The effect of temperature on certain aspects of the metabolism of psychrophiles

SUMMARY

The refrigeration of foodstuffs at 0 - 10° does not necessarily stop bacterial growth and the spoilage of chilled milk, meat, poultry, eggs and vegetables over extended periods of storage is due to the growth of psychrophilic micro-organisms. The most important bacteria in this respect appear to be the psychrophilic Gram-negative rods and while these organisms have been studied from many aspects the effect of temperature on the growth and biochemical activity of such strans has received comparatively little attention. In general the examination of small numbers of strains has provided conflicting results and has not provided adequate information of the ability of psychrophiles in general.

This study comprised the isolation of strains at 5° from dairy sources and a selection of these strains together with representative strains from other environments, 24 strains in all, were used throughout the study.

In a preliminary experiment the morphological, cultural and biochemical characteristics and reactions of the strains were examined at 5°, 15°, 25°, 30° and 37°. On the basis of this experiment growth at 5° to 35° was studied critically by nephelometry, and expressed in terms of growth parameters and Q_{10} values. The influence of 0.5% to 4.5% sodium chloride on the growth response was then examined at 5° to 35°.

The ability to utilise and degrade individual amino acids over the

same temperature range was examined and the important aspects of proteinase activity were studied with particular reference to casein breakdown using the technique of casein precipitation and the electrophoretic separation of casein breakdown products. The tributyrinas activity of the strains was examined over the temperature range using a quantitative zone technique.

The study revealed the marked diversity in the characteristics and reactions of the strains at different temperatures. The individuality of the strains was shown in terms of growth parameters and Q_{10} values and while certain strains were able to be classed as psychrophiles or mesophiles on the basis of Q_{10} values at 5° - 15°, 15° - 25° and 25° - 35° other strains displayed intermediate growth-temperature patterns. The addition of sodium chloride to a medium indicated that the growth-temperat patterns of strains may be altered in different environments.

The relationship between the production of ammonia from amino acids and their utilisation for growth was shown to vary between strains and for individual strains at different temperatures. This observation could have important implications regarding the spoilage significance of strains at particular temperatures.

The proteolytic activity of strains at different temperatures was often found to bear little relationship to the proteolytic activity at 25°, a temperature normally employed for the examination of the biochemica reactions of such strains. It was also shown that such changes were not necessarily related to growth at the same temperature.

Thus the study demonstrated that in order to assess the potential of a strain to cause spoilage at a temperature of practical importance it

is necessary to examine the metabolic activities at that particular temperature and that it is of little value to determine the metabolic activities at a higher although possibly optimum temperature of growth.

THE EFFECT OF TEMPERATURE ON CERTAIN ASPECTS OF THE METABOLISM OF PSYCHROPHILES

A thesis submitted to the University of Glasgow for the degree of Doctor of Philosophy in the Faculty of Science.

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INTRODUCTION

The aesthetic implication and practical consequences of the growth of micro-organisms in foodstuffs held under refrigeration for extended periods makes a fuller understanding of these micro-organisms of far-reaching importance. The interest in this field of study has resulted in the publication of much original work and of a number of review articles on different aspects of psychrophiles. The response of micro-organisms to low temperature in terms of the fundamental aspects of kinetics and physiology has been reviewed by Ingraham (1958, 1962, 1963), Ingraham and Stokes (1959), Michener and Elliott (1964) and Farrell and Rose (1965). The practical importance of psychrophiles has been reviewed relative to a range of foodstuffs by Borgstrom (1955), Mossel and Ingram (1955), Ayres (1960), Shewan (1962), Elliott and Michener (1965) and in a Symposium held by the Campbell Soup Company (1962) Reviews concerning psychrophiles in relation to milk and milk products have been published by Davis (1951), Doetsch and Scott (1951), Thomas (1958) and Witter (1961).

Definition

The term psychrophile was first applied by Schmidt-Nielsen (1902) to those micro-organisms, described by Forster (1887) and Fischer (1888), which were capable of growth at low temperatures. The term has been criticised many times since Müller (1903) indicated that while such isolates produced appreciable growth at 0°, more rapid growth occurred

at higher temperatures. Terms including psychro-tolerant (Kruse, 1910), psychro-carterious (Rubentschick, 1925), oryophile (Jezeski and Macy, 1946) thermophobe (Edsall and Wetterlow, 1947), frigophile (Hempler, 1955) and psychroauxan (Thomas et al., 1960) have not replaced the general use of psychrophile. Eddy (1960) proposed the term 'psychrotroph' which has been applied to those bacteria able to grow better at higher temperatures, but which nevertheless produce good growth at 0°.

Further difficulties have been encountered in attempts to define the term psychrophile and the definition is relative to the temperature of the environment from which the organism was isolated. Thus a strain isolated from milk refrigerated at 5° may not be regarded as psychrophilic as an isolate from arctic sludge (McDonald et al., 1963). Thomas et al. (1949) suggested that bacteria isolated at low temperature from soil, water and milk were 'facultative psychrophiles.' The term 'obligate psychrophile' may only be applicable to those bacteria of marine origin (Zobell and Feltham, 1938; McDonald et al., 1963; Morita and Albright, 1965) which grow below 0° and quickly died at 25° or 30°. Other differentiations between 'obligate' and 'facultative' psychrophiles have been attempted by Lawton and Nelson (1954), Van der Zant and Moore (1955) and Rose (1962).

Low temperature isolates from milk and associated sources have been generally isolated at 3° to 7° and such strains have often been found to produce optimum growth at 20° to 25° (Lawton and Nelson, 1954; Roth and Wheaton, 1961) and exhibit poor or no growth at 37° (Brown, 1957; Erdman and Thornton, 1951; Greene, 1959; Colwell and Liston, 1961; Seitz, 1961). The inability of some psychrophiles isolated from milk to

withstand pasteurisation has also been reported (Rogick and Burgwald, 1950; Thomas et al., 1961; Witter, 1961) although Macauley et al. (1963) showed that psychrophiles could survive pasteurisation. The isolation of psychrophiles after 7 to 10 days incubation at 5° to 7°, as recommended in the 11th Edition of the Standard Methods for the Examination of Dairy products (1960), was shown by Scholefield (1963) to isolate bacteria (Micrococcus sp.) which were not metabolically active at low temperature. General definitions have been proposed being based on the ability to grow well at or near 0° (Greene and Jezeski, 1954; Olson et al., 1955; Ingraham, 1958; Straka and Stokes, 1960; Witter, 1961 and Barber, 1962), but a widening of the limits of acceptance may also result in the inclusion of isolates with poor activity at the temperature of practical importance.

Psychrophiles and Milk

Gram-negative rods have been shown by many workers (Reed and Reynolds 1916; Thomas and Sekhar, 1946; Davis, 1951; Thomas et al., 1959; Thomas and Druce, 1960; Schultze and Olson, 1960; Grange and Nelson, 1961; Gyllenberg et al., 1959, 1963; Barber, 1962; Macauley et al., 1963 and Scholefield, 1963, 1964) to be isolated as psychrophiles from milk and other dairy sources. The bacteria isolated comprised mainly Pseudomonas spp., Achromobacter spp., Flavobacterium spp., Alcaligenes spp. and Aeromonas spp. Members of the genus Pseudomonas have been generally found to be the most numerous and evenly distributed of all psychrophilic isolates from milk and milk products (Jezeski and Macy, 1946; Marth and Frazier, 1957; Thomas and Druce, 1960; Schultze and Olson, 1960; Grange and Nelson, 1961; Gyllenberg et al., 1963 and Scholefield, 1964).

Marth and Frazier (1957) found Ps. fluorescens, Ps. fragi and Ps. putrefaciens to be the most frequently isolated species and Higginbottom and Alexander, (1953), Orr et al. (1960) and Scholefield (1964) found Ps. fluorescens to be the predominant species isolated and to be responsible for spoilage of milk held at low temperatures. Frank (1962) reported Ps. geniculata to be the most frequently isolated psychrophile from different habitats.

Coliform bacteria have usually been isolated at low temperature from milk by several workers (Dahlberg, 1946; Hempler, 1955; Schultze and Olson, 1957, 1960; Andrey and Frazier, 1959; Thomas et al., 1961 and Scholefield, 1964) as minor isolates, and it is probable that such bacteria, unless they comprise a large section of the microflora are overgrown at low temperature by more active pseudomonads. Coliform bacteria may be predominant in milk contaminated after pasteurisation (Thomas et al., 1961).

While 'flavobacteria' have been obtained as psychrophilic isolates by many workers (Jezeski and Macy, 1946; Sekhar, 1947; Marth and Frazier, 1957; Schultze and Olson, 1960; Grange and Nelson, 1961; Andrey and Frazier, 1959; Gyllenberg et al., 1959 and Scholefield, 1963) their abilit to utilise whole casein and establish themselves in cold-stored milk is doubtful. This group of bacteria requires further investigation.

Well authenticated isolations of psychrophilic Gram-positive bacteria are few and while Thomas and Sekhar (1946), Rogick and Burgwald (1950), Marth and Frazier (1957) and Andrey and Frazier (1959) have isolate micrococci at low temperature, Gyllenberg et al. (1959) noted that their growth in milk was poor. Micrococci isolated at 5° from milk by Scholefield (1964), were subsequently found to display a much reduced

metabolic activity at this temperature.

The psychrophilic Gram-negative rods which contaminate milk appear primarily to be of soil and water origins (Morris, 1942; Thomas and Thomas, 1947; Holding, pers. comm.). Cattle feed has been found to be a source of psychrophiles (Thomas et al., 1949; Andrey and Frazier, 1959) and Labots (1961) has shown that the aerial microflora of the dairy comprises mainly Gram-negative rods. Milk utensils and containers have often been found (Thomas et al., 1959, 1961; Olson et al. 1953) to harbour psychrophiles and the specialised environment provided by watery milk residues on dairy plant is favourable for the growth of psychrophilic bacteria. Smillie et al. (1958) suggested that psychrophilic counts should be included in tests for the control of bulk milk and Orr et al. (1960) found a high correlation between psychrophilic counts of inaccessible parts of farm dairy equipment and the bulk tank milk. Due to the nature of farm pipeline installations complete draining is difficult and colonisation by psychrophiles may occur thereby providing appreciable levels of contamination of the next milk passing through the line (Andrey and Frazier, 1959).

Conn (1903) observed that putrefying bacteria affected the 'wholesomeness' of milk at 10° and Pennington (1908) showed that peptonising types grew well in milk at 0°. The intense putrid spoilage of milk often obtained at low temperatures has been related by Doetsch and Scott (1951) to Ps. putrefaciens and defects such as 'rabbito' and 'mustiness' to other Pseudomonas spp.. Ps. fragi was observed by Morrison and Hammer (1941) to cause 'May apple' taint in milk and Ps. graveolens to cause 'potato taint' in cold-stored milk. Van der Leek (1906) reported

that low temperatures favour the development of fluorescent bacteria in milk and Olson et al. (1953) observed that, after bitter, fruity, putrid, souring or thickening changes, refrigerated milk often turned green or yellow. Thomas et al. (1960) showed that coli-aerogenes types, Alcaligenes, Achromobacter and Flavobacterium spp. could produce ropiness in milk at 3 - 5° and Anderson and Hardenbergh (1932) found that lipolytic Achromobacter-Alcaligenes spp. produced a bitter spoilage of cream. The defects found in refrigerated milk are due mainly to the breakdown of milk proteins and lipids and the particular involvement of Pseudomonas and Achromobacter spp. has been shown (Gyllenberg et al. 1960, 1963; Scholefield, 1963, 1964. Pennington (1908) indicated that the proteolysis of up to 50% of milk casein occurred after several weeks at 0°. Higginbottom and Taylor (1962) showed that casein degradation products were detected after fourteen days at 5° and after two days at 30°. However, Van der Zant and Moore (1955) found that proteolysis of milk by Pseudomonas spp. was detectable after one day at 25° but not within 5 days at 5° or 10°. It is apparent that a naturally acquired mixed contamination of milk need not result in a uniform development of proteolysis during cold storage. Thomas et al. (1949) found that only 9% of psychrophiles examined were caseolytic and Babel (1953) maintained that the extent of caseolysis in milk depended on the type of organism present. Scholefield (1964) showed that 58% of Pseudomonas strains, which comprised 62% of the total psychrophilic isolates, were able to produce alkaline or alkaline-proteolytic breakdown of milk at 5°. The biochemically inert Pseudomonas strains produced growth but no observable change in milk at this temperature. This variation in ability between

strains of the same genus implies that a keeping quality test to indicate psychrophilic activity based on an assessment of casein breakdown, as suggested by Atherton et al. (1953), would not necessarily correlate with the growth of contaminating psychrophiles, which may be non-caseolytic while being metabolically active in other respects.

Davis (1951) showed that the bitter flavour of refrigerated milk was due to fat breakdown and not, as suggested by Henneberg (1931), due to the formation of peptones. The development of a bitter flavour in refrigerated cream was shown by Anderson and Hardenbergh (1932) to be due to lipolysis by an Achromobacter-Alcaligenes strain. The lipolytic activity of psychrophiles has been described by Jezeski and Macy (1946) and Overcast and Skean (1959); and Sjostrom (1959) maintained that most psychrophiles were lipolytic. Grange and Nelson (1961) found that 56 out of 71 psychrophiles hydrolysed tributyrin and Scholefield (1964) observed that 64% of Pseudomonas strains and 85% of Achromobacter strains isolated at 5° were able to hydrolyse tributyrin at 5°.

Many investigators (Greene and Jezeski, 1954; Van der Zant, 1957; Gyllenberg et al., 1959; Harmon et al., 1959; Higginbottom and Taylor, 1962; Hurley et al., 1963) have observed that the first measurable proteolytic and lipolytic changes in milk held at low temperature occur as the growth of psychrophiles enters the stationary phase. Greene and Jezeski (1954) and Nashif and Nelson (1953) found that the relationship between enzyme activity and population level was similar at all temperature but Van der Zant and Moore (1955) observed wide variation in the proteolyti activities of four psychrophile strains at 5° and 25° in relation to population level. If, as suggested by Gyllenberg et al. (1959), autolysis

of cells is reduced at low temperatures the realease of enzyme systems should be dependent on the temperature of incubation.

Kinetics of Psychrophilic Growth

The fundamental mechanisms which enable psychrophiles to depress the minimum growth temperature below that shown by mesophiles have long been studied but a coherent understanding of the phenomenon is still wanted. Foter and Rahn (1936) observed that since growth and fermentation were chemical reactions they should continue, with progressive reduction in rate, until the medium froze. Previously Berry and Magoon (1934) had suggested that growth was possibly due to 'lessened antibiosis' at low temperature and earlier, Crozier (1924) had suggested that growth and metabolic activity may be halted at a particular temperature due to the reduction in rate of a particular transformation in the sequential metabolic processes thus blocking the whole metabolism. This 'master reaction' hypothesis was considered to operate by Belehradek (1935) and Foter and Rahn (1936). The former worker suggested than an alteration in the sol-gel state of the cell together with an alteration in permeability or ion-exchange ability and an accumulation of toxic metabolites also governed growth at low temperatures. The latter factor was shown by Sinclair and Stokes (1962) however not to be a limiting factor at any temperature.

As the rates of growth and metabolic activity change with change in temperature, the effect can be measured employing one of three terms: Temperature coefficient (Q_{10}); temperature characteristic (μ) and Belehradek's exponent b.

The temperature coefficient (Q_{10}) is defined as the ratio of the

rate of growth or metabolic activity at one temperature (K_2) to that at a temperature 10° lower (K_1)

$$Q_{10} = \frac{K_2}{K_1}$$

where K is growth or activity constant (Buchanan and Fulmer, 1930). For chemical reactions Q_{10} values are usually constant and of the order 2 - 4. In biological systems Q_{10} values of this order may be obtained at the temperature of optimum growth or activity but with decrease in temperature Q_{10} values increase and eventually become infinite. Differences in the effect of temperature on Q_{10} values for growth have been shown for psychrophiles and mesophiles by Hess (1934), Foter and Rahn (1936 and Kiser (1944) and for growth, proteolysis and lipolysis by Greene and Jezeski (1954) and Jezeski and Olsen (1962). Increase in Q_{10} values with decrease in temperature for glucose oxidation was demonstrated by Ingraham and Bailey (1959) for psychrophiles but not for mesophiles.

The temperature characteristic (μ) is derived from the van't Hoff-Arrhenius equation:

$$\mu = \frac{4.6 \, (\log K_2 - \log K_1) \, T_2 \, T_1}{T_2 - T_1}$$

where K_1 and K_2 are growth or activity constants at absolute temperatures T_1 and T_2 (Porter, 1946). The μ value represents the activation energy required to raise the molecules from a normal state to an active state enabling a reaction to take place. Reference to the review of Farrell and Rose (1965) may be made for a fuller account of the derivation of the temperature characteristic. Rates of reaction with large μ values

decrease rapidly with decrease in temperature and typically, μ values become very high near the minimum growth temperature, at which high Q_{10} values also occur. The point where μ suddenly increases has been called the 'critical temperature' (Crosier, 1924) and below this temperature the reaction becomes the limiting 'master reaction.' Olsen and Jezeski (1963) considered that there were several 'critical temperatures' affecting the growth of psychrophiles which varied with the state of nutrition, aeration etc. Whereas the Q_{10} value for a particular strain may change over a given temperature range depending on the environmental factors (Jezeski and Olsen, 1962), the temperature characteristic (μ) is a property of the bacterium and has been found to be constant by Scott (1937) and Ingraham (1958) over a considerable range of temperatures, although Haines (1931) found μ to vary continuously with temperature.

Belehradek (1935) suggested 'exponent b' to be of value in describing the effect of temperature on a biological system but the biological zero, in terms of temperature, must be known in order to calculate 'exponent b.'

The majority of psychrophiles isolated have proved to be aerobes (Brown and Weidemann, 1958; Ingraham and Stokes, 1959; Ayres, 1960 and Witter, 1961) and Jezeski and Olsen (1962) showed that the oxidation of glucose was closely involved in the growth of psychrophiles at low temperature. The carbohydrate metabolism of psychrophiles has been studied closely by several workers. Brown (1957) observed that μ values for oxygen utilisation were higher for mesophiles than for psychrophiles, and Ingraham (1958) showed that a similar relationship applied to growth. Sultzer (1961) found that psychrophiles exhibited lower μ values than mesophiles for the oxidation of fatty acids and concluded that the ability

to grow at low temperature was primarily due to certain aspects of the carbohydrate metabolism of psychrophiles.

While differences in Q_{10} values and in μ values have been obtained between psychrophiles and mesophiles in terms of growth and the metabolism of whole cells differences have been found to disappear when cell-free enzyme preparations were examined. Ingraham and Bailey (1959) showed that μ values for cell-free glucose dehydrogenase systems derived from Ps. aeruginosa and a psychrophilic strain of Ps. perolens were not appreciably different although differences occurred when resting cells were studied. Since very few enzyme systems have been examined, and these have been generally related to carbohydrate metabolism, and because very few strains have so far been considered, the possibility of the existence of individual enzyme systems with different μ values in mesophiles and psychrophiles must not be precluded. Certain studies have shown that cell-free enzyme systems of mesophiles and psychrophiles may have different μ values. McDonald et al. (1963) found that proteinases of psychrophiles had much lower μ values than those of mesophiles and Burton and Morita (1965) showed that the μ value of malic dehydrogenase from Vibrio marinus was half that of E. coli. Although the 'master reaction', as postulated by Crosier (1924), presupposes a sequential order of reactions Marr and Ingraham (1962) suggested a model by which a dichotomy of pathways could operate on the basis of the limiting μ values of individual stages. These workers envisaged that such a system resulted in a general depression of enzyme synthesis near the minimum temperature of growth. Ng et al. (1962) indicated that an increase in μ value resulted in the lowering of the concentration of enzyme-forming systems thereby

causing damage to the cell.

As Belehradek (1935) and Ingraham (1963) maintained, at least part of the phenomenon of psychrophily appears to be due to the structural integrity of the cell. Belehradek suggested that 'differences in permeability or ion exchange ability' contributed to the differences between psychrophiles and mesophiles. The importance of the cytoplasmic membrane as a structure concerned with the transport of solutes and for the localisation of certain enzyme systems is, as yet, not fully understood, but evidence is accumulating as to its significance in terms of the effect of temperature on psychrophiles and mesophiles. Baxter and Gibbons (1962) indicated that the main factors accounting for the low minimum growth temperature of psychrophiles compared with mesophiles were the characteristically lower u values of the solute transport mechanisms, together with increased permeability. Quetsch and Danforth (1964) showed that the transport mechanism involving uptake of purines was inactivated below 4^{o}.

Gaughran (1947) observed that the solidification of certain lipid fractions fixed the minimum temperature of growth of thermophiles. In relation to growth at low temperatures Farrell and Rose (1965) pointed out that lipid molecules, as micelles, have great freedom of movement in the cell and any stress which alters their nature will affect the transport role of lipo-protein membranes. While Marr and Ingraham (1962) found that a reduction in temperature increased the proportion of unsaturated fatty acids in E. coli no direct relationship between lipid composition and the minimum growth temperature was observed. Kates and Baxter (1962) confirmed that organisms growing at low temperature contained less saturated lipids than at higher temperatures, and Rose (1962) considered

that changes in lipid content may account for the reduction of growth at low temperature. More significantly Wells et al. (1963) observed that a reduction in temperature increased the lipids in the cell membrane of mesophiles but not psychrophiles, and Kates and Hagen (1964) showed that the triglycerides of phospholipids in the cytoplasmic membranes of psychrophiles and mesophiles were different. Luzzati and Husson (1962) showed that changes in physical conditions could lead to rearrangements in the phospholipid molecules of cell membranes, and Hagen et al. (1964) found that the breakdown of phospholipids in the cytoplasmic membrane caused the death of a psychrophile. Silberman and Gaby (1961) showed that phospholipids comprised 80% of the total lipids of Pseudomonas aeruginosa and that these were closely involved in the uptake of amino acids. Jezeski and Olsen (1962) found that amino acids were linked with the processes which permit growth at low temperature and Shoesmith and Sherris (1960) observed that permeability of the cell membrane to amino acids was greatest under aerobic conditions. Although Gale (1947) had shown that the cytoplasmic membranes and cell walls of Gram-negative bacteria were permeable to amino acids the actual transport does not occur in the free state, and energy for this process is provided by oxidative phosphorylation (Shirley-Taylor, 1947).

While the evidence indicates a relationship between lipids and the transport of solutes at low temperature, it is probable that protein carrier molecules (Cohen and Monod, 1957) and enzymes (Farrell and Rose, 1965) undergo conformational changes during transport, in which case low temperature damage could be due to hyperfolding of the protein molecules with loss of transport ability. Rose and Evison (1965) determined μ values

for solute transfer mechanisms but observed few differences between mesophiles and psychrophiles.

Certain enzyme systems which are located within the cytoplasmic membrane may be susceptible to organisational changes of the membrane at low temperatures. In pseudomonads systems which are involved in the oxidative metabolism of carbohydrates are known to be located in the membrane (Burrous and Wood, 1959; de Ley, 1960 and Campbell et al., 1962). The immobilisation at low temperature of glucose oxidase, succinic dehydrogenase (Campbell et al., 1962) and gluconic dehydrogenase (de Ley and Doehy, 1960) in the cytoplasmic membrane could result in a 'master reaction' phenomenon, despite low u values of cell-free enzyme systems, and thereby fix the minimum temperature of growth. Since the terminal oxidase systems of fluorescent pseudomonads are also located in the cytoplasmic membrane (de Ley, 1960), and due to the importance of catalase in aerobes (McCarthey and Hinshelwood, 1959), the significance of this group of enzymes in relation to growth at low temperature requires consideration. Jezeski and Olsen (1962) concluded that the cytochrome oxidase system was temperature dependent and Frank et al. (1963) found that the catalase production of psychrophiles was greater at 2° than at 30°, although the activity of catalase was reduced at the lower temperature. The increased solubility of oxygen at low temperatures necessitated rapid elimination of the excess hydrogen peroxide formed, and it was considered that in order to survive aerobic psychrophiles required large amounts of catalase especially during the utilisation of carbohydrates. However, the isolation by Schmidt et al. (1960) and Upadhyay and Stokes (1962) of anaerobic psychrophiles suggests that

this factor may not be the only mechanism which permits growth at low temperatures. Other factors which may influence the ability of bacteria to grow at low temperature have been indicated. Azuma and Witter (1964) were able to produce a psychrophilic mutant from a mesophilic Ps. aeruginosa by irradiation with ultra-violet and it was observed that the protoplast of the mesophile was more fragile than that of the mutant. The findings of Brown and Turner (1963) that membrane stability and salt tolerance of Gram-negative bacteria were related, and that osmotic fragility and the hexosamine content of the cell wall were related, may be significant. However, Rose and Evison (1965) found that mesophiles accumulated hexosamine at temperatures below that of minimum growth.

While it would be a gross over-simplification of the obviously complex processes involved to attempt a single explanation of the differences between mesophiles and psychrophiles it appears possible that several factors contribute to the establishment of a minimum growth temperature. These include changes in solute permeability of the cytoplasmic membrane, particularly in relation to the transport of nitrogen-containing molecules, which may be due to alterations in the conformation or structure of lipid or lipid-linked components; changes in the structure of the cytoplasmic membrane which may affect the operation of membrane-bound or associated enzyme systems including terminal oxidase, an enzyme involved in energy-yielding carbohydrate metabolism; the 'master reaction' which may operate in terms of a single enzymic stage with a high u value, or may operate as a result of changes involving the solute permeability or the structure of the cytoplasmic membrane. Further examination of the stress caused by reduction in temperature on

the growth and metabolic activities of psychrophiles and mesophiles is required for a greater understanding of the differences between these groups of organisms.

Influence of temperature on metabolism

Reed and Reynolds (1916) indicated that the changes produced in milk held at -1.1° to 35° due to the growth of specific strains were not necessarily related to the level of population. Variation in enzymic activity was apparent at different temperatures. The possible derangement of the metabolism of bacteria at low temperature was suggested by Mattick (1951) and changes in metabolic activity of psychrophiles at different temperatures have been recorded by Jezeski and Macy (1946), Davis (1951), Boyd et al (1954), Marth and Frazier (1957) and Gyllenberg et al. (1960). However, few comprehensive studies have been made of the effect of variation in test temperature on the metabolic activities of psychrophiles and the findings of workers in this particular field show much conflict.

Nashif and Nelson (1953) found that lipase was produced by Ps. fragi at low temperatures whereas none was produced at 30° despite active growth and Alford and Elliott (1960) also showed that elevation of the test temperature inhibited lipase production. Greene and Jezeski (1954) found that a Pseudomonas strain was able to liquefy gelatine at 0° but not at 30°, whereas glucose breakdown occurred at 30° but not at 0°. Van der Zant (1957) and Peterson and Gunderson (1960) observed that proteolytic enzymes of Pseudomonas spp. were active at 37° but not at 5° although the yield and the stability of the enzyme systems were greater at lower

temperatures. Fluorescence produced by psychrophilic pseudomonads has been reported to be more pronounced at low temperatures by Van der Leck (1906), Hess (1934) and Davis (1951).

Observations of qualitative changes in the metabolism of bacteria with change in temperature have been less frequently observed. Lipase activity was shown by Balls et al. (1937) to be similar between 0° and 40° towards short chain glycerides whereas long chain glycerides were not attacked at low temperatures. Goldstein and Goldstein (1953) found that Ps. fluorescens produced separate tributyrinase and triacetinase enzymes.

Differences in end-products of carbohydrate metabolism have been reported by Foster (1962) who noted that a Lactobacillus strain produced lactic acid at 37° whereas dextran was formed at 15°. Several workers including Stuart et al (1942) and Greene and Jezeski (1954) have noted the loss of aerogenesis with increase in temperature during carbohydrate fermentation. The latter workers attributed the anaerogenesis of an Aerobacter sp. at 37° to the deficiency of part of the decarboxylase enzyme system. The inability of coliform strains to ferment carbohydrates with aerogenesis at low temperatures has also been observed by Azuma and Clegg (1962).

Gale (1940) found that the decarboxylation of amino acids by E. coli was more active at 27° than at 37° and Anderson (1948) found that an Achromobacter sp. developed a requirement for amino acids at temperatures greater than 29°. Nelson (1943) and Heather and Van der Zant (1957) showed that heat damaged cells were more exacting in their nutritional requirements, and in particular in their amino acid requirements.

Reactions to biochemical tests often reflect the production of end-products of metabolism or the gross effects of enzyme systems and the tests may therefore be criticised. However, the growth of psychrophilic spoilage organisms is important in terms of the total effect on the environment, e.g. milk, and the use of biochemical tests to examine for end-products and exo-enzyme activity is valid. Such tests, performed as recommended at or near the optimum growth temperature (Manual of Microbiological Methods, 1957; Oginsky and Umbreit, 1959) do not necessarily reflect the activity of the strain at other temperatures. More particularly the activities of psychrophiles at temperatures well below the optimum for growth are frequently not taken into account although these low temperatures are of practical significance. Instances of the evaluation and characterisation of psychrophiles on the basis of tests performed within the optimum temperature range of 20° to 25° include those by Jones and Thomas (1950), McLean et al. (1951), Marth and Frazier (1957), Shewan et al. (1960), Straka and Stokes (1960), Ayres (1960) and Colwell and Liston (1961). Gyllenberg et al. (1963) isolated 729 'psychrotrophs' and attempted to relate the keeping quality of milk at 4° with the biochemical reactions obtained at 22°.

Few studies have been directed towards the comparative assessment of biochemical characteristics over a range of temperatures. Kiser (1944) in an examination of marine psychrophiles at -4° to 23° found that some strains were more demanding nutritionally near the minimum growth temperature and also showed a loss of biochemical activity. A psychrophilic strain examined by Gainor and Wegener (1954) exhibited no differences in fermentative ability at 5° and 25°, and Sekhar and Walker (1947) found

differences only in the rates of biochemical reactions of psychrophiles at 3 - 5° and at 22°. Alford (1960) noted that 26 of 60 Pseudomonas and Achromobacter strains exhibited one or more differences in reactions to tests at 20°, 32° and 36°, and some tests, e.g. xylose fermentation, were most rapid at the lowest temperature. The changes produced in 16 carbohydrate media by 15 psychrophilic coliform strains isolated from milk were examined at 4° to 37° by Azuma and Clegg (1962). Enzymic differences were indicated by marked anaerogenesis at 4° compared with 15° and above, and on this basis the 'psychrophilic' designation of these isolates may be regarded as suspect. Frank (1962) examined the biochemical activities of 16 Ps. geniculata strains at 8° and 27°. While fat and carbohydrate breakdown was less and growth in the presence of salt and antibiotics and chromogenesis was greater at 8°, the classification of the strains was not affected by the findings. More recently Frank et al. (1963) maintained that more catalase was produced by psychrophiles than mesophiles at 2° than at 30°. Higoshi (1964) examined certain biochemical activities at 5°, 20° and 35° of psychrophilic Pseudomonas strains derived from milk and other foods and concluded that growth, lipolysis, proteolysis and chromogenesis were similar at 5° and 20°. An examination of a wide range of psychrophilic strains isolated from dairy sources was made by Scholefield (1963, 1964). This work revealed that the biochemical characteristics of low temperature isolates displayed at 22° were not necessarily shown at 5°. It was found that certain Pseudomonas groups were equally active at 5° and 22° whereas Micrococcus strains displayed a substantial reduction in metabolic activity at 5°, although the micrococci often grew well at this temperature. This work demonstrated

the need for further study in order to ascertain the significance of psychrophilic isolates as agents of spoilage at low temperature.

The following study was undertaken to examine certain aspects of the growth and metabolism of Ps. strains at temperatures which had not been previously considered.

The strains acquired representative psychrophilic isolates from dairy sources together with psychrophilic and mesophilic strains derived from other sources. In Part I of the Experimental Section the morphological, cultural and biochemical characteristics of each strain were examined at 5°, 15°, 25°, 30° and 37°. On the basis of this experiment, a detailed study was made in Part II of the growth of each strain at 5° to 37°. The effect of sodium chloride on growth was also examined. The utilization and breakdown of amino acids, provided as sole carbon, nitrogen and energy sources, were studied at different temperatures in Part III. Part IV of the Experimental Section comprised an examination of the effect of temperature on the proteolytic and lipase activity of the strains with particular reference to their ability to degrade casein.

EXPERIMENTAL STUDIES

STRAINS

Throughout this study strains S1 - S24 were used, which together with their sources are listed in Table 1.

Strains S1 - S11 were psychrophilic isolates from milk and other dairy sources and were selected as being representative of those genera and groups believed to be of significance as psychrophilic contaminants of milk. Milk and rinse samples were obtained in sterile 4 oz glass stoppered bottles and surfaces were sampled using the standard swab technique (Min. of Agr. and Fisheries publ. 44871-1, 1945). Decimal dilutions of samples were plated by spreading 0.1 ml evenly over prepared Milk agar (Oxoid) in 4 cm petri dishes. Plates were held at $5^{\circ} \pm 1^{\circ}C$ in a refrigerated incubator and isolations made after seven days incubation. Representative colonies were sub-cultured on to Milk agar plates and after 3 days incubation at 15° single colonies were sub-cultured at the same temperature by dilution streaking to ensure that pure cultures had been obtained. Of 85 psychrophilic isolates strains S1 - S11 were selected as being representative on the basis of an identification scheme devised by the author (Scholefield 1963, 1964).

Strains S12 - S24 comprised a range of Gram-negative bacilli which represented type species from culture collections and also included mesophilic and psychrophilic isolates from sources other than milk. These strains were selected from those used by the Pseudomonas Working Party (P.W.P) of the Society for General Microbiology of which the

author is a working member. All the strains (S1 - S24) were maintained at room temperature (14-18°C) on Milk agar slopes in screw capped $\frac{1}{4}$ oz bijou bottles. When required the cultures were plated on to Nutrient agar (Oxoid) and incubated at 25° for 18 hours prior to use as inocula.

Table 1. List of Strains with Sources

Strain	Identification	Source
S.1	*Pseudomonas* sp.	Isolated @ 5° ex raw milk
S.2	*Achromobacter* sp.	" @ 5° ex raw milk
S.3	*Pseudomonas* sp.	" @ 5° ex pipeline rinse
S.4	*Pseudomonas* sp.	" @ 5° ex milk from cooler
S.5	*Pseudomonas* sp.	" @ 5° ex pipeline milk
S.6	*Pseudomonas* sp.	" @ 5° ex bulk tank
S.7	*Aeromonas* sp.	" @ 5° ex pipeline rinse
S.8	*Pseudomonas* sp.	" @ 5° ex bulk tank
S.9	*Pseudomonas* sp.	" @ 5° ex raw milk
S.10	*Achromobacter* sp.	" @ 5° ex pipeline rinse
S.11	Coliform str.	" @ 5° ex cow teat swab
S.12	*Ps. aeruginosa*	N.C.I.B 8295
S.13	*Bact. antitratum*	N.C.T.C 7844
S.14	*Pseudomonas* sp.	P.W.P 4A/3 (soil)
S.15	*Ps. fluorescens*	N.C.T.B 9046
S.16	*Com. percolens*	N.C.I.B 8193
S.17	*Pseudomonas* sp.	P.W.P Soil (2). Pt.2
S.18	*Ps. putida*	C.C.E.B 520
S.19	Aeromonad str.	P.W.P Water (2) 472
S.20	*Pseudomonas* sp.	P.W.P J.S.63 (milk)
S.21	*Pseudomonas* sp.	P.W.P J.S.64 (milk)
S.22	*Agrobact. tumefaciens*	N.C.P.P.B 397
S.23	*Pseudomonas* sp.	P.W.P 338/56 (human)
S.24	*Xanth. campestris*	N.C.P.P.B 528

N.C.I.B - National Collection of Industrial Bacteria
N.C.T.C - National Collection of Type Cultures
P.W.P - Pseudomonas Working Party
C.C.E.B - Culture Collection of Entomogenous Bacteria
N.C.P.P.B - National Collection of Plant Pathogenic Bacteria

I. THE EFFECT OF TEMPERATURE ON MORPHOLOGICAL, CULTURAL AND BIOCHEMICAL CHARACTERISTICS AND REACTIONS

Previous work by the author (Scholefield 1963, 1964) had shown that the biochemical characteristics of psychrophilic isolates from dairy sources could be affected by a reduction in test temperature from 22° to 5° and that the effect varied with the type of psychrophile. The following study was made to establish the morphological, cultural and biochemical characteristics of strains S1 - S24 at 5°, 15°, 25°, 30° and 37°.

Materials and Methods

Inocula.

In order to provide standard inocula 18 hour growth at 25° on nutrient agar (Oxoid) was suspended in quarter strength Ringer's solution and adjusted to 0.3 x 100 units optical density (E.E.A. Nephelometer, Evans Electroselenium, Ltd.). Inoculations were made from the standardised suspension using a standard wire loop or straight wire.

Incubation.

Replicates of each inoculated medium were incubated at 5° and 15° in refrigerated incubators and at 25°, 30° and 37° in anhydric incubators. Daily incubation temperature checks were made and a variation of not more than ± 1° was maintained.

Morphological examination.

Microscopical preparations were examined with a Watson 'Bactil' binocular microscope using magnifications of x900 to x1500.

Prepared smears were heat fixed and stained employing Hucker's (1927) modification of Gram's staining method, and the Gram reaction and morphology noted.

The morphology of living organisms in wet preparations was carried out by the method described by Mackie and McCartney (1956), substituting Nigrosine solution for India ink. A loopful of peptone water culture was added to a loopful of 10% (w/v) Nigrosine solution on a microscope slide. After covering with a No.1 cover glass the preparation was examined. The presence or absence of motility was also determined in the Nigrosine preparations.

A silver precipitation technique based on a method described by Mackie and McCartney (1956) and modified by the author (Scholefield, 1964) was used for the examination of flagella.

(i) Growth from the surface of a semi-solid agar (Mackie and McCartney, 1956) stab culture was spotted gently into one end of a distilled water streak 0.5 cm long on a chromic acid cleaned slide. By a process of diffusion and motility a graded density of cells remained after the preparation had dried.

(ii) The unheated film was treated with Fontana's fixative (acetic acid, 1 ml; formalin, 2 ml; distilled water, 100 ml) for two to three minutes.

(iii) The fixed film was washed with water and treated with Kirkpatrick's mordant (5% w/v. ferric chloride - 1 part; 20% w/v. tannic

acid - 3 parts; distilled water - 4 parts) for three to five minutes.

(iv) The film was washed well, covered with Fontana's ammoniated silver nitrate solution and heated to steaming for half a minute. The ammoniated silver nitrate was prepared by the addition of 10% ammonia solution to 0.5% (w/v) silver nitrate solution until the precipitate formed just dissolved. More silver nitrate was added drop by drop until the precipitate returned and did not dissolve.

Growth determinations.

Growth was examined on Nutrient agar, in milk, peptone water, peptone water plus 2.5% (w/v) sodium chloride and in media containing 1.0% (w/v) glucose, lactose, fructose, mannitol or sorbitol. All media were formulated from Oxoid Ltd, British Drug Houses Ltd, and Lights Ltd, constituents where possible. Before inoculation media were pre-incubated at the intended test temperature to minimise false positive growth.

Nutrient agar: Standard loopfuls of standardised inocula were streaked on to quarter plates of Nutrient agar (Oxoid). After incubation the level of growth was evaluated by the semi-quantitative scoring method described in a previous study (Scholefield, 1964).

Milk: A 10% (w/v) aqueous solution of Skim milk powder (Oxoid L.31) was dispensed in 12 ml amounts in 6 x $\frac{5}{8}$ inch tubes. Following inoculation and incubation growth was measured in terms of viable counts as described by Mackie and McCartney (1956).

Peptone water: A 0.2% (w/v) peptone (Oxoid L.37) medium was dispensed in 12 ml quantities in 6 x $\frac{5}{8}$ inch tubes. After inoculation and incubation growth was measured using an E.E.L nephelometer (Evans Electroselenium, Ltd) and expressed as units of optical density (O.D) relative to an

arbitrary standard.

Peptone water plus sodium chloride: To quantities of the peptone water medium described above 2.5% (w/v) sodium chloride was incorporated. Growth was determined nephelometrically.

Carbohydrate media: The media consisted of 1.0% (w/v) glucose, lactose, fructose, mannitol or sorbitol, and 0.1% (w/v) ammonium sulphate dissolved in Pope and Skerman basal salts medium (Skerman, 1959) with 0.05% (w/v) Yeast extract (Oxoid L.20). The pH was adjusted to 7.0 and individual media were dispensed in 12 ml quantities in 6 x $\frac{5}{8}$ inch tubes. Growth determinations were made as for peptone water cultures.

Evaluation of pigment.

Fluorescent pigment, brown pigment and pyocyanine were determined in King's A and B media (King et al., 1954). Fluorescent pigment was observed using an ultra-violet light source.

Biochemical tests.

Oxidative-fermentative test: The single tube method based on that of Hugh and Leifson (1953) and modified by the author (Scholefield, 1964) was employed. The media contained 1.0% (w/v) glucose, lactose, fructose, mannitol or sorbitol, 0.1% (w/v) ammonium sulphate dissolved in Pope and Skerman basal salts medium (Skerman, 1959) and 0.05% (w/v) Yeast extract (Oxoid L.20) with 0.45% (w/v) agar (Oxoid No.3), 0.003% (w/v) bromo-thymol blue and 0.001% (w/v) acid fuchsin. The pH was adjusted to 7.0 and individual media were dispensed in 12 ml quantities in 6 x $\frac{5}{8}$ inch tubes. The media was inoculated by stabbing with a straight wire and after

incubation the degree of oxidative or fermentative change was assessed by reference to standard comparison tubes.

Arginase activity: Sherris et al. (1959) showed that Pseudomones spp. degraded arginine with the formation of ammonia and a test based on this reaction was found by Thornley (1960) to be of value for the identification of non-pigmented pseudomonads. The medium contained 0.1% (w/v) peptone, 0.5% (w/v) sodium chloride, 0.03% (w/v) dipotassium hydrogen phosphate, 1.0% (w/v) D.L. arginine monohydrochloride, 0.001% (w/v) phenol red, 0.3% (w/v) agar and the pH was 7.4. To reduce convection and diffusion, the gel strength of this medium was increased from 0.3% to 0.5% (w/v) agar. The medium was distributed in 12 ml amounts in 6 x $\frac{5}{8}$ inch tubes closed with 'Astell' seals and autoclaved at 5 lbs/sq.inch for 10 minutes. Inoculation by stabbing was used and the reaction, in terms of increase in pH, was assessed by reference to standard comparison tubes.

Changes in milk: The changes produced in Purple milk (10% (w/v) Skim milk powder, Oxoid L.31; 0.004% (w/v) bromo-cresol purple) were examined. The medium with a pH of 6.9 was dispensed in 6 x $\frac{5}{8}$ inch tubes in 12 ml amounts and after inoculation and incubation the changes, ranging from acid coagulation to alkaline proteolysis, were noted.

Gelatine liquefaction: Strains were inoculated by stabbing 12 ml amounts of Nutrient gelatine (Oxoid) contained in 6 x $\frac{5}{8}$ inch tubes. After incubation the extent of liquefaction was assessed visually.

Casein precipitation reaction: Standard inocula were streaked on to plates of Casein agar (1.0% (w/v) Sodium caseinate, Difco; 0.04 M magnesium

chloride; 0.5% (w/v) peptone; 0.3% (w/v) sodium chloride; 1.2% (w/v) agar. pH 6.7). After incubation the reaction was assessed in terms of the formation of zones of casein precipitation surrounding the area of growth.

Tributyrinase activity: Nutrient agar (Oxoid) was poured in 7 ml amounts into plates and allowed to set before layering with 3 ml of Tributyrin agar (Oxoid). After drying, standard inocula were streaked on to the surface of the medium and after incubation the ability to hydrolyse tributyrin was assessed in terms of the zones of lysis which accompanied growth.

Egg yolk reaction: The medium was prepared in the following manner: The yolk of one egg was aseptically separated and emulsified in 150 ml normal saline. After filtration through loose cotton wool the filtrate was centrifuged at 3,000 r.p.m for 30 minutes and the supernatant was Seitz filtered. The egg yolk emulsion was aseptically mixed with an equal quantity of double-strength Nutrient agar (Oxoid) immediately before pouring into plates. After drying, standard inocula were streaked on to the surface of the medium and following incubation the egg yolk reaction was assessed in terms of the extent and type of zoning which accompanied growth.

Catalase reaction: The test was performed by dropping 5 volume hydrogen peroxide solution on to the surface of growth produced on Nutrient agar (Oxoid). The extent of a positive catalase reaction was evaluated in terms of the visible evolution of oxygen.

Oxidase reaction: Gaby and Free (1958) claimed that the 'cytochrome oxidase' test was simpler although less sensitive than Kovacs (1956) oxidase test;but Steel (1961) concluded that the latter test was more reliable. In this study the Kovacs test was used in a slightly modified form to facilitate comparative evaluation. Strains grown on Nutrient agar (Oxoid) were streaked with a platinum loop on to squared Whatman No.1 filter paper. A 1.0% (w/v) aqueous solution of tetra - methyl - p - phenylenediamine dihydrochloride (B.D.H) was dropped on to the paper to give immediate contact with all streaks. The time taken for a positive reaction to occur was the basis for evaluation. A fresh solution of reagent was prepared daily, using glass distilled water and the same platinum loop as used in the test.

Treatment of Results

Determinations of morphology, growth and reactions to biochemical tests were made at intervals during incubation at each temperature. Based on the time taken for maximum growth or reaction trials showed that the following periods of incubation at each temperature were suitable for the comparative evaluation of the results.

Temperature	Time
5°	28 days
15°	14 "
25°	4 "
30°	2 "
37°	1 day

A method used by the author in a previous study (Scholefield, 1964)

For the semi-quantitative evaluation and scoring of growth and certain biochemical reactions was employed to express the results of this experiment. The following Key to Tables 2.1 - 2.4 indicates the evaluation and scoring of morphological and cultural characteristics, growth and biochemical reactions.

Key to Tables 2.1 - 2.4

Test	Level of reaction	Symbol
Gram reaction	positive	+
	negative	-
	indifferent	±
Morphology	length, medium - 1 to 3 microns	M
	" long - over 3 "	L
	" short - under 1 "	S
	width, thin - under 0.5 "	T
	" fat - over 0.5 "	F
	shape, pointed	P
	" clubbed	C
	" curved	V
Motility	active	2
	poor	1
	absent	0
Flagella	polar	Po
	peritrichous	Pe
	absent	0

Test (Cont'd.)	Level of reaction (Cont'd.)	Symbol (Cont'd.)
Growth		
Nutrient agar	very heavy	4
	heavy	3
	moderate	2
	poor	1
	absent	0
milk	viable count over 10^9 cells per ml	4
	" " 10^7-10^9 " " "	3
	" " 10^5-10^7 " " "	2
	" " 10^3-10^5 " " "	1
	" " below 10^3 " " "	0
peptone water;	optical density over 1.5 x 100 units	4
" " plus 2.5%	" " 0.75-1.5 x 100 "	3
sodium chloride;	" " 0.25-0.74x 100 "	2
glucose, lactose, fructose	" " 0.1 -0.24x 100 "	1
sorbitol and mannitol media	" "less than 0.1 x 100 "	0
Pigment		
fluorescent pigment	strong, visible in daylight	3
	strong, only under ultra-violet light	2
	weak, " " " "	1
	none	0
pyocyanine	intense	3
	moderate	2
	weak	1
	none	0
	brown pigment	BR
	none	0

Test (Cont'd.)	Level of reaction (Cont'd.)		Symbol (Cont'd.)
Oxidative-fermentative change.			
glucose, lactose, fructose, sorbitol and mannitol	Colour	pH decrease	
	carmine	over 2.5 units	4
	red	1.6-2.4 "	3
	orange	0.8-1.5 "	2
	yellow	0.1-1.7 "	1
	green	no change	0
	oxidative change: symbol only e.g. 3		
	aerogenic fermentative change: e.g. 3FG		
	anaerogenic " " : e.g. 3F		
Arginase	Colour	pH increase	
	carmine	over 1.5 units	5
	deep red	0.9-1.4 "	4
	red	0.5-0.8 "	3
	deep pink	0.3-0.4 "	2
	pink	0.1-0.2 "	1
	orange	no change	0
Changes in milk			
	extensive alkaline change with complete proteolysis		4
	extensive alkaline change with partial proteolysis		3
	extensive alkaline change without proteolysis		2
	limited alkaline change		1
	neutral proteolysis		X
	acid with coagulation		A
	acid without coagulation		a
	no change		0

Test (Cont'd.)	Level of reaction (Cont'd.)	Symbol (Cont'd.)
Gelatine liquefaction		
	complete with clearing	4
	extensive but incomplete, with clearing	3
	either moderate with clearing or complete softening without clearing	2
	superficial	1
	no change	0
Casein precipitation		
	extensive	3
	moderate	2
	limited	1
	no change	0
Tributyrinase		
	extensive, over 5.0 m.m. zone	4
	moderate, 3.0-5.0 m.m. "	3
	limited, 1.5-2.9 m.m. "	2
	poor, 0.1-1.4 m.m. "	1
	no clearing	0
Egg yolk reaction		
	double zone of dense sharply defined opalescence with zone of lysis	3
	either double zone of opalescence without lysis or single zone of opalescence with lysis	2
	either single zone of opalescence or single zone of lysis	1
	no zone	0
Catalase		
	rapid, extensive evolution of oxygen bubbles	3
	moderate evolution of bubbles	2
	sparse evolution of bubbles	1
	no activity	0

Test (Cont'd.)	Level of reaction (Cont'd.)	Symbol (Cont'd.)
Oxidase reaction (Kovacs)		
	intense, less than 5 seconds	3
	positive in 5-10 "	2
	positive in 10-20 "	1
	no reaction or delayed	0

Table 2. 1 Morphological, Cultural and Biochemical Characteristics and Reactions at 5°, 15°, 25°, 30° and 37° (Strains S1 - S6)

Strain	Temp. °C.	Gram reaction	Morphology	Motility	Flagella	Growth: Nutrient agar	Milk	Peptone water	" + 2.5% NaCl	Glucose medium	Lactose "	Fructose "	Sorbitol "	Mannitol "	Pigment: Fluorescent	Pyocyanine	Brown	Breakdown of: Glucose	Lactose	Fructose	Sorbitol	Mannitol	Arginase	Changes in milk	Gelatinase	Casein precipitation	Tributyrinase	Egg yolk reaction	Catalase	Oxidase
S.1	5	-	MFP	2	Po	3	3	4	4	4	3	3	4	4	3	0	0	4	0	1	1	1	4	1	0	3	3	2	3	3
	15	-	MFP	2	Po	2	3	4	4	3	3	3	3	4	3	0	0	4	0	1	1	2	5	2	0	3	2	2	3	3
	25	-	MFP	2	Po	2	3	4	3	3	3	3	3	3	2	0	0	4	0	1	1	1	4	1	0	2	1	2	3	3
	30	-	MFP	2	Po	2	2	2	1	3	2	2	3	3	1	0	0	3	0	1	1	1	4	0	0	1	0	1	3	2
	37	-	MVC	1	Po	1	1	1	0	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	2
S.2	5	-	SF	0	0	2	2	2	1	2	2	3	2	2	0	0	0	0	0	0	0	0	0	0	0	0	4	2	2	0
	15	-	SF	0	0	2	3	3	1	3	2	3	2	2	0	0	0	0	0	0	0	0	0	0	0	0	4	2	2	0
	25	-	SF	0	0	2	3	2	1	2	2	1	2	2	0	0	0	0	0	0	0	0	0	0	0	0	3	3	2	0
	30	-	SF	0	0	2	3	2	0	2	2	2	2	2	0	0	0	0	0	0	0	0	0	0	0	0	2	2	2	0
	37	-	SF	0	0	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0
S.3	5	-	MFP	2	Po	3	3	3	3	3	3	3	3	3	2	0	0	4	0	1	0	1	4	3	1	2	4	0	3	3
	15	-	MFP	2	Po	3	4	4	3	3	3	4	3	3	3	0	0	4	0	1	0	1	4	4	2	3	3	0	3	3
	25	-	MFP	2	Po	3	4	3	2	3	3	3	3	3	2	0	0	4	0	1	0	1	4	2	1	1	1	0	3	3
	30	-	MT	2	Po	2	3	3	1	3	3	3	3	3	1	0	0	4	0	1	0	0	4	1	0	1	0	0	3	3
	37	-	TC	1	Po	0	0	1	1	0	0	0	1	1	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0
S.4	5	-	MLFV	2	Po	3	3	4	4	3	3	3	3	3	3	0	0	3	0	1	1	1	3	4	3	3	4	3	3	2
	15	-	MLFV	2	Po	3	4	4	4	4	3	3	3	4	2	0	0	4	0	1	3	1	4	4	3	3	4	2	3	3
	25	-	MLFV	2	Po	3	3	3	3	3	3	3	3	3	2	0	0	3	0	1	2	1	4	4	3	2	2	1	3	3
	30	-	MLF	2	Po	1	3	3	2	3	3	3	3	3	1	0	0	2	0	1	1	1	4	2	3	2	1	1	2	2
	37	-	MLF	2	Po	0	0	1	1	1	1	1	1	1	0	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0
S.5	5	-	LF	2	Po	3	3	4	3	3	3	3	2	3	0	0	0	4	1	1	0	0	5	a	0	0	0	0	3	3
	15	-	LF	2	Po	3	3	4	3	4	3	3	2	3	0	0	0	4	1	1	0	0	4	a	0	0	0	0	3	3
	25	-	LF	2	Po	3	3	3	2	3	3	3	3	3	0	0	0	4	0	1	0	0	4	a	0	0	0	0	3	3
	30	-	LF	2	Po	1	2	2	1	3	3	0	0	3	0	0	0	4	0	0	0	0	4	0	0	0	0	0	3	3
	37	-	LF	2	Po	1	1	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	3	0	0	0	0	0	2	2
S.6	5	-	MF	2	Po	3	4	4	3	2	2	2	2	2	2	0	0	1	0	0	0	0	5	2	1	0	4	0	3	3
	15	-	MFP	2	Po	2	3	4	3	4	3	2	3	3	2	0	0	4	0	0	0	0	5	2	1	0	3	1	3	3
	25	-	MFP	2	Po	2	3	3	2	3	3	3	3	3	1	0	0	4	0	1	0	0	5	2	2	1	1	2	3	3
	30	-	MFP	2	Po	2	3	3	1	3	3	3	3	3	1	0	0	4	0	0	0	0	5	1	0	0	0	0	3	3
	37	-	MVC	1	Po	1	0	1	0	1	1	0	1	1	0	0	0	2	0	0	0	0	3	0	0	0	0	0	2	2

Table 2. 2 Morphological, Cultural and Biochemical Characteristics and Reactions at 5°, 15°, 25°, 30° and 37° (Strains S7 - S12)

Strain	Temp. °C.	Gram reaction	Morphology	Motility	Flagella	Growth Nutrient agar	Milk	Peptone water	" + 2.5% NaCl	Glucose medium	Lactose "	Fructose "	Sorbitol "	Mannitol "	Pigment Fluorescent	Pyocyanine	Brown	Breakdown of Glucose	Lactose	Fructose	Sorbitol	Mannitol	Arginase	Changes in milk	Gelatinase	Casein precipitation	Tributyrinase	Egg yolk reaction	Catalase	Oxidase
S.7	5	-	MF	2	Po	4	4	4	4	4	4	3	2	4	0	0	0	4FG	0	3FG	2FG	2FG	2	1	1	1	4	2	3	0
	15	-	MF	2	Po	3	4	4	4	4	3	4	4	4	0	0	0	4FG	0	4FG	3FG	3FG	3	X	2	1	4	2	3	0
	25	-	MF	2	Po	3	4	3	2	4	3	4	4	4	0	0	0	4FG	0	4FG	4FG	4FG	3	X2	3	1	3	2	3	0
	30	-	MF	2	Po	2	3	3	1	3	3	3	3	3	0	0	0	4FG	0	3FG	3FG	3FG	3	X1	3	0	3	2	3	0
	37	-	LMC	1	Po	1	1	1	0	1	0	1	1	0	0	0	0	2F	0	2F	2F	0	0	a	0	0	1	0	2	0
S.8	5	-	MFP	2	Po	3	4	4	3	3	3	3	3	3	0	0	0	4	0	1	0	1	5	0	0	0	2	0	3	3
	15	-	MFP	2	Po	2	4	4	3	3	3	3	3	3	0	0	0	4	1	1	0	1	5	2	0	0	1	0	3	3
	25	-	MFP	2	Po	2	3	3	2	3	3	3	3	3	0	0	0	4	2	1	0	1	5	1	0	0	1	0	3	3
	30	-	MFP	2	Po	1	3	3	1	0	0	2	2	2	0	0	0	0	0	0	0	1	4	1	0	0	0	0	2	2
	37	-	MFP	2	Po	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	3	0	0	0	0	0	2	2
S.9	5	-	MFV	2	Po	3	4	4	4	4	3	3	4	4	2	0	0	3	0	1	2	1	4	4	2	1	4	2	3	2
	15	-	MFV	2	Po	3	4	4	4	4	3	3	4	3	3	0	0	4	0	1	3	3	4	4	3	3	3	3	3	3
	25	-	MFV	2	Po	3	3	3	3	3	3	3	3	3	3	0	0	2	0	1	1	1	4	3	3	1	2	2	3	3
	30	-	MFV	2	Po	2	3	2	2	0	0	3	3	2	1	0	0	0	0	1	1	1	4	2	2	1	1	1	3	2
	37	-	LMC	1	Po	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0
S.10	5	-	SMF	0	0	3	3	3	2	2	2	3	2	3	0	0	0	0	0	0	0	0	0	0	0	0	4	2	3	0
	15	-	SMF	0	0	3	3	3	2	3	2	3	3	3	0	0	0	0	0	0	0	0	0	2	0	0	4	3	3	0
	25	-	SMF	0	0	2	3	2	1	2	2	2	3	2	0	0	0	1	0	0	0	0	0	1	0	0	3	3	3	0
	30	-	SMF	0	0	2	3	2	0	2	2	2	2	2	0	0	0	0	0	0	0	0	0	1	0	0	2	1	2	0
	37	-	SF	0	0	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	2	0
S.11	5	-	MF	2	Pe	1	1	2	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0
	15	-	MF	2	Pe	2	4	2	2	3	3	3	3	3	0	0	0	3F	4FG	3FG	3FG	4FG	0	a	0	0	0	0	3	0
	25	-	MF	2	Pe	2	3	2	1	4	4	4	4	4	0	0	0	4FG	4FG	4FG	4FG	4FG	A	A	0	0	0	0	3	0
	30	-	MF	2	Pe	2	3	2	1	4	4	4	4	4	0	0	0	4FG	4FG	4FG	3FG	3FG	1	A	0	0	0	0	2	0
	37	-	MF	2	Pe	1	2	1	1	2	3	2	2	3	0	0	0	3FG	3FG	2FG	2FG	2FG	1	A	0	0	0	0	2	0
S.12	5	-	SF	2	Po	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	15	-	SMF	2	Po	2	3	3	2	3	2	1	2	3	3	2	0	2	0	0	0	3	3	2	1	0	3	2	3	2
	25	-	SMF	2	Po	3	3	3	2	4	3	3	4	4	3	3	0	4	0	0	0	4	4	4	3	1	2	3	3	3
	30	-	SMF	2	Po	4	3	3	3	4	2	2	3	3	3	3	0	4	0	0	0	4	5	4	3	1	2	3	3	3
	37	-	SMF	2	Po	4	4	4	3	3	2	2	2	2	3	3	0	3	0	0	0	2	4	4	4	1	1	3	3	3

Table 2. 3 Morphological, Cultural and Biochemical Characteristics and Reactions at 5°, 15°, 25°, 30° and 37° (Strains S13 - S18)

Strain	Temp. °C	Gram reaction	Morphology	Motility	Flagella	Growth Nutrient agar	Milk	Peptone water	" + 2.5% NaCl	Glucose medium	Lactose "	Fructose "	Sorbitol "	Mannitol "	Pigment Fluorescent	Pyocyanine	Brown	Breakdown of Glucose	Lactose	Fructose	Sorbitol	Mannitol	Arginase	Changes in milk	Gelatinase	Casein precipitation	Tributyrinase	Egg yolk reaction	Catalase	Oxidase
S.13	5	-	O	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	15	-	ST	0	0	2	2	3	2	1	1	1	1	1	0	0	0	1	0	0	0	0	0	0	0	0	2	0	2	0
	25	-	ST	0	0	3	3	3	2	3	3	2	2	3	0	0	0	3	1	0	0	0	0	1	1	0	1	0	2	0
	30	-	SMT	0	0	3	4	4	3	3	2	2	2	2	0	0	0	2	1	0	0	0	0	1	1	0	0	0	2	0
	37	-	SMT	0	0	3	3	3	2	2	2	2	2	1	0	0	0	2	0	0	0	0	0	0	0	0	0	0	2	0
S.14	5	-	ST	2	Po	3	3	3	2	3	0	0	1	3	3	0	BR	3	0	0	0	1	2	0	0	0	0	0	3	2
	15	-	ST	2	Po	4	4	4	3	4	1	1	2	3	2	0	BR	3	0	0	0	2	3	0	0	0	0	0	3	3
	25	-	ST	2	Po	4	3	3	2	4	1	1	3	4	2	0	BR	3	0	0	0	2	2	0	0	0	0	0	3	3
	30	-	ST	2	Po	3	3	2	2	3	1	1	1	2	1	0	0	2	0	0	0	1	2	0	0	0	0	0	3	3
	37	-	LTC	1	Po	1	1	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	1	2
S.15	5	-	ST	2	Po	3	3	4	3	3	0	1	2	3	2	0	0	4	0	0	1	4	4	2	3	0	3	2	3	3
	15	-	ST	2	Po	4	3	4	4	4	1	2	2	3	3	0	0	4	0	1	1	3	4	3	3	0	2	2	3	3
	25	-	ST	2	Po	4	3	3	3	4	2	2	3	4	2	0	0	4	0	1	1	3	4	2	3	0	2	2	3	3
	30	-	ST	2	Po	2	1	2	2	3	1	2	2	3	1	0	0	2	0	0	0	1	3	2	2	0	1	0	3	2
	37	-	LST	1	Po	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
S.16	5	-	MTV	2	Po	2	1	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	2
	15	-	MTV	2	Po	3	2	2	2	2	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	3
	25	-	MLTV	2	Po	3	2	3	2	3	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	3
	30	-	LTV	2	Po	2	2	2	1	3	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	2
	37	-	LT	2	Po	2	1	1	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	2
S.17	5	-	ST	2	Po	2	2	3	3	3	1	0	0	0	0	0	0	2	0	0	0	0	0	1	1	1	0	0	3	3
	15	-	ST	2	Po	3	3	4	4	3	2	1	2	2	0	0	0	2	1	0	0	0	0	2	2	2	0	0	3	2
	25	-	ST	2	Po	3	4	3	2	3	2	2	2	2	0	0	0	2	1	1	0	0	0	2	1	2	0	0	3	1
	30	-	ST	2	Po	3	3	2	1	2	2	1	0	1	0	0	0	1	1	1	0	0	0	0	0	0	0	0	3	1
	35	-	LSTC	1	Po	2	1	2	0	1	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	2	0
S.18	5	-	SM	2	Po	2	2	2	1	2	0	0	0	0	0	0	0	3	0	0	0	0	0	0	0	0	0	0	3	2
	15	-	SM	2	Po	3	3	3	3	2	1	1	1	2	2	0	0	3	0	0	0	0	1	0	0	0	0	0	3	3
	25	-	SM	2	Po	4	4	3	2	3	1	1	2	2	2	0	0	4	0	0	0	0	0	0	0	0	0	0	3	3
	30	-	SM	2	Po	4	4	2	2	2	1	1	1	1	2	0	0	3	0	0	0	0	0	0	0	0	0	0	3	3
	37	-	SM	2	Po	3	3	2	1	2	0	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	3	3

Table 2. 4 Morphological, Cultural and Biochemical Characteristics and Reactions at 5°, 15°, 25°, 30° and 37° (Strains S19 - S24)

Strain	Temp °C.	Gram reaction	Morphology	Motility	Flagella	Growth Nutrient agar	Milk	Peptone water	" + 2.5% NaCl	Glucose medium	Lactose "	Fructose "	Sorbitol "	Mannitol "	Pigment Fluorescent	Pyocyanine	Brown	Breakdown of Glucose	Lactose	Fructose	Sorbitol	Mannitol	Arginase	Changes in milk	Gelatinase	Casein precipitation	Tributyrinase	Egg yolk	Catalase	Oxidase
S.19	5	-	HT	2	Po	4	4	4	3	3	2	2	3	3	0	0	0	AFG	0	AF	AFG	AFG	4	3	3	3	4	2	3	3
	15	-	HT	2	Po	4	4	4	3	4	3	3	4	4	0	0	0	AFG	1	AFG	AFG	AFG	4	4	4	2	4	2	3	3
	25	-	HT	2	Po	3	4	3	2	4	3	3	4	4	0	0	0	AFG	1	AFG	AFG	AFG	5	4	4	2	3	3	3	3
	30	-	MHT	2	Po	3	3	3	1	3	3	2	3	3	0	0	0	AFG	1	AF	AFG	AFG	4	3	3	2	3	2	3	2
	37	-	MHTC	1	Po	2	3	2	1	1	1	1	2	2	0	0	0	2	0	0	0	0	2	1	1	0	2	1	2	2
S.20	5	-	SHT	2	Po	3	3	4	4	3	1	1	3	3	3	0	0	4	0	0	4	4	4	3	1	0	2	3	3	3
	15	-	MHP	2	Po	3	4	4	4	3	2	2	2	3	2	0	0	4	0	0	4	3	5	4	4	3	3	3	3	3
	25	-	MHP	2	Po	3	3	3	3	3	2	2	2	2	2	0	0	4	0	1	4	3	4	3	3	2	3	3	3	3
	30	-	MHP	2	Po	2	3	2	1	2	2	2	2	2	1	0	0	3	0	1	2	2	3	3	3	1	2	1	3	2
	37	-	HHC	1	Po	0	1	1	0	1	0	0	0	1	0	0	0	1	0	0	0	1	1	1	0	0	0	0	0	0
S.21	5	-	SHT	2	Po	3	3	3	3	3	1	1	1	2	2	0	0	3	0	0	0	0	4	0	0	0	0	0	3	3
	15	-	HHT	2	Po	3	4	3	3	3	2	1	2	2	3	0	BR	3	1	0	0	0	4	0	0	0	0	0	3	3
	25	-	HHT	2	Po	3	3	2	2	3	2	2	2	2	2	0	BR	3	1	1	0	0	4	0	0	0	0	0	3	3
	30	-	HT	2	Po	2	2	2	1	2	1	2	1	1	1	0	0	3	1	1	0	0	3	0	0	0	0	0	3	3
	37	-	HHC	0	0	0	0	1	1	1	0	0	0	0	0	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0
S.22	5	-	HHT	2	Po	1	1	2	1	1	1	1	0	0	0	0	0	1	0	0	1	1	0	0	0	0	0	0	2	0
	15	-	HT	2	Po	4	3	4	3	2	1	1	1	1	0	0	BR	1	0	0	2	2	0	0	0	0	0	0	3	0
	25	-	HT	2	Po	3	4	3	2	3	1	1	1	1	0	0	BR	2	1	1	2	2	0	0	0	0	0	0	3	1
	30	-	HT	2	Po	3	3	3	0	3	2	1	1	1	0	0	BR	3	1	1	2	2	0	0	0	0	0	0	3	1
	37	+	HHC	1	Po	1	2	2	0	2	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1	0
S.23	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	15	-	SHT	2	Po	3	2	3	2	1	1	1	1	1	1	0	0	0	0	0	0	1	2	2	2	2	2	2	3	2
	25	-	SHT	2	Po	3	3	3	2	2	1	1	2	3	2	0	BR	1	0	0	0	2	3	2	1	1	2	3	3	3
	30	-	SHT	2	Po	3	3	3	3	3	2	2	2	3	2	0	BR	1	0	1	0	2	3	2	1	1	3	3	3	3
	37	-	SHT	2	Po	3	3	3	3	3	1	1	1	3	2	0	0	1	0	0	0	1	2	2	1	1	4	3	3	3
S.24	5	-	SHT	2	Po	1	1	2	1	1	1	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0
	15	-	SHT	2	Po	3	2	3	3	2	2	1	2	1	0	0	0	1	0	0	1	1	0	2	1	1	1	2	3	1
	25	-	SHT	2	Po	3	3	2	1	2	2	2	3	2	0	0	0	1	1	0	1	1	0	2	1	1	3	2	3	1
	30	-	SHT	2	Po	2	2	2	1	2	1	1	1	2	0	0	0	1	1	0	1	1	0	1	0	0	3	2	3	1
	37	-	SHT	2	Po	1	1	1	0	1	1	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	0

Results and Discussion

Tables 2. 1 - 2. 4 (pages 37, 38, 39 and 40) show the evaluated characteristics and reactions of strains S1 - S24 towards tests performed at 5°, 15°, 25°, 30° and 37°.

The variations shown in the response of different strains towards individual tests over the temperature range and the effect of temperature on the characteristics and reactions of individual strains required a separate examination of the results of each strain.

Strain S1

A psychrophilic isolate from milk, this Pseudomonas strain produced good growth at 5° but not at 15° to 37° on nutrient agar, although growth in milk was good at 5°, 15° and 25°. Heaviest growth was obtained in peptone water at 5°, 15° and 25° and although the addition of 2.5% sodium chloride inhibited growth at 30° and eliminated growth at 37° in this medium the level of growth was not reduced at 5° or 15°. Good growth was produced, particularly at 5°, and at 15°, 25° and 30° in glucose, sorbitol and mannitol media and at 5°, 15° and 25° in lactose and fructose media. Growth at 37° was sparse.

The morphology and motility were similar at 5° to 30°, while at 37° elongated and 'clubbed' forms with associated loss of motility were observed.

Fluorescent pigment production was greatest at 5° and 15°, being less at 25°, further reduced at 30° and absent at 37°.

The oxidative breakdown of glucose was extensive at 5° 15° and 25°, reduced at 30° and absent at 37°. Acid production from fructose, sorbitol and mannitol was generally poor at 5° to 30° and no change was produced at 37° from these carbohydrates or from lactose.

Arginase activity was maximal at 15°, appreciable at 5°, 25° and 30° and negative at 37°. Although the strain did not liquefy gelatine, casein was precipitated well at 5° and 15°, less well at 25° and poorly at 30° while no reaction was observed at 37°. An extensive alkaline change in milk without proteolysis was observed at 15°, slight alkaligenesis was found at 5° and 25° and despite moderate growth in milk at 30° ho change was observed. At 37° growth in milk was poor.

Tributyrinase activity was greatest at 5° and was progressively reduced at 15° and 25°, no activity being observed at 30° or 37°. The effect of temperature on the egg yolk reaction was less pronounced than on tributyrinase activity with moderate activity at 5°, 15° and 25° and slight activity at 30°.

Catalase was more active at 5° 15° and 25° than at 30° and 37° and the oxidase reaction was rapid at 5° to 30° and slower at 37°.

Strain S2

Isolated at 5° from milk this Achromobacter strain produced moderate growth at 5° and did not produce very heavy growth in any of the test media. Heaviest growth was observed in milk at 15° to 30°, in peptone water and glucose medium at 15° and in fructose medium at 5° and 15°. Growth on Nutrient agar and in other carbohydrate media was moderate

at 5° to 30°. The addition of sodium chloride to peptone water resulted in sparse growth at 5°, 15° and 25° and the elimination of growth at 30°. Growth at 37° was absent except in peptone water, sorbitol medium and, while not recorded in the table of results, moderate growth was produced at 37° on Tributyrin agar.

The morphology of this strain was unchanged at 5° to 37°.

The strain was inactive towards all biochemical tests except the tributyrinase and egg yolk reactions. Maximal tributyrinase activity was displayed at 5° and 15° and a progressive reduction in activity was shown with increase in temperature. A slight tributyrinase activity was observed at 37°. Moderate catalase activity was shown at 5° to 30°.

Strain S3

This psychrophilic Pseudomonas isolate from a pipeline rinse produced maximal growth in milk at 15° and 25°, and in peptone water and fructose medium at 15°. Growth in liquid media was generally good between 5° and 30° while growth on Nutrient agar at 30° was moderate. Above 15° sodium chloride caused a marked reduction in growth in peptone water. Growth at 37° was absent or sparse in all test media.

Rods of similar morphology were observed at 5° to 30° and at 37° 'clubbed' forms with reduced motility were found.

The fluorescent pigment of this strain was intense at 15°, less marked at 5° and 25°, slight at 30° and absent at 37°.

The pattern of carbohydrate breakdown in relation to temperature was similar to strain S1, except that strain S3 did not degrade sorbitol. The oxidative production of acid from glucose was maximal at 5° to 30°.

The breakdown of arginine was maximal at 5° to 30° and much reduced at 37°. The strain exhibited differences in the changes produced in milk over the temperature range. Alkaline proteolysis was greatest at 15° and somewhat less at 5°. At 25° an alkaline change was produced without proteolysis while slight alkaligenesis was observed at 30°. Moderate gelatinase activity was found at 15° while activity was poor at 5° and 25° and absent at 30° and 37°.

No egg yolk reaction was obtained but tributyrinase activity was maximal at 5°, less at 15°, poor at 25° and absent at 30° and 37°.

The oxidase reaction and catalase activity were extensive at 5° to 30°.

Strain S4

Isolated as a psychrophile from milk this Pseudomonas strain showed heaviest growth at 15° in this medium and glucose and mannitol media and at 5° and 15° in peptone water with or without sodium chloride. Growth at 5° to 30° was generally good in all liquid media but growth was poor at 30° on Nutrient agar. Growth at 37° was sparse in all media except Nutrient agar and milk in which growth did not occur. Sodium chloride did not appreciably affect the level of growth in peptone water at any temperature.

At 5° to 25° curved rods were generally observed while at 30° and 37° fewer 'vibrio' forms were found.

The production of fluorescent pigment was greatest at 5°, less at 15° and 25°, poor at 30° and absent at 37°.

The oxidative breakdown of glucose was most apparent at 15°, less at 15° and 25°, further reduced at 30° and poor at 37°. The breakdown

of sorbitol was less vigorous than glucose but followed a similar pattern throughout the temperature range. Slight acid production from fructose and mannitol was shown at 5° to 30°.

Arginase activity was maximal at 15°, 25° and 30°, slightly reduced at 5° and poor at 37°. Gelatinase activity and the precipitation of casein were appreciable at 5° to 30° and extensive alkaline-proteolysis of milk was obtained at 5° to 25° while at 30° alkaligenesis without proteolysis was produced.

Tributyrinase activity was greatest at 5° and 15°, much reduced at 25°, poor at 30° and absent at 37°. While the egg yolk reaction was less active the pattern of activity was similar to tributyrinase at 5° to 37°.

Catalase activity was marked at 5° to 25° and reduced at 30° while the oxidase reaction was instantaneous at 15° and 25° and less rapid at 5° and 30°.

Strain S5

This psychrophilic Pseudomonas strain, isolated from milk, produced heaviest growth in peptone water at 5° and 15° and in glucose medium at 15°. Growth on Nutrient agar and in milk and carbohydrate media was generally good at 5°, 15° and 25°. Growth at 30° on Nutrient agar was poorer than in milk, peptone water and some carbohydrate media but growth was not produced at 30° in fructose and sorbitol media. Sodium chloride reduced growth in peptone water at all temperatures and inhibited growth at 37° completely. Sparse growth only occurred at 37° on Nutrient agar and in milk, peptone water and mannitol medium.

The morphology and motility of this strain were similar at all temperatures.

The oxidative breakdown of glucose was maximal at 5°, 15° 25° and 30° while the production of acid from other carbohydrates was poor and observed only at 5° and 15° in lactose medium and at 5°, 15° and 25° in fructose medium.

Greatest arginase activity was displayed at 5° although appreciable activity was shown at 15°, 25° and 30°. Growth at 37° in the medium was accompanied by a moderate arginase activity. The strain displayed no precipitation of casein, gelatinase activity, lipase activity or egg yolk reaction. A slight acid change without coagulation was observed in milk at 5°, 15° and 25° but no change was observed at 30° or 37°.

The oxidase reaction and catalase activity were fully positive at 5° to 30° and only slightly reduced at 37°.

Strain 86

The growth of this psychrophilic Pseudomonas strain isolated from a bulk milk tank was heaviest in peptone water at 5° and 15°, in milk at 5° and in glucose medium at 15°. The strain produced better growth at 5° to 30° in milk and peptone water than on Nutrient agar. While growth in carbohydrate media was good at 15° to 30° growth at 5° was reduced by comparison with other media. Sodium chloride reduced growth in peptone water at all temperatures but the effect was more apparent at 25°, 30° and 37°. Growth at 37° was generally absent or sparse in all media.

Involution and 'clubbed' forms with reduced motility were observed at 37°.

Moderate fluorescent pigment was produced at 5° and 15°, less at 25° and 30° and none at 37°.

The oxidative production of acid from glucose was maximal at 15° to 30°, much reduced at 37° and poor at 5°. Fructose was the only other carbohydrate degraded by this strain, a slight acid change being observed at 25°.

Extensive arginase activity was displayed at 5° to 30° with moderate activity at 37°. Gelatinase activity was moderate at 25°, poor at 5° and 15° and negative at 30° and 37° while slight casein precipitation was observed only at 25°. Appreciable alkaligenesis without proteolysis was produced in milk at 5°, 15° and 25°, a slight alkaline change was shown at 30° while no change was observed at 37°.

Tributyrinase activity was maximal at 5°, slightly reduced at 15°, poor at 25° and absent at 30° and 37°. A moderate egg yolk reaction was shown at 25° which was reduced at 15° and no reaction was observed at 5°, 30° and 37°.

The oxidase reaction and catalase activity were strong at 5° to 30° and slightly less at 37°.

Strain 57

A psychrophilic isolate from a pipeline rinse, this Aeromonas strain produced very heavy growth at 5°, 15° and 25° in milk and in glucose and mannitol media. Growth on nutrient agar and lactose medium was heaviest at 5° but in fructose and sorbitol media was less heavy at 5° than at 15° and 25°. Growth in peptone water at 5° and 15° was equally good with and without sodium chloride but this compound reduced growth appreciably at 25° to 37°.

Involution forms and 'clubbed' variants of the normal cocco-rods

were observed at 37°, at which temperature reduced motility was also displayed.

All carbohydrates except lactose were fermented at 5° to 30° with the production of gas. Acid production from glucose was maximal at 5° to 30°, from fructose at 15° and 25° and from sorbitol and mannitol at 25° only. At 37° moderate levels of acid were produced without aerogenesis in glucose, fructose and sorbitol media.

Moderate arginase activity was shown at 15°, 25° and 30° while activity was reduced at 5° and absent at 37°. The ability to liquefy gelatine was poor at 5° and increased to a maximum at 25° and 30° but was absent at 37° while the precipitation of casein was poor at 5° to 25° and negative at 30° and 37°. In milk a slight alkaline change was observed at 5° and a neutral soft coagulation was found at 15°. Neutral coagulation at 25° and 30° was followed by a moderate alkaline change at 25° and a slight alkaline change at 30°. At 37° a slight acid change without coagulation was observed. Tributyrinase activity was maximal at 5° and 15°, appreciable at 25° and 30° and poor at 37° while a moderate egg yolk reaction was obtained at 5° to 30° with no change at 37°.

The catalase reaction was vigorous at 5° to 30° and slightly less at 37°.

Strain 88

This Pseudomonas strain isolated at 5° from the swab of a bulk milk tank was able to grow well at 5° to 25° in most media. Good growth was observed at 5° on Nutrient agar but growth was less at 15° and 25° and poor at 30° and 37°. Growth was heaviest in milk and peptone water at

5° and 15° and good at 25° and 30°. Sodium chloride reduced growth in peptone water at all temperatures. Glucose and lactose media did not support growth at 30° or 37° and other carbohydrate media did not support growth at 37°.

The morphology and motility of this strain were similar at all temperatures.

The oxidative breakdown of glucose was maximal at 5°, 15° and 25° and absent at 30° and 37°. Limited acid production from lactose occurred at 25° with slight acid at 15°. No change was observed at 5° despite good growth. A slight acid change was observed at 5° to 30° in fructose and mannitol media.

Arginase activity was very strong at 5°, 15° and 25°, strong at 30° and an appreciable activity accompanied moderate growth at 37°. The casein precipitation reaction and gelatinase activity were negative. Appreciable alkaligenesis was observed in milk at 15° with a reduced alkaline change at 25° and 30° and no change at 37°. No change was observed at 5° despite good growth in milk.

The egg yolk reaction was not shown while tributyrinase activity was limited at 5°, poor at 15° and 25° and absent at 30° and 37°.

Catalase activity was vigorous and the oxidase reaction was rapid at 5°, 15° and 25°, while reactions at 30° and 37° were slightly lower.

Strain S9

Isolated as a psychrophile from milk this Pseudomonas strain produced good growth at 5°, to 25° in all media. A similar growth pattern, with maximal growth at 5° and 15° and heavy growth at 25°, was shown in

milk, peptone water with and without sodium chloride and in glucose and sorbitol media. Growth at 37° was absent in all media except peptone water with and without sodium chloride.

Morphology and motility were similar at 5° to 30° with less motile 'clubbed' forms being observed at 37°.

Fluorescent pigment production was greatest at 15° and 25°, less at 5°, poor at 30° and negative at 37°.

The oxidative breakdown of glucose was maximal at 15°, less at 5° and further reduced at 25°. A similar pattern of acid production at 5° to 30° was observed in sorbitol and mannitol media while slight acid was produced in fructose medium at the same temperatures.

A strong arginase reaction was observed at 5° to 30° with no change at 37°. Casein precipitation was appreciable at 15° and poor at 5°, 25° and 30° while extensive gelatinase activity was shown at 15° and 25° with reduced activity at 5° and 30°. An extensive alkaline-proteolysis of milk was observed at 5° and 15° with limited alkaline-proteolysis at 25° and alkaligenesis without proteolysis at 30°. None of these reactions were shown at 37°.

Tributyrin hydrolysis was greatest at 5° and showed a progressive decrease with increase in temperature while the egg yolk reaction was maximal at 15°, reduced at 5° and 25°, was poor at 30° and negative at 37°.

The oxidase reaction was instantaneous at 15° and 25° and rapid at 5° and 30° while catalase activity was vigorous at 5° to 30°.

Strain 810

This Achromobacter strain, isolated at 5° from a pipeline rinse

produced good growth at 5° to 30° in milk and at 5° and 15° on nutrient agar, in peptone water and in fructose and mannitol media. Growth in glucose, lactose and sorbitol media was less good at 5° than in other meida and no growth was observed in any carbohydrate media at 37°. Sodium chloride inhibited growth appreciably at 5°, 15°, and 25° and completely repressed growth at 30° and 37°.

Non-motile cocco-rods were observed at 5° to 30° while at 37° a distinct coccal morphology was more prevalent.

The strain produced a slight oxidative change in glucose medium only at 25° and no other carbohydrates were degraded with the production of acid.

Arginase activity, casein precipitation and gelatinase activity were absent. An appreciable alkaline change was observed in milk at 15°, which was less apparent at 25° and 30° and absent at 5° and 37°.

Tributyrinase activity was maximal at 5° and 15° and a progressive reduction in activity was observed from 25° to 37°. Strong egg yolk reactions were obtained at 15° and 25° and the reaction was less at 5°, weak at 30° and negative at 37°.

Catalase activity was marked at 5°, 15° and 25° and less at 30° and 37°.

Strain S11

A psychrophilic isolate obtained by swabbing a cow teat, this coliform strain produced moderate growth in peptone water at 5°. Growth at this temperature was poor on nutrient agar, in milk and peptone water with sodium chloride and absent in carbohydrate media. Heaviest growth was obtained in milk at 15° and in carbohydrate media at 25° and 30° and

growth at 37° was better in these than in other media. Sodium chloride inhibited growth in peptone water at all temperatures except 15°.

The morphology and motility of this strain showed little variation at 5° to 37°.

The fermentation of glucose with acid and gas was greatest at 25° and 30° and while aerogenesis with reduced acid was produced at 37° acid production without gas was shown at 15°. Aerogenesis was observed during the fermentation of other carbohydrates at 15° to 37° although acid production was reduced at 37°.

No arginase activity was observed at 5° and 15° while a marked acid reaction was found at 25° and a slight alkaline reaction was noted at 30° and 37°. Gelatinase activity, casein precipitation, tributyrinase activity and the egg yolk reaction were negative at all temperatures. Acid coagulation of milk was observed at 25°, 30° and 37° whereas slight acid was produced without coagulation at 15°. No change was observed at 5° in milk.

Vigorous catalase activity was shown at 15° and 25° with moderate activity at 30° and 37° and poor activity at 5°.

Strain S12

Ps. aeruginosa, a mesophile, was able to grow at 5°, albeit poorly, only in peptone water and growth in carbohydrate media displayed a progressive increase with increase in temperature. Growth was heaviest in glucose medium at 25° and 30° and at 25° in other carbohydrate media. Sodium chloride reduced growth in peptone water at 15°, 25° and 37° and completely inhibited growth at 5°.

Little variation in morphology and motility was observed at 5° to 37°

Strong fluorescence was observed at 15° to 37° and pyocyanine production was marked at 25°, 30° and 37° and slightly less at 15°.

The oxidative production of acid from glucose and mannitol was maximal at 25° and 30°, being reduced at 15° and 37°. Acid was not produced from other carbohydrates.

Arginase activity was very strong at 30°, strong at 25° and 37° and less at 15°. Gelatinase activity was poor at 15° and increased with increase in temperature to 37°, while the precipitation of casein was absent at 15° and poor at 25°, 30° and 37°. Extensive alkaline proteolysis of milk was displayed at 25°, 30° and 37° and an alkaline change without proteolysis was produced at 15°.

Tributyrinase activity was maximal at 15° and declined with increase in temperature, being poor at 37°, while the egg yolk reaction was maximal at 25°, 30° and 37° and reduced at 15°.

Catalase activity was vigorous at 15° to 37° and the oxidase reaction was rapid at 25° to 37° and slower at 15°.

Strain 813

Bacterium anitratum produced heaviest growth at 30° in milk and peptone water. Growth was best in carbohydrate media at 25° while growth was poorer at 15° and 37° in these media than on nutrient agar, in milk or in peptone water. Sodium chloride reduced growth in peptone water at 15° to 37° and growth was absent in all media at 5°.

The morphology of this non-motile strain was similar at each temperature.

The oxidative breakdown of glucose was greatest at 25°, reduced

at 30° and 37° and poor at 15°. Limited lactose breakdown occurred at 25° and 30° and no change was observed at 15° or 37°.

Arginase activity and casein precipitation were not shown. Poor gelatinase activity was displayed only at 25° and 30° at which temperatures limited alkaligenesis was produced in milk.

The egg yolk reaction was negative while moderate tributyrinase activity was observed at 15° with loss activity at 25°. Tributyrin hydrolysis was not observed at 30° or 37°.

A moderate catalase reaction was obtained at 15° to 37°.

Strain 814

The growth of this Pseudomonas isolate from soil was maximal at 15° and 25° on nutrient agar and in glucose medium and at 15° in milk and peptone water. Growth in mannitol medium was heavy at 25° but lactose and fructose media supported poor growth only at 15°, 25° and 30°. Good growth was obtained at 5° on nutrient agar and in milk, peptone water, glucose and mannitol media while some growth was observed at 37° in all except mannitol medium. Sodium chloride reduced growth at 5° to 30° in peptone water and inhibited growth completely at 37°.

Morphology and motility were similar at 5° to 30° but some loss of motility, at 37° was associated with elongation and 'clubbing' of the bacteria.

Fluorescent pigment production was greatest at 5°, less at 15° and 25° and poor at 30° with none being observed at 37°. A brown pigment was produced at 5°, 15° and 30° but not at 30° or 37°.

The oxidative breakdown of glucose was moderate at 5°, 15° and 25°, reduced at 30° and absent at 37°. Acid production from mannitol

was limited at 15° and 25° and poor at 5° and 30°.

Arginase activity was appreciable at 15°, less at 5°, 25° and 30° and poor at 37°. Other biochemical reactions were negative at 5° to 37°.

Catalase activity was vigorous at 5° to 30° but poor at 37° and the oxidase reaction was rapid at 15°, 25° and 30° and less rapid at 5° and 37°.

Strain S15

This strain of Ps. fluorescens produced very heavy growth on nutrient agar and in glucose medium at 15° and 25° while growth in peptone water was maximal at 5° and 15°. The addition of sodium chloride resulted in growth at 15° to 30° similar to that obtained in peptone water, reduced growth at 5° and the complete suppression of growth at 37°. Growth in milk was good at 5°, 15° and 25° but poor at 30° and while the growth response was also good in glucose and mannitol media at 5° to 30° poorer growth was general in other carbohydrate media. Peptone water alone supported growth, albeit poor, at 37°.

Motile short rods were observed at 5° to 30° while non-motile elongated forms were found at 37°.

Fluorescent pigment was strongest at 15°, less strong at 5° and 25° and weak at 30°.

The oxidative breakdown of glucose was extensive at 5°, 15° and 25° and much reduced at 30°. Slight acid production was observed between 5° and 25° in fructose and sorbitol media and acid production

from mannitol was extensive at 5°, less at 15° and 25° and poor at 30°.

Vigorous arginase activity was noted at 5°, 15° and 25° with a reduced reaction at 30°. The casein precipitation reaction was negative although gelatinase activity was maximal at 5°, 15° and 25° and reduced at 30°. Alkaline-proteolysis of milk was observed at 15° and alkaligenesis without proteolysis was found at 5°, 25° and 30°.

Tributyrinase activity was greatest at 5° and decreased with increase in temperature while the egg yolk reaction was moderate at 5°, 15° and 25° and negative at 30°. Growth was not observed on these media at 37°.

The oxidase reaction was very rapid at 5°, 15° and 25° and slightly delayed at 30°, while catalase activity was vigorous at 5° to 30°.

Strain S16

Comamonas percolens did not produce heavy growth at any temperature in any test medium. Best growth was produced on nutrient agar at 15° and 25°, in peptone water at 25° and in glucose medium at 25° and 30°. Poor growth in other carbohydrate media was restricted to between 15° and 30°. Growth was absent at 5° and 37° in these media and in peptone water with sodium chloride, and growth was sparse at 5° and 37° in other media except nutrient agar.

Motility was apparent at 5° to 37° and the normal curved-rod type of morphology was found at 5° to 30°. At 37° elongation of the rods tended to mask the typical shape of the bacteria.

The strain was biochemically inactive at all test temperatures.

Extremely rapid oxidase reactions were obtained at 15° and 25° and

were less rapid at 5°, 30° and 37°. Although not vigorous, catalase activity was appreciable at all temperatures.

Strain S17

The heaviest growth of this Pseudomonas isolate from soil was observed in milk at 25° and in peptone water with or without sodium chloride at 15°. Good growth was found on nutrient agar at 15° to 30° and at 5° to 25° in glucose medium. Moderate growth was observed on nutrient agar and in milk at 5° while growth in carbohydrate media other than glucose was moderate or sparse and restricted to between 15° and 30°. Growth at 37° was moderate on nutrient agar and in peptone water but sparse or absent in other media. Sodium chloride reduced growth markedly at 25° and 30° and completely inhibited growth at 37°.

Some involution forms of the normal slender rods, with reduced motility were observed at 37°.

A moderate oxidative breakdown of glucose was found at 5°, 15° and 25° while acid production at 30° and 37° was poor. No change was produced in other carbohydrate media.

Gelatine liquefaction was limited at 15° and poor at 5° and 25° with no change at 30° or 37°. The precipitation of casein was appreciable at 15° and 25°, slight at 5° and absent at 30° and 37°. Alkaligenesis with proteolysis was observed at 5°, 15° and 25° with no change being determined in milk at 30° and 37°.

Tributyrinase activity and the egg yolk reaction were negative at all test temperatures.

The oxidase reaction was very rapid at 5° but progressively slower reactions were observed with increase in temperature and a delayed

reaction was obtained at 37°. Catalase activity was maximal at 5° to 30° and moderate at 37°.

Strain S18

The growth of Ps. putida was heaviest on nutrient agar and in milk at 25° and 30°, with good growth at 15° and 37° and only moderate growth at 5°. Growth in peptone water and glucose medium followed a similar pattern. In other carbohydrate media growth was moderate or poor and restricted to between 15° and 30°. Sodium chloride inhibited growth generally throughout 5° to 37°.

Morphology and motility were similar at all test temperatures.

The production of fluorescent pigment was moderate at 15°, 25° and 30°. Faint fluorescence was observed at 37° but was not detected at 5°

The production of acid by the oxidative breakdown of glucose was maximal at 25° and appreciable at 5°, 15° and 30° while only a slight change was observed at 37°. Other carbohydrates were not degraded with acid production.

The strain exhibited slight arginase activity only at 15° but in other respects was biochemically inactive.

Catalase activity was marked at all test temperatures and the oxidase reaction was rapid at 15° to 37° and slightly less rapid at 5°.

Strain S19

This Aeromonas strain produced heaviest growth on nutrient agar and in peptone water at 5° and 15° and in milk at 5°, 15° and 25° while appreciable growth was displayed in these media at 37°. Heavy growth was also produced in glucose, sorbitol and mannitol media at 15° and 25° with appreciable growth at 5° and less at 37°. Sodium chloride

reduced growth in peptone water at all temperatures and especially at 30° and 37°.

Morphology and motility were similar at 5° to 30° but elongated and 'clubbed' forms at 37° were often accompanied by a reduction in motility.

The ability of this strain to attack carbohydrates fermentatively varied with the substrate involved and with temperature. Extensive acid production with gas was obtained from glucose, sorbitol and mannitol at 5° to 30° whereas limited acid without gas was obtained only from glucose at 37°. Acid production with aerogenesis was also obtained from fructose at 15° and 25° whereas fermentation without aerogenesis was active at 5° and 30°. A slight oxidative change was noted in lactose medium only at 15°, 25° and 30°.

Arginase activity was maximal at 25° and marked at 5°, 15° and 30° with moderate activity at 37°. Gelatine liquefaction was extensive at 15° and 25° and appreciable at 5° and 30°, slight liquefaction being produced at 37°. The precipitation of casein was absent at 37°, moderate at 15°, 25° and 30° and maximal at 5°. In milk extensive alkaligenesis with complete proteolysis was observed at 15° and 25° with less complete proteolysis at 5° and 30° and only a limited alkaline change at 37°.

Tributyrinase activity was maximal at 5° and 15° and decreased with increase in temperature, being limited at 37°. The greatest egg yolk reaction was observed at 25°, the reaction being reduced at 5°, 15° and 30° and poor at 37°.

The oxidase reaction was rapid at 5°, 15° and 25° and slightly delayed at 30° and 37°, while catalase activity was vigorous at 5° to 30° and slightly reduced at 37°.

Strain S20

This Pseudomonas strain grew well at 5° in all except lactose and fructose media and growth was sparse or absent at 37° in all media. Growth was maximal at 15° in milk and at 5° in peptone water with or without sodium chloride, although this compound inhibited growth markedly at 30° and completely at 37°.

Elongated and 'clubbed' forms of the normal medium-sized pointed rods were observed at 37° and some loss of motility was associated with this change in morphology.

Fluorescent pigment production was maximal at 5°, decreased with increase in temperature and was not detected at 37°.

The oxidative production of acid from glucose and sorbitol was greatest at 5°, 15° and 25°, reduced at 30° and little or no change was observed at 37°. Mannitol was actively degraded at 5° and degradation was less with increase in temperature. A slight oxidative change was observed in fructose media only at 25° and 30°.

Arginase activity was maximal at 15°, slightly less at 5° and 25°, further reduced at 30° and slight at 37°. The precipitation of casein was greatest at 15° with reduced reaction at 25° and 30° and no reaction at 5° or 37°. The liquefaction of gelatine followed a similar pattern. The alkaline-proteolysis of milk was extensive at 15° while proteolysis was less apparent at 5°, 25° and 30°. A slight alkaline change was produced in milk at 37°.

Tributyrinase activity was marked at 15° and 25°, less at 5° and 30° and negative at 37° while an appreciable egg yolk reaction was observed at 5°, 15° and 25°, being reduced at 30° and absent at 37°.

Catalase activity was vigorous at 5° to 30° and the oxidase reaction was rapid at 5° to 25° and slightly delayed at 30°.

Strain S21

Good growth was produced by this Pseudomonas strain at 5°, 15° and 25° on nutrient agar and in milk and glucose medium. Growth in peptone water with or without sodium chloride was good at 5° and 15° and growth was generally moderate or poor in carbohydrate media other than containing glucose. Growth at 37° was sparse or absent in all media.

The strain exhibited involution and 'clubbed' forms, which were less motile of the normal bacteria at 37°.

Green fluorescent pigment was marked at 15° and less at 5° and 25°. The pigment was faint at 30° and absent at 37°. A brown pigment was also observed only at 15° and 25°.

An appreciable oxidative breakdown of glucose was found at 5° to 30° with a slight change at 37°. Weak acid production was noted in lactose medium at 15°, 25° and 30° and in fructose medium at 25° and 30° only.

A strong arginase activity was observed at 5°, 15° and 25° with a reduced reaction at 30° and a poor reaction at 37°.

This biochemically inactive strain produced no changes in protein or lipid media and milk remained unchanged despite good growth.

The catalase and oxidase reactions were strong at 5° to 30°.

Strain S22

Agrobacterium tumefaciens produced poor or no growth in most media

at 5° and 37°. Growth on nutrient agar, in peptone water, with or without added sodium chloride, was heaviest at 15°, while growth in milk was heaviest at 25°. Good growth was obtained in glucose medium at 25° and 30° but growth in other carbohydrate media was generally poor. Over the range 5° to 37° growth in peptone water was better than in other media but sodium chloride inhibited growth markedly at 5° and 25° and completely at 30° and 37°.

Motile long slender rods were observed at 5° to 30°, while 'clubbed' forms with reduced motility were found at 37°. The Gram reaction tended to be less typical at this temperature.

No fluorescence was displayed but a brown pigment was shown at 15°, 25° and 30° and not at 5° or 37°.

The oxidative breakdown of glucose was appreciable at 30°, less at 25° and negligible at 5°, 15° and 37°. Moderate acid production was observed in sorbitol and mannitol media at 15°, 25° and 30° and slight acid changes in lactose and fructose media were found at 25° and 30° only.

The strain did not exhibit arginase activity or breakdown of proteins or lipids and milk was unchanged.

Catalase activity was vigorous at 15°, 25° and 30° and reduced at 5° and 37° while delayed oxidase reactions were shown at 25° and 30°.

Strain S23

Derived from a human source this Pseudomonas strain produced heaviest growth in milk and peptone water at 30° and generally good growth was exhibited between 15° and 37° in these media and on nutrient agar. Heaviest growth in glucose and mannitol media and in peptone

water plus sodium chloride occurred at 30° and 37° but growth in all carbohydrate media was sparse at 15°. The strain was unable to grow at 5° in any medium examined.

No difference was observed in the morphology and motility of this strain at 15° to 37°.

Moderate levels of fluorescent pigment were produced at 25°, 30° and 37° but fluorescence was faint at 15°. A brown pigment was observed at 25° and 30° only.

The breakdown of carbohydrates was generally absent or weak. Acid was produced oxidatively from glucose at 25°, 30° and 37° and from fructose at 30°. A moderate oxidative change was obtained in mannitol medium at 25° and 30° with reduced reaction at 15° and 37°.

Moderate arginase activity was displayed at 25° and 30° and less at 15° and 37°. The precipitation of casein and gelatine liquefaction were appreciable at 15° and poor at 25°, 30° and 37° while marked alkaligenesis without proteolysis was observed at 15° to 37°.

Tributyrinase activity was maximal at 37° and declined with decrease in test temperature while the egg yolk reaction was appreciable at 25°, 30° and 37° and reduced at 15°.

Vigorous catalase activity was observed at 15° to 37° while the oxidase reaction was rapid at 25°, 30° and 37° and less rapid at 15°.

Strain S24

The growth of Xanthomonas campestris was not very heavy in any of the media used. The best growth was obtained on nutrient agar at 15° and 25°, in peptone water with or without sodium chloride at 15° and

in milk and sorbitol medium at 25°. Little or no growth was produced at 5° or 37° in any media except at 5° in peptone water. The inclusion of sodium chloride resulted in reduced growth in peptone water at all temperatures except 15°.

The growth response in carbohydrate media, particularly fructose, was moderate or poor throughout the temperature range.

Morphology and motility were similar at 5° to 37°.

The oxidative production of acid from glucose, lactose, sorbitol and mannitol was poor and restricted to between 15° and 30°.

Arginase activity was not observed and gelatinase activity and the precipitation of casein were weak and restricted to 15° and 25°. Extensive alkaligenesis without proteolysis was found at 15° and 25°, with reduced alkaligenesis at 30° and no change was observed at 5° or 37°.

Strong tributyrinase activity was displayed at 25° and 30° while activity was shown at 15° and 37° and none at 5°. The egg yolk reaction was moderate at 15°, 25° and 30°, reduced at 37° and negative at 5°.

Catalase activity was vigorous at 15°, 25° and 30°, less at 5° and poor at 37° while a delayed oxidase reaction was obtained at 15°, 25° and 30° with no reaction at 5° or 37°.

The results revealed the individuality of the strains in the way the temperature of incubation modified the morphological, cultural and biochemical characteristics and reactions. Many strains displayed quantitative differences in their reactions which were related to the growth-temperature pattern; other strains exhibited differences in reactions which were not apparently related to growth at the same

temperature.

Individual strains varied in the level of growth produced at the same temperature in different media and most strains grew better in liquid media than on nutrient agar, particularly at 25° and above. This observation implies the possibility of the growth of strains in milk or other liquid media at certain temperatures, which would not be detected, or regarded as significant, by a plate isolation method at the same temperature. In addition, the evaluation of growth on solid media does not appear to be an appropriate method for the comparison of growth response at different temperatures. It is, therefore, imperative that a complete standardisation of the medium be made before attempting to define a group of organisms, e.g. psychrophile, mesophile, thermophile, on the basis of growth, or absence of growth, at a certain temperature.

Low temperature isolates from milk, strains S1 - S10, and strains S14, S15, S19, S20 and S21 were, in general, able to grow well in milk and peptone water at 5° and showed sparse or no growth at 37°. The definition of these strains as psychrophiles agrees with the definitions proposed by Brown (1957), Thomas et al. (1960), Seitz (1961), Colwell and Liston (1961) and Roth and Wheaton (1961). A further group of strains which included strains S16, S17, S18, S22 and S24 as well as strain S11, isolated at 5° from a dairy source, exhibited poor or variable growth at 5° and 37° in test media and on this basis these strains are regarded as intermediates. Frankly mesophilic strains S12, S13 and S23 were easily defined due to their inability to grow at 5° and their ability to grow at 37° in most media. This observation relative to strain S12 (Ps. aeruginosa) is confirmed by Colwell (1964)

who found that of 33 Ps. aeruginosa strains none were able to grow at 5°. The psychrophilic strains S1 - S10, S14, S15, S19, S20 and S21 and intermediate strains S17, S22 and S24 generally produced heaviest growth at 5° or 15° in milk or peptone water and growth over the temperature range was often better in these than in carbohydrate media. Good growth was produced by all strains at 25° and strains S5, S15, S16, S17, S21 and S24 grew poorly in milk or peptone water at 30°. It is not easy to compare the results with those of other workers since definitions of psychrophiles on the basis of optimum growth have been related to either growth rate or total cell crop. The results of this study are more closely related to cell crops and it is acknowledged by Witter (1961) that on this basis, rather than growth rate, the optimum temperature of psychrophiles is often lower. However, although Van der Zant and Moore (1955) found that strains similar to those in the present study grew better at 21° or above, one strain grew better at 5° and 10° and not at all at 32°. Lawton and Nelson (1954) isolated a psychrophile from milk which also failed to grow at 32° and displayed optimum growth at 10° although other isolates were regarded by these workers and by Barber (1962) to exhibit growth optima at 21° to 25°. Greene and Jezeski (1954) also observed that psychrophilic Pseudomonas strains were unable to grow above 30° but Witter (1961) indicated that psychrophiles with a maximum growth temperature greater than 30° have been frequently encountered and Sinclair and Stokes (1962) indicated that a psychrophilic strain grew best at 30°.

In comparison with milk and peptone water a reduced growth response was shown in one or more carbohydrate media by strains S6, S7, S10, S14,

S15, S16, S17, S18, S20 and S21. The strains often produced comparatively less growth at extremes of the growth-temperature range, and the inability of intermediate strain S11 to grow at 5° resulted in a mesophilic growth response in carbohydrate media. The range of temperatures over which intermediate strain S22 was able to grow in carbohydrate media was narrowed considerably while mesophilic strain S23 lost much of the ability to grow at 37° shown in milk and peptone water. A general preference for a medium containing protein or protein hydrolysate, rather than carbohydrate, as carbon source was apparent although good growth was produced by many strains, and particularly fermentative strains S7, S11 and S19, in glucose medium. The reduced growth in carbohydrate media at low temperatures (strains S6, S7, S13, S14, S15, S16, S17, S18, S20, S21, S22, S23 and S24) and at high temperatures (strains S5, S9, S13, S14, S17, S18, S21, S22 and S24) may, as has been suggested by Wodzinski and Frazier (1960, 1961) be due to the additional stress of a lowered pH. However, growth was often good (strains S1, S3, S4, S5, S7, S8, S11, S12, S14, S15, S19, S20 and S21) in glucose, sorbitol or mannitol media, in which acid production was also appreciable, while growth was generally poorer in lactose and fructose media in which little acid was produced. Brown (1957), Ingraham (1958, 1962, 1963) and his co-workers, and Sultzer (1961) have examined certain aspects of carbohydrate metabolism in several strains of psychrophiles but further studies relating to the mechanisms of uptake of carbohydrates are required.

Most strains were least sensitive to the effect of sub-optimal or supra-optimal temperatures when grown in media containing protein or

protein hydrolysate as carbon, nitrogen and energy sources. This observation agrees with the conclusions reached by Jezeski and Olson (1962) whereby the ability of psychrophiles to grow at low temperature was correlated with the availability of casamino acids. However, these workers also recorded that yeast extract provided a similar stimulus as casamino acids, but in the present study it was found that the inclusion of yeast extract (0.05%, w/v) did not prevent the reduction in growth response observed at extremes of the temperature range in certain carbohydrate media.

The deleterious effect of 2.5% sodium chloride on the growth of many psychrophilic intermediate strains (strains S1, S2, S3, S5, S6, S7, S8, S10, S11, S17, S18, S20, S22 and S24) in peptone water was most apparent at 30° and 37° while intermediate strain S16 and mesophile strain S12 displayed reduced growth at 5° and 37°. The changes in the growth-temperature patterns of strains due to the inclusion of sodium chloride in peptone water indicated the requirement for a more detailed study of this phenomenon. This was undertaken in Part II of this work.

This experiment indicated the enhanced production of fluorescent pigment at 5° and 15° by strains S1, S3, S4, S6, S14, S15 and S20 which often produced little or no fluorescence at 30° and 37°. This finding confirms the observations by Van der Leek (1906), Hess (1934), White (1940) and Davis (1951) that low temperature tended to stimulate chromogenesis, although Ingraham and Stokes (1959) were not of this opinion. Of the strains examined, only mesophile strains S12 and S23 and intermediate strain S18 were more fluorescent above than below 25°. Since Thomas and Thomas (1947) reported that 50% of psychrophiles

isolated from milk were chromogenic and Scholefield (1964) found that 53% of 275 similar isolates were chromogenic the significance of enhanced pigment production under conditions of low temperature storage assumes an important aspect of food spoilage.

Aberrant morphological forms with reduced motility were produced by many psychrophiles during growth at 37°. Mesophilic strains S12, S13 and S23, intermediate strains S11, S18 and S24 and psychrophilic strain S5, which differed from other psychrophiles in its ability to produce an acid change in milk, did not show the differences in morphology even at temperatures at which growth was poor. Elongated sinuous forms were observed by the author (Scholefield, 1964) in pseudomonad cultures at low pH. These and the forms observed in this study were reminiscent of Streptobacillus moniliformis structures illustrated by Skerman (1959). It is considered that a condition of stress, such as low pH or a near-maximum growth temperature may induce sub-lethal cell damage and malformed morphology. The spirillum-like organisms recorded by Davis (1951) in dairy equipment containing traces of chemical disinfectant may also have been aberrant forms of pseudomonads or similar organisms.

Previously published work by other authors has indicated much conflict as to the extent and significance of differences in the reactions of strains to biochemical tests at various temperatures. Sekhar and Walker (1947), Gainor and Wegemer (1954), Frank (1962) and Higoshi (1964) reported few differences in the biochemical activities of psychrophilic strains at different temperatures. However, the results of the present experiment are supported by the findings of Kiser (1944), Greene and

Jezeski (1954) and Alford (1960) who, studying similar strains, observed some differential effects of temperature on the biochemical reactions.

The oxidative production of acid from glucose by most psychrophilic intermediate and mesophilic strains was related to the growth-temperature patterns in glucose media. This observation is supported by the work of Gainor and Wegener (1954) who found few differences in the fermentation of carbohydrate by psychrophiles at 5° and 25°. Few strains in the present study actively degraded sorbitol and mannitol but where appreciable levels of acid were produced by strains S4, S9, S15 and S20 at 25° or below much lower levels of acid were produced above 25°. Strains which generally produced low levels of acid from carbohydrates (strains S3, S5, S6, S8, S10, S15, S17, S20, S21, S22, S23 and S24), notably lactose and fructose, tended to produce even less or no acid at extremes of the growth-temperature range. This observation is confirmed by Kiser (1944) who reported the inability of a strain to produce acid from glucose at 7° which was weakly fermentative at 25°.

Qualitative differences in carbohydrate breakdown were observed in fermentative strains. Loss of aerogenesis during the fermentation of glucose, fructose and sorbitol at 37° was found in psychrophilic strain S7. Strain S19 lost the ability to produce acid and gas from four sugars utilised for growth at 37° and displayed an oxidative production of acid from glucose at 37°. Aerogenesis was lost at 5° and 30° during the fermentation of fructose by this strain and strain S11 also lost the ability to produce gas during the fermentation of glucose at 15°. Loss of aerogenesis during fermentation by coliform strains at high temperatures has been reported by Stuart (1941) and

the observation was made by Asuma and Clegg (1962) that 62% of psychrophilic coliform strains able to ferment carbohydrates at 15° failed at 5°. Upadhyay and Stokes (1962), however, found that the gas production by coliform strains was maintained during fermentation at low temperatures.

Van der Zant and Moore (1955) observed differences in the effect of temperature on the proteolytic activity of four psychrophile strains and no direct relationship was found between proteolysis and growth. The present study showed that strains S3, S4, S9, S12, S15, S19 and S20 displayed a general relationship between the levels of growth at 5° to 37°, alkaline-proteolysis of milk and gelatinase activity. These strains generally showed greater proteolytic activity below 30° than above, which agrees with the findings of Greene and Jezeski (1954) that a psychrophilic pseudomonad was able to liquefy gelatine at 0° to 20° but not at 30° to 35°. However, some strains in the present study displayed more variable patterns of proteolytic activity. Although correlation between gelatinase activity and casein precipitation was frequent, it was not as complete as suggested by Sandvik (1962); strains S1, S6, S13 and S15 being the exceptions. It is also difficult to reconcile the findings of Alford (1960) with the results of the present study. Alford, in an examination of Pseudomonas and Achromobacter strains found that of 15 strains able to liquefy gelatine at 20°, 10 liquefied gelatine at 35° - 37°. This study showed that only one strain, mesophilic strain S12, was proteolytic at 37°. Strains S3 and S15 were proteolytic in milk at 5° and 15° and alkaligenic at 25°, 30° and 37°, strains S4 and S9 were proteolytic only at 5°, 15° and 25° and strains S19 and S20 were proteolytic at 5° to 30°. Although psychrophilic strains showed

generally more active proteolysis at 5° or 15° than above. Hurley et al. (1963) recorded that a strain of Ps. fluorescens showed greater proteolytic activity at 25° than at 15° or 5°. In the present study this relationship only applied to strain S12 (Ps. aeruginosa) which was proteolytic at 25° to 37° and alkaligenic at 15°. Strains S8, S10, S23 and S24 retained their alkaligenic ability down to 15° and strains S8, S10 and S24 were less alkaligenic above 25°. Strain S7 showed qualitative differences in the changes produced in milk at 5° to 37°. Neutral proteolysis was observed at 15° to 30° while alkaligenesis was found at 5° and an acid change at 37°.

The relationship between the proteolytic activity of strains and temperature appears to be less simple than has hitherto been recognised. Part IV of this study deals with a more detailed examination of certain aspects of casein breakdown.

The tributyrinase activity of all psychrophilic and intermediate strains was greater at low temperatures and activity tended to decrease progressively with increase in test temperature. Mesophiles S12 and S13 also displayed this pattern of activity but strain S23 showed greatest tributyrinase activity at 37° and a decrease in activity with decrease in temperature. These observations to some extent conflict with the findings of Greene and Jezeski (1954) who observed close correlation between lipase activity and the growth of Pseudomonas spp. at 0° to 30°. The results are, however, confirmed by those of Alford and Elliott (1960) who showed that the optimum temperature for lipase activity was lower than for growth. Frank (1962) observed that of 12 Ps. geniculata strains lipolytic at 27° only 9 were lipolytic at 8°. This result is at variance

with the findings of the present study, if, as Frank suggests, that Ps. geniculata is a common psychrophilic species. By contrast the present results showed that 17 strains exhibited appreciable tributyrinase activity at 25° or below, of which 13 strains displayed little or no activity at 30° and 37°. Alford (1960) found that of 20 strains lipolytic at 20°, 9 were not lipolytic at 35° - 37° and Hashif and Nelson (1953) observed that for similar levels of growth Ps. fragi was more lipolytic at 6° than at 15° and non-lipolytic at 30°.

The egg yolk reaction paralleled tributyrinase activity at different temperatures in strains S1, S4, S7, S9, S12, S15, S20, S23 and S24 but strains S2, S6, S10 and S19 showed maximal egg yolk reaction at a higher temperature than maximal tributyrinase activity. Strains S3, S8 and S13 exhibited tributyrinase activity but no egg yolk reaction. The complex nature of this reaction has been indicated by Gillespie and Alder (1952), Willis (1960), Hobbs et al. (1961), Esselmann and Liu (1961) and Willis and Gowland (1962) and the different zone phenomena observed in this study and by Scholefield (1964) suggest the operation of lipases, leithinase C, proteolytic enzymes and non-specific precipitation and clearing.

Catalase activity and oxidase reactions of the strains did not show marked differences throughout the growth-temperature range. Sparse growth at 5° or 15° tended to exhibit more vigorous catalase activity than sparse growth at 30° or 37°. Strain S9 displayed poor oxidase reactions at both 5° and 30° while strain S17 displayed a slow oxidase reaction at 25° and 30° and no reaction at 37°. Mesophilic strains S12 and S23 showed decreased oxidase activity at 15° and strains S22

and S24 showed poor oxidase activity between 15° and 30° with no activity corresponding to poor growth at 5° and 37°. While Frank et al. (1963) observed differences in the catalase activities of psychrophiles at 2° and 30°, the relationship of the cytochrome oxidase systems with psychrophilic growth requires further investigation. Since the terminal oxidase enzymes are located in the cytoplasmic membrane (de Ley, 1960) their function may influence, or be influenced by, the permeability of this structure.

While it is not intended to examine the effect of temperature on the taxonomy of the test strains it is of interest to note that the alteration of certain characteristics and reactions due to change in test temperature could affect species identification. Klingo (1960) and Frank (1962) indicated that certain characteristics which are not stable, e.g. pigment production, are often employed in classificational schemes as primary taxa. In the present study all psychrophilic strains and most intermediate strains (strains S1, S3, S4, S6, S9, S14, S15, S20 and S21) which were fluorescent at low temperatures were poorly fluorescent at 25° and above. A temperature of 25° has been widely used for the evaluation of biochemical reactions for purposes of classification and Frank (1962) considered 27° a proper temperature for the incubation of psychrophilic Pseudomonas cultures and tests. Proteolytic activity was generally maintained at 25° but tributyrinase activity was weak at this temperature in strains S1, S3, S6, S8, S9 and S13. Strain S17 produced a slower oxidase reaction at 25° than at 5° or 15° which could result in the separation of this strain from other oxidase positive pseudomonads. Although differences in morphology were observed only during poor growth

at high temperatures the possibility of the incorrect identification of strains due to stress on the growing organism must not be precluded.

It seems, therefore, that characterisation for the purposes of taxonomy should be considered separately from the evaluation of the metabolic activities of a strain which may prove important in terms of an effect on the environment. The effect of temperature on the metabolism of psychrophiles has important practical implications regarding the spoilage of not only refrigerated milk but also meat, fish, eggs and other foodstuffs held at low temperatures for extended periods.

In the light of results obtained in this Part an examination was made, in Parts II, III and IV of the Experimental Studies, of specific aspects of the growth and metabolism of the test strains at 5^{o} to 37^{o}.

A critical examination was made in Part II of the growth in casein hydrolysate medium and of the effect of different concentrations of sodium chloride as an additional stress on growth at sub-optimal and supra-optimal temperatures. In Part III the relationship between growth at different temperatures and substrate was examined in terms of the ability of strains to utilise and degrade amino acids provided as sole carbon, nitrogen and energy sources.

The important aspects of proteolysis and lipolysis were examined further in Part IV with particular reference to the breakdown of casein at different temperatures.

II. THE EFFECT OF TEMPERATURE ON GROWTH AND THE INFLUENCE OF SODIUM CHLORIDE

GROWTH PARAMETERS AND TEMPERATURE COEFFICIENTS

Detailed studies of the growth of psychrophiles and comparisons of the growth responses of psychrophiles and mesophiles have been reported by Greene and Jezeski (1954), Brown (1957), Ingraham (1958, 1962, 1963), Ingraham and Bailey (1959), Sinclair and Stokes (1962), Olsen and Jezeski (1963), Farrell and Rose (1965) and Rose and Evison (1965).

In the experiments described by these workers small numbers of strains were examined and in view of the results of Part I of this study, which stress the individuality of the strains in their response to change in temperature, it is considered that the results obtained and the conclusions reached by previous workers may not represent the characteristics of psychrophiles or mesophiles in general.

The following experiment was designed to examine in detail the growth of the test strains at 5° to 37° in terms of Growth Parameters and to examine the effect of temperature on growth in terms of Temperature Coefficients.

Materials and Methods

Test medium: The medium which allowed satisfactory growth of the test strains S1 - S24, and which was most suitable for growth determinations by nepholometry was found to be 0.2% (w/v) Tryptone

(Oxoid L.42) water medium. The medium was dispensed in 12 ml amounts in 6 x $\frac{5}{8}$ inch nephelometer tubes.

Inocula: Standardised suspensions of strains 81 - 824 were prepared as described in Part I from 18 hour cultures grown on 0.5% (w/v) Tryptone (Oxoid L.42) water agar plates at 20°. Each strain was inoculated into triplicate tubes of the medium, which had been held at the appropriate incubation temperature to avoid subsequent false positive growth.

Incubation: The triplicate tubes of inoculated medium were incubated at each of the temperatures 5°, 15°, 25°, 30°, 35° and 37° as described in Part I.

Growth determinations: At intervals during incubation growth was measured in terms of optical density, employing an E.E.L nephelometer head and Unigalvo galvanometer (Evans Electroselenium Ltd). Every effort was made to eliminate sources of error inherent in this method of assessing growth.

Bacterial growth has been expressed in terms of optical density in studies by Greene (1959), Jezeski and Olsen (1962), Sinclair and Stokes (1962, 1965), Brown and Turner (1963) and Farrell and Barnes (1964), for relating growth with enzymic activity by Eagon (1956), Deibel (1964) and Guroff and Ito (1965) and in the study of growth in relation to water activity by Wodzinski and Frazier (1961). Shockman (1963) showed that nephelometers are more sensitive than spectrophotometers and colorimeters for the determination of optical density since they measure light reflected by suspended particles rather than transmitted

light. In accordance with Beers Law nephelometric measurements are proportional to the number of organisms in the suspension (Starke and Rose, 1959) and Sinclair and Stokes (1965) observed a close relationship between optical density values, viable count and equivalent dry weight throughout the growth cycles of psychrophiles. Rose and Evison (1965) showed that the temperature of incubation did not alter the relationship between optical density of cultures and the equivalent dry weight.

Nephelometric determinations were made relative to a ground glass standard set to 0.25, 0.5 or 1.0 x 100 units optical density and to a distilled water standard set to zero optical density. The relationship between recorded optical density and concentration of cell suspension was linear to values of 2.0 x 100 units. Values greater than 2.0 x 100 units optical density were corrected from calibration curves.

To ensure accuracy of the method the precautions indicated by Gavin (1957) and Kavanagh (1963) were closely observed during nephelometric studies and a standardised procedure was followed throughout. All test tubes, reference tubes and pipettes were ensured free from grease, residues, fibre particles and haze both internally and externally. All test tubes and reference tubes, closures, the nephelometer head cover and annulus were marked to ensure correct orientation at each reading. The zero and reference readings were checked frequently during the course of each series of readings and all tubes were polished prior to reading. Fifteen minutes before reading, the tubes were rotated rapidly in the palms. Readings made immediately following rotation were elevated due to the presence of small air bubbles. In addition to these precautions the accuracy of the technique was further assisted

by the generally uniform turbidity obtained with all strains together with the clarity and lack of sediment or colour in the uninoculated medium. Slowness in reaching recorded optical density, indicating flow birefringence of rod forms in suspension (Kavanagh, 1963) was not observed below a value of 3.0 x 100 units optical density.

Treatment of Results

The nett optical density value for each reading was derived by subtraction of the optical density value of the uninoculated medium from the value of the recorded optical density. The mean nett optical density (O.D) was obtained from triplicate values of the nett optical density and O.D values were plotted against time for each strain at each temperature.

Growth Parameters.

Gunsalus and Schuster (1961) stated that the pertinent parameters of the growth cycle were the duration of the lag phase, the rate of logarithmic growth and the maximum cell crop. In relation to the spoilage of food Elliott and Michener (1965) maintained that the duration of the lag and logarithmic phases of growth were most important since spoilage changes have usually occurred by the time the maximum cell crop is reached.

In this study the growth parameters, Apparent Lag (L), Growth rate constant (K) and Maximum cell crop (M) were used to describe the growth response of each strain at each temperature.

Apparent lag (L): The duration of the lag phase was measured by

the method of Hinshelwood (1946). The Apparent lag (L) is defined as the difference between the time at which a culture attains a certain population, chosen within the logarithmic phase of growth, and the hypothetical time at which the same population would have been reached had logarithmic growth prevailed from the time of inoculation. A more critical examination of the duration of the lag phase was not feasible in this study because of the limitations of the techniques used.

Growth rate constant (K): While the duration of the lag phase may be affected by the size, age and growth temperature of the inoculum and by the composition of the mother culture medium the rate of logarithmic growth of a given strain is acknowledged to be independent of the state of the inoculum. Under defined conditions the rate of logarithmic growth of a strain is constant. In this study a Growth rate constant (K) was derived from each growth curve and represents the increase in mean nett optical density (O.D) during the logarithmic phase of growth of a strain at one temperature over a period of twenty-four hours.

Maximum cell crop (M): As previously stated nephelometric measurements are proportional to the numbers of cells in a suspension (Starke and Rosa, 1959), therefore the Maximum cell crop (M) is represented by the maximum mean nett optical density value recorded during the incubation period of each strain at each temperature.

Temperature Coefficient.

To determine the effect of change in temperature on the growth

of strains at 5° to 35° Temperature coefficient (Q_{10}) values were calculated for 10 degree increments 5° to 15°, 15° to 25° and 25° to 35°. This coefficient is defined as the ratio of the rate of growth at a given temperature to the rate of growth at a temperature 10° lower. In the present study K_1 and K_2 are the Growth rate constants at t° and

$$Q_{10} = \frac{K_2}{K_1}$$

at t° + 10° respectively.

Results and Discussion

The values for L (Apparent lag), K (Growth rate constant) and M (Maximum cell crop) for strains S1 - S24 grown in tryptone medium at 5°, 15°, 25°, 30°, 35° and 37° together with Q_{10} values for 5° - 15°, 15° - 25° and 25° - 35° are shown in Tables 3.1, 3. 2 and 3. 3.

Growth Parameters.

The s trains showed much variation in their growth responses at 5° to 37° and the L, K and M values varied accordingly. Strains S18, S19 and S21 displayed appreciable K and M values throughout the temperature range while strains S16 and S22 exhibited high K and M values over a restricted temperature range and strains S2, S10 and S11 showed generally low K and M values.

Strains S12, S13 and S23 which were identified as mesophiles on the basis of their growth and reactions studied in Part I exhibited typically mesophilic growth parameters. Maximum K and M values were

Table 3.1 Growth parameters and Q10 values in tryptone medium (A)

Temp.°C. / Strain		Growth parameters 5°	15°	25°	30°	35°	37°	Q10 values 5°-15°	15°-25°	25°-35°
S.1	L	1.0	0.3	0	0	0	0			
	K	18	37	43	46	12.2	6.6	2.05	1.16	0.28
	M	300	398	245	138	41	15			
S.2	L	0.5	0	0	0	0	0			
	K	5	16.3	12.4	10	8.4	6.8	3.26	0.76	0.68
	M	55	50	42	27	20	18			
S.3	L	0	0.3	0	0	0	0			
	K	10.8	28.5	29.7	22.9	18.3	11.8	2.63	1.04	0.62
	M	155	320	172	125	81	40			
S.4	L	0.7	0.4	0	0	0	0			
	K	12.8	40	42.4	43	19.2	8.5	3.12	1.06	0.45
	M	203	420	225	202	130	32			
S.5	L	0.2	0	0	0	0	0			
	K	9.3	22.8	18	14.1	12	11.5	2.45	0.79	0.67
	M	180	205	104	91	71	32			
S.6	L	0.3	0.1	0	0	0	0			
	K	7.0	24.3	21.7	27	22	17.4	3.47	0.89	1.01
	M	141	186	103	98	84	71			
S.7	L	1.0	0.3	0.1	0	0	0			
	K	11.4	26.5	35	18.7	12	11	2.32	1.32	0.34
	M	260	258	201	160	63	20			
S.8	L	0	0	0	0	0	0			
	K	8.5	19.8	17	13.7	7.7	5.0	2.33	0.86	0.45
	M	193	160	102	70	32	13			

Key: L = apparent lag (days)

K = growth rate constant

M = maximum cell crop (units O.D)

Table 3. 2 Growth parameters and Q10 values in tryptone medium (A)

Temp. °C.		Growth parameters						Q10 values		
Strain		5°	15°	25°	30°	35°	37°	5°-15°	15°-25°	25°-35°
	L	0	0	0	0	0	0			
S. 9	K	12.7	30.9	35.5	46.5	19.9	11	2.43	1.15	0.56
	M	250	392	190	130	98	49			
	L	2.5	0	0	0	0	0			
S10	K	5.0	12.5	14.5	17	13	11.4	2.5	1.16	0.9
	M	89	61	53	50	50	47			
	L	0	0	0	0	0	0			
S.11	K	5.8	19	20.3	21	17	15.2	3.28	1.07	0.84
	M	90	87	63	68	60	59			
	L	∞	1.4	0	0	0	0			
S.12	K	0.1	34.2	34.8	49	78.1	104	342	1.02	2.24
	M	15	255	215	302	351	380			
	L	∞	0.6	0	0	0	0			
S.13	K	0	13.2	20.3	27.5	31	32.6	∞	1.54	1.53
	M	2.2	107	80	95	101	114			
	L	1.2	0.6	0	0	0.5	∞			
S.14	K	8.7	25.8	23.8	16.7	6.8	1.5	2.96	0.92	0.28
	M	150	193	105	110	38	4			
	L	0.6	0.4	0	0	0	∞			
S.15	K	12.2	34.4	27.8	16	4.9	1.9	2.81	0.81	0.18
	M	197	227	138	120	40	6			
	L	8.7	0.6	0	0	0	0			
S.16	K	4.2	21.5	19	16	15.5	14.5	5.12	0.88	0.81
	M	60	177	72	70	76	73			

Key: L = apparent lag (days)

K = growth rate constant

M = maximum cell crop (units O.D)

Table 3.3 Growth parameters and Q10 values in tryptone medium (A)

Temp.°C.		Growth parameters 5°	15°	25°	30°	35°	37°	Q10 values 5°-15°	15°-25°	25°-35°
Strain	L	3.4	1.5	0.9	0	0	0			
S.17	K	13	68	40	23.8	14.1	8.2	5.23	0.59	0.35
	M	236	410	352	185	110	45			
	L	1.8	0.4	0	0	0	0			
S.18	K	11.4	27.1	29.2	45.5	35	32	2.38	1.08	1.2
	M	123	113	110	260	220	197			
	L	1.6	0	0	0	0	0			
S.19	K	20	35.9	42.3	60	41	47.6	1.8	1.18	0.97
	M	317	370	165	158	160	184			
	L	1.0	0.9	0	0	0	∞			
S.20	K	15.8	58	28.7	37	13.2	0	3.67	0.49	0.46
	M	256	380	244	158	88	0			
	L	0.4	0	0	0	0	0.7			
S.21	K	11.2	19.7	17.4	24.4	21	17.3	1.76	0.88	1.21
	M	200	177	104	160	152	92			
	L	3.0	0.6	0.2	0	0	0			
S.22	K	4.2	23.1	27	31	23	18	5.55	1.17	0.85
	M	86	216	152	131	105	80			
	L	∞	1.2	0	0	0	0			
S.23	K	0.6	26.2	26.3	34.2	53	66	43.7	1.0	2.02
	M	11	165	130	178	180	196			
	L	3.1	0.6	0	0	0.5	0.6			
S.24	K	7.6	24.6	21	13.2	12.7	11.3	3.24	0.85	0.6
	M	152	265	157	120	71	45			

Key: L = apparent lag (days)

K = growth rate constant

M = maximum cell crop (units O.D)

shown at 37° although the M values of strain S12 were appreciable over the range from 15° to 37°. All three strains exhibited low K and M values and infinite L values at 5°.

The remaining 21 strains showed marked differences in growth parameters at 5° to 37° which confirmed the individuality of the strains observed in Part I. Strains S1, S4, S6, S9, S10, S11, S18, S19, S21 and S22 demonstrated maximum K values at 30° and strains S2, S5, S8, S14, S15, S16, S17, S20 and S24 showed maximum K values at 15° while strains S3 and S7 showed maximum K values at 25°. Thus, on the basis of K values, all strains, other than distinct mesophiles, showed maximum rates of growth between 15° and 30° and the grouping of these strains on the basis of K values bore little relation to the division between psychrophiles and intermediate strains indicated in Part I.

Greene and Jezeski (1954) examined three psychrophilic strains and their results indicate a correlation between high L values, low K values and high M values at low temperatures of growth and other investigators (Hess, 1934; Van der Zant and Moore, 1955) have also shown that higher cell crops may be produced by psychrophiles at temperatures below that of the maximum growth rate. In this study strains S5, S14, S15, S16, S17, S20 and S24 exhibited maximum K and M values at 15° and strain S18 showed maximum values at 30°. Other strains showed differences in the temperature at which maximum K and maximum M values were produced. Strains S1, S3, S4, S6, S7, S9, S10, S11, S19, S21 and S22 had maximum K values at 25° or 30° and maximum M values at 5° or 15° while strains S2 and S8 showed maximum K values at 15° and maximum M values at 5°.

The production of poor or no growth at 37° has been used by other workers, including Colwell and Liston (1961) and Seitz (1961), and in Part I of this study as a criterion for the definition of a psychrophile. In this experiment poor growth at 37°, as indicated by low M values (optical density less than 0.5 x 100 units), was shown by 14 strains. This total included those strains described as psychrophiles in Part I (strains S1, S2, S3, S4, S5, S7, S8, S9, S10, S14, S15 and S20) but excluded strains S6 and S21 and included strains S17 and S24 which were described as intermediates in Part I.

Strains which produced poor or no growth at 37° (strains S1, S2, S3, S4, S5, S7, S8, S9, S10, S14, S15, S17, S20 and S24) displayed L values at 5° of between zero and 3.4 days with a mean value of 1.1 days. This mean value is similar to that of 1.5 days reported by Brown (1957) for a psyohrophile grown at 5°. L values within the range obtained in the present experiment have been reported for psychrophiles at 4° to 5° by Hess (1934), 1 to 3 days; Lawton and Nelson (1954), 2 to 3 days and Ayres (1960), less than 2 days. Intermediate strains S16, S22 and S24 exhibited L values of 8.7, 3.0 and 3.1 days respectively which compare more closely with values of 4 to 5 days observed by Burgwald a Josephson (1947) for strains isolated at low temperature from milk.

Temperature Coefficients.

The Q_{10} values at 5° - 15°, 15° - 25° and 25° - 35° provide significant information regarding the relative rates of growth of the test strains at temperatures of prime importance. The temperature of 5° has practical implications, many strains grew well at 15° and 25°

and 35° is near the maximum temperature for growth of many strains isolated at low temperature yet enables the growth of mesophiles. Unfortunately direct comparisons between Q_{10} values obtained in this study with those of Hess (1934), Foter and Rahn (1936), Kiser (1944), Greene and Jezeski (1954) and Jezeski and Olsen (1962) were difficult due to individual variations in the temperatures on which the calculations were based. Elliott and Michener (1965) however, quoted results of Yamamura (1937) who calculated Q_{10} values for the growth at 5° and 17° of strains isolated from the surfaces of fish caught in waters of different temperatures. The mean Q_{10} values were 5.34 for warm water strains, 3.8 for temperate water strains and 2.87 for strains from cold water fish. These values compare with the range of Q_{10} values at 5°-15° of 1.76 to 5.55 with a mean value of 3.11 obtained in this study for strains other than mesophilic strains S12, S13 and S23. A progressive reduction in Q_{10} values with increase in temperature was obtained for strains S1, S2, S3, S4, S5, S7, S8, S9, S10, S11, S14, S15, S16, S17, S19, S20, S22 and S24 but not for strains S6, S18 and S21 or mesophilic strains S12, S13 and S23. Jezeski and Olsen (1962) also observed that relative to the growth of Ps. fluorescens in casamino acid medium at 4° to 32° Q_{10} values progressively diminished with increase in temperature.

The Q_{10} values of mesophile strains S12, S13 and S23 at 5°-15° were very high, i.e. 342, infinity and 43.7 respectively, tended to decrease sharply at 15°-25° and were either similar at 25°-35° (strain S13) or showed a twofold increase (strains S12 and S23).

On the basis of Q_{10} values and in consequence of the growth

parameter studies of strains S1 - S24 it appears that relative to growth in 0.2% tryptone medium, a Q_{10} value at 5° - 15° of less than 4, together with a Q_{10} value at 15° - 25° of not appreciably more than unity and a Q_{10} value at 25° - 35° of well below unity are features of a psychrophile. Such a definition includes strains S1, S2, S3, S4, S5, S7, S8, S9, S10, S11, S14, S15, S20 and S24 which except strain S11, were classed as psychrophiles on the basis of growth parameters but the list does not include strain S17 which was classed as a psychrophile on the basis of growth parameters. This strain displayed a Q_{10} value at 5° - 15° of 5.23 and a Q_{10} value at 15° - 25° of 0.59 but the M values were high at 5° and 15° and low at 37°. Strains S1, S7, S9 and S10 showed slightly high Q_{10} values at 15° - 25° but the accompanying low Q_{10} values at 5° - 15° and 25° - 35° reflected their essentially psychrophilic nature. Although strain S11 exhibited a psychrophilic pattern of Q_{10} values, growth in tryptone medium was not good at 5° to 37° and it is probable that more characteristic Q_{10} values would be obtained from the rates of growth in a more suitable medium. Mesophile strains S12, S13 and S23 showed characteristically high Q_{10} values at 5° - 15° together with Q_{10} values greater than unity at 15° - 25° and 25° - 35°. Strains S6, S16, S18, S19, S21 and S22 were not able to be defined as either mesophiles or psychrophiles since they displayed growth parameters and Q_{10} values which necessitated their description as intermediate strains.

INFLUENCE OF SODIUM CHLORIDE ON GROWTH

In Part I a simple study revealed marked differences in the growth response of strains at 5° to 37° in peptone medium with and without 2.5% (w/v) sodium chloride. It was therefore decided to examine more critically the effect on the growth of strains S1 - S24 of variation in the temperature of incubation from 5° to 37° and of variation in the concentration of sodium chloride from 0 - 4.5% (w/v).

The relationships between sodium chloride and the growth of non-halophiles and halophiles has been reviewed by Ingram (1957), Larsen (1962) and Scott (1962). Scott indicated that the water activity (a_w), which is a measure of the availability of water, is a fundamental property of an aqueous solution and varies with the total concentration of solutes in the environment. So long as a solution is in the liquid phase the water activity is practically independent of temperature. Thus in the present study the water activity of a medium containing a given concentration of sodium chloride would remain constant over the temperature range 5° to 37°. The bacterial cell membrane maintains equilibrium between the cell contents and the environment below a certain ionic concentration. Salt-tolerant bacteria can maintain a low water activity due to high levels of potassium (Christian and Waltho, 1961) but bacteria unable to tolerate high concentrations of salt undergo plasmolysis (Mitchell and Moyle, 1956). Henneman and Umbreit (1964) suggested that the physical detachment of the cell membrane occurs at low values of water activity and it has been shown that membrane stability

and the salt tolerance of Gram-negative rods are related (Brown and Turner, 1963). Other malfunctions of the metabolism of bacteria at low values of water activity have been proposed. Ingram (1947) reported that high concentrations of sodium chloride affected the respiration of Ps. fluorescens and the solubility of cellular proteins thereby causing enzyme denaturation. Larsen (1962) indicated that cytochrome enzyme systems were inhibited at high concentrations of salt and Scott (1957) referred to the reduction in the solubility of oxygen in media with low water activities.

Although high concentrations of sodium chloride inhibit the growth of non-halophilic bacteria it has been found that lower levels of salt may be required for optimum growth. Marshall and Scott (1958) showed that the growth of Vibrio metchnikovii in nutrient broth was stimulated by a decrease in the water activity from 0.999 to 0.995 and Ware et al., (1955) observed a similar response in E. coli. Larsen (1962) reported that the growth of slightly halophilic Pseudomonas, Achromobacter and Flavobacterium strains was inhibited in media containing greater than 5% and less than 1.5% sodium chloride. The stimulation of growth and biochemical activities by low levels of salt has also been observed by Tomlinson and McLeod (1957) in relation to the oxidative metabolism of carbohydrates, by Pratt and Happold (1960) in relation to indole formation and by Payne (1958) in relation to gluconate oxidation. Mashif and Nelson (1953), however, found that salt inhibited the lipase activity of pseudomonads.

Larsen (1962) indicated that non-halophiles grow best in media containing less than 2.0% salt and that Gram-negative rods are usually

inhibited by 5 - 10% sodium chloride, although Foter and Vaughn (1950) and Christian and Scott (1953) observed variations in salt tolerance between genera and between strains of the same genus. Brown and Turner (1963) reported that Pseudomonas spp., Achromobacter spp. and E. coli displayed optimum sodium chloride requirements of between 1.0 and 2.0% with a maximum tolerance of about 4.0%. These workers stated that no minimum requirement of sodium chloride was shown by the strains examined. Donner and Harmon (1957) found that psychrophilic strains were able to grow in a medium containing 5.0% salt and Witter (1961) indicated that all of 15 strains examined grew in 4.0% salt medium, 6 strains grew in 6.0% and only one strain grew in 8.0% salt medium.

The relationships between the temperature of growth and low levels of water activity have been studied by Wolzinski and Frazier (1960, 1961) who found that the values of the minimum water activity for the growth of Ps. fluorescens and A. aerogenes increased with reduction in temperature from 30° to 15°. In contrast Gibbons et al., (1951) showed that sodium chloride reduced the minimum temperature for growth of psychrophiles on bacon, but little study has been made of the effect of different temperatures on the growth response of psychrophilic Gram-negative rods in media containing various concentrations of sodium chloride.

Materials and Methods

Media.

To enable a comparison of the results of this experiment with

those of the preceding experiment 0.2% (w/v) tryptone (Oxoid L42) water medium was used. Wodzinski and Frazier (1960, 1961) employed a medium containing tryptone in studies concerning water activity and growth, and Christian (1955) indicated that a medium containing amino-nitrogen was advisable for studying growth at low levels of water activity. Sodium chloride was incorporated in the tryptone medium at levels of 0.5, 2.5, 3.5 and 4.5% (w/v). The media were dispensed as described on page 77.

Inocula.

Standard inocula of strains S1 - S24 were prepared and tubes were inoculated as described in Part I and on page 77.

Incubation and Growth determinations.

Triplicate tubes of each test medium were incubated at 5°, 15° 25°, 35° and 37° for each strain, as described on page 77. At intervals nephelometric measurements were made as described on pages 77-79.

Treatment of Results

Mean nett optical density values (O.D) were calculated in the manner described in the preceding experiment and these values were plotted against time. The growth parameters - apparent lag (L), growth rate constant (K) and total cell crop (M) were derived from the growth curves of each strain grown in each medium at each temperature. Temperature coefficient (Q_{10}) values over the ranges 5° - 15°, 15° - 25° and 25° - 35° were calculated from the K values of each strain in each medium.

In order to compare more easily the rate of growth of a strain in one medium with the rate of growth of the same strain in a reference medium at the same temperature a coefficient, the St value, was introduced.

The St value represents a ratio of the growth rate constant (K_2) for growth in a given medium to the growth rate constant (K_1) for growth in 0.2% tryptone water. In this experiment St values were calculated

$$St = \frac{K_2}{K_1}$$

for the growth of each strain at each temperature in each medium containing sodium chloride. Thus, an St value of less than unity indicates a lower rate of growth of the strain in the given medium compared with the rate of growth in tryptone medium, and conversely an St value greater than unity indicates a comparative increase in the rate of growth in the medium containing sodium chloride.

Results and Discussion

Values for the growth parameters L, K and M are shown in Tables 4. 1 - 4. 6, Q_{10} values are shown in Tables 5. 1 and 5. 2 and St values are shown in Tables 6. 1 and 6. 2.

Because of the nature of this experiment it was necessary to ascertain the effect of sodium chloride on the relationship between growth, in terms of optical density, and cell concentration. Kavanagh (1963) reported that increases in the ionic concentration increased the optical density of cell suspensions. This was considered by

Table 4. 1 Growth parameters in presence of NaCl (%)

% (w/v) NaCl		0					0.5					2.5					3.5					4.5				
Temp. °C.		5°	15°	25°	35°	37°	5°	15°	25°	35°	37°	5°	15°	25°	35°	37°	5°	15°	25°	35°	37°	5°	15°	25°	35°	37°
Strain	L	1.0	0.3	0	0	0	0.2	0	0	0	0	2.0	0	0.6	1.5	∞	∞	3.5	1.8	∞	∞	∞	∞	∞	∞	∞
S.1	K	18	37	43	12.2	6.6	27.4	54	56	28	11.2	20.8	31.7	39.4	7.8	0	3.2	22.7	32.5	6.8	0	0	0	0	0	0
	M	300	398	245	41	15	312	350	305	134	28	218	257	220	39	0	32	145	133	32	0	0	0	0	0	0
	L	0.5	0	0	0	0	0	0	0	0	0	0	0	0	0	∞	0	0	0	∞	∞	∞	4.3	∞	∞	∞
S.2	K	5	16.3	12.4	8.4	6.8	3.6	10.8	10.6	9.0	7.8	3.5	6.8	8.2	4	0	3.5	6.5	4.0	0	0	0	2.7	0	0	0
	M	55	50	42	20	18	27	40	26	22	19	25	22	32	20	0	23	21	16	0	0	0	13	0	0	0
	L	0	0.3	0	0	0	0	0	0.7	∞	0	0	0.5	0.3	0	0	1.0	0.5	0.6	0	0	4.5	1.6	1.2	0.5	∞
S.3	K	10.8	28.5	29.7	18.3	11.8	15.2	40.5	52.4	30.3	14.1	13.8	36	27.5	16.8	12.9	8.4	32	24.5	15.5	11.8	8.3	25.3	23	10.2	0
	M	155	320	172	81	40	164	236	223	110	37	128	148	150	73	36	97	137	130	69	29	72	125	120	62	0
	L	0.7	0.4	0	0	0	0	0	0	0	0.4	0.8	0	0.4	0.5	0.4	2.0	0.6	0.7	0.5	0.5	8.5	1.6	0.7	∞	∞
S.4	K	12.8	40	42.4	19.2	8.5	20.5	56	54.6	35.3	20.5	15.3	38.7	45	28.5	19.8	10.7	26	39	21	16.6	10.3	21.2	11.7	0	0
	M	203	420	225	130	32	270	335	276	183	45	168	268	202	170	43	104	170	146	67	35	57	128	63	0	0

Key: L = apparent lag

K = rate constant

M = maximum cell crop

Table 4. 2 Growth parameters in presence of NaCl (%)

% (w/v) NaCl		0					0.5					2.5					3.5					4.5				
Temp. C.		5°	15°	25°	35°	37°	5°	15°	25°	35°	37°	5°	15°	25°	35°	37°	5°	15°	25°	35°	37°	5°	15°	25°	35°	37°
Strain	L	0.2	0	0	0	0	0	0	0	0	0	0.4	0	1.0	0	∞	1.5	0	0	0	∞	2.5	0.6	∞	∞	∞
S.5	K	9.3	22.8	18	12	11.5	11.5	32.4	46.2	20.5	13.8	10	27.8	23.8	14	11.3	10.2	20	18.2	7.2	5.8	9.4	17.5	8.4	0	∞
	M	180	205	104	71	32	128	318	218	160	30	105	166	135	60	23	92	136	94	21	12	76	123	49	0	0
	L	0.3	0.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0.5	0	0	0	0	0.6	0.4	0	0	∞
S.6	K	7.0	24.3	21.7	22	17.4	13.5	32	26.6	23	14.6	10.8	25.3	21.2	15.6	12.8	7.8	22.3	18.8	10	7.7	6.9	15.9	13.1	5.5	0
	M	141	186	103	84	71	132	197	155	103	52	108	145	127	84	45	83	115	105	60	23	70	103	65	20	0
	L	1.0	0.3	0.1	0	0	0	0.6	0.5	0	0	0.5	0	0	0	∞	0.5	0	0	0	∞	4	1.0	0.5	∞	∞
S.7	K	11.4	26.5	35	12	11	14	28.5	39.3	14.5	10	14	26.2	22.4	6.0	0	13	19.5	18.2	4.5	0	10.2	14.3	12.3	0	0
	M	260	258	201	63	20	175	245	218	85	22	152	200	128	35	0	149	145	92	22	0	87	130	66	0	0
	L	0	0	0	0	0	0	0	0	0	0	0	0	0	0	∞	1.0	0	0	∞	∞	5.5	0.8	0	∞	∞
S.8	K	8.5	19.8	17	7.7	5.0	13.6	24	34	17.5	11.3	12	22.4	14.2	3.5	0	10.2	17.3	9.7	0	0	9.8	14.7	6.6	0	0
	M	193	160	102	32	13	176	209	197	93	33	130	150	83	26	0	114	131	58	0	0	70	109	43	0	0

Key: L = apparent lag

K = rate constant

M = maximum cell crop

Table 4. 3 Growth parameters in presence of NaCl (%)

% (w/v) NaCl		0					0.5					2.5					3.5					4.5				
Temp. °C		5°	15°	25°	35°	37°	5°	15°	25°	35°	37°	5°	15°	25°	35°	37°	5°	15°	25°	35°	37°	5°	15°	25°	35°	37°
Strain	L	0	0	0	0	0	0	0	0	0	0	0.7	0	0	0	0	2.0	0	0	0	1.5	6.5	2.0	1.0	0	∞
S. 9	K	12.7	30.9	35.5	19.9	11	17.9	39.2	37.5	20	11.2	16.1	34.5	45	23	16.1	13.6	32.7	52	9.5	4.1	9.0	31	37	9.7	0
	M	250	392	190	98	49	208	312	212	80	25	185	260	232	86	35	135	247	210	53	16	61	202	155	68	0
	L	2.5	0	0	0	0	0	0	0	0	0	0	0	0.4	0	∞	∞	0	1.0	∞	∞	∞	∞	3.0	∞	∞
S.10	K	5.0	12.5	14.5	13	11.4	6.8	11.8	11	11	10.5	4.4	8.2	9.0	4	0	0	5.5	7.5	0	0	0	0	6.2	0	0
	M	89	61	53	50	47	60	83	56	41	33	36	43	42	21	0	0	32	32	0	0	0	0	23	0	0
	L	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	∞	0	0	∞	∞	∞	∞	∞	∞	∞
S.11	K	5.8	19	20.3	17	15.2	7.2	21.6	23	18	15	3.2	11	9.9	9.3	6.3	0	6.2	7.3	4	0	0	3	4.5	0	0
	M	90	87	63	60	59	59	103	73	67	62	29	50	37	28	16.5	0	38	27	12	0	0	11	16	0	0
	L	∞	1.4	0	0	0	5.5	0	0	0	0	∞	0	0	0	0	∞	0	0	0	0	∞	2	0.6	0	0.2
S.12	K	0.1	34.2	34.8	78.1	104	4.5	35.5	42.3	83	119	0	17	27.3	62	74	0	14.5	19.8	41	58.5	0	15	19	26	39
	M	15	255	215	351	320	40	249	262	290	252	0	190	130	163	172	0	160	114	150	145	0	140	108	116	120

Table 4. 4 Growth parameters in presence of NaCl (%)

% (w/v) NaCl		0					0.5					2.5					3.5					4.5				
Temp. °C		5°	15°	25°	35°	37°	5°	15°	25°	35°	37°	5°	15°	25°	35°	37°	5°	15°	25°	35°	37°	5°	15°	25°	35°	37°
Strain	L	∞	0.6	0	0	0	∞	0	0	0	0	∞	0	0	0	0	∞	1.5	0	0	0	∞	∞	0	0	1.0
S.13	K	0	13.2	20.3	31	32.6	0	12	23.5	40	46.8	0	8.0	11.5	28.5	26	0	6.7	6.2	14.3	17.2	0	0	6.0	5.0	4.6
	M	2.2	107	80	101	114	0	92	95	108	102	0	52	62	70	60	0	38	38	50	45	0	0	30	33	27
	L	1.2	0.6	0	0.5	∞	0	0	0	0	0	0	0	0	∞	∞	5	1.0	0	∞	∞	∞	∞	2.5	∞	∞
S.14	K	8.7	25.8	23.8	6.8	1.5	9.2	23.7	25.7	12.6	8.0	5.9	16.8	11	0	0	5.3	14.2	7.5	0	0	0	0	7.0	0	0
	M	150	193	105	38	4.0	110	110	132	75	23	64	92	57	0	0	47	81	45	0	0	0	0	30	0	0
	L	0.6	0.4	0	0	∞	0	0	0	0	0	0.5	0	0	0	0	1.5	0	0.3	0	∞	5.5	1.5	1.0	∞	∞
S.15	K	12.2	34.4	27.8	4.9	1.9	15.5	35.6	39	20	10	11.8	28.2	29.8	8.6	5.2	10.9	22.5	27.3	3.0	0	9.5	20.3	19.2	0	0
	M	197	227	138	40	6.0	182	258	269	110	22	134	216	170	52	12	110	172	145	22	0	7.5	135	98	0	0
	L	8.7	0.6	0	0	0	4.0	0	0	0	0	∞	1.0	1.0	∞	∞	∞	∞	3.5	∞	∞	∞	∞	∞	∞	∞
S.16	K	4.2	21.5	19	15.5	14.5	10	18.3	23	17.4	14.7	0	6.5	7.8	3.6	0	0	0	4.2	0	0	0	0	0	0	0
	M	60	177	72	76	73	89	165	105	85	52	0	56	42	20	0	0	0	14	0	0	0	0	0	0	0

Table 4. 5 Growth parameters in presence of NaCl (%)

% (w/v) NaCl		0					0.5					2.5					3.5					4.5				
Temp. °C		5°	15°	25°	35°	37°	5°	15°	25°	35°	37°	5°	15°	25°	35°	37°	5°	15°	25°	35°	37°	5°	15°	25°	35°	37°
Strain	L	3.4	1.5	0.9	0	0	1.0	0.7	0.5	0	0	4.5	0.7	1.0	0	∞	∞	2.7	1.5	0	∞	∞	4	2.0	∞	∞
S.17	K	13	68	40	14.1	8.2	14.4	73	51.7	20	11.3	8.8	26.5	36.3	10	0	0	21.2	17.3	3.5	0	0	12	10.8	0	0
	M	236	410	352	110	45	182	440	320	171	36	81	247	211	90	0	0	157	90	48	0	0	87	48	0	0
	L	1.8	0.4	0	0	0	2.0	0	0	0	0	∞	1.7	0	0	0	∞	3.0	1.0	1.0	1.2	∞	3.5	1.5	0	∞
S.18	K	11.4	27.1	29.2	35	32	14.6	66	37.7	39.5	37	0	40.5	28.2	26	16	0	18.8	22	13	9.0	0	16.4	19.3	7	0
	M	123	113	110	220	197	107	112	126	258	132	0	115	133	159	49	0	136	98	68	27	0	75	80	52	0
	L	1.6	0	0	0	0	0.4	0	0	0	0	0.6	0	0	0	0	2.5	0	0	0.5	1.0	∞	3.0	2.5	∞	∞
S.19	K	20	35.9	42.3	41	47.6	19.5	44.5	46	38	22	11.2	25.5	16.8	15.1	15.4	6.4	10.6	11.6	10	10.1	0	5.8	7.5	0	0
	M	317	370	165	160	184	258	348	280	120	69	115	110	50	45	42	62	76	32	30	28	0	36	22	0	0
	L	1.0	0.9	0	0	∞	0	0	0	0	∞	0	0	0	0	∞	1.6	0	0.7	1.5	∞	5.5	1.5	1.3	4	∞
S.20	K	15.8	58	28.7	13.2	0	17.7	53.5	51	16.8	0	15.3	52	45.3	13.5	0	13	23.6	43	8.5	0	10.3	21.2	33.2	5.8	0
	M	256	380	244	88	0	230	367	310	105	0	192	345	275	103	0	152	195	215	42	0	82	138	145	30	0

Table 4. 6 Growth parameters in presence of NaCl (%)

% (w/v) NaCl		0					0.5					2.5					3.5					4.5				
Temp. °C		5°	15°	25°	35°	37°	5°	15°	25°	35°	37°	5°	15°	25°	35°	37°	5°	15°	25°	35°	37°	5°	15°	25°	35°	37°
Strain	L	0.4	0	0	0	0.7	0	0	0	0	0	0	0	0	0	0	1.5	0	0.5	0.5	∞	4.0	1.0	0.6	0.5	∞
S.21	K	11.2	19.7	17.4	21	17.3	10.2	19	25.8	27.3	21.2	7.7	14.2	19.5	13.1	7.6	7.1	11.5	18.1	10.1	0	6.6	11.1	11	6.5	0
	M	200	177	104	152	92	131	147	169	178	79	101	116	115	96	27	82	93	105	70	0	63	85	62	38	0
	L	3	0.6	0.2	0	0	5.0	0	0	0	0	∞	0.5	0	0	∞	∞	3.0	0	∞	∞	∞	∞	∞	∞	∞
S.22	K	4.2	23.1	27	23	18	10.3	17	23.7	18	14.8	0	13.8	14	7	0	0	12.7	7	0	0	0	0	2.5	0	0
	M	86	216	152	105	80	85	165	212	163	52	0	140	121	51	0	0	107	53	0	0	0	0	10	0	0
	L	∞	1.2	0	0	0	∞	0	0	0	0	∞	0.6	0	0	0	∞	2.3	0	0	0	∞	4.0	0.5	0	0
S.23	K	0.6	26.2	26.3	53	66	0	16.5	27	51	64	0	14.2	23	33	41.6	0	13.3	14.6	20	24.2	0	10.6	12.5	13	14
	M	11	165	130	180	196	0	115	138	169	175	0	101	120	115	105	0	107	80	68	62	0	74	62	52	47
	L	3.1	0.6	0	0.5	0.6	4.3	0	0	0	0.5	7.0	0.6	0.5	∞	∞	∞	4.4	1.0	∞	∞	∞	∞	∞	∞	∞
S.24	K	7.6	24.6	21	12.7	11.3	10.6	18.8	16	9	5	3.2	11.1	11	5	0	0	9.3	7.6	3	0	0	0	0	0	0
	M	152	265	157	71	47	95	181	109	36	14	21	120	58	24	0	0	68	43	18	0	0	0	0	0	0

Table 5. 1 Q10 values in medium A with 0 - 4.5% sodium chloride
(Strains S1 - S12)

% NaCl(w/v)	0			0.5			2.5			3.5			4.5		
Q10 value	5-15°	15-25°	25-35°	5-15°	15-25°	25-35°	5-15°	15-25°	25-35°	5-15°	15-25°	25-35°	5-15°	15-25°	25-35°
Strain															
S. 1	2.05	1.16	0.28	1.96	1.04	0.5	1.51	1.25	0.2	7.1	1.44	0.2	0	0	0
S. 2	3.26	0.76	0.68	3.0	0.98	0.85	1.96	1.2	0.45	1.85	0.61	0	0	0	0
S. 3	2.63	1.04	0.62	2.7	1.29	0.57	2.63	0.76	0.60	3.8	0.76	0.63	3.05	0.9	0.44
S. 4	3.12	1.06	0.45	2.7	0.98	0.64	2.56	1.16	0.63	2.43	1.49	0.53	2.08	0.55	0
S. 5	2.45	0.79	0.67	2.84	1.42	0.44	2.77	0.86	0.58	1.96	0.9	0.39	2.16	0.48	0
S. 6	3.47	0.89	1.01	2.38	0.83	0.86	2.32	0.84	0.74	2.85	0.84	0.53	2.32	0.82	0.42
S. 7	2.32	1.32	0.34	2.04	1.38	0.36	1.88	0.86	0.26	1.51	0.93	0.24	1.4	0.86	0
S. 8	2.33	0.86	0.45	1.75	1.41	0.51	1.88	0.63	0.24	1.69	0.56	0	1.51	0.44	0
S. 9	2.43	1.15	0.56	2.17	0.96	0.53	2.17	1.31	0.51	2.43	1.58	0.18	3.44	1.19	0.26
S.10	2.5	1.16	0.89	1.75	0.93	1.0	1.85	1.09	0.44	∞	1.4	0	0	∞	0
S.11	3.28	1.07	0.84	3.0	1.1	0.78	3.4	0.9	0.93	∞	1.2	0.55	0	1.5	0
S.12	342	1.02	2.24	7.8	1.2	1.96	∞	1.61	2.27	∞	1.37	2.08	0	1.26	1.35

Table 5.2 Q10 values in medium A with 0 - 4.5% sodium chloride
(Strains S13 - S24)

% NaCl(v/v)	0			0.5			2.5			3.5			4.5		
Q10 value	5-15°	15-25°	25-35°	5-15°	15-25°	25-35°	5-15°	15-25°	25-35°	5-15°	15-25°	25-35°	5-15°	15-25°	25-35°
Strain															
S.13	∞	1.54	1.53	∞	1.96	1.72	∞	1.44	2.48	∞	0.92	2.32	0	∞	0.83
S.14	2.96	0.92	0.23	2.56	1.08	0.49	2.85	0.65	0	2.70	0.52	0	0	0	0
S.15	2.81	0.81	0.18	2.3	1.1	0.51	2.38	1.06	0.28	2.06	1.21	0.1	2.14	0.95	0
S.16	5.12	0.88	0.31	1.85	1.26	0.75	∞	1.2	0.46	0	∞	0	0	0	0
S.17	5.23	0.59	0.35	5.26	0.7	0.38	3.03	1.37	0.27	∞	0.81	0.2	∞	0.9	0
S.18	2.38	1.06	1.2	4.54	0.57	1.05	∞	0.69	0.92	∞	1.17	0.59	∞	1.17	0.36
S.19	1.8	1.18	0.97	2.32	1.04	0.82	2.27	0.65	0.9	1.66	1.09	0.86	∞	1.29	0
S.20	3.67	0.49	0.46	3.03	0.95	0.33	3.44	0.87	0.29	1.81	1.81	0.19	2.08	1.58	0.17
S.21	1.76	0.88	1.21	1.85	1.35	1.06	1.85	1.38	0.67	1.61	1.58	0.55	1.69	0.91	0.59
S.22	5.55	1.17	0.85	1.65	1.38	0.76	∞	1.02	0.5	∞	0.55	0	0	∞	0
S.23	43.7	1.0	2.02	∞	1.63	1.38	∞	1.61	1.44	∞	1.09	1.37	∞	1.17	1.04
S.24	3.24	0.85	0.6	1.78	0.85	0.56	3.57	0.91	0.45	∞	0.81	0.39	0	0	0

Table 6. 1 St- values for growth with sodium chloride (Strains S1 - S12)

St- value	0.5:0					2.5:0					3.5:0					4.5:0				
Strain	5°	15°	25°	35°	37°	5°	15°	25°	35°	37°	5°	15°	25°	35°	37°	5°	15°	25°	35°	37°
S1	1.52	1.46	1.3	2.3	1.7	1.15	0.85	0.91	0.51	0	0.18	0.85	0.91	0.5	0	0	0	0	0	0
S2	0.72	0.66	0.92	1.5	1.14	0.7	0.42	0.66	0.47	0	0.7	0.39	0.32	0	0	0	0.16	0	0	0
S3	1.41	1.41	1.76	1.65	1.19	1.27	1.26	0.92	0.91	1.09	0.78	1.12	0.82	0.84	1.0	0.77	0.88	0.77	0.88	0
S4	1.6	1.4	1.29	1.83	2.4	1.12	0.97	1.1	1.48	2.41	0.83	0.65	0.92	1.09	1.95	0.8	0.53	0.27	0	0
S5	1.23	1.42	2.57	1.7	1.2	1.07	1.22	1.32	1.16	0.98	1.1	0.88	1.01	0.6	0.5	1.01	0.77	0.73	0	0
S6	1.92	1.32	1.22	1.04	0.84	1.54	1.04	0.98	0.71	0.73	1.1	0.92	0.87	0.45	0.44	0.98	0.65	0.6	0.25	0
S7	1.23	1.07	1.12	1.2	0.91	1.23	0.99	0.64	0.5	0	1.13	0.73	0.52	0.37	0	0.89	0.54	0.35	0	0
S8	1.6	1.21	2.0	2.27	2.26	1.41	1.13	0.83	0.45	0	1.2	0.87	0.57	0	0	1.15	0.74	0.39	0	0
S9	1.41	1.27	1.05	1.0	1.01	1.26	1.12	1.27	1.15	1.02	1.1	1.11	1.46	0.51	0.37	0.71	1.0	1.06	0.48	0
S10	1.36	0.94	0.76	0.84	0.92	0.88	0.66	0.62	0.31	0	0	0.44	0.52	0	0	0	0	0.43	0	0
S11	1.24	1.13	1.13	1.05	0.98	0.55	0.58	0.49	0.54	0.41	0	0.33	0.36	0.23	0	0	0.16	0.22	0	0
S12	45.0	1.04	1.24	1.06	1.14	0	0.49	0.92	0.79	0.71	0	0.42	0.57	0.52	0.56	0	0.44	0.55	0.33	0.37

Table 6.2 St- values for growth with sodium chloride (Strains S13 - S24)

St- value	0.5:0					2.5:0					3.5:0					4.5:0				
Strain	5°	15°	25°	35°	37°	5°	15°	25°	35°	37°	5°	15°	25°	35°	37°	5°	15°	25°	35°	37°
S13	0	0.91	1.15	1.29	1.43	0	0.61	0.56	0.91	0.79	0	0.51	0.3	0.46	0.53	0	0	0.29	0.16	0.14
S14	1.05	0.91	1.1	1.8	5.3	0.63	0.65	0.46	0	0	0.61	0.55	0.31	0	0	0	0	0.29	0	0
S15	1.27	1.03	1.4	4.08	5.26	0.96	0.82	1.07	1.75	2.73	0.89	0.65	0.98	0.61	0	0.78	0.59	0.69	0	0
S16	2.38	0.85	1.21	1.12	1.01	0	0.3	0.41	0.23	0	0	0	0.22	0	0	0	0	0	0	0
S17	1.12	1.07	1.29	1.4	1.37	0.68	0.39	0.91	0.71	0	0	0.17	0.43	0.25	0	0	0.18	0.28	0	0
S18	1.28	2.43	1.29	1.12	1.16	0	1.49	0.96	0.74	0.5	0	0.66	0.75	0.37	0.28	0	0.6	0.66	0.2	0
S19	0.97	1.24	1.08	0.92	0.46	0.56	0.71	0.39	0.37	0.32	0.32	0.29	0.27	0.24	0.2	0	0.16	0.18	0	0
S20	1.12	0.92	1.78	1.28	0	0.97	0.89	1.58	1.02	0	0.82	0.41	1.49	0.64	0	0.65	0.37	1.15	0.44	0
S21	0.91	0.96	1.48	1.3	1.22	0.69	0.72	1.12	0.62	0.44	0.63	0.58	1.04	0.48	0	0.59	0.56	0.63	0.31	0
S22	2.45	0.73	0.87	0.78	0.82	0	0.59	0.52	0.3	0	0	0.59	0.26	0	0	0	0	0.1	0	0
S23	0	0.63	1.02	0.96	0.97	0	0.54	0.87	0.62	0.63	0	0.51	0.55	0.38	0.37	0	0.4	0.47	0.24	0.2
S24	1.39	0.76	0.76	0.71	0.44	0.42	0.45	0.52	0.39	0	0	0.38	0.31	0.24	0	0	0	0	0	0

Hememan and Umbreit (1964) to be due to the compression of the cell contents and the detachment of the cell membrane. Bernheim (1963, 1964) showed that washed cells suspended in a solution of high ionic concentration developed an initial decrease in optical density followed by an increase in optical density. In a preliminary experiment in the present study washed cells were suspended in tryptone water and in tryptone water containing 2.5% and 4.5% sodium chloride. Increases in optical density were found in media containing sodium chloride but when strains were grown in the same media at 5° and 25° the relationship between viable count and optical density was similar in all three media. This observation was corroborated by the findings of Mager et al., (1956) and Avi. Dor et al., (1956) who noted that an initial increase in the turbidity of cells suspended in a medium of high salt content was followed by a return to the original turbidity with time. Bernheim (1963) also showed that the pre-incubation of cells in potassium chloride solution resulted in little increase in optical density.

It was concluded that any effect produced by sodium chloride on the strains did not affect the relationship between optical density and cell concentration during growth studies.

The combined effect on growth of variation in the salt concentration and variation in the incubation temperature will be discussed in terms of the growth parameters, Q_{10} values and St values of each strain.

Strain S1

L values were low or zero at all temperatures in 0.5% salt medium and were increased at 5° and 15° in salt-free medium and generally

increased in media containing 2.5% salt and over. The maximum M value was shown at 15° in salt-free medium although M values were highest at 25°, 35° and 37° in 0.5% salt medium. M values were much reduced at 35° and 37° in other media and growth was absent at all temperatures in 4.5% salt medium.

The psychrophilic pattern of low values for Q_{10} (5° - 15°) and Q_{10} (25° - 35°) with the Q_{10} (15° - 25°) value approximating to unity was shown in salt-free medium and in media containing 0.5% and 2.5% salt but a high Q_{10} (5° - 15°) value, a Q_{10} (15° - 25°) value greater than unity and a low Q_{10} (25° - 35°) value reflected a restriction in growth to the middle of the temperature range in the presence of 3.5% salt.

The St values of greater than unity indicated the stimulatory effect of 0.5% salt on the growth rate at all temperatures and especially at 35°, whereas 2.5% salt stimulated the rate of growth at 5°, inhibited the growth rate markedly at 35° and completely at 37°. In the presence of 3.5% salt the inhibition of growth was marked at 5° and at 15° and 37° there was a similar reduction in the growth rate as in 2.5% salt medium at the same temperatures.

Strain 32

L values were generally zero, although a high value was obtained at 15° in 4.5% salt medium. The maximum M value was shown at 5° in salt-free medium although the temperature of the highest M value in each medium varied from 15° in 0.5% and 4.5% salt media to 5° in 3.5% and 25° in 2.5% salt media. M values were generally low throughout

and exhibited a decrease at each temperature with increase in salt concentration from zero to 4.5%

The progressive reduction in Q_{10} (5° - 15°) and Q_{10} (25° - 35°) values with increase in salt concentration from 0.5% to 3.5% reflected an increased tendency towards psychrophilic growth. The addition of 0.5% salt resulted in increased Q_{10} (15° - 25°) and Q_{10} (25° - 35°) values compared with those obtained in salt-free medium.

The G_t values indicated that 0.5% salt stimulated the growth rate at 35° and to a lesser degree at 37°, thus causing a slight mesophilic shift in the growth pattern. This tendency was reversed in the presence of higher concentrations of salt with a much reduced growth rate at 15° and above in 2.5% and 3.5% salt media. Growth was absent at all temperatures except 15° in 4.5% salt medium.

Strain 83

K_t values were generally low or zero at temperatures except 15° or 25° in media containing 0.5%, 2.5% or no salt. Increased K_t values were obtained, particularly at 5°, 15° and 25°, in 3.5% and 4.5% salt media. The maximum K_t value was obtained at 15° in salt-free medium and the highest K_t values in other media were also shown at 15°. The highest K_t values at 5° and 25° were shown in 0.5% salt medium. No growth was observed at 37° in 4.5% salt medium.

The Q_{10} values indicated that the psychrophilic growth patterns shown in salt-free medium was maintained in 0.5% and 2.5% salt media. Increased Q_{10} (5° - 15°) values in 3.5% and 4.5% salt media and a reduced Q_{10} (25° - 35°) value in 4.5% salt medium reflected reduced

rates of growth at both extremes of the temperature range.

St values demonstrated the stimulation of the growth rate by 0.5% salt at all temperatures and 2.5% salt also stimulated the growth rate at 5° and 15°, but little or no effect was apparent at 25°, 35° and 37°. Slight stimulation was shown at 15° in 3.5% salt medium but the growth rate was reduced in this medium at 5° and 35° and unchanged at 37°. The St values relative to 4.5% salt medium indicated a generalised reduction in the growth rate at all temperatures.

Strain S4

L values were zero or small in 0.5% salt medium but were higher at 5° and 15° in salt-free medium and were generally increased at all temperatures in media containing 2.5%, 3.5% and 4.5% salt. The L value at 5° in 4.5% salt medium was particularly high. The maximum M value was shown at 15° in salt-free medium and in each medium the highest M value was shown at 15°. Although the M value at 15° was higher in salt-free medium than in media containing 0.5% and 2.5% salt the M values at 5°, 35° and 37° were higher in 0.5% salt medium and the values at 35° and 37° in 2.5% salt medium were higher than at corresponding temperatures in salt-free medium. In 4.5% salt medium M values were zero at 35° and 37°.

The development of lower Q_{10} (5° - 15°) and Q_{10} (15° - 25°) values in medium with the addition of 0.5% salt suggested an increased psychrophilic tendency but increases in the Q_{10} (15° - 25°) values in 2.5% and 3.5% salt media indicated a mesophilic orientation.

The St values demonstrated the appreciable stimulatory effect of

0.5% salt on the growth rates at all temperatures and especially at 35° and 37°. While the growth rates in salt-free medium and in 2.5% salt medium were similar at 5°, 15° and 25° St values greater than unity were shown at 35° and 37°. In 3.5% salt medium St values were less than unity at 5°, 15° and 25°, slightly greater than unity at 35° and appreciably greater at 37°. Thus in 0.5 - 3.5% salt media a distinct mesophilic growth pattern developed, while in 4.5% salt medium growth was absent at 35° and 37° and the growth rate was reduced least at 5° and a psychrophilic growth pattern was displayed.

Strain S5

L values were generally low in all media at most temperatures, although increased L values were associated with low M values particularly in 2.5% - 4.5% salt media. The maximum M value was obtained at 15° in 0.5% salt medium and the highest M values in each medium were obtained at this temperature. The M values at 25° and 35° were greatest in 0.5% salt medium and at 5° and 37° in salt-free medium. In media containing 2.5%, 3.5% and 4.5% salt M values were reduced markedly at 35° and 37°.

Compared with the values shown in salt-free medium the increased Q_{10} (5° - 15°) and Q_{10} (15° - 25°) values in 0.5% and 2.5% salt media revealed a less psychrophilic growth pattern in these media although reduced Q_{10} values in 3.5% and 4.5% salt media indicated a re-establishment of a psychrophilic growth pattern.

The St values demonstrated a general stimulation of the growth rate in 0.5% and 2.5% salt medium, particularly at 25°. The stimulation

effect decreased with both decrease and increase in temperature from 25° and the St value at 37° in 2.5% salt medium was virtually unity. Slight stimulation of the growth rate was observed at 5° in 4.5% salt medium but St values were generally below unity at other temperatures. Thus with increase in salt concentration a psychrophilic habit was more pronounced than in salt-free medium.

Strain S6

Negligible or zero L values were apparent in all media except 4.5% salt medium at 5°. The M values were highest at 15° in all media and the maximum value was shown at 15° in 0.5% salt medium. This medium produced the highest M values at 25° and 35° but salt-free medium produced the highest M value at 5° and 37°. M values were reduced at all temperatures in media containing 2.5% to 4.5% salt.

This strain displayed characteristics intermediate between a psychrophile and a mesophile in terms of Q_{10} values in salt-free medium. The reduction of Q_{10} (5° - 15°) and Q_{10} (25° - 35°) values and the development of Q_{10} (15° - 25°) values of less than unity in media containing 0.5 to 4.5% salt indicated a developed psychrophilic growth pattern in these media.

St values appreciably greater than unity at 5° to 25° and less than unity at 37° in 0.5% salt medium demonstrated the increased rate of growth at low temperatures. This pattern was shown in other media containing higher concentrations of salt, marked inhibition of the growth rate occurred particularly at temperatures above 25°.

Strain S7

L values in 0.5%, 2.5% and 3.5 % salt media were small or zero throughout the range 5° to 37°. An appreciable L value was shown at 5° in salt-free medium and at 5° and 15° in 4.5% salt medium. The maximum M value was obtained at 5° in salt-free medium and the highest M values in each medium occurred at either 5° or 15°. M values were greater at 5° and 15° in salt-free medium but values at 25°, 35° and 37° were greater in 0.5% salt medium.

With increase in salt concentration the Q_{10} values reflected a more pronounced tendency towards a psychrophilic growth response.

The St values indicated that 0.5% salt produced a stimulation of the growth rate at 5° to 35°, a slight inhibition occurring at 37°. In media containing 2.5% and 3.5% salt the growth rate was stimulated only at 5°, reduction in the growth rate being progressively greater from 15° to 37° in these media. St values less than unity, which were progressively reduced from 5° to 25° were shown in 4.5% salt medium.

Strain S8

L values were zero throughout the temperature range in salt-free medium and in media containing 0.5% and 2.5% salt. Increased L values were shown at 5° in 3.5% salt medium and at 5° and 15° in 4.5% salt medium. The maximum M value was displayed at 15° in 0.5% salt medium and M values were highest at 15° to 37° in this medium but the highest M value at 5° was obtained in salt-free medium. Increase in salt concentration resulted in a general reduction in M values but the inhibition of growth was more apparent at 25°, 35° and 37° than at 5°

and 15° in 3.5% and 4.5% salt media.

Although a Q_{10} (15° - 25°) value much greater than unity in 0.5% salt medium suggested the development of a mesophilic growth response compared with the psychrophilic growth response in salt-free medium low Q_{10} values at 5° - 15°, 15° - 25° and 25° - 35° in media with higher salt concentrations indicated increased psychrophilic tendencies.

The St values showed that 0.5% salt stimulated the growth rate at 5° to 37°, but the progressive increase in St values to 37° confirmed the mesophilic tendency indicated by Q_{10} values. This mesophilic tendency was reversed in media containing 2.5%, 3.5% and 4.5% salt. St values were uniformally greater than unity at all temperatures in 2.5% salt medium but were greater than unity only at 5°, 15° and 25° in 3.5% and 4.5% salt media.

Strain 59

L values were zero in salt-free medium and 0.5% salt media while values at 5° progressively increased with increase in salt concentration from 2.5% to 4.5%. L values were also appreciable at 15° and 25° in 4.5% salt medium. The maximum M value was shown at 15° in salt-free medium and M values were highest in each medium at this temperature. Highest M values at 5°, 35° and 37° were obtained in salt-free medium and at 25° in 2.5% salt medium. Appreciable M values were exhibited at 15° and 25° in all media.

The Q_{10} values in 0.5% salt medium indicated an increased psychrophilic growth response but Q_{10} (15° - 25°) values greater than

unity in other media, despite low Q_{10} (25° - 35°) values, suggested a less pronounced psychrophilic growth response.

St values greater than unity particularly at 5° and 15° demonstrated the stimulatory effect of 0.5% salt on the growth rate. St values greater than unity showed the stimulation of the rate of growth in 2.5% salt medium at 5° to 37° while stimulation in 3.5% salt medium was apparent at 5° to 25°, with the maximum increase in growth rate being indicated at 25°.

Strain S10

L values were zero at all temperatures in 0.5% salt medium and an appreciable L value was noted only at 5° in salt-free medium. L values at 25° progressively increased with increase in salt concentration from 2.5% to 4.5%. The maximum M value was shown at 5° in salt-free medium and the highest M values at 35° and 37° were displayed in this medium. M values at 15° and 25° were highest in 0.5% salt medium and with increase in salt concentration from 2.5% to 4.5% growth became restricted to the middle of the temperature range.

Reduced Q_{10} (5° - 15°) and Q_{10} (15° - 25°) values in 0.5% salt medium indicated a higher growth rate at low temperatures than in salt-free medium but an increased Q_{10} (25° - 35°) value tended to offset this pattern. The Q_{10} values in 2.5% salt medium also indicated a psychrophilic growth response.

An St value greater than unity was shown only at 5° in 0.5% salt medium and some reduction in the growth rate was shown at 15° to 37°. St values less than unity were progressively reduced from 5° to 35° in 2.5% salt medium and low St values at 15° and 25° in 3.5% salt medium

and at 15° in 4.5% salt medium reflected a great reduction in the growth rate in these media.

Strain S11

L values were zero in salt-free, 0.5% and 2.5% salt media and were infinite where growth was sparse in 3.5% and 4.5% salt media. The maximum M value was obtained at 15° in 0.5% salt medium and the highest M values at 25°, 35° and 37° were also shown in this medium. The highest M value at 5° was shown in salt-free medium. In media containing 2.5% to 4.5% salt the reduction in M values was more apparent at 5°, 35° and 37° than at 15° and 25°.

This strain was classed as an intermediate type on the basis of the Q_{10} values in salt-free medium. The Q_{10} values in media containing 0.5% - 4.5% salt, including Q_{10} (15° - 25°) values generally greater than unity, demonstrated the retention of a growth pattern intermediate between psychrophilic and mesophilic types.

While the St values relative to 0.5% salt medium clearly indicated a stimulation of the growth rate at 5° and progressively less stimulation with increase in test temperature, with slight reduction of the growth rate at 37°, this psychrophilic tendency was not shown in other media containing salt.

Strain S12

The L values at 5° and 15° were much reduced by the addition of 0.5% salt to tryptone medium, although growth at 5° was absent in media containing 2.5% to 4.5% salt. The L value at 15° in 4.5% salt medium was appreciable. The maximum M value was obtained at 37° in salt-free

medium and the highest M values at 15° and 35° were also shown in this medium. Medium containing 0.5% salt produced the highest M values at 5° and 25°. In media containing 2.5%, 3.5% and 4.5% salt, highest M values were shown at 15°.

Reduction in the Q_{10} (5° - 15°) and Q_{10} (25° - 35°) values in 0.5% salt medium reflected a reduced mesophilic growth response in this medium compared with the growth response in salt-free medium. The typically mesophilic pattern of Q_{10} values was, however, shown in media containing 2.5% to 4.5% salt.

The St values greater than unity at all temperatures, and particularly at 5°, indicated a general stimulation of the growth rate in 0.5% salt medium. St values less than unity at all temperatures in media containing higher concentrations of salt reflected a general reduction in the growth rate.

Strain S13

L values were generally zero in salt-free medium and media containing 0.5% and 2.5% salt. Appreciable L values were obtained at 15° in 3.5% salt medium and at 37° in 4.5% salt medium. The maximum M value was shown at 35° in 0.5% salt medium and the highest M value in each medium, except that containing no salt, was also shown at 35°. In salt-free medium the highest M value was shown at 37°. With increase in salt concentration from 2.5% to 4.5% M values were reduced at all temperatures.

The Q_{10} values signified the development of a more pronounced mesophilic growth response with increase in concentration of salt and

growth was restricted to 25°, 35° and 37° in 4.5% salt medium.

The St values demonstrated the marked stimulation of the rate of growth at 37° in 0.5% salt medium and the progressive decrease in stimulation with reduction in temperature, the growth rate being reduced at 15°. St values were less than unity at all temperatures in media containing 2.5% to 4.5% salt.

Strain 314

L values were generally greater in salt-free medium than in media containing 0.5% and 2.5% salt. Appreciable L values were shown at 5° and 15° in 3.5% salt medium and at 25° in 4.5% salt medium. The maximum M value was obtained at 15° in salt-free medium although M values at 25°, 35° and 37° were greatest in 0.5% salt medium. The greatest M value at 5° was shown in salt-free medium. Salt concentrations of 2.5% and 3.5% inhibited growth completely at 35° and 37°, while 4.5% salt inhibited growth at all temperatures except 25°.

While Q_{10} values in salt-free medium and in media containing 2.5% and 3.5% salt reflected psychrophilic patterns of growth the Q_{10} (15°-25°) greater than unity in 0.5% salt medium indicated a more intermediate pattern of growth.

The St values showed that the rates of growth at 5°, 15° and 25° were not appreciably influenced by 0.5% salt but that the rates of growth at 35° and 37° were markedly increased. Although higher salt concentrations resulted in St values of less than unity, the rates of growth were reduced less at 5° than at 25° in 2.5% and 3.5% salt media. Growth in 4.5% salt medium was produced, albeit poor, at 25°.

Strain S15

L values were zero in 0.5% salt medium and small values were obtained at 5° and 15° in 2.5% salt medium and salt-free medium. L values were greater at 5° and 15° in 3.5% salt medium and at 25° in 4.5% salt medium. The maximum M value was shown at 25° in 0.5% salt medium and while M values were higher at 15°, 25°, 35° and 37° in 0.5% and 2.5% salt media than in salt-free medium, the highest M value at 5° was displayed in salt-free medium. With increase in salt concentration from 3.5% to 4.5% M values decreased markedly at 35° and 37°.

Increases in the Q_{10} (15° - 25°) values in media containing 0.5%, 2.5% and 3.5% salt indicated a divergence from the psychrophilic pattern of growth shown in salt-free medium.

The St values indicated that stimulation of the growth rate occurred in 0.5% salt medium at all temperatures and particularly at 35° and 37°. This pattern was repeated in 2.5% salt medium with high St values at 37° and values less than unity at 5° and 15°. The tendency towards psychrophilic growth in 0.5% and 2.5% salt media was reversed in media containing 3.5% and 4.5% salt. The reduction in the growth rate was less at 5° to 25° than at 35° and 37° in these media.

Strain S16

L values were zero at 15° to 37° in 0.5% salt medium and the values were appreciable at 5° in this medium and in the absence of salt. At the temperatures which supported growth in 2.5% and 3.5% salt media L values were appreciable. The maximum M value was obtained at 15° in salt-free medium but the M values at 5°, 25° and 35° were greatest in 0.5% salt medium. Increases in salt concentration from 2.5% to 3.5%

reduced M values markedly at all temperatures and growth was absent at 5° and 37° in 2.5% salt medium, at 5°, 15°, 35° and 37° in 3.5% salt medium and at all temperatures in 4.5% salt medium.

A marked reduction in the Q_{10} (5° - 15°) value in medium containing 0.5% salt, compared with the corresponding Q_{10} value in salt-free media, reflected the development of a psychrophilic growth response, although a Q_{10} (15° - 25°) value of more than unity indicated an incomplete transition from the intermediate pattern of growth. The Q_{10} values in 2.5% salt medium indicated a mesophilic growth response.

St values greater than unity in 0.5% salt medium showed that the rate of growth was stimulated, particularly at 5°. In media containing higher concentrations of salt the growth rate was reduced at all temperatures.

Strain S17

L values at 5°, 15° and 25° were lowest in 0.5% salt medium. Appreciable L values were displayed at 5° to 25° in salt-free medium and in media containing 2.5% - 4.5% salt. The maximum M value was shown at 15° in 0.5% salt medium and the highest M value at 35° was also shown in this medium. The greatest M values at 5°, 25° and 37° were shown in salt-free medium. At levels of 3.5% and 4.5% salt, M values were much reduced, particularly at 5°, 25°, 35° and 37°.

As reflected in high Q_{10} (5° - 15°) values this strain maintained an intermediate growth pattern in salt-free medium and in 0.5% salt medium and a reduction in this Q_{10} value in 2.5% salt medium was offset by a Q_{10} (15° - 25°) value of greater than unity. The Q_{10} values in media containing 3.5% and 4.5% salt suggested a mesophilic pattern of growth in these media.

The St values revealed the stimulation of the growth rate at all temperatures by 0.5% salt, with the greatest values being shown at 35° and 37°. At salt concentrations of 2.5% to 4.5% reduction in the rate of growth was obtained at all temperatures with the least effect being shown at 25°.

Strain S18

L values were small or zero at 15° to 37° and appreciable at 5° in salt-free medium and 0.5% salt medium. At higher salt concentrations L values were generally greater at most temperatures. The maximum M value was obtained at 35° in 0.5% salt medium although M values were greatest at 5° and 37° in salt-free medium, at 25° in 2.5% salt medium and at 15° in 3.5% salt medium. With the increase in salt concentration from 2.5% to 4.5% M values were reduced more at 5°, 35° and 37° than at 15° and 25°.

The Q_{10} values in salt-free media indicated the intermediate growth pattern of this strain. This tendency was accentuated in 0.5% salt medium by increase in the Q_{10} (5° - 15°) value and a Q_{10} (25° - 35°) value of greater than unity. In media containing 2.5% to 4.5% salt infinite Q_{10} (5° - 15°) values reflected definite mesophilic growth patterns.

The St values showed that while 0.5% salt stimulated the growth rate throughout the whole temperature range, the greatest increase was obtained at 15°. Stimulation of the growth rate was shown at 15° in 2.5% salt medium but with increase in temperature reduction in the growth rate was shown. In media containing 3.5% and 4.5% salt the growth rate was reduced at all temperatures with least inhibition occurring at 25°.

Strain S19

In salt-free medium and in media containing 0.5% and 2.5% salt L values were zero at 15° to 37° and appreciable only at 5° in salt-free medium. Salt concentrations of 3.5% and 4.5% resulted in generally increased L values. The maximum M value was shown at 15° in salt-free medium which also produced the highest M values at 5°, 35° and 37°. The highest M value at 25° was produced in 0.5% salt medium. M values were reduced uniformally at all temperatures with increase in the salt concentration although poor growth was obtained only at 15° and 25° in 4.5% salt medium.

The Q_{10} values relative to growth in salt-free medium indicated an intermediate growth pattern and although the Q_{10} (25° - 35°) values in 0.5% and 3.5% salt media were reduced the Q_{10} (15° - 25°) values were greater than unity reflecting an intermediate growth pattern. The Q_{10} values in 2.5% salt medium indicated a characteristically psychrophilic growth pattern.

St values showed that the growth rate in 0.5% salt medium was stimulated at 15° and 25°, reduced at 5° and 35° and markedly reduced at 37°. Although reduction in the growth rate was apparent at all temperatures in 2.5% salt medium the effect was less marked at 15° and 5° than at 25°, 35° and 37° which resulted in the psychrophilic pattern of Q_{10} values in this medium. The growth rate in other media was much reduced at all temperatures.

Strain S20

L values in 0.5% and 2.5% salt media were zero at all temperatures but were appreciable at 5° and 15° in salt-free medium and at 5° to 35°

in 3.5% and 4.5% salt media. The maximum M value was obtained at 15° in salt-free medium and the highest M value at 5° was also shown in this medium. The highest M values at 25° and 35° were produced in 0.5% salt medium. Appreciable M values were demonstrated at 5°, 15° and 25° in media containing from 2.5% to 4.5%.

Q_{10} values in salt-free medium and in 0.5% and 2.5% salt media reflected psychrophilic growth patterns while in 3.5% and 4.5% salt media Q_{10} (15° - 25°) values greater than unity indicated a less psychrophilic pattern of growth.

St values greater than unity at 5°, 25° and 35° indicated the general stimulation of the growth rate by 0.5% salt while St values greater than unity occurred only at 25° in media containing 2.5%, 3.5% and 4.5% salt with values often much lower than unity at other temperatures.

Strain S21

L values were zero at each temperature in 0.5% and 2.5% salt media but were increased at 5° and 37° in salt-free medium and at most temperatures in 3.5% and 4.5% salt media. The maximum M value was shown at 5° in salt-free medium and the highest M values at 15° and 37° were also shown in this medium. The highest M values at 25° and 35° were displayed in 0.5% salt medium. Increase in salt concentration from 2.5% to 4.5% reduced M values uniformally at all temperatures.

The intermediate nature of this strain was reflected in the pattern of Q_{10} values in salt-free medium and in media containing 0.5% - 3.5% salt. Reduction below unity of the Q_{10} (15° - 25°) value

in 4.5% salt media resulted in a psychrophilic growth pattern in this medium despite generally poor growth at all temperatures.

The St values indicated that the growth rate at 25° and to a lesser extent at 35° and 37° was stimulated by 0.5% salt, while a slight reduction in the growth rate was produced at 5° and 15°. In media containing 2.5% and 3.5% salt stimulation of the growth rate was apparent only at 25° with appreciable inhibition occurring at above or below 25°. In 4.5% salt medium the growth rate was reduced less at 5°, 15° and 25° than at 35° which accounted for the psychrophilic pattern of Q_{10} values in this medium.

Strain S22

L values were zero or low at 15° to 37° in salt-free medium and in media containing 0.5% and 2.5% salt. Where growth occurred L values at 5° were appreciable, and at 15° in 3.5% salt medium. The maximum M value was shown at 15° in salt-free medium and the highest M values at 5° and 37° were also shown in this medium. The highest M values at 25° and 35° were produced in 0.5% salt medium and although M values at 15° were appreciable in 2.5% and 3.5% salt media growth at other temperatures in media containing 2.5% to 4.5% salt was generally poor or absent.

Although a marked reduction was shown in the Q_{10} (5° - 15°) value in 0.5% salt medium compared with the equivalent value in salt-free medium the Q_{10} (15° - 25°) values of appreciably greater than unity reflected the essentially intermediate character of this strain. Infinite Q_{10} (5° - 15°) values and little or no growth at 35° in 3.5% and 4.5% salt media demonstrated the inability of the strain to grow

at either extremes of the temperature range.

The St values showed that the growth rate was stimulated only at 5° in 0.5% salt media. Reduction of the growth rate was marked in media containing 2.5% to 4.5% salt.

Strain S23

L values were zero at all temperatures in 0.5% salt medium and at 25°, 35° and 37° in all other media, except at 25° in 4.5% salt medium. The maximum M value was found at 37° in salt-free medium and the highest M values at 15° and 35° were also obtained in this medium. The highest M value at 25° was shown in 0.5% salt medium. Appreciable M values were obtained at 15° to 37° in salt-free medium and in 0.5% and 2.5% salt media, and at 15° in 3.5% salt medium. M values were uniformally reduced at all temperatures in 4.5% salt medium.

The Q_{10} values in all media indicated the mesophilic nature of this strain although a progressive reduction in Q_{10} (25° - 35°) values with increase in salt concentration reflected the reduced growth response at 35°.

The St values showed that 0.5% salt stimulated the growth rate only at 25° and higher salt concentrations markedly reduced the growth rate at all temperatures and especially at 35° and 37°.

Strain S24

Strain S24

L values were zero at 15° to 35° in 0.5% salt medium and at 25° in salt-free medium. At other temperatures and in all other media L values were appreciable and often great, particularly at 5°. The

maximum M value was obtained at 15° in salt-free medium and the highest M values at all other temperatures were shown in this medium. Salt concentrations from 0.5% to 3.5% reduced M values at all temperatures and growth was absent in 4.5% salt medium.

Typically psychrophilic patterns of Q_{10} values were shown in salt-free medium and in media containing 0.5% and 2.5% salt but growth was absent at 5° in 4.5% salt medium.

An St value greater than unity indicated stimulation of the growth rate at 5° in 0.5% salt medium but increase in temperature resulted in a progressive reduction in the growth rate in this and other media

Of the 24 strains tested all produced growth at one or more temperature in salt-free medium and media containing 0.5%, 2.5% and 3.5% salt and while growth, in terms of K or M values was often poor, only three strains (S1, S16 and S24) were unable to produce growth at any temperature in 4.5% salt medium. While difficulty exists in a comparison of the results of other workers due to differences in the interpretation of 'growth' it is worthwhile to note that Bonner and Harmon (1957) found that psychrophilic isolates were able to tolerate 5.0% salt and Witter (1961) indicated that psychrophilic strains isolated from milk were able to grow in the presence of 4.0% salt. In a previous study the author (Scholefield, 1964) showed that of 275 psychrophilic isolates from dairy sources, 86% grew on 3.0% salt medium, 31% grew on 5.0% salt medium and 4.0% of the strains grew on 8.0% salt medium at 22°.

With the increase in salt concentration from 0.5% to 4.5%

extensions of the apparent lag (L) were shown by many strains. Strains S2, S6, S8, S9, S11, S12, S13, S20 and S21 often displayed little or no apparent lag in media containing up to 3.5% salt, although where shown the apparent lag was more pronounced at 5°. Strains S1, S3, S4, S7, S14, S16, S17, S18, S19, S22 and S24 often showed appreciable apparent lags even in media and at temperatures at which the rate of growth or cell crops were high. Strains S1, S2, S4, S10, S12, S14, S15, S16, S17, S19, S20, S21 and S23 exhibited greater apparent lags at certain temperatures in salt-free medium than in 0.5% salt medium, although the cell crop (M) was often higher in the former medium. The Q_{10} and St values showed that 0.5% salt stimulated the rate of growth, at one or more temperatures, of all strains except strain S23. This observation is in accordance with the findings of Marshall and Scott (1958) and Ware et al. (1955). In the present study stimulation of the growth rate of 13 strains by 0.5% salt occurred at all test temperatures and the growth rate of 18 strains was increased at 5° by this concentration of salt. Strains S2, S13 and S21 were stimulated only at 25° to 37° while the stimulation of strains S6, S9 and S18 was greatest at 5°, 15° and 25°. Strains S17 and S24 displayed stimulation of the growth rate in 0.5% salt medium although the corresponding cell crops were lower than those produced in salt-free medium.

Gibbons et al. (1951) observed that minimum temperatures of growth of psychrophiles from bacon was reduced with increase in the salt concentration of the medium. In this study mesophilic strain S12 produced some growth at 5° in 0.5% salt medium whereas no growth was

observed in salt-free medium. A progressive reduction in the growth rate and the cell crop together with increased apparent lags were found in most strains when the salt concentration was raised from 0.5% to 4.5%. Strain S9 exhibited higher rates of growth at 25°, 35° and 37° in 2.5% salt medium than in 0.5%, 3.5% and 4.5% sale media and in salt-free medium. Salt concentrations of 2.5% to 4.5% tended to restrict the range of temperatures at which strains were able to grow, and psychrophilic strains S1, S2, S7, S8, S10, S14, S16, S20, S22 and S24 showed less ability to grow at 35° and 37° in these media. At high salt concentrations certain strains were able to grow better at low rather than at high temperatures. Strains S3, S5, S6, S7, S8, S9, S15 and S20 produced appreciable cell crops at 25° and below in 4.5% salt medium while mesophilic strain S12 exhibited the highest cell crop in this medium at 15°.

This experiment has emphasised still more the diversity of the test strains in their response to change in temperature. The effect of sodium chloride on growth as a factor additional to temperature has been seen not to be a simple phenomenon. In particular, it has been shown again, as in Part I, that it is impossible to predict the growth response of a strain at a temperature on the basis of observed growth at another temperature and that under different environmental conditions the relationship between growth and temperature is variable.

III. THE EFFECT OF TEMPERATURE ON THE UTILISATION AND BREAKDOWN OF AMINO ACIDS

AND BREAKDOWN OF AMINO ACIDS

In Part I of this study the growth of strains in media containing protein hydrolysate as carbon, nitrogen and energy sources was often found to be less affected by changes in the incubation temperature than growth in certain carbohydrate media containing an inorganic nitrogen source. Part II of this study demonstrated the value of tryptone as a substrate for the growth of strains S1 - S24. Rhodes (1959) showed that peptone supported active growth of Pseudomonas strains and Gaby et al. (1962) found that Pseudomonas strains utilised casitone at a faster rate than glucose. Tryptone contains a range of amino acids and Barker (1961) indicated that many amino acids are at oxidation levels intermediate between carbohydrates and fats and that individually they may provide useful sources of carbon, nitrogen and energy for aerobes and anaerobes.

It is evident from the literature that little is known of the ability of Pseudomonas strains to utilise amino acids or of the mode of action of the enzymes responsible (Gunsalus and Stanier, 1962; Rose, 1965). The enzyme systems which have been reported to be involved in amino acid utilisation by Pseudomonas strains include decarboxylases (Edmondson, 1954; Seaman, 1960; Halpern and Umbarger, 1961; Rose, 1965), deaminases of different types (Warren et al, 1960; Palleroni and Stanier, 1964) transaminases (Feldman and Gunsalus, 1950; Umbarger and Davis, 1962), dihydrolase (Møller, 1955; Thornley, 1960) and deamidases (Stewart, 1964).

Many psychrophilic bacteria are able to degrade casein with the formation of alkaline end-products, including ammonia and related compounds, produced by the terminal metabolism of amino acids. Few studies have been made concerning the growth of, and the changes produced by Pseudomonas strains in media containing amino acids. Sherris, Shoesmith et al. (1959) showed that nearly all Pseudomonas strains examined were able to degrade arginine with the production of an alkaline change, and Thornley (1960) utilised this reaction in the development of a biochemical test for arginase. A modified arginase test was employed in Part I of this study.

It is difficult to compare the results of different studies concerning the utilisation or breakdown of amino acids due to variations in the composition of the media used. As Kornberg et al. (1955) demonstrated, changes in nutritional conditions may cause appreciable changes in the enzyme systems and metabolic pathways used in Pseudomonas spp.. Thus a casitone concentration of 1.0% in an amino acid basal medium, as used by Gaby et al. (1962) and Deibel (1964), or a peptone concentration of 0.5%, as used by Møller (1955) may result in a non-specific alkaline change unconnected with the metabolism of the test amino acid. Such high concentrations of peptone or casitone may mask acidic products of amino acid breakdown or inhibit the operation of certain enzyme systems. Alternatively, 0.5% glucose incorporated in the medium of Gaby et al. (1962), or 1.0% glucose in the medium of Burman and Oliver (1952) may result in an acid change which could mask alkaline end-products of amino acid breakdown.

Studies of the utilisation by Pseudomonas spp. of amino acids as

sole carbon, nitrogen and energy sources are few. Georgia and Poe (1931) used asparagine and Colwell (1964) examined the ability of Ps. aeruginosa to utilise six individual amino acids as sole carbon, nitrogen and energy sources. Guroff and Ito (1965) examined the changes produced by strains in defined media containing phenylalanine, tyrosine or asparagine as carbon and energy sources but an ammonium salt was included as nitrogen source.

The influence of temperature on the utilisation and breakdown of amino acids has not been studied although Jezeski and Olsen (1962) suggested that casamino acids were involved in the ability of strains to grow at low temperature. It was therefore decided to investigate the growth of strains S1 - S24 at 5°, 15°, 25°, 30° and 35° in a defined medium containing individual amino acids. In addition, parallel determinations were made of the levels of ammonia produced during utilisation of the individual amino acids.

Materials and Methods

Media: In a preliminary experiment 21 individual amino acid media were prepared, using 0.2% (w/v) of each amino acid listed below in Pope and Skerman (Skerman, 1959) mineral salts basal medium.

L-amino acids.

B	ornithine	*I	leucine	+P	D.L-alanine
+C	lysine	*J	proline	+Q	β-alanine
*D	asparagine	*K	serine	-R	citrulline
*E	phenylalanine	*L	methionine	+S	glutamine
*F	histidine	*M	glycine	T	leucinamide
+G	glutamic acid	*N	tryptophane	*U	glycyl-glycine
*H	aspartic acid	O	ε-diaminopimelic acid	V	arginine

In the main experiment 7 individual amino acid media were prepared employing 0.2% (w/v) lysine (C), glutamic acid (G), leucine (I), glycine (M), tryptophane (N), β-alanine (Q) or arginine (V) in Pope and Skerman basal salts medium. The 7 amino acids were selected for the main experiment because they comprised representative examples of different types of amino acids and together they comprised approximately 70% of the amino acid types present in casein and casein hydrolysate. These amino acids were also found in the preliminary experiment to produce growth responses by the strains which were representative of many of the 21 amino acids. A control medium containing Pope and Skerman basal salts medium without amino acids was also prepared. The media were dispensed in 12 ml amounts in nephelometer tubes.

Inocula: Test strains were grown and standardised suspensions were prepared and inoculated into test media as described in Part II. Single tubes of each medium were inoculated for each strain in the preliminary experiment and triplicate tubes were inoculated in the main experiment. Control tubes of Pope and Skerman basal medium without added amino acid were also inoculated.

Incubation: In the preliminary experiment tubes were incubated at 25° and in the main experiment triplicate tubes were incubated at 5°, 15°, 25°, 30° and 35° as described in Parts I and II.

Growth determinations: In the preliminary experiment nephelometric measurements, as described in Part II, were made after 24 hours and 96 hours incubation at 25°. In the main experiment nephelometric measurements were made at intervals on tubes incubated at each temperature.

pH determinations: In the preliminary experiment electrometric pH measurements were made at intervals of 24 hours and 96 hours.

Ammonia determinations: A method based on the Nessler technique (Lovibond Nessleriser, British Drug Houses, Ltd) was made. The technique was employed by Deibel (1964) to relate the growth of Streptococcus spp. with the breakdown of arginine. The method employed standard comparator reference discs for the following concentrations of ammonia.

Nessleriser disc	Ammonia (mg. per ml)
NAA	0.001 - 0.01
NAB	0.01 - 0.026
NAC	0.028 - 0.06
NAD	0.06 - 0.1

One reference Nessleriser glass was filled to the 50 ml mark with distilled water and placed in the left-hand compartment. Each sample of culture, aseptically removed following nephelometric measurements from each of the triplicate tubes, was added to the other paired Nessleriser glass and distilled water added to the 50 ml mark. To each sample glass was added 2 ml Nessler's solution (British Drug Houses, Ltd). With small quantities of ammonia (0.001 - 0.005 mg/ml) readings were taken after 15 minutes, to allow full colour development. Where more than 0.005 mg./ml was present colour matching was made after 5 minutes. Nessleriser glasses were well washed in distilled water between samples to prevent carry-over of ammonia. Culture samples between 0.25 and 1.0 ml were used to provide levels of ammonia measurable

within the disc ranges.

Treatment of Results

In the preliminary experiment the growth of each strain after 96 hours at 25° in each amino acid medium was scored in the manner used in Part I of this study.

Optical density x 100			Symbol
over		1.5	4
0.75	-	1.5	3
0.25	-	0.74	2
0.1	-	0.24	1
under		0.1	0

In the main experiment mean nett optical density (O.D) values were calculated and growth curves constructed as described in Part II. From these curves the growth parameters Apparent lag (L), Growth rate constant (K) and Maximum cell crop (M) were derived for each strain grown in each amino acid medium at each temperature. Temperature coefficient (Q_{10}) values were calculated at 5° - 15°, 15° - 25° and 25° - 35° as described in Part II, from the appropriate K values for each strain grown in each amino acid medium. In order to compare the growth rate of each strain in an amino acid medium with the growth rate in 0.2% tryptone medium at the same temperature the coefficient (St), employed in Part II to assess the effect of sodium chloride on the growth rate, was used. In this experiment the St value represented the ratio of a growth rate constant (K_2) in an amino acid medium, to the growth rate constant (K_1) in 0.2% tryptone medium.

In the preliminary experiment changes in the pH of inoculated amino acid media after 96 hours incubation at 25° were semi-quantitatively scored.

pH change (units)	Symbol
over 0.8	2
0.3 - 0.8	1
below 0.3	0

In the main experiment mean nett optical density (O.D) values were plotted against the corresponding levels (mg/ml) of ammonia produced by each strain at 5°, 15°, 25°, 30° and 35° in each amino acid medium.

Results and Discussion

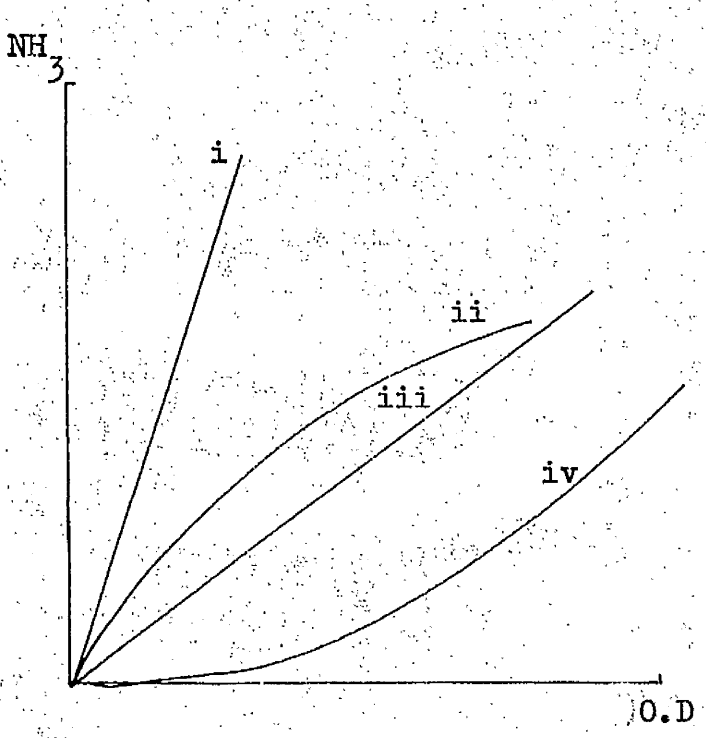
The strains showed marked differences in their abilities to utilise individual amino acids and variation in temperature affected the ability of individual strains to utilise these amino acids. No strain was able to utilise tryptophane as sole carbon, nitrogen and energy sources at any temperature and no strain was able to grow in Pope and Skerman mineral salts basal medium without amino acids.

The results concerning the utilisation of amino acids by strains S1 - S24 at 5° to 35° are shown in terms of the growth parameters (L, K and M values) in Tables 7. 1 to 7. 6, the temperature coefficients (Q_{10} values) in Tables 8. 1 and 8. 2 and the St values in Tables 9. 1 to 9. 4.

The results which relate the production of ammonia with the utilisation of individual amino acids by the strains at 5° to 35° are

shown in Figures 1. 1 to 1. 23. These results provide comparisons of (a) the abilities of each strain to produce ammonia during the utilisation of different amino acids, (b) the abilities of different strains to produce ammonia during the utilisation of each amino acid (c) the effect of temperature on the production of ammonia relative to growth.

The patterns of ammonia production often approximated to one of the types of curve shown in the accompanying figure.



Type (i) curve shows a rapid linear increase in ammonia at relatively low levels of growth with the maximum cell crop (M) often being small.

Type (ii) curve shows a rapid production of ammonia during early stages of growth with a subsequent levelling off in ammonia production

at higher cell crops.

Type (iii) curve shows a linear relationship between the production of ammonia and growth at all levels of growth.

Type (iv) curve shows an initial delay in the production of ammonia during the early stages of growth with a subsequently increased ammonia production at higher cell crops.

Growth parameters.

Apparent lag (L): In general the L values of strains S1 - S24 were greater at all temperatures in media containing glutamic acid, arginine, lysine, leucine, glycine or β-alanine than the corresponding values in tryptone medium. The only strains which exhibited lower L values than in tryptone medium were strain S10 in glutamic acid medium at 5° and strain S23 in lysine medium at 15°. The extension of the apparent lag was often more pronounced at 5° and 15° in amino acid media than in tryptone medium and strain S1 in leucine medium, Strains S3 and S4 in leucine and glycine media, strain S8 in glycine medium, Strains S14 and S17 in glutamic acid medium, strain S21 in leucine medium and strain S23 in glutamic acid and lysine media showed increased L values at both ends of the temperature range. The high L values shown by many strains were correlated with low M values in glycine, leucine, lysine or β-alanine media but many strains displayed high L values in association with high M values in media containing glutamic acid or arginine.

Growth rate constant (K): The maximum K values shown by many strains in amino acid media occurred at different temperatures to those obtained in tryptone medium. In glycine and glutamic acid media all strains except strain S23 exhibited maximum K values at temperatures similar to,

Table 7.1 Growth parameters in amino-acid media

Amino-acid 0.2% (w/v)		glutamic acid (G)					arginine (V)					lysine (C)				
Temp. °C.		5°	15°	25°	30°	35°	5°	15°	25°	30°	35°	5°	15°	25°	30°	35°
Strain																
	L	2.0	0.7	0.6	0.5	0	3.5	1.6	0.9	0	0	∞	11	5.5	3.0	1.2
S.1	K	14.4	33.7	52.7	70	23.2	11.2	28.5	40.4	52	9.8	1.0	10.5	15	5.1	3.8
	M	268	320	388	268	130	187	106	244	300	82	20	118	76	48	21
	L	2.0	1.8	∞	∞	∞	∞	∞	∞	∞	∞	∞	∞	∞	∞	∞
S.2	K	5.6	12	0	0	0	0	0	0	0	0	0	0	0	0	0
	M	105	160	0	0	0	0	0	0	0	0	0	0	0	0	0
	L	1.5	1.2	1.2	1.3	0.5	2.0	0.8	0.5	0.6	0	∞	1.2	1.6	4.0	1.7
S.3	K	8.4	23.8	31.2	29.8	13.3	6.5	23	40	31	11.3	2.0	6.0	4.2	4.8	2.2
	M	165	265	215	190	81	79	273	160	148	62	47	123	40	28	14
	L	2.0	0.8	0.6	0.5	0	2.3	1.5	0	0	0	∞	10	6.5	3.5	∞
S.4	K	10.3	37.4	53	64.8	15.5	11	29	48	39	7.9	2.0	10.4	25	5.3	0
	M	161	313	380	365	97	133	267	310	272	75	34	150	135	42	0
	L	1.5	1.2	1.2	1.5	0.7	3.2	1.4	0	0	0	∞	4.5	4.4	∞	∞
S.5	K	6.4	23	24	60	34	6.3	25	28	20	13	0.8	3.6	15.5	2.0	0
	M	150	270	148	315	250	92	243	180	196	83	20	58	94	16	0
	L	1.2	1.2	1.0	1.1	0.7	0.8	0	0	0	0		5.8	3.5	4.7	2.8
S.6	K	6.3	23	31	60	52	8.5	27	33	34	31	1.2	8.0	6.5	5.6	2.6
	M	170	370	220	213	190	112	232	200	173	184	28	112	53	39	12
	L	2.0	1.4	3.1	1.2	0.6	∞	∞	1.0	0	∞	∞	∞	∞	∞	∞
S.7	K	7.2	23.5	51	21.8	9.2	0	3.0	10	5.5	0	0	0	0	0	0
	M	140	165	242	132	51	0	12	38	48	0	0	0	0	0	0
	L	1.7	1.2	1.4	1.0	0.2	1.8	1.0	0	0.8	0	9.7	10.5	2.0	∞	∞
S.8	K	6.6	20	36.5	21	6.5	8.8	23	41	13	4.2	1.7	7.3	3.6	2.5	0
	M	162	249	235	103	41	169	208	252	67	38	32	75	34	7	0

Key: L = apparent lag (days)
K = growth rate constant
M = maximum cell crop (O.D)

Table 7. 2 Growth parameters in amino-acid media

Amino-acid 0.2% (w/v)		leucine (I)					glycine (M)					β-alanine (Q)				
Temp. °C.		5°	15°	25°	30°	35°	5°	15°	25°	30°	35°	5°	15°	25°	30°	35°
Strain																
	L	5.0	2.0	1.0	1.7	1.5	∞	∞	∞	∞	∞	∞	∞	4.7	∞	∞
S.1	K	3.3	14.1	27.5	33.5	16.5	0	0	0	0	0	0	0	22	0	0
	M	26	135	161	111	110	0	0	0	0	0	0	0	151	0	0
	L	∞	∞	∞	∞	∞	∞	∞	∞	∞	∞	∞	∞	∞	∞	∞
S.2	K	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	M	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	L	4.5	2.0	0.5	1.2	0.6	6.2	4.1	0.5	1.0	0.5	∞	∞	6.5	∞	∞
S.3	K	4.4	15.5	8.2	12.2	5.6	4.1	6.1	3.2	9.7	3.6	0	0	3.0	0	0
	M	50	152	69	95	42	70	57	35	41	23	0	0	21	0	0
	L	6.0	1.7	1.0	1.8	1.3	∞	∞	∞	∞	∞	∞	10.5	6.7	∞	∞
S.4	K	4.7	14.3	22.8	30.3	14.5	0	0	0	0	0	0	8.2	19.8	0	0
	M	35	141	131	180	105	0	0	0	0	0	0	80	101	0	0
	L	∞	∞	∞	∞	∞	∞	∞	∞	∞	∞	∞	∞	6.0	∞	∞
S.5	K	0	0	0	0	0	0	0	0	0	0	0	0	8.2	0	0
	M	0	0	0	0	0	0	0	0	0	0	0	0	47	0	0
	L	3.5	1.3	1.0	1.0	∞	∞	∞	∞	∞	∞	∞	∞	1.0	∞	∞
S.6	K	3.8	9.8	4.5	3.3	0	0	0	0	0	0	0	0	7.3	0	0
	M	95	140	32	22	0	0	0	0	0	0	0	0	76	0	0
	L	∞	∞	∞	∞	∞	∞	∞	∞	∞	∞	∞	∞	∞	∞	∞
S.7	K	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	M	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	L	5.8	3.1	2.0	2.0	∞	4.0	1.5	0.5	1.5	1.0	∞	∞	∞	∞	∞
S.8	K	3.9	12.8	6.8	5.0	0	4.3	16.6	8.6	7.4	2.1	0	0	0	0	0
	M	79	149	35	31	0	74	104	51	35	22	0	0	0	0	0

Table 7.3 Growth parameters in amino-acid media

Amino-acid 0.2% (w/v)		glutamic acid (G)					arginine (V)					lysine (C)				
Temp. °C.		5°	15°	25°	30°	35°	5°	15°	25°	30°	35°	5°	15°	25°	30°	35°
Strain																
	L	1.5	0.8	1.3	1.4	1.0	2.1	1.6	1.5	0.8	1.0	∞	4.0	3.2	3.7	2.5
S.9	K	7.7	21.8	41.7	52	24.5	4.3	11.5	23	29.7	10.5	1.8	3.2	2.8	3.1	1.5
	M	12.3	197	264	270	120	32	35	170	152	83	39	55	25	26	10
	L	2.0	0.7	0.6	0.6	0.5	3.0	2.3	1.8	1.0	0.8	∞	∞	∞	∞	∞
S.10	K	5.3	22.4	33.5	40.2	24.2	2.3	7.8	9.8	12.2	4.8	0	0	0	0	0
	M	135	232	247	290	135	19	32	38	49	31	0	0	0	0	0
	L	∞	3.5	2.0	0.5	0	∞	4.0	2.1	1.6	0	∞	∞	∞	∞	∞
S.11	K	0	8.2	13.3	26	18.7	0	6.2	0.7	9	4	0	0	0	0	0
	M	0	48	122	98	73	0	23	41	25	21	0	0	0	0	0
	L	∞	2.0	0.8	0.5	0	∞	1.6	0.2	0	0	∞	∞	3.5	2.8	1.0
S.12	K	0	21.2	43.6	49	59	0	25.2	39.5	46	53	0	0	22.4	31.2	25.3
	M	0	256	260	340	300	0	226	250	308	325	0	0	190	192	165
	L	∞	1.6	1.0	1.4	0	∞	2.8	1.2	0.8	0	∞	∞	∞	∞	∞
S.13	K	0	20.8	48	66	75	0	16	41	53.5	59.5	0	0	0	0	0
	M	0	255	220	353	312	0	240	210	290	323	0	0	0	0	0
	L	2.5	0.6	0	0.8	0.4	3.0	1.6	0	0	0	∞	2.7	2.2	2.6	1.0
S.14	K	6.4	20.4	23.5	30.7	12.3	5.6	19	22.5	25.2	9.8	2.0	11.4	22.5	11.2	3.8
	M	136	246	188	208	83	96	210	192	210	86	44	184	162	77	31
	L	2.0	1.0	1.0	1.0	0.5	2.5	1.0	0	0	0	11.6	7.5	6.0	∞	∞
S.15	K	12.0	30	41.5	46.3	21	11.0	29	39	38.3	18.5	3.0	9.5	5.0	1.3	0
	M	135	284	270	265	128	106	252	192	150	78	50	130	50	12	0
	L	∞	∞	∞	∞	∞	∞	∞	∞	∞	∞	∞	∞	∞	∞	∞
S.16	K	0	0.9	2.8	1.5	0	0	0	0	0	0	0	0	0	0	0
	M	0	9	16	12	0	0	0	0	0	0	0	0	0	0	0

Table 7. 4 Growth parameters in amino-acid media

Amino-acid 0.2% (w/v)		leucine (I)					glycine (M)					β-alanine (Q)				
Temp. °C.		5°	15°	25°	30°	35°	5°	15°	25°	30°	35°	5°	15°	25°	30°	35°
Strain																
	L	3.0	1.6	1.7	1.7	0.7	∞	∞	∞	∞	∞	∞	9.5	4.5	∞	∞
S.9	K	2.9	13.1	16.6	23	13	0	0	0	0	0	0	4.3	4.8	0	0
	M	62	14.3	105	131	70	0	0	0	0	0	0	49	35	0	0
	L	∞	∞	∞	∞	∞	∞	∞	∞	∞	∞	∞	10	2.5	∞	∞
S.10	K	0	0	0	0	0	0	0	0	0	0	0	1.9	20.2	0	0
	M	0	0	0	0	0	0	0	0	0	0	0	20	187	0	0
	L	∞	∞	∞	∞	∞	∞	∞	∞	∞	∞	∞	∞	4.5	∞	∞
S.11	K	0	0	0	0	0	0	0	0	0	0	0	0	23.4	0	0
	M	0	0	0	0	0	0	0	0	0	0	0	0	171	0	0
	L	∞	3.2	1.3	1.7	1.2	∞	∞	∞	∞	∞	∞	∞	2.3	∞	∞
S.12	K	0	8.8	7.8	16	19.5	0	0	0	0	0	0	0	38.4	0	0
	M	0	118	42	120	149	0	0	0	0	0	0	0	212	0	0
	L	∞	3.5	1.5	1.2	0.5	∞	∞	∞	∞	∞	∞	∞	2.0	1.7	0.8
S.13	K	0	8.0	16.3	20.3	22.3	0	0	0	0	0	0	0	10.8	16.5	18.7
	M	0	112	105	139	171	0	0	0	0	0	0	0	104	111	117
	L	4.3	1.2	1.2	0.4	0	10	5	3	1.0	∞	∞	10	6	∞	∞
S.14	K	3.7	13.3	14.2	12	6.5	3.5	2.5	3.2	4.2	0	0	2.7	13.6	0	0
	M	81	156	102	89	61	21	18	13	19	0	0	30	78	0	0
	L	4.0	1.5	1.7	2.7	1.3	∞	∞	∞	∞	∞	∞	10.5	7.0	∞	∞
S.15	K	4.0	9.1	11.1	25.3	12.3	0	0	0	0	0	0	16	25.3	0	0
	M	76	109	71	154	81	0	0	0	0	0	0	163	120	0	0
	L	∞	∞	∞	∞	∞	∞	∞	∞	∞	∞	∞	∞	∞	∞	∞
S.16	K	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	M	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Table 7. 5 Growth parameters in amino-acid media

Amino-acid 0.2% (w/v)		glutamic acid (G)					arginine (V)					lysine (C)				
Temp. °C.		5°	15°	25°	30°	35°	5°	15°	25°	30°	35°	5°	15°	25°	30°	35°
Strain																
	L	∞	∞	0.5	1.5	∞	∞	2.8	0.4	0	0	∞	∞	∞	∞	∞
S.17	K	0.6	2.0	3.0	3.2	2.0	2.5	5.3	15	8.5	2.3	0	0	0	0	0
	M	12	32	25	24	16	16	36	53	68	18	0	0	0	0	0
	L	5.0	1.0	0.7	0.8	0.4	6.2	2.1	0	0	0	∞	9.6	2.0	2.0	0.7
S.18	K	8.0	23.8	61	77.5	64	9.0	24	58	63	50	0	23	28.7	51	52
	M	150	302	285	340	290	75	250	270	320	146	0	271	290	350	420
	L	1.8	1.3	0.6	0.9	0.3	2.3	0.8	0.2	0	0	∞	∞	∞	∞	∞
S.19	K	7.3	34	39.6	52.2	36	7.5	21	35	53	32	0	0	0	0	0
	M	197	339	259	342	240	115	202	230	287	220	0	0	0	0	0
	L	1.9	0.9	0.6	0.8	0.4	2.1	1.3	0.3	0	0	∞	5.7	2.4	1.5	0.6
S.20	K	8.6	29.8	40.2	46.5	11.7	6.5	18.3	38	43	10.5	2.4	10.6	15.8	16.3	8.3
	M	150	294	257	278	80	153	173	238	201	91	49	163	168	105	70
	L	2.0	0.7	1.4	1.6	0.6	2.6	1.5	0.8	1.8	0	∞	1.7	1.0	1.5	∞
S.21	K	8.3	20.5	32.2	44	38.4	7.2	30	29.5	26.5	17.4	1.0	3.2	2.8	2.2	0
	M	197	169	197	273	302	171	160	182	231	125	20	61	31	20	0
	L	∞	2.3	1.2	1.2	0.3	5	1.8	0.3	0	0	∞	∞	∞	∞	∞
S.22	K	2.7	34.3	33.2	39.4	24.8	1.9	27.3	36	39.2	23	0	0	0	0	0
	M	60	270	195	202	130	80	286	192	202	155	0	0	0	0	0
	L	∞	2.4	0.4	1.5	0.6	∞	3.2	0.8	0	0	∞	1.0	1.0	2.5	1.2
S.23	K	0	37.2	18	28	34.2	0	23.2	24.5	29.6	38.5	0	4.8	4.0	5.8	8.0
	M	0	362	106	170	230	0	200	142	261	310	0	84	22	25	28
	L	∞	∞	3.0	2.3	1.0	∞	∞	2.3	1.2	∞	∞	∞	∞	∞	∞
S.24	K	0	0	8.7	9.4	6.0	3.0	6.0	9.7	6	0	0	0	0	0	0
	M	0	0	36	83	42	18	25	63	40	0	0	0	0	0	0

Table 7. 6 **Growth parameters in amino-acid media**

Amino-acid 0.2% (w/v)		leucine (I)					glycine (H)					β-alanine (Q)				
Temp. °C.		5°	15°	25°	30°	35°	5°	15°	25°	30°	35°	5°	15°	25°	30°	35°
Strain																
	L	∞	4.2	∞	∞	∞	∞	∞	∞	∞	∞	∞	∞	∞	∞	∞
S.17	K	1.0	6.7	0	0	0	0	0	0	0	0	0	0	0	0	0
	M	20	91	0	0	0	0	0	0	0	0	0	0	0	0	0
	L	6.0	1.7	2.6	1.8	1.0	∞	2.4	1.6	0.3	0	∞	∞	4.7	∞	∞
S.18	K	3.0	13.7	23	31.6	35.5	2.4	19	22.8	21.4	30	0	0	24	0	0
	M	69	166	123	195	245	25	174	122	160	210	0	0	170	0	0
	L	5.2	1.6	1.5	1.8	1.3	∞	∞	∞	∞	∞	∞	∞	∞	∞	∞
S.19	K	4.5	10.7	23.7	49.8	34.2	0	0	0	0	0	0	0	0	0	0
	M	66	137	113	268	225	0	0	0	0	0	0	0	0	0	0
	L	3.3	1.5	1.5	1.5	0.5	∞	∞	∞	∞	∞	5.5	3.5	1.6	1.5	0
S.20	K	4.0	16.6	32.8	51	18.6	0	0	0	0	0	4.1	15.7	15.8	40.7	8.7
	M	88	143	215	350	130	0	0	0	0	0	67	274	120	267	63
	L	3.5	2.0	1.5	3.0	∞	∞	∞	∞	∞	∞	∞	6.5	3.7	3.4	1.0
S.21	K	3.5	10.2	9.4	6.2	1.2	0	0	0	0	0	0	10	10	13.1	5.1
	M	77	111	39	35	16	0	0	0	0	0	0	140	69	73	38
	L	∞	∞	∞	∞	∞	∞	∞	∞	∞	∞	∞	∞	∞	∞	∞
S.22	K	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	M	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	L	∞	∞	∞	∞	∞	∞	∞	∞	∞	∞	∞	∞	∞	∞	∞
S.23	K	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	M	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	L	∞	∞	∞	∞	∞	∞	∞	∞	∞	∞	∞	∞	∞	∞	∞
S.24	K	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	M	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

or higher than, those shown in tryptone medium. In lysine medium all strains except strains S18 and S20 exhibited maximum K values at temperatures similar to, or lower than, those obtained in tryptone medium. In comparison with tryptone, glutamic acid and glycine media a wider variation in the temperature of maximum K was apparent in leucine, arginine and β-alanine media. Strains S1, S2, S4, S10 and S11 displayed a variation of 5° or less in the temperature at which the K value was maximal in different amino acid media. A variation of 10° was shown by strains S8, S12, S17 and S18 and a variation of 15° was shown by strains S3, S5, S6, S9, S14, S15, S20, S21 and S24. Strains S7, S13, S19 and S22 showed no variation in the temperature of maximum K value in tryptone medium and different amino acid media.

Thus on the basis of K values the optimum temperature for growth of the test strains varied widely, being clearly dependent on the nature of the substrate provided.

Maximum cell crop (M): Total cell crops greater than those obtained in tryptone medium were shown by strains S2, S10 and S11 in glutamic acid medium, by strains S5, S6, S8, S13, S14, S15, S21, S22 and S23 in glutamic acid and arginine media and by strain S18 in glutamic acid, arginine and lysine media. Strains S1, S3, S4, S7, S9, S12, S16, S17, S19, S20 and S24 displayed maximum M values in tryptone medium which were greater than in any amino acid medium. Lysine, except for strain S18, leucine, glycine and β-alanine did not support cell crops of the magnitude obtained in tryptone medium. The temperatures of the maximum cell crops of individual strains varied depending on the medium.

Strains S1, S3, S5, S6, S13, S14, S15, S17, S18 and S22 displayed similar temperatures of maximum M value in most amino acid media and in tryptone medium. Strains S2, S4, S7, S8, S9, S10, S11, S16, S19, S20, S21 and S24 generally showed maximum M values in amino acid media at higher temperatures than in tryptone medium and strains S12 and S23 often showed maximum M values in amino acid media at lower temperatures than in tryptone medium.

Temperature coefficients, St values and ammonia production.

Glutamic acid.

Increased Q_{10} (5° - 15°) values, indicative of increased sensitivity to low temperatures, were displayed by strains S1, S3, S4, S5, S6, S7, S8, S9, S10, S11, S12, S14, S16, S18, S19, S21, S22 and S23 grown in glutamic acid medium and in terms of the definition proposed on page 88. strains S10, S11, S19 and S24 would be regarded as mesophiles on the basis of growth in glutamic acid medium. Lower Q_{10} (5° - 15°) values were shown by strains S15, S17 and S20, although the implication of a higher rate of growth at 5° was offset by higher Q_{10} (15° - 25°) values than in tryptone medium. Strain S2 exhibited a reduced Q_{10} (5° - 15°) value which, together with no growth at 25°, 30° and 35°, indicated a definitely more psychrophilic growth pattern in glutamic acid medium than in tryptone medium. Strains S3, S4, S7, S8, S10, S18 and S19 displayed increased Q_{10} (5° - 15°) values and reduced Q_{10} (25° - 35°) values which reflected reduced growth rates at both extremes of the growth temperature range. Mesophilic strains S12, S13 and S23 and strains S16 and S22 showed large or infinite Q_{10} (5° - 15°) values

Table 8. 1 Q10 values in tryptone and amino-acid media

Amino-acid 0.2%(w/v)	tryptone (A)			glutamic acid (G)			arginine (V)			lysine (C)			leucine (I)			glycine (M)			β-alanine (Q)		
Q10 value	5–15°	15–25°	25–35°	5–15°	15–25°	25–35°	5–15°	15–25°	25–35°	5–15°	15–25°	25–35°	5–15°	15–25°	25–35°	5–15°	15–25°	25–35°	5–15°	15–25°	25–35°
Strain																					
S1.	2.05	1.16	0.28	2.34	1.53	0.44	2.55	1.42	0.24	10.5	1.42	0.25	4.28	1.96	0.6	0	0	0	0	∞	0
S.2	3.26	0.76	0.68	2.14	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
S.3	2.63	1.04	0.62	2.66	1.32	0.43	3.53	1.73	0.28	3.0	0.7	0.52	3.52	0.53	0.69	1.5	0.52	1.12	0	∞	0
S.4	3.12	1.06	0.45	3.63	1.42	0.29	2.63	1.65	0.16	5.2	2.4	0	3.04	1.59	0.64	0	0	0	∞	2.41	0
S.5	2.45	0.79	0.67	3.59	1.04	1.42	3.97	1.12	0.46	4.5	4.31	0	0	0	0	0	0	0	0	∞	0
S.6	3.47	0.89	1.01	3.65	1.35	1.68	3.18	1.22	0.94	6.66	0.81	0.4	2.58	0.46	0	0	0	0	0	∞	0
S.7	2.32	1.32	0.3	3.26	2.17	0.18	∞	3.33	0	0	0	0	0	0	0	0	0	0	0	0	0
S.8	2.33	0.86	0.45	3.03	1.83	0.18	2.61	1.78	0.1	4.29	0.49	0	3.54	0.49	0	3.86	0.52	0.24	0	0	0
S.9	2.43	1.15	0.56	2.83	1.91	0.59	2.67	2.0	0.46	1.78	0.87	0.54	4.51	1.27	0.78	0	0	0	∞	1.1	0
S.10	2.5	1.16	0.9	4.23	1.5	0.72	3.39	1.26	0.49	0	0	0	0	0	0	0	0	0	∞	10.6	0
S.11	3.28	1.07	0.84	∞	1.39	1.4	∞	1.56	0.41	0	0	0	0	0	0	0	0	0	0	∞	0
S.12	3.2	1.02	2.24	∞	2.05	1.35	∞	1.57	1.34	0	∞	1.13	∞	0.89	2.5	0	0	0	0	∞	0

Table 8. 2 Q10 values in tryptone and amino-acid media

Amino-acid 0.2%(w/v)	tryptone (A)			glutamic acid (G)			arginine (V)			lysine (C)			leucine (I)			glycine (M)			β-alanine (Q)		
Q10 value	5–15°	15–25°	25–35°	5–15°	15–25°	25–35°	5–15°	15–25°	25–35°	5–15°	15–25°	25–35°	5–15°	15–25°	25–35°	5–15°	15–25°	25–35°	5–15°	15–25°	25–35°
Strain																					
S.13	∞	1.54	1.53	∞	2.31	1.56	∞	2.56	1.46	0	0	0	∞	2.04	1.14	0	0	0	0	∞	1.73
S.14	2.96	0.92	0.28	3.18	1.15	0.52	3.39	1.18	0.43	5.7	1.97	0.17	3.59	1.07	0.46	0.71	1.28	0	∞	5.1	0
S.15	2.81	0.81	0.18	2.5	1.38	1.11	2.63	1.34	0.47	3.17	0.53	0	2.27	1.21	1.11	0	0	0	∞	1.7	0
S.16	5.12	0.88	0.81	∞	3.11	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
S.17	5.23	0.59	0.35	3.33	1.5	0.66	2.16	2.77	0.15	0	0	0	6.7	0	0	0	0	0	0	0	0
S.18	2.38	1.08	1.2	2.97	0.24	1.05	2.66	2.41	0.86	∞	1.25	1.81	4.56	1.68	1.34	7.91	1.2	1.32	0	∞	0
S.19	1.8	1.16	0.97	4.65	1.16	0.91	2.8	1.67	0.91	0	0	0	2.38	2.21	1.44	0	0	0	0	0	0
S.20	3.67	0.49	0.46	3.47	1.35	0.29	2.82	2.06	0.28	4.42	1.49	0.52	4.15	1.97	0.57	0	0	0	3.83	1.0	0.59
S.21	1.76	0.88	1.21	2.47	1.57	1.19	4.15	0.98	0.59	3.2	0.87	0	2.91	0.92	0.13	0	0	0	∞	1.0	0.51
S.22	5.55	1.17	0.85	12.7	0.97	0.75	14.4	1.32	0.64	0	0	0	0	0	0	0	0	0	0	0	0
S.23	43.7	1.0	2.02	∞	0.48	1.9	∞	1.05	1.57	∞	0.83	2.0	0	0	0	0	0	0	0	0	0
S.24	3.24	0.85	0.6	0	∞	0.69	2.0	1.32	0	0	0	0	0	0	0	0	0	0	0	0	0

Table 9. 1 Sb-values for growth on amino-acid media

Sb-value	G:A					V:A					C:A				
Temp. °C.	5°	15°	25°	30°	35°	5°	15°	25°	30°	35°	5°	15°	25°	30°	35°
Strain															
S.1	0.8	0.91	1.22	1.52	1.9	0.62	0.77	0.94	1.13	0.8	0.06	0.28	0.35	0.11	0.31
S.2	1.12	0.74	0	0	0	0	0	0	0	0	0	0	0	0	0
S.3	0.78	0.83	1.05	1.3	0.73	0.6	0.81	1.35	1.35	0.61	0.18	0.21	0.14	0.21	0.12
S.4	0.8	0.93	1.25	1.51	0.84	0.86	0.72	1.13	0.91	0.44	0.16	0.26	0.59	0.12	0
S.5	0.69	0.93	1.33	4.25	2.83	0.68	1.09	1.5	1.42	1.08	0.09	0.16	0.86	0.14	0
S.6	0.9	0.93	1.42	2.22	2.35	1.21	1.09	1.52	1.26	1.44	0.17	0.32	0.3	0.21	0.12
S.7	0.63	0.89	0.85	1.16	0.77	0	0.11	0.28	0.29	0	0	0	0	0	0
S.8	0.78	1.01	2.15	1.53	0.84	1.03	1.16	2.41	0.95	0.54	0.2	0.37	0.21	0.18	0
S.9	0.6	0.7	1.17	1.12	2.23	0.33	0.37	0.66	0.64	0.95	0.14	0.1	0.08	0.07	0.14
S.10	1.06	1.79	2.31	2.36	1.66	0.46	0.62	0.68	0.72	0.37	0	0	0	0	0
S.11	0	0.43	0.66	1.24	1.1	0	0.33	0.48	0.59	0.23	0	0	0	0	0
S.12	0	0.62	1.25	1.0	0.75	0	0.74	1.13	0.94	0.68	0	0	0.64	0.64	0.32

G:A glutamic acid : Tryptone
V:A arginine : Tryptone
C:A lysine : Tryptone

Table 9.2 St-values for growth in amino-acid media

St-value	I:A					M:A					Q:A				
Temp. °C.	5°	15°	25°	30°	35°	5°	15°	25°	30°	35°	5°	15°	25°	30°	35°
Strain															
S.1	0.18	0.38	0.64	0.73	1.35	0	0	0	0	0	0	0	0	0	0
S.2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
S.3	0.41	0.54	0.3	0.53	0.31	0.38	0.31	0.11	0.42	0.2	0	0	0.14	0	0
S.4	0.37	0.36	0.54	0.7	0.75	0	0	0	0	0	0	0.2	0.47	0	0
S.5	0	0	0	0	0	0	0	0	0	0	0	0	0.46	0	0
S.6	0.54	0.4	0.21	0.12	0	0	0	0	0	0	0	0	0.33	0	0
S.7	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
S.8	0.46	0.7	0.4	0.36	0	0.5	0.84	0.51	0.54	0.27	0	0	0	0	0
S.9	0.23	0.42	0.47	0.49	1.18	0	0	0	0	0	0	0.14	0.13	0	0
S.10	0	0	0	0	0	0	0	0	0	0	0	0.15	1.4	0	0
S.11	0	0	0	0	0	0	0	0	0	0	0	0	1.1	0	0
S.12	0	0.26	0.22	0.33	0.25	0	0	0	0	0	0	0	1.1	0	0

I:A leucine : Tryptone
M:A glycine : Tryptone
Q:A alanine : Tryptone

Table 9. 3 St-values for growth in amino-acid media

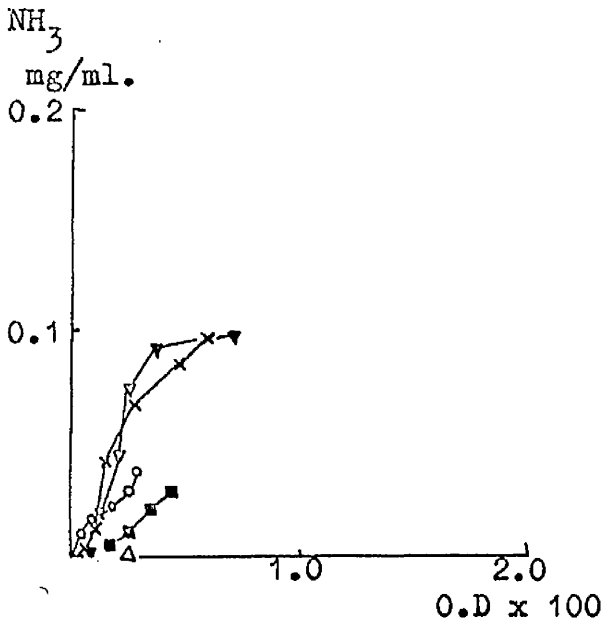
St-value	GtA					VtA					CtA				
Temp. °C.	5°	15°	25°	30°	35°	5°	15°	25°	30°	35°	5°	15°	25°	30°	35°
Strain															
S.13	0	1.57	2.36	2.44	2.42	0	1.21	2.02	1.98	1.92	0	0	0	0	0
S.14	0.73	0.79	0.99	1.24	1.81	0.64	0.74	0.94	1.51	1.44	0.23	0.44	0.94	0.67	0.56
S.15	0.98	0.87	1.49	2.89	4.28	0.9	0.84	1.4	2.41	3.77	0.24	0.28	0.18	0.08	0
S.16	0	0.4	0.15	0.1	0	0	0	0	0	0	0	0	0	0	0
S.17	0.05	0.03	0.07	0.13	0.14	0.19	0.08	0.37	0.36	0.16	0	0	0	0	0
S.18	0.7	0.88	2.08	1.7	1.83	0.79	0.88	1.92	1.38	1.42	0	0.85	0.98	1.12	1.48
S.19	0.36	0.95	0.94	0.87	0.88	0.37	0.58	0.83	0.88	0.78	0	0	0	0	0
S.20	0.54	0.5	1.4	1.26	0.89	0.41	0.32	1.32	1.16	0.79	0.15	0.18	0.55	0.44	0.63
S.21	0.74	1.04	1.85	1.8	1.82	0.64	1.52	1.69	1.1	0.83	0.09	0.16	0.16	0.09	0
S.22	0.64	1.48	1.23	1.27	1.88	0.46	1.18	1.33	1.26	1.0	0	0	0	0	0
S.23	0	1.41	0.68	0.81	0.64	0	0.89	0.93	0.86	0.72	0	0.18	0.15	0.17	0.15
S.24	0	0	0.41	0.71	0.47	0.39	0.24	0.37	0.45	0	0	0	0	0	0

Table 9. 4 St-values for growth in amino-acid media

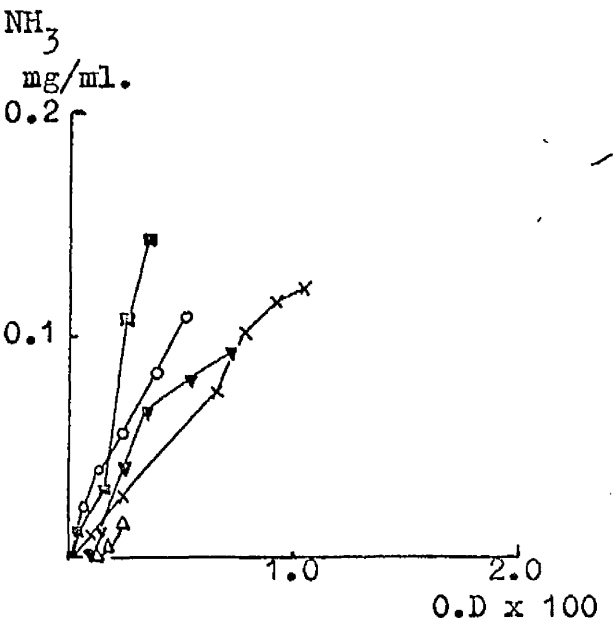
St-value	I:A					II:A					Q:A				
Temp. °C.	5°	15°	25°	30°	35°	5°	15°	25°	30°	35°	5°	15°	25°	30°	35°
Strain															
S.13	0	0.61	0.8	0.75	0.73	0	0	0	0	0	0	0	0.53	0.61	0.6
S.14	0.42	0.51	0.6	0.71	0.95	0.4	0.1	0.14	0.25	0	0	0.1	0.57	0	0
S.15	0.33	0.26	0.4	1.58	2.51	0	0	0	0	0	0	0.46	0.91	0	0
S.16	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
S.17	0.08	0.1	0	0	0	0	0	0	0	0	0	0	0	0	0
S.18	0.26	0.5	0.79	0.69	1.0	0.21	0.7	0.78	0.54	0.35	0	0	0.82	0	0
S.19	0.22	0.3	0.56	0.83	0.83	0	0	0	0	0	0	0	0	0	0
S.20	0.25	0.29	1.1	1.38	1.4	0	0	0	0	0	0.26	0.27	0.55	1.1	0.66
S.21	0.31	0.52	0.54	0.25	0.06	0	0	0	0	0	0	0.51	0.57	0.54	0.24
S.22	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
S.23	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
S.24	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Fig. 1.1 Growth and ammonia formation in defined amino-acid medium

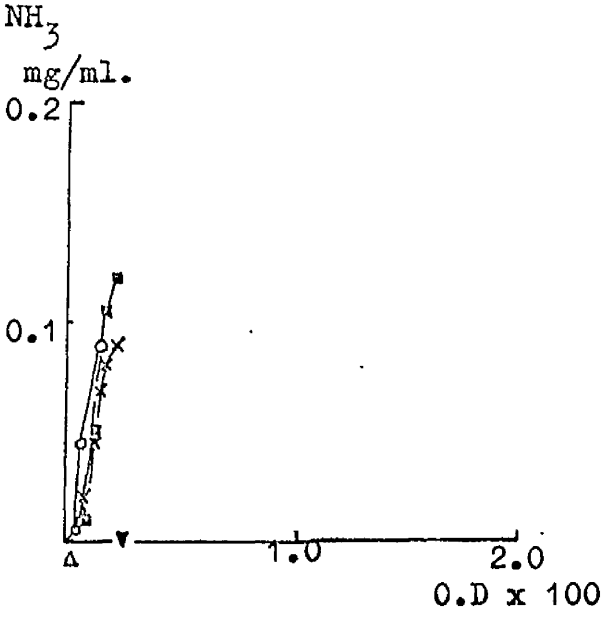
Amino-acid: glycine



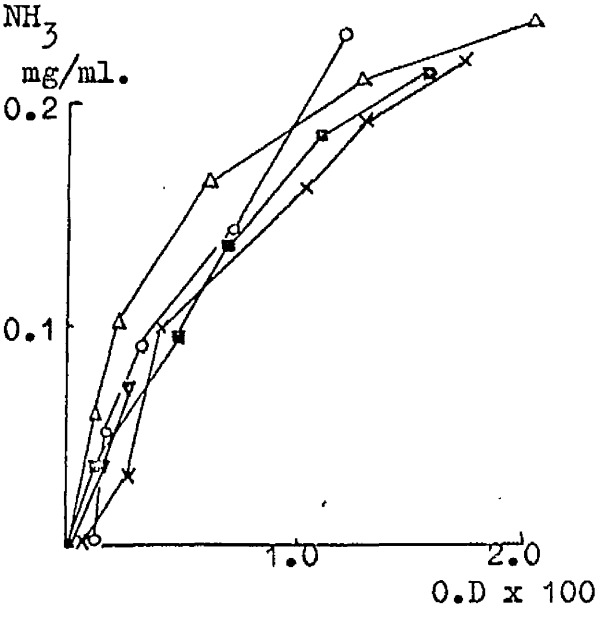
(a) Strain S.3



(b) Strain S.8



(c) Strain S.14



(d) Strain S.18

5° ▼
15° ×
25° o
30° ■
35° Δ

Fig. 1.2 Growth and ammonia formation in defined amino-acid medium

Amino-acid: leucine

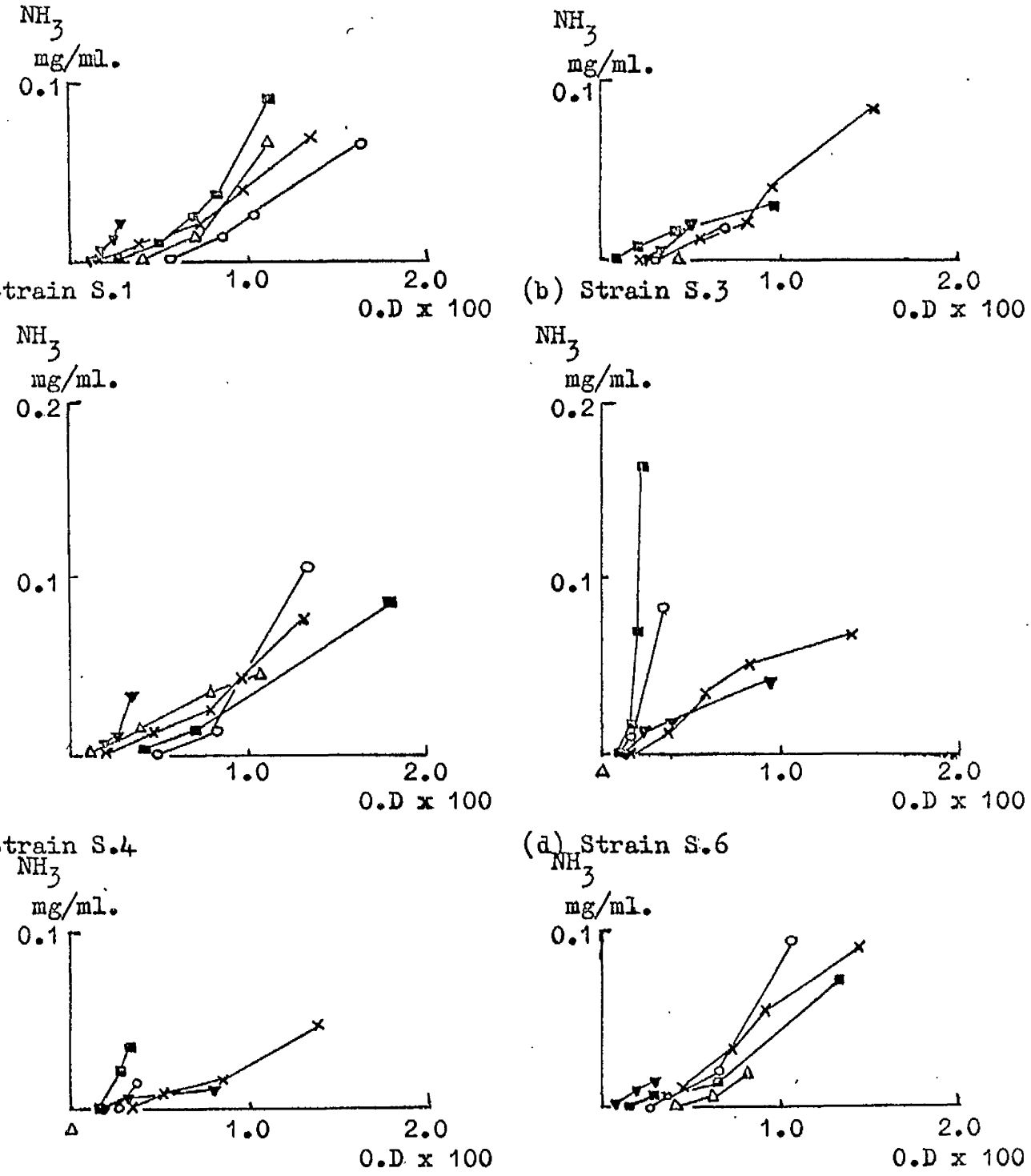
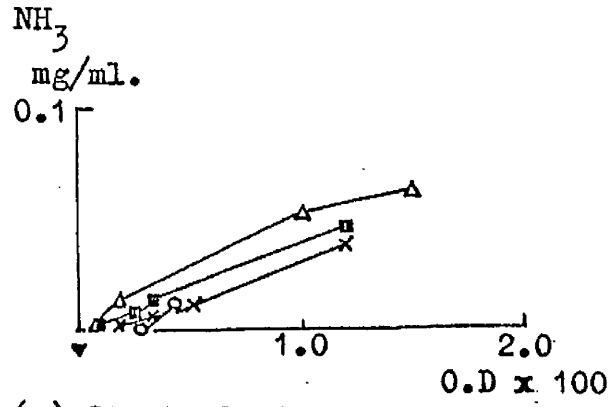
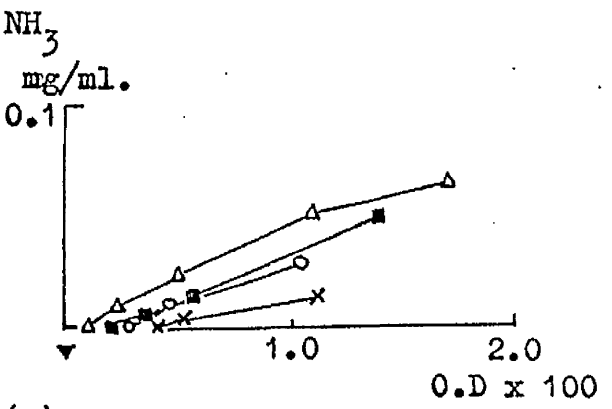


Fig. 1.3 Growth and ammonia formation in defined amino-acid medium

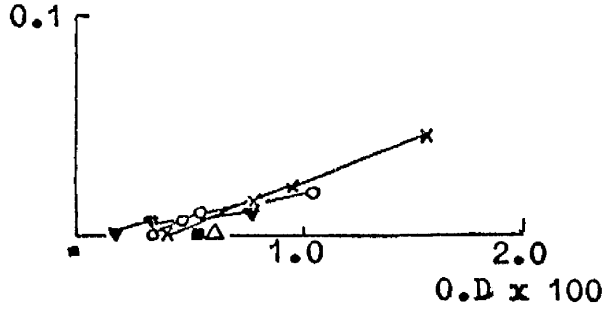
Amino-acid: leucine



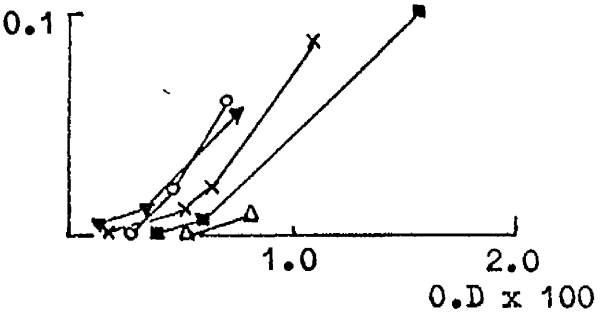
(a) Strain S.12



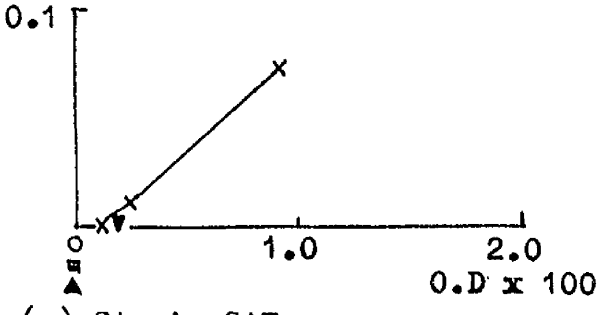
(b) Strain S.13



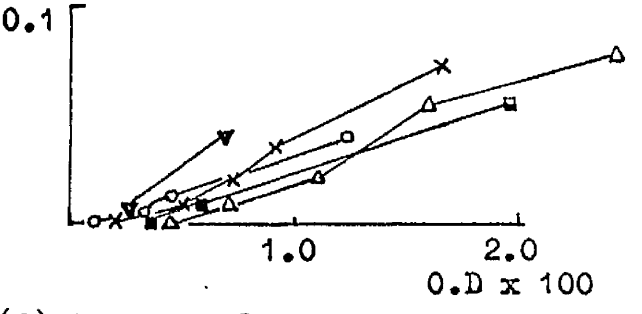
(c) Strain S.14



(d) Strain S.15



(e) Strain S17

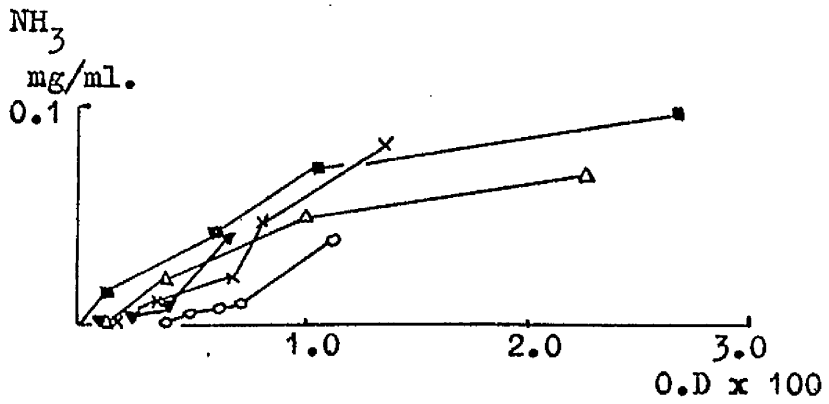


(f) Strain S18

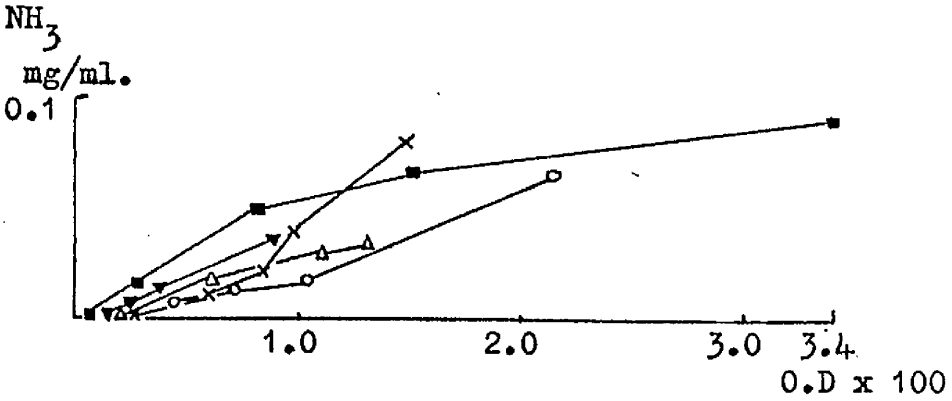
5^{o} ▼
15^{o} ×
25^{o} o
30^{o} ■
35^{o} Δ

Fig. 1.4 Growth and ammonia formation in defined amino-acid medium

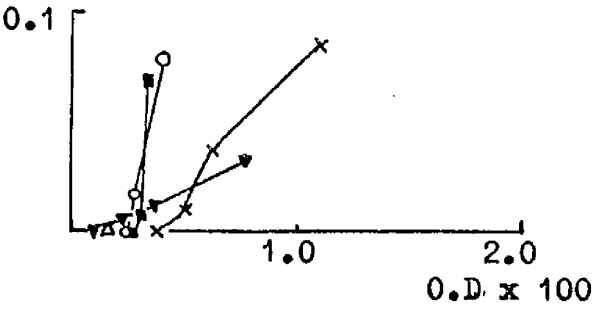
Amino-acid: leucine



(a) Strain S.19



(b) Strain S.20



(c) Strain S.21

5° ▼
15° ×
25° ○
30° ■
35° △

Fig. 1.5 Growth and ammonia formation in defined amino-acid medium

Amino-acid: lysine

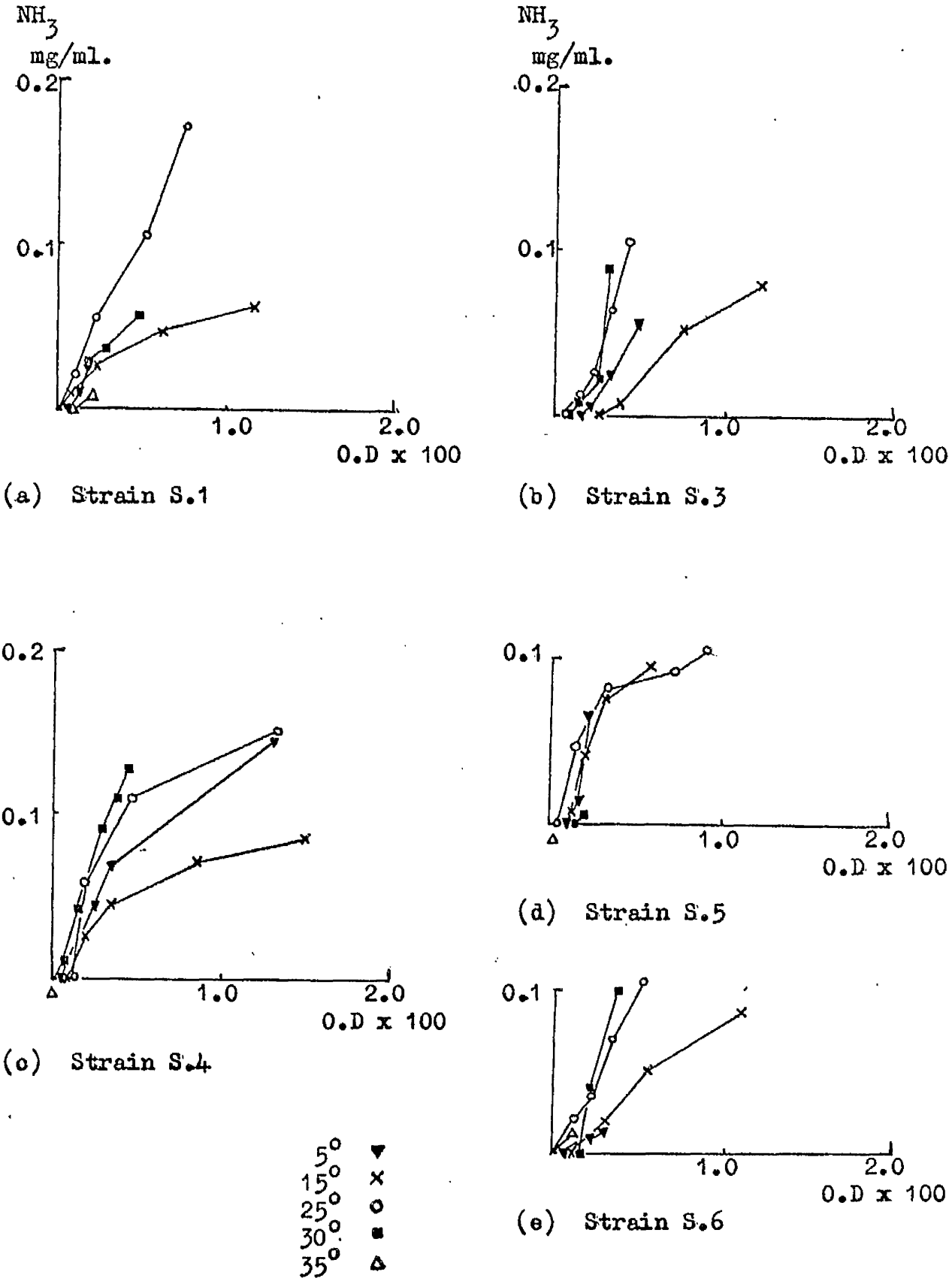
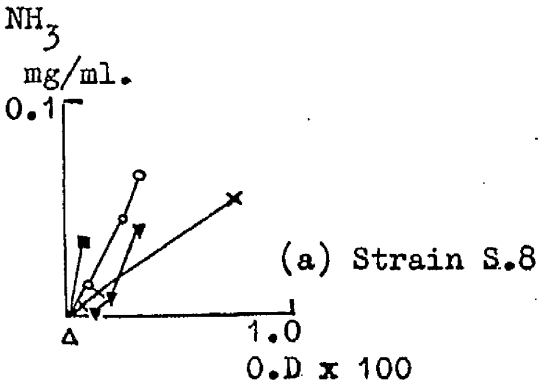
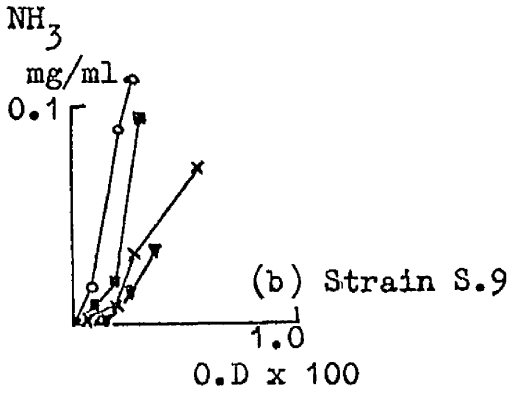


Fig. 1.6 Growth and ammonia formation in defined amino-acid medium

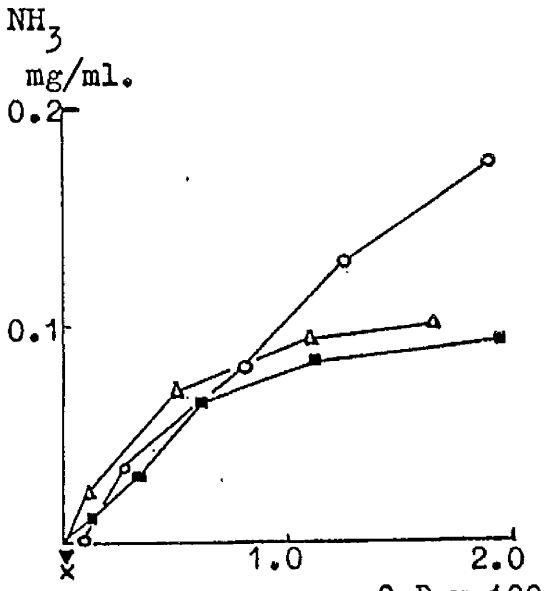
Amino-acid: lysine



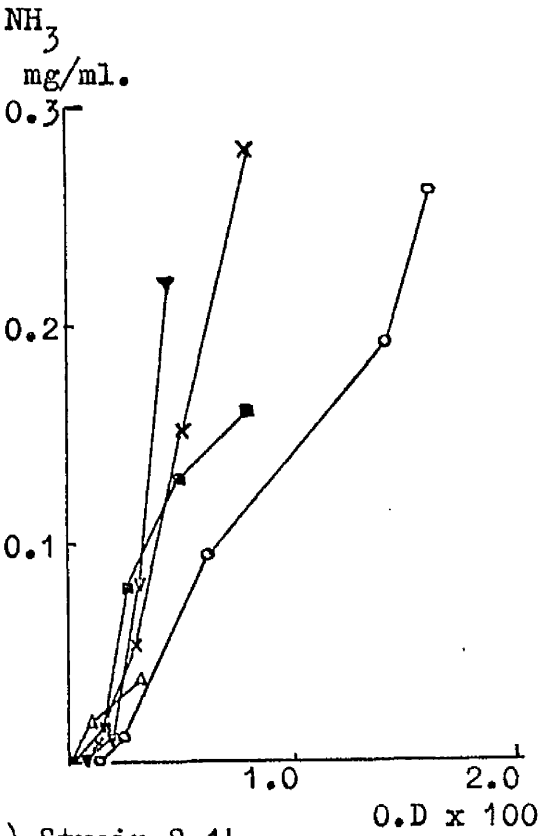
(a) Strain S.8



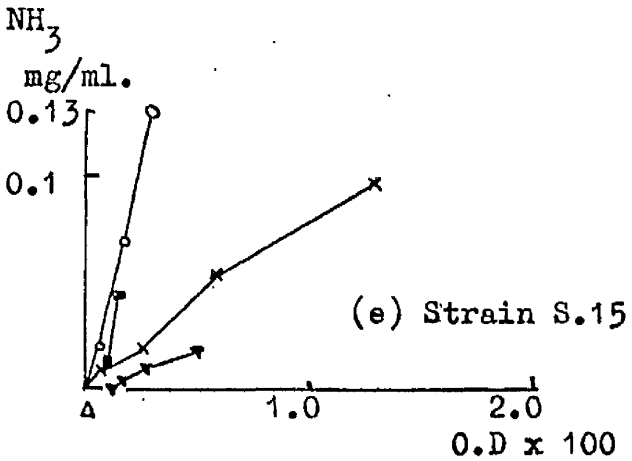
(b) Strain S.9



(c) Strain S.12



(d) Strain S.14



(e) Strain S.15

5° ▼
15° x
25° o
30° Δ
35°

Fig. 1 .7 Growth and ammonia formation in defined amino-acid medium

Amino-acid: lysine

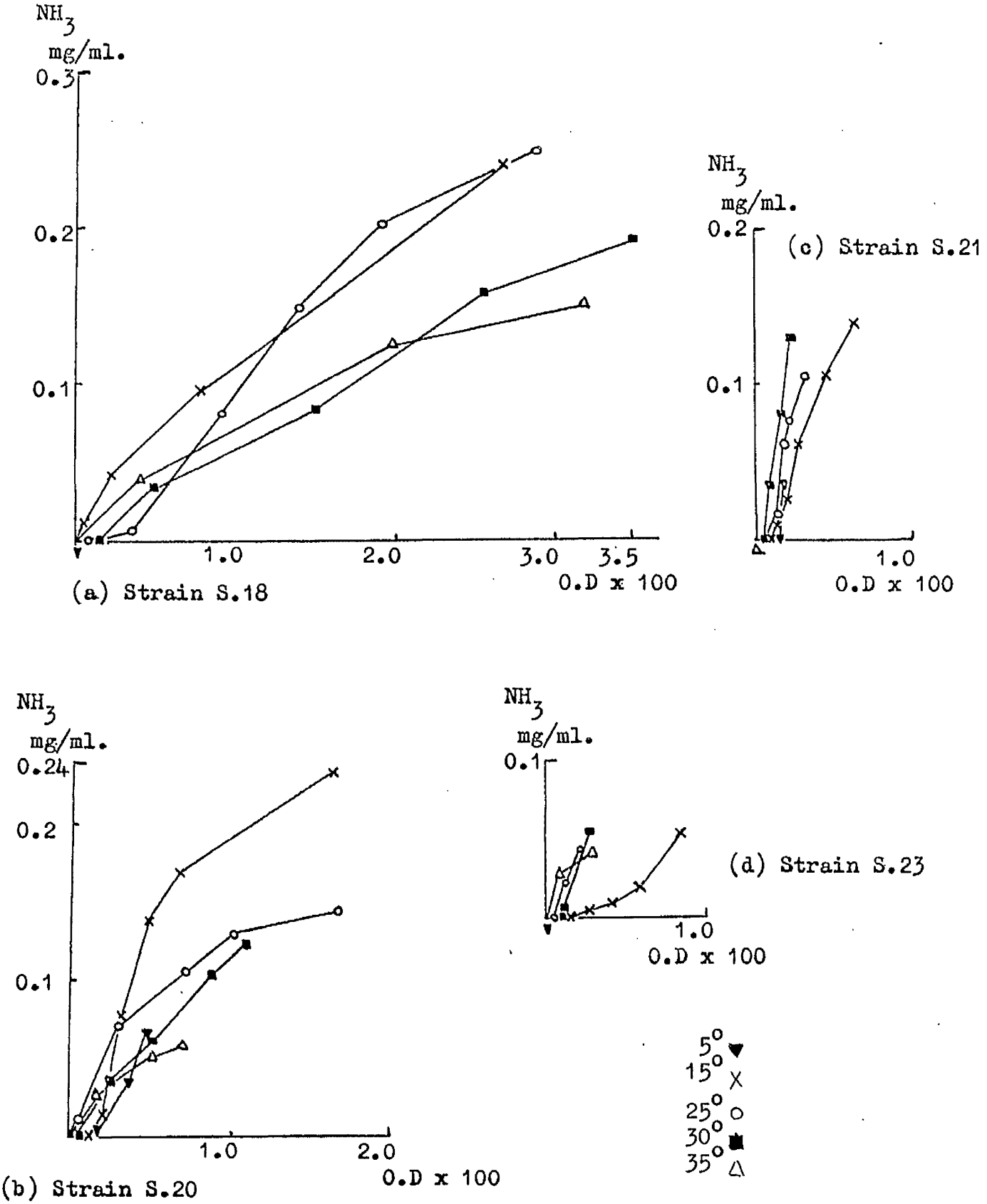
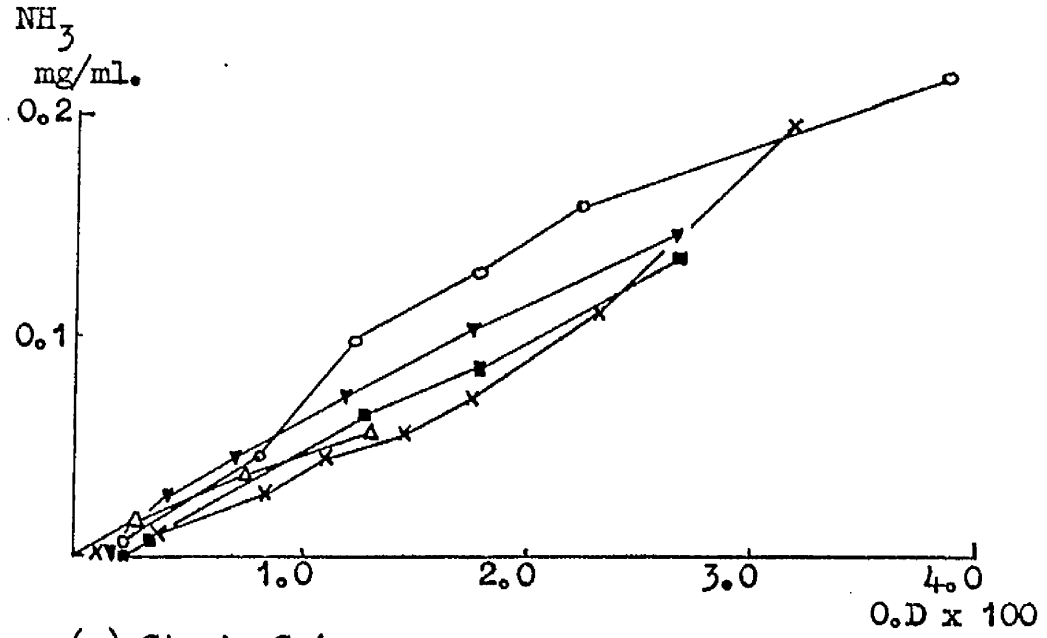
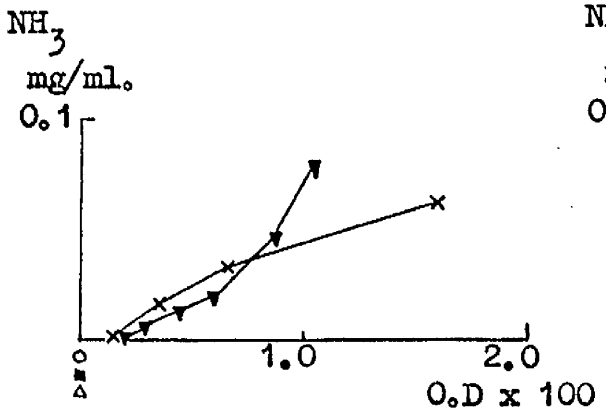


Fig. 1.8 Growth and ammonia formation in defined amino-acid medium

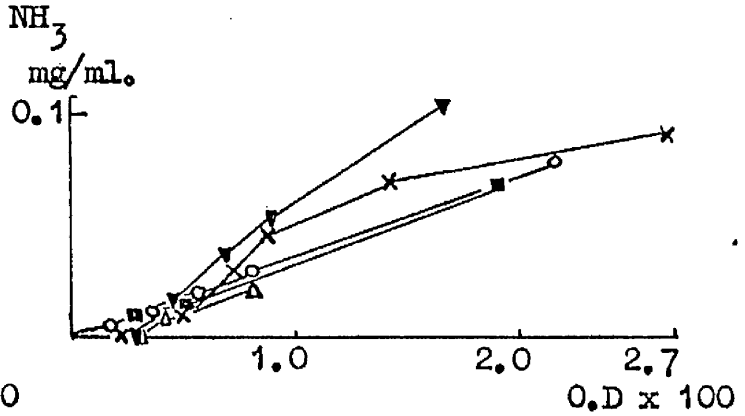
Amino-acid: Glutamic acid



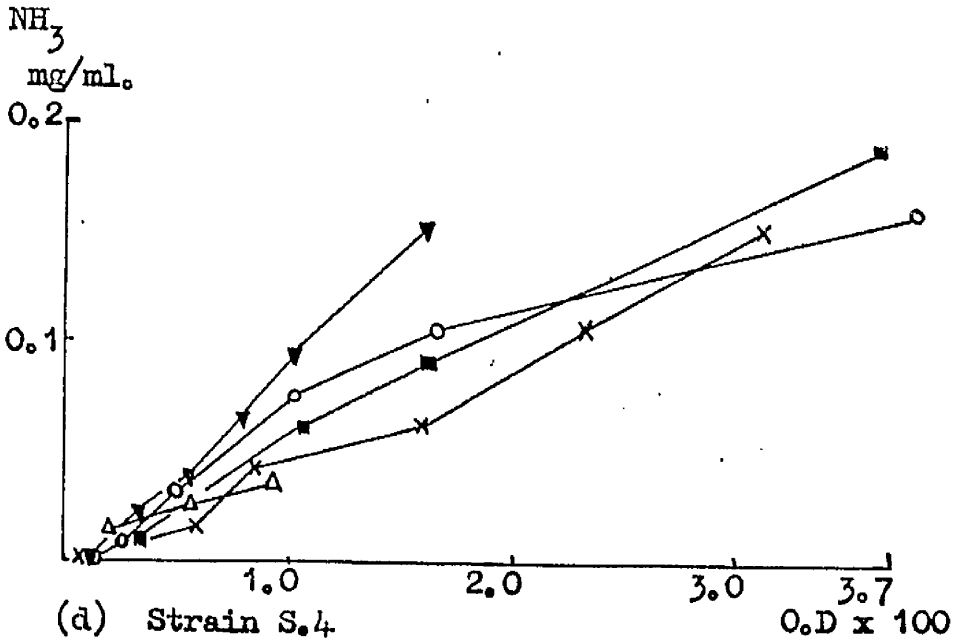
(a) Strain S.1



(b) Strain S.2



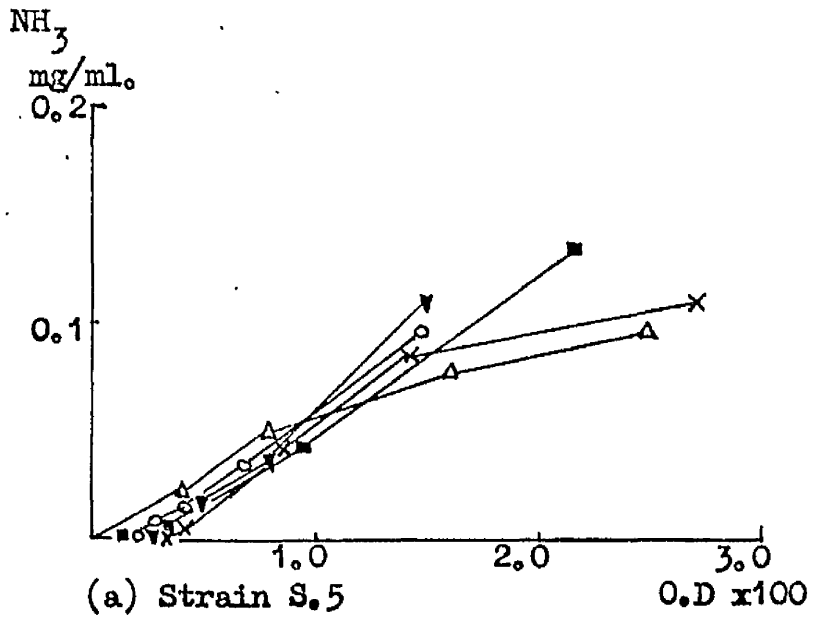
(c) Strain S.3



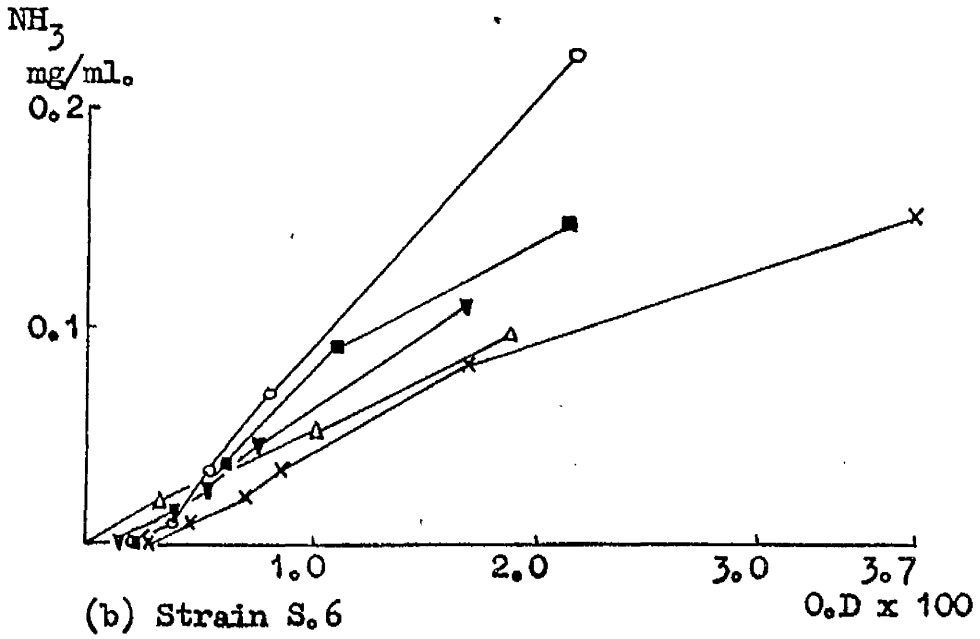
(d) Strain S.4

Fig. 1 .9 Growth and ammonia formation in defined amino-acid medium

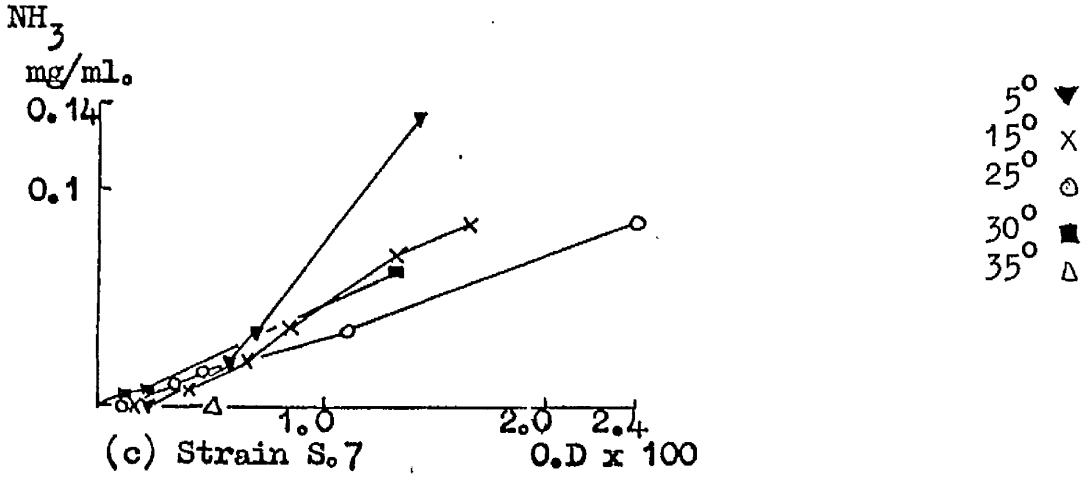
Amino-acid: Glutamic acid



(a) Strain S.5



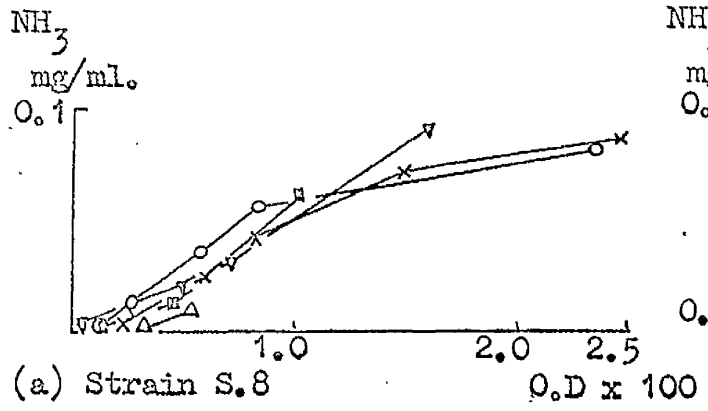
(b) Strain S.6



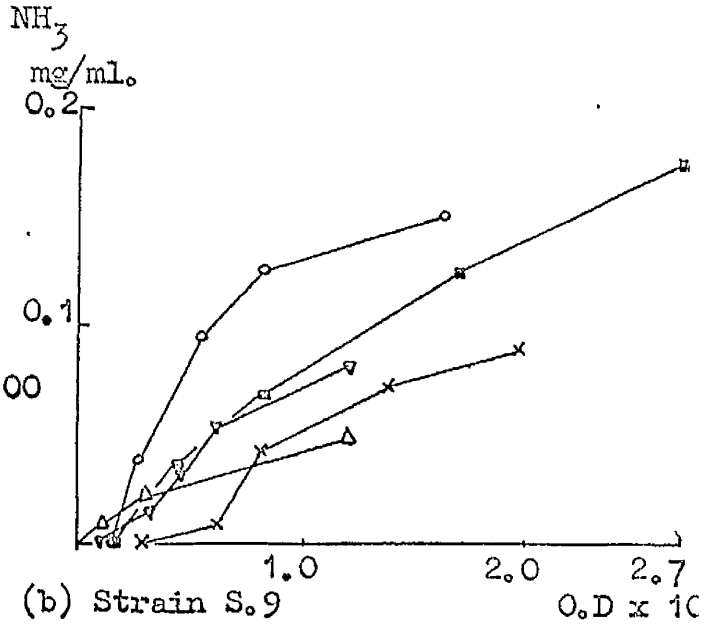
(c) Strain S.7

Fig. 1 .10 Growth and ammonia formation in defined amino-acid medium

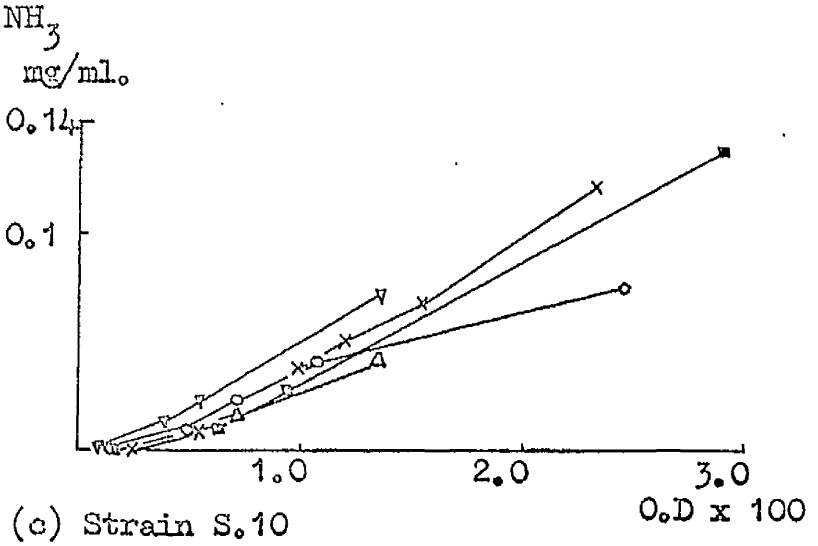
Amino-acid: Glutamic acid



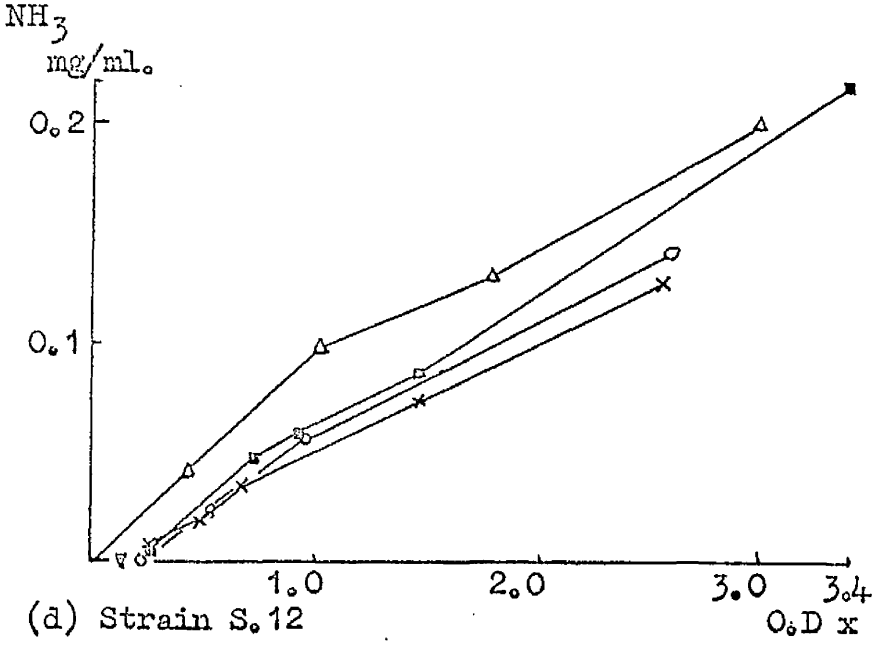
(a) Strain S.8



(b) Strain S.9 O.D x 10



(c) Strain S.10



(d) Strain S.12

Fig. 1 .11 Growth and ammonia formation in defined amino-acid medium

Amino-acid: Glutamic acid

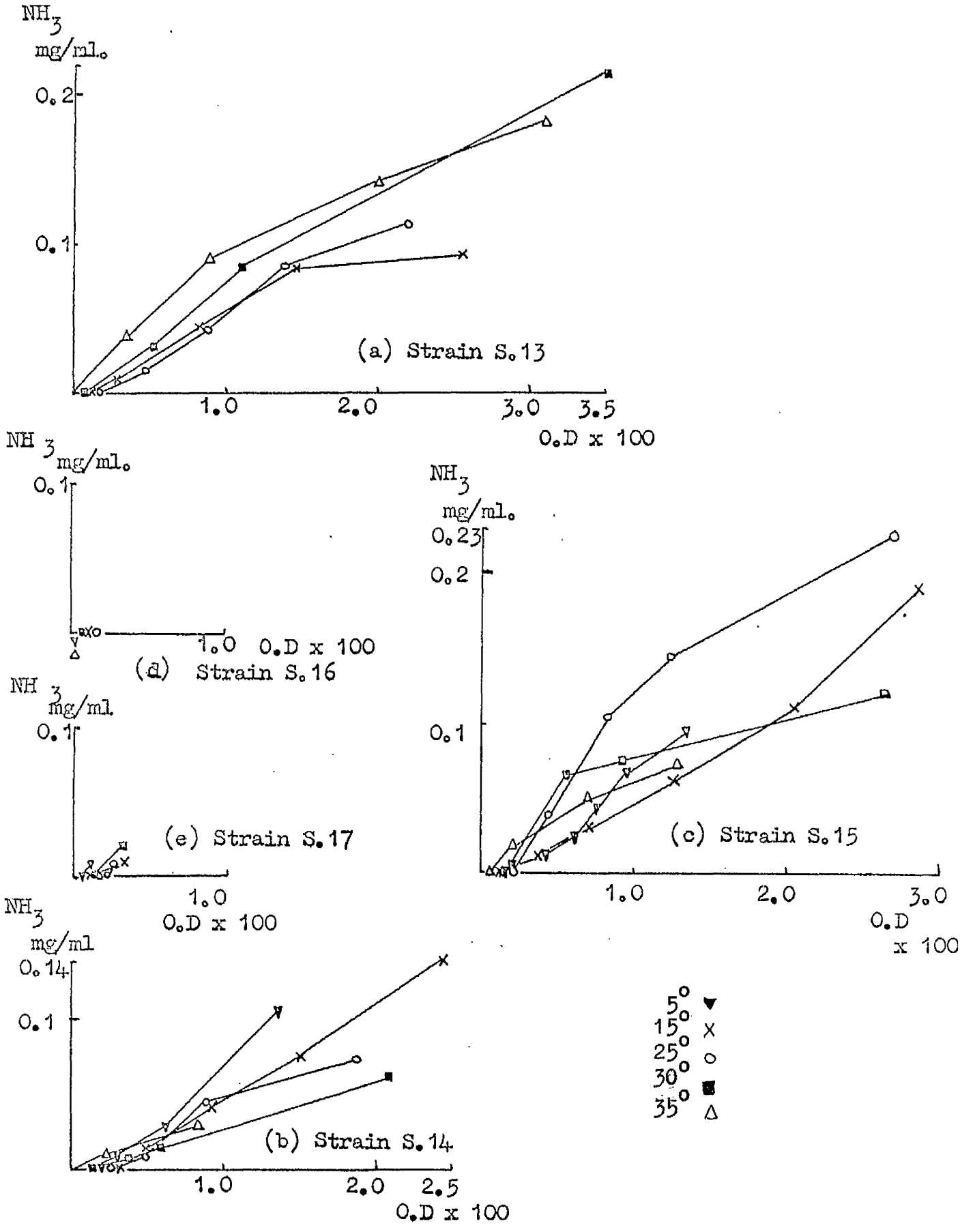


Fig. 1.12 Growth and ammonia formation in defined amino-acid medium

Amino-acid: Glutamic acid

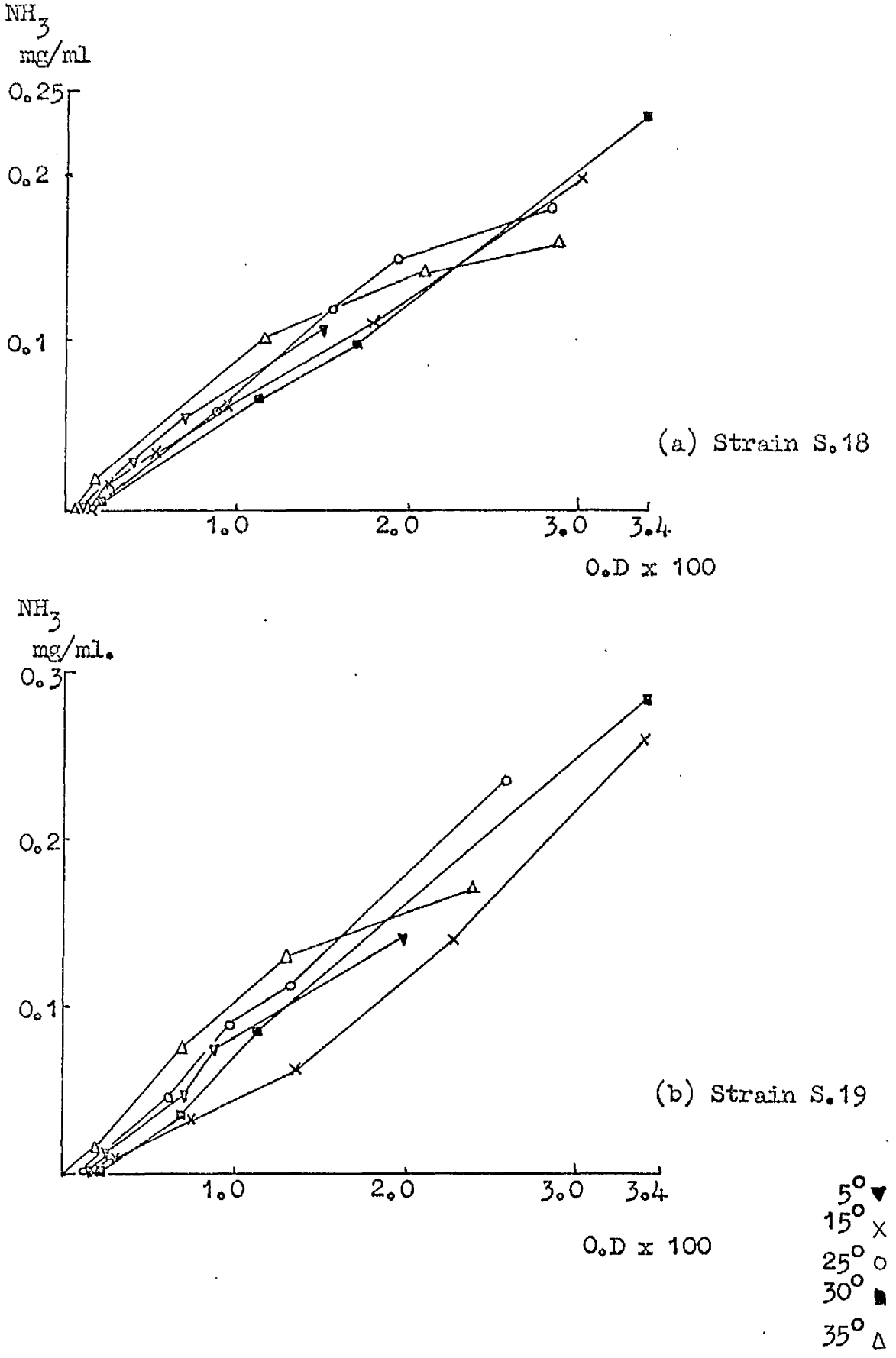
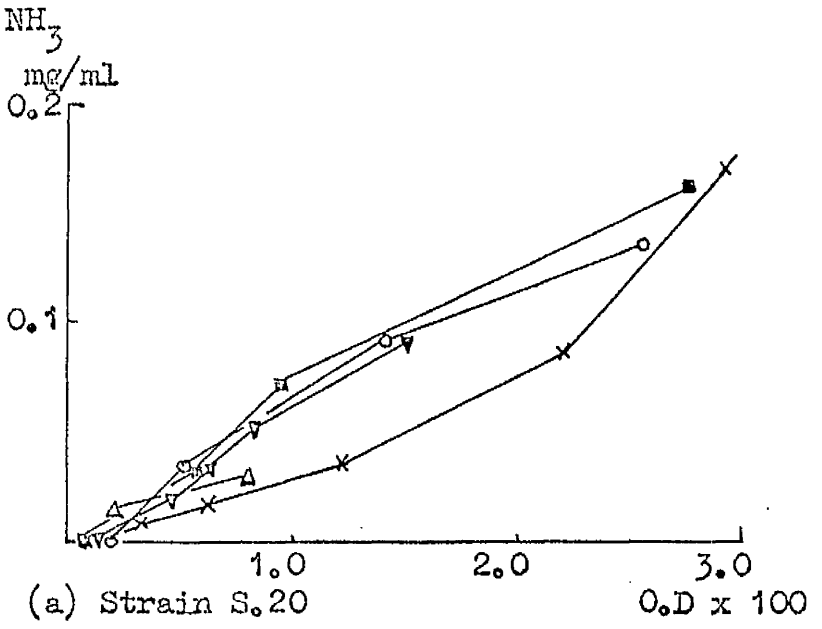
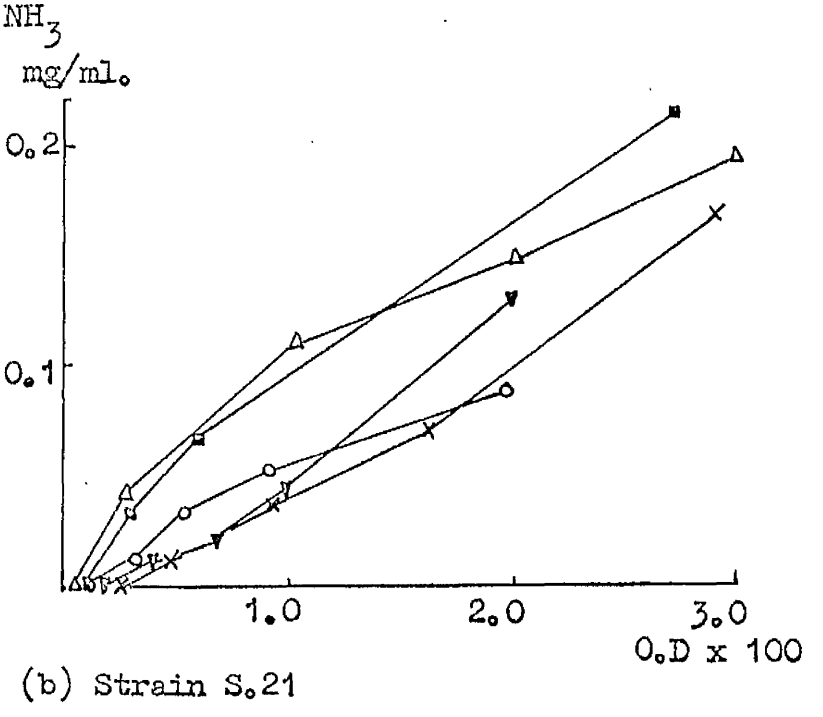


Fig. 1.13 Growth and ammonia formation in defined amino-acid medium

Amino-acid: Glutamic acid



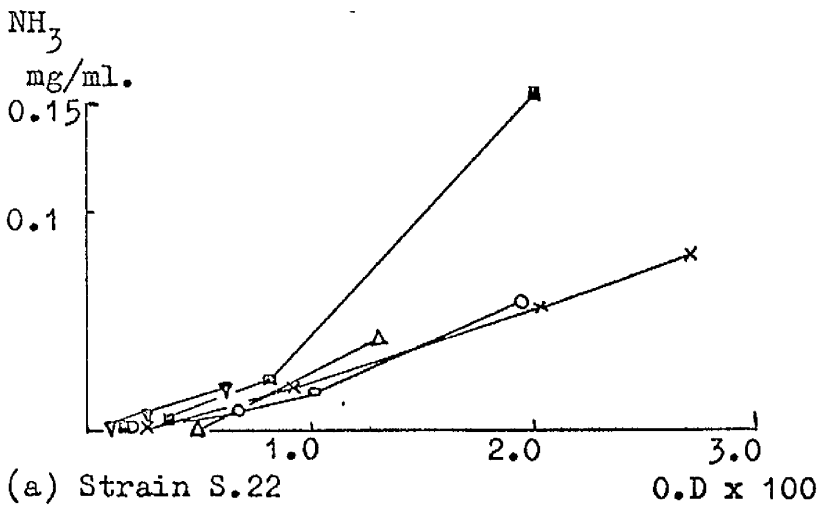
(a) Strain S.20



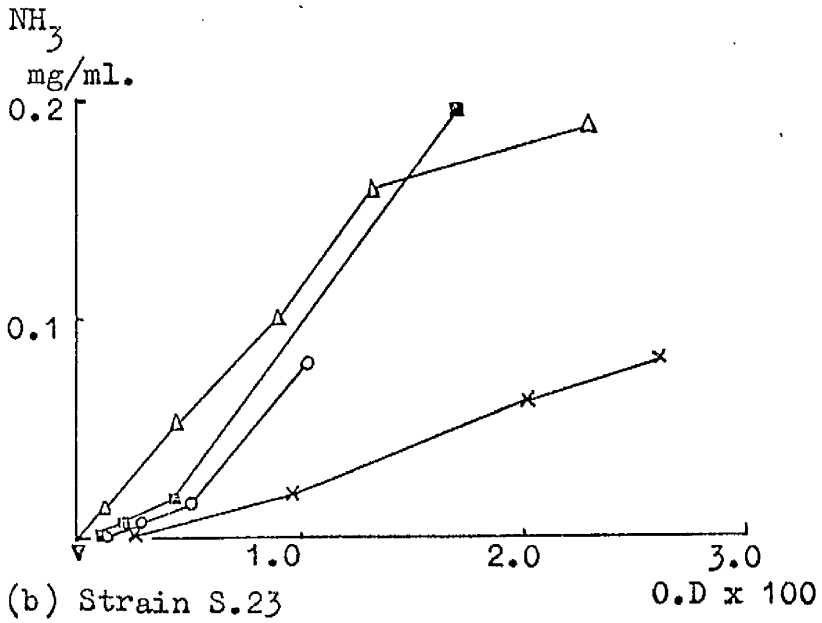
(b) Strain S.21

Fig. 1 .14 Growth and ammonia formation in defined amino-acid medium

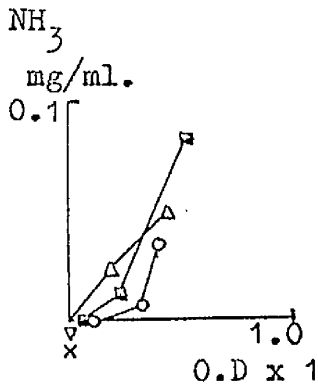
Amino-acid: Glutamic acid



(a) Strain S.22



(b) Strain S.23



(c) Strain S.24

5° ▼
15° ×
25° ○
30° ■
35° △

Fig. 1.15 Growth and ammonia formation in defined amino-acid medium

Amino-acid: Arginine

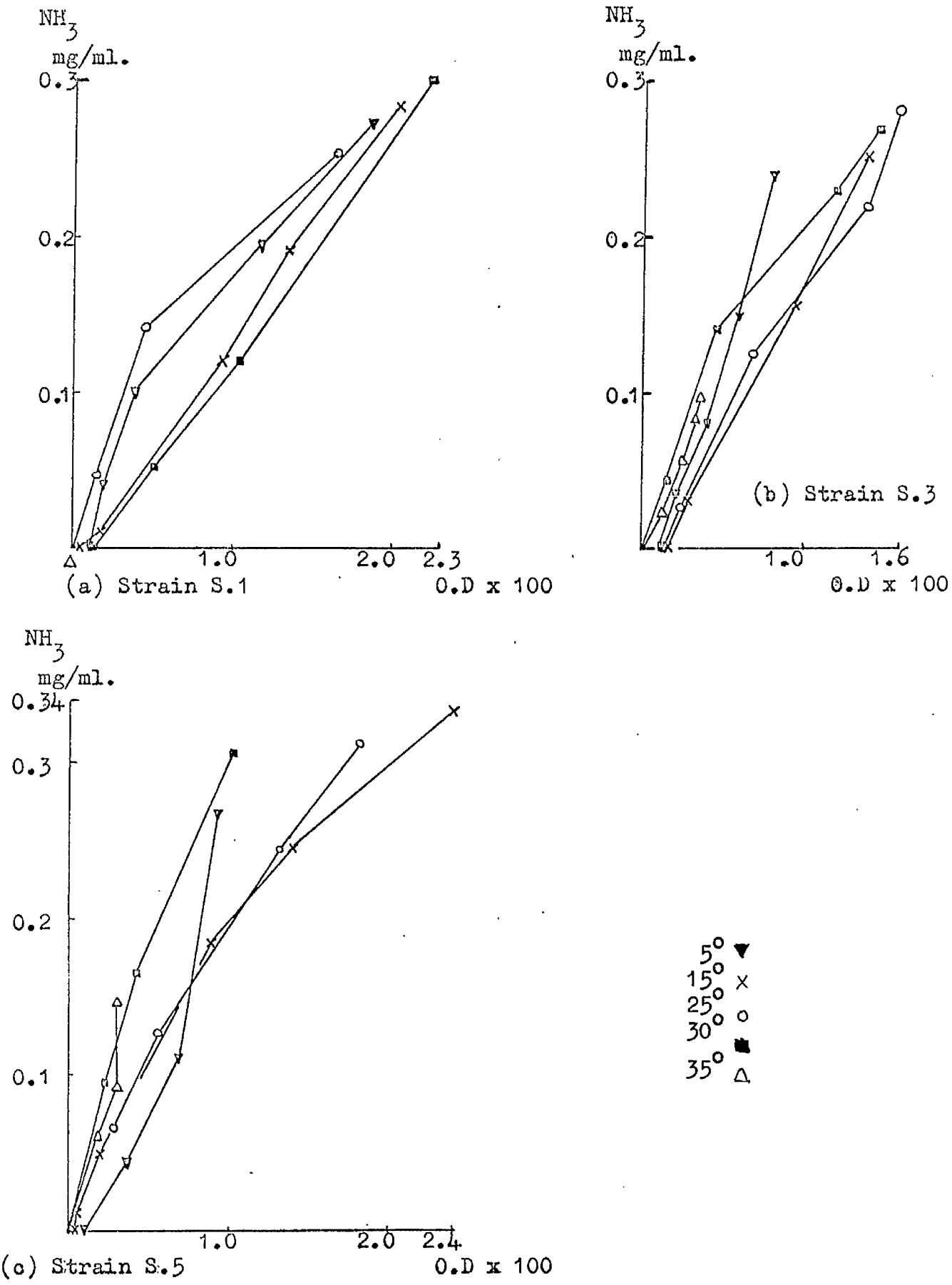


Fig. 1.16 Growth and ammonia formation in defined amino-acid medium

Amino-acid: Arginine

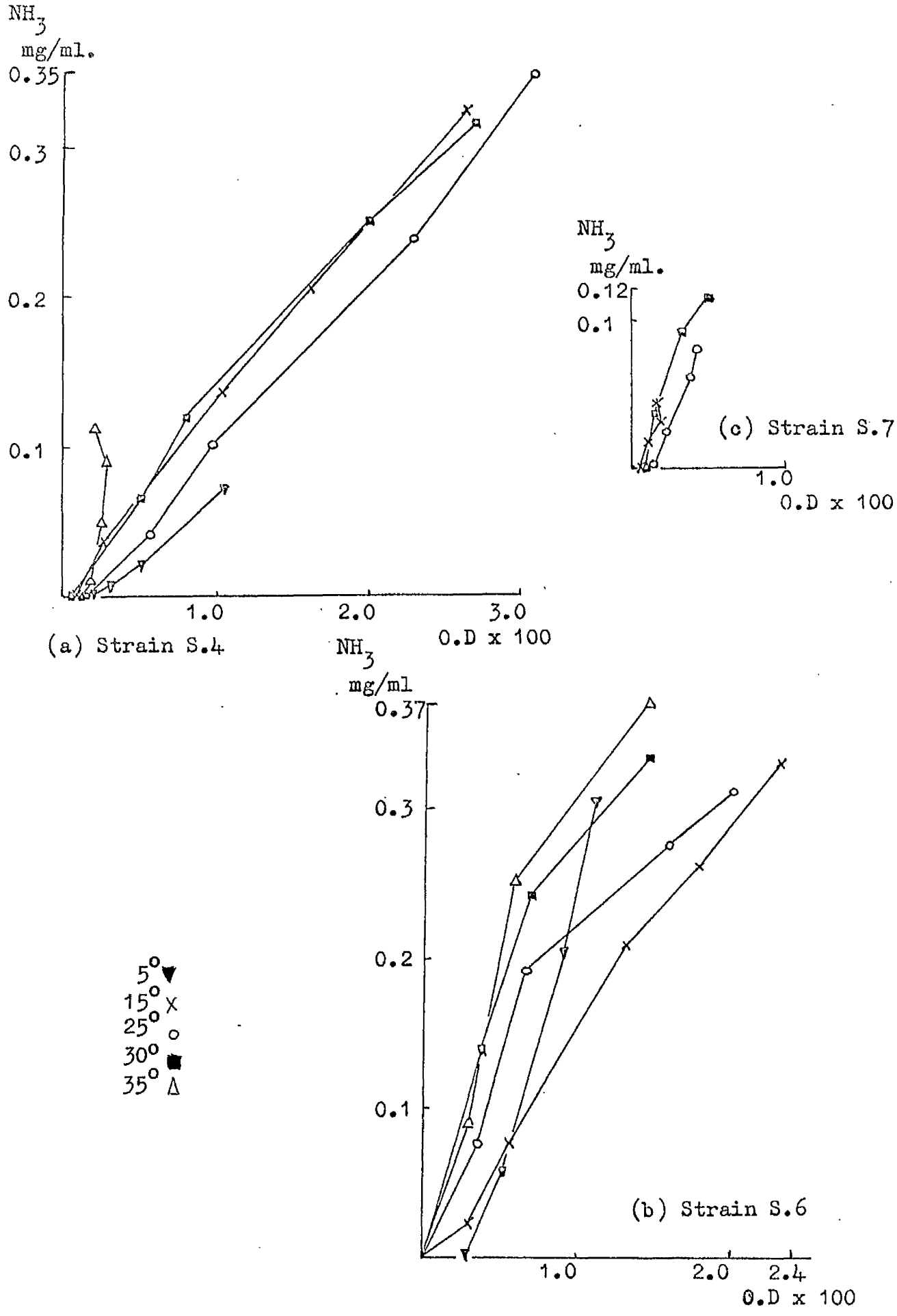


Fig. 1.17 Growth and ammonia formation in defined amino-acid

Amino-acid: Arginine

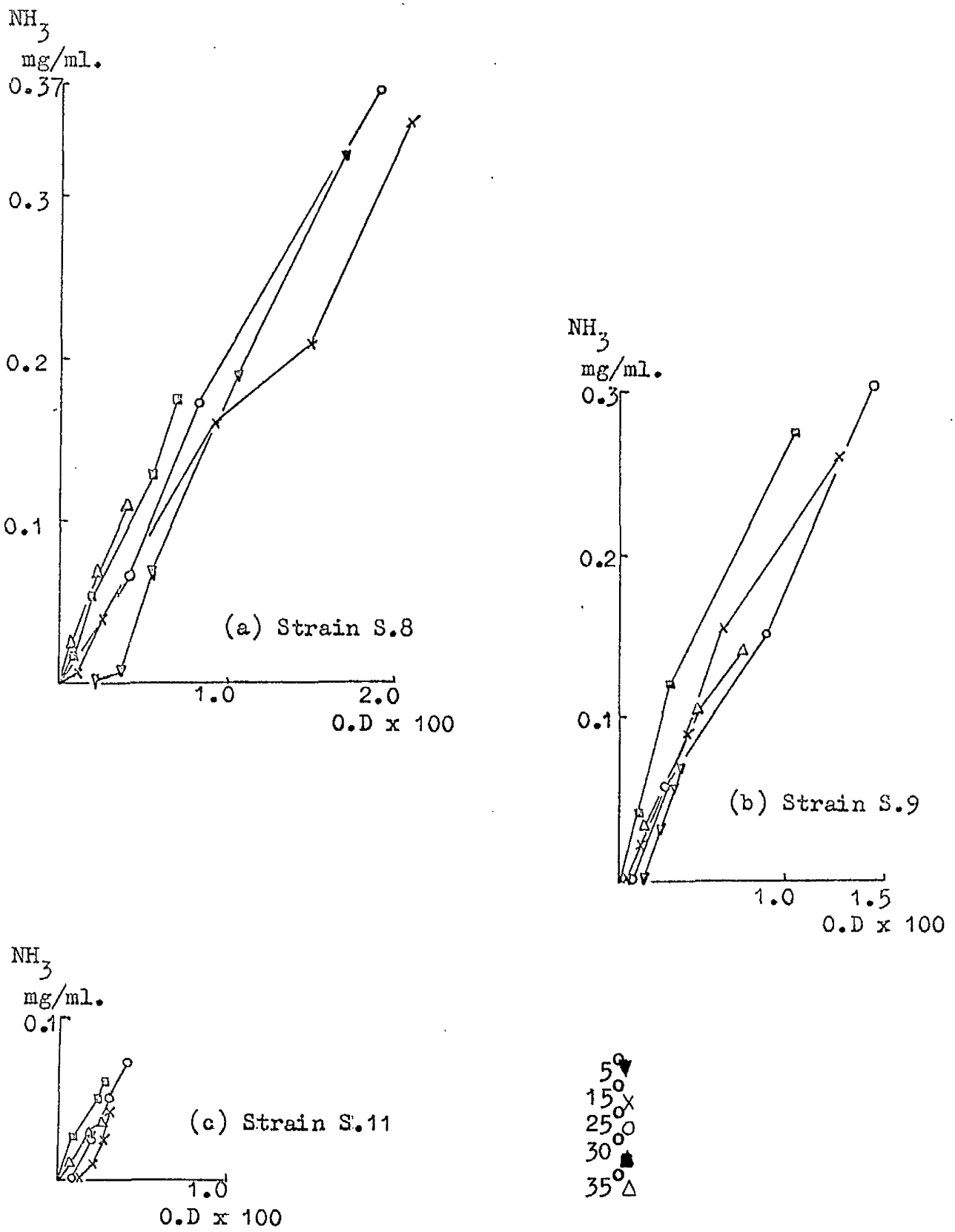


Fig. 1.18 Growth and ammonia formation in defined amino-acid medium

Amino-acid: Arginine

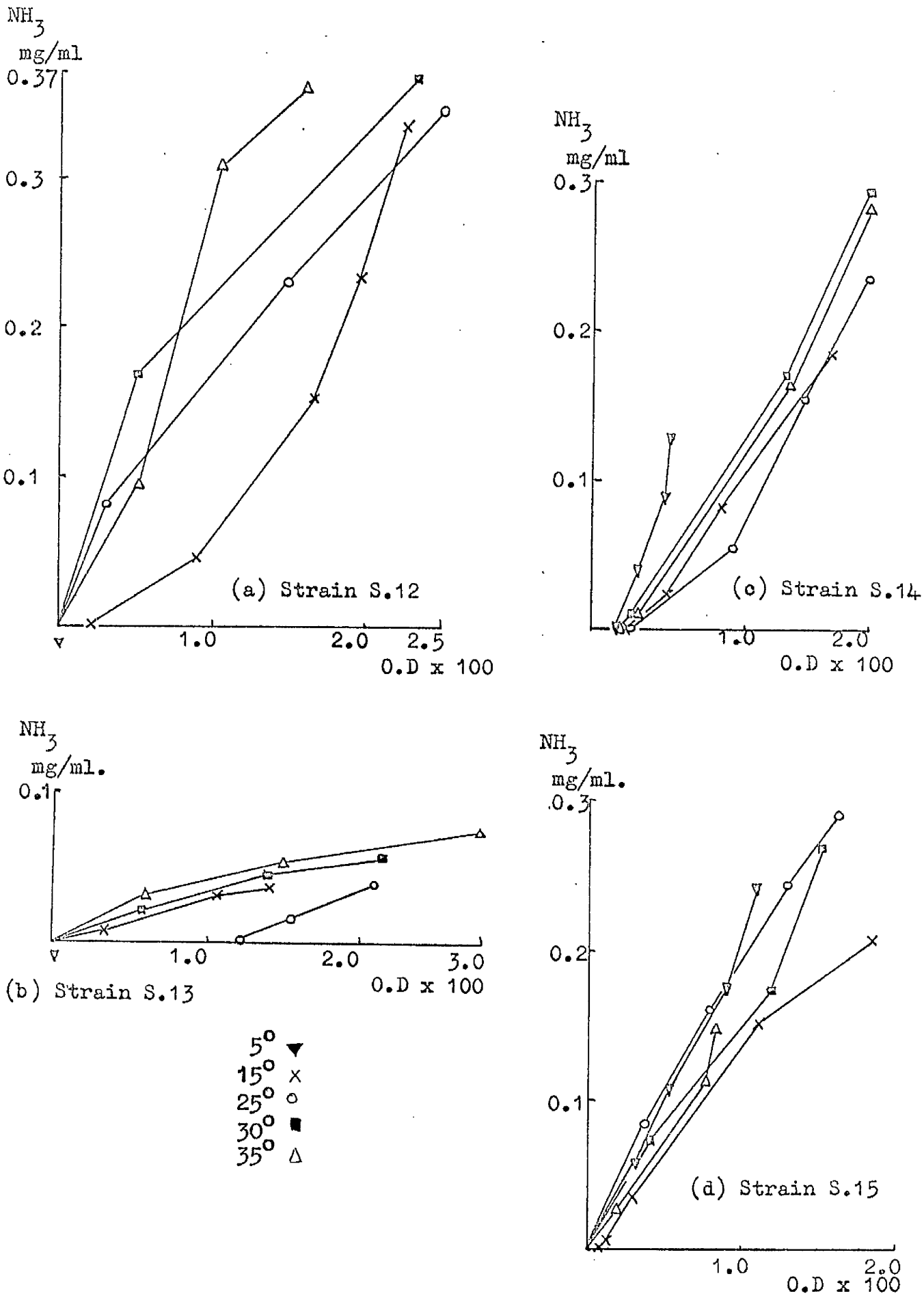
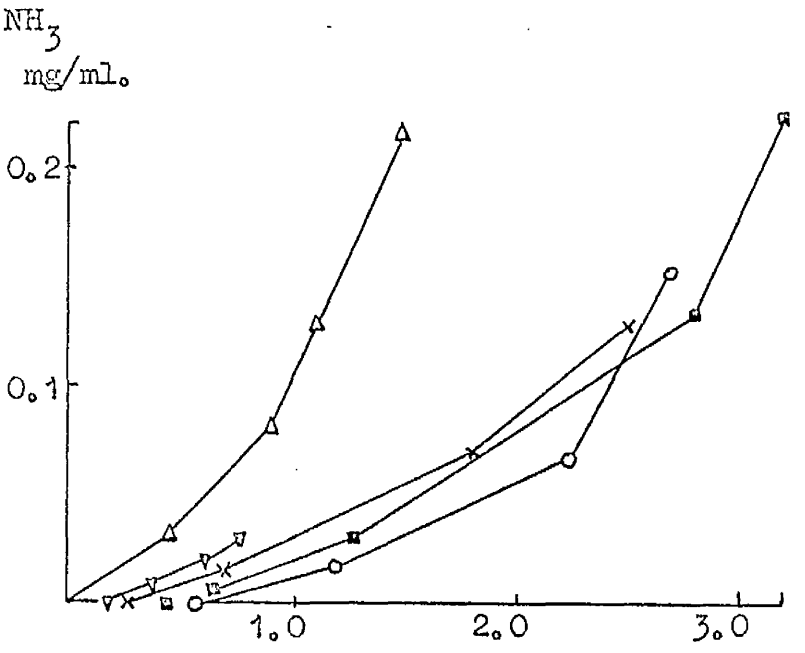
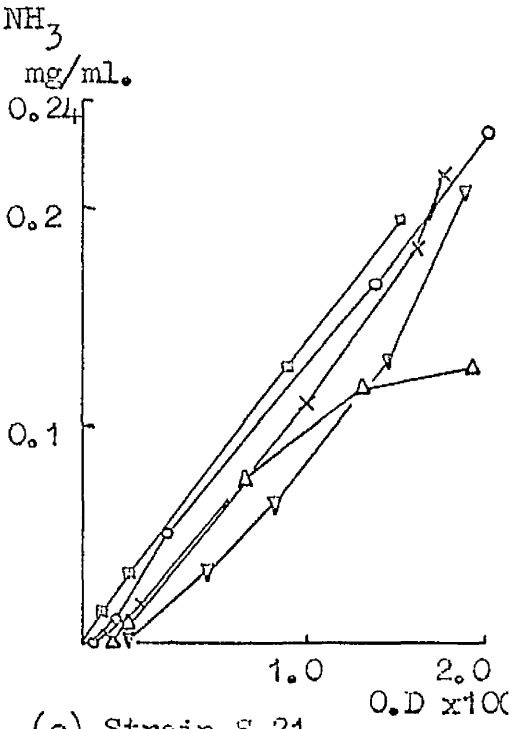


Fig. 1.19 Growth and ammonia formation in defined amino-acid medium

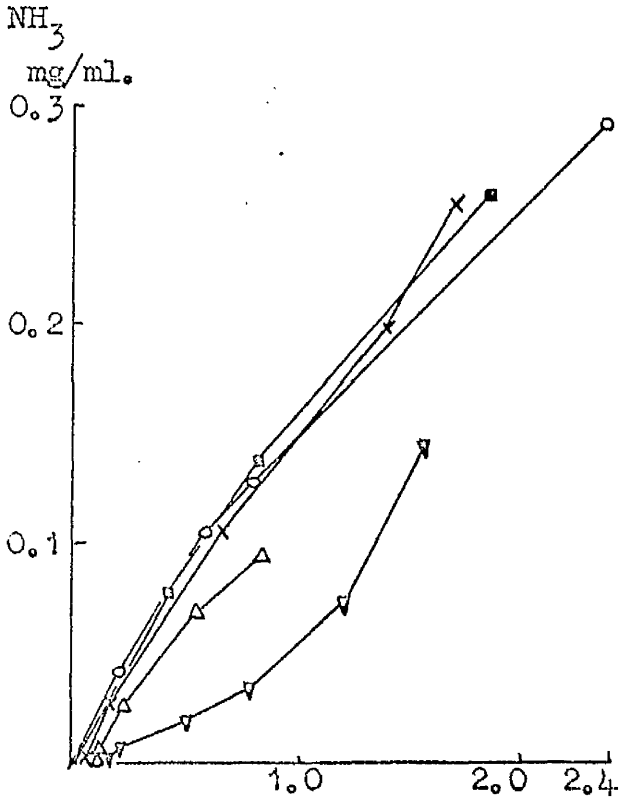
Amino-acid: Arginine



(a) Strain S.18



(c) Strain S.21

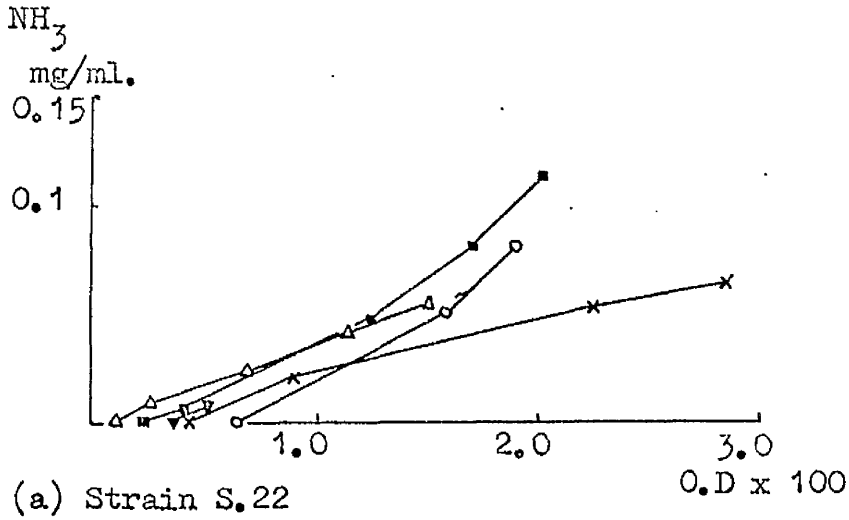


(b) Strain S.20

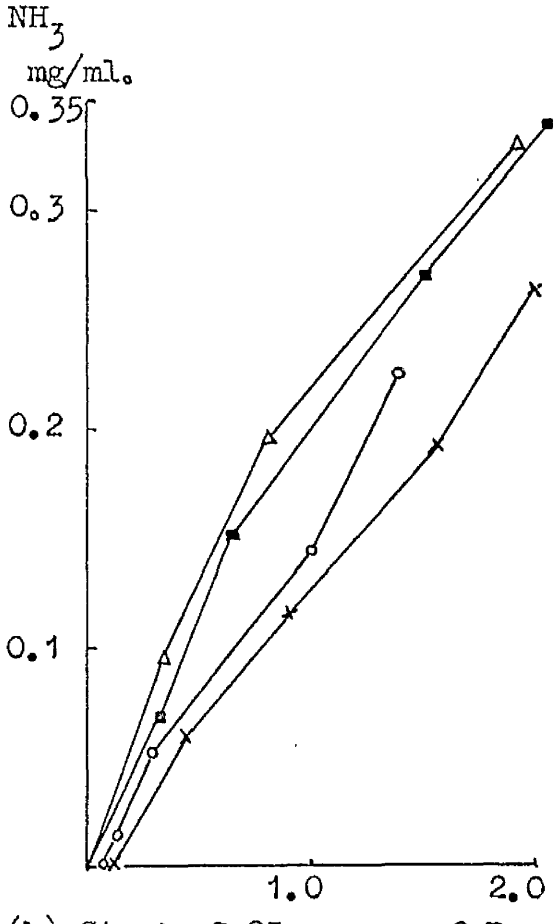
5° ▼
15° X
25° o
30° ■
35° Δ

Fig. 1.20 Growth and ammonia formation in defined amino-acid medium

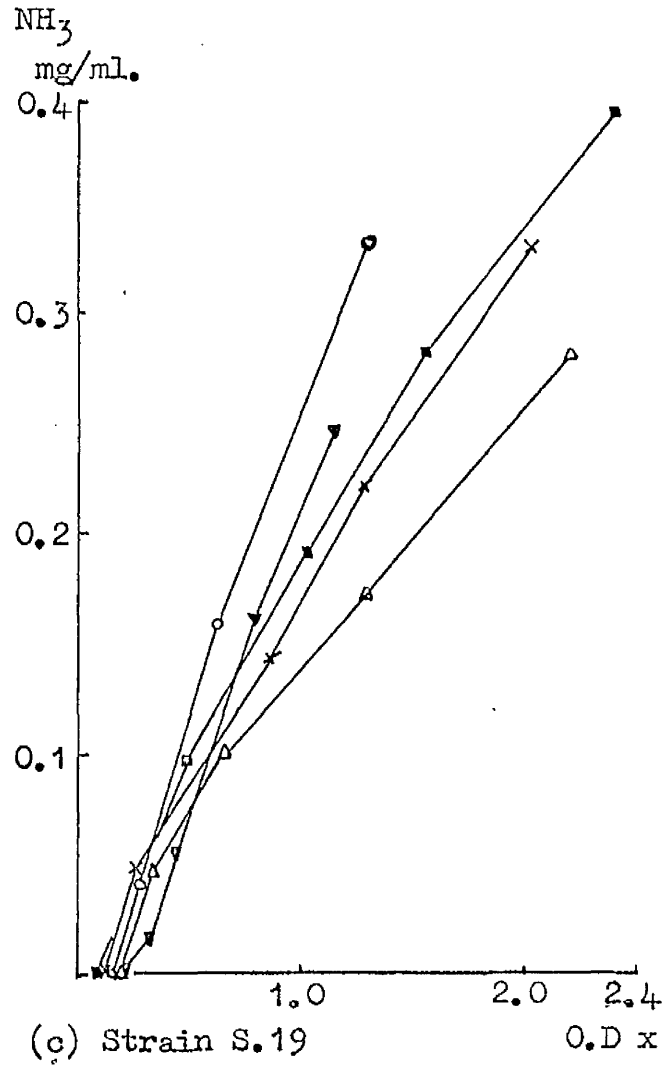
Amino-acid: Arginine



(a) Strain S.22



(b) Strain S.23



(c) Strain S.19

5° ▼
15° X
25° o
30° ■
35 Δ

Fig. 1.21 Growth and ammonia formation in defined amino-acid medium

Amino-acid: β- Alanine

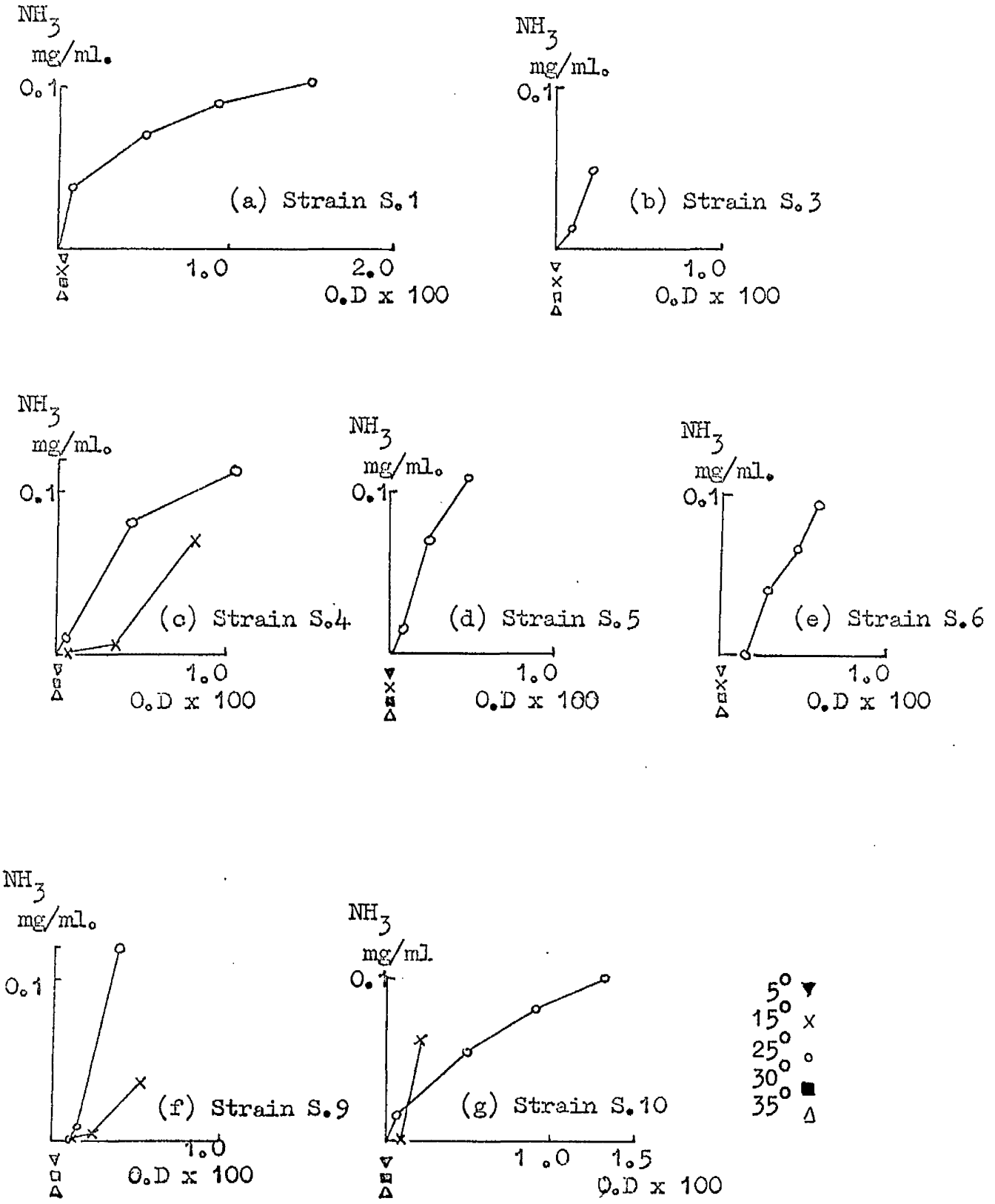


Fig. 1.22 Growth and ammonia formation in defined amino-acid medium

Amino-acid: β - Alanine

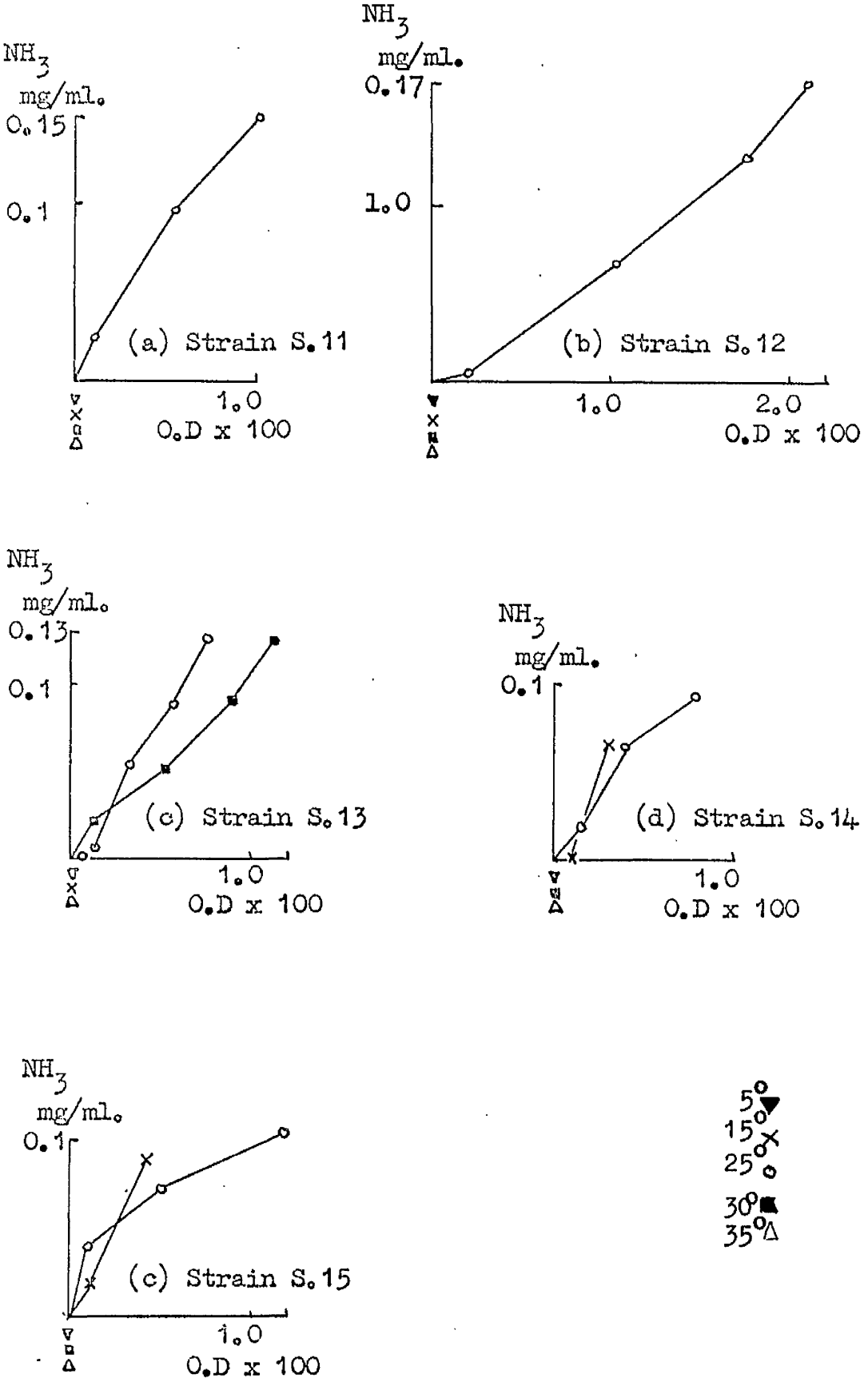
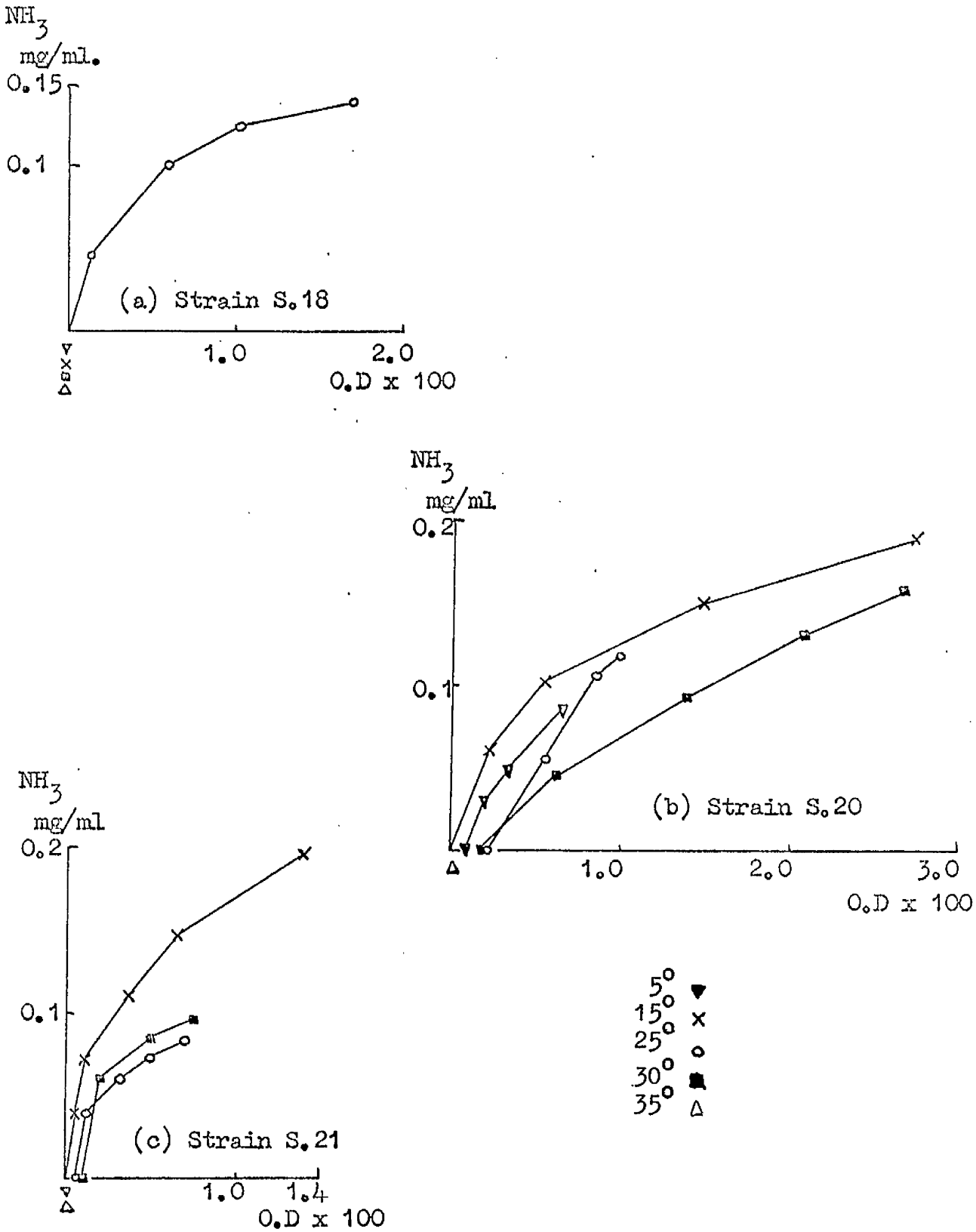


Fig. 1.23 Growth and ammonia formation in defined amino-acid medium

Amino-acid: β - Alanine



and increased Q_{10} (15° - 25°) values which indicated the reduced growth response of these strains at low temperatures in glutamic acid medium compared with tryptone medium.

St values greater than unity at 5° signified that strains S2, and S10 and S21 exhibited a higher growth rate in glutamic acid medium than in tryptone medium. Strains S8, S10, S13, S21, S22 and S23 displayed St values greater than unity at 15° and the same applied to 15 strains at 25°, 18 strains at 30° and 12 strains at 35°. This indicates that glutamic acid may be superior to tryptone as sole sources of carbon, nitrogen and energy, especially at 25° to 35°. The growth rates of the strains were more often reduced than increased at 5° and 15° and the stimulation of the growth rate was less marked at 35° than at 25° or 30°. The St values confirmed the increased psychrophilic growth response of strain S2 in glutamic acid medium. Strain S24, which displayed psychrophilic tendencies in tryptone medium, lost the ability to grow at 5° or 15° in glutamic acid medium and low St values at 25° and 35° indicated that the growth rate was much reduced. Strains S1, S3, S4, S5, S6, S9, S10, S11, S13, S14, S15 and S21 displayed a progressive increase in St values from 5° to 30° or 35°, indicating a marked general development of mesophilic tendencies in glutamic acid medium in comparison with the growth response in tryptone medium.

Ammonia production during the utilisation of this amino acid was demonstrated by 22 strains (Figures 1. 8 - 1. 14). Strains S1, S3, S10, S18 and S19 exhibited relationships between growth and ammonia production at all temperatures which approximated to the linear type (i) or (iii) curves. Other strains showed this linear relationship between ammonia

production and growth at one or more temperatures. Most strains showed the type (ii) pattern of ammonia production at one or more temperatures. Strains S1, S4, S8, S9, S10, S15, S18, S20 and S21, particularly at 25°, showed this initially rapid production of ammonia during the early stages of growth with subsequent levelling off of ammonia production at higher cell crops. Strains S6, S9, S15, S20 and S21 showed this pattern of ammonia production at 30°, as did strains S5, S9, S12, S13, S18, S19, S21 and S23 at 35°. This pattern was less apparent at 5° or 15° generally, but was shown by strains S8, S9 and S13 at 15° and by strain S9 at 5°. At 5° and 15° the production of ammonia either displayed an almost linear relationship with growth or ammonia production was initially slow and followed by a marked increase in the production of ammonia which continued to increase until the maximum cell crop was reached. Strains S1, S15, S19 and S20 exhibited this type (iv) curve at 15° and strains S2, S7, S14, S15 and S21 demonstrated the same pattern at 5°. Strains S3, S5, S7, S8, S9, S10, S14, S21 and S23 displayed the type (iv) pattern of ammonia production at temperatures other than 5° and 15° and strain S22 showed this relationship at all test temperatures. A profound effect of temperature on the production of ammonia during the utilisation of glutamic acid was apparent in strains S6, S7, S9, S14, S15, S21, S22 and S23 and the effect of temperature was most marked in strains S6, S7, S14 and S22 at high cell crops. Although strain S9 displayed the type (ii) pattern of ammonia production at most temperatures the production of ammonia was much greater at 25° than at 15° for equivalent levels of growth. Strain S23 produced much greater amounts of ammonia at 35° than at 15° for equivalent levels

of growth. At 25°, 30° or 35° strains S1, S4, S5, S8, S9, S14, S15, S19, S20 and S21 produced ammonia rapidly during the early stages of growth in glutamic acid medium and the ammonia production levelled off at higher cell crops (type (ii) curve) whereas at 5° or 15° an initial delay in ammonia production was followed by a rapid increase during the later stages of growth (type (iv) curve).

Arginine.

Except for strains S2 and S16, all strains were able to utilise arginine as sole carbon, nitrogen and energy sources. Strains S1, S3, S5, S7, S8, S9, S10, S11, S12, S14, S18, S19, S21, S22 and S23 showed Q_{10} (5° - 15°) values higher than those obtained in tryptone medium which reflected an increased sensitivity to growth at low temperature in arginine medium. However, strains S4, S6, S15, S17, S20 and S24 exhibited reduced Q_{10} (5° - 15°) values, compared with those in tryptone medium, which suggested increased psychrophilic tendencies but this conflicted with the increased Q_{10} (15° - 25°) values which were shown by 22 strains. Compared with values in tryptone medium Q_{10} (25° - 35°) values in arginine medium were only higher in strains S14 and S15. Other strains exhibited reduced Q_{10} (25° - 35°) values which, together with the generally increased Q_{10} (5° - 15°) values, indicated a marked lack of ability to grow in arginine medium at both extremes of the temperature range compared with tryptone or glutamic acid media.

St values greater than unity were fewer in arginine medium than in glutamic acid medium at 5°, 25°, 30° and 35° although the values in arginine medium showed wide variation. Strains S6 and S8 exhibited

St values greater than unity at 5°, strains S5, S6, S8, S13, S21 and S22 at 15°, 12 strains at 25°, 11 strains at 30° and 7 strains at 35°. Only strain S6 displayed St values greater than unity at all test temperatures, and although the values were greater in arginine medium than in glutamic acid medium at 5°, 15° and 25° they were less at 30° and 35°. Strains S3, S4, S5, S6, S7, S8, S9, S11, S21, S23 and S24 showed marked differences in the patterns of St values over the test temperature range, although St patterns were similar in glutamic acid and arginine media for strains S12, S13, S14, S15, S18, S19, S20 and S22. Thus, differences in the strain patterns of St values in arginine medium emphasises the profound effect of specific substrates on the rates of growth of strains at different temperatures. A progressive increase in St values from 5° to 30° or 35° was shown by strains S1, S3, S9, S10, S11, S14, S15 and S19 but strains S4, S5, S6, S8, S12, S13, S18, S20, S21, S22 and S23 generally showed maximum St values at 25° with decreased values at higher and lower temperatures.

Strains often produced greater amounts of ammonia from this diamino-monocarboxylic amino acid than from any other amino acid examined (Figures 1. 15 - 1. 20). A linear relationship between the production of ammonia and cell crop , as indicated by type (i) and (iii) curves, was shown by strains S1, S3, S4, S7, S8, S9, S11, S15, S19, S20, S21 and S23 at one or more temperatures. The relationship between ammonia production and growth of strain S4 was similar at 15°, 25° and 30° whereas for equivalent levels of growth much less ammonia was produced at 15° than at 35°. A linear relationship between ammonia production and growth was shown by strain S20 at 15°, 25°, 30° and 35°, whereas

the production of ammonia was slow during the initial stages of growth at 5° and, according to the type (iv) curve, increased markedly during the later stages of growth. The rapid production of ammonia during the early stages of growth with a subsequent levelling off in ammonia production, as in type (ii) curve, was found in strains S1 and S5 at 15° and 25° and in strains S6, S12 and S23 at 25°, 30° and 35°. Strains S1, S4, S5, S6, S12, S18, S19 and S20 showed appreciable variation in the patterns of ammonia production for similar levels of growth at different temperatures. Mesophile strain S12 displayed a marked delay in the production of ammonia at 15° despite appreciable early growth while the subsequently increased production of ammonia resulted in a final level of ammonia similar to that obtained at 30°, at which temperature a type (ii) curve was displayed. Strain S18 produced much ammonia during growth at 35°, while the type (iv) curve was apparent at 15°, 25° and 30°. The initial delay in ammonia production by this strain was most pronounced at 5° although, as shown by strains S5, S6, S8, S18, S19 and S22, the delay was followed by a marked increase in the production of ammonia during the later stages of growth. While 19 strains were able to produce ammonia during utilisation of arginine as sole carbon, nitrogen and energy sources only Pseudomonas and Aeromonas strains S1, S3, S4, S5, S6, S8, S9, S12, S14, S15, S18, S19, S20, S21 and S23, which were shown in Part I of this study to demonstrate arginase activity, were able to produce large quantities of ammonia in the present experiment. It is important to note the effect of temperature on the production of ammonia by strain S18 (Ps. putida), which in Part I was shown to be the only

Pseudomonas strain incapable of producing a reaction to the arginase test at 25°. This experiment showed that ammonia production was poor at 25° despite appreciable growth, while for much lower levels of growth at 35° ammonia production was appreciable. This observation stresses the difficulties presented by the selection of a temperature for the performance of a test when the reaction varies with increase or decrease in temperature. Strains other than pseudomonads were either not able to utilise arginine for growth (strains S2 and S16), produced poor growth and low levels of ammonia (strains S7 and S11) or produced appreciable growth and negligible ammonia (strains S13 and S22).

Lysine.

Most of the 14 strains which utilised lysine grew less well than in tryptone medium at all temperatures and compared with tryptone, glutamic acid and arginine media all strains, except strain S9, displayed higher Q_{10} (5° - 15°) values in lysine medium. Strains S3, S9, S15 and S21 exhibited Q_{10} values at 5° - 15°, 15° - 25° and 25° - 35° which reflected psychrophilic patterns of growth in this medium but strains S1, S4, S5, S6, S8, S14, S18 and S20 displayed reduced psychrophilic tendencies. Low Q_{10} (25° - 35°) values indicated the marked reduction in growth of strains S1, S6 and S14 at 35° and strains S4, S5, S8, S15 and S21 did not grow in lysine medium at this temperature despite often appreciable growth in tryptone, glutamic acid and arginine media.

Low St values were shown by 13 strains which reflected the generally reduced ability to utilise lysine. St values greater than unity were shown only by strain S18 at 30° and 35°. The St values of

this strain increased progressively from 15° to 35° indicating the development of a mesophilic growth response in lysine medium compared with trypsone medium. The St values of other strains able to grow at 35° were generally low at this temperature and the St values of most strains were zero or low at 5° and 15°. Excluding strain S18 St values approaching unity were demonstrated at 25° only by strains S5 and S14.

Ammonia was produced from this diamino-monocarboxylic amino acid by all 14 strains (Figures 1. 5 - 1. 7) and appreciable variation was observed between the abilities of the strains to produce ammonia from lysine. Strains S1, S3, S4, S6, S14, S15, S18, S20 and S23 showed marked differences in the effect of temperature on the relationship between the production of ammonia and growth. The linear type (i) or (iii) curve was exhibited at one or more temperature by strains S1, S4, S6, S8, S12, S15, S18, S20 and S21 while the type (ii) curve, signifying the rapid production of ammonia during the early stages of growth, was shown by strains S1, S4, S5, S12, S14, S18 and S20. Strain S4 displayed the type (ii) curve at 5°, 15° and 25° while other strains often showed this type of curve at 25° or 30°. Marked initial delays in the production of ammonia were shown by strains S3 and S9 at all test temperatures, being pronounced at 5° in strains S5, S8, S14 and S20. This type (iv) curve was most apparent in strain S23 at 15° despite comparatively heavy growth at this temperature and in strain S18 at 25°. The subsequent increase in ammonia resulted in a final level being produced by strain S18 comparable to that obtained at 15°.

Leucine.

The Q_{10} (5° - 15°) values of strains S1, S4, S6, S8, S14, S15, S18, S20 and S21 were less in leucine medium than in lysine medium and strains S6, S15 and S19 showed lower Q_{10} (5° - 15°) values in leucine medium than in tryptone, glutamic acid, arginine or lysine media. The Q_{10} (15° - 25°) values of 9 of the 15 strains able to utilise leucine were greater than unity and strains S1, S3, S4, S9, S12, S15, S19 and S20 displayed Q_{10} (25° - 35°) values which were higher than in tryptone, glutamic acid, arginine or lysine media. Thus on the basis of Q_{10} values these strains were apparently able to grow well over the test temperature range in leucine medium. The absence of growth at 35° and low Q_{10} values at 5° - 15° and 15° - 25° indicated the increased psychrophilic tendencies of strains S6 and S8 in leucine medium.

The St values confirmed the observations made on the basis of Q_{10} values that, in general, leucine was better than lysine for supporting growth over a wide range of growth temperatures. The St values were not as high as those in glutamic acid or arginine medium, except at 35° for strains S1 and S9 and at 30° and 35° for strain S20. Strains S1, S4, S9, S15, S18, S19 and S20 displayed mesophilic tendencies in leucine medium, indicated by progressive increases in the St values with increase in temperature. The St values of strains S6 and S8 generally decreased with increase in temperature.

All 15 strains able to utilise this branched chain amino acid were able to produce ammonia at one or more temperatures although the effect of temperature on the production of ammonia during growth varied widely

between strains (Figures 1. 2 - 1. 4). Strains S1, S3, S4, S9, S12, S13, S14, S15 and S18 displayed initial delays in ammonia production at most temperatures with subsequent increases in the production of ammonia at higher cell crops. This type (iv) curve was most apparent in strains S1, S4, S9 and S15. Strain S6 produced large quantities of ammonia for relatively little growth at 25° and 30° while for similar cell crops at 5° and 15° ammonia production was poor, and even at higher cell crop values the amounts of ammonia produced at 5° and 15° were low compared with 25° and 30°. Similar, although less marked, effects of temperature on ammonia production were shown by strains S8 and S21. Strains S12, S13 and S18 displayed almost linear relationships between ammonia production and growth, type (iii) curves being particularly apparent at 30° and 35°. Strain S17 produced ammonia only at 15° while strains S19 and S20 displayed type (ii) curves at 30° and 35° and type (iv) curves at 15° and 25°.

Glycine.

Strains S3, S8, S14 and S18 were able to utilise glycine as sole carbon, nitrogen and energy sources. The Q_{10} (5° - 15°) values of strains S3 and S14 were less than in tryptone, glutamic acid, arginine, lysine or leucine media. The corresponding values for strains S8 and S18 were higher than those obtained for growth in tryptone, glutamic acid, arginine or leucine media but were lower than those for lysine medium. Q_{10} (25° - 35°) values were greater than unity for strains S3 and S18 and strains S14 and S18 showed Q_{10} (15° - 25°) values greater than unity. Only strain S8 displayed Q_{10} values indicative of a

psychrophilic growth pattern in glycine medium.

Low St values reflected the poor rates of growth of strains S3 and S14 at all temperatures in glycine medium and the values were lower than shown in glutamic acid, arginine, lysine or leucine media. The St values of strain S8 were higher at 5° to 35° in glycine medium than in lysine or leucine media and the values of strain S18 were similar to those obtained in leucine medium. Strain S18 was the strain best able to utilise glycine as carbon, nitrogen and energy sources at 15°, 25° and 35°.

Marked differences were shown in the abilities of strains S3, S8, S14 and S18 to produce ammonia from this simple mono amino-monocarboxylic amino acid (Figure 1. 1). Strain S14 produced appreciable amounts of ammonia during poor growth at 5°, 15°, 25° and 30°, although for similar cell crops at 35° no ammonia was produced. Strain S3 displayed slight initial delays in the production of ammonia at 5° and 15°, followed by rapid increases and subsequent levelling off of ammonia production during later stages of growth. At similar cell crop values at 25° and 30° ammonia production was less than at 5° or 15° and ammonia was not produced at 35°. Strain S8 displayed linear type (i) curves at 25° and 30° and a linear type (iii) relationship was shown at 15°. At 5° an initial delay was followed by an appreciable increase in the production of ammonia in the manner shown by strain S3 at 5° and 15°. Strain S8 produced low levels of ammonia at 35°. Type (ii) curves were displayed by strain S18 at 15°, 30° and 35° and linear type (iii) curves were shown at 5° and 25°, although little ammonia was produced at 5°.

β-Alanine.

The 15 strains able to grow in β-alanine medium displayed unusual effects of temperature. Strains S1, S3, S5, S6, S11, S12 and S18 were able to utilise this amino acid only at 25° and it was observed that these strains also showed tendencies to grow poorly at extremes of the temperature range or showed mesophilic tendencies in other amino acid media. Strains S4, S9, S10, S14 and S15 utilised β-alanine only at 15° and 25° although these strains displayed psychrophilic patterns of growth in tryptone medium and other amino acid media. On the basis of Q_{10} values strain S20 alone showed psychrophilic tendencies and the ability to grow at 5°, 15°, 25° and 30°. An infinite $Q_{10}(5°-15°)$ value indicated the inability of strain S21 to grow at 5° although growth occurred at 15°, 25° and 30°. An infinite Q_{10} (15° - 25°) value reflected the mesophilic growth response of strain S13.

The St values of strains S3, S4, S5, S6, S9, S13, S14 and S21 were generally low and values greater than unity were shown only by strains S10, S11 and S12 at 25° and by strain S20 at 30°, although strains S15 and S18 displayed St values slightly less than unity at 25°. As previously indicated the Q_{10} values of strain S20 reflected a psychrophilic growth pattern in β-alanine medium, but the progressive reduction in St values from 30° to 5° reflected a tendency towards mesophilic growth compared with growth in tryptone medium.

All strains able to utilise this mono amino-monocarboxylic amino acid produced ammonia (Figures 1. 21 - 1. 23). At 25° strains S3, S5, S11, S12, S13 and S14 displayed almost linear type (i) or (iii) curves while strains S1, S4, S10, S15, S18 and S21 showed type (ii) curves.

This latter type of curve was shown at 5°, 15° and 30° by strain S20 and at 15° to 30° by strain S21. Strains S9, S10 and S14 displayed an initial delay in ammonia production at 15° with a subsequent increase at higher cell crops.

This experiment has shown that certain amino acids, e.g. glutamic acid and arginine, may effectively replace and in certain cases be superior to tryptone as sole carbon, nitrogen and energy sources. However, the utilisation of single amino acids was found to be generally more sensitive to extremes of temperature, as reflected in extension of the apparent lag, reduction of the rate of growth or reduction in the maximum cell crop. Jezeski and Olsen (1962) observed that casamino acids were involved in the ability of psychrophiles to grow at low temperature. The results of the present experiment indicate that a combination of amino acids, as found in tryptone, is generally superior to a single amino acid substrate for growth at high and low temperatures. Despite this observation individual amino acids differed widely in their ability to support growth over the range 5° to 37° and the effect of temperature was most marked on the utilisation of β-alanine. In comparison with values obtained in tryptone medium extensions of the apparent lag were shown by many strains in single amino acid media, particularly at low temperatures. This indicates an increased period of adaptation by the strain, involving reorganisation of the metabolic pathways, to the utilisation of single carbon, nitrogen and energy sources, or the conversion of the substrate to an intermediate which was more rapidly utilised by the strain, as suggested by Meister (1957).

Wide variations were observed in the abilities of strains to produce ammonia during the utilisation of amino acids. The patterns of ammonia production differed not only between strains, as was indicated by Deibel (1964) in his study of two Streptococcous spp., but the present experiment further showed that temperature had a profound effect on the relationship between ammonia production and the growth of individual strains. The findings confirm the view that the ability of a strain to cause spoilage at a particular temperature need not be related directly to growth at that temperature, nor need the ability to cause spoilage at one temperature reflect the ability to cause spoilage at another temperature despite similar levels of growth.

Results and Discussion of Preliminary Experiment

While the preliminary experiment was designed primarily to provide information for the selection of amino acids for the main experiment the results were considered to be sufficiently important to merit discussion. The abilities of strains S1 - S24 to utilise 21 amino acids as carbon, nitrogen and energy sources at 25° are recorded in Table 10.1 and the changes accompanying growth in these media are recorded in Table 10.2.

Aspartic acid (H) supported the growth of all strains except S16, S17 and S24 and alkaline changes were produced by 20 strains. Strain S19 (*Aeromonas* sp.) produced an alkaline change aerobically and an acid change anaerobically. Gaby *et al.* (1962) found that all *Pseudomonas* strains examined displayed alkaline changes during growth in aspartic acid medium. These workers found that *E. coli* produced an acid change whereas in the present study coliform strain S11 produced a slight alkaline reaction during growth in aspartic acid medium. It is considered that the observation by Gaby and his co-workers was due to the production of acid by the fermentation of glucose (0.5%) incorporated in the test medium. The general ability of the test strains to utilise aspartic acid as sole carbon, nitrogen and energy sources in the present study confirms the opinion of Rose (1965) that this amino acid is a suitable starting material for the synthesis of other amino acids.

Table 10.1 Growth of strains S1 - S24 in amino-acid media at 25°

Amino-acid	P	S	V	G	H	D	J	F	R	B	O	K	Q	I	C	T	L	U	E	M	N	
Strain S.18	2	2	3	3	2	2	3	2	1	1	1	1	2	2	3	1	1	1	1	2	0	20
S. 6	3	2	3	3	3	3	3	3	1	2	2	1	1	1	1	1	1	1	1	0	0	19
S. 1	2	3	3	4	4	4	4	2	1	3	3	2	2	3	1	2	1	1	0	0	0	18
S. 4	3	3	4	4	3	4	4	4	3	3	1	3	2	2	1	4	1	1	0	0	0	18
S.20	3	3	3	3	3	3	3	3	3	3	1	2	2	3	2	2	1	1	0	0	0	18
S. 5	3	3	3	3	3	3	4	4	2	4	2	2	1	0	1	1	1	1	0	0	0	17
S.12	3	3	3	3	3	3	3	3	1	2	1	0	3	1	2	2	1	1	0	0	0	17
S. 9	3	2	2	3	3	3	2	3	3	2	1	3	1	2	1	3	0	0	0	0	0	16
S. 3	4	3	2	3	3	4	3	3	0	2	0	2	1	1	1	0	1	1	0	1	0	16
S.14	2	2	2	2	3	3	1	3	2	0	1	2	1	2	2	3	0	3	0	0	0	16
S.15	2	2	3	3	3	3	3	3	1	0	1	2	2	1	1	3	0	0	1	0	0	16
S. 8	3	2	3	3	2	3	3	3	1	2	1	2	0	1	1	0	0	0	0	1	0	15
S.21	2	3	2	2	3	2	3	3	1	2	1	0	1	1	1	0	1	0	0	0	0	15
S.19	1	2	3	3	3	3	2	3	1	2	1	3	0	2	0	0	1	0	0	0	0	14
S.23	1	1	2	1	1	2	1	2	1	1	2	0	0	0	0	0	1	0	0	0	0	12
S.13	2	3	2	3	2	3	2	2	0	0	0	0	2	2	0	2	0	0	3	0	0	12
S.22	1	1	2	2	2	3	2	2	0	1	1	0	0	0	0	0	0	0	0	0	0	10
S.11	1	3	1	2	3	3	3	3	1	0	0	0	2	0	0	0	0	0	0	0	0	10
S.10	1	2	1	3	3	2	3	3	0	0	0	1	2	0	0	0	0	0	0	0	0	10
S. 7	0	3	1	3	3	2	4	1	0	2	0	2	0	0	0	0	0	0	1	0	0	10
S.24	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4
S. 2	1	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3
S.16	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	1	0	0	4
S.17	0	0	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	2
	22	22	22	21	21	20	20	20	16	15	15	15	15	14	13	12	11	9	6	3	0	

Refer to page 128 and results for interpretation

Table 10.2

Amino-acid	Alkaline reaction H	D	V	R	S	K	F	U	P	Q	G	M	No.	Acid reaction C	I	B	J	O	T	E	L	No.	Total
Strain																							
S.18	2	1	0	2	2	0	2	1	0	0	1	2	8	1	1	1	1	1	1	2	0	7	15
S. 4	1	1	2	2	2	1	1	1	1	1	0	0	10	1	1	1	0	1	0	0	0	4	14
S. 1	1	1	2	2	2	1	1	1	0	0	0	0	8	1	1	1	0	1	0	0	0	4	12
S.20	1	1	1	1	0	1	0	0	*	0	2	0	6	1	1	1	1	0	1	0	0	6	12
S.14	1	1	1	2	0	1	0	1	0	0	0	0	6	1	1	0	1	1	2	0	0	5	11
S. 6	1	1	2	0	0	0	0	0	1	1	0	0	5	1	1	2	1	1	0	1	0	6	11
S. 3	1	1	1	0	2	0	1	0	1	0	0	0	6	1	1	1	1	0	0	0	0	4	10
S.12	2	1	2	0	0	0	0	1	0	1	0	0	5	1	1	1	1	1	0	0	0	5	10
S.21	1	0	2	*	0	0	1	0	1	1	0	0	5	1	1	1	1	0	0	0	0	5	10
S. 9	1	1	2	2	0	1	0	0	1	0	0	0	6	1	1	1	0	0	0	0	0	3	9
S. 5	2	1	2	0	2	0	1	0	0	0	1	0	6	1	0	1	1	0	0	0	0	3	9
S. 8	2	1	2	0	0	0	0	0	0	0	0	2	4	1	1	1	1	0	0	0	0	4	8
S.15	1	1	2	2	0	1	0	0	0	0	0	0	5	1	0	0	0	1	0	0	0	2	7
S.13	2	2	0	0	2	0	0	0	0	0	0	0	3	0	0	0	1	0	1	2	0	3	6
S.22	2	1	0	0	2	0	0	0	0	0	0	0	4	0	0	0	0	•	0	0	0	0	4
S.23	1	1	2	0	0	0	0	0	0	0	0	0	3	0	0	0	0	0	0	0	0	0	3
S.10	2	1	0	0	0	0	0	0	0	1	0	0	3	0	0	0	0	0	0	0	0	0	3
S.11	1	2	0	0	0	0	0	0	0	0	0	0	2	0	0	0	1	0	0	0	0	1	3
S. 7	0	0	0	0	0	*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1
S. 2	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1
S.16	0	0	0	*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1
S.24	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
S.17	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
S.19	1*	1*	2	1	*	*	0	0	*	*	0	0	4	0	2	1	1	1	0	0	1	9	13
	20	18	14	8	7	6	5	5	5	5	3	2		13	12	12	12	8	4	3	1		

* acid reaction

• alkaline reaction

Refer to page 132, and results for interpretation

Asparagine (D) was utilised by 20 strains excluding strains S2, S16, S17 and S24 and alkaline changes were produced by 18 strains. Strain S19 produced changes similar to those observed in aspartic acid medium and strain S21 was able to utilise asparagine but did not produce an observable change. Except strain S2, which did not grow in asparagine medium, the strains which utilised, and the changes produced in, asparagine and aspartic acid media were identical. This similarity may be due to the normal pathway of asparagine breakdown being deamidation to aspartate prior to a common pathway for utilisation. On this basis it is probable that strain S2 (Achromobacter sp.) although able to grow in aspartic acid medium lacks the asparagine deamidase necessary for the utilisation of asparagine.

Arginine (V) supplied carbon, nitrogen and energy sources for 22 strains, strains S2 and S16 being unable to utilise this amino acid. Alkaline changes were produced by 14 strains which included all Pseudomonas strains examined except S18 (Ps. putida) and the atypical pseudomonad strain S17. In Part I of the study strain S18 was found to react weakly to the arginase test and it is probable that the alkaline change was due to breakdown of peptone in the medium. Gaby et al. (1962) observed that certain Pseudomonas strains were unable to produce an alkaline change in an arginine medium. As Steel and Midgley (1962) indicated the metabolism of arginine may occur via arginase, decarboxylase or dihydrolase systems and combinations of these pathways may be involved.

Citrulline (R) supported the growth of 16 strains which excluded strains S2, S3, S7, S10, S13, S17, S22 and S24, although 11 strains grew poorly. Alkaline changes were produced by 8 strains and strains S16 and S21 produced acid changes. In comparison with arginine fewer strains produced an alkaline change and the level of growth was lower throughout. If the arginine dihydrolase system is the mechanism whereby pseudomonads degrade arginine, the breakdown of citrulline as an intermediate in the dihydrolase pathway with the formation of A.T.P should be facilitated. Since citrulline is poorly utilised as sole carbon, nitrogen and energy sources it is feasible that either the arginase or arginine decarboxylase pathway are the main pathways of arginine utilisation by pseudomonads.

Glutamine (S) was utilised by 22 strains excluding strains S16 and S17. Only 7 strains were alkaligenic which included strain S18 (Ps. putida), Pseudomonas strains S1, S3, S4 and S5 and other strains S13, S19 and S22. Strain S19 produced an acid change in glutamine medium.

Serine (K) provided carbon, nitrogen and energy sources for 15 strains, strains S1, S4, S9, S14, S15 and S20 produced alkaline changes and strains S7 and S19 produced acid changes. The alkaligenic types were all Pseudomonas strains but strain S12 (Ps. aeruginosa) was unable to utilise serine. Gaby et al. (1962) found that Pseudomonas strains produced alkaline, acid or no changes in a complex medium containing serine.

Histidine (O) supported the growth of 20 strains which excluded strains S2, S16, S17 and S24, an identical distribution of strains as shown for the utilisation of asparagine medium. In histidine medium Pseudomonas strains S1, S3, S4, S5, S18 and S21 produced alkaline changes. Peterkowsky (1962) indicated that under alkaline conditions histidine is degraded by Pseudomonas spp. by desaturation deamination to urocanic acid but the widespread ability of the test strains to utilise histidine without extensive alkaligenesis suggests the involvement of alternative pathways.

Glycyl-glycine (U) was utilised as sole carbon, nitrogen and energy sources by 9 strains, all of which were Pseudomonas strains and all grew poorly except strain S14. Strains S1, S4, S12, S14 and S16 produced a slight alkaline change.

D,L-Alanine (P) provided for the growth of 22 strains which excluded strains S7 and S17. Strains S3, S4, S6, S9 and S21 were alkaligenic and strains S19 and S20 produced acid. Gaby et al. (1962) observed alkaline or acid changes or no change during the growth of different strains of Ps. aeruginosa in an alanine medium and Ps. fluorescens produced acid during breakdown of this amino acid. In the present experiment neither species (strains S12 and S15) produced any change during the utilisation of D,L-alanine as carbon, nitrogen and energy sources.

β-Alanine (Q) supported the growth of 15 strains, excluding strains

S2, S8, S16, S19, S22, S23 and S24 which were able to utilise D.L-alanine. Strains S4, S6, S10, S12 and S21 produced alkaline changes in β-alanine medium, of which strains S4, S6 and S21 displayed similar changes in D.L-alanine medium. Strain S19 showed similar acid changes in D.L-alanine and β-alanine media.

Glutamic acid (G). While this amino acid was utilised by 21 strains, excluding strains S2, S16 and S17, only strains S18 (Ps. putida) and Pseudomonas strains S5 and S20 were alkaligenic. The ability of most test strains to utilise glutamic acid as sole carbon, nitrogen and energy sources confirms the observation by Rose (1965) that this amino acid is widely used for the synthesis of other amino acids.

Glycine (M) was utilised as carbon, nitrogen and energy sources by strains S3, S8 and S18 of which strains S8 and S18 were alkaligenic.

Lysine (C) provided for the growth of 13 strains all of which were Pseudomonas strains but excluded strains S17 and S23. All 13 strains produced an acid change in lysine medium. In a lysine medium Gaby et al. (1962) found that 19 Ps. aeruginosa strains produced a slight alkaline change, 2 strains produced an acid change and one strain produced no change, while other Pseudomonas strains produced an acid change or no change. It is considered that the variation in changes produced by Pseudomonas strains in the lysine medium of Gaby et al. (1962) reflects the breakdown of casitone as well as lysine. The results

of the present experiment indicates that Pseudomonas strains normally produce acidic breakdown products from lysine. This reasoning is supported by Stewart (1964) who maintained that lysine decarboxylase operates in Pseudomonas spp., although Steel and Midgley (1962) considered that this enzyme was operative in Ps. maltophila but not in other Pseudomonas spp..

Leucine (I) supported the growth of 14 strains of which 12 produced an acid change. Strain S19 (Aeromonas sp.) showed a marked acid change in this medium. The mechanism for the utilisation of leucine as sole carbon, nitrogen and energy sources is not clear but the absence, as shown in the present experiment, of alkaline breakdown confirms the observation by Rose (1965) that the oxidative deamination of leucine does not give a directly usable energy source.

Ornithine (B) was utilised by 14 strains of which 12 produced acid. These comprised Pseudomonas and Aeromonas strains. The mechanism for utilisation of this amino acid by pseudomonads is not clear although Steel and Midgley (1962) maintained that ornithine decarboxylase was absent in Pseudomonas spp..

Proline (J) supplied carbon, nitrogen and energy sources for 20 strains excluding strains S2, S16, S17 and S24. The strains able to utilise this amino acid were identical to those able to utilise asparagine and similar to those able to utilise glutamic acid which Meister (1957) regarded as an intermediate of proline breakdown. Acid

changes were produced from proline by 12 strains which notably excluded alkaline-proteolytic Pseudomonas strains S1, S4, S9 and S15.

ϵ- α- Diaminopimelic acid (O) supported the growth of 15 strains although the level of growth was generally poor except for strain S1. Acid was produced by strains S1, S4, S6, S12, S14, S15, S18 and S19 and an alkaline change was produced by strain S22. Stewart (1964) indicated that ϵ-γ-diaminopimelic acid decarboxylase occurred in E. coli but in this experiment coliform strain S11 was unable to utilise this amino acid.

Leucinamide (T) provided carbon, nitrogen and energy sources for the growth of 12 strains which included 10 Pseudomonas strains together with strains S13 and S16. Acid was produced by Pseudomonas strains S14, S17, S18 and S20 while strains S12 (Ps. aeruginosa) and S15 (Ps. fluorescens) produced no change. These findings conflict with those of Gaby et al. (1962) who reported that Ps. aeruginosa and Ps. fluorescens produced alkaline changes in a leucinamide medium whereas other Pseudomonas spp. produced an acid change or no change.

Phenylalanine (E). Strains S6, S7, S13, S15, S16 and S18 were able to utilise this amino acid although only strain S13 produced good growth. Strain S13 (Bact. anitratum) and Pseudomonas strains S6 and S18 produced acid changes in phenylalanine medium. The

general inability of strains to produce alkaline changes in this medium was confirmed by Steel and Midgley (1962) who noted that Pseudomonas and Aeromonas strains did not display phenylalanine deaminase, although Guroff and Ito (1965) found that, in the presence of an ammonia-nitrogen source Ps. fluorescens hydroxylated phenylalanine. Phenylalanine did not support the growth of Ps. aeruginosa (S12) which agrees with the findings of Colwell (1964).

Methionine (L) was utilised by 11 strains which except strain S19 (Aeromonas sp.) were Pseudomonas strains. All strains produced poor growth and only strain S19 displayed an acid change. These findings conflict with those of Gaby et al. (1962) who reported that Ps. aeruginosa strains generally produced alkaline changes in a methionine medium whereas other Pseudomonas spp. produced acid changes or no change.

Tryptophane (W) was unable to provide sole sources of carbon, nitrogen and energy for any strain. This finding is supported by the observation that Pseudomonas spp. do not produce indole (Stewart, 1958) and that growth is poor during the oxidation of tryptophane (Stanier and Hayaishi, 1951). Rose (1965) also indicated that opening of the aromatic ring requires oxygenase systems to entrap energy and Palleroni and Stanier (1964) showed that tryptophane utilisation by Pseudomonas spp. required the induction of necessary enzymes.

The difference between strain S18, which utilised 20 amino acids,

and strain S17, which utilised two amino acids, reflects the variation exhibited by the test strains to utilise individual amino acids as sole carbon nitrogen and energy sources. With the exception of strains S17 and S23 the remaining 13 Pseudomonas strains were able to utilise 15-20 (73.3 - 95.2%) of the 21 amino acids tested. Fluorescent, proteolytic and lipolytic strains S3, S4, S6, S9, S12, S15 and S20 and fluorescent, non-proteolytic and often non-lipolytic strains S1, S14, S18 and S21 which conformed respectively to Pseudomonas groups I and II (Scholefield, 1964) utilised a wide range of amino acids and produced marked alkaline or acid changes. Non-fluorescent and non-proteolytic strains S5 and S8 which conformed to Pseudomonas group III (Scholefield, 1964) produced fewer alkaline or acid changes in amino acid media than most other Pseudomonas strains.

Aeromonas strains S7 and S19 were dissimilar in the number and types of amino acids utilised and strain S19 displayed a markedly different pattern of amino acid breakdown compared with other strains. Achromobacter strains S2 and S10 were unable to produce acid or alkaline changes in most amino acid media and strain S2 grew poorly and in only three media. Comamonas percolens (S16) utilised few amino acids and the inability to produce changes in these amino acid media confirmed the generally poor metabolic activity shown in Part I and observed by de Ley (1964).

Discrepancies in the comparison of the results of this experiment with those of Burman and Oliver (1952), Møller (1955), Gaby et al. (1962), Deibel (1964) and Guroff and Ito (1965) are unavoidable due to the incorporation into media used by these workers of carbon, nitrogen or energy sources other than the test amino acids.

It is apparent that correlation between the response of strains to tests performed in Part I and patterns of amino acid utilisation and breakdown is incomplete. However, the potential value of tests based on the utilisation and breakdown of amino acids has been indicated and it is considered that a wide range of Gram-negative bacteria need to be examined when the amino acid utilisation pattern may be tied in with other accepted taxonomic criteria.

IV. THE EFFECT OF TEMPERATURE ON PROTEINASE AND LIPASE ACTIVITIES

The spoilage of milk held at low temperatures for extended periods, as described in the Introduction, has often been related to the proteolytic and lipolytic activities of psychrophiles. However, few studies have been made of the breakdown of specific constituents of milk by bacterial enzymes, or of the effect of temperature on the breakdown of such compounds by psychrophilic bacteria.

Casein comprises 79 - 80% of the total nitrogen in milk (Davis and McDonald, 1952) and contributes much to the structure of milk. Mellander (1939) demonstrated the heterogeneity of casein, naming the fractions α, β, γ-caseins. Waugh and Hippel (1956) identified K-casein as part of the α-casein fraction and Waugh (1961) showed that K-casein serves to maintain the stable micellar structure of the whole casein complex. Molecules of the calcium sensitive K-casein free fraction of α-casein, termed α_s-casein, are orientated about the rod-shaped K-casein molecules under normal conditions but proteolytic enzymes cause irreversible changes in the structure of α-casein and the whole casein complex.

The action of proteolytic enzymes on casein has been studied relative to rennin (Linqvist, 1963; Alais and Jolles, 1962; Kiermeier and Kirschmeier, 1964), trypsin (Ganguli et al., 1962; Livrea et al., 1964; Fox and Kosikowsky, 1964), other non-bacterial enzymes (Derechin, 1962; Hill, 1964; Bang-Jensen et al., (1964); Huang et al., 1964) and bacterial enzymes (Sandvik, 1962; Merkel et al., 1964; Poznanski et al., 1965; McDonald and Gibbons, 1955; Knaut and Bruderer, 1965). Although

the specificities and relative activities of proteinases differ it is apparent (Linqvist, 1963; Sandvik, 1962) that the breakdown of casein by many proteinases is essentially similar and proceeds in three phases. During the primary phase non-protein nitrogen (glycomacro peptide) is removed from the K-casein which destroys the α_s-K-casein relationship. The secondary phase involves disorganisation of the micellar structure of the casein complex which results in aggregation and coagulation. This phase may occur in conjunction with the primary phase. The tertiary phase involves lysis of precipitated casein and proteinases differ in the extent and rate at which caseolysis is effected; e.g. rennin exhibits a much slower tertiary phase than trypsin.

The primary and secondary phases of casein proteolysis may be examined by the casein precipitation technique devised by Sandvik (1962). This worker showed that although mammalian and bacterial proteinases are heterogenous the casein precipitation reaction appears to be fundamental to all proteinases and enables comparison of their activities.

The tertiary phase of proteolysis by bacterial enzymes has been studied using several analytical methods. Casein breakdown and the breakdown of whole milk has been estimated in terms of tyrosine liberation or ammonia production (Greene and Jezeski, 1954; Van der Zant and Moore, 1955; Van der Zant, 1957; Peterson and Gundarson, 1960; Higginbottom and Taylor, 1962) and Poznanski et al. (1965) expressed casein degradation in terms of the accumulation of non-protein and soluble nitrogen. However, as Pollock (1962) indicated, the analysis of end-products of protein breakdown reflects little of the intermediate stages of proteolysis.

The technique of electrophoresis has been used to study the heterogeneity of casein and other milk proteins by many workers including Mellander (1939), Cherbuliez and Baudet (1950), Waugh and Hippel (1956), Wake and Baldwin (1961), Aschaffenburg (1961), Neelin *et al.* (1962), Manson (1962), Kalar and Brunner (1963), Peterson (1963) and Thompson *et al.* (1965). Little use has been made of the technique for the examination of casein degradation by bacterial proteinases. Skean and Overcast (1960) employed electrophoresis to examine the changes in skim milk proteins after the growth of three *Pseudomonas* strains but few conclusions were reached. The technique was also used by Knaut and Bruderer (1965) who briefly reported the changes produced in milk by a single *Pseudomonas* sp..

In Part I of this study it was shown that the temperature had a marked effect on the proteolytic and lipolytic activities of the test strains. The following experiments were devised to examine in some detail the effect of temperature on the abilities of the test strains to degrade casein and tributyrin and to examine differences between strains. In the first experiment the casein precipitation reaction used by Sandvik (1962) was employed to study the primary and secondary phases of caseolysis and the technique was modified to examine tributyrinase activity. In the second experiment the tertiary phase of caseolysis was examined, using polyacrylamide gel electrophoresis to study changes in the patterns of normal whole casein following the growth of strains at different temperatures.

CASEIN PRECIPITATION AND TRIBUTYRIN HYDROLYSIS

Materials and Methods

Medium: The 0.2% (w/v) tryptone medium employed in Part II was used, which was similar to the peptone medium found by Sandvik (1962) to be suitable for the elaboration of bacterial proteolytic enzymes. Alford and Pierce (1963) also showed that peptone stimulated lipase production. The medium was dispensed in 12 ml amounts in 6 x $\frac{5}{8}$ in. nephelometer tubes.

Inocula: Cultures of strains S1 - S24 were grown, standardised suspensions prepared and triplicate tubes of medium inoculated as described in Part II. The tubes of media were pre-incubated at the appropriate test temperatures prior to inoculation.

Incubation: The inoculated tubes of media were incubated at 5°, 15°, 25° and 37° as described in Part I.

Growth determinations: At intervals during incubation nephelometric measurements were made as described in Part II.

Treatment of cultures: At intervals 0.2 mm samples of culture were aseptically withdrawn and the triplicate samples pooled. To arrest growth and preserve proteinases and lipases 0.05 ml aqueous 0.1% (w/v) Merthiolate (Merck) was added to 0.5 ml of each pooled culture (Sandvik, 1962), and held for one hour at room temperature.

Preparation of test plates.

Casein agar: The test medium used by Sandvik (1962) comprised 1.4% (w/v) agar, Difco; 1.0% (w/v) sodium caseinate, as 4% (w/v)

solution pH 6.2; 0.01% (w/v) Merthiolate, Merck; 0.004 M magnesium chloride, as 10% (w/v) solution; distilled water to 100%. pH 6.2.

After preliminary trials the medium was modified and comprised 1.0% (w/v) agar, Oxoid Ionagar; 1.0% (w/v) sodium caseinate, Difco, as 4.0% (w/v) solution pH 6.2; 0.01% Merthiolate, Merck; distilled water to 100%. pH 6.2.

Sandvik (1962) did not give complete details for the preparation of casein agar and the following procedure was adopted. (a) A 4.0% (w/v) sodium caseinate solution was prepared by grinding 4 grams casein with 20 ml 0.1N sodium hydroxide solution using a mortar and finally a Griffiths tube. The volume was made up to 100 ml with distilled water and the pH adjusted to 6.2. The solution was held overnight at 4°, centrifuged at 900 g. to remove insoluble particles and heated to 50°. (b) One gram of agar was dissolved in 75 ml distilled water and cooled to 50°. (c) 25 ml sodium caseinate solution was added to 75 ml agar base, the pH was adjusted to 6.2 and 1.0 ml 1.0% Merthiolate solution added.

Tributyrin agar: The test medium comprised 1.5% (w/v) agar, Oxoid; 0.5% (w/v) peptone (Oxoid); 0.3% (w/v) yeast extract, Oxoid; 1.0% (w/v) glyceryl tributyrate; 0.01% (w/v) Merthiolate, Merck; distilled water to 100%. pH 7.4.

Test media and test plates were held at 50° prior to pouring since flocculation of casein occurred above this temperature and at less than 50° media tended to spread unevenly on the test plates. The test plates comprised 20 cm x 20 cm plate glass surfaces with 5 m m perspex sides and close fitting glass lids. Plates were placed on a

flat level surface and media poured to provide a uniform depth of 2 m m.

Inoculation of test plates: Circular wells, 6 m m in diameter, were bored in the test media using a cork borer. To each well was added 0.025 ml of the appropriate Merthiolated culture using a chromatographic pipette (Shandon, Ltd). Controls of distilled water, Merthiolate solution and tryptone medium were inoculated into wells on each test plate.

Incubation of test plates: Sandvik (1962) performed the casein precipitation reaction at 37°. However, proteinases and lipases of Pseudomonas spp. were shown by McDonald et al. (1963) to be labile above 30° and Hurley et al. (1963) found that the activity of proteolytic enzymes of Ps. fluorescens was greater at 25° than 15°. Preliminary experiments showed that incubation at 25° for 42 hours was better than 37° for 19 hours and 25° was also relevant due to the psychrophilic or mesophilic nature of the strains examined.

Measurement of zones: The diameters of zones of casein precipitation and tributyrin hydrolysis were measured using a transparent metric graticule. The method was found to give accurate and reproducible results, comparable with the more lengthy single determination by the magnification method of Sandvik (1962).

Treatment of Results

Sandvik (1962) showed that under standard test conditions proteinase present in merthiolated cultures diffuses centrifugally through the medium to produce a circular zone of precipitation, the diameter of which is proportional to the amount of enzyme present. In preliminary

experiments the direct relationship between zone diameters and two-fold dilution of the merthiolated cultures confirmed that zone diameter could be used as a measure of proteinase or tributyrinase concentration. Mean nett optical density (O.D) values were calculated for the growth of each strain at each test temperature as described in Part II, and corresponding mean values were calculated from triplicate determinations of the casein precipitation and tributyrin hydrolysis zone diameters. As described in Part II growth curves were constructed by plotting O.D. values against time for each strain at each temperature and corresponding curves were constructed by plotting the mean zone diameters for casein precipitation and tributyrin hydrolysis against time. From these curves, and as described in Part II, growth rate constants (K_g) were calculated for each strain at 5°, 15°, 25° and 37°, together with the constants which represent the rates of production of casein precipitating enzyme (K_{cp}) and tributyrinase (K_{tb}).

To depict the important relationships between growth and casein precipitation and between growth and tributyrinase hydrolysis and to show the effect of temperature on these relationships curves were constructed by plotting the mean zone diameters for casein precipitation and tributyrin hydrolysis against the corresponding O.D values.

Results and Discussion

Preliminary experiments.

From the results of preliminary experiments the casein test medium and the test temperature as used by Sandvik (1962) were modified.

Test temperature: A comparison of the casein precipitation reactions at 5°, 15°, 25° and 37° of strains S3, S6, S9, S10, S12, S18, S20 and S21 grown at 20° showed that precipitation was often non-specific at 5° and 15°. Opacity of the medium interfered with precipitation at 37°. Incubation at 25° for 42 hours was selected for routine use.

Test medium: Sandvik (1962) found that magnesium chloride stimulated certain proteinases and was required to stabilise media containing certain types of agar. Difco agar, No.3 agar (Oxoid) and Ionagar (Oxoid) were compared and 1.0% (w/v) Ionagar without magnesium chloride resulted in a clearer medium which developed little opacity but allowed the production of distinct zones of casein precipitation during tests.

Main experiment.

The K values for growth (K_g), casein precipitation (K_{cp}) and tributyrin hydrolysis (K_{tb}) are shown in Table 11, and the relationships between casein precipitation and growth and tributyrin hydrolysis and growth are shown in Figures 2. 1 to 2. 9.

Strains S2, S5, S10, S11, S13, S14, S15, S16, S18, S21 and S22 did not precipitate casein or hydrolyse tributyrin at 5°, 15°, 25° or 37°.

Strain S1

The maximum growth rate of this psychrophilic Pseudomonas strain was shown at 25° (K_g = 42.8) although the maximum cell crop was greatest at 15°. The highest rates of production of casein precipitating enzyme (C-P enzyme) and tributyrinase were displayed at 15° (K_{cp} = 5.3) and at 25° (K_{tb} = 0.5) respectively.

Table 11 Growth and enzyme rate constants at 5°, 15°, 25° and 37°.

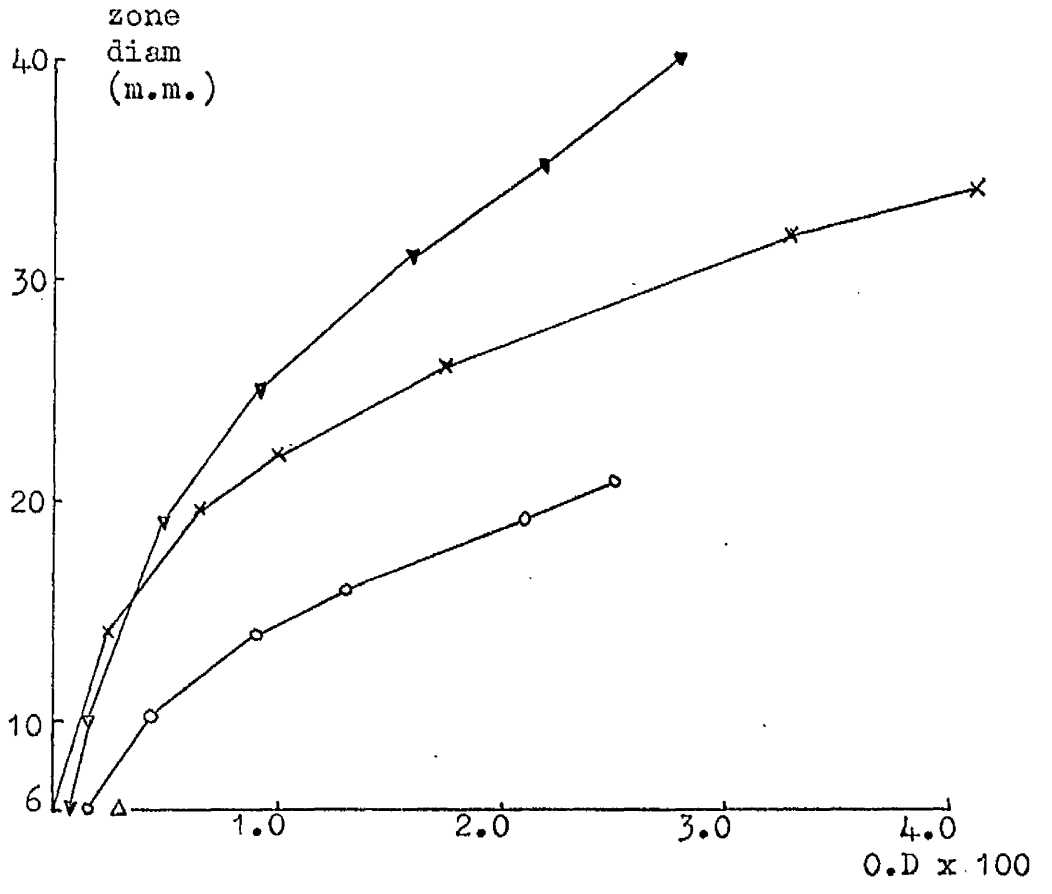
Strain		S1	S3	S4	S6	S7	S9	S12	S17	S19	S20	S23	S24
Temp. °C.	K_g	18.0	10.5	12.8	7.0	11.0	12.7	0.1	13.5	19.7	15.8	0.6	7.6
5°	K_{cp}	3.05	2.5	4.6	0	0.37	0.55	0	0.1	2.5	0	0	0
	K_{tb}	0.35	0.69	0.65	0	0.2	0.45	0	0	0.5	0.15	0	0
	K_g	36.6	28.5	39.5	24.3	26.0	31.0	34.0	68.0	36	58.5	26.2	25.0
15°	K_{cp}	5.3	3.9	5.8	0	0.42	2.2	0	1.03	2.9	2.2	0.7	0.18
	K_{tb}	0.25	1.13	0.5	0	0.26	0.7	0.62	0	0.7	1.05	0.38	0.35
	K_g	42.8	30.0	42	21.7	34.6	35.5	35.0	40.5	42.0	28.8	26.3	21.0
25°	K_{cp}	3.55	2.0	4.2	1.23	0.68	1.3	0.9	1.5	3.5	1.7	0.72	0.95
	K_{tb}	0.5	0.55	0.55	0	0.13	1.0	0.8	0	1.2	1.9	0.75	0
	K_g	6.5	12.0	8.5	17.0	11.0	11.0	104	8.2	47.5	0	66.0	11.0
37°	K_{cp}	0	0	0	0	0.2	0	2.7	0	0	0	1.3	0
	K_{tb}	0	0	0	0	0	0	1.25	0	0	0	1.35	0

Key: K_g = growth rate constant
K_{cp} = enzyme rate constant (casein precipitation)
K_{tb} = enzyme rate constant (tributyrin hydrolysis)

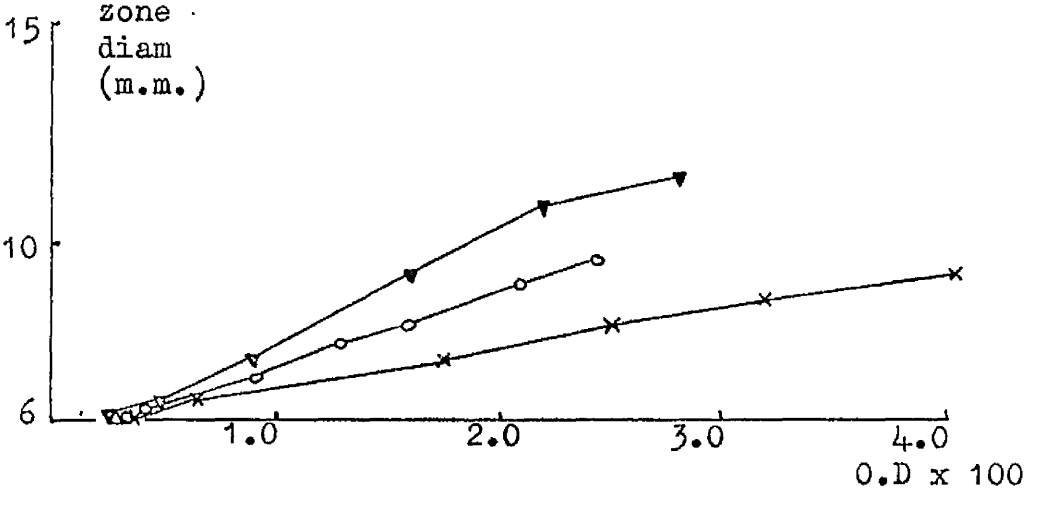
Fig. 2 .1 Growth and exo-enzyme production

Strain S.1

(a) Casein precipitation



(b) Tributyrin hydrolysis



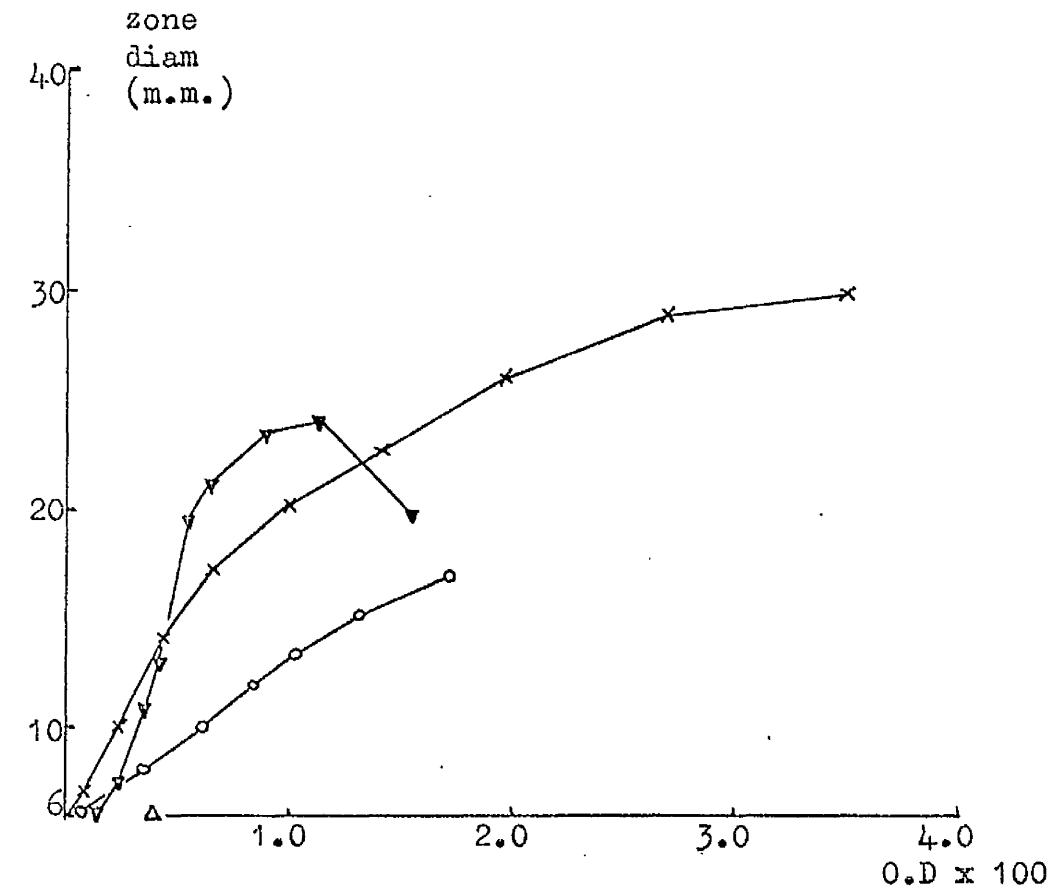
Key:

5°	▼
15°	X
25°	o
37°	Δ

Fig. 2 .2 Growth and exo-enzyme production

Strain S.3

(a) Casein precipitation



(b) Tributyrin hydrolysis

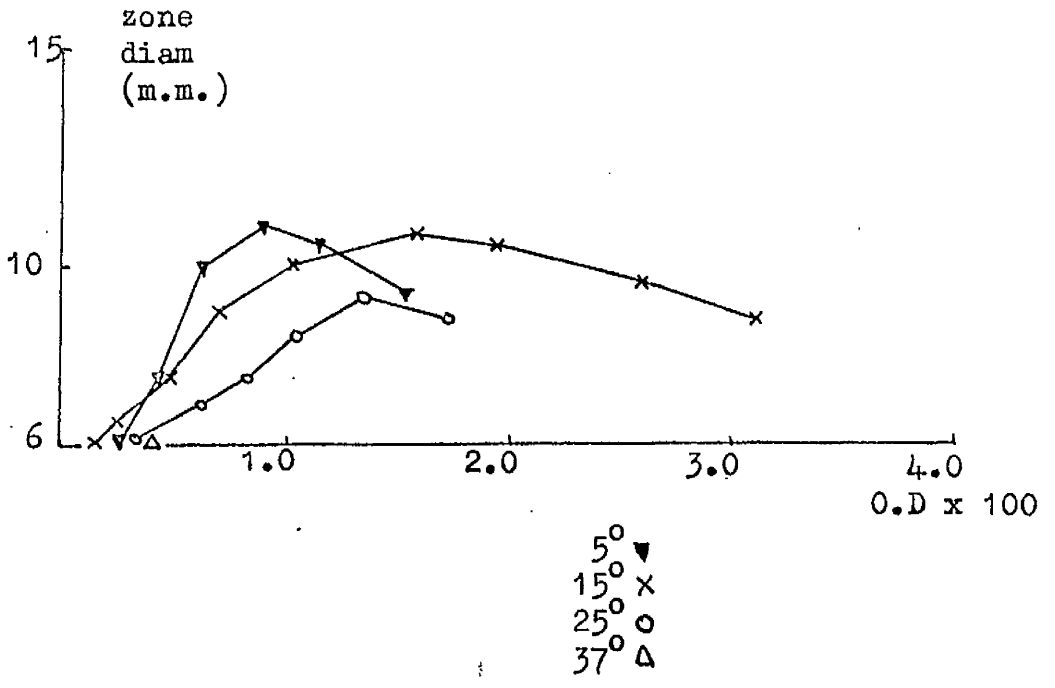
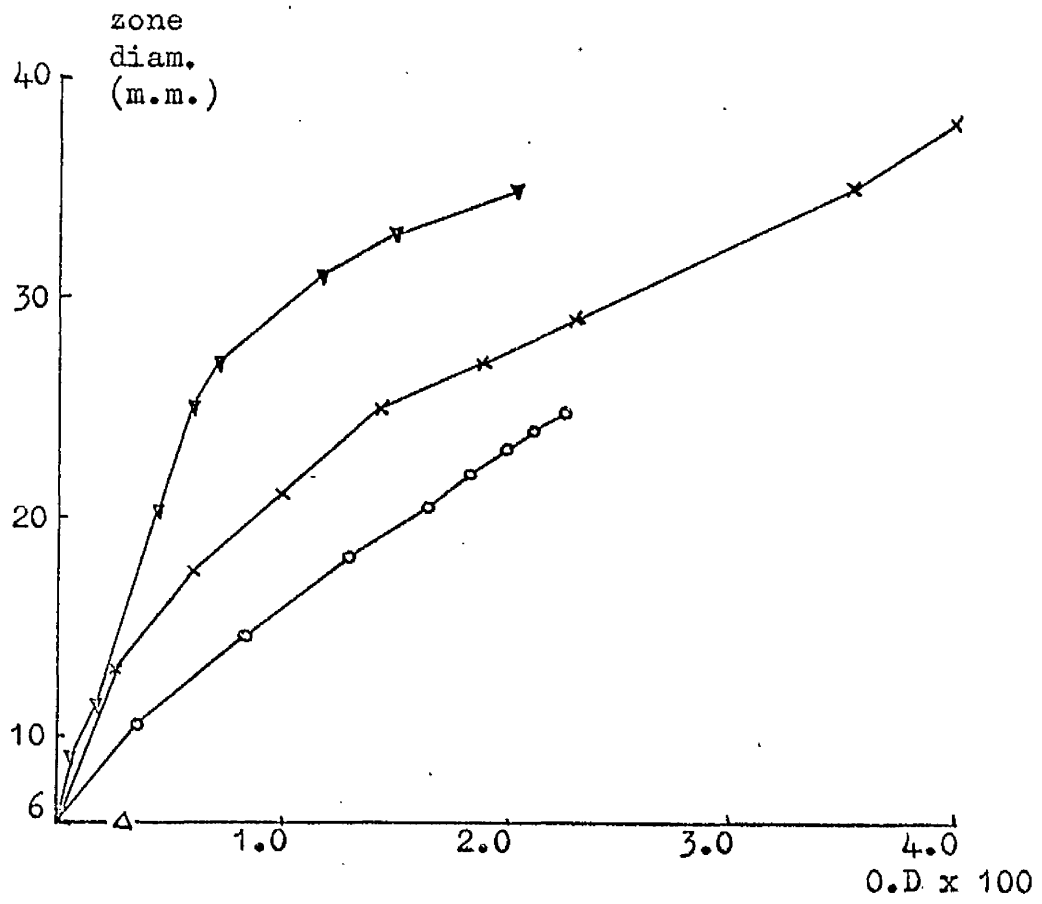


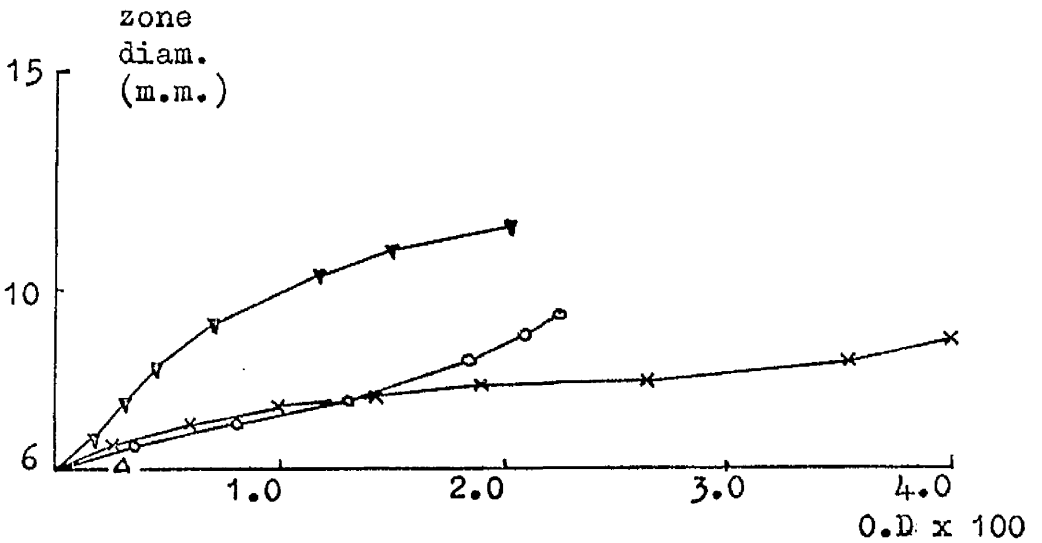
Fig. 2 .3 Growth and exo-enzyme production

Strain S.4

(a) Casein precipitation



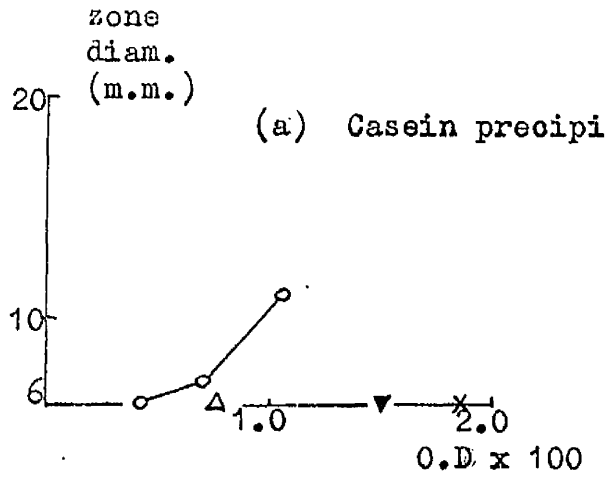
(b) Tributyrin hydrolysis



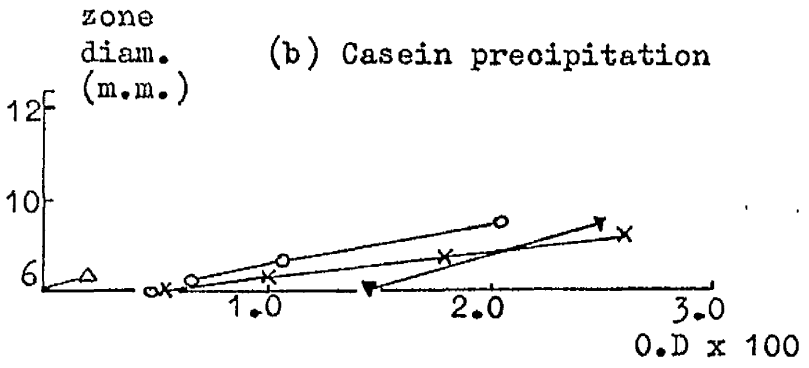
5° ▼
15° x
25° o
37° Δ

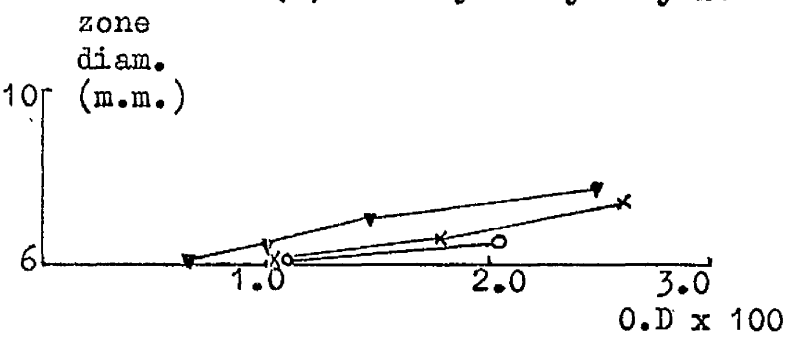
Fig. 2 .4 Growth and exo-enzyme production

Strain S.6



Strain S.7



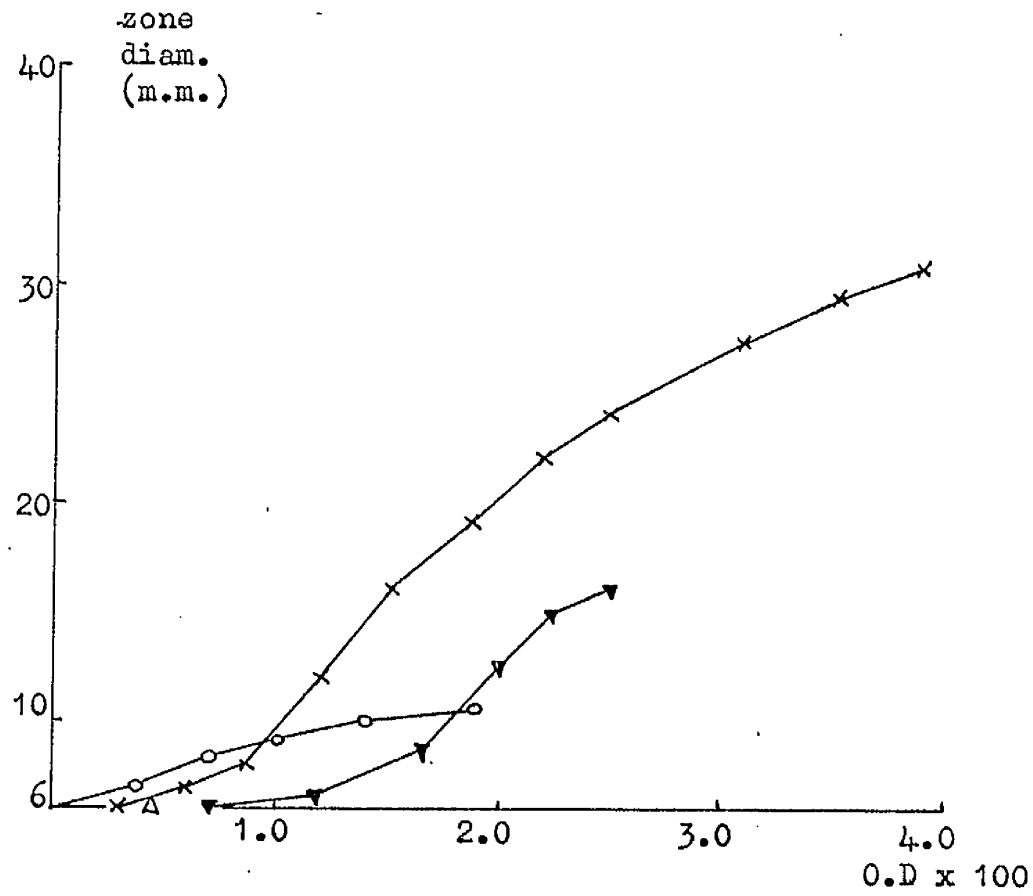


5° ▼
15° ×
25° o
37° Δ

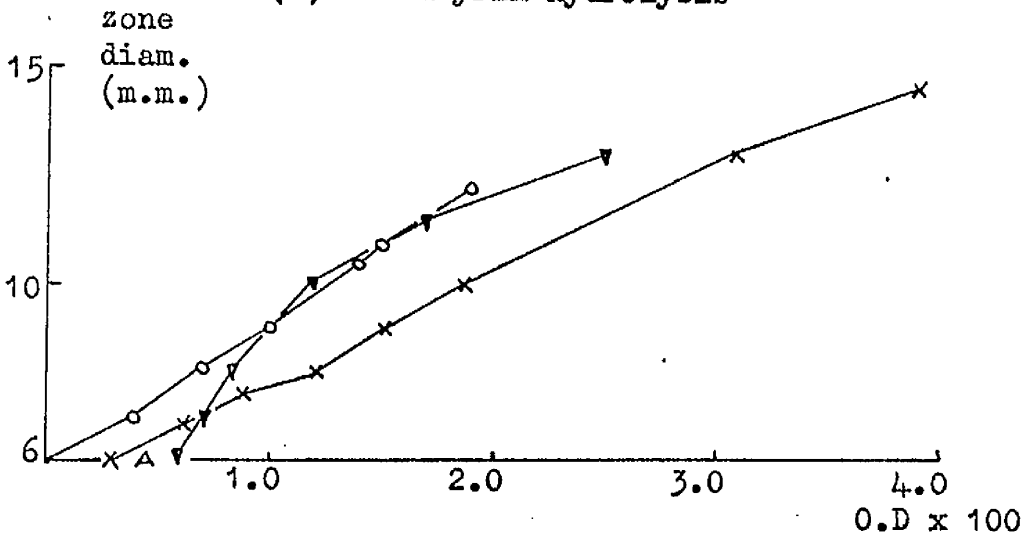
Fig. 2 .5 Growth and exo-enzyme production

Strain S.9

(a) Casein precipitation



(b) Tributyrin hydrolysis

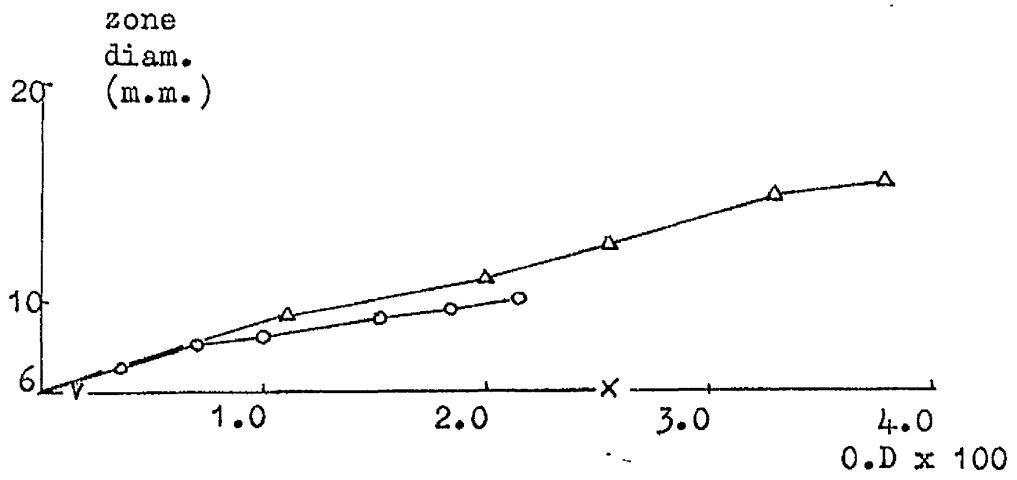


5° ▼
15° ×
25° o
37° Δ

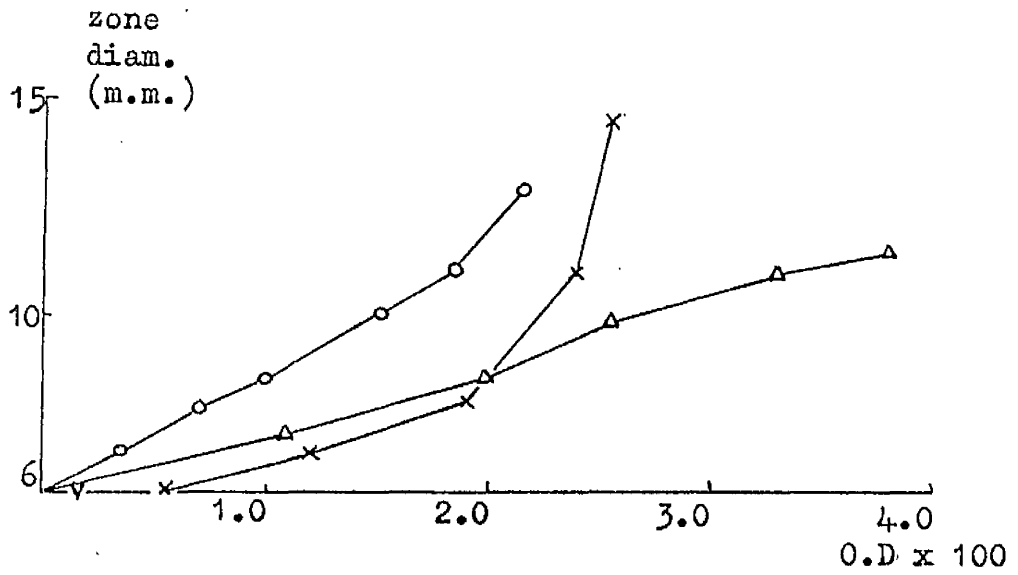
Fig. 2.6 Growth and exo-enzyme production

Strain S.12

(a) Casein precipitation



(b) Tributyrin hydrolysis

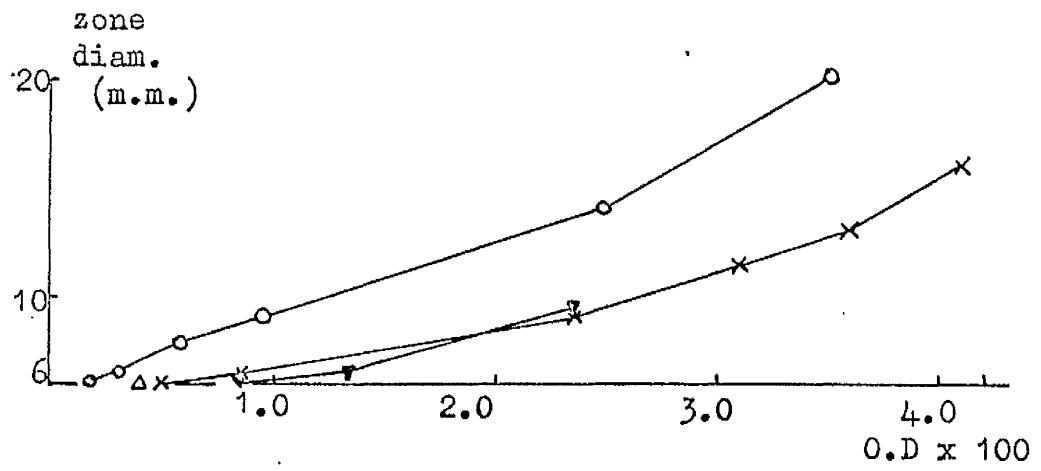


5° ▼
15° X
25° o
37° Δ

Fig. 2 .7 Growth and exo-enzyme production

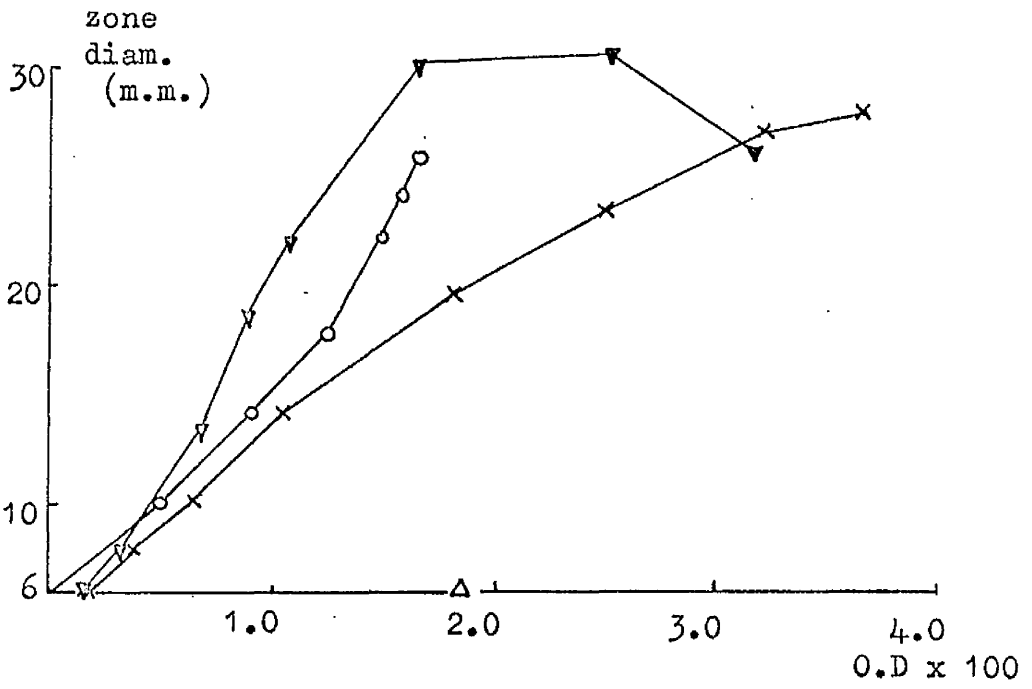
Strain S.17

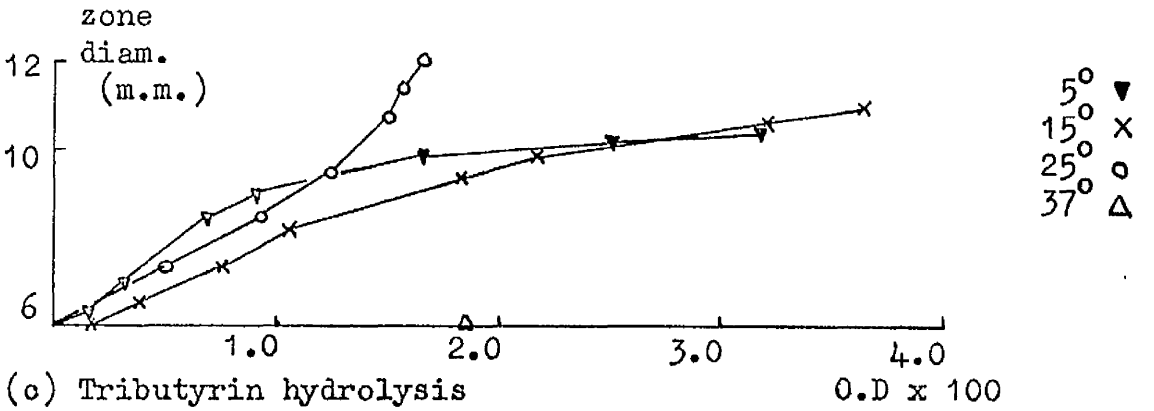
(a) Casein precipitation



Strain S19

(b) Casein precipitation



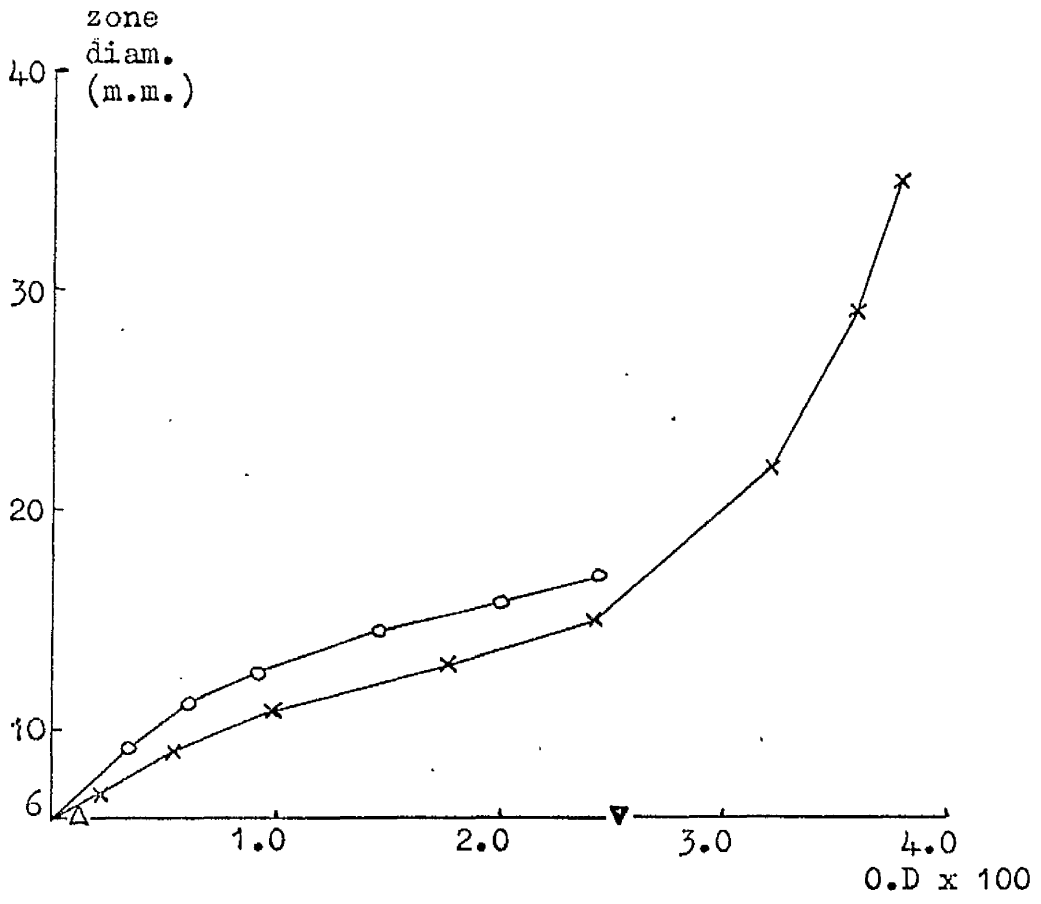


(c) Tributyrin hydrolysis

Fig. 2.8 Growth and exo-enzyme production

Strain S20

(a) Casein precipitation



(b) Tributyrin hydrolysis

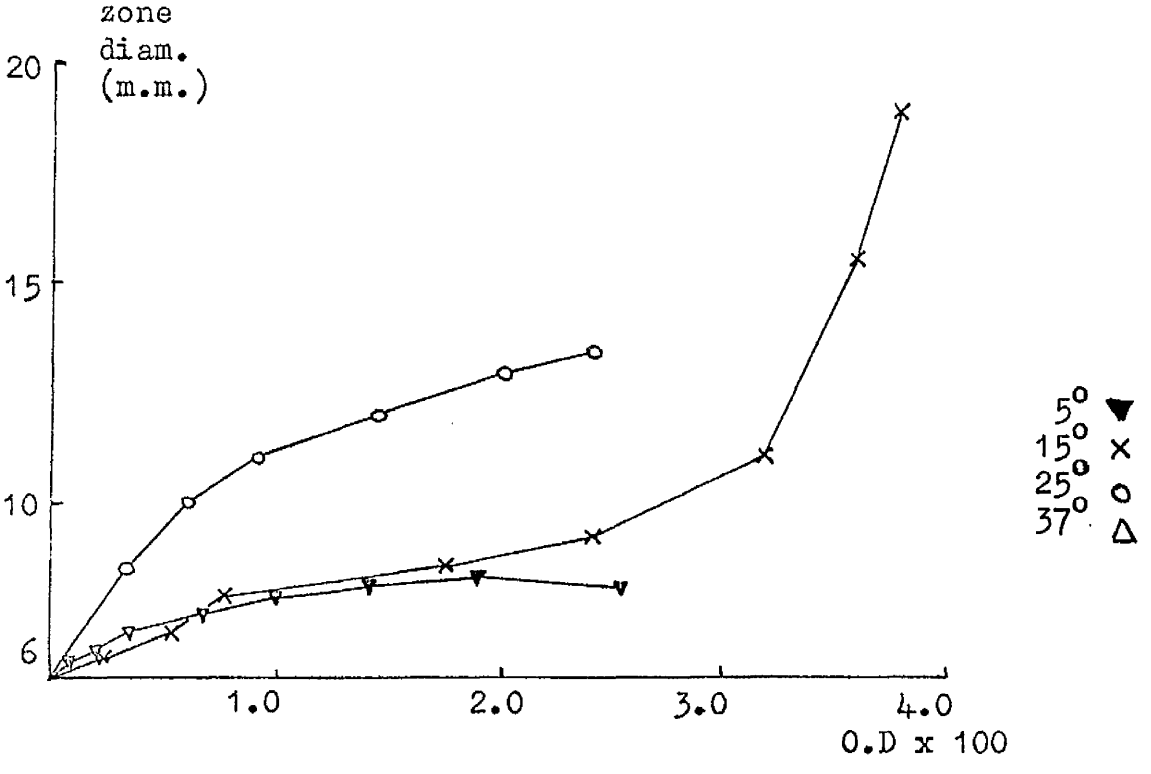
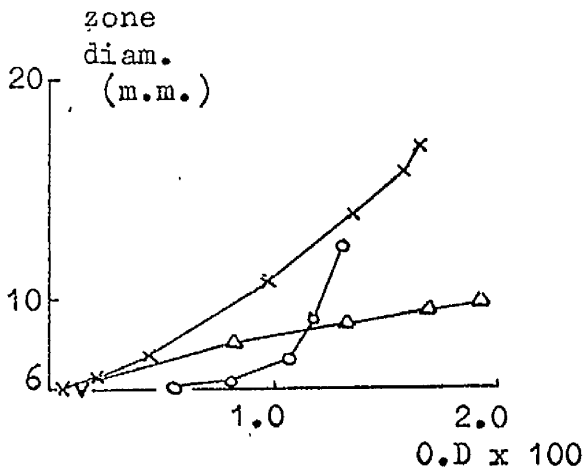


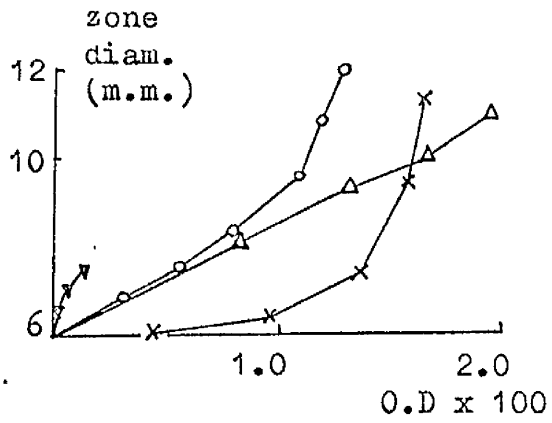
Fig. 2.9 Growth and exo-enzyme production

(a) Casein precipitation

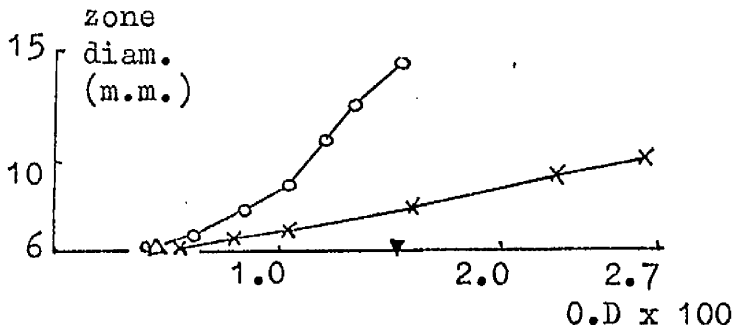


Strain S.23

(b) Tributyrin hydrolysis

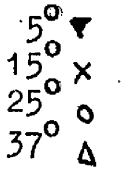


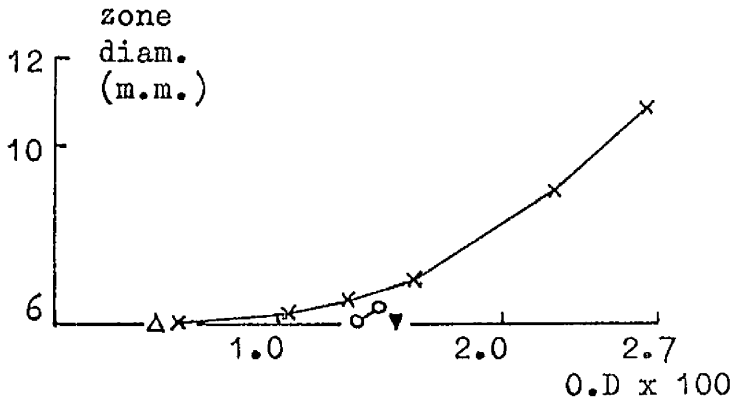
(c) Casein precipitation



Strain S.24

(d) Tributyrin hydrolysis





The production of C-P enzyme was similar during the early stages of growth at 5° and 15° but as growth increased the production of enzyme was appreciably higher at 5° than at 15°. For the same levels of growth at 25° the casein precipitation reaction (C-P reaction) was less than at 5° or 15° although the pattern of C-P enzyme production during growth was similar at 5°, 15° and 25°. The enzyme was produced more rapidly at low levels of growth than at higher levels of growth (Figure2.1a).

The production of tributyrinase was initially delayed during growth at 5°, 15° and 25° although at higher levels of growth tributyrinase production was greatest at 5°, less at 25° and least at 15° (Figure2.1b) Growth was poor at 37° and the C-P reaction and tributyrinase activity were negative.

Strain S3

The rates of growth of this psychrophilic Pseudomonas strain were similar at 15° (K_g = 28.5) and 25° (K_g = 30) although the maximum cell crop was highest at 15°. The rates of production of C-P enzyme (K_{cp} = 3.9) and tributyrinase (K_{tb} = 1.13) were highest at 15°. Despite an initially high rate of growth at 37° (K_g = 12) the maximum cell crop was low and C-P enzyme and tributyrinase were not observed at this temperature. The development of C-P enzyme production during growth at 15° and 25° followed a pattern similar to that shown by strain S1. During growth at 5° an initial delay preceded a sharp increase in the production of C-P enzyme which was followed by a marked decline in production of the enzyme. At the same levels of growth C-P enzyme production was less at 25° than at 5° or 15° (Figure2.2a).

After initial delays in the production of tributyrinase at 5°, 15° and 25° the enzyme levels increased sharply and then declined on further increase in growth. Tributyrinase production was reduced throughout growth at 25° compared with values at 5° and 15° (Figure2.2b).

Strain S4

The rates of growth of this psychrophilic Pseudomonas strain and the patterns of enzyme production were comparable to those of strain S1. The growth rates of strain S4 were similar at 15° (K_g = 39.5) and 25° (K_g = 42) and the maximum cell crop was highest at 15°. The highest rates of production of C-P enzyme and tributyrinase were displayed at 15° (K_{cp} = 5.8) and 5° (K_{tb} = 0.65) respectively.

The production of C-P enzyme was rapid at low levels of growth at 5° but as growth increased enzyme production levelled off. For similar growth at 15° and 25° less C-P enzyme was produced than at 5° and, as observed for strain S3, an almost linear relationship existed between growth and the production of C-P enzyme. (Figure2.3a).

As for strain S1 the production of tributyrinase at all stages of growth was higher at 5° than at 15° or 25°, despite the much higher maximum cell crop at 15° (Figure2.3b).

Growth was poor at 37° and the C-P reaction and tributyrinase activity were negative.

Strain S6

This psychrophilic Pseudomonas strain exhibited a maximum rate of growth (K_g = 24.3) and the highest maximum cell crop at 15° but the C-P reaction was displayed only at 25° (K_{cp} = 1.23). An initial delay preceded an appreciable increase in the production of this enzyme

towards the end of the logarithmic phase of growth (Figure2.4a). Tributyrinase activity was not detected at 5°, 15°, 25° or 37°.

Strain S7

The maximum rate of growth of this psychrophilic Aeromonas strain was shown at 25° (K_g = 34.6) although the maximum cell crop was highest at 15°. The rates of C-P enzyme and tributyrinase production were greatest at 25° (K_{cp} = 0.68) and 15° (0.26) respectively although the final levels of both enzymes were low at all temperatures.

Marked delays were apparent in the production of C-P enzyme and tributyrinase during growth at all temperatures (Figure2.4b,c).

Strain S9

The rate of growth of this psychrophilic Pseudomonas strain was highest at 25° (K_g = 35) and the maximum cell crop was highest at 15°. Maximum rates of C-P enzyme and tributyrinase production were displayed at 15° (K_{cp} = 2.2) and 25° (K_{tb} = 1.0) respectively.

After an initial delay the production of C-P enzyme increased markedly during growth at 15°. Enzyme production was delayed to a greater extent during initial growth at 5°, although the production of C-P enzyme increased rapidly towards the end of the logarithmic phase of growth. During early growth the production of C-P enzyme was comparatively greater at 25° than at 5° or 15° but the level of enzyme did not increase appreciably during later growth at 25° (Figure2.5a).

The production of tributyrinase was also appreciably delayed during early growth at 5° after which the production of this enzyme was similar during growth at 5° and 25°. Enzyme production at 15° was lower than at 5° and 25° at the same levels of growth although more tributyrinase

was eventually produced at 15° (Figure2.5b).

Growth was poor and the C-P reaction and tributyrin hydrolysis were not observed at 37°.

Strain S12

The highest rate of growth (K_g = 10.4) and maximum cell crop of this Ps. aeruginosa strain were shown at 37°. The rates of C-P enzyme (K_{cp} = 2.7) and tributyrinase (K_{tb} = 1.25) production were also highest at 37°.

The relationships between growth and the production of C-P enzyme were similar at 25° and 37° but this enzyme was not produced at 15° despite appreciable growth. (Figure2.6a).

Relative to growth tributyrinase production was greater at 25° than at 37°. A linear relationship was displayed between the production of tributyrinase and growth at 25° and 37° but at 15° an initial delay in the production of enzyme was followed by a marked increase in production towards the end of the logarithmic phase of growth (Figure2.6b).

Growth was poor and no enzyme reactions were exhibited at 5°.

Strain S17

The rate of growth (K_g = 68) and the maximum cell crop of this Pseudomonas strain were highest at 15°, although the rate of C-P enzyme (K_{cp}) = 1.5) production was highest at 25°.

The production of C-P enzyme was delayed during appreciable growth at 5° and 15° and more enzyme was produced at 25° at all levels of growth than at 5° or 15°. (Figure2.7a).

Tributyrinase was not detected at any temperature.

Strain S19

This Aeromonas strain showed the highest rate of growth (K_g = 47.5)

at 37° and the highest maximum cell crop at 15°. The rates of production of C-P enzyme (K_{cp} = 3.5) and tributyrinase (K_{tb} = 1.2) were displayed at 25°.

The development of C-P enzyme production was similar during early growth at 5°, 15° and 25°. Subsequently enzyme production increased slightly with increase in growth at 25°, while at 15° a linear relationship was maintained between the production of enzyme and growth. At 5° a slight initial lag in the production of C-P enzyme was followed by a rapid increase during the logarithmic phase of growth, after which enzyme production became static and then declined towards the maximum cell crop. (Figure2.7b).

The production of tributyrinase was also similar during the early stages of growth at 5°, 15° and 25°. At higher levels of growth enzyme production became static at 5° and 15° while at 25°, as observed for the production of C-P enzyme, tributyrinase production increased sharply (Figure2.7c).

Despite appreciable growth at 37° neither enzyme activity was displayed.

Strain S20

The growth rate (K_g = 58.5) and the maximum cell crop of this psychrophilic Pseudomonas strain were highest at 15°. The rates of C-P enzyme (K_{cp} = 2.2) and of tributyrinase (K_{tb} = 1.9) production were greatest at 15° and 25° respectively.

The relationship between the production of C-P enzyme and growth were similar at 15° and 25° but at the higher levels of growth subsequently attained at 15° there was a sharp increase in the

production of this enzyme. Despite heavy growth at 37° the C-P reaction was not shown. (Figure2.8a).

During the early stages of growth at 25° the production of tributyrinase was appreciable, although with increase in growth enzyme production tended to stabilise. Throughout growth at 5° and at the same levels of growth at 15° tributyrinase production was poor. Near the maximum cell crop the production of tributyrinase tended to stabilise at 5° while at 15° the level of tributyrinase increased sharply as observed during the production of C-P enzyme at this temperature (Figure2.8b).

Strain S23

This Pseudomonas strain displayed the highest rate of growth (K_g = 66), the highest maximum cell crop, and the highest rates of production of C-P enzyme (K_{cp} = 1.3) and tributyrinase (K_{tb} = 1.35) at 37°.

The production of C-P enzyme was highest throughout growth at 15° and at higher levels of growth, enzyme production at 37° was poor. An appreciable delay in the production of C-P enzyme during early growth at 25° was followed by a rapid increase in the amounts of enzyme produced towards the end of the logarithmic phase of growth. (Figure2.9a).

Tributyrinase production was similar during the early stages of growth at 25° and 37°. At higher levels of growth the relationship between enzyme production and growth at 37° was linear, whereas towards the end of the logarithmic phase of growth at 25° the production of enzyme increased sharply. The production of tributyrinase at 15° was delayed until the end of the logarithmic phase of growth when the levels

enzyme rapidly increased. (Figure2.9b).

Growth was poor, C-P enzyme was not detected and only a slight tributyrinase reaction was obtained at 5°.

Strain S24

The rate of growth (K_g = 25), the maximum cell crop and the rate of tributyrinase production (K_{tb} = 0.35) were highest at 15° while the rate of C-P enzyme production was highest at 25°.

Delays in the production of C-P enzyme were shown at 15° and 25° and despite heavier growth at 15° the final level of enzyme was greater at 25°. The C-P reaction was not observed at 5° or 37° although growth was appreciable at 5°. (Figure2.9c).

Tributyrin hydrolysis was poor at 25° and absent at 5° and 37° despite good growth at both 5° and 25°. Tributyrinase production was initially delayed during growth at 15° but increased markedly towards the end of the logarithmic phase of growth.

The results show that major differences exist in the relationships between growth of the test strains and the production of C-P enzyme and tributyrinase. Strains differed not only with respect to the extent of enzyme production but also in their patterns of enzyme production during growth. Temperature was found to exert a profound effect on the production of enzymes by individual strains.

The development of proteolysis was shown by Greene and Jezeski (1954) and Van der Zant (1957) to occur towards the end of the logarithmic phase of growth and Hurley et al. (1963) found that a proteolytic enzyme

of Ps. fluorescens was liberated in quantity only when the cell population had reached a maximum. However, Van der Zant and Moore (1955) observed much variation in the relationships between proteinase liberation and the growth of psychrophilic bacteria. Alford and Elliot (1960) and Alford and Pierce (1963) reported that the production of lipase was not directly related to the number of cells although Lawrence (1967) maintained that for certain bacteria a linear relationship existed between lipase production and cell numbers.

In the present study strains S6, S9, S17, S19, S20, S23 and S24 displayed initial delays in the production of C-P enzyme at one or more temperatures with marked increases in enzyme production during later stages of growth. This relationship was also exhibited by strains S4, S9, S12, S19, S20, S23 and S24 for the production of tributyrinase. This pattern conforms to that observed by Gorbach and Pirch (1936) whereby exo-enzyme is liberated from the autolysed cell after the period of logarithmic growth. Pollock (1962) did not consider that such enzymes were truly extracellular and he indicated that to be described as extracellular enzymes should be produced during the early stages of growth and their pattern of production should follow the growth pattern of the strain. These requirements are fulfilled by strains S1, S3, S4, S9, S12, S17, S19 and S23 which, particularly at 25^{0}, displayed a linear relationship between enzyme production and growth. Strains S1, S3, S4, S19 and S20 also produced rapid increases in the production of enzyme during early growth at one or more temperature with the tendency for enzyme production to stabilise at higher levels of growth. Strains S3 and S19 produced similar patterns

of enzyme production except that the levels of enzyme substantially declined at higher levels of growth. The reduction or cessation of enzyme production when autolysis of the cells commences at the end of the logarithmic phase of growth suggests the operation of an extracellular exo-enzyme. This pattern of enzyme production does not conform to the theory suggested by Rodgers (1961) who considered that the steady production of proteinase may be due to the controlled autolysis of cells throughout the growth cycle. In this event massive autolysis of cells late in the growth cycle would liberate much enzyme. Strain S20 did, however, display this pattern during the production of C-P enzyme and tributyrinase at 15° but not at 5° or 25°.

The effect of temperature on the relationships between enzyme production and growth of the test strains was not simple, as was indicated by Greene and Jezeski (1954) and Peterson and Gundarson (1960). The latter workers stated that the elaboration of an extracellular proteinase of Ps. fluorescens was inversely proportional to the temperature over the range 0° to 30°. By contrast Van der Zant and Moore (1955) reported considerable variation in the production of proteinase by 4 psychrophiles and could determine no definite relationship between enzyme production and growth at different temperatures.

In the present experiment psychrophilic strains S1, S3, S4 and S9 produced higher levels of C-P enzyme during growth at 5° and 15° than at 25° and 37° but psychrophilic strains S7, S17 and S24 produced more C-P enzyme throughout growth at 25° than at 5° or 15°. Intermediate strain S6 displayed the C-P reaction only at 25° despite good growth at 5° and 15°, while strain S19 produced C-P enzyme at 5°, 15° and 25°

but not at 37°. Mesophilic strain S12 exhibited maximum C-P enzyme production at 37° and no enzyme was formed at 15° despite good growth while mesophilic strain S23 produced more C-P enzyme during growth at 15° than at 25° or 37°.

The production of tributyrinase by psychrophilic strains S1, S3, S4, and S7 was greatest at 5° and by strain S24 at 15°. Psychrophilic strain S9 and intermediate strain S19 produced similar levels of tributyrinase during growth at 5° and 25°. Mesophilic strains S12 and S23 together with psychrophilic strain S23 produced the most tributyrinase at 25° although the enzyme was produced in large quantities at high levels of growth at 5° or 15°.

The results have revealed the appreciable differences in the abilities of the strains to produce proteinase and tributyrinase during growth and particularly, temperature has been shown to have a significant differential effect on the elaboration of these enzymes.

ELECTROPHORETIC STUDY OF CASEIN BREAKDOWN

The following experiment was devised to examine the changes which occur in the polyacrylamide gel electrophoretic patterns of casein as a result of the growth and proteolytic activities of selected test strains at 5°, 15°, 25° and 37°.

Materials and Methods

Casein medium: On the basis of the results of a preliminary examination of four commercial casein samples (Caseins A, B, C and D) and of two samples prepared from fresh skim milk (Caseins E and F) Isoelectric casein D (Difco) was selected for use. Pope and Skerman mineral salts basal medium (Skerman, 1959) containing 0.05% (w/v) L-glutamic acid was prepared in 500 ml amounts and sterilised by autoclaving. Sodium caseinate was prepared by grinding 1.0 gm casein with 5 ml 0.1N sodium hydroxide solution in a mortar and adding a further 15 ml 0.1N sodium hydroxide solution gradually to dissolve the casein. Sodium caseinate solution was aseptically added to the basal medium. The complete medium was adjusted to pH 6.2 and aseptically dispensed in 50 ml amounts in 250 ml conical (Monax) flasks. The flasks of media were held in a water bath at 65° for 30 minutes on each of three successive days and afterwards incubated at 30° for three days and at the intended test temperature prior to inoculation to allow the growth of possible contaminants. Sterility checks of all flasks of media were made prior to inoculation and suspect media

was rejected.

Milk medium: Purple milk medium used in Part I was inoculated in parallel with the casein medium.

Inocula: Following preliminary experiments and from the results of Part I, strains S3, S4, S9, S10, S12, S18, S20 and S23 were selected for the main experiment. Strains were grown on nutrient agar (Oxoid) at 20° for 24 hours, standard suspensions of cells were prepared as described in Part II, and 0.1 ml inocula were transferred to flasks of casein medium and tubes of milk medium. This level of inoculation provided a $\log_{10}$ viable count per ml of between 2.48 and 2.86.

Incubation: Inoculated media were incubated at 5°, 15°, 25° and 37° as described in Part I.

Growth determinations: Less than 15 minutes after inoculation and at intervals during incubation 0.5 ml samples of culture were withdrawn from the casein medium and the viable count was determined by the surface plating technique described in Part I.

Changes in milk medium: At intervals the changes produced in Purple milk were recorded as described in Part I.

Rennin and trypsin activities: Preliminary experiments were performed to study changes in the electrophoretic pattern of casein due to the action of rennin and trypsin. To 10 ml amounts of casein medium was added 2 ml 0.004% (w/v) rennin or trypsin solution. At intervals during incubation at 37° 2.5 ml amounts were withdrawn and 0.5 ml 4 M urea added to quench the enzyme-casein reaction. Samples were then examined by electrophoresis.

Electrophoretic techniques.

Polyacrylamide gels are easier to prepare and handle than starch gels, are transparent and have the advantage of adjustable pore size depending on the concentration of the monomer. The absence of carboxyl linkages, as obtained in starch gels has been found to give better resolution of protein fractions (Peterson, 1963). The incorporation of urea was shown by Libbey and Ashworth (1961), Kolar and Brunner (1963) and Thompson et al. (1965) to facilitate the resolution of casein fractions.

Preparation of polyacrylamide gel: The gel buffer comprised 4.59 gm Tris (hydroxy methyl amino-methane), 0.53 gm citric acid, 240 gm urea (4M), 1,000 ml glass distilled water. To 7 gm Cyanogum 41 (British Drug Houses, Ltd) in a 500 ml conical flask was added 93 ml gel buffer, 1 ml 10% (w/v) ammonium persulphate solution and 1 ml β-dimethyl-amino propionitrile. Solutions of the two catalysts were held at 4 - 5° when not in use. Dissolved air was quickly removed from the mixture by evacuation which was then gently poured into a mould taking care to exclude air bubbles throughout. The mould comprised a plate glass surface with $\frac{1}{4}$ inch square perspex side formers held by vaseline. A perspex top plate with 4, 5 or 6 slit formers ($\frac{3}{4}$ in. x $\frac{1}{4}$ in. x 1 m m). The gel measured from 4 to 6 in. wide and 7 in. long. After one hour at room temperature the top plate was removed, leaving the slits ready to receive samples. It was found that separate side formers were preferable to fixed sides. In plates with fixed sides the slight normal expansion of the gel during electrophoresis resulted in loss of adhesion of the gel and compression of the samples slits.

Apparatus: A modification of Smithies (1959) starch gel electrophoresis chamber was constructed from 'Perspex' with coiled platinum electrodes. The plate holding the prepared gel was placed on the bed of the chamber and gel-tank buffer contact was effected by surgical lint (B.P.) soaked in tank buffer. The tank buffer (pH 8.6) comprised 18.5 gm boric acid; 2.0 gm sodium hydroxide; 1,000 ml glass distilled water.

Electrophoresis was performed at a constant voltage of approximately 10 volts per cm using a Vokam (Shandon, Ltd) stabilised supply unit. Each run was continued until the buffer front, indicated by a brown line, was 10 cm from the point of application.

Preparation and application of sample: At intervals during incubation 0.5 ml samples of casein medium culture were taken aseptically and to 0.1 ml 4M urea was added to each to arrest growth and enzyme activity. 0.15 ml of sample was applied to each sample slit and the levels were topped with tank buffer. Samples of casein medium and enzyme-casein mixtures were applied similarly. Casein medium, suspensions of test strains and solutions of rennin and trypsin were run as controls. A thin polythene sheet was laid over the gel surface to prevent evaporation and care was taken to eliminate air bubbles.

Development of gels: In preliminary experiments the gels were dyed in 10% (w/v) Amido Black 10B in 10% (v/v) acetic acid solution for 18 hours but subsequent difficulty in clearing lead to the routine use of 5% (w/v) Amido Black 10B in 10% (v/v) acetic acid solution for 18 hours. No difference was detected in the densities of bands stained by these two methods. Clearing was performed by replacing the gel in the electrophoresis chamber and applying a constant current of 20 milliamps

for 12 - 16 hours using 5% (v/v) acetic acid solution in place of tank buffer. This method enabled a more rapid and uniform clearing of the gel than obtained by serial washing in acetic acid solution.

Gel scanning and photography: An Eel densiometric scanner (Evans Electroselenium, Ltd) was used to record the characteristics of the casein fractions and their breakdown products in stained and cleared gels. Sections of gels were cut, using formers and a cutting wire. A 'Perspex' gel holder was constructed measuring 170 x 50 x 4 mm with one long side open. The centre gel section was fitted into the holder and 10% (v/v) glycerol solution added. This technique eliminated air bubbles and rendered the gel background completely transparent in contrast with the stained bands. Gels were photographed using a 35 mm S.L.R camera (Wray, Ltd) and Pan F. Film. (Ilford, Ltd).

Treatment of Results

Growth in casein medium: Mean viable counts were calculated from triplicate determinations and $\log_{10}$ values were derived.

Changes in milk medium: The changes accompanying growth in milk medium were evaluated as described in Part I.

Electrophoretic patterns: The densiometric measurements of each gel separation were plotted against the gel length and expressed graphically as continuous scans. On completion of the experiment, peaks were numbered according to their position relative to α_s and β-casein fractions on the basis of a standard electrophoretic run of 10 cm.

Results and Discussion

The growth of strains S3, S4, S9, S10, S12, S18, S20 and S23 in casein medium at 5°, 15°, 25° and 37° and the corresponding changes produced in milk medium are shown in Table 12.

Electrophoretic patterns of casein and changes in the patterns of casein accompanying the action of rennin and trypsin and accompanying the growth of strains S3, S4, S9, S10, S12, S18, S20 and S23 at 5°, 15°, 25° and 37° are shown in Figures 3. 1 - 3. 13.

Preliminary experiments.

Composition of casein medium: Fat and vitamin-free casein A (British Drug Houses, Ltd) Soluble casein B (Hopkins and Williams, Ltd) and Technical casein C (Thomson, Skinner and Hamilton, Ltd) and caseins E and F, prepared from skim milk were also examined. Caseins A, B and C were incompletely soluble, contained impurities and tended to precipitate from the final medium on standing. Caseins D, E and F gave normal whole casein electrophoretic patterns while caseins A. B and C gave incomplete resolution. Caseins E and F were difficult to dissolve by the standard method and since it was difficult to ensure a supply of standard skim milk over a period Isoelectric casein D (Difco) was selected for routine use. To enable the early establishment of growth in casein-mineral salts medium 0.05% (w/v) L-glutamic acid was added. Several methods of sterilisation of the complete medium were examined. Autoclaving at 105° for 10 minutes and heating in a boiling water bath for 5 minutes caused precipitation of the casein and Seitz filtration removed much of the casein. Routinely a combination of techniques was

adopted involving strict aseptic preparation, autoclaving of the basal medium and heating of the final medium at 65° for 30 minutes on three successive days.

Polyacrylamide gel: Libbey and Ashworth (1961) and Payens (1961) showed that the level of urea in buffer affects the heterogeneity and relative rate of migration of K-casein in whole casein. At a level of 0 - 2.5 M urea K-casein migrates in the α_s - casein peak and at 6 - 7 M urea migration of the K-casein occurs in the β-casein peak. In this experiment a urea level of 4 M was incorporated into the polyacrylamide gel to resolve K-casein between the K_5 -casein and β-casein peaks.

Electrophoresis of casein D medium: The electrophoretic patterns of 0.5% (w/v) and 1.0% (w/v) casein D are shown in Figures 3. 1(a) and (b). At a level of 1.0% casein, seven peaks were resolved densiometrically. In order of increasing mobility and in accordance with the findings of Libbey and Ashworth (1961), Lahav and Babad (1964) and Thompson et al. (1965) peaks 1, 2 and 3 were tentatively identified as γ -casein fractions, peak 4 as the β-casein fraction, peak 5 as the K-casein fraction, peak 6 was unidentified and peak 7 as the α_s- casein fraction. In 0.5% casein the K-casein fraction was resolved as a single peak 5 but peak 3 was not detected.

Effect of remin and trypsin on casein: The opacity of the remin-casein reaction mixture increased to a maximum at 6 hours and the opacity remained similar between 6 and 18 hours with the development of little clearing. The electrophoretic pattern of the remin-casein system at 30 minutes was little different from the whole casein pattern except for the appearance of minor peaks 8 and 10. After six hours incubation

of the rennin casein system changes in the β-casein and κ-casein fractions (peaks 4 and 5) were observed (Figure 3 2a). There was an indication of one broad peak being developed in the κ-casein position. In addition fast moving peaks 8 and 10 were present. After 18 hours incubation the complex fraction between peaks 4 and 7 increased and in addition to increases in fast running fractions (peaks 8 and 10) complex peaks 9, 11 and 12 were observed. (Figure 3. 2b).

The trypsin —casein system exhibited opacity within 30 minutes but the opacity decreased on further incubation and the reaction mixture was clear at 18 hours. Figure 3. 3a shows that after 30 minutes of trypsin activity the electrophoretic pattern of casein was similar to that of whole casein, although two fast running fractions (peaks 11 and 12) were evident. At 6 hours the β, κ and α_s- casein fractions (peaks 4, 5 and 7) were reduced and peaks 6 and 9 appeared in addition to peaks 11 and 12. At 18 hours β, κ and α_s-casein fractions were further reduced and the fast running fractions (peaks 9, 11 and 12) were increased (Figure 3. 3b).

The increase in opacity of the enzyme-casein mixture has been shown by Sandvik (1962) to be due to the primary and secondary phases of proteolysis and that subsequent lysis of precipitated casein is due to the tertiary phase of proteolysis. In this experiment opacity of the rennin-casein mixture was displayed throughout incubation. This corresponded to the limited breakdown of the major casein fractions (α_s and β-caseins). Opacity in the trypsin-casein mixture was transistory and was followed by clearing of the mixture. Electrophoretic examination revealed that the major casein fractions were extensively reduced and fast running

fractions were progressively increased in quantity. These fractions may correspond to the high molecular weight compounds found by Zittle and Gerbulis (1958) and Cheeseman (1963) to be produced during caseolysis. The observed disorganisation of the K-casein fraction (peak 5) by remain and trypsin is supported by Kalav and Babad (1964) who found that K-casein disappeared after 14 hours reaction with remain. Dennis and Wake (1965) showed, using starch gel electrophoresis plus urea at pH 8.6 that three proteolytic enzymes each produced two peaks of para K-casein from K-casein. In the present study the complex fractions formed between peaks 4 and 7 during remain activity may represent para K-casein, which is the component remaining after the removal of glyco-macro peptide from normal K-casein (Jolles and Alais, 1961; Garnier, 1962).

Casein breakdown at 5°, 15°, 25° and 37° by strains: As a preliminary experiment the changes produced by strains S3, S9, S10 and S12 in casein medium were examined by electrophoresis after seven days incubation at 5°, 15°, 25° and 37°.

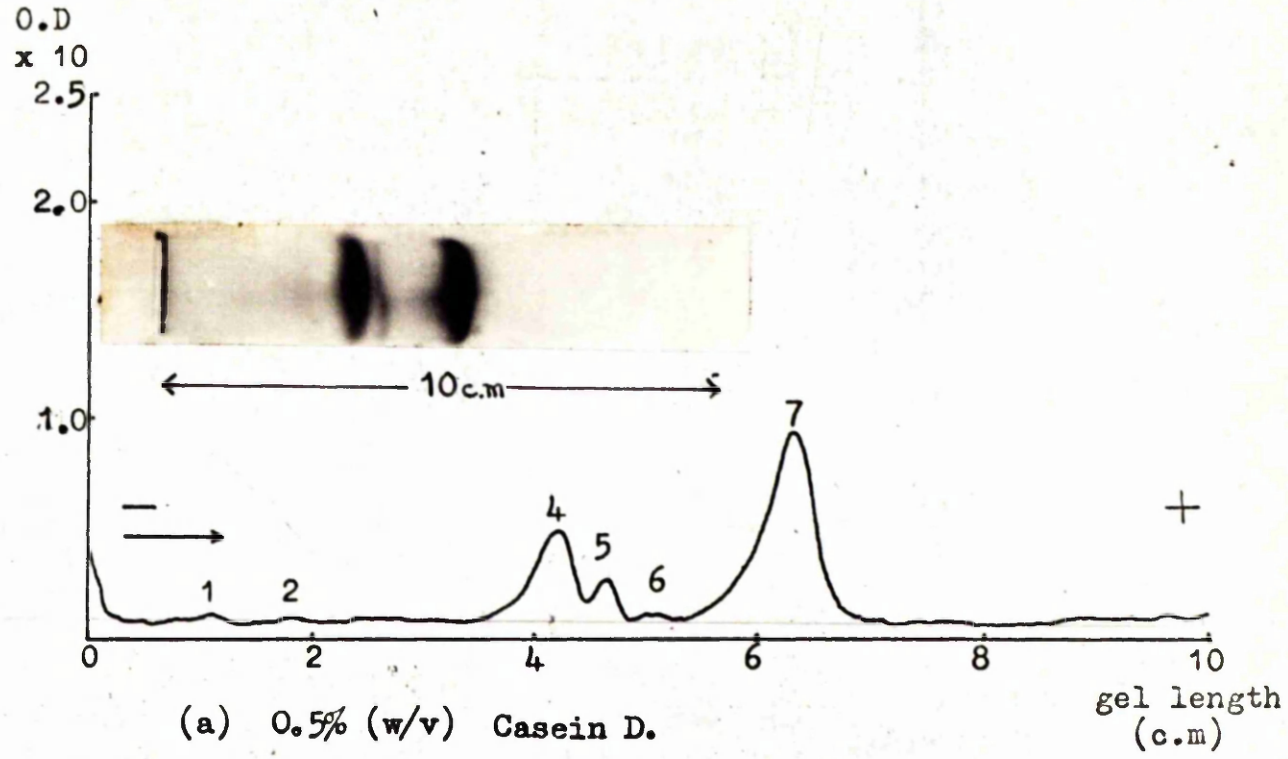
Strain S3

At 25° and 37° the casein cultures displayed normal electrophoretic patterns of whole casein and little change was found at 5°. At 15° the α_s-casein fraction (peak 4) and β-casein fraction (peak 7) were reduced. The K-casein fraction (peak 5) was not resolved but apart from a small peak 6 no new fractions were observed. (Figure 3. 4a).

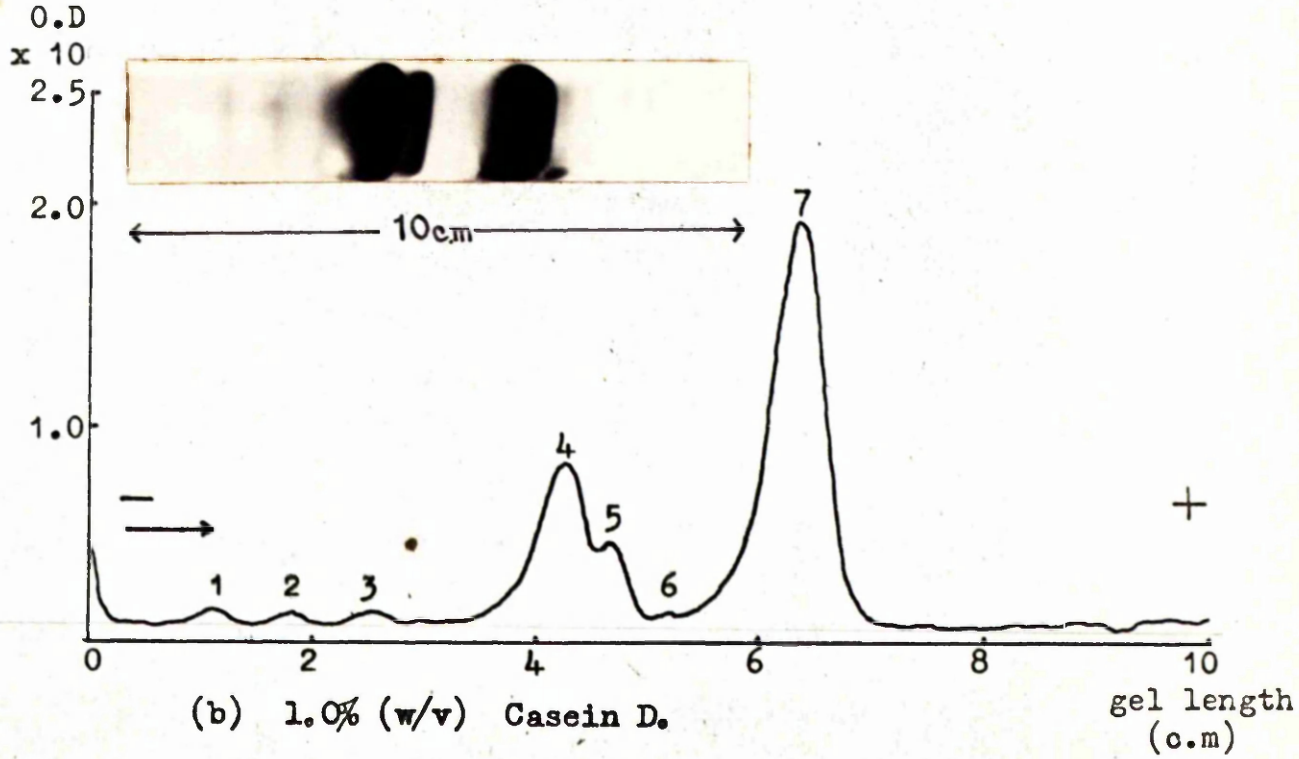
Strain S9

No change was produced in the electrophoretic pattern of casein

Fig. 3.1 **Polyacrylamide gel electrophoresis patterns of whole casein**

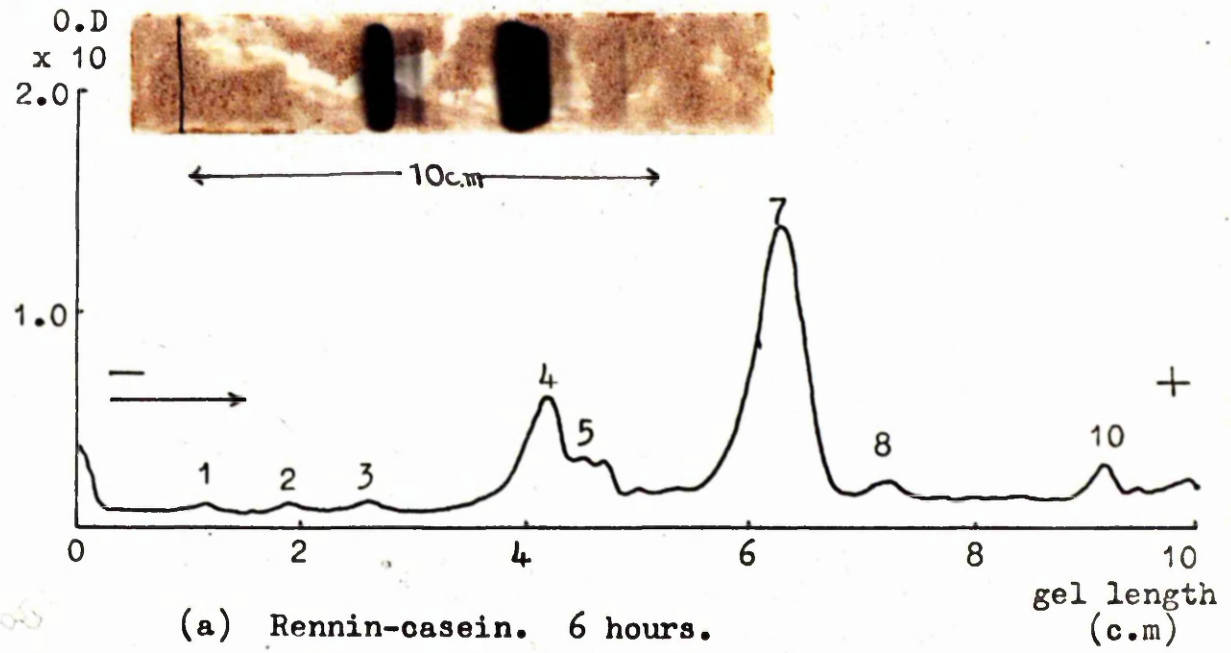


(a) 0.5% (w/v) Casein D.

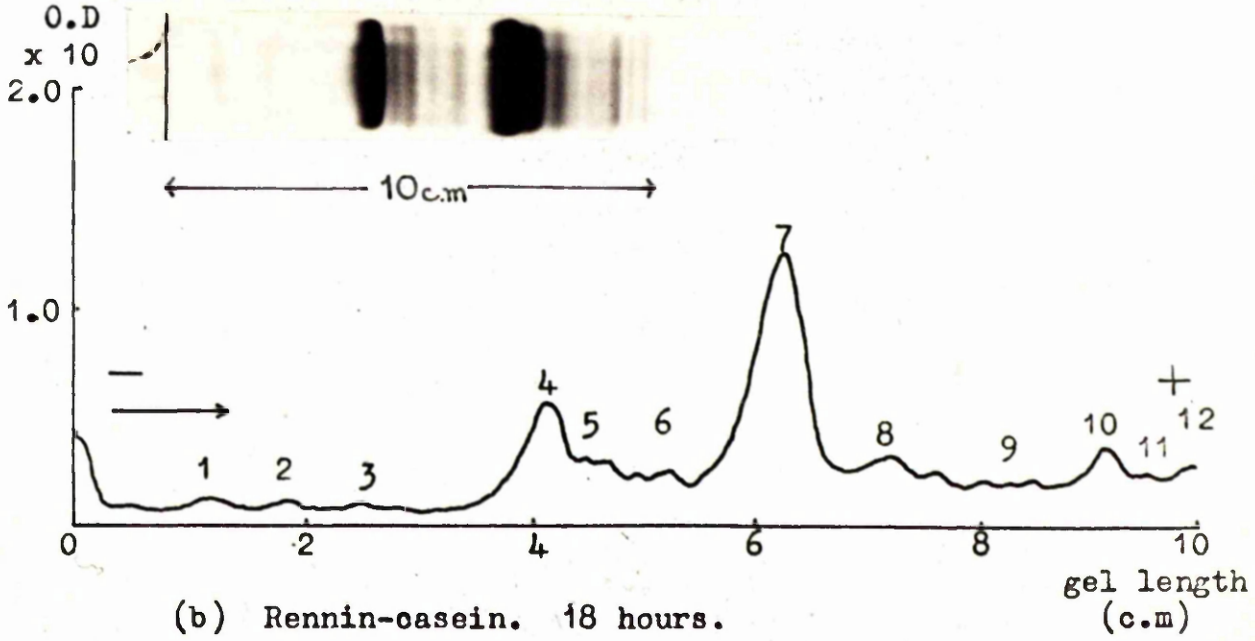


(b) 1.0% (w/v) Casein D.

Fig. 3.2 Polyacrylamide gel electrophoresis patterns of casein breakdown products

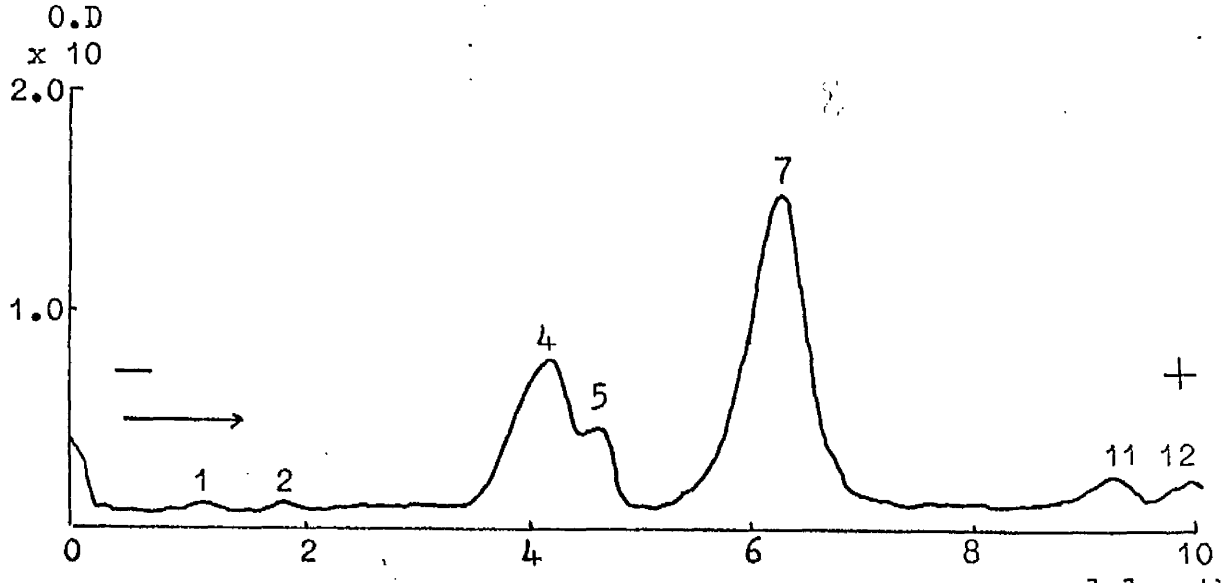


(a) Rennin-casein. 6 hours.

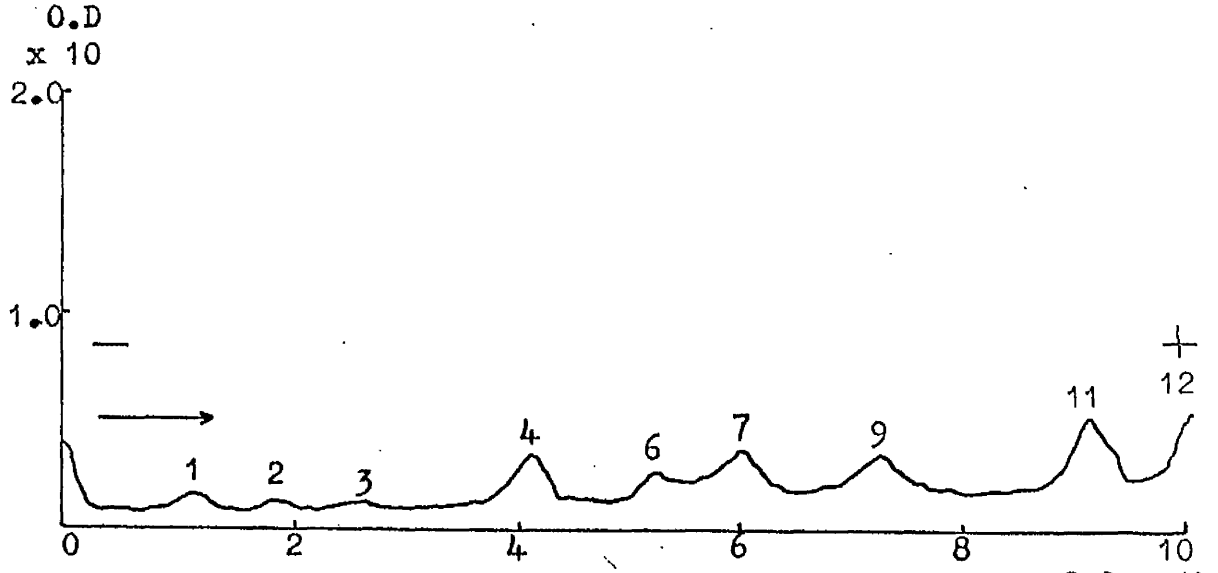


(b) Rennin-casein. 18 hours.

Fig. 3.3 Polyacrylamide gel electrophoresis patterns of casein breakdown products

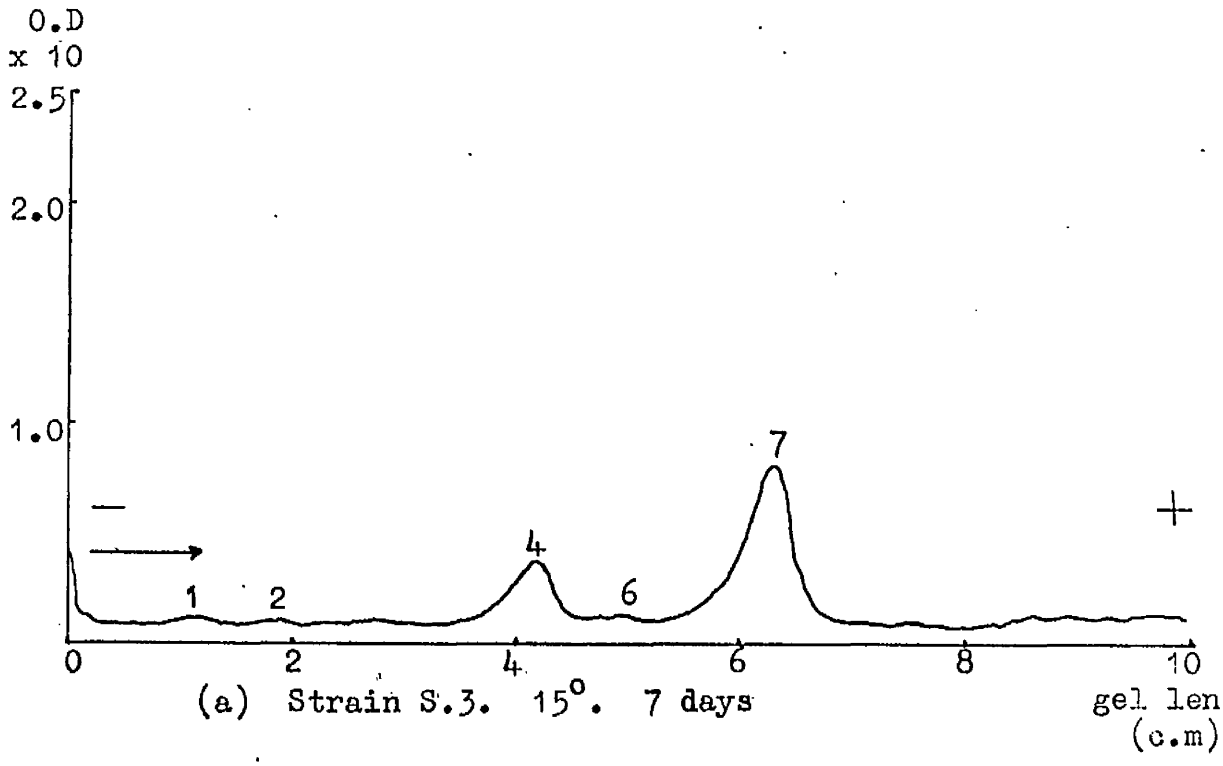


(a) Trypsin- casein 30 minutes.

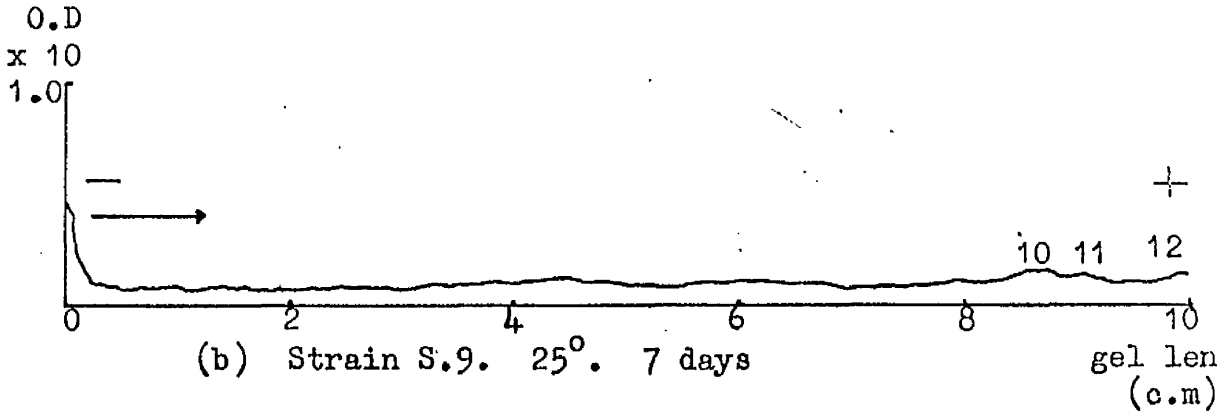


(b) Trypsin- casein 18 hours.

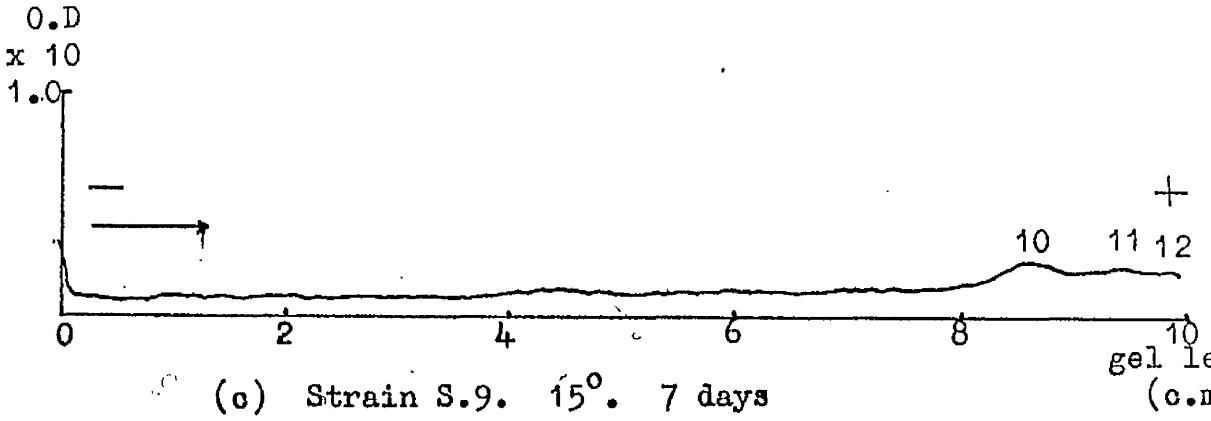
Fig. 3.4 Polyacrylamide gel electrophoresis patterns of casein breakdown products



(a) Strain S.3. 15°. 7 days

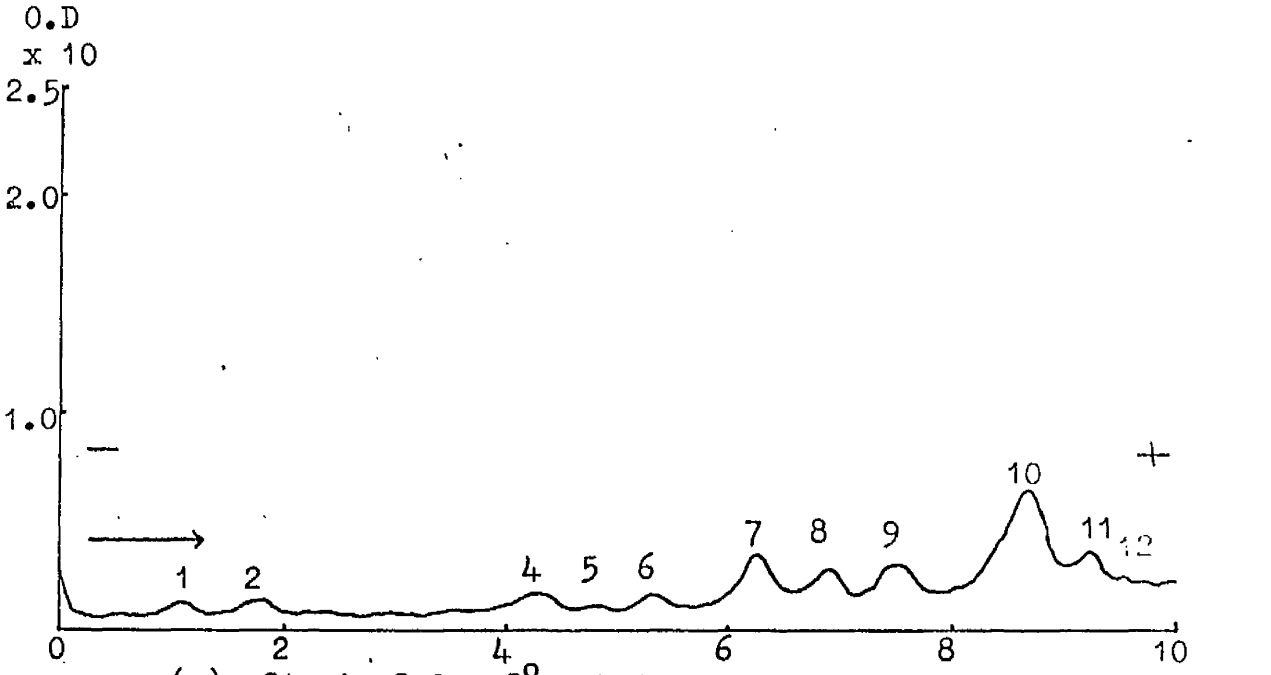


(b) Strain S.9. 25°. 7 days

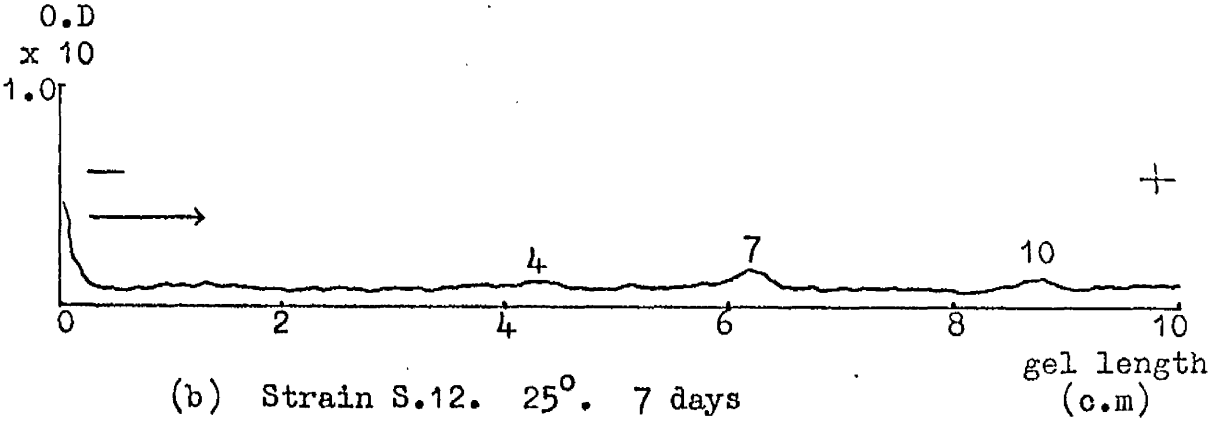


(c) Strain S.9. 15°. 7 days

Fig. 3.5 Polyacrylamide gel electrophoresis patterns of casein breakdown products



(a) Strain S.9. 5°. 7 days



(b) Strain S.12. 25°. 7 days

at 37°. Complete lysis of the casein medium was exhibited at 25° after 7 days and the corresponding electrophoretic pattern demonstrated the absence of α_s, β and K-casein fractions (peaks 1, 2, 3, 4, 5 and 7) but traces of the fast moving fractions (peaks 10, 11 and 12) were shown (Figure 3. 4b). At 15° the same fast moving fractions (peaks 10, 11 and 12) were shown although the normal casein fractions (peaks 1, 2, 3, 4, 5 and 7) were similarly absent (Figure 3. 4c). At 5° the casein culture was not lysed after 7 days incubation and the corresponding electrophoretic pattern (Figure 3. 5a) revealed traces of fractions in the positions of β-casein and α_s-casein (peaks 4 and 7) and γ-casein (peaks 1 and 2). In addition small peaks were obtained in the K-casein position (peak 5) and in positions 6, 8 and 9. A large fast moving fraction (peak 10) was observed together with a complex fraction (peaks 11 and 12).

Strain S10

This strain produced no visible change in the casein medium throughout 7 days at 5°, 15°, 25° or 37° and no change was produced in the electrophoretic pattern of normal casein at any temperature.

Strain S12

No change was produced in the electrophoretic pattern of the casein medium at 5° or 15°. After 7 days at 37° the casein medium was clear and no casein or other fraction was resolved by electrophoresis. At 25° the medium was not completely clear and the corresponding electrophoretic pattern (Figure 3. 5c) demonstrated traces of fractions in the β-casein and α_s- casein positions (peaks 4 and 7) together with traces of a fast moving fraction (peak 10).

These results showed that polyacrylamide gel electrophoresis of casein medium revealed differences in the caseolytic abilities of strains and that incubation temperature affected the electrophoretic patterns produced by strains. The main experiment was designed to investigate these differences more fully.

Main experiment.

Strain 83

The preliminary experiment indicated that this strain produced no change in casein medium within 7 days at 37°. This was confirmed and the $\log_{10}$ count showed that the numbers of viable cells declined over the incubation period. At 25° growth was appreciable increasing to a maximum in 7 days. Although the electrophoretic pattern of casein did not alter appreciably (Figure 3. 6c) evidence of a fast running fraction (peak 9) was observed at 14 days. This strain produced an appreciable alkaline change without proteolysis in milk medium within 14 days.

The viable count in casein medium exceeded 10^9 per ml at 15° within 14 days and was accompanied by the development of extensive alkaline-proteolysis of the milk medium. The electrophoretic pattern at 7 days (Figure 3. 4a) showed the reduction of β-casein (peak 4) and α_s-casein (peak 7) while at 21 days (Figure 3. 6b) the pattern showed further reductions in the β-casein and α_s-casein fractions (peaks 4 and 7) with some accumulation of fast running minor fractions (peaks 8, 9 and 10).

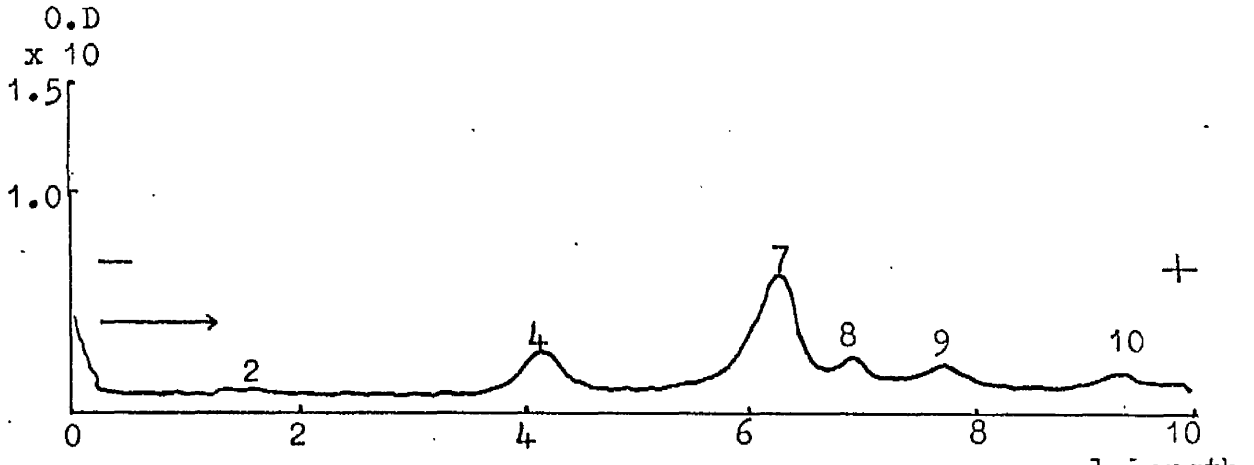
The viable count at 5° in casein medium increased to over 10^8 cells

Table 12 Growth in casein medium and changes produced in milk

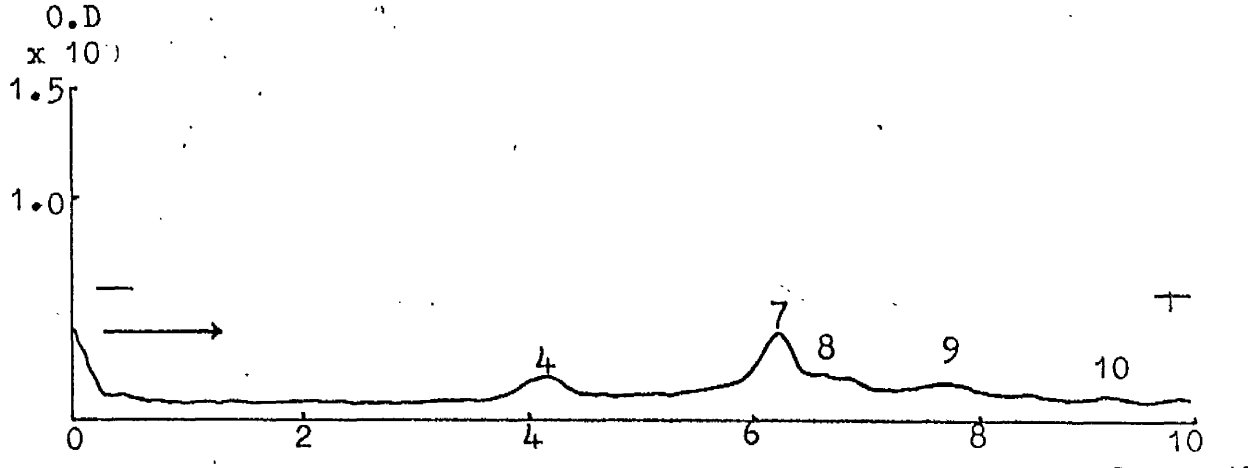
Strain	Temp. °C.	5°					15°					25°				37°		
	Incubation (days)	3	7	14	21	28	2	4	7	14	21	1	3	7	14	1	3	7
S3	Log_{10} viable count/ml	3.42	4.83	6.92	7.85	8.23	6.36	7.73	8.85	9.28	9.21	6.53	8.12	8.78	8.13	3.32	3.07	2.61
	changes in milk	0	1	2	3	3	1	2	3	4	4	1	2	2	2	0	0	0
S4	Log_{10} viable count/ml	4.24	7.36	9.11	9.87	10.13	6.87	8.51	9.14	9.73	9.56	7.24	8.32	8.78	8.83	2.41	2.43	1.78
	changes in milk	1	2	3	4	4	2	3	4	4	4	3	4	4	4	0	0	0
S9	Log_{10} viable count/ml	5.12	7.58	8.89	9.37	9.49	7.42	8.65	9.36	9.87	10.18	7.48	8.64	9.18	8.85	2.33	3.18	3.22
	changes in milk	0	3	4	4	4	2	3	4	4	4	3	3	4	4	0	0	0
S10	Log_{10} viable count/ml	3.86	5.45	6.37	7.64	7.79	4.95	6.52	7.67	8.26	8.38	6.09	8.18	8.63	8.42	4.38	5.15	4.61
	changes in milk	0	0	0	0	0	0	0	1	2	2	0	1	2	2	0	0	0
S12	Log_{10} viable count/ml	3.51	3.36	2.58	2.63	2.48	4.19	6.28	7.73	7.95	8.13	7.53	8.69	9.23	9.46	8.41	9.69	8.92
	changes in milk	0	0	0	0	0	0	0	1	2	2	2	4	4	4	4	4	4
S18	Log_{10} viable count/ml	2.62	3.89	5.07	6.28	6.83	4.93	6.46	7.21	7.59	7.97	7.23	8.86	9.31	8.37	7.83	7.68	6.72
	changes in milk	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
S20	Log_{10} viable count/ml	5.52	7.26	8.39	8.78	9.21	7.08	8.75	9.66	10.35	10.07	7.19	8.70	9.53	9.26	4.96	3.32	2.30
	changes in milk	0	1	2	2	2	1	3	4	4	4	1	3	4	4	1	1	1
S23	Log_{10} viable count/ml	3.83	4.08	4.28	3.56	2.12	3.85	5.37	6.86	7.48	7.06	5.63	8.16	7.24	6.13	6.93	8.54	7.76
	changes in milk	0	0	0	0	0	0	1	2	2	2	1	2	2	2	2	2	2

Refer to page 34 and text for interpretation

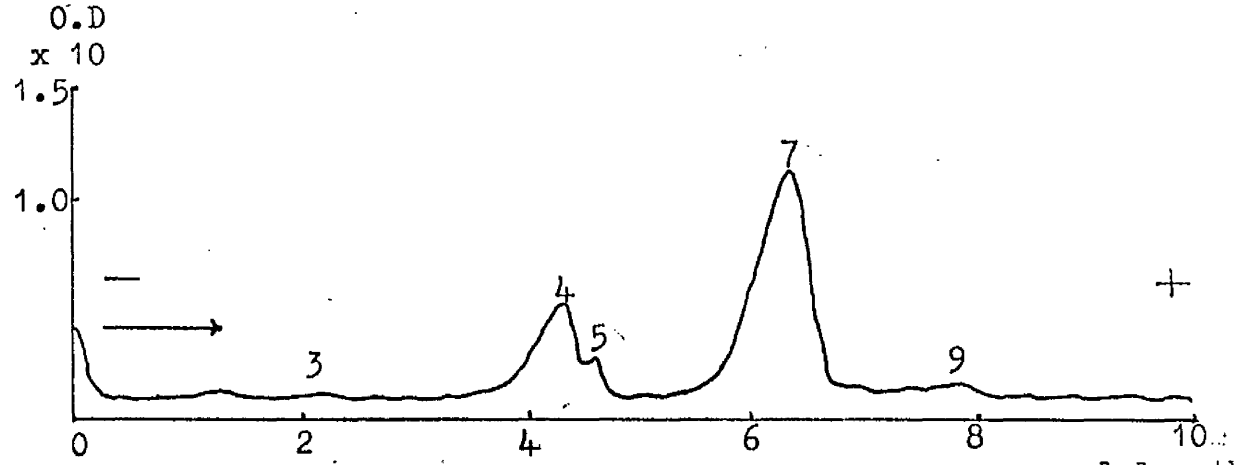
Fig. 3.6 Polyacrylamide gel electrophoresis patterns of casein breakdown products



(a) Strain S.3. 5°. 28 days

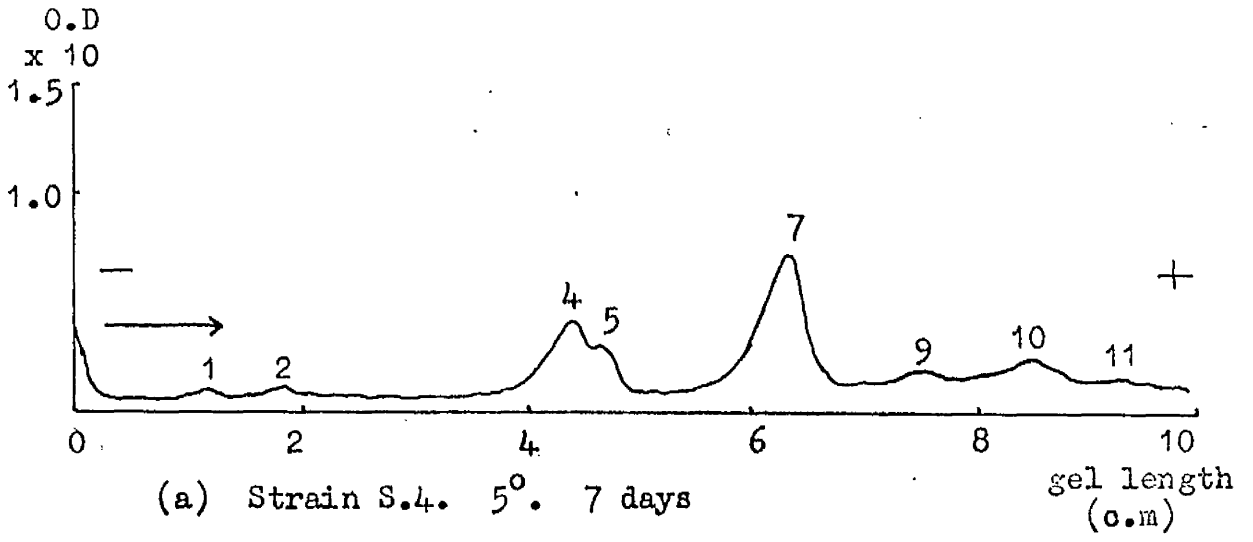


(b) Strain S.3. 15°. 21 days

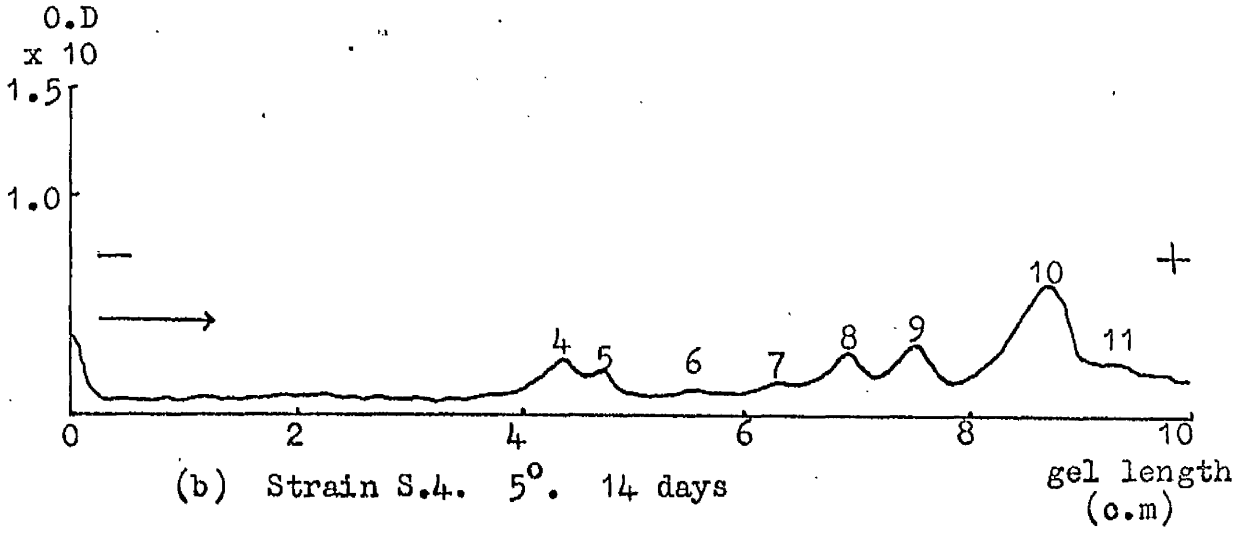


(c) Strain S.3. 25°. 14 days

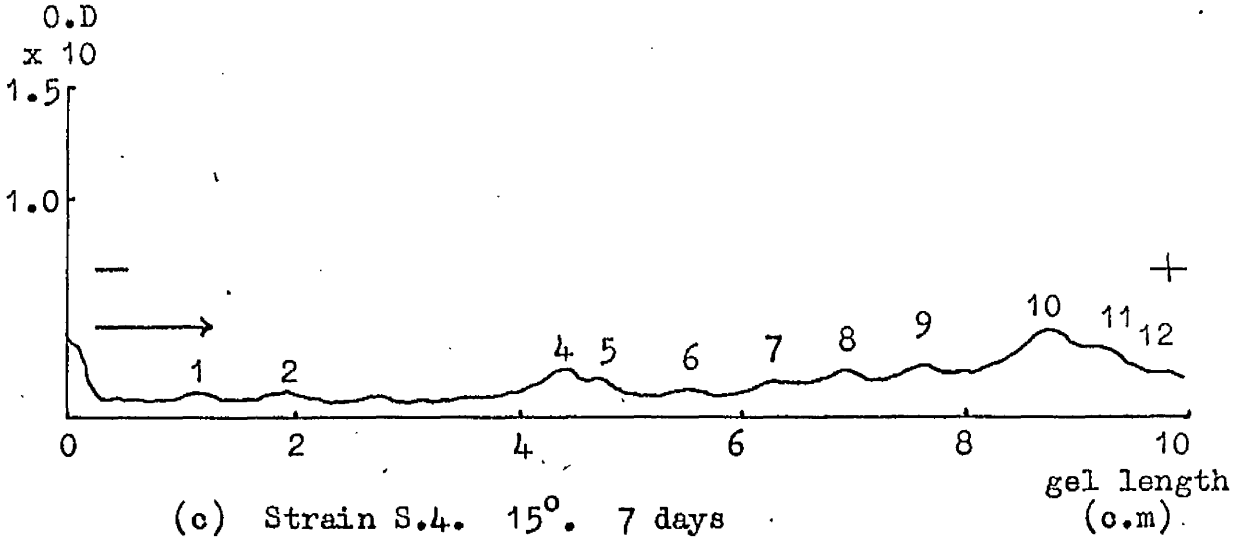
Fig. 3.7 Polyacrylamide gel electrophoresis patterns of casein breakdown products



(a) Strain S.4. 5°. 7 days

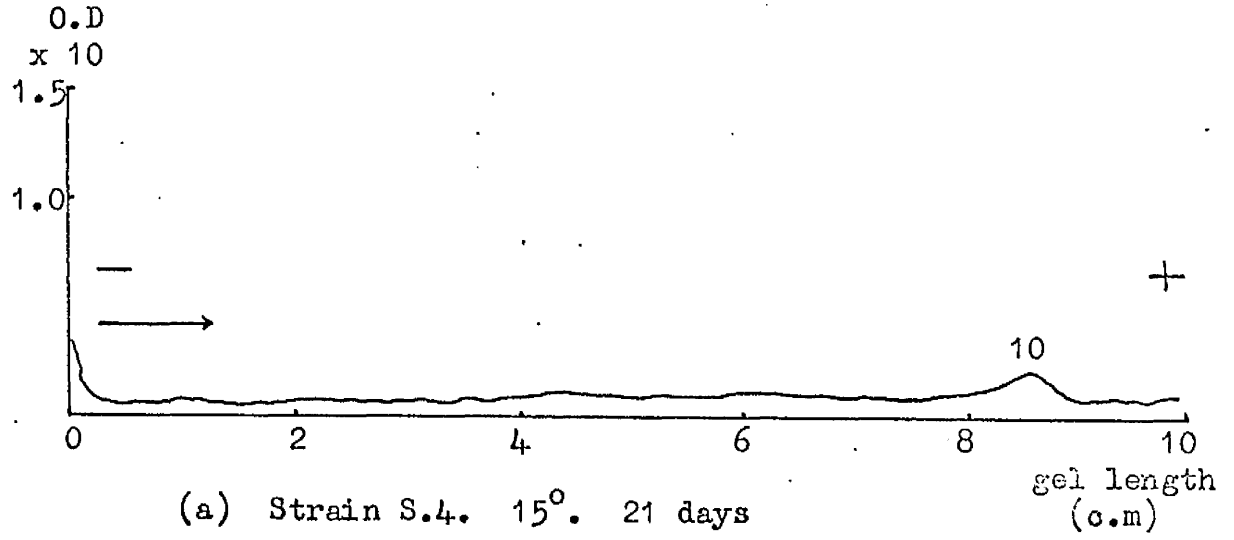


(b) Strain S.4. 5°. 14 days

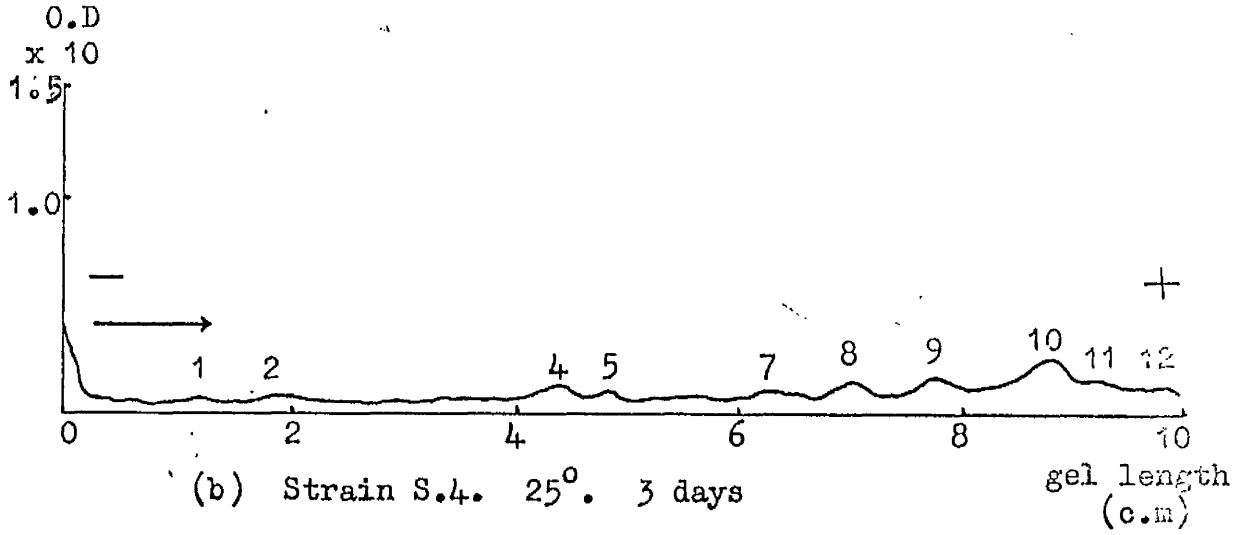


(c) Strain S.4. 15°. 7 days

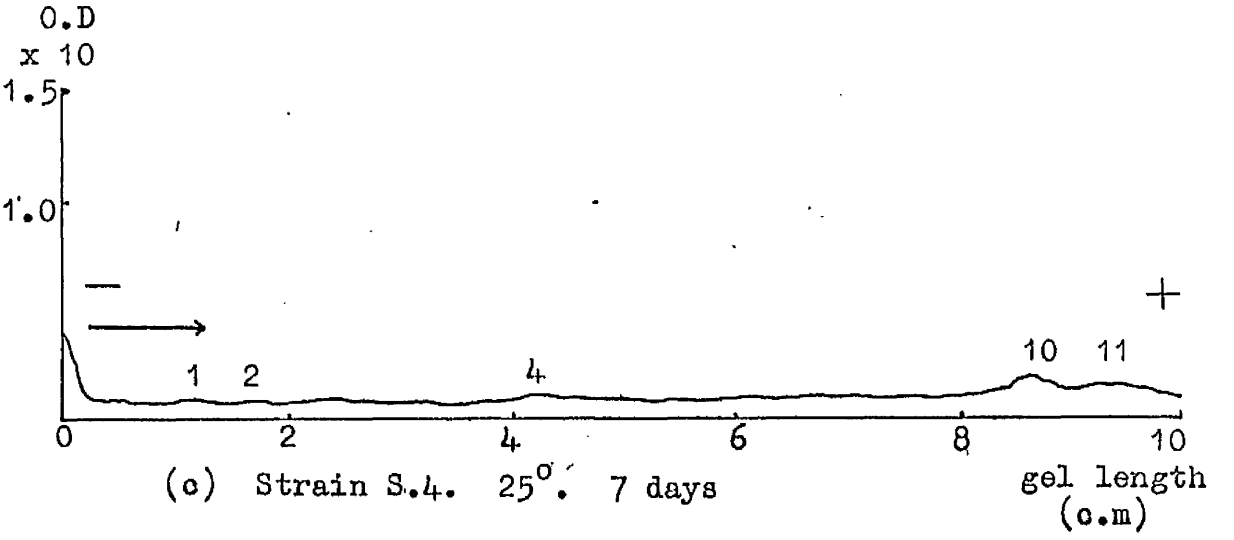
Fig. 3.8 Polyacrylamide gel electrophoresis patterns of casein breakdown products



(a) Strain S.4. 15°. 21 days

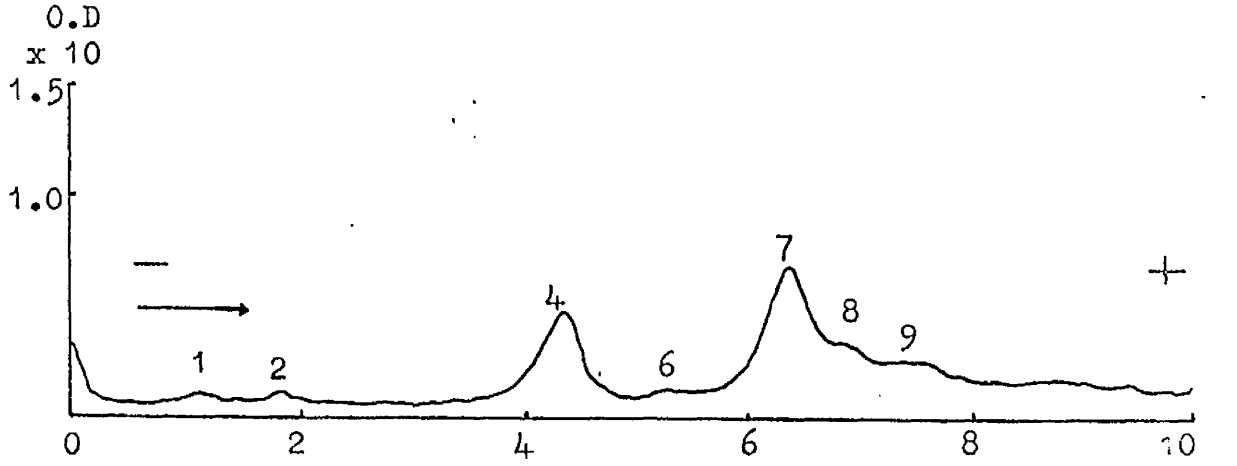


(b) Strain S.4. 25°. 3 days

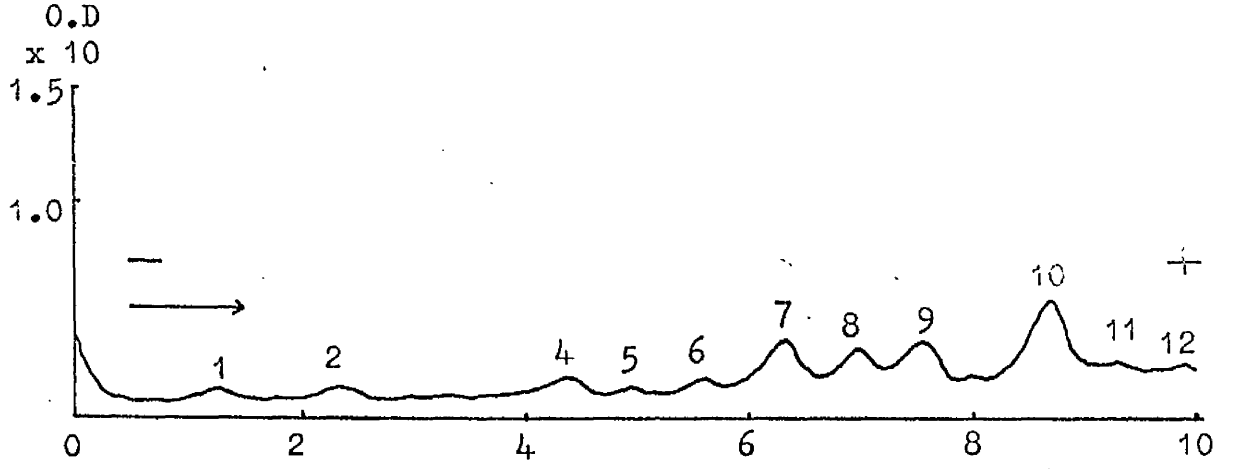


(c) Strain S.4. 25°. 7 days

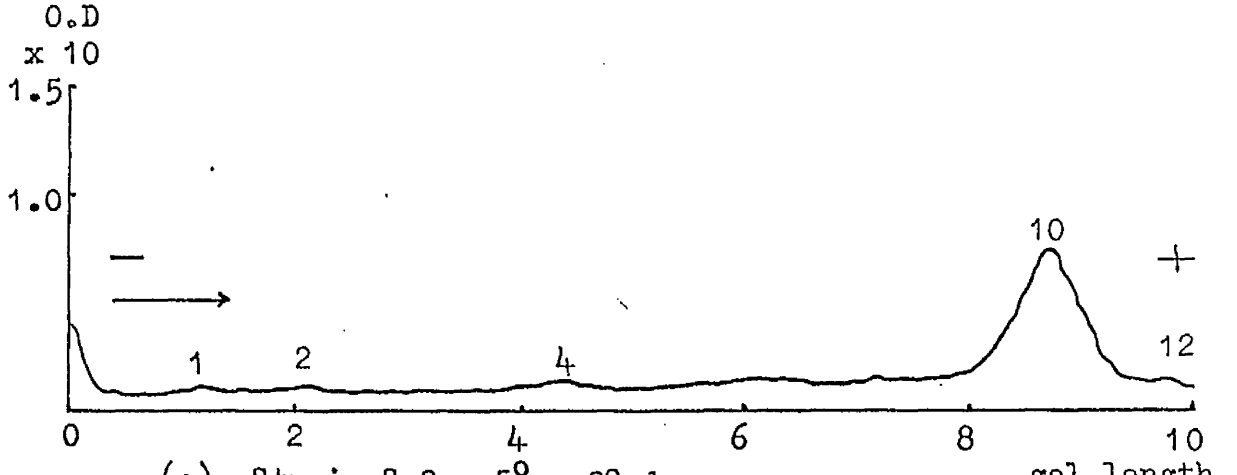
Fig. 3.9 Polyacrylamide gel electrophoresis patterns of casein breakdown products



(a) Strain S.9. 5°. 3 days.

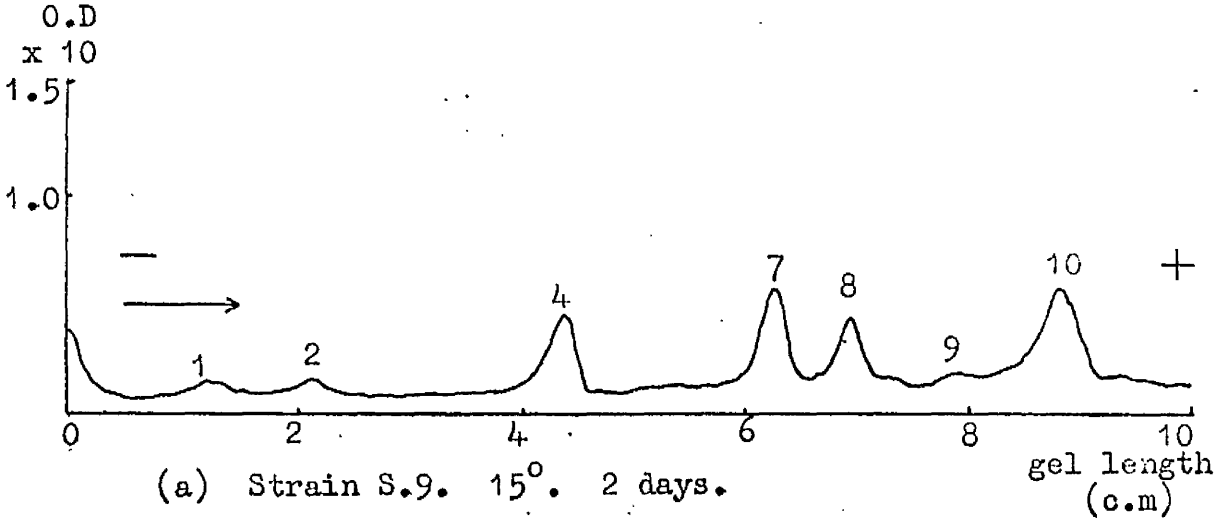


(b) Strain S.9. 5°. 14 days.

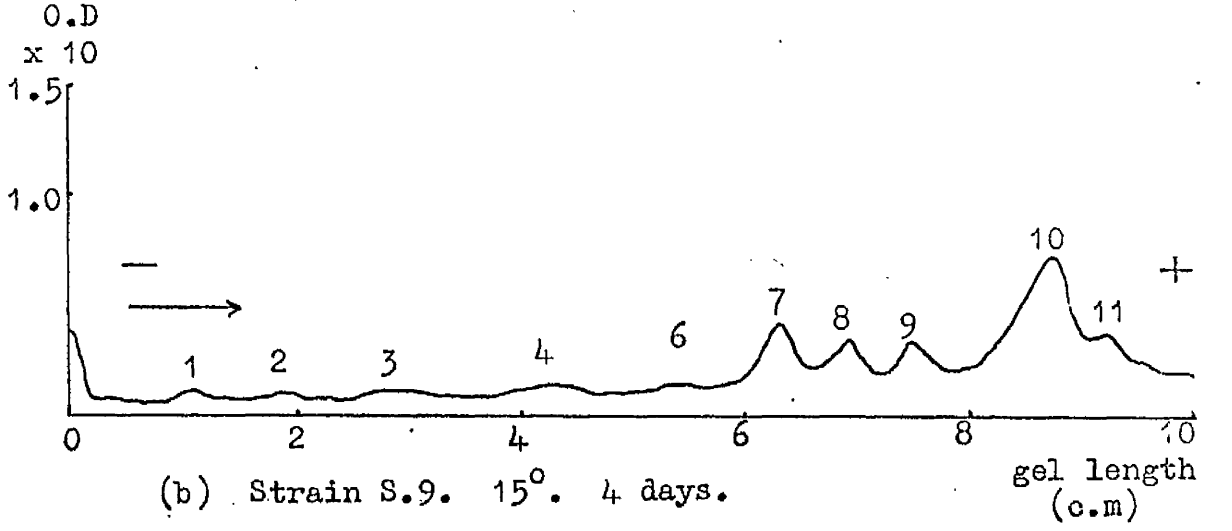


(c) Strain S.9. 5°. 28 days.

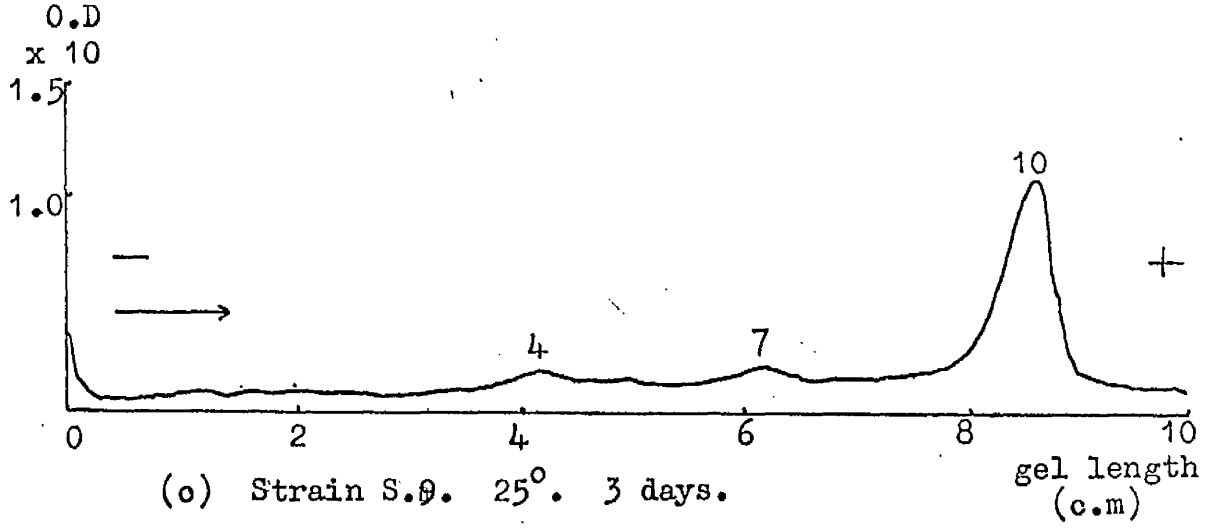
Fig. 3.10 Polyacrylamide gel electrophoresis patterns of casein breakdown products



(a) Strain S.9. 15°. 2 days.

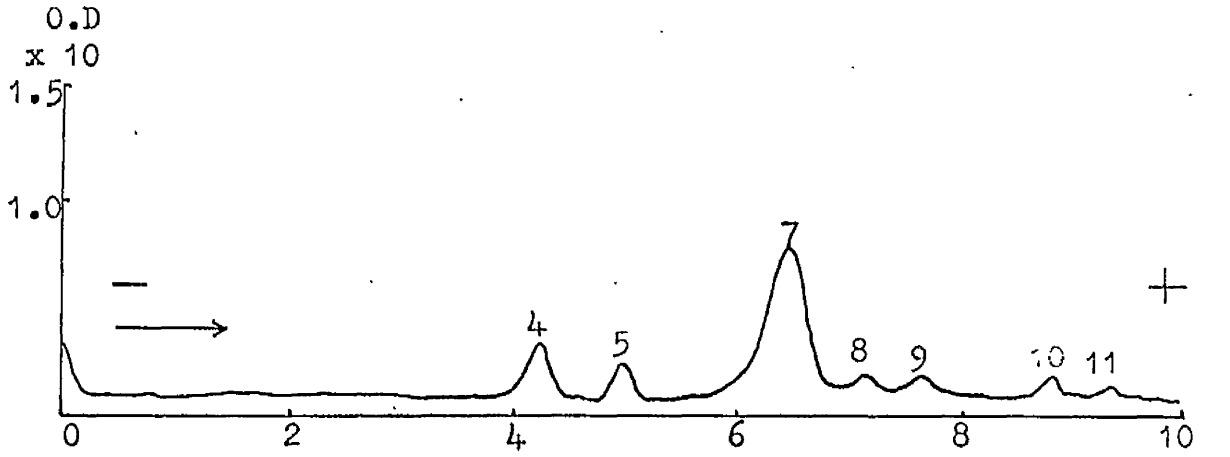


(b) Strain S.9. 15°. 4 days.

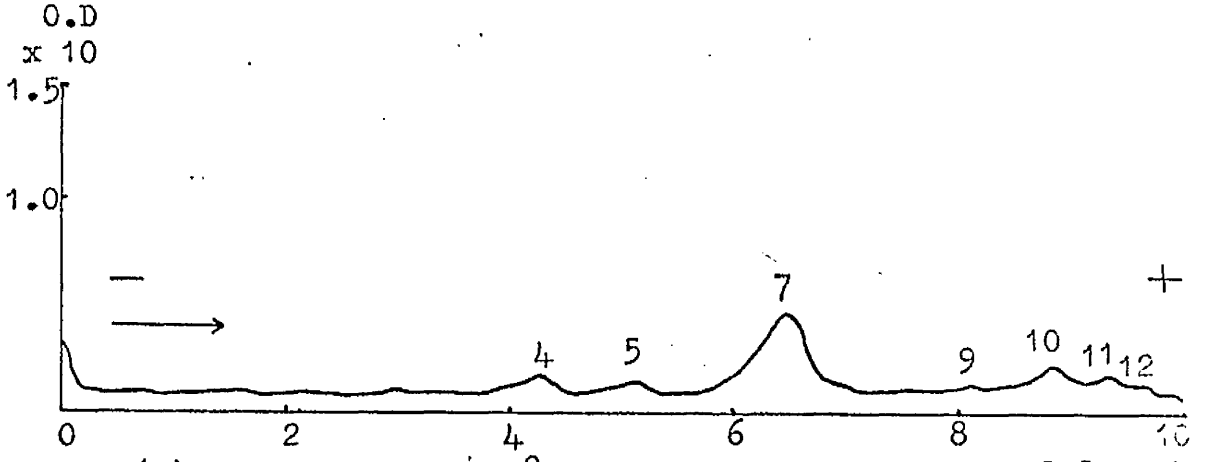


(c) Strain S.9. 25°. 3 days.

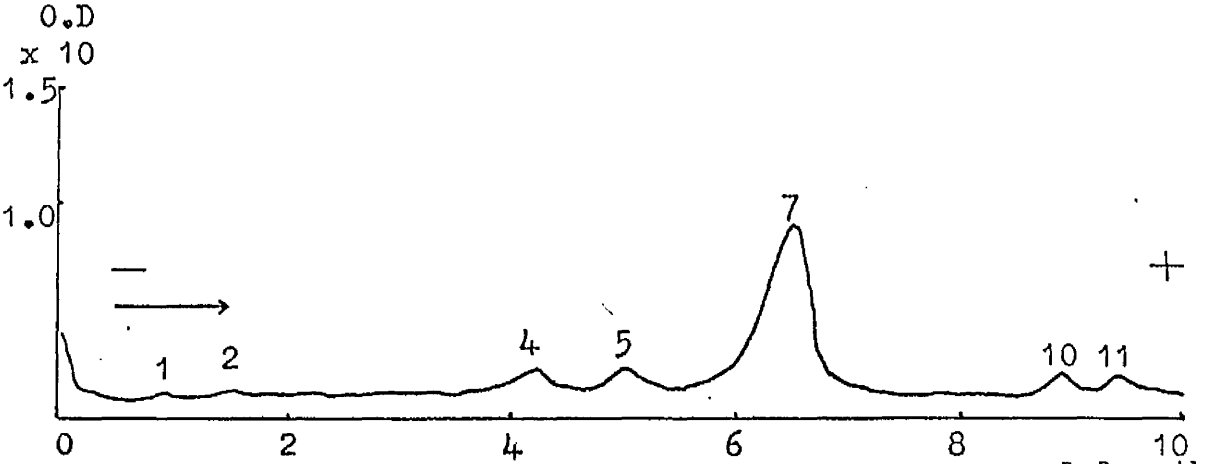
Fig. 3.11 Polyacrylamide gel electrophoresis patterns of casein breakdown products



(a) Strain S.12. 25°. 1 day.

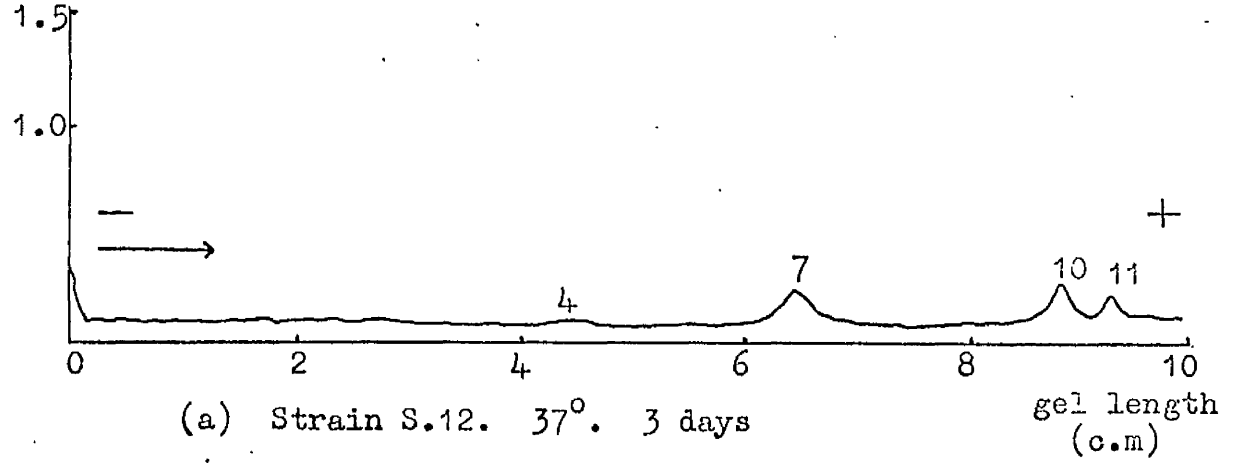


(b) Strain S.12. 25°. 3 days.

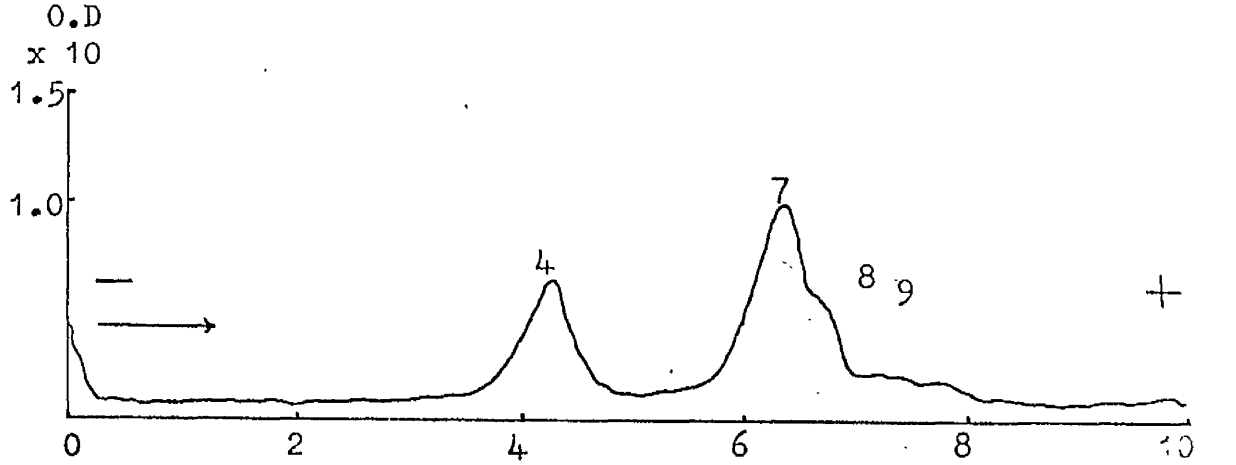


(c) Strain S.12. 37°. 1 day.

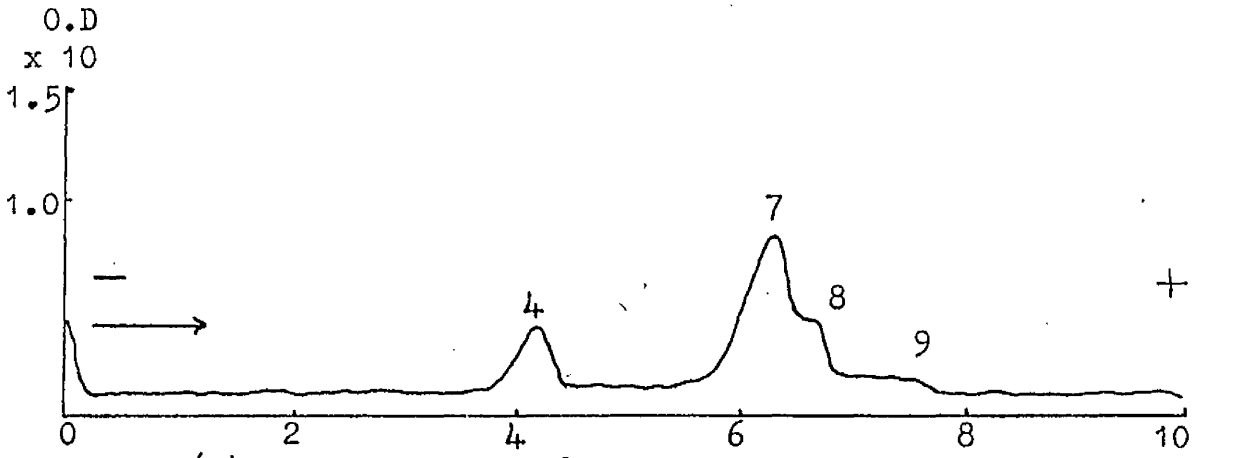
Fig. 3.12 Polyacrylamide gel electrophoresis patterns of casein breakdown products



(a) Strain S.12. 37°. 3 days

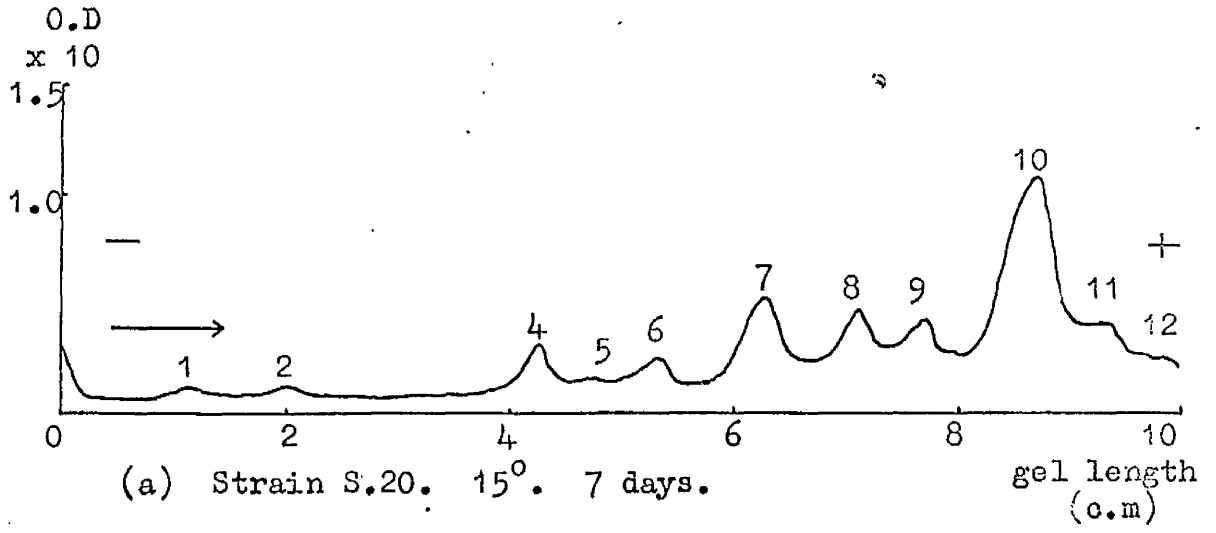


(b) Strain S.20. 5°. 14 days

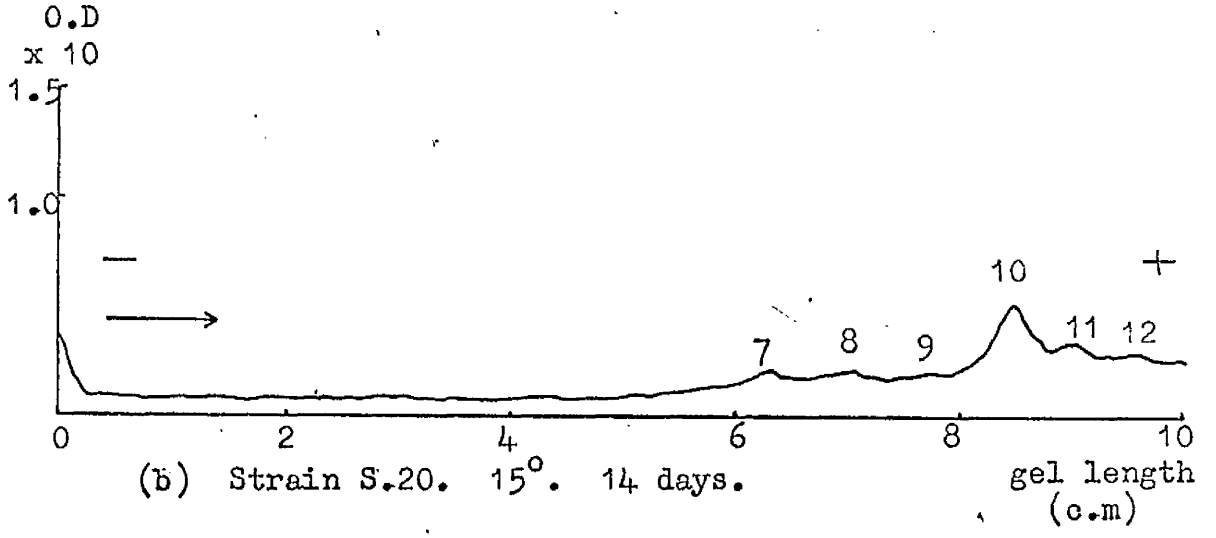


(c) Strain S.20 5°. 28 days

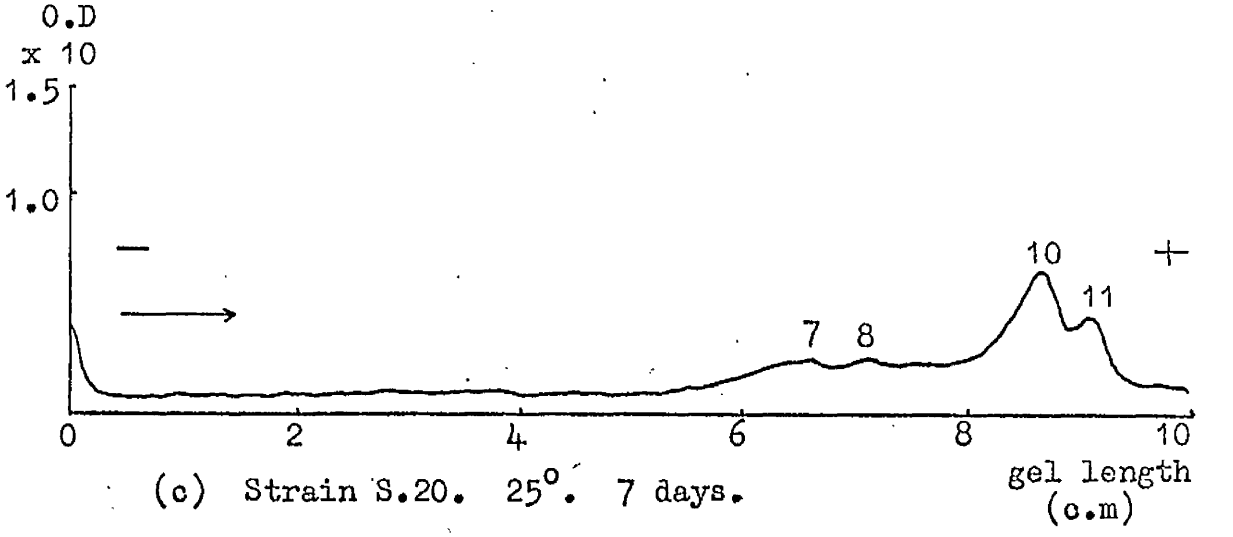
Fig. 3.13 Polyacrylamide gel electrophoresis patterns of casein breakdown products



(a) Strain S.20. 15°. 7 days.



(b) Strain S.20. 15°. 14 days.



(c) Strain S.20. 25°. 7 days.

per ml in 28 days. At 7 days little change was observed in the electrophoretic pattern of casein although at 21 and 28 days, which corresponded to alkaligenesis with slight proteolysis in milk medium, β-casein and α_s-casein (peaks 4 and 7) were reduced. As observed at 15° fast running fractions (peaks 8, 9 and 10) were shown at 5° in 14 and 28 days. (Figure 3. 6a).

Strain 84

The viable count of this psychrophilic Pseudomonas strain decreased during incubation at 37° and no changes were produced in casein medium or in milk medium.

A high viable count was produced in casein medium within 14 days at 25°. An alkaline-proteolysis of milk medium was produced in one day and the medium was completely clear in 7 days. The electrophoretic patterns (Figure 3. 8b) indicated the extent of casein breakdown in 3 days. The β-casein (peak 4) and α_s-casein (peak 7) were much reduced and fast running fractions (peaks 8, 9, 10, 11 and 12) were shown. After 7 and 14 days almost complete caseolysis was reflected in the resolution of only fast running fractions (peaks 10 and 11) in electrophoretic patterns (Figure 3. 8c).

The strain produced heavy growth in casein medium at 15° and alkaligenesis was apparent in milk medium on the second day of incubation. Proteolysis of milk was shown after 4 days and complete within 14 days. The electrophoretic pattern of casein breakdown at 15° was similar to that shown at 25° although less rapid. Within 7 days (Figure 3. 7c) β-casein (peak 4), K-casein (peak 5) and α_s-casein (peak 7) were much reduced and minor fractions (peaks 6, 8 and 9)

were formed. A complex fraction including peaks 10, 11 and 12 was also evident. At 21 days all fractions except that corresponding to peak 10 (Figure 3. 8a) were absent.

Growth at 5° in casein medium increased appreciably between 7 and 28 days and alkaligenesis in milk medium within 7 days was followed by the development of limited proteolysis in 14 days and complete proteolysis in 28 days. The electrophoretic pattern of the casein culture at 7 days (Figure 3. 7a) indicated the limited breakdown of β-casein (peak 4), K-casein (peak 5) and α_s-casein (peak 7) with the formation of fast moving fractions (peaks 9, 10 and 11). At 14 days (Figure 3. 7b) β, K and α_s-casein fractions were much reduced, the largest fraction being peak 10 with other fast running fractions also being prominent (peaks 8, 9 and 11). These fractions corresponded to those observed at 15° and 25° and after 21 and 28 days at 5° the electrophoretic patterns, showing traces of peak 10 only, were similar to those in Figure 3. 8a.

Strain S9

This psychrophilic Pseudomonas strain displayed negligible growth at 37° in casein medium and no changes were observed in casein medium or in milk medium.

Growth at 25° was rapid with over 10^9 viable cells per ml being produced in 7 days. Limited alkaline proteolysis of milk was shown in one day and proteolysis was complete within 7 days. The electrophoretic pattern of casein medium after 3 days (Figure 3. 10c) indicated the almost complete elimination of the β-casein (peak 4) and α_s-casein (peak 7) fractions with no K-casein (peak 5) being shown. A large fast running fraction (peak 10) was itself reduced on further incubation

at 25° (Figure 3. 4b).

Growth at 15° in casein medium was heavy and the viable count was in excess of 10^{10} cells per ml at 21 days. An appreciable alkaline change in milk within two days was followed by the progressive development of alkaline proteolysis. After two days (Figure 3. 10a) the electrophoretic pattern indicated the reduction of β-casein (peak 4) and α_s-casein (peak 7) and the removal of K-casein (peak 5). Fast moving major fractions (peaks 8 and 10) and a minor fraction (peak 9) were obtained. In 4 days (Figure 3. 10b) β-casein (peak 4) was almost absent, α_s-casein (peak 7) was further reduced and fractions in peaks 9, 10 and 11 increased. At 7 days, as indicated in the preliminary experiment (Figure 3. 4c), only the fast running fractions (peaks 10, 11 and 12) remained, which were also progressively reduced on further incubation.

Good growth was produced at 5° in casein medium but the viable counts did not attain those at 15°. While no change was evident in milk medium at 3 days, limited alkaline proteolysis was shown at 7 days which became progressively more extensive on further incubation. The electrophoretic pattern at 3 days (Figure 3. 9a) indicated the removal of the K-casein fraction (peak 5) and the reduction of β and α_s-casein. A complex fraction was formed between peaks 7, 8 and 9. The reduction of the β-casein (peak 4) and α_s-casein (peak 7) fractions and the production of fast running fractions (peaks 10, 11 and 12) and a slow running fraction (peak 6) was shown at 7 and 14 days. (Figure 3. 9b). After 21 days all fractions except peak 10 were much reduced and this large fast running fraction remained after 28 days. (Figure 3. 9c).

Strain S10

Although good growth was produced in casein medium at 5°, 15° and 25° this Achromobacter strain was unable to produce proteolysis in casein medium or in milk. An alkaline change was produced in milk at 15° and 25°.

Strain S12

The maximum viable count of this strain of Ps. aeruginosa in casein medium was obtained at 37° in 3 days. Extensive alkaline proteolysis of milk was shown after one day and proteolysis was complete within 3 days. The electrophoretic pattern at one day (Figure 3. 11c) revealed the marked reduction in the β-casein fraction (peak 4) although the α_s- casein fraction (peak 7) was not greatly reduced. Two fast running minor fractions (peaks 10 and 11) and a further minor fraction (peak 5) were resolved. After 3 days (Figure 3.12a) the pattern showed vestigial β-casein and α_s-casein fractions (peaks 4 and 7) together with the two fast moving fractions (peaks 10 and 11). The pattern at 7 days showed traces of peaks 4, 7 and 10.

At 25° the viable count increased throughout the incubation period. Alkaligenesis was shown in milk after one day and extensive alkaline proteolysis was apparent in three days, proteolysis being complete within 7 days. After one day the electrophoretic pattern (Figure 3. 11a) indicated that although the α_s-casein fraction (peak 7) was little affected the β-casein fraction (peak 4) was much reduced. A fraction was resolved in the K-casein position (peak 5) and four fast running minor fractions (peaks 8, 9, 10 and 11) were also resolved. After 3 days (Figure 3. 11b) the β-casein and α_s-casein fraction

(peaks 4 and 7) were further reduced, as were the minor fractions (peaks 5, 8 and 9) and a complex fraction covering peaks 10, 11 and 12 was present. The pattern after 7 days (Figure 3. 5b) showed vestigial traces of peaks 4, 7 and 10 while at 14 days no fractions were resolved.

The viable count at 15° in casein medium increased appreciably throughout the 21 day incubation period. After a delay alkaligenesis was demonstrated in milk but proteolysis did not occur at this temperature. The electrophoretic pattern of casein remained unchanged throughout the incubation period.

The strain was unable to grow at 5° in casein medium or in milk and no changes were observed in the electrophoretic pattern of casein.

Strain S18

This strain of Ps. putida produced good growth in casein medium at 15°, 25° and 37° and growth at 5° was less heavy. No change was produced in milk and the electrophoretic pattern of casein was unchanged throughout.

Strain S20

The viable counts of this psychrophilic Pseudomonas strain decreased throughout 7 days at 37°. Although a slight alkaline change was produced in milk the electrophoretic pattern of casein was unchanged.

At 25° the viable count increased to a maximum in 7 days and thereafter decreased. Slight alkaligenesis was shown in milk after one day, alkaline proteolysis was evident in 3 days and was complete in 7 days. After one day little change was observed in the electrophoretic pattern of casein but after 3 days the breakdown of

β-casein and α_s-casein fractions (peak 4 and 7) was evident with the production of fast running fractions (peaks 8, 9, 10, 11 and 12) similar to those shown in Figure 3. 13a. After 7 days the patterns showed the complete reduction of β-casein (peak 4) and the almost complete reduction of α_s-casein (peak 7) with the formation of a complex fraction covering peaks 7, 8, 10 and 11. These fractions were further reduced after 14 days.

The viable count at 15° increased appreciably to a value in excess of 10^{10} cells per ml in 14 days. A slight alkaline change was observed in milk after two days and limited alkaline proteolysis was shown after 4 days. Extensive proteolysis was obtained in 7 days and complete proteolysis in 14 days. Little change was shown in the electrophoretic pattern of casein after two days but at 4 days some reduction in the β-casein and α_s-casein fractions (peaks 4 and 7) were observed with the production of faster running minor fractions (peaks 8, 10 and 11). After 7 days the pattern (Figure 3. 13a) indicated the further reduction of the β-casein and α_s-casein fractions (peaks 4 and 7) with the appearance of a minor fraction (peak 6). Fast running fractions (peaks 8, 9, 10, 11 and 12) were also resolved. After 14 days (Figure 3. 13b) the reduction of the β-casein fraction (peak 4) was complete and a much reduced complex fraction incorporating peaks 7, 8, 9, 10, 11 and 12 was resolved. At 21 days only traces of peak 10 remained.

At 5° the viable count increased appreciably throughout the 28 day incubation period. A slight alkaline change was produced in milk after 7 days which became extensive on further incubation without proteolysis. No change was observed in the electrophoretic patterns

of casein at 3 or 7 days. After 14 days (Figure 3. 12b) the β-casein fraction (peak 4) was reduced and peak 5 in the K-casein position was not resolved. In addition the α_s-casein fraction (peak 7) showed some separation and a complex fraction was resolved between peaks 7, 8 and 9. The pattern after 28 days (Figure 3. 12c) showed the further reduction of the β-casein fraction (peak 4) and the continued presence of the complex fraction between peaks 7, 8 and 9.

Strain S23

This mesophilic Pseudomonas strain produced moderate growth at 15°, 25° and 37° but growth was negligible at 5°. Although alkaline changes were observed in milk at 15°, 25° and 37° no change was observed in the electrophoretic patterns of whole casein at any temperature.

It is recognised that the changes displayed by electrophoretic patterns represent only part of the total changes involved in the proteolysis of whole casein and the proteolysis of milk. However, certain conclusions may be drawn from the results of this experiment. The value was shown of polyacrylamide gel containing urea for the resolution of fractions associated with the breakdown of casein by rennin, trypsin and bacterial proteinases. The incomplete tertiary phase of caseolysis by rennin was shown to be related to little change in the normal electrophoretic pattern of the major casein fractions although faster running fractions were produced. The action of rennin and other proteinases on the fraction tentatively identified as K-casein (peak 5) is difficult to assess. The production of two peaks

and subsequently a complex fraction (peaks 5 -6) by rennin may confirm the identification of this fraction as K-casein since Dennis and Wake (1965) also found that rennin produced two fractions from similarly located K-casein fraction. These workers identified the two fractions as para K-casein which is the fraction remaining after the removal of glyco-macro peptide from K-casein by proteinases. In contrast with the electrophoretic patterns shown by rennin activity the rapid tertiary phase of proteolysis shown by trypsin and most bacterial proteinases resulted in the rapid reduction of α_s-casein and β-casein and the temporary accumulation of faster running fractions. These unidentified fractions (peaks 8 - 12) may represent either slightly modified major casein fractions or high molecular weight products of casein breakdown. The may comprise both types of compounds but the general accumulation of these fractions during the early stages of the tertiary phase of proteolysis corresponding to marked visible proteolysis of milk suggests that they are intermediate products of casein degradation. The subsequent disappearance of these fractions as a result of further bacterial enzyme activity may indicate that they comprise large molecular weight peptides. Zittle and Cerbulis (1958) showed that casein hydrolysis results in the formation of large peptides and Merkel et al. (1964) reported that isolation of such peptides during caseolysis by Pseudomonas spp.

Differences were found between the electrophoretic patterns of casein breakdown by trypsin and the patterns due to the activity of the test strains. The fast running fractions produced by trypsin were located in peaks 9, 11 and 12, whereas peak 10 was generally prominent

as a fraction resulting from the proteolytic activity of the strains, although fractions denoted by peaks 8, 9 and 11 were also evident.

Strains exhibited differences between each other in the electrophoretic patterns. These differences were usually related to relative differences in the disappearance of α_s-casein and β-casein fractions and the corresponding increase in the fast moving fractions. Strains S3 and S12 often displayed the disappearance of the major casein fractions without large increases in the fast moving fractions whereas strains S4, S9 and S20 displayed the accumulation of large amounts of fast moving fractions during the reduction of α_s-casein and β-casein fractions. The extended incubation of all proteolytic strains, except strain S9, resulted in the reduction of all the fast moving fractions. This observation implies the operation of enzymes which were able to degrade intermediate products of protcolysis which were not observed during trypsin activity.

The temperature of incubation of the casein medium cultures had an effect on the electrophoretic patterns shown by certain strains. The active growth of strain S3 at 25° resulted in little change in the pattern of casein which indicates an inability to elaborate proteinase at this temperature but the patterns of casein breakdown were similar at 5° and 15°. Strain S20 displayed a pattern of restricted casein breakdown at 5° despite good growth but the patterns denoting appreciable casein breakdown were similar at 15° and 25°. The patterns of casein breakdown by strains S4, S9 and S12 were essentially similar at all temperatures of growth.

Few experiments have been performed by other workers with which

the results of the present experiment can be compared. Skean and Overcast (1960) examined the breakdown of refrigerated skim milk by three Pseudomonas spp. using paper electrophoresis. The conclusions reached were limited by the poor resolution of casein fractions, whey protein fractions and breakdown products but it was shown that reduction of the total protein changes in the relative amounts of protein fractions and the formation of new fractions occurred. Knaut and Bruderer (1965) used free boundary electrophoresis to follow the breakdown of casein in milk at 25° by a Pseudomonas sp. These workers showed that β-casein was hydrolysed first followed by breakdown of α-casein with the production of two faint fast moving fractions, one of which later disappeared. Kisza and Sobina (1963) found that the β-casein fraction of mastitic milk was reduced more than the α-casein fraction. These observations corroborate certain of the findings made in the present experiment. The preferential degradation of the β-casein fraction was shown by strains S3, S9, S12 and S20 at one or more temperature but strain S4 degraded α-casein and β-casein fractions equally.

It has been shown that the method of electrophoresis used enabled the breakdown of casein by single enzymes and growing cultures to be followed. Differences were found between enzymes and strains in their ability to degrade casein and qualitative and quantitative differences were also demonstrated due to the effect of incubation temperature on the ability of individual proteolytic strains.

GENERAL DISCUSSION AND CONCLUSIONS

The study of psychrophilic bacteria has often been directed either towards the detailed examination of particular aspects of growth or metabolism of few strains (Ingraham and his co-workers, 1958, 1963; Rose and his colleagues, 1962, 1965; Jezeski and Olsen, 1962), or towards the superficial examination of large numbers of strains (Gyllenberg et al. 1963; Higoshi, 1964).

This study attempted to examine in some detail various aspects of the growth and metabolic activities of representative strains of this important group of bacteria. The strains comprised isolates from dairy sources together with strains from other sources and environments.

In an initial experiment the morphological, cultural and biochemical characteristics were examined at 5^{o}, 15^{o}, 25^{o} and 37^{o} and the results were scored in a semi-quantitative manner previously used by the author (Scholefield, 1964).

The reactions displayed by many strains to certain tests, including the oxidative production of acid from glucose often corresponded to the levels of growth at the same temperature. The levels of certain reactions or characteristics shown, including catalase and oxidase activities and morphology, were generally unaffected by incubation temperaturealthough aberrant morphological forms of psychrophilic strains were demonstrated at 37^{o}.

The pattern of reactions of strains to other tests was often dissimilar to the pattern of growth over the temperature range.

Pigment production, lipase activity and the egg yolk reaction were generally more marked at 5° and 15° than above and the production of acid by certain strains from carbohydrates other than glucose was frequently poor or absent at 5° or 37° compared with production at 15° to 30°.

Qualitiative differences in reactions produced by strains at different temperatures were observed with respect to carbohydrate breakdown and the changes produced in milk. Strains which were aerogenic during the fermentation of carbohydrates at 25° displayed no gas production at certain temperatures above and below 25° during the fermentation of at least one of the test carbohydrates. These findings are confirmed in part by those of Azuma and Clegg (1962) although Upadhyay and Stokes (1962) found that gas production by coliform strains was maintained during fermentation at low temperatures. Certain strains displayed either alkaline-proteolysis or alkaligenesis without proteolysis in milk at different temperatures and strains which produced acid changes in milk varied in their ability to produce coagulation at different temperatures. Of the seven strains, isolated at 5° from milk, which produced alkaline or alkaline-proteolytic changes in milk, the strains S1, S8 and S10 displayed a much reduced reaction at 5° compared with the reaction at 15° or 25°. Conflict exists in the literature with regard to the importance of temperature on the biochemical activities of psychrophilic bacteria and some workers (Sekhar and Walker, 1947; Alford, 1960; Higoshi, 1964) have discounted differences in the reactions of strains to tests performed at different temperatures although other workers (Kiser, 1944; Azuma and Clegg, 1962;

Frank, 1962) have, as in the present study, noted differences.

It is important to realise that small changes in reaction to individual tests such as glucose breakdown, tributyrin hydrolysis and protein breakdown may correspond to appreciable differences in the changes produced in complex media which contain similar individual substrates. Such differences may be displayed by strains as a result of temperature variation. If changes in the exo-enzyme activities of strains occur on lowering of the temperature the relationship between the organoleptic changes produced and growth breaks down.

For the purposes of classification and identification wide use is made of the gross biochemical changes produced by exo-enzymes liberated from autolysed cells and 25° is often used for the incubation of tests on strains similar to those examined in their study. It was found that variation in the temperature from 25° could markedly affect the response of strains to certain tests; including the production of pigment, tributyrin hydrolysis, proteinase activity and the production of acid, or acid and gas, from carbohydrates. The significance of this observation depends on the use of identification schemes. The danger lies not in the use of the results of tests for the sake of classification but in the interpretation of the tests in terms of the practical significance of the strain concerned. The assumption made by Gyllenberg et al. (1963) that the keeping quality of milk at 4° could be related to the biochemical reactions of psychrophilic isolates at 22° appears to be fundamentally unsound. To assess the keeping quality of milk or any other foodstuff in terms of the biochemical activities of the bacteria responsible it is necessary to perform the necessary tests at the

temperature of practical importance.

A detailed examination was made in Part II of the growth of the strains throughout the temperature range. Growth parameter and temperature coefficient values were derived and the strains were divided into three groups on the basis of Q_{10} values. Relative to growth in 0.2% tryptone medium a typical psychrophile exhibited a Q_{10} (5° - 15°) value of less than 4, a Q_{10} (15° - 25°) of not appreciably greater than unity and a Q_{10} (25° - 35°) value of well below unity. A typical mesophile exhibited a high Q_{10} (5° - 15°) value, a Q_{10} (15° - 25°) greater than unity and a Q_{10} (25° - 35°) greater than unity. Exceptions to these two groups were placed in an 'Intermediate' group because they displayed various growth responses throughout the temperature range and did not conform to either of the other defined groups. The strains grouped as psychrophiles generally grew poorly, or were unable to grow, at 37° which agrees with the simple definition of a psychrophile proposed by Thomas *et al.* (1960) and Colwell and Liston (1961).

The addition of 0.5 - 4.5% sodium chloride to the tryptone medium introduced a stress on the growth of the strains, additional to that caused by variation in the temperature, which was reflected in changes in growth parameter and temperature coefficient values. The rates of growth at each temperature in individual salt media were compared with the rates of growth in tryptone media using a derived factor, the St value. Strains were shown to display individual responses to the combined variation of temperature and salt content of the medium. In general the apparent lag increased and the growth rates and maximum cell crops decreased with increase in salt concentration from 0.5% to

4.5% and greater lags were shown in the salt-free medium than in 0.5% salt medium. Certain strains were shown to tolerate salt better at low temperatures than at temperatures closer to their optimum.

In Part III an examination was made of the ability of strains to utilise single amino acids as carbon, nitrogen and energy sources. On the basis of growth parameters and St values certain amino acids, notably glutamic acid and arginine, supported growth comparable to growth in tryptone medium. Strains differed markedly in the range of amino acids utilised. Tryptophane was not used by any strain and few strains utilised glycine. Temperature had a variable effect on the utilisation of amino acids and the Q_{10} patterns were often similar in glutamic acid, arginine and tryptone media, but the growth of psychrophilic strains tended to be retarded at low temperatures in lysine, leucine and glycine media. The effect of temperature was most marked in the utilisation of β-alanine. The growth of several strains was restricted to 15° and 25°, or to only 25° in this medium and strain S20 was the only isolate able to display growth throughout the temperature range.

The effect of temperature on the relationship between ammonia production and the growth of strains in these amino acid media was also examined. The liberation of ammonia during growth generally approximated to one of four types of curves which were shown in different combinations by individual strains throughout the temperature range. Since the breakdown of amino acids corresponds to the ultimate phase of protein degradation the differences shown between strains and by single strains at different temperatures have important practical implications.

Part IV comprised a study of the proteolytic activities of the strains at 5° to 37°. The casein precipitation technique of Sandvik (1962) was employed to examine the primary and secondary phases of caseolysis and electrophoresis was used to examine the early tertiary phase. As was shown for the production of ammonia during the growth of strains in amino acid media, strains also exhibited variations in the relationships between casein precipitation and growth and between tributyrin hydrolysis and growth. Temperature was shown to have a profound effect on the production of exo-enzymes during the growth of individual strains. The conclusion reached by Greene and Jezeski (1954), Van der Zant (1957) and Hurley et al. (1963) that proteinases are liberated towards the end of the logarithmic phase of growth was shown to be true only for certain strains at certain temperatures.

Electrophoretic studies revealed some differences in the fast running fractions produced by different strains during caseolysis and variations were also found between the fractions produced by proteolytic strains and those produced due to the activity of rennin and trypsin on casein. Certain strains displayed some differences in the fractions produced at various temperatures. Strain S20 produced good growth at 5°, 15° and 25° and although breakdown of casein was virtually complete at 25° little change was produced at 5°. Conversely, strain S3 produced appreciable caseolysis during growth at 5° and 15° yet caseolysis did not occur during appreciable growth at 25°. Therefore, the variation, observed by Punch et al. (1960), in the numbers of cells of individual psychrophilic bacteria required to cause organoleptic changes in milk may be seen to be due, not only to the individual enzymic activities of

the strains, but also to the differential effect of temperature on the activities of the strain concerned.

The growth of psychrophilic bacteria in milk or other foodstuffs held at low temperature may have ramifications which have been little investigated. Sandvik and Fosgum (1963) showed that proteinases produced by psychrophiles in bulk-tank milk at 4° rendered pasteurisation. The possibility is therefore presented of the caseolysis of market milk by preformed enzymes and the production of such enzymes would depend on the ability of the psychrophilic bacteria involved.

A further aspect concerns the influence of the growth and enzyme activity of psychrophiles on the subsequent growth of other micro-organisms in the foodstuff. Protein breakdown by psychrophiles may provide substrates for the growth of other bacteria at the same or at higher temperatures of storage. Marth and Frazier (1957) observed that psychrophilic Flavobacterium strains did not grow well in whole milk but required the products of milk protein breakdown. Poznanski et al. (1965) showed that Strep. thermophilus and M. caseolyticum strains were better able to utilise casein hydrolysate than whole casein and Tokita (1966) also demonstrated that peptides provided for the rapid growth of lactic acid bacteria.

A field of study is indicated involving the influence of bacteria on each other. The interaction of strains as mixed cultures has been little studied (DiGiacinto and Frazier, 1966) and never in relation to the ecological study of the spoilage of foodstuffs. In this context a particular study of the effect of temperature on the

relationships between psychrophiles and other groups of bacteria is indicated.

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