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# A POLARIMETRIC METHOD FOR COLLAGENASE ACTIVITY MEASUREMENT

by

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## ABSTRACT

A polarimetric method for monitoring the rate of soluble collagen breakdown by collagenase enzyme action has been developed. The method represents an extension of previous physicochemical techniques based on viscometry, but is simpler and easier to carry out, particularly in the case of reaction rate studies.

The method was developed arising from reports of collagenase activity measurement on inappropriate substrates such as gelatin, modified collagens and synthetic polypeptides. The optical method depends on measurement of the loss in optical rotation in solutions of soluble calfskin collagen resulting from initial enzymic cleavage of the collagen triple-helix, followed by spontaneous unwinding of the resultant unstable helical fragments. Specific assay conditions were chosen to ensure that the loss in optical rotation following enzymic cleavage was rapid and complete.

The method is specific since in the absence of collagenase, non-specific proteinases produce only a limited decrease in solution optical activity. The method has also been compared with established physicochemical assay techniques and compares favourably with both viscometric and titrimetric collagenase assays.

The availability of a rapid, sensitive and quantitative procedure for measurement of collagenase activity provides a convenient means for detecting the presence of collagenase in solution and examination of hide bacterial cultures for collagenase production. In addition, a study of biocidal compounds of potential interest in hide preservation for possible inhibitory effects on collagenase is conveniently carried out with the method. Fundamental research into synergistic action in enzymic hydrolysis of collagen is now possible, providing valuable insight into the mechanism of raw hide biodeterioration.

# CONTENTS

Page

List of Figures vii
List of Tables ix

1. Introduction

1.1 General 1

1.2 Brief review of collagen 2
1.2.1 Collagen types 2
1.2.2 Collagen synthesis, secretion and structure 2
1.2.3 Specificity of degradation of collagen types 3
1.2.4 Type I collagen extraction 6
1.2.5 Effect of lyotropic agents on collagen solutions 7

1.3 Collagenases 8
1.3.1 Definition of true collagenases 8
1.3.2 Mode of collagen hydrolysis 9
1.3.3 Role and applications of prokaryotic and eukaryotic collagenases 9
1.3.4 Inhibition of collagenase activity 10
1.3.5 Known bacterial collagenase producers 11
1.3.6 Conditions required for bacteria collagenase production 11

1.4 Collagenase Assays 13
1.4.1 General Assay design criteria 13
1.4.1.1 Substrate relevance 13
1.4.1.2 Assay specificity 13
1.4.1.3 Kinetic parameter determination 14
1.4.2 Assays based on the hydrolysis of synthetic peptides 14

1.4.3 Assays employing collagen in insoluble form 15
1.4.3.1 Direct visualisation of collagen lysis 15
1.4.3.2 Spectrophotometric assay of dye uptake/release from a collagen-dye complex 16
1.4.3.3 Other spectrophotometric assays of solubilised collagen 16
1.4.3.3.1 Hydroxyproline assay 16
1.4.3.3.2 Folin-Lowry and Rosen-ninhydrin total protein assays 17
1.4.3.4 Solubilisation of radiolabeled collagen 17
1.4.4 Assays based on the hydrolysis of soluble collagen 17
1.4.4.1 Viscometry 17
1.4.4.2 Titrimetry 18
1.4.4.3 Polarimetry 18
1.4.4.4 Soluble radiolabeled collagen-collagenase assays 18
1.4.5 Other collagenase assays 19

1.5 Research Objectives 20

2. Experimental

2.1 Materials 21

2.2 Methods 22
2.2.1 Preparation of stock collagen solutions 22
2.2.2 Preparation of stock collagenase solutions 22
2.2.3 Inhibition of fibrillogenesis and gelation 23
2.2.4 Collagenase assays 24

2.2.4.1 Development of polarimetric collagenase assay 24
2.2.4.2 Viscometric collagenase assay 26
2.2.4.3 Titrimetric collagenase assay 26
2.2.5 Collagenase assay application 27
2.2.5.1 Kinetic parameter determination 27
2.2.5.1.1 Influence of substrate and enzyme concentration 28
2.2.5.1.2 Influence of buffer 29
2.2.5.2 Synergistic collagen hydrolysis 30
2.2.5.3 Biocide inhibition of collagenase activity 32

3. Results and Discussion

3.1 Polarimetric collagenase assay development 33
3.1.1 Inhibition of fibrillogenesis 33
3.1.2 Effect of temperature on assay response 35
3.1.3 Effect of wavelength on assay response 37
3.1.4 Effect of active and denatured collagenases on optical activity of the collagen molecule 39
3.1.4.1 Active and denatured crude collagenase 39
3.1.4.2 Active and denatured purified collagenase 43
3.1.5 Relationship between initial optical rotation change and collagenase concentration 45
3.1.6 Comparison between polarimetric and viscometric collagenase assays 46

3.2 Kinetic parameter determination 47
3.2.1 Polarimetry 49
3.2.1.1 Crude collagenase 49
3.2.1.2 Purified collagenase 52
3.2.2 Titrimetry 52

3.2.3 Effect of buffer concentrations 53

3.2.3.1 Polarimetry 54

3.2.3.2 Viscometry 55

3.3 Assay applications. Collagenase activity studies 59

3.3.1 Synergistic activity 59

3.3.1.1 Influence of general proteinases on collagen 59

3.3.1.2 Prior collagenase exposure to general proteinases 61

3.3.1.3 Combined proteinase/collagenase activity 63

3.3.2 Biocide inhibition of collagenolytic activity 65

4. Conclusions 69

5. Appendix 72

6. References 75

## List of Figures


| | | Page |
|---|---|---|
| 1. | Arangement of alpha-chains into a helix. | 4 |
| 2. | Schematic representation of the origin of collagen fibre networks. | 5 |
| 3. | Effect of assay temperature on enzymic reaction rate. | 36 |
| 4. | Effect of assay wavelength and degree of collagenase purity on initial gradients at 20°C. | 38 |
| 5. | A. Effect of active crude collagenase on optical rotation of a native collagen solution. | 40 |
| 5 | B. Effect of heat-denatured crude collagenase on thermal transition temperature of a native collagen solution. | 40 |
| 6. | Logarithm of percentage change in collagen optical rotation amplitude. | 42 |
| 7. | A. Effect of active purified collagenase on optical rotation of a native collagen solution. | 44 |
| 7 | B. Effect of heat-denatured purified collagenase on thermal transition temperature of a native collagen solution. | 44 |
| 8. | A.C. Specificity of the polarimetric collagenase assay. | 48 |

8. B.D. Specificity of the viscometric collagenase assay. 48

9. Polarimetric kinetic parameter determination by Lineweaver-Burk method. 50

10. Influence of buffer concentration on $K_m$. 56

11. Effect of Tris buffer on the polarimetric collagenase assay. 57

12. Effect of non-specific proteinases on collagen solution OR. 60

13. Effect of 1 h pre-exposure of collagenase to non-specific proteinases. 62

14. Effect of various concentrations of non-specific proteinases on initial gradients. 64

15. Effect of various concentrations of proprietory biocides and non-proprietory chemicals on collagenolytic activity. 68

# List of Tables


| | | Page |
|---|---|---|
| 1. | Collagenolytic microorganisms. | 12 |
| 2. | Effect of perturbant type and concentration on fibrillogenesis. | 34 |
| 3. | Km-values for clostridial collagenase determined by various assay methods. | 51 |
| 4. | Polarimetric kinetic parameter determination with crude and purified collagenase. | 52 |
| 5. | Titrimetric kinetic parameter determination with purified collagenase. | 53 |
| 6. | Effect of Tris buffer on collagenase kinetic parameters using the polarimetric assay. | 54 |
| 7. | Effect of Tris buffer on collagenase kinetic parameters using the viscometric assay. | 58 |
| A1. | Specific levorotation of an approximately 1,0 $mg.m\ell^{-1}$ native collagen solution at various wavelengths. | 72 |
| A2. | Collagen concentration expressed in µM. | 74 |

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# 1. INTRODUCTION

## 1.1 General

Green (raw) hides and skins are generated as natural byproducts of the South African meat industry. The disposal of these hides and skins has led to the creation of a multifaceted allied leather industry, concerned with the collection, curing, storage, shipping and eventual tannage of raw hides/skins.

It is known that the freshly-flayed and uncured hide/skin, being rich in proteins, serves as an excellent nutrient source to over 150 different bacterial genera introduced from numerous environmental sources. To prevent hide decay, green hides and skins are cured with salt or by chemical means, or green material is placed directly into tannage processing to render non-putrescible leather. However, any undue delay between flaying and curing, or between desalination soak-back and subsequent tanning allow these bacteria to grow and reproduce, all-the-while secreting protein-degrading enzymes into their immediate surroundings. It has been postulated that general protein-degrading enzymes produced by these bacteria might trigger the production of the collagenase enzyme by certain hide contaminant bacteria, ultimately responsible for any damage to the leather-making collagen matrix (Thompson, 1988).

As the quality (and therefore the eventual price obtained) of finished leather depends largely on limitation of damage to the collagen matrix prior to tannage, knowledge of the bacterial species involved and conditions conducive to their growth as well as the characteristics and mode of collagen destruction by collagenase, becomes vital. The former will not be covered in the present study; instead, emphasis will be placed on the aspect of collagen degradation by collagenase.

## 1.2 BRIEF REVIEW OF COLLAGEN

### 1.2.1 Collagen types

There are currently twelve recognised collagen types, designated type I to XII. All vary in composition to some extent and represent unique gene products. This large gene pool, in conjunction with post-translational modifications of the collagens accounts for the large spectrum of chemical and structural features exhibited by the collagens (Weiss, 1984; Miller and Gay, 1987; Burgeson, 1988). Three collagen types make up the bulk of hide and skin collagen. Type I collagen is the dominant form in hides and skins, constituting between 80-90 % of all protein present, and is thus of special interest to the leather chemist. Small quantities of type III collagen, accounting for 10 - 20 % of the total collagen of hides and skins, are usually found in association with type I molecules (Kemp, 1983). A third collagen specie, type IV, is found mainly in basement membranes and the stromal region of the cornea. This collagen type differs from type I collagen in that it contains cysteine residues and non-helical regions within the collagen helix (Liotta *et al*, 1981; Kemp, 1983; Weiss, 1984; Miller and Gay, 1987).

The other collagen types have a widespread distribution throughout the animal body or cells, e.g. occurring in various cartilages (types II, IX and X), placental villi, bone, corneal stroma and dermis (type V), placental membranes (type VII) and in association with cultured endothelial cells (type VIII) (Miller and Gay, 1987).

### 1.2.2 Collagen synthesis, secretion and structure

Type I, III and IV collagens are synthesised by fibroblast cells and are secreted into the extracellular matrix in the form of precusor molecules, the procollagens (Na *et al*, 1989; Gerard *et al*, 1983). A procollagen type I molecule consists of three separate amino acid chains each over 1000 residues long, called alpha-chains. Two of the chains

are identical and are termed 1 (I), whilst the third chain is designated 2 (I) i.e. a heterotrimeric molecule. The alpha-chains are twisted about one another in a left-handed helix (Figure 1), whilst the helix itself is arranged around a common axis to form a superhelix or polyproline-II-helix with an overall right-handed twist. Type III collagen differs from type I in consisting of three identical I (III) chains (a homotrimeric molecule). Type IV collagen, a heterotrimeric collagen, consists of two 1 (IV) chains and an 2 (IV) chain (Miller and Gay, 1987; Otter et al, 1987). Attached to the helical procollagen molecules are non-helical peptides known as telopeptides (Figure 2) (Hayashi and Nagai, 1974).

Telopeptides are found on both the amino as well as the carboxyl terminals of the helix. Disulfide bonds in the carboxyl-terminal ends hold the three chains together prior to formation of the helix. The incompletely formed polypeptide molecule is transported through the cell membrane to the extracellular matrix where enzymatic cleavage of the telopeptides must take place before self-assembly into collagen fibrils, bundles and eventually macro-aggregates can occur (Bruckner and Prockop, 1981; Dombi and Halsall, 1985; Bateman et al, 1987; Kadler et al, 1987; Burgeson, 1988; Dombrowski and Prockop, 1988; Kadler et al, 1990).

The triple-stranded collagen molecule has an abundance of glycine, proline and hydroxyproline (unique to collagen) residues. Every third amino acid is glycine, and it is the absence of side-chains on this residue which allow the three alpha-chains to pack so closely together, allowing the formation of a polyproline-II-helix. Once formed, collagen molecules are progressively crosslinked by covalent bonds between individual molecules and between individual alpha-chains (Weiss, 1984; Otter et al, 1987).

### 1.2.3 Specificity of degradation of collagen types

Native type I collagen molecules remain resistant to degradation by all common proteolytic enzymes (Stark and Kühn, 1968; Mandl, 1972; Weiss,

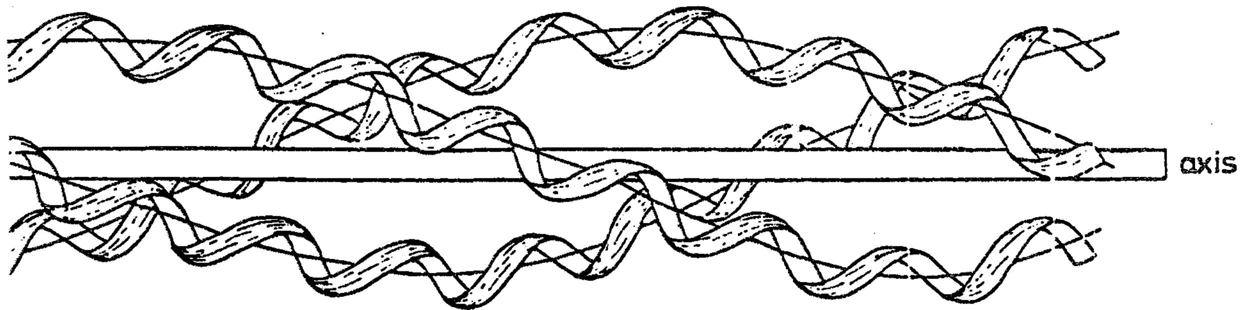


Figure 1 Arrangement of alpha-chains into a helix.

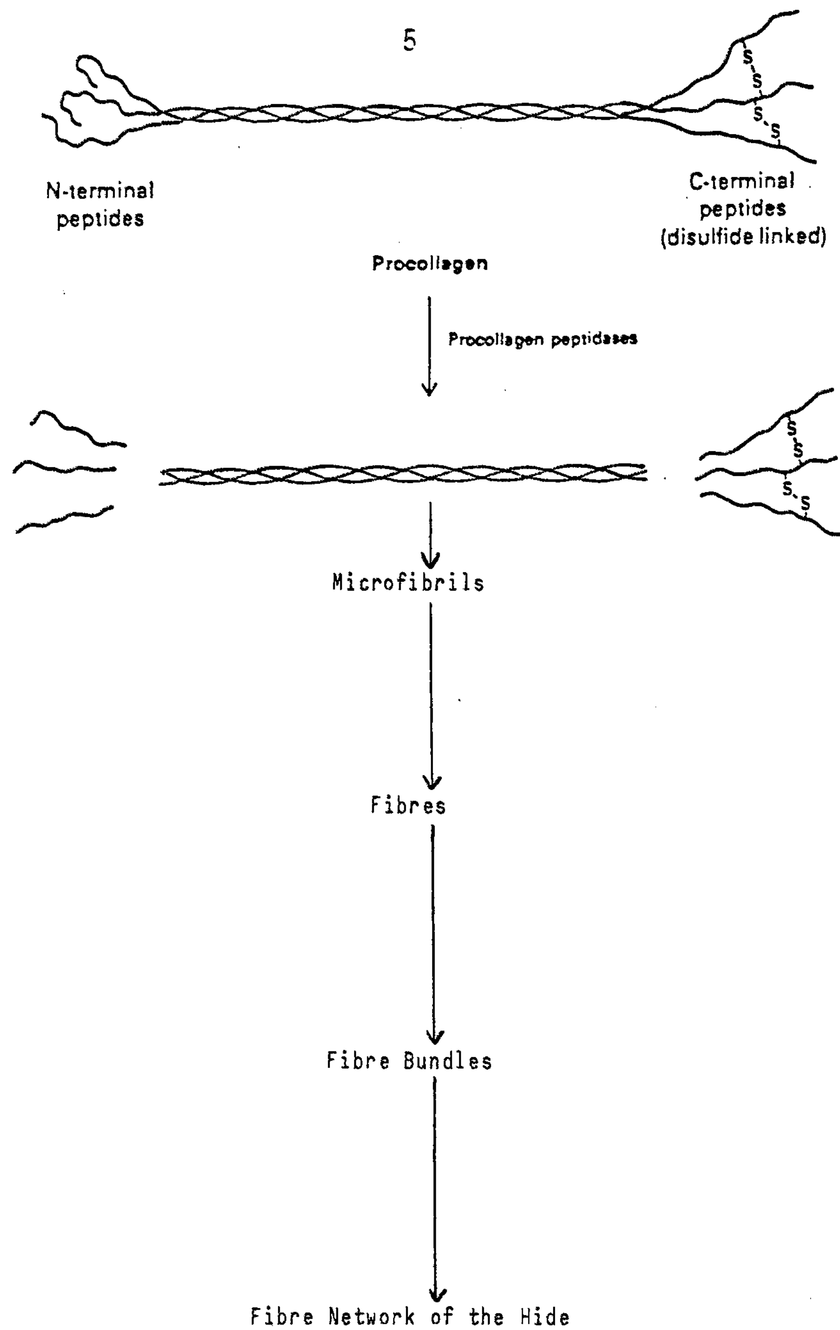


Figure 2 Schematic representation of the origin of collagen fibre networks (Seifter and Harper, 1971).

1984; Campbell et al, 1987; Fields et al, 1987; Chakraborty and Eghbali, 1989). There is, however, considerable evidence to show that collagen types III and IV are susceptible to degradation by non-specific proteinases. For example, type III collagen is particularly susceptible to degradation by trypsin, whose cleavage sites are located near to collagenase cleavage sites, which may lead to erroneous observations of collagenolytic activity (Miller et al, 1976). Type IV collagen can be degraded by proteinases such as pepsin, trypsin and chymotrypsin, suggesting the presence of regions in the peptide vulnerable to non-specific enzyme degradation (Liotta et al, 1981).

### 1.2.4 Type I collagen extraction

It is thought that collagen types III and IV are removed from hides and skins by pre-tannage operations such as soak-back, liming, deliming and bating with proteolytic preparations. Since type I collagen is the leather-making substance (Kemp, 1983), all future reference to collagen will imply type I, unless otherwise stated.

Fractions of monomeric collagen can be extracted from immature hides or skins where crosslinking of collagen molecules is incomplete. Collagen fibres become increasingly more difficult to extract the older the source becomes, probably due to the formation of stable covalent bonds (Davison et al, 1972; Weiss, 1984).

Collagen may be extracted from hide or skin by using one of three common methods:

(a) Neutral salt solutions, e.g. 1,0 M NaCl in 0,05 M Tris pH 7,5. The addition of enzyme inhibitors such as EDTA, DFP or others is considered advisable due to the possible presence of tissue proteinases active at neutral pH. Proteolytic degradation of proteinase-sensitive extra-helical regions will thereby be minimised.

(b) Dilute organic acid solutions, e.g. 0,5 M acetic acid. These solvents are efficient in removing non-crosslinked molecules from immature hide or skin. The inclusion of enzyme inhibitors (e.g. pepstatin) is desirable.

(c) Organic acid solution plus pepsin. This extraction method is as mentioned in (b), but the proteinase will promote the selective degradation of portions of the collagen molecule (telopeptides) (Miller and Rhodes, 1982).

Cooper and Davidson (1965) describe a sequential neutral salt and dilute acid extraction method; other extractant systems and conditions have been reported elsewhere in the literature.

### 1.2.5 Effect of lyotropic agents on collagen solutions

Lyotropic agents (structural perturbants or denaturants) may be defined as compounds capable of disrupting the higher order structure of collagen molecules in solutions without hydrolysis of the peptide chains; they can also affect intermolecular interactions and hence the rate of collagen fibril formation (Banga and Balo, 1965). Collagen fibril formation *in vitro* consists of a molecular nucleation process followed by fibril growth, with the rate of nucleation dependent on collagen monomer concentration and the growth phase influenced by both the concentrations of preformed nuclei and of monomer (Hayashi and Nagai, 1974). The solubilisation of collagens by lyotropic agents is brought about by the destruction of hydrogen bonds, van der Waals forces and crosslinks (Gustavson, 1956, Russell and Cooper, 1969).

The following perturbant ions are ranked in order of decreasing lyotropic effect as follows (Gustavson, 1956):

Cations: Ca > Sr > Ba > Mg > Na, K

Anions : CNS > I > Br > Cl > $SO_4$, $S_2O_3$.

The neutral salts such as bivalent metal chorides of calcium and barium are the most effective perturbants, effectively lowering the transition temperature (Tm) at which soluble collagen will undergo the collagen -

gelatin conversion (von Hippel, 1967). Urea and $CaCl_2$ perturbants reportedly do not increase collagen susceptibility to non-specific proteinases, whilst KI, NaI and $NaClO_4$ do (Banga and Balo, 1965). Collagen fibrillogenesis inhibition has been accredited to the following compounds: 1,0 M glucose as well as 0,15 M phosphate (Hayashi and Nagai, 1973), urea at a concentration exceeding 0,5 M (Russell and Cooper, 1969).

## 1.3 COLLAGENASES

### 1.3.1 Definition of true collagenases

True collagenases, whether mammalian or bacterial in origin, are defined as metalloproteinases containing zinc in the active centre and requiring calcium for protein structural stability. These metalloproteinases must be capable of hydrolysing native collagen at or near physiological pH and temperature (Merkel *et al*, 1975; Cawston and Murphy, 1981).

However, collagenases from different organisms may differ markedly with regard to subunit structure, electrophoretic mobility and activity levels on a variety of proteinaceous substrates such as casein, gelatin and native collagen. There appears to be some confusion in the literature concerning collagenase multiplicity, latency and the role of these characteristics in collagenase synthesis and collagen degradation. Bond and van Wart (1984) found that *Clostridium histolyticum* produced six distinctive collagenases which could be grouped into two separate classes based on differences in amino acid composition, stability and activity. Tong *et al* (1986) isolated three different active collagenases from *Vibrio alginolyticus.* Collagenase multiplicity may arise as a product of different genes or may occur as a result of autolysis of a parent collagenase molecule. Multiple forms of collagenases may assist in the synergistic degradation of collagens (Lwebuga-Mukasa *et al*, 1976). Mammalian collagenases may be secreted in latent forms, becoming active on demand. Such activation can be achieved *in vitro* by exposure of latent collagenase to other proteinases

(e.g. trypsin), or by the addition of perturbants (e.g. $SCN^-$), addition of mercurials and in some cases, by spontaneous activation at physiological temperature (Cawston and Murphy, 1981; Stricklin *et al*, 1983).

### 1.3.2 Mode of collagen hydrolysis

A distinction may be made between mammalian and bacterial collagenases on the basis of cleavage specificity: bacterial collagenases have multiple cleavage sites on the triple-helix whilst mammalian collagenases have a single cleavage locus. In both cases, the result of such cleavage is the generation of two triple-helical fragments known as the N-terminal three-fourths $TC^A$ and the C-terminal one-fourth $TC^B$ (Sakai and Gross, 1967; Danielsen, 1987; Imhoff *et al*, 1990). Hydrolysis of synthetic peptides and native collagen by bacterial collagenases occurs at the X-Y bond in an Pro-X-Y-Pro sequence (Vencill *et al*, 1985; Yiotakis *et al*, 1988), where X represents any amino acid and Y is alanine or glycine.

### 1.3.3 Role and applications of prokaryotic and eukaryotic collagenases

The collagenases play a key role in both normal and abnormal connective tissue metabolism. Matrix components such as collagen, elastin, proteoglycans and laminin usually act as barriers to the movement of cells but these are degraded by invading tumour cells under abnormal conditions (Nethery and O'Grady, 1989). Evidence for the role of collagenases in areas as diverse as bone, postpartum uterus, alkali - burned cornea and skin wounds comes from the relationship between loss of collagen in the living tissue and the appearance of enzyme and degradation products. There is even evidence suggesting that collagen *in vivo* may have small amounts of collagenase bound to its fibres in an inactive form (Woessner and Ryan, 1973; Gross, 1976; Stricklin *et al*, 1977; Cawston and Murphy, 1981).

The vertebrate collagenases thus chiefly serve a remodeling function during normal connective tissue turnover, with an invasive function

under abnormal conditions (for example, tumour growth). The prokaryotic and lower eukaryotic collagenases have a digestive as well as host - tissue invasive function. For example, the mosquito - parasitizing fungus Lagenidium giganteum secretes an extracellular collagenolytic enzyme that assists in penetration of the zoospore germ tube during initiation of infection. A further example is the cysteine proteinase secreted by the parasitic protozoon Entamoeba histolytica, assisting in host connective tissue damage (Keil, 1979, Dean and Domnas, 1983, Schulte et al, 1987).

Laboratory uses of bacterial collagenases include the dispersal and dissociation of animal tissue to obtain cells/materials of interest. The isolation of cells from tissue such as heart muscle (Mandl, 1972) and pancreas (McShane et al, 1989) has been documented. The potential commercial applications of bacterial collagenases is best illustrated by the following example : Achromobacter iophagus collagenase was injected into freshly slaughtered beef in an attempt to improve meat tenderness. The bacterial collagenase only slightly improved the collagen solubility of muscle, probably because the beef pH of 5,5 units was below enzyme optimum. However, inclusion of the endoglycosidase alpha - amylase increased the exposure of insoluble muscle collagen to the bacterial collagenase by cleavage of carbohydrate/collagen linkages (Cronlund and Woychik, 1987). Successful clinical trails using collagenases in burn wound therapy and in the treatment of dermal lesions have been reported ( Mandl, 1972).

### 1.3.4 Inhibition of collagenase activity

Collagenases are reliant on specific environmental conditions and specific substrate conformation for optimal activity. Since they are metalloproteinases, inhibition of such activity may best be achieved by interference with enzyme metal requirements, or by alteration of enzyme and/or substrate configuration. Therefore, bacterial collagenases are susceptible to any metal sequestrant such as EDTA (mainly $Ca^{2+}$ removal), cysteine and hydroxamic acid (zinc-ligating compounds). In addition, bacterial collagenases are inhibited by phosphoric and phosphonic amides

of peptides as well as by aldehyde- and ketone-substrate analogues. Collagenase inhibition is dependent on factors such as time, concentration of inhibitor and pH; the level of inhibition may be partial or total, reversible or irreversible (Mandl et al, 1964; Harper, 1972; Vencill et al, 1985; Makinen and Makinen, 1988; Mookhtiar et al, 1988).

### 1.3.5 Known bacterial collagenase producers

An important point to bear in mind when making comparisons between studies claiming to have isolated collagenolytic bacteria is that few of the assay methods employed are directly comparable (Bond and van Wart, 1984). In addition, earlier workers in this field may have been misled by the use of inappropriate substrates with which to screen for and quantify collagenolytic activity. Collagenase production may be difficult to detect, as collagenase may be associated cellularly and may only become evident upon cellular destruction or by cell exposure to detergents (Cawston and Murphy, 1981; Birkedal-Hansen et al, 1988).

Table 1 lists some collagenolytic microorganisms, the majority of which are bacteria.

### 1.3.6 Conditions required for bacterial collagenase production

The *in vitro* production of bacterial collagenases is induced by cell growth in the presence of collagen or the degradation products of collagen, such as gelatin or even peptone (Merkel et al, 1975; Reid et al, 1978; Ishii et al, 1988). However, a detectable basal level of extracellular collagenase has been noted in *Vibrio alginolyticus* culture filtrates, suggesting constitutive production (Hare et al, 1983). Collagenase production may be sensitive to the type of carbon source available; Reid et al (1978) concluded that glucose, sucrose and glycerol suppressed collagenase production by a hide bacterium isolate (later reclassified as *V. alginolyticus*). Collagenase levels may decrease in culture filtrates upon standing, due to proteolytic degradation (Tong et al, 1986).

Table 1 Collagenolytic microorganisms

| Microorganism | Assay Method | Reference |
|---|---|---|
| Clostridium histolyticum | azocoll, BAT hydrolysis | Mandl et al, 1953 |
| | azocoll, viscom, colorim, syn pep | Yoshida and Noda, 1965 and others |
| Achromobacter iophagus (reclassified as Vibrio alginolyticus by Reid et al, 1980) | syn pep | Lecroisey et al, 1975 |
| | syn pep, colorim, ORD | Keil-Dlouha, 1976 |
| | syn pep | Reid et al, 1978 |
| | syn pep | Long et al, 1981 |
| | radioact, syn pep | Hare et al, 1983 |
| | syn pep, fibril, electrophor | Tong et al, 1986 |
| Vibrio B-30 | collagen plates, colorim, syn pep | Merkel and Dreisbach, 1978 |
| Bacillus cereus | colorim, syn pep, radioact | Makinen and Makinen, 1987 |
| Aeromonas proteolytica | collagen plates, BAT hydrolysis, syn pep | Merkel, 1972 |
| Bacteriodes sp | fibril, viscom, electrophor | Birkedal-Hansen et al, 1988 |
| Bacteriodes sp Actinobacillus actinomycetemcomitans | radioact | Robertson et al, 1982 |
| Candida albicans | hypro | Hagihara et al, 1988 |
| Entomophthora coronata | syn pep, ORD | Keil, 1979 |
| Pseudomanas sp | colorim, syn pep, | Hisano et al, 1989 |
| Pseudomonas aeruginosa[a] Staphylococcus aureus[a] | syn pep, hypro, colorim | Waldvogel and Swartz, 1969 |
| Streptomyces sp | hypro | Chakraborty and Chandra, 1984 |
| Mycobacterium tuberculosis | viscom, colorim | Seifter and Harper, 1970 |

where a = only when grown anaerobically
- azocoll = hide powder coupled to an azo dye
- BAT hydrolysis = bovine achilles tendon hydrolysis
- colorim = colorimetry, e.g. ninhydrin
- syn pep = synthetic peptide hydrolysis
- radioact = radioactivity
- ORD = optical rotation dispersion
- fibril = collagen fibrillar assays
- electrophor = electrophoresis
- collagen plates = reconstituted collagen hydrolysis
- viscom = viscometry
- hypro = hydroxyproline release

## 1.4 COLLAGENASE ASSAYS

Collagenase assays may be subdivided into three types, based on the type of substrate employed. These are the following:

(a) Assays based on the hydrolysis of synthetic peptides.
(b) Assays employing collagen in insoluble form.
(c) Assays based on the hydrolysis of soluble collagen.

Many variations on a common theme will exist within any one class, each with certain advantages and limitations.

### 1.4.1 General Assay Design Criteria

#### 1.4.1.1 Substrate relevance

In any assay, the relevance of the substrate used is critical, as it may not always be possible to extrapolate from a synthetic or modified substrate to the native substrate (Alexander and Walker, 1987). This statement applies equally well to collagen and synthetic peptides mimicing collagen conformation. The state in which a collagen molecule exists will depend on numerous environmental conditions such as temperature, pH and ionic strength as well as on collagen source and type, and whether it has been chemically modified or radiolabeled (Birkedal-Hansen, 1987). For example, chemically modified or radiolabeled fibrillar collagens may be sensitive to ionic strength, resulting in a very low background ionic strength requirement. Even physiological salt levels may result in redissolution, which would clearly be disadvantageous in the majority of fibrillar collagenase assays as these usually measure collagen solubilisation rather than initial peptide bond cleavage (Mallya et al, 1986; Nethery et al, 1986).

#### 1.4.1.2 Assay Specificity

It has been alleged that, in general, synthetic peptide collagenase assays

are non-specific. Therefore, the observation of enzymatic cleavage of a synthetic peptide cannot be taken as absolute proof of true collagenolytic activity. Such activity should always be confirmed by enzyme exposure to native collagen substrate (Keil et al, 1975; Woessner, 1979; Biondi et al, 1988).

In the case of native collagen substrates, whether soluble or insoluble, specificity depends on a number of factors such as, for example, assay temperature and the presence of contaminant proteinases. Hydrolysis of collagen in solution also does not necessarily indicate the ability to dissolve the fibrillar collagen form (Birkedal-Hansen, 1987).

#### 1.4.1.3 Kinetic parameter determination

Ease of kinetic parameter determination varies between the three assay types. Assays employing collagen in soluble form are single phase reactions which more readily allow determination of parameters such as $K_m$, $V_{max}$ and others. Assays in which insoluble collagen serves as substrate may lead to errors in parameter determination as these do not easily lend themselves to estimations of true initial enzyme reaction rates (Mallya et al, 1986). This applies equally to synthetic peptide assays (van Wart and Steinbrink, 1981).

### 1.4.2 Assays based on the hydrolysis of synthetic peptides

This assay class is based on the hydrolysis of a synthetic amino acid sequence, mimicing cleavage site conformation of the native collagen molecule. Different strategies exist for the quantification of cleavage products; for example, these may be determined spectrophotometrically after extraction into an organic phase (Wünsch and Heidrich, 1963) or by the measurement of fluorescence generation (Stack and Gray, 1989; Barrett et al, 1989). Allegations of non-specificity are the most serious shortcoming of this assay class, although Biondi et al (1988) claim to have overcome this problem by the incorporation of the selective High Performance Liquid Chromatography (HPLC) technique in the assay method in lieu of spectrophotometry, as well as by the elimination of the extraction

step.

### 1.4.3 Assays employing collagen in insoluble form

#### 1.4.3.1 Direct visualisation of collagen lysis

The simplest way to screen for and quantify collagenase presence is direct visualisation of collagen gel or film lysis. Several strategies exist, based on collagen overlay on agar spread plates (Robbertse et al, 1978), or on collagen incorporation in an agar matrix (Yankeelov et al, 1977) and collagen film staining (Giacomello et al, 1979).

The overlay screening method described by Robbertse et al (1978) was designed for the detection of collagenolytic bacteria. The method calls for the preparation of agar spread plates onto which cold, reconstituted collagen was poured and allowed to gel. Collagenase-positive bacteria were identified by clear hydrolysis zones, following a suitable incubation period. Yankeelov et al (1977) incorporated lathyritic collagen in an agar matrix, followed by agar gelation at room temperature which was sufficiently low to prevent collagen fibril formation. Test solutions were spotted on the agar surface, followed by a lengthy incubation at 28°C. The agar/collagen plates were transferred to a 37°C incubator, where normal collagen fibril formation was encouraged. Digested collagen gave rise to clear hydrolysis zones, surrounded by opaque fibrillated collagen.

Test solutions may be spotted onto dried films of reconstituted collagen, prepared on solid supports such as a glass slide (Giacomello et al, 1979). Incubation of slides took place in a moist environment and test solutions were carefully washed off the slide after a suitable time interval. Undigested collagen was stained by the application of a dye such as Coomassie-blue. The amount of collagenolytic activity could be related to the extent of the hydrolysis zone.

### 1.4.3.2 Spectrophotometric assay of dye uptake/release from a collagen - dye complex

Nethery *et al* (1986) developed a collagenase assay in which test solutions were added to a collagen-coated microtitre well. Following incubation, test solutions were removed by washing and all residual collagen was stained by Coomassie-blue. The amount of staining was quantified by means of an automatic microtitre spectrophotometer. Limitations of this assay included susceptibility to trypsin, which was as high as 18 % of collagen solubilised by trypsin after 6 hours, as well as interference from collagen-binding proteins. However, claimed advantages included economy, reproducibility and sensitivity.

A further example is provided by Kuttan and di Ferrante (1980). The polyazo dye Sirius Red (SR) preferentially stains collagen and can thus be used for its quantification even in the presence of other proteins. These authors describe an assay based on the spectrophotometric measurement of SR release from the SR-collagen complex due to collagenase activity.

### 1.4.3.3 Other spectrophotometric assays of solubilised collagen

#### 1.4.3.3.1 Hydroxyproline assay

The imino acid hydroxyproline is unique to collagen, thereby permitting measurement of hydroxyproline release as an indicator of collagenolytic activity. Hydroxyproline containing peptides are released as a result of enzyme (collagenase) activity. Following separation and removal of unhydrolysed collagen, hydroxyproline can be liberated by acid hydrolysis and be oxidised by chloramine T to produce a pyrrole-type compound. The addition of p-dimethylaminobenzaldehyde (Ehrlich's reagent) results in chromophore production with a wavelength maximum at 558 nm. Hydroxyproline assays are highly specific, but can suffer from certain limitations, one of which is non-reproducibility due to contaminant oxidisable compounds (Jamall *et al*, 1981).

1.4.3.3.2 Folin-Lowry and Rosen-ninhydrin total protein assays

Hydrolytic activity by collagenase will result in the production of a mixture of di-, tri-, tetra- and larger polypeptides, which may be quantified by ninhydrin or Folin-Lowry assays (Rosen, 1957; Lowry et al, 1951). However, these assays may be subject to interference by assay components, e.g. the Lowry procedure is sensitive to the presence of potassium and magnesium ions, other proteins, EDTA, Tris, thiol reagents and carbohydrates (Bradford, 1976).

1.4.3.4 Solubilisation of radiolabeled collagen

Radiolabeled collagenase assays operate on the principle of separation of labeled hydrolysis fragments from undigested collagen. This separation may be brought about by selective precipitation of soluble radiolabeled collagens, using dioxane or ethanol, or by physical separation of solubilised collagen from undigested, insoluble collagen as described by Johnson-Wint (1980). The assay technique described by this author employs principles representative of other insoluble, radiolabeled collagenase assays. Microtitre plates with films of dried $^{14}C$-labeled collagen are exposed to test solutions, followed by incubation. Physical removal of solubles from the separate microwells is followed by collagenolytic activity quantification by scintillation counting.

1.4.4 Assays based on the hydrolysis of soluble collagen

This assay class consists of well-established physicochemical techniques as well as newer radiometric assays.

1.4.4.1 Viscometry

Viscometric assays are based on the inherently high solution viscosity of the native collagen molecule. The high viscosity of native collagen solutions and the relatively low viscosity of such solutions after exposure to collagenase make this method of assay highly sensitive and

viscometric assays have been claimed to be well suited to kinetic studies (Gallop et al, 1957; Wang et al, 1978).

#### 1.4.4.2 Titrimetry

Cleavage of collagen by collagenase results in the liberation of acidic carboxyl groups, which may be titrated to neutrality by constant addition of alkali, most conveniently by means of pH-stat. The course of the enzymatic reaction is followed by calculation of the number of acid equivalents produced at various times (Veis, 1964).

#### 1.4.4.3 Polarimetry

The collagen molecule is characterised by high optical activity, due to specific asymmetry of the amino acid residues comprising the polypeptide chains as well as asymmetry arising from the arrangement of individual chains into an ordered, triple-helical conformation (von Hippel, 1967). It was this characteristic which gave rise to an early attempt at a polarimetric assay, described by Nordwig and Jahn (1968). These authors isolated a proteinase, aspergillopeptidase, from the culture filtrate of the fungus *Aspergillus oryzae*. After noting that the isolated proteinase cleaved synthetic peptides at a site similar to the cleavage site conformation in native collagen, these authors investigated the hydrolysis of native collagen substrate by monitoring loss of optical rotation during an extended 170 h incubation at 30°C. The conclusion that *A. oryzae* produces collagenase should be viewed with caution, as the relatively high assay temperature and extended incubation period will increase native collagen denaturation and vulnerability to non-specific proteinase hydrolysis (Birkedal-Hansen, 1987).

#### 1.4.4.4 Soluble radiolabeled collagen-collagenase assays

Collagens may either be radiolabeled *in vivo*, by injecting animals with [$^{14}$C]-glycine or [$^{14}$C]-proline, or *in vitro* by one of the following methods:

(a) Reductive methylation by addition of $[^3H]$-formaldehyde and $NaBH_4$ to collagen.

(b) Propionylation of collagen by the addition of succinimidyl 2,3-$[^3H]$ propionate.

(c) Acetylation of collagens with $[^3H]$-acetic anhydride.

It is claimed that none of these labeling techniques results in the radical alteration of the collagen molecule (Gisslow and McBride, 1975; Cawston and Barrett, 1979; Mookhtiar et al, 1986).

The assay described by Gisslow and McBride (1975) is representative of the principles of soluble radiolabeled collagenase assays. The assay was initiated by the addition of bacterial collagenase to the neutral, radiolabeled collagen substrate. At appropriate time intervals an aliquot of the assay mixture was removed, followed by addition of phosphotungstic and hydrochloric acid to inactivate the enzyme. Undigested collagen was removed by centrifugation and scintillation counting of digested supernatant was performed.

1.4.5 Other collagenase assays

An electrophoretic collagenase assay has been described by Birkedal-Hansen (1987). In addition, numerous immunological techniques exist to detect and quantify collagenase (Birkedal-Hansen 1987). Ngo and Lenhoff (1983) describe the covalent bonding of glucose oxidase to insoluble particulate collagen. An amperometric method is used to detect and quantify glucose oxidase released from the collagen due to collagenase activity.

## 1.5 Research Objectives

1. To describe the development of a qualitative and quantitative polarimetric collagenase assay which is easy to perform, specific, accurate and sensitive.

2. To directly compare the novel polarimetric assay with established collagenase assays. Central to this objective would be the balanced evaluation of assays selected for comparative purposes, with critical assessment of assay advantages and limitations.

3. To further characterise clostridial collagenase activity in terms of kinetic constants, using the polarimetric assay method in comparison with other established physicochemical collagenase assays. The parameters obtained as a result of variations in assay conditions would serve to illustrate the viability of the polarimetric technique as an alternative collagenase assay method.

4. To illustrate polarimetric collagenase assay potential by selected application of the assay technique. Assay application would concentrate on two areas of interest to the South African leather industry. These are

   (a) the degree of collagenase inhibition afforded by biocides used as green hide preservatives ("chemical curing").

   (b) an investigation of the synergistic degradation of collagen by collagenase in the presence of other non-specific proteinases.

# 2. Experimental

## 2.1 Materials

The following lyophilized proteinases were supplied by Sigma Chemical Company (USA):

i) Crude collagenase from *Clostridium histolyticum.*
Batch activity varied from 300 - 600 $U.mg^{-1}$ solid.

ii) Chromatographically purified collagenase from *C.histolyticum.*
Batch activity varied from 1600 - 2000 $U.mg^{-1}$ solid.

iii) Protease from *Streptomyces griseus.*

iv) Thermolysin from *Bacillus thermoproteolyticus.*

v) Bromelain from pineapple stems.

In addition, the following lyophilized proteinases were used in this study:

a) trypsin (Seravac).
b) alpha-chymotrypsin (Miles Laboratories).
c) pronase (Calbiochem).

Calcium chloride ($CaCl_2$), Sodium chloride (NaCl), boric acid ($H_3BO_3$) and ethylenediamine tetra-acetic acid (disodium salt) ($C_{10} H_{14} O_8 N_2 Na_2 .2H_2O$) were general purpose reagents supplied by BDH, PAL Chemicals and Hopkin and Williams.

Tris (hydroxymethyl)-aminomethane ($C_4 H_{11} NO_3$) sodium hydroxide (NaOH) and urea $(NH_2)_2CO$ were analytical reagents supplied by Merck. Dextrose and hydrochloric acid (HCl) were analytical reagents supplied by NT Laboratory Supplies and BDH respectively. The prorietory formulation STA is an organosulphur liquid microbiocide supplied by SA Paper Chemical Company. Distilled water was used throughout this investigation.

## 2.2 Methods

### 2.2.1 Preparation of stock collagen solutions

Lyophilized acid-soluble calfskin collagen (ASC) used throughout this study was prepared according to Cooper and Davidson (1965).

Collagen stock, in the absence of buffer, was prepared as follows: 120 or 140 mg ASC (approx.) was added to 50 mℓ acidified distilled water pH 3. The water/collagen solution was kept at 4°C for 4 days with constant stirring to aid collagen swelling and redissolution in the presence of acid. The cold, reconstituted collagen solution was centrifuged at 4°C for 0,5 h at 9600g in a MSE-18 centrifuge and the pellet, representing undissolved collagen, was discarded. The resultant clear, viscous supernatants had nominal concentrations of 2,0 or 2,5 $mg.mℓ^{-1}$ respectively and collagen aliquots for experimental purposes were withdrawn from these refrigerated stocks, after polarimetric verification of collagen concentration at 365 nm (see Appendix 2).

Collagen stock, in the presence of buffer, was prepared as follows: 120 mg ASC (approx.) was added to 50 or 100 mM Tris-HCl buffer pH 3. Reconstituted collagen was centrifuged after 4 days at 4°C as described. The resultant clear, viscous collagen solution had a nominal concentration of 2,0 $mg.mℓ^{-1}$ and was kept refrigerated.

### 2.2.2 Preparation of stock collagenase solutions

The activity of clostridial collagenase solutions, whether prepared from crude or chromatographically purified source, was measured in terms of units per mℓ ($U.mℓ^{-1}$), where 1 unit is defined as that amount of enzyme which will liberate peptides from collagen equivalent in ninhydrin colour to 1,0 μ mole of L-leucine, in 5 h at pH 7,4 and 37°C (Sigma catalogue, 1984). Denatured enzyme solutions were obtained by the immersion of enzyme aliquots in boiling water for 15 minutes.

Crude or chromatographically purified Clostridium hystolyticum collagenase stock solutions, in the absence of buffer, were prepared in distilled water at the following approximate concentrations, either by serial dilution or by individual weighings of lyophilized collagenase: 0; 37,5; 75; 150; 300 and 600 $U.m\ell^{-1}$ Stock solutions were immediately refrigerated at 4°C and were used soon after preparation, to minimize activity loss due to bacterial growth.

C. histolyticum collagenase stocks in the presence of buffer were obtained from crude or chromatographically purified lyophilized collagenase individually added to or serially diluted with one of the following Tris-HCl buffer concentrations (pH 7,6): 25, 50 and 100 mM. Collagenase concentrations thus prepared ranged from approximately 0, 15, 30, 60, 120, 180 and 240 $U.m\ell^{-1}$.

$CaCl_2$ was either added separately to the assay solution (as in Section 2.2.4.3) or incorporated at 200mM concentration in the collagenase/buffer solutions.

2.2.3 Inhibition of collagen fibrillogenesis and gelation

Collagen solutions at neutral pH show heat gelation/fibril formation on warming which would interfere with optical rotation measurement.

Urea, dextrose and $CaCl_2$ were examined for prevention of fibril formation and thermal gelation and possible lyotropic effects in neutral, reconstituted collagen solutions. The following concentration series were prepared in distilled water:

(a) urea - 0; 25; 50; 75; 100; 150; 200 and 300 mM.
(b) dextrose - 0; 1000 mM.
(c) $CaCl_2$ - 0; 20; 40; 200; 1000 mM.

5,0 $m\ell$ of perturbant solution was added 1:1 to 2,0 $mg.m\ell^{-1}$ collagen solution prepared in 100 mM Tris-HCl pH 3, after which pH was adjusted

to 7,6 units by the rapid immersion and withdrawal of a NaOH pellet from the stirred collagen/perturbant solution. Aliquots of these solutions were incubated in test tubes at 22°C (and 37°C in the case of urea). In addition, $CaCl_2$ and dextrose perturbant/collagen solutions were transferred to polarimeter cells maintained at 20°C (see Section 2.2.4.1). The onset of thermal gelation was monitored and manifested as an increase in turbidity of test solutions when viewed against a dark background. Fibril dissolution temperature was determined by immersion of test tubes in a beaker of heated water ($0,5\text{-}1°C.min^{-1}$) and the temperature of incipient dissolution of fibres as gelatin was recorded (Russell, 1973).

### 2.2.4 Collagenase assays

#### 2.2.4.1 Development of polarimetric collagenase assay

Preparation of final assay solutions was performed on ice, to minimize proteolytic activity. The assay solution used throughout polarimetric assay developmental work was an equal addition of 2,0 mℓ of stock 2,0 $mg.m\ell^{-1}$ collagen solution dissolved in 50 mM Tris-HCl pH3 to active or heat-denatured collagenase solution prepared in 50 mM Tris-HCl pH7,6 containing 200 mM $CaCl_2$. Adjustment of pH to 7,6 units, if necessary, was performed by the rapid immersion and withdrawal of a NaOH pellet from the stirred assay solution; in this way, dilution of assay solution was prevented. Assay solutions were transferred as rapidly as possible to jacketted polarimeter cells of 1 dm light-path length, whose temperature was kept at a constant 20°C or 25°C (± 0,2°C) by means of coolant circulation through external Colora Ultra-Thermostat NB-34778 waterbaths. Absolute optical rotation values were obtained over a 24 h period at 365, 436 and 546 nm wavelengths generated by sodium- and mercury-lamps in a Perkin-Elmer 141 digital polarimeter. Specific degrees of levorotation were calculated by application of the following formula:

$$[\alpha]^{a}_{b} = \alpha \times 1000 \text{ (for a 1 mg.m}\ell^{-1}\text{ collagen solution)}$$

where $\alpha$ = absolute optical rotation.
a = temperature in °C
b = wavelength in nm

Specific degrees of levorotation for a 1 mg.m$\ell^{-1}$ collagen solution at 365 nm and 15°C is -1330, 56° (Russell, 1973; Kanfer, 1977).

Zero-time specific optical rotation values at the point of enzyme addition to collagen substrate, were obtained either by back-extrapolation to primary data graph y-axis intercept or by means of linear regression analysis on a Hewlett- Packard HP-85 programmable calculator.

Denatured collagenase solutions at various concentrations, incubated in parallel with corresponding active enzyme assay solutions over the initial 24 h period, were subjected to a further thermal transition (or "melting" curve) experiment and the characteristic midpoint ("melting") temperature of the collagen-to-gelatin thermal denaturation, $T_m$, was determined. Thermal denaturation of the 1,0 mg.m$\ell^{-1}$ collagen solution was induced by incremental temperature increases of between 2°-4°C of coolant circulating through polarimeter cells, with a 30 minute pause at each new temperature to permit thermal equilibration, before obtaining an absolute levorotation reading at 436 nm (von Hippel and Wong, 1963). Denaturation to gelatin was complete at 45°C.

Polarimetric readings were performed at 436 nm wavelength (unless specified otherwise) in order to minimize interference by assay solution colour/turbidity at the lower, more sensitive wavelengths (see Appendix for the relationship between assay wavelengths). The % change in amplitude was calculated as the difference between levorotation readings and a baseline value, expressed as a percentage.

2.2.4.2 Viscometric collagenase assay

The viscometric collagenase assay method of Gallop *et al* (1957) was used as guideline. Stock solutions of non-specific trypsin, alpha-chymotrypsin and protease were prepared at 0,4 $mg.m\ell^{-1}$ and crude collagenase (300 $U.mg^{-1}$ solid) was prepared at 0,2 and 0,4 $mg.m\ell^{-1}$ concentrations in 50 mM Tris-HCl, pH 7,6 containing 200 mM $CaCl_2$. 5,0 $m\ell$ of each enzyme stock was added to an equal amount of 2,0 $mg.m\ell^{-1}$ collagen stock dissolved in 50 mM Tris-HCl pH3. The pH was adjusted to 7,6 units (if necessary) as described above and an aliquot of this solution was immediately withdrawn and transferred to Canon-Fenske capillary flow-cells suspended in a Gallenkamp viscometer bath equipped with an external regulatory Colora Ultra-Thermostat NB-34778 waterbath set to 20°C (± 0,2°C). The balance of assay solution was transferred to the polarimeter as described (Section 2.2.4.1.). Viscometer cell flow times were measured at convenient intervals and specific viscosity, in centistokes, calculated by application of the following formula:

$$V = ct - {}^{b}/t$$

where V = kinematic viscosity in centistokes
t = observed flow times in seconds
b and c = calibration constants varying for individual capillary flow-tubes.

Parallel determinations of specific levorotation were carried out at 436 nm and 20°C as described (Section 2.2.4.1.). The percentage change in amplitude for both viscometric and polarimetric assays was calculated as described.

2.2.4.3 Titrimetric collagenase assay

The pH-stat method of von Hippel and Harrington (1959) was used as guideline. 10,0 $m\ell$ of stock 2,5 $mg.m\ell^{-1}$ collagen solution pH 3, reconstituted in acidified distilled water, was pipetted into a jacketed

reaction vessel, connected to an external Colora Ultra-Thermostat NB-34778 waterbath set to 20°C (+/-0,2°C). 10,0 mℓ of 250 mM $CaCl_2$ solution was added to the collagen solution, followed by correction of pH to 7,6 units by immersion and withdrawal of a NaOH pellet from the stirred solution. The final assay solution was obtained by the addition of 5,0 mℓ collagenase, dissolved in distilled water, to the reaction vessel, followed by correction of pH to 7,6 units as rapidly as possible. pH was maintained at a constant 7,6 units by 0,01 M KOH titrant addition from a 10,0 mℓ reservoir attached by gear-driven linkage to a Metrohm Herisau Potentiograph E 436 titrimeter. The amount of acidity generated as a result of collagenolytic activity was expressed as follows:

Milli-equivalents acid produced (P)

$$= \frac{\text{m}\ell \text{ 0,01M KOH delivered} \times 1000 \times 56{,}11}{1000}$$

$$= \text{m}\ell \text{ 0,01 M KOH delivered} \times 56{,}11$$

where MW (KOH) = 56,11

### 2.2.5 Collagenase assay application

#### 2.2.5.1 Kinetic Parameter determination

Serial dilutions of collagen stocks, prepared as described in Section 2.2.1., were performed either with acidified distilled water or Tris-HCl buffer acidified to pH 3, to obtain the following concentration series:

$$0{,}125;\ 0{,}25;\ 0{,}375;\ 0{,}50;\ 1{,}0 \text{ and } 1{,}25\ \text{mg.m}\ell^{-1}$$

Collagen solutions prepared in acidified distilled water were subsequently added to Tris-HCl buffer pH 7,6 solutions of differing molarities, if appropriate to the assay method of choice.

The different strategies described for the preparation of final assay solutions depend on the assay method employed, i.e. polarimetric, viscometric or titrimetric collagenase assays. Collagenase concentration ($U.m\ell^{-1}$) and $CaCl_2$ addition point were varied to accomodate different assay strategies. The investigation of the influence of Tris-buffer molarity on kinetic parameters was performed with Tris-buffers pH 7,6 of the following molarity, each containing 200 mM $CaCl_2$:

25; 50; and 100mM

2.2.5.1.1 Influence of substrate and enzyme concentration

Different final assay solutions are described depending on the assay method employed:

i) Polarimetric assay:

2,0 mℓ of a stock 2,0 mg $m\ell^{-1}$ collagen solution reconstituted in acidified distilled water was added to an equal amount of 100mM Tris-HCl buffer pH 7,6 containing 200mM $CaCl_2$ and 60 or 240 $U.m\ell^{-1}$ crude collagenase. The collagen concentration series was obtained by varying the amount of stock collagen brought to 2,0 mℓ volume by the addition of distilled water diluent. Loss of optical activity was determined polarimetrically at 436 nm and 20°C as previously described. Gradient extraction of the initial linear assay response curve was as described and results were proportioned to correspond to a final 30 or 120 $U.m\ell^{-1}$ crude collagenase concentration. Initial gradient values obtained at various collagen concentrations were used in the generation of reciprocal plots (Lineweaver-Burk) for kinetic parameter determinations. The experimental procedure was repeated with purified collagenase at the same concentrations and data were obtained as described.

ii) Viscometric assay:

It was necessary to work with larger assay volumes in the viscometric collagenase assay, due to viscometer flow-cell requirements. Therefore, 5,0 mℓ of stock 2,0 mg.mℓ$^{-1}$ collagen reconstituted with 50mM Tris-HCl buffer pH 3 was added to 5,0 mℓ of 50mM Tris-HCl buffer pH 7,6 containing 200mM $CaCl_2$ and 60 or 240 U.mℓ$^{-1}$ purified collagenase. Collagen concentration of final assay solution was varied by dilution of the stock collagen aliquot with 50mM Tris-HCl buffer pH 3 diluent. An aliquot of the final assay solution was transferred to Canon-Fenske capillary cells suspended in a Gallenkamp viscometer maintained at 20°C. The initial decrease in solution viscosity was monitored as previously described and initial gradients obtained at various collagen concentrations were used in the generation of Lineweaver-Burk graphs as described in (i).

iii) Titrimetric assay:

The minimum working volume of the titration vessel used in the titrimetric collagenase assay method was 20mℓ. Accordingly, 10,0 mℓ of stock 2,5 mg.mℓ$^{-1}$ collagen reconstituted in acidified distilled water was added to 10,0 mℓ 250mM $CaCl_2$ solution. Collagen concentration of final assay solution was varied by dilution of stock collagen aliquot with distilled water. Solution pH was adjusted to 7,6 units prior to and after the addition of 5,0 mℓ of 150 or 600 U.mℓ$^{-1}$ crude collagenase prepared in distilled water. Collagenolytic activity was monitored at 20°C by the titrimetric method as described (Section 2.2.4.3).

2.2.5.1.2 Influence of buffer

i) Polarimetric assay:

The polarimetric investigation of the influence of Tris buffer at 20°C and a constant 30 U.mℓ$^{-1}$ collagenase concentration on kinetic parameter determination was undertaken as follows: 8,0 mℓ of stock 2,5 mg.mℓ$^{-1}$

collagen solution reconstituted in acidified distilled water was added to 2,0 mℓ distilled water diluent and 10,0 mℓ Tris buffer of varying molarity containing 200mM $CaCl_2$ and 150 $U.m\ell^{-1}$ individually weighed chromatographically purified collagenase. These larger volumes described were necessary as a result of the individual weighings of highly-active purified collagenase. Thus, greater assay volumes were chosen to compensate for the minimum amount of collagenase that could be weighed accurately. Collagen concentration of the final assay solution was varied by dilution of the stock collagen aliquot with distilled water. Solution pH was adjusted to 7,6 units, if necessary. Initial gradient values were extracted from primary data as described, and were used in the generation of Lineweaver-Burk plots for kinetic parameter determinations.

ii) Viscometric assay:

The viscometric determination of buffer influence on kinetic parameter determination at 20°C and a constant 30 $U.m\ell^{-1}$ collagenase concentration was undertaken as follows: 5,0 mℓ of stock 2,5 $mg.m\ell^{-1}$ collagen solution reconstituted in acidified distilled water was added to 5,0 mℓ Tris-HCl buffer of varying molarity, containing 200 mM $CaCl_2$ and 60 $U.m\ell^{-1}$ individually weighed chromatographically purified collagenase. Collagen concentration of the final assay solution was varied by dilution of the stock collagen aliquot with distilled water. Final assay solution pH was adjusted to 7,6 units (if necessary), as previously described. Decrease in solution viscosity was monitored and initial gradients extracted from primary data as described.

#### 2.2.5.2 Synergistic collagen hydrolysis

The non-specific proteinases trypsin, alpha-chymotrypsin, thermolysin, protease and bromelain were prepared in 50 mM Tris HCl pH 7,6 containing 200 mM $CaCl_2$, at the following concentrations:

0; 0,75; 1,00; 1,50; 2,00; 2,75 and 3,00 $mg.m\ell^{-1}$

120 $U.ml^{-1}$ chromatographically purified collagenase was dissolved in 50 mM Tris-HCl pH7,6 buffer containing 200 mM $CaCl_2$. Denatured 2,0 $mg.m\ell^{-1}$ and 120 $U.m\ell^{-1}$ non-specific proteinases and collagenase stocks respectively, were prepared by immersion of enzyme aliquots in boiling water for 15 minutes. Enzyme solutions consisting of a 1:1 addition of 2,0 mℓ non-specific proteinase solution to stock collagenase were prepared, whilst the final assay solution was an equiproportional mix of 2,0 mℓ enzyme solution to a stock 2,0 $mg.m\ell^{-1}$ collagen solution reconstituted in 50 mM Tris-HCl pH 3. The pH was adjusted to 7,6 units as previously described. Loss of optical rotation was monitored by polarimetry at 436 nm and 20°C over a 24 h period. Controls for the synergistic hydrolysis of collagen solutions consisted of the following:

(a) Heat-denatured non-specific proteinases at a final assay concentration of 0,5 $mg.m\ell^{-1}$, in the presence of heat-denatured collagenase at a final assay concentration of 30 $U.m\ell^{-1}$.

(b) Heat-denatured non-specific proteinases in the presence of active collagenase (concentrations as in [a]).

(c) Active non-specific proteinases in the presence of heat-denatured collagenase (concentrations as in [a]).

(d) One hour pre-exposure of active collagenase (60 $U.m\ell^{-1}$) to active non-specific proteinases (1,0 $mg.m\ell^{-1}$) at 20°C, prior to the addition of an equiproportional collagen substrate volume.

Gradient extraction of initial linear portion of assay response was achieved by linear regression analysis on a Hewlett-Packard HP-85 programmable calculator. The percentage change in amplitude was calculated as described in Section 2.2.4.1.

2.2.5.3 Biocide inhibition of collagenase activity

The following concentration series of proprietory biocides/non-proprietory chemicals were prepared in 50 mM Tris-HCl buffer pH 7,6 containing 200 mM $CaCl_2$:

(a) STA - 0; 0,8; 1,0; 1,6; 3,0; 4,0 and 6,0 % (v/v)
(b) Boric acid - 0; 0,8; 1,6; 4,0 (m/v)
(c) Boric acid: STA in 1:1 admixture - 0; 0,8; 1,6; 2,0; 3,0; 4,0 and 6,0 % (sum of % m/v and % v/v)
(d) EDTA-disodium salt - 0; 25; 50; 100 and 200 mM

5,0 mℓ biocide stock containing 0,2 $mg.m\ell^{-1}$ crude collagenase (300 $U.mg^{-1}$ solid; individual weighings) was added to an equal volume of 2,0 $mg.m\ell^{-1}$ collagen stock reconstituted in 50 mM Tris-HCl pH 3. Adjustment of pH, if necessary, was brought about by NaOH pellet as described, or by careful droplet addition of 0,1 N HCl to the stirred solution. Care was taken not to allow the pH to drop below 6 or exceed 9 units, as clostridial collagenase may be irreversibly damaged beyond these levels (Mandl, 1972). Inhibition of collagenolytic activity was monitored by the polarimetric method at 20°C and 436 and 546 nm. It was necessary to record polarimetric readings at the higher wavelength, due to colour interference generated by the STA/collagen solutions. Gradient extraction of initial linear assay response curve was as previously described (Section 2.2.4.1.).

# 3. RESULTS AND DISCUSSION

## 3.1 Polarimetric collagenase assay development

### 3.1.1 Inhibition of fibrillogenesis

The development of an optical polarimetric collagenase assay utilising soluble collagen as the substrate, calls for the retention of collagen in soluble form at neutral pH and a temperature which is conducive to fibril formation. Collagen fibril formation is an endothermic process involving the formation of hydrophobic and electrostatic interactions between collagen molecules. Fibril formation can be affected by factors such as pH, perturbant ionic species, ionic concentration, presence of non-collagenous perturbants of high or low molecular weight, pressure and temperature, which may lead to the alteration of intermolecular interactions (Hayashi and Nagai, 1973 and 1974; Dombi and Halsall, 1985). The results of the experimental evaluation of three perturbants for their inherent ability to prevent collagen fibril formation at neutral pH and various temperatures, are summarised in Table 2.

Urea addition up to 150 mM failed to prevent collagen gelation. However, upon determination of the liquefaction temperature necessary to solubilize the resultant heat-gelled collagen, it was noted that increased urea concentrations generally decreased the temperature at which solubilisation took place. Urea is known to be a powerful lyotropic agent able to interrupt and interact with internal hydrogen and hydrophobic bonds respectively, thereby leading to irreversible disruption of native peptide-chain alignments (Veis, 1964). For instance, it is documented that concentrated urea solutions (>4,0M) will bring about a conversion of native collagen to gelatin at ambient temperatures (von Hippel and Wong, 1963), as well as inhibit helix formation in the gelatin-collagen transition (Russell and Cooper, 1969). The current data indicate that urea does indeed have a lyotropic effect on collagen, but that prevention of fibrillogenesis would only occur at

Table 2 Effect of perturbant type and concentration on fibrillogenesis

| Perturbant | Concentration (M) | Incubation temperature (°C) | Gelation (yes/no) | Dissolution temperature (°C) |
|---|---|---|---|---|
| Control | | 22<br>37 | yes<br>yes | 55,5 |
| Urea | 0,0125 | 22<br>37 | yes<br>yes | 55 |
| | 0,025 | 22<br>37 | yes<br>yes | 54,5 |
| | 0,0375 | 22<br>37 | yes<br>yes | 53,5 |
| | 0,05 | 22<br>37 | yes<br>yes | 52,5 |
| | 0,075 | 22<br>37 | yes<br>yes | 53 |
| | 0,1 | 22<br>37 | yes<br>yes | 52 |
| | 0,15 | 22<br>37 | yes<br>yes | 50 |
| Dextrose | 0,5 | 20 | no | N.D. |
| $CaCl_2$ | 0,01<br>0,02<br>0,1<br>0,5 | 20<br>20<br>20<br>20 | yes<br>yes<br>no<br>no | N.D.<br>N.D.<br>N.D.<br>N.D. |

NOTE: ND = not determined

a much higher urea concentration than practically desirable.

Fibrillogenesis was inhibited by 500mM dextrose, but further experiments were not carried out as dextrose contributed strongly to the optical activity of the collagen/dextrose solution. Hayashi and Nagai (1973) report complete inhibition of fibril formation by 1000 mM dextrose and state that collagenase activity was unaffected by this dextrose concentration.

$CaCl_2$ was found to be effective in preventing gelation at 500mM and 100mM concentrations, the latter being optimal for collagenase activity (Chakraborty and Chandra, 1989). Von Hippel and Wong (1963) found that 100mM $CaCl_2$ was effective in retaining collagen in solution; accordingly, further assay developmental work was carried out using a background 100mM $CaCl_2$ concentration.

3.1.2. Effect of temperature on assay response

Figure 3 summarises the results of preliminary polarimetric collagenase assay experiments in which the effect of assay temperature on assay response was gauged. It is clear from the graph that the higher assay temperature (25°C) results in a faster rate of optical rotation (OR) loss, due to enhanced enzymic activity at elevated temperatures.

Generally, a twofold increase in enzymic reaction rate may be expected for every 10°C temperature increase, but only if enzyme optimal temperature is not exceeded (Morris, 1970).

However, two factors prevented the selection of 25°C as the assay temperature of choice. These were:

(a) Rate of optical rotation loss at 25°C was very rapid. For example, the decrease in OR within an initial 1,5 h incubation period immediately after addition of 0,2 $mg.m\ell^{-1}$ collagenase to collagen solution was approximately 17 % (from -900° to -770°) at 20°C,

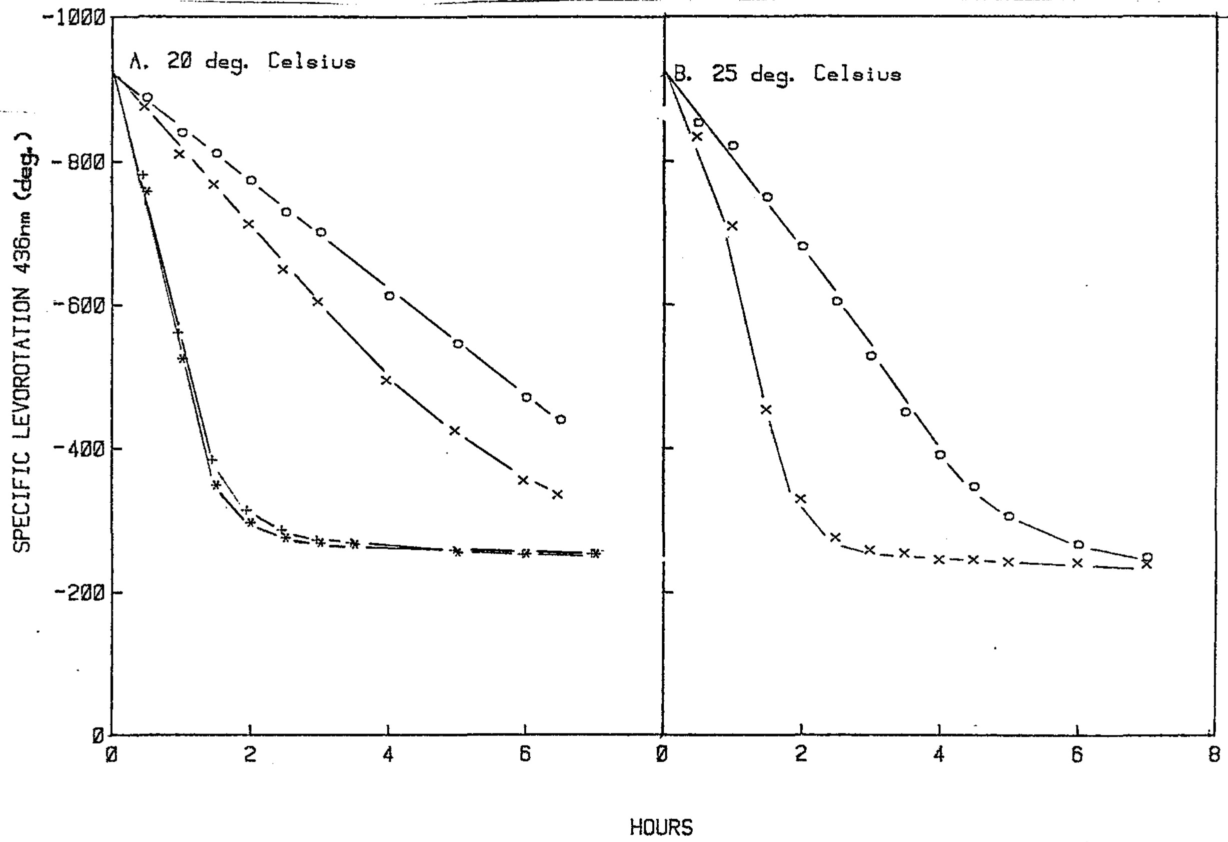


Figure 3 Effect of assay temperature on enzymic reaction rate

Key: 0 = 0,1 mg.mℓ$^{-1}$ crude collagenase
x = 0,2 mg.mℓ$^{-1}$ crude collagenase
+ = 0,3 mg.mℓ$^{-1}$ crude collagenase
* = 0,4 mg.mℓ$^{-1}$ crude collagenase

whilst the OR loss at 25°C was approximately 90 % for the corresponding period ( -900° to -470°), at 436 nm. OR values obtained for 0,3 and 0,4mg.mℓ$^{-1}$ collagenase have not been plotted as no distinction could be made between these concentrations; the OR decrease was complete within 0,5h.

(b) The 25°C assay temperature may approach a critical temperature range in which incipient denaturation and vulnerability of the collagen molecule to non-specific proteolytic attack could become more pronounced (Sakai and Gross, 1967). As the specificity of collagenase assays employing native collagen as substrate is dependent on the retention of intact, uncleaved molecules prior to enzymic hydrolysis, it is important to bear in mind that native collagen becomes increasingly susceptible to non-specific proteolysis within in a temperature interval extending from approximately 10°C below collagen denaturation temperature ($T_m$). This statement is supported by a large body of evidence which suggests that resistance of type I collagen to non-specific proteinases is a function of temperature and enzyme/collagen ratio (Birkedal-Hansen, 1987). Collagen-gelatin $T_m$-values in the literature range from approximately 37°C (Hayashi et al, 1979) to 39,8°C (Danielsen, 1984).

Therefore, subsequent assay developmental work was carried out at 20°C, where a more controlled reaction rate was obtained.

3.1.3 Effect of wavelength on assay response

Von Hippel and Harrington (1959) state that the specific rotation of an ichthyocol gelatin solution increased with decreasing wavelength. Figure 4 summarises similar results obtained with purified collagenase/native collagen solution optical activity measured at 365 and 436nm. Assay response at 365nm, gauged as a function of initial gradient (0,127° min$^{-1}$/U.mℓ$^{-1}$) was approximately 47% more sensitive than the response obtained at 436nm (0,067° min$^{-1}$/U.mℓ$^{-1}$).

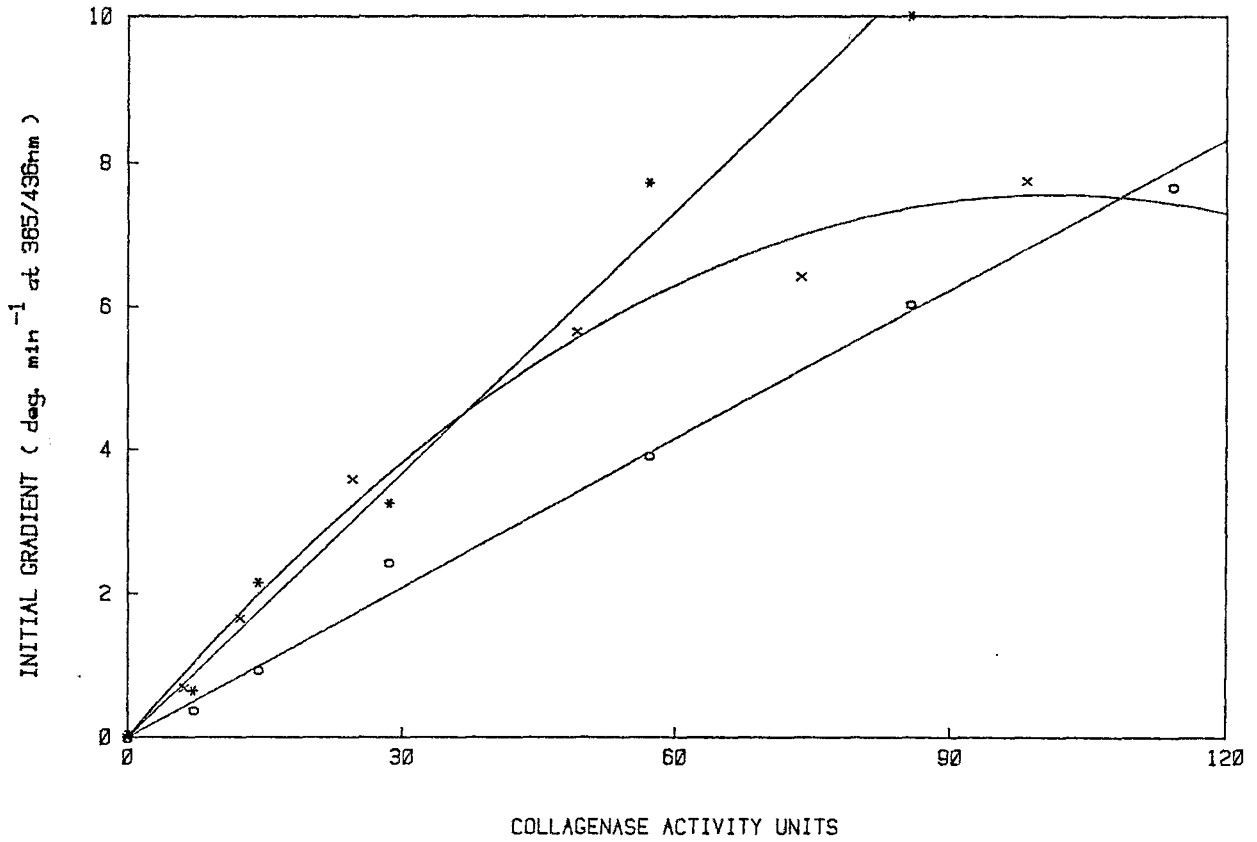


Figure 4 Effect of assay wavelength and degree of collagenase purity on initial gradients at 20°C

Key: * = purified collagenase at 365 nm
x = crude collagenase at 436 nm
o = purified collagenase at 436 nm

The increased sensitivity of assay response at 365nm was a disadvantage under certain circumstances, e.g. when the assay solution was strongly coloured. Assay response at 436nm was affected to a lesser extent by this factor, and the majority of assay developmental and application studies were performed at 436nm, unless otherwise stated (see Appendix for an explanation of the relationship between optical rotary values obtained at various wavelengths).

### 3.1.4 Effect of active and denatured collagenases on optical activity of the collagen molecule

#### 3.1.4.1 Active and denatured crude collagenase

The effect of various crude collagenase concentrations on the optical activity of collagen solutions is shown in Figure 5A. The change in optical rotation (OR) of a control collagen solution in the absence of added collagenase was monitored over a 24 hour period. A relatively small decrease in OR was noted (2 % decrease in OR over 24 hours). However, upon addition of active crude bacterial collagenase to collagen substrate, a marked decrease in optical activity was observed.

The decrease in optical activity may be attributed to two reactions occurring in solution. Firstly, the clostridial collagenase used throughout this study is known to cleave collagen at multiple sites (Keil, 1979), in contrast to vertebrate and _Achromobacter_ collagenase which have a single, specific cleavage site (Imhoff _et al_, 1990). Multiple cleavage of the collagen molecule by clostridial collagenase will result in the generation of smaller, helical polypeptides with only a limited initial loss of optical activity due to disruption of internal asymmetry in the collagen molecule (Seifter _et al_, 1958; Gross and Nagai, 1965; Stark and Kühn, 1968).

The second reaction occurs as a result of the inherently lower stability of short peptide fragments (Gross and Nagai, 1965; Stark and Kühn, 1968). Under the assay conditions adopted, these unstable peptides

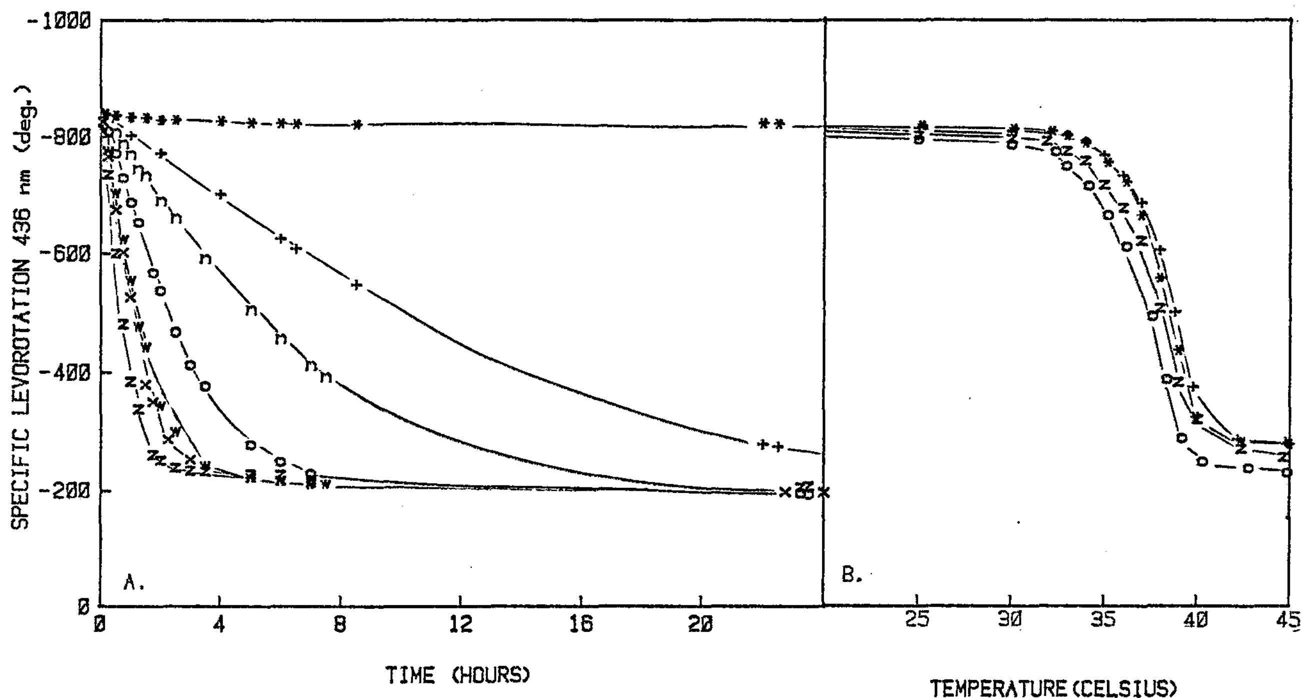


Figure 5 A Effect of active crude collagenase on optical rotation of a native collagen solution

B Effect of heat-denatured crude collagenase on thermal transition temperature native collagen solution

Key: * = 0 $U.m\ell^{-1}$ crude collagenase
+ = 6,14 $U.m\ell^{-1}$ crude collagenase
n = 12,29 $U.m\ell^{-1}$ crude collagenase
o = 24,57 $U.m\ell^{-1}$ crude collagenase
w = 49,14 $U.m\ell^{-1}$ crude collagenase
x = 73,71 $U.m\ell^{-1}$ crude collagenase
z = 98,28 $U.m\ell^{-1}$ crude collagenase

spontaneously undergo thermal unfolding (Seifter *et al*, 1958), thereby causing a large overall loss of optical activity in solution. The difference between initial OR values (representing 100 % helical content) and baseline OR values (0 % helix) in Figure 5A represents, by enzyme definition, the total substrate available for collagenase hydrolysis. The reaction rate at each collagenase concentration may be obtained from gradient values extracted from the initial linear portion of the response curve. The relative rate at which the highly ordered triple helix is converted to peptide fragments and random coils is dependent on collagenase concentration, with the rate of helix conversion increasing with increasing collagenase concentration (Figure 5A). Assay response decreases as substrate depletion becomes a factor, eventually reaching baseline OR values at which point the enzymatic reaction is complete.

Seifter *et al* (1958) followed the hydrolysis of ichthyocol (fish collagen) by measuring specific viscosity decrease. These authors found that log of specific viscosity decrease was a linear function of time of degradation and that the initial gradient was directly proportional to specific activity of the collagenase. Comparable graphical results are presented in Figure 6, where log of percentage change in amplitude (a measure of intact helical content) is proportional to both time of degradation and specific activity of crude clostridial collagenase. Harrington and von Hippel (1961), in their review of pH-stat kinetics of ichthyocol degradation, report that collagenase activity on this substrate may be categorised as the sum of two apparent first order reactions, one slow and the other faster. This two-step reaction rate was attributed to two types of collagenase-sensitive peptide bonds occuring in ichthyocol, with differing levels of cleavage susceptibility. Calfskin collagen hydrolysis does not, however, follow the two-step trend reported by Harrington and von Hippel (1961) (Figure 6). Differences in susceptibility to crude clostridial collagenase hydrolysis exhibited by calfskin and ichthyocol collagens were attributed by Mandl (1961) to the increased sensitivity of ichthyocol to contaminant proteinases.

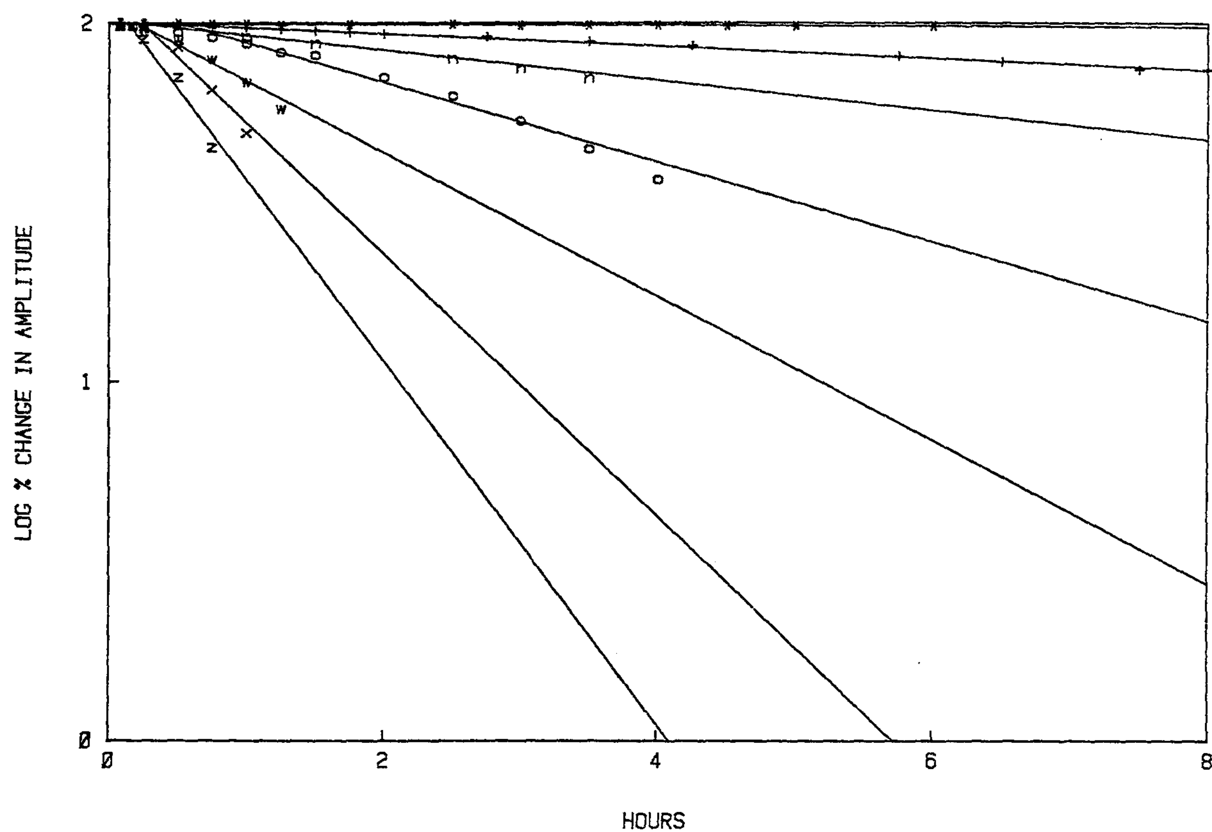


Figure 6 Logarithm of percentage change in collagen optical rotation amplitude

Key: * = 0 $U.m\ell^{-1}$ crude collagenase
+ = 6,14 $U.m\ell^{-1}$ crude collagenase
n = 12,29 $U.m\ell^{-1}$ crude collagenase
o = 24,59 $U.m\ell^{-1}$ crude collagenase
w = 49,14 $U.m\ell^{-1}$ crude collagenase
x = 73,71 $U.m\ell^{-1}$ crude collagenase
z = 98,28 $U.m\ell^{-1}$ crude collagenase

Figure 5B summarises results of thermal transition (melting curve) experiments in which the effect of increasing concentrations of heat-denatured collagenase on melting temperature, $T_m$, was assessed. $T_m$ serves as a measure of the stability of collagen in a given environment consisting of a solvent, the collagen solute capable of undergoing the helix-random coil transition and a third component. Variation in concentration of the third component, in this case heat-denatured collagenase, will result in the proportional displacement of the thermal transition along the temperature axis (von Hippel and Wong, 1963). Only selected trend-lines have been included, for purposes of graphical presentation. Generally, increased levels of denatured collagenase led to a progressive $T_m$ reduction ($T_m$-values decreased from 37,8°C to 35,5°C), indicating that a destabilising effect was operative. Such an effect could be attributed to competitive denatured enzyme binding to collagen, resulting in a lyotropic destabilisation of the collagen molecule.

3.1.4.2 Active and denatured purified collagenase

Since the crude collagenase preparation used in Section 3.1.4.1 is a first ammonium sulfate precipitate of Clostridium histolyticum culture supernatant and therefore contaminated with proteinases and other ill-defined compounds (Sigma catalogue, 1984), it was decided to verify the assay response using chromatographically purified clostridial collagenase. Figure 7A summarises results of these experiments; trends similar to those obtained with crude collagenase were noted. These results confirm that measurement of loss of optical rotation of a collagen solution was a valid basis for the development of a polarimetric collagenase assay.

Figure 7B illustrates that increased levels of denatured purified collagenase lowers thermal transition temperature $T_m$, as was the case with crude denatured collagenase.

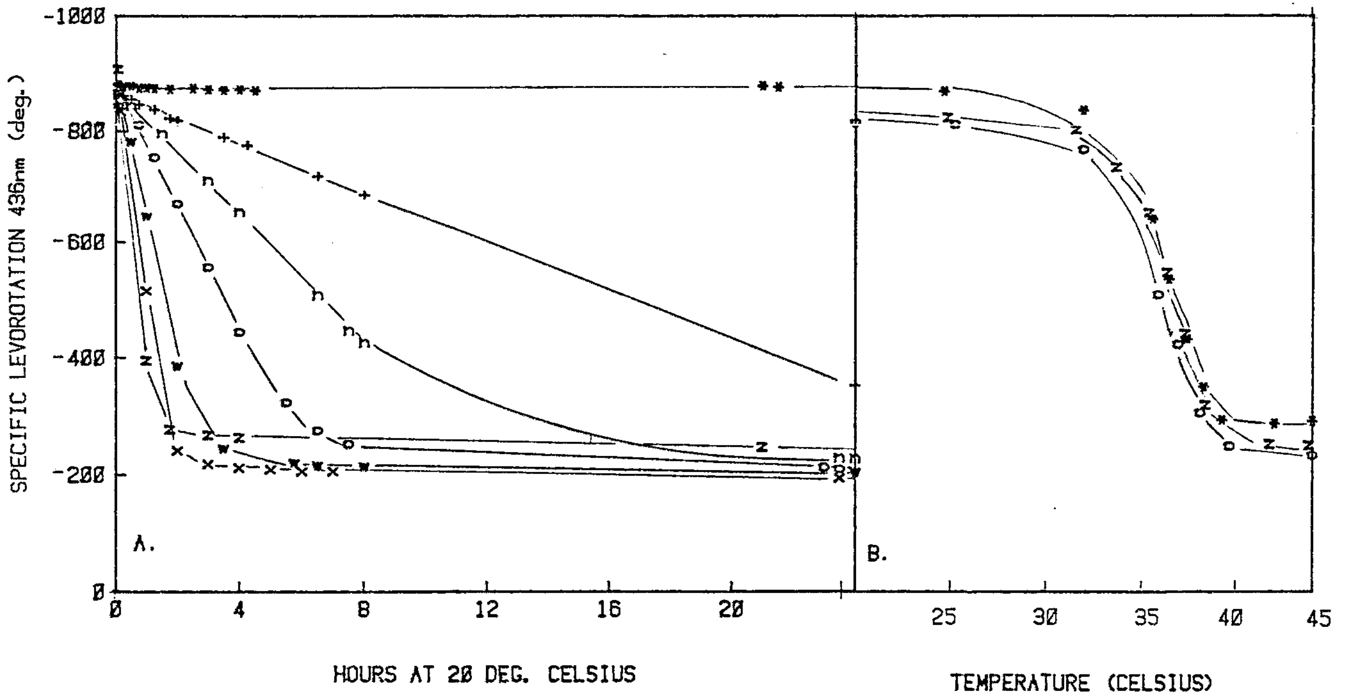


Figure 7 A Effect of active purified collagenase on optical rotation of a native collagen solution

B Effect of heat-denatured purified collagenase on thermal transition temperature of a native collagen solution

Key: * = 0 $U.m\ell^{-1}$ purified collagenase
+ = 7,14 $U.m\ell^{-1}$ purified collagenase
n = 14,30 $U.m\ell^{-1}$ purified collagenase
o = 28,60 $U.m\ell^{-1}$ purified collagenase
w = 57,1 $U.m\ell^{-1}$ purified collagenase
x = 85,70 $U.m\ell^{-1}$ purified collagenase
z = 114,3 $U.m\ell^{-1}$ purified collagenase

### 3.1.5 Relationship between initial optical rotation change and collagenase concentration

An enzymatic assay is useful for quantifying the amount of enzyme present or for kinetic studies only if initial rates vary linearly with enzyme concentrations (Mallya, 1988). The relationship between initial rate of change in OR, extracted by calculation of gradient of the initial linear portion of each response curve and crude or purified collagenase concentration, is shown in Figure 4.

The assay of purified collagenase resulted in a linear relationship between reaction rate and enzyme concentration, characteristic of first order reactions with respect to enzyme concentration (Holton *et al*, 1983; Mallya *et al*, 1986; Barrett *et al*, 1989). Crude collagenase, on the other hand, showed an initial linear relationship followed by a curvilinear response curve. This effect may be ascribed to the relative impurity of this enzymic preparation, resulting in the dark brown colour observed at higher collagenase concentrations, compared with the corresponding colourless purified collagenase solutions. Such strongly coloured solutions will result in some absorbance of a beam of polarized light, with concomitant loss in sensitivity of assay response.

Direct comparison of assay response between crude and purified collagenase highlights an interesting finding, namely that crude collagenase at lower concentrations appears to degrade collagen more rapidly than purified collagenase can. This may be deduced from the steeper initial gradient of crude collagenase in Figure 4. The crude collagenase used in this study contains contaminant neutral proteases, tryptic enzymes and clostripain (Sigma catalogue, 1984) and these enzymes may synergistically aid native collagen digestion in the presence of true collagenase. The synergistic hydrolysis of collagen leads to an apparent increase in the rate of collagen degradation compared with the rate obtained by purified collagenase alone (van Wart and Bond, 1982). Synergistic collagen hydrolysis was investigated further (Section 3.3.1).

### 3.1.6 Comparison between polarimetric and viscometric collagenase assays

The non-specific proteinases trypsin, alpha-chymotrypsin and protease were assayed for collagenolytic activity by the polarimetric and viscometric collagenase assay methods. Substrate and assay specificity was demonstrated by the finding that neither trypsin, nor alpha-chymotrypsin hydrolysed collagen when assayed by the polarimetric method, whereas collagenase completely destroyed the collagen triple helix (Figure 8A). Protease data are not shown as results similar to non-specific proteinases were obtained; these data have not been plotted for purposes of graph clarity. In contrast, the non-specific proteinases appear able to hydrolyse collagen to a limited extent when assayed by the viscometric method (Figure 8B).

Figure 8C and 8D show polarimetric and viscometric changes on a comparable percentage basis. It can be seen that the viscometric assay appears more sensitive to bond cleavage by collagenase, as evidenced by the steeper gradient and more rapid attainment of reaction completion (no further change in amplitude). A similar response was obtained by non-specific proteinases, although not to the extent noted for bacterial collagenase. The polarimetric collagenase assay (Figure 8C), in contrast, was characterised by a slower reaction rate when exposed to collagenase, whilst the non-specific proteinases appeared to have very little effect on assay response. The polarimetric collagenase assay therefore seems less sensitive than the viscometric assay.

However, the viscometric collagenase assay results represent a complex response and do not necessarily provide a reliable measure of actual reaction rate. Seifter et al (1958) monitored ichthyocol hydrolysis at 20°C by simultaneous polarimetric and viscometric assay methods and found that the rate of change of specific viscosity was greater than the rate of change of specific levorotation. These authors conclude that since viscosity is mainly a function of axial ratio of a molecule and optical rotation reflects spatial configuration, the enzymatic reaction

had resulted in the generation of polypeptides of relatively low axial ratio but with intact helices. This was supported by Stark and Kühn (1968) who proved by thermal denaturation temperature evaluation that the peptide fragments produced by collagenase hydrolysis of collagen have an intact triple helix. In addition, Gross and Nagai (1965) experimentally confirm an increased flexibility of the collagen rod-like molecule brought about by enzymic activity. The nett effect of the loss of collagen rigidity and polypeptide generation is a sharp drop in solution viscosity not accompanied by an equivalent gross destruction of the triple helix, this fact being reflected in the relatively small equivalent loss of optical activity (Harrington and von Hippel, 1961).

It is unlikely that the non-specific proteinases are hydrolysing the native collagen triple-helix as indicated by the viscometric assay method; cleavage of non-helical telopeptide fragments is the most acceptable explanation for the limited drop in solution viscosity shown in Figure 8B and D (Gross, 1976). As the non-helical telopeptides contribute very little to overall optical activity of the native collagen molecule, cleavage by proteinases would add little to the loss in OR of a collagen solution.

Thus, the viscometric collagenase assay primarily provides a measure of loss of higher order, tertiary collagen structure whereas the polarimetric assay method reflects destruction of the characteristic triple helical collagen conformation and is a closer indication of actual enzymic activity.

### 3.2. Kinetic Parameter Determination

The validity of the polarimetric collagenase assay as an alternative collagenase assay technique was assessed by determining the effect of collagen, collagenase and buffer concentration on kinetic parameters. $K_m$ and $V_{max}$ values under different reaction conditions were obtained by application of the Lineweaver-Burk equation:

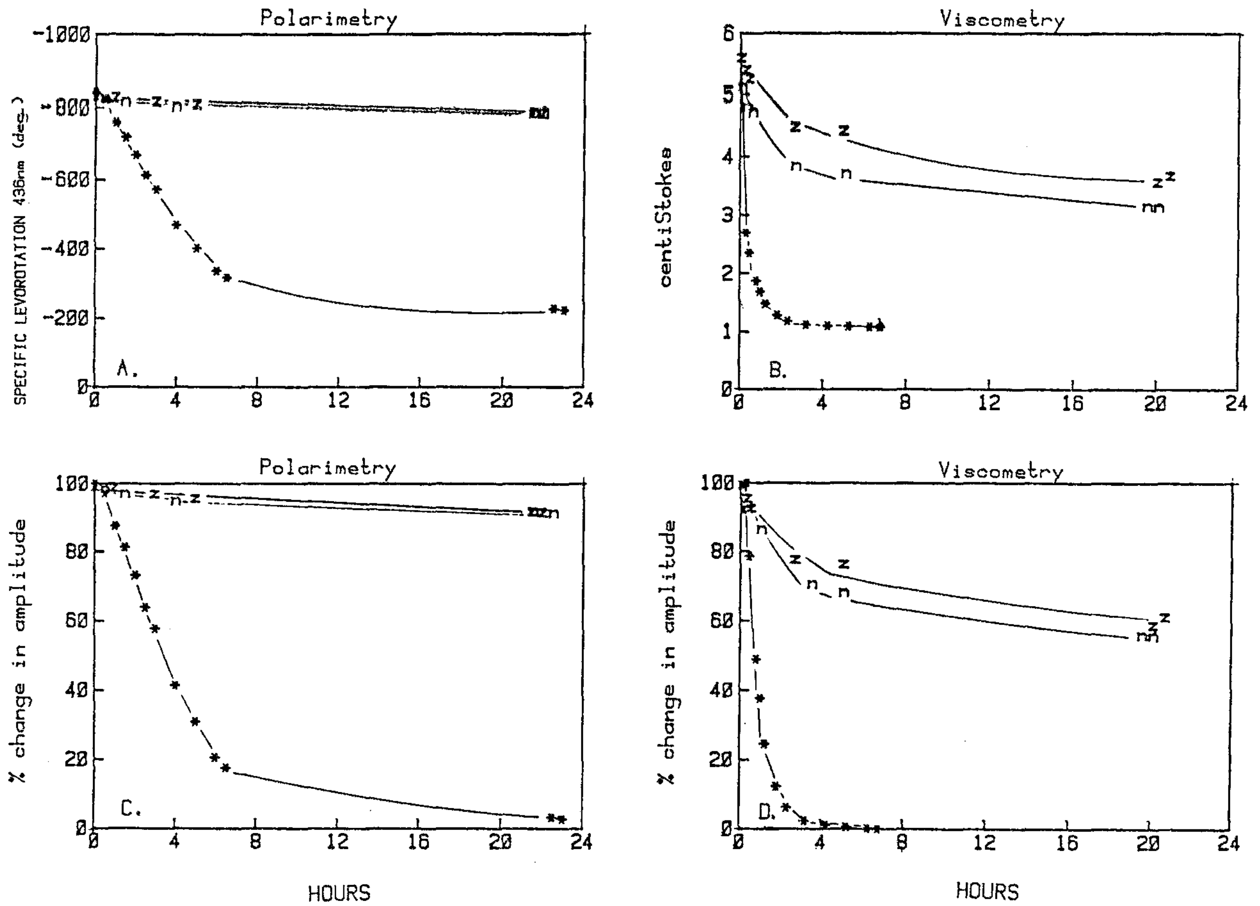


Figure 8 A, C Specificity of the polarimetric collagenase assay

B, D Specificity of the viscometric collagenase assay

Final proteinase and collagenase concentration - 0,2 mg.mℓ$^{-1}$

Key: *-* = collagenase
z-z = trypsin
n-n = alpha-chymotrypsin

$$\frac{1}{V_o} = \frac{1}{V_{max}} + \frac{K_m}{V_{max}} \times \frac{1}{[S]} \quad \text{where}$$

$V_o$ = reaction rate at $t_o$

[S] = substrate concentration

$V_{max}$ = maximum reaction rate in the presence of excess substrate

$K_m$ = Michaelis constant representing that concentration of substrate where $V_o = \frac{1}{2} V_{max}$ [41]

$K_m$ may be defined as the dissociation constant of the enzyme-substrate (ES) complex. In addition, $K_m$-values can serve as a measure of the affinity of an enzyme for its substrate, although this is not always the case as factors such as pH, buffer composition, ionic strength and temperature may all play a role in enzyme-substrate interaction. $V_{max}$-values reflect the fact that the decomposition of the ES-complex is considered rate-limiting (Morris,1970).

### 3.2.1 Polarimetry

#### 3.2.1.1 Crude collagenase

The results of an experiment performed at 436 nm with 30 enzyme activity units $.m\ell^{-1}$($U.m\ell^{-1}$) and 120 $U.m\ell^{-1}$ crude collagenase, in the presence of various collagen concentrations, are shown in Figure 9 and Table 4. The $K_m$-values of 4,3μM and 5,1μM respectively compare favourably with $K_m$-values in the literature (Table 3). The large range of $K_m$-values reported is indicative of the difficulties experienced with the accuracy of collagenase assay systems and the variety of assay methods used. $V_{max}$ values of 0,12 and 0,48°.minute$^{-1}$ respectively were obtained, reflecting proportionately enhanced ES-complex decomposition at the 120 $U.m\ell^{-1}$ collagenase concentration.

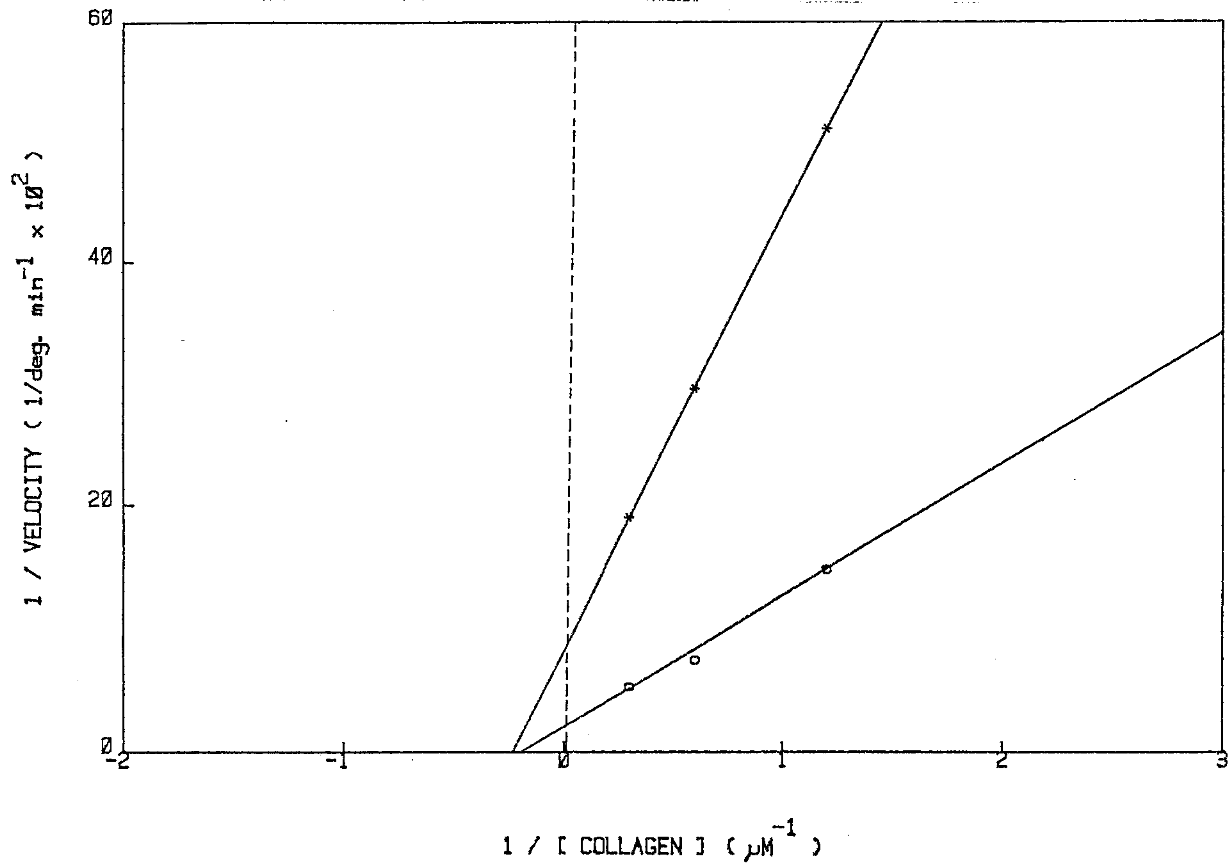


Figure 9 Polarimetric kinetic parameter determination by Lineweaver - Burk method

Key: * = 30 U.mℓ$^{-1}$ crude collagenase
0 = 120 U.mℓ$^{-1}$ crude collagenase

Table 3 $K_m$-values for clostridial collagenase determined by various assay methods

| $K_m$ | Substrate and Assay | Reference |
|---|---|---|
| 5,4 mM | Synthetic Pz-peptide. Spectrophotometric | Evans, 1981 |
| 0,5 mM | Synthetic FALGPA-peptide. Spectrophotometric | van Wart and Steinbrink, 1981 |
| 1,5 µM | Sirius red-dye release. Spectrophotometric | Hassanein *et al*, 1984 |
| 0,14-1,6 mM | Several synthetic peptides. Fluorometric, pH-stat, other spectrophotometric assays | Vencill *et al*, 1985 |
| 7,5 mM | Synthetic Pz-peptide. HPLC | Biondi *et al*, 1988 |
| 3,1-5,5 µM | Native collagen type I. Scintillation counting | Mallya, 1988 |
| 1,1 mM | Synthetic FALGPA-peptide. Spectrophotometric | Mookhtiar *et al*, 1988 |
| 17,0 µM | Synthetic Pz-peptide. Fluorometric | Barrett *et al*, 1989 |

Pz-peptide = Phenylazobenzyloxycarbonyl-Pro-Leu-Gly-Pro-D-Arg
FALGPA = 2-Furanacryloyl-L-Leucylglycyl-L-Prolyl-L-Alanine
HPLC = High Performance Liquid Chromatography

### 3.2.1.2 Purified collagenase

The experiment performed in Section 3.2.1 was repeated, using chromatographically purified clostridial collagenase (Table 4). Graphical results of $K_m$-determination are not presented as these were virtually superimposable on crude collagenase trends. The mean $K_m$ of 4,3μM compares favourably with the mean crude collagenase $K_m$ 4,7μM, suggesting that $K_m$ is independent of the degree of enzyme preparation purity and concentration. The 3,3 and 3,5 - fold proportional difference between gradients obtained at 30 $U.m\ell^{-1}$ and 120 $U.m\ell^{-1}$ enzyme concentration for crude and purified preparations respectively support this assumption.

Table 4 Polarimetric kinetic parameter determination with crude and purified collagenase

| Collagenase concentration ($U.m\ell^{-1}$) | $K_m$ (μM) | | $V_{max}$ ($^{\circ}.min^{-1}$) | | Gradient = $K_m/V_{max}$ (μM/$^{\circ}.min^{-1}$) | |
|---|---|---|---|---|---|---|
| | Crude | Purified | Crude | Purified | Crude | Purified |
| 30 | 4,3 | 4,4 | 0,12 | 0,13 | 35,7 | 35,7 |
| 120 | 5,1 | 4,1 | 0,48 | 0,40 | 10,7 | 10,2 |
| Mean | 4,7 | 4,3 | - | - | - | - |

### 3.2.2 Titrimetry

The pH-stat collagenase assay method does not monitor liberation of protons as a consequence of an "unmasking" of side-chain residues, but is considered a direct measure of peptide bond cleavage by collagenase (von Hippel and Harrington, 1959; von Hippel et al, 1960; Veis, 1964). The results of the titrimetric kinetic parameter determination with 30 and 60 $U.m\ell^{-1}$ collagenase in the absence of Tris-buffer are summarised

in Table 5. A 60 $U.m\ell^{-1}$ collagenase concentration was used instead of a final 120 $U.m\ell^{-1}$, as it was more convenient with this assay technique to avoid higher collagenase concentrations due to the amount of time needed to adjust the final pH. The mean $K_m$-value of 1,4 µM is lower than the mean $K_m$-values recorded with crude and purified collagenase using the polarimetric method (4,7 µM and 4,3 µM respectively), reflecting enhanced bond-cleavage detection by the titrimetric assay method and/or interference by the buffer component present in polarimetric and viscometric assay solutions.

Table 5 Titrimetric kinetic parameter determination with purified collagenase

| Collagenase concentration ($U.m\ell^{-1}$) | $K_m$ (µM) | $V_{max}$ (milli-equiv. $min^{-1}$) a | Gradient = $K_m/V_{max}$ (µM/milli-quiv. $min^{-1}$) |
|---|---|---|---|
| 30 | 1,7 | 0,030 | 56,6 |
| 60 | 1,1 | 0,056 | 19,4 |
| Mean | 1,4 | - | - |

(a) milli-equiv.$min^{-1}$ = milli-equivalents acid produced per minute

Although the mean $K_m$-value obtained by titrimetry is lower than those calculated for polarimetry they are of similar order of magnitude and not, for instance, 10-fold higher or lower. It is not possible to compare $V_{max}$ or gradient values generated by the two assay techniques, due to differences in the unit expression used.

### 3.2.3 Effect of buffer concentrations

During developmental work on a spectrophotometric collagenase assay, based on the hydrolysis of the 2-furanacryoyl-leu-gly-pro-ala peptide

(FALGPA), van Wart and Steinbrink (1981) found that the reaction was inhibited by Tris buffer above pH 7,5.

The slight difference between titrimetric and polarimetric $K_m$-values found in this study led to an investigation of the influence of Tris buffer on reaction kinetics in polarimetric and viscometric collagenase assays.

3.2.3.1 Polarimetry

The results of an experiment to determine the influence of Tris buffer concentration on kinetic parameters at a fixed crude collagenase concentration (30 $U.m\ell^{-1}$) and various collagen concentrations, are shown in Table 6.

The Km of 2,7 µM obtained in the presence of 50 mM Tris buffer (Table 6) is approximately 60% of that reported in Section 3.2.1 under identical assay conditions (Km = 4,3 µM). Mallya (1988) reports a Km of between 3,1 - 5,5 µM for native collagen hydrolysis by clostridal collagenases (Table 3), illustrating variation in parameter determination achieved by the same assay method under identical assay conditions.

Table 6 Effect of Tris buffer on collagenase kinetic parameters using the polarimetric assay

| Tris buffer concentration (mM) | $K_m$ (µM) | $V_{max}$ ($^o.min^{-1}$) | Gradient = $K_m/V_{max}$ (µM/$^o.min^{-1}$) |
|---|---|---|---|
| 12,5 | 3,8 | 0,095 | 40,3 |
| 25 | 2,4 | 0,059 | 40,4 |
| 50 | 2,7 | 0,051 | 52,9 |
| Mean | 3,0 | 0,068 | 44,5 |

The $K_m$-values listed in Table 6 were plotted on a graph of Tris concentration versus $1/K_m$ and the $K_m$-value at zero buffer was obtained by back-extrapolation to graph-axis intercept (Figure 10). A Km of 3,6 μM was obtained, approximately double the Km of 1,7 μM obtained by pH-stat with 30 $U.m\ell^{-1}$ purified collagenase in the absence of Tris (Section 3.2.2 Titrimetry). This discrepancy between Km-values obtained in the absence of Tris buffer may be ascribed to the use of different assay methods, since it has been shown in Section 3.2.1 that the degree of enzyme purity had little effect on Km-values.

Tris buffer has previously been shown to inhibit collagenase activity during synthetic substrate hydrolysis (van Wart and Steinbrink, 1981). It is therefore reasonable to assume that a similar effect may occur during native collagen hydrolysis. The data shown in Table 6 support this assumption. $V_{max}$-values decrease and gradient values increase with increasing Tris buffer concentration, whilst $K_m$-values do not alter markedly. The Lineweaver-Burk plot of this data (Figure 11) is characteristic of non-competitive inhibition of an enzyme-catalysed reaction. The Tris assay component, acting as an inhibitor, reacts reversibly with the enzyme at a site different from its collagen-binding site. The result is an inhibitor-enzyme (IE) complex, leading to the formation of an IES-complex which does not hydrolyse. Therefore the non-competitive Tris inhibitor reduces maximum velocity of the reaction and this cannot be reduced by an increase in substrate concentration (Morris, 1970).

In summary, it appears that the difference in $K_m$-values for 30 $U.m\ell^{-1}$ collagenase solutions, assessed by the polarimetric and titrimetric collagenase assays, may be ascribed to the use of two different assay methods as well as the presence of Tris buffer acting as a non-competitive inhibitor of enzyme activity in the polarimetric method.

3.2.3.2 <u>Viscometry</u>

Kinetic parameters were determined at a constant 30 $U.m\ell^{-1}$ collagenase

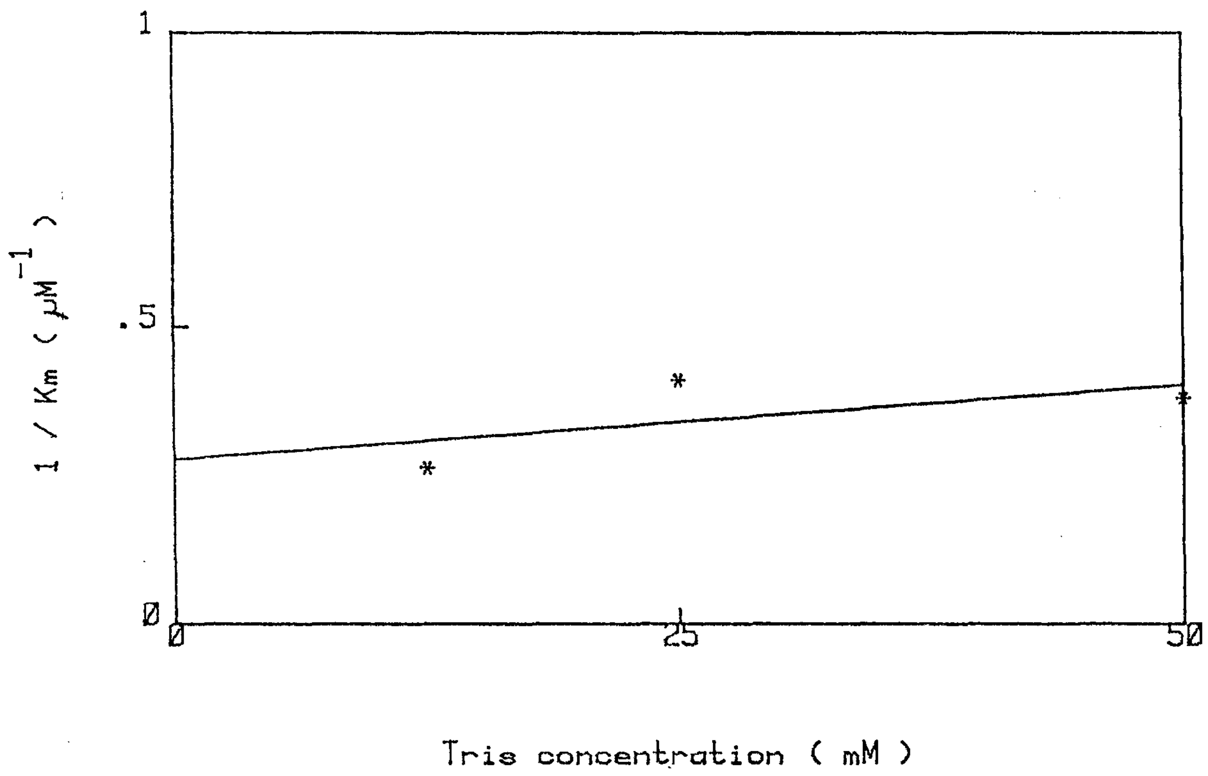


Figure 10 Influence of buffer concentration on $K_m$

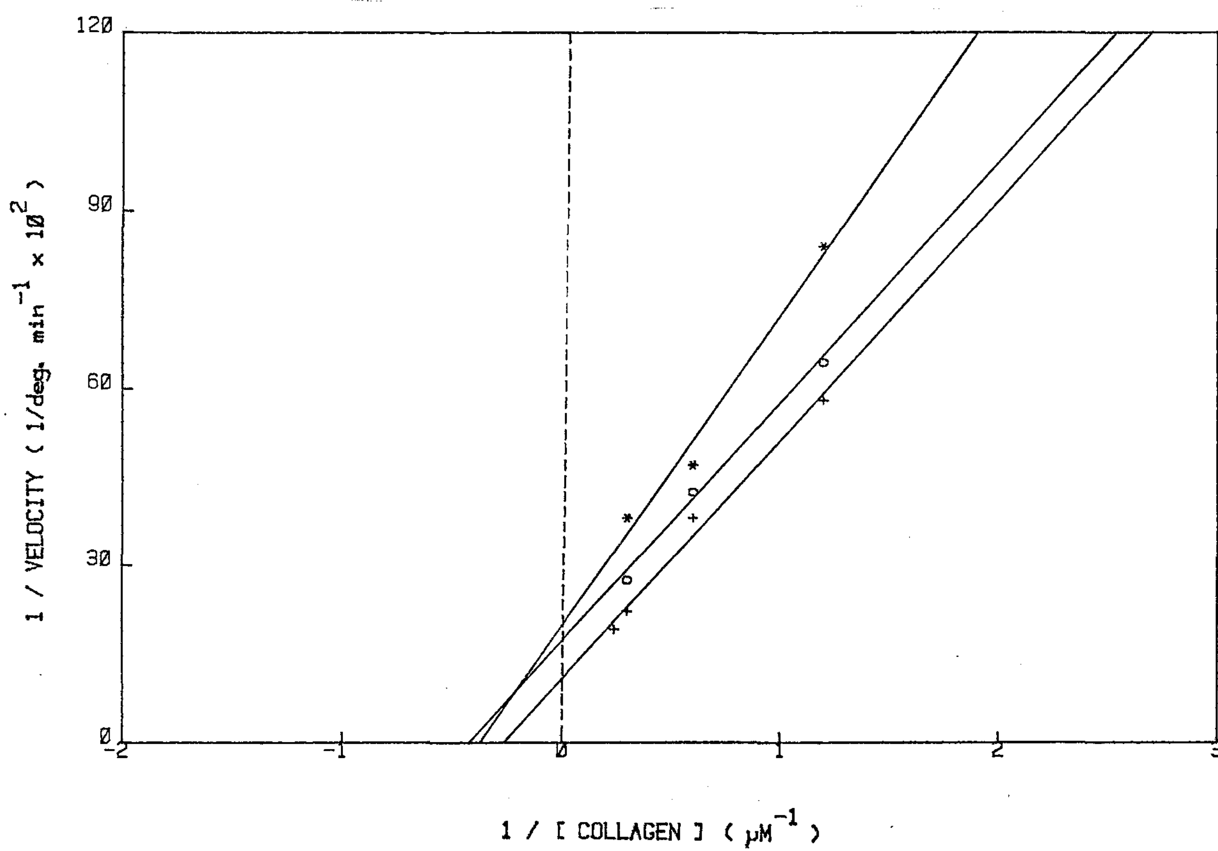


Figure 11 Effect of Tris buffer on the polarimetric collagenase assay

Key: + = 12,5 mM Tris
o = 25,0 mM Tris
* = 50,0 mM Tris

concentration and various Tris concentrations, using the viscometric assay (Table 7).

Table 7 Effect of Tris buffer on collagenase kinetic parameters using the viscometric assay

| Tris buffer concentration (mM) | $K_m$ (µM) | $V_{max}$ (centi Stokes.min$^{-1}$) | Gradient = $K_m/V_{max}$ (µM/centi Stokes.min$^{-1}$) |
|---|---|---|---|
| 12,5 | 2,7 | 0,32 | 8,4 |
| 25 | 2,4 | 0,17 | 14,4 |
| 50 | 3,1 | 0,18 | 17,0 |
| Mean | 2,7 | 0,22 | 13,3 |

Although the $V_{max}$-value in 25mM Tris buffer does not adhere to trends reported in Section 3.2.3.1, it is still possible to conclude the following from data presented in Table 7 ; there is a decline in $V_{max}$-values and an increase in gradient value with increasing Tris buffer concentration. The mean Km of 2,7 µM is comparable with that obtained by polarimetry (3,0 µM) and is approximately double the mean Km of 1,4 µM obtained in the absence of Tris buffer by titrimetry.

A graph of Tris concentration versus $1/K_m$ was drawn to obtain the $K_m$-value at zero buffer by back-extrapolation to graph axis intercept (not shown). A $K_m$ of 2,4 µM was obtained, approximately 1/3 more than the $K_m$ of 1,7 µM obtained by pH-stat and approximately 2/3 that determined graphically for the polarimetric assay ($K_m$ = 3,6 µM). These results confirm earlier conclusions reached on the role of the buffer component in appropriate assays and also serve to emphasise variations in kinetic parameter determination that will be achieved by different assay methods under identical conditions.

## 3.3 Assay applications. Collagenase activity studies

### 3.3.1 Synergistic activity

Under normal physiological conditions, the collagen molecule is highly resistant to non-specific hydrolysis. However, it has been suggested that more than one component may be involved in collagenolysis. Non-specific proteinases present in bacterial culture supernatants may synergistically assist true collagenases by hydrolysing collagen fragments produced by the specific enzyme (Mandl *et al*, 1964; van Wart and Bond, 1982; Bond and van Wart, 1984). Thompson (1988) postulates that green hide decay may be the result of a bacterial succession, in which non-specific proteinases secreted by non-collagenolytic bacteria could trigger collagenase production by certain bacterial species under appropriate environmental conditions. In addition, the non-specific proteinases would assist in the synergistic digestion of collagen.

Therefore, a group of non-specific proteinases, from diverse sources, was investigated for their ability to enhance native collagen digestion, as this could have important implications in green hide decay. As a result of differences in unit definitions and assay strategies, all experiments were performed with the non-specific proteinase concentrations defined on a mass-per-unit-volume basis.

#### 3.3.1.1 Influence of general proteinases on collagen

The results of an experiment to determine the effect of non-specific proteinases on the OR of a native collagen solution is shown in Figure 12.

It has previously been shown that alpha-chymotrypsin cleaves native type I collagen between telopeptide crosslink sites and the helical domain. As trypsin cannot cleave at this site, it is ideally suited as a control for non-specific attacks on the collagen helix. Bromelain, a plant thiol proteinase, was included as it has previously been shown capable

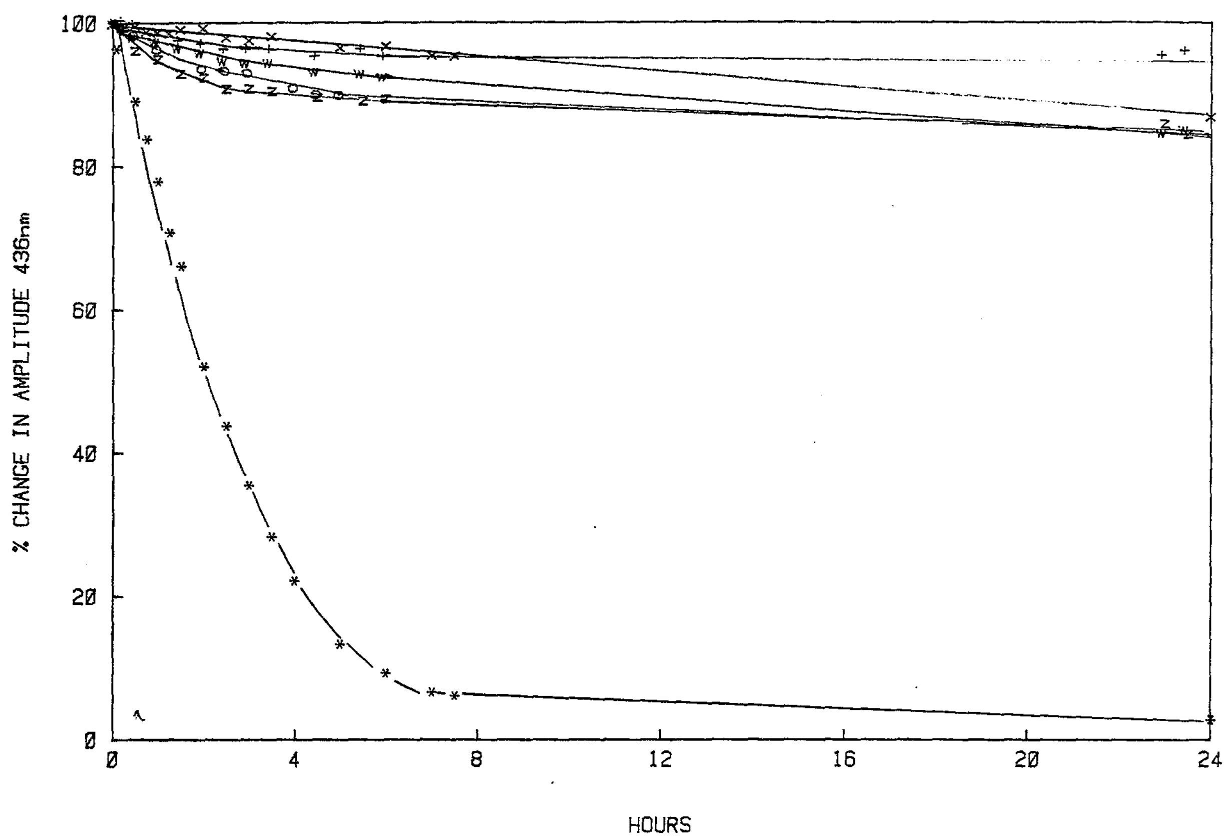


Figure 12 Effect of non-specific proteinases on collagen solution OR

Key: * = 30 U.mℓ$^{-1}$ collagenase control
x = thermolysin
o = bromelain
+ = alpha-chymotrypsin
w = protease
z = trypsin
Final proteinase concentration = 0,5 mg.mℓ$^{-1}$

of partially solubilising certain acid-insoluble collagen preparations (Mallya et al, 1986; Birkedal-Hansen, 1987).

The specificity of the substrate was confirmed, as only active collagenase (30 $U.m\ell^{-1}$) was able to destroy the triple helix completely. The slight drop in OR noted with non-specific proteinases over a 24 h period is probably due to telopeptide cleavage.

Van Wart and Bond (1982) found that 10 $\mu g.m\ell^{-1}$ thermolysin could cleave collagen to a limited extent at 35°C (initial rate 5,6 x $10^{-3}$ pKats), while 0,16 $\mu g.m\ell^{-1}$ collagenase generated an initial rate of 9,5 x $10^{-3}$ pKats. In addition, Birkedal-Hansen (1987) states in his review that 1,0 $mg.m\ell^{-1}$ thermolysin and protease (pronase) were capable of a 80 - 90 % reconstituted fibril (30 µg collagen) solubilisation at 35°C. Under present assay conditions, however, thermolysin appeared unable to hydrolyse collagen at lower temperature than optimal for thermolysin activity.

### 3.3.1.2 Prior collagenase exposure to general proteinases

Collagenase itself is a protein and, as such, is susceptible to proteinase cleavage with partial or complete loss of proteolytic activity. The results of an experiment to determine the effect of non-specific proteinases on active collagenase, prior to the introduction of native collagen substrate is shown in Figure 13.

Collagenase pre-exposure to protease resulted in a marked drop in collagenolytic activity. The protease preparation, recovered from *Streptomyces griseus* culture supernatant, is described as an "unusually non-specific protease ..." (Sigma catalogue, 1984) and it is reasonable to expect that exposure of collagenase to an ill-defined proteinase preparation will result in loss of collagenolytic activity. The results obtained with thermolysin are in contrast to those reported by van Wart and Bond (1982). They found that pre-exposure of 0,16 $\mu g.m\ell^{-1}$ collagenase to 1,0 $\mu g.m\ell^{-1}$ thermolysin over a 24 h period led to no

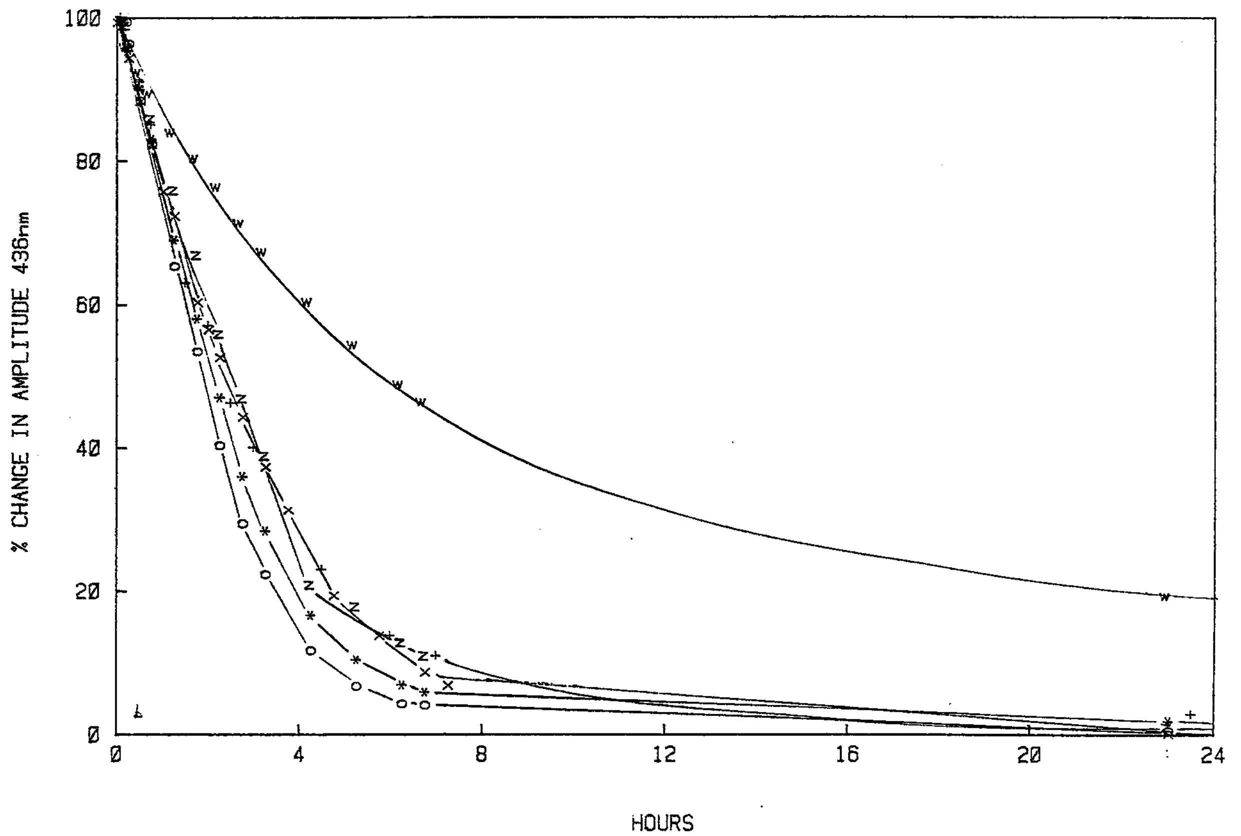


Figure 13 Effect of 1h pre-exposure of collagenase to non-specific proteinases

Final assay concentrations: collagenase 30 U.mℓ$^{-1}$ and proteinases 0,5 mg.mℓ$^{-1}$

Key: * = 30 U.mℓ$^{-1}$ collagenase control
x = thermolysin
o = bromelain
+ = alpha-chymotrypsin
w = protease
z = trypsin

detectable loss of collagenase activity. The present study indicates that the exposure of roughly 0,033 mg.mℓ$^{-1}$ collagenase (60 U.mℓ$^{-1}$) to 1,0 mg.mℓ$^{-1}$ thermolysin, alpha-chymotrypsin and trypsin for 1 h, led to a slight reduction in collagenase activity. Bromelain is the only non-specific proteinase that does not affect collagenase under these experimental conditions.

3.3.1.3 Combined proteinase/collagenase activity

The results of the investigation of synergism are presented in Figure 14. Protease and alpha-chymotrypsin inhibit collagenolytic activity, probably due to proteolytic degradation of the collagenase molecule. Ishii *et al* (1988) could not detect synergistic digestion of collagen with pronase (recovered from *Streptomyces griseus*) and collagenase. Bromelain did not appear to inhibit nor enhance collagenolytic activity markedly.

However, both trypsin and thermolysin enhanced collagen digestion, when added to substrate in conjunction with collagenase. Trypsin and thermolysin probably assist in the hydrolysis of small collagen fragments produced by collagenase, thereby increasing the apparent collagenase activity. The synergistic hydrolysis of collagen can more accurately reflect collagenase activity and accounts for the apparent decrease in collagenolytic activity noted for purified collagenase solutions (van Wart and Bond, 1982; Birkedal-Hansen, 1987).

Van Wart and Bond (1982) report a synergistic ratio of 4,6 for thermolysin at 35°C. The synergistic ratio is defined by these authors as "... the initial rate in the presence of both collagenase and the second proteinase divided by the sum of the rates obtained for each alone." This ratio is a characteristic of a collagenase and a second proteinase and will be influenced by extrinsic factors (pH, temperature and enzyme purity) as well as the intrinsic ability of a collagenase to cleave collagen into fragments suitable for hydrolysis by the second proteinase. According to the above-mentioned definition, the

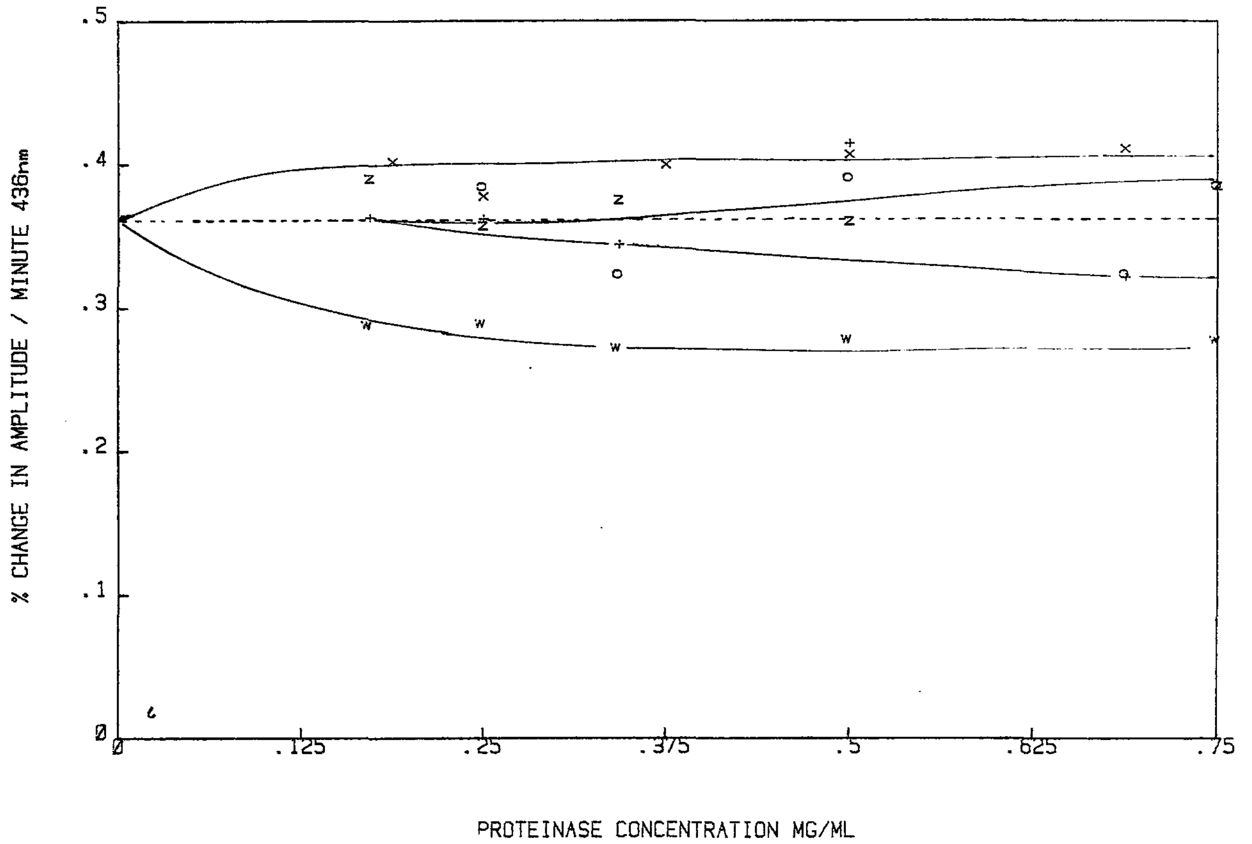


Figure 14 Effect of various concentrations of non-specific proteinases on initial gradients

Key: --- = 30 $U.m\ell^{-1}$ collagenase gradient value
x = thermolysin
o = bromelain
+ = alpha-chymotrypsin
w = protease
z = trypsin

polarimetric synergistic ratio may be calculated as follows:

(a) Initial gradient (rate) of active 30 U.mℓ$^{-1}$ purified collagenase = 0,361 % change in amplitude/minute.

(b) Initial gradient (rate) of active 0,5 mg.mℓ$^{-1}$ thermolysin in the presence of denatured 30 U.mℓ$^{-1}$ collagenase = 0,018 % change in amplitude/minute.

(c) Initial gradient (rate) of both active collagenase and thermolysin (30 U.mℓ$^{-1}$ and 0,5 mg.mℓ$^{-1}$ respectively) = 0,409 % change in amplitude/minute.

$$\text{Polarimetric synergistic ratio} = \frac{c}{a + b}$$

$$= 1{,}08$$

A contributory factor responsible for the difference in reported synergistic ratios may be temperature. Van Wart and Bond (1982) investigated the role of temperature and found that thermolysin had little effect on initial hydrolysis rates in the presence of collagenase at 25°C (synergistic ratio roughly 1,0), but as the temperature was raised to 35°C the synergistic ratio peaked at 4,6. The higher temperature is closer to the thermolysin optimal temperature at which the denatured collagen fragments will rapidly be hydrolysed, but it also is within a critical temperature range in which the intact collagen triple helix may become more susceptible to non-specific proteolytic attack (Birkedal-Hansen, 1987).

3.3.2 <u>Biocide inhibition of collagenolytic activity</u>

Laboratory investigations on the inhibition of collagenolytic activity have revealed that the collagenase molecule may be inhibited by numerous compounds, ions and metal sequestrants. In order to demonstrate the potential of the polarimetric method as an alternative collagenase

assay, it was decided to investigate a related area of interest in the leather industry, namely chemical short-term hide preservation using biocides. "Chemical curing" of green hides by application of proprietory biocides and non-proprietory chemicals has rapidly gained momentum as a means of minimising dependence on the environmentally less acceptable traditional salt curing with its attendant effluent problems (Russell et al, 1982).

The effect of certain selected biocides and non-proprietory chemicals on the proteolytic activity of a collagenase solution was examined, as this may be a factor of some importance in the assessment of biocide suitability for selection as a chemical preservative agent. The results of such an experiment are summarised in Figure 15. Boric acid, the organo-sulphur biocide STA (proprietory name) and a boric acid:STA admixture all resulted in loss of hydrolytic activity by a collagenase solution, as seen by the decrease in initial gradients. It is interesting to note that the boric acid:STA admixture had the most detrimental effect on collagenolytic activity. This result parallels technical trial results in which boric acid:biocide admixture application to green hide resulted in increased storage potential, when compared with single biocide application (Russell and Kohl, 1988). Such potentiation (or synergistic) effects obtained with boric acid:biocide admixtures commonly occur with the organo-sulphur biocide type (Russell and Kohl, 1988), but the precise mechanism of potentiation is as yet unexplained. The reversible inhibition of collagenase activity by $Ca^{++}$ removal by the metal sequestrant EDTA has been well-documented (Mandl, 1972).

This application of the polarimetric collagenase assay emphasised certain potential limitations of the optical assay method. The organo-sulphur biocide STA is a strongly-coloured liquid which partially precipitates on addition of boric acid, thereby hindering optical activity measurement of the turbid solution until the precipitate had settled. In addition, the yellowish-brown colour of the STA/collagenase solution necessitated OR readings to be carried out at 546 nm. Optical

rotation measurement response at 436 nm was erratic due to polarized light scatter; this situation was somewhat improved at 546 nm. The polarimetric assay is thus sensitive to turbidity of test solutions, as well as to the use of strongly-coloured solutions which should be avoided as far as possible.

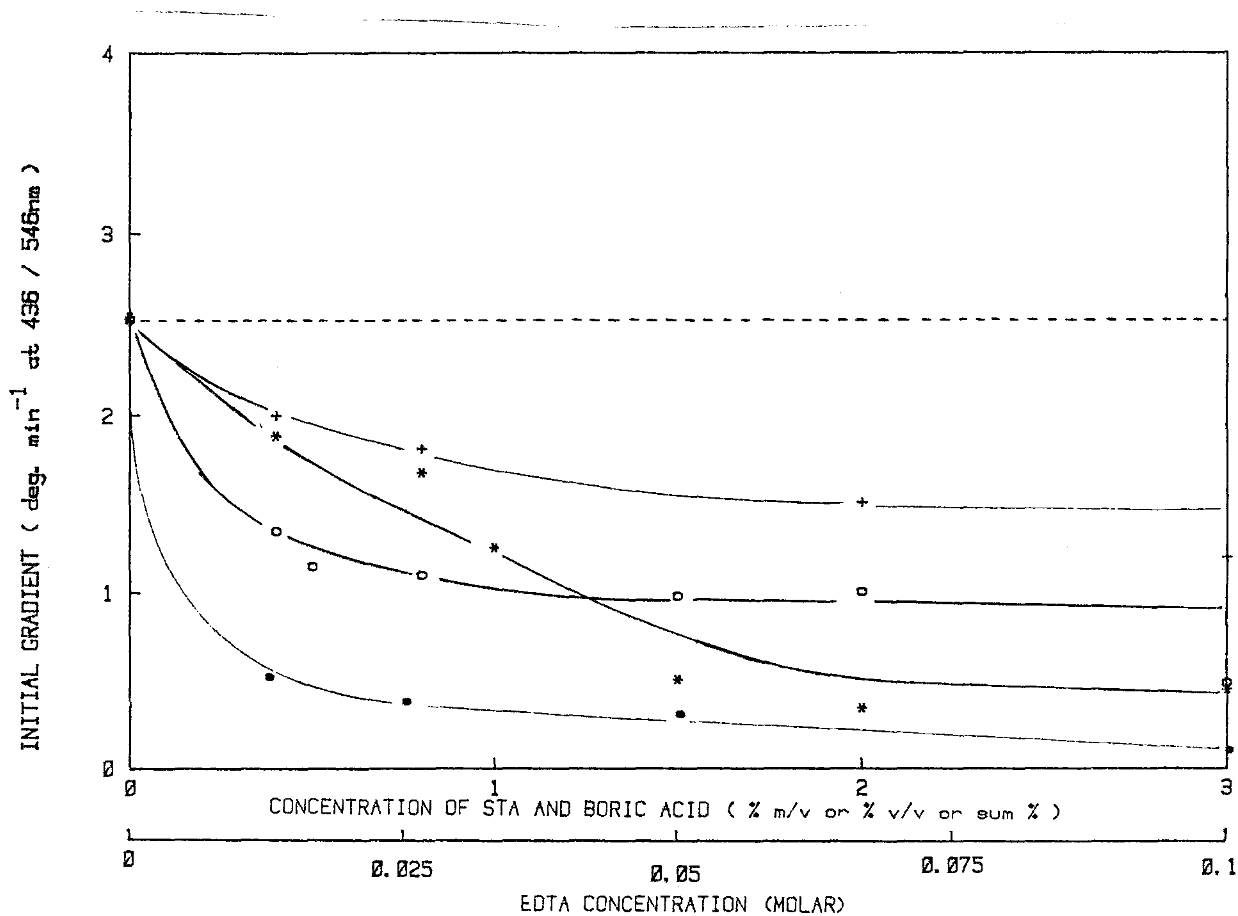


Figure 15 Effect of various concentrations of proprietory biocides and non-proprietory chemicals on collagenolytic activity

Key: --- = 0,1 mg.mℓ$^{-1}$ collagenase control
* = boric acid : STA in 1:1 admixture
o = STA alone
● = EDTA
+ = boric acid

## 4. CONCLUSIONS

The polarimetric assay method described in this study provides an alternative, specific and direct approach to the detection and measurement of collagenase activity in solutions of native collagen. In the past, collagenase assays have relied upon the use of various dubious substrates, such as gelatin, or chemically-modified collagens such as azocoll. In addition, reliance was placed on the hydrolysis of synthetic peptides as an indication of collagenolytic activity. Limitations of most of these methods have been demonstrated and the logical conclusion was that the most appropriate substrate to assess collagenolytic activity was native collagen itself.

The earliest collagenase assays employing native collagen as substrate were based on the hydrolysis and liquefaction of insoluble fibrous collagen. The discovery and description of ions and more complex molecules capable of retarding collagen fibrillogenesis created new possibilities for the development of a collagenase assay utilizing soluble native collagen as a substrate. Such an assay system was important for more fundamental research into the mechanism of collagen degradation, as soluble assays may be monitored continuously.

The collagen molecule in solution has numerous hydrodynamic and other interesting properties, the most important of which for purposes of this study, is its ability to rotate a beam of polarised light. The inherent degree of optical rotation depends on the integrity of the molecule; should the structure of the collagen molecule be damaged in any way (for example by heat or exposure to collagenase), the degree of rotation will decrease. It is this principle which was exploited in the present study.

The polarimetric collagenase assay offers a number of advantages when compared with other assay methods. Some optical methods are reliant on separate multi-step colorimetric determinations of degradation products and thus can only offer discontinuous measurement of collagen

degradation. Polarimetry permits continous monitoring of changes in solution. In addition, conditions in the present study were specifically chosen to enhance assay response by promoting spontaneous unwinding of cleavage products. The detection and quantification of collagenolytic activity in solutions containing collagenase can be carried out rapidly as all that is required is an initial linear gradient value from which a quantification of collagenase activity may be obtained by means of a standard graph. The method was shown to be specific when exposed to general proteinases from diverse sources. The polarimetric response was found to offer a direct measure of triple-helix destruction, with a sensitivity comparable to that of the absolute pH-stat (titrimetric assay) method. However, the absence of Tris buffer in the titrimetric method was a disadvantage as it called for additional adjustment of substrate pH to neutrality, prior to and immediately following collagenase addition. The viscometric assay method was found to represent a more complex reaction as it reflects destruction of higher order collagen structure as well as destruction of the triple helix itself.

The polarimetric assay method also offers advantages over radiometric procedures dependent on collagen substrate modification during radiolabeling, which may alter substrate susceptibility to enzymic hydrolysis. Nevertheless, sensitive radiometric collagenase assays have won widespread acceptance in the medical and scientific fields and must be seen as method equivalent to the polarimetric technique in terms of ease of operation and sensitivity of response. Radiometric procedures do, however, call for the need to work with expensive and potentially hazardous radio-isotopes and the continuous polarimetric measurement of intact residual collagen obviates the necessity to remove unreacted substrate as required by some radiometric methods. Therefore, a potential source of experimental error has been eliminated from the polarimetric method.

A disadvantage of the polarimetric collagenase assay is its loss in sensitivity due to the use of strongly-coloured solutions or to protein

precipitation in the assay solution. However, it can be said that this, in general, is a limitation of optical assay methods utilizing proteins in solution. This potential problem may be circumvented to a certain extent by using longer wavelengths for monitoring loss of optical rotation in coloured assay solutions; however, such solutions and solutes should be avoided as far as possible.

The potential of the polarimetric assay method has been demonstrated in the present study, by the practical application of the technique to two areas of interest in leather technology. The synergistic action in collagen hydrolysis by non-specific proteinases and collagenase has provided further insight into the mechanisms of hide biodegradation. Fundamental research into the mechanisms of hide decay is presently being carried out in these laboratories and the polarimetric assay method will be employed in this research to the benefit of the industry. Inhibition of collagenolytic activity by chemical preservatives (biocides) has to date been under-emphasised. More research is at present being undertaken into this aspect of raw-hide preservation.

# 5. APPENDIX

## 1. Optical rotation dispersion

The effect of various wavelengths on optical activity measurements was assessed as follows: an approximately 1,0 $mg.m\ell^{-1}$ native collagen solution was prepared in 50 mM Tris-HCl buffer pH 7,6 containing 100mM $CaCl_2$ and was transferred as rapidly as possible to a jacketted polarimetric cell of 1dm light-path length, whose temperature was kept at a constant 20°C (+/- 0,2°C) by means of water circulation through a Colora Ultra-Thermostat NB-34778 waterbath. Absolute optical rotation values were obtained at 365, 436, 546 and 578nm wavelengths. Table A1 summarises the results of specific levorotation measurement over a 16 hour period.

Table A1 Specific levorotation of an approximately 1,0 $mg.m\ell^{-1}$ native collagen solution at various wavelengths

| Time (hours) | Specific levorotation[b] at wavelength (nm)[a] 365 | 436 | 546 | 578 |
|---|---|---|---|---|
| 0,08 | -1412 | -883 | -511 | -449 |
| 0,15 | -1412 | -884 | -509 | -447 |
| 16,0 | -1382 | -865 | -500 | -435 |
| Mean | -1402 | -877 | -507 | -444 |
| Factor[c] | - | 1,599 | 2,765 | 3,158 |

(a) Based on $[\alpha]^{15}_{365}$ = -1330,56° for a 1,0 $mg.m\ell^{-1}$ native collagen solution (Russell, 1973; Kanfer, 1977).

(b) Reported value is the mean of duplicate measurements.

(c) Conversion factor to 365 nm.

A graph of wavelength versus conversion factor indicates that a linear relationship exists between the conversion factor and the wavelength of measurement (not shown). This implies proportionality between wavelengths, so that optical activity values obtained by necessity at a less sensitive, higher wavelength (due, for example, to colour interference) may be related back to the more sensitive, shorter wavelengths.

2. Polarimetric verification of stock collagen concentration

Stock collagen solution concentrations were determined by polarimetry at 365 nm, using the following as a guideline:

$[\alpha]^{15}_{365}$ = -1330,56° for a 1,0 mg.ml$^{-1}$ native collagen solution (Russell, 1973; Kanfer, 1977)

Procedure

2,0 ml of an approximately 2,0 mg.ml$^{-1}$ reconstituted collagen was added to an equal amount of Tris -HCl buffer pH 7,6 of appropriate molarity and containing 200 mM $CaCl_2$, thus compensating for the redissolution of collagen in the presence or absence of buffer (Section 2.2.1.). Triplicate specific levorotation determinations were performed at 20°C and the mean value was used in the following calculation:

$$V_1 \times C_1 = V_2 \times C_2 \text{ where}$$
$$V_1;\ V_2 = 4,0 \text{ ml}$$
$$C_2 = 1,0 \text{ mg.ml}^{-1}$$

Therefore $C_1$ = mean levorotation in degrees/1330,56°x 1,0 mg.ml$^{-1}$

Since the true stock collagen concentration could now be calculated, dilution of stock to an absolute 2,0 mg.ml$^{-1}$ concentration required the addition of an appropriate volume of either acidified distilled water or Tris -HCl buffer of correct molarity. Stock collagen concentration after dilution was verified in triplicate as described above.

The procedure used for the nominal 2,5 mg.ml$^{-1}$ collagen stocks was as described, except that 1,6 ml of the collagen stock was made up to 2,0 ml volume by the addition of an appropriate diluent, prior to polarimetric verification of specific levorotation at 365 nm.

3. Collagen concentration expressed in μM

The collagen molecule has a molecular weight of 300 000 (Rubin *et al*, 1965, Dombi and Halsall, 1985). Therefore, a 1mg.mℓ$^{-1}$ collagen solution is equivalent to 3,33 μM. Collagen concentration in assay solution for kinetic parameter determinations was expressed as shown in Table A2.

Table A2 Collagen concentration expressed in μM

| Collagen concentration mg.mℓ-1 | Collagen concentration μM | 1/[collagen] μM$^{-1}$ |
|---|---|---|
| 0 | - | - |
| 0,125 | 0,42 | 2,38 |
| 0,250 | 0,83 | 1,20 |
| 0,375 | 1,25 | 0,80 |
| 0,500 | 1,67 | 0,60 |
| 1,000 | 3,33 | 0,30 |
| 1,250 | 4,17 | 0,24 |

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