STUDIES ON *ACHROMOBACTER IOPHAGUS* AND OTHER COLLAGENOLYTIC HIDE BACTERIA

by

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Above all I thank my wife and my parents.

CONTENTS

CHAPTER ONE

GENERAL INTRODUCTION

Unless otherwise indicated, the information in this general introduction is taken from the works of Mandl (1961 and 1972) and Eisen, Bauer and Jeffrey (1970) on collagenases, from reviews of the structure of collagen by Bailey (1968) and Steven (1972), and from a chapter on the microbiology of hides and skins by Turner (1967).

A. COLLAGENASES

Collagenases are enzymes capable of specifically attacking the native collagen helix under non-denaturing conditions at physiological conditions of pH, temperature and salt concentration. They are active only on collagen or its breakdown products and are without effect on any other fibrous or globular protein.

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; also they are 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.

The best sources of collagenases are bacteria (Sizer, 1972) but they also occur in actinomycetes and fungi (Rippon and Lorincz, 1964) as well as in certain animal and human tissues. Bacterial

collagenases have been known for several decades but animal and human collagenases have been discovered only in recent years. The reason for this is that the latter enzymes are not stored in appreciable amounts *in vivo* and are not detectable in tissue extracts or homogenates; they are discernible only in growing tissues. Gross and Lapiere (1962) demonstrated the first animal collagenase when they developed a technique capable of visualizing the action of collagenase from metamorphosing tadpole tissue. Since then collagenases have been shown to occur in a variety of animal and human tissues including skin, gingiva, synovia and bone. However, the scope of this dissertation will be limited mainly to bacterial collagenases.

Bacterial collagenases

The digestion of tendon by anaerobic organisms from soil was described by Mall (1896) in what was probably the first report implying collagenase activity. Later, Weinberg and Randin (1931) showed that native horse Achilles' tendon was completely digested in three to five days at 37°C by eight strains of *Clostridium histolyticum* and one strain of *Bacillus anthracoides*, but not by other gelatinases. Although they named their enzyme "ferment fibrinolytique", they were obviously describing the activity of a collagenase. Subsequently, Weinberg and Randin (1932) found that the culture filtrates were as active as the organism, thereby establishing that the collagenase was an extracellular enzyme. The actual term "collagenase" was first used by Ssadikow (1927) but it referred to the action of a pancreatic enzyme on denatured collagen. Thereafter "collagenase" was used to describe the extra-cellular gelatin-splitting enzymes produced by *Cl. perfringens*, *Cl. septicum*, *Cl. histolyticum* and *Cl. chauvoei* (Maschmann, 1937, 1938a, 1938b and 1938c) but the term was later withdrawn (Maschmann 1938d) because of a lack of definite

proof of activity against collagen rather than its breakdown products (Maschmann, 1943).

MacFarlane and MacLennan (1945) reintroduced the term "collagenase" to describe an enzyme from Cl. perfringens type A culture filtrates which dissolved fresh Achilles' tendon and destroyed collagen muscle framework. At about the same time, Jennison (1945) claimed collagenase activity in B. brevis, B. mycoides and B. mesentericus and in Cl. lentoputrescens, Cl. sporogenes, Cl. bifermentans and Cl. histolyticum. He proposed the restriction of the name "collagenase" to enzymes capable of degrading collagen as opposed to enzymes able to break down gelatin but not collagen. Ironically, Jennison used dry heat to sterilize his collagen preparations and his reports of collagenase activity are therefore regarded as unreliable. Cl. perfringens types A,C,D and E were subsequently shown to produce collagenase (Oakley, Warrack and van Heyningen, 1946; Guillaumie et al., 1957) and MacLennan, Mandl and Howes (1953) found collagenase activity in twenty out of thirty Cl. perfringens strains (all the A strains) and in all of the eighty-two Cl. histolyticum strains tested. Tancous (1961) reported that a strain of Cl. capitovale produced collagenase and caused severe hide damage.

At this stage, Mandl (1961) concluded in an extensive review of collagenases that collagenolytic activity was associated only with Cl. histolyticum and Cl. perfringens and possibly Cl. capitovale, all anaerobes. Subsequently, anaerobic collagenolytic activity was reported in Bacteroides melaninogenicus, three other Bacteroides spp., Cl. tetani and Staphylococcus aureus (Gibbons and MacDonald, 1961; Waldvogel and Swartz, 1969). Collagenase activity among aerobic bacteria was reliably reported for the first time in Streptomyces madurae (Rippon and Lorincz, 1964) and has been found more recently in species of Pseudomonas, Achromobacter, Bacillus, Vibrio and Aeromonas

(Adamcic and Clark, 1970; Thomson, Woods and Welton, 1972; Merkel, 1972).

The production of collagenases by bacteria is thus a relatively uncommon phenomenon, occurring anaerobically in three genera and aerobically in six genera.

B. COLLAGEN

Collagen is the dominant structural material of the animal kingdom, occurring among both invertebrates and vertebrates. In mammals collagen comprises 25-33% of the total protein and is the main constituent of skin, tendon and cartilage, as well as being 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), though phyletic differences are greater. Further discussion in this dissertation will be concerned mainly with mammalian collagen.

Collagen is synthesized in fibroblasts as individual soluble molecules called tropocollagen molecules. These pass from the cell to the intercellular space where they aggregate to form fibrils. This is followed by stabilization of the fibrils through intermolecular cross-linking, which renders the collagen insoluble. Soluble native collagen may be extracted from homogenized collagenous tissue (usually calf-skin or tendon) using either neutral salt solutions or acid buffers. Generally the neutral salt soluble collagen is extracted first using 0.5 M NaCl and subsequently the less soluble collagen is extracted by 0.1 M acetate or citrate buffers. The acid extraction procedure yields the purer collagen as the neutral extracts may contain non-collagenous proteins. Only a limited amount of native collagen, about 2% from calf-skin, can be dissolved by these mild treatments.

The Triple Helix

The tropocollagen molecule has a molecular weight of about 300 000 and contains three individual polypeptide chains (designated α-chains), each consisting of approximately 1 000 amino acid residues. Two of the polypeptide chains are identical (except in cod fish collagen) and the third differs slightly in amino acid composition. 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 inter-chain hydrogen bonds and hydrophobic interactions. This is the "triple helix" of the collagen molecule (Fig. 1.1).

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 is shown in Table 1.1, from which it is clear that the most frequently-occurring triplet sequence in the collagen molecule will be gly-pro-X, where X may be any amino acid residue but is often hydroxyproline or alanine.

Table 1.1 Relative amino acid composition of bovine collagen (Fraenkel-Conrat, 1963).

Gly	Pro	Hyp	Ala	Other
363	131	107	106	362

Along the length of the triple helix, polar and apolar regions alternate, the polar regions providing the means for molecular aggregation during fibril formation. The apolar regions, which contain a predominance of the typical collagen sequences gly-pro-hyp and gly-pro-ala, play an important part in rendering the triple helix resistant to the action of common proteolytic enzymes. Such enzymes generally have activities directed towards polar or aromatic residues (Dixon and Webb, 1958) and in the native configuration of the collagen molecule, the polar regions are shielded by the alternating apolar regions. Collagenases alone have the specificity requirements to digest the triple helix. Should denaturation cause opening of the helical structure, however, the polar areas become exposed and are then susceptible to attack by almost any of the common proteolytic enzymes. This fact has led to numerous unreliable reports of collagenase activity, for collagen is very easily denatured, thereby being converted to gelatin. There are two stages of its denaturation. Initially the triple helix unwinds with the formation of randomly-coiled polypeptide chains. At this stage, which may be accomplished merely by heating to 40°C, partial recovery of the triple helix may be regained by careful reannealing. The second step is irreversible and involves the fragmentation of the polypeptide chains.

The Telopeptides

Attached to each end of the triple helix (Leibovich and Weiss, 1970) and situated external to it are short, randomly-coiled peptide appendages called telopeptides. The telopeptides comprise about 1% of the tropocollagen molecule and interact during the polymerization of tropocollagen to form fibrils. Initially they are responsible for the orientation of the aggregating molecules and later they are the sites of the intermolecular peptide bonds which stabilize the fibril; they also appear to restrict the diameter of the newly-formed fibril.

The non-helical telopeptides do not conform to the gly-pro-X sequence typical of the triple helix but are strikingly rich in polar residues and poor in pro and hyp. As a result, these limited regions of the tropocollagen molecule are susceptible to the action of proteolytic enzymes other than collagenases, e.g. pronase, pepsin, trypsin and chymotrypsin.

Removal of the telopeptides from tropocollagen leaves the triple helices intact but greatly reduces their ability to aggregate and form native fibrils. Moreover, selective enzymatic digestion of the telopeptides from fibrous collagen, as a consequence of the removal of the intermolecular links, results in depolymerization to soluble tropocollagen-like fragments. Both pronase and pepsin readily remove the crosslinks, the former being the more effective. Insoluble collagen can be solubilized by prolonged treatment of the fibres with pronase and pepsin at room temperature. Trypsin and chymotrypsin, though removing some crosslinks, are less effective and will not achieve solubilization.

In addition to the intermolecular crosslinks, intramolecular (inter-chain) peptide bonds also form in the telopeptides. Their role is considered unimportant, however (Harrington and McBride, 1966). Two α-chains joined in this manner comprise a β-component and three linked α-chains form a γ-component. All three components may be present in gelatin.

The Action of Collagenases on Collagen.

Almost all known collagenases are capable of degrading collagen fibrils in tissue to peptide fragments. (An exception is human granulocyte collagenase.) However, the specific actions of the collagenases on the tropocollagen molecule vary according to the origins of the enzymes.

Cl. histolyticum collagenase attacks the tropocollagen molecule

at many points, its specificity requirement being pro-X-gly-pro-Y and the enzymatic split occurring between the X and gly residues (Harper, 1972). The knowledge of this specificity requirement has led to the development of synthetic substrates containing the above sequence for use in the estimation of collagenase activity. The peptides produced by the action of Cl. histolyticum collagenase on collagen are mostly in the region of three to six residues long but the polar regions are left intact as larger peptides of five to twenty-five residues. In general, the initial action of human and animal collagenases is to cleave the tropocollagen molecule through all three chains at a single locus, thereby producing two helical fragments of 75% (the TC^A fragment) and 25% (the TC^B fragment) of the length of the original molecule. Under suitable conditions, further degradation reduces the primary fragments to small peptides. It would seem therefore, that collagen has a point of particular susceptibility to cleavage by a collagenase.

The various features of tropocollagen are summarized diagrammatically in Fig. 1.2, which is largely copied from Steven (1972).

C. THE HIDE

Leather is a valuable commodity on the national and international markets and the hide and skin industry is one of South Africa's largest commercial concerns. Leather is prepared from the skins of many animals, particularly cattle, by the process of tanning. Freshly-removed (raw) hides are not considered a suitable source of supply for the tanner, however, due to their being so highly putrescible. Unavoidable delays between the time of flaying and the beginning of tanning make it necessary to preserve the hides over this period and one of the most common ways of achieving this is to use a process of salt-curing (Everett and Gordon, 1956). Salt-curing may be done by stack-salting, i.e. stacking the hides between layers of salt, or more

rapidly by an overnight soak in saturated brine followed by stack-salting. To improve the efficiency of curing, the salt is frequently supplemented with a small proportion of an antiseptic. Hides cured by these procedures are known as wet-salted hides and a well-cured wet-salted hide contains approximately 14% salt. Cured hides are transported to the tannery where they may be stored prior to being tanned.

A cross-section of a raw hide is depicted in Fig. 1.3, which is adapted from Turner (1967). The two main layers are the surface "grain" layer and the corium. The corium consists of large collagen fibres, held together by fibrous reticulin, and soluble interfibrillary protein; the grain layer contains finer collagen fibres, elastin which lines the blood vessels and hair follicles, and the erector pili muscle fibres. The collagen is the basic leather-making material (Nandy, 1966) and the quality of the finished leather depends greatly upon the extent of removal of the non-collagenous components of the raw hide, viz. hair, epidermal tissue, sebaceous glands, as well as those mentioned above. The sequence of events from the slaughtering of the animal to the completion of the tanning process is summarized in Fig. 1.4. Generally, the hides are cut down the line of the backbone into halves or "sides" after liming, the sides then being processed further into leather. The greatest demand on the market is for the soft upper leather obtained by splitting off the upper section of the tanned leather (as shown in Fig. 1.3 (b)) prior to drying and oiling; the lower section provides suede leather.

It is well-known that some bacteria attack hides and skins, causing a considerable amount of leather devaluation (Tancous, 1961). The stages during which this damage is most likely to occur are before the raw hide is cured, during prolonged storage in salt, and during the desalination soak; leather is not susceptible to bacterial action.

As the leather-making substance is collagen, which has a limited susceptibility to common proteases, only bacteria which produce collagenases can cause decay of a hide which will adversely affect the finished leather. Bacteria attacking the flayed hide penetrate through the flesh side only, invading even the hair follicles and epithelium via this route, apparently because of the loose fibre weave and exposed blood vessels. The region most sensitive to bacterial damage is the junction of the grain layer and the corium, and degradation in this area causes loosening of the valuable grain layer in the finished leather. The degree of loose grain layer is therefore an index of the amount of "leather decay", the term which specifically denotes bacterial damage to the leather-making part of the hide.

Leather decay is assessed by subjectively examining each side of leather and according it a "Decay Grading". The decay shows up as loose grain starting in the belly and flank (giving the grade called "slight decay") and moving up through the butt towards the backbone in extreme cases ("bad decay"). In a badly-decayed side, the grain layer may be so loosened that it lifts free of the structure below. Despite its being a subjective procedure, leather decay grading as described above has been shown to give statistically consistent results (Cooper, Russell and Galloway, 1971).

As leather decay is evident only in the finished product, the industry is faced with a great problem and economically there are two desirable objectives. Firstly, it would be advantageous to be able to assess the quality of hides prior to their being tanned, for they could then be accordingly channeled for appropriate processing. The second and more important objective is an efficient method of preventing the breakdown of collagen in hides. Such were the goals at the outset of this research project.

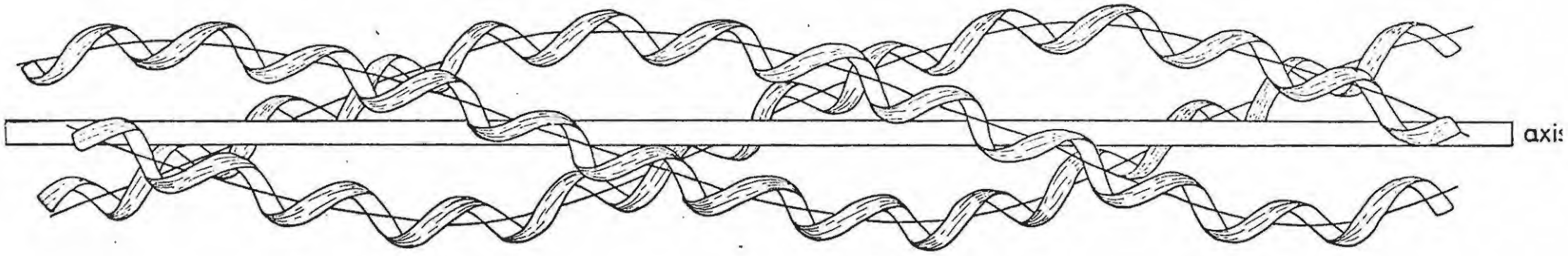


FIG. 1.1. THE TRIPLE HELIX

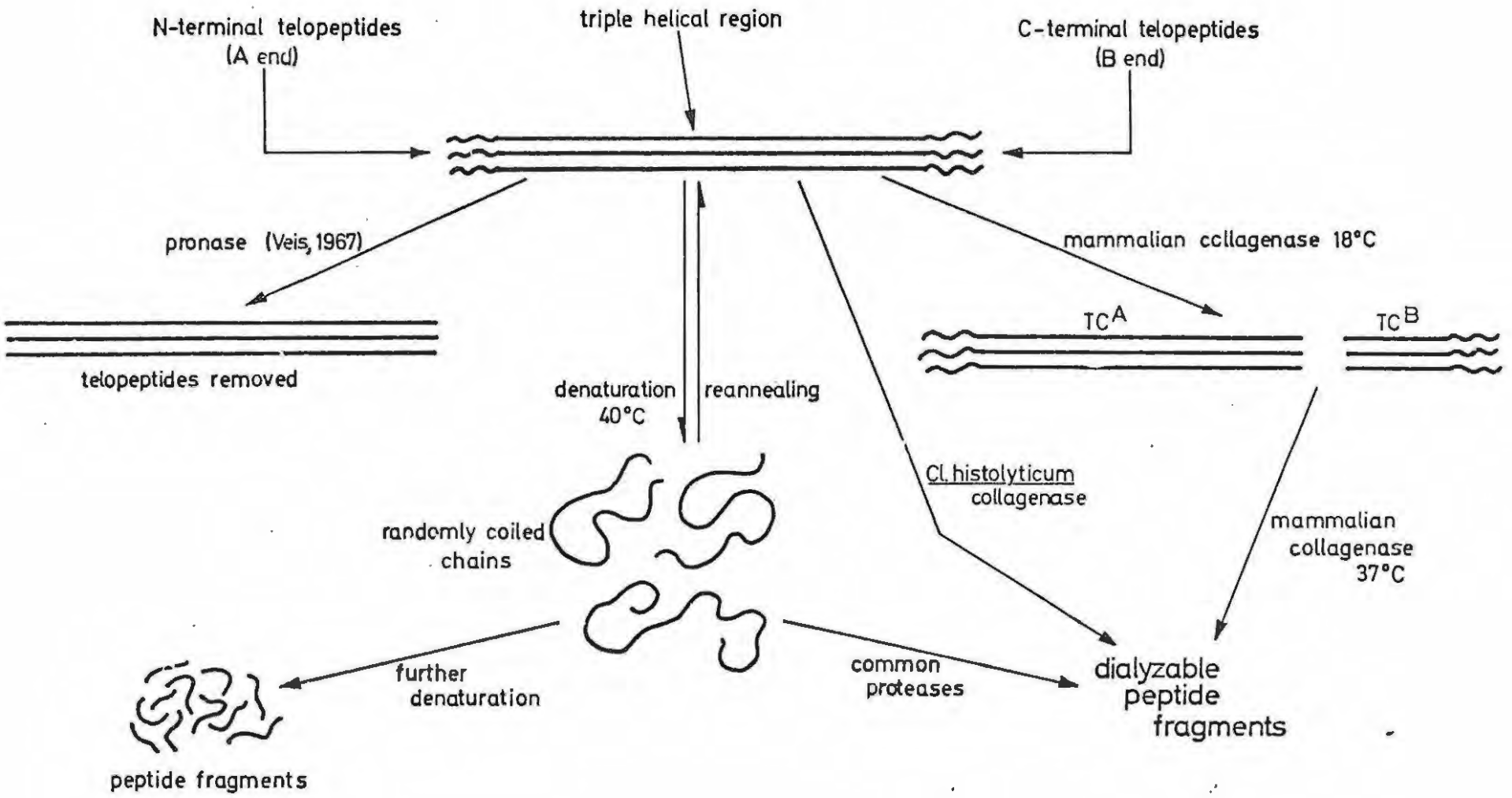


FIG. 1.2. THE STRUCTURAL FEATURES OF TROPOCOLLAGEN

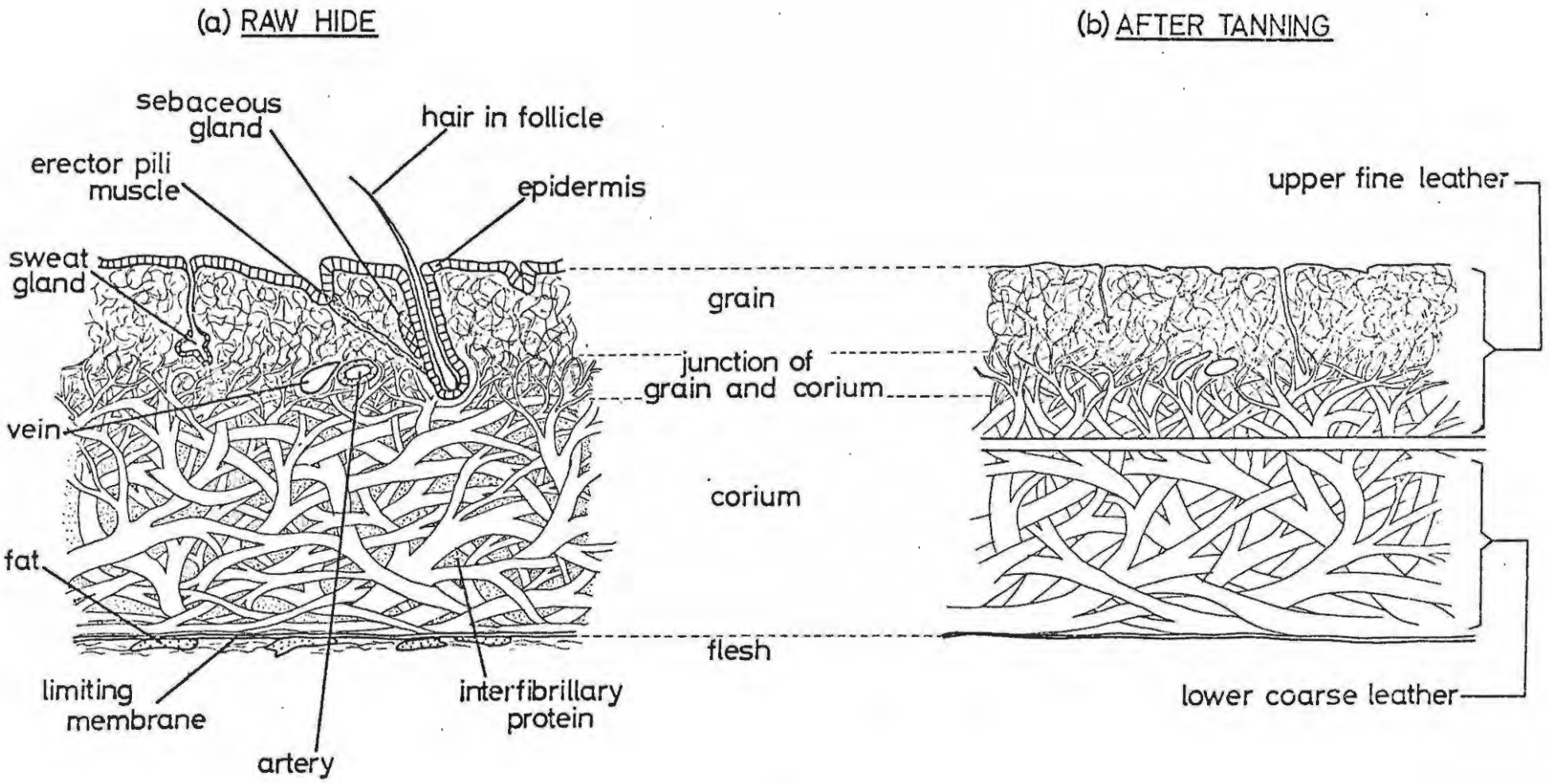
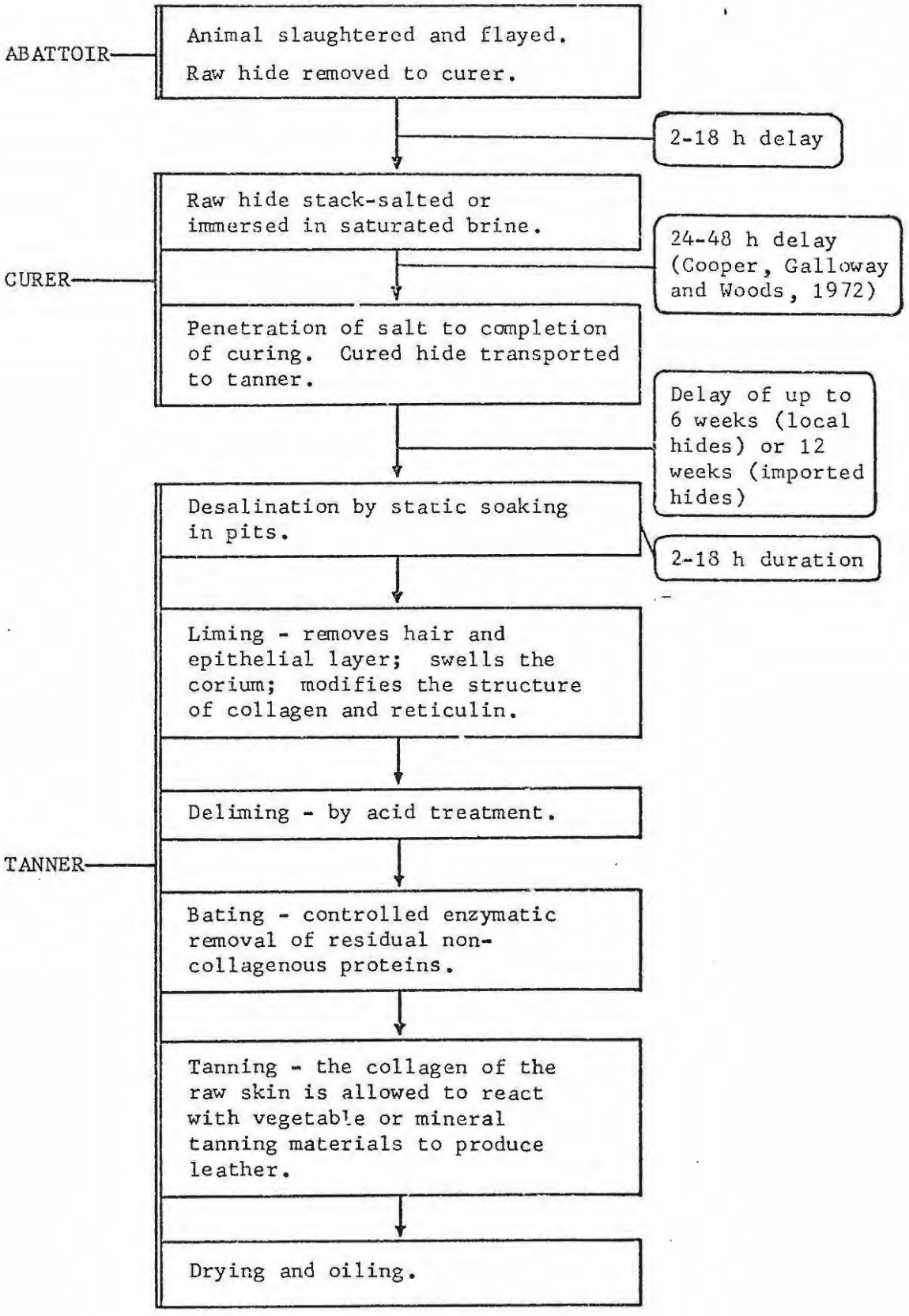


FIG. 1.3. TRANSVERSE SECTION THROUGH HIDE BEFORE AND AFTER TANNING

FIG. 1.4. THE PROCESSING OF A HIDE



CHAPTER TWO

COLLAGENOLYTIC ACTIVITY OF HIDE BACTERIA AND LEATHER DECAY

SUMMARY

An attempt was made to relate the collagenolytic activity of cured hide bacterial populations to the decay in the leather produced from the hides, with a view to providing a quality control procedure for the industry. Also, collagenolytic activity assays were made of raw and cured hide bacterial populations as well as of tannery desalination soak pit water in order to determine the stage of processing when hides are most susceptible to bacterial decay. The bacterial populations of three raw and seven cured hide batches were shown to possess differing degrees of collagenolytic activity under aerobic conditions as measured by their action on native collagen. Only one hide population showed activity under anaerobic conditions. The optimal NaCl concentration varied but activity generally decreased as NaCl concentration increased above 2.34%. No direct correlation was found between the collagenolytic activity data of the cured hide bacterial populations and the decay in the leather produced from the hides. Although allowing that decay might occur during storage of cured hides and desalination, the time of greatest bacterial damage appeared to be the period prior to the curing of the flayed hides. This would explain the above lack of correlation.

INTRODUCTION

The need for a quality control procedure for use in the hide and skin industry has been discussed (Chapter 1). Early attempts to assess hide quality prior to tanning were based on chemical methods involving determination of extractable nitrogen (Somer, 1942; Whitmore, Downing and Sherrard, 1942). Volatile nitrogen can be lost during exposure of

hides, however, so the residual amount found at any given time does not necessarily reflect the total degree that has occurred (Hauck and Lollar, 1957). Ornes and Roddy (1963), after accumulating a comprehensive set of data relating the contents of moisture, ash, hide substance and soluble nitrogen in hides to physical tests on leather, concluded that there was no correlation between the chemical composition of green salted hides and the physical strength of their leather. Subsequently, Nandy (1966) attempted to restrict chemical analysis to measuring the degraded collagen in hides and he claimed good correlation between the amount of extractable hydroxyproline and the quality of raw and cured hides. Unfortunately his grading techniques did not extend to analysis of the leather, but were based on visual, olfactory and tactile examinations of the hides. Thus there is as yet no proved leather-quality control procedure based on chemical testing of hides.

Microscopic analysis has been shown to yield valuable information regarding the quality of hides, but it has been ruled out as a quality control survey tool due to the prohibitive amount of work required for adequate sampling (Anderson, 1945).

A quality control method based on proteolytic activity present in hides was proposed by Schmitt and Deasy (1963) who assayed hide extracts for gelatinolytic enzymes. Their method is considered unreliable firstly because the enzymatic activity they measured was not specific for collagen and secondly because the level of gelatinolytic enzymes present in a hide may fluctuate.

As decay of hides is due to their microbial flora, which consists mainly of bacteria (Turner, 1967), attention was turned to the bacterial populations present in hides. Cured hides were selected for initial bacteriological studies in view of the long periods for which they may be stored (cf. Fig. 1.4) and hence exposed to bacterial action. Methods were developed for sampling and enumerating bacteria present

in cured hides (Woods et al., 1970a; 1970b; 1970c) and preliminary investigations showed that no direct relationship existed between aerobic and anaerobic viable counts of these bacteria and the amount of decay in the hides (Woods, Welton and Cooper, 1971); nor was a relationship found between the gelatinolytic activity of cured hide bacterial populations and the degree of leather decay (Woods et al., 1970d). However, extension of these methods to the assaying of hide bacterial populations specifically for collagenolytic activity should provide an index of potential leather decay and hence a test which could form the basis of a reliable quality control procedure. Furthermore, the amounts of collagenolytic activity present in the bacterial populations of raw and cured hides and of desalinating soak pit waters should indicate when a hide is most likely to sustain damage during the course of its processing. This survey was undertaken in an attempt to verify these theories.

MATERIALS AND METHODS

Hide Batches. Details of the hide batches investigated are set out in Table 2.1. Batches B, C and D were screened for collagenolytic activity both before and after curing whereas the other batches were tested only in the cured state.

Desalination Soak Pit Water. Prior to the soaking of hides, a sample of water was taken from a tannery's soak pits. A 10 ml portion of this was treated as described below for the hide bacterial suspensions.

Preparation of Collagen. Neutral salt soluble collagen was extracted from calf-skin and purified by the method of Cooper and Davidson (1965). It was stored at 4°C in the lyophilized form. A 0.2% solution of the collagen was prepared according to Waldvogel and Swartz (1969) by stirring the lyophilisate into phosphate buffer (I=0.4 M; pH 7.6) at 4°C overnight and then dialyzing against 0.01 M Tris-HCl (pH 7.6)

containing 0.4 M NaCl and 2 mM $CaCl_2$. After purifying the resultant solution by centrifuging at 105 000 x g for one hour, it was diluted tenfold in 0.01 M tris-HCl (pH 7.6) containing 2 mM $CaCl_2$ and the appropriate amounts of NaCl to produce final concentrations of 0.85, 2.34, 7.00 and 10.00% NaCl. Calcium chloride was included with reaction mixtures in these and all other experiments concerning collagenase activity because of the specificity requirement of collagenases for Ca^{++} (Eisen, Bauer and Jeffrey, 1970).

Screening for Collagenolytic Activity. Five 2 cm diameter hide plugs, one each from hides selected randomly from a batch, were cut into small pieces and shaken in 50 ml of 10% NaCl solution for one hour (Woods et al., 1970c). The bacteria in 10 ml of the resultant suspension were washed and resuspended three times in 10 ml of 10% NaCl and then tested for collagenolytic activity by a method based on that of Adamcic and Clark (1970). Four 3 ml volumes of each NaCl concentration of the 0.02% collagen solution were inoculated with 0.1 ml aliquots of washed bacterial suspension. Reaction mixtures were incubated at 35°C, two of them aerobically and the other two anaerobically in Brewer jars under 98% N_2 and 2% H_2. Controls, kept at all NaCl concentrations, consisted of (a) uninoculated collagen solution, (b) bacteria in buffer without collagen, and (c) Clostridium histolyticum collagenase Form II in the proportion 1:10 enzyme:collagen. Precipitation of collagen upon incubation of reaction mixtures and controls was disregarded as collagenases react with both soluble and insoluble collagen (Mandl, 1972).

At times zero, three, seven and eleven days, 0.5 ml aliquots of the reaction mixtures were removed and the ninhydrin-positive materials were assayed by the colorimetric method of Rosen (1957). To each 0.5 ml sample was added 0.25 ml acetate-cyanide buffer and 0.25 ml ninhydrin solution. This mixture was heated for 15 min in a

boiling-water bath and then immediately diluted with 5 ml iso-propanol-water diluent and vigorously shaken. The final mixture was allowed to cool to room temperature and was read colorimetrically at 570 nm against a reference solution of 0.5 ml of the collagen solvent buffer, 0.25 ml acetate-cyanide buffer and 0.25 ml ninhydrin solution, heated diluted and cooled as described above. The absorbance values were converted to glycine equivalents per ml by reference to the calibration data shown in Table 2.2 and Fig. 2.1. This calibration data was prepared by assaying standard solutions of glycine in 0.01 M Tris-HCl (pH 7.6) after ascertaining that varying the NaCl concentration from 0.85 to 10.00% did not affect the colour development.

Reagents for Colorimetric Analysis. (Rosen, 1957).

1. Stock sodium cyanide: 0.01 M (490 mg/litre).

2. Acetate buffer: 2700 g $CH_3COONa.3H_2O$ plus 2 litres H_2O plus 500 ml glacial CH_3COOH, made up to 7.5 litres with H_2O: pH 5.3 to 5.4.

3. Acetate-cyanide buffer: 0.2 mM NaCN in acetate buffer; 20 ml of solution 1 made up to 1 litre with solution 2. This final buffer solution usually obviates the necessity for preliminary pH adjustment of samples. In new procedures, however, a check should be made that the reaction mixture is at pH 5.0 to 5.2 before proceeding with the analysis.

4. Ninhydrin solution. 3% ninhydrin in 2-methoxyethanol. The 2-methoxyethanol should be free from peroxides as indicated by the absence of a yellow colour on mixing with an equal volume of 10% KI solution and should give a clear solution when mixed with an equal volume of water (Moore and Stein, 1948). If it fails to comply with these requirements, the 2-methoxyethanol should be redistilled over 1% by volume of a solution containing 30 g of hydrated $FeSO_4$ and 3 ml of concentrated H_2SO_4 in 55 ml of water. The first 5% of the distillate should be rejected and the final 10% of the mixture left undistilled (Eastoe and Courts, 1963).

5. Diluent: iso-propanol:water 1:1.

Leather Decay Grading. The finished sides of leather were graded according to their respective degrees of decay by representatives of the Leather Industries Research Institute, Grahamstown.

RESULTS

For clarity, time zero symbols have been omitted from graphs where necessary.

Collagenolytic Activity Control Data. The aerobic and anaerobic control data for uninoculated collagen and collagen plus collagenase are shown in Fig. 2.2 (a-d). Whereas the anaerobic uninoculated control data remained unchanged throughout, this was not the case for their aerobic counterparts. All the aerobic uninoculated collagen solutions showed an increase in ninhydrin-positive materials after seven days incubation, while the solution containing 0.85% NaCl showed a slight increase even before seven days. These effects, which may have been due to some thermal denaturation of the collagen, necessitated care in interpretation of the results obtained with the bacteria. Activity which manifested itself only when the controls showed their increase might have been due to the action of proteases on denatured collagen.

The digestion of collagen by collagenase was rapid at 0.85 and 2.34% NaCl, the latter being the optimal NaCl concentration. Activity occurred at 7.00 and 10.00% NaCl at reduced rates and to reduced degrees.

For the controls consisting of bacteria in buffer, only one NaCl concentration was used, viz. 2.34%. The results for each different bacterial population are presented with the relevant activity graphs in Figs 2.3(a-c), 2.4(a-g), 2.5 and 2.6.

Collagenolytic Activity of Hide Bacterial Populations.

(a) Aerobic Collagenolytic Activity of Raw Hide Bacteria.

The raw hide bacterial populations of batches B, C and D were examined at two NaCl concentrations. Under aerobic conditions all three bacterial populations rapidly digested collagen at 2.34% NaCl with activities approaching that of the collagenase control; at 7.00% NaCl their activities were somewhat slower but were nevertheless quite considerable. These results are shown in Fig. 2.3(a-c) There was no activity under anaerobic conditions.

(b) Aerobic Collagenolytic Activity of Cured Hide Bacteria.

The collagenolytic activities of the bacterial populations from cured hide batches A-G at 0.85, 2.34, 7.00 and 10.00% NaCl are presented in Fig. 2.4(a-g). Hide batch E showed the highest collagenolytic activities at 2.34 and 7.00% NaCl and was the only hide batch with unequivocal collagenolytic activity at 10.00% NaCl. The activity at 2.34% NaCl was similar to that of the collagenase control and the 7.00% NaCl activity exceeded the control data. There was no lag period from 0.85 to 7.00% NaCl.

The bacterial populations of batches A, B, C and D exhibited various degrees of collagenolytic activity at 0.85 and 2.34% NaCl but there was no significant activity at 10.00% NaCl. At 7.00% NaCl the bacteria from batches A, B and D showed significant activity and the batch C bacteria had the highest activity at this concentration of all the hide bacterial populations, equalling that of the collagenase control.

No significant activity was found in the bacterial populations of either batch F or the Australian batch (batch G) at any of the NaCl concentrations.

(c) Anaerobic Collagenolytic Activity of Cured Hide Bacteria.

The only hides whose bacteria showed collagenolytic activity under

anaerobic conditions were those of batch E (Fig. 2.5). Digestion of collagen occurred at 2.34% NaCl only.

Collagenolytic Activity of Desalination Soak Pit Water.

Under aerobic conditions digestion of collagen occurred rapidly at 0.85% NaCl and slowly at 2.34% NaCl; no activity occurred at 7.00 or 10.00% NaCl (Fig. 2.6). There was no activity under anaerobic conditions.

Leather Decay Grades.

These are shown in Table 2.1. Batch E produced the best leather with the fewest badly-decayed sides and batch F was the second-best. Batches A, B, C and D each contained a relatively large proportion of sides with bad decay.

DISCUSSION

The results demonstrated that both raw and cured hide bacterial populations may be capable of significant collagenolytic activity. The optimum NaCl concentration for collagenolytic activity varied but there was a general trend towards a decrease in activity with increasing NaCl concentration above 2.34%. Five out of the six South African hide batches (batches A-F) showed collagenolytic activity under aerobic conditions but only one out of six (batch E) showed activity under anaerobic conditions. Moreover, the soak pit water and the raw hide bacteria showed collagenolytic activity under aerobic conditions only. Therefore the anaerobic data from these studies were considered to be unimportant.

Although a direct correlation between collagenolytic activity of the cured hide bacteria and decay in the leather produced from the hides was not apparent, this could be explained by taking other factors into account. For instance, the collagenolytic activity of hide batch E bacteria, though being high at 2.34 and 7.00% NaCl, was zero at 10.00%

NaCl for the first three days. These hides were washed after flaying, thereby removing excess bacteria, and salted with minimum delay; hence both the period for collagenolytic activity at the lower salt concentrations and the "starting inoculum" were minimal. Furthermore, although the bacteria from these hides were able to degrade collagen at a high NaCl concentration, this was of no consequence as the hides were tanned immediately after curing. These facts provide a feasible explanation for the low incidence of decay in the batch E leather.

The leather from the Vleissentraal hide batches (batches A, B, C and D) showed more decay than indicated by the collagenolytic activity data of the cured hide bacterial populations. However, the results obtained with the raw hide populations provide the answer for this, because before batches B, C and D were cured their bacteria showed rapid digestion of collagen at 2.34 and 7.00% NaCl. Moreover, as the raw hides were not washed before being salted, excess bacteria will not have been removed; and as curing was not commenced immediately after flaying, there will have been an increased period before the rising salt concentration inhibited collagenolytic activity.

The good leather produced from batch F hides was to be expected, for the bacterial population showed no significant collagenolytic activity. In addition, the raw hides were washed and cured with the minimum of delay. Although batch G leather was not graded for decay, the absence of collagenolytic activity in the bacterial population from these Australian hides accords with a report that Australian hides generally produce uniformly good leather with little decay (Hendry, Cooper and Woods, 1970). Other Australian hide batches have been found to be free of collagenolytic bacteria (Thomson, Woods and Welton, 1972).

The discovery of collagenolytic activity in desalination soak pit water prior to the soaking of hides indicates a need for decontamination

of soak pits between hide batches. It also suggests that cured hides may contain latent bacteria capable of collagenolytic activity at lower salt concentrations, so that even at this late stage of processing there may be decay in the hide.

The high collagenolytic activities in the Vleissentraal raw hides and the considerable reduction of these collagenolytic activities after curing, when related to the high incidence of bad decay in the leather, show that the main danger period for hide decay lies between the flaying of the animal and the stage when the hide is cured. The observation that delayed curing increases the incidence of decay (Nandy, 1966) supports this finding. So does the fact that cured hide batches A, B, C and D showed no variation in leather quality due to different periods of storage, despite having similar collagenolytic activities; in other words, there was no significant decay after these hides had been cured. However, having discovered collagenolytic activity at 10.00% NaCl in batch E, decay of cured hides in general cannot be ruled out, for such NaCl-tolerance may extend to 14.00% and above.

The difference in collagenolytic characteristics between the Vleissentraal raw and cured hide populations also means that cured hide collagenolytic activity data do not necessarily provide a reliable picture of the degree of decay in hides. Such cured hide data might yield indications of the potential decay during prolonged storage, but it is the raw hides which should be assayed for indications of damage due to delayed curing. As delayed curing is the major cause of leather decay, the collagenolytic activities of raw hide bacterial populations are likely to correlate directly with the amounts of decay found in the finished leather. Further studies are needed to establish this relationship.

Origin of Bacteria	Batch	LIRI Code	Number of Hides in Batch	Curer	Hides Washed Before Curing	Delay Before Curing (Hours)	Storage Before Tanning	Collagenolytic Activity		Leather Decay Grades (% of batch)		
								Aerobic	Anaerobic	Good	Fair	Bad
Raw Hides	B	131	110	Vleissentraal	No	Not Applicable		++	-	See Below		
	C	132	110	Vleissentraal	No			++	-			
	D	130	110	Vleissentraal	No			++	-			
Cured Hides	A	128	100	Vleissentraal	No	5 to 6	3 months	+	-	49	35	16
	B	131	110	Vleissentraal	No	5 to 6	31 days	+	-	40	32	28
	C	132	110	Vleissentraal	No	3	28 days	++	-	56	28	16
	D	130	110	Vleissentraal	No	4 to 5	27 days	+	-	52	27	21
	E	134	100	National Meat	Yes	Minimum	Nil	+++	-	76	18	6
	F	129	Not known	Country Type	Yes	Minimum	3 months	-	-	63	28	9
	G	CW	Not known	Australian	Details not known			-	-	Usually Good		

Table 2.1. Hide Batches, Processing Details, Collagenolytic Activities and Leather Decay Grades.

Table 2.2. Calibration data for Rosen's colorimetric assay of amino acids.

Concentration of glycine in standard solution (μmol/ml)	Absorbance at 570 nm		
	1	2	Mean
0	0.04	0.04	0.04
0.1	0.18	0.18	0.18
0.2	0.31	0.32	0.31
0.3	0.47	0.41	0.44
0.4	0.60	0.52	0.56
0.5	0.73	0.69	0.71
0.6	0.85	0.82	0.84
0.7	1.00	0.99	1.00
0.8	1.16	1.18	1.17
0.9	1.33	1.27	1.30
1.0	1.48	1.44	1.46
1.1	1.61	1.59	1.60
1.2	1.69	1.75	1.72
1.3	1.84	1.82	1.83

Linear regression analysis of the above data produced the results:

$$a_o = 0.023; \quad a_1 = 1.410; \quad r^2 = 0.998;$$

where x = concentration of glycine (μmol/ml),

y = absorbance at 570 nm,

and the equation for the regression line was

$$y = a_o + a_1 x.$$

Absorbance values were converted to glycine equivalents per ml according to the expression:

$$\text{glycine equivalents } (\mu\text{mol/ml}) = \frac{\text{absorbance}_{570} - 0.02}{1.41}$$

(The graph in Fig. 2.1 was plotted from the mean data above.)

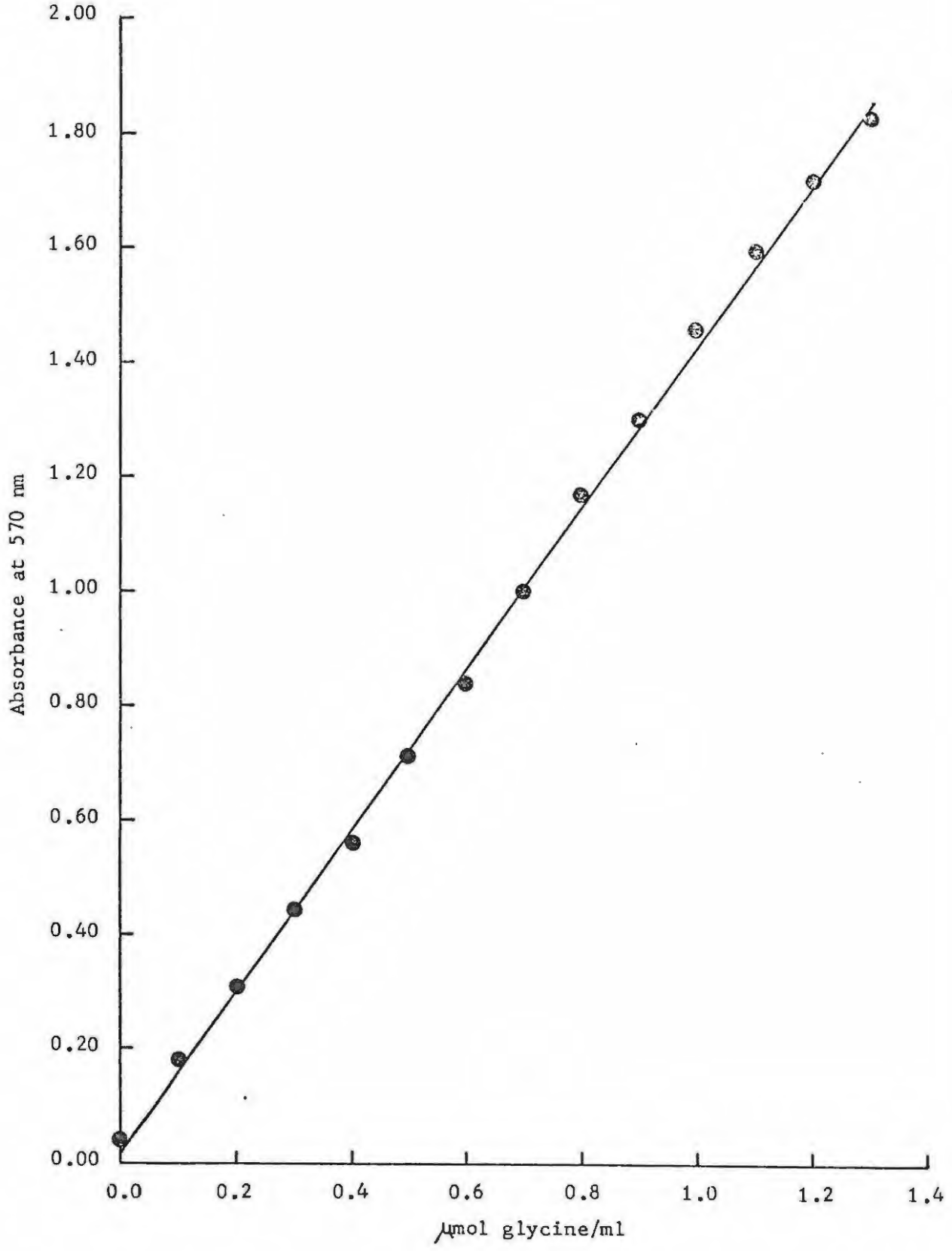


Fig. 2.1. Calibration graph for Rosen's colorimetric assay of amino acids.
This graph is plotted from the mean data in Table 2.2.

Key to Figs. 2.2 to 2.6

 , 0.85% NaCl

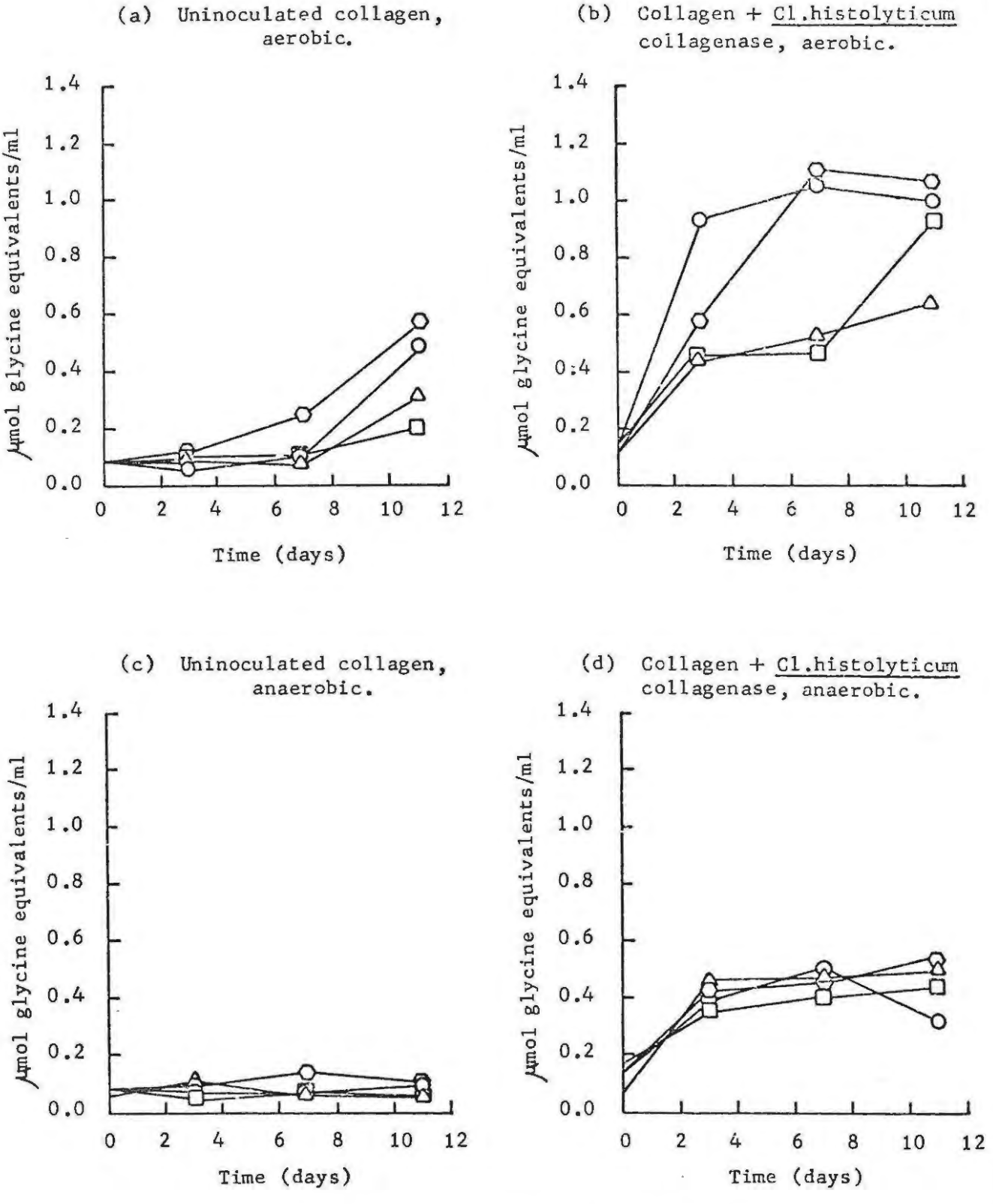
 , 2.34% NaCl

 , 7.00% NaCl

 , 10.00% NaCl

 , Bacterial control

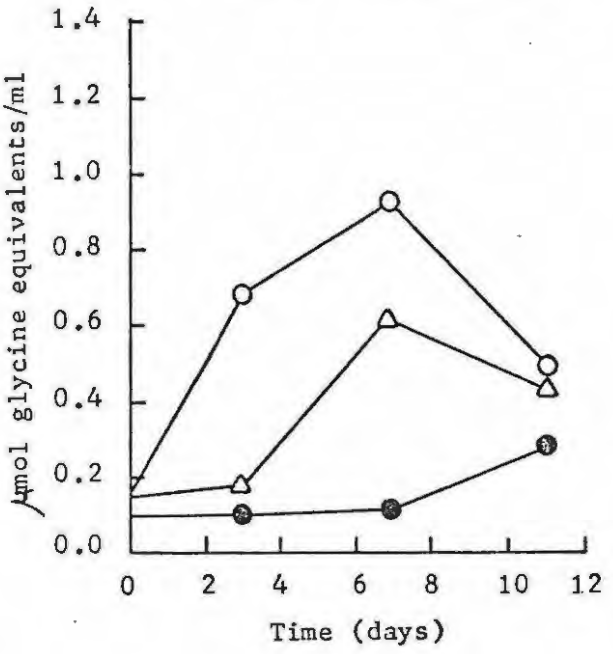
Fig. 2.2. Control data for collagenolytic activity assays.



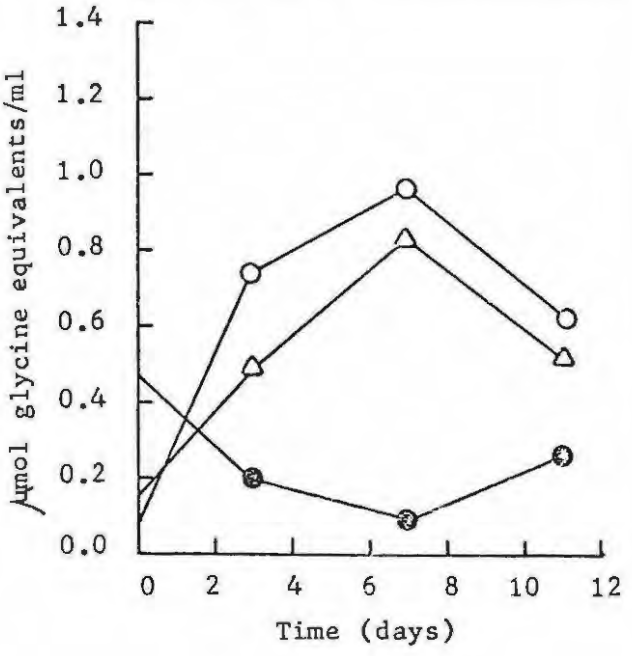
(The key to these graphs is on page 28.)

Fig. 2.3. Aerobic collagenolytic activities of raw hide bacterial populations.

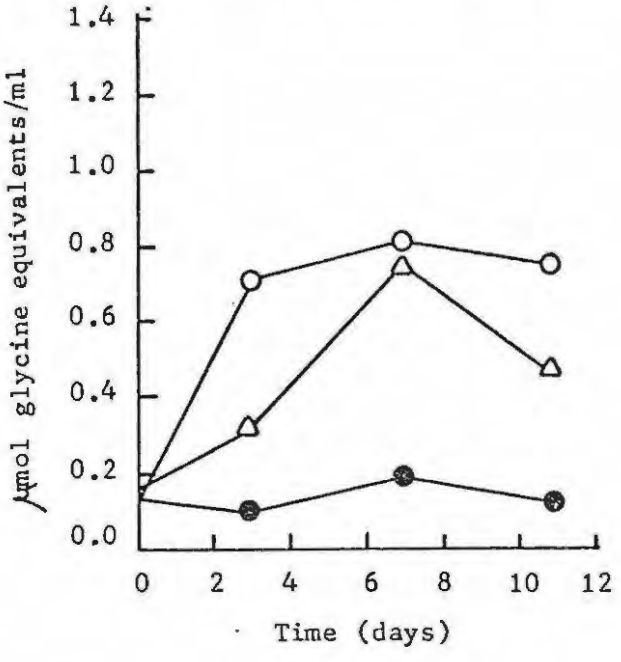
(a) Batch B (Vleissentraal).



(b) Batch C (Vleissentraal).

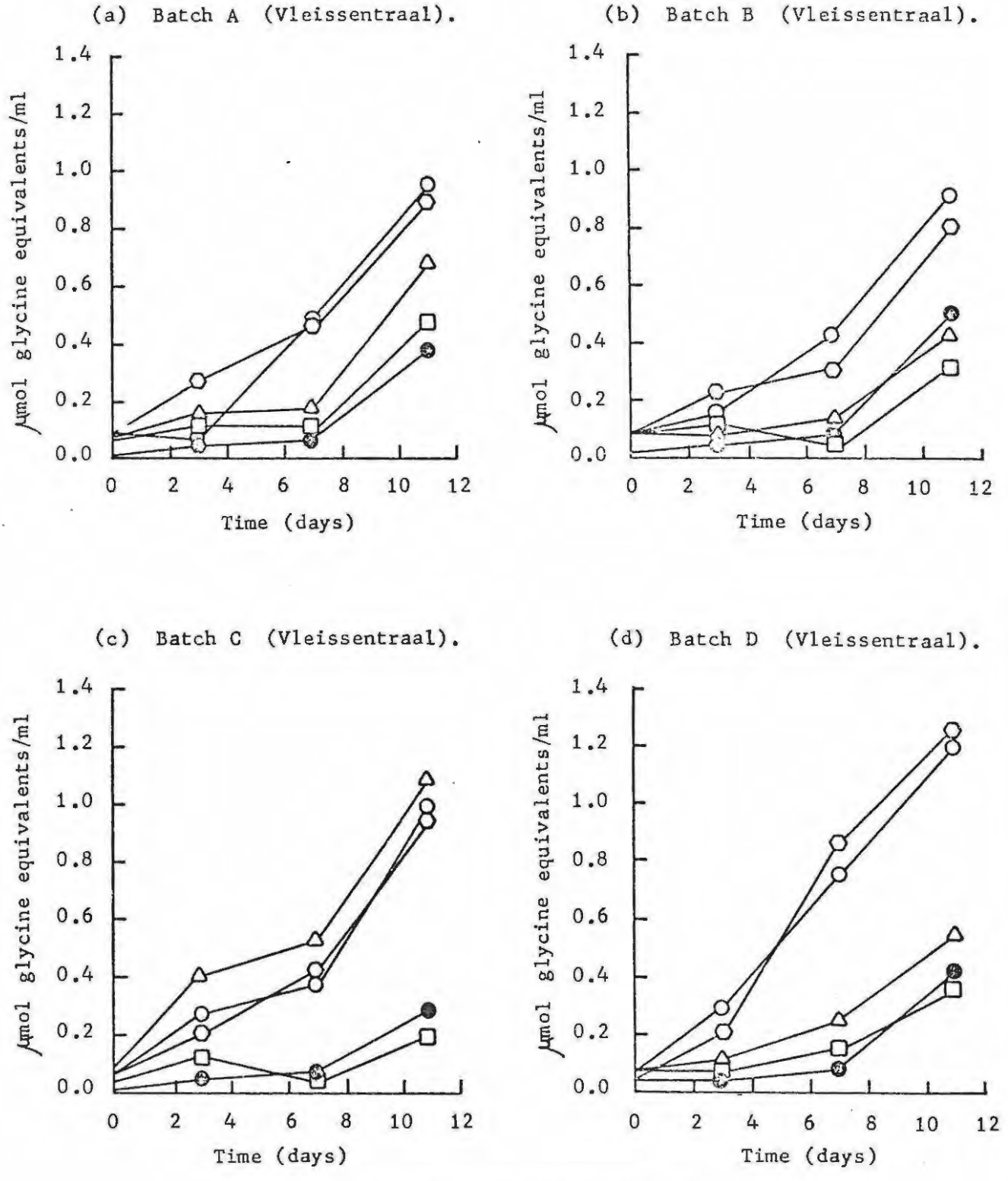


(c) Batch D (Vleissentraal).



(The key for these graphs is on page 28.)

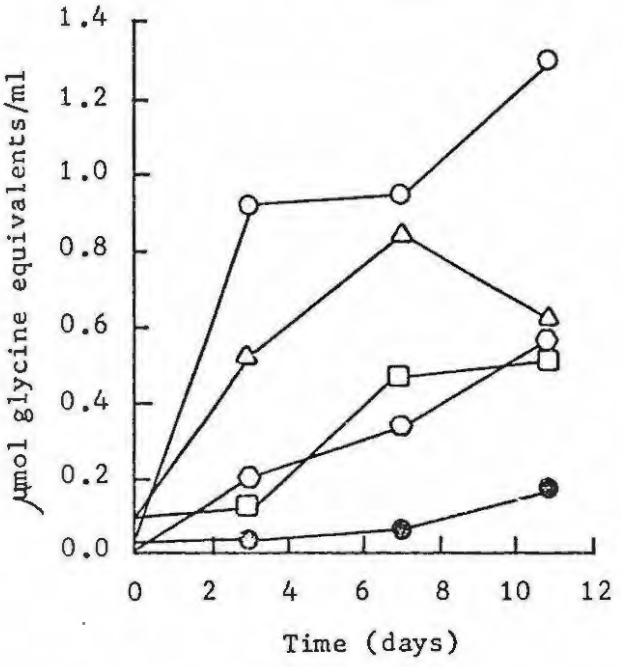
Fig. 2.4. Aerobic collagenolytic activities of cured hide bacterial populations.



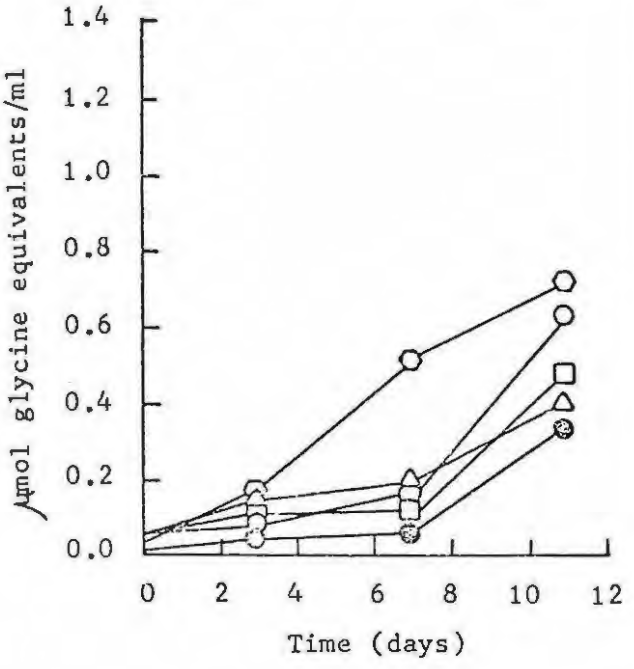
(The key to these graphs is on page 28.)

Fig. 2.4 (continued) Aerobic collagenolytic activity of cured hide bacterial populations

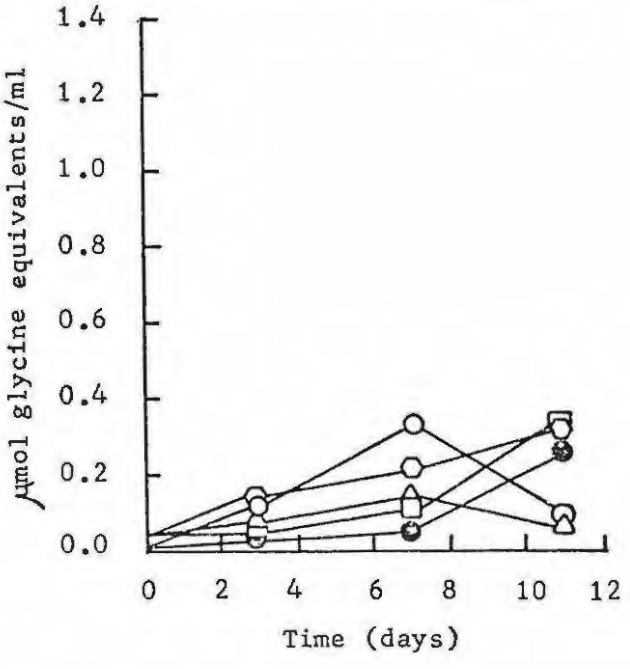
(e) Batch E (National Meat).



(f) Batch F (Country Type).



(g) Batch G (Australian).



(The key for these graphs is on page 28.)

Fig. 2.5. Anaerobic collagenolytic activity of the bacterial population from cured hide batch E (National Meat).

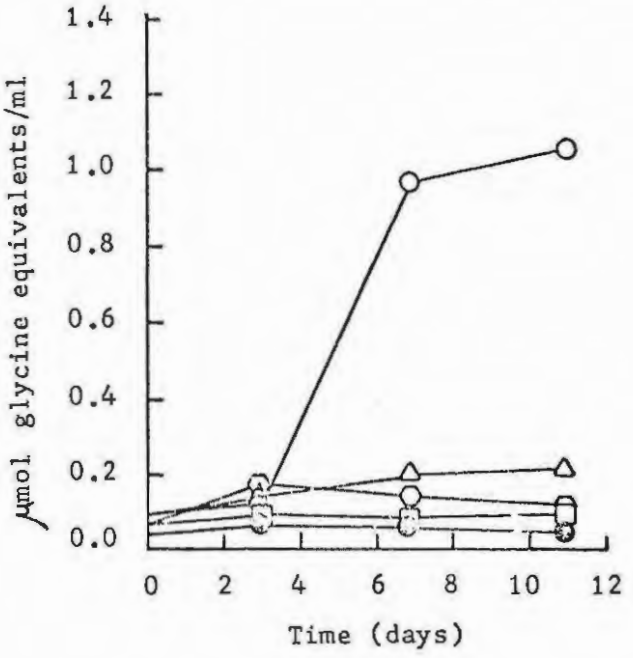
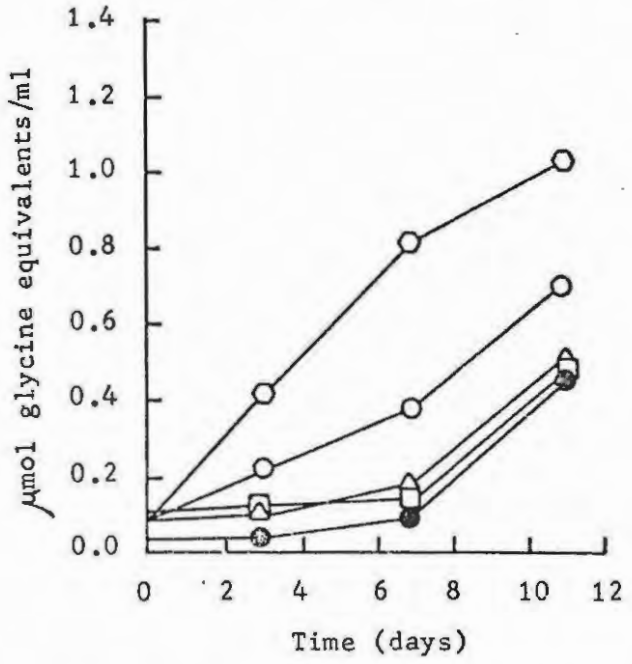


Fig. 2.6. Aerobic collagenolytic activity of the bacteria in soak pit water.



(The key for these graphs is on page 28.)

CHAPTER THREE

COLLAGENOLYTIC ACTIVITY OF ACHROMOBACTER IOPHAGUS

SUMMARY

A strain of Achromobacter iophagus isolated from cured hides was shown to lyse collagen rapidly under aerobic conditions. Changes of temperature, pH, or NaCl concentration affected either the maximum rate of collagen lysis or the lag before this rate was attained, or both. Collagenolytic activity occurred at NaCl concentrations of 0.85 to 10.00%.

INTRODUCTION

Hides frequently contain collagenolytic bacteria (Chapter 2) and the need to prevent degradation of the collagen in hides has been discussed (Chapter 1). There may be several widely-differing bacteria involved in such collagen degradation, for the microbial flora of hides is dense and varied (Turner, 1967); but the highly-specific interaction between their collagenases and collagen, because of its complexity, is likely to show only minor variations. Hence it is possible that the prevention of collagenolytic activity in hides may be more readily accomplished by collagenase-inhibition than by bactericidal or bacteristatic methods. This prospect indicates that the collagenase produced by a hide bacterium should be characterized in the hope of finding a way to efficiently inhibit it which could be used on an industrial scale. However, it should be pointed out that although inhibition of collagenolytic action in hides would prevent leather decay, this would not obviate the need to cure hides of general bacterial action so as to prevent their being rendered unsalable through putrefaction. There are two problems involved, the latter being beyond the scope of this dissertation.

The present work was undertaken with the aim of isolating a

collagenolytic hide bacterium and optimizing conditions for its collagenolytic activity as a preliminary to studying its collagenase. At the same time it was hoped that the findings would benefit the industry by providing them with the knowledge of conditions which favour collagenolytic activity in hides.

METHODS AND MATERIALS

Source and identification. The bacterium was isolated from the National Meat cured hides (batch E) which showed the highest collagenolytic activity in the survey described in Chapter 2. It was identified according to Bergey's Manual of Determinative Bacteriology (Breed, Murray and Smith, 1957) and A Guide to the Identification of the Genera of Bacteria with Methods and Digest of Genetic Characteristics (Skerman, 1967).

Medium. The medium used for maintaining the bacterium contained (g/l): casein hydrolysate, 17.0; glucose, 5.0; glycerol, 10.0; NaCl, 23.4; Na_2SO_3, 0.1; nutrient broth, 8.0; soytone, 3.0; tryptone, 0.5; vitamin-free casamino acids, 0.5; yeast extract, 2.0; and agar, 15.0. The pH was adjusted to 7.6 with NaOH. Owing to the rapid spreading of the bacterium, this medium was not suitable for isolating clones. Discrete colonies were obtained by including 1 mM $ZnCl_2$ with the above.

Collagenolytic activity assays. Collagen reaction mixtures were prepared with insoluble bovine Achilles' tendon collagen instead of the soluble collagen described in Chapter 2. Little difference was observed when the activity of A. iophagus on the two substrates was compared (Fig. 3.1) and the insoluble collagen was used for all further measurements of collagen hydrolysis in this thesis excepting viscosity studies. Reaction mixtures consisted of suspensions of the above insoluble collagen in 6 ml volumes of Tris-HCl or Tris-maleate buffer containing NaCl and 2 mM $CaCl_2$. Substrate concentrations used were

0.2, 0.5, 1.5, 3.5 and 5.5 mg/ml and NaCl concentrations were 0.85, 2.34, 4.00, 7.00, 10.00 and 13.00%. Tris-maleate buffer was used to achieve pH values of 6.0, 6.4, 6.8, 7.2 and 7.6 while Tris-HCl was used for values of 7.2, 7.6, 8.0, 8.4, 8.8 and 9.2. The final concentration of Tris in all buffers was 0.1 M.

From an overnight plate culture of the A. iophagus strain a buffered, washed suspension containing approximately 10^9 bacteria/ml was prepared. Samples (0.2 ml) of this suspension were added to reaction mixtures and incubated aerobically at 15, 20, 25, 30 and 35°C. Experimental controls comprised collagen suspensions without bacteria and bacteria in buffer without collagen. All reaction mixtures and controls were duplicated. Collagenolytic activity was measured by determining the increase with time of ninhydrin-positive materials, expressed as µmol glycine equivalents/ml, by the procedure of Adamcic and Clark (1970). Each assay was performed by withdrawing 0.2 ml of reaction mixture into 1.0 ml of 0.1 M HCl to stop the reaction and estimating the ninhydrin-positive materials in the resultant solution using the colorimetric method of Rosen (1957). This colorimetric method was described in Chapter 2, though the acid-dilution step introduced here required the preparation of fresh calibration data (Table 3.1 and Fig. 3.2) to enable the absorbance values to be converted into glycine equivalents/ml. Because distilled water was used as the reference for absorbance measurements, a blank correction had to be subtracted from each experimental reading. This correction was always made after conversion of absorbance to µmol glycine equivalents/ml, in order to avoid the complications due to the non-linearity of the calibration graph at low concentrations of glycine.

Statistical analysis. An example of the tabulated results obtained by varying one of the experimental conditions is shown in Table 3.2. The source of variation in such data was analyzed using the Hewlett-Packard programme entitled "Two way AOV with replicates" (Hewlett-Packard

Model 10 Stat Pac 2:I-2). Results of the analysis of variance for Table 3.2 are set out in Table 3.3. In the graphical representation of results (Figs 3.4 to 3.7), where each point plotted was the mean result obtained from the duplicate reaction mixtures, an estimate of the least significant difference (L.S.D.) between any two plotted points was calculated using the expression

$$L.S.D. = t_{0.05}\sqrt{\frac{2 \times RV}{r}}$$ (Steel and Torrie, 1960)

where t was Student's t-statistic, RV was the residual variance obtained in the analysis described above, and r was the number of observations per mean, i.e. 2.

RESULTS

Identification

The bacterium is shown in Fig. 3.3 and its characteristics are listed in Table 3.4. It was identified as Achromobacter iophagus.

Collagenolytic activity

The activity graphs in Figs 3.4 to 3.7 were plotted from the mean results of duplicate reaction mixtures; control values shown were the highest values at each time interval of all the controls kept. For clarity, all time zero symbols have been omitted. The L.S.D. values for Figs 3.5 and 3.6 were based on data for 4 days while those for Figs 3.4 and 3.7 were based on data for 3 and 5 days respectively.

Effect of collagen concentration. Activity at different collagen concentrations is shown in Fig. 3.4. Collagenolytic activity increased as the collagen concentration was raised from 0.2 to 5.5 mg/ml. The 1.5 mg/ml concentration was selected as suitable for testing the other experimental conditions.

Effect of temperature. The effect of incubation at different temperatures is shown in Fig. 3.5. There was no significant difference between 30 and 35°C. These temperatures produced the highest maximum rate of collagen lysis and the shortest lag period before this rate was attained. As the temperature was reduced the maximum rate of lysis decreased and the lag period increased. Even at 15°C, however, a rapid rate of collagen breakdown was reached within approximately 6 days.

Effect of pH. Fig. 3.6 shows the effect of pH over the range pH 6.0 to 9.2. No significant differences were discerned in the overall data for pH 7.2, 7.6 and 8.0, at which pH values the maximum rate of collagen breakdown was highest and the lag period was shortest. With increasing deviation from pH 7.2 to 8.0 the maximum rate of collagen lysis decreased and the lag period increased.

Effect of NaCl concentration. These results are presented in Fig. 3.7. Collagenolytic activity occurred between 0.85 and 10.00% NaCl but was inhibited by 13.00% NaCl. The maximum rate of collagen degradation occurred at 2.34% NaCl, as did also the shortest lag period. At NaCl concentrations above 2.34% the maximum rate decreased and the lag period increased. The collagenolytic activities at 0.85 and 4.00% were not significantly different.

DISCUSSION

Under optimal conditions this strain of Achromobacter iophagus exhibited an extremely rapid aerobic degradation of collagen with a short lag period. When experimental conditions were varied one at a time, the bacterium was collagenolytic at 15 to 35°C, at 0.85 to 10.00% NaCl and at pH 6.4 to 8.8. Hence it is likely that this organism could cause significant damage to the collagen of a hide if the curing of the hide was poor or delayed. Furthermore, various workers have reported the widespread occurrence of Achromobacter spp. in raw hides (Turner, 1967) and collagenolytic Achromobacter spp. have been found in cured

hide batches from various curers in South Africa (Thomson, Woods and Welton, 1972). Therefore this hide bacterium is a suitable source of collagenase for the further study envisaged.

This is the first report of a hide bacterium with collagenolytic activity at high NaCl concentrations, which implies a halotolerant collagenase.

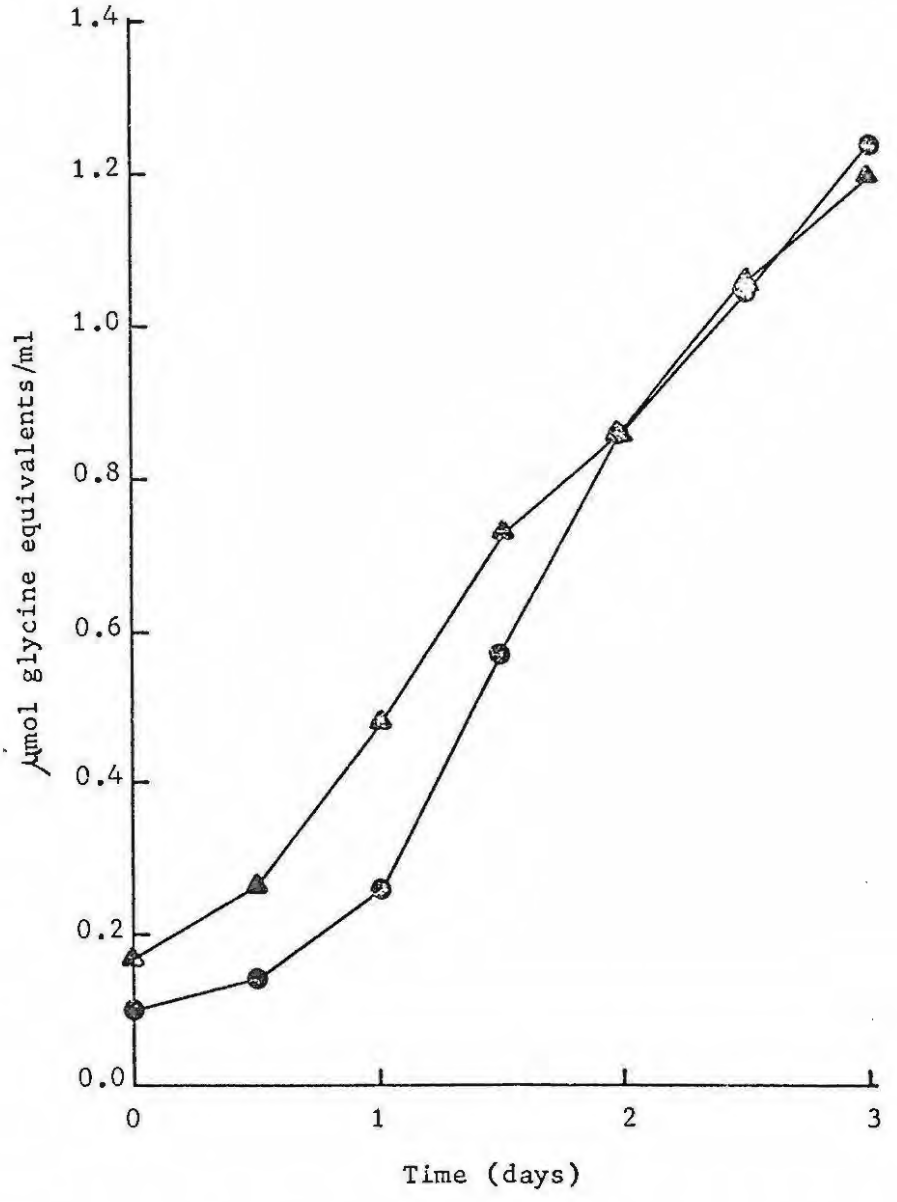


Fig. 3.1. Activity of A. iophagus on neutral salt soluble collagen and on insoluble bovine Achilles' tendon collagen. Reaction mixtures contained 1.5 mg collagen in 3 ml of 0.01 M Tris-HCl (pH 7.6) containing 0.4 M NaCl and 2 mM $CaCl_2$. Assays were performed as described in Chapter 2.

●——●, soluble collagen; ▲——▲, insoluble collagen.

Table 3.1. Calibration data for the colorimetric assay of amino acids by the procedure of Adamcic and Clark (1970).

Concentration of glycine in the standard solution (μmol/ml)	Absorbance at 570 nm		
	1	2	Mean
0	0	0	0
1	0.01	0.01	0.01
2	0.04	0.03	0.04
3	0.21	0.25	0.23
4	0.39	0.45	0.42
5	0.68	0.62	0.65
6	0.87	0.85	0.86
7	0.99	1.05	1.02
8	1.26	1.32	1.29
9	1.48	1.56	1.52
10	1.68	1.72	1.70
11	1.92	1.92	1.92

Linear regression analysis of the above data from 2 to 11 μmol glycine/ml produced the results:

$$a_o = -0.408; \quad a_1 = 0.211; \quad r^2 = 0.997;$$

where x = concentration of glycine (μmol/ml),

y = absorbance at 570 nm

and the equation for the regression line was

$$y = a_o + a_1 x.$$

Absorbance values between 0.04 and 1.92 were converted to glycine equivalents per ml according to the expression:

$$\text{glycine equivalents } (\mu\text{mol/ml}) = \frac{\text{absorbance}_{570} + 0.41}{0.21}$$

(The graph in Fig. 3.2 was plotted from the mean data above.)

Fig. 3.2. Calibration graph for the colorimetric assay of amino acids by the procedure of Adamcic and Clark (1970). The graph is drawn from the mean data in Table 3.1. Standard solutions of glycine in buffer were diluted six-fold with 0.1 N HCl and the resultant mixtures were assayed by the procedure of Rosen (1957), as described in Chapter 2. Absorbance values at 570 nm were read against a reference of distilled water. Varying the composition of the buffer within the limits described in the text did not affect colour development.

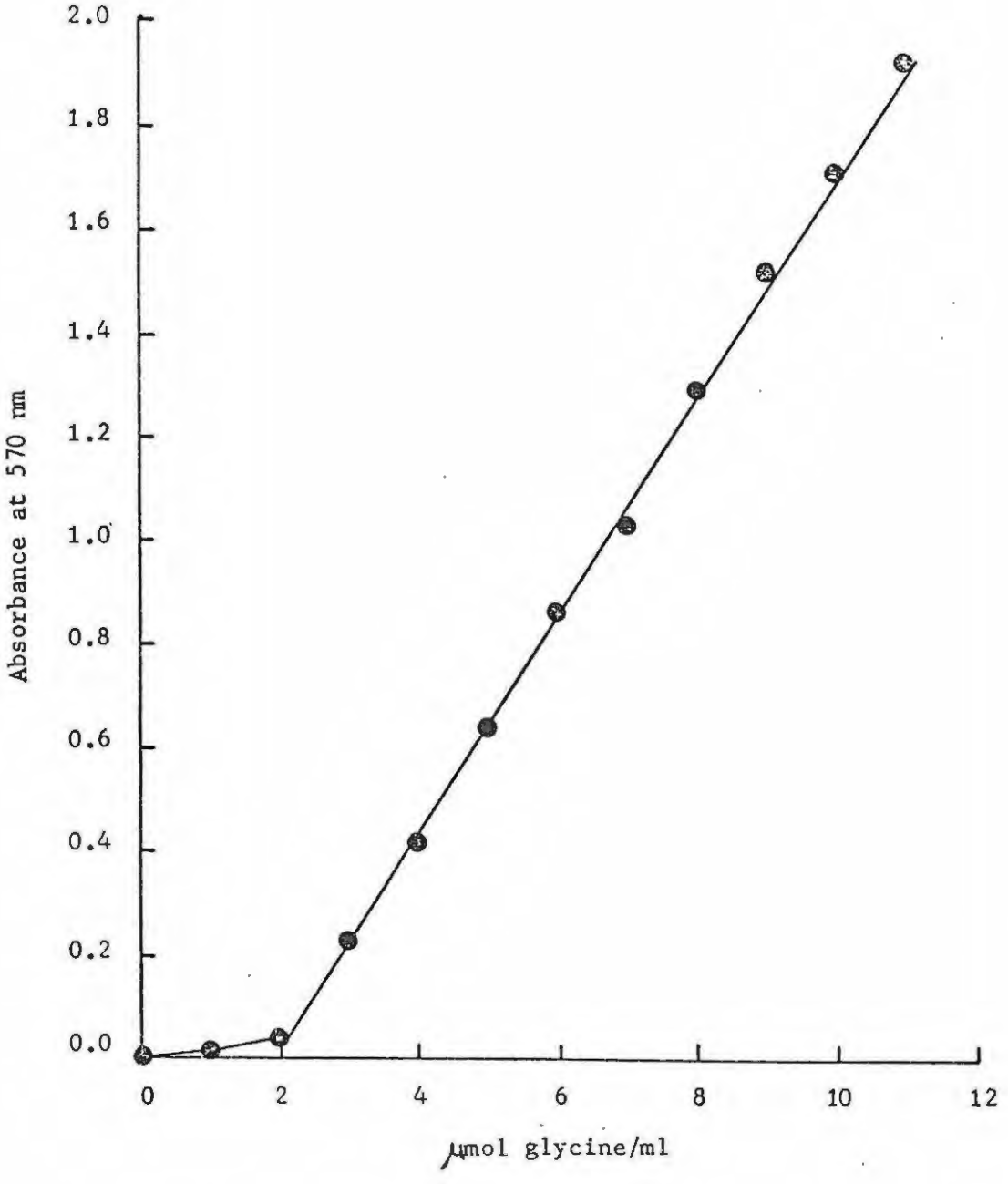


Table 3.2. Data from the experiment testing the effect of NaCl concentration on the collagenolytic activity of *A. iophagus*.

The figures in the body of the table are the amounts of ninhydrin-positive materials, expressed as μmol glycine equivalents/ml, at six different times in reaction mixtures containing six different NaCl concentrations. R_1 and R_2 are replicates.

			NaCl concentration (%)					
			0.85	2.34	4.00	7.00	10.00	13.00
Time in days	0	R_1	0.50	0.79	0.60	0.55	0.50	0.41
		R_2	0.50	0.79	0.50	0.55	0.64	0.45
	1	R_1	0.74	0.69	0.64	0.41	0.31	0.36
		R_2	0.79	0.93	0.93	0.36	0.36	0.36
	2	R_1	2.49	4.34	2.59	0.93	0.27	0.31
		R_2	2.73	3.91	2.68	0.93	0.36	0.31
	3	R_1	5.53	6.47	5.10	1.69	0.31	0.31
		R_2	5.57	7.04	4.34	2.40	0.27	0.27
	4	R_1	7.09	9.22	7.18	3.54	0.88	0.31
		R_2	7.14	8.89	6.76	4.72	0.83	0.31
	5	R_1	7.33	9.57	7.61	6.09	0.69	0.31
		R_2	8.04	10.19	7.71	5.95	0.88	0.27

Table 3.3. Analysis of Variance for Table 3.2.

Source of variance	Sum of squares	Degrees of freedom	Variance	F	Significance
Rows	269.60	5	53.92	864.82	* * *
Columns	232.55	5	46.51	745.99	* * *
Interaction	137.73	25	5.51	88.35	* * *
Residual	2.24	36	0.06		
Total	642.12	71			

Estimate of the Least Significant Difference between any two points in Fig. 3.6:

$$L.S.D._{t\ 0.05} = t_{0.05}\sqrt{\frac{2 \times RV}{2}}$$

$$= 2.03\sqrt{\frac{2 \times 0.06}{2}}$$

$$= \pm 0.50$$

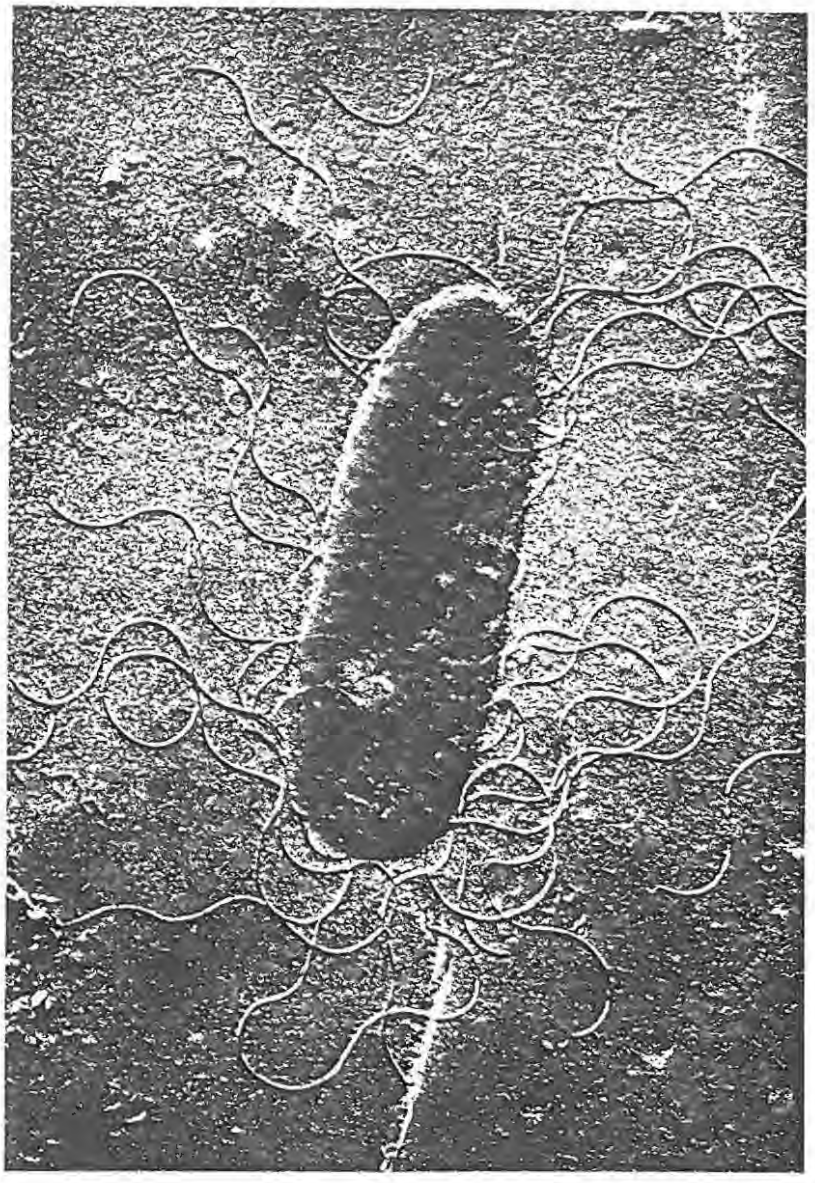


Fig. 3.3. Electron micrograph (shadowed) of A. iophagus showing peritrichous flagella. Magnification x 21 840.

Table 3.4. Characteristics of Achromobacter iophagus

1.	**Cell morphology**	
	(a) Shape	Rod
	(b) Length	2.2 to 7.2 microns
	(c) Width	1.0 to 1.8 microns
	(d) Flagella	Many, peritrichous
2.	**Staining**	
	(a) Acid fast	-
	(b) Gram's	-
	(c) Spore	-
	(d) Capsule	-
3.	**Pigmentation**	None
4.	**Growth**	
	Tested on the medium described in the text, with the NaCl concentration adjusted to 0.00, 0.85, 2.34, 7.00 and 10.00%:	
	(a) Aerobic	Growth from 0.00 to 10.00%; slight at extremes, increasing to very rapid and abundant at the optimum of 2.34%
	(b) Anaerobic	Growth at 2.34% only; slight
5.	**Biochemistry**	
	(a) Indole production	+
	(b) Catalase production	+
	(c) Oxidase production	-
	(d) Reduction of nitrates to nitrites	+
	(e) Action on litmus milk:	
	(i) acid	-
	(ii) alkali	-
	(iii) clotting	+
	(f) Gelatin hydrolysis	+
	(g) Starch hydrolysis	+
	(h) Glucose utilization	+ (Acid produced, but no gas)
	(i) Mannose utilization	+ (Acid produced, but no gas)
	(j) Sucrose utilization	+ (Acid produced, but no gas)
	(k) Lactose utilization	-
	(l) Oxidation or fermentation of glucose	fermentation

(+, -, positive or negative reaction)

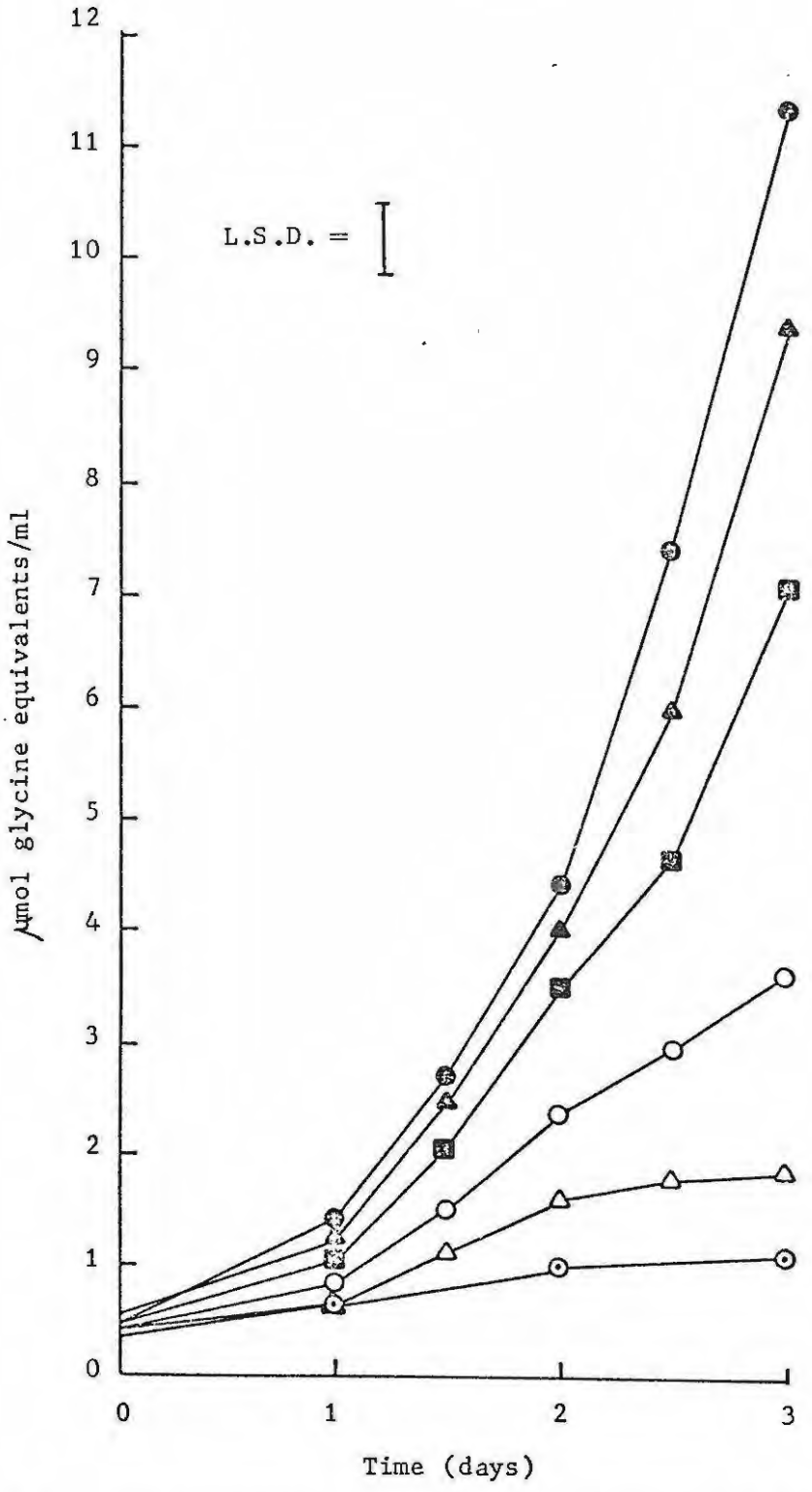


Fig. 3.4. Collagenolytic activity of A. iophagus at different collagen concentrations. Reaction mixtures, incubated at 30°C, contained 2.34% NaCl and were adjusted to pH 7.6. △——△, 0.2 mg/ml; ○——○, 0.5 mg/ml; ▣——▣, 1.5 mg/ml; ▲——▲, 3.5 mg/ml; ●——●, 5.5 mg/ml; ⊙——⊙, control.

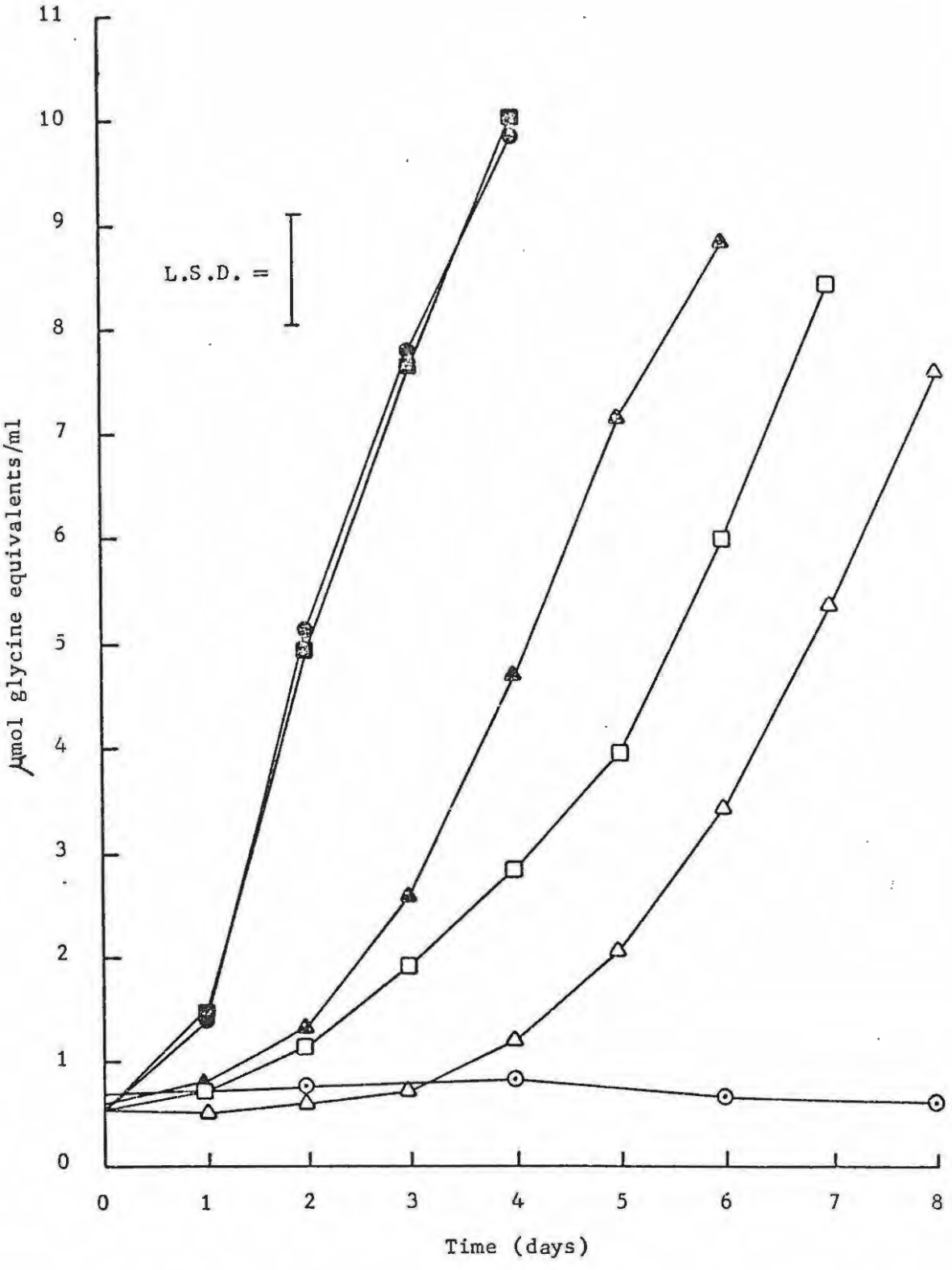


Fig. 3.5. Collagenolytic activity of A. iophagus at different temperatures. Reaction mixtures contained 2.34% NaCl and 1.5 mg collagen per ml of buffer at pH 7.6. △—△, 15°C; □—□, 20°C; ▲—▲, 25°C; ▣—▣, 30°C; ●—●, 35°C; ⊙—⊙, control.

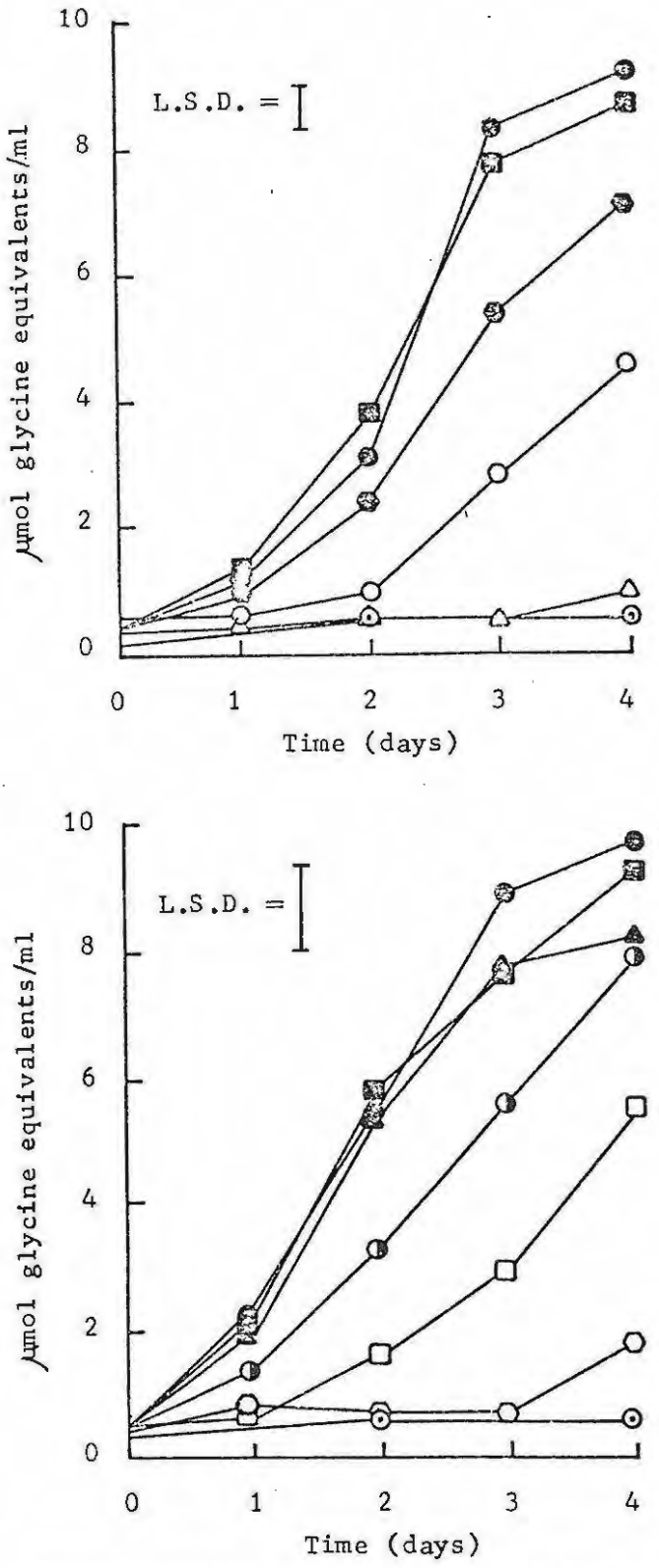


Fig. 3.6. Collagenolytic activity of A. iophagus at different pH values. Reaction mixtures, containing 2.34% NaCl and 1.5 mg collagen per ml of buffer, were incubated at 30°C. △—△, pH 6.0; ○—○, pH 6.4; ◑—◑, pH 6.8; ●—●, pH 7.2; ■—■, pH 7.6; ▲—▲, pH 8.0; ◐—◐, pH 8.4; □—□, pH 8.8; ○—○, pH 9.2; ⊙—⊙, control. Tris-maleate buffer was used for for pH values 6.0 to 7.6 (upper graph) and Tris-HCl for pH values 7.2 to 9.2 (lower graph).

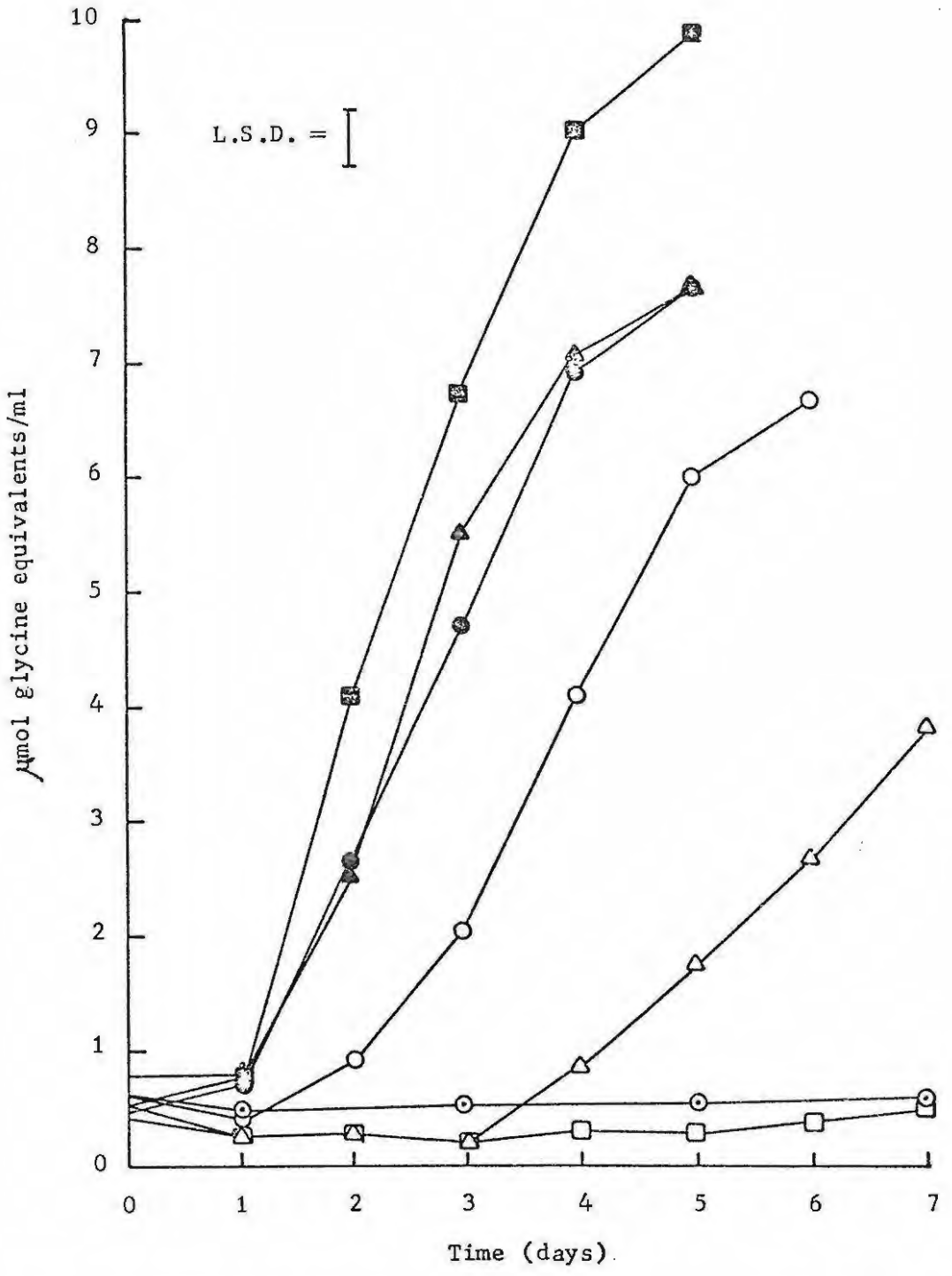


Fig. 3.7. Collagenolytic activity of A. iophagus at different NaCl concentrations. Reaction mixtures, incubated at 30°C, contained 1.5 mg collagen per ml of buffer at pH 7.6.
▲——▲, 0.85%; ■——■, 2.34%; ●——●, 4.00%;
○——○, 7.00%; △——△, 10.00%; □——□, 13.00%;
⊙——⊙, control.

CHAPTER FOUR

PRODUCTION AND PURIFICATION OF COLLAGENASE FROM ACHROMOBACTER IOPHAGUS

SUMMARY

Achromobacter iophagus produced collagenase when cultured aerobically in buffer containing 5% peptone. The bacterium is non-pathogenic and tests on rabbits indicated that the culture medium was atoxic. The collagenase was purified by $(NH_4)_2SO_4$ precipitation, starch block electrophoresis, and gel-filtration. It was shown to hydrolyze insoluble and soluble native collagen as well as the synthetic substrate PZ-pro-leu-gly-pro-arg, and to be serologically distinct from Clostridium histolyticum collagenase.

INTRODUCTION

Notwithstanding indications from workers such as Weinberg and Randin (1931; 1932) and Maschmann (1938b) that Cl. histolyticum produced a collagenase, early workers concentrated on the Cl. perfringens collagenase. Only after Neumann and Tytell (1950) and MacLennan, Mandl and Howes (1953) reported that the Cl. histolyticum collagenase was the more powerful, did the emphasis shift. Since then, the Cl. histolyticum enzyme has been exhaustively studied and as recently as 1972 it was the only commercially-available collagenase (Mandl, 1972).

For production of collagenase from Cl. histolyticum, it is generally agreed that best results are achieved with a medium containing 5% peptone (Mandl, 1961) and a variety of vitamins and mineral salts (MacLennan, Mandl and Howes, 1953), though casein hydrolysate has been used instead of peptone (Warren and Gray, 1961).

Isolation of Cl. histolyticum collagenase from culture filtrates was first described by Tytell and Hewson (1950) and Mandl, MacLennan

and Howes (1953), who used methanol and $(NH_4)_2SO_4$ precipitation respectively. Subsequent purification procedures included fractional precipitation with $(NH_4)_2SO_4$ (DeBellis et al., 1954), adsorption elution on collagen and alumina (Gallop, Seifter and Meilman, 1957; Seifter et al., 1959), paper electrophoresis (Mandl, Zipper and Ferguson, 1958) and starch block electrophoresis (Gallop, Seifter and Meilman, 1957), but none of these methods produced collagenase which was completely free of other proteolytic activity. This degree of purity was achieved only with the advent of newer methods of purification, in particular, gel-filtration techniques (Keller and Mandl, 1963; Grassmann, Strauch and Nordwig, 1963; Yoshida and Noda, 1965; Strauch and Grassmann, 1966). Mandl (1972) described gel-filtration as the most convenient method of obtaining Cl. histolyticum collagenase completely free of activities against other proteins.

Gel-filtration was decided upon as the technique to be used for the attempted purification of A. iophagus collagenase, with $(NH_4)_2SO_4$ precipitation and starch block electrophoresis as preliminary steps. Yoshida and Noda (1965) used the same procedure to purify Cl. histolyticum collagenase prior to their ion-exchange investigations.

METHODS AND MATERIALS

Organism. The collagenolytic strain of A. iophagus isolated from cured hides (Chapter 3) was used as the source of collagenase. The bacterium was maintained in 0.1 M Tris-HCl-0.4 M NaCl-2 mM $CaCl_2$ (pH 7.6) containing 1.5 mg collagen per ml. Prior to use for collagenase-production, a 24 h culture of the bacterium was prepared on a plate of the complex agar described in Chapter 3.

Production of collagenase. Two experiments were performed in order to find a suitable medium for producing collagenase from A. iophagus. In the first, production of collagenase was compared in four different

media consisting of 30 ml volumes of 0.1 M Tris-HCl-0.4 M NaCl-2 mM $CaCl_2$ (pH 7.6) supplemented with:

(a) 20 mg hide powder per ml;

(b) 1.5 mg collagen per ml;

(c) 1.5 mg collagen per ml plus 5% peptone;

(d) 5% peptone.

Each medium was inoculated with 0.5% by volume of a cloudy suspension in buffer of the 24 h plate culture of A. iophagus, and incubated at 30°C. The levels of collagenase activity in the cultures were monitored using the synthetic substrate phenylazobenzyloxycarbonyl-L-prolyl-L-leucyl-glycyl-L-prolyl-D-arginine (PZ-pro-leu-gly-pro-arg).

Medium (d) above was found to be the most suitable of the four, and the second experiment was conducted to see whether the collagenase yield in this medium could be improved by increasing the $CaCl_2$ concentration to 0.1 M or by adding vitamins. The following vitamins were added (μg/ml): meso-insitol, 20; thiamin, 4; nicotinic acid, 4; riboflavin, 4; calcium pantothenate, 4; p-aminobenzoic acid, 2; pyridoxine hydrochloride, 0.00032; D-biotin, 0.01. The cultures, whose volumes were increased to 250 ml, were inoculated and assayed as before. Incubation was at 30°C with aeration. As a fermentation apparatus was not available, a stream of filtered air was bubbled vigorously through the cultures to aerate them. This less efficient method imposed a severe limitation on the volume of cultures, 250 ml being the maximum starting volume that was used. For routine collagenase-production, batches of 12 x 250 ml volumes of 5% peptone in 0.1 M Tris-HCl-0.4 M NaCl-0.01 M $CaCl_2$ (pH 7.6) were inoculated and incubated as described above. Cultures were incubated for 3½ days, during which up to 25% of the starting volume was lost through evaporation; thereafter, cells were removed by centrifuging at 10 000 rpm for 15 min.

Ammonium sulphate precipitation. A pilot run was made to determine the percentage saturation required for optimal precipitation of A. iophagus collagenase. Ammonium sulphate crystals were added to 8 ml aliquots of culture supernatant to give various percentage saturations. After 4 h at 4°C, each precipitate was pelleted by centrifuging at 10 000 rpm for 10 min and resuspended in 8 ml of distilled water. The resultant solutions were dialyzed against distilled water at 4°C overnight and then assayed for collagenase activity and protein concentration. For each fraction, the specific collagenase activity was calculated by dividing the collagenase activity by the protein concentration.

Routine harvesting of crude collagenase from culture supernatants was performed by the addition of crystalline $(NH_4)_2SO_4$ to 60% saturation, i.e. approximately 390 g of $(NH_4)_2SO_4$ was added to each litre of supernatant. After 18-24 h at 4°C, the precipitate was collected by centrifuging at 10 000 rpm for 10 min and redissolved in distilled water. Following dialysis against 2 changes of distilled water at 4°C overnight, any further precipitate was removed by centrifugation, and the crude collagenase in the final solution was lyophilized.

Starch block electrophoresis. The crude collagenase was subjected to zone electrophoresis on a block of granular starch (Kunkel, 1954; Yoshida and Noda, 1965). A thick slurry of potato starch was prepared in 0.32 M barbital sodium-0.029 M sodium acetate-2 mM $CaCl_2$, adjusted to pH 9.0 at 4°C with normal HCl. Preliminary treatment of the starch included one wash with 0.05 N NaOH, two with distilled water, and one with buffer. A 40.0 x 2.5 x 1.0 cm perspex trough, the dimensions used by Nagai (1961), was closed off at the ends with wads of filter paper, lined with plastic film, and filled with starch slurry. The filter paper wads were allowed to draw off excess liquid from the starch mixture until all liquid disappeared from the surface. Then a

long sheet of filter paper was applied to the top of the block and pressure was exerted with a perspex plate to remove further buffer and compress the starch. The block was pre-electrophoresed for 30 min. Two hundred mg of the crude A. iophagus collagenase lyophilisate resulting from the $(NH_4)_2SO_4$ precipitation were dissolved in approximately 1 ml of buffer, mixed with dried starch (Nagai, 1961), and applied in a narrow trough gouged out of the centre of the block. After folding over the plastic lining to completely sheathe the starch block, electrophoresis was carried out at 4°C for 24 h with a constant voltage of 300 V and 6-8 mA. Changes in pH during electrophoresis were avoided by using an Ag-AgCl electrode system (Stelos, 1967) connected to the tank buffer by 1.5 cm diam salt bridges made of 3% ion agar in tank buffer; six thicknesses of Whatman No. 3 chromatography paper connected the starch block to the tank buffer. A diagram of the electrophoresis apparatus is presented in Fig. 4.1.

After completion of the electrophoresis, the starch block was allowed to dry for 30 min and then divided into 1.0 cm segments on either side of the centre. Each segment was eluted by mashing it in a sintered-glass filter funnel, adding 5 ml of 0.1 M Tris-HCl-0.4 M NaCl-2 mM $CaCl_2$ (pH 7.6) and drawing off the liquid by suction. Up to 100% recovery of the separated materials may be obtained in this way (Kunkel, 1954). The fractions were assayed for collagenase activity using PZ-pro-leu-gly-pro-arg and their relative protein concentrations were measured spectrophotometrically at 280 nm. Fractions from each activity peak were pooled, dialyzed against distilled water and lyophilized. Any precipitate which appeared during dialysis was removed by centrifugation prior to lyophilizing.

Gel-filtration. The collagenase preparations obtained by starch electrophoresis were purified by gel-filtration, the procedure followed being similar to that of Yoshida and Noda (1965). Gel columns were

prepared, eluted and maintained according to Reiland (1971). Sephadex G-200 was allowed to swell for 3 days at room temperature in an excess of eluant buffer supplemented with 0.02% sodium azide. After removal of the "fines", the gel slurry was degassed and poured into a 100 x 2.5 cm jacketed column which was partially filled with buffer (approximately 20% of its volume) and fitted with a gel reservoir. A settling time of 10 min was allowed, whereupon the flow valve of the column was opened and the column was washed for 18 h with a pressure-head not exceeding 8 cm. The column was then fitted with a flow adaptor (plunger) and a further 500 ml of buffer was passed through it. A column prepared in this way was used for four or five consecutive runs, the direction of flow being reversed with each run.

The eluant buffer used was 0.02 M Tris-HCl-2 mM $CaCl_2$ (pH 7.6). It was membrane-filtered before use, for this is reported to prolong the life of Sephadex G-200 when used with Tris buffers (Williams and Chase, 1968). The length of the gel column varied from 88 to 95 cm. Fractions of 5 ml were collected, a flow rate of 10 ml per h being maintained with a peristaltic pump, and tap water was circulated through the column jacket. The void volume of each column was determined by the elution of Blue Dextran 2000, which procedure also served to check that the gel was properly packed.

Samples, containing up to 150 mg of collagenase preparation resulting from starch electrophoresis, were dissolved in 5-10 ml of eluant buffer and clarified by centrifugation before they were loaded onto the column. Fractions were assayed for collagenase activity and the relative protein concentrations were monitored spectrophotometrically at 230 nm. Fractions with constant specific activity were pooled, dialyzed against distilled water at 4°C overnight, and lyophilized.

Collagenase assays. Three methods were used:

(1) Hydrolysis of PZ-pro-leu-gly-pro-arg. The method was that of

Wünsch and Heidrich (1963). Ten mg of PZ-pro-leu-gly-pro-arg were dispersed in 0.1 ml of methanol and made up to 10 ml with veronal-acetate buffer (Michaelis, 1931) adjusted to pH 7.1 and supplemented with 2 mM $CaCl_2$. For a quantitative activity determination, 0.25 ml of collagenase solution and 1.0 ml of substrate solution, both prewarmed, were mixed and gently shaken in a water bath at 37°C. After exactly 15 min, 0.5 ml of the reaction mixture was removed and vigorously shaken 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 0.3 g of anhydrous sodium sulphate, and its absorbance at 320 nm was read in a spectrophotometer. By reference to a calibration graph (Table 4.1 and Fig. 4.2) the absorbance at 320 was converted to units of enzymic activity.

Wünsch and Heidrich (1963) defined one unit of collagenase activity on PZ-pro-leu-gly-pro-arg as that amount of enzyme which produced 0.01 μ mol PZ-pro-leu per 5 ml of ethyl acetate under the above conditions. However, the unit of enzymic activity currently recommended by the Enzyme Commission is the katal (abbreviated kat), where one katal is the amount of activity that converts one mole of substrate per second (Florkin and Stotz, 1973). The relationship between the katal and the Wünsch-Heidrich unit is as follows:

$$1 \text{ kat} = 9 \times 10^{10} \text{ Wünsch-Heidrich units;}$$

$$1 \text{ Wünsch-Heidrich unit} = 11.11 \times 10^{-12} \text{ kat,}$$

$$= 11.11 \text{ pkat.}$$

In this dissertation, all quantitative measurements of collagenase activity were made on PZ-pro-leu-gly-pro-arg and expressed in terms of katals or suitable multiples thereof. For consistency, the above method was strictly adhered to even though activity was increased more than two-fold if the veronal-acetate buffer was replaced with 0.1 M Tris-HCl-0.4 M NaCl-2 mM $CaCl_2$ (pH 7.6).

(2) Hydrolysis of insoluble collagen. Substrate suspensions consisting of 9.0 mg of insoluble bovine Achilles' tendon collagen in 3.0 ml 0.1 M Tris-HCl-0.4 M NaCl-2 mM $CaCl_2$ (pH 7.6) were prepared 18 h before use so as to allow the dry collagen to be thoroughly soaked. After prewarming enzyme solutions and substrate suspensions, appropriate amounts of enzyme were added to substrate preparations and the reaction mixtures were incubated at 30°C. Collagenolytic activity was measured by determining the release of ninhydrin-positive materials as described in Chapter 3. In addition to the usual enzyme-free control, the substrate was shown to be undenatured by treatment with pronase at an enzyme:substrate ratio of 1:50.

(3) Hydrolysis of soluble collagen. Acid-soluble collagen, prepared by the method of Cooper and Davidson (1965), was dissolved in 0.1 M Tris-HCl-0.5 M $CaCl_2$ (pH 7.6) by gently stirring at 4°C overnight. The solution was purified by centrifugation at 105 000 x g for 1 h and the final concentration of collagen was about 1 mg/ml. Enzyme solutions were prepared in 0.1 M Tris-HCl-2 mM $CaCl_2$ (pH 7.6) and both enzyme and substrate solutions were preincubated at 20°C. Hydrolysis of the soluble collagen was measured by following the decrease in its specific viscosity. At time zero, 0.5 ml of enzyme solution or control buffer was mixed with 6.0 ml of substrate solution and the required volume of the resultant reaction mixture was pipetted into a Cannon Fenske viscometer suspended in a water bath at 20°C. The viscometer had a tube size of 100 and a flow rate with distilled water at 20°C of 54 sec. Flow rates were determined for the solvent buffer, collagen solution, collagenase-collagen mixtures, and pronase-collagen mixed 1:30, the last-mentioned having been included as a check on the native state of the collagen. The kinematic viscosity of each solution was calculated from the relationship:

$$V = Ct - \frac{B}{t}$$

where V = Kinematic viscosity in centistokes,

t = observed flow rate in seconds,

C = 0.01735 centistokes per second,

B = 1.1 centistoke seconds.

(constants B and C were provided by the manufacturer).

Kinematic viscosity values were converted to relative viscosity values and then to specific viscosity values as follows:

$$\text{Relative Viscosity} = \frac{\text{Kinematic viscosity of solution}}{\text{Kinematic viscosity of solvent}};$$

$$\text{Specific Viscosity} = \text{Relative Viscosity} - 1.$$

Protein determination. Protein was assayed using the method of Lowry et al. (1951) as described by Chase and Williams (1968). Reagents consisted of:

Solution 1: 2% Na_2CO_3 in 0.1 N NaOH.

Solution 2: 0.5% $CuSO_4.5H_2O$ in 1% sodium tartrate, freshly prepared from equal volumes of double strength stock reagents.

Solution 3: Alkaline copper solution - 50 parts of solution 1 plus 1 part of solution 2, freshly prepared.

Solution 4: Phenol reagent - 5 parts of Folin-Ciocalteu phenol reagent plus 4.7 parts of distilled water.

The assay procedure was as follows:

Step 1: 2.5 ml of solution 3 was thoroughly mixed with 0.2 ml of sample containing 10 to 100 µg of protein. This was allowed to stand for 12-15 min at room temperature.

Step 2: 0.25 ml of the diluted phenol reagent, solution 4, was rapidly added and thoroughly mixed within seconds. Colour was allowed to develop for 30 min at room temperature.

Step 3: Colour was read at 690 nm in a spectrophotometer and the absorbance values were converted to protein concentrations

by means of calibration data prepared with bovine albumin (Table 4.2 and Fig. 4.3).

Owing to the fact that Ca^{++} is reported to interfere with colour development (von Hippel et al., 1960), the above method was used only with aqueous solutions.

Polyacrylamide gel electrophoresis. Analytical polyacrylamide gel electrophoresis was performed using the high pH discontinuous buffer system of Davis (1964). The procedure followed was based on that described by Maizel (1971). Stock solutions consisted of:

1. Separation gel buffer: 48 ml of normal HCl plus 36.3 g of Tris, dissolved and diluted to 100 ml with distilled water (pH 8.9);
2. Spacer gel buffer: 5.98 g of Tris dissolved, adjusted to pH 6.7 with normal HCl, and diluted to 100 ml;
3. Electrode buffer: 6.0 g Tris plus 28.8 g glycine, dissolved and diluted to 1 litre with distilled water. This was diluted tenfold before use.

Gels were prepared as follows:

Separation gel: (10% Cyanogum): 2.0 g of Cyanogum plus 2.5 ml of separation gel buffer stock solution were dissolved and diluted to 19.9 ml with distilled water. After de-aerating this solution, 0.008 ml of TEMED and 0.1 ml of 10% ammonium persulphate were added with mixing. Gel tubes with a 5 mm internal diam were filled to the required height with the final mixture and were overlayered by spraying with distilled water. Gelling was usually complete within 20 min. Thereafter the liquid above the separation gel was poured off and the face of the gel was rinsed with spacer gel buffer.

Spacer gel: (3% Cyanogum): 0.3 g of Cyanogum plus 1.25 ml of spacer gel buffer stock solution were dissolved and diluted

to 9.9 ml with distilled water and de-aerated. After adding 0.005 ml of TEMED and 0.1 ml of ammonium persulphate, 0.2 ml of the final mixed solution was placed on top of the separation gel in each gel tube and overlayered as before. Gelling was usually complete within 35 min.

Samples to be analyzed contained 1-2 mg of collagenase lyophilisate, 0.1 ml of 1% aqueous phenol red, 0.1 ml glycerol and 0.8 ml spacer gel buffer diluted tenfold. Gels were loaded with 15-200 µl of sample preparation containing 15-400 µg of protein. Electrophoresis was carried out at 100 V and 2 mA per gel for 10 min, followed by 200 V and 2-3 mA per gel for about 90 min. After completion of the run, gels were removed with the aid of a syringe, stained for 3-18 h in 0.5% Amido black in 7.0% (v/v) acetic acid, and destained in 7.0% (v/v) acetic acid either by diffusion or by the electrophoretic method of Ward (1970).

In addition to the foregoing method, the cationic systems of Reisfeld, Lewis and Williams (1962) and Taber and Sherman (1964) were used with samples of collagenase II.

Hydrolysis of casein. Collagenase preparations were assayed for caseinolytic activity by the method of Yoshida and Noda (1965) as modified by Rippon (1968). Reaction mixtures consisted of 24 mg of casein in 4.0 ml of 0.1 M Tris-HCl (pH 7.6) and 0.2 ml of enzyme solution or control buffer. They were gently shaken for various intervals in a water bath at 37°C. Reaction was stopped by adding 2.0 ml of 25% trichloroacetic acid and precipitation was allowed to proceed for 30 min. After filtering the material, the released protein in the filtrate of each reaction mixture was determined by comparing its absorbance at 280 nm with that of a control. Absorbance values were converted to mg protein per ml by means of calibration data prepared with bovine albumin (Table 4.3 and Fig. 4.4). Caseinolytic activity was expressed as mg protein released per mg of enzyme preparation per h.

Serological comparison of collagenases. A 0.1% solution of purified A. iophagus collagenase I in 0.02 M Tris-HCl-2 mM $CaCl_2$ (pH 7.6) containing 0.2% formalin, was used to immunize a male rabbit as follows: (1) 2 mg injected intravenously; (2) one week later, intramuscular injection of 4 mg in emulsion prepared with Freund's incomplete adjuvant; (3) 4 weeks later, 1 mg injected intravenously. The rabbit was bled 4 days after the final injection. The blood was allowed to stand at room temperature for 1 h, after which the clot was gently freed from the walls of the container with the aid of a spatula. Following further storage at 4°C for 18 h, the serum was decanted and clarified by centrifuging at 10 000 rpm for 10 min. For long-term storage, serum was kept at 4°C after the addition of 0.1% sodium azide.

Using the double-diffusion-in-gel method of Ouchterlony (1958), the above antiserum was tested for reactivity with purified A. iophagus collagenase I, and for cross-reactivity with the starch-electrophoresed preparation of A. iophagus collagenase II and with Cl. histolyticum collagenase forms I and II. Ouchterlony plates were prepared using 1% ion-agar in 0.05 M sodium phosphate buffer (pH 7.2) containing 0.85% NaCl. The strained molten agar was poured into formvar-treated glass petri dishes and 5-well patterns were punched out of it once it had set. Serial dilutions of the antiserum were tested against various dilutions of the collagenase solutions, the initial collagenase concentrations being 1 mg/ml. After filling the centre well of each well-pattern with antiserum and the outer wells with the collagenase solutions, plates were kept at 30°C for 12-18 h. Reaction of the antiserum with a collagenase was indicated by a precipitin band.

Toxicity test. To test for the presence of a toxin, 2 ml aliquots of culture supernatant were injected sub-cutaneously into each of three rabbits. The rabbits were kept under daily observation for 3 weeks.

RESULTS

The comparisons of culture media (Figs 4.5 and 4.6) established that 5% peptone in 0.1 M Tris-HCl-0.4 M NaCl-0.01 M $CaCl_2$ (pH 7.6) was a suitable medium for the production of collagenase from A. iophagus; no advantage was gained by the inclusion of collagen or vitamins. This medium was therefore adopted for routine collagenase-production and from Fig. 4.6, 3½ days was selected as a suitable incubation period for 250 ml cultures. The $(NH_4)_2SO_4$ precipitation pilot run (Fig. 4.7) revealed that 60% saturation was the proportion of $(NH_4)_2SO_4$ suitable for harvesting the collagenase from culture supernatant. Routine precipitation of crude collagenase was therefore done using this % saturation.

Starch block zone electrophoresis of the crude collagenase resulted in the separation of two peaks of collagenase activity (Fig. 4.8). The activity which migrated towards the anode was produced in greater quantity and was designated collagenase I; the cathodically-migrating enzyme was designated collagenase II. Both collagenases were accompanied by some brown pigment. Upon gel-filtration of the collagenase preparations from the starch electrophoresis, each collagenase was well separated from the other major protein components (Fig. 4.9). The brown pigment was removed from both collagenases, eluting in the trailing peaks.

A typical purification is summarized in Table 4.4, though the yields and specific activities of the different preparations of the enzymes varied from batch to batch and seemed to be highly dependent upon the particular lot of peptone used to prepare the medium. The highest specific activity recorded for the collagenase I was 154 451 μkat/kg.

When the enzyme preparations produced by starch electrophoresis and gel-filtration were analyzed by polyacrylamide gel disc

electrophoresis, the results were rather surprising! As expected, the collagenase I preparations produced patterns of protein bands in the anionic system of Davis (1964); but not a single component of the collagenase II preparations was found to migrate in either of the two cationic systems tried. The collagenase II preparations were therefore applied to the system of Davis (1964), and their behaviour was found to be similar to that of their collagenase I counterparts! The actual collagenases from preparations I and II exhibited identical migration, and only slight differences were evident in the distribution of the contaminating proteins in the starch-electrophoresed preparations. Fig. 4.10 compares the distribution of components in the two collagenase preparations from the starch electrophoresis; Fig. 4.11 shows a collagenase I preparation before and after gel-filtration, and establishes that the gel-filtration is an efficient purification technique.

As hydrolysis of PZ-pro-leu-gly-pro-arg is not conclusive proof that an enzyme is a true collagenase (Heidrich, Prokopová and Hannig, 1969), the two A. iophagus enzymes were tested for the ability to hydrolyze native collagen. They showed rapid degradation of insoluble collagen as judged by the ninhydrin analysis (Fig. 4.12) with equal amounts of enzyme producing equal rates of digestion of the collagen. The pronase control showed no significant increase in ninhydrin-positive materials, indicating that the substrate was native collagen. When tested on soluble collagen (Fig. 4.13), pronase produced a drop in specific viscosity of about 16% in 30 min, which is attributable to removal of telopeptides (Drake et al., 1966). The small quantities of the A. iophagus collagenases produced reductions in specific viscosity of about 46% for the collagenase I and 34% for the collagenase II These results establish beyond doubt that the A. iophagus enzymes are true collagenases.

The antiserum prepared with purified A. iophagus collagenase I

reacted with its antigen, producing a single precipitin line (Fig. 4.14). When tested for cross-reactivity, A. iophagus collagenase II produced a reaction of identity to the A. iophagus collagenase I, but neither of the Cl. histolyticum collagenases showed any reaction (Fig. 4.15).

As the genus Achromobacter is non-pathogenic to warm-blooded animals (Skerman, 1967), it was considered unlikely that A. iophagus would elaborate any toxin. This assumption was supported by the absence of any ill effects in the rabbits injected with culture supernatant.

DISCUSSION

During starch block electrophoresis under the specific conditions, the collagenase produced by this strain of A. iophagus separated into a major anionic and a minor cationic portion. However, no difference between the two collagenases could be discerned by polyacrylamide gel disc electrophoresis, gel-filtration, Ouchterlony plate serological comparison, action on collagen, or action on PZ-pro-leu-gly-pro-arg. Therefore, evidence for a multiplicity of forms of the enzyme was considered to be insufficient and the minor cathodically-migrating portion of collagenase was disregarded for the purposes of this thesis. All further reference herein to A. iophagus collagenase indicates the enzyme which migrates towards the anode during the starch electrophoresis.

When cultured in a simple medium, this strain of the hide bacterium A. iophagus produced collagenase which could be isolated by the well-established techniques of $(NH_4)_2SO_4$ precipitation, zone electrophoresis on granular starch, and gel-filtration. The collagenase resulting from this procedure produced a single band in polyacrylamide gel disc electrophoresis, was without activity on casein, and was not closely related serogically to Cl. histolyticum collagenase forms I and II. This strain of A. iophagus thus proved

to be a highly satisfactory source of a collagenase which contributes to hide spoilage.

In addition to the aspect applying to the hide and skin industry, however, there is also the clinical and laboratory usage of collagenase to be considered. Cl. histolyticum is currently regarded as the best source of collagenase, but because of the lethal clostridial toxin, careful purification of the enzyme is necessary before it may be used clinically (Sizer, 1972). Furthermore, Cl. histolyticum produces collagenase only under anaerobic conditions (Waldvogel and Swartz, 1969) and requires a complex medium for good yields (MacLennan, Mandl and Howes, 1953; Warren and Gray, 1961). The production of collagenase using A. iophagus instead of Cl. histolyticum has the advantages of aerobic culture, simple nutrient requirements, and apparent absence of toxicity. Compared with Cl. histolyticum, this strain of A. iophagus is therefore a potentially better source of collagenase for large-scale production and clinical usage.

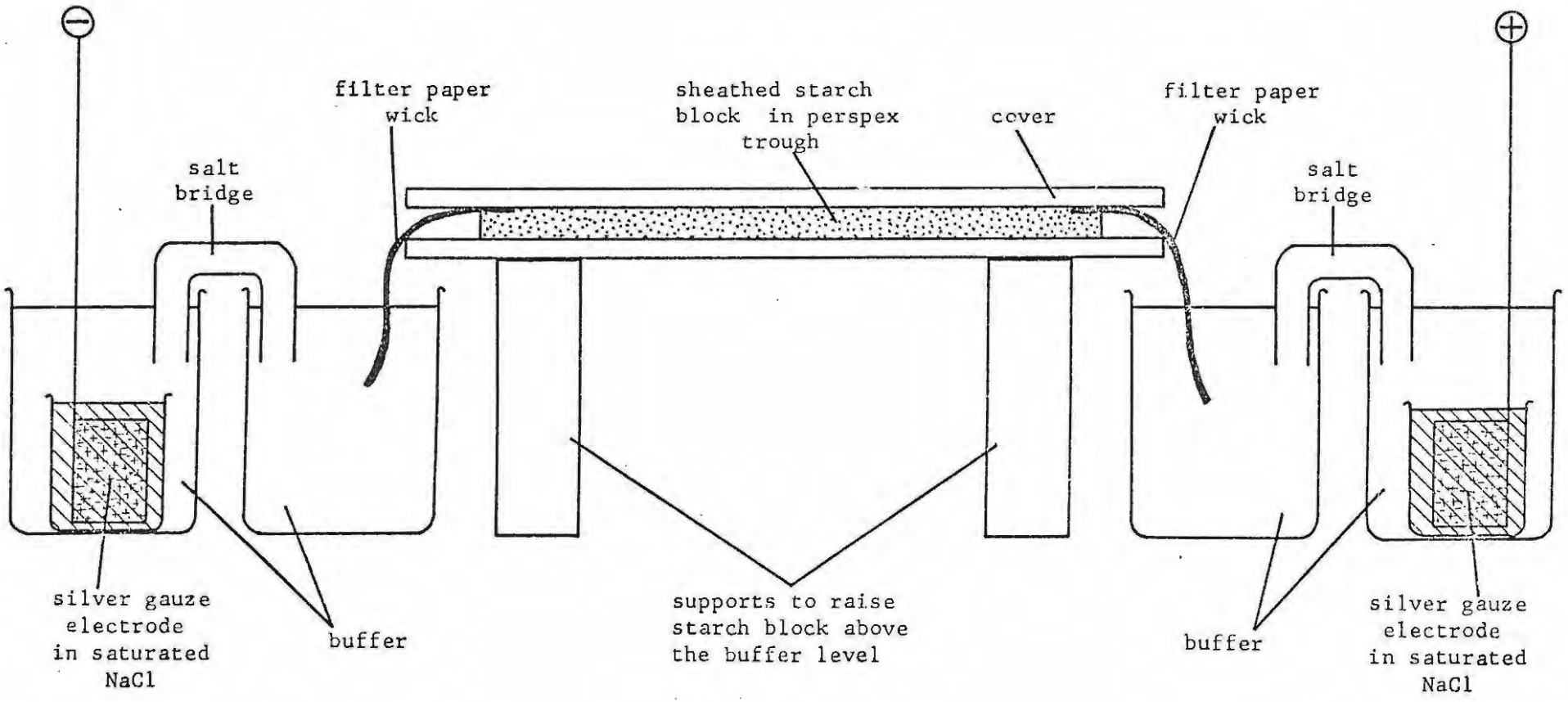


Fig. 4.1. Apparatus for starch block zone electrophoresis

Table 4.1. Calibration data for the assay of collagenase activity using PZ-pro-leu-gly-pro-arg.

Standard solutions consisted of serial dilutions of phenylazobenzyloxycarbonyl-L-prolyl-L-leucine (PZ-pro-leu) in ethyl acetate; their absorbances at 320 nm were measured in a spectrophotometer. Using the molecular weight for PZ-pro-leu of 455.54, concentrations in mg/ml were converted to μmol/ml. Wünsch-Heidrich units and picokatals were calculated as described in the text.

Concentration of PZ-pro-leu in the standard solutions			Absorbance at 320 nm	Wünsch-Heidrich units	pkat
mg/ml	μmol/ml	μmol/5ml			
0	0	0	0	0	0
0.005	0.0107	0.054	0.24	5.36	59.5
0.010	0.0214	0.107	0.45	10.72	119.1
0.015	0.0321	0.161	0.65	16.07	178.4
0.020	0.0429	0.214	0.85	21.43	238.1
0.025	0.536	0.268	1.06	26.79	297.7
0.030	0.0643	0.321	1.29	32.15	357.2
0.035	0.0750	0.375	1.50	37.50	416.6
0.040	0.0857	0.429	1.74	42.86	476.1
0.045	0.0964	0.482	1.93	48.22	535.7

Linear regression analysis of the absorbance and pkat data above produced the results:

$$a_o = 0.015; \quad a_1 = 0.004; \quad r^2 = 0.999;$$

where x = amount of activity in pkat,

y = absorbance at 320 nm,

and the equation for the regression line was $y = a_o + a_1 x$.

Absorbance values between 0 and 1.93 were converted to pkat by means of the expression:

$$\text{amount of collagenase activity (pkat)} = \frac{\text{absorbance}_{320} - 0.015}{0.004}$$

(The graph in Fig. 4.2 was plotted from the data above)

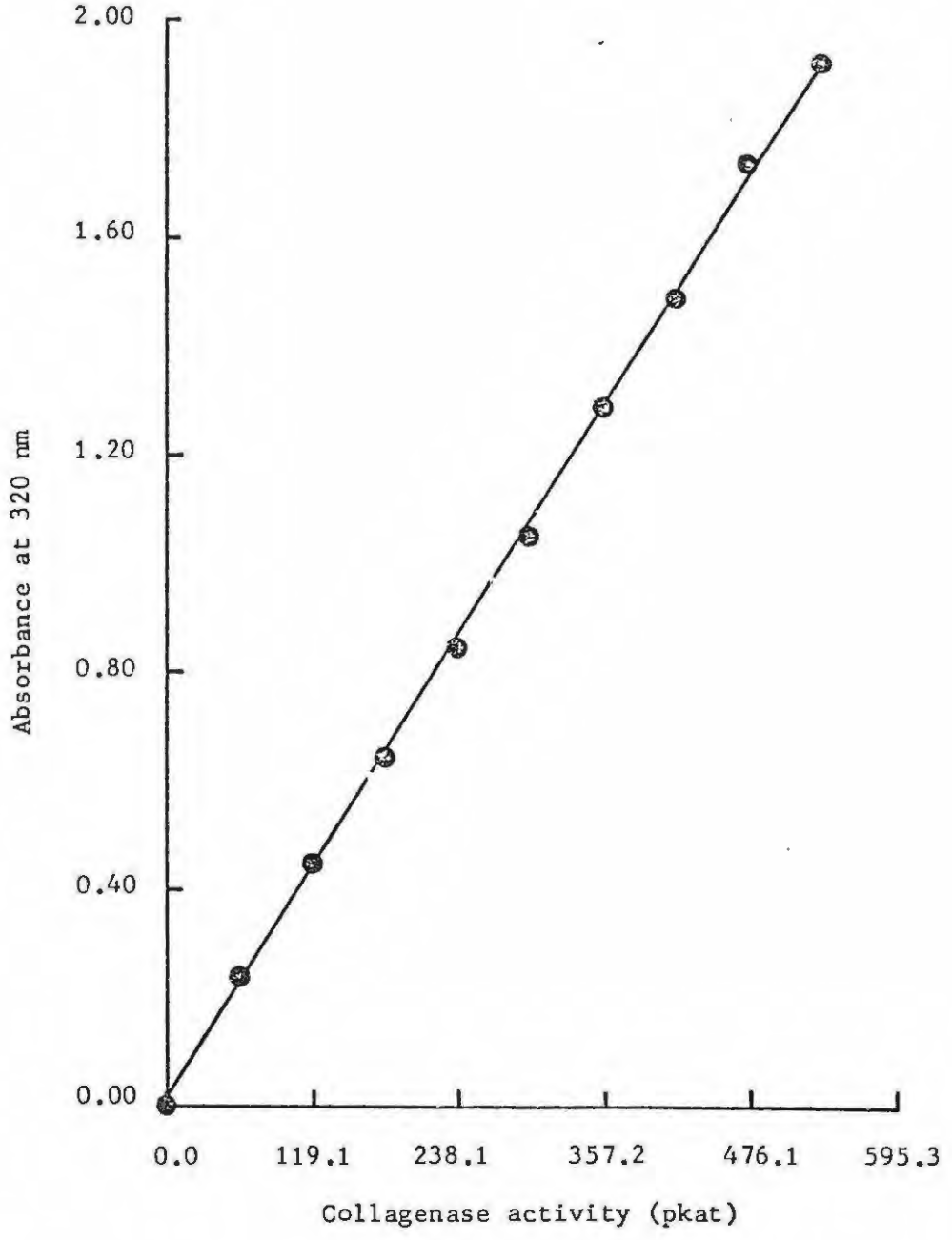


Fig. 4.2. Calibration graph for the assay of collagenase activity using PZ-pro-leu-gly-pro-arg. The graph is drawn from the data in Table 4.1.

Table 4.2. Calibration data for protein determination by the method of Lowry et al. (1951).

Duplicate standard solutions were prepared in distilled water using bovine albumin corrected for 8.3% moisture content. Moisture determinations were made by the method of Eastoe and Courts (1963). Serial dilutions of the standard solutions were processed according to Lowry et al. (1951) as described in the text.

Corrected concentration of bovine albumin (mg/ml)	Mean absorbance at 690 nm
0	0.05
0.18	0.25
0.37	0.44
0.55	0.62
0.73	0.81

Linear regression analysis of the above data produced the results:

$a_o = 0.056$; $a_1 = 1.033$; $r^2 = 1.000$;

where x = concentration of protein (mg/ml),

y = absorbance at 690 nm;

and the equation for the regression line was

$$y = a_o + a_1 x.$$

Absorbance values between 0.05 and 0.81 were converted to protein concentrations according to the expression:

$$\text{protein concentration (mg/ml)} = \frac{\text{absorbance}_{690} - 0.06}{1.03}$$

(The graph in Fig. 4.3 was plotted from the above data)

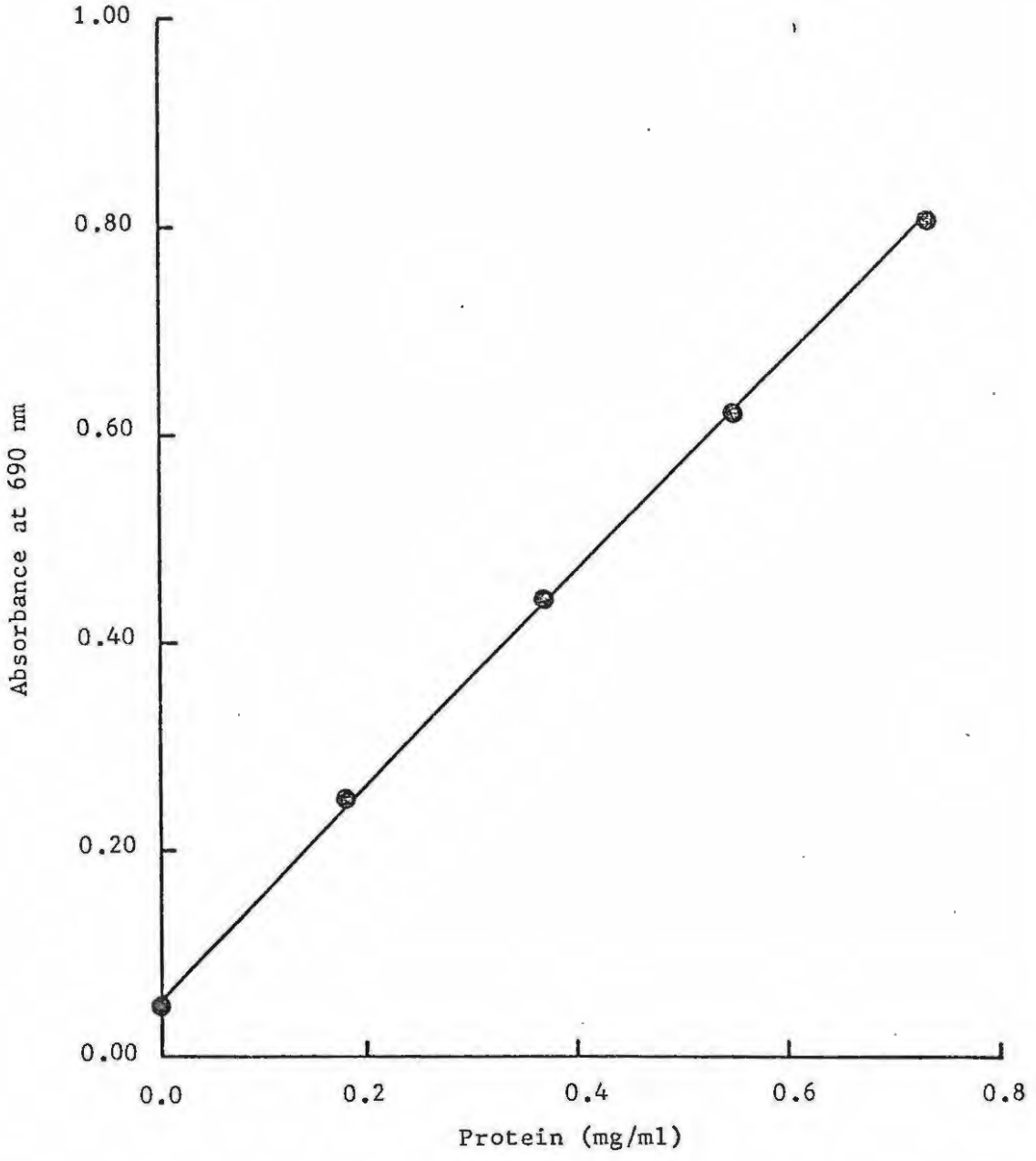


Fig. 4.3. Calibration graph for protein determination by the method of Lowry et al. (1951). The graph is drawn from the data in Table 4.2.

Table 4.3. Calibration data for the conversion of absorbance at 280 nm to protein concentration in mg/ml.

Duplicate standard solutions of bovine albumin in 0.1 M Tris HCl (pH 7.6) were prepared. The weights of bovine albumin were corrected for 8.3% moisture content (cf. Table 4.2). Absorbance values for serial dilutions of the initial standard solutions were read at 280 nm in a spectrophotometer.

Corrected concentration of bovine albumin (mg/ml)	Absorbance at 280 nm
0	0
0.60	0.41
1.21	0.77
1.81	1.14
2.42	1.52
3.02	1.88

Linear regression analysis of the above data produced the results:

$a_o = 0.039$; $a_1 = 0.610$; $r^2 = 1.000$;

where x = concentration of protein (mg/ml),

y = absorbance at 280 nm,

and the equation for the regression line was

$$y = a_o + a_1 x.$$

Absorbance values between 0 and 1.88 were converted to protein concentrations according to the expression:

$$\text{protein concentration (mg/ml)} = \frac{\text{absorbance}_{280} - 0.04}{0.61}$$

(The graph in Fig. 4.4 was drawn from the above data)

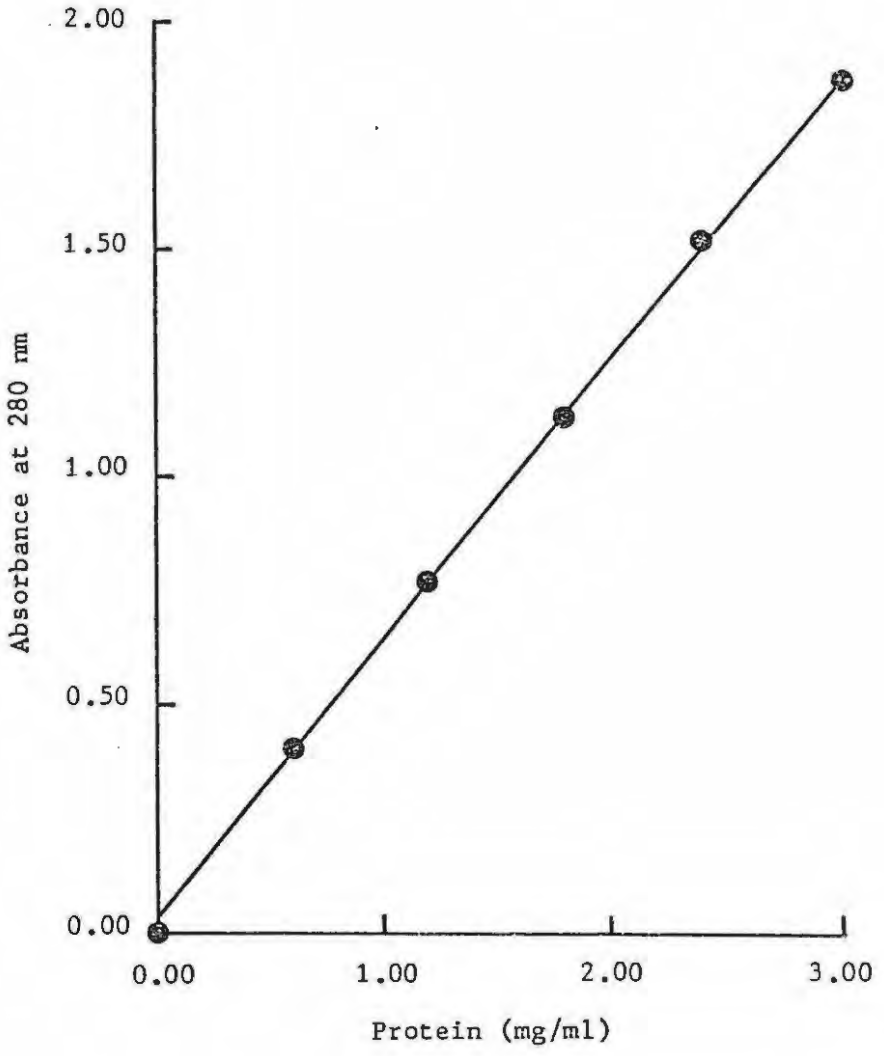


Fig. 4.4. Calibration graph for the conversion of absorbance at 280 nm to protein concentration. The graph was drawn from the data in Table 4.3.

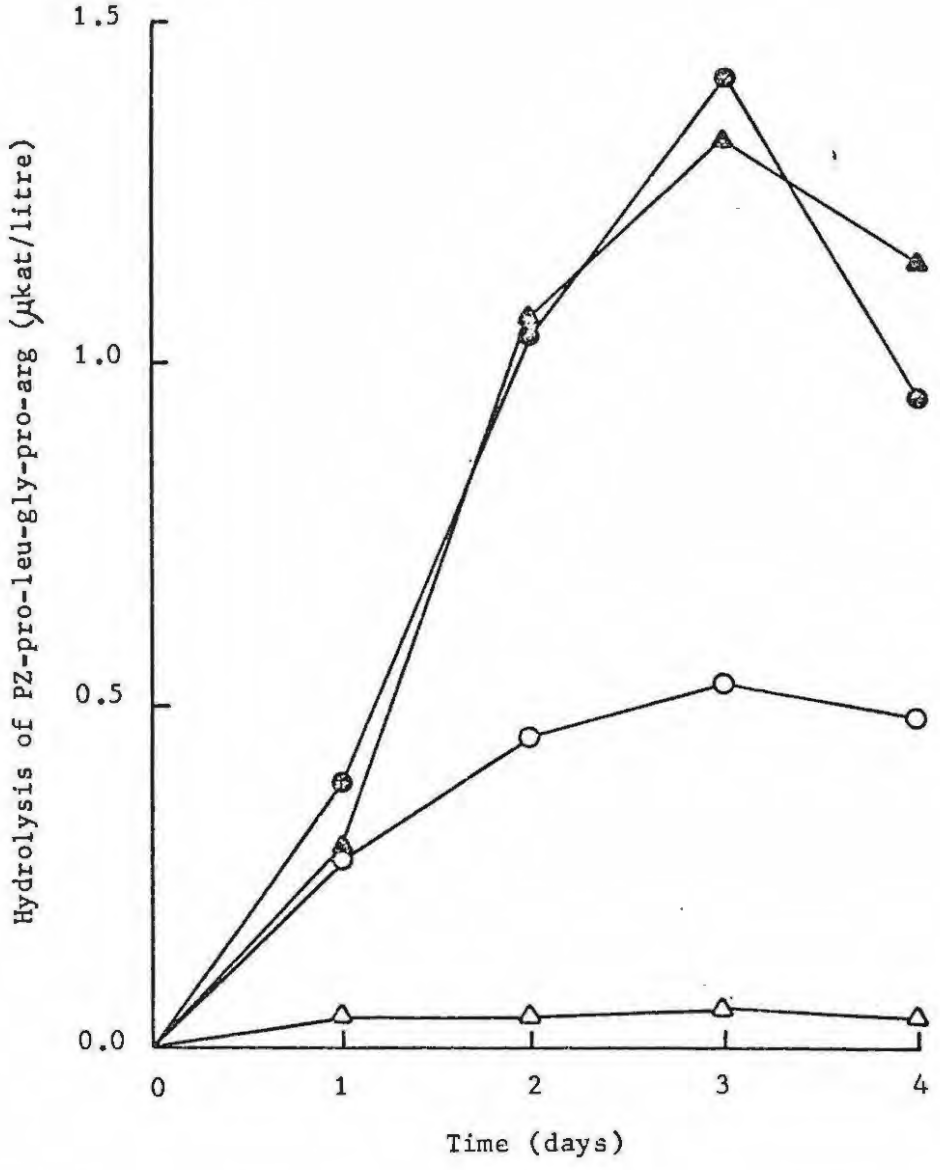


Fig. 4.5. Production of collagenase by A. iophagus: Effect of different media. △—△, hide powder; ○—○, collagen; ⊕—⊕, collagen plus peptone; ▲—▲, peptone.

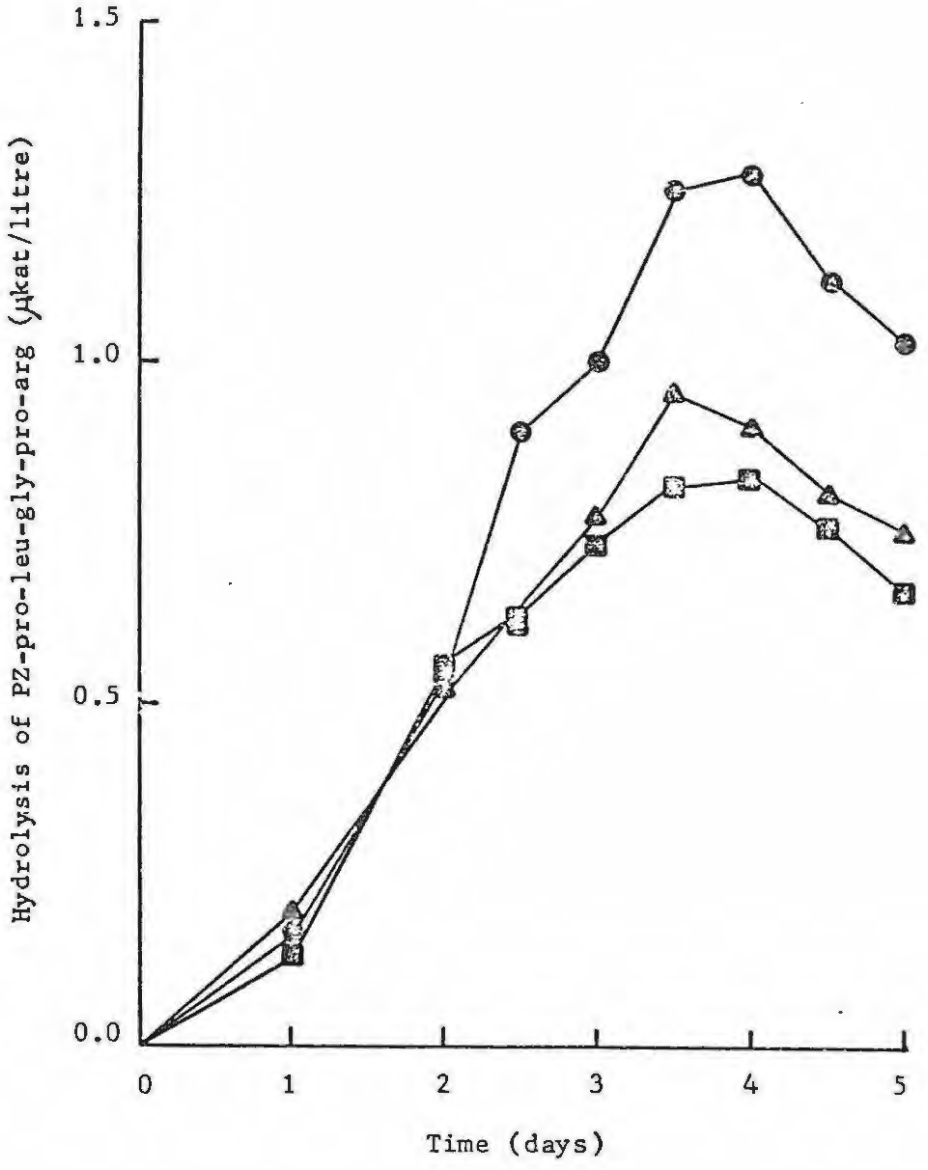


Fig. 4.6. Production of collagenase by A. iophagus: Effect of added vitamins and increased $CaCl_2$ concentration. ▲——▲, 2 mM $CaCl_2$; ●——●, 0.01 M $CaCl_2$; ■——■, 2 mM $CaCl_2$ plus vitamins.

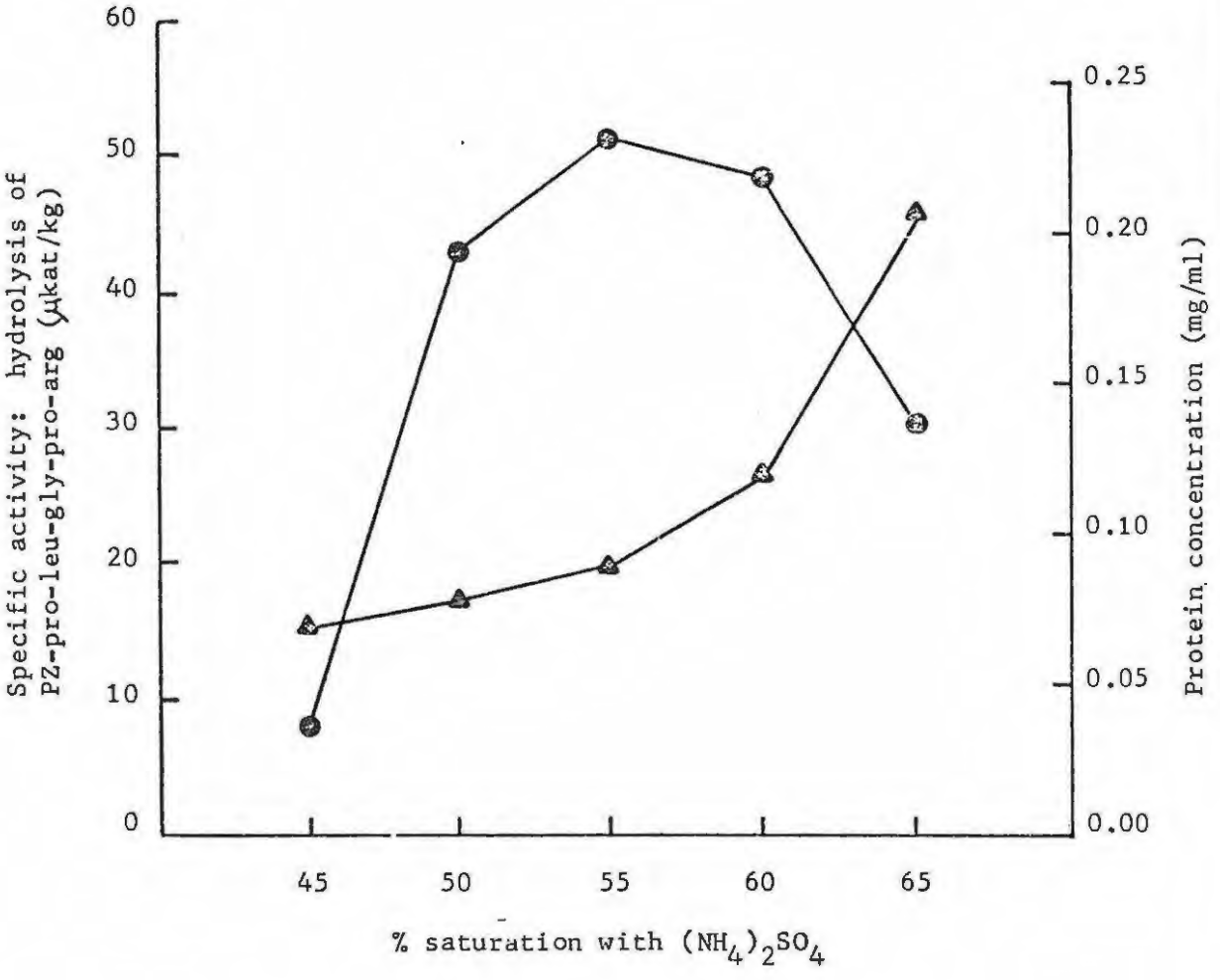


Fig. 4.7. Ammonium sulphate precipitation of A. iophagus collagenase: pilot run. △——△, protein (mg/ml); ○——○, specific activity on PZ-pro-leu-gly-pro-arg.

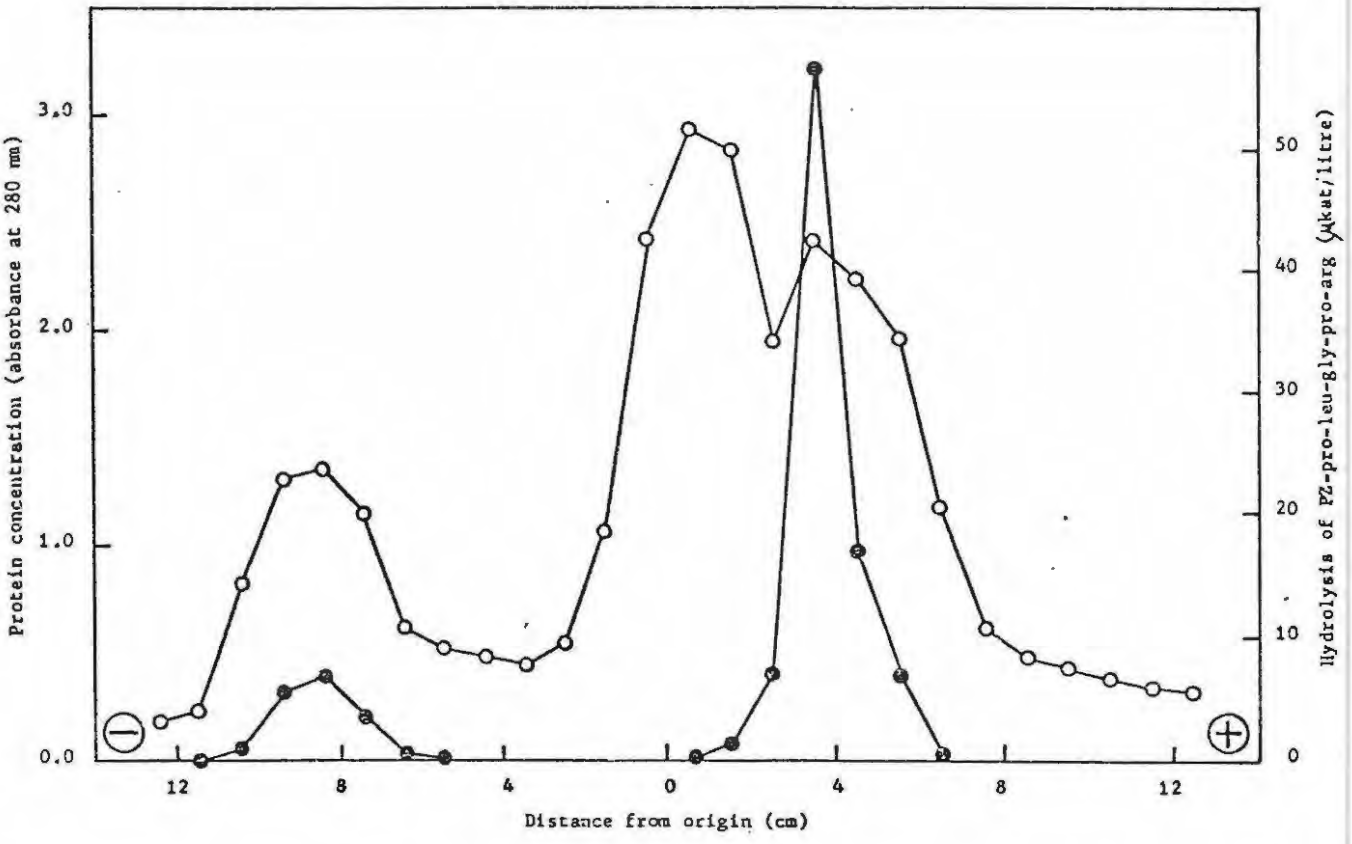


Fig. 4.8. Starch block zone electrophoresis of 200 mg of crude *A. iophagus* collagenase. O——O, protein concentration; ●——●, activity on PZ-pro-leu-gly-pro-arg. Only those fractions indicated contained activity against PZ-pro-leu-gly-pro-arg.

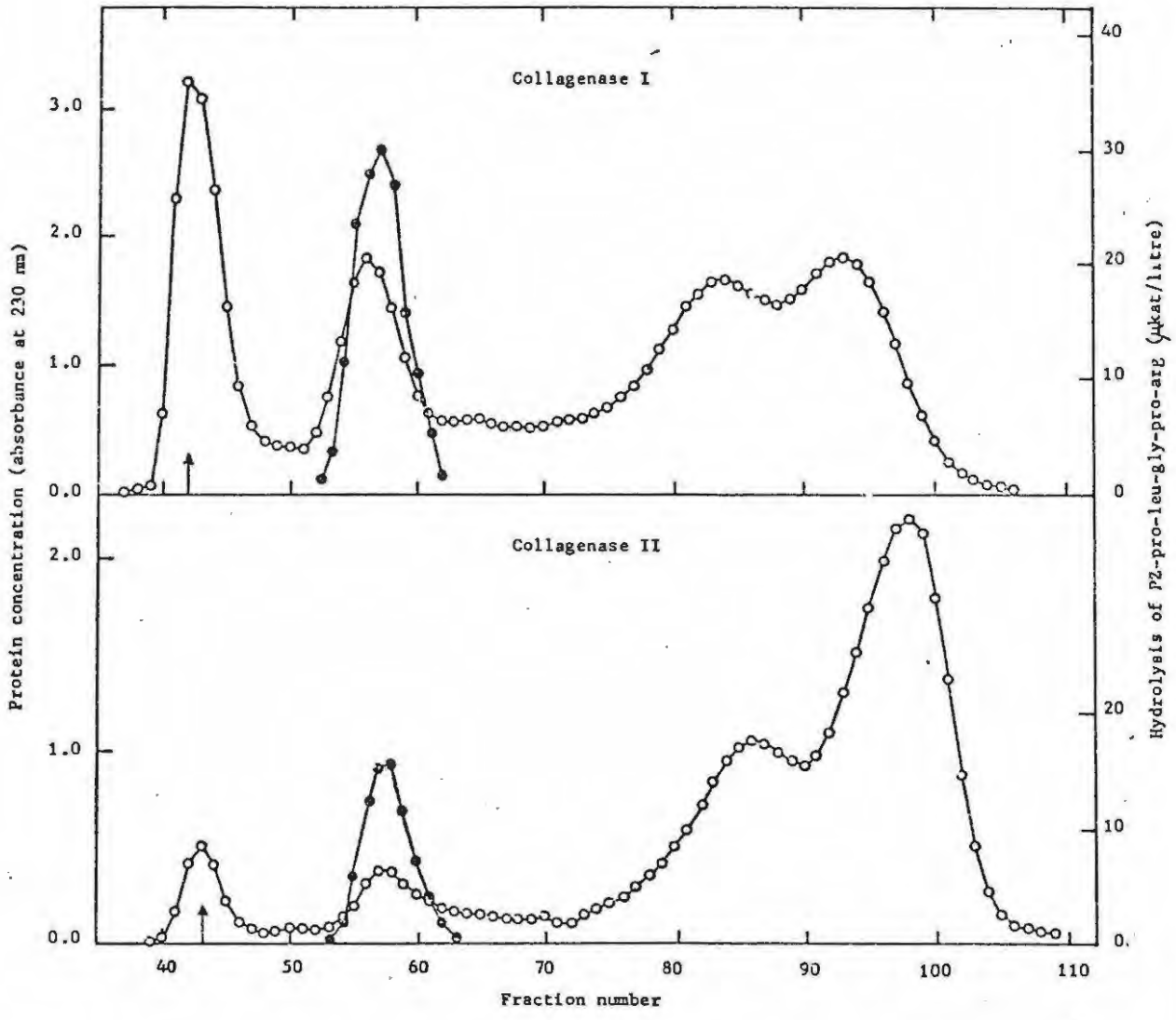


Fig. 4.9. Gel-filtration of A. iophagus collagenases I and II. Columns were loaded with material obtained from the starch block electrophoresis. Collagenase I: elution of an 80 mg sample from a column 93 cm long. Collagenase II: elution of a 53 mg sample from a 95 cm column. The arrows indicate the void volumes. O——O, protein concentration; ●——●, activity on PZ-pro-leu-gly-pro-arg. Only those fractions indicated contained activity against PZ-pro-leu-gly-pro-arg.

Table 4.4. Typical purification of *A. iophagus* collagenases from a starting culture volume of 3 litres.

Preparation	Yield (mg)	Specific activity (μkat/kg)	Yield of activity (%)	Casein hydrolysis (mg mg^{-1} h^{-1})
Culture supernatant	38 480	122	100	0.02
Ammonium sulphate	558	5 111	61	0.55
Starch electrophoresis:				
Collagenase I	213	9 643	43	1.25
Collagenase II	56	3 044	4	2.50
Gel-filtration:				
Collagenase I	10	107 401	23	0.00
Collagenase II	1	103 078	2	not tested

Fig. 4.10. Polyacrylamide gel disc electrophoresis of *A. iophagus* collagenase preparations obtained by starch block electrophoresis. Gels were loaded with 200 µg of collagenase I (left) and collagenase II (right). The method was that of Davis (1964).

Fig. 4.11. Polyacrylamide gel disc electrophoresis of *A. iophagus* collagenase I. Gels were loaded with 200 µg of the material obtained by starch block electrophoresis (left) and 15 µg of the material after gel-filtration. The method was that of Davis (1964).

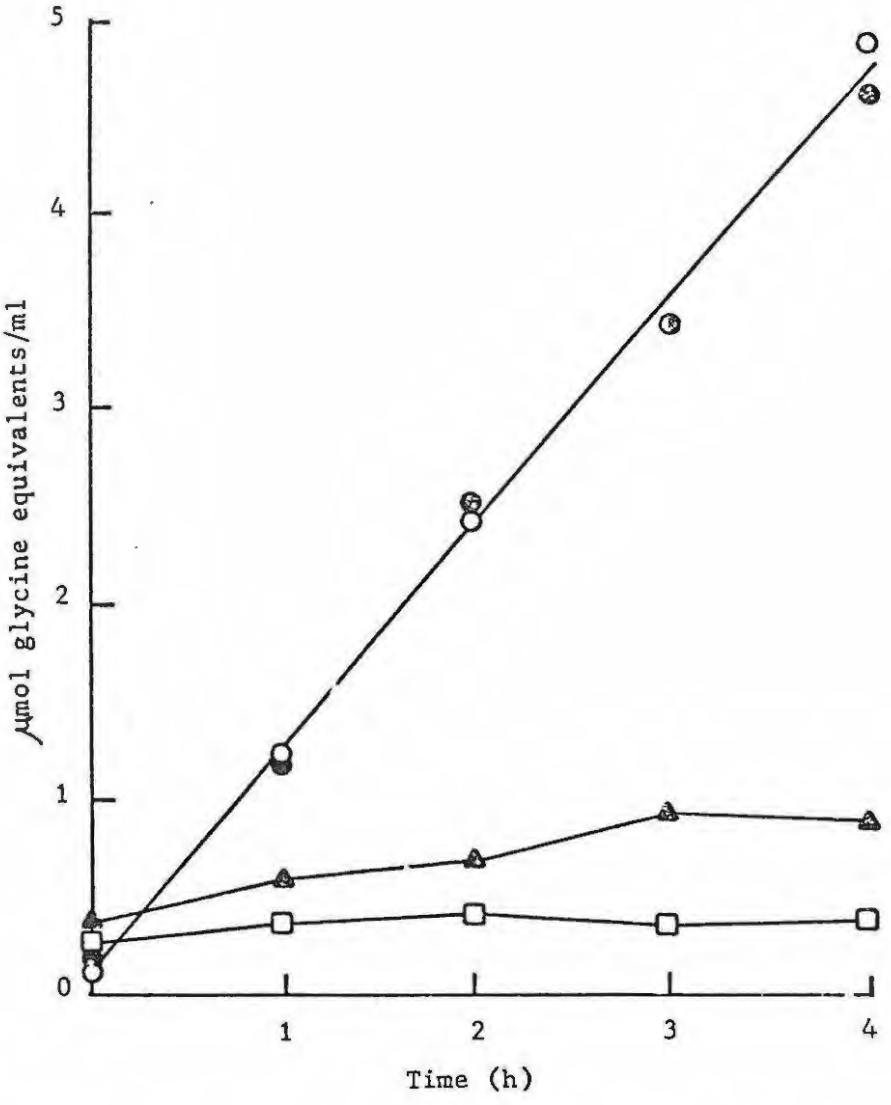


Fig. 4.12. Hydrolysis of insoluble collagen by A. iophagus collagenases I and II. O——O, collagen I (4 444 pkat of activity on PZ-pro-leu-gly-pro-arg); ●——●, collagenase II (4 444 pkat of activity on PZ-pro-leu-gly-pro-arg); ▲——▲, pronase; □——□, control.

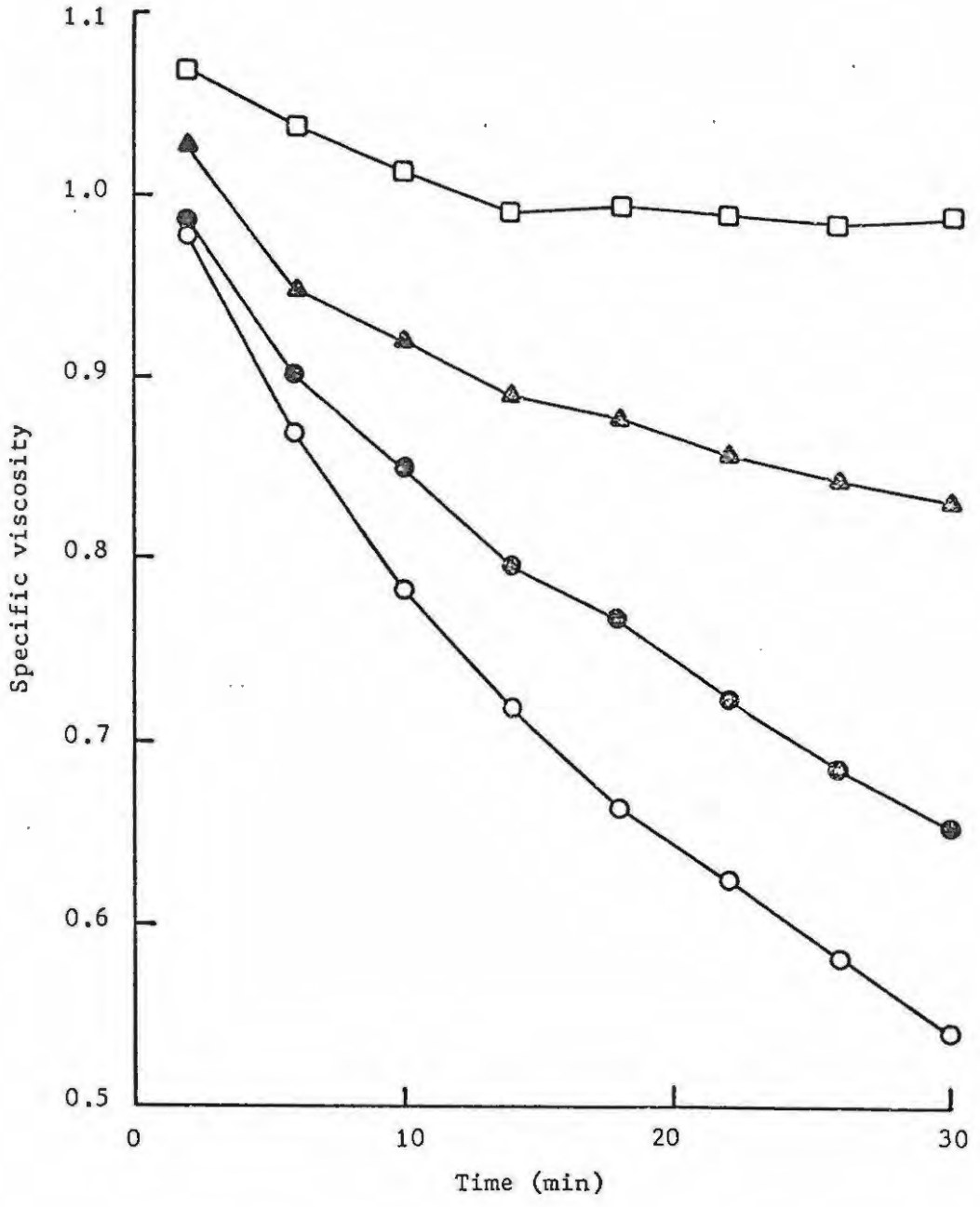
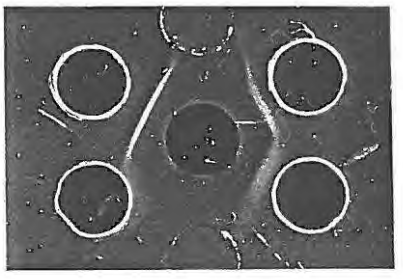


Fig. 4.13. Hydrolysis of soluble collagen by A. iophagus collagenases I and II. O——O, collagenase I (2 089 pkat of activity on PZ-pro-leu-gly-pro-arg); ●——●, collagenase II (1 933 pkat of activity on PZ-pro-leu-gly-pro-arg); ▲——▲, pronase; □——□, control.

Fig. 4.14. Ouchterlony serological reaction between A. iophagus collagenase I and its antiserum. Outer wells contained antigen solutions at the following concentrations (mg/ml): upper left, 0.2; upper right, 0.1; lower right, 0.05; lower left, 0.01. The centre well contained a ¼ dilution of the antiserum.



Actual size of well-pattern:

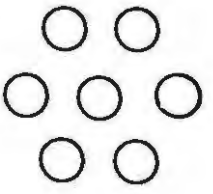
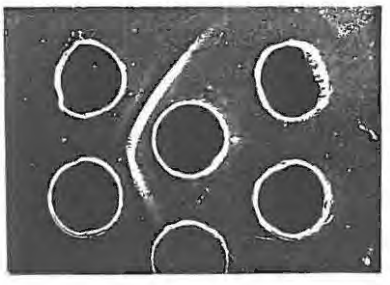
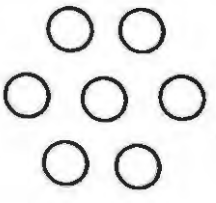


Fig. 4.15. Serological comparison between *A. iophagus* collagenases I and II, and *Cl. histolyticum* collagenase forms I and II. Collagenase solutions at concentrations of 0.75 mg/ml were placed in the outer wells as follows: upper left, *A. iophagus* collagenase I; lower left, *A. iophagus* collagenase II; upper right, *Cl. histolyticum* collagenase form I; lower right, *Cl. histolyticum* collagenase form II. The centre well contained undiluted antiserum.



Actual size of well-pattern:



CHAPTER FIVE

PARTIAL CHARACTERIZATION OF ACHROMOBACTER IOPHAGUS COLLAGENASE

SUMMARY

A. iophagus collagenase was active from 0-20% NaCl but was inhibited by low concentrations of EDTA. It was found to have the following resemblances to Cl. histolyticum collagenase: molecular weight 112 000; sedimentation coefficient 5.3 S; diffusion coefficient 5.1 Fick units; activity over a wide pH range with optimum activity between pH 7 and pH 8; inhibition by certain metal ions and chelating agents; dependence on Ca^{++} for activity. Unlike Cl. histolyticum collagenase, A. iophagus collagenase was not inhibited by sulphydryl-containing reagents.

INTRODUCTION

The characteristics of the action and inhibition of A. iophagus collagenase were examined with the problems of the industrial processing of hides in mind. The physical characterization of A. iophagus collagenase was done in order to compare it with its Cl. histolyticum counterpart.

METHODS AND MATERIALS

Effect of temperature, pH and NaCl concentration on enzymic activity. Collagenase activity was measured by following the release of ninhydrin-positive materials from insoluble collagen using the procedure of Adamcic and Clark (1970) as described in Chapter 3. Reaction mixtures consisted of 9.0 mg collagen in 3.0 ml of 0.1 M Tris-HCl or 0.1 M Tris-maleate buffer containing NaCl and 2 mM $CaCl_2$. NaCl concentrations were 0.00, 0.85, 2.34, 4.00, 7.00, 10.00, 13.00, 16.00 and 20.00%, and incubation temperatures were 15, 20, 25, 30 and 35°C. Tris-maleate buffer was used to achieve pH values of 6.0, 6.4, 6.8,

7.2 and 7.6, while Tris-HCl was used for values of 7.2, 7.6, 8.0, 8.4, 8.8 and 9.2. The above variations in experimental conditions embraced those used in Chapter 3 to study the collagenolytic activity of the bacterium.

Each reaction mixture was treated with 4 000 pkat of A. iophagus collagenase. Assays were made at time zero and after 4 h, and collagenase activity was expressed as μmol glycine equivalents released per ml in 4 h. All reaction mixtures were duplicated.

Inhibition studies. Various chemicals were tested for ability to inhibit the collagenase. Substrate preparations consisted of 9.0 mg of insoluble collagen in 3.0 ml of 0.1 M Tris-HCl-0.4 M NaCl-2mM $CaCl_2$ (pH 7.6). They were treated with 3 000 - 4 000 pkat of collagenase dissolved in the above buffer, and gently shaken in a water bath at 35°C for 4 h. Collagenase activity was measured by determining released ninhydrin-positive materials as described above.

The procedure for testing inhibitors was to pre-incubate either substrate and inhibitor or enzyme and inhibitor for 30 min at 35°C, prior to mixing enzyme and substrate. The former procedure was preferred as it paralleled the method which the hide and skin industry would use in attempts to prevent collagenolytic action in hides. Inhibition was expressed as the percentage reduction in activity as compared to a control. Inhibitors tested were:

1. Metal ions: Ni^{++} and Zn^{++} (from $NiCl_2$ and $ZnSO_4$ respectively);
2. Sulphydryl reagents: L-cysteine and dithiothreitol (DTT);
3. Metal chelators: o-phenanthroline and EDTA (disodium salt).

The $CaCl_2$ was omitted from the substrate preparations for testing the chelating agents; a similar control was kept as well.

Molecular weight estimation by SDS-polyacrylamide gel electrophoresis. The procedure followed was that of Maizel (1971) which is based on the method of Shapiro, Vinuela and Maizel (1967).

Gel preparation. Gels (4%) were prepared by dissolving 0.8 g of Cyanogum in 19.8 ml of 0.1 M sodium phosphate buffer (pH 7.2). After de-aerating this solution, 0.1 ml of 10% sodium dodecylsulphate (SDS), 0.010 ml of TEMED and 0.1 ml of 10% ammonium persulphate were added with mixing. Gel tubes of 5 mm internal diameter were filled to the required height (10 cm) with the final mixture and were overlayered by spraying with distilled water. Gelling was usually complete within 20 min. Extrusion of the 4% gels during electrophoresis was prevented by means of a 2 cm plug of 13% gel at the bottom of each gel tube.

Sample preparation. Initial sample preparations contained the following (molecular weights in parentheses):

1. 2 mg of purified collagenase;
2. 2 mg each of chymotrypsinogen A (25 700), ovalbumin (43 000) and bovine albumin (67 000);
3. 6 mg of β-galactosidase (135 000).

In each case the protein was dissolved in 0.05 ml of sodium phosphate buffer, 0.3 ml of 10% SDS, 0.1 ml of 2-mercaptoethanol and 0.45 ml of distilled water. Each solution was placed in boiling water for 1 min, cooled and mixed with 0.1 ml of glycerol. The final sample preparation was made by mixing one volume of each of the initial marker preparations with two volumes of the collagenase preparation.

Gels were loaded with 10-20 µl of the final sample preparation and electrophoresis was performed at 20 V and 2 mA/gel for 10 min followed by 80-90 V and 9-12 mA per gel for approximately 2 h. After completion of the run, gels were removed with the aid of a syringe, stained overnight in 0.2% Coomassie Blue in 50% (v/v) methanol containing 7% (v/v) acetic acid, and destained by diffusion in 7% (v/v) acetic acid. Gels were scanned in a densitometer and markers were identified by individual electrophoresis of each protein. The migration of each

marker relative to chymotrypsinogen A was plotted against the logarithm of its molecular weight. The relationship between relative migration and molecular weight was obtained by linear regression analysis and the molecular weight of the collagenase was then calculated from its relative migration.

Molecular weight estimation by gel-filtration. The method of Andrews (1965) was applied to the gel-filtration procedure described in Chapter 4. Gel columns measured 2.5 x 94.0 cm and the markers used were cytochrome c (11 700), chymotrypsinogen A (25 700), ovalbumin (43 000), bovine albumin (68 000) and catalase. Andrews (1965) reported that upon gel-filtration on Sephadex G-200, catalase behaved as a globular protein of molecular weight 195 000 although its actual molecular weight was approximately 240 000. Therefore the value of 195 000 was used for the molecular weight of catalase when calibrating the columns.

For each estimation of the molecular weight of the collagenase, two samples were eluted. One contained 5 mg each of cytochrome c and bovine albumin, and the other contained 5 mg each of chymotrypsinogen A, ovalbumin and catalase together with 1 mg of purified collagenase. The elution profiles of the markers were read at 230 nm and that of the collagenase was determined by assaying its activity on PZ-pro-leu-gly-pro-arg as described in Chapter 4. Linear regression analysis of the elution volumes and the logarithms of molecular weights of the markers enabled the molecular weight of the collagenase to be calculated from its elution volume.

Sedimentation coefficient estimation. The method of Martin and Ames (1961) was used to estimate s for the collagenase. A linear 5-15% sucrose gradient was prepared by successively layering 1 ml each of 5.0, 7.5, 10.0, 12.5 and 15.0% solutions of sucrose in 0.1 M Tris-HCl-2mM $CaCl_2$ (pH 7.6), and allowing overnight diffusion at 4°C. The gradient

was loaded with 200 µl of sample containing 200 µg of catalase and 1 mg of collagenase preparation. The collagenase preparation used was that obtained from starch block electrophoresis.

The loaded sucrose gradient was centrifuged for 12 h at 36 000 rpm and 4°C in a Beckman SW50L rotor, after which it was fractionated by manually collecting 4-drop fractions. Fractions were assayed for collagenase activity on PZ-pro-leu-gly-pro-arg (cf. Chapter 4) and for catalase activity by following the decrease in absorbance at 240 nm of a 3 ml reaction mixture containing 30 µmol of potassium phosphate buffer (pH 7.5), 18 µmol of H_2O_2 and 5-25 µl of enzyme fraction. From the relative positions of collagenase and catalase, and the sedimentation coefficient of catalase (11.3 S), an estimate of s for the collagenase was calculated according to the relationship

$$\frac{\text{Distance travelled from meniscus by collagenase}}{\text{Distance travelled from meniscus by catalase}} = \frac{\text{s of collagenase}}{\text{s of catalase}}$$

Diffusion coefficient estimation. The antiserum of A. iophagus collagenase, whose preparation was described in Chapter 4, was used to obtain an approximate value for the diffusion coefficient of the collagenase by the quantitative gel precipitin method (Polson, 1958; van Regenmortel, 1966). The method involved double diffusion in tubes using the perspex apparatus depicted in Fig. 5.1. The sections of the apparatus were greased at the interfaces and clamped together as shown, after which they were laterally manipulated so as to allow the filling of the tubes as follows:

Centre section: 1% ion-agar in 0.05 M sodium phosphate buffer (pH 7.2) containing 0.85% NaCl, filtered through cotton wool;

Upper section adjacent to centre: 2-fold serial dilutions of antigen solution;

Lower section adjacent to centre: antiserum diluted 1:1 with the above buffer.

After the agar had gelled the sections were moved laterally so as to align the holes in each section, thereby forming a series of tubes with an agar column of constant length in the middle. Diffusion was allowed to occur at 25°C and positions and widths of precipitin bands were measured after 3 and 6 days using a microcomparator. The above data were plotted against antigen concentration and the position of the precipitin band at optimal proportions was determined. Since precipitin bands are stationary and narrowest when the reactants are present in optimal proportions, the points at which the 3 and 6 day graphs intersect indicate this position. An approximate value for the diffusion coefficient D_g of the collagenase was calculated from the relationship

$$D_g = D_b \times \left(\frac{X_g}{X_b}\right)^2$$

where D_b was the diffusion coefficient of gamma globulin and equal to 4.8 Fick units (Largier, 1958) and X_g and X_b were the distances from the antigen and antibody interfaces at which the sharpest precipitin band was located by interpolation.

RESULTS

Effect of temperature, pH and NaCl concentration on collagenase activity. The relative collagenase activities under the various conditions of temperature, pH and NaCl concentration are shown in Figs 5.2, 5.3 and 5.4 respectively. Activity increased with increasing temperature from 15-35°C and showed a wide tolerance to pH, being active over the entire range studied, with optimum activity at pH 7.6. The collagenase also showed great tolerance to NaCl, with activity occurring at 0.00-20.00% NaCl and the optimum NaCl concentration covering the range 0.85-7.00%.

Inhibition studies. Results of the inhibition studies are shown in

Table 5.1. A further result which emerged from these studies was that the activity of the controls containing Ca^{++} was significantly greater than that when Ca^{++} was excluded. This indicated that the collagenase was dependent upon Ca^{++} for activity, and the inhibition by the chelating agents was presumed to be due to removal of the Ca^{++}.

Physical characteristics. The molecular weight of the collagenase as estimated by SDS-polyacrylamide gel electrophoresis was 111 700 ± 800 S.D. (4). Results from one of the gels are shown in Figs 5.5 and 5.6. In the gel-filtration studies, the molecular weight was calculated to be 106 900 ± 1 400 S.D. (3), the results of one run being those presented in Figs 5.7 and 5.8. The molecular weight value obtained by SDS-polyacrylamide gel electrophoresis is likely to be the more accurate, as this technique is generally considered to be more reliable than gel-filtration for molecular weight estimation. Furthermore, although the relationship between the gel-filtration markers was constant from run to run, it was not quite linear (cf. Fig. 5.8); the correlation coefficient for the linear regression plot of elution volume against logarithm of molecular weight was 0.95 for each run.

A typical sucrose density gradient run is depicted in Fig. 5.9; the value obtained by this technique for the sedimentation coefficient of the collagenase was 5.3 S ± 0.1 S.D. (4). For the diffusion coefficient, only one experiment was performed, the results being those in Fig. 5.10. The estimate for D of the collagenase was 5.1 Fick units.

DISCUSSION

It is most significant from the point of view of the hide and skin industry that A. iophagus collagenase is still about 46% active at 20% NaCl. This emphasises the need to prevent the action of the enzyme in hides by suitable inhibition. In studying possible inhibitors, one of the prime considerations was that an inhibitor for use on an industrial scale should present no problem with regard to the processing

of industrial effluent. Therefore heavy metal ions were largely avoided.

The finding that a low concentration of EDTA inhibits A. iophagus collagenase, which seems to be a feature common to bacterial collagenases (Mandl, 1972; Rippon, 1968), suggests a possible means of preventing collagenolytic action in hides. Immediate treatment of freshly-flayed, washed hides with EDTA and inclusion of EDTA in the curing process should help to prevent leather decay. Also, low concentrations of EDTA should not affect the processing of effluent. Tancous and Jayasimhulu (1973) showed that hide cuttings cured in 10% NaCl supplemented with 0.5% EDTA (i.e. about 13 mM) produced leather of good tensile strength and free of loose grain layer, indicating that bacterial decay had been avoided. From Table 5.1 it seems likely that effective inhibition of collagenolytic action in hides could be achieved by a much lower concentration of EDTA than 0.5%, although the above authors cited a suggestion that the excess of Na^+ from the curing salt prevented EDTA from acting at its full capacity.

Tancous and Jayasimhulu (1973) also reported that antiseptics such as sodium pentachlorophenate, when used at the same concentration as EDTA, were more effective than EDTA in preventing autolysis of hide cuttings. Nevertheless, it is felt that the treatment of hides with EDTA as a part of the curing process warrants further investigation as a means of preventing leather decay.

Characterization of Cl. histolyticum collagenase has been reviewed by Mandl (1972). A number of different forms of Cl. histolyticum collagenase have been reported, with molecular weights ranging from about 100 000 to about 50 000 and with varying specificities. It has been suggested, however, that these are modifications or subunits of one collagenase and that the various forms are convertible into each other. The most commonly-isolated form of Cl. histolyticum collagenase has a molecular weight of about 100 000, a sedimentation coefficient of 5.4 S (Seifter et al., 1959) and a diffusion coefficient of 4.3 to 5.0 Fick

units (Seifter et al., 1959; Levdikova et al., 1963). These data parallel the results obtained with A. iophagus collagenase, indicating great similarity between the two enzymes. Other characteristics which the two enzymes have in common are: activity over a wide pH range with optimum activity between pH 7 and pH 8; inhibition by Ni^{++}, Zn^{++} and the metal chelating agents tested (Soru and Zaharia, 1972); and dependence upon Ca^{++} for activity. A difference between the two enzymes is that Cl. histolyticum collagenase is partially inhibited by sulphydryl-containing reagents, whereas A. iophagus collagenase is not. As the inhibition of the clostridial collagenase by sulphydryl-containing compounds is due to sequestration of a metal ion other than Ca^{++}, specifically Zn^{++} (Seifter, Takahashi and Harper, 1970), the implication is that A. iophagus collagenase has no such requirement for a second metal component.

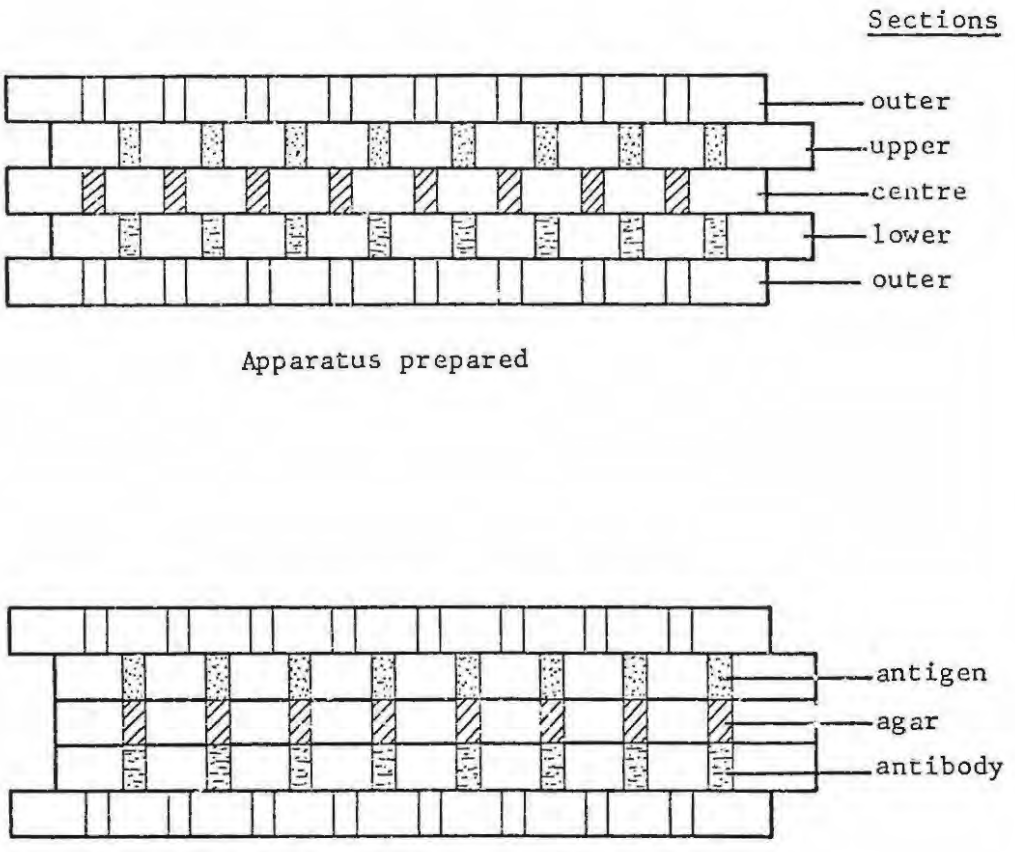


Fig. 5.1. Apparatus for estimation of the diffusion coefficient of A. iophagus collagenase by the quantitative gel precipitin method. The apparatus consists of a series of rectangular perspex bars (sections) measuring approximately 15 x 1 x 1 cm through which holes 0.4 cm in diameter have been drilled.

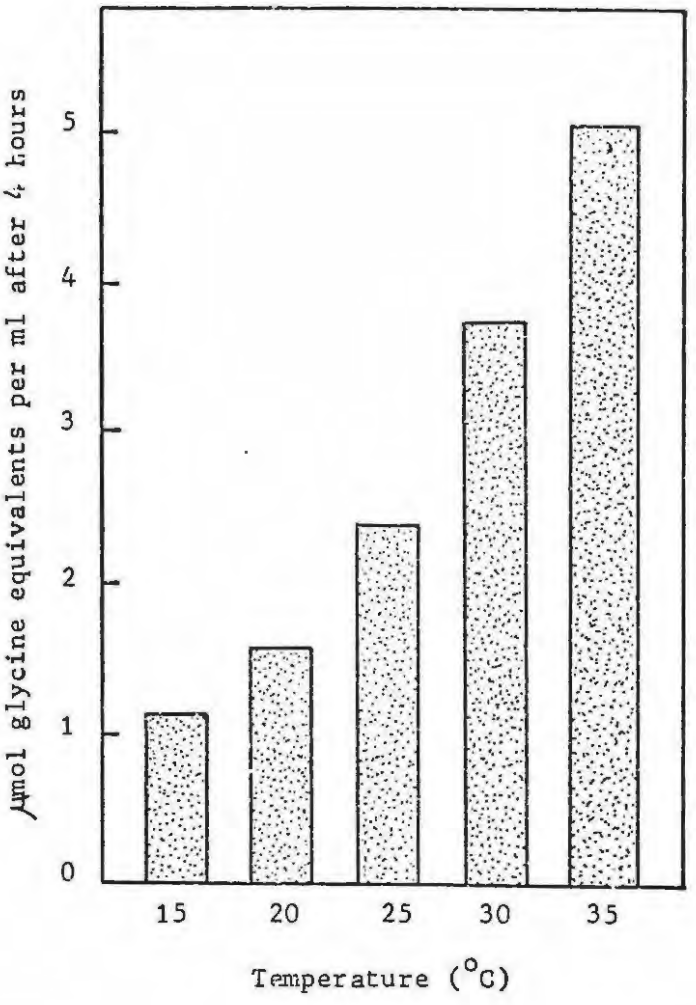


Fig. 5.2. Hydrolysis of insoluble collagen by A. iophagus collagenase at different temperatures. Reaction mixtures contained 2.34% NaCl and were adjusted to pH 7.6.

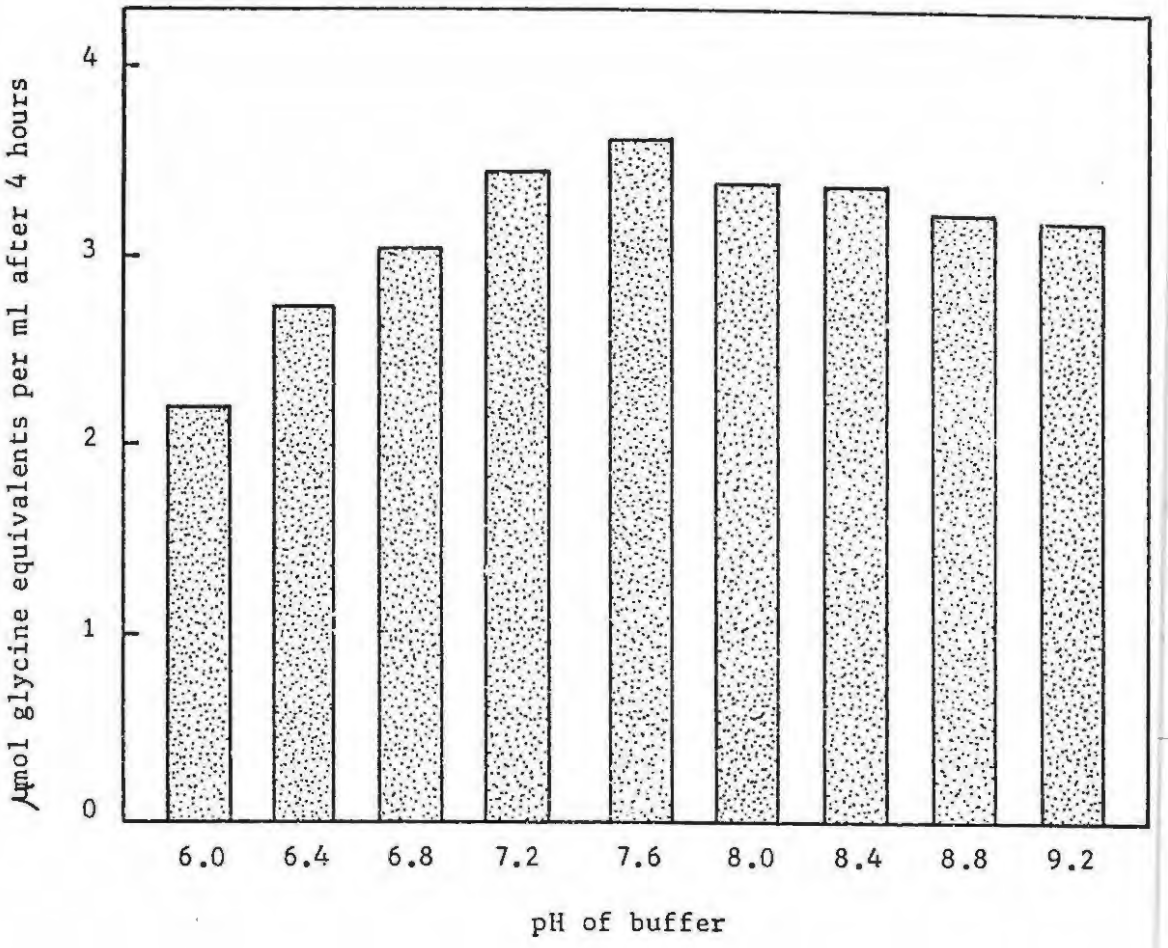


Fig. 5.3. Hydrolysis of insoluble collagen by A. iophagus collagenase at different pH values. Reaction mixtures contained 2.34% NaCl and were incubated at 30°C. Activities at pH 7.2 and pH 7.6 were the same for both the buffers used.

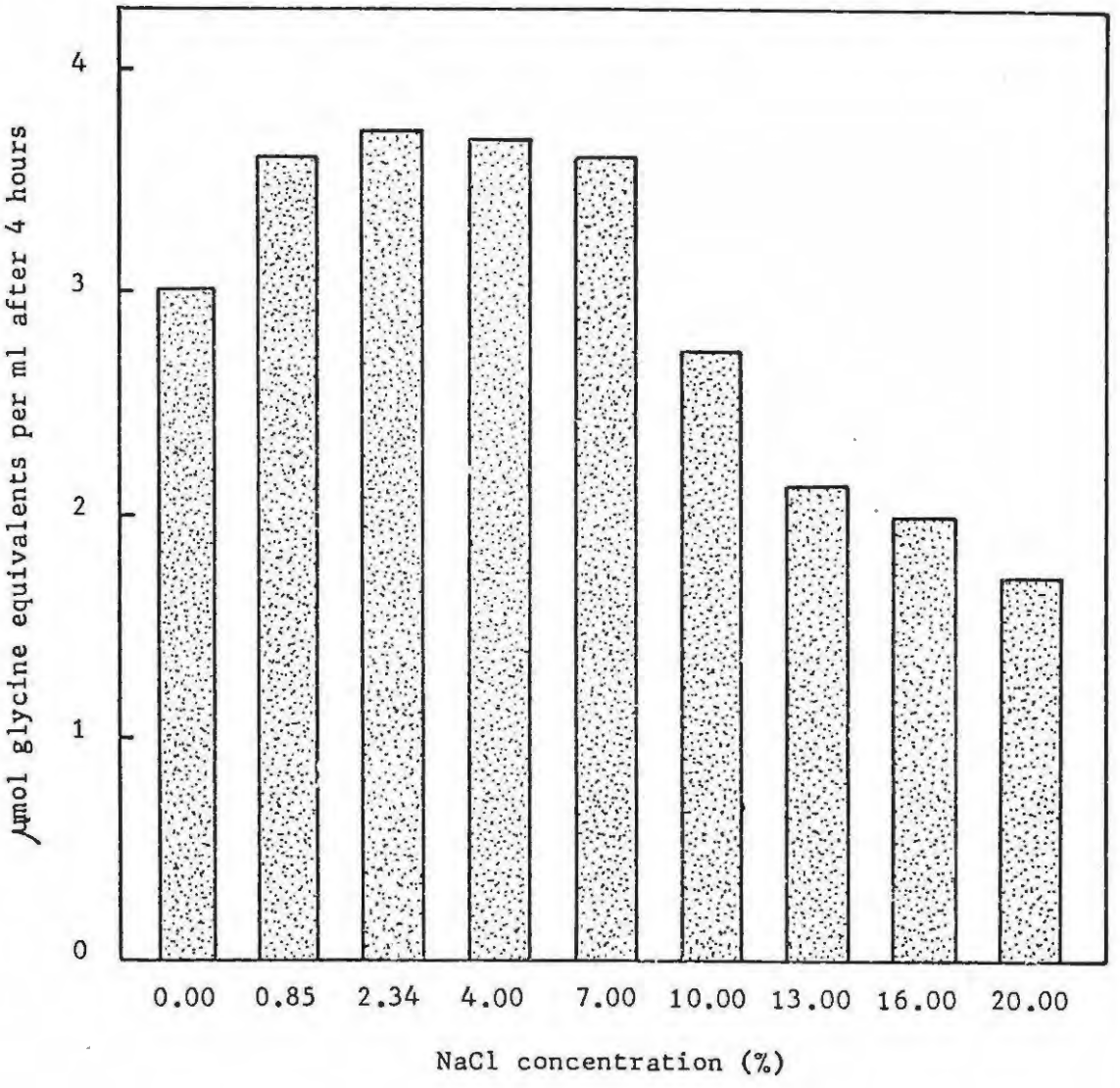


Fig. 5.4. Hydrolysis of insoluble collagen by A. iophagus collagenase at different NaCl concentrations. Reaction mixtures were adjusted to pH 7.6 and incubated at 30°C.

Table 5.1. Effect of inhibitors on the activity of A. iophagus collagenase.

Treatment	Inhibitor	Concentration of inhibitor (mM)	Inhibition (%)
Inhibitor added to substrate	Ni^{++}	1.0	83
	Zn^{++}	2.0	87
	o-phenanthroline	0.1	100
	EDTA	0.1	100
Inhibitor preincubated with enzyme	L-cysteine	1.0	0
	DTT	1.0	0

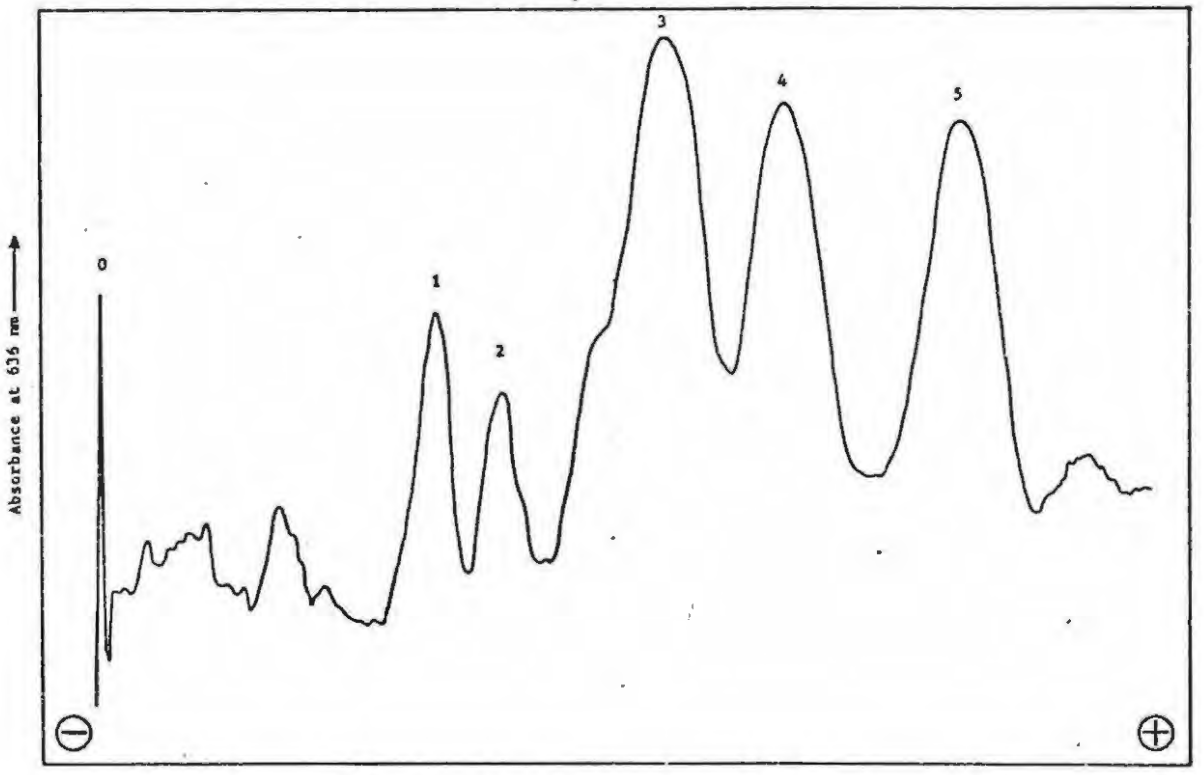


Fig. 5.5. Estimation of the molecular weight of A. iophagus collagenase by SDS-polyacrylamide gel electrophoresis: densitometer scan of a gel showing the relative positions of the stained protein bands. Peaks were: 0, origin; 1, β-galactosidase; 2, collagenase; 3, bovine albumin; 4, ovalbumin; 5, chymotrypsinogen A. Fig. 5.6 was prepared from the above data.

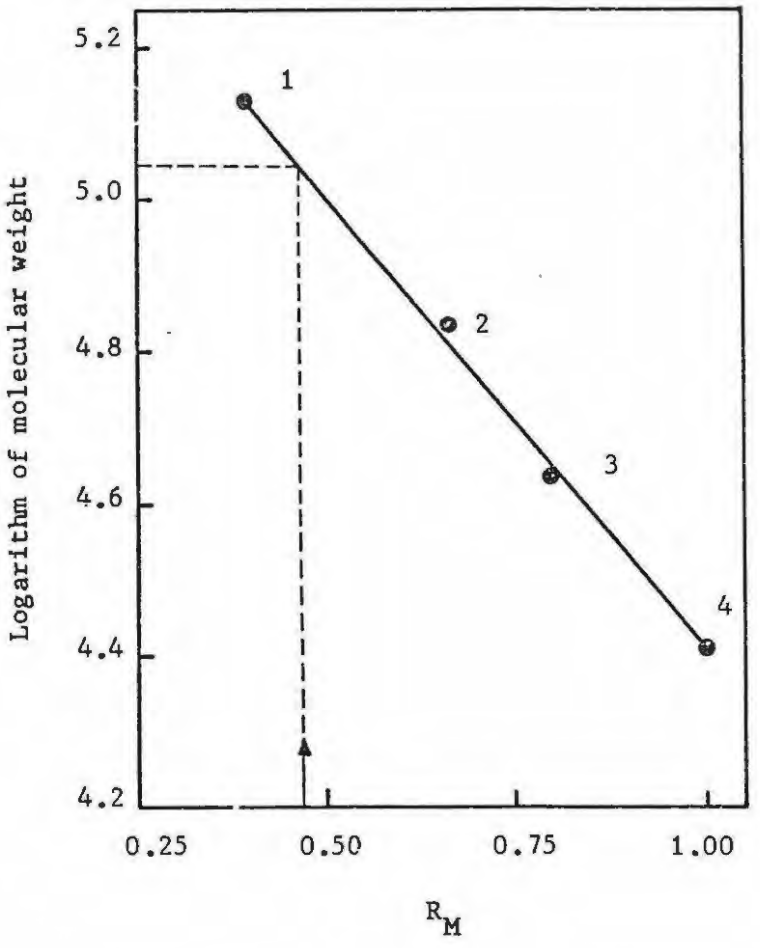


Fig. 5.6. Estimation of the molecular weight of A. iophagus collagenase by SDS-polyacrylamide gel electrophoresis: linear regression plot of logarithm of molecular weight against relative migration. The graph was drawn from the data in Fig. 5.5. Markers were: 1, β-galactosidase; 2, bovine albumin; 3, ovalbumin; 4, chymotrypsinogen A. The arrow indicates the position of the collagenase.

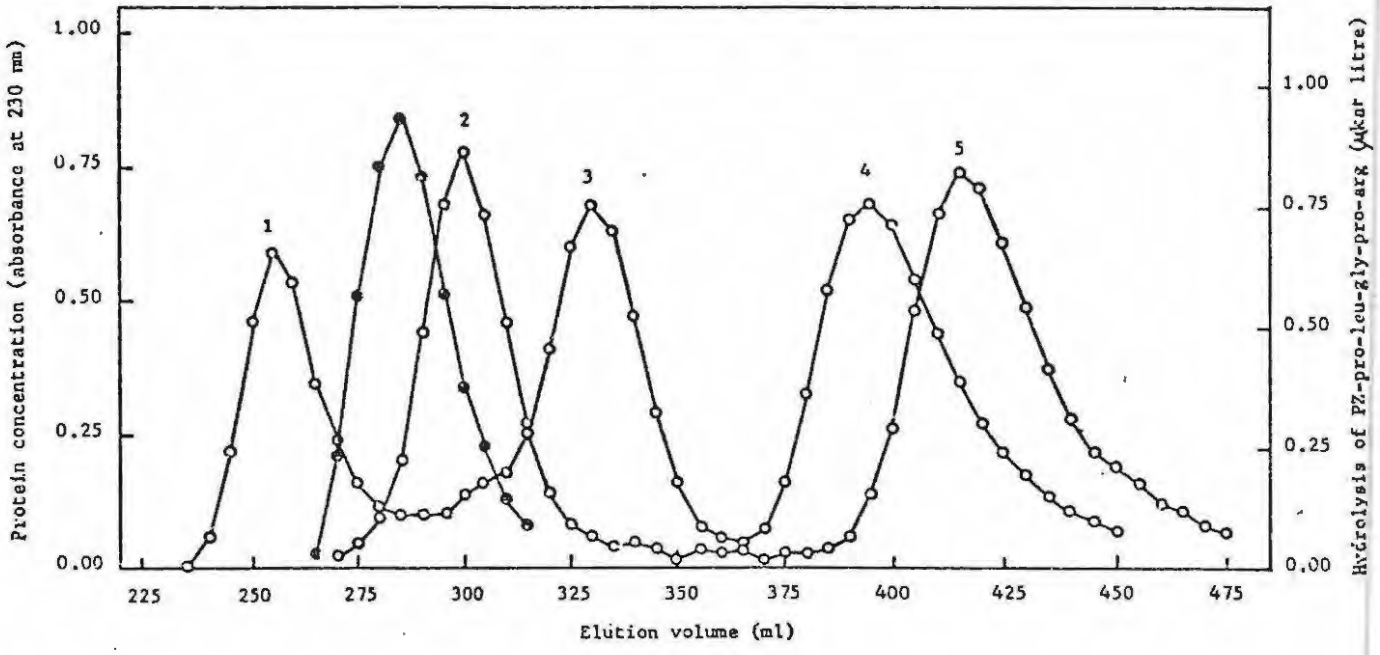


Fig. 5.7. Estimation of the molecular weight of A. iophagus collagenase by gel-filtration on Sephadex G-200. ○——○, protein concentration; ●——●, collagenase activity on PZ-pro-leu-gly-pro-arg. Markers were: 1, catalase; 2, bovine albumin; 3, ovalbumin; 4, chymotrypsinogen A; 5, cytochrome c. Fig. 5.8 was prepared from the above data.

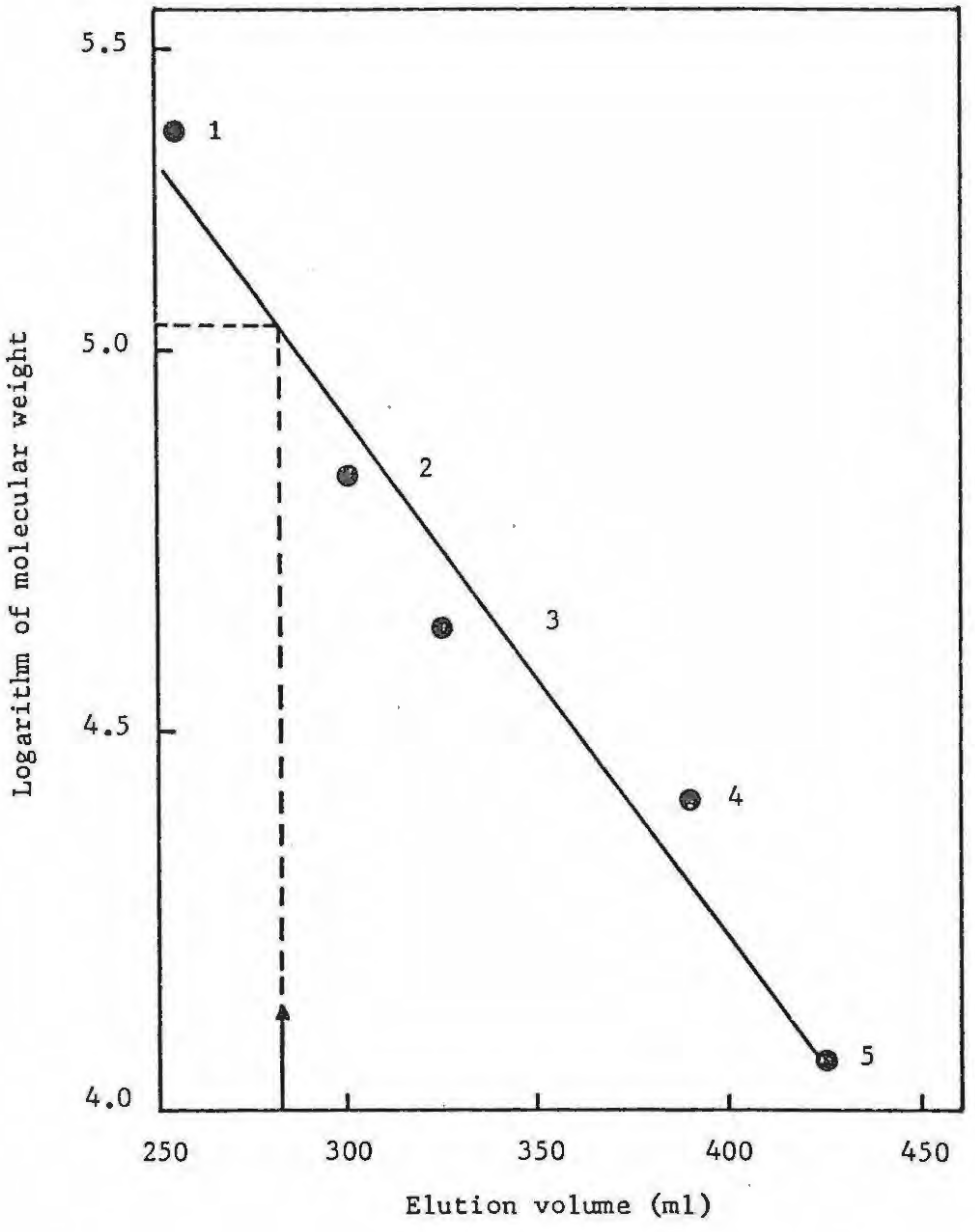


Fig. 5.8. Estimation of the molecular weight of A. iophagus collagenase by gel-filtration on Sephadex G-200: linear regression plot of logarithm of molecular weight against elution volume. The graph was drawn from data in Fig. 5.7. Markers were: 1, catalase; 2, bovine albumin; 3, ovalbumin; 4, chymotrypsinogen A; 5, cytochrome c. The arrow indicates the position of the collagenase.

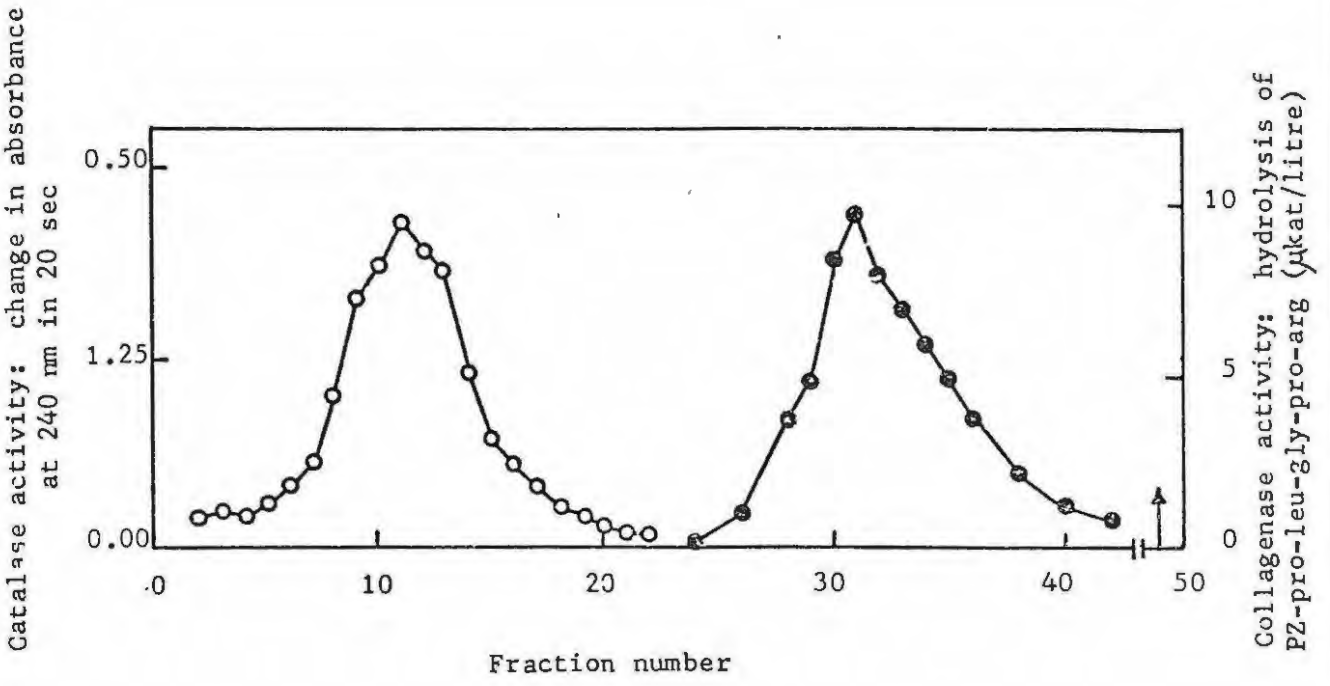


Fig. 5.9. Estimation of the sedimentation coefficient of A. iophagus collagenase by sucrose density gradient centrifugation. The graphs show the sedimentation of catalase (○——○) and collagenase (●——●) relative to the meniscus (arrow). Fractions of 4 drops were collected.

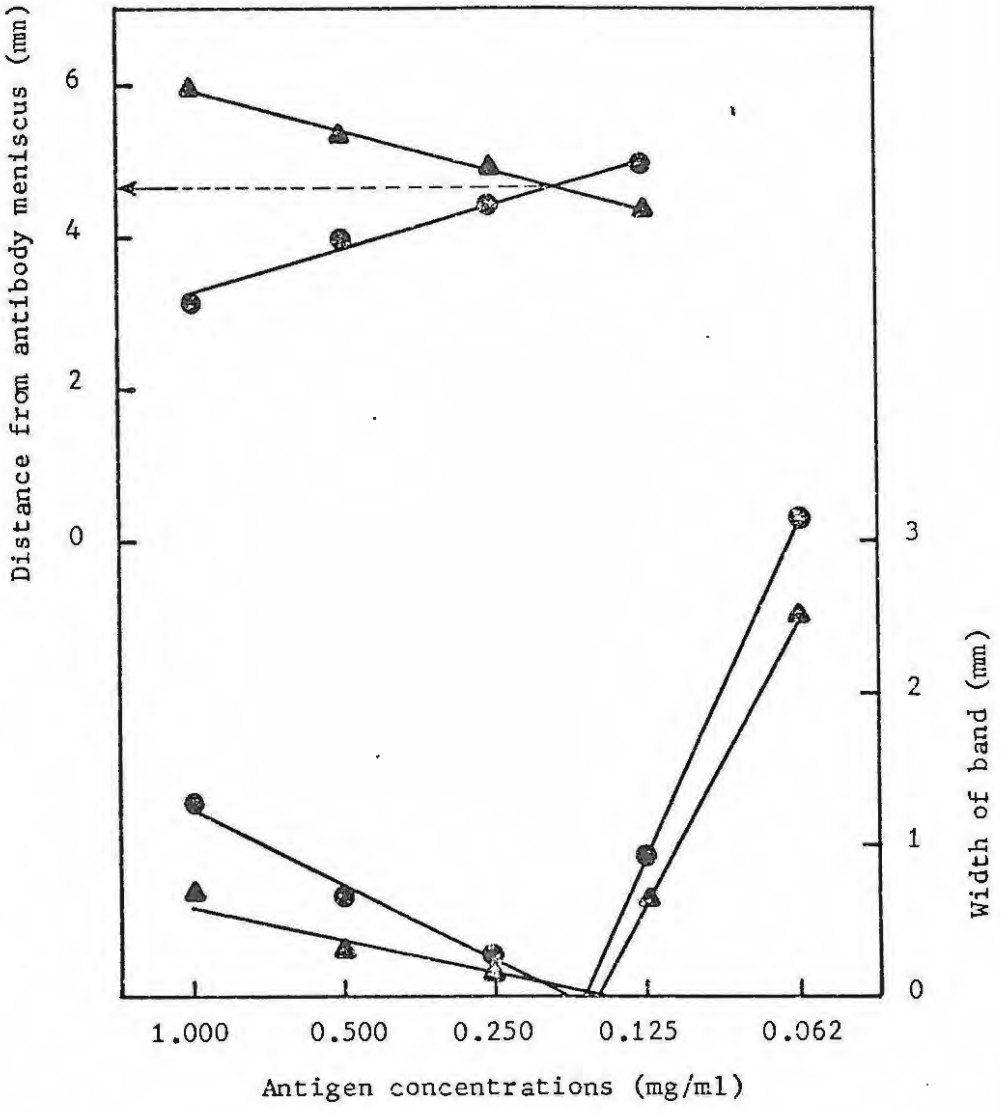


Fig. 5.10. Estimation of the diffusion coefficient of A. iophagus collagenase by the quantitative gel precipitin method. Measurements were taken after 3 days (▲——▲) and 6 days (●——●). The arrow indicates the distance of the precipitin band from the antibody meniscus at optimal proportions of antibody and antigen.

CHAPTER SIX

GENERAL DISCUSSION

The attempt to correlate collagenolytic activity of cured hide bacteria with leather decay, thereby providing a quality control procedure for the industry, was unsuccessful. However, it did indicate that hides sustain bacterial damage mainly before they are cured and that the collagenolytic activities of raw hide bacterial populations should therefore reflect potential leather decay. This indication was confirmed in subsequent work by Woods et al. (1973) who assayed the collagenolytic activities of 14 hide batches before and after curing and compared these findings with the leather decay gradings. They found that both the collagenolytic activities and the viable counts of the raw hide bacterial populations correlated with leather decay, but obtained no such correlations with the cured hide bacteria.

A. iophagus proved to be a highly suitable representative for studying the production of collagenase by hide bacteria and its control. Not only does the bacterium occur widely in hides, but it rapidly produces a halotolerant collagenase which is active over the range 0-20% NaCl. This collagenase resembles that of Cl. histolyticum in several respects. From these investigations of A. iophagus collagenase it is clear that the curing of hides must include treatment with a collagenase inhibitor if leather decay is to be avoided. EDTA may be suitable for this purpose and its use as a supplement to curing treatments is to be investigated at the Leather Industries Research Institute, Grahamstown.

Production of A. iophagus collagenase for the world market has begun at the Pasteur Institute, Paris, who purchased the bacterium and the sole rights to commercial production of the enzyme from this laboratory. Researchers at the Pasteur Institute have confirmed

that A. iophagus is highly suited to large-scale production of collagenase and also that its crude collagenase completely lacks any toxicity. However, in contrast to the results obtained here, they report that the collagenase contains a small amount of caseinolytic activity even after purification by ion-exchange chromatography and gel-filtration. This discrepancy may be due to differences in the preparation and purification procedures. For instance, anomalous behaviour was observed in this laboratory when certain batches of peptone were used. In the gel-filtration purification step, the protein peak containing the collagenase was relatively larger and eluted closer to the void volume than usual. Fractions from the topmost region of this peak contained caseinolytic activity and analysis by polyacrylamide gel disc electrophoresis showed that two distinct components were present. Another possible explanation for the above discrepancy is that at the Pasteur Institute, protease activity in impure preparations of the collagenase was prevented by inclusion of suitable inhibitors. No such step was taken in this laboratory, so there may have been proteolytic modification of the collagenase to a stable partially-degraded active form which resulted in the final purified preparation being free of caseinolytic activity.

In addition to the A. iophagus collagenase described in this dissertation and discussed above, a second, different form of collagenase was produced by A. iophagus under the growth conditions used by the Pasteur Institute. This different form of the enzyme digested collagen but was without effect on PZ-pro-leu-gly-pro-arg. Such an enzyme would not have been detected in the isolation procedure used in this laboratory, since PZ-pro-leu-gly-pro-arg was used for the initial indication of collagenase activity. However, the gel-filtration profiles in Fig. 4.9 show no other protein peaks which are likely to be due to a collagenase without activity on PZ-pro-leu-gly-pro-arg. This suggests that such a collagenase is either not produced

under the growth conditions used in this laboratory, or it is degraded by proteases before it can be isolated.

Results of the investigations of A. iophagus collagenase at the Pasteur Institute will be reported in a future publication from that quarter (Keil and Woods, 1974). The following papers have been published from the content of this dissertation:

1. The Microbiology of Curing and Tanning Processes. VI. Collagenolytic Activity of Bacteria from Raw and Cured Hides. Woods, D.R., Welton, R.L., Thomson, J.A. and Cooper, D.R. (1972) Journal of the American Leather Chemists Association 67, 217-225.
2. Collagenolytic Activity of Cured Hide Bacteria. Woods, D.R., Welton, R.L., Thomson, J.A. and Cooper, D.R. (1972) Journal of Applied Bacteriology 35, 123-128.
3. Halotolerant Collagenolytic Activity of Achromobacter iophagus. Welton, R.L. and Woods, D.R. (1973) Journal of General Microbiology 75, 191-196.
4. Collagenase production by Achromobacter iophagus. Welton, R.L. and Woods, D.R. Biochimica et Biophysica Acta. In press.

ADDENDUM

STUDIES ON A CRYSTALLINE METABOLITE PRODUCED BY ACHROMOBACTER IOPHAGUS

When A. iophagus was cultured on plates of rich nutrient media containing 2.34% NaCl, the bacterium spread rapidly and covered the entire plate in 12-18 h. After 3-4 days, tiny spots appeared in the culture on the agar surface which on closer examination proved to be agglomerates of red-brown crystals. Uninoculated control plates produced no such crystals. The agglomerates were distinctively-shaped bodies, frequently with a hollow centre and occasionally with further large crystals on top of them (Fig. A.1). They seemed to develop progressively from a single large crystal, for various "developmental stages" could be found in a plate culture at any one time (Fig. A.2). The appearance of a fully-developed agglomerate when examined by scanning electron microscopy is shown in Fig. A.3. Attempts were made to identify this crystalline metabolite because of the possibility that its presence in a culture of hide bacteria might indicate growth of A. iophagus and hence potential leather decay, and that it could therefore be used as a diagnostic feature in a quality control procedure.

Collection of agglomerates. Batches of 30-40 agar plates on which agglomerates had formed were allowed to dry completely at room temperature. A few ml of water were added to each petri-dish to loosen the agar and the contents were poured into a litre measuring cylinder half-filled with water. The sheets of agar were removed and the agglomerates purified by washing with water and decantation. Yields varied from 7-25 mg per 100 plates of 8.5 cm diam.

Tests on the agglomerates. The crystals were insoluble in water, ethanol and benzene, and on heating they charred without melting. Lassaigne's sodium fusion test produced a positive reaction for nitrogen

and negative reactions for sulphur and the halogens, but a negative Biuret reaction indicated that the material was not a protein. X-ray diffraction analysis of a single large crystal from the top of an agglomerate (cf. Fig. A.1) proved unsatisfactory due to the presence of NaCl.

In an attempt to purify the substance, agglomerates were dissolved in dilute HCl, the solution was filtered, and the substance was reprecipitated by addition of Na_2CO_3 to neutrality. The product was a very fine light-brown precipitate which was collected by centrifugation and dried. The infrared spectrum of this powder in a paraffin mull yielded little information; the very small peaks due to NH and CO absorption confirmed that it was not a protein. The ultraviolet spectrum of a solution of the powder in 0.05 M HCl showed an absorption peak at 248 nm.

Purification of the agglomerate through the formation of a crystalline acetate. An attempt was made to purify the agglomerate through a derivative, the acetate proving very effective. Approximately 20 mg of the agglomerate were refluxed for 2½ h with 0.5 ml of acetic anhydride and 2 drops of pyridine. The solvent was removed by distillation at 70°/0.2 mm and the residue was crystallized from 15 ml of boiling water to give approximately 16 mg of tan-coloured crystals. The colour of the product was not affected by recrystallization from water.

Tests on the acetate derivative. Elemental analysis produced the results C:40.0, H:4.0, N:33.1 and O:22.9%. In melting point studies the acetate softened at 342° and melted at 367°. The ultraviolet spectrum of an ethanolic solution of the acetate showed absorption maxima at 259 nm and 205 nm which did not shift upon acidification with HCl. The peak at 259 nm was close to that obtained with the solution of the agglomerate in HCl.

The mass spectrum obtained at an inlet temperature of 250° on an MS9 instrument (N.C.R.L., C.S.I.R., Pretoria) showed a base peak at $^{m}/_{e}$ 151, with a large peak at $^{m}/_{e}$ 193 (32%) and what looked like the molecular ion peak at $^{m}/_{e}$ 303 (0.16%). Accurate mass determinations carried out at the University of Stellenbosch gave values of 150.9908 and 192.9828 but no peak at $^{m}/_{e}$ 303 could be observed.

$C_2H_2N_2O_6$ = 150.991

$C_5N_3O_3$ = 150.994

$C_7N_2O_5$ = 192.981

An attempt was made to grow large crystals of the acetate from aqueous solution by careful control of the temperature in a special crystallizing apparatus. Unfortunately it formed fine leaves resembling cardboard tear-off matches, which were unsuitable for accurate crystallographic work because they showed extreme thermal motion or static disorder. They were orthorhombic with cell constants of 11.78, 6.45 and 24.37 Å and were c centred with therefore a minimum of 8 molecules per cell. Their density was 1.56 g/cc which gives a molecular weight of 218 although this value would be doubled if the molecule were symmetrical (N.P.R.L., C.S.I.R., Pretoria).

An unsuccessful attempt was made to prepare an o-bromobenzoyl derivative of the agglomerate by refluxing with o-bromobenzoyl anhydride. In spite of this failure it is considered that further attempts should be made to prepare a suitable derivative containing a heavy atom since the determination of structure by the X-ray method is undoubtedly the best way of proving the structure of the compound.

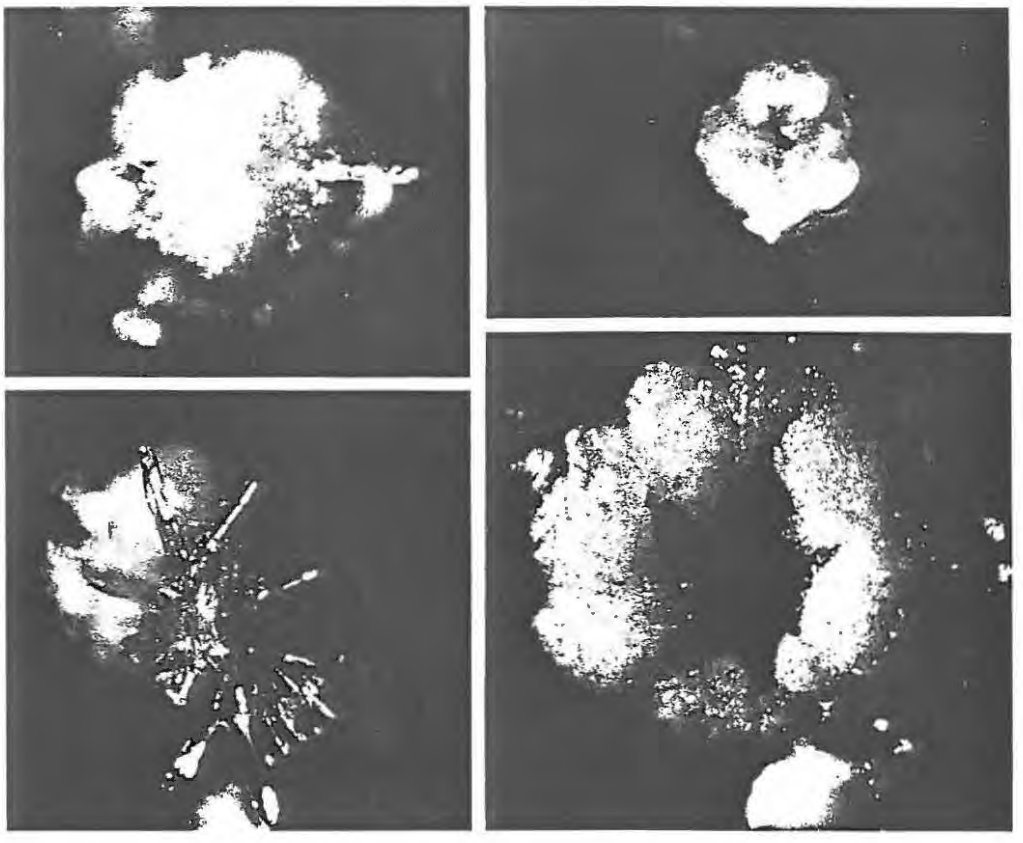


Fig. A.1. Crystalline agglomerates produced by A. iophagus.

The agglomerates were photographed in situ in a 6-day-old plate culture with illumination from above. Approximate magnifications: upper left x 50; lower left x 50; upper right x 25; lower right x 100.

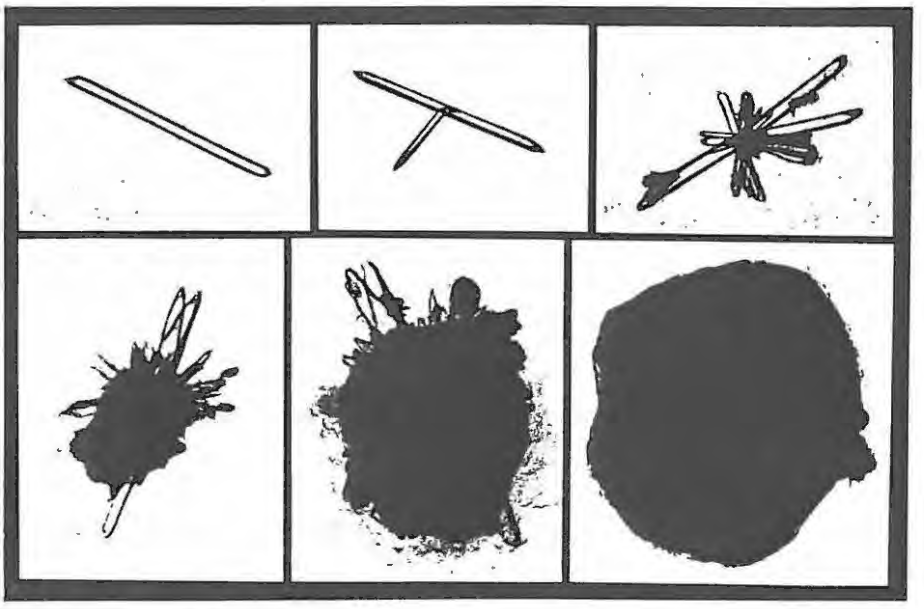
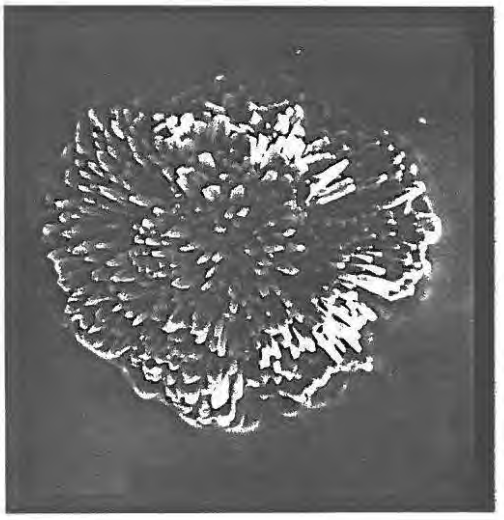


Fig. A.2. Agglomerates at various stages of development.

The agglomerates were photographed *in situ* in a 6-day-old plate culture of *A. iophagus* with illumination from beneath. Approximate magnification x 60.



Magnification x 240

Magnification x 840

Fig. A.3. Scanning electron micrographs of fully-developed agglomerates coated with gold:palladium 38:62.

APPENDIX

LIST OF THE MORE IMPORTANT MATERIALS USED AND THEIR SUPPLIERS

Note: In the text, the use of % to describe the concentration of a solution indicates w/v unless otherwise specified.

Agar: 'Oxoid' Agar No. 3 (Oxoid)

Amido Black 10B (Hopkin and Williams)

Blue Dextran 2000 (Pharmacia)

Bovine albumin (British Drug Houses)

Casamino acids, vitamin-free (Difco)

Casein, vitamin-free and fat-free (British Drug Houses)

Casein hydrolysate (Merck)

Catalase (Sigma)

Chymotrypsinogen A (Miles-Seravac)

Clostridium histolyticum collagenase, forms I and II (Koch-Light)

Collagen, bovine Achilles' tendon (Boehringer)

Coomassie Blue RL (Michrome, Edward Gurr)

'Cyanogum' 41 (British Drug Houses)

L(+)-cysteine (Merck)

Cytochrome c (British Drug Houses)

DTT: Dithiothreitol (Miles-Seravac)

EDTA: Ethylenediaminetetraacetic acid disodium salt (Merck)

Folin-Ciocalteu's phenol reagent (Merck)

β-galactosidase (Koch-Light)

D-glucose (British Drug Houses)

Glycerol (Merck)

Glycine (Merck)

Hide powder (Leather Industries Research Institute)

Ion-agar: Oxoid 'Ionagar' No. 2 (Oxoid)

2-Mercaptoethanol (Merck)

Ninhydrin (May and Baker)

Nutrient broth (Difco)

Ovalbumin (Miles-Seravac)

Peptone: Bacto-peptone (Difco)

o-phenanthroline (British Drug Houses)

Potato starch (Hopkin and Williams)

Pronase (Miles-Seravac)

PZ-pro-leu-gly-pro-arg: 4-phenylazobenzyloxycarbonyl-L-prolyl-L-leucyl-glycyl-L-prolyl-D-arginine-dihydrate (Fluka)

PZ-pro-leu: 4-phenylazobenzyloxycarbonyl-L-prolyl-L-leucine (Fluka)

Sephadex G-200 (Pharmacia; lot no. 9844)

SDS: Sodium dodecylsulphate = sodium laurylsulphate (Hopkin and Williams)

Soytone: Bacto-soytone (Difco)

TEMED: N,N,N',N'-tetraethylmethylenediamine (Eastman-Kodak)

Tryptone: Bacto-tryptone (Difco)

Yeast extract (Difco)

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