polysaccharide which could represent in the order of 20% of the total cell dry wt. The polysaccharide was rapidly degraded soon after full growth had been reached and it is possible that the dark granules (Fig. 24) could correspond to this polysaccharide. If this alternative interpretation is correct the reason for the association of the granules with the growing tip poses an interesting problem.

#### Polyphosphate Granules

Terminal or polar bodies observed in negatively stained preparations of N. corallina (Fig. 18, 19, 20, 21) were assumed to be polyphosphate granules. Such granules have been frequently reported in members of the Order Actinomycetales. The granules in N. corallina showed a marked tendency to increase in size with cell age. While log phase

cells showed polar bodies of approximately 170  $\mu$ . in diameter, cells which had respired endogenously for 7 days showed granules of up to 315  $\mu$ . in diameter. This increase in size occurred in both resuspended and non-resuspended cells during starvation. However in log phase cells there tended to be more than one polar body per cell (Fig. 19). Formation of the bodies in N. corallina (Fig. 19) seemed to occur simultaneously with, or soon after, septum formation; they were frequently observed on each side of a newly formed septum. Polar bodies appeared as small protrusions in the terminal regions of shadowed cells (Fig. 22) and they were sometimes observed as bead like structures in freeze-etched preparations (Fig. 33). In this case the polar body has been lifted out of the cytoplasm to leave a rounded depression. With thin sections the polar body was sometimes observed to have fragmented or to have been pushed out by the knife to leave a hole in the section (Fig. 25).

Polyphosphate granules have been isolated from N. leishmannii (Arai, Kuroda and Koyama, 1961) and they have been reported in the growing tips of N. rubra by Adams and McLung (1962). In 1957 Hagedorn reported the presence of opaque bodies in N. corallina while Winkler in 1953 noted the existence of polyphosphate bodies in granules in species of *Corynebacterium* and *Mycobacterium*. Winkler reported that the bodies were small at the beginning of the exponential phase, being only visible by electron microscopy, but towards the end of the exponential phase they became visible in the light microscope and further increased in size during the stationary phase.

One can but speculate whether a formed polyphosphate granule moves forward with the growing tip of the cell or remains in the same position relative to the cell wall.

### Nuclear Material

Nuclear material has been observed as an electron transparent area occupying a central portion of the cytoplasm (Fig. 26, 27). The area included a sparse network of fibrils of between 2 and 5  $\mu$ . in diameter. These observations were similar to those reported by Kawata and Inoue (1965) for N. asteroides. By contrast Hagedorn (1959b) reported nuclear fibrils of 10 to 12  $\mu$ . in diameter for N. corallina. The differences are probably due to the different methods used in preparing the cell sections. Fig. 27 shows a relatively thick section in which some nuclear fibrils occur which are approximately 8  $\mu$ . in diameter. No obvious differences were noted in the nuclear areas of cells of different sizes or ages. Intracytoplasmic membranes were sometimes observed to penetrate deeply into the nuclear areas (Fig. 26).

### Cell Division

Cell division during log phase growth was initiated by formation of a septum which formed as a ring on the inside of the cell wall and grew inwards (Fig. 27). Each septum consisted of two adjacent layers of cell wall material bounded by cytoplasmic membranes. The cell wall layers constituting the septum (Fig. 27) occasionally showed small granular inclusions. From a study of Figures 20 and 27 it appeared that a cytoplasmic membrane system might form across the cell prior to the formation of the septum. However Figure 26 and many other micrographs showed a partially formed septum consisting of two membrane layers separated by two cell wall layers, with no continuation of the membrane layers into a vesicular system (Fig. 27). Serial sectioning is required to give a more complete picture of the relationship

of the intracytoplasmic membrane system to septum formation. In no micrographs of thin sections were septa observed which consisted only of parallel membranes with no intervening cell wall material. However in Fig. 19 a whole cell (negative stained) can be observed with a division line which corresponds approximately to the thickness of two membranes.

The separation of daughter cells at a newly-formed septum seemed to be preceded by a phase in which an electron transparent region appeared between the opposing cell walls constituting the septum (Fig. 26). This phase may correspond to a period of enzyme breakdown of cell wall constituents.

Septa were generally formed towards the centre of cells ( $4 \mu$ . in length) and between nuclear areas. However, in agreement with the findings of Hagedorn (1959b) there was no indication that nuclear division and cell division were coupled functions. In some cases, the formation of more than one septum could be observed in a single cell, and it was quite common to find the two septa within 200  $m\mu$ . of each other (Fig. 26). A form of division resembling constriction was occasionally observed (Fig. 25). In this case it was considered possible that the phase of cell wall degradation which preceded the separation of the cells may have overtaken the first phase of septum formation. Possibly the formation of septa is more dependent on the supply of nutrients than is the subsequent separation of the cells.

#### Summary

From an investigation of the ultrastructure of N. corallina grown on liquid media with continual agitation it was shown that the organism did not develop the characteristic hyphal system which had

been observed on agar plates. By contrast, the organism showed continuing division into coccoid and bacillary cell forms covering a wide range of cell sizes and shapes. Cell division occurred by septum formation which was demonstrated to be associated in most cases with the formation of an extensive intracytoplasmic membrane system. Some dissolution of the septum seemed to occur prior to separation of the daughter cells. Some cells or parts of cells were found to be covered by a fibrous material which seemed distinct from the cell wall. This fibrous material was not firmly bound to the cells. Polyphosphate granules, ribosomes and either polyribosomes or glycogen granules were observed in the cytoplasm. The cytoplasmic membrane system was shown to be a triple layered structure which was continuous with the intracytoplasmic membrane systems. Freeze-etching techniques provided information concerning the granular nature of the various parts of the cells; cytoplasm, cell membrane and cell wall.

Changes which could occur with increasing cell age were largely deductions from impressions. The extensive pleomorphism of the organism and the presence of almost every type of cellular feature in every cell preparation irrespective of culture age made it difficult to define exact correlations between age and morphology. Polyphosphate granules increased in size during aging but there were fewer granules in each cell. There appears little doubt that fibre formation is a characteristic of ageing cells or at least of the older regions of cells and it appears that cell walls thicken with increasing age. The presence of polyribosome (or glycogen) granules certainly seems to be a characteristic of growing cells.



Figure 18

Negative-stained preparation of whole cells incubated for 7 days after full growth at 30° with shaking (140 rev./min.) in clefted flasks. Negative staining was carried out using 0.3% P.T.A. The cells show typical variations in size and shape. The small particles are latex spheres (264 mμ.). Magnification: x 5,250.

Figure 19

Negative-stained preparation of whole cells from a culture in the mid-log phase (clefted flask) 30 hr. after inoculation. Negative staining was carried out using 0.2% P.T.A. after dialysis against distilled water. Cells show polar bodies, septa and fibrous outer coat material. Zones of cells covered with fibrous material tend to be thicker than non fibrous regions (growing tips). Magnification: x25, 000.

Figure 18



Figure 19





Figure 20

Negative-stained preparation of whole cells from a culture in the mid-log phase (cleated flask) 24 hr. after inoculation. Negative staining was carried out using 0.3% P.T.A. The cells show considerable shrinkage. Polar bodies and intracytoplasmic membranes are visible. Cells appear electron transparent and lack outer coat material. Magnification: x 25,000.

Figure 21

Negative-stained preparation of whole cells from a culture (cleated flask) incubated at 30° and 140 rev./min. for 5 days after reaching full growth. Negative staining was carried out using 0.3% P.T.A. Large polar bodies are clearly seen in the pointed tips of the cells and the cells are heavily coated with fibrous material. Magnification: x25,000.

Figure 20

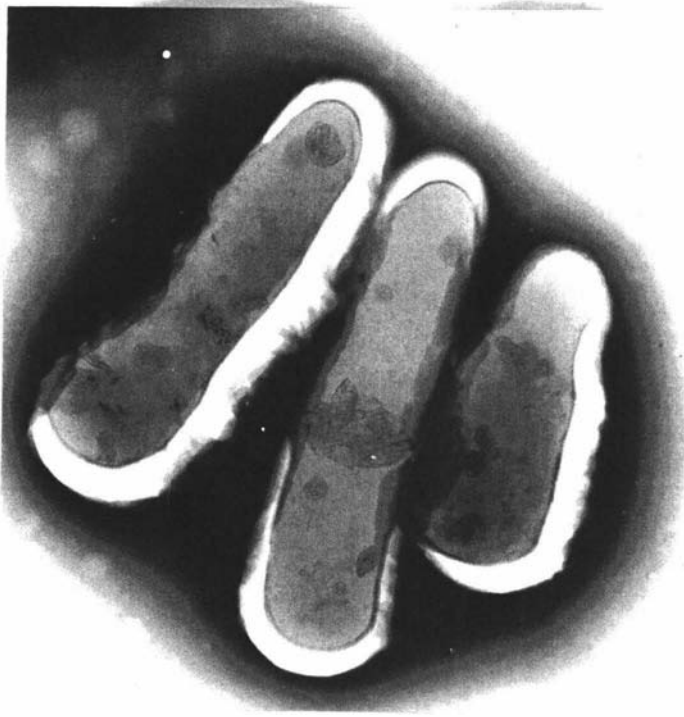


Figure 21

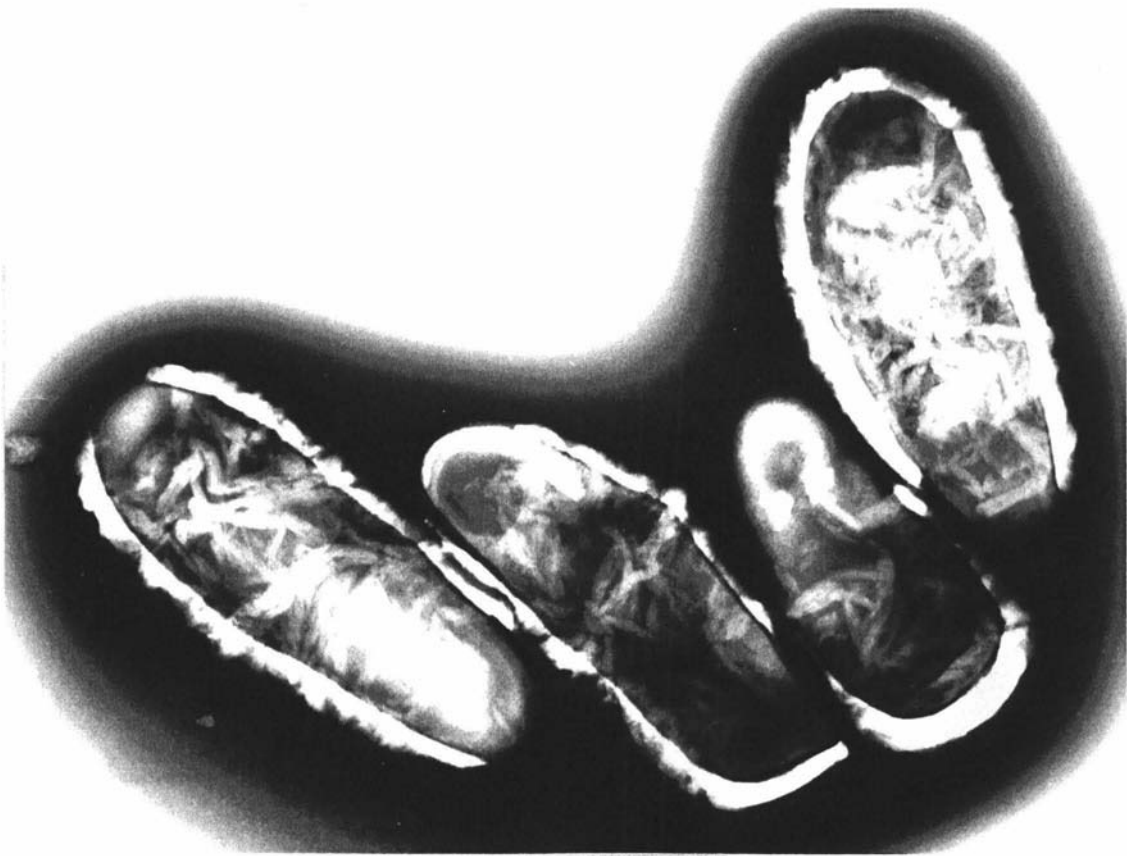




Figure 22

Shadowed preparation of cells from a culture left to incubate for 7 days after reaching full growth (cleated flask,  $30^{\circ}$ , 140 rev./min.). A thin-section preparation of cells from the same culture is presented in Fig. 25. The outer coat material occurs as a loose cover. A polar body occurs as a pellet in the terminal region of one cell. Magnification: x 21,000.

Figure 23

Shadowed preparation of cells from a culture grown in acetate medium and left to incubate for 24 hr. after reaching full growth (cleated flask,  $30^{\circ}$ , 140 rev./min.). Samples of cells were suspended in distilled water and heated at  $100^{\circ}$  for 20 min. prior to shadowing. Outer coat material appears to have separated from the cells in long coiled fibres. Magnification: x 64,000.

Figure 22

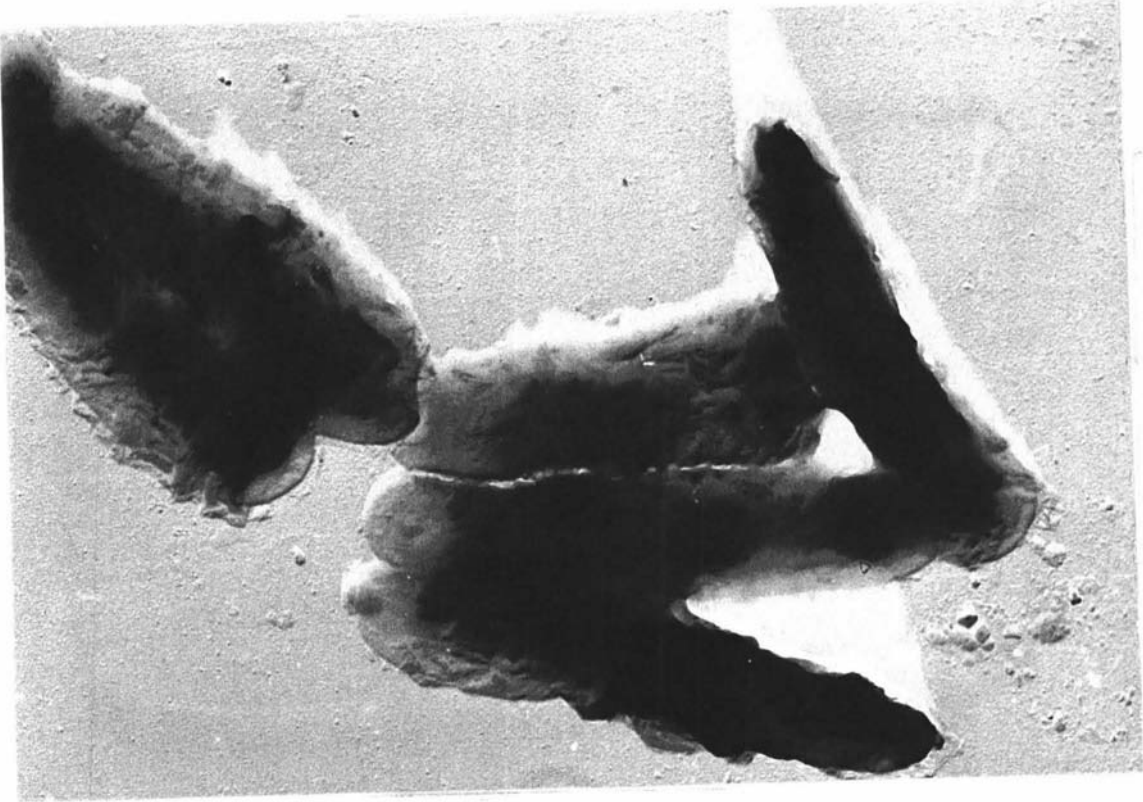


Figure 23

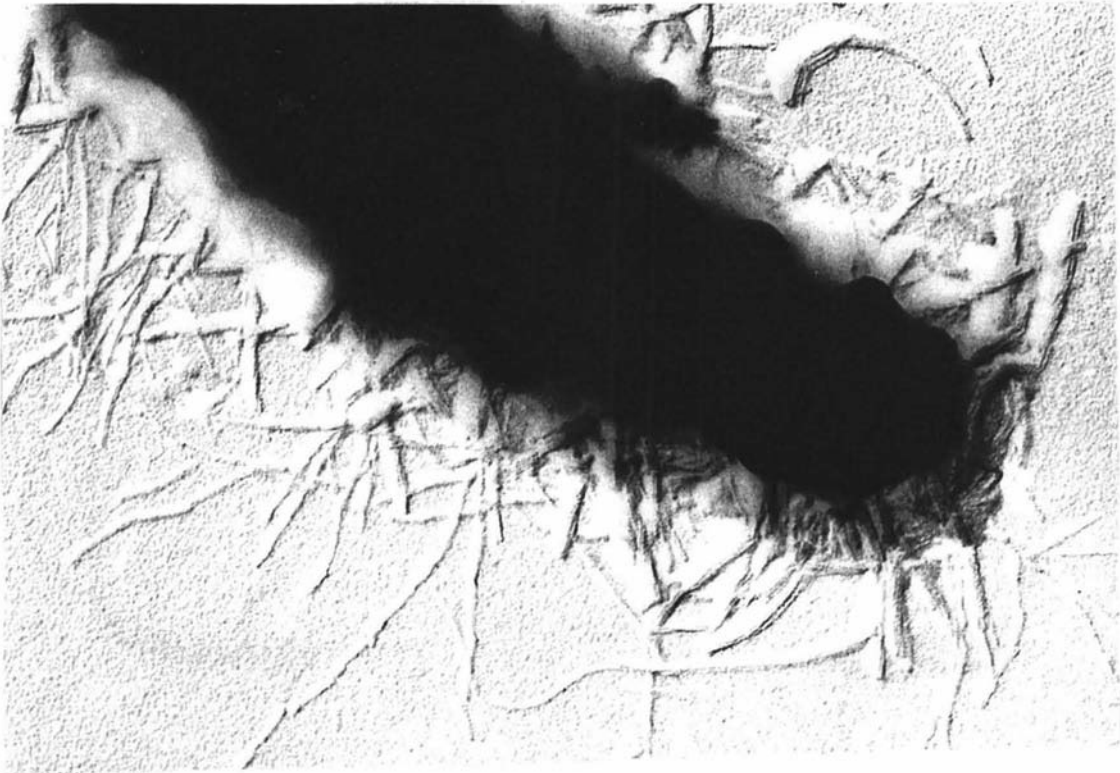




Figure 24

Longitudinal section through two cells divided by a septum. Cells were taken from a log phase culture (cleated flask) and fixed, prior to sectioning, in 3% glutaraldehyde and 2% OsO<sub>4</sub>. The cell wall is relatively thin compared with walls in cells from cultures subjected to periods of endogenous incubation (Fig. 25). Nuclear areas and granules (polyribosomes or glycogen granules) occur. Magnification: x 64,000.

Figure 25

Section through cells from a culture incubated for 7 days after reaching full growth (cleated flask, 30°, 140 rev./min.). Cells were fixed prior to sectioning, in 3% glutaraldehyde and 2% OsO<sub>4</sub>. A shadowed preparation of cells from the same culture is presented in Fig. 22. A diffuse region borders the cells. Below this there is an electron dense zone. In some cases a lighter layer occurs between the dense zone and the cell membrane. The membrane can be clearly seen as a trilayered structure. A mode of cell division suggesting constriction and a vesicular intracytoplasmic membrane system occur. Magnification: x 64,000.

Figure 24

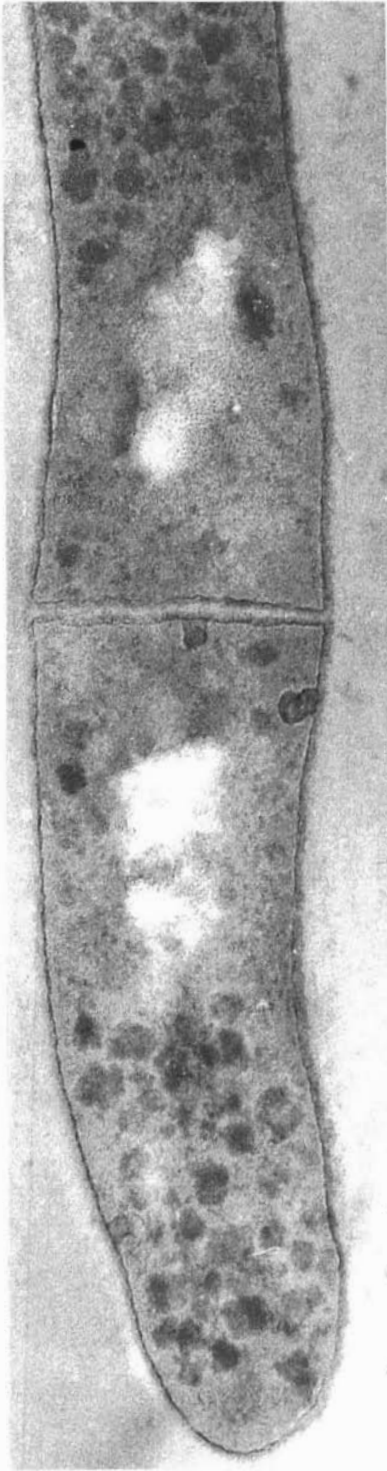


Figure 25

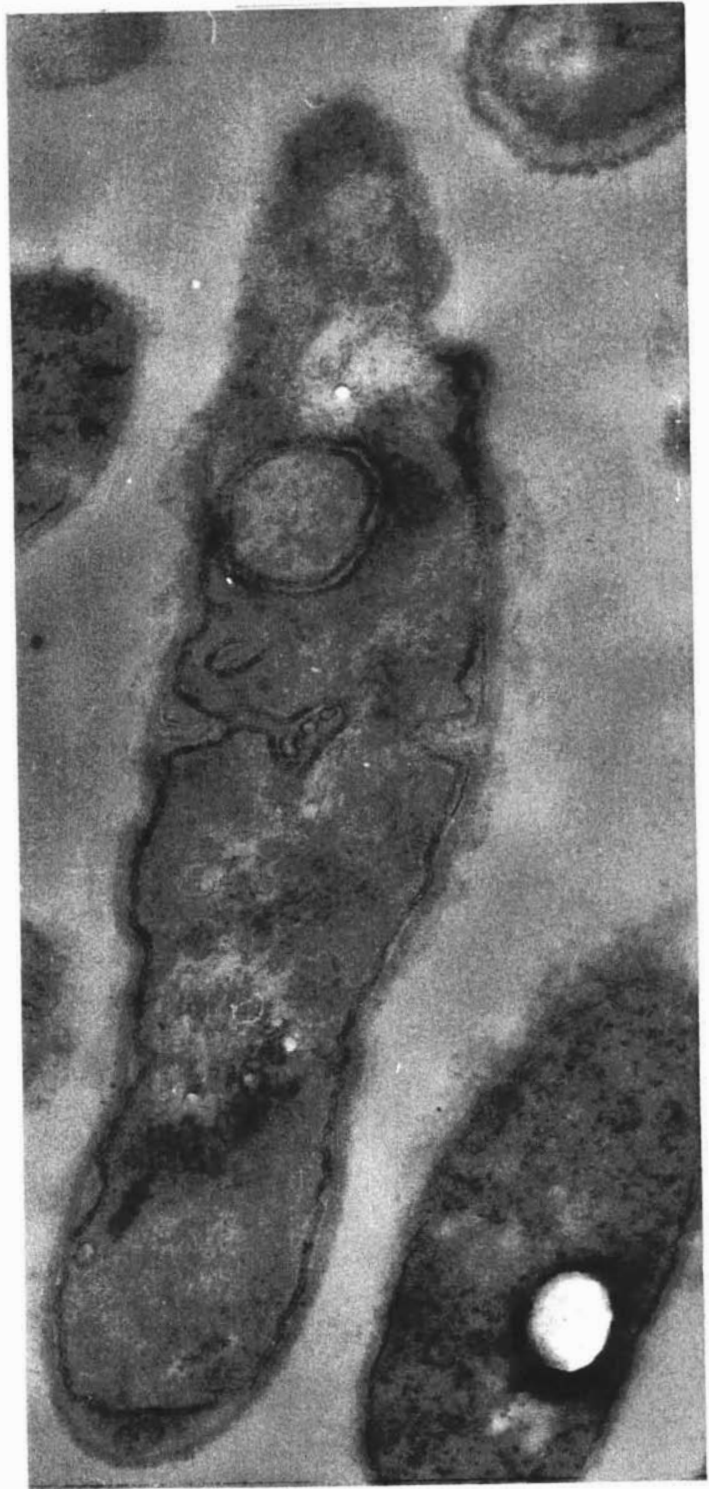


Fig. 26, 27

Figure 26

Section through cells from a suspension in phosphate buffer. The suspension was incubated for 24 hr. (cleated flask, 30°, 140 rev./min.) after resuspension of a culture (cleated flask) which had reached full growth. Cells were fixed with 1% glutaraldehyde and 1% OsO<sub>4</sub>. The small dark granules are possibly ribosomes. The nuclear area occurs as an electron transparent region filled with fibrils. Magnification: x 64,000.

Figure 27

Longitudinal section through a cell from a culture 24 hr. after full growth had been reached (cleated flask, 30°, 140 rev./min.). Cells were fixed with 3% glutaraldehyde and 2% OsO<sub>4</sub>. Probable structures include ribosomes, a partially formed septum, intracytoplasmic membranes and a nuclear region. Magnification: x 116,000.

Figure 26



Figure 27

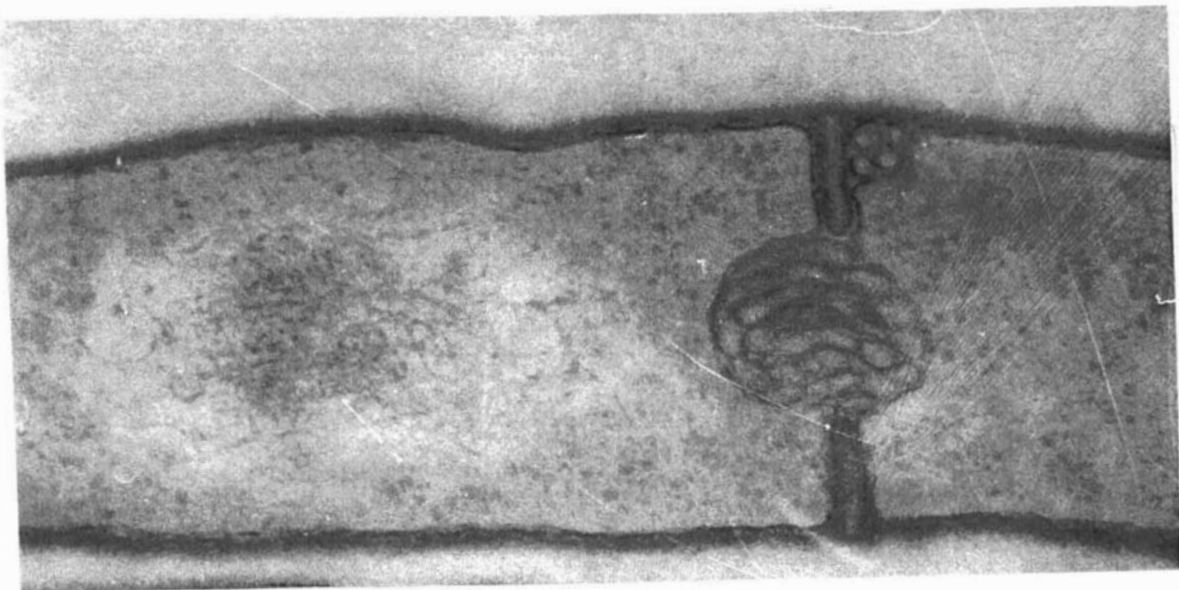




Figure 28

Freeze-etched preparation showing the outside of a cell from a mid-log phase culture (cleated flask, 30<sup>o</sup>, 140 rev./min.). The granular surface is considered to be the outside surface of the cell wall, while the striated material is seen as corresponding to the fibrous material or cell coat. Magnification: x 64,000.

Figure 29

Freeze-etched preparation of the tip of a cell from a culture left to incubate for 10 days (cleated flask, 30<sup>o</sup>, 140 rev./min.) after reaching full growth. The smooth surface partially covered with plaques could represent an area in the membrane which has split between the lipid monolayers. The plaques could correspond to pieces of one monolayer sticking to the surface of the other. The particulate regions could represent the outer surface of the membrane. Magnification: x 85,000.

Figure 30

Freeze-etched preparation showing a cross section of a cell from a culture left to incubate for 10 days after reaching full growth (same preparation as in Fig. 29). The cytoplasm appears as a highly granular structure and the cell wall appears to contain embedded particles. Magnification: x 85,000.

Figure 28

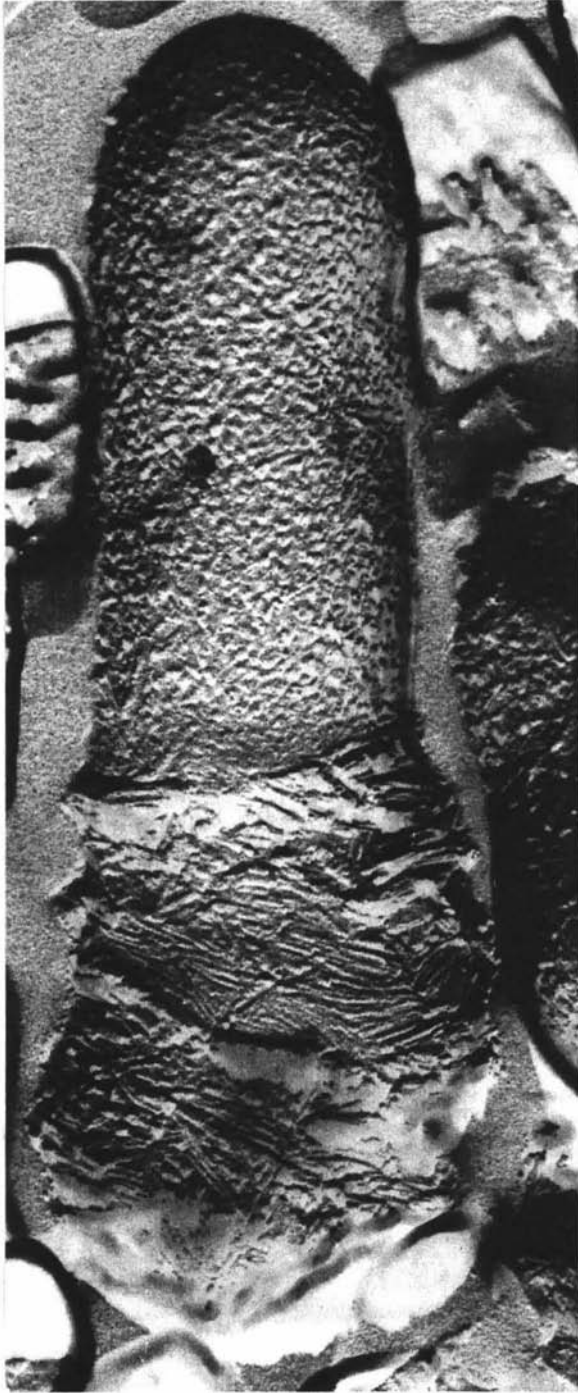


Figure 29

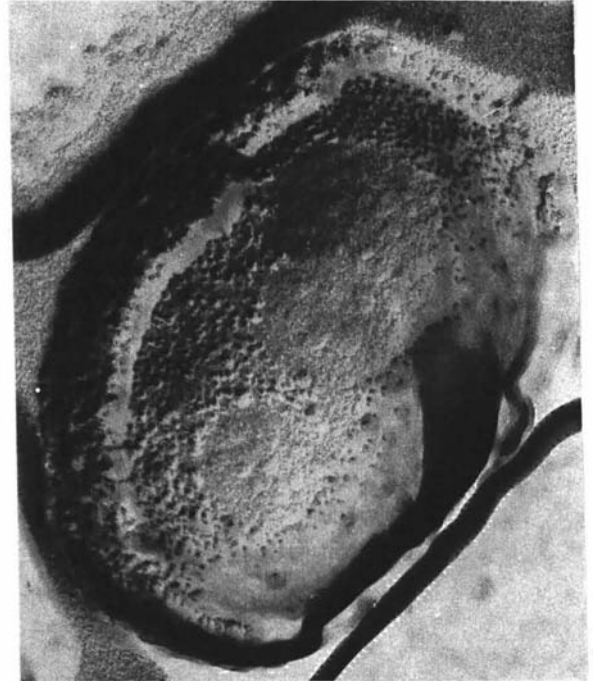


Figure 30

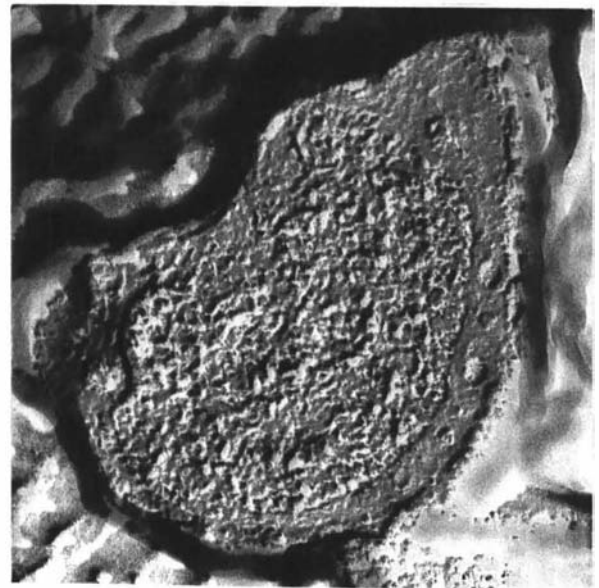


Fig. 31, 32, 33

Fig. 31, 32, 33

Figure 31

Freeze-etched preparation of a cell from a log phase culture (same preparation as for Fig. 28). The cell has broken at one end in longitudinal section to reveal the granular cytoplasm. The small inclusions embedded in the cytoplasm are considered to correspond to intracytoplasmic membrane systems. The outside surface of the cell wall is granular with a suggestion of fibre formation. Magnification: x 64,000.

Figure 32

Freeze-etched preparation of a cell from a culture left to incubate for 10 days after reaching full growth (same preparation as for Fig. 29). The cell wall has been left as a hollow cast. The particulate layer is interpreted as corresponding to the inside surface of the cell membrane. The plaque covered region could represent an area in the membrane which has split between lipid monolayers leaving small pieces of one monolayer sticking to the surface of the other. Magnification: x 64,000.

Figure 33

Freeze-etched preparation of a cell from a culture left to incubate for 10 days after reaching full growth (same preparation as for Fig. 29). The cell has split to leave a boat-like structure. The round pit has been left by the removal of a polar body (polyphosphate granule). Magnification: x 64,000.

Figure 31

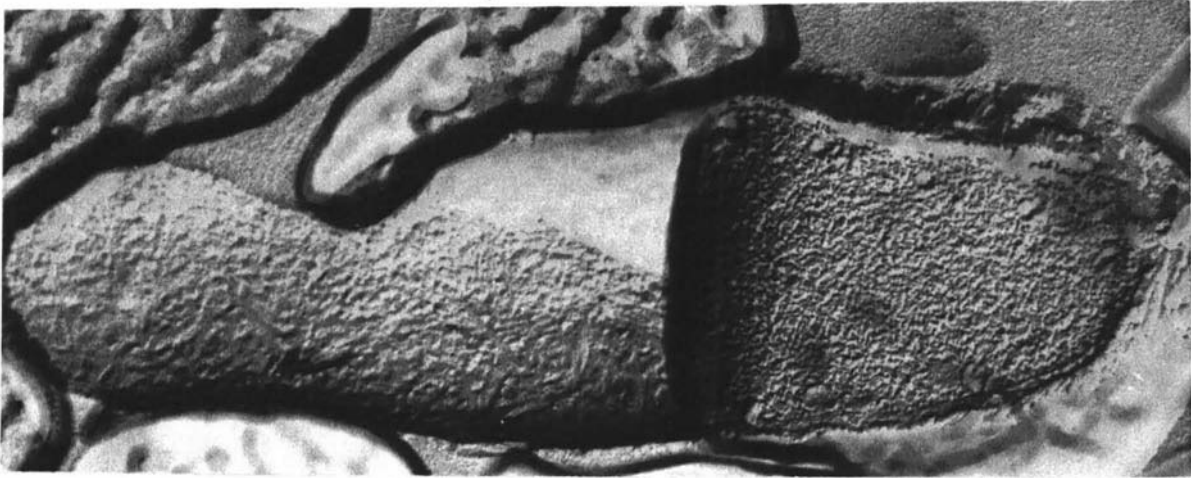


Figure 32

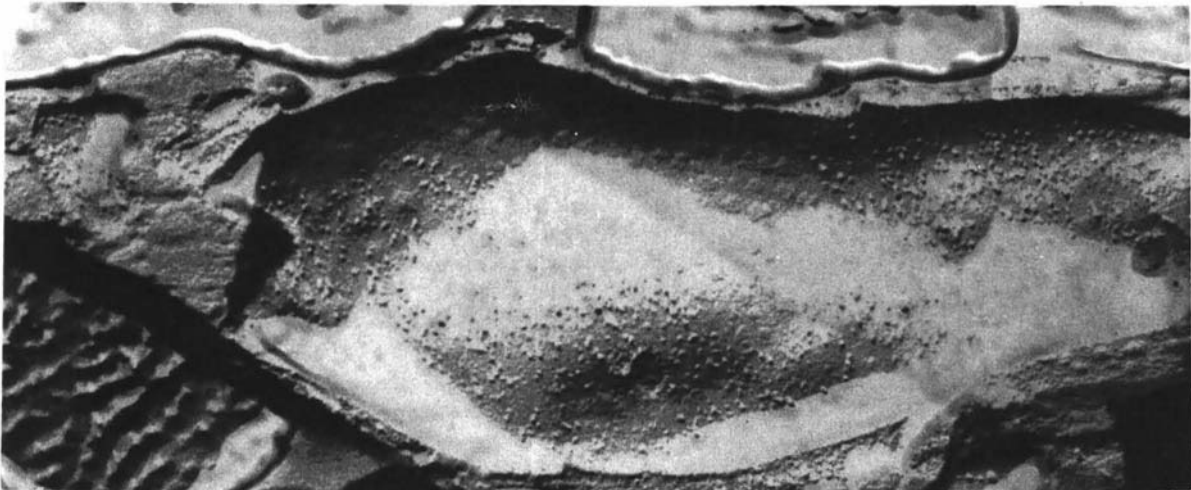
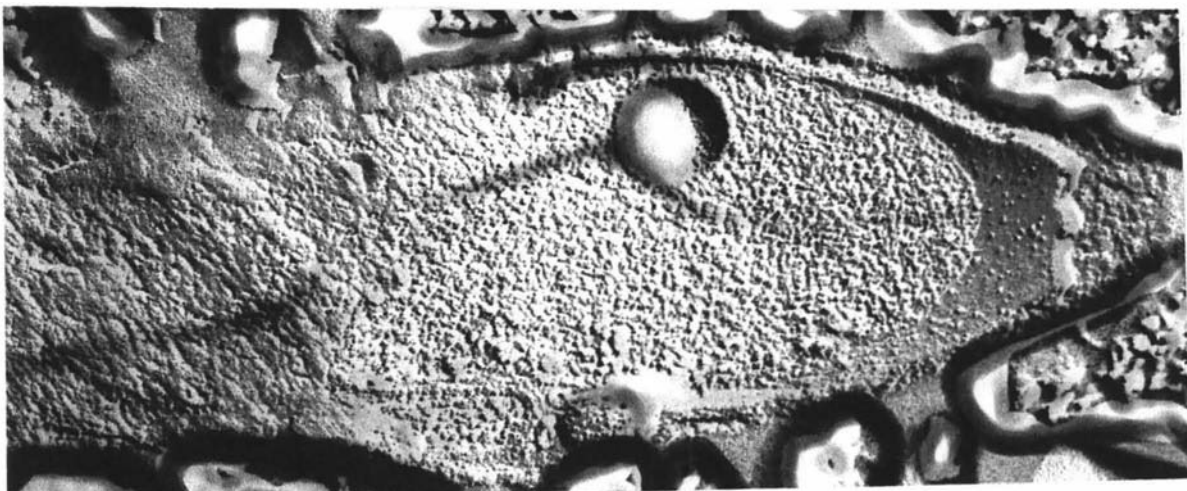


Figure 33



SECTION III

DISCUSSION

## DISCUSSION

The present study of the endogenous metabolism of N. corallina was aimed at a detailed examination of lipid catabolism and its relation to the survival capacity of the organism. This approach was based on findings from a previous investigation which "indicated that lipid and protein catabolism each contribute energy for maintenance in the absence of exogenous substrate" (Midwinter, 1962).

Following initial studies defining the conditions of growth and resuspension the project was developed in two related sections. Firstly, levels of total lipids and total fatty acids were examined in relation to changes in cell viability, and secondly structural analyses of lipids, considered to be important as endogenous reserves, were carried out. As these studies developed, it became clear, that for the conditions of growth and resuspension chosen, the fall in total lipids or total fatty acids, did not make a large contribution to the total fall in cell dry wt. which occurred during periods of endogenous metabolism. These results prompted an examination of the changes in levels of other major intracellular components. The electron microscope study, originally intended to centre on cellular lipids, developed into an intensive study of the ultrastructure of the organism with an emphasis on morphological changes during growth and starvation.

Growth Characteristics of N. corallina

Growth of N. corallina on agar slides was characterised by a period of hyphal development, (hyphae 8 - 12  $\mu$  in length, Fig. 3)

followed by fragmentation yielding coccoid and bacillary forms of the organism (2 - 4  $\mu$ . in length). In a similar examination of N. corallina, Brown and Clark (1966) described the development of hyphae up to 16  $\mu$ . in length prior to fragmentation.

Hyphae did not form when the organism was grown in liquid medium under conditions of continual agitation. Instead, coccoid cells approximately 2  $\mu$ . in length grew by elongation at both ends to form cells approximately 4  $\mu$ . in length which formed septa and subsequently divided (Fig. 24). These findings confirm the report of Clark and Frady (1957) that bacillary cells of N. corallina in liquid culture, divide in a manner similar to binary fission.

In order to reduce the experimental problems associated with excessive clumping of N. corallina grown in liquid medium Clark (1958) subjected cultures to grinding with glass beads and then removed the residual clumps by centrifugation. In the present study it was considered that this procedure would be too drastic for the type of physiological examination undertaken. In studies aimed to improve the aeration efficiency of culture flasks, it was found that the insertion of cleats in the lower walls of the flasks reduced clumping by, in effect, providing continuous homogenising conditions. (At the same time the cleats greatly improved aeration efficiency. Aeration efficiency was further improved by the use of milk filters instead of cotton wool bungs as flask closures.) The reasons for clumping were not ascertained but presumably it was due to a property of some component of the cell wall or outer coating. The use of detergents (Teepol and Tween 80) further reduced the size of clumps to a point where the majority of organisms occurred in suspension as clusters of one, two or three cells. Total and viable counts on these suspensions gave

reproducible results although viable counts gave estimates of the cluster viability only. The individual cell viability was determined from the cluster viability and the cluster size distribution using formulae, the derivation of which does not appear to have been previously reported (Appendix 2).

### Lipid Studies

Previous studies (Midwinter, 1962; Stephenson and Whetham, 1922) indicated that N. corallina and M. phlei utilised lipids when starved. To obtain more information about the lipids of N. corallina, extraction and characterisation procedures were extended from those previously described (Midwinter, 1962; Robertson, 1964).

The extraction of freeze-dried N. corallina with chloroform-methanol gave a total unbound lipid content of approximately 15% of the cell dry wt. When these lipids were hydrolysed, trehalose, mannose, inositol and glycerol were detected as water soluble components. These compounds have been previously detected as hydrolysis products of other species of Actinomycetes (Asselineau, 1966).

The assumption that triglycerides occurred in the neutral lipid fraction obtained by silicic acid column chromatography of the total unbound lipids (Batt, unpublished results; Robertson, 1964) was examined further. Total lipids were fractionated by silicic acid column and thin-layer chromatography to yield a triglyceride fraction. Alkaline hydrolysis of the triglyceride gave glycerol as the only water soluble component. Analysis of the triglyceride fatty acids by gas liquid chromatography indicated the presence of saturated and unsaturated acids in the ratio of approximately 2:1. It is possible that the triglycerides of this organism may have been formed with an unsaturated acid in the  $\beta$

position of the glyceride molecule.

Previous studies (Batt, unpublished results; Robertson, 1964) demonstrated that N. corallina assimilated long chain fatty acids into the neutral lipid fraction. In the present study experiments carried out using U-<sup>14</sup>C palmitate showed that a large portion of the palmitic acid was incorporated into the triglyceride fraction. The assimilation of fatty acids has previously been noted for species of Actinomycetes (Oginsky, Smith and Solotorovsky, 1950; Lindsay, O'Donnell and Edson, 1950). However, there appear to be no reports of a preferential incorporation into triglycerides. This ability to assimilate lipophilic substances may have some significance under natural conditions. Turfitt (1944) has shown that species of Nocardia are predominantly responsible for the degradation of sterols in soils and Raymond and Davis (1960) have demonstrated the ability of these organisms to degrade hydrocarbons.

Evidence from a thin-layer chromatography examination of the total lipids suggested that triglycerides made up only a small proportion of the total lipids in cells grown on glucose medium, therefore precluding the possibility that these compounds functioned as a major carbon reserve during starvation. Midwinter (1962) in obtaining evidence for the importance of lipids during endogenous metabolism studied changes in the levels of total fatty acids obtained in diethyl ether extracts of acid-hydrolysed cells. Attention was focused, therefore, on the levels of total fatty acids in starved cells and on the composition of the material in the diethyl ether extract. A thin-layer chromatography study of the diethyl ether extract of acid- or alkali-hydrolysed cells showed the presence of three major fatty acid groups. The least polar group

corresponded to acids of chain lengths  $C_{10} - C_{20}$ , a more polar group corresponded to the nocardic acids while the third very polar and relatively minor group was not studied.

The presence of nocardic acids in M. rhodochrous (N. corallina see Introduction) had previously been reported by Laneele, Asselineau and Castelnuovo (1965). These authors suggested that the identification of  $\alpha$ -branched  $\beta$ -hydroxy acids (nocardic or nocardomycolic acids) of carbon number  $C_{50}$  pointed to M. rhodochrous being more closely related to the Nocardia which contain  $C_{50}$  nocardomycolic acid than to the Mycobacterium which contain  $C_{80}$  mycolic acids. In recent papers, Gordon (1966) suggested that N. corallina, including the species used in the present study should be renamed M. rhodochrous.

Mass spectrometric analysis (Table 6) of the fractions obtained by gas chromatography of derivatives of nocardic acids from N. corallina showed that the acids consisted of a mixture ranging from  $C_{38} - C_{48}$ . This result confirms that N. corallina is not closely related to the Mycobacterium on the basis of the structure of the nocardic acids. However it suggests that the organism is closely related to the Corynebacterium which characteristically contain  $C_{32}$  Corynomycolic acids.

#### Changes in Viability, Total Counts, Dry Weight and Respiratory Activity in Starved Cell Suspensions

Before interpretations could be made of the relative importance of any cellular components during endogenous respiration it was necessary to define the survival capacity of the organism. The viability of N. corallina starved at  $30^{\circ}$  in phosphate buffer (containing magnesium ions) fell from 100% to 90% over a 5 - 10 day period. After a period of 20 days incubation the viability had reached 50% (Fig. 12). These

results are reasonably consistent with those reported by Webb and Clark (1957) who showed that germination of N. corallina on the surface of nutrient agar approached 100% even from cultures which were several weeks old.

During the first 48 hr. of starvation there was a fall of 30 - 40% in cell dry wt. (Fig. 11) with a subsequent small but continuous decrease in dry wt. The viability of the organism was not affected by this change in the rate of breakdown of cellular material (Fig. 11). The initial fall in dry wt. corresponded to a period of high respiratory activity which declined over a period of 36 hr. to a basal  $O_2$  uptake of 1  $\mu$ l./mg./hr. (Table 12). Respiratory quotients, estimated at intervals after resuspending the cells, were approximately 1.1 and at no time were values near 0.7 recorded. These results are in contrast to those of Midwinter (1962) who reported that after resuspending N. corallina cells in phosphate buffer, the RQ values decreased from approximately 1.0 to 0.72.

During the first 5 days of starvation a 13% increase in the total cell count was observed (Fig. 11) due possibly to continuing cell division. A similar increase in numbers in starved suspensions was reported by Schaechter (1961) and Dean (1967). An alternative explanation of the increase in total count could be an appreciable fragmentation of organisms in which septa had formed prior to resuspension. This has been observed to occur with batch cultures of N. corallina by Webb and Clark (1957) and with N. erythropolis by Adams (1965).

The observation that the total cell count increased on starvation (Fig. 11) indicated that complete cell lysis was probably not prominent during these experiments. In electron microscope studies, cells devoid of cytoplasm were very rarely observed even in cultures several weeks old.

Changes in Levels of Intracellular Components During Endogenous Metabolism

The demonstration of a sharp fall in dry wt. of cells during the first phase of endogenous respiration focussed attention on quantitative changes in cell constituents.

N. corallina harvested at full growth, contained 20 - 25% carbohydrate estimated as total hexose. The value for total reducing sugar was higher and represented a carbohydrate content of approximately 35% of the cell dry wt. During the initial period of starvation, both total hexose and reducing sugar values decreased to 5 - 10% of the cell dry wt. The fall in total hexose accounted for approximately 40% of the total fall in cell dry wt. Webley, Duff and Anderson (1962) reported that a reserve polysaccharide (probably glycogen) was stored and subsequently degraded in the late log phase by N. opaca. Midwinter (1962) concluded that polysaccharide was not degraded during starvation of N. corallina although carbohydrate (as total reducing sugar) was found to represent up to 20% of the cell dry wt. This was in contrast to an earlier report (Midwinter and Batt, 1960) that carbohydrate was probably a nutrient reserve in this organism, a conclusion which has been confirmed experimentally in the present study. It is not clear why polysaccharide accumulates during the growth of N. corallina. Intracellular polysaccharides generally accumulate under conditions where growth is limited in the presence of carbon-excess; for example when nitrogen, phosphorus or sulphur nutrients are limiting (Wilkinson and Munro, 1967). None of these substances were limiting in the growth medium used. Although thiamine was included in the medium as a growth stimulant (Martin and Batt, 1957) it is quite possible that the absence of other factors, such as amino acids might limit the

rate of growth. The effect of the concentration of inorganic ions in the growth medium used and the lack of trace elements must also be considered. The effect of growth stimulating substances on the accumulation of polysaccharide in N. corallina could form a basis for future study.

During the phase in which cellular carbohydrate was degraded rapidly no ammonia was released from the cells (Fig. 16, 17) but subsequently ammonia was released at an almost constant rate for many days. A similar result reported for starved suspensions of E. coli (Dawes and Ribbons, 1965) was interpreted as showing that carbohydrate catabolism prevents ammonia release by promoting resynthesis. The present data supports this interpretation since free intracellular nitrogenous compounds (ammonia and amino acids) fell sharply on starvation (Fig. 17) without any concomittant rise in extracellular ammonia or amino acids. A similar fall in intracellular amino acids without release of these compounds was observed by Burleigh and Dawes (1967) with starved suspensions of Sarcina lutea.

Following the breakdown of cellular carbohydrate, protein and RNA were degraded. For protein, the rate of degradation seemed to take place at a fairly constant rate (Fig. 15, 16). In this connection Bardi and Boretti (1958) have demonstrated that protein accounts for most of the dry wt. loss from N. rugosa respiring endogenously.

The yield of ammonia (Table 17, 18) was slightly greater than would be expected if the protein was catabolised to carbon dioxide and ammonia. The discrepancy in equating ammonia produced to protein breakdown could be accounted for from RNA catabolism. In most cases the release of free amino acids from respiring cells was negligible.

Total fatty acids fell from approximately 13% to 9% of the initial bacterial dry wt. over a period of 10 days starvation. Fatty acids accounted for only 5% of the total fall in dry wt. compared with carbohydrates (determined as total hexoses) 40%, protein 25% and RNA 6%. Midwinter (1962) reported that the total fatty acids of glucose-grown N. corallina decreased from 17% to 14% of the cell dry wt. after 33 hr. starvation. The total bacterial dry wt. loss in the same period was not given. However from the oxygen consumed, the lipid degraded and the ammonia excreted Midwinter (1962) concluded that protein and lipid each accounted for approximately 50% of the total endogenous oxygen uptake. In the present study, using the conditions of growth and incubation described, carbohydrate probably accounts for the major portion of the oxygen consumed in the first 24 - 36 hr. Following this period protein appears to be the major endogenous substrate. The conclusion that lipid was not catabolised to any appreciable extent by starved cells is consistent with other studies carried out with Actinomycete species (Bardi and Boretti, 1958; Frouin and Guillaumie, 1928; Cassagne, 1939). However, under conditions where lipids occur in the growth medium, M. kansasii is capable of storing fat and subsequently degrading it under starvation conditions (Schaefer and Lewis, 1965, personal communication). The high rate of assimilation of fatty acids by N. corallina (Robertson, 1964), together with indications from the present study that the proportion of triglycerides in the total lipids increased when cells were incubated with long chain fatty acids, suggests that growth on lipids or lipid precursors could produce cells containing a high proportion of lipid. This lipid could quite possibly be degraded under conditions of starvation. Further studies on the function of

lipids in N. corallina should perhaps be directed towards determining the localization of the lipids within the cells. Possibly the major portion of the total lipid extracted from glucose-grown cells occurs in the cell wall or the cell membrane. It is likely that lipids will prove to play a vital role in the maintenance of the starving cells. However, an accurate measure of the lipid utilized for the supply of maintenance energy will require more sensitive techniques than have been used in the present investigation.

#### Correlation Between Cell Survival and Endogenous Metabolism

From experiments carried out on starved N. corallina it is clear that this organism has a survival capacity considerably greater than that of vegetative forms of other bacteria studied (Table 1). Despite the differences in its ability to survive under starvation conditions, the pattern of endogenous metabolism observed in N. corallina is very similar to the pattern observed in organisms which lose viability more quickly. There is, however, one major region of difference, namely in the rates at which the intracellular components are catabolised. When the results obtained with E. coli (Dawes and Ribbons, 1965), grown in glucose-ammonium sulphate medium, are compared with results for N. corallina marked differences are found in the growth rates, rates of metabolism, and rates of loss of viability. The ratio of these rates for the two organisms are of the same order of magnitude.

Organism	<u>E. coli</u>	<u>N. corallina</u>
Growth rate (approx. doubling time hr.)	1	8
Basal $Q_{O_2}$ ( $\mu$ l./mg./hr.)	7	1
Rate of carbohydrate breakdown ( $\mu$ g./mg. dry wt./hr.)	55	10
Rate of ammonia release ( $\mu$ g./mg. dry wt./hr.)	1.2	0.2

A correlation between slow growth and slow death has been described by Harrison and Lawrence (1963). When Aerobacter aerogenes was shifted from growth to starvation conditions a selection of slow growing mutants appeared. These authors suggested that in natural habitats the selection of slow growing cells during periods of starvation might offset the selection of fast growing cells during periods of rapid proliferation. Comparative studies examining the minimum growth rates (Tempest, Herbert and Phipps, 1967) of different microorganisms could be significant in future studies of the survival capacity of vegetative cells.

Zevenhuizen (1966) has claimed that for Arthrobacter species a direct correlation exists between the slow rate at which intracellular material is utilized during starvation, and prolonged viability. In this case, the rate at which intracellular carbohydrate was utilized

was directly related to the availability of free ammonia. When cells were starved in the absence of carbon or nitrogen substrates the rate of net carbohydrate utilization was dependent on the rate of catabolism of intracellular nitrogenous compounds. The addition of ammonium ions to the starvation system caused rapid breakdown of intracellular carbohydrate and accelerated death. A starved cell containing a high intracellular level of polysaccharide may therefore be compared with a cell growing on an exogenous carbon source in a nitrogen limiting medium.

The rate of net degradation of the cellular constituents of a starving cell will depend primarily on the rates of catabolism of these constituents. Schlessinger and Ben-Hamida (1966) postulated that protein hydrolysis in starved E. coli is controlled by the intracellular level of amino acids or amino-acyl sRNA. The size of the amino acid pool is considered to be related to the rate of protein synthesis which in turn depends on the rate of the availability of glucose. In further studies on the survival of microorganisms more emphasis should perhaps be placed on the control and activity of proteases. For N. corallina at least, it seems possible that a low level of metabolic activity and especially a slow rate of protein catabolism may be important factors in sustaining viability.

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## APPENDIX 1.

1) Liquid culture medium.

$\text{KH}_2\text{PO}_4$	20.41gm.
$(\text{NH}_4)_2\text{SO}_4$	4.50gm.
thiamine HCL (Vit. B <sub>1</sub> )	0.037gm.

were dissolved in approximately 1350 ml. glass distilled water. After neutralisation (pH.7.0<sup>±</sup>0.05) with 5N NaOH

$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	0.15gm.
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previously dissolved in a few ml. of glass distilled water was added. The total solution was adjusted to 1425 ml. and dispensed evenly into 5 conical flasks (1L). fitted with muslin-coated cotton wool plugs or milk filters.

glucose	13.5gm.
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was dissolved in 90ml of glass distilled water in a 250ml. conical flask which was plugged with muslin-coated cotton wool. All reagents were Analytical Grade. All solutions were sterilised by autoclaving (15 min. 121°) After cooling, 15 ml. glucose solution was pipetted aseptically into each conical flask giving a final volume of 300ml. of 0.1M phosphate buffered medium/flask.

2) Phosphate buffer (0.1M) for cell suspensions

$\text{KH}_2\text{PO}_4$	20.41gm.
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was dissolved in glass distilled water (1.4L). To this solution after neutralisation with 5N NaOH was added

$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	0.15gm.
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previously dissolved in a few ml. of glass distilled water. All reagents were Analytical Grade. The final volume was adjusted to 1.5L., evenly dispensed among 5, 300ml. conical flasks which were plugged with muslin-coated cotton wool and autoclaved. (15 min. 121°)

3) Osmotic characteristics of culture medium and phosphate buffer.

The osmotic characteristics of the liquid medium and phosphate buffer were determined using a Fiske Osmometer (Model G62)

Total medium	315 mOsm./Kg.	distilled water
Total medium (excluding glucose)	269	"
Phosphate buffer (0.1M)	214	"
NaCl solution (0.9% W/V)	285	"

4) Agar media for slopes and slide cultures.

$\text{KH}_2\text{PO}_4$	3.40 gm.
$(\text{NH}_4)_2\text{SO}_4$	3.00 gm.
Oxoid yeast extract	0.50 gm.
thiamine (Vit. B <sub>1</sub> )	0.025 gm.
glucose	7.5 gm.

were dissolved in approximately 900ml. of glass distilled water and neutralised with 5N NaOH

$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	0.10 gm.
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was dissolved in a few ml. glass distilled water and added. The final volume was adjusted to 1 L. and divided equally between two conical flasks (1 L.)

Agar (Davis)	10.0 gm.
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was added to each of the flasks. The preparation was heated, and prior to autoclaving, was poured into McCartney bottles for slopes, or into 12cm. x 1.5 cm. test tubes (cotton wool bungs) for slide cultures.

APPENDIX 2.

Derivation of Formulae for Estimating Cell Viability from Cluster Viability and Cluster Size Distribution.

Aqueous suspensions of bacteria often contain cell aggregates of various sizes in addition to single cells. Viable counts carried out using these suspensions give an estimate of cluster viability, where a cluster is defined as a single cell, or any aggregate of cells. Assuming that viable cells are distributed randomly through clusters, it is possible to obtain a mathematical expression which gives an estimate of the cell viability from figures for cluster viability and cluster size distribution. The following was derived by Dr. N.A. Doughty.

If  $m_1, m_2, \dots, m_{j_{\max}}$  are the numbers of clusters with 1, 2, ...  $j_{\max}$  cells observed in a grid on an agar slide on which no bacterial growth has occurred, then the proportion of clusters with  $j$  cells can be expressed in a normalised form

$$n_j = \frac{m_j}{\sum_{j=1}^{j_{\max}} m_j} \dots\dots\dots(1)$$

and the normalised distribution can be expressed as  $n_1, n_2, \dots, n_{j_{\max}}$  where

$$\sum_{j=1}^{j_{\max}} n_j = 1 \dots\dots\dots(2)$$

The cluster viability ratio for a single grid is determined after bacterial growth has occurred by

$$P = \frac{\text{Number of Live Clusters}}{\text{Total Number of Live and Dead Clusters}} = \frac{N_a}{N_a + N_d}$$

Considering the case in which all clusters are of a single size  $j$  then for this restricted case  $P_j$  is the cluster viability ratio. It is required to find  $p$ , the individual cell viability ratio.

Suppose  $p$  is the probability of one cell staying alive then  $(1 - p)$  is the probability of one cell dying and  $(1 - p)^j$  is the probability of  $j$  cells dying.

Two situations occur with respect to cluster viability.

1) At least one cell stays alive in a cluster and therefore the cluster is viable.

2) All cells in the cluster die. The sum of these probabilities is 1.

$$P_j + (1 - p)^j = 1 \dots\dots\dots(3)$$

This equation is valid for all values of  $j$  from 1 to  $j_{\max}$  and gives  $p$  knowing  $P_j$  and  $j$ .

In the general case where cluster sizes vary,  $P$  will equal the sum of the cluster viabilities for each cluster size weighted by the proportion of clusters of each size.

$$P = \frac{m_1 P_1 + m_2 P_2 \dots m_{j_{\max}} P_{j_{\max}}}{m_1 + m_2 \dots m_{j_{\max}}} \dots\dots\dots(4)$$

$$P = \frac{\sum_{j=1}^{j_{\max}} m_j P_j}{\sum_{j=1}^{j_{\max}} m_j} \dots\dots\dots(5)$$

Substituting equation (1) in equation (5) gives

$$P = \sum_{j=1}^{j_{\max}} n_j P_j \dots\dots\dots(6)$$

Substituting equation (3) in equation (6) gives

$$P = \sum_{j=1}^{j_{\max}} n_j [1 - (1 - p)^j] \dots\dots\dots(7)$$

and considering condition (2)

$$P = 1 - \sum_{j=1}^{j_{\max}} n_j (1 - p)^j \dots\dots\dots(8)$$

which can be rearranged as

$$\sum_{j=1}^{j_{\max}} n_j (1 - p)^j = (1 - P) \dots\dots\dots(9)$$

This result is a polynomial of order  $j_{\max}$  in the complement of the cell viability  $(1 - p)$ , which must be solved for  $p$  from the given values of the complement of the cluster viability  $(1 - P)$ , and the fractions  $n_j$  representing the distribution of clusters among different sizes of  $j$ . No explicit solution to this equation can be written down in terms of  $P$  and  $n_j$ , and in fact, such an equation has  $j_{\max}$  solutions. However, the number of solutions is

restricted in the present case by taking into account the obvious condition that

$$0 \leq p \leq P \leq 1 \dots\dots\dots(10)$$

In the data to be analysed there was a preponderance of single and double clusters. Equation (9) was therefore solved, satisfying condition (10) by grouping all the terms for triples and higher clustering in a common term  $C$  which itself depends on  $p$ .

$$n_2(1 - p)^2 + n_1(1 - p) + C = 0 \dots\dots\dots(11)$$

where

$$C = \sum_{j=3}^{j_{\max}} n_j (1 - p)^j - (1 - P) \dots\dots\dots(12)$$

The quadratic equation (11) can be formally solved

$$1 - p = \frac{\sqrt{(n_1^2 - 4n_2C)} - n_1}{2n_2} \dots\dots\dots(13)$$

where the positive square root sign is chosen to satisfy condition (10). Note that from equation (11),  $C \geq 0$  since  $(1 - p) \geq 0$ , hence the arrangement of the square root is always positive. Since  $C$  itself depends on  $p$ , equation (13) is not an explicit solution for  $p$ , however  $p$  may be obtained from  $P$  and  $n_j$  as follows

- 1) Assume an approximate initial value  $p_1$  for  $p$ , by setting  $p_1$  equal to  $P$ . Substitute  $p_1$  for  $p$  in equation (12) and determine  $C_1$ .

- 2) Substitute  $C_1$  into equation (13) and determine  $p_2$  the second approximation for  $p$ .
- 3) Substitute  $p_2$  for  $p$  in equation (12) and determine  $C_2$  which is in turn substituted into equation (13).

This process of iteration is repeated until successive values,  $p_n$  and  $p_{(n+1)}$  differ by less than a tolerable error in  $p$ . It is found that the method converges to an adequate answer in three or four iterations.

Example.

In the following example a study was made of two agar slide cultures, one in which no bacterial growth had occurred for the distribution of cluster sizes (slide 1), and the other in which bacterial growth had occurred for the number of live and dead clusters (slide 2). In both slides clusters occurring in five grids were counted.

Slide 1 (Cluster Size Distribution)

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RAW DISTRIBUTION (Number of Clusters of each cluster size in each grid)

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Grid	<u>CLUSTER SIZE</u>					
	(Cells per Cluster)					
	$m_1$	$m_2$	$m_3$	$m_4$	$m_5$	$m_6$
1	5	8	0	2	0	0
2	12	7	1	1	0	0
3	14	7	2	1	0	0
4	12	5	3	1	1	0
5	16	7	1	0	0	1

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NORMALISED DISTRIBUTION (Proportion of clusters of each size in each grid)

Grid	<u>CLUSTER SIZE</u>						(Cells per cluster)					
	$n_1$	$n_2$	$n_3$	$n_4$	$n_5$	$n_6$	$n_1$	$n_2$	$n_3$	$n_4$	$n_5$	$n_6$
1	0.3333	0.5333	0.0	0.1333								
2	0.5714	0.3333	0.0476	0.0476								
3	0.5833	0.2917	0.0833	0.0417								
4	0.5455	0.2273	0.1364	0.0455	0.0455							
5	0.6400	0.2800	0.0400	0.0	0.0	0.0400						
Mean	$\bar{n}_1$	$\bar{n}_2$	$\bar{n}_3$	$\bar{n}_4$	$\bar{n}_5$	$\bar{n}_6$	0.5347	0.3331	0.0615	0.0536	0.0091	0.0080

Slide 2 (Cluster Viability)

Grid	Number of Live Clusters ( $N_a$ )	Number of Dead Clusters ( $N_d$ )	Cluster Viability (P)
			$(P = \frac{N_a}{N_a + N_d})$
1	22	9	0.7097
2	24	5	0.8276
3	22	7	0.7586
4	20	4	0.8333
5	15	6	0.7143
Mean ( $\bar{P}$ ) =			0.7687

To determine the mean cell viability, ( $\bar{p}$ ) from the mean cluster viability ( $\bar{P}$ ) and the mean normalised cluster distribution ( $\bar{n}_1, \bar{n}_2$ , etc) let  $p_1 = \bar{P} = 0.7687$  and substitute  $p_1$  for  $p$  in equation (12) giving  $C_1 = -0.2303$ . Substitute  $C_1$  in equation (13) giving  $p_2 = 0.6468$ . The results of a series of iterations are tabulated.

Iteration Number	$p_n$	$-C_n$
1	$0.7687 = \bar{P}$	0.2303
2	0.6468	0.2277
3	0.6503	0.2279
4	0.6500	0.2280
5	$0.6500 = \bar{p}$	

In the above example, if the probability of a cluster or cell remaining viable is expressed as a percentage, then for a mean cluster viability of 76.87% and a mean normalised cluster distribution,  $\bar{n}_1 = 0.5347$ ,  $\bar{n}_2 = 0.3331$  etc, a mean cell viability of 65.00% is obtained.