A STUDY OF THE HEAT-STABLE CASEIN PROTEOLYTIC ENZYME SYSTEMS PRODUCED BY SOME STRAINS OF PSYCHROPHILIC BACTERIA

by

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DECLARATION

Seven of the bacterial cultures used in this work were generic classified and were provided by the Hannah Research Institute, selected from a computer list of organisms collected during a bacteriological survey of the raw milk refrigerated supplies in the West of Scotland.

The work involved in section 1 in chapter V was carried out with operational help from Dr. O' Donnell and Mr. G. Davies.

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ABBREVIATIONS

The standard abbreviations, as recommended by the Editors of the Biochemical Journal (Biochem. J., 1973, 131, 1-20) are used throughout this thesis, with the following additions:

BSA = Bovine Serum Albumin; (see Miller, 1959).

BSM = Basal Salts Medium; (see Skerman, 1967).

bsm = basal salts medium; (" " ").

D = D-value as defined by Driessen and Stadhouders (1974).

D.F. = Dilution Factor; (see page 96 and Sandvik, 1962).

D.U. = Diffusion Unit; (see Sandvik, 1962).

DU = " " "

E' = Energy = ΔH^{o}; as used in the Van't Hoff Isochore equation (d ln K/d T = $\Delta H^{o}/RT^{2}$).

%H = Estimation of % hydrolysis (see page 102).

N.C.T.C. = National Collection of Type Cultures.

O.D. = OD = Optical Density = Turbidity Units

R.A. = RA = Residual Activity; (see page 104).

R' = Multiple Correlation Coefficient = r^{2}.

r = Linear " "

S1 to S8 = Abbreviations for Strains as listed on Table 1 (following page 27).

T = Temperature in ^{o}K.

U.F. = Ultrafiltration.

w = Distilled Water

Z.D. = ZD = Diffusion Zone Diameter in mm.

SUMMARY

This study comprised an investigation of eight strains of Gram-negative psychrophilic bacteria with respect to their growth and protease production at 5° C. Each of the eight strains was inoculated into basal salts medium (BSM) supplemented with 0.5% of a commercial bacteriological medium as source of organic nitrogen. Eleven commercial media were assayed in this way with each of the cultures. Tryptone supplements supported satisfactory levels of protease production and growth in relation to the remaining test commercial media. Protease production did not always correlated with growth levels, nevertheless, good correlations were observed for all the experimental strains when tryptone was used to supplement BSM. The early growth phases displayed by the strains were enhanced by moderate convectional aeration at 5°.

Extracellular protease preparations were recovered from 5 days aerated cell free growth media using BSM supplemented with 0.5% tryptone. These preparations were used to study the electrophoretic patterns of skim milk casein hydrolysis. Protease activity was generally directed against both α_s- and β-caseins, nevertheless, α_s-casein was often less susceptible to hydrolysis. Both α_s- and β-caseins became equally susceptible to hydrolysis when solutions of isoelectric casein/protease preparations were heated to 142° C for one minute.

The heat stability properties of the protease systems were also studied. The protease system produced by Pseudomonas fluorescens was affected by the stabilizing activity resulting from the presence of certain ions in BSM. In cell free growth media at pH 6.8, the heat inactivation curves were shown to have two distinct inactivation stages at temperatures below 100° C and a single inactivation stage at temperatures higher than 110° C. At 98° C and under heating time exposures of 43 to 76 minutes, the inactivation reactions of all test proteases progressed readily and led to levels of 10% of residual activity. At temperatures of 110° C or above the protease systems were easily inactivated during heating/time exposures of 110° C for 16 to 20 minutes or 140° C for 3 to 4 minutes and resulting in 10% residual activity. The heat inactivation curves for those temperatures ranging from 110° C to 140° C followed an exponential inactivation pattern.

These results are further discussed in relation to problems of milk spoilage.

INTRODUCTION

PSYCHROPHILIC BACTERIA

Terminology

The property of certain organisms to grow at or near 0° C was recognized by Forster (1887), being described as phosphorescent bacteria from fish preserved at low temperatures. Forster (1892) discovered a much wider distribution of these organisms in nature and referred to them as "glaciale bakterien". The term "psychrophile" was originally used by Schmidt-Nielsen (1902) to define organisms capable of growing and surviving at 0° C. Müller (1903) opposed this term since these organisms were able to grow better at the mesophilic temperature range. Kruse (1910) used the term psychrotolerant for those bacteria capable of growing at or below 0° C. Rubentschick (1925) preferred to define them as psychrocartericus. Various alternative nomenclature of psychrophiles appear in the literature. These include the terms psychrobe (Horowitz-Wlassowa and Grinberg, 1933); cryophile (Jezeski and Macy, 1946); thermophobic bacteria (Edsall and Wetterlow, 1947); frigophile (Hempler, 1955); and psychroauxan (Thomas *et al.*, 1960). Hucker (1954) used the term facultative and obligate psychrophile to differentiate between those able to grow at 0° C but not at 32° C and those capable of growing at both temperatures, respectively. Opposing this Eddy

(1960) proposed the term psychrotrophic to describe those bacteria capable of better growth at higher temperatures but, which would still produce some activity at or near 0° C.

Traditionally microbiologists and diagnosticians have related new isolated organisms to the most common natural habitat in which they can be found. Psychrophiles are widely distributed in nature (Morrison and Hammer, 1940,1941; Morris, 1942) a factor which has probably contributed to the difficulty of grouping them either in relation to an optimum temperature or to their natural source in the environment. Thomas et al. (1949) suggested that bacteria isolated from refrigerated milk, soil and water be defined as facultative psychrophiles. The term obligate psychrophile would be used to describe those organisms isolated from marine environments which were capable of growth below 0° C and which would quickly die at temperatures between 25° C and 30° C (Zobell and Feltham 1938; McDonald et al., 1963; Morita and Albright, 1965). The differentiation between facultative and obligate psychrophiles has gained considerable acceptance (Lawton and Nelson, 1954; Van der Zant and Moore, 1955; Rose, 1962). Definitions based on their ability to grow at or near 0° C have been used by Greene and Jezeski (1954), Olson et al. (1955) and Barber (1962). Baig and Hopton (1969) have attempted to define them by their temperature differential cha-

racteristics derived from the Van't Hoff Arrhenius equation.

Harder and Veldkamp (1967) have isolated psychrophiles with a maximum growth temperature of 20° C and Bedford (1933) has demonstrated the ability of some strains to grow at -7.5°C. Ron and Davis (1971) have shown that the presence of specific growth factors can affect the optimum growth temperature of certain microbial species. Thus, further difficulties can be encountered in defining psychrophiles on the basis of their growth rate since nutritional requirements can often change with temperature, giving rise to a new demand for a particular nutrient related to the new temperature (Scholefield, 1967). In general, when the temperature is near the minimum for growth of a certain organism there is a much greater nutritional requirement (Kiser, 1944).

The controversy involving the definition of psychrophiles led to the adoption of the term psychrotrophic (Eddy, 1960) by the dairy microbiologists. However, recent investigations have shown the existence of obligate psychrophiles with optimum temperatures for growth between 12° C and 20° C (Morita, 1966; Harder and Veldkamp, 1968; Christian and Wiebe, 1974; Morita, 1975; Gunnar and Matches, 1976).

Facultative psychrophiles are probably able to adapt themselves to lower temperatures (Olsen and Metcalf, 1968) and can thus be differentiated from

obligate psychrophiles. The latter can not survive exposure to 42° C for one minute, and the material used for handling their cultures must be refrigerated (Azuma et al., 1962; McDonald et al., 1963; Morita and Albright, 1965; Morita, 1966).

In this work the term psychrophile is used to describe either those organisms which have been considere obligate or facultative with respect to their optimum temperature for growth. Thus, the term psychrophile has been best defined by Splittstoesser (1976) as "certain of the Gram-negative rods 'which' have the ability to grow at low temperatures and have long been recognized as the principal spoilage agent of refrigerated food such as red meat, poultry, fish, eggs and dairy products. The organisms are common contaminants of fresh and sea water and once were commonly referred to as water bacteria".

Sources and Types of Psychrophiles

Psychrophilic microorganisms are ubiquitous; they occur in temperate as well as polar regions (Forster, 1892; Straka and Stokes, 1960) and in the air (Müller, 1903). They are common contaminants of fresh and sea waters (Morita, 1966; Splittstoesser, 1976) or may be found in soil and harbored by animals and plants (Morrison and Hammer, 1941). They can be isolated from specimens obtained from poikilothermic animals (Fors-

ter, 1892; Colwell et al., 1973; Trust, 1975; Liston, 1976) and have been isolated at 0° C, 18° C and 36° C (Sieburth, 1964; Sieburth, 1968). These isolates were recognized as psychrophiles from marine waters. It is believed that planktons play an important role on the seasonal distribution of at least one type through out the sea water columns (Colwell et al., 1973). Certain dairy isolates have been shown to be subject to the seasonal predominance of individual types (Morrison and Hammer, 1941; Kozlova and Block, 1973). Since the manufacture of many foods, including dairy products, often involves the utilization of ingredients such as salts, stabilizers, air, water and enzyme preparations, the cumulative effect on the bacteriological quality of the final product from indirect sources can not be overlooked. The ecological distribution of psychrophiles and their significance are part of a review published by Morita (1975). Psychrophiles have been subject of an exhaustive review by Witter (1961) and their growth effects on raw milk and market milk have been reviewed by Crawford (1968).

Although psychrophiles are known to be harbored by the milking animal, their presence in milk or in dairy products is an indication of environmental contamination. The importance of these organisms as spoilage agents in milk and dairy products is well documented and includes the genera Pseudomonas, Flavobacterium, Alcaligenes, Aeromonas and Achromobacter

(Sherman et al., 1941; Jezeski and Macy, 1946; Boyd et al., 1953; Thomas, 1953; Thomas and McQuillin, 1953; Scholefield, 1963,1967; Nakanishi and Tanabe, 1971; Kiuru et al., 1971; Kielwein, 1972; Juffs, 1973b; Braatz, 1975). Members of these genera are invariably present in all raw milk samples (Foster et al., 1957). Although those types belonging to the genus Pseudomonas appear to be the most important, a high total count of psychrophiles in raw milk is frequently associated with a comparatively lower number of mesophiles and Gram-positive organisms such as micrococci (Naylor, 1976). Druce and Thomas (1972) have shown that a temperature induced competitive advantage is displayed by psychrophiles in the rinse water from milk pipelines and equipment. This temperature advantage has also been demonstrated by Swartling (1968) and Bockelmann (1969). The fact that certain strains of psychrophiles, especially Pseudomonas sp. (Woolford, 1972), and Achromobacter sp. (Troller and Frazier, 1963) are reported to be able to produce "antibiotic-like" metabolites, could account for their predominance in raw refrigerated milk. The predominance of Pseudomonas spp. can also be accounted for by their ability to inhibit the growth of Achromobacter and Alcaligenes strains (Chai et al., 1968; Vanderzant, 1968; Vanderzant and Custer, 1969). Some observations have shown that the inhibitor factor was due to the presence of extracellular metabolites in the cell-free filtrates of Pseudomonas spp.

Similar observations were made by Sandvik and Fossum (1963). This also may be favored by the presence of casein hydrolase systems, intensely produced by pseudomonads (Sandvik, 1962; Scholefield, 1967; Fallon, 1972), making them able to grow rapidly, without sole dependence on energy through the Embden Meyerhof path way, for growth at lower temperatures, and possiblely favored by a more effective electron transport system. Another possible contributing factor for the predominance of certain strains of psychrophiles in raw milk is the degree of dissolved oxygen which is influenced by lower temperatures (Lockhart and Squires, 1963; Meynell and Meynell, 1970). The lower the temperature of the milk in contact with a cold air surface, the lower the partial pressure of oxygen required to reach a certain saturation equilibrium between the liquid and the air. Fallon (1972) studied eight strains of Pseudomonas, under aerated and static conditions at 25° C, showed a relatively small difference between aerated lag phase and static lag phase for most of the strains. However, O'Donnell (1975) has demonstrated a much greater difference between static lag phase and aerated lag phase at 5° C. During storage and transport of refrigerated milk a similar increase in dissolved oxygen level is likely to occur, especially if the storage time is longer than an average of 38 hours (Sandvik and Fossum, 1963). The level of aera-

tion of refrigerated milk, to a large extent, influences the predominance of certain types of psychrophiles (Hamamoto and Kanauchi, 1973). When raw milk is rapidly refrigerated to 4° C (normally within one hour or less), a good microbiological quality can be maintained for a period up to 24 hours at the farm (Majewski, 1975). Conversely, when raw milk is stored for longer periods the number of psychrophiles increases. Psychrophiles have been isolated from raw milk (Godbille, 1971; Juffs, 1973b; Adams et al., 1975; Yano and Morichi, 1977), from pasteurized milk (Boyd et al., 1953; Kielwein, 1972; Juffs, 1973a; Hamamoto and Kanauchi, 1973), from milk pipe line systems and equipment (Long and Hammer, 1941; Morrison and Hammer, 1941; Kiuru et al., 1971), from milk held at low temperatures (Hamamoto and Kanauchi, 1973), and from a variety of dairy products (Jezeski and Macy, 1946; Arnott et al., 1974; Fox et al., 1975).

Pseudomonas spp. have been found to be the most frequently isolated psychrophiles from raw milk samples, followed by Achromobacter, Aeromonas and coliforms (Scholefield, 1967). Most psychrophiles described are Gram-negative rods (Brown and Weidemann, 1958; Yano and Morichi, 1977) and include Flavobacterium, Alcaligenes and Acinetobacter-Moraxella spp. (Splittstoesser, 1976). Milk as the main raw material used for the manufacture of dairy products

may be contaminated with these organisms either from water sources (Morris, 1942; Castell and McDermott, 1942) when the concentration of free available chlorine is lower than 2 p.p.m. (Witter, 1961) or from equipment not properly cleaned at the farm and in the dairy plant (Morrison and Hammer, 1940, 1941).

Important Species

The difficulties encountered in many studies of species predominance in raw milk are either related to the seasonal changes in the relative numbers of bacterial contaminants (Kozlova and Block, 1973), or to variations in the isolation and enrichment procedures adopted. Marth and Frazier (1957) made enrichments in milk held at 7° C while Juffs (1973b) plated initially in penicillin agar followed by incubations at 30° C and 7° C. Also the fact that some of the psychrophiles, such as Moraxella-Acinetobacter, may appear described using different names (Henriksen, 1973) and may show great nutritional and biochemical versatility; the differentiation among their species is rather difficult and not very well established.

Pseudomonas fluorescens, Pseudomonas fragi and Pseudomonas putrefaciens have been identified as common contaminants of milk and dairy products (Marth and Frazier, 1957; Samagh and Cunningham, 1972).

Samagh and Cunningham (1972) have also included species listed as "psychrophilic Pseudomonas aerugino - sa-like organisms", Pseudomonas putida and Pseudomonas alcaligenes. Juffs (1973b) has listed the pseudomonads found in milk in the following order of importance: Pseudomonas fluorescens, Pseudomonas aeruginosa, Pseudomonas maltophilia, Pseudomonas pseudoalcaligenes, Pseudomonas cepacia and Pseudomonas alcaligenes. Fox et al. (1975) studied the incidence and types of phospholipase C producing bacteria in fresh and spoiled homogenized milk; 34 isolates from 25 homogenized milk samples resulted in 62% Pseudo - monas, 18% Alcaligenes and Enterobacter, 18% Acinetobacter together with Bacillus and Flavobacterium, and 3% Citrobacter. Samagh and Cunningham (1972) have isolated a total of 653 psychrophilic strains from milk and milk products. Of these 182 were pseudomonads consisting of 53.8% Pseudomonas fluorescens or Pseudomonas aeruginosa-like strains, 43.9% Pseu - domonas putida, Pseudomonas fragi and Pseudomonas putrefaciens and 2.7% Pseudomonas alcaligenes strains. Kielwein (1972) isolated 200 different strains of Pseudomonas from market pasteurized milk, of which 56.5% were identified as Pseudomonas fluorescens.

ENZYME SYSTEMS OF PSYCHROPHILES

Terminology

Due to the rapid growth in the science of enzymology, the International Union of Biochemistry (IUB, 1964) has classified enzymes in six main groups on the basis of the reactions which they catalyse. This report was prepared to serve as a basic guide for standard enzyme nomenclature.

The enzymes which are capable of degrading either food fat or protein are listed under sub-groups 3.1 and 3.4:

3.1 . Acting on ester bonds:

- 3.1.1 . Carboxylic ester hydrolases.
- 3.1.2 . Thiolester hydrolases.
- 3.1.3 . Phosphoric monoester hydrolases.
- 3.1.4 . Phosphoric diester hydrolases.
- 3.1.5 . Triphosphoric monoester hydrolases.
- 3.1.6 . Sulphuric ester hydrolases.

3.4 . Acting on peptide bonds:

- 3.4.1 . Alpha-amino-acyl hydrolases.
- 3.4.2 . Peptidyl-amino acid hydrolases.
- 3.4.3 . Dipeptide hydrolases.
- 3.4.4 . Peptidyl-peptide hydrolases.

Many psychrophilic bacteria are able to secrete proteolytic and lipolytic enzyme systems (Dempster, 1968). This fact is important in the spoilage of re-

frigerated milk and dairy products, since both enzyme systems (acting on ester bonds and acting on peptide bonds) may show significant high activity at low temperatures (Peterson and Gunderson, 1960).

Protein hydrolases have also been classified according to their point of attack on the primary structure of the protein substrate, being designated exo- or endo- (Bergmann, 1942). This classification can create confusion since these prefixes have also been used to indicate whether or not an enzyme is held within the cell wall. With regard to the concept of enzyme location, the terms intra-, inter- and extra- are often used to describe whether the enzyme is contained within the cell, between the plasma membrane and the cell wall or released outside of the cell, respectively (Davis et al., 1973).

Microbial proteases have also been classified on the basis of pH dependence, effect of inhibitors, metal involvement in the active site, and certain catalystic properties towards peptide, amide or ester bonds (Feder and Matsubara, 1971).

The extracellular proteolytic enzyme systems produced by psychrophiles have been variously designated as "casein precipitating enzymes" by Sandvik (1962) and Fallon (1972); "caseolytic enzymes" by Forschner and Pózvári (1977); "azocasein proteolytic enzymes" by Langner et al. (1973) and "casein clearing" proteolytic enzymes by Innerfield et al. (1970).

Many of the difficulties encountered in the classification of psychrophilic exoenzymes arise from the fact that most of the strains are able to release more than one enzyme (Sandvik, 1962; Driessen and Stadhouders, 1974), or, as in the case of Bacillus subtilis, an exoenzyme system which could be considered to be either an ester hydrolase or a peptide hydrolase (Sierra, 1964). Many ester analogues of amino acid substrates have been found to be hydrolysed by certain proteolytic enzymes (Hofstee, 1957).

In this study reference is made to a work in which enzyme hydrolases, collectively classified as exoenzymes by Pollock (1962), are named proteases, proteinases or proteolytic enzymes.

Proteolytic Spoilage of Milk and Dairy Products

The detrimental effect on milk quality of the action of psychrophilic extracellular enzymes is considered to be of major significance to the modern dairy industry (McCaskey, 1968; Bockelmann, 1970; Kiuru et al., 1971; Nakanishi and Tanabe, 1971; Mikawa and Hoshino, 1974; Purschel and Pollack, 1974; Yanagiya et al., 1974; Chapman et al., 1976; Kozlova, 1976; Kielwein, 1976; DeBeukelar et al., 1977; Marth, 1977; Cousin and Marth, 1977b; Miura et al., 1978).

Among the psychrophiles which have been found to be important producers of these extracellular enzymes, a higher number of strains was able to digest casein than the number of fat hydrolysing strains (Bockelmann,

1970). Mikawa and Hoshino (1974) and Purschel and Pollack (1974) reported the number of psychrophiles active against casein increases at lower temperatures. Kiuru et al. (1971) observed that changes in caseins of refrigerated raw milk required a cell concentration as high as 6×10^7 psychrophiles per ml. Nakanishi and Tanabe (1971) did not observed any change in the casein of milk stored at 0° C to 5° C for 10 days. Miura et al. (1978) have shown that the growth of Pseudomonas and Flavobacterium spp. in milk at 5° C produced proteases capable of almost 100% hydrolysis of casein in 14 to 28 days. The whey proteins (Yanagiya et al., 1974), except β-lactoglobulin (Sasano et al., 1977), have been shown to resist proteolysis to a greater degree compared to the other milk proteins.

Although changes in proteins caused by the active growth of psychrophiles in raw milk are well known, their effect on the stability of milk to processing seems rather more complex. Cousin and Marth (1977a) have shown that the growth of psychrophiles in milk causes changes in protein heat stability. Adams et al. (1976) demonstrated that a very low psychrophilic population was needed to detect proteolytic activity and their growth seriously affected the heat stability of the milk proteins. The visual effect of milk stability changes resulting from psychrophilic proteolysis may appear as "sweet-curdling" formation (Atmaram, 1974) or gel formation (Law et al., 1977a,b,c). Furthermore, an

excessive growth of Pseudomonas spp. in raw skim milk prior to processing at 80° C for 15 seconds has been shown to stimulate the growth of psychrophilic Bacillus cereus strains., The Bacillus cereus spp. reached counts of 40 to 60 million c.f.u./ml with ten days of refrigerated storage and lead to the appearance of the sweet-curdling defect in the processed milk (Atmaram, 1974). Law et al. (1977a) have shown that the time required for the onset of gelation in commercially sterilised milk was dependent on the previous rate of growth of Pseudomonas sp. in the raw milk. In UHT sterilised milk the problems of psychrophilic growth in the raw milk, sometimes resulting in gelation and/or bitterness in the processed product, have been studied by Lawton and Nelson (1954), Mayerhofer (1972), Hamamoto and Kanauchi (1973), Melo et al. (1973), Biryukova et al. (1974), Cheng and Gelda (1974), Bottazzi and Battistotti (1975), Adams et al. (1976), Langsrud and Hadland (1977), Nakanishi et al. (1977) and Law et al. (1977a,b,c). Bitterness in milk (Driessen, 1976; Langsrud and Hadland, 1977) and in cheese (Kishonti and Sjöstrom, 1970; Richardson and Creamer, 1973; Law and Sharpe, 1977) has been reported to be due to the growth of psychrophiles. Bitterness in cheese has also been reviewed by Crawford (1977) and Swaginga (1977). The bitterness has been shown to result from protein hydrolysis and the presence of small peptide fractions (Moreno and Kosikowski, 1972; Richardson and Creamer, 1973). Emmons et al.

(1960) reported that bitterness was negatively correlated with free amino acid content.

Although certain strains of Pseudomonas have been reported to be able to increase the coagulation rate of milk during cheese making (Cousin and Marth, 1977d), the growth of psychrophiles in milk is also known to increase the time required for rennet coagulation (Cousin and Marth, 1977a). The proteolytic spoilage of raw milk during prolonged storage may reduce the yield of cheese curd (Nelson and Marshall, 1977). Cousin et al. (1977) have suggested that the proteolytic spoilage of raw milk may result in a lower cheese yield.

In milk the α_{s1}-casein component tends to be less susceptible to proteolysis because of a greater degree of organization predicted from its low content of prolyl residues. Similarly, the κ-casein component is probably more susceptible to proteolysis than α_{s1}-casein. The β-casein component seems to be the most susceptible to proteolysis as predicted from its high prolyl residue content and also from the fact that stabilization seems to depend on hydrophobic clusters rather than extensive α-helix stabilization (Mercier et al., 1972). A cluster of phosphoseryl residues homologously present in α-casein and β-casein, seems to play a role in the calcium binding ability of the casein micelles. The multiple esterase systems as described by Lawrence (1967), and/or microbial phosphatase which has a very low substrate specifici-

ty (Lehninger, 1971), are possibly responsable for making the casein micelles less stable or for the complete hydrolysis of casein when suficient storage time is allowed (Travia and Veronese, 1940-41).

The proteolysis of casein components is influenced by different concentrations of sodium chloride (Fox and Walley, 1971). The rate of proteolysis of α-casein by rennet and by pepsin increases at higher ionic strength (5 to 10% NaCl) and is contrasted by a decrease in the rate of β-casein proteolysis under similar conditions.

Proteases produced by Pseudomonas spp. show a greater tendency to hydrolyse β-casein as compared to α_{s1}-casein (Tsugo and Yamauchi, 1959; Kiuru et al., 1970, 1971; Yanagiya et al., 1974; Purschel and Pollack, 1974; Cousin and Marth, 1977b,c). A similar proteolytic susceptibility was found for Bacillus cereus protease (Choudhery and Mikolajcik, 1970). The hydrolysis of α_{s1}-casein and β-casein, in the homogenous state, have been shown to increase 12 to 15 times as compared to the same hydrolysis carried out using a heterogenous sodium caseinate system (Fox and Guiney, 1973). Downey and Murphy (1970) demonstrated that at temperatures below 30° C there is an increase in the amount of dissociated β-casein which, according to Fox and Guiney (1973), makes it more readily available for hydrolysis. The rennin hydrolysates obtained from β-casein were bitter in flavor whereas those obtained from α_s-casein were not (Fox and Wal-

ley, 1971). Moreno and Kosikowski (1972) studied the hydrolysis of β-casein and have also observed the liberation of bitter peptides. Some of these peptides have been identified as the segments Tyr (193) and Val (209) from the carboxyl end of β-casein (Visser et al., 1975). Also, six less susceptible possible cleavage sites were identified. These appeared to have a strong tendency to associate and were thought to be responsable for the tendency of β-casein to aggregate. Bitter peptides can be obtained from β-, α_{s1}- or k-casein components by hydrolysis, and bitterness seems to be dependent on the overall hydrophobicity of the released peptide (Mercier et al., 1972). In general α_{s1}-casein is considered a hydrophobic protein, but to a lesser degree if compared to β-casein.

Heat-Stable Enzyme Systems

The production of heat-stable enzymes by psychrophiles in a variety of media, milk and dairy products, together with claims regarding their reactivation following heat treatment, have been reported throughout the literature (Wright and Tramer, 1953; Sandvik, 1962; Pinheiro et al., 1965, 1966; Kresheck and Harper, 1967; Juffs and Doelle, 1968; Peereboon, 1969; Driessen and Stadhouders, 1971, 1973, 1974; White, 1972; Knaut and Mech, 1972; Fallon, 1972; Mayerhofer, 1972; Mayerhofer et al., 1973; White and Marshall, 1973;

Cheng and Gelda, 1974; Malik and Swanson, 1974, 1975; O'Donnell, 1975; Adams et al., 1976; Kishonti, 1975; Malik, 1976; Kielwein, 1976; Speck and Adams, 1976; Barach et al., 1976a,b; Cousins et al., 1977).

Cogan (1977) has reviewed some of the reports of heat resistant lipases and proteases in relation to the quality of dairy products.

Wright and Tramer (1953) first reported the reactivation of microbial phosphatase following heat treatment at 77° C. The enzyme was produced by Pseudomonas sp. and required 30 minutes at 77° C for complete inactivation when disodium p-nitrophenyl phosphate was used as substrate. Two separate explanations to account for this reactivation have been published by Kresheck and Harper (1967) and Peereboon (1969).

The presence of thermostable proteases produced by Pseudomonas fluorescens and strains of aerobic and facultative anaerobic organisms was reported by Sandvik (1962). Sandvik and Fossum (1963) suggested that all psychrophiles capable of growth in farm refrigerated milk would be able to produce heat resistant enzymes.

Juffs and Doelle (1968) have reported a heat resistant protease produced by Pseudomonas aeruginosa ATCC 10145 which showed 6%, 36% and 100% inactivation following heat treatments of 63° C for 30 minutes, 72° C for 15 seconds and boiling for 2 minutes, respectively. The enzyme was active in a pH range of 5.5 to 9.0 and stable at 2° C up to 30 days.

Pinheiro et al. (1965) demonstrated the stability of lipases following heat treatment of milk, and the presence of the enzymes was also detected in cheese made from pasteurized milk. Heat resistant lipases produced by selected organisms have also been reported by Driessen and Stadhouders (1971, 1973, 1974). Driessen and Stadhouders (1973) indicated that part of the lipase system produced by Pseudomonas fluorescens could be made inactive at a relatively low temperature (52.5° C to 57.5° C), while the remainder was highly heat resistant, having a D value (the time required to produce 90% inactivation at a certain temperature) of 16 minutes at 130° C. In a further research report, Driessen and Stadhouders (1974) observed that the heat resistance of the unpurified extracellular lipase system demonstrated a biphasic relationship between temperature and activity at or above 50° C. The D values were, for the first stage of inactivation, 4.8 minutes at 60° C and for the second stage 200 minutes at 85° C or 4.8 minutes at 150° C. The first stage of inactivation was considered to be due to the denaturation of the native enzyme form and the second stage to a chemical inactivation. This type of biphasic inactivation profile for lipases has also been observed by O'Donnell (1975).

White (1972) claimed that heat resistant proteases produced by Pseudomonas fluorescens remained inactive when added to raw milk which was then immediately

pasteurized by the HTST system, but increased the Hull values (μg of Tyr and Trp released per ml) for milk when the enzymes were added after pasteurization.

Knaut and Mech (1972) observed the heat resistance of proteases produced by Pseudomonas fluorescens cultured in 10% milk solids nonfat solutions. Negligible enzyme inactivation was observed at 63° C, 50% at 75° C for 10 minutes, 70% at 85° C for 5 minutes and 100% inactivation after heating for 5 minutes at 100° C.

Mayerhofer (1972) and Mayerhofer et al. (1973) have studied the heat stable proteases produced by Pseudomonas fluorescens P26 and reported D values of 15 hours at 62.8° C, 8 hours at 71.4° C and 9 minutes at 121° C. The organism itself gave a D value of only 2.6 minutes at 62.8° C. They also showed that milk, whey and casein each had a protective effect on the rate of inactivation of the enzymes by heat, compared to that shown in bacteriological broth.

White and Marshall (1973) conducted experiments to evaluate the effect on cheddar cheese flavour scores of the addition of heat stable Pseudomonas fluorescens protease system. Significant quantities of enzymes were added to milk prior to pasteurization at 63° C for 30 minutes. They reported, however, that only 16.5% of the variation in flavour score of cheese made from this milk could be attributed to the effect of enzyme addition.

The majority of the proteases originating from psychrophiles studied by Adams et al. (1976) were shown to withstand a temperature of 149° C for 10 seconds. Speck and Adams (1976) have suggested various methods for controlling the problems resulting from heat stable proteases. Various workers have reported psychrophilic proteases capable of withstanding temperatures of 130° C for 5 to 10 minutes (Kishonti, 1975), 149° C for 7 seconds (Barach et al., 1976a), 149° C for 10 seconds (Barach et al., 1976b) and 140° C for 3.5 seconds (Cousins et al., 1977). Barach et al. (1976a) have demonstrated the stabilization of a Pseudomonas sp. protease by divalent metal ions such as Zn^{2+} and Ca^{2+}. In a subsequent paper these protease were shown to remain inactive in milk subject to a heating process of 55° C for 10 minutes (Barach et al., 1976b). This was considered to be due to complex combinations of the enzymes with either proteins or lipids.

Although the various references sited in this section would suggest that extracellular proteolytic and lipolytic enzyme systems produced by psychrophiles are highly heat resistant there is insufficient information to show that this is a general phenomenon. The majority of the papers sited above relate to the genus Pseudomonadaceae and in particular Pseudomonas fluorescens. That this thermostability is a common feature of enzymes produced by such psychrophilic genera as Acinetobacter, Alcaligenes, etc., is still to be firmly established.

SCOPE OF THE INVESTIGATION

Psychrophilic microorganisms are of importance in food spoilage and, in particular, in relation to the spoilage of milk and dairy products. The fact that these organisms are capable of growth at refrigeration temperatures indicates their importance in the storage life of perishable food products at near zero temperatures. Also, of particular significance, is the fact that many of these organisms are capable of producing active proteolytic and lipolytic extracellular enzyme systems at these temperatures (Nashif and Nelson, 1953; Juffs *et al.*, 1968; Kielwein, 1976; Nakanishi *et al.*, 1977).

In the dairy industry the importance of these organisms has increased over the past 10 to 20 years because of the modern trends in milk handling, transportation and storage. Milk processing operations have become more centralised, resulting in the milk being held for longer periods prior to processing. This has resulted in the dependence primarily on refrigeration to control bacterial growth during the storage period. The final consequence has been the emergence of psychrophilic bacteria as the major constituents of the bacterial flora of milk. Marth (1977) has stated that "the presence of psychrophilic bacteria in raw milk and in pasteurized milk products is the single grea-

test microbiological problem that is faced by today's dairy industry."

The production of heat resistant proteolytic and lipolytic enzyme systems by psychrophiles presents the possibility of spoilage occurring in dairy products without the presence of the producing organisms. Thus, standard microbiological methods of assessing product quality may not relate to the physical and/or organoleptic changes in the product (Hankin and Dillman, 1968). Growth of psychrophiles and proteolysis of the protein components of milk may result in gelation of UHT milk either during the heat treatment (Bengtsson et al., 1973; Adams et al., 1976; Law et al., 1977a,b, c) or during the storage of the finished product (Andrews and Cheeseman, 1972; Cheeseman and Knight, 1974; Andrews, 1975). This proteolysis in milk can also have far reaching effects in many areas of milk processing (Juffs, 1973a; Biryukova et al., 1974; DeBeukelar et al., 1977; Cousin and Marth, 1977a,b,c, d), including cheese manufacture (Moreno and Kosikowski, 1972; Driessen, 1976; Chapman et al., 1976; Nelson and Marshall, 1977).

In this study it is proposed to examine the growth and proteolytic activity of a number of psychrophilic bacteria at 5°C. This investigation will also include a detailed study of the heat stability of their protease systems and the extent of the activity of the enzymes on the native caseins in skim milk (casein in its native form found in skim milk).

CHAPTER I

MATERIALS AND METHODS

SECTION 1. MICROBIOLOGICAL METHODS

(a) Strains and Growth Media

Eight strains of psychrophilic microorganisms were examined in this study. Of these, seven cultures were provided by the Hannah Research Institute (HRI) from a computer list of organisms collected during a bacteriological survey of the raw, refrigerated milk supplies in the south west Scotland (Muir and Kelly, 1978). These organisms were selected on the basis of their ability to produce proteolytic enzymes as measured by the clearing of casein agar plates and by the water agar plate test of Taylor (1967, 1971). On the basis of Gram stain, motility and biochemical behaviour (Appendix A), the organisms were generically classified as shown in Table 1. The remaining organism chosen for this study was *Pseudomonas fluorescens* (N.C.T.C. 10038), a species frequently associated with the proteolytic and lipolytic spoilage of milk and dairy products (Thomas, 1974; Law *et al.*, 1976; Miura *et al.*, 1978).

The strains used were referred to throughout this work as S1 to S8 (see Table 1).

The strains were maintained on Nutrient Agar slopes. When required for inoculations the strains were subcultured onto fresh nutrient slopes and incubated at 21° C for 18 hours. This procedure was then

Table 1. Nomenclature of Test Strains.

Strains	Computer Identification Code	Generic Classification
S1	M 6 - 1 (HRI)	*Pseudomonas* sp.
S2	M 1 -13 (HRI)	*Pseudomonas* sp.
S3	KM6 - 3 (HRI)	*Enterobacillus*, *Pasteurella*, *Aeromonas* or *Vibrio*.
S4	KM1 - 5 (HRI)	*Flavobacterium* sp.
S5	KM1 - 7 (HRI)	*Acinetobacter*, *Brucella*, or *Moraxella*.
S6	S 1 - 2 (HRI)	*Alcaligenes* sp.
S7	M 3 - 1 (HRI)	*Chromobacterium* sp.
S8		*Pseudomonas fluorescens* (N. C.T.C. 10038)

repeated to ensure complete reactivation of the cultures. An incubation temperature of 21° C was used since it is known to fulfill the optimum growth conditions for both facultative and obligate psychrophiles (Ingraham and Stokes, 1959; Morita, 1966; Mayerhofer, 1972; Mayerhofer et al., 1973; Oliveira, 1976). After the final incubation period the cells were suspended in sterile glass distilled water, transferred to sterile 150 mm. x 13 mm. optical matched test tubes and the suspensions adjusted to 0.3 x 100 units of optical density (E.E.L. Nephelometer, Evans Electroselenium Ltd., Essex). The cultures prepared in this manner were used as standard inocula.

The basal medium used in this study was Pope and Skerman's mineral salts (Skerman, 1967; see Appendix B) supplemented with 0.5% (w/v) of commercial media. The combined media were sterilised at 121° C for 15 minutes and cooled to the incubation temperature prior to inoculation.

(b) Growth Conditions and Determinations

All the strains were treated in an identical fashion. A variety of commercial media were tested for each strain in order to select, if possible, a single medium which would support satisfactory growth and protease production for all the strains studied. In order to obviate the problems associated with enzyme

system isolation from solid media, liquid culture technique were used in this study (Johnson et al., 1967). Furthermore, shallow layer culturing was adopted since it has been shown that enzyme production by many obligate aerobic microorganisms is optimal under these conditions (Nashif and Nelson, 1953; Sodex and Hofman,1970; O'Donnell, 1975).

The BSM supplemented with 0.5% (w/v) of a synthetic medium was dispensed in 100 ml volumes in 500 ml Erlenmeyer flasks. The final pH was adjusted to 7.00 $\pm$ 0.05 prior to sterilization at 121^{o} C for 20 minutes. The flasks were cooled to 25^{o} C and incubated with 0.025 ml of the appropriate cell suspension prepared as previously described. The inoculated flasks were incubated at 25^{o} C under thermostatic controls. The rate of growth and protease production were measured at 24 hours intervals over a five days period, by aseptically withdrawing samples from each flask. Growth was stopped by the addition of 0.1% (w/v) Thimerosal (Koch-Light Laboratories) to the samples, after growth measurements and before enzyme assays.

Growth was determined by nephelometry. Shockman (1963) demonstrated that nephelometers were more sensitive than spectrophotometers and colorimeters for determining optical density, since they measure light reflected by suspended particles rather than transmitted light. Optical density (O.D.) measurements as an index of cell growth have been used by Sinclair and Stokes (1962), Scholefield (1967) and Fallon (1972). Sinclair

and Stokes (1962) observed a close relationship between optical density, dry weight of the cells and viable cell count. Scholefield (1967) observed a linear relationship between optical density and cell number up to the equivalent of 2.0 x 100 O.D. units. All measurements were made in optically matched 150 mm. x 13 mm. test tubes relative to a ground glass standard set to 0.5 x 100 O.D. units or 1.0 x 100 O.D. units and distilled water standard set to zero O.D. units. Although inaccuracies in the reading can result from the presence of air bubbles either on the top of the liquid or on the inside wall of the tube, good results can be obtained if the precautions listed by Scholefield (1967) are taken.

Following growth measurements, the bacteria were removed by centrifugation in a bench centrifuge (M.S. E. Ltd.) and the supernatant used for enzyme assay. Protease production was measured by the zone diffusion method of Sandvik (1962).

(c) Growth and Protease Production

In these experiments the organisms were cultured in the basal salts medium supplemented with 0.5% (w/v) Oxoid Tryptone (OTNE), since in preliminary experiments it was shown to be a good supporting source of organic nitrogen for growth and protease production.

The standard inocula were prepared as previously described. The liquid medium was dispensed in 1200 ml

volumes in five litre Erlenmeyer flasks and sterilized at 121° C for 20 minutes, and cooled to 5° C. A volume of 0.35 ml of standard inoculum was added to each 1200 ml volume of medium. All cultures were inoculated in replicate under identical conditions.

The flasks were incubated at 5° C for 120 hours with low speed agitation being supplied using magnetic stirring tables. Low levels of agitation or aeration have been claimed to greatly increase the rate of growth and enzyme production by psychrophiles at low temperatures (Fallon, 1972; O'Donnell, 1975). To illustrate this effect flasks inoculated with all strains were incubated at 5° C for 12 days under static conditions and a parallel experiment for 5 days under agitation. Growth and protease production were determined at 24 hour intervals. Prior to the growth determinations samples were removed from the flasks using aseptic technique and allowed to warm to room temperature for one hour to remove air bubbles.

The extracellular proteases produced during the logarithmic phase of growth of the test strains at 5° C under agitated conditions were used for further study. The heat stability of these enzyme systems was investigated in the cell-free culture media and after concentration of the enzyme system by U.F. (Ultrafiltration). Electrophoresis relating to the studies of the action of protease systems on casein

were also undertaken.

SECTION 2. BIOCHEMICAL METHODS

(a) Protease Assay Technique

Protease assay methods have been reviewed by Fallon (1972). The majority of assay procedures for the evaluation of protease activity are based on the measurement of the relative amounts of end product resultant from the hydrolysis of protein substrates (Anson, 1938; Winnock et al., 1940; Hull, 1947; Kunitz, 1947; Rick, 1963; Reimerdes and Klostermeyer, 1976). Protein hydrolysis has been measured using Folin and Ciocalteu (1927) reagent, the hydrolysis being expressed in terms of the release of phenolic groups (Anson, 1938), this representing the first stages of protein break down. Hull (1947) used Anson's technique to demonstrate that the activity of pancreatin added to milk samples could be estimated using Folin's reagent. He expressed protease activity in terms of mg. of Tyr released after 10 minutes of hydrolysis. The activity was measured in a 0.72 N trichloroacetic acid filtrate of the reaction mixture. Since this type of method does not indicate activity during intermediate steps, but phenolic compounds, it has been criticised by Pollock (1962). Protein dye-binding techniques (Savage and Thompson, 1970) and methods based on the "Aldehyde Value" as proposed by Steinegger (1906) are capable of assessing proteolytic intermediate steps. However, they either require the utilization of

a small amount of dye, which may be difficult to measure accurately, or the use of special equipment designed for formaldehyde titration.

The utilization of casein as a substrate for the determination of protease activity has been proposed by Sandvik (1962). The method is based on a three-phase sequence degradation of casein components followed by lysis of the precipitated caseins. Casein degradation has been reviewed by Lindqvist (1963) and the hydrolysis appears to depend primarily on the break down of κ-casein leading to destabilization of the casein micelle.

The casein precipitation method of Sandvik (1962) utilizes the principle of gel diffusion of the enzyme samples through a casein agar gel. The assay method has been used by Scholefield (1967) and Fallon (1972) to estimate the protease activity in a variety of bacterial culture media and has been shown to be useful in estimating enzyme concentrations when the casein concentration is 1% . Since different proteases may show one or more zones of precipitation the method can be modified by replacing the casein substrate with gelatine (Cheeseman, 1963; Ganguli and Bhalerao, 1965).

The standard assay technique of Sandvik (1962) was adopted in this work because it required small volumes of enzyme solution, was simple and reproducible. Preliminary trials demonstrated a correlation factor of 0.995 between the zone diameter and the log of

the inverse scale of the dilution factor.

The standard protease assay plates were prepared as follows: Isoelectric casein (4 g; B.D.H.) was weighed into a 100 ml volumetric flask. Twenty ml of 0.1 M sodium hydroxide was added, the contents agitated quickly for five minutes and the volume adjusted to 95 ml with distilled water. Solubilization of the casein was achieved by three successive heatings and coolings to 50° C and 10° C respectively, each step taking approximately 15 minutes. The solution was then brought to room temperature, 1 ml of 0.1% (w/v) Merthiolate added and the volume adjusted to 100 ml. This solution was stored at 4° C for a maximum period of 3 days before use.

When required, 25 ml of the caseinate solution was warmed to 50° C and mixed with 75 ml of 1.33% (w/v) sterilized, purified agar (Oxoid) at 50° C. The final pH of the mixture was adjusted to 6.2 by the addition of 0.45 to 0.50 ml of orthophosphoric acid 0.74 M. The medium was poured into 170 mm x 170 mm plate glass molds with 5 mm high prespex sides to a depth of 2 mm. Tightly fitting glass lids were placed on the top and the medium allowed to cool.

When the gel had set wells 6 mm in diameter were cut. An inoculum of 0.025 ml was added to each well, the inoculum being prepared by the addition of 1 ml of a 0.1% (w/v) Merthiolate solution to 10 ml of the culture medium. Distilled water and Merthiolate blanks

were also included among the assays. The plates were incubated at 30° C for 42 hours and the proteolytic activity was expressed as zone diameter of precipitation (Z.D.) in mm. Standard graphs relating mm Z.D. and log diffusion units (log D.U.) were prepared for the enzyme preparations in order to express the level of proteolytic activity in terms of diffusion units (Appendix C). Enzyme concentrate systems prepared by ultrafiltration were treated in a similar fashion(see also Appendix I.).

(b) Protein Determination

Protein determinations were carried out using Miller's (1959) modification of the method of Lowry et al. (1951). Other methods of protein determination such as those based on u.v. absorption, Biuret, ninhydrin or Kjeldhal were not chosen because of the reasons outlined by Lowry et al. (1951). The use of the formaldehyde titration, on the other hand, requires the removal of certain compounds which may interfere with the determination of protein (Wolfschoon and Vargas, 1977).

Immediately prior to use 50 mg. of copper sulphate was dissolved in 10 ml of 1% (w/v) alkaline sodium tartrate. One ml of this mixture was added to 10 ml of a 10% (w/v) sodium carbonate solution. One ml of the resulting mixture was added to one ml of the sample under study and allowed to stand for 10 minutes at room temperature. To the solution was then added 3 ml

of a 1 to 11 dilution of Folin-Ciocalteu reagent, the mixture quickly agitated, incubated at 37° C for 20 minutes and then allowed to cool to room temperature. After cooling for 25 to 30 minutes the extinction (E) of the solution was measured at 650 nm. Protein concentration was calculated from a standard graph drawn up in a similar manner using bovine serum albumin (Koch-Light Laboratories). The unknown protein samples were diluted so that the readings fell within the linear portion of the standard graph (Appendix D. and I.).

(c) Electrophoresis

The technique of electrophoresis is based on the principle that a charged particle or molecule in solution will migrate towards one of the electrodes when placed in a electric field.

The most widely used electrophoretic technique is that of zone electrophoresis. In this technique, the samples to be separated is applied to a relatively inert support material, and, when separated, the charged particles migrate as discrete zones. The zones thus produced can then be detected and quantified by chemical and/or physical analytical techniques. Over the past 40 or so years a variety of support media have been developed. The first of these was paper, which is readly available and economical but suffers

from the disadvantage that yields blur the electrophoretograms. Kohn (1957,1958) developed the use of cellulose acetate membranes as support media. These have a high resolving power, are sensitive and suitable for rapid microscale work and do not require the lengthy preparatory procedures associated with starch and polyacrylamide techniques. However, cellulose acetate electrophoresis is less effective for complex mixtures containing proteins of similar mobility. This inherent problem associated with the separation of complex mixtures was overcome by Smithies (1955). He made use of starch gels as the supporting medium, thereby achieving protein separation according to both charge and size. Starch gels have been largely superceeded by the use of polyacrylamide gels which are easier to handle, are transparent and have the added advantage of adjustable pore size. Additional information relating to the principles and techniques of electrophoresis can be obtained from Chrambach and Rodbard (1971) and O'Donnell (1975).

The fractions separated by electrophoresis can be detected by u.v. absorption. Alternatively, the fractions can be detected either by staining for proteins, or, in the case of enzymatically active material, by testing the supporting material for zonal activity. Any staining solution, not containing cellulose solvent, can be used successfully. A survey of suitable stains has been made by Bhargava and Sceeni-

vason (1963). Other techniques available for the location of protein bands include u.v. absorption, fluorescent staining (Holiday and Johnson, 1949) and radioactive tracing (Winteringham et al., 1952).

The purpose of this study was to demonstrate the electrophoretic pattern resulting from the digestion of native caseins by the crude enzyme concentrate systems obtained from the experimental cultures. Changes in milk proteins have been characterized using starch gel electrophoresis (Law et al., 1977a; Visser and Slangen, 1977). Thompson et al. (1964) has compared electrophoretograms of β-casein using both polyacrylamide and starch gels. They demonstrated that the use of polyacrylamide was superior in terms of both resolution and simplicity. Polyacrylamide gel electrophoresis has been used for the study of milk proteins (Fox and Guiney, 1973; Davis, 1974; Adams et al., 1976; Davies and Law, 1977). Peterson (1963) improved the polyacrylamide method described by Raymond and Nakamichi (1962) and published a high resolution method for α_{s1}- and β-caseins.

Polyacrylamide gel electrophoresis was used in this study. The method described by Peterson (1963) was chosen because of its high resolution for the two main casein components in milk. κ-casein was not demonstrated in orther to minimize the possibility of rearrangements of disulfide bridges resulting from the reaction of mercaptoethanol with disulfide (Light, 1974). This disulfide cleavage is particular favored at pH(s) higher than 7.0.

The polyacrylamide gels used contained 4.5 M urea and 7.5% (w/v) Cyanogum 41 (B.D.H.) in borate buffer at pH 8.6 . With the exception of the gel staining and destaining procedure, all the experimental details, including the sample preparation using skim milk, were identical to those described by Peterson (1963). After electrophoresis, the gels were stained by immersion for 24 hours in 0.1% (w/v) naphthalene Black in 10% (v/v) acetic acid. Destaining was carried out by the immersion of the gels in acetic acid at 7% (v/v) for 6 hours. The destaining rate was increased by the use of an electric potential supplied by a low voltage battery charger.

A densitometer Eel (Evans Electroselenium Ltd., Essex) was used to estimate the relative amounts of the main protein fractions present in the gels. Gels were cut vertically, alongside of each slot, using a glass rule and sharp blades. A fine surface layer of approximately 1 mm. was cut and removed from both sides of the gel, so that its final dimension was 120 mm. x 11 mm. x 4 mm. A "Perspex" slot holder measuring 120 mm. x 11 mm. x 5 mm was used as an aid to facilitate the removal of a uniform surface layer. This eliminated the surface layer effects on scanning resulting from protein fractions spreading. The gel strips were positioned in a gel holder measuring 120 mm. x 35 mm. x 4 mm. with one long side open. The centre gel section was fitted into the holder with 5

ml of a 10% (v/v) acetic acid solution previously added, to render a transparent gel background and to eliminate side air bubbles.

(d) Heat Stability

Heat stability studies relating bacteria or bacterial enzymes, either in solutions or in complex mixtures have been made using single test tubes (Chaudhary et al., 1960), in thin wall ampoules (Sandvik, 1962), in capillary glass tubes (Resende et al., 1969; Barach et al., 1976b), in Pasteur pipettes (Beyer, 1970) and in preheated glass tubes (Navani, 1971; Fallon, 1972). Heat stability of enzymes have also been conducted using batch pasteurizer (Pinheiro et al., 1965) or micro-pasteurization equipment (Driessen and Stadhouders, 1974). O'Donnell (1975) has conducted heat resistance studies for crude enzyme preparations in solution and for its purified form. A double tube method was used to increase the surface area exposed to the heating medium and to decrease the come-up time necessary to reach the required temperature. The come-up time for a similar tube dimension has been estimated to be between 20 to 25 seconds (Chaudhary et al., 1960).

In this study the method described by O'Donnell (1975) was chosen for temperatures up to the boiling point of water. The volume of 1.5 ml of enzyme solution was displaced by the insulated tube. A series of

replicates were placed simultaneously in the water bath adjusted to 65° C, 75° C and 98° C and 25 seconds were allowed for the come-up time, after which timing started. At regular time intervals, sets of tubes were removed from the water bath and quickly cooled to 5° C in cold water bath. Enzyme activity was measured for heated and unheated blanks.

This method was used because of the relatively short time required for the temperature to reach the experimental conditions without much surface area exposed to air. Thus, the evaporation of water or the concentration of serum solids present in the solution was rather limited. Plastic caps were used to cover both tubes to reduce the concentration even further. The possibility of heating only 1.5 ml of the enzyme solution facilitated the handling of the material and allowed sufficient volume for replicate enzyme activity assays. This procedure was also used for temperatures up to 140° C, except that a metal replica of the glass tubes with a hermetic sealing system was used instead and heating was carried out by complete immersion in Polyethylene glycol (M.W. 200, Koch-Light Laboratories Ltd) bath in a thermostatically controlled bath (Grant Instruments., Ltd. - Cambridge). The hermetic sealed system consisted of a double stainless steel tube, capable of displacing the enzyme solution into a fine layer of 0.5 mm uniformly distributed (see Plates 1, 2 and the complementary diagram of Plate 2).

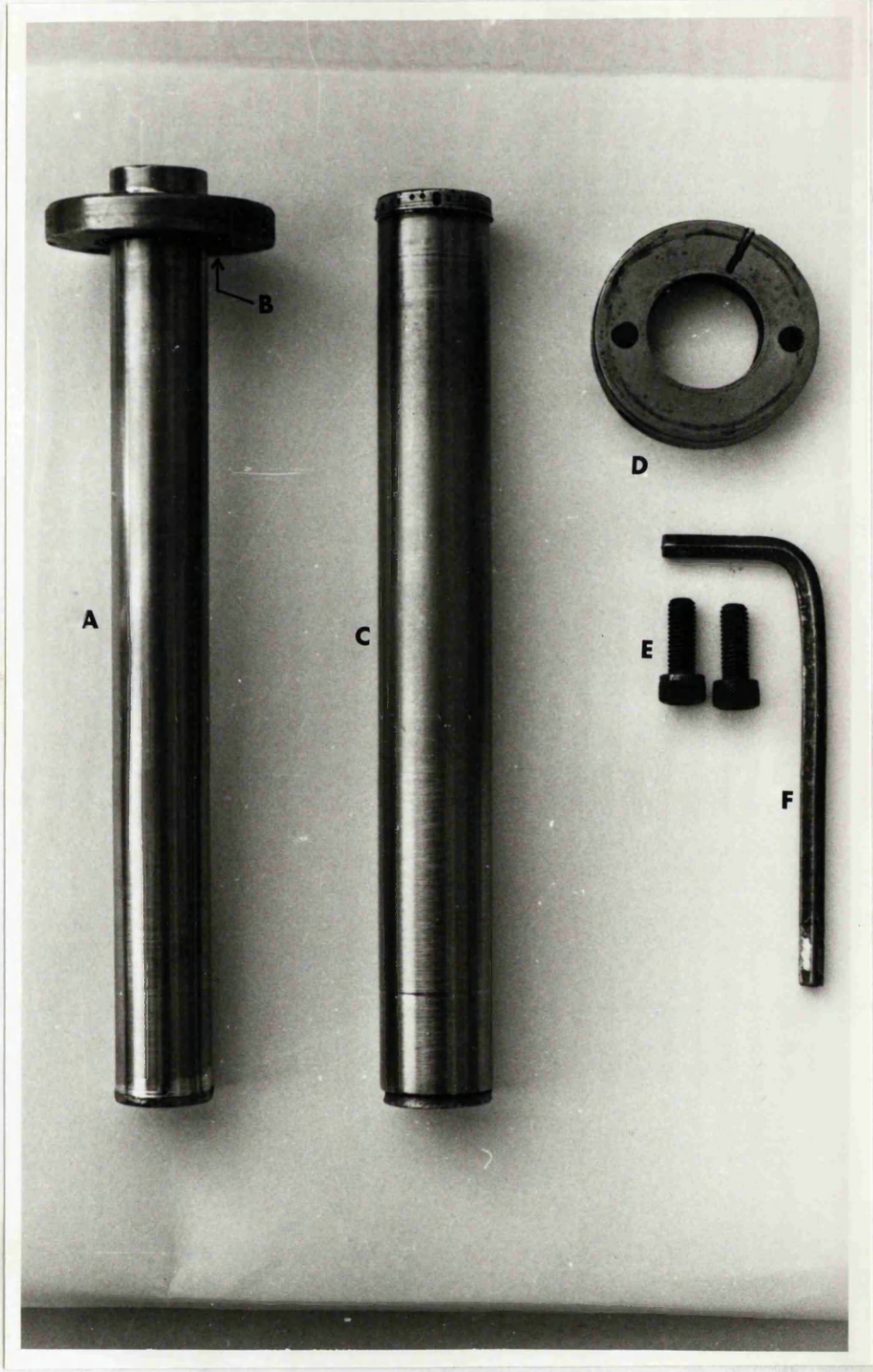


Plate 1. Parts of the Hermetic Sealed System Consisting of (A) Displacing Inner Tube, (B) Heat Resistant Gasket, (C) Outer Tube, (D) Outer Tube Support, (E) Set Screws and (F) Tool.

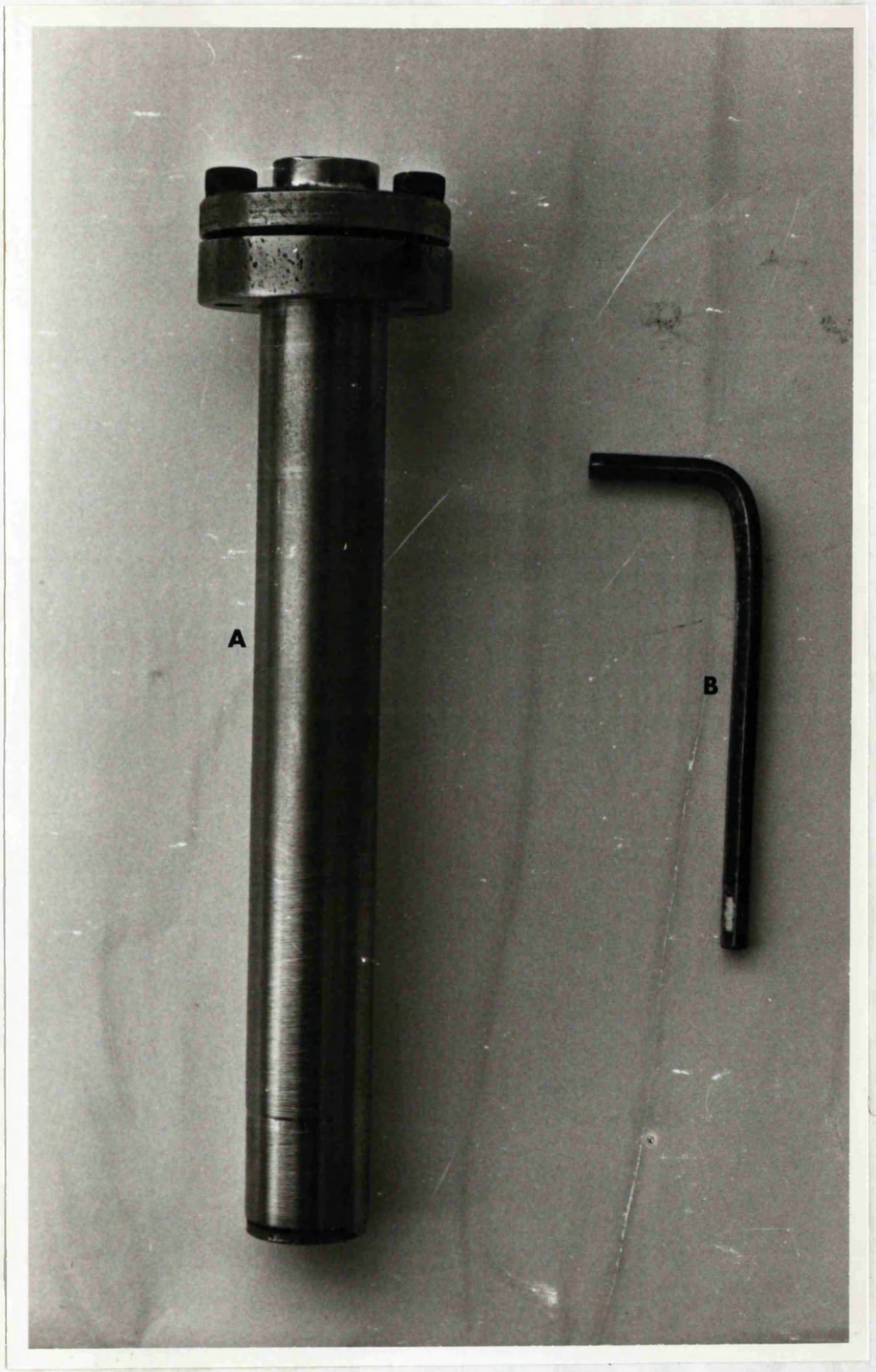


Plate 2. Assembled Parts of the Hermetic Sealed System Consisting of (A) a Double Stainless Steel Tubes with 0.5 mm. Inter-clearance and (B) Set Screw Tool.

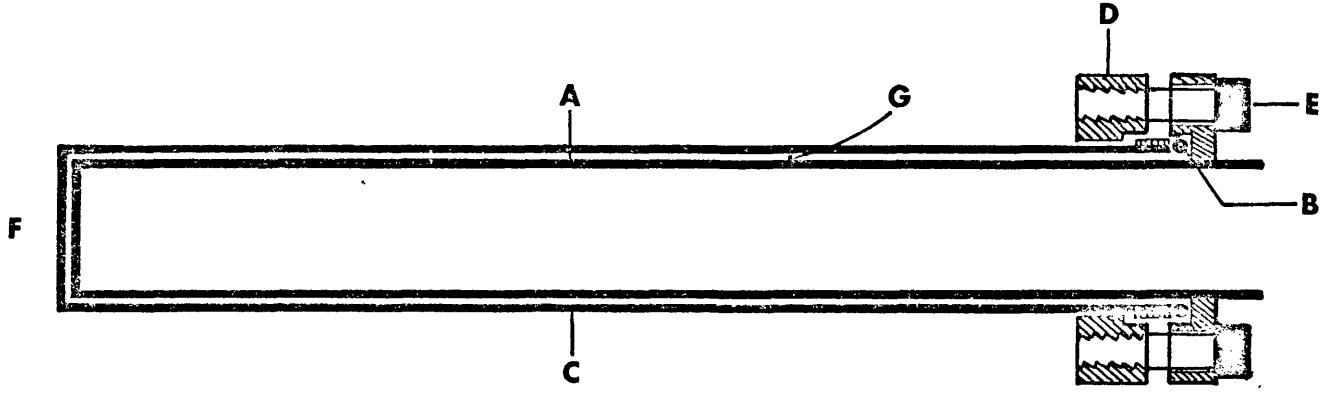


Plate 2. Complementary Diagram of Plate 2: (A) Displacing Inner Tube, (B) Heat Resistant Gasket, (C) Outer Tube, (D) Outer Tube Support, (E) Set Screw and (F) Assembled Double Stailess Steel Tubes, (G) Annular 0.5 mm. Clearance.

SECTION 3. CONCENTRATION OF THE PROTEOLYTIC ENZYME SYSTEMS

(a) Methods for Concentration of Protease Enzyme Systems

The most usual initial step in the concentration of bacterial extracellular enzyme systems is the removal of the cell bio-mass from the culture medium. As a preliminary to cell removal Merthiolate may be added to the medium to a final concentration of one to ten thousand in order to arrest bacterial growth (Sandvik, 1962). Merthiolate arrests cell growth without the occurrence of cell lysis, thus obviating the possibility of introduction of intracellular enzymes into the medium (Fallon, 1972; O'Donnell, 1975). Cell removal may be achieved by filtration (Sandvik, 1962; Fallon, 1972) or by centrifugation (Juffs *et al.*, 1968; Juffs and Doelle, 1968; Fallon, 1972; White and Marshall, 1973; Mayerhofer *et al.*, 1973; Adams *et al.*, 1975; O'Donnell, 1975; Barach *et al.*, 1976a). Fallon (1972) considered that cellulose acetate membranes were only applicable to the removal of cells from small culture volumes due to the rapid loss in membrane porosity. When large culture volumes are required high speed continuous centrifugation would appear to be the method of choice.

After cell separation from the culture medium, concentration and/or purification of the required enzyme system is necessary. Concentration and/or purifi-

cation can be achieved using the proper choice of methods. Exhaustive crystallisation, adsorption and desorption, ultrafiltration, dialysis and precipitation methods are common techniques that may be applied to the concentration and purification of enzyme systems. Extreme conditions of pH, temperature and ionic strength must be avoided (Fallon, 1972) and the chosen technique must function within the stability range of the native enzyme "to produce a purified preparation whose in vitro functions accurately reflect the activity of the molecules in the living system" (O'Donnell, 1975).

To evaluate any improvement of specific activity in the enzyme preparation an appropriate biochemical assay technique must be applied before and after the relevant purification step. Changes in specific activity (Activity/mg. of protein) can be used to evaluate the efficiency of the purification achieved between two or more steps. The techniques available for the enrichment or concentration of enzymes may be classified as follows:

(1) Selective precipitation from a solution;
    (i) Salt and/or solvent concentration,
    (ii) Isoelectric precipitation and
    (iii) Crystallisation.

(2) Recovery by solvent removal;
    (i) Flash and vacuum evaporation,
    (ii) Lyophilisation and
    (iii) Freeze-thaw procedures.

(3) Fractionation systems;

(i) Gel exclusion,

(ii) Adsorption chromatography and

(iii) Membrane moderated procedures.

Purification of psychrophilic protease systems has been achieved using various combinations of the above techniques. In some instances, as in the works of Juffs et al. (1968) and Juffs and Doelle (1968), the behavior of psychrophilic proteases was studied in cell-free extracts. On the other hand, membrane filtration, ammonium sulphate precipitation and Sephadex gel filtration procedures have been used by Mayerhofer et al. (1973) and White and Marshall (1973). Barach et al. (1976a) purified bacterial proteases on chromatography columns equilibrated with simulated milk serum buffer. O'Donnell (1975) demonstrated that psychrophilic extracellular lipases could be concentrated by ultrafiltration. Scholefield et al. (1978) have reported procedures for the isolation of lipases by ultrafiltration. Since an objective of the present study was related to the heat stability of the proteases recovered from the test strains cell-free extracts, this gentle, non-destructive method of enzyme concentration was adopted. Furthermore, this type of procedure would allow for the production of an enzyme concentrate which could be readily freeze dried into a powder form for storage and further study.

(b) Removal of Cells

The initial stage in the concentration of the

extracellular protease systems was the removal of cells from the growth media. Growth was arrested by the addition of 0.1% (w/v) Merthiolate at a point in the logarithmic phase concommitant with good protease production in order to avoid cell lysis and intracellular enzyme contamination. Cell removal was carried out at 40,000 g using a Cepa Laboratorium Type LE Centrifuge. For efficient cell removal the throughput was limited to 100 ml/minute. The temperature of the medium was controlled at approximately 10° C by the circulation of cold water. The centrifugate was stored at 5° C for periods up to 24 hours before further processing.

(c) Ultrafiltration and Freeze-Drying

Enzyme concentration was carried out using a Model 202 ultrafiltration cell (Amicon Corporation) fitted with Type PM 10 Diaflo membranes which gave retention of compounds of molecular weight 10,000 or above (according to the specifications provided by the manufacturer). The temperature of the medium was kept at 5° C $\pm$ 3° C during ultrafiltration. The ultrafiltration was speeded up by applying constant pressure of 4 kg/cm^2 N_2.

The volume of 220 ml of cell-free medium was reduced down to 30 ml. The N_2 pressure was released and an additional 190 ml of the cell-free medium introduced into the ultrafiltration cell. The pressure was reapplied and the volume again reduced to 30 ml of con-

centrate. These operations were repeated using two successives 190 ml of distilled water at 5° C in order to reduce the salt concentration to a minimum. The final 30 ml of concentrate volume was freeze-dried for 48 hours at a sublimization coil temperature of -40° C. The enzyme yields were recorded before the batches were stored at -20° C for further utilization. The overall scheme for the concentration of extracellular protease system was similar to that reported by Scholefield *et al*. (1978). Plate 3 shows the set up used during protease concentration. Plate 4 shows the Model 202 ultrafiltration cell.

Plate 3. Ultrafiltration Cell Model 202 (B) Assembled Within a Cold Water Bath (A) and Connected by a Pressure Tube (C) to a N_2 Source.

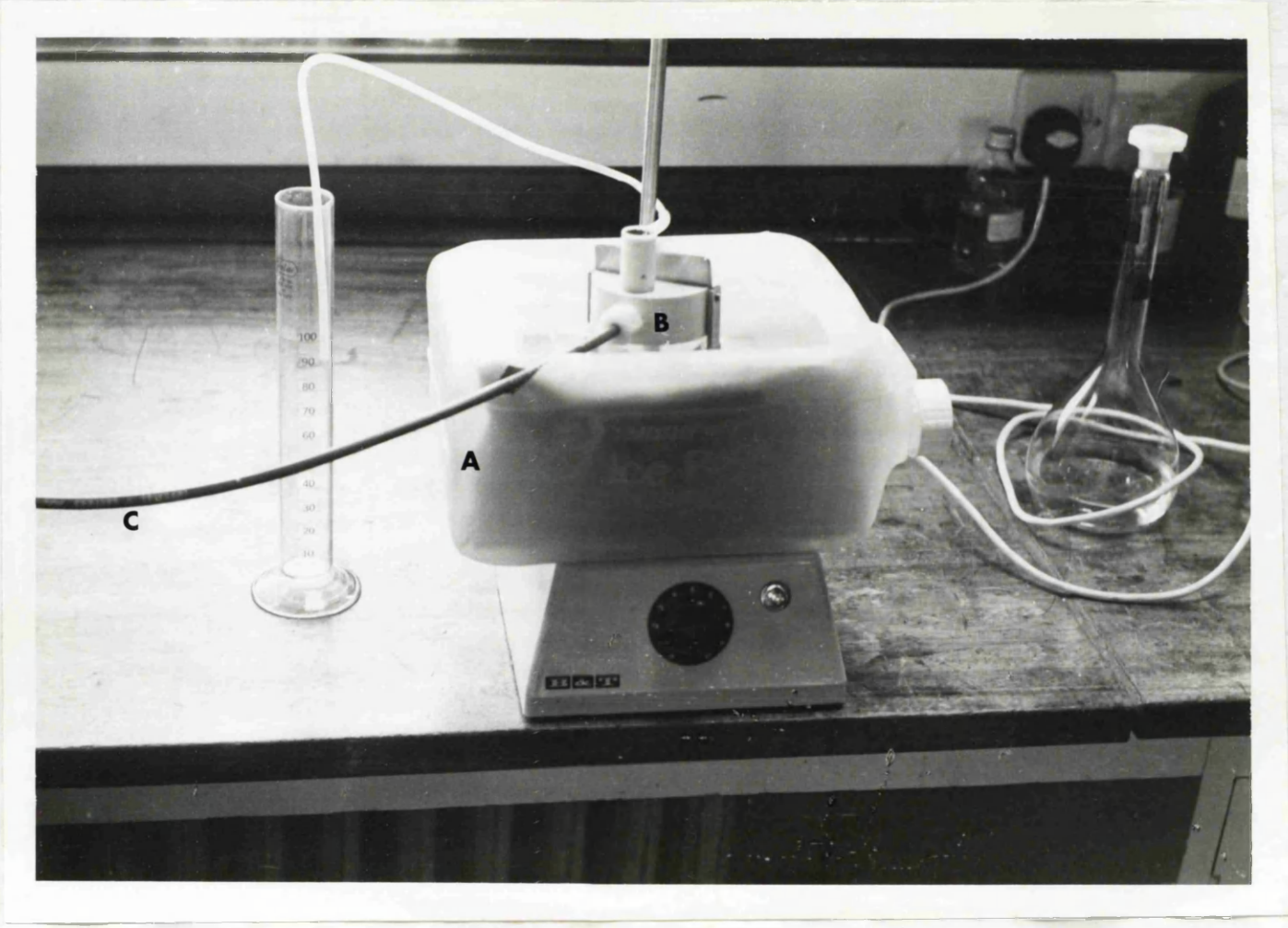
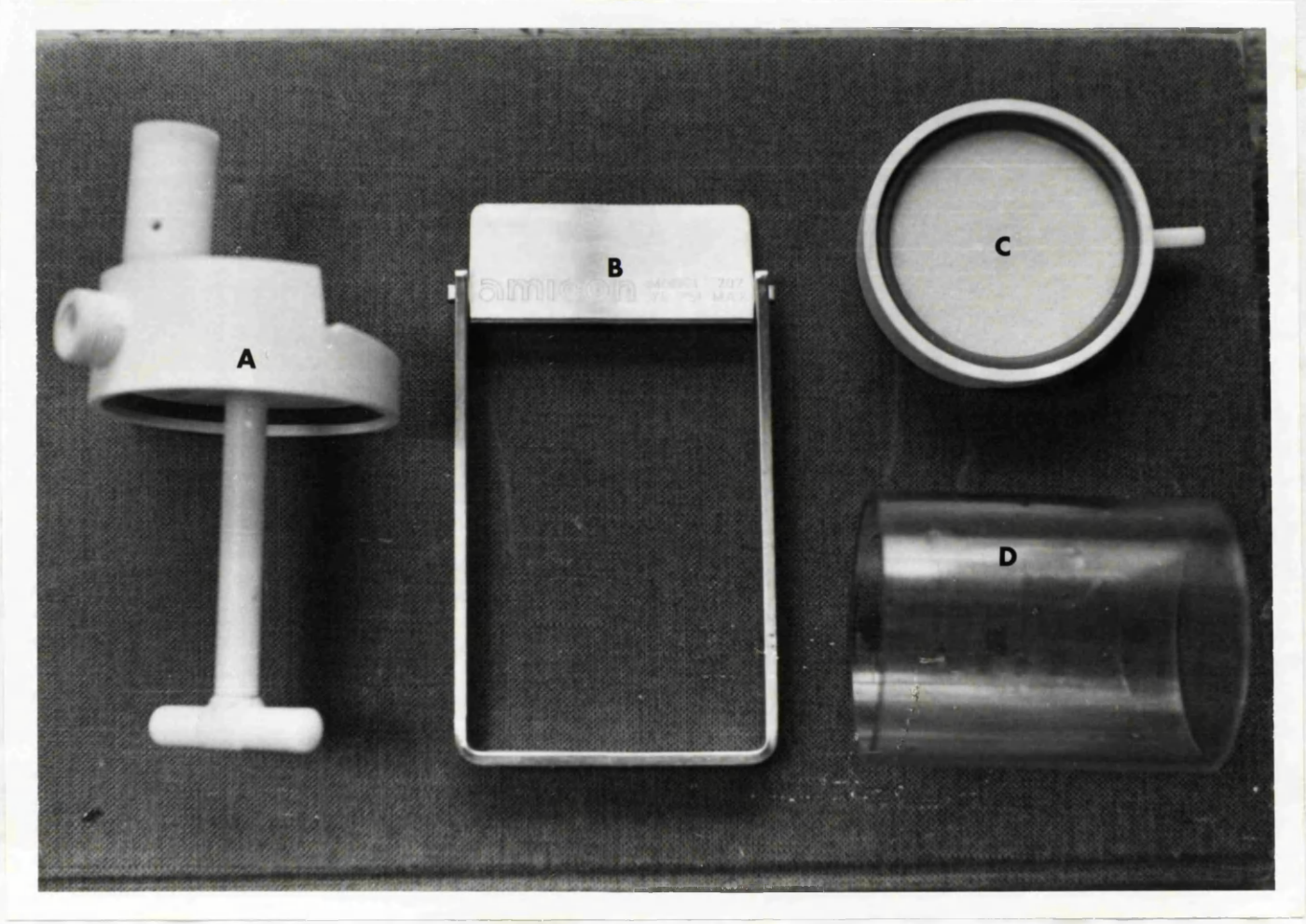


Plate 4. Ultrafiltration Cell Components: (A) Upper Sealing System, (B) Tight Fitter, (C) Membrane Support and (D) Tube Support.



CHAPTER II

GROWTH AND PROTEASE PRODUCTION UNDER AERATED AND STATIC CONDITIONS AT 5° C

SECTION 1. GROWTH AND PROTEASE PRODUCTION UNDER AERATED CONDITIONS

(a) Experimental

The effects of aeration on growth and protease production were studied using inoculations of the test strains incubated under conditions of moderated convection (magnetic field of the stirrer adjusted to mark 1). Growth and protease production were observed at 24 hour intervals following the procedure described in sub-section (c) on pages 30 and 31.

(b) Results and Discussion

The results of growth and protease production under aerated conditions are presented in Table 2. Protease systems were measurable only after the second or the third day of incubation. Significant growth developments were detected after 48 hours of incubation. A considerable amount of proteases were produced by strains S3 and S8 after the first 24 hours of incubation. This was also consistent with a greater degree of growth observed for these strains. In general protease systems could be measured on the second day and maximum concentrations were observed after six to eight days of incubation. Maximum growth levels were reached on the eighth day of incubation. These results served to indicate that the late logarithmic phase occurred after

Table 2. Growth and Protease Production Under Aerated Conditions at 5° C Expressed in Terms of Optical Density (O.D. = ●) and mm. Zone Diameter (Z.D. = ⊘).

Strains	Time (days)					
	2	4	6	8	10	12
● S1	0.20	1.08	1.90	2.10	1.97	1.80
S2	0.25	1.10	1.95	2.20	2.00	1.85
S3	0.30	1.12	2.00	2.10	2.00	1.90
S4	0.20	1.00	1.80	2.00	1.85	1.70
S5	0.28	1.18	2.05	2.30	2.08	1.84
S6	0.15	0.95	1.85	2.06	1.95	1.86
S7	0.18	1.00	1.95	2.14	2.00	1.88
S8	0.24	1.20	2.10	2.35	2.15	2.00
⊘ S1	-	16.0	23.0	22.5	22.0	22.5
S2	6.5	16.5	24.0	24.5	20.0	21.0
S3	7.5	18.0	24.5	25.0	24.0	24.0
S4	7.0	16.0	22.0	23.0	20.0	19.0
S5	6.5	15.0	20.0	21.0	21.0	19.0
S6	6.5	14.0	18.0	19.0	19.0	18.0
S7	6.5	16.0	21.0	22.0	21.0	20.0
S8	6.5	18.0	26.0	24.0	23.0	24.0

five days of incubation. These observations are important because any extension of the incubation period beyond five days may lead to intracellular enzyme contamination due to cell lysis.

(c) Conclusion

A comparison among the ranges of variation observed under aerated conditions (Figs. 1 and 2) has demonstrated that protease production shows a greater tendency to vary among the eight test strains. The results also served to indicate the limit of five days under aerated conditions does not lead to any significant intracellular enzyme contamination due to cell lysis.

SECTION 2. GROWTH AND PROTEASE PRODUCTION UNDER STATIONARY CONDITIONS

(a) Experimental

The effects of stationary conditions on growth and protease production were studied using inoculations of the test strains incubated under static shallow layer conditions. Growth and protease production were observed at 24 hour intervals following the procedure described in sub-section (c) on pages 30 and 31.

(b) Results and Discussion

The results of growth and protease production are presented in Table 3. Protease systems were measurable for strain S3 following the fourth day of incubation, for some strains following the sixth day and for

Table 3. Growth and Protease Production Under Stationary Shallow Layer Conditions at 5° C Expressed in Terms of Optical Density (O.D. = ●) and mm. Zone Diameter (Z.D. = ⊘).

Strains	Time (days)					
	2	4	6	8	10	12
● S1	-	0.08	0.12	0.56	1.00	1.33
S2	-	0.09	0.13	0.50	0.85	1.10
S3	-	0.10	0.17	0.52	0.90	1.16
S4	-	0.09	0.14	0.43	0.75	0.98
S5	-	0.06	0.10	0.38	0.65	0.85
S6	-	0.05	0.10	0.32	0.60	0.80
S7	-	0.07	0.10	0.44	0.80	1.08
S8	-	0.05	0.10	0.40	0.70	0.93
⊘ S1	-	-	6.5	8.0	12.0	16.0
S2	-	-	-	7.0	11.0	14.0
S3	-	6.5	8.0	11.0	16.0	18.0
S4	-	-	6.5	9.0	15.0	17.0
S5	-	-	-	7.5	14.0	15.0
S6	-	-	-	8.0	12.5	17.0
S7	-	-	7.0	9.0	13.0	18.0
S8	-	-	7.5	11.0	15.0	17.0

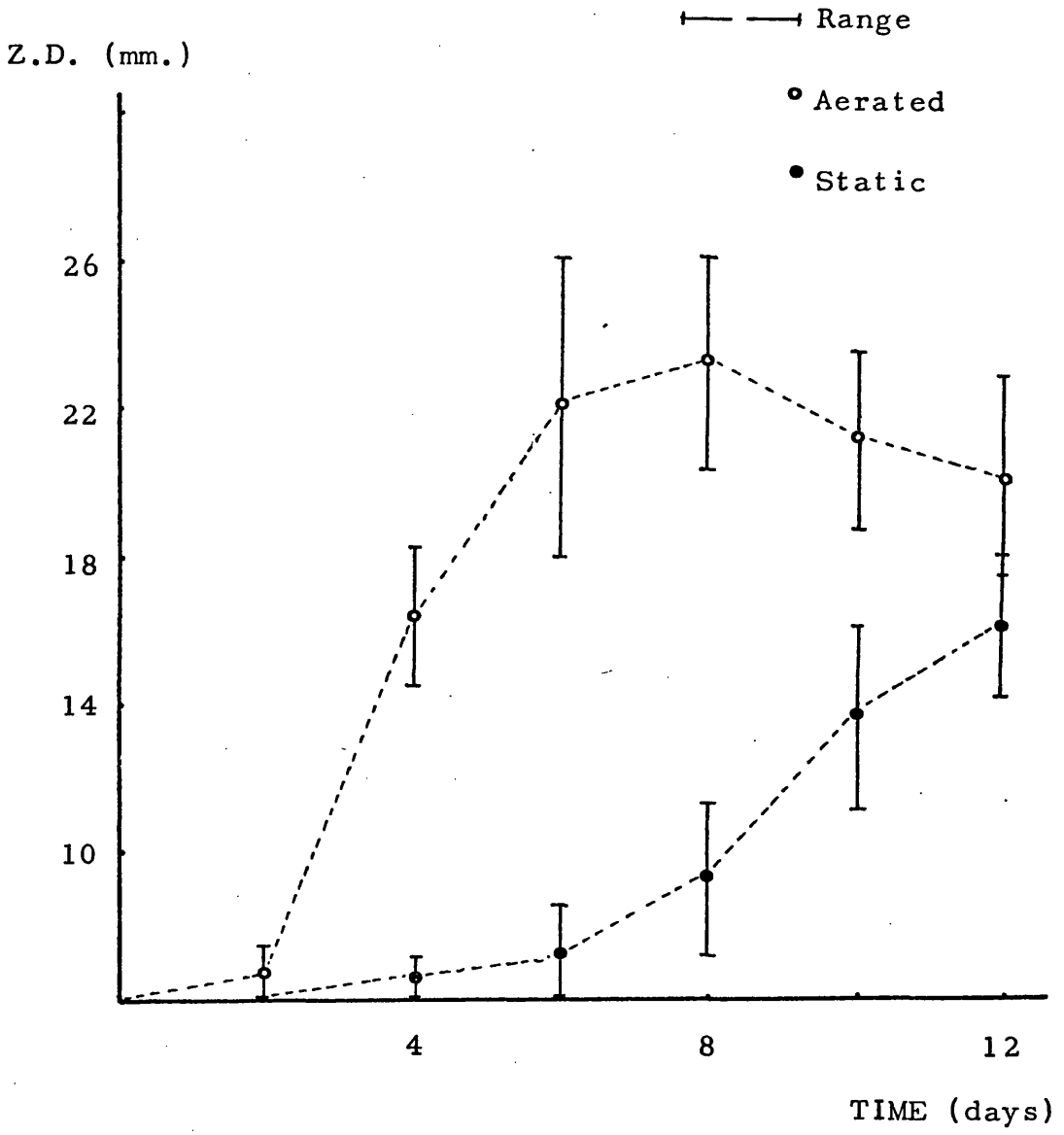


Figure 1. Protease Production Patterns Under Stationary and Aerated Conditions at 5° C Expressed in mm. Zone Diameter as the Mean Values and Their Respective Ranges for the Test Strains S1 to S8.

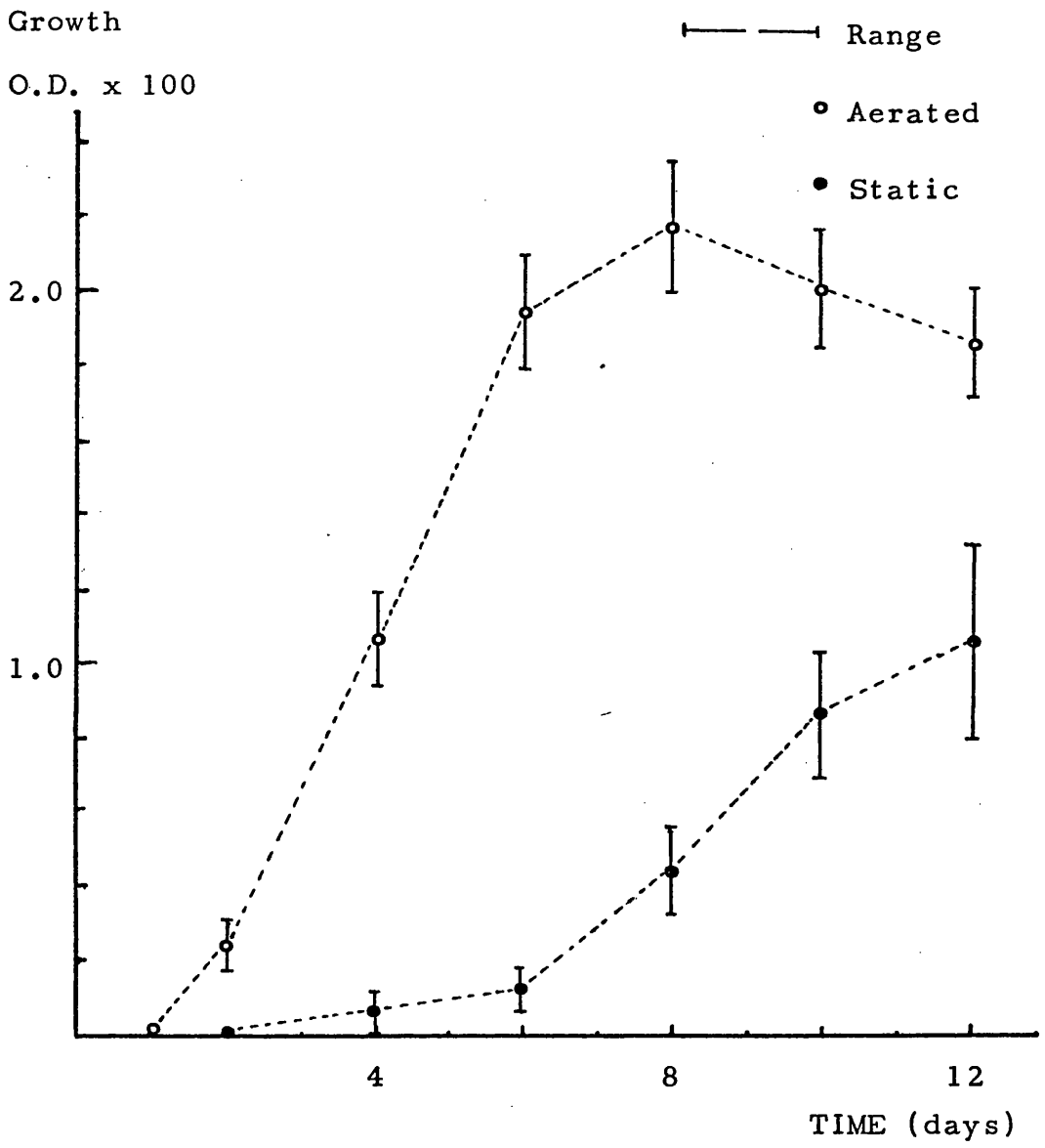


Figure 2. Growth Under Stationary and Aerated Conditions at 5° C Expressed in Units of Optical Density as the Mean Values and Their Respective Ranges for the Test Strains S1 to S8.

all strains following the eighth day. Growth and protease production by all test strains under static conditions over a period of 12 days at 5° C was less than that obtained by the same strains grown in aerated conditions for the same time at the same temperature.

(c) Conclusion

The experimental stationary conditions delayed the onset of growth and protease production by all test strains at 5° C. Neither measurable growth nor measurable protease production were detected after three days incubation at 5° C of all test strains. The expected maximum growth and protease production levels observed under aerated conditions were not reached within a period of 12 experimental days under stationary conditions.

SECTION 3. CONCLUSION

The initial rate of growth of all test strains were clearly enhanced by agitation. Protease produced during the same period, despite showing a considerable wider range of variation for different strains, was also enhanced by agitation. Although the results have demonstrated protease production was affected by aeration, the actual amount of protease produced was also dependent of the property of individual strains in response to higher levels of dissolved gases. Since the adjustment of agitation was manually controlled,

variations of the amount of dissolved gases in the liquid media may have contributed to a small degree, to certain discrepancies during the late experimental period. Thus oxygen having a very low solubility in most of the usual liquid media (Meynell and Meynell, 1970) is considered a limiting factor of both growth and protease production. Sodek and Hofmann (1970) have reported that the production of penicillopepsin at 25° C was inhibited by inadequate aeration, although good growth levels were reached experimentally. Fallon (1972) has demonstrated that inadequate aeration during stationary growth conditions at 5° C caused an initial lag period of 5 to 10 days prior to the appearance of protease activity. However, under agitated growth conditions this lag period was shown to be reduced to about three days. It was also shown that stationary conditions supported a relatively lower maximum level of protease production, although growth proceeded discretely to a maximum level on the 16th day of incubation. O'Donnell (1975) has shown a difference of 3 days between the two lag phases of aerated and stationary growth conditions at 5° C, for the production and appearance of protease activity by Pseudomonas fluorescens. His observations also indicated that the logarithmic growth phase under aerated conditions at 5° C does not normally end before the 5th day of incubation.

CHAPTER III

GROWTH AND PROTEASE PRODUCTION IN DIFFERENT COMMERCIAL MEDIA UNDER STATIONARY SHALLOW LAYER CONDITIONS AT 25° C

SECTION 1. GROWTH AND PROTEASE PRODUCTION IN DIFFERENT COMMERCIAL MEDIA

(a) Experimental

A total of eleven commercial media were tested for their ability to promote growth and protease production. Patterns illustrating the development of protease activity relative to growth for the test strains are illustrated in Figs. 3 - 10 and Tables 4 - 11. As shown below the synthetic media used are identified throughout in abbreviated form:

DPPNE - Difco Proteose Peptone
DBPNE - Difco Bacteriological Peptone
DBTNE - Difco Bacteriological Tryptone
DBTSE - Difco Bacteriological Tryptose
DYE - Difco Yeast Extract
OPPNE - Oxoid Proteose Peptone
OTNE - Oxoid Tryptone
OCH - Oxoid Casein Hydrolysate
OTSE - Oxoid Tryptose
OBPNE - Oxoid Bacteriological Peptone
OYE - Oxoid Yeast Extract

These media were recently ordered and the containers opened just before the

time the experiments were carried out.

The strains, specified S1 - S8, were grown at 25° C under stationary conditions in Pope and Skermans mineral salts medium supplemented with 0.5% (w/v) of commercial media just as described on pages 27 - 30. Growth was recorded as units of O.D. and protease activity as the diameter of the zones of precipitation on casein agar (see Appendix I.).

In order to demonstrate the relationship between the growth and protease production by the test strains, calculation of the correlation coefficients (r^2) for 88 sets of results was undertaken (Table 12). The calculations were carried out using a pre-programed magnetic card (Curve Fitting SD-03A) and a Hewlett Packard 97 Calculator (Robox Ltd., Glasgow) selected for logarithmic fit. This procedure allows for the evaluation of the degree of consistency displayed between growth and protease production independent of the level of growth achieved or the amount of protease released. As shown in Table 12, the r^2 values were particularly helpful in deciding on a common medium which supported growth and protease production by all the test strains.

(b) Results and Discussion

Protease production was apparent during and after the logarithmic phase of growth of strain S1. Although DBTNE supported one of the highest growth levels for the media tested, only low levels of protease activity were apparent. In contrast OYE medium supported a rela-

Table 4. Growth and Protease Production by Strain S1 in Commercial Media at 25° C (*)

Media	Units	Time (days)				
		1	2	3	4	5
DPPNE	O.D. x 100	0.73	1.64	1.90	2.00	2.04
	Z.D. (mm.)	11.5	13.0	13.5	14.0	14.5
DBPNE	O.D. x 100	0.45	0.98	1.42	1.54	1.62
	Z.D. (mm.)	12.0	15.0	16.5	16.0	19.0
DBTNE	O.D. x 100	1.73	2.25	2.45	2.45	2.29
	Z.D. (mm.)	11.0	12.0	13.5	14.0	15.5
DBTSE	O.D. x 100	1.57	1.83	1.95	1.93	1.90
	Z.D. (mm.)	16.5	15.0	10.0	12.5	15.0
DYE	O.D. x 100	1.93	2.00	2.12	2.02	1.97
	Z.D. (mm.)	17.0	18.0	18.5	19.0	21.0
OPPNE	O.D. x 100	1.00	1.50	1.88	1.93	1.75
	Z.D. (mm.)	16.0	17.0	18.0	18.0	20.0
OTNE	O.D. x 100	1.80	2.10	2.20	2.14	2.20
	Z.D. (mm.)	19.5	21.0	22.0	22.5	22.5
OCH	O.D. x 100	1.02	2.00	2.02	1.79	1.73
	Z.D. (mm.)	6.5	10.0	10.0	11.0	13.5
OTSE	O.D. x 100	1.38	2.08	2.17	2.12	1.90
	Z.D. (mm.)	12.0	12.0	14.5	15.0	16.0
OBPNE	O.D. x 100	1.23	1.77	1.92	1.95	1.75
	Z.D. (mm.)	18.0	20.0	21.5	22.0	23.5
OYE	O.D. x 100	1.29	1.50	1.54	1.45	1.40
	Z.D. (mm.)	9.0	11.0	14.0	15.0	16.0

(*) See page 51 and abbreviations at the beginning (x).

Table 5. Growth and Protease Production by Strain S2 in Commercial Media at 25° C (*)

Media	Units	Time (days)				
		1	2	3	4	5
DPPNE	O.D. x 100	0.52	1.66	2.02	2.08	2.04
	Z.D. (mm.)	7.0	12.0	9.0	11.5	13.0
DBPNE	O.D. x 100	0.41	0.95	1.33	1.62	1.65
	Z.D. (mm.)	7.0	10.0	12.0	12.5	14.0
DBTNE	O.D. x 100	0.64	2.25	2.35	2.42	2.42
	Z.D. (mm.)	6.0	12.0	8.0	12.0	15.0
DBTSE	O.D. x 100	0.84	2.10	2.10	2.15	2.02
	Z.D. (mm.)	10.5	18.0	8.0	12.0	14.0
DYE	O.D. x 100	0.55	2.10	2.17	2.17	1.95
	Z.D. (mm.)	6.0	14.0	15.5	16.0	18.0
OPPNE	O.D. x 100	0.77	1.58	1.92	2.08	2.12
	Z.D. (mm.)	11.0	14.0	14.0	16.0	16.5
OTNE	O.D. x 100	0.73	2.00	2.12	2.25	2.25
	Z.D. (mm.)	11.5	19.0	19.0	19.5	21.0
OCH	O.D. x 100	0.45	1.79	2.02	1.95	1.79
	Z.D. (mm.)	6.0	6.0	10.0	11.0	13.5
OTSE	O.D. x 100	0.61	2.02	2.10	2.12	2.17
	Z.D. (mm.)	6.5	7.0	10.0	12.0	13.0
OBPNE	O.D. x 100	0.73	1.65	1.90	1.92	1.90
	Z.D. (mm.)	13.0	18.0	18.0	18.5	19.5
OYE	O.D. x 100	0.70	1.42	1.50	1.52	1.48
	Z.D. (mm.)	6.5	8.0	8.0	12.0	14.5

(*) See page 51 and abbreviations at the beginning (x).

Table 6. Growth and Protease Production by Strain S3 in Commercial Media at 25° C (*)

Media	Units	Time (days)				
		1	2	3	4	5
DPPNE	O.D. x 100	0.68	1.50	1.77	1.92	2.00
	Z.D. (mm.)	10.5	13.0	12.0	12.0	12.0
DBPNE	O.D. x 100	0.41	0.75	1.10	1.50	1.62
	Z.D. (mm.)	12.0	15.5	15.5	15.5	17.0
DBTNE	O.D. x 100	1.61	2.10	1.98	2.23	2.25
	Z.D. (mm.)	13.5	11.0	11.0	12.0	16.5
DBTSE	O.D. x 100	1.29	1.65	1.90	1.95	1.98
	Z.D. (mm.)	14.5	8.5	8.0	9.5	14.0
DYE	O.D. x 100	2.02	1.92	2.20	2.20	2.02
	Z.D. (mm.)	15.5	16.5	16.5	17.0	19.0
OPPNE	O.D. x 100	1.04	1.67	2.00	2.12	2.08
	Z.D. (mm.)	14.5	15.5	15.5	17.0	18.0
OTNE	O.D. x 100	1.41	1.79	1.92	2.10	2.37
	Z.D. (mm.)	16.5	18.0	19.0	19.0	22.0
OCH	O.D. x 100	0.98	1.70	2.10	2.17	1.92
	Z.D. (mm.)	7.5	8.0	10.0	10.5	15.0
OTSE	O.D. x 100	1.23	1.77	2.00	2.08	2.12
	Z.D. (mm.)	12.0	11.0	11.0	11.5	13.0
OBPNE	O.D. x 100	0.82	1.37	1.62	1.77	1.83
	Z.D. (mm.)	16.0	19.0	19.0	19.0	22.0
OYE	O.D. x 100	1.25	1.17	1.45	1.50	1.48
	Z.D. (mm.)	14.0	12.0	11.5	11.5	16.0

(*) See page 51 and abbreviations at the beginning (x).

Table 7. Growth and Protease Production by Strain S4 in Commercial Media at 25° C (*)

Media	Units	Time (days)				
		1	2	3	4	5
DPPNE	O.D. x 100	0.41	1.82	2.00	2.10	2.20
	Z.D. (mm.)	18.0	21.5	20.0	20.0	23.5
DBPNE	O.D. x 100	0.27	0.73	1.15	1.62	1.75
	Z.D. (mm.)	15.0	16.0	18.0	19.0	22.5
DBTNE	O.D. x 100	0.48	2.12	2.27	2.29	2.33
	Z.D. (mm.)	12.0	16.5	16.0	17.0	19.0
DBTSE	O.D. x 100	0.45	2.00	2.10	2.15	2.08
	Z.D. (mm.)	12.0	7.0	10.0	6.0	8.0
DYE	O.D. x 100	0.43	2.04	2.15	2.12	2.08
	Z.D. (mm.)	13.0	20.0	16.5	16.0	19.5
OPPNE	O.D. x 100	0.64	1.29	1.52	1.79	2.02
	Z.D. (mm.)	18.0	21.5	20.0	19.0	23.0
OTNE	O.D. x 100	0.68	1.85	1.95	2.10	2.02
	Z.D. (mm.)	15.0	18.5	19.0	19.0	21.5
OCH	O.D. x 100	0.61	1.52	1.67	1.87	1.92
	Z.D. (mm.)	10.0	7.0	10.0	6.0	14.0
OTSE	O.D. x 100	1.00	1.83	1.90	2.10	2.25
	Z.D. (mm.)	17.0	19.0	17.5	17.5	19.5
OBPNE	O.D. x 100	0.66	1.42	1.52	1.79	1.77
	Z.D. (mm.)	16.0	18.5	19.0	19.0	21.5
OYE	O.D. x 100	0.25	1.35	1.48	1.52	1.45
	Z.D. (mm.)	12.5	14.0	10.0	9.0	11.5

(*) See page 51 and abbreviations at the beginning (x).

Table 8. Growth and Protease Production by Strain S5 in Commercial Media at 25° C (*)

Media	Units	Time (days)				
		1	2	3	4	5
DPPNE	O.D. x 100	0.48	1.37	1.60	1.65	1.40
	Z.D. (mm.)	11.5	14.0	13.5	13.0	15.0
DBPNE	O.D. x 100	0.27	0.77	1.02	1.22	1.25
	Z.D. (mm.)	9.0	14.0	13.0	13.0	15.5
DBTNE	O.D. x 100	1.15	2.25	2.35	2.22	2.12
	Z.D. (mm.)	15.0	12.0	9.0	13.0	11.0
DBTSE	O.D. x 100	1.05	2.00	2.17	2.20	2.10
	Z.D. (mm.)	14.5	14.0	9.0	8.0	12.5
DYE	O.D. x 100	0.98	2.02	2.10	2.10	2.05
	Z.D. (mm.)	16.0	16.5	14.5	10.0	15.0
OPPNE	O.D. x 100	0.73	1.50	1.75	1.80	1.65
	Z.D. (mm.)	13.0	15.0	13.5	17.0	15.0
OTNE	O.D. x 100	0.90	1.90	1.97	1.95	1.87
	Z.D. (mm.)	14.5	17.5	16.0	18.0	17.5
OCH	O.D. x 100	0.67	1.85	2.07	1.95	1.85
	Z.D. (mm.)	6.0	8.0	6.0	6.0	6.0
OTSE	O.D. x 100	0.85	1.75	1.92	1.80	1.80
	Z.D. (mm.)	12.0	12.0	9.0	8.0	10.0
OBPNE	O.D. x 100	0.65	1.40	1.55	1.55	1.45
	Z.D. (mm.)	14.5	16.5	16.0	16.0	16.0
OYE	O.D. x 100	0.65	1.40	1.42	1.45	1.35
	Z.D. (mm.)	13.0	10.5	6.0	6.0	7.0

(*) See page 51 and abbreviations at the beginning (x).

Table 9. Growth and Protease Production by Strain S6 in Commercial Media at 25° C (*)

Media	Units	Time (days)				
		1	2	3	4	5
DPPNE	O.D. x 100	0.17	0.60	1.05	1.40	1.85
	Z.D. (mm.)	6.0	9.0	18.5	16.0	16.5
DBPNE	O.D. x 100	0.15	0.40	0.62	0.97	1.32
	Z.D. (mm.)	6.0	13.5	18.0	16.0	16.0
DBTNE	O.D. x 100	0.50	1.25	2.10	2.37	2.30
	Z.D. (mm.)	6.0	7.0	16.0	10.0	9.0
DBTSE	O.D. x 100	0.15	0.55	1.27	2.20	2.32
	Z.D. (mm.)	6.0	6.0	13.5	10.0	10.0
DYE	O.D. x 100	0.12	0.50	1.47	2.12	2.10
	Z.D. (mm.)	6.0	6.0	13.0	16.0	15.0
OPPNE	O.D. x 100	0.21	0.72	1.40	1.80	2.00
	Z.D. (mm.)	6.0	7.5	19.0	16.0	16.5
OTNE	O.D. x 100	0.15	0.65	1.50	2.00	2.07
	Z.D. (mm.)	6.0	7.5	18.0	16.5	17.0
OCH	O.D. x 100	0.20	1.05	1.32	1.32	1.35
	Z.D. (mm.)	6.0	6.0	6.0	6.0	6.0
OTSE	O.D. x 100	0.27	0.97	1.57	2.00	2.10
	Z.D. (mm.)	6.0	7.0	15.5	13.0	11.0
OBPNE	O.D. x 100	0.12	0.57	1.17	1.65	1.80
	Z.D. (mm.)	6.0	6.0	17.0	16.5	18.5
OYE	O.D. x 100	0.05	0.87	1.27	1.35	1.30
	Z.D. (mm.)	6.0	6.0	6.0	6.0	6.0

(*) See page 51 and abbreviations at the beginning (x).

Table 10. Growth and Protease Production by Strain S7 in Commercial Media at 25° C (*)

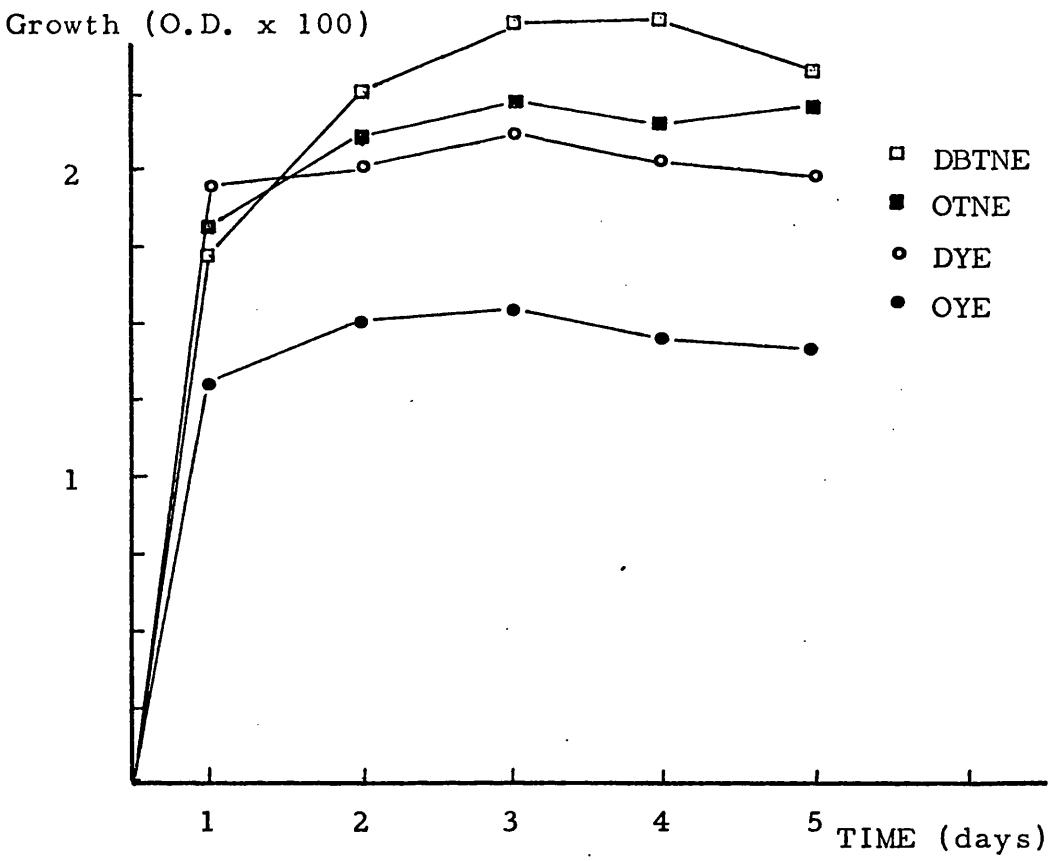
Media	Units	Time (days)				
		1	2	3	4	5
DPPNE	O.D. x 100	0.27	0.75	1.15	1.77	1.95
	Z.D. (mm.)	6.0	21.0	25.0	24.0	25.0
DBPNE	O.D. x 100	0.17	0.55	0.72	1.12	1.55
	Z.D. (mm.)	6.0	18.5	22.5	21.0	22.5
DBTNE	O.D. x 100	0.23	1.52	1.55	1.80	2.30
	Z.D. (mm.)	6.0	21.0	23.5	22.0	22.5
DBTSE	O.D. x 100	0.20	1.17	1.40	1.52	1.95
	Z.D. (mm.)	6.0	21.0	25.0	24.0	23.5
DYE	O.D. x 100	0.25	1.57	1.75	2.02	2.05
	Z.D. (mm.)	6.0	23.0	23.5	23.0	22.0
OPPNE	O.D. x 100	0.23	1.00	1.35	1.60	1.72
	Z.D. (mm.)	6.0	18.0	21.0	19.0	20.5
OTNE	O.D. x 100	0.23	1.15	1.55	1.72	1.95
	Z.D. (mm.)	6.0	17.5	21.5	20.0	20.0
OCH	O.D. x 100	0.23	1.17	1.75	1.77	1.82
	Z.D. (mm.)	6.0	12.5	19.5	17.0	14.0
OTSE	O.D. x 100	0.25	1.17	1.40	1.57	2.00
	Z.D. (mm.)	6.0	21.5	24.5	23.0	23.5
OBPNE	O.D. x 100	0.23	0.80	1.15	1.45	1.57
	Z.D. (mm.)	6.0	14.5	19.0	17.0	18.0
OYE	O.D. x 100	0.08	0.87	1.07	1.10	1.17
	Z.D. (mm.)	6.0	17.0	21.0	20.5	19.5

(*) See page 51 and abbreviations at the beginning (x).

Table 11. Growth and Protease Production by Strain S8 in Commercial Media at 25° C (*)

Media	Units	Time (days)				
		1	2	3	4	5
DPPNE	O.D. x 100	0.40	1.15	1.67	1.97	2.00
	Z.D. (mm.)	6.0	6.0	11.0	12.5	13.0
DBPNE	O.D. x 100	0.15	0.65	1.07	1.52	1.57
	Z.D. (mm.)	6.0	6.0	8.0	8.0	12.0
DBTNE	O.D. x 100	0.54	2.02	2.27	2.35	2.12
	Z.D. (mm.)	6.0	6.0	11.0	9.0	8.0
DBTSE	O.D. x 100	0.29	1.65	2.05	2.07	1.82
	Z.D. (mm.)	6.0	6.0	10.5	8.0	7.5
DYE	O.D. x 100	0.5	1.87	1.97	1.85	1.55
	Z.D. (mm.)	6.0	6.0	10.0	8.0	7.5
OPPNE	O.D. x 100	0.25	1.25	1.62	1.90	1.95
	Z.D. (mm.)	6.0	6.5	13.0	12.5	13.0
OTNE	O.D. x 100	0.62	1.82	2.07	2.12	1.97
	Z.D. (mm.)	6.0	8.0	13.5	12.0	12.0
OCH	O.D. x 100	0.60	1.52	1.90	1.47	1.40
	Z.D. (mm.)	6.0	6.0	6.0	6.0	6.0
OTSE	O.D. x 100	0.60	1.47	1.85	2.05	2.00
	Z.D. (mm.)	6.0	6.0	11.0	10.0	11.0
OBPNE	O.D. x 100	0.42	1.42	1.77	1.82	1.67
	Z.D. (mm.)	6.0	13.0	17.0	15.5	15.5
OYE	O.D. x 100	0.52	1.30	1.32	1.30	1.00
	Z.D. (mm.)	6.0	6.5	10.5	6.0	6.0

(*) See page 51 and abbreviations at the beginning (x).



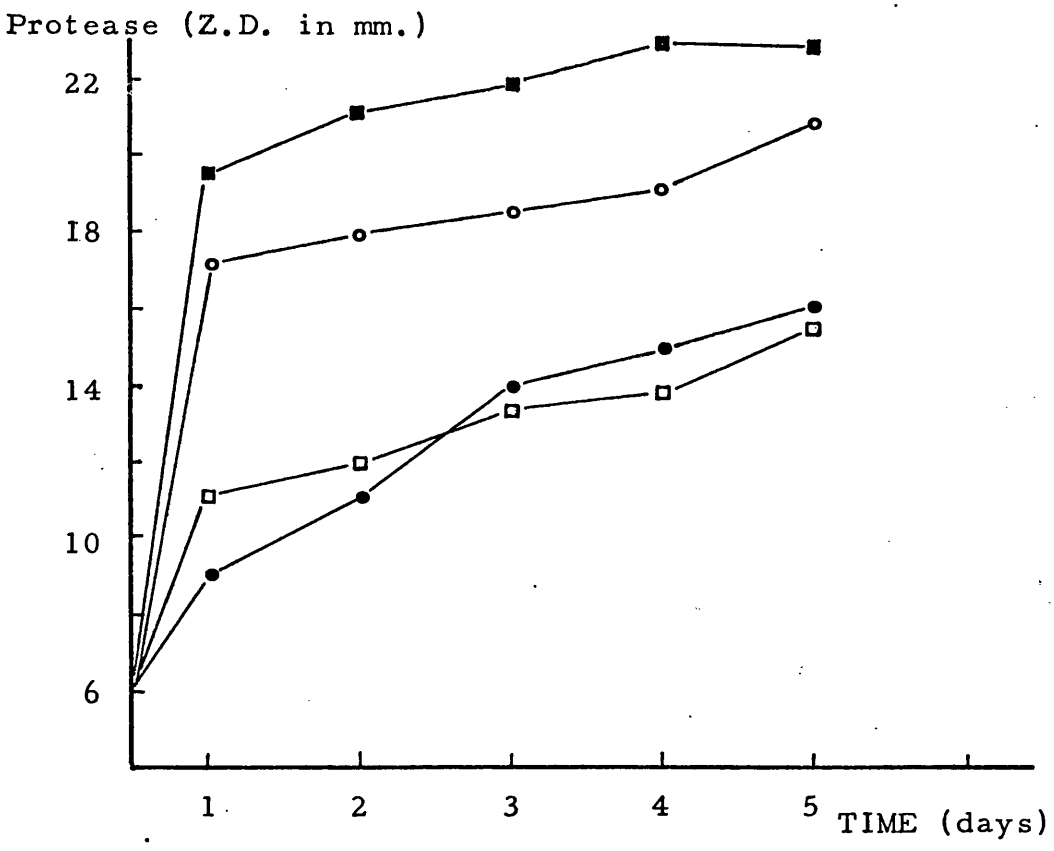
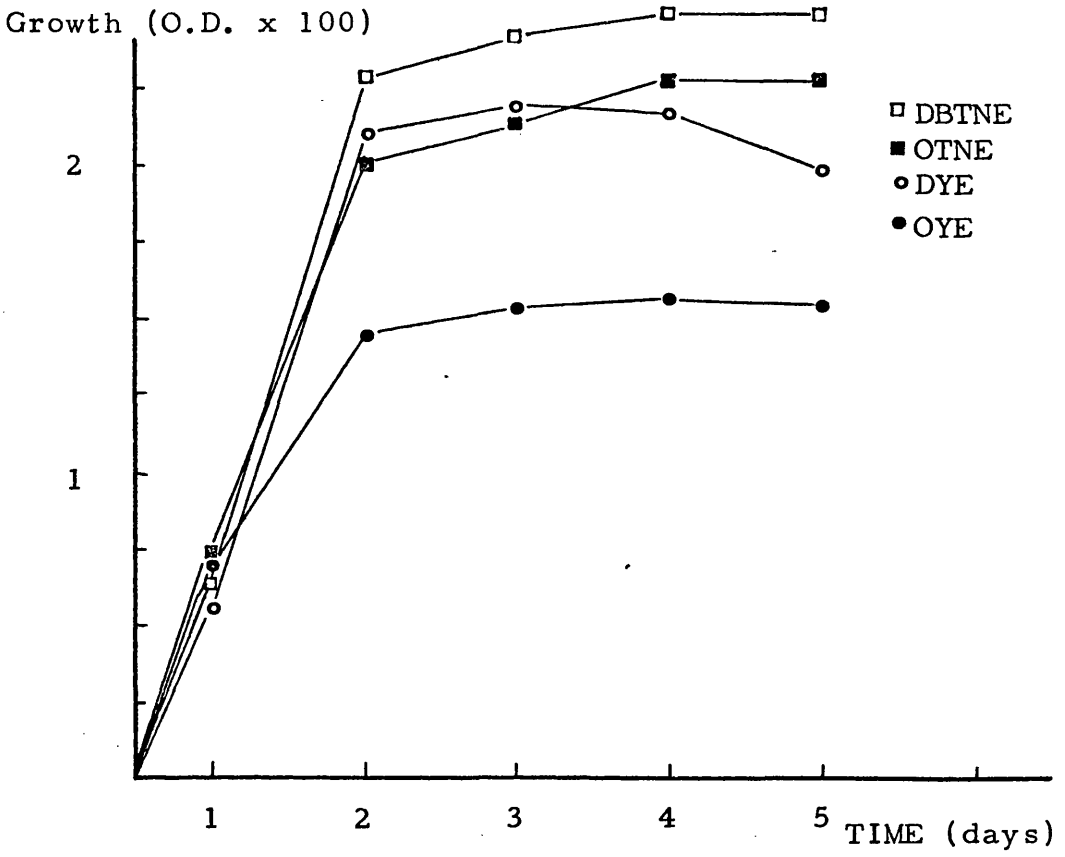


Figure 3. Growth and Protease Production by Strain S1 in Commercial Media at 25° C.



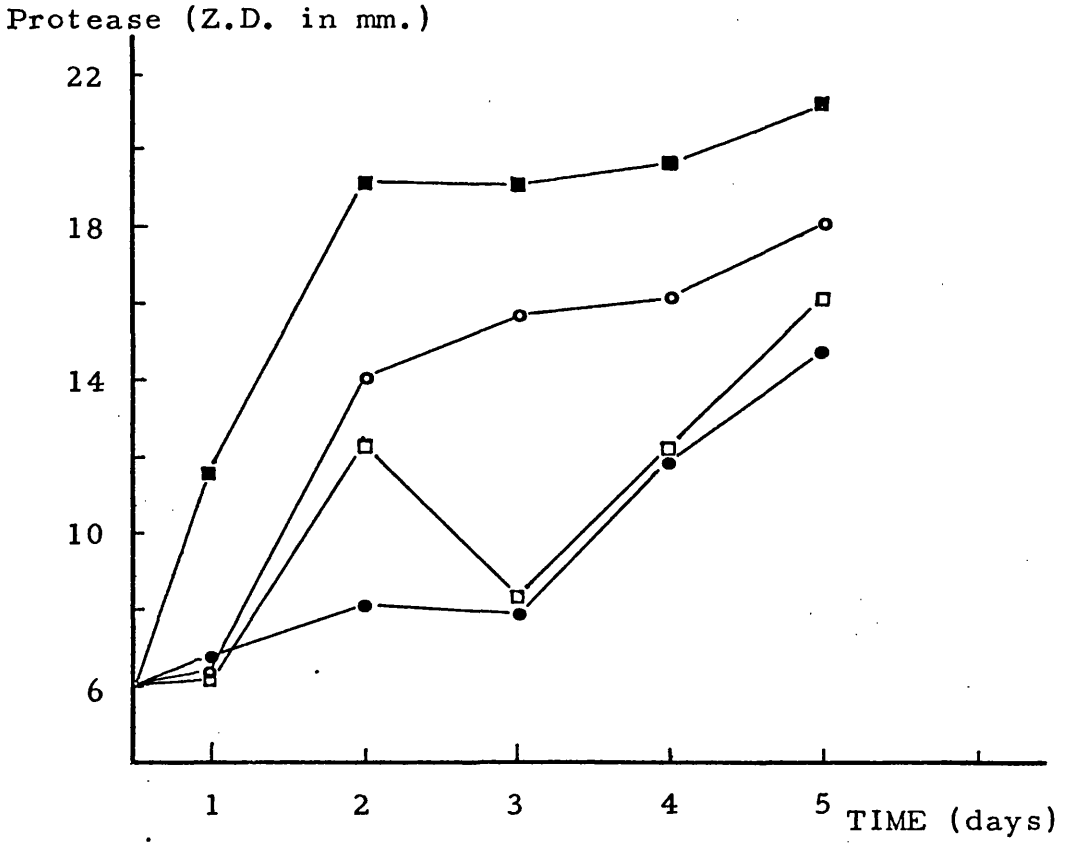
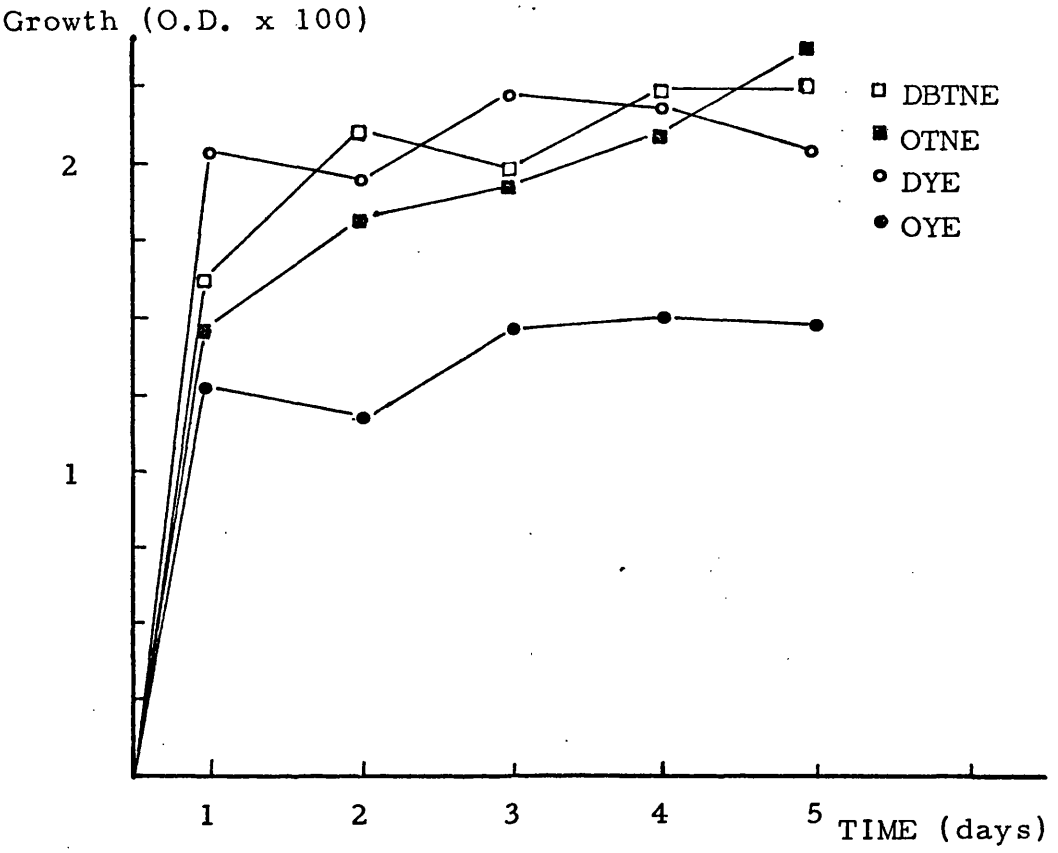


Figure 4. Growth and Protease Production by Strain S2 in Commercial Media at 25° C.



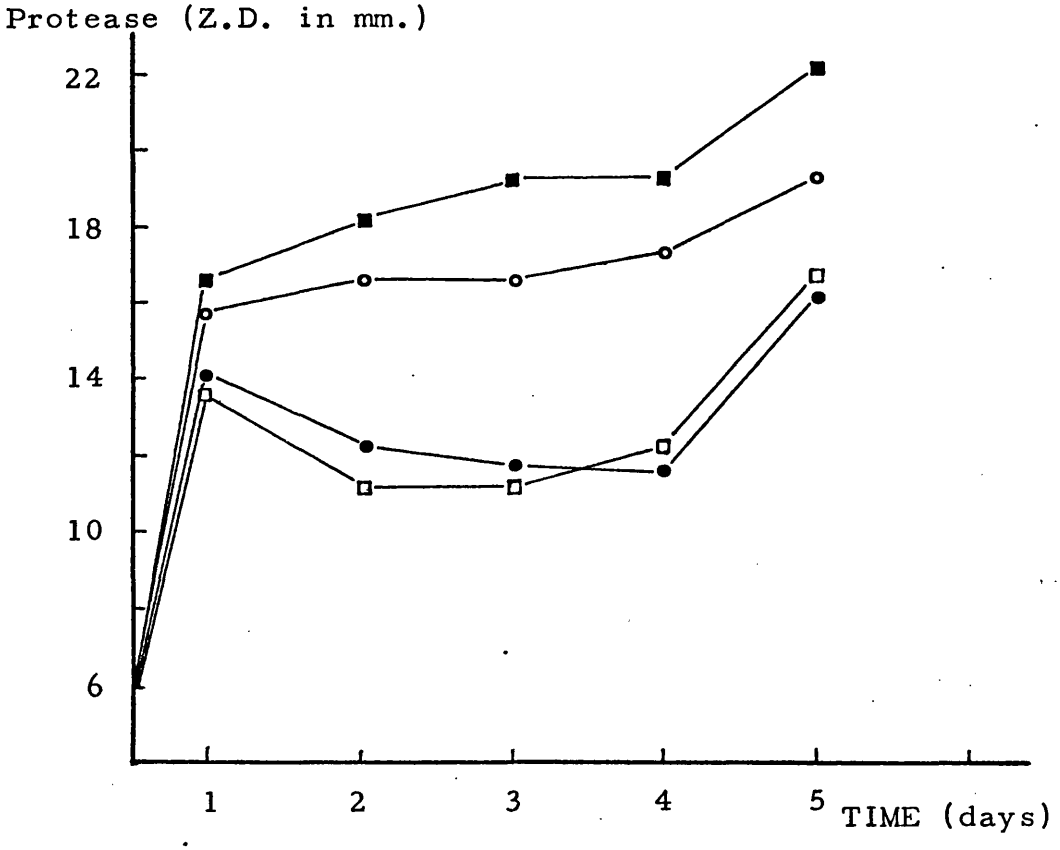
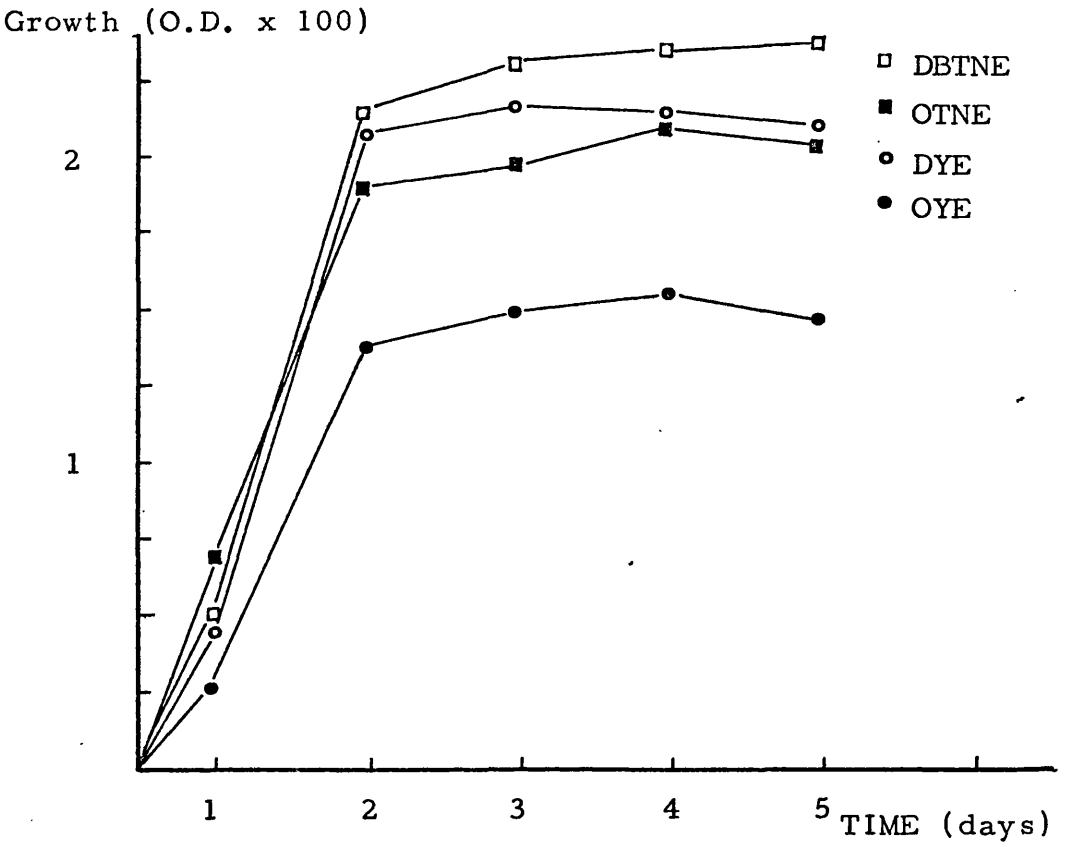


Figure 5. Growth and Protease Production by Strain S3 in Commercial Media at 25° C.



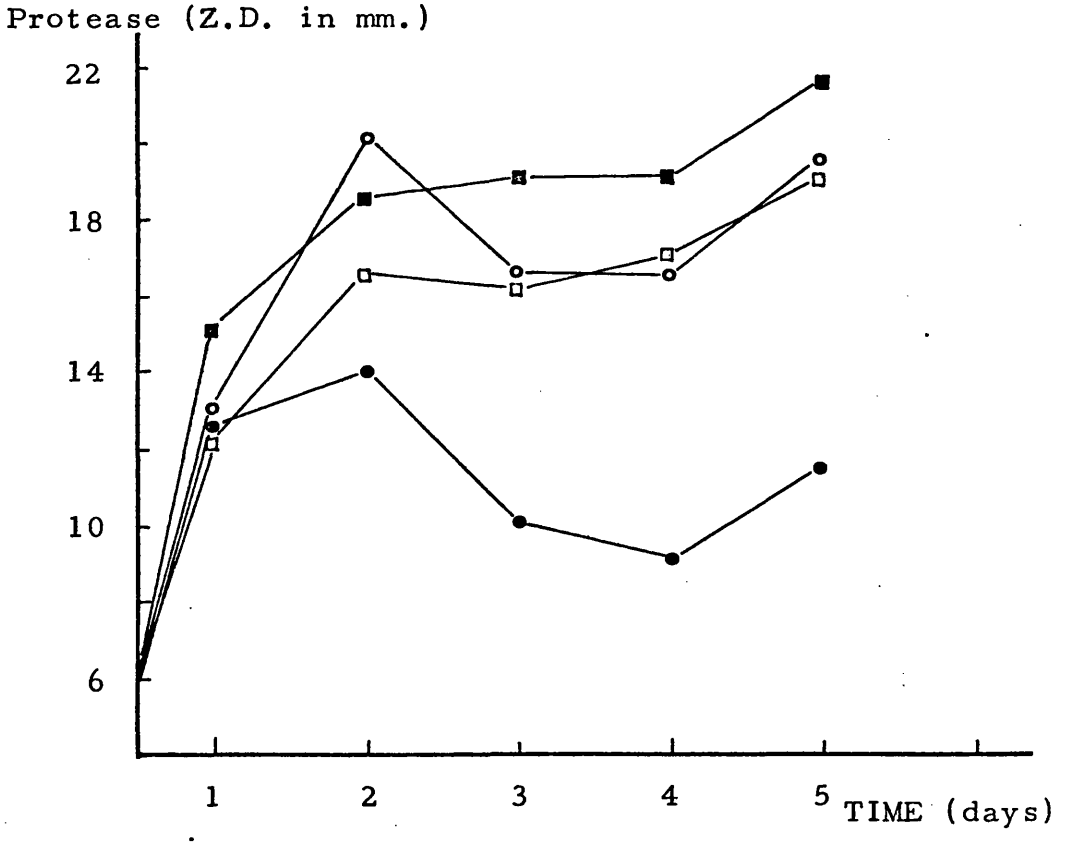
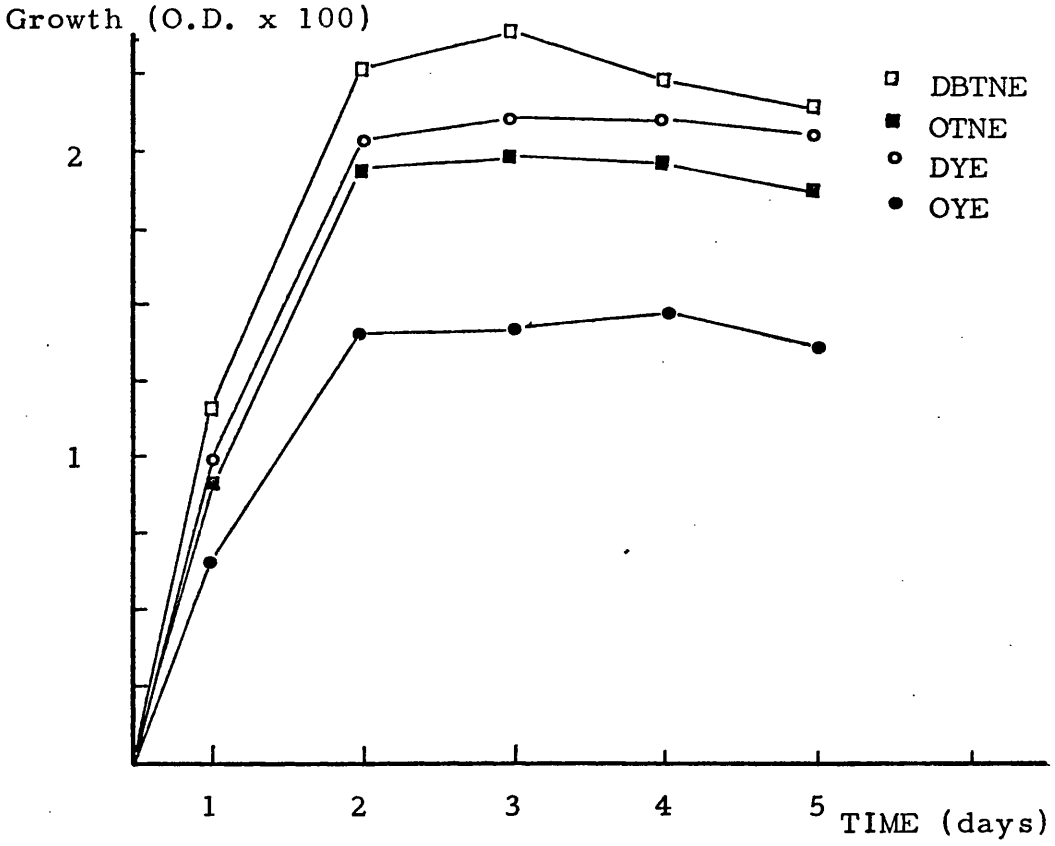


Figure 6. Growth and Protease Production by Strain S4 in Commercial Media at 25° C.



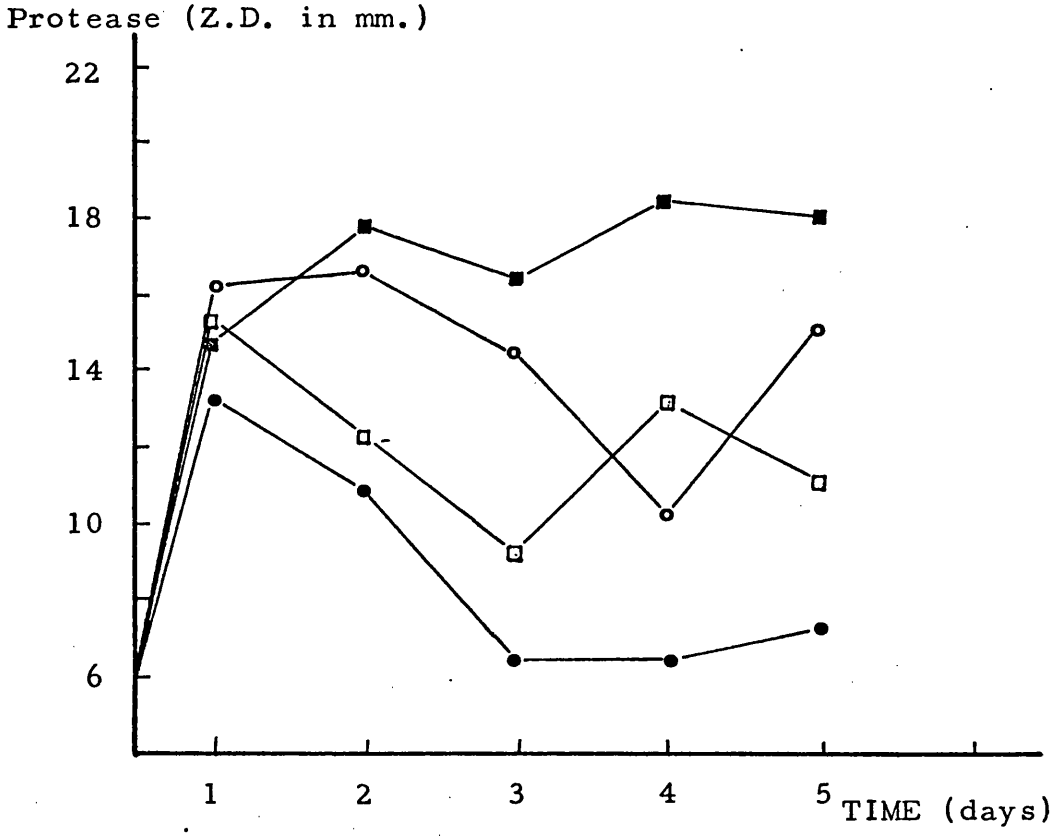
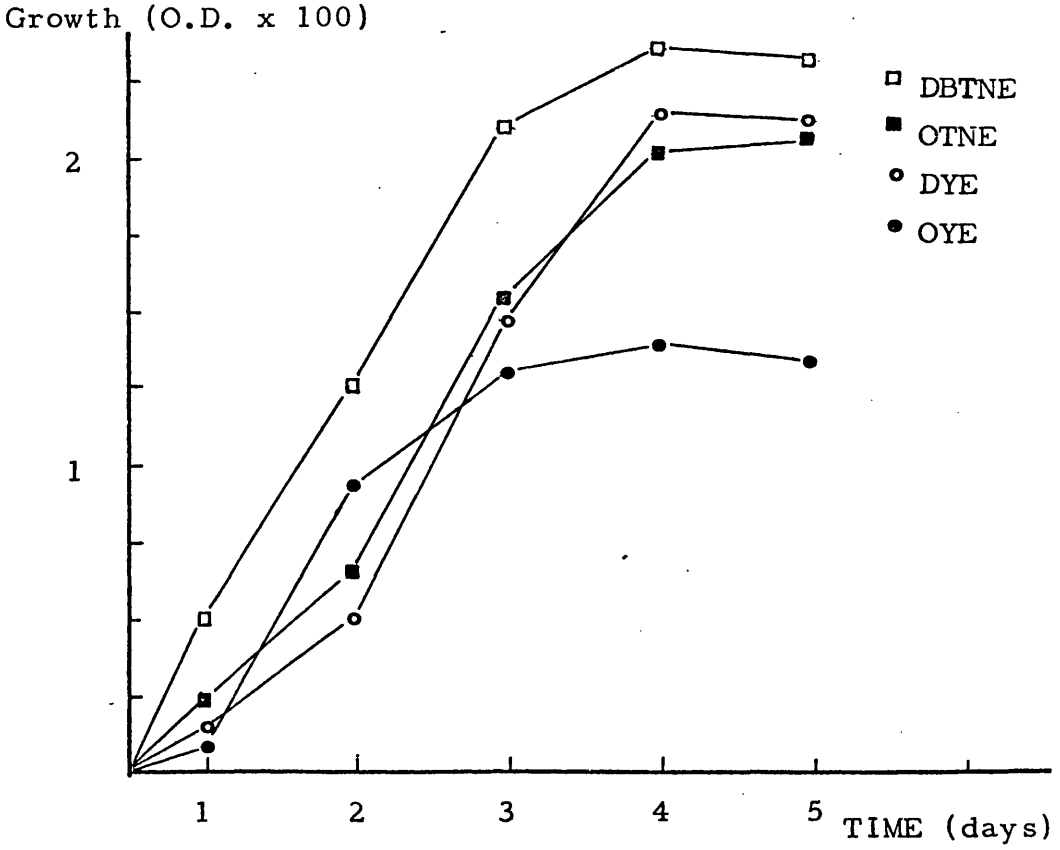


Figure 7. Growth and Protease Production by Strain S5 in Commercial Media at 25° C.



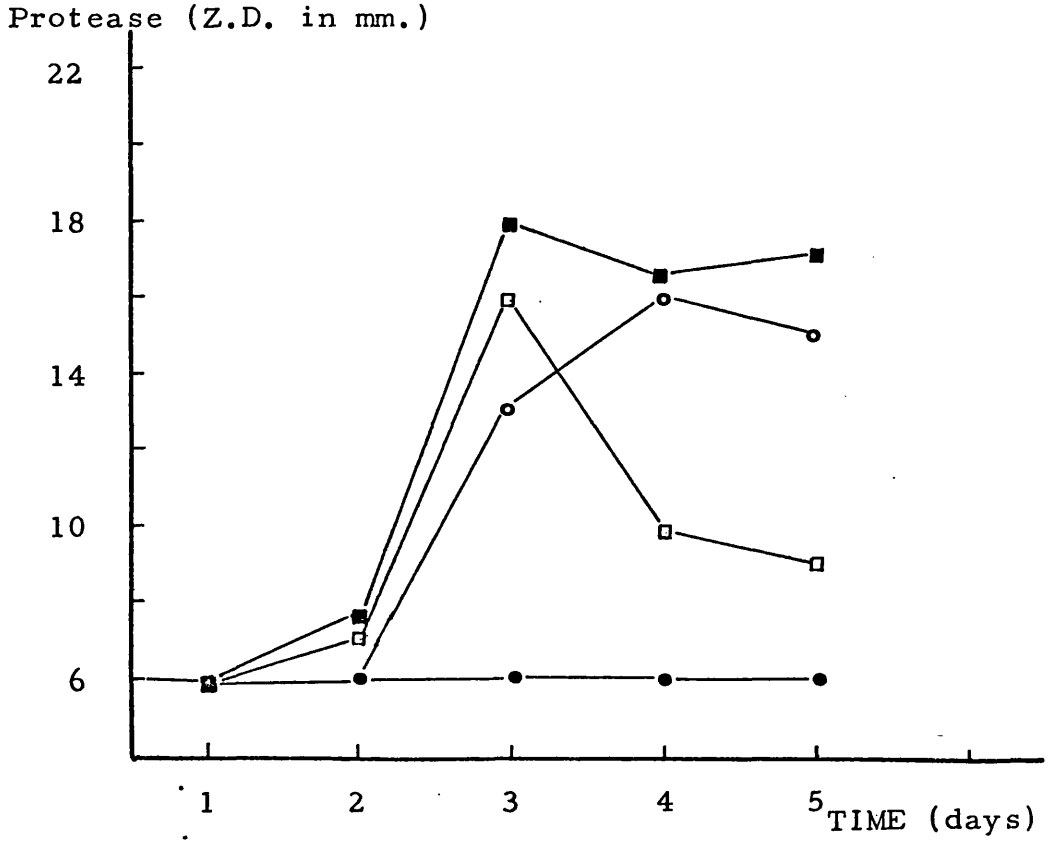
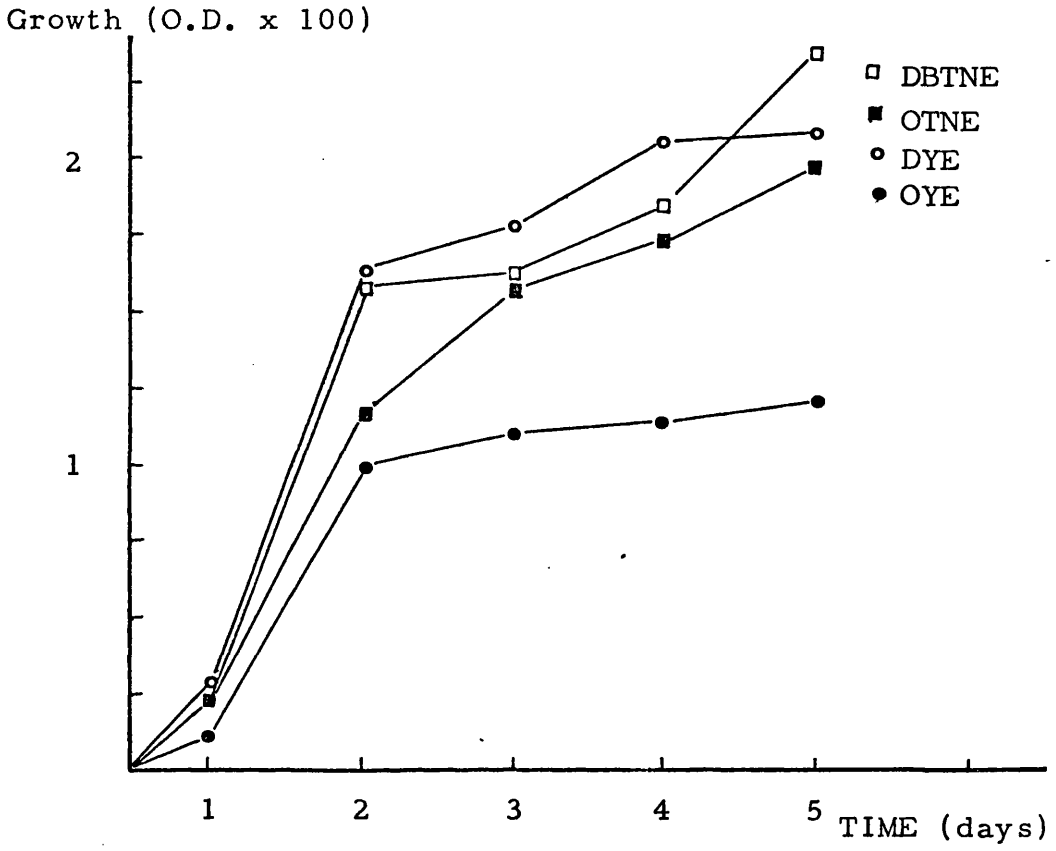


Figure 8. Growth and Protease Production by Strain S6 in Commercial Media at 25° C.



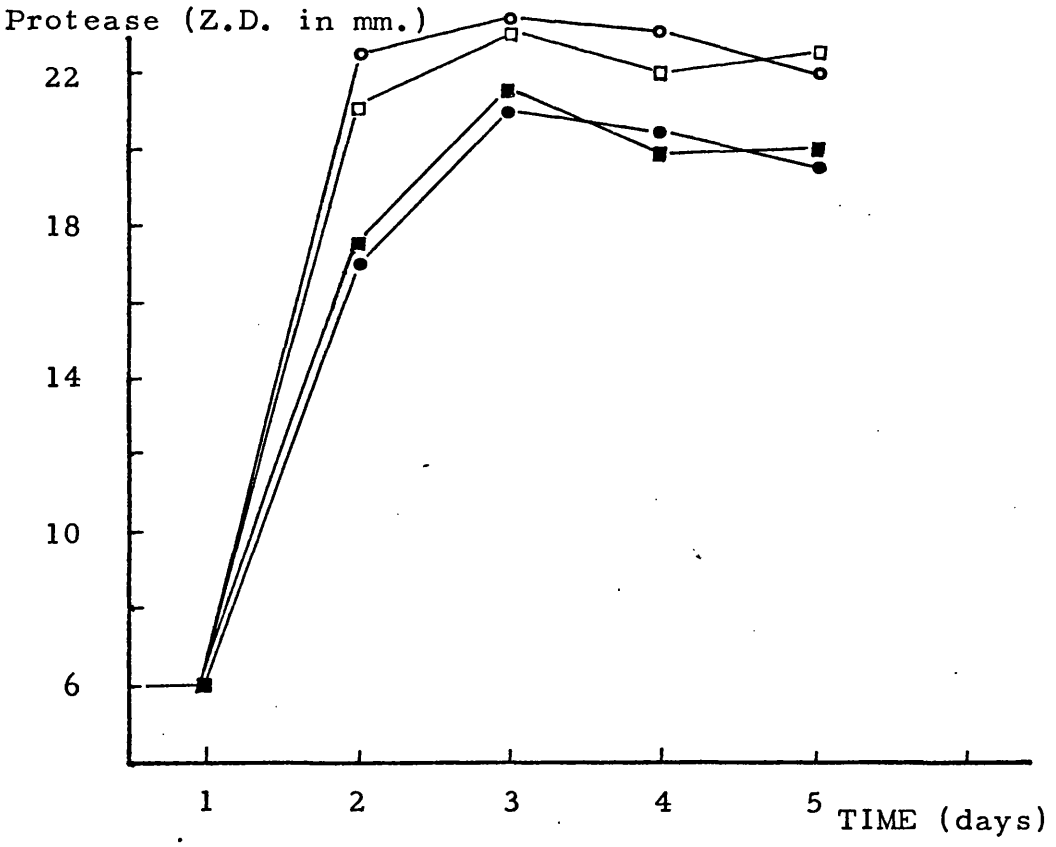
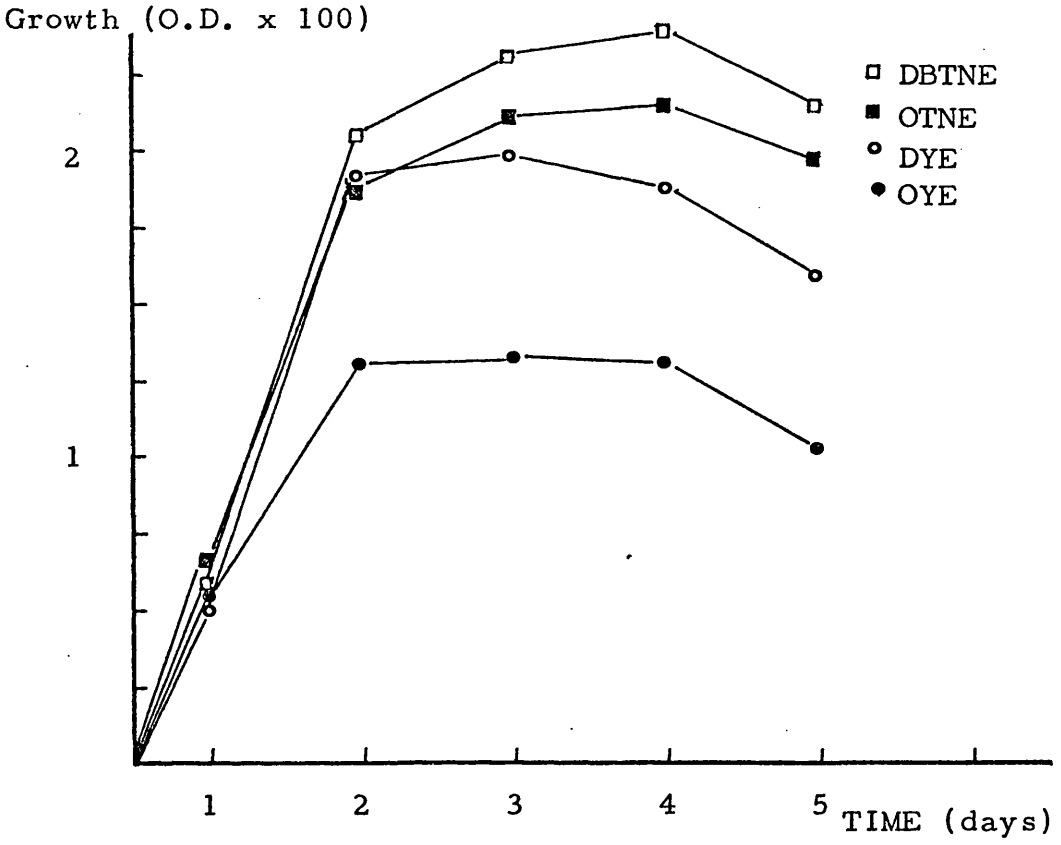


Figure 9. Growth and Protease Production by Strain S7 in Commercial Media at 25° C.



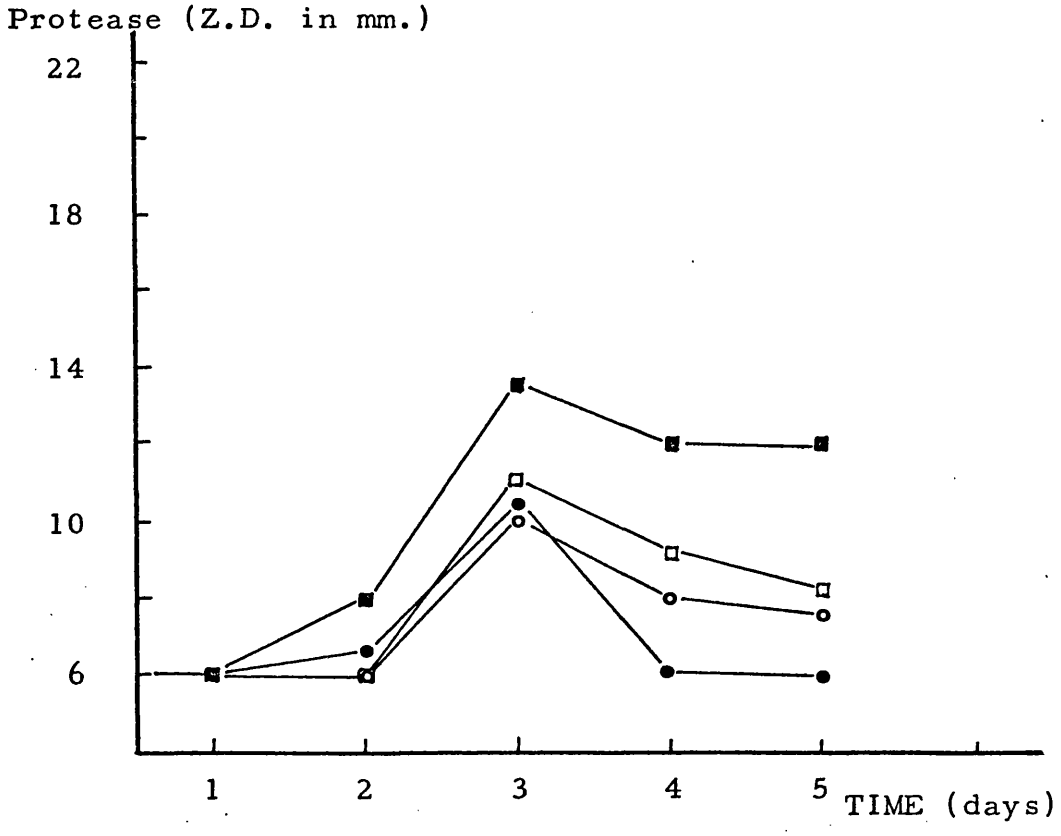


Figure 10. Growth and Protease Production by Strain S8 in Commercial Media at 25° C.

tively low level of cell growth in respect to the level of protease production (Fig. 3). High levels of growth and protease production were observed for DYE, OPPNE, OTNE and OBPNE (Table 4). Based on the highest r^2 value presented in Table 12, DYE medium was considered the best choice for the growth of this organism.

The basic patterns of protease production relative to growth of strain S2 in the various media were similar to those demonstrated by strain S1. When S2 was cultured in DBTNE and OYE a temporary decrease in protease activity was observed as the cultures entered the stationary growth phase (Fig. 4). The subsequent increase in activity may be due in part to cell lysis, intracellular proteolytic activity being released into the culture fluid. Good growth and protease production was observed when the organism was cultured in DYE, OTNE and OBPNE (Table 5). However, on the basis of the r^2 value, DBPNE was considered the most suitable culture medium.

The level of protease production by strain S3 was depressed after entering the stationary growth phase on day 1. This was most apparent when the basal salts medium was supplemented with either DBTNE or OYE (Fig. 5). Such losses of protease activity were observed within one to four days of the commencement of the experimental growth period. Good growth and satisfactory protease production was observed for the DYE and OTNE growth cultures (Table 6). Based on the r^2 values pre-

sented in Table 12, both DYE and OTNE were considered the best choices of synthetic growth media.

The test strain S4 produced high levels of enzyme activity at relatively low growth levels. Protease activity was depressed towards the end of the logarithmic growth phase, this observation being most apparent with reference to the media illustrated in Fig. 6. Good growth levels and protease production were observed with DBTNE and OTNE (Table 7). Based on the highest r^2 value, DBTNE was considered the best medium for S4 since it supported a good balance between growth and protease production.

Protease production by strain S5 was largely confined to the early logarithmic growth phase. Noticeable loss of enzyme activity occurred after 24 hours growth in DBTNE, DYE, OTNE and OYE media, and was accompanied by a decrease in growth rate (Fig. 7). Medium OTNE supported good growth and protease production and gave the highest r^2 value (Table 12). Although moderate growth of S5 was achieved using OCH media, only trace amounts of protease activity were detected after 48 hours. In comparison poor growth but reasonable protease production was detected using DBPNE as the synthetic medium (Table 8).

Maximum protease activity was demonstrated by S6 in the middle/late logarithmic growth phase (Fig. 8). A longer logarithmic growth phase, lasting until day 4, was apparent with this culture in comparison to the

previous test organisms (Table 9). No proteolytic activity was detected when the test organism was cultured in OCH or OYE media. On the basis of r^2 values OTNE was considered the best choice on medium for growth and protease production.

Good protease production was displayed in all the test media by strain S7. Enzyme production paralleled the growth of the test cultures, the greatest increase in proteolytic activity being observed in the early logarithmic growth phase. As illustrated in Fig. 9 high and low growth levels were not synonymous with high and low levels of proteolytic activity. Medium OYE, for example supported poor growth although protease production was equivalent to that observed in medium OTNE. Both good growth and protease production were apparent in media DPPNE, DYE, OTNE and OTSE (Table 10). On the basis of r^2 value OBPNE was considered the most suitable growth medium.

Protease production by strain S8 was greatest in the late logarithmic and stationary phase of growth (Fig. 10 and Table 11). Wide variations were observed in the ability of the growth media to support protease production. No proteolytic activity was detected for the growth of strain S8 after 24 hours in all test media. In general strain S8 showed relatively low levels of protease production in most of the test media. Media OYE and DYE were slightly repressive with respect to the maximum growth observed in DBTNE medium. The r^2 indicated OYE as the best choice of medium.

Table 12. Correlation Coefficients (r^2) Calculated for the Relationship Between Growth and Protease Production by the Test Strains Under Stationary Shallow Conditions at 25° C.

Media	Strains								
	S1	S2	S3	S4	S5	S6	S7	S8	Average
DPPNE	0.86	0.80	0.77	0.75	0.75	0.82	0.81	0.75	0.77
DBPNE	0.60	0.98	0.71	0.68	0.85	0.64	0.72	0.70	0.73
DBTNE	0.94	0.72	0.76	0.84	0.32	0.62	0.50	0.90	0.70
DBTSE	0.65	0.48	0.29	0.01	0.16	0.56	0.51	0.90	0.44
DYE	0.97	0.92	0.96	0.77	0.47	0.96	0.42	0.95	0.80
OPPNE	0.87	0.93	0.87	0.77	0.81	0.87	0.79	0.90	0.85
OTNE	0.95	0.96	0.96	0.83	0.86	0.92	0.80	0.92	0.90
OCH	0.69	0.47	0.63	0.12	0.07	0.00	0.00	0.93	0.36
OTSE	0.88	0.63	0.80	0.80	0.25	0.75	0.67	0.88	0.70
OBPNE	0.94	0.91	0.87	0.82	0.80	0.88	0.96	0.88	0.88
OYE	0.73	0.54	0.81	0.20	- 0.005	0.00	0.19	0.98	0.43

(c) Conclusion

Juffs et al. (1968) studied the effects of nutritional conditions on the growth and protease production by Pseudomonas spp. In their study the elaboration of extracellular protease appeared to require the presence of amino acids or peptides in the growth medium. Proteases were not produced in mineral salts medium containing inorganic nitrogen and, either citrate or citrate and casein. The absence of certain metals, especially Mg^{++} and Ca^{++} have been shown to inhibit protease production either directly or indirectly through growth inhibition (Sandvik, 1962). Denatured protein and proteose are often not attacked by certain proteolytic organisms without the addition of peptone or other nutritive substrate to the medium (Rettger et al., 1916), which are probably able to transport certain metal ions or which are absorbed by the active help of such metal ions. The presence of these ions together with organic nitrogen seems to be essential for both protease production and normal growth of the tested strains. These essential conditions appeared to be more consistently met when basal salts medium was supplemented with OTNE. Furthermore, tryptone (OTNE) was also selected by Scholefield (1967) to study the effect of sodium chloride concentrations up to 4.5% on the growth of psychrophiles.

The results obtained for the effect of organic nitrogen on growth and protease production by the

test strains are in agreement with those obtained by Juffs et al. (1968) and Juffs (1976). Juffs (1976) has also found that protease production is affected by the source of organic nitrogen in the growth medium.

The medium used throughout this work was the basal salts supplemented with OTNE, selected on the basis of its consistent ability to support growth and protease production by all the test strains (Table 12).

SECTION 2. OBSERVATIONS ON THE EFFECTS OF MEDIA COMPOSITION, GROWTH AND PROTEASE ACTIVITY

(a) Differences in Media Composition

The average r^2 values were taken for all strain responses in terms of growth and protease production (Table 12) in each commercial medium. The medium OTNE accumulated a average score (average r^2) of 0.90 and was contrasted by a score of 0.36 for OCH. The major compositional differences between OTNE and OCH were reproduced from analysis certificates provided by medium manufacturers (see Appendix E). Certain metals, such as calcium, magnesium and zinc were more readily available for the test strains in OTNE. The medium OCH contained lower assayed Trp and chlorides were considerable higher. The amount of His was significantly higher in OTNE.

(b) Discussion

Variations in the mineral composition of the commercial media may contribute to different levels of protease production by the test strains. Protease production may be stimulated either indirectly through growth stimulation or directly by affecting the mechanism of enzyme production and activation (Sandvik, 1962). The variations in salt composition of individual medium were not important source of differences of osmotic strength of the final solution of the test media, since only 0.5% (w/v) of each medium was used. Thus, the osmotic strength of the final mixture did not vary significantly with respect to salt composition in the test media. The lack of calcium was supplemented in the basal salts medium according to the actual quantity present in each commercial medium. So, the differences in growth and protease production displayed by the test strains were certainly caused by the differences of organic nitrogen and/or calcium contents, specially for the comparison between OTNE and OCH (Appendix E).

(c) Conclusion

Although the amount of salts present in the test media was slightly different in each case, the combination with the BSM solution helped to minimize this difference. Calcium was not present in the basal salts medium but was present in variable proportions in each of the commercial supplements. Thus, the mixed media may have balanced the deficiency or surplus of essential nutrients.

SECTION 3. CONCLUSION

The effects of different commercial media on growth and protease production by the test strains were investigated. The results obtained for the effects of organic nitrogen present in mixtures of salts and commercial media agree with those obtained by previous investigators. The psychrophilic strains used to study these effects were able to grow best in Pope and Skerman's basal salts medium when OTNE was the added supplementation mixture. The medium OTNE either provided additional organic nitrogen together with certain essential metals or helped to prevent the formation of insoluble phosphate salts during sterilization. This precipitation is specially known to occur when glucose is added to the medium and sterilization is carried out at 122° C for 15 minutes (Meynell and Meynell, 1970). For this reason and also the fact that most of the psychrophiles are able to grow in the absence of carbohydrates, glucose was not added to the media. In addition to this, certain psychrophilic pseudomonas are able to produce considerable amounts of acid when glucose is present, especially if shallow growth layer conditions are used (Sinclair and Stokes, 1962). When this is the case, the pH of the medium lowers down to a point in which the organism is inhibited early before the depletion of certain critical nutrients such as $(NH_4)_2\ SO_4$. On the contrary, the medium OTNE used to-

gether with BSM and with no glucose addition, supported growth under aerated conditions leading to increases in pH. This was evident after 5 days of incubation at 5^{o} C during preliminary trials using the test strain S8.

CHAPTER IV

STUDY OF THE ELECTROPHORETIC PATTERNS OF CASEIN DIGESTION

SECTION 1. PATTERNS FOR NATIVE CASEIN DIGESTION USING RAW SKIM MILK SAMPLES AS SUBSTRATE

(a) Experimental

Test enzyme preparations, obtained according to the procedure described on pages 45-46, were added to 10 ml of fresh raw skim milk (2,000 g/30 minutes) preheated to 30° C. Five portions of 1.5 ml were quickly dispensed into five presterilized test tubes (150 x 17 mm.) preheated to 30° C and stoppered with cotton plugs. The skim milk/enzyme samples were kept at 30° C in a water bath and removed at intervals of 1, 3, 6 and 24 hours. A blank sample was also incubated under identical conditions with each group of five test tubes. At the end of the incubation period the enzyme activity was stopped by adding 3 ml of concentrated buffer as recommended by Thompson *et al*. (1964). Electrophoresis was carried out according to the procedure described on pages 38-40.

The amount of added enzyme was standardized at approximately 2,000 D.U. per ml in each case. Table 13 shows the calculated D.U./ml ratio for each case. The actual amounts of enzyme preparation used were as follows: S1 to S8 respectively 3.1, 1.3, 0.3, 2.2, 0.5, 2.4, 1.6 and 0.5 mg./10 ml of the skim milk sample. Since the enzyme preparations used in this experiment

were concentrated by ultrafiltration, the changes of activity were observed for the various steps of the procedure. These changes of "specific" activity during the preparation of enzyme concentrates are shown in Appendix F. Losses of activity could be recovered when the freeze dried enzyme preparations were dissolved in Pope and Skerman's basal salts medium. As the enzyme preparations were dissolved directly into the skim milk samples, it was assumed that, in each case, the milk provided those cofactors washed out during concentration by ultrafiltration.

(b) Results and Discussion

A typical electrophoretic pattern observed for a fresh skim milk sample is illustrated in Fig. 11. Although both α_s- and β-caseins have been shown to be heterogeneous (Thompson et al., 1964; Fox and Guiney, 1972), under the electrophoretic conditions used in this experiment and using milk samples obtained from the College farm, they appear as single bands. Their comparative rates of electrophoretic mobility were approximately 1.0 for α_s- and 0.7 for β-casein fractions. Also, certain minor protein fractions of low mobility rate (approximately 0.2, 0.3 and 0.5) could often be detected. These were possibly either minor fractions of γ-casein together with whey proteins, red proteins (Peterson, 1963), or whey proteins alone.

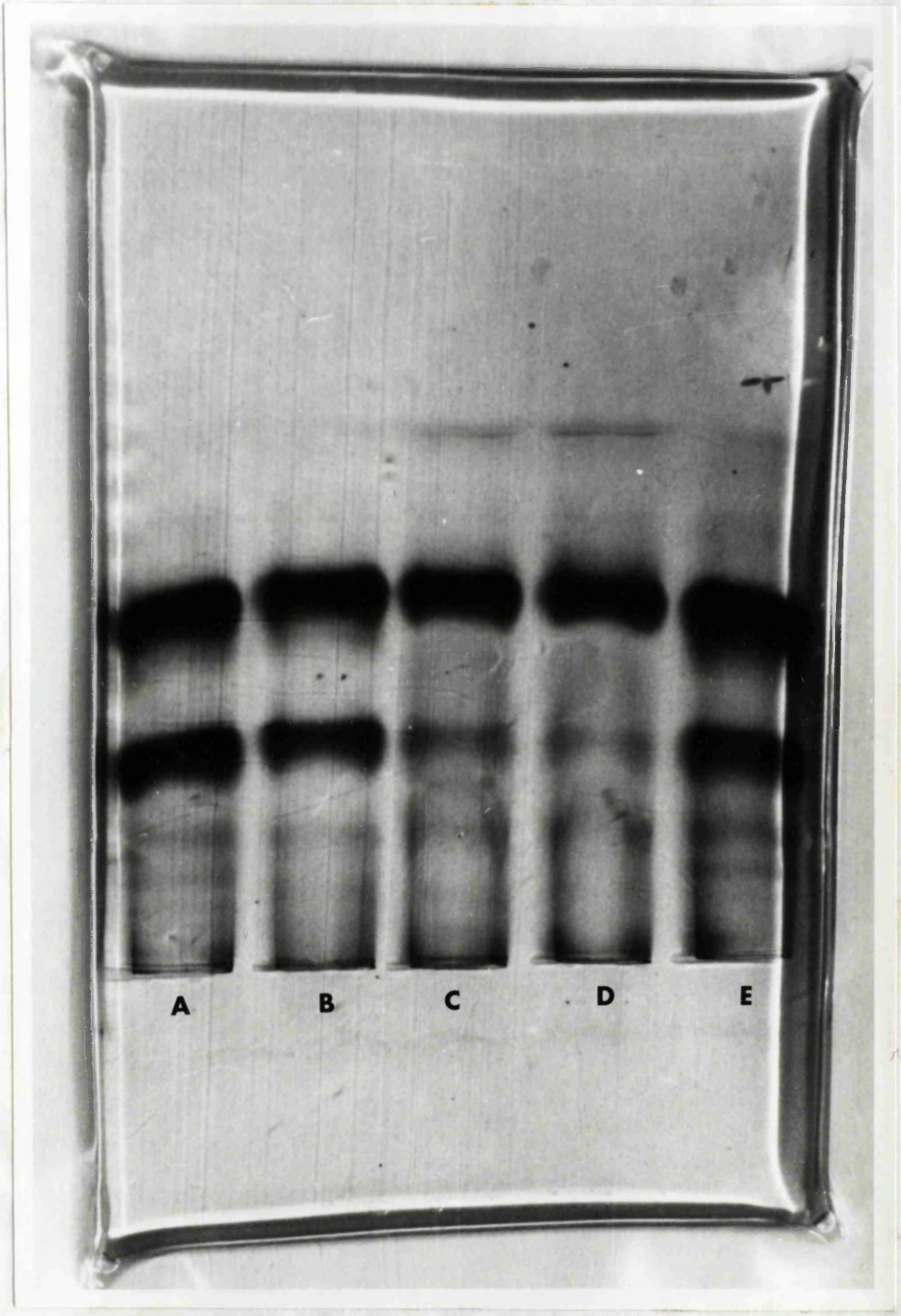


Plate 5. Electrophoretic Patterns of the Protease System of Strain S8, Activity on Skim Milk Casein Substrate at 30° C (A = 1 hour, B = 3 hours, C = 6 hours, D = 24 hours, E = 24 hours blank).

The ratio between the peak areas of α_s- to β-caseins was found to be on the region of 1.75 for fresh skim milk samples.

Plate 5 shows a sequence of patterns for the digestion of β-casein by Pseudomonas fluorescens's protease system at different intervals, extended up to 24 hours (1, 3, 6 and 24 hours at 30° C). The results for the scanning of stained protein bands in polyacrylamide gels are shown in Figs. 12-19 and Table 13. These results were obtained after 24 hours of incubation of the skim milk samples with added enzyme systems and of their respective blanks. Their peak areas were calculated and used to estimate the relative percentage hydrolysis of α_s - and β-caseins taking into account both effect of growth and effect of added enzyme preparations. These estimations are related to the observed ratio of 4 to 7 for the relative peak areas exhibited between β- and α_s-caseins respectively (Fig. 11), obtained for fresh skim milk samples under identical conditions.

Although the problem of bacterial growth during incubations caused considerable masking effect on the true degree of preferential hydrolysis of α_s- or β-fractions by the various enzyme systems, there was a tendency for preferential hydrolysis of β-casein rather than of α_s-casein. This tendency to hydrolyse preferentially β-casein was notably greater for the protease systems obtained from strains S2, S3, S6,

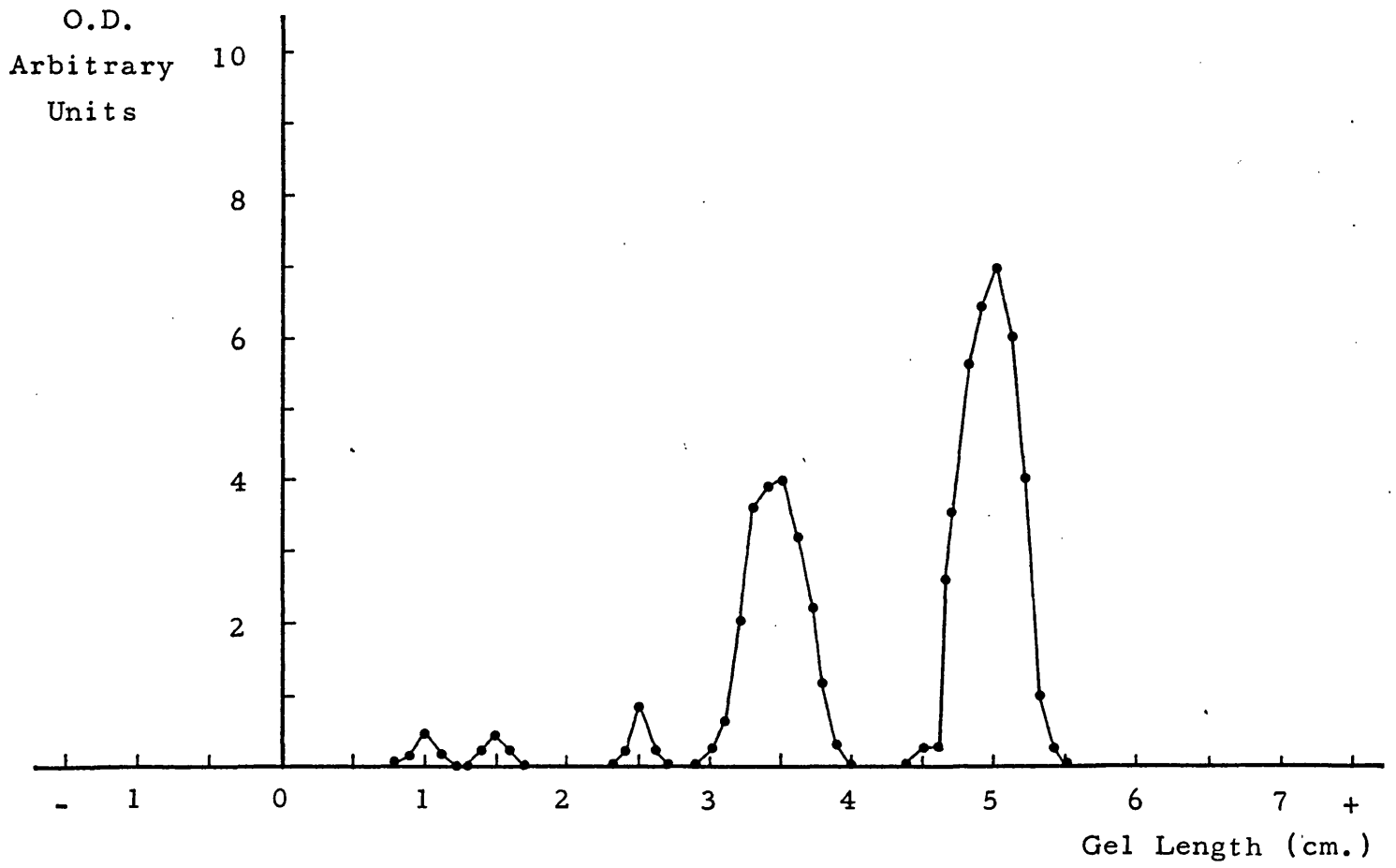


Figure 11. Electrophoretic Pattern for a Fresh Skim Milk Sample in Polyacrylamide Gel Containing 4.5 M Urea and 7.5% (w/v) Cyanogum at pH 8.6.

S7 and S8. Fast moving bands (mobility of 1.13 or higher) as well as release and degradation of minor slow moving bands were also observed. In most cases para-κ-casein was released and appeared either as a single or as a double fraction which migrated toward the negative electrolytic contact. These fractions together with those of low electrophoretic mobility (0.2, 0.3 and 0.5 relative to α_{s}-casein) were invariable completely digested after 24 hours incubation, in some cases, however a small proportion of para-κ-casein remained (see Figs. 12-19).

(c) Conclusion

The degree of preferential hydrolysis of α_{s}- and β-caseins displayed by the test enzyme systems was studied. The results indicate that for most of the tested protease systems a greater tendency to hydrolyse β-casein. This tendency was notably greater for the hydrolysis of native skim milk casein by the protease systems obtained from strains S2, S3, S6, S7 and S8. Para-κ-casein appeared to be progressively released and often complete hydrolysed. These results are in agreement with those reported by Adams et al. (1976) and Law et al. (1977a). In the work of Adams et al. (1976) κ-casein displayed a high susceptibility to hydrolysis and was followed by a rapid decline in β-casein concentration. According to the data presented in this study, α_{s}-casein appeared to be hydrolysed

Table 13. Estimation of Percentage Hydrolysis by Electrophoretic Scanning of the Stained Alpha and Beta Caseins After 24 Hours Incubation at 30° C for the Test Strains.

Enzyme System	Units of Activity	Casein Fractions			
		Alpha		Beta	
		(a)	(b)	(a)	(b)
S1	0	6.50	7.0	2.60	35.0
	1,280	3.30	46.0	0.60	50.0
S2	0	6.82	2.5	3.24	19.0
	2,523	4.20	37.5	1.10	53.5
S3	0	5.75	18.0	3.70	7.5
	1,592	2.59	45.0	0.96	68.5
S4	0	6.05	13.5	3.60	10.0
	2,249	2.52	50.0	1.44	54.0
S5	0	5.86	16.3	2.68	33.0
	1,419	1.59	61.0	0.00	67.0
S6	0	5.50	21.0	3.70	7.5
	1,280	2.72	40.0	0.60	77.5
S7	0	6.00	14.3	3.15	21.2
	2,523	2.62	48.3	0.70	61.2
S8	0	5.08	24.5	3.00	25.0
	1,282	1.90	45.0	0.35	66.0

(a) Relative Peak Area = Width x Height (cm), (b) Percent Hydrolysis (see Appendix I on page 102).

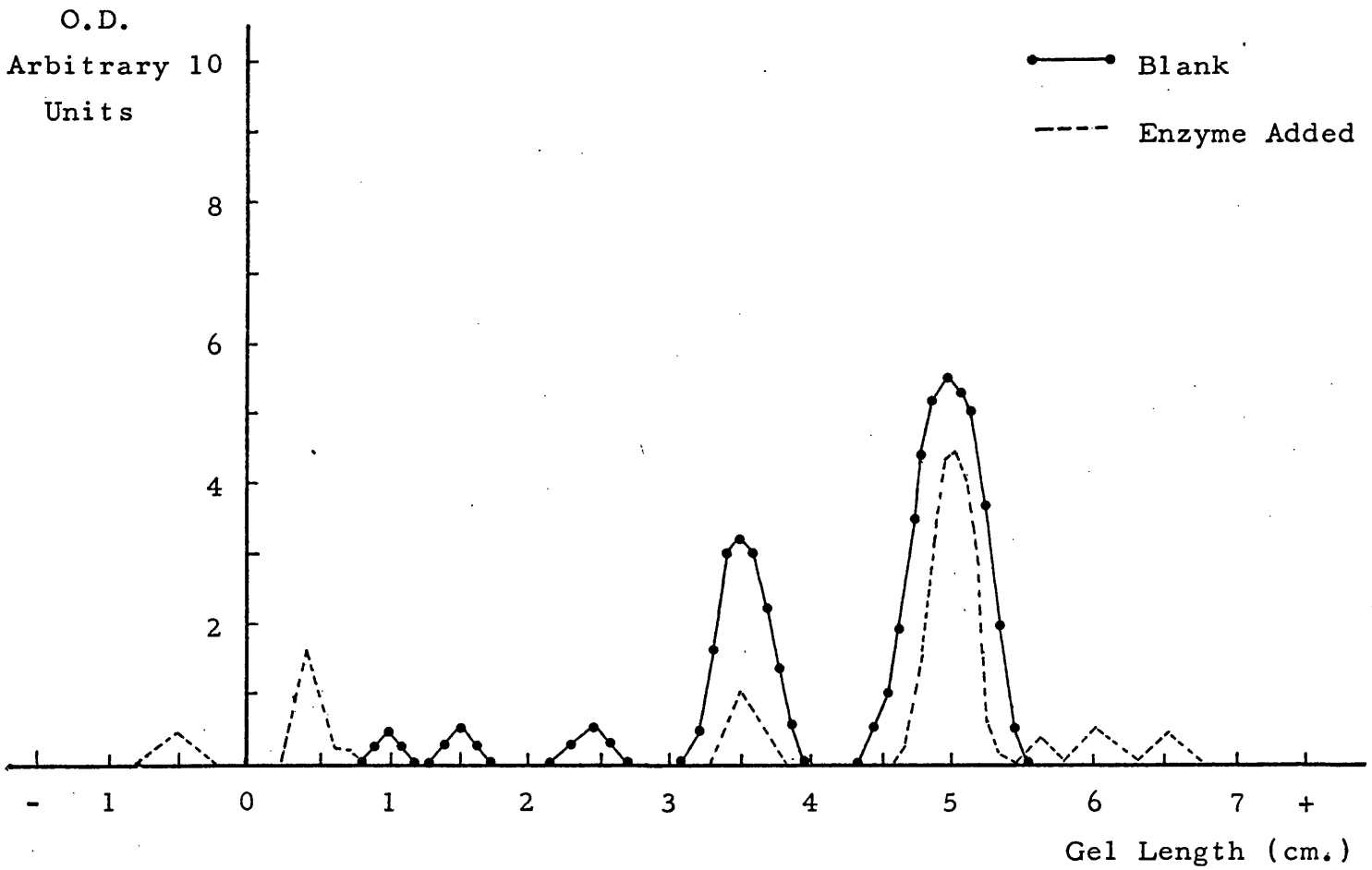


Figure 12. Electrophoretic Patterns of the Protease System of Strain S1, Activity on Skim Milk Casein Substrate at 30° C for 24 Hours.

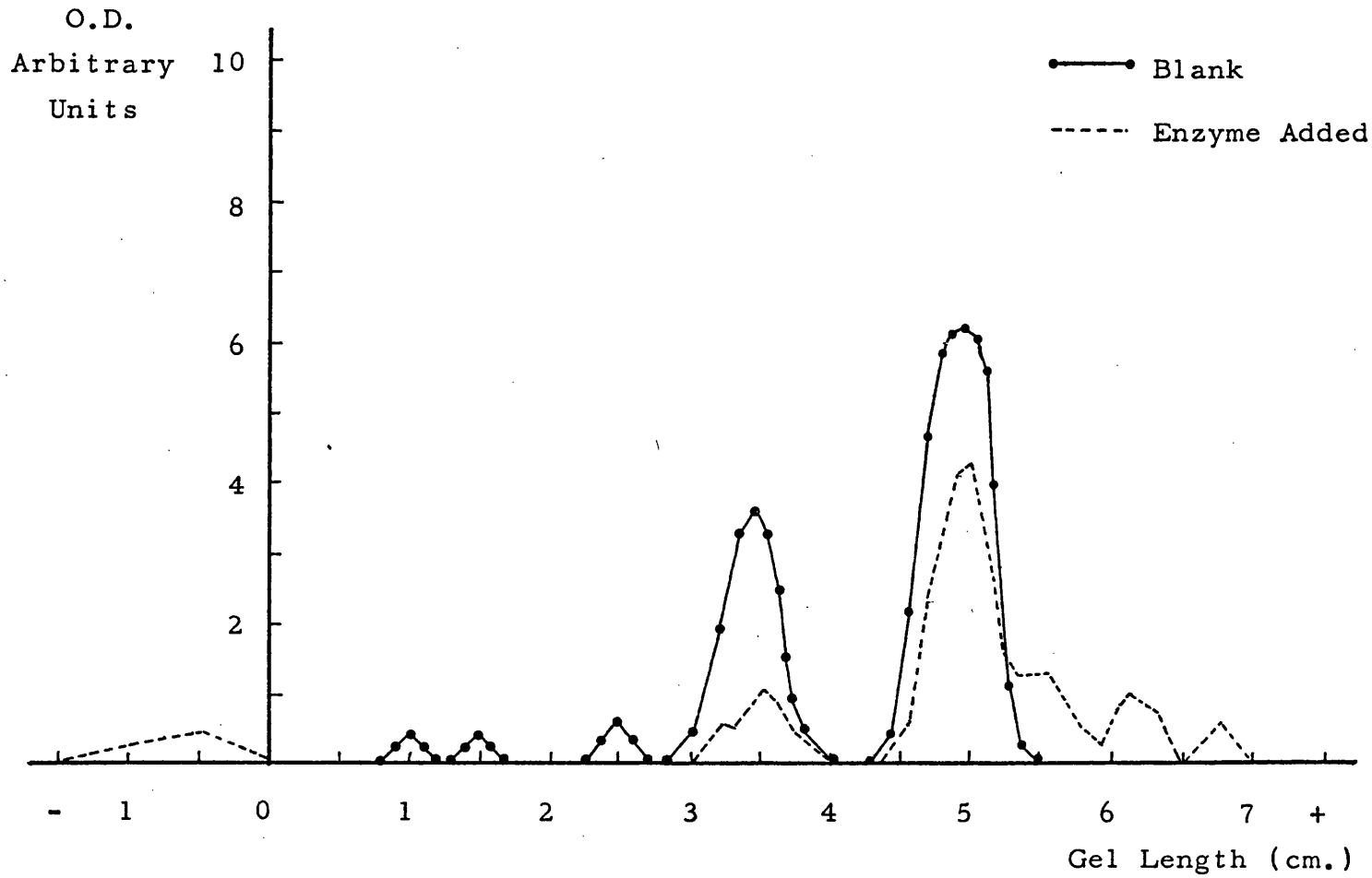


Figure 13. Electrophoretic Patterns of the Protease System of Strain S2, Activity on Skim Milk Casein Substrate at 30° C for 24 Hours.

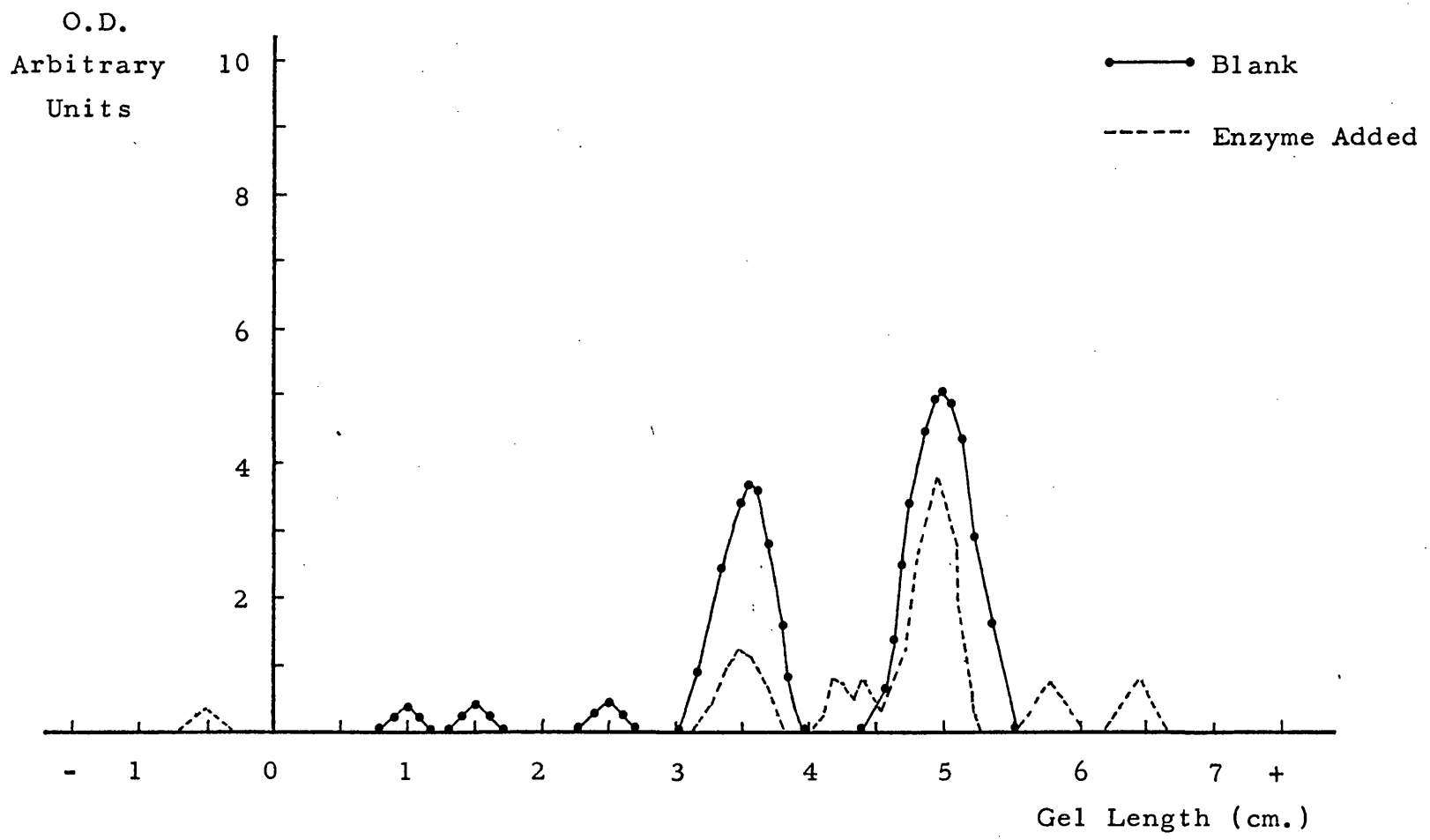


Figure 14. Electrophoretic Patterns of the Protease System of Strain S3, Activity on Skim Milk Casein Substrate at 30° C for 24 Hours.

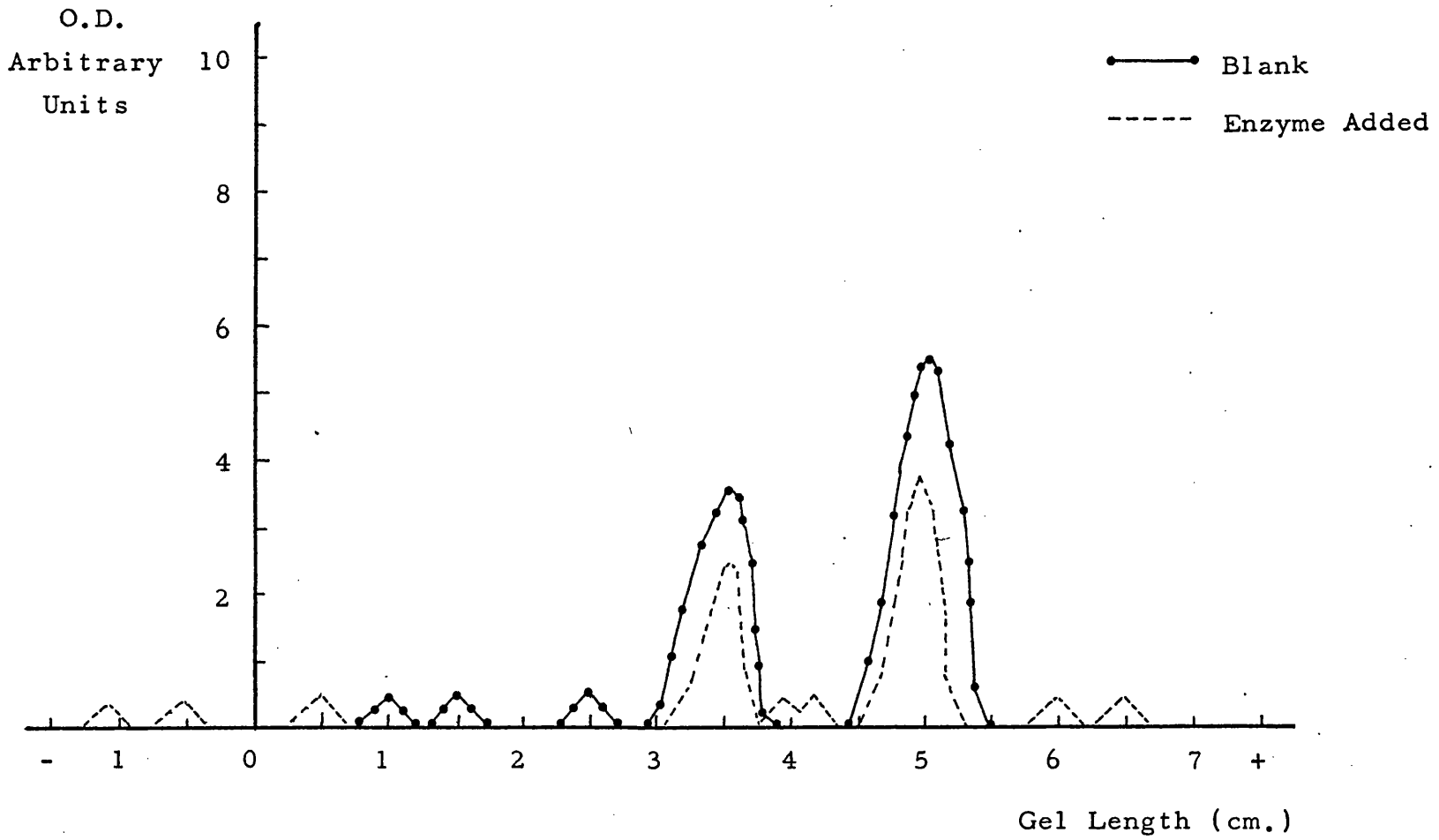


Figure 15. Electrophoretic Patterns of the Protease System of Strain S4, Activity on Skim Milk Casein Substrate at 30° C for 24 Hours.

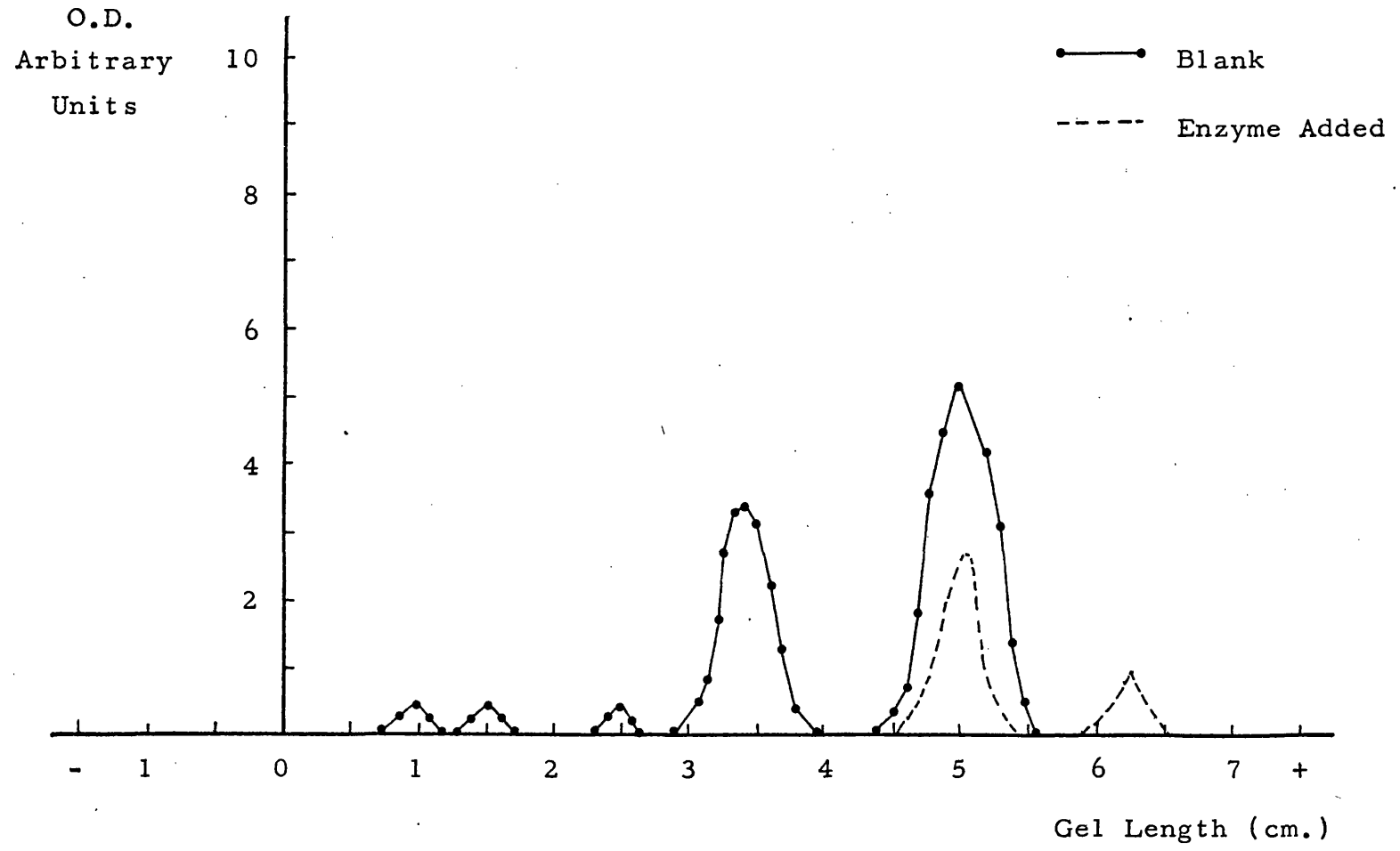


Figure 16. Electrophoretic Patterns of the Protease System of Strain S5, Activity on Skim Milk Casein Substrate at 30° C for 24 Hours.

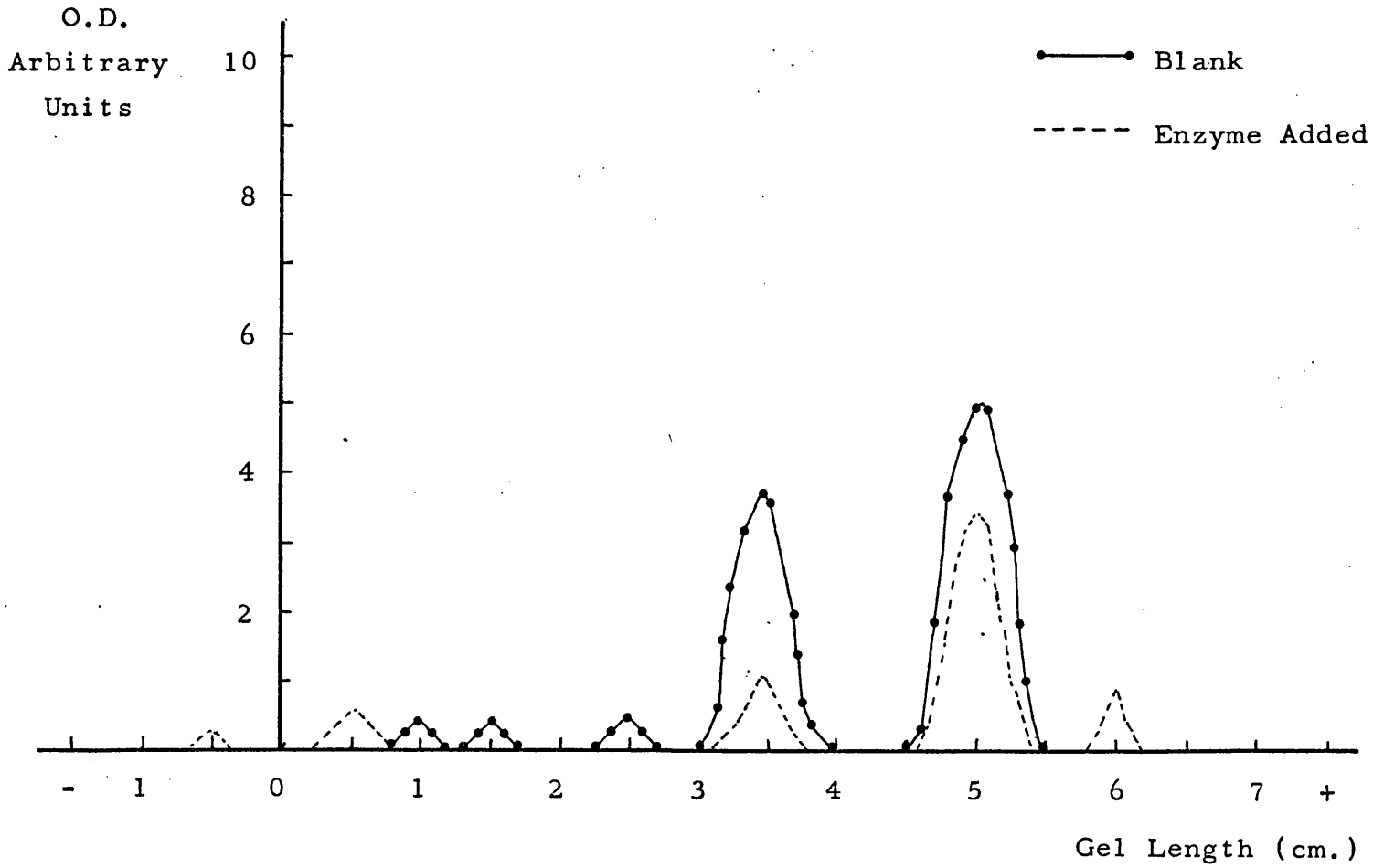


Figure 17. Electrophoretic Patterns of the Protease System of Strain S6, Activity on Skim Milk Casein Substrate at 30° C for 24 Hours.

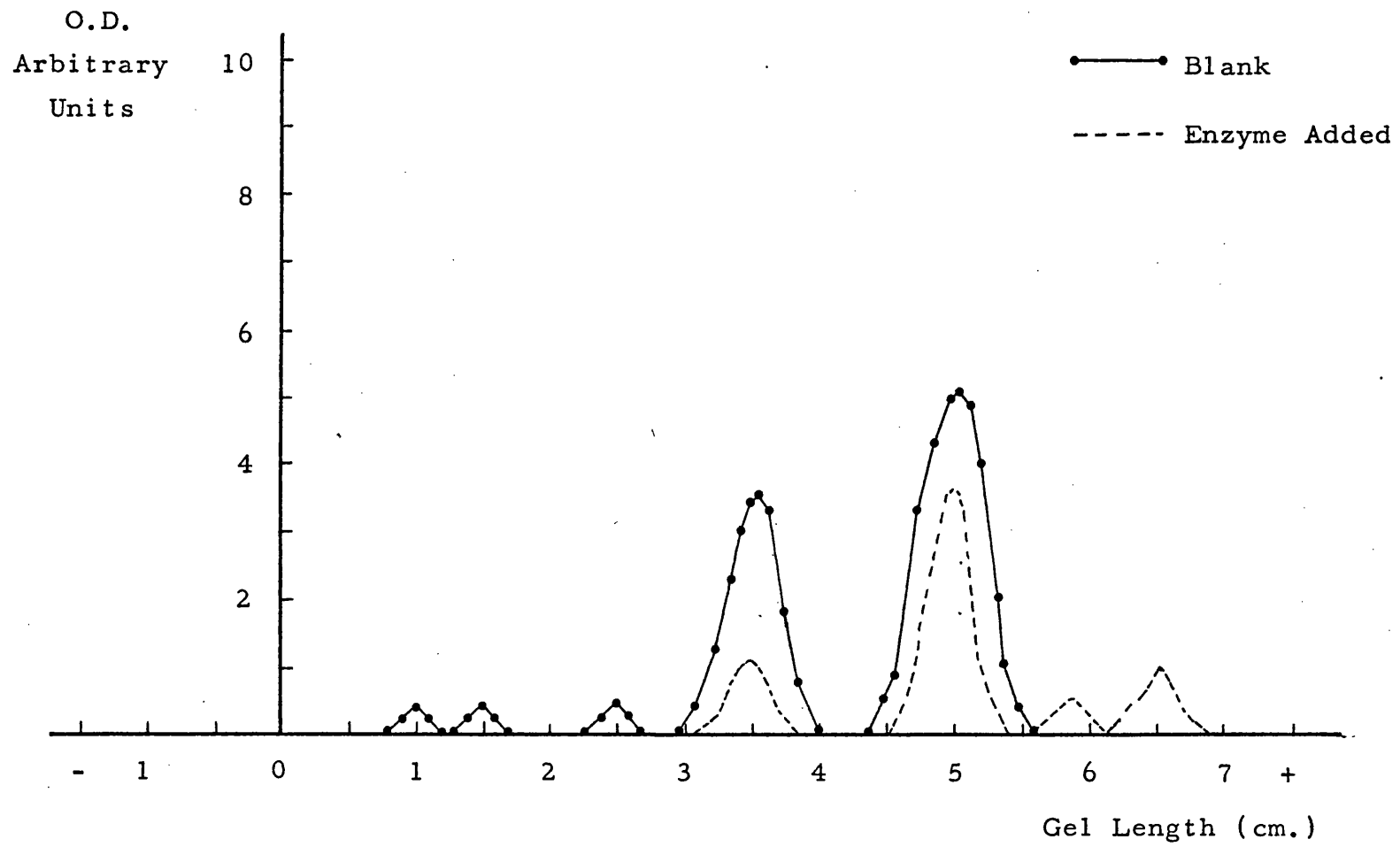


Figure 18. Electrophoretic Patterns of the Protease System of Strain S7, Activity on Skim Milk Casein Substrate at 30° C for 24 Hours.

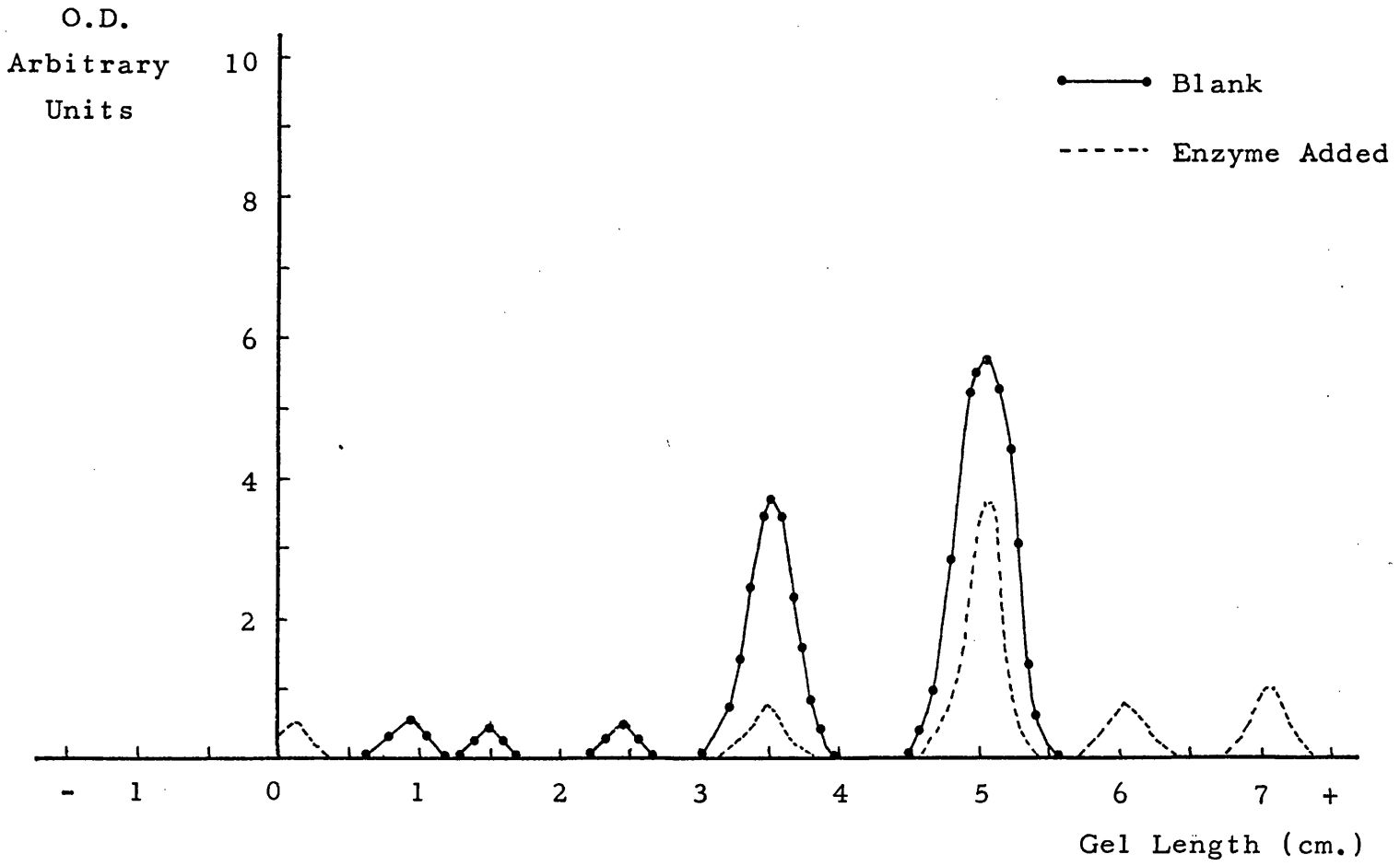


Figure 19. Electrophoretic Patterns of the Protease System of Strain S8, Activity on Skim Milk Casein Substrate at 30° C for 24 Hours.

at a reduced rate in relation to that of β-casein hydrolysis. The hydrolysis of these major fractions was followed by the release of products of higher and lower electrophoretic mobility. Their precursor with respect to either α_s- or β-caseins can not be discussed further, since their origin can not be traced within the framework and technique used in this study. This is so firstly because the electrophoretic method used is only capable of resolving α_s- and β-caseins and secondly because the enzyme preparation used in each case was not purified. Thus, the significance of of the degrading activity exhibited by the psychrophilic protease systems on caseins was best summarized by Cousin and Marth (1977b): " The decrease in the various casein fractions could present problems in products whose manufacture is dependent on the content of casein in milk. It could also affect the functional properties of the protein during and after the product is manufactured ".

SECTION 2. PATTERNS FOR THE ENZYME/CASEIN SYSTEM HEATED TO 142° C FOR ONE MINUTE AND FOLLOWED BY INCUBATION AT 30° FOR 24 HOURS

(a) Experimental

A fresh solution of isoelectric casein (B.D.H.) 4% (w/v) was prepared according to the method described on page 34. To 3 ml of the casein solution one ml of the enzyme solution was added. This procedure was re-

peated for all the test protease systems and the protease preparations were assayed for activity before being mixed with casein solutions. These assays indicated that enzyme activity (D.U./ml) varied between 748 and 3,460 units (Table 14). Although difficult to assess, the recovered activity was also estimated using the casein precipitation technique.

Approximately 3 ml of the casein enzyme solution was displaced in the heating system shown on Plate 1. The solution was quickly heated up to 142° C for one minute and cooled to 5° C by immersion in a large beaker containing approximately 10% (v/v) hydrogen peroxide. The beaker was placed in a cold water bath thermostatically controlled at 4° C. The activity of the heated mixture was estimated according to the procedure described on pages 34-35. One ml of the heated solution was aseptically transferred to a sterile glass tube covered with cotton plug using presterilized disposable pipettes. After all tubes were heated they were incubated at 30° C for 24 hours. Electrophoresis was conducted as described on pages 38-40 and the gel was photographed.

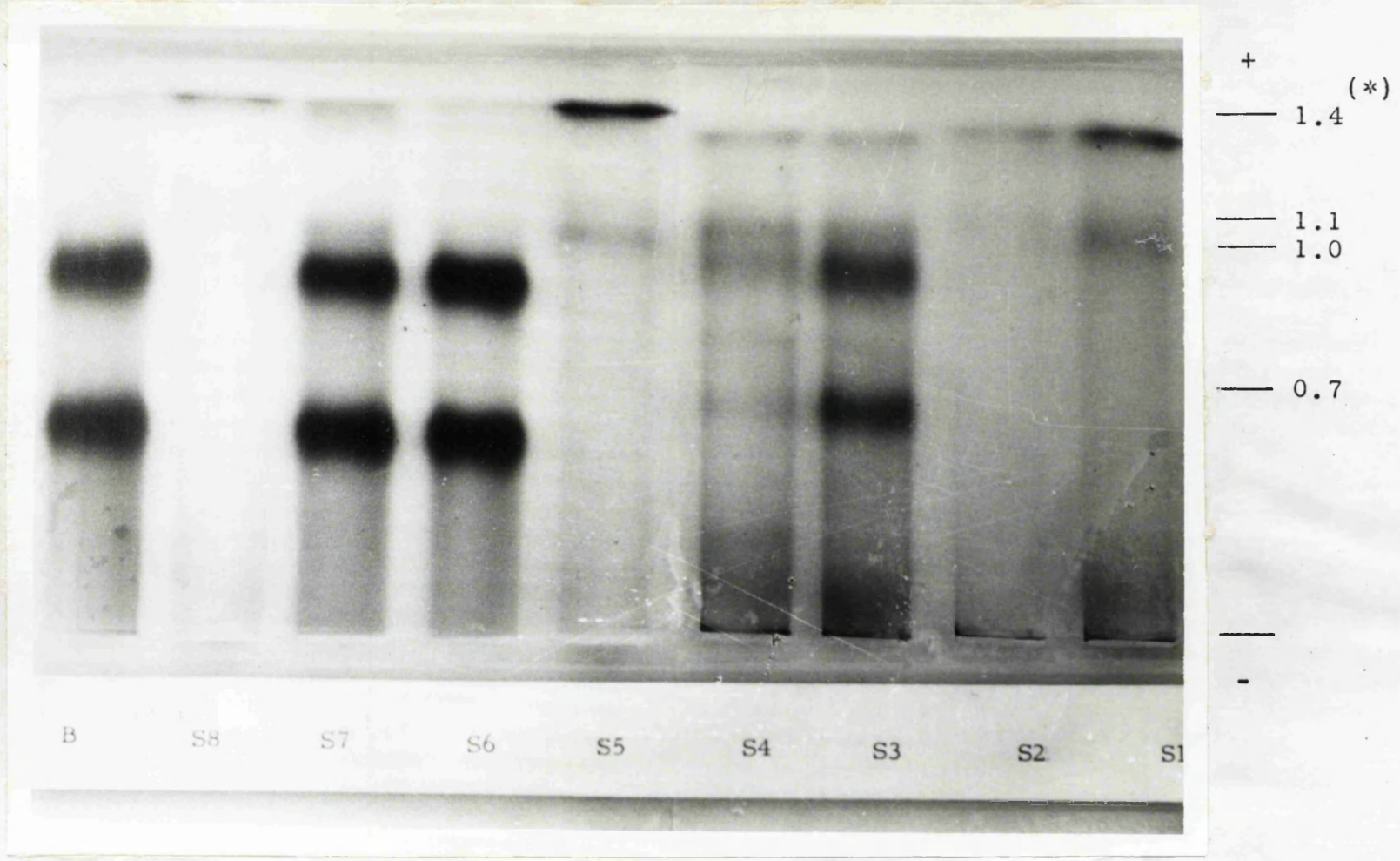
(b) Results and Discussion

The results are presented in Table 14 and the electrophoretic patterns are shown on Plate 6. Varying degrees of hydrolysis were observed for all casein/protease preparations in relation to the pure blank

Table 14. Activity of Protease Preparations Added to Casein Solutions and Activity Recovered After Heating, Assayed at 30° C for 48 hours.

Sources	Activity (D.U./ml)			
	Added		Recovered	
	Z.D. (mm.)	D.U./ml	Z.D. (mm.)	D.U./ml
S1	17.0	748	13.0	273
S2	16.0	1,139	13.0	454
S3	20.0	3,460	6.0	Non Diffusible
S4	17.5	1,436	14.0	495
S5	20.0	2,270	16.0	713
S6	15.0	1,448	6.0	Non Diffusible
S7	18.0	1,859	6.0	" "
S8	19.0	1,734	15.0	554

Plate 6. Electrophoretic Patterns of Heated Casein/Enzyme Solutions After 24 hours at 30° C in Polyacrylamide Gels at pH 8.6 (Mobility With Respect to α_s-Casein = 1.0)*



labelled as "B". Preparations S1 and S5 showed nearly 100% hydrolysis of β-casein while α_s-casein was not completely hydrolysed (see Plate 6, 1.1). The formation of thick fast moving bands were evident in both cases (see Plate 6, 1.4). Preparations S2 and S8 showed nearly 100% hydrolysis of both α_s- and β-caseins, resulting in the formation of fast moving bands (Plate 6, 1.4). Although a lower level of hydrolysis was apparent for preparation S4 both α_s- and β-caseins were attacked. Preparations S3, S6 and S7 showed greatly reduced enzyme activities. The intensity of the α_s- and β-casein bands were similar to those of the pure casein blank (B). Their assay on casein/agar plates after heating was not possible because the solutions did not diffuse out (see Table 14, S3, S6 and S7). Casein/enzyme aggregation may have contributed to this reduced diffusibility by creating an insoluble blockage in the casein/agar gel. For the remaining preparations the Z.D. of the precipitated zones were somehow blurred but still visible. Preparation S3 showed the highest activity in solution and condiderable reduced activity in the heated enzyme/casein system. This result seems to suggest that either the enzymes became heat sensitive or some form of aggregation took place when these preparations were heated in the presence of casein. Barach et al. (1978) have shown that inactivation by protease/casein aggregation can occur under certain conditions. They have also shown that the native form of the pro-

tease can undergo autolysis. When the protease is present in aggregated/casein form it remains inactive but may be reactivated completely by heating at 55° C using 6 M urea as dissociating agent.

(c) Conclusion

This experiment comprised an examination of the effect of heat on post-heat activity of casein/protease preparations. The presence of isoelectric casein in the protease solutions subject to heating at 142° C for one minute, caused different digestion patterns with respect to comparable experiments using raw skim milk casein. This was particularly true of Alcaligenes type proteases (see Plate 6, S6). The formation of casein/protease aggregates may explain these phenomena. Possibly these proteases are similar to those reported by Warner and Polis (1945), when a casein/enzyme association was demonstrated. Their statement was made on the basis of the following evidence: (a) the enzyme activity could be destroyed by heat; (b) an optimum pH was observed for the enzyme; (c) the enzyme activity could be concentrated; (d) proteolytic activity would proceed in sterile solutions. Recently a certain pseudomonad protease was shown to be stabilized by calcium against thermal inactivation (Barach et al., 1976a). According to the data presented in Plate 6 (S1, S2 and S8), pseudomonad proteases did not form any casein/protease aggregation since considerable enzyme activities were demonstrated. According to Barach et al. (1976b)

purified MC60 pseudomonad protease can be inactivated at 55° C for one hour in raw skim milk. Barach et al. (1978) stated that pseudomonad MC60 protease, "at more realistic concentrations in milk, would be inactivated only by aggregation ". This aggregation would be energetically favored at certain time/temperature exposures (Barach et al., 1978). The aggregation of protease to casein is also thought to be favored by an increase in the number of hydrophobic groups exposed as a result of increasing temperature, leading to a more stable configuration with less exposed hydrophobic groups in the complex aggregated form (Barach et al., 1978).

SECTION 3. CONCLUSION

The enzyme systems produced by the psychrophilic test strains showed activity against both α_s- and β-caseins. The α_s-casein fraction appeared to be less readily hydrolysed than the β-casein component when raw skim milk was used as substrate. The reduced susceptibility to hydrolysis displayed by α_s-casein may be due to its greater degree of organization. This can be predicted from a higher proportion of Pro present in β-casein in relation to that of α_s-casein. The proportion of Pro to total amino acid content has been shown to be greater in β-casein (Mercier et al., 1971, 1972; Grosclaude et al., 1973). Since Pro lacks the planar peptide bond and its ring structure tends to favor configuration bending (Light, 1974), a greater

susceptibility to hydrolysis is normally expected to be displayed by the β-casein fraction. Previous reports relating to protease preparations obtained from Pseudomonas spp. have also shown that β-casein is preferentially hydrolysed (Tsugo and Yamauchi, 1959; Kiuru et al., 1970, 1971; Yanagiya et al., 1974; Purschel and Pollack, 1974; Cousin and Marth, 1977b,c).

The susceptibility to hydrolysis displayed by β-casein appeared to be partially lost when the enzyme preparations were heated together with casein solutions. This was evident from the electrophoretic patterns displayed by the reaction mixtures after 24 hours of incubation at 30° C. The test protease preparations either showed indifferent hydrolysis toward both α_s- and β-casein (Plate 6, S2 or S8) or remained partially inactive (S3, S6 or S7). Nearly 100% inactivation was observed for the heated casein protease system S6. The formation of casein/protease aggregate as a result of heat may explain this phenomenon (Warner and Polis, 1945; Barach et al., 1976a,b; Barach et al., 1978).

CHAPTER V.

STUDY OF THE HEAT-STABLE ENZYME SYSTEMS PRODUCED UNDER AERATED CONDITIONS AT 5° C

SECTION 1. EFFECTS OF REMOVAL OF SALTS ON THE HEAT-STABILITY OF *Pseudomonas fluorescens* ENZYME SYSTEM

(a) Experimental

In this study, both enzyme concentration and reduction of salt content were carried out using the technique described by O'Donnell (1975) and by Scholefield *et al*. (1978). The assay procedure for protease activity described on pages 34-35 was used to follow the changes in protease titre either during the concentration procedure or during the application of heat. Protein content was determined as described on pages 35-36. Appendix G shows the changes in specific activity during the concentration process. The main objective of this experiment was to demonstrate the effects of salts removal on the heat-stability of the enzyme systems. The enzyme preparation used was obtained from strain S8.

(b) Results and Discussion

The results are presented in Fig. 20 and Table 15. The inactivation reaction showed a definite change of order (see Appendix H) between the two experimental processes (Fig. 20, B.S.M and Water). This change can

Table 15. Effect of the Removal of Salts on the Heat-stability Pattern, D(values) and Inactivation Constants for the Protease System S8.

Heating Time	Residual Activity		D (values)		Constants	
	BSM	H_2O	BSM	H_2O	BSM	H_2O
	(DU_2/DU_1)		(s)		(s^{-1})	
0	1.00	1.00				
15	0.96	0.50 *		27		0.0853
30	0.94	0.44				
60	0.93	0.43				
240	0.85	0.42				
480	0.78 *	0.37	2,101		1.096×10^{-3}	
720	0.67	0.37				
960	0.64	0.37				
1,200	0.60 *	0.37 *	4,640	5,229	4.963×10^{-4}	4.4×10^{-4}
1,920	0.40	0.37				
2,400	0.25	0.12				
3,000	0.08 *	0.05 *	2,924	2,716	7.876×10^{-4}	8.48×10^{-4}

* Heating Stages

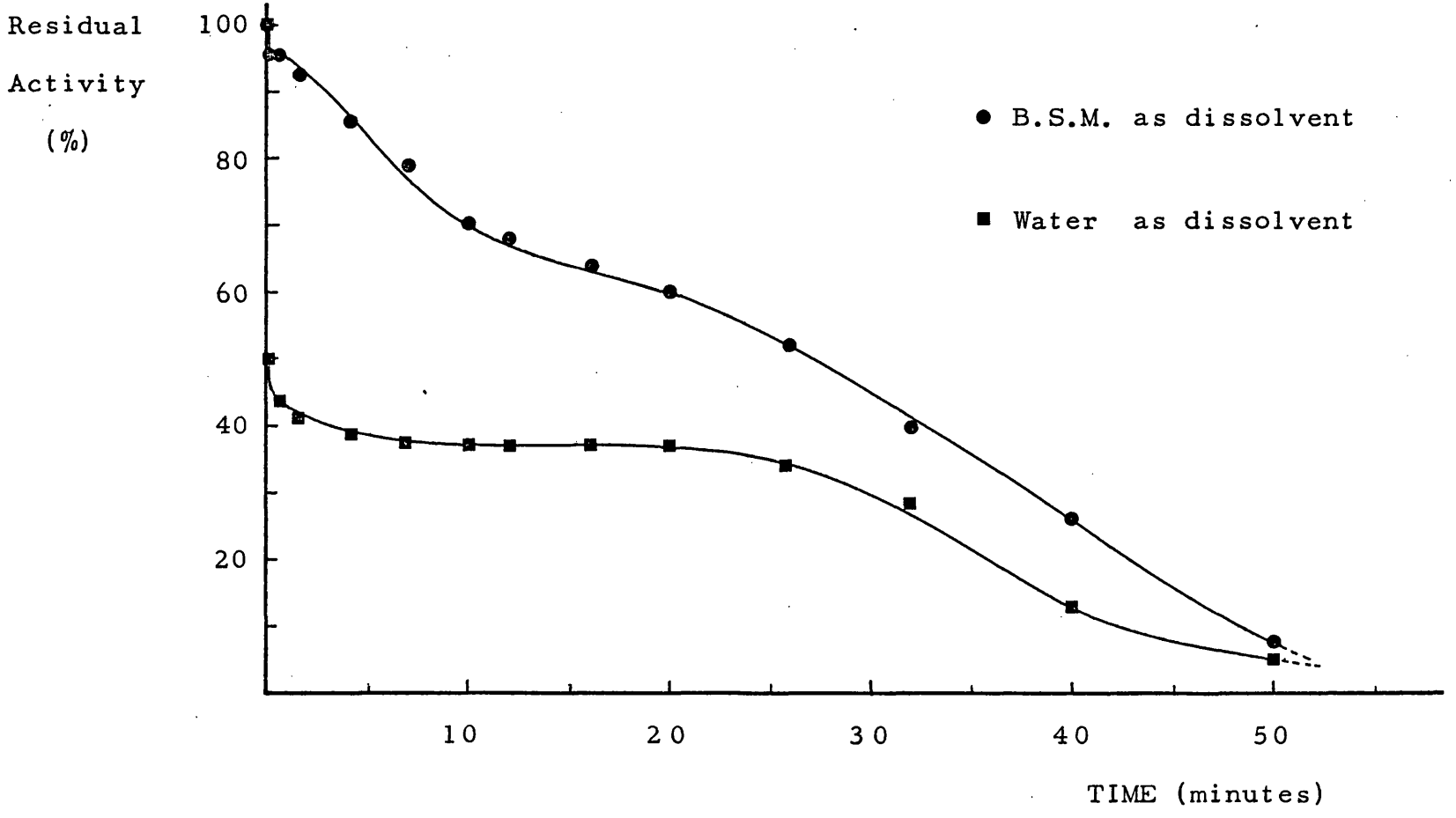


Figure 20. Effect of the Removal of Salts on the Heat-stability Pattern Displayed by Pseudomonas fluorescens Protease System (● and ■ heating of 100° C/0-50 min.).

be easily seen from difference observed in the inactivation rates (Table 15). In a study carried out by Driessen and Stadhouders (1974) the existence of two inactivation stages was demonstrated. The thermal activation and inactivation of bacterial extracellular enzymes have also been studied by Dahlquist et al. (1976), Barach et al. (1976a,b) and Barach et al. (1978). Although in these studies different substrates have been used, the enzymes involved showed notable similarities with respect to their heat stability properties. They have been shown to be stabilized by calcium ions (Barach et al., 1976a; Dahlquist et al., 1976) and under certain conditions may lose their activity at a relatively low temperature (50° C to 55° C). Driessen and Stadhouders (1974) demonstrated their heat inactivation display a biphasic loss of activity curve coupled with two stages of thermal denaturation/inactivation. Denaturation appeared to proceed quickly and was followed by inactivation eventually leading to a form nearly complete inactive. Dahlquist et al. (1976) have demonstrated that: (1) the native enzyme contains four calcium ions and one zinc ion bound to the protein in such a manner that enable it to be thermally denatured by autolysis; (2) calcium binding is coupled to a cooperative transition in the protein molecule which may lead to autolysis at temperatures above 50° C; (3) the replacement of a double calcium ion site by terbium (Zn, Tb, 2Ca; or Zn, 3Tb) does not alter either the catalytic or the stabilizing proper-

either the catalytic or the stabilizing properties of the enzyme; (4) the depletion of calcium in the enzyme molecule, carried out at temperatures lower than 50° C is invariably followed by loss of activity and the maintenance of an organized structure which is not stabilized by calcium ions; (5) the replacement of the double calcium ion site by terbium (Zn, Tb) following treatement with EDTA results in 40% residual activity and the removal of the calcium thermal stabilizing property.

A model for the stabilization of the enzyme system was proposed by Dahlquist et al. (1976). This model has been used in an attempt to explain the results shown in Fig. 20 and Table 15. The following scheme is thought to be representative of the changes observed for residual activity after temperature/time exposures:

$$E\,(Ca)_z \underset{}{\overset{K_x}{\rightleftharpoons}} E \overset{K_c}{\rightleftharpoons} E' \longrightarrow \text{autolysis}$$

$$+$$

$$xCa$$

The configuration changes would be related to a equilibrium system E (native active) and E' (inactive denatured). The native form E is also considered coupled to the binding of xCa ions leading to the stabilized form $E\,(Ca)_z$. Under certain conditions, e.g., pH, temperature and osmotic strength (Dahlquist

et al., 1976; Barach et al., 1978) it may undergo irreversible autolysis. This appeared to be true when the enzyme system was heated using distilled water as dissolvent (Fig. 20).

In the following analysis it is assumed that the critical temperatures at which the transition in the protein occurred were at 55° C (T_w=55° C where ΔG^o_w = 0) for the situation where heating was conducted using distilled water as dissolvent and at 92° C (T_{bsm}=92° C where ΔG^o_{bsm}=0) for the case where basal salts medium was used as dissolvent. The value for the change in enthalpy (ΔH_{eff}) associated with both conformational changes in the protein molecule and calcium binding is assumed to be -x(130,000 cal/mole) (Dahlquist et al., 1976). The x value is considered either 1 or 2, one for the situation where water was used and 2 for the case where BSM was used. According to the observations presented in Table 15 two distinct intermediate equilibria, $(DU_2/DU_1)_w$=0.37 and $(DU_2/DU_1)_{bsm}$=0.85= K_c, were used as ΔG^o estimates. Based on these assumptions some values relating the thermodynamics of the thermal inactivation were presented in Appendix H. Thus the author's view in relation to the heat-stability behavior (Fig. 20) displayed by the enzyme system can be summarized as follows: The equilibrium of the inactivation reaction tends to completion at any temperatures above 53.39° C when heating is conducted in enzyme/water solutions or in low osmotic

strength systems which lack important stabilizing factors. In this case the number of stabilizing calcium ion was apparently one (ΔH^{o} = -130,000 cal/mole). The net inactivated form (E') of the enzyme system becomes easier to achieve at temperatures higher than 53.39° C since ΔG_{w} becomes negative (see Appendix H). This situation is contrasted by the inactivation reaction in BSM. The number of stabilizing ions increased (being hypothetically considered to be two Ca, ΔH^{o}_{bsm} -260,000 cal/mole). The net inactivated for (E') is only favored when the temperature is higher than 92° C when ΔG_{bsm} becomes negative. Intrinsic energy appeared to be released in both cases since ΔH_{w} and ΔH_{bsm} were negative. Dahlquist *et al.* (1976) stated that this is probably due to a highly cooperative transition resulting from stabilization when calcium is available in the system. The fact that ΔS_{bsm} was almost equivalent to double the value estimated for ΔS_{w} indicates the two distinct order in the heating conditions (see Appendix I.).

(c) Conclusion

The activation/inactivation reaction displayed by the test protease system was dependent on the stabilizing properties of certain salts (or ions not defined in this work) present in the basal salts medium. Both calcium and zinc have been shown to play an important role in the thermal stability of protease systems (Dahlquist *et al.*, 1976; Barach *et al.*, 1978).

SECTION 2. HEAT STABILITY OF DIFFERENT ENZYME SYSTEMS AT TEMPERATURES BELOW 100° C.

(a) Experimental

Heat inactivation experiments were conducted at temperatures of 65° C, 75° C and 98° C. Cell free growth media from 5 days growth at 5° C under aerated conditions were adjusted to pH 6.80 ± 0.05 by the addition of 0.74 M orthophosphoric acid. Volumes of 1.5 ml taken from each cell free broth were displaced into fine layers of liquid using the double glass tube method (pages 40 and 41). Thirty seconds were allowed for the come up time before timing commenced. The residual activity of heated and unheated samples were determined according to the procedure described on pages 34 and 35. The results in terms of mm. zone diameter were converted into D.U. (see Appendix C). See Appendix I for further information on treatment of the results.

(b) Results and Discussion

The results of the heat stability experiments conducted at three temperatures below 100° C are presented in Table 16 and Figures 21-36. Table 17 shows calculations of the D-value and ln of the inactivation constant . These are related to the first (0 to 60 seconds) and second (61 to 3,840 seconds) inactivation stage intervals. In a few cases the calculations of the D-value were not applicable, since there were net over-

Table 16. Heat Inactivation of Test Strain Proteases in Cell Free Media at pH 6.8 at Constant Temperatures of 65° C, 75° C and 98° C (in Ratio of DU_2/DU_1).

Heating Time	S1			S2			S3			S4		
(in Seconds)	65°	75°	98°	65°	75°	98°	65°	75°	98°	65°	75°	98°
Blank 0	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
15	0.88	0.82	0.94	0.74	0.80	0.82	0.85	0.90	0.92	0.80	0.79	0.94
60	0.88	0.82	0.94	0.52	0.85	0.90	0.95	0.90	0.85	0.75	0.89	0.89
120	0.88	0.82	0.90	0.69	0.80	0.80	0.85	0.80	0.90	0.71	0.79	0.84
240	0.82	0.80	0.85	0.60	0.90	0.80	0.72	0.80	0.80	0.80	0.89	0.79
480	0.77	0.82	0.80	0.60	0.90	0.80	0.72	0.90	0.75	0.90	0.89	0.69
960	0.82	0.85	0.55	0.60	0.90	0.71	0.76	0.90	0.54	0.85	0.79	0.52
1,920	0.77	0.82	0.16	0.60	0.85	0.54	0.59	0.80	0.26	0.80	0.54	0.08
3,840	0.52	0.55	0.00	0.52	0.85	0.21	0.67	0.90	0.00	0.71	0.60	0.00

Table 16. (Continuation) Heat Inactivation of Test Strain Proteases in Cell Free Media at pH 6.8 at Constant Temperatures of 65° C, 75° C and 98° C (in Ratio of DU_2/DU_1).

Heating Time		S5			S6			S7			S8		
(in Seconds)		65°	75°	98°	65°	75°	98°	65°	75°	98°	65°	75°	98°
Blank	0	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
	15	0.90	0.89	0.90	0.93	0.93	1.00	0.84	0.73	0.84	0.95	0.95	1.00
	60	0.85	0.95	0.80	0.74	0.64	0.73	0.79	0.89	0.84	0.90	0.85	0.85
	120	0.90	0.79	0.75	0.45	0.45	0.49	0.90	0.73	0.84	0.85	0.81	0.81
	240	0.85	0.84	0.71	0.45	0.51	0.44	0.94	0.78	0.84	0.85	0.85	0.81
	480	0.90	0.75	0.62	0.51	0.46	0.34	0.84	0.78	0.79	0.80	0.76	0.77
	960	0.80	0.66	0.46	0.93	0.46	0.20	0.89	0.78	0.74	0.71	0.72	0.60
	1,920	0.75	0.66	0.16	0.00	0.36	0.12	0.84	0.73	0.40	0.75	0.67	0.27
	3,840	0.62	0.57	0.00	0.00	0.41	0.00	0.84	0.78	0.03	0.71	0.67	0.00

Table 17. Treatment of the Results(*) for Heat Inactivation Patterns Displayed by the Test Proteases at Temperatures Below 100° C ;

Strains	Stages	$\frac{10^3}{T}(K^{-1})$	D-value (s^{-1})	ln (k)
S1	First	2.96	558.00	- 5.49
		2.87	363.00	- 5.06
		2.69	1,143.00	- 6.21
	Second	2.96	9,294.23	- 8.30
		2.87	11,264.24	- 8.49
		2.69	3,000.19	- 7.17
S2	First	2.96	114.72	- 3.91
		2.87	495.00	- 5.37
		2.69	950.29	- 6.02
	Second	2.96	17,706.80	- 8.95
		2.87	163,065.02	-11.17
		2.69	4,562.25	- 7.59
S3	First	2.96	6,525.00	- 7.95
		2.87	675.00	- 5.68
		2.69	385.79	- 5.12
	Second	2.96	17,963.91	- 8.96
		2.87	(- 49,392.00)**	- **
		2.69	3,110.86	- 7.21
S4	First	2.96	241.67	- 4.65
		2.87	909.00	- 5.98
		2.69	531.00	- 5.44

(*)Results from Table 16; (**)Not Determinable.

Table 17. (Continuation) Treatment of the Results[*] for Heat Inactivation Patterns ;

Strains	Stages	$\frac{10^3}{T}(K^{-1})$	D-value (s^{-1})	ln (k)
S4	Second	2.96	32,889.06	- 9.57
		2.87	9,144.05	- 8.29
		2.69	2,888.86	- 7.13
S5	First	2.96	399.70	- 5.16
		2.87	2,565.00	- 7.02
		2.69	285.00	- 4.82
	Second	2.96	10,883.24	- 8.46
		2.87	11,030.46	- 8.47
		2.69	2,952.27	- 7.16
S6	First	2.96	208.39	- 4.51
		2.87	149.99	- 4.18
		2.69	192.14	- 4.42
	Second	2.96	3,041.79	- 7.19
		2.87	16,013.71	- 8.85
		2.69	2,593.58	- 7.03
S7	First	2.96	288.39	- 4.83
		2.87	1,336.30	- 6.36
		2.69	411.75	- 5.19
	Second	2.96	43,606.60	- 9.85
		2.87	(-278,733.00)[**]	- [**]
		2.69	3,480.81	- 7.32

[*] Results from Table 16; [**] Not Determinable.

Table 17. (Continuation) Treatment of the Results(*) for Heat Inactivation Patterns.

Strains	Stages	$\frac{10^3}{T}(K^{-1})$	D-value (s^{-1})	ln (k)
S8	First	2.96	577.50	- 5.52
		2.87	367.10	- 5.07
		2.69	340.71	- 4.99
	Second	2.96	21,109.19	- 9.12
		2.87	16,551.77	- 8.88
		2.69	3,169.50	- 7.23

(*) Results from Table 16.

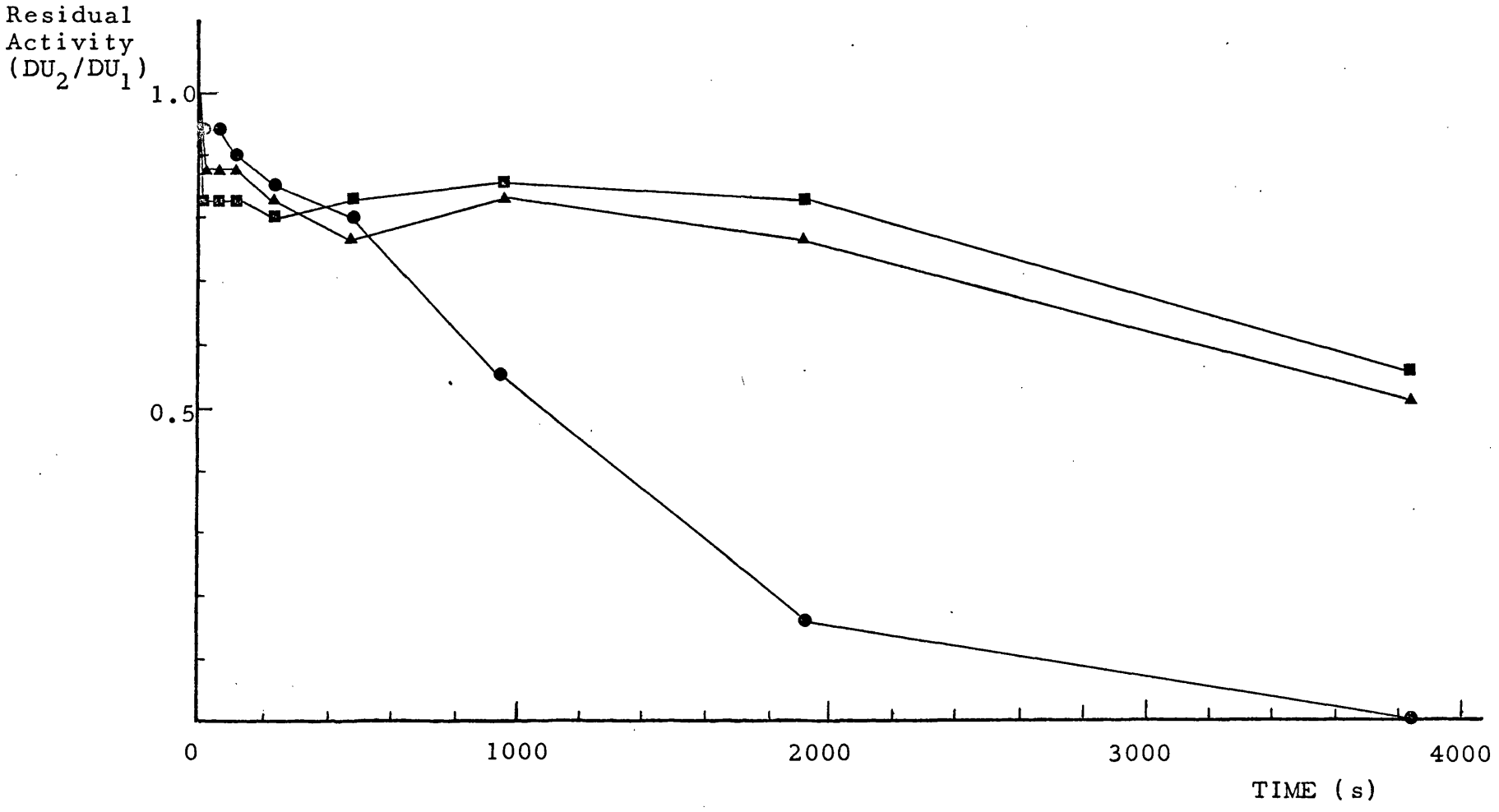


Figure 21. Heat Inactivation of Strain S1 Protease in Cell Free Media at pH 6.8 (▲=65° C, ■=75° C and ●=98° C)

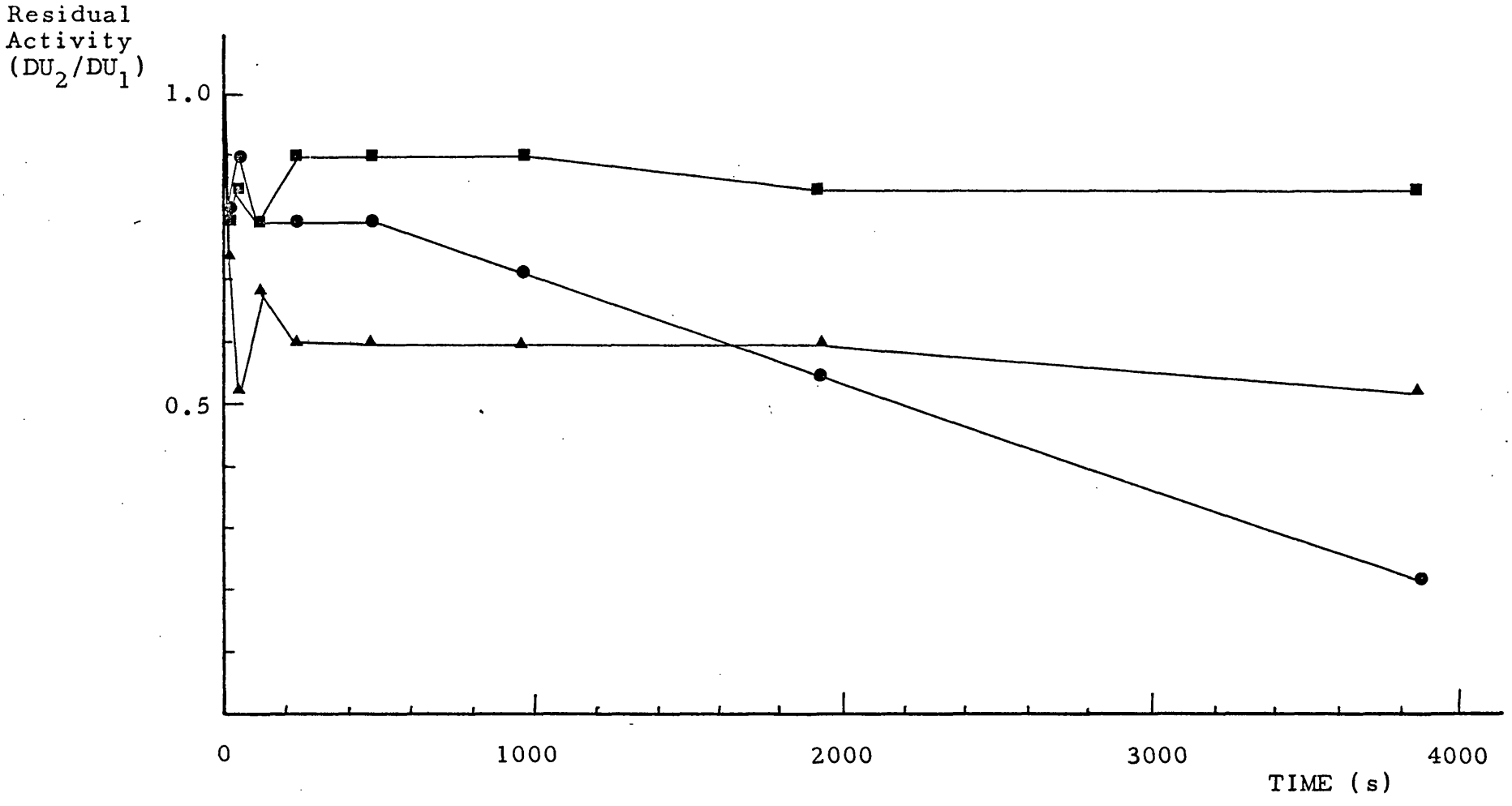


Figure 22. Heat Inactivation of Strain S2 Protease in Cell Free Media at pH 6.8 (▲=65° C, ■=75° C and ●=98° C).

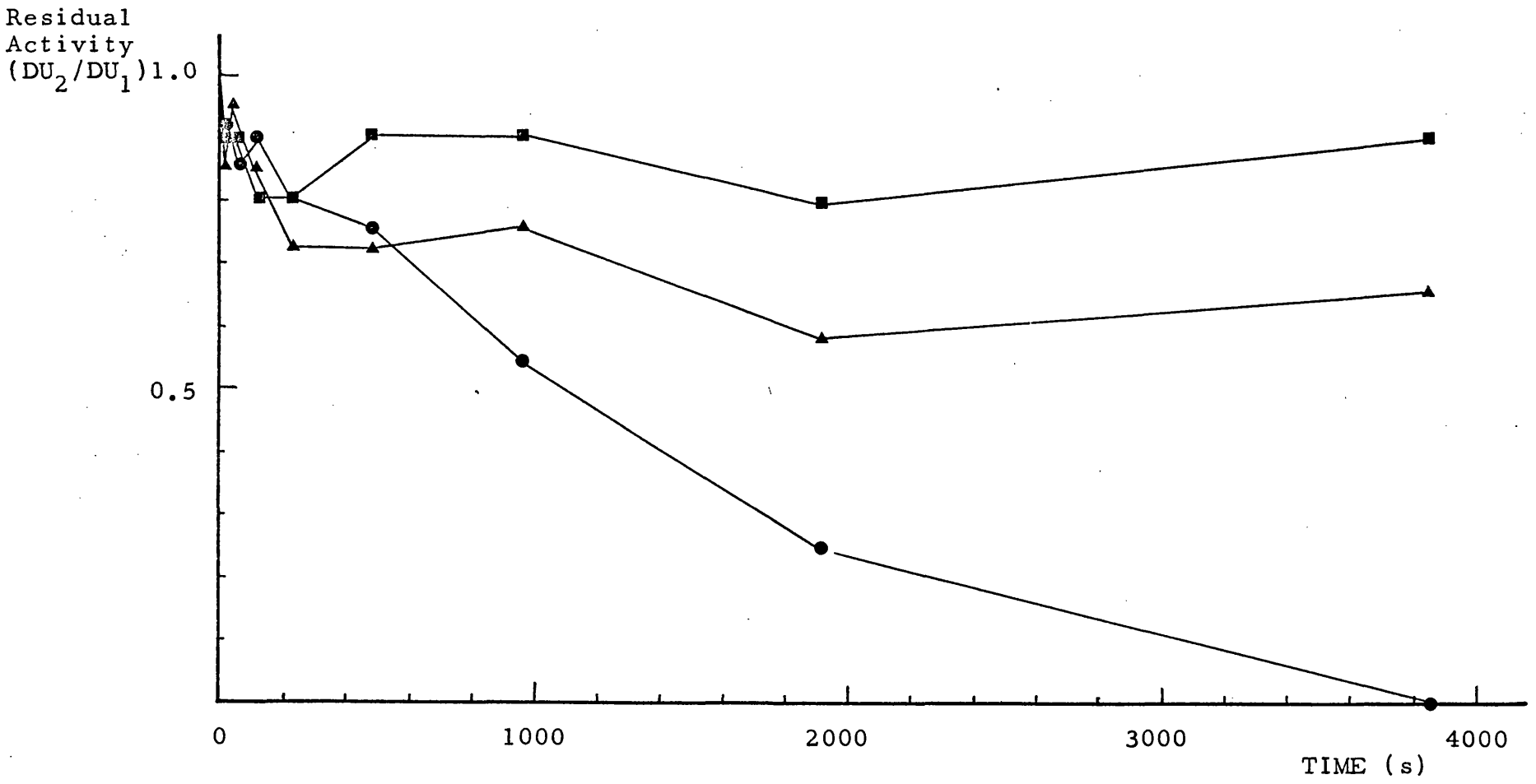


Figure 23. Heat Inactivation of Strain S3 Protease in Cell Free Media at pH 6.8 (▲=65° C, ■=75° C and ●=98° C).

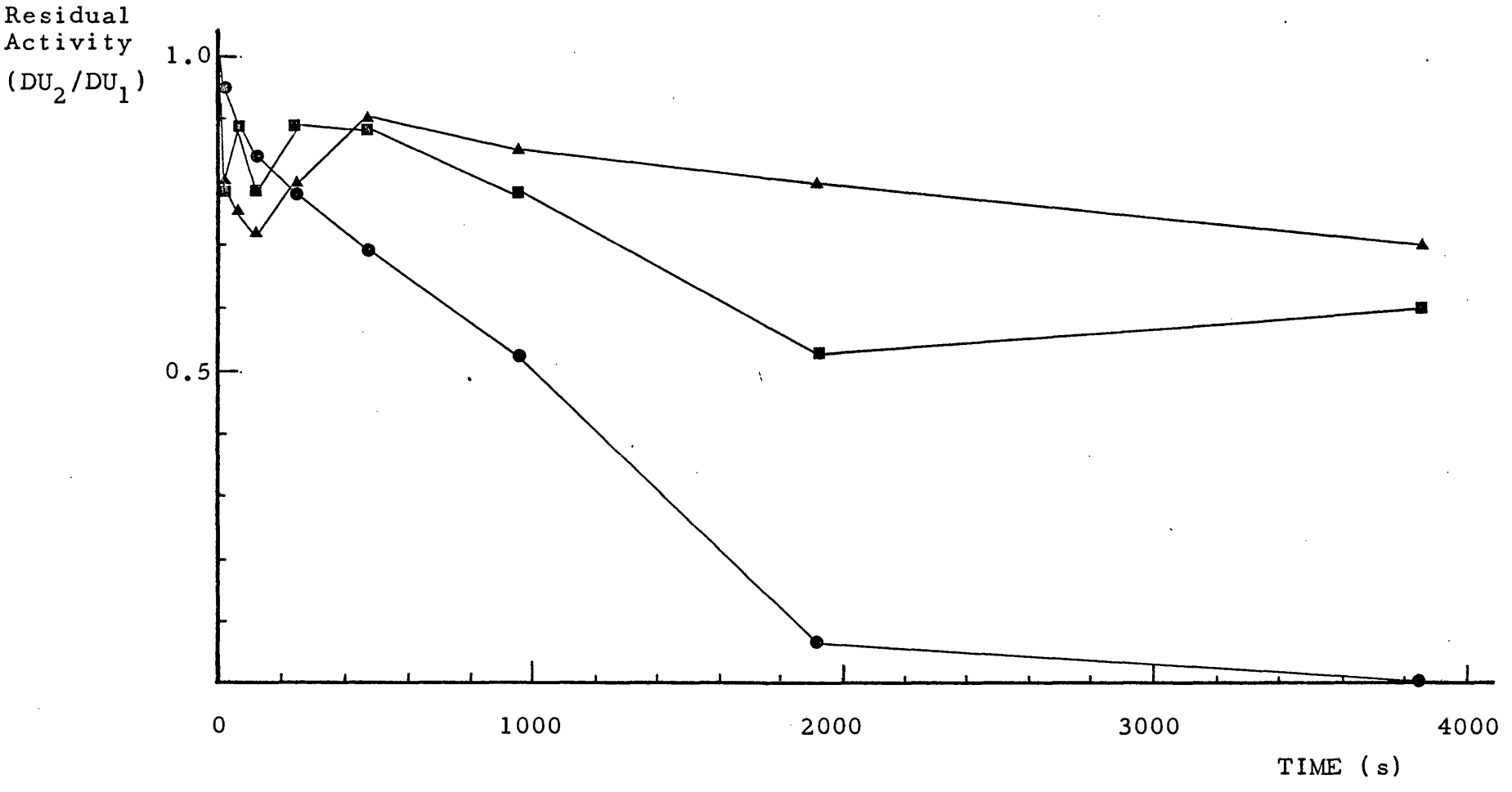


Figure 24. Heat Inactivation of Strain S4 Protease in Cell Free Growth Media at pH 6.8 (▲=65° C, ■=75° C and ●=98° C).

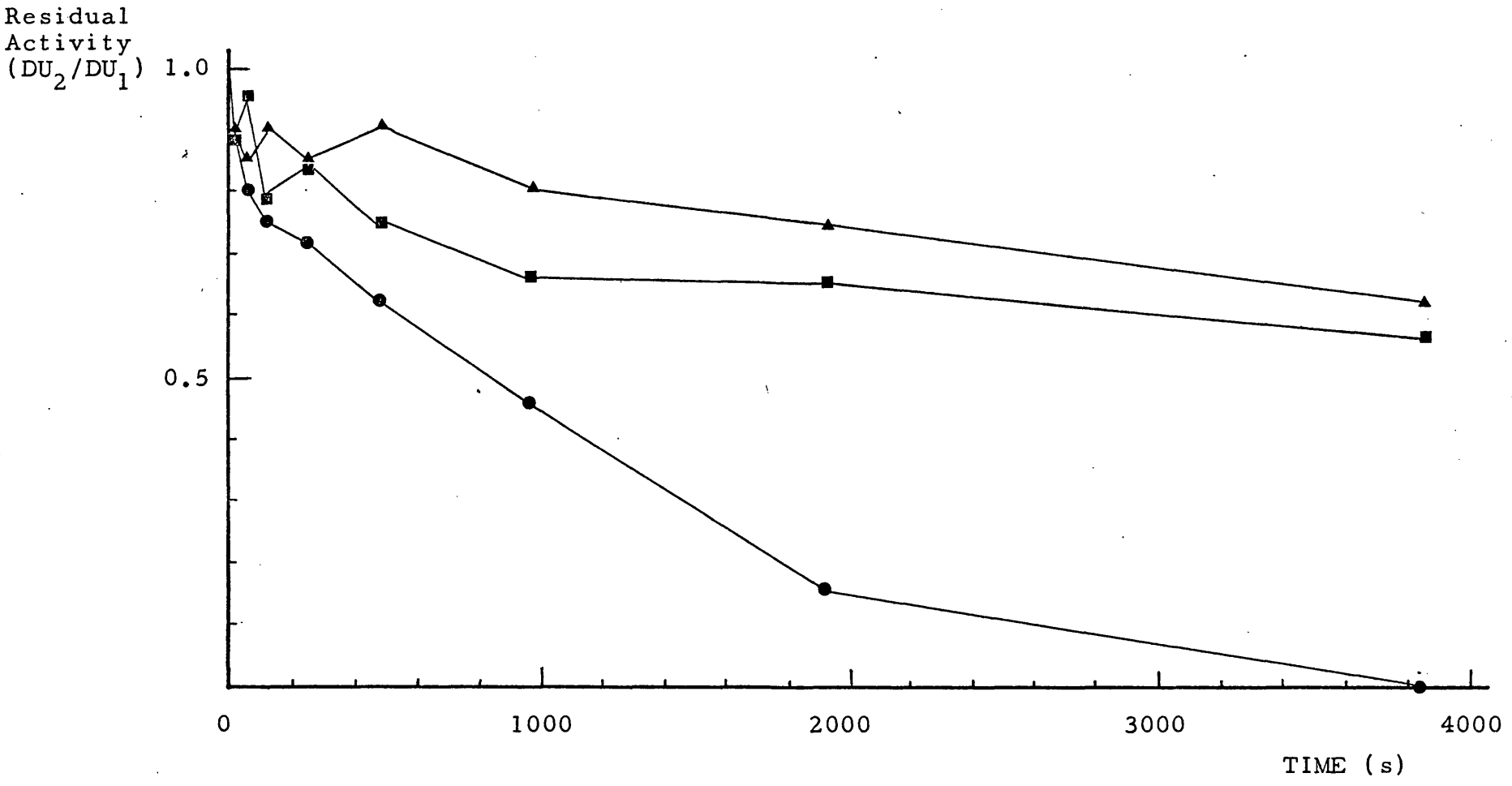


Figure 25. Heat Inactivation of Strain S5 Protease in Cell Free Growth Media at pH 6.8 (▲=65° C, ■=75° C and ●=98° C).

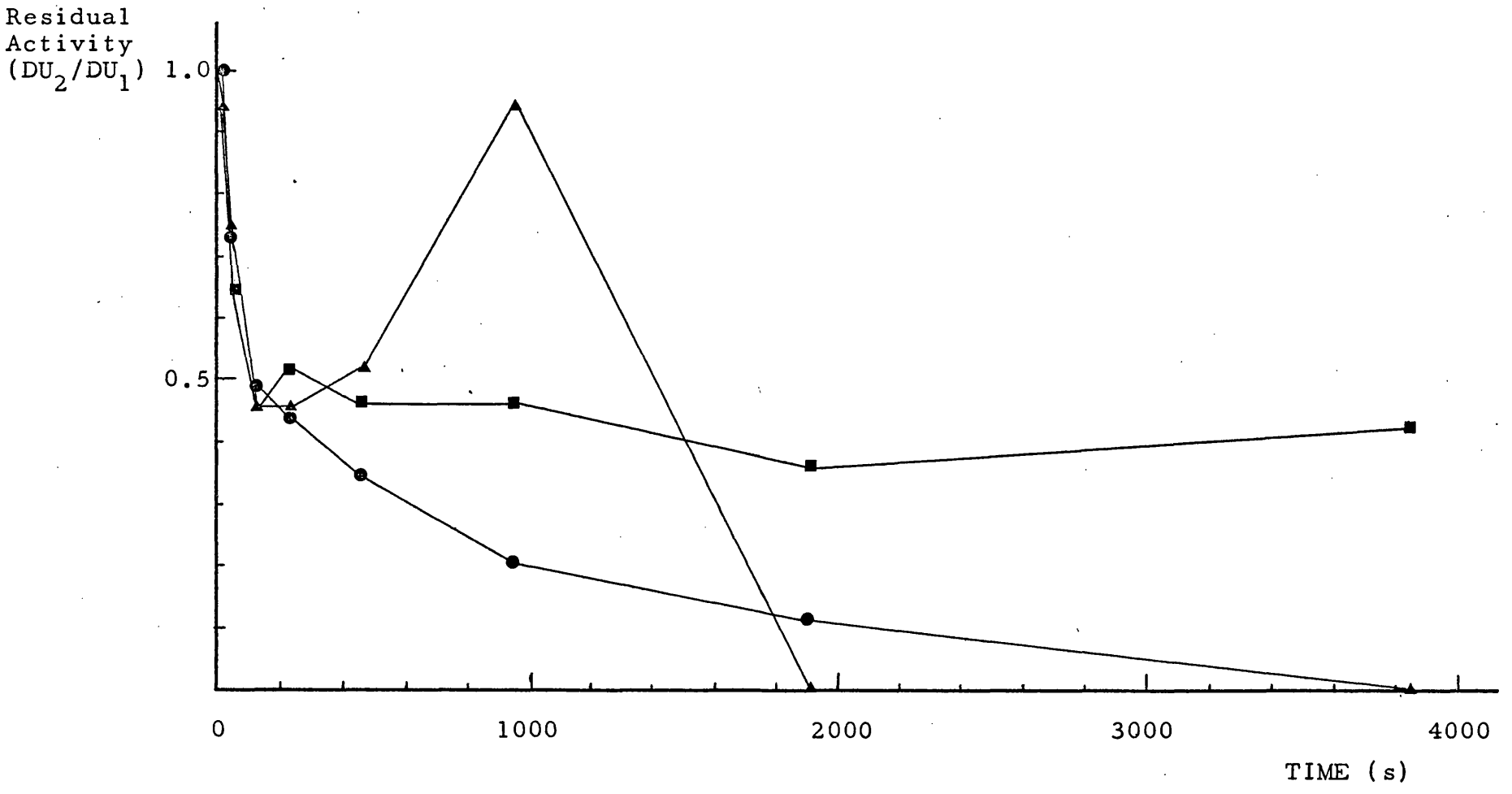


Figure 26. Heat Inactivation of Strain S6 Protease in Cell Free Growth Media at pH 6.8 (▲=65° C, ■=75° C and ●=98° C).

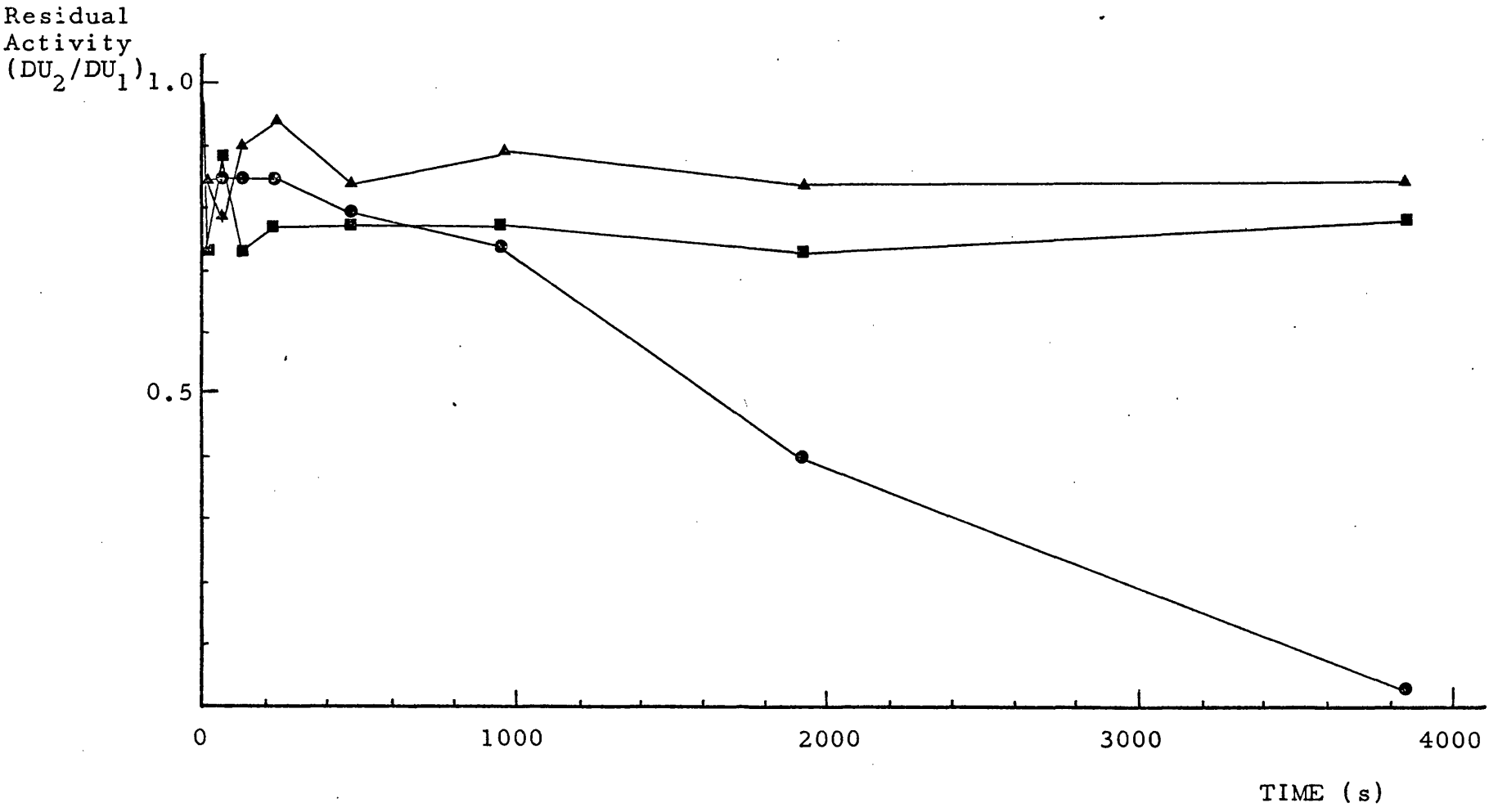


Figure 27. Heat Inactivation of Strain S7 Protease in Cell Free Growth Media at pH 6.8 (▲=65° C, ■=75° C and ●=98° C).

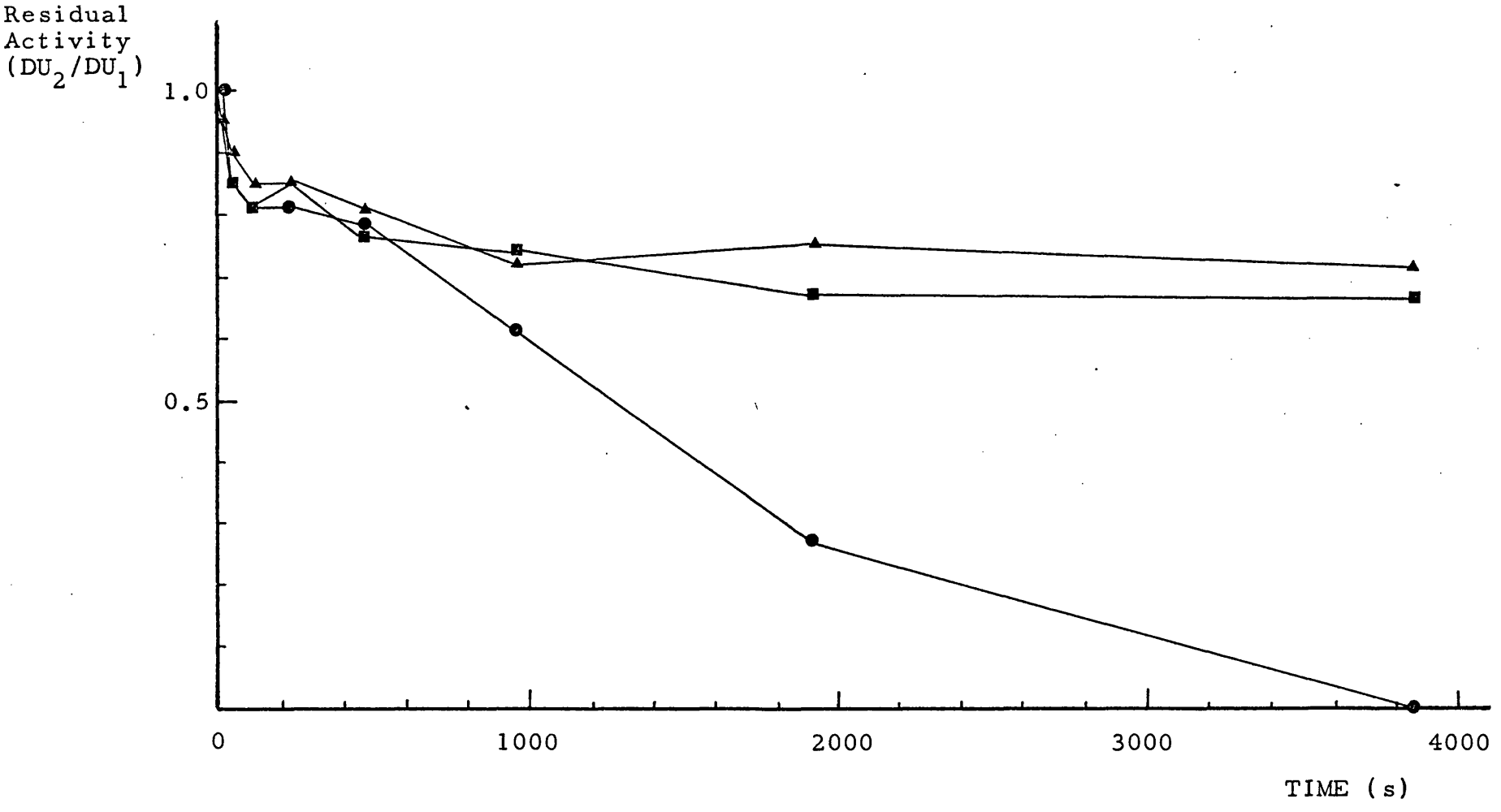


Figure 28. Heat Inactivation of Strain S8 Protease in Cell Free Growth Media at pH 6.8 (▲=65° C, ■=75° C and ●=98° C).

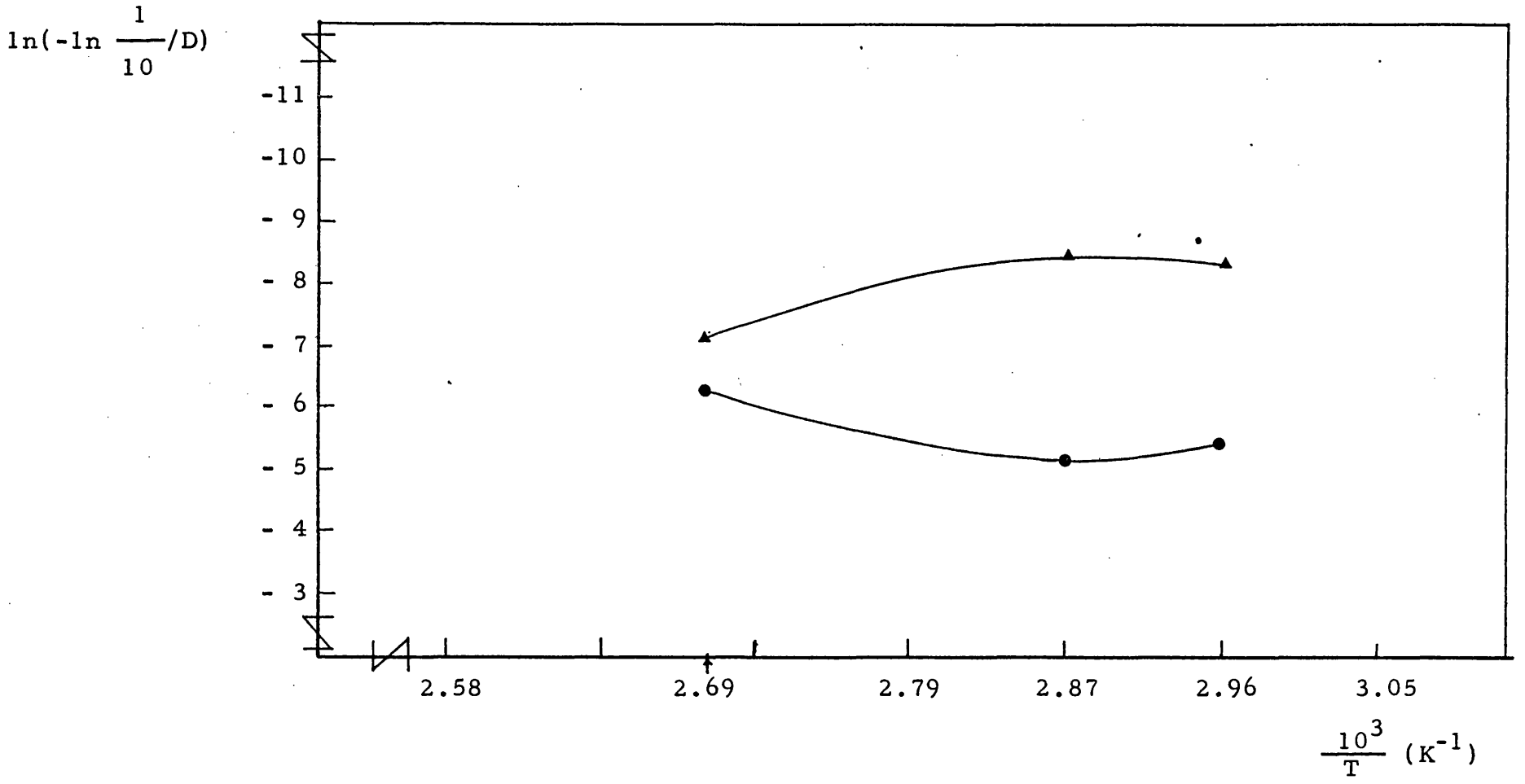


Figure 29. Arrhenius Plot of the First (●) and Second (▲) Stages of Inactivation of the Strain S1 Protease in Cell Free Growth Media at pH 6.8.

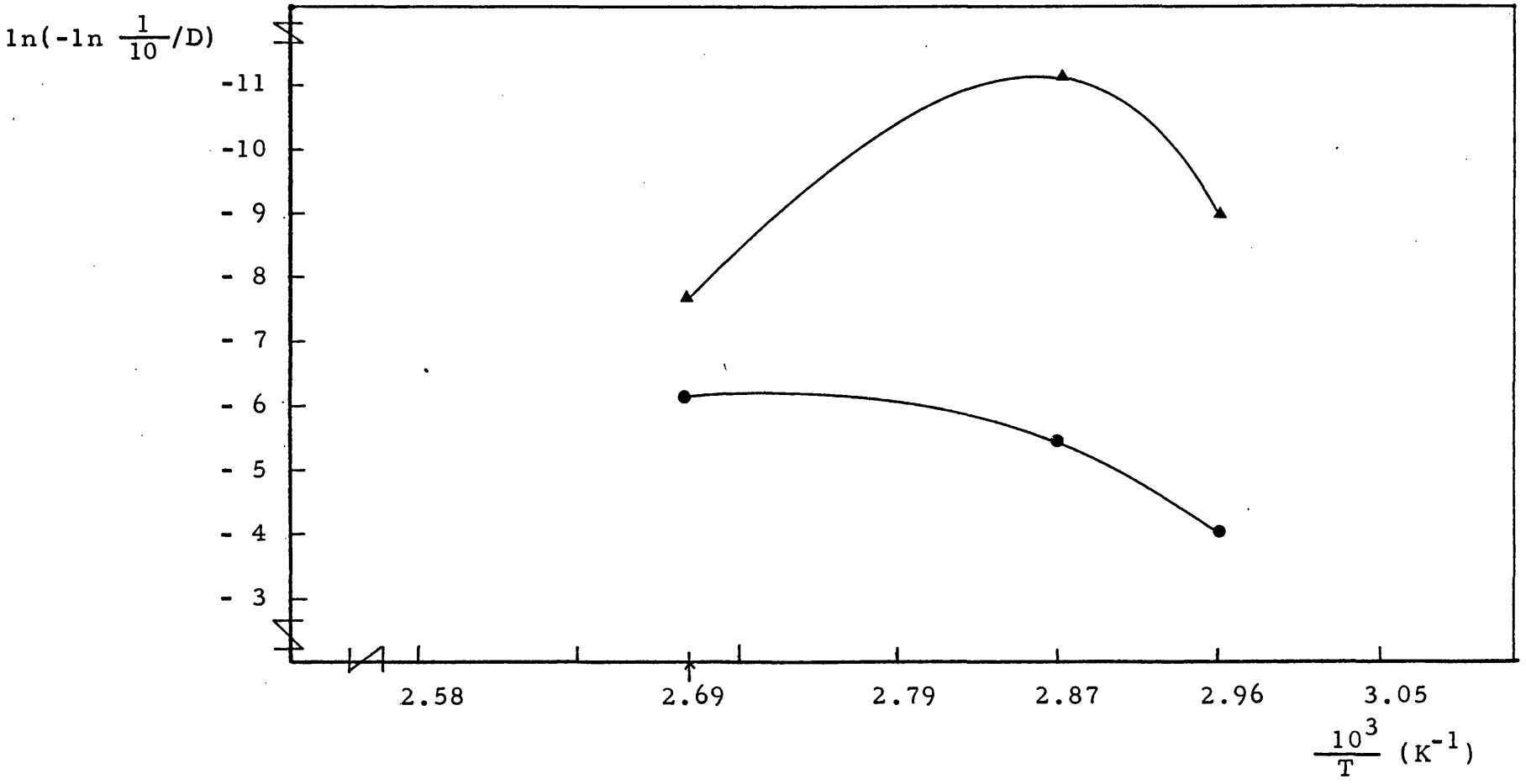


Figure 30. Arrhenius Plot of the First (●) and Second (▲) Stages of Inactivation of the Strain S2 Protease in Cell Free Growth Media at pH 6.8.

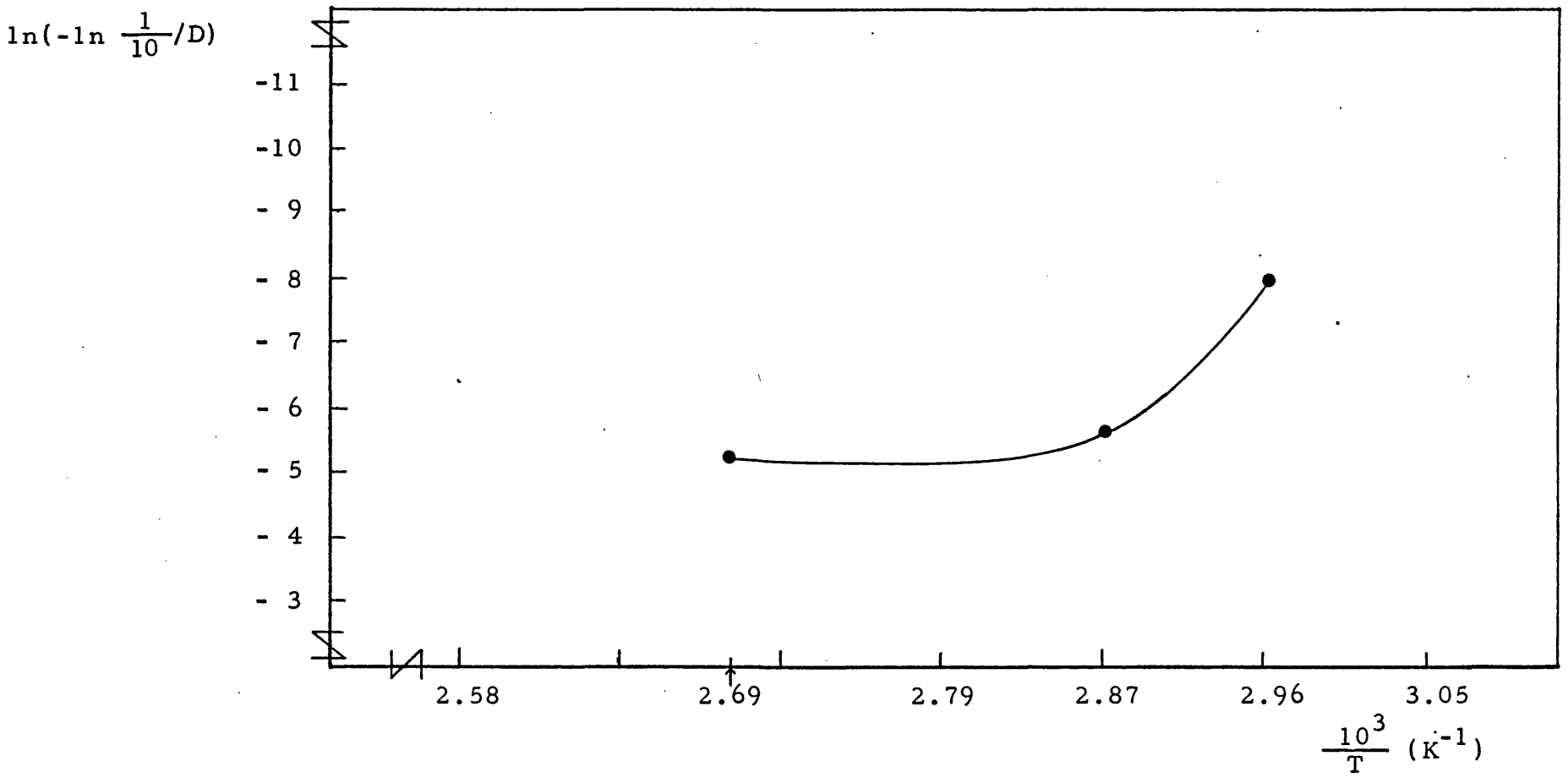


Figure 31. Arrhenius Plot of the First (●) and Second ( ) Stages of Inactivation of the Strain S3 Protease in Cell Free Growth Media at pH 6.8.

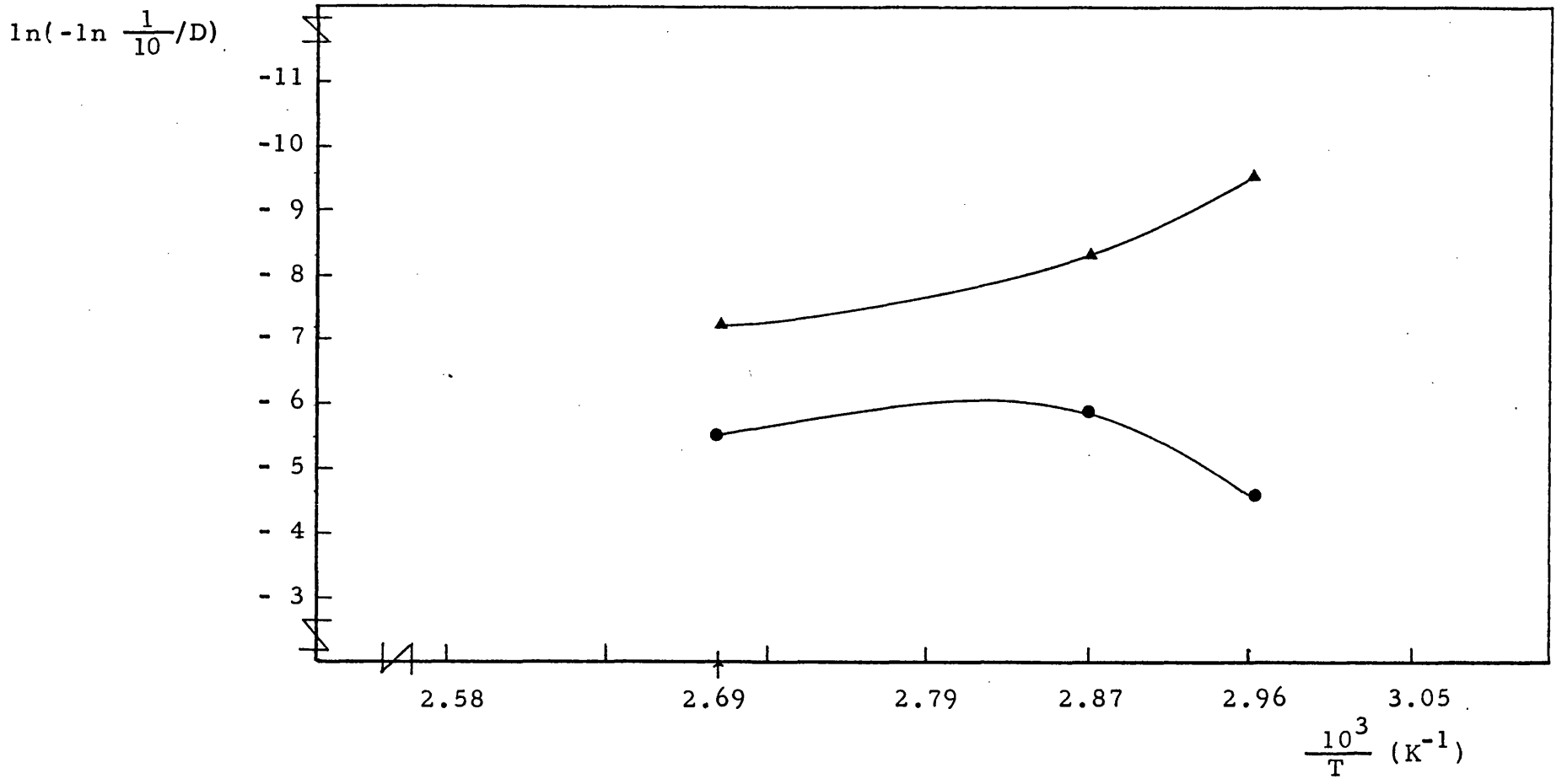


Figure 32. Arrhenius Plot of the First (●) and Second (▲) Stages of Inactivation of the Strain S4 Protease in Cell Free Growth Media at pH 6.8.

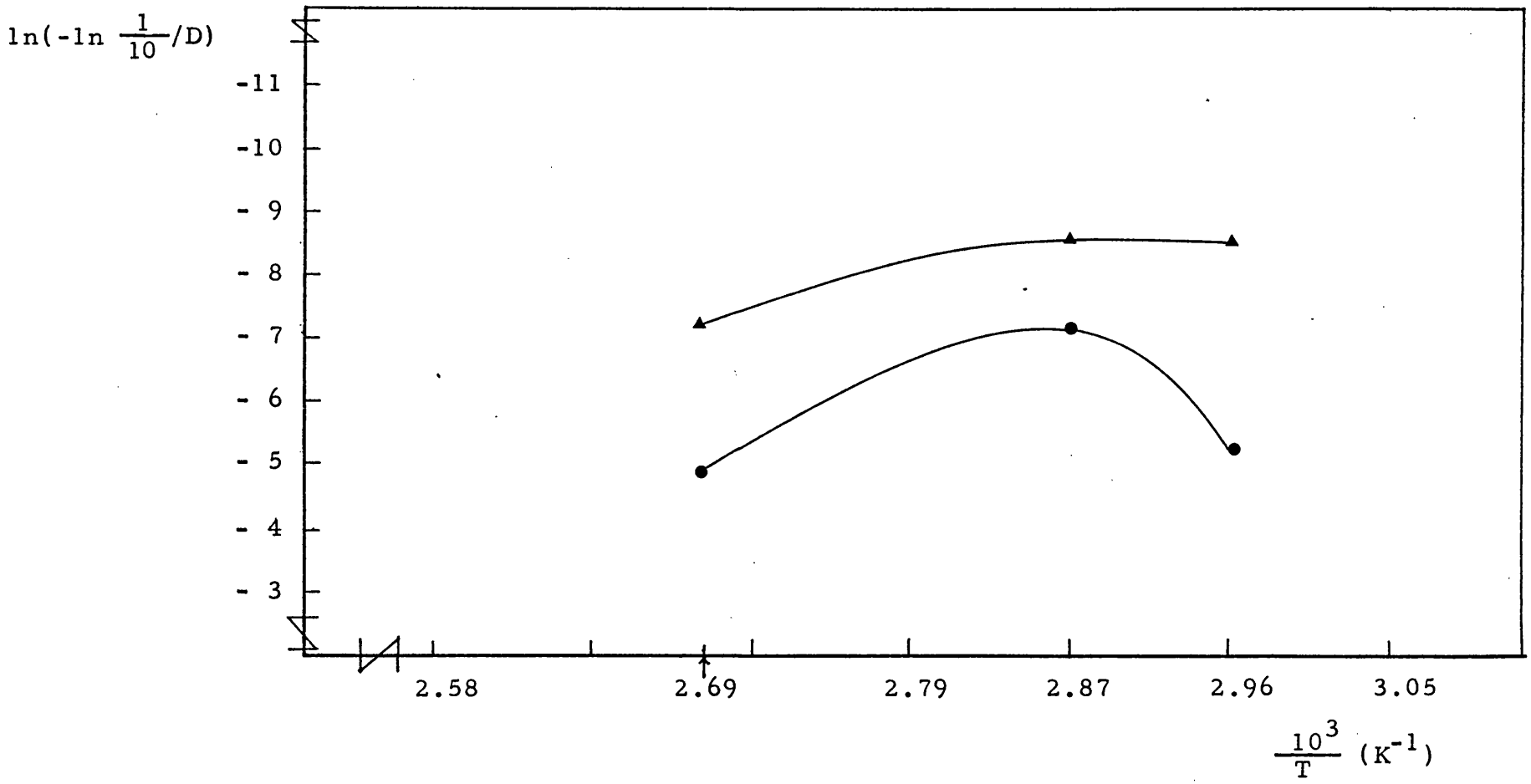


Figure 33. Arrhenius Plot of the First (●) and Second (▲) Stages of Inactivation of the Strain S5 Protease in Cell Free Growth Media at pH 6.8.

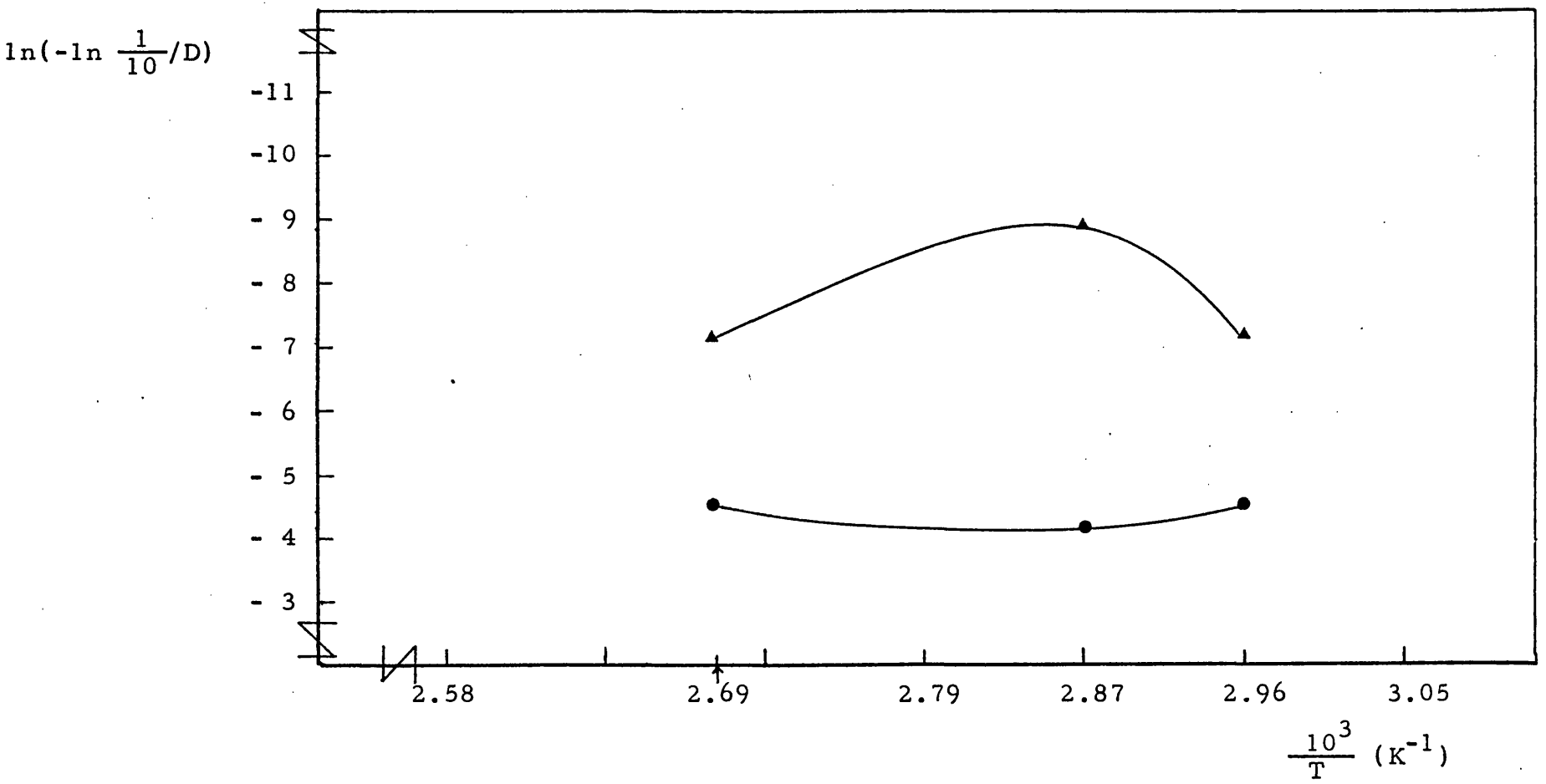


Figure 34. Arrhenius Plot of the First (●) and Second (▲) Stages of Inactivation of the Strain S6 Protease in Cell Free Growth Media at pH 6.8.

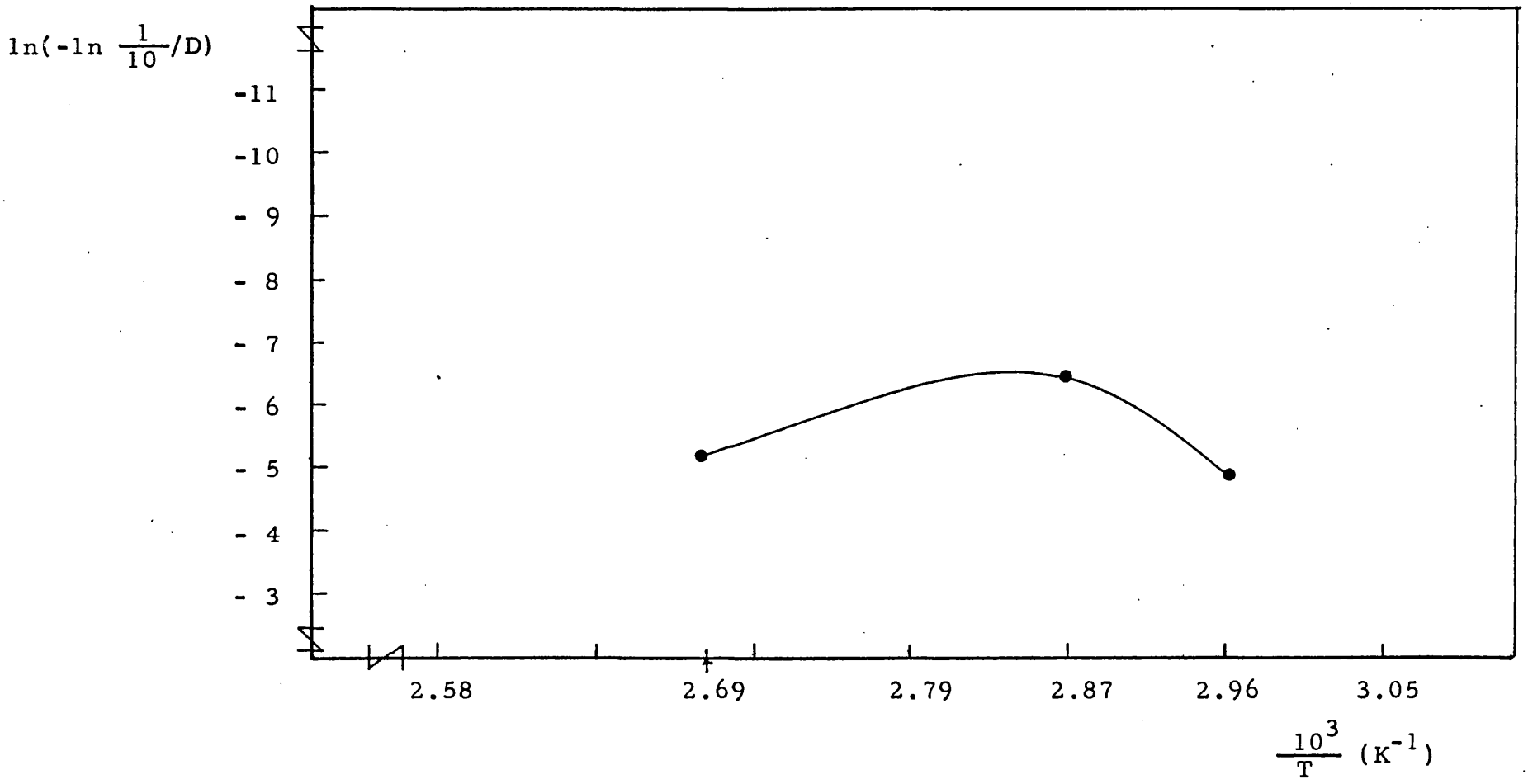


Figure 35. Arrhenius Plot of the First (●) and Second ( ) Stages of Inactivation of the Strain S7 Protease in Cell Free Growth Media at pH 6.8.

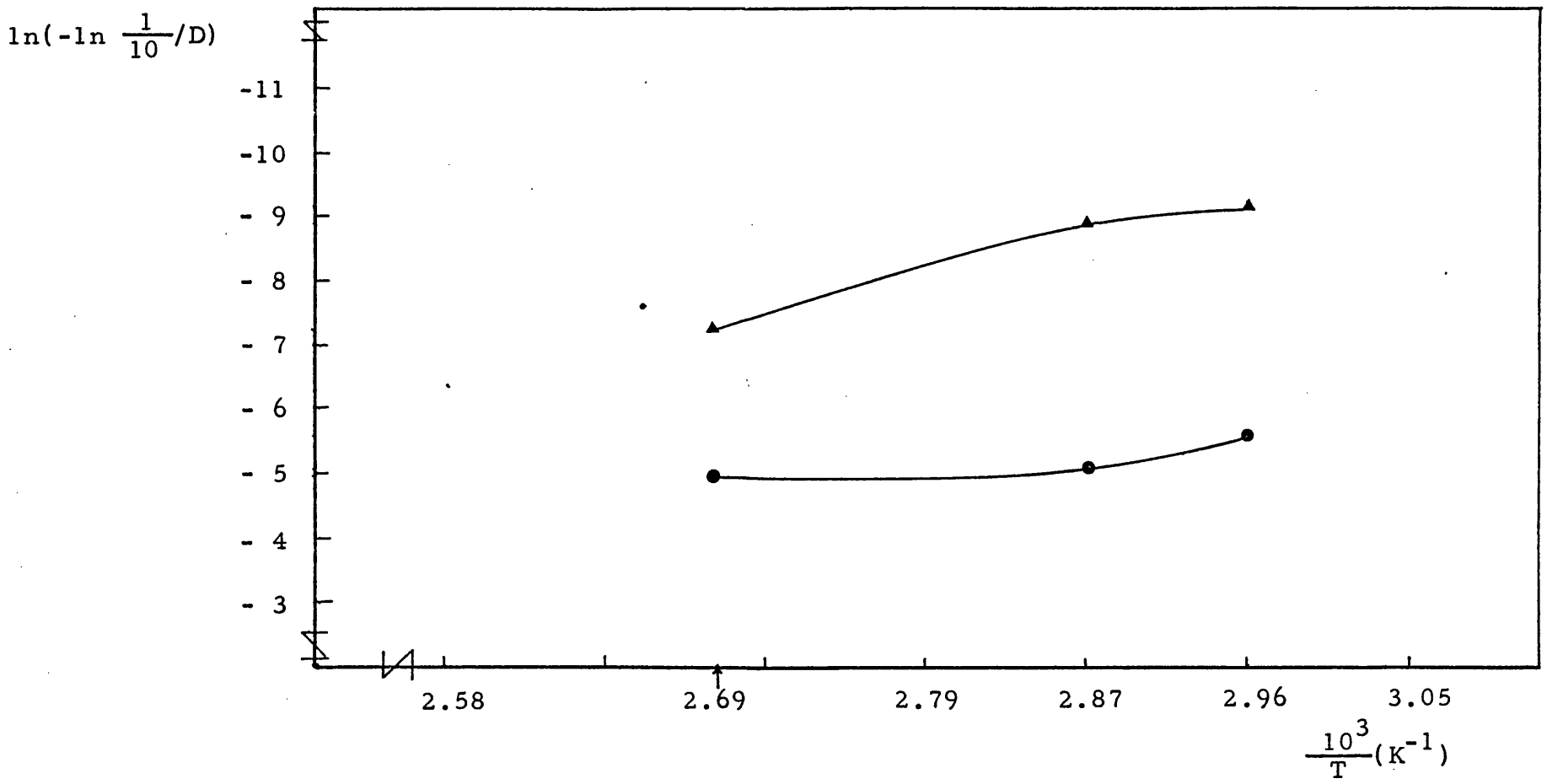


Figure 36. Arrhenius Plot of the First (●) and Second (▲) Stages of Inactivation of the Strain S8 Protease in Cell Free Growth Media at pH 6.8.

all balances favoring activation rather than inactivation (see Table 17, S3 and S7 both during the second stages of inactivation at 75° C). Calculations of the D-value would lead to a negative number since the lines were positively sloped within those stages (S3, 2nd stage = -49,392 and S7, 2nd stage = -278,733). As discussed on pages 72 - 75, this can occur as a result of calcium stabilization and may lead to a net gain of activation at 65° C for 16 minutes (Fig. 26) or at 75° C for about 3 to 6 minutes (Figs. 22 - 27). Apparently when stabilizing ions (BSM) and calcium are available in the heating medium the resulting heat-stabilized enzyme system may show a modified activity mode, since "apoenzyme" aggregation into units equivalent to tetrameter has been shown by Dahlquist et al. (1976). This change in inactivation profile was also observed when the enzyme preparations S2, S5 and S8 were heated to 142° C for one minute in the presence of casein (see pages 66 to 67 or Plates 5 and 6).

The natural logarithm (ln) of the inactivation constants related to 10% residual activity were ploted against the inverse of the absolute temperatures equivalent to 65° C, 75° C and 98° C (2.96, 2.87 and 2.69). This plot was carried out separately for the first and second inactivation stages to test: (1) the nature of the heat inactivation reactions within those intervals; (2) the differences in inactivation constants and activation energies displayed by different enzyme systems when subjected to heat treatments. Although only three

temperatures were examined, linear relationships were observed for both stages of inactivation by enzyme preparation S8. The remaining enzyme preparations exhibited at least two distinct phases of inactivation. This may suggest that most of the extracellular enzyme systems produced by the test strains included two or more enzymes. It is also possible to consider heat stabilization by metal ions partially responsable for this behavior, either resulting from enzyme aggregation or from autolysis (Dahlquist et al., 1976). A comparison of the rate constants for the first and second stages of inactivation led evidence to this type of arrangement. As demonstrated in Figs. 29-36, the inactivation rates were invariable lower for the second heating stage. After the application of heat for the first 0 to 100 seconds a stabilized enzyme form is thought to predominate. Thus based on the results presented in Figs. 29-36, the first stage may be seen as a stabilization process occurring as a result of divalent metal ions binding coupled with a cooperative configurational change in the protein molecule. Since the inactivation process was carried using cell free growth medium, such a stabilization may be involved not only with calcium or zinc but also with other divalent ions (see Appendix B). Thus the stabilization of the enzymes during the initial inactivation stage may either be governed by the strength of individual ion binding to specific sites or by the existence of such sites in the protein molecule. Thus, both composi-

tion of the medium containing the enzyme together with the pH, which will affect the acid/base balance may be important factors contributing to the initial stages of inactivation/activation. In addition the second inactivation stage seems to be dependent on the nature of the stabilized site and on the ions involved. Indications for this can be seen by compairing the heat stability patterns displayed by enzyme preparations S2 and S6 at 65° C (Figs. 22 and 26). Recent reports have indicated that some of these proteases may undergo inactivation at 55° C (Barach et al., 1976b; Barach et al., 1978). Fig. 26 suggests that this may be true for the protease system produced by the Alcaligenes sp. This property was not demonstrated at 65° C for any of the remaining enzyme preparations. The enzyme preparation from strain S6 was found to be the least heat-stable among the preparations obtained from the remaining strains.

The heat stability of enzyme systems produced by the test Gram negative bacteria is supported by reports published by Wright and Tramer (1953), Pinheiro et al. (1965, 1966), Driessen and Stadhouders (1971, 1973, 1974), Kishonti (1975), O'Donnell (1975) and Barach et al. (1978). The heat stability patterns displayed by pseudomonad proteases have been shown to be thermodynamicaly similar to the heat stability displayed by thermolysin (Barach and Adams, 1977).

(c) Conclusion

The heat stability of the test enzyme systems were

investigated at temperatures of 65° C, 75° C and 98° C. Heat stability measurements were conducted in cell free growth media in order to assess their heat stability in an *in vivo* situation similar to that occurring in a liquid foodstuff such as milk. Under these conditions the enzyme systems exhibited a considerable degree of thermostability, even when subjected to a temperature of 98° C. During the initial stages of heat treatment the enzyme systems may be stabilized by ions in the heating media. This was considered as the first inactivation stage. The second stage of inactivation proceeded very slowly, with the exception of the enzyme preparation S6. In general the first inactivation stage was associated with very little loss of enzyme activity (85% to 95% residual activity) after the first 60 seconds plus 30 seconds allowed for the come up time. Further loss of enzyme activity usually required heating of 98° for 43 to 76 minutes to achieve 10% residual activity. Exponential loss of activity was observed for the heat inactivation of the enzyme preparation S6.

SECTION 3. HEAT STABILITY OF DIFFERENT ENZYME SYSTEMS AT TEMPERATURES ABOVE 100° C

(a) Experimental

Heat inactivation experiments were conducted at temperatures of 110° C, 120° C, 130° C and 140° C.

Cell free growth media were obtained as this text previously described (page 76). Volumes of 3 ml taken from each cell free broth were displaced into fine layers of liquid using a double stainless steel tubes (see Plates 1 and 2). This displacement was carried out using an internal fitting tube tightly packed with glass wool. In order to avoid humidity within the internal tube, both the glass wool and the tube were placed at 140° C in a dry heat oven for 2 hours until just before the packing was carried out. The internal tube was then sealed with a rubber stopper. The tubes were cooled to 5° C, 3 ml of the samples were introduced and they were fitted together through a hermetical sealing system as shown in Plate 2. Heating and cooling were conducted according to the methods described on pages 41 and 66 respectively. The allowed cooling period was one minute at 5° C. The residual activity of heated and unheated samples were determined according to the procedure described on pages 34 and 35. See Appendix I for further information on the procedure used for treatment of the results.

(b) Results and Discussion

The results of the heat stability experiments conducted at temperatures above 100° C are presented in Table 18 and Figs. 37 to 44. Table 19 shows calculations of the D-value and the ln of the inactivation constant

Table 18. Heat Inactivation of Test Strain Proteases in Cell Free Media at pH 6.8 at Constant Temperatures of 110° C, 120° C, 130° C and 140° C (*)

Heating Time (in Seconds)	S1		S2				S3		S4	
	130°	140°	110°	120°	130°	140°	130°	140°	130°	140°
Blank 0	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
30	0.32	0.32	0.54	0.86	0.86	0.73	0.65	0.56	0.54	0.47
120	0.15	0.03	0.40	0.63	0.46	0.09	0.27	0.09	0.19	0.07
240	0.03	0.00	0.30	0.25	0.07	0.00	0.05	0.00	0.03	0.00
480	0.00		0.16	0.09	0.00		0.00		0.00	
960			0.04	0.00						

(*) In Ratio of DU_2/DU_1.

Table 18. (Continuation) Heat Inactivation of Test Strain Proteases in Cell Free Media at pH 6.8 at Constant Temperatures of 110° C, 120° C, 130° C and 140° C. (*)

Heating Time	S5		S6				S7		S8			
(in Seconds)	130°	140°	110°	120°	130°	140°	130°	140°	110°	120°	130°	140°
Blank 0	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
30	0.31	0.36	0.33	0.59	0.69	0.48	0.72	0.61	0.37	0.42	0.21	0.37
120	0.13	0.05	0.29	0.23	0.16	0.04	0.52	0.14	0.08	0.18	0.07	0.09
240	0.03	0.00	0.23	0.16	0.02	0.00	0.07	0.00	0.24	0.10	0.04	0.00
480	0.00		0.11	0.02	0.00		0.00		0.21	0.02	0.00	
960			0.02	0.00					0.07	0.00		

(*) In Ratio of DU_2/DU_1.

Table 19. Treatment of the Results(*) for Heat Inactivation Patterns Displayed by the Test Proteases at Temperatures Above 100° C.

Strains	Temperatures		D-value	ln (k)
	°C	$\frac{10^3}{T}(K^{-1})$	(s^{-1})	$\ln(-\ln\frac{1}{10}/D)$
S1	130	2.48	150	-4.17
	140	2.42	100	-3.77
S2	110	2.61	650	-5.64
	120	2.54	450	-5.27
	130	2.48	230	-4.60
	140	2.42	115	-3.91
S3	130	2.48	210	-4.51
	140	2.42	119	-3.94
S4	130	2.48	175	-4.33
	140	2.42	118	-3.94
S5	130	2.48	135	-4.07
	140	2.42	90	-3.66
S6	110	2.61	555	-5.48
	120	2.54	320	-4.93
	130	2.48	155	-4.21
	140	2.42	85	-3.61
S7	130	2.48	235	-4.62
	140	2.42	135	-4.07
S8	110	2.61	550	-5.47
	120	2.54	240	-4.64
	130	2.48	105	-3.82
	140	2.42	115	-3.91

(*) Results from Table 18.

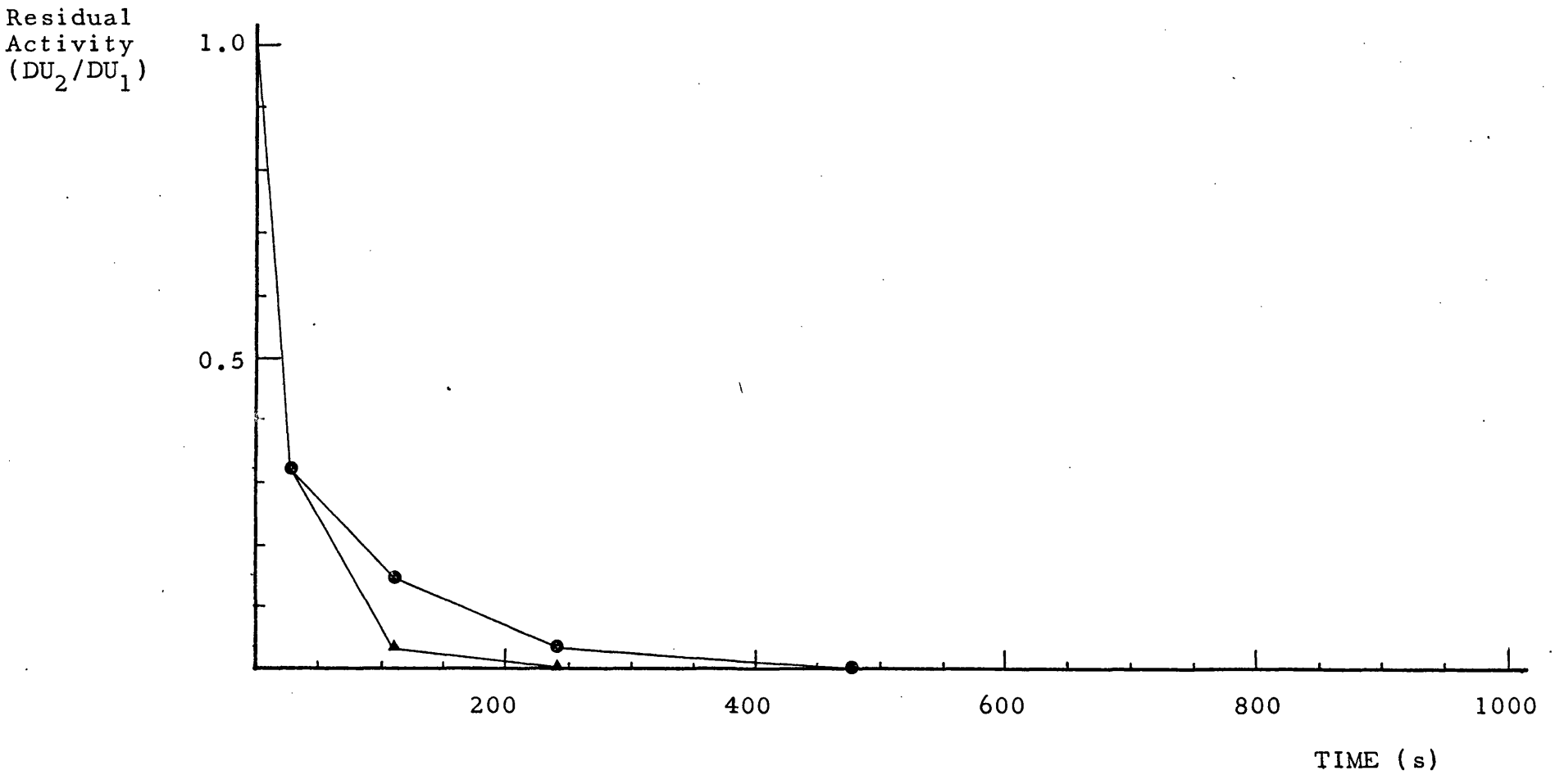


Figure 37. Heat Inactivation of Strain S1 Protease in Cell Free Growth Media at pH 6.8 (● =130° C, ▲=140° C).

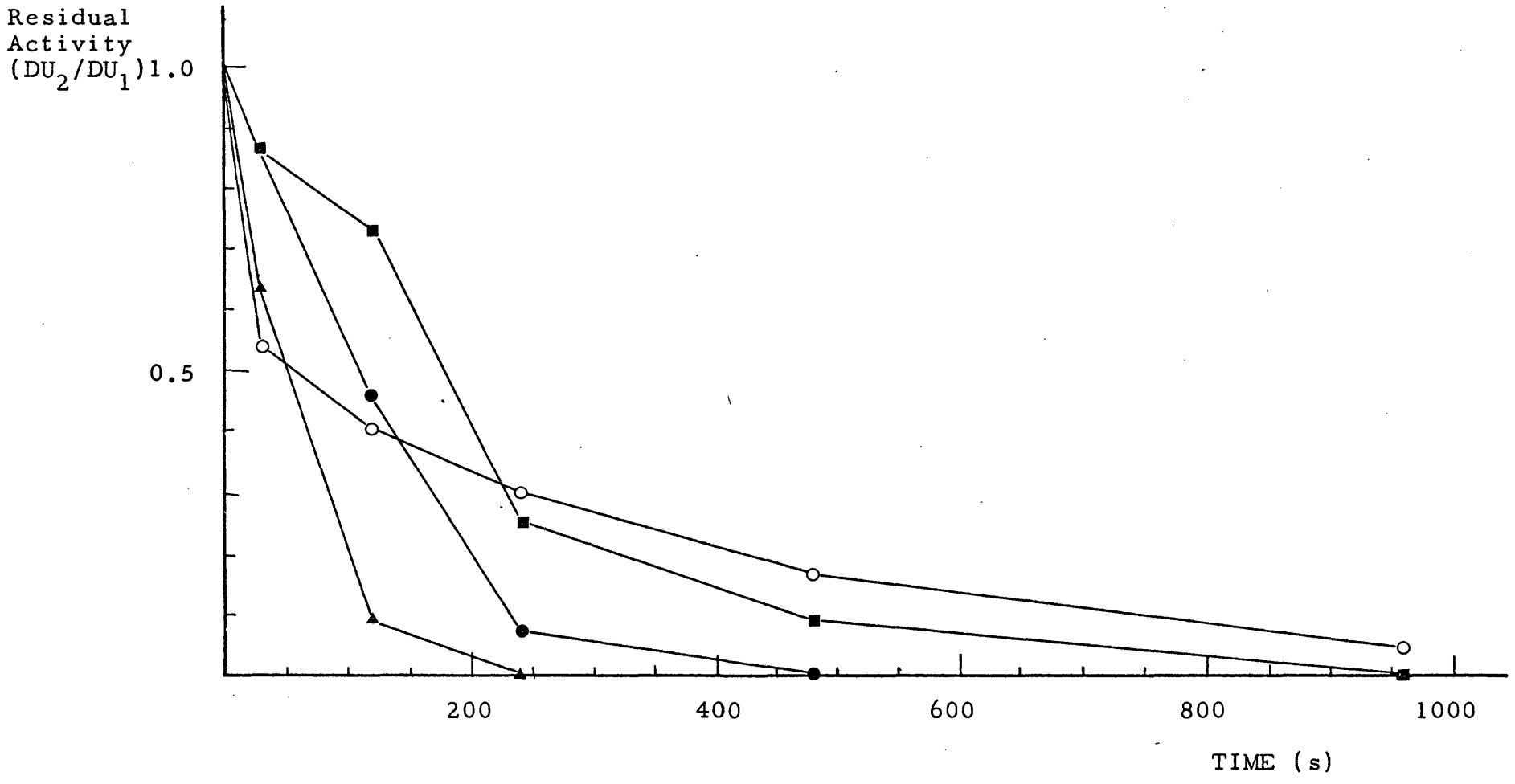


Figure 38. Heat Inactivation of Strain S2 Protease in Cell Free Growth Media at pH 6.8 (○ =110° C, ■=120° C, ●=130° C and ▲=140° C).

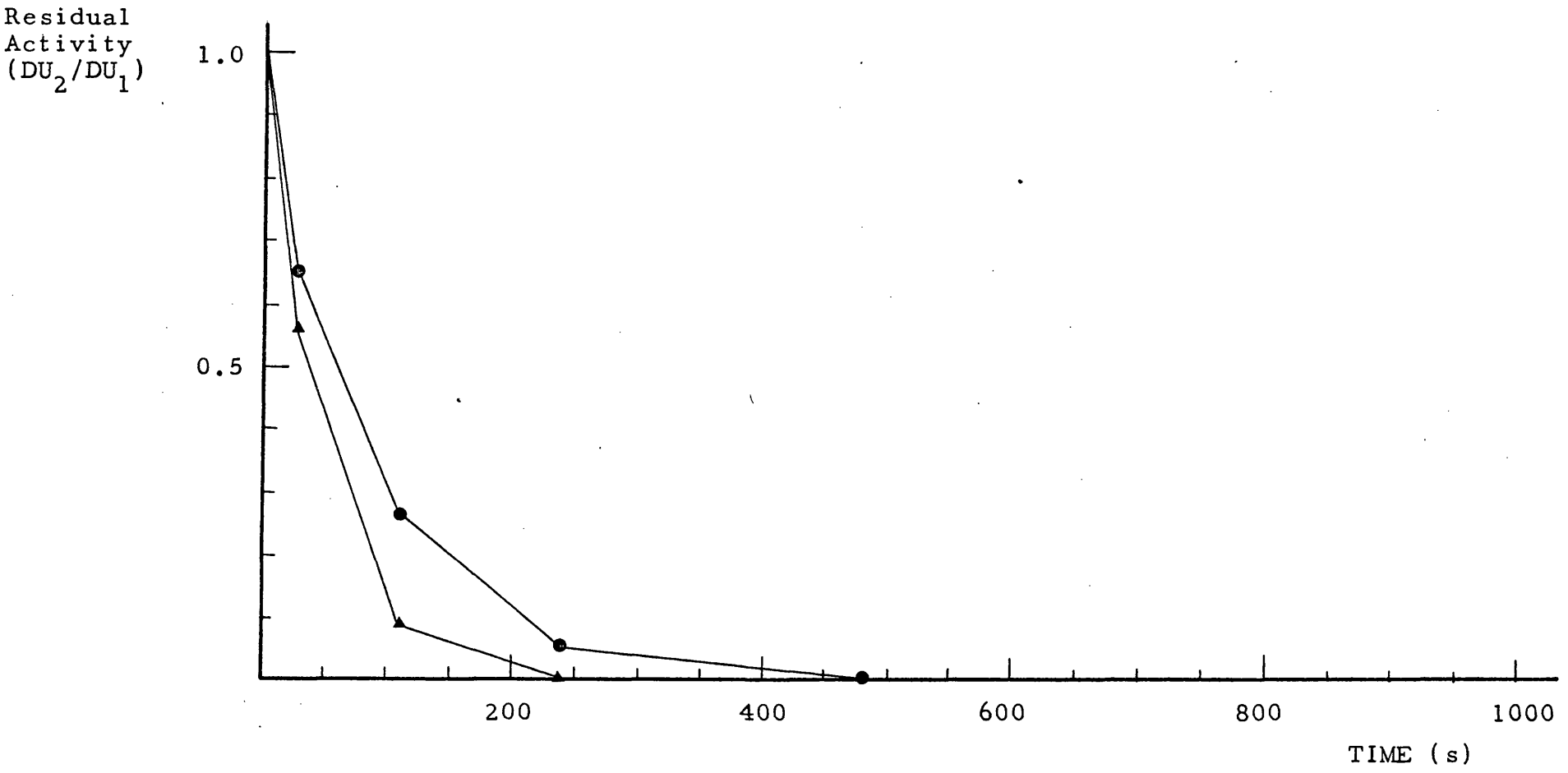


Figure 39. Heat Inactivation of Strain S3 Protease in Cell Free Growth Media at pH 6.8 (●=130° C and ▲=140° C).

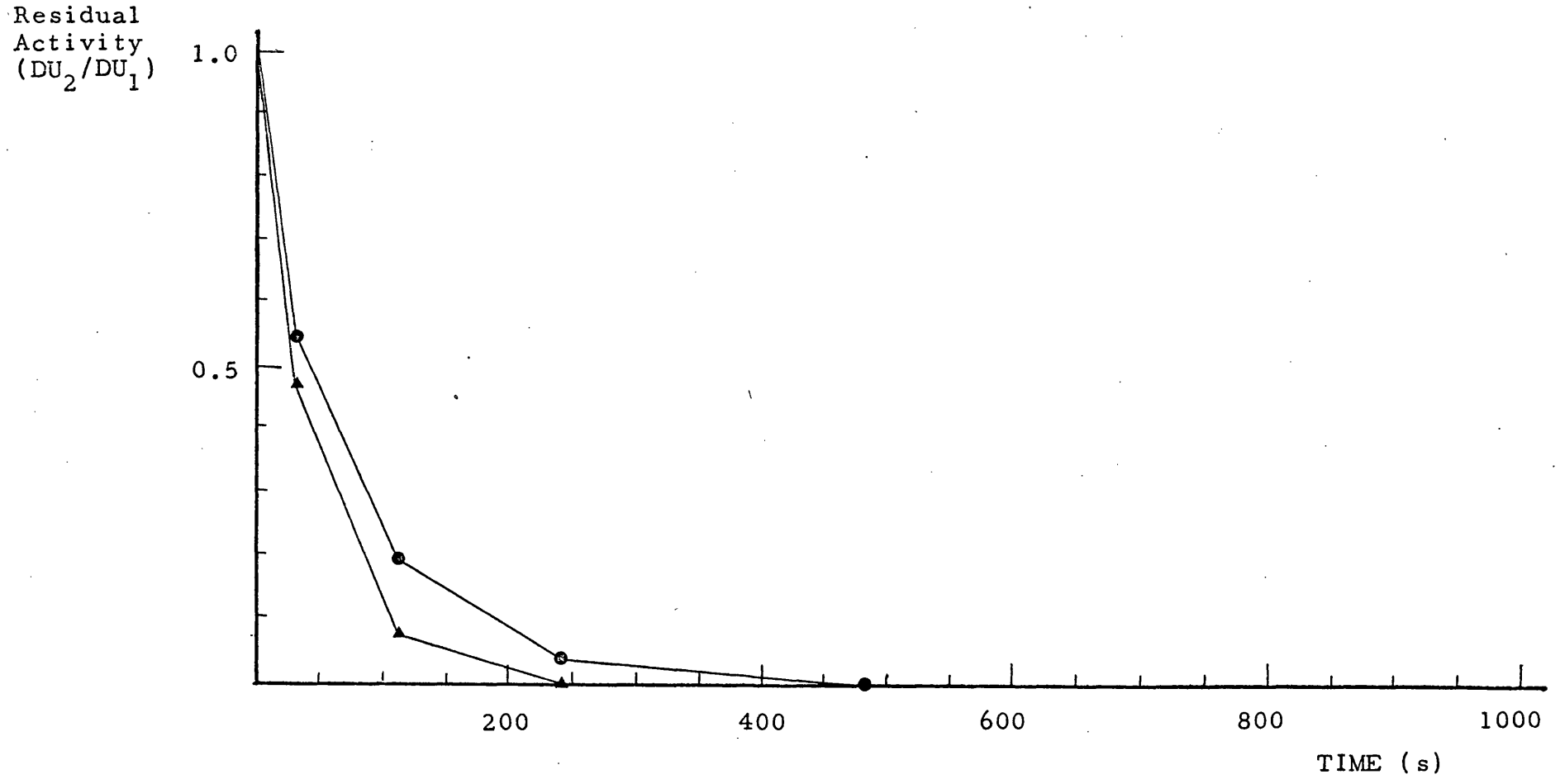


Figure 40. Heat Inactivation of Strain S4 Protease in Cell Free Growth Media at pH 6.8 (●=130° C and ▲=140° C).

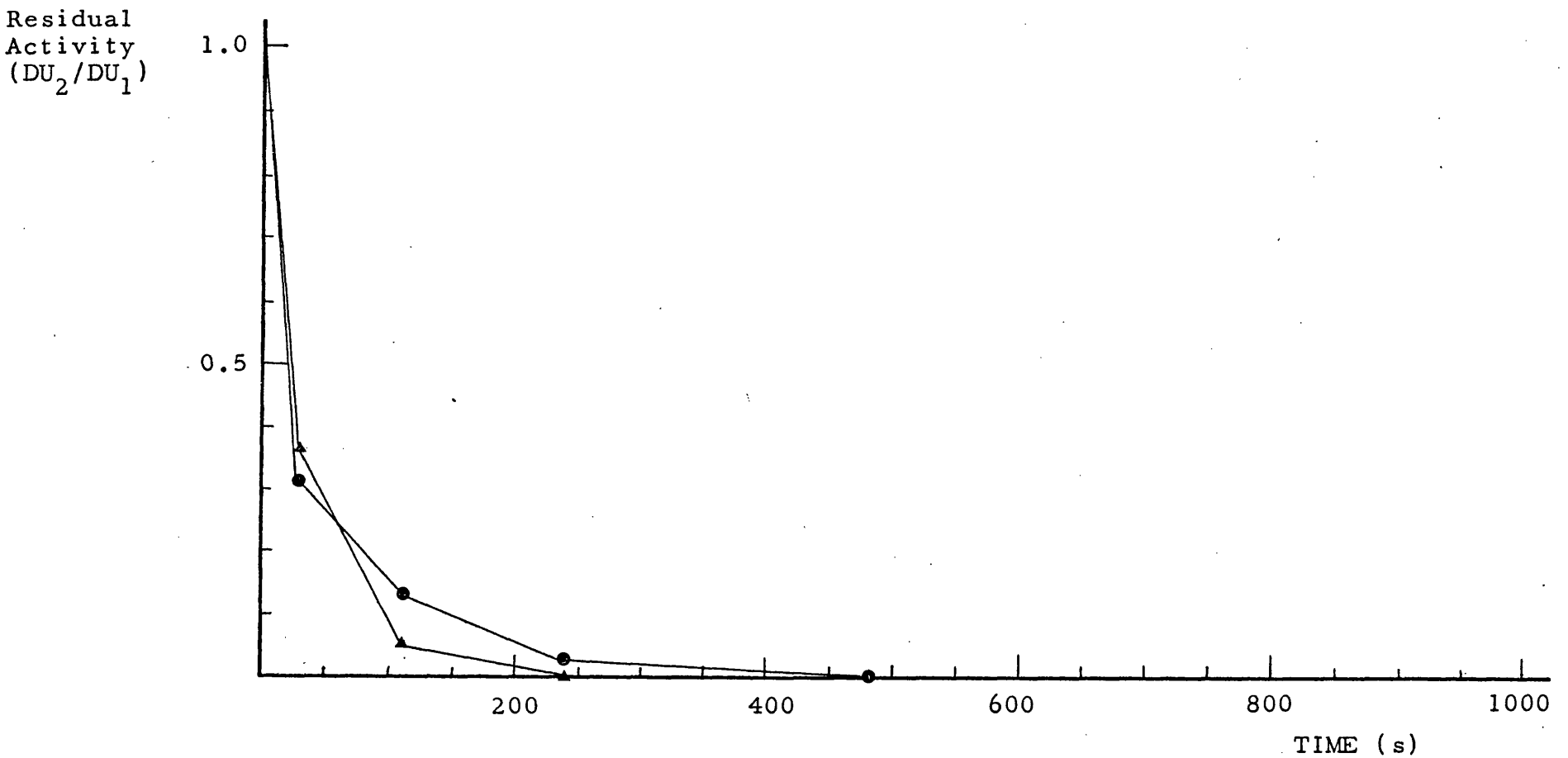


Figure 41. Heat Inactivation of Strain S5 Protease in Cell Free Growth Media at pH 6.8 (●=130° C and ▲=140° C).

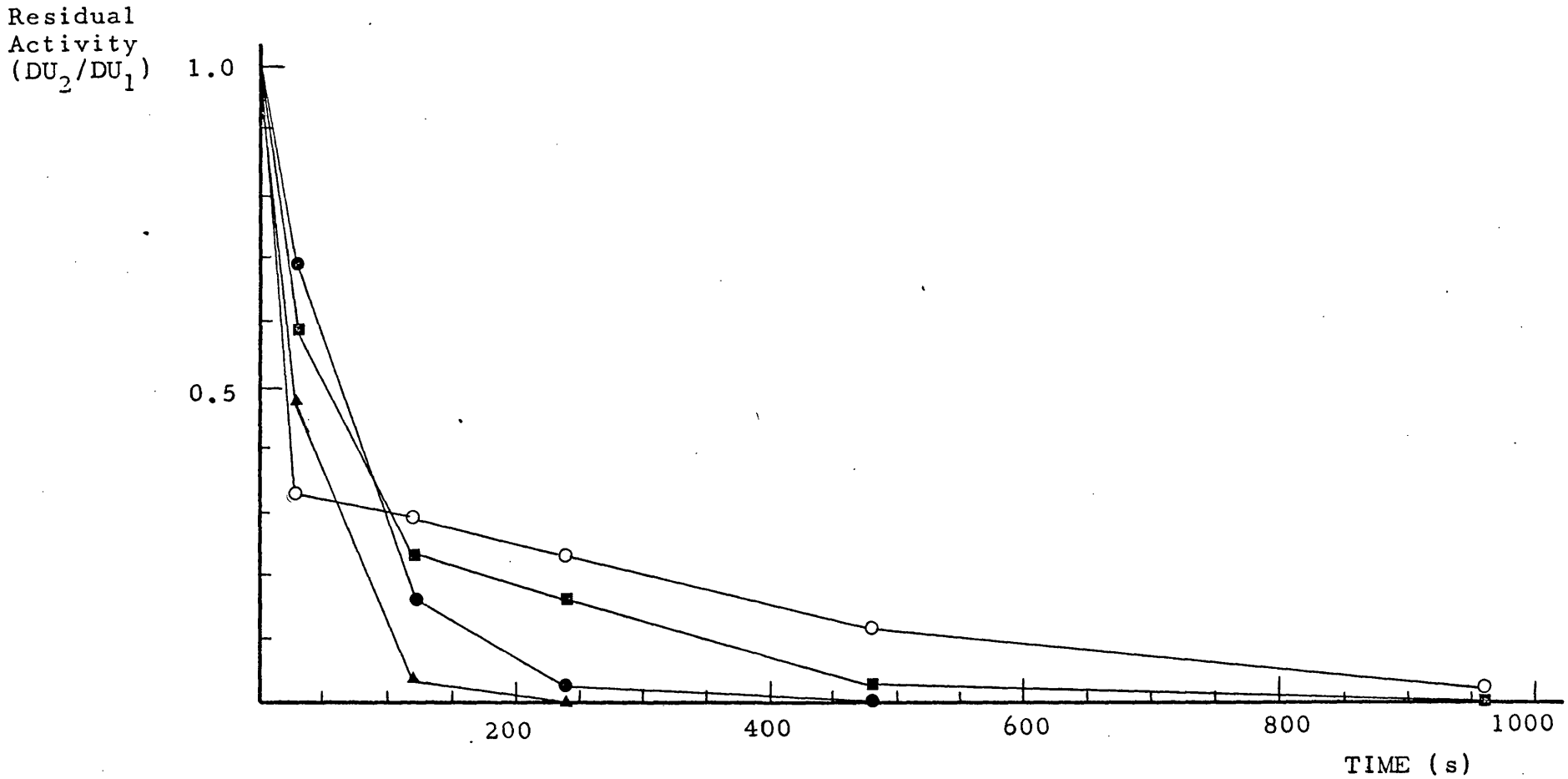


Figure 42. Heat Inactivation of Strain S6 Protease in Cell Free Growth Media at pH 6.8 (○ =110° C, ■ =120° C, ● =130° C and ▲ =140° C).

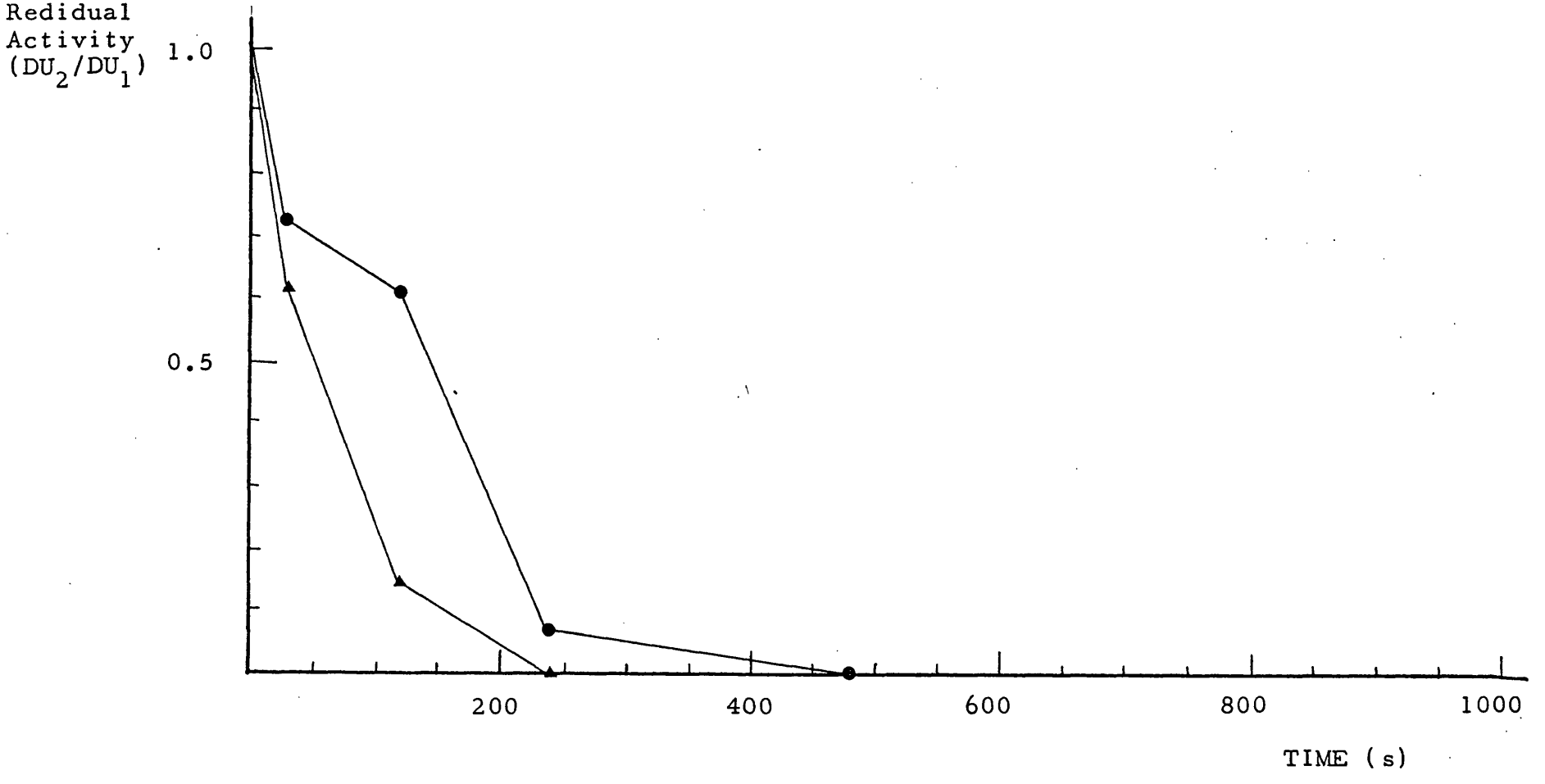


Figure 43. Heat Inactivation of Strain S7 Protease in Cell Free Growth Media at pH 6.8 (● =130° C and ▲=140° C).

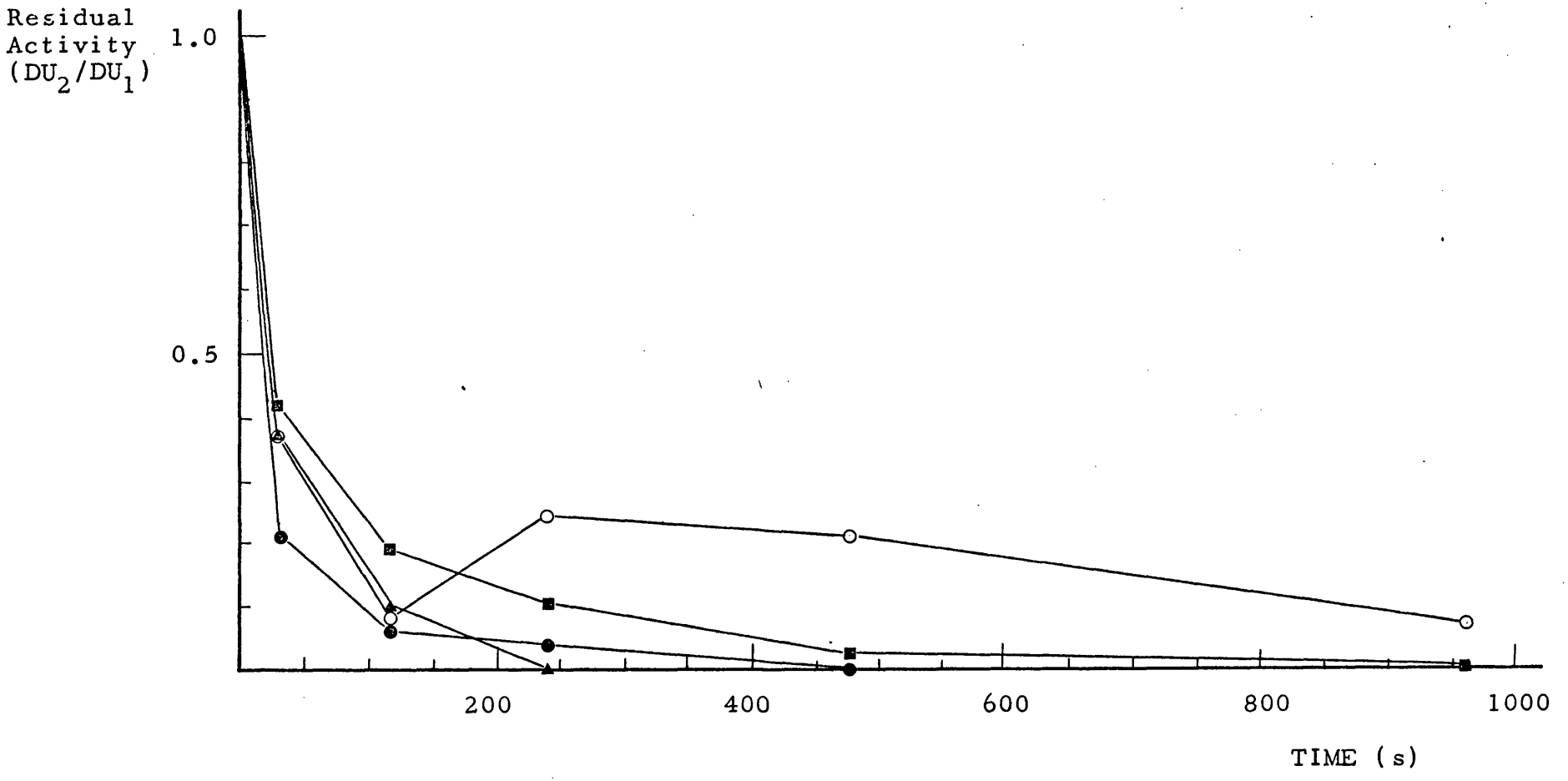


Figure 44. Heat Inactivation of Strain S8 Protease in Cell Free Growth Media at pH 6.8 (○ =110° C, ■ =120° C, ● =130° C and ▲ =140° C).

at the point where 90% loss of the initial activity was achieved by heating. At temperatures of 110° C or above the inactivation reactions were favored toward an inactive enzyme form (Figs. 37 to 44). Their residual activity fell toward values close to zero within the heating time interval of 200 to 1000 seconds. There was no significant increase in the activation rate with respect to inactivation rate since most of the heat inactivation curves followed continuous losses of enzyme activity. These losses of enzyme activity followed a decreasing rate pattern and were exponentially related with respect to heating time. Two exceptions were noted, the enzyme preparations S7 at 130° C and S8 at 110° C (Figs. 43 and 44). For the remaining enzyme preparations the exponential relationship between their respective D-values and heating temperatures was demonstrated as illustrated in Fig. 48. Although such relationships were generally better correlated when the experimental points for single test strains were considered separably, the overall correlation for all the experimental points put together was -0.94. The shortest D-value (85s) was observed for the heat inactivation experiment at 140° C for the enzyme system S7. The average D-values taken within each temperature resulted in 585 s at 110° C, 337 s at 120° C, 174 s at 130° C and 110 s at 140° C. The Arrhenius plots (Figs. 45 to 47) showed good degree of linearity for most of the heat inactivation experiments within a single inactivation stage. This relationship has been

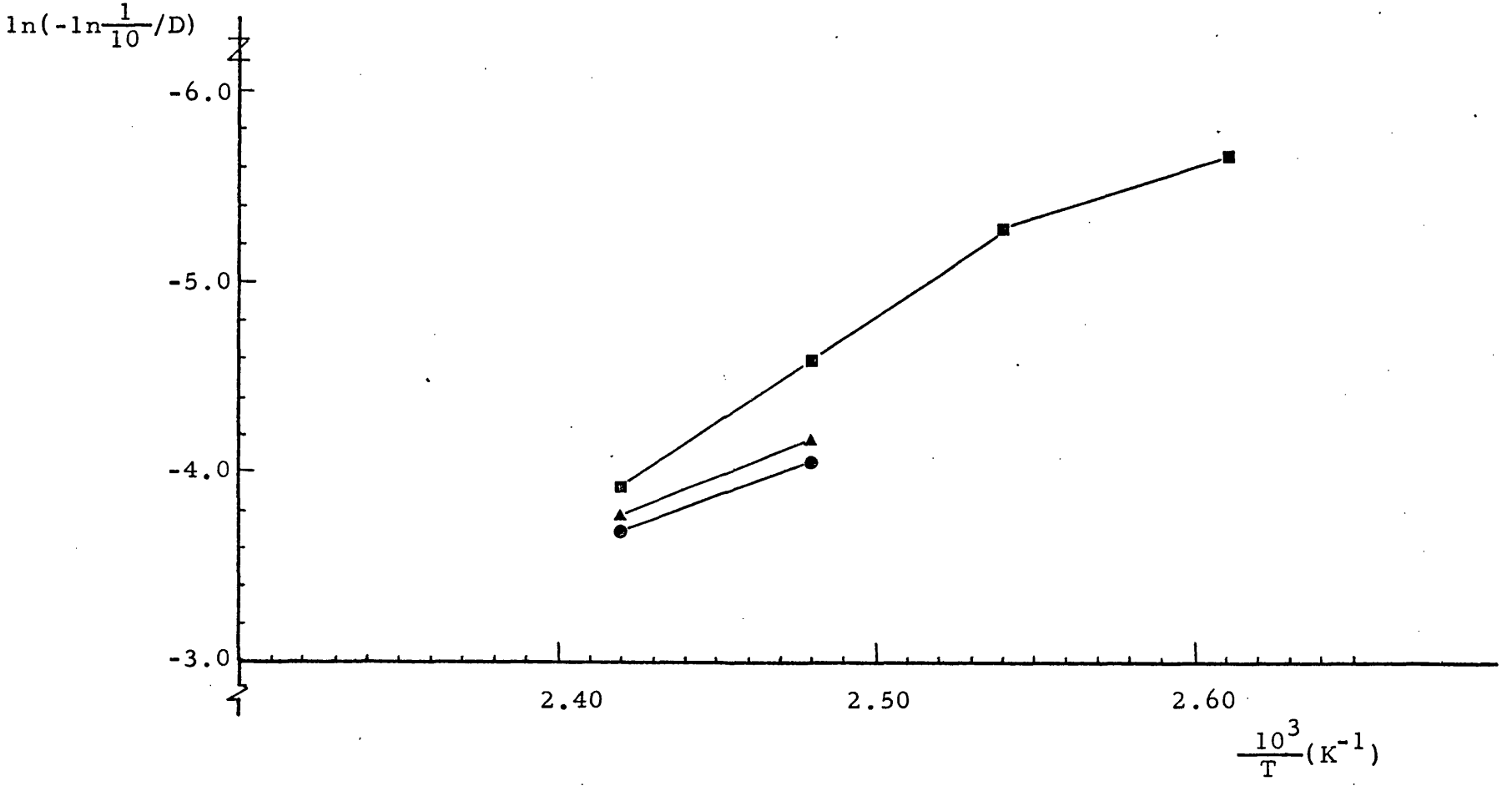


Figure 45. Arrhenius Plot of the Strains S1 (▲), S2 (■) and S5 (●) Proteases in Cell Free Growth Media at pH 6.8.

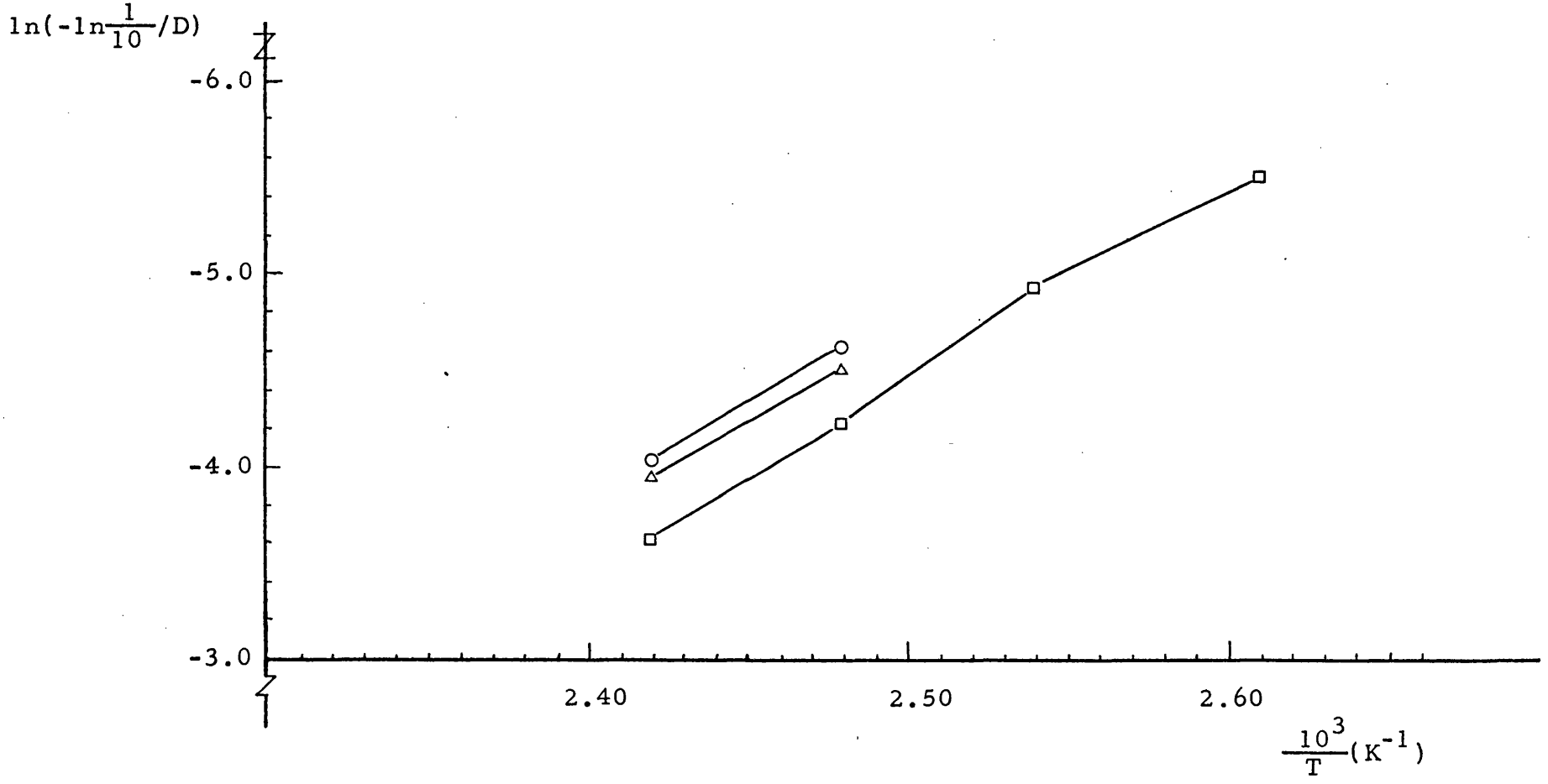


Figure 46. Arrhenius Plot of the Strains S3 (△), S6 (□) and S7 (○) Proteases in Cell Free Growth Media at pH 6.8.

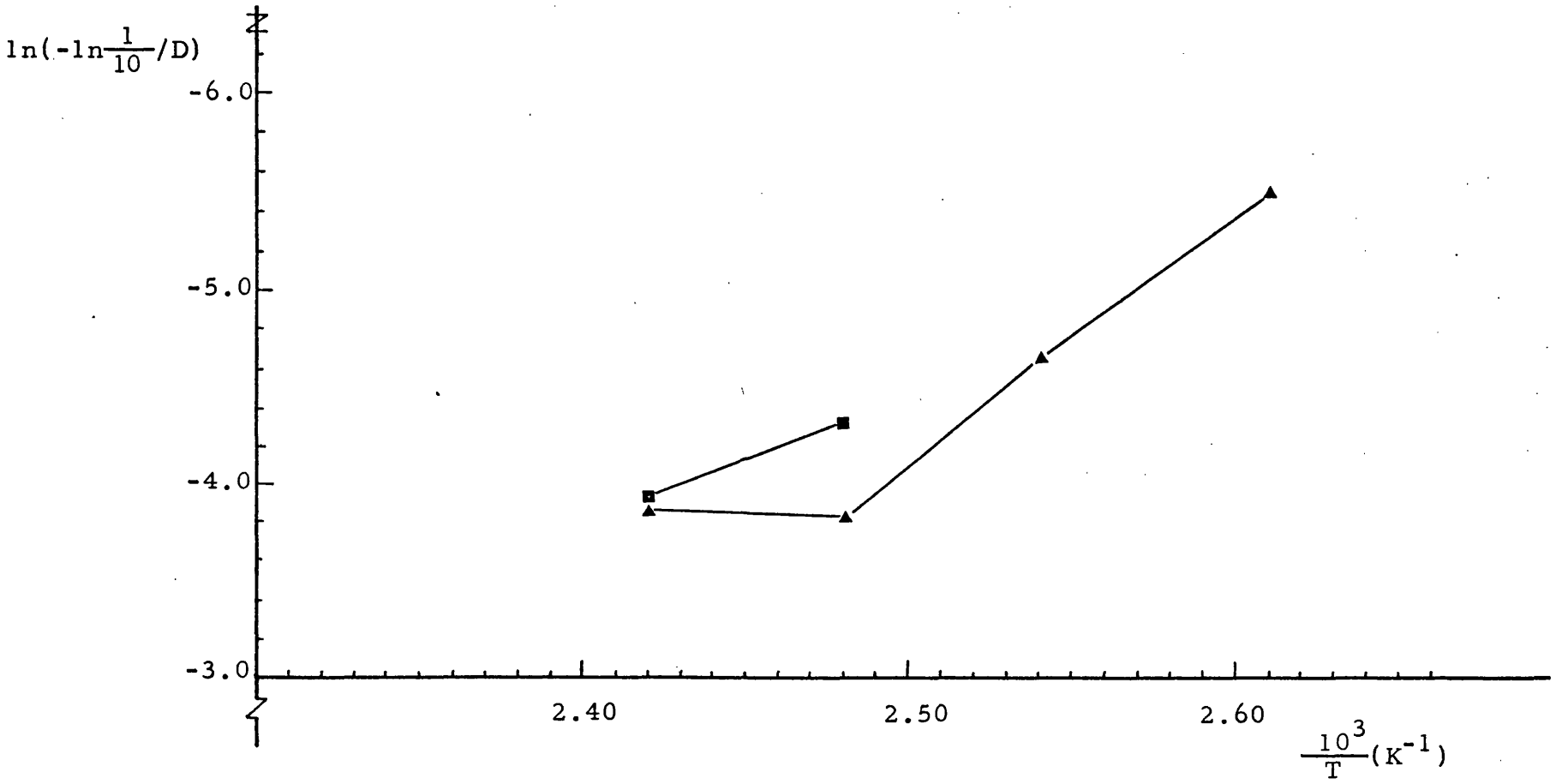


Figure 47. Arrhenius Plot of the Strains S4 (■) and S8 (▲) Proteases in Cell Free Growth Media at pH 6.8.

demonstrated by Adams et al. (1975), Kishonti (1975) and Barach et al. (1976a). Adams et al. (1975) have shown that the D-values for a Pseudomonas sp. (MC60) protease were approximately 90 to 100 s at 150° C and over 1,000 s at a temperature of 110° C. Kishonti (1975) has reported D-values of 90 s at 150° C and over 2,000 s at 110° C.

Some reports relating to the heat stability of protease preparations in their purified forms, have indicated a generally lower degree of stability. Fallon (1972) has demonstrated that Pseudomonas spp. proteases were very unstable at temperatures higher than 65° C when partially purified enzyme preparations were tested. Barach et al. (1976a) have shown that the half-life of the protease MC60 was only 7 s at 149° C. They have also indicated that there was no difference between the half-life in skim milk or in milk-salts buffered with calcium. Lower heat stability patterns may be expected when purification steps are included (O'Donnell, 1975) or when heating is conducted in a saltless medium. This problem is further complicated since the denatured form of the enzyme protein molecule may become themselves good substrates for the remaining native enzyme conformation (Dahlquist et al., 1976).

(c) Conclusion

At temperatures of 110° C or above enzyme inactivation was generally easily achieved during time expo-

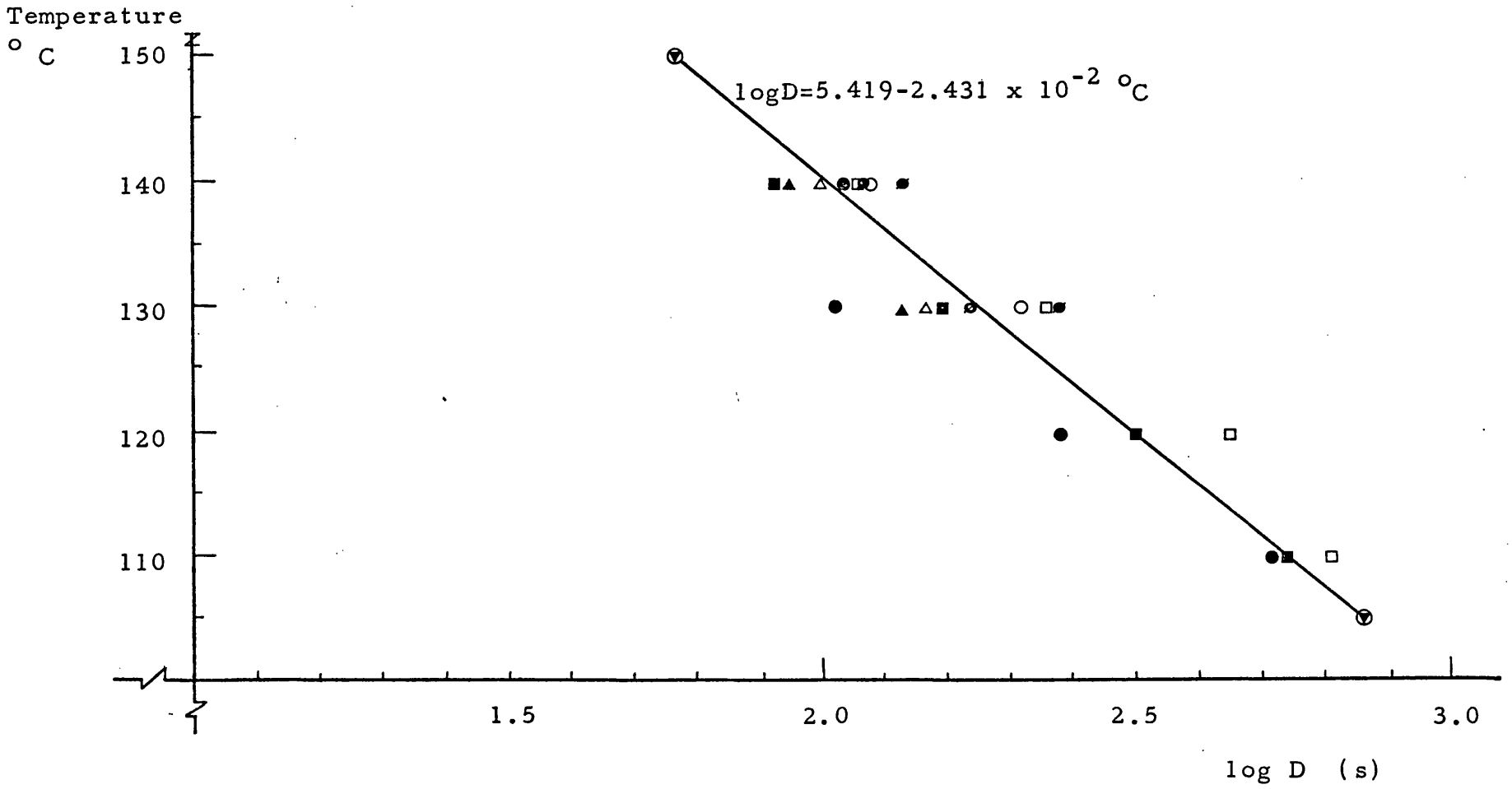


Figure 48. Relationship Between Observed D-values and Temperature for All Test Strain Proteases; (△=S1, □=S2, ○=S3, ⌀=S4, ▲=S5, ■=S6, ⌀=S7 and ●=S8; ⛛=Estimated).

sures of 3 to 16 minutes, leading to less than 10% residual activity levels. At temperatures ranging from 110° C to 140° C there were no significant changes of the inactivation rate leading to intermediate positive slopes. In most of the cases the inactivation rate fell continually toward zero and became asymptotic to the time axis as the heating time progressed. The heat inactivation curves generally followed an exponential pattern. This exponential relationship between temperature and D-value was demonstrated by ploting temperatures against the logarithm of their respective D-values. The overall correlation for this linear relationship was -0.94. The average D-values taken for the various protease systems at each temperature were 585 s at 110° C, 337 s at 120° C, 174 s at 130° C and 110 s at 140° C.

SECTION 4. CONCLUSION

The heat stability of the test protease systems were studied at temperatures ranging from 65° C to 140° C. Their stability to heat inactivation at temperatures of 65° C, 75° C and 98° C were notable dependent of the stabilizing properties of certain ions present in the heating media. This has been previously shown to be true for Pseudomonas sp. pro-

teases (Barach et al., 1976a) and for the endopeptidase "thermolysin" produced by Bacillus thermoproteolyticus (Dahlquist et al., 1976). The stabilizing property of the ions present in Pope and Skerman's basal salts medium (Skerman, 1967) was evident when the heat inactivation of the enzyme preparation S8 dissolved in BSM and dissolved in distilled water were compaired. Thus, operations that tend to remove these ions or activators (such as those of the allosteric type) will affect the stabilizing properties of the protease when subjected to heat inactivation experiments (Yudkin and Offord, 1973). The reduced levels of ions and low osmotic strength conditions resulting from the ultrafiltration concentration procedure used caused an acceleration of the inactivation rate during the first stage at 98° C. This situation has also been observed for the heat inactivation of lipase produced by some psychrophilic Pseudomonas spp. (O'Donnell, 1975).

Since milk proteins normally are carriers of certain ions if they are to exhibit their full nutritive or biological fuctions (Alais and Blanc, 1975), any study of enzyme inactivation by heat must be carried out either in milk itself or in a simulated milk serum having milk stabilizing properties. In this study the heat stability of the test protease systems were carried out in cell free growth media at pH 6.8. Under these conditions the heat inactivation reactions showed two distinct inactivation stages at temperatures below 100° and a single inactivation stage at

temperatures above 100° C. At temperatures below 100° C the inactivation reactions were only favored at 98° C for 43 to 76 minutes leading to residual activity levels of 10%. At temperatures of 110° C or above the test protease systems were relatively easily inactivated during heating/time exposures of 110° C for 16 to 20 minutes or 140° C for 3 to 4 minutes leading to residual activity levels of 10%.

Therefore, most of the conventional heat process operations (HTST, High Temperature Short Time; HHST, High Heat Short Time; UHT, Ultra High Temperature) applied to pasteurization of milk are not capable of inactivating psychrophilic proteases solely as a result of heating. This is also true with respect to the UHT-Long Life Milk (142° C for 0.01 to 5.00 s). Nevertheless, homogenization under high pressure (1,000 to 8,000 psi. or 70.3 to 562.4 kg per sq cm) is known to cause physical-chemical changes in the milk system (Harper, 1976). Since homogenization under high pressure is an important component of the UHT-process, the psychrophilic protease systems may have their activity impaired during long life storage as a result of: (1) adsorption of casein on the fat surface, (2) formation of sulphydryl compounds resulting from the migration of phospholipids to the skimmilk phase, (3) changes of the acid/base equilibrium, (4) aggregation of protease/casein being favored by high pressure. Also the absence of oxygen in the final packed UHT-milk seems to be vital for its long storage life.

CHAPTER VI

GENERAL CONCLUSION

In this study eight psychrophilic bacteria were investigated with respect to growth and protease production at 5° C. The results have shown that the logarithmic phases of growth displayed by the test strains were limited to five days under aerated conditions and twelve days under stationary conditions. The lag phases of growth and of protease production were shown to be extended from 1 - 2 days under aerated to 2 - 6 days under stationary conditions. These results agreed with previous reports whereby protease production and growth have been shown to be enhanced by aeration(Sinclair and Stokes, 1962; Sodek and Hofmann, 1970; Fallon, 1972; O'Donnell, 1975). Optimum levels of protease production in liquid media required a convective stirring to support the oxygen demand by the test cultures. Meynell and Meynell (1975) have stated that the oxygen demand of aerobic cultures growing in liquid media can not be met without taking steps to increase the rate at which O_2 is replenished.

Assays conducted using the test strains in eleven commercial media showed that medium composition affects protease production. Strains which were able to produce large quantities of proteases in tryptone were often not able to produce them in casein acid hydrolysate. Juffs *et al.* (1968) have shown that protease production requires complex organic nitrogen in the growth medium. Juffs (1976) has shown that protease production is enhanced by the addition of peptone and

is inhibited by the addition of glucose and lactates to the growth medium.

The levels of protease production were shown to vary for different strains under the same temperature or for a single strain under different temperatures. Thus, protease production appeared to be dependent on certain metabolic advantages displayed by individual test strains under the experimental conditions at 25° C and 5° C. This is supported by Scholefield (1967) and by Bannerjee (1967). They have shown that psychrophilic metabolism is also a function of temperature in addition to the individuality of a particular strain. For example, Pseudomonas spp. display comparative metabolic advantages with respect to coliforms under aerated conditions at 5° C. Arafa and Chen (1975) have shown that this phenomenon enable certain psychrophilic pseudomonads to outgrow both coliform and Enterobacter on the surface of unpacked chicken pieces.

This study has suggested that pseudomonad protease systems are especially important because: (1) they often include lecithinase, protease and multiple esterase exoenzymes (Lawrence, 1967; Schmidt et al., 1969; Fox et al., 1975), (2) they may include exoenzyme systems containing phospholipase C and protease with elastase activity (Johnson et al., 1967; Schmidt et al., 1969) and (3)electrophoretic assays of heated (142° C for one minute) casein/enzyme preparations have shown they are not inactivated by ag-

gregation under the experimental conditions adopted in this study. Protease inactivation by aggregation to casein was observed for the enzyme preparations recovered from strains S6 and S7. On the contrary, strains S1, S2 and S8 produced protease systems which remained active after heating (142° C for one minute) of casein-enzyme preparations and during 24 hours of incubation at 30° C. Since the isoelectric casein used to assay protease activity during the incubation period did not have any specification with respect to vitamin contamination, these results can not be regarded as representative of an *in vivo* situation found in raw milk casein.

The presence of inactive protease in casein has been suggested by Warner and Polis (1945). It is possible that raw milk normally contains small quantities of protease associated with casein. In addition to this certain bacterial proteases may be inactivated by casein association as a result of heat. Mechanisms for the inactivation of bacterial proteases by aggregation and for the role of calcium on protease thermal stabilization have been recently published (Barach *et al.*, 1976a; Barach *et al.*, 1978).

All test protease systems were found to display considerable heat stability when they were heated in cell-free growth media at pH 6.8. The protease systems recovered from pseudomonad strains were particularly heat stable since their inactivation by aggregation or by autolysis could not be demonstrated by heat experi-

ments conducted at 142° C for 60 s.

The heat stability of the test protease systems showed different inactivation stages at temperatures below 100° C and a single inactivation stage at temperatures above 100° C. At 98° C and under heating/time exposures of 43 to 76 minutes, the inactivation reactions of all test protease proceeded readily and led to levels of 10% residual activity. At temperatures of 110° C or above the protease systems were easily inactivated during heating/time exposures of 110° C for 16 to 20 minutes or 140° C for 3 to 4 minutes and resulting in 10% residual activity. The heat inactivation curves for those temperatures ranging from 110° C to 140° C followed a decreasing exponential inactivation pattern. These results agree with previous reports (Driessen and Stadhouders, 1974; Kishonti, 1975; Dahlquist et al., 1976).

There are considerable implications relating these results with those usual heating methods applied to milk. Protease activity during storage may lead to gelation of UHT-products (Bengtsson et al., 1973). Psychrophiles are capable of producing protease during raw milk storage and according to Adams et al. (1975) all strains are able to produce heat-stable proteases and 70% to 90% of raw milk samples may contain psychrophilic bacteria capable of producing them at refrigeration temperatures. Speck and Adams (1976) have stressed that the ability to produce heat-stable proteases is likely to be a common

property of psychrophiles including Gram-negative bacteria.

Kishonti (1975) has indicated that low levels of enzyme activity may cause significant spoilage of dairy products, especially those which are subjected to long time of postprocess storage. Either the health aspects or the economical implications resulting from this postprocess spoilage are yet to be fully investigated.

In general the test protease systems showed activity against both α_s- and β-caseins, although α_s-casein was found to be less susceptible to digestion. The preferential susceptibility displayed by β-casein appeared to be partially lost when the enzyme preparations were heated (142° C/60 s) together with 1% (w/v) isoelectric casein. Psychrophilic proteases have been shown to hydrolyse both α_s- and β-caseins, nevertheless, β-casein has been shown to be preferentially hydrolysed in raw milk (Kiuru et al., 1970, 1971; Yanagiya et al., 1974; Purschel and Pollack, 1974). Possibly, the susceptibility displayed by β-casein is due to its high content of Pro residues. The content of Pro residues found in α_s-casein has been shown to be about one half of that of β-casein (Mercier et al., 1971, 1972; Grosclaude et al., 1973).

Pseudomonad protease systems are unlikely to contain rennin type enzymes (EC 3.4.23.4). Schmidt et al. (1969) did not observe hydrolysis of the endopeptide Ser-Leu by pseudomonad purified enzyme preparations.

On the contrary, Ser-Leu endopeptide has been shown to be hydrolysed by rennin (Visser and Slangen, 1977). Indeed, Barach and Adams (1977) have demonstrated a high degree of likeness existing between a pseudomonad protease and thermolysin (EC 3.4.24.4). Both enzymes contained Zn and Ca, but they were slightly different with respect to their Tyr content.

This study allowed the author to make a few conclusive statements: (1) all test psychrophilic bacteria were able to produce casein precipitating proteases, (2) pseudomonad protease systems were found to be particularly important with respect to the problem of spoilage of milk and dairy products and (3) all test protease systems were considerable heat stable under the adopted experimental conditions.

These results call for a greater emphasis to be placed on the study of the marketability life time of milk and dairy products. In addition to this, the dairy entrepreneurs must be directly provided with improved methods of milk preservation and utilization.

The problems of psychrophiles in milk and dairy products can be remedied by: (1) minimization of contaminations from all sources, (2) improvements of cleaning method applied to dairy equipments, (3) time limitations for cold milk storage and (4) minimization of aeration of raw and processed dairy products.

An evaluation of the overall utility gained from the homogenization of fluid milk in the various process operations is suggested.

APPENDIXES

Appendix A. First-Stage Diagnostic Table for Gram-negative Bacteria (Cowan and Steel, 1966).

	a			c		d		e				f				
	a_1	a_2	b	c_1	c_2	d_1	d_2	e_1	e_2	e_3	e_4	f_1	f_2	f_3	g	g
Shape	S	S	R	R	R	R	R	R	R	R	R	R	R	R	R	R
Motility	-	-	+	-	-	-	+	+	+	-	-	-	-	+	-	d
Growth in Air	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-
Catalase	+	-	+	+	-	+	+	+	+	+	+	+	+	+	NT	d
Oxidase	+	-	-	-	-	+	+	+	-	+	-	-	+	+	d	-
Glucose (acid)	d	+	+	+	+	+	+	+	+	+	+	-	-	-	NT	d
O-F Test	0/-	F	F	F	F	F	F	0	0	0	0	-	-	-	NT	NT

(+) 100 - 80% strains positive; (d) 79 - 21% strains positive; (-) 20 - 0% strains positive; (F) fermentation; (0) oxidation; (NT) not testable; (S) sphere; (R) rod.

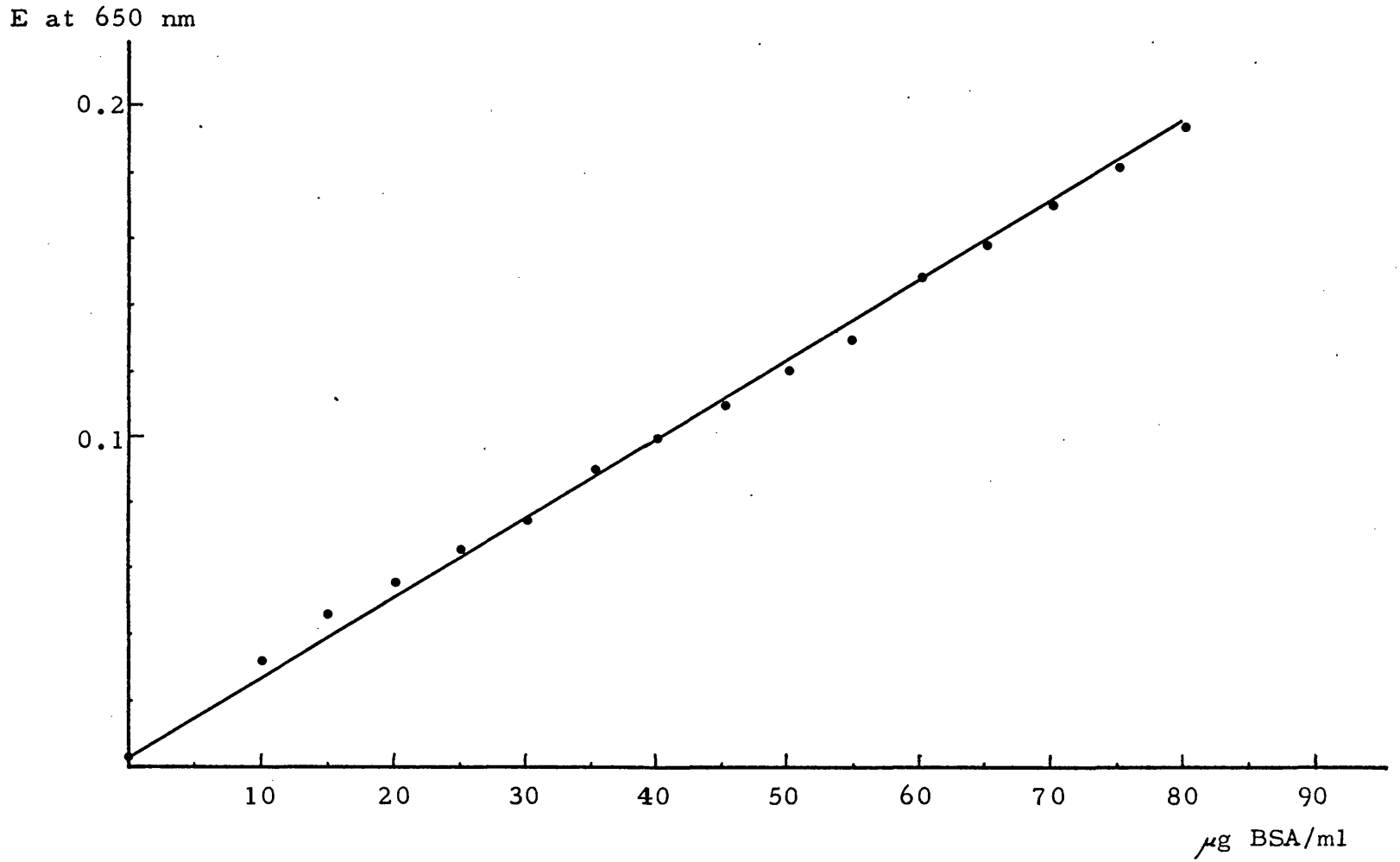
Appendix B. Composition of the Basal Salts Medium Prepared According to Skerman (1967).

Salts	Required Final Concentration μg/litre
NaCl	300,000
$(NH_4)_2SO_4$	660,000
$LiCl_2$	21
$CuSO_4.5H_2O$	80
$ZnSO_4.7H_2O$	106
H_3BO_4	600
$Al_2(SO_4)_3.18H_2O$	123
$NiCl_2.6H_2O$	110
$CoSO_4.7H_2O$	109
$TiCl_4$	60
KBr	30
KI	30
$MnCl_2.4H_2O$	629
$MgSO_4.7H_2O$	140,000
$SnCl_2.2H_2O$	36
$FeSO_4.7H_2O$	300

Appendix C. Serial Dilution of Protease Preparations and Zone Diameter in mm. for the Experimental Cultures.

D.F.	mg/ml (a)	Zone Diameter in mm.							
		S1	S2	S3	S4	S5	S6	S7	S8
1:1	1.23	19.0	19.0	24.0	18.5	24.5	15.0	18.5	24.5
1:2	0.82	16.5	16.5	22.0	17.0	22.0	13.0	17.0	22.5
1:4	0.49	14.0	14.0	19.0	16.0	20.5	10.0	15.0	21.0
1:8	0.27	10.5	11.0	16.0	13.0	19.0	8.0	13.0	18.5
1:16	0.14	7.5	9.5	13.5	10.0	16.5	7.5	10.0	16.0
1:32	0.07	6.0	7.0	11.0	7.5	13.5	6.0	8.5	13.0
1:64	0.04		6.0	9.0	6.0	11.0		6.0	10.0
1:128	0.02			7.0		7.5			8.0
1:256	0.01			6.0		6.0			6.0

(a) Although the average weight of 2.46mg/ml was taken as a common initial concentration only for illustration purpose, the actual weight for individual source varied within 4%.



Appendix D. Relationship Between Extinction (E) at 650 nm. and Concentration for Bovine Serum Albumin in Water Dilutions (E measured at 25° C).

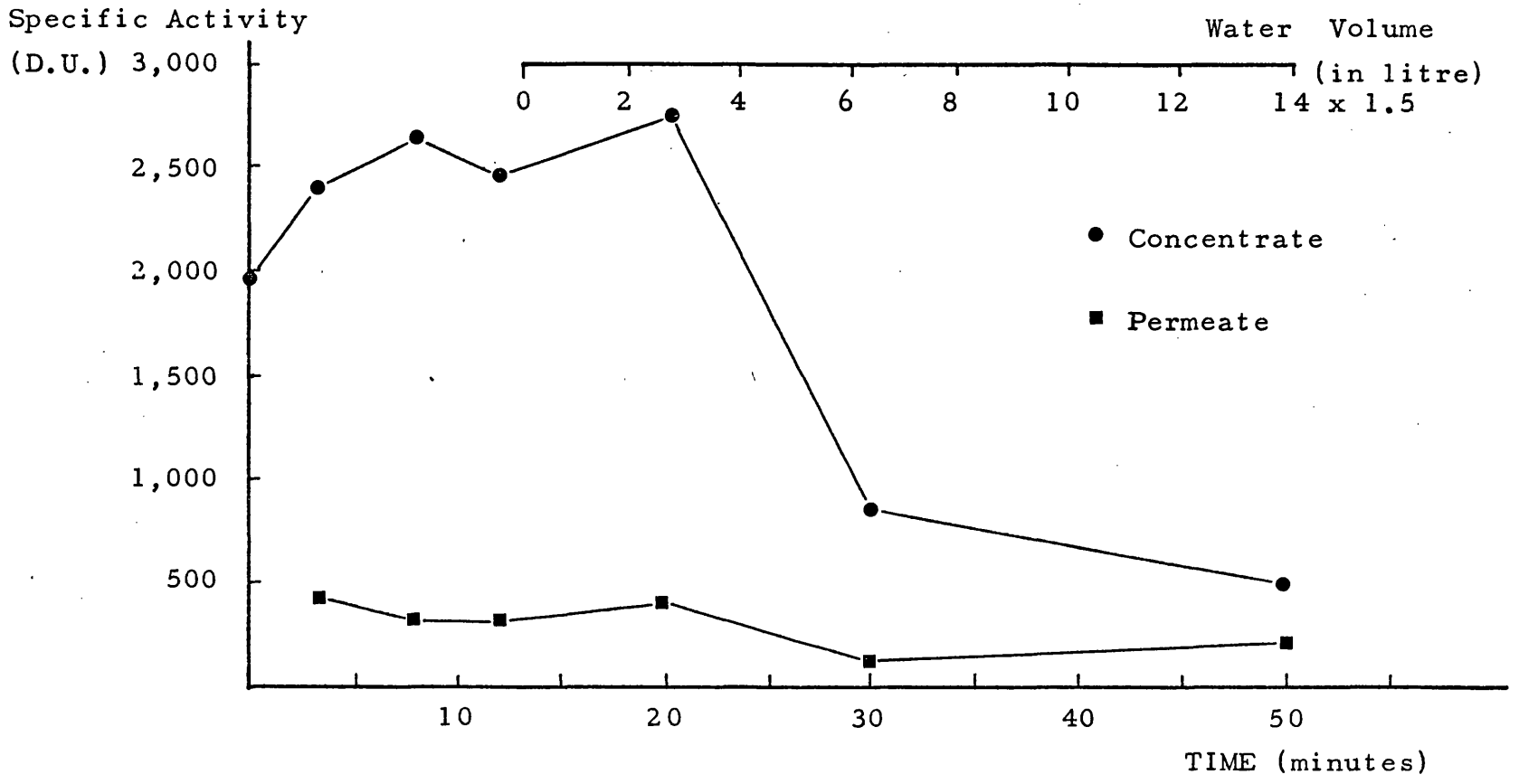
Appendix E. Summary of the Major Compositional Differences Between OTNE and OCH Media*

Components	Units	Typical Analytical Values	
		OTNE	OCH
General:			
Total N	%(w/w)	12.0	7.9
Ash	%(w/w)	14.0	40.0
Chlorides	%(w/w)	4.0	30.8
Amino N/Total N	%(w/w)	26.7	51.9
Amino Acids:			
His	%(w/w)	1.83	0.42
Trp	%(w/w)	1.11	-
Metals:			
Calcium	p.p.m.(w/w)	3,460	243
Magnesium	p.p.m.(w/w)	283	143
Zinc	p.p.m.(w/w)	49	25

*Summarized from the certificates of analysis provided by the manufacturers.

Appendix F. Changes of Activity Displayed by Protease Systems During Ultrafiltration Concentration Procedure.

Steps	D.U./mg. Protein							
	S1	S2	S3	S4	S5	S6	S7	S8
Cell Free Media	2,854	5,970	2,886	4,047	2,539	2,091	4,099	2,950
41 to 3 - Concentrate	228	1,422	1,106	181	1,001	77	286	772
- Permeate	1,737	1,690	84	291	192	84	64	44
Wash 1 - Concentrate	260	1,094	1,358	391	1,154	1,218	423	609
- Permeate	89	85	59	45	121	409	54	38
Wash 2 - Concentrate	452	844	785	876	1,087	4,798	2,750	900
- Permeate	22	24	94	11	194	2,128	530	81
Solution 1 mg./ml in Basal Salts Medium	6,376	15,528	68,000	9,074	38,603	8,350	12,098	38,363



Appendix G. Concentration of Pseudomonas fluorescens Protease System by Molecular Sieving Using a De Danake Sukkerfabrikker (D.D.S.) Plate-and-frame Module With a effective membrane area of 1.8 m^2 (D.D.S., Type 600 M.W. 20,000).

Appendix H. Estimated Values for the Thermodynamics of the Thermal Denaturation of the Protease System Produced by Strain S8.

°C	°K	Water as Dissolvent			BSM as Dissolvent		
		ΔG_w	ΔH_w	ΔS_w	ΔG_{bsm}	ΔH_{bsm}	ΔS_{bsm}
10	283	17,281	- 95,438	-398.3	58,320	-143,346	-712.6
20	293	13,298	-103,404		51,193	-157,599	
30	303	9,315	-111,370		44,067	-171,851	
40	313	5,532	-119,136		36,940	-186,104	
50	323	1,349	-127,302		29,814	-200,259	
53.39	326.39	- 1	-130,002		27,398	-205,187	
60	333	- 2,634	-135,268		22,687	-214,609	
70	343	- 6,617	-143,234		15,561	-228,861	
80	353	-10,600	-151,200		8,434	-243,114	
90	363	-14,583	-159,166		1,308	-257,366	
91.8	364.8	-15,300	-160,600		25	-259,931	
100	373	-18,566	-167,132		- 5,819	-271,619	

w = water; BSM or bsm = basal salts medium .

Appendix I. Analytical Procedures Applied Through out the Thesis

Chapters	Procedures

II. Protein $\mu g/ml = \frac{(E - 0.007)}{0.00233} \times DF$

$$D.U./ml = \frac{\text{antilog } (a + bx)}{0.025}$$

a=y intercept

b=slope of y=a+bx

III. Linear r:

$$r = \pm \frac{\sum XY - \frac{(\sum X)(\sum Y)}{N}}{\sqrt{\left[\sum X^2 - \frac{(\sum X)^2}{N}\right]\left[\sum Y^2 - \frac{(\sum Y)^2}{N}\right]}}$$

Multiple r^2

$$R = \frac{\sqrt{(r^2_{X_1Y} + r^2_{X_2Y} - 2r_{X_1Y} \cdot r_{X_2Y} \cdot r_{X_1X_2})}}{(1 - r^2_{X_1X_2})}$$

IV. Estimation of % Hydrolysis

$$\%H = \frac{\overline{\text{Skim Milk}} - \overline{\text{Enzyme Added}}}{\overline{\text{Standard for } \alpha \text{ or } \beta}} \times 100$$

Appendix I. (Continuation) Analytical Procedures Applied Through out the Thesis

Chapters	Procedures

V. (a) Changes in Gibbs Free Energy

$$\Delta G^{o} = \Delta H^{o} - T\Delta S^{o}$$

$$\Delta G = - RT \ln K$$

G = cal/mole

H = cal/mole

S = cal/deg/mole

R = 1.98 cal/deg/mole

K = Equilibrium Constant

T = ^{o}Kelvin

$$\Delta G^{o}_{w} = - RT \ln K_{w} = 645.7 \text{ cal/mole}$$

$$\Delta G^{o}_{bsm} = - RT \ln K_{bsm} = 117.4 \text{ cal/mole}$$

$$\Delta S^{o}_{w} = \frac{H^{o}_{w} - G^{o}_{w}}{T_{w}} = -398.3 \text{ cal/deg/mole}$$

$$\Delta S^{o}_{bsm} = \frac{H^{o}_{bsm} - G^{o}_{bsm}}{T_{bsm}} = - 712.6 \text{ cal/deg/mole}$$

Appendix I. (Continuation) Analytical Procedures Applied Through out the Thesis.

Chapters	Procedures

V. (b) Arrhenius Equation

$$\frac{d \ln K}{d T} = \frac{E'}{R T^2}$$

$$\ln \left(\frac{1}{10}\right) = - KD$$

$$D = \frac{0.1 - a}{b}$$

a=y intercept

b=slope of y=a+bx

$$K = \frac{- \ln 1/10}{D}$$

(c) D is defined as the time required to achieve 90% inactivation.

(d) Residual Activity (RA)

$$RA = \frac{\text{DU of the Heated Sample}}{\text{DU of the Unheated One}}$$

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