Observations on the Different Substrate Behaviour of Tropocollagen Molecules in Solution and Intermolecularly Cross-Linked Tropocollagen within Insoluble Polymeric Collagen Fibrils

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Bacterial collagenase was used to compare the extent of digestion of tropocollagen monomers in solution and in reconstituted fibrils with that of tropocollagen molecules intermolecularly cross-linked within insoluble polymeric collagen fibrils obtained from mature tendons at given time-intervals. The extent of digestion of tropocollagen monomers in solution was directly proportional to the enzyme concentration (a range of enzyme substrate molar ratios 1:200 to 1:10 was used). The extent of digestion of polymeric collagen was followed by measuring the solubilization of fluorescent peptides from fluorescent-labelled insoluble polymeric collagen fibrils. The extent of digestion of tropocollagen within polymeric collagen was linear over a very small range of enzyme concentrations, when the enzyme/substrate ratio in the reaction mixture was less than 1:400 on a molecular basis. The behaviour of tropocollagen in the form of reconstituted collagen fibrils, which had been matured at 37°C for 8 weeks, was intermediate between the behaviour of solutions of tropocollagen and insoluble polymeric collagen fibrils. The significance of the results is discussed in terms of the structure of polymeric collagen fibrils and the protection against enzymic attack provided by tropocollagen molecules on the circumference of the fibril. The results suggest that assays of collagenase activities based on tropocollagen as substrate cannot be directly related to the ability of these enzymes to degrade mature insoluble collagen fibrils.

The assay procedures for bacterial and mammalian collagenase depend on the use of tropocollagen molecules as the substrate, whether these be in the form of (a) true solution (viscosity changes; Gallop *et al.*, 1957), (b) the prevention of collagen-fibril formation from solutions of tropocollagen (Nagai *et al.*, 1966) or (c) the solubilization of ¹⁴C-labelled peptides from ¹⁴C-labelled reconstituted collagen fibrils (Nagai *et al.*, 1966). The purpose of the present paper is to draw attention to the fact that tropocollagen molecules in solution behave quite differently from tropocollagen molecules that are intermolecularly cross-linked in polymeric collagen fibrils, when these two forms of collagen are compared as substrates for collagenase. This difference in substrate behaviour is related to the maturation of collagen fibrils. An explanation for this difference in substrate behaviour is proposed on the basis of the structures of tropocollagen and intermolecularly cross-linked tropocollagen within the polymeric collagen fibrils.

Mature connective tissue consists of 1–2% of extractable tropocollagen and 98% of insoluble collagen bundles, composed of polymeric collagen fibrils (Steven & Jackson, 1967). Bacterial collagenase

has been used in this study to illustrate the difference in substrate behaviour between tropocollagen and polymeric collagen fibrils. The bacterial enzyme was chosen because of its commercial availability compared with mammalian collagenase. Results from studies with crude preparations of mammalian collagenase are similar to those obtained with bacterial collagenase (F. Steven, unpublished work). Highly purified bacterial collagenase, prepared by chromatography and by affinity chromatography was found to be identical in behaviour with the commercially purified CLSPA collagenase used largely in this study.

The data presented in this paper suggest that it may be misleading to use tropocollagen as a substrate for the assay of collagenase involved in tissue remodelling, if the results are to be interpreted in terms of the ability of this enzyme to degrade insoluble collagen, i.e. polymeric collagen fibrils.

Materials

Polymeric collagen fibrils were prepared from 2-year-old bovine extensor tendons after pre-treatment with EDTA, pH 7.5 (Steven, 1967). Tropo-

collagen was prepared from rat skin by solution in 0.2M-acetic acid followed by purification of the tropocollagen by repeated reconstitution of collagen fibrils (Gross & Kirk, 1958).

Two types of fluorescent-labelled polymeric collagen fibrils were prepared by coupling rhodamine iso-thiocyanate (BDH Chemicals, Poole, Dorset, U.K.) and fluorescein iso-thiocyanate (BDH) to accessible amino groups in polymeric collagen fibrils to yield the rhodamine-labelled and fluorescein-labelled polymeric collagen fibrils (Steven, 1975).

Twice-crystallized papain and purified bacterial collagenase, CLSPA, were obtained from Worthington Biochemical Corp., Freehold, NJ, U.S.A. Highly purified bacterial collagenase, free of proteolytic activity, was prepared by chromatography (Lee-Own & Anderson, 1975) and generously provided by these authors. Bacterial collagenase was also purified by affinity chromatography on insoluble elastin (Serafini-Fracassini *et al.*, 1975) at 4°C, followed by selective binding of collagenase to a column of polymeric collagen and finally elution with 0.75M-NaCl in 50mM-Tris/5mM-CaCl₂ buffer, pH7.5.

Methods

In these experiments, the fluorescein-labelled polymeric collagen fibrils contained seven fluorescein residues per tropocollagen molecule. The degree of substitution was calculated by determining the absorbance or the percentage fluorescent emission of the total papain digests of weighed samples of the labelled polymeric collagen fibrils (Steven, 1975; Steven & Lowther, 1975), with fluorescein-labelled lysine and rhodamine-labelled lysine as standards. The fluorescent labelling technique was shown to label all molecules of tropocollagen within the polymeric collagen fibrils to the same extent, rather than selectively labelling those tropocollagen molecules on the surface of polymeric collagen fibrils (Steven *et al.*, 1975). Substitution of tropocollagen under the same experimental conditions also yielded monomers with seven substituted amino groups per molecule of tropocollagen, suggesting that the maximum number of available ϵ -lysine residues in tropocollagen had been substituted in fluorescein-labelled polymeric collagen fibrils to provide uniform labelling.

Viscometric analysis of bacterial collagenase digestion of tropocollagen solutions

Solutions of rat skin tropocollagen (0.5mg/ml) were prepared in 50mM-Tris/HCl buffer containing 5mM-CaCl₂, pH7.5, and the viscosity measurements were made at 20°C with an Ostwald viscometer with a flow time for water of 85s. To 5ml of tropocollagen solution in the viscometer were added a range of concentrations of bacterial collagenase

(0–100 μ g/5ml of reaction mixture) by means of a microsyringe. The contents of the viscometer were rapidly mixed and after exactly 2min the flow time of the collagen plus collagenase system was measured. The choice of a 2min reaction time was made on the basis of the need to record the extent of the initial reaction and the fact that the rate of change in viscosity of a tropocollagen solution in the presence of collagenase is maximal during the first 10min.

Bacterial collagenase digestion of reconstituted collagen fibrils

Collagen fibrils were formed by taking a series of tubes into which had been placed 5ml of a tropocollagen solution containing 4mg of tropocollagen, adjusted to pH7.5 and containing 50mM-Tris/5mM-CaCl₂; gels were allowed to mature for periods of 1–48h at 37°C before digestion with the enzyme. A further series of tubes containing 6.9mg of tropocollagen/5ml of the above buffer system, plus 1000i.u. each of streptomycin and penicillin, was equilibrated at 37°C for 8 weeks to observe the effect of prolonged maturation on the collagen fibrils. The gels were disrupted with a spatula, and bacterial collagenase (Worthington CLSPA collagenase) over a range of 0–80 μ g of enzyme per tube was added by means of a microsyringe. After exactly 5min incubation at 37°C, the enzymic digestion was inhibited by adding 0.1ml of 1M-EDTA, and the contents of the tubes were centrifuged. The supernatant fractions were removed and assayed both for soluble hydroxypropyl peptides (Woessner, 1961) and for free amino groups by the fluorescent assay procedures with 'fluram', based on the method of Weigle *et al.* (1972). In the present assay, 10 μ l of the collagenase digest was mixed with 1ml of 0.2M-sodium borate buffer, adjusted to pH9.0, and caused to react with 150 μ g of 'fluram' dissolved in 0.5ml of acetone by vigorous mixing. The resultant fluorescent solution was determined on the Aminco-Bowman spectrofluorimeter by using the 0.03 and 0.3 scales with an excitation wavelength of 390nm and an emission wavelength of 490nm. Standard solutions of L-leucine were used to prepare a calibration scale of fluorescent emission for μ mol of free amino groups dissolved in 5ml of reaction mixture.

On the Aminco-Bowman spectrofluorimeter the fluorescent emission can be measured on a range of scales, e.g. 0.01, 0.03, 0.1, 0.3 and 1.0. On each scale the fluorescent emission is measured as a percentage of the full-scale deflexion. Thus a fluorescent emission of 12% measured on scale 0.3 is equivalent to 120% on scale 0.03.

Bacterial collagenase digestion of rhodamine-labelled and fluorescein-labelled polymeric collagen fibrils

Two series of capped plastic tubes were set up, each tube containing 7.0mg of labelled polymeric collagen

fibrils. In series (A), a known quantity of bacterial collagenase (0–50 μg) was added plus 50mM-Tris/HCl/5mM- CaCl_2 buffer, pH7.5, to bring the final volume to 5ml. In series (B) the buffer contained 20mM-EDTA, which is known to inhibit collagenase (Mandl, 1961), and these tubes (B) served as a control series for the test series (A). This technique is described by Steven & Lowther (1975). The tubes were shaken at 37°C for 1 h, centrifuged if necessary to remove particles and the supernatant fractions assayed for soluble fluorescein-labelled peptides or rhodamine-labelled peptides by direct spectroscopy or by fluorimetry. Fluorescein-labelled peptides were determined by measuring their E_{495} alternatively, after dilution by 50-fold, the supernatants were assayed by the percentage fluorescent emission on an Aminco-Bowman spectrofluorimeter at an excitation wavelength of 495nm and an emission wavelength of 520nm. The rhodamine-labelled peptides were determined by direct spectroscopy of the supernatant fractions at 365nm or by fluorimetry of the undiluted supernatant fractions at an excitation wavelength of 553nm and an emission wavelength of 582nm.

Effect of time on the digestion of rhodamine-labelled polymeric collagen by bacterial collagenase

A similar series of tubes was set up containing a range of concentrations of bacterial collagenase and incubated as above. At 10min intervals, the tubes were removed and the quantity of solubilized rhodamine-labelled peptides were quantified by measuring the E_{365} of the supernatant. The tubes were then returned to the bath for further incubation. This procedure is not ideal because of the time required to make measurements; during this period the reaction mixture is not shaken or heated to 37°C.

Effect of both time and collagenase concentration on the digestion of fluorescein-labelled polymeric collagen fibrils

The test system was set up with 7.0mg of substrate in 5ml of buffer, containing a range of collagenase concentrations and incubated at 37°C. At intervals of 0.5h, 100 μl samples were removed from each tube and diluted to 5ml before fluorimetric assay.

Distribution of bacterial collagenase bound to the substrate and in free solution in the test system

The test system was set up as above with 7.0mg of fluorescein-labelled polymeric collagen fibrils and 1–7 μg of bacterial collagenase in 5ml of buffer. The tubes were preincubated at 37°C for 15min, and then the supernatant fractions were rapidly removed from the residual substrate. The quantities of fluorescein-labelled peptides solubilized during the preincubation were measured by fluorimetry. Duplicate samples of the supernatants were then reincubated with fresh fluorescein-labelled polymeric

collagen fibrils, and the volumes made up to 5ml with buffer. After 1h the total collagenase activity in each of the original preincubation supernatants was determined by measuring the increase in fluorescein-labelled peptides solubilized during this hour of digestion. At the same time the insoluble residues of fluorescein-labelled polymeric collagen fibrils remaining from the 15min preincubation were resuspended in 5ml of buffer and assayed for collagenase activity after 1h at 37°C. The percentage distributions of the bacterial collagenase binding to the solid substrate and present in the aqueous phase were then determined.

Bacterial collagenase digestion of fluorescein-labelled polymeric collagen fibrils after 5min at 37°C

Tubes were set up with 7.0mg of fluorescein-labelled polymeric collagen fibrils in 5ml of Tris/HCl/ CaCl_2 buffer containing 0–10 μg of bacterial collagenase and shaken for 5min at 37°C. The reaction was stopped by adding EDTA, and the total supernatant fraction was removed for fluorimetric analysis.

Results and Discussion

Bacterial collagenase digestion of tropocollagen in solution

The change in initial viscosity of the tropocollagen solution after 2min digestion with an increasing range of collagenase concentrations is shown in Fig. 1. The change in viscosity was found to be directly related to the amount of enzyme added, over the range 0–100 μg of collagenase per 5ml of reaction

Fig. 1. Decrease in viscosity of tropocollagen solution caused by bacterial collagenase

Decrease in the viscosity of tropocollagen solution (2.5mg of tropocollagen/5ml) in Tris/ CaCl_2 buffer, pH7.5, at 20°C in the presence of 0–100 μg of bacterial collagenase was measured. The initial extents of digestion were measured as the change in flow time after 2min incubation with the enzyme.

Fig. 2. Solubilization of reconstituted collagen fibrils by bacterial collagenase as determined by solubilization of hydroxyprolyl peptides

The solubilization of hydroxyprolyl peptides after 5 min digestion of 4 mg of reconstituted collagen fibrils/5 ml of reaction mixture at 37°C. The results are expressed as mg of collagen solubilized.

mixture containing 2.5 mg of tropocollagen, and producing a change in viscosity of up to 33% of the initial value. This linearity is expected since this technique is the basis of bacterial collagenase assays (Gallop *et al.*, 1957). On a weight basis, 100 μg of collagenase represents an enzyme/substrate ratio of 1:25 at the time of mixing. If the mol.wt. of tropocollagen is approx. 270000 (Holmes *et al.*, 1973) and bacterial collagenase is approx. 100000 (Mandl *et al.*, 1964) then at 100 μg of collagenase, the molar ratio of enzyme/substrate is approx. 1:10.

Bacterial collagenase digestion of freshly reconstituted collagen fibrils

The extent of solubilization of reconstituted collagen fibrils by bacterial collagenase after 5 min

digestion, determined by the solubilization of hydroxyprolyl peptides (Fig. 2) and by soluble peptide amino groups detected by the 'fluram' assay procedure (Fig. 3) demonstrated linearity over the range 0–80 μg of enzyme per 5 ml of reaction mixture containing initially 4 mg of collagen fibrils. At the level of 80 μg, the enzyme/substrate ratio is approx. 1:18, calculated on a molar basis. At this concentration the enzyme had solubilized half the total substrate in 5 min; it was not considered profitable to increase the enzyme concentration above this level. The extents of digestion shown in Figs. 2 and 3 were identical for collagen fibrils which had matured for 1 h and for 48 h at 37°C before enzymic digestion. The results suggest that bacterial collagenase attacks tropocollagen in solution (Fig. 1) and freshly

Fig. 3. Solubilization of reconstituted collagen fibrils by bacterial collagenase as determined by the 'fluram' assay

The solubilization of peptides measured by the 'fluram' assay of free amino groups after 5 min digestion of 4 mg of reconstituted collagen fibrils/5 ml of reaction mixture at 37°C. The results are expressed as the total free amino groups solubilized in 5 ml of reaction mixture, on the basis of their leucine equivalent values.

prepared reconstituted collagen fibrils prepared from tropocollagen solutions (Figs. 2 and 3) in essentially the same manner. The introduction of intermolecular cross-linkages, which may have taken place during 2 days maturation of the collagen fibrils at 37°C, had no marked effect on the extent of collagenase digestion.

Solubilization of fluorescein-labelled peptides from fluorescein-labelled polymeric collagen fibrils by bacterial collagenase during 5h

It was observed that for low concentrations (3 µg/5ml) of bacterial collagenase the rate of solubilization of fluorescein-labelled peptides was linear over 5h. It was convenient to measure the extents of digestion of polymeric collagen fibrils, fluorescein-labelled polymeric collagen fibrils and rhodamine-labelled polymeric collagen fibrils after 1h.

Distribution of bacterial collagenase bound to fluorescein-labelled polymeric collagen fibrils and in free solution in the test system

Over the range 0–7 µg of collagenase per 7.0mg of fluorescein-labelled polymeric collagen fibrils suspended in 5ml of buffer it was found that 46% of the added collagenase was associated with the solid substrate and 54% was present in the supernatant fraction. Thus on a weight basis, i.e. 7 mg of substrate and 5g of buffer, the collagenase partitioned with an affinity for fluorescein-labelled polymeric collagen fibrils 609 times that for the same weight of buffer.

Comparison of the digestion of fluorescein-labelled polymeric collagen fibrils with pure bacterial collagenase and CLSPA collagenase

Commercially available bacterial collagenase may contain non-specific proteolytic enzymes as impurities (Miyoshi & Rosenbloom, 1974; Peterkovsky & Diegelmann, 1971), which could contribute to the release of soluble products from the non-helical regions of the collagens used in this study. Such non-specific enzymes would have little effect on the viscosity of tropocollagen solutions or on the solubilization of freshly reconstituted collagen fibrils, although they would be expected to solubilize fluorescein-labelled peptides from the non-helical regions of fluorescein-labelled polymeric collagen fibrils (Steven *et al.*, 1975). In this study, with fluorescein-labelled polymeric collagen fibrils as the substrate for a bacterial collagenase assay system, there was always found to be a small amount of non-specific proteolytic activity associated with the CLSPA collagenase, i.e. 5–10% of the total fluorescein-labelled peptides solubilized were not due to collagenase but to impurities in the enzyme preparation. This was taken into account in the assay system, where the collagenase digestion was measured as the difference in

absorbance or percentage fluorescent emission between the total (Series A, collagenase plus non-specific proteolytic enzymes) and the collagenase-inhibited reading (Series B, non-specific proteolytic enzymes), as described in the Methods section. Two highly purified collagenase preparations (see the Methods section) were shown to possess no measurable non-specific proteolytic activity. When these highly purified bacterial collagenases and CLSPA collagenases were compared by measuring the solubilization of fluorescein-labelled peptides and hydroxyproline from fluorescein-labelled polymeric collagen fibrils under the standard conditions over a range of 0–14 µg of bacterial collagenase, the results were identical, provided that the precautions described above were observed. This comparison confirmed the validity of the test system as a collagenase assay which was independent of the non-specific proteinases present in trace amounts in CLSPA collagenase.

Bacterial collagenase digestion of fluorescein- and rhodamine-labelled polymeric collagen fibrils

An amount of bacterial collagenase of 10 µg was capable of solubilizing almost completely 7.0mg of the labelled substrates in 48h, demonstrating that the intermolecular cross-linkages and replacement of selected lysine residues with fluorescent markers did not prevent bacterial collagenase from attacking the helical regions of tropocollagen within these fluorescent-labelled polymeric collagen fibrils. Kinetic studies of collagenase digestion of polymeric collagen fibrils and both the fluorescein- and rhodamine-labelled polymeric collagen fibrils (Steven, 1975) demonstrated that the introduction of the label had no effect on the rate of enzyme attack.

It was observed, however, that the extent of solubilization of fluorescein-labelled and rhodamine-labelled peptides by bacterial collagenase was linear with collagenase concentration over a very low range, reaching a limiting value at 6–7 µg of collagenase per 7.0mg of substrate (Fig. 4). At higher enzyme concentrations, little increase in the extent of digestion was observed. It appeared that all the available sites for a productive enzyme-substrate complex on the tropocollagen molecules within the polymeric collagen fibrils had been occupied when 6–7 µg of enzyme was added. For simplicity this limiting enzyme concentration will be referred to as the 'point of equivalence', at which the linearity relating to products formed and enzyme concentration is lost. At concentrations below the point of equivalence, the enzyme is digesting tropocollagen molecules within polymeric collagen fibrils with maximal efficiency.

If this interpretation of the observed data is correct, then when a fixed quantity of enzyme (e.g. 50 µg) is used and the concentration of substrate

Fig. 4. Bacterial collagenase digestion of rhodamine- and fluorescein-labelled polymeric collagen fibrils

Release of soluble peptides from 7.0mg of labelled substrates by 0–10 μg of bacterial collagenase in 5ml of Tris/CaCl₂ buffer, pH 7.5, after 1 h at 37°C. E_{365} was measured for rhodamine-labelled peptides (○) and E_{495} for fluorescein-labelled peptides (●).

is increased in a series of tubes, it should be possible to demonstrate an increased extent of solubilization, which is directly proportional to the quantity of substrate used. This effect is shown in Fig. 5 in which every tube contained 50 μg of collagenase and a range 0–20mg of labelled substrate, the incubation time being 1 h. The results of Fig. 5 indicate that at enzyme concentrations above the point of equivalence, the quantity of substrate in the reaction mixture controls the rate of solubilization of products.

An alternative way of demonstrating this effect is shown in Fig. 6 when the enzyme concentration is in excess of the point of equivalence. In this case the progress of digestion of rhodamine-labelled polymeric collagen fibrils was followed during 1 h. Curves (A) and (B) represent the digestion of 2mg of substrate by 10 μg of bacterial collagenase and 100 μg of bacterial collagenase respectively. A tenfold increase in enzyme concentration has little effect on the initial rate of soluble-product formation (compare this with an equivalent situation for tropocollagen in solution, Fig. 1). Similarly, curves (C) and (D) represent the digestion of 4mg of rhodamine-labelled polymeric collagen fibrils by 10 μg and 100 μg of collagenase respectively. Again the tenfold increase in enzyme concentration had little effect on the initial solubilization rate. In contrast, doubling the quantity of substrate approximately doubled the rate of product formation [compare curves (A) and (C) in Fig. 6 and also curves (B) and (D)]. The results presented in Fig. 6 were obtained by direct reading of absorbance values, which necessitated the removal of the tubes from the incubation tank for each reading. This fact may account in part for the curvature of the graphs.

Effect of both time and collagenase concentration on the digestion of fluorescein-labelled polymeric collagen fibrils

In this experiment a range of bacterial collagenase concentrations (3–12 μg/5ml of buffer) was used, and the solubilized fluorescein-labelled peptides were measured on samples removed at intervals of 0.5 h for 5 h (Fig. 7). The results show linearity over 5 h for the solubilization of fluorescein-labelled peptides when 3 μg of collagenase was used. This linearity with time was not found for higher concentrations of collagenase after 2 h. At all concentrations below 12 μg of collagenase/5ml, the solubilization of fluorescein-labelled peptides was linear for the first hour. After 1 h, the effect of increasing the collagenase concentration above 7 μg/5ml can also be seen to be negligible, i.e. the point of equivalence has been reached. The lack of linearity of the quantity of product formation between 2 and 5 h with 6–12 μg of collagenase suggests that, during this phase of the digestion of polymeric collagen fibrils, most of the readily available or susceptible bonds have

Fig. 5. Effect of substrate concentration on the extent of digestion of fluorescein-labelled polymeric collagen fibrils by bacterial collagenase

Solubilization of fluorescein-labelled peptides from various quantities of fluorescein-labelled polymeric collagen fibrils by 50 μg of bacterial collagenase acting for 1 h at 37°C in Tris/CaCl₂ buffer. E_{495} was measured, and the results are for 5ml of reaction mixture.

Fig. 6. Effect of enzyme and substrate concentration on solubilization of rhodamine-labelled polymeric collagen fibrils, when the enzyme concentration is greater than found at the point of equivalence

Release of soluble peptides from rhodamine-labelled polymeric collagen fibrils by bacterial collagenase measured over 1 h at 37°C in Tris/CaCl₂ buffer; E_{365} /5ml of reaction mixture. Curves (A) and (B) were obtained with mixtures containing 2mg of rhodamine-labelled polymeric collagen fibrils, and curves of (C) and (D) with 4mg of rhodamine-labelled polymeric collagen fibrils. Mixtures for curves (A) and (C) contained 10 μ g of enzyme and those for curves (B) and (D) contained 100 μ g of enzyme.

been hydrolysed by the enzyme, thus accounting for a decrease in the amount of products solubilized.

At enzyme concentrations above the point of equivalence the extent of product formation is directly proportional to the weight of substrate used (Fig. 5), and, since polymeric collagen fibrils have a fairly uniform cross-sectional area (Dixon *et al.*, 1972), it can be assumed that the weight of fluorescein-labelled polymeric collagen fibrils is directly proportional to the circumference and total surface area of the fibrils within the bundles of collagen fibres, present at pH 7.5. At very low concentrations of collagenase (Fig. 4) normal enzyme kinetics apply and the extent of solubilization depends only on the quantity of enzyme added. When 6–7 μ g of collagenase are caused to react with 7 mg of labelled substrate in 5 ml of reaction mixture the point of equivalence is reached. If one can assume a mol.wt. for bacterial collagenase of approx. 100000 and tropocollagen of 270000, at the point of equivalence 1 molecule of collagenase is equivalent to approx. 430 or 390 molecules of tropocollagen within the polymeric collagen fibrils, depending on whether 6 or 7 μ g of enzyme is considered to be the point of equivalence. It has been calculated that, under the

experimental conditions used in this study, only 46% of the total added collagenase is associated with the insoluble substrate at the point of equivalence. This would mean that the enzyme/substrate ratio on a molar basis, present within the solid polymeric collagen fibrils, would be nearer 1:934 or 1:848.

It appears that the surface of the polymeric collagen fibrils contained a limited number of molecules of intermolecularly cross-linked tropocollagen which were initially accessible to bacterial collagenase. The interior of the fibrils contained tropocollagen molecules which are initially inaccessible to the enzyme. Thus the surface layer of tropocollagen molecules on polymeric collagen fibrils would

Fig. 7. Effect of both time and collagenase concentration on the digestion of fluorescein-labelled polymeric collagen fibrils

Release of soluble fluorescein-labelled peptides from fluorescein-labelled polymeric collagen fibrils (7.0mg) in 5 ml of buffer, 37°C over 5 h by a range (3–12 μ g) of collagenase. Portions (100 μ l) of the soluble fraction were removed and added to 5 ml of water before fluorimetric analysis at an excitation wavelength of 495 nm and an emission wavelength of 520 nm, with the scale reading set at 0.03. $\circ$, 3 μ g of collagenase; $\blacksquare$, 6 μ g; $\bullet$, 7 μ g; $\square$, 12 μ g.

effectively protect the inner tropocollagen molecules from the initial attack by collagenolytic enzymes. As the digestion proceeds, all the substrate is eventually exposed and attacked by the enzyme to produce solubilization of the fluorescein-labelled polymeric collagen fibrils.

The extent of attack by bacterial collagenase on tropocollagen molecules in true solution (Fig. 1) and in freshly reconstituted fibrils (Figs. 2 and 3) are directly proportional to the quantity of enzyme added and no such point of equivalence was observed, even when an enzyme-substrate molecular ratio of 1:10 was used. Clearly tropocollagen molecules and polymeric collagen fibrils have entirely different properties as substrates for bacterial collagenase.

Bacterial collagenase digestion of unlabelled polymeric collagen fibrils

The extent of collagenase digestion of unlabelled polymeric collagen fibrils after 1 h of the standard assay condition is presented in Fig. 8 for the range 0–14 μg of CLSPA collagenase/5 ml of reaction mixture. In this assay the 'fluram' analysis was conducted on 50 μl of the supernatant fraction, and the total quantity of 'fluram'-reacting free amino groups per 5 ml of reaction mixture is expressed as leucine equivalents. When two highly purified preparations of bacterial collagenase were used in this experiment, graphs identical with that shown in Fig. 8 were obtained.

The evidence presented in Fig. 8 confirms the observations obtained with fluorescein- and rhodamine-labelled polymeric collagen fibrils that a point

Fig. 8. Solubilization of peptides from polymeric collagen fibrils by bacterial collagenase

'Fluram' analysis of peptide groups, expressed as leucine equivalents solubilized by bacterial collagenase after 1 h digestion at 37°C of 7 mg of polymeric collagen fibrils per 5 ml of reaction mixture.

of equivalence is reached at approx. 6–7 μg of collagenase in the presence of 7.0 mg of insoluble substrate. The evidence of Fig. 8 also refutes any suggestion that the point of equivalence observed with collagenase digests of fluorescein-labelled polymeric collagen fibrils and rhodamine-labelled polymeric collagen fibrils is a result of dye-labelling, which might be considered to be a possible explanation for this difference in behaviour observed between the digestion of the fluorescein-labelled polymeric collagen fibrils and the soluble unlabelled form of tropocollagen molecules.

Bacterial collagenase digestion of 8-week-matured collagen fibrils

When collagen fibrils were matured at 37°C for 8 weeks before collagenase digestion, it was

Fig. 9. Solubilization of 8-week-matured collagen fibrils by bacterial collagenase

Solubilization of hydroxyprolyl peptides by 0–100 μg of bacterial collagenase acting on 6.9 mg of tropocollagen molecules per 5 ml of reaction mixture for 5 min at 37°C. Results are expressed as mg of tropocollagen solubilized.

Fig. 10. Solubilization of 8-week-matured collagen fibrils by bacterial collagenase

'Fluram' assay of free amino groups on peptides solubilized from 6.9mg of tropocollagen per 5ml of reaction mixture by bacterial collagenase after 5 min at 37°C. Results are presented as leucine equivalents per 5ml of reaction mixture.

observed that the quantity of solubilized peptides (measured as hydroxyprolyl peptides in Fig. 9 and as 'fluram'-reacting peptides in Fig. 10) was linearly related to quantity of enzyme added over the range 0–60 μg of collagenase/5ml of reaction mixture. In this experiment using 6.9mg of tropocollagen/5ml and with 60 μg of collagenase at the point of equivalence, the enzyme/substrate ratio was approx. 1:43 on a molecular basis. The quantity of collagen solubilized in 1 h by 60 μg of collagenase acting on the 8-week-matured collagen fibrils was approx. 71% of the collagen solubilized under identical conditions by using freshly prepared fibrils (Figs. 2 and 9), when estimated by hydroxyproline analysis, and 76% (Figs. 3 and 10), when estimated by the 'fluram' technique.

It appears that maturation of reconstituted fibrils results in (a) a decreasing rate of collagenase digestion of the fibrils, (b) the loss of linearity between the amount of enzyme added and the

amount of soluble products formed, and (c) as a result of (b) a point of equivalence can be detected at 60 μg of collagenase in this instance. This point of equivalence was not observed in studies on tropocollagen molecules in solution or with freshly reconstituted fibrils (Figs. 1, 2 and 3). Clearly the 8-week period of maturation caused a shift in substrate behaviour towards that observed in the polymeric collagen fibrils. The results obtained with 8-week-matured fibrils refute the possible criticism that the effects described in this study are due to the substitution of polymeric collagen fibrils with fluorescent labels, which may have altered the collagen or be located only on surface molecules, thus yielding complex kinetic data.

Bacterial collagenase digestion of fluorescein-labelled polymeric collagen fibrils after 5 min at 37°C

When the total soluble products were determined by fluorimetry after 5 min of incubation at 37°C (Fig. 11), the extent of hydrolysis was found to be linear over the range 0–7 μg of collagenase/7.0mg of substrate. Above the point of equivalence (7 μg) addition of further quantities of the enzyme had little effect on the amount of product formed. These data confirm the results obtained with the fluorescein-labelled polymeric collagen fibrils after 1 h digestion, and clearly show the point of equivalence during the

Fig. 11. Solubilization of fluorescein-labelled peptides from fluorescein-labelled polymeric collagen fibrils by bacterial collagenase after 5 min at 37°C

After 5 min digestion at 37°C, the reaction was terminated by addition of EDTA, and the undiluted supernatant fractions were analysed by fluorimetry by using the 0.1 emission scale.

initial stages of digestion when there can be no question of a lack of suitable substrate.

In conclusion it can be stated that solutions of tropocollagen and freshly reconstituted collagen fibrils behave entirely differently from aged polymeric collagen fibrils and 8-week-matured collagen fibrils as substrates for bacterial collagenase. Work with crude mammalian collagenase preparations suggests a similar relationship holds for this enzyme. The very high specific activities of mammalian collagenase assayed with ^{14}C -labelled collagen fibril suspension and the very low activity of the same enzyme preparation when assayed with insoluble collagen has been reported by Woolley *et al.* (1975), although they did not comment on this finding. Since 98% of the collagen present in mature connective tissue is in the form of polymeric collagen fibrils it is suggested that the results of mammalian collagenase assays based on the solubilization of ^{14}C -labelled peptides from freshly reconstituted fibrils should not necessarily be extrapolated to calculate the rates at which the enzyme can degrade insoluble collagen unless similar studies are carried out with polymeric collagen fibrils or some other suitable form of cross-linked insoluble collagen.

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