STUDIES ON THE REGULATION OF EXTRACELLULAR COLLAGENASE PRODUCTION BY *VIBRIO ALGINOLYTICUS*

by

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CERTIFICATION OF SUPERVISORS

In terms of paragraph eight of "General regulations for the degree of Ph.D." we, as supervisors of the candidate, G.C. Reid, certify that we approve of the incorporation in this thesis of material that has already been published or submitted for publication.

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ABSTRACT

Vibrio alginolyticus synthesized extracellular collagenase in a highly aerated peptone medium at the late-exponential and early-stationary phases of growth. Collagenase synthesis was subject to end-product repression and was repressed by various amino acids and ammonium ions. Glutamine caused severe repression of collagenase production. Collagenase synthesis was sensitive to catabolite repression by glucose and a number of carbon sources. Cyclic AMP, dibutyryl cyclic AMP and cyclic GMP did not relieve catabolite repression. Glucose and 2-deoxyglucose caused a severe transient repression. No intracellular preformed collagenase was detected and collagenase production ceased when induced cells were washed and resuspended in buffer. Trypsin and α-chymotrypsin had no effect on collagenase production by cells or sphaeroplasts.

The inducers of collagenase production in peptone were shown to have a broad molecular weight range between 1,000 and 60,000. The peptone inducers supported slow growth of *V. alginolyticus* when supplied as the sole nitrogen source in minimal medium. Digestion of the peptone inducers with purified *V. alginolyticus* collagenase resulted in a decrease in their inducing ability, whereas digestion with trypsin or α-chymotrypsin did not. Peptone acted as an inhibitor of collagenase. A minimal medium induction system was developed which involves resuspending cells at high density in a medium containing succinate, $(NH_4)_2SO_4$, KH_2PO_4 and the peptone inducers.

Collagenase production was shown to occur for 30 to 60 min in the presence of rifampin at levels which completely inhibit the incorporation of [^{3}H] uracil into trichloracetic acid-precipitable material. Chloramphenicol completely and immediately abolished collagenase production, which together with labelling studies has confirmed that collagenase production involves de novo synthesis of the enzyme. Rifampin-insensitive collagenase production was partially repressed by the addition of glucose or Casamino Acids, although synthesis of the enzyme continued for 30 to 60 min after their addition. The amount of repression was the same whether glucose or Casamino Acids were added separately or together with rifampin.

An in situ immunoassay for the detection of collagenase producing clones of V. alginolyticus was developed. By utilizing a double counter-selection mutagenesis technique, for the selection of auxotrophic mutants, a glutamate auxotroph was selected. No plasmid DNA could be detected in V. alginolyticus. Attempts to show plasmid transfer by conjugation of V. alginolyticus with E. coli J53 (RP4) and P. aeruginosa PA08 (R68.45) were unsuccessful. Attempts to show in vitro protein synthesis utilizing V. alginolyticus S30 extracts were unsuccessful.

CHAPTER I

INTRODUCTION

1.1 General

Microbial extracellular enzymes are important from both an applied and an academic point of view. The biochemical diversity of microorganisms makes them logical sources of a wide variety of exoenzymes for use in food and other biotechnological systems, whereas the unique process of synthesis and secretion of exoenzymes provides an interesting field of study.

Extracellular enzymes are generally regarded as those which exist free in the medium or those found in the periplasmic space. Glenn (1976) suggested that transport through the cytoplasmic membrane represents the primary secretion event. Thus the difference between gram-positive and gram-negative organisms has generally been found to be that with the former, the enzyme is found free in the medium, whereas with the latter the protein is confined to the periplasmic space. However, there are exceptions in both cases and in some instances the same enzyme may exist in either a cell bound form or free in the medium (Priest, 1977). The definition, therefore, rests more on the similarities between the modes of secretion than on the cellular locations of the enzymes, although for practical purposes the distinction between the two is of considerable importance.

Since many extracellular proteins are produced in large quantities and are relatively easy to purify, there have been many studies on the properties of exoproteins, particularly the proteases. The exoproteins produced by bacteria have molecular weights ranging from 12,000 to 50,000. Most of them are in the 20 to 40,000 range (Glenn, 1976).

Many exoenzymes are hydrolytic, acting on large insoluble substances such as starch, protein, cellulose, pectins or nucleic acids. Production of these enzymes, when inorganic or small organic nutrients are in short supply, suggests an obvious role for these enzymes in the life of the cell, that is, a means whereby organisms are able to utilize macromolecules in the environment (Glenn, 1976). However those exoenzymes that possess cellular substrates, e.g. proteases, cell wall lytic enzymes, nucleases, etc., have also been implicated in a number of physiological processes, including sporulation, cell wall turnover, growth and DNA-mediated transformation (Priest, 1977).

1.2 Mechanism of Secretion

The excretion of protein molecules (enzymes, cell wall components, hormones, toxins, etc.) that are too large or hydrophilic to pass readily through the lipid diffusion barrier of the plasma membrane is an important physiological feature of virtually all cells. The first major developments in studies on the mechanisms of secretion were made with the more complex eukaryotic cells rather than the simpler prokaryotes (reviewed by Palade, 1975 and Rothman and Lenard, 1977).

A molecular model for bacterial protein secretion has emerged that is derived from many different sources, with similarities to the molecular model for initial synthesis and secretion of eukaryotic secretory protein into the intracisternal space of the endoplasmic reticulum (Davis and Tai, 1980). This model incorporates the "signal sequence" hypothesis of Blobel and Sabatini (1971) and the models for transfer of protein across membranes proposed for eukaryotes by Blobel and Dobberstein (1975) and for prokaryotes by Both _et al_. (1972).

The signal sequence hypothesis proposes that mRNA's for secretory proteins possess a unique sequence of codons located immediately adjacent to the initiation codon. These signal codons are not present in the mRNA's coding for cytoplasmic proteins. Translation of the signal codons results in a unique amino acid sequence (15 to 30 residues) on the amino terminal of the polypeptide chain, the signal sequence. When the nascent polypeptide chain bearing the signal sequence emerges from the large ribosomal subunit it is recognised by mobile receptors in the membrane. This leads to the formation of membrane bound polyribosomes. The nascent protein would then begin to traverse the membrane. Soon after the amino terminal end (signal peptide sequence) has emerged from the membrane, a protease removes the signal peptide sequence from the amino terminal end. The nascent protein continues to be secreted and, following or during secretion of the protein, the protein folds and assumes its final three-dimensional conformation. This model implies that synthesis and secretion of a secretory protein is one process and that there is never any intracellular pool of completed protein accumulated for later secretion.

Lampen and his associates (Lampen, 1978) have undertaken an extensive study on the synthesis and secretion of penicillinase by *Bacillus licheniformis* 749/C (a constitutive mutant for the production of penicillinase) and have provided substantial evidence for the above model. *B. licheniformis* 749/C produces both an extracellular and a membrane-bound form of penicillinase. Lampen *et al*. have been able to demonstrate that the extracellular form of penicillinase (MW 29,000) is derived from the precursor membrane bound form of penicillinase (MW 33,000) by a specific proteolytic clevage (penicillinase releasing protease; PR protease) of a 25 amino acid peptide from the amino terminal end (Yamamoto and Lampen, 1976; Aiyappa *et al*., 1977; Aiyappa and Lampen, 1977). The membrane-bound form of penicillinase was found to have phosphatidic acid covalently bound to the serine of its amino terminal end. Since the other amino acids of the peptide are hydrophilic, it is suggested that the acyl fatty acids of the phosphatidylserine-penicillinase enable hydrophobic binding of the precursor form of penicillinase to the cytoplasmic membrane and presumably assists its secretion through the cytoplasmic membrane.

Recently Smith, Tai and Davis in collaboration with Lampen and associates (as quoted by Ramaley, 1979) obtained evidence for a larger molecular weight precursor of the *B. licheniformis* penicillinase. They examined the product of synthesis from membrane bound polysomes. In the absence of penicillinase-releasing protease they observed major protein peaks at 29,000 MW (free penicillinase), 33,000 MW (membrane-bound penicillinase) and ∿ 37,000 MW (precusor of penicillinase?). The 37,000 MW form was shown to be even more hydrophobic than the secreted product. In addition the 33 K product was separated into a highly hydrophobic and a hydrophilic fraction; only the hydrophobic 33 K fraction contained [^{3}H]-glycerol (presumably in phospholipid) when derived from cells carrying this label (Davis and Tai, 1980).

Evidence for the presence of a precursor form of Escherichia coli alkaline phosphatase comes from studies with the product of an alkaline phosphatase cell-free protein-synthesizing system (Inouye and Beckwith, 1977). The product of this system was precipitated by E. coli alkaline phosphatase antibody. However, the product had a slightly higher molecular weight and could be converted to the conventional molecular weight by incubation with E. coli membrane fraction. The presumed precursor can dimerize to form an active enzyme without being processed, and the resultant enzyme appears to be more hydrophobic than the mature enzyme. Further examples of where precursors have been found for bacterial exoenzymes are the outer membrane lipoprotein (Inouye et al., 1977) and the periplasmic leucine-specific binding protein (Oxender et al., 1980) of E. coli. However, not all secreted proteins have precursors e.g. colicin E1 and E3 (Jakes and Model, 1979) and evidence suggests that chicken ovalbumin contains an internal signal sequence (Lingappa et al., 1979).

Convincing evidence for cotranslational secretion of exoenzymes was provided by Smith et al. (1977) with sphaeroplasts of E. coli. They used [^{35}S] acetylmethionyl methylphosphate sulfone (AMMP) which does not penetrate the cytoplasmic membrane but does react with the emerging polypeptide of secretory protein by reacting with free amino groups of protein and phospholipid. When E. coli sphaeroplasts, treated with [^{35}S] AMMP, were disrupted and fractionated, 6% of the radio-active label was found in the polysome fraction. More recently, the use of [^{125}I] diazoiodosulphanilic acid, a non-penetrating reagent that labels only tyrosine and histidine residues and not phospholipid, has increased about four fold the proportion of cellular label that is recovered on polysomes (Smith et al., 1979). The label on the polysomes (from either reagent) is evidently attached via peptidyl- tRNA : it was released by low Mg^{2+}; subsequent cleavage by dilute alkali decreased the average molecular weight by about that of tRNA; and the label was also released by puromycin, or by chain completion in vitro.

The demonstration of cotranslational secretion has provided a role for membrane bound ribosomes in bacteria. In thin sections of bacterial cells, unlike eukaryotic cells, it has not been possible to distinguish membrane-bound and free polysomes by electron microscopy, because the population of ribosomes is too dense (Davis and Tai, 1980). A functional attachment was previously suggested by Cancedda and Schlesinger (1974) who found that the membrane-bound polysome fraction of E. coli produced a somewhat greater abundance of the secreted protein, alkaline phosphatase, than the free fraction. In addition, the synthesis of extracellular enzymes by bacteria has been shown to be more sensitive to inhibitors of protein synthesis than has general cell protein synthesis and this has been interpreted as evidence for a membrane site for extracellular enzymes (Glew and Heath, 1971; Both *et al.*, 1972; Stinson and Merrick, 1974).

Evidence that exoenzymes are secreted in an unfolded form was provided by Saunders and May (1975) and Bettinger and Lampen (1975), who showed that during secretion, exoenzymes of *Bacillus amyloliquefaciens* and *B. licheniformis* were sensitive to proteolytic degradation whereas the native enzymes were totally resistant. This work is discussed more fully in Chapter II.

Cotranslational secretion, although widespread is not the only mechanism for protein export and in prokaryotes cytoplasmic synthesis, in precursor form has been demonstrated for subunit A of cholera toxin (which is later combined with subunit B and secreted) (Nichols *et al.*, 1979 as quoted by Davis and Tai, 1980).

In eukaryotes the small subunit of a chloroplast enzyme, ribulose bisphosphate carboxylase, is synthesized as a precursor of higher molecular weight and is taken up into intact isolated chloroplasts and cleaved to its final size in the absence of protein synthesis (Highfield and Ellis, 1978). Precursors for the three largest mitochondrial F_1-ATPase subunits of yeast have also been shown to be synthesized in a precursor form before transport across both mitochondrial membranes and conversion to the "mature" subunits (Maccecchini et al., 1979).

The genetic manipulation of bacteria, to yield mutants or recombinants altered in various functions, provides a powerful tool for the study of the secretory process. Beckwith, Silhavy and associates (Silhavy et al., 1977), using the gene fusion system of E. coli, were able to replace a portion of the amino-terminal sequence of the cytoplasmic protein β-galactosidase with various amounts of the amino-terminus of the lam B protein. The lam B protein is located in the outer membrane and is involved in the transport of maltose and maltodextrins, and also serves as a receptor for several bacteriophages, including λ. In a strain where the hybrid protein contained about 200 amino acids of the lam B protein, the major amount of the β-galactosidase activity was present in the outer membrane. However, in another lam B -β-galactosidase strain containing only a small portion of the lam B protein, all the β-galactosidase activity remained in the cytoplasm (Bassford and Beckwith, 1979). Recently Moreno et al. (1980) have constructed a hybrid strain which produces a β-galactosidase which remains cytoplasmic even though it possesses the complete signal sequence of lam B protein precursor at the amino-terminal end. They propose that the signal sequence alone might not have sufficient information to initiate the transfer of a polypeptide through the cytoplasmic membrane or that the signal sequence does suffice to initiate the transfer but that severe restrictions exist as to which amino acid sequences can be extruded through a membrane.

Combining various observations, Ramaley (1979) proposes that the gene for some secretory proteins may have the following functional sequence areas:

H_2N————|————|————|————COOH

SIGNAL SEQUENCE	HYDROPHOBIC AREA	LOCATION AREA	CATALYTIC AND STRUCTURAL SEQUENCE AREA
Orientation on membrane pore	needed to cross membrane	Binding to cellular site, periplasmic outer membrane	

The study of signal sequences for secretion has recently found practical applications. For example, to increase the yield of insulin in recombinant bacteria the gene for insulin has been fused with the beginning of the gene for E. coli periplasmic β-lactamase; the resultant hybrid protein is secreted and it can then be recovered by osmotic shock and cleaved to yield the hormone (Villa-Komaroff et al., 1978). In addition, the bacterial mechanism of secretion can evidently recognise a mammalian signal, since bacteria containing the ovalbumin gene secrete its product (Fraser and Bruce, 1978; Mercereau-Puijalon et al., 1978).

These gene fusion studies indicate that it should be possible to genetically create a secretory form of practically any enzyme that is desired. This might be especially valuable in purification of desired intracellular enzymes or some cloned DNA gene products where the gene product could be placed into the cell's periplasmic space or even the extracellular medium. This would greatly facilitate its purification since the initial material would not contain the numerous cytoplasmic enzymes found in the usual cell-free extract of disrupted cells.

1.3 Collagen and Collagenases

Unless otherwise specified the information in this section is taken from the works of Seifter and Harper (1971), Mandl (1972) and Keil (1979).

The best sources of collagenases are bacterial species (e.g. Clostridium, Bacteroides, Pseudomonas, Vibrio, Streptomyces etc.) but they also occur in fungi as well as in human and animal tissues. True collagenases are enzymes capable of solubilizing insoluble collagen by peptide bond cleavage under physiological (non-denaturing) conditions of pH, temperature and salt concentration. This definition eliminates many proteases which cleave readily denatured collagen or which cleave collagen chains in their non-helical peripheral parts. Vertebrate collagenases cleave usually only one or two out of more than a thousand peptide bonds in the collagen chains, whereas the bacterial collagenases are described as proteases of very low specificity effecting simultaneous or stepwise degradation of hundreds of peptide bonds in the same substrate. It has been shown that collagenases also possess a limited ability for the digestion of proteins other than collagen, at sites of common character.

The main function of collagenases produced by prokaryotes and lower eukaryotes is to help the invasion in the connective tissues of the host by rapid degradation of native collagen. The vertebrate collagenases play an essential role in the regulatory mechanisms of growth, tissue remodelling, resorption and wound healing.

Collagen comprises 33% of the total protein in mammalian organisms and is the main constituent of skin, tendon and cartilage, as well as the organic component of teeth and bone. Amino acid sequence analysis has indicated great similarity between collagens from different tissues within a species (e.g. skin and tendon) and from different classes within a phylum (e.g. avian and mammalian), although phyletic differences are greater. The collagen molecule forms a triple helix of 3 peptide chains (designated α-chains), each of which contains approximately 1,000 amino acids. The molecular weight of the collagen molecule is about 300,000. Each α-chain is arranged in the form of a left-hand (i.e. clockwise) helix and these three minor helices are arranged in a right-hand spiral about a common axis, thus forming a major or super helix which is stabilized by interchain hydrogen bonds and hydrophobic interactions.

The arrangement of the triple helix is such that the α-carbon atom of every third residue lies on the inside of the major helix and only glycine, with no side chain on its α-carbon atom, can sterically fit into this position. Thus glycine accounts for approximately one third of all the amino acids of the collagen molecule. Next to glycine, the most commonly occurring residue is proline, followed by hydroxyproline (whose occurrence is unique to collagen) and alanine. The relative proportions of these amino acids in bovine collagen is: 363 (glycine):131 (proline): 107 (hydroxyproline):106 (alanine):362 (other amino acids) (Fraenkel-Conrat, 1963).

In the laboratory, collagenases are used in investigations of the biosynthesis of collagen and for structural and immunochemical studies of collagens and collagen-like proteins. They are also proving their worth as agents for facilitating tissue transplantation and for cell-dispersion in tissue cultures. Established clinical applications of collagenases include the treatment of burns and dermal lesions; in addition they are being evaluated as agents for the removal of undesirable tissues such as herniated intervertebral discs and the sloughs resulting from cryogenic or cauterizing procedures. Moreover, as human collagenases are implicated in various pathological disorders involving connective tissue degradation, the roles played by these collagenases are being investigated in the hope of finding ways to arrest, control or treat the diseases.

1.4 Characterisation of the *Vibrio alginolyticus* collagenase

Welton and Woods (1973, 1975) described the isolation from cured hides of an aerobic, halotolerant, collagenolytic, gram-negative bacterium which was originally classified as an *Achromobacter iophagus* strain. This strain has subsequently been reclassified as a *Vibrio alginolyticus* strain (NCIB 11038) by M. Hendrie of the National Collection of Industrial Bacteria, Aberdeen, Scotland.

This strain is of interest because it produces an inducible extracellular collagenase (EC 3.4.24.3) with the highest specific activity for a collagenase (Lecroisey *et al.*, 1975). The collagenase is induced by peptone, gelatin and collagen or its high-molecular-weight fragments and is synthesized as the culture enters stationary phase (Welton, 1974; Keil-Dlouha et al., 1976). Keil-Dlouha et al. (1976) showed that the presence of collagenase-digestible peptide bonds in the macromolecular inducer fragments from collagen were essential for induction and suggested that the tertiary conformation of the α-helix plays an important role in the collagenase induction process. In addition an extracellular neutral protease was shown to be induced by β-casein but this enzyme has subsequently been shown to be produced constitutively at low levels (Long et al., 1980. Submitted).

Depending on the nature of the growth medium, i.e. peptic hydrolysate of denatured collagen or gelatin, two homogenous forms of V. alginolyticus collagenase, with molecular weights of 70,000 and 80,000 respectively, have been found (Keil-Dlouha and Keil, 1978). The more highly active form of collagenase (specific activity 2 μkat mg^{-1}) has a molecular weight of 70,000 and is composed of two identical subunits of molecular weight 35,000 (Keil-Dlouha and Keil, 1978). Each subunit consists of a single polypeptide chain and the dissociation of the dimer results in the complete and irreversible loss of enzyme activity. The second form of collagenase (specific activity 1.64 μkat mg^{-1}) has a molecular weight of 80,000. It is also a dimer in which each of the two subunits of molecular weight 35,000 binds non-covalently a peptide of molecular weight 5,000. The dissociation of this form is also accompanied by irreversible loss of enzymic activity. The amino acid composition of the subunits which were isolated from both 70,000 MW and 80,000 MW collagenases is the same.

Keil-Dlouha and Keil (1978) point out that the two types of bonding, subunit-peptide and dimer, are very tight and suggest that the ability of collagenase to bind certain peptides could explain the difference in molecular weight found by them and the value of 106,000 to 111,000 for the same enzyme isolated from a peptone medium by Welton and Woods (1975).

The amino acid composition of V. alginolyticus collagenase differs from that of Clostridium histolyticum mainly in the sulphur containing amino acids (Keil-Dlouha, 1976). It has a calcium requirement for activity and binds one mole of zinc per mole of enzyme. The purified enzyme is inhibited by EDTA, cysteine and histidine, whereas p-chloromercuribenzoate and diisopropyl-flurophosphate are without effect. It has thus been suggested that the V. alginolyticus collagenase belongs to the group of hydrolytic enzymes containing metal in their active site (Lecroisey et al., 1975).

V. alginolyticus collagenase cleaves the x-gly bond in the sequence pro-x-gly-y where y is generally proline or alanine and x is a neutral amino acid. The collagenase splits this kind of bond in the helical regions of native collagen as well as in a number of synthetic peptide substrates (Keil et al., 1975). Highly purified Vibrio collagenase contains traces of caseinolytic activity; however this activity has been shown to correspond to the specificity of the collagenase. In β-casein, which is rich in proline, four bonds are cleaved by Vibrio collagenase, three of them involving the amino group of glycine, one of alanine; in each case the next residue is proline.

The bonds x-gly cleaved thus correspond well to the specificity of Vibrio collagenase on collagen and on synthetic substrates (Gilles and Keil, 1976). During the autodegradation of collagenase, the bonds cleaved correspond to the specificity of collagenase and result in the formation of at least three active fractions (Keil-Dlouha, 1976).

Although there are many reports of true exoprotein production by gram-positive bacteria (Priest, 1977), there are few reports on the production of exoproteins by gram-negative bacteria (Glenn, 1976), in contrast to numerous reports on periplasmic space proteins. Thus the purpose of this thesis was to study the regulation of collagenase production by Vibrio alginolyticus.

CHAPTER II

REGULATION OF COLLAGENASE PRODUCTION

Summary: *V. alginolyticus* synthesized extracellular collagenase in a highly aerated peptone medium at the late-exponential and early-stationary phases of growth. Collagenase synthesis was subject to end-product repression and was repressed by various amino acids and ammonium ions. Glutamine caused severe repression of collagenase production. Collagenase synthesis was sensitive to catabolite repression by glucose and a number of carbon sources. Cyclic AMP, dibutyryl cyclic AMP and cyclic GMP did not relieve catabolite repression. Glucose and 2-deoxy-D-glucose caused a severe transient repression. No intracellular preformed collagenase was detected and collagenase production ceased when induced cells were washed and resuspended in buffer. Trypsin and α-chymotrypsin had no effect on collagenase production by cells or sphaeroplasts.

2.1 INTRODUCTION

Although extracellular enzymes of microbial origin have been studied extensively with respect to their isolation and characterisation, the regulation of these enzymes has received less consideration (Glenn, 1976). The study of the regulation of extracellular enzymes also has some economic importance since according to Wodzinski (1979); "many extracellular enzymes would become articles of commerce if they could be produced in high enough concentrations to make them economically feasible". For the production of an exoenzyme to be economically feasible the synthesis of the enzyme as well as the process catalysed by the enzyme must be optimized.

Synthesis of an enzyme may be optimized by varying the cultural conditions (e.g. temperature, pH, carbon source, nitrogen source, aeration, agitation and induction of inducible enzymes) and genetic approaches which remove restrictive control mechanisms may also be utilized.

Although there are conflicting reports, two definite patterns of exoenzyme synthesis during the growth cycle are apparent: (i) a very low rate of secretion during active growth followed by increased synthesis in the late exponential and early stationary phases of growth (idiophasic) and (ii) an increase in the synthesis and secretion of the exoenzyme accompanying growth which decreases as the culture enters stationary phase (trophophasic)(Priest, 1977.). Some extracellular enzymes e.g. penicillinase, levansucrase, cellulase and collagenase are synthesized in response to a specific inducer molecule and in the absence of this inducer the basal activity is barely detectable.

In the case of enzymes which are produced in direct association with microbial growth greater amounts of these enzymes should be produced through application of continuous culture techniques which maximize the growth rate. However, industrial enzymes are rarely produced in continuous culture, probably due to the instability of the strains and to higher costs of enzyme recovery and of instrumentation (Eveleigh and Montenecourt, 1979). The differences in the physiological state of bacteria growing in batch and continuous culture can be partially resolved by the use of multistage continuous systems. The multistage chemostat consists of a growth vessel into which the medium enters and from which the culture is fed into a second vessel. The transferred culture is, to a certain extent, "shifted down", and a part of the growth curve after the exponential phase is adopted (Priest, 1977).

Many factors have been shown to affect exoenzyme production and although there are clearly marked differences among organisms, it has been found that the synthesis of many exoproteins is influenced by the levels of individual nutrients in the extracellular environment and they may also require induction. Specifically, many exoenzymes are subject to control by end product repression and catabolite and/or transient repression.

End product repression. A widely reported example of end product repression is the repression of exoprotease synthesis by amino acids; this has been reported in a number of bacterial genera including Bacillus spp. (Chaloupka and Křečková, 1966; May and Elliott, 1968); Arthrobacter (Hofsten and Tjeder, 1965) and Sarcina sp. (Glenn, 1976). In some cases the particular repressive amino acids have been identified; isoleucine and proline appear to be among the most common (Glenn, 1976).

The site affected by amino acids is still unknown. It has been suggested that amino acids bring about exoprotease repression only when entering the cell;

probably during transport through the cytoplasmic membrane, since in Bacillus megaterium the intracellular level of amino acids remained relatively constant while the extracellular level was increased (Chaloupka et al., 1963). Glenn et al. (1973) provide evidence that amino acid repression of exoprotease in Bacillus amyloliquefaciens occurs at the level of transcription.

It has yet to be shown whether or not amino acid repression of exoenzymes is mediated through an increase in intracellular ammonia. Intracellular urease synthesis is strongly repressed by ammonia or NH_4^+ ions in various organisms (Vogels and van der Drift, 1976), and in Klebsiella aerogenes the repression of urease by ammonia is caused by the repression of glutamine synthetase by ammonia (Friedrich and Magasanik, 1977). The mechanism of regulation of the "hut" operon in K. aerogenes by glutamine synthetase, mediated via the intracellular levels of ammonia is well established (Magasanik, 1977). It has also been shown that glutamine synthetase activates the formation of a periplasmic L-asparaginase in K. aerogenes (Resnick and Magasanik, 1976). It is possible that similar mechanisms of control involving glutamine synthetase might apply in the regulation of exoenzymes which are repressed by ammonia.

The influence of inorganic phosphate levels on the synthesis of extracellular alkaline phosphatase and ribonuclease in several bacterial species is another example of end product repression (Glenn, 1976). However in all these examples the mechanism of end product repression remains obscure.

Catabolite Repression. In addition to end-product repression, some exoenzymes are subject to catabolite repression and/or transient repression by various carbon sources. For example in Bacillus subtilis the exoenzyme α-amylase is repressed by glucose, NH_4^+ and amino acids (Heineken and O'Connor, 1972). In the marine bacterium Aeromonas proteolytica the exoenzyme endopeptidase is repressed by glucose, glycerol, sucrose, acetate and individual amino acids (Litchfield and Prescott, 1970). In Pseudomonas maltophilia exoprotease secretion is repressed by pyruvate, L-malate, succinate and α-ketoglutarate, which are preferred substrates for growth of this organism (Boethling, 1975). In Vibrio parahaemolyticus an extracellular "gelatinase" is repressed by amongst others, galactose, fructose, mannose, mannitol, maltose and L-arabinose (Tanaka and Iuchi, 1971).

Catabolite and transient repression have been studied most extensively in E. coli and it has been concluded that they are distinct from one another (Tyler et al., 1967). In E. coli the addition of glucose to induced, glycerol grown cells, represses their ability to synthesize β-galactosidase. The first 10-30 minutes of repression is complete (transient repression) after which there is partial recovery (catabolite repression) (Wayne and Rosen, 1974). Non-metabolizable glucose analogues such as 2-deoxy-D-glucose or α-methylglucoside which are phosphorylated but not catabolized by the cell, cause transient repression, whereas these substances do not elicit catabolite repression (Tyler et al., 1967).

Cyclic adenosine 3',5' -monophosphate (cyclic AMP) overcomes both catabolite and transient repression by glucose of the synthesis of many inducible enzymes in E. coli and in low concentrations stimulates enzyme synthesis. It has been proposed that, since glucose lowers the intracellular concentration of cyclic AMP, the intracellular level of cyclic AMP regulates the rate of synthesis of many inducible enzymes in E. coli and other microorganisms (De Crombrugghe et al., 1969).

Cyclic AMP exerts its influence through a protein, the catabolite gene activator protein (CAP) or cyclic AMP receptor protein (CRP). The cyclic AMP - CAP complex facilitates binding of RNA polymerase to the promoter regions of catabolite-sensitive operons (Rickenberg, 1974). However, in studies on the regulation of catabolite repression of β-galactosidase in E. coli, mutants deleted for the adenyl cyclase gene (cya^-), which are unable to synthesize cyclic AMP, it has been found that, provided a second mutation occurs either in the CAP gene or in the lactose region, these mutants exhibit catabolite repression (Dessein et al., 1978a). If the catabolite repression seen in the mutant strains corresponds to the mechanism operating in wild-type cells, the results would suggest that the intracellular concentration of cyclic AMP cannot be the unique regulator of catabolite repression. In this context Dessein et al. (1978b) have postulated that a catabolite modulator factor (CMF) specifically inhibits the expression of operons sensitive to catabolite repression.

Priest (1977) has reviewed the role of cyclic AMP in the genus Bacillus and concludes that it is not the universal regulator molecule it was once thought to be as it cannot be detected in several bacilli. Cyclic guanosine 3', 5' -monophosphate (cyclic GMP) has been detected in B. licheniformis and it is possible that this cyclic nucleotide may play a role similar to cyclic AMP in Bacillus spp. (Bernlohr et al., 1974). In E. coli however cyclic GMP has been strongly implicated as a regulator of chemotaxis (Black et al., 1980).

Since peptone is used for the industrial production of collagenase by V. alginolyticus, the regulation of collagenase in a peptone medium was investigated.

2.2 MATERIALS AND METHODS

2.2.1 Maintenance of V. alginolyticus.

Media are listed in Appendix A.

For the long term maintenance of V. alginolyticus, the strain was kept in the Tris-HCl buffer (0.4M NaCl) containing approx. 5 mg ml^{-1} of bovine Achilles' tendon collagen. For routine use the culture was maintained on the complex medium of Welton and Woods (1973).

2.2.2 Growth conditions and collagenase production.

Aeration was shown to be critical for collagenase production and to facilitate better aeration of the culture flasks (Ehrlenmeyer) loosely fitting aluminium foil caps were used instead of cotton wool "bungs". The culture volumes were 5 to 10% of the flask volume.

At time 0 h a 10 ml sample of an overnight aerated

V. alginolyticus culture in the Casamino Acids medium was inoculated into 100 ml of 2.5% peptone medium and incubated in a Gallenkamp orbital incubator at 140 rev. min^{-1} at $30^{o}C$. To ensure parallel cultures, incubation was continued for 2.5 h before the 100 ml culture was divided into 10 ml aliquots. Growth was followed turbidimetrically at 600 nm on a Bausch and Lomb "Spectronic 20" spectrophotometer.

Supernatant fractions obtained by centrifugation of the cultures were assayed for collagenase at different time intervals. However, when collagenase activity was assayed at 15 min time intervals cultures were assayed without centrifugation as this did not affect the accuracy of the assay.

2.2.3 Collagenase assay.

Collagenase activity was assayed using the synthetic substrate phenylazobenzyloxycarbonyl-L-prolyl-L-leucyl-glycyl-L-prolyl-D-arginine (PZ-Pro-Leu-Gly-Pro-Arg) (Fluka, Buchs, Switzerland). The method used was adapted from that of Wünsch and Heidrich (1963) and was as follows: 100 mg of PZ-Pro-Leu-Gly-Pro-Arg was dispersed in 1 ml of methanol and made up to 100 ml with the Tris-HCl buffer. Culture supernatants (0.25 ml) and 1.0 ml of the substrate solution, prewarmed for 10 min at $37^{o}C$, were mixed in a water bath at $37^{o}C$. After exactly 15 min, 0.5 ml of the reaction mixture was removed and vigorously mixed on a Fisons Whirlimix with 1 ml of 0.5% citric acid plus 5 ml of ethyl acetate for 15 sec. When the inorganic phase had settled, 4 ml of the overlying organic phase was withdrawn, briefly dried with approx. 0.3 g anhydrous sodium sulphate, and the absorbance read at 320 nm.

This method was adapted slightly to conserve the expensive substrate: 200 μl of the substrate solution was added to 50 μl of culture at 37°C and incubated for 5 min. The reaction was stopped by the addition of 0.5 ml of 0.5% citric acid. The total reaction volume was then extracted with 2.5 ml of ethyl acetate as above. This method permitted a large number of samples to be assayed at close intervals and allowed the use of the same calibration curve. Each sample was assayed in duplicate, and experiments were repeated at least twice. The standard error for the collagenase assays was less than 10% but usually lower (<5%).

By reference to a calibration curve the absorbance at 320 nm was converted to units of enzyme activity. The unit of enzymic activity currently recommended by the Enzyme Commission is the katal (symbol kat), where one katal is the amount of activity that converts one mole of substrate per second (Florkin and Stotz, 1973). Enzyme activity was expressed in nkat ml^{-1} absorbance unit at 600 nm^{-1} [nkat $ml^{-1}(A_{600}$ unit$)^{-1}$] or as nkat ml^{-1} in experiments where inhibitors were added. In order that the substrate did not become limiting, enzyme samples were diluted in the Tris-HCl buffer so that the resulting absorbance at 320 nm remained below 0.5.

2.2.4 <u>Repression of collagenase synthesis</u>.

The effects of the addition of carbon sources, amino acids, ammonium ions, methionine sulfone and cyclic nucleotides on collagenase production were investigated.

Unless otherwise specified carbon sources were added at 0.2% and amino acids at 0.5%. Additions were made to peptone-grown cultures 3.5 h after inoculation and collagenase activity was assayed at 6 and 8 h after inoculation.

2.2.5 Ultrasonic disintegration and toluene treatment of cells.

In order to determine whether collagenase was preformed within the cell, 4 ml samples of culture were disintegrated with a MSE ultrasonic disintegrator (5 min at 21 K Hz) and the collagenase activity of the disintegrated cells was compared with the activity of untreated control samples. Cells were also treated with toluene to determine whether they would release preformed collagenase or become permeable to the substrate and indicate the presence of preformed collagenase : 0.2 ml toluene was mixed with 0.5 ml samples of culture and supernatant and the collagenase activity was determined in the supernatant and in uncentrifuged samples.

2.2.6 The effect of Trypsin and α-chymotrypsin on collagenase production by *V. alginolyticus* cells and sphaeroplasts.

V. alginolyticus sphaeroplasts were made by the addition of penicillin G to a 1% peptone medium containing 0.6 M NaCl and 2g l^{-1} $MgSO_4.7H_2O$ (sphaeroplasting medium). Penicillin was added as follows: 1000 units ml^{-1} at time 0 h and 500 units ml^{-1} at times 3 h and 5 h. Trypsin and α-chymotrypsin were added at concentrations of 100 μg ml^{-1} at 3.5 h. The effect of trypsin and α-chymotrypsin on the activity of purified collagenase was determined by incubating purified collagenase (9.5 nkat ml^{-1}) with trypsin and α-chymotrypsin at concentrations of up to 1 mg ml^{-1} for 21 h while assaying for collagenase activity.

Trypsin and α-chymotrypsin were tested for protease activity in 0.4 and 0.6 M NaCl using an azocasein assay: azocasein (1 ml, 2%) in 0.1M Tris HCl buffer pH 7.6 made 0.4 or 0.6 M with NaCl was incubated with 1 ml of enzyme solution (made 0.4 or 0.6 M with NaCl) for 30 min at 37°C. The reaction was stopped by the addition of 2 ml 10% trichloracetic acid and cooled on ice for 30 min before filtering. NaOH (1.5 ml, 0.5M) was added to 1.5 ml of the filtrate and the absorbance read at 440 nm.

2.3 RESULTS

2.3.1 Kinetics of collagenase production.

Collagenase was produced in the 2.5% peptone medium when the culture reached the late-exponential and early-stationary phases of growth (6 to 12 h) and maximum yields were obtained after 10 to 12 h (Fig. 2.1). Production of collagenase was affected by aeration and optimum yields were only obtained under highly aerated conditions (Fig. 2.2).

2.3.2 Glucose repression of collagenase synthesis.

Addition of 0.4% glucose to the peptone culture before 6 h, the time at which collagenase production normally starts, completely inhibited the synthesis of collagenase (Fig. 2.1). The glucose analogue 2-deoxy-D-glucose at the same concentration (0.4%) had a transient inhibitory effect on the production of collagenase when added at 3.5 h (Fig. 2.1). Addition of 2-deoxy-D-glucose at 2 or 3 h.,

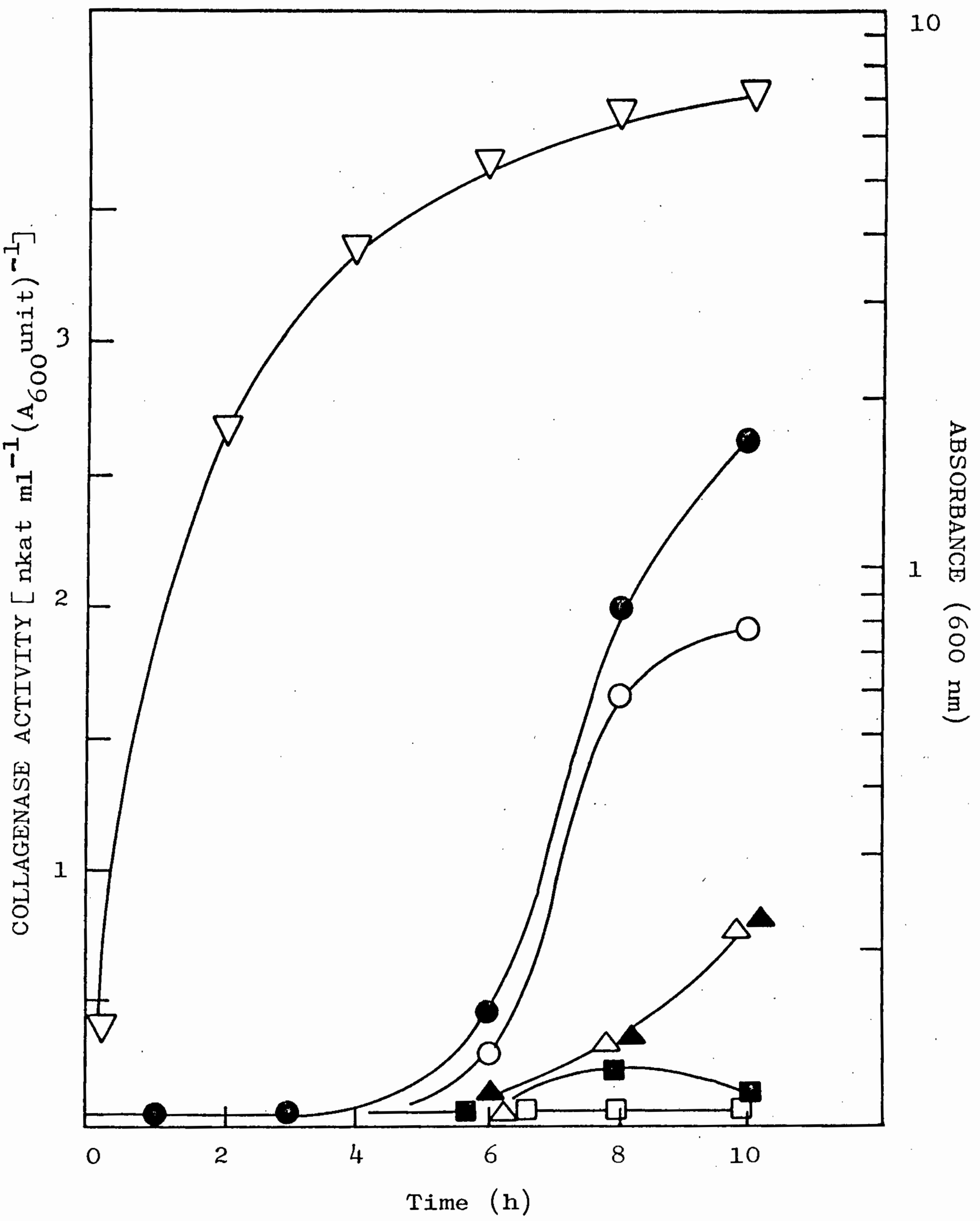

Fig. 2.1: Effect of glucose and 2-deoxy-D-glucose on collagenase production in 2.5% peptone medium. No additions (●); 0.4% glucose added at 2 h (□); 4 h (■) and 6 h (○); 0.2% glucose added at 3.5 h (▲); 0.4% 2-deoxy-D-glucose added at 3.5 h (△). Absorbance of control culture (▽).

at concentrations of 0.2% and 0.4%, also resulted in a transient inhibitory effect, with 0.4% 2-deoxy-D-glucose causing more inhibition than 0.2% (results not shown). This transient effect was also observed with 0.2% glucose (Fig. 2.1) and 0.05% glucose had no effect on collagenase production. The addition of 0.4% glucose at 6 h caused only a slight reduction in collagenase production (Fig. 2.1).* Various other carbon sources repressed the synthesis of collagenase (Table 2.1). Lactose, which the bacterium is unable to utilize (Welton and Woods, 1973), caused little repression after 6 h and no repression after 8 h.

2.3.3 Effect of cyclic nucleotides.

Repression of collagenase synthesis by glucose (0.2%) was not relieved by 5 mM cyclic AMP, 5 mM dibutyryl cyclic AMP or 5 mM cyclic GMP (Fig. 2.3). The addition of 5 mM cyclic AMP actually inhibited collagenase production whereas 1 mM cyclic AMP or 2 mM cyclic GMP had no effect (Fig. 2.3). Increasing the concentrations of cyclic AMP or dibutyryl cyclic AMP to 10 mM did not relieve glucose repression of collagenase (results not shown).

2.3.4 Effect of amino acids.

Casamino Acids (0.5 and 1%) and various individual amino acids (0.5%) inhibited collagenase production (Table 2.1). Glutamine caused severe repression of collagenase synthesis but other amino acids inhibited collagenase production to a lesser extent. Ammonium ions (50 or 100 mM) also repressed collagenase synthesis.

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* This effect is discussed more fully in the discussion of Chapter IV.

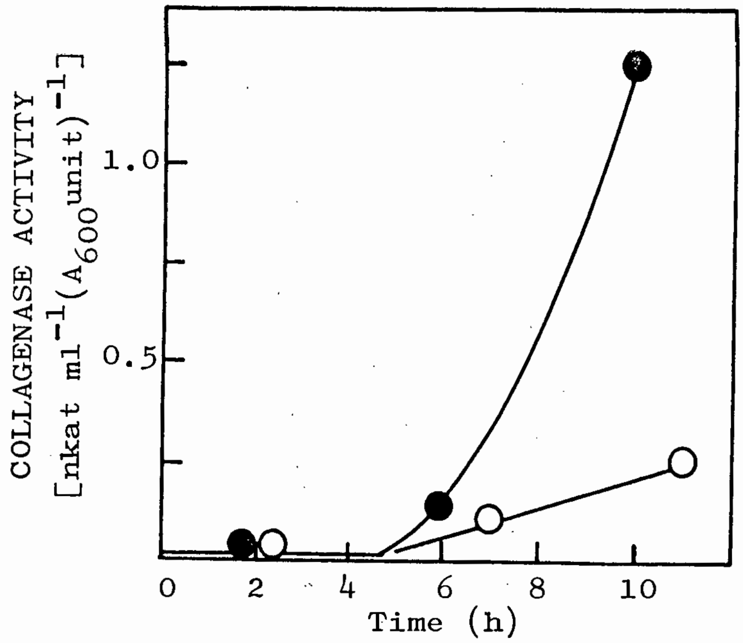

Fig. 2.2: Effect of reciprocal and orbital shaking on collagenase production. Reciprocal shaking (○); orbital shaking (●).

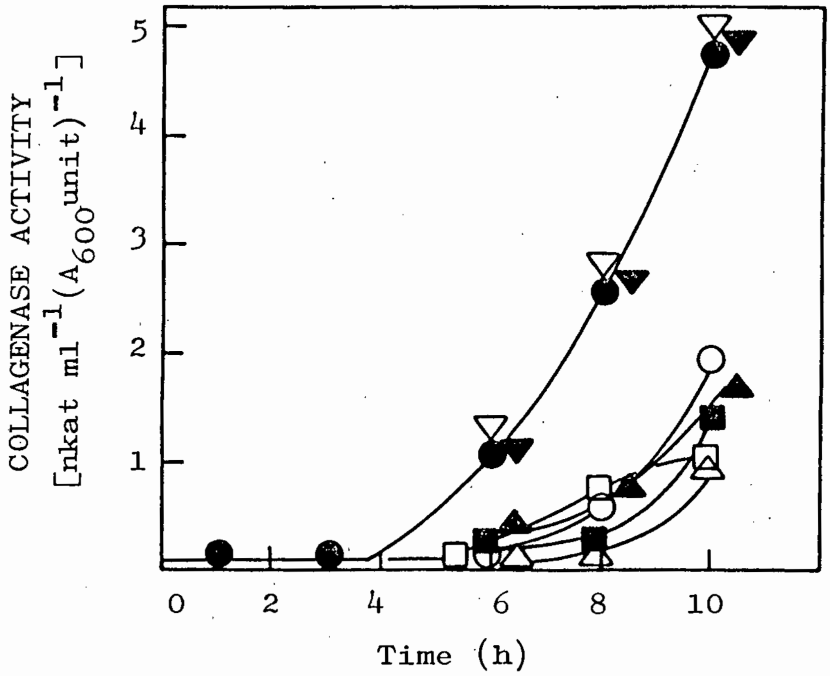

Fig. 2.3: Effect of cyclic nucleotides on glucose repression of collagenase production. Glucose and the cyclic nucleotides were added at 3.5 h. No additions (●); 0.2% glucose (○); 0.2% glucose + 5 mM cyclic AMP (△); 0.2% glucose + 5 mM dibutyryl cyclic AMP (■); 0.2% glucose + 5 mM cyclic GMP (□); 5 mM cyclic AMP (▲); 1 mM cyclic AMP (▼); 2 mM cyclic GMP (▽).

Table 2.1: Effect of various carbon sources, amino acids and ammonium ions on collagenase production. Carbon sources were added at 0.2% and amino acids at 0.5%. Additions were made to peptone grown cultures 3.5 h after inoculation and collagenase activity was assayed at 6 and 8 h after inoculation.

Carbon Source	Activity (% of Control)	
	6 h	8 h
No additions	100	100
glucose	0	9
fructose	5	17
sucrose	0	18
D (+)-mannose	0	19
mannitol	10	22
maltose	9	24
glycerol	0	28
disodium succinate	24	28
pyruvic acid (sodium salt)	8	38
arabinose	50	63
α-ketoglutarate	80	70
lactose	70	100

Amino acid	Activity (% of Control)	
	6 h	8 h
No additions	100	100
Casamino Acids (1%)	1	4
Casamino Acids (0.5%)	3	47
glutamine	3	5
L-proline	38	28
histidine.HCl	17	31
glycine	14	33
tryptophan	53	49
iso-leucine	100	60
L-hydroxyproline	100	60
leucine	64	61
β-alanine	80	100
$(NH_4)_2SO_4$ (50 mM)	65	100
$(NH_4)_2SO_4$ (100 mM)	40	42

Methionine sulfone derepressed collagenase synthesis in the presence of NH_4^+ (Table 2.2).

2.3.5 Ultrasonic disintegration and toluene treatment.

Ultrasonic disintegration or toluene treatment of the cells did not result in an increase in collagenase activity (Table 2.3).

2.3.6 Release of collagenase by washed cells.

Experiments were carried out to determine whether induced cells which were producing collagenase in the peptone medium would continue to release collagenase in buffer after washing. Washed induced cells did not produce collagenase in buffer.

2.3.7 Effect of trypsin and α-chymotrypsin on collagenase production by cells and sphaeroplasts.

Although the addition of penicillin G to cultures in 2.5% peptone resulted in the formation of sphaeroplasts, little collagenase was produced. However, when the peptone concentration was 1% the repressive effect of excess nutrients on collagenase production by sphaeroplasts was eliminated. The addition of penicillin to the culture medium resulted in the formation of 100% sphaeroplasts after 2 h and the continued addition of penicillin at 3 and 5 h maintained up to 90% sphaeroplasts for 9 h.* The sphaeroplasts were osmotically stabilized by 0.6 M NaCl and collagenase production was the same as that of cells at this NaCl concentration; 0.7 or 0.8 M NaCl resulted in decreased collagenase production by sphaeroplasts (Fig. 2.4). Collagenase production by cells was unaffected by 0.6, 0.7 or 0.8 M NaCl.

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* Sphaeroplasts were observed using a Zeiss photomicroscope fitted with phase contrast optics.

Table 2.2: Derepression of collagenase in the presence of ammonium ions by methionine sulfone.

	Activity (% of control)			
	6 h	8 h	10 h	12 h
Control	100	100	100	100
$(NH_4)_2SO_4$ (100 mM)	17	10	12	15
$(NH_4)_2SO_4$ (100 mM) + methionine sulfone ($25\ \mu g\ ml^{-1}$)	55	30	67	78

Table 2.3: Effect of ultrasonic disintegration and toluene treatment on collagenase activity.

Ultrasonic disintegration

Age of culture (h)	Collagenase activity (nkat ml^{-1})	
	Untreated	Treated
4	0.58	0.58
6	9.11	7.95

Toluene-treatment

Age of culture (h)	Collagenase activity (nkat ml^{-1})		
	Untreated	Toluene treatment	
		Supernatant	Uncentrifuged
4	0.47	0.55	0.55
6	3.53	2.89	2.84
8	6.46	7.20	5.79
10	10.60	8.20	7.21

Sonication and toluene treatment did not affect cell free colagenase activity. In addition no inhibitor of colagenase activity is released by these treatments.

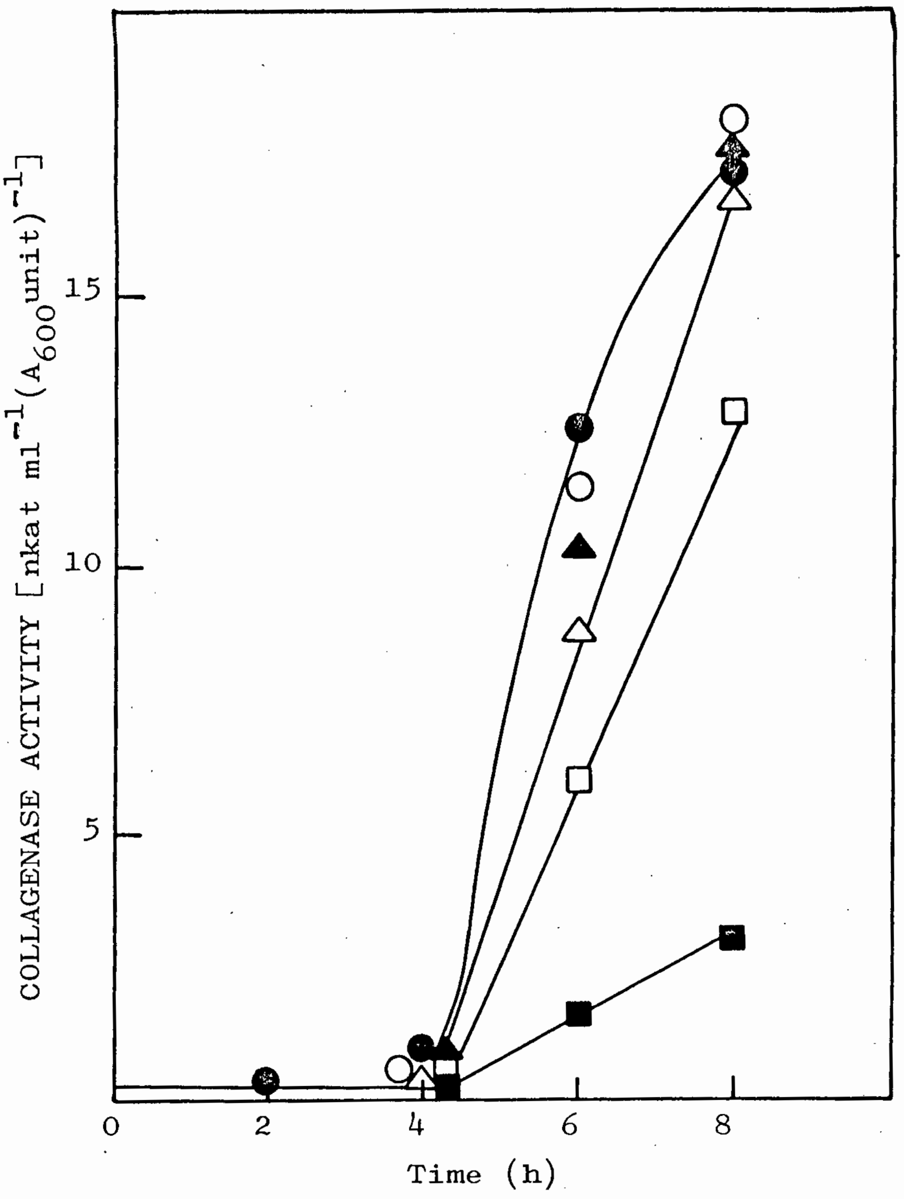

Fig. 2.4: Effect of NaCl concentration on collagenase production by cells and sphaeroplasts. Collagenase production by cells in 0.6 (●), 0.7 (○) and 0.8 M NaCl (△). Collagenase production by sphaeroplasts in 0.6 (▲), 0.7 (□) and 0.8 M NaCl (■).

The addition of trypsin (100 μg ml^{-1}) and α-chymotrypsin (100 μg ml^{-1}) to cultures of cells or sphaeroplasts had no effect on the levels of collagenase produced (Fig. 2.5). However the addition of these enzymes resulted in the production of collagenase marginally earlier than in untreated cells or sphaeroplasts (Fig. 2.5). This effect was consistently observed in a number of repeat experiments.

Trypsin and α-chymotrypsin at concentrations of 1 mg ml^{-1} had no effect on purified collagenase activity after 4 h at 30°C and reduced collagenase activity by 8 and 6% respectively over 21 h (Table 2.4). Both enzymes were fully active under conditions of 0.4 and 0.6 M NaCl as determined by the azocasein assay over 30 min.

2.4 DISCUSSION

The production of collagenase by V. alginolyticus requires a specific inducer (see Chapter III) and only occurs at the late-exponential and early-stationary phases of growth in highly aerated cultures. The growth of cells to late-exponential phase and the precise time of collagenase synthesis at 6 h, facilitated studies of the regulation of enzyme production.

In common with exo-protease synthesis by bacteria of a number of genera (Glenn, 1976), collagenase production is subject to control by end-product repression. Various amino acids and ammonium ions repressed collagenase production. The severe repression by glutamine suggests that the enzymes involved in glutamine metabolism may be implicated in the regulation of collagenase (Streicher et al., 1974; Friedrich and Magasanik, 1977). The

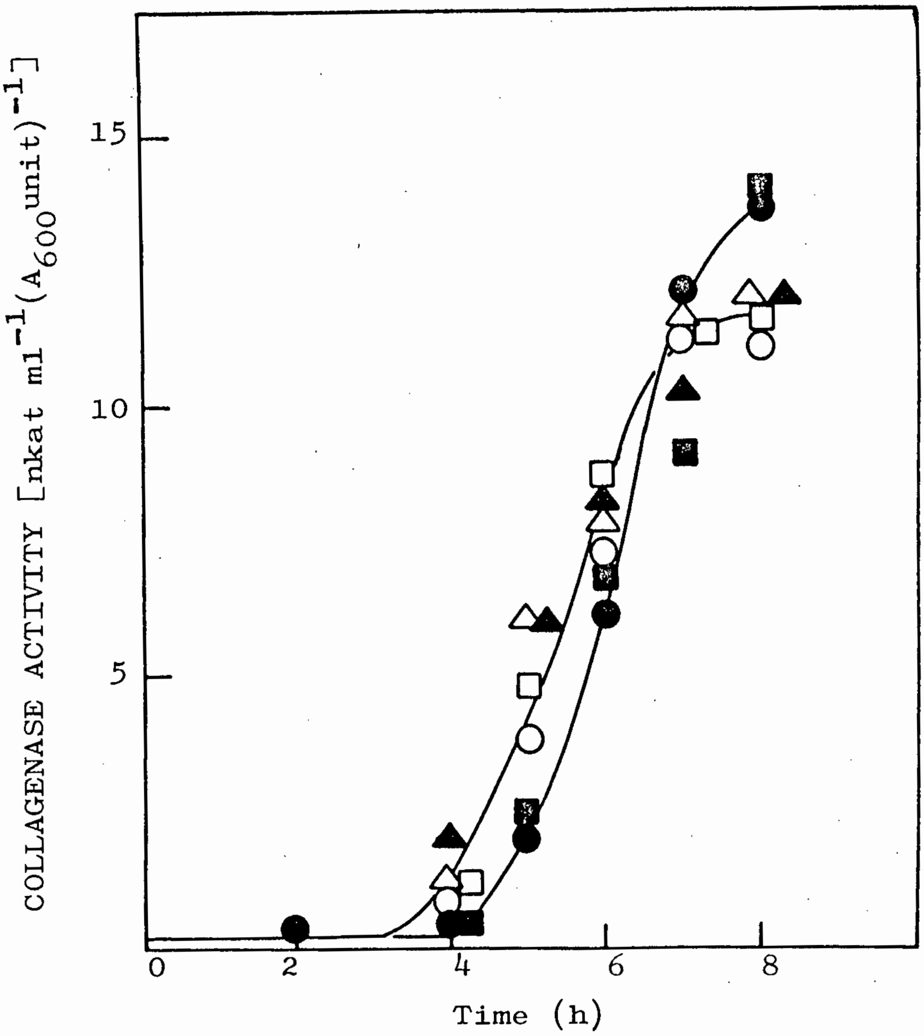

Fig. 2.5: Effect of trypsin and α-chymotrypsin on collagenase production by cells and sphaeroplasts. The NaCl concentration was 0.6 M. Collagenase production by cells (●); cells + α-chymotrypsin (○); cells + trypsin (△); sphaeroplasts (■); sphaeroplasts + α-chymotrypsin (□); sphaeroplasts + trypsin (▲). Trypsin and α-chymotrypsin were added at 3.5 h each at a concentration of 100 μg ml^{-1}.

Table 2.4: Effect of trypsin and α-chymotrypsin on the activity of purified collagenase. Trypsin and α-chymotrypsin were incubated with collagenase as described in the text.

Enzyme	% Collagenase activity			
	0 h	2 h	4 h	21 h
Trypsin (1 mg ml^{-1})	100	99	103	92
α-Chymotrypsin (1 mg ml^{-1})	100	98	105	94
Collagenase control	100	101	109	102

derepression of collagenase synthesis by methionine sulfone, in the presence of ammonium ions further supports this hypothesis. Methionine sulfone, a glutamine analogue, has been shown to derepress nitrogenase in the presence of ammonium ions in *K. aerogenes* (Brenchley, 1973) and *Spirillum lipoferum* (Okon *et al*., 1976) and acts by supressing the adenylation of glutamine synthetase (Bishop *et al*., 1975).

It is interesting that glycine, which constitutes one-third of all the amino acids in collagen, strongly repressed collagenase synthesis. Lecroisey *et al*. (1975) reported that histidine inhibited purified collagenase and part of the apparent repression caused by histidine may be due to the inactivation of the collagenase. Isoleucine and proline, which have been identified as the most common repressive amino acids in other bacteria (Glenn, 1976), also repressed collagenase synthesis but not as severely as glutamine.

In addition to end product repression the synthesis of collagenase is subject to catabolite repression by a number of different growth substrates. However, the addition of exogenous cyclic AMP, dibutyryl cyclic AMP [which stimulates the synthesis of β-galactosidase in *Caulobacter crescentus* (Shapiro *et al*., 1972)] or cyclic GMP did not relieve catabolite repression. The failure of cyclic AMP to overcome the glucose effect has been reported for extracellular enzyme synthesis in *V. parahaemolyticus* (Tanaka and Iuchi, 1971), *P. maltophilia* (Boethling, 1975), *Pseudomonas lemoignei* (Stinson and Merrick, 1974), *Staphylococcus aureus* (Yoshikawa *et al*., 1974) and many

Bacillus species (Priest, 1977). Cyclic AMP actually inhibited the synthesis of collagenase. A similar inhibitory effect of cyclic AMP was reported for the synthesis of extracellular 1,3-β-glucanase in a Streptomyces strain (Lilley et al., 1974).

As well as inducing catabolite repression the addition of 0.2% glucose or 0.4% 2-deoxy-D-glucose resulted in a severe transient repression similar to the transient repression observed with the lac operon in E. coli (Tyler et al., 1967). Higher concentrations of glucose (0.4%) resulted in the complete repression of collagenase synthesis.

The isolation by Robbertse et al. (1978) of a col G mutant, which is insensitive to glucose but repressed by amino acids, and a col A mutant, which is insensitive to glucose but requires amino acids, suggests that the glucose and amino acid effects are mediated differently.

The finding that ultrasonic disintegration or toluene treatment of cells did not result in an increase in collagenase activity, indicates that there is no intracellular accumulation of the enzyme. Keil-Dlouha et al. (1976) also reported that no zymogen or cell accumulated enzyme is present during exponential growth.

Enzyme secretion across membranes by both eucaryotes and procaryotes is not yet fully understood. Although secretion of enzymes across single membranes has been studied quite extensively the secretion of enzymes across double membranes has yet to receive attention (Lodish and Rothman, 1979). The study of the secretion of extracellular enzymes by gram-negative bacteria should provide a means whereby this phenomenon may be more fully understood. To this end preliminary studies on the secretion of collagenase by V. alginolyticus were undertaken.

Secretion by protoplasts of extracellular α-amylase and protease by B. amyloliquefaciens (Saunders and May, 1975) and of penicillinase by B. licheniformis (Bettinger and Lampen, 1975) have been shown to be sensitive to the proteolytic enzymes trypsin and α-chymotrypsin, whereas the native enzymes were totally resistant to proteolytic degradation. This evidence has been used to support the hypothesis that extracellular enzymes are secreted through the cytoplasmic membrane in an unfolded form which is sensitive to proteolytic degradation.

Considering the exclusion limit of the outer membrane to be of the order of 1,000 daltons (Nakae and Nikaido, 1975; DiRienzo et al., 1978) it might not be unreasonable to assume that extracellular enzymes of gram-negative bacteria are extruded through both cytoplasmic and outer membranes in an unfolded or partially unfolded form. However, trypsin and α-chymotrypsin at concentrations of 100 μg ml^{-1} had no effect on the secretion of collagenase by V. alginolyticus cells or sphaeroplasts, suggesting that a different mechanism of secretion might apply. Perhaps this result could be expected as gram-negative bacteria would normally have to have a mechanism of enzyme secretion which would not result in proteolytic degradation of secreted enzymes.

Removal of the cell wall in Bacillus stearothermophilus (Welker and Campbell, 1963a) and Streptococcus faecalis (Hammel and Zimmerman, 1966) causes exoenzyme synthesis to cease, whereas the results in this chapter suggest that the cell wall of V. alginolyticus does not play a significant role in the induction or in the secretion of collagenase.

CHAPTER III

CHARACTERIZATION OF THE PEPTONE INDUCERS AND THE DEVELOPMENT OF A MINIMAL MEDIUM INDUCTION SYSTEM

Summary: The inducers of collagenase production by V. alginolyticus in peptone were shown to have a broad molecular weight range between 1,000 and 60,000. The peptone inducers supported slow growth of V. alginolyticus when supplied as the sole nitrogen source in minimal medium. Digestion of the peptone inducers with purified V. alginolyticus collagenase resulted in a decrease in their inducing ability, whereas digestion with trypsin or α-chymotrypsin did not. Peptone acted as an inhibitor of collagenase. A minimal medium induction system was developed which involves resuspending cells at high density in a medium containing succinate, $(NH_4)_2SO_4$, KH_2PO_4 and the peptone inducers.

3.1 INTRODUCTION

It was demonstrated at the beginning of the century that certain enzymes of yeast are formed only in the presence of their specific substrates; this effect was subsequently termed enzyme adaptation and is now known as "induction". Most studies of induction have been carried out in bacterial systems in which the effect is considerable. The inducible β-galactosidase system of E. coli is the classic example of induction (Jacob and Monod, 1961). E. coli grown in the absence of a galactoside contains only about 5 molecules per cell of the enzyme β-galactosidase, whereas cells grown in the presence of the substrate may contain up to 5,000 molecules of enzyme. Enzyme activity can be detected very rapidly (within 2-3 minutes) after addition of substrate; and removal of substrate results in an equally rapid cessation of activity.

Bacterial exoenzymes may be inducible, partially constitutive or completely constitutive and although a substantial proportion of exoenzymes appear to be inducible (Priest, 1977), there have been few studies on the induction of bacterial exoproteases.

Pollock (1962) outlined the problems involved in studying induction of extracellular enzymes: Inducers are usually the substrate for the enzyme or some structurally related compound and since exoenzymes have large molecules as substrates, the inducers themselves are usually large (e.g. hyaluronic acid, starch or chitin). However, it can be argued that there must be a limit to the size of molecule which can enter the cell and therefore the question which arises is whether the large molecular weight inducers act directly at the cell surface or whether the inducers are smaller degradation products which can enter the cell. It is difficult to distinguish between the two since basal levels of enzyme, present before induction, might be responsible for hydrolysis of the substrate to produce a low molecular weight inducer.

As yet it has not been conclusively demonstrated that high molecular weight substrates alone are the active inducers and it appears that low molecular weight compounds are efficient in inducing some exoenzymes. For example, the regulation of penicillinase synthesis in <u>Bacillus cereus</u> has been studied in some detail and is induced by benzylpenicillin (Aten and Day, 1973). The penicillinase system is possibly unique in that the inducer does not enter the cell but is bound to the cell wall. The most effective inducer of α-amylase in Bacillus stearothermophilus (Welker and Campbell, 1963b) and B. licheniformis (Saito and Yamamoto, 1975) has been shown to be maltotetraose whereas maltotriose and higher maltooligosaccharides are less efficient inducers. Pollock (1962) found similar results with a streptococcal hyaluronidase. He found that purified tetrasaccharide and trisaccharide were as efficient inducers as the undegraded hyaluronic acid (molecular weight approx. 10^6).

Collagenase production by V. alginolyticus has been shown to be inducible by denatured collagen and its high molecular weight fragments (Keil-Dlouha et al., 1976). Peptone also induces collagenase (Welton and Woods, 1975) and the nature of the inducers in peptone have been investigated in this study. A minimal medium induction system utilizing the peptone inducers was also developed.

3.2 MATERIALS AND METHODS

3.2.1 Fractionation of Peptone

Peptone (2g) was dissolved in 5ml of 0.01M NH_3-NH_4Cl buffer (pH 8.5) and layered on a Sephadex G25 column (80 by 2.5 cm). Samples (10ml) were eluted with the same buffer at a flow rate of 30 ml h^{-1}. Fractions under the same peaks were pooled, dialysed* against two changes of distilled water at 4°C, and lyophilized. The pooled fractions were tested for their ability to induce collagenase by including the fractions at concentrations of 0.5% in

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* (membrane retention ~1 000 to 8 000 mw)

2.5% Casamino acids medium and assaying for collagenase activity. The collagenase-inducing fraction was termed the peptone inducer. The molecular weight of the peptone inducer was determined by applying 0.7g in 2 ml of buffer (as above) to a Sephadex G100 column (80 by 2.5 cm) and eluting 3 ml fractions at a flow rate of 10 ml h^{-1}. The column was calibrated with ovalbumin (molecular weight 43,000), cytochrome C (molecular weight 11,700), and insulin (molecular weight 6,000).

3.2.2 Peptone Inducer as a Substrate for growth.

The peptone inducer was tested for its ability to support growth of V. alginolyticus. An overnight Casamino acids culture was washed twice in 0.4 M NaCl and then used to innoculate the following: 2.5% peptone in the Tris-HCl buffer; 2.5% peptone inducer in the Tris-HCl buffer; Minimal Medium; 0.1% peptone inducer in minimal medium without the $(NH_4)_2SO_4$; 0.1% peptone inducer in minimal medium without the $(NH_4)_2SO_4$ and the glucose. Growth was followed turbidimetrically at 600 nm.

3.2.3 Optimization of collagenase induction in minimal medium using the peptone inducer.

The induction of collagenase in V. alginolyticus by the peptone inducer was tested under various conditions. The specific details of each experiment are included with the appropriate figures. Cells were resuspended in 10 ml SNP medium (which contained succinate, $(NH_4)_2SO_4$, KH_2PO_4) at high cell density after growth in different media. Cells were washed once, by centrifugation and resuspension, with the medium in which they were to be resuspended. The peptone inducer was included at a concentration of 0.5%. Collagenase activity was assayed as in Chapter II. The effect of including the peptone inducer at concentrations of 0.1, 0.05 and 0.025% on collagenase induction in low SNP medium was also determined.

3.2.4 Enzymatic digestion of peptone and peptone inducer.

Unfractionated peptone and the peptone inducer in low SNP medium were subjected to digestion by purified *V. alginolyticus* collagenase, trypsin, or α-chymotrypsin at enzyme-to-substrate ratios of 1:50 or 1:100 for 13 to 39 h at 30°C. After digestion the enzymes were inactivated by placing the flasks in a boiling-water bath for 30 min. These flasks were then inoculated with washed minimal medium-grown cells to give an absorbance at 600 nm of 8. The collagenase activity was expressed as a percentage of the control activity at the peak of production. The control consisted of heat-inactivated enzyme added to undigested peptone or peptone inducer. The method of collagenase purification is described in Chapter V.

3.2.5 Inhibition of collagenase by peptone.

Purified collagenase at a concentration of 4.2 nkat ml^{-1} was incubated with various concentrations of peptone (0.25% to 2.5%) in Tris-HCl buffer at 30°C. Samples were withdrawn at intervals and assayed for collagenase activity.

3.3 RESULTS

3.3.1 Fractionation of peptone.

Peptone fractionated on a Sephadex G25 column gave three major peaks (Fig. 3.1). Samples from peak 1 induced collagenase, whereas samples from peaks 2 and 3 did not induce the production of the enzyme. Samples from peak 1 were pooled and added to a Sephadex G100 column (Fig. 3.2). A broad peak was obtained, and the resolution was not improved by the addition of smaller quantities of material to the column. The molecular weight ranged between 1,000 and 60,000. Samples taken from different positions within the peak all induced collagenase production with equal efficiency. This material was used for subsequent induction experiments and is referred to as the peptone inducer.

3.3.2. Peptone inducer as a substrate for growth.

The peptone inducer (0.1%) supported slow growth of V. alginolyticus when supplied as the sole source of nitrogen in minimal medium (Fig. 3.3). When the peptone inducer was included in minimal medium without both $(NH_4)_2SO_4$ and glucose no growth occurred over 10 h and only slight growth was detected after 24 h. Tris-HCl buffer containing 2.5% peptone inducer supported slight growth over 7.5 h (Fig. 3.3).

3.3.3. Optimisation of collagenase induction in minimal (SNP) medium utilizing the peptone inducer.

The standard error for collagenase assays was less than 10% but usually lower (<5%).

The induction of collagenase by the peptone inducer was determined after resuspending the cells in SNP medium containing 0.5% inducer. No collagenase was produced in the absence of the inducer.

Initial experiments showed no significant differences in the rate and the amount of collagenase produced by

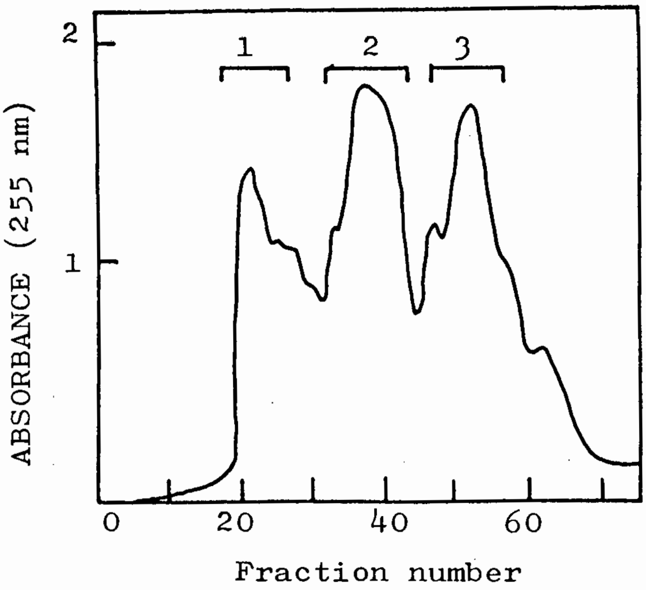


Fig. 3.1: Gel filtration of peptone on Sephadex G-25. Fractions under each peak were pooled as shown in the figure (brackets). Only the first peak induced collagenase. Void volume, fraction 17; fully included volume, fraction 72.

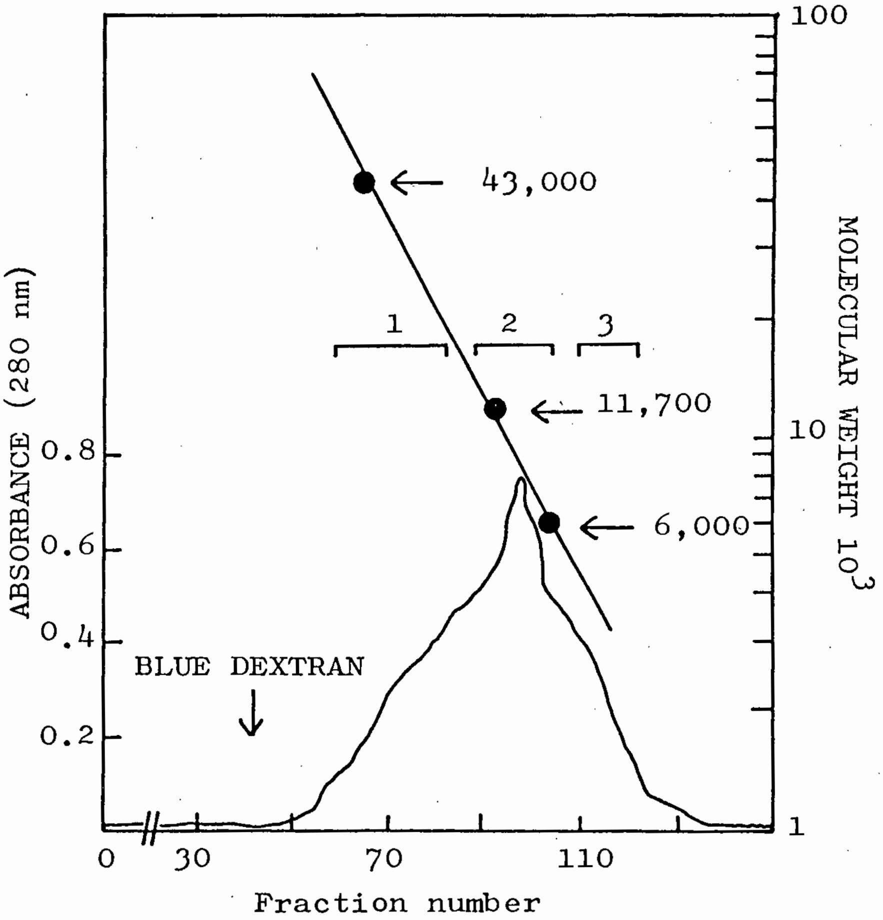


Fig. 3.2: Gel filtration of the peptone inducer on Sephadex G-100. Fractions under the brackets were pooled. Fractions 1,2, and 3 all induced collagenase equally.

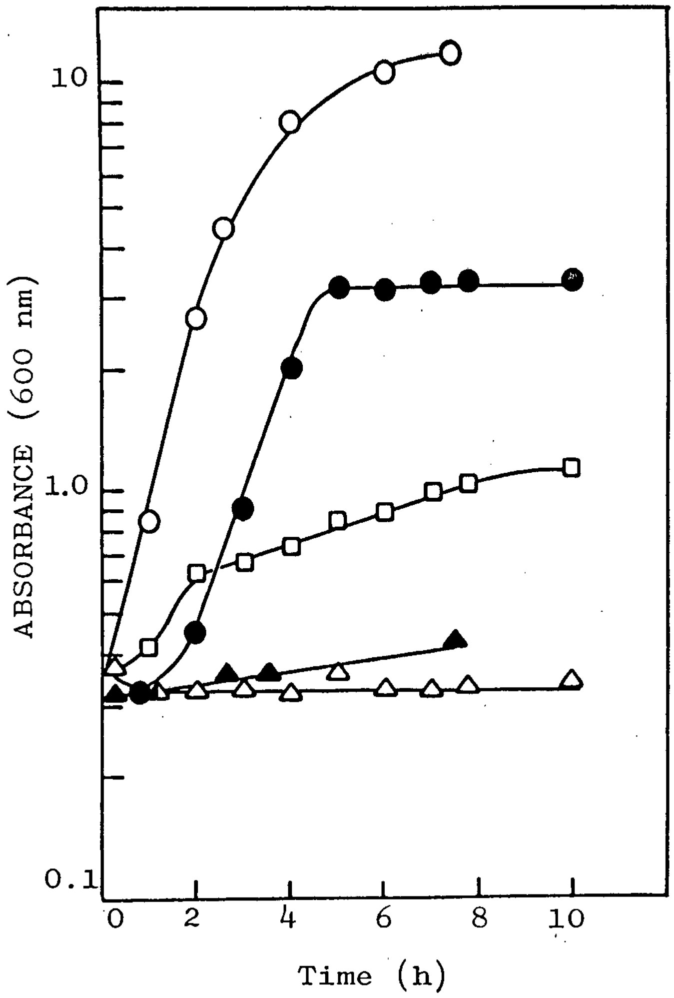


Fig. 3.3: Growth curves of V. alginolyticus in various media. Growth in 2.5% peptone medium (○); minimal medium (●); minimal medium without $(NH_4)_2SO_4$ containing 0.1% peptone inducer (□); 2.5% peptone inducer in the Tris-HCl buffer (▲); minimal medium without $(NH_4)_2SO_4$ and glucose containing 0.1% peptone inducer (△).

cultures, grown on peptone, when resuspended in SNP medium containing 20 mM succinate and 10 mM $(NH_4)_2SO_4$ (high SNP medium) or 5 mM succinate and 2.5 mM $(NH_4)_2SO_4$ (Fig. 3.4). However, when the concentrations of succinate and $(NH_4)_2SO_4$ were reduced to 2 mM and 1 mM respectively (low SNP medium), significant increases in collagenase production were observed (Fig. 3.4). The addition of 1 mM $MgSO_4$ and 0.5 mM $FeCl_3$ resulted in decreased collagenase production (Fig. 3.4).

Small but inconsistent differences were observed in collagenase production by cultures grown on peptone and harvested at 4, 5 or 6 h (Table 3.1), or by cultures resuspended at cell densities of 5, 7 or 14 absorbance units (Fig. 3.5). However, cultures harvested at 11 h, before resuspension, showed reduced collagenase production (Fig. 3.4). Subsequently cells were harvested at 4 h and resuspended at an absorbance at 600 nm of 8 units; this being the cell density of a fully induced culture in 2.5% peptone. The cell density increased from 8 to 11 - 12 absorbance units over the duration of the experiments (3 to 4 h).

The production of collagenase was affected by the nature of the growth medium before cells were washed and resuspended in the SNP induction medium. Cells grown in minimal medium induced more rapidly and gave higher yields of collagenase than cells grown in peptone, Casamino Acids or tryptone media (Fig. 3.6). High SNP medium repressed collagenase production whereas low SNP medium enhanced collagenase production (Fig. 3.6). The addition of succinate, $(NH_4)_2SO_4$ and KH_2PO_4 (i.e. low SNP) to the 0.5% inducer in Tris-HCl buffer markedly enhanced the production

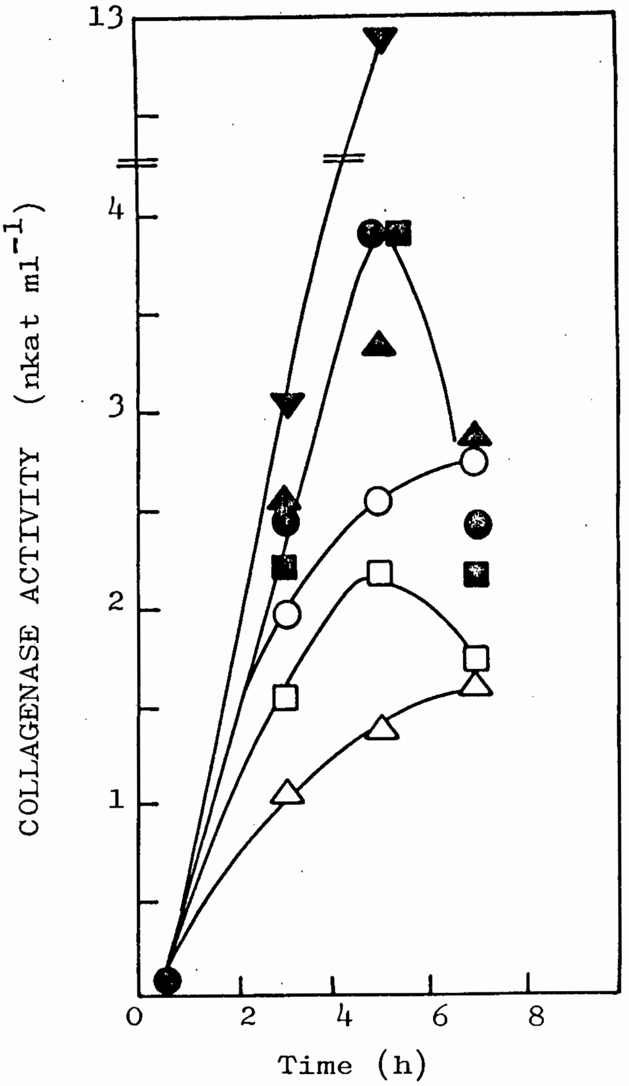


Fig. 3.4: Collagenase induction in SNP media by the peptone inducer. A culture grown in 2.5% peptone was harvested at 11 h and resuspended at a cell density of 10 absorbance units in the following SNP media containing 0.5% peptone inducer, with 1 mM $MgSO_4$ and 0.5 mM $FeCl_3$ (open symbols) and without (closed symbols): 20 mM succinate, 10 mM $(NH_4)_2SO_4$ and 1 mM KH_2PO_4 (●,○); 10 mM succinate, 5 mM $(NH_4)_2SO_4$ and 1 mM KH_2PO_4 (■,□); 5 mM succinate, 2.5 mM $(NH_4)_2SO_4$ and 1 mM KH_2PO_4 (▲,△).

A culture harvested at 4 h and resuspended at a cell density of 8 absorbance units in 2 mM succinate, 1 mM $(NH_4)_2SO_4$ and 1 mM KH_2PO_4 (low SNP medium) (▼).

Table 3.1: Effect of the age of peptone cultures on their inducibility by the peptone inducer. Cultures grown in 2.5% peptone were harvested at 4, 5 and 6 h and resuspended at a cell density of 8 absorbance units in high SNP medium containing 0.5% peptone inducer.

Time of assay	Collagenase activity (nkat ml^{-1})		
	4 h	5 h	6 h
2 h	7.5	7.0	6.6
4 h	7.0	9.2	8.6

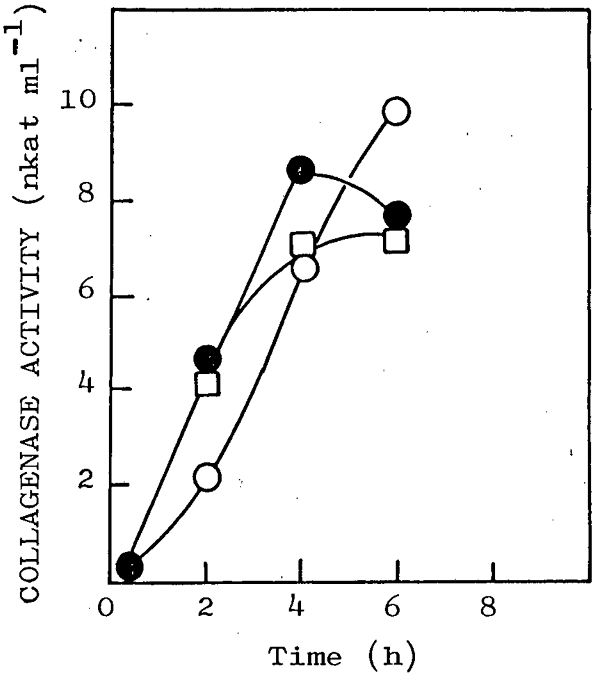


Fig. 3.5: Induction of collagenase at different cell densities. A 2.5% peptone culture was harvested at 4 h and resuspended in high SNP medium containing 0.5% peptone inducer at cell densities having absorbances of 14 (○), 7 (●) and 5 (□) units.

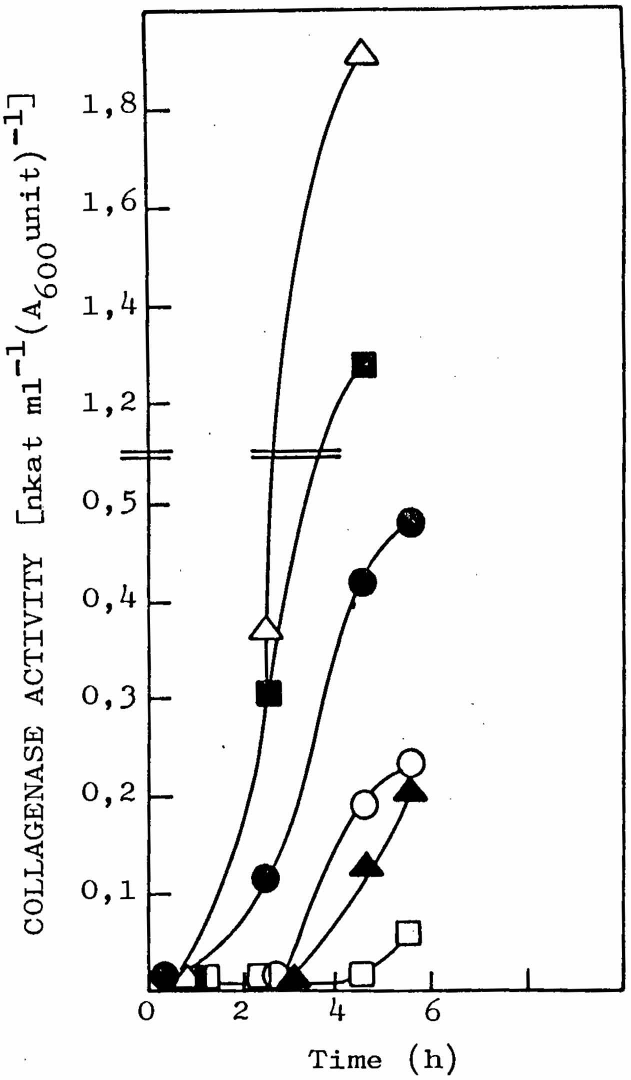


Fig. 3.6: Induction of collagenase by the peptone inducer after growth in different media. Cultures were harvested at 4 h and resuspended at a cell density of 8 absorbance units. Cells were resuspended in high SNP medium after growth in minimal medium (●), peptone (○), Casamino Acids (▲) and tryptone (□). Cells were resuspended in low SNP medium after growth in minimal medium (△) and peptone (■). The peptone inducer was included at a concentration of 0.5%.

of collagenase, although the rate of collagenase production in the absence of low SNP medium is the same as in the presence of low SNP medium but a longer lag period occurs in the former medium (Fig. 3.7). There was a lag of approximately 1 h before collagenase production began (Figs. 3.6 and 3.7).

When the inducer concentration was reduced from 0.5 to 0.1% the collagenase activity decreased by 60%; 0.05% inducer showed slight induction of collagenase (0.27 nkat ml^{-1} absorbance unit at 600 nm^{-1}), whereas induction by 0.025% inducer was negligible (0.02 nkat ml^{-1} absorbance unit at 600 nm^{-1}).

3.3.4. Enzymatic digestion of peptone and peptone inducer.

Digestion of the peptone inducer and unfractionated peptone by purified V. alginolyticus collagenase caused a marked decrease in the inducing ability of both the inducer and peptone (Table 3.2). Digestion by collagenase (35 nkat ml^{-1}) for 39 h did not completely destroy the inducing ability of the peptone inducer. Collagenase was stable and lost no activity under these conditions over 22 h (Fig. 3.8). Digestion with α-chymotrypsin and trypsin had little or no effect on the inducing ability of the inducer or peptone (Table 3.2). Both α-chymotrypsin and trypsin were active under the same conditions as used for the digestion of peptone, when assayed by the azocasein assay used in Chapter II.

3.3.5. Inhibition of collagenase by peptone.

Collagenase activity, when assayed with the synthetic substrate PZ-Pro-Leu-Gly-Pro-Arg, was inhibited by

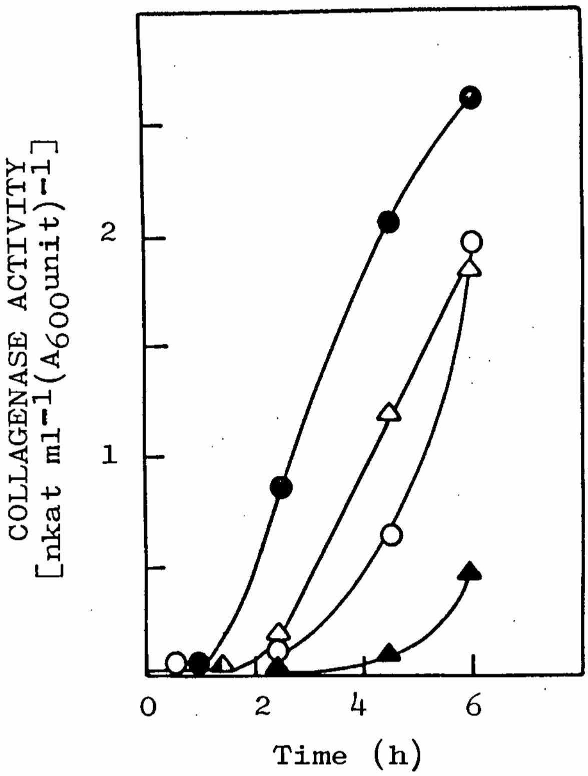


Fig. 3.7: Induction of collagenase by the peptone inducer in the presence and absence of low SNP medium. Cultures were harvested at 4 h and resuspended at a cell density of 8 absorbance units. Cells were grown in minimal medium and resuspended in low SNP medium (●) or Tris-HCl buffer (○). Cells were grown in peptone and resuspended in low SNP medium (△) or Tris-HCl buffer (▲). The peptone inducer was included at a concentration of 0.5%.

Table 3.2: Effect of enzymatic digestion of peptone and the peptone inducer on their ability to induce collagenase.

Substrate	Digestion Time (h)	Enzyme/ Substrate Ratio	% Control collagenase activity after digestion of substrate with: Collagenase	α-Chymotrypsin	Trypsin
0.5% peptone inducer	13	1:100	68	94	107
0.5% Peptone	20	1:100	51	102	90
0.25% Peptone	39	1:50	44	111	-

The standard error of this data is 2.4% and duplicate samples were assayed in each case.

increasing concentrations of peptone (Fig. 3.8). At an enzyme concentration of 4.2 nkat ml^{-1}, no inhibition was observed by 0.25% peptone compared with 65% inhibition by 2.5% peptone. Decreasing the collagenase concentration to 0.86 nkat ml^{-1} resulted in 35% inhibition by 0.25% peptone. The inhibitory effect of peptone can be removed by continued incubation with collagenase (Fig. 3.8). Thus, 65% inhibition by 2.5% peptone was decreased to 5% inhibition by incubation with 4.2 nkat of collagenase ml^{-1} for 4 h.

3.4. DISCUSSION

The collagenase inducer molecules in peptone have a molecular weight between 1,000 and 60,000 which is similar to that reported by Dreisbach and Merkel (1978) for collagen fragments (molecular weight 1,000 to 10,000) required for the induction of a Vibrio B-30 strain isolated from seawater. Keil-Dlouha *et al.*, (1976) showed that collagenase induction in *V. alginolyticus* required a macromolecular fraction of collagen (molecular weight approx. 8,000 to 30,000). The collagenase inducers in peptone are presumably degradation products of collagen or collagen-like molecules.

The inability of the peptone inducer to support rapid growth of *V. alginolyticus* suggests that the ability of *V. alginolyticus* to utilize the high molecular weight nutrients might depend on its ability to secrete hydrolytic enzymes into the medium. Although many inducible enzymes are present in non-induced cultures at basal levels (Priest, 1977), no such basal levels of collagenase in

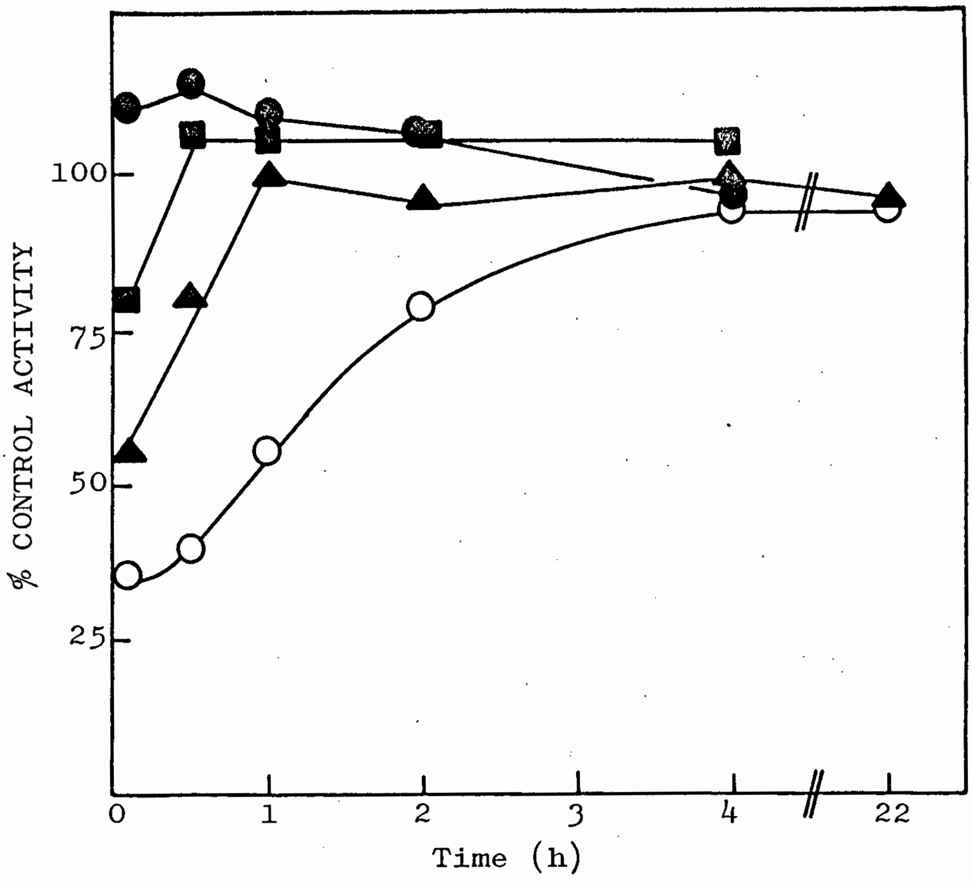


Fig. 3.8: Inhibition of collagenase by peptone. Peptone at final concentrations of 2.5% (○), 1% (▲), 0.5% (■) and 0.25% (●) was incubated with purified V. alginolyticus collagenase (final concentration, 4.2 nkat ml^{-1}) at 30°C. Collagenase activity was assayed at intervals as described in the text.

V. alginolyticus have been detected. However, Dreisbach and Merkel (1978), found that collagen hydrolysis products had an improved ability to induce collagenase and suggest that proteases (not necessarily collagenases) might hydrolyse collagen to produce a low molecular weight inducer molecule. V. alginolyticus has at least one such protease (Keil-Dlouha et al., 1976) which is synthesized in the absence of an inducer, although its production is increased by induction with various peptides (Long et al., 1980, submitted). In contrast to collagenase this protease is synthesized immediately when stationary phase washed cell suspensions are resuspended in new media (Hare and Woods, personal communication).

Further examples of low molecular weight compounds inducing or stimulating extracellular enzymes are: stimulation of proteases by amino acids in a marine bacterium (Daatselaar and Harder, 1974) and a Micrococcus sp. (McDonald and Chambers, 1966); cellulase induction in Trichoderma viride by sophorose (2-O-β-D-glucopyranosyl-D-glucose) (Mandels et al., 1962); induction of chitosanase by glucosamine in a Streptomyces sp. (Price and Storck, 1975). Protease production by V. alginolyticus is stimulated by the addition of histidine to low SNP medium (Long et al., 1980, submitted). On the other hand Keil-Dlouha et al., (1976) found that the addition of the low molecular weight substrate (PZ-Pro-Leu-Gly-Pro-Arg) or inhibitor (Pro-Leu-Sar-Pro) of collagenase did not stimulate enzyme synthesis.

The finding that collagenase was produced in greater quantities and more rapidly by cells harvested at 4 to 6 h than cells harvested at 11 h agrees with that of Keil-Dlouha et al., (1976) who showed that induction of collagenase in V. alginolyticus is associated with a particular phase of growth and that the later the inducer is added the less the amount of collagenase produced.

Induction of collagenase appears to be a relatively slow process since there is a delay of approximately 60 min from the time the cells are exposed to the inducer before collagenase can be detected in the medium. Dreisbach and Merkel, (1978) found that when using collagen as an inducer in a Vibrio B-30 strain maximum production occurred 3.5 to 4 h after its addition, whereas when using hydrolysed collagen maximum production occurred 1.5 h after its addition. Keil-Dlouha et al., (1976) showed that the rate of collagenase production in V. alginolyticus was greatest when denatured collagen was added early in the growth cycle but when added in early stationary phase collagenase production began almost immediately but at a reduced rate of synthesis.

The differences in the kinetics of collagenase induction and the amount of collagenase produced when cultures grown in different media and harvested at different times are resuspended in the same induction medium, suggests that collagenase production by V. alginolyticus is regulated by a sensitive control mechanism and depends on the nature of the inducer, the presence of repressors and the stage of the growth cycle.

Digestion of either peptone or the peptone inducer by purified V. alginolyticus collagenase for 13 to 39 h did not completely eliminate its inducing ability but reduced it by 32 to 56%. In contrast Keil-Dlouha et al., (1976) reported that digestion of high-molecular-weight fragments of collagen with collagenase at a lower enzyme-to-substrate ratio for 2 h prevented the induction of collagenase in the same strain. They suggested that the collagenase sensitive bond and tertiary structure of collagen are required for induction. The reduction in the inducing activity of peptone by digestion with collagenase supports this suggestion, but peptone differs from collagen as an inducer in that its inducing ability is not completely eliminated by extensive digestion with collagenase. Dreisbach and Merkel, (1978) observed that digestion of collagen improved its inducing ability, but this could be due to incomplete digestion which generates collagen fragments with an increased capacity to induce.

Keil (1978) showed that a number of synthetic peptides act as competitive inhibitors of collagenase, including the product of the cleavage of the substrate Z-Gly-Pro-Leu-Gly-Pro i.e. Z-Gly-Pro-Leu. The finding that peptone acts as an inhibitor of collagenase and that continued incubation of peptone with collagenase results in the elimination of the inhibitory effect, suggests that the mechanism of inhibition by peptone may also be competitive. However, kinetic studies are necessary to confirm this. Since incubation with collagenase relieves the inhibitory effect of peptone it is presumably the high molecular weight molecules which inhibit collagenase. This contrasts with inhibition by Z-Gly-Pro-Leu, a low molecular weight compound.

CHAPTER IV

RIFAMPIN-INSENSITIVE COLLAGENASE PRODUCTION

Summary: Collagenase production was shown to occur for 30 to 60 min in the presence of rifampin at levels which completely inhibit the incorporation of $[^3H]$ uracil into trichloracetic acid-precipitable material. Chloramphenicol completely and immediately abolished collagenase production, which together with labelling studies has confirmed that collagenase production involves *de novo* synthesis of the enzyme. Rifampin-insensitive collagenase production was partially repressed by the addition of glucose or Casamino Acids, although synthesis of the enzyme continued for 30 to 60 min after their addition. The amount of repression was the same whether glucose or Casamino Acids were added separately or together with rifampin.

4.1 INTRODUCTION

The phenomenon of rifampin-insensitive exoenzyme synthesis has been shown for a few bacterial species e.g. *B. amyloliquefaciens* (Both *et al.*, 1972), *B. subtilis* (Semets *et al.*, 1973), *P. maltophilia* (Boethling, 1975) and *P. lemoignei* (Stinson and Merrick, 1974). Most studies of this phenomenon have been done on the synthesis of extracellular protease by *B. amyloliquefaciens*, which shows unusual responses to the transcriptional inhibitors rifampin and actinomycin D.

When washed cells of *B. amyloliquefaciens* are resuspended in a medium containing low concentrations of amino acids, protease production occurs almost linearly, but when cells are resuspended in a medium containing high concentrations of amino acids there is a rapid phase (phase 1) of production for 30 min followed by a levelling for 50 min after which synthesis resumes linearly (phase 2) (Both et al., 1972). Phase 1 synthesis is not inhibited by rifampin or actinomycin D whereas phase 2 synthesis is promptly inhibited by both antibiotics. Phases 1 and 2 are however both sensitive to chloramphenicol, an inhibitor of protein synthesis. Labelling studies have confirmed that de novo synthesis of the enzyme is involved.

To explain these findings Both et al. (1972) proposed that postexponential-phase cells contain sufficient preformed mRNA to enable protease synthesis to be maintained for an extended period of time in the absence of RNA synthesis. The inhibition of phase 2 synthesis by rifampin indicated that the protease mRNA had a normal short half-life and that phase 1 synthesis must therefore be supported by a pool of mRNA composed of many short-lived species, rather than long-lived mRNA. Glenn et al. (1973) suggested that the excessive production of mRNA may represent a primitive prokaryote messenger transport system to ensure that sufficient intact mRNA reaches membrane localised translational sites, to support extracellular enzyme synthesis, despite its rapid degradation.

The extracellular α-amylase and ribonuclease of B. amyloliquefaciens (Gould et al., 1973) and the extracellular protease of B. subtilis (Semets et al., 1973) have been shown to behave in an identical manner, indicating that the accumulation of mRNA for extracellular protease, supported by rapid transcription, is a biologically meaningful phenomenon related to extracellular enzyme synthesis.

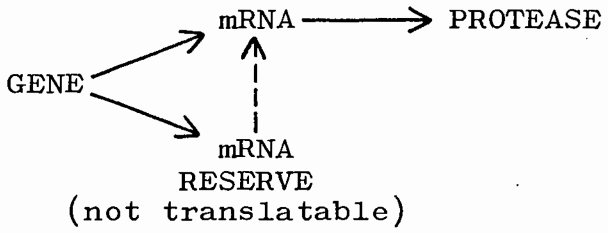
Although Coote _et al_. (1973) have criticized the use of rifampin in the determination of the lifetime of mRNA because of its non-specific cytotoxic effects, Brown and Coleman (1975a) have validated its use with reasonable confidence at concentrations of 10 $\mu g\ ml^{-1}$ in _B. amyloliquefaciens_. Utilizing a DNA to RNA hybridization technique they found the half-life of mRNA in both exponential- and postexponential-phase cells to be 6 min. A method which involves studying the loss of ability to incorporate L- $[^{14}C]$ leucine into protein in the presence of rifampin (10 $\mu g\ ml^{-1}$) gave half-lives of 4.5 and 6 min for exponential - and postexponential-phase cells respectively. These half-lives are of the same order as the half-life of 4.2 min found for _E. coli_ mRNA when growing in the same medium at 30°C (Gray and Midgley, 1970), but considerably shorter than the half-life of 11.5 min found for the _E. coli_ outermembrane lipoprotein (Hirashima _et al_., 1973). However, Brown and Coleman (1975a) suggested that the half-life of 6 min for postexponential-phase cells could be made up of 50% cellular mRNA having a half-life of about 4 min and 50% exoenzyme mRNA having a half-life of 8 min. The contribution to the half-life of 4.5 min of mRNA in exponential-phase by exoenzyme mRNA would be negligible, as it only constitutes 4% of this mRNA. In addition, Brown and Coleman (1975b) found no evidence for the accumulation of a specific component of the messenger RNA in exponential- or post-exponential-phase cells when using a serial hybridisation technique. However these findings are inconclusive and Priest (1977) suggests that although the discrepancy in the half-lives of mRNA in exponential- and postexponential-phase cells needs explanation, they may be due to the high concentration of rifampin used in these studies or due to some changes in the translational apparatus in stationary phase cells. He also expresses reservations about the hybridization technique used by Brown and Coleman (1975a).

Coleman et al. (1975) have proposed an alternative explanation for the regulation of exoenzyme synthesis in B. amyloliquefaciens, which involves two seperate effects and may be superimposed. The first involves both the modification and an increase in the availability of the RNA polymerase during stationary phase, resulting in the increased transcription of extracellular enzyme mRNA. The second involves an increase in the nucleotide precursor pool as a result of increased turnover of rRNA during stationary phase, causing in turn a further increase in exoprotein mRNA and thus exoprotein synthesis. However this evidence for the availability of nucleotide precursors is very much circumstantial and the regulation of RNA metabolism and precursor pools must be studied in greater depth before such a model for the regulation of exoenzyme synthesis can be accepted.

As yet there is no direct evidence for either long-lived or short-lived extracellular protease mRNA in B. amyloliquefaciens although the fact that phase 2 synthesis is sensitive to rifampin and actinomycin D, is strong evidence for a pool of short-lived mRNA. Glenn et al. (1973) suggest that although it is conceivable that the same mRNA molecules are more stable in phase 1 than in phase 2, no precedent for such a change is known and it therefore seems unlikely.

Semets et al. (1973) emphasized that there is no obligatory requirement for a mRNA pool for exoenzyme synthesis and phase 2 synthesis is thought to be the result of coupled transcription and translation in the absence of a mRNA pool. Amino acids appear to affect neither the translation nor the stability of the mRNA since in the presence of rifampin the pool disappears at approximately the same rate in high and low amino acids (Glenn et al., 1973). These authors concluded that transcription of the protease mRNA is repressed by high amino acids and that the pool is the product of a dynamic equilibrium between synthesis and degradation. O'Connor et al. (1978) postulate that the mRNA pool also causes repression of transcription and that both the mRNA pool and amino acids are required for maximal repression.

In this more recent work O'Connor et al. (1978) have shown that when cells, which have been exhausted of their capacity to synthesize protease in the presence of rifampin by incubation in high amino acids, are transferred to low concentrations of amino acids, they progressively regain the capacity for rifampin-actinomycin-insensitive protease production. Measurement of the apparent mRNA pool size over 12 h shows a cyclical rise and fall, and these changes correlate with a periodic variation in the rate of protease production. They suggest that protease gene transcription is alternatively active and inactive and during the former period mRNA is produced for immediate translation and in addition a reserve pool of mRNA is laid down in a nontranslatable form. During periods of protease gene inactivity they envisage that protease synthesis continues at a slightly different rate and is supported by conversion of the reserve mRNA to a translatable form. They propose the following working hypothesis to account for their observations:



However they acknowledge that this is not the only mechanism which could be operating and that conclusive demonstration of the presence of a mRNA pool requires more direct measurement.

4.2 MATERIALS AND METHODS

4.2.1. Growth conditions

Initial experiments on the effect of rifampin and chloramphenicol on collagenase production were performed utilizing the growth conditions described in Chapter II, i.e. continuous growth in 2.5% peptone for 10 h. Subsequently, growth conditions were similar to those described in Chapter III, except that cells were induced with 0.25% peptone and not the peptone inducer.

V. alginolyticus cells were grown in the Tris-HCl buffer containing 2.5% peptone for 4 h (absorbance of 3 at 600 nm, early stationary phase). Cells were harvested rapidly by centrifugation (12,000 g for 2.5 min), washed once in 0.25% peptone and finally resuspended in 10 ml of 0.25% peptone to give an absorbance at 600 nm of 8. Any variations from this method are mentioned with the appropriate figures.

4.2.2. Effect of rifampin and chloramphenicol on collagenase production

Rifampin and chloramphenicol were added to give final concentrations as indicated with the appropriate figures.

4.2.3. Effect of glucose and Casamino Acids on collagenase production

The effect of 0.4% glucose and 0.5% Casamino Acids on collagenase production under conditions of induction by 0.25% peptone were investigated. Glucose and Casamino Acids were added at 2 h seperately or together with rifampin (100 μg ml^{-1}).

4.2.4. RNA and protein synthesis

RNA and protein synthesis were determined by the incorporation of [^3H] uracil (2μg ml^{-1}, 1 μCi ml^{-1}) and [^3H] leucine (2 μg ml^{-1}, 0.6 μCi ml^{-1}), respectively, into trichloracetic acid-precipitable material by the method of Eichenlaub and Winkler (1974). Rifampin and chloramphenicol were added to give final concentrations as indicated. Samples (0.5 ml) were removed from cultures, added to 0.5ml 10% trichloracetic acid (containing 1 mg ml^{-1} of either unlabelled leucine or uracil) and allowed to stand on ice for 30 min. The samples were then filtered, washed with 20 ml 5% trichloracetic acid and 10 ml 1% acetic acid and the filters were dried before counting in a Beckman liquid scintillation counter.

4.2.5. Incorporation of [^{3}H] leucine into collagenase in the presence of rifampin

A washed 4 h peptone culture was resuspended in 0.25% peptone and incubated with shaking for 1.5 h before the addition of rifampin (100 μg ml^{-1}). Incubation was continued for 15 min before the addition of [^{3}H] leucine (2 μCi ml^{-1}) (specific activity, 146 Ci mmol^{-1}). Incubation was continued for 1 h before collagenase was purified as described in Chapter V. Collagenase was eluted stepwise from a DE 52 cellulose column (7.5 by 1.5 cm) by subsequent application of three Tris-HCl buffers (pH 7.0) : 0.2 M - 0.3 M - 0.3 M made 1.0 M in NaCl. The flow rate was 12 ml h^{-1}, and 2 ml fractions were collected. Fractions were assayed for collagenase activity as described in Chapter II. Radioactivity was assayed by the addition of 100 μl samples of each fraction to 10 ml "Insta-Gel" before counting in a Beckman liquid scintillation counter.

4.3 RESULTS

4.3.1. Induction of collagenase by 0.25% peptone.

The induction of collagenase by resuspending washed cells in 0.25% peptone in the Tris-HCl buffer resulted in the production of high (up to 25 nkat ml^{-1}) levels of collagenase. Induction by 0.1% or 0.5% peptone resulted in lower levels of collagenase being produced (2 to 6 nkat ml^{-1}). The addition of low SNP medium to 0.25% peptone decreased the levels of collagenase produced (2 nkat ml^{-1}).

4.3.2. Effect of rifampin and chloramphenicol on collagenase production

When collagenase was induced in cultures containing 2.5% peptone, the addition of rifampin (200 µg ml^{-1}) and chloramphenicol (500 µg ml^{-1}) at 3 h prevented the production of collagenase (Fig. 4.1). When these antibiotics were added at 6 h, collagenase production was not inhibited completely and significant increases in activity occurred. However the effect of rifampin (300 µg ml^{-1}) and chloramphenicol (300 µg ml^{-1}) on the incorporation of [^{3}H] uracil and [^{14}C] leucine, respectively, into trichloracetic acid-precipitable material when added at 6 h indicated that rifampin inhibited RNA synthesis and chloramphenicol inhibited protein synthesis immediately (Fig. 4.2).

Washed cell suspensions resuspended in 0.25% peptone synthesized collagenase linearly for 2 to 3 h after an initial lag of approximately 1 h (Fig. 4.3). When rifampin (100 µg ml^{-1}) was added at 1, 2 or 3 h after resuspension, collagenase synthesis continued for 30 to 60 min after its addition (Fig. 4.3). When rifampin was added immediately after resuspension, no collagenase was produced. These and other similar results indicate that there is an increase in the amount of rifampin-insensitive collagenase produced the later rifampin is added, although as yet it has not been possible to show a linear increase with time. During induction from 1 to 3 h, the absorbance at 600 nm increased from 8.6 to 9.6,

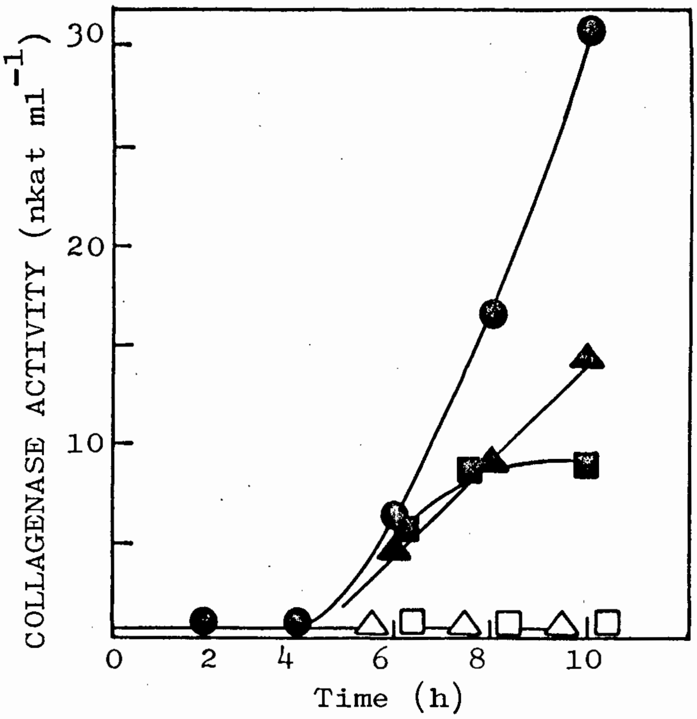


Fig. 4.1: Effect of rifampin and chloramphenicol on collagenase production in 2.5% peptone. No additions (●); 200 µg rifampin ml^{-1} added at 3 h (△) and 6 h (▲); 500 µg chloramphenicol ml^{-1} added at 3 h (□) and 6 h (■).

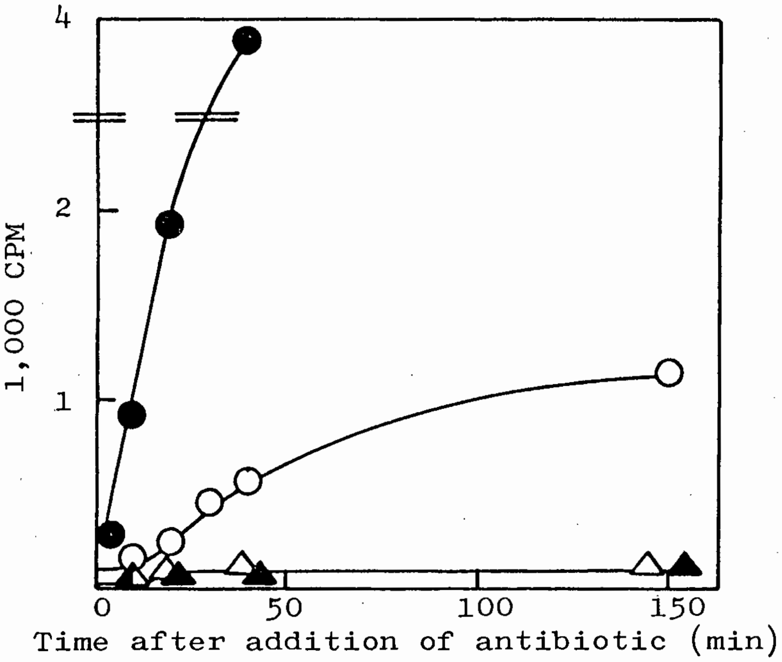


Fig. 4.2: Effects of rifampin and chloramphenicol on the incorporation of [^{3}H] uracil (2 µg ml^{-1}, 0.4 µ Ci ml^{-1}) and [^{14}C] leucine (2 µg ml^{-1}, 0.4 µCi ml^{-1}), respectively, into TCA-precipitable material. [^{3}H] Uracil incorporation: no additions (○); 300 µg rifampin ml^{-1} added at 6 h (△). [^{14}C] leucine incorporation: no additions (●); 300 µg chloramphenicol ml^{-1} added at 6 h (▲).

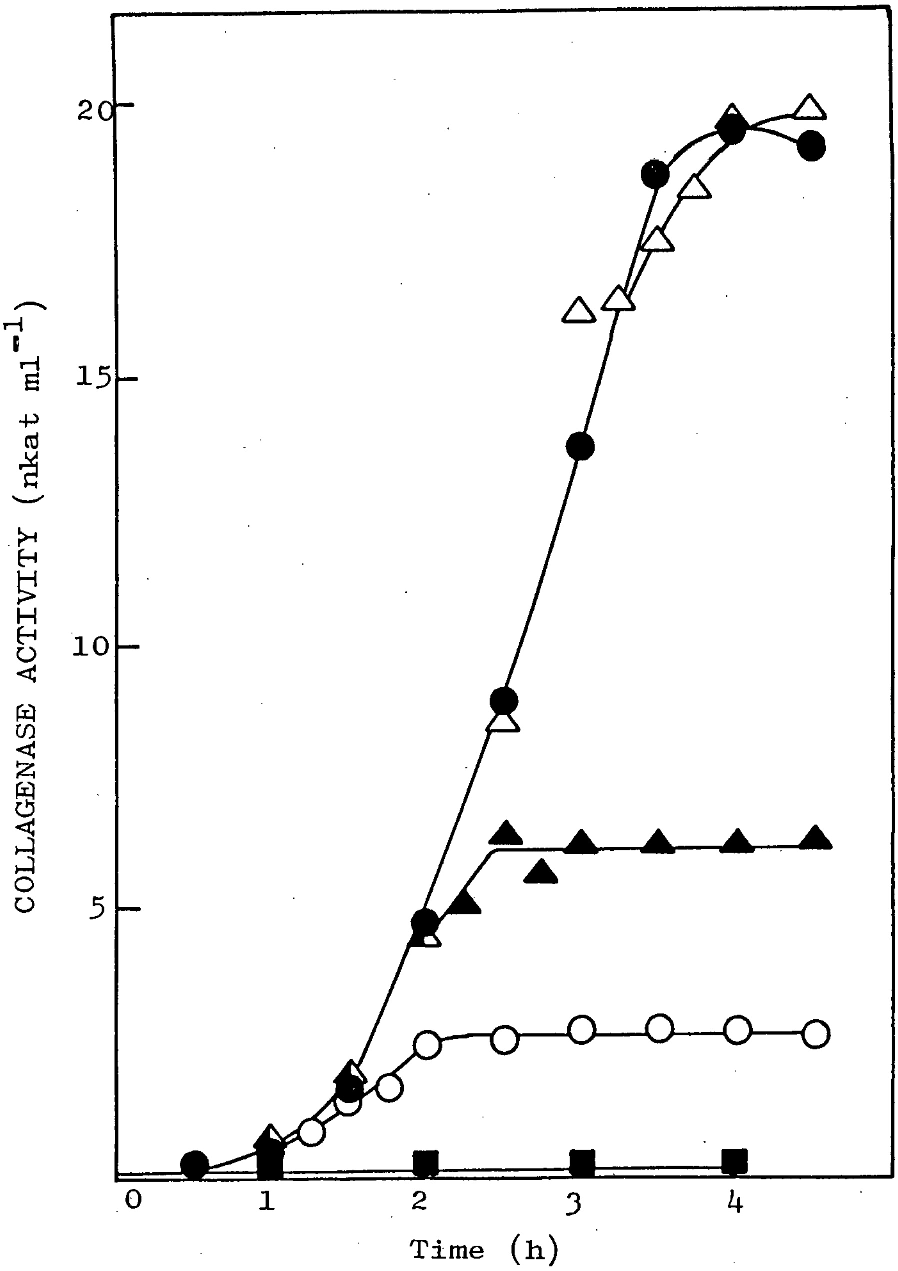


Fig. 4.3: Effect of rifampin on collagenase production in 0.25% peptone. No additions (●). Rifampin (100 μg ml^{-1}) was added at 0 (■), 1 (○), 2 (▲) and 3 h (△) after the resuspension of washed cells.

whereas the amount of rifampin-insensitive collagenase produced during the same period almost doubled. This increase in cell density cannot fully account for the increase in rifampin-insensitive collagenase production.

The addition of chloramphenicol at 0, 1, 2 and 3 h after washed cell suspensions were resuspended in 0.25% peptone medium, completely and immediately abolished the synthesis of collagenase (Fig. 4.4).

Rifampin (100 μg ml^{-1}) added at 1 and 3 h completely inhibited the incorporation of [^{3}H] uracil into trichloracetic acid-precipitable material after washed cell suspensions were resuspended in 0.25% peptone (Fig. 4.5). Chloramphenicol (100 μg ml^{-1}) added at 2 h completely inhibited the incorporation of [^{3}H] leucine into trichloracetic acid-precipitable material (Fig. 4.6). *V. alginolyticus* was resistant to actinomycin D, and this antibiotic therefore could not be used as an inhibitor of mRNA synthesis.

When cultures grown on 2.5% peptone were washed twice with 0.1% peptone and resuspended in 0.1% peptone, collagenase was induced although at a lower level (Fig. 4.7). When rifampin (100 μg ml^{-1}) and chloramphenicol (100 μg ml^{-1}) were added under these conditions of induction the same results were found i.e. chloramphenicol inhibited collagenase synthesis immediately whereas collagenase synthesis continued for 30 min after the addition of rifampin (Fig. 4.7).

No significant difference in the amount of rifampin-insensitive collagenase produced was observed when rifampin was added at concentrations of 10, 50, 100 and

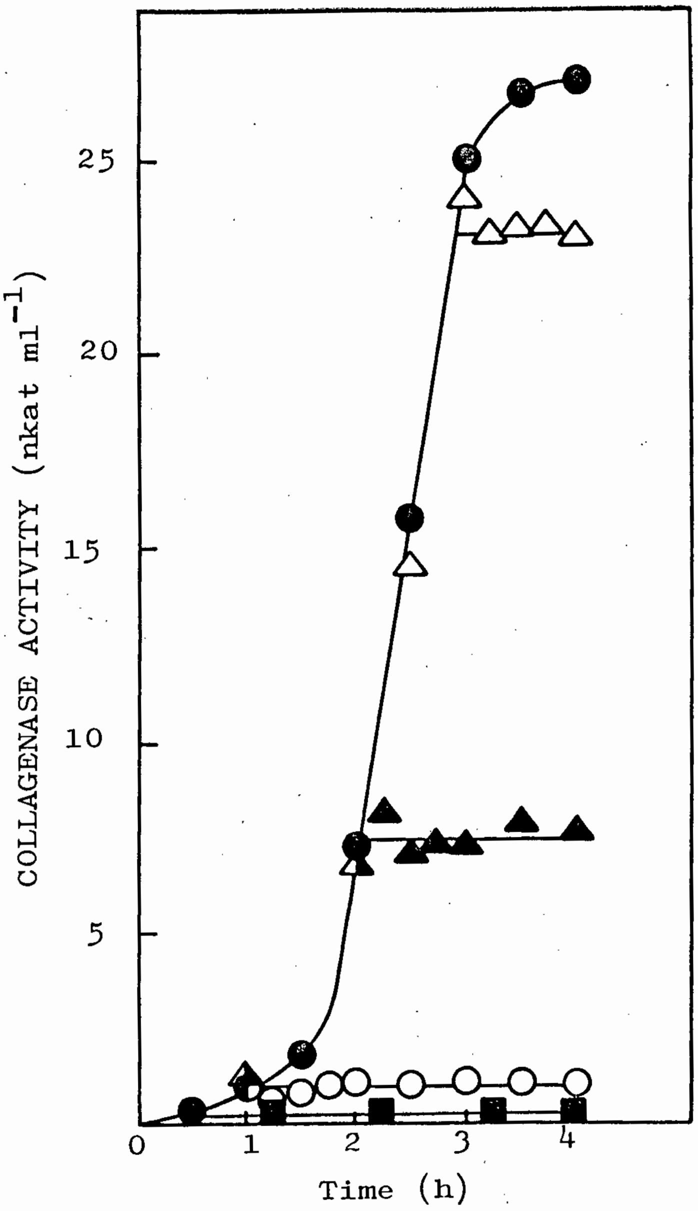


Fig. 4.4: Effect of chloramphenicol on collagenase production in 0.25% peptone. No additions (●). Chloramphenicol (100 µg ml^{-1}) was added at 0 (■), 1 (○), 2 (▲) and 3 h (△) after the resuspension of washed cells.

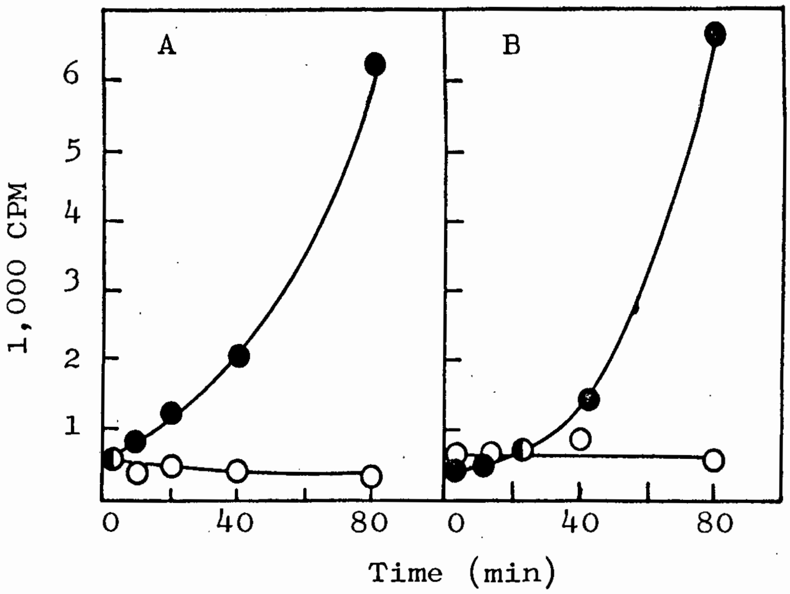


Fig. 4.5: Effect of rifampin on [^{3}H] uracil incorporation as described in the text. (A) [^{3}H] uracil was added at 1 h. No addition of drug (●); rifampin (100 μg ml^{-1}) was added at 1 h (○). (B) ^{3}H uracil was added at 3 h. No addition of drug (●); rifampin (100 μg ml^{-1}) was added at 3 h (○).

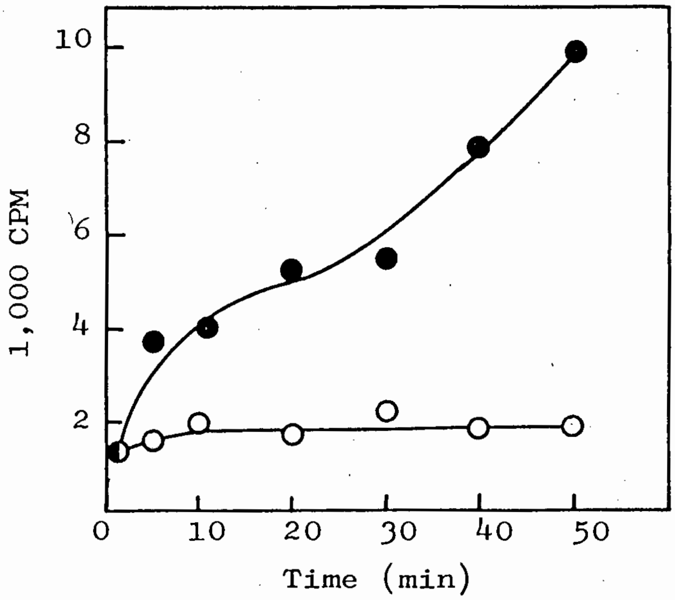


Fig. 4.6: Effect of chloramphenicol on [^{3}H] leucine incorporation as described in the text.

[^{3}H] leucine was added at 2 h. No addition of drug (●); chloramphenicol (100 μg ml^{-1}) was added at 2 h (○).

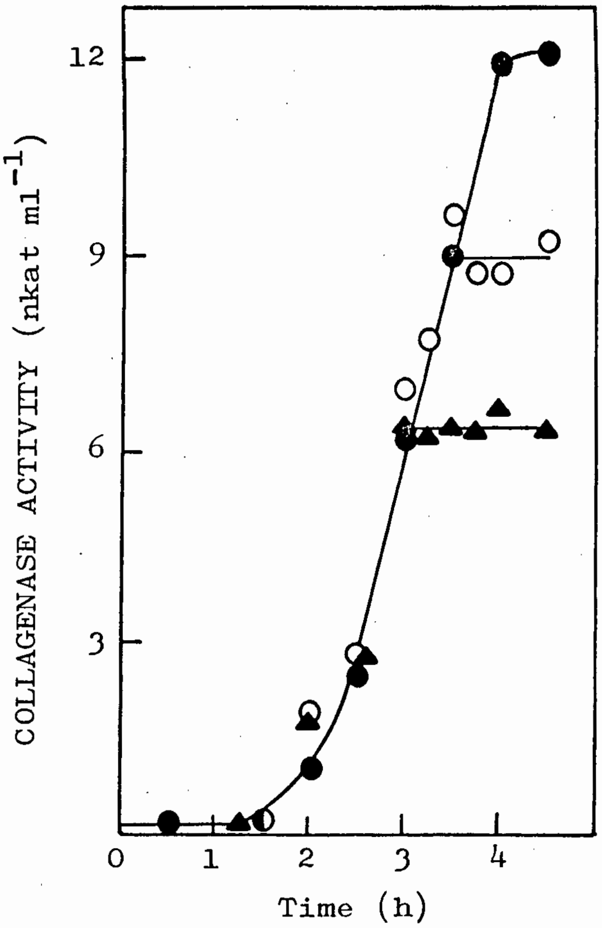


Fig. 4.7: Effect of rifampin and chloramphenicol on collagenase production in 0.1% peptone. No additions (●); rifampin (100 μg ml^{-1}) was added at 3 h (○); chloramphenicol (100 μg ml^{-1}) was added at 3 h (▲).

200 μg ml^{-1} (Fig. 4.8).

4.3.3. Incorporation of [^{3}H] leucine into collagenase in the presence of rifampin.

The elution pattern of [^{3}H] leucine-labelled collagenase gave three peaks of radioactivity and one peak of collagenase activity (Fig. 4.9). The first peak of radioactivity coincided with the elution position of the neutral protease produced by V. alginolyticus (Keil-Dlouha et al., 1976). The second peak of radioactivity coincided with the elution position of an autolytic degradation product of collagenase which shows reduced collagenase activity (Keil-Dlouha, 1976). The third peak of radioactivity coincided exactly with the elution position of collagenase.

4.3.4. Effect of glucose and Casamino Acids on collagenase production

Glucose (0.4%) and 0.5% Casamino Acids added 2 h after cells were resuspended in 0.25% peptone partially repressed rifampin-insensitive collagenase synthesis (Fig. 4.10). However this repression was not immediate and synthesis of the enzyme continued for 30 to 60 min after their addition. The amount of collagenase produced after the addition of glucose or Casamino Acids was less than after the addition of rifampin but more than after the addition of chloramphenicol. Glucose (0.4%) consistently caused greater repression than 0.5% Casamino Acids. The amount of repression was the same whether glucose or Casamino Acids were added separately or together with rifampin (100 μg ml^{-1}) (Fig. 4.11). This repression was not increased by the addition of 0.8% glucose or 1% Casamino Acids.

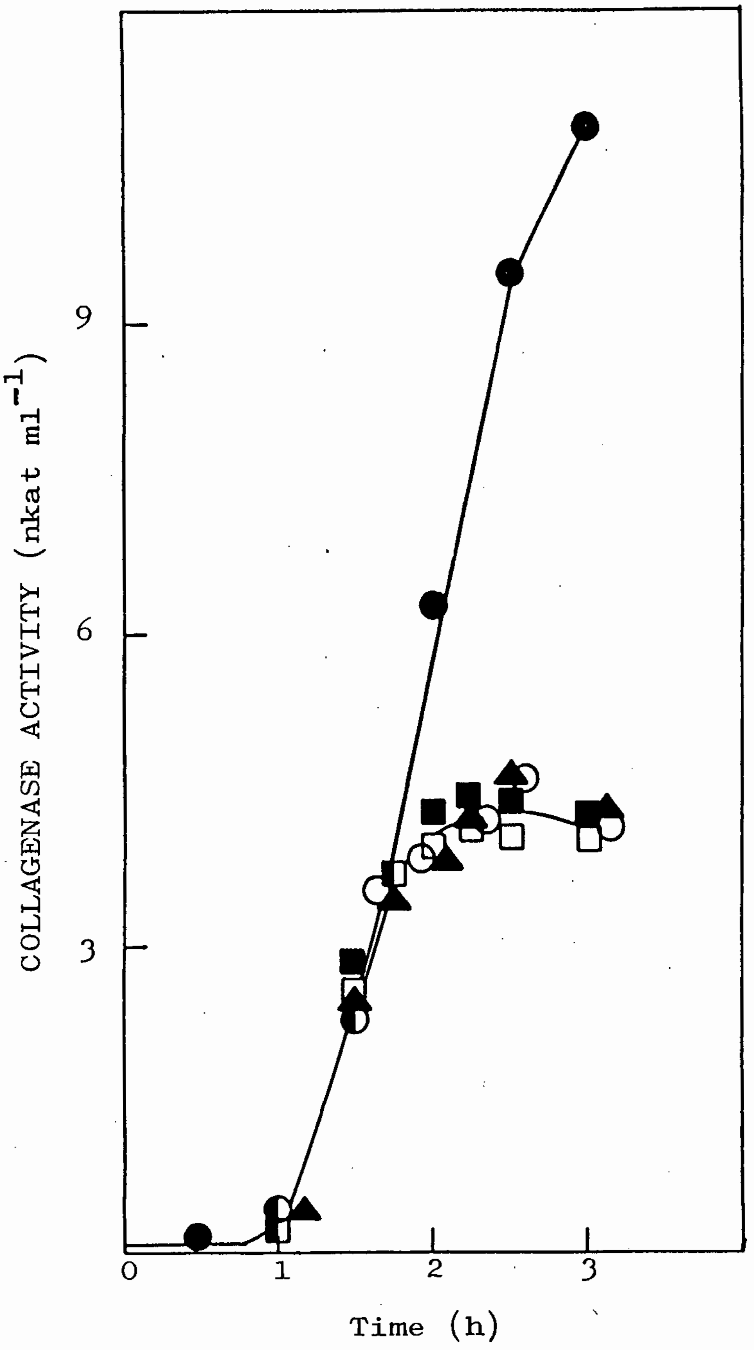


Fig. 4.8: Effect of different rifampin concentrations on collagenase production in 0.25% peptone. No additions (●). Rifampin was added at 1.5 h at concentrations of 10 (○), 50 (□), 100 (▲) and 200 μg ml^{-1} (■).

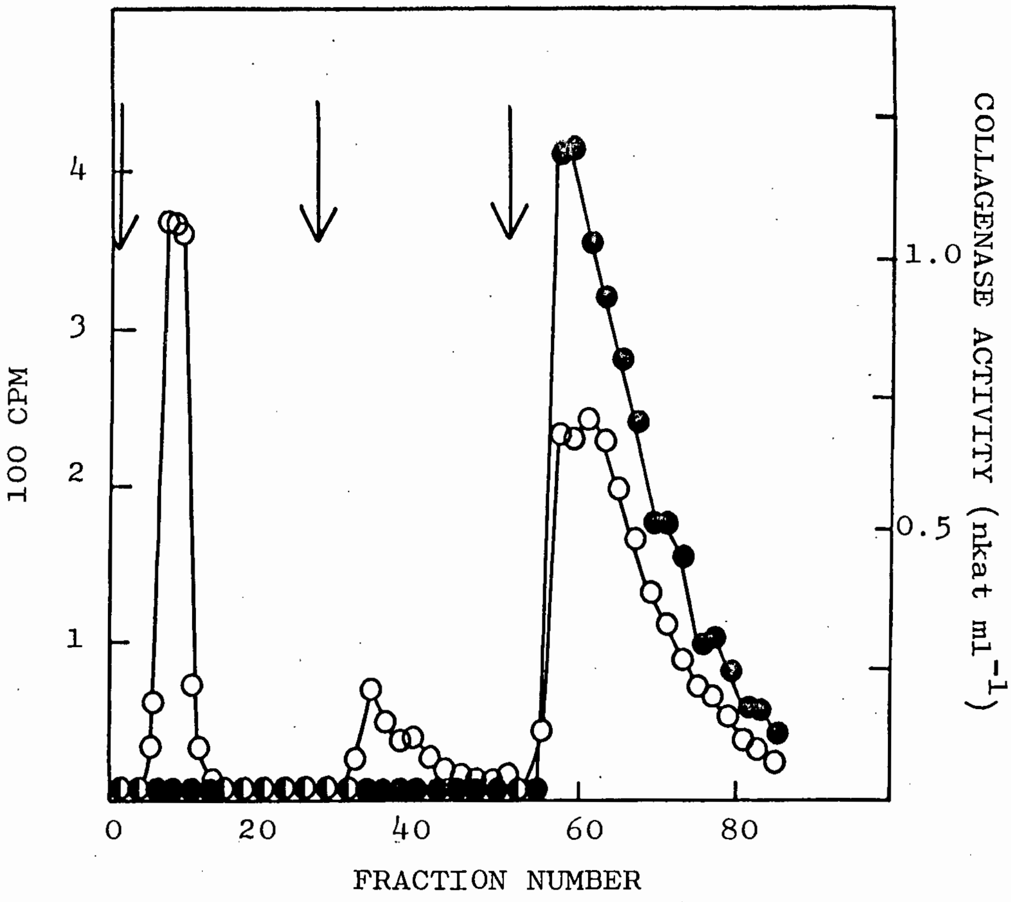


Fig. 4.9: Purification of labelled collagenase on DE 52 cellulose. The arrows mark the stepwise elution as described in the text. Collagenase activity (●), counts per minute (○). The first radioactivity peak coincides with the elution position of the neutral protease, whereas the second coincides with the elution position of an autolytic degradation product of collagenase. The third peak is pure collagenase.

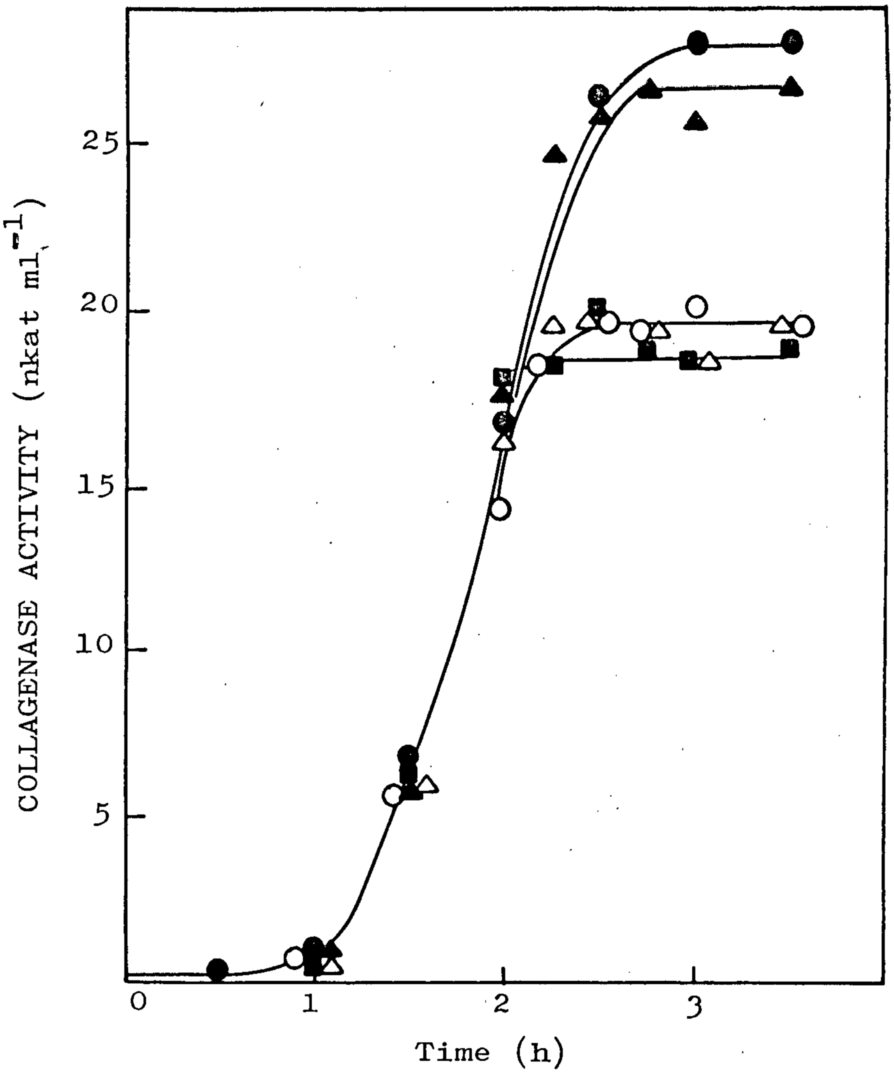


Fig. 4.10: Effect of glucose and Casamino Acids on collagenase production in 0.25% peptone. No additions (●); glucose, 0.4% (△); Casamino acids, 0.5% (○); chloramphenicol, 100 μg ml^{-1} (■); and rifampin, 100 μg ml^{-1} (▲), were added at 2 h, after the resuspension of washed cells.

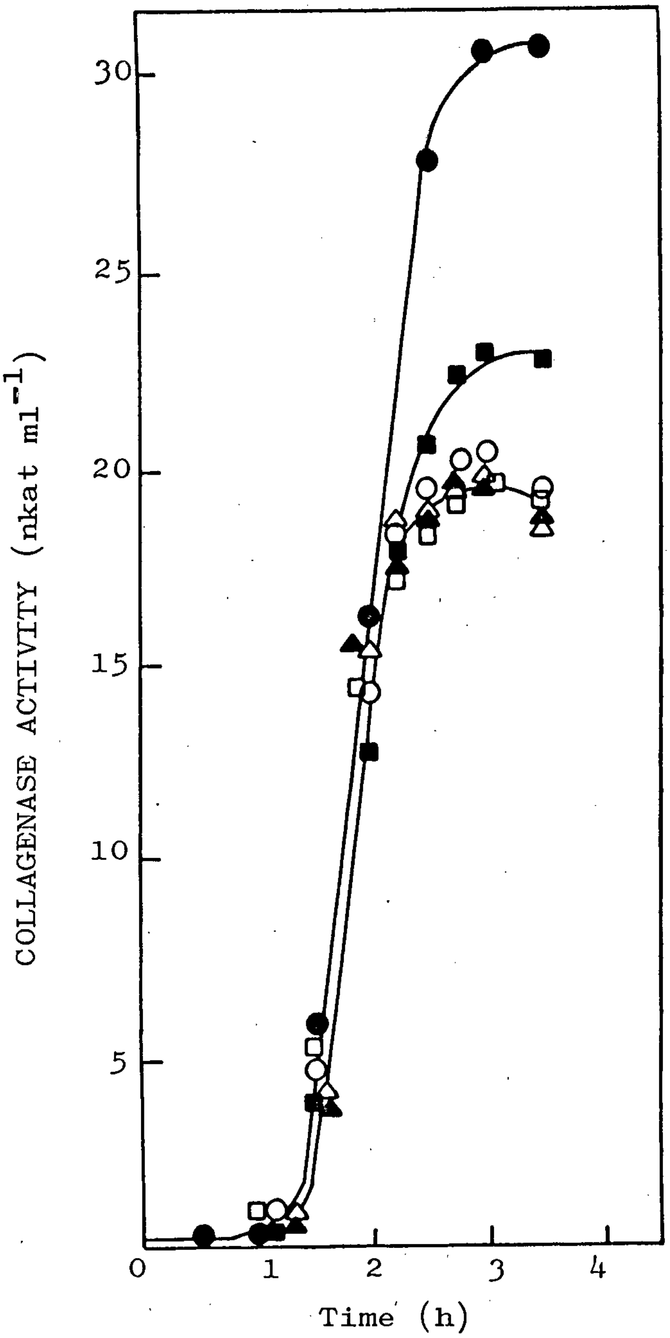


Fig. 4.11: Effect of glucose and Casamino Acids on collagenase production in 0.25% peptone, when added together with rifampin. No additions (●); glucose, 0.4% (△); Casamino Acids, 0.5% (○); rifampin, 100 μg ml^{-1} (■); glucose, 0.4% + rifampin, 100 μg ml^{-1} (▲); Casamino Acids, 0.5% + rifampin, 100 μg ml^{-1} (□), were added at 2 h after the resuspension of washed cells.

4.4 DISCUSSION

The method of resuspending V. alginolyticus cells in 0.25% peptone facilitated studies on the effect of rifampin and chloromphenicol on collagenase production. This method bypassed the need to purify the peptone inducer and consistently induced high yields of collagenase.

Rifampin and chloramphenicol added at 6 h to cultures growing in 2.5% peptone failed to inhibit collagenase production although rifampin inhibited RNA synthesis and chloramphenicol inhibited protein synthesis immediately. It was initially thought that the increase in activity in the culture to which chloramphenicol was added could have been due to the release of preformed enzyme. However this seemed unlikely as ultrasonic disintegration or treatment of cells with toluene did not result in an increase in collagenase activity (Chapter II). The finding that peptone acts as an inhibitor of collagenase (Chapter III) explains these initial results in which linear increases in collagenase activity were observed for up to 4 h after the addition of rifampin or chloramphenicol at concentrations of 300 $\mu g\ ml^{-1}$. The use of 0.25% instead of 2.5% peptone to induce collagenase ensured that the increase in collagenase activity after the addition of rifampin cannot be due to the digestion of peptone, which inhibits collagenase. These results emphasize the care which must be taken when studying the regulation of inducible exoenzymes in complex media.

Inhibition of translation by chloramphenicol and transcription by rifampin affected collagenase production in a way similar to exoenzyme production in late log phase in B. amyloliquefaciens (Both et al., 1972), B. subtilis (Semets et al., 1973), P. maltophilia (Boethling, 1975) and P. lemoignei (Stinson and Merrick, 1974). Chloramphenicol rapidly inhibited collagenase production and protein synthesis, indicating that de novo protein synthesis is required for normal collagenase production. Collagenase can be labelled 15 min after the addition of rifampin, which indicates that rifampin-insensitive collagenase synthesis also involves de novo synthesis of the enzyme. There is a peak of radioactivity, without any collagenase activity at a position coinciding with the elution position of an autolytic degradation product of collagenase. This could be expected as the degradation products of collagenase show reduced collagenase activity (Keil-Dlouha, 1976) and the detection of radioactivity is far more sensitive than the collagenase assay.

V. alginolyticus cells continued to produce collagenase for 30 to 60 min in the presence of rifampin at levels which inhibit RNA synthesis. This type of observation in B. amyloliquefaciens has been used to support a hypothesis which involves a pool or reserve of mRNA (Both et al., 1972; O'Connor et al., 1978). The results obtained with our Vibrio strain are similar to those obtained with B. amyloliquefaciens, where it was shown that the later rifampin was added, the greater was the amount of rifampin-insensitive protease produced. When rifampin was added after 3 h of incubation, at which time maximum rifampin-insensitive collagenase production occurs, [^{3}H] uracil incorporation was no less sensitive to the drug than at 1 h. This indicates that the cells did not become progressively more resistant to rifampin. O'Connor et al. (1978) showed that when B. amyloliquefaciens is transferred from a 0.5% Casamino Acids medium to a 0.025% Casamino Acids medium a mRNA pool (rifampin-insensitive secretion) builds up and reaches in 75 min a value several times greater than that found in the harvested cells. The results obtained with V. alginolyticus suggest that an apparent mRNA pool builds up during the first hour after resuspension and increases over 1 to 2 h.

These results on the production of collagenase also support the hypothesis of O'Connor et al. (1978) which postulates that short-lived mRNA is produced for immediate translation and, in addition, a reserve of stable mRNA is laid down in a nontranslatable form. In the absence of transcription, reserve mRNA is converted to translatable mRNA and accounts for the continued production of enzyme in the presence of rifampin.

Results in Chapter II showed that collagenase production was subject to repression by glucose and Casamino Acids. However when glucose was added at 6 h to a culture growing in 2.5% peptone there was only a slight repression of collagenase synthesis, compared to the almost complete repression when added at 4 h. These findings can be explained in terms of the results in Chapter III and the results in this chapter i.e. when V. alginolyticus is grown in 2.5% peptone the peptone acts as an inhibitor of collagenase and levels of enzyme activity increase with continued incubation, even after the addition of an inhibitor or repressor of enzyme synthesis. In addition, the results in this chapter show that once collagenase production is in progress the repressive effect of glucose and Casamino Acids is not immediate. These findings explain the inability of glucose to repress collagenase synthesis when added at 6 h to a culture growing in 2.5% peptone.

The partial repression of rifampin-insensitive collagenase production by glucose and Casamino Acids suggests that both glucose and Casamino Acids repress collagenase synthesis at a post-transcriptional but pre-translational stage. It cannot, however, be excluded that glucose or Casamino Acids do not inhibit transport or release of collagenase. Priest (1975) showed that glucose represses α-amylase synthesis in B. subtilis at the level of transcription but does not repress rifampin-insensitive α-amylase synthesis. This repression by glucose was not relieved by cyclic AMP. Boethling (1975) however, showed that rifampin-insensitive protease secretion by P. maltophilia could be repressed by either α-ketoglutarate or chloramphenicol, suggesting that α - keto-glutarate is coupled to a translational or posttranslational stage. If the effect of α-ketoglutarate on protease secretion is a reflection of a mechanism similar to cyclic AMP-mediated catabolite repression, this finding is anomalous since catabolite repression is primarily thought to be a transcriptional control. These and the results in Chapter II suggest that collagenase synthesis, like other exoenzymes, is not regulated by classical catabolite repression.

These results support the hypothesis that mRNA accumulation is a general property of bacterial extracellular enzyme synthesis. This applies to both constitutive and inducible exoenzymes produced by gram-positive or gram-negative bacteria. However conclusive proof of the mRNA pool hypothesis still requires direct measurement of the mRNA pool.

CHAPTER V

STUDIES ON THE DEVELOPMENT OF A GENETIC SYSTEM FOR V. ALGINOLYTICUS

Summary: An *in situ* immunoassay for the detection of collagenase producing clones of *V. alginolyticus* was developed. By utilizing a double counter-selection mutagenesis technique, for the selection of auxotrophic mutants, a glutamate auxotroph was selected. No plasmid DNA could be detected in *V. alginolyticus*. Attempts to show plasmid transfer by conjugation of *V. alginolyticus* with *E. coli* J53 (RP4) and *P. aeruginosa* PA08 (R68.45) were unsuccessful. Attempts to show *in vitro* protein synthesis utilizing *V. alginolyticus* S30 extracts were unsuccessful.

5.1 INTRODUCTION

Although genetic manipulation is highly developed, studies on the genetics of exoenzymes have lagged behind those of intracellular enzymes. This is probably because of the lack of true extracellular enzymes in *E. coli*. However there have been considerable advances with studies on the genetics of the outer membrane proteins of *E. coli* and in particular the outer membrane lipoprotein (DiRienzo *et al*., 1978). The greatest understanding of genetic regulation of extracellular enzyme synthesis has been obtained from transformation studies with *Bacillus* spp., (Glenn 1976; Priest 1977). Specifically, the α-amylase, proteases and penicillinase have received most attention, and the α-amylase of *B. amyloliquefaciens* H, has been cloned into B. subtilis via the specialized transducing phage Ø3T (Yoneda et al., 1979). The bacteriophage carrying the gene(s) encoding α-amylase lysogenized in B. subtilis with normal efficiency and resulted in the production of the B. amyloliquefaciens α-amylase which is electrophoretically different from that of B. subtilis.

Implicit in any consideration of gene cloning is the selection of a suitable cloning vector. Initial studies, therefore, were directed at determining whether or not V. alginolyticus harboured a plasmid of its own. A naturally occurring plasmid should be more stable in and more readily transferable between mutants of this strain than plasmids isolated from other sources.

R plasmids belonging to the compatibility class P have been shown to be transferable between different genera of bacteria (Sykes and Richmond, 1970; Datta et al., 1971; Datta and Hedges, 1972; Beringer, 1974; Towner and Vivian, 1977) and thus transfer of the P group plasmids RP4 and R68.45 from E. coli J53 and P. aeruginosa PA08 respectively to V. alginolyticus was also attempted.

The isolation of various mutants is essential in any genetic study and for this purpose a double counter-selection technique was developed. This method was used in an attempt to isolate glutamine auxotrophs in order to further implicate glutamine synthetase in the regulation of collagenase.

An in situ immunoassay was developed for the detection of collagenase mutants. This technique also has the advantage of being able to detect non-functional cloned gene products and as such is a powerful tool in this type of experiment (Skalka and Shapiro, 1976).

The development of a defined cell-free translation system would also facilitate studies on the regulation of collagenase synthesis or the cloning of the collagenase gene(s). In vitro synthesis of several exoenzymes including the alkaline phosphatase of E. coli (Dohan et al., 1971), the penicillinase of B. licheniformis (Dancer and Lampen, 1975) and the α-amylase of B. amyloliquefaciens (Coleman, 1970) has been reported. In addition, the mRNA for the outer membrane lipoprotein of E. coli which is relatively long-lived has been extensively purified and translated in a cell-free system (Hirashima et al., 1974). However the major technical problem in experiments involving the isolation of RNA is one of preventing RNA hydrolysis by ribonucleases (Iynedjian, 1979) and thus the isolation of short-lived bacterial mRNA species is difficult.

The experiments in this chapter were undertaken with the view of developing a genetic system for further studies on the regulation of collagenase synthesis. In the absence of a genetic system for V. alginolyticus the possibility of eventually cloning the collagenase gene(s) into a host such as E. coli was also borne in mind. However it is not known exactly how many genes are responsible for the regulation of collagenase synthesis and the linkage or lack thereof between them and therefore the cloning of "collagenase" might prove to be somewhat difficult.

5.2 MATERIALS AND METHODS

5.2.1 Purification of Collagenase

The method of collagenase purification was adapted from the methods of Lecroisey et al. (1975), Keil-Dlouha (1976) and Keil-Dlouha and Keil (1978).

V. alginolyticus cultures (200 ml of 1% peptone in the Tris-HCl buffer in 2 l boiling flasks) were grown for 9h before centrifugation at 16,000 g for 10 min. The supernatant was made 60% saturated with $(NH_4)_2SO_4$ by the addition of 390 g $(NH_4)_2SO_4$ l^{-1}, and allowed to precipitate overnight at 4°C. The precipitate was collected by centrifugation at 16,000 g for 30 min and resuspended in stabilizing buffer (0.3 M Tris-HCl pH 7.0, 1 M NaCl, 10 mM $CaCl_2$, 0.1 M histidine and 10^{-4} M p-chloromercuribenzoate [pCMB]). Crude collagenase was stable for up to 3 weeks in stabilizing buffer at 4°C.

All purification steps were performed at 4°C. Collagenase was precipitated out of the stabilizing buffer by 60% saturation with $(NH_4)_2SO_4$, resuspended in a small volume of 50 mM Tris-HCl buffer pH 7.0 containing 10 mM $CaCl_2$ and 10^{-4} M pCMB, and dialysed against three changes of the same buffer. The insoluble fraction was removed by centrifugation and discarded. The crude collagenase prepared from 10 l of culture (approximately 200 mg) was then applied to a DE 52 cellulose column (1.5 by 15 cm) equilibrated with the same buffer. The flow rate was 6 ml h^{-1} and 4 ml fractions were collected. A stepwise elution was made at pH 7.0 by subsequent application of three Tris-HCl buffers: 0.2 M - 0.3 M - 0.3 M made 1 M in NaCl. All buffers contained 10 mM $CaCl_2$ and 10^{-4} M pCMB. The collagenase activity of the eluted fractions was assayed as described in Chapter II.

Fractions under the peak of collagenase activity eluted by the third buffer (0.3 M Tris-HCl, 1 M NaCl) were pooled and the collagenase was precipitated by 60% saturation with $(NH_4)_2SO_4$. The precipitate was resuspended in 2 ml of the third buffer, clarified by centrifugation and layered on a Sephadex G100 column (2 by 60 cm) equilibrated with the same buffer. The flow rate was 10 ml h^{-1} and 5 ml fractions were collected. Fractions under the peak of collagenase activity were concentrated and desalted by ultrafiltration using an "Amicon X M 50 Diaflo Ultrafilter", before lyophlization. Collagenase used for the preparation of antiserum was dialysed overnight against 10 volumes of 0.2% formalin before being lyophilized.

5.2.2 Preparation of Antiserum

Rabbits were immunized by weekly intramuscular injections of an emulsion of 0.5 mg of formalinised collagenase in Freunds' incomplete adjuvant. The rabbits were bled at weekly intervals and the serum was stored at -20°C. The titre of the antisera were monitored utilizing the double-diffusion-in-gel method of Ouchterlony (1958). Ouchterlony plates were prepared using 1% "Oxoid" agar No. 2 in 50 mM Tris-HCl buffer (pH 7.0) containing 0.85% NaCl. Serial dilutions of the antisera were tested against various dilutions of pure collagenase, the initial collagenase concentration being 1 mg ml^{-1}.

5.2.3 Inactivation of collagenase by antiserum.

Inactivation of collagenase by antiserum was determined in 0.04 M Tris-HCl buffer (pH 7.6) containing 0.85% NaCl. The synthetic peptide substrate was made up in the same buffer. Although collagenase is more stable in 2.34% (0.4 M) NaCl, this concentration of salt interferred with the collagenase-antibody reaction. Antiserum with a titre of $^1/16$ (determined against a collagenase concentration of 0.25 mg ml^{-1}) was diluted two-fold with a solution of purified collagenase (443 nkat mg^{-1}) to give final concentrations of collagenase from 2.5 to 25 µg ml^{-1}. Collagenase activity was assayed after incubation for 5 min at room temperature.

5.2.4 Development of an *in situ* immunoassay for collagenase producing clones of *V. alginolyticus*

V. alginolyticus has a tendency to swarm on agar plates containing various nutrients and therefore initial experiments involved determining a suitable nutrient which would both induce collagenase and maintain isolated colonies. Optimum conditions were obtained by reducing the NaCl concentration to 0.5% and by using 0.05 to 0.1% peptone supplemented with 1 to 2% of the macromolecular fraction of peptone. This fraction was prepared by exhaustive dialysis of peptone followed by lyophilisation. The effect of including 0.4% glucose or 0.4% Casamino Acids in the agar plates was also investigated. Agar plates were made up with 1% "Oxoid" agar no. 2 in 50 mM Tris-HCl buffer (pH 7.6).

Antiserum with a titre of $^1/16$ was included at a concentration of 20% just before the plates were poured. Plastic petri dishes with a diameter of 55 mm were used in order to conserve antibody. Agar (3ml) was added to each plate to provide a layer of approximately 2 mm thick to allow for the sensitive detection of antibody-antigen precipitates around the colonies (Skalka and Shapiro, 1976). Plates were allowed to dry (shut) for 2 h at 37°C. Wild type V. alginolyticus and a spontaneous mutant which produced reduced levels of collagenase in liquid medium were stabbed onto agar plates and incubated at 30°C for up to 4 days. In order to prevent the agar plates from drying out, they were incubated in plastic containers lined with moist filter paper. Plates were examined daily for the presence of zones of precipitation around colonies and were photographed with indirect lighting against a black background.

5.2.5 Mutagenesis of V. alginolyticus

Cells from a log phase minimal medium (as in Appendix A) culture were harvested by centrifugation and resuspended in 0.2 M Tris-Maleic buffer pH 7.6 containing 0.4 M NaCl, 0.4 mM $MgSO_4$, 7.5 mM $(NH_4)_2SO_4$ and 2 mM sodium citrate. NTG (N-Methyl-N'-Nitro-N-Nitrosoguanidine) was added to give a final concentration of 50 or 100 $\mu g\ ml^{-1}$ and the cells were incubated at 30°C for 20 min. Samples were removed at intervals, washed twice in the Tris-Maleic buffer, diluted and plated on tryptone agar plates (as in Appendix A).

5.2.6 Induction of auxotrophic mutants

In attempts to isolate glutamine auxotrophs, V. alginolyticus cells were treated as above with 100 µg ml^{-1} of NTG for 15 min. NTG was removed by washing with Tris-Maleic buffer and glutamine auxotrophs were selected by a double counter-selection technique using both D-cycloseine and ampicillin, adapted from the methods of Gorini and Kaufman (1960), Curtis et al. (1965), Hopwood (1970), Tubb (1974) and Streicher et al. (1975).

(i) The NTG treated cells were inoculated into minimal medium containing 0.5% glutamine (absorbance at 600 nm of 0.15, approximately 2 x 10^8 cells ml^{-1}) and incubated at 30°C for 6 h (absorbance of 1.2). (ii) Cells were washed twice in minimal medium before inoculation into minimal medium containing 0.1% glutamate (absorbance of 0.2). (iii) After 3 h the cells were diluted in minimal medium containing 0.1% glutamate (absorbance of 0.2) and ampicillin and D-cycloserine were added each at a final concentration of 100 µg ml^{-1}. (iv) Incubation was continued for 1 h to allow the growing cells to be converted to sphaeroplasts which are stabilized in 2.34% NaCl. (v) Sphaeroplasts were lysed by washing in 0.85% NaCl and resuspending in 0.85% NaCl for 15 min. (vi) Unlysed cells were then harvested and resuspended in 2.34% NaCl and steps (i) to (vi) were repeated. Finally cells were plated on minimal medium agar plates containing a limiting amount of glutamine (0.5 µg ml^{-1}). The smaller colonies were then replica-plated onto minimal medium and minimal medium supplemented with 20 μg glutamine ml^{-1}. Glutamine solutions were freshly prepared, filter sterilized and added to the agar plates just before use.

5.2.7 Plasmid isolation studies

The method of Grinsted *et al*. (1977) was used to determine whether *V. alginolyticus* contains a plasmid. Cells were harvested from an overnight 500 ml 1% peptone culture and resuspended in 25 ml of 25% sucrose in 0.05 M Tris-HCl buffer (pH 8). Lysozyme (1.75 ml of a 10 mg ml^{-1} solution in 0.25 M Tris-HCl, pH 8) was then added and the cells were incubated at 30°C for 30 min. Cells were kept on ice for 5 min. EDTA (6.5 ml of a 0.25 M solution) was then added and cells were kept on ice for a further 5 min. Triton X-100 (12.5 ml of a 2% solution in 0.05 M Tris-HCl, pH 8) was added and the solution kept on ice for 20 min. The solution was then centrifuged at 37,000 g at 4°C for 30 min and the supernatant carefully removed. NaCl (0.1 volume of a 5 M solution) was added to the supernatant. Polyethylene glycol (PEG) 6000 (the gram equivalent of 0.1 volume) was added and the solution left at 4°C overnight. The solution was shaken gently to dissolve the PEG. The PEG precipitate was harvested by centrifugation (3,000 g for 10 min) and resuspended in 4 ml of SSC buffer (0.15 M NaCl, 0.015 M sodium citrate). PEG was removed by gently mixing with 2% octanol in chloroform. The solution was centrifuged at 10,000 g for 15 min and the upper aqueous phase removed. CsCl (1 g ml^{-1}) was added and the refractive index adjusted to approximately 1.392.

Ethidium bromide was then added to a final concentration of 0.2 mg ml^{-1} and the solution was centrifuged at 145,000 g at 6 to 10°C for 48 h. The DNA bands were visualised under ultra violet (UV) irradiation. E. coli J53 (RP4) was used as a positive control. When no plasmid band was visible under UV irradiation the fraction of the CsCl gradient where a plasmid would be expected to band, was subjected to restriction analysis by agarose gel electrophoresis (Grinsted et al., 1977) or alternatively labelled with $[^{32}P]$ by nick translation (Maniatis et al., 1975) before agarose gel electrophoresis and autoradiographed as below.

The ethidium bromide was removed by extraction with isopropanol saturated with CsCl. The DNA was precipitated by adding to a 5 ml volume, 0.6 ml of 3 M sodium acetate, 1 mM EDTA (adjusted to pH 6 with glacial acetic acid) and 3 ml isopropanol. The solution was mixed by inverting several times and placed at -20°C overnight. The DNA precipitate was resuspended in 0.5 ml 10 mM Tris-HCl (pH 8), 0.1 mM EDTA and stored at 4°C.

The plasmid DNA was digested by incubation of 20 µl DNA with 10 µl 150 mM Tris-HCl (pH 7.6), 15 mM $MgSO_4$ and 1 µl Eco R1 (30 units ml^{-1}) at 37°C for 30 min. The reaction was stopped by the addition of 15 µl 40% sucrose, 0.1 M EDTA, 0.15 mg ml^{-1} bromophenol blue.

Samples (20 µl) of plasmid DNA were electrophoresed in a 1% agarose gel in 0.09 M Tris-Borate buffer, pH 8.6, containing 2.5 µg ethidium bromide ml^{-1} (Tris-Borate buffer x 10 contained : Tris 54 g, EDTA 4.6 g, boric acid 27.5 g in 500 ml H_2O). Electrophoresis was at 25 V for 18 h. Gels were visualised under UV irradiation.

Plasmid DNA was labelled using a nick translation kit (Bethesda Research Laboratories, Inc.). To 10 µl 10 x translation buffer was added 2 µl of each dTTP, dCTP, dGTP and dATP, 5 µl [^{32}P] ATP, 75 µl DNA and 1 µl DNase I. The solution was incubated for 10 min at 15°C before adding 2 µl of <u>E. coli</u> DNA polymerase I. Incubation was continued for 1 h at 15°C. The reaction was terminated by the addition of 10 µl BRL stop buffer. Gel electrophoresis of the labelled DNA was performed as above and gels were autoradiographed using Kodak X-Omat MA X-ray film. The film was clamped together with the agarose gel and placed at -20°C for 48 h. After exposure the film was developed according to the manufacturers instructions.

5.2.8 Conjugation of V. alginolyticus rif^{r} with plasmid carrying strains of E. coli and P. aeruginosa.

A rifampin resistant mutant of <u>V. alginolyticus</u> was isolated by plating unmutated cells on rifampin gradient plates. A mutant was obtained which was resistant to 100 µg ml^{-1} of rifampin. This mutant produced normal levels of collagenase when compared to the wild type strain.

The effect of NaCl concentration on the ability of <u>V. alginolyticus</u> rif^{r} to plate on minimal and Luria agar plates containing kanamycin was determined. This was done by plating 0.1 ml of a cell suspension containing 5×10^{9} cells ml^{-1} on plates containing various concentrations of kanamycin (15 to 55 µg ml^{-1}) and 1 or 2.34% NaCl under similar conditions to those used in the conjugation experiments.

V. alginolyticus rif^r was crossed with E. coli J53 (RP4) (obtained from Prof. M.H. Richmond, Bristol University, U.K.) and P. aeruginosa PA08 (R68.45) (obtained from Prof. J.A. Thomson, University of the Witwatersrand, R.S.A.) by the filter technique and examined for the transfer of R plasmids by selection for antibiotic resistance. The P group plasmids RP4 and R68.45 both carry resistance markers to the antibiotics kanamycin, ampicillin and tetracycline.

Log phase cultures of donor and recipient strains were mixed (2 ml each of donor and recipient) and filtered on a 0.45μ Millipore filter. Donors were grown in Luria broth (1% NaCl) and the recipient was grown in Luria salt broth (2.34% NaCl). After filtration the filter discs were incubated on Luria agar plates (1% NaCl) at 30°C. When the donor was P. aeruginosa the filter disc was transferred after 2.5 h from Luria agar to minimal medium agar plates containing 50 μg ml^{-1} rifampin and 100 μg ml^{-1} kanamycin. Incubation was continued overnight. When the donor was E. coli the filter disc was left on Luria agar overnight. Controls consisted of donor and recipient strains treated similarly. After overnight incubation the cells were scraped off the filter discs, diluted and plated on the appropriate selection media. When selecting for exconjugants, dilutions were performed in 2.34% NaCl, whereas in the control experiments, donors were diluted in 0.85% NaCl and V. alginolyticus was diluted in 2.34% NaCl. All agar plates contained 1% NaCl.

P. aeruginosa and E. coli were selected for on Luria agar containing 50 µg ml^{-1} kanamycin. V. alginolyticus was selected for on minimal medium containing 50 µg ml^{-1} rifampin. Exconjugants were selected for on minimal medium containing (a) 50 µg ml^{-1} rifampin and 100 µg ml^{-1} kanamycin or (b) 50 µg ml^{-1} rifampin and 25 µg ml^{-1} tetracycline or (c) 50 µg ml^{-1} rifampin, 50 µg ml^{-1} kanamycin and 25 µg ml^{-1} tetracycline.

5.2.9 In vitro protein synthesis.

The preparation of S-30 extracts of V. alginolyticus.

The method of Legault-Demare and Chambliss (1974) was followed. Cells were grown for 2 h in 1% peptone, poured over ice and harvested by centrifugation. All further manipulations were performed at 4°C. Cells were washed twice with Buffer I (0.01 M Tris-HCl pH 7.5, 0.01 M $MgCl_2$, 1.0 M KCl, 5 mM EDTA [dipotassium salt], 0.01 M mercaptoethanol and 10% glycerol) and once with Buffer II (Buffer I with the KCl concentration reduced to 0.05 M). The cells were resuspended (2 to 3 times the wet weight of cells) in Buffer III (0.01 M Tris-HCl pH 7.5, 0.01 M $MgCl_2$, 0.06 M NH_4Cl, 5 mM EDTA [dipotassium salt], 5 mM mercaptoethanol, 10% glycerol and 3.45 mM phenylmethylsulfonylfluoride). The cells were broken by passage through a "Yeda press" at 20,000 psi. Deoxyribonuclease I (Sigma) was added to 1 µg ml^{-1} after which the lysate was centrifuged once at 15,000 g for 10 min and then twice at 30,000 g for 20 min each time. The supernatant fluid (S-30 extract) of the last centrifugation was dialysed against Buffer III for 4 h frozen and stored in liquid nitrogen until use.

Extraction of RNA from log and stationary phase cells

The method of Legault-Demane and Chambliss (1974) was followed. Cells from log and stationary phase cultures were poured over ice and harvested by centrifugation before being resuspended in $^1/100$ of the original culture volume of Buffer IV (0.01 M Tris-HCl pH 7.5, 0.01 M KCl, 5 mM $MgCl_2$ and 0.01 M NaN_3). After the addition of sodium dodecyl sulphate to 1% and heparin to 0.1% the cell suspension was mixed with an equal volume of water-saturated meta-cresol. The cells were broken by passage of the meta-cresol-cell suspension mixture through a chilled "Yeda-press" (20,000 psi). After centrifugation (27,000 g for 5 min) of the resultant lysate, the upper aqueous phase was decanted,made to 0.1 M NaCl, precipitated by the addition of 2 volumes of ethanol, and stored at -20°C for several hours. The precipitated RNA was collected by centrifugation and dissolved in 0.1 M sodium acetate buffer, pH 5. This was then extracted twice at 37°C for 10 min with equal volumes of water-saturated phenol. The RNA was precipitated from the aqueous phase by making it to 2 M NaCl, adding 1.5 volumes of ethanol and storing the solution at -20°C overnight. The precipitated RNA was washed three times with 0.01 M Tris-HCl pH 7.5 containing 0.01 M NaCl and 70% ethanol, dissolved in water and clarified by centrifugation at 27,000 g for 20 min. After dialysis against deionized water for 4 h the concentration of RNA was adjusted to 2 mg ml^{-1} and the solution was frozen in small samples and stored in liquid nitrogen.

In Vitro protein synthesis.

The method used was adapted from those of Nirenberg and Matthaei (1961) and Legault-Demare and Chambliss (1974). The reaction mixture was 72 mM Tris-HCl pH 7.5, 72 mM NH_4Cl, 12 mM magnesium acetate, 2.4 mM dithiothreitol, 2 mM adenosine triphosphate (ATP), 0.1 mM guanosine triphosphate (GTP), 5 mM phosphoenolpyruvate, 20 µg of pyruvate kinase ml^{-1}, a mixture of 20 amino acids (each at 72 µM), 18 µM [^{35}S] methionine (34 counts min^{-1} picomole^{-1}), 50 µg of calcium folinate ml^{-1}, 0.6 mM EDTA (dipotassium salt). The final volume was 0.2 ml per tube. The amount of mRNA and S-30 extract added was varied between 10 to 40 µl per assay and the NaCl concentration was varied between 0 and 0.4 M.

Incubations were for 60 min at 30°C. The extent of amino acid incorporation was determined by measuring the radioactivity remaining insoluble after trichloracetic acid-precipitation. Samples (50 µl) were removed at intervals and added to 0.5 ml 10% TCA (containing 1 mg ml^{-1} of unlabelled methionine) at 0°C for 30 min. Samples were then filtered, washed with 20 ml 5% TCA and 10 ml ethanol and counted in a liquid scintillation counter.

5.3 RESULTS

5.3.1 Purification of Collagenase

Collagenase activity in 200 ml cultures of 1% peptone reached levels of about 13 nkat ml^{-1} after 9 h incubation (Table 5.1). This level of activity could not be increased by increasing or decreasing the peptone concentration and was comparable with the levels of collagenase activity obtained in the better aerated 10 ml cultures containing 1% peptone but was about half of that in 10 ml cultures containing 2% peptone (Table 5.1). Cultures larger than 200 ml resulted in reduced levels of collagenase activity.

Purified collagenase with a specific activity of 450 to 750 nkat mg^{-1} was obtained. The yield was approximately 2 mg per litre of cell culture.

5.3.2 Inactivation of collagenase by antiserum

Antisera with a titre of $^{1}/16$ were obtained when tested against collagenase at a concentration of 0.25 mg ml^{-1}. A two-fold dilution of this antiserum completely inhibited the collagenase activity of a solution containing 2.5 μg ml^{-1} (specific activity 443 nkat mg^{-1}) (Table 5.2). This antiserum caused 86% inhibition of the collagenase activity of a solution containing 25 μg ml^{-1} (Table 5.2).

5.2.3 Development of an *in situ* immunoassay for collagenase producing clones of *V. alginolyticus*.

The development of immunoprecipitates as halos around mutant and wild type colonies of *V. alginolyticus* is shown

Table 5.1: Effect of peptone concentration on collagenase production in 10 and 200 ml culture volumes.

Time of assay (h)	Collagenase activity (nkat ml^{-1})							
	Culture volume							
	10 ml				200 ml			
	Peptone concentration (% w/v)				Peptone concentration (% w/v)			
	2	1	0.5	0.25	2	1	0.5	0.25
7	8.1	6.9	5.2	2.8	7.3	8.5	4.9	2
8	16.5	12.2	6.3	2.6	11.4	12.1	5.7	1.8
9	23.2	12.3	6.4	2.4	11.6	13	0.52	1.1

Table 5.2: Inhibition of collagenase with antiserum. Antiserum, with a titre of $^{1}/16$, was diluted two-fold with a solution of purified collagenase as described in the text.

Collagenase concentration (µg ml^{-1})	% Inhibition of collagenase activity
2.5	100
5	96
25	86

in Fig. 5.1. Halos were visible after two days incubation but became more distinct after 4 days. Agar plates containing both 0.1% peptone and 2% of the macromolecular fraction of peptone (dialysed peptone), promoted more rapid growth and induced halo formation earlier than did plates containing 0.05% peptone and 1% dialysed peptone (Fig. 5.1). Plates containing only 1% peptone as nutrient and inducer gave isolated colonies but the halos were indistinct (results not shown).

Halo formation by the mutant of V. alginolyticus which produced reduced levels of collagenase in liquid medium, was induced more readily in plates containing 2% dialysed peptone than 1% (Fig. 5.1). In addition, zones of precipitation were also observed to form more readily in the area between two adjacent colonies of this mutant or between adjacent mutant and wild type colonies than around isolated mutant colonies. Isolated colonies of the mutant did not produce zones of precipitation after 4 days incubation on plates containing 0.05% peptone and 1% dialysed peptone.

Glucose (0.4%) completely repressed halo formation around mutant and wild type colonies whereas 0.4% Casamino Acids had little effect (Fig. 5.2). Both glucose and Casamino Acids enhanced the growth of colonies considerably.

5.3.4 NTG mutagenesis of V. alginolyticus

The lethal effect of 50 and 100 $\mu g\ ml^{-1}$ NTG is shown in Fig. 5.3. NTG (100 $\mu g\ ml^{-1}$) caused 90% killing in 17 min and this dose was used for mutagenesis.

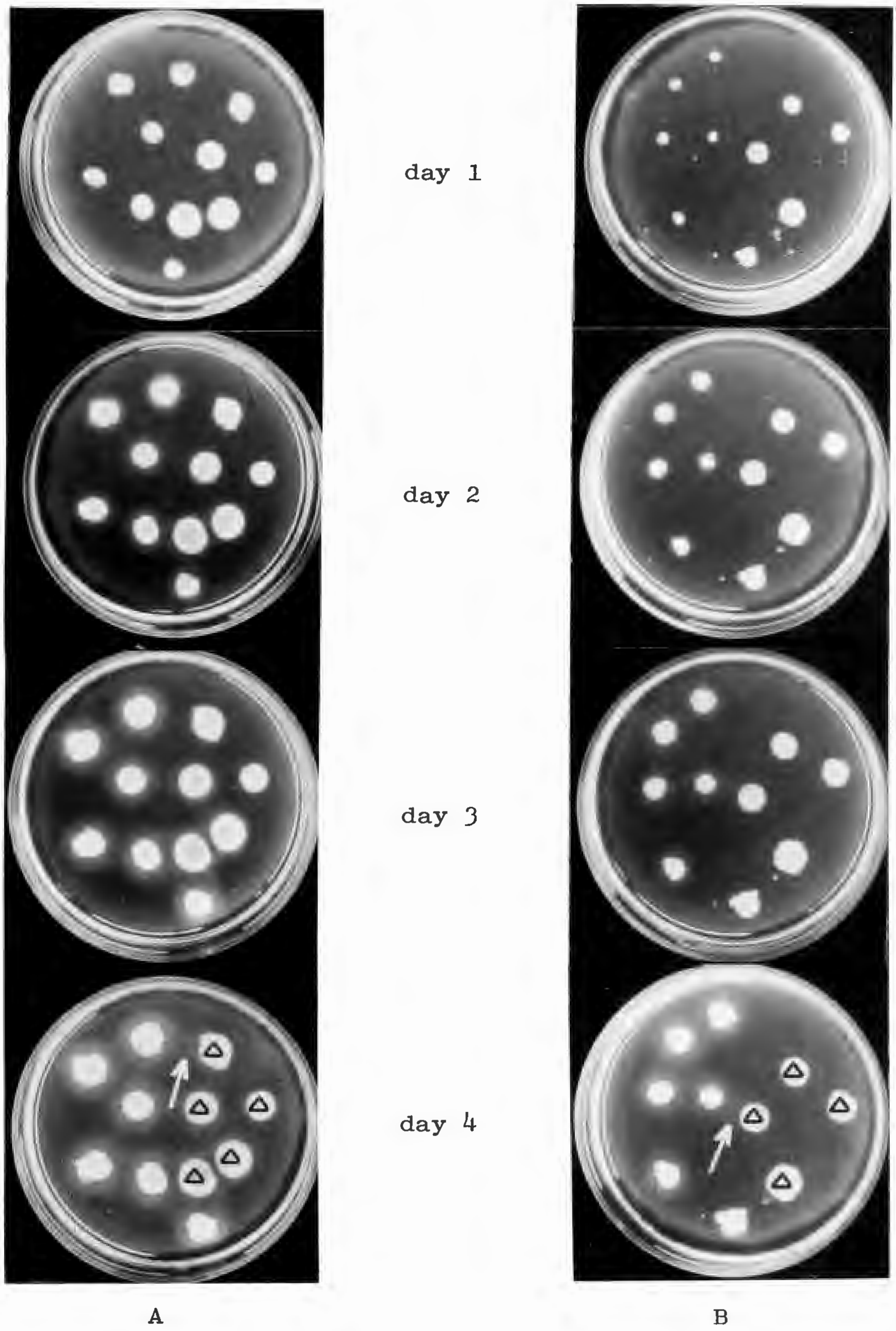
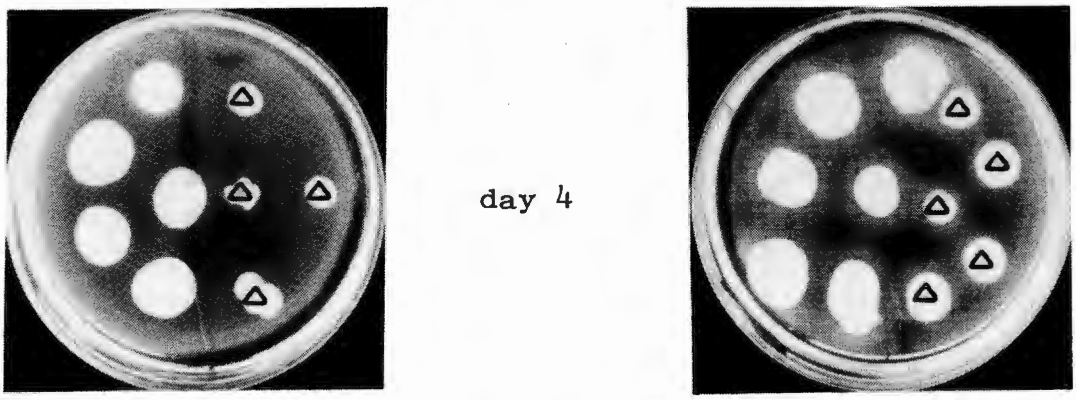


Fig. 5.1: The development of immunoprecipitates around colonies of V. alginolyticus. Agar plates were photographed at daily intervals. The plate in column A contained 0.1% peptone and 2% dialysed peptone, whereas the plate in column B contained 0.05% peptone and 1% dialysed peptone. Mutant colonies on day 4 are marked with a △ ; wild-type colonies are unmarked. The arrows point out the areas of precipitation between mutant and wild-type colonies.



day 4

A

B

<u>Fig. 5.2</u>: The effect of glucose and Casamino Acids on the formation of immunoprecipitates around colonies of *V. alginolyticus*. The agar plate in fig. A contained 0.4% glucose whereas that in fig. B contained 0.4% Casamino Acids. Both plates contained 0.05% peptone and 1% dialysed peptone. Mutant colonies are marked with a △; wild-type colonies are unmarked. Photographs were taken after 4 days.

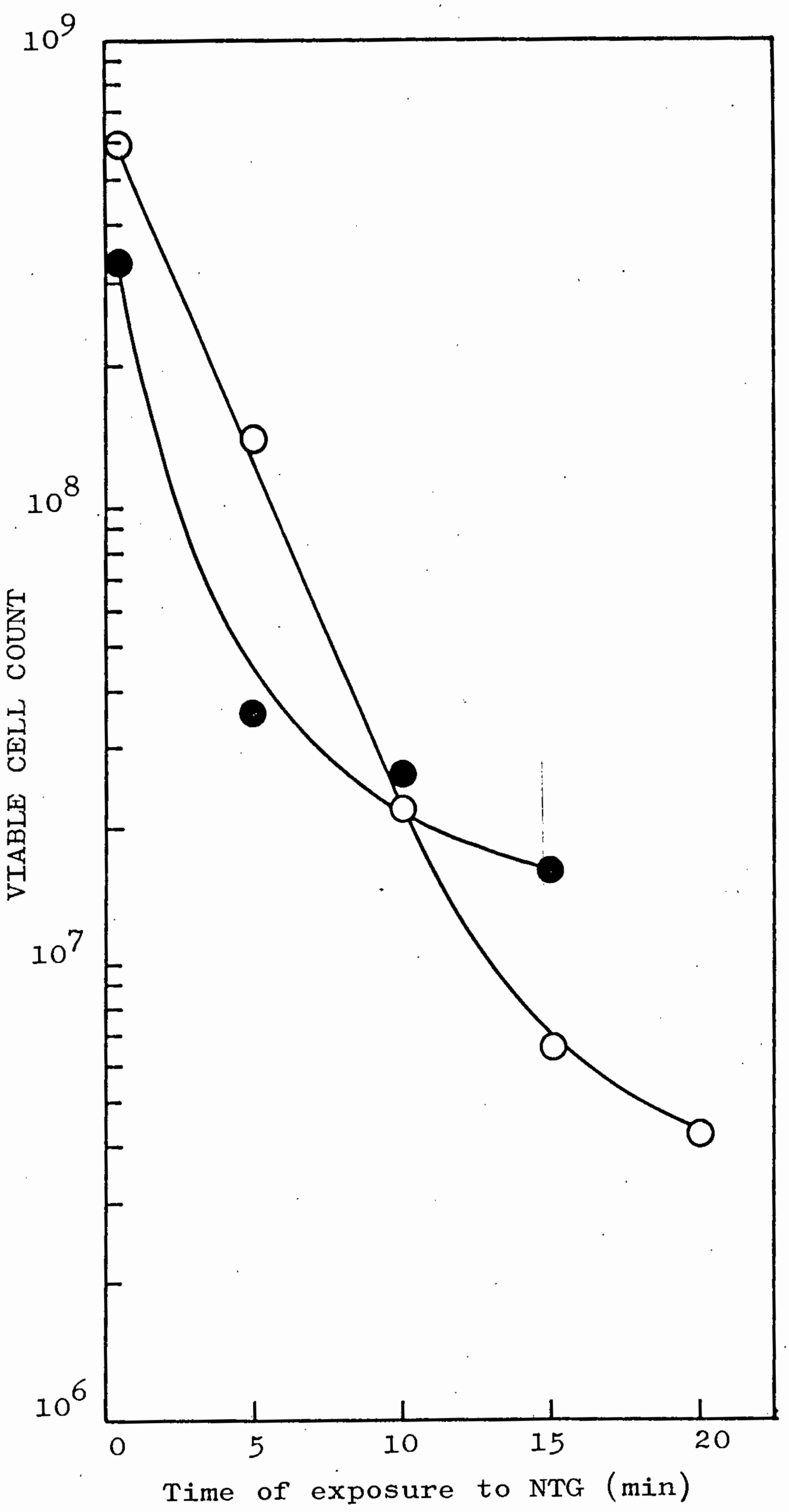


Fig. 5.3: The lethal effect of NTG on *V. alginolyticus*. Cells were incubated with 50 (●) and 100 μg NTG ml^{-1} (○).

5.3.5 Induction of glutamine auxotrophs of *V. alginolyticus*

No glutamine auxotrophs were obtained. However a glutamate auxotroph was obtained which grew on minimal medium plates supplemented with either glutamine or glutamate. This mutant produced reduced levels of collagenase when compared to the wild type strain.(3 nkat ml^{-1} compared to 25 nkat ml^{-1}). Supplementation of the peptone medium with 200 µg ml^{-1} glutamate repressed the collagenase levels even further (1.8 nkat ml^{-1}). The production of reduced levels of collagenase by this mutant could be due to the fact that it did not grow as well as the wild type strain.

5.3.6 Plasmid isolation studies

No plasmid DNA was detected in *V. alginolyticus* by any of the methods used. Plasmid DNA purified from *E. coli* J53 (RP4) formed a distinct band below that of the chromosomal DNA when subjected to dye-buoyant CsCl density gradient centrifugation, gave a typical pattern during agarose gel electrophoresis when digested with the restriction endonuclease Eco R1 and also gave a plasmid band on autoradiographs of agarose gels when labelled with $[^{32}P]$ by nick translation.

5.3.7 Conjugation of *V. alginolyticus* rif^r with plasmid carrying strains of *E. coli* and *P. aeruginosa*.

Transfer of antibiotic resistance carrying plasmids between *E. coli* J53 (RP4), *P. aeruginosa* PA08 (R68.45) and *V. alginolyticus* was not achieved.

The NaCl concentration as well as the composition of the agar plates (minimal medium or Luria agar) had a considerable effect on the ability of V. alginolyticus rif^r to plate or kanamycin containing plates (Table 5.3). Dilution of V. alginolyticus rif^r in 1% NaCl resulted in a 10-fold reduction in the number of cells compared to dilution in 2.34% NaCl. Subsequently all dilutions were performed in 2.34% NaCl. Inhibition of growth by kanamycin was greatest on minimal medium agar plates containing 1% NaCl (Table 5.3).

Because of the high rate of spontaneous mutation of V. alginolyticus rif^r to kanamycin (25 μg ml^{-1}) resistance exconjugants were selected for on minimal medium agar plates containing (a) 50 μg ml^{-1} rifampin and 100 μg ml^{-1} kanamycin or (b) 50 μg ml^{-1} rifampin and 25 μg ml^{-1} tetracycline or (c) 50 μg ml^{-1} rifampin, 50 μg ml^{-1} kanamycin and 25 μg ml^{-1} tetracycline. E. coli J53 (RP4) and P. aeruginosa PA08 (R68.45) were both resistant to 110 μg ml^{-1} kanamycin and 55 μg ml^{-1} tetracycline.

5.3.8 In vitro protein synthesis

Repeated attempts, utilizing a number of different RNA and S30 preparations, failed to show incorporation of [^{35}S] methionine into protein, under any of the conditions used for in vitro protein synthesis.

Table 5.3: Effect of NaCl concentration on the ability of *V. alginolyticus* rif^r to plate on minimal medium and Luria agar plates containing various kanamycin concentrations. A cell suspension containing 5×10^{10} cells ml^{-1} was diluted ten-fold in 1 or 2.34% NaCl before being plated.

Minimal medium agar plates						
		Number of colonies per plate				
% NaCl in:		Kanamycin concentration (μg ml^{-1})				
dilution	agar plate	15	25	35	45	55
1	1	∞	92	3	0	1
2.34	1	∞	401	15	5	1
1	2.34	∞	496	35	23	5
2.34	2.34	∞	587	211	66	30
Luria agar plates						
1	1	84	67	34	20	2
2.34	1	330	203	150	60	30
1	2.34	∞	261	123	56	53
2.34	2.34	∞	430	311	212	121

5.4 DISCUSSION

Collagenase was purified from 200 ml cultures of 1% peptone because higher concentrations of peptone or larger culture volumes decreased the levels of collagenase produced. This was probably due to inefficient aeration in the larger volumes and repression by unutilized nutrients. The use of fermentors with their better aeration facilities would probably allow the use of larger volumes and higher concentrations of peptone.

The development of an *in situ* immunoassay proved to be a simple and sensitive method for the detection of collagenase producing clones of *V. alginolyticus*. Although the titre of antibodies directed against the purified collagenase was only $^1/16$, this titre could probably be increased by enlarging the antigen by crosslinking the collagenase molecules. A limitation of this technique is the large quantity of antiserum required to screen a large number of colonies for collagenase production.

The *in situ* immunoassay could also serve as a direct method for detecting collagenase which has been cloned and would be independent of the expression of collagenase activity. It could also be used for the detection of a collagenase gene product which was not excreted, by utilizing the colony lysis method of Skalka and Shapiro (1976). Other methods for the detection of cloned gene products include restriction endonuclease analysis of cloned DNA (Hamer and Thomas, 1976), *in situ* nucleic acid hybridization (Grunstein and Hogness, 1975; Jones and Murray, 1975; Kramer *et al*., 1976) or assay for specific functions encoded in the cloned genes (Hershfield et al., 1974; Clarke and Carbon, 1975; Struhl et al., 1976). These methods are more tedious than the *in situ* immunoassay which has as an advantage the ease with which a large number of clones may be screened.

Glucose (0.4%) completely prevented halo formation whereas 0.4% Casamino Acids had little effect when included in the immunoassay agar plates. A similar effect was noted in liquid medium (Chapter II) where glucose caused far greater repression of collagenase production than did Casamino Acids.

Zones of precipitation occurred more readily in the area between two mutant colonies or between a wild type and a mutant colony than was observed around isolated mutant colonies. These results suggest that isolated mutant colonies produce insufficient collagenase to cause precipitation but when two colonies are in close proximity the combined collagenase concentration is sufficient to cause precipitation.

An unsuccessful attempt was made to isolate glutamine auxotrophs in order to obtain direct evidence for the involvement of glutamine synthetase in the regulation of collagenase synthesis. A pleiotrophic mutation which resulted in glutamine auxotrophy and altered collagenase control would support the involvement of glutamine synthetase in collagenase regulation. Similar results, implicating glutamine synthetase involvement in the regulation of the histidase (Prival et al., 1973) and the L-asparaginase (Resnick and Magasanik, 1976) of *K. aerogenes* have been found.

No naturally occurring plasmid was found in *V. alginolyticus* and therefore attempts were made to show plasmid transfer between *E. coli* J53 (RP4), *P. aeruginosa* PA08 (R68.45) and *V. alginolyticus* rif^{r}. However no plasmid transfer was shown, even though these P group plasmids have been successfully transferred between a variety of different genera, as mentioned in the introduction to this chapter. Presumably the correct conditions for plasmid transfer into *V. alginolyticus* have yet to be found.

No *in vitro* protein synthesis was detected in *V. alginolyticus* extracts. A major difference between the conditions for *in vitro* protein synthesis of *E. coli*, *B. subtilis* and *V. alginolyticus* may be the requirement for NaCl by *V. alginolyticus* which *in vivo* grows optimally in 0.4 M NaCl. However this requirement will have to be determined once some form of *in vitro* protein synthesis has been achieved in *V. alginolyticus* extracts. In addition, more stringent precautions may have to be taken against the *V. alginolyticus* proteases, than those required to reduce the proteolytic activity in *B. subtilis* extracts.

It would appear that this strain of *V. alginolyticus* is somewhat recalcitrant to genetic analysis since repeated attempts by members of this laboratory at transformation and attempts to isolate bacteriophages specific for *V. alginolyticus* have also been unsuccessful. Thus, in view of the difficulties experienced with these preliminary genetic studies it seems to be that the way to proceed would be to persevere with the *in vitro* system. The collagenase gene(s) could be cloned by synthesizing complementary DNA from collagenase mRNA.

CHAPTER VI

CONCLUSION

The aim of this work was to study the regulation of extracellular collagenase production by V. alginolyticus. In many respects this study has yielded considerable information as to how collagenase production is regulated but many areas still require further investigation.

The initial studies on collagenase regulation revealed that collagenase synthesis is subject to catabolite and end product repression. However the results indicate that collagenase synthesis is not regulated by classical catabolite repression and the roles of cyclic AMP or other regulatory nucleotides need further investigation. As with many other extracellular enzymes, the exact mechanisms of regulation by glucose and amino acids remain unknown.

As yet no relatively small homogenous inducer molecules for collagenase synthesis have been found and the minimum size of inducer molecule has yet to be determined. It does however appear that the collagenase sensitive bond is a prerequisite for induction. The minimal medium induction system developed in this thesis will prove useful in studying the ability of various inducer molecules to induce collagenase production. It would probably be more feasible to search for a small inducer molecule in the degradation products of collagen which should be more homogenous than peptone. The findings in this thesis suggest that a relatively small molecule (MW approx. 1,000) might be able to induce collagenase.

Results in Chapter IV suggest that the production of collagenase under certain growth conditions can be supported by a pool of mRNA (rifampin-insensitive production). This hypothesis still requires confirmation by direct measurement of the mRNA pool. It is possible that the mRNA is relatively long-lived. In the absence of a collagenase DNA probe these questions will probably remain unanswered. The development of an in vitro transcription and translation system should provide the means whereby the mRNA pool hypothesis may be more fully studied and understood, in addition to facilitating studies of the numerous other regulatory aspects of collagenase production.

The in situ immunoassay developed will facilitate the isolation of both constitutive and other regulatory mutants of collagenase production which are essential in any study of this nature. Although the attempts at plasmid transfer by conjugation and those to develop an in vitro protein synthesizing system were largely unsuccessful, perseverence with these techniques should yield more fruitful results. The methods followed were essentially those developed for E. coli and B. subtilis and whereas these methods provide a useful starting-point for studies in other bacterial groups the method which eventually works might differ considerably from that used at the start. It might be appropriate to quote Priest (1977) who wrote; "experiments that would be relatively simple when using E. coli are virtually impossible in B. subtilis"! The synthesis of collagenase by V. alginolyticus does however provide an ideal model for studying the regulation of exoenzyme production which requires specific induction by a macromolecule such as collagen.

APPENDIX A

Media

Casamino Acids Medium

2.5% (w/v) vitamin free Casamino Acids in the Tris-HCl buffer pH 7.6 (as below).

Complex medium agar

Casein hydrolysate	17 g
NaCl	23.4 g
glycerol	10 g
Na_2SO_3	0.1 g
nutrient broth	8 g
soytone	3 g
tryptone	0.5 g
vitamin free Casamino Acids	0.5 g
yeast extract	2 g
glucose	5 g
Difco agar	15 g
distilled water	1000 ml

Adjust the pH to 7.6 with NaOH.

Immunoassay agar

NaCl	0.5 g
peptone	0.05 or 0.1 g
dialysed peptone	1 or 2 g
50 mM Tris-HCl buffer (pH 7.0)	80 ml
antiserum	20 ml

The antiserum was added just before the agar plates were poured. Dialysed peptone was prepared by exhaustive dialysis of peptone. Agar plates were dried (shut) for 2 hr at 37°C.

Luria media

tryptone	10 g
yeast extract	5 g
NaCl: Luria broth	10 g
or Luria salt broth	23.4 g
distilled water	1000 ml

Agar plates contained 15 gl^{-1} Difco agar and 10 gl^{-1} NaCl.

Minimal medium

Salts solution:	K_2HPO_4	52.5 g
	KH_2PO_4	22.5 g
	sodium citrate	2.35g
	$(NH_4)_2SO_4$	5 g
	distilled water	500 ml
	$MgSO_4.7H_2O$ (added cold in solution)	0.5 g
Glucose:	20% (w/v) in distilled water.	
Water agar:	Oxoid agar no. 2	13 g
	NaCl	23.4 g
	distilled water	1000 ml
To make up:	water agar	210 ml
	salts solution	24 ml
	glucose	3 ml

Peptone media

2.5% (w/v) Difco peptone in the Tris-HCl buffer pH 7.6 (as below), unless specified otherwise. Collagenase was purified from 1% peptone medium, whereas 0.25% peptone was usually used for experiments in which cells were resuspended.

SNP media

High SNP medium:

disodium succinate (hexahydrate)(20 mM)	5.4 g
$(NH_4)_2SO_4$ (10mM)	1.32g
KH_2PO_4 (1 mM)	0.136 g
Tris-HCl buffer pH 7.6 (as below)	1000 ml

Low SNP medium:

disodium succinate (hexahydrate)(2 mM)	0.54 g
$(NH_4)_2SO_4$ (1 mM)	0.132 g
KH_2PO_4 (1 mM)	0.136 g
Tris-HCl buffer pH 7.6 (as below)	1000 ml

Sphaeroplasting medium

peptone	10 g
$MgSO_4.7H_2O$	2 g
NaCl (in addition to that in the buffer)	11.7g
Tris-HCl buffer pH 7.6 (as below)	1000 ml

Tris-HCl buffer

NaCl	23.4 g
$CaCl_2.2H_2O$	0.29 g
Tris (0.1 M)	12.1 g
distilled water	1000 ml

The pH was adjusted to 7.6 with concentrated HCl.

Tryptone agar

tryptone	10 g
yeast extract	5 g
NaCl	5 g
glucose	1 g
Difco agar	15 g
distilled water	1000 ml

Agar plates were well dried at room temperature in order that isolated colonies may be maintained.

Tryptone broth

2.5% (w/v) tryptone in the Tris-HCl buffer pH 7.6 (as above).

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