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Study of Collagen and its Reaction with Bacterial Collagenase by Circular Dichroism

by

Fung-Hwei Valeria Chu

A Dissertation Presented to the
Faculty of the Department of Biochemistry of
the City University of New York

In Partial Fulfillment of the Requirement for
the Degree of Doctor of Philosophy

1976

This manuscript has been read and accepted for the Graduate Faculty in Biochemistry in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

9-1-76
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Date

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Executive Officer

Abstract

Abstract

STUDY OF COLLAGEN AND ITS REACTION WITH
BACTERIAL COLLAGENASE BY CIRCULAR DICHORISM

by

Fung-Hwei Valeria Chu

Adviser: Professor Aaron Lukton

CD Spectrum of native collagen shows a maximum around 220 nm. The maximum changed after heat denaturation or collagenase hydrolysis. It is, therefore, possible to study the collagenase reaction by examining the rate of change of the maximum. Collagenase can be purified by collagen-sepharose 4B affinity chromatography. Collagen-collagenase reaction is a two first order reactions. There are one fast reaction and one slow reaction. Collagenase activity was enhanced by Ca^{++} and Co^{++} ions. Collagen-collagenase reaction was inhibited by EDTA, but the enzyme activity could be restored by adding Ca^{++} ions. Hydroxyproline induces a higher stability in the collagen molecules. Therefore, collagen with higher hydroxyproline content is less susceptible to the collagenase digestion. Collagen with higher hydroxyproline content is more

stable and shows higher Tm, due to the hydrogen bonding formed through the hydroxyl groups. In the presence of Ca^{++} ions Tm is lowered. It is assumed firstly due to an ionic disorganization of the water contacting the polypeptide chains and secondly to their mutual electrostatic repulsion. At neutral pH mucopolysaccharides have no effect on either the Tm of collagen or on the collagen-collagenase reaction.

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I would like to thank Professor Aaron Lukton for his many years of advice and guidance in the preparation of this work.

DEDICATION

To My Parents

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Chapter I

Introduction

Collagen

The skin of animals have long been used for the making of leather; their skin, bones, and tendons have been used for the making of gelatin and glue. The word collagen, meaning glue former, came into use during the nineteenth century to designate the material of these tissues responsible for the formation of glue when the tissues were boiled in water and the solution evaporated. Collagen now has a more specific meaning: it is the particular mammalian protein, the dominant protein of derma, bone, cartilage, and tendon, that in its most common form is insoluble in water. It occurs in tissues as a fibrous material that is entirely extracellular and that is one of the principal structural elements of the animal body: It has a function in the animal somewhat similar to that of cellulose in plants. It is the main component of the framework in and on which are arranged the living cells and organs of an individual animal. It defines the individual as all the parts held together by this fibrous frame. In animals other than mammals there are similar proteins, sometimes differing in composition, sometimes with different names: ichthyocol of fish swim bladder and skin, elastoidin of elasmobranch fins, spongin and gorgonin of some invertebrates. For fibers of mammalian tissues words other than collagen are also sometimes used; vitrosin for the fibrils of the vitreous body, and assein for those of bone. It is

perhaps better to refer to these varied forms as the collagens, related members of a family of proteins with similar compositions and having similar properties and functions.

The fibrous character and the insolubility in water are outstanding properties of collagen. Collagen is so different from the water-soluble proteins of cells and plasma that is to be put into a separate class called scleroproteins or proteinoids, as if it were only almost a protein. Until three decades ago particularly only leather chemists studied collagen. Protein chemists attended to the soluble proteins that could be fractioned and purified. With the sudden development of interest in the rheumatic disease and connective tissue chemistry, collagen could no longer be ignored. In addition, two incidents gave immense impetus to the study of collagen. The rediscovery that from some tissues a water-soluble form could be separated; and the development of the electron miscroscope which immediately led to the finding of the characteristic banding pattern of the fibrils.

Proteins consist of chains of amino acid residues linked together by peptide bonds, -CO-NH-. Individual protein chains may form specific aggregates of larger size by various types of interchain bonding, including covalent cross-links, hydrogen bonds, salt bridges, and hydrophobic

interactions. Proteins that are soluble in water have chains that are generally neither extended nor randomly coiled, but are quite specifically folded into neat, compact bundles with hydrophilic groups tending to be collected at the surface of the molecule, and with hydrophobic groups collected within the folds of chains so they are not exposed to the water. The chains of insoluble proteins may be more extended in their conformation and so expose more of their hydrophobic groups to the solvent.

Collagen fibers are entirely extracellular. They form coherent woven structure such as membranes, tubes, pockets, and packings to compartmentalize the body, to separate or to connect organs, and generally to hold the body together. The arrangements of fibers in different tissues is often characteristically different and apparently functional. In tendons, fibers are generally nearly parallel giving rope-like structures. In other tissues such as the cornea, the collagen fibrils are arranged in laminated sheets or they may occur as a fine network of individual fibers holding the soft tissues and glands together. The mechanisms involved in the organization of the collagen fibrils in the tissues are completely unknown.

The fundamental structural unit of collagen appears to be a tropocollagen molecule, 15 Å in diameter and 2800 Å long, with a molecular weight of about 300,000. "Appears to be", for although the tropocollagen theory

explains all the evidence at hand, no one has yet seen an isolated tropocollagen molecule in the electron microscope (1, 2).

The current view is that the tropocollagen molecule is composed of three separate primary protein chains, each helically coiled as indicated in Fig. 1.1. (3), and all three held together at specific locations by a large number of hydrogen bonds. Of the three protein chains of a tropocollagen molecule, two are nearly identical in amino acid composition, and are called α_1 chains, and the third more distinctly different is called an α_2 chain.

The tropocollagen molecules are thought to pack with a displacement of one quarter of their length to form an overlapping collagen fibril, having a characteristic banding pattern with electron microscope stains every 640 Å.

Of the eighteen amino acid residues found in many proteins collagen lacks tryptophan and cysteine, and in addition to those commonly found in other proteins, it contains 4-hydroxyproline, δ-hydroxylysine, and traces of 3-hydroxyproline. A striking feature of collagen composition is its high content of glycine which makes up a third of all amino acid residues. Another third is made up of proline, hydroxyproline and alanine. That two-thirds of all amino acid residues is made up of these four is of

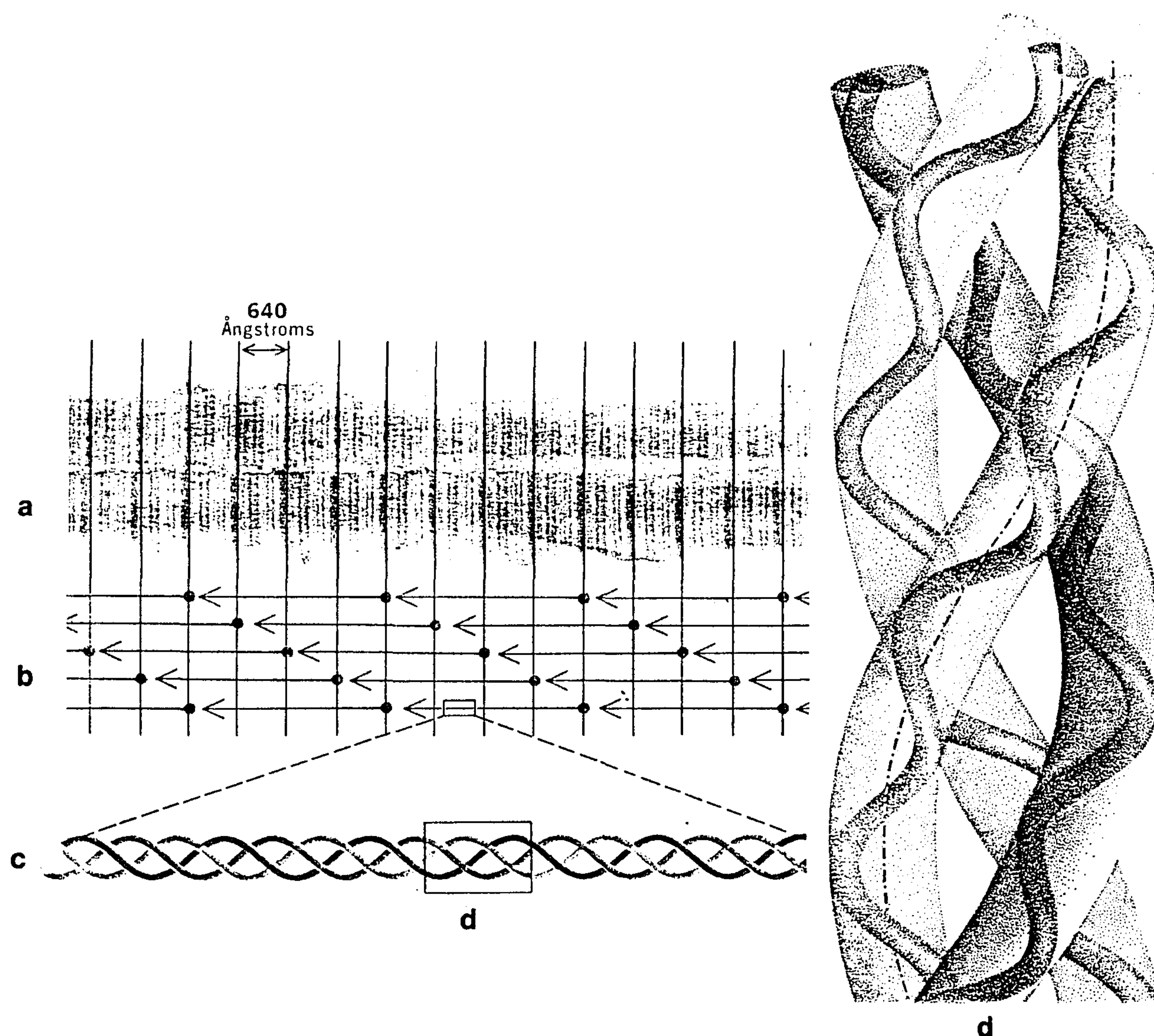


Fig. 1.1. (a) Higher magnification (approximately 100,000 times) of calfskin collagen fibrils stained with phosphotungstate. (Both photographs by courtesy of Dr. Alan Hodge.)

(b) One tropocollagen molecule, represented as an arrow, is believed to extend through four of the 640-Å long sets of cross-striations.

(c) The triple helix of the tropocollagen molecule.

(d) The section above, enlarged at the right, shows how three left-handed helices are given a right-handed twist to form a three-fold superhelix.

Ref. (3).

some significance for the configurations of the chains in the tropocollagen molecule. The high proportion of glycine and alanine, which have the smallest side chains, allows close packing of adjacent chains. The high proportion of proline and hydroxyproline may have a great influence on chain conformation because of the limitation on rotation around the bond between the N and the C which is part of the pyrollidine ring which in turn is part of the peptide chain of collagen:

```
   H     H      H     H     H
   |     |      |     |     |
-N-C-C-N-C-C-N—C-C-N-C-C-N-C-C-
 | | ‖ | | ‖ |  | ‖ | | ‖ | | ‖
 H R O H R O C  C O H R O H R O
              \/
              C
```

Table 1.1. shows the amino acid composition of different collagens (4).

The triple-helix structure of tropocollagen was worked out by Rich & Crick (5) and by Ramachandran and his group (6). Poly-proline, poly-glycine, and poly-hydroxyproline all give excellent fiber X-ray patterns indicating a three-residue extended helix, with amides and carbonyls extending perpendicular to the other. Three such parallel left-handed three residues helices, removed from the crystalline environment and given a gentle twist to the right, form the basis of the collagen structure. Two of every three amides

Table 1.1.

Amino Acid Composition of Vertebrate Collagens and Gelatins[a]

Amino acid	Ox-skin (C)	Calf-skin (C)	Ox bone (C)	Pig-skin (G)	Sheep ten-don (C)	Rat-skin (C)	Hu-man ten-don (E)	Whaleskin (G)	Chicken tendon (G)	Crocodileskin (G)	Py-thon-skin (G)	Toadskin (G)	Lungfishskin (G)	Sharkskin (G)	Stur-geon swim bladder (C)	Carp swim blad-der (C)	Carp-skin (G)	Cod-skin (G)	Pike-skin (G)
Alanine	99.6	112	109.7	110.8	99.9	106	110.7	110.5	114.6	114.0	125.0	98.0	128.0	119.0	118.9	126	120	107	114
Glycine	338	320	314	326	327	327	324	326	331	324	315	301	311	333	337	325	317	345	328
Valine	27.1	20	21.2	21.9	25.1	22	25.4	20.6	19.8	15.4	20.2	21.9	21.3	21.9	18.0	18	19	19	18
Leucine	39.9	25	27.9	23.7	25.4	25	26.0	24.8	23.8	20.1	25.7	28.8	25.2	23.9	17.7	21	25	23	20
Isoleucine	—	11	12.3	9.6	12.7	10	11.1	11.0	10.9	11.4	11.7	14.0	12.2	19.4	11.4	10	12	11	9.2
Proline	122.3	138	118.8	130.4	120.0	117	126.4	128.2	129.5	127.9	119.4	109.7	126.0	113.4	102.2	116	124	102	129
Phenylalanine	14.1	13	16.3	14.4	13.6	13	14.2	13.0	14.3	17.7	14.2	19.3	15.3	13.9	14.1	14	14	13	14
Tyrosine	5.1	2.6	2.9	3.2	4.8	3.2	3.6	3.6	3.4	3.3	1.8	6.1	1.1	1.4	2.4	2.0	3.2	3.5	1.8
Serine	29.9	36	37.8	36.5	27.9	41	36.9	41.0	28.6	42.1	43.6	66.3	43.7	44.5	50.5	37	43	69	41
Threonine	17.9	18	19.7	17.1	20.6	20	18.5	24.0	19.1	22.0	17.9	26.4	26.1	25.8	29.2	29	27	25	25
Methionine	5.0	4.3	5.1	5.4	5.7	6.3	5.7	4.7	6.2	6.5	6.1	8.7	4.0	10.0	8.8	13	12	13	12
Arginine	46.0	50	49.0	48.2	49.9	49	49.0	50.1	44.8	49.5	49.9	49.2	51.0	50.3	52.4	53	53	51	45
Histidine	4.5	5.0	5.8	6.0	4.2	5.1	5.4	5.7	4.5	4.7	4.7	6.5	5.1	7.4	4.8	3.8	4.5	7.5	7.4
Lysine	28.6	27	26.2	26.2	35.5	29	21.6	25.9	19.0	25.3	27.6	29.1	24.2	24.3	21.8	26	27	25	22
Aspartic acid	44.0	45	49.8	46.8	49.2	47	48.4	46.3	48.1	45.5	48.0	54.9	48.6	42.6	47.5	47	47	52	54
Glutamic acid	71.7	72	75.8	72.0	76.4	74	72.3	69.6	74.1	72.8	62.4	77.9	78.9	65.8	70.5	71	74	75	81
Hydroxyproline	99.6	94	100.8	95.5	102.4	100	92.1	89.1	98.5	92.8	102.0	77.5	73.1	78.5	82.0	81	73	53	70
Hydroxylysine	6.3	7.4	6.4	5.9	—	5.7	8.9	5.8	9.6	4.9	4.0	4.3	5.3	4.7	10.7	7.4	4.5	6.0	7.9
Amide groups	43.9	46	41.8	40.8	—	51	44.0	25.6	40.1	25.5	22.1	53.9	46.8	29.4	41.0	38	26	33	42
Total N	18.6	—	18.3	18.3	17.9	—	17.9	18.6	17.8	18.3	16.2	18.0	18.2	18.2	18.5	—	—	—	—
Mean residue weight	91.3	—	92.4	91.3	92.4	—	91.6	91.1	91.1	91.4	91.2	93.3	91.4	90.8	90.7	—	—	—	—

[a] Residues of amino acids per 1000 total residues.   [b] Abbreviations: C = collagen; G = gelatin; E = extract.

Ref. (4).

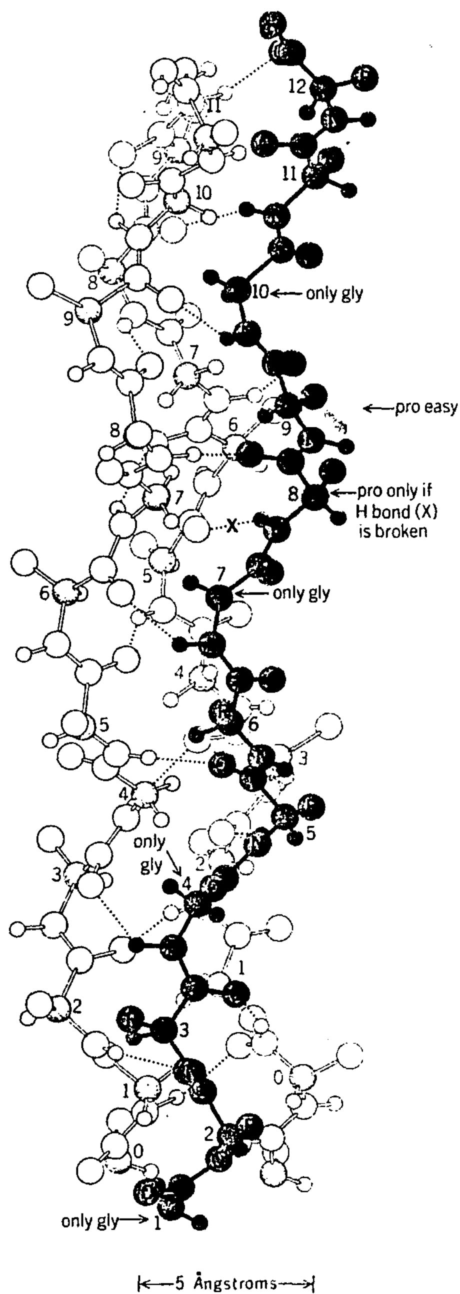


Fig. 1.2. The basic coiled-coil structure of collagen, three left-handed single-chain helices wrap around one another with a right-handed twist. Ref. (2).

and carbonyls are hydrogen-bonded to one of the other two chains. In Fig. 1.2. (2), all the carbons are numbered. Those marked 1,4,7,......to the left are too close to the other two chains for side groups and can only be Gly, explaining the observed 33 percent Gly (Glycine) composition. The bulky pyrrollidine rings of Pro (Proline) and Hyp (Hydroxyproline) can be accomodate at carbons 3,6,9,.... without strain, and without eliminating an amide involved in hydrogen bonding. The sequences (-Gly-X-Pro-) or (-Gly-X-Hyp-) where X is any residue, are thus particularly favored. Little distortion is required to accomodate Pro at the intermediate carbons 2,5,8,..... although one hydrogen bond is lost for each Pro present.

The pattern of interchain hydrogen bonding makes the collagen triple-chain coiled coil strong and rigid, although the individual chains will collapse if the hydrogen bonding is broken. It is obvious that this particular structure is induced by amino acid sequences such as $(\text{-Gly-X-Pro-})_n$, $(\text{-Gly-X-Hyp-})_n$, and $(\text{-Gly-Pro-Hyp-})_n$, since poly-glycine, poly-proline, and poly-hydroxyproline each prefer a folding very much like that of collagen (7, 8 and 9). The fact that the tropocollagen molecules line up in the fibril with just the right quarter-length displacement indicates that there must be some long-range regularity in amino acid sequence beyond that which has been described here. Although many suggestions have been made for this, the

matter is still unsettled. The amino acid sequence responsible for the triple helix is known; that which produces the higher level of organization is yet to be found.

The triple helical chain of collagen can be separated in part by warming the solution. The product formed is called parent gelatin. It results from a partial separation of the three helically coiled chains of tropocollagen as a result of breaking the many hydrogen bonds that stabilize the stiff triple helix. The rupture of the hydrogen bonds by heat seems to occur in two stages: in the first the break up of the rods is accompanied by a rapid decrease in the angular dependence of light scattering, a rapid drop in viscosity, and a rapid rise in optical rotation; in the second a slower dissociation into smaller components is accompanied by a slower drop in molecular weight measured by light scattering (10). Addition of urea to solutions of tropocollagen causes a similar drop in viscosity and rise in rotation, supporting the idea that formation of parent gelatin does not involve rupture of covalent bonds (11). High concentrations of $CaCl_2$, or KI also cause such changes in optical rotation and viscosity of collagen solution (12).

This separation is not simply a dissociation into the three distinct protein chains of which tropocollagen is composed. It appears that in addition to the large number

of hydrogen bonds linking these chains there are also some covalent bonds which are not disrupted by the gentle conditions that produce parent gelatin. The existence of some covalent bonds, together with two or three kinds of α chains give rise to the large number of products found in parent gelatin than would be expected if tropocollagen simply dissociated into its three component chains. This is shown in Fig. 1.3. where there are represented four possible situations depending on the locations of the covalent bonds, represented by solid lines between chains while hydrogen bonds are represented by dotted lines. In C1 there are no covalent bonds, in C2 covalent bonds occur only between α 1 chains, in C3 only between one α 1 and the α 2 chains. In a tropocollagen solution each of these possibilities occurs in different molecules. On heating to produce parent gelatin, hydrogen bonds are broken and C1 give rise to two 1 and one 2 chains, C2 gives a dimeric form consisting of an α 1 and an α 2 chain called β 12, and C4 gives a trimeric form called .

Preparation of purified collagen have persistently been reported to contain small amounts of carbohydrate, of the order of a half of 1%. Because of the close association in tissues of collagen fibrils with ground substance polysaccharides, it was not easy to decide whether a small amount of carbohydrate was really combined with collagen which could be more satisfactorily purified retained

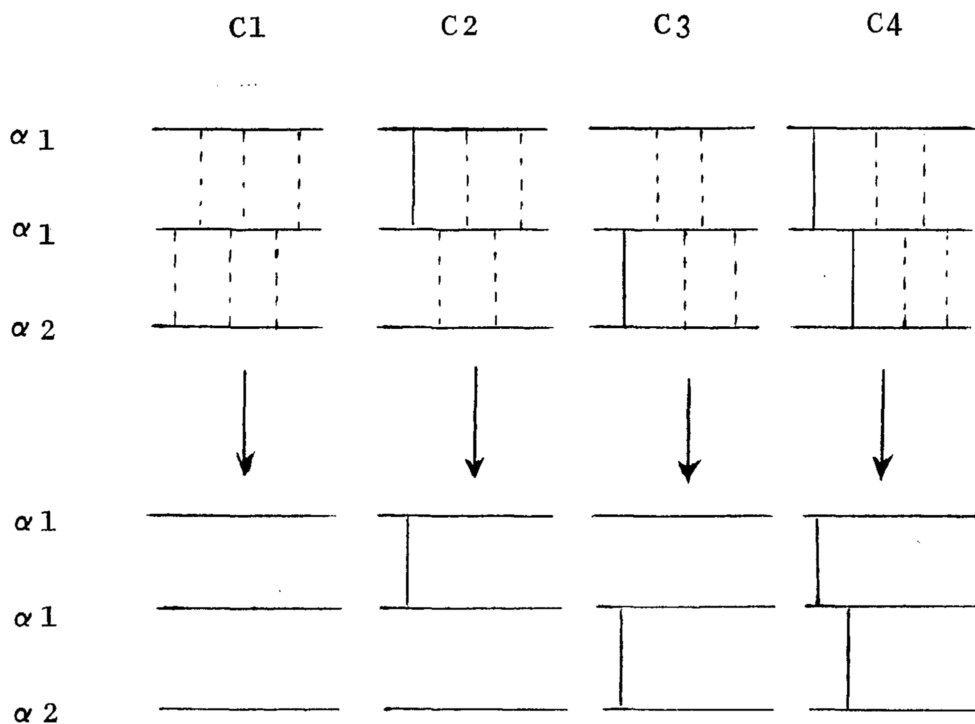


Fig. 1.3. Diagrammatic representation of cross linking of α1 and α2 chains in tropocollagen with hydrogen bonds (dotted cross-links) and covalent bonds (solid cross-links) to show the formation of 3 α chains at C1; a β11 and α2 chain at C2, a β12 and α1 chain at C3; a γ chain at C4.

about 0.5% hexose identified as glucose and galactose (13, 14).

Circular Dichroism & Collagen

The electronic transitions of the peptide group give rise to a number of spectral bands in the wavelength region below 240 nm. The strong rotatory power of these bands can be determined either from ORD Cotton effects or from CD absorption bands.

If an optically active compound is exposed to alternating left-& right- hand polarized light in the absorption region of its spectrum, then one of the components will be absorbed to a greater extent than the other. As a result the emergent light will be elliptically polarized. A diagrammatic representation fo this situation is shown in Fig. 1.4. (15). Where, the left-and right-hand components precess and the resultant is vector OM, which represents the intensity of transmitted light. Optically active materials by definition have different refractive indices for left-and right-hand polarized light, which is equivalent to saying that the velocity of left- and right-hand polarized light is different. Hence OL' and OR' precess at different rates and their vector sum, OM, traces an elliptical path. The ellipticity per unit length of sample, θ , is then defined in terms of the minor and major axes of this ellipse, $\tan\theta = OB'/OA'$. The angle is the optical rotation, and is related to the refractive index of the medium by the Fresnel equation:

$$\alpha \text{ (radians)} = \pi \ell (n_L - n_R) / \lambda$$

where ℓ is the path length of the light in the medium, n is refractive index. The difference in absorbance ($A_L - A_R$) is used to calculate the dichroism of the sample, expressed as molar ellipticity, as follows:

$$[\theta]_\lambda = 3300 (\varepsilon_L - \varepsilon_R).$$

ε is molar extinction coefficient.

Table 1.2 shows CD data for polypeptides (15). Fig. 1.5. illustrate the CD spectra of poly-lysine in three different conformations (16). Fig. 1.6. shows CD spectrum of triple-helix calf skin collagen (17). Since only the native collagen shows a peak around 220 nm of its CD spectrum (18,19, 20). The change of this peak after collagenase degradation can be used to study the kinetics of collagen-collagenase interaction (21).

Fig. 1.4. Action of optically active medium on plane-polarized light. Right circularly polarized light absorbed to a greater extent than left, and index of refraction for left greater than that for right, as depicted.

Ref. (15).

Table 1.2.

Circular Dichroism Data for Polypeptides

PPII (poly-L-proline II)

Spectral characteristics	β sheet	Helices			Random
		α	PPII	calf skin collagen	
Max (+)	196	191	226	223	
Min (-)	219	221	207	198	205
		209			
Crossover	207	207	220	215	

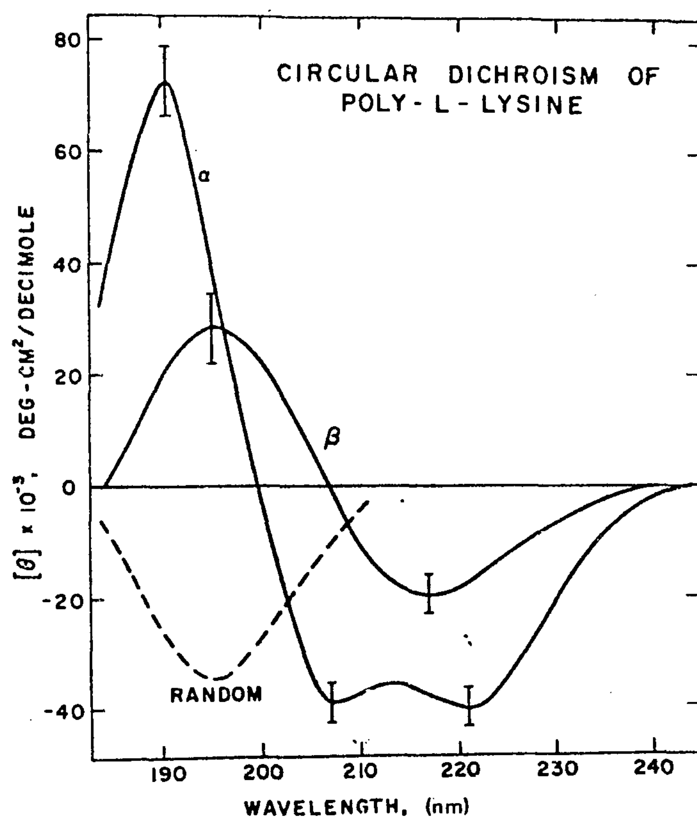


Fig. 1.5. CD spectrum of poly-lysine in the α helical, β, and random coil conformations.

Ref. (16).

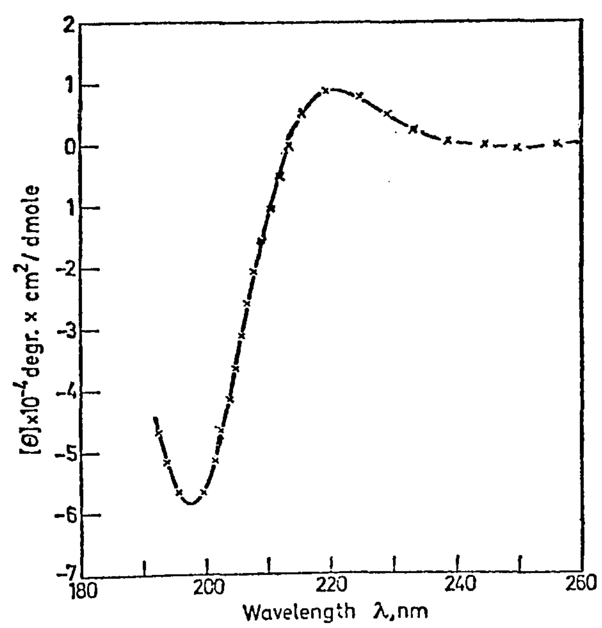


Fig. 1.6. CD spectrum of calf skin collagen.

Ref. (17).

Collagenase

To qualify as a collagenase an enzyme must be capable of causing hydrolytic cleavage of molecules of collagen in their native conformation. A further restriction in the definition is that cleavage must occur in the domains of the substrate pervaded by the collagen helix. This qualification is made necessary by the fact that native molecule of collagen have regions of their polypeptide chains that remain outside the collagen fold, and these are amenable to scission by general protease such as trypsin or chymotrypsin (22, 23). Accordingly, a discussion of collagenases must be prefaced with some consideration of the nature of the substrate.

Collagen from a variety of sources, vertebrate and invertebrate, have been studied as substrate for collagenases. All are made up of polypeptide chains that as a consequence of special amino acid composition and sequence assume a characteristic helical structure for which a proteolytic model is that of poly-L-proline (form II) (8, 9). The responsible composition is the occurrence, in those regions bearing the characteristic helix, of a glycine residue as every third residue, and a content of proline plus hydroxyproline residues that approximates 20% or more of the total residues. Relative to the residues of glycine, those of the imino acids are spaced so that triad sequences

of -Gly-Pro-X- and -Gly-Pro-Hyp- occur with sufficiently great frequency to cause the characteristic helix to pervade the major part of the polypeptide chain. Therefore a collagenase is defined as an enzyme capable of causing hydrolytic scission of peptide bonds located in the characteristic poly-L-proline type of helical regions when the substrate is in the undenatured state. This definition permits inclusion of enzyme action in these regions and perhaps making only one cut per α chain, as in the case of some tissue collagenases (24, 25), and other enzymes acting in the same regions but making multiple scissions, as in the case of clostridial collagenases. The definition does not require that the enzyme cleave bonds in the repeating "nonpolar", "crystalline" regions of the α chains responsible for the typical collagen structure. In fact it allows inclusion of an enzyme, if such is discovered, that acts on the more "polar" regions located in the helical domains. So far little is known of the specificities of the many collagenases described to state whether these indeed act only in nonpolar regions. On the contrary side of this requirement, a peptidase that cleaves bonds in a synthetic oligopeptide containing sequences identical with those in a nonpolar region of collagen cannot be classified as a collagenase unless it also acts on undenatured collagen (22, 23). Furthermore, a general protease such as trypsin that readily digests parent gelatin extensively cannot be considered a collagenase since it does not attack

the same bonds held in the context of the typical helical regions of the undenatured substrate. Yet many general proteases, as mentioned before, can conduct limited proteolysis of undenatured collagen, but only in the terminal regions of α chains outside the helical domains.

In summary, then, an enzyme does not qualify as collagenase if it digests native collagen in terminal regions of the chains outside the collagen fold, if it digests denatured collagen, or if it cleaves sequences in oligopeptides resembling sequences found in the helical regions of α chains. It does qualify, however, if in addition to one or all of the above it cleaves sequences in the typical helical regions when the collagen is undenatured.

At present, approximately 20 enzymes have been described that classify as collagenases. Broadly there are two groups of collagenases. One group is elaborated by microorganisms that in themselves do not contain collagen. A second group of collagenases, the so-called tissue collagenases, are produced by multicellular organisms that in fact have collagen as a major extracellular component of their tissues. The eventuality that a third group of collagenases will be recognized as serving strictly nutritional (digestive) functions in multicellular organisms is presaged by the discovery of an extractable collagenase

of the heptopancreas of the crab (26). There are no rigorous demonstrations of mammalian pancreatic collagenases, although one should anticipate that such enzymes, homologous with the known pancreatic proteases, should have evolved. These might be expected to occur in carnivores.

One can infer two sorts of functions for the collagenases of microorganisms, those associated with mechanisms of invasion of a host and those that are nutritional. An example of the first would seem to be the collagenases of clostridia. These enzymes are capable of weakening and destroying the connective tissue barriers of a host. Most of the collagenases of this sort are truly extracellular, i.e., they are secreted and do not appear in the growth media merely as the result of cell death and lysis. The second function of collagenases of microorganisms could be digestive in the nutritional sense. The collagen of the host would be cleaved to smaller peptides by the collagenases, and associated peptidases could act to provide amino acids for nutritional purposes.

The action of most tissue collagenases, however, appears to be more limited and directed to different ends. The collagenase such as that of the resorbing tail of the tadpole or the involuting postpartum uterus are exquisitely controlled. They function in a program of remodeling of specialized tissues at a particular time of development or

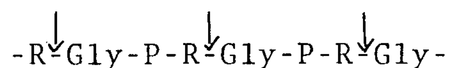
physiological expression. Other tissue collagenases are associated with a mechanism of repair of tissues, removing injured collagen and perhaps promoting regeneration or wound healing in an orderly manner. When searching for collagenolytic activity in tumor tissues, Robertson and Williams (27) could report only minimal breakdown effects. Collagenases were definitely demonstrated, however, in tumors of both epithelial and mesenchymal origin by the tissue culture assay (28, 29). The question of collagenase playing a role in malignant tumor invasion is of special interest.

All tissue collagenases, whether part of a system of general turnover of collagen or part of a mechanism of remodeling or repair of tissues, may provide secondarily a means of conservation of amino acids and in this sense serve a nutritional function as well.

The overall action of collagenases is to initiate disruption or destruction of the native collagen molecule. The disrupted molecule may suffer a decrease in stability of its helical structures and thereby become more susceptible to the action of general proteases or peptidases that may be present. Clostridial collagenases, and perhaps other collagenases of microorganisms, not only initiate the disruption of collagen but also carry out its extensive degradation as well. They are sufficient for the function

of invasion. For the nutritional function, their action would have to be supplemented by the actions of other proteases and peptidases.

The occurrence of an extracellular collagenase of clostridium histolyticum (E.C.3.4.4.19) was first demonstrated rigorously by Maschmann (30). This investigator clearly differentiated between "gelatinase" activities of general proteases acting on denatured collagen and highly specific collagenases acting on an insoluble, undenatured collagen substrate. Following these observations, Bidwell et al. (31), MacLennan et al. (32), and DeBellis et al. (33, 34), made significant contributions in the preparation of what is now known as clostridiopeptidase A. Michaels et al., and Kasakova et al., established that the action of collagenase on collagen resulted in formation almost solely of peptides with a glycyl residue in the amino-terminal position (35, 36). This indicated that the minimum requirement for specificity for the enzyme is a peptide bond involving the amino group of glycine. The specificity requirement for the sequence was as following:



where P stands for proline or hydroxyproline and where R represents a position which may be occupied by many amino acid residues. The arrows indicate the peptide bond cleaved. The specificity requirement for clostridiopeptidase A indicated above was also verified by Harper using

synthetic tripeptide polymers (22).

Gallop and Seifter (37) reported that Ca^{++} ion was required for both the binding of the enzyme to the collagen and for full catalytic activity. Magnesium ions couldn't substitute for Ca^{++} ions. Thus agents such as EDTA inhibited collagenolytic activity in the first instance by binding Ca^{++}. Takahashi and Seifter (38) showed that collagenase was able to be photoinactivated in the presence of methylene blue, and that enzymatic activity was fully lost after destruction of four of the total sixteen residues of histidine; calcium ions were shown to offer strong protection against the inactivation. Also there was much evidence that an intrinsic metal component, most likely a zinc atom, was present at the active site of collagenase. Maschmann (30) originally described inhibition of collagenase by cysteine. Harper and Seifter (39) studied details of the inhibition. In addition to its inhibition by cysteine, it was inhibited by reduced glutathione, by 2,3-dimercaptopropanol and by dithiothreitol. It was poorly inhibited by -SH containing substances that did not contain a second functional group capable of chelating strongly. Seifter suggested that cysteine and similar reagents inhibited by their properties of chelation and that they were acting on a probable zinc component in the enzyme. Seifter et al. (40) were used to prepare collagenase from a clostridial culture medium in which Zn^{65} was incorporated. The Zn^{65}-collagenase was

first treated with 2,3-dimercaptopropanol and then passed through a sephadex column that resulted in a Zn^{65}-free protein, enzymatically inactive, and Zn^{65}-thiol complex. The zinc-free protein was then treated with S^{35}-cysteine and this solution then passed through a column of sephadex. The elution pattern showed recovery of nonradioactive enzymatically inactive collagenase and of all the cysteine added. Thus, prior removal of zinc from collagenase rendered it incapable, subsequently, to bind with cysteine.

The collagenases have proved to be of considerable use to biochemists and biologists in general as investigative tools. Laboratory applications of collagenases have been limited largely to clostridialpeptidase A. In the field of collagen research, the highly specific clostridial collagenase has proved of inestimable value in mapping out the repeating nature of amino acid sequences in α - chains (41, 42 and 43). Also it has been used effectively to study the unfolding and refolding of the collagen helix. The enzyme also has helped locate specific structural features of the collagen molecule such as the aldol cross-linkages (44, 45) and the hydroxylamine-sensitive linkages (46). The tissue collagenases have served to provide larger fragments of α chains that permit a study of the self-assembly process in collagen (47, 48). Collagenases are useful as a specific means of identifying collagen. Collagenases are being used with considerable effect as a

means of loosening cells held in a net of connective tissue, thereby allowing subsequent isolation of specific cell types. Thus, in studies relating to the biosynthesis of insulin; collagenases have been applied successfully for the preparation of islet cells of the pancreas (49). Finally, collagenases may be employed in the study of certain disease process and of wound-healing mechanisms. Collagenase preparations incorporated into ointments were used for topical application in the treatment of burns and the debridement of ulcera and decubiti (50, 51 and 52). Further clinical applications appear quite feasible and are under investigation.

In this work a new method is developed to study the collagen-collagenase interactions. Since the circular dichroism spectrum of native collagen shows a maximum around 220 nm. The maximum will change after heat denaturation or collagenase hydrolysis. It is, therefore, possible to study the collagenase reaction by examining the rate of change of the maximum.

In order to study if Ca^{++}ions are necessary for the collagenase-collagen interaction, EDTA is added to the reaction mixture. If the enzyme activity is inhibited then increase the Ca^{++}ions and observe if the enzyme activity can be restored.

Previously, it was found that imino acid content is the determinant of stability of collagen. Since hydrogen bonding can be formed through the hydroxyl group of hydroxyproline, the effect of hydroxyproline content to the stability of collagens and to the susceptibility of collagenase digestion are studied.

At acidic pH mucopolysaccharides stabilize collagen by charge interaction. Since collagen-collagenase interaction is studied under neutral pH, therefore mucopolysaccharides effect on Tm of collagen and on collagen-collagenase interaction are investigated at neutral pH.

Chapter II

Material and Methods

Materials

1. Proteins

Clostridium histolyticum collagenase (E.C.3.4.4.19) CLSPA grade was obtained from Worthington Biochemical Co. The acid extracted collagen of carp swim bladder (ichthyocol) was prepared by the procedure described previously (53, 54). Calf skin collagen was obtained from Sigma Chemical Co. Bovine serum albumin was from Pentex. Lathyritic rat skin collagen was a generous gift of Dr. Coffey of Hoffman LaRoche.

2. Reagents

Tris-(hydroxymethyl)-amino methane, calcium chloride dihydrate, succinic acid, formic acid were obtained from Fischer Scientific Co. Disodium EDTA dihydrate was from Baker Chemical Co. Anthrone and thiourea were obtained from Matheson Coleman & Bell. Rare earth metal chlorides were obtained either from ALFA Inorganics Ventron or BDH chemicals. Magnesium chloride, cobalt chloride, manganeous chloride and cupric chloride were obtained from Fisher. Acrylamide, N.N'-methylene bisacrylamide, N,N,N,N'-tetramethylethylenediamene (TEMED), ammonium persulfate and other chemicals used for disc gel electrophoresis came from Canalco.

3. Column Chromatography

Sepharose 4B was obtained from Pharmacia Inc.

Instrumentation

For all pH determinations, Corning pH meter model 7 was used. Difference spectra were measured by a Cary model 17 spectrophotometer. Collagenase kinetic studies were performed with JASCO spectropolarimeter model J-20 and Beckmann DU spectorphotometer with Gilford attachment. Equipment for disc gel electrophoresis was from Canalco. Preparative centrifugation was done with the Sorvall model RC-2-B. Automatic Freeze dryer model #10-010 from Virtis Co. was used for lyophilization.

Methods

Determination of protein concentration

Collagenase

Collagenase was dissolved in distilled water or in 0.05M Tris buffer, 0.5M $CaCI_2$ at PH 7.0. Absorbances were measured at 280 nm and 260 nm and plugged into the following equation. Collagenase concentration was calculated accordingly (55).

$$\text{Protein concentration mg/ml} = 1.55\ D_{280} - 0.76\ D_{260}$$

Collagen

It was found that the ellipticity of collagen solution at 220 nm was proportional to its concentration. Therefore a standard graph relating concentration and ellipticity was prepared and subsequently was used as a method for determining the concentration (21).

Preparation of ichthyocol from carp swim bladder

Ichthyocol was prepared accordingly to the methods by Seifter and Gross (53, 54).

Approximately 500g of swim bladder external tunica were

blended in a Waring blender for 1-2 mins. at 5° with pre-chilled 0.5M sodium acetate solution and chips of dry ice. The volume of homogenate was then brought to 3 liter with the same reagent. The mixture was stirred mechanically overnight and then centrifuged at 200 r.p.m. at 5° for 1 hr. The supernatant was discarded. The pasty residue was transferred to a clean towel and squeezed until most of the liquid was removed. This process was performed two times more, yielding finally a residue from which non-collagenous solutble proteins and polysaccharides were removed; a fraction of soluble collagen was also removed by the slightly alkaline medium. The residue was next suspended in 2ℓ of cold distilled water and centrifuged at 2000 r.p.m. at 5° for 1 hr. The supernatant was discarded, and the precipitate was again suspended in 2ℓ of water and centrifuged. Repeated once more. The precipitate was then transferred to a clean towel and the liquid was squeezed out. The residue was then suspended in 2ℓ of cold 0.1M sodium citrate buffer, pH 4.3 , and the mixture was stirred at least 24 hrs. It was then centrifuged, and the supernatant was stored in the cold. Citrate buffer extraction was carried out two times more. Supernatant from each were combined and centrifuged at 18000 r.p.m. at $0\text{-}5^{o}$ for 1 hr. To remove residual particles, the supernatant was filtered through glass wool. The filtrate was dialyzed with stirring at 5° C against large volume of 0.02M disodium hydrogen phosphate solution. The dialysis medium was replaced

frequently by fresh solution so that its pH continued to be basic. When the pH of the protein solution within the sacks became neutral or slightly alkaline, thick, rigid, needlelike fibrils of collagen appeared. This process usually occurred after 8 h. but the time varied with efficiency of dialysis. Dialysis was then continued for 48 hrs., during which time the external medium was replaced several times by fresh solution. The collagen was collected by centrifuged at 2000 r.p.m., and the precipitate was resuspended in distilled water and centrifuged again. This procedure was repeated two times. 25% TCA was added to the collagen water suspension dropwise, until the solution was at pH 3.5. After standing in the cold for 30 mins. any residual particles were removed by centrifugation. The collagen rich supernatant was dialyzed immediately against phosphate buffer to remove TCA and to adjust to neutral pH. After dialysis, absolute alcohol was added dropwise to a final concentration of 14 percent. The solution was at 4°C overnight. The collagen precipitate which was formed in the centrifuge redissolved by dispersing in small successive volumes of 0.5% acetic acid and stored in the freezer or dialyzed against phosphate buffer and distilled water and then stored as an aqueous suspension in the freezer.

Determination of carbohydrate content of collagen

1. Sample preparation (56)

10-20 mg collagen was hydrolyzed in 2 N H_2SO_4 for 4 hrs. at 100 C in nitrogen gas sealed tube.

2. Anthrone reagent (57)

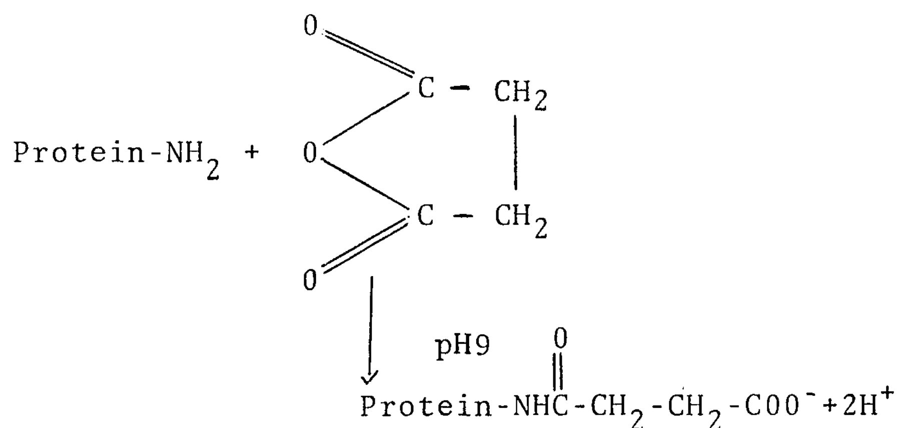
A solution containing 0.05% anthrone, 1% thiourea and 66% by volume H_2SO_4 was used.

3. Standard curve

A standard 0.25% glucose solution was prepared. A linear curve in the concentration range of 50-250 μg/ml was obtained at 620 nm.

Succinylated collagen

Succinylated collagen was prepared according to the method of Rantuberg and Kühn (58). A 5.5% collagen in 0.1 N acetic acid was adjusted to pH 9.5-10 by adding 10% NaOH at which time collagen fibers were formed. 100 mg succinic anhyride in 5 ml acetone were added to the collagen solution dropwise with stirring. The collagen solution was kept at pH 9-10 by adding 10% NaOH, stirred for 1h at this pH, then adjusted to pH 4.2-4.5 with 1N HCl. Succinylated collagen precipitated at this pH, which was washed thoroughly with distilled water (58, 59 and 60).



Disc gel electrophoresis

1. Collagen

The separation gel contained 7.5% acrylamide in a 0.438 M acetate buffer pH 4. The separating gel catalyst was 0.15% ammonium persulfate. The upper and lower electrode buffer was 0.0038 M acetate buffer pH 4.0 (61). 50-100 μl a 1-10 mg/ml proteinsolution was applied on top of the gel. 4-phenylazo-1-naphthylamine was used as tracking dye. A current of 2.5 mA per gel was applied for one hour, in the cold room (5° C). The gels were removed from their tubes stained overnight in 1% amid amido black in 7% acetic acid, then destained electrophoretically in the quick gel destainer and stored in 7% acetic acid. The destaining solution was H_2O : Glacial Acetic Acid : Ethanol = 73 : 7 : 20 by volume.

2. Collagenase

The gels were prepared according to the instructions of the Canalco manual. The separating gel contained 7.5% acrylamide in a 0.375 M Tris buffer pH 8.8-9.0. The separating gel catalyst was 0.15% ammonium persulfate. The upper and lower electrode buffer was 0.005 M Tris-glycine pH 9.5 (62).

Collagenase was dissolved into saturated sucrose solution, to the concentration of 0.2 mg/ml. Bromophenol blue was used as tracking dye. 75 μl of sample was applied to each gel. A current of 4 mA was applied to each gel for 50 mins. at 5° C. Gels were stained with 1% amido black, destained electrophoretically and stored in 7% acetic acid.

Affinity chromatography

Cyanogen bromide activation of sepharose was based on procedures previously described (63, 64 and 65).

Sepharose 4B (decanted) was mixed with an equal volume of water, and cyanogen bromide (100 mg per ml of settled sepharose) was added in an equal volume of water. The pH was immediately adjusted to and maintained at 11 by titration with 4 N NaOH. The temperature was maintained at about 20° by adding ice as needed. The reaction was completed in 8 to 10 mins., as indicated by the cesation of proton release. A large amount of ice was then rapidly added to the suspension, which was transferred quickly to a Büchner funnel and washed under suction with 0.2 M $NaHCO_3$ pH 9. Approximately 30 ml of cyanogen bromide activated sepharose 4 B was suspended in 60 ml 0.2 M $NaHCO_3$ (pH 9.0) and 100 mg of calf skin collagen in 30 ml of 0.4 M NaCl was added immediately. The mixture was stirred gently for 18 h at 4°, filtered, washed with water and equilibrated with 0,05 M Tris-HCl (pH 7.0) containing 0.005 M $CaCl_2$.

Affinity chromatography was carried out by applying 19 mg of collagenase to a column (1.2x10 cm). The buffer, 0.05 M Tris - HCl, 0.005 M $CaCl_2$, pH 7.5 was passed through the column until the absorbance at 280 nm remained at the base line. Elution was accomplished by addition of 1.0 M

NaCl to the same buffer. Eluant fractions were dialyzed against water and lyophilized. After reconstitution of the enzyme powder in buffer, samples were assayed for collagenase activity and examined by poly-acrylamide gel electrophoresis on 7% gels.

Tm determination of collagen in different solvents or in carbohydrate solutions by circular dichroism

Collagen in solution of 0.01 N HCl, acetic acid, citric acid, succinic acid, formic acid with and without $CaCl_2$ were tested. Collagen in Tris-HCl buffer with 0.5 M $CaCl_2$ was tested also. Ichthyocol (0.5 mg/ml)-chondroitin-6-sulphate (0.22 mg/ml) mixtures with r = 2.7, 5.5, 11 respectively, were investigated. r was defined as the number of disaccharide residues per 100 amino acids (66, 67 and 68).

Circular dichroism (CD) spectra were recorded on a JASCO J-20 recording spectropolarimeter using a jacketed cell of path length of 1 cm. The temperature was controlled by circulating water. The data was reported in terms of ellipticity, θ in deg. The collagen melting curves were obtained by plotting the ellipticity at 220 or 222 nm as a function of temperature.

Difference spectra of collagen

Collagen in Tris buffer containing $CaCl_2$ were denatured in boiling water for 10 mins. and quick cooled in an ice bath. Difference spectra of native and denatured collagen were measured using a Cary 17 Spectrophotometer.

Assay of collagenase

Method 1

Collagen and collagenase were mixed and the increase of absorbance at 220 nm were recorded as function of time (69, 70) at 20° C.

Method 2

A JASCO spectropolarimeter model J-20 was used. The change in ellipticity at 220 nm was recorded as a function of time. Aliquots of enzyme solution were added to the collagen solution at 20°C and 1 ml of the reaction mixture was transferred to the jacketed fused cell 1 cm in cell length and maintained at 20°C for the CD measurement.

The mixing and transferring was accomplished in less than 20 sec (56).

With the CD method, the kinetics of the collagenase reaction was determined by following the decrease of θ with time. The effects of EDTA, Ca^{++} heparin, hyaluronic acid and chondroitin 6-sulfate on collagenase activity were determined.

Chapter III

Ultraviolet Spectroscopy of Collagens

Ultraviolet Absorption spectroscopy

Results

Since collagens contains no tryptophanyl and only a few phenylalanyl and tyrosyl residues, it has essentially no absorption in the 280 nm region. Thus, conventional difference spectroscopy, designed to detect protein conformational changes by changes in absorption in this region, could not be used with collagen. However, Imahori and Tanaka (71) and Rosenheck and Doty (72) have shown that the peptide chromophore shows a marked hypochromism on transfer from an α-helical environment into a random-coil state.

In Fig. 3.1., 3.2. and 3.3. the absorption spectra of native and denatured collagen in the range 210 to 250 nm is shown. There is a peak around 210 nm. In Fig. 3.4., 3.5. and 3.6. the difference spectra of heat denatured collagen relative to the native collagen shows a prominent peak about 223 nm (69, 70). The concentration of collagen used was 0.117 mg/ml for swim bladder collagen, 0.17 mg/ml for calf skin collagen and 0.195 mg/ml for lathyritic collagen.

Fig. 3.1. Absorption spectrum of a solution of 0.117 mg/ml carp swim bladder collagen with 0.5 M $CaCl_2$ in 0.05 M tris buffer at pH 7 at 20° C o---o. A sample of the same solution was denatured by heating to 100°C for 10 mins. and immediately cooled, ▫----▫ .

Fig. 3.2. Absorption spectra of a 0.17 mg/ml calf skin collagen solution in the native and denatured form. Experimental condition was the same as in Fig. 3.1.

Fig. 3.3. Absorption spectra of 0.195 mg/ml lathyritic rat skin collagen in the native and denatured form. Experimental condition was the same as in Fig. 3.1.

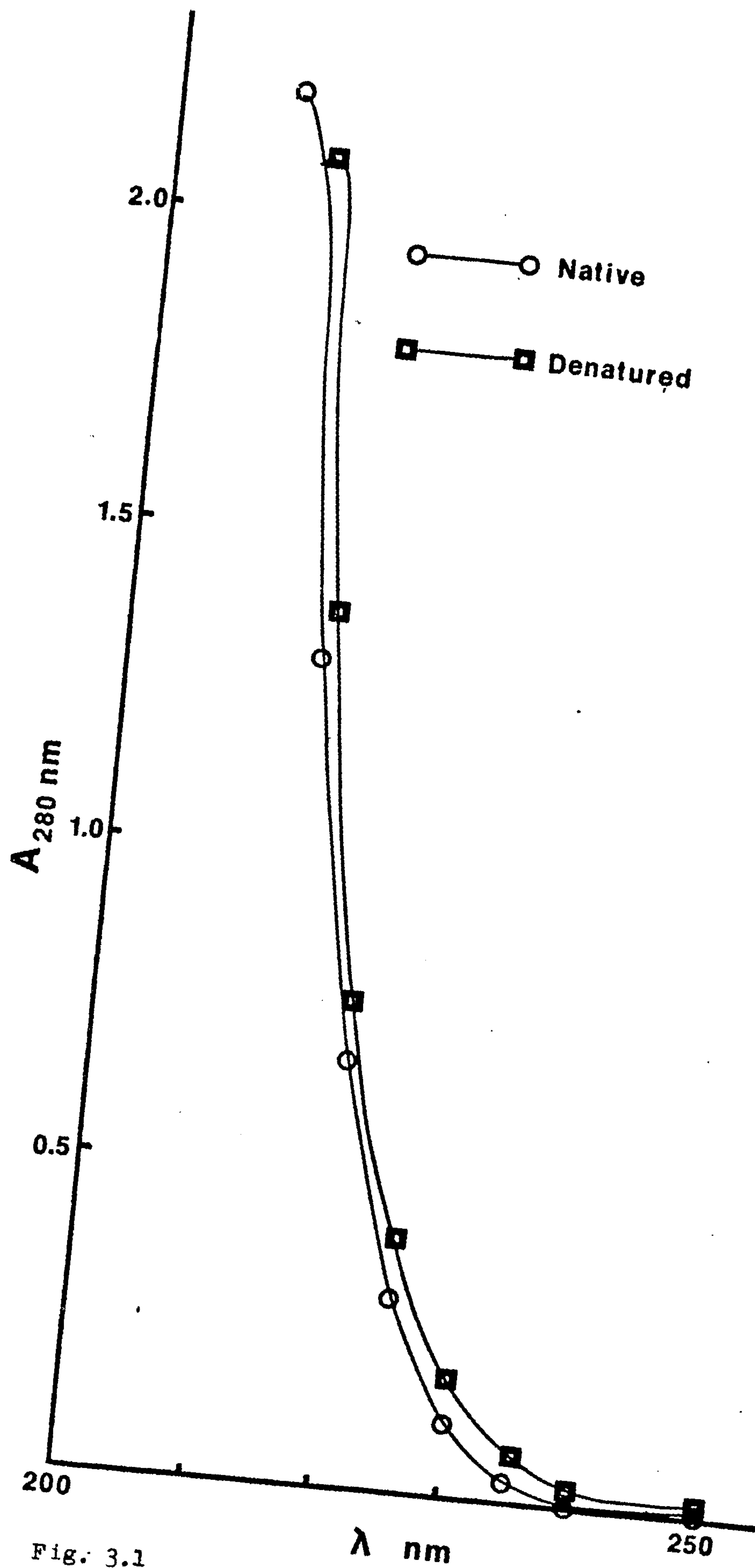


Fig. 3.1

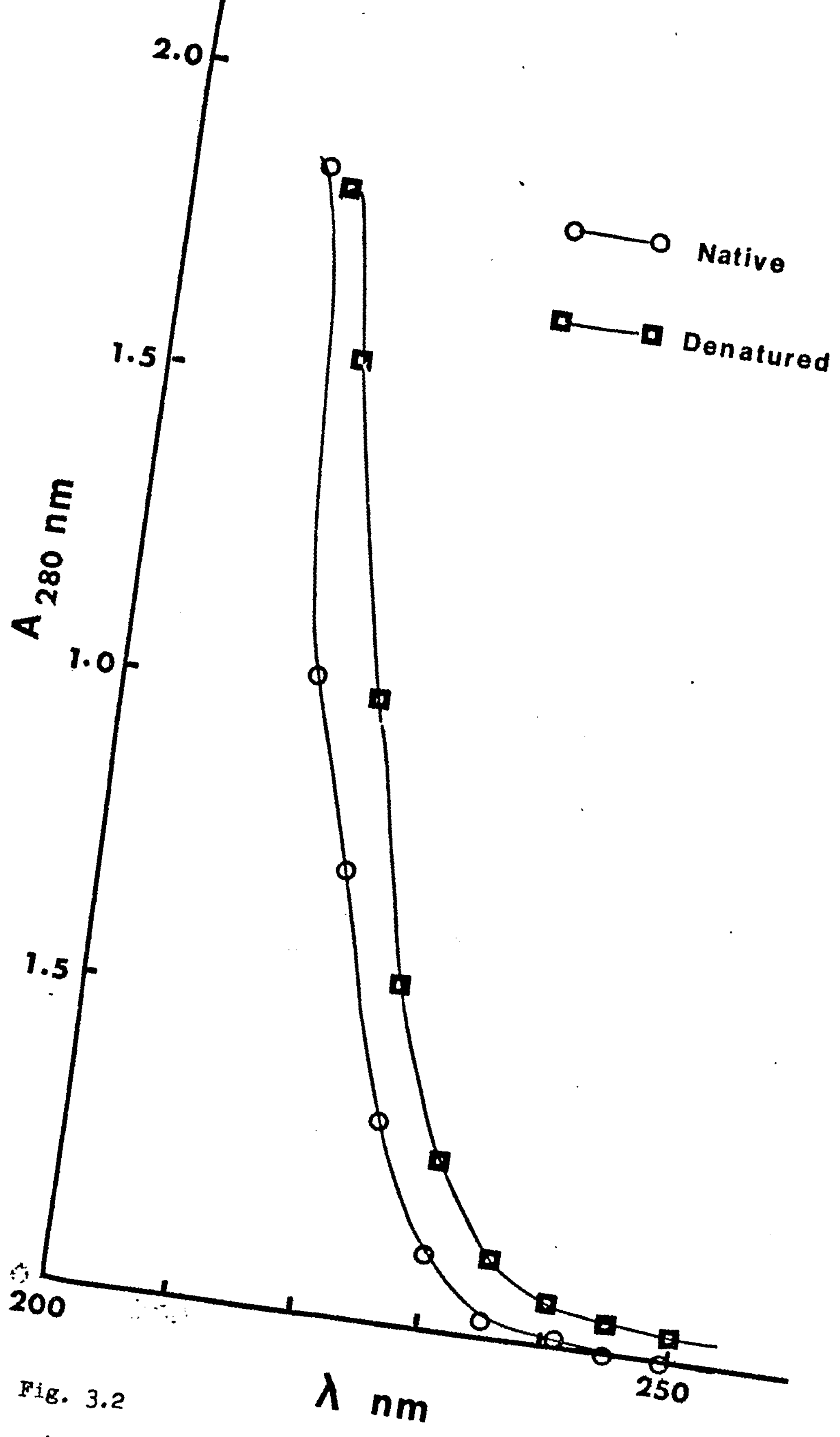


Fig. 3.2

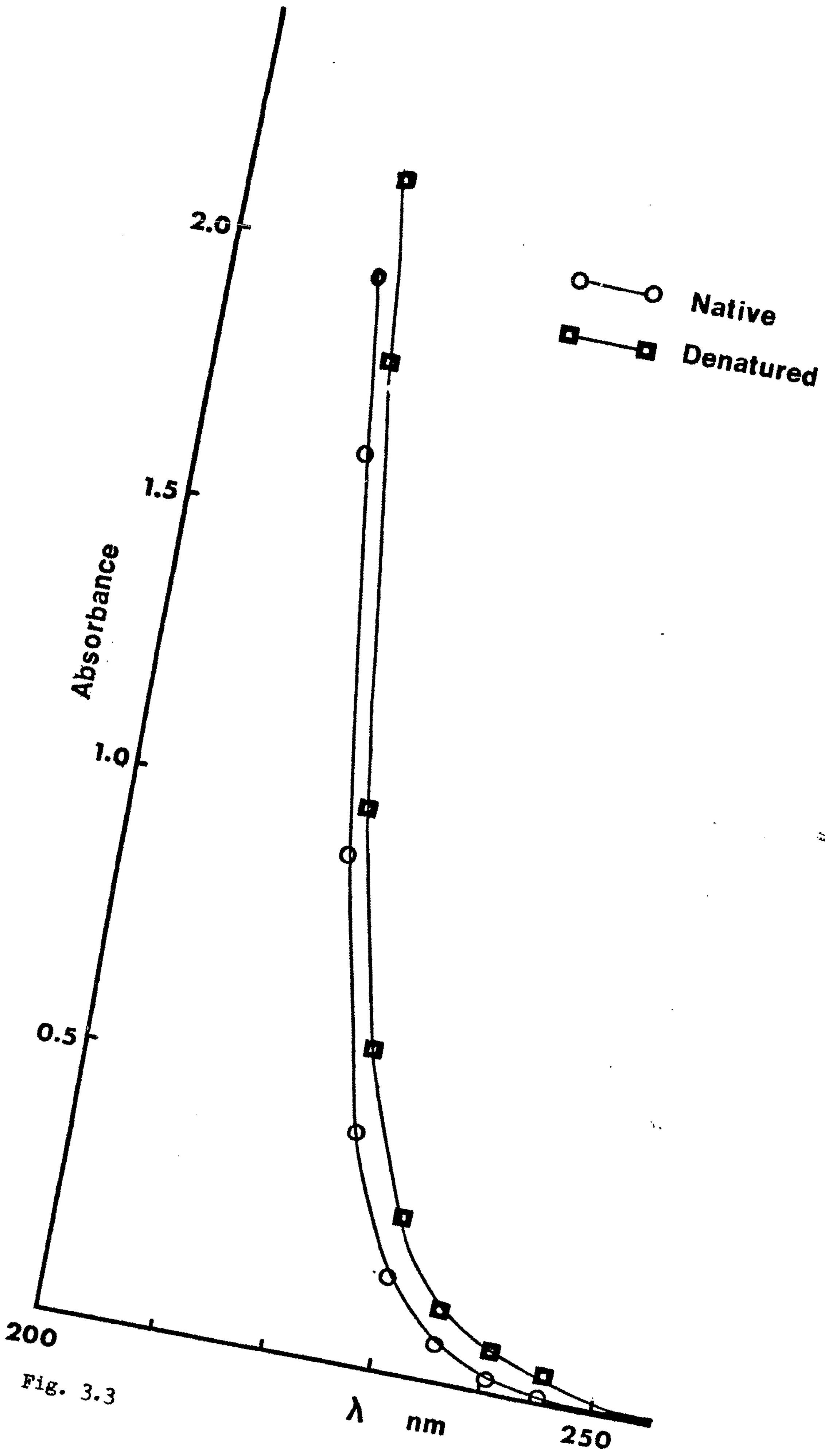


Fig. 3.3

Fig. 3.4. Difference spectrum at 20° C of a solution of 0.117 mg/ml carp swim bladder collagen with 0.5 M $CaCl_2$ in 0.05 M tris buffer at pH 7, heated to 100°C for 10 mins. and immediately cooled. Reference cell contained unheated collagen.

Fig. 3.5. Difference spectrum at 20° C of 0.17 mg/ml calf skin collagen solution. Experimental condition was the same as in Fig. 3.4.

Fig. 3.6. Difference spectrum at 20° C of 0.195 mg/ml lathyritic rat skin collagen. Experimental condition was the same as in Fig. 3.4.

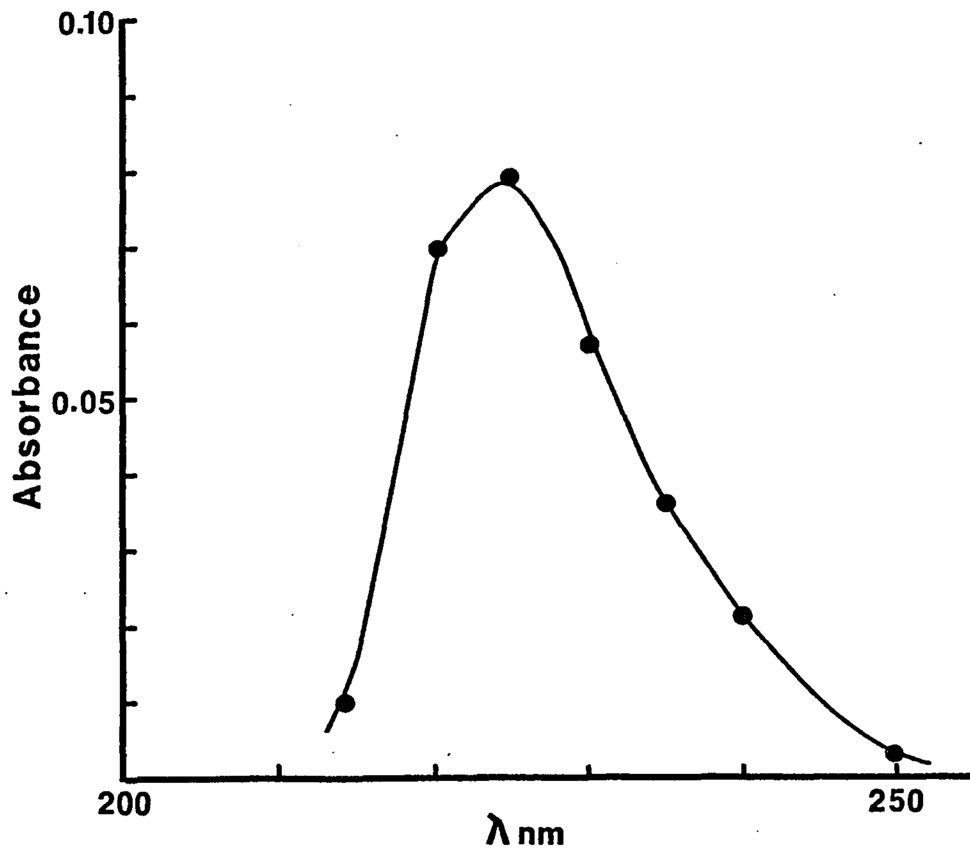


Fig. 3.4

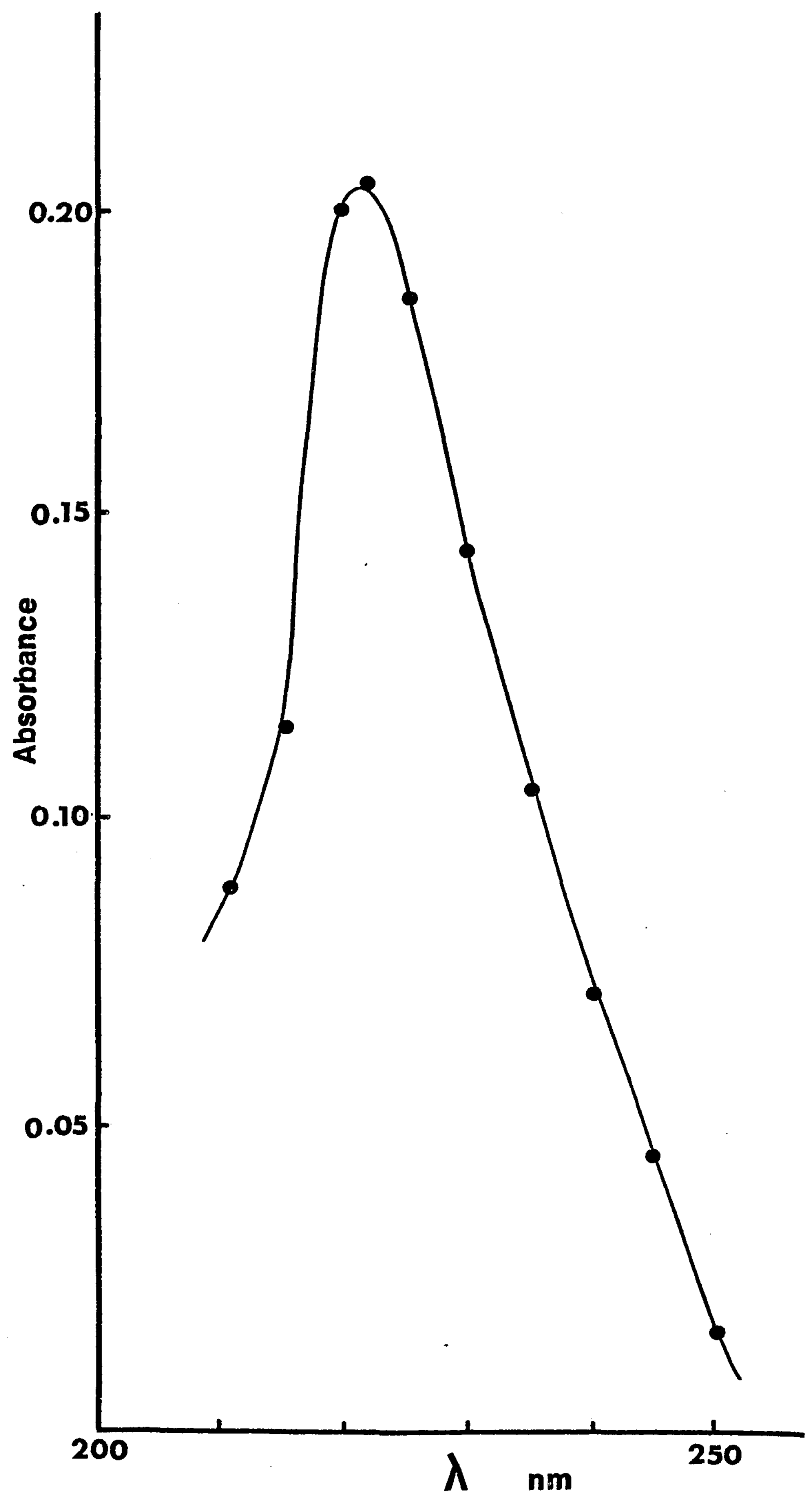


Fig. 3.5

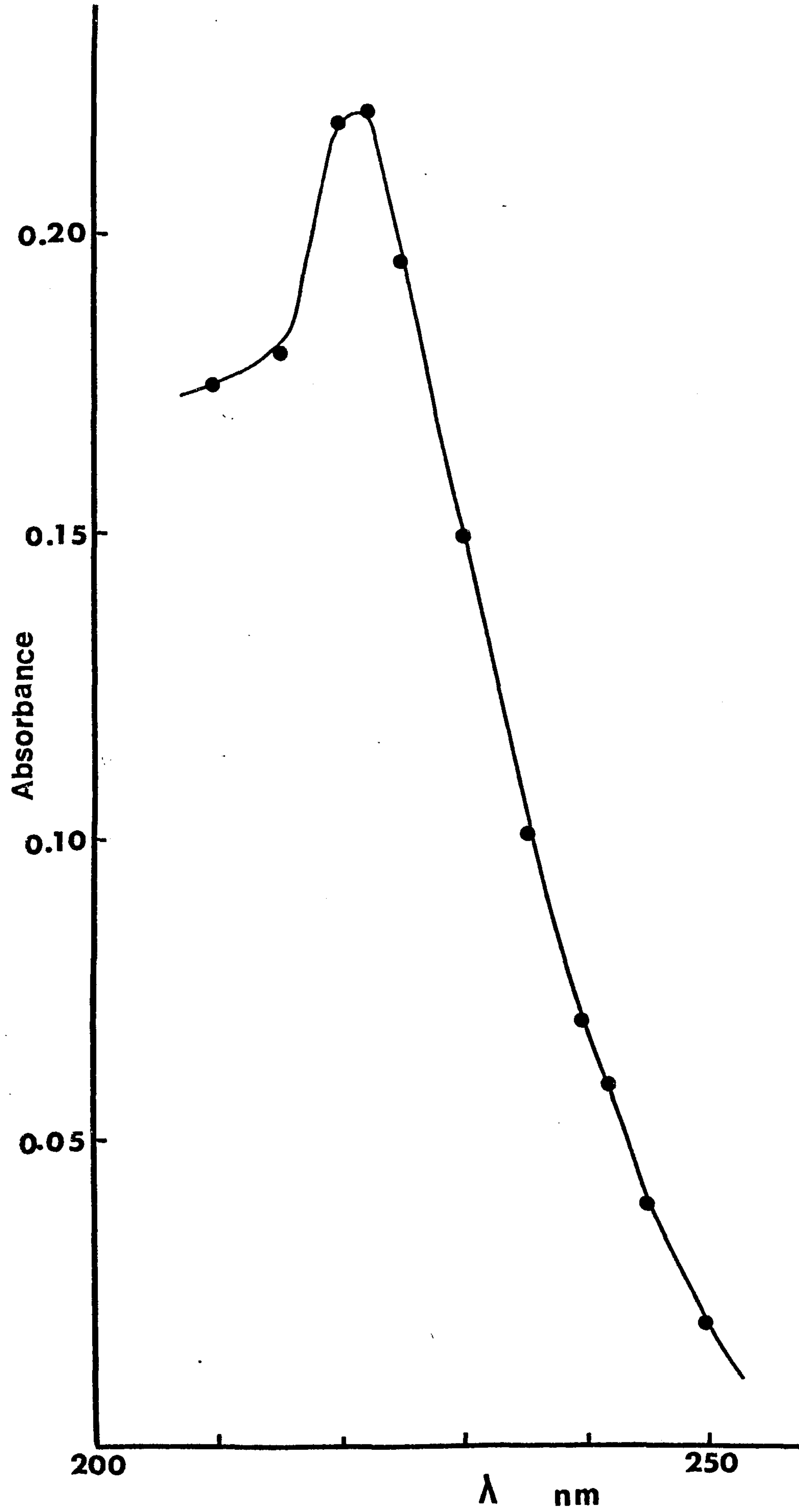


Fig. 3.6

Discussion

Doty, Wanda, and Yang (73) found that at pH 4 poly-L-glutamate exists as an α-helix. As the pH was raised and the carboxyl group ionized, repulsion between adjacent carboxylate ions resulted in a transformation of the helix into a random coil. It was shown that the change of helix to random coil followed very closely the ionization curve of the γ carboxyl of glutamic acid. The finding of a difference spectrum peak associated with the helix-coil transformation of poly-glutamic acid at 225 nm suggested that changes in the conformation of the peptide backbone were reflected in this region of the spectrum.

Tyrosine and tryptophan residues contribute substantially to the absorption of proteins in the region of 230 nm. Glazer & Smith (74, 75) studied the denaturation of several proteins and found that no correlation could be shown between the tyrosine and tryptophan content of the proteins studied and the magnitude of the 230 to 235 nm peak obtained on denaturation. They found that changes in the environment of aromatic side chains need not give rise to the difference peak at 230 nm which was given by the difference spectrum obtained by comparing pepsin at pH 5.7 to the protein at pH 7.3.

Exposure to pH 7.3 altered the native structure and inactivated it. The difference spectrum showed peaks at 278, 286 and 292 nm. These peaks represented changes in the environment of the tryptophan and tyrosine residues. Denatured pepsin failed to reveal any significant change in the region 225 to 245 nm. Therefore, changes in the tyrosine and tryptophan absorption associated with protein denaturation were not reflected in the 225 to 245 nm region of the difference spectrum. The imidazole group of histidine and the carboxyl groups of the dicarboxylic amino acids absorb in the region of 225 to 230 nm. However, the $\Delta\mathcal{E}_M$ value obtained for the changes in ionization of the imidazole group and the carboxyl group were not of the same order of magnitude as the changes observed on protein denaturation. Furthermore, as in the case of tyrosine and tryptophan, no correlation was observed between the histidine or carboxyl content of the protein studied and the difference peak in the 230 nm region. Mora and Elodi studied the near and far ultraviolet denaturation difference spectra of lactic dehydrogenase and glyceraldehyde-3 phosphate dehydrogenase. They suggested that the far ultraviolet denaturation difference spectra of the two dehydrogenases might be attributed to changes in the environment of aromatic chromophores, particularly tryptophan (76). These studies indicate that the collagen difference spectra at about 230 nm is not due to changes of the aromatic chromophores environment.

After denaturation, swim bladder and calf skin collagens showed hypochromism below 215 nm, Fig. 3.1., 3.2., but lathyritic rat skin collagen showed hyperchromism, Fig. 3.3. It is known that lathyritic collagen has less crosslinkage than collagen (77, 78). Therefore, it could be suggested that not only changes in the conformation of the peptide backbone may be reflected in the far ultraviolet region.

The nature of these spectral changes were yet unknown but one might speculate that they were a result of conformational changes in the polypeptide chains.

Chapter IV

Disc Gel Electrophoresis of Collagens and Carbohydrate Content of Collagens

Results and discussions

Disc gel electrophoresis

Typical electrophoretic pattern of denatured swim bladder collagen, calf skin collagen and lathyritic rat skin collagen are shown in Fig. 4.1. The monomers (α - components), dimers (β -components), and trimers (γ -components) plus larger intermolecular aggregates of denatured collagen are clearly separated into three distinct bands. The monomer moves faster than the dimers owing to the molecular sieving of the gel. The γ components and larger aggregates remain at the top of the gel. The electrophoretic pattern of denatured lathyritic collagen has the characteristic feature of thicker α bands. These results are consistent with those obtained from the CM-cellulose column chromatography and ultracentrifuge analysis on unfractionated samples (78).

The electrophoretic pattern of succinylated swim bladder collagen is given in Fig. 4.2. Besides three major bands as in the unmodified collagen, the succinylated collagen also shows some light bands on the top of the gel. Probably during the succinylation process there were intermolecular aggregates formed with covalent linkage through carboxyl groups of succinic anhydride and amino groups on collagen molecule.

Carbohydrate content of collagen

Results and discussion

The anthrone reagent was used to detect the carbohydrate content of the various collagen preparations. Fig. 4.3. shows that the reaction with glucose follows Beer's law up to 200 μg. Using this as a standard, it was found that calf skin collagen contains 0.6% hexose, swim bladder collagen contains 0.3% hexose and lathyritic rat skin collagen contains 0.7% hexose.

Carbohydrates in collagen are attached to hydroxylysine residues in the form of galactosylhydroxylysine and glucosylgalactosylhydroxylysine. The content of mono- and disaccharides is different in collagens not only from various species but also from diverse tissues (79, 80).

Fig. 4.1. Disc gel electrophoretic pattern of denatured collagen

a) swim bladder collagen

b) lathyritic rat skin collagen

c) calf skin collagen

Current was applied for 1.5 hr. 5 mA/gel.

Fig. 4.2. Disc gel electrophoretic pattern of

a) succinylated swim bladder collagen

b) swim bladder collagen

Current was applied for 1.5 hr. 5 mA/gel.

Fig. 4.3. Anthrone-glucose color yields follow the Beer's law.

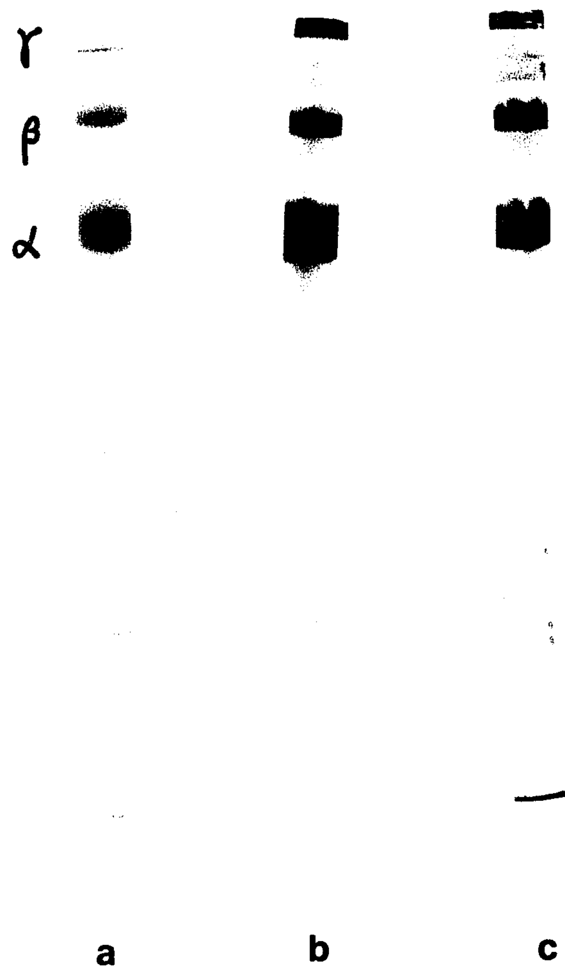


Fig. 4.1

Fig. 4.2

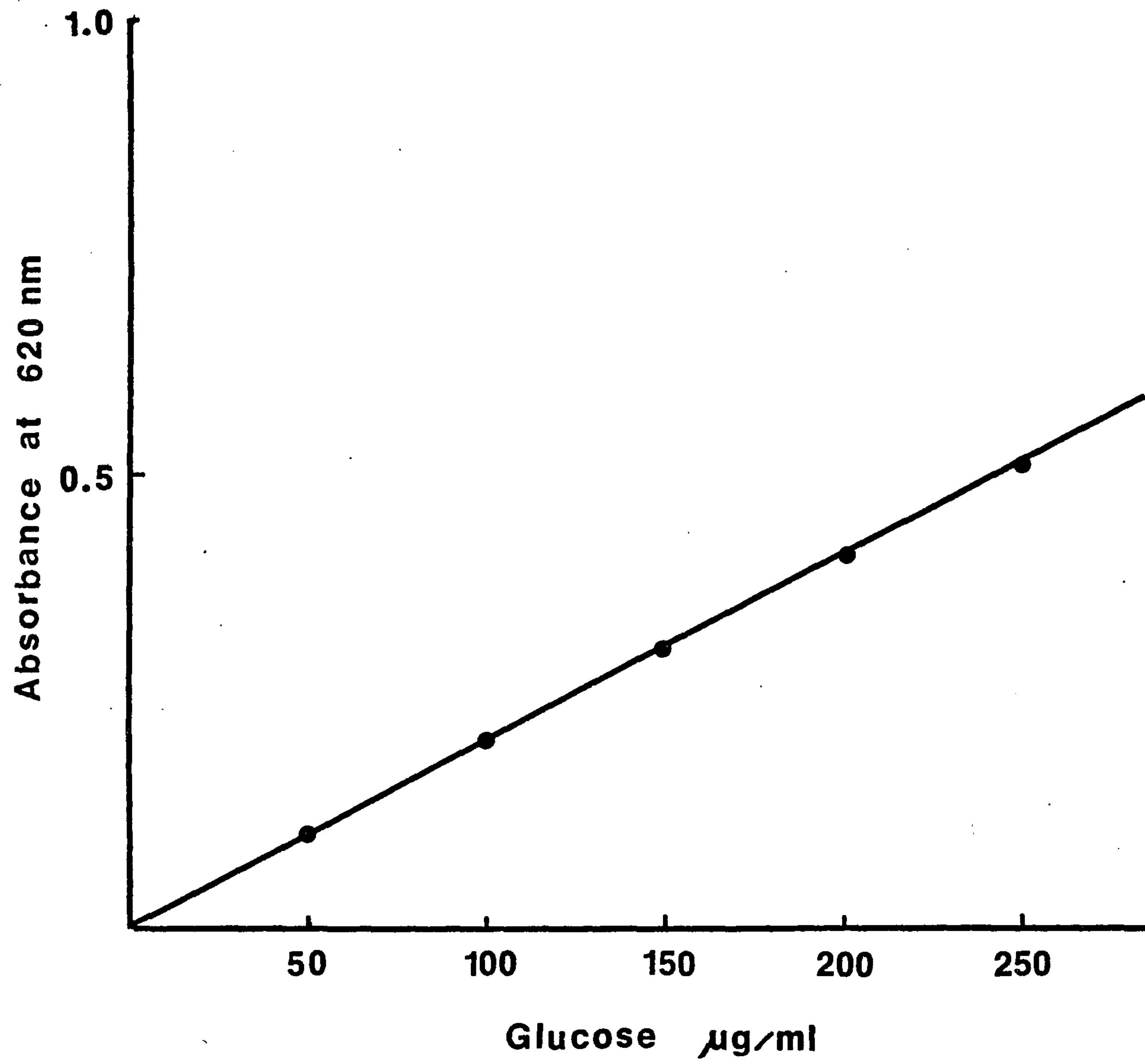


Fig. 4.3

Chapter V

Collagenase Induced Changes in the Circular Dichroism Spectrum of Collagens

Collagenase Induced Changes in the Circular Dichroism Spectrum of Collagen

The CD spectrum of collagen is characteristic of polymers that have a left-handed three-fold helix conformation (19, 20). In citrate buffer, 0.3 M, PH 3.5, it has a positive band at 223 nm, a negative minimum band at 198 nm with a crossover at 213 nm, and the $[\theta]_{Max} = 2.1 \times 10^3$ deg cm^2/ d mol (18). There is essentially no difference in the CD spectrum of collagen dissolved in ethylene-glycol-water 2:1 v/v at 24° c and at -112° c (81, 82). However at denaturing temperatures or in high concentration salt solutions, the positive band at 220 nm disappears and the curve gradually becomes more negative from zero at about 250 nm to a minimum at 198 nm that is less negative than that in native collagen (17). It was also shown that solvents affect the positions of the positive and negative bands and the molar ellipticity (18).

The CD spectra of native collagens, denatured collagen, and collagenase treated collagen are given in Fig. 5.1. In 0.05 M tris buffer PH 7.0 and 0.5 M $CaCl_2$, $[\theta]_{220}$ for carp swim bladder is 5.7×10^3 deg cm^2/d mol, $[\theta]_{223}$ for calf skin collagen in 5.2×10^3 deg cm^2/d mol and $[\theta]_{223}$ is 5×10^3 deg cm^2 d mol for lathyritic rat skin collagen.

Fig. 5.2. shows the linear relationship between the ellicipity value at 220 nm or 223 nm and the concentration of collagens. Each point is the average of the three measurements.

The action of collagenase at pH 7.0 results in the decrease of the positive 220 nm maximum for swim bladder collagen, or 223 nm maximum for calf skin or lathyritic rat skin collagen, reflecting the disruption of the triple-fold helix. Thus, by following the rate of decrease of ellipticity at 220 nm or 223 nm a collagenase assay was obtained. A typical time course of collagenolytic reaction wherein the peak at 220 nm or 223 nm decreased is given in Fig. 5.3. and 5.4. A semilogarithmic plot of these data shows that there were two first order reactions, as shown in Fig. 5.5. and 5.6. The reaction was run at various initial collagen concentrations and the slopes of the resulting lines were measured at the beginning of the reaction. By plotting log $v_{initial}$ against log S as shown in Fig. 5.7. A line was obtained with a slope of n = 0.98. Thus, within the limits of error the reaction was found to be first order in swim bladder collagen concentration in the range of 0.623×10^{-4} g/ml to 2.7×10^{-4} g/ml. At the higher substrate concentrations, the zero order region obtained and thus the slope approach zero. Calf skin collagen reacted with collagenase similarly to that of swim bladder collagen. In Fig. 5.8. an enzyme

time course plot, showed that at substrate concentrations above 2.4×10^{-4} g/ml, there were two zero order reactions while at low substrate concentration it was not a straight line but curved as in Fig. 5.4. In Fig. 5.6. a semi-logarithmic plot showed that there were two first order reactions if collagenase reacted with calf skin collagen in low concentrations. On the other hand, in the same concentration range used for calf skin and swim bladder collagen, lathyritic rat skin collagen gave zero order reaction. These data are shown in Fig. 5.9.

For swim bladder collagen the Km was 7.8×10^{-7} M and at an enzyme concentration of 0.14 mg/ml, the Vmax was 1.05×10^{-4} g/ml/min as determined from the reciprocal plot in Fig. 5.10. The initial rate was linear with respect to enzyme concentration as shown in Fig. 5.11.

For calf skin collagen the Km was 2.4×10^{-7} M and at an enzyme concentration of 0.20 mg/ml, the V max was 6.25×10^{-5} g/ml/min as determined from the reciprocal plot in Fig. 5.13.

The hyperchromicity of collagen at 220 nm of the ultra-violet absorption spectrum results from the disruption of the native collagen structure by heating at or above the denaturation temperature (69, 70). The time course of enzyme induced swim bladder collagen hyperchromicity is

shown in Fig. 5.12. The specific rates are kf = 0.3825 min^{-1} and k_s = 0.0835 min^{-1} giving a k_f/k_s ratio of 4.6.

These results are similar to the CD results, indicating that the two methods are measuring the same physical process. It appears that ultraviolet absorbing groups become exposed as the collagen unfolds; however, the specific chromophores have not yet been identified.

If higher concentrations of calf skin collagen were used 2.6 x 10^{-4} g/ml, two zero order reactions were obtained thus at specific rate constants of 2.5 x 10^{-8} M/min and 1.5 x 10^{-8} M/min at an enzyme concentration of 0.20 mg/ml, were obtained.

For lathyritic collagen in the concentration range of from 1 x 10^{-4} g/ml to 3 x 10^{-4} g/ml and at an enzyme concentration of 0.20 mg/ml, zero order reactions were obtained. The specific rate constant was 1.7 x 10^{-8} M/min.

Fig. 5.1. CD spectra of swim bladder collagen in 0.5 M Tris buffer pH 7, 20° C in the native state, heat denatured (100° C, 10 mim), and after 1 hour of the collagenolytic reaction.

Fig. 5.2.a. Linear relationship of ellipticity and concentration of swim bladder collagen at 220 nm in 0.05 M Tris pH 7 and 0.5 M $CaCl_2$, 20° C.

Fig. 5.2.b. Linear relationship of ellipticity and concentration of calf skin collagen at 223 nm in 0.05 M Tris pH 7 and 0.5 M $CaCl_2$, 20° C.

Fig. 5.2.c. Linear relationship of ellipticity and concentration of lathyritic rat skin collagen at 223 nm in 0.05 M Tris pH 7 and 0.5 M $CaCl_2$, 20° C.

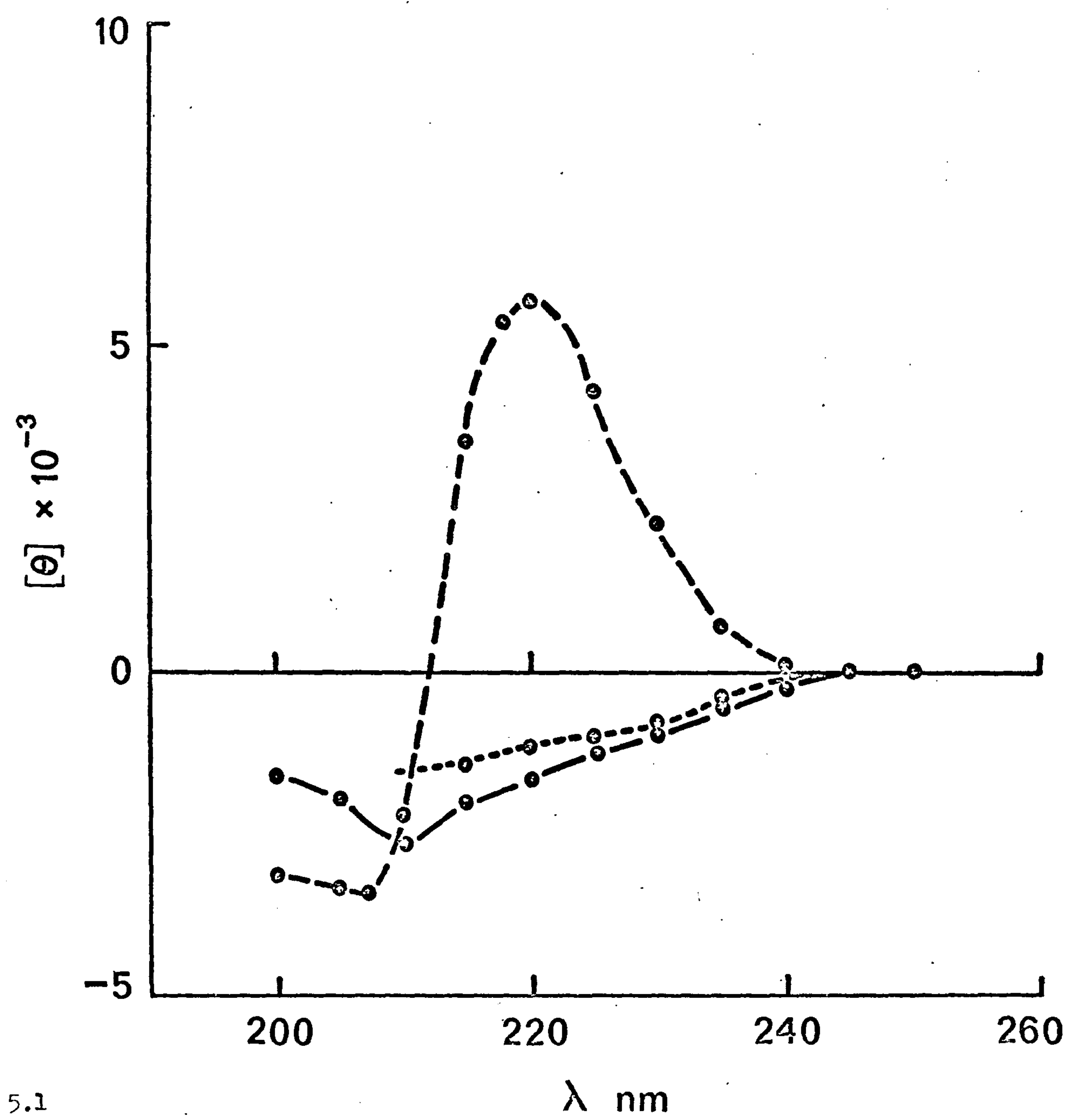


Fig. 5.1

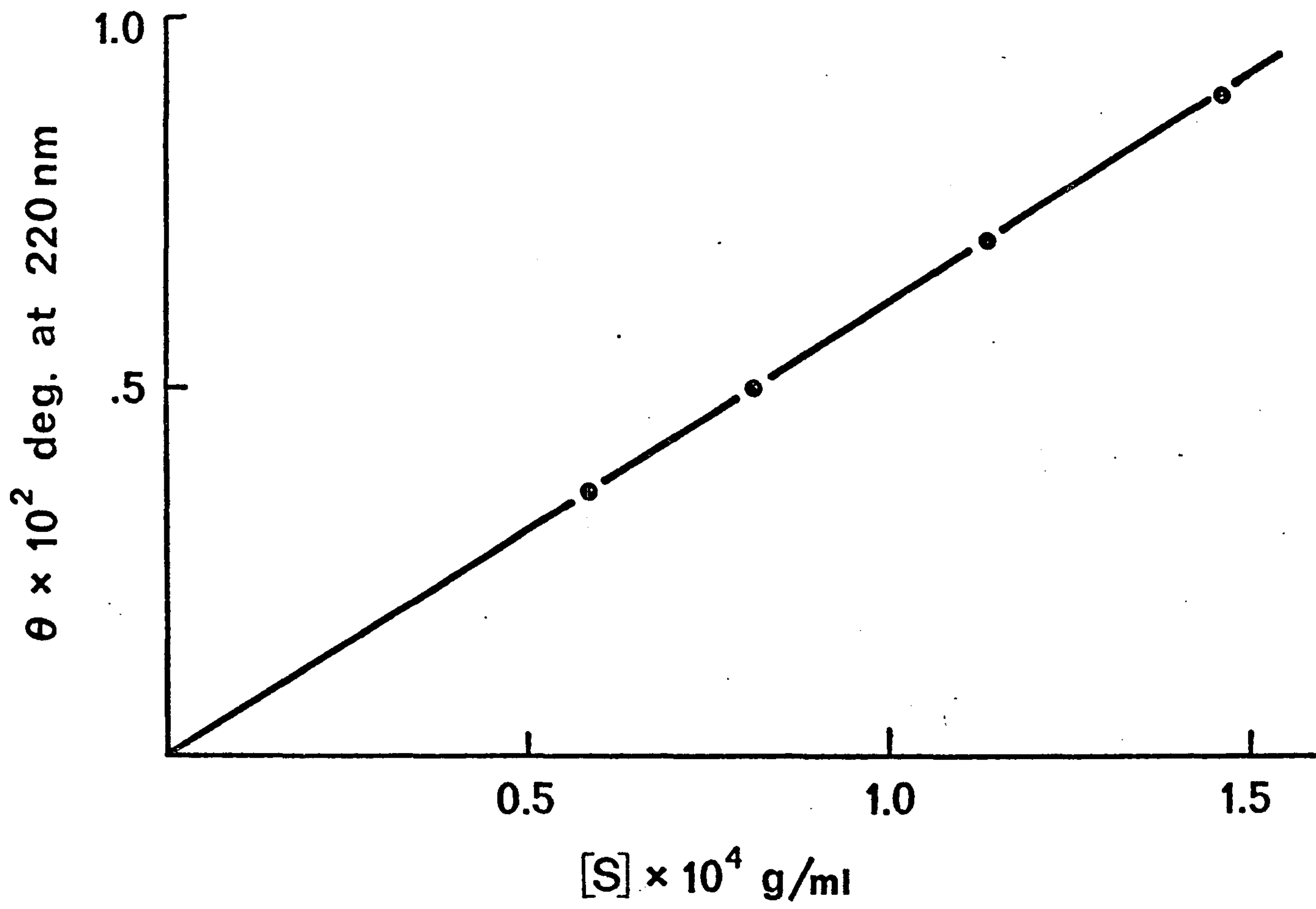


Fig. 5.2a

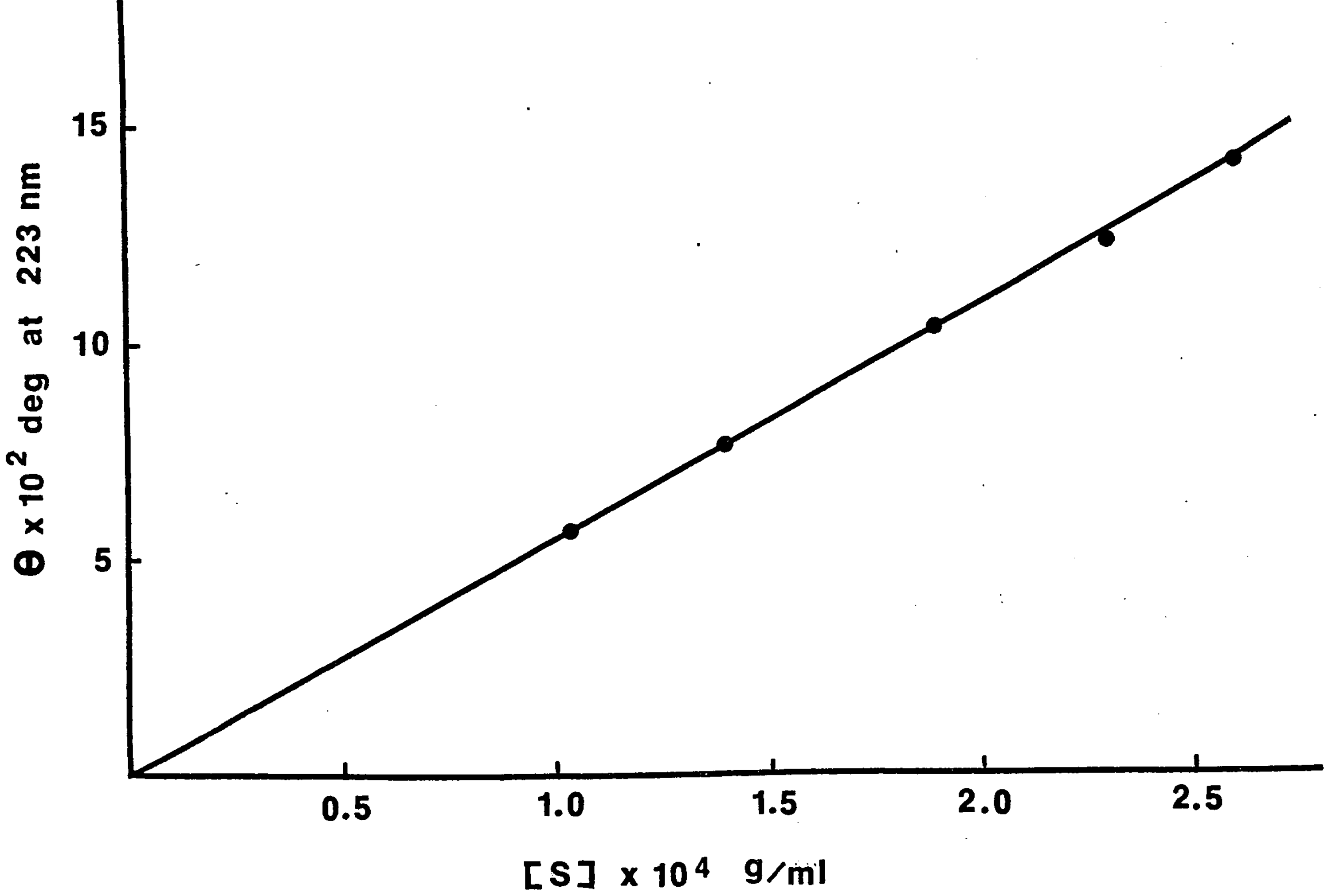


Fig. 5.2b

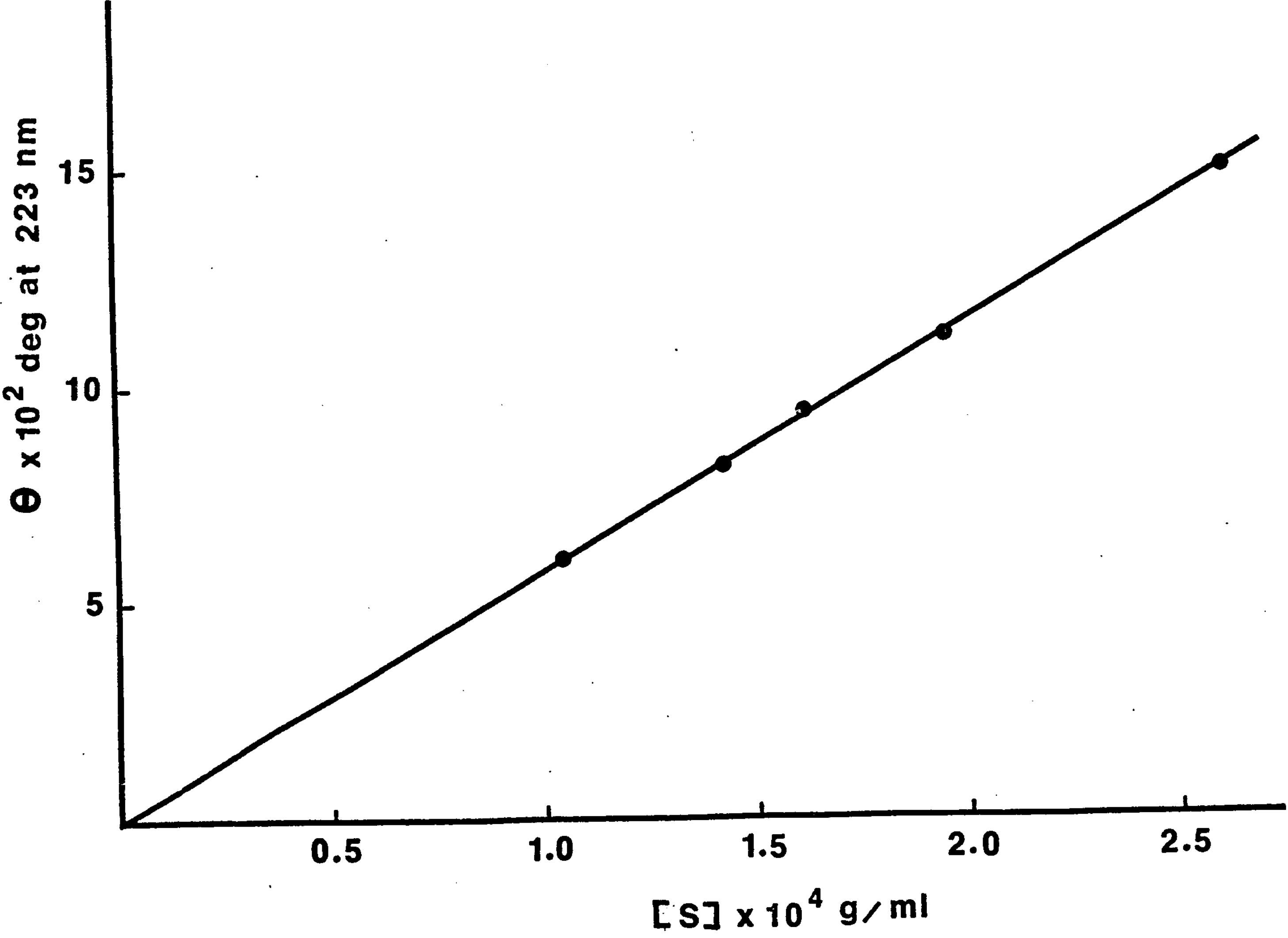


Fig. 2c

Fig. 5.3. Molar ellipticity changes at 220 nm as a function of time during the collagenolytic reaction at 20° C; swim bladder collagen concentration 1.63×10^{-4} g/ml, 4 ml, collagenase concentration 1.4×10^{-4} g/ml, 0.5 ml.

Fig. 5.4. Molar ellipticity changes at 223 nm as a function of time during the collagenolytic reaction at 20° C; calf skin collagen concentration 1.3×10^{-4} g/ml, 4 ml, collagenase concentration 2×10^{-4} g/ml, 0.5 ml.

Fig. 5.5. Fractional molar ellipticity at 220 nm of swim bladder collagen (in log scale), as a function of time during collagenase hydrolysis; collagen concentration 1.1×10^{-4} g/ml, collagenase concentration 1.4×10^{-4} g/ml.

Fig. 5.6. Fractional molar ellipticity at 223 nm of calf skin collagen (in log scale), as a function of time during collagenase hydrolysis; collagen concentration 1.84×10^{-4} g/ml, collagenase concentration 2×10^{-4} g/ml.

Fig. 5.7. log $v_{initial}$ against log [S].

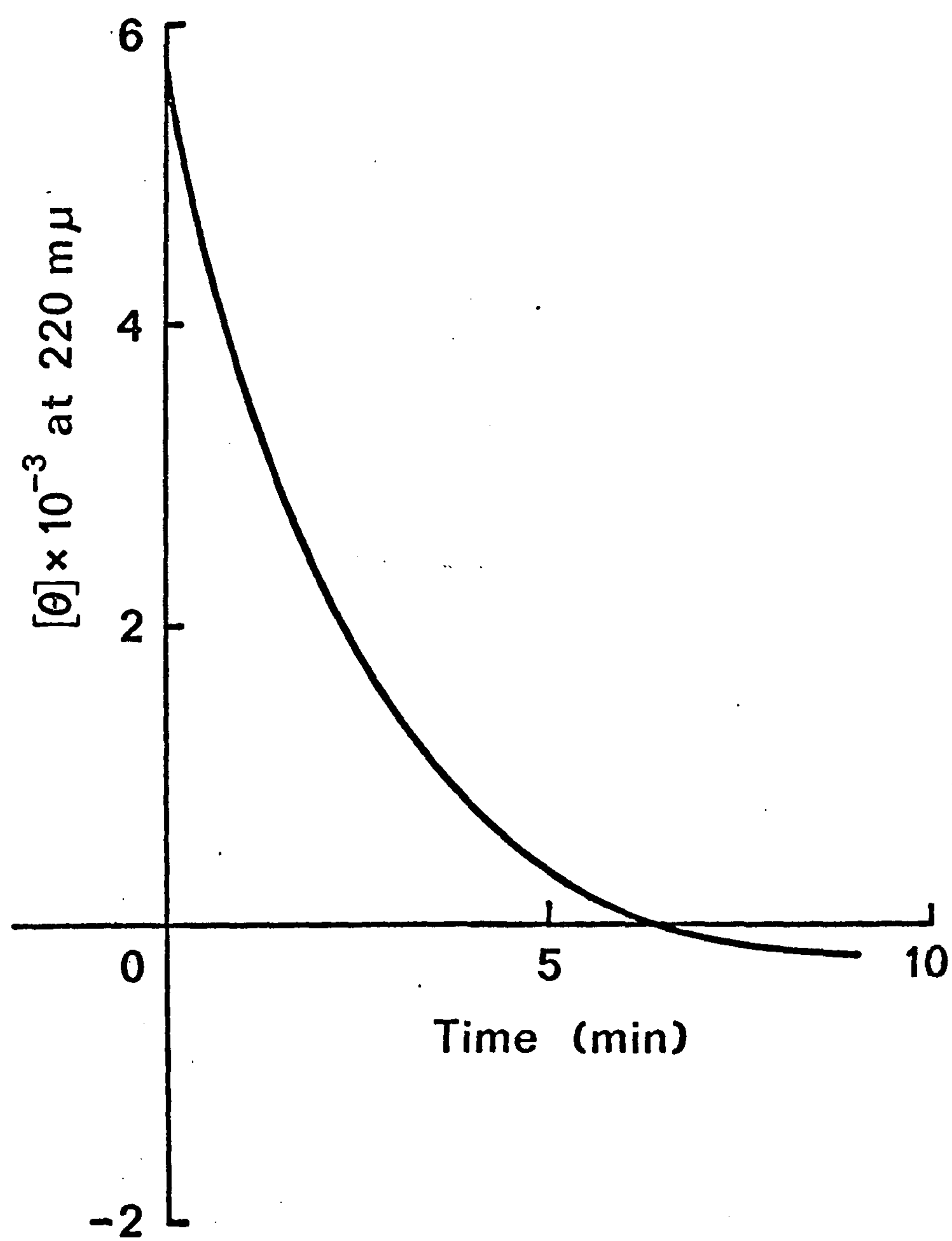


Fig. 5.3

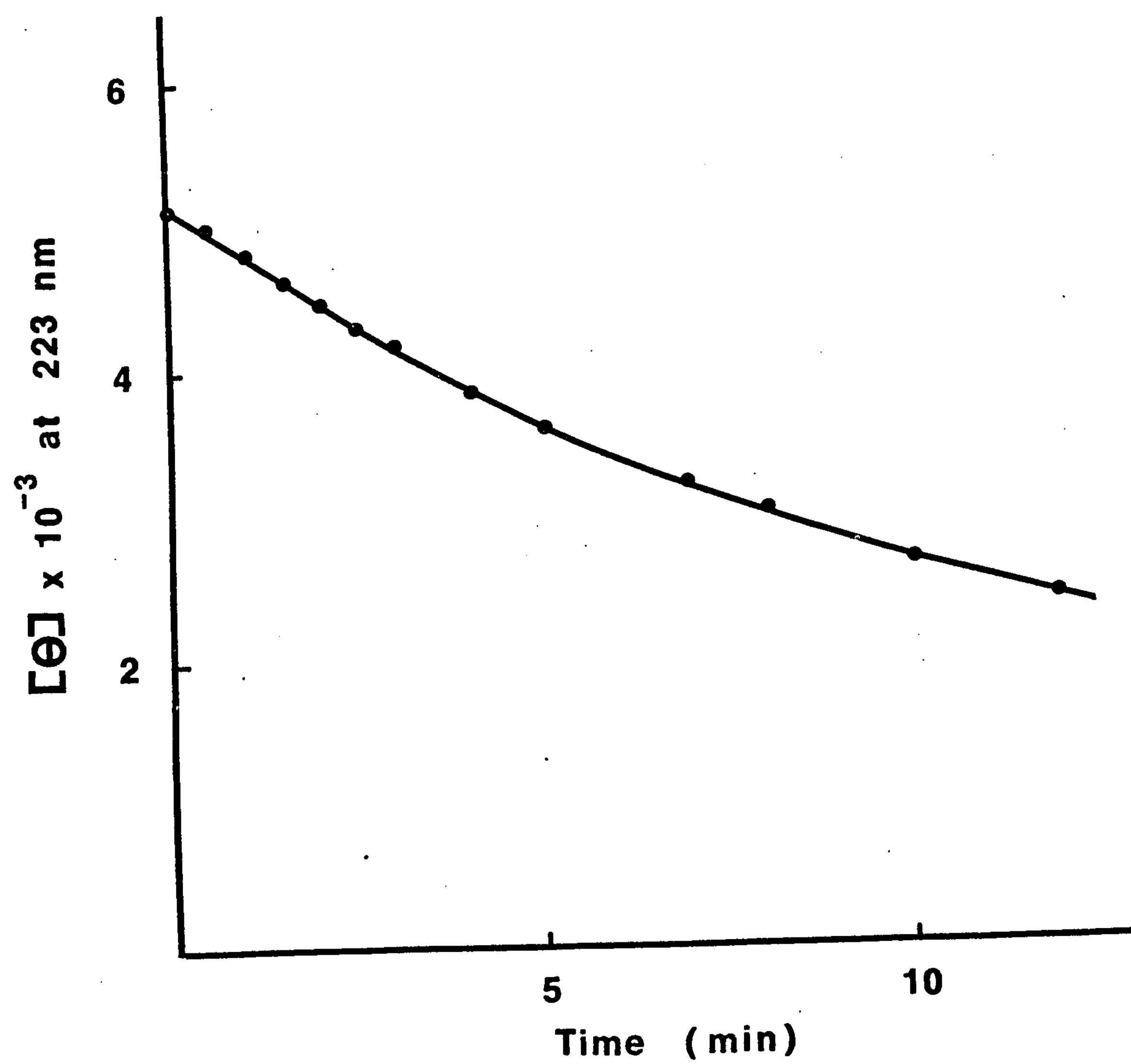


Fig. 5.4

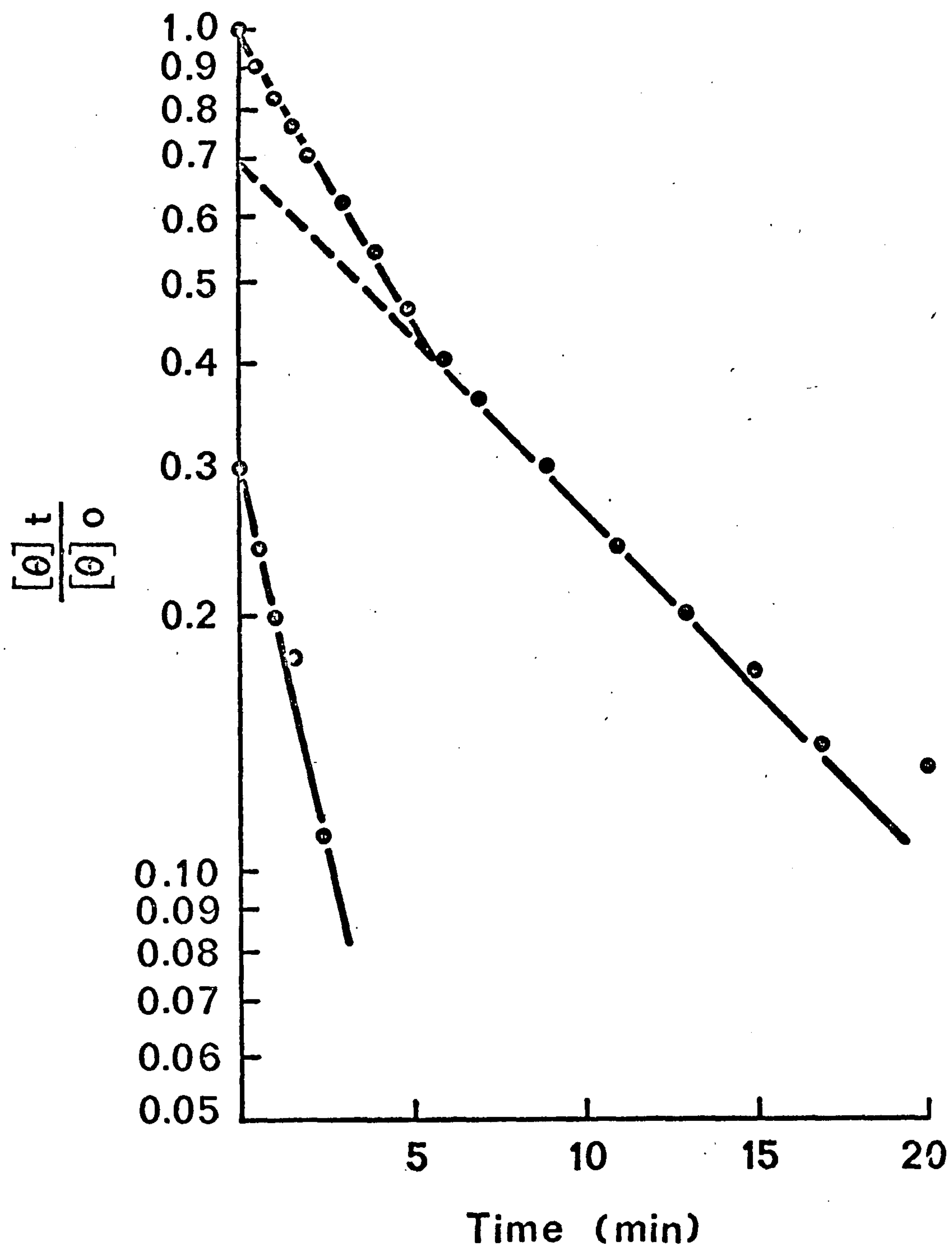


Fig. 5.5

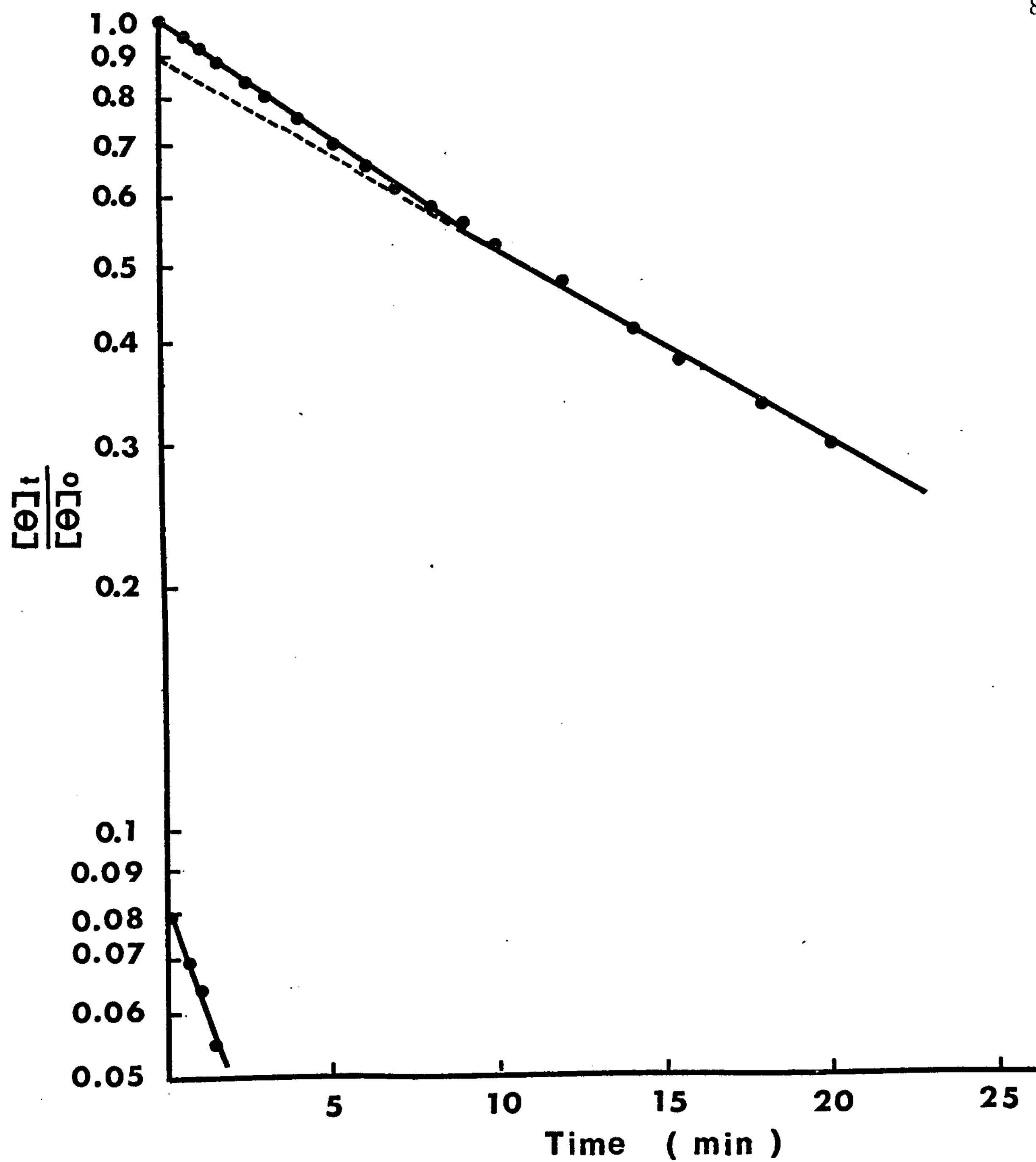


Fig. 5.6

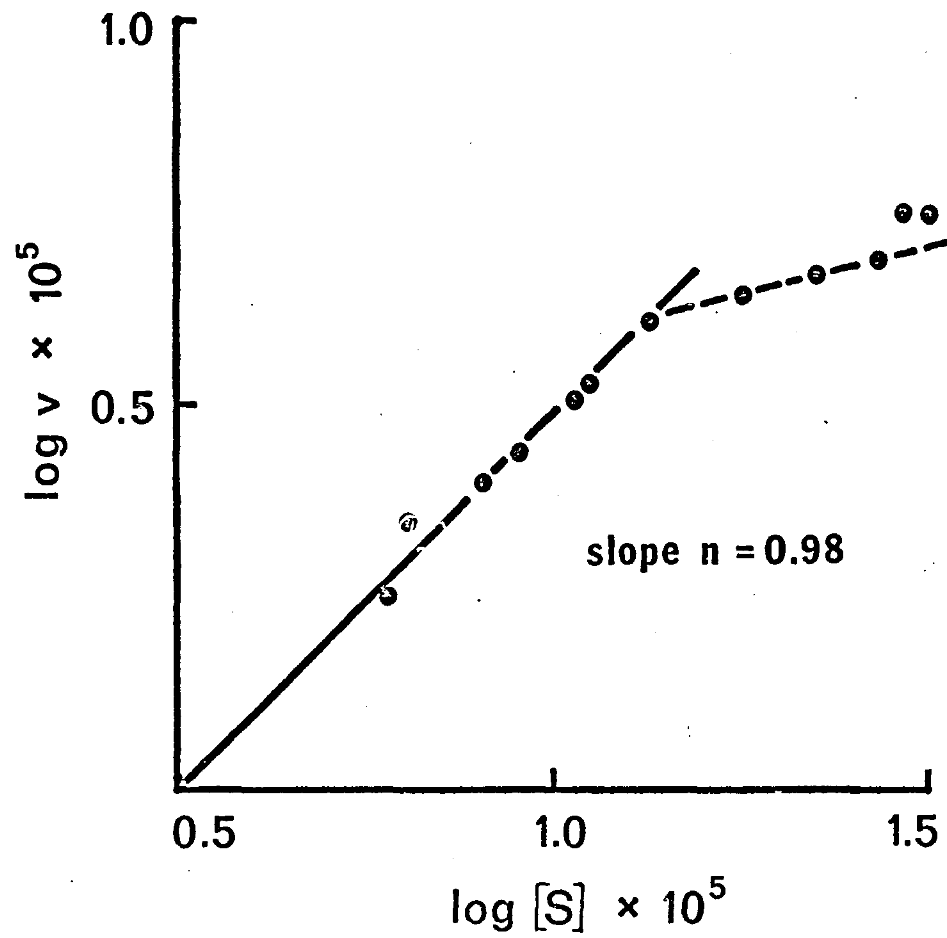


Fig. 5.7

Fig. 5.8. Molar ellipticity changes at 223 nm as a function of time during the collagenolytic reaction at 20° C; calf skin collagen concentration 2.6×10^{-4} g/ml, 4 ml collagenase 2×10^{-4} g/ml, 0.5 ml.

Fig. 5.9. Ellipticity changes at 223 nm as a function of time during the collagenolytic reactions at 20° C; lathyritic rat skin collagen with different concentrations were used, collagenase concentration 2×10^{-4} g/ml.

Fig. 5.10. Lineweaver Burk plot of the collagenase reaction on swim bladder collagen.

Fig. 5.11. Effect of the collagenase concentration on the initial rate of collagen hydrolysis experiments carried out at pH 7, 0.05 M Tris and 0.5 M $CaCl_2$, at 20° C.

Fig. 5.12. Fractional hyperchromicity (in OD) at 220 nm of collagen (in log scale) as a function of time during collagenase hydrolysis. Collagen concentration 1.1×10^{-4} g/ml, collagenase concentration 1.4×10^{-4} g/ml.

Fig. 5.13. Lineweaver Burk plot of the collagenase reaction on calf skin collagen.

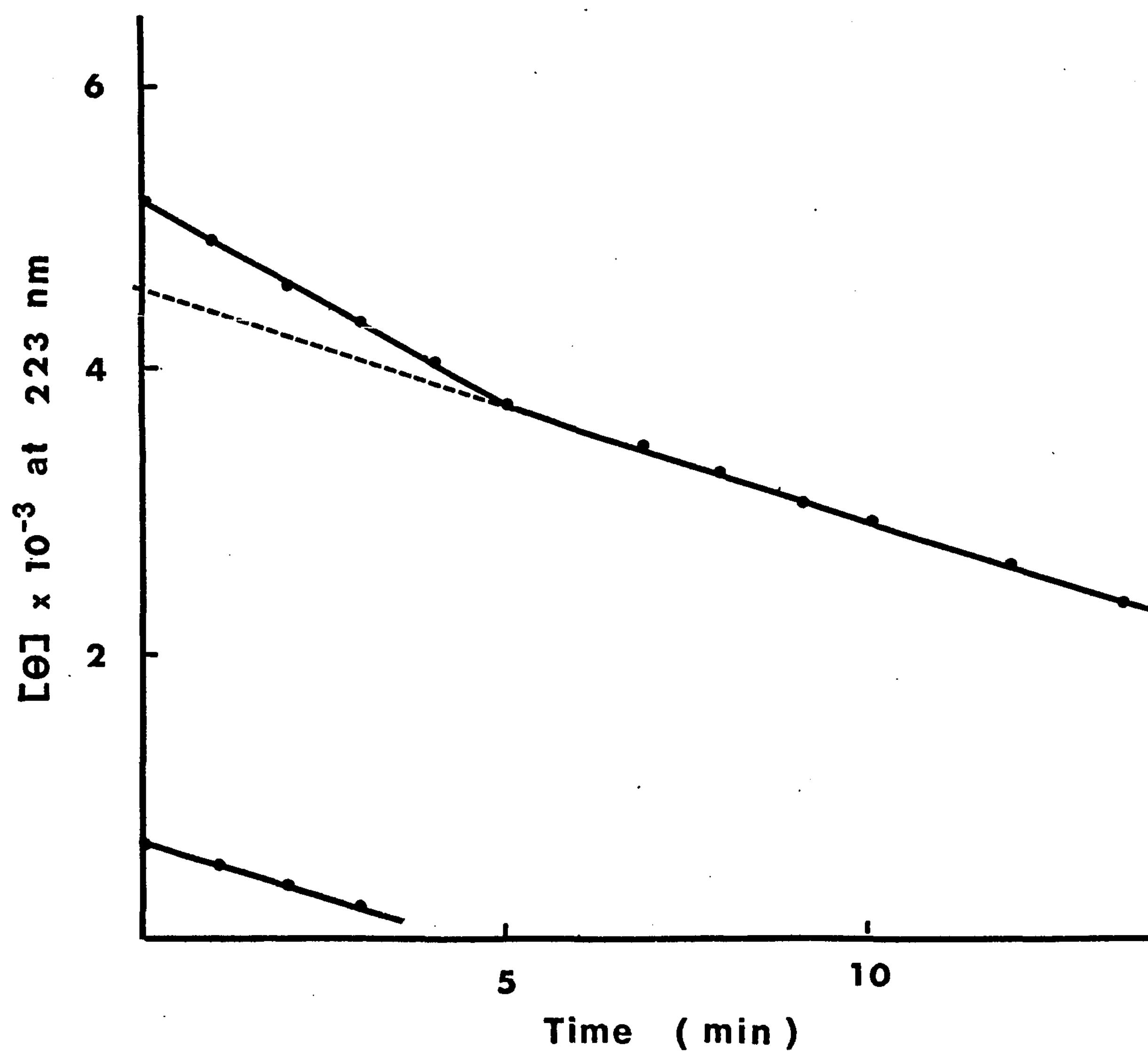


Fig. 5.8

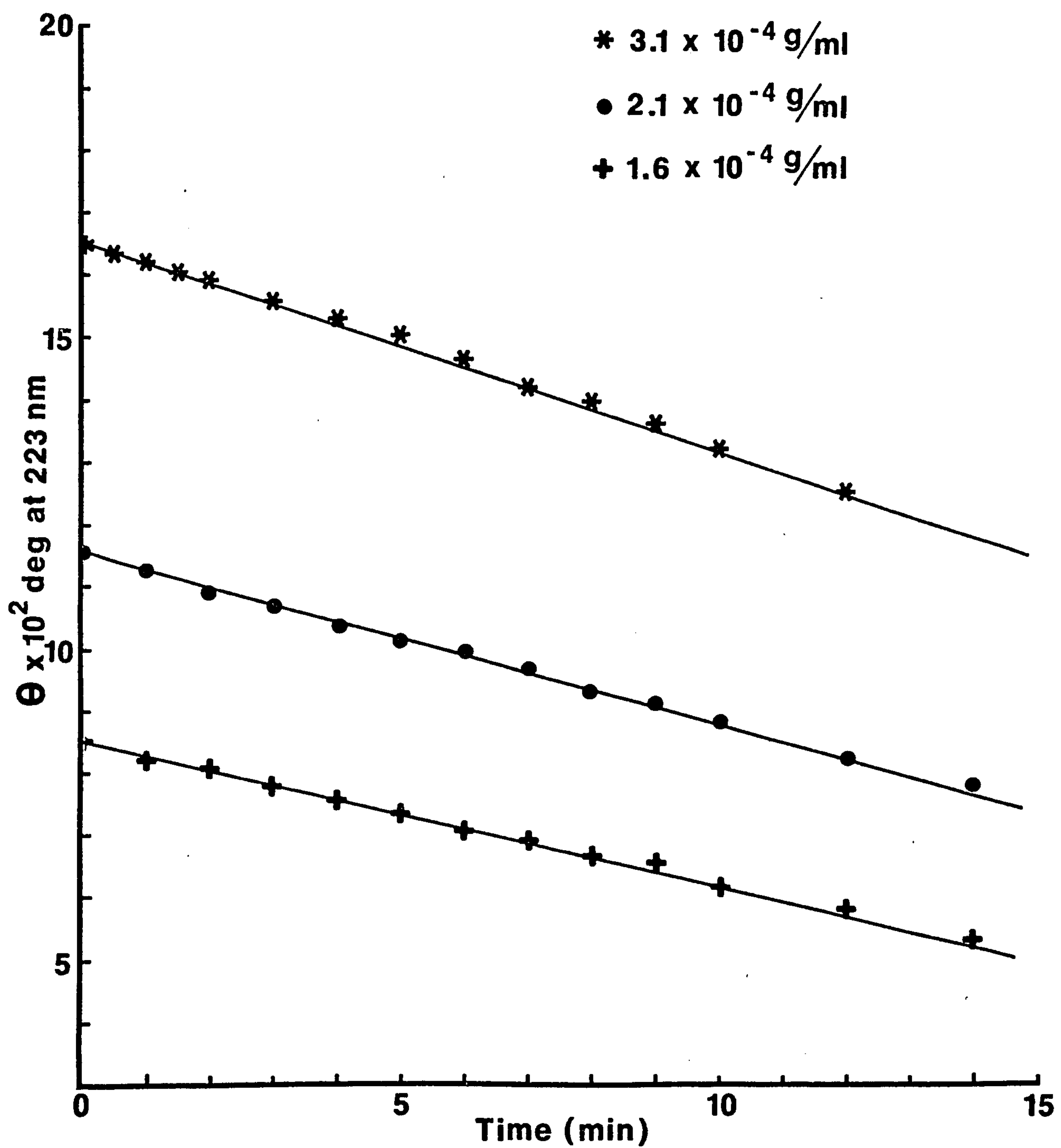


Fig. 5.9

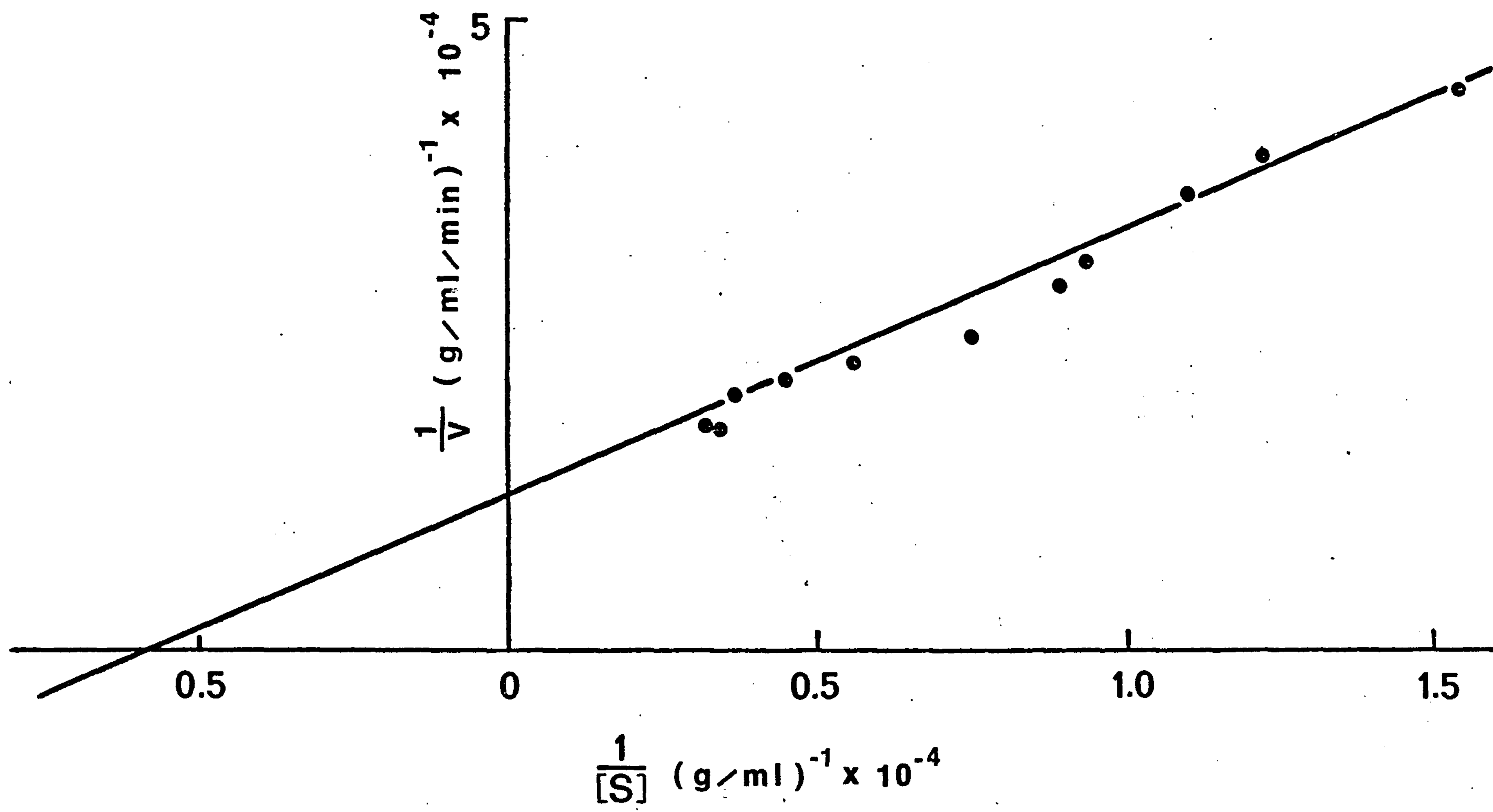


Fig. 5. 10

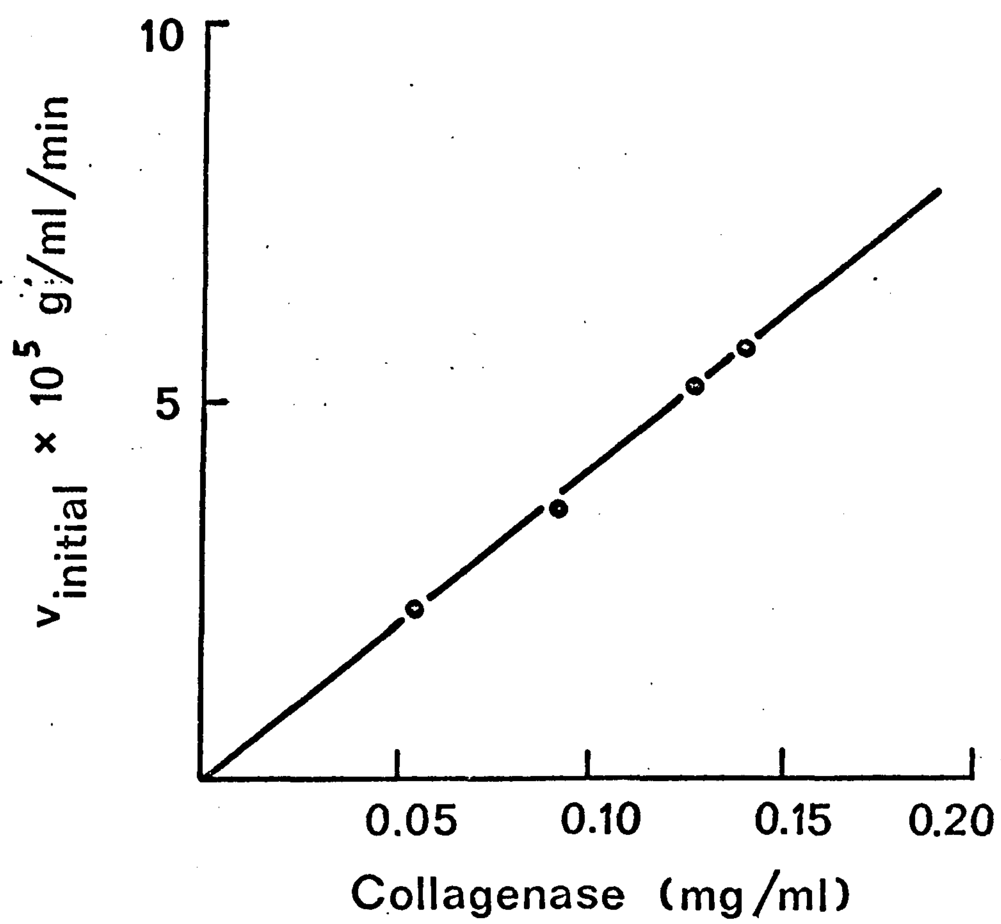


Fig. 5. 11

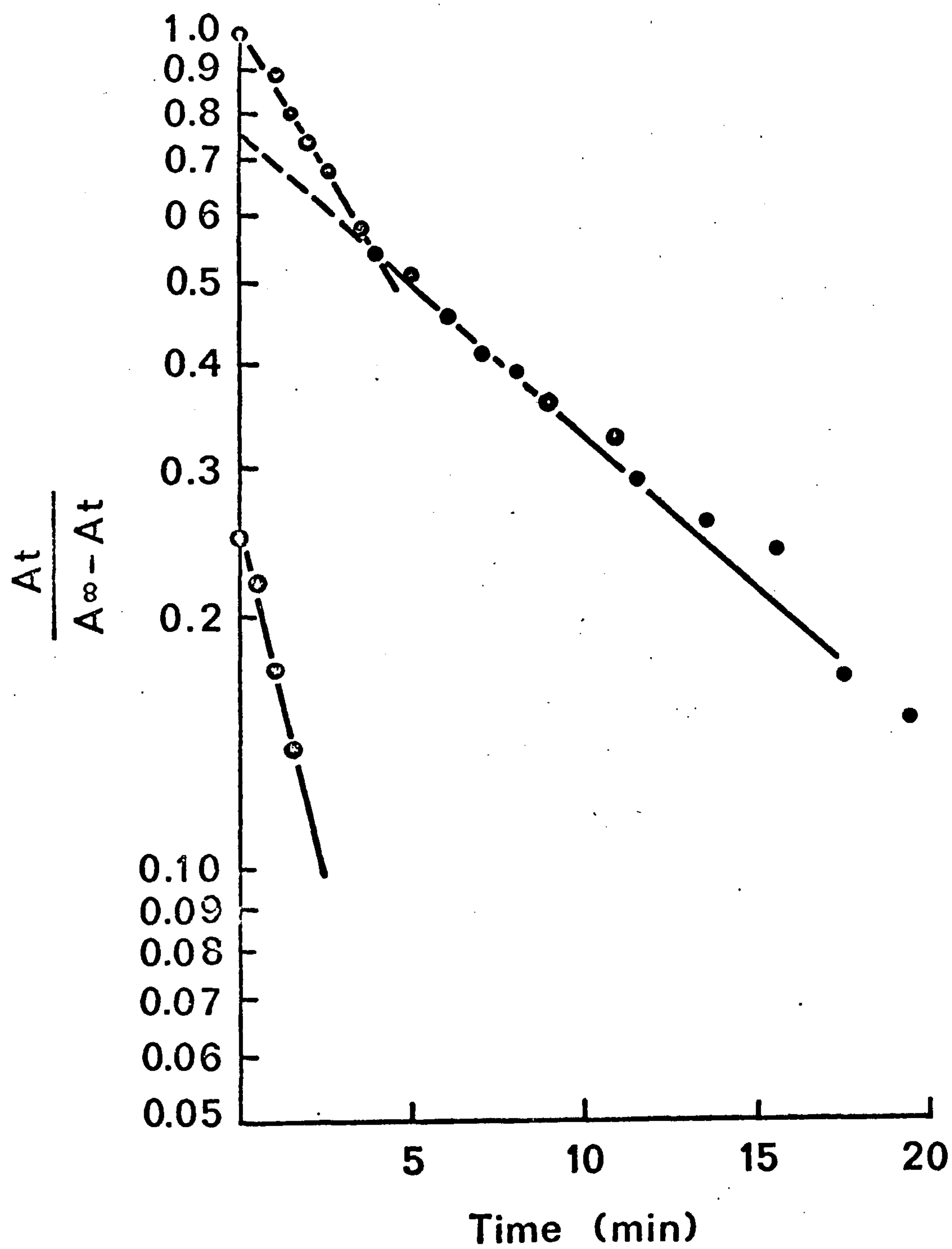


Fig. 5.12

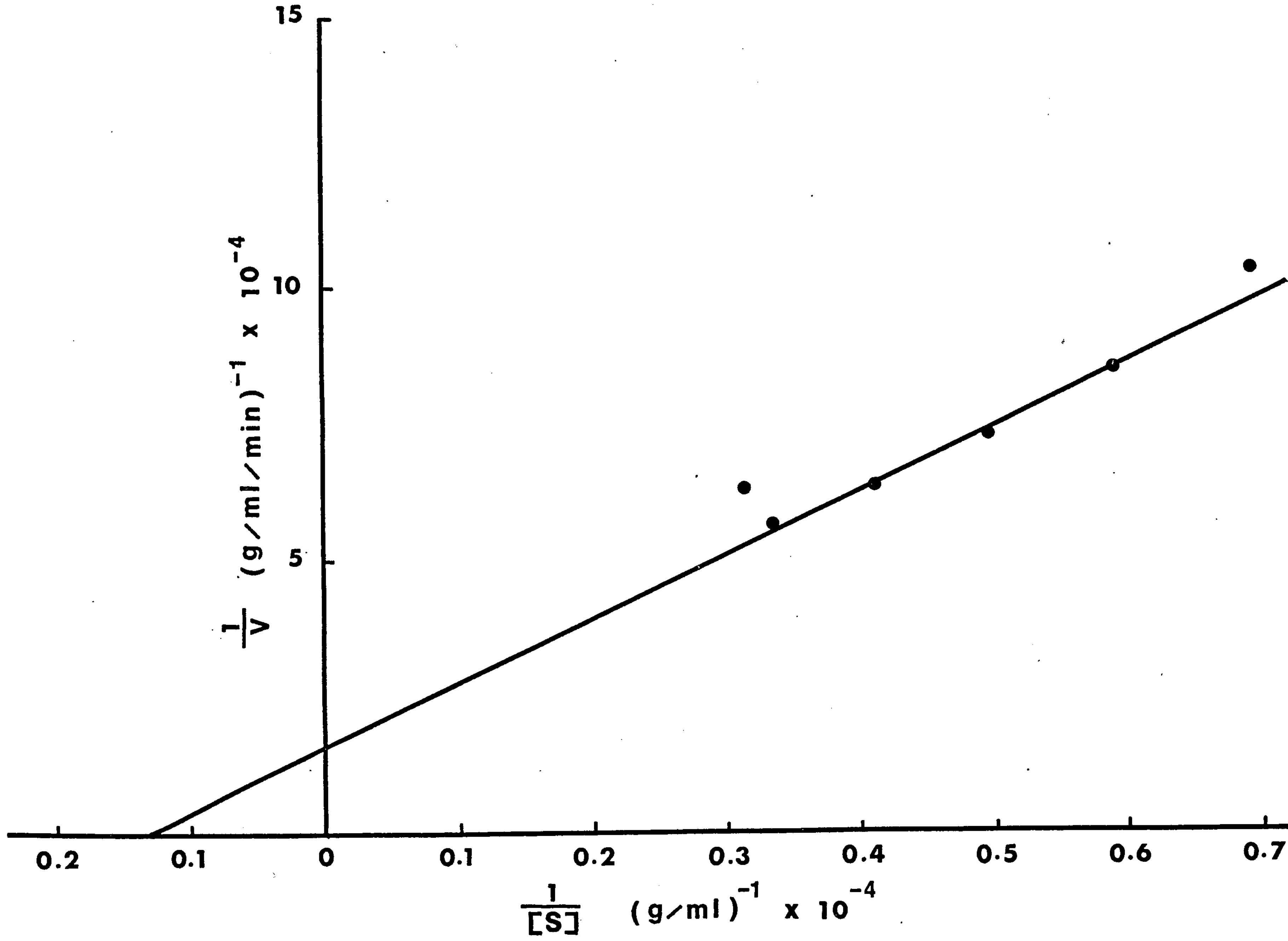


Fig. 5.13

Discussion

Extracellular collagenase from clostridium histolyticum catalyzes the hydrolysis of peptide bonds in native collagen and in gelatin (34, 22). Although there is some doubt concerning the exact specificity (83), it appears to require a sequence -P-Y-Gly-P where P may be Pro or Hyp and Y is any other amino acid residue. Cleavage occurs between Y and Gly (22, 42, 84 and 85). The specific tertiary structure requirements are unclear.

The action of collagenase on soluble icthyocal has previously been investigated with the view of obtaining information about the structure of collagen in solution (86, 87). Using colorimetric ninhydrin and PH-stat methods the overall proteolysis kinetics on native collagen were found to be the sum of two concurrent reactions. About 20% of the bonds were split in the fast reaction while 80% were cleaved in the slow reaction. The ratio k_{fast}/k_{slow}, k_f/k_s, was 4.3/1 at 19.6^o C (88). The rate of proteolysis of denatured collagen, however, followed single first order kinetics. The interpretation of these data was related to local differences in polypeptide chain configuration at the susceptible peptide bonds. On the basis of measurements of viscosity, optical rotation, and molecular weight changes (as seen in the result of light scattering and nondialyzable protein concentration), the

initial action of collagenase was hypothesized to be on the collagen triple helix whereby cleavage of single strands leaves the molecule relatively intact while inducing partial structural change. A slower rate of cleavage then proceeds resulting in an average molecular weight of about 500 at the end of collagenolysis (88).

Later investigations have revealed that the enzyme cleaves randomly along the substrate chain (86).

The data presented in Fig. 5.5. revealed that the enzyme induced change of the collagen triple helix is an apparent single first order process. However, the reaction is the sum of two apparent first order processes with respect to time. Two rate constants for the fast reaction k_f and the slow reaction k_s were determined from the data in Fig. 5.5. They are 0.385 min^{-1} and 0.0962 min^{-1}, respectively. The ratio k_f/k_s is thus 4.1, when compared favorably with that of 4.3 referred to above. In Fig. 5.6. showed that if calf skin collagen was used, the two rate constants were 0.248 min^{-1} and 0.061 min^{-1} respectively. The ratio k_f/k_s is thus 4.

These data are in agreement with the concept that the enzyme cleaves collagen by a two step process. They provide direct evidence that the enzyme acts on the triple-helix form of the substrate in the first fast reaction.

The data, however, do not explain the nature of the slow reaction. It could result from differential rates of hydrolysis of new groups as they become exposed by structural changes that follow the initial attack.

A single tropocollagen molecule consists of three individual strands (α chains). α -strands within each tropocollagen molecule are considered to attached covalently to one another (i.e. cross linked intramolecularly) to form aggregates designated as β and γ (89).

Dramatic malformations of mesenchymal tissues may be induced in a variety of growing animals by a series of simple compounds (lathyrogenic agents) such as β - amino-propionitrile (BAPN) (90, 91). The ability of BAPN to induce the appearance of extractible collagen in the skin of mature and aging guinea pigs has been described by Levene and Gross (92, 93). Later, it has been found that soluble collagen from lathyritic animals is deficient in dimeric β components (77, 89).

The identification of a cross-linking sites has required highly selective methods of degradation of tropo-collagen and of the separated α and β chains derived from it. Cyanogen bromide has been useful for this purpose. It cleaves an isolated polypeptide chain selectively at the carboxyl end of a methionine residue (89, 94).

Isolated α_1 and α_2 chains and β_{12} double chains from rat skin collagen were cleaved with CNBr and the digests were chromatographed on phosphocellulose. Studies on the peptides obtained indicated that a specific lysyl residue, located in a nonhelical region near the N-terminus of each α chains, participates in the formation of the intramolecular interchain cross-link in collagen (89, 95). It was concluded that the lysine side chain can be converted to an aldehyde identified as the δ-semialdehyde of α-amino adipic acid. Two of these groups in separated chains combine to form an intramolecular crosslink possibly through an aldol-type condensation with subsequent dehydration to produce an α, β- unsaturated aldehyde.

Lathyritic collagen has been reported to bind less 2,4 -DNP hydrazine than normal collagen, suggesting that lathyrogens block aldehydic groups which normally function to cross-link collagen chains, (89, 96) α chains of lathyritic collagen from animals fed BAPN are normally synthesized but are deficient in aldehydic groups because specific lysyl residues are not connected to the intermediate aldehyde. This suggested that lathyrism is due to the inhibition of an enzymatic process by which lysine in peptide linkage is connected to the aldehyde.

The lack of cross linking may effect the enzyme activity and it may provide an explanation for the

observation that lathyritic rat skin collagen as substrate results in only one zero order reaction.

Swim bladder and calf skin collagen when reacted with collagenase showed two first order reactions, while lathyritic rat skin collagen showed only one zero order reaction. It could be explained that the fast first order reaction for swim bladder and calf skin collagen was the recognition of collagenase to or close to the crosslink region and meanwhile in the triple helical body the Gly-P-Y-Gly-P-Y-peptide sequence was cleaved by the collagenase. Since the crosslink region was outside the helical area of collagen (92, 97), the recognition of it by collagenase might help the "enzyme-substrate fixation" to give the best orientation for the enzymatic digestion in the triple helix to occur. The slow reaction could result from differential rates of hydrolysis of new groups as they become exposed by structural changes that follow the initial attack. Lathyritic rat skin collagen has very few crosslink, that might result in failing to obtain the proper enzyme substrate interaction. Also, lathyritic rat skin collagen has more hydroxyproline residues, it is more resistant to the collagenase digestion (98, 99 and 100). These might be able to explain that lathyritic rat skin collagen reacted with collagenase showed zero order reaction.

Chapter VI

Purification of Collagenase by Affinity Chromatography

Results and discussion

Purification of collagenase by affinity chromatography

Fig. 6.1. shows a typical affinity chromatogram obtained by passing clostridial histolicum collagenase through a collagen-sepharose column. Two fractions containing enzyme activity were obtained at fraction I and fraction II. Although only partial adsorption of collagenase activity occurs, that fraction which binds could be eluted with a buffered solution of 1.0 M NaCl (64, 65).

The enzymatically active eluant, fraction II appears as a single band on polyacrylamide gel electrophoresis as shown in Fig. 6.2. Starting with 19 mg of partially purified collagenase a single chromatographic procedure yielded only 1.4 mg of the purified enzyme, that is fraction II. The specific activity has increased 1.6 times.

The use of affinity chromatography with collagen-sepharose column after partial purification of the enzyme reduces the likelihood that non-specific adsorption of components present in the crude mixtures will occur. The electrophoretic criteria of enzyme purity indicated the feasibility of using affinity chromatography as a simple and reproducible method for obtaining a pure preparation of the collagenase.

The purified enzyme showed the same reaction pattern as before affinity chromatography purification. There were two first order reactions. Therefore the two reactions were not due to more than one enzyme.

Either with or without the end product of collagenase digestion in the reaction media, the collagenolytic reaction showed two first order reactions. Therefore the two first order reactions were not due to product inhibition. It is unique for the collagenolytic reaction within the concentration used in this work.

Fig. 6.1. Affinity chromatography of clostridium histoliticum collagenase on collagen-sepharose column. A sample of 19 mg of partially purified enzyme protein was applied to a column (1.2 x 10 cm) and effluent fractions of 3 ml were collected at a rate of 9 ml/hr. Elution was accompanied by the addition of 1.0 M NaCl to the eluant buffer.

Fig. 6.2. Polyacrylamide gel electrophoresis of clostridium histoliticum collagenase purified on collagen-sepharose column. 7.5% gels were used at 5 mA per gel. The gel on the left shows the pattern of the partially purified enzyme and fraction I as well. The gel on the right shows the purified collagenase fraction II.

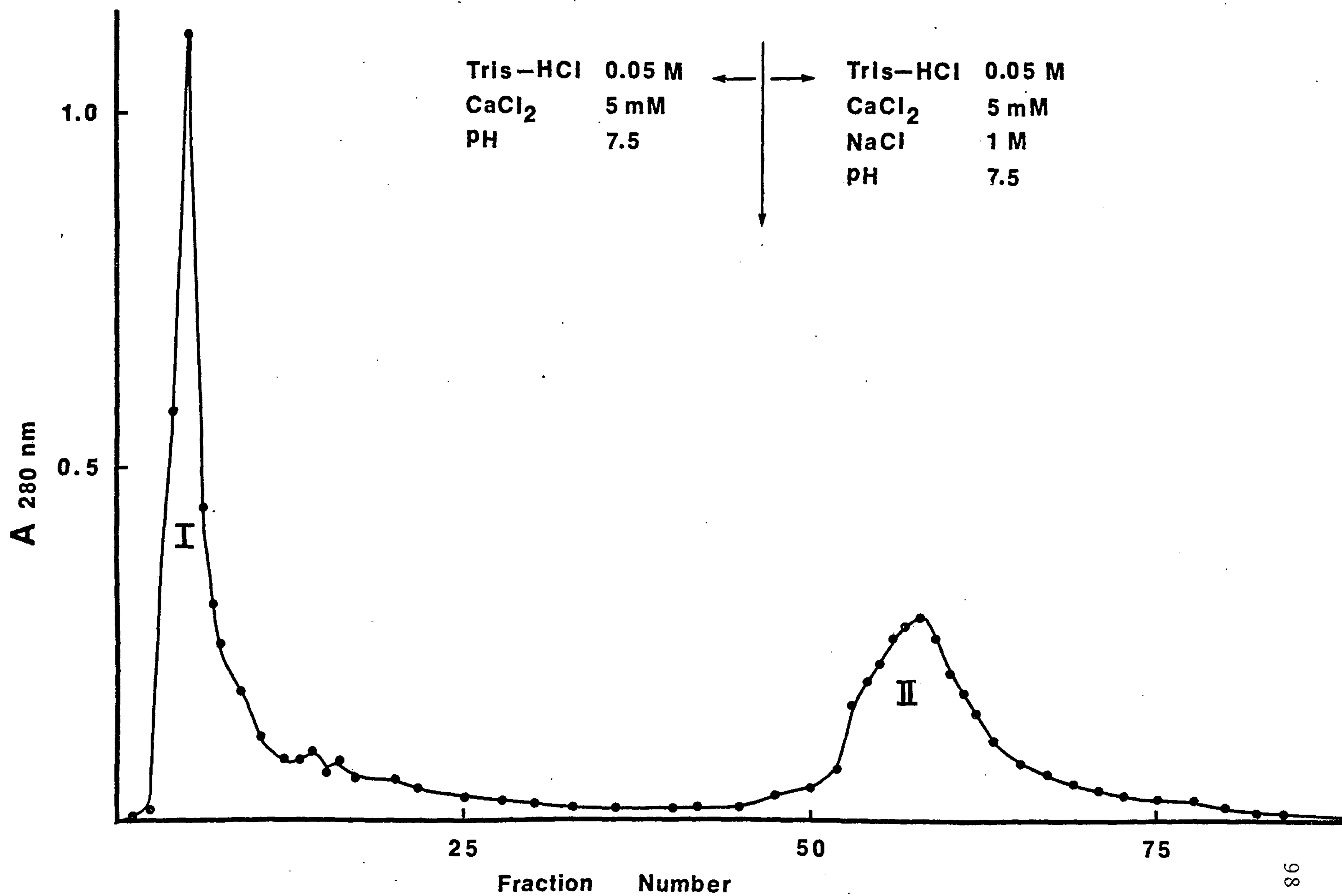


Fig. 6.1

Fig. 6.2

Chapter VII

Effect of Metal Ions on Collagenolytic Reaction

Effect of metal ions on collagenolytic reaction

Results

The relationship between ellipticity and concentration of succinylated swim bladder collagen is the same as that of unmodified collagen. Fig. 7.1. shows that in the presence of calcium ion, succinylated swim bladder collagen reacted with collagenase and the rate of change indicated that the reaction pattern was similar to that for unmodified collagen. It shows two first order reactions as for unmodified collagen. k_f/k_s is about 3 for both native and modified collagen.

The reaction of succinylated collagen react with collagenase in the absence of Ca^{++} ions is shown in Fig. 7.2. Unmodified collagen was reacted with collagenase under the same condition as that of succinylated collagen but with Ca^{++} ions only, Fig. 7.3. The rates are presented in Table 7.1. The concentration of collagen used was 1.5×10^{-4} g/ml and that of collagenase was 0.17 mg/ml.

Since succinylated collagen can be dissolved in Tris buffer without Ca^{++} ions. It was used to test the effect of different metal ions on the enzyme activity. The concentration of collagen used was 1.6×10^{-4} g/ml and that of collagenase was 0.17 mg/ml. The final concentration of each ion is listed in Table 7.2.

Fig. 7.1. Fractional molar ellipticity at 220 nm of succinylated collagen (in log scale), as a function of time during collagenase hydrolysis; collagen concentration 1.5 x 10^{-4} g/ml, collagenase concentration 0.17 mg/ml. Solvent used was 0.05 M Tris buffer pH 7.0 with 0.5 M $CaCl_2$.

Fig. 7.2. Fractional molar ellipticity at 220 nm of succinylated collagen (in log scale), as a function of time during collagenase hydrolysis; collagen concentration 1.5 x 10^{-4} g/ml, collagenase concentration 0.17 mg/ml. Solvent used was 0.05 M Tris buffer pH 7.0.

Fig. 7.3. Fractional molar ellipticity at 220 nm of unmodified collagen (in log scale), as a function of time during collagenase hydrolysis; collagen concentration 1.5 x 10^{-4} g/ml, collagenase concentration 0.17 mg/ml. Solvent used was 0.05 M Tris buffer pH 7.0 with 0.5 M $CaCl_2$.

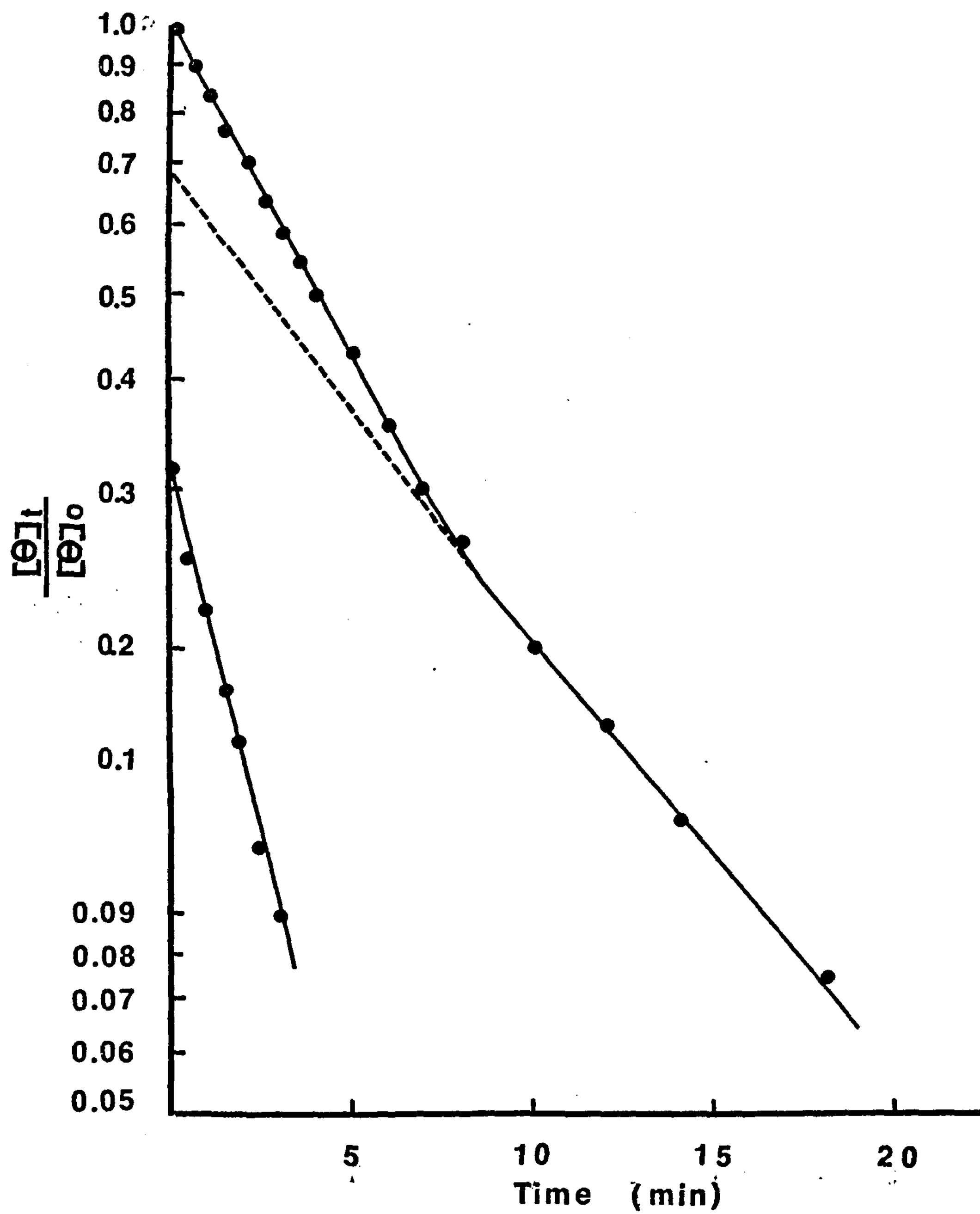


Fig. 7.1

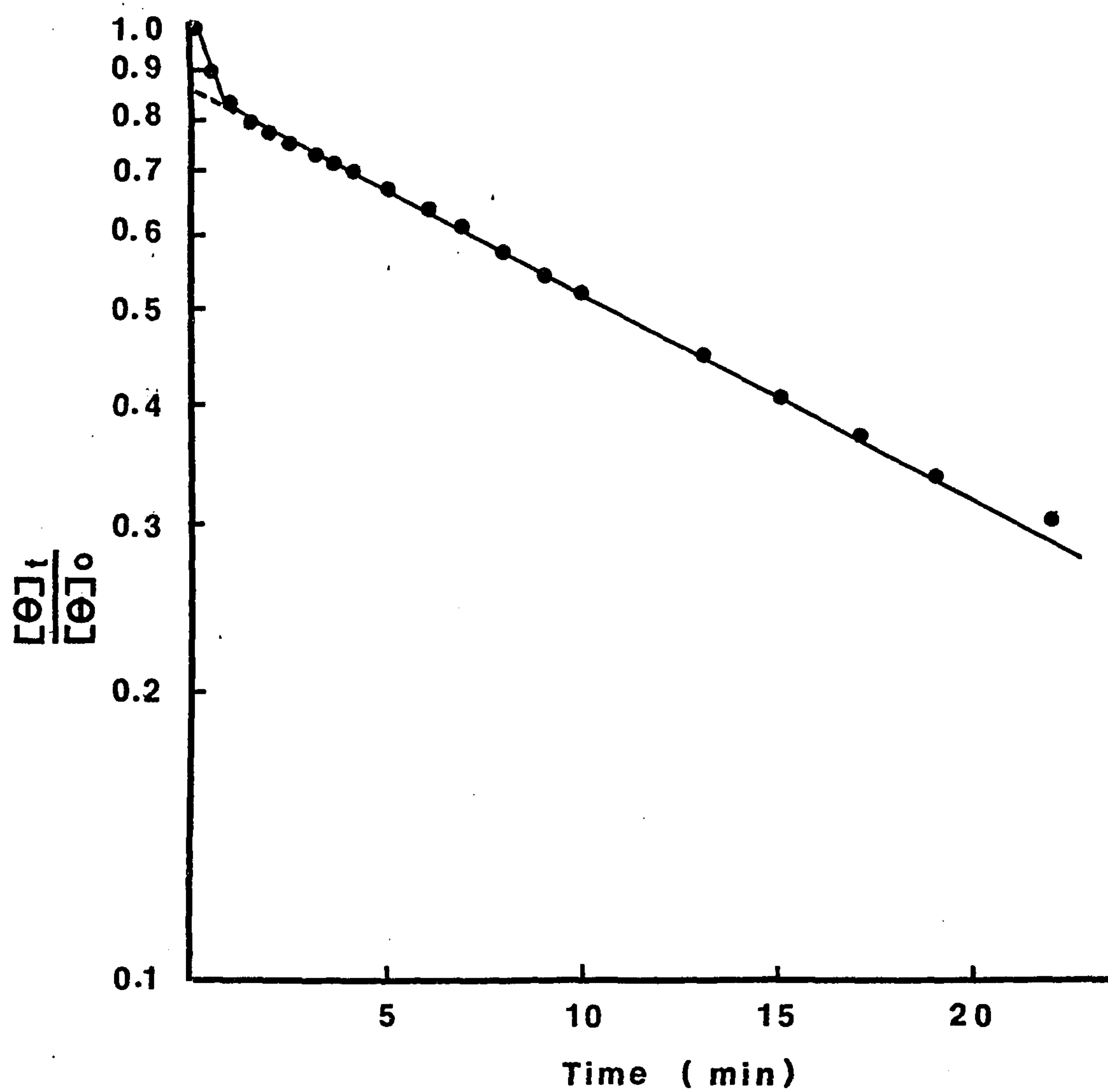


Fig. 7.2

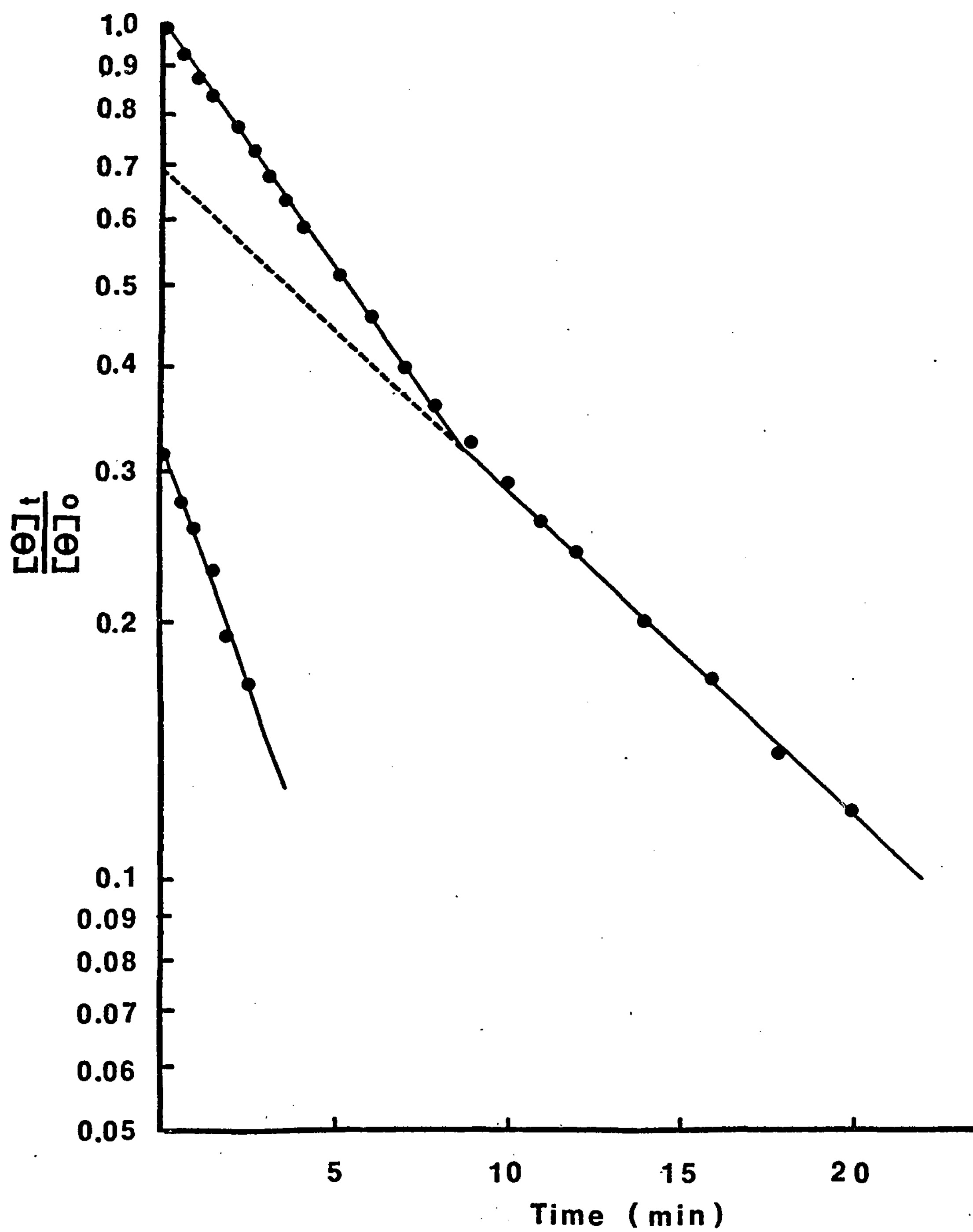


Fig. 7.3

Table 7.1.

Kinetic data of collagen reacting with collagenase with or without Ca^{++} ions

	Succinylated collagen		Original collagen
	0.5 M Ca^{++}	0 Ca^{++}	0.5 M Ca^{++}
t_f 1/2	2 min	2.5 min	2.5 min
t_s 1/2	5.9 min	13.5 min	7.7 min
k_f	0.346 min^{-1}	0.277 min^{-1}	0.277 min^{-1}
k_s	0.117 min^{-1}	0.052 min^{-1}	0.089 min^{-1}
k_f/k_s	3	5.4	3.1

Table 7.2.

Kinetic data of Succinylated collagen reacted with different metal ions

	0 Ca^{++}	0.005M Ca^{++}	0.5M Ca^{++}	0.005M Mn^{++}	0.005M Mg^{++}	0.005M Co^{++}
t_f 1/2	2.7 min	2.5 min	2 min	2.6 min	2.6 min	1.7 min
t_s 1/2	15 min	7.5 min	5.9 min	7.9 min	11 min	6 min
k_f	0.256 min^{-1}	0.231 min^{-1}	0.346 min^{-1}	0.266 min^{-1}	0.266 min^{-1}	0.407 min^{-1}
k_s	0.046 min^{-1}	0.092 min^{-1}	0.117 min^{-1}	0.087 min^{-1}	0.063 min^{-1}	0.115 min^{-1}
k_f/k_s	5.6	3	3	3.04	4.2 min	3.5

Discussion

Acid extracted and purified collagen has a very low solubility without salt at neutral pH (9, 101 and 102). Thus collagen solution for collagenase assays are prepared in 0.05 M Tris buffer pH 7.0 with 0.5 M $CaCl_2$.

It has been reported that Ca^{++} ions are required for both the binding of the enzyme to the collagen substrate and for full catalytic activity and that Mg^{++} ions cannot substitute for Ca^{++} ions (103, 104 and 105).

Since succinylated collagen showed a kinetic pattern similar to that of unmodified collagen and could be dissolved into Tris buffer without salt, it was used for the study of effects of ions on collagenase activity.

Table 7.1. shows if succinylated collagen was used with or without Ca^{++} ions and if original collagen was used with Ca^{++} ions, the half-life of the fast reaction were almost the same. While the half-life of the slow reactions were quite different. It depends on if Ca^{++} ions were added. If data for collagenolytic reaction with succinylated collagen without Ca^{++} ions was handled as that with Ca^{++} ions that was to extrapolate the slow reaction to time zero, then the reaction rate would be too fast and impossible. Therefore, it might be assumed that without Ca^{++} ions, the fast

and slow reaction would not occur coincidentally.

There were two first order reactions either with or without added ions. When the reaction was without Ca^{++} ions or with 0.005 M Ca^{++}, Mn^{++}, Mg^{++}, the half-life for the fast reaction was almost the same, about 2.6 mins. While that of slow reaction was quite different in each case. Mn^{++}ions enhanced the collagenolytic activity and Co^{++} ions, even at concentration as low as 0.005 M, had almost the same effect as 0.5 M Ca^{++}ions. Yagisawa et al. (85) had suggested some degree of activation of collagenase by Co^{++}ions. Takahashi and Seifter (38) had studied the effects of metals on the enzyme activity and observed that if Co^{++}ions are added to the culture medium, enzyme with specific activity 1.8 times higher than that obtained in the usual culture medium can be prepared. Significant, but less striking results were obtained with Mn^{++}, Fe^{++} in place of Co^{++}. It seems that metal ions have, if any, very little effect on the fast reaction but shows significant effect on the slow reaction.

It was mentioned before that the enzyme binds to the undenatured substrate and possibly resulting in a reorganization of the molecule, or part of it, as a prelude to proteolysis (81).

It might be that the first attack of enzyme on the

substrate is Ca^{++} ions independent and the following hydrolysis of the peptide, Ca^{++} ions are helpful activator.

Chapter VIII

Studies of EDTA Inhibition on Collagenase

Studies of EDTA inhibition of collagenase

Results and discussion

Succinylated swim bladder collagen was used to study the inhibitory effect of EDTA on the enzyme activity. With either 0.25 M or 0.5 M Ca^{++} ions in the reaction mixture, the same k_f/k_s was obtained which was 3.6. EDTA was added to the reaction mixture to the final concentration of 4.46 x 10^{-4} M. The collagenolytic reaction was completely inhibited. If Ca^{++} ions was added to a final concentration of 0.25 M, the collagenolytic activity was fully restored and gave k_f/k_s equal to 3.2.

It had been reported that Ca^{++} ions are required for both the binding of the enzyme to the collagen substrate and for full catalytic activity (103). Thus agents such as EDTA inhibit enzyme activity in the first instance by binding Ca^{++}. But it was found in this work that without Ca^{++} or with a low Ca^{++} ion concentration, such as 0.005 M, the collagenolytic reactions still can be seen, but at a slower rate. It might be that Ca^{++} ion is not necessary for the binding of the collagenase to the substrate. The effect is but rather after binding of enzyme and substrate. Takahashi and Seifter (38, 40), Harper and Seifter (39, 106) found that an intrinsic metal component, most likely a zinc atom, is present at the active site of collagenase. It

might be that Ca^{++}ions make the enzyme proteins conform around the putative zinc atom so that the latter is chelated with two or three amino acid residues in a manner resembling that of zinc in carboxypeptidase A. If Ca^{++}ions are not present in sufficient amount the protein conformation will be different, which will effect the rate.

Maschmann (107, 108) originally described the inhibition of collagenase by cysteine, Seifter and Harper (39, 106) studied the details of the inhibition. In addition to its inhibition by cysteine, clostridial collagenase is inhibited by reduced glutathone, by 2,3-dimercaptopropanol, and by dithiothreitol. It is poorly inhibited by -SH containing substance that do not contain a second functional group capable of chelating strongly. The EDTA inhibition might be due to the chelation to the zinc atom, and if enough Ca^{++}ions were added the active site of the enzyme was freed and the enzyme activity restored.

Chapter IX

Tm of Collagens in Different Solvents with or without Ca^{++}ions

Tm of collagen in different solvents with or without Ca^{++}ions

Results and discussion

Table 9.1. gives the Tm values of calf skin collagen, swim bladder collagen and lathyritic rat skin collagen in different solvents with or without Ca^{++}ions. The solvents used were 0.05M Tris buffer pH 7.0, 0.01N HCl, 0.01N succinic acid, 0.01N acetic acid, 0.01N formic acid and 0.01N citric acid. Figures 9.1. to 9.6. show the corresponding melting curves.

Maser and Rise (109, 110), von Hippel and Wong (111), Josse and Harrington (112) concluded that the only possible correlation between collagen composition and the Tm is the total pyrrolidine residue content. But more recently, evidence was obtained that hydroxyproline makes a specific contribution to the thermal stability of synthetic polypetides, apparently via hydrogen bonding involving the hydroxyl group as does collagen (113, 114 and 115). Ramachandran et al. have studied the possibility of hydroxyproline residues stabilizing the collagen triple-helical structure by the formation of additional hydrogen bonds through their γ-hydroxyl group. It is not possible for this hydroxyl group to form a direct hydrogen bond with a suitable group in a neighboring chain of the triple-helical protofibril. However, in the modified one-bonded

structure, which is stabilized by additional hydrogen bonds being formed through water molecules as intermediaries, it is found that the γ -hydroxyl group of hydroxyproline can form a good hydrogen bond with the water oxygen as acceptor. It is proposed that, in addition to stabilizing the collagen triple-helical structure due to the stereochemical properties of the pyrrolidine ring, hydroxyproline gives added stability by the formation of an extra hydrogen bond.

Table 9.2. gives imino acids content of collagens. Rat skin collagen contained 213 imino acid residues (92 Hyp, 121 Pro) per 1000 amino acid (116). Calf skin collagen has 220.8 imino acid residues (86.1 Hyp, 134.7 Pro) per 1000 amino acid (117). Carp swim bladder collagen contains 192 imino acid residues (76 Hyp, 116 Pro) per 1000 amino acid (118). Therefore as expected, swim bladder collagen shows the lowest Tm. It was found that Tm of collagen was pH independent between pH 3-11 in the same solvent system (119, 120). In the lower or higher pH region there are stronger charge repulsions which occur intramolecularly and a decreased Tm would be expected (121). Therefore, at pH 2.0, 0.01N HCl, a lower Tm was observed.

It was known in the collagen structure side chain groups are readily disposed on the cylindrical surface of the elongated molecule, accessible for interaction with the solvent environment and/or neighboring molecules (120,

122). The binding of ions to charged groups of collagen is not responsible for the effects of these compounds on Tm, but the extent to which these ions are bound by charged groups seems to be qualitatively correlated with their competence as Tm affectors (121, 124). In Table I it shows that with and without Ca^{++}ions in the same solvent the Tm of collagen was quite different. These differences are due to lyotropic effect of Ca^{++}ions (121, 124 and 125). The lowering of Tm with Ca^{++}ions is assumed firstly due to ionic disorganization of the water contacting the polypeptide chains and secondly to their mutual electrostatic repulsion (126).

The melting temperature of normal swim bladder collagen and a swim bladder collagen that has been artificially cross linked through formaldehyde was compared (127). It was found that the introduction of about 10 cross links per collagen monomer raises the Tm only 1.4 °C . Thus cross-linking has, at most a second order effect on the thermal stability of collagen. Therefore the Tm of calf skin collagen and lathyritic rat skin collagen are very close to each other, with similar imino acid residues, in solvents with Ca^{++}ions. There is at the most, a 1 °C difference between them. Since lathyritic rat skin collagen has more hydroxyproline residues for hydrogen bonding formation through hydroxyl group and water molecules, therefore without Ca^{++}ions lathyritic collagen shows a higher

Tm value than calf skin collagen. There is about a 3°C difference in each solvent.

Fig. 9.1. Tm of swim bladder collagen in different solvents with 0.5 M Ca^{++} ion.

Fig. 9.2. Tm of swim bladder collagen in different solvents without Ca^{++} ion.

Fig. 9.3. Tm of calf skin collagen in different solvents with 0.5 M Ca^{++} ion.

Fig. 9.4. Tm of calf skin collagen in different solvents without Ca^{++} ion.

Fig. 9.5. Tm of lathyritic rat skin collagen in different solvents with 0.5 M Ca^{++} ion.

Fig. 9.6. Tm of lathyritic rat skin collagen in different solvents without Ca^{++} ion.

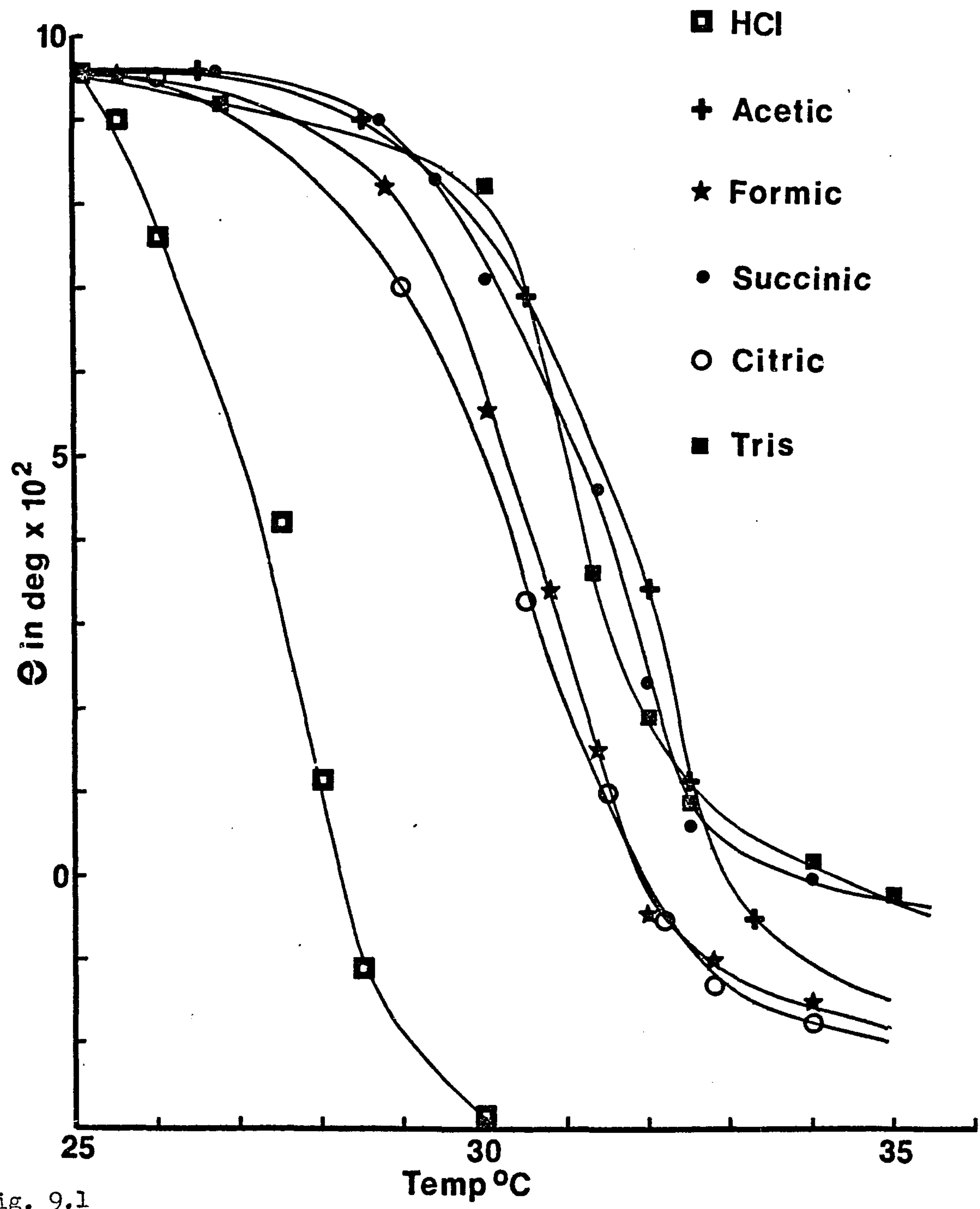


Fig. 9.1

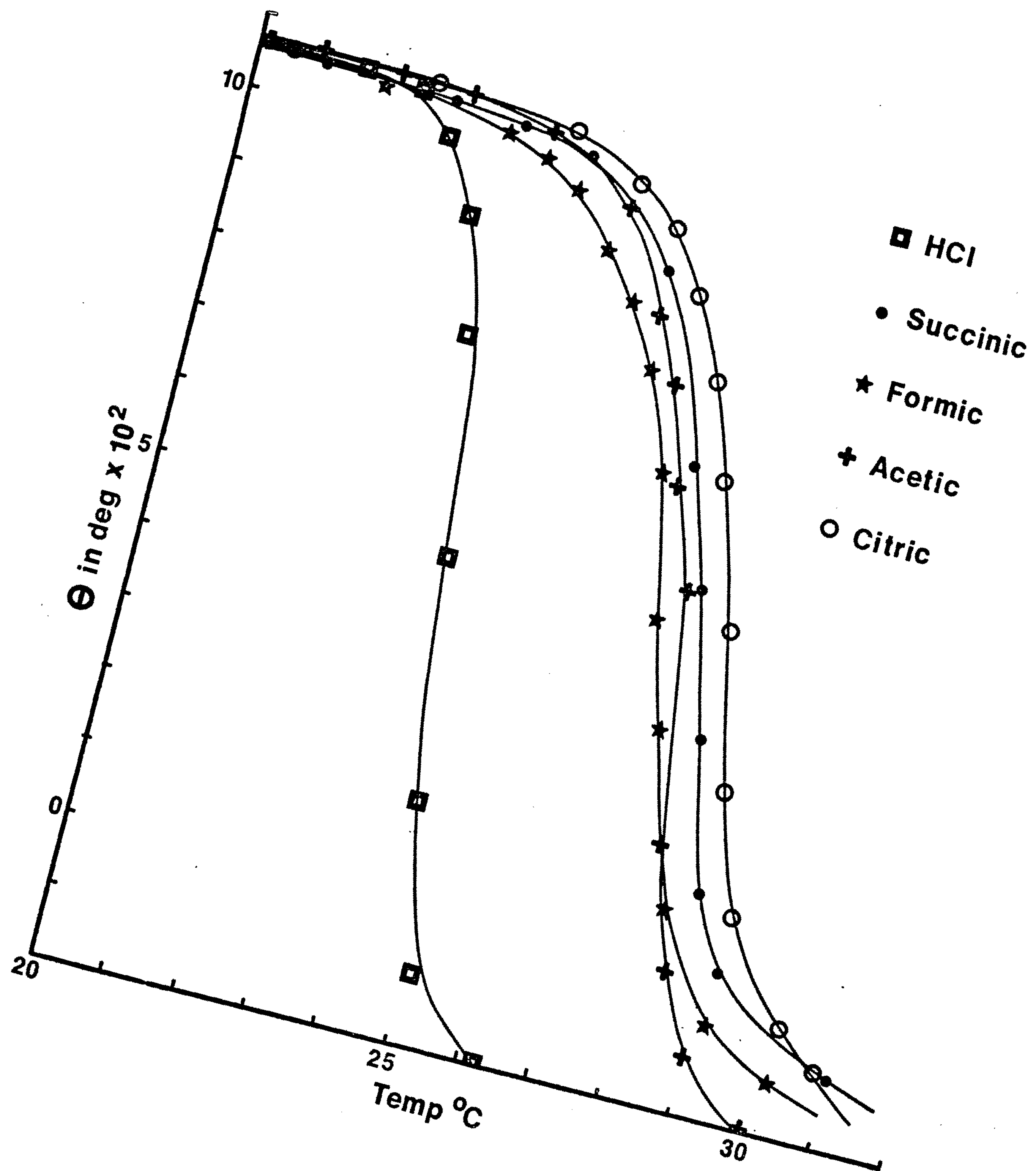


Fig. 9.2

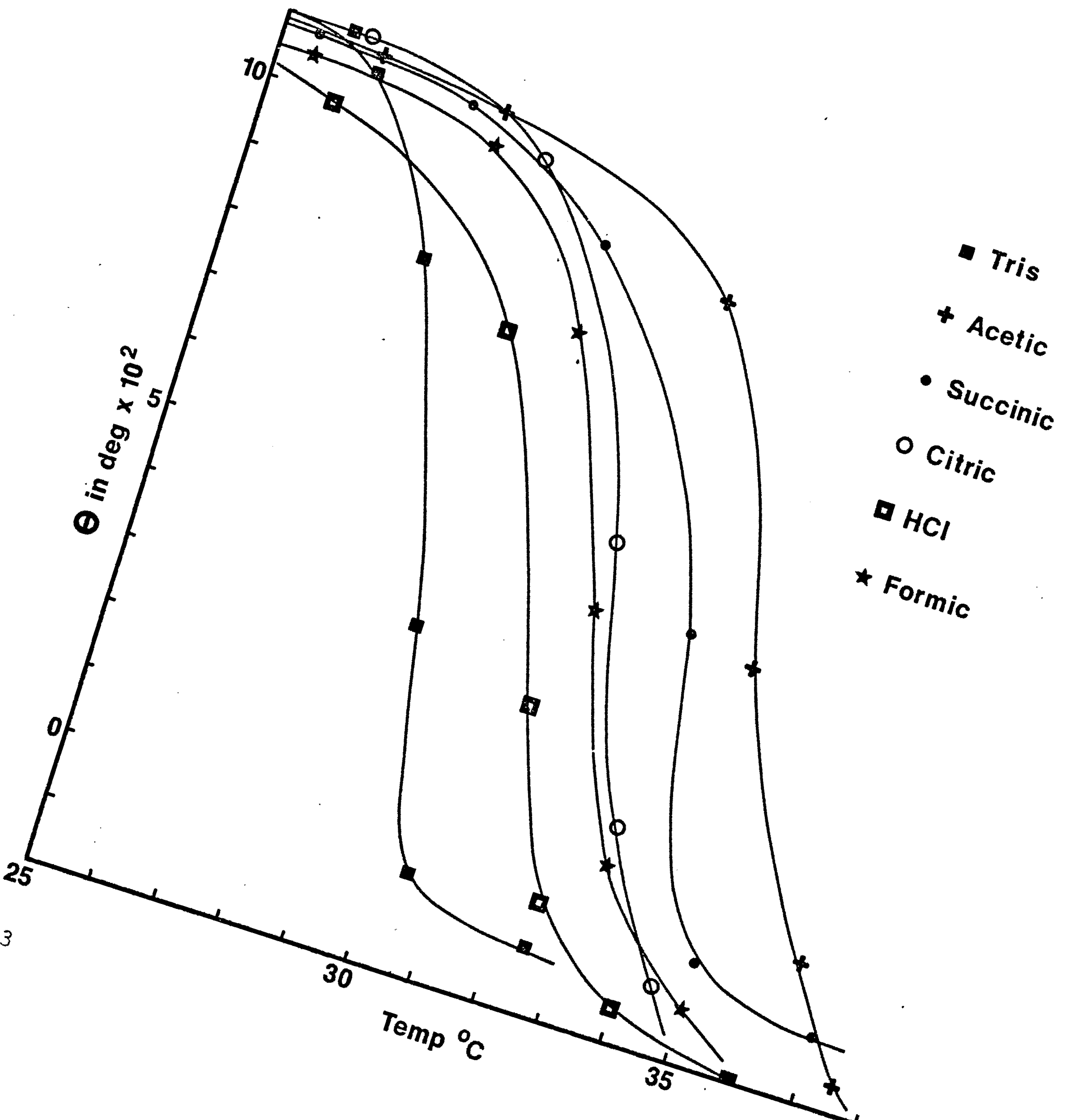


Fig. 9.3

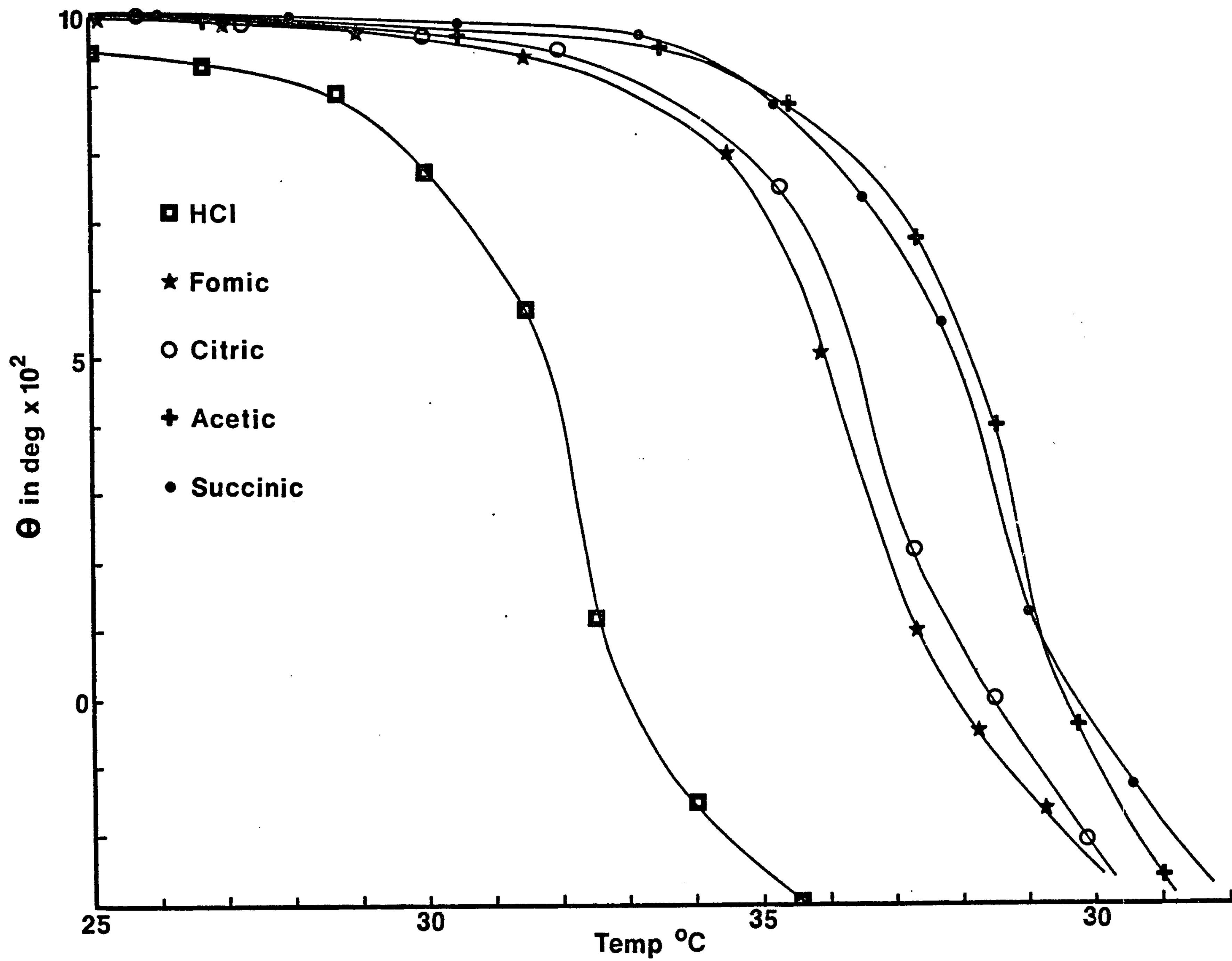


Fig. 9.4

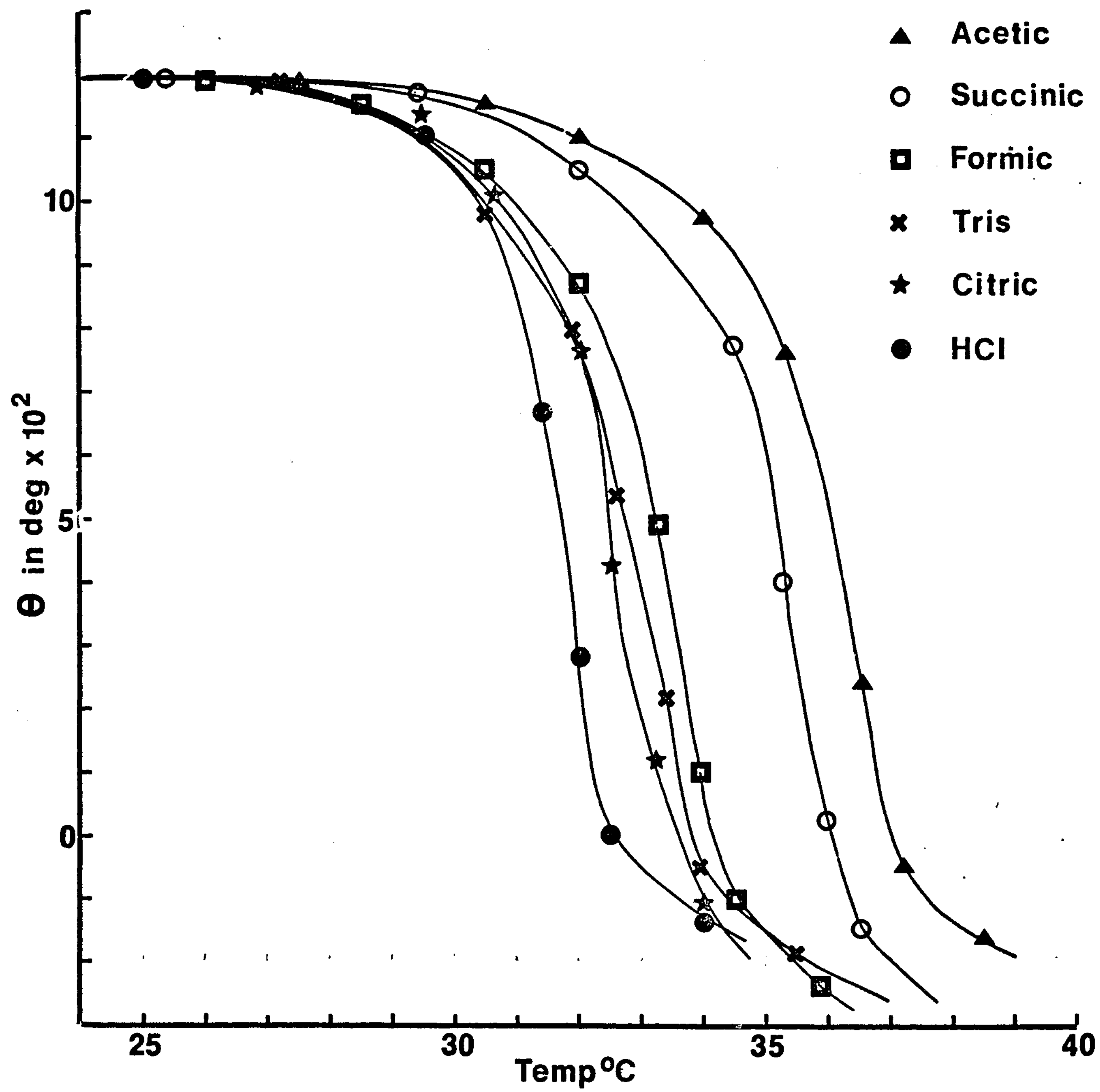


Fig. 9.5

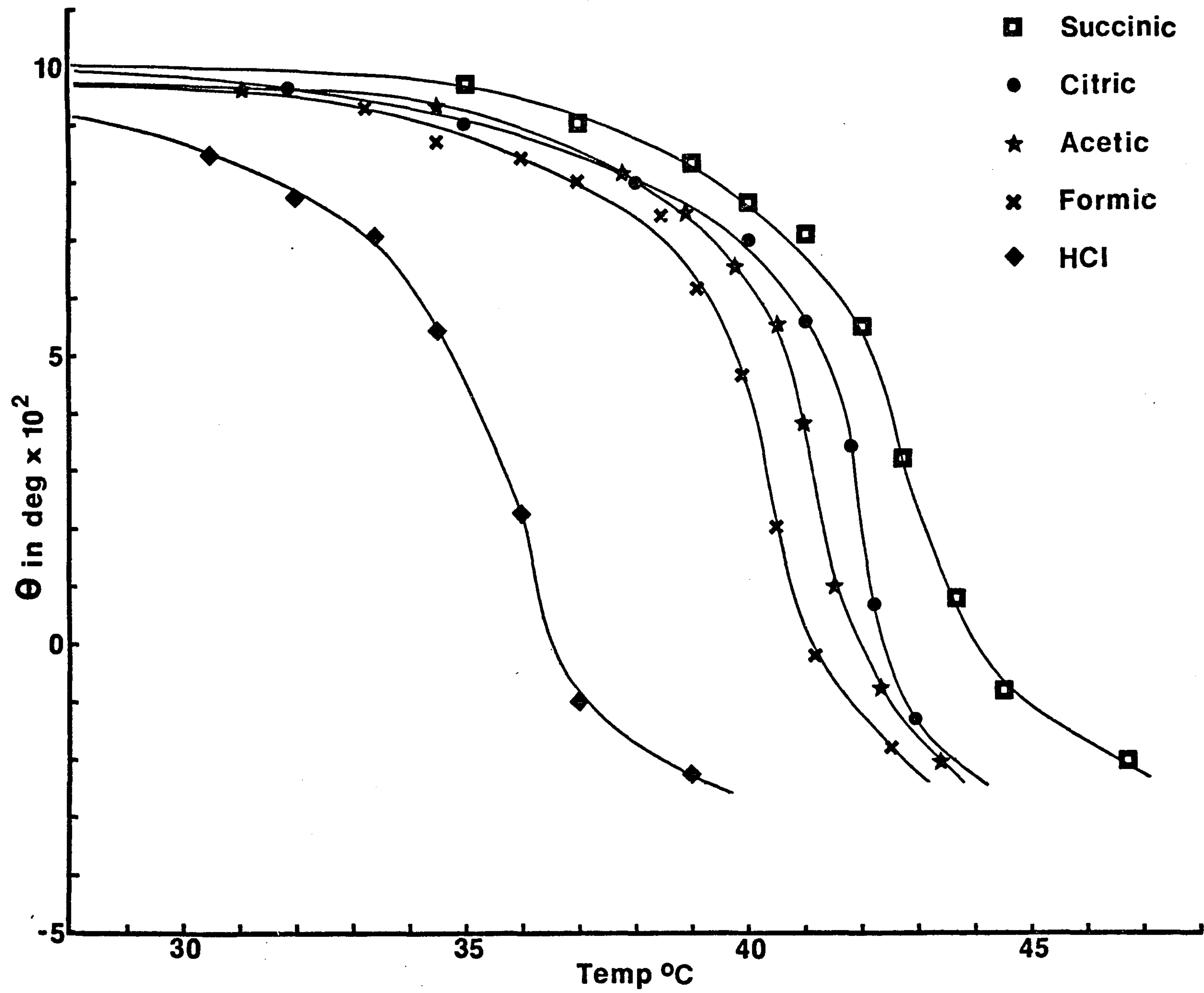


Fig. 9.6

Table 9.1.

Tm of collagen in different solvents

with or without Ca^{++} ions

Collagen / Solvents		Tris	HCl	Acetic	Formic	Succinic	Citric
			Tm °C				
Calf skin collagen	0.05 M Ca^{++}	30	32	35.5	32.6	34.3	32.9
	no Ca^{++}		32	38.6	38.3	39.8	39.1
Lathyritic collagen	0.05 M Ca^{++}	33.9	31.8	36.8	33.7	34.9	33.3
	no Ca^{++}		36.5	41.3	40.1	42.4	42.3
Swim bladder collagen	0.05 M Ca^{++}	30.8	27	32.3	31.7	31.7	31.8
	no Ca^{++}		27	33.7	34.3	34.4	34.9

Table 9.2.

Imino acid residues per 1000 amino acid in collagen

Imino acid / Collagen	Rat skin	Calf skin	Carp swim bladder
Proline	121	134.7	116
Hydroxyproline	92	86.1	76
Total imino acid	213	220.8	192
% Hydroxyproline	43%	39%	39%

Chapter X

Effect of Mucopolysaccharides on Tm of Collagens and on Collagenolytic Reaction

I. Mucopolysaccharides effect on Tm of collagen

Results and discussion

Gelman and Blackwell have reported that mucopolysaccharides such as chondroitin-6-sulfate, chondroitin-4-sulfate combined with collagen at PH 4.3. The collagen molecule was thus stabilized and it showed a higher Tm (the melting temperature) (66, 128).

Studying the interaction between cationic polypeptides, such as poly (L-arginine), poly (L-lysine) and poly-(L-ornithine), and mucopolysaccharides, Gelman and Blackwell found that there is a conformation-directing effect on mucopolysaccharides on polypeptides by the interaction between the positive amino acid residue side chains and the sulfate and carboxyl group of mucopolysaccharides (67, 68, 129 and 130).

In 0.05M Tris buffer PH 7.0 with 0.5M $CaCl_2$ the interactions between mucopolysaccharides and collagen were studied. The r was defined as the ratio of disaccharide residues per 100 amino acid residues (131). It was reported that the melting point of calf skin collagen in acetic acid, which was normally 38° c was increased to 46° c in the presence of chondroitin-

6-sulfate at a concentration ratio in excess of 5.5 disaccharide residues per 100 amino acid residues. Thus an interaction occurs between the collagen and mucopolysaccharides, which increases the stability of the former. At $r < 5.5$, biphasic melting curves were obtained with transitions at 46° c and 38° c indicating the presence of both complexed and uncomplexed collagen. Therefore the Tm value can be indicative of interactions (67).

Table I gives the Tm value of the mucopolysaccharides and collagen interaction in Tris buffer pH 7.0 as determined using the CD. Table II gives the T value for the mucopolysaccharides and succinylated collagen interaction in Tris buffer at pH 7.0.

Data in Table I and II show that there is no stabilization effect of mucopolysaccharides on collagen at neutral pH. Instead, there is a Tm decrease from 30.8 °C to 27°C , for the original collagen, while succinylated collagen remains almost the same, at 26°C . Since succinylated collagen is more negatively charged in solution than the original collagen it has a lower Tm value and the introduction of negative charges from mucopolysaccharides could not change its Tm as much as the original collagen.

II. Mucopolysaccharides effect on collagenolytic reaction

Results and discussion

It has been reported that heparin was used in the culture medium for animal tissue explants as a collagenase activator (131). Therefore it was interesting to study if mucopolysaccharides have any effect on the bacterial collagenase during the collagenalytic reaction.

The concentration of collagen or succinylated collagen used was 1.76 x 10^{-4} g/ml with r = 0, 2.7, 5.5 and 11 for chondroitin-6-sulfate. Collagenase was at a concentration of 0.172 mg/ml. All the reactions gave the same k_f and k_s, they were 0.277 min^{-1} and 0.103 min^{-1} respectively.

The concentration of collagen used was 1.65 x 10^{-4} g/ml and that of collagenase was 0.245 mg/ml in the studies of heparin and hyaluronic acid effect on collagenolytic reaction. In each case, r = 11 was studied. Either with or without mucopolysaccharides k_f and k_s were the same. They were 0.533 min^{-1} and 0.216 min^{-1} respectively.

From the data above it can be concluded that

heparin, hyaluronic acid and chondroitin-6-sulfate have no effect on bacterial collagenolytic activity.

Table 10.1 Tm of unmodified swim bladder collagen in the presence of different ratio of mucopolysaccharides (in °C)

mucopolysaccharide/r	0	2.7	5.5	11
Chondroitin-6-sulfate	30.8	27	27	27
Hyaluronic acid	30.8	27	27	27
Heparin	30.8	27.1	27	27.3

Table 10.2 Tm of succinylated swim bladder collagen in the presence of different ratio of mucopolysaccharides (in °C)

mucopolysaccharide/r	0	2.7	5.5	11
Chondroitin-6-sulfate	26.8	26.2	26.2	26.2
Hyaluronic acid	26.8	26.2	26.2	26.2
Heparin	26.8	26.2	26.2	26.2

Conclusion

Conclusion

The electronic transitions of the peptide group give rise to a number of spectral bands in the wavelength region below 240 nm. The strong rotatory power of these bands can be determined either from ORD Cotton effects or from CD absorption bands.

The maximum around 220 nm in the circular dichroism (CD) spectrum of native collagen solution is characteristic of the triple-folded helix (18, 19 and 20). It changes to a negative value after heat denaturation or collagenase hydrolysis (21). By examining the rate of change of the 220 nm maximum as a direct measuring of triple-helix disruption, it is, therefore, possible to study the nature of collagenase action on its substrate.

The collagenase induced rate of CD change at 220 nm was shown to be two first order reactions. One fast reaction and one slow reaction. If succinylated collagen is used as substrate, the reaction mixture with Ca^{++} or without Ca^{++} can be compared. The rate of fast reaction maintained almost the same either with or without Ca^{++}. The rate of slow reaction is highly activated with added Ca^{++}. If EDTA is added to the reaction mixture the collagenase activity is completely inhibited but can be restored with more added Ca^{++}. It implies that EDTA not

only can chelate the Ca^{++} but also it might chelate the intrinsic metal ion in the active center of collagenase. Succinylated collagen has more negative charges but when reacted with collagenase shows the same reaction pattern as unmodified collagen. Therefore probably carboxyl groups or ε-amino groups do not give too much effect on collagenase collagen interaction.

It was reported previously (109-112) that the stability of collagen molecule depends on imino acid content. Recently, the effect of hydroxyproline has been emphasized (113-115). The γ-hydroxyl group of hydroxyproline can form extra hydrogen bonds to stabilize the collagen molecule. In this work added proof of effect of hydroxyproline has been found. Rat skin collagen contained 213 imino acid residues (92 Hyp, 121 Pro) per 1000 amino acid (116). Calf skin collagen has 220.8 imino acid residues (86.1 Hyp, 134.7 Pro) per 1000 amino acid (117). Carp swim bladder collagen contains 192 imino acid residues (76 Hyp, 126 Pro) per 1000 amino acid (118). Rat skin collagen with the highest Hyp content, 43%, shows the highest Tm in different solvent as shown on tables 9-1 and 9-2. Calcium ion will lower the Tm of collagen. It is assumed firstly due to ionic disorganization of the water contacting the polypeptide chains and secondly to their mutual electronic repulsion (126).

Also, in this work, it is found that collagen containing more Hyp residue shows higher resistance to collagenase digestion. Thus, the rate of hydrolysis of rat skin collagen is slower than calf skin collagen or carp swim bladder collagen.

Previously, it was reported that at acidic pH there is interaction between collagen and mucopolysaccharides (66, 128). The reaction might be through the positive amino acid residue side chains and the sulfate and carboxyl groups of the mucopolysaccharides (67, 68, 129 and 130). It results in stabilizing the collagen molecule by increasing the Tm. In this study, the interaction of collagen and mucopolysaccharides is investigated under neutral pH. Tm of collagen has not been found to increase but decrease a little bit.

It has been reported that heparin was used in the culture medium for animal tissue explants as a collagenase activator (131). In this work, mucopolysaccharides do not show effect on activity of bacterial collagenase.

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